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THE EFFECTS OF BUFFERS AND CARBON DIOXIDE DEFICIENCY ON THE MULTIPLICATION OF HERPES SIMPLEX AND VACCINIA VIRUSES IN HELA CELL CULTURES

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THE EFFECTS OF BUFFERS AND CARBON DIOXIDE DEFICIENCY

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF TABLES (CON.)

LIST OF ILLUSTRATIONS

LIST OF ILLUSTRATIONS (CON.)

LIST OF ILLUSTRATIONS (CON.)

THE EFFECTS OF BUFFERS AND CARBON DIOXIDE DEFICIENCY ON THE MULTIPLICATION OF HERPES SIMPLEX AND VACCINIA VIRUSES IN HELA CELL CULTURES

CHAPTER I

INTRODUCTION

Mammalian cells cultivated *in vitro* have been used successfully in the study of host cell-virus relationships. With such cells, certain reactions, uncomplicated by those factors associated with an animal's normal defense mechanisms, can be studied in a carefully controlled environment. In *in vitro* systems, a definite number of genetically homogeneous cells can be infected with a known number of infectious virus particles; the events which take place within the host cell can be studied more easily than in systems which utilize intact animals. It is necessary to know as much as possible about the normal attributes of uninfected, *in vitro* cultivated mammalian cells before a study of the host cell-virus relationship is undertaken.

There are three types of monolayer cell cultures: primary, serially propagated, and clonal cell lines. When animal tissues are grown *in vitro* for the first time, the initial growth is termed a primary culture. Although many cells or tissues of adult animals cannot be grown successfully *in vitro*, very often primary cultures can be

initiated from the same type of tissue when it is taken from an embryo or fetus. Serial propagation of primary cultures, in many instances, is unsuccessful. The cells may, however, proceed to undergo mitotic changes and alteration of normal chromosome pattern takes place to form a stabilized culture. Thereafter, the cells are capable of indefinite multiplication under favorable conditions. Such an established culture is designated as a serially propagated cell line or strain. In such a cell culture only one cell type is usually evident, but to insure a homogeneous cell population, techniques are used to isolate and propagate a single cell. The progeny from a single cell, which is theoretically genetically homogeneous, is called a clone or a clonal cell strain.

Nutrition of Serially Propagated Mammalian Cell Strains

The nutritional requirements for primary cultures, serially propagated, and clonal cell strains may differ. Before studying needs of cells for virus proliferation, it is necessary to understand the nutritive requirements of normal host cells. Until recent years media employed for the cultivation of mammalian cells *in vitro* were derived from body fluids of homologous species and consisted primarily of blood plasma, blood serum, ascitic fluid, and other body exudates or tissue extracts. These natural materials are complex and variable and it is difficult to determine which constituents are required by cells. Completely synthetic media are needed.

Tissue Culture Media

Early attempts to prepare a chemically defined medium were crude. White (1946) made the first serious attempt to cultivate animal cells in

a solution of known composition, i.e., completely free of serum or other biological fluid. His feeding solution which consisted of many well defined chemical ingredients supported chick embryo heart tissue in a state of functional survival for several weeks. Subsequently, many synthetic media have been devised, most of which are maintenance media and do not permit sustained growth of cells, although they do preserve the capacity of cells to carry out many of their normal metabolic activities. The length of time that cells can survive is dependent on the medium and the cell strain. Addition of serum may enable a chemically defined maintenance medium to become a growth medium and to support long term growth of cells. There are many examples of such media being used routinely. Medium 199 is chemically defined and contains a complete complement of amino acids, vitamins, certain intermediary metabolites, growth factors, and several nucleic acid constituents (Morgan et al., 1950). CMRL-1066 (Parker et al., 1957) is also a maintenance medium when not supplemented with serum. Serum is not an absolute requirement for all mammalian cells; a serum-free medium designated NCTC-109 (McQuilkin et al., 1957) supported for several years growth of L-2071 cells, a strain of mouse cell fibroblasts which is considered to be a nutritional variant of strain L-929 (Swim, 1959). A specific medium is devised for a particular strain of cells; many of the so called essential metabolites in one medium may be needed for one cell but not needed for another. Many media now in common use probably contain nonessential components.

The careful and methodical work of Eagle (1955a, 1955b, 1955c; Eagle et al., 1956) has done much to define in chemical terms the

nutritional requirements of cultured mammalian cells. Thirteen amino acids, eight vitamins, certain inorganic salts, glucose, and whole or dialyzed serum are the minimal essential nutrients for most of the serially propagated cell lines which have been investigated.

Although a cell may have a limited capacity to synthesize one or more of the essential amino acids, most cell lines require arginine, cysteine, glutamine, histidine, tyrosine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, and valine for maintenance of nitrogen balance and growth (Rose et al., 1955).

Choline, folic acid, nicotinamide, pantothenate, pyridoxal, riboflavin, and thiamine are required vitamins (Eagle, 1959). Trace amounts of other vitamins may be required, and these could be supplied by other components of the medium, particularly serum. Many essential vitamins are known to form integral moieties of co-enzymes (Levintow and Eagle, 1961). Most cell strains also require inositol.

A carbohydrate source is needed in the medium, and glucose is routinely used although it has been shown that a number of carbohydrates can be utilized effectively. There is an apparent lack of correlation between carbohydrate utilization and growth (Bryant et al, 1958). Some studies indicate an inverse relationship between cell population and metabolism of glucose (Zwartouw and Westwood, 1958). The efficiency of carbohydrate utilization and anaerobic glycolysis which takes place are closely interrelated. The rate at which hexose phosphate becomes available to the cell is thought to be extremely important for carbohydrate utilization (Eagle et al., 1958). The addition of purines and pyrimidines or their derivatives is not essential for replication

of mammalian cells in vitro. It is known that these compounds can be synthesized from simpler chemical intermediates. The actual contribution of whole or dialyzed serum to the nutritional requirements of the cell is difficult to assess. Most media designed for supplementation with serum are completely inadequate for cell survival in its absence. This suggests that the role of serum is a major one (Morgan et al., 1956). Eagle's basal medium and many other chemically defined media require addition of five to 20 per cent serum for sustained growth of cells. These media, which are considerably less complex than those to which no serum is added, have been used with success to demonstrate specific nutritional deficiencies and their correction. Serum protein is believed to function in the attachment of cells to glass or to other cells (Lieberman and Ove, 1958). Eagle (1960) suggested that serum protein acts as a carrier of low molecular weight, dialyzable nutrients. He demonstrated that human carcinoma cells (strain HeLa) and mouse fibroblast cells (strain L) could grow in suspension cultures in a chemically defined, protein-free medium if the culture were equilibrated, across a cellophane membrane, with a medium which contained one to five per cent dialyzed serum and a dialyzed enzyme solution prepared from pancreatic extract. Only slow and sparse growth was obtained when the enzyme preparation was omitted. He postulated that protein provides essential, small molecular weight growth factors which were originally bound to the protein or were derived from protein by enzymic proteolysis. Other substances, such as nutritionally inert methylcellulose, have been shown to have a protective influence on cell multiplication. When L-M cells (a strain of mouse fibroblast) were

grown in Eagle's basal medium without serum supplement, cell counts were 70,000 to 200,000 higher when cells were harvested with methylcellulose than when methylcellulose was omitted. This suggests that protein may function as a physical protective agent since it can be replaced in certain instances by this macromolecular substance (Merchant and Hellman, 1962). When protein or peptides were not present a two-fold increase in amino acids, vitamins, and glutamine was necessary for rapid cell multiplication. A change of medium at three day intervals was also necessary for maximum growth.

Balanced Salt Solutions

Synthetic media used for *in vitro* cultivation of mammalian cells are routinely prepared in balanced salt solutions (Tyrode, 1910; Gey and Gey, 1936; Earle *et al.*, 1943; Hanks and Wallace, 1949). Such solutions have three primary functions: they provide water to irrigate the cells and maintain their tonicity; they furnish inorganic ions which are necessary for metabolic activities; they serve as buffers which maintain the physiologically compatible pH range of 7.2 to 7.6. The salt content of these solutions is similar to that of the cultured cells and, hence, to that of serum which bathed the cells. Osmotic pressure is closely related to the transport of metabolites in and out of cells so that if isotonicity is not maintained within narrow limits cellular metabolism cannot proceed normally. In carefully controlled experiments, Eagle (1956) showed that, in addition to maintenance of isotonicity, certain ions (sodium, potassium, magnesium, calcium, chloride, and phosphate) were essential for growth of HeLa and L cells in a

minimal growth medium. The function of these ions is not completely understood, but it is known that sodium and chloride ions are required for the maintenance of osmotic pressure. Potassium may serve a similar purpose within cells, but it probably has a more specific, though yet undescribed, function. Calcium and magnesium are necessary for the activities of many intracellular enzymes. Calcium may be concerned with the alteration between the sol and gel state of the cytoplasm. Calcium and magnesium also influence spreading of cells over glass surfaces. The phosphate ion is involved in energy transport via high energy phosphate bonds (Paul, 1960).

There is little definite information on the role of trace elements, although they probably operate in certain enzyme systems. Waymouth (1960) suggested that iron, aluminum, zinc, cobalt, molybdenum, and manganese were essential.

The pH of cell cultures is normally regulated by a carbon dioxide-bicarbonate buffering system modeled after that present in the blood of living animals. In cell cultures CO_2 is available from air, metabolic activity of cells, and the medium. Bicarbonate is normally added to medium as part of the balanced salt solution. The pH of the medium is then regulated by the amount of CO_2 present in the gas phase. In open cultures CO_2 pressure can be maintained at a constant level in special incubators in which a mixture of CO_2 in air (usually 95 per cent air and five per cent CO_2) is allowed to flow continuously. Cells also can be cultivated in airtight containers where metabolically-produced CO_2 is allowed to accumulate in order to maintain the optimal pH.

The Role of Carbon Dioxide in Nutrition of *In Vitro* Cultivated Cells

Early studies dealt with the effects of gassing cells with CO_2 and established the fact that high concentrations of CO_2 were toxic for cells (Bauer, 1925; Mottram, 1927; Mottram, 1928). More recently, it was shown (Brues and Naranjo, 1948; Brues and Buchanan, 1948) that CO_2 was more than an end product of cellular metabolism. Bicarbonate which was labeled with C_{14} and incorporated into the growth medium was taken up rapidly by chick embryo tissues and retained in an organic form. After CO_2 fixation by bacteria had been demonstrated (Werkman, 1951), it was suggested that CO_2 could be a requirement for all living cells. Harris (1954) undertook an investigation of the role of bicarbonate in the *in vitro* outgrowth of chick embryo heart fibroblasts. All biological materials used in cultivation of heart fragments were depleted of bicarbonate by dialysis. Metabolic CO_2 and that present in the gas phase of cultures were removed by adsorption. To remove the CO_2, cups filled with 0.15 N sodium hydroxide were incorporated in the systems in which tissues were cultivated. Increases in surface area were recorded with a projectoscope and measured with a planimeter. At a pH of 7.4 in a medium which contained 0.2 per cent bicarbonate without a CO_2 trap, there was a flourishing outgrowth of cells. At a similar pH without added bicarbonate and with a CO_2 trap cellular outgrowth was completely inhibited. Growth, however, could be reinitiated at any time up to six days upon removal of the CO_2 trap and addition of bicarbonate. Partial restoration of the outgrowth could demonstrated even in a bicarbonate-free medium if the pH were elevated to about 9.0. Harris concluded

that bicarbonate was essential for maintenance of an intracellular, physiologically compatible pH. Eagle (1956) demonstrated that HeLa or L cells did not require added bicarbonate. When, in a closed system, the organic buffer tris[1] was substituted for bicarbonate in the medium and the medium was changed daily to prevent damage due to acidification, cells grew as well as those in bicarbonate-containing media in closed cultures. These apparently different observations concerning the role of bicarbonate, exclusive of its buffering action, have been explained by more recent studies.

Swim and Parker (1958) studied the growth and maintenance of six strains of fibroblasts derived from human, mouse, and rabbit tissues. They used a chemically defined medium which was supplemented with dialyzed serum and dialyzed embryo extract and was buffered with phosphite, phosphite-bicarbonate, or bicarbonate. Regardless of the buffer employed growth of individual strains in stoppered flasks was the same. Growth of all strains decreased, however, when the medium was buffered with phosphite and flasks were left open to the air by means of loosely fitted metal caps. Here cells failed to proliferate at a significant rate and began to degenerate within seven to 10 days. The pH of the phosphite medium in closed flasks did not differ significantly from that in the open containers at any time during the experiment. Since growth in stoppered flasks which contained phosphite-buffered medium was the same as that obtained in the bicarbonate medium, it was concluded that cells could produce adequate CO_2 metabolically if it were

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[1]tris (hydroxymethyl) aminomethane

allowed to accumulate within the closed flasks.

Geyer and Chang (1958) chose conjunctival and HeLa cells for experiments to determine the effect of low concentrations of bicarbonate on in vitro growth of human cells. One of the two media used consisted of 10 per cent dialyzed horse serum in Eagle's basal medium which was modified by the addition of 20 μM mesinositol and 5 mM Tris while bicarbonate concentration was reduced to 5 mM. The second medium was like the first except bicarbonate was omitted. Potassium hydroxide traps for use with bicarbonate-free medium were made by fitting rubber stoppers of tissue culture tubes with a glass tube which contained a filter paper onto which 10 per cent potassium hydroxide solution could be introduced directly with a hypodermic needle and syringe. Cultures were incubated at 36 C for 24 hours in stationary racks and thereafter at 36 C on a roller drum. The medium was changed on the third day and subsequently on alternate days. In some experiments cells were incubated in the absence of CO_2 through the use of potassium hydroxide traps. After designated time intervals, CO_2 was readmitted by removal of the trap, and medium containing 5 mM bicarbonate was substituted for the bicarbonate-free medium. In the absence of bicarbonate no net increase in cell count was obtained with either type of cells. The fact that conjunctival cells were able to resume multiplication when bicarbonate was reintroduced indicated that irreversible degeneration did not occur during a period of 14 days. When the medium was not changed on cultures which contained potassium hydroxide traps the pH fell from 7.8 to 7.0 and cell counts diminished. When the medium was changed regularly cell counts remained stationary. Transient

exposure of cells to bicarbonate while the medium was being changed could account for this difference in cell growth.

The gas phase of the culture was found to be of prime importance when the watch glass technique of organ culture was used for cultivation of human neoplastic tissues (Rovin, 1962). Neoplasms were cultivated in three media: (a) a chemically defined medium CMRL-1066, (b) Eagle's medium plus 20 per cent human serum, and (c) a clot composed of chicken plasma and chick embryo extract. Cultures were incubated at 35 to 37 C in three different gas phase conditions: (a) incubator atmosphere of approximately 20 per cent oxygen and 0.03 to 0.04 per cent CO_2, (b) a candle jar containing approximately one per cent oxygen and five per cent CO_2 and (c) an atmosphere of 95 per cent oxygen and five per cent CO_2. When they were cultivated in Eagle's medium plus 20 per cent horse serum in a candle jar six neoplasms survived for seven days and two were viable at 17 and 19 days, respectively. In CMRL-1066 medium in a candle jar, none of the tumors survived more than three days. In no instance was a change to an alkaline pH range observed in any of the media. In additional studies, prolonged survival time of tissues was related to increased CO_2 content rather than low oxygen concentration in the gas phase.

In an effort to supply the factor which interrupted multiplication of CO_2-deficient cells, Geyer and Neimark (1958) added extracts of normal and CO_2-deficient cells to HeLa and conjunctival cell cultures grown in the presence of a potassium hydroxide trap. The sole buffer for CO_2-deficient cells was 1 mM phosphite. These cells were grown in Eagle's medium plus 10 per cent inactivated horse serum until the total

number of cells equaled 3 to 4 x 10^6. This medium was then removed and the cells were incubated overnight in Eagle's medium without serum, magnesium chloride, or calcium chloride. In the absence of magnesium and calcium ions cells disintegrated rapidly. Cellular debris was not removed, and the extract was reconstituted with dialyzed horse serum, calcium and magnesium chloride. This medium was agitated with the alkaline CO_2 trap for four to five hours to remove traces of CO_2 and was then transferred to roller tube cultures. Potassium hydroxide traps were always used with bicarbonate-deficient cells. Both HeLa and conjunctival cells failed to multiply in the absence of CO_2. Net multiplication of both strains was restored by addition of similarly prepared, normal cell extracts, although such an extract had no effect on the multiplication of normal cells. Extracts of CO_2-deficient cells had an adverse effect on deficient cells. Even when net multiplication was completely arrested in the absence of CO_2, conversion of C^{14} labeled acetate-1 to $C^{14}O_2$ was normal at the end of five days. This finding indicated that general metabolism was still high and corroborated the observations of Geyer and Chang (1958) that a state of irreversible degeneration of CO_2-deficient cells did not occur for some time.

In the case of CO_2-dependent bacteria, oxaloacetic acid, an intermediate in the Krebs cycle, is known to serve, in part, for CO_2. For this reason oxaloacetic acid was selected as a possible substitute for CO_2 in the metabolism of single mammalian cells (Gwatkin and Siminovitch, 1960). Stock cultures were grown in defined medium CMRL-1066 which was supplemented with 20 per cent dialyzed horse serum for L cells and with 20 per cent whole sheep serum for HeLa cells. Primary cultures

of rhesus monkey kidney cells were grown in Hanks' balanced salt solution with 0.5 per cent lactalbumin hydrolysate and 1.0 per cent bovine serum. For single cell plating experiments double strength CMRL-1066 was made without bicarbonate. To half of the medium sodium bicarbonate was added to a concentration of 2.2 grams per liter and to the other portion, sodium chloride was substituted for bicarbonate. Both media were supplemented with 20 per cent bicarbonate-depleted serum. A 0.25 M solution of oxaloacetic acid in bicarbonate-free CMRL-1066 was added to each petri dish culture which contained 100 cells in 5.0 ml of medium. Cultures with bicarbonate were incubated for two weeks at 37 C in a gas phase of five per cent CO_2. Cultures without bicarbonate were incubated in air for the same length of time. The proportion of cells which formed colonies, or the efficiency of plating (EOP), varied from 0 to 61 per cent in bicarbonate-free systems. With oxaloacetic acid in the medium, the EOP was consistently high in the case of both HeLa and L strains; the number of cells increased and they lacked the necrotic appearance of cells grown in bicarbonate- and oxaloacetate-free medium. Primary cultures of monkey kidney cells failed to develop in bicarbonate-free medium and oxaloacetic acid had no apparent beneficial effect.

Oxaloacetic acid has been found to be as good or better than CO_2 for multiplication of CO_2-deficient cells in the presence of ribonucleosides (Chang et al., 1961). In their studies, conjunctival and HeLa cells were cultivated in a closed system in Tris-buffered Eagle's basal medium, no bicarbonate was added, and the medium was exposed to an alkaline trap before each experiment to minimize adsorption of CO_2. Bicarbonate labeled with C^{14} was incorporated into the medium 24 to 48

hours after which the cells were washed with normal saline, and dissolved in saline which contained sodium lauryl sulphate. The resulting cell solutions were pooled and then separated into five fractions: acid soluble, lipids, ribonucleic acid, desoxyribonucleic acid, and proteins. Approximately 90 per cent of the C^{14} activity was found in the acid soluble and nucleic acid fractions. Chromatographic separation of the purines and pyrimidines of cells grown in C^{14} labeled sodium bicarbonate showed the C^{14} activity in spots formed by guanine, adenine, cytosine, uracil, and thymine. Total activity of purines was higher than that of pyrimidines. Ribonucleosides were partially effective as a substitute for CO_2, whereas oxaloacetic acid alone was ineffective. The combination of ribonucleosides and oxaloacetic acid was as good as or better than CO_2. Thus, under these experimental conditions, CO_2 was shown to be necessary for synthesis of purines, pyrimidines, and oxaloacetate by human cells grown in vitro.

Nutrition of Virus-Infected Cell Cultures

Under controlled conditions with chemically defined media it has been possible to study the effect of extracellular growth medium on virus propagation within infected cells. Hare and Morgan (1954) found that sustained, long term growth of psittacosis virus did not occur in cultures of minced chick embryo tissue maintained in a balanced salt solution only. Addition of either beef embryo extract or synthetic medium 199 greatly stimulated virus production. On the contrary, Burr et al. (1954) studied propagation of mumps and influenza viruses in chick embryo chorioallantoic tissue cultivated in synthetic media and

found that extracellular medium had little effect on virus production. Many other investigators have studied various aspects of the metabolism of virus-infected cells. Koppelman and Evans (1959) and Darnell and Eagle (1960) presented excellent reviews of various aspects of biosynthesis of viruses in cell culture, metabolic changes in cells due to chemical or physical agents, and implications of the effects of some of these agents on virus formation. Results of these and other investigations (Rappaport, 1956; Eagle and Habel, 1956; Levine et al., 1956) demonstrated that the effect of the extracellular medium on virus propagation may vary with each virus: in fact, it may be characteristic of each virus. These authors, however, considered carbohydrate, protein, amino acid, and nucleic acid metabolism with little reference to the influence of ionic species and buffers on virus replication.

The Role of Carbon Dioxide in Nutrition of Virus-Infected Cell Cultures

Because bicarbonate is an essential metabolite for the in vitro cultivation of mammalian cells and is specifically associated with nucleic acid synthesis, the development of infectious virus particles in CO_2-deficient cells should be studied. Since virus multiplication involves the synthesis of a certain type of nucleic acid, the CO_2-deficient cell in culture offers an excellent tool for use in the investigation of specific steps in the replication of a virus. During the last decade the first results of experiments elucidating this aspect of virus growth were published. Vogt et al. (1957) described mutants of poliomyelitis virus, designated d- (delayed) mutants, in which the d-

character was associated with a gradual decrease in susceptibility of host cells to the d- mutant under an acid agar overlay, and a narrow pH range for optimal growth. Rhesus monkey kidney cell cultures were grown, in petri dishes, in Earle's saline plus 0.5 per cent enzymatic lactalbumin hydrolysate and six per cent ox serum. Agar overlays consisted of 0.9 per cent agar, 1/40,000 neutral red, 0.1 per cent yeast extract, 0.1 per cent bovine albumin, and varying amounts of sodium bicarbonate to produce, in different plates, an alkaline, intermediate, and acid agar of pH 7.5, 6.9, and 6.8, respectively. Plates were maintained in a humid atmosphere with increased CO_2 in the gas phase. To determine the efficiency of plating, virus was assayed in duplicate on alkaline and acid plates and titers were expressed as plaque forming units per ml. The wild type (d+) was able to produce plaques under either acid or alkaline agar with equal facility. In contrast, d- mutants produced 100 to 1,000 times fewer and smaller plaques under acid agar overlays. Under alkaline agar overlays, differences from the wild type were evident in the size of plaques, but the number of plaques was similar. Single cycle and multiple cycle growth experiments in acid liquid medium failed to delineate any differences between d- and d+ strains. Under acid agar overlays, one cycle curves were similar for d- and d+ strains, but multiple cycle curves, after the initial 10 to 12 hours of the first growth cycle, showed a 12-fold decrease in overall growth rate for d- mutants compared to a three-fold decrease for d+ strains. Other experiments in the same series showed that reduction of the growth rate under acid agar was due to decreased cell susceptibility which was evident only after several

hours incubation. This change in cell susceptibility was thought to be due to the effect of differences in pH. All of the d- mutants which were studied appeared to be attenuated, and they showed decreased neuropathogenicity for monkeys.

Hsiung and Melnick (1958) investigated a number of strains of poliovirus in an attempt to correlate the occurrence of the d+ or d- character with neuropathogenicity for rhesus monkeys as a differentiation between virulent and attenuated strains. Since they intended to use tightly sealed bottle cultures of rhesus monkey kidney cell monolayers in their work, they conducted numerous preliminary experiments to investigate the conditions under which d- mutants replicated in this system. After adsorption of virus strains onto cells, agar overlays which contained varying amounts of bicarbonate were added. Virus plaques were counted on the third and fourth days, and it was noted that the size of plaques produced by virulent strains grown under agar overlays with low concentrations of bicarbonate (as low as 0.11 grams per cent) were smaller than those in cultures overlaid with higher bicarbonate concentrations (up to 0.9 grams per cent). No plaques of attenuated strains developed in the presence of low bicarbonate concentrations. Both virulent and attenuated strains produced large plaques in 48 hours in high bicarbonate-containing overlays. Variation in the bicarbonate concentration in liquid medium did not affect virus adsorption to the cells or growth curves of attenuated or virulent strains. In other experiments, Hsiung and Melnick employed a low level of bicarbonate (0.11 grams per cent) in agar overlays and varied the pH by addition of sodium hydroxide or by bubbling CO_2 through the medium. When

the pH was raised no plaques were formed by any of the attenuated strains. Although virulent strains produced the expected number of plaques in acid media, they were smaller than normal. It was concluded that the d- character was related to bicarbonate concentration rather than to pH of the medium.

Chang (1959) investigated virus replication of Coxsackie virus (group B, type 1), poliovirus (type 1), and a strain of vaccinia virus in CO_2-deficient HeLa and conjunctival cells. When cells were grown in medium depleted of bicarbonate by an alkaline CO_2 trap Coxsackie virus failed to produce cytopathic effects in five days, whereas typical viral-induced cell changes were seen in two days in media which contained bicarbonate. Ribonucleic acid and desoxyribonucleic acid were added to bicarbonate-depleted cultures infected with Coxsackie virus to determine their effect on the yield of virus. When ribonucleic acid was added to the medium the appearance of the cytopathic effects and the amount of virus yield were comparable to those obtained in experiments in which the CO_2 trap had been removed. Addition of desoxyribonucleic acid caused cells to appear granular and vacuolated, and had no beneficial effect on either virus yield or cytopathic changes. Additions of purine and pyrimidine bases were also ineffective with or without the addition of ribose. Inclusion of nucleotides and nucleosides was practically as effective as addition of ribonucleic acid or bicarbonate. No correlation could be found between the resumption of cell multiplication and enhancement of virus synthesis following reintroduction of ribonucleic acid into cultures of bicarbonate-depleted cells. Addition of ribonucleic acid, ribonucleosides, or ribonucleotides reversed the suppressive

effect caused by bicarbonate depletion of the poliovirus cultures. In contrast to Coxsackie virus, poliovirus replication was not completely suppressed in bicarbonate-depleted medium. Results with vaccinia virus were much the same as with Coxsackie virus. Nucleosides enhanced virus multiplication and appearance of cytopathic effect without causing a net increase in cell numbers. Coxsackie and poliomyelitis viruses are composed of ribonucleic acid, while vaccinia virus contains desoxyribonucleic acid. Since it is ribonucleic acid, not desoxyribonucleic acid which expedites the appearance of cytopathic changes in bicarbonate-depleted cells, Chang postulated that suppression of virus synthesis is not due to a lack of nucleic acid precursors. Chang (1959) postulated a more indirect cause:

> The virus induces the formation in the infected cells of new proteins, presumably enzymes, which are necessary for the formation of virus precursors; any interference with cellular ribonucleic acid metabolism would result in the suppression of new protein synthesis which could in turn interfere with viral synthesis.

Since poliovirus multiplication was not completely suppressed, it is likely that the alkaline traps were not capable of removing all metabolically-produced CO_2 and that the synthesis of poliovirus proceeded at lower levels of CO_2 than that of Coxsackie and vaccinia viruses.

Mosley and Enders (1962) confirmed observations of several earlier investigators and presented a simple technique for depletion of CO_2 from cell cultures. Monkey kidney cells were grown in a liquid medium from which bicarbonate was omitted. The CO_2 produced by cellular metabolism was allowed to escape by replacing screw caps of culture tubes with cotton plugs. In closed cultures CO_2 accumulated even

though none was added to the medium. With increasing concentrations of CO_2 in the gas phase of cultures the CO_2 concentration in the medium rose to levels adequate for virus growth. Cotton plugs permitted the CO_2 in the medium to equilibrate with the gas phase. Mosley and Enders used 10 mM Tris and 0.133 grams per cent sodium dihydrogen phosphate to enhance the buffering capacity of medium 199 to which two per cent serum had been added. The medium in cotton plugged cultures (CPC) contained no added bicarbonate, whereas the medium in screw capped cultures (SCC) included 0.22 grams per cent sodium bicarbonate. The reaction of CPC and SCC was adjusted to pH 7.2 and 7.0, respectively, with hydrochloric acid. In growth studies of several strains of poliovirus, cells were placed in the appropriate medium 24 hours prior to infection and incubated at 37 C on a roller drum in a humidified incubator. After 24 hours incubation the pH in SCC and CPC had fallen to 6.8 and 6.7, respectively. Medium was changed daily to avoid cell damage due to low pH. After infection representative tubes were frozen and thawed three times, pooled, and stored at -20 C until assayed for infectivity. When bicarbonate was omitted from the medium and screw caps were replaced with cotton plugs there was no rise in pH, the cells survived and increased slightly in number, some glucose was utilized, and lactate was produced. Multiplication of virus in CPC was consistently retarded as compared with SCC, but the degree of inhibition of poliovirus growth in CPC varied with the lot of monkey kidney cells and the strain of virus used. When experimental conditions were as previously described except that CPC were incubated in an atmosphere which consisted of five per cent CO_2 in air, production of virus and the cytopathic changes were the same for

CPC as for SCC. In order to determine the effect on virus replication screw caps were replaced by cotton plugs at different times in relation to infection of cells. When cotton plugs were substituted for screw caps 24 hours prior to infection, cell cultures failed to support virus growth. Substitution of cotton plugs at the time of infection had a marked suppressive effect, but virus was replicated. When cotton plugs were used 24 hours after infection there was a slight, but definite, suppression of virus multiplication. Replacing cotton plugs of cultures with screw caps three days after infection resulted in the resumption of virus multiplication at a rate equal to that of cultures which had not been cotton plugged. No virus could be detected in cotton plugged cultures on the fourth day after infection, and the substitution of screw caps for cotton plugs on the fifth day failed to stimulate virus multiplication. Experiments with media of various pH values in CPC and SCC illustrated that the effect of cotton plugging was greater than that of pH alteration. Thus, decreased poliovirus synthesis in CPC appeared to be related to bicarbonate concentration rather than to pH. It was found that conditions produced by cotton plugs did not alter adsorption of virus or its release, but separate assay of intracellular and extracellular virus demonstrated that there was delayed intracellular synthesis of virus in CPC. Intracellular virus could be detected in SCC five hours after infection, but did not appear in CPC until 48 hours after infection. Mosley and Enders (1962) confirmed the observation that oxaloacetate in combination with ribotides enhanced the growth of CO_2-deficient cells (Chang et al., 1961) and found that under these growth conditions replication of poliovirus was stimulated. Contrary

to the observation of Chang (1959), Mosley and Enders found that ribonucleic acid bases, as well as ribosides and ribotides, promoted multiplication of virus strains which were sensitive to bicarbonate depletion. They postulated that these differences could be due to the cell strains employed. The same strains exhibited delayed growth in monkey kidney cell monolayers under acid agar overlays, as well as in cotton plugged, bicarbonate-depleted liquid media. In addition, ribotides and oxaloacetate reversed the delayed effect under acid agar overlays. The authors concluded that the difference in effect of bicarbonate depletion on virulent and attenuated strains of poliovirus-infected cells may be due to differences in the ability of d- and d+ strains to utilize host cell materials in synthesis of ribonucleic acid when bicarbonate is not available, or to differences in cellular production of poliovirus nucleic acids at CO_2 levels below that needed for multiplication of the cells.

