LATENT VIRAL INFECTION OF CELLS IN TISSUE CULTURE

VI. Role of Amino Acids, Glutamine, and Glucose in Psittacosis Virus Propagation in L Cells*

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Plates 42 and 43

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Latent infections with psittacosis virus (6BC strain) can be established in chick embryo tissues (1) or in mouse fibroblasts (L cells) (2) by maintaining such cells in culture with a medium containing only inorganic salts and glucose prior to infection. Virus propagation cannot be demonstrated while the cells remain in this deficient medium and no infectious virus can be demonstrated in the cells. The addition of a more complete nutrient medium subsequently induces the production of virus in these infected cultures. The important nutrient components of this growth-stimulating medium were shown to be amino acids and water-soluble vitamins (3), and the specific role of certain amino acids in the stimulation of growth of psittacosis virus in minced chick embryo tissue cultures has been established (4). The L cell system, in providing a uniform population of cells suitable for direct microscopic examination and a shorter experimental period, has made possible a more thorough investigation of the metabolites necessary for the conversion of a latent infection with psittacosis virus to an active one *in vitro* (2). Observations on the effects of amino acids, glutamine and glucose on the stimulation of virus growth in this system are reported here.

Materials and Methods

Virus.—The 6BC strain of psittacosis virus, maintained in this laboratory by yolk sac passage, was used. Virus suspensions were prepared as previously described (1). The stock virus suspension was diluted in Earle's balanced salt solution (BSS) to give a final concentration of $10^{2.0}$ to $10^{3.0}$ LD_{50} for chick embryos per ml., and this fluid was used to infect the tissue cultures.

Virus Titrations.—The single dilution method of Golub (5) was used to determine the amount of virus in the tissue culture fluids. One-quarter of a milliliter of a 10^{-1} dilution of culture fluid was inoculated into the yolk sac of each of a dozen 7-day-old chick embryos and virus titers expressed as the $\log_{10}$ LD_{50} (1).

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Test Media.—The media employed, derived from the synthetic medium 635 of Parker (6), contained only amino acids, water-soluble vitamins, glutamine, glucose, and inorganic salts (Table I). Each test medium was deficient in a single metabolite, except in the case of cysteine

TABLE I

Constituents of Synthetic Medium

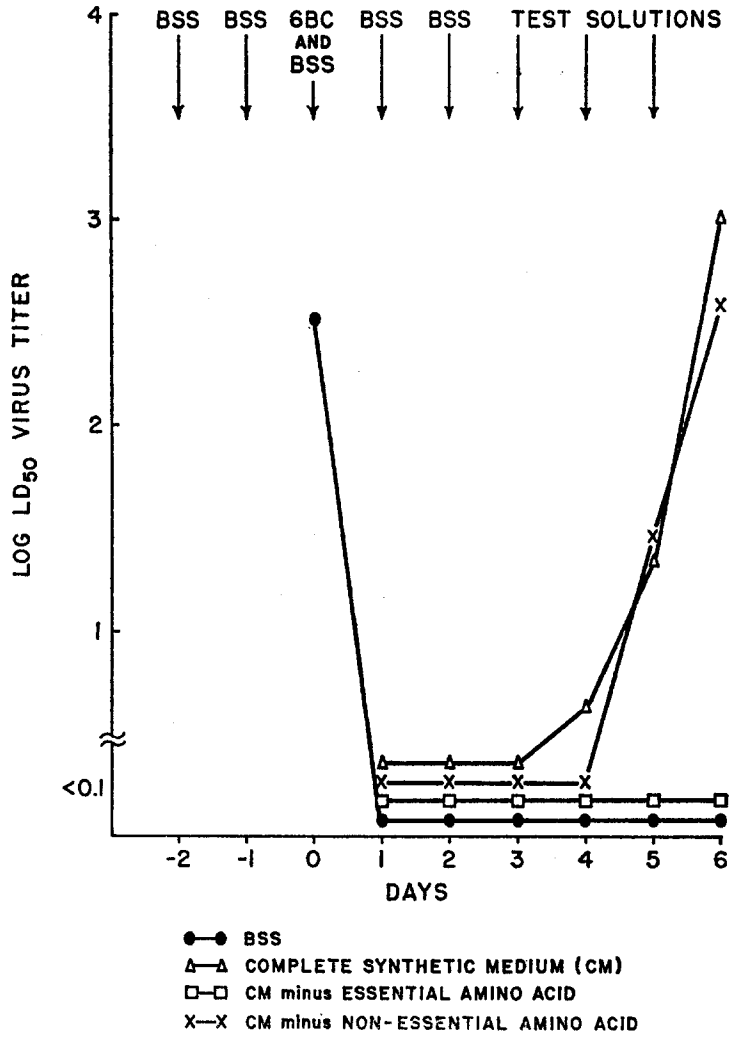
A. Amino Acids	*mg./liter*	*B.*	*mg./liter*
DL-Aspartic acid	60	Glutamine	100
DL-Glutamic acid	150	Glucose	1000
L-Proline	40		
DL-Alanine	50		
L-Hydroxyproline	10		
L-Arginine	70		
L-Lysine	70		
L-Histidine	20		
DL-Serine	50		
DL-Threonine	60		
DL-Methionine	30		
L-Isoleucine	40		
DL-Leucine	120		
DL-Phenylalanine	50		
L-Tyrosine	40		
DL-Tryptophan	20		
DL-Valine	50		
L-Cystine	20		
Cysteine	100		
Glycine	50		
C. Vitamins	*mg./liter*	*D. Inorganic Salts*	*mg./liter*
Niacin	0.025	NaCl	6800
Niacinamide	0.025	KCl	400
Pyridoxal	0.025	$MgSO_4$	200
Pyridoxine	0.025	$NaH_2PO_4H_2O$	140
PABA	0.050	$CaCl_2$	200
Folic acid	0.100	$NaHCO_3$	700
Inositol	0.050		
Choline	0.500		
Ca pantothenate	0.010		
Riboflavin	0.010		
Thiamin	0.010		
Biotin	0.010		

