HEMODIALYSIS CULTURE OF BACTERIA

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ABSTRACT

HEMODIALYSIS CULTURE OF BACTERIA

by

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Hemodialysis culture was implemented and tested as a new technique for growing microorganisms entirely on nutrients obtained by steady-state dialysis with the mammalian blood stream. The technique allowed a culture to grow in direct, continuous communication via an external carotid-jugular shunt with the blood of a living animal, and thus provide a new model system for studying the host-parasite relationship of bacterial septicemia.

Two types of culture systems were investigated. The first was comprised of a goat, an artificial-kidney hemodialyzer, and a modular fermentor, and was used to grow large volumes (800 ml) of culture with control of temperature, agitation and aeration. *Serratia marcescens* grew both aerobically and anaerobically in a two-phase growth cycle. Initially the population multiplied exponentially at a maximum rate of about 2 generations/hr. At a cell density of $10^{9.0}$ to $10^{9.5}$ viable cells/ml, the culture shifted to a linear growth rate of about 0.2 generations/hr, apparently because diffusion of nutrients through the dialyzer membrane became limiting. The linear growth phase continued to a maximum of about $10^{10.5}$ viable cells/ml, at which point the maintenance maximum of the system was reached and viable cell density declined. A toxemia was observed in the host goat,

apparently related to the shift in growth phase or the attainment of a particular cell density. Signs of the toxemia were consistent with those of endotoxemia, including severe leukopenia, pyrexia, and general malaise. Dialysis devices with membranes of nominal molecular weight retentions of 300, 8,000, and 30,000 were used as primary hemodialyzers and as differential dialyzers to obtain cell-free fractionated culture dialysates. The devices which passed the two larger classes of molecules allowed good growth of *Serratia*, but the 300 molecular weight retention device failed to support growth. When cell-free dialysates of these classes were tested for endotoxic activity by chick embryo lethality and Limulus coagulation tests, the results were equivocal. However, the 8,000 and 30,000 MW dialysates, but not the 300 one, produced profound hypothermia in precooled mice.

The artificial kidney-fermentor system did not, however, fully simulate in vivo growth conditions as evidenced by in vitro-like characteristics of *Bacillus anthracis* grown in the system. This was believed caused at least in part by the exclusion of very low (gaseous) and high molecular weight blood components by the dialysis membranes.

A second hemodialysis system, comprised of a newly designed prosthetic hemodialysis culture unit, was also tested. This self-contained unit allowed use of various types of membranes, singly or in combination, and was designed to remain attached to an animal's neck for extended time periods of several days. The unit measured 3.5 x 3.5 x 2.0 cm and contained 3.3 ml of culture. Tests with

B. anthracis indicated that this system more closely simulated the in vivo environment than the other system, but restrictions imposed by limited membrane diffusion were believed to prevent a full duplication of in vivo conditions. Although a virulent strain of *Treponema pallidum* did not multiply in the system, other less fastidious organisms (*B. anthracis*, *S. marcescens*, *Streptococcus pyogenes*, and *Blastomyces dermatitidis*) did grow. No toxemia was observed with *Serratia*, probably due to the small volume of the culture and restrictions of diffusion. Trials with protoplasts of *Bacillus megaterium*, goat peripheral blood cells, and mouse spleen cells demonstrated the prosthetic culture unit has applicability for both delicate microbial and mammalian cells.

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By

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3. GENERAL INTRODUCTION

In vivo dialysis culture was pioneered in 1896 by Metchnikoff, who implanted a collodion sac containing *Vibrio cholera* into the peritonal cavity of an animal to demonstrate the production of a diffusible toxin (88). Since then, implanted chambers of various designs have been used for in vivo studies of bacteria, parasites and tissue cells, and diverse other techniques have been developed for culturing organisms in vitro by means of dialysis. These culture systems fundamentally are governed by exchange dialysis: the diffusion of molecules in two directions through a membrane, which separates two solutions with different substances of unequal concentrations. A culture of microbes growing in a chamber on one side of a membrane is provided nutrients from a chamber on the opposite side of the membrane. As the culture grows, products from it conversely pass through the membrane to the nutrient chamber. The principles and applications of dialysis culture have been reviewed recently (119) and are updated in another portion of the thesis (Sections 4.1 and 4.2).

The determination of growth rates and growth characteristics of bacteria in vivo is difficult. Experimental design and errors inherent in the techniques often make the final results less quantitative than desired. Some of the techniques used for establishing

rates in vivo, and the differences between in vivo and in vitro grown cells, are reviewed in Section 4.3. One of the problems in conducting quantitative studies of bacteria in an animal is that of phagocytic clearing and destruction. Implanted chambers with cell-impermeable membranes provide one means of circumventing this problem.

An implanted chamber, however, is limited in use for in vivo culture. The chamber is small, thus limiting the volume of culture, and may be inaccessible or difficult to sample. Because of location inside the host, the entire chamber becomes "walled off" by adhering macrophages, and diffusion between culture and host thus becomes reduced or eliminated. The most important limitation is inherent in the fact that the chamber must be implanted in the peritoneal cavity, beneath the skin, or in a similar place where only secondary communication is established with the blood.

Direct diffusional communication with the blood stream can be accomplished by means of an "artificial kidney"[1], which in recent years has come into routine use in medicine for treatment of chronic kidney failure. Such a hemodialyzer is connected into a bypass of the blood circulation to permit diffusion of body wastes from the blood through membranes to an external reservoir of dialysate solution. The literature on hemodialysis in medicine is reviewed in another portion of the thesis (Section 4.4).

"Hemodialysis would seem to offer considerable promise for extracorporeal study of septicemia" (Schultz and Gerhardt, 1969).

Implementation of this idea was the goal of the research reported in this thesis. The specific objectives were (1) to design and construct hemodialysis culture systems entirely supportive of bacterial growth yet atraumatic to the host, (2) to evaluate hemodialysis culture as a model for in vivo septicemic growth of bacteria, and (3) to determine the applicability of the two hemodialysis culture systems that eventuated to diverse organisms, including mammalian as well as microbial cells.

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[1]It should be recognized that the term "artificial kidney" is a misnomer. In practice, these devices commonly do nothing more than dialyze the blood indiscriminately, and thus only partially duplicate the many and complex activities of a healthy kidney. However, it is common practice to use the terms more or less interchangeably and this will also be the case in this thesis.

4. GENERAL HISTORICAL REVIEW

4.1 In vitro dialysis culture (1969-1973)

4.1.1 *Introduction*

The development of procedures and equipment, theory, and applications of dialysis culture were comprehensively reviewed in 1969 by Schultz and Gerhardt (119) and in 1970 by Humphrey (67).

The major advantages of growing cells in a dialysis culture system, rather than in a conventional manner, include: the prolongation of active reproduction, thus allowing the attainment of high densities of viable cells; the stabilization of the maximum stationary phase of the growth cycle; the removal or dilution of diffusible inhibitory growth products; the production of culture liquors free of macromolecules from the media; and the capability for study of interactions between separated populations of microbial or mammalian cells.

A dialysis system is composed of three functional regions: a reservoir, a dialyzer, and a culture chamber. To achieve maximum efficiency and to allow maximum flexibility and control, the three components are best separated. They may be of almost any size and specific characteristics, depending on the particular goals of the experiment. Generally they are joined through a system of tubing and pumps so that temperature, flow rates, aeration, and agitation of solutions in the nutrient and culture vessels are controlled individually.

The dialyzer itself is the key instrument for efficiency in dialysis culture. A plate-and-frame dialyzer, with multiple repeating chambers of membrane separating nutrient from culture solution, was designed and tested by Gallup and Gerhardt (51), Schultz and Gerhardt (119), and Humphrey (67). This "Biodialyzer" was much more suitable for biological work than the chemical dialyzers then available. A redesigned version of this dialyzer is in process of manufacture and should soon be available commercially from BioTec AB, Stockholm, Sweden. Plate-and-frame types of artificial kidneys also may be used effectively for in vitro dialysis culture. For example, the Dialung hemodialyzer (Cardiovascular Electrodynamics, Baltimore, Maryland) was applied with good success in dialysis fermentations by Abbott and Gerhardt (1970). Several types of hollow-fiber dialyzers have been introduced recently by commercial sources (Cordis-Dow Corporation, Miami, Florida; Amicon Corporation, Lexington, Massachusetts). These dialyzers are designed primarily for the separation and concentration of molecules, but should be applicable to dialysis culture systems. To date, their application to culture of microorganisms has not been thoroughly studied or reported, but preliminary studies by Quarles and Gerhardt (unpublished) have demonstrated the successful use of Cordis-Dow beaker dialyzers and artificial kidneys for dialysis culture of *Serratia marcescens* and *Bacillus anthracis*.

Schultz and Gerhardt, in their 1969 review (page 37, reference 119) listed 50 different algae, bacteria, fungi, protozoa,

and tissue cells which had been studied with dialysis culture in vitro to that time. This review of dialysis culture, including some applications of the technique since 1969, is updated in the following sections.

4.1.2 *Microorganisms*

Pan and Umbreit (97) used dialysis culture techniques to demonstrate that presumably obligate autotrophic bacteria will grow on glucose rather than inorganic energy sources if metabolic products are prevented from accumulating by use of dialysis. *Nitrosomonas europaea, Nitrobacter agilis, Thiobacillus denitrificans, T. neapolitanus,* and *T. thioparus* were grown on glucose-salts media in the absence of the specific inorganic energy sources. Pyruvic acid was the metabolic product found to inhibit *N. agilis,* but the toxic product for *N. europaea* was not identified. Results with the *Thiobacillus* species indicated that pyruvate (or related keto acids) may be more inhibitory to the organism when growing on glucose than when growing on a specific nutrient. This work was important in that it showed a qualitative as well as a quantitative difference between dialysis and non-dialysis culture, and also showed that redefinition of the term "obligate autotroph" may be necessary.

Pan and Umbreit (98) also studied growth of autotrophs and heterotrophs in mixed cultures (both with conventional and dialysis cultures) and found that the effects of autotrophs on heterotrophs, and the effects of heterotrophs on autotrophs, were highly specific. For example, *Escherichia coli* and *Pseudomonas aeruginosa* had

essentially no effect on *Nitrobacter agilis; Streptococcus faecalis* caused slight inhibition; *Serratia marcescens, Hydrogenomonas eutropha* and *Saccharomyces cerevisiae* caused slight stimulation; and *Candida albicans* caused significant stimulation on the growth of *Nitrobacter*. Dialysis culture showed less effect and was considered less successful for the demonstration of interactions than the use of ordinary mixed cultures. Technical considerations such as inadequate diffusion and aeration may have influenced the results, however.

A double dialysis method was used by Edwards (46) to produce antigenic material from the mold that causes "farmer's lung disease." Nutrient medium within dialysis tubing was equilibrated with an aqueous solution of sodium chloride, and mycelia of *Micropolyspora faeni* were inoculated into the resulting dialysate. After growth of the organism, the growth medium (containing organisms, dialyzable material from the original nutrient broth, and products of microbial metabolism) was placed in other dialysis tubing and a second dialysis was conducted to separate dialyzable molecules from the mycelium and macromolecular end products. The resultant organisms and macromolecules constituted the antigens for further studies.

Dialysis culture was used by Friedman and Gaden (49) to study growth and lactic acid production by *Lactobacillus delbrueckii*. They confirmed the inhibitory effect of lactate by dialyzing the product away from the culture medium. Inhibition of the culture

by lactate occurred after the log phase. By maintaining low lactate concentrations at that time, they obtained higher than usual specific growth rates and maximum cell concentrations. Additionally, overall acid production in dialysis culture was significantly higher than in conventional non-dialysis batch culture.

Wyrick and Gooder (144) grew L-phase variants of *Streptococcus faecium* on membrane filters placed on solid agar to demonstrate that L-elements passed through filters with 0.22 μ pores. They also studied reversion of protoplasts, but found that protoplasts could not pass the filter and form colonies under it. However, in some cases covering the filter with solid L-phase medium before inoculation of protoplasts gave rise to colonies. Protoplasts could not form colonies on the membrane without a covering of agar, thus indicating that the three-dimensional effect of agar was important.

In similar studies, Clive and Landman (28) investigated the reversion of protoplasts and L-phase variants of *Bacillus subtilis* on membrane filters and reported similar results. They also reported that the reversion of protoplasts was enhanced by layering wall material on the filters.

Several oceanic phytoplankton species were grown in dialysis culture, both in the laboratory and in the field, by Jensen et al. (69). They tested the technique with eleven species of algae and obtained good growth and dense cultures with artificial media and non-enriched sea water. For studying growth characteristics, they

constructed a dialysis culture flask based on the flask apparatus of Gerhardt and Gallup (54). A second system consisted of bags of dialysis tubing suspended on a rotary support in appropriate media. Sartorius membrane-filters of various porosities and dialysis tubing of regenerated cellulose were used as dialysis membranes. From preliminary work, they concluded that dialysis culture was well suited to studying marine microorganisms and could likely be used for obtaining high densities of algae, assessing the nutritive quality of sea water for phytoplankton production, monitoring biotic and abiotic factors in water, bioassaying pollutants, and studying species interactions. Two important suggested advantages to dialysis culture were that it allows cells to accumulate trace substances from large volumes of water and it can eliminate grazing in field studies on phytoplankton growth.

In a series of three articles on dialysis fermentation, Abbott and Gerhardt (1,2,3) demonstrated that dialysis culture yields a dramatic increase in the amount of a diffusible product and cell mass. They first studied the conversion of naphthalene to salicylic acid by *Pseudomonas fluorescens* and found that the cumulative total amount of both cell mass and salicylate continued to increase throughout the 15 days of the experiment. At termination, product formation and total cell mass were still increasing although less than 10% of the cells were viable (1). A kinetic analysis showed the important role of maintenance metabolism in salicylate production in the system, with maintenance accounting for about 84% of

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the total salicylate produced. Dialysis fermentation yielded a 20-fold increase in the production of salicylate compared to a conventional non-dialysis fermentation, and a 2.6-fold increase compared to an optimally recycled non-dialysis control (2). In companion studies (3), an attempt was made to use dialysis to alleviate product control over threonine production by an auxotroph of *Escherichia coli*. Abbott and Gerhardt unexpectedly found an inhibition instead of an enhancement. This was explained by depletion of α-ε-diaminopimelic acid as the limiting factor rather than threonine inhibition of its own synthesis. The imbalance was not corrected by exogenous replacement of diaminopimelate.

Trypanosoma lewisi was cultured in media composed of a "gel phase" of protein, agar, and defibrinated rabbit blood in dialysis tubing by Dusanic (45). He found agar necessary for good growth of the organism. The agar presumably acted as a sink to absorb inhibitory products from the medium. In addition to yielding satisfactory growth, the system provided a good means for studying amino acid utilization by the organism.

Lysed blood in dialysis tubing was used by Crook et al.(37) as a nutrient source for growing *Leishmania mexicana* for antigen production. These workers considered dialysis culture the method of choice for eliminating the contamination of antigen by medium components.

The technique of growing bacteria on dialysis membranes spread over agar medium was reported to provide a good yield of

essentially all the extracellular products of *Staphylococcus aureus* by Bamen and Haque (10). Although not all products were present in concentrated form, the significant finding was that the entire complement of products could be detected.

Concentration of a bacterial growth product via dialysis culture was demonstrated by Bissell et al. (15) and Sarner et al. (116). They grew a *Sarcina* strain (coccus P) in dialysis bags suspended in media to achieve a highly concentrated proteinase. A cell density of about 24 x 10^8 cells/ml was obtained in dialysis culture, as compared to 6 x 10^8 in flasks. The proteinase was retained by dialysis membranes and therefore was concentrated.

Chet, Fogel and Mitchell (27) studied the chemical detection of microbial prey by bacterial predators. They found that an exudate of the phycomycete prey, *Pythium debaryanum*, was dialyzable. However, a chemotactic exudate of a diatom prey, *Skeletonema costatum*, was not. In both cases a highly motile pseudomonad species was the predator. In these studies, dialysis was used only to help characterize the exudates of the prey which attracted the predator. Dialysis growth systems were not used, although the possibility seems obvious.

The use of dialysates of various protein substrates as media for growth of bacteria in flask culture is common. Pollock (102, 103) found that dialyzable nutrients support growth of mycoplasma. Dialysates of peptone, yeast extract, or hydrolyzed casein were used by Elmer and Nicherson (47) to study the nutritional requirements of *Mucor rouxii*. They demonstrated the presence of a

dialyzable factor, termed Y-factor for "yeast promoting," which was active in changing the culture from a mycelial phase to a yeast phase. The molecular weight of this substance was estimated as less than 700. A dialysate of brain heart infusion was used by Parisi and Kiley (99) to study chromogenic variants of *Staphylococcus aureus*. They suggested that differences in amino acid metabolism between parent and mutant account for differences in chromogenic characteristics in at least some cases.

4.1.3 *Mammalian cells*

Dialysis culture techniques have also been used for in vitro studies with mammalian cells and tissues. Rose and colleagues, over a period of some 20 years, have developed and refined equipment for the in vitro dialysis growth of various types of tissue cultures (110, 111). Their equipment has evolved from a single multipurpose chamber to a 12-chamber circumfusion system which allows control of nutrient and gas flow rates, pressure, agitation and temperature. This system was designed to yield not only growth of tissue cells but also differentiation. Using the system, they observed morphological differentiation and maintenance of the differentiated state in fetal rat ovary cells and thyroid cells.

A less elaborate, multipurpose, microperfusion chamber for dialysis culture of microorganisms as well as tissue cells was reported by Poyton and Branton (104). This chamber was designed to allow observation of the organisms via phase microscopy while the cells were growing. The chamber was used for studying various organisms which included *E. coli*, a blue-green alga *(Nostoc)*, a

marine amoeba, and several fungi. The article also included references and a comparison and summary of the most important features of some 14 different perfusion chamber designs.

The primary immune response of spleen cells from unimmunized mice was studied by growing the cells on dialysis membranes above a reservoir of medium (82). Three to five days after exposure to sheep or horse erythrocytes, significant numbers of antibody-producing cells were detected. This system had the advantage of allowing the spleen cells to grow undisturbed by agitation or mixing, so that foci of antibody-producing cells were not disturbed.

In an article with important implications for dialysis and hemodialysis culture of both microbial and mammalian cells, Knazek et al. (75) reported the use of an artificial capillary device to perfuse both gas and medium into tissue cultures. The capillaries provided a matrix for cell attachment so that secreted products could be harvested without disturbing the culture. The technique of combining both nutrient-passing and gas-passing membranes in a single device is a significant step because dialysis bmembranes are essentially impermeable to gases such as oxygen and carbon dioxide. The system as devised and tested by these workers allowed cells to attain solid tissue densities in vitro similar to those found in vivo.

A variety of inhibitory and stimulatory products of tissues, tissue cultures, and media have been detected and studied by dialysis techniques. However, the reported studies did not seem to

demonstrate any trend in the probability that a given dialysate or retentate would contain either a stimulatory or an inhibitory factor. Each instance of medium and cells seemed a separate case, and extrapolation from one to another was difficult. For example, Robinson et al. (109) found that a non-dialyzable factor from the perfusion of porcine livers and spleens stimulated colony formation from bone-marrow cells. Metcalf (87) found that dialysates of tissue culture medium from several types of normal cells and leukemic cells inhibited colony formation by mouse bone-marrow cells. The inhibition was not species specific, and both leukemic and normal cells produced about equal inhibition. A dialyzable substance from tissue culture medium which could replace the active ingredient of calf serum and stimulate the mitotic activity of BHK cells in vitro was reported by Shodell (124). Taylor et al. (135) found that a dialyzable material from Bacto-peptone could act as a serum substitute for mammalian cells. The dialysate was used as a supplement in conventional medium for growing rabbit myocardial cells. The active factor in the dialysate was not identified, but was something other than free amino acids.

In all of these cases, the investigators did not use dialysis culture systems but rather studied only dialysates or retentates of dialysis. The theory of dialysis culture and the applications of other workers suggest that at least some of these studies might have produced better results, in terms of increased cell mass and products, if dialysis culture had been used.

4.2 In vivo dialysis culture (1969-1973)

4.2.1 *Introduction*

The peritoneal cavity was first used for in vivo dialysis culture (88) and it remains the most widely used today. The most common technique involves implantation of sealed chambers or "sacs" comprised of some type of membrane. Intestinal ligation in situ also has been used for a type of dialysis culture, most frequently for relatively short-term growth experiments and studies on toxin elaboration by bacteria. Examples of this technique are the studies of De et al. (40, 41) and Kasai and Burrows (71) with *Vibrio cholera*, Taylor et al. (134) and Smith and Halls (129) with *Escherichia coli*, and Hauschild et al. (63) with *Clostridium perfringens*. However, intestinal ligation appears less suitable than cell-impermeable diffusion chambers for a wide variety of studies, especially those requiring an extended time period.

An historical review of in vivo dialysis techniques and a summary of the major achievements up to 1969 is included in the review of Schultz and Gerhardt (119). Some of the more important studies using in vivo dialysis since that review are summarized in the following paragraphs.

4.2.2 *Microorganisms*

A particularly promising area for using diffusion chambers and in vivo dialysis is for the culturing of fastidious or slow growing parasitic microbes. In 1960, Huff et al.(66) showed that the exoerythrocytic stages of malaria could be cultured in implanted

chambers within chickens, turkeys, ducks, chick embryos and mice. Chickens were especially capable of encapsulating the chambers, which often is one of the major disadvantages of the system. Another finding was that *Plasmodium gallinaceum*, although about 0.5 to 1.5 μ in size, could pass through 0.45 μ Millipore membranes. In later studies with parasites, Petithory and Raussed (101) immunized mice to *Trypanosoma gambiense* via growth in cellulose diffusion chambers.

Relatively fastidious bacteria have also been grown in implanted chambers. Rightsel and Wiygul (108) cultured *Mycobacterium lepraemurium* in cell-impermeable chambers. They found a relationship between growth of the organism and susceptibility of the host. Another important finding was that the organism grew well in chambers in the absence of other tissue cells. That is, living cells were not essential, and multiplication occurred in a cell-free environment. However, inclusion of macrophages in the chamber seemed to yield better results. The organism had a generation time of 6 to 8 days with macrophages and 11 days in the cell-free environments. Other studies by these workers (143) showed that *Mycobacterium lepraemurium* grew well in chambers within animals containing cells (human embryonic skin) from a species other than the natural host (mice). In fact, the human skin cells enhanced growth. Chambers without skin cells gave greater yields in mice, the normal host, than in guinea pigs.

Less fastidious organisms frequently have been grown in dialysis chambers. Dent et al. (43) found that bacterial products

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released from contaminated bursa chambers enhanced antibody production in chickens. Osebold and colleagues (94, 95, 96) conducted a series of experiments on cellular immunity to *Listeria monocytogenes* in mice. They reported that live bacteria must contact macrophages for development of good cellular immunity. Humoral substances produced in response to diffusible antigens were not able to inactivate the organism. In several instances, sham chambers without bacteria increased the host's resistance to later challenge with *Listeria,* thus indicating that nonspecific resistance is associated with the presence of a foreign body. This was a very important finding, in that it showed that resistance studies must be carefully interpreted to separate the role of the chamber from that of the enclosed organism. Osebold et al. (95, 96) also found that tissue reactions progressed around the chambers to the point that a chronic abscess was formed, and that pleomorphic mutants of *Listeria* appeared during prolonged cultivation in implanted chambers.

