HERPES SIMPLEX VIRUS: AMINO ACID REQUIREMENTS IN HUMAN SKIN CELL CULTURE

Thesis for the Degree of M. S.

MICHIGAN STATE UNIVERSITY
JAMES X. HARTMANN

1967

HERPES SIMPLEX VIRUS: AMINO ACID REQUIREMENTS IN HUMAN SKIN CELL CULTURE

By

James X. Hartmann

A THESIS

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

MASTER OF SCIENCE

Department of Microbiology and Public Health

1967

DEDICATION

To my parents, whose support and guidance made this work possible.

ACKNOWLEDGMENTS

Due appreciation is extended to Dr. Walter N. Mack for counseling and guidance received throughout this work. The author is also indebted to Dr. Virginia H. Mallmann and Dr. Richard W. Luecke for a critical review of the manuscript. Sincere gratitude is extended to Mrs. Eadene B. Winterton for technological assistance. The patience and understanding of my wife is received with the utmost appreciation.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABSTRACT

HERPES SIMPLEX VIRUS: AMINO ACID REQUIREMENTS IN HUMAN SKIN CELL CULTURE

by James Hartmann

The amino acid requirements for production of herpes virus in tissue cell cultures were studied. Each of thirteen essential amino acids (L-isomers) in minimum essential medium (MEM) or basal medium Eagle (BME) were individually omitted and the amount of virus produced was assayed by plague technique. The requirements for the AU (human skin epithelium) were studied in particular; HEp-2 (human epidermoid carcinoma) was investigated in comparison to results with AU cells. To provide a complete chemically defined medium methycellulose replaced serum.

Methionine was the most and histidine the least required for AU cells in MEM. The methionine requirement was less marked with BME than MEM. Cystine was least required by both AU and HEp-2 when BME was employed. Demonstration of an arginine requirement for viral replication depended on its concentration in the medium employed, either BME or MEM.

AU cells and HEp-2 cells had similiar requirements for twelve of thirteen amino acids in BME. In a glutamine deficient medium, HEp-2 cells produced one seventh the amount of virus produced by AU cells, when compared to the amount produced in complete medium.

The necessity of determining the optimal time of assay for virus from infected cells in deficient media, and the importance of recognizing what media to employ were discussed.

INTRODUCTION

A model system for the study of human tumor virus and tumor cell relationship was needed. Since the wart virus causes a tumor (verruca vulgaris) in humans, isolation and characterization of the virus and a host cell was highly desirable.

Hayashi (22) demonstrated wart virus infection by inoculation of particulate wart tissue onto cultured human skin epithelium designated AU by Wheeler et al (50). Twelve other cell lines were tested but only the AU line supported growth of the wart virus, detected by degeneration of the cells. The virus could be subcultured only by passage of infected cells onto normal cells, and it was not isolated from cell-free tissue culture medium. Latent or masked viral growth would sometimes occur, exhibiting infection only on repeated passage. A minimally enriched medium afforded best growth of the agent.

The presence or absence of certain amino acids in tissue culture medium has been shown to markedly effect the amount of virus produced (15) (36) (44), and the development of latency (29) (30) (36). The particular amino acids required by the AU cell for virus production were unknown prior to this study. Elucidation of these requirements may provide a medium for increased virus production and perhaps eventual isolation of infective wart virus particles from tissue culture. The virus could then be more readily studied in its relation

to host cell infection and tumor (wart) production.

Since the wart virus was not readily isolated or quantitated on the AU cell line, another related virus, herpes simplex, had to be used. Another cell line was tested for amino acid requirements as a comparison to those of the human skin epithelium, AU. A unique requirement by the AU cell line may indicate why it was the only one of thirteen cell lines which was susceptible to infection by the wart virus.

REVIEW OF LITERATURE

I. Studies on the Presence or Absence of Amino Acids and Related Compounds in Various Tissue Cell Systems in Relation to Viral Replication.

Investigations on the role of amino acids in viral multiplication have utilized amino acid analogues, an excess of the natural amino acid, or a deficiency of the amino acid in the medium. Early studies employed analogues, chemical compounds similiar to the metabolite but with different substituted chemical groups. It was assumed the analogue competed with the natural amino acid for utilization.

Thompson (45), studied vaccinia virus growth in chick embryo tissue cells. Methoxinine, the analogue of methionine was used in varying concentrations. Viral multiplication was inhibited, but it was not determined if methoxinine was competing or toxic. Ackerman (1) found that methoxinine was not destroying virus or host cells, nor did it prevent infection. He suggested the analogue reduced the concentration of the essential metabolite or competed for enzymatic sites.

Vaccinia virus growth was inhibited in chick embryonic cell culture when B-2-thienyalanine was added without the natural metabolite phenylalanine (46). Since inhibition was reversed by addition of phenylalanine, the analogue was competitive. Whether B-2-thienyalanine acted directly on the cell or on the virus was not determined.

Excess lysine at one mg/ml allowed very little reproduction of Theiler's virus in minced mouse brain (35) (39). Histidine and tryptophan had to be present at three mg/ml to inhibit; other amino acids were not inhibitory even at this high concentration. The lack of radioactive phosphate incorporation into phospholipid and protein-bound phosphate fraction suggested that an excess of amino acids antagonized incorporation of phosphate into host tissue.

Excess amino acids inhibited mumps and influenza virus replication on chorioallantoic membrane suspended in Hanks' balanced salt solution (17). The basic amino acids arginine, lysine, ornithine and some others were inhibitory in concentrations varying from 1 to 10 mg/ml. Amino acids with two basic groups were most active, and the effect was augmented by high pH.

Lysine serine and ornithine were inhibitory at one mg/ml for Theiler's virus in mouse tissue cell culture (34). Phenylalanine and others were inhibitory but at 3 mg/ml concentration. Lysine was found to be the most inhibitory in this system; even .5 mg/ml showed activity. Lysine inhibition was reversed not only by methionine but by leucine and tyrosine as well.

Reversal or augmentation of lysine inhibition by other amino acids has been related to transport of lysine across the cell membrane (13). Krebbs 2 ascites tumor cells were chosen as the ideal cell for this study and infected with influenza virus. When virus infected cells

were placed in a minimal media with an inhibitory concentration of lysine, addition of histidine caused increased uptake of lysine into the free amino acid pool of the cell and also increased lysine inhibition of viral replication. Other amino acids decreased transport of lysine and reversed its inhibitory effect. However, it was later shown (19), that reversal of inhibition was not related to the prevention of the amino acid from entering the cell. For instance, proline and alanine reduced transport of ^{14}C phenylalanine into Krebbs 2 ascites tumor cells and also reversed phenylalanine inhibition of hemagglutination (protein coat) formation. Serine which also reverses phenylaline inhibition, did not reduce phenylaline transport but increased it.

A possible mode of action of lysine, once inside the cell at inhibitory concentrations (18), was a damaging effect on synthesis of a viral precursor protein or enzyme formed during the first three hours of infection. Lysine added at 15 millimolar concentration several hours after infection had little effect on replication of virus protein coat. If added during the first three hours, it was inhibitory. Labeling lysine with ^{14}C indicated an interaction with the protein-nucleic acid fraction of cells, perhaps the viral precursor.

Viral replication inhibition by amino acid excess has been extended to include leucine, tryptophane and phenylaline (19). Their ability to inhibit, with the exception of leucine, could be reversed by addition of each of 18 other amino acids

at non-inhibitory concentrations. Perhaps the presence of excess amino acids "in nature" may play a role in regulating the amount of virus a cell can synthesize. If a cell was present in an organism at a site where the surrounding medium contained an excess of an amino acid, this cell would be an unfavorable host. The finding that D-amino acids were much less effective as inhibitors or reversors indicated a specific site of attachment for the natural L-isomers. Uptake of ^{14}C-leucine into cell protein also indicated that excess amino acids inhibited cellular as well as viral protein synthesis.

Of the amino acids discussed above, lysine and histidine were related to virus growth in mouse brain in vivo (40). Amounts of these two amino acids were reduced in virus infected tissue, indicating a role in cell metabolism during viral propagation. Furthermore, virus infection stimulated incorporation of ^{14}C from glucose into most amino acids except lysine and histidine.

Perhaps analogues were not indicating a true requirement for an amino acid. In the case of phenyalanine (34), analogues were inhibitory at 0.1 mg/ml, whereas the metabolite had to be at thirty times this concentration.

The first evidence indicative of some of the limitations in the use of analogues was presented by Brown (4). In growth studies of poliomyelitis virus in monkey testicular cell culture, five out of nine amino acid analogues were inhibitory. Four were analogues of aspartic, methionine or phenylalanine and might indicate their essentiality, but

many of these same analogues inactivated virus in the supernatant fluid. This indicated the compounds not only may not be useful in determining amino acid requirements, but may also render the cells incapable of viral multiplication.

Most of the work up to this point suggested little correlation between amino acid requirements of a particular virus and certain cell lines. Lysine and methionine, when in excess, seemed to be the prominent inhibitors. Morgan (32) indicated common needs among various host-virus systems. His finding that the analogues B-2-thienylalanine, 6-methyl tryptophan and ethionine inhibited psittacosis virus multiplication in minced chick embryo tissue, correlated with common needs in other systems. The analogue ethionine indicated a need for methionine, proven essential for vaccinia (44), influenza (1), and Lansing poliomyelitis viruses (4) (30). The requirement for phenylalanine correlated with a need by Theiler's GD VII mouse encephalitis (34), vaccinia (45), and Lansing poliomyelitis viruses (4). Morgan's determination of the tryptophan requirement for psittacosis multiplication was not shared with other viruses in tissue culture.

