CHEMICAL STUDIES IN HOST-VIRUS INTERACTIONS*

IV. A Method of Determining Nutritional Requirements for Bacterial Virus Multiplication

By CATHERINE B. FOWLER and SEYMOUR S. COHEN, Ph.D.

(From The Children's Hospital of Philadelphia (Department of Pediatrics), and the Department of Physiological Chemistry, University of Pennsylvania, School of Medicine, Philadelphia)

(Received for publication, August 30, 1947)

Many studies with multicellular organisms have shown that the multiplication of virus depends on the nutrition of the organism. A certain dependency was to have been expected, but the specific character of this dependency has proven unpredictable in many cases. For example, thiamine deficiency diminishes the susceptibility of mice to some strains of poliomyelitis virus (1), but on the other hand lowers the resistance of pigeons to psittacosis (2). A study of these specificatio ns may be expected to provide important clues to the mechanism and contro*l* of a particular viral infection.

The purpose of the present studies has been the investigation of methods by which nutritional requirements can be detected. The knowledge that a metabolite is required suggests the use of an antimetabolite which will interfere with the process. This thesis has been well developed by Wooley (3) and others, and provides a rational approach for chemotherapeutic investigation. In the present series of studies, the effect of two antimetabolites specific for determined requirements has been shown to interfere with virus multiplication, illustrating again the validity of this approach. Some studies of the effects of organic components of a medium on bacteriophage production have already been made by Wahl (4) and Spizizen (5, 6). These studies, in addition to the previous experience in this laboratory, made it expedient to use the technically favorable and uncomplicated *Escherichia coli*-T2 bacteriophage system.

Evidence of the dependence of virus synthesis on the constituents of the medium of the host cell has been presented (7). It has been shown that *E. coli* infected with T2 virus assimilate the nitrogen and phosphorus of the simple medium F at a considerable rate. The metabolism of phosphorus is directed exclusively to the production of the phosphorus-containing compound of T2, desoxyribose nucleic acid. Using radioactive phosphorus, it was also shown that most of the phosphorus in the synthesized virus is derived from the medium

* The work described in this paper has been aided by the Office of Naval Research.

after infection. Other studies (7) including those described below indicate that similar relationships prevail for the utilization of the carbon and nitrogen of the medium.

It has been shown previously (8) that *E. coli* do not multiply after infection. Therefore, it is considered, pragmatically, that those compounds found to increase the number of virus particles produced or rate of production are those which are used in virus synthesis. This does not preclude a rapid organization or turnover by the bacterial cell of the material which, under normal conditions, might be used for its own growth and multiplication.

Two methods of determining whether a specific compound is involved in virus synthesis have been previously described. First, analysis of the T2 virus showed desoxyribose nucleic acid, arginine, tryptophane, and tyrosine to be virus constituents (8). A more complete analysis may be presumed to give a wider scope to the choice of an antimetabolite. Second, an antimetabolite was tested for inhibition of virus synthesis. Reversal of the inhibition with the specific metabolite indicates that it is required for synthesis (9). In the course of studies with 5-methyl tryptophane, a specific antagonist for tryptophane utilization (9), two interesting effects were observed in addition to the confirmation that tryptophane was essential for virus synthesis. First, it was shown that it is possible to determine at what time during the latent period of multiplication within a host cell a compound is required, and secondly, infectious centers were irreversibly destroyed by the action of the compound after a certain period of inhibition of virus multiplication.

This paper presents a third method of determining whether a specific compound is required for virus synthesis; *i.e.*, the effect of addition of a specific compound to the system in a condition suboptimal for virus synthesis. The times required for multiplication of T2 virus within a host cell are almost the same if the cell is grown and infected in either a complex medium such as nutrient broth or in a simple synthetic medium containing glucose (10) or lactate (9) as a carbon source. This does not mean, however, that the latent period is independent of the nutrients present in the medium, since these systems contain two independent and compensating variables. It is possible that bacteria grown in the absence of external amino acids have greater abilities to synthesize these compounds than bacteria grown in a complex medium, such as broth. That the latent periods for these two systems are identical may mean that the rates at which components of virus are made available for organization, in the one case by synthesis, in the other by absorption, are identical.

When the multiplication of virus is followed in a system with just one variable, *i.e.* the composition of the medium, the dependence of virus synthesis on the composition of the medium is readily observed. *Escherichia coli* grown in broth and transferred and infected in a simple synthetic medium show a much longer latent period and a much smaller burst size than the same bacteria in-

fected in broth. The simple medium may be supplemented with various compounds. A study of the time required to liberate virus and of the number of virus particles synthesized, as compared to the unsupplemented medium, indicates whether or not the particular compound is a rate-determining factor in virus synthesis under these conditions. Many substances were found to stimulate synthesis, but no single supplementary compound was found which approximated the rate and amount of virus synthesis characteristic of broth.

It has been proposed (11, 12) that virus synthesis is the autocatalytic conversion of preformed cellular protein into virus. If this were the case, the number of steps in the process of synthesizing active virus from inactive precursor would be expected to be very few and to involve the specificities of large molecules. The control of this type of process might be very difficult. We have found, however, that synthesis of virus depends on the presence of simple compounds in the external environment of the host cell. It would seem that a great many syntheses of relatively simple compounds are essential stages in virus multiplication. Such a process should be susceptible to inhibition by a wide variety of antimetabolites.