Guthrie and Nunez (60) studied delayed hypersensitivity to 1-fluoro-2,4-dinitrobenzene (DNFB) and *Mycobacterium tuberculosis* by implanting chambers containing peritoneal exudate cells from guinea pigs sensitized to these agents into unsensitized animals. The recipients developed specific skin-test reactions to DNFB but not to old tuberculin or to purified protein derivative. Apparently there were basic differences in the release of these factors from cells.

In an interesting modification of the in vivo dialysis technique, Arko (8) implanted a polyethylene practice golf ball (a hollow plastic ball with numerous holes) in the subcutaneous tissue of rabbits and allowed encapsulation by connective tissue to form an artificial chamber and natural membrane. *Neisseria gonorrhoeae* grew and retained virulence in these chambers, and the chambers were usable for a period of several weeks. The success of this system raised the possibility of studying in vivo interactions between various factors such as antibody, leukocytes, complement and bacteria. In addition, a relatively simple system was provided for culturing virulent *N. gonorrhoeae*. It should be noted that the "membrane" enclosing the organisms was tissue and not an artificial membrane.

It seems likely that most bacteria can be grown in some type of in vivo dialysis system, and, indeed, a variety have been cultured in implanted cell-impermeable chambers. Three possible limitations to the implanted chamber technique should be realized however: (1) chambers may elicit a nonspecific immune response exclusive of that caused by the contained bacteria, (2) chambers may be "walled off" to such an extent that diffusion is minimal or eliminated, and (3) chambers may be difficult or impossible to sample without sacrificing the host.

4.2.3 *Mammalian cells*

Normal mammalian cells, antibody-producing cells, and tumor cells have been grown in diffusion chambers. Alekseeva and Yunker (5) implanted 1 x 2 cm envelopes of membrane filters, with 0.1 to

0.3 μ pores, into animals to grow mouse and rabbit cells. Transplantation immunity reactions were investigated by Ambrose et al. (7), who found that a diffusible toxic antibody was involved in tumor transplantation rejection. This factor appeared to be an IgG immunoglobulin and present only in hamsters immunized specifically to SV40 tumor specific transplants. Adenovirus 31 tumor cells in diffusion chambers were not inhibited in hamsters immunized against SV40 tumor specific transplantation antigen. Borella (19) used diffusion chambers to study the regulating mechanism of IgM and IgG antibody-forming cells. He showed that the expression of IgM memory was inhibited by the appearance of IgG-producing cells as early as 4 to 10 days following primary antigenic stimulation. In other studies (18, 20), Borella showed that the antibody response of spleen cells cultured in diffusion chambers suppressed virus replication. An interesting change in the type of host was used by Tucker (137). He grew a variety of tumor cells in chambers implanted in chick chorioallantois.

Growth and differentiation of liver and spleen cell homografts were studied by autoradiography and diffusion chambers in the abdominal cavity of newts (58). This work demonstrated that diffusion chambers can be implanted into animals other than traditional laboratory animals. Harrison and co-workers (62) investigated histocompatibility interactions between nixed types of mouse spleen cells by use of diffusion chambers.

The effect of radiation on mammalian cells has been studied with implanted diffusion chambers by a number of investigators.

Makinodan and co-workers published a series of articles on the effect of radiation on the growth and antibody synthesis of various cells in immunodiffusion chambers (59, 92, 113, 114, 138, 139). The role of thymus cells in restoring resistance to radiated thymectomized mice was studied by Schneiberg et al. (118), who found that thymus-bearing chambers implanted in thymectomized mice restored radiation resistance. Spertzel and Pollard (130) conducted similar studies on spleen cells by implanting membrane-filter chambers in mice. They reported the presence of a humoral factor that increased recovery of the animals.

Blood cell cultures have been investigated by several workers. Rasmussen and Hjortdal (107) grew homologous blood and buffy-coat cells for 3 week periods in diffusion chambers in the peritoneum of rats. Microscopic examination showed that neither fibroblasts nor connective tissue fibers developed inside the chambers if contamination with extraneous connective tissue was prevented. They concluded, therefore, that the development of fibroblasts in blood and buffy-coat culture is due to contamination with connective tissue cells during sampling of the blood. Benestad (12) maintained mouse bone-marrow cells and blood leukocytes in implanted chambers. He found that granulocyted and macrophages were formed but lymphocytes and mature end cells were lost. Additionally, the mouse bone-marrow cells synthesized DNA actively when placed within in vivo chambers, as opposed to a rapid decline reported by others for in vitro studies. Kuralesova (78) also used diffusion

chambers to culture and compare bone-marrow cells from mice which had received post irradiation injections of syngenic bone-marrow cells from mice without these injections.

Kitsukawa (74) culture cells of rabbit aortic endothelium in diffusion chambers that were implanted (in 1 to two weeks) in rabbit abdominal cavities, in an attempt to study the biologic nature of the cells. The cells often showed ring formations of two to three cells, and tended to form "alveolar-like" arrangements around clusters of erythrocytes.

Chamber cultures of up to 10 days duration were used by Laerum and Bayum (79) to study the viability of hairless-mouse epidermal cells. They reported that the cells appeared intact for the first 24 hr, but then a significant cell loss occurred. Cell loss outweighed proliferation under their experimental conditions.

In summary, it seems apparent that in vivo growth of tissue cells of various types is successful in implanted diffusion chambers, and that many functions of the cells can be studied by this technique.

4.3 Growth of bacteria in vivo vs. in vitro

4.3.1 *Introduction*

It is a truism that microbes grow differently in vivo than in vitro, but there are very few experimental models for studying these assumed differences. Most studies which purport to show different characteristics fail to withstand close scrutiny. Very few bacteria actually have definite, easily scored, unequivocal markers

that distinguish the two types of growth. Even the determination of a growth rate in vivo becomes a major problem, with an error in counting viable cells greater than that in vitro, because in most instances the errors of in vivo sampling compound the errors of in vitro plate counts. Usually a study on the growth of a bacterium in a host simply uses a given cell density as an original inoculum and uses death or clinical signs at a particular time as the end point, instead of viable cell density in time intervals.

The importance of using in vivo grown bacteria to determine the biochemical mechanisms of microbial disease was pointed out by Smith in several significant articles (125, 126, 127). His underlying idea is that virulent strains possess genetic differences from avirulent strains. These differences, possibly minor or subtle, are fully expressed only in vivo. Also, the host tissues are continually changing when under microbial attack, and this spectrum of change is not easily reproduced in vitro. These differences in the organism are often difficult to detect and document, but some informative models are available.

The following discussion will review the growth of bacteria in vivo in terms of the differences between in vitro and in vivo characteristics, and the techniques for determination of in vivo growth rates.

4.3.2 *Experimental models*

Most approaches have involved a search for physiologic or antigenic differences between virulent and avirulent strains or a search for toxic substances produced by the virulent strain.

Segal and Bloch (121, 122) and Bloch and Mizuno (17) demonstrated that cells of *Mycobacterium tuberculosis* grown in vitro have biochemical and pathologic differences from those grown in vivo. In vitro grown cells have a higher hydrogen transfer rate, and respiration is more stimulated by substrates such as glucose, glycerol, lactate, acetate and pyruvate. In vivo grown cells respond to salicylate, which is considered by some workers as correlated with pathogenicity. There are no differences in morphology, staining properties, colonial characteristics, or rate of growth on oleic acid-albumin agar. The degree of aggregation is important in determining virulence: small units (single cells or small aggregates) are less virulent than larger ones. When the two types of organisms are prepared similarly aggregated, the in vivo grown cells are more virulent for mice than the in vitro grown ones. A phenol-killed vaccine prepared from in vitro grown organisms produces better immunity in mice than that from in vivo grown organisms, thus indicating an immunologic difference.

One important factor leading to virulence is often that of resistance to phagocytosis. In studies on *Yersinia pestis*, Burrows and co-workers (24, 25, 26) showed that virulent and avirulent strains are indistinguishable in vitro, but are easily identified

in vivo in mice. Avirulent strains are phagocytosed completely, but virulent strains rapidly become phagocytosis-resistant and kill the host. Later studies indicated that resistant strains can be grown in vitro, and that resistance seems associated at least partially with the so-called V- and W-antigens which are produced in vivo. Smith and his colleagues (126, 127) grew *Y. pestis* in guinea pigs and collected the plasma and body fluids to demonstrate that in vivo grown organisms produce toxin which kills guinea pigs and mice. Fukui et al. (50) sought to detect metabolic differences between in vivo and in vitro grown *Y. pestis* cells, but found no significant differences which could account for pathogenicity. However, the in vivo grown cells acquire the ability to oxidize gluconate, which was suggested as possibly important in the production of virulence antigen.

Cells of *Bacillus anthracis* are both morphologically and metabolically different in vivo than in vitro. In vivo grown cells tend to encapsulate to a greater extent, form no chains or only short chains, have a square-ended morphological appearance, and fail to sporulate (55, 125, 127). Smith and Tempest (128) reported that the in vivo grown organism used large quantities of glutamine, threonine, tryptophan, and glycine, but did not use histidine, lysine, tyrosine, phenylalanine, methonine, or alanine for growth. In general, amino acids which are important antimetabolites in vivo are not in vitro. Thus, there seem to be different, but unexplained, utilizations of amino acids in the two environments.

The brucellae provide a good example of tissue specificity in vivo caused by the presence of a particular nutrient. Erythritol isolated from fetal and maternal tissues (placentae, fetal fluids, chorions) of susceptible species was shown to be highly stimulatory for growth of the organism (127). Cells of *Brucella abortus* in vitro preferentially use erythritol instead of glucose even if 1000 times more concentrated. Primary invasion by the organism is a separate problem, not controlled by erythritol.

Beining and Kennedy (11) compared *Staphylococcus aureus* cells grown in vitro on trypticase soy agar or broth to those grown in vivo in guinea pigs. The two types of cells are similar in a number of characteristics: morphology, size, staining, bound and soluble coagulases, bacteriophage type, antibiotic sensitivities, common fermentation reactions, DNA base composition, qualitative tests for hemolysins, deoxyribonuclease, ribonuclease, staphylokinase, protease, lipase, and phosphatase. However, the in vivo grown cells are significantly different in certain other characteristics: respiratory rates, virulence in mice and guinea pigs, agglutinability, agar gel diffusion tests, growth on tellurite-glycine agar, and the quantitative production of deoxyribonuclease, alpha-hemolysin, leukocidin and hyaluronidase. Gellenbeck (52) showed that in vitro grown *S. aureus* cells have a lower exogenous respiratory rate than in vivo grown cells.

Metabolic differences between the two types of cells of *Streptococcus* were reported by Gordon and Gibbons (56). They found

that *Streptococcus mitis* cells that are grown in vivo in gnotobiotic rats and mice have a 3 to 5 times greater glycolytic activity on a per cell basis than those grown in vitro.

4.3.3 *In vivo growth rates*

Accurate determinations of growth rates in vivo present a greater problem than determination of differences between in vivo and in vitro grown cells. An in vivo growth rate is actually the net of growth rate less clearance rate. The most obvious and straightforward method to obtain a growth rate is to inject identical inocula into a number of animals and periodically count viable organisms from the entire body of one or more animals. Generally, this requires either the use of germ-free animals or a selective and differential medium to eliminate normal flora. The experimental procedure is usually to remove the skin and feet, grind the weighed carcass in a blender, and plate out a known sample of the suspension.

Berry and co-workers used essentially this technique to study *Salmonella typhimurium* in mice (13, 14). They found that approximately 10^9 viable bacterial cells were recoverable from the animal at time of death. The generation time of the organism in mice is about 58 min, as compared to approximately 22 min in brain heart infusion medium.

Another approach involves the removal of only selected organs, e.g., the blood, lungs, liver, spleen, and kidneys. The organ is weighed, ground up to release the bacteria, suspended in a diluent,

and then a representative sample is used for viable cell determinations. This technique has been used frequently for both qualitative and quantitative studies.

Collins (30, 31, 32, 33, 34) determined growth rates and yields in both normal and immunized or multiply infected animals by removal and quantitative culture of selected organs. Several *Salmonella* species were studied in this manner. He found that the bacterial population in the intestine tended to stabilize at about 10^4 to 10^5 organisms after oral inoculation. Oral inoculation was most often used in an attempt to duplicate closely the normal sequence of events during infection. Increasing the size of challenge doses had little influence on the final outcome of the infection, but did increase the rate of spread throughout the body. Representative growth rates were difficult to obtain, but the rates were relatively slow. Maximum numbers of organisms per organ were about 10^3 to 10^4 after 6 days growth. Several hours per generation was common. Problems of phagocytosis and spreading throughout the body made rate studies more difficult in these experiments.

Srivastava and Thompson (131) suggested that best results are obtained if the organism of interest is not mixed with other organisms, as is the case with most total body experiments. Consequently they used local lesions in the thighs of mice to enumerate the growth rates of *E. coli* and to study the effect of streptomycin in the organism in vivo. They stripped off the skin, amputated the thigh, and homogenized the tissue. There was a loss of recoverable bacteria from 5.8×10^7 viable cells to 1.6×10^7 cells

immediately after inoculation, and a 1- to 1½-hr lag before an overall exponential growth of approximately one generation per 90 min for about 12 hr. A maximum of about 10^{10} viable cells/ml of homogenate was reached at 12 hr and maintained for 3 days before the viable cell count decreased. Young cells were much more sensitive to streptomycin than old ones. It is obvious that comparing this type of experiment with one involving organs or total body studies is very difficult. The environmental conditions are different and great differences are to be found in rates and maximum numbers attained.

Meynell (90) and Maw and Meynell (84) proposed a method for measuring division and death rates of bacteria in vivo. This technique, termed the superinfecting phage method, involves lysogenizing the infecting bacterium with a temperate phage and superinfecting with a differentially marked mutant prophage before inoculation into a host. The superinfecting phage neither lysogenizes most of the bacteria nor replicates during bacterial multiplication. The proportion of bacteria with superinfecting phage thus decreases with each generation of bacteria in a predictable manner. Therefore, the proportion of bacteria carrying superinfecting phage can be determined at each generation and a count obtained. The proportion of superinfected cells is determined by inducing vegetative phage growth by exposure to ultraviolet light and plating on bacterial indicator strains specific for the prophage and superinfecting phage respectively. The technique and analytic procedures are somewhat

complicated and tedious, but were used for *E. coli* and *S. typhimurium*. In these studies, the division rate of the salmonella in mouse spleen was only 5 to 10% of the maximum in vitro rate. Generation times were 8 to 10 hr in vivo and about 30 min in vitro.

Matsuo (83) studied the growth of *Mycobacterium leprae* in mouse foot pads and found that multiplication of the organism depended considerably on the ambient temperature. Multiplication was faster at 20 C than at uncontrolled or partially controlled room temperatures. Growth rates were very slow in all cases, as an original inoculum of 10^4 organisms per foot pad increased to only about 3×10^6 after 44 wk. His enumeration technique was to mince and grind foot pad tissue, and then to remove and count the acid-fast bacteria.

Saymen et al. (117) published a method for quantitatively determining viable bacteria in infected tissues or wounds. This method involved placing a known number of bacteria into a standardized surface wound created by surgery. Bacteria were obtained from the wound by surgical removal of the area and washing or swabbing the tissue. Other workers have discussed similar techniques (22, 53, 123).

For bacteria which cause a septicemia, determination of viable cells in the blood may be satisfactory for growth rates and total yields. This method is simple and has the advantage of not requiring destruction of the animal, so that repeated samples can be obtained from an individual host. Of course, large numbers of bacteria may be sequestered in various organs and cells of the body

and thus missed by sampling the blood. This approach can provide useful information, however, as shown by the studies of Lincoln and his colleagues with *Bacillus anthracis* (80, 81). They examined growth in separate tissues and whole animals and also conducted quantitative studies on the in vivo growth of the anthrax bacillus by determining the number of organisms per ml of blood. They prepared a mathematical model of the septicemia, based on their in vivo studies, which agreed well with actual experimental data from infected animals.

4.4 Hemodialysis in medicine

4.4.1 *Historical development*

The basic concept of hemodialysis was put into medical practice some 60 years ago. In 1913, Abel, Rowntree and Turner (4) published the first article on a hemodialyzer, or "artificial kidney." They envisioned the technique as a means of providing aid in emergencies such as renal failure. The possibilities of using hemodialyzers in chronic cases were unrealized until fairly recently, and relief from acute renal failure remained the main use for many years. In fact, in 1949, one of the leading dialysis centers in Boston suggested that hemodialysis in chronic renal disease was useful only for acute cases or in preparing uremic cases for surgery (85). It was 10 years later before prophylactic or repeated dialysis came into routine use.

Frequent dialysis is required for chronic patients because levels of retained products quickly build up to toxic levels. The

unsolved problems of the early days of dialysis treatment made repeated dialysis difficult or impossible. These problems were mainly technical and were related to the inability to establish and maintain permanent circulatory shunts. Access to the artery and vein required a surgeon to insert and remove the cannulae each time, and bleeding at the surgical site following heparinization often resulted. These factors combined to use up the available blood vessels of patients rapidly and made repeated, long-term dialysis unattractive. Also, the equipment was large, difficult to set up and sterilize, and expensive to use and maintain.

Other problems were not technical but rather were a matter of practice and experience. It was soon realized that patients with chronic renal difficulties and slowly progressing renal failure had gradually adjusted to the situation and their systems had compensated to some extent. Rapid, efficient dialysis often actually made them worse than before treatment, as demonstrated in the "disequilibrium syndrome" (136, 141).

The situation remained much the same through 1959. That year, two schools of thought were represented by two articles in the *Transactions of the American Society for Artificial Internal Organs*. One article reflected the previous years of difficulties and stated the prevailing view that dialysis was not the answer to chronic uremia (115). The other reported on the use of daily hemodialysis via polyvinyl cannulae (93). Cannulae in the latter situation were established in vessels (radial artery and antecubital vein) and maintained patent throughout the treatment period. Thus, repeated

dialysis was possible without surgical cannulation each time. More significantly, this allowed prophylactic and not just emergency dialysis. That is, the chronic patient could be dialyzed periodically, before a toxic crisis occurred.

During the 1960's, techniques and equipment developed and improved rapidly. Quinton et al. (105, 106) published a paper on chronic cannulation for prolonged hemodialysis. Reports were also published on intermittent hemodialysis (120) and indwelling cannulae and by-passes (64, 91, 105, 142). They and other workers developed the basic procedures which are used with only minor modifications today.

Solving the technical and medical problems of hemodialysis made possible the saving of lives which otherwise were certain to be lost. The choice between the goals of either maintenance or rehabilitation of life raised difficult financial, legal, and moral problems which are beyond the scope of this review, but which have not been satisfactorily resolved to date.

The technology of equipment and delivery systems also developed rapidly. Multipatient facilities, which allowed dialysis of several patients at a time, were initiated. Central systems for delivery of dialysate and monitoring equipment and patients were developed. Home dialysis became possible as a means of reducing costs and placing fewer restrictions on the patient (16, 38, 86). Techniques for putting shunts in the foot or ankle were developed and tested, thus allowing the patient to conduct self dialysis (9). In all these

cases, a combination of improving equipment and procedures had to be combined with increased experience.

The first practical clinical dialyzer employed cellophane membranes (44), and similar membranes are still the basic type in use. Although a variety of hemodialyzers have been successfully tested for both chronic and acute dialysis, the most commonly used ones can be classed into three main types: (1) the flat filter press or plate-and-frame type, of which the Kiil dialyzer is probably the best known (72, 73), (2) the coil or membrane-tubing type, with either single or twin coils, of which the Kolff hemodialyzer is the best example (76), and (3) the more recently developed hollow-fiber type, represented by the Cordis-Dow dialyzer (56, 132).

Each of these types of hemodialyzers has particular characteristics, advantages, and disadvantages. Generally the choice of a dialyzer depends on a combination of features and requirements, including personal preferences of the user. A short description of an example of each of these types is given below:

4.4.2 *Plate-and-frame (Kiil) dialyzer* (72, 73)

A representative Kiil dialyzer is rectangular in shape and 100 x 40 x 20 cm in size. Its weight is about 27 KG. Blood is circulated through parallel channels between sheets of cellophane. A dialysate solution is circulated countercurrently on the opposite side of the membranes. The two solutions flow along the long axis of the dialyzer. Membranes are supported by a series of ridges and grooves in plates on the dialysate side of each membrane sheet.

Blood flows through the dialyzer in a thin film to increase the contact with membranes and to increase the efficiency of dialysis.

This type of dialyzer commonly is made of stainless steel and machined polypropylene. About 400 to 500 ml of blood is required to fill it and the accompanying tubing, but most of the blood can be returned to the patient at the end of dialysis. The most serious disadvantage is probably the difficulty in assembling and "sterilizing" the dialyzer. It must be washed, assembled, pressure tested for leaks, liquid "sterilized", and rewashed before use. Proper stretching and placement of previously moistened cellophane membranes on the grooved board requires great care and some practice. About 1½ hours work, part of which requires two persons, is usually needed for best results.

Dialysis efficiency is good and the unit can be used for chronic or acute dialysis. A two layer system is most often used, but more layers can be added to increase the membrane area. Smaller models, requiring less blood, are available for small patients.

4.4.3 *Coil (Kolff) dialyzer* (76, 77)

This dialyzer is basically a length of cellulose tubing wrapped around a central cylinder and surrounded by a fiber glass or plastic supporting screen. The entire assembly is immersed in a tank of dialysate solution. Two parallel coils are often used to increase membrane area and efficiency, thus reducing dialysis time. Blood is circulated through the tubing, and dialysate is circulated crosswise to the blood. A blood pump is required to maintain satisfactory blood flow. These dialyzers come in three sizes (small, medium,

and large) with coil lengths of 5.20, 8.00, and 10.75 meters and dialysis surfaces of 9,000, 14,500, and 19,000 sq cm respectively. Relatively large amounts of blood are required for priming and filling the system (550 to 950 ml). The coil dialyzers are efficient and satisfactory for both chronic and acute dialysis.

Coil dialyzers are considered easy to assemble and prepare for use, especially in comparison to Kiil type dialyzers. Coil hemodialyzers can be mass produced, presterilized, and discarded after use. Their main disadvantages are in the requirement for mechanical pumping of blood with consequent possible trauma, and in the large priming volume.

4.4.4 *Hollow-fiber (Cordis-Dow) dialyzer* (57, 132)

The hollow-fiber dialyzer is a relatively newly developed type which is very compact and efficient and seems to offer several advantages over both Kiil and Kolff types of hemodialyzers. The hollow-fiber dialyzer is a bundle of approximately 11,000 hollow membrane fibers surrounded by a plastic jacket. The fibers are constructed of regenerated cellulose, and are 285 μ in diameter (225 μ inner diameter) and about 13.5 cm in length. The unit is cylindrical, about 21.6 cm long x 7.0 cm diameter in overall size, and weighs only 680 g, when filled. Blood flows through the inside of the fibers, and dialysate is circulated countercurrently through the outside. The fibers give a total effective membrane surface area of 1 to 1.3 sq meters, depending on the model.

