EFFECT OF AMINO ACID DEPRIVATION IN UNINFECTED AND SHOPE FIBROMA VIRUS INFECTED RABBIT KIDNEY CELLS

by 6402

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B.S., University of Massachusetts, 1966

A MASTER'S THESIS

submitted in partial fulfillment of the

requirements for the degree

MASTER OF SCIENCE

Division of Biology

Microbiology

KANSAS STATE UNIVERSITY
Manhattan, Kansas

1971

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DEDICATION

To my husband, Danforth, for without him it would have been impossible.

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INTRODUCTION

Shope fibroma virus, a member of the poxvirus group, was isolated from tumors in wild cottontail rabbits. The virus can be propagated in several cell lines and produces characteristic cytoplasmic B-type inclusions which are the sites of viral macromolecular synthesis (Kato et al., 1963; Scherrer, 1968). The virus produces benign tumors in young adult rabbits (Thompson, 1938).

Rabbit kidney cell cultures infected with the virus show an initial depression of host macromolecular synthesis. However, an increase in the host synthetic activity leads to rapid proliferation and morphological alteration of the infedted cells (Tompkins et al., 1969). Several enzymes coded for by the viral genome have been demonstrated in poxvirus infected cells (Joklik, 1966). Munyon et al., (1967) and Pogo and Dales (1969a and 1969b) have demonstrated some early enzymes associated with purified poxvirus.

An understanding of some of the control mechanisms of virus replication may be obtained by studying the nutritional requirements during virus replication. Macromolecular synthesis in bacteria as well as cultured mammalian cells under conditions of amino acid deprivation has been studied

(Schachtele and Rogers, 1968; Copeland, 1969; Inouye and Pardee, 1970; Rogers and McConnell, 1970; Hare, 1970; Ley and Tobey, 1970).

The effects of amino acid deprivation on some of the control mechanisms of several intranuclear DNA viruses i.e. herpesvirus, adenovirus, SV40 and polyoma virus have been investigated (Rouse and Schlesinger, 1967; Goldblum et al., 1968; Winters and Consigli, 1969; Courtney et al., 1970).

The purpose of this investigation was to study the effect of amino acid deprivation on host DNA synthesis, viral DNA synthesis and progeny formation and the interrelationship between the early proteins and viral DNA synthesis in rabbit kidney cell cultures.

REVIEW OF LITERATURE

The unique characteristic of Shope fibroma virus to produce tumors in rabbits (Shope, 1932a and 1932b) has made it one of the interesting models for the study of oncogenic viruses. Lloyd and Kahler (1955) by electron microscopy and Scherrer (1968) by autoradiographic studies have observed multiplication of the virus within B-type cytoplasmic inclusion bodies. In tissue cultures, host macromolecular synthesis is inhibited during viral replication (Ewton and Hodes, 1967; Scherrer, 1968; Minocha and Maloney, 1970).

Fibroma virus can be propagated in rabbit kidney cells (Hinze and Walker, 1964; Israeli and Sachs, 1965; Roby et al., 1965; Verna, 1965) and Hela cells (Ewton and Hodes, 1967).

The *inhibitor*, 5-fluoro-2'-deoxyuridine (FUdR), has been used to study the relationship between protein synthesis and viral DNA synthesis in DNA viruses (Salzman et al., 1963; Jungwirth and Joklik, 1965; Kates and McAuslan, 1967). This drug completely inhibits the synthesis of many DNA viruses (Consigli et al., 1968; Loh and Payne, 1965; Flanagan and Ginsberg, 1961).

McAuslan, in his review of vaccinia virus replication (1969) has detailed the relationships between early and late protein synthesis and viral DNA synthesis learned from inhibitor studies. Poxvirus DNA synthesis required concurrent protein synthesis. The protein(s) required accumulated in the presence of FUdR

and are therefore, an early viral function. The normal course of events during infection was the simultaneous synthesis of the required protein(s) with the viral DNA so that there was no appreciable accumulation. The protein(s) needed are coded for by a relatively unstable messenger RNA with a lifetime of approximately 45 minutes (Kates and McAuslan, 1967).

The presence of FUdR (12 ug/ml) completely inhibits the synthesis of fibroma virus and the inhibition is rapidly reversed with the addition of an exogenous supply of thymidine (Minocha and Maloney, 1970). Minocha (unpublished data) has observed that fibroma virus DNA synthesis also requires concurrent protein synthesis. These proteins accumulated in the presence of FUdR and were stable for several hours after their synthesis, however their messenger RNA was relatively unstable and had a lifetime of 2 to 3 hours.

The essential nutritional requirements for the growth of tissue culture cells in vitro include many amino acids, salts, vitamins and some sugars (Morgan et al., 1950; Eagle, 1955a and 1955b; Eagle, 1959). A recent report by Ley and Tobey (1970) has shown the specific requirement of isoleucine in several mammalian cell lines was needed to initiate DNA synthesis and cell division. The amino acid requirements for the propagation of poliovirus in HeLa cells (Eagle and Habel, 1956) and vaccinia virus in L cells (Holtermann, 1969) have been shown

to be similar to those needed for the growth of the host cell.

Recent studies have shown specific amino acid requirements, namely arginine and lysine, for the replication of several animal viruses. Intranuclear DNA viruses such as polyoma (Winters and Consigli, 1969), SV40 (Goldblum et al., 1968), adenovirus (Rouse and Schlesinger, 1967) and herpesvirus (Courtney et al., 1970) as well as a few intracytoplasmic RNA viruses such as measles virus (Romano and Brancato, 1970), reovirus (Loh and Oie, 1969) and influenza virus (Becht, 1969) have been shown to require arginine for virus synthesis.

The most detailed study of a specific amino acid requirement for virus replication has been examined in the adenoviruses. Rouse et al., (1963) were first to show the specific requirement of arginine for plaque formation. The time course of maximum uptake of arginine was shown to coincide with the virus synthesis (Bonifas, 1966). Amino acid analysis of adenoviruses by Polasa and Green (1967) showed that twice as much arginine was present than that found in RNA viruses. It was postulated that adenoviruses might contain an arginine-rich internal component associated with the viral DNA. Prage et al., (1968) isolated two basic proteins from adenovirus cores which appeared to be rich in arginine, but Laver (1970) isolated and purified only one arginine-rich core protein. When arginine deprived

infected cell cultures were refed with labeled arginine medium, virus synthesis was immediately stimulated and the label was found incorporated into the newly formed particles indicating the need for an arginine-rich maturation factor (Rouse and Schlesinger, 1967 ; Russell and Becker, 1968). Infected cells deprived of arginine could synthesize early antigens, viral DNA and structural capsid proteins which also suggested that the early steps in the replication of adenovirus were not inhibited (Rouse and Schlesinger, 1967; Mantyjarvi and Russell, 1969). It was concluded that arginine plays a specific role late in the assembly process of adenoviruses.

Similarly, Winters and Consligli (1969) reported the synthesis of defective virions of polyoma virus under arginine deprived conditions. These virions were sensitive to nuclease digestion suggesting a loss in the structural integrity of the virus.

Requirement of arginine for synthesis of herpesvirus was demonstrated by Tankersley (1964), Sharon (1966) and Inglis (1968). Becker et al.(1967) observed that viral DNA synthesis was not affected by arginine deprivation; however, the coating of the viral DNA was inhibited. The nucleoplasmic fraction of arginine deprived infected cells did not contain capsids or capsomeres as did infected cells in complete medium. Subak-Sharpe et al.(1966) reported an arginyl-transfer RNA, specified

by the viral genome, in herpes infected cells. Complement fixation and immunofluorescent studies indicated that herpes' virus antigens were synthesized in the absence of arginine and accumulated outside the nuclear membrane (Courtney et al.,1970). These antigens could be detected within the nucleus 2 hours after release from arginine deprivation.

The intranuclear DNA virus, SV40, also requires arginine for viral assembly. Goldblum et al.(1968) studied the effect of the thirteen essential amino acids on SV40 virus replication. Synthesis of the "T" antigen was inhibited slightly when arginine was removed from the medium, whereas deprivation of any of the other amino acids did not affect its synthesis. However, synthesis of other viral antigens was influenced by the particular amino acid removed from the medium, and could be grouped according to the extent of their inhibitory effect. Deprivation of group I (glycine, serine and lysine) did not decrease the number of cells producing viral antigen. Deprivation of group II (histidine, methionine, cystine, isoleucine, tryptophan and phenylalanine) produced a slight reduction in the number of cells synthesizing viral antigen. Deprivation of group III (threonine, valine and leucine) produced a marked reduction in the percent cells synthesizing viral antigens, but their effect was not as marked when compared to arginine deprivation which produced the greatest inhibitory effect. Viral progeny

was inhibited also under arginine deprived conditions, but normal yields of virus were obtained when the arginine inhibition was reversed. Thus all the intranuclear DNA viruses studied require arginine for a late step in virus maturation.

Several RNA viruses have also demonstrated a specific requirement for arginine and/or lysine for virus synthesis. Omission of arginine from the medium inhibited reovirus synthesis by 99 percent, whereas lysine deprivation produced a 60 percent inhibition of progeny yield (Loh and Oie, 1969). Structural proteins and double-stranded RNA were synthesized in the absence of lysine, but the formation of empty capsids was enhanced. The reversal of the inhibitory effect could only be achieved when lysine was added to deprived cultures 2 hours after infection, suggesting that an early protein, rich in lysine was necesssry for viral assembly.

Influenza virus is an RNA virus that requires the participation of host DNA during its replication i.e. requires an intranuclear replicative step. Becht (1969) observed the viral induced synthesis of an arginine-rich component in nucleii which is later transported to the cytoplasm for incorporation into mature virus in cells infected with fowl plague virus. Cultures deprived of arginine but not lysine or leucine, reduced virus yields by 85 percent.

Measles virus, an intracytoplasmic RNA virus, also requires arginine for growth (Romano and Brancato, 1970). The presence of mycoplasmas in measles-infected cells prevented virus synthesis by 93 percent, however an arginine supplement to the growth medium completely reversed the inhibitory effect of the mycoplasmas. Schimcke (1967) has shown that non-fermenting mycoplasmas are dependent on arginine as their energy source for growth.