Materials and Methods

Escherichia coli B was subcultured monthly to Difco agar nutrient broth slants. From these, weekly subcultures were made. Cultures were made by inoculating broth medium, containing 8 gm. of Difco nutrient broth and 5 gm. of NaCl per liter of distilled water, with bacteria from a slant and aerating at 37°, overnight. At the end of this time, the culture contained about 5×10^9 viable bacteria per cc. 0.05 cc. was transferred to 10 cc. of fresh broth and grown to about 5×10^7 bacteria per cc. with aeration at 37°. The broth cultures were then centrifuged, washed twice in a simple defined (8) medium (F), and resuspended in F, F plus a supplement, and N. Bacteria treated in this way were designated B_N-F, and B_N-N, the subscript indicating the medium in which the bacteria were grown and the large letter the medium in which they were resuspended. The resuspended bacteria were infected with a purified T2r$^+$ concentrate prepared from F medium or broth lysates (13, 14). All T2 virus referred to in this paper was the r$^+$ type. The liberation of virus in the same host cells in different media was followed by the one-step growth technique of Delbrück and Luria (15) using the spreading and layer modification of Hershey *et al.* (16).

EXPERIMENTAL

Bacteria grown in N and infected in N (B_N-N) liberate virus consistently at 20 to 22 minutes with a burst size or yield of virus particles per infected cell of from 80 to 200. The same bacteria resuspended in F (B_N-F) do not start liberating virus until at least 40 minutes and in some cases as late as 70 minutes after infection. The burst size in F has been found to vary from 5 to 30. An experiment of this type is presented in Fig. 1.

Other evidence that B_N-F are deficient in their synthetic capacities is obtained from studying the turbidity curves of B_N grown in N, washed, and resuspended in N and F. Fig. 2 shows that B_N in F has a much slower growth rate than the same bacteria in broth.

The effect of the different constituents in F medium was determined by preparing a series of F media each lacking a single constituent. The total concentration of all other ions was kept approximately constant by the addition of appropriate salts. Table I gives the details of this experiment.

It may be seen in Fig. 3 that an external source of nitrogen and carbon is essential for virus synthesis. Virus liberation and yield seem to be almost inde-

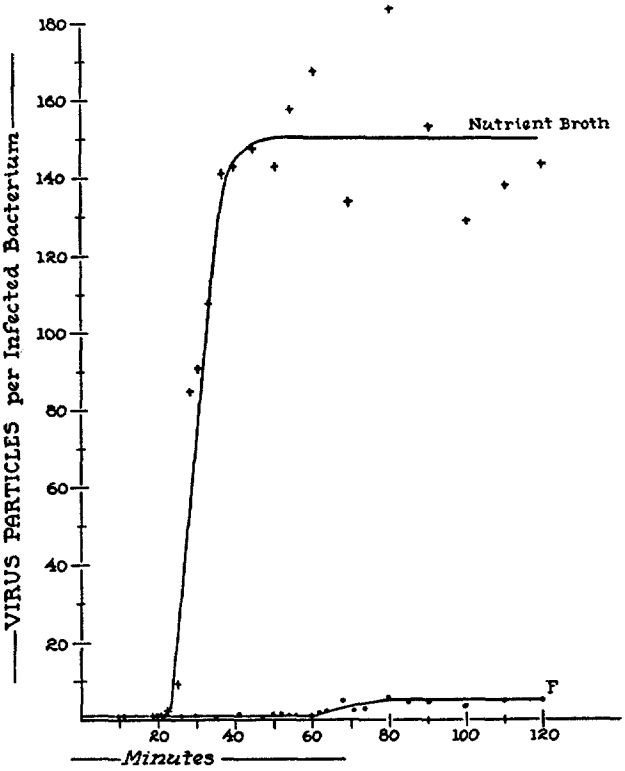

FIG. 1. One-step growth curves for broth-grown bacteria in the broth and F media.

pendent of added sulfate. The absence of external phosphate severely limited virus multiplication, decreasing the yield of virus and significantly increasing the latent period. The absence of magnesium was also found to limit virus synthesis.

In connection with the apparent lack of a sulfur requirement, it must be noted that the other constituents of the medium may be sufficiently contaminated with sulfate to obscure a minimal need. Chemical test for sulfate in the deficient medium was negative, however. Nevertheless, methods to be reported (17) have shown a definite methionine requirement in this system.

