STUDIES ON AMINO ACID REQUIREMENTS OF T - 3 BACTERIOPHAGE FOR ADSORPTION TO ITS HOST

Thesis for the Degree of M. S.

MICHIGAN STATE UNIVERSITY

Aina Valdmanis

1956

STUDIES ON AMINO ACID REQUIREMENTS OF T-3 BACTERIOPHAGE FOR ADSORPTION TO ITS HOST

by

AINA VALDMANIS

A THESIS

Submitted to the College of Science and Arts
Michigan State University of Agriculture and
Applied Science in partial fulfillment of
the requirements for the degree of

MASTER OF SCIENCE

Department of Microbiology and Public Health

1956

9/25/52
9

ACKNOWLEDGMENT

Sincere appreciation is expressed to Dr. Walter N. Mack for the suggestion of the problem and guidance in the work.

TABLE OF CONTENTS

LIST OF TABLES

INTRODUCTION

The bacteriophages, like all viruses, require a living cell for their propagation. In this host--parasitic interaction, we can distinguish four distinct phases:

1. The attachment of the virus onto its susceptible host.
2. The entry of the bacteriophage protein into the host.
3. The production of prophage in lysogenic bacteria or,
4. the production of lysis and bacteriophage.

Bacteriophages are designated by a code, symbol or letter, followed by a number to distinguish them. The T-bacteriophages, which infect Escherichia coli, strain B, are designated as T-1, T-2, etc., to T-7 (Luria, 1953). The seven T-bacteriophages can be grouped into four classes by physical and serological methods. Although each specific bacteriophage can be distinguished serologically, the individual type can be classified in one of four groups. The even-numbered bacteriophages T-2, T-4, T-6, are indistinguishable in morphology and give serological cross-reactions. They are capable of genetic recombinations in mixed-infected hosts (Evans, 1952). The body of the particles are prismatic and measure 80 x 60 mu with 20 x 100 mu processes or "tails."

The T-1 bacteriophage appears spherical, or nearly so, and measures 50 mu in diameter with a 10 x 120 mu "tail." The largest of this group of bacteriophages is T-5, which has a head 90 mu with a 15 x 170 mu "tail." The final group consists of the T-3 and T-7 bacteriophages. These two viruses are spherical and the smallest. Measurements of both T-3 and T-7 indicate that they have an average diameter of 45 mu. The majority of the virus particles consists of a simple sphere, but an occasional particle, besides the spherical "head," possesses a very minute process or "tail."

Because of their larger sizes, the even-numbered viruses (T-2, T-4 and T-6) have been studied extensively. As compared with the even-numbered viruses, very little is known about the smaller T-3 and T-7 viruses.

Studies on the chemical structure of the various bacteriophages have resulted in quite uniform results. In general, the viruses consist of protein and nucleic acid, the composition depending upon the media used to produce the virus and host cells. On the other hand, Putnam and Kozloff (1949) have shown that virus prepared on various media give identical results when carefully purified. The protein composition of the bacteriophages consists of approximately 40% of their dry weight; however, N^{15} studies

indicated that only 18% of the parent protein is present in the bacteriophage progeny.

Once attachment occurs, between the bacteriophage and bacterial cell, and the nucleic acid has been taken into the host cell, the host provides everything necessary to produce the virus progeny. As already stated, one of two things happens at this time. Lysis of the bacterial cell occurs or the infection may produce a latent tendency (so-called lysogenesis) in which the virus fails to produce lysis of the host. In the latter event, there is every reason to believe that the prophage exists within the host cell, without interfering with the host economy. Liberation of the bacteriophage can be produced upon maturation of the prophage and has been done experimentally by Lwoff, in 1953, using irradiation, hydrogen peroxide and nitrogen mustard. Resistance to bacteriophage infection by the host cells occurs. The bacteriophage in this event fails to become adsorbed to the host cells. The resistance is a characteristic of the host, as heat-killed bacteriophage will readily become attached to susceptible cells; however, no progeny is produced. The number of units of bacteriophage that can be attached to a single bacterium has been estimated by Luria (1953) to be from a single virus particle to as many as 300 particles per cell. In the latter case, the bacterium-phage system is one of extreme susceptibility.