These units are commercially produced, presterilized and stored in formaldehyde, and sold ready for washing and use. Resistance to blood passage is very low and blood pumps are not required. No assembly is necessary prior to use. The dialyzers are considered disposable, but may be flushed, resterilized and reused several times under usual conditions. Only about 100 ml of blood is required to fill the dialyzer and most of this can be returned to the patient.

Advantages of the hollow-fiber dialyzer are that no assembly and only a minimum of preparation (primarily washing away formaldehyde) are required before use, and the units are compact and efficient. Additionally, only a small amount of blood is required to fill the unit, and pumping is not necessary. Significant disadvantages have not become apparent, and the units are coming into widespread use.

5. EXPERIMENTAL RESULTS

5.1 Vascular surgery and prosthesis

5.1.1 *Introduction*

Hemodialysis is dependent on access to a major circuit of the blood circulation, for example shunting the blood supply from an artery through an external bypass to the dialyzer and back into a vein. In human clinical usage, an artery and vein of the wrist or ankle are most commonly used. With experimental animals, vessels of the legs or neck are more convenient.

There are two primary techniques for achieving the required access to the blood circulation. The older and most frequently used is the external prosthetic shunt. The other technique involves the creation internally of an arterilized vein (i.e., an arterial-venous fistula), with access through repeated venipuncture (23). The external shunt is more vulnerable to clotting, to possible infection and (especially in experimental animals) to unplanned opening and resultant exsanguination. An external shunt also may restrict activities of the patient or animal. The internal arterial-venous fistula overcomes these limitations and is reported to be preferred by some patients. It does, however, require venipuncture with relatively large needles for each dialysis, and the surgery is more complicated than that required for the external shunt.

We decided to use the external shunt for hemodialysis culture, recognizing possible shortcomings, for the following reasons: (1) limitations in surgical competence, (2) ease of connecting and disconnecting the variety of equipment to be tested, (3) ease of monitoring and sampling from the shunt, and (4) preferred use of the carotid artery and jugular vein.

5.1.2 *Materials and methods*

The arterial-venous cannula was established by surgical techniques like those used for human patients. The shunt was similar to those originally reported and modified by Scribner and co-workers (105, 106).

The animal was anaesthesized and an incision of approximately 10 cm made on the right side of the freshly shaved skin of the neck. The incision was parallel to the direction of the major blood vessels, about halfway between the animal's head and shoulder. The jugular vein and carotid artery were isolated, with the vagus nerve carefully separated from the artery. Beginning with the artery, each vessel was ligated distally. The vessel was then temporarily constricted proximally to prevent blood loss, and a small opening was made (with scissors) between the temporary constriction and the permanent ligation. A Teflon vessel tip attached to Silastic tubing was inserted into the blood vessel and tied in place. Double or triple ties were used, with cross ties around the vessel and tubing. Other ties were used to attach the process to nearby muscle or fascia. The procedure is shown diagrammatically in Fig. 1.

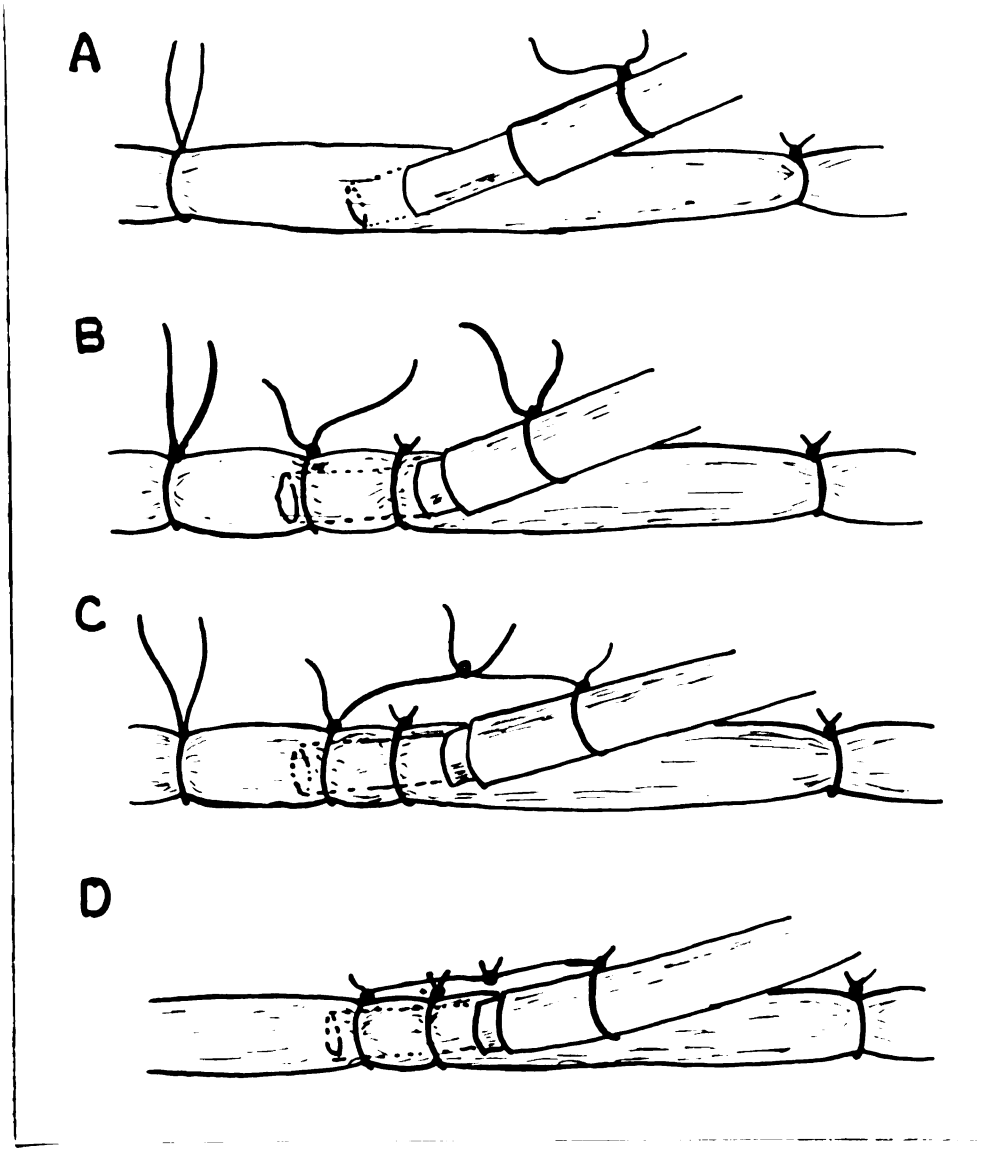

FIG. 1. *Surgical technique for establishment of vascular shunt. The vessel is permanently ligated distally (right) and temporarily constricted proximally (left). A vessel tip with attached tubing is inserted (A) and tied in place (B). A double tie is placed around the area of the blood vessel over the inserted tip and cross-tied to the tubing (C). With the vessel tip firmly in place inside the vessel, the temporary constriction is removed to allow blood flow through the tubing (D).*

The Silastic tubing was aligned parallel to the vessels for about 5 cm and then brought outside the body through openings in the skin about 1 cm to either side of the incision. The arterial tube was above the incision and the venous tube, below. The incision was closed with sutures and allowed to heal.

The two externalized tubes were connected with a Teflon connector or drug infusion "T" to establish extracorporeal arterial-venous circulation. In practice, an infusion "T" was usually left in place for 24 to 48 hr after surgery to allow periodic flushing of the shunt, and was then replaced with a connector for the duration of the shunt.

Several hours after surgery, heparin anticoagulant therapy was initiated and maintained as long as the shunt remained patent. About 8,000 to 10,000 USP units of heparin (as a sterile solution of sodium heparin, Upjohn Company) were given subcutaneously every 12 hr to maintain the blood clotting time about 3 times normal.

The shunt was put into use for hemodialysis by clamping shut the Silastic tubing on each side of the connector, removing the connector, and attaching a hemodialyzer to the arterial and venous tubes. The clamps were removed and blood was allowed to flow from the animal, through the dialyzer, and back into the animal. Heparin was continuously infused into the hemodialyzer as required to prevent clotting.

The experimental animals were all young-adult female goats of mixed breed, in weights from 50 to 125 lb. The animals were

maintained unrestrained indoors, in rooms approximately 10 x 12 ft. Prior to surgery, the goats were checked for parasites and the general state of health, and various normal values (clotting times, blood cell numbers) were determined. Following surgery, the shunt was covered with a light dressing of gauze and tape, and the entire neck was covered with a canvas collar for protection of the shunt.

5.1.3 *Results*

A summary of surgical results and maintenance of the arterial-venous shunt for all the experimental animals used in the study is given in Table 1. The weight for each animal is the weight at time of surgery.

5.1.4 *Discussion*

Goats reportedly are poor anaesthetic risks during surgery (48) and our results reflected this observation. Three of the fifteen goats died during surgery, apparently due to the anaesthetic. In our experience, the usual anaesthetic of choice, sodium pentabarbitol, must be used with caution. Better and very promising results were obtained with an experimental drug designated CI-744, recommended and obtained through the courtesy of Dr. R. W. Coppock of the Parke-Davis Company. According to the company ("CI-744 Brochure for Investigators," Feb. 29, 1972), the drug is a combination of two ingredients: tiletamine hydrochloride (CI-634) and a diazepinone (CI-716). Tiletamine is a central nervous system depressant which produces analgesia and cataleploid anaesthesia. The diazepinone is a tranquilizer and CNS depressant with anticonvulsant and anti-anxiety activity.

TABLE 1. *Surgery and maintenance of arterial-venous shunts in goats*

Animal number	Weight (lb)	Duration of shunt (days)	Total dialysis time (hr)	Number of dialyses	Reason for termination
1	125	3	0	0	Clotted
2	68	69	6	7	Exsanguinated
3	88	69	68	8	Exsanguinated
4	74	--	--	-	Died, surgery
5	58	46	66	7	Clotted
6	51	--	--	-	Died,surgery
7	80	47	39	4	Exsanguinated
8	72	58	20	3	Clotted
9	50	16	4	2	Clotted
10	52	--	--	-	Died, surgery
11	52	65	92	14	Exsanguinated
12	100	23	46	6	Clotted
13	50	24	39	6	Clotted
14	80	32	66	6	Clotted
15	60	51	209	8	Clotted

After the prosthesis was established, the primary problems were in preventing accidental exsanguination via unplanned opening of the shunt and in preventing clotting in the shunt. The first problem was solved reasonably well by use of protective canvas collars over the shunt, hobbles on the animal's legs, and removal of all protruding objects from the room. The second problem was not completely solved, despite anticoagulant therapy with heparin, and clotting remained somewhat a matter of chance throughout the study. However, the minimum time the shunts remained patent was only once less than three weeks (the first attempt is excluded) and was usually four weeks or greater. This was considered an acceptable duration.

5.2 Fermentor - artificial kidney - goat system

The hemodialysis culture system as initially designed was comprised of an animal, a commercial artificial kidney, and a modular fermentor, connected through rubber and tygon tubing. A biological pump (Maisch metering pump, Tuthill Pump Company, Chicago) circulated the culture, and the animal's heart circulated the blood. This system allowed the use of several hundred milliliters of culture with precise control over temperature, aeration and agitation of the culture suspension. The design was similar in concept to the fermentor-dialysis system described for in vitro dialysis culture by Schultz and Gerhardt (119).

A Dialung artificial kidney (obtained through the courtesy of Dr. W. G. Esmond, Baltimore, Maryland) was initially tested for use in the hemodialysis culture system. This plate-and-frame type of

dialyzer was designed for use with human kidney patients. In our use with the goat, however, it caused hemolysis and rapid clotting of the blood to the extent that blood flow in the dialyzer ceased. It was thus considered unacceptable for our work.

A Cordis-Dow hollow-fiber artificial kidney (Cordis Corporation, Miami, Florida) then was tested and successfully used for hemodialysis with the goat. Therefore, it was chosen as the hemodialyzer for development of the system.

The basic characteristics of the fermentor - artificial kidney - goat system, its application as a model of septicemic growth, and the demonstration in vivo of a dialyzable toxic product of *Serratia marcescens* are described in the following manuscripts. The manuscripts were written in the format for and will be submitted for publication in *Infection and Immunity* or a comparable journal.

5.2.1 *Goat hemodialysis culture of Serratia marcescens as a model of septicemia: Growth characteristics. (Manuscript)*

PHILIPP GERHARDT, JOHN M. QUARLES and RALPH C. BELDING

Manuscript for submission to

Infection and Immunity

(ABSTRACT)

Hemodialysis was employed to simulate growth conditions in mammalian blood but without phagocytosis. The blood stream was shunted surgically via prosthetic tubing from a carotid artery through the hollow-fiber membranes in an artificial-kidney hemodialyzer and back into a jugular vein. Culture in the dialysate solution concurrently was pumped from a modular fermentor through the hemodialyzer jacket outside of the membranes and back into the fermentor. Hemodialysis between the two circuits was maintained continuously. With the goat and *Serratia marcescens* selected as a host-parasite model, this new culture system allowed the inoculum initially to multiply at the maximum exponential rate and then at a lesser linear rate, equally well under aerobic or anaerobic conditions. Beaker hemodialyzers were equally effective with membrane porosity equivalent to nominal molecular weights of 10,000 or 30,000, but not 300.

(INTRODUCTION)

We have devised a new way to grow organisms by in vivo dialysis, with the experimental rationale derived from the fact that the interior milieu in animals is maintained essentially by circulation of the blood. Dialyzers connected directly with the circulatory system are in common clinical use as artificial kidneys for humans. It appeared that such a hemodialyzer could be used to establish continuous communication between the blood stream of an animal and a fixed volume of dialysate solution inoculated with an organism. In this way, dialyzable molecular constituents of the blood would diffuse into the culture and so feed it, yet the blood cells and macromolecules would be too large to diffuse through the membrane, so phagocytosis and immunological reactions against the culture would be prevented. Conversely, metabolic products of small molecular size from the culture would diffuse through the membrane barrier into the blood and so relieve the feedback inhibition that often limits growth in a closed culture system. Also, diffusible toxins could exert effects. Furthermore, membranes of different porosity could be employed in the system, for example, to distinguish between the exchange of macro- and micromolecules in hemodialysis. The system would be analogous to the batch fermentor - continuous reservoir mode of operating an in vitro dialysis culture (see Fig. 8, reference 7), but with the blood supply of an animal used as the nutrient reservoir.

Historically, in vivo dialysis culture first was attempted by implanting a collodion membrane sac into the peritoneal cavity — in 1896, Metchnikoff grew cholera bacteria in this way and demonstrated

the production of a diffusible toxin (4). Dialysis chambers subsequently were implanted in the peritoneal cavity and elsewhere for a number of purposes with a number of organisms, including animal cells and tissues, as reviewed by Schultz and Gerhardt (7). The use of implanted chambers, however, is limited by the different environment in the peritoneal or other body fluid than in the blood itself, the occluding growth of macrophages on the membrane surface, the restricted size, and the difficulty in sampling.

We conceived the use of hemodialysis to offset these limitations and essentially to transpose septicemic conditions extracorporeally. The feasibility of hemodialysis culture was demonstrated in tests with the domestic goat and *Serratia marcescens* selected as the host-parasite model. The methodology, an unusual bimodal pattern of bacterial growth, and the effects of three porosities of membrane in hemodialysis culture are described in the present paper. The toxemic host response is reported in a succeeding one.

MATERIALS AND METHODS

The hemodialysis culture system is depicted and diagrammed in Fig. 1. Blood from an experimental animal was circulated by the heart through one side of an artificial-kidney hemodialyzer (the blood circuit), and dialysate culture from a modular fermentor was circulated by a pump through the opposite side (the dialysate-culture circuit).

The goat was selected as the experimental animal because of its convenient size, long neck for accessibility in vascular surgery, placid disposition, and hardiness. The goat has disadvantages in its relative sensitivity to anaesthesia and hemolysis (1). Short-haired domestic goats of mixed breed were used. All were females, 1 to 3 years of age, and weighed from 50 to 100 pounds. They were maintained indoors in stalls, unrestrained except for a rear-leg hobble. During dialysis trials the goat was partially harnessed into a caged platform but was free to stand, lie, eat, drink, and excrete wastes.

A permanent external prosthetic shunt was established between a carotid artery and jugular vein in the neck by vascular surgery, based on the techniques of Quinton et al. (5,6). Shunts, vessel tips, and tubing were made of medical grade Teflon, Tygon, or silicone rubber (Cobe Laboratories, Inc.), as used for humans.

Sodium heparin (Upjohn Co.) was used to prevent the blood from clotting, with subcutaneous injections of 15,000 to 20,000 USP units/24 hr. During dialysis, about 1,500 USP units/hr were

continuously infused into the arterial shunt (Sage Infusion Pump, Model 240; Orion Instrument Co.). Ethyl isobutrazine hydrochloride was administered as a tranquilizer in early experiments, but later was obviated by training the animal. Neither drug affected growth of the test bacterium. The possibility of blood clotting in the hemodialyzer was monitored visually and by a thermometer in the effluent tubing, where the temperature went down if the flow was reduced by clotting.

A given goat became usable for hemodialysis experimentation about a week after surgery and remained so for about 1 to 3 months, when accidental exsanguination by the goat or the formation of a blood clot in an artery or vein (despite heparinization) caused termination.

A hollow-fiber artificial kidney was selected as the hemodialyzer (Cordis-Dow Model 2 or 3, Cordis Laboratories Inc.). The jacketed cylindrical unit measures 7.0 cm in diameter and 21.6 cm in height, and contains a bundle of 11,000 hollow-fiber membranes fabricated from regenerated cellulose, through which the blood is circulated. Each fiber is 13.5 cm in length and 225 μm in inside diameter, and the bundle provides about 1 m^2 of total membrane surface area. The dialyzer was sterilized with 1.5% formaldehyde and rinsed thoroughly with water before use. The culture-dialysate was circulated outside the hollow-fiber membranes, through the dialyzer jacket.

Beaker chemical dialyzers with a looped bundle of hollow-fiber membranes (Cordis Laboratories Inc.) also were employed as

hemodialyzers. The beakers measure 7 cm in diameter and 14 cm in height, and provide a total membrane surface area of about 1,000 cm^2. Beaker dialyzers with three different retention porosities were used, identified in terms of the nominal molecular weight (MW) threshold for dialysis: MW 300 ("Osmolyzer", Model b/HFU-1), MW 10,000 ("Dialyzer", Model b/HFD-1, with the same porosity as the artificial-kidney dialyzer), and MW 30,000 ("Ultrafilter", Model b/HFU-1). The beaker dialyzers first tested caused hemolysis and toxemia, and so were unsatisfactory for use as hemodialyzers. However, the embedding material used to secure the fiber bundles subsequently was changed in manufacture, and more recent products proved satisfactory as hemodialyzers.

A modular 1-liter glass fermentor with control of temperature, agitation and aeration (Microferm Model MF 102, New Brunswick Scientific Co.) was used to contain the dialysate-culture suspension. Anaerobic conditions were achieved by sparging the fermentor contents with sterile argon, which was scrubbed free of oxygen by passage over hot copper wire kept reduced with a stream of hydrogen gas. Aerobic conditions were achieved by sparging the fermentor contents with filter-sterilized air at a rate of 2.4 l/min and by driving the impellor at a rate of 300 rev/min.

A sterilizable gear pump (Maisch metering pump, Tuthill Pump Co.) was used to circulate the dialysate-culture from the fermentor through

the tubing to the dialyzer and back. Thick-walled rubber tubing was used for the dialysate-culture circuit, but the tubing in the blood circuit was medical-grade Tygon or silicone rubber.

The dialysate-culture circuit routinely was charged with 800 ml of a glucose-salts solution approximately balanced in makeup to that of goat blood (1). The solution was constituted as follows per 100 ml: 50 mg glucose, 310 mg Na^{+} (as NaCl and Na acetate), 15 mg K^{+} (as KCl), 11 mg Ca^{++} (as $CaCl_2$) and 3.7 mg Mg^{++} (as $MgCl_2$). The solution volume represented about 40 to 50% of the total blood volume of a goat.

S. marcescens strain 8UK was selected as the test organism because of its previous use in developmental studies on in vitro culture (2,3). This gram-negative bacterium occurs mostly as single cells, grows rapidly on synthetic or natural media under either aerobic or anaerobic conditions, and is red pigmented when grown on most media at about 30 C. *S. marcescens* commonly is thought of as saprophytic, but is quite capable of parasitic growth in the body fluids, produces endotoxin and may cause serious diseases in man. The inoculum was prepared from an aerated exponential culture at 35 C in trypticase soy broth (BBL), which was sedimented and resuspended in the dialysate solution to an appropriate concentration.

Hemodialysis was initiated and the system allowed to equilibrate for 1 hr prior to introducing the inoculum. The culture was maintained at 39 C (the approximate normal temperature of goats) and consequently was not pigmented. Samples of the culture were removed periodically by syringe-and-needle through a self-sealing rubber

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diaphragm positioned in the circuit tubing. Cell populations were measured by optical density and by viable cell counts that were performed by surface plating on trypticase soy agar (BBL).

Cell-free samples of the culture for biochemical analyses were obtained by membrane filtration. Samples of blood were collected in ethylenediamine tetraacetic acid (EDTA) to obtain plasma, or were allowed to clot to obtain serum. The plasma and serum samples were immediately stored at -20 C or -70 C until assayed. Assays for the following routinely were made on the samples with a flame photometer and autoanalyzer (Model SMA 12, Technicon Corp.) by use of standard procedures: sodium, potassium, calcium, magnesium, inorganic phosphate, glucose, urea nitrogen, uric acid, total protein, albumin, bilirubin, cholesterol, alkaline phosphatase, lactic dehydrogenase, and glutamic-oxaloacetic transaminase.

RESULTS

During hemodialysis in the absence of culture, dialyzable molecules in the blood reached and then maintained equilibrium with the dialysate solution. In Table 1 are listed the greatest and least values obtained from periodic sampling of the blood and dialysate during 9 hr of control dialysis. All of the values remained essentially within normal ranges. The lower values in the dialysate solution usually were those in the original dialysate, before equilibrium was established. Large molecules in the blood, such as the proteins, did not pass through the dialysis membrane. Subsequently it was demonstrated that the equilibrium was attained within 1 hr.

The original glucose-salts solution was inadequate to support growth of *S. marcescens*, mainly because of the absence of a nitrogen source. Even after attainment of equilibrium with blood, the dialysate solution supported only a slight amount of bacterial multiplication if samples of the dialysate were withdrawn into separate culture tubes and inoculated.

