NUTRITIONAL REQUIREMENTS FOR THE PRODUCTION OF HERPES SIMPLEX VIRUS[1]

I. Influence of Glucose and Glutamine on Herpes Simplex Virus Production by HeLa Cells

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ABSTRACT

Lewis, Vester J., Jr. (University of Oklahoma School of Medicine, Oklahoma City) and L. Vernon Scott. Nutritional requirements for the production of herpes simplex virus. I. Influence of glucose and glutamine on herpes simplex virus production by HeLa cells. J. Bacteriol. **83**:475–482. 1962.—The importance of glucose and glutamine in herpes simplex virus production by HeLa cells was evaluated by variation of the composition of Eagle's basal medium. Simultaneous omission of glucose and glutamine from the serum-containing medium resulted in marked curtailment of viral synthesis. The effect was attributable neither to decreased survival time of infectious particles or of cells, nor to decreased rate of viral adsorption or penetration in the presence of the deficient medium. Therefore, the effect was probably on intracellular viral replication.

When the requirements for glucose and glutamine were determined separately, it was found that viral production was reduced in glucose-deficient medium. In contrast, a medium which was complete except for restriction of the glutamine content to that occurring naturally in the serum component supported significantly greater viral multiplication than did the complete medium. Limited viral replication occurred with medium which lacked both glutamine and serum. Viral multiplication in the presence of serum-free medium was stimulated by the addition of glutamine.

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Tissue culture techniques offer unique opportunities for determination of the specific nutritional requirements of cells for the production of viruses. Eagle and Habel (1956) found that synthesis of poliovirus type I by HeLa cells which were nourished with Eagle's (1955) basal medium (EBM) was decreased markedly upon omission of glucose from the medium. Even less virus was synthesized when glutamine was omitted from the otherwise complete medium. Darnell and Eagle (1958) showed that Earle's (1943) balanced salt solution (EBSS), which regularly contains 0.1% glucose and to which was added 2 mM glutamine, supported replication of poliovirus type I almost as well as did Eagle's medium. In addition to glutamine and glucose, the complete basal medium of Eagle contains 12 amino acids and 8 vitamins in Earle's solution. Tyndall and Ludwig (1960) reported that balanced salt solution with glutamine and glucose supported near maximal production of poliovirus type II by HeLa cells.

This study is concerned with the influence of glutamine and glucose on the synthesis of herpes simplex virus by HeLa cells.

MATERIALS AND METHODS

Virus. The viral pool used was prepared by inoculation of monolayers of HeLa cells with the HF strain of herpes simplex virus. The inoculated monolayers, covered with lactalbumin medium, were incubated at 35 C until viral cytopathic effect was well advanced. The medium from several bottles was pooled at this time and stored at −62 C until used. The viral content was shown to be $10^{8.2}$ $TCID_{50}$ per ml.

Cells. HeLa (Gey) cells, which had been cultivated in human serum when obtained from Tuskegee Institute, Alabama, were adapted to calf serum in this laboratory before use. These

cells were shown to be free of pleuropneumonia-like organisms (PPLO) by eight serial propagations in antibiotic-free medium. Failure of these organisms to grow when cells were placed in an enriched medium was added proof of the absence of PPLO.

Media. The medium used in preparation of the viral pool was of the following composition: enzymatic lactalbumin hydrolysate (Nutritional Biochemicals Corp.), 0.25%; Yeastolate (Difco), 0.05%; calf serum, 5%; EBSS, 94.70%. Growth medium was EBM, purchased from Microbiological Associates, to which 2 mM of glutamine and 10% calf serum were added. Experimental media consisted of modifications of the commerical medium, unless the effect of glucose on viral synthesis was tested. In the latter instance, it was necessary to make EBM in this laboratory because of the presence of glucose in the commercial medium. The EBM prepared in this laboratory was, except for the omission of glucose, of the same composition as the commercial EBM. All experimental media except EBSS contained 5% calf serum unless otherwise noted. All media in the study contained 100 units of penicillin G and 100 μg of streptomycin per ml, and were sterillized by Seitz filtration.

