BACTERIOLOGICAL REVIEWS, Mar. 1969, p. 1–47
Vol. 33, No. 1

Dialysis Culture of Microorganisms: Design, Theory, and Results

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INTRODUCTION

Regulation of a constant *milieu interieur* is a prime characteristic of life systems (71), with the control often exercised by a membrane. Although best exemplified with individual cells, the principle applies as well to multicellular organisms—an illustrative example occurs in the placental envelopment of the embryo. One may also think of analogous situations where a population of growing microorganisms is nurtured within a membrane-enclosed environment—a classic instance is seen with bacterial peritonitis.

Efforts to simulate this disease situation led to the first uses of artificial membranes for culturing microorganisms. In 1896, Metchnikoff et al. (98) implanted collodion sacs containing cultures of cholera vibrios into the peritoneal cavity of animals to establish *tout d'abord* that there existed a diffusible cholera toxin. This new and simple technique was promptly exploited by Nocard and Roux (110) to achieve cultivation of the "peripneumonia" organism (mycoplasma), and by Novy (112) to enhance virulence of pneumococci by animal passage without phagocytosis.

A logical modification of these in vivo experiments was the suspension of a collodion sac containing bacterial culture in a laboratory flask with ordinary medium. This step was promptly taken by Carnot and Fournier (20) in 1900 in their search for a diffusible toxin from pneumococci; they reported that properties different from those in ordinary culture were acquired, such as greater capsulation, prolonged viability, and more persistent virulence. In the same year, Ruffer and Crendirpoulo (142) described a similar culture arrangement, but only proposed possible uses. Shortly thereafter, in 1904, Frost (41) exploited the technique to study the antagonism of soil or water organisms toward typhoid bacteria, observing what now would be called antibiotic effects.

These pioneering uses of semipermeable membranes in culturing microorganisms involve several principles that distinguish the method from ordinary ones. The primary feature is that the microbial population is sequestered into a milieu on one side of the diffusion barrier. On the other side is the greater environment, which contains the nutrients for metabolism and growth. These nutrients diffuse through the barrier into the culture compartment, and diffusible metabolic products diffuse away from the culture—that is, exchange dialysis occurs.

With in vitro dialysis culture systems, the culture compartment contains a population of microbial cells that usually is in a liquid medium and is in some way mixed so as to obtain a homogeneous suspension. For the system to be effective, the volume of the reservoir must be relatively large in comparison with that of the culture compartment, or else it must be replenishable. Regulation of the system is achieved through alteration of the ratio of culture-to-reservoir volume and through selection of the type of inert membrane and adjustment of its area. These factors are irreducibly controlling in any dialysis culture system. But other parameters must also be considered, such as the rate of liquid flow, liquid turbulence, concentrations of essential nutrients and diffusible products, and osmotic effects. Refinements on simple dialysis (such as electrodialysis, adsorption dialysis, and ultrafiltration) also seem applicable but as yet appear not to have been studied.

What we seek to do first in this article is to present briefly the evolution in the design of apparatus for managing dialysis culture. The technical limitations of the original membrane-sac technique have largely been overcome, and presently available systems enable a larger scale of size, better control of the essential elements, and the use of different types of membrane material in membrane sheets. It will become evident that dialysis culture can be operated in a batch cycle, continuously under steady-state conditions, or in combined modes. The principles of dialysis can be extended to systems that employ a filter membrane, a solution-transport membrane, or an interface, rather than the usual dialysis membrane.

With development of a generalized design, it became possible for us to derive new mathematical models that describe the expected behavior of some simplified dialysis culture systems. This theoretical analysis represents the second objective of this article. The simplifying assumption that allows such a theoretical analysis is the limiting nutrient concept (102), since it provides the common feature that enables one to relate dialysis kinetics to growth kinetics. The resulting equations and their graphical solutions lead to attractive predictions about dialysis culture, some of which are verified from existing experimental data and others of which are remarkable but untested. Perhaps the most important purposes of a simplified mathematical analysis are to focus attention on the more important parameters deserving attention in experiments, and to challenge investigators to greater exploitation of the method.

Our third objective is to describe the differences in growth response that distinguish dialysis culture from ordinary propagation systems, and to define some of the more important controlling variables. The remarkable consequences of dialysis culture have led to application of the technique for a range of useful purposes, which we will review, and have opened prospects for a number of new uses, which we will attempt in part to preview. If through design, theory, or application of dialysis culture the course of further research is illuminated, then the central purpose of this effort will be accomplished.

DESIGN AND APPARATUS

Membrane Dialysis Culture

The surprising fact about the evolution of the dialysis culture technique is that so little advancement was made in basic designs for so many years. Only recently have significant improvements ap-

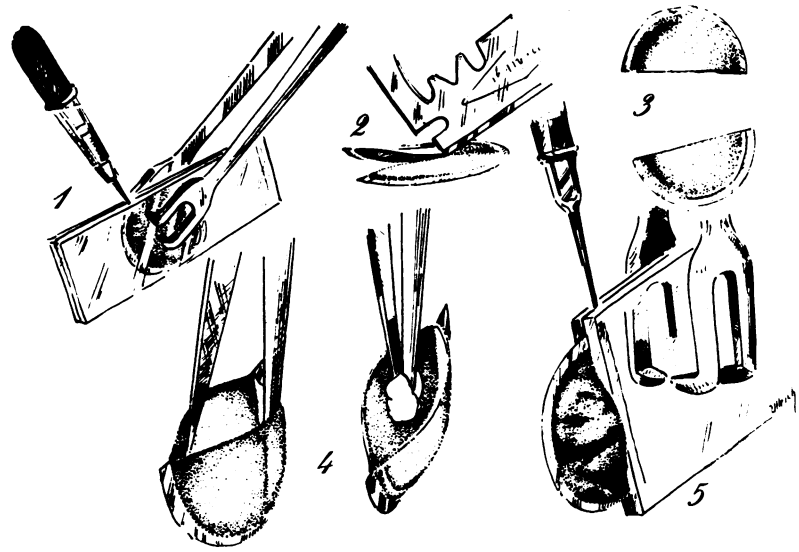


FIG. 1. *Preparation of implantable filter-membrane envelopes for in vivo dialysis culture. Acetone is employed to fuse the edges. Reproduced from Wong et al. (166).*

peared with in vitro systems, and these have greatly increased the applicability and study of the method.

For in vivo studies, however, the original implantable "diffusion chamber" method still persists with little modification. Algire (6) described several effective arrangements for peritoneal insertion, in which filter membranes are sealed with solvent to small Plexiglas rings so as to provide an implantable chamber. Wong et al. (166) devised a neat method for preparing envelopes made from cellulose acetate filter membranes fused around the edges with acetone (Fig. 1). Such implantable envelopes or chambers are easily multiplicable but do not allow access for sampling while in place. This can be accomplished with the "Vivar" diffusion chamber developed by Fina et al. (37), which they applied to a study of the bovine rumen. The device is shaped like a drum, with the two heads comprised of membrane held in place by nuts and rubber washers. An access tube for sampling extends as a side arm and is passed through a fistula in the animal. A comparably employed device (28) is designed as a long insertable tube of dialysis membrane supported inside and out by cylindrical polythene netting.

In the first in vitro experiments, the arrangement mimicked that used for in vivo studies, except that a glass vessel of medium replaced the peritoneum or rumen. The membrane sac was made by coating a tube or thimble with collodion and then removing the mold, or by tying the end of a tube of parchment, cellophane, or similar membrane material. The simplest arrangement, however, is to employ a length of dialysis membrane tubing that is intussuscepted so as to form a double-walled tube, in the annular space of which the culture is contained (165). The scale of size attainable with such a device obviously is limited, although Sterne and Wentzel (155) managed a toxin production system of this type with 3.5 liters of culture surrounded by 35 liters of medium in a carboy (Fig. 2).

The first really basic change in design introduced the distinctive concept of carrying out growth in a culture vessel that is remote from but in communication with a nutrient reservoir vessel (44). This separation of the principal compartments of a dialysis culture system makes it possible for each to be controlled independently and more effectively. The concept was at first applied by coiling dialysis membrane tubing around a support in either the culture or the reservoir vessel, with the respective contents continuously circulated through the tubing by means of a pump. Both of these systems, however, had inherent limitations in capability for control, scale-up, and use with different types of membranes.

To overcome these limitations, all three regions—culture, reservoir, and dialysis—were separated. In the system thus evolved (Fig. 3), the culture vessel and the nutrient reservoir both can be of conventional fermentor design without any special modifications, and in principle any type of vessel could be employed. Remote from these vessels but connected with them by tubing and pumps is a dialyzer through which the culture and medium are continuously circulated. An

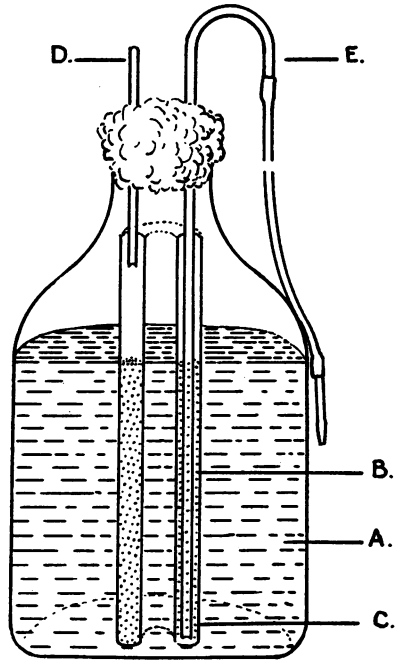


FIG. 2. *Representative sac-dialysis culture system. A tube of dialysis membrane is intussuscepted so as to form a double walled sac (C), the annular space of which contains culture (B). The medium (A) is contained in a carboy. There also is a tube for sampling and inoculating (D) and a harvesting siphon (E). Reproduced from Sterne and Wentzel (155).*

important attribute of this system is that it allows the use of membranes in sheet form, which are commercially available in a range of materials and types. Experiments demonstrated that this new "dialyzer-dialysis system" is inherently capable of independent control of the component operations; for example, the culture can be agitated and aerated as much as necessary while the reservoir is held quiescent to help guard against contamination. Furthermore, the system apparently could be adapted to any size simply by scaling up each part in proportion. An example is illustrated in Fig. 4, where a 14-liter glass fermentor with 10 liters of culture is shown attached to a 250-liter steel fermentor charged with 100 liters of medium.

The key to effective operation of the above dialyzer-dialysis culture system is a suitably designed dialyzer. The best principle of design appears to be one resembling a plate-and-frame filter press, but holding a series of membrane sheets. An industrial chemical dialyzer was employed initially (44). Subsequently, Gerhardt and co-workers (Abstract, Meeting Am. Chem. Soc., New York, 1966) designed and constructed a multimembrane dialyzer especially suitable for dialysis culture and other biological applications. The resulting instrument (Fig. 4) consists of two end plates that compress an alternating series of thin metal frames, sheet membranes, and molded rubber separators. All of the parts withstand repeated autoclaving and are not toxic to microorganisms or mammalian cells. The plates are made of stainless steel or Pyrex glass and the frames, of stainless steel. The separators (Fig. 5) are custom molded from medical-grade silicone rubber. Each separator in one piece provides gasketing, entry holes and ports, and fields of membrane-support elements. Each such element is an equilateral pyramid which causes a minimum of masking yet provides uniform support of the membrane and also promotes turbulence in the flow of liquid, with consequent increase in dialysis efficiency and prevention of the lodgment of cells. The flow of liquids through the dialyzer can be upward or downward, cocurrent or countercurrent, and slow or fast. Any type of sheet dialysis or filter membrane can be employed.

With the development of more functional fermentor systems, there also became evident a need for more convenient flask dialysis culture systems, so that a number could be used at one time. The result was an efficient, twin-chambered dialysis flask (Fig. 6), constructed mainly from stock components and designed for mounting on a shaking machine (51). A supported membrane is clamped between a reservoir of medium in the bottom and a small volume of culture above. The lower reservoir is stirred by the rotation of a Teflon-coated steel ball, and is accessible by hypodermic needle through a rubber diaphragm. The culture in the upper chamber is aerated and agitated by baffled swirling, and is accessible through a removable cotton closure on the top opening. The flask is constructed of stock fittings of commercial Pyrex pipe and can be made in any desired size.

Nurmikko (115) also had designed a flask dialysis system, in which the membrane is held between flanged side arms in the base of each of two adjoined flasks. A stagnant pocket of liquid can occur in the side arms and the relative area of membrane is low, so that the efficiency of dialysis probably is low. Furthermore, adequate mixing and aeration appear difficult to achieve in this system. However, dialysis flasks of similar design are commercially available.

A simple and effective dialysis flask can be assembled from two L tubes of stock Pyrex pipe, with a gasketed membrane positioned between the opposing arms by means of stock clamps (Zaccharias, *personal communication*).

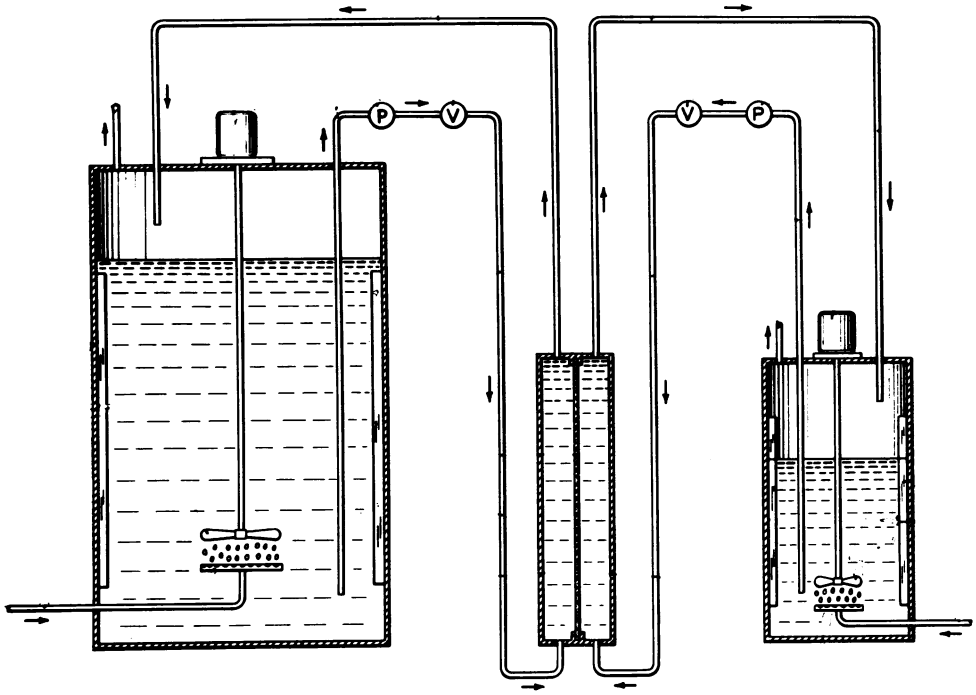


FIG. 3. *Dialyzer-dialysis culture system. The nutrient reservoir is on the left, the dialyzer in the center, and the culture fermentor on the right. P designates a pump and V designates a valve. The arrows show the direction of circulation of medium and culture. Reproduced from Gallup and Gerhardt (44).*

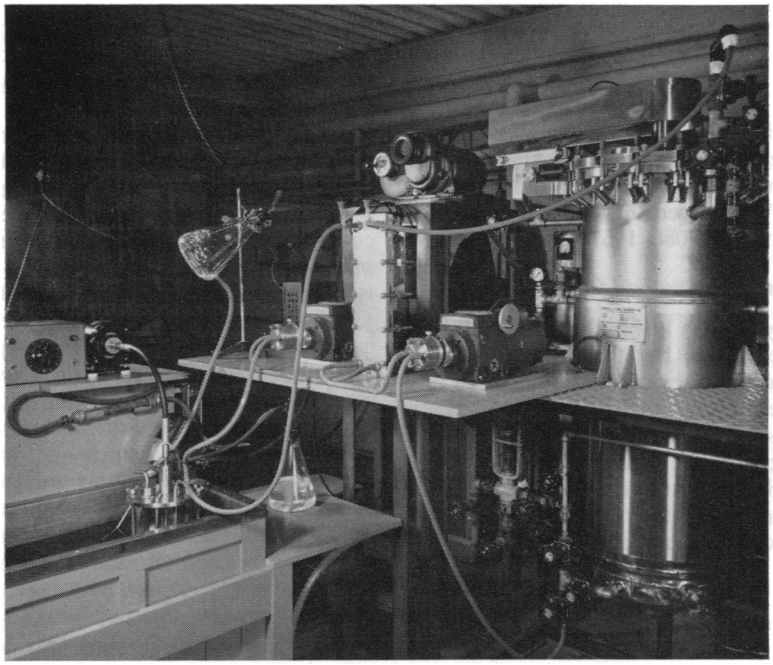


FIG. 4. *Pilot-plant adaptation of dialyzer-dialysis culture system. An ordinary glass-jar fermentor assembly containing 10 liters of culture is at the left; the dialyzer and two circulating pumps are at center; and a stainless-steel reservoir containing 100 liters of sterile medium is at the right. Reproduced from Gerhardt and Schultz (53).*

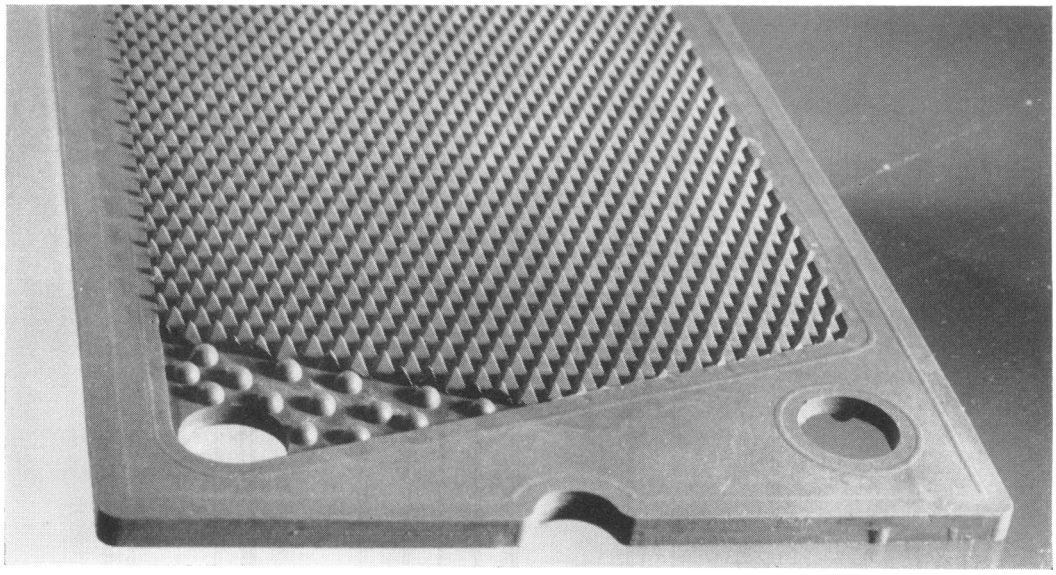


FIG. 5. *Enlarged dimensional view of the silicone rubber separator used in the Gerhardt dialyzer.*

Another dialysis flask was devised by Langlois et al. (83) for propagation of chick myeloblast cell suspensions. It embodied the useful feature of a separate medium reservoir, which was connected by tubing and a pump to the base of the flask so that the medium could be replenished easily (Fig. 7). The flask could be mounted on a shaker.

Yet another design inventively made use of the Venturi principle to induce turbulence around dialysis tubing held in an hourglass-shaped flask, which is mounted on a reciprocating shaker (J. N. Dews, G. W. Schmersahl, and W. C. Patrick III, U.S. Patent 3,321,086, 1967).

Interface Dialysis Culture

One usually thinks of dialysis as diffusion through a membrane, but the diffusion barrier also can be an interface between two physically different phases. For example, an ordinary broth culture in a test tube may be considered a dialysis system in which a nutrient (gaseous oxygen) from a large reservoir (the atmosphere) diffuses across a barrier (the gas-liquid interface) and into solution in the culture; carbon dioxide similarly dialyzes away as a product. Interfacial dialysis situations can be visualized as useful in microbial growth not only in such a gas-liquid biphasic system but also in solid-liquid and gas-solid systems. Furthermore, liquid-liquid systems are possible if two immiscible solutions are employed, and a solid-solid system, in a sense, is exemplified by ordinary colonial growth upon nutrient agar.

This extended concept of dialysis has been applied to microbial propagation in solid-liquid systems directly analogous to the membrane dialysis flask system of Gerhardt and Gallup (51). Protozoologists apparently have employed the principle for years, without clearly explaining its effects or systemically studying its basis. The origin of the technique may be found in the work of McNeal and Novy (90), who employed the liquid of syneresis on blood-agar slants to grow trypanosomes. This was followed by a series of rediscoveries that an overlay of liquid on an agar base provides a useful culture system. An early example is in the work of Drbohlav and Boeck in 1924 (30), who described a biphasic medium for the cultivation of *Entamoeba*. Whole egg was mixed and inspissated in a tube, thus providing a solid reservoir of nutrient. This or blood-agar was overlaid with Ringer's or Locke's solution plus diluted serum, into which the protozoa were inoculated. The result was a solid-liquid dialysis culture system, with the solid phase serving as a reservoir and the interface serving as a diffusion barrier. The method was extended to obtain growth of trypanosomes by Kelser (79), with the necessary blood restricted to the agar-solidified base. Chang and Negherbon (24) described typical growth curves. Although the protozoa are known to be obligate aerobes, active aeration of

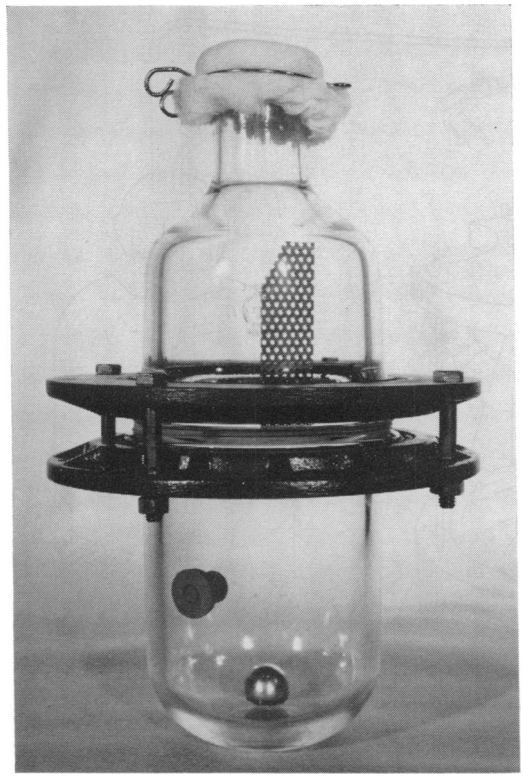


FIG. 6. *Dialysis culture flask, designed to be held on a carriage mounted on a rotary shaking machine. The bottom compartment is filled with sterile medium which is stirred by the rotating ball. The top compartment holds the culture, which is turbulently aerated by swirling and baffling. Reproduced from Gerhardt and Gallup (51).*

cultures (e.g., placing a biphasic flask on a shaking machine) apparently has not been attempted. A convenient review of the cultivation of trypanosomes has been provided by Tobie (158).

