Effects of L-Glutamine Deprivation on Growth of HVJ (Sendai Virus) in BHK Cells

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L-Glutamine requirement for viral maturation was found in BHK-HVJ cells, a cell line of baby hamster kidney cells persistently infected with HVJ (Sendai virus). Synthesis of envelope protein in BHK-HVJ cells was markedly suppressed by deprivation of L-glutamine, whereas development of nucleocapsid (S) antigen was less affected. More detailed examination of this phenomenon was carried out by using a cytolytic system. Growth of HVJ in BHK cells cultured in media deprived of various amino acids was investigated, and omission of L-glutamine from culture medium resulted in a marked inhibitory effect on the release of infectious virus and synthesis of envelope protein, although synthesis of virus-specific RNA and nucleocapsid antigen in the cells was readily detected. When L-glutamine was restored to the culture medium, infectious virus and envelope protein could be detected. L-Glutamic acid, L-aspartic acid, or L-alanine could be substituted for L-glutamine. Effects of L-glutamine deprivation on HVJ growth in several other cells were also investigated. The growth of HVJ in the cells other than BHK and FL cells was not suppressed by lack of L-glutamine. Growth of Sindbis virus in BHK cells was also markedly retarded in the absence of L-glutamine.

It has been shown that specific amino acids are essential for replication of different viruses, for example: L-arginine for herpesvirus (2, 4, 17, 18), adenovirus (16), respiratory syncytial virus (RSV) (10), and Newcastle disease virus (NDV) (3); L-lysine for reovirus (11); phenylalanine for RSV (10); and L-serine for NDV (5).

We accidentally cultured one series of BHK-HVJ cells, a cell line of baby hamster kidney cells persistently infected with HVJ (Sendai virus; see reference 13), in Eagle minimal essential medium (MEM) devoid of L-glutamine, and found that neither hemadsorption nor cell-associated hemagglutination could be detected. These observations prompted us to perform a series of experiments to clarify the L-glutamine requirement of BHK-HVJ cells to exhibit these properties. More detailed examination of this phenomenon was also carried out with the HVJ-BHK cytolytic system. This investigation is concerned with the effect of L-glutamine deprivation on HVJ growth and on the inhibition of envelope protein synthesis.

MATERIALS AND METHODS

Cell lines and culture media. A continuous line of BHK cells was used throughout this study. In addition, Vero, L, RK-13, HeLa, FL, rat embryo, hamster embryo, chicken embryo, and human embryo cells

were used. Cells were grown in MEM supplemented with 10% calf serum, 10% tryptose phosphate broth, and antibiotics. MEM containing 2 mmol of L-glutamine per liter will be referred to as glut(+) medium, and that devoid of L-glutamine glut(-) medium.

A cell line of BHK cells persistently infected with HVJ, designated as BHK-HVJ cells, was originally obtained by prolonged cultivation of BHK cells which had survived HVJ infection (13). The surviving cells were subcultured at 34°C in growth medium and MEM supplemented with 5% calf serum, and persistent infection of this cell line with HVJ was maintained to date, after 282 subcultures over 3 years. In most passages, more than 80% of the cells exhibited hemadsorbing capacity when tested after the cells had been grown to confluence. The viral products released from the cells were noninfectious components (13). The calf serum used in this study contained L-glutamine (less than 3 µg/ml). Thus, L-glutamine concentration in the medium devoid of L-glutamine supplemented with 10% calf serum is less than 2×10^{-3} mM.

Viruses and virus assay. The Nagoya strain of HVJ was used. Stock virus was prepared by allantoic inoculation of 10- to 11-day-old embryonated eggs with 0.1 ml of 10^{-1} or 10^{-2} dilution of infected allantoic fluid. After incubation for 3 days at 35°C, allantoic fluid was harvested and stored at -80°C. Infectivity titration of HVJ was carried out by intra-allantoic inoculation of chicken embryos with 0.2 ml of each 10-fold serial dilution of test material. After 3

days of incubation at 35 C, allantoic fluids were tested for hemagglutination. The 50% infectivity end points (EID_{50}) were calculated by the method of Reed and Muench. Miyadera strain of NDV, New Jersey strain of vesicular stomatitis virus (VSV), and Sindbis virus were also used.

