CARBOHYDRATE METABOLISM IN CULTURED HUMAN AMNION CELLS INFECTED WITH ADENOVIRUS

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ABSTRACT

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Cultured Human Amnion Cells
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Evidence is presented indicating that an increase in glucose disappearance and lactic acid accumulation occurs in secondary amnion monolayer cell cultures and in FL monolayer cell cultures infected with Adenovirus type 3. This change in glycolysis was found to begin at a time close to the beginning of the logarithmic phase of virus growth and it was not accompanied by any change in the oxygen consumption. Addition of oxanic acid, a competitive inhibitor of lactic dehydrogenase, to culture fluids caused an inhibition of glucose disappearance, lactic acid accumulation and virus synthesis. This suggests that lactic dehydrogenase is important in the formation of infective virus particles.

Robert James Hay, August, 1961.

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INTRODUCTION

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The exact chemical nature of virus growth is just beginning to be understood. Most work done to date cannot be interpreted in precise chemical terms but certain changes in the virus-cell complex have been shown to occur at given stages in its development. In order to build up a more complete picture of the intracellular events leading to the production of virus particles, it is necessary to establish whether changes in metabolism of cells following infection are intimately associated with virus synthesis. If this can be established, further studies may be carried out to reveal the significance of the changes, in relation to virus production.

Recently, considerable attention has been focused on the adenovirus infected cell as a model system for the study of virus replication. It has been shown that an increase in aerobic glycolysis occurs, following infection with this virus, but no study has been made relating this change to Adenovirus replication. This study was designed to relate the increase in aerobic glycolysis observed following infection of human amnion cell cultures with type 3 Adenovirus to the multiplication of this virus and to attempt to determine its importance in Adenovirus replication. It was also proposed to investigate the oxygen consumption of adenovirus infected cells, at various stages in the development of the virus-cell complex.

REVIEW OF THE LITERATURE

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Investigation of the multiplication of viruses in cell cultures permits the observation of various stages of a growth cycle. Although the duration of each phase in the cycle may vary from one virus to another, the overall picture of reproduction is similar. The growth cycle involves the adsorption of virus to the cell surface, penetration of the cell membrane, a lag period during which only a very small amount of virus is recoverable, and a period of exponential increase of virus infectivity. (Dulbecco, 1955; Luria, 1959; Isaacs, 1959; Cooper, 1959; Walker, 1960).

A great deal of work has been done on metabolic and morphologic changes in tissue and cell cultures infected with various viruses. (Pearson, 1953; Enders, 1954; and Lynn, 1954). Until recently, however, little has been done on cultures in which the virus type being studied is multiplying in a one step growth pattern. Since very many changes undoubtedly occur as the infected cell passes from early stages of infection to complete disruption, it is of prime importance to be able to relate any change observed to the phase of virus growth with which it is related.

Extensive studies relating biochemical changes to the phase of development of the virus-host cell complex have been carried out with poliovirus infected cells, the emphasis being placed on nucleic acid metabolism. Relatively little work has been done on the metabolism of cell cultures infected with other animal viruses. This review is primarily concerned with obser-

vations which have been made on metabolic changes in adenovirus infected cells (as they relate to virus replication), but because of the imperfect state of knowledge concerning metabolic changes in virus infected cells, an attempt is made to provide perspective by including an account of findings in similar studies with other virus-host cell systems.

Aspects of Adenovirus Growth

The growth curve and cytopathic effect associated with Adenovirus multiplication have been studied in a number of laboratories. (Boyer, 1957; Ginsberg, 1958; and Pereira, 1959). The lag period varies with the serological type of virus, being about 17 hours for Adenovirus types 1 and 2, and 14 to 15 hours for Adenovirus types 3 and 4. The latent period for Adenovirus type 5 appears to be about 12 hours. The incremental period of intracellular virus reaches a peak sometime between the twenty-third and the twenty-sixth hour after infection. Release of virus from cells has been shown to be very slow and incomplete. (Ginsberg, 1958). Adenoviruses are adsorbed to the host cell at a comparatively slow rate (Ginsberg and Dixon, 1959). Observations on the appearance of antigen and of the cytological changes accompanying the stages in virus growth suggest that some variation in the time taken for initiation of infection must exist among cells. The variation in time taken for cells to reach a particular stage in viral synthesis is least pronounced towards the end of the growth cycle.

With Adenovirus type 5, the formation of viral antigen, as detected by the fluorescent antibody technique, occurred at about 12 hours after infection. (Pereira, 1959). The intensity of fluorescence increased thereafter, becoming a maximum at 24 hours. At 12 hours only about 20% of the cells showed any fluorescence, while by the 24th hour after infection, almost 100% of the cells showed it. This latter observation supports the supposition that all of the cells in the culture were infected initially, while the former observation emphasizes the lack of completely synchronous development of virus among individual cells. The first changes in the morphology of the nuclei of fixed and stained cells occur at the twelfth hour after infection by Adenovirus type 5. At just about the same time, virus specific antigen is detectable in the nucleus by the fluorescent antibody technique. No inclusion corresponding in position to the fluorescent dots, which are presumably virus precursor, could be detected in fixed and stained cells. Nuclear alterations are distinct by the time measurable levels of infective virus appear, and these changes increase thereafter.

Since the most prominent signs of cell damage are noted in the nucleus of the cell, and since evidence from electron microscopic and fluorescent antibody studies indicates the presence of crystalline arrays of virus particles in the nucleus, it seems very probable that the nucleus is the primary site of virus synthesis.

"Toxins" are known to be liberated by cells infected with adenoviruses. (Pereira and Kelly, 1957; Pereira, 1958; Everett and Ginsberg, 1958; Rowe et al, 1958.) A factor, termed a cell-detachment factor, is produced by all serologic types of adenovirus excepting 10, 12, 13 and 15. Types 3, 4 and 7, and a simian type M-3 produce this factor but to a lesser extent than do other types. Most work has been done on the factor produced by types 1, 2 and 5. (Rowe et al. 1958.) Its effect is demonstrable by agitating the medium over the cell sheet some time after inoculation with fluid containing this factor. This causes the cell sheet to become detached from the glass.

Other studies (Pereira and Kelly, 1957; Pereira, 1958; Everett and Ginsberg, 1958) describe a "toxic" substance which causes an early cytopathic effect 3 to 4 hours after infection. This is possibly the same substance which causes cell detachment. It does not cause the characteristic nuclear alterations noted in adenovirus infected cells and does not prevent or inhibit cell division. The effect observed is reversible and cells showing it may carry virus and may support virus growth. The infective and toxic components may be separated by ultracentrifugation, the infective component sedimenting more rapidly than the toxic. The toxic component is more stable to heat and ultra-violet irradiation than the infective component, is not destroyed by ribonuclease or deoxyribonuclease, is not dialysable, and is not affected by

diethyl ether. It is, however, destroyed by trypsin and this has been used as a method for removing it from viral inocula. It has been characterized as a ribonucleoprotein (Wilcox and Ginsberg, 1959).

Antigenic studies indicate the toxin to be one of three complement fixing antigens associated with types 2 and 5 (Pereira et al. 1959; Klemperer and Pereira, 1959). The biological role of these components is not known. It was shown to be detectable first after 20 hours of infection and also found to follow closely the changes seen in the complement fixing titer.

It has been suggested (Walker, 1960) that the toxin may have some effect on the cell permeability, thus allowing final release of virus particles. Although this suggestion has little supporting evidence, it seems a plausible hypothesis since significant quantities of the toxin appear only relatively late in the reproductive cycle.

Metabolic Changes Associated with Adenovirus Growth

Levy and his associates (Levy et al. 1957) investigated the effects of infection of HeLa cells with type 2 Adenovirus on the uptake of radioactive glycine, on the uptake of radioactive inorganic phosphate, and on the production of lactic acid at selected times after infection. They used cells cultured in bottles, and a high multiplicity of virus to cells was employed in order to obtain as nearly a synchronous infection as possible. They found that an increase in the rate of uptake of P^{32} and C^{14} occurred, even in the

cultures which had been infected for only two or three hours. This effect was still manifest after 48 hours and possibly longer. No increase in total phosphorus content of the cells and no effect on the concentrations of RNA[1] or DNA[2] could be shown at an interval five hours after infection. This is contrary to the finding of Ginsberg and Dixon (1959).

These workers, however, were using the HeLa cell--adenovirus type 4 system, and they examined the cells and cell extracts after 4 days of infection. They found a marked increase in the DNA and protein content of infected cells. Ginsberg also found that the greater part of the DNA produced, is not incorporated into infectious viral particles, and that a large proportion of the accumulated DNA differs from the host cell DNA in its solubility properties and nucleotide composition.

Neither of these studies was planned so as to take samples at regular intervals in the viral growth cycle.

Green and Daesch (1961), working with KB cells growing in suspension, and infected with adenovirus type 2, showed that multiplication of the infected cells was limited to the first 12 hours, and over a 48 hour period after infection was only about one-third that of control cells. The first appearance of virus occurred at 14 hours after infection and virus content reached a peak 36 to 48 hours after infection. Measurement of adsorption, infective center formation

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1. RNA - Ribonucleic acid.
2. DNA - Deoxyribonucleic acid.

and surviving cells indicated that essentially all cells were infected.

A continuous increase in mass was shown to occur by dry weight determination, and this increased to double that of control cells by 36 hours. Protein, DNA and RNA accumulated continuously in infected cells starting at 12 hours after infection, and at 36 hours after infection, the cell content of these was double that of controls.

Ginsberg and Dixon (1959) did not find a significant increase in RNA of HeLa cell monolayers after 36 hours of infection with type 4 Adenovirus. This difference may be due to the cell type used, the virus type used, or it may also be that the lack of RNA increase in Ginsberg's data is more apparent than real in that some early increase may have been masked by a subsequent loss of RNA from the cells.

Green (1959) found that infection of KB monolayers with Adenovirus type 2 resulted in a greater incorporation of P^{32} into RNA between the ninth and eighteenth hours after infection and a greater incorporation of P^{32} into DNA both between nine and eighteen hours after infection and eighteen to twenty-seven hours after infection. He interprets his data as indicating that infection has stimulated the incorporation of P^{32} into the phosphate pool of the cell between the ninth and eighteenth hour. At the same time, the acid soluble nucleotide and phospholipid fractions were also found to be more highly labelled in infected than in control cell cultures. Green

feels the prolonged increased uptake of P^{32} into DNA of infected cells may reflect a stimulated synthesis of a new DNA, possibly virus precursor.

Earlier studies on the characteristics of adenovirus infection indicated that the fluids of HeLa cell cultures infected with these viruses were more acidic than those from companion control cultures. (Rowe et al. 1955).

Fisher and Ginsberg (Fisher and Ginsberg, 1957) investigated this aspect of adenovirus infection using HeLa cells infected with Adenovirus type 4. It was found that lactic, α-ketoglutaric, pyruvic and acetic acids were produced in greater quantities by the infected cell cultures as compared to control cultures. Glucose was used at a greater rate in infected cultures. No study was made of the state of the virus at the time samples were taken for analysis, so it is not possible to correlate the changes reported with any phase of the growth cycle. Moreover, no glucose determinations were done in the experiments measuring the production of acids, other than lactic acid, so no quantitative comparisons among acids produced (presumably from glucose) can be made. The complexity of the media used in these experiments also makes quantitative comparisons unreliable.

Rozee et al. (1957), working with HeLa cell cultures infected with Adenovirus type 7, examined the effects of sodium fluoroacetate on the citric acid levels in infected and control cell lysates. The citric acid content of infected

cell lysates was found to be lower than in those of control cells, but it is higher in infected cell lysates, previously poisoned with fluoroacetate, than in control cells treated similarly. Rozee also showed that infected cell cultures took up glucose at a greater rate than did controls--for the first 24 hours after infection, but thereafter this rate of uptake decreased until by 60 hours, the rate of glucose uptake by infected cells was about one-half that of control cells. It was also shown that the pyruvate levels in the medium of infected cultures was lower than in controls for the first 30 hours after infection. This then began to increase to a value slightly higher than controls. Lactic dehydrogenase activity was found to be higher in infected cell homogenates than in those of control cells at the period 48 hours after infection.

In all of his experiments Rozee used a low titer inoculum (50 TCD_{50}) so it is not likely that synchronous infection was approximated. He interprets his results as indicating that the glycolytic processes are more active in the infected cells. The citric acid-fluoroacetate experiments present presumptive evidence of increased activity of the citric acid cycle. Since less citric acid was present in infected cells than in the control cells, there was presumably a greater turnover of citric acid in the infected cells. In support of this, it was found that more citric acid is present in the fluoroacetate poisoned, infected cells, than

in control, poisoned cells. It would be interesting to know at what point in the growth cycle of the virus these changes occurred. Also, it would be interesting to determine at what point the lactic dehydrogenase activity increased in relation to the content of pyruvic acid in the culture medium.

Metabolic Changes Associated with the Growth of Other Viruses

Poliovirus.

Maassab and his associates have carried out detailed studies on metabolic alterations of HeLa cells following infection with massive doses of Poliovirus type 1. (Maassab and Ackermann, 1957; Maassab, Loh and Ackermann, 1957; Ackermann, 1958; Ackermann, Loh and Payne, 1959).

An increased rate of incorporation of P^{32} into nuclear RNA and DNA and into cytoplasmic RNA was detected after one hour of infection. The enhanced rate of incorporation of P^{32} into nuclear RNA continued until the fourth hour, at which time an abrupt decline in activity was observed. At about this time, infective virus starts to accumulate in the cytoplasm. Incorporation of P^{32} into nuclear DNA began to fall off after about the second hour and fell below normal by the fifth hour after infection. The enhanced rate of incorporation of P^{32} into cytoplasmic RNA increased until the sixth hour. There was then a decline just before virus release began.