With this susceptibility, the bacteriophage is adsorbed to the host cell and is influenced by the physiological condition of the medium. The rate at which the bacteriophage becomes attached to the host cell also depends upon the growth phase of the host cells. It is generally agreed that adsorption occurs rapidly when the bacterial cells are in their logarithmic phase. The physical state is also important for adsorption. The optimum temperature is 37° C and collision between bacteria and bacteriophage must occur. The rate of attachment also depends upon the viscosity of the medium. Anderson (1950) has shown that violent stirring not only prevents adsorption, but it capable of removing particles of bacteriophage that have already become attached.

In pure water, there is no attraction between the virus and host. Adsorption will occur in the presence of certain cations and the optimum concentration of cations varies with the bacteriophage. The ion requirement for T-3 as given by Luria (1953) is 10^{-3} M Na, K or NH_4. The T-7 bacteriophage, which is physically similar to the T-3 bacteriophage, will attach to host cells in the above ion as well as 10^{-3} Mg.

Together with the ion requirements, some of the T-viruses require molecular co-factors. Anderson (1948), working with T-4 and T-6, showed that l-tryptophane and

cations are necessary medium constituents before the viruses could be adsorbed to susceptible host cells. The l-tryptophane was the most effective co-factor for adsorption; other amino acids were much less effective. Garen and Puck (1951) interpreted the role of co-factor and cation as affecting the bacteriophage surface. The co-factor modifies the surface of the virus on which ions become attached and from a certain pattern of electrical charges, which must be present to permit contact between the bacteriophage and the specific bacterial receptors. Although tryptophane acts as a co-factor with the T-4 and T-6 viruses, the kinetics of tryptophane action is complex, as was illustrated by Delbrück (1948). Indole, a tryptophane analogue is a competitive inhibitor of tryptophane reaction. The activity of indole as a tryptophane inhibitor is remarkable, in that it provides an example of a potential, specific defense mechanism against viruses. Indole is a product of the metabolism of tryptophane by Escherichia coli, the host bacterium for the tryptophane requiring phages T-4 and T-6. With these two strains of virus, tryptophane is required, as is the presence of cations. However, an excess of tryptophane in the medium produces indole, which can act to prevent adsorption of these bacteriophages to the host's cells.

Experiments in this laboratory with the T-3 bacteriophage require that large volumes of virus in high concentration be produced. Virus is best produced in nutrient broth, which contains all known, essential growth factors for both the virus and the E. coli cells. Infective virus concentration of 10^8 to 10^9 virus particles per ml can be obtained using nutrient broth. Concentrations of virus produced in nutrient broth have resulted in satisfactory virus titers. However, the concentration procedures also accumulate other protein fractions besides virus protein. The resulting product contains high protein content and this protein is not associated with the virus. When synthetic medium is substituted for the nutrient broth medium, the nitrogen content of the virus concentrate is decreased, as well as the virus content.

The work reported in this thesis is an attempt to determine if the addition of one or more amino acids to a synthetic medium would act as co-factors and produce an increase in virus adsorption. To test the efficiency of the specific amino acid to act as co-factor, the experiments were designed to determine the number of virus particles remaining unadsorbed when virus of concentration was placed together with susceptible host cells.

MATERIALS AND METHODS

The purity of the bacterial culture was tested by growing the organisms on eosin-methylene blue agar plates. Typical colonies of Escherichia coli, strain B, were isolated and used throughout this study. The bacterium is Gram negative and non-motile. Stock culture of the organism were made on nutrient agar slants containing o.4 per cent gentian violet to prevent the growth of Gram positive organisms. Seeded stock cultures were incubated 28 hours at 37° C and then stored until used at 4° C. E. coli, strain B, is susceptible to infection with all of the T-group bacteriophages.

Lysates were produced by infecting large numbers of E. coli cells with the T-3 virus. The bacterial cells contained on the surface of an agar slant were removed from the culture by adding 5 ml of sterile distilled water to the tube. The cells were removed by lightly rubbing a wire loop over the surface of the agar. The resulting heavy bacterial suspension was then used to seed Petri plates containing synthetic agar. From 1 to 2 ml of the bacterial suspension was spread evenly over the surface of the agar by a small, glass triangle spreader. Upon incubating the seeded plates for 6 to 8 hours at 37° C, an

even, heavy growth of bacterial cells was found to completely cover the surface of the agar.