However, when hemodialysis was maintained continuously for an extended period, an inoculum of *S. marcescens* in the dialysate circuit of the system multiplied extensively and in a characteristic bimodal pattern (Fig. 2). Multiplication of the inoculum proceeded, usually after a short lag period, at an exponential rate (about 2 generations/hr) until a population density of about $10^{9.5}$ viable cells/ml was reached (Fig. 2A). At this juncture, the multiplication

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rate changed from an exponential to a linear function at about one-sixth the generation time (Fig. 2B). The shift in growth kinetics was explained by the bacterial population reaching a density for which the nutrient demand exceeded the steady-state supply of nutrients from hemodialysis. Because the diffusion of nutrients occurred at a linear rate, bacterial multiplication was limited similarly.

The effect of bacterial multiplication on glucose concentrations in the dialysate and in the blood is shown in Fig. 3. The dialysate glucose decreased rapidly to low values as the culture approached a density of about 10^9 viable cells/ml and became undetectable by the time a density of 10^{10} viable cells/ml was reached. The blood glucose also decreased, but not so greatly.

When a larger inoculum was employed, the bimodal pattern of multiplication also occurred, but with the exponential rate persisting only for a short period (Fig. 2C). The rate of multiplication soon became linear (Fig. 2D), and the population continued to increase in this fashion for several hours. A maximum was reached at about $10^{10.6}$ viable cells/ml, followed by a decline in the population.

At first the cultures were managed with highly aerobic conditions in the fermentor because of knowledge that relatively little oxygen from the blood can diffuse through a cellulose dialysis membrane (2,7). However, strictly anaerobic conditions in the fermentor resulted in the same bimodal growth pattern and supported essentially

the same exponential and linear rates of multiplication (Fig. 4). Apparently the limitation on growth rate of *S. marcescens* by oxygen supply (2,3,8) occurs only in artificial media or at population densities in excess of those attained with hemodialysis culture. The rate of exponential multiplication in hemodialysis culture, about 2.0 generations/hr, was essentially as great as the maximum that had been attained by any other means of culture or type of medium.

The artificial-kidney hemodialyzer was available only with one membrane porosity, which is equivalent to retention of molecules larger in nominal molecular weight than about 10,000. However, chemical beaker dialyzers, which were constructed similarly with a bundle of hollow membrane fibers and available with three different types of dialysis membrane, were found satisfactory for use as a hemodialyzer. In Table 1 are included the concentrations of some blood components in the beaker dialysates after equilibrium hemodialysis without culture.

The growth response in goat hemodialysis culture of *S. marcescens* with beaker dialyzers of three different membrane porosities is shown in Fig. 5. The MW 300 beaker dialyzer did not support a significant change in bacterial population over an extended time period (Fig. 5A and B) although dialysate glucose decreased slightly during the trial, indicating metabolic activity by the culture. In vitro dialysis with the MW 300 beaker and trypticase soy broth medium in the reservoir also failed to support growth. The MW 10,000 beaker, which is constructed of the same membrane as in the artificial kidney, and the MW 30,000 one

both supported a bimodal pattern of growth (Fig. 5C and D, Fig. 5E and F, respectively) much the same as that with the artificial kidney (compare with Fig. 2). Representative rates were 1.7 generations/hr during exponential growth and 0.2 generations/hr during linear growth with the MW 10,000 beaker, and 1.8 generations/hr (exponential) and 0.3 generations/hr (linear) with the MW 30,000 beaker.

DISCUSSION

A bacterial septicemia in vivo, in terms of growth kinetics, usually represents a steady state between parasite multiplication in the blood serum and clearance by phagocytosis, with any net growth considerably less than the maximum rate. Only when the host defense mechanisms are overwhelmed can the parasite population increase rapidly and exponentially. Such a fulminating septicemia was simulated by the conditions of hemodialysis culture, in which assimilable nutrients from the serum were continuously available to the bacterial culture but phagocytes were excluded. The results during the primary growth phase of *S. marcescens* demonstrated that even an organism capable of rapid multiplication can attain its maximum exponential rate from the continuous supply of dialyzable blood constituents.

The secondary phase of linear growth, i.e., first-order kinetics, ensued when the rate of nutrient demand by the bacterial population exceeded the steady-state rate of nutrient diffusion through the membrane, which then became limiting. This situation seems unlikely

to happen with an in vivo septicemia, and would be delayed in hemodialysis cultures with smaller inocula (compare Fig. 2B and 2D) or those with organisms that multiply more slowly. The onset of linear growth is primarily a function of the effective surface area of membrane relative to the amount of organisms.

Only a limited number and type of hollow-fiber membranes were available for use in hemodialysis culture. The MW 300 beaker membrane proved incapable of supporting growth, apparently because essential nutrient molecules were retained. The MW 10,000 membrane in the beaker or artificial kidney and the MW 30,000 beaker membranes both supported a similar pattern of growth. Both regulate the passage of much the same size class of small molecules from the serum, and both retain serum proteins.

Experiments usually were terminated after 12 to 15 hr of continuous hemodialysis culture because the animals showed signs of acute physiological stress, such as pyrexia and leukopenia, which were presumed to result from toxins produced by the culture and continuously diffused into the animal. This toxemia is characterized in a succeeding paper.

ACKNOWLEDGMENTS

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TABLE 1. *Concentrations[a] of molecular components in goat blood and those in dialysate solution during extended periods[b] of continuous hemodialysis without culture*

Component	Blood serum	Dialysate solution			
		Artificial kidney	MW 300 beaker	MW 10,000 beaker	MW 30,000 beaker
Sodium	366 - 390	310 - 390	-	-	-
Potassium	14.4 - 23.6	17.6 - 20.7	-	-	-
Calcium	5.0 - 7.6	2.4 - 7.2	4.3 - 5.8	4.5 - 5.6	2.8 - 4.1
Magnesium	2.3 - 3.1	1.8 - 3.3	-	-	-
Inorganic phosphate	5.0 - 8.1	4.1 - 7.7	0.3 - 0.4	0 - 5.2	0.3 - 4.5
Glucose	65 - 88	70 - 84	58 - 62	62 - 74	40 - 57
Urea nitrogen	17 - 22	9 - 21	0 - 7	0 - 13	0 - 14
Uric acid	0	0	0	0	0
Total protein	4.6 - 6.2	0	0	0	0
Albumin	2.5 - 3.2	0	0	0	0
Bilirubin	0.2 - 0.4	0	0	0	0
Cholesterol	100 - 200	0	0	0	0
Alkaline phosphatase	0 - 12	0	0	0	0
Lactic dehydrogenase	80 - 152	0	0	0	0
Glutamic-oxaloacetic transaminase	80 - 125	0	0	0	0

[a]Concentrations are expressed as mg/100 ml, except albumin and protein as g/100 ml, lactic dehydrogenase as Wroblewski units, alkaline phosphatase as King-Armstrong units, and the transaminase as Karmen units.

[b]9 hr with artificial kidney and 10 to 12 hr with beaker hemodialyzers.

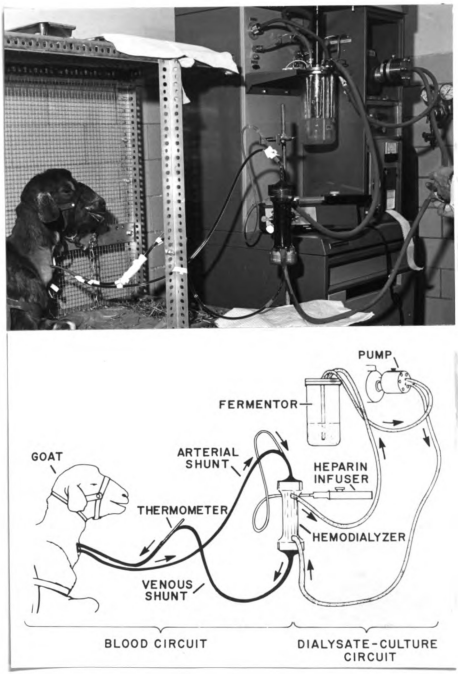

FIG. 1. *Hemodialysis culture system. The major components of the experimental system shown in the photograph (top) are identified in the tracing (bottom). An artificial kidney is shown, but beaker dialyzers also were employed as the hemodialyzer.*

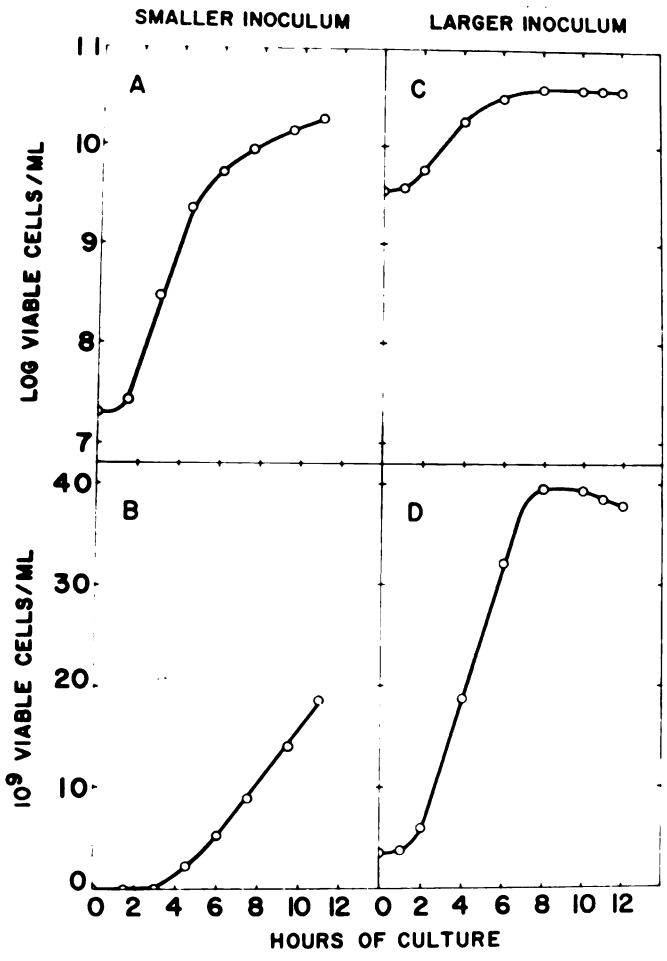

FIG. 2. *Growth curves of S. marcescens in goat hemodialysis culture with an artificial kidney, with a smaller inoculum (A and B) and a larger one (C and D). The results are plotted with both an exponential scale (A and C) and a linear scale (B and D) on the ordinates.*

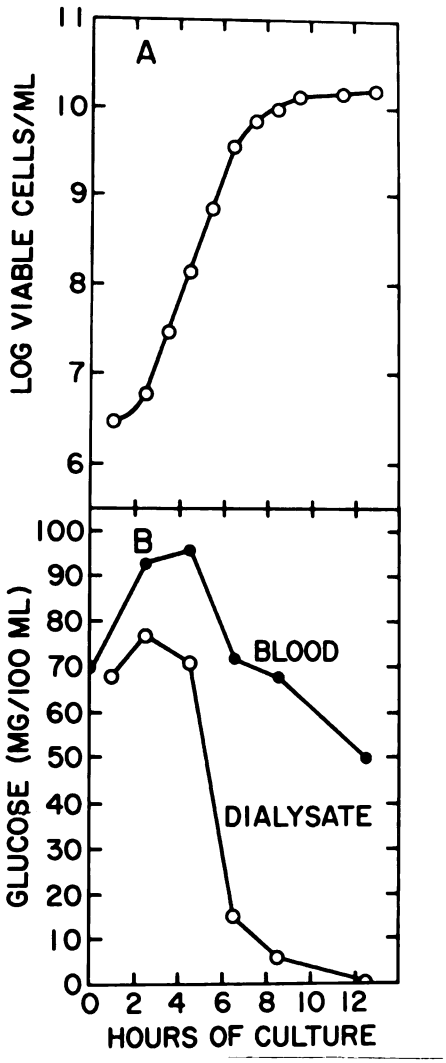

FIG. 3. *Effect of goat hemodialysis culture of S. marcescens on glucose concentration in the dialysate (open circles) and in the blood (closed circles).*

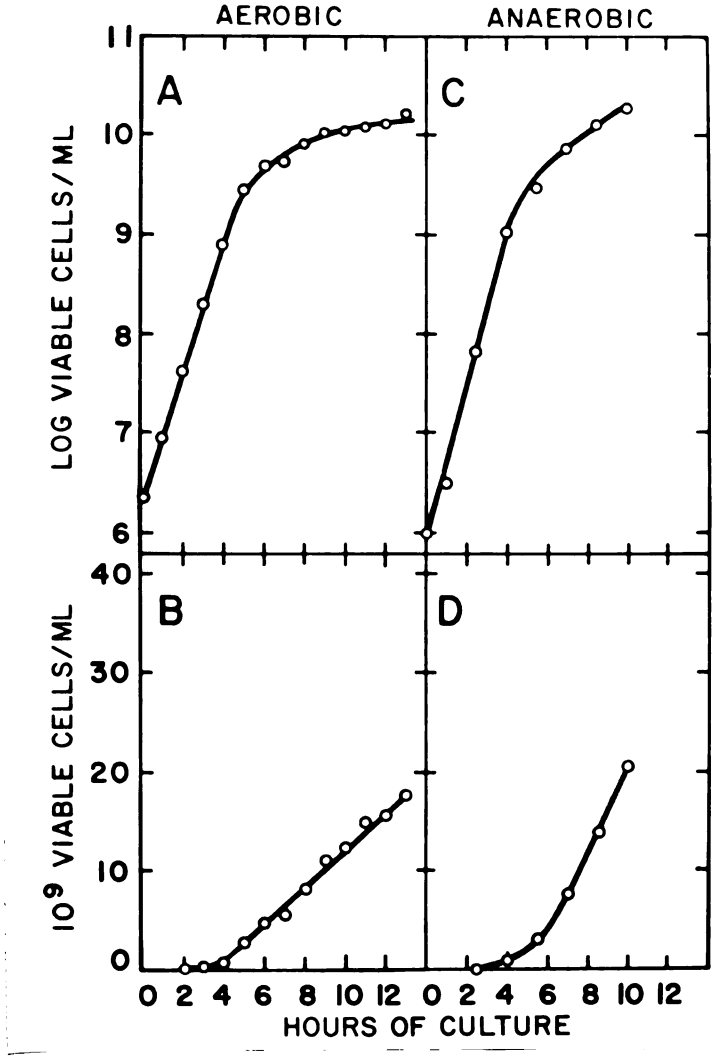

FIG. 4. *Growth curves of S. marcescens in goat hemodialysis culture with highly aerated conditions (A and B) and strictly anaerobic conditions (C and D) in the dialysate-culture circuit. The results are plotted with both an exponential scale (A and C) and a linear scale (B and D) on the ordinates.*

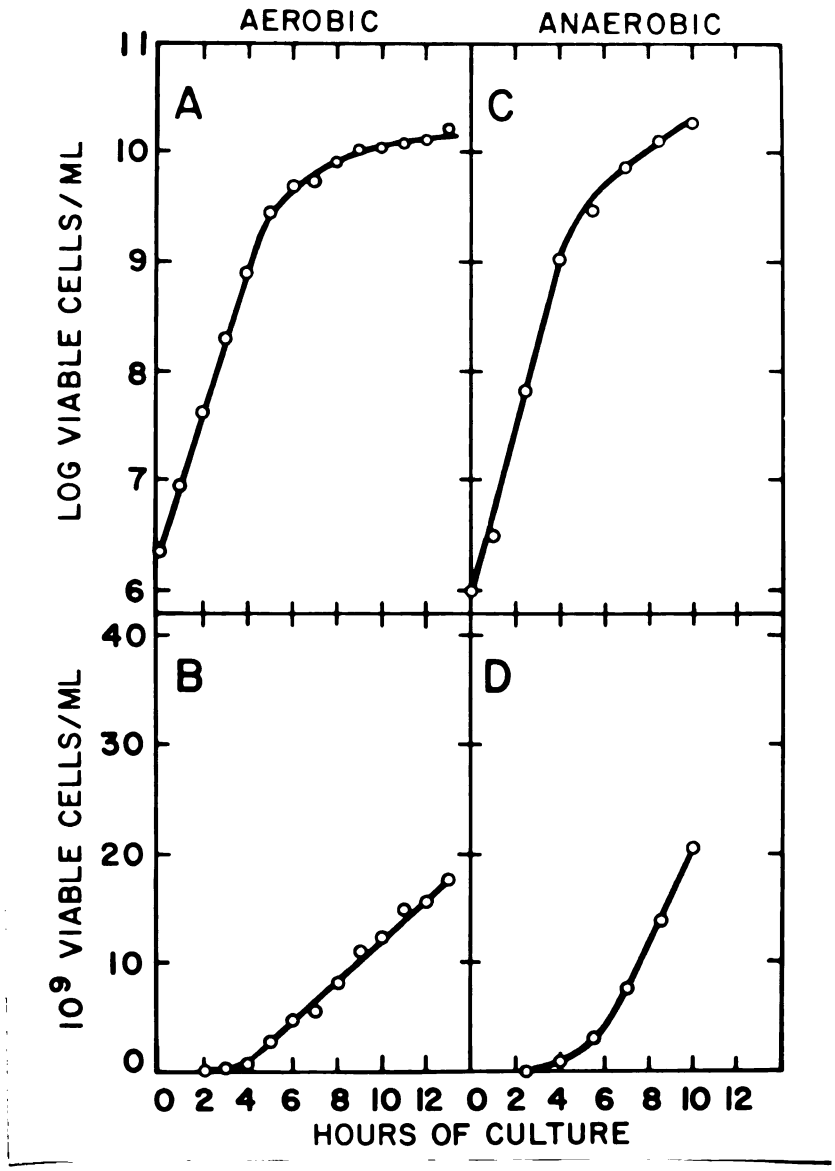

FIG. 4. *Growth curves of S. marcescens in goat hemodialysis culture with highly aerated conditions (A and B) and strictly anaerobic conditions (C and D) in the dialysate-culture circuit. The results are plotted with both an exponential scale (A and C) and a linear scale (B and D) on the ordinates.*

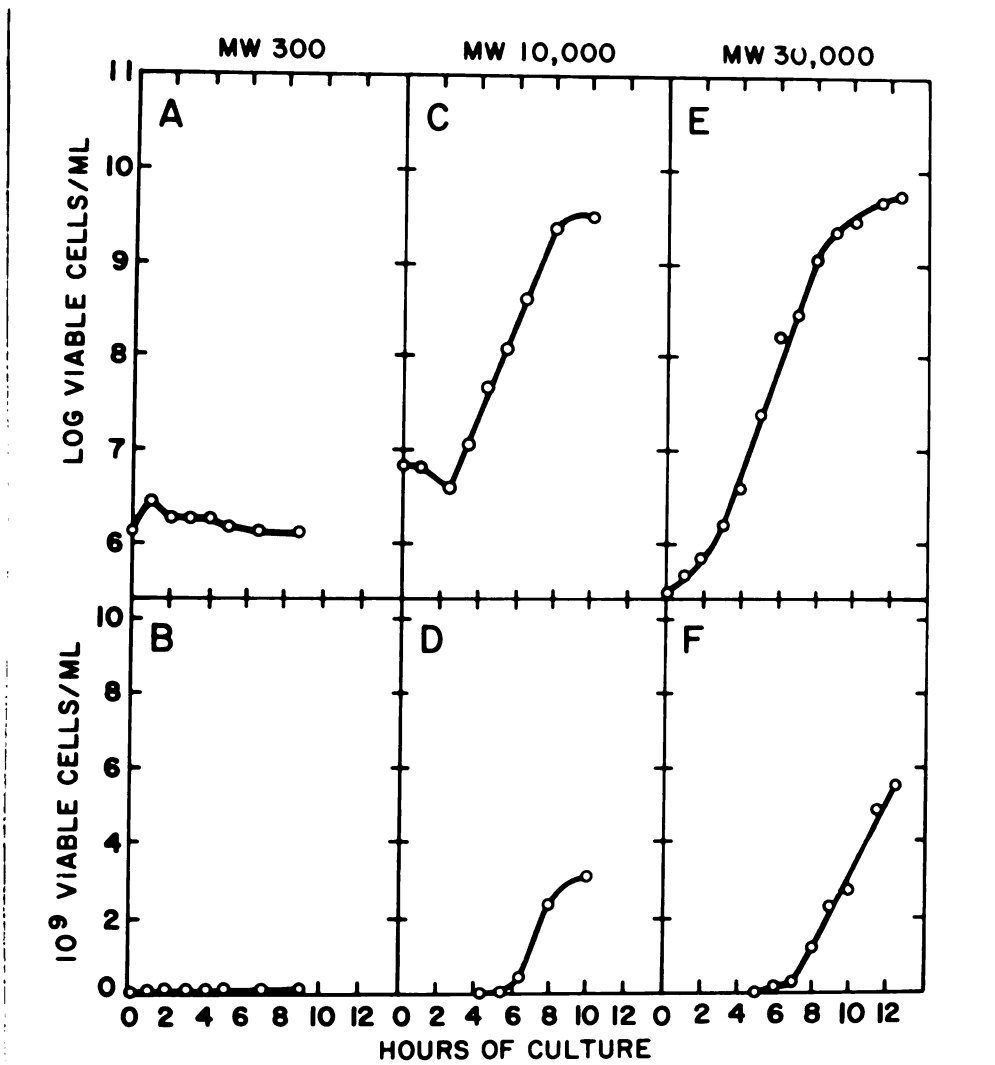

FIG. 5. *Growth curves of S. marcescens in goat hemodialysis culture with a beaker hemodialyzer having a membrane porosity equivalent to retention of nominal molecular weight 300 (A and B), 10,000 (C and D), and 30,000 (E and F). The results are plotted with both an exponential scale (A, C and E) and a linear scale (B, D and F) on the ordinates.*

5.2.2 *Goat hemodialysis culture of Serratia marcescens as a model of septicemia: Toxemia. (Manuscript)*

JOHN M. QUARLES, RALPH C. BELDING, TEOFILA C. BEAMAN, and PHILIPP GERHARDT

Manuscript for submission to

Infection and immunity

(ABSTRACT)

Serratia marcescens grown by continuous hemodialysis in a fermentor - artificial kidney system caused a general toxemia with acute pyrexia and leukopenia in the host goat, more so with aerobic than anaerobic culture conditions. A large amount of purified *S. marcescens* endotoxin in the system produced similar effects, but only transiently and to much less an extent. The dialyzable toxic culture material depressed the body temperature of precooled mice, even after boiling the sample, but produced equivocal results with chick-embryo lethality and *Limulus* coagulation tests. The use of beaker dialyzers with different membrane porosities indicated that the size of the dialyzable toxic material was equivalent to a nominal molecular weight between approximately 300 and 10,000. By analysis of the membrane diffusion threshold, the maximum molecular size was further defined relative to a rigid globular protein of 15,000 in molecular weight and 1.9 nm in hydrodynamic radius or a flexible fibrous polyglycol of 5,500 in molecular weight and 2.6 nm in hydrodynamic radius.

(INTRODUCTION)

Hemodialysis culture is a way to obtain growth conditions in vitro simulating those in mammalian blood, but unrestrained by phagocytosis. The blood stream is surgically shunted via prosthetic tubing from a major artery through the hollow-fiber membranes of an artificial-kidney hemodialyzer and back into a major vein of the animal. Concurrently, the dialysate culture is pumped from a modular fermentor through the hemodialyzer jacket outside the membranes and back into the fermentor. In this way, the culture and blood are in continuous diffusional communication but the cells of each are separated.