MATERIALS AND METHODS

A detailed description for the preparation of media and reagents used for this investigation can be found in the appendix.

Preparation of Rabbit Kidney Tissue Cultures

Primary rabbit kidney (PRK) cell cultures were prepared from 5- to 10-day old albino rabbits. Rabbits were asphyxiated with carbon dioxide for 20 min and the kidneys removed aseptically and cleaned of fibrous tissues. The kidneys were then minced and washed twice with sterile phosphate buffered saline pH 7.2, 0.01 M (PBS) and once with sterile trypsin solution (0.25%) to remove red blood cells. The tissue was trypsinized for 10 to 15 min at 25 C and the cell suspension centrifuged 10 min at 1,000 x g. The pellet of cells was resuspended and dispersed in Medium 199 plus 8% fetal calf serum (FCS). This procedure was repeated until all the tissue had been trypsinized. The resuspended cells were stirred on a magnetic stirrer at medium speed for 2 min to disperse cell aggregates. The cell suspension was filtered through sterile gauze, counted in a Neubauer counting chamber, and seeded in 32 oz prescription bottles at a concentration of 18 x 10^6 cells in 25 ml of Medium 199 plus 8% FCS per bottle.

Preparation of Secondary Rabbit Kidney Cell Cultures

Media was removed from bottles of PRK monolayers, washed twice with sterile PBS and 1.5 ml of a mixture of 0.06% trypsin plus 0.01% ethylene diamine tetraacetic acid (EDTA) was added for 10 to 15 minutes. The cells were then scraped off the glass surface with a rubber policeman, resuspended in Medium 199 plus 8% FCS and counted. Cells were seeded at a concentration of 5×10^4 cells per ml on 9 x 9 mm^2 coverslips (Bellco, Vineland, New Jersey) in Shell vials (Kimble Products, Owens, Illinois) to give sparse cultures in 24 hours. Falcon plastic dishes (15 x 60 mm; Falcon Plastic Company, Los Angeles, California) were seeded with 5 ml each of cell suspension (2×10^5 cells/ml) to obtain confluent monolayers in 24 hours.

Virus Propagation

The Patuxent strain of Shope fibroma virus was obtained from the American Type Culture Collection (ATCC), Rockville, Maryland. The virus was propagated in PRK cells. Stock virus was diluted one to three in Hanks basal salt solution (BSS) and 2 ml added to primary monolayer cultures in 32 oz prescription bottles. Following adsorption for 3 hr, 25 ml of Medium 199 plus 5% FCS was added to each 32 oz bottle. Monolayers were harvested after 3 days' incubation at 36 C by scraping cells into 2 ml Hanks BSS per 32 oz prescription bottle. Each set of 10 bottles was washed with an additional 5 ml of Hanks BSS

and pooled with the scraped cells. The suspension was centrifuged for 15 min at 1,500 x g at 4 C, and the pellet resuspended in 15 ml of the supernatant. The suspension was sonicated at 40 amps for 30 seconds with a Branson sonifier, Model 140-C (Heat Systems Company, Melville, New York). The sonicate was centrifuged for 10 min at 1,500 x g and the pellet resuspended in 15 ml of the original supernatant fluid. Sonication of the pellet was repeated as before, all supernatants pooled and kept frozen at -70 C. This stock virus was assayed for infectivity in secondary rabbit kidney (SRK) cell cultures. The virus preparations producing cytoplasmic inclusions in 70 to 80% of the cells were distributed in 5 ml aliquots and frozen at -70 C until used.

Virus Assay

The virus was assayed for infectivity by scoring the formation of labeled inclusion bodies in sparse cell cultures. Sparse cell cultures on coverslips, prepared as previously described, were washed once with Hanks BSS and infected with 0.1 ml of virus. Following 1.0 hr adsorption on a mechanical rocker in a CO_2 incubator, excess virus was removed with two washes of Hanks BSS and the cells refed with 1.0 ml Eagles' Minimal Essential Medium (MEM) containing 3% dialyzed fetal calf serum (DFCS). Seven hours after infection (the time of maximum DNA synthesis) cells were pulsed for 1.0 hr with thymidine-^{3} H (^{3}H-TdR) (0.5 ml/vial of Hanks BSS containing 1.5 uC/ml^3 H-TdR). Radioactive

pulse medium was removed and coverslips washed three times with Hanks BSS in the vials to remove unincorporated label. The coverslips were removed from the vials and passed through a series of 8 beakers containing 100 ml Hanks BSS each to further remove any extraneous label. Cells were fixed in 2.5% glutaraldehyde solution in Hanks BSS in plastic trays (Linbro Chemical Company, Inc., New Haven, Connecticut) for 2 hr at 25 C or overnight at 4 C. Glutaraldehyde was removed with 3 washes of deionized water and the coverslips air-dried in the plastic trays. They were mounted, cell side up, on 1 x 3 inch microscope slides with Permount (Fisher Scientific Company, Fair Lawn, New Jersey) and allowed to set overnight before dipping in photographic emulsion. All experiments with sparse cell cultures on coverslips were treated as described above, unless otherwise stated.

Autoradiography

Mounted slides were dipped into Kodak Nuclear Track Emulsion (NTB-3) diluted one to one with distilled water and kept in a 45 C water bath. Dipped slides were placed in black slide boxes where they were air-dried, sealed in a light-tight box and incubated for 3 days at 4 C. The autoradiographs were developed for 10 min in Kodak Microdol X developer, rinsed in tap water for 30 min and stained for 15 min in Giemsa staining solution, pH 5.75. Following a brief rinse in distilled water,

slides were wiped free of excess stain and emulsion and air-dried for counting of labeled cells. (All Kodak products were obtained from Eastman Kodak Company, Rochester, New York).

Counting

A total of 500 cells were counted on each coverslip using a Leitz phase microscope to determine the percent cells showing nuclear and/or cytoplasmic incorporation of the label.

Progeny Virus Synthesis in Amino Acid Deprived Cell Cultures

Monolayers of SRK cells in 60 mm dishes were washed once with Hanks BSS to remove traces of complete growth medium. One set of dishes for each of the amino acid deprived medium was used. Each set of dishes was pretreated for 16 hr with 3 ml of the appropriate medium (lacking one of the amino acids) to deplete the intracellular pool of the specific amino acid. All dishes were infected with 0.5 ml of the virus per dish. Following adsorption of the virus for 2 hr at 37 C in a humidified CO_2 incubator on a mechanical rocker, cultures were washed twice with Hanks BSS to remove unadsorbed virus and 3 ml of the appropriate amino acid deprived media was replaced. At various times following infection, duplicate dishes from each set were harvested by scraping the cells into 1.0 ml of Hanks BSS per set of dishes. Each set of dishes was washed with an additional 1.0 ml of Hanks BSS, pooled in a small screw cap vial and stored at -70 C. Samples were sonicated with a Bran-

son sonifier (Model 140-C) in the vials for 30 sec at 40 amps using the microtip. Sonicated samples were assayed for infectivity on sparse cell cultures on coverslips, prepared for autoradiography and the labeled cells scored as previously described.

Quantitative DNA Synthesis of Cultures in Media Deprived of an Amino Acid.

Sparse cell cultures on coverslips in vials were grown in Medium 199 plus 8% FCS for 16 to 24 hr at 37 C in a humidified CO_2 incubator. Cultures were washed once in Hanks BSS to remove excess growth medium and divided into sets, each set pretreated for 16 hr with media deprived of one of the thirteen amino acids. All sets were infected with 0.1 ml of the virus, as previously described, and each set of coverslips refed with appropriate media lacking in one of the amino acids. At 2 hr intervals cells were pulse-labeled with ^{3}H-TdR for 1.0 hr and autoradiographs prepared. Cells were scored for quantitative, as well as qualitative DNA synthesis. For quantitative data, the mean number of cytoplasmic or nuclear grains per cell was determined on the basis of counts made on 25 cells per coverslip.

Threshold Concentration of Arginine for DNA and Progeny Virus Synthesis.

Different sets of monolayer cultures of SRK cells in 60 mm

dishes were pretreated for 16 hr with media (3.0 ml/dish) containing different concentrations of arginine (0 to 200 ug/ml). At intervals following infection, duplicate dishes from each set were harvested, and titrated for infectivity on sparse cell cultures as previously described.

The above experiment was repeated in sparse cell cultures on coverslips for quantitative DNA synthesis. Different sets of cultures on coverslips were pretreated for 16 hr and maintained after infection in media containing different concentrations of arginine. Seven hours following infection, cells were pulse-labeled with ^{3}H-TdR for 1.0 hr and processed for autoradiography as described before. Cells were scored for quantitative DNA synthesis.

Release of Arginine from Secondary Rabbit Kidney Tissue Cultures During Arginine Deprivation

Secondary RK cells (1.5 x 10^{6} cells/ml) were seeded in 60 mm dishes for 16 hours. Media was removed and replaced with a medium containing 53 ug arginine per ml and 1.0 uC L-4-arginine-^{3}H per ml (specific activity 13.0 C/Mmole) for 24 hours. Cultures were washed twice with Hanks BSS to remove the radioactive medium and incubated in Eagles' MEM plus 3% DFCS for 24 hr to remove unincorporated radioactive arginine from the pools. Cultures were then infected and divided into two sets, one set of dishes was maintained in complete MEM, the other

set was maintained in medium lacking arginine for the duration of the experiment. At intervals following infection, samples of supernatant media from each set were removed and precipitated with cold trichloroacetic acid (5% final concentration). The precipitated samples were stored at 4 C and kept in an ice bath for 3 hr prior to centrifugation. The precipitate (acid insoluble) was sedimented by centrifugation at 20,000 x g for 15 minutes. Monolayers of the cultures were hydrolyzed in 1.0 N NaOH at 25 C for 30 minutes. Samples of the acid soluble, acid insoluble and hydrolyzed cells were assayed for radioactivity in a Packard Tricarb scintillation counter.

