The Relation between the Synthesis of Deoxyribonucleic Acid and the Synthesis of Protein in the Multiplication of Bacteriophage T2

By K. BURTON

Department of Biochemistry, University of Chicago, and Medical Research Council Unit for Research in Cell Metabolism, Department of Biochemistry, University of Oxford*

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Cohen & Fowler (1947) and Cohen (1948) showed that 5-methyltryptophan, which inhibits the biosynthesis of tryptophan (Beerstecher, 1954), can prevent the development of bacteriophage and the net synthesis of deoxyribonucleic acid (DNA) in *Escherichia coli* infected with bacteriophage T2, indicating that the synthesis of DNA is, in this system, dependent on the synthesis of protein. This paper is concerned with a further study of this relationship between the two synthetic processes. In some of the experiments, 5-methyltryptophan has been used to inhibit the synthesis of tryptophan and hence of protein. In most experiments, however, bacterial mutants which require either histidine, leucine, tryptophan, tyrosine or purines for growth have been used as host cells for the bacteriophage. The primary effect of a deficiency in any of the four amino acids is expected to be the inability to synthesize proteins.

Bacteriophage T6r⁺ was used in the earlier experiments, but in the multiply infected cultures necessary for the metabolic studies considerable difficulty was experienced owing to a variable and unpredictable premature lysis which occurred without liberation of phage (cf. Hershey, Dixon & Chase, 1953). With the closely related bacteriophage T2r this phenomenon is much less frequent, and this bacteriophage was therefore used for the majority of the experiments.

MATERIALS AND METHODS

Bacteria. *Esch. coli* B and its nutritional mutants were used. The tyrosine-requiring strain was isolated by Dr J. Ceithaml of the University of Chicago from ultraviolet-irradiated cultures of *Esch. coli* B/1. The tryptophan-requiring strain (B/1, *t*) was obtained from Dr A. Novick of the University of Chicago and is a mutant of *Esch. coli* B selected by its resistance to bacteriophage T1. B-33 (leucine⁻), B-62 (histidine⁻), B-38 (adenine⁻) and B-96 were obtained from Dr J. S. Gots of the University of Pennsylvania. Strain B-96 is unable to synthesize purines and accumulates 4-amino-5-imidazolecarboxamide and its derivatives in the growth medium (Gots, 1950, 1953); adenine, guanine, hypoxanthine or xanthine can satisfy the purine requirement of this strain.

Bacteriophage. The T2r and T6r⁺ strains did not require tryptophan for adsorption to the host cell. The stocks were obtained by confluent lysis of *Esch. coli* B (cf. Adams, 1950) on the surface of agar medium in 9 cm. diameter Petri dishes. Two layers of agar were used; the lower layer (20 ml.) contained 0.8% Bacto-tryptone (Difco), 0.5% NaCl and 2% agar. The upper layer (3 ml.), which was seeded with about 5×10^7 bacteria and about 10^4 phage, contained the same amounts of tryptone and NaCl but only 0.8% agar. The bacteriophage was purified by two cycles of differential centrifuging (Kozloff & Putnam, 1949).

5-Methyltryptophan. This was kindly supplied by Dr S. S. Cohen.

Infective centres. Infective centres (i.e. plaque-forming particles) were determined after suitable dilution in buffered saline, a double-layer technique (cf. Adams, 1950) being used. The agar layers were similar to those used in the preparation of the bacteriophage and the same amount of bacteria (5×10^7 cells) was added to the upper layer. The plaque count does not differentiate between free phage particles and infected cells which are capable of liberating phage.

Growth media. 'Lactate' medium was the 'F' medium of Cohen & Anderson (1946). It contained sodium lactate and NH_4Cl as sole sources of carbon and nitrogen respectively. 'Mannitol' medium was similar, except that the sodium lactate was replaced by 1% mannitol plus 0.1M-NaCl. Individual L-amino acids were added as required for the appropriate mutants at the following concentrations: tyrosine, 100 mg./l.; histidine-HCl, 10 mg./l.; leucine, 50 mg./l. The purine-requiring mutants were grown with either adenine (7 mg./l.) or adenosine (15 mg./l.) as a supplement.

The mannitol medium was very suitable for experiments involving multiply infected cells (about 5 phage per bacterial cell) since there was little tendency for premature lysis. The medium was not, however, suitable for experiments with low multiplicities of infection (<0.05 phage per cell) as the singly infected cells gave very variable yields and latent periods. In the lactate medium, although premature lysis tended to occur on multiple infection, the yields and latent periods were more reproducible. In general, therefore, the lactate medium was used for experiments at low multiplicities of infection, and the mannitol medium for experiments involving multiply infected cells.

