DIALYSIS CULTURE OF BACTERIA: DIALYZER DESIGN, NUTRIENT TRANSFER, GROWTH, AND DIALYSIS AERATION

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This is to certify that the

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AND DIALYSIS AERATION

presented by

Harold E. B. Humphrey

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ABSTRACT

DIALYSIS CULTURE OF BACTERIA:
DIALYZER DESIGN, NUTRIENT TRANSFER, GROWTH AND DIALYSIS AERATION

By

Harold E. B. Humphrey

A new and versatile dialyzer designed for biological application has been constructed. This instrument resembles a rectangular filter press with stainless steel end plates, one containing entry and exit fittings. Clamped between these is an alternating series of sheet membranes, stainless steel frames, and molded silicone rubber separators. Each separator integrally provides gasketing, entry ports, and a field of pyramidal elements which support the membrane with minimal masking and effectively induce turbulent flow within each dialysis chamber. The dialyzer measures 43 cm x 12.7 cm x 15 cm exteriorly, has an effective area of 288 cm^2 per membrane, has a resident volume of 65 cc per dialysis chamber, and may be expanded by addition of chambers and membranes.

The dialyzer was incorporated into a dialyzer-dialysis culture system which utilized a fermentor and separate reservoir of 3 to 10 liters, respectively. The efficiency of this system for nutrient transfer was evaluated by determination of half equilibrium times (ET_{50}) and overall permeability coefficients (P_m). Mean values of 144 $\pm$ 15 min (ET_{50}) and 4.7 $\pm$ .47 x 10^{-3} cm/min (P_m) were obtained with a 1% glucose solution and a single dialyzer membrane. Continuous operation, autoclaving, dialyzer position, and fluid direction had no effect on dialysis performance. Optimum rates were obtained with an area of .2880 m^2 and bulk

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flow rates above .5 L/min. The variations in performance associated with large overall liquid volumes and volume ratios and the significance of internal fluid dynamics were discussed.

The effectiveness of this dialysis culture system was demonstrated by the growth of Serratia marcescens. Liquid cultures were circulated from a conventional 5 liter fermentor through one side of the dialyzer while nutrient media from a separate reservoir was circulated through the opposite side. A series of growth trials established the extent of variation between repeated results and demonstrated the superiority of dialysis cultures, in terms of culture viability, culture concentration, and extension of the exponential and stationary growth phases, over comparable nondialysis cultures. Fluid circulation velocities had no effect on dialysis growth. A 1:10 fermentor to reservoir volume ratio, obtained by increasing the reservoir volume, produced optimal biological performance. The addition of dialyzer membranes resulted in improved culture concentrations with 15 membranes (.4320 m^2) producing a maximum viable cell concentration of 283 billion cells/ml. Diffusional access to the nutrient reservoir was shown to be instrumental in maintaining a culture environment which permitted the high dialysis growth yields, an extension of the stationary phase for over five days, and the concentration of cells on a relatively weak growth medium. In several instances the dialysis growth response to operational conditions was similar to that shown previously for nutrient transfer.

Culture aeration by dialysis gas transfer was demonstrated by incorporating silicone rubber membranes into the dialyzer. Liquid cultures of Serratia marcescens were circulated from a conventional 5 liter unsparged fermentor through the dialyzer past one face of the

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membranes(s) while humidified gas (air) was circulated past the opposite face. This aeration system enabled the use of non-sterile air, eliminated bubble-liquid interfacial effects, and reduced the power and antifoam requirements. The membrane represented the greatest resistance to oxygen transfer. This was reduced by the addition of membranes at the expense of operational efficiency. A single membrane (.0288 m^2) produced a K_La of 6.2 x 10^{-2} min^{-1} and an OTR of .0018 m moles 0_2/L/min. Six membranes (.1728 m^2) increased these values to 28 x 10^{-2} min^{-1} and .0250 m moles 0_2/L/min respectively. Increases in liquid velocity especially, gas velocity, oxygen partial pressure, and overall gas pressure improved the dialysis transfer of oxygen. Although the rate of oxygen transfer with six membranes was only 1/20th of that for a well sparged fermentor, dialysis-aerated cultures attained equal or greater population densities. In addition, comparable results were obtained when dialysis-aeration was combined with a nutrient-dialysis culture system.

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DIALYSIS CULTURE OF BACTERIA:

DIALYZER DESIGN, NUTRIENT TRANSFER, GROWTH, AND DIALYSIS AERATION

By

Harold E. B. Humphrey

A THESIS

Submitted to
Michigan State University
in partial fulfillment of the requirements
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Department of Microbiology and Public Health

1970

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Harold E. B.

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I wish to dedicate this thesis in memory of my father, Harold E. B. Humphrey, Sr., whose example of patience and persistence has been indispensable.

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ACKNOWLEDGMENTS

I wish to express my gratitude to my wife, Jane, for her help and encouragement during the course of this work; to our families for their faith and patience; and to Dr. Philipp Gerhardt for providing the opportunity.

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2. GENERAL INTRODUCTION

Dialysis has been defined as the selective transport of solutes through a semipermeable membrane (interface) in the direction of and in proportion to a concentration gradient (102). The principle was first employed by Thomas Graham over 108 years ago for the effective separation of colloids from crystalloids in urine (70). These pioneering experiments showed that the quantity of solute diffused in a given time was proportional to the concentration of the original solution, a principle from which Fick's law of diffusion has evolved. The dialysis process continues to be used for the separation, purification, and identification of solutes in research laboratories. The commercial economies afforded by dialysis of spent process liquors for recovery of processing chemicals constituted the primary focus and application of the dialysis process during the first half of this century (10). More recently the basic principle of dialysis has been extended to a number of unique applications such as artificial organs, ultrafiltration, fuel cells, packaging films, reverse osmosis, solute concentration, food processing, and administration of anesthesia.

Except for some isolated applications at the beginning of this century, dialysis was largely ignored in microbiology. However, since the early 1950's a surprisingly large number of microorganisms have been studied with <u>in vitro</u> dialysis culture; see Schultz and Gerhardt (141). The motives for employing the dialysis principle were as varied

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At first the dialysis culture equipment designed for use in most of the above investigations involved implantation of membrane sacs, suspension of dialysis sacs in vessels containing nutrients, fabrication of flasks with membrane bottoms, or other schemes. Frequently these were either poorly designed, adapted for a specific application, could not be duplicated, or were not adequately described. The lack of uniform or sound equipment design, as well as little or no reported data on the performance characteristics of the system employed, has made evaluation of the experimental results difficult and subject to question on the basis of the unknown influence of the equipment or system itself.

Recently, a dialysis culture system based on the separation and independent operation of the fermentor, reservoir, and exchanger regions has been developed by Gallup and Gerhardt (57). Such an arrangement affords a high degree of operational flexibility and control, utilizes conventional fermentation equipment, can be scaled to a larger

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In this thesis I seek to refine and extend the basic concept developed by Gallup and Gerhardt. The goal is to present a comprehensive demonstration and analysis of the operational parameters and the biological performance of dialysis culture within the identifiable confines of a system utilizing a new and specifically designed dialyzer and to extend, convincingly, the dialysis concept to a new application, culture aeration.

The first objective (Section 3) is to describe thoroughly the new dialyzer used in this work. This instrument represents the culmination of a design specific for biological application, which has evolved through several preliminary models over several years.

With the development of a workable and satisfactory dialyzer, it became possible to analyze the nutrient transfer characteristics of the entire dialysis culture system and the dialyzer itself under various operational modes similar to those which might be employed during propagation of microorganisms. The glucose-transfer data gathered (Section 4) will provide the background information necessary for the identification and explanation of operational optimums and the operational limitations of this system, permitting a more meaningful evaluation of the dialysis growth trials.

The third objective (Section 5) is to demonstrate by a series of dialysis growth trials the capacity of the system for propagation of a bacterial culture and its superiority over conventional methods. An

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The final objective (Section 6) is to extend, successfully, the dialysis concept to the problem of culture aeration. Features which have made dialysis attractive for culture solute transfer also make the concept potentially applicable to gas transfer. Recently developed membranes with high gas permeability have made this practicable. Successful development of the dialysis aeration process will open a new approach to supplying a gas environment to liquid cultures, especially heretofore sensitive species or those with complex or changing gas demands. It also represents a system by which oxygen transfer from gas to liquid could be investigated with a fixed and defined interfacial area and resistance.

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3. DIALYZER DESIGN

3.1 Introduction and Historical

Gallup and Gerhardt (57) have outlined several schemes for operating a dialysis culture of which the dialyzer-dialysis system appeared most practical and compatible with conventional fermentation equipment. The system may be operated in four modes: fully batch, fully continuous, batch fermentor and continuous reservoir, or continuous fermentor and batch reservoir basis; see Figure 8 in Schultz and Gerhardt (141). In all of these the key to successful operation is a suitably designed diffusion exchanger, or dialyzer.

A plastic and stainless steel laboratory dialyzer designed for chemical application (Graver Water Conditioning Company) was tried first (57). Although this unit successfully enabled the demonstration of a prototype dialysis system, its usefulness was limited by construction materials, membrane capacity, and fluid dynamics. Other commercial dialysis devices are available but most suffer from similar negative characteristics. This situation with chemical dialyzers apparently results from the historical application of dialysis to chemical separations such as caustic soda from rayon steep liquor, sugar extraction, drug purification, and acid recovery from metal plating liquors (10).

The most nearly satisfactory commercial exchangers for microbiology are those developed as artificial lungs and kidneys. Such dialyzers have

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been designed in three basic forms: the coil type, in which membrane tubing is spirally wrapped around a mesh cylinder (93); the plate-and-frame type, in which a series of flat membranes are sandwiched together with support units (144, 56, 46); and the capillary type, in which a bundle of fine membrane tubes is used (153). The rectangular plate-and-frame configuration was found to be mathematically the most preferable geometry (168) and seems most applicable to a microbiological culture system because of its simplicity, capacity, and durability. However, even the best of these hemodialyzers lack certain features necessary for successful culture application: namely sterilizability, bulk flow capacity, and full turbulence on both sides of the membrane.

After unsuccessful efforts to modify a chemical dialyzer, and before suitable hemodialyzers became available, Gerhardt, Pederson and the author undertook to design a dialyzer explicitly for dialysis culture applications. The design criteria for such a dialyzer included the following requirements: variable membrane area; adaptability for using different types of membranes; a geometric form which could be scaled up to industrial dimensions; minimal priming volume (and therefore minimum fluid holdup); positive gasketing; sterilizability; construction from nontoxic materials; induction of turbulence at the membrane face; and capacity for fluids of various viscosities. Construction of a series of models and prototypes culminated in the design and assembly of a dialyzer which satisfied these design criteria. This instrument is described below.

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3.2 Description

The dialyzer (Figure 1) basically resembles a rectangular filter press and consists of two end plates which compress an alternating series of molded separators, sheet membranes, gaskets, and frames. Each end plate is constructed from type 316 stainless steel (4-M)* cut 1/2 inch thick, 17 inches long, and 5 inches wide. The faces are machined to flatness. Holes that accommodate 1/4 inch diameter stainless steel clamping bolts are equally spaced around the perimeter to distribute compression uniformly and to align components during assembly. The back plate is blank except for the clamping holes. The front plate has threaded holes in each corner to accommodate four Swagelok entry fittings (14-M), which are sealed with O-ring gaskets and teflon thread tape. A back plate (not shown) of 3/4 inch pyrex glass (13-M) may be used alternatively, although this back plate requires the addition of 5 teflon-cushioned steel bars to accommodate the clamping bolts. It permits visual inspection of the fluids as they pass through the dialyzer. This feature was useful during physical evaluation of the dialyzer and in monitoring cultures.

Each of the repeating functional units consists of a flat membrane, gaskets and metal frames on both sides of the faces of two separators.

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*References designated with an "M" are found in the material reference citations.

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This unit may be visualized as a rectangular box 1/4 inch thick with the separators as top and bottom, gaskets and frames for the sides and ends, and the membrane bisecting the box into two dialysis chambers. The volume of each chamber is 65 ml and the exposed area of the membrane is .0288 m^2. The total membrane area may be increased simply by adding additional units between the end plates. Fifteen units, with a total membrane area of .5320 m^2, have been successfully employed. Since a single unit is only 1/4 inch thick, the 15-unit assembly still remains compact and easy to handle, measuring 17 inches long, 5 inches wide and 4 3/4 inches thick, and weighing approximately 15 pounds.

Any type of sheet membrane material may be used in this instrument. In continuity with previous work (57), membrane sheets were cut from regular Visking regenerated cellulose dialysis tubing of 3 in. flat width, .0016 in. dry thickness, and 5 mμ nominal pore diameter (28-M). Neither of these membrane materials are self sealing, necessitating use of gaskets. These are 1/32 inch thick, 30 durometer S-2000 silicone rubber (15-M), cut by means of a die in the shape of the stainless steel separator support frames.

The separator support frames (Figure 2), made of type 316 stainless steel sheets, are 16 inches long, 4 inches wide, and .05 inches thick. They are cut to fit over, and match, the perimeter sealing edges of the molded silicone rubber separators, as illustrated. These frames give rigidity to the perimeter sealing edges of the separators, bridge the triangular entry ports, and provide an even surface for seating the membrane (and membrane gaskets) over these critical areas.

Figure 1. Exte

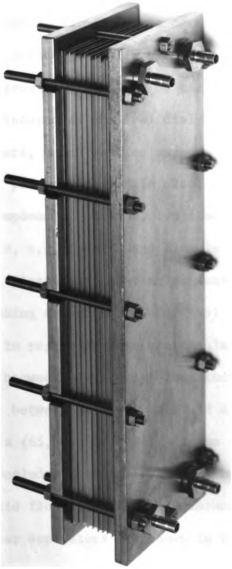

Figure 1. Exterior view of the dialyzer assembled with ten membrane units.

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The separator, a combination membrane support and fluid chamber, has been a difficult but essential design feature. Industrial dialyzers have metal or plastic separator plates with appropriately placed fluid conduits and wire screens (136), rubber coated extruded metal mesh (152), machined grooves (6, 85, 94, 139), or blade-like grids (76, 162) fastened or molded in the central area for membrane support. These separator styles generally are successful in promoting fluid turbulence and providing support for the thick and rugged membranes typical of the industrial chemical dialysis processes.

Hemodialyzers, which require support for thin delicate membranes and require the passage of fragile blood cells, are designed with separator units composed of screens, longitudinally grooved mats, or mats with cone fields, e.g. Leonard and Bluemle (102). In representative circular types, plastic or neoprene screens are wrapped adjacent to the membrane tubing around a cylinder (99), or are placed inside the tubing (138). In representative rectangular types, sheet membranes are compressed between plates with longitudinal grooves or ridges (89, 30, 46) or between mats with a field of staggered cone or column support elements (65, 84, 17, 122). In an evaluation of these styles, Peirce (120) concluded that the staggered cone design provided superior efficiency, fluid flow, and minimal membrane masking.

Our dialyzer separators are shown in Figures 2 and 3. They are custom molded from silicone rubber (15-M), comparable to Dow Corning Corporation medical grade S-2000 of 50-durometer hardness. The separator in one piece provides gasketing, entry holes and ports, and fields of membrane-support elements. It is completely reversible. The four entry holes in a series of separators and frames correspond with the

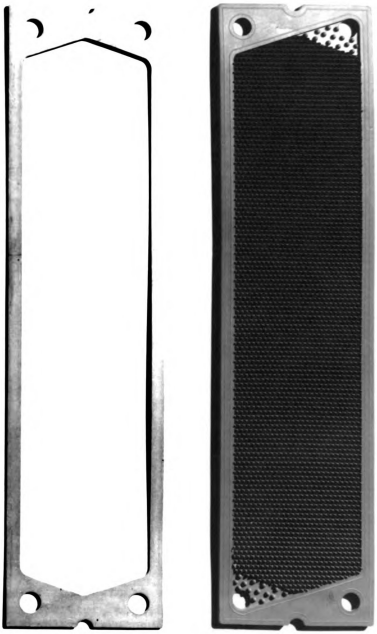

Figure 2. A stainless steel separator support frame (left) and molded silicone rubber separator (right). Gaskets also are used, which are shaped the same as the steel frame.

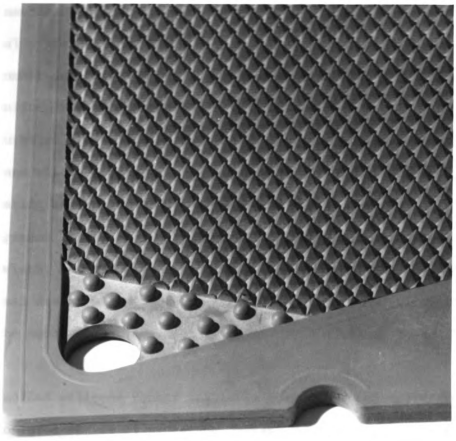

Figure 3. Dimensional view of a corner portion of the molded silicone rubber separator, magnified about 10 x. The gasketing beads, entry hole, triangular entry port with hemispherical supports, and pyrimidal membrane support elements are visible.

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entry taps in the front end plate. Separator entry holes lead into triangular entry ports (Figure 3) which contain a number of hemispherical supports for the bridging portion of the steel frame positioned over them. This area is critical with respect to potential leakage. The perimeter of the separator has two molded .003 inch high beads for sealing this surface.

The membrane support elements are unique. They consist of snubbed equilateral pyramids, each 1/8 inch in height, set in a field with lateral alignment and longitudinal staggering (Figure 3). These elements provide uniform membrane support with minimum surface masking and develop maximum turbulence as fluid strikes their faces and is diverted against the membrane with a scrubbing action. Fluid also passes around their edges creating eddies, as on the downstream side of a weir. In addition, the arrangement of the entire field provides even distribution of fluid over the width, but a tortuous path over the lenth, of the separator. This unusual design minimizes stagnant fluid movement and reduces laminar flow at the membrane surface, while maximizing bulk fluid mixing and flow capacity.

The molded silicone rubber separator just described represents the culmination of a series of prototype designs developed over the past five years. It supersedes a preliminary prototype which was used in some of the initial solute transfer evaluations reported in the following section. The preliminary separator has been fully described (see H. E. B. Humphrey, M.S. Thesis, University of Michigan, 1965). It was composed of two metal frames, similar to that shown in Figure 2, a 1/16 inch thick silicone rubber spacer gasket with fluid entry channels cut in opposing corners, and a molded neoprene mat for membrane support and

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turbulence induction. The mat was trimmed to fit into the central cavity formed by the above frames and spacer gasket. The current prototype separator combines the form and function of the former spacer gasket and mesh mat into a single molded unit.

A cost analysis for construction of an operational dialyzer is presented in the appendix.

The dialyzer is designed to accommodate the problems associated with propagating microbial cultures. It may be sterilized by steam, maintains sterility by positive seals, is nontoxic to growing cells, accommodates increasing culture viscosities associated with cell metabolism of nutrient media, allows high bulk fluid flow without undue internal pressures or holdup, and is easily assembled and integrated into a culture system.

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3.3 Summary

A new and versatile dialyzer designed for biological use has been described. It resembles a rectangular filter press with stainless steel end plates, one of which contains entry and exit fittings. Clamped between these are a series of sheet membranes, thin stainless steel frames, and molded silicone rubber separators. Each side of a separator forms, in conjunction with a frame and membrane, a dialysis chamber. The separator in one piece provides gasketing, entry ports, and membrane support. The field of pyrimidal support elements on each face induces turbulence as well as supports the membrane, .0288 m^2 effective area, with minimal masking. Dialyzer area is expanded by installation of additional separators and membranes. This instrument represents a reliable and efficient exchanger which may be incorporated into a functional dialyzer-dialysis culture scheme.

4. NUTRIENT TRANSFER CHARACTERISTICS

4.1 Introduction

The key to a successful dialysis culture system is separation of the three process regions (fermentor, reservoir, and exchanger) to enable independent control of each (57). Although a pilot dialyzer-dialysis system was successfully demonstrated utilizing conventional fermentor vessels and a commercial chemical dialyzer, unsuccessful efforts to modify the exchanger to the necessary specifications have prevented a complete analysis of the system. The essential function of an exchanger, is to exchange diffusible materials from one process region through the semi-permeable membrane to another region and to retain nondiffusible materials in the desired region under culture conditions. The dialyzer described in the preceding section satisfied such specifications.

Possession of a satisfactory dialyzer permitted a critical and complete analysis of physical, physiological, and culture influences on the solute dialysis characteristics of this dialyzer-dialysis system. The effect of these operational procedures, which are indicative of dialyzer performance, must be known in order to establish the parameters, limitations, and optimums for efficient dialysis culture operation. Ultimately such information will aid in the selection of satisfactory operational schemes and specifications for further dialysis applications. Information of this type, with the exception of membrane testing (61), has been lacking. Consequently, an analysis of solute transfer

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characteristics for our dialyzer and dialysis culture system under potential operating conditions is presented below. All operational procedures were examined, even those in which the results appear obvious, in order to thoroughly document and confirm the influence of these on performance. In addition, an explanation was sought for the selection of and the results observed with certain operational conditions and procedures. Although many of the conclusions were anticipated from the laws of diffusion, this work contributes a meaningful and necessary evaluation of nutrient dialysis within the system proposed for dialysis culture of microorganisms.

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4.2 Materials and Methods

The dialyzer was evaluated under conditions which simulated those for a growth trial with the dialyzer-dialysis culture system diagrammed in Figure 4. A 2-liter filter flask or a 5-liter fermentor and a 14-liter fermentor (24-M) served as the culture and reservoir vessels respectively. These were situated in a water bath equipped with a mixer (22-M), thermo-regulator (3-M), and immersion heater (2-M) for temperature control. The dialyzer was equipped with presoaked Visking regenerated cellulose dialysis membranes (28-M) and connected to the vessels by 1/4 inch I.D. rubber tubing (25-M). A positive displacement variable speed pump (27-M) inserted in the reservoir and fermentor circuits circulated the fluids from their respective vessels through the dialyzer and back. Glass "T's" capped with vaccine bottle stoppers were also placed in the fluid circuits to allow sample removal by syringe and needle. Unless noted otherwise, the reservoir was charged with one liter of a 1% glucose solution (which approximates the concentration in a growth medium), the fermentor contained 300 ml of distilled water, the liquid flow rates were 2 liters per minute, the dialyzer held one membrane (.0288 m^2), and the temperature was maintained at 30° C.

The dialyzer employed in most of these tests as well as all subsequent experiments and trials in this thesis was the model described in the preceding section. A preliminary prototype dialyzer, referred

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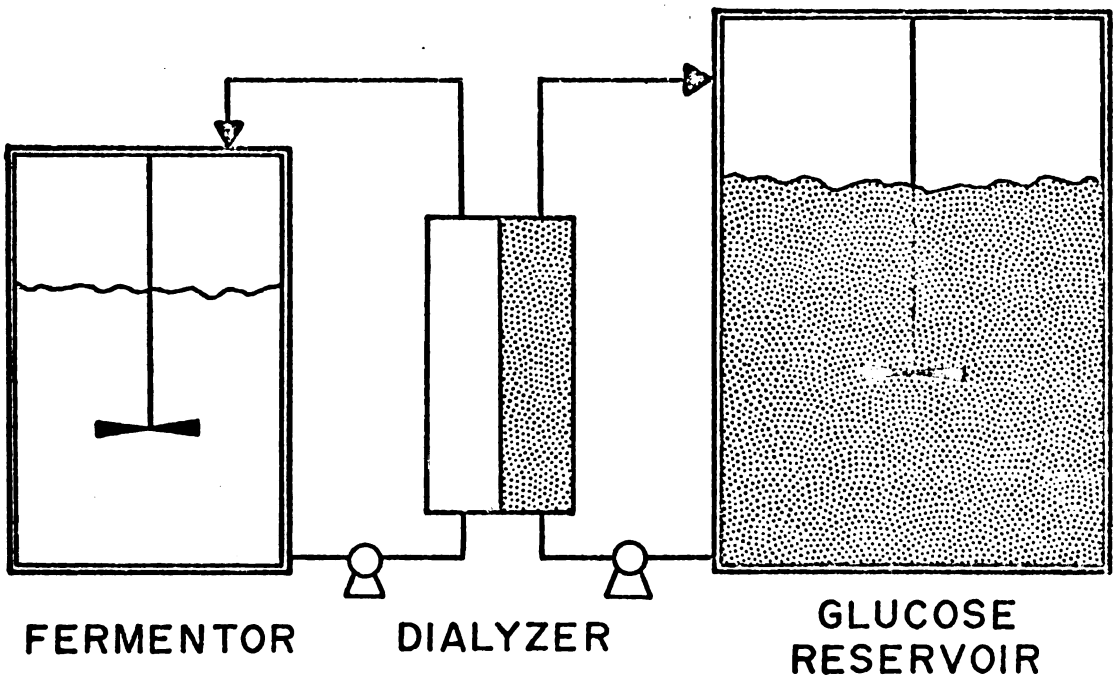

Figure 4. Schematic diagram of the dialysis system used for determining glucose transfer.

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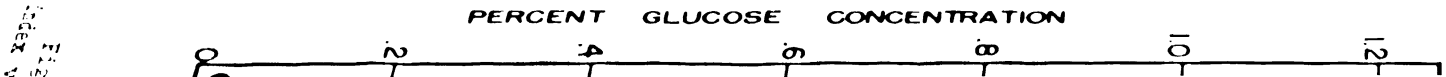
to in the same section, was used for tests measuring the influence of autoclaving (Table 1), duration of operation (Table 2), dialyzer position and flow direction (Table 3), membrane hydration (Table 4), membrane lots (Table 5), and temperature (Table 9 and Figure 13). These trials were reported in a previous thesis (H.E.B. Humphrey, M.S. thesis, University of Michigan, 1965) and are included here in order to provide complete documentation of dialyzer performance. This work was not repeated with the current model dialyzer, which utilizes molded silicone rubber separators, because separator selection would not appreciably alter the conclusions derived from the above trials.

Each experiment involved filling the fermentor and reservoir with their respective fluids (in a 1:3 ratio unless specified otherwise), assembling the dialyzer, connecting the necessary conduits, selecting pump and mixing speeds, and removing an initial sample of the fluids. The system was then set into operation and fluid samples removed periodically and analyzed for glucose concentration. The quantity of glucose transferred by dialysis from the reservoir to the fermentor was determined indirectly by measuring the refractive index (5-M) or directly by the colorimetric anthrone analysis. Glucose concentration is proportional ($\pm$ .05%) to refractive index, when no other solutes are present, from .1% to 1.2% (Figure 5). The anthrone method was employed for solutions containing less than .1% glucose or for situations where interfering solutes were present.

Hydraulic pressure measurements were made by inserting glass tubes with water or mercury columns into the conduits near the dialyzer entry and exit taps. The columns also could be connected via syringe needles inserted through the separator wall into an individual dialysis chamber.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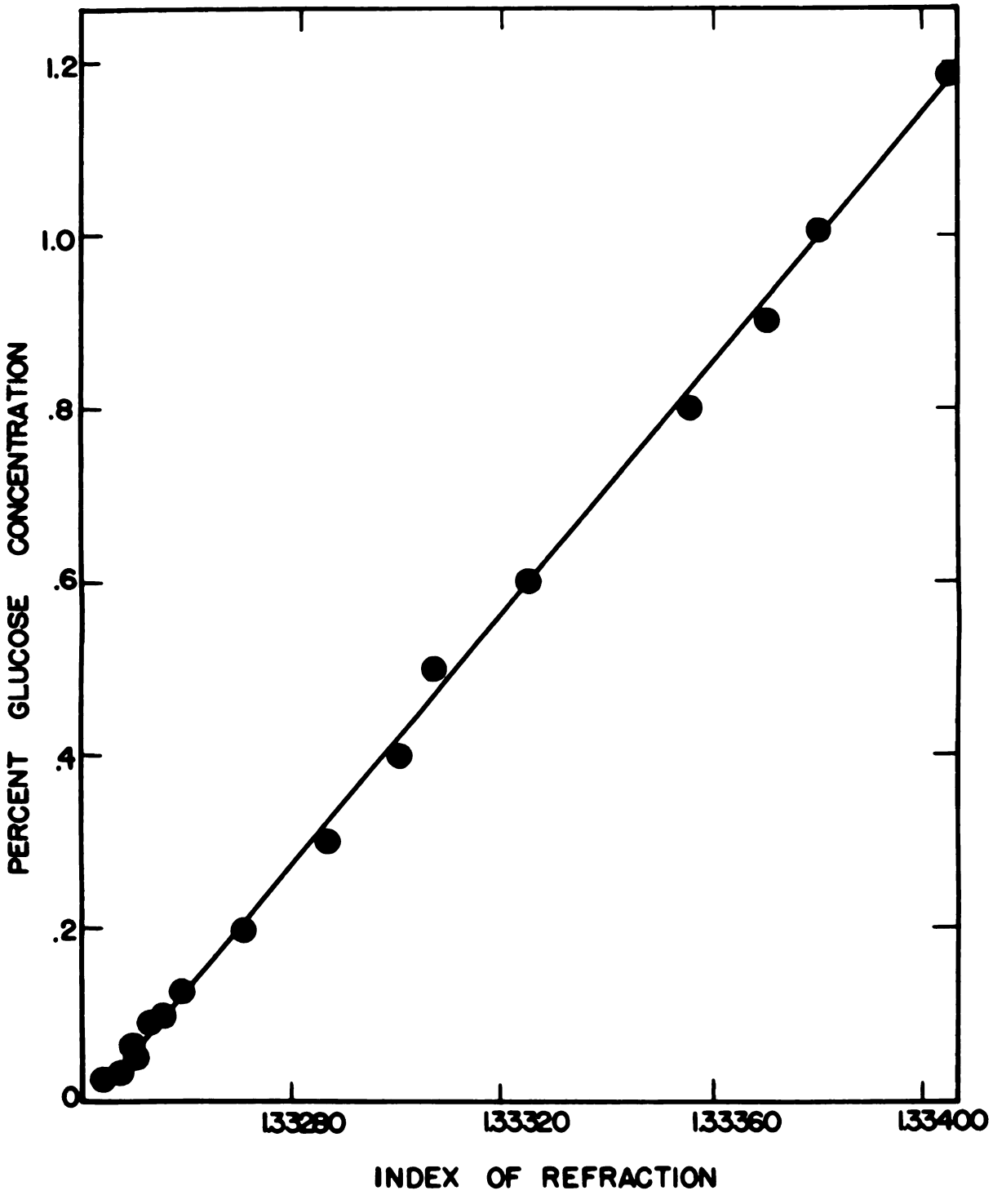

Figure 5. Least squares fit of the correlation curve for refractive index values (n_{25}^{D}) and glucose concentrations.

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Measurement of column heights indicated pressure gradients across the area being tested.

The parameters used for evaluating glucose dialysis rates in this dialyzer-dialysis system were the half-equilibrium concentration time (ET_{50}, in minutes) and the calculated permeability coefficient (P_m, in cm/min). These were determined from glucose concentration data for the reservoir and fermentor regions during operation, and are defined as follows:

ET_{50} = Number of minutes required for the solute concentration in the fermentor to reach one half of the equilibrium concentration for the whole system.

P_m = The coefficient describing the permeation (dialysis) rate for a particular solute and membrane material within a system of defined volume and interfacial area. It is related to the ET_{50} by:

$$P_m = \frac{\ln 2}{(1/V_1 + 1/V_2)\, A_m\, ET_{50}} ,$$

Where V_1 and V_2 = the fermentor and reservoir volumes respectively and A_m represents the dialyzer membrane area.

The ET_{50} notation is a convenient means for evaluation of the dialysis process because it accounts for membrane factors, solute gradients, bulk fluid flow, and fluid turbulence and because it is easily

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determined. The ET_{50} and P_m notations apply only to the diffusional transfer of solutes as determined in this section. These could be developed for application in a fermentation system if factors considering the rate and extent of cell growth as well as growth inhibitory product formation were included in the basic equations. For each dialysis experiment an equilibrium and half-equilibrium concentration are calculated for the system and the solute receiving vessel from the concentration of the initial sample. Samples removed during the dialysis run are analyzed for glucose concentration. A time plot is made (Figure 6 is an example of such a plot) for each experiment from which the rate of solute transfer can be determined and reported as the ET_{50} or the calculated P_m value. The influence of an imposed experimental variable on the solute transfer rate between the reservoir and fermentor is reflected in ET_{50} and P_m values. Diminishing ET_{50} values indicated more rapid transfer and increasing P_m values indicated superior efficiency.

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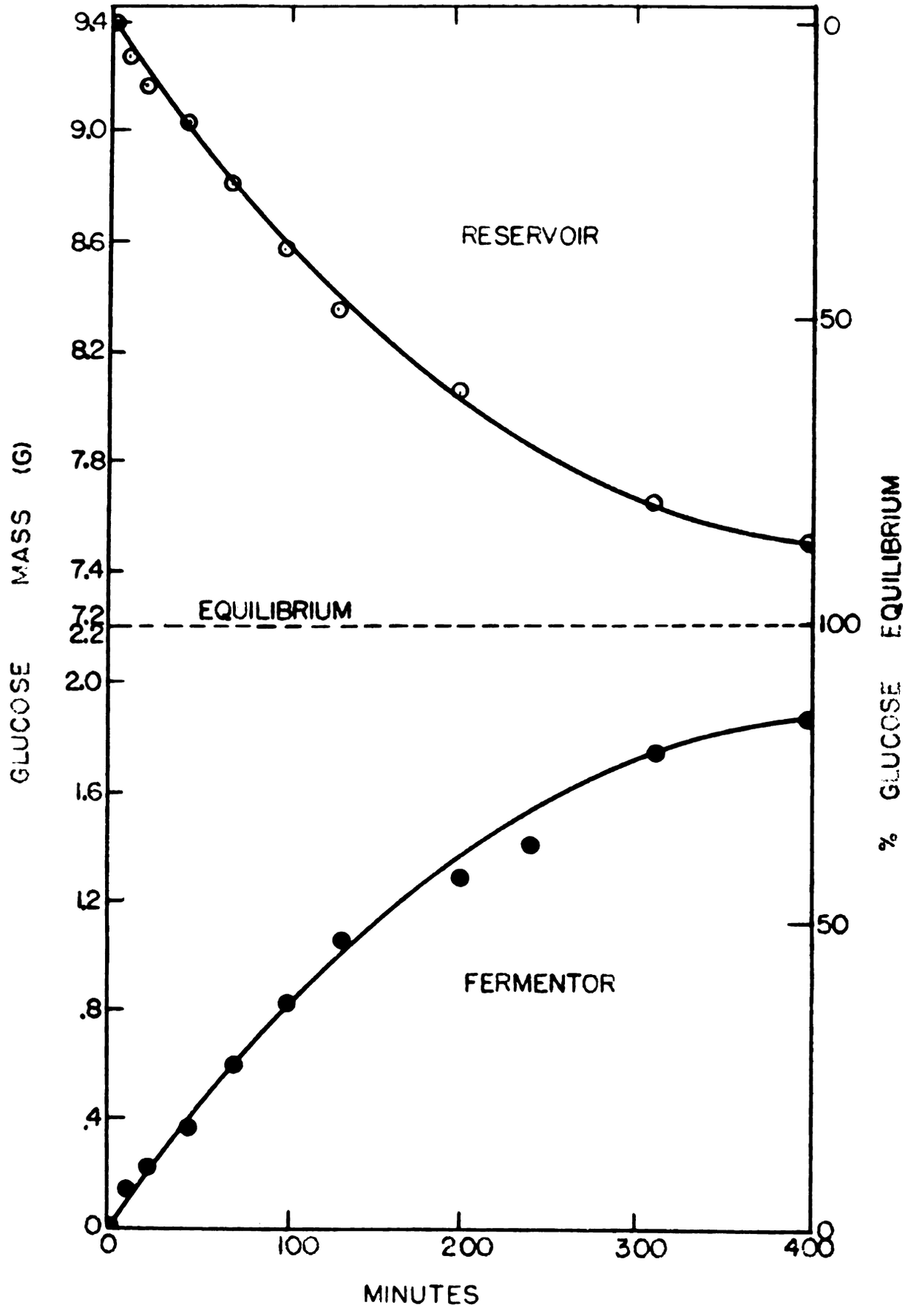
4.3 Results

The process employed in all of the experimental work was equilibrium dialysis. This involved continuous circulation of the respective fluids through the dialyzer. The ongoing transfer of solute produced a continuously diminishing concentration gradient. Therefore, both the mass and concentration of solute gradually increased in the fermentor and decreased in the reservoir until the system approached a concentration equilibrium end point (Figure 6). The illustration, a typical dialysis trial, shows an asymptotic approach to the quilibrium condition and a greater total mass of solute present in the reservoir than in the fermentor, throughout the process and at the end point. This reserve quantity of solute, a consequence of the larger (3.3 times) reservoir volume, represents one of the advantages dialysis holds for biological applications as will be shown in a later section.

Each dialysis trial was analyzed for glucose transfer to the fermentor, and the data were plotted as illustrated in Figure 7. As shown, the ET_{50} (half equilibrium time) value for the trial was determined by extrapolation from the calculated half-equilibrium concentration for the system in question. The dialyzer was equipped with one membrane and the dialysis system operated at: 2 liters/min liquid flow rates; 1 to 3.3 fermentor to reservoir volume ratio; 1% initial glucose concentration in the reservoir; and 30° C temperature. The mean ET_{50} for twelve duplicate runs was 144 min $\pm$ 15 min over a range of 110 min to 170 min.

Figure 6. Correlation of glucose concentration in the fermentor and reservoir with time during an equilibrium dialysis trial with a 300 ml fermentor and a 1000 ml reservoir.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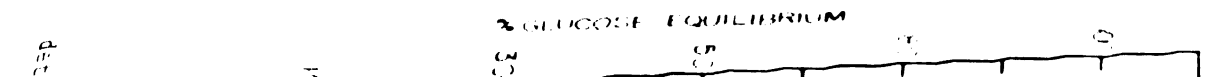
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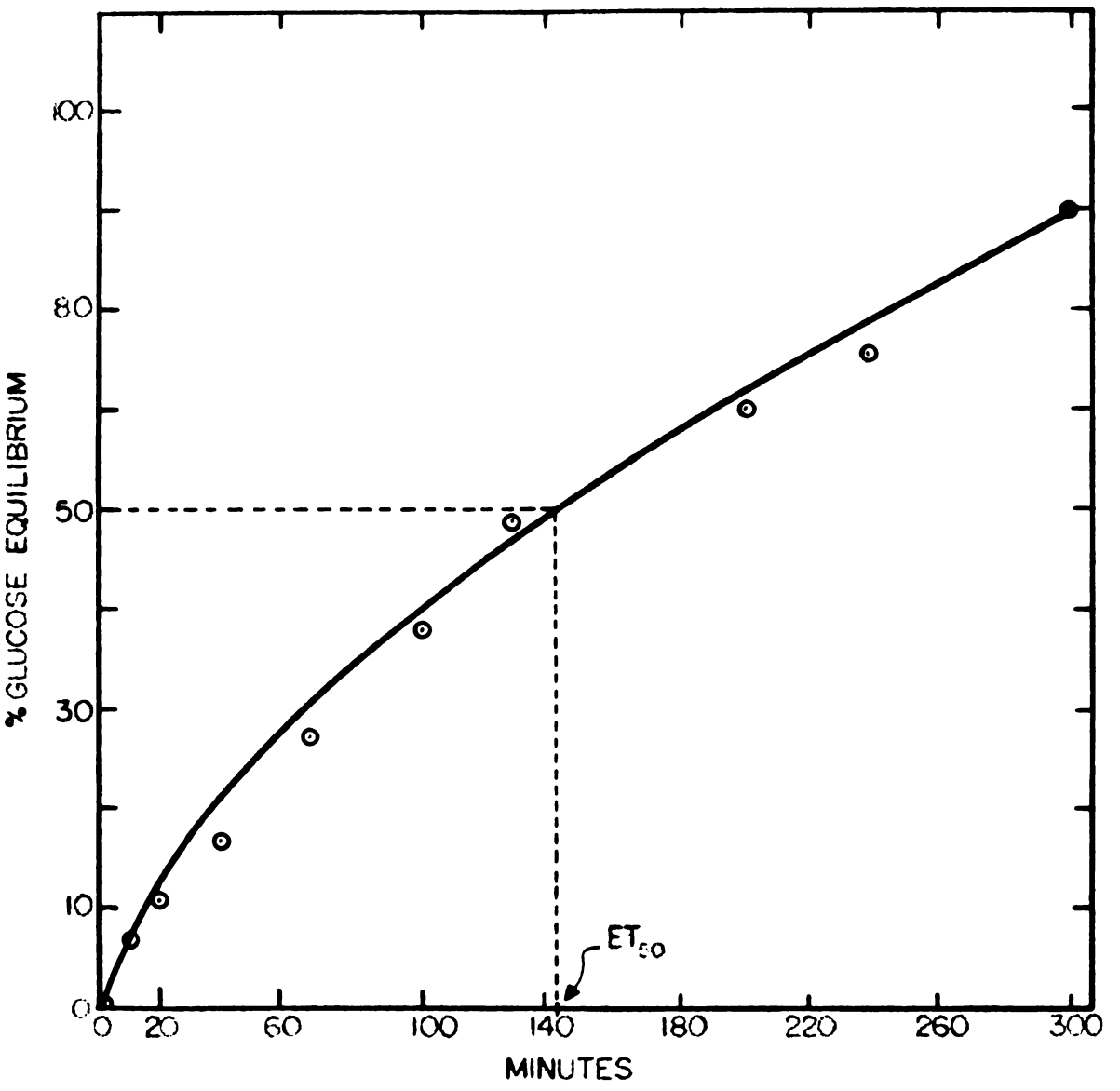

Figure 7. Example of a glucose dialysis rate plot used for ET_{50} determinations.

The mean P_m was 4.69×10^{-3} cm/min $\pm$ $.47 \times 10^{-3}$ cm/min over a range of 4.25×10^{-3} to 5.68×10^{-3} cm/min. The operational conditions given above and the mean values obtained for them represent the standards against which all subsequent data in this section were compared.

The influence of steam sterilization (Table 1), length of operation (Table 2), and fluid flow direction within the dialyzer and its position during operation (Table 3) were evaluated. These manipulations did not change the solute dialysis rates within the standard deviations shown above. Membranes should be hydrated for at least ten minutes before use (Table 4). Differences in dialysis rates were noted with different manufacturer's lots of membranes (Table 5).

Enlarging the dialyzer membrane area resulted in superior ET_{50} values but poorer efficiency (P_m) until an optimum area was reached (Figure 8). For the 1% glucose system, 7-10 membranes (.202 to .288 m^2) produced optimum ET_{50}'s of 40 minutes (for a 1:3 fermentor to reservoir volume ratio) and 60 minutes (for a 1:1 ratio). Further addition of area did not significantly change these values. Increasing the dialyzer area also increased the internal volume and fluid holdup time (Table 6). Predicted and measured holdup times did not correspond at the largest areas.

A leveling trend in dialysis rate was observed as the liquid flow rates through the dialyzer were increased (Figure 9). With a single membrane in the dialyzer, a significant reduction in ET_{50} (from 268 minutes to 226 minutes) occurred as the flow rate was increased from 1/2 L/min to 1 L/min. Above 1 L/min, the ET_{50} did not change significantly. Additional membrane area lowered the overall values but did not change this trend. Fluid turbulence, measured by visual

Table 1. Influence of steam sterilization on glucose dialysis.

EXTENT OF AUTOCLAVING (Hours)	ET_{50} (Min)	P_m (Cm/Min)
0	150	.00453
2	166	.00426
4	156	.00435
6	158	.00430

Table 2. Influence of duration of dialyzer operation on glucose dialysis.

LENGTH OF OPERATION (Hours)	ET_{50} (Min)	P_m (Cm/Min)
0	140	.00472
24	122	.00557
70	145	.00468
109	154	.00441
135	150	.00453

Table 3. Influence of dialyzer operating position and fluid flow direction on glucose dialysis.

DIALYZER POSITION	COUNTERCURRENT FLOW		CONCURRENT FLOW	
	ET_{50}(Min)	P_m(Cm/Min)	ET_{50}(Min)	P_m(Cm/Min)
Horizontal	148	.00459	152	.00447
Vertical	152	.00447	148	.00459

Table 4.

Table

Table 4. Influence of membrane hydration time on glucose dialysis

LENGTH OF HYDRATION BEFORE USE	ET_{50} (Min)	P_m (Cm/Min)
3 Minutes	194	.00350
4 Minutes	182	.00363
10 Minutes	139	.00488
2 Days	142	.00480

Table 5. Influence ofmanufactured lots of membranes* on glucose dialysis

MEMBRANE LOT	ET_{50} (Min)	P_m (Cm/Min)
June 1964	135 to 160	.00503 to .00425
September 1964	160 to 180	.00425 to .00377

* Both lots were Visking regenerated cellulose membranes (Union Carbide Corporation); regular type, .003 μ pore diameter.

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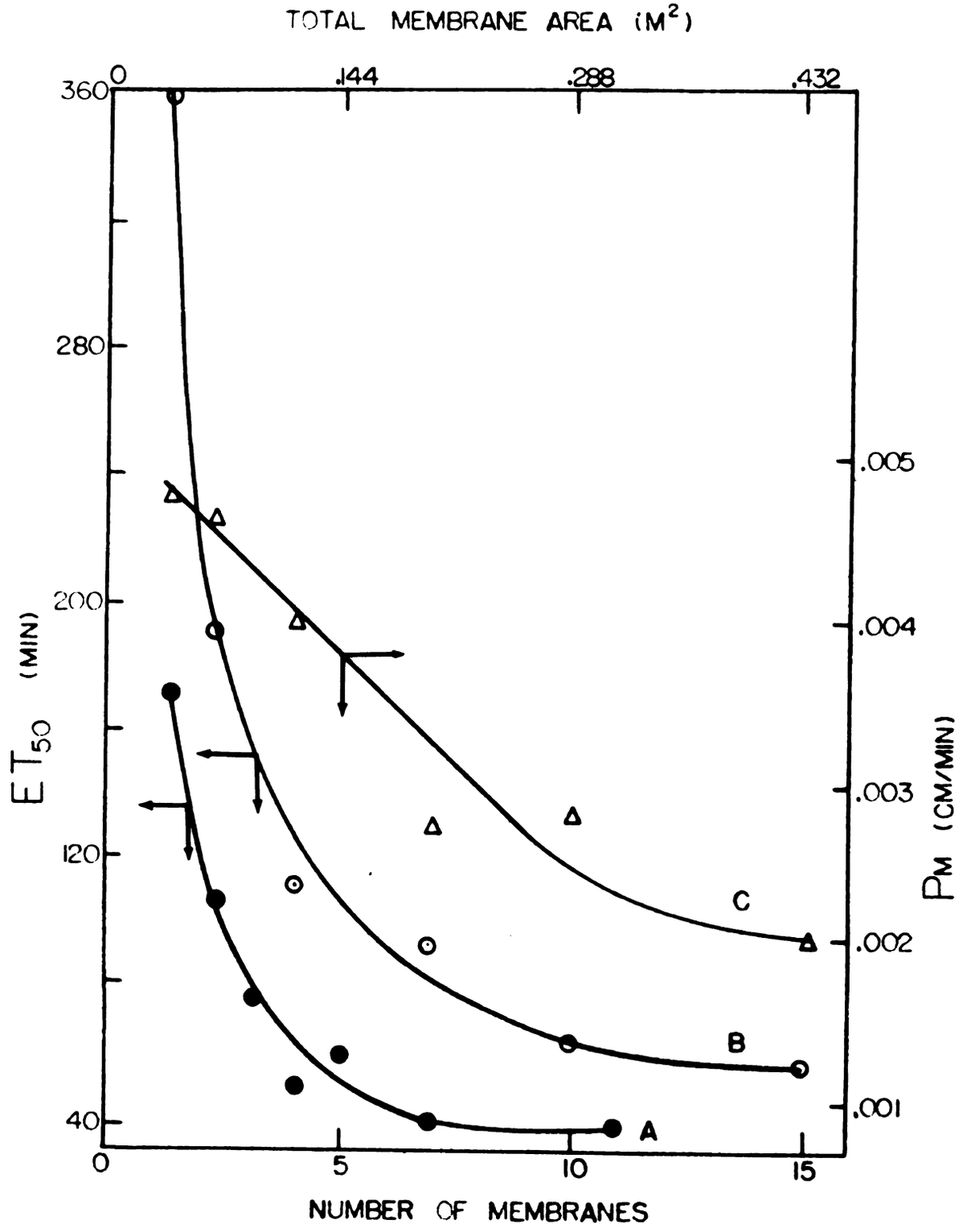

Figure 8. Influence of the number of membranes on glucose dialysis. Fermentor to reservoir volume ratios of 1:3 (curve A) and 1:1 (curves B and C) were used.

Table 6. Influence of number of membranes on dialyzer volume and fluid holdup time.