Early Macromolecular Synthesis in Cultures Deprived of Arginine

Sparse cell cultures on coverslips in vials were pretreated with media deprived of arginine and infected with virus as previously described. One set of vials was refed with media lacking arginine plus 15 ug per ml of 5-fluoro-2'-deoxyuridine (FUdR) (Hoffman-LaRoche, Inc., Nutley, New Jersey). At intervals following infection, FUdR inhibition was reversed by the addition of 5.0 ug per ml of cold thymidine. Cells were pulse-labeled with deoxycytidine-^{3}H (^{3}H-CdR) for 1.0 hr at intervals following reversal. Autoradiographs were prepared as previously described and cells scored for quantitative and qualitative DNA synthesis.

RESULTS

The effect of amino acid deprivation on the synthesis of DNA and fibroma virus progeny in rabbit kidney (RK) cells was investigated by autoradiography. All cell cultures were pretreated for 16 hr prior to infection with media lacking one of the 13 essential amino acids present in complete Eagles' MEM medium to deplete the cell pool of the amino acid. All experiments in sparse cell cultures were carried out for a period of one replication cycle of the virus (12 hr post infection).

Pulse experiments were done using a tritium-labeled precursor of DNA. Cells showing cytoplasmic or nuclear incorporation of the DNA precursor, which is indicative of viral and host DNA synthesis, respectively, were scored.

The Effect of Amino Acid Deprivation on the Synthesis of DNA and Virus Progeny

Figure 1 demonstrates DNA synthesis measured by quantitative autoradiography over a 12 hr period in cultures deprived of individual essential amino acids. Viral (cytoplasmic) and host (nuclear) DNA synthesis was inhibited to the same relative extent in infected and uninfected deprived cultures with respect to the individual amino acid studied. The total DNA synthesized over a period of one replication cycle of the virus ranged between 35 to 75% of the DNA synthesized in control cultures maintained in Eagles' MEM medium. Glutamine, isoleucine or methionine deprived cultures showed the least amount of DNA synthesis,

EXPLANATION OF FIGURE 1

Different sets of sparse SRK cells on coverslips in vials were pretreated for 16 hr with media lacking in one of the essential amino acids. At intervals following infection, cells were pulse-labeled with ^{3}H-TdR. Autoradiographs were scored for total quantitative nuclear (host) and cytoplasmic (viral) DNA synthesis over a 12 hr period as compared to the controls (MEM).

Different sets of SRK monolayer cultures were pretreated for 16 hr in media lacking in one of the essential amino acids. Cultures were harvested 24 hr following infection and assayed for infectivity on sparse SRK cell cultures. Progeny yields are graphed as percent of the controls (MEM).

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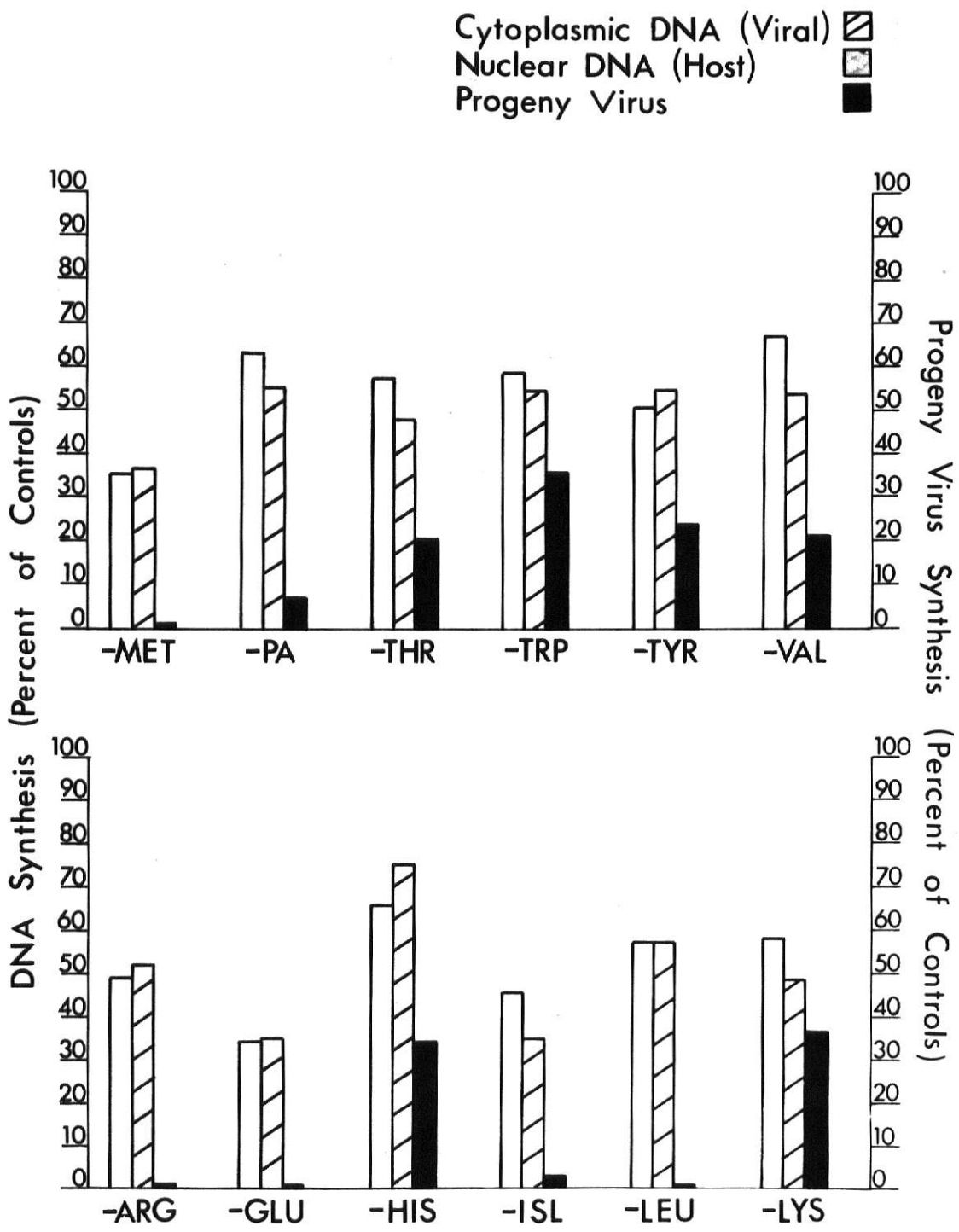
THIS IS AS RECEIVED FROM CUSTOMER.

FIGURE 1


Cytoplasmic DNA (Viral)
Nuclear DNA (Host)
Progeny Virus
DNA Synthesis (Percent of Controls)
Progeny Virus Synthesis (Percent of Controls)
100
90
80
70
60
50
40
30
20
10
0
-MET
-PA
-THR
-TRP
-TYR
-VAL
-ARG
-GLU
-HIS
-ISL
-LEU
-LYS

while the maximum viral DNA synthesis occurred in histidine deprived cultures. Synthesis of infectious virus was inhibited in cultures deprived of arginine, glutamine, leucine and methionine, while less than 10% of the virus progeny was synthesized in the absence of isoleucine and phenylalanine. Cultures deprived of the remaining essential amino acids (histidine, lysine, threonine, tyrosine, tryptophan and valine) showed 20 to 40% synthesis of infectious virus.

The data presented on the synthesis of viral DNA as related to progeny virus formed demonstrates that 35% viral DNA synthesis in minus isoleucine cultures permitted 3% virus production, however 35% viral DNA synthesis in minus methionine or glutamine cultures did not give rise to any progeny virus. Likewise 52 and 57% viral DNA synthesis in minus arginine and minus leucine cultures, respectively, did not allow any synthesis of infectious progeny, whereas comparable amounts of DNA synthesis in any of the other amino acid deprived cultures permitted 20% (threonine, tyrosine, valine) to 35% (lysine and tryptophan) synthesis of infectious virus.

Although cultures deprived of histidine allowed 75% viral DNA synthesis, only 34% progeny was made. In contrast to this 40% viral DNA synthesis in minus lysine cultures produced the maximum virus yield (37%) observed in any of the deprived amino

acid systems. Similarly phenylalanine deprived cultures synthesized 7% progeny virus in the presence of 55% viral DNA synthesis.

The Effect of Amino Acid Deprivation on the Initiation and Time-Course Synthesis of DNA in Secondary Rabbit Kidney Cells