By variation of cellular metabolism, Quersin-Thiry (1961) isolated a small, plaque-forming mutant of Western equine encephalitis virus from a culture of the large plaque forming, wild type parent. Further investigations revealed that these strains were associated with different requirements for bicarbonate in the agar-overlay medium. The wild type virus grew best when no CO_2 was added to the medium, although some CO_2 was supplied in the gas phase, since plates were incubated in an atmosphere of five per cent CO_2. High concentrations of bicarbonate were less inhibitory for the small plaque mutant, and its optimal bicarbonate level was 0.4 per cent.

Bicarbonate levels have also been shown to influence replication

of SE polyoma virus in primary mouse embryo cell cultures (Eddy and Stewart, 1958). The usual maintenance medium contained 0.125 per cent bicarbonate. A decrease from this amount directly affected the detectable degenerative changes in virus-infected cells and hemagglutination titers. In virus-infected cultures maintained in a medium which contained 40 per cent more sodium bicarbonate than the usual amount, extreme cytopathic changes and increased hemagglutination titers were observed.

Substitution of an Organic Buffer for Bicarbonate in Cell Culture Media

Many substances have been used to substitute for bicarbonate in the buffer system of tissue culture media. An organic buffer, tris (hydroxymethyl) aminomethane, for use in pH range of 6.5 to 9.6, was first introduced by Gomori (1946). No inhibitory effects due to Tris were evident in the enzyme systems which he studied. Swim and Parker (1955) were the first to use Tris in tissue culture media. They wished to avoid maintenance of cultures in closed systems or in a gas phase enriched with CO_2. Swim and Parker found that HeLa and other cell cultures initiated with a small inoculum did not replicate as well in Tris as in bicarbonate. Cells appeared more granular and had a greater tendency to clump on the glass. The authors stated that Tris had a slight inhibitory effect. The depression of cellular multiplication was later reported to be a result of bicarbonate depletion rather than an effect of Tris (Swim, 1961). Growth of HeLa and L strains was inhibited when Tris was used in the medium at a concentration greater than 0.03 M. Cells proliferated well for many months when maintained in either 0.02 M

Tris- or sodium phosphite-buffered media in closed cultures. The growth rate of Tris-buffered cultures which were cotton plugged or fitted with loose metal caps was significantly less than that in tightly sealed flasks. In the former cultures the cells degenerated after six to eight days and eventually died. Swim (1961) reported that the reduced growth rate was due to bicarbonate depletion rather than to the inhibitory effect of Tris, since, in closed cultures, cells multiplied at a rate less than, but the total number was comparable to that obtained in a similar medium which contained bicarbonate.

Many studies on the activity of viruses in cell cultures which employed Tris have been reported. Porterfield and Allison (1960) observed that Tris, in addition to its buffering capacity, exerted an influence on vaccinia, herpes simplex, ectromelia, and cowpox viruses in agar overlay cultures. Plaques of vaccinia on monolayers of chick embryo cells with bicarbonate agar overlays were small even after five days incubation, and plaque counts were lower than pock counts on chick embryo chorioallantoic membranes. With the substitution of Tris buffer for bicarbonate, easily countable plaques of vaccinia, cowpox, and herpes simplex viruses were obtained after three to four days incubation, and of ectromelia virus after five to six days incubation. Tris-buffered agar overlays gave excellent plaque formation with several strains of herpes simplex virus. Adsorption of herpes simplex virus was maximal at 31 C at a pH of 6.5. Allison and Valentine (1960) established the fact that adsorption of vaccinia virus was promoted by certain amines. Porterfield and Allison (1960) postulated that Tris may influence virus multiplication by making the adsorption process more

rapid and efficient.

The initial aim of the present study was to determine the optimal conditions for replication of herpes simplex virus in HeLa cell cultures. In agreement with Stoker (1959), this virus-cell system was considered to be well suited for study of virus multiplication at the cellular level. During the development of this problem, and following the report of Porterfield and Allison (1960), Tris buffer was included as a substitute for the bicarbonate buffer system since these authors reported an enhanced ability of herpes simplex virus to form plaques under agar which contained Tris. Since CO_2 had been shown to affect the replication of several viruses and is closely associated with buffers and buffering activity, the growth of herpes simplex virus in CO_2-deficient cells was studied according to the methods of Chang (1959) and Mosley and Enders (1962). Preliminary studies indicated that this virus behaved differently from those studied by Chang (1959). Therefore, vaccinia virus, which Chang found to be markedly affected by CO_2 depletion and which is similar to herpes simplex virus in nucleic acid composition, was included to serve as a control for the techniques of creating CO_2-deficient cells. The object was to determine if herpes simplex and vaccinia viruses react differently to alterations in the buffers of cell culture media or in gas phase conditions influenced by various CO_2-depletion techniques.

CHAPTER II

MATERIALS AND METHODS

Viruses

Two strains of herpes simplex virus and two of vaccinia were used in these studies. The HF strain of herpes simplex virus and the Downie (D) strain of vaccinia virus represent laboratory-adapted strains. For comparison, two recently isolated viruses were utilized in several experiments. The histories of these viruses are presented in Table 1. Serological examination of these viruses has confirmed their identification. In the presence of hyperimmune herpes simplex rabbit antiserum, both herpes simplex virus strains were shown to fix complement; neutralization of virus-antiserum mixtures was demonstrated by intracerebral inoculation of mice and in replicate tube cultures of HeLa cells. The vaccinia virus strains were also neutralized by hyperimmune vaccinia rabbit antiserum when virus-antiserum mixtures were inoculated onto HeLa cells. Further confirmation of the identity of these viruses was supplied by Dr. P. A. Brunell[1].

Cells

Preliminary experiments were conducted with several types of

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[1]Virus Reference Unit, Communicable Disease Center, Atlanta, Georgia.

TABLE 1

HISTORY OF VIRUSES

Virus	Initial Isolation	Source	HeLa Cell Passages[a]
Herpes simplex HF strain	Flexner and Amoss, 1925	C.D.C.[b]	18 to 20
Herpes simplex M strain	O.U.M.C.[c]	Oral mucocutaneous lesion	3 to 5
Vaccinia D strain	Downie, 1939	Fort Dedrick[d]	3 to 5
Vaccinia O strain	O.U.M.C.[c]	Smallpox vaccine	3 to 5

[a]Passage history in this laboratory.

[b]Communicable Disease Center, Atlanta, Georgia.

[c]University of Oklahoma Medical Center, Oklahoma City, Oklahoma.

[d]Supplied through the courtesy of Mr. N. Hahan, Fort Dedrick, Maryland.

cell cultures in an effort to determine which was best suited for the purposes of this research. The sources of the primary and serially propagated cell cultures are given in Table 2. Primary cell cultures were prepared in this laboratory from the tissues described. Serially propagated cell strains were maintained in this laboratory during these studies. Periodically, all of the serially propagated cell strains were tested for the presence of L-forms and pleuropneumonia-like organisms, but all attempts to isolate these agents on PPLO agar[1] were unsuccessful. To insure complete freedom from such contamination all routinely propagated cell strains were cultivated once every two months in media which contained 2.5 micrograms of chlortetracycline[2] per ml (Carski and Shepard, 1961).

Reagents

All solutions were prepared in distilled water which was passed through a deionizing column and redistilled through a glass distilling apparatus. Chemicals were from several sources, and all were of reagent grade.

The balanced salt solutions of Earle *et al*. (1943) and Hanks and Wallace (1949) are commonly used as bases for preparation of tissue culture media. Earle's balanced salt solution (EBSS), which contains glucose and several inorganic salts, is buffered exclusively by a bicarbonate-carbon dioxide buffer system (0.22 g sodium bicarbonate per 100 ml). A physiologically compatible pH value of 7.2 to 7.6 is

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[1]Difco Laboratories, Detroit, Michigan.

[2]Lederle Laboratories, Pearl River, New York.

TABLE 2

SOURCE OF CELL CULTURES

Type of Cell	Source	Local Passage History
Primary		
Chick embryo fibroblasts from embryos incubated 9 to 12 days	local hatchery	primary
Young adult hamster kidney cells	local dealer	primary
Serially propagated		
FL normal human amnion cells[a]	M.A.[b]	5 to 10
HeLa-MA Epidermoid cells cancer of cervex[c]	M.A.[b]	5 to 10
HeLa-10 Epidermoid cells cancer of cervex[c]	Carver Foundation[d]	More than 150 over 3 years

[a]Fogh, J. and Lund, R. O., 1957.

[b]Microbiological Associates, Bethesda, Maryland

[c]Gey et al., 1952.

[d]Brown, R. W., Carver Foundation, Tuskegee, Alabama.

normally maintained in an air atmosphere which contains five per cent CO_2. Hanks' balanced salt solution (HBSS) also contains glucose and inorganic salts, but the buffering capacity is maintained by a chemically complex, phosphate buffering system. Since the bicarbonate concentration is low in HBSS (0.035 g sodium bicarbonate per 100 ml), no drastic pH changes are produced in ordinary atmospheric conditions, where CO_2 pressures are normally low. All cell culture media were made in either HBSS or EBSS. Methods of preparation of balanced salt solutions from stock solutions are presented in Tables 3 and 4. Modifications of the original formulae for these studies can be noted by a comparison of column 5 with column 6 in these tables. Table 4 shows that EBSS, which contains only seven ingredients excluding the phenol red pH indicator, was prepared from six stock solutions. This procedure was necessary since many of the experiments utilized media which contained different amounts of bicarbonate or no bicarbonate. The tonicities of solutions were kept constant by alterations of the sodium chloride concentration to compensate for differences in sodium bicarbonate content.

Phosphate buffered saline (DPBS) was prepared according to the method of Dulbecco and Vogt (1954), phenol red (10 mg per liter) was added as a pH indicator, and the solution was sterilized by autoclaving.

Virus diluent consisted of sterile DPBS supplemented with five per cent sterile calf serum which had been inactivated by heating at 56 C for 30 minutes. Dialyzed calf serum of the same concentration was added to this diluent for use with CO_2-deficient media.

TABLE 3

PREPARATION OF HANKS' BALANCED SALT SOLUTION (MODIFIED)

Chemical	Stock Solution Number	Stock Solution Amount[a]	Dilution for use	Final Concent.[a]	Original Formula[a]
NaCl	1	16.00	1:20	0.800	0.800
KCl	1	0.80	1:20	0.040	0.040
$MgSO_4 \cdot 7H_2O$	1	0.20	1:20	0.010	0.020
KH_2PO_4	1	0.12	1:20	0.060	0.060
Na_2HPO_4	1	0.12	1:20	0.060	0.060
Glucose	1	2.00	1:20	0.100	0.100
$CaCl_2$	2	1.40	1:100	0.014	0.014
$MgCl_2 \cdot 6H_2O$	2	1.00	1:100	0.010	-
$NaHCO_3$[b]	3	8.40	1:200	0.042	0.035
Phenol red[b]	-	0.40	1:400	1.0[c]	2.0[c]

[a]Grams per 100 ml.

[b]Stock solution also used for the preparation of EBSS.

[c]Expressed as mg per 100 ml.

TABLE 4

PREPARATION OF EARLE'S BALANCED SALT SOLUTION (MODIFIED)

Chemical	Stock Solution Number	Stock Solution Amount[a]	Dilution for use	Final Concent.[a]	Original Formula[a]
NaCl	1	25.0	variable	variable	0.680
KCl	2	4.0	1:100	0.040	0.040
$CaCl_2$	2	2.0	1:100	0.020	0.020
$MgCl_2 \cdot 6H_2O$	3	2.0	1:100	0.020	-
$NaH_2PO_4 \cdot H_2O$	4	1.5	1:100	0.015	0.0125
Glucose	5	10.0	1:100	0.100	0.100
$NaHCO_3$[b]	6	8.4	variable	variable	0.220
Phenol red	-	0.4	1:400	1.0[c]	2.0[c]
$MgSO_4$	-	-	-	-	0.010

[a]Grams per 100 ml.

[b]Stock solutions also used for the preparation of HBSS.

[c]Expressed as mg per 100 ml.

Stock solutions of trypsin[1] (2.5 per cent) were prepared in DPBS which contained no calcium or magnesium salts. They were sterilized by Seitz filtration and stored in 10 ml portions at -25 C until needed. For use in dispersion of cells, stock solutions of trypsin were thawed and diluted so that one part of trypsin was contained in nine parts of calcium- and magnesium-free DPBS which had been further modified by the addition of 0.25 per cent gelatin[1]. The final concentration of trypsin was 0.25 per cent.

Penicillin[2] and streptomycin[3] stock solutions were prepared in sterile distilled water and stored at -25 C in 0.5 or 1.0 ml amounts. They were thawed and added to media and reagents as required (penicillin 100 units per ml; streptomycin 50 mcg per ml).

A solution of 0.1 per cent crystal violet in 0.1 M citric acid was used to enumerate cells by nuclei counts (Sanford et al., 1951). Viable cell counts were performed in a one per cent, aqueous solution of either nigrosin[4] or trypan blue[5], and 0.1 ml of the dye solution was added to each ml of cell suspension.

A 1 M stock solution of tris (hydroxymethyl) aminomethane[6] buffer was made in distilled water and sterilized in an autoclave at 15 pounds

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[1]Difco certified, Difco Laboratories, Detroit, Michigan.

[2]Potassium penicillin-G, Parke, Davis & Co., Detroit, Michigan.

[3]Streptomycin Sulfate, E. R. Squibb & Sons, New York, New York.

[4]Coleman and Bell Co., Norwood, Ohio.

[5]Allied Chemical Corporation, New York, New York.

[6]Sigma 121, Sigma Chemical Company, St. Louis, Missouri.

pressure for 10 minutes. Dilutions of Tris were made in the reagents or media and the pH of these solutions were adjusted to the desired level with 0.3 N NaOH or 0.3 N HCl (Hanks, 1955).

Phenol red was prepared according to the method of Melnick (1956) and added to media and reagents to make a final concentration of 10 mg per liter.

A sterile 1 M stock solution of sodium bicarbonate[1] was prepared in distilled water.

Tissue Culture Media

Media were sterilized by Seitz filtration; penicillin and streptomycin were added to all media. Several media were adopted for different purposes. An inexpensive medium (HLY) was used to cultivate cells for virus assay. It was prepared in HBSS and contained 0.5 per cent enzymatic lactalbumin hydrolysate[2], 0.1 per cent yeastolate[3], and five per cent heat inactivated calf serum. A similar medium (ELY) was prepared in EBSS and contained 0.2 per cent sodium bicarbonate and 0.2 per cent dextrose in addition to the serum, lactalbumin hydrolysate, and yeastolate. It was used as a maintenance medium for assay of cells.

The chemically defined, minimum essential medium[4] (MEM) of Eagle (1959) was supplemented with 2 mM sterile glutamine[5], and five

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[1]Matheson, Coleman and Bell, Cincinnati, Ohio; East Rutherford, New Jersey.

[2]Nutritional Biochemicals Corporation, Cleveland, Ohio.

[3]Difco Laboratories, Detroit, Michigan.

[4]Microbiological Associates, Bethesda, Maryland.

[5]Chromatographically pure, Mann Research Laboratories, Inc., New York 6, New York.

per cent sterile, heat inactivated calf serum. The glutamine was prepared as a 100 x concentrated stock solution in water, sterilized by Seitz filtration, and stored at -25 C in 10 ml quantities until needed. This medium was used to cultivate experimental cells. When ionic components and buffers were to be altered, a modified minimum essential medium (MEM-M) was prepared in EBSS from stock solutions of essential amino acids[1] and vitamins[1] in the same concentrations as in MEM plus heat inactivated calf serum to make a complete medium. When bicarbonate was to be omitted, the serum component was supplied as dialyzed, heat inactivated, calf serum.

Calf Serum

The blood, which was collected from several young calves at a local abattoir, was allowed to clot and the serum removed to obtain two large pools. The pooled sera were centrifuged at 2500 rpm for 20 minutes in an International PR-2 refrigerated centrifuge. The clear, supernatant fluid was removed, heat inactivated at 56 C for 30 minutes, distributed in 25 ml amounts in vaccine bottles, and stored at -25 C. When needed the serum was thawed and sterilized by Seitz filtration. Dialyzed serum was prepared by dialysis against 0.85 per cent saline at 4 to 6 C for 24 hours. Dialysis was continued for an additional 48 hours against EBSS which lacked sodium bicarbonate and glucose. The dialysate was renewed every 18 hours.

Human Herpes Simplex Virus Antiserum

Antiserum for herpes simplex virus was obtained by pooling normal

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[1]Microbiological Associates, Bethesda, Maryland.

human sera. Neutralization tests in replicate HeLa cell cultures showed that this serum, diluted one to 10, would neutralize 10^3 to 10^4 $TCID_{50}$ of the HF or M strains of herpes simplex virus. In control cultures it was not toxic for normal HeLa cells. To compare virus assay methods, $TCID_{50}$, and plaque forming units, this serum was incorporated into all media used to cultivate herpes simplex-infected HeLa cells.

Preparation of Primary Cell Cultures

Aseptic techniques were employed to prepare two types of tissues used in primary cultures. Chick embryos of nine to 12 days incubation were used as the source of chick fibroblasts. After removal of the head, appendages, and viscera, the carcasses were minced into small fragments and washed in large volumes of sterile HBSS. Kidneys were removed from exsanguinated hamsters, and after stripping the capsules, cortical tissues were pooled and washed with sterile HBSS. Hamster kidney and chick embryo monolayer cultures were prepared in the following manner: tissues from each source were placed in 0.25 per cent trypsin solution and incubated at 37 C on a continuous stirring apparatus (Magmix) for 15 to 30 minutes. The supernatant fluid was discarded, fresh trypsin solution was added to the fragments, and dispersion was continued on a Magmix apparatus at 4 to 6 C for 18 hours. Suspensions were filtered through several thicknesses of cheesecloth and cells were sedimented at 600 rpm for 15 minutes in an International PR-2 refrigerated centrifuge at 4 C. After the supernatant fluid was discarded, cells were washed in several volumes of HLY and resuspended in this medium. Samples were removed, diluted, and cell count determined after which

appropriate dilutions were made and cells were seeded into replicate, rubber stoppered tissue culture tubes which measured 16 by 150 mm. Approximately 3 x 10^6 cells per ml and 5 x 10^5 cells per ml were used to initiate chick embryo and hamster kidney cultures, respectively. All cultures were incubated at 37 C as stationary cultures in racks[1] at an angle of about 10 degrees from the horizontal. Chick embryo cell cultures became monolayers within 48 hours after seeding and were used for virus studies at which time the virus was introduced. Hamster kidney cell cultures required eight to 12 days to develop monolayers. Growth medium was changed on these cultures at four to five day intervals.

Cultivation of Serially Propagated Cell Strains

Stock cultures of serially propagated cell strains were cultivated in HLY, MEM or both. Growth was initiated in 250 ml Kimax bottles incubated at 37 C in a horizontal position. When HLY was used, it was necessary to change the medium only every five to seven days; MEM cultures required a medium change every two to four days. Monolayers were usually obtained seven to 10 days after seeding. Media were decanted and cells were harvested by addition of eight to 10 ml of 0.25 per cent trypsin solution. A cell suspension was obtained within 10 to 20 minutes at room temperature. Cells were mixed with a pipette and sedimented with low speed centrifugation for one to two minutes. Cells were washed one time with the appropriate growth medium and a sample was taken for cell count. Dilutions were made so an inoculum of 1.5 x 10^6 viable cells was added to each bottle containing 12 to 15 ml of growth

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[1]Seelye Craftmen, Minneapolis, Minnesota.

medium. When MEM was employed, it was necessary to flush the bottles briefly with a mixture of five per cent CO_2 in air to create a suitable pH for the initial seeding of cultures. Gassing was not necessary once cultures were established.

Cell Counts

For preliminary cell counts a nuclei counting technique (Sanford et al., 1951) was used. Cells were diluted in 0.1 per cent crystal violet and counted in a hemocytometer (five squares on each chamber). Duplicate or triplicate counts of each sample were made and the number of cells per ml of suspension was calculated by multiplying the average of the total number of cells counted per 10 cmm by the dilution factor and then by 10^3. Viable cell counts were made in a similar manner. Cell suspensions were diluted in HLY and 0.1 ml of a nigrosin or trypan blue solution was added per ml of each cell sample. The number of viable cells was determined per ml in a hemocytometer.

In growth experiments the number of cells in replicate tube cultures was determined. Individual cell counts were made from two or three cultures at each time interval. For this purpose the growth medium was removed and 0.5 to 1.0 ml of trypsin solution was added. An incubation period of 15 minutes at room temperature with frequent agitation was required to remove the cells from the glass. Appropriate quantities of HLY were added directly to each culture to be counted. To minimize clumping, 0.1 per cent sodium citrate was incorporated into the HLY. Trypan blue, 0.1 ml per ml of cell suspension, was added. After mixing with a capillary pipette cells were counted in a hemocytometer. Each count represented the average of two or three cultures in

which the enumeration was done in duplicate or triplicate.

Preparation of Virus Suspensions

All virus suspensions were prepared from young monolayer cultures of HeLa-10 cells. Herpes simplex virus, HF strain (pool #3) was used for the preliminary studies. After monolayer-cultures in bottles were inoculated with virus, a maintenance medium, ELY, was added and the cultures were incubated at 36 C for three to four days or until maximum cytopathic effects (CPE) were seen. The supernatant fluid was removed and centrifuged lightly to sediment the cells. The virus-containing supernatant fluid was distributed in 1.0 ml quantities in small test tubes which were sealed with a gas-oxygen burner, frozen quickly in an alcohol bath at -65 C, and stored at this temperature until needed. Repeated titrations of this suspension in HeLa cell cultures gave values of approximately $10^{5.2}$ $TCID_{50}$ per 0.1 ml. In later studies separate bicarbonate-free pools of herpes simplex and vaccinia viruses of high infectious titers were used. To obtain these seed virus suspensions, large amounts of infectious virus were added to cell monolayers in small volumes so that each cell had maximum opportunity for infection. After adsorption at room temperature for three hours, the unadsorbed virus was discarded and the infected monolayers were overlaid with 12 ml of ELY maintenance medium, and were incubated at 36 C for 24 hours. At this time CPE was apparent, but little virus was evident in the supernatant fluid, which was withdrawn and replaced with fresh maintenance medium. After an additional 18 hours incubation at 36 C the cultures began to degenerate. It appeared that all cells were morphologically infected

but still intact. The supernatant fluid was again discarded and replaced with eight ml of DPBS supplemented with five per cent dialyzed, heat inactivated calf serum. Following an additional incubation period of six to eight hours at 36 C all cultures were frozen at -65 C and thawed at room temperature. This cycle was repeated twice to disrupt cells and liberate intracellular virus. After low speed, refrigerated centrifugation, the supernatant fluid was found to contain high concentrations of infectious virus. The virus suspensions were frozen quickly and stored at -65 C in 0.5 ml volumes in small, sealed test tubes. The average titers of these suspensions are presented in Table 5. Since DPBS contained no added bicarbonate, virus pools of low bicarbonate content had been prepared.

Virus Titrations

Optimal conditions for titration of herpes simplex virus (HF strain) were investigated and the methods selected were those used throughout this experimental work. For assay purposes, HeLa-10 cells were cultivated exclusively in HLY. Young monolayer cell cultures were harvested in a routine manner with 0.25 per cent trypsin solution. Cells were collected by low speed centrifugation, washed in a small amount of HLY, and resuspended in this medium. Cell suspensions were maintained on a Magmix stirrer to insure homogeneous distribution of cells while replicate tube cultures were prepared. The number of viable cells was determined from a sample and the original suspensions diluted so that 3 to 4 x 10^5 viable cells were present per ml of medium, which was distributed in 0.5 ml amounts (1.5 to 2 x 10^5 cells per tube) into rubber stoppered tissue culture tubes. All tubes were incubated in

TABLE 5

TITERS OF VIRUS POOLS

Virus	Pool #	$TCID_{50}$ per 0.1 ml
Herpes simplex, HF strain	3[a]	$10^{5.2}$
Herpes simplex, HF strain	4	$10^{6.4}$
Herpes simplex, M strain	1	$10^{6.5}$
Vaccinia, D strain	1	$10^{6.4}$
Vaccinia, O strain	1	$10^{6.6}$

[a]Other pools (#1 and #2) had lower infectious titers and were not used in this study.

stationary racks described previously. The number of cells per tube was about 3.5 to 5.5 x 10^5 after three to four days incubation at which time they were used for virus assay.

Samples to be assayed for virus content were thawed quickly in a 37 C water bath and serial 10-fold dilutions were made in DPBS which was supplemented with five per cent heat inactivated calf serum. All tubes and reagents were maintained in an ice-water bath; separate pipettes were used to prepare each dilution. Replicate tube cultures were divided into groups of four to six, growth medium was aspirated, and 0.1 ml amounts of each dilution for virus assay were added to each of the appropriate cultures. A two-hour adsorption period at 30 to 32 C was allowed, during which time tubes were agitated frequently to prevent drying of those cells not covered by the small volume of fluid. After the adsorption period, 0.5 ml of ELY was added to each tube. Stationary cultures were incubated at 36 C and examined microscopically at three, five, and six days after infection. When characteristic cytopathic changes were noted in cells the culture was recorded as infected and discarded. All tubes were discarded as negative if specific cytopathic changes were not seen within five to six days. The $TCID_{50}$ was calculated according to the formula of Reed and Muench (1938). All $TCID_{50}$ data were expressed as the positive $\log_{10}$ of the dilution which produced infection in 50 per cent of the inoculated cultures.

In some preliminary studies the $TCID_{50}$ was compared with results of the plaque forming unit (PFU) method of Farnham (1958). Preparation of virus dilutions, inoculation of cultures, and virus adsorption were

identical for both methods. When the PFU was to be calculated, ELY was modified by replacing calf serum with five per cent human serum which contained nuetralizing antibodies for herpes simplex virus. In such media, plaques which developed in three to five days were due to initially adsorbed virus. The number of plaques which developed in infected tubes were counted four and five days after infection. The average number of plaques was calculated from the two highest 10-fold dilutions which showed countable plaques. This average was multiplied by the dilution factor and was expressed as the lowest whole number of plaque forming units per 0.1 ml of inoculum.

Cell Monolayers for Experimental Studies

Cells used for experimental studies were cultivated in chemically defined MEM. Since cells grew rapidly, it was necessary to change the medium every three to four days to prevent their degeneration. Cells were harvested with trypsin, sedimented, and washed with MEM-M which contained 10 mM sodium bicarbonate. With this medium, flushing with a CO_2-air mixture was not necessary. The number of viable cells was determined in trypan blue. Then the suspension was diluted so that the inoculum for each culture tube was 1.0 ml of MEM-M containing approximately 4×10^5 viable cells. All tubes were incubated in stationary racks at 36 C for 24 hours. At this time, when cells had attached to the glass and multiplication had begun, cultures were placed on roller drums[1] which made 12 revolutions per hour. The constant mixing and bathing of cells assured equilibrium of gases between the liquid and

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[1]Wyble Engineering Development Corp., Silver Spring, Maryland.

gas phases of the culture and minimized the chance of local accumulation of metabolic end-products. After 24 hours on the roller drum, the medium was aspirated from all cultures and the appropriate experimental medium was added to respective groups of cultures. For the remainder of the study all cultures were incubated at 36 C on the roller drum. The medium was changed daily to maintain optimum pH, to assure adequate nutrition, and to avoid accumulation of deleterious metabolic products. Most experiments were designed so that the experimental medium was in contact with cells for 36 to 48 hours before addition of virus.

When inoculated onto cells, the frozen virus suspensions were thawed, and diluted with DPBS containing five per cent dialyzed calf serum to a final dilution of 10^{-4}. The DPBS contained no added bicarbonate, and the serum had been dialyzed to remove as much CO_2 as possible. After aspiration of the experimental medium each tube was infected with 0.2 ml of a suspension which contained approximately 2.5 $TCID_{50}$ (log_{10}) per 0.1 ml. Following the adsorption period unadsorbed virus was aspirated, experimental medium was reintroduced, and incubation of the cultures was continued. Appropriate controls were included and will be described later. When samples were taken for virus assay the contents of two or three cultures were frozen quickly at -65 C, thawed rapidly in a 37 C water bath, pooled, refrozen, and stored at -65 C. Samples were never stored more than three weeks before assaying. Under such storage conditions loss in virus titers was minimized.

