

## THE NUTRITIONAL REQUIREMENTS FOR THE PROPAGATION OF POLIOMYELITIS VIRUS BY THE HeLa CELL

By HARRY EAGLE, M.D., AND KARL HABEL, M.D.

*(From the Section on Experimental Therapeutics and the Section on Basic Studies,  
Laboratory of Infectious Diseases, National Institute of Allergy and  
Infectious Diseases, National Institutes of Health,\* Bethesda)*

(Received for publication, May 3, 1956)

The specific amino acid, vitamin, and salt requirements of the HeLa cell have been described in previous papers (1-5). On the omission from the medium of a single essential amino acid, salt or vitamin, or of glucose, the cells stopped multiplying and eventually died. Depending on the specific growth factor, from 1 to 14 days were required for the cytopathogenic effects of the specific deficiency to become fully evident. In contrast, the maximum evolution of poliomyelitis virus from HeLa cultures after a suitably large viral inoculum was usually observed in 24 to 48 hours. It thus became possible to determine which of the nutrients known to be necessary for the survival and growth of the cell were essential also for the propagation of poliomyelitis virus.

As will be here shown, the omission from the medium of vitamins, 12 of the 13 essential amino acids, and of protein had only a minor effect on the amount of the virus formed, even when the cells had been deprived of these factors for 12 hours prior to inoculation. Under the conditions of the present experiments, only 2 components of the medium other than salts were demonstrably essential for the optimal propagation of poliomyelitis virus: glucose and glutamine.

### *Methods*

Cultures of the HeLa cell were grown as a cellular layer adherent to the flat surface of a glass container, overlaid with a medium embodying the essential metabolites, each in the concentration previously found to be optimal for growth (1-5), and supplemented with 10 per cent human serum. Prior to inoculation with virus, the cultures were washed 2 or 3 times with medium free of human serum, and overlaid with an experimental medium deficient in 1 or more of the metabolites under study. When serum was needed, 3 to 5 per cent whole or dialyzed horse serum was added. After varying periods of incubation to deprive the cells of the specific metabolite(s) omitted from the medium, the viral inoculum was added, usually in 1/10 to 1/20 the volume of the fluid overlay. In most of the experiments a type II strain (No. 6468)<sup>1</sup> was used; a few of the experiments were carried out with the Saukett and Mahoney

\* Public Health Service, United States Department of Health, Education, and Welfare.

<sup>1</sup> Isolated in monkey kidney tissue culture from a human case of poliomyelitis by A. I. Shelokov and K. Habel.

strains. After  $\frac{1}{2}$  to 2 hours at 37°C. to allow fixation of virus, the cultures were washed 4 to 5 times, either with the appropriate medium, or with Earle's balanced salt solution containing neither glucose nor glutamine, and the experimental medium was added. The fluid was then replaced at varying times, and the *successive* harvests titrated for viral content in tube cultures of monkey kidney epithelium. In other experiments, however (*e.g.* 5 and 6 of Table II), the fluid was not replaced, and the medium was harvested when the cytopathogenic effect was complete.

Preliminary experiments indicated the need for a viral inoculum as free as possible from amino acids, vitamins, glucose, glutamine, and serum protein, in order to minimize the possible uptake of these compounds by the cell during the period of incubation with virus. Accordingly, virus inoculum was prepared by adding the standard viral suspension at a final concentration of  $10^{-8}$  to bottles previously washed in Earle's balanced salt solution containing neither glucose nor glutamine, and overlaid with the same solution. In this medium, smaller amounts of virus were produced ( $10^8$  to  $10^5$  infectious doses per ml.), but the harvest contained only minute amounts of amino acids and no demonstrable glucose, and could be used even at a 10 per cent level with no danger of feeding the previously starved cells with these metabolites.

Quantitation of the virus contained in the various harvests was carried out in monkey kidney epithelial cells obtained by trypsinization of fresh monkey kidney and grown as monolayer cells in 16 x 100 mm. roller tubes. Serial 10-fold dilutions of harvested fluids were made in Hanks's balanced salt solution, and 5 tubes were inoculated with 0.1 ml. of each of at least 4 dilutions. Cultures were maintained without change of medium in Earle's salt solution to which had been added either 25 per cent serum ultrafiltrate and 1 per cent horse serum, or 0.5 per cent lactalbumin hydrolysate and 1 per cent calf serum. The roller tubes were read microscopically at 2 or 3 days, and again at 6 to 7 days following inoculation. The  $ID_{50}$  dose (concentration necessary to produce a definite cytopathogenic effect in half the tubes) was determined by the Reed and Muench method, and the results are expressed in the tables and figures as the number of  $ID_{50}$  doses per milliliter of fluid tested.

#### EXPERIMENTAL

##### *A. The Importance of Glucose and Glutamine for the Elaboration of Poliomyelitis Virus by HeLa Cell*

Four "basal" media were prepared consisting of (a) glucose-free balanced salt solution; (b) salts and dialyzed serum; (c) salts, dialyzed serum, 12 of the 13 essential amino acids (omitting only glutamine), and 7 essential vitamins; and (d) the foregoing medium (c) further supplemented with 6 non-essential amino acids, purines, pyrimidines and  $NH_4Cl$  (*cf.* Table I). To each of these 4 basal media was added either (1) no supplement; (2) 5 mM glucose; (3) 2 mM glutamine; or (4) both glucose and glutamine, to give 16 media varying in deficiency in essential and non-essential growth factors.

Fully grown HeLa cultures were washed twice with Earle's balanced salt solution containing neither glucose nor glutamine, and overlaid for 12 hours with one or another of the 16 media described above. Fresh medium of the same composition was then added, and the cultures were inoculated with 1/10 volume of the specially prepared viral inoculum (*cf.* text above). After 1 to 2 hours at 37°C., the cultures were washed, re-fed with the same medium, and the viral harvests collected as described in a previous section. The results of a number of experiments are summarized in Tables II and III, and a single experiment (No. 4) is illustrated in Fig. 1.