Apparently the first use for interfacial dialysis culture in bacteriology was devised by Hestrin et al. (68). *Aerobacter levanicum* was grown in a thin layer of aqueous sucrose over a base of nutrient agar in a flask. The cells were then harvested, washed, and autolyzed to obtain endocellular levansucrase.

A solid-liquid biphasic flask system for growing bacteria in interfacial dialysis culture was developed and studied in principle by Tyrrell et al. (161). In their system, an Erlenmeyer flask is partially filled with hot medium containing 2% agar. After the agar base has solidified, it is overlaid aseptically with a small volume of broth, which then is inoculated. The flask thus prepared can be clamped on a shaking machine to provide agitation and aeration during incubation, can be made in numbers, and is adaptable to any size of flask up to several liters. Its effectiveness is limited mainly by the immobilization of the nutrient within a solid matrix. The movement of nutrients is thus dependent on internal diffusion. Consequently, in practice the agar base must be limited to a depth of 5 cm or less. The agar also is subject to breakup from shaking, and some care must be taken to prevent hydrolysis from overheating. Breakup also can be minimized by immobilizing the agar by use of a square or indented flask, and by precoating the flask with a film of agar in order to obtain better bonding. If all of the medium components are incorporated into the agar base, the overlay can be distilled water. After an equilibration period, the organisms can be inoculated into the resulting clear diffusate. For bacteria such as gonococci, which normally must be grown in a turbid medium enriched with blood and starch, a relatively clean crop of cells can thus be harvested (52).

The above solid-liquid dialysis culture systems may be confused with the well-known double medium of Castaneda (21), which was devised for simplifying blood cultivation procedures in the diagnosis of brucellosis. In this method, liquid medium is placed in the base of an upright culture bottle, which is periodically tipped to inoculate a layer of agar medium on one of the side walls. The principle of dialysis, however, is not involved.

The feasibility of using a liquid-liquid biphasic culture system was explored by Puziss and Heden (132), in an approach based on the extensive use of immiscible polymer solutions for separation procedures (5). A 2% solution of dextran (molecular weight, 480,000) separates as a lower phase from a 12% solution of polyethylene glycol (molecular weight, 4,000). Incorporated into an agitated culture system for *Clostridium tetani*, the solutions were kept dispersed as droplets during the course of growth and toxin formation. Upon completion of the production cycle, the cell-containing dextran phase was permitted to coalesce to the bottom and then was withdrawn. Toxin was equally distributed in the two layers, but was recovered mainly in the top glycol phase because of its greater volume. The main feature of this liquid-liquid biphasic culture system, therefore, appears to be dialytic removal of a macromolecular product into a dispersed reservoir of extracting solvent.

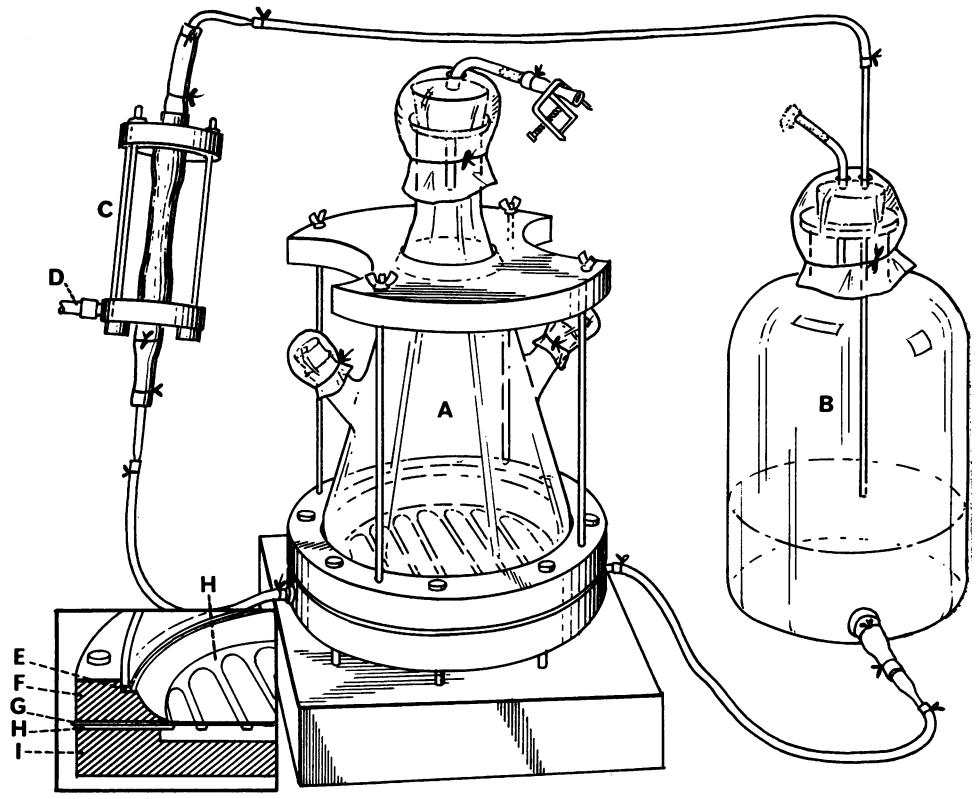


FIG. 7. *Dialysis flask (A) used for production of virus from myeloblast cell culture, with provision for circulation of medium from a separate reservoir (B). The pump (C) consists of a rubber tube with check valves, which is positioned within a glass chamber and activated by air pressure delivered by a reciprocating motor. The remaining components are self explanatory. Reproduced from Langlois et al. (83).*

Colonial Growth with Dialysis

The discussion thus far has tacitly assumed that the microbial populations were homogeneously suspended in liquid culture. Within or on a solid medium, however, microorganisms generally develop as a localized mass of cells. This colonial growth usually occurs in a situation governed by dialysis, but the nature of growth is quite different from that in liquid culture.

Initially, cells in a colony are largely physiologically homogeneous and multiply exponentially with time. But after a few generations, the population becomes more heterogeneous and the order of growth may become very complex, probably because of the close proximity of the cells and their relative location within the colony. Since nutrients from the substratum can reach cells only by diffusion, any given cell at the top of the colony is in competition for nutrients with the entire metabolizing mass of cells beneath it. Conversely, any given cell at the bottom must compete with the respiring barrier of cells above it. These two cells in a colony only 1 mm thick may be separated by hundreds of competing cells, and even greater competition gradients exist between cells in the center and on the periphery of the colony. Consequently, colonial growth poses severe limitations in the design of controllable systems, the development of rational theory, and even the application to practical needs.

Despite these limitations, there are a number of methods for colonial growth with dialysis, many of which are cited in a recent monograph on miniaturized methods (64). Only selected and exemplifying techniques will be cited here.

The classical method of growing microorganisms on medium solidified with agar or other gels may be considered as an interfacial dialysis culture system. Although mainly used in petri plates or as slants in test tubes, agar microcultures also have been devised in ingenious variety by "hanging-block" techniques (64).

Placed on the surface of agar medium, an inoculated limiting membrane can subsequently be

lifted off, and the colonial growth on it can be readily examined in situ and even preserved. Apparently introduced by Birch-Hirschfeld (13), the technique was redescribed and applied to various uses in morphology (64). The familiar membrane-filter technique is a modification in which the organisms strained from a large and dilute sample are subsequently cultured by placing the membrane on nutrient agar.

The feasibility of employing membranes or other porous solid surfaces for producing masses of microorganisms in colonial growth has been explored from time to time (126, 134). Invariably, these have proven impractical; in principle, they are inefficient, and the resulting cells are physiologically heterogeneous. However, colonial surface cultures may be eminently useful in certain industrial fermentation processes, notably, trickling filter beds for sewage and the "quick process" generator for vinegar.

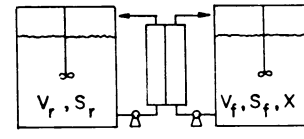
Colonial growth on membranes in contact with a reservoir of liquid medium has unexcelled merit for certain microscopic applications. Both simple (119) and elaborate (128) techniques have been devised by Powell and his colleagues to permit the examination of microcultures. The latter technique permits circulation of the medium beneath the membrane, variation in the gaseous environment above the membrane (including anaerobiosis), control of the amount of liquid around individual cells, micromanipulation of the cells, and use of reflected, transmitted, or phase-contrast optics. Hillier et al. (69) described a method for growing microcultures on a collodion membrane so that they could be transferred undisturbed for examination in an electron microscope.

A natural membrane dialysis system is provided by the chorioallantoic membrane of the embryonated chicken egg. This serous surface with its underlying reservoir of nutrient has long been employed for propagating colonies of a number of microorganisms. An ingenious and useful extension of this method has been made by implanting on the natural membrane a diffusion chamber with a cellulose-acetate filter membrane. Human leukocytes grow better in this environment than in vitro, reaching a maximum mitotic rate 72 hr after implantation (117), and the immune response elicited by other in vivo environments is avoided. The method also has been found valuable for growing a number of different types of embryonic tissues and for obtaining differentiation of neural tissue (12).

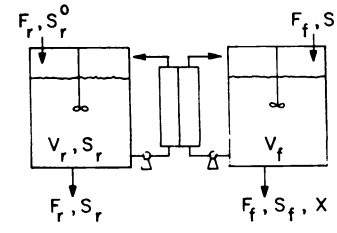
Although these techniques for obtaining colonial growth with dialysis have been very useful in specific circumstances, all of the following discussion will be directed toward the more unrestricted situations where the growing microbial populations are homogeneously suspended in dialyzed liquid culture.

Methods of Operation

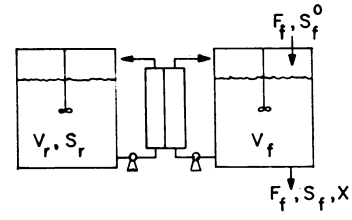
The classical method of growing microorganisms (e.g., a broth culture in a cotton-plugged test tube) may be said to be operated as an approximation of a closed system, in which all reactants, products, and energy are contained and insulated within a completely closed vessel. It is obvious that a microbial culture system only roughly



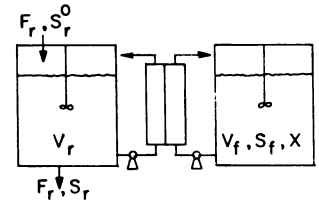
a) BATCH RESERVOIR AND FERMENTOR



b) CONTINUOUS RESERVOIR AND FERMENTOR



c) BATCH RESERVOIR, CONTINUOUS FERMENTOR



d) CONTINUOUS RESERVOIR, BATCH FERMENTOR

FIG. 8. *Four modes of operating a dialysis culture. Although a dialyzer-dialysis system is diagrammed, the principles are intrinsically applicable to any design. The symbol F is the flow rate, S is the substrate concentration, V is the volume, and X is the cell concentration into, within, and out from the reservoir and fermentor vessels. Reproduced, with revisions, from Gerhardt and Schultz (53).*

approximates this ideal, because gaseous nutrients and products are free to exchange with the atmosphere through the plug and also because heat is freely lost from the system. In common parlance, this is a batch culture. It is characterized by the familiar population growth cycle and a pattern of nutrient depletion and product accumulation.

Dialysis cultures usually have been operated as batch cultures, as illustrated diagrammatically in Fig. 8a. A fixed volume of medium is charged into the fermentor (V_f) and reservoir (V_r), with an initially fixed concentration of limiting substrate in each (S_f, S_r). Microorganisms are inoculated into the fermentor, also at an initially fixed concentration (X). The resulting growth cycle in dialysis culture appears similar to that seen in an ordinary batch culture, differing mainly in a prolongation of the exponential phase so that the maximal stationary phase occurs at a higher level (*see below*, section on In Vitro Systems)

It is now well known that microorganisms also can be grown in an open system, in an approximation of a steady state. In common parlance, this is a continuous culture. A constant concentration of organisms and a constant rate of growth occur when dynamic equilibrium is reached.

Dialysis culture also can be operated as a continuous culture, although the experimental trials have been limited (58, 59, 65; Gallup, Ph.D. Thesis, Univ. of Michigan, Ann Arbor, 1962). As illustrated in Fig. 8b, both the fermentor and the reservoir can be operated in steady state, but the option also exists to operate in a partially continuous mode with the fermentor in steady state and the reservoir not (Fig. 8c), or vice versa (Fig. 8d). Continuous operations are directly amenable to mathematical analysis. The equations for the batch situation can be handled best by computer simulation or by an approximating analytical approach. The above four possible modes of operating a dialyzer-dialysis fermentor system are treated in the section on Theory (*see below*, Growth in Dialysis Culture).

Types of Membranes

Three main types of membranes applicable to dialysis culture presently are available commercially. The type usually considered is ordinary dialysis membrane, exemplified by the tubing made of regenerated cellulose by the Visking process. The rated average pore size is in the order of 5 nm in diameter but varies with the size of tubing, which is available in various diameters. This type of membrane retains large molecules such as enzymes and toxins but permits the passage of small molecules such as sugars and salts. Wall thickness varies with size of the tubing, the percentage of void space differs, and the porosity changes with the degree of hydration. This type of membrane may be fabricated from collodion, parchment, cellophane, or a variety of plastics. A recently introduced type of membrane is described as anisotropic, but essentially is a very thin (and therefore efficient) film of ultramicroporous polymer cast on a supporting layer of coarsely porous material. This permselective film can be cast with pore sizes from 0.3 to 10 nm. It will be convenient to refer to such ultramicroporous membranes in general as *dialysis membranes*.

A second principal type of membrane is microporous. The rated mean pore diameter for microbiological use is in the order of 200 nm, but the available range of porosity extends down to about 25 nm. This type of membrane retains particles such as bacteria but permits the passage of most solutes, including macromolecules. These membranes are exemplified by several on the market made from cellulose-acetate, and conveniently may be called *filter membranes* (or membrane filters). Materials of construction may vary widely and recently have tended toward plastics such as polyvinyl alcohol. Asbestos, unglazed porcelain, and sintered metal or glass also have been used to fabricate membrane filters, as well as the more usual filter candles and funnels. The disadvantages for dialysis applications lies mainly in the relative thickness and coarse porosity of membranes made from such materials.

A third type of membrane has a more restricted applicability in dialysis culture, because only gases can penetrate it. This type, made from materials such as silicone rubber or Teflon, shows no evidence of porosity but instead behaves as a solvent in which dissolved molecules migrate by diffusion. Such membranes will be called *solution-transport membranes*.

Selection from the above membrane types is, of course, determined by those properties which best serve experimental requirements. In general, however, microbiological applications require that a membrane be capable of withholding organisms during the culture period, sterilizable by autoclaving, durable in construction, undigestible by bacterial enzymes, and efficient in the rate of solute diffusion. Although membrane porosity, void space, and thickness mainly govern diffusion rates, it is desirable to make direct comparisons with glucose or some other representative nutrient as a test or reference solute. Such a representative comparison of membranes has been published (51). Theoretical aspects of membrane characteristics, permeability measurements, po-

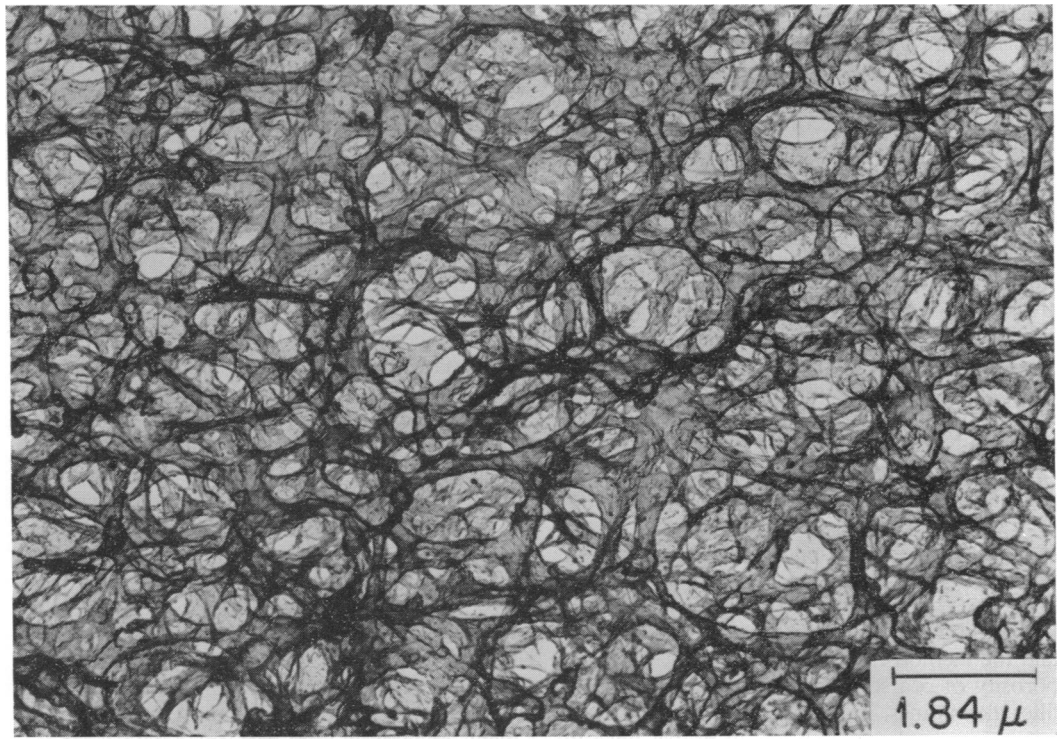


FIG. 9. *Electron micrograph showing the ultrastructure of a typical filter membrane. Reproduced with permission of Gelman Instrument Co., Ann Arbor, Mich.*

rosity, and hydrodynamics are presented later in this article (*see* Principles of Dialysis).

Membrane Permeability and Solute Sieving

In both dialysis and filter membranes, the mechanisms for permeation of solutes are related to membrane ultrastructure. Both are made from essentially linear polymers, the macromolecular fibrils of which are cross-linked by chemical bonds to form a tertiary heteroporous matrix. Considerable study has been made of membrane ultrastructure by means of electron microscopy (18, 91). A dimensional representation of this ultrastructure is depicted in Fig. 9. It is important to remember the spongelike or foamlike nature of porous membranes, because quite a different image often is conjured, e.g., something like a perforated sheet. Our model embodies openings or pores of various sizes distributed randomly in the membrane and creating a tortuous passageway for penetration. It would be desirable, of course, if the pores were uniform in size, but such isoporous membranes are not known to be available except in much coarser porosity, as with screens or fabrics, or in much finer porosity, as with crystal lattices.

Since the openings of membranes usually are filled with water, small solute molecules can either diffuse through the passageway or be carried through by net flow of water. This latter mechanism of transport in dialysis may not be as commonly recognized as diffusion, but it accounts for a substantial part of the total, especially if pressure differentials occur.

In solution-transport membranes, a completely different mechanism of permeability prevails. The membrane is not a gel with water-filled openings, but is instead a sol. The solute penetrates, therefore, by dissolving in the substance of the membrane, passing through by diffusion, and then leaving on the other side. The solute can enter or leave the membrane either from the gaseous state or liquid solution.

The withholding or sieving of molecules is related to solubilities in the case of solution-transport membranes and to maximum pore size in the other two types of membrane. Although electrostatic charge and other factors have an influence, it is mainly the largest passageway in the dialysis or filter membrane which determines the maximum size of molecule or particle that can penetrate. Molecules large enough to be withheld

by a dialysis membrane usually will be polymers, typically linear ones. Such long macromolecules tend to coil or agglomerate in solution and behave as spheres or rods; their equivalent diffusion diameter can be determined by the Einstein-Stokes formulation.

Membrane Penetrability by Microorganisms

It is obvious that special precaution must be exercised against the possibility of penetration of microorganisms through rents or holes in defective membranes. Each piece of membrane to be used can be examined for pinpoint leaks by flooding the membrane with alcohol while it rests on a dark blotter, or by flooding the membrane with water while it lies on an indicator mixture of 98% sugar and 2% crystal violet (63). Small leaks also can be detected by bubble-testing in a device arranged so that above one side is water and under the other is an air pressure chamber; if the membrane is adequately supported with a flat screen or a sintered plate, a pressure of 15 psi will disclose flaws. At high pressures, a relationship exists between bubble-threshold pressure and porosity, and the method can be employed to calibrate the porosity of coarsely porous membranes, such as filter membranes.

Given a flawless membrane of proper porosity, it is generally assumed that cellular microorganisms such as bacteria cannot penetrate it. The ability to filter bacteria is dependent not only on a pore structure smaller in size than the organisms (usually less than about 0.2 μm) but also on the use of a pressure differential. This causes the organism to become impinged mainly on the surface of the filter, but also in the tortuous passageways. The mechanism by which bacteria are filtered has been examined by electron microscopy of bacteria-clogged filter membranes (101).

The filtration process is not absolute, however. It is common knowledge among those who employ filter membranes for sterilizing biologicals that bacteria can be forced through the barrier if the filtration time is prolonged. It also has been discovered that deformable organisms such as mycoplasma will pass through a filter membrane directly in proportion to the pressure applied (Morowitz, *unpublished results*).

In dialysis culture (and in many other situations in which membranes are employed in microbiology), the critical factor of pressure-induced impingement is not present, and consequently much greater concern must be given to the possibility of penetrability of the unclogged membrane by microorganisms. This seemingly has not been a practical limitation in earlier systems, but in a differential dialysis flask system it was discovered that a bacterium such as *Staphylococcus aureus* apparently can penetrate a filter membrane with pores far smaller than the size of the cells (67). Besides the absence of a pressure differential, the situation was distinctive in the requirement for an extended incubation period and perhaps also in the membrane supporting structure, which could trap organisms in contact with the membrane. This occurrence prompted a literature search for other experimental evidence that organisms can penetrate membranes with pores seemingly much too small.

The phenomenon of apparent dialyzability of bacteria was traced, with some diligence, first to a Letter to the Editor in the *Journal of the Indonesian Medical Association* by Gan (45), and thence to a full report with experimental results (46). In these accounts, Gan told of controlled experiments in which some 21 different bacteria and common yeast were found to be capable of penetrating intact dialysis (Visking) tubing This astonishing observation, if verified, would have basic implications not only for dialysis culture but also for a number of tenets in microbiology. Consequently, Gan's findings will be summarized in some detail and his method will be scrutinized.