Since over 50% of the infective virus is formed

between the sixth and the seventh hours; that is, after the synthesis of cytoplasmic RNA has ceased, it is apparent that the synthesis of RNA is not the rate limiting reaction in formation of infective particles. No net increase in the nuclear RNA or DNA occurred in the infected cell but a drametic net synthesis of cytoplasmic RNA occurred. Net synthesis of protein in all cytoplasmic fractions was also found.

The authors point out that the increases in protein and RNA are much greater than can be accounted for by the virus particles produced. They also showed that the major portion of the RNA formed must be of a composition different from that of poliovirus.

Work done in other laboratories lends some support to the findings summarized above. There are, however, a number of reports which are not in agreement with Maaseab's findings.

Becker and his associates (1958) studied the effect of poliovirus type 2 infection on the phosphorous uptake and glucose utilization of human amnion cells. They report that infected cultures take up more phosphorus than do control cultures for the first ten hours after infection. This phosphorous uptake then decreases to values below those for controls.

Miroff et al. (1957) reported an increased uptake of radiophosphorus into the total nucleic acid fraction of

HeLa cells infected with Poliovirus type 1 for periods up to four hours.

Goldfine et al. (1958) found that the incorporation of labelled cytidine into HeLa cells infected with massive doses of Poliovirus type 3, was diminished during the first 5½ hours after infection.

Rothstein and Manson (1959), working with type 2 Poliovirus and HeLa cells, were not able to confirm the reports of increased RNA synthesis and increase of P^{32} incorporation into the nucleic acid fractions. They were, however, incubating the cells at 25°C in order to inhibit viral release, and the cultures were examined after 48 hours, rather than at regular intervals during the virus growth cycle.

Salzman and Lockart (1959) found no significant increase in the RNA of infected cultures. They also failed to observe a more rapid turnover of RNA or DNA in infected as compared to non-growing, control cultures. Since this work was carried out using suspension cultures, it is possible that this disagreement with the findings of Maassab, who used monolayer cultures, may reflect differences in the metabolic state of the cultures used. Another point which might contribute to differences in results is the medium used. Some workers, including Maassab and his associates, held cells in a medium which does not allow continuous growth and cell division, while others used a medium which could support continuous growth. Graham (1959) points out the

necessity of controlling the physiological state of cells used for study of metabolic alterations caused by virus infection. These differences could perhaps be explained on that basis.

Recently work has been done on the source of Poliovirus RNA and protein. These have been shown to originate from the soluble intracellular pool of nucleotides and amino acids. (Salzman and Sebring, 1961; Darnell and Levintow, 1960).

Darnell et al. (1961) investigated the time course of synthesis of poliomyelitis RNA in HeLa cell suspension cultures. They found that viral RNA synthesis precedes appearance of mature virus by no more than one-half hour, and at no time is there a large amount of free viral RNA. This is in sharp contrast to studies made on other viruses. They also showed that the synthesis of virus protein and RNA began at about the same time and proceeded in parallel. The authors point out that the synthesis of viral macromolecules and maturation of virus are more closely coordinated in time than for any other animal virus system so far studied.

This evidence of the close coordination in time between virus protein and nucleic acid synthesis, together with evidence that Poliovirus contains 60 protein subunits (Finch and Klung, 1959) and likely only one RNA molecule (Boeye, 1959) is used by Darnell et al. (1961) as a basis for the hypothesis that the original RNA of the infecting particle does not serve as a template for virus protein replication. The authors point out that if each RNA molecule served as template in the formation of virus

protein, one might expect a considerable lag in time between RNA and protein synthesis. They state that the template function could be performed by the first viral RNA synthesized, by a fragment of this, or even by RNA of a different molecular species entirely. In this latter case, the synthesis of RNA, which would serve as template for virus protein, might be stimulated, along with synthesis of viral RNA, by the original RNA species entering the cell. The concept that viral RNA is formed first and then acts as a template for protein synthesis seems unlikely, since the bulk of the RNA and protein appear to be formed simultaneously.

It is interesting to note here that the work of Maassab and Ackermann (1957) lends some additional support to this hypothesis. They found that the increase in cytoplasmic RNA appeared to occur at one rate for the first four hours after infection; then this rate increased from the fourth to the sixth hours. This might suggest the synthesis of two different types of RNA.

Investigations of carbohydrate metabolism of Poliovirus infected cells were carried out by Becker and his associates (1958). They found that infected cultures took up more glucose than did uninfected cultures for the first ten hours after infection. This uptake then dropped to a value similar to that of control cultures by 24 hours after infection.

They also report work done with metabolic inhibitors. Potassium cyanide (10^{-3} Molar) inhibited neither virus growth nor the development of cytopathic changes in the cells, but it did stimulate the uptake of glucose by infected and by control cultures. Sodium azide and sodium fluoroacetate also stimulated glucose uptake but no mention is made of their

effect on virus synthesis. Iodoacetate was found to inhibit the production of virus completely and to depress glucose utilization in both infected and control cultures. Sodium fluoride also was found to depress glucose utilization, but here again no report of its effect on virus production was made.

The authors interpret these results as indicating that glycolysis is very important in the synthesis of Poliovirus.

In support of this, Gifford and Syverton (1957) reported that Poliovirus replication can occur in HeLa and monkey kidney cells under strict anaerobic conditions. Levy and Baron (1957) reported that poliomyelitis type 3 infection of monkey kidney cell cultures causes an initial stimulation of lactic acid production under both aerobic and anaerobic conditions.

Concerning the relation between cellular respiration and virus synthesis Gifford, Robertson and Syverton (1954) report that Poliovirus synthesis is greatly inhibited under anaerobic conditions. In a later report (Gifford and Syverton 1957), it was shown that the inhibition mentioned above was not complete. The latent period of virus development under anaerobic conditions is extended, as compared to that under aerobic conditions. This perhaps explains the "apparent" inhibition reported in the first article. It is interesting that the yield of virus per cell was shown not to be altered by removal of oxygen from the system. This delay in virus production shows that a limiting reaction is involved.

Gifford (1960) presents results which indicate that no change in the respiration of poliovirus infected cells, as compared to controls, occurs until the seventh hour after infection. At this time, most of the virus has been produced and the release of virus has begun. Thus, the change can be considered as a result of cell damage rather than one intimately connected with virus reproduction.

Herpes simplex.

Fisher and Fisher (1959, 1961) studied the effect of infection of HeLa cell cultures by Herpes simplex virus on glucose utilisation and organic acid production. The initial study indicated that herpes-infected HeLa cells produce more organic acids than controls when glucose is the principal substrate.

This work was extended in the latter study to include C^{14} labelled glucose as the substrate. The infected cultures (DMB-nasal epithelial cells) showed increased lactic acid production over controls but the specific activity of the lactate recovered, was the same for both control and infected cultures. This implies that the lactate is produced by the same pathway in both cases. Acetate and pyruvate were labelled only in trace amounts suggesting that they did not accumulate as a result of glucose dissimilation. The specific activity of CO_2 liberated by infected cultures was greater than that of uninfected cultures when glucose $C_1{}^{14}$ was used as substrate.

This would indicate a stimulation in pentose shunt metabolism following infection. This finding was reinforced by studies on specific activity of CO_2 liberated when glucose $C_6{}^{14}4$ was the substrate. In these cases the specific activities were low.

The authors did not investigate the state of the virus at the sampling intervals, and, although they felt the inoculum was sufficiently concentrated (about 0.25 pock forming units per cell) to initiate one-step growth conditions, no results are presented which confirm this.

Ackermann and Francis (1950) present results of a study of biochemical changes which occur in host tissue during infection by herpes simplex. They found that nucleic acid synthesis was stimulated and a change in the normal ratio of succinoxidase to α-ketoglutaric acid oxidase activity occurred. The samples for this study were taken three days after exposure to virus and the tissues used were heart and liver of the chick embryo. These observations cannot be related to the cycle of virus multiplication.

Newton and Stoker (1958) investigated the nucleic acid content of HeLa cells at various intervals after infection with a high multiplicity of Herpes virus. While no significant increase in RNA occurred, the DNA content of the infected cells rose 6 to 9 hours after infection, before any increase in infective virus was detectable. By 72 hours the cells contained nearly double their normal content of DNA. This increase was confined to the nucleus. The latent period

for herpes virus in HeLa cells was found to be more than 9 hours with a rise in infectivity at 12 hours. The release phase began after 16 hours. Again it is pointed out that the DNA formed in infected cells is far in excess of that accounted for as virus.

Smith and Kun (1954) showed that infection of the chorioallantoic membrane of chick embryos with herpes simplex did not alter the oxygen upteke. It did, however, cause increased production of lactic acid by the membrane and caused an increase in the activity of hexosediphosphate fermentation by homogenates of the membrane.

Pseudorabies.

Pseudorabies, a virus closely related to herpes simplex, Kaplan and Vatter (1959) was studied by Kaplan and BenPorat (1959). They investigated the effect of this virus on the nucleic acid content of rabbit kidney cells. The results indicate that a net synthesis of DNA is detectable toward the end of the latent period and continues during the exponential rise in virus infectivity. This increase in DNA was shown to be accompanied by nuclear proliferation.

Myxoviruses.

Magill and Francis (1936) reported that influenza will not multiply in minced chick embryo tissue maintained under anaerobic conditions. Parodi et al. (1948) found no significant difference in the oxygen consumption of hen's

eggs infected with Influenza virus. Pinkerton et al. (1950) found an increased oxygen consumption by influenza infected eggs, for the first 24 hours after infection. Greiff et al. (1950) reported that a toxin is liberated into the allantoic fluid of Influenza virus infected eggs. This toxic agent was shown to affect oxygen consumption of a new series of eggs.

Results such as these are difficult to interpret because of the complexity of the systems used. Ackermann (1951) infected intact chorioallantoic membranes with Influenza A (PR8 strain), and investigated the effect on the metabolism and respiration of the whole membrane. No significant increase in virus titer occurred in the absence of oxygen. Malonate and antimycin A, substances which inhibited the respiration of the tissue, but not glucose utilization at the concentrations used, were found to inhibit viral multiplication also. This suggests that reactions of the Krebs cycle are important in virus synthesis. Eaton and Perry (1953) and Ackermann and Johnson (1953) found that exposure of influenza infected chorioallantoic membranes to dinitrophenol reduced their capacity to produce virus. Since this substance is known to stimulate adenosine-triphosphatase with the release of inorganic phosphate, these results were interpreted as indicating that the energy required for virus synthesis is derived from the oxidative phosphorylation activity of the host tissue.

It is, however, difficult to interpret results such as these since the effect of the inhibitor on the ability of the cell to survive is uncertain. Tamm (1956) reports macroscopic damage of cells of the chorioallantoic membrane being held under anaerobic conditions. Thus, energy of oxidative phosphorylation in the form of ATP may be essential for chorioallantoic cell survival. If the addition of an uncoupling agent, such as dinitrophenol, causes cell death, surely it is not surprising that no virus synthesis occurs. It would not be valid, then, to conclude on the basis of this work, that oxidative phosphorylation is directly essential for the synthesis of this virus.

Gifford and Syverton (1957) propose that the question of whether or not a given virus can multiply under anaerobic conditions may be dependent on the cell system used, rather than on the virus. They suggest that some cells may behave as obligate aerobes, whereas others may be capable of living as facultative anaerobes. The cells of the chorioallantoic membrane, then, might be classed as obligate aerobes, while HeLa cells and monkey kidney would be facultative anaerobes since they are capable of surviving under completely anaerobic conditions (Gifford and Syverton 1957). An hypothesis such as this, if proven, could explain many of the disagreements between results obtained regarding the requirement of oxygen for the synthesis of a number of viruses.

Wielgosz (1957) found that influenza A infected

chorioallantoic membranes took up more pyruvate than did uninfected membranes. No study of the point in the growth cycle at which this change occurred was reported.

Klemperer (1960) studied the effect of infection of chick embryo cells with Influenza virus on the metabolism and respiration. Within one and one-half hours after infection, when the complement fixing antigen first appeared, the rate of aerobic lactate formation was increased 50 to 100 percent without any change in the oxygen uptake. Aerobic glycolysis of control cells was stimulated approximately 100 percent by the addition of phosphate and 200 percent by the addition of phosphate plus adenosine monophosphate. Compared with control cells, aerobic lactate formation by infected cell cultures was 50 percent greater in presence of added phosphate but about the same in the presence of phosphate and adenosine monophosphate. The results are interpreted as indicating that the increase in glycolysis by infected cells is due to an increased supply of phosphate acceptor.

Experiments with C_1^{14} and C_6^{14} labelled glucose (klemperer, 1960) indicate that less than 10 percent of the glucose taken up by the control cells is oxidized via the pentose phosphate pathway and that this pathway is not an important pathway of ribose synthesis for RNA in either infected or control cells. However, within 3 hours after infection, when the viral haemagglutinin appeared, the rate of direct oxidation of

glucose-phosphate increased by 30 to 60 percent. There was no increased dehydrogenase activity for glucose-6-phosphate or 6 phospho-gluconate in infected cells. Methylene blue greatly increased the rate of direct oxidation of glucose. This is interpreted as indicating that glucose breakdown via this pathway is limited by the rate of regeneration of TPN[3] from the reduced coenzyme.

Lepine and Bonissol (1960) found that KB cells infected with Parainfluenza virus exhibited a marked increase in aerobic glycolysis with no significant difference in respiration.