As there shown, glucose, glutamine and salts were both sufficient and necessary for the propagation of poliomyelitis virus by the HeLa cell. The absence from the medium of all amino acids except glutamine, of serum protein, vitamins, purines, pyrimidines or  $\text{NH}_4^+$  did not greatly affect the viral output, even if the cells had been deprived of these factors for 12 hours prior to inoculation. On the other hand, if the cells were depleted of their glucose and glutamine reserves, their capacity to form virus was greatly reduced, and in some experiments almost totally abolished.

TABLE I  
*Composition of "Basal" Media Used in Present Experiments*

| A      | B                       | C   | D  |
|--------|-------------------------|---|--|
| Salts* | Salts<br>Serum protein† | Salts<br>Serum protein<br>12 essential amino acids§<br>7 essential vitamins | Salts<br>Serum protein<br>12 essential amino acids<br>7 essential vitamins<br>6 non-essential amino acids¶<br>4 purines**<br>4 pyrimidines‡‡<br>$\text{NH}_4\text{Cl}$ (0.05 mM) |

\* Earle's balanced salt solution.

† Supplied as 3 to 5 per cent dialyzed horse serum.

§ Arginine, cystine, histidine, isoleucine, lysine, leucine, methionine, phenylalanine, threonine, tryptophan, tyrosine, valine, each at the concentration providing for maximum growth of the HeLa cell (2).

|| Choline, folic acid, nicotinamide, pantothenate, pyridoxal, riboflavin, thiamin, each at a concentration providing maximum growth of the HeLa cell (3).

¶ Alanine, aspartic acid, glutamic acid, glycine, proline, serine, each at 0.1 mM.

\*\* Adenine, guanine, hypoxanthine, xanthine, each at 0.05 mM.

‡‡ Cytosine, orotic acid, thymine, uracil, each at 0.05 mM.

(a) No matter what else was present in the basal medium, if the cells were depleted of glucose and glutamine for 12 hours, prior to inoculation, only minute amounts of virus were formed. Even if the medium contained an otherwise complete complement of salts, vitamins, amino acids, purines, pyrimidines,  $\text{NH}_4^+$ , and serum protein, no more virus appeared than in a simple salt solution (*cf.* left-hand portion of Fig. 1 and column (1) of Table II). The maximum titers of virus formed by such glucose- and glutamine-deficient cells varied in individual experiments from  $10^{1.7}$  to  $10^{4.0}$  tissue culture doses per ml., averaging  $10^{2.6}$ . Despite the low yields of virus the cytopathogenic effect of viral inoculation was much more rapid in these cultures than normally. Ackermann, Rabson, and Kurtz (6) have pointed out a similar lack of correlation between cellular injury and viral increase. A contributory factor to the rapid deterioration in the present experiments was the harmful effect of the glucose and glutamine

deficiency itself, evident in control uninoculated cultures; but the superimposed toxic effect of viral inoculation was nevertheless rapid and pronounced.

TABLE II  
*The Effect of the Composition of the Medium on the Yield of Type II Poliomyelitis Virus from HeLa Cells*

| Composition of basal medium†  | Exp. No. | Viral titer* when basal medium was supplemented with |                           |                                 |                                    | Viral titer in complete growth medium |
|---|----------|--|---------------------------|---------------------------------|------------------------------------|---------------------------------------|
|   |          | (1)<br>0   | (2)<br>Glucose§<br>(5 mM) | (3)<br>Gluta-<br>mine<br>(2 mM) | (4)<br>Glucose<br>+ gluta-<br>mine |                                       |
| Salts only  | 1        | 4.0  | 5.6                       | 6.8                             | 8.0                                | 8.5                                   |
|   | 2        | 1.7  | 3.5                       | 4.7                             | 4.5                                | 5.6                                   |
|   | 3        | 2.5  | 5.5                       | 6.0                             | 5.8                                | 7.7                                   |
|   | 4        | 2.0  | 4.4                       | 5.6                             | 6.3                                | 7.5                                   |
|   | 5        | —  | —                         | —                               | 6.4                                | 7.3                                   |
| Salts + serum protein   | 3        | 3.5  | 4.8                       | 7.0                             | 7.0                                | 7.7                                   |
|   | 4        | 2.6  | 4.8                       | 6.0                             | 7.5                                | 7.5                                   |
|   | 5        | 2.2  | 4.4                       | 6.5                             | 7.7                                | 7.3                                   |
| Salts, essential amino acids, vitamins, serum protein   | 2        | 2.4  | —                         | —                               | 4.6                                | 5.6                                   |
|   | 3        | 2.8  | 5.0                       | 6.6                             | 7.5                                | 7.7                                   |
| Salts, essential and non-essential amino acids, vitamins, purines, pyrimidines, and serum protein | 4        | 3.0  | 6.5                       | 7.3                             | 7.5                                | 7.5                                   |
|   | 5        | —  | 6.5                       | 6.3                             | 8.0                                | 7.3                                   |
| Average increase in log virus titer as compared with glucose- and glutamine-deprived cells....    | —        | —  | 2.2                       | 3.6                             | 4.6                                | 4.6                                   |
| Average decrease in log virus titer as compared with complete growth medium.....                  | —        | 4.6  | 2.3                       | 1.15                            | 0.3                                | —                                     |

\* Log of ID<sub>50</sub> tissue culture doses per ml. In Experiments 1 to 4, in which the culture fluid was replaced at varying intervals after inoculation, the titer given is the maximum observed in the several harvests.

† Cf. Table I.

§ Cf. pages 278-279 regarding slow elaboration of virus in medium supplemented with glucose, but lacking glutamine.

|| Medium containing 13 essential amino acids, 7 essential vitamins, glucose, and 5 per cent whole horse serum.