Amino Acids.—All of the naturally occurring amino acids were tried, singly, as supplements to F. Those amino acids which consistently gave stimulation were *l*(+)-isoleucine, *l*(−)-phenylalanine, *l*(+)-aspartic acid, *l*(−)-proline, *l*(+)-lysine, *l*(+)-valine, *l*(+)-arginine, and *l*(+)-glutamic acid. Some of these results are presented in Figs. 4 and 5. At 25 mg. per cc. these compounds or their racemic mixtures caused a decrease in latent periods and slight and somewhat variable increases in burst size. The most marked stimulatory effect was noted with glutamic acid. The latent period in F supplemented with 25

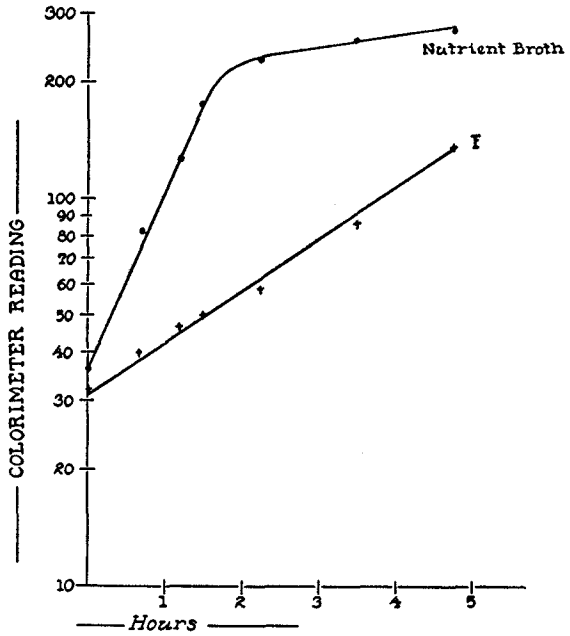

FIG. 2. Increase in turbidity of cultures of broth-grown bacteria in the broth and F media.

mg. per cc. of *l*(+)-glutamic acid was about 35 minutes, which in some cases was nearly 40 minutes shorter than the time required for infected cells in F medium alone. The burst size was frequently not increased but with a few cultures of bacteria was 4 or 5 times the control in F. Glutamine did not appear to be stimulatory.

l-tyrosine, *l*-histidine, and *d*(−)-valine, or *dl*-valine did not give reproducible stimulation. Certain amino acids were without effect. These included glycine, *l*(+)-alanine, *dl*-threonine, *dl*-methionine, and *l*(−)-tryptophane.

In contrast to these, *l*(−)-leucine and *l*(−)-serine inhibited virus synthesis. *l*(−)-cystine and *l*(−)-cysteine were toxic; *i.e.*, not only was virus not liberated but the number of infectious centers rapidly decreased.

The inhibition by *l*(−)-leucine was completely overcome by *dl*-valine, *dl*-

isoleucine, or *dl*-norleucine (Fig. 6). When one of these amino acids was added at 25 γ per cc. to infected bacteria in the presence of 25 γ per cc. of *l*(−)-leucine, even more virus was liberated and more rapidly than in infected bacteria in F.

TABLE I

The Effect of Omission of Single Constituents of F on Virus Multiplication in B_N-F

Time		
min.		
90	0.10 cc. 24 hr. culture of B_N inoculated into 10 cc. N. Incubated at 37° with aeration. (B)	
	Removed six 2 cc. aliquots. Centrifuged 10 min. at 4000 R.P.M. Bacteria were suspended in: Tube 1 in 2 cc. F Tube 2 in 2 cc. F without Mg^{++} Tube 3 in 2 cc. F without $lactate^-$ Tube 4 in 2 cc. F without $SO_4^=$ Tube 5 in 2 cc. F without $PO_4^{\equiv}$ Tube 6 in 2 cc. F without NH_4^+	
	Centrifuged 10 min. Repeated twice. Assayed B	
0	0.90 cc. tubes 1–6 + 0.10 cc. $T2r^+$	Tubes a–f
5	0.10 cc. a–f + 9.9 cc. appropriate medium	Tubes 5a–5f
6	0.10 cc. 5a–5f + 9.9 cc. appropriate medium	Tubes I–VI
9	1.0 cc. I and III in centrifuge. Centrifuged 5 min.	
10	Assayed I–VI	
14	0.8 I–VI + 3.2 cc. appropriate medium	Tubes VII–XII
15	Assayed I–VI	
18	Assayed supernates I and III Assayed periodically on nutrient agar	

Inhibition by serine, cysteine, and cystine was not overcome by methylated compounds such as thymine or methionine.[1]

Glutamic Acid and the Antimetabolite, Methionine Sulfoxide

The marked stimulatory effect of glutamic acid suggested an essential rôle in the elaboration of virus. To demonstrate this, the effect of a specific glutamic

[1] We are indebted to Dr. Jack Schultz and Dr. Gerrit Toennies of the Lankenau Hospital Research Institute for some of the amino acids used in these experiments.

antimetabolite, *dl*, *dl*-methionine sulfoxide (i-MSO) was tested (18). For the following group of experiments, *E. coli* were grown in F medium. They were

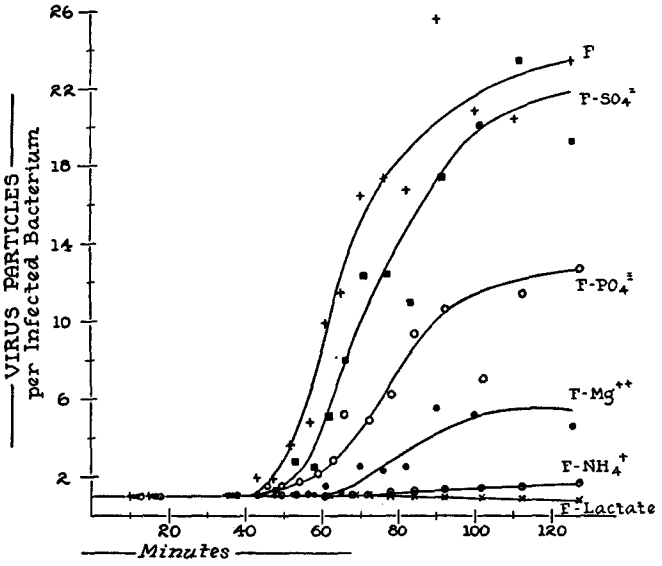

FIG. 3. The effect of omission of single constituents of F on virus production in F by broth-grown bacteria.

