DIALYSIS CULTURE OF MAMMALIAN CELLS

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Chi-Byi C. Horng
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DIALYSIS CULTURE OF MAMMALIAN CELLS

By

Chi-byi C. Horng

A THESIS

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

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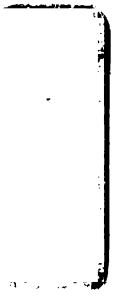

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ABSTRACT

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Chi-byi C. Horng

In order to determine the feasibility of applying the dialysis technique to mammalian cell culture systems, a growth study was made with L-strain mouse-fibroblast cells in a coil dialyzer system. To minimize the possible deteriorating effect of dialysis, a small reservoir-to-culture volume ratio of 2:1 was utilized, with intermittent renewal of the reservoir medium. Observations relating to dialysis were limited to the late logarithmic or early stationary phase in the growth cycle.

A lag period of about 12 hours, observed after dialysis was initiated, was interpreted as being the result of the diluting out of the dialyzable growth factors present in the conditioned medium. The presence of this lag period would not necessarily affect the efficiency of the dialysis application.

Following the lag period, logarithmic growth resumed, the glucose concentration increased to a higher level, and accumulated lactic acid decreased in the culture. It was thus demonstrated that stress derived from dialyzable nutrients and metabolic products can be released and cell growth promoted by dialysis. Furthermore, since the serum component of the medium was not necessary in the large medium reservoir, a significant cost reduction appeared feasible.

However, subsequent dialysis with intermittent renewal of the reservoir medium did not significantly affect growth. The cells at this stage showed low viability, decreased glucose consumption and diminished lactic acid production. It was concluded that dialysis culture has limitations but these restrictions might be eliminated by improving the physical construction of the culture system. Modification of the physical design of the culture vessel and its relevant units apparently is necessary before a final assessment can be made of dialysis culture for the mammalian cell.

It was suggested that consideration be given to the applicability of dialysis systems to monolayer, as opposed to suspension, culture of primary cells. Problems such as cell dissociation, nutritional environment, culture surface and environmental regulatory systems were discussed. It was proposed that a model system be employed for further investigation: Mammary secretory cells could be employed as the cell type; the cell dissociation procedure could be improved; and regulatory factors, such as hormones, could be incorporated into the medium in place of serum so that the specialized cell functions might be maintained or triggered in the in vitro culture environment.

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TO MY WIFE

HUEY-YU

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TABLE OF CONTENTS

TABLE OF CONTENTS (CONTINUED)

TABLE OF CONTENTS (CONTINUED)

1.1 LIST OF TABLES

1.2 LIST OF FIGURES

2. INTRODUCTION

2.1 Historical

2.1.1 Early development of tissue culture

In early methods of cultivation of mammalian cells, a fragment of fresh tissue, generally embryonic, was placed in a drop of plasma (11) or saline solution (61), and for a few days thereafter migration and multiplication of cells might be observed. However, the phenomenon was irregular and of short duration, and no increase in the mass of the tissue was observed.

Subsequently, culture of animal tissue cells developed, as Earle has suggested (32), into three general groups: static matrix cultures, static surface substrate cultures, and agitated fluid suspension culture.

The first and earliest group, static matrix cultures, owes its development to Carrel's work during the 1910's and 1920's. The problems of providing the cells with the necessary food and removing the catabolic substances from the medium were solved by the use of a biphasic medium composed of plasma-clot and fluid medium, by the transfer of tissue sections every 48 hours (12) and by the construction of containers and instruments permitting the aseptic handling of the cultures (13).

However, disadvantages of the plasma-clot substrate, such as clouding and liquefying of the culture and size limitation, led to the

development of the second group, static surface substrate cultures. As the culmination of a long series of experiments, Earle's group grew cells successfully first under perforated cellophane (33) and then directly on the glass floor of the flask (28). Instead of transferring the culture by excising a fragment of the cell sheet and implanting it in a new culture flask, the cell suspension could be prepared from the cell sheet and used as the inoculum (27). Through these efforts, the mass culture of cells as monolayers on glass could be done routinely and reproducibly (30). Since that time these procedures have been generally adopted as conventional tissue culture techniques.

While there was no inherent size limitation in the solid substrate culture method, the advantages to being able to entirely eliminate the surface substrate as a complicating factor in the growth of large cultures were so significant that a new group of experiments was initiated to explore the possibility of obtaining rapidly-proliferating cultures with the cells freely in suspension in the nutrient fluid. Owens and Gey made the first attempt in 1953 by providing the culture tubes with a rapid rotatory tumbling action so as to maintain the medium in constant motion and the cells--a lymphoblastic strain of tumor origin. which never did grow on glass--in continuous suspension (78). Earle and his co-workers elaborated on this idea of "tumbling cell culture" to establish rapidly proliferating fluid-suspension cultures of pure strain L cells from the mouse (11). Cultures were maintained in rotating roller tubes. Factors such as viscosity, circulation and initial

cell population were studied in these fluid-suspension cultures.

2.1.2 Agitated fluid suspension culture

Subsequent developments in the agitated fluid suspension culture can be reviewed from three aspects: design of culture vessels, increase in culture volume and control of culture environment.

Various designs in the culture vessel appeared during the early stage of development. Graham and Siminovitch reported the propagation of monkey kidney cell strain in roller tubes rotated around their horizontal axis at 40 to 50 rpm (45); these roller tubes were similar to those used by Earle and his associates for cultivating mouse connective tissue cells (L strain) in suspension. Concurrently, the roller tube was modified by Powell into a hexagonal-sided roller tube so that the fluid medium successively collected in and was discharged from the angled space between the longitudinal faces of the tubes (82). Good growth of ascites tumor cells was thus demonstrated. Making an imaginative parallelism between mammalian cell suspension and fermentation in the antibiotics industry, Earle and his colleagues successfully grew L cells in an Erlenmeyer flask mounted on a New Brunswick type shaker (30). Similar techniques have been used by Kuchler and Merchant for studying growth cycle of L cells (57). The shaker system was further developed by Earle et al. who, instead of an Erlenmeyer flask, used a flat-bottom boiling flask with a gas inlet filter and outlet filter provided for continual

gassing (31). In addition to the shaker flask cultures, a technique employing a suspended magnetic stirrer bar for agitation was introduced by Cherry and Hull in 1956 (14). A spinner culture apparatus was then developed by McLimans and his colleagues in 1957 (65). The apparatus consisted of a teflon-covered magnetic bar suspended in a Pyrex vessel. The submerged culture thus employed showed an actively proliferating mammalian cell growth, able to support viral growth (23), and the capacity to be extrapolated to larger types of equipment (65).

As a result of the increasing demand for kinetic studies of mammalian cells from the standpoint of nutritional, immunological, biochemical and growth characteristics as well as an interest in the feasibility of obtaining cell products, attempts were made to scale up culture systems. Scale-up prototypes were developed with all-glass culture vessels by making minor modifications to increase the size. Thus, modification of Cherry's magnetic stirring bar culture resulted in the development of the stirrer flask, centrifuge stirrer flask and filter bottom stirrer flask with a capacity to 2 liters (15). The original roller tube was modified into a 6-liter roller bottle rotated on a ball mill (15). The suspended magnetic stirring bar was placed to rest on the floor of carboy-type bottles which had a volume of up to 9 liters (100). Conversely, the spinner apparatus was modified and described as "minispinner" of a volume from 5 to 50 milliliters; "orthospinner" types of which are commercially available; and "magnaspinner" the capacity of which is 7 and 15 liters (73). McLimans et al. pointed out

that this process is difficult to scale up or to extrapolate to larger vessels. Through initial investigation of the relative toxicity of types of construction used in larger fermentor systems as well as determination of satisfactory antifoam agents (41), they proposed that a 5-liter New Brunswick type fermentor be used for scale-up studies (64). A 20-liter stainless fermentor used in antibiotic fermentations was soon adapted by them for the culture of mammalian cells (102). Many investigators have based their studies on this proposal. Rightsel, McCalpin and McLean have studied cell growth in 5, 7.5 and 30-liter fermentors (83). Among recent developments in use of these large-scale fermentors, two groups are of particular interest. In Pirbright, England, a culture vessel with automatic pH control was scaled up from 5 liters (90) to a pipe-line connected 30-liter fermentor used for the culture of hamster kidney cells (91) and the production of Semiliki Forest virus from these cells (92). At Roswell Park Memorial Institute, in Buffalo, New York, a pilot plant, initiated with a 6-liter vibromixer (97) and scaled up to capacities of 20, 50, 250 and 1250 liters, was established for the culture of human leukemia (74).

Initial success with scale-up cultures revealed new problems and questions which had not received serious study. The delicate nature of mammalian cells and their inferior growth rate, compared with that of the microbial system, called attention to the necessity of improving the culture environment so that a better understanding of cell functions might be achieved. Various factors, including medium composition,

gas aeration or overlay, agitation, sterilization, pH, oxidation-reduction potential, osmolarity, viscosity and temperature have been assessed, the culmination of which has been the development of complete monitor and automatic control systems for both batch culture and continuous cultures. The experimental studies have been well documented, and only selected examples will be cited for illustration.

The majority of the culture systems used Eagle's medium (25) with some modification. The medium contains a variety of amino acids, vitamins, glucose, and balanced salts, with the addition of 1-to-10 per cent serum. Methylcellulose was used by Earle and his associates (29), and its protective effect was confirmed by Koza and Motejlova (56). Whole or dialyzed serum seems to be a necessary component of the medium for a consistent growth pattern; the growth factor activity appears to be carried by small micelles of serum proteins (94). Attempts have been made to minimize or eliminate the serum requirement; success apparently depends on cell type (72). It has been suggested that the serum component may be replaced by peptone in autoclavable medium (77). Chemically defined medium has been formulated with 0 per cent serum; in the presence of methylcellulose, the medium was able to provide apparent growth of L cells (48).

The importance of controlling the overlaying gas phase of the culture was pointed out as early as 1956 by Earle et al. (31). Having realized that pH drift was largely due to CO_2 generated by the cells and CO_2 equilibration between liquid and gas phase, these investigators

circulated a constant, slow flow of gas containing CO_2 through each culture flask (31). Another important component of the gas phase is O_2. Its influence on cell growth rate was established by Cooper et al. in 1958 and assessed to be related to liquid-phase oxygen level or oxidation-reduction potential (ORP) (17). Various devices include manual adjustment of gas flow rates of independent supplies of CO_2 and air (9); automatic control over the surface with adjustable mixtures of CO_2 and air, with another gas mixture, used as a control, entering the culture liquid directly beneath the impeller (90); and sensitive feedback monitor systems for both pH and pO_2. Among the latter are a system which controls ambient conditions, described by Thompson et al. (93); a gas monitor and control unit, the Meta-Stat, described by Harris et al. (46); and an aeration apparatus described by Daniels and Browning (19). Daniels et al. further emphasized the importance of ORP (21). Results of recent experiments demonstrated that incubation of medium prior to inoculation induces desirable qualities reflected in better growth (22). Higher levels and increased rates of cell growth are associated with the initial QRP before inoculation (21).

Physical factors have a critical effect. Temperature is generally controlled at 37°C by use of an incubator (21); a direct thermistor control (20); or a circulating water jacket (46). Agitation has been achieved by use of a ball mill (15); a shaker (30); a suspended magnetic stirring bar (65); a magnetic stirring bar resting on the floor (100); a non-suspended magnetic stirring bar rotating at one point

on bottom bulges (93); a suspended impeller (64); and a vibromixer utilizing the Bernoulli effect (97). Attention has been directed to viscosity since suspension techniques came into use. The effect of osmolarity was noted when it was discovered that different cell types exhibit specific optimum tonicity (79).

Concomitant with these development were attempts to control the nutritional substrates via the continuous introduction of fresh nutrients as well as the removal of cellular waste products. One of these systems employs a constant population as maintained by the balanced addition and removal of medium and the continuous elimination or harvesting of the cell crop. The other, however, maintains growth constancy without loss of cell mass. The former is the so-called "continuous cell culture" and the latter is "dialysis cell culture." Both have been described in relation to their use for propagation of microorganisms.

In 1950 Monod advanced his concept and theory concerning continuous culture in microbial systems (71); the feasibility of continuous culture of mammalian cells was first reported by Cooper, Burt and Wilson in 1958 (17). Sustained growth in a constant environment was achieved by the continuous introduction of fresh medium at a fixed rate and the simultaneous removal of spent medium and cells. For control of the "fixed rate," three methods were designed by Cooper et al. (18): constant rate of medium flow in the chemostat; constant cell density in the turbidostat; and a combination in the chemo-turbidostat.

The chemostat method was studied further by Cohen and Eagle (16) and by Pirt and Callow (80).

2.1.3 Development of dialysis culture in microbial systems

Historically, in both microbial systems and mammalian cell culture, dialysis techniques were applied before continuous culture techniques. As early as 1896 Metchnikoff, Rox and Salimbeni demonstrated the diffusibility of cholera toxin by implanting cellophane sacs containing Cholera vibrios in the peritoneal cavity of animals (70). Shortly thereafter, this in vivo application was extrapolated to an in vitro situation by Carnot and Fournier (10); a collodion sac containing pneumococci cultures was suspended in a laboratory flask containing ordinary growth medium, and the presence of diffusible toxin was further demonstrated. In addition, they reported that properties were acquired which were different from those found in ordinary culture, such as greater capsulation, prolonged viability and more persistent virulence.