(b) The presence of glucose at a concentration of 5 mM (approximately 0.1 per cent) partially preserved the capacity of the cell to elaborate virus, the viral titers ranging between 100- and 1000-fold greater than those obtained in the glucose- and glutamine-free medium, the increase averaging 170-fold (cf. columns 1 and 2 in Table II and Fig. 1). The glucose effect was observed

TABLE III  
*The Effect of the Composition of the Medium on the Rate of Elaboration of Poliomyelitis Virus by the HeLa Cell*

|                       | Supplement          | Composition of basal medium* | Time in hrs. after inoculation                                |     |      |      |      |      |  |
|-----------------------|---------------------|------------------------------|---|-----|------|------|------|------|--|
|                       |                     |                              | 12  | 18  | 24   | 36   | 48   | 60   |  |
|                       |                     |                              | Viral titer in supernatant fluid harvested at indicated time† |     |      |      |      |      |  |
| Exp. No. 3            | 0                   | Salt solution                | 3.8   | —§  | 2.5  |      |      |      |  |
|                       |                     | Salt + serum protein         | 2.8   | 3.5 | 3.5  | 3.2§ | 2.3  |      |  |
|                       |                     | Growth medium                |   | 2.5 | 2.8  | <2§  |      |      |  |
|                       | Glucose             | Salt solution                | 4.6   | 5.5 | 5.4§ |      |      |      |  |
|                       |                     | Salts + serum protein        |   |     | 4.2  | 4.8  | 4.8§ |      |  |
|                       |                     | Growth medium                |   |     | 4.4  | 3.5  | 4.7  | 5.0§ |  |
|                       | Glutamine           | Salt solution                | 4.8   | 6.0 | 5.8§ |      |      |      |  |
|                       |                     | Salts + serum protein        | 4.7   | 5.5 | 5.8  | 7.0§ |      |      |  |
|                       |                     | Growth medium                |   | 4.8 | 5.8  | 6.6§ |      |      |  |
|                       | Glucose + glutamine | Salt solution                | 5.2   | 5.8 | 5.5§ |      |      |      |  |
| Salts + serum protein |                     | 5.3                          | 5.5   | 5.6 | 7.0§ |      |      |      |  |
| Growth medium         |                     |                              | 5.8   | 6.8 | 7.5§ |      |      |      |  |
|                       | Control             |                              |   | 5.5 | 7.7§ |      |      |      |  |
| Exp. No. 4            | 0                   | Salt solution                | 2§  |     | 1.4  |      |      |      |  |
|                       |                     | Salts + serum protein        | 1.7   |     | 2.6§ | 1.6  |      |      |  |
|                       |                     | Growth medium                | 1.6   |     | 2.3  | 3.0§ |      |      |  |
|                       | Glucose             | Salt solution                | 3.6   |     | 4.4§ | 4.4  |      |      |  |
|                       |                     | Salts + serum protein        | 3.3   |     | 4.5  | 4.8§ |      |      |  |
|                       |                     | Growth medium                | 2.6   |     | 3.7  | 6.5¶ |      |      |  |
|                       | Glutamine           | Salt solution                | 4.4   |     | 5.6§ |      |      |      |  |
|                       |                     | Salts + serum protein        | 4.3   |     | 4.6  | 6.0§ |      |      |  |
|                       |                     | Growth medium                | 4.3   |     | 5.4  | 7.3§ |      |      |  |
|                       | Glucose + glutamine | Salt solution                | 4.5   |     | 6.6§ | 6.3  |      |      |  |
|                       |                     | Salts + serum protein        | 4.8   |     | 5.6  | 7.5§ |      |      |  |
|                       |                     | Growth medium                | 4.6   |     | 5.5  | 7.5§ |      |      |  |
|                       | Control             | 5.0                          |   | 6.5 | 7.5§ |      |      |      |  |

\* Cf. Tables I and II for composition of growth medium in individual experiments.

† Fresh medium was added to the flask at the time of each harvest.

§ = more than 75 per cent of cells either off the glass or showing marked cytopathogenic effects of viral infection.

|| 14 hour harvest.

¶ Less than 75 per cent degenerated even after 36 hours.

whether the cells were incubated in a simple glucose-salt solution, or whether glucose was added to media containing all the essential growth factors except glutamine, and in some experiments, the non-essential amino acids, purines,

pyrimidines, and  $\text{NH}_4^+$  as well. Kuwata and Shiba (7) have shown a similar stimulatory effect of glucose on the propagation of ornithosis virus in surviving chick embryonic tissue. The provision of glucose alone did not, however, restore the virus-propagating capacity of the cells to its normal level.

The delayed elaboration of virus in media supplemented with glucose, but lacking glutamine, is discussed in the following section.

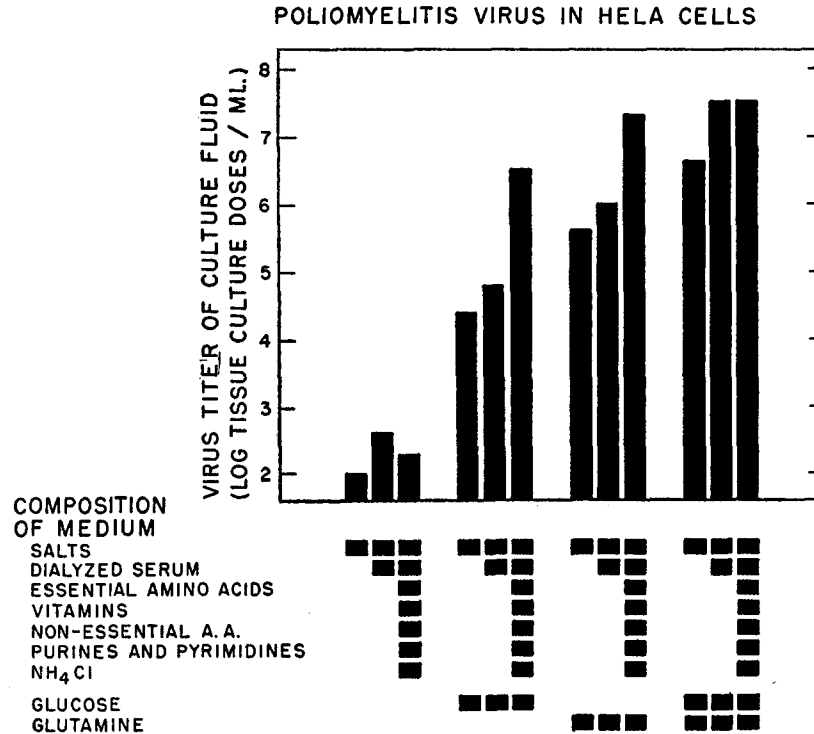


FIG. 1. Illustrating the marked effects of glucose and of glutamine on the production of poliomyelitis virus by the HeLa cell, and the relatively minor effect of other components of the medium.