Smith and Kun (1954) found that infection of the chorioallantoic membrane of the chick embryo with Newcastle disease virus did not affect the oxygen consumption of the membrane, but caused stimulation of lactate formation and an activation of hexose diphosphate fermentation. This was also found following infection of the chorioallantoic membrane with fibroma, herpes simplex, vaccinia, Rous sarcoma, swine influenza (Smith and Kun, 1954), and myxoma (Kun and Smith, 1950). In the latter case it was shown that the accumulation of lactate was accompanied by a greater activity of aldolase and the glycolytic enzymes causing the formation of 3-phosphoglycerate from fructose-1,6-diphosphate. No significant difference in the activities of glucose-6-phosphate dehydrogenase or 6-phosphogluconate dehydrogenase could be detected between

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3. Triphosphopyridine nucleotide.

control and infected cultures.

Green (1958) found that cells carrying Newcastle disease virus exhibited a marked increase in aerobic glycolysis with no significant change in respiration.

Avian pox virus.

Kun et al. (1960) investigated the effect of infection of the chorioallantoic membrane with Avian pox virus. Again they found an increase in aerobic glycolysis. They also showed, by experiments measuring liberation of $C^{14}O_2$ from glucose $C_1{}^{14}$ that the pentose phosphate pathway is operating at a greater rate in virus infected membranes than in the normal ones. Homogenates of infected as compared to control membranes also showed these differences. Studies of this system revealed that in addition to the DPN[4] linked lactic dehydrogenase activity of chorioallantoic membrane, a TPN linked lactic dehydrogenase activity exists in both infected and control membranes. The results are interpreted as indicating that the TPN linked activity of lactic dehydrogenase is more active in the infected than in the control membranes.

It was observed that the evolution of CO_2 and the activity of the lactic dehydrogenase increase in parallel in infected membrane homogenates. The authors state that the reoxidation of reduced TPN is the rate limiting reaction of the pentose phosphate pathway. They present the hypothesis that

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4. Diphosphopyridine nucleotide.

the increase in TPN linked lactic dehydrogenase activity increases the amount of exidized TPN available and therefore causes increased turnover of the pentose phosphate pathway. The result of this increased turnover would be increased synthesis of pentoses which could be used for the synthesis of virus nucleic acids. Klemperer's report (1960) that the pentose pathway is not important in ribose synthesis for RNA in either influenza virus infected or uninfected chick embryo cells argues against this hypothesis, but additional evidence is necessary before a more definite conclusion can be made regarding other virus-host cell systems.

Experimental studies of the metabolic alterations occurring in cell cultures infected with viruses have thus revealed a number of features which are common to the majority of virus-cell systems. Stimulation of RNA or DNA synthesis seems to follow infection with RNA or DNA viruses respectively. However, since studies of this kind have been restricted to a few viruses only, the acceptance of this as a general rule would be somewhat premature.

The stimulation of glycolysis following virus infection is a much more common observation. It is highly possible that this could be due, at least in part, to an increase in activity of several glycolytic enzymes, as shown by Kun (1950) in the case of infection with Myxoma virus.

Most studies carried out so far, have reported no difference in the oxygen consumption of infected as compared to uninfected cells. While this does not eliminate oxygen as an essential element for viral synthesis, it has been shown (Gifford and Syverton, 1957) that Poliovirus, at least, can replicate in cells held under anaerobic conditions.

The observation that direct oxidation of glucose-phosphate increases after infection has been reported for three animal virus-host cell systems. Both Kun (1960) and Klemperer (1960) present evidence suggesting that the rate limiting reaction of this pathway, as operating in virus infected cells, is the regeneration of reduced TPN. If the increase in activity of the TPN linked lactic dehydrogenase, as reported by Kun (1960) for avian pox virus, is common to virus infected cells in general, the increase in glycolysis and the increase in direct oxidation of glucose-phosphate may be interdependent upon the relative amounts of reduced or oxidized TPN present. The increase in "concentration" of TPN linked lactic dehydrogenase and several glycolytic enzymes, the synthesis of which is presumably stimulated by intracellular presence of the virus, would perhaps cause a shift in the regulatory mechanisms of the normal cell to a state more suitable for the synthesis of virus particles.

These observations add substance to the hypothesis (Rivers, 1928; Newton and Stoker, 1958; Rivers, 1959; Ackermann, 1959) that infection of a cell stimulates the

metabolic mechanisms in some manner, causing the cell to synthesize large quantities of material both of an infectious and noninfectious nature. It is probably this upset of the controlling mechanisms of the cell, leading to diversion of essential cell components to compounds not utilizable by the cell, that causes the cell destruction which is so frequently observed.

MATERIALS AND METHODS

MATERIALS AND METHODS

Preparation of Cell Cultures.

First and second generation dispersed cell cultures were prepared from human amniotic membranes as described below. A transformed line of the human amnion cell (strain FL[1], Fogh and Lund, 1957) was also used in this study.

Human placentae were collected from the maternity ward of the Winnipeg General Hospital and were treated by technicians in the Virus Laboratory as follows. The amniotic membrane was stripped off and washed several times in Hank's basal salt solution (See Appendix A). It was then placed in 30 to 40 milliliters of 0.25 percent trypsin solution (Appendix A) and incubated at 37° C. for 30 minutes. Following this "predigestion," the membrane was removed from the trypsin and adhering mucous was stripped off. The membrane was then placed in 400 to 500 millileters of 0.25 percent trypsin and incubated at 37° C. for 3 to 4 hours. The suspension was agitated manually at frequent intervals to promote removal of cells from the membrane. Following this digestion period, the cell suspension was decanted off and centrifuged at 1000 r.p.m. for 10 minutes. The supernate was discarded and the cell pellet was transferred to propagating medium and magne-stirred. The resulting cell suspension was dispensed into screw-cap milk dilution bottles. These milk dilution bottles were of 160 milliliter capacity,

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1. Obtained from Microbiological Associates Incorporated, Bethesda, Maryland.

10 milliliters of suspension being placed in each bottle. No cell counts were performed, but a crude estimate of the number of cells present was made by determining the packed cell volume of the cells present in the original suspension. One milliliter of packed cells would suffice for about 15 bottles. The cultures were incubated at 37° C. for two or three days. The fluid was then removed and replaced with fresh propagating medium. After an additional three to five days incubation, a confluent sheet of cells had usually formed and the cultures were ready for further manipulation. It was these primary cell cultures which were used in experiments measuring respiration.

Secondary amnion cell cultures were prepared as follows: The fluid was removed from primary cell cultures and 0.25 percent or 0.5 percent trypsin solution (10 milliliters per bottle) was added. When the cell sheets had detached from the bottle, the fluids containing these were pooled and the cell sheets dispersed by drawing the fluid up into a syringe and expelling it quite forcibly about 10 times. Alternatively, the trypsin solution containing the cell sheets was thoroughly mixed by drawing the mixture up into a pipette and expelling the fluid, the procedure being repeated about 10 times. The suspension was then centrifuged at 1000 r.p.m. for 10 minutes and the supernate was discarded. The cells were then suspended in propagating medium and magne-stirred. A Cornwall automatic syringe was used to dispense this cell

suspension into roller tubes, 0.5 milliliters per tube. No cell counts were done on the suspension but 20 to 30 tubes could generally be made from one bottle of cultured cells. The tubes were stoppered with rubber corks, placed in stationary racks and incubated at 37° C. at a slight angle to the horizontal. After 3 or 4 days the cultures were ready for use.

The FL cells were serially propagated, the number of cells doubling with each passage. The transfer was carried out in exactly the same way as was the change from primary amnion cell cultures to secondary amnion. The only differences were the composition of the propagating medium and the transfer of the FL cells to bottles rather than tubes. Difficulties in culturing these cells were, however, frequently encountered.

Cell Counts.

In every experiment a cell count on 2 to 4 randomly selected cultures was carried out. The fluid was removed from the cultures and 1 milliliter of 0.25 percent trypsin solution was added to each tube. The cultures were then incubated at 37° C. for 30 minutes. Following this period, the tubes were cooled in an ice water bath to inhibit the action of trypsin. The cell sheets were broken up as described above and counts were performed on the resulting suspension, using a Spencer Bright-Line Haemacytometer. The number of

cells in bottle cultures was estimated in exactly the same manner, but ten milliliters of 0.25 percent trypsin solution were added instead of one milliliter.

Counts on individual tubes varied by 20 to 50 percent from the mean, while counts on bottles of cultured cells, in which the population is very much greater, agreed to within 20 percent of the mean.

Media.

Human amnion cells were propagated in a medium consisting of 60 percent Hank's basal salt solution, 20 percent horse serum, 20 percent tryptose phosphate broth, 0.2 percent glucose and .05 percent yeast extract. The medium used to propagate FL cells differed only in the concentration of Hank's basal salt solution (70 percent), in the serum concentration (10 percent) and in the concentration of yeast extract (.01 percent).

Scherer's maintenance medium, (Syverton et al., 1954) (with parenamine, glycerol and pyruvate omitted) supplemented with 5 percent horse serum, was used to maintain human amnion cell cultures. Eagle's minimal essential medium (Eagle, 1959) with 2.5 percent horse serum, was used to maintain both human amnion and FL cell cultures. Details of the preparation of these media are presented in Appendix A.

Penicillin and streptomycin were added to all media, the final concentrations being 100 units per milliliter and 50 micrograms per milliliter respectively.

Preparation of Virus Pools.

The GB strain of Adenovirus type 3 was obtained from the American Type Culture Association. A stock virus was prepared from this and maintained by serial passage in cell cultures, as follows: Several bottle cultures of human amnion cells were infected with a dilution of the virus in Scherer's maintenance medium (See Appendix A). The cultures were incubated at 37° C. until maximal cytopathogenic effects were noted. This took from 4 to 8 days. The cultures were then frozen and thawed three times to release intracellular virus, the fluids pooled and centrifuged at about 1,500 r.p.m. for 30 minutes to remove cell debris. Aliquots of the supernate were then stored at -20° C. Pools prepared in this manner were used for all experiments in which infected human amnion cells were under study.

A separate virus pool was made in similar fashion by infecting bottle cultures of FL cells with a dilution of the virus in Eagle's maintenance media. This pool was used for all experiments in which FL cells were under study.

Virus Assay.

Samples to be assayed for virus infectivity were stored at -20° C. until titrations could be carried out. For estimation of the virus titer, serial whole log (1:10) or half log (1:3.2) dilutions of the sample were made using Scherer's maintenance medium as diluent. Four amnion cell cultures, from which the fluid had previously been removed,

were infected with each dilution, one milliliter per tube. The cell cultures were incubated at 37° C. for 10 days and were then examined under low power lens for signs of cytopathic effect. The criterion for a positive cytopathic effect was the presence of at least one easily discernable zone of cellular degeneration, typical of adenovirus infection. Fifty percent end points were calculated according to the method of Kärber (1931), and were expressed in cytopathogenic doses per milliliter (CPD_{50} units).

Chemical Analyses.

Samples for glucose and lactic acid determination were stored at -20° C. until analysis could be carried out. They were then thawed and deproteinized (Somogyi, 1952) by adding 7 milliliters of distilled water, 1 milliliter of 10 percent sodium tungstate and 1 milliliter of 7 percent copper sulphate pentahydrate to each milliliter of maintenance medium being analysed. The resulting suspension was then thoroughly mixed using a glass plunger and allowed to stand for 30 minutes or longer. The mixture was centrifuged at 1500 r.p.m. for 10 minutes and an aliquot of the supernate was used directly for lactate estimation. An aliquot of a 1:2 dilution of this deproteinized sample was used for glucose estimation.

In each experiment, samples of the maintenance medium before and after addition of glucose were saved and frozen at -20° C. The former served as a blank sample in glucose estimation, while the latter served as blank for

lactic acid determination and as a sample for estimation of the initial glucose concentration.

Lactate Determination. Lactic acid was determined according to the method of Barker and Summerson (1941), as modified by Umbreit et al. (1957). One milliliter of the protein free solution was transferred to a 150 by 16 millimeter test tube containing one milliliter of 20 percent copper sulphate pentahydrate and the volume was made to 10 milliliters with distilled water. Approximately 1 gram of powdered calcium hydroxide was added and the mixture was stirred thoroughly using a glass plunger. After 30 minutes or more, the mixture was centrifuged at 1500 r.p.m. for 10 minutes and one milliliter of the supernate was removed with care so as not to include any of the precipitate. This aliquot was carefully layered over the surface of 6 milliliters of concentrated sulfuric acid in another 150 by 16 millimeter test tube. The acid had been cooled previously by immersing the lower half of the tube in an ice water bath. With the tube remaining in this ice bath, the two layers were quickly and thoroughly mixed. The tubes were covered with Morton stainless steel culture tube caps, heated in a boiling water bath for 5 minutes and immediately cooled to about 15° C. After the caps were removed from the tubes, 0.1 milliliters of para-hydroxydiphenyl reagent (see Appendix B) and 0.1 milliliters of 4 percent copper sulphate pentahydrate were added. These reagents were then thoroughly dispersed in the sulfuric acid

using a glass plunger, and the mixture was incubated at 30° C. for 20 minutes. During this interval the para-hydroxydiphenyl was dispersed once again. The tubes were then capped and heated in a boiling waterbath for 90 seconds to destroy excess para-hydroxydiphenyl. The tubes were immediately cooled and the samples were decanted to colorimeter tubes. The optical density was then determined using a Klett-Summerson photoelectric colorimeter containing a number 54 filter which transmits light of wavelengths from 520 to 580 millimicrons. A standard curve was constructed with lithium lactate. Care was taken to ensure that pipettes, tubes and glass plungers had not been contaminated with lactic acid due to handling. Duplicate determinations were done on each sample and differed by less than 0.3 micromoles of lactic acid.