It should at once be recognized that the experiments apparently were performed with great care and with anticipation of incredulity (criticism was obtained from two eminent biophysicists before publication of the results). The test apparatus was crude but suitable: hydrated dialysis tubing was inverted on itself, forming a double-walled cylindrical sac which was hung in the bulb of a Smith fermentation tube (Fig. 10). The test organism and medium were inserted into the membrane sac, and the dialyzing fluid was placed in the tube proper. Appropriate precautions apparently were taken to prepare, assemble, sterilize, inoculate, and sample in the experiments.

The demonstration experiments were conducted with a good representation of organisms: gram-positive and gram-negative, motile and nonmotile, large and small, and morphologically different. At least 2, and on occasion as many as 23, replications were made with a given organism. With dialysis into distilled water, penetration of different organisms was observed within 5 days with 11 to 100% consistency. The presence of organisms in the dialysate was determined by inoculating a 0.1-ml sample onto an agar medium.

Most importantly, control experiments were conducted with protein molecules and virus particles. Hemoglobin, vaccinia virus, or bacteriophage, present in the dialysis sac together with a test bacterium, did not penetrate, whereas the organism did. Moreover, dialysis tubing did

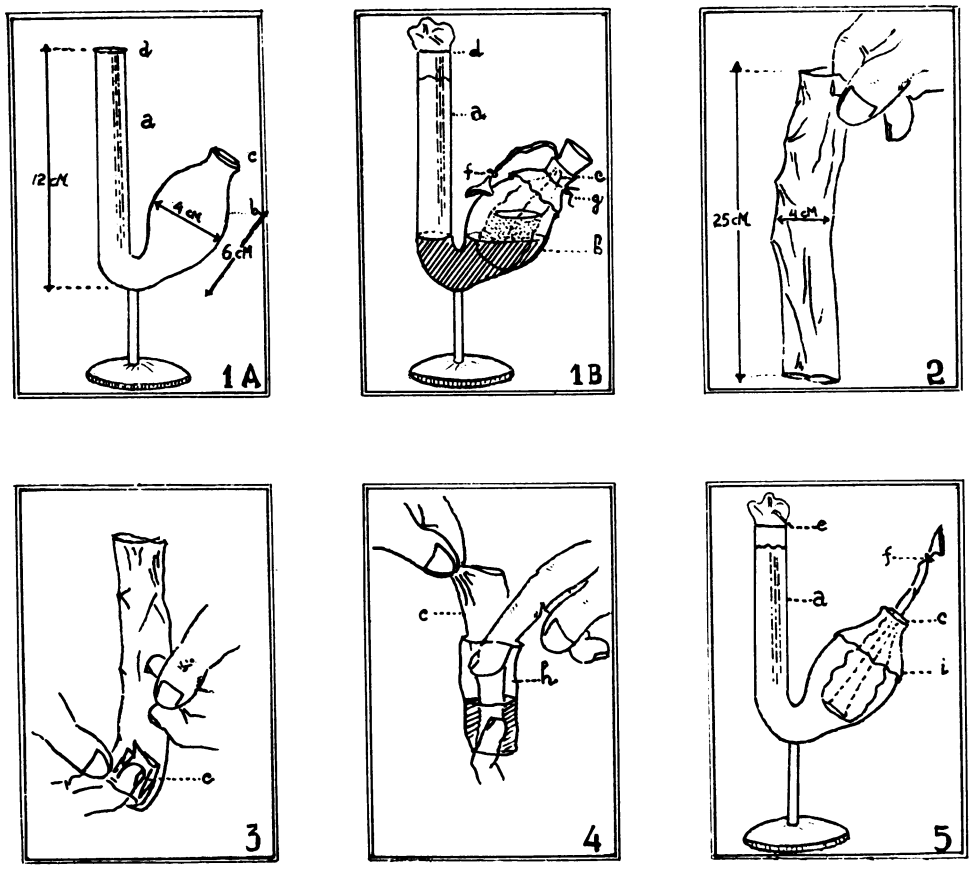


FIG. 10. *Apparatus used to test the penetration of dialysis membrane by bacteria. Reproduced from Gan (45).*

not become permeable to hemoglobin after exposure to six test bacteria, singly or together, for 15 days. One might criticize these experiments on the basis that one bacterium penetrating the membrane would multiply and so would be observed, whereas one penetrating virus might not be included in the sample transferred to a plaque test and so might escape observation.

Several variables were examined by Gan, including the culture age, the quantity and type of diluting medium used with the test cells, and the *p*H and type of the dialyzing fluid. In retrospect, the last variable is believed especially critical. Distilled water and phosphate solution, in about three times the volume of the culture medium, were found to be the most effective in causing cell penetration of the membrane. Unfortunately, the control situation was apparently not included in which the medium employed with the cells was also used as the dialyzing fluid.

We have come tentatively to accept Gan's conclusions that several kinds of bacteria in fact have the ability to penetrate the pores of dialysis tubing under certain experimental conditions and that the results are not attributable to leakage. We further accept his admonition that the findings require confirmation by other workers.

Two later and entirely independent studies on permeability of membranes to bacteria were found in the literature on food technology. Hartman, Powers, and Pratt (63) reported that cellulose-acetate or polyethylene membranes seemed permeable to bacteria but cellophane membranes did not. They felt it necessary, however, to use unsterilized membranes and therefore had to resort to a statistical estimate of the probability of penetration after test sacs were incubated for 72 hr. Furthermore, a considerable number of pinhole leaks were detected after the growth tests were completed. The cellophane bags were perforated more often than any of the others. This observation deserves attention in evaluating Gan's

results (*above*), although his use of protein and virus as controls would seem to preclude the possibility.

Ronsivalli et al. (140) subsequently also investigated food-packaging films and concluded that all were impermeable to bacteria. However, their actual data seem inconsistent with their conclusions, e.g., two of six defect-free polyethylene sacs were indicated to have allowed bacteria to pass into and grow in the surrounding medium.

If the dialyzability of bacteria is tentatively taken as fact, the mechanism of passage is immediately in question. Gan (46) interpreted his findings to indicate that the transfer of the bacterial multiplying activity through the membrane must occur by units smaller than the normal bacterial cell. In further studies, Gan (47) employed *Shigella sonnei* at low temperature in an attempt to exclude active multiplication, and alleged that 3-hr "dialysates" (diffusates) contained submicroscopic viable units different in nature from the parent test bacteria. These particles were purported to be filterable through a collodion filter and not to be sedimented after centrifugation for 2 hr at 16,000 to 20,000 rev/min. Morphological examination was said to reveal the presence, after incubation of the diffusate for 1 hr, of minute (0.20 to 0.35 μm) undifferentiated granules; after 3 hr of incubation, these increased in diameter to 1 to 2 μm and became pleomorphic; after 4 hr, typical bacterial growth occurred. Obviously, critical confirmation is needed.

An alternative mechanism also might account for bacterial dialyzability, namely, an infiltrating growth process by diapedesis through the openings of the membrane. Capalbo et al. (19) found that mouse peritoneal cells were excluded from implanted diffusion chambers only if the filter membranes were 0.1 μm or less in average porosity, which is far smaller than the size of the cells. Sectioned and stained membranes of coarser porosity were observed to have cell processes penetrating them. Keller and Sorkin (78) further noted that the presence of a chemotactic agent enhanced the migration of such cells through a filtering barrier. This could explain the observed growth of staphylococcal L forms through filter membranes of a pore size as small as 0.05 μm when placed on agar medium (100). In the situations in which Gan (46) reported membrane penetration by a number of bacteria, the cells were dialyzed against distilled water or phosphate solution. The ionic and osmotic gradients thus established across the membrane conceivably could have caused plasmoptysis of the bacteria, with resulting formation of a spheroplast or bleb. These plastic forms conceivably could have traversed the membrane and generated normal cells on the other side.

Although the occurrence and mechanism of the reported penetration of membranes by bacteria needs critical confirming study, the likelihood of such an occurrence clearly must be taken into account in the practice of dialysis culture and in other comparable situations in microbiology.

THEORY

Principles of Dialysis

Membrane characteristics. Even if the microgeometry of all the tortuous paths through a membrane were known exactly, it still would be impossible to predict precisely the excursion of molecules through the membrane. Therefore, a semiempirical approach, based on Fick's law of diffusion, has been adapted to characterize membrane permeability. Fick's law states that the rate of diffusion through a uniform film of liquid is related to the concentration gradient across the film and to other parameters as shown below:

$$N = \frac{D^\circ A}{L_p} p\Delta S \qquad (1)$$

where N is the rate of permeation (g/hr), D° is the diffusivity of the solute in the liquid (cm^2/hr), A_p is that part of the total membrane area (i.e., pore area) available for diffusion (cm^2), L_p is the length of the diffusion path in the pores (cm), and ΔS is the concentration difference across the film (g/cm^3).

A dialysis membrane may be regarded as a thin film of liquid retained within the porous matrix of membrane material, but usually the diffusivity of solute within the membrane and the distributions of pore areas and lengths are not known. Therefore, these unknown factors are combined to give an empirical factor, the permeability coefficient (P_m), which must be experimentally determined for each membrane and solute.

Equation 1 is rewritten (160) as:

$$N = P_m A_m \Delta S \qquad (2)$$

which defines P_m as

$$P_m = \frac{N}{A_m \Delta S} \qquad (3)$$

where A_m is the total area of the membrane (which is larger than A_p, defined above), and the permeability coefficient (P_m) has the units cm/hr.

Equation 2 states that *the rate of molecular diffusion through an inert porous membrane is directly related to the permeability coefficient, membrane area, and concentration difference across the membrane.*

Measurement of permeability. A method for measuring the overall permeability coefficient of a membrane for a particular compound under given hydrodynamic conditions is suggested by equation 3. Experimentally, one can separate two chambers with the membrane, and then measure the rate at which the compound diffuses from one side to the other and the concentration of the compound on each side of the membrane. If two well-mixed closed chambers are used, only one concentration needs to be measured as a function of time. The following formula gives the relationship of concentration and time to permeability (94):

$$\ln\left[\frac{S_1\left(\frac{1}{V_1}+\frac{1}{V_2}\right)-\left(\frac{S_1^\circ}{V_2}+\frac{S_2^\circ}{V_1}\right)}{\left(\frac{S_1^\circ - S_2^\circ}{V_1}\right)}\right] = -\left(\frac{1}{V_1}+\frac{1}{V_2}\right) P_m A_m t \quad (4)$$

where S_1 is the concentration in chamber 1 at any time, S_1° and S_2° are the initial concentrations in chambers 1 and 2, and V_1 and V_2 are the volumes of chambers 1 and 2.

If the concentration in one of the chambers is initially zero ($S_1^\circ = 0$), the left-hand side of equation 4 is simplified so that

$$\ln\left(1-\frac{S_1}{S_e}\right) = -\left(\frac{1}{V_1}+\frac{1}{V_2}\right) P_m A_m t \quad (5)$$

Where S_e is the final equilibrium concentration in both chambers,

$$S_e = S_2^\circ V_2/(V_1 + V_2) \quad (6)$$

The time to reach half of the equilibrium concentration ($t = ET_{50}$) is sometimes used (27, 51). This number is related to the permeability coefficient by the following formula, when $S_1 = S_e/2$:

$$\ln\left(1-\frac{S_e/2}{S_e}\right) = \ln \tfrac{1}{2} = -\left(\frac{1}{V_1}+\frac{1}{V_2}\right) P_m A_m ET_{50} \quad (7)$$

Rearranging, we find

$$ET_{50} = \frac{\ln 2}{\left(\frac{1}{V_1}+\frac{1}{V_2}\right) P_m A_m} \quad (8)$$

Thus, the half-equilibration time is dependent on the chamber volume and the membrane area. Therefore, it has limited usefulness in characterizing membrane permeability. For example, Craig (27) and Gerhardt and Gallup (51) give ET_{50} values of 3.5 and 2.5 hr, respectively, for glucose through Visking dialysis membranes. On the basis of these data, it would seem that there was not much difference in the permeability of glucose through these two membranes. But if one calculates P_m for each membrane by equation 8, P_m for Gerhardt and Gallup's membrane was 9.69 cm/hr, whereas P_m for Craig's membrane was 0.23×10^{-2} cm/hr; i.e., they in fact differed by a factor of 300. This result is not surprising, because Craig purposely treated his membranes to reduce their pore size and so to separate glucose and sucrose by dialysis, but the effect of this treatment on membrane permeability is not apparent from the reported ET_{50} data.

Porosity. There are several methods for estimating the effective pore size of a membrane. These methods are based on resistance to liquid flow, capillary surface tension, void content, and diameter of diffusing molecules (118, 135). None of these methods is entirely satisfactory, but, from the work that has been done in this field, it is certain that the passage of molecules through a membrane is impeded as the size of the molecule approaches the dimensions of the pore opening.

For example, Manegold (94) showed that the permeation of sucrose through a series of membranes increases as the average pore size of the membrane increases. Some of Manegold's data are replotted in Fig. 11. It can be seen that the relative permeability of membranes with the smallest pores is about 40% of the maximum. Experiments by Spandau and Gross (151) showed that the converse is also true; that is, the perme-

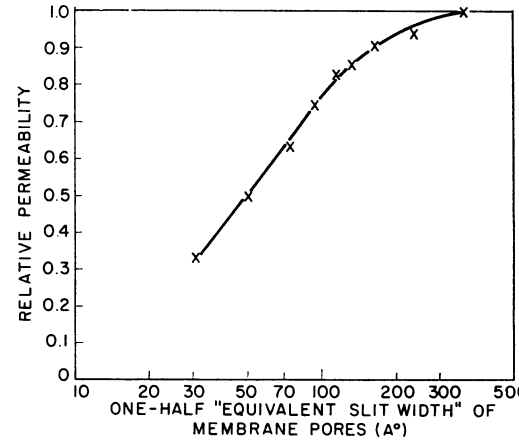


FIG. 11. *Permeation of sucrose through a series of membranes graded in porosity. Data from Manegold (94), replotted.*

ability of a given membrane to a series of increasingly larger molecules decreases more rapidly than does the diffusivity of the molecules in solvent alone.

Renkin (135) and Craig (27) have corroborated this effect. Thus, for a given membrane, P_m decreases with increasing molecular size. If a mixture of compounds is dialyzed, the individual components will diffuse through the membrane at different rates, even with the same concentration driving force across the membrane. Craig (27) developed this principle for the separation of small protein fragments, and Siggia et al. (149) used it to measure the relative amounts of sugars or amino acids in a mixture.

The various explanations that have been proposed to account for the effect shown in Fig. 11 are all related to pore size and membrane structure. Three main explantations exist: restricted diffusion of a molecule, decreased effective open area, or increased length of the diffusion path. All three properties can be affected when the pore size approaches molecular dimensions.

It has been proposed in the first case that diffusion is hindered when a molecule diffuses through a capillary pore, an effect similar to the reduction in sedimentation velocity of particles in small tubes. Correlations for the wall effect on sedimentation have been used for predicting the effect of pore size on hindered diffusion (82, 118). Two such correlations, suggested by Faxén (34) and Francis (40), are

$$f = \frac{D_m}{D^\circ} = 1 - 2.104\left(\frac{r}{R}\right) + 2.09\left(\frac{r}{R}\right)^3 - 0.95\left(\frac{r}{R}\right)^5 \quad (9)$$

and

$$f = \frac{D_m}{D^\circ} = \left(1 - \frac{r}{R}\right)^{2.5} \quad (10)$$

where f is the ratio of the permeation rate of a substance through the membrane to the permeation rate through an equivalent thickness of pure solvent, D_m is the effective diffusivity of the substance in the membrane, r is the molecular radius of the substance, and R is the effective radius of the pore in the membrane.

A second possibility is that the effective open area available for diffusion decreases with increasing molecular size because of the low probability that an incident molecule will actually penetrate the pore. Ferry (35) suggested that the effective available area for diffusion is related to the true open area by the following formula:

$$f = \frac{A_e}{A_p} = \left(1 - \frac{r}{R}\right)^2 \quad (11)$$

where A_e is the effective open area of the pore and A_p is the true open area of the pore. Note the resemblance of this equation to the empirical formula given in equation 10.

The two previous explanations are based on an isoporous membrane model. However, a membrane is made up of many interconnected passageways of various sizes. All of the holes would be effectively open for very small molecules, but for large molecules the number of suitable passageways would be reduced, and the average distance a large molecule would have to travel to get through the membrane would be longer.

In other words, for larger molecules the effective open area of the pores will be smaller and also the effective diffusion path will be longer. This can be expressed as

$$f = \frac{\left(1 - \frac{r}{R}\right)}{\left(1 + \frac{nr}{R}\right)} \quad (12)$$

where the numerator represents the decrease in open area, the denominator represents the increase in diffusion path for decreasing pore size, and n is an empirical constant which is found by curve fitting.

Equations 9–12 are plotted in Fig. 12, and they all exhibit the same form shown in Fig. 11. Each

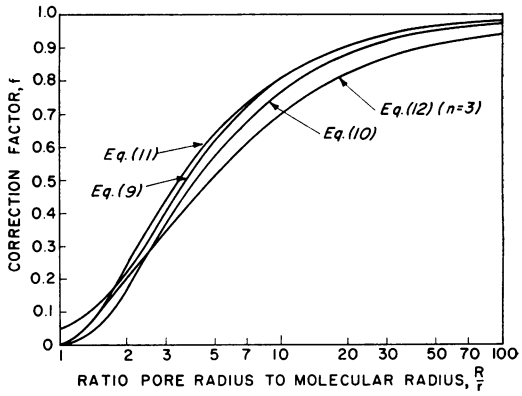


FIG. 12. *Calculated models for the effect of membrane pore size on permeability. The correction factor, f, is the ratio of the actual diffusion rate through the membrane to the diffusion rate (by Fick's law) calculated for a film of solvent having equal geometric thickness and open area.*

TABLE 1. *Calculated effect of membrane pore size on the separation of molecules A and B, where the molecular size of B is twice as large as that of A*

Radius of pore/radius of A	Radius of pore/radius of B	Ratio of permeation rates, f_A/f_B[a]	Relative permeation rate of A, f_A[a,b]
100	50	1.03	0.98
20	10	1.14	0.88
10	5	1.35	0.77
5	2.5	2.04	0.57
3.3	1.67	4.50	0.41
2.5	1.25	15.5	0.28

[a] Calculated from equation 10.

[b] Relative to an equivalent thickness for solvent alone.

of the above equations for the correction factor f, or combinations of them, can be used to correlate experimental data on membrane coefficients (P_m) for various substances relative to the diffusion rates of the same substance in solvent alone. They should, therefore, be looked upon as semiempirical rather than fundamental formulations.

There are several interesting consequences of these correlations insofar as dialysis culture is concerned. One of the potential advantages of dialysis culture is the differential segregation of molecules of different sizes (51). It can be seen from Fig. 11 and 12, however, that an effective separation of molecules of similar size by a single membrane is not practical. For example, consider two molecules A and B where the diameter of B is twice as large as that of A. The relative diffusion rates of these two molecules are a function of pore size, as shown in Table 1. To achieve an appreciable difference in the rates of penetration of A and B through a membrane (column 3), a membrane has to be selected with a mean pore size about five times the molecular diameter of A. However, as shown in column 4, this separation is obtained at the expense of a considerable reduction in the penetration rate of A. If A were glucose (molecular weight, 180), then B would correspond to a compound with a molecular weight of about 1,440 (assuming the molecular diameter is proportional to the cube root of molecular weight).

In addition to molecular size, there may be other factors which influence the passage of molecules through membranes. Ryle (143) showed that polyethylene glycol ($\overline{M}_n$ about 15,000) passed through dialysis tubing which retained hemoglobin. However, this polymer appeared at the same holdup volume as hemoglobin when passed through a gel chromatography column (Sephadex G-100). Since both dialysis and gel filtration depend on the principle of hindered diffusion, there must be some interaction between the diffusing molecules and the porous media.

From these considerations, it is apparent that *the use of a single membrane to separate microbial products produced in dialysis culture will be practical only if there are large differences in the molecular sizes of the products.*

The use of membranes with small pores, relative to the molecular size of the nutrients in the reservoir, may result in different permeation rates into the culture chamber and a corresponding imbalance in the dialyzed medium. *Furthermore, if the prime purpose of the membrane is to separate the microbial cells from the nutrient supply, then the durability of the membrane is probably a more important consideration than the pore size, as long as the pore size is more than about 15 times the diameter of the largest nutrient molecule.* From Fig. 12, it is seen that there is little difference in diffusion rate if the pore size is between 15 and 100 times the molecular diameter.

Hydrodynamics. The rate of dialysis is strongly dependent on the liquid velocity at the membrane surface. A typical experimental curve, for the dialysis of sucrose (54), is shown in Fig. 13. This behavior is rationalized by postulating the existence of a thin zone of nonturbulent liquid (Nernst film) in the immediate vicinity of the membrane surface, as pictured in Fig. 14. The thickness of this liquid film decreases as the bulk liquid velocity near the membrane increases. As far as the permeant molecule is concerned, there

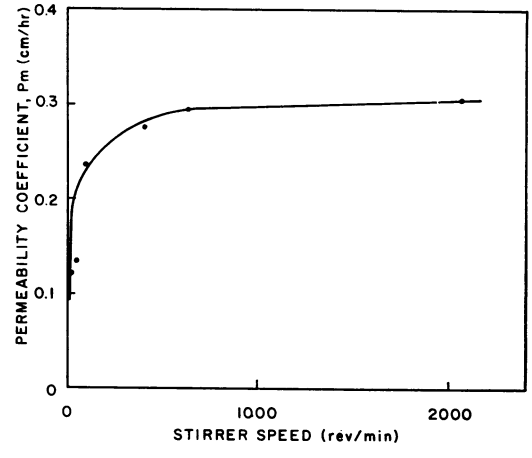


FIG. 13. *Effect of liquid agitation on membrane permeability to sucrose. Data from Ginzburg and Katchalsky (54), replotted.*

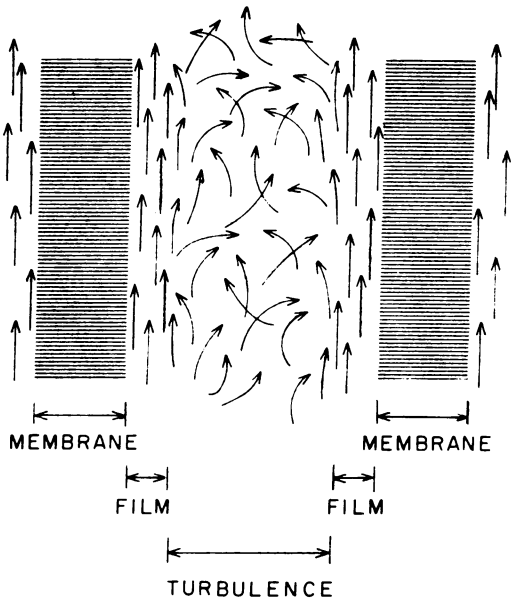


FIG. 14. *Representation of fluid flow past the opposing faces of two membranes, with a laminar film immediately along each surface and turbulent bulk flow in the central channel.*

are effectively three barriers that must be penetrated: a liquid film on each side of the dialysis membrane, and the dialysis membrane itself.