Dialysis culture was subsequently employed in microbial systems in a wide variety of culture processes which included cell production, formation of nondiffusible and diffusible cell products and interbiotic culture systems (87). Among these are important contributions by Gerhardt and his associates. Their efforts were directed primarily toward achieving concentrated cultures of bacteria. A biphasic system consisting of a layer of solid agar medium overlaid with a small volume

of broth was first employed (96). The solid phase served as a reservoir both for supplying nutrients and for removing metabolic products. The system was demonstrated to successfully increase cell concentration of various bacterial species (96) and of gonococci (39). This concept of a culture system employing a large reservoir phase and a small culture phase was succeeded by a dialysis flask technique in which a semipermeable membrane served to separate a reservoir liquid medium and a culture liquid medium (40). In order to achieve independent control of the component operations and to adapt to the larger scale of size, dialysis fermentor systems were developed in which growth was achieved in a fermentor remote from a nutrient reservoir, but connected with it by conduits and pumps. Dialysis was accomplished with membrane tubing in either the fermentor or the reservoir or, most satisfactorily, with a membrane sheet in a plate-and-frame dialyzer which was remote from, but connected with, both vessels (38). A thorough review of dialysis culture of microorganisms as well as a theoretical treatment of the subject has been reported (87). Some further developments are: a differential dialysis culture employing a small intermediate product chamber separated from the culture chamber by a membrane filter and from the reservoir chamber by a semipermeable dialysis membrane for concentrating macromolecular products (47); a demonstration of the applicability of dialysis culture to the production of diffusible cell products (1) and their kinetic analysis (2); an attempt made to employ dialysis as a means to alleviate the product feedback control (3); and

an extrapolation of liquid medium dialysis to gas phase dialysis (50).

Dialysis culture seems to be particularly feasible for some poorly growing organisms. The fact that cultivation of fastidious microbes can be facilitated by using dialysis techniques was recently illustrated by Rightsel and Wiygul. Mycobacterium lepraemurium was demonstrated to grow in a cell-free environment in either an in vivo implanted or an in vitro maintained cell-impermeable diffusion chamber (84).

2.1.4 Dialysis culture of mammalian cell suspensions

Essentially, dialysis culture is a technique by which cell populations can be cultivated by supplying nutrients from and removing metabolic products to a reservoir medium through a dialysis membrane while preventing the cells from being diluted out by the medium. This principle was employed in mammalian cell culture by Graff and McCarty as early as 1957, the same year that the spinner apparatus was employed for suspension cell culture. In their cytogenerator, instead of a dialysis membrane, they employed fritted glass candles to introduce nutrients and to remove cellular products. Growth constancy was maintained without loss of cell mass (44). However, in contrast with the well documented culture techniques employed for mammalian cell suspensions, the dialysis system seems to have received less attention than it merits. Among the few early reports was that of Mount and Moore who

claimed that incorporating a continuous flow dialyzer to conventional suspension culture vessels provided a satisfactory proliferation for extended periods of time with a concomitant increase in cell yield and in cell viability over that of non-dialysis control cultures. They reported this achievement at a Tissue Culture Association meeting (75), but no detailed data were published thereafter and the dialysis system was not employed in their later work. Gori, in an effort to establish continuous culture of HeLa cells in the chemostat (42) and continuous culture of virus from HeLa cells in the lysostat (43), reported promising effects from a dialysis system, although this culture system was not detailed. By employing a dialysis culture chamber, Langlois et al. were able to achieve a high level of virus concentration elaborated by leukemic myeloblasts (58). In these reports, no information was provided about the dialysis culture of mammalian cell suspensions in terms of details of culture techniques and growth patterns.

On the other hand, discouraging results were reported by Sommer regarding the possibility of concentrating a mammalian cell population by dialysis. A decreased population of L cells, as compared to the control, was observed in a dialysis flask with a reservoir; the culture volume ratio of the reservoir to the flask was either 4:1 or 10:1 (89).

2.2 Motives and Objectives

Dialysis culture of mammalian cells appears not to have been thoroughly treated. Thus a careful evaluation of the potential of this system, employing precise cell culture techniques, is warranted. Cultivation of mammalian cells is more difficult than cultivation of the more rapidly proliferating microorganisms. Yet, the application of dialysis to mammalian cell culture systems would appear more urgent and meaningful than in the instance of microbial culture.

Interesting aspects which support this view include:

1. One of the puzzling aspects in mammalian cell culture is the limited cell population that can be achieved. It would be of both practical and theoretical interest to determine if this limit can be extended by adapting a dialysis system. This rationale was derived from Gallup and Gerhardt's work which demonstrated that Serratia marcescens can be cultivated with the dialysis system to a virtually unlimited population with viable cell counts in excess of 10^{12} cells/ml and partial cell volume of 50 per cent (38).

2. Theoretically, the efficiency of dialysis culture is described by the formula:

$$X/Xnd = Sr^{o}/Sf^{o} \ (1 + Pm\ Am/Vf\ \mu_m) \qquad (87)$$

where X is the cell density for dialysis culture, Xnd is that of non-dialysis culture, Sr^{o} is the initial substrate

concentration in the reservoir, Sf^o is that in the fermentor, Pm is the permeability coefficient, Am is the total area of the membrane, Vf is the volume of the fermentor, and μ_m is the maximum growth rate constant. The relative benefit of dialysis, as revealed by the term $Pm\ Am/Vf\ \mu_m$, favors particularly the slower-growing mammalian cells. With a lower μ_m, the efficiency of the system should be higher for mammalian cells than for microorganisms.

3. Among the various components of the mammalian culture medium, serum presents the greatest obstacle in terms of cost and variability of growth response. It has been suggested that the growth-promoting factors of serum may be non-dialyzable (94). Applicability of dialysis culture to mammalian cells would mean that the serum component of the medium may be limited to smaller volumes, with considerable decrease in cost. Efforts to eliminate the serum component have not been routinely successful. However, effort has not been directed toward the incorporation into the culture system of a membrane impermeable to serum factors.

The present work is an initial attempt to apply a dialysis system to mammalian cell culture. The first and main objective was to determine the feasibility of applying the dialysis concept to cell culture systems.

Sophisticated culture systems as employed in current culture techniques were not available; therefore, a rather primitive type of culture vessel and dialysis design were adapted. Moreover, the experimental design was directed toward providing a minimum stress condition to negate the possible deteriorating effect of dialysis. The study was therefore not primarily concerned with achieving maximum efficiency.

The second objective was to clarify a previously-described failure (89), so that the mechanisms involved might be revealed. L-mouse-fibroblast cells were chosen for this study.

The success achieved thus far with dialysis culture techniques can be attributed to developmental work with microbial systems. The present work is an attempt to extrapolate techniques for microbial culture to mammalian cell culture. Consequently, comparisons will be made between the results obtained from this work and those pertaining to the use of microbial systems. This was the third objective.

Although suspension culture is an ideal system for culture of mammalian cell lines, it is of little value in the instance of primary cultures. To determine the applicability of dialysis techniques to mammalian cell culture in general, a further extrapolation should be made from suspension culture to monolayer culture. This constituted the fourth objective.

The final objective was to discuss the possibility of employing the dialysis culture technique to assist in maintaining cell function and/or differentiation in vitro. Although the dialysis technique alone cannot

maintain cell differentiation, its intrinsic character of separating dialyzable molecules from non-dialyzable ones permits an interesting approach to the in vitro study of cell differentiation.

Only through further extrapolation can dialysis culture of mammalian cells be applied to biochemical engineering and/or the utilization of mammalian cells in the fermentation industry. This is the ultimate interest of the author.

3. MATERIALS AND METHODS

3.1 Cell Strain and Stock Culture

3.1.1 Cell strain

Earle's strain L mouse fibroblast was used in the study. This strain was originated from normal subcutaneous connective tissue of an adult C3H strain mouse. It had been treated with a carcinogen, 20-methyl-cholanthrene, for 111 days and then subcultured without the carcinogen (26). The strain was shown capable of producing sarcomas on injection into C3H strain mice (26).

L cells have been widely employed as a model cell type since the initiation of agitated fluid suspension culture.

Two sources of L cells were used. First, a LB cell strain which had been adapted to suspension culture, was obtained from Dr. W. Munyon of Roswell Park Memorial Institute. The cells were subcultured in spinner flasks as stock culture and were employed for some preliminary studies such as effect of centrifugation on inoculation and toxicity of medium. Subsequent to an accidental contamination, the stock culture was terminated. Although the source of the contamination was afterwards identified as the serum in the medium, this strain was replaced by a second strain. Both strains had comparable growth patterns.

The second strain, L-929, was kindly provided by Dr. S. Weiss of the Monsanto Company, St. Louis. This strain is the first cloned

mammalian cell isolated from Earle's L strain mouse fibroblast (86). The cells were adapted to suspension culture and proven to be PPLO-free.

3.1.2 Stock culture

For the stock culture of L-929, both monolayer and suspension subcultures were routinely established.

RPMI 1X Eagle 1955 (7 M) was the medium used for monolayer culture. It is a modification of Eagle's Minimum Essential Medium. The medium contained 10 per cent inactivated fetal calf serum and 100 U/ml of penicillin and streptomycin.

The dissociating agent employed for subculture was 0.2% trypsin in PBS (8 M). For routine subculture, the T-30 flask was used. The G-90 was used for mass culture. Aliquots of used medium were mixed with fresh medium to initiate a subculture. Neither HCl nor $NaHCO_3$ was used for pH adjustment; the latter was achieved by raising with aeration or fresh medium and lowering with 5 per cent CO_2 in compressed air. For routine subculture, a culture split of 1:4 was practiced. A split of 1:2 was sometimes employed to ensure luxuriant growth.

Eagle's Minimum Essential Medium (9 M) was used for suspension culture. The medium differs from that used for monolayer culture in its high concentration of phosphate buffer and the absence of

calcium salt. The medium was prepared by adding glutamine to a final concentration of 29.2 mg/l, penicillin and streptomycin to 100 U/ml, and inactivated fetal calf serum to 10 per cent.

Routine culture was carried out in a spinner flask at a culture volume of 100 ml. Inoculation was done without centrifugation. With an initial cell population between 1.5×10^5/ml to 2.0×10^5 and with viability of the inoculum above 95 per cent, cell growth began after inoculation without an extensive lag phase. Adjustment of pH was achieved by aeration; exchanging a rubber stopper for a gauze stopper resulted in a rise of pH. The culture was maintained continuously with sufficient medium replenishment to keep the cells at a logarithmic growth phase. Cell counting and pH measurement were performed daily.

3.2 Culture and Dialysis System

The dialysis system was essentially a coil dialyzer placed inside its special fermentor. Culture vessels were designed to meet the objectives of feasibility for dialysis operation and maintenance of sterile conditions.

The culture vessel is shown diagrammatically in Figure 1. It has been described by McLimans et al. (66) as monitor, dialyzer and chemostat. The flask is a 500-ml. water-jacketed, pyrex glass vessel. The top is constructed of 316 stainless steel, with appropriate holes provided for insertion of sampling, feeding, circulating and temperature sensoring elements mounted in No. 4, 6, or 7 silicone stoppers. The stopper units are held in place by simple "Y" clamps. Appropriate connectors are provided for gas flow by means of stainless steel tube-ports. The stainless steel top is securely fitted to the flange neck of the flask by a collapsible under-ring. A gas-tight seal is assured by means of a recessed Teflon gasket on the underside of the steel head.

Dialysis tubing--Visking regenerated cellulose (16 M) with an average pore size of 24 Å, flat width of 1 cm, total length of 170 cm, surface area of 340 cm^2--was inserted on a type 316 solid stainless steel coil. The coil dialyzer rested on or was suspended in the culture vessel. The two ends of the dialysis tubing were connected with rubber tubing sleeves to glass tubings mounted on silicone stoppers. Tie straps (11 M) were used around the rubber tubing sleeves so that a tight

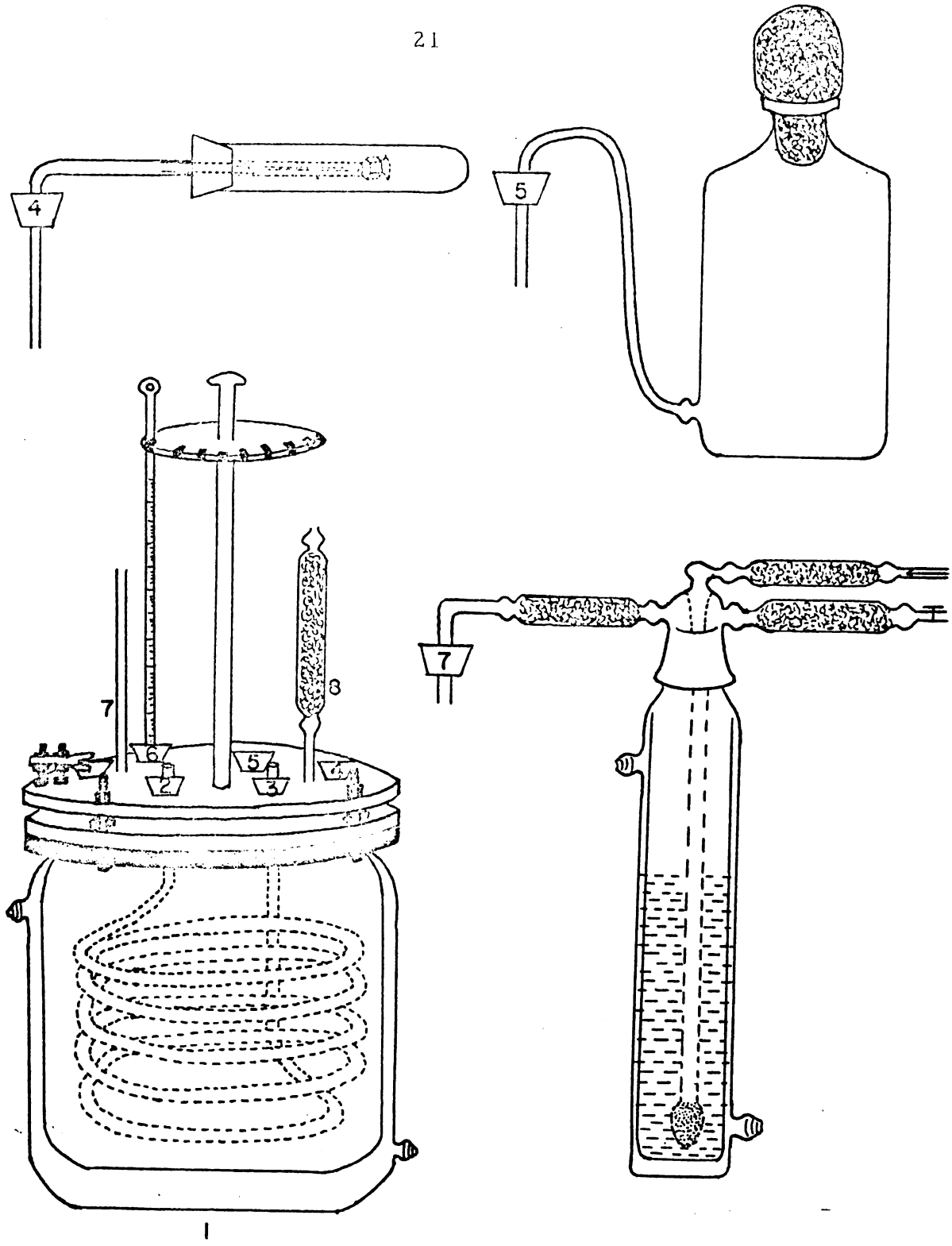

Figure 1. Schematic of culture vessel assembly showing: 1. culture vessel; 2. reservoir medium outlet; 3. reservoir medium inlet; 4. sterile connector for sampling; 5. feeding and inoculation unit; 6. thermometer; 7. gassing unit; 8. gas vent.

connection could be ensured. For suspension of the coil dialyzer, four of the silicone stoppers inside the culture vessel were cut into grooves. The coil was suspended under the silicone stoppers by use of 4 Silk 00 Sutpacks (5 M) which were fastened around the grooves.