Titration of HA and hemadsorbing activity. Hemagglutinin (HA) was titrated by twofold dilutions of test materials in 0.25-ml amounts in plastics trays. Chicken erythrocytes were added as one drop of a 2.5% suspension. Titers were read by the pattern method after 60 min at 4 C. The highest dilution showing partial agglutination contained 1 HA unit.

Tests for the capacity of cells to adsorb erythrocytes to their surface were performed by using guinea pig erythrocytes at 4 C, and the extent of erythrocyte adsorption was observed under a microscope. Hemadsorption was graded from (-) to (++++), depending on the percentage of cells exhibiting hemadsorption. The maximal reaction (++++), signifies that over 75% of the cells exhibited hemadsorption.

Nase assay. Cell-associated neuraminidase (Nase) was assayed by a modification of the thiobarbituric acid method of Warren by using a fetuin substrate (9). A 0.2-ml amount of cell homogenate in phosphate-buffered saline (PBS) was mixed with 0.1 ml of fetuin, and the pH was adjusted to 5.0 by adding 0.1 ml of 0.4 M sodium acetate buffer. After 60 min of incubation at 37 C, 0.2 ml of the reaction mixture was used for the assay of free *N*-acetylneuraminic acid. Optical density was read at 549 nm (OD_{549}) against a blank tube containing fetuin plus normal BHK cell homogenate.

Preparation of anti-V and anti-S antisera. Virus materials employed for the preparation of anti-V antiserum were obtained as follows. HVJ was grown in the allantoic cavity of 10- to 11-day-old chicken embryos. Infected allantoic fluid was cleared by centrifugation at 3,000 rpm for 30 min. Virus was pelleted by centrifugation at $80,000 \times g$ for 60 min, suspended in PBS, and used for immunization. Such virus preparations contained about 10^4 HA units/ml. A purified nucleocapsid suspension for immunization was prepared from HVJ-infected BHK cells prepared by the method of Compan and Choppin (1). Anti-V and anti-nucleocapsid (anti-S) antisera were prepared in rabbits by intravenous or intramuscular injection of the envelope protein (V) and virus nucleocapsid (S) antigen preparations, respectively.

CF test. Complement fixation (CF) tests were carried out for detection and titration of S antigen and V antigen by the method of Kolmer (3). Briefly, 0.1 ml of twofold serial dilutions of antigen preparation, 0.1 ml (4 units) of antiserum, and 0.2 ml (2 units) of guinea pig complement were mixed and allowed to stand overnight at 4 C. A 0.2-ml amount of 2.5% sheep erythrocytes was then sensitized with 3 units of hemolysin and was added and allowed to stand for 30 min more at 37 C. The CF titers were expressed as the reciprocal of the highest dilution of antigen exhibiting more than 75% fixation of the complement.

Virus inoculation of cells in the presence or absence of L-glutamine. Monolayers of BHK cells and other cells that had been grown in MEM supplemented with 10% calf serum were washed twice with

prewarmed glut(-) medium. After washing, fresh glut(-) medium was added to the cultures, and they were incubated for 24 h at 35 C before infection. This period of starvation was intended to reduce the cellular glutamine pool. These glutamine-starved cells were inoculated with HVJ at an input MOI of approximately 20 EID_{50} per cell, NDV at an MOI of 10, VSV at an MOI of 10, and Sindbis virus at an MOI of 8. After adsorption of virus for 1 h, the cells were maintained in either glut(+) or glut(-) medium.

Measurement of radioactivity incorporated into macromolecules. To study protein or RNA synthesis in cells under various conditions, 1.0 μ Ci of 14 C-leucine per ml instead of unlabeled leucine or 3.0 μ Ci of 3 H-uridine per ml and 2.5 μ g of actinomycin D per ml were added to culture media, respectively. After various times of incubation, the incorporation of labeled precursors was stopped by the addition of cold 10% trichloroacetic acid to the culture. After washing five times with cold 5% trichloroacetic acid, the cells were dissolved in 1 N NH_4OH . A sample was put into a vial containing Bray scintillation fluid, and the radioactivity was counted in an Aloka scintillation spectrometer. The method of Lowry et al. (12) was used for protein determination.