CO_2-Deficient Cultures

For experiments in which CO_2-deficient cells were studied, two methods of bicarbonate depletion were employed. Potassium hydroxide

traps (Geyer and Chang, 1958) were placed in the gas phase of cultures to absorb CO_2. The traps, consisting of a small, sterile glass tube containing absorbent cotton, were attached to the inside of rubber stoppers and projected into the tubes, but did not touch the culture medium. After adding bicarbonate-free medium to cultures, the rubber stoppers and traps were inserted, and 0.5 ml of 10 per cent potassium hydroxide was added to the absorbent cotton with a needle inserted through the rubber stoppers. Changes of medium were accomplished daily by inserting a needle through the stopper outside the potassium hydroxide trap. Fresh bicarbonate-free medium was added in a similar manner. At no time during incubation were the rubber stoppers removed from the cultures.

A much simpler method (Mosley and Enders, 1962) for creating CO_2-deficiencies was by replacing rubber stoppers with cotton plugs. When this technique was used medium was changed daily.

CHAPTER III

RESULTS

Quantitation Methods

Since the present investigation involved relationships between a virus and its host cell, the need for accurate and reproducible methods for enumerating both components of the system was paramount.

Cell Counts

During early phases of these studies the crystal violet-citric acid method of Sanford et al. (1951) was utilized for cell nuclei counts. Because suspensions of HeLa cells contained an excess number of clumps, counts were not uniformly reproducible. Since enumeration of viable cells seemed to be of more value than knowing only the total number of cells, the use of nigrosin stain (Kaltenbach et al., 1958) was investigated. Cell suspensions were made in trypsin solution and suitably diluted in nigrosin solution for cell enumeration. Initial counts were appreciably higher than subsequent counts of the same specimen. When growth medium containing lactalbumin hydrolysate, yeastolate and five per cent calf serum in Hanks' balanced salt solution (HLY) was utilized as the cell diluent instead of Dulbecco's phosphate balanced salt solution (DPBS), cell counts were stabilized, but clumping was still a problem. Incorporation of 0.1 per cent sodium citrate into the diluent

minimized this source of error; however, since the dark brown to black color of nigrosin made counting somewhat difficult, a comparison of crystal violet, nigrosin, and trypan blue stains was made in counts of three cell suspensions. Cell counts were reproducible with all three stains, as may be seen in Table 6. Trypan blue was the method of choice due to the ease in visualizing cells and the advantage of knowing the number of viable cells.

Virus Assay Method

Several primary and serially propagated cell culture systems were investigated to determine which type of cell was most convenient and sensitive for use in virus assay. Primary cultures were prepared from developing chick embryos and from kidneys of young adult hamsters. Serially propagated cell lines, FL and HeLa strains, were obtained from commercial sources. After monolayers had developed, samples of herpes simplex virus, pool #3, were diluted serially in HLY, and 0.1 ml amounts of the virus dilutions were inoculated into replicate tube cultures. The adsorption period was one hour at 37 C. Various media were evaluated for maintenance of cells during the virus titrations. Results of these studies are presented in Table 7. Chick embryo cells proved to be the most sensitive, but they were difficult to maintain. In the first experiments utilizing chick embryo cell cultures, the maintenance medium which contained five per cent head inactivated calf serum and 0.1 per cent glucose, proved inadequate; the cells became detached from the glass after about 48 hours and had completely peeled after four days. For the next group of chick embryo cells ELY maintenance medium

TABLE 6

A COMPARISON OF METHODS FOR COUNTING CELLS

	Cell Suspension 1			Cell Suspension 2			Cell Suspension 3		
	Nuclei Count	Nigro-sin	Trypan Blue	Nuclei Count	Nigro-sin	Trypan Blue	Nuclei Count	Nigro-sin	Trypan Blue
	231	196/238[a]	200/235[a]	110	100/113	104/110	152	138/143	144/150
	238	198/232	201/232	112	100/113	109/118	158	139/146	150/160
	240	200/233	205/242	112	102/111	109/120	160	142/150	152/160
	245	204/240	208/240	113	107/120	111/130	161	154/159	155/160
	246	207/250	212/242	113	110/125	115/128	162	154/161	158/161
	247	211/247	213/252	115	110/122	117/129	165	155/164	160/165
	250	213/251	215/240	117	115/125	118/125	171	159/168	161/170
	258	218/248	218/253	122	117/130	120/130	172	166/168	161/169
	263	221/262	220/248	131	118/131	121/131	174	167/169	168/179
	264	225/252	229/260	136	126/134	121/128	180	176/179	172/178
Cell Count x 10^5	2.5	2.1/2.5	2.1/2.4	1.2	1.1/1.2	1.2/1.3	1.7	1.6/1.6	1.6/1.7

[a]Ratio of viable cells (unstained) to total number of cells (stained and unstained).

TABLE 7

COMPARATIVE TITRATIONS OF HERPES SIMPLEX VIRUS[a] IN FOUR CELL CULTURE SYSTEMS

Cell Culture	Growth Medium	Days for Monolayer Development	Maintenance Medium	Virus Titer[b]
Chick embryo, P[c] 10 day incubation	HLY	2	ELY-M1[d]	6.5
Chick embryo, P 12 day incubation	HLY	2	ELY-M2[e]	6.8
Hamster kidney, P	HLY	10	ELY-M1	5.6
Hamster kidney, P	HLY	8	ELY-M1	5.5
FL, S[f]	MEM	4	ELY-M1	5.0[g]
FL, S	MEM	4	MEM	4.7[g]
HeLa-MA[h], S	MEM	4	ELY-M1	4.2[g]
HeLa-MA, S	MEM	4	MEM	4.5[g]
HeLa-10[i], S	HLY	4	ELY-M1	5.2[g]
HeLa-10, S	HLY	4	HLY	5.0[g]

[a]HF strain, pool #3.

[b]$TCID_{50}$ expressed as positive $\log_{10}$ of dilution per 0.1 ml.

[c]Primary culture.

[d]ELY-M1 contained 0.1 per cent glucose.

[e]ELY-M2 contained no calf serum; 0.1 per cent glucose.

[f]Serially propagated.

[g]Average of two experiments which differed less than 0.4 $\log_{10}$.

[h]From Microbiological Associates.

[i]From Tuskegee Institute.

contained the same amount of glucose but was not supplemented with serum; cytopathic changes (CPE) developed within 24 hours after inoculation and peeling of cells from the glass was no problem for five or six days during which time the cells were observed. Isolated areas of cytopathic changes developed rapidly over the entire cell sheet. Hamster kidney cell cultures were also shown to be sensitive to herpes simplex virus. Cytopathic changes developed more slowly (48 to 72 hours after infection) but the changes were distinct and easily recognized. The cells remained in good condition for seven days without a medium change. Although both of the primary cell cultures proved to be sensitive, certain disadvantages were found when compared to serially propagated cultures. Preparation of cell cultures directly from tissues was time consuming and also entailed the necessity of having available a constant source of animals or embryos of proper ages. Chick embryo cell cultures were at least 10 times more sensitive than other cells tested but difficulty in handling and maintaining the cells offset this advantage. The slow development of monolayers of hamster kidney cell cultures prohibited their use in these studies.

Serially propagated cell lines were desirable because of the ease with which replicate tube cultures could be prepared at any convenient time. FL, a normal human amnion cell line, and two strains of HeLa, a human carcinoma cell line, were compared for virus sensitivity. In Table 7 it can be seen that small, but significant, differences in susceptibility were evident. Because of its hardiness, the HeLa-10 strain was chosen for additional study. It was cultivated routinely in HLY growth medium in which good cell morphology was retained for

four to six days without a medium change.

After the HeLa-10 cell strain was chosen, it was decided to investigate the feasibility of using 0.5 ml, instead of the usual 1.0 ml, cell inoculum for seeding replicate tube cultures. This would have the advantage of allowing the preparation of twice the number of tube cultures for assay from the same number of cells, since the number of cells per unit of volume remained constant. Cell suspensions were used to prepare groups of replicate cultures of 0.5 ml containing 2×10^5 cells per tube and groups of 1.0 ml containing 4×10^5 cells per tube. After four days incubation, serial, 10-fold dilutions of herpes simplex virus were prepared in HLY, and 0.1 ml was inoculated onto each monolayer. After one hour adsorption at 37 C, maintenance medium ELY (0.1 per cent glucose) was added to each tube in a volume (0.5 or 1.0 ml) equal to that of the original cell inoculum. No significant difference was noted in $TCID_{50}$ values of the two groups in repeated experiments (Table 8). For this reason, 0.5 ml volumes of cell inoculum were utilized in subsequent studies. Figure 1 shows the results of several HeLa-10 growth experiments in HLY medium. Cell counts were done daily. Although growth medium was not changed during this study, numbers of viable cells increased to a maximum at six to seven days after seeding and remained constant to about 10 days.

To determine if cultures of different ages which contain different numbers of cells would exhibit equal sensitivity for virus titration, replicate cultures were prepared in 0.5 ml volumes of HLY. At 24 hour intervals viable cell counts were performed on uninfected cell cultures. The original medium was not changed on cultures until

TABLE 8

TITRATION OF HERPES SIMPLEX VIRUS[a] IN 0.5 ML AND 1.0 ML MONOLAYER CULTURES OF HELA-10 CELLS

Experiment Number	Volume in ml.	Initial Cell Count[b]	4 Day Cell Count[b]	Virus Titer[c]
1	0.5	2.0	4.0	5.4
	1.0	4.0	8.6	5.2
2	0.5	2.0	4.6	5.4
	1.0	4.0	9.0	5.4
3	0.5	2.0	4.3	5.2
	1.0	4.0	8.6	5.2

[a]HF strain, pool #3.

[b]Cell count x 10^5.

[c]$TCID_{50}$ expressed as positive $\log_{10}$ of dilution per 0.1 ml.

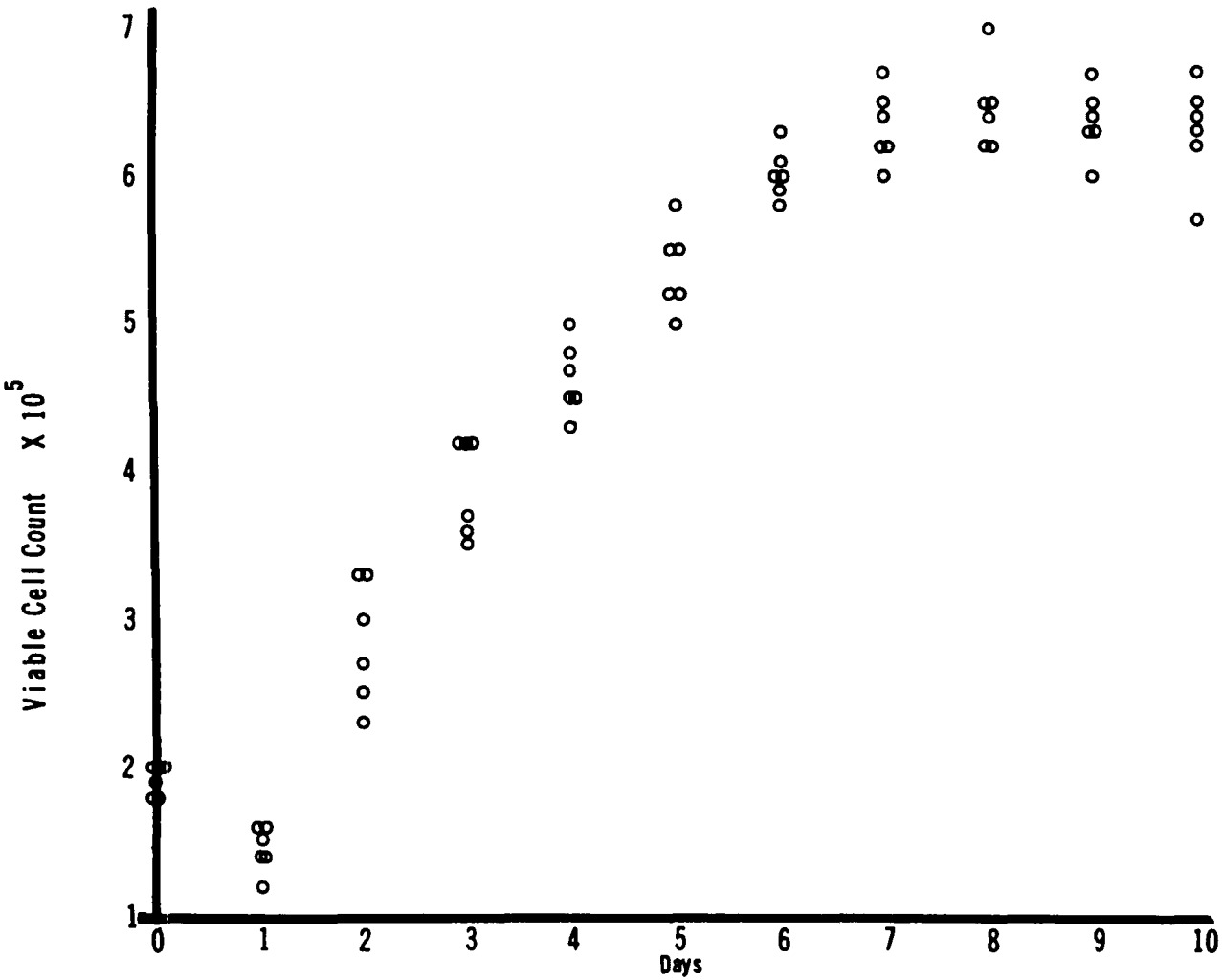


Fig. 1. Growth of HeLa cells in HLY[a] medium, six representative experiments.

[a]HLY contains lactalbumin hydrolysate and yeast extract in Hank's balanced salt solution.

the time of infection. From day three to day eight after seeding, HF herpes simplex virus, pool #3 and pool #4, were inoculated onto separate groups of replicate cultures. Virus dilutions were prepared routinely in HLY. After the 0.1 ml inoculum was introduced into the cell cultures and an adsorption period of one hour at 37 C had elapsed, 0.5 ml of ELY (0.1 per cent glucose) was added to each culture. As may be noted in Table 9 viable cell counts increased daily, even though no change of medium was made. However, maximum virus titers were obtained only in those cultures infected three to five days after seeding. When cell cultures were more than five days of age at the time of infection virus titers tended to be lower. Thus, in ensuing titrations replicate cultures were used which were three to five days of age and which contained 3.5 to 5.5 x 10^5 viable cells per tube.

Up to this point in the study, the routine virus diluent had been HLY, a nutrient medium which contained lactalbumin hydrolysate and yeast extract, as well as bicarbonate. For the bicarbonate depletion experiments which were contemplated, it was important to have an alternate, bicarbonate-free diluent which had little nutritive value, yet was buffered and would protect the infectivity of the virus. Dulbecco's phosphate buffered saline (DPBS), unsupplemented or supplemented with five or 10 per cent calf serum, was used to dilute HF herpes simplex virus, pool #3. These dilutions were assayed in a routine manner in HeLa-10 cells. As seen in Table 10, the titers obtained with DPBS containing either five or 10 per cent serum supplements were comparable to those obtained with HLY. The much lower titers obtained with DPBS unsupplemented with serum illustrate that a protein factor is necessary

TABLE 9

TITRATION OF HERPES SIMPLEX VIRUS IN HELA-10 CELLS INFECTED AT DIFFERENT AGES

Age of Culture	HF Pool #3		HF Pool #4	
	Viable Cells[a]	Virus Titer[b]	Viable Cells[a]	Virus Titer[b]
0	2.0	---	2.0	---
3	2.7	5.2	3.0	6.4
4	3.6	5.4	4.1	6.2
5	4.6	5.4	5.0	6.2
6	5.5	4.8	6.1	5.8
7	6.2	4.6	6.6	5.5
8	6.4	4.2	6.8	5.4

[a]Cell count x 10^5.

[b]$TCID_{50}$ expressed as positive $\log_{10}$ of dilution per 0.1 ml.

TABLE 10

EFFECT OF THE DILUENT ON TITRATION OF HERPES SIMPLEX VIRUS[a] IN HELA-10 CELLS

Experiment	Virus Titer[b] in HLY	DPBS	DPBS-5[c]	DPBS-10[d]
1	5.2	4.4	5.4	5.4
2	5.4	4.6	5.4	5.6

[a]HF strain, pool #3.

[b]$TCID_{50}$ expressed as positive log_{10} of the dilution per 0.1 ml.

[c]Supplemented with five per cent heat inactivated calf serum.

[d]Supplemented with ten per cent heat inactivated calf serum.

to protect the infectivity of herpes simplex virus. All subsequent samples for virus assay were diluted with DPBS supplemented with five per cent, heat inactivated calf serum (DPBS-5).

Up to this point a one hour adsorption period at 37 C had been set arbitrarily. Experiments were conducted to determine the optimal time and temperature for adsorption of HF herpes simplex virus onto HeLa-10 monolayer cell cultures. Serial 10-fold dilutions of the virus in DPBS-5 were inoculated onto HeLa-10 cell cultures. Virus adsorptions were conducted as depicted in Table 11. Maintenance medium was added to one set of cell cultures (0) immediately after the virus dilutions were added. Adsorption temperatures were 30 to 32 C or 37 C, for one to four hours. Results showed that a period of adsorption was necessary to obtain maximal virus titers. When the adsorption temperatures were 30 to 32 C titers were slightly higher than in those cultures held at 37 C. There were only slight differences in titers with adsorption periods from one to four hours. Adsorption at 30 to 32 C for two hours was, therefore, incorporated into the virus assay method.

Virus titers to date had been calculated numerically and expressed as $TCID_{50}$, the positive $\log_{10}$ of the virus dilution which would infect 50 per cent of the inoculated cultures. The plaque forming unit (PFU), an expression of the number of infectious virus particles which produce plaques in a susceptible cell culture is calculated from the number of plaques which develop per volume of virus suspension, on the assumption that each virus particle produces one plaque (Fazmham, 1958). This should represent accurately the number of infectious virus particles present. Replicate cultures were prepared and virus dilutions

TABLE 11

OPTIMAL TIME AND TEMPERATURE FOR ADSORPTION OF HERPES SIMPLEX VIRUS[a] TO HELA-10 CELLS

	Virus Titer[b] after Adsorption Time in Hours				
Temperature	0	1	2	3	4
37 C	4.2	4.8	5.2	5.4	5.2
30 to 32 C	4.4	5.2	5.4	5.5	5.4
30 to 32 C	4.5	5.4	5.5	5.5	5.8

[a]HF strain, pool #3.

[b]$TCID_{50}$ expressed as positive log_{10} of the dilution per 0.1 ml.

were made as described previously. One group of cultures received the usual maintenance medium, ELY (0.1 per cent glucose) supplemented with five per cent calf serum; a second group received ELY (0.1 per cent glucose) supplemented with five per cent human serum which was known to contain herpes simplex neutralizing antibodies. The adsorption period for both groups was the same. All cultures were examined for cytopathic changes and in the cultures containing herpes simplex antiserum, the number of plaques developing from each virus dilution were counted. From this data, the $TCID_{50}$ and PFU of the same virus solution could be determined. In Table 12 data are presented which compare the amount of virus present as determined by two different methods. Repeated titrations demonstrated the reproducibility of both methods. The time involved in counting individual plaques precluded routine use of this method because a large number of samples would be required for this investigation. Since calculation of $TCID_{50}$ was an acceptable and reproducible method which required only a brief examination of each tube to determine the presence or absence of CPE, this method was adopted for routine use.

Throughout this work it had been noted that after ELY (0.1 per cent glucose) medium had been on cells for five or six days, excessive, nonspecific, degeneration of cells occurred. Since glucose is an essential nutrient which can be readily exhausted from the medium, the amount of glucose was doubled in the next experiments in order to obviate cell damage. The experimental design remained as described except for glucose content. As shown in Table 13, titers were consistently higher in medium which contained 0.2 per cent glucose. In addition, cells were

TABLE 12

A COMPARISON OF THE $TCID_{50}$ AND PFU METHODS OF VIRUS ASSAY

Experiment Number	HF Pool #3			HF Pool #4		
	ELY-M1[a]	ELY-M1H[b]		ELY-M1[a]	ELY-M1H[b]	
	$TCID_{50}$[c]	$TCID_{50}$	PFU[d]	$TCID_{50}$	$TCID_{50}$	PFU[d]
1	5.2	5.0	0.9	5.8	5.8	6.2
2	5.2	5.2	1.1	6.2	6.2	6.7
3	5.2	5.2	1.2	6.4	6.2	6.9
4	5.4	5.2	1.3			
5	5.5	5.4	1.5			

[a]Supplemented with five per cent heat inactivated calf serum and 0.1 per cent glucose.

[b]Supplemented with five per cent heat inactivated human serum which contained herpes simplex antibodies.

[c]$TCID_{50}$ expressed as positive $\log_{10}$ of the dilution per 0.1 ml.

[d]Plaque forming unit expressed as the average number of plaques per 0.1 ml x 10^5.

TABLE 13

EFFECT OF INCREASED GLUCOSE CONCENTRATION ON THE TITER OF HERPES SIMPLEX VIRUS[a] IN HELA-10 CELLS

Experiment Number	Virus Titer[b]	
	Glucose, 0.1 per cent[c]	Glucose, 0.2 per cent
1	5.8	6.4
2	5.8	6.5
3	6.2	6.5
4	6.2	6.6
5	6.2	6.6
6	6.4	6.8

[a]HF strain, pool #4.

[b]$TCID_{50}$ expressed as positive $\log_{10}$ of the dilution per 0.1 ml.

[c]Glucose included in ELY.

morphologically in better condition than in medium which contained 0.1 per cent glucose.

Preparation of Samples for Titrations of Infectivity

In these studies it was important to determine the total amount of infectious virus present in a cell culture. This includes the virus present in the supernatant fluid and the virus associated with the host cells. A method which utilized alternate cycles of freezing and thawing of the cultures was studied to determine if it would be effective in liberation of virus without being detrimental to infectivity. Herpes simplex (HF strain)-infected cultures of HeLa-10 cells which showed gross CPE were placed at -65 C. When complete freezing had occurred (five to 10 minutes), the contents of the bottles were thawed rapidly in a 37 C water bath. This first cycle of freezing and thawing completely freed the cells from the glass and resulted in a turbid suspension. After the suspensions from several bottles were pooled a sample was removed for virus assay. Additional samples were taken after second and third cycles of freezing and thawing and all samples were assayed immediately for total infectious virus content. Results presented in Table 14 indicate that a single cycle of freezing and thawing was inadequate for complete liberation of the cell-associated virus. A second cycle was apparently required for producing maximal titers. Freezing and thawing appeared to have no detrimental effects upon infectivity of virus preparations. In all subsequent studies cultures to be assayed for virus were frozen quickly at -65 C, thawed rapidly in a 37 C water bath, pooled, frozen a second time at -65 C, and stored at that temperature.

TABLE 14

AMOUNT OF VIRUS[a] LIBERATED FROM HELA CELLS FOLLOWING CYCLES OF FREEZING AND THAWING

Number of cycles[b]	Virus Titers
1	5.8[c]
2	6.7
3	6.5

[a]Herpes simplex virus, HF strain.

[b]Frozen in alcohol bath (-65 C) and thawed quickly in water bath (37 C).

[c]$TCID_{50}$ expressed as the positive $\log_{10}$ of the dilution per 0.1 ml.

Effect of Bicarbonate on Cell Proliferation and Virus Replication

Initial experiments were conducted to determine the optimal growth conditions for HeLa-10 cells maintained in chemically defined MEM. Suspensions of HeLa cells which had been cultivated in MEM were prepared in MEM-M containing 10 mM bicarbonate. This amount of bicarbonate created no serious problem with pH control as visually determined by the color of phenol red indicator, and gassing of the cultures with CO_2 was unnecessary. Replicate cultures containing 1.0 ml of the cell suspensions were sealed tightly with rubber stoppers and incubated as stationary cultures at 37 C for 24 hours. The cells settled and attached firmly to the glass. They became smooth, flattened and hyaline, and continued their normal development. After the initial incubation period of 24 hours, all cultures were transferred to roller drums at 36 C for the remainder of experiments. Forty-eight hours after seeding and 24 hours after cultures were placed on the roller drum, the tubes were divided into two groups. One group was incubated without a change of the original growth medium, whereas the second group had daily medium changes of MEM. Viable cell counts made at 24 hour intervals showed that daily medium changes were optimal for normal development of the cultures (Figure 2). In cultures incubated without changing the medium cell degeneration began within five to six days after the cultures were initiated, and viable cell counts were low after an additional three to four days. When Figure 2 is compared with Figure 1 differences in cell growth can be noted; HLY supported sustained growth of the cells without a medium change, whereas MEM required frequent changes of medium.

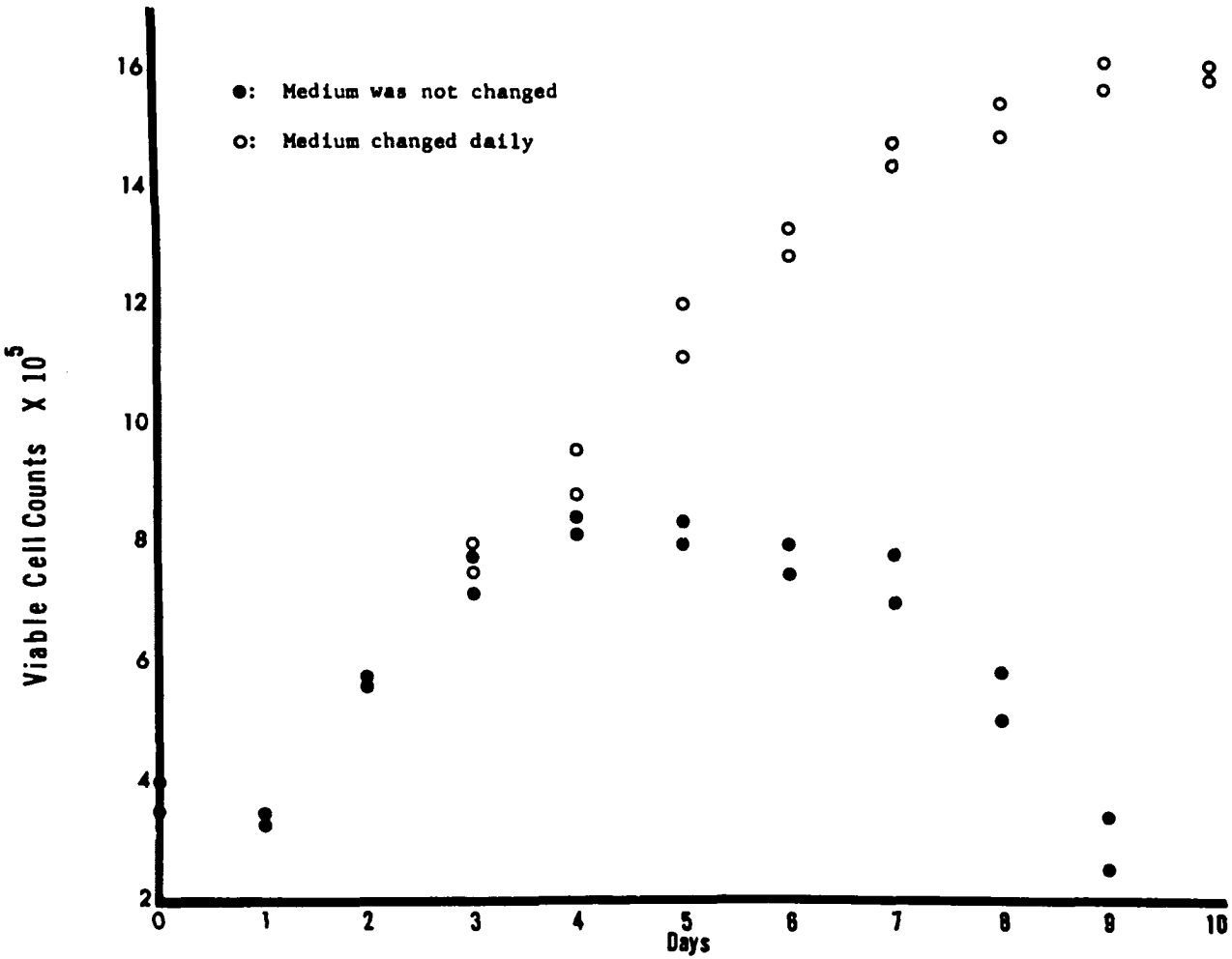


Fig. 2. Growth of HeLa cells in MEM[a], two representative experiments.

[a]Eagle's minimum essential medium.

Further experiments were designed to determine the effect of different concentrations of bicarbonate on host cell development and virus replication. Since multiplication of vaccinia virus had been shown to be retarded in bicarbonate-depleted cultures (Chang, 1959), replication of the O strain of vaccinia virus and the HF strain of herpes simplex virus was compared. Cultures were seeded and maintained for 48 hours as described in the previous experiments. After that time the cultures were divided into four groups, the medium was discarded and replaced with MEM-M which contained five per cent dialyzed calf serum and varying concentrations of sodium bicarbonate. Before addition, pH of these media was adjusted to 7.6 with either 0.3 N HCl or 0.3 N NaOH. At 24 hour intervals thereafter, the average number of viable cells in two or three cultures was determined and the medium of the remaining tubes was changed. Cell counts and approximate pH values of cultures during growth of uninfected HeLa-10 cells are presented in Tables 15 and 16. The data from two experiments which are recorded in Table 15 show that the cells developed normally up to the time when experimental media were added. Table 16 shows data from two experiments pertaining to the development of cultures in the presence of different concentrations of bicarbonate. In the medium containing 40 mM sodium bicarbonate, growth of HeLa cells was greatly retarded and the cell numbers diminished rapidly. No effort was made to control the elevated pH which resulted from use of such a medium. Cell growth was sustained in media containing 5, 10, and 20 mM sodium bicarbonate. There were no apparent differences in the total numbers of cells from cultures which were grown in media containing 5, 10, or 20 mM

TABLE 15

HELA CELL DEVELOPMENT IN GROWTH MEDIUM[a]

Experiment Number	Cell Count and pH by Hours[b]					
	-48[c]		-24[d]		0[e]	
	CC[f]	pH[g]	CC	pH	CC	pH
1	2.6	7.1	2.2	7.3	4.0	7.1
2	3.0	7.1	2.5	7.3	4.4	7.1

[a]Minimum essential medium (Eagle)-modified (MEM-M) was used (bicarbonate 10 mM). All cultures initiated in rubber stoppered tubes.