NUMBER OF MEMBRANES	TOTAL AREA (M^2)	DIALYZER VOLUME (Ml)	CALCULATED FLUID HOLDUP (Sec)	EXPERIMENTAL FLUID HOLDUP (Sec)
0	0	65	2	9
1	.0288	130	4	8
5	.1440	390	12	16
10	.2880	715	21	16
15	.4320	1040	31	16

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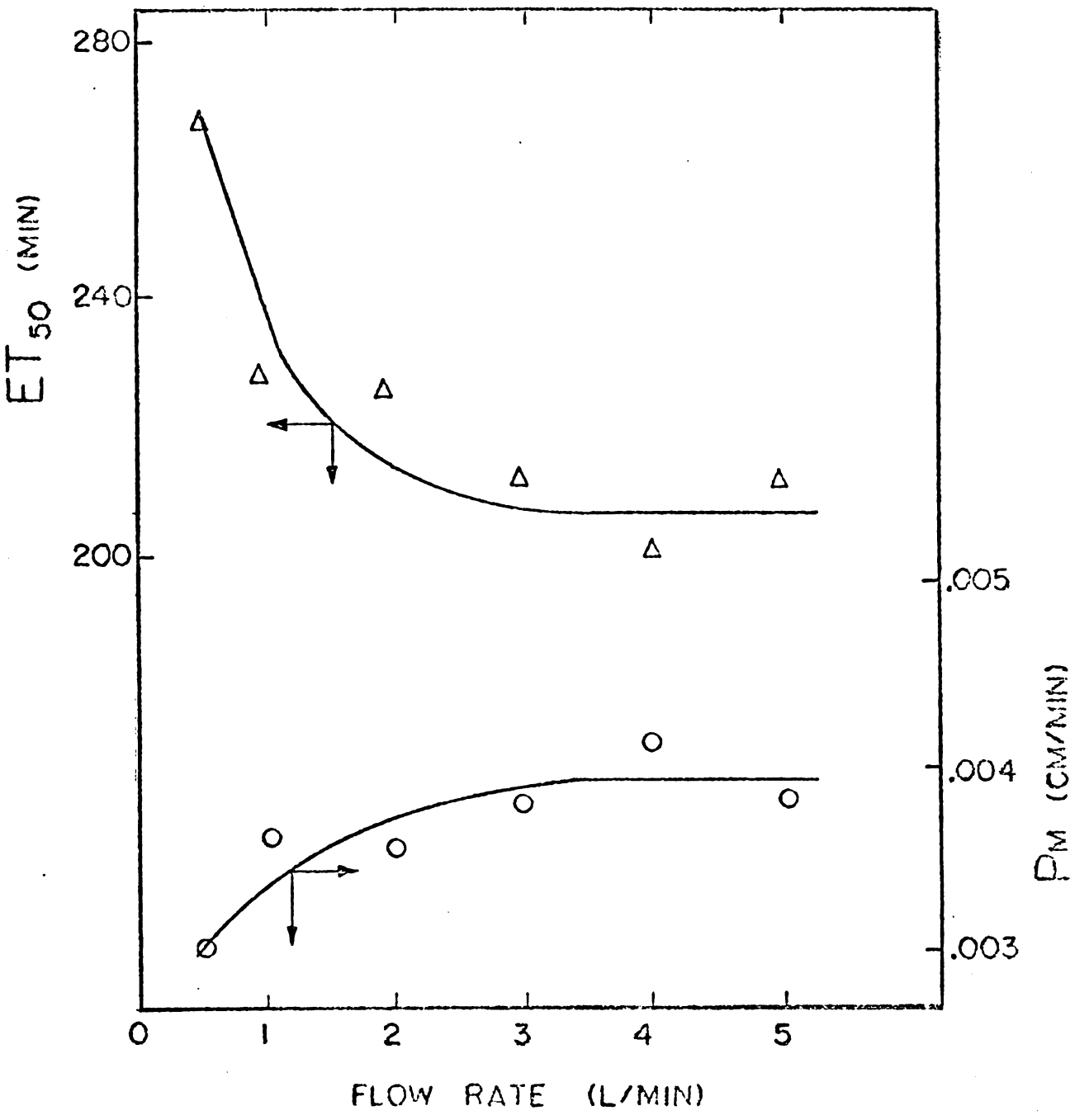

Figure 9. Correlation of glucose dialysis with the fluid flow rate through the dialyzer.

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observation of gas bubble movement within the dialyzer, first occurred at 1/2 L/min and continued to the maximum flow of 5 L/min. The pressure drop (ΔP) across each of the dialysis chambers adjacent to a membrane rose, but not identically, as flow rates were increased (Figure 10). This increase in pressure gradient across a given chamber was small (0-80 cm H_2O), was reduced as additional membranes were added to the dialyzer, and was similar at flow rates of 1 and 2 L/min when five or more membranes were present in the dialyzer (Figure 11). However, the pressure drop for 5 and 10 membranes differed when flow rates greater than 2 L/min were used (Figure 12). The pressure drop across individual chambers was less than across the whole dialyzer, but the response to applied conditions was similar for both.

The temperature under which the dialysis system was operated had a nearly linear effect on glucose dialysis rates, which rose with temperature from 4°C to 30°C (Figure 13). The best transfer and efficiency occurred at 60°C.

When the initial reservoir glucose concentration was increased above 10 g/L (1%) slower dialysis rates and poorer efficiency were observed (Figure 14). The diffusivity of glucose declined as concentration rose (Figure 15). In contrast to ET_{50} values, the initial dialysis rates (over the first 100 minutes) rose when the same increases in concentration were used (Figure 15).

The dialysis system was easily enlarged by use of larger vessels and/or liquid quantities. The resulting total volume increase, from .6 liters to 6 liters for example, resulted in higher ET_{50} and P_m values (Table 7). Although the initial concentration (10 g/L) was kept constant, an increase in volume caused an overall increase in the total

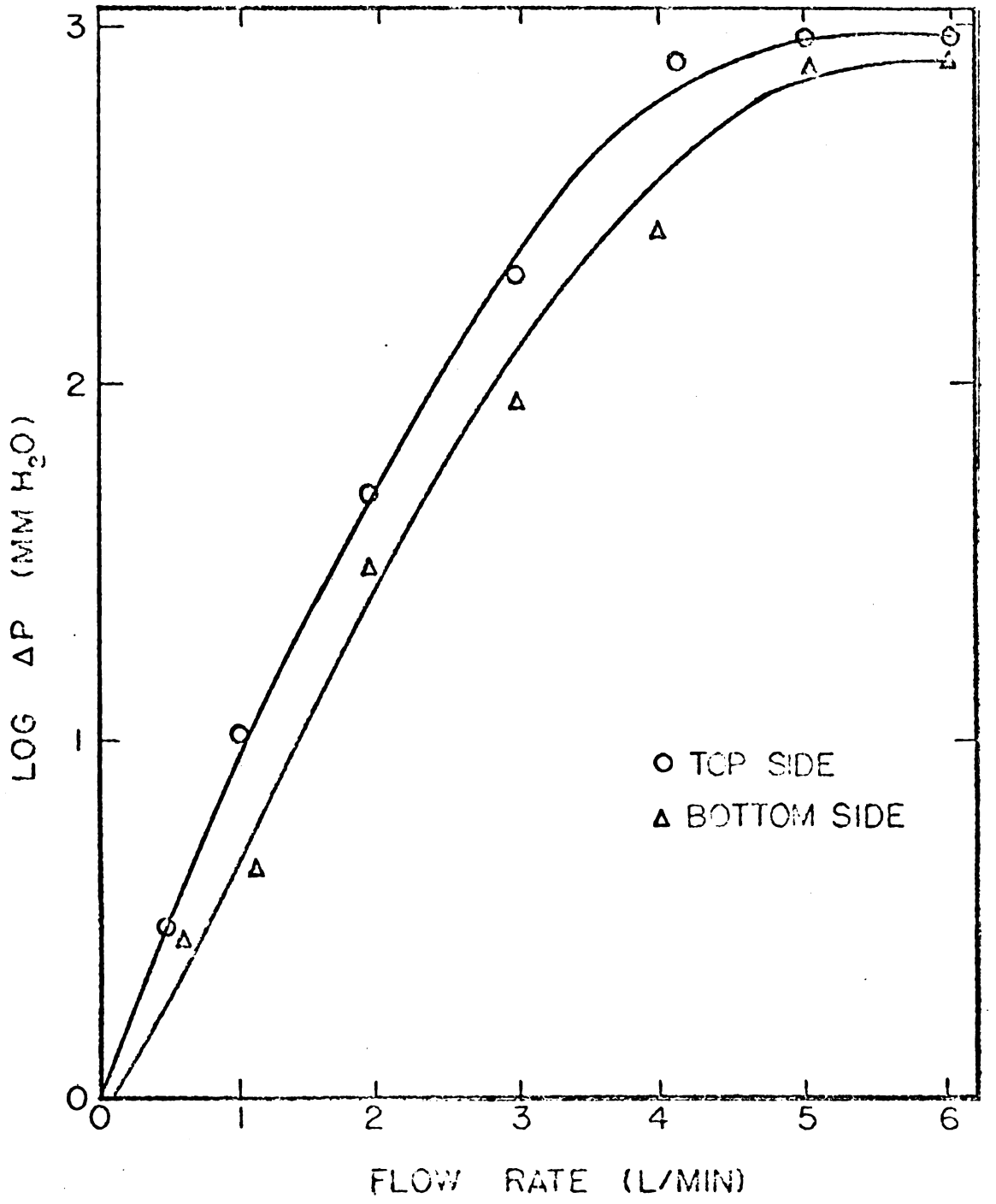

Figure 10. Correlation between fluid flow rate through the dialyzer and pressure drop in the dialyzer chambers adjacent to the membrane.

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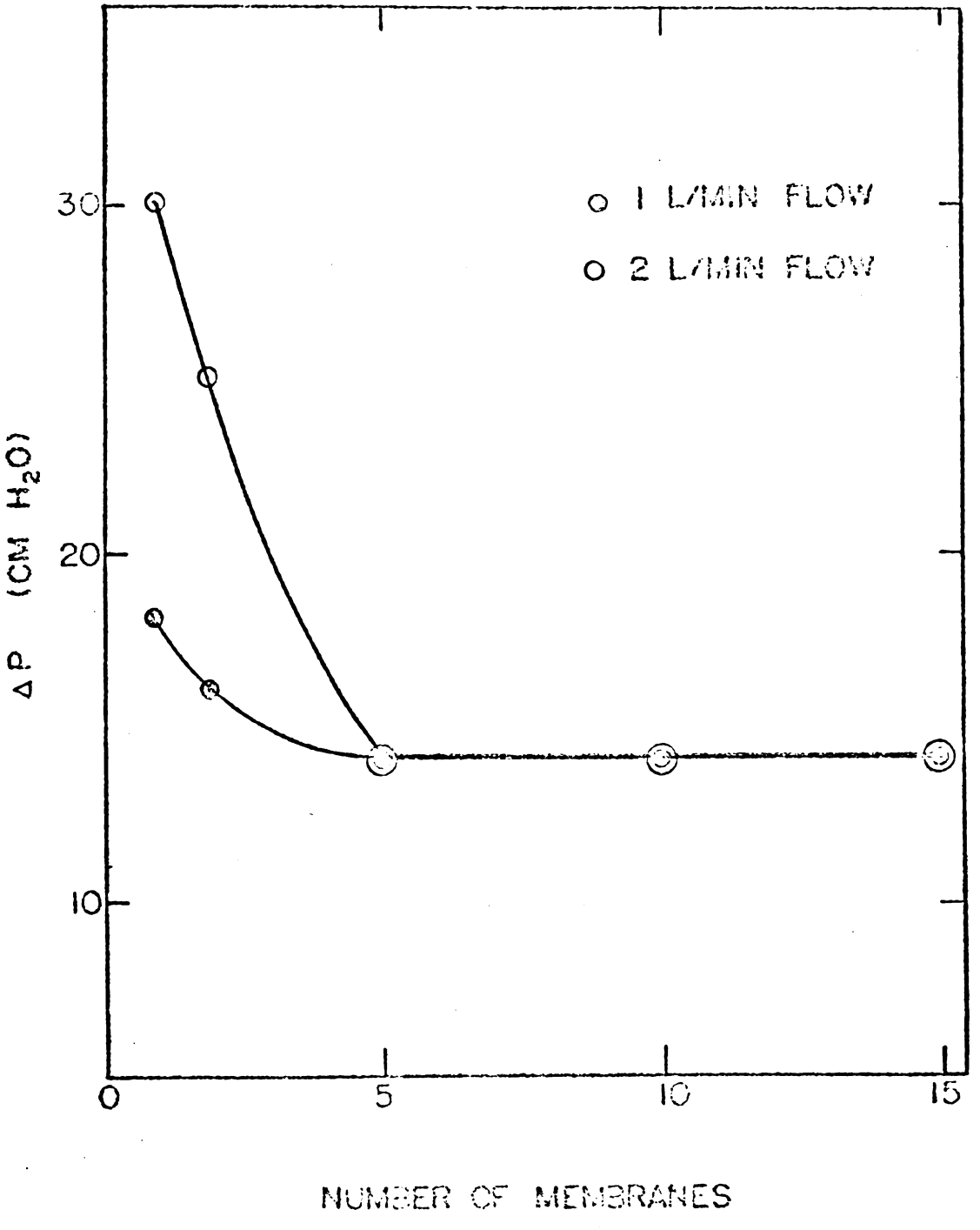

Figure 11. Influence of dialyzer membrane area on the pressure drop across a given dialyzer chamber at fluid flow rates of 1 and 2 L/min .

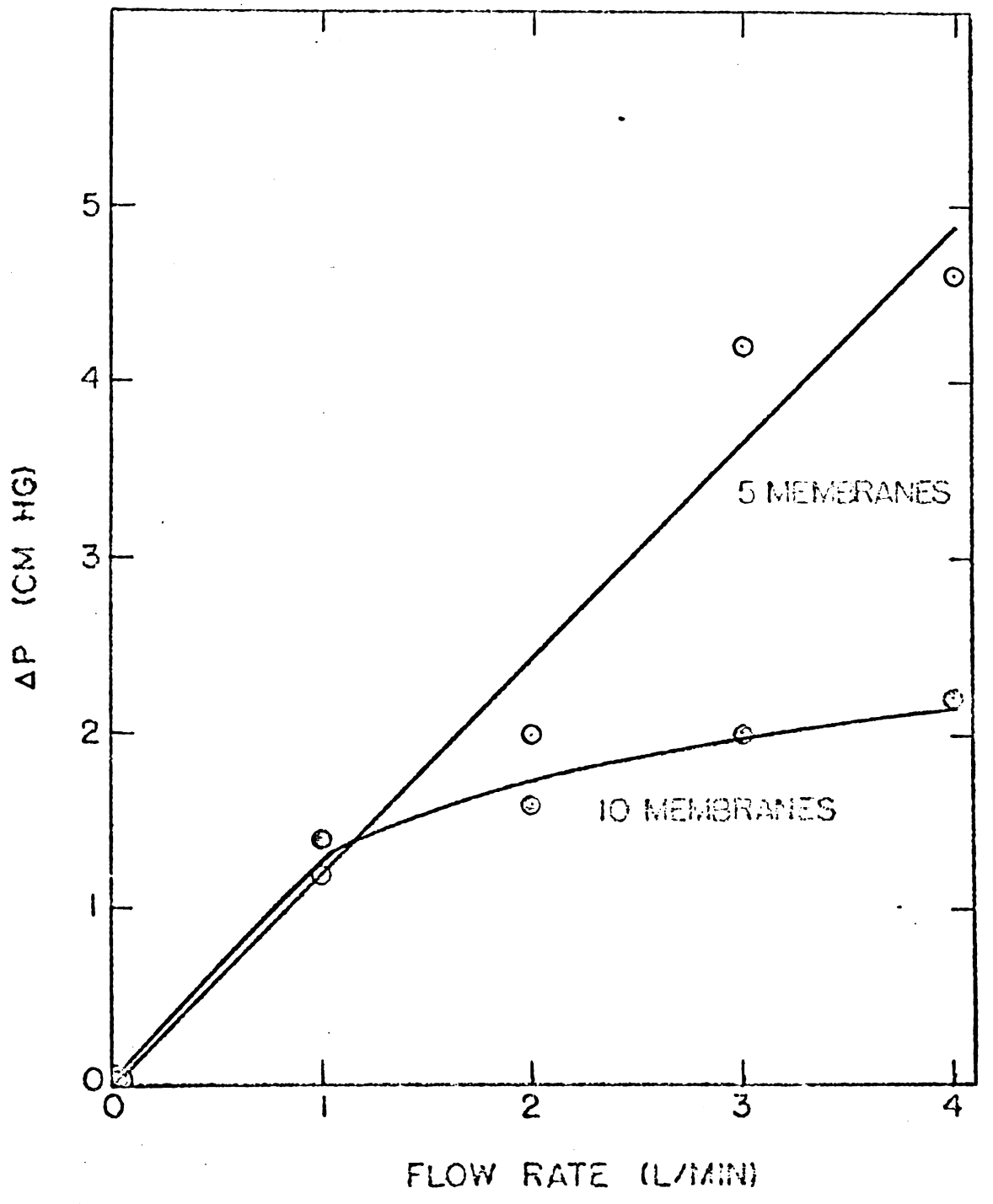

Figure 12. Influence of flow rate through the dialyzer on the pressure drop across a given dialyzer chamber with 5 and 10 membranes installed.

Figure

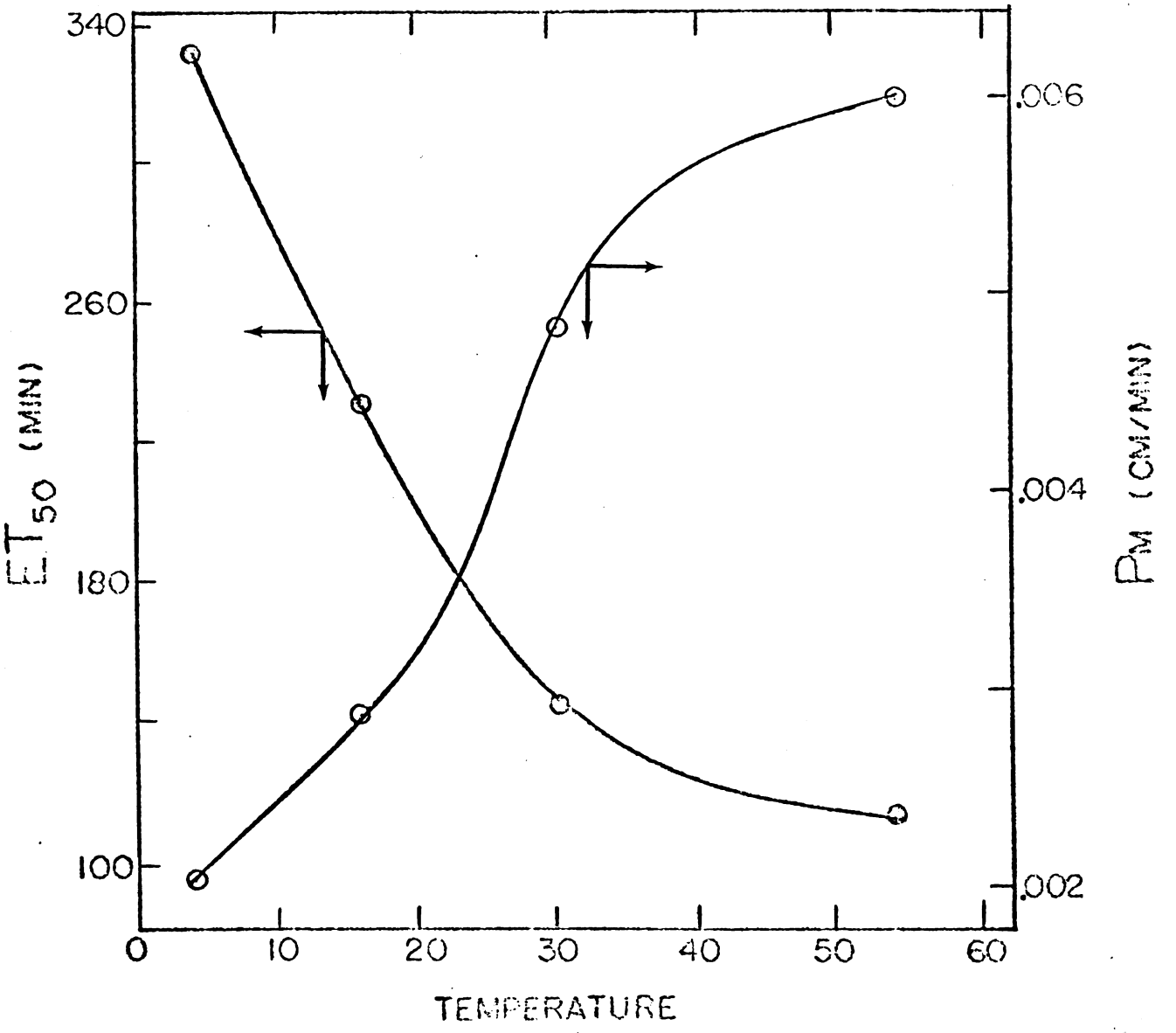

Figure 13. Influence of operating temperature on glucose dialysis.

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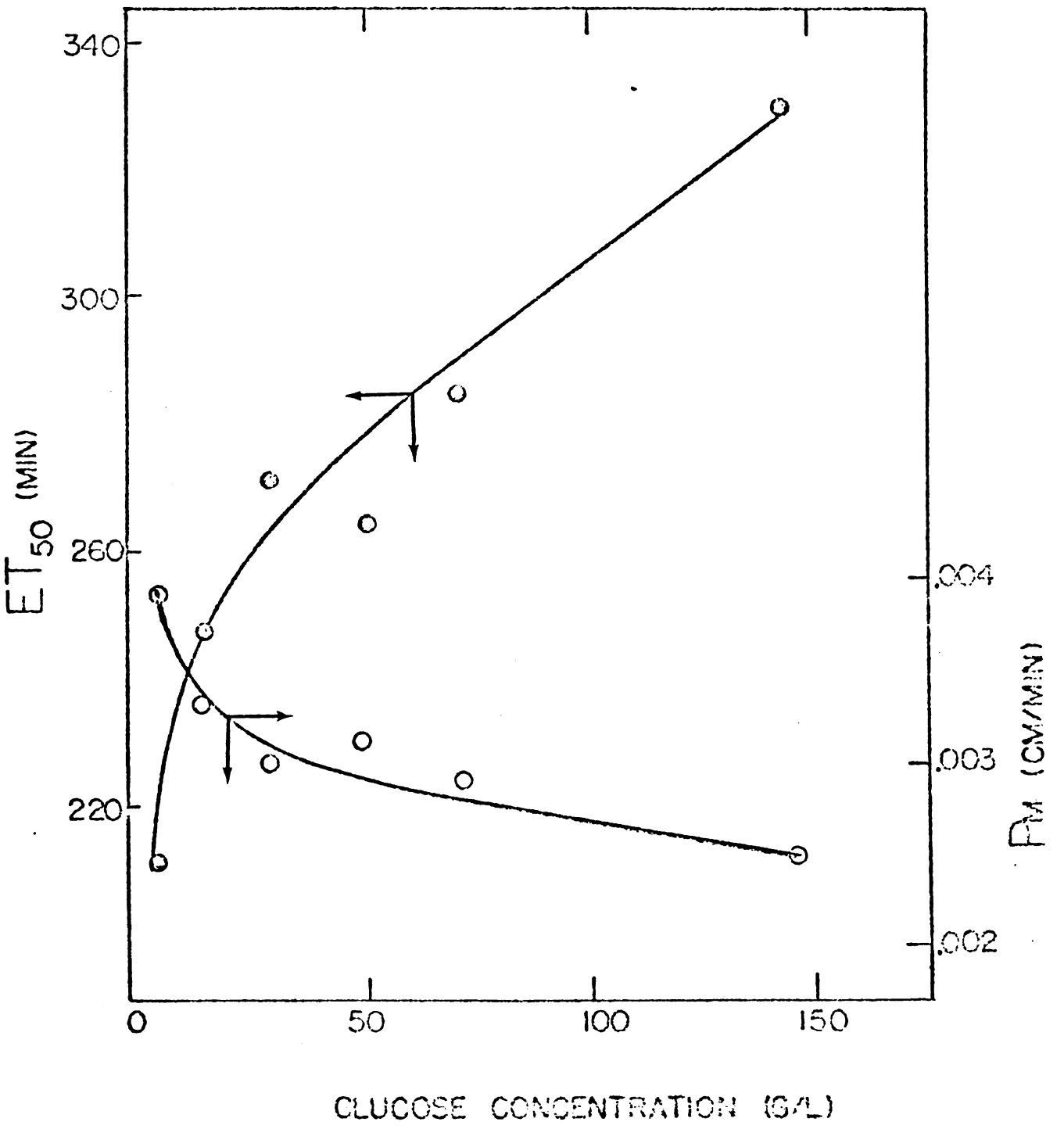
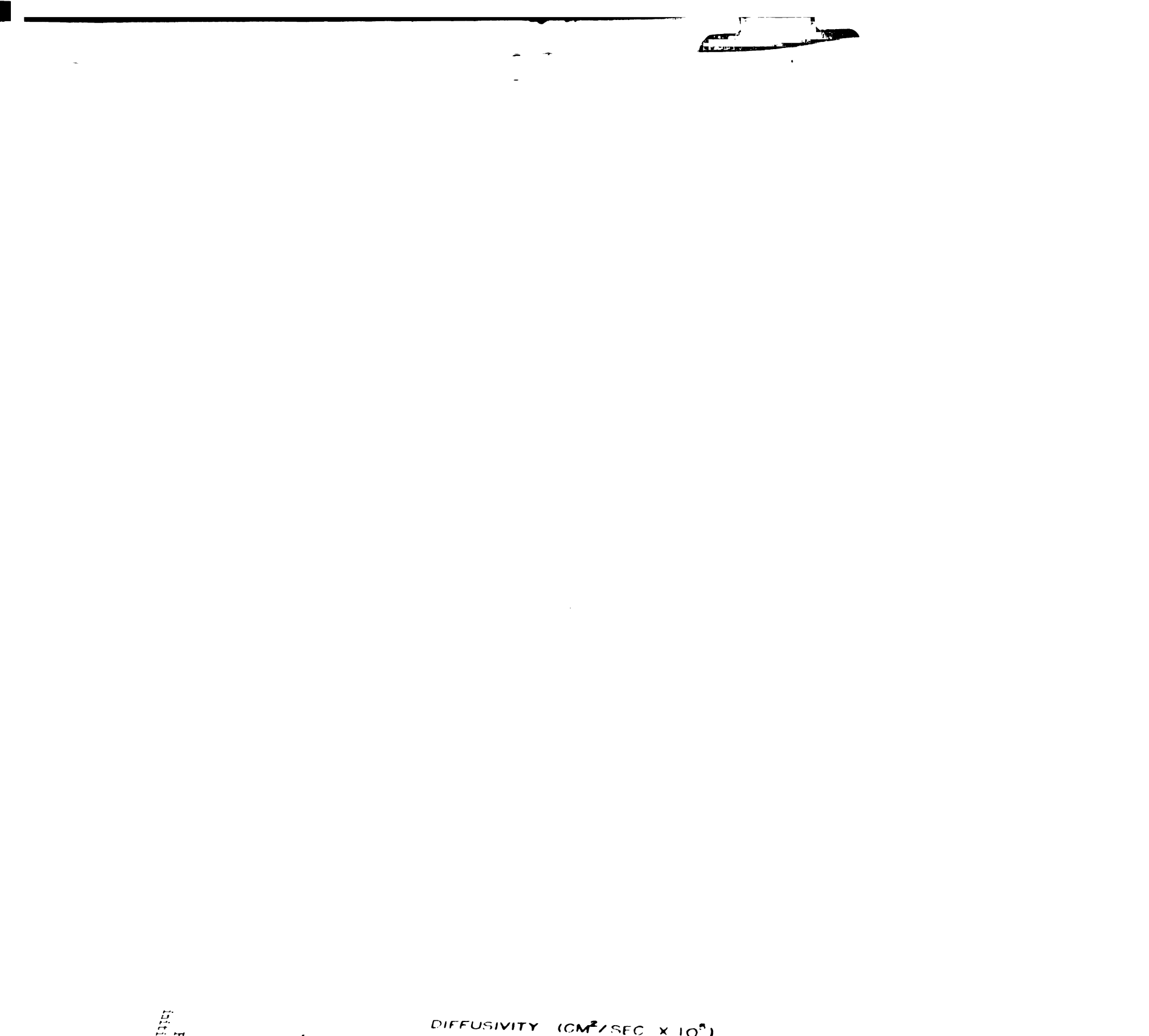


Figure 14. Influence of initial reservoir concentration on glucose dialysis.


DIFFUSIVITY (CM²/SEC × 10⁵)

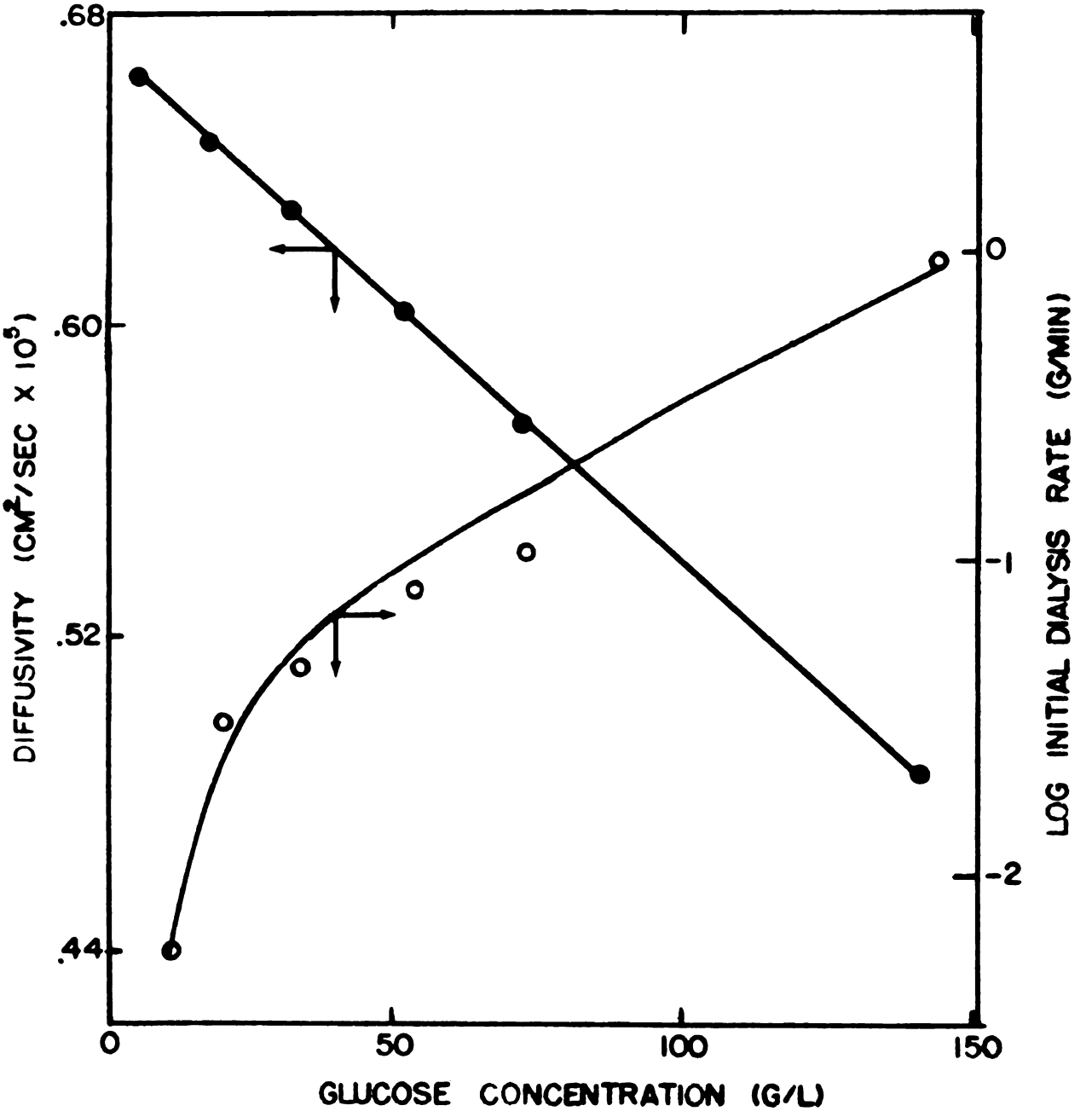


Figure 15. Influence of concentration on glucose diffusivity and initial dialysis rate.

Table 7. Influence of the total volume of the dialysis system on glucose mass transfer and dialysis rates.

VOLUME (Ml)			RESERVOIR	FERMENTOR	ET_{50}	P_m
Fermentor	Reservoir	Total	(g) Glucose Initially	(g) Glucose Received To Reach Half Equilibrium	(Min)	(Cm/Min)
300	300	600	3.2	.80	126	.00284
700	700	1400	7.2	1.80	232	.00356
1000	1000	2000	9.7	2.45	290	.00415
1500	1500	3000	14.1	3.52	424	.00418
3000	3000	6000	30.3	7.60	680	.00520

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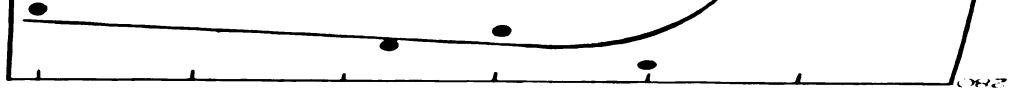
mass of glucose initially present in the reservoir. This resulted in an increase in the mass transfer necessary to reach half equilibrium.

Manipulation of vessel size or liquid volumes also made changes in the fermentor to reservoir volume ratios possible. A low ratio (1:1) and small overall volume (600 ml) produced the fastest dialysis rate (126 min). When the ratio was increased to 1:10 by enlarging the reservoir, the ET_{50} values increased markedly. Further ratio increments produced no significant change in the values (Figure 16). The mass of glucose present in the reservoir both initially and at half equilibrium correspondingly rose as the ratio was increased. However the mass transfer necessary to produce a half equilibrium concentration increased up to a 1:10 ratio but not above (Figure 16 and Table 8). Thus the dialysis rate and necessary mass transfer to the fermentor corresponded over the range of volume ratios tested.



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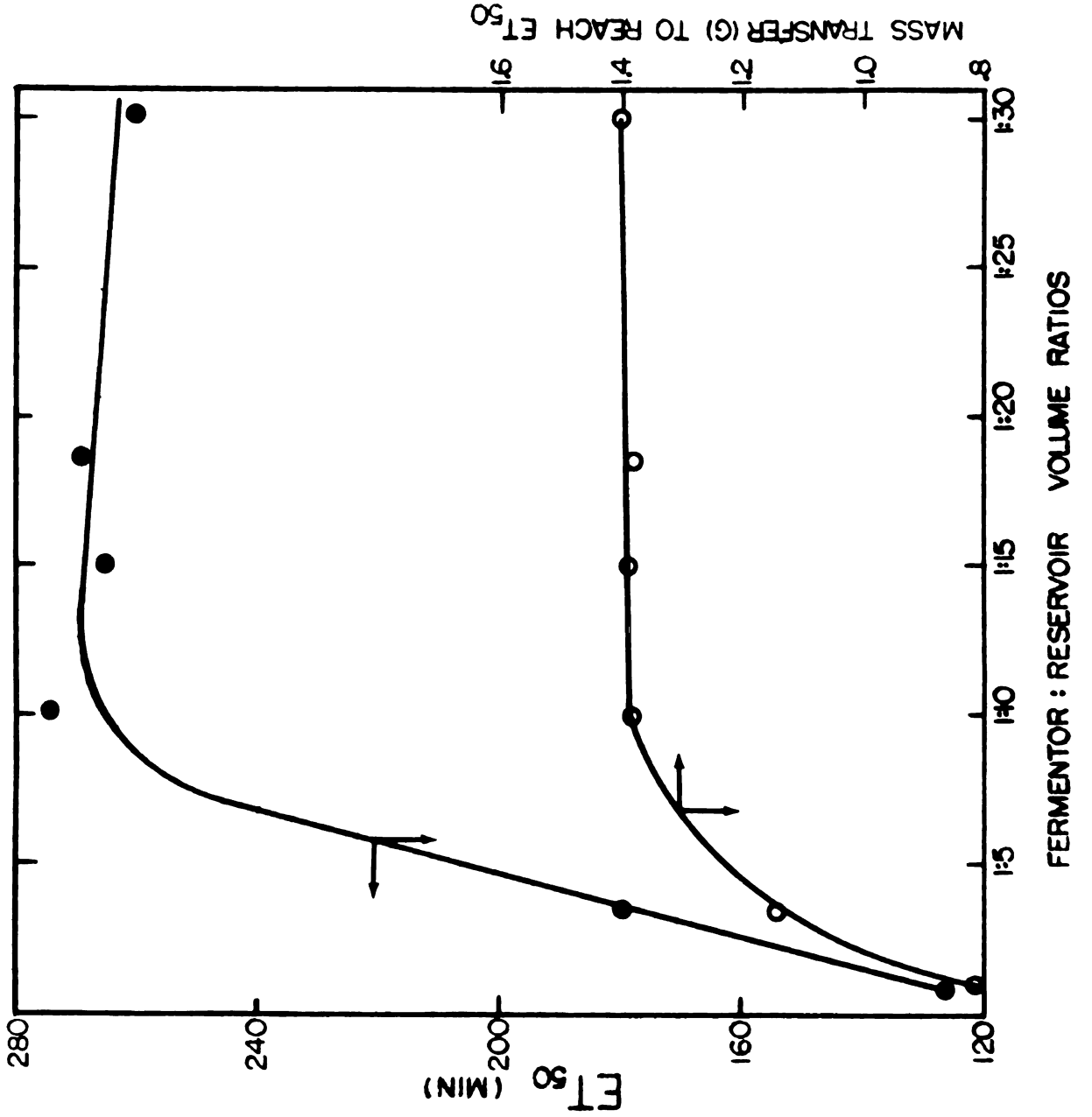

Figure 16. Influence of fermentor-to-reservoir volume ratios on glucose dialysis and mass transfer necessary to reach half equilibrium concentration.

Table 8. Influence of fermentor-to-reservoir volume ratios on glucose mass transfer and dialysis rates.

VOLUME (Ml)		RATIO	RESERVOIR (G) Glucose		FERMENTOR (G) Glucose Received To Reach Half Equilibrium	ET_{50} (Min)
Fermentor	Reservoir		Initially	At Half Equilibrium		
300	300	1:1	3.2	2.4	.8	126
300	1000	1:3.2	10.2	9.0	1.2	180
300	3000	1:10	32.8	31.4	1.4	275
300	4500	1:15	44.5	43.1	1.4	266
300	5550	1:18.5	53.3	51.9	1.4	270
300	9000	1:30	90.0	88.6	1.4	260

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4.4 Discussion

The dialyzer and the dialyzer-dialysis system were designed for cultivation of microorganisms. Because of this objective, solute dialysis was evaluated under conditions resembling, as closely as possible, those necessary for growth of bacteria or allied with microbiological processes. For example, a temperature of 30°C, circulation rates of 1 to 2 liters per minute, and Visking regenerated cellulose membranes have been shown to be satisfactory for growth of Serratia marcescens and other bacterial species (57, 61). Consequently they were adopted as standard testing conditions. Similarly, glucose was employed as the test solute because a fast assay method was available and because this sugar is a required growth-limiting nutrient for most bacterial species. Glucose is included in both a chemically defined medium (at 2% concentration) (145) and in Trypticase soy broth (at .25% concentration), both of which have proven satisfactory for culturing the standard test organism, Serratia marcescens.

Glucose dialysis was reported as ET_{50} (half equilibrium time) and P_m (permeability coefficient) values. The ET_{50} notation was used previously (61, 34) as a means of evaluating dialysis membranes. The concept originated as a factor for indicating electrolyte penetration of algal vacuoles (31) and was used as a coefficient for evaluation of transport in artificial kidney devices (69). The ET_{50} designation represents a meaningful criterion for evaluation of dialyzer performance but

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is limited by dependance on the system volume and membrane area (141). The overall permeability coefficient (P_m) which includes the above and all other performance parameters was developed and adopted in order to provide a second and all encompassing factor for evaluation of the dialyzer.

The overall coefficient, P_m, was derived (J. Schultz, personal communication) from Manegold's formula (109) for diffusion of solutes through a membrane film and relates solute concentration, time and volume to permeability:

$$\ln\left[\frac{C_1\ (1/V_1 + 1/V_2) - (C_1/V_2 + C_2/V_2)}{\left(\frac{C_1^o - C_2^o}{V_1}\right)}\right] = -\ (1/V_1 + 1/V_2)\ P_m\ A_m\ t; \quad (4.1)$$

Where C_1 is the solute concentration in chamber 1 (fermentor) at any time t, C_1^o and C_2^o are the initial solute concentrations in chambers 1 and 2 (fermentor and reservoir respectively), V_1 and V_2 are the volumes of chambers 1 and 2, and A_m the membrane area. Manegold's formula was developed to measure the permeability of membrane materials and was based on the transfer of an identifiable solute from one side to the other in accordance with the basic laws of diffusion. It was assumed that A represented the effective membrane surface available for solute transfer, the chamber volumes were constant, the fluids were well mixed, the initial concentrations in chambers 1 and 2 were known, and the concentration in one of the chambers could be measured as a function of time.

If the concentration in one of the chambers (fermentor) is initially zero ($C_1^o = 0$), the left-hand side of the above equation 4.1 is simplified so that

$$\ln\ (1 - C_1/C_e) = -\ (1/V_1 + 1/V_2)\ P_m\ A_m\ t\ ; \quad (4.2)$$

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Where C_e is the final equilibrium concentration in both chambers,

$$C_e = \frac{C_2 V_2}{(V_1 + V_2)} \tag{4.3}$$

When the system reaches half equilibrium, $t = ET_{50}$ and $C_1 = C_e/2$, and the left hand side of equation 4.2 becomes

$$\ln \frac{1 - C_e/2}{C_e} = \ln 1/2. \tag{4.4}$$

Equation 4.1 then is written:

$$\ln 1/2 = - (1/V_1 + 1/V_2) P_m A_m ET_{50}. \tag{4.5}$$

Rearranging:

$$ET_{50} = \frac{\ln 2}{(1/V_1 + 1/V_2) P_m A_m} \quad \text{or} \quad P_m = \frac{\ln 2}{(1/V_1 + 1/V_2) A_m ET_{50}} \tag{4.6}$$

Thus, two criteria exist for reporting solute transfer from experimental data: an ET_{50} value encompassing membrane thickness and material factors, fluid flow rates, turbulence, and solute concentration; and a P_m value accounting for the volume and membrane area. These provide a basis for evaluation, modification, and scale up of the dialysis system.

Design of the dialyzer for operation with cultures implied that several important criteria be met: compatibility with metabolizing cells, sterilization and maintanance of sterility, consistent performance over long periods of operation, freedom to place the instrument anywhere within the pilot plant, choice of fluid flow direction within it, and information concerning membrane treatment and consistency. The results verified that these conditions were satisfied. Moreover, the durability and reliably consistent performance of the dialyzer was demonstrated by the absence of equipment deterioration by steam sterilization and by the unaltered solute transfer characteristics during continuous operation, for over six days. These were important because steam represents the only dependable method for biological sterilization and the continuity of

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operation is essential for microbial propagation in either batch or continuous culture.

Independent control and placement of equipment components, especially the dialyzer, is a valuable and convenient asset of this culture system. Historically, dialysis exchangers were constructed with vertical membranes and chambers to take full advantage of the counter current curculation produced at the membrane surfaces by gravity stratification of the solutions as they moved through the exchanger (36). This scheme maintained the maximum concentration gradients necessary for effective dialysis but limited bulk fluid flow and reduced the freedom of exchanger placement within the system. Counter current flow, which maintains a maximal concentration gradient linearly along the full surface of the membrane, facilitates efficient mass transfer. This has been achieved in hemodialyzers positioned either horizonally or vertically by fluid pumping (101, 102). The physical characteristics of a microbial culture, such as gas bubbles, viscosity, particulate nutrient suspensions and sedimentation of cells, may supersede the importance of maintaining maximal mass transfer conditions and will influence dialyzer placement and operation. For example, aerated broths containing air bubbles require a vertical fluid flow to prevent entrapment of the bubbles within the dialysis chamber. The flexibility of the dialysis system facilitates satisfaction of such demands without jeopardizing solute transfer performance.

Visking regenerated cellulose membranes provide good glucose diffusion rates, 8-10 grams per hour (167), are sufficiently durable, and were shown superior to other membrane materials for dialysis applications (61). Preparation and selection of these membranes was necessary for consistent performance within the established range. A minimum hydration

time was necessary and differences were observed between manufactured membrane lots. Although these variations were not of a great enough magnitude to affect the dialysis transfer of solutes in principle, they were sufficient to influence the precision of individual experiments or groups of comparable experiments. Therefore the membrane factor must be recognized and accounted for during dialysis investigations.

Independent control of the dialyzer affords a means of increasing dialysis capacity. This was accomplished by addition of dialysis chambers and membranes to the instrument. Expansion of membrane area permits adaptation of the dialyzer to a fermentation system of any desired size. If an excessively large area is required, two or more dialyzers may be added in parallel. Addition of membrane area (.0288 m^2 for each membrane) improved dialysis of 1% glucose solutions until an optimum was reached above which no substantial improvement was observed. This effect has been observed before (D. M. Gallup, Ph.D. Thesis, University of Michigan, 1962) and represents the point at which membrane area ceases to limit dialysis. Seven membranes were optimal for a 1:3.3 fermentor to reservoir volume ratio (1.3 liters total volume) and ten membranes for a 1:1 ratio (6 liters total volume). Because the second system had a larger volume it required a greater mass transfer to reach half equilibrium (7.6 g vs 1.8 g, see Table 7) and, therefore, required a larger area to achieve optimum dialysis. These results are in accord with Fick's diffusion law which states that solute transfer rates are proportional to the exposed membrane area. Moreover they substantiate the principle that membrane surface should be as large as possible in relation to the volume of liquid to be dialyzed (36).

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The surprising decrease in dialyzer efficiency with additional membrane area was believed to be associated with a change in internal fluid dynamics as dialysis chambers were added. Enlargement of the dialyzer increased the internal volume and the fluid holdup time as measured by the time required for an injected swarm of bubbles or dye to clear the dialyzer (Table 6). However the holdup times did not correspond directly with the area used. Bubble swarms began to clear the dialyzer within 4-5 seconds regardless of the area. It was concluded that a given volume of fluid was not distributed evenly among all of the dialysis chambers and therefore was not only unexposed to some of the available membrane area but also left the dialyzer sooner than expected. This effect multiplied as chambers were added, resulting in reduced efficiency. Moreover, the increased internal volume associated with additional dialysis chambers also reduced efficiency by reducing the flow rate across a given membrane surface. This premise was substantiated with a 15 membrane dialyzer where an increase in P_m from 2×10^{-3} to 2.7×10^{-3} cm/min was observed when the pump rate was raised from 2 to 6 L/min respectively. A similar reduction in efficiency with addition of membrane area has been attributed to unequal flow through the separate chambers in hemodialyzers of similar design (52).

The influence of operational conditions on solute dialysis was analyzed. In particular fluid velocity, temperature, solute concentration, and fermentor and reservoir volumes and volume ratios were examined.

Fluid velocity past a membrane surface generally determines the thickness of the laminar film immediately adjacent to it. In addition, fluid velocity through a dialysis chamber governs the degree of turbulence and bulk mixing within the vicinity of the membrane. The fluid dynamics

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within the dialyzer are depicted in Figure 17. Both separator design and fluid velocity contribute to optimization of the above conditions. Adequate mixing maintains the solute concentration gradient and thin laminar films reduce membrane diffusion resistance. Both of these aid dialysis transfer rates. The success of separator design was shown by observance of fluid turbulence at fluid flow rates of 1/2 L/min or more. Achievement of optimized fluid dynamics within the dialyzer was shown by stabilization of ET_{50} and P_m values at fluid velocities of 2 L/min or above.

Increments in pump rates increased the pressure drop across the whole dialyzer and across the dialysis chambers on either side of a membrane. The observed pressure gradients were relatively small, similar for both chambers, and similar in chambers positioned adjacent to or distant from the front plate of the dialyzer. These results suggested that hydrodynamic conditions were similar for all dialysis chambers within the dialyzer and that the dialyzer presents an efficiently small resistance to fluid flow. The hydraulic gradients, although analogous to those observed in smooth pipes, could not be used as criteria for determining turbulence within a dialysis chamber. The fluid dynamics within the rectangular dialysis chamber were not the same as those within a round pipe. Therefore visual observation through the glass back plate of the dialyzer (as indicated above) was used for this determination. The hydraulic pressure gradients diminished when membranes and dialysis chambers were added. This was attributed to the expanded internal volume and constant pump rate. Resoration of the gradient values to those characteristic of the dialyzer with a few membranes was accomplished by raising the pump rate. The resultant improvement in dialysis efficiency (see

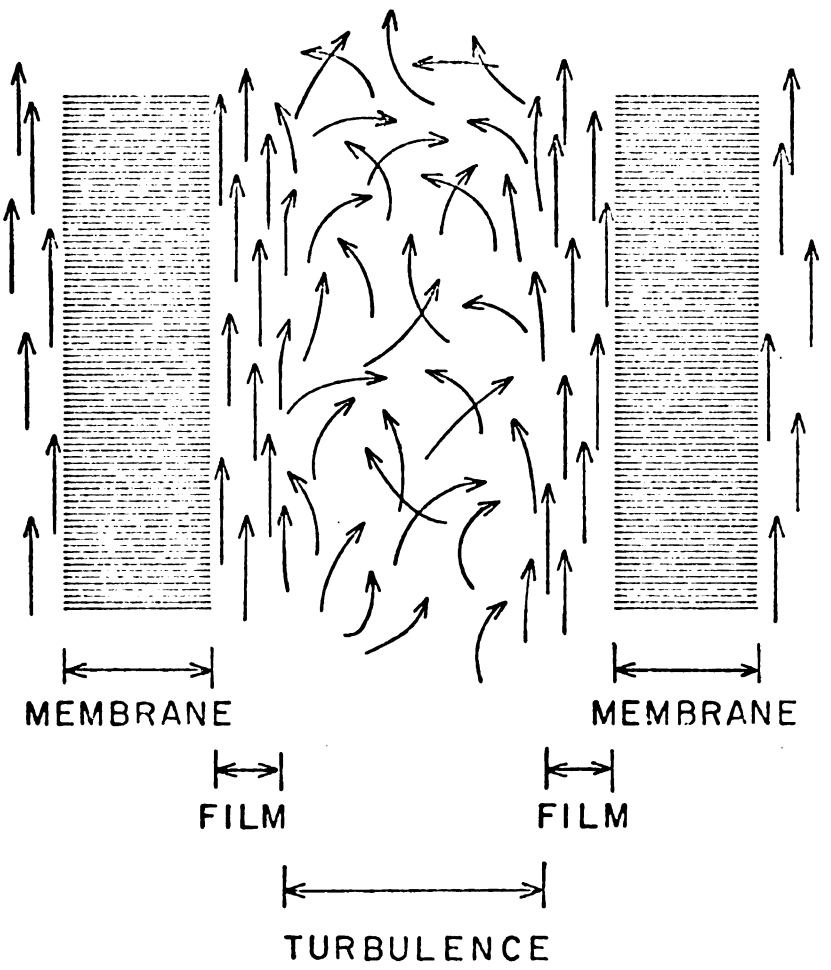

Figure 17. Representation of fluid flow past the opposing faces of two membranes, with a laminar film immediately along each surface and turbulent bulk flow in the central channel. Reproduced from Schultz and Gerhardt (141).