Five-tenths ml of T-3 bacteriophage was added to each of the Petri plates containing the bacterial cells. Again, even distribution of the virus over the entire area was insured by employing the glass spreader. The Petri plates were again incubated at 37° C for 18 to 24 hours. The resulting crude lysate on the agar surface contained virus, bacterial cellular debris and occasionally virus-resistant colonies of E. coli. The lysate was removed from the Petri plates by adding 5 ml of sterile distilled water to each Petri plate. The glass spreader was used to rub the agar surface and separate as much virus as possible from the agar. The bacterial cellular debris and virus-resistant organisms were separated from the virus by centrifugation. Suspensions of the crude lysate were placed in the International, multi-speed, refrigerated centrifuge and centrifuged at 9000 r.p.m. for 15 minutes at 4° C. The resulting clear, supernatant fluid was removed from the sediment and constituted as the stock virus lysate. The stock virus is relatively stable at 4° C.

The virus was assayed by the soft agar technique described by Gratia (1936). Ten-fold dilutions of the virus were made, using nutrient (Difco) broth as diluent. One-tenth ml of the diluted virus was mixed with 0.5 ml of the

heavy bacterial suspension on a melted 0.7 per cent agar diluent and poured over the surface of standard agar plates. The base agar consisted of 1.5 per cent agar and was used for a foundation. The efficiency of the assay method has been discussed by Adams (1950) and, providing the bacterial cells are actively growing at an exponential rate and the virus is viable, a plaque is the result of a single viable virus particle. The resulting plaques were counted on each plate, the number of plates per dilution averaged, and the total number of virus particles per ml computed, considering the original virus dilution.

The amino acids used in these experiments were purchased from Nutritional Biochemicals Corporation, Cleveland 28, Ohio. Seven amino acids were tested in this study and the choice of amino acids used were those commonly employed in bacteriological laboratory media. Each amino acid was tested independently as an adsorption co-factor by adding the amino acid to synthetic medium. The ability of the bacteriophage to attach to the bacterial cells was then determined. Using this same method, all seven amino acids were combined with either synthetic medium or water, and the adsorption of virus to cell determined. For comparison, the adsorption of virus to host cells was determined with the virus and cells suspended in Difco nutrient broth and synthetic liquid medium.

There is no information available regarding the amount of any specific amino acid required for adsorption of host and virus. The selective amino acid medium described in the ninth edition of the Difco manual (p. 230) was consulted and comparable amounts of each specific amino acid were incorporated into the synthetic medium (see Table 1).

TABLE 1

CONCENTRATION OF AMINO ACIDS INCORPORATED INTO SYNTHETIC MEDIUM, AS COMPARED TO NUTRIENT BROTH

Amino Acids	Per Cent Amino Acid in Nutrient Broth	Per Cent Amino Acid in Synthetic Medium
Arganine	8.0	0.04
Glutamic acid	11.0	0.02
Leucine	3.5	0.05
Lysine	4.5	0.05
Phenylalanine	2.3	0.02
Valine	3.2	0.05
Tryptophane	0.29	0.01

Less amino acid was added to the synthetic (E) medium than is found in nutrient broth, to prevent an excess of amino acid breakdown products from becoming inhibitors.

When the seven amino acids were pooled and tested in synthetic medium and water, the amino acids were used in the same concentration as when single amino acids were used.

The synthetic (F) medium has been used for bacteriophage production extensively and is discussed by Adams (1950) in his review on bacteriophage. The composition of his medium is as follows:

NH_4Cl	1.0 Gm.
$MgSO_4$	0.1 Gm.
KH_2PO_4	1.5 Gm.
Na_2HPO_4	3.5 Gm.
Lactic Acid	9.0 Gm.
H_2O	1000.0 ml.

Adjusted pH to 6.8 with NaOH.

When solid medium was required, 15 gm. of agar were added to the above synthetic medium.

Difco nutrient broth contains the essential growth nutrients to support growth of E. coli cells. When T-3 is permitted to infect bacterial cells growing in nutrient broth, the resulting lysate contains greater virus concentration per volume than lysates produced in either liquid or solid synthetic medium.

It was, therefore, decided that any tests on the adsorption of virus on cells should be compared to the adsorption taking place on cells suspended in Difco's nutrient broth.