Another vessel, of the same size but without the coil dialyzer, served as the reservoir vessel.

Feeding of medium and inoculation were performed through another glass feeding bottle. The feeding bottle was connected with the culture vessel by silicone tubing. Sampling was carried out with a 5 ml syringe via a sterile connector (Figure 1).

Circulation of the medium between the reservoir and the dialysis tubing was achieved by use of a peristaltic pump (1 M) with a flow rate of 55 $\pm$ 5 ml per minute.

The vessel was gassed with 5 per cent CO_2 in compressed air (10 M). The gas passed through a cotton filter, a gas washing bottle and a filtered manifold before entering the culture vessel. Another cotton filter acted as a gas vent. Flow of gas was adjusted manually to achieve an optimum pH level.

By use of a circulating water bath (Radiometer 12 M), temperature was maintained at 37°C in the water-jacketed units, including the culture vessel, the reservoir and the gas washing bottle. A thermometer was inserted in both vessels for constant temperature check.

Connection between the culture vessel and the reservoir, sampling unit, feeding bottle and gas unit was achieved by use of I.D. 1/8

inch silicone tubing (3 M) with the exception of the piece inserted into the pump. For the latter, rubber tubing of 1/8 inch I.D. was used. Because of the abrasive action of the pump, daily replacement of the rubber tubing was necessary.

Agitation was achieved by use of a teflon-coated magnetic stirring bar, 1.5 inches in length, resting on the floor of the culture vessel. The culture vessel was mounted on a magnetic stirrer (4 M) and the stirring bar rotated at 100 to 150 rpm.

The entire unit, after being cleaned and assembled, was tested for air leaks. It was then autoclaved as an assembled unit at 121°C for 45 minutes. To ensure adequate sterilization, glass-distilled water was added to each vessel. Thus, the vessels generated their own steam. After autoclaving, the condensed water was withdrawn with a syringe and evaporated to dryness with flowing gas.

To initiate a culture, a portion of medium was introduced into the culture vessel and gassed with 5 per cent CO_2 overnight. After the proper pH was achieved, the vessel was inoculated with cells and the volume adjusted with medium to give a desired cell population.

3.3 Measurements

Routine measurement was made of pH and cell concentration. The former was measured by a Micro Electrode Unit pH meter (13 M), and the latter by trypan blue (6 M) vital staining and a Rosenthal counting chamber (2 M).

An autoanalyzer (15 M) was used for simultaneous automatic analysis for both glucose and lactic acid. For glucose analysis, the method described by Frings, Ratliff and Dunn was employed (35). Aliquots were quantitatively sampled and separated from each other by air and were drawn through flow tubing. O-Toluidine reagent was added, and they were passed through a single mixing coil, incubated in a heating bath for 40 minutes and cooled through a water-jacketed single coil. Their adsorbance was measured at 630 mu and recorded.

For lactic acid analysis, an enzymatic method without dialysis of the samples, developed by Hochella and Weinhouse (49), was carried out. Samples were drawn and mixed with pH 9.6 glycine buffer. A dye solution, 3-p-Nitrophenyl-2-p-iodophenyl-5-phenyltetrazolium chloride (INT), and the enzyme reagent--containing diaphorase, $\overline{NAD^+}$, and lactic dehydrogenase--were added. During an incubation time of 6.5 minutes, the dye was reduced and the adsorbance which developed was measured at 500 mu. The principle employed is that under the catalysis of lactic dehydrogenase, NAD^+ is reduced by lactic acid to NADH. Instead of measuring the ultraviolet adsorbance of the NADH, the NADH is used to reduce the INT in another enzyme reaction catalyzed by diaphorase.

4. RESULTS

4.1 Preliminary Establishment of Experimental Conditions

4.1.1 Cell clumping and stock culture

The experiments performed during the course of this study are essentially considered as "growth trials," the term used by Humphrey (50) in his dialysis study. For reproducible "growth trials" a stock culture maintained in an actively growing state was used as the inoculum so that the "growth trials" would have a standardized starting point--which is of ultimate importance (52). The experimental operating procedure employed in this study for preparation of the inoculum was based upon the following criteria:

1. The L-929 had been adapted to suspension culture.
2. It was PPLO-free.
3. It showed a generation time of less than 24 hours.
4. The inoculum had a viability higher than 95 per cent.
5. It initiated growth above 1.5×10^5/ml.

In meeting these criteria, the first difficulty encountered was that of cell clumping. Cell clumping is a well-established problem. Although the objective of agitated fluid suspension culture is to grow the cell as an individual unit, cell clumping frequently occurs and has been described as groups of 2 to 4 cells or as large matrices (65).

At the beginning of the study, it was found that when a confluent monolayer culture was maintained in an incubator for an additional two

days beyond the confluent stage, the cells associated themselves to fibrous material which was visible after trypsinization. The cell line seemed to maintain its originality as a fibroblast and elaborated fibrous material. When these cells were inoculated as a suspension culture, cell clumping occurred. Cell number in the clump varied from 2 to 20. Clumped cells generally showed lower viability. Some clumps even showed entirely dead cells. As a result, the generation time was usually longer than 30 hours, and cell counting could not be performed precisely.

The situation was improved by diluting the cells with fresh medium and increasing the rate of stirring, but the clumps could not be completely removed. Finally, an attempt was made to transfer the cells back to the monolayer culture. After several subcultures, with a split ratio of 1:2 to encourage luxuriant growth and with subculture at the early stage of the confluent monolayer, the cells were transferred to suspension with satisfactory results. The cells appeared in suspension in the fluid medium as discrete units, with a generation time of about 20 hours. Shown in Figure 2 is a typical growth curve of L-cells in a spinner flask. The generation time indicated in this example was 20 hours; viability was maintained above 96 per cent up to the 75th hour, when the cells were already in the stationary phase. The pH of the culture was maintained generally between 7.1 and 7.6. Adjustment of the pH was achieved by simply exchanging the silicone stopper for a sterilized gauze stopper for a few hours. The accumulated CO_2, which caused the lowering of the pH, equilibrated with the atmosphere. As an

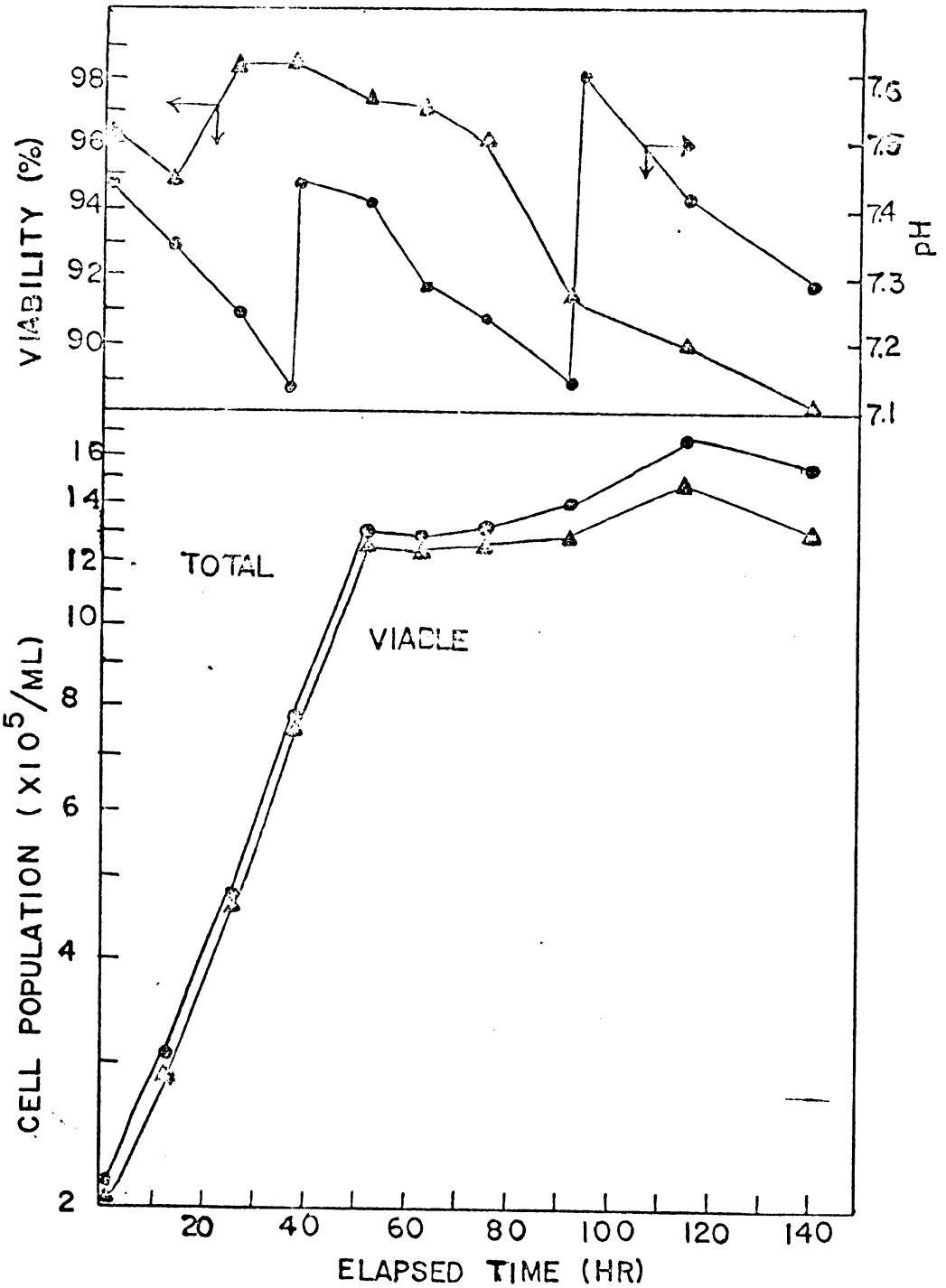

Figure 2. Growth curve of L-cell suspension culture in spinner flask.

illustration, adjustment of pH from the 38th hour to the 40th hour caused a rise in pH of from 7.13 to 7.42. Because the conventional pH adjusting agents (HCl and $NaHCO_3$) were not used, an interfering effect was not evident in the adjustment process. Release from the pH stress resulted in a better proliferation, as shown in the 92nd hour in Figure 2.

For the stock culture, the cells were generally maintained in the logarithmic phase of growth so that they could be kept in a highly uniform state. Figure 3 shows the maintenance of logarithmic growth, together with desirable viability and pH levels. The inoculum used for the "growth trials" was from the late stages of the logarithmic phase, with a cell population of about 1×10^6/ml.

4.1.2 Bacterial contamination

Another difficulty encountered in the beginning stage in the "growth trial" was bacterial contamination. Procedures employed to locate the source of contamination and to maintain sterile conditions were as follows:

1. Improvements in the culture system were made, including the use of a sterile connector for sampling and a sterile aspirator as the gas washing unit; autoclaving the connected system as a whole unit; and routinely testing its air-tightness.