and cystine, in which both were excluded. All media were sterilized by filtration through sintered glass filters and stored in the refrigerator until used.

L Cell Tissue Cultures.—Cultures of L cells were grown in T-15 flasks[1] with a medium containing 25 per cent horse serum, 0.2 per cent lactalbumin hydrolysate,[2] 0.07 per cent

[1] Obtained from Kontes Glass Co., Vineland, New Jersey.

[2] Obtained from Nutritional Biochemicals Corp., Cleveland.



TEXT-FIG. 1. Virus titers of tissue culture fluids from L cells depleted with BSS, infected with psittacosis virus (6BC strain), and treated with complete synthetic medium (CM), media deficient in an amino acid necessary for stimulation of virus growth (CM minus essential amino acid) or media deficient in an amino acid not required for stimulation of virus growth (CM minus non-essential amino acid).

yeast extract,[3] and Earle's BSS as previously described (2). When a uniform sheet of cells had grown over the surface of the glass, the nutrient medium was replaced with Earle's BSS. In all subsequent changes the fluids were completely removed before the addition of fresh media. BSS was added again the following day, and on the 3rd day psittacosis virus was in-

[3] Obtained from Difco Laboratories, Detroit.

cluded in the replacement fluid to infect the cells. After one or two additional changes with BSS, depending on the state of the cells as determined microscopically, the media to be tested were added to the cultures and replaced each day for two more days. Virus titers of tissue culture fluids were determined daily.

RESULTS

Stimulation of Virus Proliferation.—When L cells were incubated with only BSS for 2 days before infection, they showed no evidence of propagation of psittacosis virus (2). However, if the complete synthetic medium was added to cultures after infection, virus proliferation occurred (Text-fig. 1). Each experiment contained a group of flasks to which only BSS was added and a group to which complete medium was added after infection. It was found that the test media could be divided into two main groups on the basis of stimulation of viral growth. One group gave no detectable virus titers, while the other produced virus titers which were of the same magnitude as the complete medium controls. Typical responses to complete medium or media deficient in an amino acid which was not required for virus stimulation compared to the responses of the BSS controls or to a medium deficient in an essential amino acid are seen in Text-fig. 1. Virus usually appeared in the tissue culture fluids on the 2nd day after the addition of the test solutions and by the 3rd day the amount of virus in these fluids was often greater than that of the original inoculum. This is evidence that the virus was actually proliferating and not merely being absorbed and subsequently released into the fluids. Titers of two flasks for each substance tested, which are typical for all flasks in the same group, are presented in Table II. The amino acids, tyrosine, threonine, methionine, isoleucine, phenylalanine, tryptophan, leucine, valine, and cysteine or cystine were necessary in the synthetic medium for the stimulation of viral proliferation. Lysine, arginine, histidine, hydroxyproline, proline, glutamic acid, aspartic acid, serine, alanine, and glycine were not essential for stimulation of viral growth in this system.

A glutamine-deficient medium also gave stimulation of viral multiplication comparable to that seen with complete medium, and glutamic acid did not substitute for glutamine since elimination of both glutamine and glutamic acid from the medium gave similar results (Table II).