[b]Hours before experimental conditions initiated.

[c]Cells incubated in stationary cultures at 37 C.

[d]Cultures placed on roller drum at 36 C.

[e]Experimental conditions initiated.

[f]Cell count x 10^5.

[g]Approximate pH visually determined by color of phenol red indicator.

TABLE 16

EFFECT OF BICARBONATE ON HELA CELL GROWTH

Hours[a]	Bicarbonate 5 mM[b]		Bicarbonate 10 mM[b]		Bicarbonate 20 mM[b]		Bicarbonate 40 mM[b]	
	CC[c]	pH[d]	CC	pH	CC	pH	CC	pH
24	7.8	6.8	7.5	7.3	6.2	7.7	4.2	7.7
	7.2	6.9	8.1	7.3	6.8	7.7	5.0	7.8
48	10.1	6.7	10.2	7.3	9.3	7.6	3.1	7.9
	10.4	6.7	11.0	7.4	10.0	7.5	4.5	7.9
72	11.8	6.6	12.4	7.2	11.5	7.5	2.0	7.9
	12.3	6.6	13.2	7.1	11.8	7.4	2.6	7.9
96	14.0	6.5	13.7	7.3	13.1	7.4	0.0	8.0
	14.3	6.5	14.4	7.1	13.6	7.4	0.0	8.0
120	14.6	6.4	15.0	7.1	14.4	7.3	-[e]	-
	15.1	6.4	15.5	7.1	14.0	7.3	-	-
144	15.7	6.4	16.1	7.0	14.6	7.4	-	-
	16.0	6.4	16.8	7.1	15.0	7.3	-	-
168	16.5	6.4	17.3	7.0	15.2	7.3	-	-
	17.3	6.4	18.0	7.0	15.7	7.3	-	-
192	17.4	6.4	18.1	6.9	15.9	7.3	-	-
	18.1	6.4	18.4	6.9	16.4	7.3	-	-

[a]Time after experimental conditions initiated.

[b]Minimum essential medium (Eagle)-modified (MEM-M) was used. All rubber stoppered cultures incubated at 36 C on roller drum.

[c]Cell count x 10^5.

[d]Approximate pH visually determined by color of phenol red indicator.

[e]No samples taken.

bicarbonate, although differences in pH were demonstrated. Cultures containing 10 mM bicarbonate maintained pH values of approximately 7.0, while cultures grown in 20 mM bicarbonate medium had pH values above 7.0. Figure 3 illustrates graphically data from Tables 15 and 16 on growth of HeLa cells in various concentrations of bicarbonate.

In experiments to determine virus replication in cells cultivated in the presence of varying amounts of sodium bicarbonate, a diluted virus suspension was added to replicate cultures 96 hours after they were seeded and 48 hours after each bicarbonate-containing medium was added. At this time, exclusive of the medium containing 40 mM bicarbonate, approximately one million cells were contained in each culture. The medium from each culture was discarded and 0.2 ml volumes of 10^{-4} dilutions of seed viruses (HF herpes simplex or 0 vaccinia) were added to replicate cultures grown in the different bicarbonate containing media. In each instance, after adsorption, unadsorbed virus was removed, and 1.0 ml of the appropriate bicarbonate medium was again added to each culture. Samples representing total infectious virus content at zero time were taken from each group of cultures, prepared by freezing and thawing, and stored at -65 C. Similar samples were taken at 12 or 24 hour intervals throughout the experiments. Titrations of seed virus were included as a control in the assay of virus sample from each culture maintained in four bicarbonate-containing media. Results of the titrations of vaccinia and herpes simplex viruses are presented in Tables 17 and 18, respectively. Figure 4 graphically depicts these combined data. No virus was produced in cultures maintained in 40 mM bicarbonate medium. In 5, 10, and 20 mM bicarbonate-containing media,

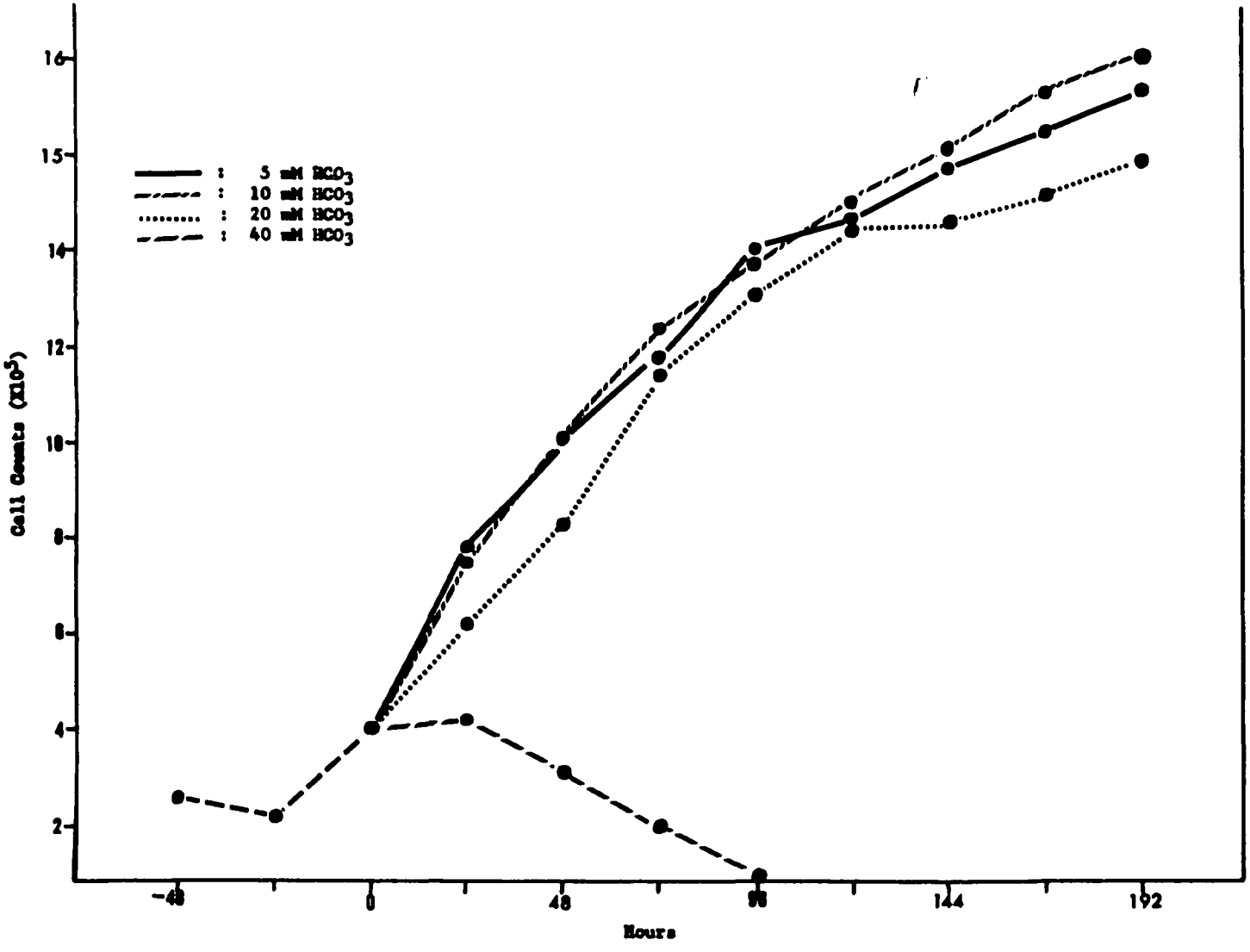


Fig. 3. Effect of bicarbonate on HeLa cell growth.

TABLE 17

EFFECT OF BICARBONATE ON VACCINIA VIRUS[a] PRODUCTION BY HELA CELLS

Growth Conditions[b]	Hours After Adsorption When Virus Samples Were Taken							
	0	12	24	48	72	96	120	144
Bicarbonate 5 mM	1.2[c]	2.0	3.8	4.6	4.8	5.4	5.6	5.5
Bicarbonate 10 mM	0.8	3.2	4.5	5.2	5.6	6.0	6.4	6.8
Bicarbonate 20 mM	1.2	2.8	4.2	5.5	5.5	6.2	6.2	6.5
Bicarbonate 40 mM	0.6	0.8	0.2	0.0	-[d]	-	-	-

[a]O strain.

[b]Minimum essential medium (Eagle)-modified (MEM-M) was used. All rubber stoppered cultures incubated at 36 C on roller drum.

[c]$TCID_{50}$ expressed as positive $\log_{10}$ of dilution per 0.1 ml.

[d]No sample taken.

TABLE 18

EFFECT OF BICARBONATE ON HERPES SIMPLEX VIRUS[a] PRODUCTION BY HELA CELLS

Growth Conditions[b]	Hours After Adsorption When Virus Samples Were Taken						
	0	12	24	48	72	96	120
Bicarbonate 5 mM	0.6[c]	1.6	3.6	4.5	5.2	5.8	5.2
Bicarbonate 10 mM	0.8	2.2	3.8	5.2	6.4	6.5	4.2
Bicarbonate 20 mM	0.5	1.4	3.2	4.6	5.5	5.8	4.8
Bicarbonate 40 mM	0.6	0.0	0.0	-[d]	-	-	-

[a]HF strain.

[b]Minimum essential medium (Eagle)-modified (MEM-M) was used. All rubber stoppered cultures incubated at 36 C on roller drum.

[c]$TCID_{50}$ expressed as positive $\log_{10}$ of dilution per 0.1 ml.

[d]No sample taken.

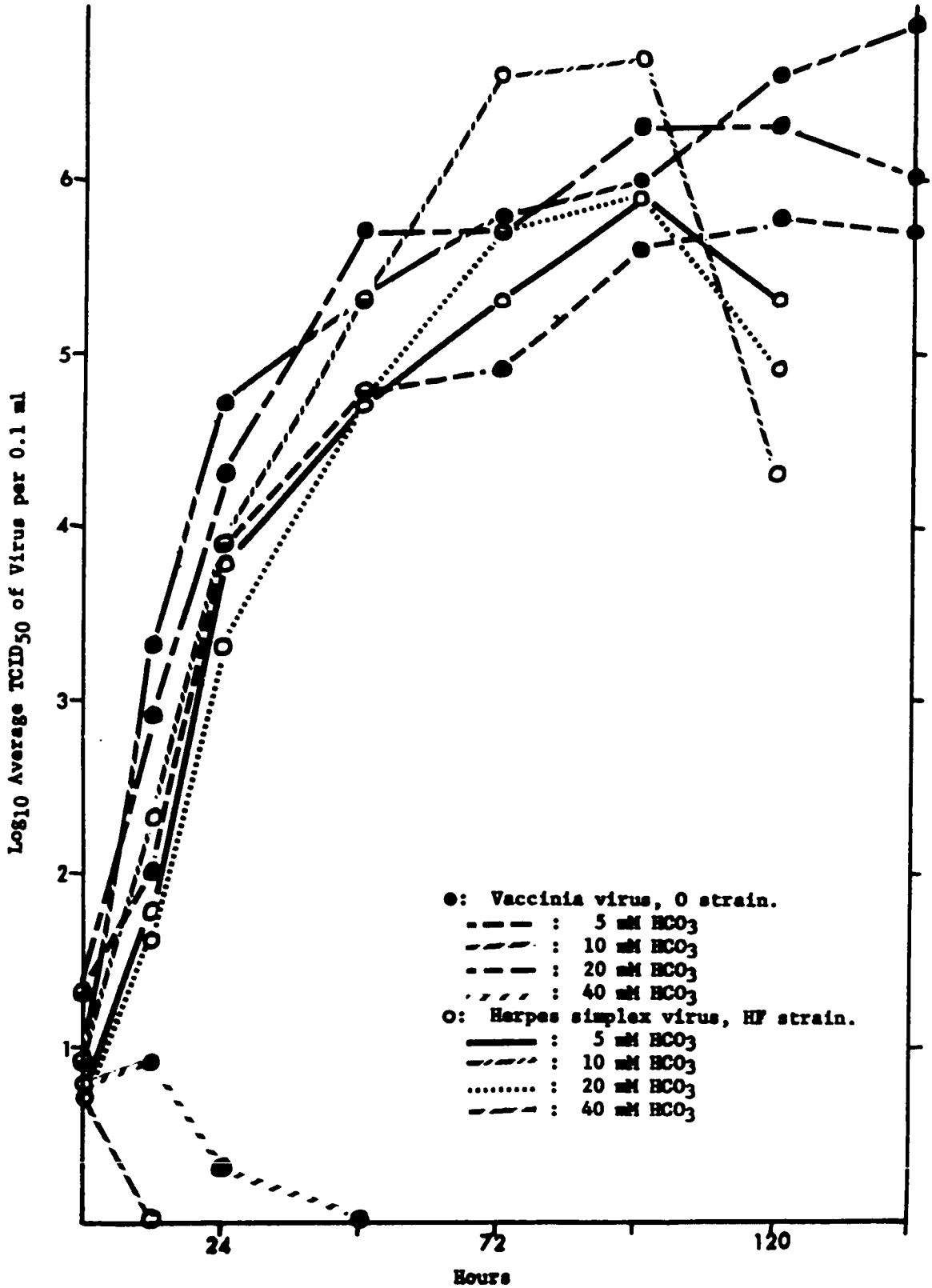


Fig. 4. Effect of bicarbonate on viral replication in HeLa cells.

replication of vaccinia virus continued to completion of the experiment (144 hours). The amount of vaccinia virus produced was slightly lower in the 5 mM bicarbonate-containing medium than in the 10 and 20 mM media. Herpes simplex virus production reached its maximal level at 72 to 96 hours, by which time most of the cells had been destroyed by virus activity. Optimal production of herpes simplex virus occurred in the 10 mM bicarbonate medium.

Effect of Tris on Cellular Proliferation and Virus Replication

Since Chang et al. (1961) and Mosley and Enders (1962) showed that certain types of cells could be maintained in vitro in Tris-buffered media which did not have an atmosphere of increased CO_2, studies were undertaken to determine the effects of Tris on the growth of HeLa cells. To do this open and closed cultures were used. Attempts to initiate cultures in Tris-buffered media as described by Geyer and Chang (1958) were completely unsuccessful. In such media, the cells remained rounded, were granular, had marked tendencies to remain clumped, and sustained growth was erratic. Therefore, 1.0 ml volumes of suspensions of cells in MEM-M containing 10 mM bicarbonate were used to initiate replicate tube cultures. All tubes were tightly closed with rubber stoppers. Preparation of cells and subsequent growth conditions for 48 hours after seeding were the same as in the previous bicarbonate studies. After 48 hours the cultures were divided into two equal groups, the medium was discarded and 1.0 ml quantities of MEM-M, containing five per cent dialyzed, heat-inactivated calf serum and buffered with various concentrations of Tris, were added to designated cultures of each group. The

initial pH values of all media were adjusted to 7.6 with HCl or NaOH, as in the bicarbonate study. One group of cultures was incubated in tubes sealed with rubber stoppers and a second group was incubated as open cultures, i.e., rubber stoppers were replaced with loosely fitted cotton plugs. All cultures were kept on roller drums at 36 C. Medium change and viable cell counts were performed daily. In Table 19 data concerning development of cultures prior to institution of experimental conditions are recorded. Table 20 gives data of three experiments on the development of HeLa cells in Tris-containing media in closed cultures. Medium containing 20 mM Tris was toxic for the cells, as shown by rapid decrease in the number of viable cells, their granular, rounded appearance, and rapid detachment from the glass surfaces. In cultures grown in medium containing 1, 5, or 10 mM Tris, the number of viable cells continued to increase even where pH values were reduced to about 6.5, as occurred in media containing 1 and 5 mM Tris. In a medium containing 10 mM Tris the pH was maintained at more nearly physiological levels and optimal growth of cells occurred. Microscopically, the cells could not be distinguished from those grown in bicarbonate-buffered media. The development of HeLa cells in Tris-buffered media in open cultures is given in Table 21. As in closed cultures, 20 mM Tris proved to be toxic for cells. The number of viable cells continued to increase for about 24 hours in the open cultures which contained 1, 5, or 10 mM Tris. Thereafter, and for the remaining five days of the experiment, cell numbers in cotton plugged cultures remained remarkably constant, whereas cells maintained in a closed culture continued to multiply. The pH values were uniformly higher in open cultures

TABLE 19

HELA CELL DEVELOPMENT IN GROWTH MEDIUM[a]

Experiment Number	Cell Count and pH by Hours[b]					
	-48[c]		-24[d]		0[e]	
	CC[f]	pH[g]	CC	pH	CC	pH
1	3.0	7.1	2.5	7.4	4.5	7.2
2	3.1	7.1	3.0	7.4	5.0	7.2
3	3.5	7.1	3.1	7.4	5.2	7.1

[a]Minimum essential medium (Eagle)-modified (MEM-M) was used (bicarbonate 10 mM). All cultures initiated in rubber stoppered tubes.

[b]Hours before experimental conditions initiated.

[c]Cells incubated in stationary cultures at 37 C.

[d]Cultures placed on roller drum at 36 C.

[e]Experimental conditions initiated.

[f]Cell count x 10^5.

[g]Approximate pH visually determined by color of phenol red indicator.

TABLE 20

EFFECT OF TRIS ON HELA CELL GROWTH IN CLOSED CULTURES

Hours[a]	Tris 1 mM[b]		Tris 5 mM[b]		Tris 10 mM[b]		Tris 20 mM[b]	
	CC[c]	pH[d]	CC	pH	CC	pH	CC	pH
24	6.6	6.4	6.8	6.4	6.8	6.9	4.4	7.9
	6.6	6.4	7.0	6.4	7.2	7.0	4.8	7.8
	7.0	6.4	7.1	6.4	7.4	6.9	5.1	7.7
48	9.5	6.4	10.0	6.4	10.3	6.9	1.0	7.7
	9.8	6.4	9.9	6.4	10.6	6.9	2.3	7.9
	10.4	6.4	10.8	6.4	11.1	6.9	2.5	7.7
72	11.2	6.4	11.0	6.4	12.0	6.9	0.0	7.9
	11.5	6.4	11.6	6.4	12.6	6.9	0.0	7.9
	12.0	6.4	12.6	6.4	13.0	6.9	0.0	7.9
96	13.6	6.4	14.4	6.4	15.5	6.7	-[e]	-
	14.1	6.4	14.9	6.4	16.2	6.7	-	-
	15.0	6.4	14.7	6.4	16.5	6.7	-	-
120	14.5	6.3	15.0	6.4	16.3	6.7	-	-
	15.1	6.2	15.8	6.4	16.8	6.7	-	-
	15.5	6.3	16.4	6.4	17.1	6.7	-	-
144	15.0	6.3	14.6	6.4	17.1	6.7	-	-
	15.5	6.3	16.2	6.4	16.6	6.7	-	-
	16.1	6.3	16.5	6.4	17.0	6.7	-	-

[a]Time after experimental conditions initiated.

[b]Minimum essential medium (Eagle)-modified (MEM-M) was used. All rubber stoppered cultures incubated at 36 C on roller drum.

[c]Cell count x 10^5.

[d]Approximate pH visually determined by color of phenol red indicator.

[e]No samples taken.

TABLE 21

EFFECT OF TRIS ON HELA CELL GROWTH IN OPEN CULTURES

Hours[a]	Tris 1 mM[b]		Tris 5 mM[b]		Tris 10 mM[b]		Tris 20 mM[b]	
	CC[c]	pH[d]	CC	pH	CC	pH	CC	pH
24	6.8	6.6	6.8	6.7	6.2	7.2	4.0	7.9
	6.5	6.7	6.8	6.7	6.5	7.1	3.6	7.9
	7.5	6.6	7.2	6.7	6.9	7.1	4.8	7.8
48	7.0	6.7	7.1	6.7	6.8	7.2	0.0	8.2
	7.1	6.7	7.5	6.7	7.5	7.2	0.0	8.2
	7.8	6.7	7.5	6.7	8.1	7.2	0.0	8.2
72	7.1	6.7	7.6	6.7	7.5	7.2	-[e]	-
	7.3	6.7	7.8	6.7	7.8	7.1	-	-
	7.5	6.7	8.1	6.7	8.0	7.1	-	-
96	6.8	6.8	7.0	6.9	7.6	7.1	-	-
	6.7	6.7	7.6	6.8	8.0	7.1	-	-
	7.0	6.7	8.0	6.8	7.8	7.1	-	-
120	6.5	6.8	7.2	6.8	7.8	7.0	-	-
	6.8	6.8	7.2	6.7	8.2	7.1	-	-
	7.0	6.8	7.6	6.8	8.3	7.1	-	-
144	6.4	6.9	7.1	6.9	7.5	7.1	-	-
	6.6	6.8	7.3	6.8	8.0	7.0	-	-
	6.7	6.8	7.4	6.7	8.1	7.0	-	-

[a]Time after experimental conditions initiated.

[b]Minimum essential medium (Eagle)-modified (MEM-M) was used. All cotton plugged cultures incubated at 36 C on roller drum.

[c]Cell count x 10^5.

[d]Approximate pH visually determined by color of phenol red indicator.

[e]No samples taken.

than in closed cultures. Microscopically, the cells in open cultures became delicate and hyaline in appearance and larger than normal. In other, long term experiments, under these same conditions, cells retained these characteristics for seven to 10 days, after which time degeneration occurred rapidly. In Figure 5 data from Tables 19, 20, and 21 are combined to show the growth of HeLa cells in Tris-containing, open and closed cultures. The distinctly different growth patterns of the cells in the two gas phase conditions are readily seen. At 48 hours after introduction of medium containing 1, 5, or 10 mM Tris, cell counts of cultures grown in the closed system and in the open system were approximately 1×10^6 and 7×10^5 respectively. Representative cultures from each group in the experiments just described were infected with either HF herpes simplex or D vaccinia virus. Techniques used for the preparation, inoculation, and adsorption of virus and for the collection of samples for assay of total virus content were identical to those described in the earlier study on effects of bicarbonate concentration on replication of herpes simplex and vaccinia viruses by HeLa cells. In media buffered with 20 mM Tris, a concentration which had previously been shown to be toxic for HeLa cells, neither herpes simplex nor vaccinia virus was replicated, regardless of the conditions of incubation. As may be seen in Table 22, production of vaccinia virus by HeLa cells was essentially the same in cultures maintained in a closed system in the presence of 1, 5, and 10 mM concentrations of Tris. Essentially the same results were obtained with herpes simplex virus in a closed system (Table 23).

When the development of these viruses in an open system was

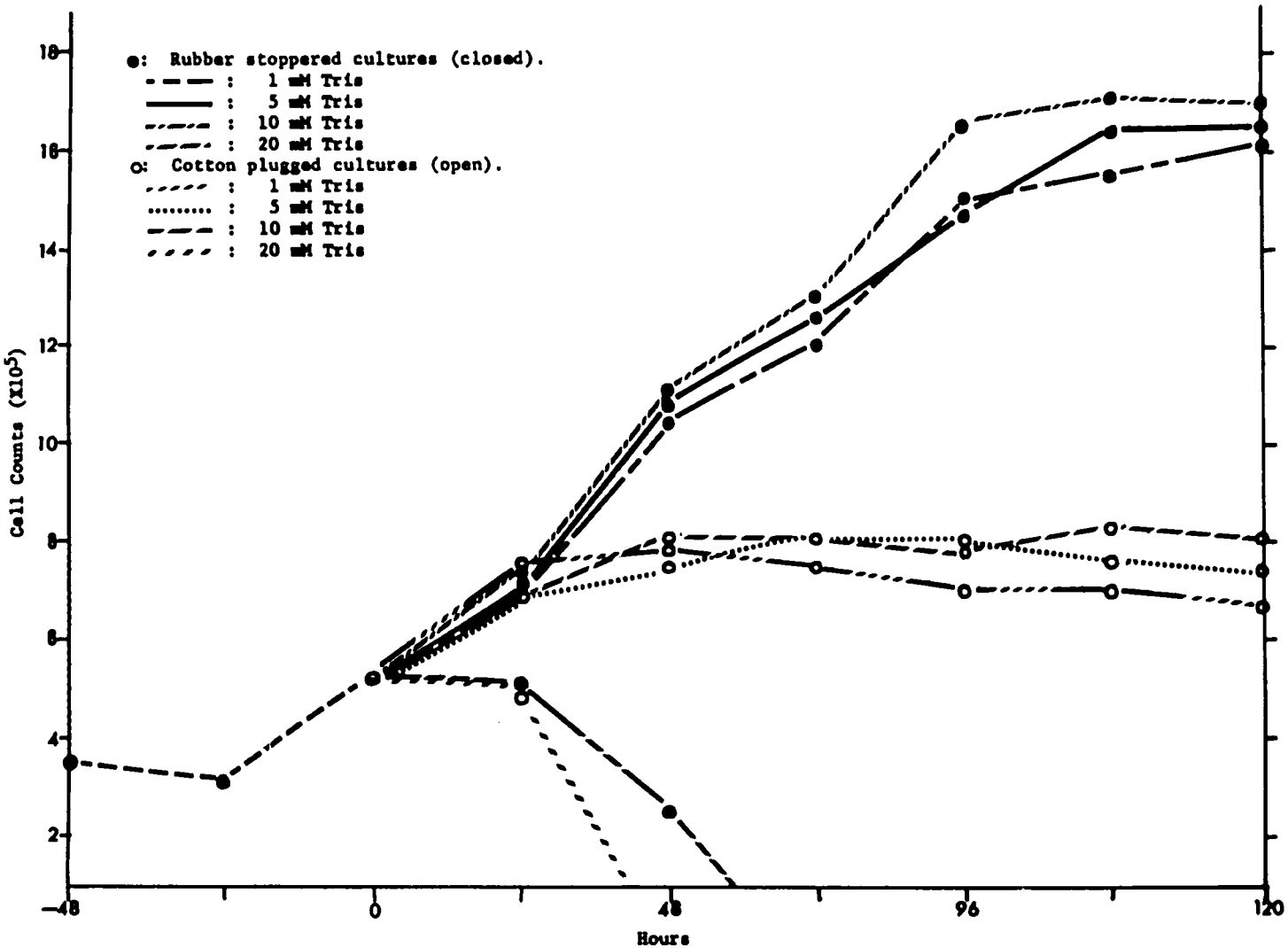


Fig. 5. Effect of Tris on HeLa cell growth in open and closed cultures.

TABLE 22

EFFECT OF TRIS ON VACCINIA VIRUS[a] PRODUCTION BY HELA CELLS IN OPEN AND CLOSED CULTURES

Hours after Adsorption	Tris 1 mM[b]		Tris 5 mM[b]		Tris 10 mM[b]		Tris 20 mM[b]	
	closed[c]	open[d]	closed	open	closed	open	closed	open
0	0.6[e]	0.8	0.5	0.6	0.6	0.8	0.5	0.5
12	2.2	0.6	2.4	0.5	2.2	0.8	0.4	0.2
24	2.5	0.8	2.6	0.8	2.5	1.2	0.0	0.0
36	3.2	1.5	3.5	1.4	3.6	1.8	0.0	0.0
48	3.5	1.2	3.8	1.5	4.2	1.8	-[f]	-
60	4.5	1.8	4.6	2.4	4.7	2.2	-	-
72	5.2	2.4	5.2	2.6	5.4	2.5	-	-
96	4.8	2.6	5.4	2.5	5.4	2.6	-	-
120	5.2	2.8	5.6	2.8	5.8	2.5	-	-
144	5.5	2.8	6.0	3.0	6.2	2.8	-	-
168	5.6	3.2	6.2	3.4	6.5	3.4	-	-

[a]D strain.

[b]Minimum essential medium (Eagle)-modified (MEM-M) was used. All cultures incubated at 36 C on roller drum.

[c]Rubber stoppered cultures.

[d]Cotton plugged cultures.

[e]$TCID_{50}$ expressed as positive $\log_{10}$ of dilution per 0.1 ml.

[f]No samples taken.

TABLE 23

EFFECT OF TRIS ON HERPES SIMPLEX VIRUS[a] PRODUCTION BY HELA CELLS IN OPEN AND CLOSED CULTURES

Hours after Adsorption	Tris 1 mM[b]		Tris 5 mM[b]		Tris 10 mM[b]		Tris 20 mM[b]	
	closed[c]	open[d]	closed	open	closed	open	closed	open
0	0.8[e]	0.8	0.8	0.6	0.6	0.8	0.4	0.4
12	0.8	0.5	0.8	0.6	1.2	1.4	0.5	0.2
24	2.4	2.0	2.5	2.4	2.8	2.5	0.2	0.0
36	2.8	2.5	2.8	2.6	3.2	2.8	0.0	0.0
48	3.2	2.8	2.8	3.0	3.8	3.4	-[f]	-
60	3.8	3.6	3.8	4.2	4.6	4.4	-	-
72	5.8	5.2	5.4	5.2	6.5	6.2	-	-
96	6.2	5.8	6.2	5.6	6.6	6.4	-	-
120	5.5	5.6	5.4	5.5	5.8	6.2	-	-

[a]HF strain.

[b]Minimum essential medium (Eagle)-modified (MEM-M) was used. All cultures incubated at 36 C on roller drum.

[c]Rubber stoppered cultures.

[d]Cotton plugged cultures.

[e]$TCID_{50}$ expressed as positive $\log_{10}$ of dilution per 0.1 ml.