Immunofluorescence staining. For immunofluorescence staining of intracellular antigens, cells were grown on cover slips in bottles, air dried, and fixed in acetone for 20 min at room temperature. The cover slips were covered with fluorescein-labeled rabbit anti-V or anti-S antiserum, and were incubated for 30 min at 37 C. They were then washed three times, rinsed, and mounted in buffered glycerol. Microscopy was carried out with an Olympus fluorescence microscope.

RESULTS

Effect of L-glutamine deprivation on BHK cells persistently infected with HVJ. BHK-HVJ cells were cultured in growth medium for 2 days. After confluent monolayers had formed, cells were washed with PBS, and glut(+) medium or glut(-) medium was added. At various times of incubation, HA in culture fluid and cell-associated HA and Nase were assayed. BHK-HVJ cells produced a considerable amount of HA in culture fluid when cultured in glut(+) medium, but none in glut(-) medium (Fig. 1). When L-glutamine was restored to BHK-HVJ cells incubated in glut(-) medium for 2 days, hemagglutinin appeared in culture fluid. Titers of cell-associated hemagglutinin and neuraminidase increased steadily during the next 2 days of incubation in glut(+) medium, but were unchanged in glut(-) medium.

After BHK-HVJ cells were grown to confluence for 2 days, monolayer cultures were maintained for another 2 days in the presence or absence of L-glutamine, and hemadsorbing activity and intracellular viral antigens were investigated (Table 1). The cells incubated in

FIG. 1. A, Hemagglutinin production on the culture medium of BHK-HVJ cells incubated in glut(+) or glut(-) medium. Symbols: ●—●, glut(+) medium; ○—○, glut(-) medium; ●—●, addition of L-glutamine(1) to glut(-) medium. B, Cell-associated hemagglutinin and neuraminidase production by BHK-HVJ cells. Symbols: —, hemagglutinin; ---, neuraminidase; ●, glut(+) medium; ○, glut(-) medium.

both media exhibited distinct hemadsorption. CF tests were used for the detection of S (nucleocapsid) antigen. There was almost the same amount of S antigen in the cells incubated in glut(-) medium as in glut(+) medium (Table 1).

The presence of intracellular viral antigens was also examined by fluorescent-antibody staining. Both S antigen and V antigen were detected in the cytoplasm of the cells incubated in both media (Table 1).

When BHK-HVJ cells were cultured in glut(-) medium supplemented with 10% calf serum, they formed confluent monolayers in about 10 days. The monolayers thus obtained,

and hereafter referred to as glut(-)-BHK-HVJ cells, were incubated in glut(+) or glut(-) medium for another 2 days and examined for cell-associated HA, cell-associated Nase, hemadsorbing activity, and viral antigens. Glut(-)-BHK-HVJ cells maintained in glut(-) medium showed no cell-associated HA, cell-associated Nase, or hemadsorbing activity. When glut(-)-BHK-HVJ cells were maintained in glut(+) medium, however, cell-associated HA, cell-associated Nase, and hemadsorbing activity were found within 24 h after the glut(-) to glut(+) shift. On the other hand, titers of S antigen, detected by the CF test, were as high in glut(-)-BHK-HVJ cells maintained in glut(-) medium as in glut(-)-BHK-HVJ cells maintained in glut(+) medium. These results suggest that the viral component which is dependent on L-glutamine may not be a nucleocapsid, but may be an envelope protein. However, since the HA test and the Nase assay are more sensitive to alterations in titer than is the CF test, it is doubtful that the CF test was capable of detecting changes in the amount of S antigen produced. Thus, more detailed examination of this phenomenon was carried out by using a cytolitic system.