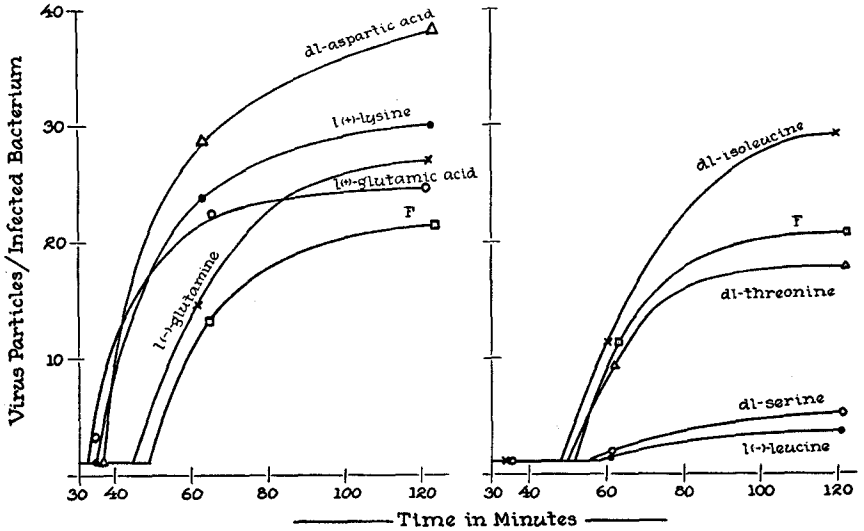

FIG. 4. The effect of some amino acids on the production of virus.

infected in the presence of i-MSO, or i-MSO was added at different times during the latent period of multiplication within the host. It was found that at high

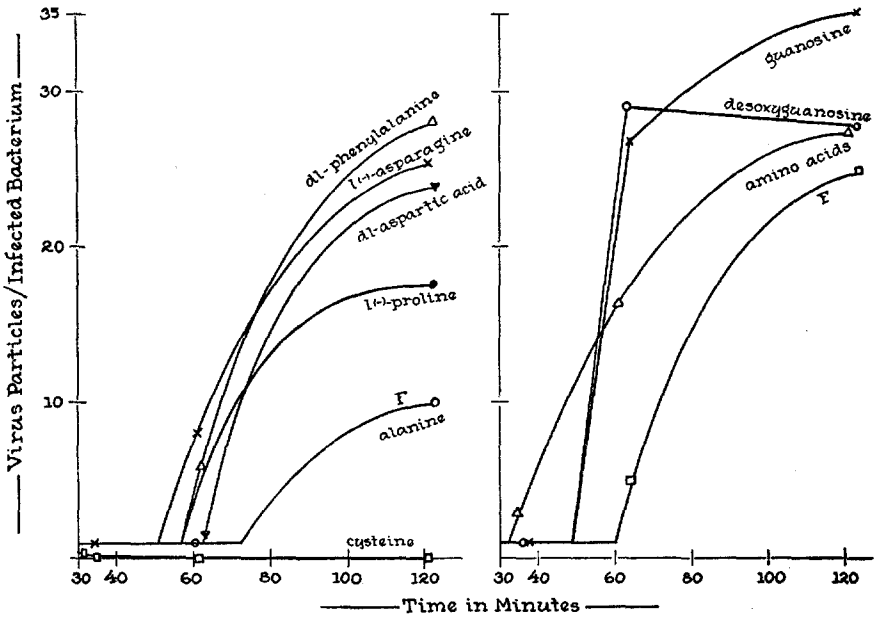

FIG. 5. The effect of some amino acids and nucleosides on virus production.

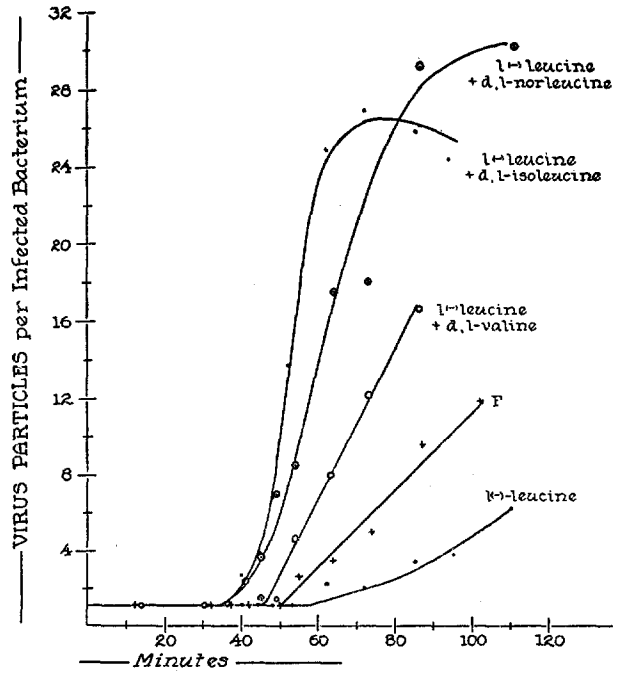

FIG. 6. The effect of some amino acids on the inhibition of virus production by leucine.