[f]No samples taken.

studied, markedly different results were obtained with herpes simplex virus and vaccinia virus. The amount of vaccinia virus recovered from open cultures was as much as 100 times less than that recovered from closed cultures. No such difference was evident in the case of open and closed cultures inoculated with herpes simplex virus. There were no significant differences in amounts of herpes simplex virus produced in open cultures with media containing 1, 5, or 10 mM Tris. This was also the case with vaccinia virus in corresponding media. The effect of Tris on replication of vaccinia virus in open or closed HeLa cell cultures is illustrated in Figure 6; the effect of Tris on herpes simplex virus replication under similar cultivation conditions is presented in Figure 7 (data from Tables 22 and 23, respectively).

The influence of Tris in the medium on the infectivity of these viruses was studied. Duplicate, serial dilutions of HF herpes simplex virus and D vaccinia virus were prepared. For each virus, one set of dilutions was made as usual with DPBS-5; the second set was made in DPBS-5 containing 10 mM Tris. The pH of the Tris-containing diluent was adjusted to 7.2 with 0.3 N HCl prior to preparation of the dilutions. After 30 minutes incubation at room temperature, 0.1 ml volumes of the respective virus dilutions were inoculated to replicate tube cultures of HeLa cells, and titrations were performed in the routine manner. The presence of Tris in the virus diluent did not influence the infectivity of either virus (Table 24).

In earlier studies with Tris-buffered medium, in which it was found that vaccinia virus, but not herpes simplex virus, replicates to a lesser extent in open than in closed cultures, the only variable had

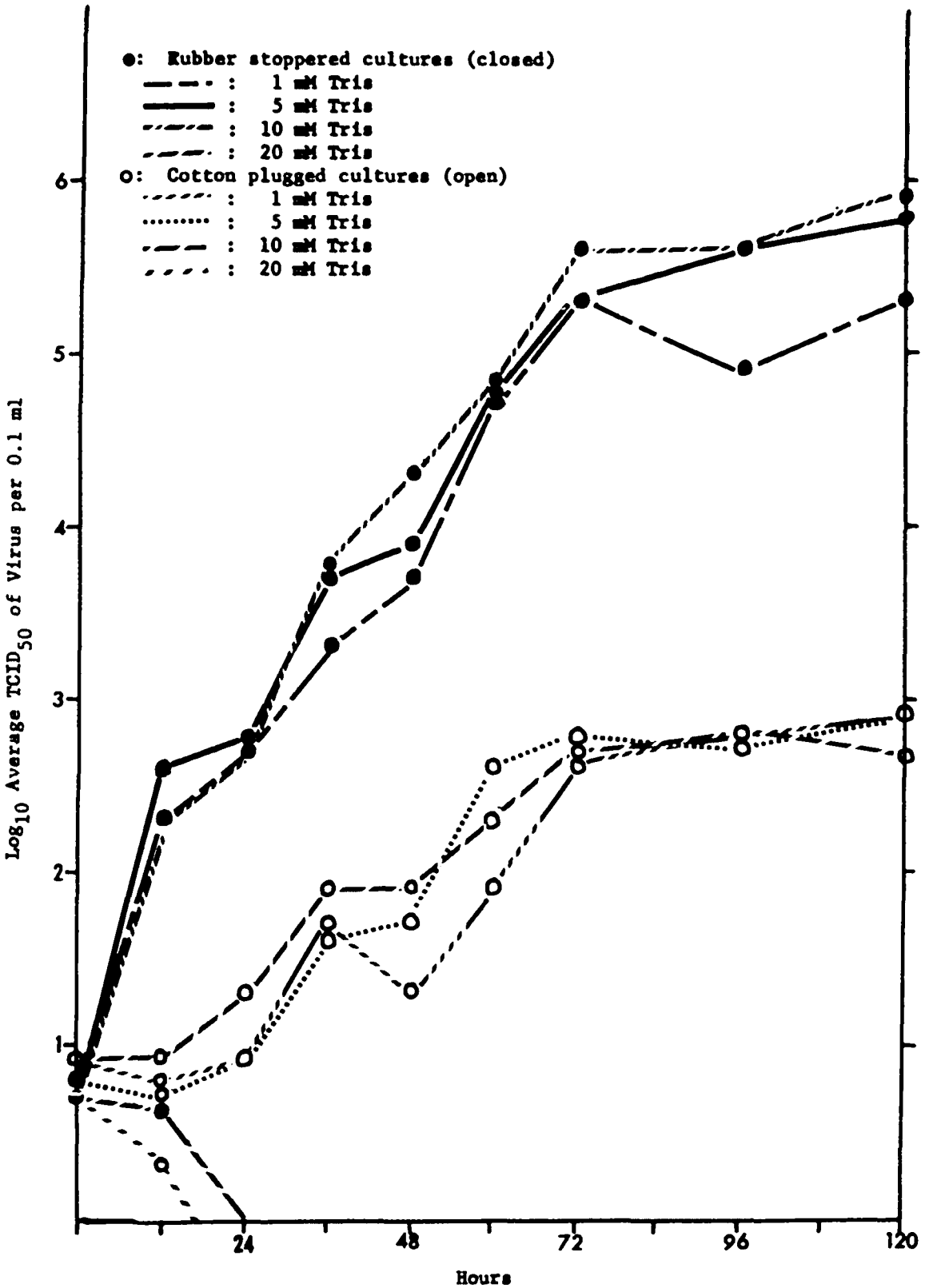


Fig. 6. Effect of Tris on vaccinia virus (D strain) production by HeLa cells in open and closed cultures.

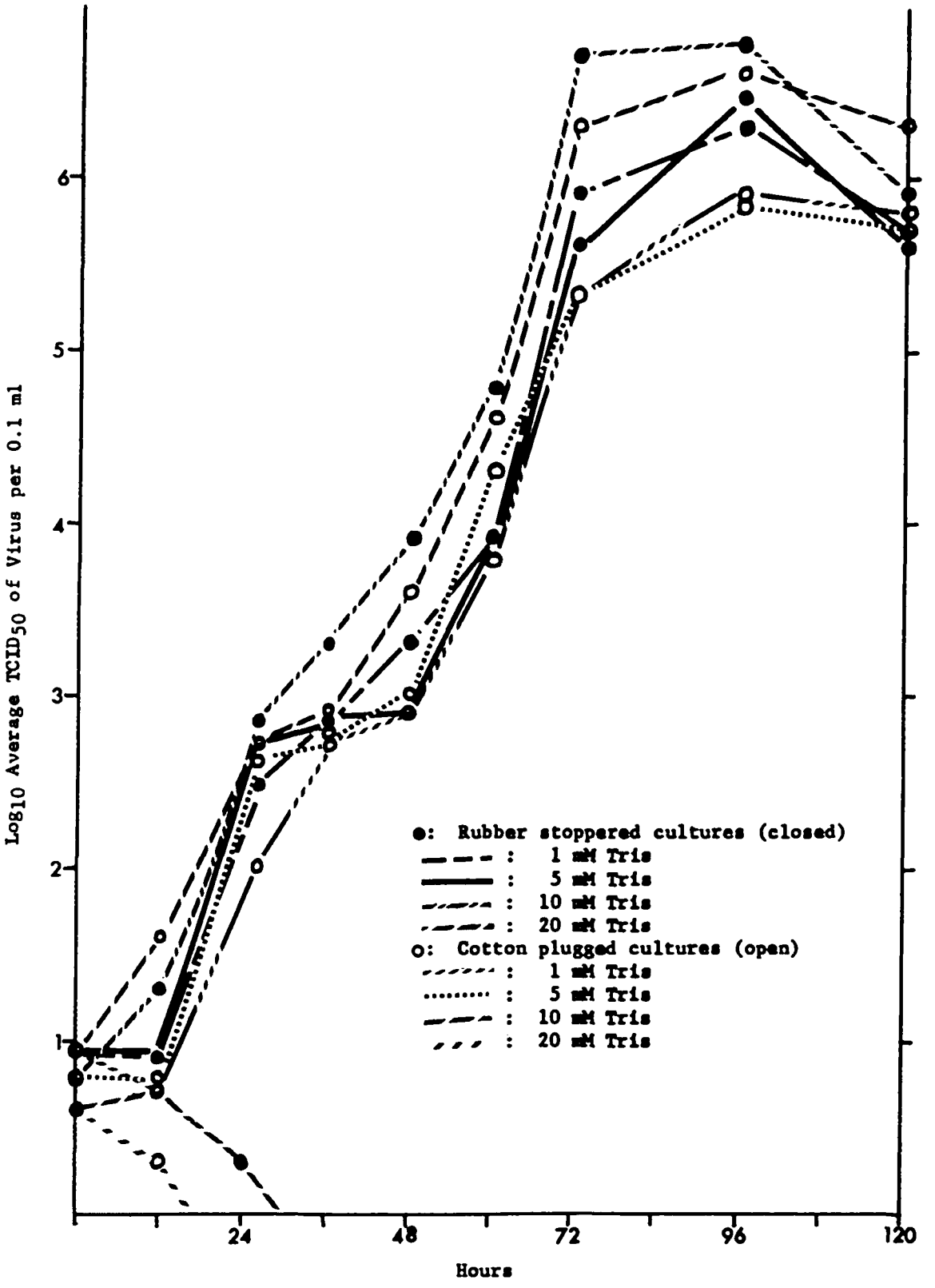


Fig. 7. Effect of Tris on herpes simplex virus (HF strain) production by HeLa cells in open and closed cultures.

TABLE 24

EFFECT OF TRIS ON VIRUS INFECTIVITY IN HELA CELLS

Virus	Tris 10 mM[a]	No Tris
Herpes simplex[b]	6.2[c]	6.4
Herpes simplex[b]	6.2	6.4
Vaccinia[d]	6.6	6.7
Vaccinia[d]	6.5	6.5

[a]Virus diluent was Dulbecco's phosphate buffered saline which contained five per cent heat inactivated serum. Tris (10 mM) was added to one diluent.

[b]HF strain, pool #4.

[c]$TCID_{50}$ expressed as positive $\log_{10}$ of dilution per 0.1 ml.

[d]D strain, pool #1.

been the manner in which the culture tubes had been closed, i.e., with cotton plugs or rubber stoppers. The most obvious distinction between these two types of cultures would be the difference in gaseous exchange. In a tightly sealed (rubber-stoppered) culture any metabolically-produced volatile substance would accumulate. When cultures were opened to the normal atmospheric conditions such as was the case with cotton plugs, any gaseous substance would rapidly diffuse into the surrounding environment.

Effect of Carbon Dioxide Deficiency on Cellular Proliferation and Virus Replication

The next experiments were designed to test the effect of CO_2 deficiency on the growth of HeLa cells, and on the replication of vaccinia and herpes simplex viruses by HeLa cells in bicarbonate-depleted cultures. Four cultural conditions were compared. Series of replicate HeLa cell cultures were initiated and maintained in the usual manner for the first 48 hours. After aspiration of the initial growth medium, an experimental medium which contained either 20 mM bicarbonate or 10 mM Tris was added to selected groups of cultures. In earlier studies, the 10 mM concentration of Tris was found to be optimal for pH control in closed cultures without adversely affecting host cell development or virus replication. A medium containing 20 mM bicarbonate, although not optimal for either growth of cells or virus replication, was chosen for the proposed studies because in it pH values were maintained above 7.0. Furthermore, this concentration of bicarbonate was similar to that routinely used in virus maintenance media. All cultures receiving bicarbonate-buffered media were sealed with rubber stoppers. Sets of similar,

Tris-containing cultures were incubated as (a) closed cultures, (b) open cultures, and (c) closed cultures with a KOH trap to absorb metabolically-produced carbon dioxide. Once these conditions were established, all cultures were maintained on roller drums at 36 C for the remainder of the experiments. Viable cell counts were determined on two or three cultures daily, and at the same time the medium was changed on all remaining tubes. Forty-eight hours after experimental conditions were begun, diluted virus suspensions were added to all cultures except those of each set which were left uninfected in order that viable cell counts could be continued throughout the study. These experiments utilized two strains of herpes simplex and two strains of vaccinia virus. The amounts of virus used, plus methods used for inoculation, adsorption, and assay of virus, were similar to those in earlier experiments. Data concerning growth and development of HeLa cells prior to institution of experimental conditions are presented in Table 25. One of the experiments is graphically illustrated in Figure 8. From the number of viable cells, it is evident that initially the cultures developed normally. Once the experimental conditions were instituted (Table 26), cultures grown in bicarbonate- or Tris-buffered media and incubated in closed systems continued to develop normally. In Tris-buffered cultures incubated in open tubes or in closed tubes with CO_2 traps, the number of cells diminished after 24 hours, but, thereafter, viable cell counts remained constant for the duration of the experiments. No significant differences in viable cell counts were noted between the open cultures and the closed cultures with CO_2 traps. In cultures in which the cells continued to grow it was impossible to distinguish morphologically

TABLE 25

HELA CELL DEVELOPMENT IN GROWTH MEDIUM[a]

Experiment Number	Cell Counts by Hours[b]		
	-48[c]	-24[d]	0[e]
1	3.5	3.5	5.8
2	3.8	3.7	6.0
3	3.8	3.4	5.6
4	4.0	3.9	6.2

[a]Minimum essential medium (Eagle)-modified (MEM-M) was used. All cultures initiated in rubber stoppered tubes.

[b]Hours before experimental conditions initiated.

[c]Cells incubated in stationary cultures at 37 C.

[d]Cultures placed on roller drum at 36 C.

[e]Experimental conditions initiated.

[f]Cell counts x 10^5.

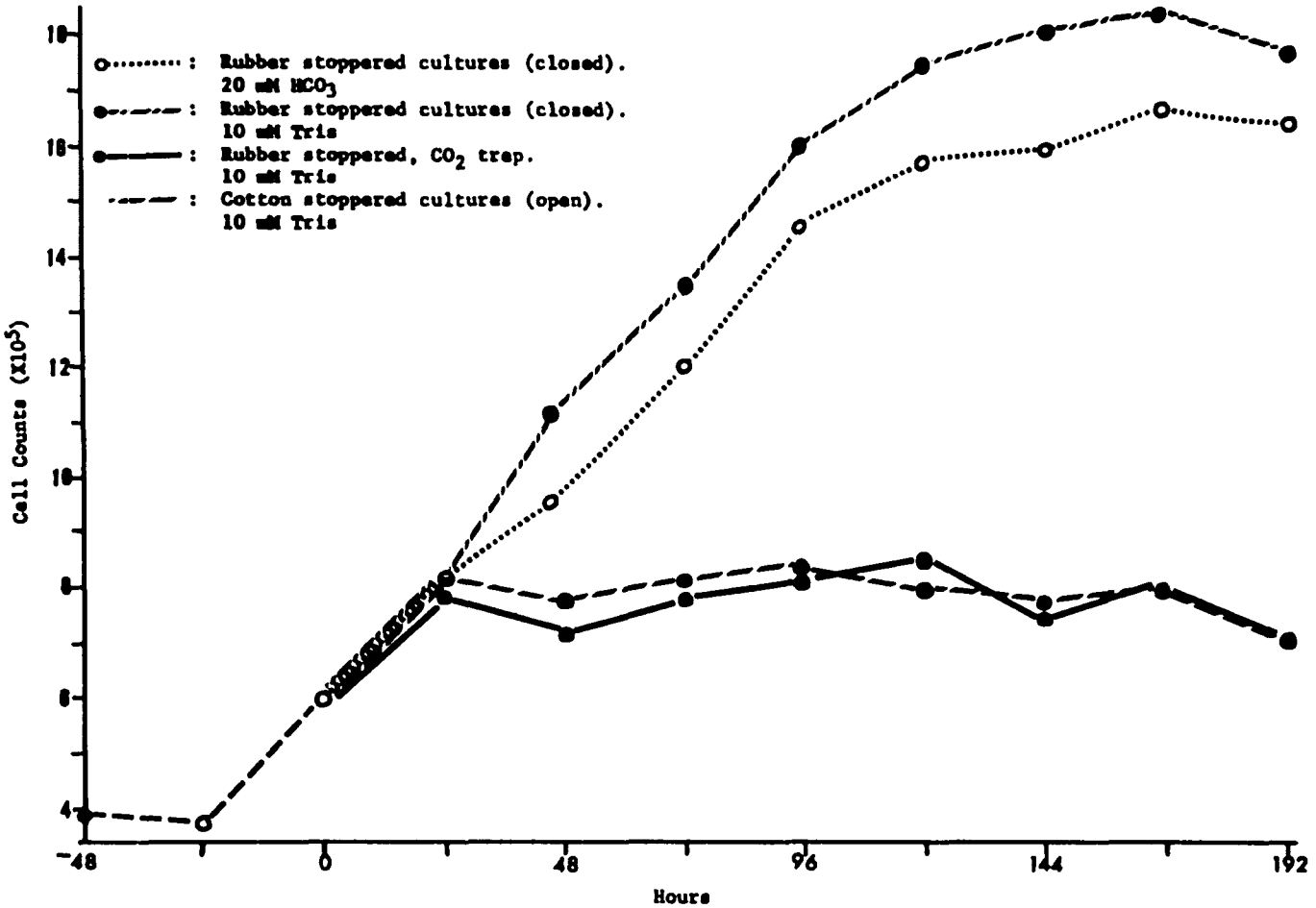


Fig. 8. Growth of HeLa cells under varying conditions.

TABLE 26

GROWTH OF HELA CELLS UNDER VARYING CONDITIONS IN MEM[a]

Hours[b]	Closed[c] Bicarbonate 20 mM	Closed Tris 10 mM	Closed Tris 10 mM CO_2 Trap[e]	Open[d] Tris 10 mM
24	7.9[f]	7.4	7.4	7.2
	8.2	8.2	7.8	8.1
	7.6	7.2	7.2	6.9
	8.6	8.0	7.9	7.6
48	10.0	11.1	7.5	7.3
	9.6	11.2	7.2	7.8
	10.2	11.9	7.6	8.2
	11.1	13.2	8.4	8.8
72	11.2	14.0	7.8	7.8
	12.1	13.5	7.8	8.2
	12.3	14.6	8.0	8.8
	13.0	15.1	8.8	9.2
96	13.1	18.1	8.2	7.5
	14.6	16.1	8.2	8.4
	14.2	17.3	7.8	8.5
	14.9	17.8	8.5	8.9
120	14.4	17.6	8.0	8.3
	15.8	17.6	8.6	8.0
	15.6	16.8	8.4	8.3
	16.2	18.2	8.2	9.0
144	14.6	17.2	7.6	8.0
	16.2	18.2	7.5	7.8
	17.0	17.4	8.2	7.4
	16.8	18.5	7.6	8.2
168	14.4	17.0	7.5	7.6
	16.8	18.5	8.0	8.0
	17.6	18.1	8.2	7.8
	17.0	18.0	7.1	7.6
192	14.8	16.6	7.0	6.8
	16.5	17.8	7.2	7.2
	16.8	17.6	7.6	6.5
	17.4	17.2	7.2	7.2

TABLE 26--Continued

[a]Minimum essential medium (Eagle)-modified (MEM-M) was used. all cultures incubated on roller drums at 36 C.

[b]Hours after experimental conditions were initiated.

[c]Rubber stoppered cultures.

[d]Cotton plugged cultures.

[e]Potassium hydroxide CO_2 trap.

[f]Cell counts x 10^5.

between bicarbonate- and Tris-buffered cultures. Similarly, the cells of cultures in which growth ceased could not be differentiated morphologically.

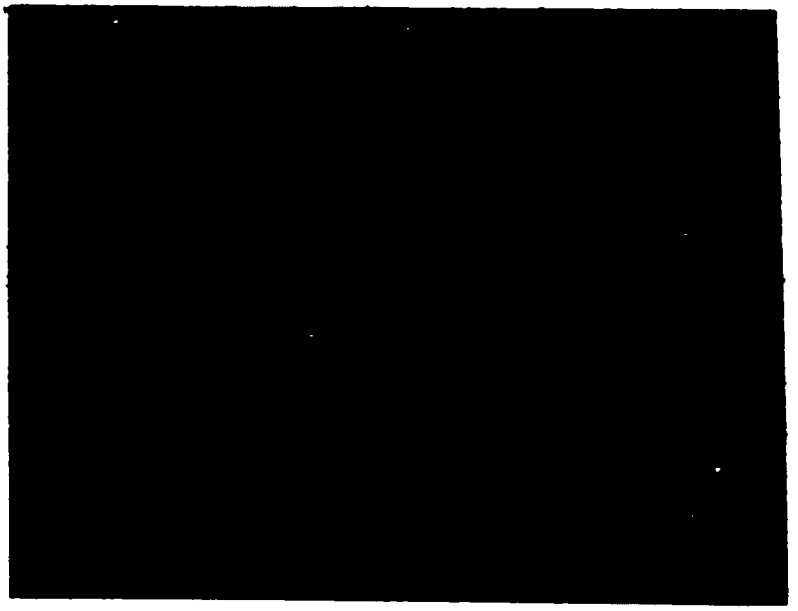
It had been noted earlier that there were differences in the cytopathic effects produced by virus activity in Tris- or bicarbonate-buffered media. Uninfected HeLa cells grown in closed cultures in a medium containing 20 mM bicarbonate and in a medium containing 10 mM Tris are shown in Plate I. Similar cells grown in 10 mM Tris in an open culture are also shown.

Plate II presents photomicrographs of HeLa cells inoculated with the two herpes simplex virus strains and grown in closed cultures. In bicarbonate-containing media, HF herpes simplex virus produced characteristic cytopathic changes. Infected HeLa cells appeared to lose their individual identities and merged to form large syncytia and multinucleated giant cells, as described by Gray et al. (1958). In Tris-buffered media, regardless of the conditions of incubation, the infected cells did not lose their individualities; rather the cell outlines appeared more distinct than the neighboring uninfected cells. Formation of giant cells and large syncytia was not observed. The M strain of herpes simplex virus produced the proliferative type of CPE, which consisted of areas of rounded, clumped cells with a three dimensional effect (Gray et al., 1958). Very little change from this type of CPE was noted in Tris media.

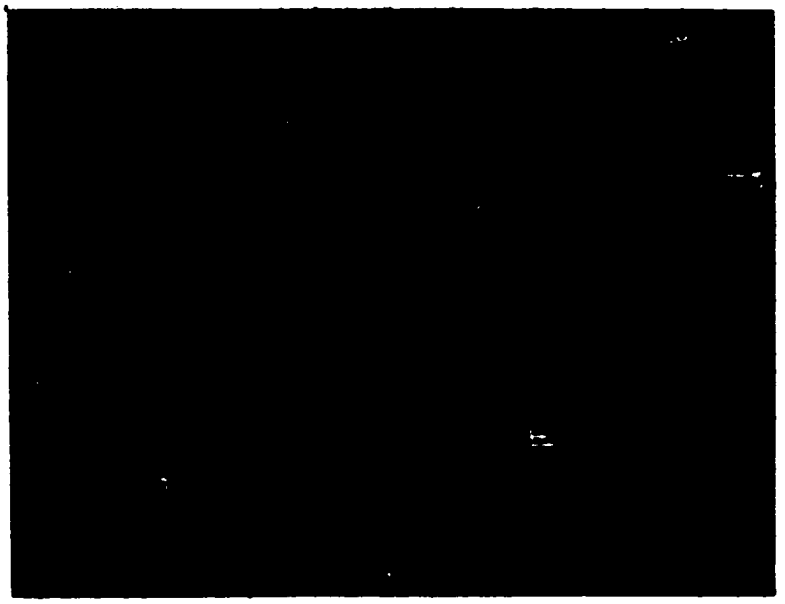
The type of CPE produced by the D and O strains of vaccinia virus were quite similar. Photomicrographs of vaccinia virus-infected HeLa cells grown in closed cultures in a bicarbonate-buffered medium

PLATE I

UNINFECTED HELA CELLS CULTIVATED UNDER VARYING CONDITIONS



Ia. HeLa cells in Eagle's minimum essential medium containing 20 mM bicarbonate incubated for four days as closed cultures (X 120).



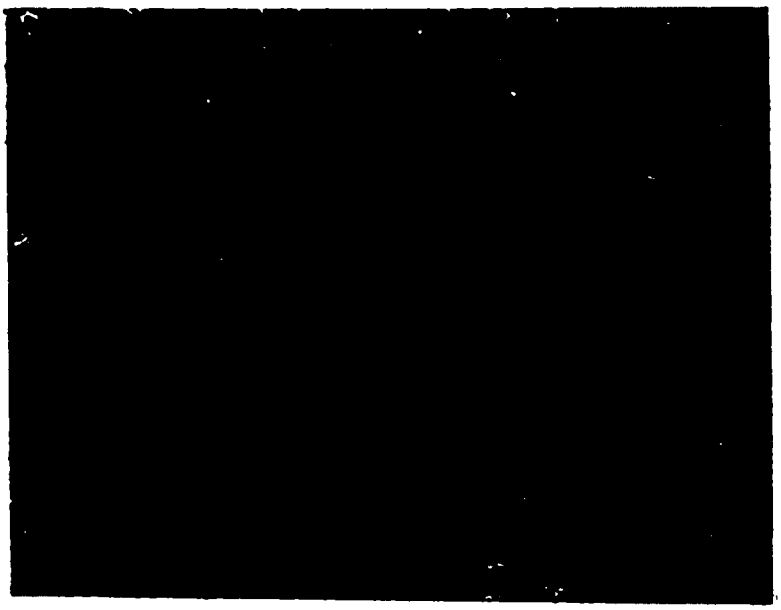
Ib. HeLa cells in Eagle's minimum essential medium containing 10 mM Tris incubated for four days as closed cultures (X 120).

PLATE I--Continued

Ic. HeLa cells in Eagle's minimum essential medium containing 10 mM Tris incubated for four days as open cultures (X 120).

PLATE II

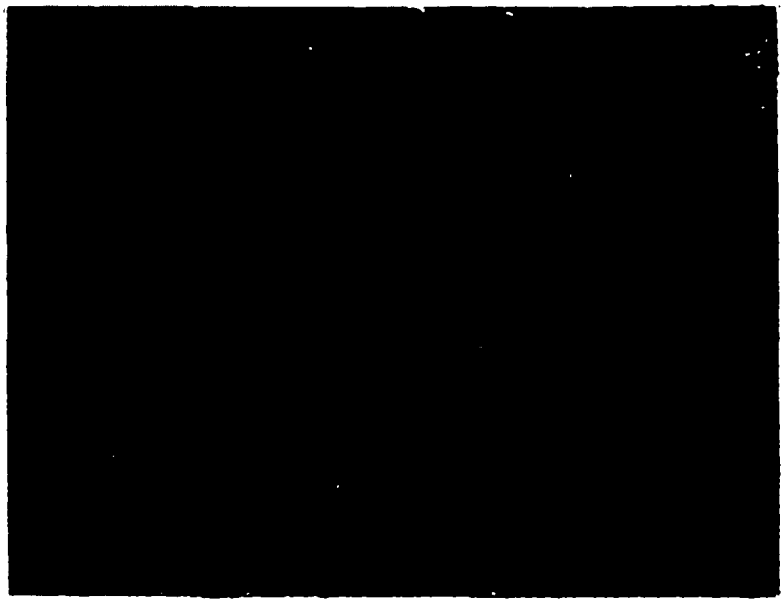
HELA CELLS INFECTED WITH HERPES SIMPLEX VIRUS



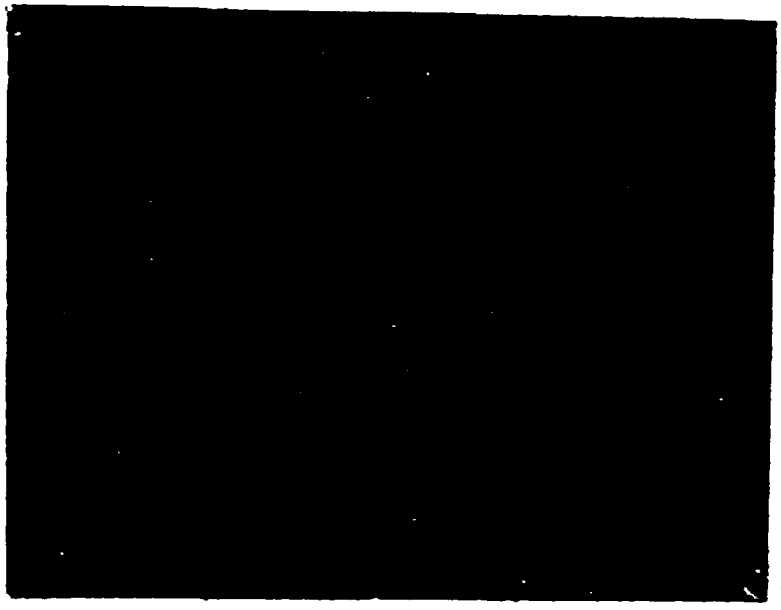
IIa. Cells infected with HF strain, maintained in Eagle's minimum essential medium containing 20 mM bicarbonate and incubated as closed cultures for two days (X 120).

IIb. Cells infected with HF strain, maintained in Eagle's minimum essential medium containing 10 mM Tris and incubated as closed cultures for two days (X 120).

PLATE II--Continued



IIc. Cells infected with M strain, maintained in Eagle's minimum essential medium containing 20 mM bicarbonate and incubated as closed cultures for two days (X 120).



IId. Cells infected with M strain, maintained in Eagle's minimum essential medium containing 10 mM Tris and incubated as closed cultures for two days (X 120).

and in a Tris-buffered medium are shown in Plate III. In bicarbonate-containing media, compact nodules formed; the area of infected cells became dense, piled up, and sloughed off of the glass. Conversely, in Tris-buffered media the infected areas were not compressed, but spread over well delineated areas. Plaques formed and large holes in the cell sheet were microscopically discernable.