Glucose Estimation. Glucose was estimated using Somogyi's copper reagent (Somogyi, 1945) and Nelson's arsenomolybdate color reagent (Nelson, 1944) (see Appendix B). One milliliter of the 1:2 dilution of deproteinized sample was placed in a colorimeter tube. One milliliter of copper reagent was added and the tubes were covered with stainless steel culture tube caps. The tubes were heated for 10 minutes in a boiling water bath and immediately cooled to about 15° C. One milliliter of the color reagent was added and the solutions were mixed using a glass plunger. Distilled water was used to rinse the plunger of traces of the solution and to make the volume in the tubes up to 10 milliliters.

After mixing again, using a glass plunger, the optical density was determined using a Klett-Summerson photoelectric colorimeter containing a number 50 filter which transmits light of wavelength 470 to 530 millimicrons. Duplicate determinations were done on each sample and differed by less than 0.3 micromoles of glucose.

Inhibitor Studies.

Oxamic acid, a competitive inhibitor for lactic dehydrogenase (Hakala et al., 1953; Novoa et al., 1959; Winer and Schwert, 1959; Papaconstantinou and Colowick, 1961) was employed at concentrations of 8×10^{-2} Molar and 4×10^{-2} Molar in Eagle's minimal essential medium. (See Appendix A). The pH of this solution was adjusted to 7.0, as judged by the phenol red indicator, by the addition of 1 Normal sodium hydroxide. The isotonicity was maintained by reducing the sodium chloride concentration of the Hank's basal salt solution, used to make up the Eagle's medium. Thus, the final solution differs from Eagle's minimal essential medium, only in the presence of oxamate and in the lesser concentration of chloride ion.

Manometric Methods.

The oxygen uptake of cells in suspension was determined using Warburg's apparatus[2] and conventional manometric techniques described by Umbreit et al. (1957). The suspending medium was Krebs-Ringer salt solution buffered

2. Bronwill Warburg-Apparatus, Model UV.

at pH 7.4 with phosphate (See Appendix A). In experiments which included carbon dioxide in the atmosphere of the flask, bicarbonate was also added to the Krebs-Ringer-phosphate solution (See Appendix A). Cells cultured in bottles were used in each experiment. A uniform cell suspension was prepared from the monolayer cultures as described previously. The cells suspended in trypsin were centrifuged at 800 r.p.m. for 5 minutes and washed once in Krebs-Ringer-phosphate or Krebs-Ringer salt solution buffered with phosphate and bicarbonate. In each case, the cell suspension to be placed in the Warburg vessels was magne-stirred continuously as aliquots were taken. A 1:10 dilution of these cell suspensions was made and the number of cells present was counted in a Spencer Bright-Line Haemacytometer.

The temperature of the water bath of the Warburg apparatus was maintained at 37° C. and the vessels were agitated at an amplitude of 4 centimeters at 140 strokes per minute.

EXPERIMENTAL PROCEDURES AND RESULTS

Glucose Consumption and Lactic Acid Production by Infected Cultures.

Although earlier studies have shown that adenovirus infected HeLa cell cultures produce more lactic and other organic acids and utilize glucose at a greater rate than do uninfected cultures, no similar work with the human amnion cell--Adenovirus type 3 system has been reported. Because of this, it was necessary to establish, by preliminary experimentation, whether or not an increased rate of glucose uptake and lactic acid production occurred following infection of amnion cell cultures with Adenovirus type 3.

Tube cultures of primary amnion cells, prepared in the same manner as bottle cultures, described earlier, were employed in this preliminary experiment. Each tube was infected with 1 milliliter of a 1:10 dilution of an Adenovirus type 3 pool, the diluent being Scherer's maintenance medium. Companion tube cultures received 1 milliliter of Scherer's maintenance medium containing no virus. The tube cultures were placed in stationary racks and incubated at 37° C. at a slight angle to the horizontal. At 5 and 10 day intervals after infection, 18 infected and 18 control tubes were removed from the incubator and frozen and thawed three times to release intracellular virus. Following this, the culture fluids from each set of tubes were pooled and lactic acid, glucose, and virus were assayed.

Glucose uptake was calculated by subtracting the glucose present at the selected interval after infection, from the glucose concentration of the original maintenance

medium (2000 micrograms per milliliter). In this experiment, as in following experiments, the blank determination for lactic acid estimation consisted of an assay of the lactic acid present in an aliquot of the maintenance medium (less than 10 micrograms per milliliter).

The virus multiplied since the initial concentration was $10^{4.5}$ CPD_{50} per milliliter while after 5 and 10 days the concentrations were $10^{4.75}$ CPD_{50} per milliliter and $10^{5.0}$ CPD_{50} per milliliter respectively.

Results of the calculation of glucose utilized and lactate produced by infected as compared to control cultures, are presented in Table I. As was reported for the Adenovirus--HeLa cell systems (Levy et al., 1957; Fisher and Ginsberg, 1957; and Rozee, 1957) a marked increase in lactic acid production and glucose consumption occurred in adenovirus infected amnion cell cultures as compared to uninfected ones.

TABLE I

GLUCOSE CONSUMED AND LACTIC ACID PRODUCED BY INFECTED AND UNINFECTED AMNION CELL CULTURES

	Days	µM Glucose Consumed	µM Lactate Produced
INFECTED	5	4.44	3.56
	10	4.86	4.56
CONTROL	5	3.06	1.67
	10	3.89	2.89

Glycolysis in Infected Cultures as Related to Virus Synthesis.

The preliminary experiment did not provide any information of metabolic change which could be assiciated with the cycle of virus replication. A number of experiments were therefore carried out to examine the glucose consumption and lactic acid production at various intervals during the growth cycle of the virus. The titer of virus inocula varied among experiments but was selected to be as high as possible to approximate one step growth conditions.

Typical experiments were carried out as follows: Secondary amnion cell cultures to be used in these experiments were examined using a low power microscope containing a 5.1 power objective lens and a 10 power ocular lens. Those cultures which were not suitable, due either to contamination or obviously poor growth, were discarded. The fluid present in the remaining cultures was removed. A 1:10 dilution of the virus pool to be used was made, using Scherer's maintenance medium as diluent. One milliliter of this was added to each tube to be infected. The cultures were then incubated at 37° C. to allow for adsorption of virus.

Earlier studies (Hannan, unpublished data) with a different strain of Adenovirus type 3 and human amnion cell cultures, indicated that between 80 and 98 percent adsorption could be achieved during a 2 hour incubation period, the extent of adsorption varying with the strength of the virus inoculum. The results were not confirmed with the GB strain of

Adenovirus type 3 since a wide variation in the extent of virus adsorption was noted among experiments. It was found necessary to allow an adsorption period of 4 to 8 hours to approximate one step growth conditions.

At the end of the adsorption period, the medium remaining on five cultures was removed, pooled and saved for titration. The residual virus was removed from the remaining infected cultures and they were washed twice with 1 milliliter of Hank's basal salt solution (without glucose) to remove traces of unadsorbed virus. It has been found (Hannan, unpublished data) that two washings are sufficient to remove all remaining virus. Either Scherer's maintenance medium or Eagle's minimal essential medium was then added to each of the infected cultures and they were incubated at 37° C. Glycerol and pyruvate were omitted from Scherer's maintenance medium since these substances could be used as precursors of lactic acid.

Control cultures were treated in a similar manner, the conditions differing only in two respects. First, the medium placed on the control cultures during the period allowed for viral adsorption to the infected cultures, contained no by-products of other infected cultures. Secondly, the control cultures were washed only once with Hank's basal salt solution without glucose. This was done to remove traces of glucose present in the medium originally covering the cells.

In each experiment the titer of the virus inoculum and the titer of the residual virus were determined by making

serial half log dilutions of the respective samples. The virus adsorbed was then calculated by subtracting the amount of the residual virus (antilogarithm of virus titer) from that of the viral inoculum. Since the virus dose and the mean number of cells per culture had been determined, the virus dose per cell could be calculated.

Cultures were then removed from the incubator at various intervals after infection and were frozen at -20° C. After all the samples had been taken, they were frozen and thawed three times to free intracellular virus. Following this, the samples were pooled, and glucose, lactic acid and virus were assayed. Results of these experiments are presented in Figures 1 to 5.

Scherer's Maintenance Medium. Experiments 2, 3, and 4 were carried out using Scherer's medium to maintain the cell cultures. The results are illustrated in Figures 1, 2, and 3. In experiments 2 and 3, (Figures 1 and 2) a 2 hour adsorption period was allowed and the amount of virus adsorbed per cell was low, being .003 CPD_{50} per cell and .01 CPD_{50} per cell respectively. The differences in glucose consumption and lactic acid production illustrated in Figure 1 are slight and since the experimental error of the assays for these substances approaches the magnitude of the differences, their significance is doubtful. In the second experiment in which there was likely a greater number of infected cells per culture, the differences tetween control and infected cultures were more pronounced. The glucose consumption of infected cultures was less than that of control cultures for the first

24 hours and equalled that of control cultures by the forty-eighth hour after infection. The glucose consumption thereafter increased until the fifth day. Lactic acid production by infected cultures exceeded that of control cultures during the second, third, and fourth days following infection.

Prior to the metabolic study of adenovirus infected FL cells, it was decided to investigate the glucose consumption and lactic acid production of adenovirus infected amnion cells maintained on Eagle's medium. The results are illustrated in Figures 4 and 5. While one-step growth conditions, as judged by the viral growth curve, were not approximated in either of these experiments, it is apparent from Figure 2, that the change in glucose and lactic acid metabolism by infected cultures, occurs during the logarithmic phase of virus growth.

Figure 3 illustrates the results of experiment 4 in which one-step growth conditions were more closely approximated. A four hour period was allowed for viral adsorption in this experiment. The amount of virus adsorbed per cell was much higher than in the previous experiments, being 0.67 CPD_{50} per cell. The glucose consumption was greater in infected cultures than control cultures and the lactic acid production was greater for infected cultures after the first 24 hours of infection. It is even more apparent in this experiment that the increased glucose utilization and lactic acid production by infected cultures, coincides with the