Prior to each adsorption test, fresh bacterial cultures were prepared. The surface organisms from the stock culture of E. coli were removed by adding 5 ml of sterile, distilled water to the slant culture. The resulting heavy suspension of the organisms was used to seed a nutrient broth culture, which was incubated for 18 hours at 37° C. At the end of the incubation period, such cultures were found to contain approximately 10^{9} bacterial cells per ml, many in the growth phase. To remove the bacterial cells from the nutrient broth, the culture was centrifuged in the multi-speed, refrigerated centrifuge at 9000 r.p.m. for 15 minutes. The supernatant fluid was discarded and the bacterial sediment was resuspended in 10 ml of sterile, distilled water. A second cycle of centrifugation sedimented the cells. The sediment was finally resuspended in nutrient broth, synthetic medium or in water, depending on the tests performed. Regardless of the suspending media, the tubes containing the bacterial cells were vigorously shaken and aspirated forcibly from a pipette to break up bacterial clumps. The sediment was suspended into approximately 15 ml. of the desired media. Only 13.5 ml from the above sus-

pension was then used in the test. The tubes containing the bacterial cells suspended in the test medium were placed in a 37° water bath and allowed to come to constant temperature, before 1.5 ml of T-3 bacteriophage was added to the bacterial cells. The titer of the virus was so adjusted that 1 ml of suspension contained 10^6 virus particles. Immediately after adding the virus to the bacterial cells and mixing, 2 ml of the virus cell mixture was removed from the tube. This 0 time sample was placed into a test tube and quickly plunged into a dry ice, alcohol bath to prevent further adsorption of the virus to the cell. Similar samples were collected at 5, 10, 15, and 20 minutes.

The samples were allowed to liquefy at 4° C and then centrifuged at 9000 r.p.m. for 10 minutes to separate the unadsorbed virus from the bacterial cells and adsorbed virus. The supernatant fluids were assayed for their content after dilution by the soft agar technique.

RESULTS

The results of these experiments are shown collectively in Table 2. Each individual adsorption experiment was repeated three times, and the results shown in the Table are averages of the experiments.

Immediately after mixing the virus and host cells, a sample of the mixture was removed and adsorption inhibited by reducing the temperature of the sample to 0° C. This first sample represents the virus concentration in particles per ml at 0 minutes. The per cent adsorption of virus to the cells was computed on the samples collected at 0 and 15 minutes, based on the concentration of virus remaining in the supernatant fluid of the samples. In every experiment, regardless of amino acid or medium used, the sample taken at the 15 minute period contained the least amount of virus remaining unadsorbed. Within the next 5 minutes, i.e., 20 minutes, the progeny began to develop, resulting in an increase in virus concentration.

As can be seen in Table 2, the most suitable medium for adsorption of the virus to the cell was nutrient broth in which forty-nine per cent of the virus was removed from the supernatant fluid. Only about half as much adsorption took place (21%) when the synthetic medium was used without

TABLE 2

CONCENTRATION OF T-3 BACTERIOPHAGE ADSORBED ON *ESCHERICHIA COLI* IN 15 MINUTES

Media or Amino Acids Used in the Adsorption Test	Virus Titer of Supernatant Fluid Time in Minutes 0	5	10	15	20	Per Cent Adsorption
Nutrient Broth	1.2×10^6	9.0×10^5	7.1×10^5	6.2×10^5	7.8×10^5	49
Synthetic Medium	1.4×10^6	1.4×10^6	1.3×10^6	1.1×10^6	1.2×10^6	21
Synthetic Medium with Seven Amino Acids	1.2×10^6	1.1×10^6	1.0×10^6	6.6×10^5	8.5×10^5	45
Aqueous Solution of Seven Amino Acids	1.3×10^6	1.1×10^6	1.0×10^6	9.8×10^5	1.1×10^6	25
L-Arganine in Synthetic Medium	1.2×10^6	1.1×10^6	1.0×10^6	9.3×10^5	1.1×10^6	22.5
D-Glutamic Acid in Synthetic Medium	1.4×10^6	1.2×10^6	1.1×10^6	9.5×10^5	1.1×10^6	31
L-Leucine in Synthetic Medium	1.9×10^6	1.8×10^6	1.5×10^6	1.1×10^6	1.7×10^6	42
L-Lysine in Synthetic Medium	1.4×10^6	1.3×10^6	1.1×10^6	1.0×10^6	1.3×10^6	28
DL-Phenylalanine in Synthetic Medium	1.9×10^6	1.8×10^6	1.6×10^6	1.27×10^6	1.8×10^6	33
DL-Valine in Synthetic Medium	1.2×10^6	1.1×10^6	1.0×10^6	9.4×10^5	1.0×10^6	21
L-Tryptophane in Synthetic Medium	1.9×10^6	1.3×10^6	1.2×10^6	1.1×10^6	1.6×10^6	42