It should be noted that the correlation among viruses for certain amino acid requirements was not absolute. A common need, as reflected by an antimetabolite, may indicate the metabolite was more crucial for cell function and survival than viral synthesis. The antimetabolite commonly used may have caused temporary or unnoticeable damage to the cell.

Both these parameters made it difficult to assess how valuable an amino acid was in viral reproduction or general functioning of the cell.

The determination of similar amino acid requirements among viruses became more complicated by the use of different cell types. There was evidence that an amino acid pool existed within cells and supplied amino acids for virus propagation (6). Therefore, as the intracellular pool of a cell line varied, so did its capacity for virus production. Another variable was introduced when different media were used to grow the cells because the intracellular concentration of amino acids was dependent on their concentration in the extracellular medium.

Almost three hundred amino acids and amino acid analogues were tested for inhibition of influenza A & B viruses grown in embryonic chick lung tissue (26). Toxicity of the compounds was determined by microscopic examination of tissue incubated with the analogue and virus. If the cells were altered or their growth depressed, the compound was judged toxic.

The study showed inhibition of influenza virus by norleucine and other methionine analogues. The inhibition was reversed competitively by methionine and resembled inhibition by methoxine and ethionine shown by Ackermann (1). B-phenylserine, canavanine flavianate and S-ethylcysteine were also inhibitory and were competitively reversed by phenylalanine, l-arginine and cysteine or cystine respectively.

Reversal of viral inhibition by addition of the natural metabolite was accompanied by a paralled reversal in toxicity

of the inhibitor to the tissue. The relatively large concentrations required to inhibit virus formation suggested the analogues were not directly inhibiting sites of virus formation but may have been incorporated into virus protein. The compounds found inhibitory to the viruses were tested in mice infected with vaccinia but none were effective.

Viral latency (a hidden infection) can be produced by growing cells in deficient media (23). When minced chick embryo was placed in a salt plus glucose medium there was cell growth. When psittacosis virus was added to the culture, no viral multiplication occurred. Both cells and virus were viable, but the virus remained latent or hidden. When amino acids and vitamins were added to this latent system, viral multiplication was induced. This suggests that the cell can obtain amino acids necessary for survival from either protein degradation or biosynthetic pathways. However, higher intracellular concentrations of amino acids were required before viral replication occurred. In this system, the presence of phenylalanine and tryptophan were found to be the most essential in reactivating the latent state into viral replication. These same amino acids are also required for mammalian cell growth.

Eagle and Habel (15) and Darnell and Eagle (5) found little correlation between amino acid requirements for cell growth and those for virus production. When HeLa cells, starved for 12 hours of all components but glucose, glutamine and salts, were placed in a medium containing only these three components and infected with poliovirus type I, virus

was synthesized. Cell growth had been arrested and cell injury had occurred due to the absence of other amino acids, vitamins, and serum. Without glutamine, viral production was delayed, suggestive of latency. If only glutamine alone were added, there was a two thousand fold increase in virus production. With only glucose alone there was a 170 fold increase and a delay in virus production. Decreased virus production was not due to lack of adsorption to host cells. Thus a cell line which needed 13 amino acids for growth, required none except glutamine for viral synthesis. This probably reflected synthesis of viral protein from amino acids pre-existing in the cell or from breakdown of the cells own protein. The cell can not synthesize any of the other 12 omitted from the medium.

Because cell injury was evident it might be assumed that absence of glucose and glutamine in connection with reduced virus output reflected cell death. But since addition of glucose and glutamine restored virus production, impaired cell function rather than death was suggested. The obvious corollary to this observation was that glucose and glutamine might be preserving cell integrity and not be actively involved in viral synthesis. This was not true because there was obvious cell degeneration. Another possibility was that glucose and glutamine served either as sources of energy, precursors for synthesis of viral nucleic acid or both.

Glucose was also demonstrated essential for herpesvirus growth in the HeLa cell (27). Unlike the results with

poliovirus (5), glutamine was not. When glutamine was omitted from the extracellular medium there was an enhancement of virus production. The reason for this is unknown. It was not attributed to removal of glutamine as an inhibitor because higher concentrations were not inhibitory. Serum or an inhibitor in serum may play a role in this phenomenon because stimulation occurs only in the absence of serum.

Poliovirus types I, II and III; had different requirement than was found with type I in HeLa cells (15). When monkey kidney cells were infected and a medium deficient in cystine added, cytopathic effect (cell degeneration) was delayed. Adsorption was eliminated as the cause. A requirement for cystine which was not shown by Eagle and Habel (15) was indicated. This system also suggested a latent state since the virus had a longer eclipse phase than was usual.

Rappaport (41) had previously shown that cystine replaced all other amino acids for the multiplication of poliovirus in monkey heart cells. Dubes (7) therefore suggested that an antimetabolite to cystine and/or cystine be used as a therapeutic agent against poliovirus.

The requirement for L-cystine in virus-host cell systems was extended by Tyndall and Ludwig (47). Coxsackie B_3 and vaccinia virus were found to require cystine when grown in rabbit kidney, chick embryo, vascular endothelium, monkey heart, and to some extent in HeLa cell culture. Poliovirus ·pe II also required cystine when grown in monkey heart but ·bbit kidney only balanced salt solution, glucose, and

glutamine were needed. The inability of glutathione, ascorbic acid or thioglycollate to effectively replace the requirement for cystine indicated its function was not as a hydrogen acceptor or environmental stabilizer, since these compounds function as such. It may mean that it was an actual structural unit in the synthesis of virus coat or in enzymes involved in production of new virus.

The cystine requirement was highly specific (43). Cystine analogues with various substituted chemical groups would not effectively replace cystine. The analogues demonstrated the specific need for the presence and spatial orientation of the amino group, sulfur atom, carbonyl group, and the three carbon chain. If cells were first starved for twenty-four hours and then infected, the virus remained latent or hidden. Addition of cystine activated viral multiplication. No virus could be found during the latent stage, perhaps it was present inside the cell as viral ribonucleic acid and not intact particles.

Pelmont and Morgan (36) were the first to complete a comprehensive study of the thirteen essential amino acids as requirements for herpes virus. They employed the L strain of mouse fibroblasts for virus growth, and titrated the virus by counting lesions produced after inoculation of chick chorioallantoic membrane.

The cells and virus were grown in a synthetic medium composed of the DL forms of the amino acids, not the natural L-isomers. Omission of phenylalanine, tryptophan, arginine,

threonine, leucine, valine and histidine markedly decreased virus growth. Isoleucine, lysine, alanine and glycine were removed without any effect on viral replication. The remaining amino acids showed a slight decrease in viral production when omitted.

It was also determined that herpes proliferated in a synthetic medium which did not allow growth of the L cells. If cells were infected while in Earle's balanced salt solution, no multiplication occurred. The virus was activated from this latent state when the salt solution was replaced with a complete medium containing horse serum. Latency was induced by the addition of ethionine, the analogue of methionine, at 60 mg/ml concentration.

Tankersley (44) also studied the thirteen essential amino acids as requirements for herpes virus multiplication, using natural L-isomers of the amino acids. Another improvement over Pelmont and Morgan's work (36) was the replacement of the serum requirement by methylcellulose, allowing complete chemical definition of the test medium. A more typical host cell than mouse fibroblasts, human esophageal epithelium, was employed.

Minimally infected cells were first placed in a complete medium with serum for 24-48 hours to assure infection. Infected sheets of cells were then rinsed with salt solution, and basal medium Eagle deficient in one amino acid was added. After seventy-two hours further incubation

the supernatant fluid was discarded and the monolayer assayed for virus by the standard plaque method.

Eleven of the thirteen essential amino acids and glutamine were required. Lysine was not needed and was even partially inhibitory. Omission of histidine caused the greatest decrease in viral replication. In the absence of arginine and presence of lysine, no virus was formed and no cytopathic effect was detected.

Roizman, Spring and Roane (42) grew a macroplaque strain of herpesvirus in human epidermoid carcinoma cells and confirmed that omission of the essential amino acid arginine markedly reduced replication of herpesvirus. Arginine was not needed during the first four hours of infection but was required six hours after infection, and thereafter, for virus multiplication. This was shown by addition of arginine from 0-4 hours after infection, withdrawal the remaining 4-16 hours, and a subsequent titer of 2.7×10^2 polykaryocyte forming units per ml. If arginine was omitted 0-6 and added 6-15 hours after infection, the titer was 2.4×10^4 polykaryocyte forming units per ml. The virus was assayed directly in the first series of experiments by count of polykaryocyte forming units.

The second series of experiments was performed with the knowledge that arginine was required 6-15 hours after infection. Cells grown on coverslips were arginine starved for 0-6 hours and then infected. Cells on one half the coverslips received arginine, the other half did not.

Eighteen hours after infection cells were stained with fluorescent human γ-globulin (demonstrating cytoplasmic virus) and rabbit hyperimmune serum against boiled infected cells (demonstrating nuclear virus). Addition of arginine caused a 30-fold increase in the number of cells containing cytoplasmic and nuclear fluorescent granules respectively.