With the goat and *Serratia marcescens* selected as a host-parasite model, this new system allows the bacterial inoculum initially to multiply at the maximum exponential rate. Eventually the rate of diffusion becomes limiting, and the multiplication of bacteria then changes to a lesser and linear rate. Populations in the order of $10^{10.5}$ viable cells/ml are attained in a 12-hr period (2).

The response of the goat host to this simulated fulminating septicemia with *S. marcescens* was examined in the studies reported below. Because the membrane in the artificial kidney prevents the passage of molecules larger in nominal molecular weight than approximately 10,000, including macromolecular toxins, a toxemic response was not expected and did not occur initially as the culture grew. However, an acute toxemia with signs of pyrexia and leukopenia developed secondarily. This host response to culture was compared with that to purified lipopolysaccharide endotoxin in the same situation, the dialyzable toxic

material was separated and its heat stability and biological activity were assayed with tests considered indicative of endotoxin, and the maximum size of the dialyzable toxic material was defined relative to two types of molecules.

MATERIALS AND METHODS

The hemodialysis culture system, with a modular fermentor and either an artificial kidney or a beaker dialyzer, was the same as described previously (2) except for the use in certain trials of three beaker dialyzers also in the dialysate-culture circuit (Fig. 1). In this arrangement, three separate samples of dialyzable culture product were obtained simultaneously while the animal was hemodialyzed with the culture. Unless otherwise stipulated, the culture in the fermentor was aerated and stirred.

Vascular surgery and management of the goat for hemodialysis were the same as described previously (2). The body temperature was measured with a rectal thermometer as frequently as needed. Samples of blood for biochemical analyses were prepared and analyzed as before (2). Samples of blood for cell counts were treated with EDTA to prevent clotting. Leukocyte and erythrocyte counts then were made by microscopic examination of the cells by use of counting chambers, and differential cell counts by use of Wright-stained smears.

Tests for endotoxin-like activity were made by chick-embryo lethality (4) and *Limulus* coagulation assays (5,6). Activity also was assayed by the effect on the temperature regulation of precooled mice held at 4 C, with 0.5 ml of sample injected intraperitoneally. Rectal

temperatures were taken every 30 min for 3 hr (YSI model 42SC Tele-Thermometer with physiological probe, Yellow Springs Instrument Co.). Ten mice were used for each sample, and the average temperature change was determined.

Purified lipopolysaccharide endotoxin was studied by allowing the hemodialysis culture system (with the artificial kidney) thoroughly to equilibrate for 4 hr and then introducing the endotoxin into the fermentor at a final concentration of 0.05 mg/ml. The endotoxin was a commercial preparation from *S. marcescens* (Lipopolysaccharide W, Difco Laboratories).

Membrane diffusion thresholds of the Cordis-Dow hollow-fiber artificial kidney were determined by conducting dialysis of polyethylene glycols (PEG, Union Carbide) of known molecular sizes and molecular weights. Polyethylene glycol 4000 (41.0 osmolal), with number average molecular weight ($\overline{M}_n$) 3350 was dialyzed against 20.5 osmolal PEG 20,000 ($\overline{M}_n$ 17,500). Polythylene glycol 1540 ($\overline{M}_n$ 1540) and PEG E 9000 ($\overline{M}_n$ 9,500, Dow Chemical Co.) also were dialyzed against the high molecular weight glycol of the dialysis membranes to maintain osmotic stability.

The volume of glycol was 400 ml and dialysis was conducted for 4½ hr at 23 C. The change in concentration of polyethylene glycol was measured by refractometry (Bausch and Lomb Precision Refractometer, Model 33-45-01, sodium light source) and by dry weight determinations. The molecular weight distribution of PEG 4000 before and after dialysis was determined using Biogel P-10 (Biorad Laboratories) by the method of Scherrer and Gerhardt (7). The mass of the polymer in each elution fraction (1.5 ml) was determined by dry weight measurements.

RESULTS

Hemodialysis without culture. During hemodialysis in the absence of culture, body temperature of the goat remained within a normal range, 102 to 103.5 $\pm$ 1 F (Fig. 2A). However, total leukocytes in blood samples from the animal increased in number during the first few hours of control hemodialysis and then remained essentially constant at the higher level (Fig. 2B). Erythrocytes remained at an approximately constant level from the outset (Fig. 2C), and biochemical constituents also were unaffected control hemodialysis (see Table 1 in reference 2).

Blood samples also were removed from the inlet and outlet of the artificial kidney at various times during control hemodialysis. The results (Table 1) indicated the hemodialyzer itself did not significantly affect the numbers of erythrocytes, leukocytes or total cells. Similar studies with differential cell counts indicated that the passage of blood through the dialyzer did not have a selective effect on either of the two most numerous types of leukocytes (neutrophils and lymphocytes).

Hemodialysis with culture. When an inoculum of *S. marcescens* was introduced into the dialysate solution, after the usual equilibration period, the host goat at first remained quite normal. This lack of host response persisted for a number of hours, depending on the size of the inoculum, even though the bacterial population in the dialysate-culture circuit may have risen to 10^9 viable cells/ml. An acute episode of fever then ensued with dramatic suddenness. The general picture of this pyrexia is shown in Fig. 3, in which the

average temperature change in six trials was normalized as a function of the number of viable bacterial cells in the dialysate culture.

The time courses of this acute fever and other host responses in a representative trial are shown in Fig. 2, together with control responses of the same goat during hemodialysis without culture. The body temperature (Fig. 2E) started to rise after about 7 hr of hemodialysis culture, which coincided with the time when a population of 10^9 viable cells/ml was exceeded (also see Fig. 3) and when multiplication changed from an exponential to a linear rate in the culture (Fig. 2D). An acute reduction in the number of peripheral blood leukocytes was a second main sign of host toxemia (Fig. 2F), and typically the onset of leukopenia preceded that of the pyrexia by about 2 hr. The number of erythrocytes remained within the normal range (Fig. 2G). Among the other physiological parameters monitored, only the concentration of blood glucose changed significantly with hemodialysis culture (see Fig. 3 in reference 2).

In addition to and at about the same time as these host responses in the second phase of hemodialysis culture, the goat evidenced general signs of toxemia with violent shivering and lethargy that persisted for 2 hr or more. In some instances bloody urine was passed, but in other cases kidney function appeared blocked. Feeding and drinking also stopped during an episode of toxemia.

The fever and leukopenia observed with aerated hemodialysis cultures were less apparent with cultures maintained under strictly anaerobic conditions, although the cultures multiplied similarly in

the two situations (Fig. 4). The reason for the difference in host responses was not apparent.

It might be thought that the host responses were caused by some nonspecific effect of bacterial growth. However, goat hemodialysis culture of *Bacillus anthracis* (the avirulent Sterne vaccine strain) did not result in a toxemic host response even though massive bacterial growth occurred with either aerobic or anaerobic conditions in the fermentor.

Hemodialysis of endotoxin. The host response to hemodialysis culture of *S.marcescens* was compared with that to a large amount of purified lipopolysaccharide endotoxin from the same bacterium (Fig. 5). After the equilibration period and about 1 hr after the introduction of endotoxin into the dialysate solution, the body temperature increased by about 1.5 F and the number of peripheral leukocytes decreased by about $3000/mm^3$. The animal also showed signs of discomfort, but not as marked as with hemodialysis culture of *S. marcescens*. These effects with pure endotoxin persisted for less than 2 hr, then returned to normal and remained constant for the rest of the trial. The small and transient nature of the responses suggested either that the endotoxin preparation was not pure or that biologically active endotoxin represents a wide distribution of molecular sizes, some of which are dialyzable. Apparently only a small part of the total amount participated in the reaction.

Differential hemodialysis culture and tests for endotoxic-like activity. Beaker dialyzers with three different porosities were employed to separate and estimate the molecular size of the dialyzable toxic material produced during hemodialysis culture of *S. marcescens*. Although the artificial-kidney hemodialyzer was available only in the one porosity of membrane [maximum retention equivalent to nominal molecular weight (MW) of about 10,000], chemical beaker dialyzers were available not only in the same membrane porosity but also in a finer one (MW 300) and a coarser one (MW 30,000). Each type was substituted for the artificial kidney in the hemodialysis culture system, and the results are shown in Fig. 6. The total surface area of membrane in each beaker dialyzer was only one-tenth of that in the artificial kidney, and consequently the time courses of the culture and the host responses were somewhat different than before.

The MW 300 beaker as a hemodialyzer failed to support any multiplication of the inoculum (Fig. 6A), and no significant responses were observed in the host (Fig. 6B and C). The MW 10,000 beaker as a hemodialyzer supported much the same patterns of culture growth (Fig. 6D) and host responses (Fig. 6E and F) as did the artificial kidney (compare with Fig. 2). A longer lag period than before was observed in the culture, and the fever response of the goat occurred about in the middle of the exponential phase of culture growth. The MW 30,000 beaker as a hemodialyzer also supported a typical pattern of culture growth (Fig. 6G), although the fever (Fig. 6H) and leukopenia (Fig. 6I) occurred earlier in the time course than usual,

probably because a high bacterial population density was reached earlier as the result of a larger inoculum in this particular trial.

The differential dialysis system shown in Fig. 1 then was employed to collect three different size classes of culture product in the beakers simultaneously with artificial-kidney hemodialysis. After the goat evidenced acute toxemia, the three beaker-dialysate samples were removed and assayed, as follows.

Injection of the MW 10,000 beaker-dialysate sample into another goat (3 ml, intravenously) partially duplicated the results obtained by direct hemodialysis culture with either the artificial kidney or the same beaker dialyzer. Even though the amount of toxin was much less than that transferred over a period of time in continuous hemodialysis, the MW 10,000 beaker sample evoked pyrexia, but without measurable leukopenia. Similar results were obtained with the MW 30,000 beaker sample. However, the MW 300 beaker sample (and also the original salts solution, employed as a control) did not evoke any measurable host response.

The three beaker-dialysate samples also were tested by intraperitoneal injection into precooled mice and measurement of body temperature depression, a test which is considered indicative of endotoxin (1,3). The results are shown in Fig. 7. Neither the original salts solution (Fig. 7A) nor the dialysate sample from the MW 300 beaker (Fig. 7C) had a significant effect. The cell-free hemodialysis culture medium had the greatest effect, and lowered the temperature in the mice by about 2.5 C (Fig. 7B).

A depression of about 1 F was obtained with the MW 10,000 beaker sample and about 2 F with the MW 30,000 beaker sample. Diarrhea occurred in each case during the time of temperature depression. All of the mice were observed for at least 48 hr afterwards, without further signs of illness. The beaker-dialysate solutions appeared free of bacterial growth throughout the culture trial and were sterile when tested by inoculation of 0.5 ml samples into trypticase soy broth at termination, so clearly the results were attributable to dialyzable toxic molecules.

The three beaker-dialysate samples also were assayed by two other tests considered indicative of endotoxin, those for chick-embryo lethality (4) and *Limulus* coagulation (5,6). Only marginal effects occurred, and the results were considered equivocal.

The toxic material was found to be heat stable by heating the active beaker-dialysate samples at 100 C for 5 min and then measuring the body-temperature response of precooled mice to injections of the heated sample. Results like those in Fig. 7D and E were obtained.

Molecular size of the dialyzable toxic material. The results from use of the beaker dialyzers with different membrane porosities indicated that the size of the dialyzable toxic material was equivalent to a nominal molecular weight more than about 300 and less than about 10,000. The latter definition of size is relative to the molecular weight of globular proteins that reportedly just diffuse through the membrane in the artificial kidney and MW 10,000 beaker dialyzer (Bulletin No. 175-1187-71, Dow Chemical Co.). Of the proteins employed, cytochrome c (presumably of bovine heart origin,

molecular weight 13,370) lies closest within the dialysis threshold of the membrane and has an equivalent hydrodynamic radius (r_{ES}) of 1.88 nm, determined with the Einstein-Stokes formulation and based on an experimentally determined diffusion coefficient of $D_{20,w}$ = 11.4 x 10^7 (8). Myoglobin (presumably of horse heart origin, molecular weight 16,890) lies just outside the dialysis threshold of the membrane and has an r_{ES} = 1.90 nm, based on $D_{20,w}$ = 11.3 x 10^7 (8). Consequently, the MW 10,000 membrane has a threshold porosity equivalent to a rigid globular molecule approximately intermediate in size between the above two proteins, i.e., molecular weight 15,000, r_{ES} = 1.89 nm.

However, the dialyzable toxic material from *S. marcescens* might be more fibrous and flexible in its molecular configuration, and its size might be better related to the dialysis threshold of a more comparable molecule. A long flexible molecule (like a polyethylene glycol) in solution coils randomly and loosely into a spherical form that is large relative to its molecular weight. Scherrer and Gerhardt (7), using polyglycol samples, have devised a distribution-analysis method to determine the porosity threshold of bacterial cell walls.

By use of this new method, the exclusion threshold of the artificial-kidney membrane was successfully determined with a polydisperse polyethylene glycol sample of 3,350 mean in number-average molecular weight ($\overline{M}_n$), whereas smaller and larger polyglycol

samples proved unsatisfactory. The results (Fig. 8) showed that the size of a just-excluded molecule, determined from the intersection point of the two distribution curves, was equivalent to a quasi-monodisperse polyethylene glycol of M_n = 5,500 and r_{ES} = 2.6 nm.

Thus, the maximum molecular size of the dialyzable toxic material from *S. marcescens* was further defined relative to two different types of molecules. If its molecular configuration were like a rigid globular protein, the toxic material was less than molecular weight 15,000 and r_{ES} = 1.9 nm. If like a flexible fibrous polyglycol, it was less than molecular weight 5,500 and r_{ES} = 2.6 nm.

DISCUSSION

The nature of the dialyzable toxic material demonstrated by goat hemodialysis culture of *S. marcescens* is unclear, but the most likely possibility seemed that of an endotoxin or endotoxin-like substance. Bacterial endotoxins produce diverse responses in animals, including fever, transient leukopenia (followed by leukocytosis), hyperglycemia, circulatory system disturbances, altered resistance to bacterial infections, hemorrhage, and (in sufficiently large doses) irreversible shock and death. The fever, leukopenia, and general physiologic response of the goat, and the thermostability of the toxic material, all were consistent with endotoxemia. The hypothermic

reaction of mice obtained with the dialyzable toxic material was considered especially characteristic of endotoxin (1,3), and the results were similar to those reported by Dennis (1) for *Vibrio fetus* endotoxin.

Native endotoxin usually is considered to be comprised of a macromolecular complex of protein, lipid and polysaccharide with a nominal molecular weight of about 1,000,000. Molecules of this size could not permeate the membranes of any of the dialyzers used in this study. However, the smallest moiety of endotoxin that yields some or all of the typical reactions is unclear. Milner and Finkelstein (4) reported that the state of dispersion of endotoxin particles profoundly affects pyrogenicity and chick-embryo lethality tests but does not influence mouse lethality tests. In the present experiments, continuous hemodialysis allowed the passage of relatively small molecules in a natural state. It is possible that these unaltered molecules then aggregated or became adsorbed to blood constituents, and thereby had activities which would be lost or altered during the traditional chemical and physical procedures used in the purification of endotoxin.

It is possible that the dialyzable toxic material produced in hemodialysis culture of *S. marcescens* was not endotoxin. Proliferating bacteria produce many types of metabolic products, including pyrogenic ones of small size. It seems improbable that an organic acid or other ordinary metabolite would cause the range or severity of reactions that were observed. But it is possible that a small moiety of the lipopolysaccharide molecule caused the results, e.g., lipid A or conjugated

or simple protein (9,10). This also would account for the similar but lesser response obtained by hemodialysis of purified endotoxin.

The argument also might be advanced that the results were caused by intact bacterial cells passing through an undetected opening in the membrane. However, in control experiments in vitro with artificial-kidney dialysis cultures and a reservoir of nutrient medium, the reservoir remained sterile for at least 24 hr. Furthermore, in the control hemodialysis trials, blood proteins did not pass into the dialysate circuit.

ACKNOWLEDGMENTS

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TABLE 1. *Effect of passage through the artificial-kidney hemodialyzer on the numbers of blood cells*[a]

Blood sample	Leukocytes (no./mm^3)	Erythrocytes (no./mm^3)	Packed-cell volume (%)
Inlet (arterial)	15,000	9,450,000	21
Outlet (venous)	14,200	9,050,000	21

[a] Representative measurements taken after 3 hr of hemodialysis without culture in the dialysate circuit.

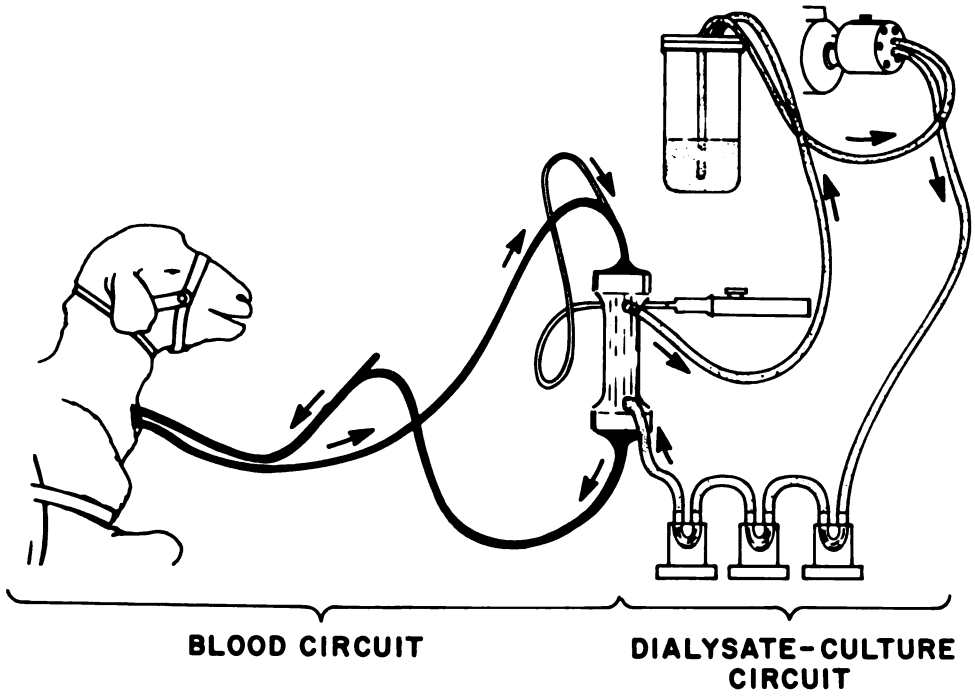

FIG. 1. *Differential hemodialysis culture system, with three beaker dialyzers of different porosity inserted in series into the dialysate-culture circuit. In all other respects, the system was the same as that depicted in Fig. 1 of Gerhardt, Quarles and Belding (1973).*

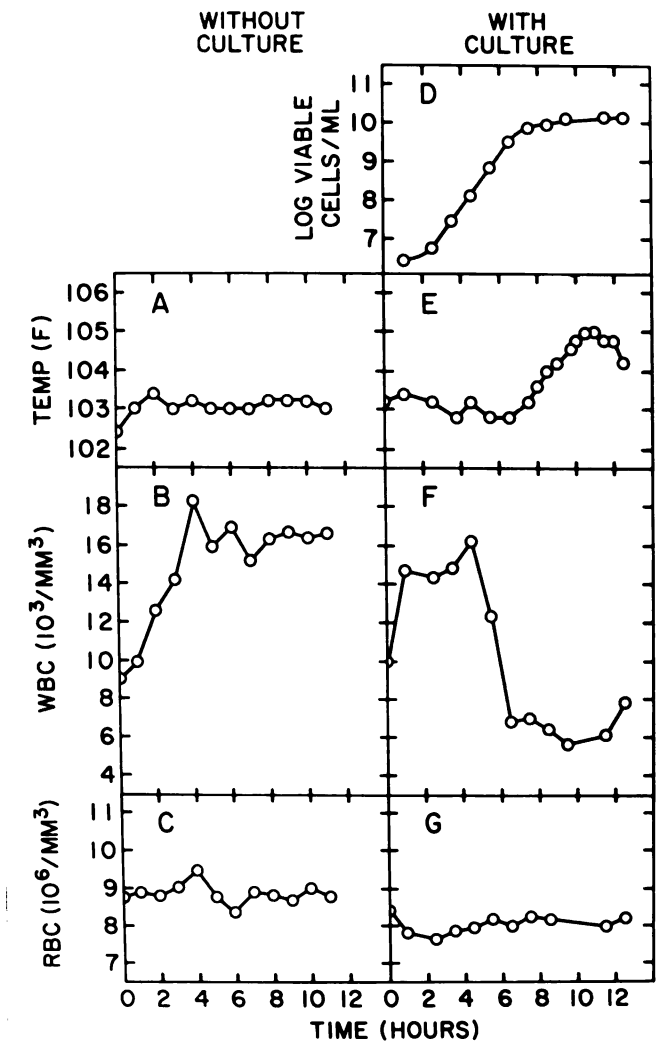

FIG. 2. *Time course of changes in the responses of a representative animal to hemodialysis without culture (left) and with culture (right): A and E, body temperature; B and F, white blood cells; C and G, red blood cells. In D there is shown the corresponding growth curve of Serratia marcescens in the dialysate-culture circuit.*

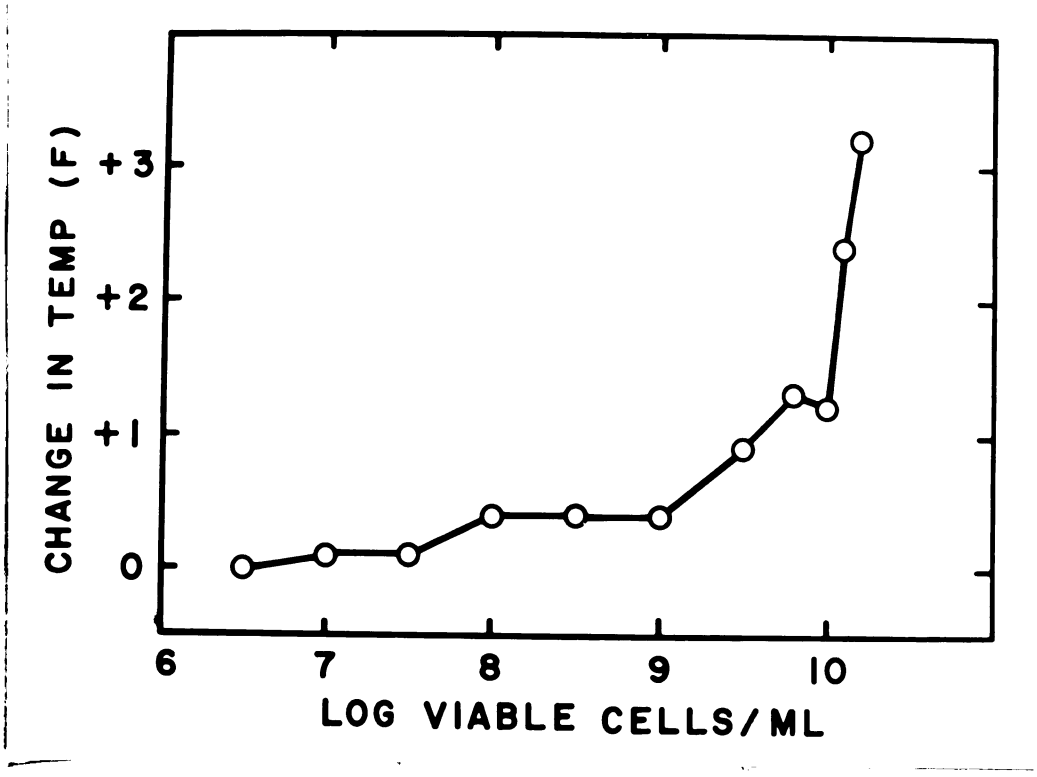

FIG. 3. *Normalized change in host body temperature as a function of the numbers of viable cells of S. marcescens in hemodialysis cultures, averaged from six cultures with four animals.*