A medium which contained the full complement of amino acids but lacked glucose did not produce stimulation of virus growth (Table II).

The possibility that one non-essential amino acid could substitute for another in this system was investigated. A medium lacking all of the non-essential amino acids and glutamine was prepared and placed on infected cells after BSS depletion (Text-fig. 2). Some virus growth resulted after 3 days' exposure to this medium, and by the 4th day large quantities of virus were demonstrable in the tissue culture fluids. All of the non-essential amino acids could thus be

eliminated and the media still retained the capacity to support virus growth. Although the essentiality of an amino acid for stimulation of psittacosis virus

TABLE II

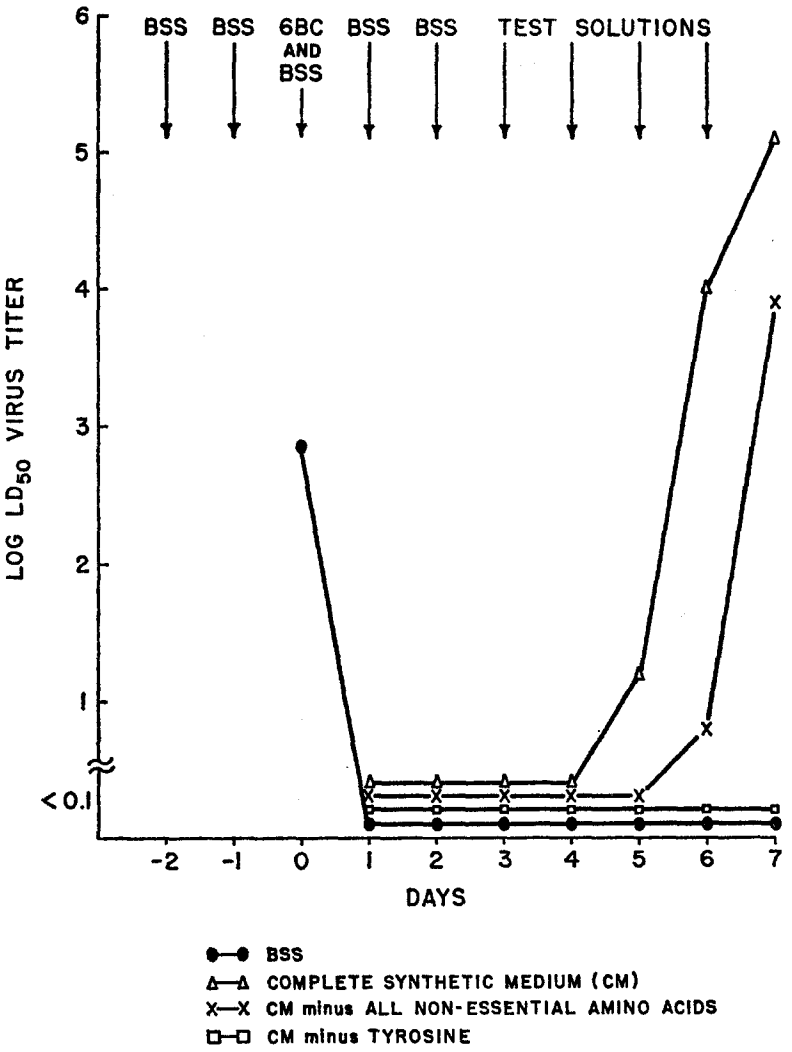
Psittacosis Virus Titers and Conditions of Cells 3 Days after Addition of Test Media

	Virus titers	Morphological condition of cells*
	log_{10} LD_{50}	
Complete medium	3.2, 2.9	++++
BSS	<0.1, <0.1	+
‡Minus tyrosine	<0.1, <0.1	+++
" threonine	<0.1, <0.1	+++
" methionine	<0.1, <0.1	++++
" isoleucine	<0.1, <0.1	++++
" phenylalanine	<0.1, <0.1	++
" tryptophan	<0.1, <0.1	++
" leucine	<0.1, <0.1	+++
" valine	<0.1, <0.1	+++
" cysteine and cystine	<0.1, <0.1	+++
Minus lysine	4.0, 2.6	++
" arginine	2.2, 2.2	++
" histidine	3.0, 2.6	+++
" hydroxyproline	3.2, 1.9	++++
" proline	2.6, 2.2	++++
" glutamic acid	3.0, 2.4	++++
" aspartic acid	3.1, 2.4	++++
" serine	3.1, 2.3	++++
" alanine	2.3, 1.9	++++
" glycine	3.0, 2.4	++++
Minus glutamine	2.6, 1.9	++++
" glutamine and glutamic acid	2.9, 2.2	++++
Minus glucose	<0.1, <0.1	+

‡ Medium contained all other listed constituents of the complete synthetic media.