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the example cited previously) indicated that efficient internal fluid conditions could be maintained by manipulation of the fluid flow rates within the dialyzer and that the expanded internal volume associated with additional membrane area could be compensated for.

Dialysis efficiency was closely related to the temperature of operation. An increase in dialysis rates with temperature was expected since diffusion is directly related to temperature (21). Warmer conditions produce greater molecular activity and reduced viscosity. Both of these increase glucose diffusivity (see Table 9), which explains the correlation of dialysis performance with temperature. Thus the dialysis culture system would achieve its best efficiency when used to propagate thermophilic organisms. On the other hand reduced dialysis efficiency must be considered if the same system were to be used with psychrophiles. Most of the experimental data was obtained at 30°C (mesophilic optimum) because this group of microorganisms are the most numerous and commercially the most useful.

A biological growth medium may contain glucose in concentrations ranging from 1.5 g/L (Trypticase soy broth) to 141 g/L (synthetic medium) depending on the requirements of the organism used or the metabolic process being explored. Glucose concentrations in this range were placed in the reservoir and dialyzed against a fermentor containing an equal volume of water to demonstrate the capacity of the dialysis system for these conditions. Initial dialysis rates (over the first 100 minutes) increased as the reservoir mass increased from 3.8 g to 141 g. This was expected since the rate of dialysis, like diffusion, increases as the concentration gradient across the membrane becomes greater (Fick's law). However, the half equilibrium time became longer and dialysis efficiency

Table

Table 9. Influence of temperature on glucose dialysis and diffusivity.

TEMPERATURE (°C)	ET_{50} (Min)	P_m (Cm/Min)	DIFFUSIVITY (10^{-5} Cm^2/Sec)
4	331	.00205	.365
16	233	.00291	.540
30	144	.00472	.793
54	114	.00596	1.326

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poorer as the concentration gradient (reservoir concentration) rose over the same range. This had not been anticipated and suggested that increased reservoir concentration produced apparently opposing effects on dialysis. The larger glucose concentrations increased fluid viscosity, decreased solute diffusivity, and increased mass of solute which must diffuse across the membrane to achieve the half equilibrium concentration as well as the increased concentration gradient. These effects are tabulated in Table 10. Thus it appears that, at the onset of dialysis, mass transfer was rapid in response to the solute concentration gradient. As dialysis proceeded toward equilibrium the gradient was reduced and its influence on mass transfer was lessened, allowing the influence of diffusivity, viscosity, and especially required mass transfer to prevail. The reduction in dialyzer efficiency resulting from the greater required mass transfer associated with increased reservoir solute concentration could be improved by additional membrane area.

Independent control of the fermentor and reservoir regions permits expansion of the dialysis system to accommodate any desired operating volume. When these were increased in 1:1 ratios, the total system volume rose and correspondingly, the reservoir solute mass as well as the mass transfer required to reach the half equilibrium concentration, rose. The latter increase explains the observed reduction in glucose dialysis rates as the total volume rose from .6 L to 6 L. The corresponding increase in dialysis coefficients suggested that the dialyzer remained relatively efficient in transferring the additional volume and solute mass involved. Again the addition of membrane area, which would maintain the necessary area to dialysate volume relationship, would improve performance in large volume applications.

Table 10. Influence of the initial reservoir concentration on glucose dialysis rates, diffusivity, and mass transfer.

RESERVOIR GLUCOSE MASS (G)	MASS TRANSFER TO FERMENTOR AT HALF EQUILIBRIUM (G)	ET_{50} (Min)	P_m (Cm/Min)	INITIAL TRANSFER RATE (G/Min)	DIFFUSIVITY (10^{-5} Cm^2/Sec)
3.8	.95	212	.00395	.006	.662
13.6	3.40	248	.00333	.031	.648
27.7	6.90	272	.00304	.045	.630
50.4	12.60	264	.00313	.080	.622
62.3	15.50	286	.00293	.098	.576
141.0	35.00	330	.00250	.196	.486

The ratio between fermentor and reservoir volumes was increased by increasing the latter while the former was held constant. Because the reservoir region would be the simplest and least expensive region to expand in a cell propagation system, the ratios were increased in this manner allowing retention of the same fermentor in each case. This permitted analysis of the ratio effect on a key feature of dialysis culturing: the concentration of culture by confinement within a limited volume which is possible only by exchange of dialyzable nutrients from a reservoir. Dialysis performance became decidedly poorer when the ratio increased from 1:1 to 1:10 but remained constant at larger ratios. The explanation again resided in the mass of solute which had to be transferred through the dialyzer to attain half equilibrium. For 1:1, 1:3.2, and 1:10 ratios the required mass transfer rose from .8 g to 1.4 g (Table 8). However, ratios above 1:10 showed no significant increase in required mass transfer which was reflected by the constant ET_{50} values observed at these ratios. This effect was due to the fermentor volume, being constant, becoming an increasing smaller portion of the total system volume as the ratio rose by increasing the reservoir volume. Thus, half-equilibrium could be established in a 1:30 system as quickly as in a 1:10 system because only 2% to 4% of the reservoir mass needed to be dialyzed. A 1:1 ratio in constrast, required 25% of the reservoir mass to be transferred. Since the reservoirs of large ratio systems retain 96% - 98% of their solute mass at half equilibrium, a large reservoir of additional solute is provided for transfer into the fermentor if equilibrium conditions are upset, as occurs during growth. Comparing Tables 7 and 8 reveals, as just shown, that the total volume effect predominated over the volume ratio effect. For example, the 1:1 system with a 6 L total volume required 680 minutes

to transfer 7.6 g of glucose while a 1:18.5 system with nearly the same volume required only 270 minutes to reach half equilibrium because a transfer of only 1.4 g of glucose was required. Thus the system with a larger volume and the larger required mass transfer will either have slower performance, or will require additional dialyzer area to attain equal performance. These results demonstrate the importance of identifying the conditions under which the dialysis system will operate, so necessary adjustments may be made to attain the most efficient or effective performance.

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4.5 Summary

The previously described dialyzer was incorporated into a dialyzer-dialysis culture system which utilized a fermentor and separate reservoir of 3 to 10 liters and 10 to 30 liters, respectively. Nutrient transfer (glucose) for this system was evaluated by determination of half-equilibrium times (ET_{50}) and overall permeability coefficients (P_m). Mean values of 144 $\pm$ 15 min (ET_{50}) and 4.7 $\pm$ .47 x 10^{-3} cm/min (P_m) were obtained with a 1% glucose solution and a single dialysis membrane. Continuous operation, autoclaving, dialyzer position, and fluid direction had no effect on dialysis performance. Optimum rates were obtained with an area of .2016 m^2 to .2880 m^2, depending on the volume ratio used, and bulk flow rates above .5 L/min. The decrease in performance associated with large overall liquid volumes or fermentor-to-reservoir volume ratios and the significance of internal fluid dynamics were discussed.

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5. GROWTH OF A TYPE BACTERIUM

5.1 Introduction and Historical

5.1.1 Introduction

Microorganisms are traditionally grown in conventional fermentors when large yields are desired. Although these are relatively simple to assemble and operate the efficiency of the process is low and the operational flexibility limited, precluding adoption of new or novel culture schemes. The continuous culture technique improves the culture environment within a conventional system but the costly control and monitoring equipment make it impractical for routine use. The dialysis culture technique can not only improve the culture environment but also increases the operational flexibility of a conventional culture system.

The application of dialysis to the culture of microorganisms began with the use of simple equipment. Seventy years ago it was discovered that a pneumococcal culture confined within a dialysis membrane sac not only grew well but exhibited enhanced virulence and toxin production (24). Since those initial experiments, the dialysis culture technique has been widely exploited to achieve results superior to those of conventional fermentations. A historical survey of the literature pertinent to the evolution of the dialysis culture technique and the major contributions concerning its design, apparatus, theory, and application was recently compiled by members of this laboratory, including contributions by this author, and was published as a review (141). This survey,

including results from this laboratory, revealed that major consequences of growing cells in dialysis culture were: the prolongation of active multiplication to reach higher maximum populations which had high viability and often extraordinarily high densities; the stabilization of the maximum stationary and terminal phases; the production of cells free from macromolecular constituents of the medium; the removal and dilution of diffusible growth inhibiting solutes; the increased production and accumulation of culture products such as extracellular enzymes, toxins, or spores; and the opportunity to prepare and study unique interbiotic relationships such as symbiosis and antagonism. A surprisingly large variety of organisms have been studied in dialysis culture: 23 genera of bacteria, 6 genera of fungi, 6 genera of protozoa and 6 lines of tissue cells.

Most of the investigations to date were made with the same basic dialysis culture design used in the initial investigation seventy years ago, a dialysis sac containing the culture suspended within a nutrient reservoir, or with small custom built dialysis chambers (141). These systems, while sufficient for specific investigations, are inherently limited in capacity, capability for control, scale-up, and operational modifications. The closest approach to a large scale system using the dialysis sac scheme was devised by Sterne and Wentzel (151) who used a large intussuscepted dialysis sac (3.5 liters of culture) surrounded by 35 liters of medium in a carboy to produce high yields of pure and potent botulinum toxin. Hedén (75) produced high molecular weight extracellular products by using a 15 meter long dialysis bag, containing a culture which was circulated and aerated by an external pulse-aeration pump, suspended in a fermentor of nutrient. Both of these devices although

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The aim of the investigations in this laboratory have been to improve the basic design of dialysis culture equipment, to test the applicability of membranes, to analyze the performance (chemical and biological) of the dialysis system, and to develop a functional dialysis fermentation system. Several important studies preceded those reported in this thesis. The first was the development of a flask dialysis culture system (61). This twin-chambered flask is constructed of stock Pyrex pipe fittings, with a supported membrane clamped between the lower medium reservoir portion and the upper culture portion and can be mounted on a shaking machine. Several of these units could be operated simultaneously for experimental duplication and control. The dialysis flask system was used to evaluate the suitability and diffusion properties of membranes, to assess the variables of dialysis culture, and demonstrate the general usefulness of the concentrating effect of dialysis culture with a variety of microorganisms.

Next, the dialysis culture concept was extended to a pilot scale fermentation system where the culture was remote from, but connected by conduits and pumps with, the nutrient reservoir (57). Initially coiled dialysis tubing and conventional fermentor vessels were used to construct a fermentor-dialysis system, where the nutrients from a remote reservoir were circulated through tubing placed in the fermentor. In a reservoir-dialysis system, the culture fermentor was separate and the tubing was placed within the reservoir vessel. The third and best scheme was a dialyzer-dialysis culture system where the principle regions -- culture, reservoir, and dialysis -- were separated and could be

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controlled independently. This system necessitated the use of a dialyzer. A plate-and-frame industrial chemical dialyzer, which used sheet membranes, was employed. The dialyzer-dialysis system demonstrated the potential and usefulness of dialysis for large scale fermentations when growth trials revealed high densities of Serratia marcescens cultures and showed that operational manipulations such as supplemental feeding and osmotic dehydration of the culture for further concentration could be successfully employed. These studies also showed that the key to the successful operation of a dialysis culture system is a suitably designed dialyzer. The design, construction, and analysis of such a dialyzer represents the initial portion of the current study, is presented in the preceding sections (Sections 3 and 4), and was reported earlier (Abstract, 152nd Meeting, American Chemical Society, New York, 1966).

The prototype dialyzer (Section 3) was incorporated into the dialyzer-dialysis culture scheme in the experiments which follow. The objectives of these investigations were: (a) to examine the suitability of the dialyzer for dialysis culture; (b) to examine the influence of operational variables on dialysis growth and to compare their influence on solute transfer; (c) to demonstrate and compare the characteristics of dialysis and nondialysis growth; and (d) to illustrate and analyze some of the attributes of dialysis culture. The test organism Serratia marcescens was chosen in order to retain continuity with previous investigations and to permit comparison.

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5.1.2 Historical

The selective transport of solutes through a membrane barrier makes dialysis a potentially useful technique for biological processes as a means by which nutrients could be made available to and metabolic products removed from a confined microbial culture. This was first recognized nearly seventy-five years ago when Metchnikoff, Roux and Salimbeni demonstrated the solubility of cholera toxin by implanting collodion sacs containing cholera vibrios in the peritoneal cavity of animals (113). Shortly thereafter Carnot and Fournier (24) extended the use of dialysis to an in vitro situation by suspending a collodion sac containing pneumococci cultures in a laboratory flask containing ordinary growth medium, also in an effort to demonstrate the presence of a diffusible toxin. Subsequently the dialysis principle has been employed in numerous biological investigations including: a variety of in vivo and in vitro schemes for propagating a large variety of microorganisms; the production, purification, and/or concentration of both diffusible and nondiffusible culture products; membrane testing; surface growth and filtration; the study of interbiotic relationships and effects such as synergism and antagonism; gas exchange; and peritoneal and hemodialysis (141). In addition, the availability of new types of synthetic membranes with a greater range of permeabilities has extended application of the dialysis process into the administration of anesthesia (51), the prolonged administration of drugs (9, 50) and of deficient enzymes (25) by capsule

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implants in animals, and osmotic pressure filtration such as "reverse osmosis" for water purification (60).

The membrane interface represents the key element of a dialysis culture system. The most commonly used schemes employ membrane sheets, tubes, or sacs which sequester the microbial population while maintaining diffusional access to a larger nutrient reservoir. The integrity of the membrane is both assumed and essential, especially when a pure culture is desired, interbiotic relationships are to be studied, or cell free diffusible products are to be recovered from the reservoir. Typically regenerated cellulose membranes "ordinary dialysis membrane" with a mean pore diameter of 5 mμ are used although other materials such as plastic or polyvinyl chloride membrane filters with a range of pore diameters as small as 25 mμ are becoming popular for specific applications. A third and relatively new type of membrane, a nonporous synthetic membrane film, has been applied to gas diffusion. However one of these, a co-polymer material based on co-polyether-ester compounds (poly-oxyethylene glycolmethylene bis 4 phenyl isocyanate), also has been proven useful for the separation of related solutes (106, 107). As shown for other non-porous membranes, mass transfer through these films is related to solute-membrane interactions.

Generally a membrane is assumed suitable for dialysis culture if it can be sterilized, has an adequate solute transfer rate, is not destroyed during the culture process, has a porosity smaller than the organism used, and is free of rips, holes or flaws. Several tests are available to insure the latter (141). Penetration of the dialysis membrane by the microbial culture apparently has not been a problem for either in vivo or in vitro dialysis. Our own experience with dialysis culture has not

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revealed such a problem (57, 61, current results) and a dialyzer-dialysis culture has been operated for as long as seven days without contamination of the reservoir (see the results presented in this Section). Reservoir contamination, when it did occur, was readily attributable to a demonstratable hole or tear in the membrane, an unsealed gasket, or a contaminating organism from an external source.

It is generally recognized that membrane filters and other fine porosity materials when used in pressure filtration will eventually pass bacteria. The length of retention is related to the concentration of organisms, duration of the filtration process, and the filtration pressures and flow rates (143, 155). Singer recently showed that Pseudomonas cells did not penetrate membrane filters for a least 7 days (143). During the demonstration of a differential dialysis flask system it was discovered that Staphylococcus aureus cells penetrated the membrane filter but not the dialysis membrane (79). This was considered unusual because no pressure differential existed across either membrane barrier. Even more remarkable were the studies of Gan which revealed that over twenty species of bacteria and yeast were capable of "dialyzing through" normal dialysis membranes (58). A review and discussion of bacterial penetratability was included in the recent review by Schultz and Gerhardt (141). Because this represents a critical aspect of the dialysis culture process additional related evidence will be presented here.

Gan attributed membrane penetration to submicroscopic viable units of the parent bacteria. He recently supported this supposition by demonstrating, at growth-discouraging temperatures, that such dialyzable units of Shigella sonnei passed through collodion filters, could not be

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sedimented, and increased in size and changed in morphology until they became recognizable bacteria (59). He claimed this supported the theory that the bacterial life cycle included a filterable "invisible" form such as an extracorporal gene or reproductive unit.

Two alternate explanations for penetration were proposed by Schultz and Gerhardt (141). The first suggested the formation of plastic forms (e.g. spheroplasts or L-forms) by plasmoptysis of the bacteria as a result of the osmotic and ionic gradients across the membrane. The second involved the growth or diapedetic transfer of the organisms through the membrane openings. The demonstrated penetration of membrane filters by bacterial L-forms and illustrated growth of mouse peritoneal cell processes through membrane filters were cited as supporting examples. The appearance of bone tumor cells on the exterior surface of millipore filter growth chambers was recently shown to be the result of the growth of cell processes through the filter (54). Photomicrographs of embedded and sectioned specimens illustrated that cytoplasmic extensions and collagin fibrils had grown through the tortuous pore channels apparently without deforming them. Significantly, this study showed that diapedesis occurred with membranes which have a pore diameter (10 mμ) which is only twice as large as that of a regenerated cellulose membrane (5 mμ).

The growth or diapedesis of organisms through the pores represents a more plausible explanation of membrane penetration than the passive diffusion of submicroscopic viable units. Cliver showed that such submicroscopic particles, 30-85 mμ viruses for instance, do not necessarily penetrate a membrane but instead absorb to the surface even though the mean pore diameter is 285 times the virus

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diameter (28). This phenomenon, observed under pressure filtration, was related to the chemical composition of the membrane material, except for porosities close to the virus diameter where penetration was influenced by the diluent used. These results pose the possibility that Gan's bacterial units might behave in a similar manner and would not penetrate a dialysis membrane, which is thicker and has much smaller pores, by passing through it especially in the absence of a pressure gradient. The significance of the failure of Gan's control particles to pass through the dialysis membrane which had been penetrated by the bacterial submicroscopic units could be criticized on the basis of the above phenomenon. This would permit speculation that dialyzability might still be simply due to the presence of some large pores in the dialysis tubing.

Examination of various membranes by transmission and stereoscanning electron microscopy has revealed that the cross sectional channels and cavities are quite tortuous, have random widths, and have numerous blind pockets (43, 95, 54, 77). These structural features have been cited as an explanation for the entrapment of particles smaller than the rated pore size (54). Further, the examination of membrane filters after use showed that the organisms are concentrated on and within the upper half of the membrane thickness, with few if any being found beyond the mid point (77). Thus, intact bacteria do not readily penetrate membranes and it would be reasonable to speculate again that Gan's presumed submicroscopic units would have difficulty successfully dialyzing through the narrow tortuous channels of the relatively thick dialysis membrane under the passive pressure conditions described. However, these same structural conditions have not retarded the growth

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of tissue cells within and through membranes and the illustrated ability of this process to occur with porosities approaching that of dialysis membranes lends support to the growth and diapedesis explanation of bacterial dialyzability. It is possible that the environmental conditions of Gan's tests stimulated such processes within the parent culture. Certainly electron microscopic examination of Gan's dialysis membranes would be a logical extension of his investigations and could be valuable in establishing the validity of the growth and diapedesis mechanism of membrane penetration.

Dialysis culture has been employed in a wide variety of culture processes which include cell production, formation of nondiffusible and diffusible products, mammalian cell and virus production, interbiotic culture systems, gas exchange, periotoneal and hemodialysis, culture chamber implants, and rumen symbiodialysis (141). Since this publication, additional investigations concerning the application of the dialysis culture technique have appeared. These are presented below and should serve as an updating for the above literature review and the Supplemental List of References on Dialysis Culture of Microorganisms (available from P. Gerhardt by request).

Schultz and Gerhardt (141) compiled a table (Table 5) listing the investigations in which nondiffusible products were obtained with dialysis culture in vitro. Two additional studies have been published and should be included in the above list. The first concerns the production of thermostable hemolysin from Staphylococcus epideomidis cultures grown on a layer of cellophane covering the surface of a brain heart infusion agar plate (90). Higher yields of hemolysin were recovered from the surface dialysis cultures than from cultures grown directly

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on the semi-solid medium. The second investigation utilized the suspended dialysis-bag technique to concentrate and recover an extracellular enzyme, proteinase, from Streptococcus faecalis cultures (114). The single dialysis apparatus, a cluster of dialysis tubes suspended in a six liter flask of growth medium, did not represent an advancement in equipment design and was similar to that used by previously for the production of highly potent tetanus toxin (91). Both of these schemes exploited the semipermeability of the dialysis membrane as a means for confining and concentrating a viable culture and its enzyme product while allowing diffussional access to the necessary nutrients in the flask.

Schultz and Gerhardt found only a few reports where dialysis culture had been used as a means to enhance the production of diffusible compounds. The technique has merit because it would allow metabolic products which are lethal or inhibiting to be continuously removed and diluted in the reservoir, resulting in higher growth and productivity as well as simplified product recovery from the reservoir. This principle was recently used to remove a "growth inhibitory factor," tentatively identified as dialyzable lauric acid, from yeast cultures (4). Dilution of the inhibiting fatty acid by dialysis permitted markedly improved growth in hexadecane or decane medium. A similar culture response has been found for the production of salicylic acid from naphthalene (1). Dialysis cultures of Pseudomonas fluorescens allowed dialytic removal of the cellular product, salicylic acid, which resulted in a greatly enhanced production of cells and consequently a 20-fold improvement in salicylic acid production over non-dialysis cultures. In addition, the product could be easily recovered

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from the cell free reservoir during the fermentation. Finally, it has been discovered that membrane phase separation and product removal by diffusion are useful for the examination of enzyme -- substrate -- product formation reactions (T. A. Butterworth, D. I. C. Wang, and A. J. Sinskey. Abstract, 158th National Meeting, American Chemical Society, New York, 1970). Judicious selection of ultrafiltration membrane porosity allowed an active enzyme preparation, α-amylase from Bacillus subtilis, to be retained and continually re-used within a diffusion chamber which served as a reaction vessel. Substrate, continually pumped into the chamber under constant ultrafiltration pressure, reacted with the enzyme and the reaction products, di-oligo-and polysaccharides, passed through the membrane for collection while the enzyme and unreacted substrate were retained within the chamber. This technique prevented the loss of enzyme permitting its continual re-use for product formation.

An increase in culture density is a proven attribute of dialysis culture. Diffusional access to a nutrient reservoir is largely responsible for the enhanced growth. This was aptly demonstrated by Marino who reported that dialyzing Norcardia salmonicolor cultures after batch culturing vastly improved the cell yield (110). This organism achieved a culture density of 4-5 g/L when grown conventionally on a hydrocarbon -- mineral salts medium. When the same culture was then dialyzed, by circulating it through dialysis tubing coiled within a 40 L reservoir, against fresh medium the culture resumed growth and achieved densities of 6, 26, and 47 g/L after 1, 7, and 16 days respectively. The improvement was attributed to the removal and/or dilution by dialysis of growth inhibiting products which had accumulated.

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Sortland and Wilke achieved high culture densities of Streptococcus faecalis by utilizing a quasi-dialysis-continuous culture process (146). They designed a rotating microfiltration (millipore membranes) fermentor which could be operated on a continuous nutrient feed basis, batchwise, and with or without filtration. The desire to improve the time efficiency of continuous culture, to study the effect of concentrated cell density on growth characteristics, and to analyze the culture products free of cells prompted the development of this culture system. Essentially, this scheme allowed retention of the cells normally washed out during continuous culture while still permitting a constant feed of nutrients through the fermentor. Theoretically this eliminated interferring fluctuations in nutrients and growth rates while maintaining a high culture density. The system apparently met expectations. High cell densities, up to 40% packed cell volume, were obtained which were 45 times greater than achieved in simple batch culture. A practically cell free (filter efficiency was high but not absolute) culture effluent allowed precise chemical analysis which showed that glucose was converted almost stoichiometrically to lactic acid with no consumption for cell maintenance. In addition high cell densities had no apparent influence on growth characteristics and changes in cell yield per mole of glucose consumed unexplainably changed abruptly during growth. As found with most dialysis processes, this system could prove useful on an industrial scale if costs were lower.

Schultz and Gerhardt cited examples where the dialysis culture technique has been applied toward propagation of mammalian cells and tissues both in vitro and in vivo, the latter by dialysis chamber implantations. The reported investigations were directed toward

propagation of the cells and tissues themselves, virus production, and examination of cell differentation, antibody production, homgraph survival, and other interbotic relationships. Several additional studies concerning in vitro dialysis culture of mammalian cells have appeared since the review. One involves the modification of a tissue culture chamber by inserting a membrane partition which allows diffusional access to the cell clones (11). The construction of a dialysis chamber is not new but the use of Nuclepore membranes (the .5μ to .8μ pores are formed by α particle bombardment) is. The membrane was autoclavable, nontoxic, unpenetratable by the cells, and diffused radioiodinated serum albumen molecules with equilibrium between the chambers being reached in 7-9 hours. The authors felt that this culture system would enable certain essential metabolites from a feeder culture in one chamber to diffuse through the membrane to supply and enhance cell cloning in the other chamber. They also proposed the use of this device for small scale continuous perfusion.

A far more complex dialysis tissue culture system described as a multichamber circumfusion system has been under development by Rose and co-workers for over ten years (132). First they noted that differentiation in growing tissue explants was superior when the multipurpose culture chambers were overlayed with dialysis membranes which improved cell anchorage to the glass cover plate (135). Then they created the circumfusion system by modifying twelve of these chambers for the continuous perfusion of nutrient fluids and connected them with the necessary pumps, reservoirs, and conduits to form a compact self-contained system enclosed in a plexiglass incubator (132). Nutrient fluid, circulated by pumps, was separated from the tissue explants by the membranes which permitted

diffusional exchange but not direct contact with the culture environment. Oxygen was supplied to the fluids by exchange through the teflon conduits and carbon dioxide from an exchanger, composed of teflon tubes, submerged in the nutrient reservoir. Detailed descriptions of the equipment and its operation have been illustrated (132). This dialysis system was shown to successfully propagate various lines of tissue cells, enhance cellular differentiation, and, in a separate study, allow continuouse propagation of mouse melanoma cells in a serum free environment for 418 days (133). Continuous and indirect contact with the culture environment by in vitro dialysis more closely resembled a natural in vivo environment than conventional or individual culture chambers. It was felt that this enabledsuperior control of a constant physiological pH and the provision and retention of basic and vital nutrients and cellular secretions respectively creating a growth enhancing microenvironment. In addition this system provided the interconnection of multiple culture units, the use of a reliable respiratory system, and the containment of all the components in a temperature controlled air circulated incubator. Although attributes of dialysis exchange have been applied to bacterial nutrition and aeration (57, 61, and Section G of this thesis) this represented an advance in technique and scale size for the application of dialysis to mammalian tissue culture.

Recently Rose further improved the circumfusion system for multipurpose culture chambers by redesigning the individual culture chambers for better membrane seals and by adding the physical dimensions of chamber rotation, additional culture chambers, alternating intrachamber hydrostatic pressures, and fluid switching mechanisms (134). This work represented a refinement of the previous system and was reportedly

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superior for the maintenance of cellular differentiation. The major design changes eliminated membrane wrinkling in the culture chambers, reduced sedimentation deposits, simulated in vivo venous and arterial capillary pressures, enlarged the capacity of the system, and provided for the selective direction of fluid nutrients through a choice of culture units. The elimination of sediments and closer simulation of in vivo conditions reportedly enhanced the results for the tissue lines illustrated. The additional chambers and fluid switching capability permitted continuous or intermittent membrane mediated fluid contact for the study of such interbiotic relationships as symbiosis or host-tissue reactions. The work represents further refinement of hardware with illustrated examples of its operation.

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5.2 Materials and Methods

Dialysis growth trials were conducted in a dialyzer-dialysis culture system (Figure 18 and 19) similar to the one developed by Gallup (61) and the same as the one described in the preceding section (Section 4.2). The culture vessel was a conventional 5 liter fermentor (24-M) which was charged with 3 liters of Trypticase soy broth (30 g/L) (9-M), synthetic medium (145), or distilled water. The reservoir was charged with 10 to 26 liters of one of these media. Depending on the volume desired it consisted of either one or two 14 liter fermentors (24-M). The latter were connected in series by conduits and a pump (Figure 19). The fermentor and reservoir vessels were situated in a 30" x 20" x 15" water bath equipped with a mixer (22-M), thermoregulator (3-M), and 1000 watt immersion heater (2-M) for maintaining a constant temperature of 30°C. The prototype dialyzer described previously (Section 3.2) contained 1 to 15 (.0288 m^2 to .4320 m^2 area) presoaked Visking regenerated cellulose dialysis membranes (28-M) and was connected to the vessels by 1/4 inch I.D. rubber tubing (25-M). A positive displacement variable speed pump (27-M) inserted in the reservoir and fermentor circuits circulated the fluids from their respective vessels through the dialyzer and back. The stainless steel heads of these pumps were removable which permitted steam sterilization of the entire dialysis culture system as a unit. Glass "T's" capped with vaccine bottle stoppers were also placed in the fluid circuits to allow asceptic inoculation and

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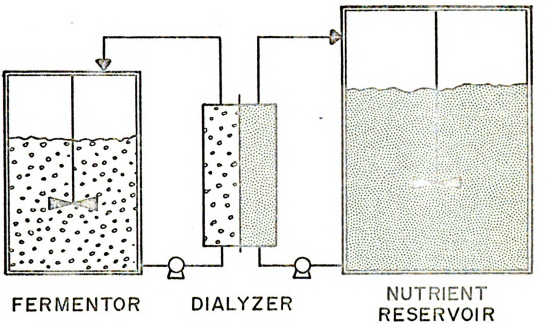


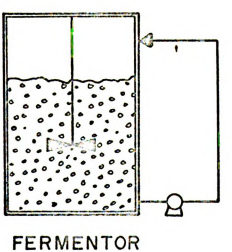
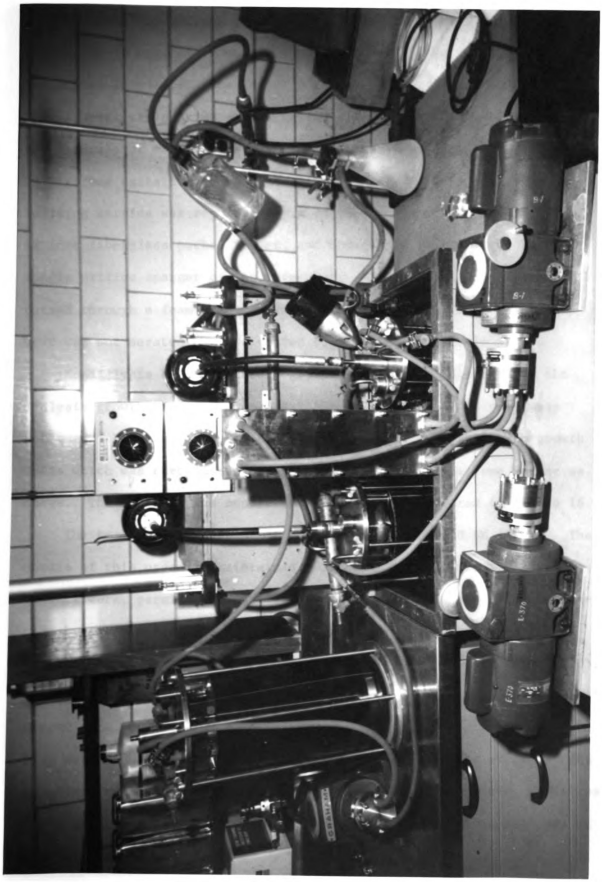


Figure 18. Schematic diagrams of the culture systems used. Top; A dialyzer-dialysis culture system. Bottom; A nondialysis "control" culture system.

Figure 19. Assembly of the experimental dialysis growth system. Two 14 liter fermentors are joined to form a 30 liter medium reservoir (left) which is connected to the dialyzer (center) by rubber tubing. The culture fermentor (right) is connected to the dialyzer in the same way. Pumps (foregound) circulate the respective fluids through the dialyzer.



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sample removal by syringe and needle. The contents of the fermentor and reservoir were mixed by means of the pumped fluid circulation and the vessel impellors. The latter were connected to 1/8 H.P. motors (10-M) by flexible drive shafts (21-M) with speed being controlled by speed regulator power units (17-M). Culture aeration was accomplished by vigorous agitation and sparging. Prefiltered (12-M) air from the building service was regulated by a flowmeter (18-M), sterilized by a 12 inch fiberglass packed filter, and humidified before entering the single orifice sparger of the fermentor. Gases exiting the fermentor passed through a foam trap and another fiberglass filter. The reservoir was not aerated but was vented by a filter and slowly mixed.

Nondialysis batch "control" growth trials were identical to the dialysis growth trials, except that the reservoir and dialyzer were not used. In this case, the culture fermentor was charged with growth media which was circulated, sampled, and aerated in the same manner as above. The dialysis and nondialysis schemes are compared in Figure 18.

The test organism was Serratia marcescens strain 8 UK (29-M). The choice of this organism maintained continuity with previous dialysis culture work, permitted identification of equipment leaks or contamination by its pigmentation, and allowed meaningful evaluation of growth results because its nutrition and growth has been documented (145, 100). A uniformly-pigmented colony was picked from a streak plate and transferred to a 500 ml flask containing 100 ml of Trypticase soy broth. This was incubated on a rotary shaker (23-M) for 10 hours at 30°C. The resulting culture served as an inoculum for the growth trials. This was approximately a one percent by volume or 70 to 200 million cells per ml concentration.

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The initiation and follow-though of all growth trials was similar. The equipment was assembled as described and illustrated. Preparation of inoculum and media was the same in companion experiments. Several types of trials were used: (a) "control" where the fermentor contained 3 liters of growth medium; (b) dialysis where both the fermentor and reservoir initially received growth media; and (c) dialysis where the fermentor was initially charged with water, the reservoir charged with growth medium, and a 10 hour dialysis exchange period run before inoculation of the fermentor.

Upon selection of the type of trial to be run, the necessary equipment was assembled, filled, and steam sterilized as an integral unit for 40-50 minutes at 121°C. Routinely 3 or 2.6 liters were used in the fermentor and 9 or 26 liters in the reservoir, equivalent to fermentor to reservoir ratios of 1:3 and 1:10, respectively. All fluids were sterilized within their respective vessels, except for glucose which was added asceptically prior to inoculation. Upon cooling, the system was assembled as illustrated in Figure 19. After temperature equilibration to 30°C., the fermentor was inoculated with 2.6 or 3 ml of the shake culture to provide an initial cell concentration of approximately 70 million cells per ml in the fermentor. Unless specified otherwise, the culture system was operated at pump velocities of 2 L/min., air velocity of 9 9 L/min, agitation of 365 r.p.m. (fermentor) and 50 r.p.m. (reservoir), a pH of 7, and a temperature of 30°C.

During the 48 hour growth trials, culture samples were periodically removed and assayed for total and viable cell numbers. The amount of cell matter was assayed by dry weight and optical density. Total counts were obtained by direct microscopic observation using a conventional Petroff-Hauser counting chamber and appropriate dilutions.

Viable counts were obtained by the conventional surface plating technique on Trypicase soy agar. Dry weights were determined by centrifuging, washing, resuspending and oven drying (11-M) culture samples at 105°C to a constant weight. Optical density was measured at 620 mμ by a colorimeter (6-M) on 1:10 sample dilutions. All samples were corrected for liquid loss. Initial volumes were maintained by addition of sterile water from an attached reservoir or by syringe as needed. Foam was controlled by addition of approximately 50 ppm of polypropylene glycol (16-M) prior to sterilization. Finally, all growth trials were monitored for pH (7-M), contamination, and loss of volume due to evaporation, sampling, foaming, or leaks.

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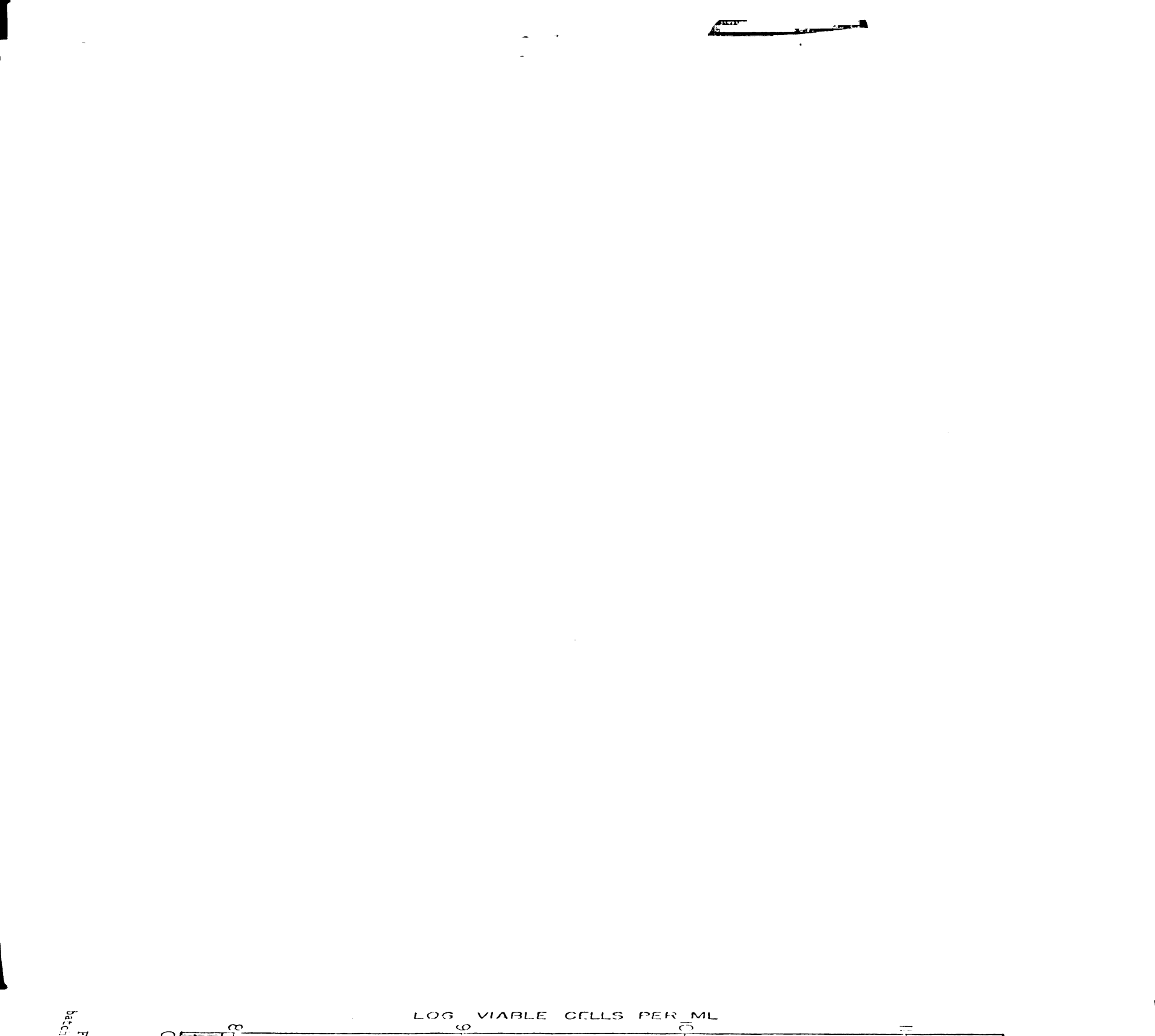
5.3 Results

The experimental growth trials reported here were intended to illustrate the attributes of a dialysis culture system, demonstrate the suitability of this prototype dialyzer, and show the influence of some operating variables on the growth of Serratia marcescens cultures. Analysis of the growth characteristics of the test organism in nondialysis batch culture provided a standard reference against which subsequent dialysis growth trials could be compared. A series of duplicated nondialysis cultures were grown in 3 liters of Trypticase soy broth under the routine conditions listed previously. A composite growth curve (Figure 20) revealed that these achieved a mean viable cell concentration of 55 billion cells/ml after 36 hours, which decreased to a mean of 46 billion cells/ml after 48 hours. The peak cell concentrations ranged from 43 to 88 billion cells/ml with a standard deviation of $\pm$ 22 billion cells/ml between repeated trials. During the exponential growth phase the organism had a mean generation time of 30.1 minutes $\pm$ 4 minutes (s.d.).

A similar series of duplicate growth trials with dialysis were conducted using 3 liters and 10 liters of Trypticase soy broth in the fermentor and reservoir respectively (a 1:3.2 fermentor to reservoir ratio), ten dialyzer membranes (.288 m^2 area), and the same operating conditions as above. Significantly superior viable cell concentrations were reached and a similar repeatability of results were observed (Figure 21). These dialysis growth trials achieved a mean peak cell concentration of 120 billion


LOG VIABLE CELLS PER ML

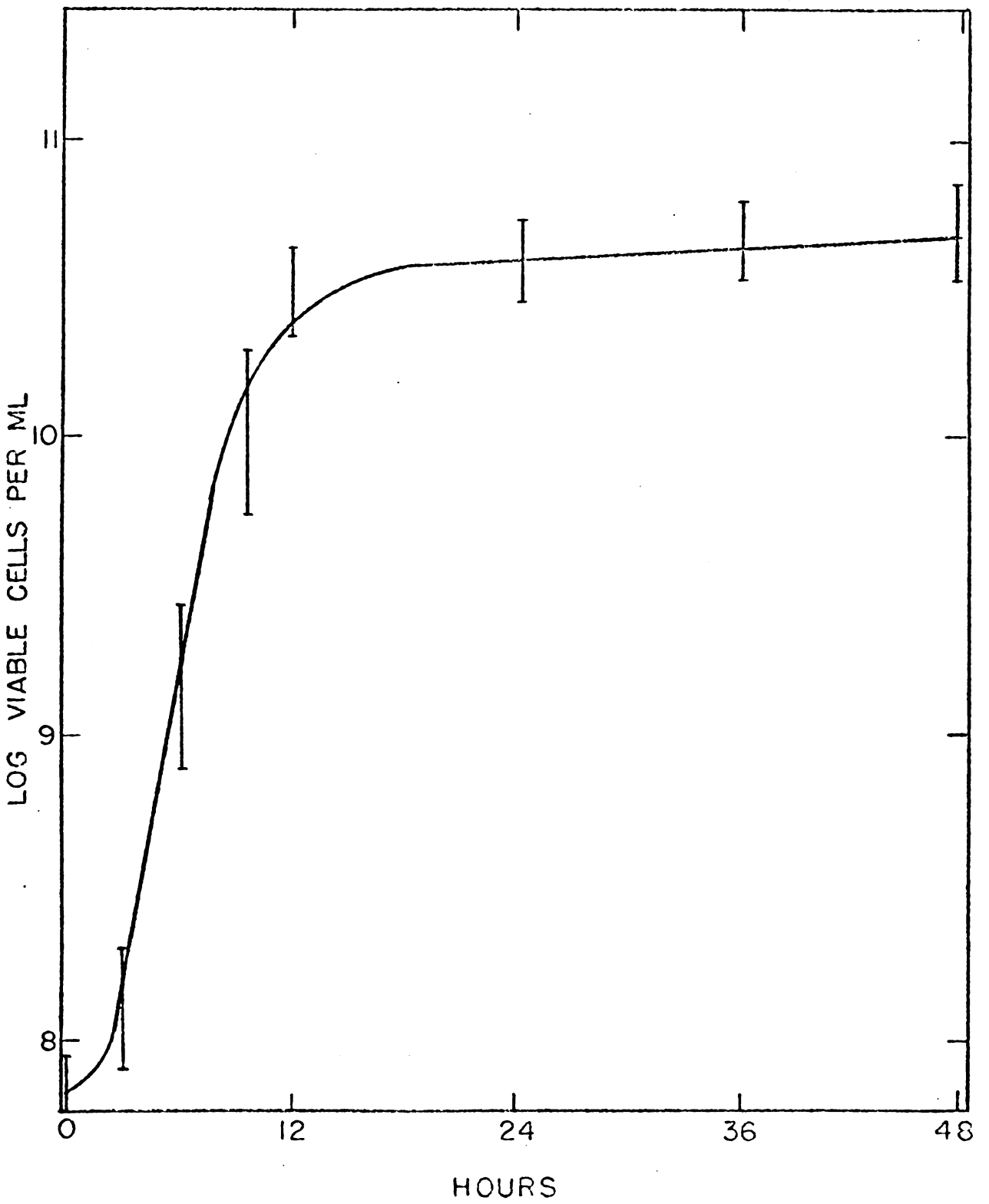
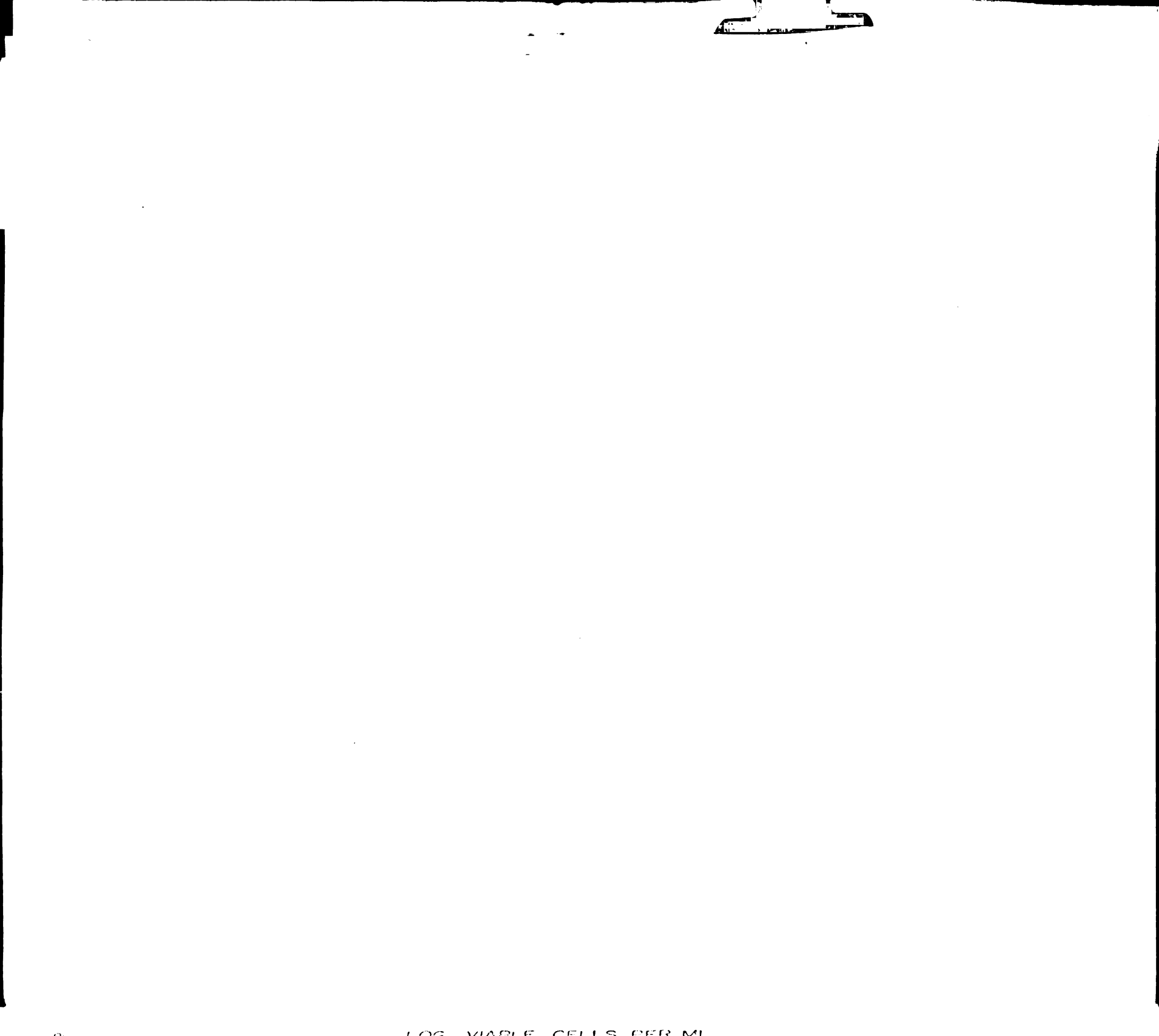


Figure 20. Growth curve of Serratia marcescens; mean of four duplicated batch nondialysis cultures.