(c) Glutamine alone was even more effective than glucose in promoting the formation of virus (*cf.* columns 1 and 3 in Table II, and Fig. 1). When added at a concentration of 2 mM, glutamine effected an average 4000-fold increase (3.6 logs) in the amount of virus elaborated, the viral titers attaining levels within 0.2 to 2 logs of those observed in a complete growth medium, containing glucose as well.

(d) When both glucose and glutamine were present in the medium, the amount of virus formed was increased an average of 4.6 logs (40,000-fold) beyond the amounts formed by cells depleted of their glucose and glutamine

reserves. Given glucose and glutamine, it made but little difference in the amounts of virus formed whether the medium additionally contained only salts, or a complete supplement of amino acids, vitamins, purines, pyrimidines, serum protein, and  $\text{NH}_4^+$  (*cf.* Fig. 1). In the simplest medium, containing only glucose, glutamine and salts, the amounts of virus formed averaged 1 log less than in a complete growth medium, which in addition included the essential amino acids, vitamins and whole serum. This difference was, however, not due to a decreased rate of viral elaboration (*cf.* following section), but rather to the fact that in the glucose-glutamine-salt medium the cells degenerated before the maximum amounts of virus had been formed. When cell degeneration was only slightly delayed, as by the addition of serum protein, the amounts of virus formed were essentially equal to those formed in a complete growth medium (*cf.* second section of Table II).

*B. The Rate of Viral Propagation as a Function of the Composition of the Basal Medium*

The striking effect of glucose and of glutamine in preserving the virus synthesizing capacity of the HeLa cell, even when added to simple salt solution (top section of Table II) was all the more surprising in view of the fact that in such a manifestly deficient medium the cells had already undergone profound degenerative changes by the time of their inoculation with virus. Indeed, the virus-inoculated cells deteriorated in such salt solutions only slightly faster than control, uninoculated, cultures. Such rapidly deteriorating cultures nevertheless produced virus actively after inoculation with poliomyelitis virus. Experiments to show the rate of virus formation as a function of the composition of the basal medium are summarized in Table III and in Fig. 2. The effects of glucose and glutamine depletion on the amounts of virus elaborated are again evident. It is, however, to be noted that with a given glucose or glutamine supplement, just as much virus was formed in the first 12 to 24 hours by cells overlaid with a simple salt solution (curves  $\circ-\circ$  in Fig. 2) as was formed by cells maintained in a medium containing all the factors necessary for cellular growth, and a number of stimulatory non-essential factors as well ( $\bullet-\bullet$ ). Indeed, in some experiments, the virus initially evolved even more rapidly<sup>2</sup> in cells overlaid with salt solution than when the more complete media were used. The larger yields of virus ultimately obtained in the more complex growth media were associated with, and perhaps due to, the longer survival of the cells (*cf.* † in Fig. 2), and not necessarily to a more active initial biosynthesis of virus.

The elaboration of virus, and its cytopathogenic effect, were significantly

<sup>2</sup> The amounts of virus present in these experiments at the earliest time period tested were several logs greater than those present in the last wash fluid after inoculation, and therefore reflect actual viral multiplication rather than residual inoculum.

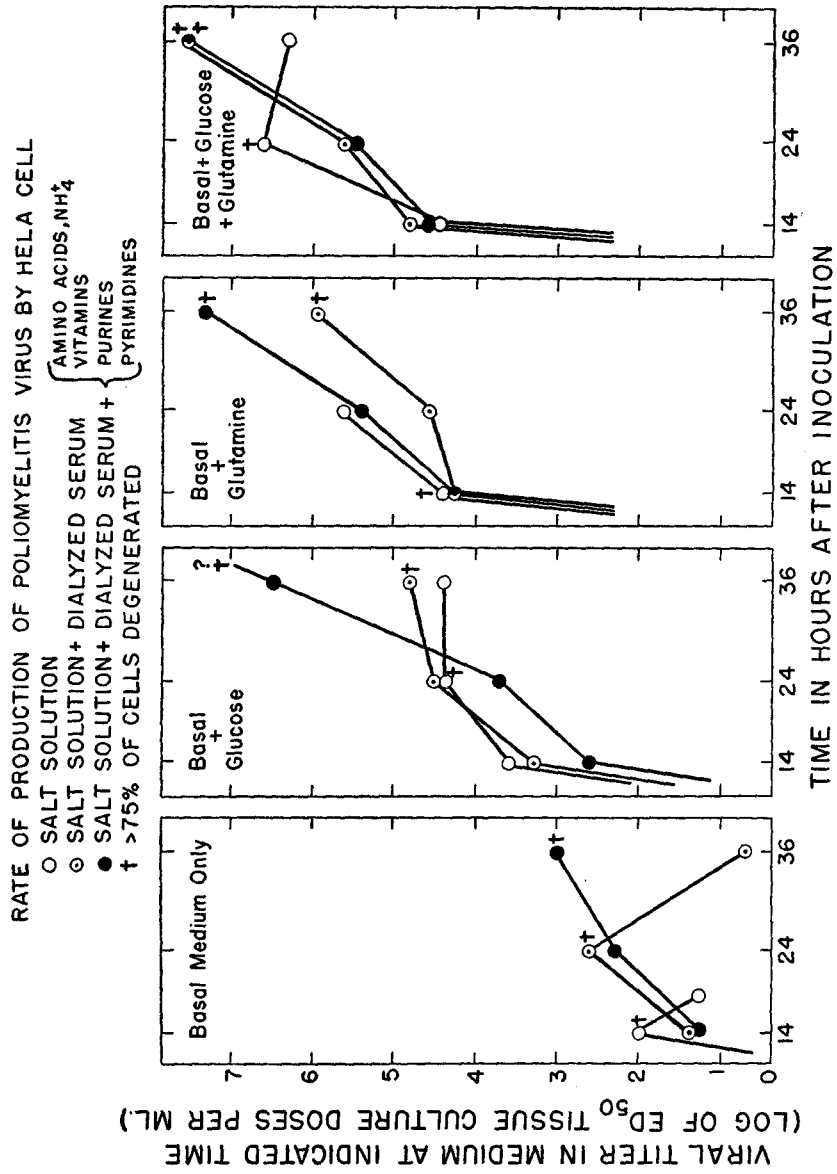


FIG. 2. The rate of production of poliomyelitis virus by the HeLa cell as a function of the composition of the medium. (The marked effects of glucose and glutamine, as contrasted with the minor effects of the other components of the medium. Experiment 4 of Tables II and III.)