concentrations (5×10^{-2} M) i-MSO completely inhibited the liberation of T2 even if added after 17 minutes of the normal 25 minute latent period. At 5×10^{-3} M i-MSO, there was normal liberation of virus. The inhibition was reversed with glutamic acid or by diluting out the i-MSO in F. If bacteria were infected and allowed to incubate 6 minutes in the presence of i-MSO before ad-

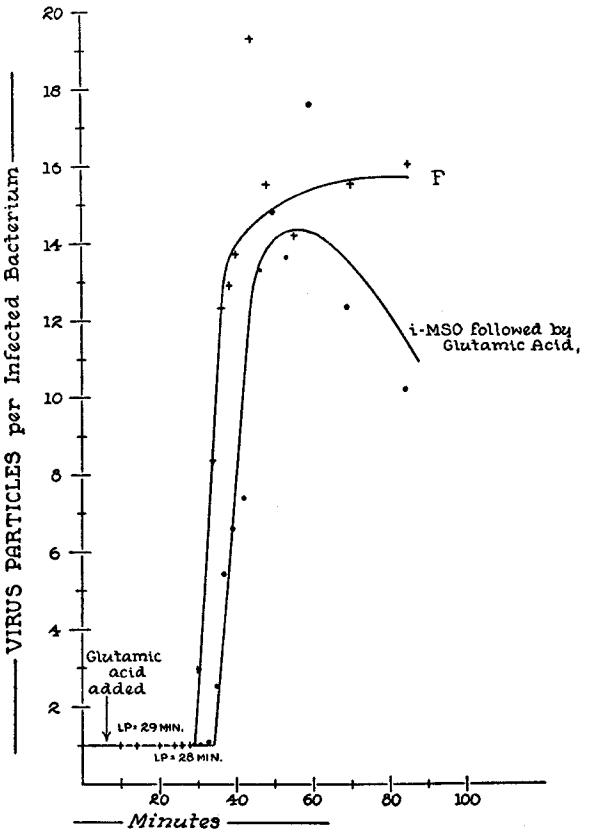

FIG. 7. The inhibition of virus production by i-MSO and the reversal of inhibition by the addition of glutamic acid.

dition of glutamic acid, it was found that virus was liberated 28 minutes after this reversal. The latent period of the control infected bacteria was 29 minutes (Fig. 7). There was a somewhat variable irreversible reduction in the number of infectious centers when the bacteria were left in i-MSO. These two effects, (1) the blocking of the start of virus synthesis, and (2) the "killing" of infected bacteria on continued exposure had previously been found to be characteristic of 5-methyl tryptophane. The effect of interrupting the latent period, as in studies with 5-methyl tryptophane (9), was further investigated. B_F were infected in F and after 10 minutes of the normal latent period, were inhibited

with i-MSO for 10 minutes. The i-MSO was diluted in F, and F containing 5×10^{-3} M glutamic acid. It was found that in either case virus was liberated a few minutes after the burst of the control bacteria, instead of the full 10 minutes required to complete the total normal latent period. The yield of virus from the interrupted bacteria about one-half the burst size of the normal bacteria. This may be explained by the "killing" of some of the infectious centers observed in this experiment during the 10 minute incubation with i-MSO. 5-methyl tryptophane, on the other hand, was shown to cause an absolute interruption of virus synthesis within the system, which on addition of tryptophane required the residual latent period and gave a normal burst size.[2]

Indole-3-Acetic Acid.—We have reported (19) that certain preparations of indole-3-acetic acid obtained from Eastman Kodak Company stimulated the production of T2 in B_N-F. Such stimulation was not found using samples from other sources (20) nor did possible contaminants such as indole, skatole, indolyl-1,3-diacetic acid, or Cu^{++}, which was used in the preparation of Eastman material (21), cause any stimulation. No other single substance, among more than 60 tested, produced as great a stimulation as we found for these particular preparations. 5×10^{-3} M indole-3-acetic acid itself, regardless of origin, inhibited virus multiplication.

Nucleic Acid Derivatives.—Fig. 5 shows the stimulatory effects of guanosine and desoxyguanosine. There were marked decreases in latent period and a somewhat variable increase in burst size in the presence of these compounds at 10 γ/cc. Depolymerized (*i.e.*, purified after alkaline extraction) preparations of ribose and desoxyribose nucleic acid showed similar effects on virus synthesis. The purine and pyrimidine bases, singly, at 10 γ/cc. were without marked effect.