* ++++ Excellent.
+++ Good.
++ Fair.
+ Poor.

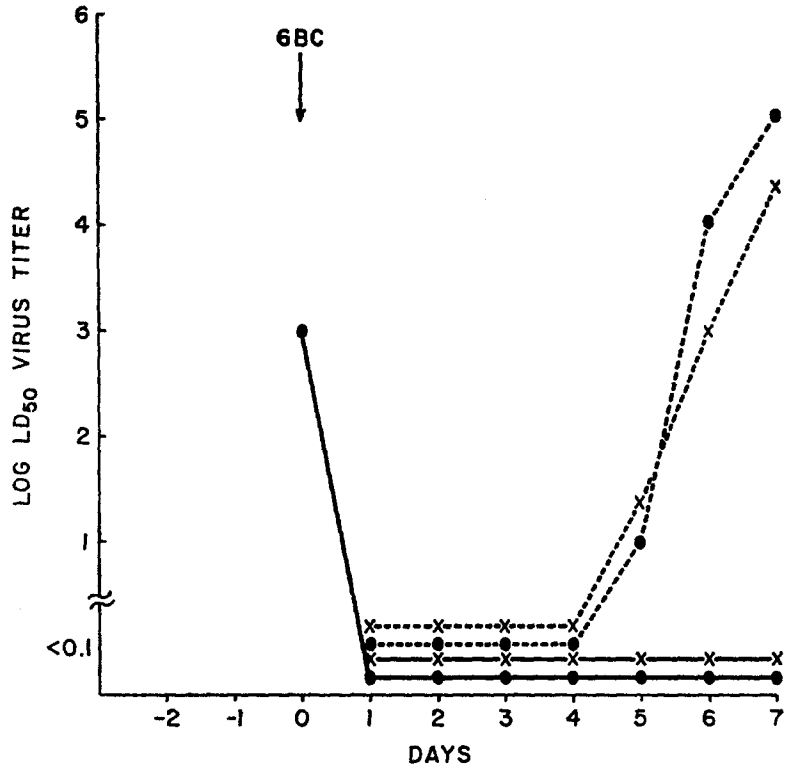
as described earlier is based in some cases on only 3 days' contact of infected cells with the deficient medium, resulting in no detectable virus growth, extending this time to 4 days still did not result in the appearance of virus in the fluids (*e.g.* tyrosine, Text-fig. 2).



TEXT-FIG. 2. Virus titers of tissue culture fluids from L cells depleted with BSS, infected with psittacosis virus (6BC strain) and treated with complete synthetic medium (CM), media deficient in all amino acids not required for stimulation of virus growth, or media deficient in tyrosine, which is necessary for stimulation of virus growth.

Cell Morphology with Complete and Deficient Media.—When the nutrient medium on the L cells is replaced with BSS, notable changes in cellular morphology as observed microscopically were seen over the next few days. After 4 or 5 days' treatment of the cells with BSS, the cytoplasm diminished considerably, although typical fibroblastic bipolar processes were still apparent. The

various media when added produced different changes in these depleted cells. The complete medium and most of the media deficient in single non-essential amino acids brought about an increase in the amount of cellular cytoplasm



TEXT-FIG. 3. Virus titers of tissue culture fluids from L cells maintained for 2 days before infection with psittacosis virus (6BC strain) on phenylalanine- or tryptophan-depleted media, complete medium added 4, 5, and 6 days after infection.

within 24 hours and maintained the cells in good condition during the remainder of the experimental period, although no evidence of cellular multiplication was ever seen. Conversely, cells which were treated with media lacking an amino acid required for virus stimulation usually showed little cytoplasmic expansion and profound cytopathic effects were observed after a few days. Soon after

the period of the experiment, the cells maintained on these media became granular, then rounded, and soon washed away from the glass surface. However, there were important exceptions to both of these types of response. Introduction of media deficient in methionine or isoleucine allowed the cells to attain a morphological condition similar to that seen with the cells receiving complete medium, though virus production was not stimulated in these cells. Arginine and lysine, on the other hand, were not necessary for virus stimulation, but when media deficient in either of these amino acids were placed on depleted, infected cells, cytopathic effects were seen which resembled those noted for media lacking amino acids essential to virus growth. The microscopic condition of cells treated with various media are illustrated in Figs. 1 to 7. The comparative condition of cells maintained in these media are presented in Table II.