LOG VIABLE CELLS PER ML

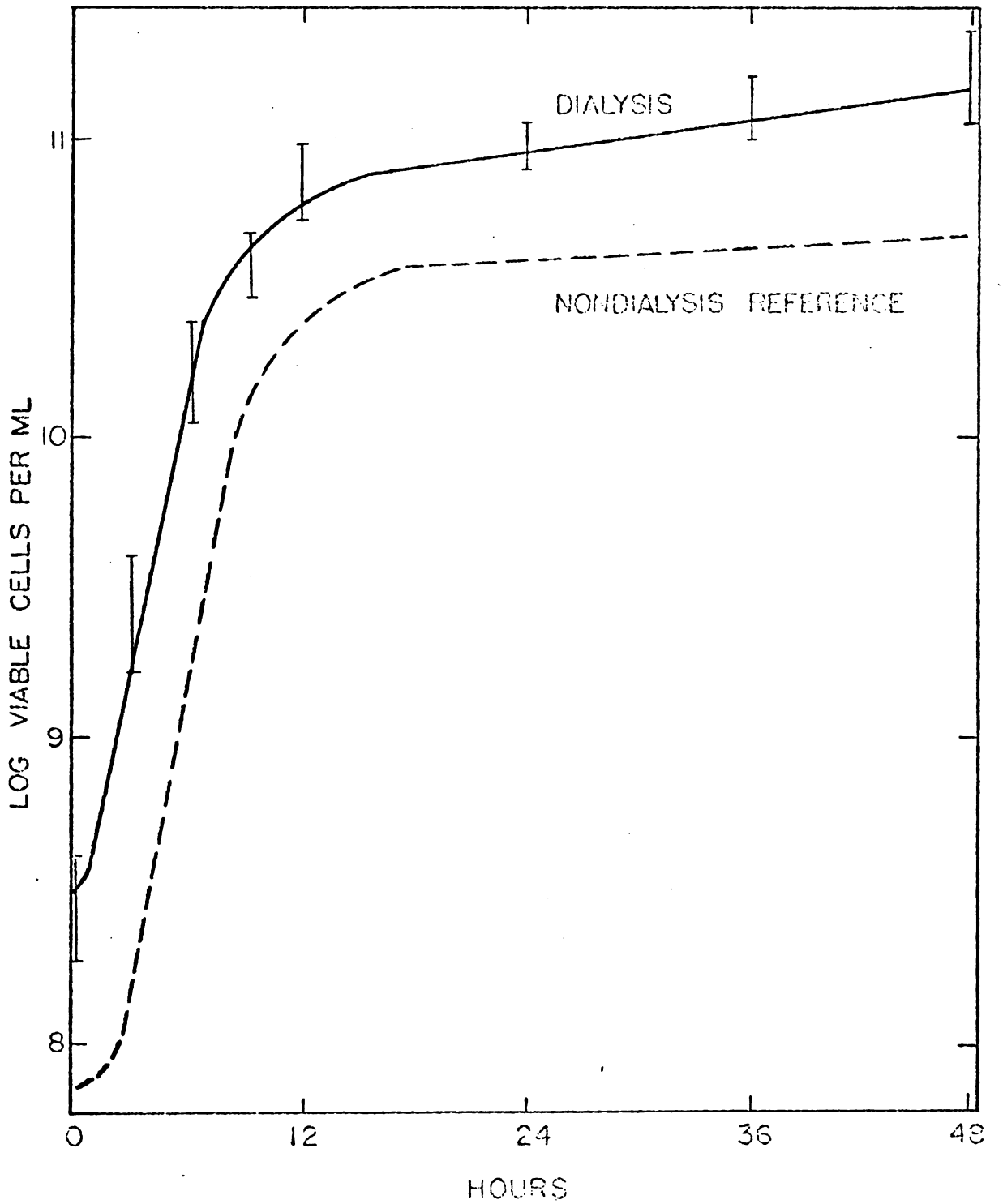


Figure 21. Growth curve of Serratia marcescens; mean of duplicated dialysis cultures with ten membranes (.288m^2) in the dialyzer.

cells/ml after 48 hours, had a range of peak values from 100 to 140 billion cells/ml. The exponential dialysis growth rate, the mean generation time, 35.4 minutes $\pm$ .6 minutes (s.d.), and the standard deviation, $\pm$ 20 billion viable cells/ml between repeated trials were similar to those for the nondialysis cultures. Although there was a difference in inoculum concentration between the two sets of growth trials (Figure 21) this was shown, in subsequent experiments, to have no significant effect on final culture concentrations. Thus the repeated superiority of dialysis cultures, an extended exponential growth phase and a greater final culture density, could be accepted with confidence.

Another attribute of dialysis culture was the maintenance of a greater proportion of viable cells during a 48 hour growth trial (Table 11). The examples illustrated show that: the correlation between total and viable cells was superior for dialysis culture in all cases; the proportion of viable cells in a culture decreased as growth progressed; dialysis culture with a small membrane area, series A, produced cell concentrations similar to those of comparable nondialysis but with a superior proportion of viable cells per total (89% vs 75%) after 48 hours; and dialysis culture, utilizing an optimal membrane area and a water diffusate medium, series B, produced both superior cell concentrations (283 billion vs 8 billion) and a markedly greater proportion of viable cells per total (85% vs 42%) after 48 hours of growth.

The preceding data demonstrates the successful operation of this dialysis cultue system with the prototype dialyzer and confirms two potential advantages of dialysis culture, increased culture density and improved culture viability. Microbial propagation in a clear particulate-free medium is desirable in certain fermentations, especially when

Table 11. Comparison of total and viable cell concentrations in dialysis and nondialysis growth trials with Serratia marcescens.

SERIES*	CULTURE SYSTEM	BILLIONS OF CELLS PER ML					
		AFTER 24 HOURS			AFTER 48 HOURS		
		Total	Viable	Percent Viable	Total	Viable	Percent Viable
A	Dialysis, 2 Membranes ($.0576m^2$)	37	35	95	45	40	89
	Comparable Nondialysis	53	48	90	56	42	75
B	Dialysis, 2 Membranes ($.4320m^2$)	175	162	97	330	283	85
	Comparable Nondialysis	18	11	61	19	8	42

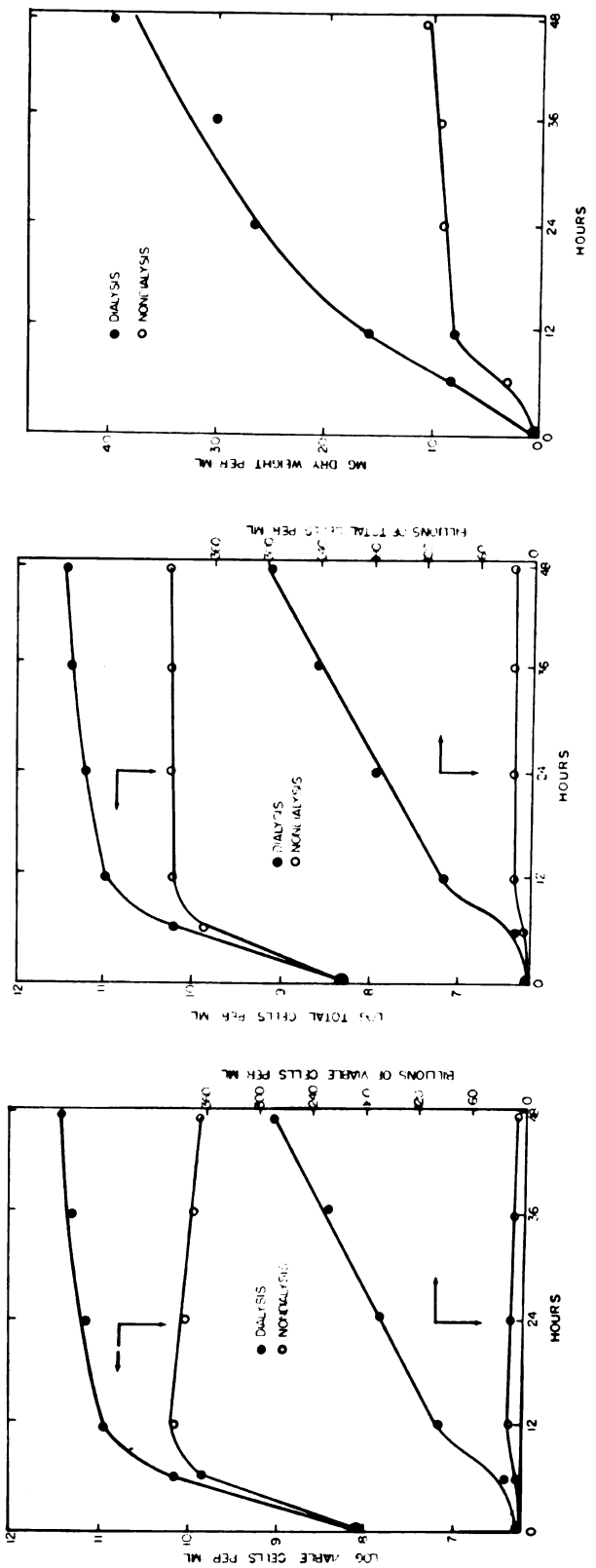
* A, dialysis growth trials where both fermentor and reservoir initially contained Trypticase soy broth medium.

B, dialysis growth trials where the fermentor initially contained only the water diffusate dialyzed from the reservoir containing Trypticase soy broth medium.

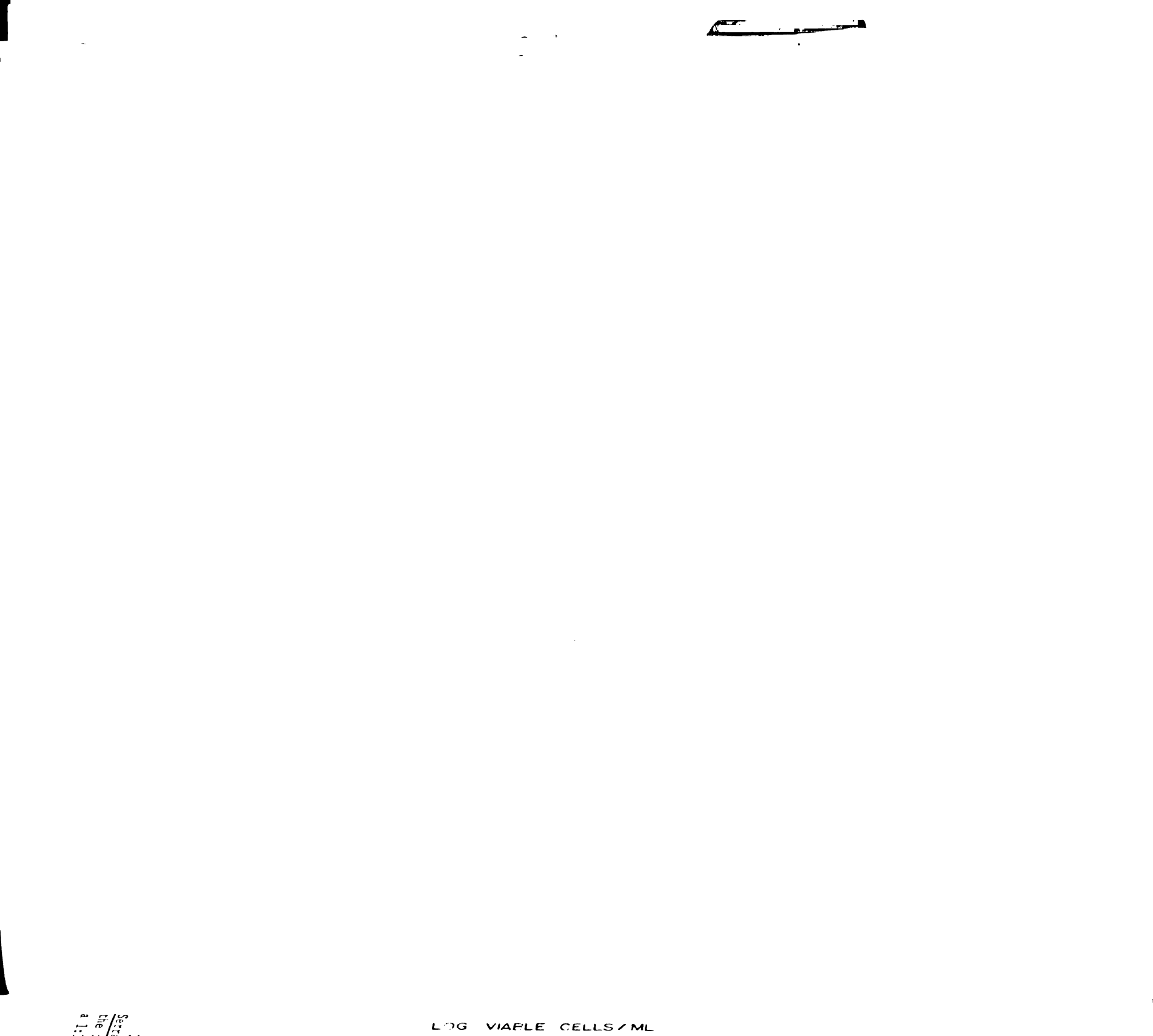
recovery of a metabolic product or a "clean" suspension of cells is sought. Such a fermentor environment can be obtained and a continual adequate nutrient supply maintained in dialysis culture. Such dialysis cultures, when operated optimally and with a large membrane area (15 membranes, .4320 m^2 area), achieved high population densities and cell yields (Figures 22, 23, 24). As illustrated, the significant superiority of dialysis culture was demonstrated in terms of higher viable cell density, total cell density, and cell mass. The comparable nondialysis culture in this instance was initially identical, the fermentor containing 2.6 liters of Trypticase soy broth diffusate, but did not receive the benefit of continuous nutrient exchange by dialysis after inoculation. The advantage of dialyzing a large nutrient reservoir was clearly shown by the longer exponential phase and by the continuous increase in viable cell concentrations and cell mass in the later stages of the trial (48 hours). In contrast these parameters in nondialysis declined or remained stationary.

Probably the most important variable of the dialysis culture system was expansion of dialyzer membrane area. When a 2.6 liter fermentor containing the water diffusate of the Trypticase soy broth in the 26 liter reservoir (1:10 ratio) was inoculated with Serratia marcescens, dialyzer membrane areas of .0288 m^2 (1 membrane), .0864 m^2 (3 membranes) and .1728 m^2 (6 membranes) produced similar rates of growth and only slightly increasing viable cell concentrations after 48 hours (Figure 25). As shown an increase in area to .2880 m^2 (10 membranes) significantly improved cell concentrations from 80 billion cells/ml for six membranes to 210 billion cells/ml. Further expansion to .4320 m^2 (15 membranes) produced only a small additional increment in final viable cell concentration to 283 billion cells/ml,

Figures 22, 23 and 24. Growth curves of Serratia marcescens, in dialysis and comparable nondialysis culture. Operational conditions were: 2.6 liters of Trypticase soy diffusate in the fermentor and 26 liters of Trypticase soy broth in the reservoir (a 1:10 ratio), 15 membranes in the dialyzer, 30 C, 9 L/min sparging, 365 r.p.m. agitation, and 2 L/min liquid flow rates.



Figures 22, 23, 24 .


LOG VIABLE CELLS / ML

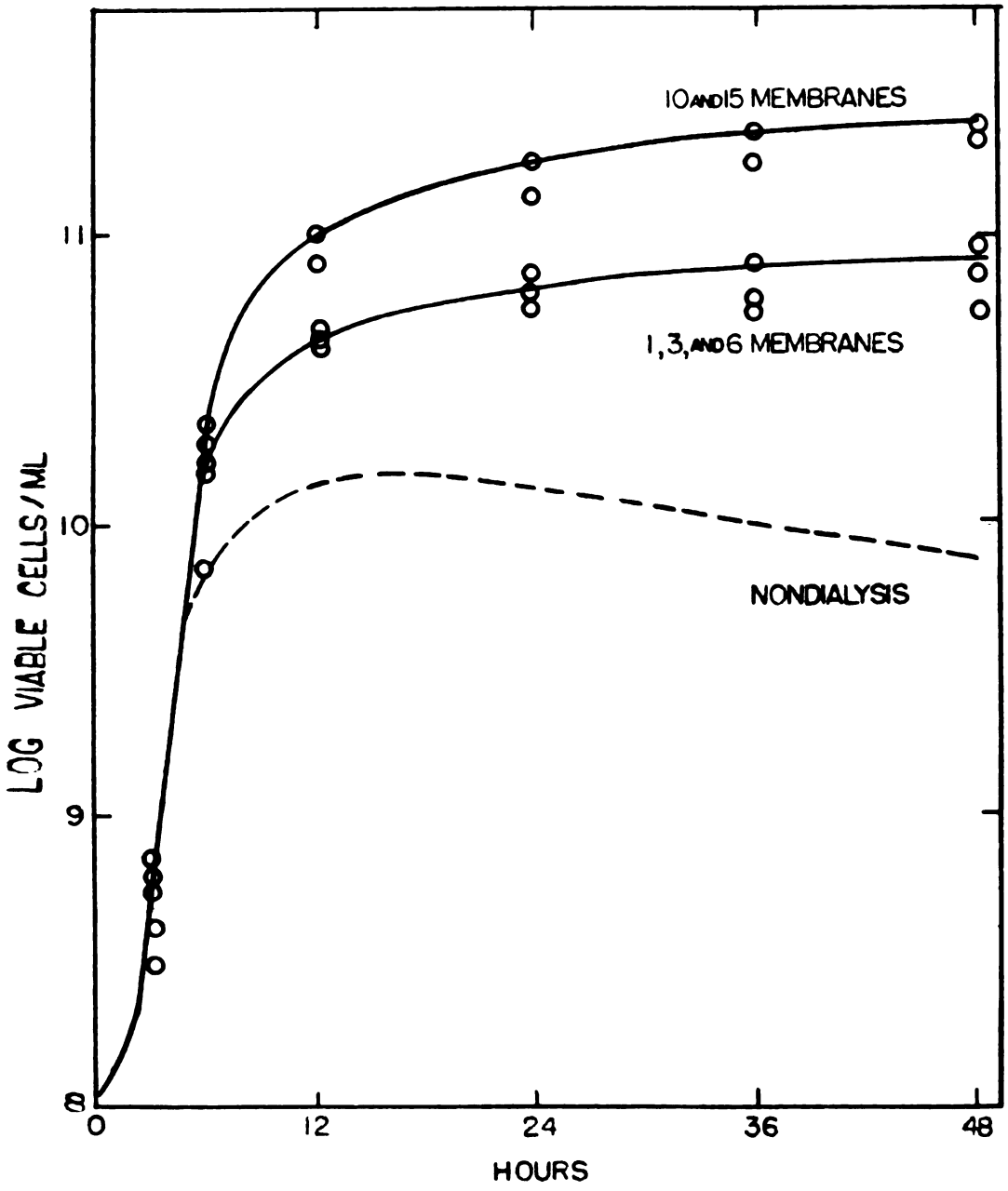


Figure 25. Influence of dialyzer membrane area on growth of Serratia marcescens. Each membrane has an effective area of .0288m^2, the fermentor contained water diffusate of Trypticase soy broth, and a 1 : 10 fermentor to reservoir volume ratio was used.

the highest value recorded for the system. These latter results compared favorably with the earlier observation that nutrient transfer rates optimized at a dialyzer area of .288 m^2 (10 membranes) (Figure 8). An increase in membrane area appeared to extend the duration of the exponential growth phase enabling the attainment of higher culture concentrations. Area enlargement did not however increase the proportion of viable cells within the culture (Table 11). The advantage of additional area was further illustrated by the final cell mass (dry weight) for the growth trials (Figure 26). Here the addition of area above ten membranes produced a more decided increase in yield than shown by viable cell counts. This difference was because mass measurements included both viable and nonviable cells. Dialysis cultures in all cases significantly exceeded the results obtained in comparable nondialysis cultures. In addition, dialysis under these conditions enable a prolonged linear-stationary growth phase in contrast to the decline in culture viability observed in nondialysis.

The preceding growth trials revealed that dialysis cultures consistently achieved superior culture concentrations and viability, extended exponential growth phases, and prolonged linear and/or stationary growth phases. In addition, these results were enhanced by an increase in membrane area. These trials indicated that adequate diffusional access to a nutrient reservoir was the key to the success of the dialysis culture system. It was shown earlier that this process, by virtue of the continuous circulation of the culture and reservoir fluids through the dialyzer, represented equilibrium dialysis (Figure 6). As shown, solutes dialyzed from the reservoir into the fermentor until the system reached equilibrium. The same process occurs in a culture situation (Figure 27).

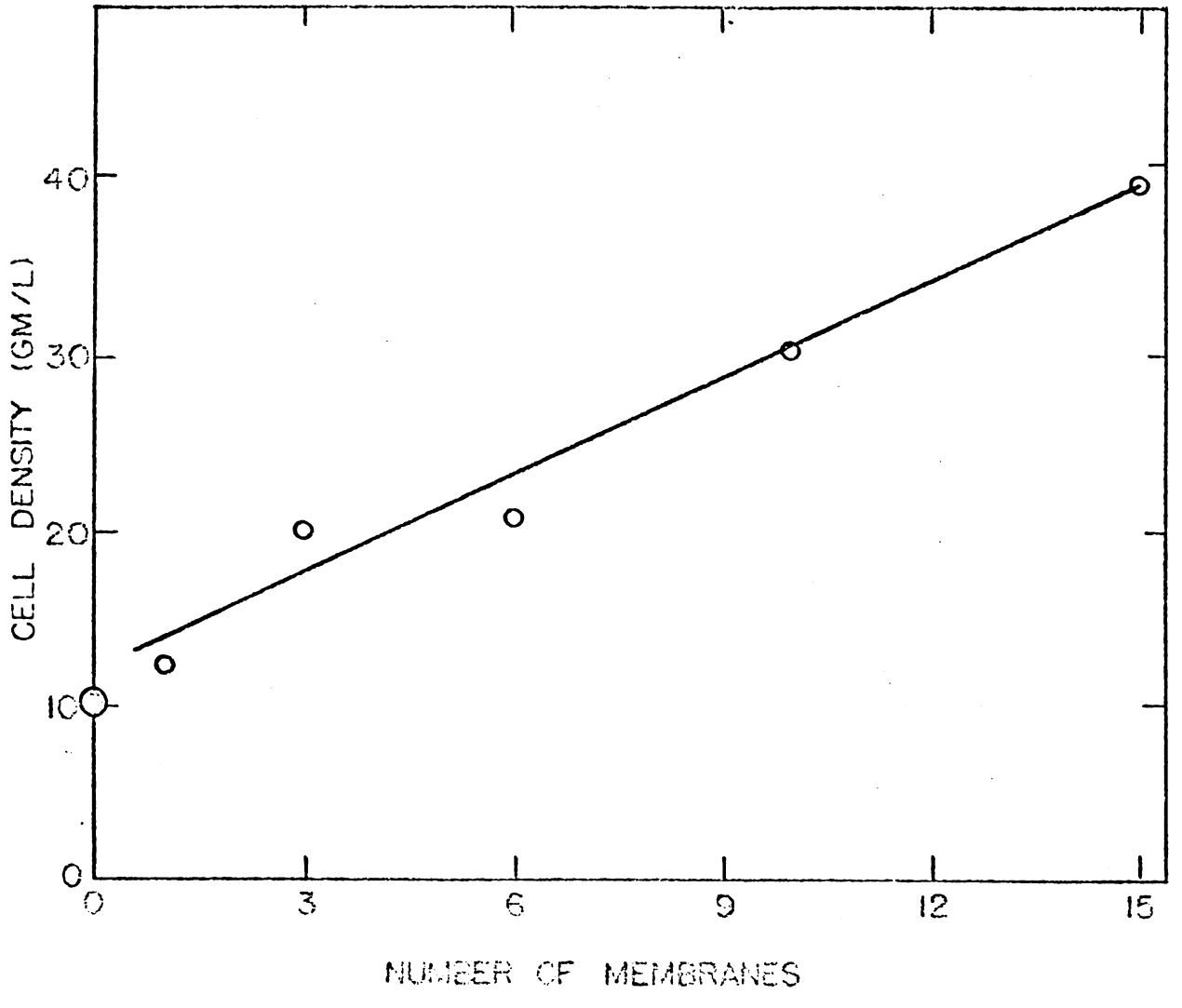


Figure 26. Influence of dialyzer membrane area on cell mass of Serratia marcescens after 48 hours growth under the same conditions given in the preceding figure. The data point on the absissa represents the value for a nondialysis growth trial.

Here the nutrients, a glucose-citrate salts synthetic medium containing 2.5 g/L glucose which was the solute measured, transferred from the 30 L reservoir to the 3 L fermentor containing water, during a 12 hour equilibration period. In this example the dialyzer had a single membrane and the system had not reached equilibrium (which was 2.2 g/L or 86% of the initial reservoir concentration) at the time of culture inoculation. However, the fermentor had received 1.8 g/L of glucose (80% of equilibrium or 69% of the initial concentration) which was adequate for rapid growth. Additional membrane area would enhance the rate of glucose transfer prior to inoculation. Figure 27 shows that culture growth, especially during the exponential phase, caused a pronounced decrease in fermentor nutrients. Diffusional access to the reservoir prevented the decrease in the fermentor from extending below 30% of the initial glucose concentration and allowed the nutrient concentration to gradually recover during the growth trial. Reservoir nutrients were not expended by the end of the trial. They had only decreased to 70% of their initial value, and a new fermentor-reservoir equilibrium of 1.5 g/L or 57% of the initial concentration, was in the process of being established. In contrast, a comparable nondialysis growth trial with the same growth medium experienced a 90% decline in nutrient concentration within 12 hours (Figure 28). This limited the extent of growth (26 vs 139 billion cells per ml) and caused the appearance of a decline growth phase. Diffusional access to the nutrient reservoir appeared to be an important attribute of the dialysis culture system. This permitted sufficient transfer to prevent the exhaustion of fermentor nutrients and allowed attainment and maintenance of superior culture concentration. In addition these growth trials

Figure 27. Correlation between dialysis growth and solute (glucose) concentration in the fermentor and reservoir; the time period -12 to 0 hours represents the equilibration of the 3 liter fermentor and 30 liter nutrient reservoir prior to culture inoculation. A single membrane (.0288 m^2) was used in the dialyzer.

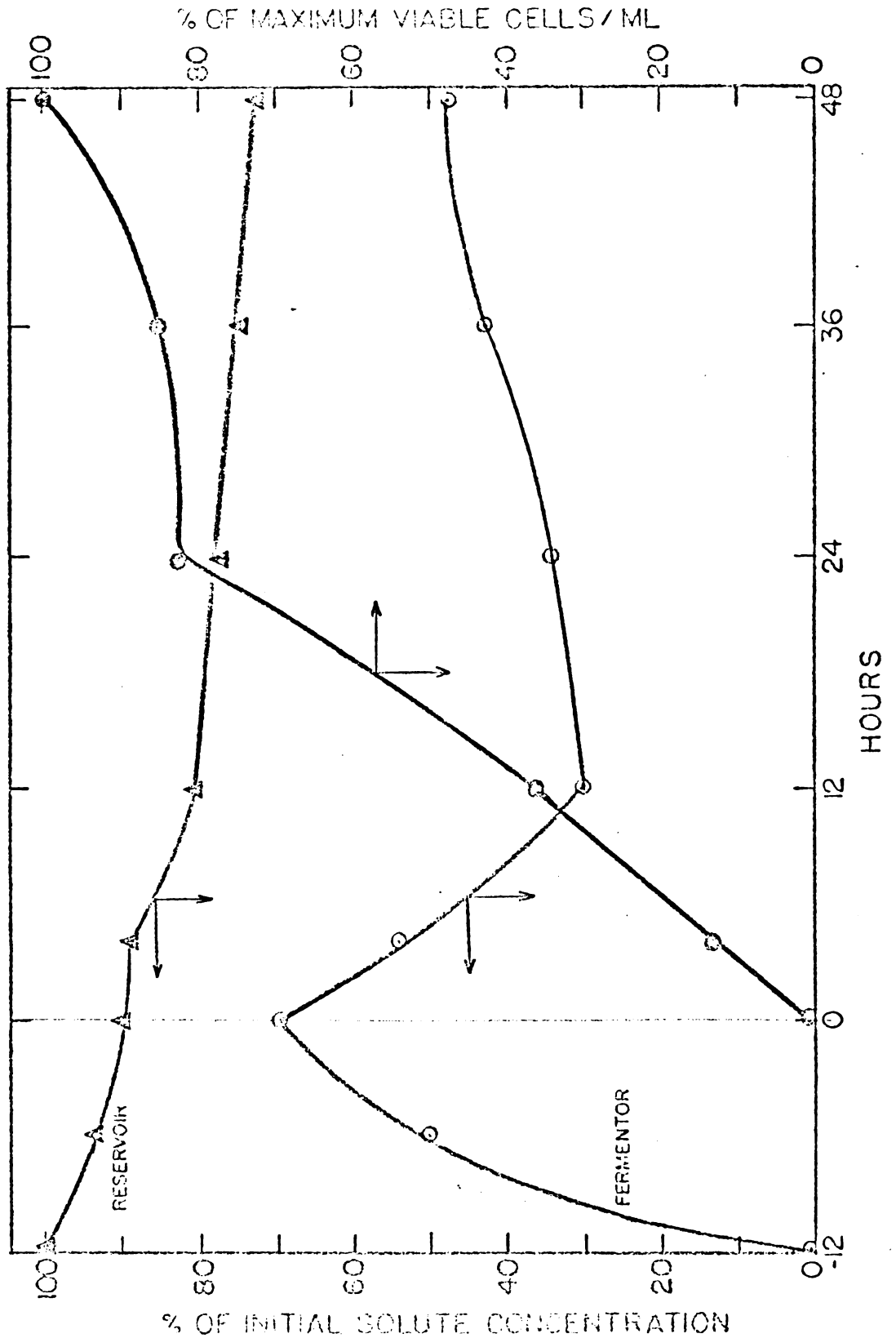

% OF MAXIMUM VIABLE CELLS / ML
100
80
60
40
20
0
48
36
24
12
0
-12
HOURS
RESERVOIR
FERMENTOR
% OF INITIAL SOLUTE CONCENTRATION

Figure 27.

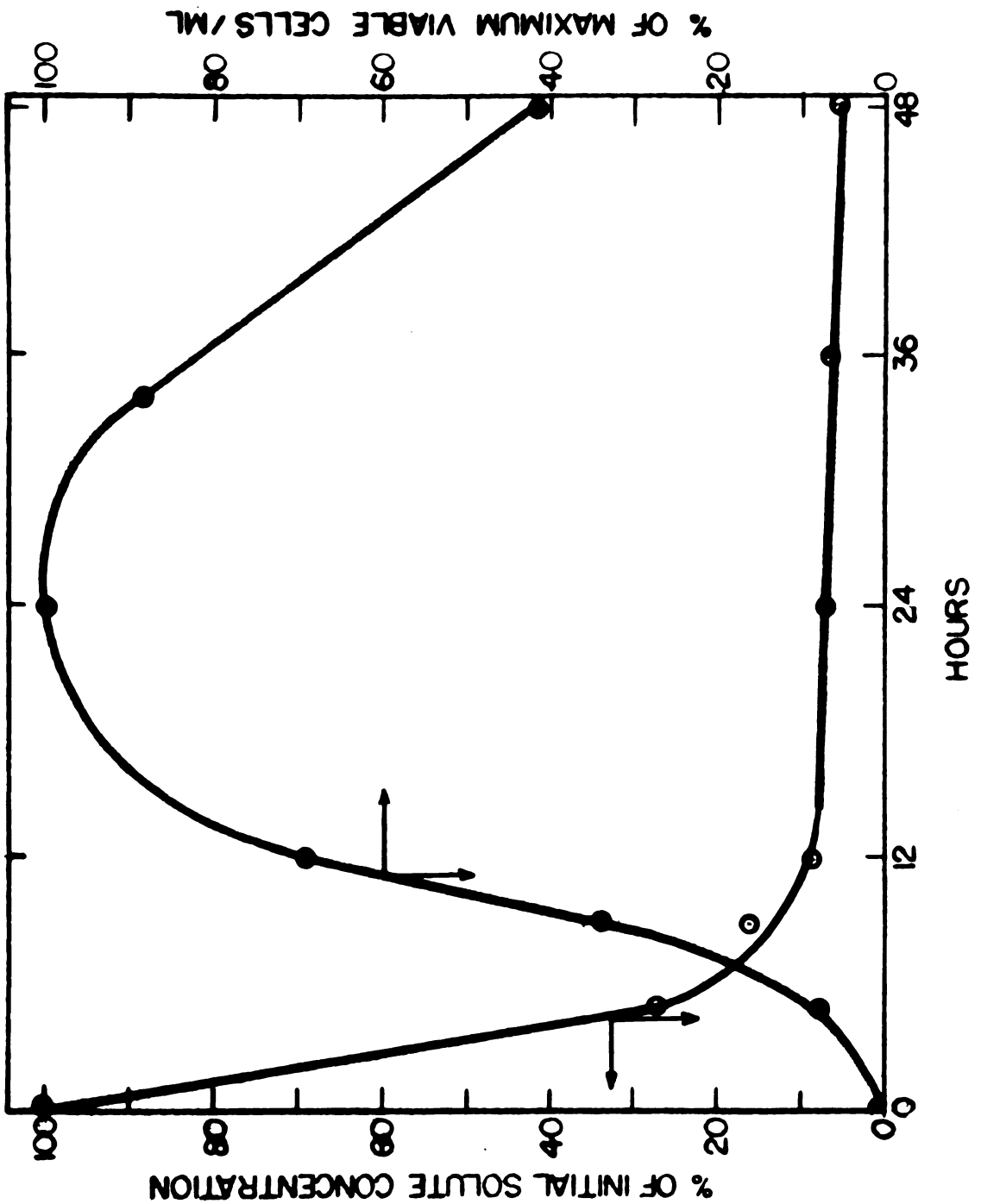
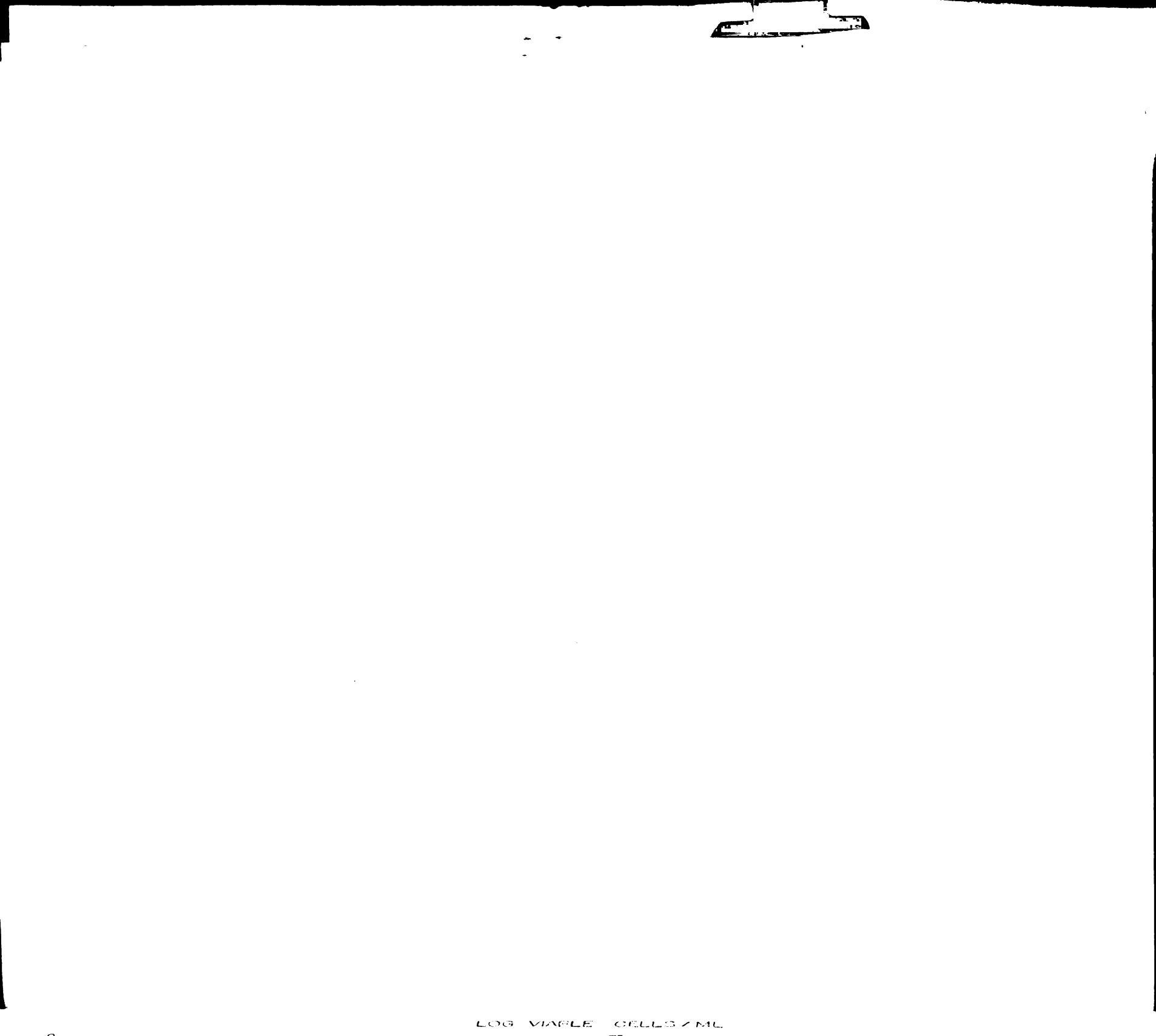


Figure 28. Correlation between nondialysis growth and solute (glucose) concentration.

demonstrated that the presence of metabolizing cells had no effect on the dialysis mass transfer process other than to shift the equilibrium end point of the system.

The benefit derived from diffusional access to a nutrient reservoir was vividly illustrated when growth trials were extended to five days or more (Figure 29). Dialysis cultures of Serratia marcescens achieved substantially higher culture densities which were maintained in a prolonged stationary growth phase for up to five days. An enlarged dialyzer membrane area had no influence of the stationary phase other than to raise the level of cell concentration during this period. In contrast, nondialysis cultures had a substantial reduction in viable cell concentration over the same time span and showed little or no stationary growth. Sufficient nutrients remained in the reservoir after six days to further extend dialysis growth an additional six days. These results demonstrate the value of the dialysis culture reservoir for the supply of nutrients and the dilution of growth limiting solutes within the culture. As a result highly concentrated viable cultures were consistently maintained for long periods. This characteristic could prove advantageous for the production of intermediary metabolic products which generally are produced most rapidly during the stationary growth phase.

Growth media, on the basis of glucose concentration, appeared to influence nondialysis cell yields (Table 12). The greatest culture densities occurred in the medium with the greatest glucose concentration, with progressively poorer yields occurring in weaker media. This dependance on nutrient strength was not observed with dialysis cultures, which achieved higher cell yields in all the growth media. The


LOG VIABLE CELLS / ML

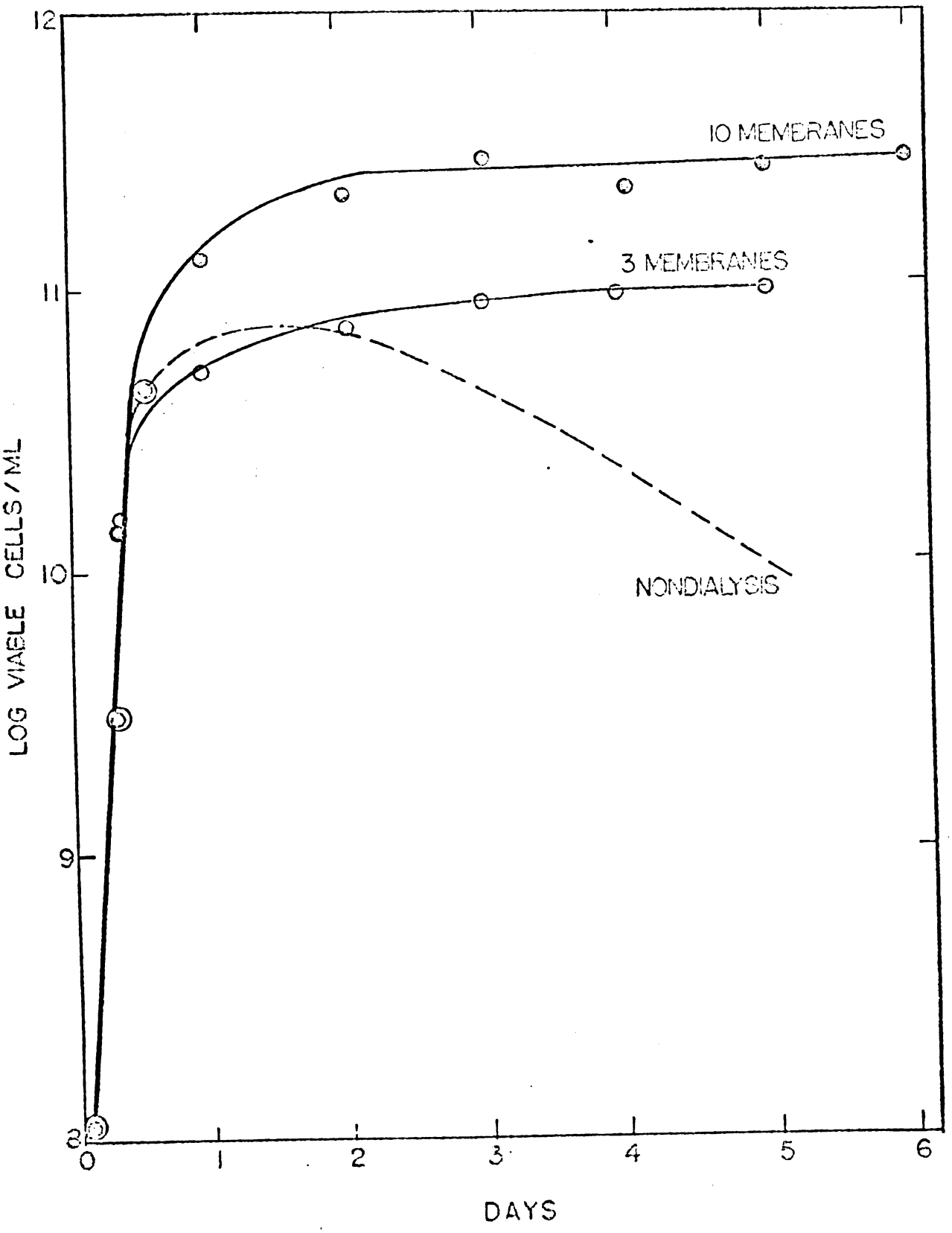


Figure 29. Influence of dialysis culture and membrane area on the viable concentration of Serratia marcescens cells over an extended growth period.

Table 12. Influence of selected growth medium on cell production in dialysis (D)* and nondialysis (C) cultures.

	MEDIUM IN CULTURE FERMENTOR	GLUCOSE CONCENTRATION (G/L)	VIABLE CELLS (Billions/Ml)			
			D_6	D_{10}	C	D/C
1	Water Diffusate of Trypticase Soy Broth	1.9	90	140	10	9 & 14
2	Trypticase Soy Broth	2.5	120		56	2.1
3	Smith-Johnson Synthetic Medium	10.0	120		70	1.7

* Subscripts designate the number of membranes in the dialyzer.

superiority of dialysis over nondialysis was especially apparent with the weakest growth medium, water diffusate of Trypticas soy broth, in which dialysis produced 9 times more cells. This yield was further improved, to the highest shown in the table, by an increase in membrane area. The results again exemplify the advantage gained by diffusional access to a nutrient reservoir. In this case dialysis of a large reservoir of dilute growth media prevented the growth limiting depletion of the fermentor nutrients during the fermentation. As expected when a small concentration gradient is involved, the process responded favorably to increased membrane area by providing additional nutrients sufficient for greater cell growth.

The separate fermentor, reservoir, and dialyzer regions of the dialysis culture system allowed independant control of each. A number of fermentor-to-reservoir volume ratios were obtained by changing the volumes used in these vessels. Solute transfer experiments indicated that a 1:10 fermentor to reservoir volume ratio was optimal (Figure 16). Previously published growth trials with Serratia marcescens in a reservoir-dialysis culture system showed that the same ratio produced the best culture yields (57). Growth trials with the same organism demonstrated that a 1:10 ratio achieved higher culture yields than smaller (1:4) ratios in the present dialyzer-dialysis culture system (Table 13). These results correlate with the calculated differences in culture yields anticipated for different ratios in this system (Figure 30). The fermentor-to-reservoir ratio could be changed either by reducing the fermentor volume (series A, Table 13) or by increasing the reservoir volume (series B, Table 13). The results illustrate that the greatest cell densities and culture yields were obtained with the latter adjustment.

Table 13. Influence of fermentor-to-reservoir volume ratios on viable cell concentration during dialysis culture of Serratia marcescens.

SERIES*	VOLUME (L)		RATIO	BILLIONS OF VIABLE CELLS PER ML		CELL YIELD
	Fermentor	Reservoir		After 24 Hours	After 48 Hours	(Trillions/ Fermentor)
A	3	12	1:4	90	120	360
	1.3	13	1:10	130	155	201
B	3	12	1:4	90	120	360
	2.6	26	1:10	147	200	520

* Series A, ratio change due to reduction of fermentor volume.

Series B, ratio change due to an increase in reservoir volume.

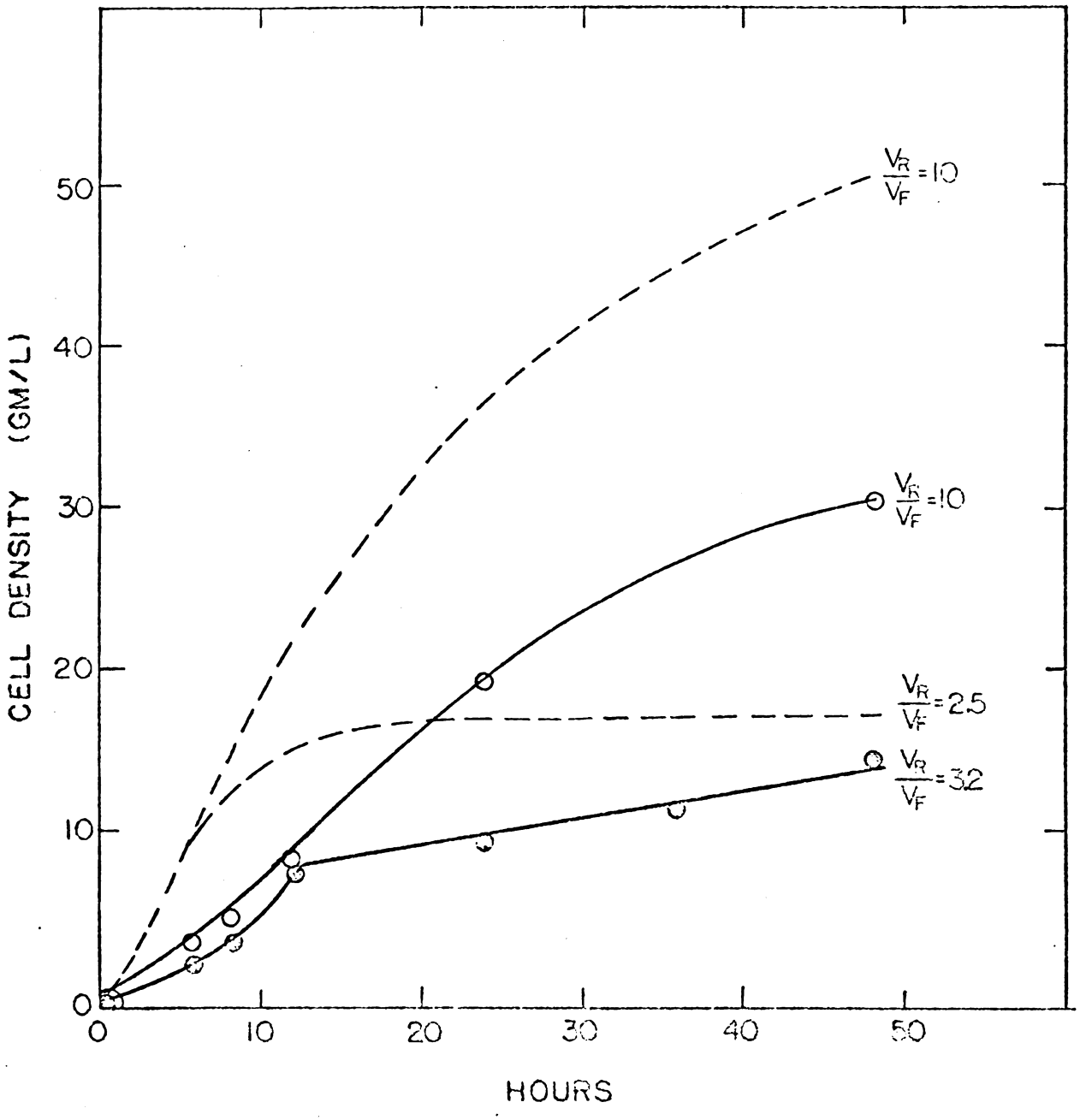


Figure 30. Computed and experimental effect of the reservoir-to-fermentor volume ratio on the cell density of dialysis cultures.

Although reduction of the fermentor volume achieved a 1:10 ratio, the beneficial effect of the proportionately larger reservoir was lost due to the inefficiency of the smaller 1:3 liter volume in the 5 liter fermentor. Consequently this scheme although able to produce a higher cell concentration did not achieve as great a culture yield as anticipated (Series A). The same ratio, 1:10, when obtained by enlargement of the reservoir while retaining the original fermentor volume, 3 liters, produced both a higher cell density and culture yield (series B). This data further demonstrated the biological advantage gained from the nutrient supply and toxic dilution capacity of a large reservoir in the dialysis culture system. Reservoir enlargement to ten times the fermentor volume appears to be the most convenient, most practical, and most productive operational scheme for this dialysis culture system.