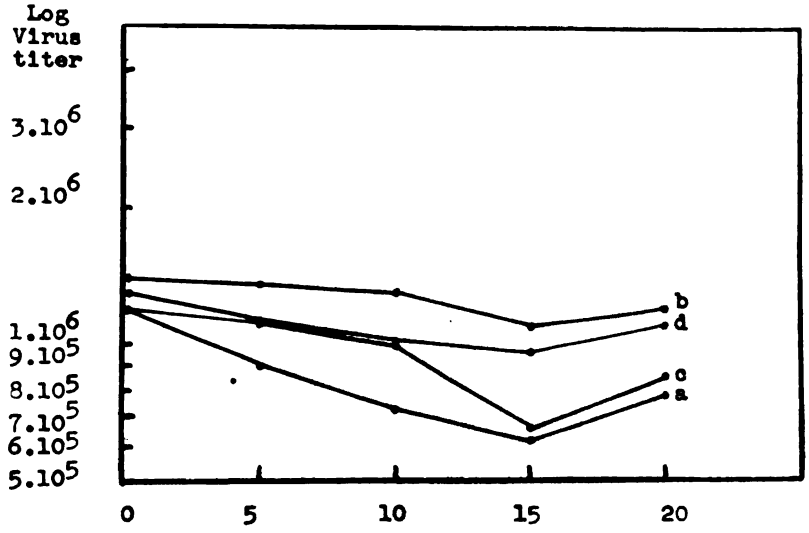
added amino acids. When the seven amino acids were combined with the synthetic medium, however, the efficiency of the adsorption increased to 45% (Table 2), which, when considering the experimental errors, would be equal to the results obtained by the use of nutrient broth. The presence of carbon and ions in the synthetic medium is important for the adsorption process as can be seen by the results of the next experiment. In medium composed of the seven amino acids in distilled water, 25% adsorption of the virus occurred. The results of these four experiments are illustrated as log-dilutions in Table 3.

The addition of a single amino acid to the synthetic medium produced progressively less adsorption than a complete medium, depending upon the specific amino acid used. L-Arganine permitted only 22.5% adsorption, while D-glutamic acid allowed 31% adsorption. The concentration of these amino acids in the synthetic medium was 0.04% and 0.02%, respectively. When 0.05% L-leucine was added to the synthetic medium, 42% of the virus particles were found to be adsorbed. Leucine was one of the two most effective single amino acids in enhancing the adsorption process (Table 4).

L-Lysine (0.05%), DL-Phenylalanine (0.02%), and DL-valine (0.05%) produced 28, 33 and 21% adsorption, respectively.

TABLE 3.

Adsorption of T-3 on E.coli in Nutrient broth, Synthetic medium, Synthetic medium with seven amino acids and Aqueous solution of seven amino acids.