delayed in cells depleted of glutamine, but supplied with all other factors necessary for growth, including glucose. Illustrative experiments are summarized in Table IV. The aggregate amounts of virus ultimately formed in such media by glutamine-deficient cells were usually within 1 to 2 logs of the amount produced with both glucose and glutamine available; but the rate of virus formation was significantly reduced, and the survival time of the cells correspondingly prolonged.

TABLE IV  
*The Delayed Cytopathogenic Effect and Delayed Elaboration of Virus in Cells Depleted of Glutamine, but Supplied with All Other Factors Necessary for Growth, Including Glucose*

| Exp. No. | Basal medium                                  | Glucose | Glutamine | Approximate per cent of cells degenerated (and viral titer), after |             |               |          |               |               |
|----------|---|---------|-----------|--|-------------|---------------|----------|---------------|---------------|
|          |   |         |           | 14 to 18 hrs.  | 24 hrs.     | 32 to 40 hrs. | 48 hrs.  | 60 to 72 hrs. | 90 to 96 hrs. |
| 3        | Growth medium*                                | 0       | 0         | 35 (2.5)   | 50-75 (2.8) | 90 (1.8)      | 100      |               |               |
|          |   | +       | 0         | 0 (4.2)  | 15 (4.4)    | 25 (3.5?)     | 25 (4.7) | 30 (5.0)      |               |
|          |   | 0       | +         | ± (4.8)  | 50-75 (5.8) | 100 (6.0)     |          |               |               |
|          |   | +       | +         | 25 (5.8)   | 50-75 (6.8) | 100 (7.5)     |          |               |               |
| 4        | Growth medium + non-essential growth factors† | 0       | 0         | 50 (1.6)   | 50 (2.3)    | 75-90 (3.0)   |          |               |               |
|          |   | +       | 0         | 0 (2.6)  | 0 (3.7)     | 25-35 (6.5)   |          |               |               |
|          |   | 0       | +         | ± (4.3)  | 35 (5.4)    | >90 (7.3)     |          |               |               |
|          |   | +       | +         | 0 (4.6)  | 25 (5.5)    | 90 (7.5)      |          |               |               |
| 5        | Growth medium + non-essential growth factors  | 0       | 0         | 90   | >90         | 100 (-)       |          |               |               |
|          |   | +       | 0         | ±  | 10          | 35 (4.5)      |          | 50 (5.5)      | 90 (6.5)      |
|          |   | 0       | +         | 15-25  | 90          | >90 (6.3)     |          |               |               |
|          |   | +       | +         | ±  | 35          | >90 (8.0)     |          |               |               |
| 6        | Growth medium + non-essential growth factors  | 0       | 0         | 100  |             | - (4.0)       |          |               |               |
|          |   | +       | 0         | 15   | 25          | 50 (-)        |          | 90 (6.6)      | 100 (7.0)     |
|          |   | 0       | +         | 90   | 100         | - (6.5)       |          |               |               |
|          |   | +       | +         | 25-35  | 50-75       | 100 (7.5)     |          |               |               |

\* Essential amino acids and vitamins, salts, and dialyzed serum (*cf.* Table I).

† Essential amino acids and vitamins, salts, dialyzed serum, non-essential amino acids, purines, pyrimidines, and NH<sub>4</sub>Cl (*cf.* Table I).

### C. The Effect of Glucose and Glutamine on the Fixation of Poliomyelitis Virus by the HeLa Cell

Experiments were undertaken to determine whether glucose and glutamine were necessary for the fixation of poliovirus by the HeLa cell, or whether they were necessary instead for the propagation of virus after it had been fixed.

Cells previously washed and starved for 6 to 12 hours, either in Earle's salt solution, or salt solution supplemented with 4 per cent dialyzed serum (but in either case containing no added glucose or glutamine), were inoculated with a viral suspension similarly devoid of added glucose or glutamine. (a) After incubation for 1 to 1½ hours at 37°C., the supernatant fluid was removed, the flasks were washed in Earle's salt solutions 5 times, and refed with a medium containing glucose and glutamine. (The final wash titrated at <10<sup>6</sup> to 10<sup>1.5</sup>, indicative of the essentially complete removal of free, unbound virus.) (b) In 3 experiments, a monkey anti-

serum titrating at 1:1000 vs. 100 tissue culture doses of virus was added to the washed cultures at a final antiserum concentration of 1:100 in order to neutralize any residual traces of virus, prior to the addition of glucose and glutamine. The medium containing antiserum was removed after 1 hour at 37°C., the flask washed and refed with a medium containing glucose and glutamine. (c) In 2 experiments, the glucose and glutamine were not added immediately

TABLE V

*Showing That the Essential Role of Glucose and Glutamine in the Formation of Poliomyelitis Virus by the HeLa Cell is Not in the Fixation of Virus by the Cells*

| Exp. No. | Time for which cells were starved in salt solution prior to inoculation | Additional time for which cells were kept in salt solution prior to addition of glucose and glutamine* | Viral harvest          |        | Titer in control cells kept in growth medium throughout |
|----------|---|--|------------------------|--------|---|
|          |   |  | Time after inoculation | Titer† |   |
| 2        | 12<br><i>hours</i>  | 0<br><i>hours</i>  | 24<br><i>hours</i>     | 5.4    | 5.6   |
|          |   | 2  | 24                     | 5.0    |   |
|          |   | 4  | 24                     | 5.2    |   |
|          |   | 6  | 24                     | 4.3    |   |
|          |   | 12   | 24                     | 1.6    |   |
| 4        | 11  | 0  | 36                     | 6.5    | 7.5   |
| 4 a§     | 11  | 0  | 36                     | 5.2    | 7.5   |
| 5§       | 7   | 0  | 40                     | 7.5    | 7.3   |
| 6§       | 6   | 6  | 40                     | >8.4   | 8.0   |

\* In experiments 4, 4 a, and 5, glucose and glutamine were added in simple salt solution; in experiments 2 and 6, glucose and glutamine were added in complete growth medium. There was no important difference in viral output in these experiments.