II. "Amino Acids in Relation to the Growth of Mammalian Cells and their Function as Hosts for Viral Synthesis."

Early developmental media for the growth of tissue cells in vitro were usually extracts from tissues or other sources rich in growth factors generally chemically undefined. To study the effect of certain nutrilites on cell metabolism, especially virus infection and multiplication, each growth factor present in the medium should be known. Such chemically defined media have been developed with the demands of the cell for each nutrilite estimated (12). It was thus possible to precisely vary the medium composition and observe the subsequent effect on cell function. Unfortunately it was still necessary to add an undefined component, serum, in order for the cells to proliferate rapidly (10). Methylcellulose may replace serum in protecting cells and keeping them viable, but it did not permit rapid growth (2).

A typical chemically defined medium contains a balanced salt solution, a sugar, vitamins and amino acids.

The amino acids used have been most extensively studied since they are the building blocks of cell protein. Established cell lines utilize and liberate amino acids in a characteristic way. It was possible to differentiate cell lines on this basis (29).

The amino acid requirements for a particular cell type have been divided into essential and non-essential. The essential amino acids were those which must be provided in the medium for the cell to grow while non-essential amino acids can be synthesized by the cell.

Eagle (11) (12) (13) found that thirteen L-amino acids of the natural twenty-one are essential for growth of human carcinoma and mouse fibroblast cells. Omission of any of these thirteen from the medium, caused microscopic changes in the cells within two to three days. The changes reflected cellular injury and differed according to particular amino acids, probably indicative of their cellular function.

The requirement of thirteen essential amino acids by most cell lines was correlated with the cell's intracellular amino acid pool (37). Most essential amino acids, were actively transported into the cell and concentrated five to eleven times greater than in the extracellular medium. Lysine and arginine were concentrated only two to three times, cystine was not concentrated. The non-essential amino acids had a very high intracellular concentration ratio since they were synthesized internally by the cell. The free amino acids of the intracellular pool were generally

the same in most cell lines, although there were significant differences (37).

The role of the intracellular amino acid pool in viral replication has been demonstrated by labeling experiments. Darnell and Levintov (6) have shown that the protein coat of poliovirus was made from twelve amino acids in the intracellular pool. Thus in order to study the amino acid requirements for production of virus protein coat, the medium the cells were grown in could be depleted of an essential amino acid (25). This depletion was reflected in the intracellular pool, and the protein coat might either be deficient in the amino acid or not synthesized at all. The cell could compensate for this deficiency by breakdown of its own protein or use of dipeptides.

An amino acid deficiency can probably effect viral replication in many other ways. Many are unknown. Some may be so intimately associated with cell function that they are inseparable. Viral directed synthesis of early enzymes needed for the biosynthetic machinery involved in replication can be adversely effected. The studies of amino acid deficiencies rely on a decrease in the amount of virus produced. The specific effect at the molecular level is unknown.

MATERIALS AND METHODS

I. Materials

A. Virus

Herpes simplex virus (HSV) McIntyre strain, American type culture six, was obtained from Michigan State Health Laboratory, East Lansing, Michigan.

B. Tissue Cultures

1. AU, a normal human skin type, derived by Wheeler et. al. (50).
2. RK-13 normal rabbit kidney, Beale et. al. (3) was procured from Henry Ford Hospital, Detroit, Michigan, courtesy of G. La Grippo, M.D.
3. HEP-2, human epidermoid carcinoma of the larynx, Moore et. al. (31) was obtained from Flow Laboratories, Rockville, Maryland.

C. Stock Virus

The procedure for the propagation of HSV was a modification of Kaplan's (24). Rabbit kidney cells were grown to confluency in a 160 ml milk dilution bottle with a final medium volume of 10 ml. Minimal essential medium with .3% methocel (methylcellulose, 15 cps. premium, Dow Chemical Co., Midland, Michigan) supplementing serum was employed. One tenth ml of untitrated virus stock was inoculated onto a RK-13 cell monolayer and

allowed to adsorb to the cells for one hour. The inoculating fluid was then decanted.

After a total 48 hours incubation at 37°C, the culture was frozen* and thawed three times, and centrifuged at 550 maximum relative centrifugal force (International centrifuge model V-size 2) for three minutes. Five ml aliquots of the supernatant fluid, with a virus titer of 10^5 pfu/ml, were placed into screw cap tubes, sealed with plastic tape, and frozen.* The stock was re-titered just before use to obtain the desired inoculum.

D. Balanced Salt Solution

1. Earle's balanced salt solution (EBSS-10X) (16).

	Component	Amount	Preparation
Unit #1	NaCl	68.00 gm	Dissolve in 900 ml water+
	KCl	4.00 gm	
	$NaH_2PO_4 \cdot H_2O$	1.25 gm	
	$MgSO_4 \cdot 7H_2O$	2.00 gm	
	Glucose	10.00 gm	
	Phenol Red	0.20 gm	
Unit #2	$CaCl_2$	2.00 gm	Dissolve in 100 ml water

Two ml of chloroform was added to unit #1 and both samples refrigerated. For a 1X solution, 9 ml of unit #1 and 1 ml of unit #2 were added to 90 ml

---

* All materials were frozen at -20°C.

+ All water used was glass distilled twice.

of water and sterilized by autoclaving at 15 lbs. 120°C for 15 minutes.

2. Hanks' balanced salt solution (HBSS-10X) (21).

	Component	Amount	Preparation
Unit #1	NaCl	80.0 gm	Dissolve in 900 ml water
	KCl	4.0 gm	
	$MgSO_4 \cdot 7H_2O$	2.0 gm	
	$Na_2HPO_4 \cdot 2H_2O$	0.6 gm	
	Glucose	10.0 gm	
	KH_2PO_4	0.6 gm	
	Phenol red	0.2 gm	
Unit #2	$CaCl_2$	1.4 gm	Dissolve in 50 ml

When unit #1 was completely dissolved, unit #2 was added to it, and the final volume brought to 1 liter. Two ml of CCl_4 was added and the stock refrigerated.

E. Vitamin Stock

Basal Eellun Eagle (12).

Component	Gm/L
Biotin	0.001
Folic acid	0.001
Choline HCL	0.001
Nicotinamide	0.001
Ca-D-Pantothenate	0.001

(continued on next page)

Component	Gm/L
Pyridoxal HCL	0.001
Thiamine HCL	0.001
Riboflavin	0.0001
Inositol	0.0013

Purchased frozen as 100X from Microbiological Associates, Bethesda, M.D. The solution was thawed, dispensed into 5 ml quantities, and refrozen at -20°C until used.

F. Amino Acid Stock

Solutions for minimal essential medium (MEM) and basal medium Earle (BME).

	Component	gms/ liter-MEM	gms/ liter-BME
Unit #1	L-arginine HCL	0.126	0.021
	L-cystine	0.026	0.012
	L-histidine HCL	0.042	0.0093
	L-isoleucine	0.0525	0.026
	L-leucine	0.0524	0.026
	L-lysine HCL	0.073	0.0365
	L-methionine	0.015	0.0075
	L-phenylalanine	0.033	0.0165
	L-threonine	0.048	0.024
	L-tryptophan	0.010	0.004
	L-tyrosine	0.036	0.018
	L-valine	0.047	0.0235
Unit #2	L-glutamine	0.292	0.292

All of the amino acid used were purchased as the natural L-form from Nutritional Biochemical Company, Cleveland, Ohio. Unit #1 was prepared as 50X and sterilized by autoclaving at 120°C for 15 min. Cystine, tyrosine, and methionine were first dissolved separately in .25 N HCl, then added to a water solution of the remaining amino acids. The stock solution was held at -20°C until used.

Unit #2 was prepared as 100X in water, sterilized by Millipore membrane filtration, and frozen (-20°C) until used.

C. Antibiotic Stock

A final concentration of 100 units penicillin and 100 ug streptomycin per ml of tissue culture medium was employed.

D. Tissue Culture Media

1. Minimal essential medium (MEM-1X) (11).

Component	Amount
Hanks' BSS-1X or Earle's BSS-1X as desired	93.5 ml
Vitamin stock-100X	1 ml
Amino acid stock-MEM-50X	2 ml
L-glutamine-100X	1 ml
Stock antibiotics	1 ml
7% $NaHCO_3$	1.5 ml
	100 ml

The balanced salt solution was sterilized by autoclaving at 120°C. 15 min. and the remaining sterile ingredients added aseptically. The amount of $NaHCO_3$ varied ±.5 ml. depending on the desired pH.

2. Basal Medium Eagle (BME-1X) (12).

The composition and preparation was identical to that for MEM except BME amino acid stock was supplemented for the MEM amino acid stock.

3. Experimental MEM and BME media deficient in one amino acid.

These were made identical to the preparations given above except for substitution of a stock amino acid solution 50X, made up from individual amino acids (as described in part F) with one amino acid omitted.

Methocel (Dow Company, Midland, Michigan) at .3% concentration, 15 centipoise viscosity was added to media as a supplement for the usual serum requirement (20). This allowed complete chemical definition of a medium. The material was added to boiling water, made into a slurry, and autoclaved at 120°C for 15 minutes. Final hydration was accomplished by first cooling at room temperature for two hours, then refrigerating (-4°C) overnite. The components of the medium were then added aseptically.

I. Plaque Assay Overlay (42).

Methocel, 2% concentration, 4,000 centipoise viscosity, was included in MEM medium with Hanks' BSS. The procedure for hydrating the product was identical to that given for experimental media part C section 3. Agar was not employed since it inhibits herpes virus (49).

J. Trypsin.

A .25% solution in Hanks' BSS was achieved by warming at 37° for ½ hour. Sterilization was effected by Seitz filtration.