The permeability coefficient, as calculated by equation 3, reflects the overall resistance of the composite barrier. It can be shown that the overall permeability coefficient is related to the true membrane permeability coefficient ($P_m°$) by the following equation

$$\frac{1}{P_m} = \frac{1}{P_1} + \frac{1}{P_m°} + \frac{1}{P_2} \qquad (13)$$

where

$$P_1 = \frac{D°}{\delta_1}, \qquad P_2 = \frac{D°}{\delta_2} \qquad (14)$$

$D°$ is the diffusivity of the substance in water, and δ is the thickness of the unstirred liquid film.

As the bulk liquid velocity near the membrane increases, the liquid film decreases (δ_1 and δ_2 become smaller) and P_m approaches $P_m°$ (the true permeability of the membrane). It can be seen in Fig. 13 that *the overall permeability coefficient, P_m, can be increased by a factor of two or three by vigorous stirring or high flow rates near the membrane. A point of diminishing returns is reached insofar as the value of providing extremely high turbulence near the membrane*, however, because the added resistance of the liquid films is probably never completely eliminated. Ginzburg and Katchalsky (54) estimated that a limiting film thickness of 25 nm remains, even at extreme stirring rates. These authors also stated that the relative importance of the liquid film decreases as larger permeant molecules are considered because $P_m°$ is more sensitive to molecular size than P_1 and P_2.

Osmosis. Whenever a permeable membrane is interposed between different solutions, there is a possibility of water transport across the membrane by osmosis. In dialysis culture, concentration gradients of nutrients and products across the dialysis membrane will occur and therefore cause some movement of water either into or out of the culture chamber. Water will tend to move toward the region of higher osmotic pressure. The rate of water flow through a membrane is given by equation 15 (77, 152):

$$Q = K_f(\Delta P - RT \sum_i \sigma_i \Delta S_i) \qquad (15)$$

where Q is the filtration rate of water through the membrane (cm³/hr cm² of membrane area), K_f is the filtration coefficient for the membrane (cm³/hr atm cm²), ΔP is the hydrostatic pressure across the membrane, R is the gas constant [82.05/cm³ atm/(g mole °K)], T is the absolute temperature (°K), ΔS_i is the concentration gradient of a solute across the membrane (g mole/cm³), and σ_i is the reflection coefficient for the solute.

Usually, dialysis cultures are carried out under conditions where there is no net external pressure across the membrane (i.e., $\Delta P = 0$), and then equation 15 becomes

$$Q = -K_f RT \sum_i \sigma_i \Delta S_i \qquad (16)$$

The reflection coefficient takes into account the fact that membranes are not ideally semipermeable, and that lower osmotic pressures are exerted by freely diffusable solutes. For nonpermeant molecules, $\sigma = 1$. For permeable substances, σ is less than one and approaches zero for freely permeating molecules. Ginzburg and Katchalsky (54) give values for σ with 0.1 M sucrose, 0.1 M glucose, and 0.15 M urea solutions, respectively, of 0.114, 0.072, and 0.013 with a Visking dialysis membrane.

A sample calculation of the magnitude of water flow one might obtain in dialysis culture is instructive. The filtration coefficient (K_f) for dialysis tubing is about 6×10^{-2} cm³/atm hr (54, 135). If a glucose gradient of about 0.1 M is maintained between the reservoir and culture cham-

bers, then, from equation 16

$$Q = 6 \times 10^{-2} \frac{\text{cm}^3}{\text{atm hr cm}^2} \times 82.05 \frac{\text{cm}^3 \text{ atm}}{\text{g mole } ^\circ\text{K}} \times 298 \times 0.072 \times 0.1 \times 10^{-3} \frac{\text{g mole}}{\text{cm}^3} = 1.06 \times 10^{-2} \frac{\text{cm}^3}{\text{hr cm}^2} \quad (17)$$

If the membrane area were 100 cm², and the duration 50 hr, then the total volume of water leaving the culture chamber would be $1.06 \times 10^{-2} \times 100 \times 50 = 50$ ml. This amount of fluid would make quite a difference if the culture chamber contained 100 to 250 ml initially. Actually, large osmotic transfers of water are not found in practice, probably because metabolic products in the culture chamber tend to minimize the osmotic pressure gradient and also because it is common practice to place saline in the culture chamber initially to counterbalance the osmotic pressure of the reservoir chamber. However, transfers of water may occur if disparate amounts of protein in the nutrient reservoir exert a Donnan effect or if positive pressure from aeration in the culture chamber causes mass solvent flow with ultrafiltration.

Growth in Dialysis Culture

Basic considerations. A valid approach toward quantitatively characterizing the proliferation of microorganisms requires that only one factor at a time be considered, with the others invariant. This requirement can be approximated with a culture in which a metabolic steady state is maintained by means of an open system, i.e., in a continuous culture. Although this technique dates far back in the art of fermentation, it was not until 1942 that Monod (102) introduced the important modification of limiting the rate of growth and metabolism by regulating the concentration of a single required nutrient. A finite relationship exists between nutrient concentration and growth rate of the organism, and also between nutrient utilization and the amount of cell substance produced. Consequently, growth can be characterized in simple mathematical expressions (103, 111):

$$r_g = \mu X \quad (18)$$

$$\mu = \left(\frac{S_f}{K_s + S_f}\right) \mu_m \quad (19)$$

where r_g is the rate of increase of cells due to multiplication of the organism, (g/cm³ hr). We use the symbol r_g instead of the more conventional dX/dt to emphasize the fact that equation 18 is a kinetic rate expression which reduces to dX/dt only for the specific case of a completely batch system. S_f is the concentration of substrate, X is the concentration of cells (g/cm³), μ is the specific growth rate constant (hr^{-1}), μ_m is the maximum growth rate constant (hr^{-1}), and K_s is a proportionality factor for the particular limiting substrate.

A number of other mathematical models for growth have been derived from this concept (26, 42, 102, 105, 129, 131, 141, 156). As will be shown below, many of our conclusions are independent of the growth model chosen.

Although the limiting-nutrient concept is particularly adaptable to continuous culture, it also is exemplified in a number of other situations. In ordinary batch culture, unless all the nutrients required by an organism are supplied in exactly the right proportions, a condition is soon reached where one of the nutrients begins to limit growth and metabolism. With aerobic bacteria in a test tube, for example, the supply of oxygen rapidly becomes depleted and growth becomes limited by the relatively slow diffusion of air through the tube closure and dissolution in the medium. In some instances, different nutrients may successively become the limiting growth factor. An example is the familiar diauxie phenomenon, exhibited by yeast grown in a medium containing both glucose and maltose (102): first, the cells preferentially utilize glucose until it is depleted, and then a second growth cycle results after maltose-degrading enzymes are induced.

The limiting-nutrient concept is the common feature that allows one to relate dialysis kinetics to microbial growth kinetics, since dialysis is simply another method by which nutrient can be fed to a culture. The basic equations for molecular diffusion through inert porous membranes can be combined with the mathematical models that describe cell proliferation as a function of nutrient concentration, first for continuous (steady state) culture and then for batch (nonsteady state) culture. The resulting equations prove difficult to solve explicitly but can, however, be solved easily with an analogue computer (*see below*).

To relate dialysis kinetics to growth kinetics, one has to establish not only the relationship between nutrient concentration and growth rate (equation 19) but also the relationship between nutrient utilization and cell production. A useful model was proposed by Marr et al. (95) and subsequently used by others (2, 133):

$$-r_s = \frac{r_g}{Y_x} + Y_E X \quad (20)$$

where $-r_s$ is the rate of substrate utilization due to growth and metabolism, r_g is the rate of growth of the organism, and Y_x and Y_E are empirical rate constants. The first term on the right of equation 19 represents the portion of the substrate that is used in cell growth, and Y_x is the yield coefficient. The second term is the portion that is used by the organism to maintain itself, and Y_E is called the "specific maintenance rate" (95). If the first term is zero, with no cell growth, then the second term is equivalent to the substrate utilized in maintenance metabolism. The maintenance requirement becomes significant at low growth rates or high cell densities.

An idealized diagram of a dialysis culture system operated in the four possible ways is shown in Fig. 8. The four basic modes correspond to the following: (a) batch reservoir and fermentor, where F_r and $F_f = 0$; (b) continuous reservoir and fermentor, where F_r and $F_f > 0$; (c) batch reservoir and continuous fermentor, where $F_r = 0$ and $F_f > 0$; and (d) continuous reservoir and batch fermentor, where $F_r > 0$ and $F_f = 0$.

The mathematical analysis consists of making suitable material balances on both chambers for the generalized situation. First, *a balance on the limiting substrate in the reservoir chamber* results in the following equation:

$$\text{In} + \text{Production} = \text{Out} + \text{Accumulation}$$

$$F_r S_r^\circ + 0 = F_r S_r + P_m A_m (S_r - S_f) + V_r \frac{dS_r}{dt} \quad (21)$$

where S_r° is the concentration of substrate in the reservoir influent, S_r is the concentration of substrate in the reservoir effluent, P_m is the permeability of the membrane to substrate, A_m is the area of the membrane, and V_r is the volume of the reservoir. $F_r S_r^\circ$ and $F_r S_r$ represent bulk flow of substrate into and out of the reservoir, and $P_m A_m (S_r - S_f)$ represents the removal of substrate by dialysis. In these derivations, it is assumed that circulation through the dialyzer is rapid enough so that the same concentrations exist in the dialyzer as in the chambers.

A similar *balance on the limiting substrate in the fermentor chamber* can be established:

$$F_f S_f^\circ + P_m A_m (S_r - S_f) + V_f r_s = F_f S_f + V_f \frac{dS_f}{dt} \quad (22)$$

where S_f° is the concentration of substrate in the fermentor influent and V_f is the volume of the fermentor.

Third, *a balance on cells in the fermentor chamber*, when cells enter in the feed stream at a concentration, X°, and no transport of cells across the dialysis membrane occurs, results in the following equation:

$$F_f X^\circ + V_f r_g = F_f X + V_f \frac{dX}{dt} \quad (23)$$

In the usual situation, no cells are present in the feed stream and $F_f X^\circ$ reduces to zero. Cells may be present in the feed stream, however, if the fermentor is part of a multistage cascade or if cells are recycled in a single-stage unit.

These three material balance equations plus model equations 18, 19, and 20 are mathematically sufficient to determine cell and substrate concentrations in dialysis culture.

Completely continuous operation. The subject of ordinary continuous culture has been thoroughly reviewed by several authors (50, 70, 92, 93, 97, 150). A simplified theoretical analysis of continuous dialysis culture was attempted by Gori (58), but the specific effects of membrane area and membrane permeability were not taken into account.

Although this is the most difficult operation to achieve in practice, it is the simplest type of dialysis culture to solve mathematically, since a steady state can be assumed to occur in both chambers. In steady state continuous culture, the time derivatives dS_r/dt (equation 21), dS_f/dt (equation 22) and dX/dt (equation 23) are all equal to zero.

From equation 23, the growth rate can be related to the flow through the fermentor and cell concentration in the fermentor,

$$r_g = \frac{F_f}{V_f} (X - X^\circ) = D(1 - R_x)X \quad (24)$$

where $D(\text{hr}^{-1})$ is the dilution rate defined as the flow rate into the fermentor divided by the fermentor volume, and R_x is the ratio of the cell concentration in the feed to that in the outlet ($R_x = X^\circ/X$). When no cells are present in the fermentor feed stream, $R_x = 0$.

Similarly, from equations 20 and 24 the substrate utilization rate can be related to the flow rate and cell concentration,

$$r_s = -\frac{(1 - R_x)}{Y_x} \frac{F_f}{V_f} - Y_E X \quad (25)$$

This value for r_s can be substituted into equation 22 so as to obtain,

$$F_f S_f^\circ + P_m A_m (S_r - S_f) - V_f X \left[\frac{(1 - R_x)}{Y_x} \frac{F_f}{V_f} + Y_E \right] = F_f S_f \quad (26)$$

Also, equation 21 can be solved for S_r in terms of S_f, and the results substituted into equation 26. The following equation is obtained for the cell concentration in terms of the operating parameters and S_f, the substrate concentration in the fermentor:

$$X = \frac{\left[\dfrac{S_r^\circ - S_f}{\dfrac{1}{P_m A_m} + \dfrac{1}{F_r}} + F_f(S_f^\circ - S_f)\right]}{\left[\dfrac{F_f(1 - R_x)}{Y_x} + Y_E V_f\right]} \quad (27)$$

Note that equation 27 was derived without recourse to the growth models given in equations 18 and 19. The only assumptions in its development are those corresponding to the substrate-utilization model, equation 20. To use this equation in a predictive fashion, S_f must be related to the operating parameters.

Now, from equations 24 and 18,

$$\mu = \frac{F_f}{V_f}(1 - R_x) = D(1 - R_x) \quad (28)$$

which shows that the specific growth rate constant, μ, is numerically equal to the dilution rate when no cells are present in the feed stream (i.e., $R_x = 0$).

The fermentor substrate concentration is determined by combining equation 28 and the growth model equation 19:

$$D(1 - R_x) = \mu_m \frac{S_f}{K_s + S_f} \quad (29)$$

Rearranging, we find that

$$S_f = \frac{K_s}{\dfrac{\mu_m}{D(1 - R_x)} - 1} \quad (30)$$

The equations given above are quite general for the behavior of cells in continuous culture, subject to the validity of the models chosen. Effects of dialysis, maintenance metabolism, and cells in the feed stream on effluent cell and substrate concentration can be evaluated. Simpler, more restrictive equations can be obtained by giving a zero value to the appropriate constant. For example, the interrelations between flow rate, feed cell concentration, and maintenance metabolism for nondialysis continuous culture can be calculated from these equations by allowing P_m to become zero. To emphasize the effects of dialysis in the discussion and equations that follow, we will restrict our considerations to a single-stage fermentor with no cells in the feed stream (i.e., $R_x = 0$)

Note that the fermentor substrate concentration term (S_f) given in equation 30 was derived without recourse to equations 21 and 22, which have dialysis terms, nor to equation 20, the substrate-utilization model. Therefore, the substrate concentration in the fermentor (S_f) and in the fermentor effluent is independent of membrane permeability (P_m) and membrane area (A_m), and is altogether independent of the dialysis operation. Also, it is independent of yield coefficients Y_x and Y_E. The substrate concentration in the fermentor is in fact exactly the same as would be obtained in normal continuous culture at the same dilution rate.

However, the maximum or critical dilution rate (D_c), i.e., where washout occurs, is definitely a function of dialysis. The flow rate for washout can be found by letting $R_x = 0$ and solving equations 27 and 29 simultaneously:

$$D_c = \frac{\mu_m}{K_s\left[\dfrac{1 + F_f\left(\dfrac{1}{P_m A_m} + \dfrac{1}{F_r}\right)}{S_r^\circ + F_f S_f^\circ\left(\dfrac{1}{P_m A_m} + \dfrac{1}{F_r}\right)}\right] + 1} \quad (31)$$

For the particular situation where the same medium is used in the fermentor and the reservoir, i.e. $S_r^\circ = S_f^\circ$, equation 31 reduces to

$$D_c = \frac{\mu_m}{\dfrac{K_s}{S_f^\circ} + 1} \quad (32)$$

This is the same condition for washout as in normal continuous culture (66).

Another important point is that the reservoir volume, V_r, does not appear in any of the expressions 27 to 31. This means that *the performance of fully continuous dialysis culture is completely independent of the reservoir volume, and the reservoir can be made as small as is operationally feasible. This remarkable result has important implications for the design of large-scale continuous dialysis culture systems, since the reservoir need not be scaled-up in the same proportion as the fermentor or dialysis unit.*

A comparison of continuous dialysis culture with normal continuous culture is best served by a numerical example. The example is based on the same hypothetical organism used by Herbert et al. (66) in their discussion of continuous culture. If glucose is the limiting substrate, the permeability coefficient, P_m, of cellulose-acetate membranes is approximately 0.42 cm/hr (82).

A realistic value to assume for A_m is 1 cm² per ml of culture volume, based on the following line of reasoning. If a separate dialyzing unit is used,

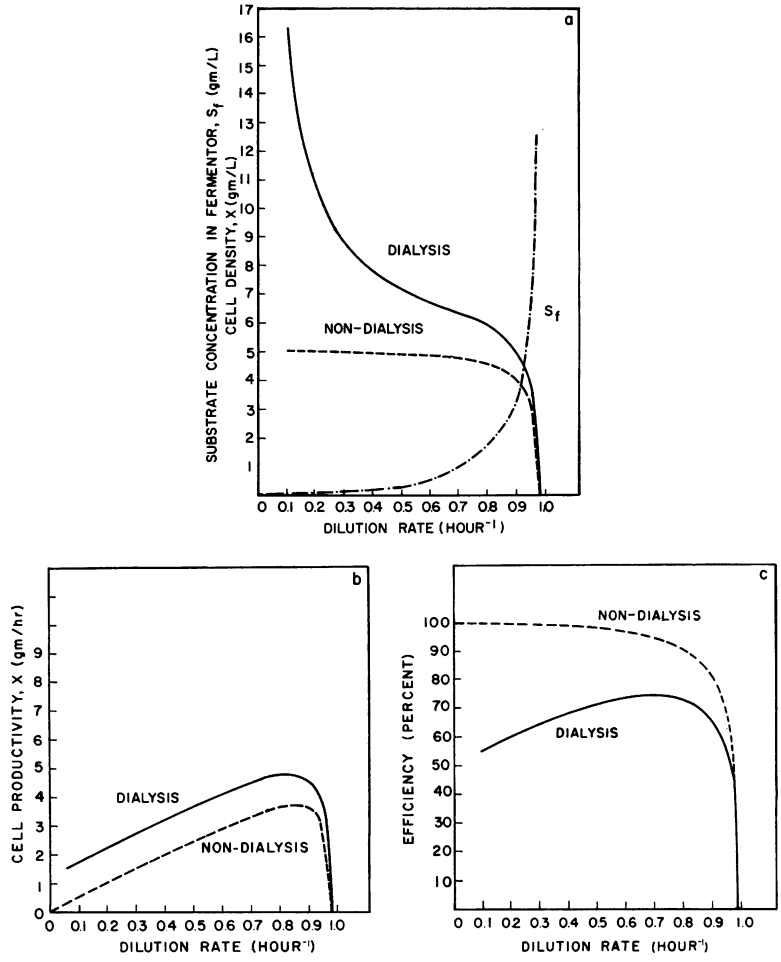


FIG. 15. *Calculated comparison of dialysis and nondialysis continuous culture with respect to cell density (a), productivity (b), and efficiency (c). The following conditions were assumed: fermentor volume, V_f = 1 liter; growth rate constant, μ_m = 1 hr^{-1}; growth constant, $K_s = 2 \times 10^{-4}$ g/ml; yield constant, Y_x = 0.5; maintenance constant, Y_E = 0; substrate concentration in fermentor and reservoir feed, $S_r^\circ = S_f^\circ = 10^{-2}$ g/ml; flow rate through reservoir, F_r = 500 ml/hr; product of membrane permeability and area, $P_m A_m$ = 420 cm^3/hr.*

the holdup in the dialyzer probably should not exceed 10% of the fermentor volume, or 100 ml per liter of fermentor volume. A dialyzer can reasonably be fabricated with 1 mm clearance between the membrane and supporting structure. This would result in 0.1-ml holdup per cm^2 of membrane area. A 100-ml holdup would allow 1,000 cm^2 of membrane area per liter of fermentor volume. If an integral type unit is used, the membrane area might be as high as 10 cm^2 per ml of culture fluid.

The calculated example based on these assumptions is shown in Fig. 15.

The most striking and important difference between nondialysis and dialysis continuous culture (Fig. 15) is the much higher cell densities attainable in dialysis culture, especially at low dilution rates. The reason for this behavior is easily seen if one realizes that in normal continuous culture all the substrate enters the fermentor with the feed stream. Therefore, at low dilution rates, the cell population is limited by the substrate concentration in the feed stream. In fully continuous dialysis culture, however, an additional supply of substrate is made available to the fermentor through the dialysis membrane. This essentially increases the net substrate concentration available. Consequently, a much higher cell concentration can be established. The rate at which cells are produced in a continuous dialysis culture is obtained simply by multiplying the flow rate by the concentration of cells in the effluent stream and by taking

$R_x = 0$:

$$\text{Production rate} = XF_f = \left[\frac{F_f}{\frac{F_f}{Y_x} + Y_E V_f}\right]\left[\frac{S_r^\circ - S_f}{\frac{1}{P_m A_m} + \frac{1}{F_r}} + F_f(S_f^\circ - S_f)\right] \quad (33)$$

where S_f is obtained from equation 30.

In Fig. 15b, one also sees that the productivity of fully continuous dialysis culture will be higher than that of a normal continuous culture throughout the entire range of dilution rates, with the same medium flowing through both chambers.

The flow rate through the fermentor which corresponds to a maximum production rate is found by differentiating equation 33 with respect to the flow rate through the fermentor (F_f) and setting the result equal to zero. If we neglect maintenance metabolism ($Y_E = 0$), the following equation results:

$$F_{f\max} = V_f \mu_m \times \left[1 - \sqrt{\frac{K_s}{K_s + S_f^\circ}\left(1 + \frac{P_m A_m F_r}{\mu_m V_f P_m A_m + F_r}\right)}\right] \quad (34)$$

In comparing this equation with that given by Herbert et al. (66) for a normal continuous fermentation, it is seen that *the maximum production rate in continuous dialysis culture is achieved at a lower dilution rate than in nondialysis continuous culture.*

The efficiency of cell production, defined as the actual production rate divided by the production rate equivalent to complete utilization of the substrate supplied, is given by:

$$\text{Efficiency} = \frac{XF_f}{Y_x(F_r S_r^\circ + F_f S_f^\circ)} = \frac{\frac{F_f}{F_f + Y_E Y_x V_f}\left[\frac{S_r^\circ - S_f}{\left(\frac{1}{P_m A_m} + \frac{1}{F_r}\right)} + F_f(S_f^\circ - S_f)\right]}{F_r S_r^\circ + F_f S_f^\circ} \quad (35)$$

An example of the variation in efficiency with dilution rate is also shown in Fig. 15c. *The high cell densities obtainable in continuous dialysis culture at low dilution rates are achieved at the expense of lower efficiencies in converting substrate to cells.* Also, the efficiency of continuous dialysis culture is always less than that of nondialysis continuous culture. The difference between the two is attributable to the unused substrate which passes out of the system with the reservoir effluent stream.