Vitamins and Other Growth Factors.—Single vitamins were without significant stimulatory effect on B_N-F. Each vitamin was tested at only one concentration, with the exception of pyridoxine which was tested in the range of 0.005 to 5.0 γ per cc. Biotin—0.5 γ/cc., choline—5.0 γ/cc., cozymase—5.0 γ/cc., folic acid—0.5 γ/cc., glutathione—5.0 γ/cc., inositol—0.5 γ/cc., 2-methyl-1, 4-naphthoquinone—0.5 γ/cc., nicotinamide—2.0 γ/cc., para-aminobenzoic acid—0.2 γ/cc., Ca pantothenate—0.5 γ/cc., riboflavin—0.2 γ/cc., thiamine—3.0 γ/cc., sodium α-tocopherol phosphate—0.5 γ/cc. were used. It is possible that an optimal concentration which would cause stimulation was missed in this survey.

Inorganic Ions.—Cu^{++} and Fe^{+++} were toxic in concentrations as low as 10^{-5} M. The toxicity was overcome by dilution. At no concentration, however, was a stimulation of virus production found with these ions. Ca^{++} was also without effect in this system at concentrations below 10^{-3} M.

Complex Supplements.—On adding, at 500 γ per cc., a commercial casein hy-

[2] We wish to thank Dr. Heinrich Waelsch of the Department of Biochemistry of the New York State Psychiatric Institute, and Dr. Theodore Levine of the Lankenau Hospital Research Institute for samples of i-MSO and *l*-methionine *dl*-sulfoxide, respectively.

drolysate (Stearns "parenamine") fortified with 1.5 per cent tryptophane, a latent period of less than 30 minutes was always found, as well as an increase in burst size to nearly 50 per cent of that of B_N-N. Increasing the concentration

TABLE II

The Amino Acid, Purine, and Pyrimidine Composition of the Complete Defined Medium

Substance*	Concentration‡	Source
	γ/cc.	
Alanine	9.25	Bios
Arginine	18.6	Bios
Aspartic acid	30.0	Bios
Cystine	2.10	Bios
Glutamic acid	108	Merck
Glycine	2.25	Merck
Histidine	12.5	Pfanstiehl
Hydroxyproline	1.15	Bios
Isoleucine	48.3	Bios
Leucine§	58.0	—
Lysine	31.3	Merck
Methionine	16.3	Bios
Phenylalanine	19.0	Bios
Proline	43.3	Bios
Serine	25.0	Bios
Threonine	17.5	Bios
Tryptophane	5.00	Pfanstiehl
Tyrosine	26.6	Bios
Valine	39.7	Bios
Cytosine	10.0	Dougherty
Thymine	10.0	Schwartz
Adenine	10.0	Schwartz
Guanine	10.0	Schwartz

* All amino acids in this table were the natural isomers (*l* series).

‡ The concentrations of some amino acids were given by Stearns for parenamine. The concentrations of the other amino acids were taken from the values given for casein by Cohn and Edsall (22).

§ We wish to thank Dr. Joseph Fruton of the Department of Physiological Chemistry, Yale University, for the *l*(−)-leucine.

of parenamine did not cause further stimulation. It was found that parenamine was more effective than a mixture of naturally occurring amino acids at a total concentration of 500 γ per cc., and mixed in the proportions comparable to parenamine (Table II). There is considerable variation in the latent period and burst sizes in these two systems. Toward the end of these studies, subcultures of our original strain of *E. coli* B appeared to utilize the amino acid mixture more effectively than at the beginning. Parenamine, however, was always found to effect a speedier and greater synthesis of virus than the amino acid mixture alone (Fig. 8).

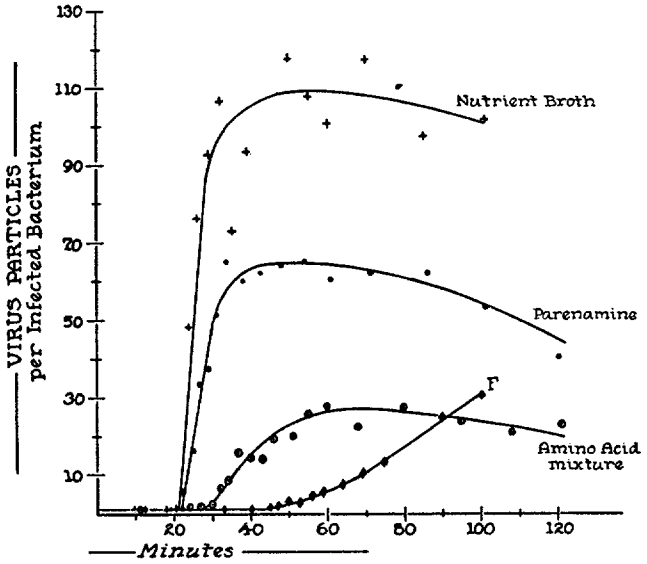

FIG. 8. A comparison of the stimulatory effects of parenamine and an amino acid mixture on virus production in F by broth-grown bacteria.