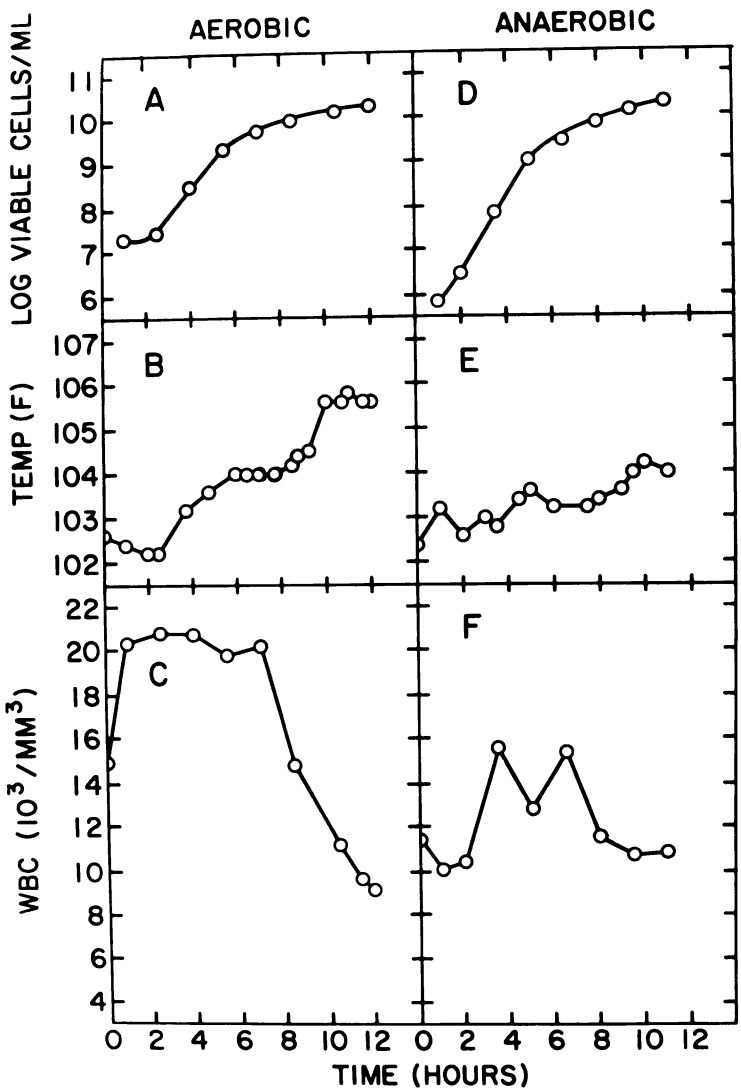

FIG. 4. *Comparison of anaerobic with aerobic hemodialysis culture conditions on body temperature and blood leukocytes of the animal.*

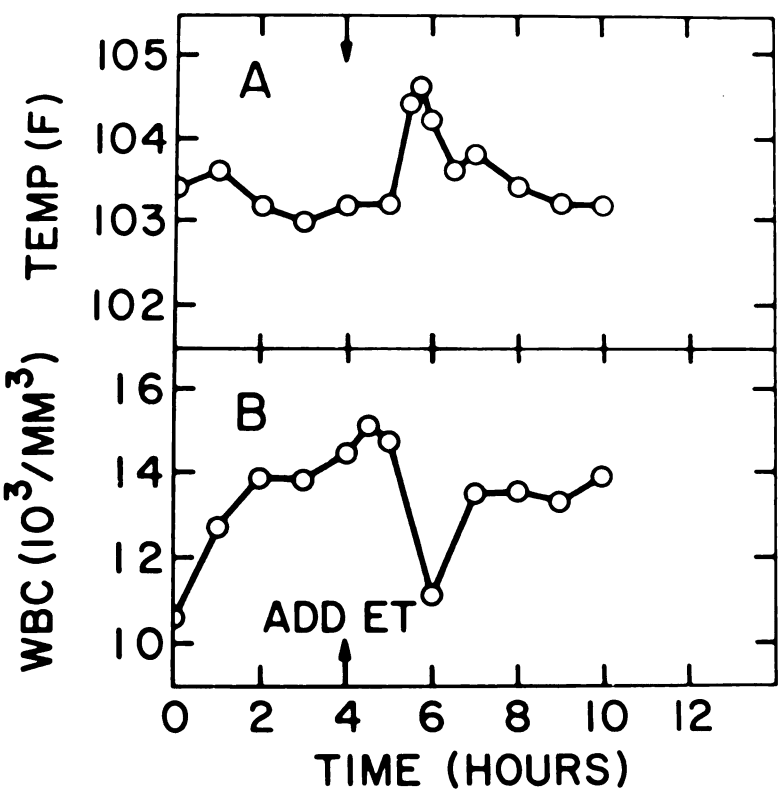

FIG. 5. *Time course of changes in the response of a goat to hemodialysis of purified lipopolysaccharide endotoxin. In A is shown the body temperature and in B the peripheral leukocyte count. The arrows indicate the time at which the endotoxin was added.*

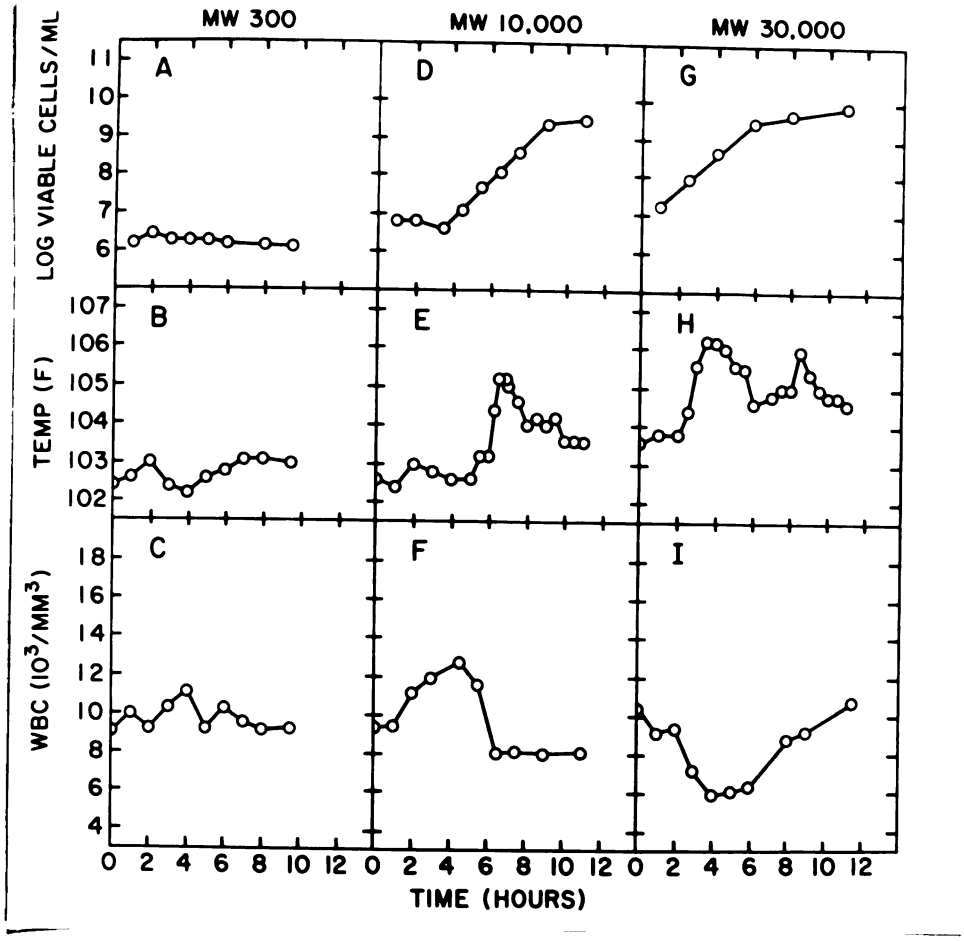

FIG. 6. *Time courses of hemodialysis cultures and host responses with beaker dialyzers of different porosity used as the hemodialyzer, each identified by the porosity threshold in nominal molecular weight of 300 (left), 10,000 (center), and 30,000 (right).*

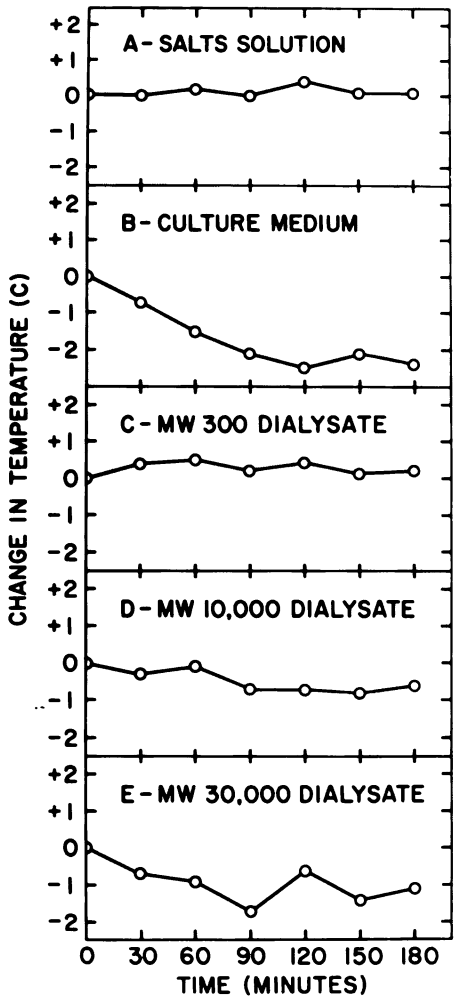

FIG. 7. *Body-temperature response of precooled mice (average of 10) to injection (0.5 ml, intraperitoneally) of salts solution (A), cell-free hemodialysis culture medium (B), MW 300 beaker-dialysate sample (C), MW 10,000 beaker-dialysate sample (D), and MW 30,000 beaker-dialysate sample (E).*

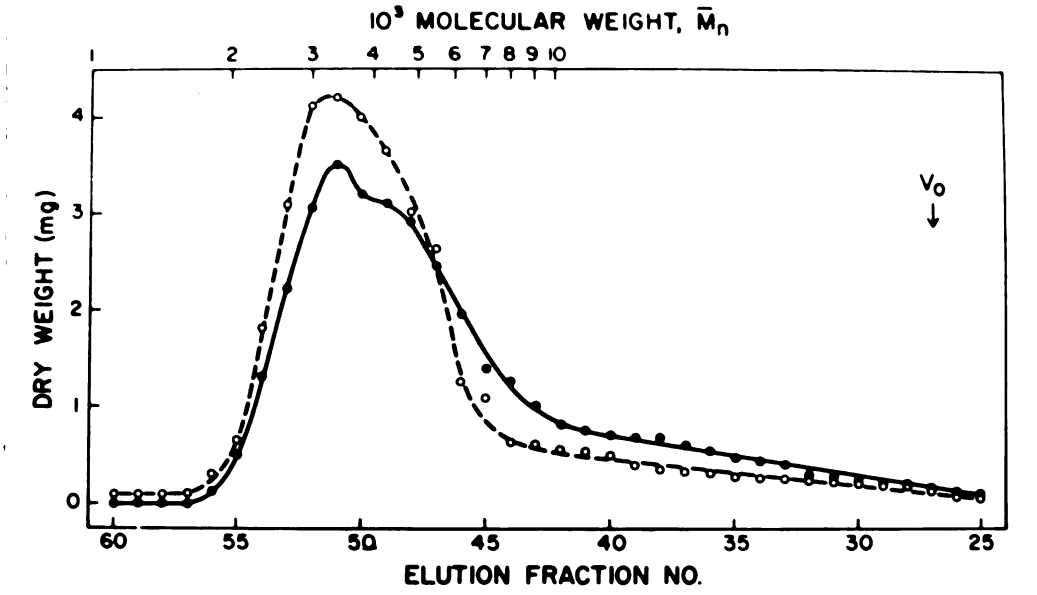

FIG. 8. *Molecular weight distribution of polyethylene glycol* $\bar{M}_n$ *3,350 before (o) and after (●) equilibrium dialysis through the artificial-kidney membrane. The intersection point represents the porosity exclusion threshold.*

5.3 Prosthetic hemodialysis culture unit

5.3.1 *Introduction*

The studies with the fermentor - artificial kidney - animal system indicated that in vivo conditions were not fully simulated by hemodialysis. This shortcoming was considered caused mainly by two factors: (1) the artificial-kidney membrane was impermeable to molecules of nominal molecular weights greater than about 10,000, including most blood proteins, and (2) the membrane was poorly permeable to gases, including CO_2 and O_2. Membranes that pass larger molecules and gases are available in the form of hollow-fiber dialyzers, but these are not designed for hemodialysis and are limited in the availability of different types of membranes. Furthermore, the design of the system and the associated equipment (fermentor, pumps, and tubing) is complex. Finally and importantly, the requirement for constant attendance with the animal restricted dialysis culture with an artificial kidney to about 15 hr.

Consequently, considerable effort was spent in the conception of a workable design and eventually in the construction and testing of prototype models of a hemodialysis culture unit which would allow the use of a nutrient-passing membrane simultaneously with a gas-transport membrane, which could be mounted on an animal, and which would enable dialysis culture over an extended time period. The aid of B. M. Stutsman in constructing the prosthetic hemodialysis unit is gratefully acknowledged.

Because prosthetic shunts of Teflon and Silastic rubber tubing were maintained for periods of several weeks without causing clotting or lysis of the animal's blood, we incorporated this design concept for the blood flow channel of the new hemodialysis culture unit. That is, the blood flowed through a single piece of Silastic rubber tubing, with an opening in one side of the tubing exposed to the membrane. This design had the advantage of causing minimal physical restrictions in the path the blood followed as it passed from the artery, through the dialyzer, and back into the vein. Also, the flow of the blood through the dialyzer swept across the membrane, thus helping keep it free of deposits and assuring more effective dialysis. Two separate "windows" (2 x 7 mm) of membrane were aligned longitudinally between the blood and dialysate, and these allowed molecular communication between the two regions. The design was such that the two windows may be of the same or different membrane material. Only a small amount of membrane (about 28 mm^2) was in contact with the blood. However, this membrane area was sufficient to supply nutrients to the culture chamber because the unit was designed for continuous dialysis with a small volume of culture (about 3.3 ml). The size of the entire unit was made so that it could easily be mounted on a goat's neck when attached to the arterial-venous shunt and impose only minimal restrictions on the animal's movements. The design employed also eliminated the need for continuous infusion of anticoagulant and the requirement for continuous monitoring of the animal and equipment. Satisfactory agitation of the culture was achieved via the animal's normal movement.

This prosthetic hemodialysis culture unit likely will be changed and improved in the future. The results with the prototype unit reported in the following sections are incomplete and are intended as a progress report to aid in further development of the device and application of it in hemodialysis culture.

5.3.2 *Materials and Methods*

The prosthetic hemodialysis culture unit in present state of development is depicted in Fig. 1. The assembled dialyzer consists of four main pieces: (1) the blood chamber, consisting of a plastic block fitted with silicone rubber tubing with a slotted opening, (2) membranes, (3) supporting metal plate, and (4) dialysate-culture chamber. The unit is self-contained, with the blood, membranes and culture all in close proximity. The two chambers were machined from Lucite plastic, and measured about 2 cm x 3.5 cm x 3.5 cm when assembled.

The dialysate or culture chamber was 3.5 cm x 3.5 cm x 0.9 cm in outside dimensions. Inner dimensions of the chamber were 0.7 cm x 2.1 cm x 2.1 cm, which provided a working volume of approximately 3.3 ml. Sampling from the dialysate-culture chamber was achieved in a first model by puncture of a self-sealing rubber vaccine-bottle stopper, and in a subsequent model by a similar but smaller stopper at the end of tubing extending from the sides of the chamber. This change made sampling easier while the unit was attached to the animal and also protected the membranes from possible puncture. In use, one of the two adaptors was used for sampling and the other for

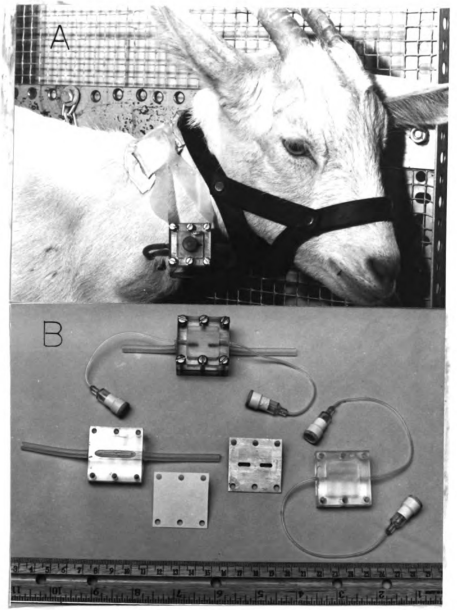

FIG. 1. *Prosthetic hemodialysis culture unit. A. The unit is shown mounted on the neck of a goat and connected with tubing to an arterial-venous shunt. B. The unit is shown assembled at the top and beneath it (left to right) are the component parts: blood chamber with silicone rubber tubing, a sheet of cut and punched membrane, a stainless steel support plate, and the dialysate-culture chamber with two sampling extensions.*

equilization of air pressure. Three non-threaded holes were drilled in each of two sides of the culture chamber, through which screws passed into corresponding threaded holes in the blood chamber block.

The blood chamber block was approximately 0.9 cm x 3.5 cm x 3.5 cm in size. An angled hole was drilled through the block from two of the (opposite) smaller faces so that exit and entry ports were formed on these sides and a slot was formed on one of the larger faces. Silicone rubber tubing was threaded through this hole and firmly attached to the block with Dow-Corning Silastic medical adhesive silicone Type A (catalog #891). The tubing was then trimmed flush with the face of the block, thereby forming a silicone rubber-lined blood flow channel with a longitudinal opening (see Fig. 1). The membrane covered this opening when the unit was in use.

A metal supporting plate was constructed of polished 24 gauge stainless steel (about 0.05 cm in thickness). Two separate holes (2 mm x 7 mm and 4 mm apart) in the center of the plate were aligned longitudinally over the slotted opening of the blood channel in the blood block. This steel plate provided support for the membrane and the two holes allowed the use of two different types of membrane.

Any type of sheet membrane material which is compatible with blood may be used in the hemodialyzer, either alone or in combination with another type. Examples of tested materials include dialysis membranes of cellophane (DuPont 315), filter membranes of

cellulose acetate (Millipore Ha 0.45 μ and Gelman GA 8), filter membranes of polycarbonate (Nuclepore GE 10, 0.1 μ) and gas-transport membranes of silicone rubber (Dow-Corning Silastic). Membranes of two different types of material were prepared by cutting a hole in one (properly aligned with the blood channel and hole in the supporting plate) and covering the opening with the second material. Dow-Corning Silastic medical adhesive was used to join the two membranes. The adhesive is non toxic and produces a strong bond between any combination of the membranes listed above.

The unit was connected to the external arterial-venous shunt by means of Teflon connectors. For dialysis trials, the assembled unit was attached to the animal's neck in a variety of ways. At first it was held on the side of the neck in a tape-and-gauze sling, which allowed the shunt and connector tubing to flex and bend without crimping and reducing blood flow. The unit also was mounted on the back of the neck by use of longer connecting tubing, but this increased the resistance to blood flow. The last method was to tie the unit to a supporting platform constructed of rubber-covered fiber mesh, which was sutured to the animal's neck. This provided a firm but flexible support from which the unit could be quickly removed and re-attached. The open mesh nature of the support allowed circulation of air and prevented irritation and infection.

The unit as presently constructed was not steam sterilizable but instead had to be sterilized with ethylene oxide (Bard Sterilizer System 2279, D. R. Bard, Inc.). Prior to use, the dialyzer

was loosely assembled with the membrane in place and exposed to the gas for 6 to 18 hr. However, the unit could be machined of steel, polycarbonate, or other steam sterilizable material if desired.

Routinely, a solution of 0.85% NaCl was used as the initial solution in the chamber. A saline solution was used because it is non-nutritive and allowed determination of electrolytes dialyzed into the chamber from the animal's blood, while also preventing hemolysis of erythrocytes. In some instances whole blood or tissue was placed in the chamber. With the artificial kidney - fermentor system, it was necessary to use a more complex, balanced solution because the large volume used in the dialysis chamber (800 ml) would upset the animal's electrolyte balance if saline were the dialysate. The small volume (3.3 ml) of the plastic hemodialysis culture unit allowed the use of a less complex solution.

For periodic sampling, as in growth studies, specimens of 0.1 ml were removed and the volume replaced with saline. For equilibrium studies, specimens of varying amounts up to the entire contents of the chamber were removed as required. This was necessitated primarily by the fact that the autoanalyzer used for biochemical tests required 4 ml specimens, and generally not greater than about 1 to 5 dilutions, for maximum reliability. Biochemical tests, hematologic studies, and growth curves were conducted as described for the artificial kidney - fermentor system except in the instances as given in the materials and methods sections of the particular trials listed below.

5.3.3 Prosthetic hemodialysis without culture

5.3.3.1 *Introduction*

The prosthetic hemodialysis unit allowed the use of membranes composed of essentially any material, with the limitation only that the material be compatable with blood and in sheet form. In order to obtain information on the types of molecules from blood which traverse the three main types of membrane (dialysis, filter and gas-transport membranes) under conditions of equilibrium hemodialysis, samples of these materials were tested in the unit without culture. These experiments were designed to provide both qualitative data on the types of molecules which passed (or were retained by) the membranes and also a rough comparison of rates and amounts.

5.3.3.2 *Materials and methods*

The dialysate chamber of the miniature hemodialysis unit was filled with sterile 0.85% NaCl and the unit was connected to the arterial-venous shunt. Hemodialysis was allowed to proceed for 1 hr (2hr in the example of DuPont 315 dialysis membrane), and then the resulting dialysate solution was removed and tested for the blood constituents as listed in Table 1. Biochemical tests were conducted by standard procedures on an SMA 12 autoanalyzer (Laboratory of Clinical Medicine, Lansing, Mich.). In each trial, prior to use, the hemodialyzer was sterilized with ethylene oxide gas, with the designated membranes in place. Combination membranes were prepared as described in Section 5.3.2.