The relative cell density for dialysis culture (X) and nondialysis culture (X_{nd}) at the same flow rates can be calculated from equation 27. The cell density in nondialysis culture with maintenance metabolism is obtained from equation 27 by setting $P_m A_m = 0$:

$$X_{nd} = \frac{F_f(S_f^\circ - S_f)}{\left(\frac{F_f}{Y_x} + Y_E V_f\right)} \quad (36)$$

The ratio X/X_{nd} at the same dilution rate is

$$\frac{X}{X_{nd}} = \frac{S_r^\circ - S_f}{\left(\frac{1}{P_m A_m} + \frac{1}{F_r}\right) F_f(S_f^\circ - S_f)} + 1 \quad (37)$$

Notice that the maintenance coefficient, Y_E, does not appear in this equation, so that the relative effect of dialysis on cell density is the same whether or not maintenance metabolism occurs.

An examination of Fig. 15a shows that the substrate concentration in the fermentor, S_f, is much smaller than S_f° or S_r° at low dilution rates, i.e., if $D < 0.7\ D_c$. An approximate expression for the relative cell density X/X_{nd}, neglecting S_f in equation 37, is

$$\frac{X}{X_{nd}} = \frac{S_r^\circ}{F_f S_f^\circ\left(\frac{1}{P_m A_m} + \frac{1}{F_r}\right)} + 1 \quad (38)$$

Data given by Hauschild and Pivnick (65) can be used to check the validity of equation 38 (Table 2). The necessary values for operating conditions, which are needed to calculate the increase in cell concentration with dialysis, were estimated from their description of the experimental apparatus. It can be seen by comparing

TABLE 2. *Comparison of measured and calculated values for cell production of Brucella abortus in dialysis and non-dialysis continuous culture*[a]

Medium lot	Viable bacteria per ml		Measured X/X_{nd}	Calculated X/X_{nd}
	With dialysis (X)	Without dialysis (X_{nd})		
A	375×10^9	152×10^9	2.46	2.42
B	80×10^9	38×10^9	2.10	2.42

[a] The following operating conditions were used: $P_m = 0.5$ cm/hr; $A_m = 90$ cm^2; $S_r^\circ = S_f^\circ$, $F_r = 20.8$ ml/hr; $F_f = 10$ ml/hr. Data from Hauschild and Pivnick (65).

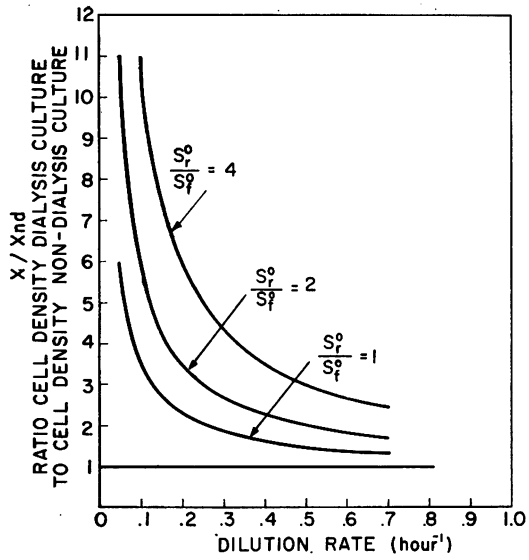


FIG. 16. *Calculated effect of the substrate concentration in reservoir feed on the cell density in continuous dialysis culture, plotted as relative cell density in dialysis culture (X) to nondialysis culture (X_{nd}) at the same dilution rates. Assumptions: product of membrane permeability and area, P_mA_m = 500 cm³/hr; substrate concentration in fermentor feed, $S_f^\circ = 10^{-2}$ g/ml; S_r°/S_f° as shown; all else the same as in Fig. 15.*

the last two columns of Table 2 that the predicted results are very close to the measured values.

Also, in the region of flow rates where S_f is negligible, the cell concentration is practically independent of the model chosen for the effect of substrate concentration on growth rate, equation 19. The cell concentration is determined principally by the total substrate available in the feed streams and the yield coefficients, Y_x, and Y_E.

Some appreciation of the effect of the substrate concentration in reservoir feed (S_r°), feed rate (F_r), and membrane permeability and areas (P_mA_m) can be obtained from the calculated examples given in Fig. 16–18. *An increase in concentration of substrate in the reservoir influent, S_r°, is reflected in an almost directly proportional increase in cell density. The cell density is less sensitive to changes in P_mA_m and F_r (Fig. 17 and 18), especially at the higher dilution rates.*

An interesting feature of fully continuous dialysis culture is that high cell densities can be obtained at low dilution rates, even with feed solutions containing very low substrate concentrations. To achieve the same effect in nondialysis continuous fermentation, a portion of the cells must be separated from the fermentor effluent stream and recirculated.

This feature of high cell densities at low dilution rates would be expected to be displayed in continuous dialysis culture even if the organism had a significant level of maintenance metabo-

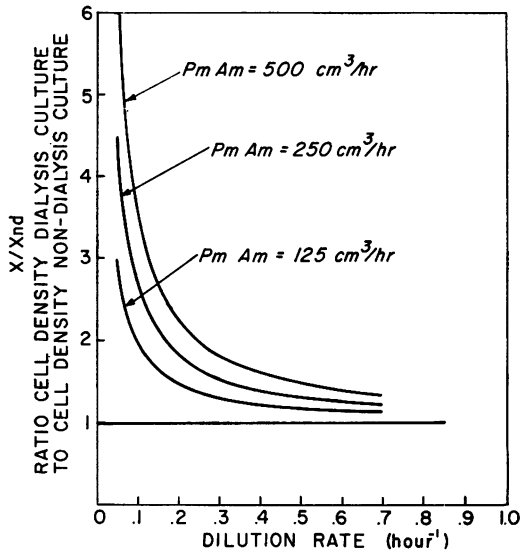


FIG. 17. *Calculated effect of the membrane permeability and area (P_mA_m) on the cell density in continuous dialysis culture, plotted as relative cell density in dialysis culture (X) to nondialysis culture (X_{nd}) at the same dilution rates. Assumptions: P_mA_m as shown; all else the same as in Fig. 15.*

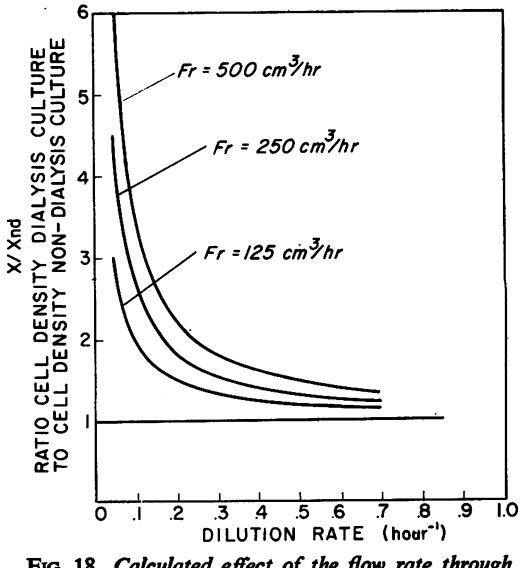


FIG. 18. *Calculated effect of the flow rate through reservoir (F_r) on the cell density in continuous dialysis culture, plotted as relative cell density in dialysis culture (X) to nondialysis culture (X_{nd}) at the same dilution rates. Assumptions: P_mA_m = 500 cm³/hr; F_r as shown; all else the same as in Fig. 15.*

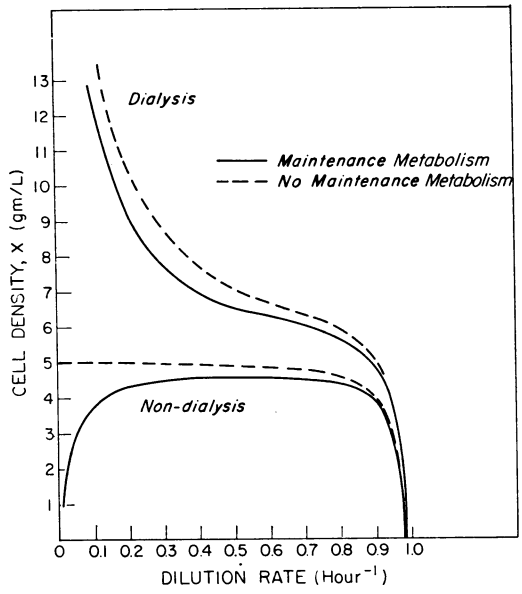


FIG. 19. *Calculated effect of maintenance metabolism on cell density in dialysis and nondialysis continuous culture with the limiting substrate present in the fermentor feed stream.*

lism. This is in contrast to the behavior of such an organism in normal continuous culture, where the cell density drops to zero at low dilution rates. For example, Marr et al. (95), in continuous culture studies, showed that the specific maintenance rate, Y_E, had the numerical value of 0.028 hr^{-1} for a strain of *Escherichia coli* with a growth rate constant of 1.0 hr^{-1}. The expected behavior if such a culture is grown in a continuous dialysis system is shown in Fig. 19.

In situations where none of the limiting substrate is presented in the feed to the fermentor, as when cells are grown on the dialysate of a complex medium, dialysis culture is not as advantageous for growing concentrated cultures. Figure 20 shows the response to dilution rate (D) expected for this type of operation. High cell concentrations are only obtained at very low dilution rates. Cell densities below "normal" are obtained through a wide range of flow rate, unless of course the substrate concentration in the reservoir is several times higher than "normal." A common experimental situation analogous to the condition of no substrate in the fermentor feed is that which occurs with aerobic continuous cultures when oxygen is the growth-limiting nutrient (123).

Completely batch operation. The remaining three basic modes of operating dialysis culture are essentially nonsteady-state in nature. Because the defining equations are nonlinear, they are best solved by computer (*see below*).

Completely batch operation results when there is no flow through either the fermentor or reservoir chambers (Fig. 8a). Mathematically, this is equivalent to letting F_f and F_r equal zero in equations 21 to 23.

The expected growth pattern for this type of operation is shown in Fig. 21. The growth cycle can be separated into two phases: first, cells will grow exponentially until the substrate concentration in the fermentor falls to a level which limits

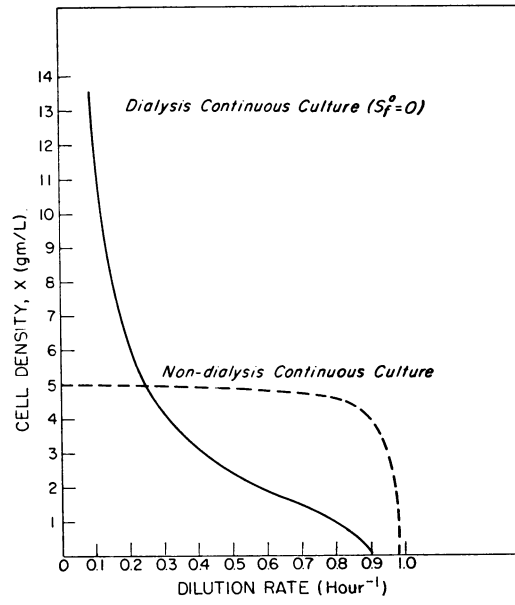


FIG. 20. *Calculated comparison of nondialysis continuous culture with the dialysis situation when the limiting substrate is not present in the fermentor feed stream and the culture has no maintenance requirement.*

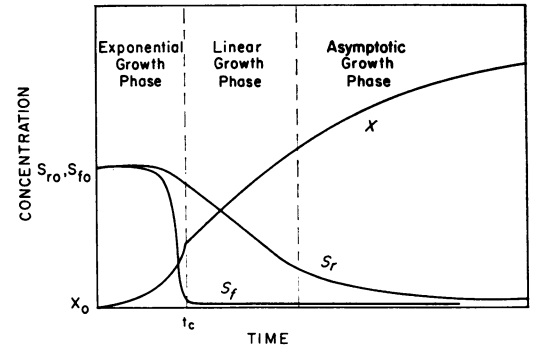


FIG. 21. *Expected changes in cell density and limiting substrate concentrations for dialysis culture system operated with batch fermentor and reservoir. X, S_f, and S_r, respectively, are cell density, fermentor-substrate concentration, and reservoir-substrate concentration during culture period. The critical time when substrate diffusion through membrane becomes limiting is shown as t_c.*

the growth rate; afterwards, the growth rate will be governed by the diffusion rate of substrate into the fermentor from the reservoir, and so become linear.

Ideally the concentration of cells formed in batch dialysis culture should be directly related to the amount of nutrients available to the culture from the fermentor and reservoir chambers. By material balance and neglecting maintenance metabolism, the final cell concentration is given by the equation

$$V_f(X - X^\circ) = Y_x(V_f S_f^\circ + V_r S_r^\circ) \quad (39)$$

where X° is the concentration of cells present initially as inoculum. For ordinary (nondialysis) batch culture, the final cell yield is given by the balance

$$(X_{nd} - X^\circ) = Y_x S_f^\circ \quad (40)$$

Therefore, the concentration ratio achieved by dialysis is

$$\frac{X}{X_{nd}} = 1 + \frac{V_r S_r^\circ}{V_f S_f^\circ} \quad (41)$$

where X is the cell concentration in the culture chamber of the dialysis flask, X_{nd} is the cell concentration in an equivalent-volume, nondialysis flask, and X° is small relative to the final cell concentrations. The concentration ratio is seen to depend on the relative nutrient concentration in the reservoir and culture chambers, S_r°/S_f°, and on the ratio of chambers, V_r/V_f. In the experiments reported by Gerhardt and Gallup (51), the same medium was used in both chambers (i.e., the ratio $S_r^\circ/S_f^\circ = 1.0$) and the cell density in dialysis culture was proportional to $1 + V_r/V_f$. Thus, *if the ratio of reservoir volume to fermentor volume (V_r/V_f) is 10:1, it is possible to attain a cell density in dialysis culture which is 11 times greater than in a nondialysis batch culture. However, higher cell densities also can be obtained by using higher nutrient concentrations in the reservoir.*

The period of exponential growth in batch dialysis culture will depend upon the rate of cellular growth relative to the rate of substrate diffusion through the dialysis membrane. If the generation time of the cells is short, diffusion may become limiting early in the fermentation. On the other hand, if the cells grow slowly or the membrane area is very large, diffusion may never become limiting. Usually it is desirable to employ excess membrane area so that substrate diffusion does not limit the growth rate.

With dialysis culture, the exponential growth phase is extended because of the additional nutrient which is provided by the reservoir via the membrane. An approximate expression for the increase in exponential-phase cells that can be obtained in dialysis culture relative to nondialysis culture is

$$\frac{X}{X_{nd}} = \frac{S_r^\circ}{S_f^\circ}\left(1 + \frac{P_m A_m}{V_f \mu_m}\right) \quad (42)$$

where S_r° is the initial substrate concentration in the reservoir for dialysis culture, and S_f° is the initial substrate concentration in the fermentor for nondialysis batch culture.

This result, which is independent of whether or not the cells have a large maintenance metabolic component, is important for cases where it is desirable to obtain high concentrations of exponentially growing cells. For example, the enzymes involved in protein synthesis are often found in highest concentration during the exponential growth phase, and so large amounts of exponential cells are required to isolate these enzymes.

The increased exponential phase in dialysis culture also means that the cells are maintained in a more constant environment for a longer period of time than in nondialysis culture. The relative benefit of dialysis culture mainly stems from the term $P_m A_m/V_f \mu_m$. The ratio is related to the diffusion rate of substrate through the dialysis membrane per unit volume of fermentor. The larger this value is in comparison to the specific growth rate constant, μ_m, the greater will be the benefit of dialysis culture. For fast-growing (e.g., bacterial) cells, one might obtain only twice the amount of exponential growth, but for slower-growing (e.g., mammalian) cells, the amount of exponential growth might be increased 10-fold.

The influence of membrane permeability and area on the kinetics of batch dialysis culture is shown in Fig. 22. As the magnitude of the term $P_m A_m$ is made smaller, a longer period of time is required for a given cell yield to be obtained. For a given cell yield, the relationship is approximately

$$t = \frac{\text{Constant}}{P_m A_m} \quad (43)$$

Thus, if $P_m A_m$ is halved, it takes about twice the time to reach the same cell yield.

The effect of changing the reservoir volume alone is shown in Fig. 23. Two consequences are apparent: *first, reducing the reservoir volume curtails the maximum cell yield; second, the final cell yield is approached faster with lower reservoir volumes,* because a larger portion of the cells is formed during the exponential-growth phase.

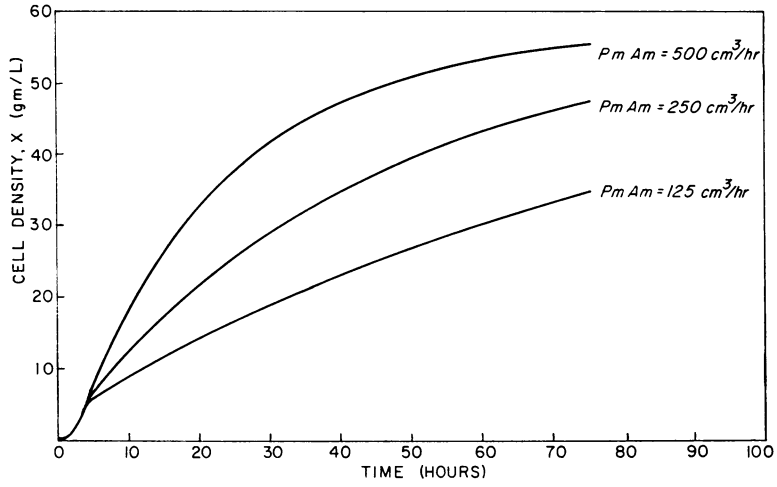


FIG. 22. *Computed effect of membrane permeability and area (P_mA_m) on cell density in fully batch dialysis culture. Assumptions: ratio of reservoir volume to fermentor volume, $V_r/V_f = 10$; fermentor volume, $V_f = 1$ liter; initial substrate concentration in reservoir and fermentor, $S_r^\circ = S_f^\circ = 10^{-2}$ g/ml; cell inoculum, $X^\circ = 5 \times 10^{-5}$ g/ml; P_mA_m as shown; growth constants as given in Fig. 15.*

Both of these predictions have been confirmed experimentally (44, 51; *unpublished data*).

The efficiency of batch dialysis culture should approach 100% if the fermentation is allowed to run long enough. But, if the membrane permeability or area is too small relative to the fermentor volume, a point may be reached when the rate of substrate diffusion can only satisfy the maintenance metabolism of the organism and not the growth requirements. On the other hand, the cells might begin to autolyze before all the substrate in the reservoir is utilized.

The effect of maintenance metabolism on batch-culture kinetics is striking. Figure 24 shows the decrease obtained in final cell yield for various values of the specific maintenance rate, Y_E.

Relatively low levels of maintenance metabolism have a profound effect on the maximum cell yield obtainable, because at high cell densities an increasing proportion of the substrate is used in maintenance rather than for building more cell substance. Even if the reservoir were continually replenished with fresh substrate (Fig. 8d), the cell population in the fermentor would reach a final stable level when the amount of substrate diffusion into the fermentor became equal to the amount used for maintenance. The maximum cell density obtainable is calculated (by letting dX/dt and $dS_f/dt = 0$):

$$P_mA_m(S_r^\circ - S_{fmin}) = V_fY_EX_{max} \quad (44)$$

and, if the substrate concentration in the reservoir is much larger than in the fermentor ($S_r^\circ \gg S_{fmin}$) when the cell concentration becomes constant,

$$X_{max} = \frac{P_mA_mS_r^\circ}{V_fY_E} \quad (45)$$

Equation 45 suggests another unique use for dialysis culture, namely as an experimental method for determining the specific maintenance rate, Y_E. By growing a culture in a batch dialysis system with continual replenishment of the reservoir fluid, one can experimentally determine the values of X_{max} and S_{fmin}. With a knowledge of the other quantities, P_m, A_m, S_r°, and V_f, a

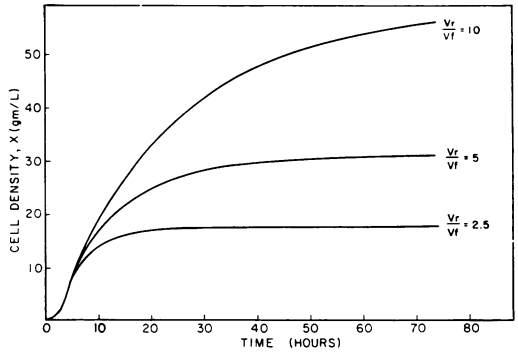


FIG. 23. *Computed effect of reservoir volume on cell density in fully batch dialysis culture, plotted with the ratio of reservoir volume to fermentor volume (V_r/V_f) as a parameter. Assumptions: product of membrane permeability and area, $P_mA_m = 500$ cm³/hr; V_r/V_f as shown; all else as given in Fig. 15.*

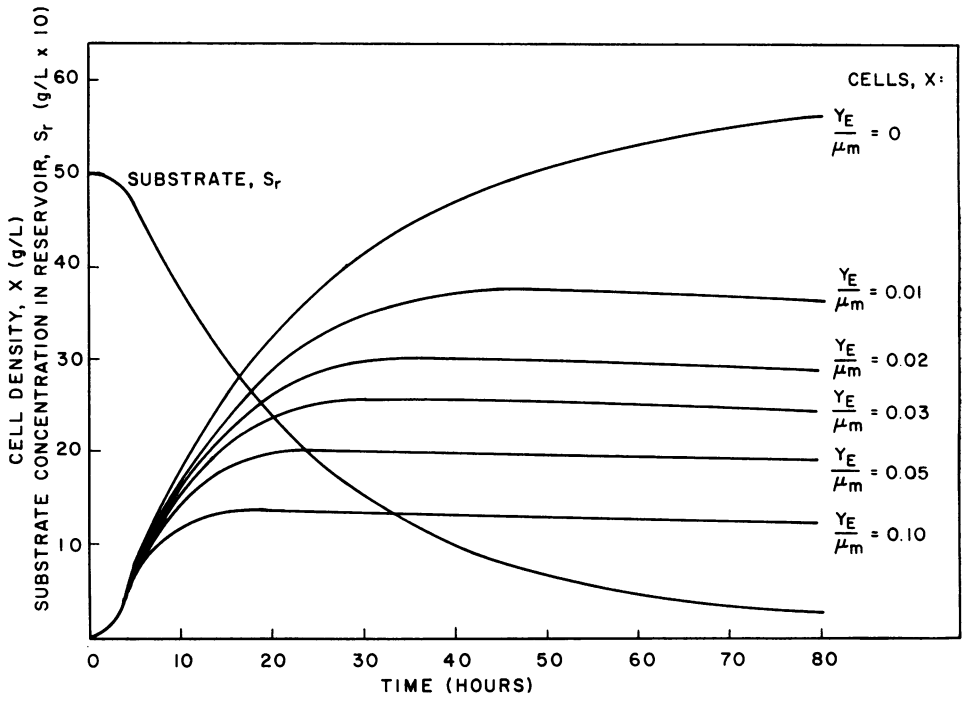


FIG. 24. *Computed effect of maintenance metabolism on final cell yield in batch dialysis culture. Assumptions are the same as for Fig. 15 except that values of the maintenance coefficient, Y_E, were assumed to give the values of the ratio, Y_E/μ_m, shown as the parameters on the curves.*

value for Y_E can be calculated from equation 45. A series of such experiments with different environmental conditions and a variety of substrates would reveal important information about maintenance metabolism.