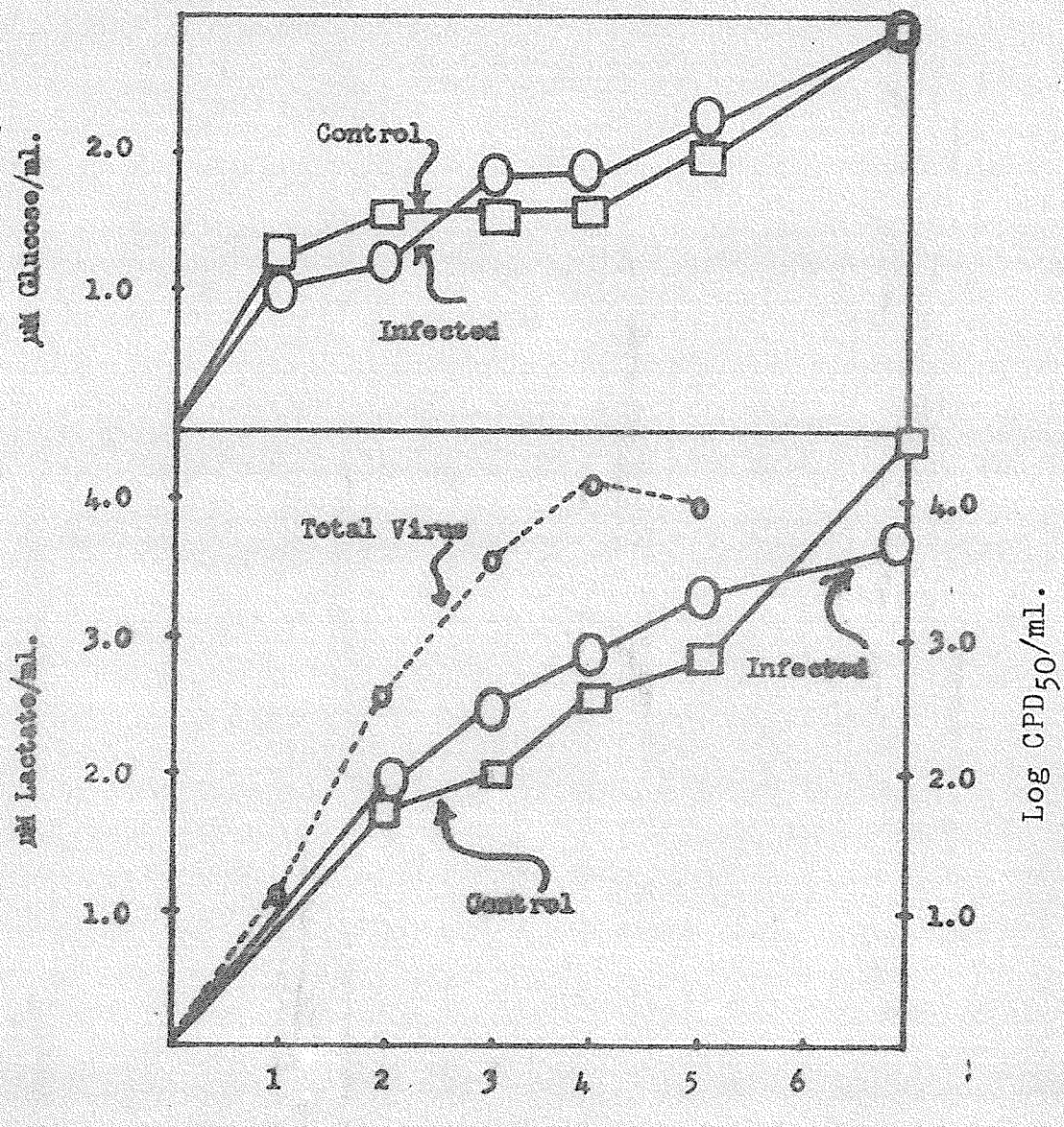

FIGURE 1

GLUCOSE UTILIZED, LACTIC ACID PRODUCED AND VIRUS PRESENT IN INFECTED AS COMPARED TO UNINFECTED CULTURES

45,000 CELLS/TUBE

.003 CPD_{50}/CELL

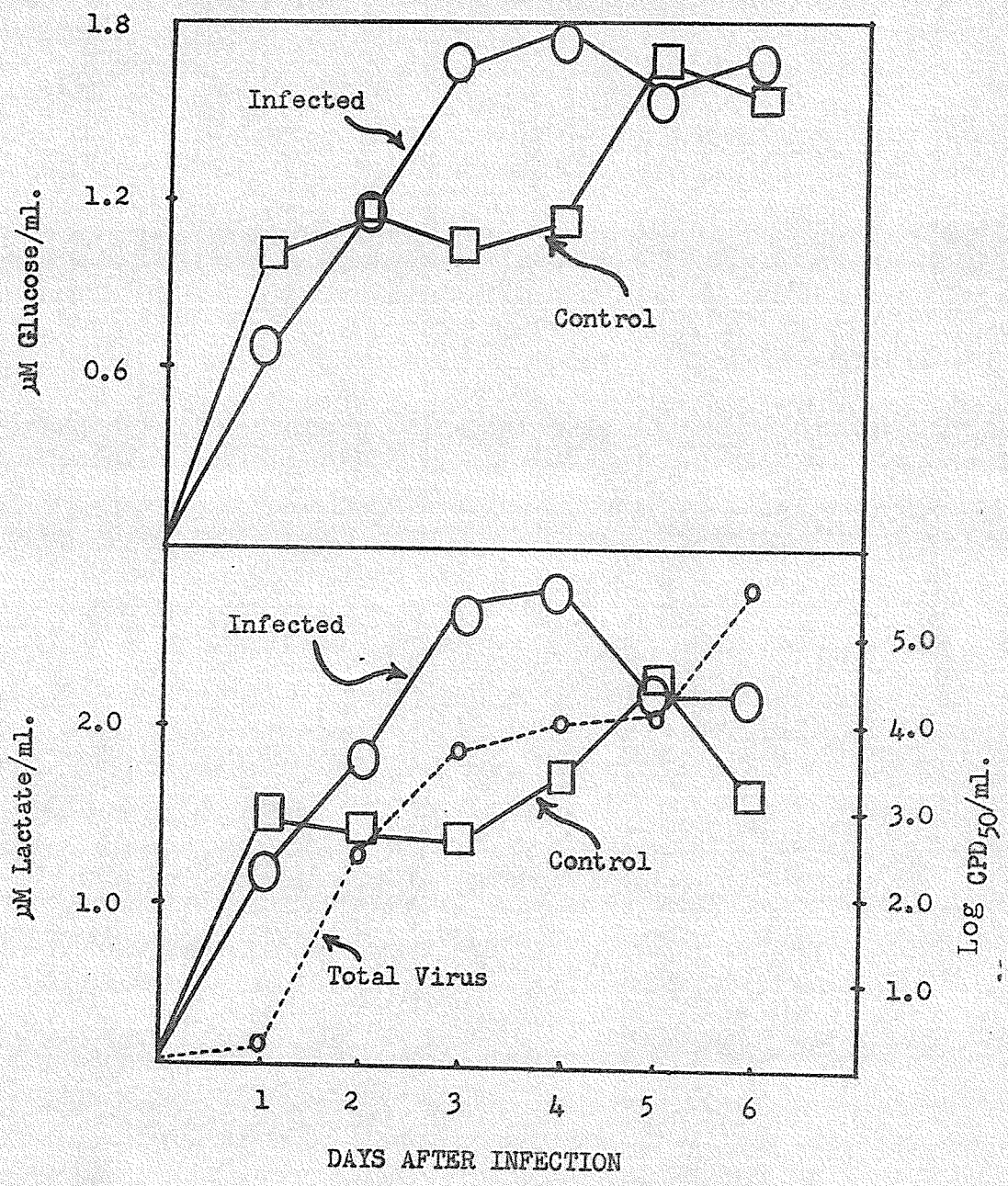

FIGURE 2

GLUCOSE UTILIZED, LACTIC ACID PRODUCED AND VIRUS PRESENT IN INFECTED AND CONTROL CULTURES

18,700 CELLS/TUBE

.01 CPD_{50}/CELL

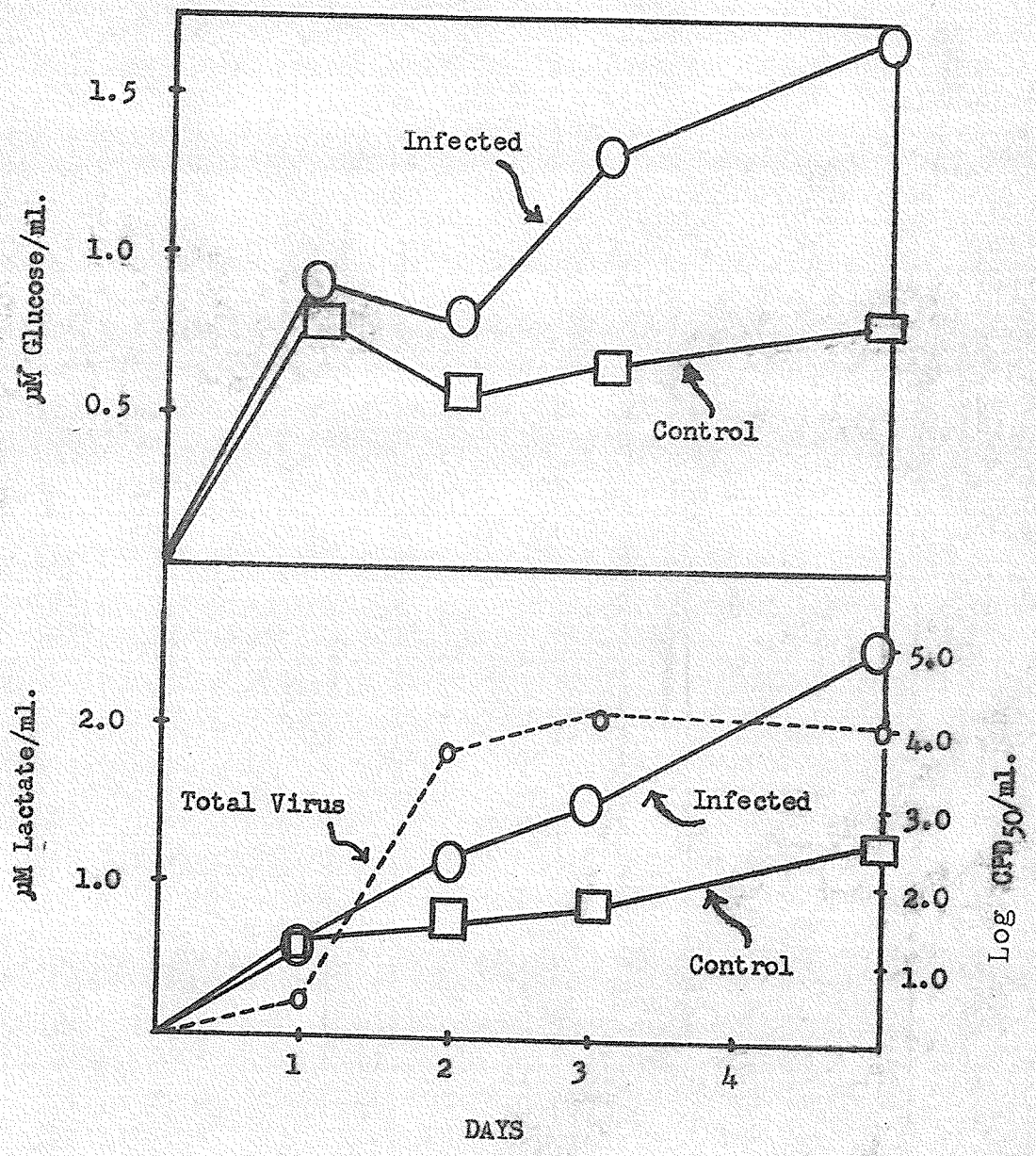

FIGURE 3

GLUCOSE UTILIZED, LACTIC ACID PRODUCED AND VIRUS PRESENT IN INFECTED AS COMPARED TO CONTROL CULTURES

75,000 CELLS/TUBE

0.67 CPD_{50}/CELL

beginning of the logarithmic phase of virus replication.

Eagle's Minimal Essential Medium. Experiments investigating the respiration of amnion cells were being carried out simultaneously with the metabolic experiments cited. Through these studies it became apparent that the cell numbers necessary for experiments measuring respiration and glycolysis at given intervals after infection, were extremely high. It was therefore decided to use the transformed line of the human amnion cell, strain FL, in metabolic and respiratory experiments. Preliminary work with this strain indicated that Scherer's medium would not maintain FL cell cultures but it was found that Eagle's minimal essential medium would serve this purpose.

Prior to the metabolic study of adenovirus infected FL cells, it was decided to investigate the glucose consumption and lactic acid production of adenovirus infected amnion cells maintained on Eagle's medium. The results are illustrated in Figures 4 and 5.

Experiment 5 (Figure 4) was carried out in exactly the same manner as were earlier experiments, the only difference being the replacement of Scherer's maintenance medium with Eagle's minimal essential medium. While the amount of virus adsorbed per cell was less in this experiment (.07 CPD_{50} per cell) than in experiment 4, one step growth was still approximated. This may indicate that the dose of virus per

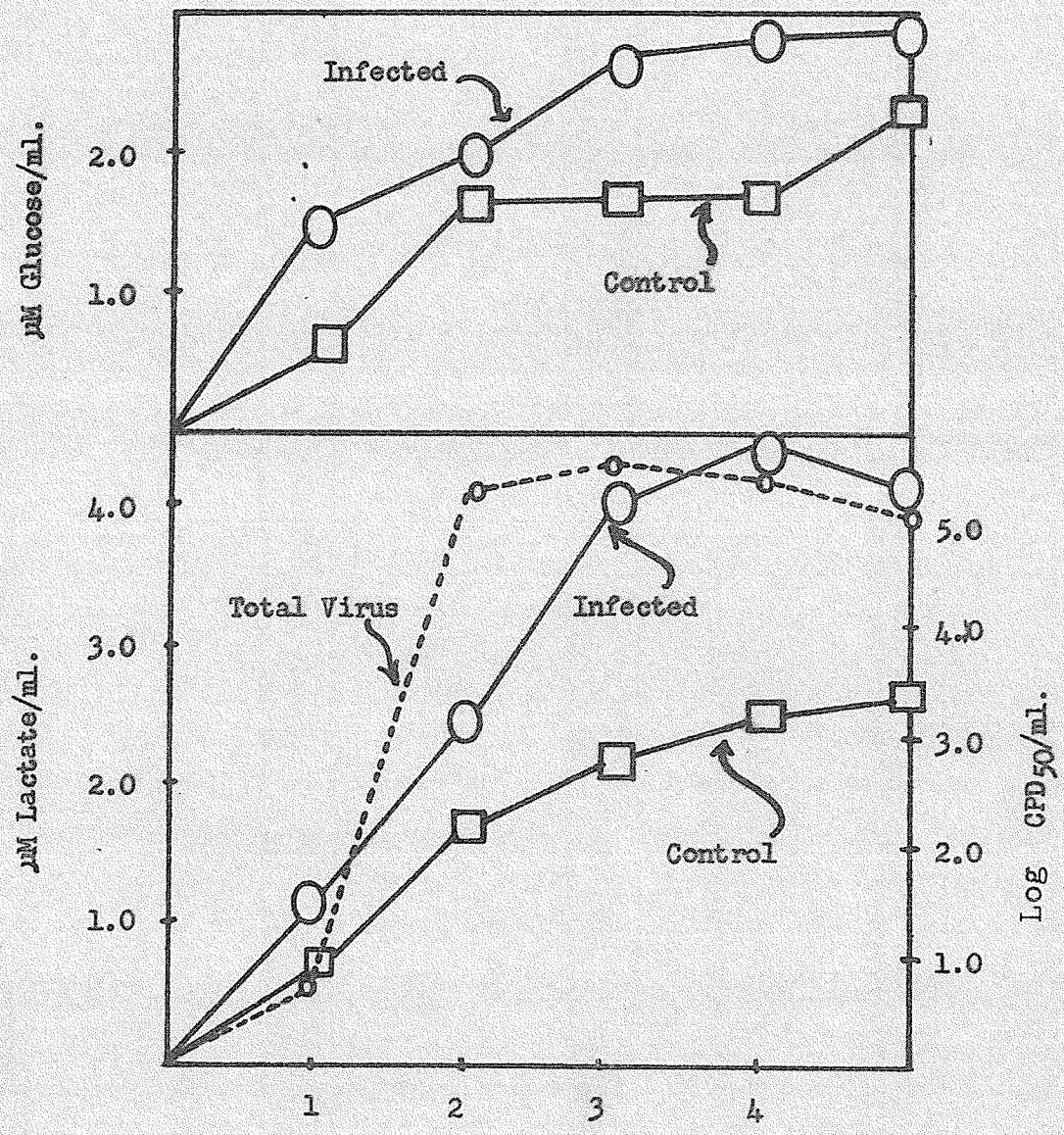

FIGURE 4

GLUCOSE UTILIZED, LACTIC ACID PRODUCED AND VIRUS PRESENT IN INFECTED AS COMPARED TO CONTROL CULTURES

94,000 CELLS/TUBE

.07 CPD_{50}/CELL

cell, achieved in experiment 4, is much higher than that necessary to approximate one-step growth conditions. The glucose consumption and lactic acid production were greater for infected than control cultures, the difference being detectable at 24 hours after infection, close to the beginning of the logarithmic phase of virus growth. By the seventy-second hour after infection, when the maximum amount of virus had been synthesized, the differences in glucose uptake and lactic acid production between control and infected cultures, had become comparatively larger.