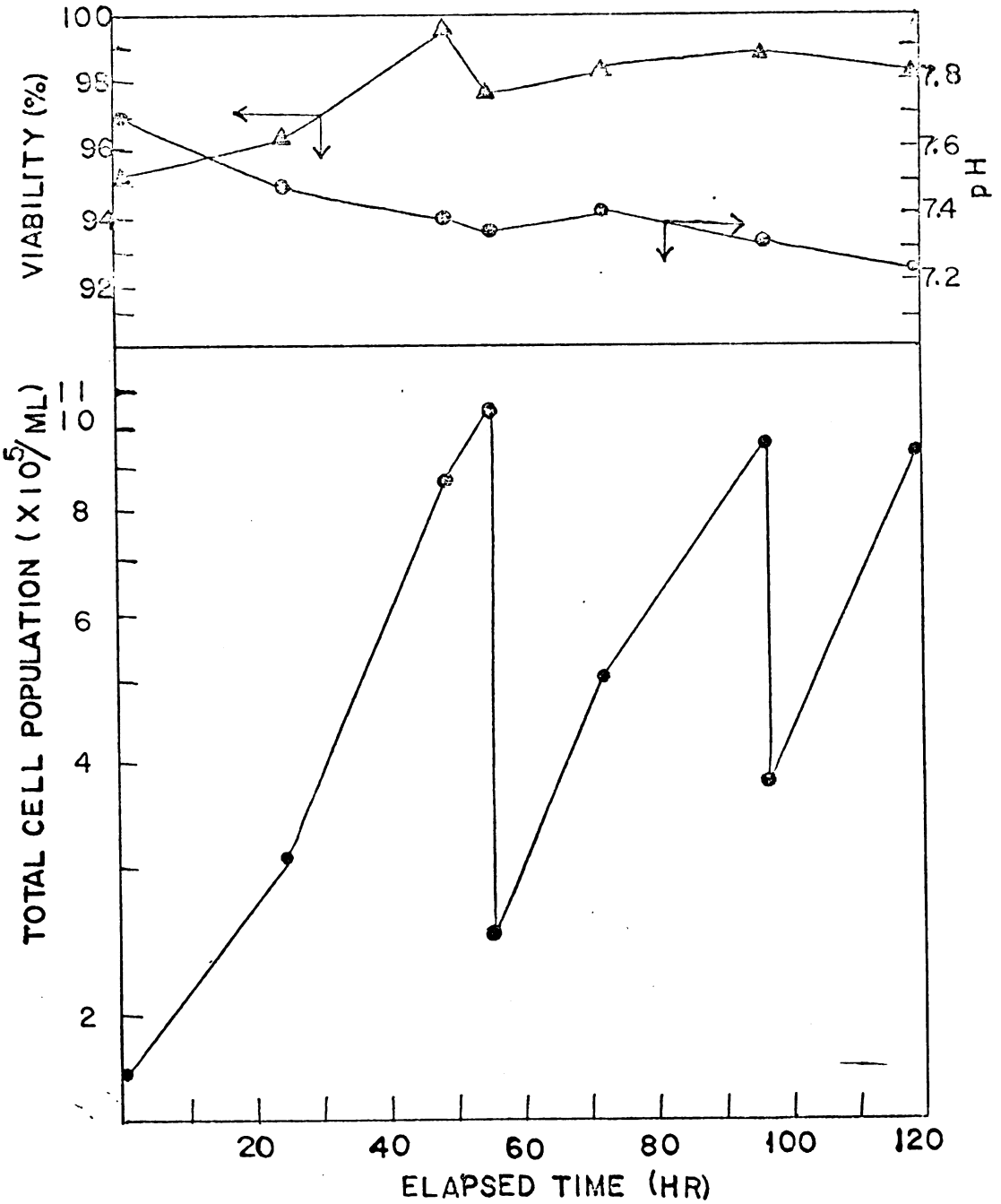

Figure 3. Maintenance of logarithmic growth in the stock suspension culture of L-cells.

2. The improved culture system was tested for sterility by running the system with sterile nutrient broth for a day or two.

3. The original LB strain was exchanged for a PPLO-free L-929 strain.

4. Fluid-thioglycolate medium and brain-heart-infusion broth were employed as a routine sterility test for each batch of medium, stock culture and "growth trial" culture.

The source of contamination was thereafter traced by means of a sterility test and was found to be in the serum. The medium has since been sterilized by Millipore filtration and dispensed in 100-ml sterile vaccine bottles.

4.1.3 Culture volume

The culture volume employed for a particular vessel appears to influence both the effectiveness of agitation and the efficiency of gassing. The former arises because different culture volumes give different characteristics of agitation. The latter, being determined by the ratio of surface to volume, influences, in turn, the oxidation-reduction potential and pH adjustment. The culture volume conventionally employed is between one-half to one-fourth of the total capacity of the culture vessel. For example, Earle et al. used 400 ml for a 1.5-liter flat-bottom boiling flask in the shaker culture (31); McLimans et al. used volumes

up to 3 liters in the 5-liter impeller-agitated fermentor (64).

Because of restrictions imposed by the limited availability of culture vessels and dialyzer coils, attempts were made to employ a culture volume of 400 ml so that the entire coil dialyzer could be submerged in culture medium. The result is shown in Figure 4. The "growth trials" were done without the coil dialyzer. In contrast to a desirable growth pattern obtained with a culture volume of 150 ml, the 400-ml culture volume failed to achieve satisfactory growth.

4.1.4 Centrifugation of the inoculum

Conventional techniques of mammalian cell suspension culture use an inoculum of seed cells together with the used medium of the stock culture. In order to have an initial precise growth environment--e.g., a high glucose concentration in fresh medium and a zero concentration of lactic acid--efforts were made to initiate the "growth trial" without used medium. Aliquots of 21 ml of LB cells from the spinner culture, with a cell population of 7.17×10^5/ml and a viability of 88.6 per cent, were centrifuged at 1,000 rpm for 5 minutes and suspended in 10 ml of fresh medium. The cell suspension was inoculated in a culture vessel without a dialyzer, at a total culture volume of 100 ml. As shown in Figure 5, both the cell population and the viability decreased sharply during the first two hours. Although both were recovered later, centrifugation of the inoculum brought about a lag period as long as 60 hours. Inoculation has since been done without centrifugation.

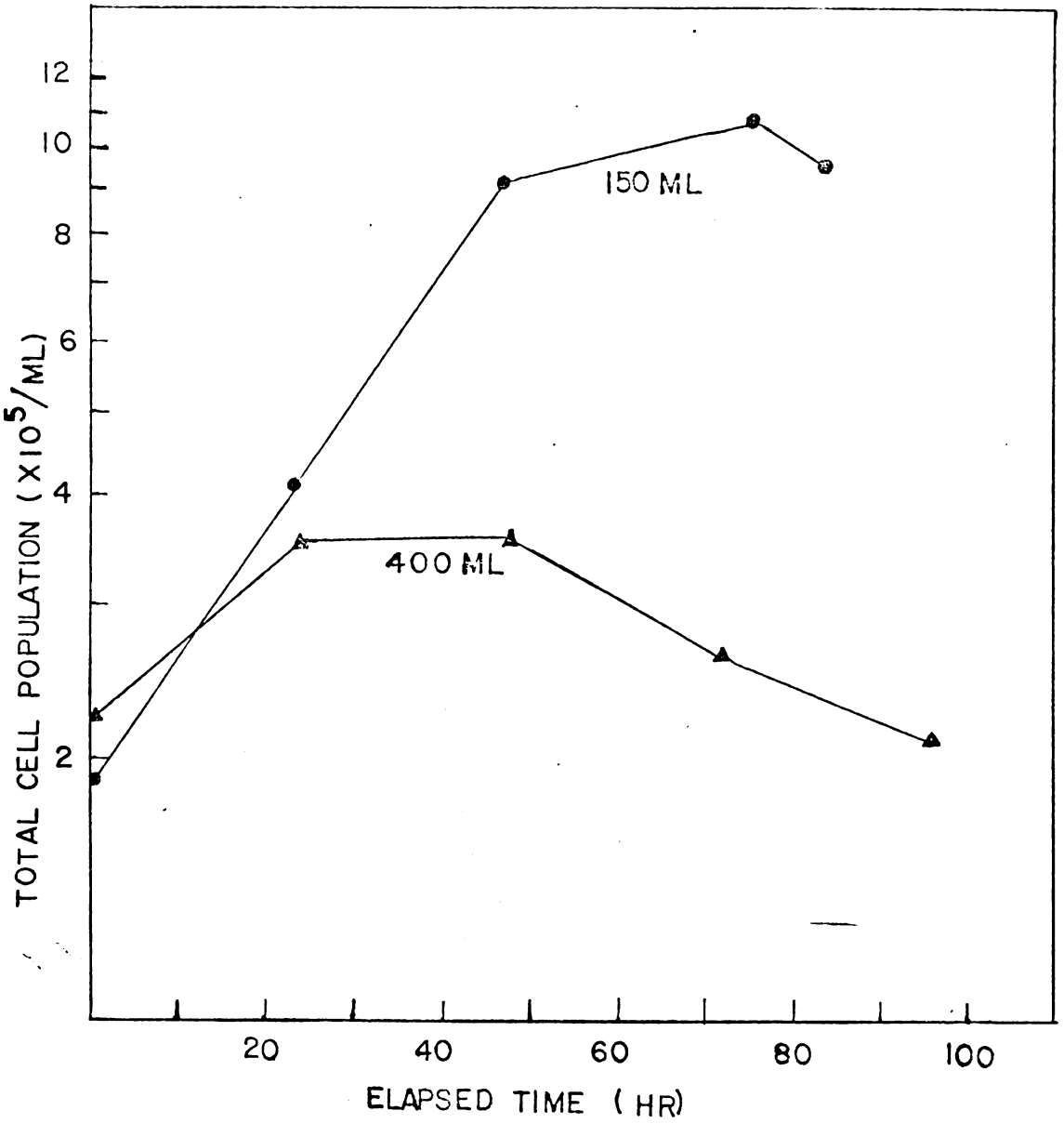

Figure 4. Effect of culture volume on cell growth.

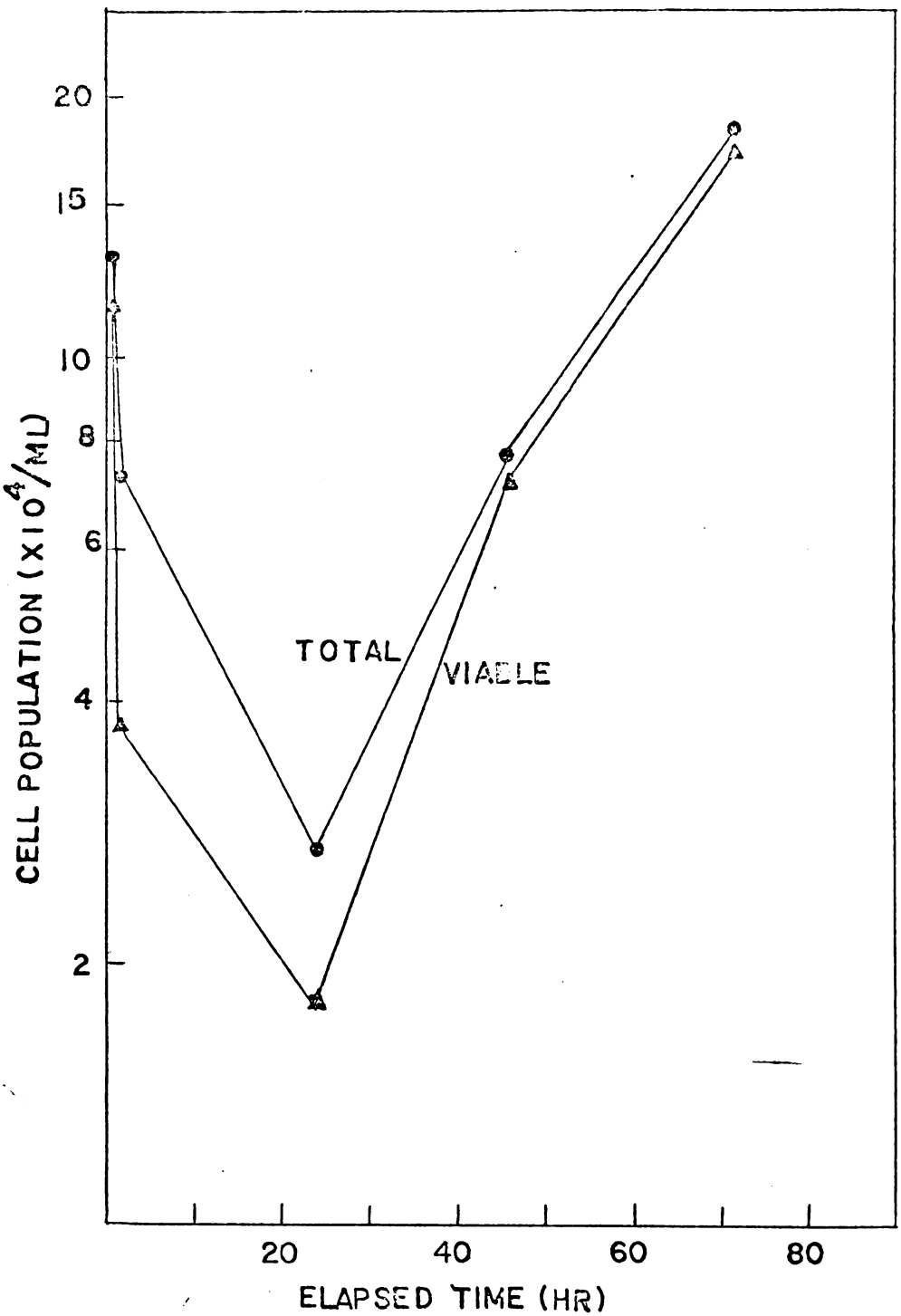

Figure 5. Effect of centrifugation of inoculum on cell growth.

4.1.5 Improvement of agitation

The agitation system employed in the "growth trials" was achieved by means of a teflon-coated stirring bar resting on the floor of the culture vessel and rotated via a magnetic stirrer located below the culture vessel. This system appeared satisfactory for growth in a culture vessel without a coil dialyzer. However, the presence of a coil dialyzer in the same vessel greatly interfered with apparent cell proliferation. Repeated attempts to obtain growth in the absence of a dialyzing medium were made using a culture vessel containing a dialyzer coil. As shown in Figure 6, the coil appeared to have an inhibiting effect on cell growth. It was suspected that this might be due either to cell attachment on the dialyzer membrane or to the toxicity caused by the dialysis tubing and/or by the inside steel coil. Conclusive evidence could not be found for the former possibility; microscopic examination revealed little cell attachment on the membrane. To check the latter possibility, a sample of used medium was extracted from the culture vessel and separated from the cells by centrifugation. The medium was added to a spinner flask; growth was measured and compared to a control. As shown in Figure 7, no toxicity was evident.