Effects of amino acid deprivation on growth of HVJ in BHK cells. Amino acid-starved BHK cell monolayers that were incubated in amino acid-deprived medium (Earle BSS plus vitamins) for 24 h were infected with HVJ and further incubated with medium from which an individual essential amino acid or all amino acids except L-glutamine were omitted. The amino acids examined were those that were found essential for cell growth. Virus yields are shown in Table 2. Although the deprivation of amino acids other than L-glutamine permitted virus production, no virus production could be detected in the absence of L-glutamine. It was also noted that a medium containing only inorganic salts, glucose, and L-glutamine supported multiplication of the virus. These results show that L-glutamine plays an important role in HVJ growth in BHK cells.

Effects of L-glutamine deprivation on growth of HVJ in BHK cells. Growth of HVJ in BHK cells was studied in both glut(+) and glut(-) medium. HVJ release into culture fluid was assessed by titration of HA and by egg infectivity (Fig. 2). When BHK cells infected with HVJ were incubated in the glut(+) medium, logarithmic virus growth was observed, which began at 12 h and reached a plateau at 36 to 48 h. In contrast, no detectable amount of progeny virus was produced in the cells cultured in the glut(-) medium. The almost full produc-

TABLE 1. *Characteristics of BHK-HVJ cells incubated in glut(+) medium or glut(-) medium*

Culture medium ^a	Cell-associated ^d			Hemadsorbing act ^e	HA medium	Immuno-fluorescence ^h	
	CF-S	HA	Nase			V	S
glut(+) ^a	NT ⁱ	8	0.202	++++	<2	+	+
glut(+) → glut(+) ^b	8	32	0.714	++++	16	+	+
glut(+) → glut(-) ^c	8	8	0.198	++++	<2	+	+
glut(-) → glut(-) ^d	8	<1	0.020	-	<2	+	+
glut(-) → glut(+) ^e	8	16	0.286	++++	2	+	+

^{a-e} Confluent monolayers of BHK-HVJ cells (a) grown with glut(+) medium supplemented with 5% calf serum were incubated in glut(+) medium (b) or glut(-) medium (c) for 2 days. Glut(-)-BHK-HVJ cells which were cultured in glut(-) medium supplemented with 10% calf serum for 10 days were further incubated in glut(-) medium (d) or glut(+) medium (e) for 2 days.

^f For measurement of S antigen, cell-associated hemagglutinin and neuraminidase, the cells in two tissue culture dishes detached by EDTA treatment were suspended in 2.0 ml of PBS and were sonically treated for 30 s.

^g Hemadsorption was graded from (-) to (++++), depending on the percentage of cells exhibiting hemadsorption. The maximal reaction, (++++), signifies that over 75% of the cells exhibited hemadsorption.

^h For fluorescent-antibody staining of intracytoplasmic V and S antigen, cells grown on cover slips were used after fixation by acetone for 30 min at room temperature.

ⁱ NT, Not tested.

TABLE 2. *Effects of individual amino acid deprivation on the growth of HVJ in BHK cells*

Amino acid deprivation ^a	Virus yields ^b		
	Cell-associated		Culture fluid
	HA/0.25 ml	Nase/0.1 ml	HA/0.25 ml
Glutamine	<2	0.042	<1
Leucine	256	0.788	6
Tryptophan	256	0.940	8
Histidine	256	0.976	8
Phenylalanine	384	0.864	12
Cystein	256	0.816	8
Tyrosine	384	0.846	12
Isoleucine	384	1.082	12
Threonine	384	1.176	8
Methionine	256	1.044	12
Valine	512	1.140	12
Arginine	512	1.178	16
MEM	256	0.960	8
Salts, glucose, and glutamine	64	0.446	4

^a Amino acid-starved BHK cells were infected with HVJ and they were incubated in medium from which individual amino acid or all amino acids except glutamine were omitted.

^b Virus yields at 24 h after infection were determined.

tion of HVJ, however, was restored by the addition of 2 mmol of L-glutamine per liter to the glut(-) medium.