Psittacosis virus itself has a profound cytopathic effect on L cells when actively proliferating (7), but it is doubtful if the virus played much part in the cytopathogenicity observed with these various deficient media, since the greatest disturbances were usually seen in cells which were not actively producing psittacosis virus.

Cells treated with glutamine-deficient media seemed to recover from their starved condition, but when glucose was absent from the medium the cells degenerated rapidly.

Depletion with Media Lacking a Single Amino Acid.—By exposing L cell cultures to media deficient only in phenylalanine or tryptophan for 2 days before infection it was also possible to obtain a latent infection with psittacosis virus (Text-fig. 3). The addition of a complete medium 4 days after infection resulted in the appearance of virus in the culture fluids. Thus, an induced deficiency in a single essential amino acid was sufficient to keep the virus in the latent state and to prevent its multiplication. These cells treated from the beginning with deficient medium rather than only BSS, did not show the extreme pathogenic changes that were seen when cells are originally depleted with BSS and the deficient medium added later.

DISCUSSION

It was demonstrated previously that chick embryo cells rendered nutritionally deficient *in vitro* by maintenance in balanced salt solution were incapable of supporting the growth of psittacosis virus (1). If at any time up to 15 days after infection a nutrient medium was placed on such infected cells, virus reappeared in the tissue culture fluid. A similar phenomenon has been observed in L cells (2). It was found that a synthetic medium containing only amino acids, water-soluble vitamins, glutamine, glucose, and inorganic salts was sufficient for stimulation of virus production in both systems (3). This permitted a study of the role of each amino acid and these studies show nine amino acids (tyrosine, threonine, methionine, isoleucine, phenylalanine, tryptophan, leucine, valine, and cysteine or cystine) are necessary for the stimulation of psittacosis virus growth in a system where L cells are rendered nutri-

tionally depleted before infection. Ten amino acids (lysine, arginine, histidine, hydroxyproline, proline, glutamic acid, aspartic acid, serine, alanine, and glycine) are not essential.

Heggie and Morgan (4) have shown that phenylalanine and tryptophan are essential for stimulation of viral growth in depleted chick embryo tissue cultures and that aspartic acid, hydroxyproline, and lysine are not. The results obtained in the L cell system corroborate their findings.

The importance of phenylalanine, methionine, and tryptophan for the propagation of psittacosis virus was demonstrated in previous experiments (9), utilizing the metabolic analogues B-2 thienylalanine, ethionine, and 6-methyl tryptophan which inhibited viral growth in chick embryo tissue cultures. The present observations support these earlier findings and provide more direct evidence of the important role of these amino acids in the growth of psittacosis virus.

It is of special interest that glutamine, histidine, lysine, and arginine, all of which were found to be essential for the growth of L cells by Eagle (8), are not required in a medium which will stimulate virus propagation. The fact that these four compounds have basic properties attracts special attention, but with their differences in metabolic properties no definite conclusion can be drawn to explain their non-essentiality in this system. At least four explanations are possible: (*a*) these compounds may not be depleted from the L cell metabolic pool as rapidly as other amino acids and thus are available for virus synthesis; (*b*) only minimal amounts of these compounds are utilized in the synthesis of viral protein; (*c*) the cell synthesizes small amounts of these compounds which are sufficient for virus production but not for cellular growth; (*d*) the psittacosis virus particle itself contains a factor or factors which aid in the synthesis of these compounds.

Although the mechanism is not clear, it is rather striking that compounds which are essential constituents in a medium which supports the growth of mammalian cells (strain L) are not required for the growth of a virus in these cells. The last possibility suggested above for such a difference in metabolic requirements would imply a certain degree of metabolic autonomy of the virus particle, beyond carrying the genetic information for replication. Many of the viruses, including psittacosis virus, are of sufficient size to carry enzymes or coenzymes as integral parts of the virus particle which could aid in the replication of the particle despite a metabolic deficiency in the cell. There is some evidence to suggest that psittacosis virus synthesizes folic acid (10) and the folic acid content of purified psittacosis virus has been shown to parallel infectivity of the preparations (11). Reduced diphosphopyridine nucleotide cytochrome c reductase activity was shown to be associated with meningopneumonitis virus with the suggestion that the enzymatic activity is a property of the virus itself (12). It is not unreasonable to assume, therefore, that although viruses are dependent on the organized cell for reproduction, certain

metabolic reactions, which are or are not normally associated with cellular activity, can be activated by virus constituents where cellular deficiencies in such activating compounds occur.

The failure of glucose-deficient media to stimulate the production of psittacosis virus is in agreement with studies by other workers who have emphasized the importance of this substance in virus propagation.