Batch fermentor, continuous reservoir operation. In this type of operation, a continuous stream containing substrate is passed through the reservoir, but no additions to or withdrawals from the fermentor chamber are made (Fig. 8d). The expected behavior is shown in Fig. 25. Assuming no lag, the cells grow exponentially at first; i.e., the numerical value of the substrate concentration in the fermentor will be much greater than the growth rate constant ($S_f \gg K_s$). The exponential growth period will depend on many parameters of the system, as discussed in the previous section. However, eventually the substrate concentration in the fermentor will drop to a low constant value, and growth will depend almost entirely on diffusion of nutrient from the reservoir. Under these conditions, the substrate concentration in both chambers will approach constant values; i.e., dS_f/dt and $dS_r/dt \approx 0$.

The material balance equations can be solved to obtain an approximate expression for the growth rate after the exponential phase:

$$\frac{dX}{dt} = \frac{Y_x P_m A_m (S_r - S_f)}{V_f} - Y_E Y_x X \quad (46)$$

If the maintenance factor (Y_E) is negligible, the second term will drop out and growth will be linear with time. Thus, under these conditions, one should be able to control the linear growth rate by varying the parameters P_m, A_m, V_f, and S_r. Of course, the cell density cannot increase indefinitely since eventually some other factor such as aeration or mixing effects will become limiting.

On the other hand, if maintenance metabolism is important, the second term in equation 46 cannot be neglected and the growth rate (dX/dt) will eventually go to zero. Under these conditions, the final cell yield is given by the equation

$$X = \frac{P_m A_m (S_r - S_f)}{Y_E V_f} \quad (47)$$

where the reservoir substrate concentration (S_r) can be calculated from equation 21 by letting

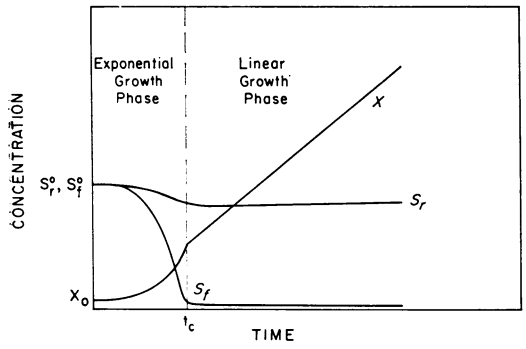


FIG. 25. *Expected changes in cell density and limiting substrate concentrations for dialysis culture systems operated with a batch fermentor and continuous reservoir. X, S_t, and S_r, respectively, are cell density, fermentor-substrate concentration, and reservoir-substrate concentration during culture period. The critical time when substrate diffusion through membrane becomes limiting is shown as t_c.*

$dS_r/dt = 0$:

$$S_r = \frac{P_m A_m S_f + F_r S_r^\circ}{P_m A_m + F_r} \tag{48}$$

If the flow rate through the reservoir (F_r) is much greater than $P_m A_m$, then $S_r = S_r^\circ$, and the final cell concentration will be given by the equation

$$X = \frac{P_m A_m}{Y_E V_f} (S_r^\circ - S_f) \tag{49}$$

Equation 49 is essentially the same as for completely batch operation (equation 45). Therefore, this mode of operation can also be used to determine the magnitude of the endogenous coefficient, Y_E.

Continuous fermentor, batch reservoir operation. In this type of operation, there would be continuous flow through the fermentor but not through the reservoir (Fig. 8c). This is inherently a nonsteady-state operation, since the concentration of substrate in the reservoir is always changing with time. The expected behavior of the system will depend somewhat on the start-up procedure. If the fermentor chamber is inoculated first and if circulation through the dialyzer is started only after the fermentor has reached a continuous steady state, the behavior will be as shown in Fig. 26.

There would seem to be little point to this method of operation, since the cell density will slowly decay towards the original steady-state value as substrate in the reservoir is utilized, except that it is easier to have a batch reservoir than a continuous-flow type. Almost fully continuous dialysis culture could be achieved if the reservoir were periodically replenished. How often the reservoir needs to be replenished can be estimated after a decision is made as to how much substrate waste is feasible.

Product Formation in Dialysis Culture

Basic considerations. The degree of complexity increases immeasurably when one considers product formation in relation to dialysis culture. The word "product" can refer to any of the thousands of materials that can be generated by microbial systems, and the detailed kinetics of formation of any particular product in relation to microbial growth may be unique. The intricacies of fitting a mathematical model to the kinetics of product formation is well illustrated by the work of Chen et al. (25) on steroid conversions, where phenomena such as substrate solubility, coprecipitation, feedback inhibition, and substrate inhibition had to be taken into account. In another study, Shu (148) showed that cell age distribution is an important factor in lysine formation. Just as there is no general formulation which accounts for the kinetics of ordinary reactions, it is clear that a general mathematical model for all microbial reactions will not be forthcoming soon.

However, if one does have a kinetic model for the production of a particular microbial product in nondialysis culture, it is not too difficult to predict the behavior in dialysis culture. The only additional information needed is the permeability characteristics of the product for the membrane

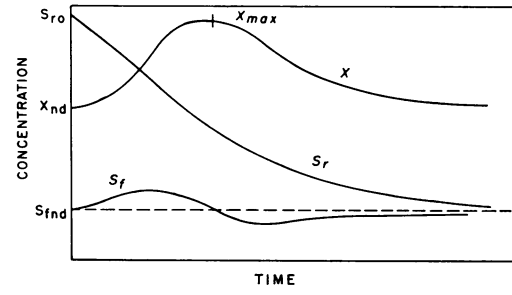


FIG. 26. *Expected changes in cell density and limiting substrate concentrations for dialysis culture system operated with continuous fermentor and batch reservoir. X_{nd} = steady-state cell density and S_{fnd} = steady-state substrate concentration in fermentor before starting of dialysis; S_{ro} = initial substrate concentration in reservoir; X, S_f, and S_r, respectively, are cell density, fermentor substrate concentration, and reservoir-substrate concentration after dialysis is initiated.*

materials that are used in the dialysis culture apparatus.

Some of the characteristics of product formation in dialysis culture can be illustrated with the semiempirical product model proposed by Luedeking and Piret (88):

$$r_p = \alpha r_g + \beta X \tag{50}$$

where r_p is the total rate of product formation due to metabolism, r_g is the cell growth rate, and α and β are empirical constants. This model states that the net rate of product formation is the sum of the product formed that is directly related to growth rate (first term) and the product formed that is directly related to cell concentration (second term). This model has been chosen here because it probably represents a good one for "main-line" metabolic products, such as lactic acid and gluconic acid, where the product is a result of energy metabolism. The model clearly has limitations in that it predicts *continued* product formation after growth ceases, i.e., after $r_g = 0$. However, this deficiency will only be important in the later stages of batch culture.

In addition to a kinetic model for product formation, a complete analysis requires an accounting for the amount of product in each of the chambers of the dialysis culture apparatus. Material balances for the reservoir and fermentor chambers are obtained in the same manner as the substrate balances given earlier in equations 21 and 22:

$$\text{In} + \text{Production} = \text{Out} + \text{Accumulation}$$

For the reservoir chamber:

$$P'_m A_m (P_f - P_r) + 0 = F_r P_r + V_r \left(\frac{dP_r}{dt}\right) \tag{51}$$

For the fermentor chamber:

$$0 + V_f r_p = P'_m A_m (P_r - P_f) + F_f P_f + V_f \left(\frac{dP_f}{dt}\right) \tag{52}$$

where P_f is the concentration of product in the fermentor chamber, P_r is the concentration of product in the reservoir chamber, and P'_m is the permeability of the membrane to product. Of course, if the product is intracellular, this situation can be handled by letting P'_m equal zero. These equations can readily be accommodated into the analogue computer program for cell formation (*see below*) to give a complete solution for any mode of operation. However, some analytical solutions are possible for particular situations, and these will be discussed to illustrate the behavior of the dialysis system.

Completely continuous operation. The equations can be solved algebraically because the time derivatives (dP_r/dt and dP_f/dt) are zero and, from equation 24, $r_g = (F_f/V_f)X$.

The product concentration within the fermentor chamber is directly related to cell concentration by the following formula:

$$P_f = \frac{XV_f(\alpha F_f/V_f + \beta)}{\left(\frac{P'_m A_m F_r}{P'_m A_m + F_f}\right) + F_f} \tag{53}$$

where the cell concentration X is given by equation 27.

If the product is nondialyzable, ($P'_m = 0$) and if $\beta = 0$ (i.e., if product formation is related only to cell growth), the equation reduces to

$$P_f = \alpha X \tag{54}$$

For this specific case, product concentration in the fermentor is directly proportional to cell concentration. The relation between product concentration and dilution rate will be the same as shown in Fig. 15–20, except that the ordinate is multiplied by α.

If $\alpha = 0$ (i.e., if product formation is only related to cell concentration), the equation reduces to

$$P_f = X \frac{V_f}{F_f} \beta \tag{55}$$

This equation indicates that, as the dilution rate decreases, i.e., as the flow rate through the fermentor (F_f) becomes smaller, the product concentration (P_f) becomes a higher and higher multiple of the cell concentration. The actual shape of the curve for product concentration versus dilution rate will depend on the relative magnitudes of α and β. But as the β term becomes dominant, the theory predicts very high product concentrations at low dilution rates. The expected behavior is shown in Fig. 27.

Productivity of a nondialyzable product is given by:

$$\text{Productivity} = P_f F_f = XF_f \left(\alpha + \frac{V_f}{F_f}\beta\right) \tag{56}$$

Since the term XF_f is the productivity of cells, the productivity of product ($P_f F_f$) is obtained simply by multiplying the cell productivity curve by the factor ($\alpha + V_f\beta/F_f$). This is shown in Fig. 27 for various magnitudes of α and β. Again, the influence of the β term is seen; as β becomes larger, productivity of product no longer goes through a maximum but actually increases at low dilution rates.

Similar equations for product formation in

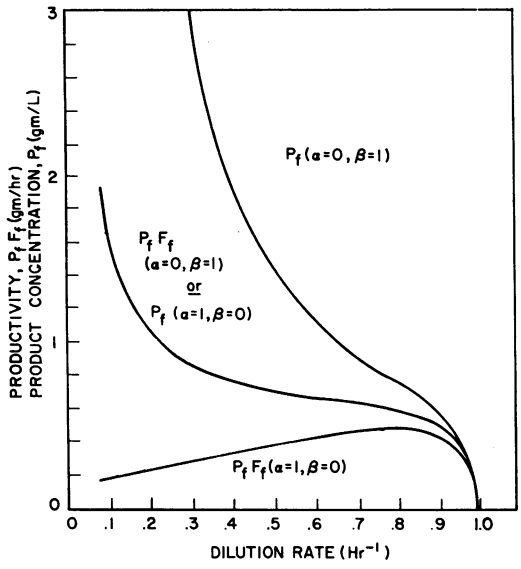


FIG. 27. *Expected changes in product concentration and productivity in continuous dialysis culture when the product is not dialyzable. Assumptions are the same as in Fig. 15.*

nondialysis continuous culture are readily obtained by using equation 36 for cell concentration (X), rather than equation 27.

If the product is dialyzable ($P'_m \neq 0$), then it will be distributed between the fermentor and reservoir chambers. The concentration of product in the reservoir chamber is given by the equation

$$P_r = \frac{P_f}{\frac{F_r}{P'_m A_m} + 1} \qquad (57)$$

where P_f is given by equation 53.

If the membrane is very permeable, or its area is large, or the flow through the reservoir is small, then $1 \gg F_r/P'_m A_m$ and the product concentration in the reservoir will approach its concentration in the fermentor. In no case can the product solution concentration in the reservoir become greater than that in the fermentor chamber, because a concentration gradient from the fermentor to the reservoir chamber must exist in order for diffusion to occur.

The fraction of the total product formed which will be removed in the reservoir effluent stream is given by $F_r P_r (F_r P_r + F_f P_f)$, and by substitution with equation 57 this fraction is given by the equation,

$$\frac{F_r P_r}{F_r P_r + F_f P_f} = \frac{1}{1 + F_f \left(\frac{1}{P'_m A_m} + \frac{1}{F_r} \right)} \qquad (58)$$

Therefore, in order to obtain a large fraction of the product in the reservoir effluent free from cells, both the permeability ($P'_m A_m$) and the reservoir flow rate (F_r) must be large numbers in comparison to the fermentor flow rate (F_f). This type of operation would result in a low concentration of product in the reservoir effluent stream and perhaps also in difficulty with recovery of the product.

However, if the product happens to be an inhibitor of metabolism, or if it is desired to maintain the fermentor chamber at relatively constant environmental conditions and essentially free from metabolic products, then the usefulness of equation 53 becomes apparent for predicting the values of $P'_m A_m$ and F_r needed to reduce the product concentration in the fermentor chamber (P_f) to any desired level.

Batch operations. Product formation in nonsteady-state dialysis culture cannot be meaningfully discussed in general terms because of the many patterns to be expected from various combinations of flow, product syntheses, and permeabilities. For any particular set of circumstances, the expected behavior can be predicted with the analogue computer program.

Completely batch operation (Fig. 8a) deserves some comments, however, because it is the most likely system to be used for experimental purposes. If the product is dialyzable, it will eventually distribute between the fermentor and reservoir chambers so that the concentration is the same in both chambers. This means that the fraction of product that will be obtained in the reservoir chamber, free from cells, will be given by the volume ratio, $V_r/(V_r + V_f)$. The fraction of product remaining in the fermentor chamber can be made as small as desired merely by increasing the volume of the reservoir. However, this is accomplished at the expense of diluting the product.

Another factor to be considered in this type of operation is that the product concentration in the reservoir will lag behind the concentration in the fermentor, because of the membrane diffusion barrier between the two chambers. Figure 28 shows an example of this type behavior. The rate of equilibration between the two chambers depends on the factor $P'_m A_m (P_f - P_r)$. If the product concentrations are very low, then a very large membrane area will be needed to obtain acceptable rates of equilibration.

Implications of Theory

In the preceding analysis, we have shown, on the basis of a few simple models, how the behavior of dialysis culture can be predicted for a variety of operational systems. The extent to

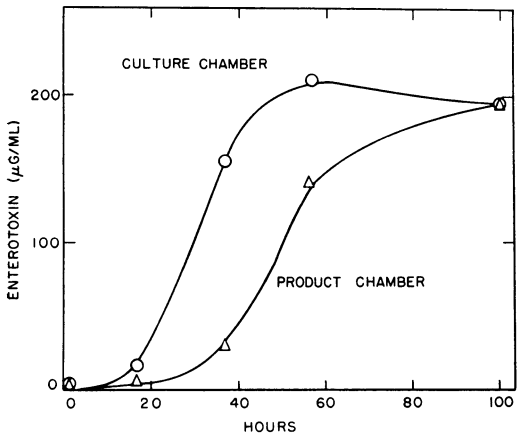


FIG. 28. *Comparison of the changes in concentration of a product, staphylococcal enterotoxin, in the product and culture chambers of a differential dialysis flask. Reproduced from Herold, Schultz, and Gerhardt (67).*

which the performance of any real dialysis culture matches that predicted by the analysis will depend on how closely the models reflect reality.

It is apparent that the behavior of these systems is more closely linked to the operation of the fermentor chamber than that of the reservoir chamber. Therefore, the first step in designing a dialysis culture system is to choose between batch and continuous culture. The relative advantages of each type of operation for normal culture methods have been discussed at length by Maxon (97), Ellsworth et al. (33), and Gerhardt and Bartlett (50). The arguments can be summarized as security and flexibility of batch culture as opposed to uniformity and economy of continuous culture.

There are additional factors to be considered for dialysis culture. Fully continuous dialysis culture requires two systems to be simultaneously operable, one for the fermentor and the other for the reservoir. Therefore, the chances for mishaps are compounded. In theory, the reservoir need not be maintained sterile if a dialysis-type membrane is used, but growth of an unwanted organism in the reservoir would reduce the nutrient supply to the fermentor and deleteriously affect cell yield.

Fully batch dialysis culture has the inherent advantage that higher efficiencies can be attained in the conversion of substrate to cells. But, if the process is to be scaled up to industrial standards, extremely impractical reservoir volumes may be required. For example, if a 10:1 increase in cell density were desired over that attainable in normal batch culture, the reservoir would have to be nine times as large as the fermentor. If the fermentor were 20,000 gallons, the reservoir would have to be a staggering 180,000 gallons. As mentioned previously, continuous dialysis culture does not require proportional scale-up of the reservoir.

The equations presented provide a point of departure for experimentation and design. Theoretically, one needs to determine the values of only five parameters, P_m, μ_m, K_s, Y_x, and Y_E, to design a dialysis system to produce cells. Membrane permeability, P_m, can be measured independently by normal dialysis, and the constants for the cell metabolism and growth models can be found by ordinary continuous-culture studies. However, the model parameters are not really constant but are a function of environmental conditions, especially *p*H and dissolved oxygen concentration. Unless these conditions are controlled, the prevailing environment in dialysis culture may differ from that in an ordinary culture. Other factors such as toxic product formation may lead to differences between the growth model for dialysis and ordinary culture methods.

A sounder approach to dialysis culture design would be to use the equations developed here as a guide in experimental pilot-scale studies. Depending upon the objective, whether it be high cell densities, high productivity, or efficient utilization of substrate or growth on dilute media, one can estimate the effect of the many independent variables and map out an experimental region which will provide design information for scale-up studies.

An examination of the equations shows that scale-up can be achieved by increasing volumes, flow rates, and membrane area proportionally, with the concentrations of substrate and inoculum kept constant. Not all of the independent variables are of equal importance in scale-up. For instance, in continuous dialysis culture at low dilution rates, cell density is more sensitive to changes in flow rate than in membrane area. But at high dilution rates the opposite is true. In batch dialysis culture it is more important to maintain the ratio of fermentor to reservoir volume than the ratio of fermentor volume to membrane area. If, as is often the case, equipment limitations prevent equivalent scale-up of all components, the given equations allow one to choose operating conditions to attain the desired objectives.

Summary of Mathematical Abbreviations

The mathematical abbreviations used throughout the section on Theory are defined in Table 3.

Analogue Computer Program

A general analogue computer program was devised to solve simultaneously for cell concentration, substrate concentrations, and product con-

TABLE 3. *Summary of mathematical abbreviations*

Abbreviation	Definition	Units	Initial equation
A_e	Effective open area of membrane for diffusion	cm^2	11
A_m	Geometric area of membrane	cm^2	2
A_p	Cross-sectional pore or void area of membrane	cm^2	1
D	Dilution rate	hr^{-1}	24
D_c	Critical dilution rate for cell washout	hr^{-1}	31
D°	Free diffusion coefficient of substrate in water	cm^2/hr	1
D_m	Hindered diffusion coefficient of substrate in membrane	cm^2/hr	9
ET_{50}	Measure of membrane permeability, expressed as time to reach one-half equilibrium diffusion concentration	hr	7
f	Ratio of actual diffusion rate through a membrane to rate expected for solvent of equal thickness and area		9
F	Flow rate through a chamber	cm^3/hr	21
F_{fmax}	Flow rate for maximum cell production rate	cm^3/hr	34
K_f	Filtration coefficient for water flow through membrane	$cm^3/(hr\ atm\ cm^2)$	15
K_s	Cell growth model constant	g/cm^3	19
L_p	Length of diffusion path in pores	cm	1
N	Permeation rate through membrane	g/hr	1
n	Empirical constant		12
P_m	Overall membrane permeability coefficient for substrate	cm/hr	2
P'_m	Overall membrane permeability coefficient for product	cm/hr	51
P_m°	True permeability coefficient for membrane alone	cm/hr	13
$P_{1,2}$	Effective permeability coefficient of "unstirred liquid layers"	cm/hr	13
ΔP	Hydrostatic pressure gradient across membrane	atm	15
P	Product concentration	g/cm^3	51
Q	Filtration rate of water through membrane	$cm^3/(hr\ cm^2)$	15
r	Molecular radius	nm	9
R	Pore radius	nm	9
R	Gas constant	cm^3 atm/gmole $^\circ K$	15
R_x	Ratio of cell concentrations into and out of continuous fermentor, X°/X		24
r_g	Rate of cell growth	g/cm^3 hr	18
r_p	Rate of product formation	g/cm^3 hr	50
r_s	Rate of substrate "formation," negative value	g/cm^3 hr	20
S	Substrate or solute concentration	g/cm^3	1
S_e	Equilibrium solute concentration	g/cm^3	5
T	Temperature	°K	15
t	Time	hr	4
V	Chamber volume	cm^3	4
X	Cell concentration	g/cm^3	18
X_{nd}	Cell concentration in nondialysis culture	g/cm^3	36
X_{max}	Maximum cell concentration in batch culture	g/cm^3	44
Y_E	Specific maintenance rate	hr^{-1}	20
Y_X	Yield coefficient for conversion of substrate to cells	g of cells/g of substrate	20
α	Yield coefficient for product formation	g of product/g of cells	50
β	Specific product formation rate	hr^{-1}	50
δ	Thickness of unstirred liquid layer	cm	14
σ	Reflection coefficient for substrate by membrane		15
μ	Specific growth rate constant	hr^{-1}	18
μ_m	Maximum specific growth rate constant	hr^{-1}	19
Subscript f	Fermentor chamber		
Subscript r	Reservoir chamber		
Subscript 1, 2	Diffusion chambers		
Superscript °	Initial concentration in batch cases, or feed concentration in continuous cases		

centrations. Although applicable for any mode of operation of dialysis culture, it was especially useful for the nonlinear equations derived for batch operations. To facilitate programming, the pertinent equations were put in dimensionless form by defining new dimensionless variables: time, $\tau = \mu_m t$; substrate concentration in reservoir, $\bar{S}_r = S_r/S_r^\circ$; substrate concentration in fermentor, $\bar{S}_f = S_f/S_r^\circ$; cell concentration, $\bar{X} = X/Y_x S_r^\circ$; and product concentration, $\bar{P}_r = P_r/S_r^\circ$, $\bar{P}_f = P_f/S_r^\circ$. With these new variables, most of the concentration parameters will have numerical values between zero and one.