The velocity of the fluids circulating through the dialyzer could be varied by altering the pump speeds. Previously it was shown that a minimal velocity of .5 L/min was necessary to produce turbulent flow within the dialyzer and that flow rates greater than 2 L/min had no effect on solute mass transfer rates (Figure 9). Neither the growth rate nor the cell concentration of Serratia marcescens cultures were significantly changed when the dialysis system was circulated at velocities above 2 L/min (Figure 31).

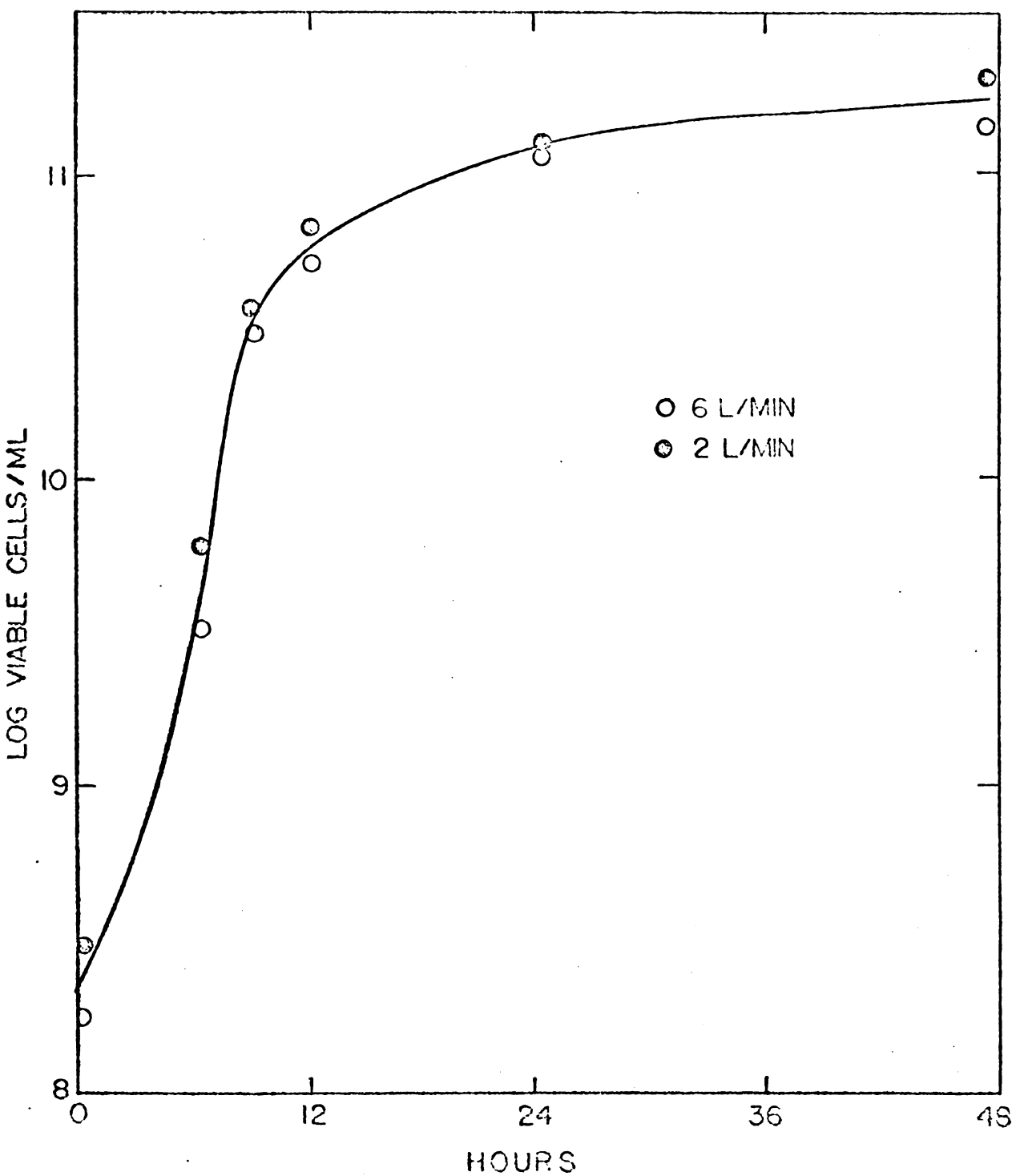


Figure 31. Influence of liquid flow rate through the dialyzer on growth of Serratia marcescens.

5.4 Discussion

The experiments presented in this section were not intended to introduce the dialysis culture concept or dialyzer-dialysis culture system. This has been accomplished (57). Instead these investigations were conducted to demonstrate the biological compatibility and performance of the dialyzer described in Section 3 when used in a dialyzer-dialysis culture system. In addition to showing the successful operation and confirming the previously claimed superiority of dialysis culture, the growth trials were also intended to define the influence of various operational procedures on biological performance. The data were examined to determine if a correlation existed between solute transfer and culture growth, and an attempt was made to delineate the key factors which contribute to the biological superiority of the dialysis culture process.

In order to critically analyze dialysis cultures, the routine procedures used in fermentation growth trials were individually examined and found to have little or no influence on interpretable results. This permitted conclusions derived from dialysis and nondialysis results to be attributed confidently to the culture process and not to the influence of operational conditions, such as pH, buffering, agitation speed, sparging flow rates, antifoam agent, or inoculum size. However culture circulation was an exception. Uncirculated nondialysis cultures showed a decline phase between the 24th and 48th hour of the growth trial. This was less pronounced in circulated nondialysis trials and absent

in dialysis trials. Although various velocities had no effect, the dynamics created by the positive displacement gear pumps apparently reduced cell clumping or improved culture aeration or culture mixing resulting in improved viable cell concentrations during the later stages of the growth trial. Thus nondialysis trials were always circulated in order to accurately compare the growth results with those of dialysis trials which, by necessity, were circulated.

The absence of a clearly defined decline phase in 48 hour nondialysis cultures was observed only when Trypticase soy broth was used. Similar nondialysis cultures using synthetic glucose citrate salts or water diffusate of Trypticase soy broth media exhibited decline growth phases. Dialysis cultures showed a prolonged stationary phase and no decline phase regardless of the nutrient medium used. Nutrient selection did, however, influence the degree by which dialysis culture densities surpassed the comparable controls. This emphasized the care which must be exercised in selecting duplicate culture procedures for both dialysis and nondialysis when comparison between growth trials are intended. Failure to do this as detected in previous reports (57) reduces the credibility of the conclusions drawn from the experimental work.

The many variables of a microbial fermentation which must be controlled or accounted for often make experimental duplication difficult. The variation observed between supposedly identical fermentations has prompted many to regard the fermentation process as an art and has made the term "growth trial" a more appropriate description of these investigations.

A series of repeated dialysis and nondialysis growth trials were made. These permitted an estimate of the standard deviation, range, and mean viable cell counts which could be expected for both types of

culture techniques. This statistical information helped evaluate data and determine the significance of comparative assays.

The duplicated nondialysis "controls" produced growth curves which were similar to those reported previously for batch growth of Serratia marcescens on Trypticase soy broth (57). However, the mean peak cell concentrations achieved in these trials were unexplainably lower than before. Duplicated dialysis cultures using the same nutrient medium produced growth curves comparatively similar to nondialysis except that the mean peak cell concentrations were significantly higher (2 fold) and the culture densities continued to increase in a linear manner throughout the later stages of the trials. Additional membrane area and a larger fermentor to reservoir volume ratio further improved the concentration of cells in dialysis culture to better than a 5 fold advantage over nondialysis. These results all agreed, in principle, with those reported previously for dialysis cultures (57). The similarity in exponential growth rates and in viable cell assay standard deviation values between the two culture systems showed that dialysis had no adverse effect on bacterial multiplication. The series of duplicated growth trials demonstrated that dialysis results were repeatable within certain limits. The trials also showed that this dialysis culture system equalled or exceeded the expected superiority over comparable nondialysis. The primary biological advantages gained from employing dialysis culture appeared to include an extention of the exponential growth phase, a continual linear increase in culture concentration in the latter stages of the growth trial, an improved culture viability, and as a result of these a greater concentration of cells within a defined fermentor volume. Although the results did not surpass those reported for the original

prototype system, the conclusions obtained were in agreement with those predicted for the dialysis culture technique (141).

Several growth trials were made to correlate the biological performance of this dialyzer and dialysis culture system with its solute transfer characteristics. Solute transfer data (Section 4) had shown that a fluid velocity of 1 L/min and a volume ratio of 1:10 produced optimal glucose transfer rates. The same was found to be true for propagation of Serratia marcescens. Increased fluid velocity to 6 L/min produced no improvement in growth over a 2 L/min rate and a 1:10 ratio produced significantly better growth, especially toward the end of the trial, than a 1:4 ratio. The demonstration that a 1:10 ratio was optimal agreed with earlier data for other dialysis culture schemes which showed that both smaller and larger ratios produced poorer growth densities (57, 61). It was noted that, with this dialyzer-dialysis culture system, the best culture densities were obtained if the fermentor volume was kept constant (3 liters) while the reservoir volume was increased. In contrast, superior culture densities had been achieved in the dialysis flask and reservoir-dialysis culture systems when the optimal ratio was obtained by a reduction in fermentor volume. In both of these the membrane interface was an integral part of the reservoir region. With the dialyzer-dialysis culture system it was separate and a reduction in the fermentor volume reduced the liquid level within this 5 liter vessel to a point where its operational efficiency declined. It is expected that this problem could be alleviated by the use of a smaller fermentor vessel. However on an industrial scale it would be simpler and more economical to increase the size of the reservoir while maintaining a constant standard fermentor size. The results indicated a direct relationship

between optimal solute transfer conditions and microbial growth. Further, it was evident that this dialyzer-dialysis culture system responded to the advantage of a proportionately larger nutrient reservoir in a manner similar to that observed for other dialysis schemes and in accordance with theoretical predictions for the system (141).

Enlarging the dialyzer membrane area improved culture yields in the same manner as it had improved soluted transfer efficiency (Section 4). A comparison of growth curves when 6 (.1728 m^2), 10 (.2880 m^2), and 15 (.4320 m^2) membranes were used suggested that the optimum area for solute transfer (10 membranes) also held true for growth since the difference in culture density between 10 and 15 membranes was only 5%. However the highest viable cell concentrations recorded for this dialysis system (283 billion cells/ml) were obtained when the larger area was used. It is presumed that, on the basis of growth, the optimum membrane area would vary with the organism and growth medium used. Further increases in area were not tested so the optimum for these culture conditions was not precisely identified. It is possible that subsequent additions beyond 10 membranes would produce increases in cell concentrations, but of dimishing magnitude as indicated with the comparison between 10 and 15 membranes. Such results indicate that either the diffusional demands of the culture were approached or the efficiency of the dialyzer declined. The first is difficult to determine since the dialysis system might well eliminate the limitations imposed by toxic accumulations or nutrient deficiencies permitting growth to increase to a point where another factor such as aeration became limiting. A reduction in dialyzer efficiency is also possible since other data (Section 4 and Section 6) has shown that the addition of dialyzer membranes resulted in an increase in

internal volume, an increased fluid hold-up time, and a decrease in oxygen transfer efficiency.

The performance of this dialysis culture system equaled or exceeded the levels expected from previous work. This was especially apparent in terms of culture concentration superiority over nondialysis, a specific attribute of the dialysis system. The growth trials showed that, on Trypticase soy broth, a 5 fold increase in culture concentration was achieved. On water diffusate of Trypticase soy broth, this rose to a surprising 35 fold advantage. These results were, in the first case, equal to and, in the second case, greater than those reported previously (57). Although reasonable high culture concentrations were achieved these did not surpass the densities reported for the original prototype system, which had a smaller membrane area (57). This difference was not necessarily attributed to a poorer efficiency for the present dialyzer because it was noted that the current nondialysis "control" cultures also achieved lower cell densities than in earlier work. It is likely that the above differences in culture yields may be associated with the organisms themselves or with subtle differences in the growth media or the culture procedures.

The dialyzer-dialysis culture technique physically separated the fermentor, reservoir, and diffusional exchange regions of the system into independently controlled units. This allowed the adjustments in volumes, volume ratios, and membrane area necessary to provide the optimal solute transfer conditions for the best use of various growth media or for the culture demands of various microorganisms. Such an operational flexibility resulted in two biological attributes of dialysis culture which have not been emphasized; the ability to produce high culture

yields on relatively weak culture media and the extension of a stationary growth phase for surprisingly long periods.

The selected nutrient medium was found to directly influence the culture densities produced in nondialysis cultures. Here the concentration of viable cells corresponded with the quantity of carbon source (glucose) contained in the medium with a low carbon water diffusate broth producing the poorest yield. Examination of the concentration of glucose remaining in the fermentor during a growth trial revealed that its reduction to a low level, possibly a critical concentration, coincided with the termination of exponential growth. Dialysis cultures with the same types of media produced greater culture yields with the glucose concentration of the medium having little influence on the extent of growth. Moreover, when the membrane area was sufficiently enlarged dialysis cultures achieved surprisingly high cell concentrations on the low glucose water diffusate broth. In these trials the glucose concentration within the fermentor fell to a level which was over three times higher than that in the above nondialysis and which remained constant or rose during the remainder of the trial. Maintenance of a higher nutrient concentration in the fermentor allowed an extension of the exponential growth phase, which resulted in the production of higher culture densities and a prolonged linear-stationary growth phase after the 24th hour of the trial. These results indicate a direct relationship between diffusional access to a nutrient reservoir and the creation and maintenance of an adequate culture environment which permits production of higher culture concentrations regardless of the strength of the selected growth medium.

The maintenance of a high concentration of viable cells in a prolonged stationary growth phase further illustrated the advantage of dialyzing a nutrient reservoir. This growth phase was continued for over six days without a loss in culture density. After the fifth day the reservoir still contained 60% of its initial glucose concentration. These trials indicated that a dialysis culture could be continuously operated for up to two weeks as a closed system without the risk of nutrient limitation or culture contamination. Conceivably a single dialysis culture operated for several days could produce as great a viable cell yield as several batch nondialysis cultures. In addition to the economies gained by such an operation, the prolonged stationary phase produced by dialysis cultures provided an extended time during which the synthesis of culture products, many of which are formed only during the stationary growth period, could continue. Long term dialysis cultures might well represent a commercially economical method for obtaining high yields of such products. In addition these dialysis cultures with or without the adaption of the reservoir to a continuous operation could represent a useful alternative to the more complex continuous culture system for some fermentation studies.

The dialyzer and the dialysis culture system as operated in these trials was not only compatible with the test organism but demonstrated biological performance which equaled or exceeded that anticipated for the system. Apparently the only influence exerted by a growing culture on the dialysis process were changes in the solute concentration gradient as nutrients were consumed and $\dot{m}_A$ the dialysis equilibrium concentration. No appreciable changes in the mass transfer characteristics of the system were noted. Diffusional access to the nutrient reservoir repeatedly

appeared to represent a key factor in the success of dialysis growth trials. The successful concentration of viable cells on a weak nutrient, increased duration of the exponential growth phase, the extension of the stationary growth phase, increased culture viability, and growth response to increased membrane area which were demonstrated characteristics of dialysis culture were largely the result of access to the nutrient reservoir. The additional nutrient supply contained in the reservoir effectively prevented or greatly delayed the onset of growth limiting conditions within the culture fermentor. The reserve solutes were continually diffused into the fermentor during the growth period. This reduced the magnitude of nutrient depletion within the fermentor during exponential growth and provided a continuing replenishment of consumed nutrients especially during the later stages of growth.

As a result, higher nutrient levels were maintained in the fermentor throughout the growth trial. These levels represented an improved culture environment which contributed to the attainment of a higher culture concentration ceiling for a given set of culture conditions within the confines of a fixed culture volume. Correspondingly the proportion of viable cells within the culture increased especially during the later stages.

The dialysis reservoir also represented a sink for removal and dilution of diffusible metabolic byproducts, some of which become toxic upon accumulation. The growth of a microbial culture is generally limited by the depletion of an essential growth factor or the accumulation of a growth inhibiting factor. It is likely that the increase in dialysis culture levels and viability were also due to the capacity of the dialysis system to remove toxic solutes from the fermentor and dilute them within the reservoir. This function may be the reason larger volume ratios (achieved by

increasing the reservoir volume) enhanced culture yields. The dilution of toxic solutes effectively improved the culture environment by preventing or slowing the rate of their accumulation within the fermentor. Thus the presence of the dialysis reservoir had the effect of enlarging, on a molecular basis, the fermentor region while maintaining a constant volume within the relatively small physical confines of a given fermentor capacity. The net effect and a valuable attribute of the dialysis culture technique was the production of higher viable cell concentrations within a small culture volume than would be possible by nondialysis.

The separation of the fermentor and reservoir regions within the dialyzer-dialysis culture system provided a convenient method for large scale symbiotic culture schemes. The former was discovered to be feasible when the accidental contamination of the reservoir by Bacillus megaterium was found to have no effect on the growth, yield, or purity of the fermentor culture. This indicated that a sufficiently large (13 to 26 liters) reservoir contained enough nutrients to support both an inoculated and a contaminating culture within its confines for at least 48 hours. Moreover, the dialysis membranes allowed diffusional exchange between the two cultures while maintaining separation of the cells insuring the purity of both cultures. Such an application of dialysis culture to the study of symbiotic cultures has been reported elsewhere. The growth of Bordetella pertussis cultures for vaccine production have been enhanced by dialysis against Corynebacterium pseudodiphtheriticum in a dialysis flask system (13). The influence of mixed cultures on a bacterial species was studied by the use of a dialysis device with membrane filters to simulate a mixed culture environment (140). A study of the "satellite phenomenon" of Hemophilus influenzae was made by dialyzing these cultures against a Staphlococcus

culture contained in a cellophane sac (130). Dialysis cells have been used to determine the associative interrelationships between various microorganisms (117). Each of the above investigations employed small scale or crude dialysis devices. The dialyzer-dialysis culture system, on the basis of the above results, could be employed for symbiotic studies and would afford the benefit of operational flexibility and a large scale to such investigations.

Finally, the lack of negative consequences caused by reservoir contamination indicated that dialysis cultures could be successfully propagated on unsterilized growth media. This could be accomplished by dialyzing sterilized water from the fermentor against the reservoir containing the unsterilized nutrients until a sufficient quantity of solute had transferred to enable inoculation and growth. Such a scheme would preclude the need to sterilize the reservoir nutrients and would allow the use of heat liable growth media without the danger of culture contamination.

5.5 Summary

A model plate-and-frame dialyzer (Section 3) containing regenerated cellulose dialysis membranes was incorporated into a dialyzer-dialysis culture system. Liquid cultures of a type bacterium, Serratia marcescens, were circulated from a conventional 5 liter fermentor through one side of the dialyzer while nutrient media from a separate reservoir was circulated through the opposite side. A series of growth trials established the extent of variation between repeated results and demonstrated the superiority of dialysis cultures, in terms of culture viability, culture concentration, and extension of the exponential and stationary growth phases, over comparable nondialysis cultures. Fluid circulation velocities had no effect on dialysis growth. A 1:10 fermentor to reservoir volume ratio, obtained by increasing the reservoir volume, produced optimal biological performance. The addition of dialyzer membranes resulted in improved culture concentrations with 15 membranes (.4320 m^2), producing a maximum viable cell concentration of 283 billion cells/ml. Diffusional access to the nutrient reservoir was shown to be instrumental in maintaining a culture environment which permitted the high dialysis growth yields, an extension of the stationary phase for over five days, and the concentration of cells on a relatively weak growth medium. In several instances the dialysis growth response to operational conditions was similar to that shown previously for nutrient transfer.

6. DIALYSIS AERATION

6.1 Introduction and Objectives

The use of membranes for the diffusional exchange of nutrients and cellular products during microbial growth can be readily extended to include the exchange of gaseous nutrients and products, namely oxygen and carbon dioxide. Dialysis aeration was first demonstrated 22 years ago by Gladstone (64) for the production of protective antigens from Bacillus anthracis cultures. Air was passed over the surface of cellophane sacs containing the culture. More recently an attempt was made to employ this technique for the growth of Serratia marcescens cultures in the bottom of a dialysis flask (61) and in a small dialysis fermentation system (62). Gas or gas-saturated liquid contacted the membranes and diffused through the aqueous channels of the membrane material. Although this system transferred sufficient oxygen when a relatively porous membrane filter was used, the resulting cell density and growth rate were less than in a conventionally aerated control. Independently, Brewer (19) developed and patented a relatively crude apparatus which employed an outer envelope made from polyethylene sheets for gas transfer to an aerobic culture contained within the device.

The membrane barrier is classically considered to be a micro-heteroporous seive with pores or open spaces which fill with the liquid solvent. Thus solute (gas) is transferred by diffusion through the bulk solvent filling the membrane not through the membrane material itself. Selective

permeability under these conditions is a function of solute dimensions (volume) and pore diameters as well as electrostatic forces, entropy factors, absorption, and membrane swelling. The rate of transport is inversely proportional to the square root of the molecular weight, diffusivity in the fluid, and bulk fluid movement (7, 159).

In the past decade a new type of membrane has been developed completely devoid of pores, channels or crystallinity (16). These membranes are typically fabricated from methyl or dimethyl silicone rubber or from fluorocarbon polymers and are called permselective films or solute transport membranes. Transport through this type of membrane is a function of solubility into and diffusion through the membrane polymer. Gases dissolve directly into one face of the membrane, diffuse through its thickness, and escape from the other face. Silicone rubber, in addition to being strong, is selectively and highly permeable to oxygen and carbon dioxide but not to water. Their usefulness for biological applications has been demonstrated in prosthetic artificial lungs (45, 38), artificial placentas (138, 172), anesthesia administration (51), and underwater life support (128).

The development and availability of permselective membranes and their successful use in blood oxygenation suggested applicability to the propagation of aerobic microorganisms. We first tested this idea in a dialysis culture system in 1965 and reported preliminary results in 1966 (H. E. B. Humphrey and P. Gerhardt, 1966, American Chemical Society 152nd Meeting, New York, Abstracts of Papers p. Q-2).

Three basic experimental schemes were used and analyzed in this study: a batch scheme utilizing conventional sparging and agitation as a control system; a dialysis aeration scheme utilizing a dialyzer and

membrane exchange as the sole source of aeration; and a full dialysis scheme utilizing one dialyzer for aeration and a second dialyzer for nutrient supply. The third scheme represents the ultimate in dialysis culturing -- a microbial population solely supported by exchange through membranes and totally isolated from the exterior environment. The systems were evaluated for oxygen transfer characteristics and then evaluated for microbial growth characteristics. The potential advantages expected for dialysis aeration are as follows:

A) Economy of Operation and Safety; (a) The need for air sterilization is eliminated, (b) the required quantity of antifoam materials is reduced, (c) the existence of positive pressure within the culture vessel is eliminated, thereby reducing the hazard of aerosol contamination, and (d) the membrane provides a known and defined gas-liquid interface, the gas and liquid films of which may be manipulated by velocity adjustments.

B) Improved Culture Environment; (a) CO_2 stripping is reduced, (b) the denaturing effect of violent agitation and direct gas-cell interfacial contact is eliminated, (c) the evaporation of culture liquid is reduced, (d) the uniform and even diffusion of gas molecules into the liquid eliminates the fluctuation in dissolved oxygen associated with sparged gas bubbles, and (e) the character of the dissolved gas environment is better controlled by partial pressure adjustment in the gas phase prior to membrane diffusion into the liquid.

The objectives of this section of the thesis were: (a) to assemble a historical and theoretical background on aeration of microbial cultures; (b) to examine and compare quantitatively the oxygen transfer rates of dialysis and sparged aeration systems; and (c) to examine the feasibility of using permselective membranes for gas exchange in mass culture of a typical aerobic bacterium.

6.2 Historical and Theory

6.2.1 Oxygen demand of microbial cultures.

Oxygen transfer is commonly regarded as a critical factor in the development of an aerobic fermentation process. An aeration system must be designed to provide an adequate dissolved oxygen environment for exponential multiplication and other physiological phases. The success of an aerated fermentation will depend on operational efficiency and on the influence which the resultant physical and chemical conditions exert upon the growth and metabolism of the organism. A large portion of industrial research has been concerned with the measurement and manipulation of these conditions in an attempt to optimize the desired microbiological process.

An equally concentrated effort has been directed toward examination of the organisms themselves with respect to oxygen requirements, since the quantity of dissolved oxygen required by a multiplying culture largely dictates the design of the aeration system. Oxygen demand is a function of both the physiological state and the concentration of the culture. Clifton (27) demonstrated that Aerobacter aerogenes or Escherichia coli cultures had the highest uptake on a per cell basis when young but that the overall culture demand was greatest later, during the decline physiological stage when the high concentration of cells dominated uptake rates. This was confirmed later by manometric measurements of Streptomyces griseus cultures (68). Zobell and Stadler (175) demonstrated that the oxygen demand of lake bacteria rose, both on the per cell and total

overall basis, when the nutrient concentration was increased. Thus optimal or super-optimal nutrient conditions can render a standard aeration supply system inadequate and create a condition in which oxygen is growth-limiting. This condition has been experienced in dialysis culture. Supplemental addition of nutrients permitted a culture concentration of 1×10^{12} organisms/ml, and the cessation of growth was attributed to insufficient oxygen transfer, which had been adequate at lower cell concentrations (57). Provision of oxygen is further complicated by changes in metabolic function. Sporulation, for instance, creates a peak demand period as adaptive enzymes begin synthesizing complex organic bonds and molecules (71). The physiologic state, the metabolic function and the population density of the organism appear, therefore, to represent important considerations in the design, operation, and apparent efficiency of a culture aeration system.

Overall oxygen demand constitutes a limiting condition which must be satisfied by fermentation equipment in order to achieve optimal aerobic growth. Finn (48) noted that relatively little was known about the peak demand of cultures actively growing in rich nutrients. He found that most of the reported values had been obtained under artificial conditions different from those of the actual fermentation. Such data did not reflect conditions within the culture nor the influence of environmental factors as growth proceeded. Factors considered important to oxygen demand were nutrient concentration, accumulation of toxic end products or loss of volatile intermediates, nitrogen source and growth factor quantities, oxygen supply, and cell accumulation. Some of these affected oxygen solubility and others, cellular respiration.

The lack of accurate demand values was primarily due to the lack of a method for measuring dissolved oxygen, and therefore oxygen demand, within the culture itself. The same problem also prevented accurate evaluation of aeration equipment. The methods which have been used until recently include: manometric analysis of removed samples, titrimetric analysis of cell-less sodium sulfite solutions, and polarographic analysis. In each case the data obtained failed to represent an accurate picture of the actual situation because a substitute solution was used, samples had to be removed, cells were not present, or the probe used was subject to deterioration by the culture.

The development of a membrane-covered, bimetallic electrode by Clark (26) represented a break-through in measuring dissolved oxygen. Subsequent improvement in the electrode and membrane materials (66, 82) and the perfection of a reliable, long-lived, steam-sterilizable oxygen electrode (86, 15) have permitted precise and continuous monitoring of the culture during a fermentation process. A "dynamic" (i.e. continuous) measurement technique for evaluation of "in situ" oxygen transfer, oxygen concentration, and culture demand has been developed (156) as a result of the availability of such oxygen probes. The method reportedly produced results as precise as those from a calibrated manometer, but without the problems and errors associated with other detection methods. The value of this "dynamic" technique is exemplified in Table 15 (below). The data, from an active Serratia marcescens culture, show both the occurrence and magnitude of oxygen demand for the culture as the fermentation progressed. This information not only identified the peak oxygen transfer but also indicated periods when such transfer could be reduced. The latter represents an operational economy and permits elimination of potentially toxic excess oxygen conditions.

Maintenance of optimal oxygen conditions as well as satisfaction of culture demands have been design goals in industrial fermentations. A tailored aeration system, which produces an optimal condition throughout the physiological stages of the culture, can result in advantageous cell or product yields. In an effort to avoid either excessive or deficient oxygenation, complex and expensive monitoring and control devices have been developed. These detect, record, analyze, and adjust aeration in order to maintain culture aeration within predetermined optimal levels. Generally the components include oxygen probes, small computors, and elaborate automated valving, flow meters, and gas selectors for delivery of the correct oxygen concentration to the sparger (72, 108). Realization of the importance of adequate supply and maintenance of dissolved oxygen for the productive success of aerobic fermentations has justified the development of more efficient fermentors and aeration monitors.

6.2.2 Oxygen states

Because oxygen functions as the primary metabolic electron acceptor, aerobic microorganisms are sensitive to excesses or deficiencies. A culture exposed to suboptimal conditions may respond with an altered physiology or submaximal growth and viability. With respect to oxygen a fermentation may experience a deficient, optimal or toxic state depending on aeration efficiency and control.

A deficient oxygen state may result in death, growth inhibition, or metabolic disruption in bacteria. Oxygen defficiency was shown to occur dramatically in Acetobacter cells within 15 seconds (48) after air flow

disruption. Oxygen supply and concentration also influences the duration of the exponential growth phase, which in turn affects the concentration of cells produced. Rahn (25) demonstrated that oxygen exhaustion restricted the growth of Pseudomonas fluorescens in an ordinary test tube to a population level of 2×10^6 cells per ml. More recently, oxygen also was shown to limit the concentration of Serratia marcescens in dialysis culture at a level of 2×10^{12} cells per ml (57).

Other manifestations attributed to insufficient oxygen conditions include a shift in operating enzyme systems, a change in physiological function (inhibition of sporulation), a decrease in product formation, or a change in growth rate (44, 71, 18, 105, 97). Such results apparently occur because the oxygen concentration falls below a minimal tolerance. This minimum has been defined as the critical oxygen tension, a term introduced by Gerard and Falk in 1931 (73) to describe the point at which growth rates cease to be independent of oxygen concentration. The value for this concentration varies with temperature, the organism, and measurement precision.

There has been little agreement on the level of the critical tension, largely because of the lack of precise oxygen determination at low concentrations. Reported values for bacteria range from .003 to .005 m moles O_2/L (48, 100). Not only has the value of the critical oxygen tension been unsure but until recently little was known about the relationship between oxygen concentration and cellular respiration rates at oxygen levels approaching and within the critical range. This relationship was recently evaluated and categorized with respect to the critical oxygen concentration value (73). Oxygen concentration above the critical value had no effect on respiration rates and represented an excess condition.

Concentrations below the critical value directly influenced respiration rates and represented a limited condition. Between these two a transition condition was detected where respiration rates and oxygen concentrations fluctuated in an unique manner. The unusual oscillations found in the transition stage led the authors to propose a new definition of the critical oxygen law; "the critical oxygen tension is that concentration above which the respiration rate of an organism is independent of changes in dissolved oxygen concentration and below which dissolved oxygen and oxygen uptake may vary" (73). If oxygen levels are not maintained above the critical value for the duration of a fermentation exponential growth rates, viability and culture density will decline (5, 165).

The optimal oxygen state represents the range of oxygen concentrations which are ideal for maximal cell growth and function. The dissolved oxygen concentrations characteristic of this state are specific for each species and occur between the critical low values and toxic high values. The principal goal of fermentation design, scale-up, and operation has been the attainment and maintenance of an optimal oxygen state, the importance of which has justified development of the elaborate oxygen sensing and control devices mentioned previously. Despite these efforts an optimal oxygen state seldom is maintained throughout aerobic liquid fermentations. The inherent inefficiency of liquid aeration and the variation in culture oxygen demand as the cells age and multiply are the causative factors. In addition, the optimum oxygen state for product synthesis or a physiological function such as sporulation is often different from that for growth (67, 71). Fermentor aeration techniques are generally insensitive and unresponsive to changing demands and therefore are unable

continually to produce the necessary optimal conditions, with a resulting loss in efficiency and product yield.

The toxic oxygen state represents conditions in which excess oxygen retards growth or inhibits a metabolic function. Although this state is less recognized than the previous two, it was implicated in restricting the length of the lag growth phase which occurred after inoculation of an aerobic fermentation, especially if the cells were young, the inocula small or the nutrients dilute (175). In addition, excessive oxygen concentrations have been shown to inhibit antibiotic synthesis in certain fermentations (18).

Oxygen toxicity has been attributed to shock or respiratory enzyme poisoning from sudden exposure to high concentrations, slow adaption of key enzyme systems to high oxygen levels, unfavorable conditions due to carbon dioxide or nitrogen stripping, or oxidation of metabolic intermediates (126, 160, 165, 35). Although use of pure oxygen instead of air generally magnifies the inhibitory effect, some organism such as Aspergillus niger are stimulated by excess oxygen concentration (173). Thus, oxygen toxicity probably represents a combination of effects which do not affect all organisms in a universal manner. However, it is possible that a toxic state may occur at some point, especially early in the fermentation process. More importantly, sparger aerated cultures may expose a portion of the culture directly to gas bubble interfaces at any given moment. This represents a source of oxygen contact at the cellular level which may be inhibitory to sensitive cells, even through the overall dissolved oxygen level is within acceptable limits. The frequency of such cell-bubble contact is regarded sufficient enough so that it has been proposed as an alternate pathway for oxygen transfer (12).

The three oxygen states and their influence on culture viability or yield take on added significance when fermentor aeration is considered at the cellular level. Under existing sparged and agitated liquid aeration techniques, an individual cell may alternately be directly exposed to air bubbles, exposed to varying concentrations of dissolved oxygen, and exposed to no dissolved oxygen. The idea that cells become oxygen starved during deep liquid fermentation was proposed as an explanation for the difference in duration of exponential growth in a shake flask versus that for deep liquid culture of Pseudomonas fluorescens (125). The authors concluded that a disproportion existed between oxygen demand and supply in deep liquid cultures, which resulted in death or slower growth. More recently the dissolved oxygen concentration was found to vary throughout an aerated fermentor broth, and the variation was attributed to inadequate bulk mixing (123). In addition, realization that an area of decreasingly concentrated oxygen trails behind sparged air bubbles as they rise led to the conclusion that bacterial cells are exposed to varying degrees of oxygen concentration as they circulate through these bubble trails. The variation in dissolved oxygen throughout the liquid, was reported actually to be stagnant under some mixing conditions (116) so that the actual critical oxygen concentration may be higher than commonly believed. Although overall aeration may appear adequate on the basis of gross dissolved oxygen analysis, it thus appears that the cells themselves alternate between regions of optimal and suboptimal oxygen conditions as they circulate through the sparged fermentor. It is difficult to compare this effect precisely against the success of a fermentation. However, the physiological influence of these suboptimal oxygen states strongly suggests that improved aeration at the cellular level would result in superior fermentation yields overall.

6.2.3 Nondialysis aeration methods

Adequate delivery of oxygen from an air stream into a fermentation broth has been one of the most critical as well as most difficult problems facing fermentation technologists. A wealth of literature has accumulated concerning the design, improvement, and operation of fermentor aeration equipment. The oxygen transfer process has been examined and theoretical equations, based on mass transfer principles, have been developed to explain observations, facilitate predictions and aid performance evaluation. No attempt will be made to review fully all aspects of fermentor aeration as several pertinent and comprehensive reviews have been published (39, 48, 149, 127, 104, 161) Early research in fermentor aeration emphasized development of mechanical devices for transfer of gaseous oxygen from the air stream into the liquid. The low solubility of oxygen in aqueous liquids makes this transfer difficult and has focused attention on development of efficient operational techniques. Liquid aerators have been classified according to the way they distribute air. Those which have practical application to industrial fermentors include fixed metallic distributors with bored holes (e.g. single or multiple-orifice, hollow-tube spargers), finely porous aerators (e.g. sintered glass or metal foam bubblers), fixed distributors with various openings other than bored holes, and mechanical air distributors (e.g. rotating hollow porous agitators) (39).

Various modifications have been made on the basic aerator designs and on the associated equipment, agitators and baffles that are found in modern fermentor aeration systems. The German-designed Waldhof fermentor represents one of the successful variations. This design popularized the

use of a central draft tube suspended over a hollow shafted agitator which dispersed air bubbles near the bottom of the vessel. Additional air and any foam created during aeration was drawn down the draft tube and redispersed in the liquid. Lower air requirements and elimination of antifoam agents make this design desirable although power requirements are greater than more conventional fermentros (142). It is believed that the liquid vortex depth which is enhanced by the draft tube is related to oxygen transfer and a key to the effectiveness of the Waldhof design (158). Another aeration system eliminated the need for compressed air by utilizing the suction created by rotating turbine blades within a hollow cylinder to draw air into the cylinder from an adjacent intake pipe (83). A similar device utilizing a rotating turbine and fixed stator blades through which air bubbles are dispersed horizontally into the liquid was recently reported (42). Other modified systems include the use of acoustic air velocities through a single orifice sparger to create very small bubbles and violent agitation (2) and the use of a sparger which may be moved vertically to better locate gas dispersion and improve gas-liquid contact time (49).

The search for superior aeration techniques has not been limited only to the modification of conventionally designed equipment. Ingenious methods have been proposed which utilize chemical reactions, electrical currents, or pumps and valves. One of the most successful techniques, one which set a precedent for circulation of a culture to an aerator on the exterior of a fermentor was devised by Strich (154). The culture broth was pumped from the upper level of the fermentor through an external helical gas exchanger and returned to the bottom level of the fermentor. Aeration was accomplished by passage of very fine gas bubbles

from a continuous air stream through a microporous, (20-60μ diameter) ceramic or metallic diaphragm into the circulating culture liquid. This system produced extraordinarily fine bubble aeration and afforded control of oxygenation rates through manipulation of the gas and liquid velocities. Another unique aeration technique employed a pulsating aerator which utilized a single air filter and a siphon device to produce positive and negative air pulses. The positive pulse forced air bubbles into the culture, the negative pulse removed foam and exhaust air from the culture, and the succeeding positive pulse forced fresh air and the foam into the culture. The culture is maintained under negative pressure, a desirable feature for growth of pathogenic organisms (74). Generation of adequate oxygen for submerged culture of Pseudomonas fluorescens by electrolysis of the culture medium has been demonstrated on a small scale (137) and an electrochemical cell employing a KOH solution has been shown to concentrate oxygen from the air into 99.5% pure oxygen at a rate of .2lb/hr (17). Finally, periodic additions of H_2 0_2 to a culture containing catalase have been shown as a satisfactory method for generation of oxygen "in situ" (169).

Although such proposals are ingenious, their application to large scale or commercial fermentations is limited by the costs involved. It has been found that, ideally, an aeration system should provide for an even distribution of gas throughout the liquid and for a maximal gas-liquid surface contact for a maximal length of time. Consideration must also be made for the design of fermentor tank geometry, baffle placement, volume and height ratios, agitator design and operation, and air velocity (112, 81, 104, 48, 53, 161). These considerations increase in significance when the design of large scale deep liquid aerators is attempted.

As shown, the principle techniques for improving aeration efficiency have involved manipulations which are designed to raise the value of the oxygen transfer coefficient ($K_L a$). This approach continues to be held in high esteem by fermentation technologists (161) and with Finn's conclusion that the principle means for aeration improvement lay in attempting to increase $K_L a$ values or raise the driving force ($C^* - C_L$) (48). The latter would require hyperbaric oxygen conditions or the use of pure oxygen, both of which are economically impractical on a larger scale.

The objective in fermentor aeration has been to assure that dissolved oxygen is maintained in excess of the critical concentration. Although the importance of this has been recognized, there has been little agreement on the value of the critical concentration (55). It has generally been assumed that oxygen uptake rates and respiration rates would be independent of fluctuations in dissolved oxygen concentration when the fermentor was operated so that the detectable liquid oxygen concentration never fell as low as the critical level (123, 127). Wise condluded that this type of operation was basically inefficient and wasteful of oxygen (166). The variations in dissolved oxygen observed both at a given point and throughout the fermentor have been attributed to the inefficiencies of mixing and have not been regarded as important to the fermentation as long as the overall level was above the critical tension (123). Although these assumptions are partially correct, they fail to recognize a fundamental consideration in microbial aeration, namely that the overall "macroscale" oxygen conditions are magnified, with respect to the respiring cells, in the "microscale" cellular environment. Thus, inefficiencies in mixing or sparging which produce varying dissolved oxygen levels in a bubble aerated fermentor actually produce pockets within

the culture which are suboptimal or even critical for aerobic growth. Cells present in these locations for even a short time may have their essential metabolic functions disrupted to some degree. In addition the repeated encounter with optimal, suboptimal, and excess oxygen conditions may produce uneven metabolic rates and potentially submaximal culture yields. It is entirely possible that a consistent and fully adequate oxygen environment at the cellular level is unattainable by bubble sparging.

6.2.4 Mechanism of oxygen transfer

The conclusion that fluctuating oxygen conditions exist within conventionally aerated fermentors is plausible in view of past and ongoing attempts to improve oxygen transfer by manipulation of agitation, sparger design, and gas velocity. If the importance of the micro-level oxygen environment is significant and if the inadequacy of bubble aeration is an acceptable premise, then there exists a need for an alternative method of delivering oxygen to the cell suspension. Dialysis aeration is proposed as a feasible alternative. In order to understand dialysis (membrane) aeration and to appreciate its relationship to conventional aeration, the oxygen transfer mechanism and theory should be examined.

The solution of oxygen into water or a culture broth is considered to be a gas absorption process in which gas molecules are transferred from gas bubbles (gas phase) through a gas-liquid interface and into the liquid phase. This represents a mass transfer in which the interface and the thin gas and liquid films adjacent to it are the principle rate-determining factors. The process is commonly explained by the Whitman

two-film transfer theory, in which the stagnant films adjacent to the gas-liquid interface are assumed to control the rate of mass transfer (3). The Whitman theory provides the basis for derivation of the familiar gas transfer laws and volumetric mass transfer coefficients from the basic mass transfer equation,

$$N = K_o (\Delta C) A \quad , \tag{6.1}$$

where; N = the mass transfer rate

K_o = the overall mass transfer coefficient

C = the oxygen concentration driving force, and

A = the interfacial area between the gas and liquid phases.

Oxygen transfer from the gas phase to cells suspended in liquid involves a series of transfer steps from the bulk gas to the gas film, the gas film to the interface, the interface to the liquid film, the liquid film to the bulk liquid, the bulk liquid to the liquid film surrounding a cell, and the cell liquid film to the interior and/or enzyme receptors of the cell (see Figure 32). This concept was first applied to oxygen transfer by Bartholomew (8) twenty years ago. A mass transfer equation, similar to equation 6.1, may be written for each of these steps. The mass transfer coefficient, k, for each step is characteristic of the transfer in that region.

The rate of transfer in each step of this process is determined by the concentration gradient and the resistance to transfer characteristic for each region. The latter is

designated as the reciprocal of the mass transfer coefficient in each case $\left(\frac{1}{k}\right)$ and the overall resistance to mass transfer is also the sum of the individual resistances $\left(\frac{1}{K_o} = \frac{1}{k_1} + \frac{1}{k_2} \ldots + \frac{1}{k_7}\right)$ (127).

As shown in the diagram (Figure 32) oxygenation of liquid involves five oxygen transfer steps with an additional two steps involved in transfer into the interior of the cell itself. As previously stated, the interface and the adjacent gas and liquid films $\left(k_2, k_3 \text{ and } k_4\right)$ are regarded as the key steps in this mass transfer process. The other steps $\left(k_1, k_5, k_6, \text{ and } k_7\right)$ are considered to be far less important in comparison. Experimental evidence has shown that the bulk gas (k_1) and bulk liquid (k_5) transfer steps are diffusional processes, obey Fick's law of diffusion $\left(J = -D \frac{dc}{dx}\right)$ and do not limit the rate of oxygen transfer (127, 15). The transfer steps associated with cells $(k_6 \text{ and } k_7)$ have no influence on oxygen absorption into the liquid and therefore are significant only to the biological aspect of aeration. Borkowski and Johnson (15) showed that the liquid film surrounding the cells had no importance to cellular aeration as long as the cells were suspended within the liquid. The transfer step (k_7) into the cellular material may be disregarded in the case of bacterial cells where the enzymatic oxygen receptors lie on the plasma membrane. With cells such as yeast where these sites lie deeper within the cell matrix, diffusion through the protoplasm may become limiting when very low oxygen concentrations are encountered (87).

The foregoing examination of the mass transfer steps supports the emphasis placed on the gas-liquid interface and its films by the Whitman

Figure 32. Diagram of the transfer of gas molecules from the gas phase to a cell suspended in the liquid phase. A mass transfer equation may be written for each step of the transfer process with k_1 - k_7 representing the mass transfer coefficient in each case.

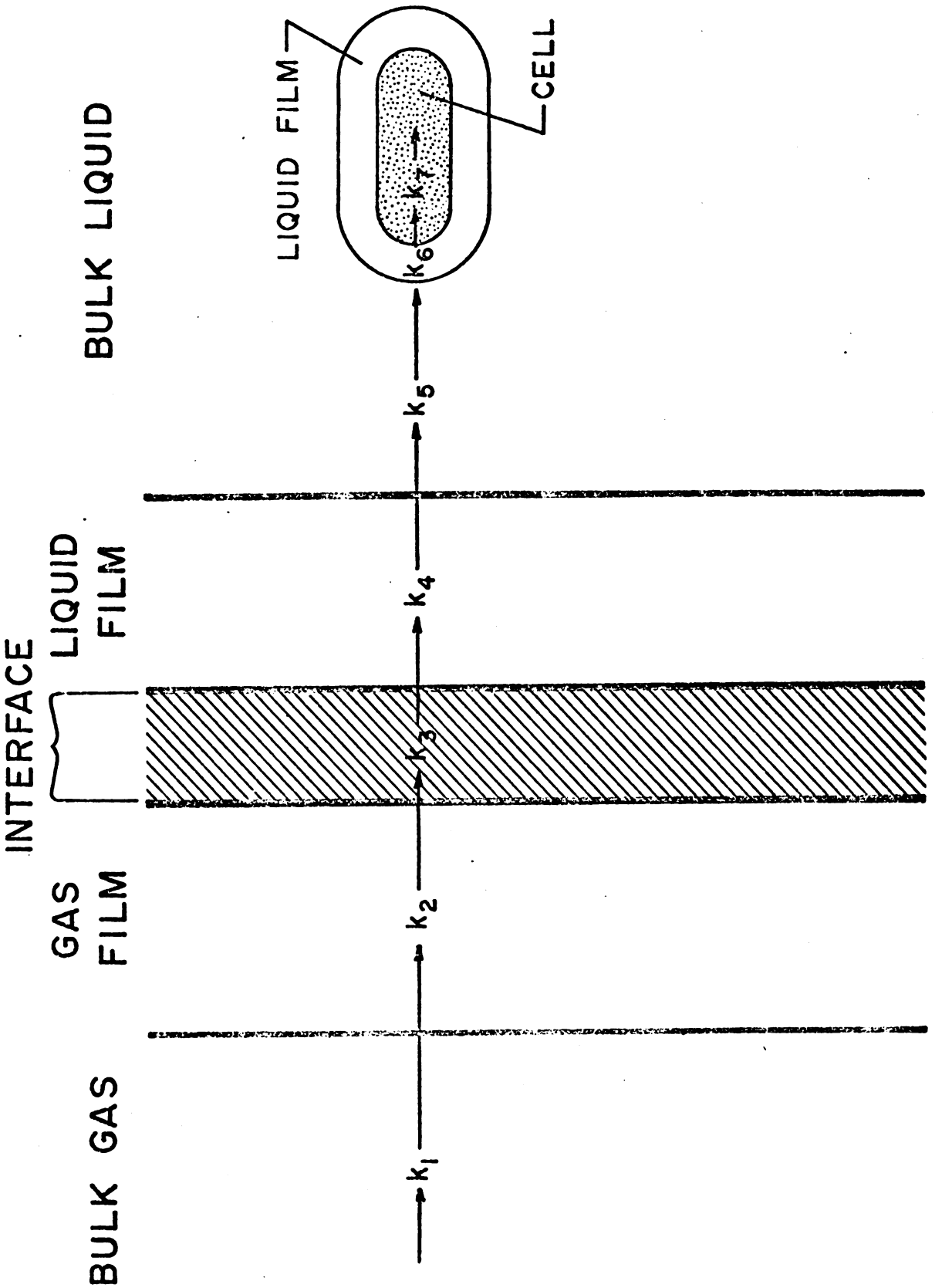

BULK LIQUID
LIQUID FILM
CELL
k_6
k_7
k_5
k_4
INTERFACE
LIQUID FILM
k_3
GAS FILM
k_2
BULK GAS
k_1

Figure 32.

theory. Numerous experimental determinations have directly confirmed that these most probably do represent the key rate limiting steps in oxygen transfer (22, 8, 48, 171, 115, 80, 127, 3). Although unaminous consensus does not exist, most investigators feel that the liquid film represents the greatest resistance to mass transfer and therefore controls the aeration process. For example, investigations concerning measured thermodynamic effects on $K_L a$ values and on diffusion coefficients for aqueous and gas phases as well as the low viscosity of gas in comparison to liquid, showed that the gas film would have to be far thicker than found at the bubble-liquid interface in order to be a significant rate limiting obstacle to gas transfer (48, 8). Thus the assumptions of the Whitman theory of gas transfer appear valid. This theory with its emphasis on the gas and liquid films is, along with interfacial considerations, essential for understanding dialysis aeration. A summary of the key features of the two-film theory and the development of the familiar gas transfer equations follows, and is based on explanations which have appeared in two texts (149, 3).