Production of virus. Bottle and stationary-tube cultures, containing approximately 7×10^6 and 2×10^4 cells per monolayer, respectively, were used to determine the ability of various media to support viral production. The mono-

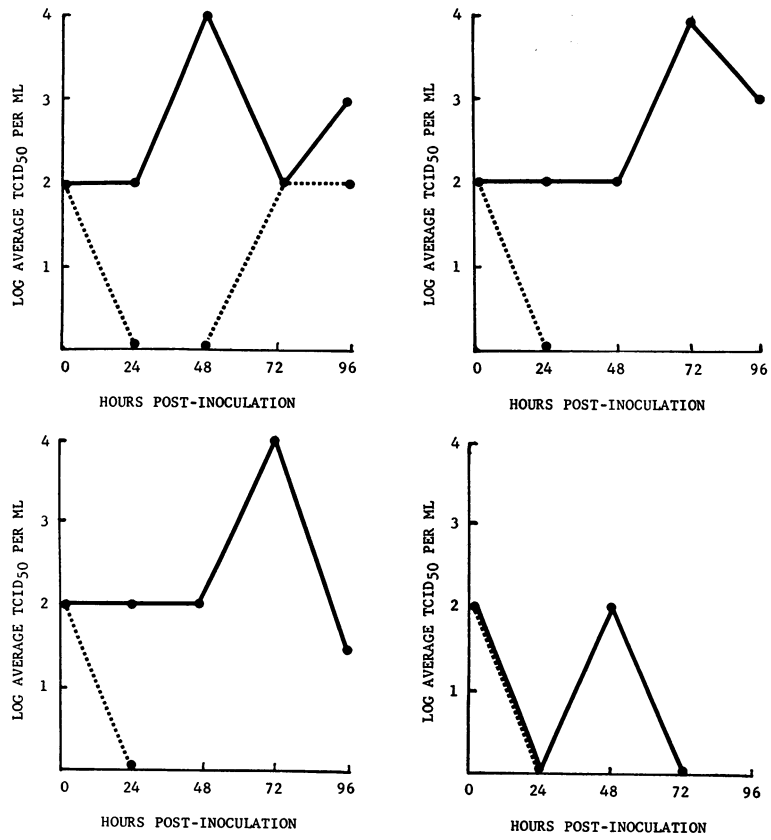

FIG. 1–4. *Virus production by cells maintained with complete or deficient media for various times before infection. Complete medium (Eagle's basal medium), solid line; deficient medium (Eagle's basal medium, less glucose and glutamine), broken line. Fig. 1 (top, left). Cells maintained in experimental media 0 hr preinoculation. Fig. 2 (top, right). Cells maintained in experimental media 12 hr preinoculation. Fig. 3 (bottom, left). Cells maintained in experimental media 24 hr preinoculation. Fig. 4 (bottom, right). Cells maintained in experimental media 36 hr preinoculation.*