It was finally discovered that many cell clumps had precipitated on the outside bottom of the coil. The presence of the coil greatly minimized the dispersing effect of the magnetic stirrer for the portion of the fluid outside the coil. As a result, cells precipitated and formed

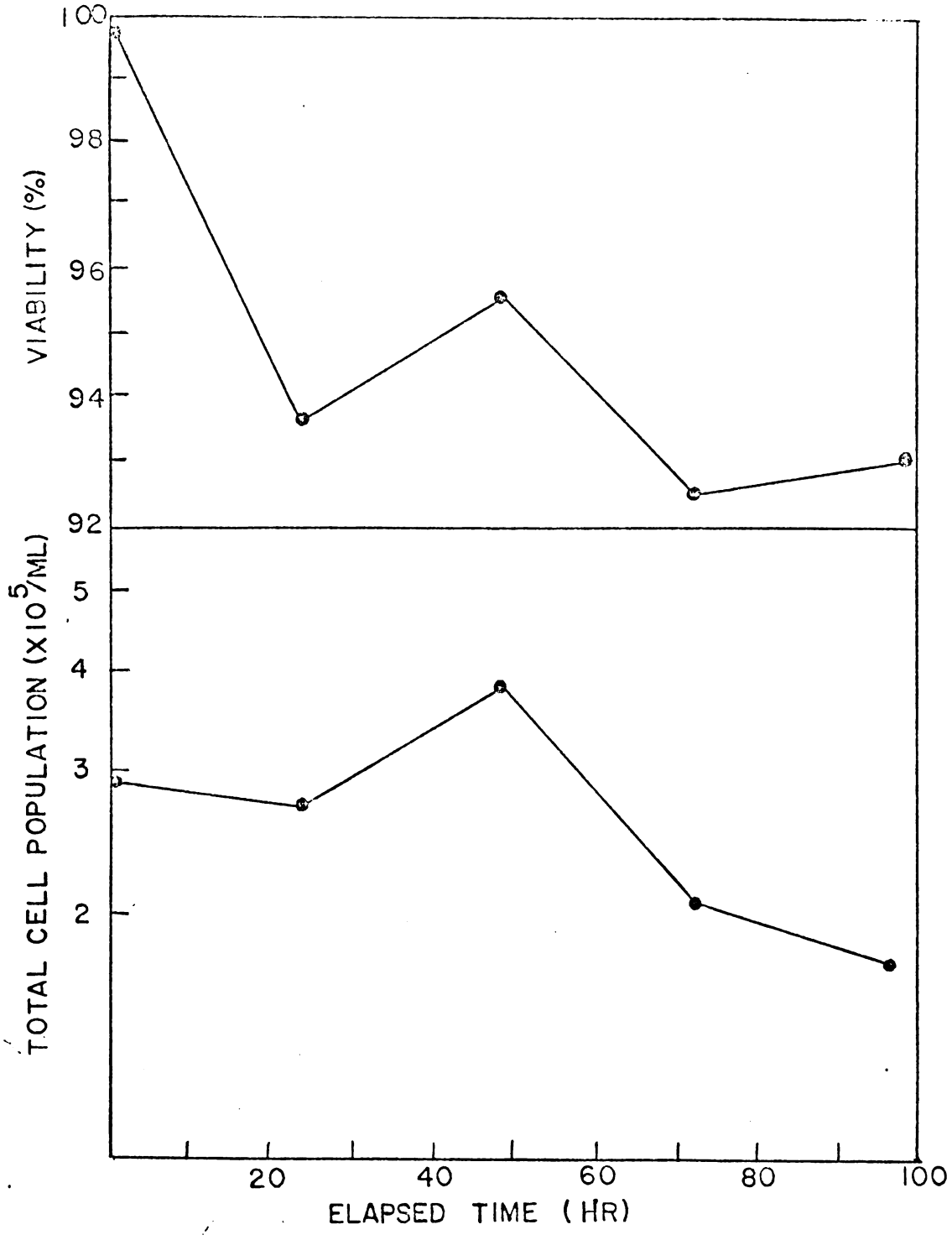

Figure 6. Cell growth in culture vessel with dialysis coil.

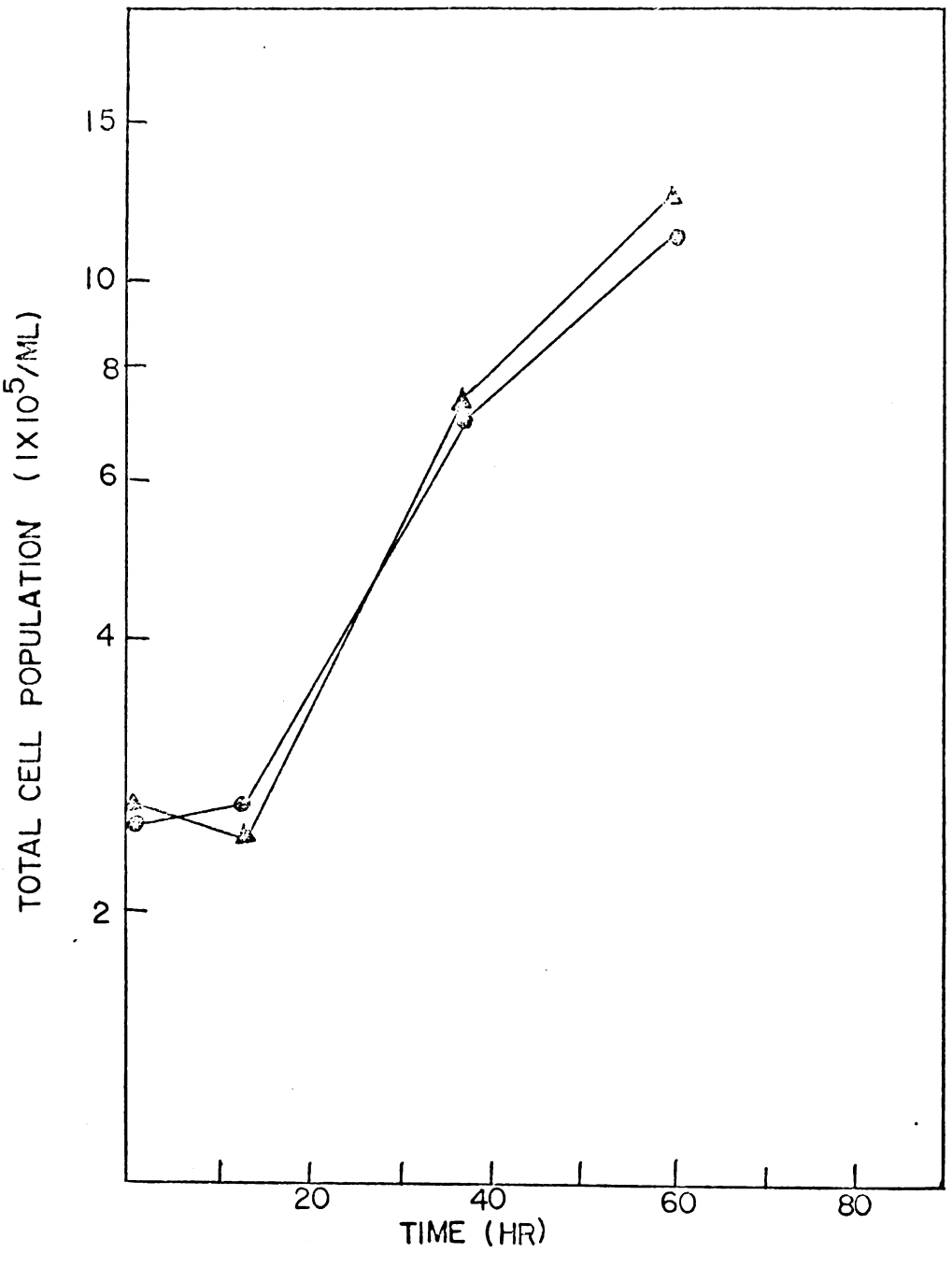

Figure 7. Checking toxicity of medium from culture vessel with dialysis coil by spinner culture.

▲——▲——▲ : Spinner culture with the medium from coil vessel. 30 ml inoculum + 30 ml medium from culture vessel + 40 ml fresh medium.

●——○——● : Control spinner. 30 ml inoculum + 70 ml fresh medium.

clumps.

An attempt was then made to improve the stirring effect by suspending the coil with four silk strings so that it would not have direct contact with the bottom of the culture vessel. This seemed to improve the situation.

The culture vessel, with a total capacity of 500 ml, is 5 cm in height. The coil dialyzer was also designed to be of the same height. It was decided to employ a culture volume of 150 ml and to raise the coil so that it was separated from the floor of the vessel by a distance of 0.5 cm. As a result, only about one-fifth of the dialyzer coil was submerged in the medium.

Figure 8 shows the growth in the culture vessel containing the coil, as compared to another without a coil. The presence of a dialyzer coil in the culture vessel no longer caused significant interference.

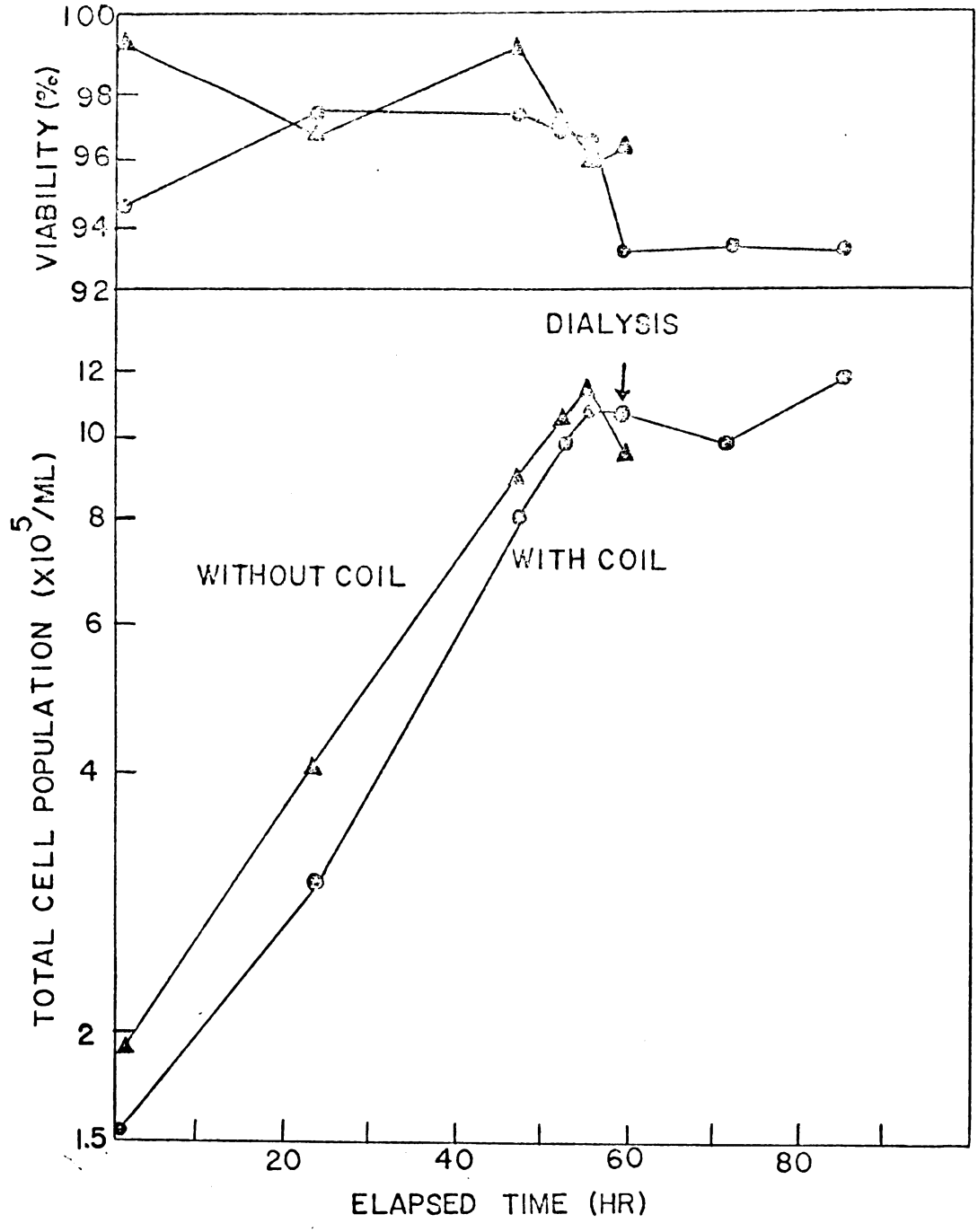

Figure 8. Growth comparison between culture vessel with dialysis coil (without running dialyzing medium) and culture vessel without the coil. Preliminary dialysis was attempted as indicated by the arrow.

4.2 "Growth Trials" of Dialysis Culture

4.2.1 Cell growth in dialysis culture

On the basis of the following, attempts were made to apply the dialysis system at the early stationary phase or late logarithmic phase of growth:

1. Essential effects of dialysis, as revealed in the microbial system, are prolongation of logarithmic growth and increase in cell viability (87). It does not significantly alter the slope of the logarithmic growth curve. In other words, before entering the stationary phase, the essential nutrients might not have been exhausted and the metabolites might not have accumulated sufficiently to influence the growth pattern. As a consequence, applying dialysis at the early stationary phase or late logarithmic phase would be as effective as applying it at the beginning.
2. The possibility was considered that there might be some essential growth factors which were not provided by the medium and which would have to be generated by the growing cells themselves. Concentration of these so-called "conditioned medium factors" should be higher in the stationary phase than in the lag phase. They might be diluted below the required concentration if dialysis were applied from the beginning. To avoid this possible deteriorating

effect of dialysis, it appeared preferable to start dialysis at either the early stationary phase or late logarithmic phase.

3. Applicability of initiating dialysis at the stationary phase was actually suggested by the work of Abbott and Gerhardt (1). In their demonstration experiment of salicylic acid fermentation, dialysis was successfully initiated at an early stationary phase and then repeated by intermittent replenishment of the dialysis reservoir.

4. To reveal the true mechanism of dialysis culture, initiating dialysis at the stationary phase and successively replenishing the reservoir is preferable to applying dialysis culture only once at the beginning. In the former case, metabolites can be analyzed so that their roles in limiting the rate of cell growth can be assessed.

The experiment shown in Figure 8 was run simultaneously with two "growth trials." One, without a coil, served as a control culture; the other had a dialyzer coil but was carried out under the same conditions. After indicating comparable growth patterns in the logarithmic phase, the cell populations of both cultures were measured every 4 hours at the late logarithmic phase in order to assess the beginning of the stationary phase. After the stationary phase had been reached, at the 59th hour, the control vessel was washed with pyrogen-free sterile distilled water and fed with 300 ml of fresh medium. It was then connected

with the dialyzer of the other culture vessel and thus served as a reservoir, with a reservoir-to-culture volume ratio of 2:1. After application of the dialysis system, growth in the dialyzed culture vessel showed a lag period of about 12 hours and then increased. A mechanical accident of pumping interfered with continuation of the "growth trial."