The important steps for oxygen absorption in an aqueous solution involve the gas film, the interface, and the liquid film. Mass transfer equations may be written to represent oxygen transport through each of these regions the sum of which represents the overall mass transfer process;

$$N = K_o (\Delta C_{o_2})A = k_g (P - P_i)A = k_i (P_i - C_i)A = k_L (C_i - C_L)A \quad , \qquad (6.2)$$

Thus the overall concentration gradient driving force , $\Delta C_{o_2} \cong (P_{o_2} - C_L)$, is a composite of the gradients across the gas film, interface, and liquid

film regions. Each region is characterized by its mass transfer coefficient which is largely determined by the resistance to mass transfer found in the region. As discussed previously the overall resistance to transfer is a composite sum of the individual steps involved

$$1/K_o = 1/k_g + 1/k_i + 1/k_L \quad . \tag{6.3}$$

In magnitude of importance the resistance to transport offered by the interface itself is considered to be negligible. Thus equation 6.2 can be simplified by elimination of the interfacial term;

$$N = K_o A (P-C_L) = k_g A (P - P_i) = k_L A (C_i - C_L) \quad . \tag{6.4}$$

Oxygen solubility in aqueous liquids is low (7.6 mg/ml at 30^oC for air saturated water, slightly less for biological broths), is dependent on the gas phase oxygen partial pressure, and obeys Henry's Law

$$C_{o_2} = 1/H \; P_{o_2} \qquad \text{or} \qquad P_{o_2} = H \; C_{o_2} \quad . \tag{6.5}$$

Because saturation of the liquid with oxygen is the object of aeration the process aims toward achieving an equilibrium between the gas and liquid phases, where $P_i = C_i$ or $P^* = C^*$, where the * represents the equilibrium values of concentration and partial pressure. Equation 6.4 may therefore be written;

$$N = K_o A (P - C_L) = k_g A (P-P^*) = k_L A (C^*-C_L) \quad . \tag{6.6}$$

Applying Henry's Law under equilibrium conditions ,

$$P^* \doteqdot H\, C_L \quad \text{and} \quad H\, C^* \doteqdot P \quad . \tag{6.7}$$

Substituting this into equation 6.6 gives a mass transfer rate equation written in terms of oxygen concentration;

$$N = K_o\, A\, (P - C_L) = k_g\, A\, H\, (C^* - C_L) \doteqdot k_L\, A\, (C^* - C_L) \quad . \tag{6.8}$$

Steel (149) showed that H 1 for air saturated broth permitting this factor to be removed in the above equation 6.8 which may be further simplified;

$$N = K_o\, A\, (C^* - C_L) = k_g + K_L\, A\, (C^* - C_L) \quad . \tag{6.9}$$

It was previously shown that the liquid film is believed to control the rate of oxygen transfer. This implies that, $\frac{1}{k_L} >> \frac{1}{k_g}$, and although

$$\frac{1}{K_o} = \frac{1}{k_g} + \frac{1}{k_i} + \frac{1}{k_L} \tag{6.3}$$

the other resistances are insignificant with respect to the liquid film allowing the following assumption to be made, $\frac{1}{K_o} \backsim \frac{1}{k_L}$ and $K_o \backsim K_L$.

Since the individual film coefficients can only be estimated by graphical interpolation the overall term K_L is commonly used. Therefore, the overall oxygen transfer equation 6.9 may be made in terms of the liquid film alone which permits evaluation of oxygen transfer rates (OTR) and oxygen transfer coefficients on the basis of bulk liquid dissolved oxygen cencentration measurement,

$$N = K_L\, A\, (C^* - C_L) \quad . \tag{6.10}$$

The gas-liquid interfacial area varies with fermentor volume and operational procedures. Since the rate of oxygen dissolution is proportional to the interfacial area and is inversely proportional to the liquid volume used, the oxygen transfer equation is commonly written in terms of the volumetric mass transfer coefficient which combines the area and volume factors, $\frac{A}{V} = a$. Thus $K_L A/V = K_L a$ where $K_L a$ is the volumetric mass transfer coefficient and the units used are; $K_L a = 1/min$, $K_L = cm/min$, $A = cm^2$, $V = cm^3$, and

$$N = \frac{dC}{dt} = OTR \quad , \tag{6.11}$$

where OTR is m Moles 0_2/L/Min . With these definitions the oxygen transfer equation 6.10 takes the form which has been commonly used by fermentation technologists ;

$$OTR = K_L a \ (C^* - C_L) \quad . \tag{6.12}$$

Olson and Johnson (118) were the first to use the $K_L a$ term as a criterion for gas transfer evaluation. It has since become the standard index for reporting the overall oxygen absorption capacity of fermentor aeration equipment and a valuable means for comparison of different fermentor designs or sizes.

6.2.5 Principles and Problems of Bubble Aeration

Aeration of liquid cultures is, as previously described, conventionally accomplished by gas bubble production and mixing within a cylindrical fermentor containing a sparger and driven agitator. The establishment of an equilibrium between dissolved oxygen in the liquid and the gas (bubbles) phase represents the goal of the process. Ideally this would be accomplished by the total dissolution of each sparger

produced gas bubble as it rose and was gently mixed to evenly distribute the dissolved oxygen throughout the liquid volume. Unfortunately such ideal oxygen transfer does not occur, mainly because bubbles released into the bottom of a liquid tank are dynamic and do not behave uniformly. Because of this, oxygen is not uniformly dispersed in the liquid, much of the gas remains in the gas phase and as a result, aeration efficiency is relatively low, about 1% to 2% (48).

The key elements in sparger aeration are the maximization of the bubble-liquid interfacial area and the maximization of the duration of contact between bubbles and liquid. This agrees with the importance placed on the interface by the Whitman two-film oxygen transfer theory, as explained previously.

In an attempt to improve oxygen transfer rates fermentation technologists have attempted to increase the interfacial area by manipulating operational procedures and/or equipment design, in order to increase the air volume input, produce smaller bubbles, or delay the escape of bubbles and improve mixing (104). Other techniques designed to improve oxygen solubility such as raising the temperatures, gas pressure, or oxygen partial pressure have not proven economical on a large scale basis.

Flushing high volumes of air through a fermentor provides a large quantity of gas, usually with some back pressure within the vessel (which raises the partial pressure of oxygen), but also is very inefficient. High flow rates cause large bubble formation, bubble coalescence, excessive foaming, creation of gas channels within the liquid, and air leakage around the agitator shaft and other seals. Thus any advantage gained by the additional oxygen is offset by the disadvantages of this procedure.

A high rate of liquid agitation will also improve oxygen transfer rates because it will improve bubble dispersion throughout the liquid, reduce bubble coalescence, delay the escape of bubbles from the liquid, and increase the effective interfacial area of the bubbles by moving them about, thereby reducing their liquid film thickness and producing surface regeneration (a net increase in interfacial area) (33, 41, 23).

Vigorous agitation reduces stagnant zones within the liquid and increases the overall interfacial area for oxygen transfer per given volume of gas sparged. However, there appears to be an optimal speed above which undesirable effects outweigh any improvements in efficiency. Excessive agitation may produce bubble coalescence and "flooding" around the impeller blades, cause excessive liquid evaporation and foaming, produce over-aeration conditions which may retard cell growth, or produce a shear effect which may damage the cells (14, 104, 23).

The total surface area exposed to the liquid and the internal partial pressure of oxygen are inverse functions of gas bubble diameter (32, 2). Pattle concluded from his studies of gas solution from rising bubbles that small gas bubbles held in suspension for as long as possible produced the most effective oxygen transfer (119). In addition, small gas bubbles tend to rise slowly and dissolve more completely (33). In order to exploit the advantages of small bubble aeration investigators have attempted to design and operate aeration systems which could produce consistently fine gas bubbles, with the ideal being about .2 and .4 cm in diameter (41). For example, a single orifice sparger was operated at acoustic air velocities, and a "P-jet" sparger was used to release small bubbles (2, 119). Although these systems are effective, they require either excessively high operating pressures or impractically small

orifice openings. Therefore the commonly used spargers produce a variety of bubble sizes and represent a compromise between efficiency and economics.

In an attempt to understand and improve bubble aeration some excellent studies have been made of bubble dynamics. Photostrobe measurements and high speed motion pictures have been used to study the fate of bubbles after release from the sparger (174). Upon release a bubble rises, dissolving as it moves and leaving a trail of oxygen rich liquid (123). Bubbles of different sizes were found to behave differently and rise at different rates. Small bubbles act as spheres and larger bubbles change shape while ascending (96). The initial size determines the height traveled before disappearance, although mixing will influence this because it changes the bubble path from vertical to a more dispersed path (119). Hydrostatic pressure, initial bubble volume, and liquid viscosity influence the rate of rise. This rate would be expected to increase as a bubble ascends because the hydrostatic pressure is reduced allowing an increase in size and lift. However Calderbank has shown that bubbles of a given size actually rise at a constant velocity (22). Bubbles suspended in liquid tend to coalesce, which reduces the net surface area and increases the rise velocity. In addition to dispersing bubbles, mixing also retards coalescence. The addition of hydrophilic organic substances such as lactic acid, glycol, and glycerol have also been successful in retarding coalescence and allowing prolonged bubble life and surface area (22, 23, 119).

Examination of the literature reveals that bubble behavior is both dynamic and unpredictable. The foregoing description of sparged bubble behavior represented idealized observations under defined experimental

conditions. The conditions within a fermentation are far more complicated and profoundly influence bubble dynamics and the efficiency of oxygen transfer. For example Calderbank observed that the rise velocity of gas bubbles was independent of the liquid viscosity (22). However, fermentation broths. ex-perience viscosity changes with the age and growth of the culture. This has been shown to cause an 85% reduction in oxygen transfer rates as compared to only an 8% reduction in cell-free medium (40). It is probable, therefore, that oxygen transfer efficiency, which is influenced by bubble dynamics and oxygen solubility, is directly related to the surface tension, the viscosity, and the presence of solutes, antifoam agents, and suspended particles which are characteristic of fermentation broths. In addition these liquid characteristics along with sparger design, fermentor geometry, and agitation influence bubble shape, rate of formation, coalescence, velocity of rise, distribution, interfacial area, and duration of existence (37).

It may be concluded that in conventional sparged and agitated fermentors adequate liquid aeration is best accomplished by the production of relatively small bubbles which are held in suspension as long as possible and are well dispersed throughout the liquid. Although careful selection of equipment design and operation may improve oxygenation efficiency up to 30%, stagnant regions continue to exist within the fermentor and sharp dissolved oxygen gradations continue to exist within the liquid. As a result, cell yields do not always correspondingly improve with improved aeration efficiency (150). Therefore it appears that the bubble aeration technique, although able to deliver a net transfer of oxygen to liquid, is inherently inefficient and may, as a result,

limit the optimal oxygenation of fermentation broths, especially at the microscopic-cellular level.

6.2.6 Mechanism of Membrane Oxygen-Transfer

The transfer of oxygen from the gas phase through a membrane into the liquid phase is similar in principle to oxygen transfer from bubbles, except that the phases are separated by a defined membrane interface which has transfer characteristics unique to the membrane material used. Transfer of solutes through solid or membrane barriers has been adequately examined and discussed by Barrer (7), Brubaker and Kammermeyer (20), and more recently by Tuwiner (159). Except for the influence of the thicker interface, oxygen transfer through a membrane is fundamentally no different than mass transfer from bubbles and may also be explained by the Whitman two-film mass transfer theory as outlined previously in Section 6.2.4. This theory accounts for the gas film, the liquid film, and the interface (membrane) factors which constitute the principle elements which control oxygen transfer in dialysis aeration. The key equations explaining membrane oxygen transfer as described in several references are reviewed below (3, 159).

Dialysis aeration involves the same transfer steps as visualized in Figure 32. The key difference between dialysis and bubble aeration lies in the importance of the membrane resistance factor $\left(\frac{1}{k_3}\right)$ and the gas and liquid dynamics adjacent to the membrane interface. The interface, regarded as a small and immeasurable factor in bubble

aeration, is tangible and has a major influence on membrane aeration. In addition, the design of the dialyzer allows a greater degree of control of the bulk gas and liquid as they flow past the membrane(s). These velocities influence the thickness of the stagnant (laminar) gas and liquid films and accordingly their resistances to transfer $\frac{1}{k_2}$ and $\frac{1}{k_4}$. The influence of fluid velocity and dialyzer operation on the fluid dynamics adjacent to the membrane were discussed in Section 4.4 of this thesis, were diagrammed in Figure 17, and will influence the dialysis aeration system in a similar manner.

As shown previously, the overall oxygen transfer process is represented by the basic mass transfer equation, (6.1), which is the summation of the mass transfer steps 1 through 5 (Figure 32);

$$N = K_o(\Delta C)A = k_{\substack{\text{bulk}\\\text{gas}}}(\Delta C)A = k_{\substack{\text{gas}\\\text{film}}}(\Delta C)A = k_{\substack{\text{membrane}\\\text{interface}}}(\Delta C)A = k_{\substack{\text{liquid}\\\text{film}}}(\Delta C)A = k_{\substack{\text{bulk}\\\text{liquid}}}(\Delta C)A. \qquad (6.13)$$

Transfer through the bulk gas and bulk liquid is a diffusion process, obeys and may be represented by Fick's Law of Diffusion, and does not represent a significant limitation to oxygen transfer. Thus, these two factors can be assumed small and neglected, as explained previously. This assumption may be made with confidence in the dialysis aeration system because the dynamics within the dialyzer were shown to produce excellent bulk turbulence, which promotes adequate mixing and aids bulk diffusion (Section 4 of this thesis). Equation 6.13 may now be reduced to;

$$N = k_g(\Delta C)A = k_m(\Delta C)A = k_L(\Delta C)A \; . \qquad (6.14)$$

Tuwiner (159) described membrane transport as a diffusional process. This makes the membrane transfer coefficient in equation 6.13 equivalent

to a diffusion coefficient;

$$N = k_m \ (\Delta C) \ a \sim N = D/X \ (\Delta C) \ A \quad \text{and} \quad k_m \cong D/X, \tag{6.15}$$

where D = membrane diffusion coefficient and X = the membrane thickness.

The object of dialysis aeration, as with bubble aeration, is the establishment of an equilibrium state between the gas and liquid phases which results in the saturation of the liquid with dissolved oxygen. Thus the reasoning used previously to develop the oxygen transfer expression in terms of steady state concentration expressions can be applied here. Thus equation 6.14 becomes;

$$N = k_g \ (P - P_i) \ A = k_m \ (P_i - C_i) \ A = k_L \ (C_i - C_L) \ A \tag{6.16}$$

and

$$N = k_g \ (P - P^*) \ A = k_m \ (P^* - C^*) \ A = k_L \ (C^* - C_L) \ A \tag{6.17}$$

where the * denotes the equilibrium values. Henry's Law is again used to write the oxygen transfer relationship in terms of liquid oxygen concentration;

$$N = k_g + k_L \ (C^* - C_L) \ A = k_{\blacksquare} \ (C^* - C^*) \ A \ . \tag{6.18}$$

Under these steady state conditions the membrane has a constant concentration gradient across its thickness and because of its fixed transfer characteristics the membrane factor in the above expression will be constant

permitting equation 6.18 to be written in terms of the liquid film equilibrium concentrations;

$$N = k_g = k_m = k_L (C^* - C_L) A \quad . \qquad (6.19)$$

As shown in equation 6.3 the overall resistance to mass transfer equals the sum of the individual resistances. Although the membrane coefficient and resistance are major elements in dialysis oxygen transfer, in contrast to the significance placed on the interface in bubble transfer, they are fixed characteristics of the material and may be calculated separately from diffusion data,

$$k_m \cong D/X \qquad (6.20)$$

allowing ommission of (k_m) from the overall oxygen transfer equation. This permits the expression of membrane oxygen transfer to be made in terms of the gas and liquid film coefficients and the liquid film oxygen concentration. The importance of the liquid film in contrast to the gas film was shown previously. This relationship,

$$\frac{1}{k_L} >> \frac{1}{k_g} \quad ,$$

also applies to the membrane transfer process permitting the overall expression of dialysis aeration to be made solely in terms of the liquid film;

$$N = K_o A (\Delta C) \curvearrowleft N = K_L A (C^* - C_L) \quad . \qquad (6.21)$$

Although the interfacial area is measurable in dialysis aeration, the use of the volumetric oxygen transfer coefficient which has been the accepted criteria for evaluation of gas transfer for twenty years, is preferred in order to facilitate comparison to previous data. Thus the proportion $K_L \frac{A}{V} = K_L a$ applies and equation 6.21 is written in familiar terms;

$$OTR = K_L a \quad (C^* - C_L) \quad . \tag{6.22}$$

With the use of equation 6.22, dialysis aeration may be evaluated in a manner similar to bubble aeration. OTR and C_L values are determined by dissolved oxygen analysis, C^* values are characteristic of oxygen saturation in the liquid used, and equations 6.21 and 6.22 are used to determine the overall transfer coefficient and the volumetric transfer coefficient, respectively.

6.2.7 Principles of Dialysis Aeration - Silicone Membranes

Oxygen is transferred to a liquid in a uniform, continuous, controllable, defined, and predictable manner by dialysis aeration. This contrasts with the unpredictable and variable transfer associated with dynamic bubble aeration, as discussed in Section 6.2.5. Unlike sparging, dialysis aeration has a gas-liquid interfacial area which is stable and of known dimensions, a degree of control over the thickness of the

gas and liquid films, a stable oxygen partial pressure in the gas phase and therefore a reasonably constant concentration gradient across the interface, an even diffusion of oxygen into the liquid phase which reduces foaming and sharp oxygen concentration variations within the liquid, and an exposure of the total liquid volume to the oxygen exchange interface by virtue of the fluid circulation and the turbulent relatively thin fluid film passing through the dialyzer.

Although the aeration of a microbial culture by membrane exchange was demonstrated on a small scale over twenty-two years ago (64), the technique was largely ignored until the oxygenation of blood was successfully demonstrated by oxygen transfer through membranes (ethyl cellulose) contained in an external independent exchanger (29). The ability to handle large volumes and the elimination of toxic, direct, blood-gas contact represent the features which spurred interest in this technique. During the ensuing years considerable effort has been expended in selecting suitable membrane materials, analyzing fluid dynamics and oxygen transfer properties, and designing efficient exchangers. The development of artificial lungs has been thoroughly recorded over the past sixteen years in the Transactions of the American Society for Artificial Internal Organs. These reports of successful applications of membrane aeration using exchangers similar to our model dialyzer served as the impetus for applying this large scale technique to microbial aeration.

The membrane interface represents the key element in dialysis aeration. Early artificial lungs utilized a variety of membrane materials including cellulose. These did not provide sufficient gas transmission rates to oxygenate blood adequately. The subsequent use of Teflon membranes and, more recently, silicone rubber membranes (which are 4 to 100

times more permeable to oxygen and carbon dioxide) has permitted the maintenance of up to 92% oxygen saturation with a reasonable membrane area (122, 38, 98). The oxygen transfer rates of these membranes, especially the silicone rubber, exceed the rate of transfer through blood. This demonstrated that these membranes do not limit oxygen transfer and confirmed that the fluid film represents the major resistance to oxygen transfer (122). This validates the use of oxygen transfer equations 6.21 and 6.22, which are based on the assumption that the liquid film constitutes the major resistance to transfer.

Investigations with a variety of membrane oxygenators has shown that membrane gas transfer involves two phenomena, intramembrane transport and phase boundary transport (88). This emphasizes the importance of the membrane and its surface films. The first involves molecular diffusion, which is expressed by Fick's Law of Diffusion $J = -D \frac{\Delta C}{X}$. The composition of the membrane material determines oxygen diffusivity, while membrane thickness and area as well as the temperature of operation influence transport rates. Membranes as thin as .0005" have been successfully employed although the increased strength associated with thicker membranes ∿.003" is preferred (98). The superior gas permeability of the newer membranes has focused interest on the second phenomena. The liquid film is considered to be the major resistance governing gas transfer and reduction of its thickness is important to improving liquid oxygenation (111, 102). Greater velocity, faster agitation, and counter current gas-liquid flow have been shown to improve oxygen transfer efficiency by as much as 50% (122, 98). These techniques improve oxygen transfer to the bulk liquid by reducing the laminar layer of liquid

adjacent to the membrane and rapidly exchanging this film, which quickly becomes saturated with oxygen, with fresh unsaturated fluid. In addition the continuous flow of fresh gas over the opposite face of the membrane maintains a constant partial pressure of gas-phase oxygen, which aids exchange efficiency and contrasts with the reduction in gas volume and partial pressure when gas bubbles rise and dissolve. Other factors which influence overall gas transport to a lesser extent include gas concentration (air vs. pure oxygen), membrane area, and membrane support and dialysis chamber design. These are discussed elsewhere in this thesis (Section 6.4).

The key to the efficient membrane oxygenation of blood and to the successful dialysis aeration of a microbial culture has been the availability of silicone rubber membranes. Its high permeability to oxygen and carbon dioxide and its biological compatibility make it superior to other materials. The permeability properties of several types of commonly used membranes are compared in Table 14.

The pheonomenon of gas movement through membranes has been examined and reviewed by Barrer (7), Brubaker and Kammermeyer (20), Tuwiner (159), Rickles (128), and Kaufmann and Leonard (88). Generally, transport through a material is a function of crystalinity, thickness, electrostatic attraction, energy gradients, tortuosity factors, temperature, and concentration gradients and is a process which obeys diffusion equations (Fick's laws). It was recently noted that the nature of the newer membrane polymers and the variety of interacting factors operating in membrane permeation make it impossible to write meaningful equations which can predict the process accurately (129). The mechanism for gas transfer through silicone rubber is reported to be analagous to transfer through solid surfaces (Dow data

Table 14. Gas permeation coefficients of membrane films *.

MEMBRANE TYPE	P_m , O_2 **	P_m , CO_2 **
Natural Rubber and Polyvinyl Chloride	1	-
Polyethylene	2	11
Regenerated Cellulose	3-9	-
Ethylcellulose	17	70
Teflon	30	78
Silicone Rubber	1210	6310

* Values were obtained from Gas Transmission Rates of Plastic Films, Dow Corning Corporation, Midland, Michigan , and from references (29, 122, 131).

** $\frac{\text{cc gas}}{\text{min m}^2 \text{ atm/mil thickness}}$

sheet, Gas Transmission in Plastic Films, 1959). This permeation has been described as a three-step process as follows: condensation on and solution into the membrane surface, which is a function of the interaction between the gas molecule and the membrane polymer; molecular diffusion through the thickness of the membrane; and dissolution and evaporation of the gas molecule at the opposite membrane surface (147, 103).

Braley (16) has described silicone rubber as a homogenous organosilicone compound consisting of a highly viscous non-crystalline polymer with linear sequences of alternating silicone and oxygen atoms and with organic methyl or ethyl molecules attached to each silicone atom:

$$\left| \begin{array}{cccc} CH3 & & CH3 & \\ -Si & - O - & Si & - O \\ CH3 & & CH3 & \end{array} \right|_n$$

The number of these sequences per polymer molecule, n, determines the viscosity of the material and is about 5000 for pliable rubber films. Here these polymers are vulcanized, forming a three dimensional film of polymer chains lightly cross-linked between the carbon atoms. The vulcanizing agent (dichlorobenzoylperoxide) haloginates the methyl groups permitting cross bonding but does not become a part of the film after vulcanization. This produces a pliable rubber film which consists solely of the cross linked organopolymer and the silica filler (SiO_2 for strength). As a result of this purity silicone rubber is nontoxic and biologically compatible with blood, tissues, and bacteria.

Because silicone rubber films have no pores or open spaces and show no appreciable bulk flow of aqueous solvents they are categorized as plastic membranes. Solute movement through these films is analogous to the three-step process explained previously, with the exception that the movement through the membrane material itself does not follow the classical

diffusional transport concept given above. Rapid gas transport in silicone rubber is attributed to the high solubility of the gas molecules in the organo-silicone polymer, the high diffusivity characteristics of the polymer, the absence of crystallinity, and the flexibility of the bonds in the silicone-oxygen-silicone chain (16, 131). Because the high permeability is related to the solubility of gases in silicone rubber, the transport process is written in terms of both solubility and diffusion;

$$P_m = S \cdot D \tag{6.23}$$

Thus, the equation for mass transfer across silicone membranes (in equations 6.17 and 6.18 given in Section 6.2.6) is written;

$$N = \frac{P_m A \ (\Delta C)}{X} \tag{6.24}$$

where the membrane transfer coefficient, k_m, and its relationship to diffusion and membrane thickness, $k_m \cong D/X$, is replaced by the permeability coefficient, P_m, and the new relationship which emphasizes solubility (45, 131),

$$P_m \cong \frac{D \cdot S}{X} \tag{6.25}$$

The selectivity of gas solubility and the exact process of gas movement through the silicone polymer is not known. It is possible that gas molecules squeeze past the pliable polymer bonds or the cross-linkage bonds, that a chemical exchange occurs between gaseous oxygen molecules and the polymer oxygen molecules, or that the methyl groups of the organo-silicone polymer rotate around its long axis opening "void spaces" which enhance the movement of gas molecules. Solubility and solute-membrane

interaction play a key role in mass transfer with this material. For instance carbon dioxide permeates the film 10 times faster than much smaller and lighter helium molecules, a condition which is contrary to a strictly diffusional model for transport (107, 131). The higher solubility shown by gases with a higher boiling point suggests that molecular energy may also play a role in molecular transmission through the silicone polymer. Despite the inability to clearly identify the mass transfer process, the characteristics of silicone rubber make it an excellent vehicle for the uniform transfer of gas molecules from the gas to the liquid phase without direct gas-liquid contact.

6.2.8 Applications of Dialysis Aeration

The development of suitable membrane films has been the major technological advance necessary for serious interest in the dialysis aeration process. Careful material selection, equipment design, and equipment construction have eliminated most of the problems associated with the assembly and operation of membrane aeration equipment. In addition membrane manufacturing has become more sophisticated, permitting the production of reliable thin films, dacron mesh-reinforced films, capillary tube membranes, and co-polymer films incorporating molded membrane support elements (16, 131, 47, 121). Recently these developments have primarily involved silicone rubber and are in response to the successful application of this material to the refinement of artificial lung devices. The potential of membrane exchange has also been reported for the following applications: prolonged drug therapy,

administration of general anesthesia, separation, purification, concentration, and/or measurement of gas mixtures, oxygen extraction from water, and artificial placenta devices (50, 51, 164, 131, 172). On-going developments concerning the application of silicone materials are periodically reviewed and summarized in the Bulletin of the Dow Corning Center for Aid to Medical Research, Midland, Michigan.

The concept of culture aeration by membraneous gas exchange was introduced before the discovery of gas permeable films. Gladstone (64) used a crudely constructed combination of cellophane sacs contained within a glass nutrient broth chamber. Air passed through a cylindrical inner sac which was surrounded by a more coarse outer sac with the culture being contained in the space between the two. The Bacillus anthracis culture was aerated by gas diffusion through the membranes. This small scale dialysis-aeration scheme prevented autolysis and the liberation of proteolytic enzymes and delayed sporulation, resulting in a 25-fold increase in antigen production. The improved yield was attributed to the elimination of the need for toxic antifoam agents, a necessity with bubble aeration, and to the elimination of the denaturing effect of direct gas-liquid and gas-cell contact. These exemplify two of the advantages which dialysis aeration can provide for culture propagation.

Another small scale dialysis-aeration scheme was reported more recently by Brewer (19). This consisted of a small dialysis fermentation system described as a closed outer envelope containing the culture and an inner semipermeable envelope which comprised the nutrient chamber. The device was provided with inlet and outlet ports for culture sampling and intermittent or continuous nutrient circulation. When the outer

envelope was constructed of thin polyethylene or polypropylene, oxygen from the atmosphere was transmitted to the culture and permitted aerobic growth which was demonstrated with Corynebacterium diptheriae and Bacillus stearothermophilus. With this scheme the culture received nutrients by dialysis from the inner envelope and oxygen by gas permeation of the outer envelope.

Both of the above dialysis aeration systems were limited in size, constructed of fragile materials, and in the second case relied upon passive oxygen transfer. Their oxygen transfer capabilities were limited by the selected membrane materials and available membrane area. In addition no provisions were made for fluid circulation in order to reduce the liquid film resistance to oxygen transfer. Although these devices demonstrated the potential value of dialysis aeration, their practicality for larger scale production of microorganisms was limited. Furthermore because no data were shown regarding the performance parameters of these aeration processes, no calculations could be made to predict their efficiency or adaptability.

Dialysis aeration was demonstrated on a somewhat larger scale when Serratia marcescens cultures were propagated in the bottom of a dialysis shake flask, a culture system specifically designed for biological applications (61). The culture was separated from the atmosphere by a membrane and a thin water layer. Aeration was provided by oxygen diffusion through the water layer and membrane interface. When a membrane filter was used, viable cell growth was observed to be markedly higher than the anaerobic control but less dense than growth achieved by conventional culture shaking. This concept was explored further with a 12-liter dialysis fermentation

system using, for the first time, an external membrane exchanger (62). In this demonstration the nutrient supply, which was aerated by conventional sparging, was circulated through the dialyzer which contained regenerated cellulose membranes. Oxygen transfer occurred from the oxygenated nutrient fluid across the membrane surface to the culture, which was circulated through the dialyzer from the fermentor. Viable cell densities of 1.4×10^{10} cells/ml were reported after 48 hours growth. Although these results were inferior to the cell densities achieved by conventional fermentor aeration, they did illustrate the principle of microbial aeration by indirect oxygenation.

The last two examples represent an advance in equipment design, reliability, and biological compatability. Although the membrane area available for oxygen transfer was greater, the potential of these systems was again limited by the membrane material used. Regenerated cellulose has a low permeability to oxygen (see Table 14) and membrane filters will not dependably confine bacteria (79). Unfortunately no examination of the oxygen transfer characteristics was reported for either system. In addition, the operation of both of these schemes was dependent upon the saturation capacity of the liquid, resulting in a much lower oxygen concentration than with air and therefore a lower oxygen concentration gradient across the membrane. The dialysis flask system relied upon passive oxygen availability to the liquid film covering the membrane, had no means of manipulating the gas or liquid phases, and had a limited culture capacity. As a result this experimental system is limited in adaptability to larger scale operations and has its primary value as a test bed for membrane evaluation. The dialyzer-dialysis culture system on the other hand was designed for

large scale use, permitted, by virtue of its independent exchanger, adjustments in liquid flow rates, membrane area, and culture volume, and could be used as a model system for further investigation of the characteristics of dialysis-aeration.

Microbial cultures are conventionally aerated by internal sparging and agitation. External aeration of the fermentation has seldom been attempted, the only notable example being an external aeration system designed by Stich which produced fine bubbles by forcing air through porous materials into the circulating fermentation broth (154). The dialyzer-dialysis culture system designed by Gallup and Gerhardt (57) provided a means of examining the principle of membrane aeration to a full scale microbial fermentation. The availability of highly permeable, leak-free silicone rubber membranes and the successful application of these to external membrane aeration of blood demonstrated the potential of this scheme for fulfilling the demands of a fragile biological system. Theoretically the dialysis-aeration technique has several features (listed in Section 6.1) which could be advantageous to a microbial fermentation, especially when oxygen sensitive or pathogenic cultures are used. The examination of these desirable features, the analysis of aeration characteristics, and the examination of the effect of uniform bubble free oxygenation on microbial growth provide reasonable justification for investigation of dialysis-aeration. The serious consideration of dialysis-aeration as a substitution for conventional aeration requires that two principle questions be answered: 1) will the experimental system perform reliably under the rigors of fermentation conditions and provide sufficient oxygen transfer rates;

and 2) will this system which has a gas-liquid interfacial area which is relatively small support cell growth approaching or exceeding culture densities obtained by conventional aeration? Preliminary investigations utilizing a prototype of the dialyzer described in Section 3 showed that, when equipped with silicone rubber membranes and using pure oxygen, the dialysis-aeration system provided sufficient oxygen transfer to theoretically support a 1.5 liter culture of Saccharomyces cervisiae (G. F. Bennett, personal communication). The preliminary analysis of the oxygen transfer characteristics for the system (presented at the 152nd American Chemical Society meeting, 1966) have been extended and an analysis of both the physical and biological characteristics of dialysis-aeration have been completed and are presented in the following parts of this section.

6.3 Materials and Methods

Dialysis aeration experiments were conducted with the same fermentation equipment described for dialysis growth trials. Adaptation for aeration required only minor operational changes and inclusion of gas permeable membranes in the dialyzer. The operational configurations used were: non-dialysis control, with conventional air sparging and agitation (Figure 18); dialysis aeration plus nondialysis nutrients (Figure 33); and dialysis aeration-dialysis nutrients (total dialysis) with one dialyzer for gas exchange and another for nutrient exchange (Figure 34).

Dialysis aeration also required pumps, conduits, a dialyzer, air flow meters (18 M), air pressure gauges (19 M), a dissolved oxygen probe and analyzer (8 M), a fabricated nylon "T", threaded to hold the dissolved oxygen probe in the fermentor liquid conduit line, a chart recorder (26 M), and most importantly, silicone rubber membranes (20-M) cut and fitted to the dialyzer. In the case where both aeration and nutrition were accomplished by dialysis, two dialyzers were assembled. One contained silicone rubber membranes separating air flow and fermentor liquid flow, and the other contained Visking regenerated cellulose membranes separating nutrient reservoir liquid flow and fermentor liquid flow.

The silicone rubber membranes chosen for dialysis aeration were .007 inches thick, dacron-weave reinforced, "Silastic" S-2000 type (produced by the Dow Corning Corporation). Assembled in the dialyzer,

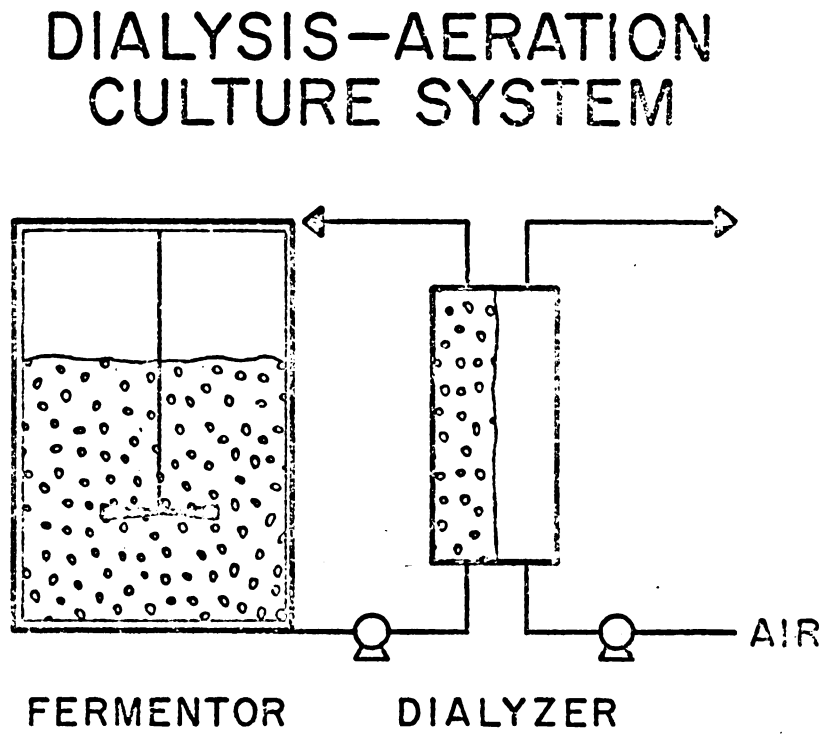


Figure 33. Diagram of the dialysis-aeration culture system.

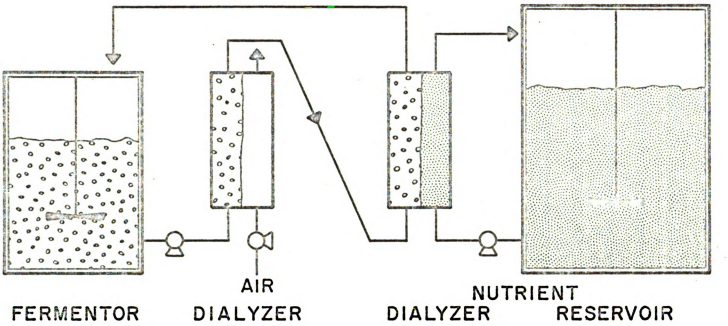


Figure 34. Diagram of the dialysis-aeration -- dialysis-nutrient culture system.

they proved to be non-toxic and autoclavable and had an exposed surface area of .0288 m^2 per membrane. Silicone rubber is highly permeable to oxygen, 40 times greater than Tetrafluoroethylene (Teflon)*, which makes it a desirable membrane material for dialysis aeration.

In order to simulate microbial culture conditions, all experiments were conducted in fermentation equipment identical to that used previously for dialysis and nondialysis growth trials. These were: a 5 liter New Brunswick culture fermentor which contained 3 liters of growth medium (Trypticase soy broth); 1/4 inch diameter rubber tubing fluid conduits which contained culture sampling ports and the dissolved oxygen probe threaded "T" connector; Maisch metering pumps for fluid circulation; a 15 liter New Brunswick fermentor which was filled with growth medium and functioned as a nutrient reservoir -- in nutrient dialysis growth trials only; a custom built water bath which contained the vessels and maintained a constant temperature; fermentor agitator drive motors and speed regulators; and the aforesaid air filters, foam trap, sampling syringes, antifoams, flow meters, pressure gauges, and air humidifier necessary for conducting analyses of equipment performance or growth trials.

Evaluation and comparison of dialysis and conventional aeration involved investigation of pressure gradients, oxygen transfer rates, and microbial growth rates and yields. Gas pressure analyses were intended to evaluate pressure drop across the dialyzer and within the growth fermentor. Measurements were made with either pressure gauges

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* Oxygen diffusion constants for Silastic and Teflon are 1210 and 30 ml/min/m^2/atm/mil STPD at 25°C respectively (data provided by Dow Corning Corporation).

(19 M) or mercury columns. Depending on the measurement to be made these devices were located as follows: on the fermentor vessel to determine pressure gradients across one side of the entire dialyzer, to 20 gauge needle probes inserted at various points into an individual dialyzer chamber to determine the pressure drop across a single chamber of the assembled dialyzer, and also to the inlet and outlet gas lines of the fermentor vessel to determine pressure differences between incoming and exiting gas during conventional aeration. After attachment of the measurement devices the system was operated as it would be in an actual fermentation situation. The effect of membrane area, gas flow rates, and fluid flow rates on pressure was measured.

The oxygen transfer characteristics of conventional aeration and dialysis aeration were evaluated by measuring the dissolved oxygen concentration in the fermentor fluid during a simulated growth trial without cells. Data was reported at OTR (oxygen transfer rates into the liquid, m moles/L/min) or $K_L a$ (volumetric oxygen transfer coefficients, min^{-1}) values. The influence of air flow rates, agitation speed, and liquid volume was determined for conventional sparged (control) aeration. The influence of the above and membrane area was determined for dialysis aeration and compared to the appropriate control. In each trial the fermentor was filled with growth medium which was maintained at $30^{o}C$ and circulated at 2 L/min through an external conduit which contained the dissolved oxygen electrode. The liquid was initially deaerated by nitrogen sparging. For conventional aeration tests the fermentor was agitated at 360 rpm and sparged at 9 L/min with a single orifice sparger unless noted otherwise. For dialysis aeration tests a dialyzer containing from 1 to 6 (.0288 to .1728 m^2 effective area) Silicone rubber

membranes was inserted into the external conduit. The fermentor was not sparged, and the system was operated at 300 rpm agitation, 2 L/min liquid velocity, and 25 L/min air velocity unless noted otherwise. Oxygenation of the fermentor liquid was detected by the previously calibrated electrode and read out on the chart recorder as percent saturation. The data was easily converted to units of concentration (ppm or mg/L) for calculations or comparison to other work by referring to a standard curve corrected for the influence of local elevation (Figure 35).

The following evaluation of a dialysis aeration trial illustrates the treatment of the data obtained in each experiment. Oxygen transfer to the fermentor liquid was recorded as percent saturation during the course of aeration (Figure 36). As shown, the greatest rate of oxygenation occurred during the initial five minutes of aeration when the oxygen concentration gradient between the gas and liquid was greatest. This was representative of the maximal OTR obtainable by the aeration system under the conditions of the test. These initial oxygen transfer rates were indicative of the system's capacity to fulfill the oxygen demands placed on it by an active aerobic culture (148). The oxygenation data shown in Figure 36 was also used to compute the volumetric oxygen transfer coefficient $K_L a$ for the aeration system (Table 15 and Figure 37). The continually changing concentration gradient between the gas and liquid phases $\left(Cg - C_L \right)$ as aeration proceeded necessitated calculation of the average bulk liquid dissolved oxygen concentrations $\left(C_L \text{ av} \right)$ over the time intervals of the trial (Table 15). These averages were then used to determine $\left(C^* - C_L \text{ av} \right)$ factors which represented the approach to liquid - gas equilibrium C^*, which is the oxygen

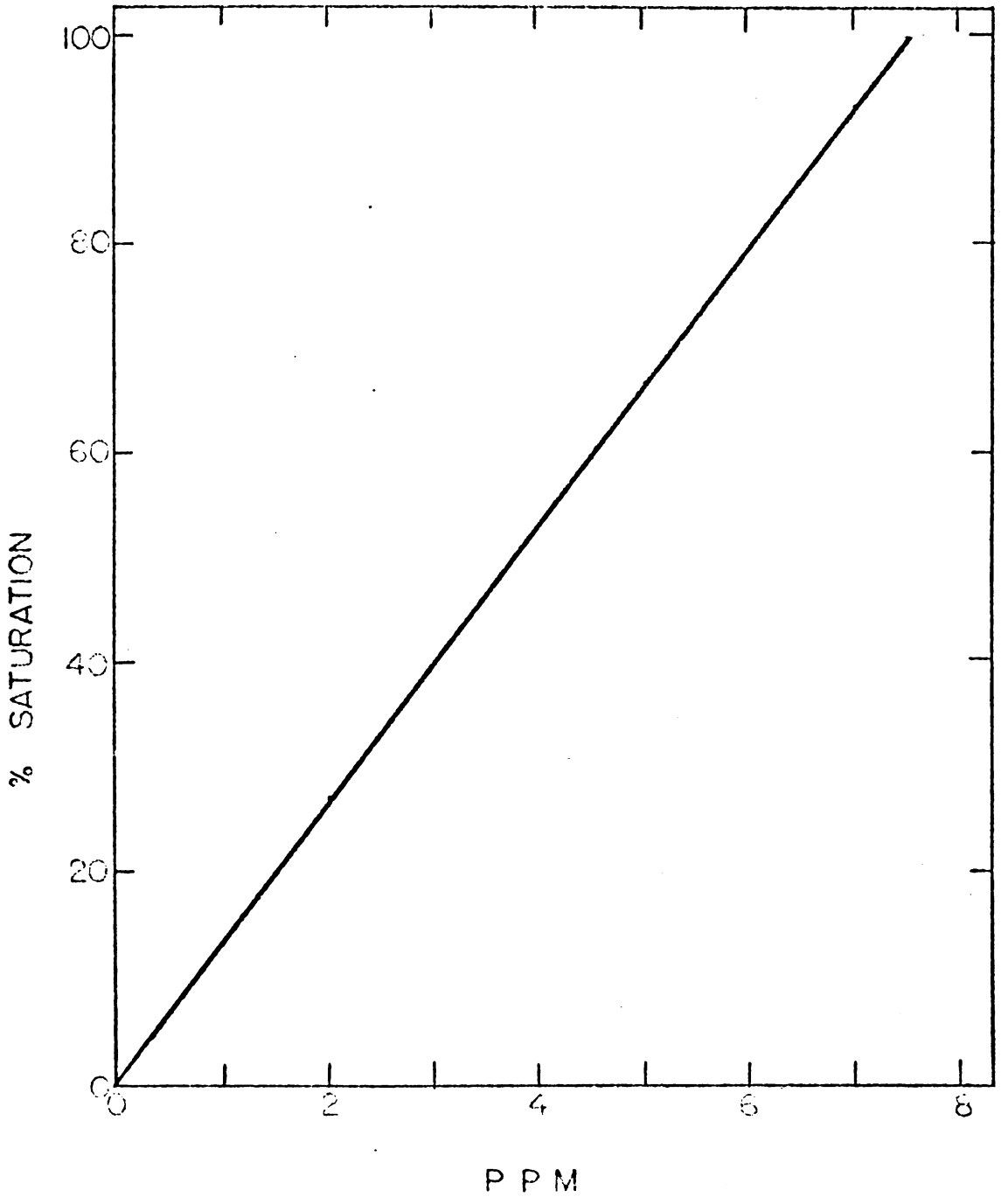

Figure 35. Relationship between percent saturation and PPM concentration of dissolved oxygen at 30°C.

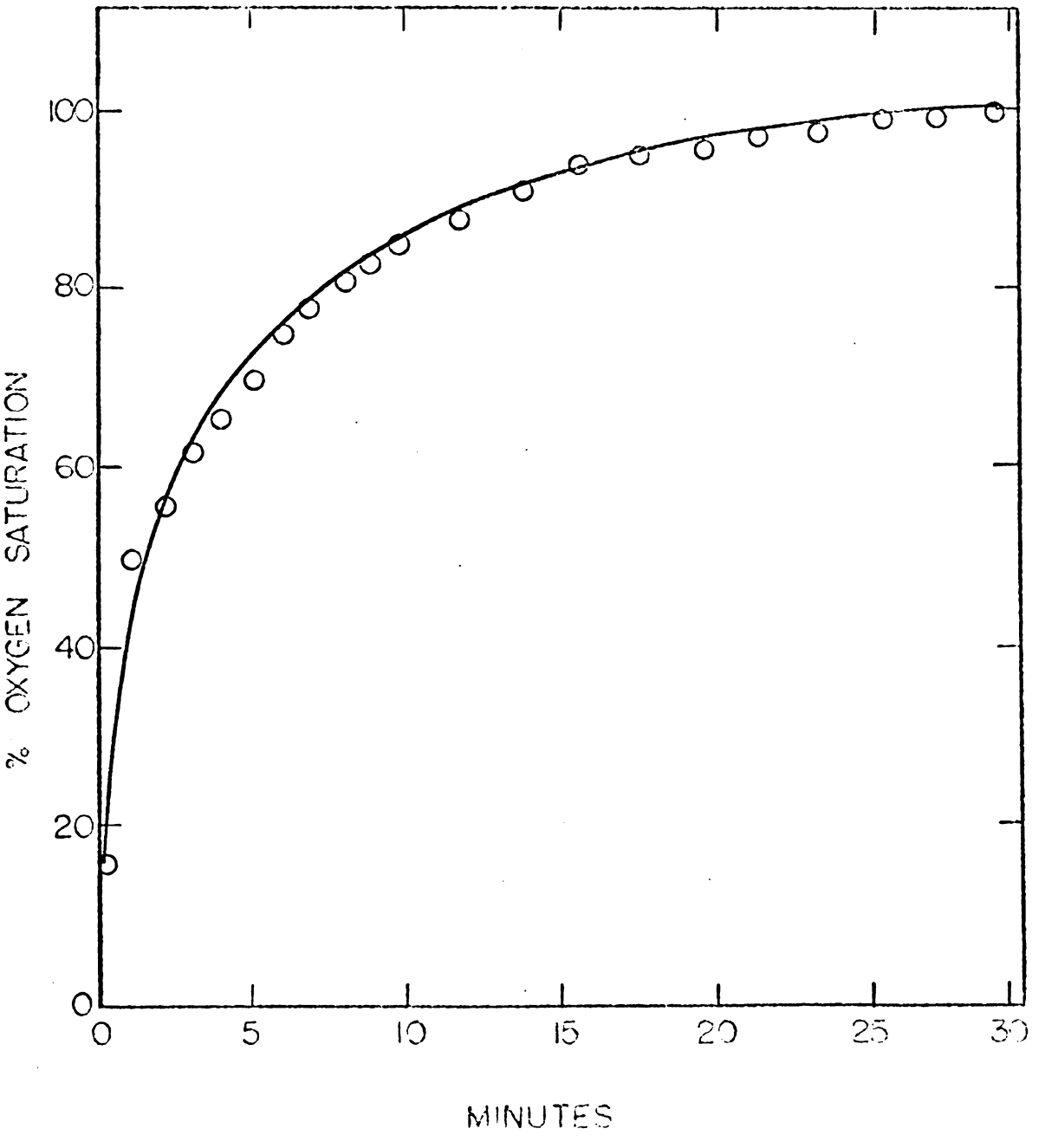

Figure 36. Oxygen uptake by 3 liters of medium in a 5 liter fermentor aerated by dialysis.

Table 15. An example of the computations necessary for the graphical determination of the overall $K_L a$ value, as illustrated in Figure 37 *.

TIME (Min)	DISSOLVED OXYGEN (% Saturation)	COMPUTATIONS	
		C_L (av)	$C_e^* - C_L$(av)
0	16	33	67
1	50		
2	56	61	39
4	66		
6	75	78	22
8	81		
10	85	86	14
12	88		
14	91	92	8
16	94		
18	95	95.5	4.5
20	96		
22	97	97.5	2.5
24	98		
26	99	99.5	.5
28	100		

* Dissolved oxygen values are from the dialysis aeration trial shown in Figure 36. C_e^* represents the equilibrium concentration end point for this gas-liquid system (100% saturation or 7.3 PPM) and C_L(av) represents the average dissolved oxygen concentration over the designated time intervals of the aeration trial.