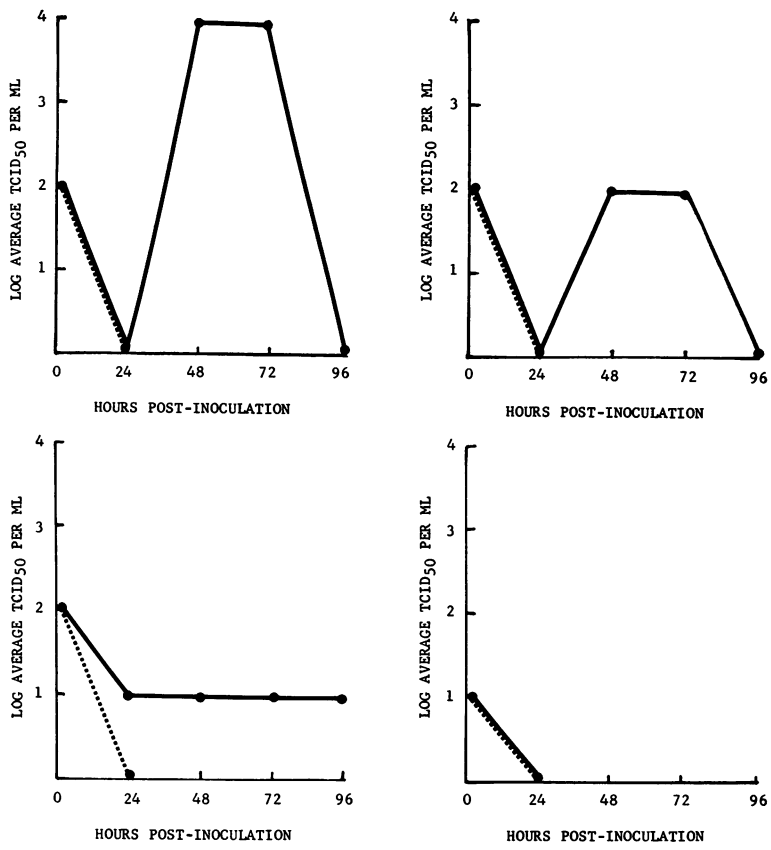

FIG. 5–8. *Virus production by cells maintained with complete or deficient media for various times before infection. Complete medium (Eagle's basal medium), solid line; deficient medium (Eagle's basal medium, less glucose and glutamine), broken line. Fig. 5 (top, left). Cells maintained in experimental media 48 hr preinoculation. Fig. 6 (top, right). Cells maintained in experimental media 60 hr preinoculation. Fig. 7 (bottom, left). Cells maintained in experimental media 72 hr preinoculation. Fig. 8 (bottom, right). Cells maintained in experimental media 84 hr preinoculation.*

layers were washed thoroughly with EBSS to remove traces of growth medium which may have contained the nutrients under test. Glucose was omitted from EBSS in experiments concerning the effect of glucose on viral synthesis. The viral inocula, suspended in just enough EBSS to cover the monolayers, were added to the washed cells at a ratio of approximately 10 infectious particles per cell. Adsorption was carried out at 35 C for 90 min. Experimental media were added, and the cells were incubated at 37 C.

Titration of samples. The viral content of samples of supernatant fluids was determined by titration in replicate stationary tubes of HeLa monolayers supplied with growth medium. Conditions of viral adsorption and of culture incubation were as described for infection of the experimental monolayers. The titration monolayers were examined at 3, 5, and 7 days after inoculation for viral cytopathic effect, and the $TCID_{50}$ of the samples were calculated by the method of Reed and Muench (1938).

RESULTS

Virus production in medium deficient in glucose and glutamine. Experiments were done to determine if HeLa cells kept for various periods of time in EBM, which lacked the usual amounts of glucose and glutamine, were capable of synthesis of herpes simplex virus. Tubes of monolayers were divided into two groups. The growth me-