K. Citric Acid-Crystal Violet Solution.

Crystal violet 0.1 gm and citric acid 2.1 gm were added to 100 ml water and autoclaved at 120°C for 15 minutes.

II. Methods

A. Production of virus in cells grown in medium deficient in one amino acid.

The particular cell type used in each experiment was first grown to a monolayer in a 160 ml milk dilution bottle, and the monolayer removed by either trypsinization or use of a rubber tipped rod. The cells were counted in a hemacytometer and properly diluted to 10^5 cells/ml with medium containing 20% heat inactivated, sterile calf serum (Flow Laboratories, Rockville, M.D.).

All tests were done in screw cap standard tissue culture tubes which were seeded with 1 ml of the

cell suspension. The cells were grown at 37°C and the medium replaced with fresh medium plus 10% serum every 24 hours. When a monolayer had formed, usually 2-3 days, the medium was decanted and 0.1 ml of the desired titer of virus added. After adsorption for one hour at 37°C the inoculating fluid was decanted. Media deficient in one amino acid was added at this time unless otherwise indicated. Each test was done in triplicate.

After the desired incubation time, 1.0 ml of Hanks' balanced salt solution, containing 5% serum was added to each tube as a stabilizer for virus activity, and to neutralize the acid pH. The tubes were sealed with plastic tape and immediately frozen; with the medium covering the monolayer until the time of assay.

B. Assay of virus produced by cells in amino acid deficient medium.

The procedure was similiar to the method used by Kaplan (24) with modifications given here. The cells were ruptured to release the virus by freezing and thawing three times. While thawing, the ice sheet was shaken inside the tubes to free the cells from the surface of the tube.

The triplicate tubes for each deficiency test were then pooled, and the cell debris centrifuged at 550 max. R.C.F. (International centrifuge model V

size - 2) for three minutes. The virus containing supernatant fluid was withdrawn and diluted through four tenfold dilutions by adding 0.5 ml to 4.5 ml of HBSS.

One tenth ml of each dilution was inoculated onto a rabbit kidney monolayer grown in a 1.0 oz. French square bottle. The virus was adsorbed for one hour at 37°C with rotation of the bottle every 15 minutes to assure even distribution. The inoculum was decanted and three ml of a 2% methocel overlay medium was added.

Following incubation for 72 hours, 6 ml of water (22°C) was added and gently mixed with the methocel so it would become less viscous and easily decanted. The cell monolayer was stained with citric acid-crystal violet solution for two minutes, rinsed with water and the plaques counted.

I. Influence of Incubation Time on Amount of Virus Produced in Deficient Media.

Preliminary studies have indicated that the human skin epithelial cells (AU) underwent degenerative changes within seventy-two hours when grown in a medium deficient in one essential amino acid. This degeneration had been observed microscopically, but the subsequent effect on viral replication within these cells was unknown.

Experiment I was performed to determine the appropriate time the virus infected cells should remain in deficient medium in order that amino acid requirements for viral replication would be best demonstrated.

All cell monolayers were simultaneously infected with 500 plaque forming units, and deficient medium containing .3% methocel added one hour after infection. After incubation for 24, 48, and 72 hours, the cultures were frozen until time of assay.

The results in table 1 are in plaque forming units (pfu) per milliliter. One plaque forming unit was shown by Dulbecco to represent an area of cell lysis caused by one virus particle (9).

The data revealed replication in the amino acid deficient media until seventy-two hours. At this time a marked drop in the number of plaques was observed. In complete medium, containing serum or methocel, viral

Table 1. Effect of incubation time on amount of virus produced in experimental media

Medium	24 hrs.	48 hrs.	72 hrs.
Arginine[a.]	314[b.]	2360	72
Cystine	640	1700	70
Glutamine	50	160	0
Histidine	330	1980	126
Isoleucine	676	2380	168
Leucine	453	1640	94
Lysine	226	120	4
Methionine	92	40	2
Phenylalanine	400	2000	72
Threonine	448	2760	28
Tryptophan	512	2560	130
Tyrosine	666	2620	66
Valine	454	1200	34
[illegible]—methocel	345	2630	t.n.t.c.
[illegible]—serum	453	3830	t.n.t.c.

a. Missing amino acid.

b. Pfu per ml at 10^{-1} dilution.

c. t.n.t.c. = too numerous to count.

multiplication continued; and at the dilution assayed the plaques were too numerous to count (t.n.t.c.).

The extreme reduction in amount of virus at seventy two hours was best explained by degeneration of the cells and development of lesions, as previously shown by Eagle (12). Not only would viral multiplication cease, but intracellular virus would be released or left unprotected by rupture of the cells.

Since herpesvirus was shown to be an extremely thermolabile virus at 37°C (33) (20), both the extracellular virus normally present and that released from injured cells could be inactivated. Thus the amount of virus would not be less, but the number of infectious particles surviving 37° would give an apparent reduction in number of virus particles as only infectious particles were assayed.

Another explanation was that the amino acid pool was depleted, especially in the particular amino acid omitted, and that viral multiplication could not occur without these present. This was likely to account for part of the reduction in titer; but if multiplication stopped without degeneration, much virus must have remained within the cell. However, this virus should have remained infectious and detected during assay, since the cells were ruptured by freezing and thawing just prior to assay.

It was apparent from microscopic evidence and marked reduction in infectious virus that gross cell damage occurred from 40-72 hours after infection. Forty-eight hours was then chosen as the appropriate time to assay all future experiments as multiplication was at a peak, amino acid pool depletion evidently occurred, and specific amino acid requirements were best demonstrated.

The 24 hour experiment was not done in triplicate at this time, but later experiments showed all deficient media with less virus than complete. Not all the amino acid requirements were evident at 24 hours, many being identical at this time, so 40 hours was chosen as the ideal time for incubation and assay.

II. Virus Production in Infected AV Cells Incubated for 40 Hours in Animal Essential Medium Deficient in One Amino Acid.

Since it had been concluded from experiment one that 40 hours was an appropriate time for assay, the next three experiments were performed with assays at this time. Each experiment was conducted in triplicate for each amino acid. Otherwise the procedures were identical to experiment one.

The average number of plaques for the three experiments are given in table 2. Virus production in deficient media was also calculated in terms of percent produced in complete medium. Complete medium was 100%.

Table 2. Amount of virus produced by AV cells in minimal essential medium deficient in one amino acid.

Medium	pfu/ml*	Percent of amt. on complete	Ave. deviation
Arginine	7.1×10^4	62	±7
Cystine	5.6×10^4	49	±4
Glutamine	1.8×10^4	16	±6
Histidine	8.5×10^4	73	±5
Isoleucine	1.0×10^4	8.8	±3
Leucine	1.5×10^4	13	±2
Lysine	2.8×10^3	2.5	± .5
Methionine	5.7×10^2	0.5	± .2
Phenylalanine	5.5×10^4	48	± .10
Threonine	6.1×10^4	54	±2
Tryptophan	3.0×10^4	26	±5
Tyrosine	3.5×10^4	31	±2.6
Valine	1.1×10^4	10	±3
Complete [illegible]	1.14×10^5	100	

* Average of three experiments done in triplicate.

$$\text{Average deviation} = \frac{X-P_1 + X-P_2 + X-P_3}{3}$$

$$\text{where } X = \frac{P_1 + P_2 + P_3}{3} \text{ and } P = \text{number of pfu.}^{**}$$

** Brumbley, May U., Quantitative Analysis, Barnes Noble, Inc., New York, 1950, p. 24.

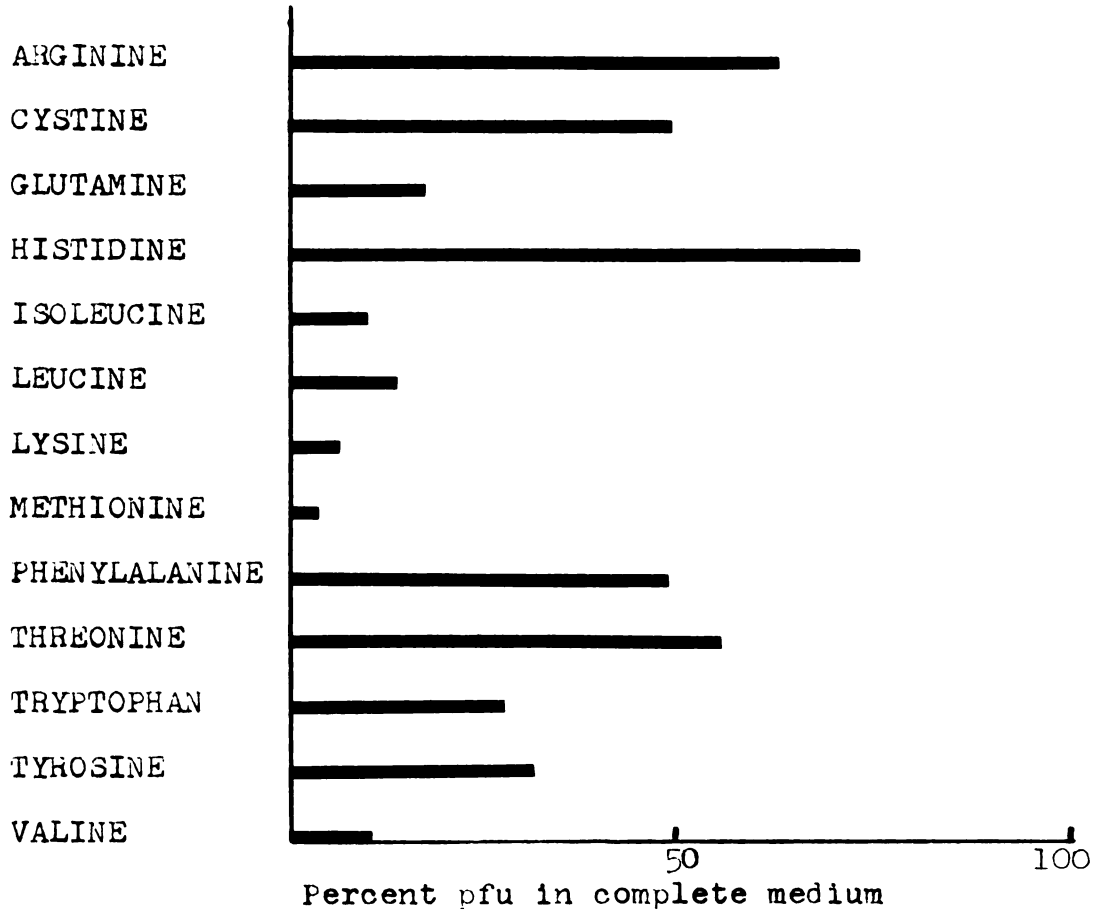

Fig. 1. Comparative virus production by AU cells in serum free medium defieient in one amino acid.