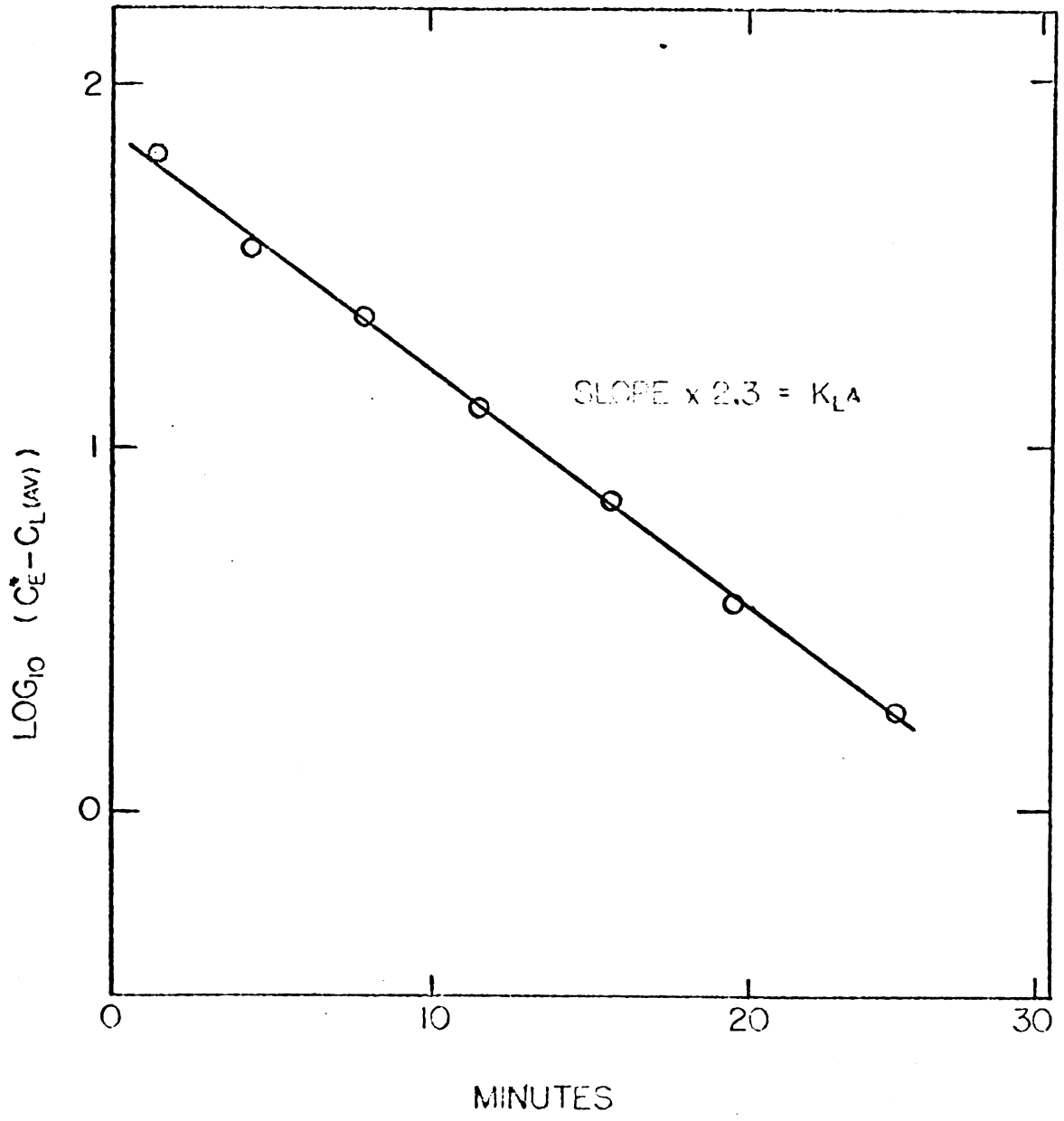

Figure 37. Graphical determination of the overall volumetric oxygen transfer coefficient ($K_L a$) for the dialysis aeration trial shown in Figure 36.

saturation end point of the liquid aeration operation ($C^* = C_L$ av = saturation = 7.6 ppm). These factors $C^* - C_L$ av were the integrated values ($\ln (C^* - C_L \text{ av}) = K_L a\ t + \text{constant}$) of the oxygen transfer equation ($\frac{dc}{dt} = K_L a\ (C^* - C_L \text{av})$) over the aeration period. They provided one of the coordinates $\ln (C^* - C_L \text{ av})$, t being the other, for the graphical solution of the overall $K_L a$ value for the experiment (Figure 37). The $K_L a$ value was obtained from the slope of the plot as shown or could be determined by solving the oxygen transfer equation for each time interval of the trial and averaging the values.

Demonstration of cell synthesis and viability in dialysis aerated cultures of Serratia marcescens represented the final and most important evaluation of this aeration technique. These growth trials were conducted with the same equipment and in the same manner as described above for oxygen transfer analysis and previously for growth analysis (preceding section of this thesis) with the exception that the sole source of oxygen was by dialysis aeration (conventional air sparging in comparative controls) and the fermentor was inoculated with viable cells. As described previously, the culture was sampled via syringe and sampling ports and was analyzed for total and viable cell concentrations and dry weight. In addition, oxygen uptake as well as culture oxygen demand were measured during growth by either the sampling method or the dynamic degassing-gassing method (156). The first method required removal of a culture sample which was placed in a stirred, oxygen-saturated beaker of broth. The utilization of dissolved oxygen by the respiring cells was detected by the dissolved oxygen electrode. The second method was more convenient and eliminated sampling errors and atmospheric oxygen contamination because it allowed oxygen uptake analysis at any time without

sampling. Instead, the culture aeration was stopped and the dissolved oxygen utilization of the culture was detected as it circulated past the electrode in the external conduit. Deoxygenation of the culture fluid was indicative of uptake and demand, and oxygenation of the fluid upon resumption of aeration was indicative of the oxygen transfer capacity of the aeration system under actual culture conditions. This technique also allowed determination of $K_L a$ values under these conditions. However one problem was observed which reduced the usefulness of this method. The electrode frequently lost accuracy toward the end of long growth periods. Presumably this was due to poisoning of the electrode by the salts in the growth medium.

6.4 Results

6.4.1 Physical Aeration Characteristics

The dialysis-aeration technique produced no observable positive gas pressure within the fermentor vessel, a characteristic which significantly reduced the possibility of air leaks at the fermentor gaskets and agitator shaft bearings. In contrast, a conventionally sparged fermentor developed an internal gas pressure of 2 lb/sq. inch to 5 lb/sq. inch depending on the gas velocity used. Thus dialysis-aeration lowered the risk of aerosol contamination, which represents an important consideration especially for the propagation of pathogenic microorganisms.

A second attribute of dialysis aeration was the elimination of the need for gas sterilization. The silicone rubber membranes completely separated the culture from direct contact with the gas phase and were impervious to bacteria. Therefore these membranes when properly sealed in the dialyzer, protected the culture from contamination by the incoming air and protected the outgoing air from contamination by the culture. This eliminated the necessity for gas sterilization at either site. In addition, dialysis aeration did not, as shown above, produce a positive pressure or gas flow within the fermentor, permitting the use of a simple and inexpensive vent on the culture vessel. Difficulty in obtaining a perfect membrane seal in the dialyzer was occasionally experienced during the investigations. This should not detract from the potential economy which dialysis-aeration affords because it represented a technological problem which has been reported for

artificial lung devices and reliably solved by the use of silicone gasket cements.

Another attribute of dialysis-aeration was the reduction in liquid loss due to evaporation and foam formation, which are associated with high volume sparging. Analysis of the liquid lost during conventional and dialysis-aeration did not however show as large a reduction in liquid loss as expected (Table 16).

Table 16

Influence of the aeration method on culture liquid loss.

CULTURE METHOD	VOLUME LOSS (ML)
Non-aerated batch control*	175
Sparged air -- Nondialysis nutrients	460
Sparged air -- Dialysis nutrients 1 membrane	750
Sparged air -- Dialysis nutrients 6 membranes	1300
Sparged air -- Dialysis nutrients 10 membranes	2300
Dialysis air -- Nondialysis nutrient+	350

*Culture vessel was not aerated or agitated

+Average of 5 trials

The large losses associated with the sparged dialysis-nutrient system were attributed to the osmotic transfer of water through the dialysis membranes to the nutrient solution. Because membrane oxygenation does not produce a high throughput of gas bubbles within the fermentor, it was believed that evaporation would be nearly eliminated. The data

suggested that a 350 ml volume loss represents a base line liquid loss over the course of the fermentation and that sparger aeration at the rate used, 9 L/min air velocity, did not cause as great a liquid loss as anticipated. Higher air velocities would be expected to produce greater evaporation in conventional aeration than in dialysis aeration. The elimination of gas bubbles should reduce foaming and permit the quantity of necessary antifoam to be reduced or eliminated altogether. This was observed to be the case when a culture was dialysis-aerated and not agitated. In contrast, sparging under the same conditions produced unmanageable foam within the fermentor. However as will be shown below in Figure 45 agitation was necessary in order to achieve the greatest cell density in a dialysis-aerated culture. Although the quantity required was comparatively lower, the initial addition of antifoam was required under such culture conditions.

The rate of oxygen transfer is directly proportional to the magnitude of the oxygen concentration gradient from the gas phase to the liquid phase. An increase in the partial pressure of oxygen in the gas phase will enlarge this gradient and therefore the driving force for mass transfer. The selection of gas used for dialysis-aeration was found to influence the oxygen transfer coefficients for the aeration system (Table 17). As shown, the gas with the greatest partial pressure, pure oxygen, produced the highest volumetric oxygen transfer coefficient. However its use was regarded as impractical for long term or large scale fermentations because of the expense involved. Humidified air showed a slightly lower oxygen transfer coefficient than dry air. This was attributed to the additional transfer resistance produced by the liquid film which formed on the gas side of the membrane by the

moist air. Humid air represented the best gas selection for the dialysis aeration method because it eliminated liquid evaporation via the membrane interface. Reduction of the gas-liquid vapor gradient, by use of moist air, has been shown to prevent the transfer of water vapor across silicone membranes (131).

The partial pressure of oxygen can also be increased by an increase in overall air pressure. Under the selected standard mode of operation, ($30^{o}C$, 2 L/min liquid velocity, and 25 L/min gas velocity) no pressure gradient existed across the dialyzer membrane (e.g. the difference in pressure between the liquid and the gas chambers of the dialyzer was zero). Insertion of an air filter in the outgoing gas conduit restricted gas flow and raised the internal pressure to .13 atm. The addition of membranes did not affect this value. With the filter attached the internal gas pressure increased when the gas velocity passing through the dialyzer was raised (Table 18). These increases in the pressure gradient were relatively small, would produce only small increments in the oxygen partial pressure, and would not appreciably improve the oxygen transfer driving force. Thus the manipulation of gas flow as a means of increasing the partial pressure of oxygen did not appear to be justified.

The pressure drop across the length of a dialyzer air chamber was very small (.021 atm @ the above standard operational conditions). Slight changes, corresponding in nature to those observed for total pressure in Table 18, were observed when the gas or liquid velocities were increased. These data demonstrated that the dialyzer as designed presented little resistance to air passage. The addition of membranes produced a small reduction in pressure drop across a given chamber which indicated that the increase in internal volume associated with additional

Table 17. Influence of the gas selected on the oxygen transfer coefficients for the dialysis aeration system.

GAS USED	$K_L a$ (Min^{-1}) *
Oxygen	4.2×10^{-2}
Air	3.4×10^{-2}
Humid Air	3.1×10^{-2}
Nitrogen	0

* With one membrane (.0288 m^2) in the dialyzer.

Table 18. Influence of the gas velocity through the dialyzer on the internal gas pressure.

GAS VELOCITY (L/Min)	GAS PRESSURE (Atm) *
7	0
15	.07
21	.10
25	.13
31	.17

* Positive pressure above the ambient atmospheric pressure.

chambers further reduced the resistance to gas flow. Variations in pressure drop were noted for different chambers within the dialyzer suggesting that the membranes and/or membrane separators flexed or bulged when gas and/or liquid velocities were changed. This was further substantiated by the observation that these variations in pressure drop were more pronounced with six membranes than with one. It is possible that the distortion of the internal dialyzer components may limit the membrane area and gas velocity which are operationally practical. The standard operational conditions adopted for these and subsequent dialysis aeration investigations did not appear to be excessive. Although no membrane failure occurred at the maximums of operation the risk of membrane rupture and gasket failure (gas leakage was occasionally observed at 31 L/min) should be considered carefully, especially if the system was to be operated under conditions approaching the maximal limits.

The separate and independently controlled membrane exchanger represented the most important characteristic of the dialysis-aeration system. This scheme permitted a combination of independent adjustments to be made on the operation of the gas and liquid phases. In addition the gas-liquid interfacial area was a measurable entity and could be changed by adding or removing membranes from the dialyzer. The influence of these operational adjustments on the oxygen transfer capability of the dialysis-aeration system were evaluated in terms of oxygen transfer rates (OTR, in millimoles of oxygen/liter/min) and/or volumetric oxygen transfer coefficients (K_La, in min^{-1}) in the following experiments.

The influence of air velocity on oxygen transfer was analyzed while the membrane area and liquid velocity were held constant. An increase in air velocity from 9 L/min to 25 L/min or 31 L/min produced only a

small increment in OTR values unless a large membrane area was used (Figure 38 and Table 19). As shown, an increase in membrane area improved the OTR at all air velocities with the greatest improvement occurring with six membranes and 31 L/min. The data values given in the table show that an increase in air velocity from 9 L/min to 25 L/min produced a 50% increase in OTR when 1, 2, 3, or 4 membranes were used, a 360% increase in OTR when 6 membranes were used, and a 5% increase in OTR for the sparger aerated control. However the OTR's for sparger aeration were over twenty times greater than that for dialysis-aeration operated with 6 membranes and at the maximum gas velocity. Corresponding improvement in dialysis-aeration K_La values have been observed (Bennett, personal communication). The improved oxygen transfer was primarily attributable to a reduction in gas-film resistance at the membrane surface and to the increase in gas pressure and corresponding oxygen partial pressure associated with greater air velocity. The more pronounced improvement in OTR when six membranes were used reflected the value of additional interfacial transfer area. The effectiveness of this area was enhanced by greater air velocity which increased dialyzer ventilation and reversed the reduction of internal pressure drop associated with added dialyzer chambers. It appeared that dialysis-aeration transferred oxygen more efficiently when operated at highest practical air velocity and the maximum possible membrane area.

The influence of liquid velocity on dialysis-aeration oxygen transfer rates was analyzed in a manner corresponding to that given above. In this case, gas velocity was held constant while the liquid velocity was varied. An increase in liquid velocity from 1 L/min to

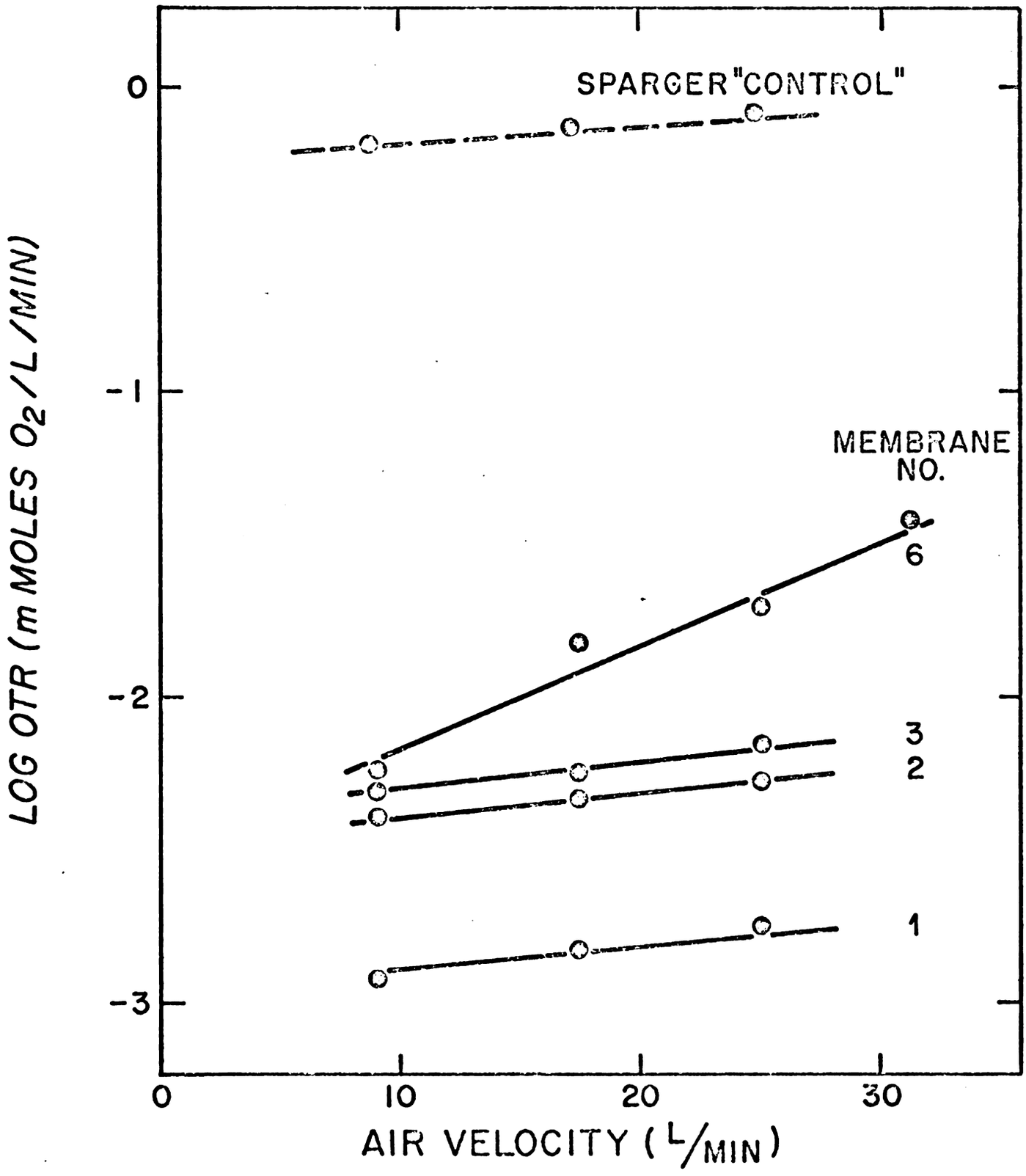

Figure 38. Influence of air velocity and membrane area on dialysis-aeration oxygen transfer rates.

Table 19. Influence of air velocity and membrane area on oxygen transfer rates for the dialysis aerator. *

AIR VELOCITY (L/Min)	OTR (m Moles/L/Min) Number of Membranes and Membrane Area (m^2) (1) .0288	(2) .0567	(3) .0864	(4) .1152	(6) .1728	SPARGED AIR CONTROL
9	.0012	.0044	.0053	.0054	.0057	.80
17	.0016	.0050	.0058	.0060	.0160	.75
25	.0018	.0055	.0073	.0099	.0205	.83
31				.0108	.0350	

* Operated at a constant liquid velocity of 2 L/Min through the dialyzer.

3 L/min produced similar 70% to 80% increases in OTR values for all the membrane areas used (Figure 39 and Table 20). An increase in membrane area resulted in a larger OTR value at each liquid velocity. Again, the OTR value for the sparged control was far greater than that recorded for dialysis-aeration at the maximal liquid flow rate. Liquid velocity increments have been shown to produce corresponding increases in dialysis-aeration $K_L a$ values (Bennett, personal communication). The improvements in oxygen transfer rates were attributed to a reduction in the liquid film thickness and resistance at the membrane surface and an improvement in bulk liquid mixing both of which are essential to efficient membrane transfer and occur with increased flow rates. These results correspond to those observed earlier for solute dialysis (Figure 9 of Section 4 of this thesis) and demonstrate that dialysis transfer of oxygen was maximized when the system was operated at the greatest practical liquid velocity and maximum possible membrane area.

The preceding results revealed that increases in gas and liquid velocities raised OTR values and that, generally, liquid velocity increments produced the largest improvements in oxygen transfer rates. This was further illustrated by increases from intermediate to maximal or near maximal operational flow rates (Table 21). Upward adjustments in liquid velocity consistently produced the greatest percent change in OTR values regardless of the membrane area selected. Liquid velocity was again confirmed as the most important factor when the transfer within a single dialysis unit was analyzed. Increments in gas and liquid velocities from the minimum to the maximum operational values, 9 L/min to 31 L/min and 1 L/min to 3 L/min respectively, produced respective 50% and 78% increases in OTR values. In addition it was

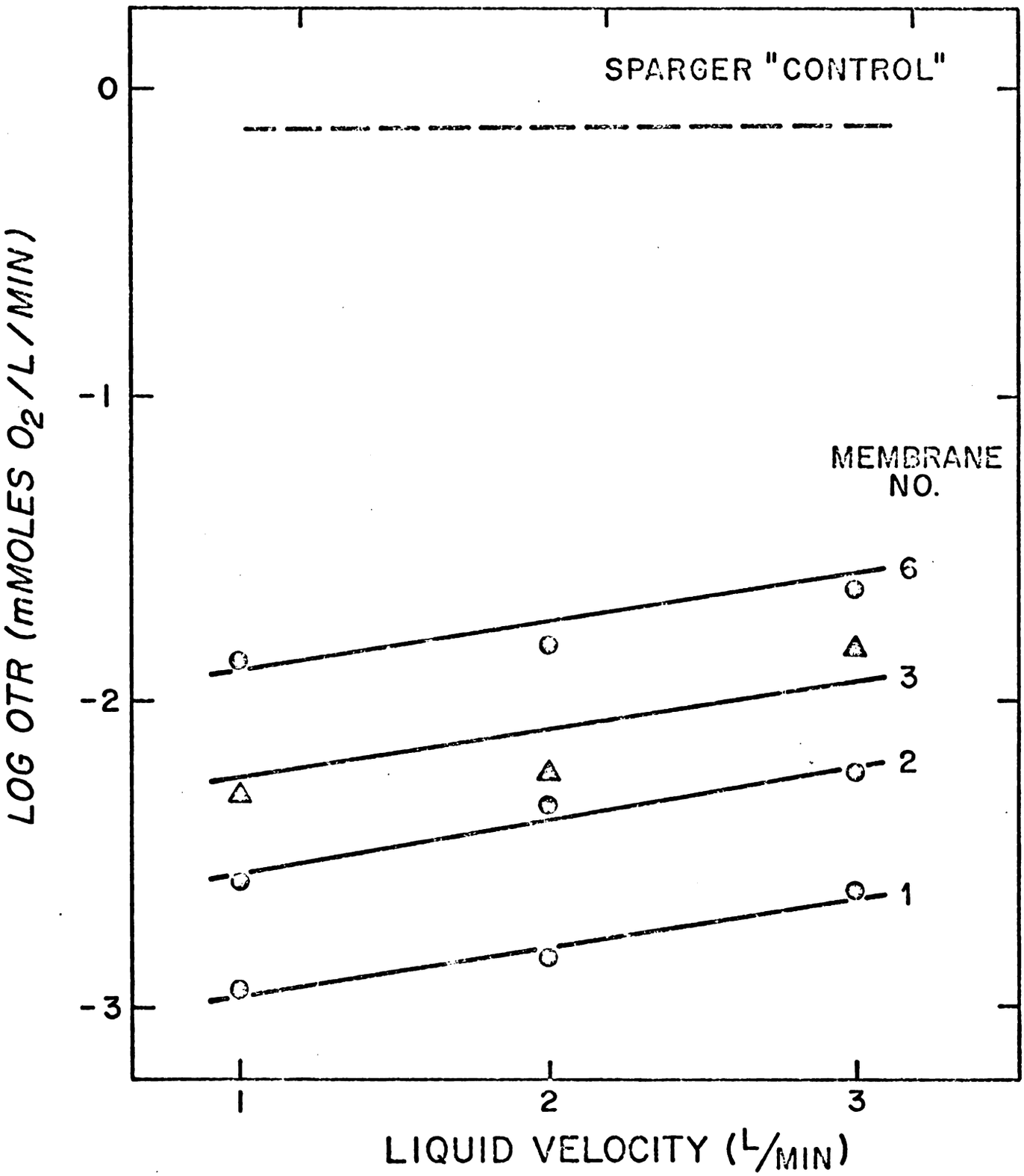

Figure 39. Influence of liquid velocity and membrane area on dialysis-aeration oxygen transfer rates.

Table 20. Influence of liquid velocity and membrane area on oxygen transfer rates for the dialysis aerator. *

LIQUID VELOCITY (L/Min)	OTR (m Moles/L/Min) Number of Membranes and Membrane Area (m^2)			
	(1) .0288	(2) .0567	(3) .0864	(6) .1728
1	.0014	.0029	.0055	.0145
2	.0016	.0050	.0058	.0160
3	.0025	.0057	.0160	.0240

* Operated at a constant air velocity of 17 L/Min through the dialyzer.

Table 21. Influence of membrane area, liquid flow rate, and air flow rate on dialysis-aeration oxygen-transfer rates. *

NUMBER OF MEMBRANES	AIR FLOW INFLUENCE ON OTR (17 L/Min)	(25 L/Min)	PERCENT CHANGE	LIQUID FLOW INFLUENCE ON OTR (2 L/Min)	(3 L/Min)	PERCENT CHANGE
1	.0016	.0018	12½	.0016	.0025	56½
3	.0058	.0073	26	.0058	.0160	176
6	.0160	.0205	28	.0160	.0240	50

* OTR, in m Moles/L/Min .

found that the OTR for the highest air velocity (.0350 m moles O_2/L/min) could be further improved (by 30%) to .0410 m moles O_2/L/min (the highest recorded value for the dialysis aeration system, using six silicone rubber membranes) by an increase in liquid velocity to 3 L/min. These results show that the dialysis-aeration system delivered the greatest oxygen transfer at the maximum operational conditions and that liquid velocity through the dialyzer was more important for improving oxygen transfer rates than air velocity.

The comparatively greater gain in oxygen transfer obtained by raising the liquid velocity, which accordingly reduced the liquid film thickness at the membrane surface, prompted an attempt to calculate the liquid film resistance and its response to fluid velocities. This was possible because, for the first time in a fermentor aeration system, the exact area and oxygen transfer properties of the gas-liquid interface were known and constant during operation. For this analysis a single silicone rubber membrane (.0288 m^2 area), a 3 liter liquid volume, a constant 17 L/min air velocity, and varying liquid velocities were used. The oxygen transfer rate (OTR), volumetric oxygen transfer coefficient (K_La), overall mass transfer coefficient (K_o), and overall, membrane, and combination gas and liquid film resistance $\frac{1}{K_o}$, $\frac{1}{k_m}$, $\frac{1}{k_L'}$ values (Table 22) were determined by the previously described methods, the equations and relationships described in Section 6.2.6, and the following relationships: $K_La = K_o \frac{A}{V}$, $\frac{1}{K_o} = \frac{1}{k_m} + \frac{1}{k_L} + \frac{1}{k_g}$, and $\frac{1}{K_o} - \frac{1}{k_m} = \frac{1}{k_L'}$; where $\frac{1}{k_L'} = \frac{1}{k_L} + \frac{1}{k_g}$ *.

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* (See footnote on page 190.)

Table 22. Calculated liquid film resistance to oxygen transfer at three liquid flow rates through the dialyzer-aerator. *

FLOW RATE (L/Min)	OXYGEN TRANSFER RATE (m Moles O_2/L/Min)	(% Change)		MASS TRANSFER K_La (Min^{-1})	K_o (Cm/Min)	TRANSFER RESISTANCE $1/K_o$	$1/k_m$	$1/k_L'$**	(% Change)	
1	.0014	+14.3	+78	.0059	.0614	302	285	17	-12	-47
2	.0016	+56.2		.0068	.0707	300	285	15	-40	
3	.0025			.0107	.1113	294	285	9		

* One 7 mil thick Silastic membrane with a calculated mass transfer coefficient (k_m) of .0035 cm/min was used.

** Represents a combined liquid and air film resistance value, air film resistance is assumed small and constant since air flow rates were held at 17 L/min in all cases.

As shown an increase in liquid velocity through the dialyzer from 1 L/min to 2 L/min produced a 14% increase in OTR and a corresponding 12% decrease in the calculated liquid film resistance to oxygen transfer. A further velocity increase from 2 L/min to 3 L/min resulted in an additional 56% increase and 40% decrease, respectively. The correlation between OTR improvement and liquid film resistance reduction supported the importance placed on liquid velocity in dialysis-aeration and substantiated the validity of the assumption made in equation 13 and reported by others (48). The liquid film clearly represents the largest and therefore most important factor in gas-liquid oxygen transfer.

Enlargement of the dialyzer membrane area represented a direct and measurable change in the gas-liquid interfacial area which is essential to oxygen transfer. When the dialysis aeration system was operated at an air velocity of 25 L/min and a liquid velocity of 2 L/min a single silicone rubber membrane (.0288 m^2 area) produced an OTR of .0018 m moles O_2/L/min and a K_La of 6.2 x 10^{-2} min^{-1} (Figure 40 and Table 23). An increase in area to six membranes (.1728 m^2) produced a 12 fold increase in OTR (to .0205 m moles O_2/L/min) and a 4 fold increase in K_La (to 25.6 x 10^{-2} min^{-1}). Previous experiments had shown similar increases in OTR

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* (From preceding page) The $\frac{1}{K_L'}$ term represented a combination gas and liquid film resistance value. The available instrumentation permitted precise dissolved oxygen analysis. However gas analysis equipment was not available preventing precise evaluation of the gas film transfer coefficient and resistance values. Because the liquid film mass transfer resistance has been reported to be far greater than the gas film resistance $1/K_L >> 1/k_g$ and because the gas velocity was great enough to minimize gas film resistance and was held constant it was felt that the data from this experiment would be primarily indicative of changes in the liquid film and its resistance to oxygen transfer.

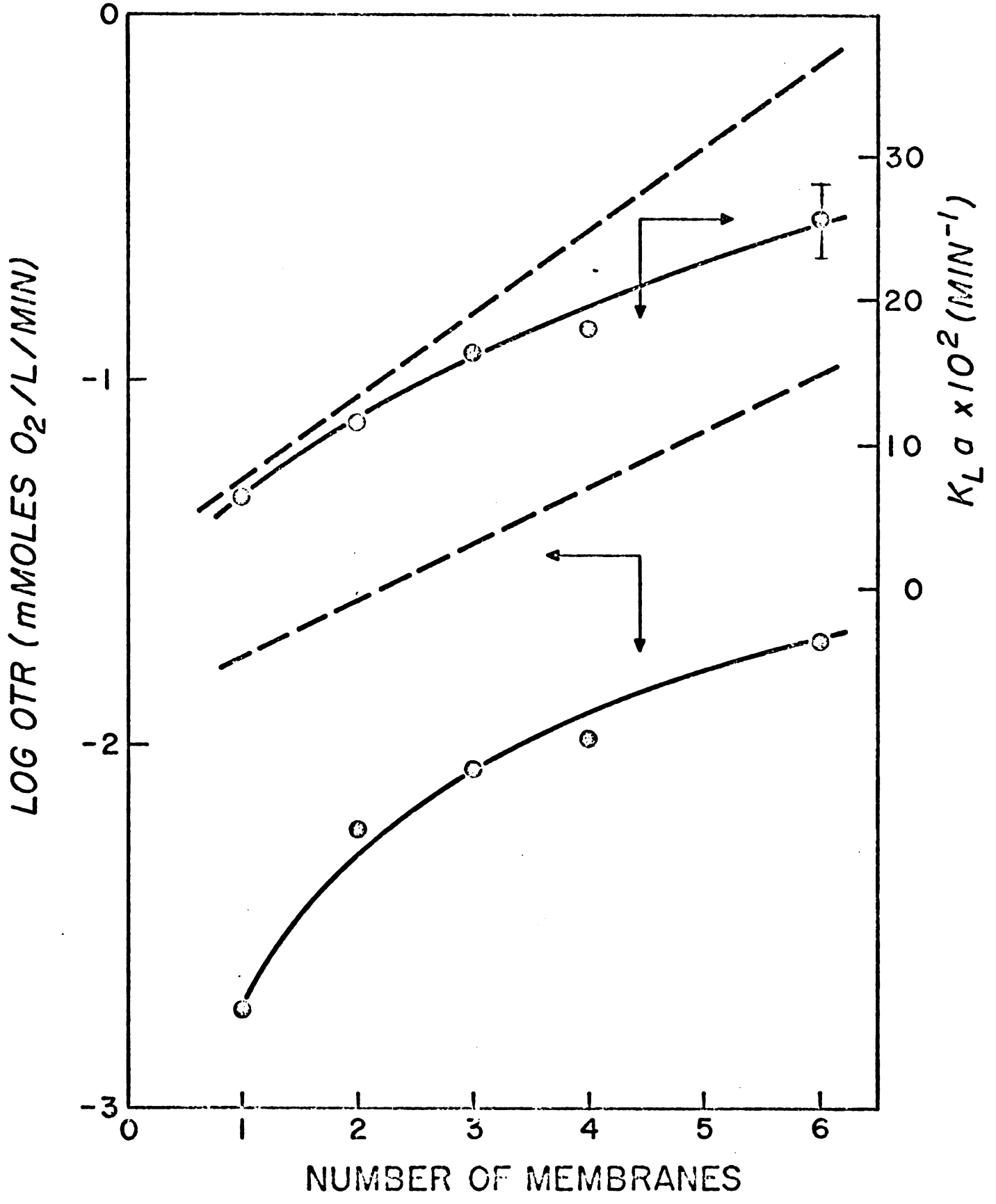

Figure 40. Influence of membrane area on experimental and calculated oxygen transfer rates and oxygen transfer coefficients for the dialysis-aeration system.

Table 23. Influence of membrane area on the calculated and experimental oxygen transfer rate and coefficient values for dialysis aeration.

NUMBER OF MEMBRANES (.0288 M^2/Membrane)	K_La (Min^{-1})		OTR (m Moles O_2/L/Min)	
	(Calculated)	(Experimental) *	(Calculated)	(Experimental) *
1	6.25×10^{-2}	6.2×10^{-2}	.0153	.0018
2	12.52×10^{-2}	11.4×10^{-2}	.0295	.0055
3	18.78×10^{-2}	15.9×10^{-2}	.0442	.0073
4	25.04×10^{-2}	18.0×10^{-2}	.0590	.0099
6	37.56×10^{-2}	25.6×10^{-2}	.0873	.0205

* Experimental values were determined at air and liquid velocities of 25 L/min and 2 L/min respectively.

when membrane area was increased (Figures 38 and 39 and Tables 19 and 20). These results clearly demonstrated the increase in oxygen transfer gained by increasing the interfacial transfer area as predicted by the oxygen transfer equations. The dialyzer has been successfully operated with up to 10 membranes installed and this system could incorporate two or more dialyzers in order to increase the total available membrane area. Unfortunately additional .007 inch thick reinforced silicone rubber membranes were unavailable from the manufacturer precluding investigations with greater than 6 membranes.

The defined area and known permeability of the aeration membranes permitted the analysis of dialysis-aeration gas transfer efficiency and the prediction of the membrane area required for microbial aeration (Figure 40 and Tables 23 and 24). A comparison of the calculated and experimental K_La and OTR values for the dialysis-aeration system operated at 25 L/min air velocity, 2 L/min liquid velocity, 3 liter liquid volume, and varying membrane areas (.0288 m^2/membrane) showed a close agreement between the K_La values when one or two membranes were used but an increasingly wider disagreement in K_La values as additional membrane area was added (Table 23 and Figure 40). In addition, the data showed a large difference between calculated and experimental OTR values, even with a single membrane. The calculated values were determined from published permeability specifications for the .007 inch thick silicone rubber membranes. The calculated K_La and OTR values of 6.25×10^{-2} min^{-1} and .0153 m moles O_2/L/min respectively for one membrane were obtained after considerations had been made for liquid volume, area, proportion of oxygen in air, and the assumption of a maximal concentration gradient driving force. Because the calculated and experimental K_La values were approximately the same,

it was assumed that the membrane specifications and the calculations were reasonably accurate. The large discrepancies between calculated and experimental OTR values were therefore attributed to errors in dissolved oxygen detection, timing, and data evaluation. As shown in the materials and methods section, values selected to represent the OTR for a given experiment were those which occurred during the most rapid increase in liquid oxygenation. Such a determination required interpretation of the portion of the dissolved oxygen trace (represented by Figure 36) with the fewest data points and was therefore subject to the greatest amount of error. In contrast the graphical determination of a K_La value utilized numerous data points (as shown in Table 15) accordingly improving accuracy, as confirmed in Table 23 for a single membrane. It may be concluded that the reported OTR values for all experiments were subject to this error and tend to be lower than the actual values. However the consistent interpretation of dissolved oxygen traces and the demonstrated duplication of resultant values offset the detracting effect of this error on the experimental trends observed or the conclusions drawn.

The greater accuracy associated with K_La values enhanced their usefulness as criteria for dialysis-aeration efficiency determinations and membrane area predictions. The addition of six membranes (.1728 m^2 area) was expected, assuming 100% efficiency, to result in a six fold increase in the volumetric oxygen transfer coefficient. The experimental data however revealed an increase of only slightly greater than 4 fold (Table 23) resulting in a lower efficiency, 75% (Table 24). Repeated experiments with six membranes established the range in K_La values which could be expected between duplicated trials, $\pm$ 2 x 10^{-2} min^{-1}, (Figure 40). The best K_La value recorded for six membranes under the operational conditions was

Table 24. Theoretical and experimental oxygen transfer coefficients for dialysis aeration, and estimation of the membrane area necessary to meet active culture requirements. *

NUMBER OF MEMBRANES USED	K_La (Min^{-1}) (Calculated)	K_La (Min^{-1}) (Experimental)	TRANSFER EFFICIENCY	K_La (Min^{-1}) (Viable Culture)	NUMBER OF MEMBRANES NEEDED (100%)	NUMBER OF MEMBRANES NEEDED Efficiency (75%)
1	6.25×10^{-2}	6.20×10^{-2}	98%	10×10^{-2} To 80×10^{-2}	13	18 - 20
6	37.56×10^{-2}	28.10×10^{-2}	75%			

* Based on the transfer of oxygen from an air stream through 7 mil Silastic membranes with a specified permeability of 235 mg $O_2/min/m^2/atm$.

28.1×10^{-2} min^{-1}, a dialysis-aeration efficiency of 75%. The reduction in efficiency associated with larger membrane areas was attributed to the previously reported changes in internal dialyzer pressures, variations in fluid and gas dynamics, reduction in bulk flow and turbulence, uneven distribution of gas and fluid through the dialysis chambers, and resultant increases in film resistances all of which contribute to the reduction in efficient mass transfer through the membranes. The results show that greater oxygen transfer occurs when larger membrane areas are used and that operational adjustments in liquid and/or air velocities would permit more effective utilization of this area and probably increase aeration efficiency.

The volumetric oxygen transfer coefficient for an active Serratia marcescens culture was determined to vary between 10×10^{-2} min^{-1} and 80×10^{-2} min^{-1} (Table 26). The membrane area necessary to satisfy the maximum oxygen transfer requirements of this organism by dialysis-aeration was predicted from the experimental K_La values and expected efficiency for this system (Table 24). As shown, highly (100%) efficient dialysis aeration would require approximately 13 membranes (.3744 m^2 area). On the basis of the lower (75%) efficiency observed for the system a larger membrane area would be needed. These membrane requirements would vary with the oxygen demand of the organism selected.

6.4.2 Culture Demand

Evaluation of the biological aspects of dialysis-aeration required the use of an aerobic test organism and knowledge of its oxygen demand. Serratia marcescens 8-UK was used previously in the dialysis culture

system, met this specification, was found to have a maximum oxygen demand of .66 m moles O_2/L/min (Table 25), and was reported to respond to increased aeration efficiency by increased cell synthesis (145). During the course of a batch culture the oxygen demand of this organism rose from a low initial value (.11 m moles O_2/L/min) to the above peak value, which occurred at the end of exponential growth and returned to a low value (.13 - .14 m moles O_2/L/min) during stationary growth (Figure 41 and Table 25). These oxygen demands were slightly greater than those previously reported for the same strain using a similar analytical technique (.075 to .377 m moles O_2/L/min) (100) and were in general agreement with the values reported for several other organism (48).

The volumetric oxygen transfer coefficients for the growing Serratia marcescens culture were also determined. The dynamic determination method (156) utilizing a dissolved oxygen probe permitted an estimation of the range of $K_L a$ values which prevailed during the propagation of a conventional control culture (Table 26). The range of these experimental values, from a high of 80.4 x 10^{-2} min^{-1} during exponential growth to a low of 10 x 10^{-2} min^{-1} during stationary growth, corresponded to those reported for other cultures (48), 156). As noted previously, the maximal $K_L a$ obtained with the six membrane dialysis-aeration system was 28.1 x 10^{-2} min^{-1} which is less than the peak value for this viable culture. The initial conclusion from these results suggest that the cell density and oxygen demand achieved with conventional aeration could not be equalled with dialysis-aeration unless a greater membrane area were used.

Table 25. Oxygen demand of a Serratia marcescens culture.

HOUR	DRY WEIGHT (mg/ml)	OXYGEN DEMAND	
		(m Moles/L/Min)	(m Moles/mg wt/Min)
2	.12	.11	.00091
6	2.17	.66	.00030
12	5.25	.33	.00006
24	7.08	.14	.00002
48	14.10	.13	.00001

Table 26. Volumetric oxygen transfer coefficients during propagation of Serratia marcescens in a conventional fermentor.

GROWTH PERIOD (Hours)	VIABLE CELLS (Billions/Ml)	K_La (Min^{-1})
2	.56	80.4×10^{-2}
5	2.50	36.0×10^{-2}
8	12.00	10.0×10^{-2}

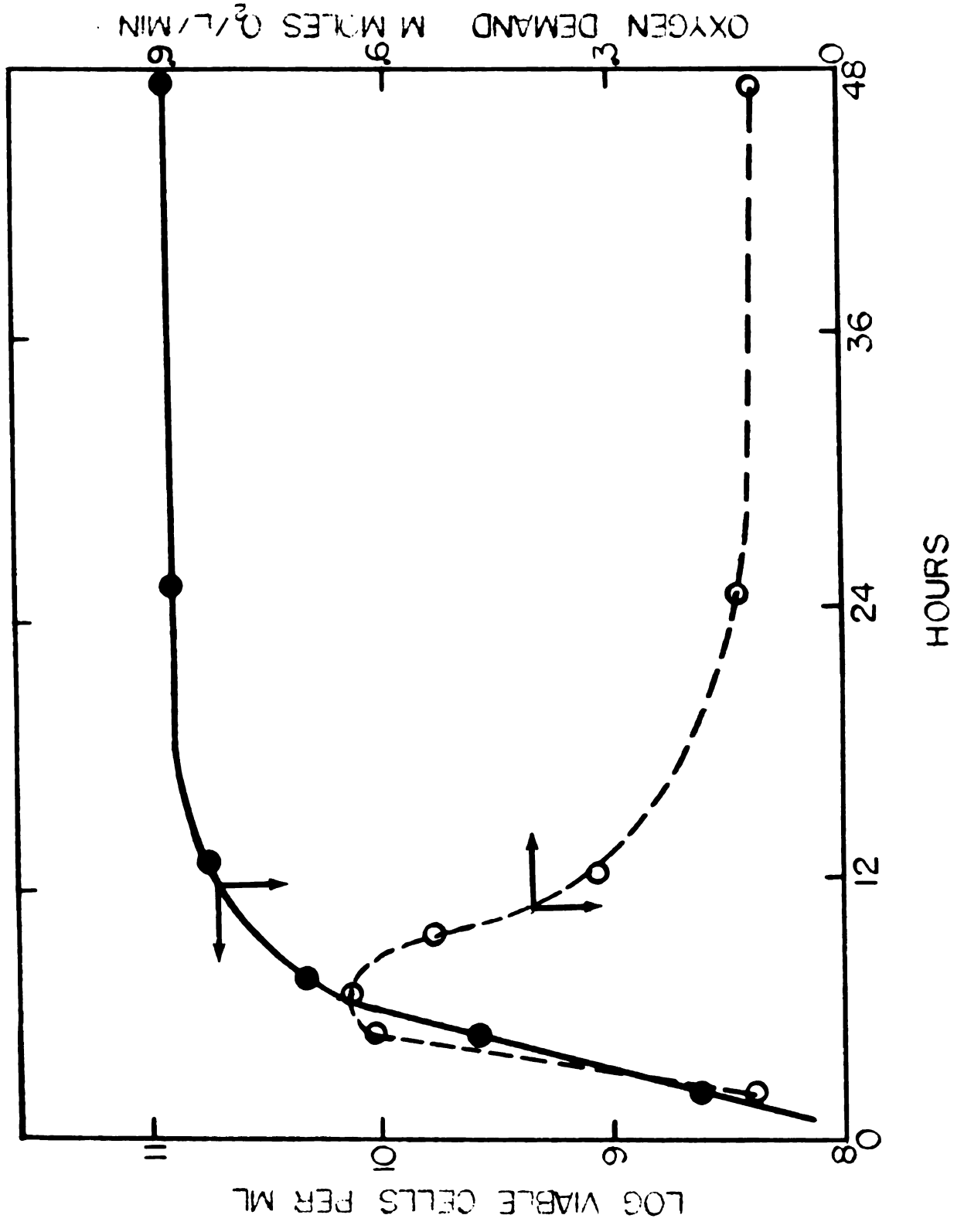

Figure 41. Correlation of the oxygen demand and growth in an air-sparged, nutrient-dialysis culture of Serratia marcescens .

6.4.3 Biological Aeration Characteristics

The previous data (Figure 41) showed that the maximal oxygen demand of a Serratia marcescens culture occurred during and toward the end of the exponential growth phase, i.e. the 6th to the 12th hour. The oxygen demand associated with concentrated cell growth had a diminishing effect on the dissolved oxygen concentration of the fermentation broth (Figure 42). This was reduced from approximately 100% saturation at the initiation of growth to a minimum of 40% saturation for sparger aeration and 10% saturation for dialysis aeration between the 6th and 12th hours of growth, the period corresponding to that for the maximal demand. As illustrated, the dissolved oxygen concentration rose after this period and remained relatively constant at 80% saturation and 12%-14% saturation respectively for the duration of the 48 hour growth trial. Dialysis-aeration resulted in a greater reduction in and poorer recovery of culture oxygen saturation levels during the course of the fermentation. This corresponded with the smaller oxygen transfer rates previously observed for this aeration process. However, dialysis-aeration provided culture oxygen levels superior to anaerobic conditions (no active aeration) and did maintain, at all times, a dissolved oxygen concentration significantly greater than the reported range of concentrations (.003 m moles O_2/L (.7% saturation) to .050 m moles O_2/L (5% saturation)) critical for microbial growth (48, 100) (Figure 42).

Dialysis-aeration (6 membranes, .1725 m^2 area) did not limit the growth or concentration of Serratia marcescens batch cultures (Figure 43). Duplicated growth trials showed that dialysis-aerated cultures attained similar but longer exponential growth phases and slightly greater mean

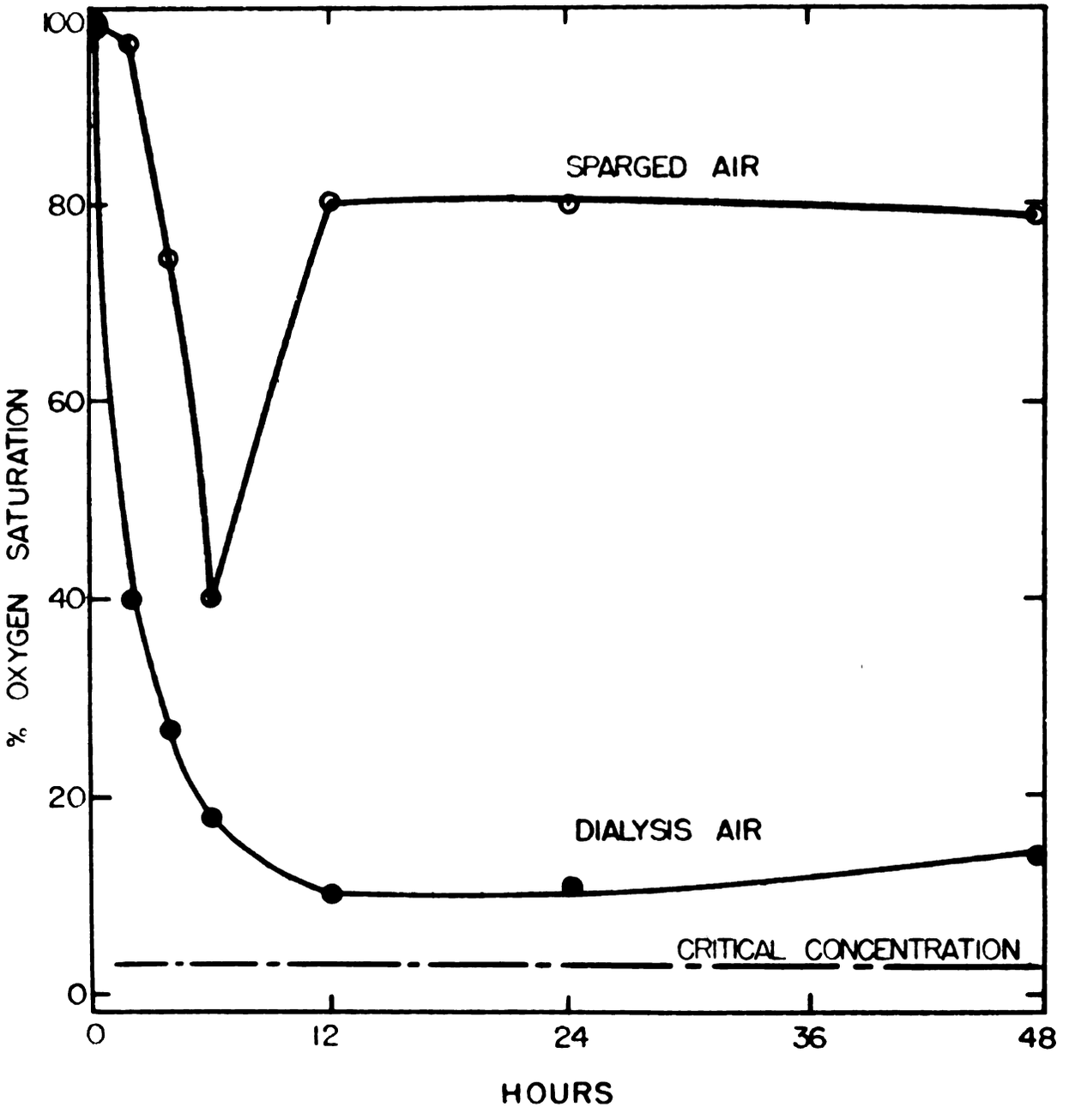

Figure 42. Dissolved oxygen concentrations in the culture medium during batch growth of Serratia marcescens under sparger and dialysis-aeration with a membrane area of .1728 m^2 (6 membranes).