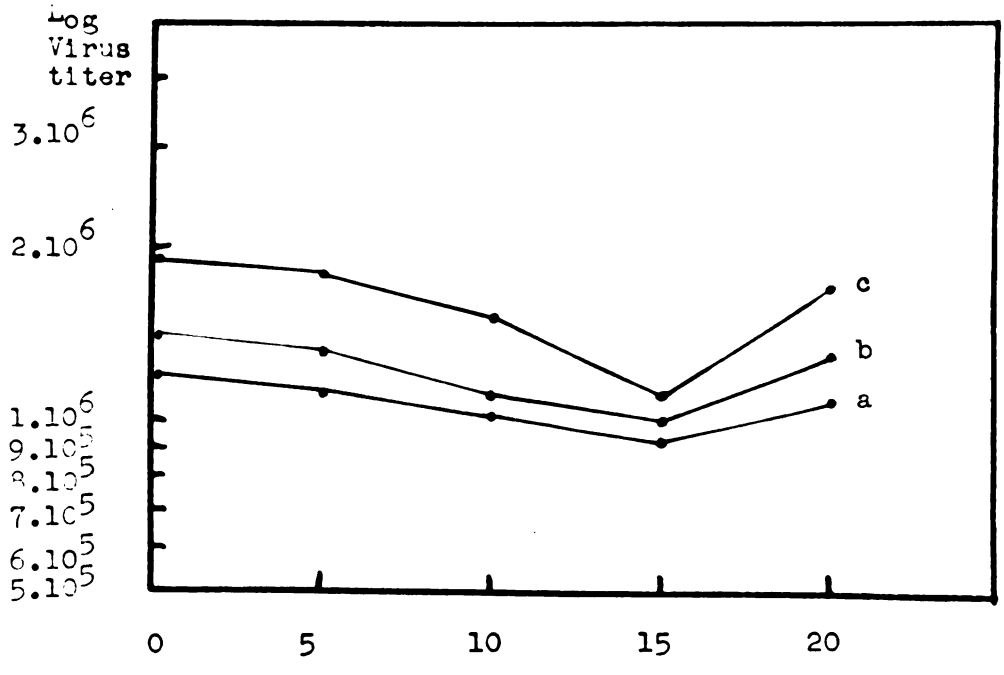


a) Nutrient broth
b) Synthetic liquid medium
c) Synthetic medium with seven amino acids.
d) Aqueous solution of seven amino acids

TABLE 4.

Adsorption of T-3 on E.coli in liquid

Synthetic medium containing single amino acids.



a) Synthetic medium with L-Arganine
b) Synthetic medium with L-Lysine
c) Synthetic medium with L-Leucine

The last amino acid tested was 0.01% concentration of L-Tryptophane in synthetic medium. Forty-two per cent of the virus particles were found to be adsorbed to their hosts within the 15 minute period. It appears from Table 5 that tryptophane reduced the free virus from the medium early in the test period. A 0.3 log. decrease was found within the first five minutes of the test with tryptophane. It also is evident that very small concentrations of this amino acid are active in enhancing adsorption.

DISCUSSION

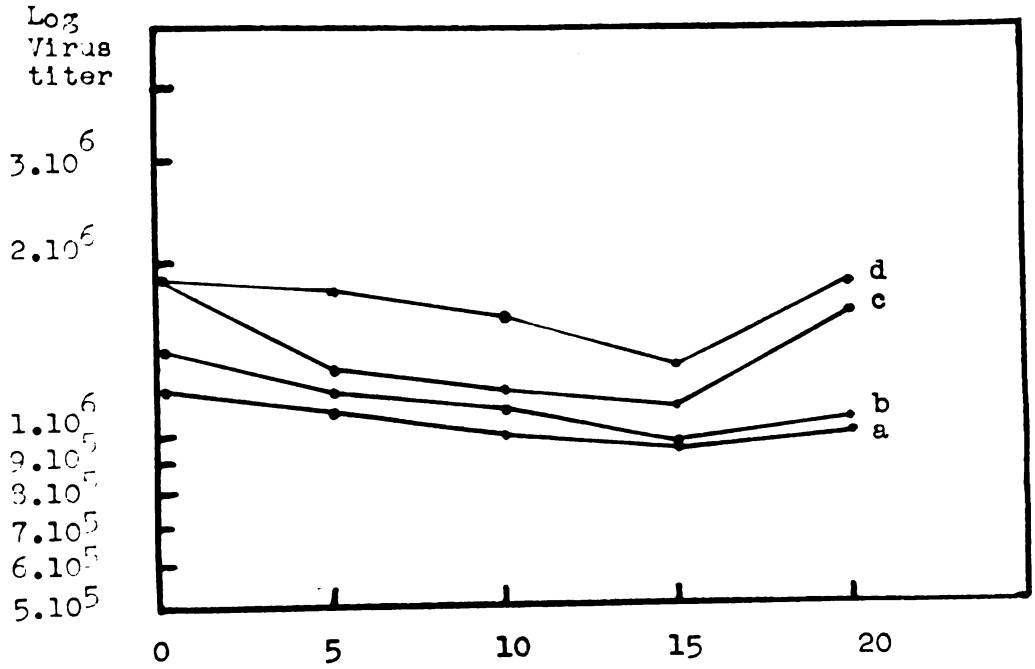
The T-3 bacteriophage does not require a co-factor, as is evidenced by the fact that adsorption will occur when the virus and host are suspended in a synthetic medium. Unlike the T-4 and T-6 viruses, which will not adsorb without trace amounts of tryptophane, the T-3 virus will be adsorbed to its host without a co-factor, but only to a limited extent. The presence of 0.01% tryptophane or 0.05% leucine enhanced adsorption considerably, but was not essential for adsorption. The mechanism by which these amino acids enhance attachment of the T-3 virus is unknown. Their presence either affects the metabolism of the host cell or in some way helps produce union between receptors of the bacterial cell and virus particle. The effect of tryptophane upon the adsorption is an immediate one, since with 5 minutes of contact between virus and host the free virus in suspension shows a decrease (Table 5) in concentration. Although leucine (Tables 2 and 4) produced an equal amount of adsorption, the period of adsorption was not evident until after 10 minutes contact between the virus and bacterial cells.