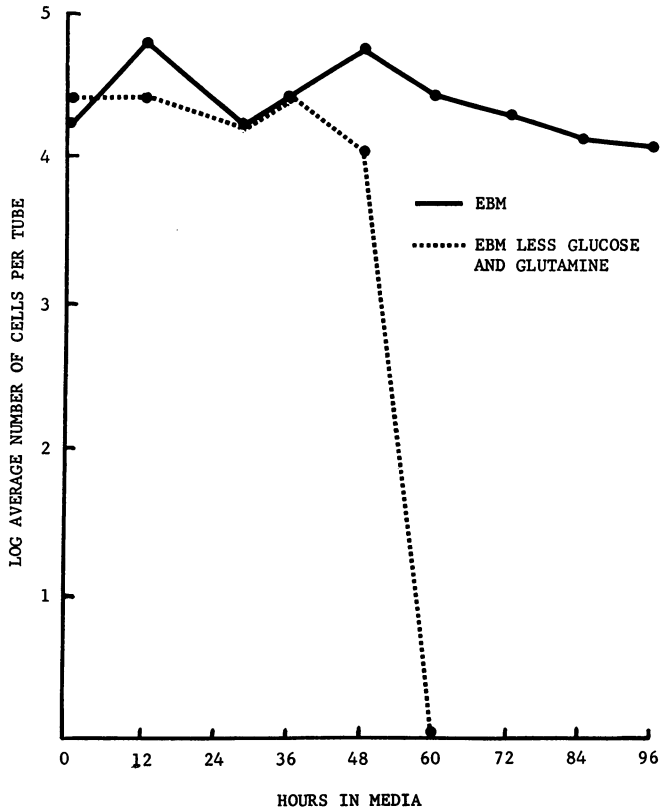

FIG. 9. *Survival of HeLa cells in complete and deficient Eagle's basal medium (EBM)*

dium of one group was replaced with modified EBM in which the only source of glutamine and glucose was the serum component. The second group received complete EBM. Immediately and at 12-hr intervals, tubes from each group were inoculated with virus. This variation in the time that cells were kept in experimental media before viral challenge was to determine the interval needed for depletion of the glucose and glutamine which were intracellular and in the serum component of the medium. At zero time and at 24-hr intervals after inoculation, several tubes from both experimental media were removed and the amounts of virus present were determined.

Typical results of titrations are depicted by Fig. 1 through 8. Samples taken at zero time were shown to contain the same concentration of virus as the inoculum. It was demonstrated in later samples that complete medium supported synthesis of virus in all instances except when inoculation of virus was delayed until 84 hr after the experimental media had been added. Cells supplied with the deficient medium produced virus only if inoculated immediately after addition of the medium, a time when small amounts of glucose and glutamine probably were present.

The lack of viral production by cells in the

TABLE 1. *Penetration of herpes simplex virus into HeLa cells in presence of complete and deficient media*

Hours for penetration	Average number[a] of plaques on monolayer	
	Complete medium[b]	Deficient medium[c]
1	8	7
2	10	17
3	22	32
4	36	35
5	35	40
6	37	38

[a] Number: average of plaque counts on monolayers 3 days after exposure to virus.

[b] Eagle's basal medium containing 5% calf serum.

[c] Eagle's basal medium containing 5% calf serum; deficient in glucose and glutamine.

deficient medium was not attributable to the failure of cells to survive in the medium. At the time each sample was taken, the numbers of cells in uninoculated monolayers maintained in the test media since the beginning of the experiment were determined. Results of such hemocytometer cell chamber counts are shown in Fig. 9. Cells survived longer in the complete medium of Eagle than in the deficient medium. During the time when both cultures contained approximately equal numbers of cells it was observed that more virus was produced in the complete medium than in the deficient medium.

The next step in this investigation was to determine if the lack of viral synthesis in the presence of glucose- and glutamine-deficient EBM was due to inhibition either of viral adsorption onto the cell or of viral penetration. Growth medium on cell monolayers in bottles was replaced with the deficient medium or with complete EBM. At hourly intervals after addition of 100 $TCID_{50}$ of virus to each bottle, experimental media were removed from replicate monolayers. The infected monolayers were thoroughly washed and covered with growth medium to which had been added pooled human serum at a final concentration of 10%. This amount of serum was shown to contain sufficient antibody to neutralize extracellular virus present. After 3 days incubation of the inoculated monolayers, the microscopic plaques which had formed as a result of viral cytopathic effect were enumerated (Table 1).