However, the mannitol medium, when supplemented with acid-hydrolysed casein (0.5% Difco casamino acids) and adenosine (15 mg./l.), gave reproducible phage yields and latent periods, and premature lysis did not follow multiple infection. Since this fortified medium was deficient in tryptophan it was used for all the experiments

* Present address.

with the tryptophan-requiring strain and is designated as 'fortified mannitol medium'. To compensate for the NaCl present in the casein hydrolysate, the amount of NaCl added in making up this medium was only 0.084M, instead of 0.10M in the unfortified mannitol medium.

Under conditions of multiple infection with T2r in the lactate or unfortified mannitol media, lysis was often delayed for 1 or 2 hr., although mature intracellular phage appeared at about 18 min. after infection. In the fortified mannitol medium there were visible signs of lysis (intense frothing followed by progressive clearing of the culture) at about 35 min. after infection provided that there was no interference with the phage multiplication by nutritional deficiency.

Buffered saline. This contained 5.85 g. NaCl, 1.5 g. KH_2PO_4 , 3.4 g. Na_2HPO_4 and 10 mg. MgSO_4 per l.

Growth conditions. For each experiment, bacteria were freshly grown at 37° with good aeration to about 7×10^8 cells/ml. The medium used for the development of the infected cells was identical with that used for the growth of the host bacteria, except for the absence of the growth factor or the presence of the analogue. In all experiments where the behaviour in the absence of a growth factor was compared with that in its presence, a culture was washed and divided into two portions, to one of which the growth factor was added. Cultures were not aerated if the bacterial concentration was less than 10^5 cells/ml.; it was sufficient that the medium had previously been saturated with air.

Washing of cells. This was performed at 4° and required 30–45 min. The culture was centrifuged at 4000 rev./min. for 5 min. in the Servall-type SS-1 centrifuge, the pellet suspended in the original volume of buffered saline, centrifuged and then resuspended. The resuspending fluid was buffered saline if the cells were to be infected with a low ratio (<0.1) of phage particles to bacterial cells. In order to minimize premature lysis when multiply infected cells were required the resuspending medium was similar to the original growth medium except that it lacked the appropriate amino acid or purine.

After decanting the supernatant fluids from the pellets, excess of fluid was removed from the internal walls of the centrifuge tube by means of absorbent tissue.

One-step growth experiments. Washed bacteria (about 7×10^8 viable cells/ml.) were infected in buffered saline with 10^7 bacteriophage per ml. and 5 min. at 37° allowed for adsorption. Measured portions were then diluted 10^3 -fold into two tubes of medium, one containing and one lacking the required growth factor. In studying the delayed addition of the growth factor tenfold dilutions were subsequently made from both tubes into fresh medium which, when necessary, contained the growth factor. Bacteriophage liberation was followed by plaque count.

Under these conditions the bacteriophage are liberated at a very low concentration of bacterial cells, so that the rate of adsorption on the uninfected cells present is very slow. A single cycle of bacteriophage growth is thus studied.

Ultraviolet irradiation experiments. Cells were infected as in the one-step growth experiments and then cooled in an ice bath. Samples were diluted 50-fold into growth media from which, at intervals, 0.1 ml. samples were diluted 20-fold into buffered saline and immediately exposed to ultraviolet light in Petri dishes of 9 cm. diameter. During exposure the dishes were gently shaken on the horizontal

platform of a rotary shaker; the platform was 33 cm. below a 15w germicidal lamp and the exposure time was usually 7 sec. The infective centres surviving the irradiation were determined by plaque count.

Chemical analyses

Nucleic acid. A 10 ml. sample of the culture containing about 5×10^9 cells was mixed with 0.22 ml. 70% (w/w) HClO_4 . After about 2 hr. at 4°, it was centrifuged in a tapered centrifuge tube and the precipitate resuspended in 3 ml. water. n-HClO_4 (0.75 ml.) was then added and the sample was recentrifuged after about 30 min. at 4°. The pellet was twice extracted with 4 ml. 0.5N- HClO_4 for 10 min. at 70° and the extracts, which contained the nucleic acids, were combined.