Another "growth trial" was made using a culture vessel with the dialysis coil but without medium running in the coil. After a normal growth period, dialysis was initiated at the late stage of the logarithmic phase. Again, there was a lag period of approximately 12 hours followed by a revival of growth at a typical logarithmic rate. As shown in Figure 9, the rate of this renewed logarithmic growth was not less than that of the first one. It can be definitely concluded that dialysis did show a growth-promoting effect.

This second logarithmic growth did not continue as long as the first one. It was soon followed by another stationary phase. After the stationary phase had been reached, dialysis was attempted by renewing the reservoir medium. There was still some effect of dialysis, although the high cell population and low viability made the effect less evident.

It should be noted that as the cell population increased, cell viability progressively decreased. This decrease in cell viability was not extensively related to the dialysis process. At the late stage of the "growth trial," cell clumps, with the cell number between 2 and 10, appeared. Most of the clumped cells were non-viable.

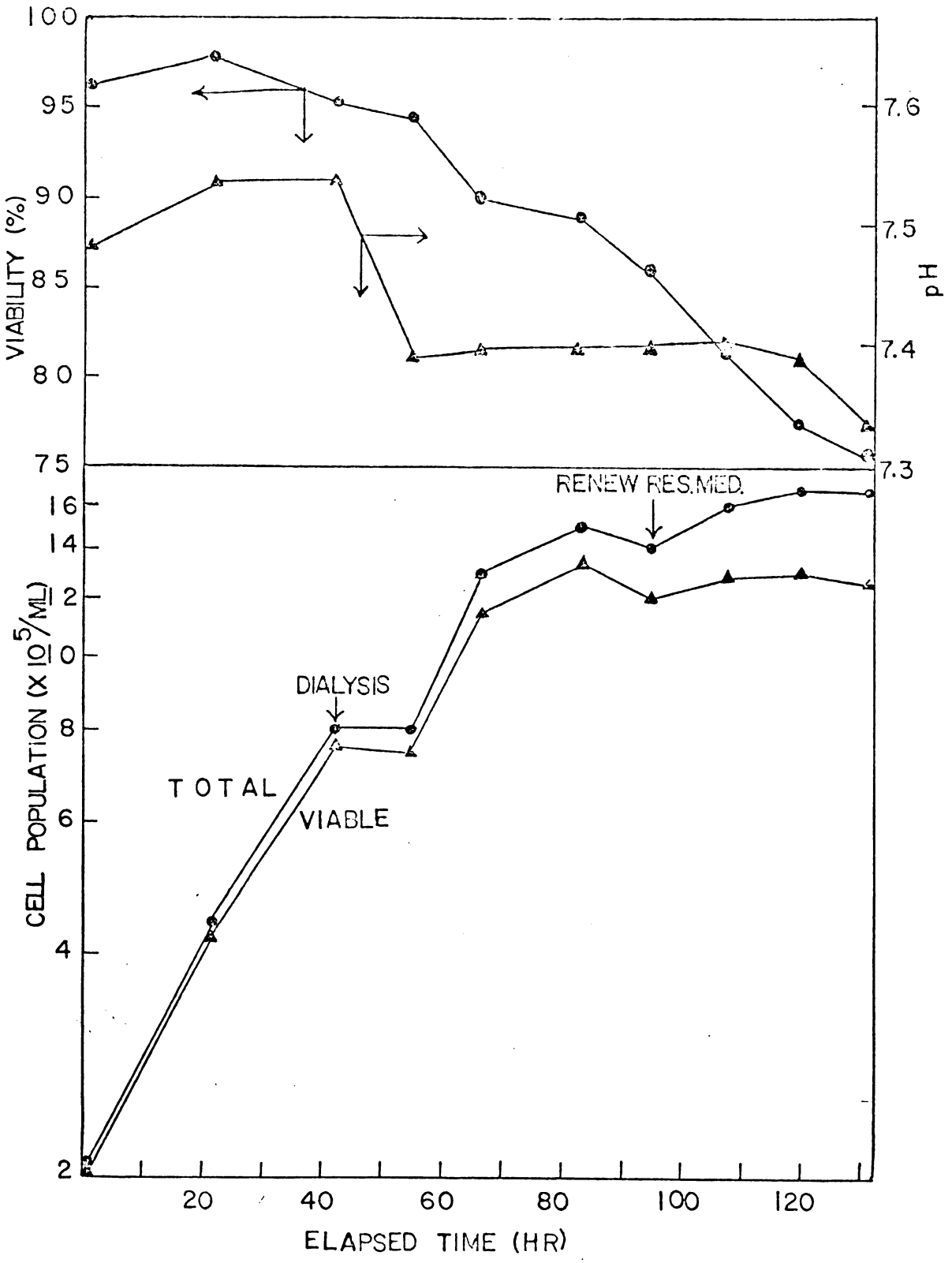

Figure 9. Dialysis culture and cell growth. Initiation of dialysis and renewing of reservoir medium are shown by arrows.

The decrease in cell viability was not a pH effect. As indicated in Figure 9, pH of the culture was maintained at a fairly stable level.

4.2.2 Dialysis effects on the exhaustion of nutrients and accumulation of metabolic products

The above-mentioned cell growth analysis can only give an overall view of the feasibility of dialysis culture. For an assessment of the true mechanism, the concentrations of the nutrients and the metabolic products in both the culture vessel and the reservoir must be measured and analyzed. This objective could not be entirely achieved because many of the factors involved are not chemically defined and, thus, assay methods are not available. However, glucose and lactic acid were selected as representatives for the nutrients and the metabolic products, respectively. Glucose is supposedly the major energy source of cell growth, whereas lactic acid is essentially a metabolic product.

Glucose concentration in the culture vessel and reservoir, correlated with cell growth, is shown in Figure 10. In the absence of dialysis, cell growth was initiated with a glucose concentration of 1.62 mg/ml. During the logarithmic growth phase the concentration dropped linearly to 0.560 mg/ml. Dialysis was initiated from this point--the late logarithmic phase. As soon as dialysis was applied, concentration gradient of glucose between the two vessels impelled the glucose to flux from reservoir to culture vessel, in which the concentration resumed a

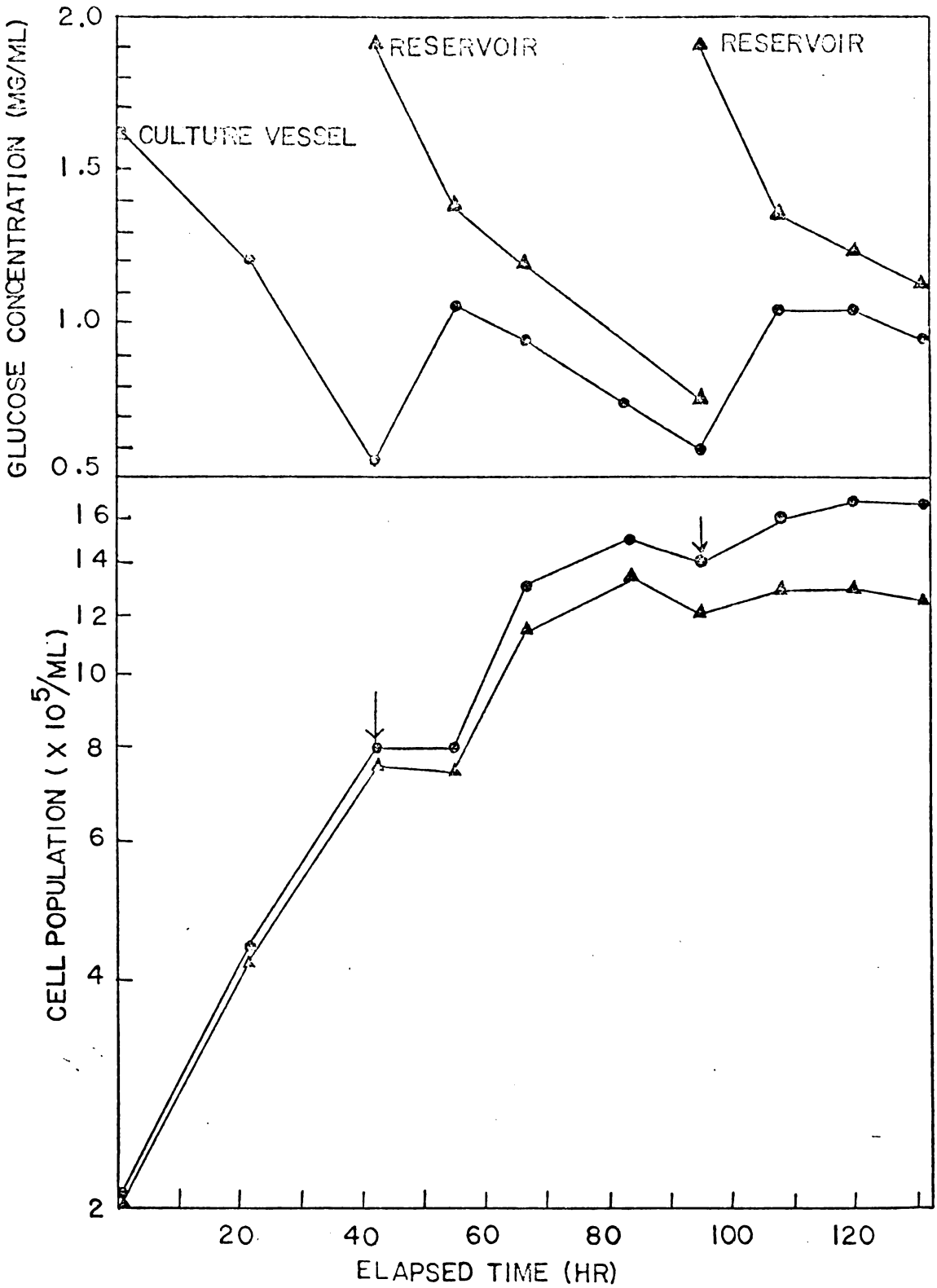

Figure 10. Correlation between cell growth and glucose concentration in the culture vessel and reservoir.

higher concentration of 1.068 mg/ml. This reflux of glucose occurred during the lag period of cell growth. The higher glucose concentration in the culture vessel, as well as the continuous diffusion of glucose from the reservoir, seemed to have been responsible for another new phase of logarithmic growth. At the stationary phase of the resumed growth, glucose in the culture vessel again dropped to a low level of 0.600 mg/ml. By renewing the medium in the reservoir, another dialysis was initiated from this point. Again, the glucose concentration in the culture vessel increased to 1.040 mg/ml. However, cell growth did not significantly correspond to the glucose concentration, and the latter did not drop so rapidly as in the previous dialysis.

Correlation between cell growth and lactic acid concentration in the culture vessel and reservoir is shown in Figure 11. It followed a similar but reverse pattern to that of glucose. The culture was initiated with a lactic acid concentration of 0.710 mg/ml. At the late logarithmic phase, it reached its highest concentration of 2.080 mg/ml. In the lag period of the first dialysis culture, the accumulated lactic acid dropped to 1.060 mg/ml in the culture vessel. Following the resumed growth, the concentration increased linearly to 1.74 mg/ml. The second dialysis brought about a decrease of the lactic acid, but the cell growth did not respond very well and lactic acid seemed to be maintained at a rather stable level.

It appeared reasonable to conclude that by providing nutrients and diluting out metabolic products through the dialysis membrane,

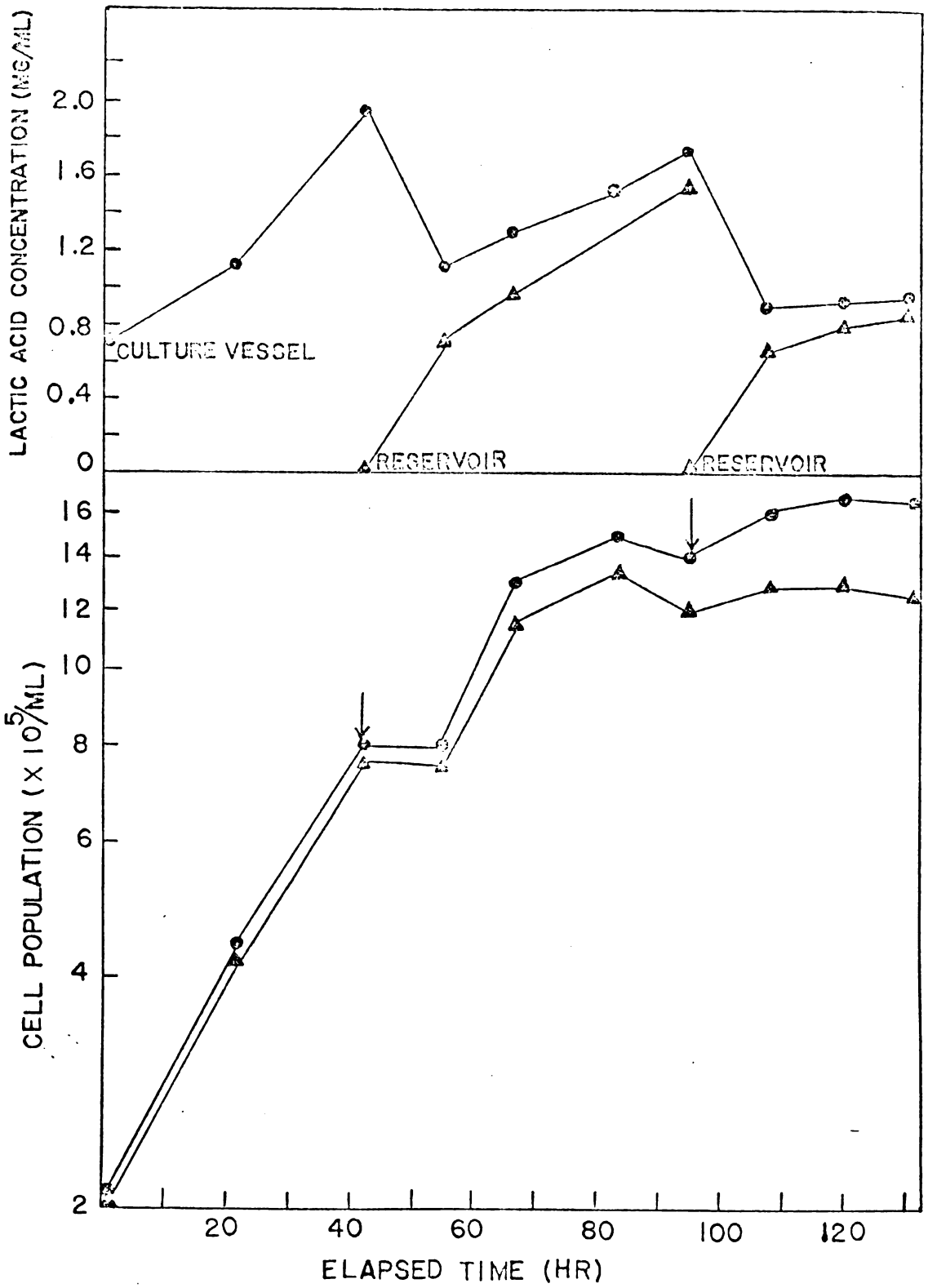

Figure 11. Correlation between cell growth and lactic acid concentration in the culture vessel and reservoir.

dialysis did show a promising effect on cell growth. This is evident from the corresponding effects of cell growth, glucose consumption and lactic acid production during the first trial of dialysis culture. The appearance of the lag period during this trial is of theoretical interest. However, during the second trial the cell growth was already in an undesirable state, as revealed by its low viability; thus, dialysis could not have had a significant effect. It appeared that some factors other than the simple nutrients or metabolic products were playing a role.