Adsorbed virus which had not penetrated by the time of addition of the antibody-containing medium was neutralized upon that addition. Farnham (1958) reported that each plaque appearing in HeLa cell monolayers within 3 days of inoculation with herpes simplex virus arises from one infectious particle in the original inoculum. Accordingly, in the present experiment the increase which was seen in the number of plaques with time before the antibody-containing medium was added represents rate of

TABLE 2. *Effect of glucose and glutamine on survival of HeLa cells and virus synthesis*

Medium		Hours at which samples were taken after virus adsorption										
		0	8	16	24	32	40	48	56	64	72	80
EBM,[a] complete	Log_{10} virus per ml	0	0	1	2	2	3	3	3	4	4	3
	Control cells[b]	100[c]	100	100	100	100	100	100	100	100	100	100
EBM, no glucose or glutamine	Log_{10} virus per ml	0	0	0	0	0	0	0	0	0	0	0
	Control cells	100	100	100	100	100	100	100	25	3	0	0
EBM, no glucose	Log_{10} virus per ml	0	0	0	0	0	0	0	0	0.5	1	2
	Control cells	100	100	100	100	100	100	100	75	10	10	10
EBM, no glutamine	Log_{10} virus per ml	0	0	1	1.5	2	3	3.5	3	2.5	2.5	1
	Control cells	100	100	100	100	100	100	100	75	70	70	50
EBSS,[d] with glutamine	Log_{10} virus per ml	0	0	0	0	0	0	0	0	0	0	0
	Control cells	100	100	100	100	100	50	0	5	0	0	0.5
EBSS, no glucose	Log_{10} virus per ml	0	0	0	0	0	0	0	0	0	0	0
	Control cells	100	100	50	0	0	0	0	0	0	0	0

[a] Eagle's basal medium.
[b] Cells of uninfected monolayers maintained on same test media as infected cells.
[c] Per cent of cells of uninfected monolayers which appeared viable.
[d] Earle's balanced salt solution.

viral penetration. Virus penetrated the cells as readily in the deficient medium as in the complete medium (Table 1). Similar experiments using medium without antiserum demonstrated that the adsorption rates in complete and deficient media were substantially equal.

These data also established that inactivation of virus did not occur more rapidly in deficient medium than in complete medium. Had this been the case, fewer plaques would have been expected to develop on monolayers which were exposed to deficient medium during adsorption than on monolayers which were supplied with complete medium during the same periods of time.

Viral production in glucose-deficient and glutamine-deficient media. Glucose and glutamine were examined separately for their effects on viral proliferation. In a representative experiment, tubes of monolayers were divided into groups and each group received one of the following: complete EBM, EBM deficient in both glucose and glutamine, EBM deficient in glucose, EBM deficient in glutamine, EBSS with the usual 0.1% glucose and to which 2 mM glutamine had been added, or glucose-free EBSS. After 12-hr incubation for depletion of endogenous cellular reserves of the nutrients under investigation, virus was allowed to adsorb onto the cells. The appropriate test media, which had been removed from the monolayers and saved during the adsorption period, were placed again on the washed cells after viral adsorption. Use of such "conditioned" experimental media, rather than fresh media, decreased the likelihood of fortuitous addition of small amounts of the nutrients under test. Samples removed at 8-hr intervals over an 80-hr period after inoculation were titrated for viral content (Table 2).

TABLE 3. *Student's t-distribution[a] analysis of virus production by HeLa cells in Eagle's basal medium with and without glutamine*

Expt. no.	Log_{10} $TCID_{50}$ of virus per ml of sample[b]		Difference $D = X_1 - X_2$	Deviation $d = D - \bar{d}$	Squared deviation d^2
	Deficient medium X_1	Complete medium X_2			
1	3.0	2.5	0.5	−1.2	1.44
2	3.0	2.0	1.0	−0.7	0.49
3	2.5	0.5	2.0	0.3	0.09
4	3.6	1.0	2.6	0.9	0.81
5	4.8	2.5	2.3	0.6	0.36
6	4.0	2.2	1.8	0.1	0.01
Totals			10.2	0	3.20
Mean			$\frac{10.2}{6} = 1.7 = \bar{d}$		$\frac{3.20}{5}$[c]

[a] Computations patterned after and symbols taken from Snedecor (1956).