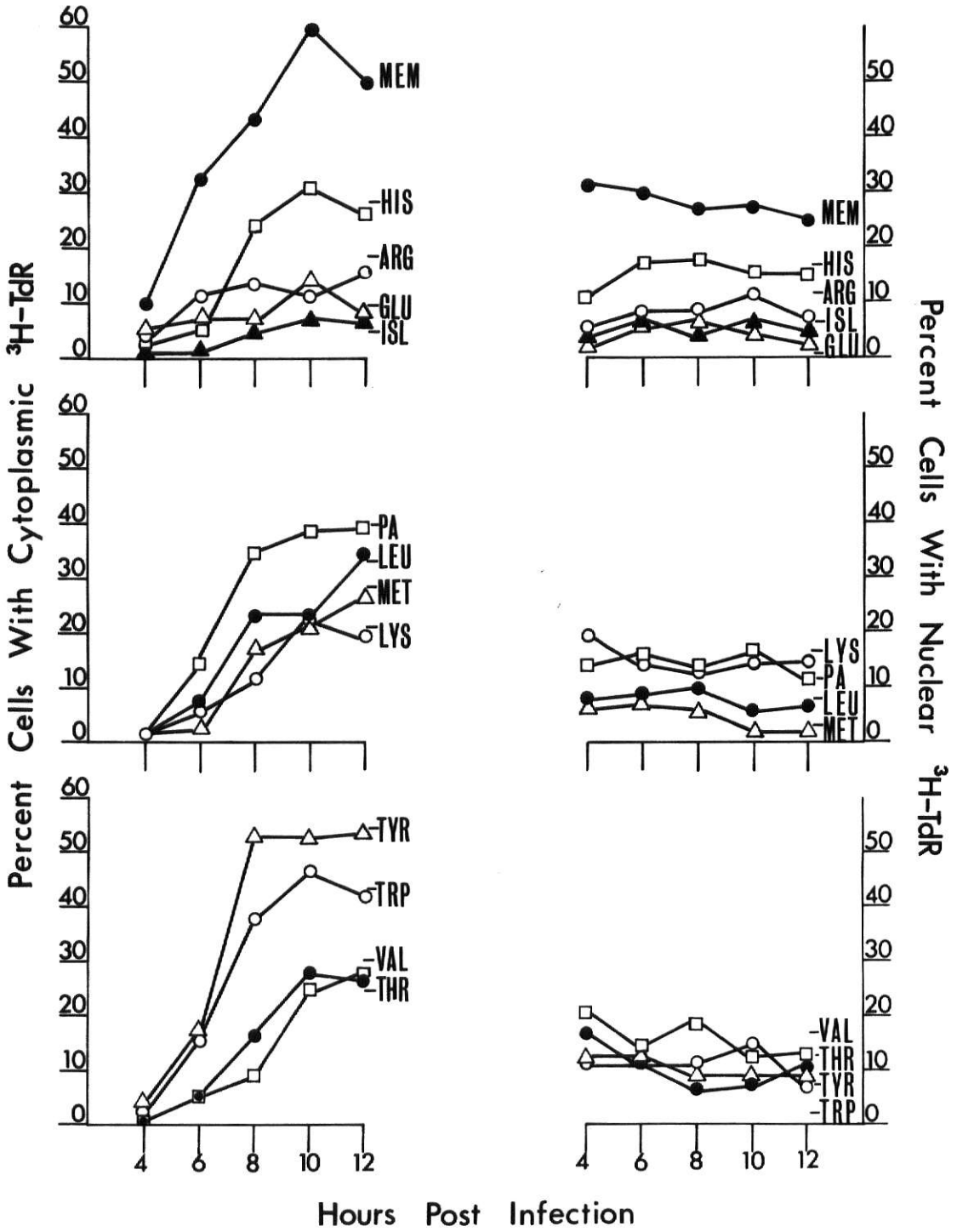
The effect of amino acid deprivation on initiation and time-course synthesis of cytoplasmic (viral) DNA in infected cells and nuclear (host) DNA in uninfected cells is shown in Figure 2. Cultures lacking arginine, methionine, leucine or valine showed a 2 hr delay in maximum DNA synthesis (12 hr post infection) as compared to the control cultures in Eagles' MEM medium (10 hr post infection). Tyrosine deprived cultures showed maximum DNA synthesis at 8 hr and maintained that level of DNA synthesis for the duration of the experiment. Deprivation of any of the other essential amino acids did not affect the time-course of DNA synthesis in infected cells.

The initiation of viral DNA synthesis was delayed in all minus amino acid systems. In the control cells (MEM) 10% of the cells showed active DNA synthesis at 4 hr post infection, whereas an additional 4 hr was required before any of the deprived cultures exhibited a comparable number of cell synthesizing DNA with the exception of phenylalanine, tryptophan or tyrosine deprived cultures which required only 2 hours.

EXPLANATION OF FIGURE 2

Different sets of sparse SRK cells on coverslips in vials were pretreated for 16 hr with media lacking in one of the essential amino acids. At various time intervals following infection, cells were pulse-labeled with ^{3}H-TdR. Autoradiographs were scored for percent cells showing nuclear and/or cytoplasmic DNA synthesis over a 12 hr period.

FIGURE 2


Percent Cells With Cytoplasmic ³H-TdR
Percent Cells With Nuclear ³H-TdR
MEM
HIS
ARG
GLU
ISL
PA
LEU
MET
LYS
TYR
TRP
VAL
THR
Hours Post Infection

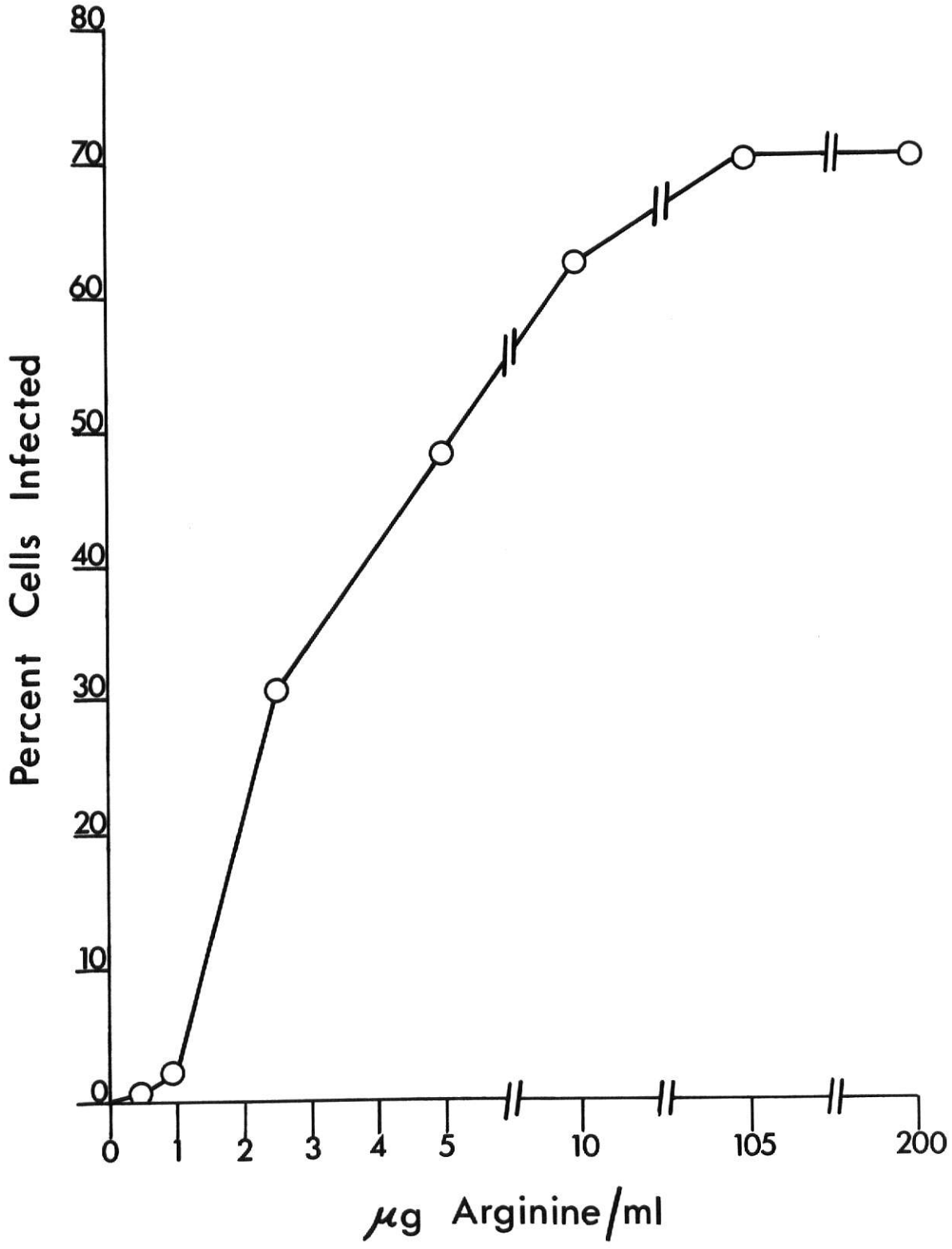
Sparse cell cultures maintained in MEM showed 60 to 80% cells infected at 10 hr post infection (Figure 2). Cultures deprived of arginine, glutamine or isoleucine were the least susceptible to infection with only 10% of the cells exhibiting DNA synthesis 10 hr post infection. Tryptophan or tyrosine deprived cultures showed maximum cells infected, 40 to 53% respectively. The remaining essential amino acids were intermediate in their inhibitory effect, since 20 to 35% of the cells were susceptible to infection.

Nuclear (host) DNA synthesis in uninfected cells maintained in MEM over a 12 hr period showed an average of 28% cells synthesizing DNA (Figure 2). The maximum inhibition of nuclear DNA synthesis was in cultures deprived of glutamine (4.1%), isoleucine or methionine (5.5%). The minimum inhibition was observed in cultures deprived of histidine, lysine or valine which allowed an average of 15% cells to synthesize host DNA. The remaining essential amino acids ranged between 7% (arginine and leucine), 11% threonine, tryptophan and tyrosine) and 13% (Phenylalanine) of cells showing host DNA synthesis averaged over a 12 hr period.

EXPLANATION OF FIGURE 3

Different sets of SRK monolayer cultures in 60 mm dishes were pretreated for 16 hr and maintained after infection in media containing different concentrations of arginine. Cultures were harvested 24 hr following infection and assayed for infectivity on sparse SRK cell cultures. Cells were pulse-labeled 7 hr post infection, and autoradiographs were scored for percent cells showing cytoplasmic DNA synthesis.

FIGURE 3


Percent Cells Infected
80
70
60
50
40
30
20
10
0
0
1
2
3
4
5
10
105
200
μg Arginine/ml

The Effect of Arginine Concentration on Virus Synthesis

Monolayer cultures of SRK cells were pretreated and maintained after infection in media containing various concentrations of arginine to determine which concentration could support maximum synthesis of infectious virus. Arginine in concentrations of 200 ug per ml and 105 ug ml (Eagles' MEM) was found to support maximum synthesis of infectious virus (Figure 3). A decrease in the arginine concentration to 10 ug per ml allowed 90% synthesis of virus progeny, while 2.5 ug per ml permitted 44% synthesis. The minimum virus synthesis (2.2 to 30%) was supported by arginine present in concentrations of 1.0 to 2.5 ug per ml, respectively.