Kuwata and Shiba (13) demonstrated the importance of glucose for propagation of ornithosis virus in chick embryo tissue cultures, Eaton (14) showed glucose to be essential for influenza virus growth in chick chorioallantoic membrane cultures, and Eagle and Habel (15) demonstrated the crucial role of glucose for the growth of poliomyelitis virus in HeLa cells. Apparently the amino acids contained in the medium employed here alone are not effective as an energy source to support viral proliferation in the infected L cell. The rapid degeneration of cells deprived of glucose demonstrates the importance of this substance in maintaining cellular integrity in this system. Glutamine does not substitute for glucose in maintaining cellular structure nor does it allow psittacosis virus growth as is suggested for the growth of poliomyelitis virus in HeLa cells (15).

The possibility that media deficient in certain combinations of non-essential amino acids might not stimulate virus production has been eliminated by demonstrating that a medium deficient in all of these amino acids is still capable of inducing virus growth. The delayed appearance of psittacosis virus from cells treated with media lacking all the non-essential amino acids is probably due, at least in part, to the decreased amount of nitrogen available to the cell, since no attempt was made to compensate for the nitrogen content of the amino acids which were eliminated from the medium. Eagle (8) has reported that media deficient in methionine, isoleucine, histidine, or glutamine, do not support the growth of L cells and that within a short time the cells show degenerative changes and lose their viability. The observations presented here differ from those observations since, after a total of 8 days' treatment with BSS and media minus any one of these substances, many cells still appear in good condition and adhere to the glass surface. These differences can probably be attributed to the inclusion in Eagle's medium of dialyzed horse serum which is not added to the synthetic media used in our experiments. This material may enhance the metabolic activity of the cells and rate of multiplication so that the cells maintained on this deficient medium are depleted of their reserves faster than when maintained on our simpler medium and thus such cells would show degenerative changes earlier. Also in the experiments reported here the cell population was allowed to reach a steady state with little cell multiplication before the cells were exposed to deficient media, so that these experiments involve "resting" cells.

Although in many cases cells on depleted media showed cytopathic altera-

tion, there seemed to be little correlation between morphologic condition and ability to support psittacosis virus growth. Cells treated with arginine- or lysine-deficient media which supported virus growth became granular and distorted, while cells treated with media deficient in methionine or isoleucine were morphologically similar to cells with complete medium, but still incapable of producing infectious virus.

Depletion of cells with media deficient in a single amino acid such as phenylalanine or tryptophan for 2 days before infection also rendered the cells incapable of supporting virus growth. This observation suggests that the free amino acid pool of the cells is rapidly depleted and that psittacosis virus protein is formed from this amino acid pool rather than from the breakdown of cellular proteins. A similar situation was noted with vitamin B_6-depleted HeLa cells (16). The failure of these HeLa cells to support growth of poliomyelitis virus was associated with the disappearance of certain amino acids from the free amino acid pool of the cell, and the addition of amino acids to the cells again permitted maximal virus growth (16).

Since L cells depleted of only single amino acids do not seem to lose their viability as quickly as those depleted in BSS, a system of this type may prove valuable in extending the time during which the latent virus infection can be examined. Such a study is now in progress in this laboratory.

The importance of the water-soluble vitamins in this synthetic medium for the stimulation of psittacosis virus reproduction in this depleted L cell system is being examined and will be reported subsequently.

In addition to the observations of latent infection with psittacosis virus in chick embryo tissue cultures previously reported, further evidence is presented here to support the concept that the viability of cells is not the only condition necessary for the propagation of virus but that these cells must be in a proper state of metabolic activity and that specific metabolites must be available for the production of the virus. It has been shown that the psittacosis virus remains in a non-infectious latent state in depleted chick embryo cells (1) or mouse fibroblasts (2) and this phenomenon may have important implications in the problem of latent psittacosis infections, in which certain conditions may alter the metabolic activity of the host, allowing the release of infectious virus with the precipitation of disease. Whether experimental latent infections similar to those described for psittacosis virus can be established for other viruses has yet to be determined.

SUMMARY

Mouse fibroblasts (L cells) fail to support the growth of psittacosis virus (6BC strain) if they are maintained on a medium containing only inorganic salts and glucose for 2 days prior to infection. Virus propagation can be stimu-

lated by the addition of a synthetic medium containing amino acids, water-soluble vitamins, glutamine, glucose, and inorganic salts. By omitting single amino acids from the complete synthetic medium, tyrosine, threonine, methionine, isoleucine, phenylalanine, tryptophan, leucine, valine, and cysteine or cystine were found to be essential for stimulation, while lysine, arginine, histidine, hydroxyproline, proline, glutamic acid, aspartic acid, serine, alanine, and glycine were not essential. The cells on deficient media showed varying degrees of degenerative changes, but there was little correlation between ability to support psittacosis virus growth and morphologic condition of the cells.