DNA was estimated by mixing 2 ml. of the extract with 0.1 ml. 0.07M acetaldehyde and 4 ml. diphenylamine reagent (7.5 g. steam-distilled diphenylamine dissolved in 500 ml. acetic acid plus 7.5 ml. conc. H_2SO_4). After 16–25 hr. at 25–30° the optical density was read in a Beckman spectrophotometer at 600 μm . and compared with that given by calf-thymus DNA of known phosphorus content. The standard DNA was heated for 15 min. at 70° in 0.5N- HClO_4 before mixing with the reagent. The intensity of colour is very nearly proportional to the amount of DNA present; 0.2 μmole DNA phosphorus or 0.1 μmole 2-deoxy-L-ribose results in an optical density of about 0.35 at 600 μm . This modified diphenylamine reaction is an improvement over the usual procedure (Dische, 1930) involving heating with the reagent at 100° for 10 min. in that it is more sensitive and specific and is much less susceptible to interference by other compounds. Five mg. of a commercial yeast RNA preparation gave the same reading as 0.011 mg. DNA, and 4 mg. each of several substances, including fructose, sucrose, starch, glycogen, glucosamine, ascorbic acid, rhamnose, ribose, acid-hydrolysed casein and tryptophan, gave less colour than 1.5 μg . DNA. Except for ascorbic acid, which suppresses the colour development, none of these compounds influenced the amount of colour obtained from DNA. The method is at least as sensitive and specific as those of Cohen (1944), Stumpf (1947) and Ceriotti (1952). Further properties of the reaction will be described in a later publication.

The results are expressed as moles DNA-nucleotide by comparison with calf-thymus DNA. Diphenylamine appears to react with the sugar derived from the purine nucleotides of the DNA, but not with that derived from the pyrimidine nucleotides, since the latter are not hydrolysed under the conditions used, whereas purine deoxyribose derivatives are very labile to acid. DNA's of different origins, including calf thymus, *Esch. coli* and T2 bacteriophage, all have a purine/pyrimidine ratio very close to unity (Chargaff, 1955); they are therefore all expected to give the same amount of colour with diphenylamine for the same amount of DNA nucleotide.

The RNA values are obtained by the orcinol method (Ogur & Rosen, 1950), with ribose as a standard and a heating time of 45 min. at 100°. To make them comparable with the DNA values they are arbitrarily expressed as RNA nucleotide on the assumption that 1 mole ribose is equivalent to 2 moles RNA nucleotide.

Purines and pyrimidines. These were determined in the DNA of the cultures by the procedure of Hershey *et al.* (1953). The procedure involves separation of the DNA

fraction after alkaline degradation of the RNA, hydrolysis by 6N-HCl followed by paper chromatography and spectrophotometric analysis of the bases.

RESULTS

Amino acid requirements for bacteriophage development

One-step growth experiments showed that each of the four amino acid-requiring mutants did not support the growth of T2r or of T6r⁺ in the absence of the required amino acid. In the presence of the amino acid the latent period and burst sizes were similar to those obtained with the wild-type strain.

Fig. 1. Phage liberation in one-step growth experiment from tyrosine-requiring *Esch. coli* infected with phage T6r⁺ in the absence of tyrosine. x, L-Tyrosine (0.1 g./l.) added at time of diluting into growth medium (0 min.); Δ, tyrosine added at 5 min.; O, tyrosine added at 11 min. Lactate medium.

Delayed addition of required amino acids. In experiments with the mutants requiring tyrosine and tryptophan (Figs. 1 and 2), the washed cells were infected with phage in buffered saline and then diluted into growth medium. If the required amino acid was initially absent but added 5 or 10 min. later, the liberation of progeny bacteriophage was delayed by almost the same time as that for which the amino acid had not been present. The results shown in Figs. 1 and 2 suggest that during a period of 10 min. in the absence of the

amino acid less than the equivalent of about 2 min. of normal bacteriophage development takes place, despite the presence of all other essential nutrients; development appears to continue normally when the amino acid is subsequently supplied. If a required amino acid is withdrawn after the infected cells have been in a complete medium for several minutes, there is again a suspension of phage development which is resumed on later addition of the amino acid. In the experiment shown in Fig. 3 the tyrosine-requiring mutant was infected and allowed to develop in complete medium for 5 min. before separating the cells and the amino acid by centrifuging. Tyrosine was required for the subsequent liberation of phage. When tyrosine was added after a period of 15 min. in medium lacking

Fig. 2. Phage liberation from tryptophan-requiring *Esch. coli* infected with phage T2r in the absence of tryptophan. x, L-Tryptophan (50 mg./l.) added at time of diluting into growth medium (0 min.); Δ, tryptophan added at 10 min.; O, no tryptophan. Fortified mannitol medium.

tyrosine, the release of phage was, in most of the cells, delayed by about 15 min.