as here the greatest amount of virus was produced. The percentage on a deficient medium indicated what proportion of the virus produced on complete medium was produced when that particular amino acid was omitted. The smaller the percent, the more required the amino acid was for viral replication.

The results are seen graphically in figure one, also on a percentile basis. It was found that upon omission of methionine from the medium least viral multiplication occurred, only 0.5% of the amount produced in complete medium.

Lysine was the next most required, the remaining amino acids required in varying degrees. Without histidine, 73% of the normal virus output was achieved. It should be noted, in relation to Tankersley's work (44) that arginine was the second least required nutrilite in this particular system.

III. Virus Production by AJ Cells in Basal Medium Eagle Deficient in One Amino Acid.

The results in the previous experiments differed greatly from the requirements shown by Tankersley (44). For this reason experiments were repeated using a procedure similiar to his.

Basal medium Eagle (BME) was substituted for minimal essential medium. The cells were grown in complete medium, then starved of the particular amino acid in question for 24 hours. After 24 hours in the deficient

medium, each monolayer was infected with 100 plaque forming units. Incubation was terminated at 43 hours although Tankersley continued incubation for 72 hours. The results from experiment one demonstrated the disadvantage incurred by incubation of this cell line in deficient medium for 72 hours.

The use of a different medium and procedure did alter some requirements for the AU cell line, as recorded in table 3. Arginine now appeared as much of a requirement as methionine. The data indicated valine was the most essential, but since the results were very close to methionine and lysine requirements, it cannot be definitely said which was more crucial for viral synthesis. A requirement for histidine was more marked in this system whereas with MEM medium it was the least required amino acid.

IV. Amino Acid Requirement for Virus Production by the AU Cell in Comparison to the HEp-2 Cell.

The amino acid requirements for the AU cell in these experiments were different from those of the human esophageal epithelium experiments done by Tankersley (44). It was contended that the discrepancies may have been due to differences in cell types rather than experimental procedure. To test this hypothesis another cell line, human epidermoid carcinoma, was employed. This cell line had been used by Holzman (42) when he confirmed Tankersley's finding (44) for an arginine requirement.

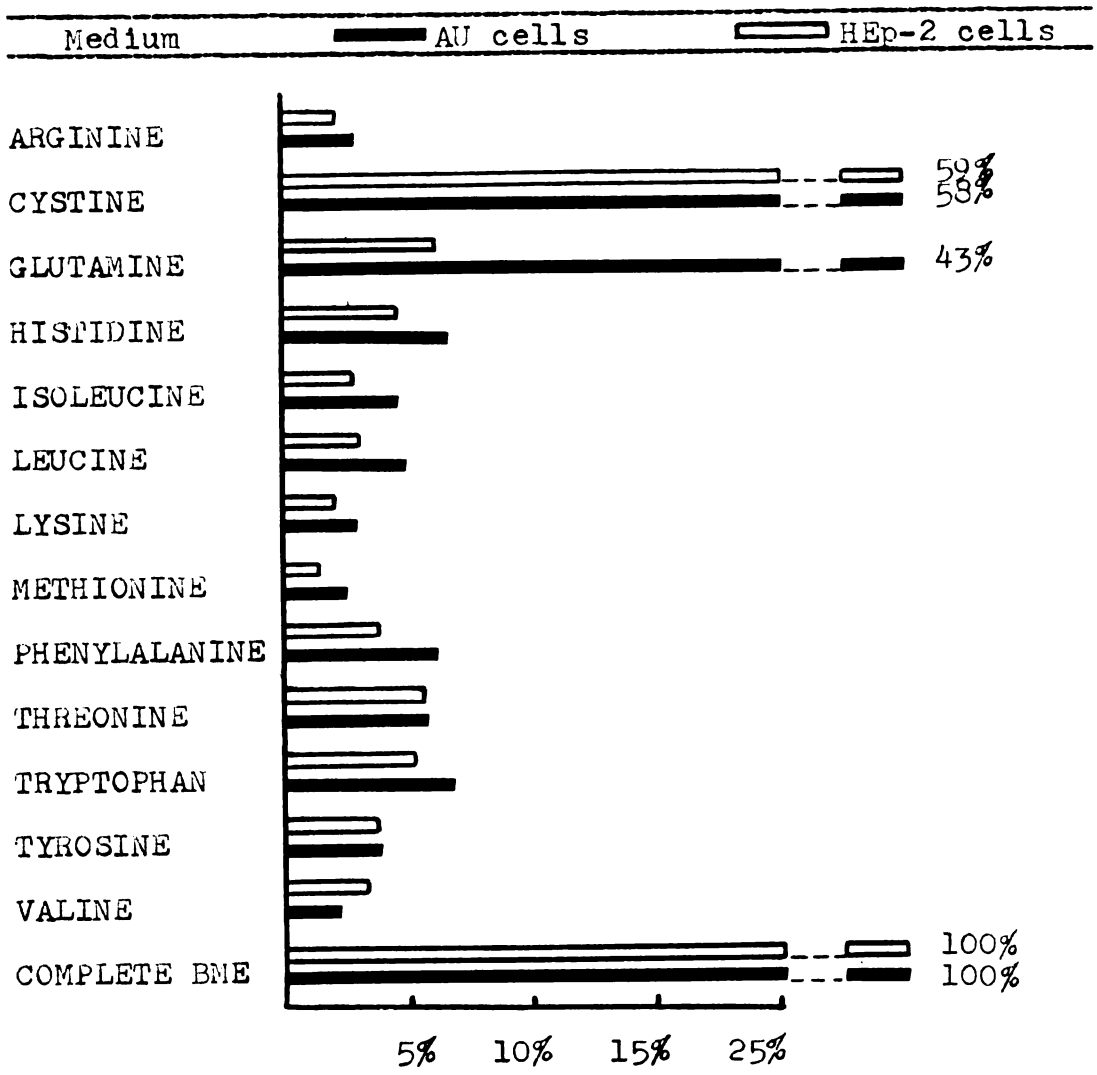

Fig. 2. Comparative virus production by AU and HEp-2 cells in basal medium Eagle deficient in one amino acid.

Table 3. Amount of virus produced by AU cells in comparison to that produced by HEp-2 cells in basal medium Eagle deficient in one amino acid.

Medium	AU cells	HEp-2 cells
Arginine	250[a]	136[b]
Cystine	5200	4000
Glutamine	3900	413
Histidine	600	313
Isoleucine	390	191
Leucine	423	210
Lysine	249	141
Methionine	220	83
Phenylalanine	550	243
Threonine	500	330
Tryptophan	600	366
Tyrosine	301	264
Valine	132	226
Complete BME	9000	6300

Results of one experiment done in triplicate

[a] ffu/ml ± 20 avg. std. dev.

[b] ffu/ml x 10^{-1} ± 25 avg. std. dev.

The Hep-2 cell line was tested simultaneously with the AU cell line. The procedure was identical for both cell lines and has been given under part III of the results.

The results of the experiment are given in table 3 and figure 2. The amino acid requirements were similiar for both cell lines except for one amino acid. Glutamine was required to a much greater extent by the Hep-2 cells. If it was omitted during viral proliferation in the AU cell, 43% of the amount replicated in complete medium was produced. However, if glutamine was omitted during replication in the Hep-2 cell, only 6% of the normal amount was produced.

DISCUSSION

Examination of the results indicated that methionine was the most required amino acid for the multiplication of herpes simplex virus in AU cells when minimal essential medium was used. Tankersley (44) reported that arginine was most required for herpesvirus growth in human esophageal epithelium. His system differed in three ways--virus strain, host cell, and medium. The strain of herpes virus was isolated in his laboratory. The McIntyre strain of American type culture six was used in this study. Different amino acid requirements can be due to different virus types and mutants as was shown with poliovirus (8) (51).

Tankersley used basal medium Eagle, a synthetic medium developed for optimal growth of mouse fibroblasts. The study reported here originally employed minimal essential medium, optimal for growth of human carcinoma (HeLa) cells. The AU cell grew optimally in MEM, which contained twice the amount of each amino acid in BME, except one. Minimal essential medium contained six times the amount of arginine in BME.