Effects of L-glutamine concentration on HVJ growth. To study effects of L-glutamine concentrations in medium on HVJ growth in

BHK cells, glutamine-starved BHK cells infected with HVJ were overlaid with a medium supplemented with varying concentrations of L-glutamine. HVJ growth was closely correlated to L-glutamine concentrations in medium (Table 3). It is remarkable that the presence of only a small amount of L-glutamine (at 0.002 mM) led to some increase in viral multiplication as indicated by HA, Nase, and CF test.

Effects of L-glutamine deprivation on protein and RNA synthesis by BHK cells. To study effects of L-glutamine deprivation on the ability of BHK cells to synthesize protein, BHK cell monolayers incubated in glutamine and leucine-deprived medium for 24 h at 35°C were overlaid with the glut(-) medium or glut(+) medium (which contained 2 mmol of L-glutamine per liter), both containing ¹⁴C-labeled leucine. The cell monolayers were then incubated at 35°C for various periods of time and measured for the radioactivity incorporated into an acid-insoluble cell fraction. Cells in the glut(-) medium synthesized protein at about 50% of the rate of those in the glut(+) medium (Fig. 3).

Effects of L-glutamine deprivation and actinomycin D concentration on RNA synthesis by BHK cells were investigated as follows. BHK cells were incubated in the glut(+) or glut(-) medium containing 9 μCi of ³H-uridine per ml, and actinomycin D was added to each culture 3 h before incubation. At 30 min after pulse labeling, cells were harvested, and the acid-insoluble radioactivity was measured (Table 4). Cells in the glut(-) medium synthesized RNA at about one-third the rate of those in medium containing L-glutamine. Actinomycin D in a

FIG. 2. Growth curves of HVJ in the presence or absence of L-glutamine. Glutamine-starved BHK cells were infected with HVJ and after washing three times with glut(-) medium, they were further incubated at 35 C in glut(+) or glut(-) medium. Twelve hours after incubation in glut(-) medium, BHK cells infected with HVJ were added to L-glutamine. At various times after incubation, 0.5 ml of the culture fluids was withdrawn and assayed for infectivity (A) and hemagglutinin (B). Infectivity titrations of HVJ were carried out by intra-allantoic inoculation of chicken embryos. Symbols: ●—●, glut(+) medium; ●---●, glut(-) medium, ×—×, addition of L-glutamine(↓) to glut(-) medium.

dose of 2.5 μg/ml suppressed more than 99% of RNA synthesis.

Development of intracellular viral components. To examine the mechanism of the inhibitory effect of L-glutamine deprivation on virus growth, synthesis of viral RNA and viral protein antigens in glutamine-starved cells were studied (Fig. 4 and 5). Glutamine-starved BHK cell monolayers were infected with HVJ and incubated in the glut(+) or glut(-) medium containing 3 μCi of ³H-uridine per ml.

Actinomycin D was added to each culture at 3 h before infection and was present throughout the incubation at a concentration of 2.5 μg/ml. The amount of radioactivity incorporated into acid-insoluble materials was determined at various times. Although RNA synthesized in the glut(-) medium was only about 10% of that in the glut(+) medium, virus-specific RNA appeared to be continuously synthesized even in the glut(-) medium as indicated by the actinomycin D-resistant incorporation (Fig. 4).

Development of S antigen, HA, Nase, and V antigen were examined (Fig. 5). Monolayers of glutamine-starved BHK cells were infected

TABLE 3. Effects of various concentrations of L-glutamine on HVJ growth in BHK cells^a

L-Glutamine concn (mM)	Virus yields ^b			
	Cell-associated			Culture fluid (HA)
	S	HA	Nase	
None	4	<2	0.004	<1
2 × 10 ⁻³	4	8	0.464	<1
2 × 10 ⁻²	4	32	0.816	<1
2 × 10 ⁻¹	8	64	1.156	2
2	16	256	1.540	8
2 × 10	16	256	1.570	8

^a Glutamine-starved BHK cells were infected with HVJ and were incubated in MEM containing various concentrations of L-glutamine.

^b Virus yields at 24 h after infection were determined.