Glucose is also an essential component of the medium for viral growth, but the absence of glutamine had no effect on stimulation of virus propagation. L cell cultures maintained on media deficient in phenylalanine or tryptophan for 2 days before infection were also found to be incapable of supporting virus growth. The implications of this study in latent viral infections are discussed.

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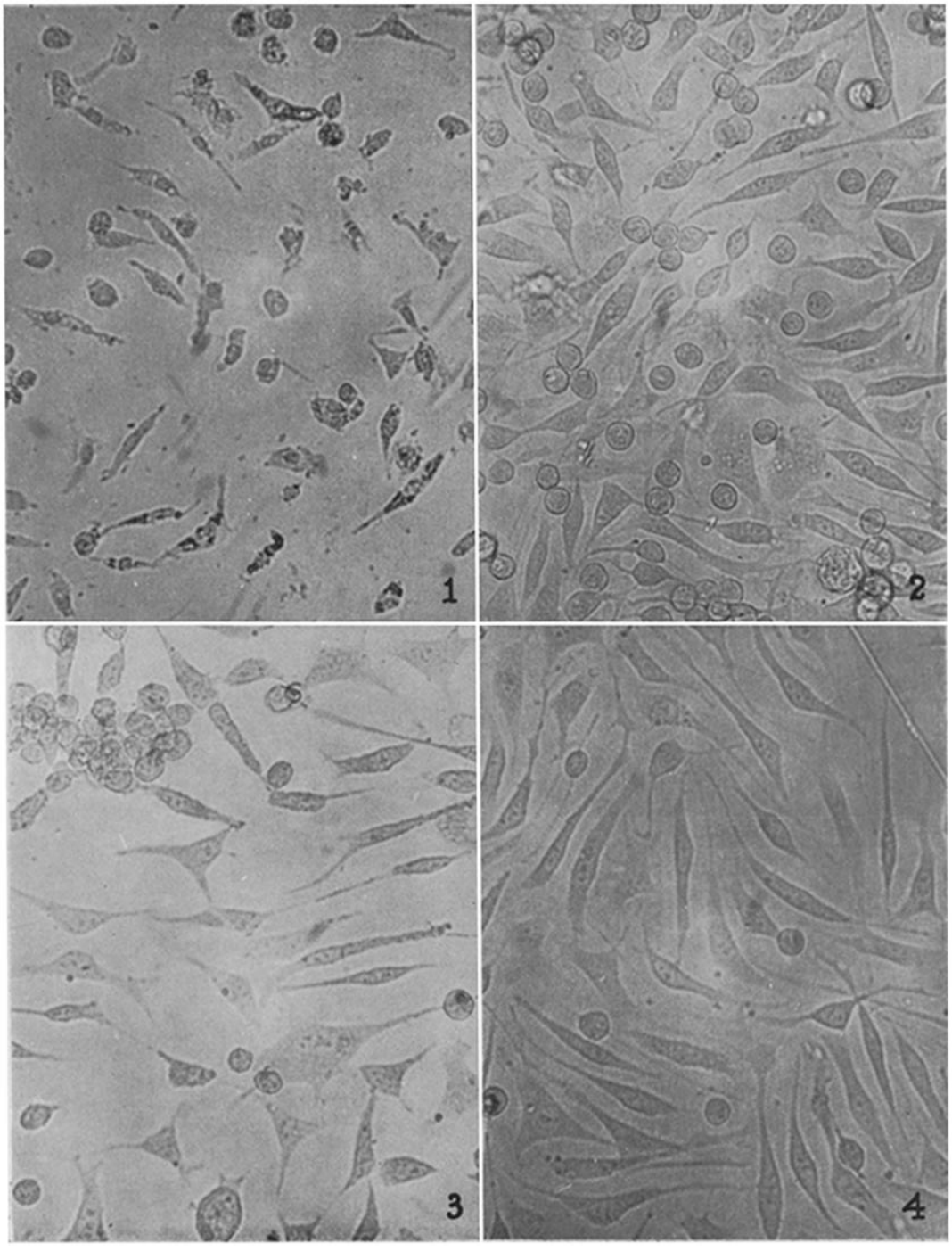
EXPLANATION OF PLATES

FIGS. 1 to 7. The effect of induced specific amino acid deficiencies in L cells infected with psittacosis virus (6BC strain). (All cells were treated with Earle's balanced salt solution 5 days before addition of a medium deficient in a single amino acid. Cultures were changed with the test media on 3 successive days, and, on the 3rd day after addition of the media, were photographed. Figs. 1 to 4, × 96. Figs. 5 to 7, × 94.)