† Log ED<sub>50</sub> tissue culture doses per milliliter.

§ In all the experiments, the cultures were washed with salt solution 4 to 5 times after inoculation, and the titer of the last wash was 10<sup>0</sup> - 10<sup>1.5</sup>. In experiments 4 a, 5, and 6, any residual traces of free uncombined virus were neutralized by the addition for 1 hour of a type II monkey antiserum at a dilution of 1:100.

|| In this experiment, 5 per cent dialyzed horse serum was added to the simple glucose- and glutamine-free salt solution.

after the removal of excess virus, but only after an additional period of starvation in the glucose- and glutamine-free medium.

The results of these several experiments are summarized in Table V. As there shown (*cf.* last 2 columns), even when glutamine and glucose were added after inoculation, and after the residual unbound virus had been removed both by thorough washing and by the addition of antiserum, the addition of glucose and glutamine initiated the formation of normal amounts of virus. This propagation of virus could be induced even when glucose and glutamine were added 6 hours after inoculation (*cf.* experiments 2 and 6). Up to that time.

the addition of these 2 compounds usually caused a rapid reversal of the microscopic degenerative changes produced by incubation in the deficient medium. With longer preliminary incubation, however, no such reversal oc-

TABLE VI

*The Effects of Glucose and Glutamine on the Elaboration of Poliomyelitis Virus by HeLa Cells Not Previously Depleted of Their Glucose and Glutamine Reserves*

| Basal Medium*   | Glucose,<br>5 mM | Gluta-<br>mine,<br>2 mM | Viral<br>titer† | Increase in viral titer<br>effected by addition of: |                |                             |
|---|------------------|-------------------------|-----------------|---|----------------|-----------------------------|
|   |                  |                         |                 | Glucose   | Gluta-<br>mine | Glucose<br>+ glu-<br>tamine |
| Salt solution   | 0                | 0                       | 4.5             |   |                |                             |
|   | +                | 0                       | 6.5             | 2.0   | 3.0            | 3.3                         |
|   | 0                | +                       | 7.5             |   |                |                             |
|   | +                | +                       | 7.8             |   |                |                             |
| Salt solution with 4 per cent dialyzed<br>horse serum   | 0                | 0                       | 4.4             |   |                |                             |
|   | +                | 0                       | 6.8‡            | 2.4   | 3.1            | 3.8                         |
|   | 0                | +                       | 7.5             |   |                |                             |
|   | +                | +                       | 8.2             |   |                |                             |
| Salt solution, 4 per cent dialyzed horse<br>serum, essential amino acids, and<br>vitamins   | 0                | 0                       | 3.6             |   |                |                             |
|   | +                | 0                       | 6.0‡            | 2.4   | 3.9            | 5.1                         |
|   | 0                | +                       | 7.5             |   |                |                             |
|   | +                | +                       | 8.7             |   |                |                             |
| Salt solution, 4 per cent whole serum,<br>essential and non-essential amino<br>acids, vitamins, purines, pyrimidines,<br>and NH <sub>4</sub> Cl | 0                | 0                       | 4.0             |   |                |                             |
|   | +                | 0                       | 6.8‡            | 2.8   | 3.6            | 4.7                         |
|   | 0                | +                       | 7.6             |   |                |                             |
|   | +                | +                       | 8.7             |   |                |                             |
| Control (4 per cent whole horse serum,<br>amino acids, vitamins, and salts)   | +                | +                       | 8.4             | —   | —              | —                           |

\* Cf. Table I for details of composition.

† Log of tissue culture effective doses per milliliter in culture fluid after 45 hours. At this time, more than 90 per cent of the cells had degenerated in all media except the ones indicated with an ‡ in the body of this Table containing glucose, but lacking glutamine.

curred, and the effects of glucose and glutamine on virus formation were correspondingly less evident (cf. experiments 2 and 6 of Table V).

*D. The Production of Poliomyelitis Virus by HeLa Cells Not Previously Depleted of Their Glucose and Glutamine Reserves*

In the experiments described in the preceding sections, the essential role of glucose and glutamine in the production of virus had been demonstrated by depleting the cells of one or both factors for 12 hours prior to their inoculation.

An illustrative experiment to determine the amounts of virus which would be formed in the absence of added glucose or glutamine by healthy cells, not previously depleted of their glucose or glutamine reserves, is summarized in Table VI.

HeLa cultures in the logarithmic phase of growth were washed and overlaid with various types of basal media, supplemented with glucose or glutamine in varying combination, as outlined in the table, and inoculated with virus. The fluids were harvested after 45 hours, when the cells had completely degenerated in all save the growth media containing glucose, but lacking glutamine (*cf.* pages 278-279).

The results paralleled those previously observed. The omission of both glucose and glutamine caused an average 4.2 log decrease in the amounts of virus formed by the unstarved cells, as compared with the average 4.6 log decrease in cells previously depleted of their glucose and glutamine reserves. The omission of glucose again had less effect on viral output than the omission of glutamine; in growth media lacking only glutamine, both the cytopathogenic effect and the elaboration of virus were significantly retarded; the effects of glucose and glutamine were again independent of the composition of the basal medium; and almost as much virus was formed in a medium consisting of only glucose, glutamine, and salts as in a complete growth medium containing essential and non-essential amino acids, vitamins, glucose,  $\text{NH}_4\text{Cl}$  and serum protein.