The Effect of Arginine Concentration on Quantitative DNA Synthesis

Sparse cultures on coverslips in vials were pretreated for 16 hr and maintained in media containing various concentrations of arginine following infection. Quantitative autoradiography was used to determine the concentration of arginine needed to support maximum DNA synthesis. Maximum host (nuclear) and viral (cytoplasmic) DNA synthesis occurred when the concentration of arginine was 105 ug per ml (Eagles'

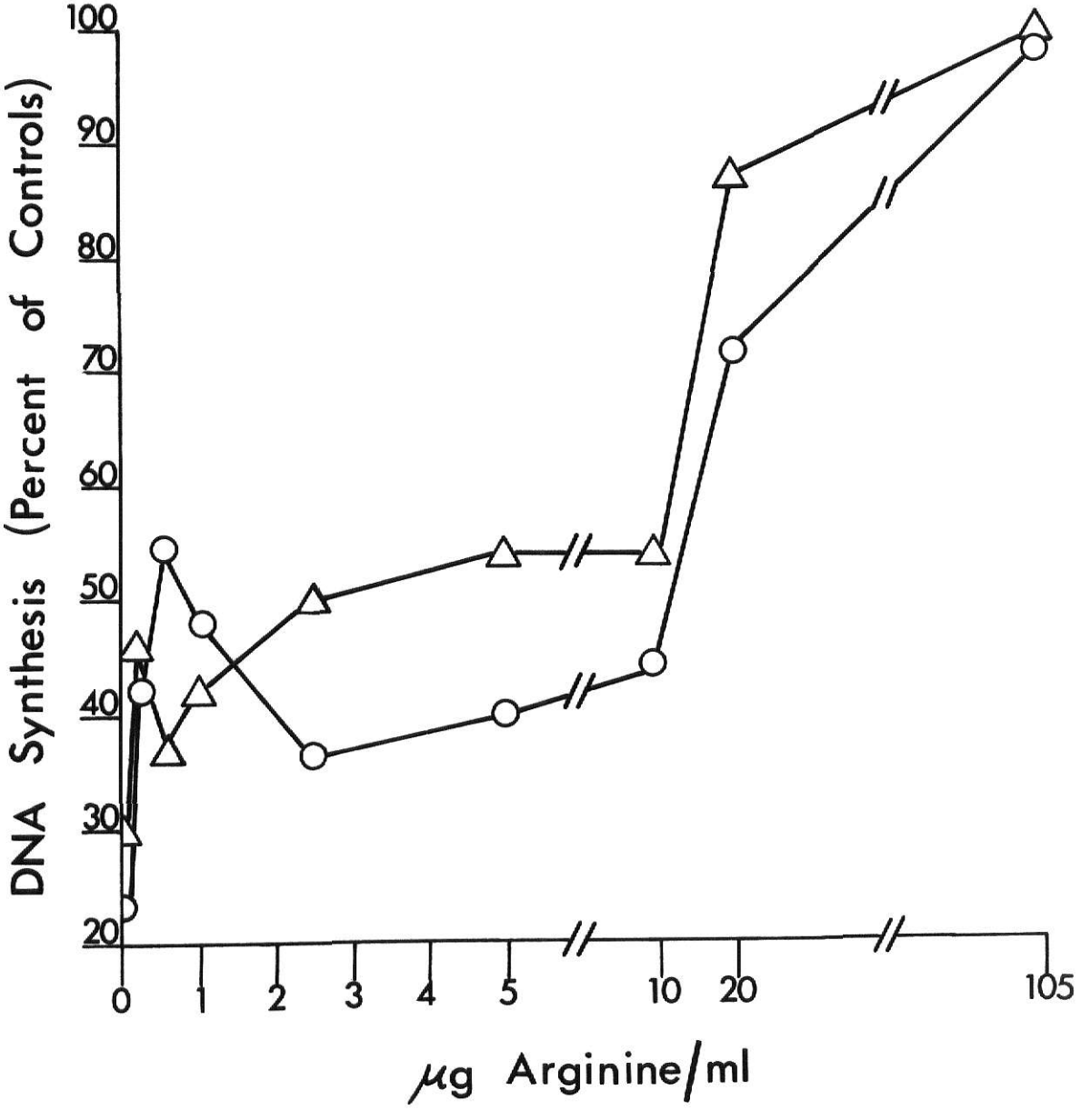
EXPLANATION OF FIGURE 4

Different sets of sparse SRK cell cultures on coverslips in vials were pretreated for 16 hr with media containing various concentrations of arginine. Cells were pulse-labeled with ^{3}H-TdR 7 hr post infection. Autoradiographs were scored for quantitative nuclear and/or cytoplasmic DNA synthesis and graphed as percent of the controls (105 ug per ml arginine).

△ : cytoplasmic (viral) DNA synthesis

○ : nuclear (host) DNA synthesis

FIGURE 4


DNA Synthesis (Percent of Controls)
100
90
80
70
60
50
40
30
20
0
1
2
3
4
5
10
20
105
μg Arginine/ml

MEM) as shown in Figure 4. Cultures deprived of arginine showed approximately 30% viral DNA synthesis and 24% host DNA synthesis. Stimulation of host or viral DNA synthesis was not observed with arginine concentrations between 1.0 to 10 ug per ml. However, 20 ug per ml of arginine increased host DNA synthesis from 40 to 70% and viral DNA synthesis from 50 to 90% as compared to the controls (Eagles' MEM, 105 ug arginine per ml).

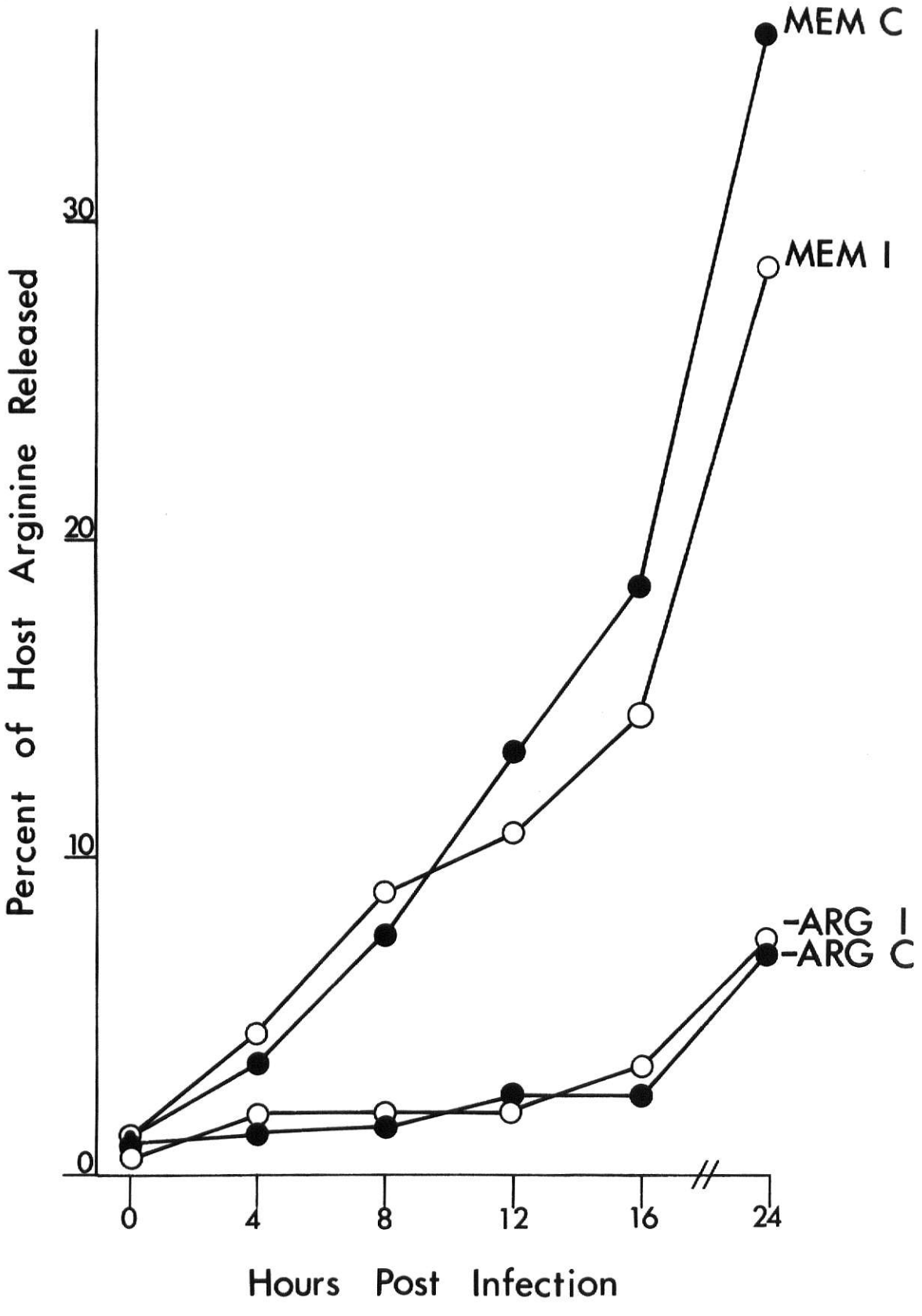
The Effect of Arginine Deprivation on the Release of Arginine from Secondary Rabbit Kidney Tissue Cultures

Monolayer cultures of SRK cells were prelabled with arginine-^{3}H and maintained in nonradioactive medium with or without arginine following infection. The release of labeled arginine into the supernatant fluid was indicative of the turnover of protein as shown in Figure 5. Uninfected and infected cultures maintained in Eagles' MEM for 24 hr had turned over 36% and 28% of their protein, respectively. Arginine deprived uninfected and infected cultures, however, had released only 7% of their protein after 24 hours. The turnover of protein began immediately in cultures maintained in MEM and continued to increase over the 24 hr period. Figure 5 indicates that arginine deprived cultures effectively prevented turnover of protein until 16 hr, when an increase from 3.5% to 7% was observed.

EXPLANATION OF FIGURE 5

Monolayer cultures of SRK cells in 60 mm dishes were prelabeled for 2 hr with medium containing 53 ug per ml arginine plus 1.0 uC per ml arginine-^{3}H. Cultures were maintained in Eagles' MEM medium for an additional 24 hr to deplete the cellular pool of unincorporated arginine-^{3}H. Following infection one set of dishes was maintained in MEM, while the other set received minus arginine medium. At intervals following infection, samples of supernatant fluid were assayed for the radioactive acid soluble fraction.