When minimal essential medium was used, the requirement for methionine was very marked in comparison to other requirements (fig. 1). When BME was employed in following experiments, five additional requirements, including arginine, were as marked as the methionine requirement. These may be requirements for general functioning of the cell rather than for viral replication.

A deficiency of individual amino acids was reflected in viral multiplication at 48 hours (table 1). Additional incubation caused cell degeneration and did not accurately demonstrate specific viral requirements. This same effect may have been achieved when BME was used, especially with prolonged incubation. Deletion of an amino acid from a medium which already had a small amino acid concentration was soon reflected in viral replication, as the amino acid pool is depleted within 12-24 hours (15) (37). Thus the requirements demonstrated by BME could have been due to a gross amino acid depletion and subsequent cell degeneration. A timed study would be necessary to see if a single deficiency had the same affect on viral multiplication both early and late in infection.

Tankersley's demonstration that lysine inhibited multiplication of herpesvirus can not be explained by use of a different medium. Both BME and MEM tests with the AU cell indicated it was second to methionine as a requirement for viral replication. Lysine has definitely been shown to be an essential amino acid for survival and growth of mammalian cells (11) (12). It inhibited certain viruses, but only when present in excess (13) (34). It is a constituent in the protein coat of several viruses (6).

Roizman et al. (42), using a different cell line, confirmed Tankersley's finding (44) for an arginine requirement in herpesvirus multiplication. Lack of arginine during multiplication resulted in decreased virus production. The

relative requirements for other amino acids were not determined. Unless comparative studies are made, the amino acid which is most required is not known.

The discrepancies between Tankersley's work and the study here could have been due to use of different cell lines. To test this possibility the HEp-2 cell line used by Holzman was compared to the AU cell. The procedures, except for a shorter incubation period, and medium were like that of Tankersley's. This modification was necessary as noted in the results on the effect of incubation time on production of virus in deficient medium (27). Each of the thirteen amino acid deficiencies, except glutamine, had a similiar effect on viral multiplication in both the AU and HEp-2 cell. Glutamine was more necessary for the HEp-2 cell than AU cell. Only one seventh of the amount of virus produced by the AU cell was produced by HEp-2 in glutamine deficient medium.

The varying requirements for replication of the same virus do not appear to depend on differing amino acid metabolism among host cells. In mammalian cells, the thirteen essential amino acids, except glutamine, are metabolized to a minimal degree; they are primarily incorporated into cell protein (13). Omission of one essential amino acid probably limits the incorporation of that amino acid into viral enzymes and viral protein coat.

When one amino amino acid was deleted from the extracellular medium, host cells maintained the concentration of the remaining

amino acids with little change for twenty-four hours (37). Because the varying amino acid requirements did not relate to different amino acid metabolism, the different amino acid requirements might have been due to the concentration of an amino acid in the intracellular pool and its effect on control mechanisms of the host cell. There was a minimal intracellular amino acid concentration, peculiar to each amino acid, that was necessary for a cell to initiate protein synthesis (14). This may also correspond to viral protein synthesis. If the concentration of an amino acid droped below a critical threshold, viral protein and thus complete virus, was not synthesized.

A minimal concentration of an amino acid may also play a role in nucleic acid synthesis. It has been shown that a supply of amino acids was necessary for nucleic acid synthesis in the bacterium E. coli (33). A similar pattern in mammalian cells has not been demonstrated. If one existed, it might indicate amino requirements for replication of viral nucleic acid.

The intracellular amino acid concentration depends on the extracellular amino acid concentration, the specific amino acid and the cell line. In this study the first two factors were constant, only the cell lines differed. Therefore, the different amino acid requirements for the HEp-2 and AU cells must reflect their capacity to concentrate a particular amino acid, in this case glutamine.

Ilez and Eagle (37) have shown that the free amino acid pools of mammalian cells do not differ significantly. One

amino acid, methionine, was not found in the free amino acid pool of liver cells but was found in the HeLa cell. If methionine were not concentrated intracellularly by the AV cells, this could account for the methionine requirement found in this study. Likewise, methionine may not have been as marked a requirement in the cell line used by Tankersley because the cell could concentrate the amino acid intracellularly.

If amino acid pools of the AV and HEp-2 cells were very similar, as shown with HeLa, liver and conjunctiva (37), the different amino acid requirements may have been due to their ability to retain an amino intracellularly when it has been omitted extracellularly. A different amount of leakage or loss has been observed with HeLa and liver cells (37).

The concept of the host cell concentrating amino acids in its intracellular pool may also explain the results in this study with cystine deficient medium. Neither AV or HEp-2 cells required cystine for viral replication to the extent of all other twelve amino acids. Since cystine could not be detected in the intracellular amino acid pool (37), viral synthesis must not have depended on the pool for its source of cystine in the viral protein coat. Cystine could be provided by four general pathways in the host cell, including de novo synthesis (13). Even though bio-synthesis does not suffice for growth of the host cell, it may be enough for viral replication. It is also possible that cystine was not a component of herpes virus and was not at all needed in

synthesis. Cystine was a component of other viruses (6) which did require it for synthesis (7).

The requirement for methionine for both AU and HEp-2 cells may be related to the inability of these cells to effectively concentrate cystine. If cystine was a component of the virus and required for its synthesis, the cell may synthesize cystine from methionine. If such a pathway existed, it would explain the requirement for methionine and lack of a requirement for cystine.

Understanding the many similarities among cell lines may be more important than an elucidation of different amino acid requirements. The similar requirements between the AU and HEp-2 cells for twelve out of the thirteen essential amino acids did not correlate with their diverse differences as cell types. The HEp-2 cell was a cancerous cell with a much faster generation time than the "normal" AU cell.

It has been shown that most cell lines have similar growth requirements (13) and amino acid pools (37). Many cell lines of different origin are indistinguishable morphologically. This may be because they have been isolated and maintained under similar environmental and nutritional conditions.

SUMMARY

1. Of the thirteen amino acids in minimal essential medium, omission of methionine caused the greatest reduction in the amount of herpesvirus produced by the AU cells. Omission of histidine caused the least reduction.
2. When basal medium Eagle was employed, additional amino acid requirements including arginine were as marked as the methionine requirement had been when minimal essential medium was employed.
3. AU cells and HEp-2 cells had similiar requirements for twelve out of thirteen amino acids in basal medium Eagle. In a glutamine deficient medium HEp-2 cells produced one-seventh the amount of virus produced by AU cells when compared to the amount produced in complete medium.
4. Cystine deficiency least affected viral replication in basal medium Eagle by both the AU and HEp-2 cells.
5. Incubation time in a deficient medium and different media affected the apparent amino acid requirements.

BIBLIOGRAPHY

1. Ackermann, W. W. 1951. Role of methionine in virus propagation. J. Exptl. Med. 93:333-343.

2. Bader, J. P. and H. R. Morgan. 1957. Latent viral infection of cells in tissue culture. VI. Role of amino acids, glutamine and glucose in psittacosis virus propagation in L cells. J. Exptl. Med. 103: 617-630.

3. Beale, A. J., G. C. Christofinis, and I. G. S. Furminger. 1963. Rabbit cells susceptible to rubella virus. Lancet. II:640-645.

4. Brown, G. C. 1952. The influence of chemicals on the propagation of poliomyelitis virus in tissue culture. J. Immuno. 69:441-443.

5. Darnell, J. E. and H. Eagle. 1953. Glucose and glutamine in poliovirus production by HeLa cells. Virology 6: 556-566.

6. Darnell, J. E. and L. Levintov. 1960. Poliovirus protein: Source of amino acids and time course of synthesis. J. Bio. Chem. 235 No. 1:74-77.

7. Dubes, George R. 1956. Cystine requirement for normal poliovirus action on monkey kidney tissue culture. Proc. Soc. Exptl. Biol. Med. 93:129-132.

8. Dubes, G. R., and Chapin, M. 1953. Poliovirus mutants with altered responses to cystine. J. Gen. Microbiol. 13:320-329.

9. Dulbecco, R., 1952. Production of plaques in monolayer tissue cultures by single particles of an animal virus. Proc. Nat. Acad. Sci. 38:747-752.

10. Dupree, L. T., K. K. Sanford, B. B. Westfall, and A. E. Kovalesky. 1962. Influence of serum proteins on determination of nutritional requirements of cells in culture. Exp. Cell Res. 28:381-405.

11. Eagle, H. 1955. The specific amino acid requirements of a human carcinoma cell (strain HeLa) in tissue culture. J. Exptl. Med. 122:37-49.

12. Eagle, H. 1955. Nutrition needs of mammalian cells in tissue culture. Science. 122:501-504.

13. Eagle, H. 1959. Amino acid metabolism in mammalian cell cultures. Science. 130:432-437.

14. Eagle, H., K. A. Piez, and M. Levy. 1961. The intracellular amino acid concentrations required for protein synthesis in cultured mammalian cells. J. Biol. Chem. 236:2039-2042.

15. Eagle, H. and K. Habel. 1956. The nutritional requirements for the propagation of poliomyelitis virus by the HeLa cell. J. Exptl. Med. 104:271-287.

16. Earle, W. R. 1943. Production of malignancy in vitro. IV. The mouse fibroblast cultures and changes seen in living cells. J. Natl. Cancer Inst. 4:165-212.