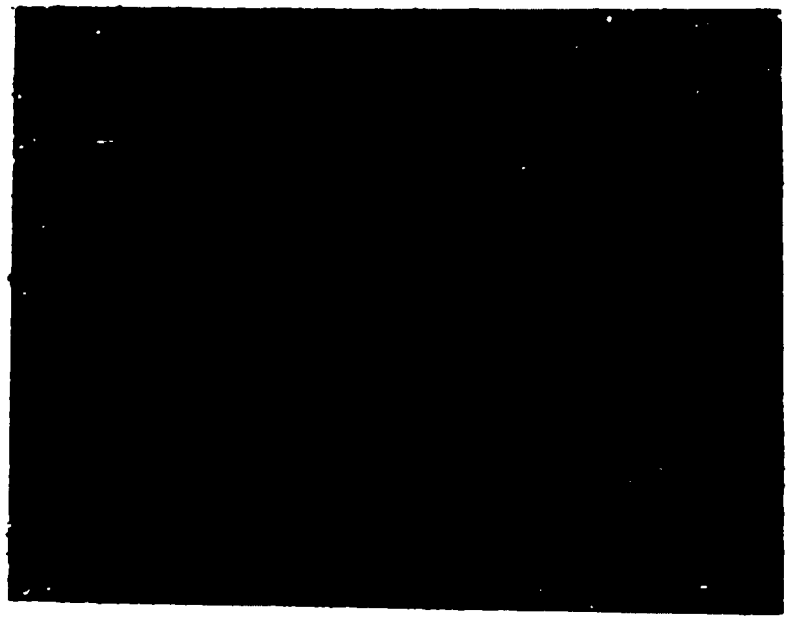
The development of the four viruses in relation to the numbers of viable cells contained in comparable, uninfected cultures is presented in Tables 27 to 30. Data from Tables 27 and 28 are represented graphically in Figures 9 and 10 for D and O vaccinia virus, respectively. Results show that optimal numbers of viable cells and optimal virus titers were consistently obtained in closed cultures. Virus titers and cell numbers were significantly lower in cultures grown under conditions of CO_2 deficiency, i.e., in open cultures or closed cultures with CO_2 traps.

In Figure 11 some of the data from Tables 27 and 28 are combined in order to visualize better the similarities of behavior of two strains of vaccinia virus under the different conditions of incubation. Their significantly reduced titers under conditions of CO_2 deficiency also can be noted.

Results with the two strains of herpes simplex virus are shown in Tables 29 and 30 and graphically illustrated in Figures 12, 13, and 14. Both of these strains were similarly affected by various conditions of incubation. Closed cultures created optimal conditions for cell replication and virus proliferation. The striking dissimilarity between the behavior of the herpes simplex and the vaccinia viruses becomes apparent upon examination of the data concerning open cultures and closed

PLATE III

CELLS INFECTED WITH VACCINIA VIRUS



IIIa. Cells infected with O strain, maintained in Eagle's minimum essential medium containing 20 mM bicarbonate and incubated as closed cultures for two days (X 120).

IIIb. Cells infected with O strain, maintained in Eagle's minimum essential medium containing 10 mM Tris and incubated as closed cultures for two days (X 120).

TABLE 27

VACCINIA VIRUS (D STRAIN) PRODUCTION IN HELA CELLS GROWN UNDER VARYING CONDITIONS

Hours[a]		Closed[b] Bicarbonate[d] 20 mM	Closed Tris 10 mM	Closed Tris 10 mM CO_2 Trap[e]	Open[c] Tris 10 mM
0	$TCID_{50}$[f]	0.8	0.6	1.2	1.2
	CC[g]	9.6	11.2	7.2	7.8
12	$TCID_{50}$	2.4	2.4	0.6	0.8
	CC	-	-	-	-
24	$TCID_{50}$	2.5	2.8	1.5	1.5
	CC	12.1	13.5	7.8	8.2
36	$TCID_{50}$	3.5	3.6	1.8	2.2
	CC	-	-	-	-
48	$TCID_{50}$	3.8	4.2	2.6	2.5
	CC	14.6	16.1	8.2	8.4
60	$TCID_{50}$	4.3	4.7	2.8	2.8
	CC	-	-	-	-
72	$TCID_{50}$	4.5	5.4	3.2	3.5
	CC	15.8	17.6	8.6	8.0
96	$TCID_{50}$	5.5	5.4	3.6	3.8
	CC	16.2	18.2	7.5	7.8
120	$TCID_{50}$	5.4	5.8	4.4	4.5
	CC	16.8	18.5	8.0	8.0

[a]Hours after experimental conditions initiated.

[b]Rubber stoppered cultures.

[c]Cotton plugged cultures.

[d]Minimum essential medium (Eagle)-modified (MEM-M) was used. All cultures incubated at 36 C on roller drum.

[e]Potassium hydroxide CO_2 trap.

[f]$TCID_{50}$ expressed as positive $\log_{10}$ of the dilution per 0.1 ml.

[g]Cell count x 10^5.

TABLE 28

VACCINIA VIRUS (O STRAIN) PRODUCTION IN HELA CELLS GROWN UNDER VARYING CONDITIONS

Hours[a]		Closed[b] Bicarbonate[d] 20 mM	Closed Tris 10 mM	Closed Tris 10 mM CO_2 Trap[e]	Open[c] Tris 10 mM
0	$TCID_{50}$[f]	0.6	0.5	1.2	0.8
	CC[g]	11.1	13.2	8.4	8.8
12	$TCID_{50}$	2.2	2.5	0.8	1.0
	CC	-	-	-	-
24	$TCID_{50}$	4.2	3.8	1.6	2.0
	CC	13.0	15.1	8.8	9.2
36	$TCID_{50}$	4.4	4.5	1.8	2.6
	CC	-	-	-	-
48	$TCID_{50}$	4.6	4.8	2.4	2.5
	CC	14.9	17.8	8.5	8.9
60	$TCID_{50}$	5.2	5.2	2.6	2.8
	CC	-	-	-	-
72	$TCID_{50}$	5.4	5.5	3.2	3.5
	CC	16.2	18.2	8.2	9.0
96	$TCID_{50}$	6.5	5.8	3.5	3.8
	CC	16.8	18.5	7.6	8.2
120	$TCID_{50}$	6.8	6.3	4.6	4.8
	CC	17.0	18.0	7.1	7.6

[a]Hours after experimental conditions initiated.

[b]Rubber stoppered cultures.

[c]Cotton plugged cultures.

[d]Minimum essential medium (Eagle)-modified (MEM-M) was used. All cultures incubated on roller drum at 36 C.

[e]Potassium hydroxide CO_2 trap.

[f]$TCID_{50}$ expressed as positive $\log_{10}$ of the dilution per 0.1 ml.

[g]Cell count x 10^5.

TABLE 29

HERPES SIMPLEX VIRUS (HF STRAIN) PRODUCTION IN HELA CELLS GROWN UNDER VARYING CONDITIONS

Hours[a]		Closed[b] Bicarbonate[d] 20 mM	Closed Tris 10 mM	Closed Tris 10 mM CO_2 Trap[e]	Open[c] Tris 10 mM
0	$TCID_{50}$[f]	0.6	0.6	1.2	0.8
	CC[g]	10.0	11.1	7.5	7.3
12	$TCID_{50}$	1.6	2.2	1.2	1.5
	CC	-	-	-	-
24	$TCID_{50}$	3.2	3.8	3.5	3.4
	CC	11.2	14.0	7.8	7.8
36	$TCID_{50}$	4.3	4.6	4.4	4.2
	CC	-	-	-	-
48	$TCID_{50}$	5.2	5.5	5.6	5.2
	CC	13.1	16.1	8.2	7.5
60	$TCID_{50}$	5.6	6.4	6.6	6.2
	CC	-	-	-	-
72	$TCID_{50}$	5.8	6.8	6.5	6.2
	CC	14.4	17.6	8.0	8.3

[a]Hours after experimental conditions initiated.

[b]Rubber stoppered cultures.

[c]Cotton plugged cultures.

[d]Minimum essential medium (Eagle)-modified (MEM-M) was used. All cultures incubated on roller drum at 36 C.

[e]Potassium hydroxide CO_2 trap.

[f]$TCID_{50}$ expressed as positive $\log_{10}$ of the dilution per 0.1 ml.

[g]Cell count x 10^5.

TABLE 30

HERPES SIMPLEX VIRUS (M STRAIN) PRODUCTION IN HELA CELLS GROWN UNDER VARYING CONDITIONS

Hours[a]		Closed[b] Bicarbonate[d] 20 mM	Closed Tris 10 mM	Closed Tris 10 mM CO_2 Trap[e]	Open[c] Tris 10 mM
0	$TCID_{50}$[f]	1.0	1.2	1.4	1.2
	CC[g]	10.2	11.9	7.6	8.2
12	$TCID_{50}$	2.2	2.4	2.2	1.8
	CC	-	-	-	-
24	$TCID_{50}$	3.8	4.2	4.2	3.6
	CC	12.3	14.6	14.6	8.8
36	$TCID_{50}$	4.4	4.8	4.8	4.2
	CC	-	-	-	-
48	$TCID_{50}$	5.8	5.6	5.6	5.5
	CC	14.2	17.3	7.8	8.5
60	$TCID_{50}$	6.2	6.4	6.4	5.8
	CC	-	-	-	-
72	$TCID_{50}$	6.8	6.8	6.5	6.4
	CC	15.6	16.8	8.4	8.3
96	$TCID_{50}$	6.5	6.4	6.6	6.4
	CC	17.0	17.4	8.2	7.4

[a]Hours after experimental conditions initiated.

[b]Rubber stoppered cultures.

[c]Cotton plugged cultures.

[d]Minimum essential medium (Eagle)-modified (MEM-M) was used. All cultures incubated on roller drum at 36 C.

[e]Potassium hydroxide CO_2 trap.

[f]$TCID_{50}$ expressed as the positive $\log_{10}$ of the dilution per 0.1 ml.

[g]Cell count x 10^5.

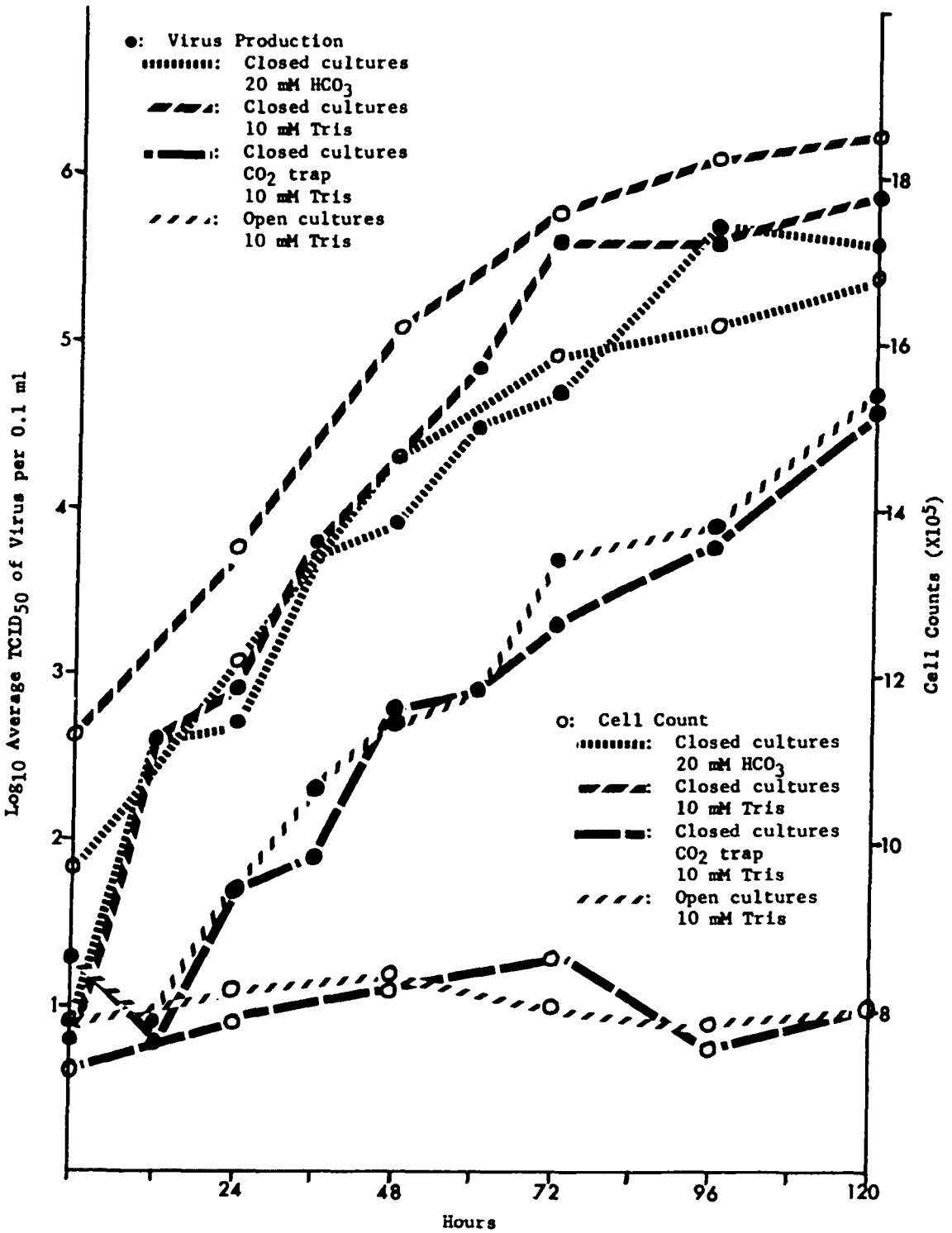


Fig. 9. Vaccinia virus (D strain) production in HeLa cells grown under varying conditions.

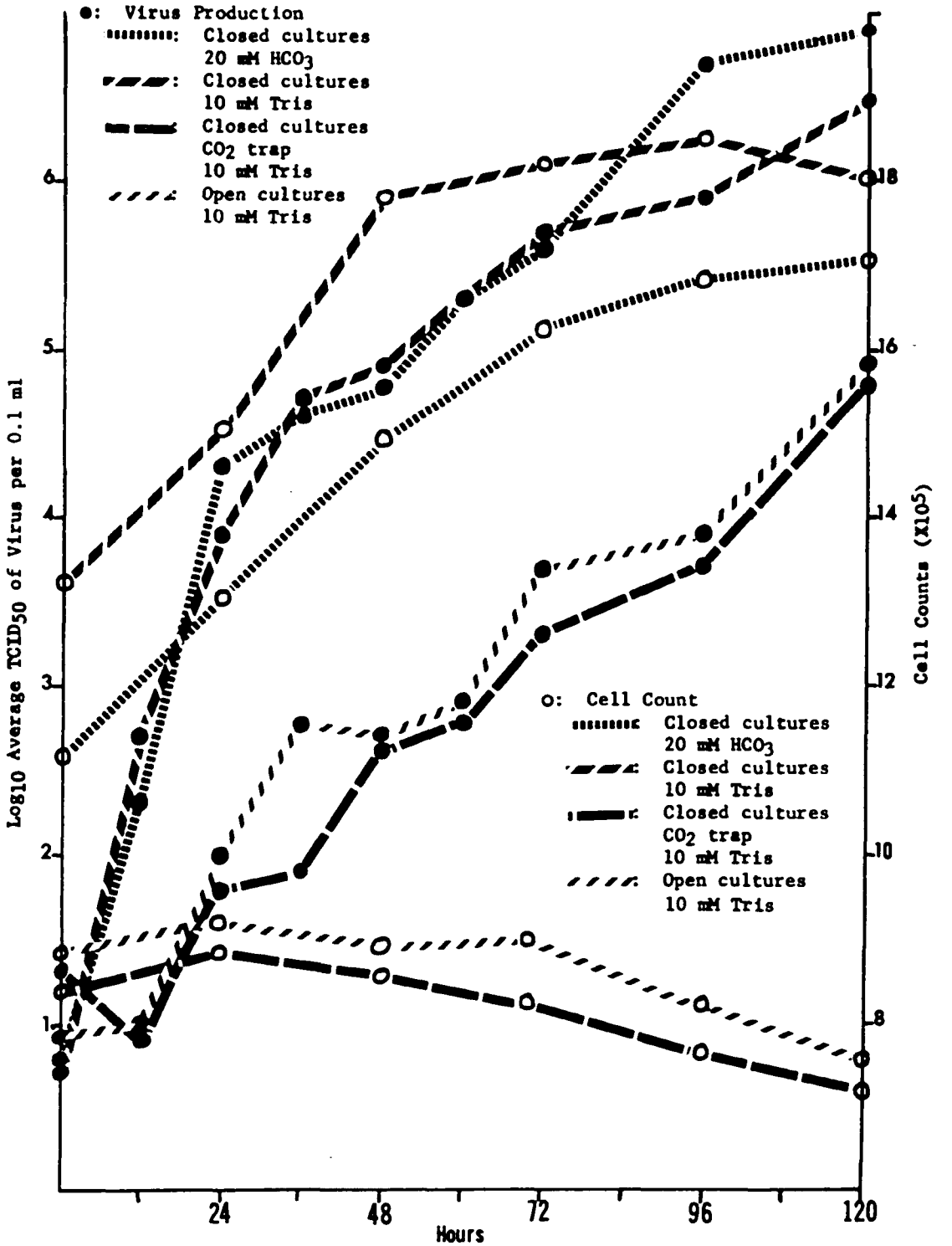


Fig. 10. Vaccinia virus (O strain) production in HeLa cells grown under varying conditions.

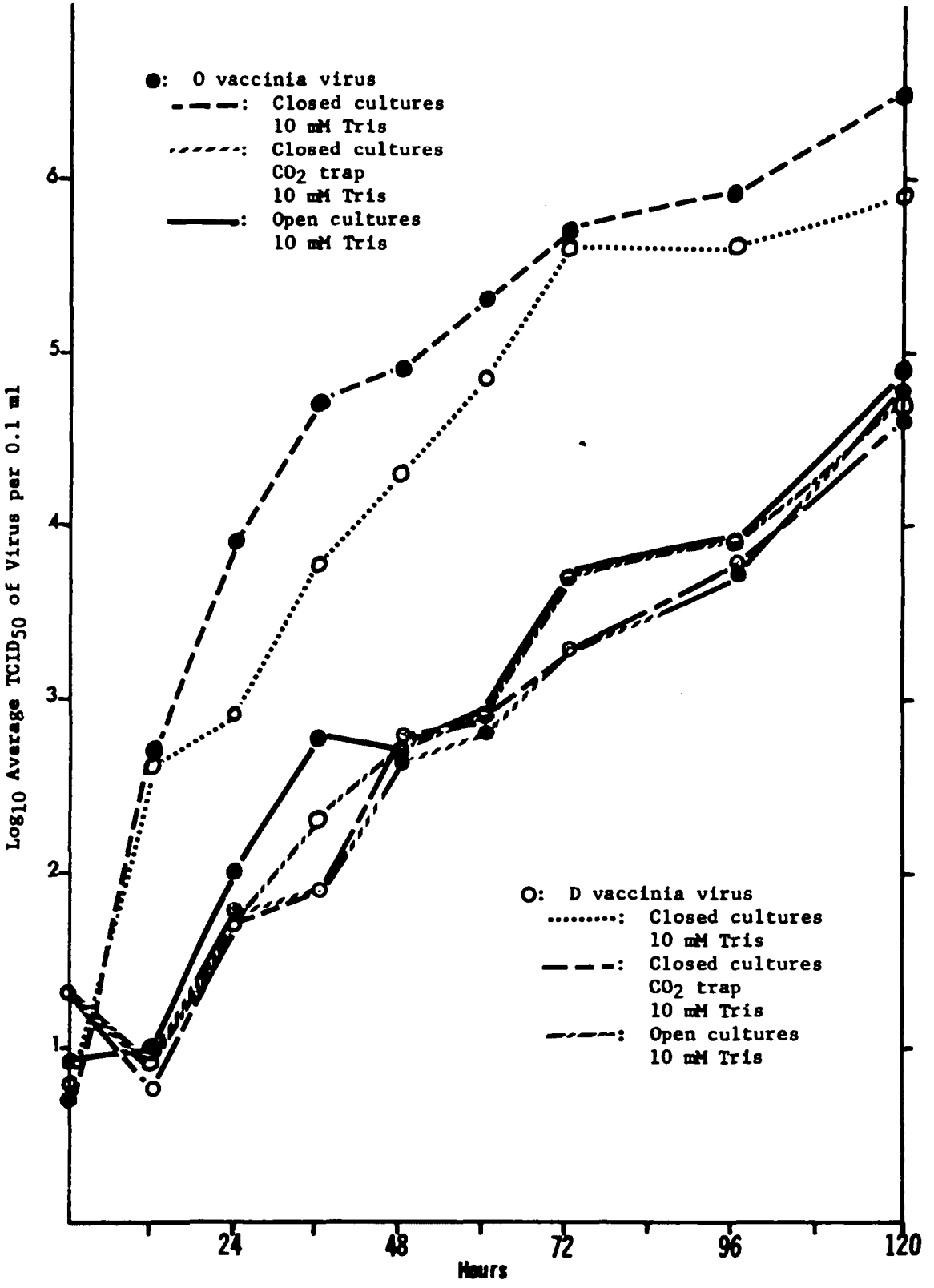


Fig. 11. A comparison of the production of D strain vaccinia virus and O strain vaccinia virus in HeLa cells grown in open and closed cultures.

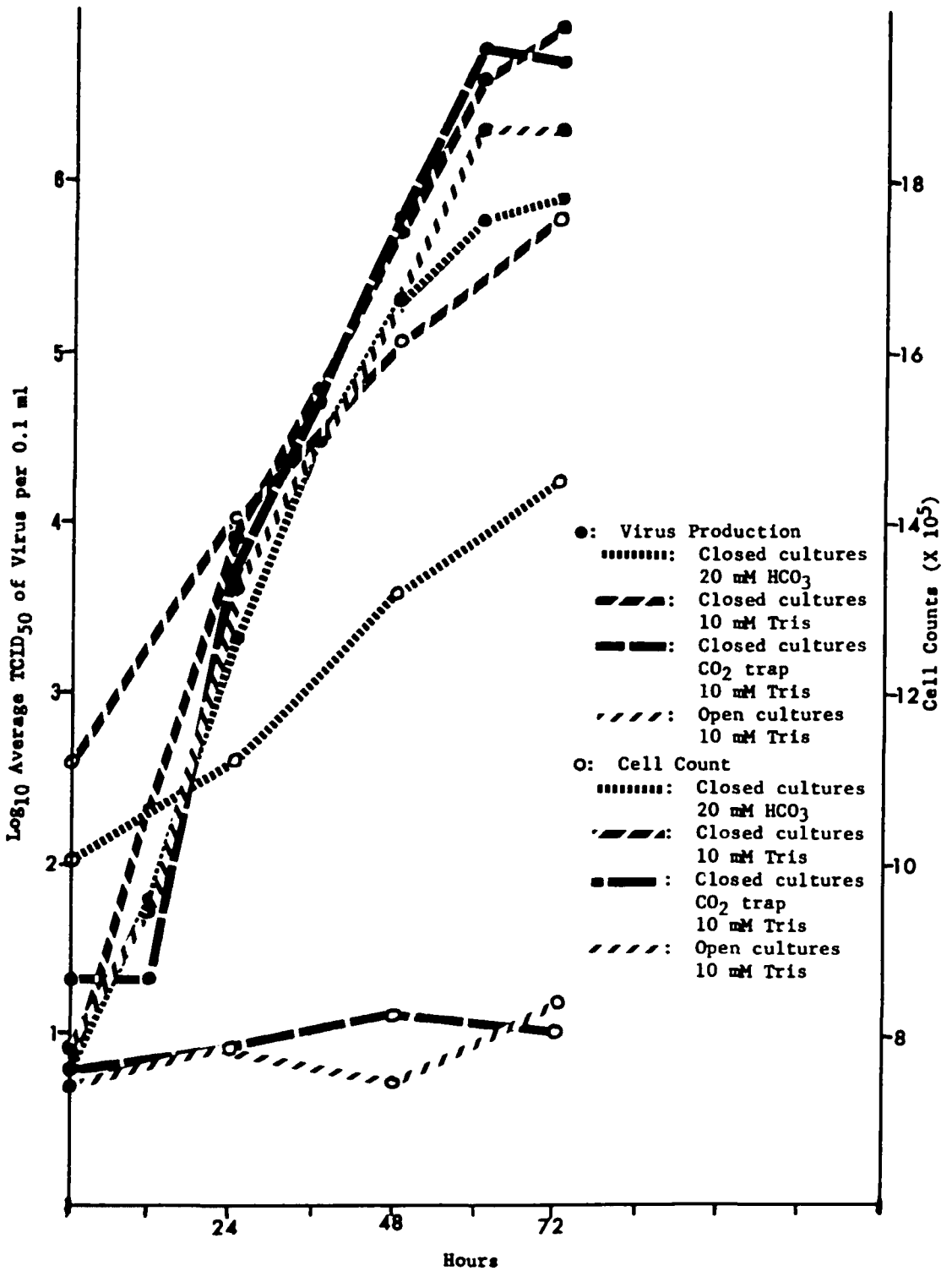


Fig. 12. Herpes simplex virus (HF strain) production in HeLa cells grown under varying conditions.

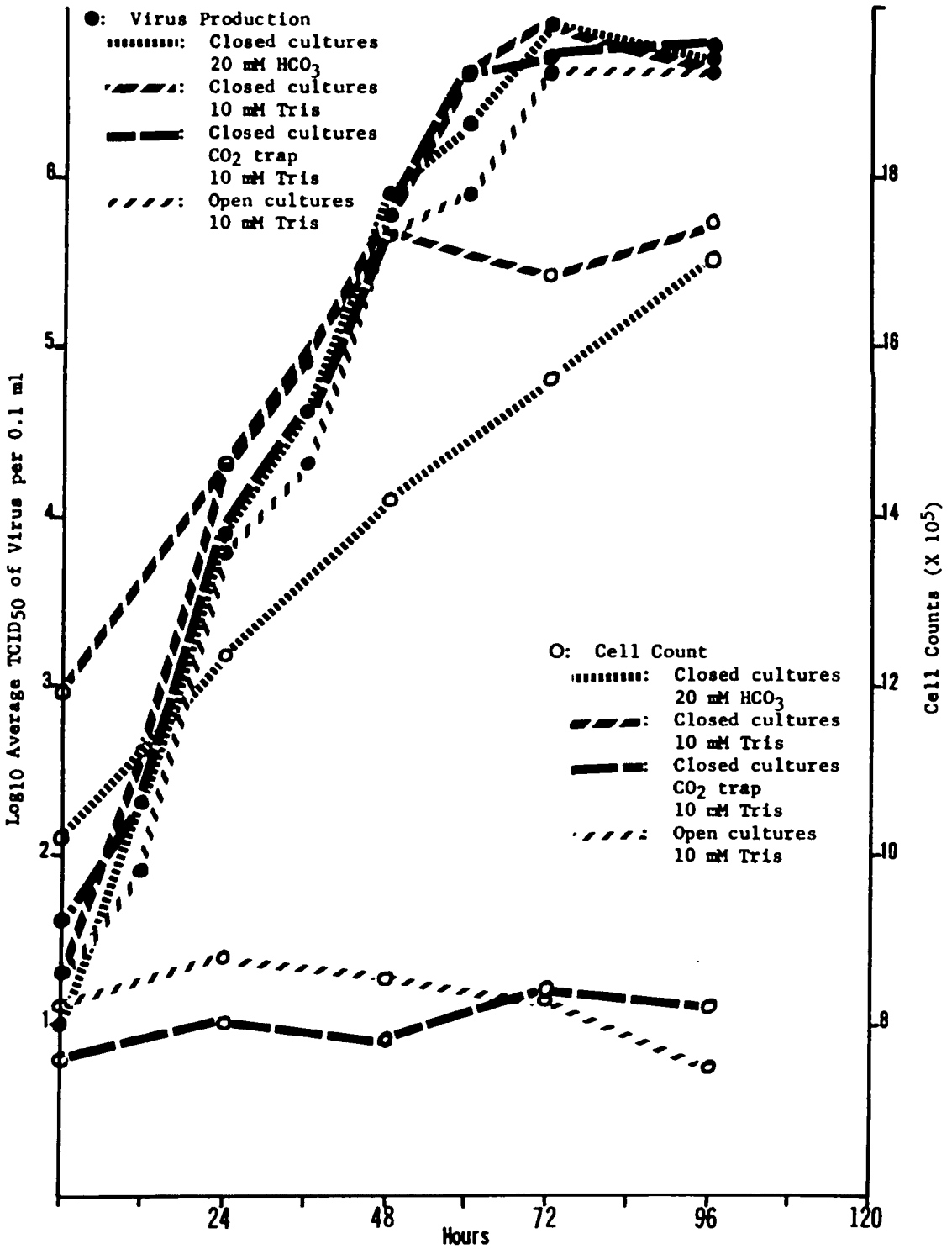


Fig. 13. Herpes simplex virus (M strain) production in HeLa cells grown under varying conditions.