The period of the second dialysis attempt, when cell growth did not respond to the dialysis effect, can be more clearly revealed by computed data of glucose consumption and lactic acid production. In order to present the computation, the original data concerning viable cell population, glucose and lactic acid concentrations are shown in Table 1. From these original data, glucose consumption and lactic acid production were computed in terms both of total quantity per unit time and total quantity per unit time per viable cell. It must be understood that this computation supplies a rather rough value for glucose consumption and lactic acid production. For more precise analysis, a continuous monitor system would have to be employed. However, as shown in Table 2, a general pattern can be followed and interpreted.

The relationship between nutrient utilization and cell production can be clearly illustrated by the model proposed by Marr et al. (63):

$$-r_s = \frac{r_g}{Y_x} + Y_E X$$

Table 1. Change of viable cell population, glucose and lactic acid concentration during dialysis culture.

Time (hr.)	Viable Cells (#/ml)	Glucose Conc. (ug/ml)		Lactic Acid Conc. (ug/ml)	
		Cult. Ves.	Reservoir	Cult. Ves.	Reservoir
0	1.97×10^5	1,620	--	700	--
21	4.28×10^5	1,200	--	1,100	--
43	7.63×10^5	560	1,920[a.]	2,080	0[a.]
55	7.56×10^5	1,068	1,400	1,060	736
67	1.16×10^6	940	1,220	1,260	992
95	1.21×10^6	600	[b.] 780–1,920	1,740	[b.] 1,580–0
108	1.32×10^6	1,040	1,360	900	700
120	1.31×10^6	1,048	1,240	912	800
132	1.26×10^6	952	1,140	920	872

a. Concentration analyzed in fresh medium.

b. Process of renewing reservoir.

Table 2. Calculated glucose consumption and lactic acid production during dialysis culture.

Time Interval (hr.)	Average Viable Cells (#/ml)	Glucose Consumption		Lactic Acid Production	
		ug/ml/hr. (I)	ug/#cell/hr. (II)	ug/ml/hr. (III)	ug/#cell/hr. (IV)
21 (0-21)	3.13×10^5	20.0	6.39×10^{-5}	19.0	6.09×10^{-5}
22 (21-43)	5.96×10^5	29.1	4.88×10^{-5}	44.5	7.47×10^{-5}
12 (43-55)	7.60×10^5	44.3	5.83×10^{-5}	37.7	4.96×10^{-5}
12 (55-67)	9.58×10^5	40.6	4.24×10^{-5}	59.3	6.19×10^{-5}
28 (67-95)	1.19×10^6	43.6	3.66×10^{-5}	59.1	4.97×10^{-5}
13 (95-108)	1.27×10^6	52.3	4.12×10^{-5}	43.0	3.39×10^{-5}
12 (108-120)	1.32×10^6	19.3	1.46×10^{-5}	17.7	1.34×10^{-5}
12 (120-132)	1.29×10^6	24.7	1.91×10^{-5}	12.7	0.98×10^{-5}

where $-r_s$ is the rate of substrate utilization due to growth and metabolism, r_g is the rate of growth of the organism, and Y_X and Y_E are empirical rate constants. The first term on the right of the equation represents the portion of substrate used in cell growth. The second term is the portion used for cell maintenance. It can be expected that as the cell population increases, a higher amount of substrate will be used for the second term. This is shown in column (I.) of Table 2. The total glucose consumption per ml per hour increased from 20 ug/ml/hr to 52.3 ug/ml/hr. Because of this maintenance term, the duration of the logarithmic phase achieved with dialysis cannot be as long as that of the first logarithmic phase. After the 108th hour the glucose consumption dropped to an abnormal level. Dividing column (I.) by viable cell concentration provided glucose consumption given in glucose consumed per cell per hour, which revealed the importance of the first term, the substrate used for cell growth. The highest value was in the beginning of growth, when the cells were in an actively dividing state. The level decreased to a more or less stable range. Again, after the 108th hour, the cells showed an abnormal nutrient consumption. It seemed that cell growth could be divided into two phases. During the first phase, nutrient consumption could be interpreted by use of a mathematical model and cell growth could be promoted by dialysis culture. During the second phase, on the other hand, nutrient consumption was abnormal and growth was unresponsive to dialysis effect.

Lactic acid production is dependent on viable cell population and

physiological state of the cells. The latter is revealed in column (IV.), the lactic acid produced per cell per hour. The values are scattered in a range between 7.47 ug/cell/hr and 3.39 ug/cell/hr, except for the rather low level revealed in the abnormal second phase.

5. DISCUSSION

5.1 Objectives Achieved in the Experiment

5.1.1 Applicability of dialysis culture to mammalian cell suspensions

Mammalian cell culture, like most biological processes, is subject to various limiting factors. These factors can be classified as follows:

1. Physical environment. Factors such as agitation, viscosity, osmolarity, etc., fall into this category. These factors, if poorly controlled, will cause cell sedimentation, cell clumping and mechanical injury.
2. Chemical environment. Included in this category are nutrients, metabolic products, pH, oxidation-reduction potential, etc. These factors are determined by the nutrients supplied in the medium, the gaseous environment over the surface of the culture and the metabolic state of the cells. The nutrients and metabolic products can be further divided into dialyzable and non-dialyzable. Furthermore, nutrients include those which can be supplied in the medium and others which have to be generated by the cells themselves.

It is unreasonable to have an overall statement as to whether or not dialysis can be applied to mammalian cell suspension culture. However, taking into consideration the above limiting factors of mammalian cell culture, the applicability of dialysis culture can easily be seen. For-

example, it cannot be expected that dialysis would have any promising effect on physical environment. In fact, the presence of a dialyzer in a culture system might be another limiting factor which would have to be critically considered in the physical construction. Likewise, for the chemical environment, dialysis will not be able to dilute out non-dialyzable metabolic products, to supply non-dialyzable nutrients or to supply nutrients which are not present in the medium. Actually, one of the limitations of dialysis is that it might be possible to dilute out some dialyzable growth factors which can only be generated by the cells.

However, within a certain range, it cannot be denied that dialysis can be applied to mammalian cell culture to promote cell growth. As demonstrated in this study, after application of the dialysis system, a new phase of logarithmic growth resumed, exhausted glucose concentration increased and accumulated lactic acid was diluted out. In other words, stress derived from dialyzable nutrients and metabolic products can certainly be released by dialysis.

5.1.2 Discovery of a lag period

In an attempt to reveal the mechanism of dialysis culture, this study utilized a small volume ratio (1:2) with intermittent renewal of the reservoir medium, and applied dialysis at the late log phase or early stationary phase rather than at the initiation of the culture. A lag period was observed after applying dialysis. This lag period was not previously

reported. It is suspected that the significance of the lag period might be related to the mechanism which would explain a prior failure of a dialysis trial in a similar experiment (89). A rationale is that there might be some growth factors generated by the cells themselves. These factors might be the ones termed "conditioned medium factors." As shown in Figure 5, centrifugation of inoculum diminished these factors so that cell multiplication waned until a sufficient concentration was again generated. For an inoculum size of 10 per cent without centrifugation, the concentration of these factors could be diluted 10 times and still be effective, although there is usually a lag phase. However, if dialysis were run from the beginning, in the case of a volume ratio of 1:10 the factors would be diluted 100 times. As a result, good proliferation would not take place. This is the same rationale that caused the author to apply dialysis at a later stage so that the more populated and highly active cells would generate a higher amount of these factors. Yet, there is still a good opportunity to observe the lag period after initiating the dialysis system.

Theoretically, although a lag phase would still be present, the application of the dialysis system at the later stages of growth should not affect the system's efficiency since it is generally at later stages that its growth-promoting effect is shown, namely, prolongation of the log phase and maintenance of higher viability at the stationary phase.

5.1.3 Limitation of dialysis culture

The concept of rate-limiting factors should be borne in mind for interpretation of the efficiency of any biological process. It has been mentioned in one of the previous sections that there are some limiting factors involved in mammalian cell suspension culture. Among these factors are some which can be released by dialysis and others upon which dialysis would not have any effect. These factors might exert their influence simultaneously, or some of them might become predominant at certain stages of growth as rate-limiting factors.

This work appears to have demonstrated that the cell population of a mammalian cell culture can be increased by a dialysis system. However, as the cell population increases, the physical construction of the culture vessel assumes a critical role. In a poorly constructed vessel, it might be more difficult to maintain cells in homogeneous suspension without resultant precipitation and clumping. A magnetic stirrer, which grinds the cells underneath it, is another critical factor. As a result of its use, viability of the cells decreases rapidly despite the application of dialysis.

Nevertheless, the difficulties encountered in the present study should not be considered an impediment to further investigations. What is emphasized here is that in the application of dialysis culture, those factors which seem to have no relationship to dialysis should nevertheless be taken into consideration. If these factors can be made

non-rate-limiting, the continuous process of dialysis might promote cell growth to an unlimited concentration. There is a high cell population of blood cells in the blood vessels, 5.2×10^9 to 4.5×10^9 for erythrocytes and 5.0×10^6 to 9.0×10^6 for leucocytes, because of a smooth flow and because of the existence of a smooth layer of endothelium; in like manner, it is essential that an improvement in the physical design of the culture vessel be made before assessing the efficacy of improving the chemical environment through a dialysis system. Among critical factors are the delicate cell membrane which is liable to be destroyed by drastic stirring and, at the same time, to clump together without efficient stirring, and a larger cell size which, again, calls for a compromise between stirring and sedimentation. All of these problems do not exist in a microbial system.

5.1.4 Economics aspect

Since serum growth-factors are usually described as being protein fractions (94) and are thus non-dialyzable, an attempt was made to use serum-free medium in the reservoir. Applicability of dialysis culture to mammalian cell suspensions, as demonstrated in the present study, would result in restriction of serum to the small volume of a fermentor. Consequently, there is a noteworthy economical reduction. A cost and yield analysis, based on 80 per cent cell yield efficiency of dialysis culture, is shown in Table 3. The cell population of dialysis,

Table 3. Cost and yield analysis--a comparison of batch vs. dialysis culture.

Culture System	Batch	Dialysis	
		Culture Vessel	Reservoir
Total Volume (l)	1,000	1,000	10,000
Max. Cell Pop. (10^6/ml)	1.4	12.32[a]	--
Total Cells Harvested ($x10^{12}$)	1.4	12.32	--
Weight of Cells Harvested[b] (gm)	1,400	12,320	--
Total Serum-free Media[c] used (l)	900	900	10,000
Total Sera Used[c] (l)	100	100	--
Total Cost Serum-free[d] media	$450.	$450.	$5,000.
Total Cost Sera[e]	$3,750.	$3,750.	--
Total Cost Media	$4,200.	$9,200.	
Media Cost per gm Cells	$3.00	$0.75	
Media Cost Ratio: 4-fold decrease			
Cost Decrease for the Batch Fermentor Cell Yield (1.4×10^{12}): $3,150.			

a. $1.4 \times 10^6 \times 11,000/1,000 \times 0.8 = 232 \times 10^7$/ml
b. 10^6/mg c. 10% serum d. $0.50/l e. $35.5/l
Figures in b. d. e. were provided by Dr. McLimans.

calculated from relevant data, is not purely imaginative. In continuous cell culture, McLimans et al. achieved a cell population to 8-10 x 10^6/ml, and Earle et al., to 20 x 10^6/ml. There is no reason, through improvement of physical environment and thus abolition of rate-limiting factors, that dialysis cannot achieve an equally high population, or an even higher value since no cell loss is involved. Further evidence was provided by Graham and Siminovitch who, using a strain of monkey kidney cells grown in a suspension system, achieved a concentration of 10^7 cells/ml through the expediency of making complete medium changes every 24 hours (45).

A four-fold decrease in cost is indicated. It should be sufficiently optimistic to encourage further attempts to improve the dialysis culture system. By employing intermittent renewal of reservoir medium, further cost reduction becomes a possibility.