#### DISCUSSION

Glucose, glutamine, and salts have been found to be the only components of the medium necessary for the propagation of poliomyelitis virus by the HeLa cell. It follows that the nutritional requirements for viral synthesis are clearly unrelated to the requirements for cellular growth or survival. Given glucose and glutamine, the HeLa cell remained capable of normal viral propagation even when growth had been completely arrested in a multiply deficient medium, and after profound cytopathogenic changes had developed in consequence of those deficiencies.

1. Cultures could be deprived of all amino acids but glutamine, of serum protein, vitamins,  $\text{NH}_4^+$ , purines, and pyrimidines, without seriously modifying their capacity to propagate poliomyelitis virus. Even after 12 hours' preincubation in a medium containing only glucose, glutamine, and salts, the cells were able to elaborate amounts of virus within 1 log of the amounts formed in a complete growth medium. The present experiments indicate that even this small difference may reflect the earlier death of the cell in the simple glucose-glutamine-salt mixture, rather than a decreased rate of viral propagation (*cf.* page 277). When the rapid degeneration otherwise observed was only slightly delayed, as by the addition of small amounts of serum protein, the amounts of

virus formed were essentially equal to those formed in a complete growth medium.

2. In marked contrast to the failure of amino acids, purines, pyrimidines, protein, or  $\text{NH}_4^+$  to affect viral production, depletion of the glucose and glutamine reserves of the cell by 12 hours' incubation in media free of these factors caused a striking decrease in their capacity to elaborate virus. The amounts of virus formed by such depleted cells averaged 1/40,000 of the amounts produced in a medium containing both glucose and glutamine. The presence of amino acids, vitamins, purines, pyrimidines, serum protein, and  $\text{NH}_4^+$  did not affect this glucose and glutamine requirement. Of the two, glutamine was the more important factor for viral synthesis. The depletion of glutamine, retaining glucose, caused an average decrease in viral titer to 1/200 its normal level; while the removal of glucose caused a decrease to 1/15 the normal output. These effects of glucose and glutamine depletion were again independent of the presence or absence of the other growth factors listed above. No explanation can be given for the delayed cytopathogenic effect, and the delayed elaboration of virus, noted in many of the experiments in growth media deficient only in glutamine. In such media, the amount of virus ultimately formed was within 1 to 3 logs of the amounts elaborated in a complete medium, containing both glucose and glutamine; but the rate of virus formation was significantly reduced.

3. Qualitatively and quantitatively similar results were obtained in cells not previously depleted of their glucose and glutamine reserves. Relatively small amounts of virus were formed if both factors were omitted from the medium. The addition of glucose again caused a significant increase in viral output, averaging 400-fold (2.4 logs); glutamine was again more effective than glucose, resulting in an average 4,000-fold (3.4 logs) increase in virus titer; and the addition of both glucose and glutamine caused a 15,000-fold increase (4.2 logs) in virus titer.

4. It is a necessary corollary of the present observations that the viral protein can be made largely from the cell's own proteins or amino acid pool. Experiments with  $\text{C}^{14}$ -labelled glutamine and glucose indicate that these compounds can be used for the synthesis of the non-essential amino acids (primarily alanine, glycine, and serine in the case of glucose, and aspartic acid, glutamic acid, and proline in the case of glutamine (10)); but none of the other 12 amino acids essential for the survival of the cell can be so synthesized. To the degree that viral protein contains arginine, cystine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, tyrosine, or valine, then in a salt-glucose-glutamine medium those amino acids must derive either from preformed cellular proteins, or from the amino acid pool. Since the cell can draw on its own substance for the essential amino acids, there is no

*a priori* reason to doubt that it can obtain the 6 non-essential amino acids similarly. Their synthesis from glucose and glutamine would therefore not appear to be the essential role of the latter compounds in viral synthesis.

The ability of the HeLa cell to elaborate poliomyelitis virus in the absence of amino acids in the medium contrasts with the need for amino acids in T2 bacteriophage production by *E. coli* K56 (8). The difference may be referable to corresponding differences in the size and stability of the amino acid pools in the HeLa cell (9), as compared with *E. coli* (13).

5. The possibility that glucose and glutamine act by facilitating the fixation of virus appears to have been excluded by the fact that these compounds were effective in initiating virus formation even when added 2, 4, or 6 hours after their inoculation with virus, and after any last traces of free, unbound virus had been removed by thorough washing and appropriate incubation with antiserum. These results indicate that cells depleted of glucose and glutamine can nevertheless fix sufficient virus to initiate normal viral propagation. The possibility that glucose or glutamine may promote viral fixation is, however, not thereby excluded.

6. It is possible that glucose and glutamine serve either to prevent the death of the cell, or to preserve the integrity of the metabolic processes necessary for viral synthesis, without however participating directly in that synthesis. This possibility cannot be wholly excluded on the basis of the present experiments. HeLa cells rapidly degenerated in a simple salt medium, and the addition of glucose and glutamine alone significantly retarded that degenerative process. It is however worth noting that even with glucose and glutamine added, there was obvious deterioration of the cells within 6 to 12 hours in this minimal medium, and that despite those degenerative changes, cells inoculated after such a period of depletion went on to form within one log of the normal amounts of virus. Conversely, when the deterioration of the cells in a glucose- and glutamine-free medium was somewhat delayed, as by the addition of dialyzed serum, there was nevertheless no significant evolution of virus unless glucose or glutamine was added.

7. Perhaps the most reasonable working hypothesis with respect to the function of glucose and glutamine is that they are used for viral synthesis, either as sources of energy, as precursors for the synthesis of viral nucleic acid, or both. When HeLa cells are grown in a limiting medium which includes only the essential amino acids, vitamins, glucose, salts, and serum protein, then the glucose and glutamine carbon, and the glutamine amide N are drawn on heavily for the synthesis of both ribo- and desoxyribonucleic acid (10).

It seems a reasonable working hypothesis that these compounds may similarly be required for the synthesis of viral nucleic acid. The degree to which the cellular nucleic acid itself, or its precursors, are used for this purpose, remains to be determined.