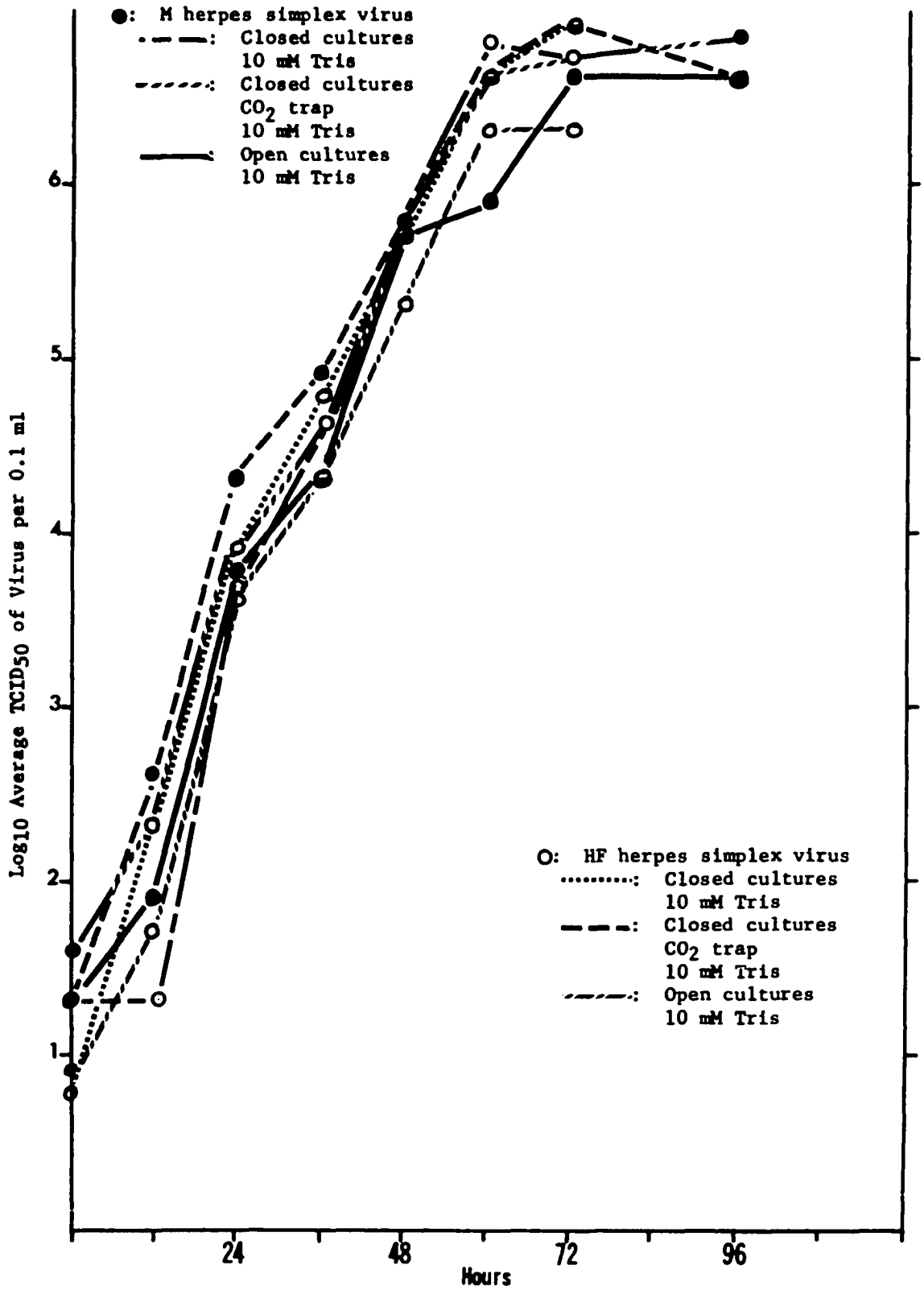


Fig. 14. A comparison of the production of HF strain herpes simplex virus and M strain herpes simplex virus in HeLa cells grown in open and closed cultures.

cultures with potassium hydroxide traps. Figure 15 depicts the titers of D vaccinia and HF herpes simplex viruses in closed cultures, closed cultures plus potassium hydroxide traps, and open cultures in which there was free gaseous exchange between the culture and the surrounding environment. Under the conditions of CO_2 depletion, the production of herpes simplex virus continued uninhibited whereas titers of vaccinia virus were considerably reduced.

It had been established that either creating a deficiency of CO_2 by the inclusion of a potassium hydroxide trap in closed cultures, or an open atmosphere by substituting cotton plugs for rubber stoppers resulted in cessation of HeLa cell multiplication. Chang (1959) concluded that HeLa cells were adversely affected by CO_2 depletion created by use of the potassium hydroxide trap. This treatment of cultures, which were infected with vaccinia virus strains, resulted in diminished virus titers compared with those of closed cultures. In the present studies herpes simplex virus titers were not adversely affected by these conditions of incubation. Since creating CO_2 deficiency by the substitution of cotton plugs for rubber stoppers was a much less complex procedure than the use of a CO_2 trap, the former method was chosen for use in the next experiments. If these observed differences in the production of vaccinia virus were due to CO_2 depletion there should be a correlation between the amount of virus produced and the time the cultures were cotton plugged relative to the time cells were infected.

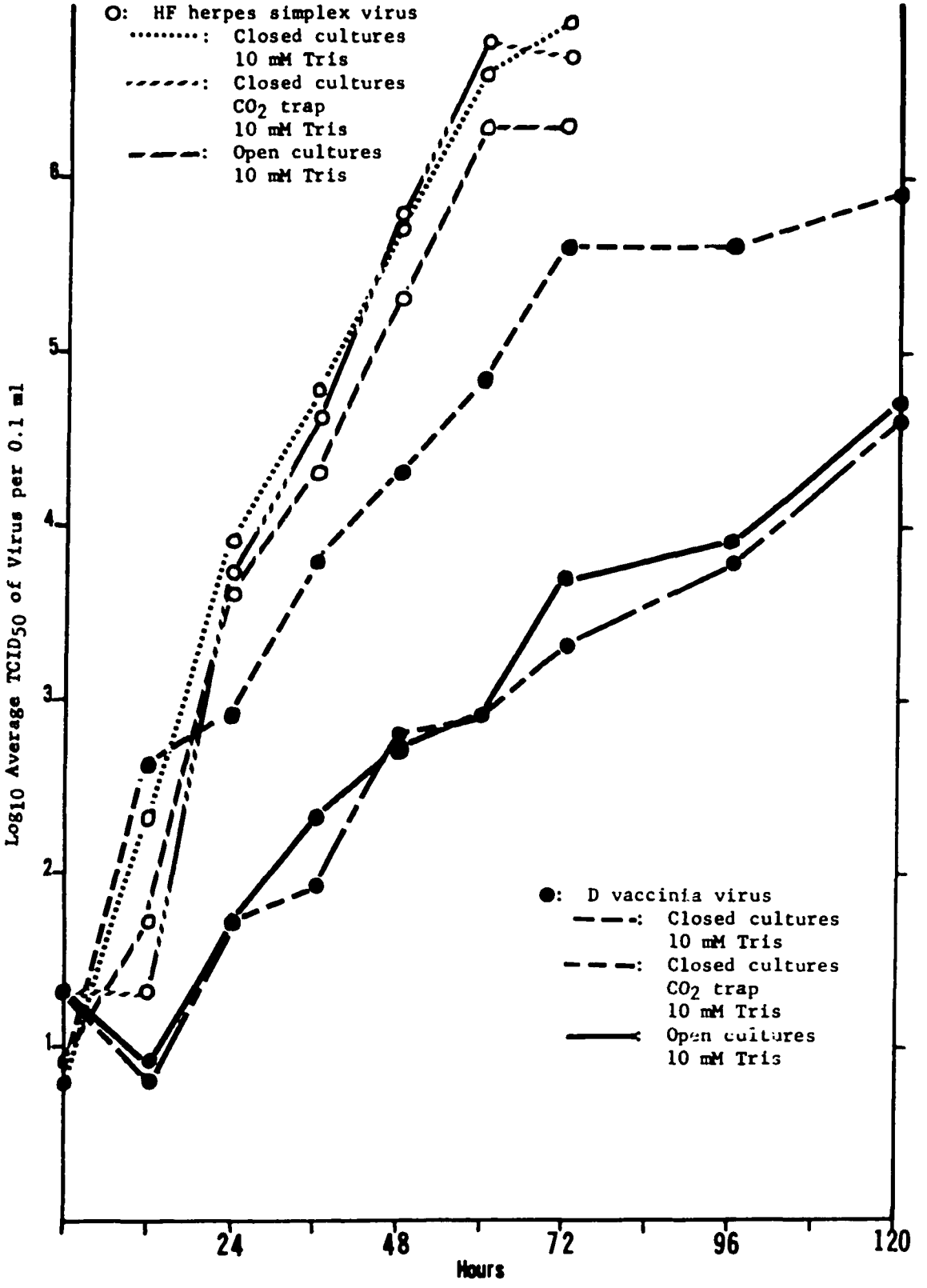


Fig. 15. A comparison of the production of D strain vaccinia virus and HF strain herpes simplex virus in HeLa cells grown in open and closed cultures.

Virus Replication in Cotton Stoppered HeLa Cell Cultures in Relation to Time When CO_2 Deficiency Was Produced

Replicate cultures were established as usual in MEM-M containing 10 mM bicarbonate. After 24 hours as stationary cultures at 37 C, the medium was changed to MEM-M containing 10 mM Tris, and the rubber stoppers of one set were replaced by cotton plugs. All cultures were placed on roller drums at 36 C for the remainder of the study; viable cell counts were performed and the medium was changed daily. Second and third sets of cultures were cotton plugged at 48 and 24 hours before the viruses were added. A fourth set was cotton plugged at zero time when the cells were infected with vaccinia or herpes simplex virus. A final control set was continued as closed cultures throughout the study. Cell counts were performed daily on uninfected cultures in each set and samples were taken from infected cultures at designated intervals for assay of infectious virus particles.

Figure 16 illustrates the time needed for inhibition of HeLa cell growth in open cultures. After the insertion of the cotton plugs a period of 24 hours was necessary before normal growth of cells was interrupted. The level at which the total number of cells became static appeared dependent on the length of time which elapsed between the seeding of the cultures and the insertion of the cotton plugs. Tables 31 and 32 present the titers of two vaccinia viruses and viable cell counts of corresponding uninfected cultures which were cotton plugged at different time intervals after infection. The replication of the vaccinia viruses is depicted in Figures 17 and 18. Similar growth patterns, maximum cellular development and virus titers were produced with

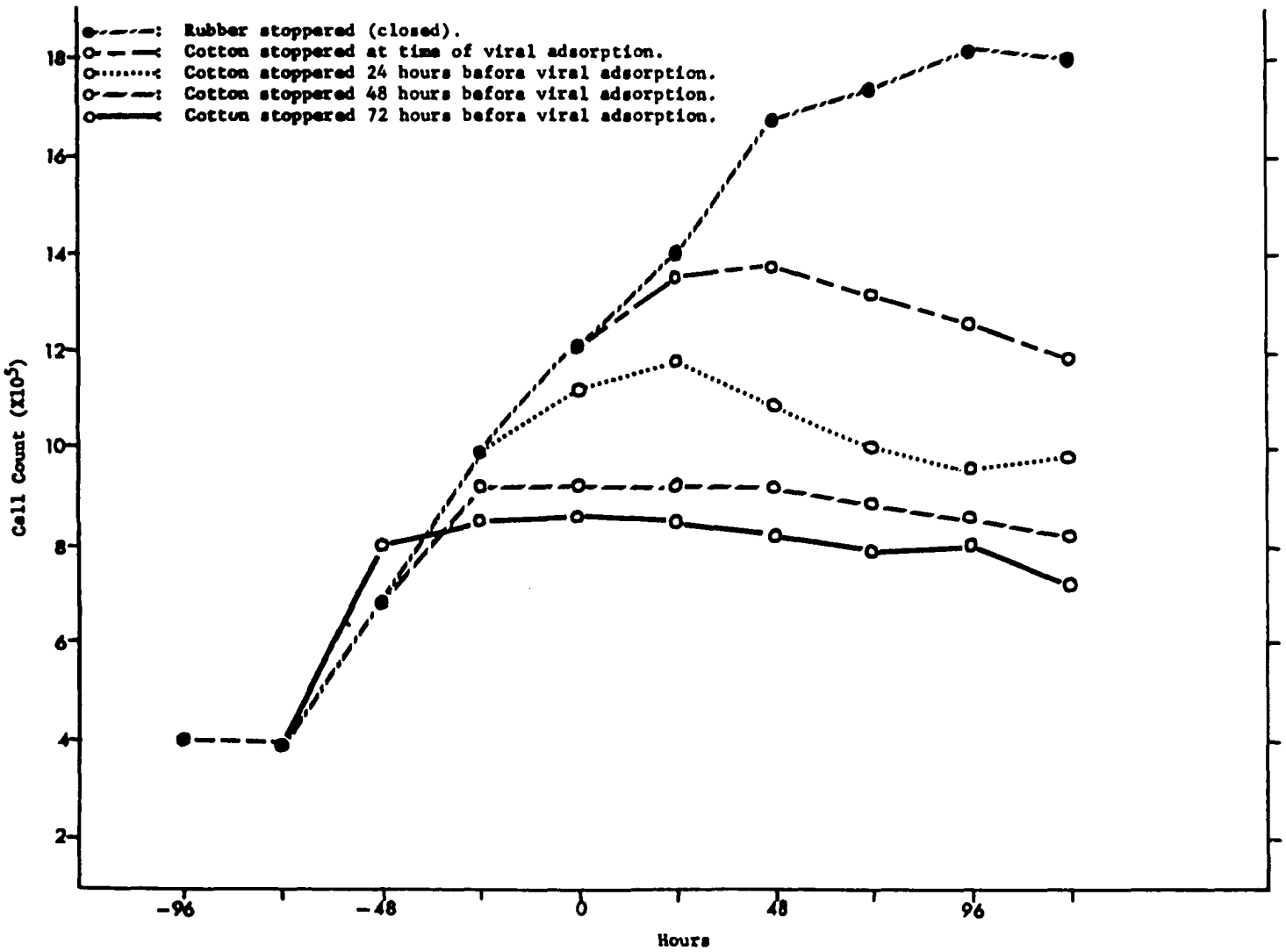


Fig. 16. Variation in the growth of HeLa cells in cotton stoppered cultures in relation to the time when a CO_2 deficiency was produced.

TABLE 31

TIME AT WHICH CREATING AN OPEN SYSTEM AFFECTS REPLICATION OF VACCINIA VIRUS (D STRAIN) IN HELA CELLS

Growth Conditions[a]		Hours after adsorption when virus samples were taken									
		0	6	12	24	36	48	60	72	96	120
Closed[b]	$TCID_{50}$[d]	0.6	1.5	2.2	2.6	3.4	4.2	4.5	5.2	5.4	5.8
	CC[e]	12.2	-	-	13.6	-	15.5	-	16.6	17.5	17.7
Opened[c] 72 hours before virus	$TCID_{50}$	0.8	0.4	0.5	1.0	1.6	2.4	2.8	3.5	3.8	4.2
	CC	7.8	-	-	7.5	-	7.8	-	8.1	7.5	7.0
Opened 48 hours before virus	$TCID_{50}$	0.6	0.4	0.8	1.2	1.5	2.2	3.2	3.5	4.2	4.8
	CC	8.8	-	-	9.0	-	8.5	-	8.2	8.3	7.9
Opened 24 hours before virus	$TCID_{50}$	0.5	0.8	1.2	1.8	2.2	2.5	3.0	3.4	4.2	4.0
	CC	12.5	-	-	11.7	-	10.9	-	10.2	10.4	10.1
Opened at time virus added	$TCID_{50}$	0.6	1.8	2.2	2.5	2.4	2.4	3.2	3.2	4.4	4.5
	CC	12.2	-	-	12.9	-	11.7	-	11.9	11.0	10.6

[a]Minimum essential medium (Eagle)-modified (MEM-M). Tris 10 mM was used. All cultures incubated at 36 C on roller drum.

[b]Rubber stoppered cultures.

[c]Cotton plugs substituted for rubber stoppers.

[d]$TCID_{50}$ expressed as positive $\log_{10}$ of the dilution per 0.1 ml.

[e]Cell count x 10^5

TABLE 32

TIME AT WHICH CREATING AN OPEN SYSTEM AFFECTS REPLICATION OF VACCINIA VIRUS (O STRAIN) IN HELA CELLS

Growth Conditions[a]		Hours after adsorption when virus samples were taken								
		0	6	12	24	36	48	72	96	120
Closed[b]	$TCID_{50}$[d]	0.8	1.5	2.5	4.2	4.7	5.2	5.5	5.8	6.5
	CC[e]	12.1	-	-	14.0	-	16.8	17.9	18.2	18.0
Opened[c] 72 hours before virus	$TCID_{50}$	0.8	0.5	1.2	1.8	2.2	3.0	3.5	4.2	4.8
	CC	8.6	-	-	8.5	-	8.2	7.9	8.0	7.2
Opened 48 hours before virus	$TCID_{50}$	0.8	0.6	1.2	2.0	2.3	2.8	3.4	4.5	5.2
	CC	9.2	-	-	9.2	-	9.2	8.9	8.6	8.2
Opened 24 hours before virus	$TCID_{50}$	0.5	1.7	2.0	2.2	2.5	2.6	3.4	3.8	-
	CC	11.2	-	-	11.8	-	10.9	10.0	9.6	9.8
Opened at time virus added	$TCID_{50}$	0.6	1.5	2.2	2.6	2.6	3.4	3.8	4.2	5.0
	CC	12.1	-	-	13.5	-	13.8	13.2	12.6	11.9

[a]Minimum essential medium (Eagle)-modified (MEM-M). Tris 10 mM was used. All cultures incubated at 36 C on roller drum.

[b]Rubber stoppered cultures.

[c]Cotton plugs substituted for rubber stoppers.

[d]$TCID_{50}$ expressed as positive $\log_{10}$ of the dilution per 0.1 ml.

[e]Cell count x 10^5.

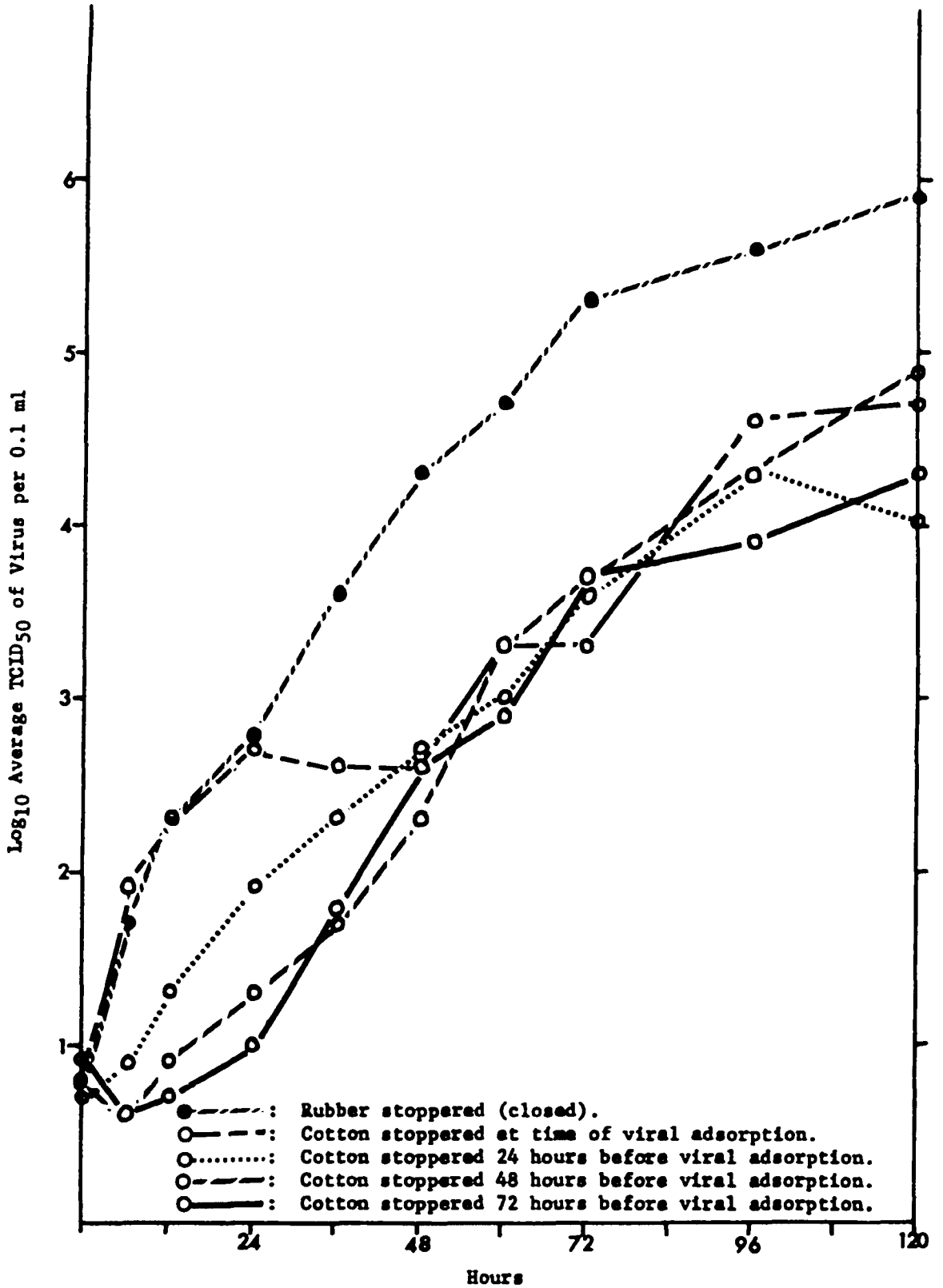


Fig. 17. The production of vaccinia virus (D strain) in HeLa cells grown in cotton stoppered cultures for varying lengths of time.

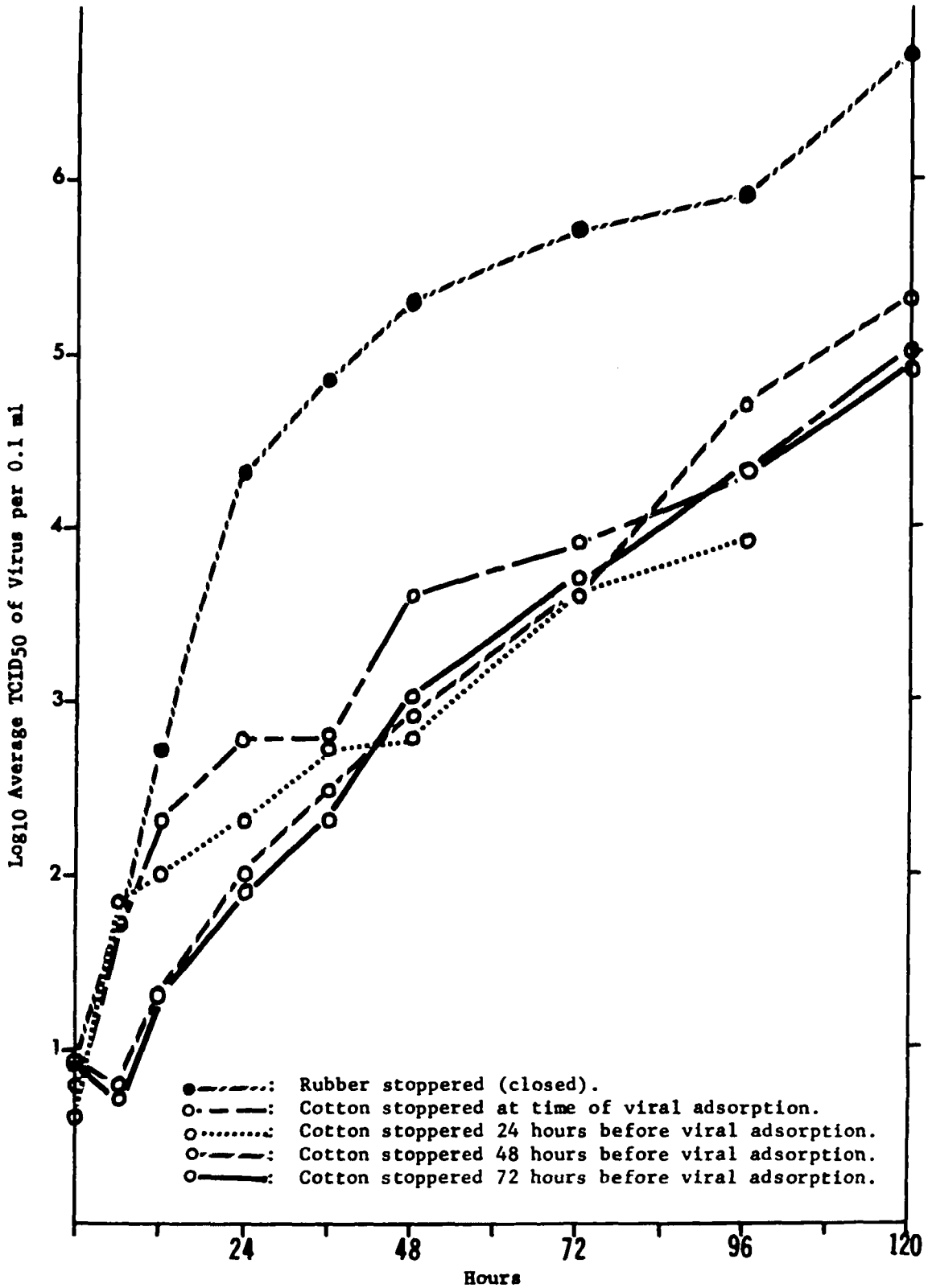


Fig. 18. The production of vaccinia virus (O strain) in HeLa cells grown in cotton stoppered cultures for varying lengths of time.

the two vaccinia viruses in closed cultures. In cultures exposed to atmospheric conditions 72 and 48 hours before the cells were infected, no difference was apparent in total virus content, although slight differences in viable cell counts were evident. The virus titers produced in open cultures were almost 100 times less than those obtained in closed cultures. Within 24 hours after infection the cultures exposed to normal atmospheric conditions at the time of virus inoculation produced amounts of infectious virus comparable to those cultivated in closed cultures. When cultures were opened for 24 hours before infection, total infectious virus content was intermediate to those in closed cultures and in cultures opened 48 to 72 hours before the introduction of the virus.

From the data in Tables 33 and 34, which are graphically depicted in Figures 19 and 20, no differences were evident among virus titers produced by herpes simplex strains in closed cultures or in those opened to the atmosphere at any time before infection.

Virus Adsorption Studies

In all of the experiments previously described, controls were included in order to determine how much virus was adsorbed by HeLa cells. At the time that cultures were inoculated with virus, comparable amounts of virus were added to clean, sterile culture tubes. These control tubes were handled in the same manner as the cell cultures during the period of adsorption. At the end of this period, the unadsorbed virus was collected from the control tubes which contained no cells and from the tubes containing cell cultures, and each sample was assayed for

TABLE 33

TIME AT WHICH CREATING AN OPEN SYSTEM AFFECTS REPLICATION OF HERPES SIMPLEX VIRUS (HF STRAIN) IN HELA CELLS

Growth Conditions[a]		Hours after adsorption when virus samples were taken							
		0	12	24	36	48	60	72	96
Closed[b]	$TCID_{50}$[d]	1.2	1.8	3.8	4.8	5.6	6.2	6.5	5.6
	CC[e]	13.1	-	14.6	-	17.1	-	18.0	18.2
Opened[c] 72 hours before virus	$TCID_{50}$	1.5	1.2	3.2	4.4	5.3	5.6	6.2	6.0
	CC	8.2	-	8.0	-	7.7	-	7.9	7.6
Opened 48 hours before virus	$TCID_{50}$	1.5	1.5	3.6	4.2	5.2	5.8	5.6	5.5
	CC	10.4	-	9.9	-	10.1	-	9.7	9.5
Opened 24 hours before virus	$TCID_{50}$	1.2	1.7	3.4	4.5	5.2	5.6	5.8	5.4
	CC	11.9	-	12.1	-	11.5	-	11.6	11.2
Opened at time virus added	$TCID_{50}$	0.8	1.4	3.5	4.5	4.8	6.2	5.8	5.8
	CC	13.1	-	13.9	-	13.2	-	12.8	12.2

[a]Minimum essential medium (Eagle)-modified (MEM-M). Tris 10 mM was used. All cultures incubated at 36 C on roller drum.

[b]Rubber stoppered cultures.

[c]Cotton plugs substituted for rubber stoppers.

[d]$TCID_{50}$ expressed as positive $\log_{10}$ of the dilution per 0.1 ml.

[e]Cell count x 10^5

TABLE 34

TIME AT WHICH CREATING AN OPEN SYSTEM AFFECTS REPLICATION OF HERPES SIMPLEX VIRUS (M STRAIN) IN HELA CELLS

Growth Conditions[a]		Hours after adsorption when virus samples were taken									
		0	6	12	24	36	48	60	72	96	120
Closed[b]	$TCID_{50}$[d]	0.4	0.5	2.0	3.3	4.3	5.5	-	6.2	5.8	4.7
	CC[e]	11.7	-	-	12.9	-	14.2	-	16.0	17.1	17.9
Opened[c] 72 hours before virus	$TCID_{50}$	0.6	0.3	1.5	2.7	3.3	5.4	-	5.8	5.5	4.5
	CC	8.2	-	-	8.0	-	7.9	-	8.2	7.5	7.1
Opened 48 hours before virus	$TCID_{50}$	0.6	0.8	1.5	3.0	4.0	5.4	-	5.6	5.2	4.7
	CC	9.4	-	-	9.5	-	9.8	-	9.1	8.7	8.2
Opened 24 hours before virus	$TCID_{50}$	0.8	0.5	1.4	2.8	3.7	5.6	-	6.2	5.6	5.2
	CC	11.9	-	-	10.8	-	10.5	-	10.1	10.2	9.8
Opened at time virus added	$TCID_{50}$	0.8	0.3	1.2	2.7	3.5	5.3	-	5.6	5.6	4.6
	CC	11.7	-	-	12.0	-	11.7	-	10.5	10.1	10.4

[a]Minimum essential medium (Eagle)-modified (MEM-M). Tris 10 mM was used. All cultures incubated at 36 C on roller drum.

[b]Robber stoppered cultures.

[c]Cotton plugs substituted for rubber stoppers.

[d]$TCID_{50}$ expressed as positive $\log_{10}$ of the dilution per 0.1 ml.

[e]Cell count x 10^5.