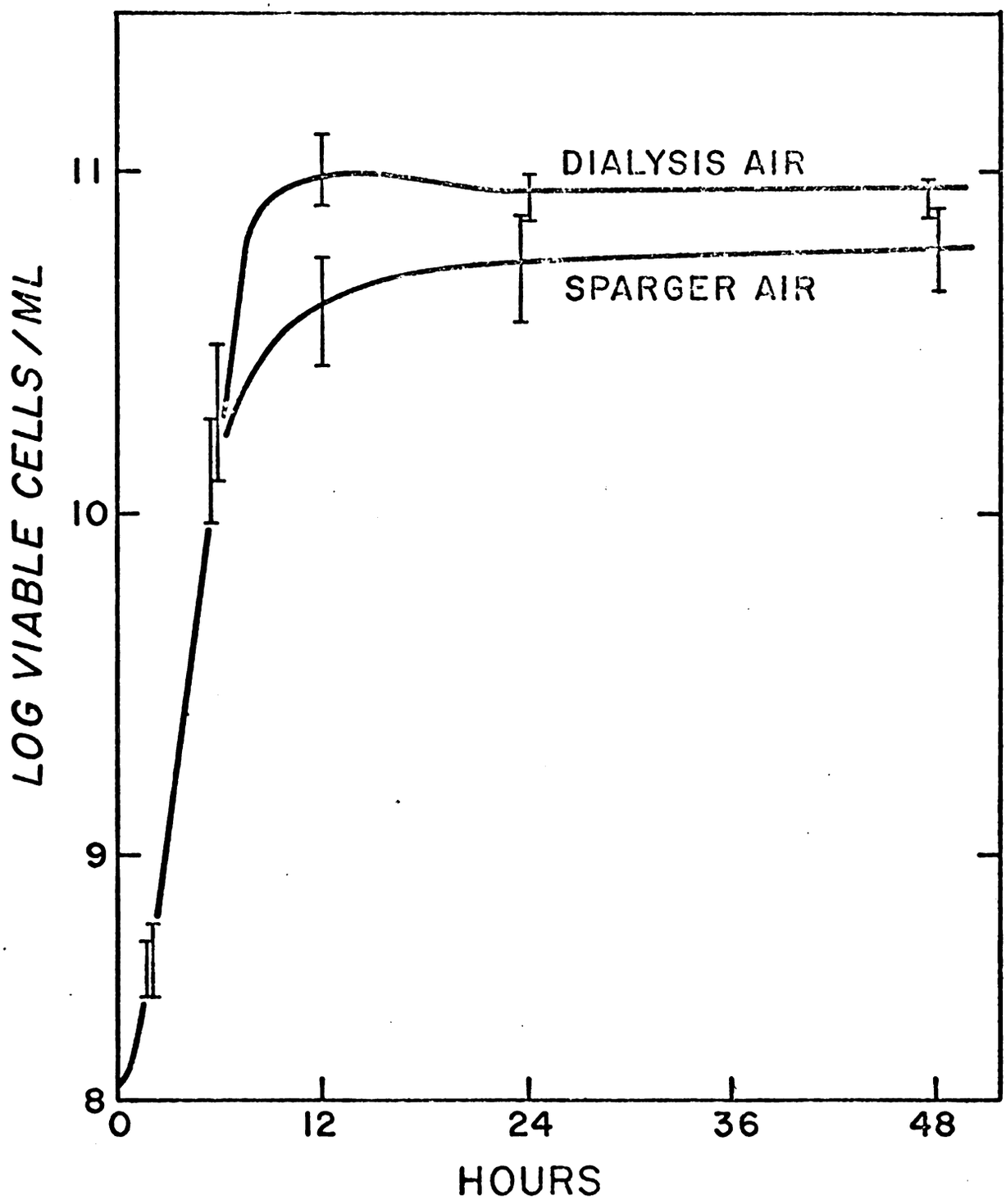

Figure 43. Growth of Serratia marcescens on Trypticasesoy broth under dialysis-aeration (6 silicone rubber membranes, .1728 m^2 area) and sparger aeration.

concentrations of viable cells than comparable conventionally aerated cultures. As illustrated, dialysis-aeration permitted a mean peak cell concentration of 107 billion viable cells/ml within a range of 86 to 120 billion cells/ml to be achieved at the 12th hour of repeated 48 hour growth trials in Trypticase soy broth. Sparger aerated cultures by comparison achieved a peak concentration of only 55 billion cells/ml within a range of 43 to 88 billion cells/ml. In both cases the viable cell concentrations declined to 86 and 46 billion cells/ml respectively during the stationary phase of growth. The results were repeatable and variations in growth data throughout the trials, the standard deviation was $\pm$ 20 - 24 billion cells/ml, were similar for both aeration methods.

The unexpected ability of dialysis-aeration, even with a suboptimal membrane area, to support high densities of Serratia marcescens cultures was further demonstrated in growth trials utilizing: 1) dialysis-aeration and dialysis-nutrients, 2) sparger-aeration and dialysis-nutrients, and 3) sparger-aeration and nondialysis-nutrients (Figure 44). The first represented a "total dialysis" culture system where the entire culture environment was provided by membrane exchange. The second system was similar to the conventional dialysis culture trials reported previously (Section 5) utilizing conventional sparger aeration. The third system represented a conventional nondialysis "control" fermentation which typified the batch culture process commonly used in the fermentation industry and served as a basis for comparison. As illustrated, the dialysis-aeration and dialysis-nutrient culture system supported a mean concentration of viable Serratia marcescens of 73 billion cells/ml after 48 hours of growth. Although this was only half as great as the cell density achieved by the sparger-aeration and dialysis-nutrient culture

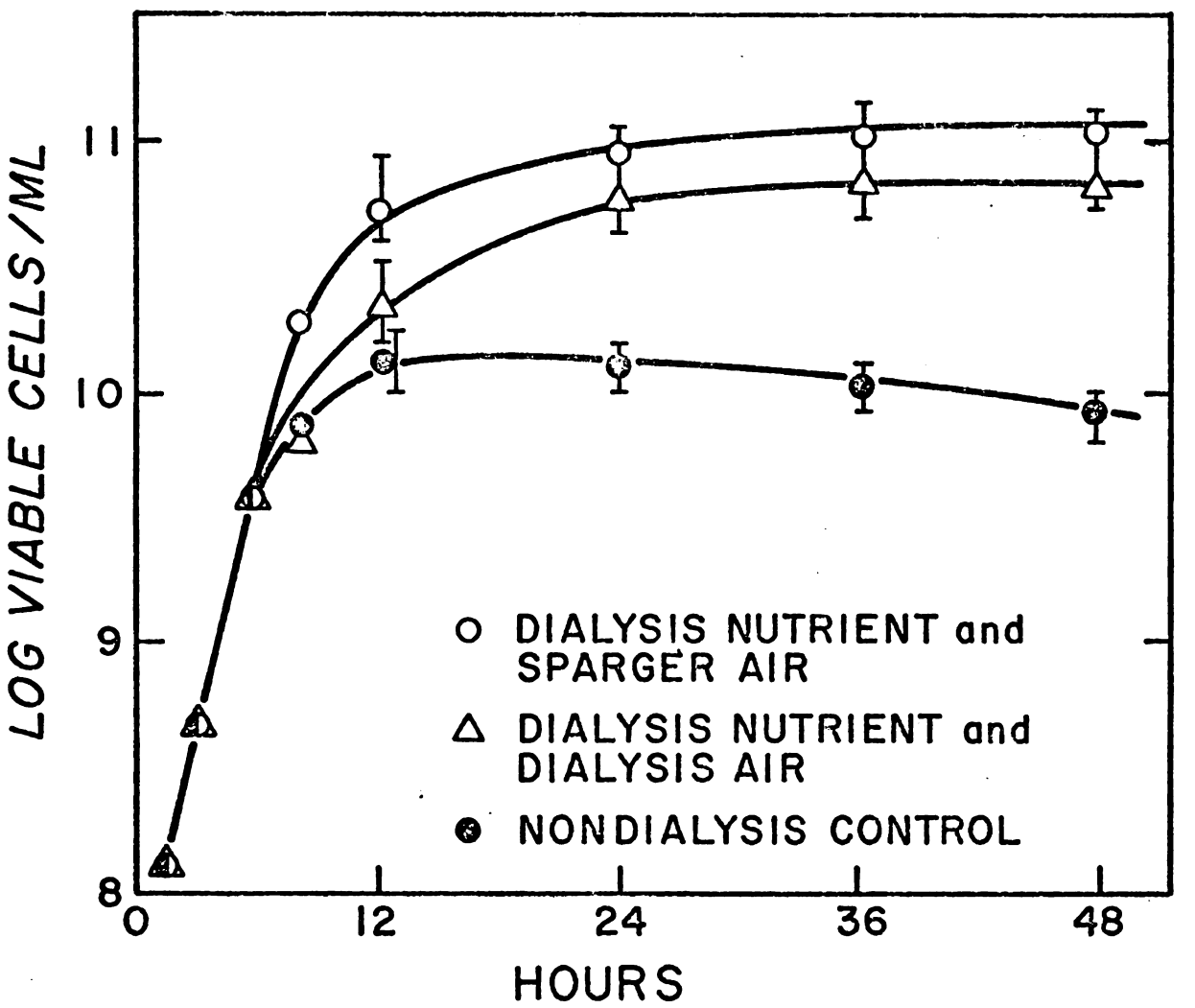

Figure 44. Growth of Serratia marcescens on water diffusate of Trypticase soy broth under; dialysis-nutrient and dialysis-aeration, dialysis-nutrient and sparger-aeration, and nondialysis-nutrient and sparger-aeration culture conditions.

system (mean of 147 billion cells/ml) after the same period of growth, the range of values observed between individual duplicated trials for each system often overlapped, indicating that the culture densities supported by the two were in closer correspondence than the mean values alone suggested. The "total" dialysis culture system produced a viable cell density over eight times greater than the comparable nondialysis "control" culture system (mean of 8.5 billion cells/ml after 48 hours), which again demonstrated the superiority of dialysis-aeration over conventional sparger-aeration. The greatly enlarged difference between the dialysis-aeration and sparger-aeration growth results shown here was due, in part, to the nutrient supply. In all of the growth trials shown in Figure 44 the nutrients were provided by dialyzing the 3 liter culture fermentor, initially containing water, for 12 hours against a 12 liter reservoir containing Trypticase soy broth. Thus at inoculation all of the systems contained a similar concentration of water diffusate medium. During growth the nondialysis sparger-aerated "control" system did not receive the additional nutrients provided in the other two nutrient-dialysis systems. This contributed therefore to lower cell concentrations which were shown for the "control" in Figure 44. The results represented by Figures 43 and 44 clearly demonstrated that dialysis-aeration met the oxygen demand of a concentrated viable Serratia marcescens culture and was equal or superior to sparger-aeration as a means of delivering sufficient oxygen to the growing cells.

Agitation of the fermentation broth was unnecessary for adequate oxygen transfer by dialysis-aeration. A greater viable cell density was achieved in a nonagitated dialysis-aerated fermentation than in a non-agitated sparger-aerated fermentation (Figure 45). Conventional

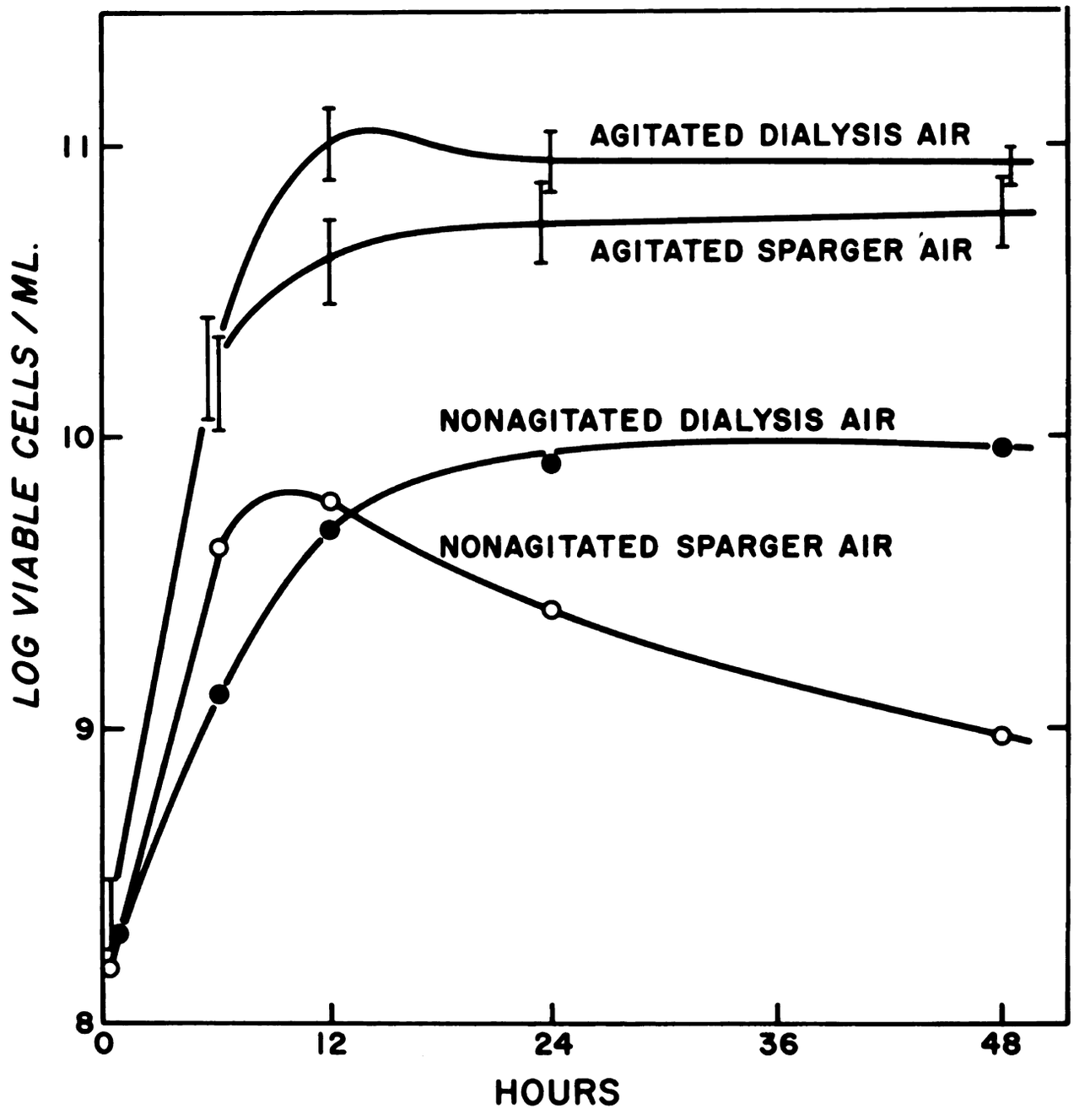

Figure 45. Correlation between growth of Serratia marcescens and the culture aeration method and agitation.

sparger-aeration produces bubbles which must be mixed and dispersed in order to facilitate oxygen transfer to the liquid. Agitation has been shown to directly affect cell growth and yield in conventional fermentations (163). Dialysis-aeration by nature of its permeation and diffusion of gaseous oxygen through the membrane interface into a relatively thin and turbulent liquid film does not require agitation for adequate oxygen transfer. Therefore elimination of agitation will not have as great an effect on oxygen availability to the cells of a dialysis-aerated culture as shown in the figure. However it was found that superior cell yields could be attained when the dialysis-aerated culture was agitated at 300 rpm (Figure 45). As shown, a sparger-aerated and agitated (365 rpm) culture also attained improved growth, as expected, but did not achieve as great a cell concentration as the comparable dialysis-aerated and agitated culture. In both instances, with and without agitation, dialysis-aeration again demonstrated superior cell yields. The results indicate that agitation was necessary in order to achieve the biological potential of the aeration system used. With sparger-aeration, agitation primarily facilitated oxygen transfer itself. In the case of dialysis-aeration, agitation represented the mixing necessary to reduce culture stagnation and to reduce the liquid film around the cells themselves. It has been shown that an agitation rate of approximately 250 rpm is necessary to reduce the liquid film resistance around the cells to a negligible amount (15). That this was probably the case with dialysis aeration was illustrated by the nonagitated growth curves in Figure 45, where sparger aeration demonstrated a greater exponential growth rate. This was attributed to the advantage gained by the mixing action as the sparged bubbles rose through the culture, an effect which was absent in the dialysis-aerated growth

trial. The necessity for agitation in order to achieve maximal biological growth with dialysis-aeration eliminated two potential advantages of this aeration method, the complete elimination of antifoam agents and elimination of agitation power requirements.

The results of the growth trials with Serratia marcescens cultures revealed that dialysis-aeration not only supplied sufficient oxygen to the culture for growth but was able to support concentrated cell densities equal to or greater than those achieved in comparable conventionally aerated trials. These results were repeatable and strongly support the feasibility of membrane aeration as a reasonable substitute for sparger-aeration, at least on the scale demonstrated in these experiments.

6.5 Discussion

This experimental work was intended to demonstrate the operational characteristics of the dialysis-aeration system and its practical potential for gassing microbial liquid cultures. The results were surprising. Dialysis-aeration not only was demonstrated to have potential but actually matched or exceeded conventional aeration in supporting high concentrations of an aerobic bacterium with high oxygen demand. These results were unexpected in view of the lower oxygen transfer rates and coefficients achieved by dialysis-aeration in comparison to conventional aeration, and the utilization of membrane areas below that predicted to be optimal. Apparently the manner by which dialysis-aeration introduced oxygen into the liquid medium (by molecular permeation and diffusion through a membrane, in contrast to diffusion from rising bubbles) was more efficient in providing an adequate supply of dissolved oxygen at the bacterial cell-liquid interface. In other words, satisfaction of the oxygen requirement of the organisms themselves could be accomplished by a means which was up to 40 times poorer in mass transfer of oxygen into liquid alone.

Analysis of the physical operation of the dialysis-aeration system revealed several features potentially useful in the fermentation process. These included the elimination of positive pressure within the fermentor, the elimination of gas sterilization, the elimination of gas bubbles within the culture liquid and subsequent reduction of liquid evaporation by high volume sparging, the reduction of necessary antifoam agents, and the reduction of high agitation rates and corresponding power requirements.

The growing culture was effectively separated and isolated from the fermentor air supply by the dialyzer membranes. The Dacron-reinforced silicone rubber membranes have been proven reliable and rupture free in artificial lung devices and performed correspondingly in these investigations. The seal between the air and culture permitted the elimination of both entering and exiting gas sterilization, thereby effecting an operational economy unique to the dialysis aeration system. This same phase separation within the dialyzer and the elimination of gas sparging reduced the pressure within the fermentor vessel to zero. The elimination of positive pressure within the fermentor represented a feature of this aeration system particularly desirable for the culture of hazardous organisms because it greatly reduced the danger of aerosol contamination of the laboratory area.

Investigation of the operational characteristics of the dialysis-aeration system revealed that the equipment capably handled gas velocities as high as 31 L/min, liquid velocities as high as 3 L/min, and up to six membranes in the dialyzer. With the exception of membrane area these represented maximums. Although no excessive pressures were detected at these values it was noted that pressure distribution was not equal in all dialyzer chambers and some flexing of internal components was observed. The risk of membrane rupture and gasket leakage represented potential drawbacks for the system, although neither proved to be a problem during these investigations. In order to insure the integrity of the system values of 2 L/min liquid velocity, 25 L/min air velocity and 300 rpm agitation were selected as operational standards. These represented a reasonable compromise between maximal oxygen transfer, economical operation with minimal internal dialyzer stress, and a practical

efficiency which permitted confidence in the long term operational reliability of the dialysis-aeration system.

The bubbless diffusion of oxygen through the dialyzer membranes eliminated, to a large extent, the conventional function of agitation in liquid aeration. Indeed, little or no improvement in OTR values was observed when a dialysis-aerated nutrient broth was agitated. Because of this it was hypothesized that culture agitation might be eliminated altogether, therby precluding the need for antifoam agents and fermentor power requirements. However, growth trials revealed that this was not possible. Elimination of culture agitation resulted in poorer growth yields for both sparger-aeration and dialysis-aerated cultures. The first was expected because agitation has been shown to be essential to the gas bubble dispersion and interfacial area maximization necessary for adequate sparger oxygenation (8, 23). Nonagitated dialysis-aerated fermentations achieved greater culture densities, demonstrating that agitation played a less important role in culture aeration by this method. Although this indicated that the above hypothesis was partially correct, the superior growth observed when dialysis-aerated cultures were agitated showed that agitation could not be totally eliminated. Evidently the fluid circulation to and from the fermentor and the fluid turbulence within the dialyzer (which were sufficient for optimal oxygen transfer to the liquid broth alone) were not sufficient to provide the bulk culture mixing necessary for optimal oxygen transfer to the cells themselves. It has been reported that culture agitation of 250 rpm (.3 watts/L) was necessary in order to keep cells suspended and minimize the formation of a stagnant liquid film, an oxygen transfer barrier, on the cell surfaces (15). Thus, at least minimal agitation

was required for maximal growth in dialysis-aerated cultures and as a result antifoam chemicals and fermentor power requirements could be reduced but not eliminated with this aeration system.

The rate of dialysis-aeration of nutrient broth was improved by increasing the air velocity, the liquid velocity, the membrane area, and the oxygen partial pressure. Increases in membrane area and liquid velocity produced the greatest improvements, results which correspond to those reported for artificial lungs (122, 56). Membrane area, since it represented the sole gas-liquid interface, was the critical variable in dialysis-aeration and the superior oxygen transfer rates observed with larger areas were expected. Unfortunately an insufficient supply of silicone rubber membranes of .007 inch thickness prevented a complete analysis of dialyzer membrane area on dialysis-aeration performance. An increase in area up to .1728 m^2 (6 membranes), the maximum dialyzer area used, produced a 12 fold increase in OTR to .0205 m moles O_2/L/min. This value, however, was only 1/40th as great as the OTR produced by sparger-aeration at the same air velocity. Additional membrane area would be expected to produce further increases in OTR values. However, the exact magnitude of improvement was difficult to assess because it was observed that dialyzer mass transfer efficiency declined with the addition of membranes. Thus the actual oxygen transfer coefficient with six membranes was only 75% of the value calculated for that area. This decline in efficiency complicated prediction of the area necessary to produce a coefficient equal to that of a growing culture. Rough estimates indicated that as few as 13 membranes (assuming 100% efficiency) or as many as 20 membranes (assuming a poorer efficiency) might be required to meet the demands of a growing culture. The operational flexibility of the dialysis-aeration system did however allow adjustments in gas

and liquid velocities which effectively improved oxygen transfer rates and efficiency. An increase in these to the operational maximums produced a 2 fold increase in OTR to .0410 m moles O_2/L/min, the best value achieved for the system.

The decline in oxygen transfer efficiency associated with the addition of membranes has been observed with artificial lungs (52) and was attributed to dynamic conditions within the dialyzer. The addition of membranes enlarged the internal volume of the dialyzer which resulted in an uneven distribution of gas and fluid through the dialysis chambers, a reduction in bulk fluid velocity and turbulence in any given chamber, an increase in the laminar film thickness at the membrane surfaces, a decrease in internal gas pressure, and a greater flexing of internal dialyzer components all of which contributed in some degree to the reduction of optimal conditions for mass transfer and therefore dialyzer efficiency. It appeared that the diminution in efficiency would limit the number of membranes which could be effectively used in a single dialyzer. However the addition of two or more dialyzers to the system represented an alternate method for increasing the overall dialysis-aeration membrane area.

Liquid velocity represented the second greatest influence on dialysis-aeration performance. Increases in liquid velocity resulted in substantially greater OTR improvement than corresponding increases in gas velocity, suggesting that the greatest resistance to oxygen transfer lay in the liquid phase. In both cases a velocity increase exhibited a larger improvement in performance when six membranes were used than when a single membrane was used. This effect was attributed to the change in internal dynamics which accompanied the addition of membranes.

As explained above, liquid and/or gas velocity increases helped to restore the optimal mass transfer conditions which prevailed when the dialyzer was equipped with one or two membranes and was most efficient. Faster flow through the dialyzer was believed to improve bulk movement, maintain a maximal gas-liquid concentration gradient, maximize turbulence and mixing at the membrane surfaces, and most importantly reduce film resistances by minimizing the thickness of the gas and liquid films at the membrane interfaces. Other investigations have shown that a direct relationship exists between flow rates and liquid turbulence and the thickness of laminar liquid films within the chambers of a dialyzer (63). These dynamic manifestations of velocity all influence the rate of oxygen transfer to some degree. The improvement in oxygen transfer gained by increasing gas and especially fluid velocities as observed with this dialysis-aeration system corresponds to the influence exhibited by velocity on solute transfer with the same dialyzer as reported in Section 4 and corresponds to the results reported elsewhere for other dialyzers of similar design (56, 102).

The results of the velocity experiments demonstrated that liquid velocity had the greatest influence on OTR values. This data suggested that the liquid film was more important to oxygen transfer than the gas film. The status of liquid film resistance as a major governing factor in oxygen transfer has been emphasized in the literature (22, 23, 48) and was assumed in the theoretical discussion presented earlier in this section. Thus, on the basis of experimental data, the use of equation

$$N \backsim (OTR) = K_L a \quad (C^* - C_L)$$

which was based on the importance of the liquid phase was valid for the

analysis of dialysis-aeration performance. Because the laminar films (gas and liquid) at the surface of the membranes represent mass transfer barriers which can be operationally reduced and are regarded as a critical factor in membrane permeability it was deemed desirable to calculate these film resistances and the effect of velocity on their magnitude. In addition the dialysis-aeration system, by nature of its defined interfacial membrane area and membrane resistance provided an opportunity to evaluate film resistance more accurately than is possible with conventional bubble aerators. Ideally both the gas and liquid phase oxygen concentrations would be analyzed so that the transfer of oxygen from the gas into the liquid would be precisely known. However gas analysis equipment was not available necessitating the use of only dissolved oxygen data, a constant gas velocity, and a modified liquid phase resistance factor $\frac{1}{k'_L}$ in order to estimate the liquid film resistance and the influence of liquid velocity on it. The results showed a good correlation between liquid velocity increases, OTR improvements, and liquid film resistance decreases. These calculations supported the principle that increased liquid velocity improved dialysis-aeration because it reduced the laminar liquid film on the membrane surface thereby reducing a resistance to oxygen transfer. The determinations were made with the same constant gas velocity, which maintained a constant gas film resistance in all cases. These considerations plus agreement of the results with other published data (22, 48) and the previous theoretical considerations lent validity to the calculations and to the conclusion derived from them. Although the resistance values obtained in these determinations could be criticized because the gas film contribution was not determined, the conclusions drawn from the principle they demonstrate should still be valid. The dialysis-aeration system may well

represent an excellent test bed for further studies of the aeration process. Additional investigations with gas analysis and dissolved oxygen analysis equipment would be a logical extension of the work begun here and might prove valuable in elucidating the magnitude and importance of the gas and liquid films in gas transfer through a membrane interface.

The separation of components and independent operation of the gas and liquid phases in the dialysis-aeration system permitted a greater degree of operational flexibility than commonly afforded with conventional fermentation equipment. Manipulations, especially with the gas phase, were possible without the development of positive pressures, foaming, or mixing problems. The low solubility of oxygen in aqueous liquids made any adjustments which would increase the partial pressure of oxygen in the gas phase desirable in that they would increase the concentration gradient driving force for oxygen transfer. The use of counter-current liquid and gas flow and pure oxygen represented possible advantageous manipulations. The first, which was employed, improved oxygen transfer on the basis of the superior longitudinal gas and liquid oxygen concentration gradients which were created in the dialysis chambers on each side of the membrane. The use of pure oxygen however was economically impractical in terms of the long duration of most fermentations. Humidified air was selected as the most desirable gas for use in this system because it is relatively inexpensive and it reduced the possibility of liquid loss from the fermentor by vapor transfer through the membranes.

Oxygen transfer could also be improved by increasing the overall pressure on the gas side of the dialyzer membranes. Generally the gas

pressure within the dialyzer was low. Restricting the effluent gas flow or increasing the overall gas velocity did increase this pressure to a small degree but the advantage gained in terms of improved oxygen partial pressure was not considered great enough to justify the risk of gasket or membrane failure or reduced gas throughput. Therefore the operational gas and liquid velocities selected for general use were chosen as a reasonable compromise between operational reliability and oxygen transfer efficiency. Upward adjustments in gas velocity were found to be valuable in restoring the pressure of the gas side of the dialyzer when six membranes were used to that when a single membrane was used and the dialyzer was most efficient. In this case faster air velocity replaced the pressure which had been disipated by the increased internal volume associated with added membranes.

The results of the physical aeration experiments demonstrated that the dialysis-aeration system does deliver oxygen to the liquid circulated through the dialyzer. The system had several characteristics advantageous to fermentations. As expected from theoretical predictions the membrane interface proved to be the principle factor in oxygen transfer with additional area providing an overall reduction in transfer resistance and surprisingly a proportionate reduction in dialyzer efficiency. Liquid velocity increases reduced the liquid film thickness at the dialyzer membrane surfaces and represented the most effective operational manipulation for improving dialysis-aeration rates. Although other operational adjustments which improved oxygen transfer were possible, the dialysis-aeration system did not quantitatively approach the oxygen transfer capacity of a conventional sparger-aeration system which produced OTR values at least 20 times greater.

Dialysis-aerated Serratia marcescens cultures produced viable cell densities at least equal to and in some cases as much as two times greater than those of comparable sparger-aerated cultures and eight times greater than previously reported results on this scale (62). The results of these dialysis-aerated growth trials were especially surprising in view of the preceding discussion and the fact that the operational conditions used (25 L/min gas velocity, 6 membranes, and 2 L/min liquid velocity) achieved an oxygen transfer rate (.0205 m moles O_2/L/min) which was approximately 40 times poorer than sparger-aeration. In addition the calculated $K_L a$ (28×10^{-2} min^{-1}) for this system was less than the maximum of the range recorded for a Serratia marcescens culture. Equally remarkable were the results for nutrient-dialysis, dialysis-aeration growth trials. These achieved culture densities as great as 73 billion cells per ml which was over half of the density achieved in a sparger-aerated, nutrient-dialysis system. The latter system has been noted for its potential for producing high concentrations of viable cells. The cell densities achieved in the nutrient-dialysis, dialysis-aeration system were again significantly greater than those achieved in a comparable sparger-aerated control culture. The growth data could be accepted with confidence because the results observed here for the control cultures corresponded closely with those recorded previously (Section 5) and the standard deviations for duplicated trials in all cases were similar to those obtained in previous growth experiments.

Examination of the dissolved oxygen data for the growth trials showed that the dialysis-aerated cultures experienced a perceptible drop in dissolved oxygen. Although this decline was greater than with sparger-aeration, the dissolved oxygen concentration did not fall below the

critical level. It appears, therefore, that a Serratia marcescens culture of reasonably high density can be supported under conditions of low dissolved oxygen concentration as long as the dissolved oxygen concentration is maintained above the critical level. This is supported by experiments which have shown that cellular oxygen uptake was independent of oxygen concentration provided it was in excess of the critical level (149). Further, the results suggest that the higher dissolved oxygen levels maintained by sparger-aeration have no beneficial influence on cell viability or growth in batch cultures. This suggests that highly sparged operations waste a large portion of the oxygen introduced into the fermentor. This contention is supported by the low efficiency, 1% - 2%, reported for conventional aeration systems (48). In addition, sparging increases evaporation losses, tends to strip CO_2 from the culture, and may produce oxygen concentrations which could be toxic to cell synthesis in some instances. These have been reported to be detrimental to cell metabolism or synthesis and are effectively avoided with dialysis-aeration (35, 67, 78, 124). It may be concluded that dialysis-aeration, although inferior in oxygen transfer capacity with the membrane area used, was able to deliver dissolved oxygen in such a manner that the critical oxygen level was exceeded at all times, thereby enabling production and maintenance of a concentration of viable cells equal to or slightly better than that produced in sparged fermentations.

The improvement in culture conditions provided by the removal of nutrient limitations and dilution of toxic accumulations by a conventional, sparged dialysis culture system resulted in improved growth and higher culture densities. Under these conditions the excess dissolved oxygen provided by sparger-aeration apparently contributed the additional oxygen

necessary for the greater viable cell density which resulted. Although the same system under dialysis-aeration achieved improved cell densities, these were less than shown above. Apparently the available membrane area was insufficient to provide the additional oxygen necessary to support such high culture populations. Nonetheless the results for dialysis-aeration, which did approach those for the conventional dialysis culture, were impressive when the small membrane area and inferior physical transfer rates are considered. It would be reasonable to expect that equal or superior growth results could be achieved in the dialysis-aerated, nutrient-dialysis system if a greater oxygen exchange interface were available by addition of more silicone rubber membranes to the dialysis aerator. Incorporation of both nutrient-dialysis and dialysis-aeration into a single "total dialysis" system represented a culture system which would be potentially capable of producing high cell yields for prolonged periods while allowing independent control of the system components and providing isolation and maintenance of the culture by membrane interfaces. The results shown here establish the "total dialysis" system as an operationally feasible and perhaps a practical method for propagation of microorganisms.

The transfer of oxygen by permeation of a gas through a highly receptive membrane interface represents the key to the success of the dialysis-aeration system and may provide an explanation to the seemingly contradictory physical transfer and biological growth results. The principle difference between dialysis-aeration and conventional sparger-aeration lies in the transfer of gaseous oxygen through a silicone rubber membrane interface instead of a gas-liquid bubble interface. Although the latter has a greater area and a smaller resistance for mass transfer, certain dynamic conditions attributed to bubbles suspended in liquid tend

to reduce the efficiency and effectiveness of this form of aeration and produce a widely varying oxygen environment for the biological system. The dialysis-aeration system eliminates introduction of gas bubbles in the fermentation and shifts the transfer of oxygen from the interface of the bubbles rising through the liquid to the dialyzer membrane interfaces which separate the thin turbulent films of gas and liquid. This method provides a continuous evenly distributed transfer of oxygen into the turbulent liquid film circulating from the fermentor by direct molecular diffusion from the membrane surface. This eliminates the sharp differences in oxygen concentration between bubbles, bubble trails, and the liquid and the rise, coalescence, and disappearance of bubbles all of which are characteristic of conventional sparger aeration. It is entirely possible that such a diffusional transfer better provides adequate dissolved oxygen throughout the entire culture volume than does sparging. It would follow that such a condition would improve and optimize the microscale oxygen environment at the cell surfaces and would result, as observed, in greater cell synthesis than expected from the macroscale oxygen transfer rates for the aeration system. In addition the elimination of sparged bubbles and reduction of foaming probably enhanced growth by reducing the metabolic shock or denaturing effects associated with exposure to large variations in dissolved oxygen concentration or violent agitation.

The liquid flow rates selected for the dialysis-aeration growth trials (2 L/min) insured that the entire culture volume was cycled through the dialyzer every 1 1/2 minutes, assuming adequate mixing within the fermentor. The culture was continually exposed to an oxygen-rich interface, the net effect of which was a more thorough distribution of dissolved oxygen and the elimination of stagnant poorly aerated areas

throughout the culture. Again this provided a more adequate transfer and retention of oxygen within the vicinity of the respiring cells than does sparging and may well explain why a dialysis-aeration system with a smaller interfacial area and poorer overall oxygen transfer capacity was able to support equal or superior culture densities.

The successful application of dialysis-aeration was dependent on the availability of a suitable membrane material. A large resistance to mass transfer or too large a porosity have made most membrane materials inadequate or inappropriate for the oxygen transfer necessary to support a viable biosystem (61, 122). The development of thin reinforced silicone rubber membranes has made dialysis-aeration feasible. The composition of this membrane's chemical skeleton is such that molecular void spaces are created by the easily rotated methyl groups within the polydimethyl siloxane polymers. Although no liquid passage or seiving occurs this characteristic aids oxygen permeability because it reduces the entropy associated with membrane diffusion. This factor is, in most membranes, two to three times greater than that of a gas-liquid interface (7). In addition to the favorable transport characteristics of silicone rubber membranes, the continuous counter current circulation of oxygen depleted liquid from the fermentor and fresh oxygen (air) through the dialyzer permitted maintenance of a maximal oxygen concentration gradient at all times. This allowed the culture to be exposed to a relatively constant and maximal oxygen partial pressure throughout the fermentation, a characteristic which contributed to the effective support of microbial growth.

Separation of the culture and gas phases also facilitated more precise control of these. The composition or partial pressure of the gas to which the culture was exposed was easily adjusted by manipulation of the gas flow through the dialyzer. Changes in the gaseous environment

to suit specific culture needs can be implemented quickly in this manner. Thus aerobic, anaerobic or intermediate conditions could be produced, maintained, or adjusted in order to satisfy culture requirements for either growth or metabolic synthesis. This operational flexibility enhances the applicability of dialysis-aeration to the wide variety of known fermentation processes.

Dialysis-aeration appears to represent a feasible alternative to conventional aeration methods. The growth trial results indicate that this system deserves serious consideration both on the basis of its ability to support concentrated growth and on the operational flexibility which permits more precise control of the culture environment. Although the dialysis transfer of oxygen was relatively low, operational adjustments and the addition of membranes sufficiently increased its magnitude so that surprisingly good growth yields were obtained. The explanation for the excellent culture yields evidently lay in the elimination of gas bubbles from the fermentation medium and in the highly efficient process of oxygen diffusion and transfer through the silicone rubber membrane interfaces in the dialyzer. The successful demonstration of the operation and the applicability of dialysis culture, dialysis-aeration, or the combination of the two, "total dialysis", to known and experimental fermentation processes warrants continued investigation of the features and performance of these membrane mediated systems.

6.6 Summary

A dialysis-aeration system was designed which incorporated the previously described dialyzer, assembled with gas-transfer membrane sheets, into a modified dialysis culture system. Liquid nutrient medium or a Serratia marcescens culture was circulated from a conventional 5 liter fermentor through the dialyzer past one face of the membrane(s). Humidified gas (air) circulated past the opposite face. Unlike conventional air sparging, this aeration system enabled the use of nonsterile air, eliminated bubble-liquid interfacial effects, and reduced power and antifoam requirements. The greatest resistance to oxygen transfer, the membrane interface, could be reduced by increasing the area. A single membrane in the dialyzer (.0288 m^2) produced a volumetric oxygen transfer coefficient (K_La) of 6.2 x 10^{-2} min^{-1} and an oxygen transfer rate (OTR) of .0018 m moles O_2/L/min. Six membranes (.1728 m^2) increased these values to 28 x 10^{-2} min^{-1} and .0205 m moles O_2/L/min, respectively, but lowered the overall transfer efficiency of the dialyzer. Increases in liquid velocity, gas velocity, oxygen partial pressure, and overall gas pressure improved dialysis transfer of oxygen, with the first showing the greatest effect. Even though the OTR with six membranes was 1/20th of that for a well sparged fermentor, dialysis-aerated cultures attained equal or greater population densities. In addition, comparable results were obtained when dialysis aeration was used in conjunction with nutrient-dialysis. Apparently the elimination of gas bubbles and the mechanism of membrane-mediated molecular oxygen transport effectively

satisfied the biological demands of a culture even though the physical transfer rates for the dialysis-aeration system indicated that it would be insufficient.

7. GENERAL SUMMARY

Contributions were made to the design and construction of a new and versatile dialyzer for use in dialysis culture of microorganisms. It is assembled as a press with two rectangular stainless steel end plates, one of which contains entry and exit fittings. The plates compress an alternating series of sheet membranes, thin stainless steel frames, and molded silicone rubber separators. Each separator forms a chamber adjacent to the membrane and in one piece provides gasketing, entry holes and chamber ports. A field of pyramidal elements, on each face of the separator, point-support the membrane and induce turbulent fluid flow within the chamber. The dialyzer measures 43 x 12.7 cm, has an effective area of 288 cm^2 per membrane, and retains a volume of 65 ml per chamber.

The dialyzer was incorporated in a dialyzer-dialysis culture system which utilized a reservoir and a culture fermentor with 10 to 30 liters and 3 to 10 liters, respectively. Dialysis efficiency for the system was evaluated by determining the half-equilibrium time (ET_{50}) and the overall permeability coefficient (P_m) for transfer of glucose solutions. Continuous membrane use, autoclaving, dialyzer position, and fluid flow direction had no effect on transfer performance. However, ambient temperature, bulk fluid flow rates, and glucose concentration significantly affected transfer. A mean ET_{50} of 144 $\pm$ 15 min and a P_m of 4.7 x 10^{-3} cm/min occurred with a 1% glucose solution and a single membrane. Optimum solute transfer rates were obtained with an area of 2,050 cm^2 (7 membranes) and bulk flow rates above .5 liters per minute. Increasing the

volume of the total system or the ratio of fermentor:reservoir volume slowed these rates. In all cases, solute transfer from reservoir to fermentor approached an equilibrium concentration for the system.

Dialysis efficiency was also evaluated by culturing Serratia marcescens, in comparison with nondialysis "control" growth trials. The bulk flow rate of the culture had no effect on the viability or cell yield. Increasing the initial nutrient concentration increased the growth rates but not the final cell yield, although the effect was more pronounced with a defined medium than with a natural broth. Continuously providing additional nutrients by dialysis maintained greater cell concentrations in the fermentor than those observed in nondialysis fermentors, where nutrients were depleted during growth. Cell densities as high as 2.85×10^{11} cells/ml were maintained by dialysis for as long as six days. Increasing the fermentor:reservoir volume ratio had little effect on final cell yield. Increasing the dialyzer membrane area only slightly affected growth rates, but significantly increased final cell yields from 12.5 mg/ml dry wt. with 1 membrane to 40 mg/ml dry wt. with 15 membranes. The dialysis system with a fermentor:reservoir ratio of 1:10 and with 15 membranes produced a maximum of 3.0×10^{11} cells/ml, with a 5% difference between viable and total cell counts. In contrast, $.08 \times 10^{11}$ cells/ml and a 58% difference were obtained with a comparable nondialysis control.

A dialyzer was equipped with silicone rubber membranes which separated culture flow (2 L/min) on one side from humidified air flow (25 L/min) on the opposite side. This method for providing air by dialysis was incorporated both into a dialysis culture system and a nondialysis culture system. Volumetric oxygen transfer coefficients ($K_L a$, min^{-1}) and oxygen

transfer rates (OTR, m-moles O_2/L/min) were near predicted values; they increased with gas and liquid velocity through the dialyzer, with p O_2, and with culture mixing; and they increased from 6.2 x 10^{-2} and .0018, respectively, with one membrane (.0288 m^2) to 28 x 10^{-2} and .025 with six membranes (.1728 m^2) in the dialyzer. Although the OTR with six membranes was 1/20 of that in a well sparged fermentor, dialysis aerated cultures consistently attained populations of Serratia marcescens at least as high (.5 - 1.5 x 10^{11} cells per ml) as those in the control situation. Comparable results were obtained when dialysis aeration was used in conjunction with nutrient dialysis. Thus, despite a lower measured rate of oxygen transfer, dialysis aeration appeared at least as efficient in meeting the oxygen demand of an aerobic culture (.003 m-moles O_2/mg cell wt/min) as conventional sparging. Furthermore, dialysis aeration enabled use of nonsterile air, eliminated air-liquid interfacial effects, and reduced antifoam and power requirements, culture evaporation, and aerosol danger as compared to conventional air sparging.

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MATERIAL REFERENCES

9. MATERIAL REFERENCES

1 M	American Instrument Company, Inc. Silver Spring, Maryland	Electronic Relay
2 M	American Instrument Company, Inc. Silver Spring, Maryland	Lolog-Flexible-type Immersion Heater, 1000 Watt
3 M	American Instrument Company, Inc. Silver Spring, Maryland	"Quickset" Bimetal Thermo-regulator
4 M	Bamel Corporation 8051 W. Chicago Blvd. Detroit, Michigan	Stainless Steel Plates and Bolts
5 M	Bausch and Lomb, Inc. 60967 Bausch Street Rochester, New York 14602	Precision Refractometer
6 M	Bausch and Lomb, Inc. 60967 Bausch Street Rochester, New York 14602	Spectronic 20 Colorimeter
7 M	Beckman Instruments, Inc. 24755 Five Mile Road Detroit, Michigan	Model 76 Expandomatic pH Meter and Frit Junction Combination Electrode 39030
8 M	Beckman Instruments, Inc. Scientific and Process Instr. Div. 24755 Five Mile Road Detroit, Michigan	Oxygen Analyzer Model 77
9 M	BioQuest, Inc. 1640 Gorsuch Avenue Baltimore, Maryland	Trypticase-Soy Broth (Agar 1.5%)
10 M	Bodine Electric Company 2500 West Bradley Place Chicago, Illinois	Bodine Type N Shunt wound D.C. Motor 1/8 H.P.
11 M	Central Scientific Company 1700 Irving Park Road Chicago, Illinois 60613	Cenco Drying Oven #95470
12 M	Chemical Rubber Company 2310 Superior Avenue Cleveland, Ohio	Air Line CRC "Pollutex" Filters

13 M	Corning Glass Works Corning, New York	#7740 Pyrex Glass Back Plates
14 M	Crawford Fitting Company 884 East 140th Street Cleveland, Ohio	Swagelok Fittings
15 M	Detroit Silicone Rubber Co. 10439 Northlawn Detroit, Michigan 48204	Silicone Rubber Separators and Gaskets
16 M	Dow Chemical Company 1000 Main Street Midland, Michigan	Polyglycol P-2000
17 M	Edon Industrial Products 4412 Fernlee Royal Oak, Michigan	Edon Power Unit Model 624-3
18 M	Gelman Instrument Company P. O. Box 1448 Ann Arbor, Michigan	Flowmeters
19 M	Marshalltown Manufacturing, Inc. Marshalltown, Iowa	Pressure Gauges
20 M	Medical Products Division Down Corning Corporation Midland, Michigan	Silastic Oxygenator Membrane
21 M	Michigan Industrial Sales 2540 Park Avenue Detroit, Michigan	Elliott Flexible Drive Shaft SB-271
22 M	Mixing Equipment Company, Inc. 135 Mt. Read Boulevard Rochester, New York	Lightnin Model L Mixer
23 M	New Brunswick Scientific Company 1130 Somerset Street New Brunswick, New Jersey	Gyrotory Tier Shaker Model G52
24 M	New Brunswick Scientific Company Somerset Street P. O. Box 606 New Brunswick, New Jersey	Fermentor Models F-05 and F-14
25 M		Rubber Tubing Used - 1/4" I. Diam. x 3/16" Wall Thickness
26 M	Sargent Company 4647 West Foster Avenue Chicago, Illinois	Recorder Model S-R

27 M	Tuthill Pump Company 1716 W. Hubbard Street Chicago, Illinois	Maisch Biological Metering Pump, Model HQDCC, 11029NM
28 M	Union Carbide Corporation Food Prod. Div. 6733 W. 65th Street Chicago, Illinois	Dialysis Tubing ("Visking") Regenerated Cellulose
29 M	U.S. Army Chemical Corps Fort Detrick, Maryland	Serratia marcescens strain 8-UK

APPENDIX

10. APPENDIX

10.1 Dialyzer Cost Analysis

An attempt was made to construct the dialyzer from commercially available materials, such as Swagelok fittings and standard size bolts and nuts, to reduce costs. However the final design included some custom built parts, such as the molded silicone separators which required design and construction of custom master dies ($3,000) and the professional molding and trimming. The values given below include the labor costs for machining, drilling, trimming, etc., for each piece but do not reflect the time and effort spent in development or purchase of manufacturing machinery (i.e. mold dies or trimmers). Twelve dialyzers have been manufactured to specifications, with the Detroit Silicone Rubber Company responsible for separator and gasket production and the University of Michigan machine shop responsible for the preparation of the metal parts.

A. Exterior Dialyzer Shell

Stainless steel front plate	$ 88.00	
Stainless steel back plate	88.00	
Alternate pyrex glass back plate		($ 30.00)
12 bolts and nuts, 4 swageloks, 5 clamping bars	20.00	
TOTAL	$196.00	($138.00)

B. Dialysis Membrane Unit

2 stainless steel separator frames	$ 12.00
2 1/32 inch silicone rubber gaskets	4.00
2 silicone rubber separators	24.00
1 dialysis membrane	0.00
TOTAL PER UNIT	$ 40.00

For each membrane used in the dialyzer the above components are necessary. Thus, as additional membranes are added a similar number of units will be required and the cost will rise proportionally.