[b] Samples taken at 72 hr.

[c] $\frac{3.20}{5} = 0.64 = sD^2$; $s\bar{d}^2 = \frac{0.64}{6} = 0.17$; $s\bar{d} = 0.41$; $t_{(5)} = \frac{1.7-0}{0.41} = 4.1$, P less than 0.01.

No virus was detected in the glucose- and glutamine-deficient EBM, in the complete EBSS, or in the glucose-free EBSS. Virus was not found in the glucose-deficient EBM before the 64th hr, and the titer in this medium never exceeded 100 $TCID_{50}$ per ml. In contrast, viral synthesis with glutamine-deficient EBM was as early and approximately as great as with the complete medium. It should be emphasized that hemocytometer counts revealed the numbers of cells in uninoculated tubes with the various test media to be approximately equal during a time when profound differences were present among these media with respect to viral production in the inoculated tubes. Later, the numbers of cells decreased in deficient media.

More virus was produced by cells in glutamine-deficient EBM which contained serum than by cells in complete medium. Representative data from several experiments are shown in Table 3. In only one of numerous experiments was the amount of virus approximately the same with the two media. The results found in this unexplained exception appear in Table 2. Upon application of Student's *t*-test to the results recorded in Table 3, it was found that increase in viral production as large as was observed upon limitation of glutamine could be expected to occur less than one time in a hundred by chance alone. Results shown in Table 2 were not included in this analysis, since the cells of the experiments shown in Table 3 were maintained in experimental media from 5 to 7½ hr before exposure to virus. The period of conditioning in the experiment recorded in Table 2 was 12 hr.

Viral production in various concentrations of glutamine. Experimental results to this point