FIG. 3. Effect of L-glutamine deprivation on protein synthesis by BHK cells. Glutamine-starved cells were incubated in glut(+) or glut(-) medium containing 1 μCi of ¹⁴C-leucine per ml instead of unlabeled leucine. At various times after incubation the amount of radioactivity incorporated into acid-insoluble material was counted. Symbols: ●—●, glut(+) medium; ●---●, glut(-) medium.

TABLE 4. RNA synthesis by BHK cells

Actinomycin D ($\mu\text{g/ml}$) ^a	Counts per min ^b	
	glut(+) medium	glut(-) medium
10	142	79
5	128	68
2.5	196	88
None	54,462	17,126

^a Actinomycin D was added 3 h before each pulse and was present throughout the incubation.

^b BHK cells were incubated in the glut(+) or glut(-) medium, and were exposed to 9 μCi of ³H-uridine per ml for 30 min. After pulse labeling, cells were harvested and trichloroacetic acid-insoluble radioactivity was counted.

with HVJ and, after being washed three times with the glut(-) medium, they were again incubated at 35 C in either glut(+) or glut(-) medium. At various intervals the cells, detached by EDTA treatment, were suspended in 2.0 ml of PBS and were sonically treated for 30 s. The resulting fluid was assayed for biological activity. Intracellular S antigen in glutamine-starved cells increased exponentially until 36 h after infection at about 25% of the rate of that in glut(+) medium. However, no increase of cell-associated HA, Nase, and CF-V antigen could be detected throughout infection (Fig. 5). Reduction of S antigen synthesis in the glut(-) medium could be largely explained by reduction of protein and RNA synthesis in the glut(-) medium. When L-glutamine was added to the glut(-) medium at 12 h after infection, synthesis of cell-associated HA, cell-associated Nase, and V antigen was restored.

Effects on HVJ growth of removal of L-glutamine at various times after infection. Effects on HVJ growth and removal of L-glutamine at various times during viral multiplication were studied. When L-glutamine was removed from the medium at 8 h after infection, HA was not detected in the medium (Fig. 6). Removal of L-glutamine at 20 h resulted in a reduced rate of viral multiplication. This finding was compatible with results obtained by addition of glutaminase (10 units/ml) instead of removal of L-glutamine. When glutaminase was added to the medium at 8, 20, and 30 h after infection, HVJ growth as indicated by HA titers was suppressed to varying degrees. These findings suggest that L-glutamine is required throughout the process of HVJ growth.

Effects of other nonessential amino acids on HVJ growth in glut(-) medium. The effect of addition of other nonessential amino acids to glut(-) medium on the growth of HVJ was

examined. L-Glutamic acid, L-aspartic acid, and L-alanine could compensate for the lack of L-glutamine (Table 5). Some glucosamine, D-glucosamine, and D-mannosamine could not be substituted for L-glutamine when tested at a concentration of 8 mM in the glut(-) medium.

Effects of L-glutamine deprivation on growth of HVJ in other cells. HVJ growth in other glutamine-starved cells was also studied. A variety of cells tested (including FL, L, HeLa, Vero, RK-13, chicken embryo, hamster embryo, rat embryo, and human embryo cells) showed that the phenomenon observed in BHK cells

FIG. 4. Effect of L-glutamine deprivation on virus specific RNA synthesis by HVJ-infected cells. Glutamine-starved BHK cells were infected with HVJ and incubated in glut(+) medium(A) or glut(-) medium(B), both containing 3 μCi of ³H-uridine per ml. A 2.5- μg amount of actinomycin D per ml was added 3 h before infection. At various times the radioactivity incorporated into acid-insoluble material was counted. Symbols: ●—●, infected cells; ●---●, noninfected cells.

FIG. 5. Intracellular development of virus structural components by glutamine-deprived cells. Glutamine-starved BHK cells were infected with HVJ and after washing three times with glut(-) medium they were incubated in glut(+) or glut(-) medium. Twelve hours after incubation in glut(-) medium, BHK cells infected with HVJ were added to L-glutamine. At various times, cells were harvested by EDTA treatment and finally resuspended in 2 ml of PBS. The cell suspension was sonically treated in an ice box for 30 s. A, S antigen; B, V antigen; C, hemagglutinin; D, neuraminidase. Symbols: ●—●, glut(+) medium; ●---●, glut(-) medium; ×—×, addition of L-glutamine (1) to glut(-) medium.

was found only in FL cells (Table 6).