5.2 Comparison with a Bacterial Culture System

In addition to being multicellular and eukaryotic as compared with the unicellular and prokaryotic microorganisms, mammalian cells exhibit some unique characteristics which must be taken into consideration if a bacterial culture system is to be applied. Among these characteristics are:

1. Naked cell membrane. Without a protective cell wall, the mammalian cell membrane is directly exposed to the culture environment. The delicate cell membrane is liable to be destroyed by drastic mechanical force, fluid dynamic effect and surface tension. Thus, agitation, which is necessary to maintain the cells in suspension, should not be too violent as this will cause mechanical and fluid shearing injury. If the dialyzer-in-reservoir dialysis or the independent-dialyzer dialysis is to be employed, circulation of the cell suspension is necessary, and prevention of damage to the cell suspension is dependent on the type of pump used and the speed of circulation. Gassing appears as an overlay on the culture surface since sparging becomes impractical as long as a media serum component is required. It has been demonstrated that damage caused by fluid shearing is of the first order of reaction with respect to cell number and that L 929 cells are more

sensitive than HeLa S_3 cells (4). More precise measurements, including tolerance to mechanical force and surface tension, appear to be necessary if large-scale cultivation of mammalian cells is to be employed for industrial purposes. Another problem related to the cell membrane is that of cell clumping. Basically, mammalian cells are multicellular. Aggregation of cells is likely to occur if conditions are favorable. Consequently, cell clumping appears as the cell suspension becomes more concentrated.

2. Larger cell size. In a static fluid suspension, a cell particle is subject to two forces: an upward buoyancy equivalent to $\frac{4}{3}\pi r^3\rho'$ and a downward gravity equivalent to $\frac{4}{3}\pi r^3\rho$, where r is the radius of the cell, ρ is the specific gravity of the cell and ρ' is the specific gravity of the suspension fluid. The net force, equal to $\frac{4}{3}\pi r^3(\rho-\rho')$, causes sedimentation of the cell. By comparing this sedimentation force of mammalian cells with that of bacteria, taking a radius ratio of 10, the difference can be well appreciated. In order to keep the cells in a suspension state, an agitation device must be employed, which creates a mobility characterized by Stokes' formula:

$$u = \frac{1}{6\pi\eta r}$$

where u is the mobility for spherical particles, moving in a continuous liquid under the influence of unit force, and η

is the viscosity of the liquid. Again, the larger mammalian cells cannot be mobilized by a given agitation system as efficiently as the smaller bacteria.

3. Nutritional requirement. The nutritional requirement of mammalian cells is much more complicated than that of microorganisms. The formulae for culture medium are comprised of the energy source, vitamins, amino acids, and inorganic salts. In addition, various amounts of serum must be employed, with consideration also given to "conditioned medium" factors. The growth-promoting factors in serum are still chemically unknown. In one suspension culture, the serum was replaced by insulin for the growth of L-cells (76) and HeLa cells (7). Conditioned medium is most readily demonstrated via cell clones. The principle was first employed by Sanford, Earle and Likely (86). By means of procedures involving restriction of the volume of culture medium and pre-conditioning of the medium, the L-929 cell strain was isolated as single, completely separated tissue cells (86). The substance in conditioned medium can be produced from either unirradiated or x-irradiated feeder cells (81). Relationship between feeder cells and clone-forming cells does not appear to be cell-type specific (8). Conditioned medium from normal leucocytes permits the growth of leukemic marrow cells (51).

A linear relationship was demonstrated between the dose of the conditioned medium and the number of colonies forming (8). There might be different entities in different cell types since the substance has been described as heat-stable in mouse embryo cells (81) but thermolabile in chick embryo cells (85). The substance seems to consist of macromolecules characterized as being removable from the medium by prolonged high speed centrifugation (85), precipitable by 50-100 per cent saturation with ammonium sulfate (8), and non-dialyzable (81). In the present study, a lag period was observed after initiating dialysis culture at either late logarithmic growth phase or early stationary phase. The lag period has been interpreted as resulting from conditioned medium. If this interpretation is correct, it appears that there exists in conditioned medium a substance which is dialyzable. Since the observation is rather indirect, its significance needs to be characterized further.

5.3 Further Extrapolation of Dialysis to Mammalian Cell Culture

The experimental work in this study has clearly demonstrated that the dialysis technique is applicable to mammalian cell suspension culture. However, the use of established cell lines for the manufacture of products designed for use in man is generally forbidden and the approved primary cell has not yet been grown in suspension culture despite extensive efforts by some workers. Consequently, it would be valuable to investigate the feasibility of extrapolation of the applicability of dialysis culture of mammalian cell suspensions to monolayer culture of primary cells.

Currently, while the in vitro cultivation of primary cells can be done without a great deal of difficulty, what is of challenge is the production of cells which would not only demonstrate luxuriant growth but which would also maintain their specific function. Achievement of this aim is a prerequisite of fermentative utilization of animal cells, which is a prospective field of biochemical engineering. Since this aspect is of interest, a preliminary effort will be made to determine if a possible approach might be conceived.

Prevalent techniques for primary cell culture are still far from being perfect. Questions have been raised about the physiological significance of the cultivated cells (68). Thus, in vitro cultivation of cells leads to dedifferentiation, survivor cells might be those more resistant ones selected from the rather barbaric culture environment, and little

is known about nutritional factors in serum--which is an essential medium component in current culture techniques. In order to produce cells comparable to the cells in the original tissue, it might be essential to mimic the in vivo system as closely as possible. By comparison of the in vivo and in vitro systems, the following major problems are revealed:

1. Cell dissociation. For the isolation of the individual cell from its original tissue, perfusion agents, mechanical devices and dissociating agents are used either alone or in various combinations. As a result, the cell membrane might be altered and key enzymes or regulating factors might be lost before the cell can be cultivated.
2. Nutritional environment. The importance of the constancy of the internal environment in regulating the activities of living tissue was pointed out by Claude Bernard in as early as 1857 (5). However, the culture environment, in terms of gaseous and liquid medium conditions, is still greatly inferior to the in vivo one. Of particular interest is the fine distribution and efficient transport of the in vivo circulatory system which it is almost impossible to reproduce in vitro.
3. Attaching surface. Tissue cells in vivo are associated with each other either directly or through connective tissue. In the in vitro system, glass or plastic is chosen rather arbitrarily

as the material to which the cells attach. It may be possible that these artificial surfaces might bring about physical or chemical changes in the cell membrane. Among naturally-occurring connective tissue, collagen has been used in the in vitro cell culture with promising effects (66).

4. Regulatory system. Another important doctrine developed by Bernard, in 1878, is the concept of homeostasis (6). According to him, the environment is not only the product of tissue metabolism; it reacts in turn upon the tissues themselves and regulates their activity. Indeed, homeostasis is the unique characteristic of multicellular organisms. It does not take a great deal of imagination to relate in vitro cell dedifferentiation and in vivo cancer to aberrations in a communication system. If tissue cells can be induced to be autonomous by disturbing the in vivo regulatory system (36), how can in vitro cells be maintained in a differentiated state without their regulatory system? It is speculated that improvement of culture environment might be essential, but not sufficient, for maintaining cell functions in vitro. Cybernetics, the study of methods of control and communications which are common to both living organisms and machines (101), must be the key language to be learned for a full understanding of both in vivo and in vitro systems.

All of these problems are not encountered in suspension culture.

For the extrapolation of dialysis techniques from suspension culture to monolayer culture, and for the cultivation of primary cells competent to maintain cell functions, each of the problems should be seriously studied. Some of the problems are obviously not pertinent to dialysis culture. However, failure to take them into consideration may create rate-limiting factors which will make dialysis culture successful but impractical.

6. CONCLUSIONS

The present work utilized a small volume ratio with intermittent renewal of the reservoir medium and applied dialysis at the late log phase or early stationary phase rather than at the initiation of the culture. The following conclusions may be reached as a result of careful interpretation of the experimental data:

A lag period of about 12 hours was observed after applying dialysis. This might be the result of a dialyzable substance present in conditioned medium. Presence of the lag period might not affect the efficiency of the dialysis application.

Applicability of dialysis culture was demonstrated by the observation of a resumed new phase of logarithmic growth, an increased higher level of glucose concentration, and a diluted lower level of lactic acid concentration. Thus, the stress derived from dialyzable nutrients and metabolic products can be released by dialysis. Furthermore, economical cost reduction was achieved by the restriction of the expensive serum component of the medium to the small culture vessel.

There has, however, been only limited success in the use of dialysis culture techniques. Nevertheless, some of the limitations can be overcome by improvement in physical construction of the culture vessel. It is essential that physical limitation factors be removed before the chemical efficiency of dialysis culture may be assessed. The present study has only indicated the existence of the physical factors and the

necessity for improvement. The results of further investigation would be of value to industry.

Another interesting potential line for further investigation is the determination of whether dialysis culture of mammalian cell suspensions can be extrapolated to primary cell monolayer culture. Problems such as cell dissociation, nutritional environment, attaching surface and regulatory systems must be considered in the investigation.

7. RECOMMENDATIONS

As a possible approach, it is proposed that a model system be developed which will be useful for further investigation of differentiation functions and response to regulatory substances, resulting in decrease in the stress of the culture environment and enabling consideration of scale-up possibilities. The proposal would hopefully lead to experimental work concerning the scale-up of in vitro cultivation of differentiated cells so that this differentiated function might be utilized in the field of fermentation. Guidelines of the model system are as follows:

1. Cell type. Criteria for a suitable cell type for the model system are responsiveness to known regulatory factors and the existence of well-characterized specialized functions. The mammary secretory cell might be a good choice. At the in vivo level, functions of the mammary gland are subject to ovary, adrenal and pituitary regulation (55). At the organ culture level, insulin alone in a chemically defined medium is capable of maintaining the cells in a healthy state. Insulin plus prolactin maintains initial mitotic activity and stimulates alveolar development (24). Among the other regulatory factors affecting its function are hormones such as hydrocortisone, progesterone, estrogen, etc. (62, 95). The uniqueness of the mammary cell in producing several products found nowhere else and

the vast literature concerning them, the differentiation of this cell in the adult animal to proliferation and then to milk synthesis, the active metabolic rate, and the obvious dependence upon external hormonal influence make this cell an attractive location in which to study the control mechanisms of cellular differentiation (60).

2. Culture technique. Among the current superior techniques used for primary culture are those which provide a thin film for gas diffusion with a large volume of medium so that a constant environment can be maintained (67). Another factor worthy of consideration is the separation of macromolecules from the other components of the medium so that the environment containing small molecular components can be constantly maintained, while macromolecule components such as hormones can be added or withdrawn at will. It is conceivable that a dialysis system with these intrinsic characteristics might be the best candidate. Thus the plate-and-frame dialyzer might be used as a culture chamber. On one side of the chamber, nutrients can be provided from and metabolic products can be diluted into a large reservoir through a dialysis membrane. On the other side, gas can be diffused through a silicone membrane. Essential for this application might be a suitable substratum for the cells to attach. However, theoretical estimation and

physical design must first be worked out.

3. Regulatory factors. Although differentiated cells can be cultivated in vitro, these cells lose function in a certain period of time (37, 59). The reason might be the loss of or diluting out of inducers, hormones, metabolic intermediates or other factors. Additionally, in vitro culture techniques might alter the cell membrane so that responsiveness to the regulatory factors might be decreased. The two aspects should be separately treated. A good culture technique should be able to maintain responsiveness. In the absence of regulatory factors, differentiated functions might not be demonstrated even though response to the factors might still be present. In vitro cultivated cells might be termed differentiated if they are potentially capable of maintaining or redeveloping their specific characteristics after exposure to the correct environment. It might be possible to use a carrier substratum in organ culture to replace the cell dissociation procedure. The carrier, such as ion exchange resin particles (98), might in turn serve as the inoculum. Regulatory factors can be employed at various culture stages in order to examine the response. A rationale of the necessity for this is that the mechanism of various hormonal activation is generally assumed to involve an allosteric interaction between the hormone and either the

adenyl cyclase or the membrane with which it is associated (53).

4. Serum factor. Serum used in the conventional culture medium might contain both essential growth factors and regulating agents. As long as the serum component cannot be replaced, culture media are not chemically defined and biochemical studies will present difficulties. Since the serum level in the medium does not affect milk synthesis nearly as much as it affects cell survival or replication, it has been speculated that, perhaps indirectly, a low serum level may enhance the maintenance of specific function (59). In fact, hormones have been added to chemically defined medium for the maintenance of the histological pattern and the alveolar secretory appearance of the mammary organ (54).

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MATERIAL REFERENCES

9. MATERIAL REFERENCES

1 M	American Instrument Company, Inc. Silver Spring, Maryland	Aminco Peristaltic Pump
2 M	C. A. Hausser & Son Max Levy	Fuchs Rosenthal Ultra Plane Hemocytometer
3 M	Cole-Parmer Instrument Company 7425 North Oak Park Avenue Chicago, Illinois 60648	Silicone Tubing
4 M	Cole-Parmer Instrument Company 7425 North Oak Park Avenue Chicago, Illinois 60648	Micro-V Magnetic Stirrer
5 M	Ethicon, Inc. Somerville, New Jersey	Silk 00 Sutupak Sterile Surgical Silk Type B U.S.P.
6 M	Grand Island Biological Company Grand Island, New York 14072	Trypan Blue Stain (0.4%) in Hank's Balanced Salt Solution
7 M	Microbiological Associates, Inc. Bethesda, Maryland	RPMI 1955 1X Eagle's Medium
8 M	Microbiological Associates, Inc. Bethesda, Maryland	0.2% Trypsin Solution for RP
9 M	Microbiological Associates, Inc. Bethesda, Maryland	Minimum Essential Medium Eagle for Suspension Cultures, without L-glutamine
10 M	National Cylinder Gas Division of Chemetron Corporation Chicago, Illinois	5% CO_2 in Compressed Air
11 M	Panduit Corporation 17301 Ridgeland Avenue Tinley Park, Illinois	Tie Straps
12 M	Radiometer Copenhagen, Denmark	Water Thermostat
13 M	Radiometer Copenhagen, Denmark	Micro Electrode Unit Type E pH Meter

14 M	Reichhold Chemicals, Inc. Cuyahoga Falls, Ohio	Latex Surgical Tubing
15 M	Technicon Corporation Ardsley, New York 10502	Auto Analyzer
16 M	Union Carbide Corporation Food Products Division 6733 W. 65th Street Chicago, Illinois	Dialysis Tubing ("Visking") Regenerated Cellulose