17. Eaton, M. D., B. Kapasankin, M. E. Ferry, and D. Karabian. 1951. Inhibition of influenza and mump virus in tissue culture by basic amino acids. Proc. Soc. Exptl. Biol. Med. 77:505-508.

18. Eaton, M. D., Scala, A. R., and H. Birnie. 1965. Lysine inhibition of cell protein and viral synthesis. Reversal by other amino acids. Proc. Soc. Exptl. Biol. Med. 119:993-1004.

19. Eaton, M. D., A. R. Scala, and I. E. Low. 1964. Amino acid imbalance and incomplete viral replication. Arch. Gesamte Virusforsch 14:583-593.

20. Farnham, A. E., and A. A. Newton. 1959. The effect of some environmental factors on herpes virus grown in HeLa cells. Virology. 7:449-461.

21. Hanks, J. H., and R. E. Wallace. 1949. Relation of oxygen and temperature in the preservation of tissues by refrigeration. Proc. Soc. Exptl. Biol. Med. 71:196-200.

22. Hayashi, K. 1961. Studies on the viral etiology verruca vulgaris in tissue cull culture. Thesis, Michigan State University.

23. Heggie, A. D., and R. R. Morgan. 1956. Latent viral infection of cells in tissue culture. Role of certain amino acids. Proc. Soc. Exptl. Biol. Med. 92:506-509.

24. Kaplan, A. S. 1957. A study of the herpes simplex virus-rabbit kidney cell system by the plaque technique. Virology 4:435-457.

37. Flex, F. A., and H. Eagle. 1953. The free amino acid pool of cultured human cells. J. Biol. Chem. 231: 533-545.

38. Plummer, G., and B. Lewis. 1955. Thermoinactivation of herpes simplex virus and cytomegalovirus. J. Bact. 72:671-674.

39. Rafelson, M. E., Jr., H. E. Pearson, and R. J. Winzler. 1953. The effects of certain amino acids and metabolic antagonists on propagation of Theiler's G.VVII virus and P^{32} uptake by minced one-day-old mouse brain. Arch. Biochem. 39:69-

40. Rafelson, M. E., R. J. Winzler and H. E. Pearson. 1951. A virus effect on the uptake of C^{14} from glucose in vitro by amino acids in mouse brain. J. Biol. Chem. 193:205-217.

41. Rappaport, C. 1956. Monolayer cultures of trypsinized monkey kidney cells in synthetic medium. Application to poliovirus synthesis. Proc. Soc. Exptl. Biol. Med. 91:464-470.

42. Roizman, B., S. E. Spring, and F. E. Roane, Jr. 1967. Cellular compartmentalization of herpes virus during viral replication. J. Virology 1:191-192.

43. Sanford, K. K., W. T. McQuilkin, M. C. Fioramonti, V. J. Evans, and W. R. Earle. 1953. Study of amino acid requirements for increase in cell population of NCTC clone 929 (strain L). J. Nat. Cancer Inst. 20:775-735.

44. Tankersley, R. W., Jr., 1964. Amino acid requirements of herpes simplex virus in human cells. J. Bact. 87:609-613.

45. Thompson, R. L. 1947. The effect of metabolites, metabolite antagonists and enzyme inhibitors on the growth of the vaccinia virus in Maitland type of tissue culture. J. Immunol. 55:345-351.

46. Thompson, R. L., and M. L. Wilkin. 1948. Inhibition of growth of the vaccinia virus by B-2-thienylalanine and its reversal by phenylalanine. Proc. Soc. Exptl. Biol. Med. 63:434-436.

47. Tyndall, R. L. and E. H. Ludwig. 1960. Nutritional requirements for the production of polioviruses type III, coxsackie B5 and vaccinia virus by continuous animal cell cultures. J. Bact. 80: 96-103.

48. Tyndall, R. L. and E. H. Ludwig. 1963. Cystine requirement for production of coxsackie B_3 virus in cultured monkey heart cells. J. Bact. 85: 1339-1345.

49. Tytell, A. A. and R. E. Herman. 1963. A medium free of agar, serum, and peptone, for plaque assay of herpes simplex virus. Proc. Soc. Exptl. Biol. Med. 113:343-346.

50. Wheeler, C. E., C. H. Canby and E. P. Conley. 1957. Long-term tissue culture of epithelial-like cells from human skin. J. Invest. Derm. 22:383-392.

51. Younger, J. S. and J. V. Hallum. 1964. Cystine-independent thermoresistant mutants of poliovirus. J. Bact. 83:265-266.

relative requirements for other amino acids were not determined. Unless comparative studies are made, the amino acid which is most required is not known.

The discrepancies between Tankersley's work and the study here could have been due to use of different cell lines. To test this possibility the HEp-2 cell line used by Holzman was compared to the AU cell. The procedures, except for a shorter incubation period, and medium were like that of Tankersley's. This modification was necessary as noted in the results on the effect of incubation time on production of virus in deficient medium (27). Each of the thirteen amino acid deficiencies, except glutamine, had a similiar effect on viral multiplication in both the AU and HEp-2 cell. Glutamine was more necessary for the HEp-2 cell than AU cell. Only one seventh of the amount of virus produced by the AU cell was produced by HEp-2 in glutamine deficient medium.

The varying requirements for replication of the same virus do not appear to depend on differing amino acid metabolism among host cells. In mammalian cells, the thirteen essential amino acids, except glutamine, are metabolized to a minimal degree; they are primarily incorporated into cell protein (13). Omission of one essential amino acid probably limits the incorporation of that amino acid into viral enzymes and viral protein coat.

When one amino amino acid was deleted from the extracellular medium, host cells maintained the concentration of the remaining

amino acids with little change for twenty-four hours (37). Because the varying amino acid requirements did not relate to different amino acid metabolism, the different amino acid requirements might have been due to the concentration of an amino acid in the intracellular pool and its effect on control mechanisms of the host cell. There was a minimal intracellular amino acid concentration, peculiar to each amino acid, that was necessary for a cell to initiate protein synthesis (14). This may also correspond to viral protein synthesis. If the concentration of an amino acid droped below a critical threshold, viral protein and thus complete virus, was not synthesized.

A minimal concentration of an amino acid may also play a role in nucleic acid synthesis. It has been shown that a supply of amino acids was necessary for nucleic acid synthesis in the bacterium E. coli (33). A similar pattern in mammalian cells has not been demonstrated. If one existed, it might indicate amino requirements for replication of viral nucleic acid.

The intracellular amino acid concentration depends on the extracellular amino acid concentration, the specific amino acid and the cell line. In this study the first two factors were constant, only the cell lines differed. Therefore, the different amino acid requirements for the HEp-2 and AU cells must reflect their capacity to concentrate a particular amino acid, in this case glutamine.

Paez and Eagle (37) have shown that the free amino acid pools of mammalian cells do not differ significantly. One

amino acid, methionine, was not found in the free amino acid pool of liver cells but was found in the HeLa cell. If methionine were not concentrated intracellularly by the AV cells, this could account for the methionine requirement found in this study. Likewise, methionine may not have been as marked a requirement in the cell line used by Tankersley because the cell could concentrate the amino acid intracellularly.

If amino acid pools of the AV and HEp-2 cells were very similar, as shown with HeLa, liver and conjunctiva (37), the different amino acid requirements may have been due to their ability to retain an amino intracellularly when it has been omitted extracellularly. A different amount of leakage or loss has been observed with HeLa and liver cells (37).

The concept of the host cell concentrating amino acids in its intracellular pool may also explain the results in this study with cystine deficient medium. Neither AV or HEp-2 cells required cystine for viral replication to the extent of all other twelve amino acids. Since cystine could not be detected in the intracellular amino acid pool (37), viral synthesis must not have depended on the pool for its source of cystine in the viral protein coat. Cystine could be provided by four general pathways in the host cell, including de novo synthesis (13). Even though bio-synthesis does not suffice for growth of the host cell, it may be enough for viral replication. It is also possible that cystine was not a component of herpes virus and was not at all needed in

synthesis. Cystine was a component of other viruses (6) which did require it for synthesis (7).

The requirement for methionine for both AJ and HEp-2 cells may be related to the inability of these cells to effectively concentrate cystine. If cystine was a component of the virus and required for its synthesis, the cell may synthesize cystine from methionine. If such a pathway existed, it would explain the requirement for methionine and lack of a requirement for cystine.

Understanding the many similarities among cell lines may be more important than an elucidation of different amino acid requirements. The similar requirements between the AJ and HEp-2 cells for twelve out of the thirteen essential amino acids did not correlate with their diverse differences as cell types. The HEp-2 cell was a cancerous cell with a much faster generation time than the "normal" AJ cell.

It has been shown that most cell lines have similar growth requirements (13) and amino acid pools (37). Many cell lines of different origin are indistinguishable morphologically. This may be because they have been isolated and maintained under similar environmental and nutritional conditions.

SUMMARY

1. Of the thirteen amino acids in minimal essential medium, omission of methionine caused the greatest reduction in the amount of herpesvirus produced by the AU cells. Omission of histidine caused the least reduction.
2. When basal medium Eagle was employed, additional amino acid requirements including arginine were as marked as the methionine requirement had been when minimal essential medium was employed.
3. AU cells and HEp-2 cells had similiar requirements for twelve out of thirteen amino acids in basal medium Eagle. In a glutamine deficient medium HEp-2 cells produced one-seventh the amount of virus produced by AU cells when compared to the amount produced in complete medium.
4. Cystine deficiency least affected viral replication in basal medium Eagle by both the AU and HEp-2 cells.
5. Incubation time in a deficient medium and different media affected the apparent amino acid requirements.