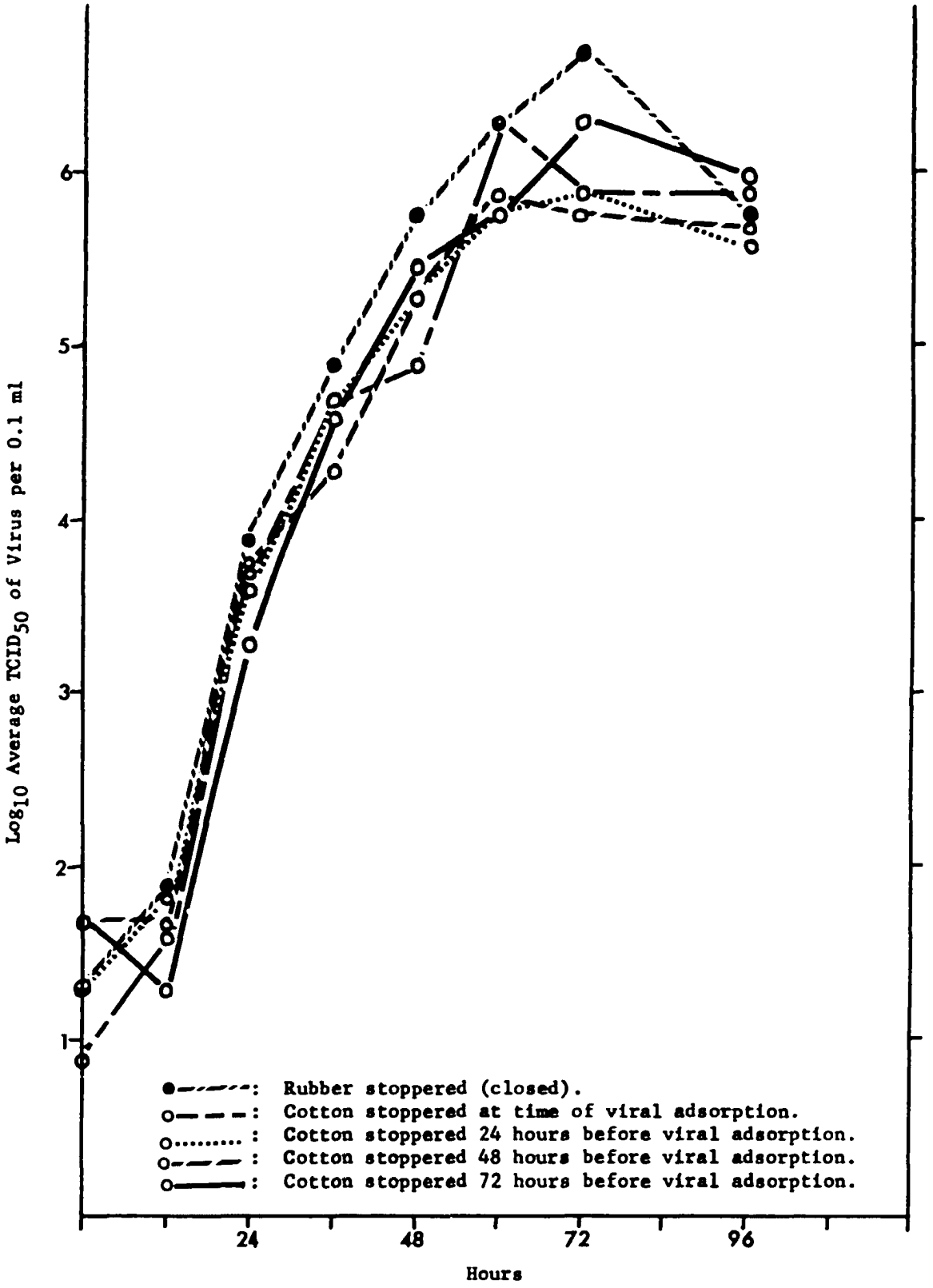


Fig. 19. The production of herpes simplex virus (HF strain) in HeLa cells grown in cotton stoppered cultures for varying lengths of time.

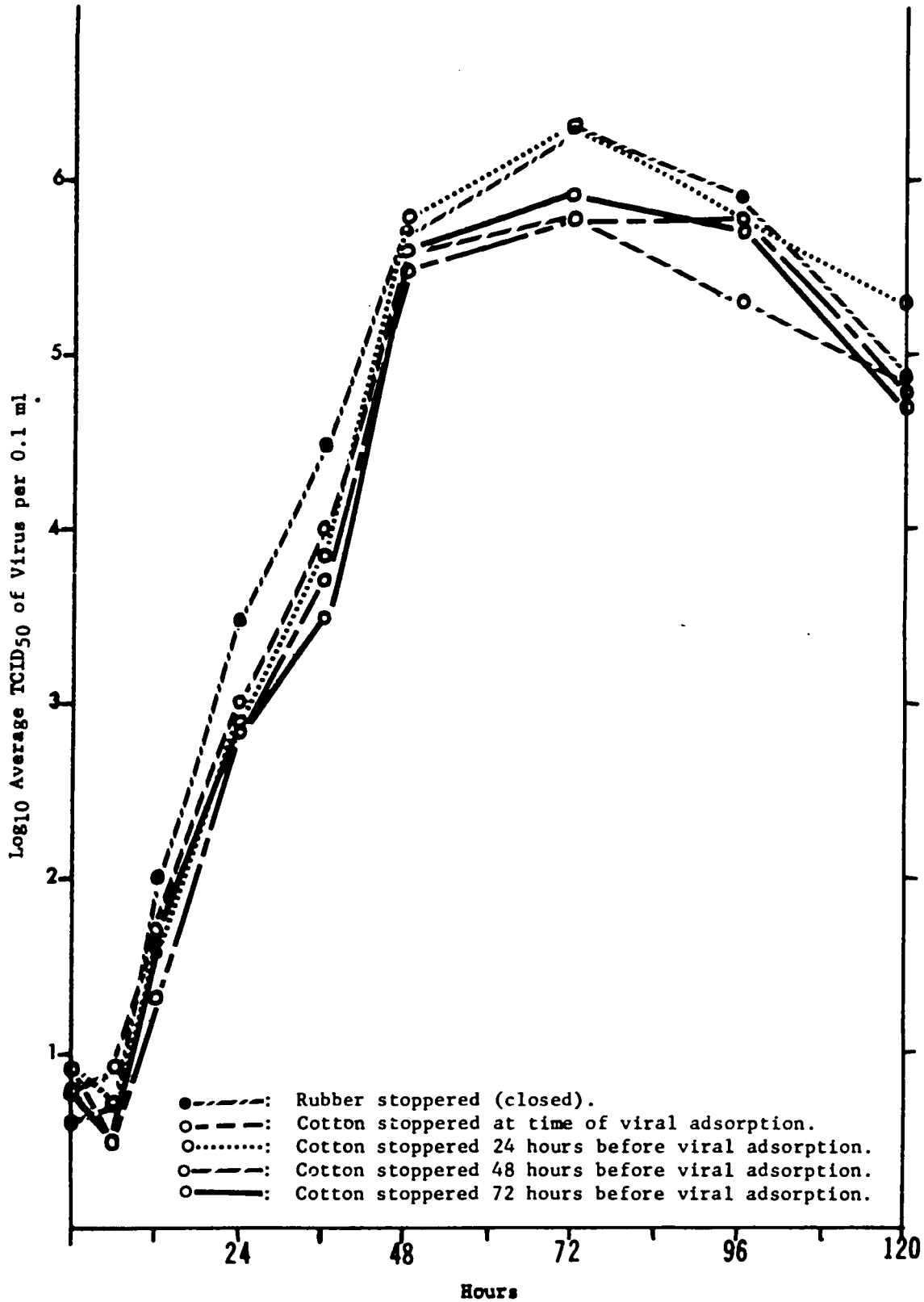


Fig. 20. The production of herpes simplex virus (M strain) in HeLa cells grown in cotton stoppered cultures for varying lengths of time.

infectious virus. Differences between the amount of virus in the inoculum and the amount of virus in the control tubes after adsorption was presumed to be due to inactivation of virus during the adsorption period. Difference between the amount of virus in the control tubes and the amount of residual virus removed from cell cultures after the adsorption period was presumed to be the amount of virus adsorbed by the cells under experimental conditions. Since it was postulated that Tris might enhance adsorption of virus (Porterfield and Allison, 1960), an additional study was made to determine if there were any detectable differences in adsorption of virus to HeLa cell cultures in bicarbonate- and Tris-containing media. Replicate tube cultures were initiated and incubated for 48 hours in the routine manner. After 48 hours the bicarbonate-containing MEM-M was discarded from all but one set of cultures and replaced with MEM-M containing 10 mM Tris and dialyzed calf serum. The bicarbonate control set was maintained in 20 mM bicarbonate MEM-M. Cultures in Tris medium were divided so that one set remained in sealed tubes, a second set was cotton plugged, and a third set was made CO_2 deficient by means of a potassium hydroxide trap. After 24 hours all media were changed, and after 48 hours under the experimental conditions virus adsorption studies were done.

Each of the four viruses was treated in a similar manner. The seed virus was diluted to 10^{-4} in DPBS-5 supplemented with dialyzed calf serum. After aspiration of the experimental medium, 0.2 ml of diluted virus suspensions were added to each culture. Clean, sterile culture tubes, previously rinsed with 1.0 ml of DPBS-5, were also inoculated with 0.2 ml of the virus suspension. Some of the control tubes were cotton

plugged during the three hour adsorption period at 30 to 32 C, while the remainder were sealed with rubber stoppers. All control tubes were agitated and treated in the same manner as the cell cultures during adsorption. Samples of the original 10^{-4} virus dilution, as well as unadsorbed virus from the control tubes and from the cultures, were frozen quickly and stored at -65 C until they could be assayed. In Table 35 it may be seen that differences in virus inactivation in either open or closed cultures were not remarkable. Titers from control tubes were comparable to those of the original virus inoculum. When comparing the amount of virus adsorbed to HeLa cells grown under different environmental conditions, only slight differences in virus titers were noted.

TABLE 35

VIRUS ADSORPTION STUDIES

Adsorption Environment	Amount of Virus Recovered after Adsorption[a]			
	Vaccinia		Herpes Simplex	
	D Strain	O Strain	HF Strain	M Strain
Initial inoculum[b]	2.4[c]	2.6	2.6	2.5
Glass tubes, no cells[d]				
Closed[e]	2.0	2.5	2.0	2.5
Open[f]	1.8	2.5	2.2	2.6
Bicarbonate cells[g]				
Closed	1.8	2.2	1.6	2.3
Tris cells[h]				
Closed	1.8	2.4	1.8	2.5
Tris cells, CO_2 trap				
Closed	1.8	2.5	1.8	2.5
Tris cells				
Open	1.8	2.4	2.2	2.4

[a]Adsorptions at 30 to 32 C for three hours.

[b]Average $TCID_{50}$ of four titrations.

[c]$TCID_{50}$ expressed as positive $\log_{10}$ of the dilution per 0.1 ml.

[d]Inoculum incubated in tubes without cells.

[e]Rubber stoppered tubes.

[f]Cotton plugged tubes.

[g]Grown in MEM-M 20 mM bicarbonate.

[h]Grown in MEM-M 10 mM Tris.

CHAPTER IV

DISCUSSION

Comparison of Methods of Cell Enumeration

Many tissue culturists employ the cell nuclei counting method of Sanford et al. (1951). Although it is convenient, such a technique includes all intact cells, both living and dead. For the purposes of this investigation the enumeration of viable cells was of more value than the total number of cells. Several compounds such as trypan blue, nigrosin, eosin, safranin, and erythrosin have been used to enumerate viable cells. All of these dyes stain dead cells and not viable cells. Kaltenbach et al. (1958) concluded that the best dye for viable cell enumeration was nigrosin. Most protocols specify the use of a balanced salt solution as a cell diluent. In the present study, when nigrosin or trypan blue were employed with DPBS as the diluent, the numbers of viable cells were consistently low and were progressively lower on repeated enumeration of the same sample. When five per cent calf serum was incorporated into the diluent, the cells were stabilized and counts were repeatedly reproducible. This observation parallels that of Phillips and Andrews (1959) that tissue culture cells rapidly lost their viability upon standing in a buffered salt solution unless the cells were protected by the addition of a colloid, such as protein or

methylcellulose. On the basis of these studies trypan blue was selected for use throughout the experimental work.

Clumping of the cells was an initial problem. Trypsin alone was not effective in producing a monodispersed cell suspension, but the addition of 0.1 per cent sodium citrate minimized this source of error.

Development of an Assay Method for Herpes Simplex Virus

Several host cell lines could have been utilized successfully in this investigation. Chick embryo cells were found to be 10 to 100 times more sensitive to infection with herpes simplex virus infection than other cell lines tested, but factors such as availability, ease of handling, maintenance of cultures, and variability were paramount in the decision to use a serially propagated cell line. Of the three serially propagated cell strains investigated, HeLa-10 proved to be the most satisfactory. The FL strain of human amnion cells and the HeLa-MA human cervical carcinoma cells have been maintained in chemically defined media supplemented with up to 20 per cent serum and both cell types were rather fragile and easily affected by adverse growth conditions. During the period that it was maintained in this laboratory, the HeLa-10 line had been adapted to a medium which contained only five per cent serum. There was essentially no nonspecific degeneration of the HeLa-10 cells for five days without a medium change, whereas FL and HeLa-MA cells degenerated rapidly in medium which contained five per cent serum. Since nonspecific degeneration was not a problem with the HeLa-10 cells, CPE appeared earlier and was more easily visualized.

Since herpes simplex virus is extremely labile, various factors were investigated to determine optimal conditions for a simple and

reproducible method of titration. The optimal time and temperature for adsorption of virus were found to be two hours at 30 to 32 C. At these temperatures adsorption of virus was maximal and inactivation of virus was minimal. This temperature was also favored by Waterson (1959), but in his system a period of four hours was necessary for maximal adsorption. An increase from 0.1 per cent to 0.2 per cent glucose in the maintenance medium resulted in slightly higher virus titers, and the cells appeared to be in better morphological condition during the period of observation. Standardization of these and other variables resulted in a convenient assay procedure which was reproducible within $\pm$ 0.5 $\log_{10}$.

Since the effects of various buffers on cell growth and virus proliferation were to be considered, it was important that the medium used in the preparation of the virus pools, and as the diluent, should be bicarbonate-free, and should protect the virus infectivity. Dulbecco's phosphate buffered saline, supplemented with five per cent, heat-inactivated, dialyzed calf serum, was satisfactory in all respects. Thus, the virus pools were free of nutrients except for the serum component and they contained no added bicarbonate.

Maintenance of Tonicities of Solutions

In previous reports which described the effects of bicarbonate concentration on viral replication, no mention was made of efforts to compensate for the alteration of bicarbonate in the media in order to maintain the tonicities of the solutions (Vogt et al., 1957; Hsung and Melnick, 1958; Chang, 1959; Quersin-Thiry, 1961; and Mosley and Enders, 1962). The tonicity of the medium and specifically the concentration

of sodium chloride was shown by Stubblefield and Mueller (1960) to affect the growth, biochemical composition, and metabolism of HeLa cells. Recently it has been suggested that the expression of the d marker of poliovirus may not be due to bicarbonate deficiency, but to the lowered tonicity of the medium when bicarbonate was omitted (Agol and Chumakova, 1962). Since, in the present investigation, tonicities were maintained at normal values of EBSS by regulation of amounts of sodium bicarbonate and sodium chloride in the media, it does not seem likely that altered tonicities influenced the results.

Preliminary studies in this investigation of the effect of bicarbonate depletion on the replication of herpes simplex virus indicated that virus production was not inhibited. This observation was completely unexplainable since Chang (1959), using cells made CO_2-deficient with alkaline CO_2 traps, showed suppression of several viruses. Vaccinia virus, shown by Chang (1959) to be inhibited by a CO_2 deficiency, was included in this study because the nucleic acid of both viruses has been shown to be desoxyribonucleic acid (DNA) (Smadel et al., 1940; Hoagland et al., 1940; and Epstein, 1962). Two strains, HF herpes simplex and D vaccinia viruses, were well documented laboratory strains. The two newly isolated viruses, M herpes simplex and O vaccinia, were included in several studies to determine if they behaved in the same manner as the established strains.

Effect of Variation of Buffers

Media buffered with bicarbonate in concentrations of 20 mM or less did not grossly affect the growth of the host cells or replication of either virus in rubber stoppered cultures. In media containing low

concentrations of bicarbonate, changing the medium daily prevented cell damage due to low pH.

When Tris was substituted for bicarbonate in cultures grown in closed systems, no deleterious effects to the host cells were discernible so long as the concentration of Tris did not exceed 10 mM. Growth of cells ceased rapidly at 20 mM concentrations of Tris, although Swim and Parker (1955) found that this concentration was not toxic for cells in their experimental system. In the studies reported here, the amount of infectious virus produced by cultures grown in Tris buffered media was comparable to that produced in bicarbonate-containing media when both types of cultures were maintained in closed systems. Tightly sealed, rubber stoppered cultures allowed metabolically-produced CO_2 to accumulate to levels which would support cellular growth, even if no bicarbonate were added to the media (Swim, 1961).

The remarkable differences in the type of CPE produced by HF herpes simplex virus in HeLa cells in Tris, as compared with bicarbonate-buffered media apparently has not been reported previously. Although Porterfield and Allison (1960) described enhanced plaque formation by both herpes simplex and vaccinia viruses under agar buffered with Tris, they did not mention morphologic changes in the infected cells. They postulated that enhancement of plaque formation might be associated with a greater efficiency in the adsorption of virus in Tris buffered media. Gray et al. (1958) described distinct differences in the type of CPE produced by different strains of herpes simplex virus in HeLa cells cultivated in bicarbonate-containing media. Hoggan et al. (1961) were able to separate the giant cell-forming variant of herpes

simplex virus from the variant which produced the proliferative type of CPE, and they demonstrated that, although the two variants produce different types of cytopathic effects, they are immunologically identical. The character appeared to be stable since the same type of CPE was produced regularly under given conditions of incubation. The HF strain of herpes simplex virus which was used in this investigation produced giant cells in bicarbonate-containing media. No typical proliferative lesions had been demonstrated previously in HeLa cells.

In the present study replicate tube cultures were grown in bicarbonate- and Tris-buffered experimental media and both groups were infected with the same inoculum of HF herpes simplex virus. In bicarbonate-containing medium, giant cell formation occurred as usual, but in Tris-buffered medium no giant cells were formed, and instead, individual cell outlines became more distinct than those of surrounding, uninfected cells. This difference in the type of CPE produced by the HF strain of herpes simplex virus in bicarbonate- and in Tris-buffered media could account for the observations of Porterfield and Allison (1960) regarding the enhanced plaque formation by the virus under Tris-buffered, agar overlay.

The M strain of herpes simplex virus produced a proliferative type of cell lesion in both bicarbonate- and Tris-containing media. Waterson (1959) reported that some strains of herpes simplex virus did not form plaques in chick embryo monolayers under agar overlays. It is possible that he was using herpes simplex virus strains which formed the proliferative type of lesions.

The D and O strains of vaccinia virus produced tight, nodular

type lesions in bicarbonate-containing media; in Tris-buffered media the lesions were diffuse and the necrotic areas were wide spread. These differences in CPE could account for the enhanced plaque formation under the experimental conditions described by Porterfield and Allison (1960).

Carbon Dioxide Deficiency

Geyer and Chang (1958) showed that alkaline CO_2 traps were effective in eliminating detectable CO_2 in Tris-buffered HeLa cell cultures; multiplication of cells ceased under these conditions. They were able to show, however, that if bicarbonate were added to the medium and the CO_2 trap removed, normal growth would resume at any time up to 14 days.

Swim (1961) described conditions of CO_2 deficiency which were created by the substitution of a phosphite or Tris buffer for bicarbonate. Cultures were closed with loosely fitted metal caps rather than with rubber stoppers. The metal caps allowed free gaseous exchange between the cultures and the surrounding atmosphere so that CO_2 diffused continuously into the atmosphere as rapidly as it was produced by the cells. Under these conditions cell growth also ceased, but it began again when the cultures were sealed with rubber stoppers, even though no bicarbonate was added to the medium. Inhibition of cell growth under normal atmospheric conditions is probably not due to unfavorable oxygen concentration since Rueckert and Mueller (1960) showed that 20 to 30 per cent oxygen, the oxygen tension of ordinary air, was optimal for HeLa cell growth. If oxygen were responsible for the difference in cell growth, cell multiplication in open cultures should proceed more rapidly than in closed cultures where oxygen is utilized continually in metabolic activities.

An extensive search of the literature revealed only two reports on the use of CO_2-deficient cells for studying replication of viruses (Chang, 1959; Mosley and Enders, 1962). Two of the three viruses studied by Chang, vaccinia and Coxsackie, were completely inhibited under conditions of CO_2 depletion created with CO_2 traps. Although the replication of poliovirus was greatly decreased under these conditions, it was not completely suppressed. These results represent the first direct evidence that there are differences in susceptibility of viruses to CO_2 deficiency. Mosley and Enders continued studies on poliovirus and showed that the d- marker was demonstrable in open monkey kidney cell cultures in liquid medium where CO_2 pressures were at low levels.

The studies presented here confirmed several observations of Chang and of Mosley and Enders. It was possible to create CO_2-deficient HeLa cells as demonstrated by cessation of cell multiplication with an alkaline CO_2 trap or by the use of open cultures. For open cultures, cotton plugs, suggested by Mosley and Enders (1962), were used in place of the loosely fitted metal caps described by Swim (1961). In the present investigation 24 to 36 hours were necessary before growth of the cells ceased. This time probably represented the time required to deplete the available reservoir of bicarbonate. Under these conditions replication of two strains of vaccinia virus was curtailed markedly, though not halted completely. Complete CO_2 depletion, such as Chang described, probably was not achieved since the medium was changed daily. Even though tubes with CO_2 traps were not opened to the air during the medium change, small amounts of CO_2 dissolved in the medium were probably introduced each day. In Chang's experiments the medium was changed

at three day intervals. Differences in the susceptibility of the strains of HeLa cells employed could be a factor in failure to obtain complete inhibition of replication of vaccinia virus.

When herpes simplex virus was exposed to the same experimental conditions there was no apparent difference between the amount of infectious virus produced by CO_2-deficient and by normal cells. Production of comparable amounts of infectious herpes simplex virus under the different conditions of incubation was difficult to explain since there were fewer cells in the CO_2-deficient cultures than in normal cultures. However, when one considers the small size of the initial virus inoculum in relation to the number of cells (approximately 2.5 $TCID_{50}$, log_{10}, to 1 x 10^6 normal or 7 x 10^5 CO_2-deficient cells), it is apparent that the same amount of virus could be adsorbed in either case. Studies on the adsorption process indicated that there was no detectable difference in adsorption of virus under the various experimental conditions.

Since the nucleic acid contained in vaccinia and herpes simplex viruses is DNA, and CO_2 fixation is necessary for purine synthesis (Buchanan and Wilson, 1953), and purine bases are in both DNA and RNA, the differences between vaccinia and herpes simplex virus replication under conditions of CO_2 deficiency are difficult to explain on the basis of what is known regarding their metabolic activity. It is interesting to speculate on the known differences in the metabolic activity of the two viruses. It has been reported that there is an initial increase in ribonucleic acid (RNA) in the cytoplasm of cells infected with vaccinia virus with no detectable increase in DNA (Loh et al., 1959;

Joklik, 1959; Joklik and Rodrick, 1959). Conversely, when cells were infected with herpes simplex virus the DNA content of the nuclei increased initially, with no increase in cytoplasmic RNA (Newton and Stoker, 1958). By the addition of RNA and RNA derivatives, Chang (1959) was able to restore the replication of vaccinia virus in CO_2-deficient cells, but DNA or DNA derivatives were not effective. He speculated that the virus initially caused the production of new protein, presumably enzymes, which were necessary for the formation of virus components. Protein synthesis is governed by RNA and since RNA or RNA derivatives alleviated the adverse effect of CO_2 deficiency, it would seem that CO_2 deficiency is more closely related to RNA than to DNA synthesis, even though purines, synthesized in part from CO_2, are incorporated into both. Mosley and Enders (1962) also showed that RNA derivatives and oxaloacetate were effective in reversing the effect of bicarbonate deficiency in monkey kidney cell cultures infected with certain strains of poliovirus. It is possible that CO_2 deficiency did not affect replication of herpes simplex virus because this virus does not depend on an initially increased synthesis of RNA and therefore is relatively independent of a need for increased concentrations of CO_2.

The facts that purine bases are integral parts of both RNA and DNA, and CO_2 is utilized in the synthesis of purine bases (Buchanan and Wilson, 1953) offer no explanation for differences in the replication of vaccinia and herpes simplex viruses under conditions of CO_2 deficiency. However, on the basis of results presented here, there apparently are differences in the synthesis of RNA and DNA by virus-infected HeLa cells which can be related to CO_2 concentration.

The present investigations leave many problems worthy of further study. The dissimilarity of the requirement for bicarbonate displayed by two viruses of similar chemical composition should be investigated in greater detail. The effects of CO_2 deficiency on other cell cultures of different origins should be examined. There were distinct differences in the reactions of HeLa cells used in the present study and those of the monkey kidney cells used by Mosley and Enders (1962). The HeLa-10 cell cultures required from 24 to 36 hours before the effects of CO_2 deficiency were operative in inhibiting virus replication. Future studies on replication of these viruses under similar conditions should be concerned especially with the 36 to 48 hour period after inoculation of cell cultures with virus. In cultures of HeLa-10 cells maintained in Tris-buffered media in closed systems, control of the pH was a major problem. Within 12 to 18 hours the pH dropped from approximately 7.5 to below 6.8. Changes in pH were less pronounced in open cultures, where the pH rarely dropped below 7.0. Mosley and Enders observed opposite effects using monkey kidney cell cultures. More glucose was used, more lactate accumulated, and a lower pH developed in their open cultures than in their closed cultures. Such observed differences in dissimilar virus-host cell systems indicate major differences in the metabolic processes and, perhaps, in synthesis of cell components. Further study of the effect of CO_2 deficiency on both the multiplication of mammalian cells in vitro and replication of virus therein might add further to the understanding of virus infection.

CHAPTER V

SUMMARY

Replication of two strains of herpes simplex virus and two strains of vaccinia virus was studied in HeLa cell cultures grown in media containing different buffers and incubated in different gas phase conditions. Relatively simple methods for reproducible quantitation of host cells and viruses were developed.

When tightly closed cultures of HeLa cells were grown in Eagle's minimum essential medium containing varying concentrations of sodium bicarbonate, marked differences in buffering capacity and growth of cells were evident. No effort was made to regulate and maintain pH values at physiologically compatible levels. Under these conditions, 40 mM bicarbonate did not sustain growth of the cells, and the pH of the cultures rose to about 8.0. Optimal growth of cells was obtained in media which contained five or 10 mM bicarbonate when the medium was changed daily. Introduction of either herpes simplex or vaccinia viruses into growing cell cultures produced essentially the same titers of infectious virus regardless of the amount of bicarbonate added to the medium.

Similar experiments in closed systems utilized varying concentrations of tris (hydroxymethyl) aminomethane (Tris) as a substitute for bicarbonate. At a concentration of 20 mM, Tris was toxic for HeLa cells.

In media containing 10 mM Tris, excellent development of cells was seen and physiologically compatible pH levels were maintained for at least 24 hours. Lower concentrations of Tris were less effective in buffering capacity, but adequate growth of the cells occurred if the medium was changed daily. HeLa cells grown in closed cultures with Tris-buffered media could not be distinguished microscopically from those grown in bicarbonate media. When HF herpes simplex virus, or either strain of vaccinia virus was added to cells grown in Tris-buffered media, changes in the cells were markedly different from those seen in bicarbonate-containing media. On the other hand, the M strain of herpes simplex virus produced similar cytopathic changes regardless of the medium used to maintain the cells. Replication of herpes simplex and vaccinia viruses in closed cell cultures was essentially the same regardless of the amount of Tris used, if the medium supported growth of the host cell. Furthermore, the amount of infectious virus produced in cultures grown in Tris medium and in bicarbonate-buffered medium was essentially the same.

The simple substitution of cotton plugs for rubber stoppers produced remarkable changes in the growth of HeLa cells in Tris-containing medium. When cultures were thus aerated, growth of cells stopped within 24 to 36 hours, but a constant number of viable cells was maintained for several days. Microscopically, these cells appeared larger than normal, quite hyaline, and delicate. When open cultures were infected with herpes simplex or vaccinia viruses, marked differences in replication of the two viruses were evident. The amount of infectious vaccinia virus produced was usually 100 times less in open cultures of HeLa cells

than in similar cultures tightly sealed with rubber stoppers, whereas comparable amounts of infectious herpes simplex virus were produced in either the open or closed cultures.

Further experiments in which cultures were made CO_2 deficient with potassium hydroxide traps confirmed these differences in the effect of CO_2 deficiency on the two viruses. Here again, replication of vaccinia virus diminished in the CO_2-deficient cultures of HeLa cells, but similar amounts of herpes simplex virus were produced in CO_2-deficient and in normal cell cultures.

Additional evidence that these differences in replication were due to CO_2 deficiency was gained when cultures were opened to the air, by substituting cotton plugs for rubber stoppers, at various times before the introduction of virus. When closed cultures were opened to the air, a period of approximately 24 to 36 hours was required before cell growth stopped. Maximum suppression of vaccinia virus replication did not occur until at least 48 hours incubation in an open atmosphere. When cultures were opened to the air at the time the virus was introduced, the replication of vaccinia virus for the first 24 to 36 hours was equal to that in closed cultures, and after that time the amount of infectious virus was less than that produced in closed cultures. Development of herpes simplex virus was not affected by opening the cultures at any time. Comparable amounts of infectious herpes simplex virus were found in closed cultures, in cultures opened at the time the virus was introduced, and in cultures opened at 24, 48, and 72 hours before the virus was added.

The demonstration that two desoxyribonucleic acid-containing

viruses, herpes simplex and vaccinia, differ in their intracellular replication in CO_2-deficient cells suggests major differences in their synthesis. Apparently, the development of vaccinia virus, but not herpes simplex virus, depends on the presence of an adequate supply of CO_2.

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