Experiment 6, illustrated in Figure 5, was carried out with bottles of cultured cells. These were treated in essentially the same manner as were the tube cultures in previous experiments. Since the population is much greater in bottles of cultured cells, than in tubes, 10 milliliters of the 1:10 dilution of virus in Scherer's maintenance medium was used as inoculum. The bottles were washed twice with 10 milliliters of Hank's basal salt solution, and 10 milliliters of Eagle's minimal essential medium were added instead of one milliliter as for tube cultures. The virus adsorbed was again .07 CPD_{50} per cell and one step growth conditions were approximated. A greater consumption of glucose and an increased lactic acid production was noted for infected as compared to uninfected cultures. As in the previous experiment, this difference was detected after 24 hours of infection, close to the beginning of the logarithmic phase

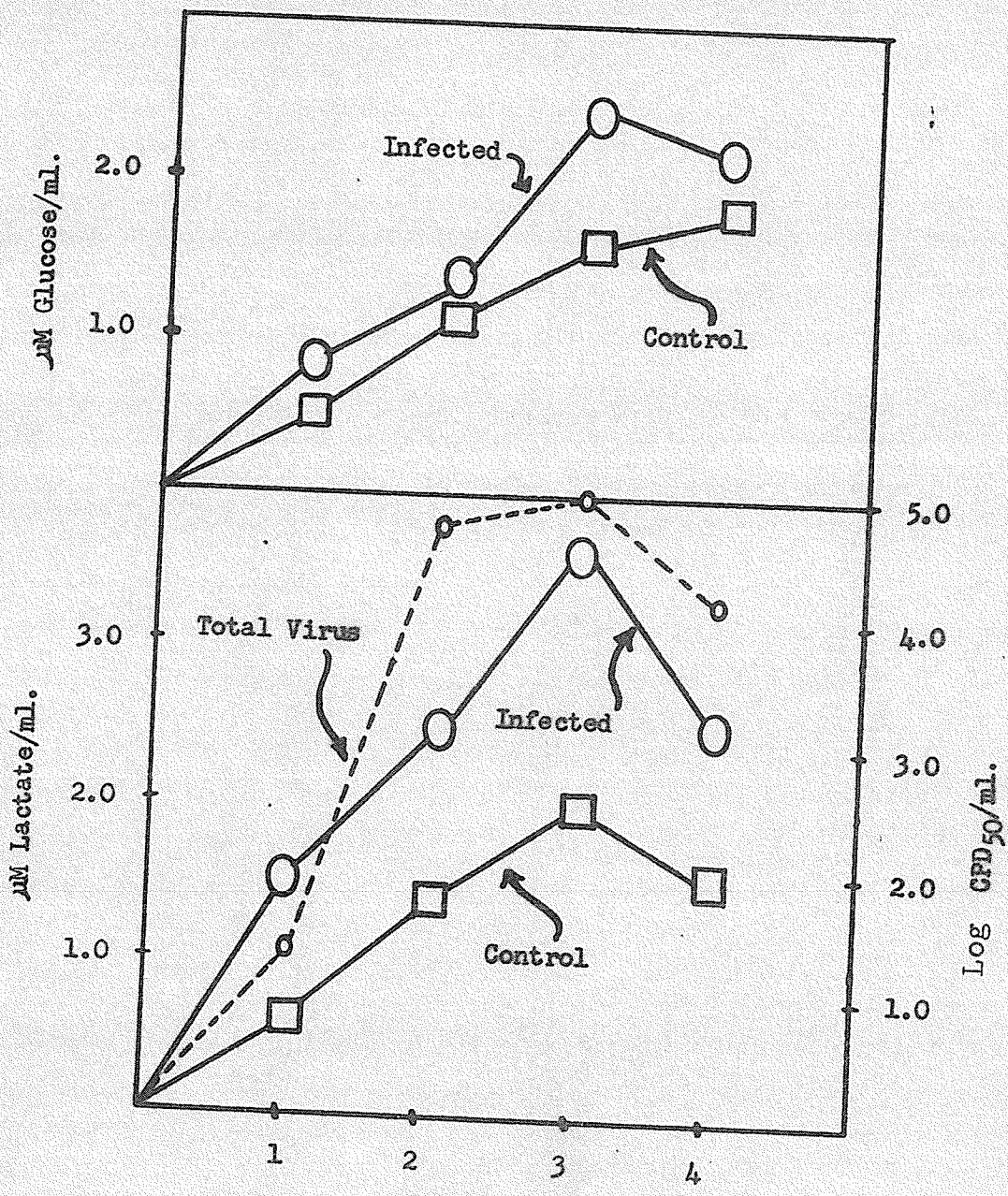

FIGURE 5

GLUCOSE UTILIZED, LACTIC ACID PRODUCED AND VIRUS PRESENT IN INFECTED AS COMPARED TO CONTROL CULTURES

2,000,000 CELLS/BOTTLE

.07 CPD_{50}/CELL

of virus growth.

In experiments 3, 4, 5 and 6 the greatest amount of virus was synthesized between the twenty-fourth and the seventy-second hours of infection. Attempts at quantitative correlation of glucose utilization, lactic acid production, or the amounts of glucose accounted for as lactate produced, with the amount of virus synthesized, did not reveal any consistent correlation among experiments. It seems very likely that the physiological state of the cultures varied among experiments since there was considerable difference in the glucose utilization and lactic acid production by control cultures. In some cases, cultures with fewer cells utilized more glucose or produced more lactic acid than did cultures with a greater number of cells in another experiment. This or some other uncontrolled factor may have masked any quantitative relationship between the extent of glycolysis and virus synthesis, which may exist.

Inhibitor Studies.

Oxamate has been shown to be a competitive inhibitor of lactic dehydrogenase. (Hakala et al., 1953; Novoa et al., 1959; Winer and Schwert, 1959; Papaconstantinou and Colowick, 1961). The addition of this inhibitor to cultures containing adenovirus infected cells would presumably inhibit the formation of lactic acid from pyruvic acid. It was proposed to investigate the effects of this inhibitor on glucose utilization, lactic acid production, and virus replication in human amnion cell cultures.

Tubes of cultured cells were treated in the same

manner as described for previous experiments. Infected cultures were divided into three lots and one milliliter of Eagle's minimal essential medium was added to one set of cultures. One milliliter of Eagle's minimal essential medium containing sodium oxamate at a concentration of 4×10^{-2} Molar (See Appendix A) was added to a second lot of cultures, and one milliliter of Eagle's minimal essential medium containing sodium oxamate at a concentration of 8×10^{-2} Molar was added to the third lot of infected cultures. Control, uninfected cultures were divided similarly into 3 lots and Eagle's medium with one of the two concentrations of sodium oxamate or without oxamate, was added to each series of cultures. The results of two experiments of this kind are presented in Figures 6 and 7.

In both of these experiments, the glucose consumption and lactic acid production by infected cultures exceeds that of uninfected cultures. It was found that oxamate inhibited glucose utilisation as well as lactic acid production in infected and in control cultures. The reasons for the inhibition of glucose utilisation, caused by oxamate, are not clear. Figure 6 illustrates the effect of oxamate on glucose consumption and lactic acid production by the infected cell cultures, while Figure 7 shows the effects of oxamate on virus replication. The addition of 4×10^{-2} Molar oxamate reduces the amount of virus synthesized after 24 and 48 hours by 70 to 95 percent. Increasing the oxamate concentration to 8×10^{-2} Molar