PLATE 42

FIG. 1. BSS only.
FIG. 2. Complete synthetic medium.
FIG. 3. Arginine-deficient cells.
FIG. 4. Glutamine-deficient cells.



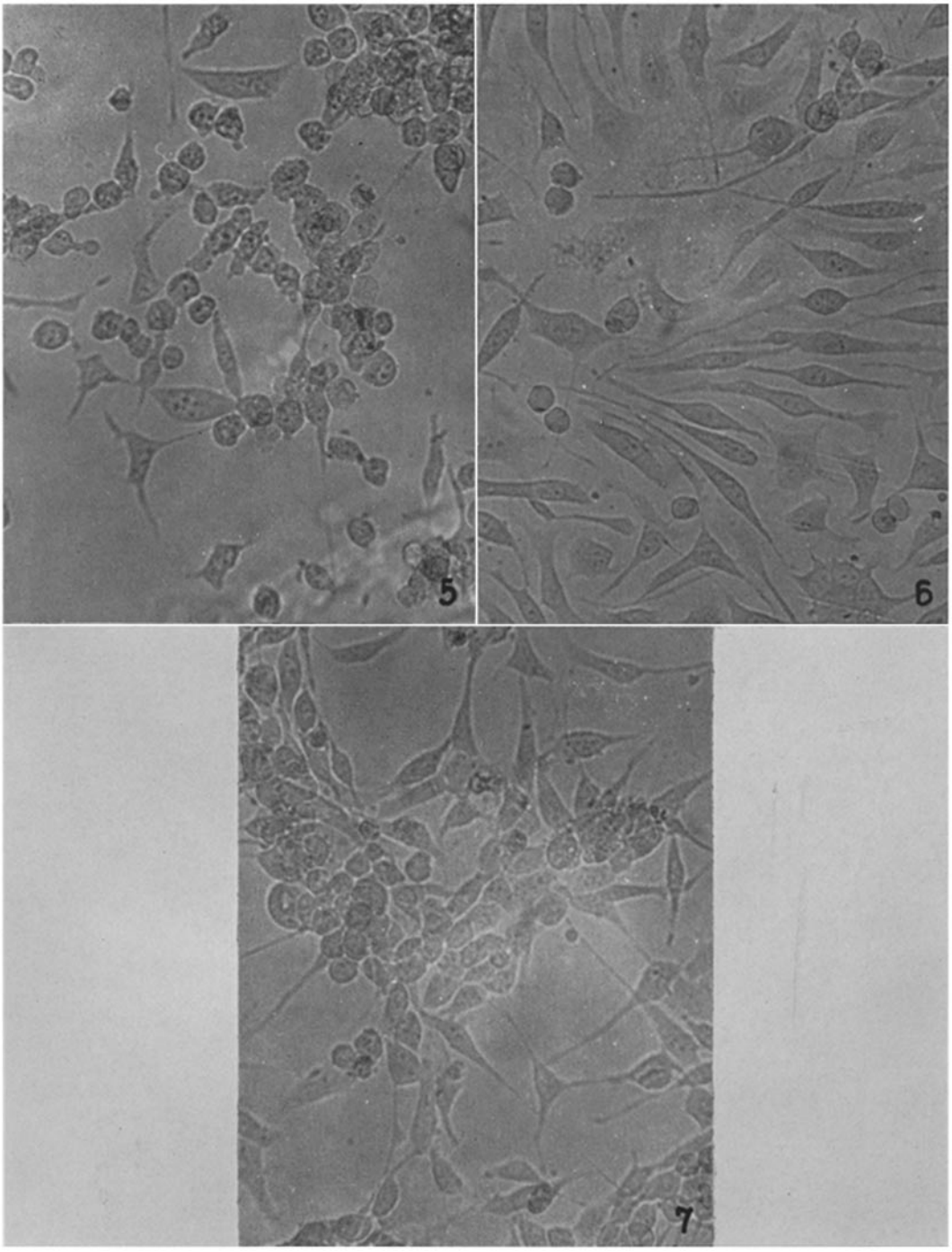
(Bader and Morgan: Latent viral infection of cells *in vitro*)

PLATE 43

FIG. 5. Phenylalanine-deficient cells.

FIG. 6. Methionine-deficient cells.

FIG. 7. Cysteine- and Cystine-deficient cells.



(Bader and Morgan: Latent viral infection of cells *in vitro*)