BIBLIOGRAPHY

1. Ackermann, W. W. 1951. Role of methionine in virus propagation. J. Exptl. Med. 93:333-343.

2. Bader, J. P. and H. R. Morgan. 1957. Latent viral infection of cells in tissue culture. VI. Role of amino acids, glutamine and glucose in psittacosis virus propagation in L cells. J. Exptl. Med. 103: 617-630.

3. Beale, A. J., G. C. Christofinis, and I. G. S. Furminger. 1963. Rabbit cells susceptible to rubella virus. Lancet. II:640-645.

4. Brown, G. C. 1952. The influence of chemicals on the propagation of poliomyelitis virus in tissue culture. J. Immuno. 69:441-448.

5. Darnell, J. E. and H. Eagle. 1953. Glucose and glutamine in poliovirus production by HeLa cells. Virology 6: 556-566.

6. Darnell, J. E. and L. Levintov. 1960. Poliovirus protein: Source of amino acids and time course of synthesis. J. Bio. Chem. 235 No. 1:74-77.

7. Dubes, George R. 1956. Cystine requirement for normal poliovirus action on monkey kidney tissue culture. Proc. Soc. Exptl. Biol. Med. 93:129-132.

8. Dubes, G. R., and Chapin, K. 1953. Poliovirus mutants with altered responses to cystine. J. Gen. Microbiol. 13:320-329.

9. Dulbecco, R., 1952. Production of plaques in monolayer tissue cultures by single particles of an animal virus. Proc. Nat. Acad. Sci. 38:747-752.

10. Dupree, L. T., K. K. Sanford, B. B. Westfall, and A. B. Povalesky. 1962. Influence of serum proteins on determination of nutritional requirements of cells in culture. Exp. Cell Res. 28:381-405.

11. Eagle, H. 1955. The specific amino acid requirements of a human carcinoma cell (strain HeLa) in tissue culture. J. Exptl. Med. 122:37-49.

12. Eagle, H. 1955. Nutrition needs of mammalian cells in tissue culture. Science. 122:501-504.

13. Eagle, H. 1959. Amino acid metabolism in mammalian cell cultures. Science. 130:432-437.

14. Eagle, H., K. A. Piez, and M. Levy. 1961. The intracellular amino acid concentrations required for protein synthesis in cultured mammalian cells. J. Biol. Chem. 236:2039-2042.

15. Eagle, H. and K. Habel. 1956. The nutritional requirements for the propagation of poliomyelitis virus by the HeLa cell. J. Exptl. Med. 104:271-287.

16. Earle, W. R. 1943. Production of malignancy in vitro. IV. The mouse fibroblast cultures and changes seen in living cells. J. Natl. Cancer Inst. 4:165-212.

17. Eaton, M. D., B. Karasanik, M. E. Perry, and D. Karabian. 1951. Inhibition of influenza and mump virus in tissue culture by basic amino acids. Proc. Soc. Exptl. Biol. Med. 77:505-508.

18. Eaton, M. D., Scala, A. R., and H. Birnie. 1955. Lysine inhibition of cell protein and viral synthesis. Reversal by other amino acids. Proc. Soc. Exptl. Biol. Med. 119:993-1004.

19. Eaton, M. D., A. R. Scala, and I. E. Low. 1964. Amino acid imbalance and incomplete viral replication. Arch. Gesamte Virusforsch 14:583-591.

20. Farnham, A. E., and A. A. Newton. 1959. The effect of some environmental factors on herpes virus grown in HeLa cells. Virology. 7:449-461.

21. Hanks, J. H., and R. E. Wallace. 1949. Relation of oxygen and temperature in the preservation of tissues by refrigeration. Proc. Soc. Exptl. Biol. Med. 71:196-200.

22. Hayashi, K. 1961. Studies on the viral etiology verruca vulgaris in tissue cull culture. Thesis, Michigan State University.

23. Heggie, A. D., and H. R. Morgan. 1956. Latent viral infection of cells in tissue culture. Role of certain amino acids. Proc. Soc. Exptl. Biol. Med. 92:506-509.

24. Kaplan, A. S. 1957. A study of the herpes simplex virus-rabbit kidney cell system by the plaque technique. Virology 4:435-457.

25. Kuchler, R. J. 1964. The flexible nature of the amino acid pool in L strain fibroblasts. Proc. Soc. Exptl. Biol. Med. 116:20-25.

26. Kundin, W. D., M. L. Robbins, and F. K. Smith. 1959. Inhibition of influenza virus multiplication in tissue culture by structural analogs of amino acids. Virology 7:1-27.

27. Lewis, V. J., Jr., and L. V. Scott. 1962. Nutritional requirements for the production of herpes simplex virus. I. Influence of glucose and glutamine on herpes simplex virus production by HeLa cells. J. Bact. 83:475-482.

28. Merchant, D. J., F. B. Hellman, H. Schneider, and E. E. Muirhead. 1962. Protection of animal cells with methylcellulose. Bacteriol. Proc., p. 141.

29. McCarty, K. M. 1962. Selective utilization of amino acids by mammalian cell cultures. Exp. Cell. Res. 27:230-240.

30. Mohanjer, S. and J. Gablike. 1966. The role of methionine deficiency in poliovirus replication in tissue culture. J. Exptl. Med. 123:17-24.

31. Moore, A. E., L. Sabachewsky, and H. W. Toolan. 1955. Culture characteristics of four permanent lines of human cancer cells. Cancer Res. 15:598-602.

32. Morgan, H. R. 1954. Factors related to the growth of psittacosis virus. IV. Certain amino acids, vitamins and other substances. J. Exptl. Med. 22:451-460.

33. Pardee, Arthur B., and Prestidge, Louise S. 1956. The dependence of nucleic acid synthesis on the presence of amino acids in Escherichia coli. J. Bact. 71:677-683.

34. Pearson, H. E., D. L. Lagerborg and R. J. Winzler. 1952. Effects of certain amino acids and related compounds on propagation of mouse encephalitis virus. Proc. Soc. Exptl. Biol. Med. 79:409-411.

35. Pearson, H. E., and R. J. Winzler. 1950. Amino acid analogues and Theiler's GD.VII virus in mouse brain tissue culture. Fed. Proc. 8:409- ; 9:389-396.

36. Pelmont, J., and H. R. Morgan. 1959. Facteurs nutrifs influencant la croissance du virus de l'herpes dans la souche de cellules L de Earle. Ann. Inst. Pasteur 96:443-454.

37. Mlez, E. A., and H. Earle. 1953. The free amino acid pool of cultured human cells. J. Biol. Chem. 231: 533-545.

38. Plummer, G., and B. Lewis. 1965. Thermoinactivation of herpes simplex virus and cytomegalovirus. J. Bact. 92:671-674.

39. Rafelson, M. E., Jr., M. E. Pearson, and R. J. Winzler. 1959. The effects of certain amino acids and metabolic antagonists on propagation of Theiler's G.V.II virus and P^{32} uptake by minced one-day-old mouse brain. Arch. Biochem. 29:69-

40. Rafelson, M. E., R. J. Winzler and M. E. Pearson. 1951. A virus effect on the uptake of C^{14} from glucose in vitro by amino acids in mouse brain. J. Biol. Chem. 193:205-217.

41. Rappaport, C. 1956. Monolayer cultures of trypsinized monkey kidney cells in synthetic medium. Application to poliovirus synthesis. Proc. Soc. Exptl. Biol. Med. 91:464-470.

42. Roizman, B., S. B. Spring, and F. E. Evans, Jr. 1967. Cellular compartmentalization of herpes virus during viral replication. J. Virology 1:191-192.

43. Sanford, K. K., W. T. McGuilkin, M. C. Fioramonti, V. J. Evans, and W. R. Earle. 1953. Study of amino acid requirements for increase in cell population of NCTC clone 929 (strain L). J. Nat. Cancer Inst. 20:775-735.

44. Tankersley, R. W., Jr., 1964. Amino acid requirements of herpes simplex virus in human cells. J. Bact. 87:609-613.

45. Thompson, R. L. 1947. The effect of metabolites, metabolite antagonists and enzyme inhibitors on the growth of the vaccinia virus in Maitland type of tissue culture. J. Immunol. 55:345-351.

46. Thompson, R. L., and M. L. Wilkin. 1948. Inhibition of growth of the vaccinia virus by B-2-thienylalanine and its reversal by phenylalanine. Proc. Soc. Exptl. Biol. Med. 68:434-436.

47. Tyndall, R. L. and E. H. Ludwig. 1960. Nutritional requirements for the production of polioviruses type III, coxsackie B3 and vaccinia virus by continuous animal cell cultures. J. Bact. 80: 96-103.

43. Tyndall, R. L. and E. H. Ludwig. 1963. Cystine requirement for production of coxsackie B3 virus in cultured monkey heart cells. J. Bact. 85: 1339-1345.

49. Tytell, A. A. and R. E. Newman. 1963. A medium free of agar, serum, and peptone, for plaque assay of herpes simplex virus. Proc. Soc. Exptl. Biol. Med. 113:343-346.

50. Wheeler, C. E., C. M. Canby and E. P. Cawley. 1957. Long-term tissue culture of epithelial-like cells from human skin. J. Invest. Derm. 29:383-392.

51. Younger, J. S. and J. V. Hallum. 1964. Cystine-independent thermoresistant mutants of poliovirus. J. Bact. 83:265-266.