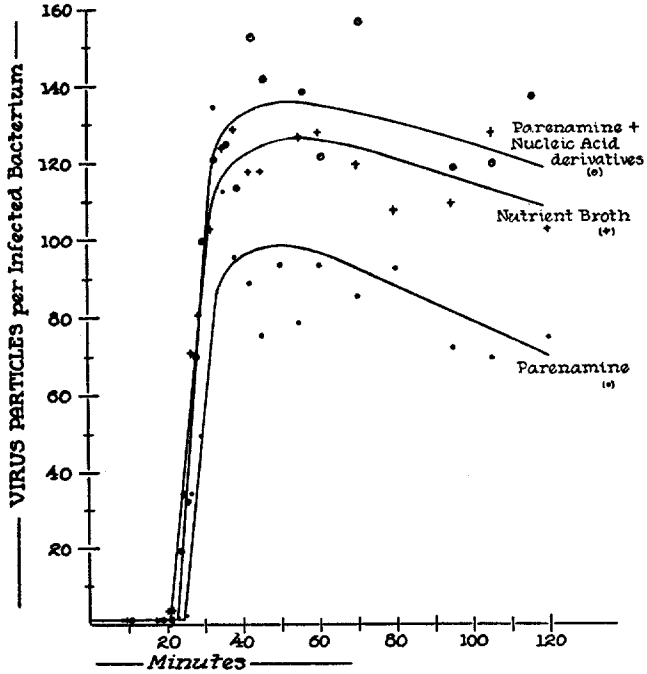

FIG. 9. The stimulatory effect of nucleic acid derivatives supplementing parenamine in the F medium.

The effect of adding a mixture containing adenine, guanine, thymine, and cytosine to parenamine is shown in Fig. 9. With such a mixture both burst size and latent period closely approximated those for B_N-N. When these purine and pyrimidine bases were added to a known amino acid mixture, there was also a marked stimulatory effect on latent period and burst size, as presented in Fig. 10.

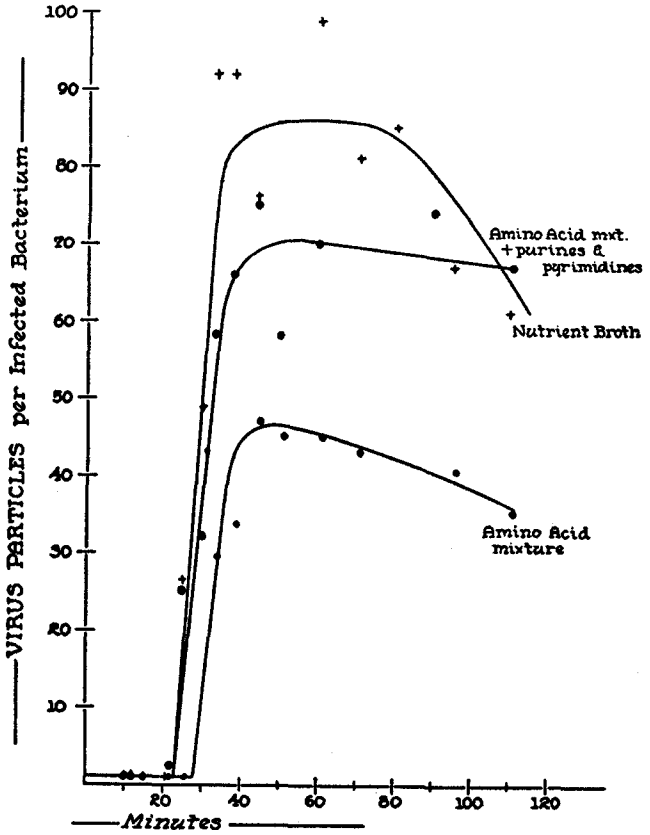

FIG. 10. The stimulatory effect of purines and pyrimidines supplementing an amino acid mixture in the F medium.

The addition of various vitamin mixtures to either of these complex supplements did not give reproducibly significant stimulatory effects.

It may be seen from Figs. 9 and 10 that the rate and amount of virus synthesized by B_N-N may be almost duplicated by a mixture of either parenamine fortified with purines and pyrimidines, or of the naturally occurring amino acids and purines and pyrimidines. With this mixture of chemically defined constituents, the latent period was within 3 minutes of, and sometimes the same as, the latent period in broth, and the burst size was rarely lower than 80 per cent of, and occasionally equalled, that of broth.

DISCUSSION

The time required to elaborate cellular components, such as proteins and nucleic acids, by *E. coli* B grown in nutrient broth (N) and transferred to the F medium was greatly increased. This is shown by the slower rate of increase in turbidity and the decreased rate of virus synthesis. It seems probable that those amino acids which, when added to B_N-F, caused a stimulation in the production of virus are the ones whose rate of synthesis in these bacteria under these conditions may be limiting factors in virus peptide synthesis. On the other hand, glutamic acid, which was the greatest stimulator of any of the amino acids, may be important in transamination, thereby facilitating the synthesis of other amino acids, or may be stimulatory because of the activity of derivative metabolites, such as α-ketoglutaric acid, as described by Spizizen (6). Thus, the stimulation by this amino acid when added as a single supplement to a simple medium may have resulted from an activity other than its function in the synthesis of virus in broth.

Other amino acids, also essential, would not necessarily be detected by this method. For example, tryptophane may be considered essential for virus synthesis since it was found in the virus, and 5-methyl tryptophane, a tryptophane antimetabolite, completely inhibited virus synthesis; however, tryptophane was not stimulatory when added as a single supplement to F. This merely signifies that other factors may have been more rate-limiting than tryptophane.