FIGURE 5


MEM C
MEM I
-ARG I
-ARG C
Percent of Host Arginine Released
30
20
10
0
0
4
8
12
16
24
Hours Post Infection

Infected cultures maintained in MEM showed a decrease in protein turnover occurring at 12 hr post infection. The difference in the turnover of protein in infected and uninfected cultures in MEM was 4% at 16 hr and 8% at 24 hours. This suggested that infected cultures were conserving arginine. However, no difference in protein turnover could be observed in infected or uninfected arginine deprived cultures.

The Effect of Arginine Deprivation on the Synthesis of Early Proteins in Cultures Treated with FUdR

Sparse cultures were pretreated for 16 hr in minus arginine medium. Following infection cultures were maintained in arginine deprived media plus FUdR. Figure 6A and 6B show the quantitative DNA synthesis in cells after reversal of FUdR inhibition at various times. Cells maintained in MEM or MEM plus FUdR and reversed at 2,4 and 6 hr (Figure 6A) demonstrated similar time-course curves of DNA synthesis and quantity of DNA synthesized. However, cells maintained in minus arginine medium plus FUdR and reversed at 2,4 and 6 hr synthesized three times more DNA than that synthesized in cells maintained in minus arginine medium alone (Figure 6B). When FUdR inhibition was reversed at 2 hr in arginine deprived cells, the time-course of DNA synthesis was delayed by 2 hr, showing a maximum at 12 hr post infection.

Figures 6C and 6D show the percent cells synthesizing DNA at any one time interval, and confirm the findings of the quantitative data.

EXPLANATION OF FIGURE 6

Sparse cell cultures of SRK cells on coverslips in vials were pretreated for 16 hr with minus arginine medium or Eagles' MEM. Following infection one set of vials was refed with MEM medium containing 15 ug per ml FUdR, while the other set received minus arginine medium plus FUdR. At various intervals, FUdR inhibition was reversed with the addition of 5 ug per ml cold thymidine. Cells were pulse-labeled with ^{3}H-CdR and autoradiographs scored for quantitative and qualitative DNA synthesis.

●-●: MEM and/or -ARG

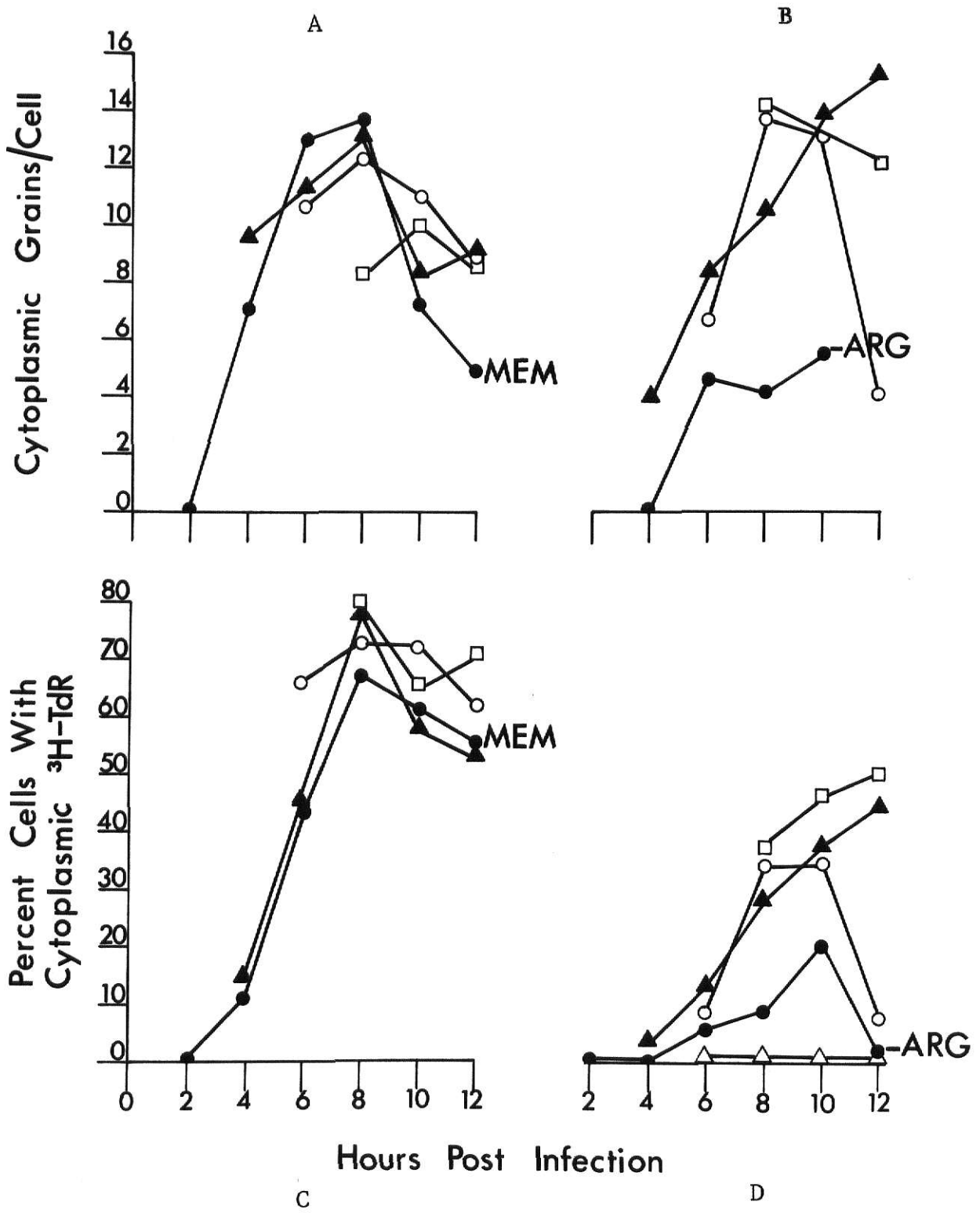
▲-▲: MEM and/or -ARG plus FUdR: plus cold thymidine at 2 hr

O-O: MEM and/or -ARG plus FUdR: plus cold thymidine at 4 hr

□-□: MEM and/or -ARG plus FUdR: plus cold thymidine at 6 hr

△-△: -ARG plus FUdR

FIGURE 6


A
B
C
D
Cytoplasmic Grains/Cell
Percent Cells With Cytoplasmic ³H-TdR
Hours Post Infection
MEM
-ARG
MEM
-ARG
0
2
4
6
8
10
12
14
16
20
30
40
50
60
70
80

DISCUSSION

Studies reported by Eagles (1959) on the nutritional requirements for growth and propagation of cultured mammalian cells in vitro demonstrated an absolute requirement for thirteen essential amino acids: arginine, cystine, glutamine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, tyrosine, and valine. Different cell lines were found to require differing concentrations of these amino acids for optimum growth i.e. HeLa cells required 0.03 mM cystine, whereas L cells required 0.01 mM (Eagle, 1955). Eagle also noted that the effect of some amino acid deprivations produced cytopathic changes in the cell that closely resembled those resulting from virus infection. In the studies reported, rabbit kidney cell cultures did not show any cytopathic changes except when cystine was omitted from the medium which produced rapid cell death in 16 hours. This is in contrast to the observations of Holtermann (1969) in L cells deprived for 72 hr of any one of the essential amino acids where there were no cytopathic changes. Winters and Consigli (1969) observed inhibition of cell proliferation under arginine deprived conditions but no loss in the viability of mouse embryo cell cultures. Arginine deprivation of RK 13 cells and HEp-2 cells inhibited normal cell division, but confluent monolayers could be maintained

for several days without the appearance of cytopathic effects (Inglis, 1968; Roizman et al., 1967).

The requirement of RK cells of cystine to maintain viability as well as the effects of arginine deprivation on the cell lines described indicate the importance of considering these effects on the host cell when studying viral replication under amino acid deprived conditions.

A specific example of amino acid control of a cellular process has recently been reported by Ley and Tobey(1970). Mouse L, Syrian hamster BHK 21 and Chinese hamster cell cultures deprived of isoleucine for 36 hr accumulated in the G_1 phase of the cell division cycle. Addition of isoleucine at 36 hr reinitiated DNA synthesis and produced a synchronous cell division in all three cell lines. Synthesis of protein during G_1 is a prerequisite for the initiation or continued replication of DNA synthesis in mammalian cells (Terasima and Yasukawa, 1966). Poxvirus DNA synthesis has also been shown to require concurrent synthesis of protein(s) (McAuslan, 1969). The data presented here demonstrated that deprivation of any one of the essential amino acids inhibited host or fibroma virus DNA synthesis to the same extent during a 12 hr period. This suggests that their synthesis is under the influence of the same control mechanisms. The level of control is most

likely the synthesis of messenger RNA (m-RNA) coded for by the virus for viral DNA synthesis, or in the case of mammalian cells a m-RNA for histone synthesis coded for by the host genome (Gallwitz and Mueller, 1969). Smulson and Thomas (1969) have shown that HeLa cells deprived of amino acids decreased their RNA synthesis due to the accumulation of deacylated transfer RNA. When the cellular pool of a single amino acid was depleted, a decrease in the rate of ribosomal RNA synthesis in bacteria (Copeland, 1969; Goodman, et al., 1969; Cleaves and Cohen, 1970; Donini, 1970) and yeast was observed (Kjellin-Straby, 1969; Phillips, 1969). The evidence suggests that in these cells either aminoacylated or nonacylated transfer RNA, rather than free amino acids alone, interacts with a cellular regulatory component which controls the synthesis of RNA. Rabbit kidney cells deprived of any of the amino acids produced relatively equal inhibitions of nuclear (host) DNA synthesis in uninfected cells and cytoplasmic (viral) DNA synthesis in infected cells. However, host and viral DNA synthesis in cells deprived of glutamine, methionine or isoleucine were inhibited by 65% when compared to controls (MEM). Lack of any of the other amino acids produced inhibitions of 50 to 25 percent. This data might suggest that these three amino acids are more involved in the initiation of host DNA synthesis which directly or indirectly influences viral DNA synthesis

by interfering with the normal division process.