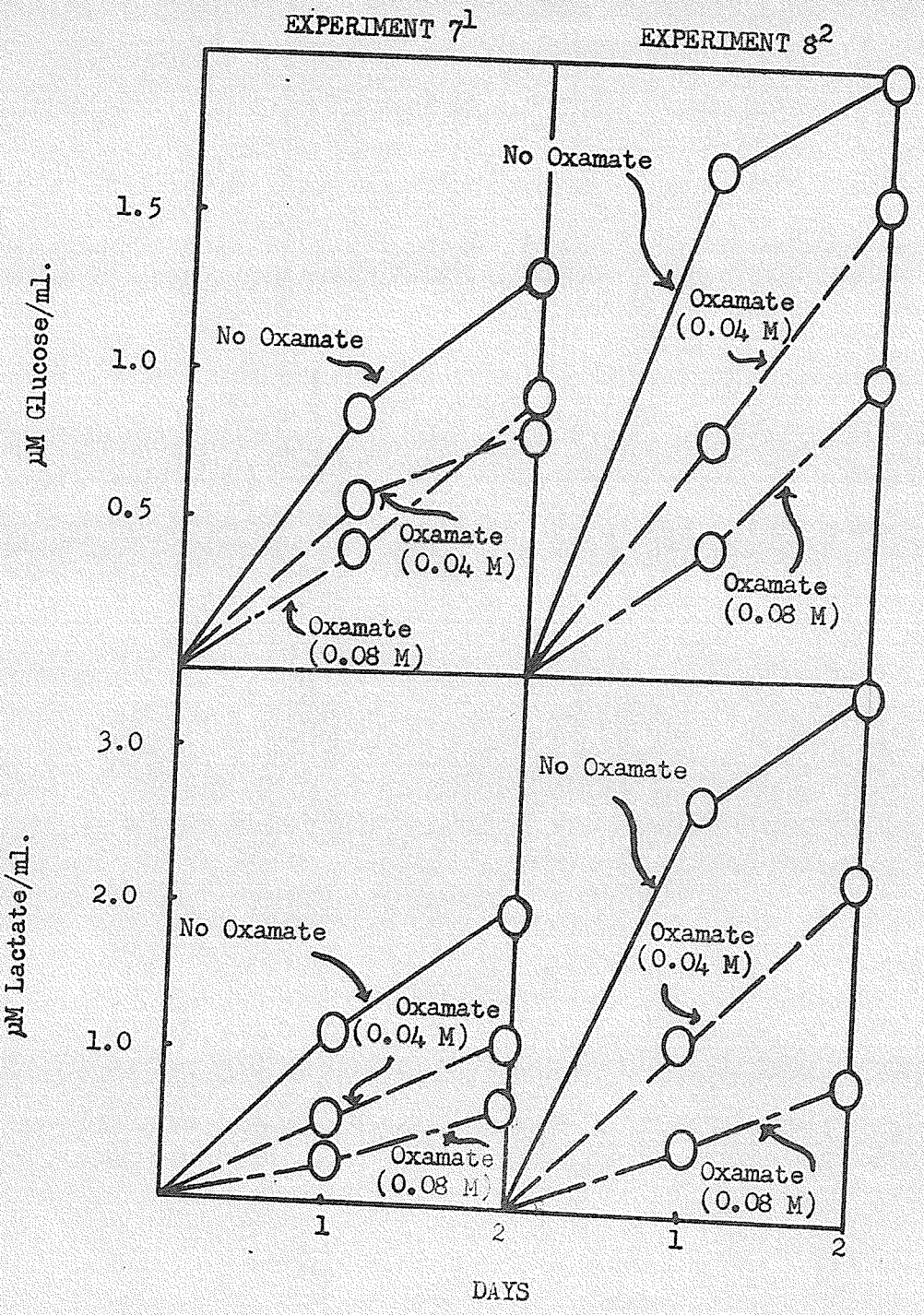

FIGURE 6

EFFECT OF OXAMATE ON GLUCOSE UTILIZATION AND LACTIC ACID PRODUCTION BY INFECTED AMNION CELL CULTURES

[1] 92,000 CELLS/TUBE

0.05 CPD_{50}/CELL

[2] 288,000 CELLS/TUBE

0.09 CPD_{50}/CELL

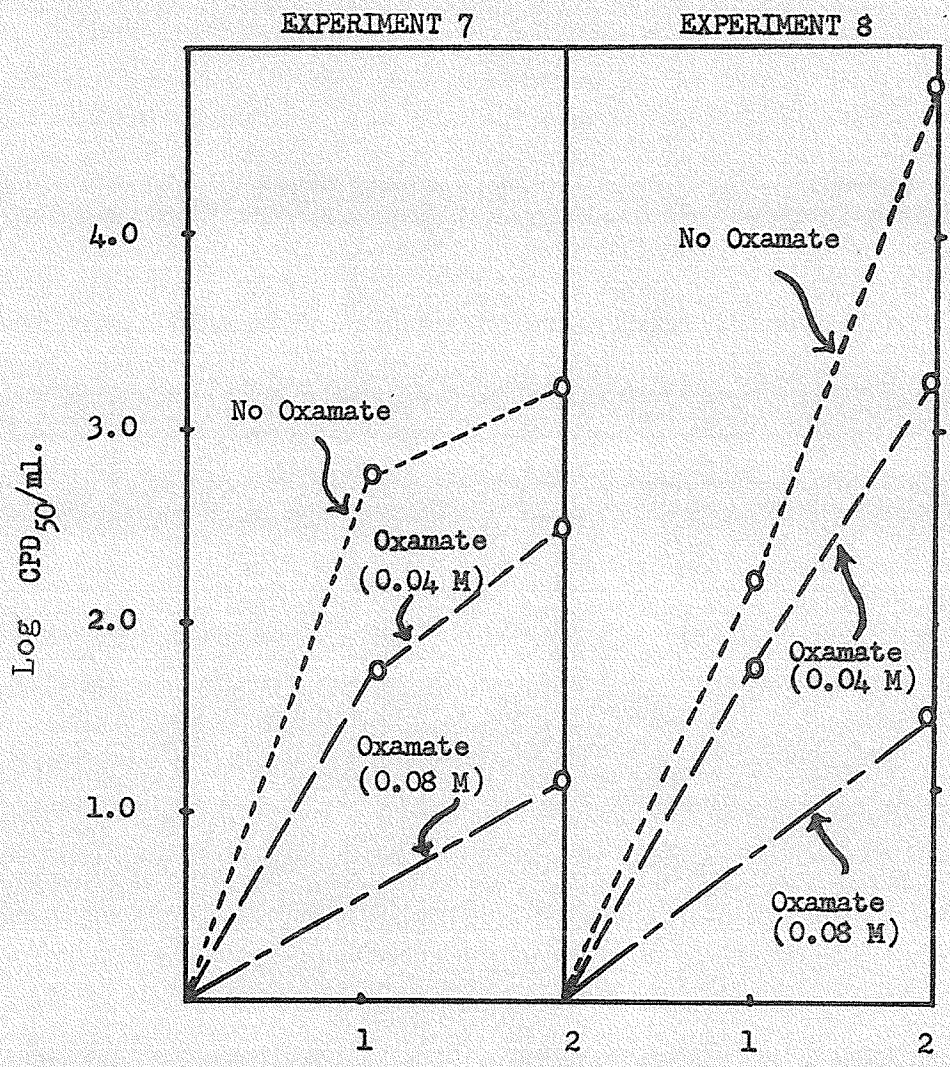

FIGURE 7

EFFECT OF OXAMATE ON ADENOVIRUS REPLICATION IN AMNION CELL CULTURES

decreased the amount of virus synthesized by more than 95 percent in each case. In both of these experiments, this inhibition of virus synthesized was accompanied by a decrease in the amounts of glucose utilized and lactic acid produced.

Manometric Studies

Findings reported by Rozee et al. (1957) indicated that the Krebs cycle was operating at a greater rate in HeLa cells infected with adenovirus type 7 than in uninfected cells. It was therefore proposed to investigate the oxygen consumption of amnion cells infected with adenovirus type 3, at various stages during the growth cycle of this virus.

Preliminary Experiments.

Since large numbers of primary amnion cells were not readily available, preliminary studies were carried out to determine the average oxygen consumption and to select the least number of cells which take up conveniently measureable amounts of oxygen. The Q_{O_2}[1] for 10^6 cells, in the presence of glucose (2 milligrams per milliliter), was found to be about 4 microliters, while those for 5×10^6 cells, 10×10^6 cells and 15×10^6 cells were found to be 9 microliters, 20 microliters and 34 microliters respectively. It was decided to use 10^7 cells per Warburg flask for further experiments.

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1. Oxygen uptake in microliters per hour, the gas phase being air.

The original intention was to determine the oxygen consumption and carbon dioxide evolution by infected as compared to uninfected cells according to the direct method (Umbreit et al., 1957). Since this measurement depends on the assumption that no difference exists between respiration in the presence and respiration in the absence of carbon dioxide, experiments were carried out to test the validity of this. The endogenous respiration of amnion cells was also determined. The results indicated that the endogenous oxygen consumption[2] of primary amnion cells is slightly greater than the oxygen consumption of cells suspended in a medium containing glucose as substrate. It was also found that the endogenous oxygen consumption is about 15 percent greater in the absence of carbon dioxide than in its presence.

Through these studies it became apparent that a comparatively large number of cells would be needed to carry out an experiment to determine the respiration of uninfected and infected cells at selected intervals during virus growth. Since the supply of primary amnion cells was very limited, the FL strain of the human amnion cell was substituted for this purpose.

A preliminary experiment indicated that, just as with human amnion cells, the endogenous oxygen consumption of FL cells is 15 to 20 percent greater than the oxygen consumption when cells are suspended in a medium containing glucose.

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2. The uptake of exogenous oxygen for the metabolism of endogenous substrate or of exogenous substrate stored within the cell.

It was decided then, to measure the endogenous oxygen consumption of adenovirus infected FL cells as compared to that of controls. These measurements were to be accompanied by glucose, lactic acid, and virus assays. It was anticipated that the rates of oxygen consumption could then be correlated with virus growth.

The experiment was set up in exactly the same way as were earlier experiments designed to determine changes in glycolysis associated with virus growth. In this case, FL cells, cultured in bottles, were used instead of the secondary amnion cell cultures used earlier. Ten milliliters of a 1:10 dilution of virus in Scherer's maintenance medium was used as inoculum. After the residual virus was removed, the cell monolayers were washed twice with 10 milliliters of Hank's basal salt solution (without glucose) and 10 milliliters of Eagle's minimal essential medium were added. With a six hour adsorption period, calculation showed that 0.008 CPD_{50} of virus was adsorbed per cell. Every 24 hours, for 96 hours, six infected and six control cultures were removed from the incubator. Four cultures were needed to provide enough cells for duplicate determinations of oxygen consumption while two cultures were needed to provide samples for glucose, lactic acid and virus assay. These latter cultures were frozen until the appropriate assay could be carried out.

The measurement of oxygen uptake was performed using cell suspensions prepared from monolayer cultures, by trypsinization, as described earlier. The washed cells were suspended

in Krebs-Ringer phosphate solution and magne-stirred. Sodium bicarbonate (2.45 percent) was added to each suspension so as to bring the concentration to 2.9×10^{-3} Molar. Pardee buffer, (See Appendix C) used in the center well of each Warburg flask, was made up to buffer the carbon dioxide concentration in the flask atmosphere, at one percent. The bicarbonate concentration and carbon dioxide concentration buffered the pH of the suspending medium at 7.4. Equal volumes of each suspension were placed in two Warburg flasks, the flasks were attached to the corresponding manometers, and placed in the waterbath at 37° C. After 30 minutes equilibration, readings were begun, the oxygen consumption over a four hour period was determined and the Q_{O_2} of 10^7 cells was calculated.

The results are presented in Table II. While there is slightly more oxygen consumption by infected cells the difference is likely within the limits of experimental error, so its significance is doubtful.

TABLE II

RATE OF ENDOGENOUS OXYGEN CONSUMPTION OF INFECTED AS COMPARED TO CONTROL FL CELLS AT VARIOUS INTERVALS AFTER INFECTION

DAYS	Q_{O_2} FOR 10^7 CELLS			
	INFECTED		CONTROL	
	Flask No. 1	Flask No. 2	Flask No. 1	Flask No. 2
1	31.9	32.7	30.2	29.1
2	28.8	26.1	25.2	25.2
3	27.7	26.1	24.2	26.5
4	25.9	31.4	25.5	----

In the preparation of the cell suspensions, it was noticed by calculation that the yield of cells from infected bottles decreased at a steady rate until by the fourth day after infection, it had decreased to about 60 percent of the original number. The number of cells in control cultures did not vary beyond limits to be expected due to experimental error in the cell count. Whether this drop in cell number is due to lysis during viral replication or to a selective action of trypsin on infected cells is not known.

The results of glucose, lactic acid and virus assays as presented in Figure 8, indicate that FL cell cultures infected with adenovirus type 3 consume more glucose and produce greater amounts of lactic acid than do uninfected cultures. Again the differences were noted to occur with the beginning of the logarithmic phase of virus growth.

Thus, glucose utilization and lactic acid production by FL cells increased after infection with adenovirus type 3 but no significant change in endogenous oxygen consumption could be detected.

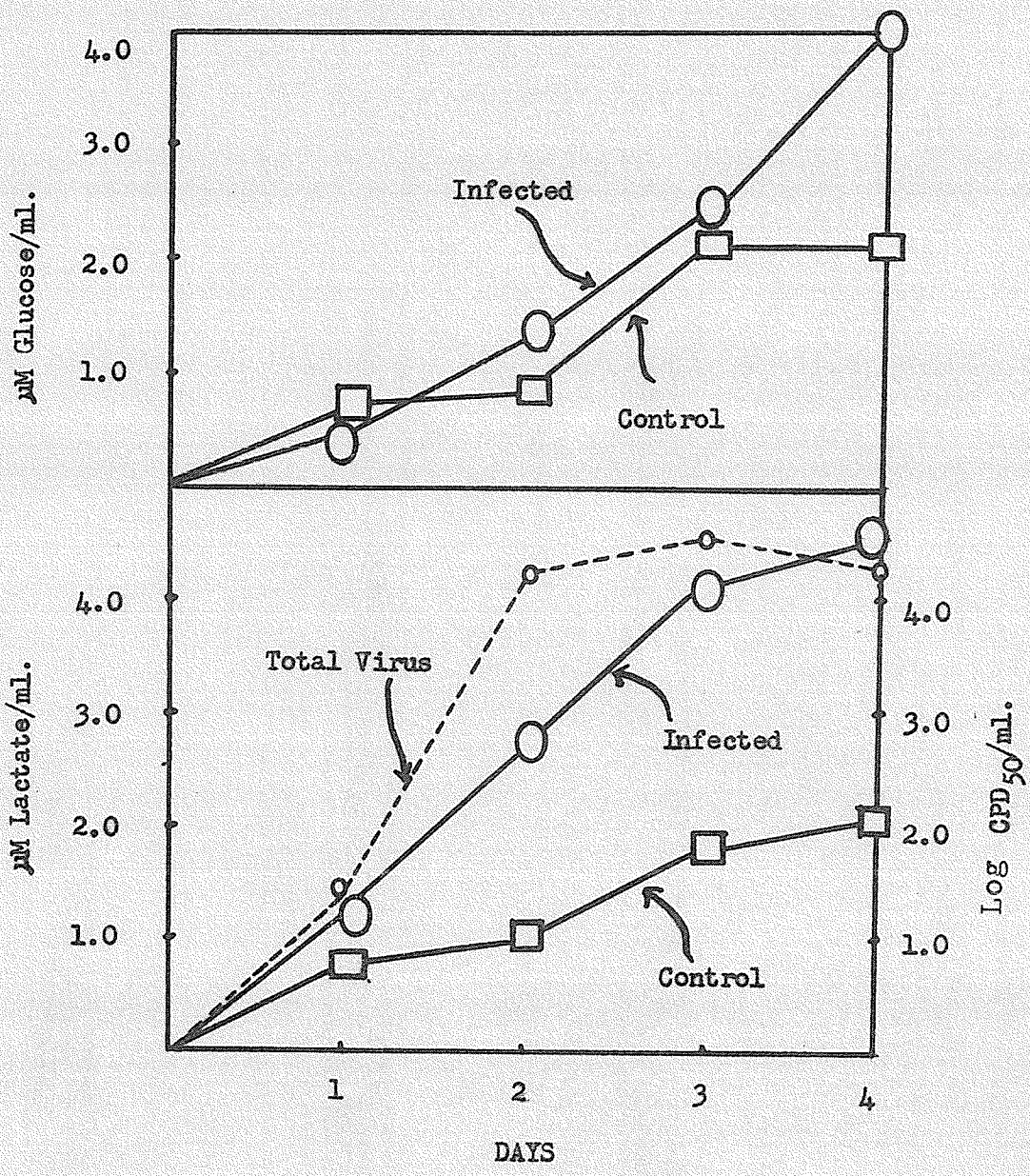

FIGURE 8

GLUCOSE CONSUMED, LACTIC ACID PRODUCED BY INFECTED AND CONTROL FL CELL CULTURES

4,870,000 CELLS/BOTTLE

.008 CPD_{50}/CELL

DISCUSSION

DISCUSSION

The results presented, agree with the findings for the adenovirus-HeLa cell systems reported by other workers, that adenovirus infected cell cultures exhibit a marked increase in glycolysis over that of uninfected cultures. The work done in other laboratories has been extended here, to include a study of this change in glycolysis in association with the stages of virus growth. This change in carbohydrate metabolism occurs very close in time to the beginning of the logarithmic phase of virus replication, as judged by the total amount of virus present in the cultures. The interpretation of the results is based on several prime assumptions. The assay of total virus present, glucose disappearance and lactic acid appearance by the culture as a whole, are assumed to reflect the events occurring in single cells. Studies on the formation of adenovirus antigen, as detected by the fluorescent antibody technique (Pereira, 1959), show that completely synchronous development of virus among individual cells, does not occur. However, this would not likely affect the interpretation of these results, since the changes found would only be detected when the majority of cells in the culture passed from the eclipse phase of the development of the cell-virus complex, to the logarithmic phase. Electron microscope studies (Pereira et al., 1958) indicated that a maximum of 100 virus particles are present per CPD_{50} unit. On this basis, the cells used in most of the experiments cited in this study,

adsorbed one or more virus particles and, judging from the virus growth curves, one step growth conditions were often approximated. It should be added, however, that while one step growth conditions were approximated in the whole of the host-virus system, some variation in the cycle of virus growth likely existed among individual cells in the culture.

It is assumed throughout this work that the assays for glucose and lactic acid are reasonably specific. Production of reducing substances by the cells would increase the reducing power of the culture extract and the calculated glucose concentration could therefore be greater than the true one.