The method used has shown the nutritional requirements for synthesis of T2 to be multiple. External sources of both carbon (lactate) and nitrogen (NH_4^+) were necessary. The absence of phosphorus in the medium was severely limiting, confirming, in general, the results of studies with radioactive phosphorus which showed that most of the phosphorus incorporated in virus synthesized under somewhat different conditions was derived from the phosphorus-containing medium. The absence of magnesium also limited virus synthesis. On the other hand, the requirement for sulfur was not demonstrated by this method. Other methods, however, have shown a methionine requirement, which may serve as a sulfur source. In any case, although the omission of sulfur was found not to be limiting for virus synthesis by host cells in a limited medium, it might prove so for the greater synthesis of virus in the same host cell in a complete medium.

No single carbon- and nitrogen-containing compound was found which could effect a rate of synthesis of T2 in B_N-F characteristic of B_N-N. Thus the rate and amount of synthesis in B_N-N were approximated only by a complex mixture of *l*-amino acids, and purine and pyrimidine bases. Bacteria which have been grown in nutrient broth and washed, may still contain vitamins sufficient for the maximal utilization of known substrates. Under these conditions, supplementation with vitamins would not be expected to cause a stimulation. However,

a critical requirement for a vitamin in this system might be detected by the antimetabolite technique.

A survey of our data shows that the liberation of virus is to some extent independent of the amount of virus synthesized. Compounds which markedly decreased latent period did not always increase burst size.

The mechanism of the inhibition of virus synthesis by cystine, cysteine, leucine, and serine in this system is obscure. The inhibition by leucine was overcome by closely related amino acids. This same type of effect was observed by Gladstone (23) in the case of growth rate of *Bacillus anthracis* inhibited with serine and leucine.

SUMMARY

Using the one-step growth technique the production of the virus T2 in its host, measured by latent period and burst size, was shown to depend on the nutritional environment of the host cell.

When *E. coli*, grown in broth, was transferred to a simple medium, single organic compounds such as some amino acids and nucleosides were found to increase or accelerate the synthesis of virus.

An antimetabolite of glutamic acid, an amino acid important for virus synthesis, was shown to be inhibitory.

Several naturally occurring amino acids, leucine, serine, and cysteine, inhibited virus synthesis in the simple medium.

A chemically defined mixture was found which supported a rate of virus synthesis very nearly comparable to that found for host cells in nutrient broth.

BIBLIOGRAPHY

1. Foster, C., Jones, J. H., Henle, W., and Dorfman, F., *J. Exp. Med.*, 1944, **79,** 2, 221.
2. Pinkerton, H., and Swank, R. I., *Proc. Soc. Exp. Biol. and Med.*, 1940, **45,** 705.
3. Woolley, D. W., *Physiol. Rev.*, 1947, **27,** 308.
4. Wahl, R., *Ann. Inst. Pasteur*, 1946, **72,** 73.
5. Spizizen, J., *J. Infect. Dis.*, 1943, **73,** 212.
6. Spizizen, J., *J. Infect. Dis.*, 1943, **73,** 222.
7. Cohen, S. S., in Cold Spring Harbor Symposia on Quantitative Biology, Cold Spring Harbor, Long Island Biological Association, 1947, **12,** in press.
8. Cohen, S. S., and Anderson, T. F., *J. Exp. Med.*, 1946, **84,** 511.
9. Cohen, S. S., and Fowler, C. B., *J. Exp. Med.*, 1947, **85,** 771.
10. Luria, S. E., and Latarjet, R., *J. Bact.*, 1947, **53,** 149.
11. Krueger, A. P., and Baldwin, D. M., *Proc. Soc. Exp. Biol. and Med.*, 1937, **37,** 393.
12. Northrop, J. H., *J. Gen. Physiol.*, 1939, **23,** 59.
13. Cohen, S. S., *J. Biol. Chem.*, 1947, **168,** 511.
14. Cohen, S. S., *J. Biol. Chem.*, 1947, in press.

15. Delbrück, M., and Luria, S. E., *Arch. Biochem.*, 1942, **1,** 111.
16. Hershey, A. D., Kalmanson, G., and Bronfenbrenner, J., *J. Immunol.*, 1943, **46,** 267.
17. Cohen, S. S., and Fowler, C. B., *J. Exp. Med.*, 1948, **87,** 275.
18. Waelsch, H., Orvades, P., Miller, H. K., and Borek, E., *J. Biol. Chem.*, 1946, **166,** 273.
19. Cohen, S. S., and Fowler, C. B., *J. Biol. Chem.*, 1947, **167,** 625.
20. Cohen, S. S., and Fowler, C. B., *J. Biol. Chem.*, 1947, **168,** 775.
21. Jackson, R. W., and Manske, R. H., *Canad. J. Research,* 1935, **13,** sect. B, 170.
22. Cohn, E. J., and Edsall, J. T., Proteins, Amino Acids and Peptides, New York, Reinhold Publishing Corporation, 1943.
23. Gladstone, G. P., *Brit. J. Exp. Path.*, 1937, **18,** 322.