Figure 1 presents the difference between viral DNA synthesis and progeny virus formation in amino acid deprived cultures. The omission of arginine, glutamine, leucine or methionine from the medium prevented virus synthesis but allowed 30 to 60% of viral DNA synthesis. However in all the other amino acid deprived cultures, viral DNA synthesis was followed by the synthesis of some virus progeny. This suggested that the amount of viral DNA synthesized was not the limiting factor for progeny formation, but some later step in the replication cycle was inhibited to the greatest degree when these four amino acids were lacking from the medium. The observed inhibition of progeny virus synthesis, whether complete or partial, could be due to defective virus particles containing incomplete viral DNA, defective structural components or lack of viral enzymes associated with the complete virus particle. Any one condition or combination of the above would be presumed to produce the observed inhibition, however further investigations must be carried out to determine the exact mechanism by which progeny virus synthesis is inhibited.

Depending on the specific amino acid removed from the medium, the number of cells showing active host or viral DNA

synthesis was greatly influenced over a 12 hr period (Figure 2). Cultures in complete medium (MEM) showed 10% cells synthesizing viral DNA at 4 hr post infection, whereas it required another 4 hr for any of the other amino acid deprived cells, except minus phenylalanine, tryptophan or tyrosine cultures which required only 2 hr to show the same percent cells synthesizing viral DNA. The observed delay in viral DNA synthesis may be a result of delayed repression of host control mechanisms determining host DNA synthesis. The increase in time may reflect the time needed to accumulate "initiator" protein synthesized under amino acid deprived conditions required to promote synthesis of viral DNA.

Isoleucine deprived cells never showed more than 8% cells synthesizing viral DNA. This might be interpreted in view of Ley and Tobey's report (1970) on the control of isoleucine on initiation of host DNA synthesis to mean that host cells in the G_1 phase are the least susceptible to viral infection. Host enzymes needed by the infecting virus for early macromolecular synthesis i.e. DNA polymerase, DNA dependent RNA polymerase, would be present in the S phase during active host DNA synthesis but not in the G_1 phase which could account for the observed lack of susceptibility to virus infection in these cells.

The decrease in the number of cells showing viral DNA synthesis in minus amino acid systems corresponded directly with the decrease in the number of cells synthesizing host DNA. This may suggest that some control mechanism on the part of the host, as previously postulated in the case of isoleucine deprivation, may determine the susceptibility of cells to infection. However, once the cell is infected, the control of host DNA synthesis is repressed. Fibroma virus infection depresses host DNA synthesis, only 5% of the cells exhibit nuclear DNA synthesis at 10 hr (Minocha and Maloney, 1970). This may occur through modification of host enzymes for viral DNA synthesis or preferential use of protein precursors by the virus for viral synthesis.

The effect of arginine deprivation on fibroma virus synthesis was studied in greater detail, since considerable evidence has shown this amino acid to be necessary for a late replication event in polyoma, SV40, herpesvirus and adenovirus synthesis, all are intranuclear DNA viruses. Synthesis of fibroma virus (2.2 to 43%) in RK cells required 1.0 to 2.5 ug per ml of arginine, respectively. The threshold concentration of arginine for herpesvirus synthesis (10%) in BSC_1 cells was 42 ug per ml (Becker et al., 1967) and in RK 13 cells was 53 ug per ml allowing 80% synthesis of herpesvirus (Inglis, 1968).

A minimum of 0.126 to 0.63 ug per ml of arginine permitted 10% synthesis of polyoma virus in mouse embryo cells (Winters and Consigli, 1969). This would suggest that different cells have different arginine pools and/or that synthesis of a particular virus requires different amounts of exogenous arginine. The data presented was obtained under different experimental conditions. Only in the case of fibroma virus synthesis were the RK cells pretreated for 16 hr prior to virus infection to deplete the intracellular pool of arginine. Controlled studies on several mammalian cell lines infected with fibroma virus would determine the variation in arginine pools and its effect on the threshold concentration of arginine for virus synthesis.

A comparison of the data presented in Figures 3 and 4 suggested that the synthesis of viral progeny was dependent on an arginine-requiring step late in the replication cycle. Thirty percent viral DNA synthesis occurred in the absence of exogenous arginine and increased to 50% when the concentration of arginine was 10 ug per ml. However, progeny virus synthesis was completely inhibited in minus arginine cultures, but had increased to 90% of controls when arginine was present at a concentration of 10 ug per ml. This was a ninety-fold increase in progeny, but only a 1.5-fold increase in viral DNA synthesis.

This would indicate that sufficient viral DNA was synthesized, but could not be packaged to give rise to viral progeny unless arginine was present in critical amounts necessary for later events in the replication cycle.

The observed inhibitory effect of arginine deprivation on virus synthesis could be due to "selective protein failure" (Spring et al.,1969). The translation of m-RNA in deprived cells would be inhibited at all codons specifying arginine when the arginine pool was depleted. The arginine pool present in infected cells maintained under deprived conditions appears to be sufficient for early protein synthesis and initiation of viral DNA synthesis. However, the arginine pool cannot sustain the synthesis of late proteins coded for by the viral genome which are rich in arginine and necessary for viral maturation. The data presented in Figure 5 demonstrated the turnover of protein in cultures maintained under arginine deprived conditions. There was four (infected MEM) to five (control MEM) times more turnover of protein in cultures maintained in MEM than that observed in minus arginine cultures. This data would support the hypothesis of "selective protein failure".

Several investigators have reported the accumulation of early enzymes during arginine deprivation of infected cells

determined by immunofluorescence (Rouse and Schlesinger, 1967; Goldblum et al., 1968; Courtney et al., 1970). Figures 6A and 6B demonstrate the accumulation of early enzymes in the presence of FUdR during arginine deprivation of fibroma infected RK cells as determined by quantitative autoradiography. The reversal of FUdR inhibition in minus arginine cell cultures allowed three times the amount of viral DNA to be synthesized. The early enzymes accumulated in the presence of FUdR when all DNA synthesis was inhibited, and reversal of the FUdR inhibition permitted immediate DNA synthesis at levels observed in cultures maintained in MEM (Figures 6A and 6B). Concurrent protein synthesis was shown to be necessary for poxvirus DNA synthesis (Kates and McAuslan, 1967; Bedson and Cruickshank, 1968). When cells were deprived of arginine, early enzymes were synthesized at a constant, but reduced level which resulted in decreased viral DNA synthesis (Figure 6B). However subsequent DNA synthesis in the presence of the accumulated enzymes occurred at the rate normally observed in cultures maintained in MEM (Figure 6A). If viral DNA synthesis occurs, then late m-RNA which code for the late proteins are made. The synthesis and accumulation of the late proteins act to shut down the synthesis of the early proteins. Therefore, early enzymes synthesized in the presence of FUdR

accumulated because all DNA synthesis was inhibited which prevented the synthesis of late proteins necessary to "switch off" early enzyme synthesis. The increased level of DNA synthesis in arginine deprived cells upon reversal of FUdR inhibition was due to this phenomenon.

The synthesis of host and viral DNA during amino acid deprived conditions appears to be under the same control mechanism. This coordinated inhibitory effect directly influences the susceptibility of the cells to fibroma virus infection. Whether or not it is due to interruption of DNA synthesis or interference with the initiation of DNA synthesis is not known.

Progeny virus was completely inhibited in four of the twelve minus amino acid systems suggesting that arginine, methionine, glutamine and leucine are particularly important for fibroma virus synthesis. Amino acid analysis of a purified preparation of fibroma virus may determine if these four amino acids are present in the greatest amount, and therefore would be particularly important for progeny synthesis.

The evidence presented in this work is in accord with studies reported by other investigators who found that early protein synthesis and viral DNA synthesis occurred during

arginine deprived conditions, but some late step was inhibited in viral replication preventing formation of complete infectious progeny.

Shope fibroma virus is the first intracytoplasmic DNA virus shown to require arginine for its synthesis and can be added to the growing list of intranuclear DNA viruses and intracytoplasmic RNA viruses whose requirement for arginine has already been demonstrated.

SUMMARY

The effect of amino acid deprivation on the host and shope fibroma virus DNA synthesis in RK cells was determined by autoradiography. Uninfected and virus infected cultures deprived of any one of the essential amino acids showed inhibitory effects on DNA synthesis. The degree of inhibition was maximum in cultures lacking glutamine, isoleucine or methionine (55-65% inhibition) whereas histidine deprived cells showed the minimum inhibitory effect (35%) as compared to control cultures in Eagles (MEM).

Synthesis of infectious virus progeny was completely inhibited in cultures deprived of arginine, glutamine, leucine or methionine. Lack of phenylalanine or isoleucine produced less than 10% virus progeny. Virus yields of the remaining essential amino acid deprived cultures ranged between 20-40%.

Cultures deprived of glutamine, arginine or isoleucine showed 10% cells or less and tryptophan or tyrosine deprived cultures demonstrated 40 and 53% cells respectively, synthesizing viral DNA.

The turnover of protein as indicated by the release of arginine from uninfected RK cells maintained in MEM media was 36%, as compared to 7% observed in uninfected cells maintained in minus arginine medium. Infected cells in MEM were shown

to conserve arginine after 12 hours, showing a decrease of 8% in the protein turned over.

Presence of arginine in concentration of 105 ug/ml of media demonstrated maximum DNA and fibroma virus progeny synthesis.

Early proteins seem to accumulate when viral DNA synthesis was inhibited in minus arginine cultures containing FUdR. However, when the inhibition was reversed by the addition of thymidine, an increased rate of DNA synthesis was observed.

APPENDIX

Media

Medium 199 (Grand Island Biological Company, New York)

Fetal calf serum (KAM Laboratories, Kansas City, Kansas)

Primary rabbit kidney (PRK) cells were grown in Medium 199 containing 0.75 g/l $NaHCO_3$ supplemented with 8% fetal calf serum (FCS). Tissue cultures were maintained in Medium 199 containing 1.5 g/l $NaHCO_3$ plus 5% FCS. This was also used for propagation of the virus.