8. Whether the small amount of virus formed by previously depleted cells in media containing no added glucose or glutamine means that limited viral synthesis can take place in their absence, or whether it reflects the presence in the presumably depleted cells of residual traces of these compounds (or compounds which can be substituted for them) is not clear from the present experiments.

In either case, however, since it has been estimated (11, 12) that 200 to 600 infectious particles of poliomyelitis virus are released per infected monkey kidney cell, then assuming a burst size of the same order of magnitude for the HeLa cell, the fact that the omission of both glucose and glutamine caused a 40,000-fold decrease in viral yield necessarily implies that only a minute proportion of the cells are then participating in viral synthesis. Whether these few cells are forming normal amounts of virus, or whether the number of viral particles formed per cell is also decreased, remains to be determined. The greatly decreased proportion of cells which form virus in the absence of glucose or glutamine reflects impaired function rather than death, for the addition of glucose and glutamine even 6 hours after inoculation restores their capacity to form virus; and if the glucose and glutamine are added in a complete medium, the cells may resume their normal microscopic appearance, prior to the cytopathogenic effects of viral infection.

9. In many experiments, the initial elaboration of virus was slower in media containing some elements necessary for growth than in media containing salt solution alone (contrast curves  $\circ$ ,  $\bullet$ , and  $\odot$  in various sections of Fig. 2).

10. The fact that normal amounts of virus were formed in the absence of added vitamins, and after preliminary incubation of the cells for 12 hours in a vitamin-free medium, does not necessarily mean that intracellular vitamins and vitamin conjugates are not required for viral synthesis. It has been shown (3) that the HeLa cell continues to multiply for 3 to 14 days in the absence of essential vitamins, presumably because of the cellular reserves; and it will be of interest to determine the effect of prolonged vitamin depletion on the virus synthesizing capacities of the cell.

11. Preliminary experiments indicate that glutamine and/or glucose are required by the HeLa cell for the propagation of the Saukett and Mahoney strains of poliomyelitis. The applicability of the results here reported to other cell-virus systems is under present study, as are the quantitative aspects of the glucose and glutamine requirement. It is to be noted in this connection that Burr, Campbell, Morgan, and Nagler (14) found that cultures of chick

allantoic membrane formed normal amounts of influenza virus when overlaid with a balanced salt solution, presumably containing glucose as the only organic supplement. On the other hand, H. R. Morgan (15) has found that chick embryo tissue maintained for 11 to 28 days in Hanks' balanced salt solution lost their capacity to support the multiplication of psittacosis virus unless *e.g.* beef embryo extract were added.

#### SUMMARY

Only minimal amounts of poliomyelitis virus were formed by HeLa cells placed in a medium free from glucose and glutamine, even if the medium contained an otherwise full complement of essential and non-essential amino acids, purines, pyrimidines,  $\text{NH}_4^+$ , and serum protein. Conversely, within one log of the optimal yield of virus was formed by HeLa cells in a medium containing only glucose, glutamine, and salts, even if the cells had been starved in this medium for 12 hours prior to their inoculation.

The presence of glucose alone caused an average 170-fold increase in viral output beyond the amounts formed by the glucose- and glutamine-depleted cells. The addition of glutamine alone caused an average 2000-fold increase; and the addition of both increased the viral formation 40,000-fold. Qualitatively similar results were obtained with unstarved cells, not previously depleted of glucose and glutamine. It follows that only a small proportion of HeLa cells are capable of forming virus unless either glucose or glutamine, or both, are present in the medium.

The elaboration of virus was significantly delayed in media containing glucose but no glutamine.

The absence of glucose and glutamine did not prevent the fixation of poliomyelitis virus by the cell. When these compounds were added to previously depleted cells even 6 hours after inoculation, and after the excess free virus had been removed by washing and by the addition of specific antiserum, normal amounts of virus were formed despite the degenerative changes caused by the previous glucose and glutamine deprivation. Possible functions of glucose and glutamine in the elaboration of virus are discussed in the text.

Such factors other than glucose, glutamine, or salts (*e.g.* amino acids, purines, pyrimidines, vitamins, protein, or  $\text{NH}_4^+$ ) as may be needed by HeLa cells for the propagation of poliomyelitis virus, need not be present in the medium and cannot be easily washed out of the cell. Even 12 hours' total deprivation of the cells in salt solution prior to inoculation only slightly decreased their virus-synthesizing capacity in a similarly deficient medium, provided only that adequate amounts of glucose and glutamine were retained.

#### BIBLIOGRAPHY

1. Eagle, H., *J. Biol. Chem.*, 1955, **214**, 839.
2. Eagle, H., *J. Exp. Med.*, 1955, **102**, 37.



3. Eagle, H., *J. Exp. Med.*, 1955, **102**, 595.
4. Eagle, H., *Arch. Biochem. and Biophysics*, in press.
5. Eagle, H., Oyama, V. I., Levy, M., Horton, C. L., and Fleischman, R., *J. Biol. Chem.*, 1956, **218**, 607.
6. Ackermann, W. W., Rabson, A., and Kurtz, H., *J. Exp. Med.*, 1954, **100**, 437.
7. Kuwata, T., and Shiba, S., *Experientia*, 1955, **11**, 269.
8. Burton, K., *Biochem. J.*, 1955, **61**, 473.
9. Piez, K. A., Eagle, H., and Fleischman, R., data in preparation.
10. Eagle, H., Piez, K. A., Salzman, N. P., Levintow, L., and Fleischman, R., unpublished observations.
11. Lwoff, A., Dulbecco, R., Vogt, M., and Lwoff, M., *Virology*, 1955, **1**, 128.
12. Youngner, J. S., *J. Immunol.*, 1956, **76**, 50.
13. Roberts, R. B., Abelson, P. H., Cowie, D. B., Bolton, E. T., and Britten, R. J., Studies of Biosynthesis in *Escherichia coli*, *Carnegie Institution of Washington Pub. No. 607*, 1955, 91.
14. Burr, M. M., Campbell, M. E., Morgan, J. F., and Nagler, F. P., *Canadian J. Microbiol.*, 1954, **1**, 158.
15. Morgan, H. R., *J. Exp. Med.*, 1956, **103**, 37.