5.3.3.3 *Results*

The results of trial dialyses with the several membranes are shown in Table 1.

5.3.3.4 *Discussion*

The silicone rubber membrane (Dow-Corning Silastic) is a gas-transporting material without porosity (119), and consequently none of the blood components assayed for in this trial dialyzed through this membrane (Table 1). Therefore, it should be recognized that in dialysis trials using silicone rubber in combination with a nutrient passing membrane, the effective membrane area for nutrient transfer is reduced by 50%.

Dialysis membranes, generally manufactured from regenerated cellulose, have an average rated pore size of about 5 nm diameter. Thus, they retain large molecules (e.g., albumin and other serum proteins or enzymes) but pass small molecules (e.g., glucose or salts). The membrane used in these trials retained albumin or the other proteins but allowed calcium, phosphorus, glucose, and urea to pass. Glutamic-oxalacetic transaminase values for this trial, and the other trials, in the approximate range of 10 to 15 units are considered of marginal significance because of variability in several negative control specimens.

The filter membranes tested, manufactured of cellulose acetate or polycarbonate, contain larger pores (in the range of 0.1 to 0.45 μ) and therefore pass many proteins and other large molecules. As shown in Table 1, Gelman, Millipore, and Nuclepore membranes passed

TABLE 1. Effect of different membranes on passage of selected blood components[a] *during 1 hr hemodialysis*

Component	Silicone rubber	DuPont 315 dialysis[b]	Gelman GA 8 filter	Gelman GA-8 filter and silicone rubber	Millipore HA filter	Nuclepore GE 10 and silicone rubber
Calcium	0	0.5	1.1	1.1	3.4	0.4
Inorganic phosphate	0	0.7	1.0	0.6	2.3	0.7
Glucose	0	35	10	5	20	10
Urea nitrogen	0	5	2	2	10	1
Total protein	0	0	0.7	0.6	2.4	0.2
Albumin	0	0	0.3	0.2	1.0	0.2
Bilirubin	0	0	0	0	0.1	0
Cholesterol	0	0	10	10	44	trace
Alkaline phosphatase	0	0	0	0	4	trace
Lactic dehydrogenase	0	15	10	5	20	5
Glutamic-oxaloacetic transaminase	0	15	18	10	30	10

[a] Original concentration in each case was 0. Concentrations expressed as mg/100 ml, except albumin and protein as g/100 ml, lactic dehydrogenase as Wroblewski units (W.U.), alkaline phosphatase as King-Armstrong units (K.-A.U.), and transaminase as Karmen units (K.U.).

[b] Two hr dialysis in this instance.

albumin and the other proteins. The lower level of glucose passed by these membranes as compared to the dialysis membrane is believed due to the fact that the results for the dialysis membrane were after 2 hr dialysis.

Some characteristics of the membranes which are important to hemodialysis must be considered. Gelman and Millipore membranes are very rough at a microscopic level. Thus, although they contain a large amount of empty space and are very porous and efficient for dialysis, they are not compatible with blood. These membranes tended to attract the blood cells and other blood constituents and cause clotting, especially during hemodialysis trials of several hours.

The Nuclepore filter membrane was found to be much more compatible with blood, and caused little if any destruction of blood cells. It did not cause clotting and remained free of extraneous materials during extended hemodialysis trials. However, it has the disadvantage of having relatively few pores per unit area, thus reducing its efficiency for dialysis. Therefore, the particular characteristics of the membranes must be considered before deciding which type is best. The membrane with the largest pores may not always provide the best results in a hemodialysis system.

5.3.4 Prosthetic hemodialysis culture of *Serratia marcescens*

5.3.4.1 *Introduction*

Serratia marcescens had been used as the primary test bacterium for the artificial kidney - fermentor hemodialysis culture system, and therefore was chosen as a test organism for the prosthetic unit, to provide a point of reference between the two systems. The DuPont 315 dialysis membrane was employed because of its similarity to the dialysis membrane of the artificial kidney. In order to make the comparison more meaningful, the dialysis membrane was not combined with any other type in the prosthetic unit.

5.3.4.2 *Materials and methods*

Unless otherwise stated, or restated for emphasis, the procedures used in this and subsequent culture trials with the miniature prosthetic unit were the same as previously described for the studies with the artificial kidney system.

Cells of *S. marcescens*, strain 8UK, grown for about 12 hr in trypticase soy broth were centrifuged for 15 min at 5000 x g, resuspended in 0.85% NaCl, and an inoculum of about 10^7 viable cells/ml introduced into the culture chamber of the miniature hemodialysis unit. Samples (0.1 ml) of culture were removed from the dialysate-culture chamber periodically, diluted in 1% peptone water, and surface plated on trypticase soy agar for viable cell determinations. The volume in the chamber removed by sampling was replaced with saline. It should be recognized that this represented approximately

a 3% dilution of the culture in the chamber, and was compounded with each sampling. For this reason, samples were taken less frequently than with the artificial kidney system. Blood specimens were taken periodically by disconnecting the arterial tube of the shunt. The temperature in the dialysate-culture chamber was approximately 35 to 36 C, as determined in previous control dialysis trials.

5.3.4.3 *Results*

The results of an 11 hr hemodialysis culture trial with *S. marcescens* are shown in Fig. 1. The exponential growth rate was approximately 0.8 generations/hr, and the maximum density was about $10^{8.5}$ viable cells/ml. Growth in the second phase was so slow as to be almost stationary. The organism was multiplying linearly, but the generation time was estimated from Fig. 1 as greater than approximately 20 to 24 hr/generation.

There was no observable fever response nor alteration of leukocyte numbers in the host goat (Fig. 1). The temperature remained between 103 and 104 F, and leukocyte numbers remained essentially constant during the entire trial. No signs of toxemia (shivering, discomfort, or other distress) were observed.

5.3.4.4 *Discussion*

Serratia grew well in the prosthetic hemodialysis culture unit. However, the generation rate was lower (about 0.8 generations/hr vs. 2 generations/hr), the maximum viable cell density was less ($10^{8.5}$ vs. $10^{10.5}$ viable cells/ml), and the phase of linear growth

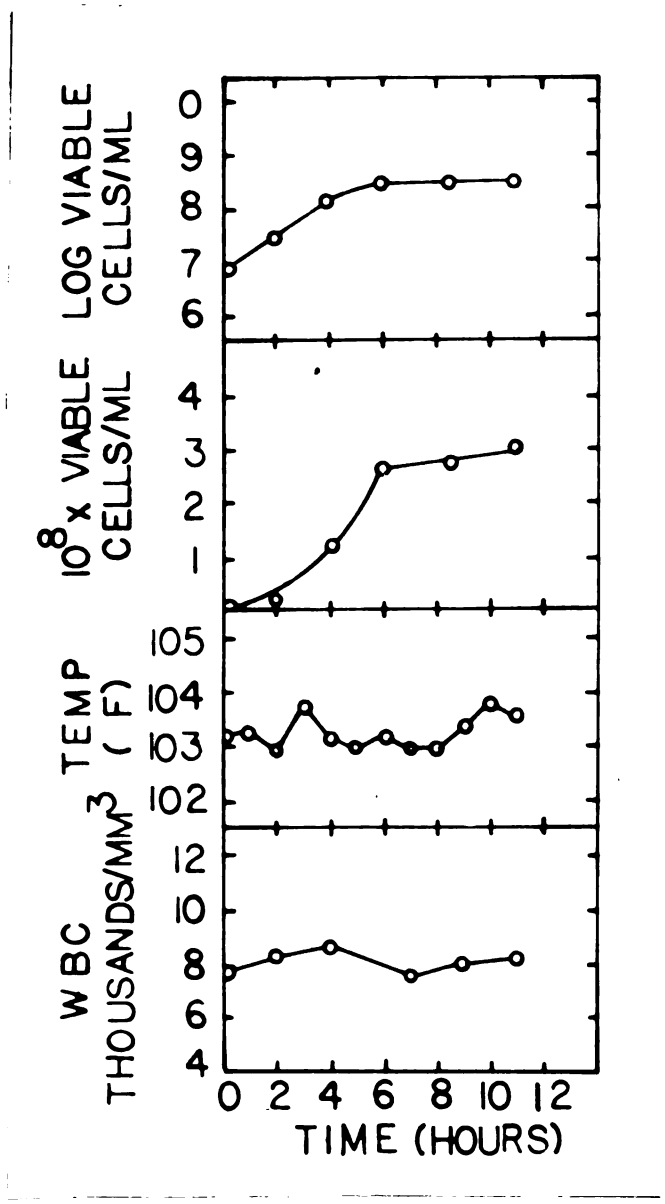

FIG. 1. *Time courses of hemodialysis culture and host response to growth of S. marcescens in the prosthetic unit.*

was at a much lower rate and less distinct (i.e., nearly stationary) than with the artificial kidney system. Significantly greater viable cell densities may be beyond the capability of the prosthetic unit, as indicated by the fact that the later (linear) growth rate was so slow (about 20 to 24 hr/generation). This was likely caused by insufficient nutritional supply from the animal, due to insufficient membrane area in the dialyzer, as discussed below.

There are several possible reasons for the differences reflected in the growth results. In the trial with the prosthetic hemodialyzer, the culture chamber was initially charged with bacteria suspended in saline instead of in a balanced salt solution in equilibrium with the animal. This was intended to show that the organism would receive all required nutrients via dialysis from the animal's blood into saline instead of into a balanced salt solution. As a direct comparison with the artificial kidney system, this was obviously a break in routine. This difference between the two techniques, however, should not affect the rate or extent of growth, because all organic nutrients required for continued bacterial multiplication came from the blood in both situations.

The problem of dilution of the culture in the chamber (about 3%/sampling) also had some effect on the growth characteristics, but this also was not considered of primary importance.

Three other factors were considered more important. The first was that the ratio of effective membrane to culture volume was

different in the two systems. The artificial kidney contains 1 m^2 of membrane surface area (10^6 mm^2) and was used with a volume of 800 ml culture, which provided a ratio of about 1,250 mm^2 of membrane/ml of culture. The miniature hemodialysis unit contains about 28 mm^2 of membrane surface area (two windows, each approximately 2 x 7 mm) and about 3.3 ml of culture volume, which provided a ratio of about 8.5 mm^2 of membrane/ml of culture. The ratio for the artificial kidney thus was about 150 times greater. This allowed much more efficient dialysis and therefore was much better able to meet the nutritional demands of the culture.

Another variable inherent in the design of the two systems concerns temperature control. The artificial kidney - fermentor system was used at an externally controlled 39 C, whereas the miniature unit depended primarily on the animal's blood and body temperature for temperature control of the culture. Even if enclosed in protective insulation, the chamber was thus influenced to some degree by changes both in the animal's temperature and ambient temperature of the surroundings. The temperature in the chamber was determined to be about 35 to 36 C during a typical dialysis trial, and this was some 3 to 4 C less than that in the fermentor system.

The most important difference between the two culture systems was in turbulence. In the artificial kidney - fermentor system, the cell suspension was greatly stirred and continuously circulated, with resulting high turbulence. In the prosthetic unit, the cell

suspension was neither stirred nor circulated, and consequently was quiescent. Thus, the liquid film around each cell was much greater in the prosthetic unit and nutrient and product transfer at the cellular level was much slower.

The absence of toxemia in the goat (Section 5.2) with the miniature hemodialyzer may have been because the volume of culture and the cell density attained were insufficient to affect the animal observably. The total number of bacterial cells involved was about 10^{13} for the fermentor system but only about 10^{9} for the miniature hemodialysis unit. This difference in total numbers may have been significant.

Therefore, it appears there are significant differences inherent in the two systems. The artificial kidney - fermentor system was designed for short-term experiments with rapidly multiplying organisms, and, due to the large volume of culture, is especially applicable to studies of toxemia. The miniature hemodialysis unit was designed for long-term experiments with organisms of low generation times, and the small culture volume limits its value in toxemia studies.

5.3.5 Simulation of in vivo conditions evaluated by growth of *Bacillus anthracis* in the two hemodialysis culture systems

5.3.5.1 *Introduction*

Bacillus anthracis was chosen as a test organism to evaluate the simulation of in vivo growth conditions in hemodialysis culture, because of the relatively easily scored markers this organism provides. In vivo, anthrax cells usually occur singly or short chains, are square ended in shape, do not sporulate, and encapsulate.

5.3.5.2 *Materials and methods*

The avirulent Sterne strain of *B. anthracis* was grown overnight in trypticase soy broth, washed free of medium by centrifugation and resuspension in saline, and about 10^6 cells/ml used as the starting population in the chamber. In selected experiments with the prosthetic unit, an initial population of 10^2 to 10^3 cells/ml was used in the chamber.

For tests in the artificial kidney - fermentor system, all experimental conditions were as previously described (Section 5.2), with both aerobic and anaerobic conditions employed in the fermentor vessel.

The prosthetic hemodialysis unit was used as described in Section 5.3.4, except that two membranes also were tested in combination. The following membranes were used: (1) DuPont 315 dialysis membrane and silicone rubber, (2) Gelman GA 8 filter membrane alone, (3) Gelman GA 8 filter membrane and silicone rubber.

Bacteria (approximately 10^6 cells/ml for large inocula and 10^2 to 10^3 cells/ml for small inocula) were suspended in saline for tests with each of the three membranes listed, and in whole heparinized goat blood (100 USP units sodium heparin/ml) for tests with the GA 8 and silicone rubber membranes in combination.

Viable cell counts, hematologic and biochemical tests were made as previously described. Microscopic examination was by phase microscopy, gram stains, and India ink wet mounts. Specimens for electron microscopy were deposited on 0.2 μ Millipore filters, layered with agar, and fixed by the Ryter-Kellenberger (112) procedure. Thin sections were cut with a diamond knife on an LKB ultramicrotome, post stained with uranyl acetate and lead citrate, and examined on a Philips 300 electron microscope.

5.3.5.3 *Results*

B. anthracis grew well in 12 to 13 hr trials in the artificial kidney - fermentor system, under both anaerobic and aerobic conditions. However, the organism chained so extensively in both cases that it was impossible to obtain reliable counts of viable cells. Microscopic examination revealed massive formation of chains, indistinguishable from those formed in vitro. There was no evidence of capsule formation. Beginning stages of sporulation were detected by electron microscopic examination of thin sections of the cells after 10 to 12 hr of culture.

It was postulated that both high molecular weight molecules and the balance of CO_2 and O_2 in the blood might be important in

duplicating the in vivo environment. The artificial kidney used in earlier studies did not allow for the diffusion of these constituents. Therefore, the prosthetic unit was provided both with a filter membrane and a gas-transport membrane. The chamber allowed the use of whole blood in the culture chamber, and this also was tested.

In the prosthetic hemodialysis culture unit, the results with a large original population (about 10^6 viable cells/ml) in the chamber were similar to those seen in the fermentor system. With dialysis membranes and silicone rubber, and with Gelman filter membranes alone or with silicone rubber, the organism chained as though growing in vitro. Inclusion of whole goat blood in the chamber as the initial solution had no significant effect. In all instances, chaining was so extensive that viable cell counts were not reliable, and the cells had the morphology typical of in vitro growth.

However, when a small original inoculum (about 10^2 to 10^3 cells/ml) was tested in the chamber with Gelman GA 8 and silicone rubber membranes, the bacilli tended to remain as short chains of 2 or 3 cells during approximately 6 hr of culture. As the cell density increased, chaining also increased. It was concluded that chaining was absent or minimal only at very low cell densities.

No evidence of capsule formation was noted in any of the samples from hemodialysis culture. Sporulation was not observed by phase contrast examination of cells cultured in the chamber of the prosthetic unit for 12 hr.

No evidence of distress or toxemia was observed in the host goats during hemodialysis culture of the anthrax bacillus in either system. Blood cell counts and temperature readings remained within normal limits, and no unusual biochemical results were noted.

5.3.5.4 *Discussion*

The environment provided by the artificial kidney - fermentor system did not simulate in vivo conditions, as evaluated by growth and morphology of *B. anthracis*. The dialysate obtained by use of a dialysis membrane alone presumably was deficient in high molecular weight molecules such as proteins, and in gases such as CO_2 and O_2.

It was thought that by using a filter membrane which passed more blood components than dialysis membranes, that in vivo conditions would be better simulated. However, the use of a filter membrane, or a filter membrane in combination with a gas transport membrane, at first did little better. The improvement was also slight when whole blood was used as the initial medium in the prosthetic culture chamber. In all cases, the organism grew well by qualitative observation, but chained extensively. However, short chains, indicative of in vivo conditions, were observed at very low cell densities. This result probably reflected a close similarity to in vivo conditions, until diffusion limitations of nutrients became restricting. As growth proceeded, more cells were found in long chains and the in vivo simulation was not maintained.

The Sterne strain of *B. anthracis* reportedly is genetically incapable of forming capsules, so this was not considered a valid test criterion.

Inclusion of whole (goat) blood in the chamber had relatively little effect on the way the organism grew. This was believed due at least partially to metabolism of the blood cells (in addition to metabolism of bacterial cells) and the subsequent nutrient limitations in the chamber (see Section 5.3.9 on control studies with blood cells).

The major problem apparently was that the chamber and membranes partially limited the availability of nutrients. As cell density increased, this limitation became so acute that environmental conditions were established which were very different from those in the blood stream, and also different from those at a very low cell density in the chamber. It was also possible that intimate contact with tissue cells was required for a true in vivo environment. It was apparent that inclusion of peripheral blood cells was insufficient to fully duplicate in vivo environmental conditions at high cell densities.

Thus, it was concluded that neither the artificial kidney - fermentor system nor the prosthetic hemodialysis culture unit fully simulated the in vivo environment of the blood, as evaluated by growth and morphology of *B. anthracis*. It seemed likely, however, that an efficient hemodialyzer comprised of membranes which pass both large molecules and gas might provide at least a partial simulation, acceptable for some investigations. Further work in this area would seem warranted.

5.3.6 Prosthetic hemodialysis culture of *Streptococcus pyogenes*

5.3.6.1 *Introduction*

Streptococcus pyogenes produces various extracellular enzymes and toxins, and the organism or antigenic material associated with it are implicated in heart and kidney disease. In order to investigate the feasibility of conducting future studies on this organism and its products by hemodialysis culture, the bacterium was grown in the prosthetic hemodialysis unit.

5.3.6.2 *Materials and methods*

Strain SS 235 of *S. pyogenes* was obtained from R. Facklam, Streptococcus Unit, Center for Disease Control, Atlanta, GA. The inoculum for hemodialysis culture was prepared by growing the organism for 18 hr in trypticase soy broth at 37 C. Cells were centrifuged at 5000 x g for 15 min, resuspended in 0.85% saline, and introduced into the hemodialysis culture unit containing DuPont 315 membranes. An inoculum of approximately $10^{6.3}$ viable cells/ml was used. Hemodialysis was maintained for 11 hr.

Samples (0.1 ml) were removed periodically for viable cell determinations and the volume replaced with saline. The samples were diluted in 1% peptone water and surface plated on sheep red blood cell-trypticase soy agar. Characteristically small, beta-hemolytic colonies were counted. Plates were incubated 48 hr at 37 C. Hematologic and biochemical tests were as previously described.

Gram-stained smears of the culture were examined by light microscopy and negatively stained preparations (phosphotungstic acid), with a Philips 300 electron microscope.

5.3.6.3 *Results*

The organism multiplied well in hemodialysis culture but formed chains. Therefore, reliable cell counts were not obtained. Increasing chaining and increasing numbers of cells occurred during the period of hemodialysis culture.

The animal's temperature remained within 1 F of the initial reading during 11 hr of culture, and this was considered essentially constant. Leukocyte counts varied by a maximum of only about 500 per mm^3 during the growth trial, and this also was considered an insignificant change. No subjective signs of toxemia or distress were observed during the culture trial.

5.3.6.4 *Discussion*

Although it was not possible to obtain quantitative data, in terms of reliable cell counts, qualitative observations indicated that cells of the *Streptococcus* multiplied. This indicated that further studies on this organism are possible with hemodialysis culture techniques.

Dialysis membranes were chosen instead of filter membranes to provide minimal conditions for growth. If the organism would grow with dialysis membranes, it should grow with membranes passing larger molecules. The implication was that some molecular screening of different size molecules may be possible with hemodialysis culture.

This should allow investigations concerning the size of molecules which can stimulate toxic or antigenic reactions in the host. This may be especially important for streptococcal heart and kidney disease.

The fact that no toxic response was observed in the host goat was not surprising, based on knowledge that dialysis membranes retain large molecules and the previous findings that even the dialyzable toxin of *S. marcescens* was not detected during growth in the miniature hemodialysis unit. Lack of toxemia did not, however, preclude the possibility of immunologic response and this may be a much more fruitful direction for future hemodialysis culture work to take.

In vivo-grown cells of *S. pyogenes* tend to occur as single or double cells. Morphologic characteristics of in vivo-grown *Streptococcus* are not as distinct as those of *Bacillus anthracis*, but may be used to aid in evaluation of the miniature hemodialysis unit as a means of duplicating the in vivo environment. As in the studies with the anthrax bacillus, the streptococcus in hemodialysis culture formed long chains of cells. This provided additional data that the prosthetic hemodialysis unit (with dialysis membranes in this example) did not fully simulate the in vivo environment.

5.3.7 Prosthetic hemodialysis culture of *Blastomyces dermatitidis*

5.3.7.1 *Introduction*

Several species of fungi have the ability to grow in alternative morphological forms. The expression of this ability, termed dimorphism, generally depends on the cultural environment and is an important consideration because most of the more pathogenic fungi are dimorphic. These fungi are usually yeast-like in infected tissue but mold-like in laboratory media.

Blastomyces dermatitidis is an example of a dimorphic pathogenic fungus. This organism usually grows like a yeast at 37 C, but produces mycelium at 25 C. Like most other fungi, it multiplies relatively slowly (generation time about 48 hr) in comparison to bacteria such as *Serratia* (generation time about 0.5 hr).

In order to test the ability of the prosthetic hemodialysis unit to support growth of a slowly reproducing dimorphic fungus, the growth of *B. dermatitidis* was examined.

The aid of A. L. Rogers in preparing the organisms and evaluating the results is gratefully acknowledged.

5.3.7.2 *Materials and methods*

Two strains of *Blastomyces dermatitidis*, Saint Joseph and KL-1, were used. The inocula were grown in CYPG medium (per liter: casamino acids, 4 g; yeast extract, 4 g; peptone, 4 g; glucose, 10 g) to obtain actively multiplying cells. The organisms were washed and diluted in 0.85% saline to a concentration of approximately 3 to 5 x

10^5 cells/ml, and introduced into the miniature prosthetic unit fitted with DuPont 315 dialysis membranes. Goat hemodialysis culture was conducted for the time periods as indicated. Samples of the cell suspension were removed at various times and examined microscopically for numbers of cells, viability, and morphological characteristics.