Effects of L-glutamine deprivation on growth of other envelope virus in BHK cells. To study the effects of L-glutamine deprivation on growth of other enveloped virus, glutamine-starved BHK cells were infected with either VSV, NDV, or Sindbis virus and were incubated in the glut(+) or glut(-) medium. The yield of Sindbis virus was markedly decreased, whereas that of VSV and NDV was not significantly affected by L-glutamine deprivation (Fig. 7).

DISCUSSION

The experimental results described in this investigation show that release of virus and synthesis of cell-associated HA and Nase in BHK-HVJ cells were markedly suppressed by deprivation of L-glutamine, but S antigen in the cells was less affected. Addition of glutaminase at a concentration of 10 units/ml resulted in an effect similar to that obtained by L-glutamine deprivation (unpublished data). It was also shown that, when L-glutamine was restored to the medium, BHK-HVJ cells resumed synthesis of cell-associated HA and Nase, and virus release.

More detailed examination of this phenomenon was carried out by using a cytolitic system. Omission of L-glutamine from culture medium resulted in a marked inhibitory effect on the release of infectious virus and synthesis of envelope protein (CF-V, HA, and Nase) in BHK cells, although synthesis of RNA and S antigen by the cells was readily detected. It was also shown that, when L-glutamine is restored to the glut(-) medium, virus production as well as synthesis of envelope protein increased.

When the L-glutamine-starved BHK cells that had been incubated in the glut(-) medium for 24 h were infected with HVJ and further incubated in the glut(-) medium, infectious virus, cell-associated HA and Nase, and V antigen could not be detected. However, despite a marked reduction in synthesis of envelope protein in the glut(-) medium, S antigen was synthesized at a rate of 25% that in control cells. This finding was confirmed by means of fluorescent antibody staining. That is, V antigen was not stainable, whereas S antigen was readily stainable (unpublished observations). Virus-specific RNA was continuously synthesized in the glut(-) medium, although at a fairly reduced rate. Observed reduction in synthesis of

FIG. 6. A, Effect on HVJ growth of the removal of L-glutamine at various times after infection. B, Effect on HVJ growth of addition of glutaminase instead of removal of L-glutamine at various times after infection. HVJ-infected BHK cells were incubated in glut(+) medium. At indicated times, the cells were washed twice time with glut(-) medium and further incubated in glut(-) medium, or glutaminase was added to culture fluid. At various times, hemagglutinin in culture fluid was assayed. Symbol: (—), removal of L-glutamine or addition of glutaminase.

both S antigen and virus-specific RNA in the glut(-) medium could be attributable mainly to the reduction of protein and RNA synthesis of the cells incubated in the glut(-) medium. However, observed reduction in envelope protein synthesis was out of proportion compared with that of S antigen and too extreme to be explained by the reduction of protein synthesis of BHK cells. These results show that marked inhibition of HVJ growth in BHK cells by omission of L-glutamine is due to a decrease of envelope protein synthesis.

There are at least two possible interpretations of inhibition of envelope protein synthesis. First, synthesis of RNA related to envelope protein synthesis may be dependent on L-glutamine. Since virus-specific RNA synthesis was somewhat reduced in the HVJ-infected cells incubated in the glut(-) medium, it is important to determine whether there is a differential effect of glutamine deficiency on a particular species of virus-specific RNA, e.g., 18S complementary RNA or 50S genomic RNA (15). A further study is now under way to clarify this point. The second possible interpretation is that envelope protein may be formed through a metabolic reaction in which L-glutamine is utilized. It was recently reported that D-glucosamine and 2-deoxy-D-glucose inhibit the formation of HA, Nase, and infective virus, whereas almost normal amounts of viral RNA and nucleocapsid protein are synthesized in some enveloped RNA virus-infected cells (6, 7). Kaluza, Scholtissek, and Rott (6) speculated that glucosamine obviously interferes with the production of the carbohydrate-containing subunits which are a part of the virus envelope. L-Glutamine is one of the most important physiological substrates for aerobic oxidation in malignant cells (8). L-Glutamic acid, L-alanine, and L-aspartic acid, all of which were found in the present study to be substituted for L-glutamine, are also