The importance of glucose in adenovirus synthesis has not been established, but Eagle and Habel (1956) have shown that it is essential for poliovirus synthesis. Lactic acid formation may be important not only in the DPN-linked glycolytic activity but also in the reoxidation of reduced TPN in the cell (Navazio et al., 1957). Pyruvate addition to ascites tumor cells has been shown in fact, to increase the direct oxidation of glucose-phosphate, and it is suggested that this may be due to an increase in oxidized TPN, produced by reduction of some added pyruvate to lactic acid (Wenner, 1959). While it may be possible that TPN as well as DPN linked lactic dehydrogenase activity may play an important role in virus synthesis (Kun, 1960) additional evidence is necessary before definite conclusions can be made. The results presented here do not conclusively tie up lactic dehydrogenase with virus

synthesis. It is indicated, however, that oxamate partially inhibits Adenovirus replication, at least over the first 48 hours of infection, during which time a large amount of virus is synthesized in cultures containing no oxamate. This inhibition is accompanied by a reduction in the amount of glucose utilized and lactic acid produced. Oxamate may affect other reactions in which pyruvate, or substances of similar chemical configuration, participate, so the results must be interpreted with caution. Since reasons for the oxamate induced inhibition of glucose utilization are not known, the inhibition of viral synthesis may be due to inhibition of a reaction remote from the formation of lactic acid. At the present time, however, no other reactions are known to be affected by this inhibitor (Papaconstantinou and Colowick, 1961). Thus, the results lend some support to the hypothesis that glycolysis is intimately connected with virus replication but additional work with this inhibitor is necessary before definite conclusions can be made.

Although no significant change in oxygen consumption by infected cells could be detected, the possibility remains that trypsin exerts some selective effect on the infected cells, thus masking any difference which does exist. The use of cells grown in suspension would eliminate the trypsinization step in preparing cell suspensions and would therefore be more desirable than monolayer cultures for studies of this kind.

By the time the first infective virus is detectable, the metabolism of the infected cell must have been altered so as to produce some viral subunits from which mature infective virus is formed. During the logarithmic phase of virus replication it is likely that the chemical processes involved in both the production of mature virus, and in the synthesis of viral subunits are in progress. For this reason, any change in the metabolism of the virus-host cell complex which occurs with the logarithmic phase of virus replication, might be connected either with the maturation process or with the synthesis of virus subunits. Although oxamic acid causes an inhibition in the synthesis of infective virus, it cannot be ascertained through these results, whether the effect of this inhibitor is exerted on the synthesis of virus precursors or on the assembly of these precursors into mature, infective virus.

In conclusion, secondary human amnion cells and the transformed line of the human amnion cell, strain FL, exhibit a marked increase in glucose utilization and lactic acid production after infection by adenovirus type 3. This increase occurs close to the beginning of the logarithmic phase of virus replication. No change in the endogenous oxygen uptake of FL cells occurs following infection with adenovirus type 3.

Oxamic acid, an inhibitor of lactic dehydrogenase, causes inhibition of glucose uptake, lactic acid production and virus synthesis, suggesting that lactic dehydrogenase is very important in the formation of infective adenovirus particles.

SUMMARY

SUMMARY

It was established that glucose utilization and lactic acid production in adenovirus type 3 infected amnion and FL cell cultures is greater than in companion uninfected cell cultures.

The growth cycle of adenovirus type 3 in amnion cell cultures consists of an eclipse phase of less than 24 hours and a phase of logarithmic increase in virus infectivity lasting 48 hours.

The increase in glycolysis, associated with adenovirus infection, is detectable at a time close to the beginning of the logarithmic phase of virus replication and the difference between glycolysis in infected and glycolysis in control cultures was greatest at the end of the virus growth cycle. Although the aim was to limit virus replication to a single cycle, this was not likely achieved.

The changes in glycolysis were noted with both low and relatively high doses of virus.

A similar pattern of glycolytic change was found when either Scherer's maintenance medium or Eagle's minimal essential medium was used.

Oxamic acid, a competitive inhibitor of lactic dehydrogenase, was found to inhibit glucose consumption and lactic acid production in both infected and uninfected cultures. Virus replication was also inhibited by oxamate, suggesting that lactic dehydrogenase is important in some stage of virus

synthesis.

The Q_{O_2} of suspensions of FL cells was determined and the endogenous oxygen consumption was found to be greater than the oxygen consumption when glucose was added as substrate.

No significant change in the endogenous oxygen consumption of adenovirus infected FL cells could be detected.

On the basis of the evidence presented, it is concluded that an increase in glycolysis occurs in adenovirus infected amnion and FL cell cultures, at a point in time close to the beginning of the logarithmic phase of virus replication, and that this increase is not accompanied by a change in the endogenous oxygen consumption. It is suggested also, that lactic dehydrogenase is important in virus synthesis.

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APPENDIX

APPENDIX A

MEDIA AND SOLUTIONS USED IN PREPARATION OF CELL SUSPENSIONS AND IN MAINTENANCE OF CELL CULTURES

Hanks' Basal Salt Solution (Hanks, 1949).

	Concentration (mg./L)
NaCl	8000
KCl	400
$MgSO_4 \cdot 7H_2O$	200
$CaCl_2$	140
$Na_2HPO_4 \cdot 12H_2O$	150
KH_2PO_4	60
Phenol red	20
Glucose	omitted

Deionized water to make 1 liter.

Maintenance Solutions

Scherer's Maintenance Medium (Syverton et al., 1954).

Stock Solutions.

1. Hanks' Basal Salt Solution (without glucose).

2.	Amino acetic acid	0.2 gm.	Dissolved in
	DL Histidine (free base)	0.2 gm.	100 ml. stock 1.
3.	L cystine	0.15 gm.	Dissolved in 100 ml. 0.1N HCl.
4.	Glycerol	4 ml.(5 gm.)	
	Sodium acetate .3 H_2O	5.64 gm.	Dissolved in
	Succinic acid	0.1 gm.	100 ml. stock 1.
	L malic acid	0.05 gm.	

5.	Adenine sulphate $.2H_2O$	0.068 gm.	
	Guanine hydrochloride $.2H_2O$*	0.01 gm.	
	Xanthine	0.01 gm.	Dissolved in
	Uracil	0.01 gm.	100 ml. stock 1.
	Thymine	0.004 gm.	
	Cytosine	0.004 gm.	
6.	Thiamine hydrochloride	0.1 gm.	
	Nicotinamide	0.04 gm.	
	Calcium pantothenate	0.04 gm.	
	Pyridoxal hydrochloride	0.04 gm.	Dissolve in 500 ml.
	Pyridoxamine dihydrochloride	0.04 gm.	stock 1.
	Ribose	0.04 gm.	
	Riboflavin	0.04 gm.	
	i - inositol	0.14 gm.	
	Choline chloride	0.14 gm.	
7.	Biotin	0.01 gm.	Dissolve in 100 ml.
	Folic acid	0.01 gm.	stock 1 after ad-
	Para amino benzoic acid	0.01 gm.	justing pH to 7.0.

Solutions 1, 2, 4, 6 and 7 were sterilized by autoclaving at a pressure of 10 pounds for 10 minutes. Solutions 3 and 5 were sterilized by millipore filtration.

To prepare the working stock solution, 10 milliliters of stocks 2, 3, 4 and 5 were combined. Five milliliters of stock 6 and one milliliter of stock 7 were added. Ten milliliters of penicillin (10,000 units per/ml.), 2.5 milliliters of streptomycin (20,000 ug per/ml.) and 20 milliliters of 10 percent glucose were added. The antibiotics

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* Dissolved in a few drops of 0.1N HCl.

had been stored at -20° C. before use.

The pH was adjusted to 7.4 by adding 1.4 percent sodium bicarbonate. To make up the maintenance solution, 7.85 milliliters of working stock, plus 5.0 milliliters of horse serum were added to 87.2 milliliters of stock solution (1).

Eagle's Maintenance Medium.

Stock Solutions.

1. Hanks' basal salt solution (without glucose).

2.	L arginine	1.05 gm.	
	L histidine	0.31 gm.	
	L isoleucine	0.52 gm.	
	L leucine	0.52 gm.	
	L lysine	0.58 gm.	Dissolve in 100 ml.
	L methionine	0.15 gm.	stock 1 by heating
	L phenylalanine	0.32 gm.	to 80° C.
	L threonine	0.48 gm.	
	L tryptophane	0.10 gm.	
	L valine	0.46 gm.	
3.	L cystine	0.24 gm.	Dissolved in 100
	L tyrosine	0.36 gm.	ml. 0.1N HCl.
4.	Choline	0.1 gm.	
	i - inositol	0.2 gm.	
	Nicotinamide	0.1 gm.	Dissolved in 1000
	Calcium pantothenate	0.1 gm.	ml. of stock 1.
	Pyridoxal	0.1 gm.	
	Riboflavin	0.01 gm.	
	Thiamine	0.1 gm.	
5.	Folic acid	0.01 gm.	Dissolved in 100 ml. stock 1 by adding a few drops of 0.5 N NaOH.

6. Glutamine	2.92 gm.	Dissolved in 100 ml. stock 1.

The Hanks' salt solution was sterilized by autoclaving at 10 pounds for 10 minutes. The other solutions were sterilized by millipore filtration. Stock solution 6 was stored at -20° C. until use.

The working stock solution was prepared by combining 10 milliliters of solutions 2, 3, 5 and 6 and one milliliter of stock 4. Twenty milliliters of 10 percent glucose, 10 milliliters of penicillin (10,000 units/ml.) and 2.5 milliliters of streptomycin (20,000 ug/ml.) were added. The antibiotics had been stored at -20° C. until use. To make up the maintenance solution, 7.35 milliliters of working stock and 2.5 milliliters of horse serum were added to 90 milliliters of stock one. The pH was adjusted to 7.4 by adding 1.4 percent sodium bicarbonate.

Trypsin.

A one percent solution of trypsin (1.300) was made up in Hanks' basal salt solution and sterilized by seitz filtration. This was diluted 1:4 in Hanks' solution before use.

Oxamate.

Oxamic acid	0.395 gms.
NaCl	0.405 gms.
KCl	0.040 gms.
$MgSO_4 \cdot 7H_2O$	0.020 gms.
$CaCl_2$	0.014 gms.
$Na_2HPO_4 \cdot 12H_2O$	0.015 gms.
KH_2PO_4	0.006 gms.
Phenol red	0.002 gms.

These were dissolved in about 80 milliliters of deionized water. One Normal NaOH was added to adjust the pH to 7.0 and the volume made to 100 milliliters. After thorough mixing, the solution was sterilized by millipore filtration. Ninety milliliters of this solution was used in place of the Hanks' salt solution used in preparation of Eagles' medium.

The resulting medium would be 0.04 M in oxamate. The 0.08 M oxamate medium was made by dissolving 0.791 grams of oxamate and reducing the sodium chloride concentration to .009 grams per 100 milliliters.

Krebs-Ringer phosphate and bicarbonate solutions.

Stock solutions.

1. 0.90 percent NaCl. (All solutions made up
2. 1.15 percent KCl. with deionized water.)
3. 1.22 percent $CaCl_2$.
4. 3.82 percent $MgSO_4 \cdot 7H_2O$.
5. 0.1 Molar phosphate buffer, pH 7.4.
   (43.4 gms. $Na_2HPO_4 \cdot 12H_2O$ + 20 ml. 1 N HCl
   diluted to 1 liter.)

The Krebs-Ringer solution was prepared by mixing the following amounts of the above solutions.

100 parts of solution 1
4 parts of solution 2
3 parts of solution 3
1 part of solution 4
0.6 parts of 0.4 percent phenol red.

To make up the Krebs-Ringer phosphate solution, 10 milliliters of solution 5 were diluted to 100 milliliters with stock Krebs-Ringer solution.

If carbon dioxide was to be present in the gas phase of the Warburg flask, sodium bicarbonate (2.45 percent)

was added to the Krebs-Ringer Phosphate solution (0.1 milliliters bicarbonate per 10 milliliters of Krebs-Ringer phosphate solution).

APPENDIX B

Reagents Used in Chemical Assays

Somogyi's Copper Reagent (Somogyi, 1945).

$Na_2HPO_4 \cdot 12H_2O$		35.3 gms.
Rochelle Salt		20.0 gms.

These were dissolved in 350 ml. distilled water.

NaOH	1 N	50 ml.
$CuSO_4$	10 percent	40 ml. (with stirring)
Na_2SO_4 (anhydrous)		90 gms.

This was diluted to 500 milliliters after the solids dissolved and allowed to stand for a week or two before use. It was then decanted from the precipitate and filtered.

Nelson's Arsenomolybdate Chromogenic Reagent (Nelson, 1944).

Ammonium molybdate	25 gms.	in 450 ml. water
H_2SO_4 (conc.)	21 ml.	
$Na_2AsO_4 \cdot 7H_2O$	dissolved in 25 ml. water and added.	

After being mixed, the solution was placed in a brown glass stoppered bottle and incubated at 37° C. for 24 to 48 hours before use.

The Para-hydroxy Diphenyl Reagent for Lactate Determination (Barker and Summerson, 1941).

p-hydroxydiphenyl	1.5 gm.	(Dissolved in 10 ml. 5 percent NaOH)

Made up to 100 milliliters with distilled water.

APPENDIX C

Preparation of Pardee Buffer (Umbreit et al., 1957).

Diethanolamine	6.0 ml.
Thiourea	15 mg.
$KHCO_3$	3.0 gms.
HCl (6 N)	2.2 ml.
H_2O	6.8 ml.

This mixture was shaken in a stoppered container and allowed to stand overnight before use.