The pertinent equations in dimensionless form become:

$$\bar{r}_g = \bar{\mu}\bar{X}$$

$$\bar{\mu} = \frac{\bar{S}_f}{\bar{K}_s + \bar{S}_f}$$

$$\frac{d\bar{X}}{d\tau} = \bar{r}_g + \frac{F_f}{V_f \mu_m}\bar{X}^\circ - \bar{X}$$

$$\frac{d\bar{S}_r}{d\tau} = -\frac{P_m A_m}{V_r \mu_m}(\bar{S}_r - \bar{S}_f) + \frac{F_r}{V_r \mu_m}(1 - \bar{S}_r)$$

$$\frac{d\bar{S}_f}{d\tau} = \frac{P_m A_m}{V_f \mu_m}(\bar{S}_r - \bar{S}_f) - \bar{r}_g - \frac{Y_E Y_x}{\mu_m}\bar{X} + \frac{F_f}{V_f \mu_m}(\bar{S}_f^\circ - S_f)$$

$$\bar{r}_p = \alpha Y_x \bar{r}_g + \frac{\beta Y_x \bar{X}}{\mu_m}$$

$$\frac{d\bar{P}_f}{d\tau} = \bar{r}_p - \frac{F_f}{\mu_m}\bar{P}_f - \frac{P'_m A_m}{V_f \mu_m}(\bar{P}_f - \bar{P}_r)$$

$$\frac{d\bar{P}_r}{d\tau} = \frac{P'_m A_m}{\mu_m V'_r}(\bar{P}_f - \bar{P}_r) - \frac{F_r}{V_r' \mu_m}\bar{P}_r$$

where $\bar{r}_g = r_g/Y_x S_r^\circ \mu_m$, $\bar{K}_s = K_s/S_r^\circ$, and $\bar{r}_p = r_p/S_r^\circ$.

A schematic drawing of the analogue computer program is shown in Fig. 29, and the physical meaning of the various potentiometers is given in the legend.

RESULTS AND APPLICATIONS

In Vitro Systems

Growth response and variables. A comparison of schematic growth curves for dialysis and nondialysis batch cultures is representative of the experimental results obtained with a number of different organisms in several different systems (Fig. 30). The growing of cells in a dialysis system does not usually affect the lag period or the rate of exponential multiplication. In some situations, dialysis seems to reduce the growth rate, probably because diffusion has been allowed to become rate-limiting (44). The main result of dialysis conditions is a prolongation of active multiplication to reach a higher maximum population, which often is extraordinarily dense. Viable-cell counts during the growth cycle have been compared with total cell counts, dry weight and deoxyribonucleic acid analyses, and turbidity measurements (44, 51). Viability approximated 100% of the total counts throughout the growth and plateau phases of dialysis cultures, but the percentage of living cells decreased rapidly after growth ceased in nondialysis cultures. A remarkable, but unexplained, two-stage curve became evident when the two measures of cell mass were plotted for the growth cycle in the dialysis fermentor system (51), but not in the flask system (44).

A second main consequence of growing cells in dialysis culture is a stabilization of the maximum stationary phase and the terminal phases of the growth cycle: viability is sustained both in the culture (161) and after the cells are washed and stored (E. A. Tyrrell, Ph.D. Thesis, Univ. of Michigan, Ann Arbor, 1962), slow but active multiplication actually may occur (161), and autolysis may be allayed (11, 55; Tyrrell, Ph.D. Thesis). The last mentioned result is particularly apparent with some organisms that are susceptible to autolysis (e.g., *Listeria monocytogenes*) but not with others (e.g., *Diplococcus pneumoniae*), probably because of differences in the causes of autolysis. In some cases, spore formation may be prevented (55, 99), but in others such morphogenesis proceeds uninterrupted, with the useful consequence that a relatively clean suspension of spores is produced (145; Tyrrell, Ph.D. Thesis).

Under usual conditions, the primary variable controlling the extent of growth attainable in dialysis culture is oxygen supply, as influenced by aeration and agitation of the culture. Final cell density is affected by oxygenation to a greater degree in dialysis than in ordinary cultures (44). Most culture systems in common use are grossly inadequate in providing for oxygenation of aerobic cultures. In keeping with modern bioengineering principles, flask dialysis systems must be usable on a shaking machine, contain baffles to cause turbulence, use a proportionally small volume of liquid culture (less than 20% of the

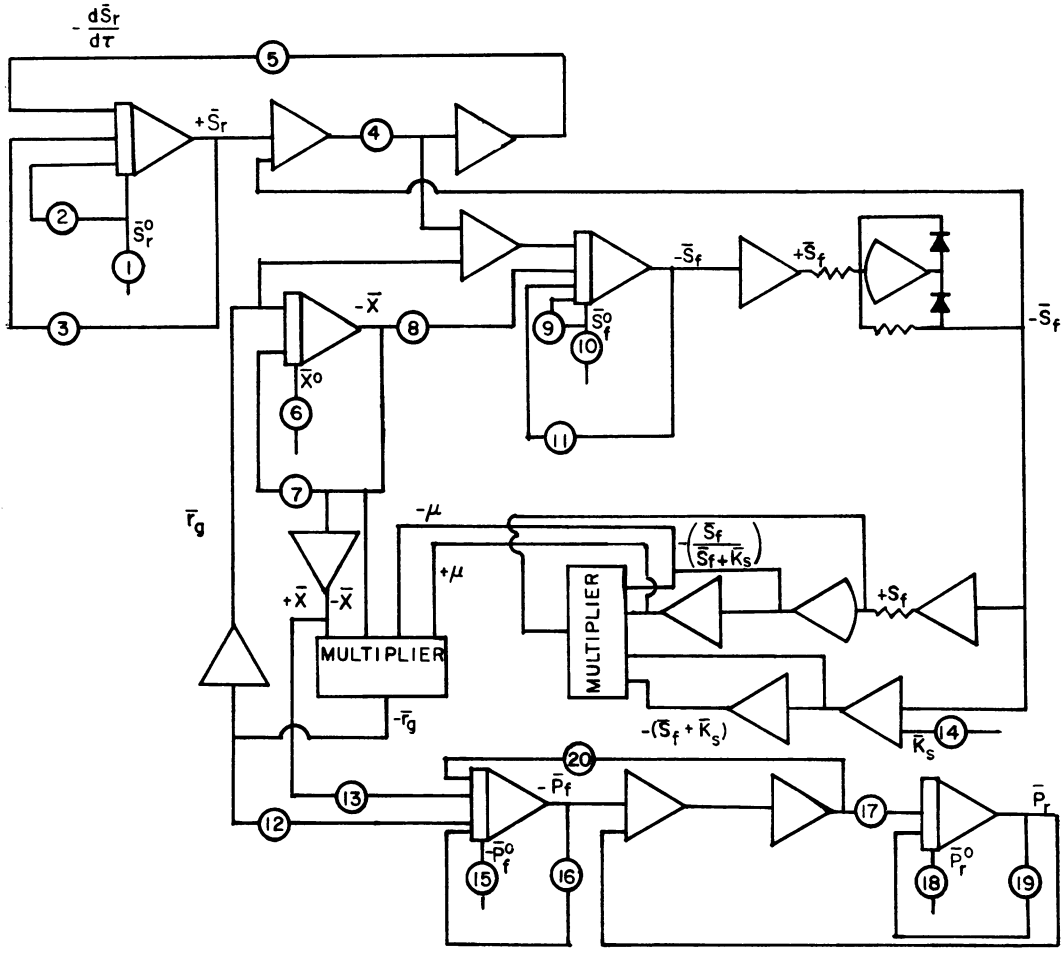


FIG. 29. *Analogue computer program for dialysis culture. Potentiometer parameters are as follows: (1)* $\bar{S}_r^\circ$, *(2)* $F_f/V_r\mu_m$, *(3)* $F_r/V_r\mu_m$, *(4)* $P_mA_m/V_f\mu_m$, *(5)* V_f/V_r, *(6)* $\bar{X}^\circ$, *(7)* $F_f/V_f\mu_m$, *(8)* Y_E/μ_m, *(9)* $F_f/V_f\mu_m$, *(10)* $\bar{S}_f^\circ$, *(11)* $F_f/V_f\mu_m$, *(12)* αY_x, *(13)* $\beta Y_x/\mu_m$, *(14)* $\bar{K}_s$, *(15)* $\bar{P}_f^\circ$, *(16)* F_f/μ_m, *(17)* $P_m'A_m/V_f\mu_m$, *(18)* $\bar{P}_r^\circ$, *(19)* $F_r/V_r\mu_m$, *(20)* $P_m'A_m/V_f\mu_m$.

flask volume), and employ a cotton-gauze pad closure having a minimal resistance to gas exchange. Fermentor dialysis systems similarly must be provided with a high rate of humidified sparged air (more than one volume of air per minute per volume of liquid culture), efficient and vigorous agitation, baffling, and an effective antifoaming agent. Under such conditions, the measured oxygen-transfer rate should exceed 1 mmole of O_2 per liter per min.

When oxygen demand is met in dialysis cultures, media thought to be optimal at the usually limiting oxygen levels may actually be suboptimal in nutrients, and carbon starvation in particular often ensues. Supplemental feeding then can produce an astonishingly high population, unobtainable in ordinary systems. With *Serratia marcescens*, for example, the viable-cell count was extended to 2.5×10^{12} cells per ml in 48 hr, with the cell pack occupying half of the volume in a tube of the culture after centrifugation (44).

The relative geometry of a batch dialysis culture system also determines the degree of concentration achievable (*see* Fig. 23). That is, as the volume of the culture compartment is reduced relative to the volume of the reservoir compartment while the efficiency of nutrient conversion remains constant, then the degree of cell concentration will increase in direct proportion. In practice, this relationship holds up to a reservoir to

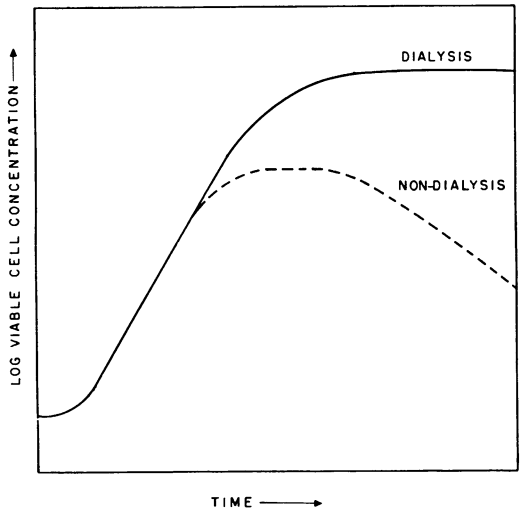


FIG. 30. *Schematic representation of growth curves in dialysis and ordinary batch culture systems.*

fermentor ratio of about 10:1, beyond which the conversion efficiency suffers (44, 51). The 10-fold ratio appears to be about optimal (58, 154).

Another obviously controlling variable in dialysis culture is the area of membrane available for diffusion (51). In general, the rate of growth should not be limited by the membrane area and rate of diffusion; if so, a linear rather than an exponential rate of growth may result, and at a lower mean rate (*see* Fig. 21). It is probably for this reason that active growth under dialysis conditions tends to change from exponential to linear when very high population densities are attained (44). That is to say, a greater area of membrane is needed to maintain exponential growth at high cell concentrations than at low ones.

Morowitz and Maniloff (104) have observed that *Mycoplasma* undergoes synchronized multiplication when passed through a filter membrane and inoculated onto Formvar-coated electron microscope grids placed on nutrient agar. This method of inducing synchronized cellular division may depend upon initial dispersion of cell aggregates and sizing of cells due to passage through the filter. Alternatively, cells may be depleted of internal primary food reserves and thereby reduced to a common base of metabolic activity. The cells then will receive nutrients from the medium at a rate limited by diffusion through the supporting membrane and, consequently, initiate synthesis and divide synchronously for a generation or more. This method has been used to study morphogenesis during the cellular division cycle.

It becomes evident from the above that the diffusional supplying of nutrient is a major controlling factor in dialysis culture. Likewise, the diffusional removal of metabolic products may directly or indirectly increase growth. The net result of dialysis is to lower the concentration of such inhibitors per cell, because the product is diluted into the reservoir volume and the cells are concentrated within the growth chamber. In many situations, the nature of the inhibitory product is not known. With bacteria such as lactobacilli, however, the observed enhancement of growth under dialysis conditions (51) is attributable to removal of inhibitory hydrogen ions and undissociated lactic acid molecules. A more dramatic and better-defined example is provided by studies on the usual inability of the obligate autotroph *Thiobacillus thiooxidans* to grow on glucose. It was reasoned that a toxic material is formed from glucose metabolism. This material subsequently was identified as pyruvate and was shown to be inhibitory at 0.2 mM, in either glucose- or sulfur-containing media. By continual dialysis of the culture to remove the toxic metabolite, however, the organism was grown readily in glucose-containing medium (17).

Adsorption may sometimes account for results seemingly attributable to dialysis. This was elegantly demonstrated in a series of experiments conducted 30 years ago by McClean (89) in an investigation of the cause of the increased production of hemolysin when staphylococci were grown in a cellophane sac. He clearly demonstrated that the result was not due to dialysis but instead to adsorption by the cellophane, which removed an unidentified substance from the medium. This substance did not affect cell growth but only affected hemolysin production. Other adsorbents (e.g., agar, kieselguhr, filter paper) were shown to duplicate the effect of the cellophane. Such an effect could account for the growth enhancement occasionally reported with use of dialyzed media (158).

A synergistic effect of agar on the yield of cells has been observed when some species of bacteria are grown in interface dialysis systems. With *Salmonella typhi* in an agar-liquid biphasic flask, for example, the density of population was increased as much as 20-fold over nondialysis yields, whereas the geometry of the system would explain only a 5-fold increase (161). Presumably, the effect could be duplicated in a dialyzer-dialysis system by including a particulate or macromolecular adsorbing agent in the liquid medium reservoir or, alternatively, the reservoir contents could be continuously circulated through an ion-exchange bed.

If the aim of a dialysis system is to produce as

concentrated a slurry of cells as possible, then advantage can be taken of osmosis and hydrophilic colloids. After the conclusion of the growth cycle in a dialyzer-dialysis system, the spent medium can be supplemented or replaced by a concentrated suspension of a material such as high molecular weight polyethylene glycol or dextran. As it is circulated opposite to the cell suspension in the dialyzer, water passes from the cell suspension into the reservoir until the culture becomes so thick that pumping is precluded. Although relatively slow, such osmotic dehydration provides a useful and safe method for terminally concentrating pathogenic microorganisms without the need for centrifugation and additional equipment (44).

Another important exploitation of the dialysis method is for the production of cells free from contamination by macromolecular constituents of the medium. This is accomplished simply by incorporating all of the medium constituents into the reservoir of a membrane dialysis system or into the solidified base of an interface-dialysis system, with only water initially in the culture zone. After allowance for a period of diffusional equilibration, the organism can be inoculated and grown in the clear diffusate. The principle has been applied to the propogation of gonococci (52), mammalian cells (32), and *Haemophilus influenzae* (51) in water diffusates of the protein-enriched media that otherwise would have been mandatory. The idea also is applicable to the production of uncontaminated macromolecular products such as enzymes and toxins (67, 125). A practical application is exemplified in the production of brucella cells for use as a live vaccine. Sterne (153) devised a semicontinuous method in which filter-sterilized medium was continuously circulated outside a dialysis tube that contained about 500 ml of a saline diffusate culture of *Brucella abortus*. This was harvested every 3 to 4 days; the material harvested was replaced with fresh saline, and another batch of cells was allowed to regenerate.

Cell production. Because of the concentrating effect during growth, which allows the attainment of extraordinarily high population density, the main use of dialysis culture has been in cell mass production. The obvious application is in the manufacture of vaccines.

Seemingly, the types of microorganism that can be propagated efficiently in dialysis culture systems are unrestricted. Table 4 lists representative genera that have been used, and provides key references. Although the general principles of the dialysis method may be expected to apply to any organism used, nonetheless special modifications will be required to accommodate certain types. Aerobic organisms require provision for adequate

TABLE 4. *Genera of microorganisms studied with dialysis culture in vitro*

Microorganism	Selected reference
Algae	
Scenedesmus	159
Bacteria	
Aerobacter	68
Bacillus	51, 55, 57, 68, 132, 137, 161
Bacteroides	51
Bordetella	15, 72
Brucella	57, 65, 108, 134, 153, 161
Clostridium	80, 132, 154, 155, 162
Corynebacterium	106, 161
Diplococcus	20, 142
Escherichia	51, 57, 115, 144, 161
Haemophilus	51, 138
Lactobacillus	51, 114, 115; Friedman, Thesis[a]
Mycobacterium	109
Mycoplasma	124
Neisseria	52
Nitrobacter	16, 157
Nocardia	Mobile Oil Corp.[b]
Pseudomonas	51, 161
Rhizobium	61
Salmonella	41, 57, 134, 161
Serratia	46, 51, 52, 161; Gerhardt and Gallup[c]
Shigella	47, 161
Staphylococcus	51, 67, 89, 107
Streptococcus	51, 139
Streptomyces	51
Thiobacillus	17
Vibrio	48, 51
Fungi	
Mycotorula	3
Neurospora	115
Oospora	115
Pencillium	51, 84, 146
Phytopthora	96
Saccharomyces	51
Protozoa	
Entamoeba	30
Eudiplodinium	31
Leishmania	24
Ophryoscolex	31
Polyplastion	31
Tetrahymena	51
Trypanosoma	24, 36, 79, 90, 113, 158
Tissue Cells	
KB, HeLa, L fibroblast	32
Sarcoma 180, L fibroblast	43
KB, HeLa, HEp	58, 59
Avian myeloblast	83

[a] Columbia Univ., New York, N.Y., 1967.
[b] British Patent 1,110,999, 1968.
[c] U.S. Patent 3,186,917, 1965.

aeration if maximum growth rate and extent are to be attained (*see* preceding section); anaerobes, growing only at a relatively reduced E_h, require the exclusion of air; and the growth of some types (e.g. *Bordetella pertussis*) is greatly improved if they are cultured symbiotically (*see below*). Provisions to insure safety of the operator have been built into systems designed for producing pathogenic microbes, especially by arranging for completely diffusional gas exchange (*see below*). Mammalian cells usually have been propagated with a view toward virus production (*see* following section). Not all types of organisms have been successfully managed in dialysis culture; e.g., inexplicably, *Streptococcus lactis* does little better in a dialysis flask system than under ordinary conditions.

Formation of nondiffusible products. The consequences of dialysis culture that apply to cells themselves in the main also apply to their nondiffusible products, i.e., particles or macromolecules that do not pass through ordinary dialysis membranes with a porosity of less than about 10 nm. (Obviously, this definition of "nondiffusible" is arbitrary because of the availability of membranes in almost a complete gradation of porosity.) For the most part, the production of protein exotoxins has been studied, but the principles seem applicable to the formation of other materials that accumulate extracellularly, e.g., endotoxins, enzymes, antigens, allergins, polymers, and even viruses and cell particles. The potential for application of dialysis culture is thus greatly extended, as indicated in Table 5.

TABLE 5. *Nondiffusible products studied with dialysis culture in vitro*

Product	Organism	Selected reference
Antigens		
Anthrax protective antigen	*Bacillus anthracis*	55
Enzymes		
Hyaluronidase	*Streptococcus haemolyticus*	139
Polysaccharides		
Levan	*B. subtilis*	68
Toxins		
Anthrax toxin	*B. anthracis*	132
Botulinum toxin	*Clostridium botulinum*	155, 162
Diphtheria toxin	*Corynebacterium diphtheriae*	106
Enterotoxin	*Staphylococcus aureus*	67, 89
Gangrene toxins	*Clostridium chauvoei*	154
Hemolysin	*S. aureus*	85, 89
	Pseudomonas sp.	86
Tetanus toxin	*Clostridium tetani*	80, 132
Viruses		
Adenovirus	KB	59
BAI avian leukosis virus	Avian myeloblast	83
Poliovirus	HeLa	59

Practical results are exemplified in the development of dialytic methods for the production of botulinum toxin, for use as toxoid. Polson and Sterne (125) first employed a regenerated-cellulose sac with saline, into which *Clostridium botulinum* was inoculated after allowing time for equilibration with the medium in the reservoir. Type D toxin was obtained in 7 to 10 days instead of 3 weeks and was 80 times more potent than that obtained from broth alone in an ordinary culture system. The seemingly faster production rate may only have been a reflecton of the concentrating effect of dialysis culture, which allowed detection earlier. The method then was scaled up by employing a sac containing 3.5 liters of saline diffusate surrounded by 35 liters of medium (155). The resulting toxin reportedly was 50 to 100 times more effective. These improvements are much greater than what is expected from the 10:1 ratio of reservoir to culture volume and probably reflects a concomitant increase in purity of the product, since potency is expressed in terms of toxicity per unit weight of isolated protein. Barron and Reed (11) reported comparable success in producing type E botulinum toxin, and the principle has been demonstrated to be applicable to the production of a number of other bacterial toxins.

In the above methods, the macromolecular product remains with the cells, withheld by the dialysis membrane. Theoretically (*see above*, section on Porosity), it is possible to separate and concentrate a macromolecular product from the cells by adding a small intermediate product chamber, with a microporous membrane filter between product chamber and culture chamber, and with an ultramicroporous dialysis membrane between product chamber and reservoir. The cells will be restricted to the culture chamber; the macromolecules will diffuse through the membrane filter and into the product chamber, but no further; nutrients and products of low molecular weight will exchange freely among the three chambers.