5.3.7.3 *Results*

The concentration of cells of the St. Joseph strain increased from an original inoculum of 2.75×10^5 cells/ml to 5.25×10^5 cells/ml during 33 hr of hemodialysis culture. At termination, the cells were clumped and the clumps contained many non-viable cells. However, the cells were all in the yeast-phase.

The initial concentration of cells in the trial with the KL-1 strain was 5.62×10^5 cells/ml. Counts obtained during hemodialysis culture were 6.00×10^5 cells/ml after 28 hr, 6.75×10^5 cells/ml after 39 hr, and 7.00×10^5 cells/ml after 46 hr. At termination (46 hr) cells were vacuolated and otherwise abnormal in appearance. Viability determinations on the final suspension (using Janus Green B) indicated that, of a total of 6.50×10^5 cells, 2.50×10^5 were alive and 4.00×10^5 were dead. Cells were in the yeast-phase.

5.3.7.4 *Discussion*

The major findings of these experiments were that the prosthetic unit was satisfactory for long-term culture of a slowly multiplying microbe, and that the diffusate of dialysis membranes

supported growth of the fungus. The fact that many cells were non-viable at the end of the culture periods was not unexpected in light of previous studies, which indicated that diffusion of nutrients was a limiting factor for high cell densities in the prosthetic unit. It is also likely that near anaerobic conditions existed in the chamber, and this may have influenced clumping of the organisms. *B. dermatitidis* tends to clump more in liquid laboratory media than on solid laboratory media, and this too may have influenced the tendency toward clumping in hemodialysis culture. The cells were expected to remain in the yeast-phase because of the relatively high temperature (35 to 36 C), and this was observed.

The important implications of this experiment are that hemodialysis culture with dialysis membranes (i.e., a minimal diffusate) will support growth of *Blastomyces* (although limited in the extent) and that the prosthetic unit can be used for growth studies for the extended times (days) necessary for slowly growing cells.

5.3.8 Attempted prosthetic hemodialysis culture of *Treponema pallidum*

5.3.8.1 *Introduction*

Treponema pallidum was chosen as an example of a very fastidious, slowly reproducing organism which seemingly is obligately parasitic. Virulent strains of this organism have not been cultivated on artificial media, so growth in hemodialysis culture would provide a breakthrough of importance. Because specialized techniques and considerable experience were considered necessary for an attempt to culture virulent treponemes, members of the Venereal Disease Unit, U.S.P.H.S. Center for Disease Control at Atlanta collaborated on these studies. J. W. Foster and W. Clark provided rabbits and organisms, and conducted and evaluated the viability, motility, serologic and virulence tests.

5.3.8.2 *Materials and methods*

The virulent Nichols strain of *T. pallidum* was harvested from infected rabbit testicles. Testicular tissue and cellular debris were separated from bacterial cells by low speed centrifugation and deposition of bacteria on Nuclepore GE 10 (0.1 μ porosity) filters. In each trial, the suspension was adjusted by darkfield microscopy to contain 10^6 to 10^7 organisms per ml. The miniature hemodialysis culture unit was fitted with a combination of Nuclepore GE 10 and silicone rubber membranes. The following experimental conditions were used:

Trial 1 The chamber initially was charged with organisms suspended in a mixture of approximately equal volumes of 0.85% saline and pooled normal rabbit serum (inactivated). Hemodialysis was maintained for 21 hr. At termination, the treponemes were counted and examined for motility by darkfield microscopy, and were tested for virulence by skin tests in rabbits.

Trial 2 The chamber was charged with organisms suspended in inactivated goat serum that was enriched with goat leukocytes and red cells. Hemodialysis was conducted for 4 hr. At termination, the culture was examined as in Trial 1.

Trial 3 The chamber was charged with organisms suspended in inactivated goat serum containing rabbit testicular tissue. Hemodialysis was maintained for 7 hr. At termination, the culture was examined as in Trial 1.

Virulence and viability were determined for each specimen by duplicate intradermal inoculation of 0.1 ml samples of three different dilutions (undilute, 1:10, and 1:100) on the backs of two different rabbits. All animals were examined periodically for 35 days before being considered negative for darkfield positive lesions.

5.3.8.3 *Results*

Treponema pallidum did not multiply in hemodialysis culture. By the end of the 21-hr culture period (Trial 1), essentially all of the organisms had lost motility and viability. Material from the culture chamber at the end of this trial failed to produce darkfield positive lesions in rabbits and failed to cause rabbits to become serologically positive for syphilis, as measured by the VDRL test.

The results of Trial 2, in which the bacteria were cultured in an environment containing goat peripheral blood cells, were identical to those of Trial 1. During the 4 hr of hemodialysis culture, the organism lost motility, and the ability to form darkfield positive lesions and to stimulate VDRL reactivity in rabbits.

Inclusion of rabbit testicular material in the chamber (Trial 3) during a 7-hr hemodialysis trial had no effect on preserving motility or in stimulating VDRL reactivity in rabbits. This material did have the capacity to form darkfield positive lesions, however, so presumably some virulent treponemes remained.

In all three trials, the original suspension of organisms was approximately 90 to 95% motile, formed darkfield positive lesions, and produced VDRL-reactive serological tests in rabbits.

5.3.8.4 *Discussion*

As attempted in these three preliminary trials, hemodialysis culture was not successful as a culture technique for *T. pallidum*. The organism not only lost motility (a sign of viability) but also

lost the ability to form darkfield positive lesions (a sign of viability and virulence) in two of the trials. In the trial that included rabbit testicular tissue in the culture chamber, the bacteria did establish darkfield positive lesions in rabbits. However, because viable organisms may have been sequestered in and protected by the rabbit tissue, this observation was not considered conclusive. It did indicate an approach for future studies.

The reason for lack of VDRL reactivity in the one trial in which darkfield positive lesions were formed was not apparent. It was possible that the organisms were damaged to such an extent that their characteristic antigenicity was lost, but this seemed unlikely in light of the nonspecific nature of the VDRL test. This organism, and serologic tests for it, are noted for variability, however.

The reasons for loss of viability, virulence and motility were not known. Possible causes included a lack of required nutrients and subsequent starvation, damage to the organism by blood components, and damage during extraction procedures. The later case seemed unlikely as the primary cause because control specimens (not incubated in hemodialysis culture) tested at the time of initiation of hemodialysis were viable, motile and virulent.

5.3.9 Prosthetic hemodialysis maintenance culture of protoplasts

5.3.9.1 *Introduction*

Bacterial protoplasts are sensitive to the osmotic conditions of their environment and act as osmometers (36). Thus protoplasts seemed a valuable tool to test the osmotic conditions in the miniature hemodialysis culture chamber. If protoplasts would withstand hemodialysis culture, it should also be possible to use the technique to study bacterial L-forms, *Mycoplasma* species, or antibiotic-damaged bacteria.

5.3.9.2 *Materials and methods*

Protoplasts of *Bacillus megaterium* KM were prepared by lysozyme treatment of vegetative cells (36). The suspension in 20% sucrose was placed in the hemodialysis chamber, which was assembled with DuPont 315 dialysis membrane. Hemodialysis was maintained for 90 min. Identical samples of the protoplasts in test tubes were incubated at 4 C and 35 C, as controls. Specimens were examined by phase-contrast microscopy initially, after 30 min, and after 90 min of hemodialysis or incubation. Subjective determinations were made by two workers (J. Quarles and T. Corner) on the relative lysis or damage to the protoplasts in each specimen.

5.3.9.3 *Results*

Under hemodialysis conditions, about 20 to 25% of the protoplasts were visibly damaged as judged optically. Protoplasts that had been incubated at 35 C were damaged to about the same extent.

Protoplasts held at 4 C, however, apparently were unaffected after 90 min. All of the protoplasts seemed intact and undamaged when examined microscopically. There was no apparent difference in the samples taken at 30 min from those taken after 90 min.

Quantitative counts were not performed, but it was estimated that there was no significant lysis under any of the three experimental conditions. Thus, protoplasts maintained in hemodialysis culture were damaged to about the same extent as those incubated in a test tube at 35 C, but neither condition caused significant lysis.

5.3.9.4 *Discussion*

Hemodialysis maintenance culture of bacterial protoplasts caused some damage and leakage of internal constituents, as compared to a 4 C control, but did not cause significant lysis. The damage seemed qualitatively comparable to that in a control test tube incubated at 35 C, which was the average temperature in the hemodialysis culture chamber during dialysis trials. It is known that protoplasts metabolize and leak at high temperatures (i.e., 35 C), and it therefore was concluded that changes in the protoplasts were due to the temperature and subsequent metabolic activity, rather than to hemodialysis.

This finding has implications for hemodialysis culture studies of other cell wall-deficient microbes. For example, it should be possible to culture *Mycoplasma* in the miniature hemodialysis chamber. Bacterial L-forms should also be able to withstand the environment

of the chamber during hemodialysis. This might provide a new approach to studying the metabolism and toxicity of osmotically sensitive forms in an environment in direct communication with a host animal.

5.3.10 Prosthetic hemodialysis maintenance culture of goat blood cells

5.3.10.1 *Introduction*

Whole goat blood had been included in the chamber during growth trials in previous studies with *Bacillus anthracis* and *Treponema pallidum* to simulate the in vivo environment better. Consequently, it was considered important to obtain information about the capability of blood cells themselves to withstand hemodialysis.

5.3.10.2 *Materials and methods*

The prosthetic hemodialysis unit was fitted with a combination of a Gelman GA 8 filter membrane and a silicone rubber membrane. Whole goat blood was removed from the arterial shunt, mixed with heparin (100 USP units / ml), and placed in the chamber. Hemodialysis was maintained for 10 hr in the usual way.

Hematological studies (total and differential cell counts) were conducted by standard counting chamber techniques. Counts of 100 or 200 cells were made on Wright-stained smears for the differential cell counts. Biochemical tests were conducted on the SMA 12 autoanalyzer, as previously described. Blood cells were removed by centrifugation as required to obtain cell-free samples for biochemical tests.

5.3.10.3 *Results*

The results of hemodialysis maintenance culture of whole goat blood are shown in Table 1. The data are the averages of two trials.

Erythrocytes in the chamber were not lysed, and the leukocytes remained essentially unchanged in both total and differential counts. The subjective observation was made that leukocytes showed some degeneration and appeared somewhat abnormal in the terminal samples.

Biochemical tests on the cell-free fluid from the chamber and on serum from the initial inoculum revealed only one result of significance. In the two trials, glucose decreased in the chamber from initial values of 70 and 78 mg/100 ml to terminal values of 8 and 12 mg/100 ml, respectively.

5.3.10.4 *Discussion*

Goat peripheral blood cells were maintained in an intact state during 10 hr of hemodialysis culture. Total and differential leukocyte counts remained constant throughout the trials. The blood cells apparently were metabolically active during hemodialysis, as indicated by the decrease in free glucose in the terminal samples. The dialysis supply of glucose was insufficient to meet the demands of the cells. This result was in agreement with the previous findings on the growth of bacteria in the chamber, and was additional evidence for the conclusion that limitations in dialysis transfer may be an important factor inherent in the design of the miniature hemodialysis unit, as discussed in Section 5.3.4 and following sections.

TABLE 1. *Effect of hemodialysis on peripheral blood cells of the goat*[a]

Sample		Leukocytes per mm^3	Erythrocytes per mm^3	Differential count	
Place	Time			Neutrophils (%)	Lymphocytes (%)
Goat	Initially	8,850	10,500,000	47	50
Goat	Terminally	12,500	10,300,000	47	51
Chamber	Initially	8,850	10,500,000	47	50
Chamber	Terminally	7,700	11,100,000	48	51

[a] Average of two trials, each with 10 hr of hemodialysis.

5.3.11 Prosthetic hemodialysis maintenance culture of mouse spleen cells

5.3.11.1 *Introduction*

It was considered important to find a means of evaluating hemodialysis culture other than the techniques previously used (i.e., growth, metabolic activity, morphological changes). Consequently, a system for detecting maintenance (or loss) of a cellular function was desired. The production of antibody by primary mouse spleen cells was chosen as this functional model, and a preliminary study was conducted to determine the feasibility of using it to evaluate the effect of hemodialysis on the system. The aid of Harold Miller and Barbara Laughter in planning and conducting these tissue culture trials is gratefully acknowledged.

Marbrook (82) reported that mouse spleen cells stimulated with sheep or horse erythrocytes produce antibody to these antigens. He cultured spleen cells in a dialysis apparatus which consisted of a cylindrical tube with dialysis membrane over one end and contained 1 ml of cell suspension. This tube was immersed in a larger test tube containing 9 ml of medium. The open ends of both tubes were plugged with cotton and the entire apparatus was placed in an incubator. Cellular function (i.e., antibody production) was assayed as the number of plaque-producing cells, as determined by the Jerne-plaque technique (70). In this technique, antibody-producing cells are detected as a clear circle, or plaque, of hemolyzed

erythrocytes on a background of intact sensitized red cells suspended in a layer of agar. Marbrook's work, and the work of others (H. Miller, personal communication), indicated that antibody production is maximal between 4 and 5 days of culture in the Marbrook chamber. This system was therefore used as the basis of the following experiments.

5.3.11.2 *Materials and methods*

Primary isolates of mouse spleen cells were obtained, exposed to 1.5% sheep red blood cells in vitro, and cultured for 4 days in Marbrook dialysis chambers (82). The spleen cells then were transferred to the prosthetic hemodialysis culture chamber, which was fitted with a combination of Nuclepore GE 10 filter membrane and silicone rubber membrane. Goat hemodialysis culture was maintained for 12 hr. The spleen cells were counted and tested for viability by microscopic examination of dye exclusion, and for antibody by a plaque technique (70). Plaques were counted as an antibody-forming unit (i.e., an antibody-producing cell) and reported as a plaque-forming unit. Concurrent comparisons were made of cells cultured by goat hemodialysis and in controls consisting of Marbrook chambers and the hemodialysis culture chamber in vitro. In each trial, and equal aliquot (number) of cells was used per chamber.

5.3.11.3 *Results*

The results of a 12-hr hemodialysis culture trial and the corresponding controls are shown in Table 1. Viability was similar in all cases. The three viability results were considered not

TABLE 1. *Effect of culture technique on viability and antibody production of mouse spleen cells*[a]

Culture technique	Viability (%)	Plaque-forming units per chamber
Hemodialysis chamber in vivo	44	1130
Hemodialysis chamber in vitro	20	2530
Marbrook tube in vitro	21	1670

[a] Results are after 4 days culture in vitro in Marbrook chambers followed by 12 hr culture in the indicated systems.

significantly different. The number of antibody-producing cells, represented as the number of plaque-forming units/chamber, was also in the same magnitude for the three conditions.

5.3.11.4 *Discussion*

Goat hemodialysis culture proved to be essentially as efficient as in vitro dialysis culture of mouse cells. Some important conclusions for further development of the system were drawn. First, neither the chamber, membranes nor dialysate were toxic to the tissue cells. This implied that tissue cells of other types might be cultured in the chamber. Related to this was the observation that, at least in the 12-hr time period, there was no cidal reaction between the components of goat blood and the mouse cells. This implied that cells of heterologous species may be cultured by hemodialysis techniques. Another important finding was that function (i.e., antibody production) as well as viability were successfully maintained.

A primary variable between the controls and the hemodialysis culture technique was the difference in temperature. The in vitro controls were at 37 C, whereas the hemodialysis system averaged about 35 to 36 C. This difference in temperature may have been enough to cause the hemodialyzed cells to be slightly less efficient in antibody production. It should be noted that results with the chamber on the goat were similar to results with the chamber in vitro at 37 C, so the temperature difference probably was not critical.

There was also a difference in membranes. Both the in vivo and in vitro hemodialysis chamber trials were conducted with a combination of Nuclepore and silicone rubber membranes, but the Marbrook chamber was used only with a dialysis membrane. The Nuclepore membrane was not successfully attached to a Marbrook chamber, but the use of the hemodialysis chamber in vitro with the combination membrane furnished a control for this aspect.

Thus, it was concluded that the hemodialysis culture system can be used for culturing tissue cells and should be further investigated for this purpose. The system can probably be evaluated with the plaque technique or a qualitative assay for similar antibody production. An important consideration for tissue culture work is to devise a means of more accurately controlling the temperature of the chamber. A heating or insulating collar around the animal's neck probably would suffice.

6. GENERAL DISCUSSION

The prosthetic hemodialysis culture unit was successfully used for growth or maintenance of a variety of cells. Although not designed for rapidly multiplying microbes, bacteria such as *S. marcescens*, *B. anthracis*, and *S. pyogenes* grew well in the system.

Comparison of growth characteristics of *Serratia* in the two hemodialysis culture systems (artificial kidney - fermentor system and prosthetic unit) demonstrated an inherent difference between the two systems: the ratio of membrane area to culture volume was less in the miniature unit than in the artificial kidney - fermentor system. The resulting reduction in nutrient diffusion limited growth in the miniature unit, and conversely the resulting reduction in product diffusion may have prevented the toxemic reaction.

Two other differences between the two systems involve membranes and temperature control. The prosthetic unit can be assembled with any sheet membrane that is compatible with blood, thus greatly exceeding the membrane versatility of commercial artificial kidneys. However, the artificial kidney - fermentor system allows more precise control of the temperature of the culture. As currently implemented, the prosthetic unit relies on the blood and body heat of the goat, and an insulating collar, to maintain temperature of the culture. Heating and temperature control could be designed into the protective collar if desired, with a subsequent increase in complexity of the system and probable loss of mobility of the animal.

Although not useful for demonstrating toxemia, the prosthetic unit more fully duplicated an in vivo environment than the fermentor system. By using a filter membrane together with a gas-transport membrane in the miniature unit, small concentrations of cells of *B. anthracis* appeared more characteristic of in vivo grown cells than those grown in the fermentor. This subjective observation was based primarily on the reduction of chaining and seemed valid only for low cell densities (before the limitation of nutrients became significant), but did provide a lead for further studies. Allowing the larger components of blood into the chamber via filter membranes seemed to help in achieving more nearly in vivo-like conditions.

The prosthetic unit also was more useful than the artificial kidney - fermentor system in long-term hemodialysis culture with slowly growing or delicate cells. The longest continuous dialysis attempted with the prosthetic unit was 118 hr, but apparently this time period could be considerably extended. Because the unit is self-contained and attached to the animal's neck, it can be left in place with relatively little monitoring for an extended time. Because pumping and vigorous stirring of the culture are not involved (as in the fermentor system), cells which would be harmed by these procedures can be cultured in the prosthetic unit. The observation that a relatively slowly reproducing microbe, *Blastomyces dermatitidis*, grew in the system suggested its applicability to other slow-growing organisms. Maintenance by hemodialysis culture was demonstrated with bacterial protoplasts, goat peripheral blood cells,

and mouse spleen cells. The syphilis treponeme was not successfully grown nor maintained in the prosthetic unit. However, improved protection of the microbe when tissue cells were included in the chamber indicated maintenance of tissue cells in the unit might be an important lead toward eventual cultivation of virulent *T. pallidum*.

7. GENERAL SUMMARY AND CONCLUSIONS

Hemodialysis culture was envisioned as a way to obtain growth conditions in vitro like those in the mammalian blood stream, but unrestrained by phagocytic clearing. The aims of the research reported in this thesis were to design, implement and test systems for putting this concept into practice.

In order to gain access to the blood stream of the experimental animal of choice, a goat, the blood was shunted surgically via prosthetic tubing from a carotid artery and back into the jugular vein. Hemodialysis was accomplished by connecting a hemodialyzer into an extension of this shunt. There were two main problems in implementing the hemodialysis culture systems: (1) the establishment and maintenance of an arterial-venous shunt, and (2) the choice and utilization of an effective yet atraumatic hemodialyzer. Experience with surgical techniques and anticoagulation therapy solved the first problem to the extent that shunts were routinely established and maintained patent for periods up to 10 weeks. A hollow-fiber artificial kidney was selected as the hemodialyzer, after investigation of the various commercially available equipment and unsuccessful trials with a plate-and-frame dialyzer. A successful design for a new prosthetic hemodialysis culture unit was achieved through experience and trials with several prototype models.

Two different hemodialysis culture systems were developed. The first was constructed with a modular fermentor vessel which contained the bacterial culture and was connected through tubing and a biological pump to a commercial artificial-kidney hemodialyzer. This system was designed for relatively short-term growth trials with large volumes of culture.

With the goat and *Serratia marcescens* selected as a host-parasite model, the artificial kidney - fermentor system allowed the inoculum initially to multiply at the maximum exponential rate and then at a lesser linear rate, under either aerobic or anaerobic conditions and completely supported by the continuous equilibrium diffusate from the blood. Hemodialyzers were equally effective with membrane porosity equivalent to nominal molecular weights of 8,000 or 30,000, but not 300. At about the time of the second phase of growth, the culture caused a general toxemia in the host goat with acute pyrexia and leukopenia. The dialyzable toxin(s) was produced more in an aerobic than an anaerobic culture of *S. marcescens*, but not by a gram-positive bacillus. The use of dialyzers with three different membrane porosities indicated that the size of the toxin was equivalent to a nominal molecular weight between 300 and 8,000. Dialysate samples depressed the body temperature of pre-cooled mice, but produced equivocal results with chick embryo lethality and *Limulus* coagulation tests. The biologic activity of the toxin in the host goat and in mice and the demonstration that goat hemodialysis (with an artificial kidney) of purified *Serratia* endotoxin

partially reproduced the toxemia, together suggested a possible relationship of the toxin to gram-negative endotoxin.

The second hemodialysis culture system was a newly fabricated prosthetic hemodialysis culture unit, all of the elements of which were self-contained and could be mounted on the neck of the animal. This unit was devised as a way to manage hemodialysis culture for extended periods of time (necessary for slowly growing cells) and with both a dialysis and a gas-transport membrane together. The unit consisted of (1) a plastic blood chamber in which was embedded silicone rubber tubing with a slotted opening, (2) the membrane(s) and a sheet stainless steel support with two openings coinciding with the one on the blood chamber, and (3) a dialysate-culture chamber with access through self-sealing samplers. This system was tested in a preliminary way with a number of different organisms to ascertain the range of applicability.

During hemodialysis culture with this prosthetic unit, *S. marcescens* at first multiplied exponentially and then linearly, but at lesser rates than observed with the artificial-kidney system. *Bacillus anthracis* and *Streptococcus pyogenes* formed chains and otherwise grew with characteristics more like those in vitro than in vivo. Applicability of the unit to slowly growing microbes was demonstrated by growth of *Blastomyces dermatitidis*. Attempted hemodialysis culture of *Treponema pallidum* was unsuccessful. However, hemodialysis with the miniature prosthetic unit enabled the maintenance of bacterial protoplasts, goat blood cells, and antibody synthesis by mouse spleen cells.

Hemodialysis culture therefore provides a new technique for growing cells extracorporeally in a simulated in vivo environment, on nutrients received entirely from mammalian blood, and thus provides a new model system for septicemic growth of cells. The technique seems applicable to a number of microbial and mammalian cells, and to problems such as population dynamics of a culture, toxemia in a host, and physiologic interactions between a host and parasite.

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