TABLE 5. Effects of addition of other nonessential amino acids and some amino sugars on HVJ growth in BHK cells^a

Amino acids or amino sugars added	Virus yields ^b		
	Cell-associated		Culture fluid (HA)
	HA	Nase	
None	<2	0.042	<1
L-Glutamine	256	1.220	8
L-Glutamic acid	64	0.948	4
L-Aspartic acid	32	0.520	<1
L-Alanine	32	0.778	<1
L-Asparagine	2	0.080	<1
Glycine	<2	0.040	<1
L-Proline	<2	0.062	<1
L-Serine	<2	0.020	<1
D-Glutamine	4	0.134	<1
D-Glucosamine	2	0.095	<1
D-Mannosamine	<2	0.072	<1

^a Glutamine-starved BHK cells were infected with HVJ and after washing three times with the glut(-) medium they were further incubated in the glut(-) medium or the glut(-) medium supplemented with 2 mM of a nonessential amino acid or 8 mM of an amino sugar.

^b Virus yields at 24 h after infection were determined.

TABLE 6. *Effects of L-glutamine deprivation on HVJ growth in other cells^a*

Cell lines ^b	Virus yields ^c					
	Glut(+) medium			Glut(-) medium		
	Cell-associated		Culture fluid (HA)	Cell-associated		Culture fluid (HA)
	HA	Nase		HA	Nase	
FL	512	1.364	64	16	0.496	<2
L	12	0.204	<2	<2	0.032	<2
HeLa	128	0.876	2	8	0.224	<2
Vero	64	0.442	32	64	0.192	32
RK-13	32	0.272	8	16	0.194	4
Chicken embryo	512	1.318	64	512	1.458	16
Hamster embryo	24	0.324	8	16	0.214	4
Rat embryo	<2	0.022	<2	<2	0.012	<2
Human embryo	<2	0.072	<2	<2	0.016	<2

^a Glutamine-starved cells were infected with HVJ and after washing three times with the glut(-) medium, they were further incubated in the glut(+) or glut(-) medium.

^b L-Glutamine requirement for HVJ growth was not limited to our laboratory line of BHK cells and was similarly observed in other lines of BHK cells maintained in other laboratories.

^c Virus yields at 48 h after infection were determined.

FIG. 7. The effect of L-glutamine deprivation on the growth of several enveloped viruses was examined. Glutamine-starved BHK cells were infected with Sindbis virus (8 MOI), VSV (10 MOI), and NDV (10 MOI), and after washing three times with glut(-) medium they were incubated in glut(+) or glut(-) medium. At various times after incubation, 0.2 ml of the culture fluids was withdrawn and assayed for virus titers. Virus titration was carried out by plaque formation in chicken embryo cells. A, Sindbis virus; B, VSV; C, NDV. Symbols: ●—●, glut(+) medium; ●---●, glut(-) medium.

intimately related to glycolysis. Oppenheimer et al. (14) reported that the teratoma cells require L-glutamine for the synthesis of amino sugars which are utilized for the formation of cell surface macromolecules involved in intercellular adhesion. It is, therefore, probable that L-glutamine deficiency may inhibit envelope protein synthesis by interfering glycolysis. This possibility is also under investigation.

Effects of L-glutamine deprivation on HVJ growth in nine other cell lines tested were inhibitory in FL cells and were insignificant in

the rest. This may be due to either insufficient glutamine deprivation in some cells or to a low degree of glutamine dependency in others.

Effects of L-glutamine deprivation on growth of other enveloped virus were also examined. The yield of Sindbis virus was markedly decreased, whereas that of VSV or NDV was not significantly affected by L-glutamine deprivation.

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