This concept of differential dialysis culture was embodied in a prototype flask, tested in principle with the production of diphtheria toxin (P. Gerhardt and D. M. Gallup, U. S. Patent 3,186,917, 1967), and then examined further with an enterotoxigenic staphylococcus (67). The total

amount of enterotoxin formed was about seven times greater than in the nondialysis control; two-thirds of this amount was separated in the product chamber, free from cells or macromolecular constituents of the medium and at about twice the concentration found in nondialysis culture. In comparison with ordinary dialysis culture, the differential system proved less concentrating (by 80%) and less productive in total toxin yield (by 20%). The choice between ordinary and differential dialysis culture will depend on the importance of separating the macromolecular product from the culture.

In principle, one might also exploit the fact that the porosity of agar gel increases with decreasing agar concentration. Consequently, a differential interfacial dialysis system probably could be devised.

Just as cell suspensions can be concentrated to a thick slurry by use of a hyrophilic colloid to replace spent medium, so also could macromolecular products be further concentrated by this supplemental method. The principle has been demonstrated with proteins (39, 81).

Use of dialysis culture for preparation of enzymes has been virtually unexploited. Rogers (139) employed a membrane dialysis system for producing hyaluronidase and also made an important ancillary observation. In studying the formation of two different types of hyaluronidase, he found not only that the concentrations of the enzymes were about six times higher than in ordinary cultures but also that the distribution of enzymes was different in dialysis culture. Just as was observed for cells grown in continuous culture, it appears that a different physiological balance is created by growth in dialysis culture. One cannot expect *a priori* to extrapolate directly from one condition of growth to another.

Another instance in the use of dialysis culture for enzyme production is afforded in a limited sense by the study of Hestrin et al. (68). They employed a solid-liquid interface system to grow *Aerobacter levanicum* and then extracted the endocellular levansucrase after autolysis. They also used a dialysis membrane system to enhance the production of levan by *Bacillus subtilis*, which excretes its levansucrase.

Several efforts have been made toward propagating mammalian cell suspensions for virus production in dialysis systems. Langlois and associates grew dialysis-cultured myeloblast cells from leukemic chickens in an effort to enhance the production of leukosis virus (83). Populations of nearly 2×10^8 viable cells per ml were maintained by a replacement technique with a shaken dialysis flask, in which the medium reservoir was continuously recirculated.

Gori (58) devised a dialysis-fermentor system in which several mammalian cell lines were propagated under conditions approaching steady-state. Populations of about 0.6×10^6 to 1.2×10^6 cells per ml were maintained with HeLa 53-1, KB, and HEp-2 lines. Subsequently, poliovirus was grown on the HeLa cells with a yield of 421 $TCID_{50}$ per cell, and adenovirus was grown in the KB cells with a yield of 116 $TCID_{50}$ per cell (59).

Production of diffusible compounds. Surprisingly, there has been relatively little work reported on the use of dialysis culture for the production of diffusible metabolic products. In principle, the technique may allow a higher productivity per unit quantity of cells because toxic products may be continuously removed. Such a product may be nonspecific in its toxic effect, as with the accumulation of an acid that increases hydrogen ion and undissociated molecular concentration, or it may be specific, as when the concentration of threonine exerts feedback inhibition on the activity of homoserine kinase.

Demonstration in principle of the former situation is afforded by results with lactic acid production. Gerhardt and Gallup (51) reported that the amount of titratable acid, as well as cell growth, was greatly increased by dialysis culturing of *Lactobacillus acidophilus*. Quantitative confirmation has been provided by a study of *L. delbrueckii* grown in ordinary batch culture, batch culture with continual nutrient addition, and batch dialysis culture (M. R. Friedman, Eng. Sc.D. Thesis, Columbia Univ., New York, N.Y., 1967). The results showed that lactate inhibits its own production at concentrations above 10 g/liter. With dialysis, lactate could be maintained below this critical level so that a maximum rate of production per cell could be achieved along with higher cell concentration. Thus, markedly higher overall acid production rates were obtained.

A convincing hypothesis for increased production of threonine has been developed by Abbott (*personal communication*). Even though tested experimentally only in a preliminary way, it deserves description as an exemplifying model for dialysis fermentation. Only threonine accumulates in cultures of *Escherichia coli* W, if appropriate auxotrophic mutants are selected that relieve repression and feedback controls on threonine synthesis and prevent diverted synthesis of lysine and methionine from aspartate (75). In this situation, a primary hindrance toward higher threonine yield appears to be a feedback inhibition by threonine on homoserine kinase. Apparently, this and comparable blocks can be relieved by dialyzing away the product as it is released from the organism, thereby maintaining the lowest possible intracellular levels of the con-

trolling metabolite. This type of approach would seem to have important implications for industrial fermentations.

Interbiosis. A natural extension of the use of dialysis culture for study of diffusible metabolic products is to bring together two or more populations of organisms on the opposite sides of one or more membranes, so that their metabolites can interchange. The situation may be beneficial to one population (parasitism) or both (symbiosis), and may be harmful (antibiosis) or innocuous (commensalism). Of course the entire application to in vivo situations represents such "interbiosis," but the concepts also have been examined with in vitro systems.

The search for a diffusible "toxin" to explain the parasitism of infectious disease agents motivated some of the pioneering research with dialysis culture (20), and Frost in 1904 used the method to show that diffusible products from saprophytic organisms inhibit typhoid bacilli (41). And, of course, the now common agar-diffusion method for detecting formation of an antibiotic compound by one organism active upon another is but a modification of the same principle.

Investigation of symbiotic relationships between population pairs has employed dialysis culture techniques in a number of different situations. Ritter (138) grew *Haemophilus influenzae* with *Staphylococcus aureus* across a membrane. Black (15) found that higher densities and prolonged survival of *Bordetella pertussis* were obtained when it was grown in a dialysis flask with *Corynebacterium pseudodiphtheriticum*. Marx and Haasis (96) discovered that sporangial formation was induced in *Phytophthora cumamoni* by diffusates from adjacently growing soil bacteria; sporangial formation was 10 times higher when a filter membrane was used instead of a dialysis membrane, suggesting that a large molecular substance was causing the effect. A symbiotic protective action was illustrated in the work of Schaumberg and Kirsch (144), who used dialysis techniques to show that *Escherichia coli* could substitute for reducing agents to stimulate the growth of anaerobic methane-forming bacteria. The *E. coli* culture was interposed between the methane bacteria and the atmosphere to scavenge oxygen and maintain a low E_h.

The most precise investigation of "symbiodialysis" was made by Nurmikko (114, 115). He grew auxotrophic mutants deficient for a specific amino acid in conjunction with strains producing it in an attempt to measure the specific formation rate. He reasoned that the amino acid was removed from the system by the symbiotic culture as it was formed, thereby eliminating feedback inhibition mechanisms. However, as shown by mathematical theory, a concentration of the amino acid must build up so as to provide a driving force for diffusion, and consequently the feedback inhibition can be alleviated but never completely eliminated.

Symbiotic mixed cultures in sewage sludge digestion were studied in dialysis culture by M. J. Hammer (Ph.D. Thesis, Univ. of Michigan, Ann Arbor, 1964). He employed a dialyzer-dialysis system to separate and optimize the acid and the gasification stages of anaerobic digestion. Raw sewage solids were fed into the primary fermentation vessel to establish a mixed enrichment culture for production of volatile organic acids, which diffused through a vinyl dialysis membrane into a secondary vessel to establish another enrichment for production of methane and other gases. The study demonstrated that the two stages could be separately controlled (e.g., in E_h and pH) and that the acid fermentation stage was rate-limiting in the process. It also nicely demonstrated the potential usefulness of dialysis culture as an enrichment method for obtaining microorganisms.

Gas exchange. Another little exploited feature of dialysis culture is the ability to provide oxygen or other required gases, and to remove carbon dioxide or other gaseous products, solely by diffusion. The principle seems especially applicable to the propagation of oxygen-requiring but fragile organisms, such as protozoan or mammalian cells, which may be injured by direct gassing or the addition of antifoam agents. It might also be useful for regulating the oxygen supply of microaerophilic organisms. An important practical consequence will be a greatly improved safety factor in the production of pathogenic microorganisms for vaccines, where a main potential hazard is aerosol formation. In current procedures, the culture vessel is usually aerated under pressure, and leaks, especially around the agitator shaft, almost inevitably arise. With dialysis aeration, the passage of air is removed from the culture to the opposite side of the membrane. Furthermore, it becomes evident that the supply of air need not be separately sterilized in such a system, thus eliminating a substantial source of contamination as well as a difficult and costly mechanical problem.

The possibility of dialysis aeration was first explored by Gladstone (55), who used the method to produce a cell-free protective antigen for anthrax without the danger of denaturation by gassing or from antifoam agents. Gerhardt and Gallup (51) further demonstrated the principle by inoculating a facultatively aerobic bacterium into the bottom chamber of a dialysis flask, rather than the top as in the usual procedure, and comparing the effectiveness of a dialysis membrane and a

filter membrane. The latter proved much the better and supported growth surprisingly near that attained with normal aeration.

The effective use of this principle depends on newly developed solution-transport membranes (*see above*, section on Types of Membranes), which selectively and rapidly transport gases. Humphrey and Gerhardt (*unpublished data*) employed silicone rubber membranes in a dialyzer–dialysis culture system, with humidified air continuously circulated through the dialyzer. Variables affecting the efficiency of oxygen transport included air velocity, culture liquid velocity, membrane area, partial pressure of oxygen, and culture mixing. Even though the measured oxygen transfer rates were less than 25% of those in well–sparged fermentors, the dialysis–aerated cultures attained population densities at least as great as those in conventionally aerated cultures. Similar results were obtained when dialysis aeration was used in conjunction with nutrient dialysis of cultures.

In Vivo Systems

Microbial growth within animal tissues is recognized often to differ from that in artificial media, as exemplified by the common observation of changes in virulence, antigenicity, metabolism, and morphology when infectious agents are transplanted into the laboratory.

Two general conditions distinguish the in vivo environment: antibacterial factors of the host and the nutritive quality of its tissues. These cannot be differentiated in tests in which the microorganism is introduced directly into the tissues, but can be if the agent is implanted within a membrane chamber. All known nutrients are small enough to diffuse through dialysis membranes, whereas phagocytic cells, antimicrobial factors associated with serum proteins, and other macromolecules are excluded.

The in vivo nutritive environment is distinguished by two further conditions that are less commonly recognized. First, there often is a flow of fluids and a limitation of nutrients that approach steady-state conditions. Second, the nutrient supply usually is regulated by diffusion through one or more membranes, surrounding either an organ or a cell in which the parasitic microorganism is localized. Thus, in vivo growth can best be simulated by continuous culture governed by dialysis.

The animal has a number of organs and tissues with a fluid environment in which dialysis culture can be managed experimentally. For the most part, this has been accomplished by implanting a sac or chamber directly into the region. In some cases, a number have been implanted so that one after another could be removed for examination. In other cases, a single device has been used, with an excess tube for sampling.

Peritoneal dialysis. The most obvious organ for in vivo dialysis culture is the peritoneum, and historically it was the first used, in the demonstration of bacterial toxigenesis (98). A considerable body of literature ensued along a similar vein of demonstrative experiment, including the culture of supposedly obligate parasites (110), alteration of virulence (112), prolongation of multiplication (49), and the role of phagocytosis (62). In virtually all of this early work, collodion sacs were used.

Cellophane, which is much more reliable than collodion and of known ultramicroporosity, came into use for in vivo propagation of microorganisms about 10 years ago. An excellent and exemplifying quantitative study was made by Gladstone and Glencross (56), who investigated growth of and toxin production by staphylococci in vivo. They employed a cellophane tube fitted with a fine nylon tube for inoculation and sampling, which was filled with saline and immersed in the peritoneal fluid. Filter-sterilized serum was used as an in vitro control. A total of 28 strains of staphylococci, both coagulase-positive and -negative, were grown in vivo with dialysis, and 5 strains were studied quantitatively in mice, rats, guinea pigs, and rabbits. In comparison with the in vitro control, the in vivo growth curves usually exhibited both a prolonged lag phase and a slower rate of exponential growth. A more rapidly growing mutant often arose and could be selected, especially with rabbits as the host animals. When the production of a hemolysin and a leukocidin were compared, however, staphylococcal strains that had failed to produce these toxins in vitro did so in vivo with dialysis. Furthermore, other strains produced toxin in amounts far exceeding those produced under the best conditions in vitro. The results nicely demonstrated the entirely different growth conditions for bacteria that prevail within an animal.

Later results demonstrated that the virulence of staphylococci was directly related to a diffusible hemolysin (74). When *S. aureus* was implanted intraperitoneally in a diffusion chamber equipped with filter membranes, necrosis usually occurred in the vicinity of the implant; but, when the chamber was fitted with dialysis membranes, no necrosis occurred. Analysis of the latter contents showed that hemolysin was still produced but evidently did not cause necrosis because hemolysin could not diffuse away. Growth curves with the two types of membranes appeared much the same, and the maximal populations were re-

ported to be about one log higher than in "typical" broth cultures.

The loss or gain of virulence after passage of infectious microorganisms through animals is a common phenomenon. Ogg et al. (116) investigated this phenomenon by implanting cellophane sacs inoculated with *Pasteurella pestis* into guinea pigs. In contrast to usual behavior, a virulent culture did not lose its virulence, nor did avirulent strains revert. Apparently, more intimate contact between the microorganisms and the host is required. In addition, they noted that a lower cell population was achieved in vivo than in an aerated in-vitro control. This last result would seem opposite to that reported by Houser and Berry for staphylococci (74). The fallacy in both comparisons, of course, is that neither employed a really comparable control situation such as was devised by Gladstone and Glencross (56).

Lorincz, Priestly, and Jacobs (87) found that mice have an interesting defense mechanism against a dermatophyte. The organism *Trichophyton mentagrophytes* was inhibited from proliferation when implanted intraperitoneally within a filter-membrane chamber, and the inhibition was not relieved when dialysis membrane was substituted for the more porous membranes. The evidence points to a dialyzable, water-soluble substance which is present in the serum and interferes with the growth of dermatophytes. Studies such as these should lead to a fuller understanding of in vivo defense mechanisms.

Mammalian cells and tissues also have been propagated in vivo within implanted dialysis chambers. The earliest attempts apparently were made about 1933 by Rezzesi (136) and Bisceglie (14), using collodion sacs and Ehrlich carcinoma cells. The method was revived and modified in 1954 by Algire and co-workers (130). Their "diffusion chamber technique" utilized microporous filter membranes to separate the cell culture from host cells, so that other noncellular growth-controlling influences of the host were allowed to act.

Quantitative measurement of cell growth in vivo was attempted first by Amos and Wakefield (10), who used direct counting methods on ascites tumor cells growing in free suspension within the diffusion chamber. The cells were found to grow exponentially only for a short period and after an initial drop in cell concentration.

A comparison of the growth rate of tissue cells in vivo and in vitro was made by Gabourel and Fox (43), employing lactate dehydrogenase activity as an index of cell numbers. A special double-membrane diffusion chamber was used to separate the implanted cells from the host cells that grew on the outer membrane surface. The growth of L-fibroblast and sarcoma 180 cells appeared to be exponential, both in vivo and in vitro, with over a 100-fold increase in cell density. However, a somewhat larger inoculum of the L-fibroblast cells was required in vivo to obtain consistent results. The generation times for both lines were similar, but they were much longer for the respective in vivo cultures. The slower in vivo growth rate may be caused by nutrient deficiencies due to low diffusion rates through the membrane surfaces and hindrance by cell growth on the outside membranes. However, directly comparable conditions in the two situations were not achieved.

The implant chamber technique has also been used to study cell differentiation in vivo. Shelton and Rice (147) presented evidence that normal leukocytes develop into plasma cells and fibroblasts within filter-membrane diffusion chambers; collagen accumulates concomitantly. These observations were substantiated by Petrakis, Davis, and Lucia (121), who used diffusion chambers implanted subcutaneously to demonstrate that human leukocytes differentiated to form macrophages and "polyblasts" initially and then developed into histiocytes and fibroblast-like cells. After 4 to 6 weeks, collagen formation was extensive and in some instances fat cells were formed. Over the same period, initially mature granulocytes underwent disintegration. In experiments with guinea pigs, in which normal and ascorbic acid-deficient leukocytes were used, Petrakis (120) further showed that ascorbic acid was required for the differentiation of leukocytes into fibroblastic cells.

The concept of the intact animal as a controlled environment has been used to study antibody production by tissue cells contained within a diffusion chamber. Holub (73) found that suspensions of spleen cells, lymph nodes, or lymphatic cells would produce antibodies against bacterial antigens when incubated within diffusion chambers in the peritoneal cavity of rabbits. He also showed, by trypan blue diffusion studies and microscopic examination, that the membrane walls were eventually blocked by occlusive cell growth on the membrane surface. Diffusion rates between the chamber and surrounding fluid dropped by a factor of about 8 over a 10-day period. Weiler (164) showed that antibody production by immunologically competent cells started 4 days after implantation intraperitoneally in a diffusion chamber and peaked between 8 and 15 days. No new exposure to antigen was needed to elicit this response. The evidence provided by titer analysis indicated that antibody was produced inside the chamber and not outside by the host cells. A further contribution, made with the

aid of diffusion chambers and important to an understanding of antibody formation, was reported by Fishman and Adler (38). They demonstrated that nonimmune lymph node cells in an implanted chamber could be stimulated to produce novel and specific antibody (to bacteriophage) by addition of a cell-free homogenate from macrophages that had been specifically stimulated in vitro. The active principle in the homogenate was found to be a low molecular weight ribonucleic acid (RNA) fraction, which was inactivated by ribonuclease. Diffusion chambers have an adjuvant effect on soluble antigens (1), however, and the possibility remains that the RNA carried bacteriophage fragments as an antigen-RNA complex. Antibody-synthesizing cells also can be explanted and maintained in vitro in a functioning state for at least 2 weeks, by use of a dialysis apparatus and a continuous flow of medium through the reservoir compartment (4).

In a series of studies on the growth and survival of tissue homografts, Algire et al. found that prolonged survival of the implanted tissue could be attained if the grafted tissue was protected from direct cellular contact with the host by peritoneal implantation within a finely porous diffusion chamber. However, if a membrane-filter material with pores large enough to allow lymphocytes to enter the diffusion chamber was used, the grafts were destroyed in a manner similar to unprotected implants. These results indicate that humoral factors are not responsible for graft rejection, but rather that antibodies transported by lymphocytes cause graft destruction (6, 9, 130, 163). In further work, in which heterografts were used, it was found that the survival time was reduced to a few days if the host was previously immunized, even when the transplant was placed in cell-impenetrable chambers (7).

Potter and Haverback (127) examined thyroid tissue within diffusion chambers implanted intraperitoneally in thyroidectomized dogs. Although the implanted tissues lasted for 10 months, they slowly deteriorated. This led the authors to conclude that the technique would not be satisfactory for clinical purposes.

Hemodialysis. A dialyzer can be connected into a bypass of the venous circulatory system to permit communication through a membrane with an external reservoir. Such hemodialysis systems have been widely employed as artificial kidneys and lungs, for detoxification, and for administration of anesthetics. The reader is referred to papers in the *Proceedings of the Society for Artificial Organs* and in a recent engineering symposium (29). Developments in this field often have application to dialysis culture of microorganisms, especially in the development of membranes and dialyzers. Although a main concern in artificial organ work is asepsis, hemodialysis would seem to offer considerable promise for extracorporeal study of septicemia. However, we have been unable to find such efforts reported in the literature and, in preliminary experimental work, have discovered only that a culture dialyzer and pump efficiently destroy erythrocytes (Molstad and Gerhardt, *unpublished results*).

Solid tissue implants. Membrane diffusion chambers containing cells can be implanted upon solid tissues as well as within the more usual liquid tissues employed in dialysis culture in vivo. Essentially colonial growth occurs. The technique has mainly been applied to the study of tissue homografts, but would seem to offer considerable promise for examination of virulence factors in infections.

Much of the study on tissue homografts and heterografts by Algire et al. (*see* preceding section) was accomplished with diffusion chambers implanted subcutaneously as well as intraperitoneally (6, 9), or only subcutaneously (8). Essentially the same results were obtained with the two sites (*see above*).

Similarly, the study of cell differentiation in vivo by Petrakis, Davis, and Lucia (121) was accomplished with subcutaneous implantation (*see* preceding section).

Castellanos and Sturgis (22) extended the work on graft transplants within diffusion chambers positioned intraperitoneally by studying the function as well as survival of endocrine gland transplants within chambers positioned subcutaneously. Ovarian tissues were transplanted to castrated rats, and uterine development was observed over a period of 2 months. They found conclusive evidence of estrogenic stimulation of the uterus, which implies that the grafted tissue maintained functional estrogenic capabilities over this period. Similar experiments with monkeys over a span of 7 months also showed continued functional performance (23).

Embryonated egg. Mention should be made of the chorioallantoic and other membranes of fowl eggs as natural dialysis systems, which were discussed earlier in the section on Colonial Growth with Dialysis. Although these have found considerable use in microbiology, the resulting microbial growth is colonial in character.

Rumen symbiodialysis. The biochemical reactions occurring in the rumen of cattle and other ruminant animals result from the digestive activity, mainly cellulolytic, of a mixed population of symbiotic microorganisms. The wall of the rumen itself acts as a dialysis barrier, so that the organ represents an enormously complex dialysis culture system. In vitro techniques applied to

study this ecosystem have been extensive (76), and include artificial rumen systems in which membrane chambers are filled with samples of rumen contents and allowed to dialyze against various media in an effort to simulate natural absorption. Some of these have incorporated the significant concept of operation under steady-state conditions (60).

The comparable in vivo studies are those in which some form of dialysis chamber is cannulated through a fistula and immersed in the rumen of an animal. Although much of the effort to date seems to have been expended in developing and testing apparatus, some progress has been made toward characterizing cellulose digestion (122), fatty acid production (37), hydrogenation of fatty acids (28), mixed microbial growth (28, 31), and protein synthesis (28).

In discussing applications of the dialysis technique to problems of rumen ecology, Fina and associates (37) pointed to vistas of study now susceptible to investigation: examination of pure cultures and defined mixtures in context with their natural environment, cultivation of fastidious organisms heretofore not cultivatable in pure form, and isolation of enzymes that are produced in the milieu. Such visions might well be extended to the dialysis culture technique in general. It remains to be seen, however, how well and how generally this promise is realized.

ACKNOWLEDGMENTS

We appreciatively acknowledge the library assistance of Lynn E. Sorensen and the resource of reference material accumulated in the theses of Elizabeth A. Tyrrell, D. M. Gallup, J. U. Pedersen, J. D. Herold, and H. E. B. Humphrey, Jr.

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The experimental work by the authors and preparation of this article were supported by contracts from the Department of the Army, Fort Detrick, Frederick, Md., and by Public Health Service grants GM-15152 and AI-06999 from the National Institutes of Health.

This is article no. 4504 from the Michigan Agricultural Experiment Station.

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