Experimental Media

10X Earles' Salt Solution

NaCl	68.0 g
KCl	4.0 g
$NaH_2PO_4.H_2O$	1.4 g
$MgCl_2.6H_2O$	0.8 g
$NaHCO_3$	22.0 g
Dextrose	10.0 g
Phenol red	0.05g

Chemicals are combined in the above order being sure that each is in solution before adding the next. It is made to be final volume of 1 liter with deionized water and millipore-filtered to sterilize.

100X $CaCl_2$

20.0 g of $CaCl_2$ per liter of deionized water was dissolved and milliporefiltered to sterilize.

100X Antibiotics

A stock solution of 10,000 units of Penicillin G (Squibb) and 0.01 g Streptomycin (Eli Lilly) per ml was prepared by adding to 100 ml of sterile deionized water 1,000,000 units of Penicillin and 1.0 g Streptomycin. One ml of the stock solution is added to 100 ml of media giving a final concentration of 100 units of Penicillin per ml and 0.1 mg Streptomycin per ml.

7.5% $NaHCO_3$

7.5 g $NaHCO_3$ per 100 ml of deionized water was dissolved and milliporefiltered to sterilize.

Dialyzed Fetal Calf Serum (DFCS)

300 to 500 ml of fetal calf serum was dialyzed against 4 to 6 changes of saline, pH 7.2 over a four-day period. The dialyzate was filtered through a Selas porcelain filter, pore size 0.22 u and stored at -20 C.

50X Amino Acid Stock Solutions

Arginine (ARG)	5.25 g in 10 ml
Cystine (CYS)	1.20 g "
Glutamine (GLU)	14.60 g "
Histidine (HIS)	1.55 g "

Isoleucine (ISL)	2.62 g in 10 ml
Leucine (LEU)	2.62 g "
Lysine (LYS)	2.90 g "
Methionine (MET)	0.75 g "
Phenylalanine (PA)	1.60 g "
Threonine (THR)	2.40 g "
Tryptophan (TRP)	0.50 g "
Tryosine (TYR)	1.80 g "
Valine (VAL)	2.30 g "

Chemically pure preparations of the above amino acids were obtained from Sigma Chemical Company (St. Louis, Missouri). The salts of the amino acids were dissolved in concentrated HCl or NaOH and made to a final volume of 10 ml in deionized water and autoclaved 10 min, 8 lb pressure at 235 F with the exception of arginine, glutamine and lysine which were filtered to prevent decomposition (Merck Index). Stock solutions (50X) were then made by combining 1.0 ml of the various 500X stocks together, omitting a different amino acid from each of the ten stock solutions and made to a final volume of 100 ml with deionized water. Stock solutions (50X) lacking arginine, valine and histidine were obtained from Grand Island Biological Company, New York. The final concentrations of these amino acids in complete media, were those found in Eagles' MEM medium.

Eagles' Minimal Essential Medium (MEM)

10X Earle's salts	10.0 ml
100X Antibiotics	1.0 ml
100X $CaCl_2$	1.0 ml
100X MEM vitamins	1.0 ml
100X glutamine	1.0 ml
DFCS	3.0 ml
50X Amino acid stock	2.0 ml

The above solutions were combined and made to a final volume of 100 ml with deionized water, adjusting the pH to 7.2 with 2.0 N HCl or 7.5% $NaHCO_3$ to make complete Eagles' MEM medium. Glutamine was omitted only when glutamine deficient media was made. 100X MEM vitamins stock solution was obtained from Grand Island Biological Company, New York.

Phosphate Buffered Saline (PBS)

Basic solutions:

1. $Na_2HPO_4.12H_2O$	176.1 g in 1.0 liter of deionized water
2. $NaH_2PO_4.H_2O$	69.0 g in 1.0 liter of deionized water

Buffer Stock

$Na_2HPO_4.12H_2O$	386 ml
$NaH_2PO_4.H_2O$	114 ml
Deionized water	500 ml

pH 7.15, 0.01 M

10X Hanks Basal Salt Solution (BSS)

Solution #1

NaCl	100.0 g
KCl	5.0 g
$MgSO_4 7H_2O$	2.5 g

Dissolve in 500 ml deionized water

Solution #2

$NaHPO_4.H_2O$	1.87 g
KH_2PO_4	0.75 g

Dissolve in 500 ml deionized water

Solution #3

$CaCl_2.2H_2O$	2.3 g

Dissolve in 250 ml deionized water

Each salt should be dissolved before adding the next. Combine solutions #1 and #2, and add 250 ml deionized water. Shake well and add solution #3. Add 0.25 g phenol red.

Complete Hanks BSS

10X Hanks BSS	100.0 ml
10% Dextrose	20.0 ml
100X Antibiotics	10.0 ml
Deionized Water	870.0 ml

Adjust to pH 7.2 to 7.4 with 7.5% $NaHCO_3$ and millipore-filter to sterilize.

Trypsin Solution 0.25%

Trypsin	10.0 g	(Nutritional Biochemical Co.)
PBS	4.0 liters	

Stir on a magnetic stirrer until dissolved, millipore-filter to sterilize. Final concentration of trypsin is 0.25% in 0.01 M phosphate buffered saline.

Giemsa Stain

Giemsa powder	0.8 g	(Matheson Coleman and Bell)
Glycerol	50.0 ml	
Methanol	50.0 ml	

Dissolve powder in mixture and place on shaker for 2 days.

Staining for Autoradiography

Stock Solutions

Giemsa stock

Citric Acid 0.1 M	(21.0 g/liter)
Na_2HPO_4 0.2 M	(28.4 g/liter)

pH 5.75 Buffer

Citric Acid 0.1 M	85 ml
Na_2HPO_4 0.2 M	115 ml
Deionized water	800 ml

Adjust pH to 5.75 with either of the stock solutions.

Stain

pH 5.75 Buffer	276 ml
Methanol	9 ml
Giemsa Stock	15 ml

Use this stain fresh within one-half hour. Stain slides for 15 min and rinse quickly in distilled water.

ACKNOWLEDGEMENTS

The author wishes to thank Dr. Harish C. Minocha for his expert guidance during the course of this investigation and for his helpful advice in the preparation of this manuscript. The helpful suggestions of my committee members Dr. Richard A. Consigli and Dr. John D. Iandolo are gratefully acknowledged.

This investigation was supported by research grant No. RO1 CA 11801-02 from the National Institutes of Health.

The author wishes to thank Mrs. A. Wilson for her help in the completion of this manuscript.

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EFFECT OF AMINO ACID DEPRIVATION IN UNINFECTED AND SHOPE FIBROMA VIRUS INFECTED RABBIT KIDNEY CELLS

by

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B. S., University of Massachusetts, 1966

AN ABSTRACT OF A MASTER'S THESIS

submitted in partial fulfillment of the

requirements for the degree

MASTER OF SCIENCE

Division of Biology

Microbiology

KANSAS STATE UNIVERSITY
Manhattan, Kansas

1971

Shope fibroma virus, an oncogenic poxvirus, can be propagated in rabbit kidney (RK) cells. The virus produces characteristic cytoplasmic B-type inclusions which are the sites of viral macromolecular synthesis. Cells infected with Patuxent strain of the virus show an initial depression of host macromolecular synthesis followed by an increased nuclear synthetic activity.

The effect of amino acid deprivation on host (nuclear) and viral (cytoplasmic) DNA synthesis was determined by quantitative autoradiography over a 12 hour period. Sparse RK cultures on coverslips were pretreated for 16 hours with media deprived of individual essential amino acids. Uninfected and infected cultures were pulse-labeled at 2 hour intervals with tritiated thymidine (3H-TdR). Cultures maintained in Eagle's (MEM) served as controls. Viral and host DNA synthesis in cultures deprived of different amino acids was inhibited to the same degree. Glutamine, isoleucine or methionine deprived cells showed maximum inhibitory effect (55-65%) on the host and the viral DNA synthesis, whereas histidine deprived cells demonstrated the minimum inhibition (35%).

Synthesis of infectious progeny assayed by the enumeration of cytoplasmic inclusions was inhibited in cultures pretreated for 16 hours and maintained for 24 hours after infection in media lacking arginine, glutamine, leucine or methionine.

However, cultures lacking isoleucine or phenylalanine showed 10% or less synthesis of virus progeny. Deprivation of any of the remaining essential amino acids allowed 20 to 40% synthesis of progeny virus made in cultures maintained in Eagle's (MEM).

The initiation of viral DNA synthesis was delayed in each of the minus amino acid systems. In the control cells (MEM), 10% or less cells showed DNA synthesis at 4 hours post infection. However, an additional 2 hour period was required before any of the amino acid deprived cultures demonstrated a comparable number of cells synthesizing DNA.

Monolayer cultures of RK cells pretreated and maintained after infection in media containing different concentrations of arginine demonstrated a maximum synthesis of infectious virus at a concentration of 105 ug arginine per ml of medium. An identical requirement of arginine for maximum DNA (host and/or viral) synthesis was also observed as determined by quantitative autoradiography. Synthesis of infectious virus in cell cultures was shown to require the presence of arginine. A concentration of 10 ug of arginine/ml allowed 1.5 times as much viral DNA synthesis as observed in cultures deprived of arginine; however a 90 fold increase in progeny virus synthesis occurred. Protein turnover in monolayer cultures prelabeled

with ^{3}H-arginine and maintained in Eagle's (MEM) or minus arginine medium for 24 hours was determined by measuring the radioactivity in the acid soluble fractions. Infected and uninfected cells maintained in minus arginine media conserved 4 to 5 times the amount of ^{3}H-arginine as compared to cells maintained in Eagle's (MEM).

Sparse cell cultures infected with the virus and maintained in minus arginine medium containing fluorodeoxyuridine (FUDR) demonstrated complete inhibition of viral DNA synthesis. Reversal of FUDR inhibition by thymidine demonstrated immediate synthesis of viral DNA indicating accumulation of early proteins in arginine deprived cultures.