None of the amino acids was as efficient in producing adsorption, nor was a pool of the amino acids compar-

TABLE 5.

Adsorption of T-3 on E.coli in liquid

Synthetic medium containing single amino acids.



a) Synthetic medium with DL-Valine
b) Synthetic medium with D- Glutamic acid
c) Synthetic medium with L- Tryptophane
d) Synthetic medium with DL-Phenylalanine

able to the nutrient broth. This was to be expected, as the nutrient broth contained all of the essentials for growth of the host's cells.

The presence of cations was not involved in these experiments, except when the amino acids were pooled in distilled water. It is surprising that as much adsorption, 25% (Table 2), took place in water solution, since the ion requirements were not present. When synthetic medium was used, the ion requirements were fulfilled, so that the increase in adsorption was the result of the presence of the amino acid under test.

SUMMARY

1. Seven amino acids, singly or pooled in synthetic medium, were tested for their ability to produce adsorption of the T-3 bacteriophage to Escherichia coli cells.

2. Two amino acids, tryptophane and leucine, were found to enhance adsorption. Tryptophane in 0.01% concentration and 0.05% leucine each produced 42% adsorption. The effect of tryptophane on adsorption was found to act within the first five minutes of contact, while leucine was slower to effect union between virus and cell.

3. A pool of seven amino acids in synthetic medium produced 49% adsorption as compared to 21% for synthetic medium alone.

4. Nutrient broth was the most effective in enhancing union between virus and cells. Forty-nine per cent of the virus was adsorbed to the cells in nutrient broth within 15 minutes.

REFERENCES

1. Adams, M. H. (1950) Methods of study of bacterial viruses. In: Methods in Medical Research, Vol. 2:1-73

2. Anderson, T. F. (1948) The role of tryptophane in the adsorption of 2 bacterial viruses on their host E. coli. Journal of Bact. 55:651.

3. _____ (1950) Destruction of bacterial viruses by osmotic shock. Journal of Appl. Phys. 21:70.

4. Delbrück, M. (1948) Biochemical mutants of bacterial viruses. Journal of Bact. 56:1-16.

5. Difco Manual of Dehydrated Culture Media and Reagents for Microbiological and Clinical Laboratory Procedures. Ninth ed. Detroit, Mich. (1953)

6. Evans, E. A. (1952) Biochemical studies of bacterial viruses. University of Chicago Press. Chicago, Ill.

7. Garen, A., and T. T. Puck (1951) The mechanisms of virus attachment to the host cells. Repr. from Journal of Exp. Medicine, Vol. 93:1 Jan.

8. Gratia, A. (1936) Numerical relation between lysogenic bacteria and particles of bacteriophage. Ann. Inst. Pasteur 57.

9. Luria, S. E. (1953) General Virology, John Wiley & Sons Inc., New York.

10. Lwoff, A. (1953) The nature of phage production. In: The nature of virus multiplication. Cambridge University Press.

11. Putnam, F. W., and L. Kosloff (1949) Biochemical studies of virus reproduction: 1. Purification and properties of Escherichia coli-bacteriophage. J. of Biol. Chem. 179:303.

Date Due

May 6 '57			