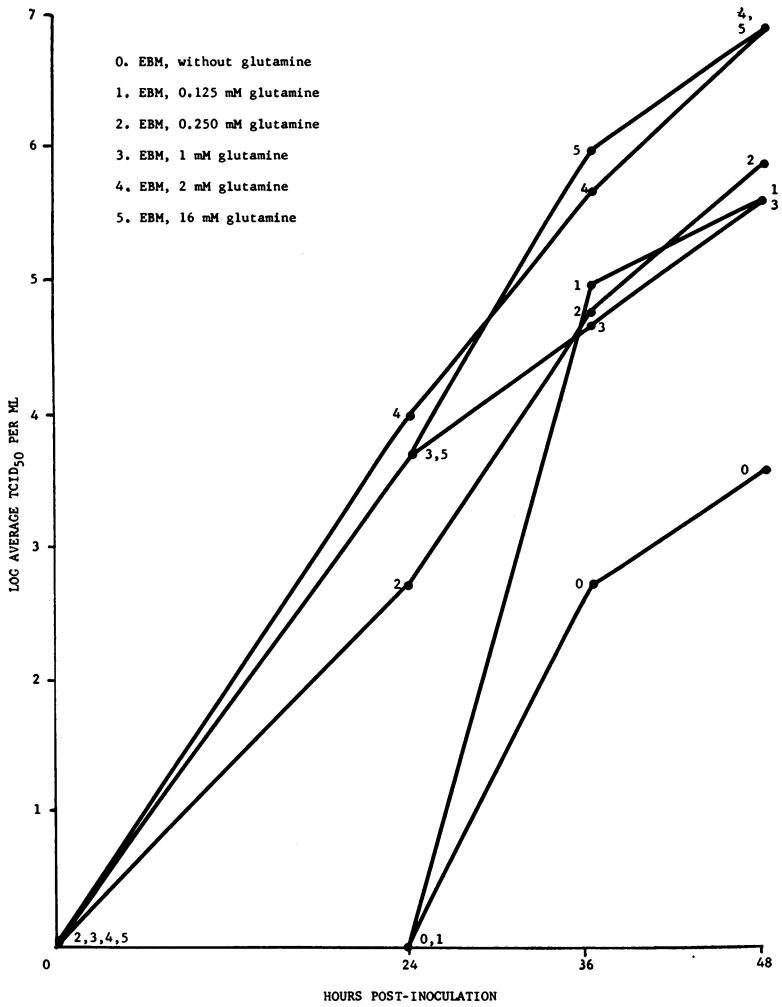

FIG. 10. *Virus production in Eagle's basal medium (EBM) containing vary ing concentrations of glutamine and no serum.*

were compatible with the hypothesis that the presence of glutamine above a certain concentration might suppress synthesis of virus. To test this possibility, viral replication by cells in EBM which contained various concentrations of glutamine was determined. The exact amounts of glutamine present were known because serum, which contains this amide, was not incorporated into the medium. Nine concentrations of glutamine were tested, but only the data obtained from six are shown in Fig. 10. The results with 0.5 mM of glutamine were essentially the same as those recorded for 0.25 mM, and the data with 4 and 8 mM of glutamine were approximately the same as those shown for 16 mM. It is apparent that there was no diminution in the amount of virus produced in the presence of increasing concentrations of glutamine in serum-free EBM. An additional observation was that virus was produced after an increased period of time and only in reduced amounts in a medium which lacked both serum and glutamine.

DISCUSSION

This investigation demonstrated that HeLa cells in the presence of EBM which lacked the amounts of glucose and glutamine routinely incorporated into this medium failed to syn-

thesize appreciable amounts of herpes simplex virus. Virus was produced by cells in complete medium and not by those in deficient medium during the time that the numbers of cells in the two media were approximately equal. Therefore, failure was not attributable to lack of survival of cells in the deficient medium. The latter medium allowed virus to become adsorbed onto cells and to penetrate them as rapidly as did complete medium. It is suggested by these observations that the curtailment of viral production in the deficient medium may reflect a failure of intracellular synthesis.

Likewise, HeLa cells in the presence of EBM which was deficient only in an oxidizable carbohydrate failed to produce virus. In addition to serving as energy source for virus production, glucose may yield compounds through intermediary metabolism which are required directly or indirectly for synthesis of virus. Darnell and Eagle (1958) found that among several carbohydrates only fructose successfully replaced glucose in EBM for the replication of poliovirus by HeLa cells. The degree of specificity of the requirement for glucose in the multiplication of herpes simplex virus has not been investigated in the present study.

In contrast to the effect of glucose-deficient medium, production of virus was enhanced when the amount of glutamine present was limited to that found in the serum component of the medium.

Statistical analysis indicated that the magnitude of the stimulation of viral production with the glutamine-deficient medium could be expected to occur by chance alone less than one in a hundred times. This effect awaits explanation. Eaton et al. (1951) demonstrated that multiplication of myxoviruses in chick embryo tissue culture was inhibited by the presence of basic amino acids. A similar role was not played by glutamine *per se* in the present investigation, since high concentrations of the amide did not decrease synthesis of virus in serum-free EBM.

The presence in calf serum of a viral inhibitor which required glutamine for activity might explain the suppression of viral replication when both glutamine and serum were present. Stimulation of viral synthesis by glutamine occurred only in the absence of serum. However, the presence of such an inhibitor is as yet unproven, and other explanations are possible.

This investigation has not shown that virus is produced in the complete absence of glutamine. In addition to the amounts of glutamine which may have been contributed by the serum used in most experiments, amounts of this amide may arise from intermediary cellular metabolism. Additional glutamine may become available to surviving cells through degradation products of monolayer cells which die during the course of an experiment. The study has demonstrated that modification of Eagle's basal medium by omission of the glutamine component enhances the replication of herpes simplex virus by HeLa cells, provided that serum is present.

ACKNOWLEDGMENTS

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