These results are analogous to those of Cohen & Fowler (1947) on the action of 5-methyltryptophan. The inhibition of phage and bacterial growth due to this compound are reversed by tryptophan in accordance with the concept that the analogue inhibits the biosynthesis of tryptophan.

Delayed addition experiments, while very suggestive are, however, ambiguous since the same results could be obtained by partial development in the absence of the amino acid followed by a slower

than normal development when the amino acid was added. For this reason the ultraviolet sensitivity of the infected cells was studied as another indication of their development.

Fig. 3. Phage liberation from tyrosine-requiring *Esch. coli* which had developed in complete medium for 5 min. after infection with phage before withdrawal of the tyrosine. 10^8 cells/ml. in buffered saline at 37° were allowed to adsorb 1.5×10^7 T2r/ml. for 5 min. One volume of lactate medium containing L-tyrosine (8 mg./l.) was added and after 5 min. aeration at 37° the culture was centrifuged at 4° and re-suspended in fresh lactate medium at 37° . x, L-Tyrosine (0.1 g./l.) added at time of re-suspending (0 min.); Δ , tyrosine added at 15 min.; O, no tyrosine. Without centrifuging the infected cells, phage liberation started at 28 min. after infection.

Ultraviolet sensitivity of infected cells

Irradiation of phage-infected cells with ultraviolet light reduces the number of cells which can yield bacteriophage, the radiation sensitivity depending on the state of development at the time of irradiation (Luria & Latarjet, 1947). After the doses of irradiation used in the present experiments, between 12 and 25 % of the infected cells are able to yield phage if irradiated immediately after the infection with phage. During the first 5 min. of development in growth medium the cells start to become much less sensitive. If irradiated with the same doses after 20–25 min. in the growth medium, more than 95 % of the infected cells are able to yield phage. As shown by Luria & Dulbecco (1949)

the increase in the resistance to ultraviolet light is greatest in cultures which have been infected with a low ratio (0.01–0.02) of phage to bacteria. The uninfected cells which are present do not interfere in the experiment since the plaque-counting technique scores only free phage or infected cells capable of liberating phage.

Fig. 4. Effect of ultraviolet light on T6r⁺-infected cells of tyrosine-requiring *Esch. coli* following different periods of development in lactate medium after infection. O, Development in the presence of L-tyrosine (0.1 g./l.); x, no tyrosine. For procedure see text under 'Materials and Methods'.

With each of the four amino acid-requiring mutants used in this work, the appropriate amino acid has been found to be essential for the change in ultraviolet sensitivity of cells infected with either the T2r or the T6r⁺ bacteriophage (e.g. Fig. 4). This is taken to indicate that an early stage in the development of phage-infected cells requires these four amino acids. The change of sensitivity requires a source of metabolic energy for the infected cells (Benzer, 1952; Benzer & Jacob, 1953).

It should be noted that Luria & Latarjet (1947) consider that the primary effect of ultraviolet irradiation is on the intracellular phage rather than on the host cell. Perhaps the most striking evidence for this view is that bacteria which have been killed by ultraviolet irradiation are still capable of supporting phage growth. Immediately after infection with a single T2 bacteriophage, the ability of the cells to form phage is much more susceptible to the irradiation than is the viability of the uninfected cells.

Adsorption and invasion

Since amino acids are needed early in the infective cycle it must be considered whether the adsorption of the infecting phage and the introduction of viral DNA into the host cell require a supply of amino acids. In most of the above experiments, it is clear that the primary attachment of the phage to the bacterial cell (Puck, 1953) must have occurred before dilution into the growth medium containing amino acids. Otherwise, the rate of attachment in the diluted cultures would have been too slow for any appreciable development to occur.

Hershey & Chase's (1952) experiments on the introduction of the phage DNA into the host cell do not exclude an amino acid requirement for this step, because the bacteria had not been washed free of the nutrient broth in which they had been grown. To investigate this possibility the tyrosine-requiring mutant was grown in mannitol medium supplemented with tyrosine, washed and resuspended at 10^8 cells/ml. in medium lacking tyrosine. Bacteriophage T2r (2×10^8 /ml.) were then added. After 4 min. at 37° for adsorption, a sample was diluted with 20 vol. of mannitol medium at 0° and treated in the Waring Blendor for 5 min. No infective centres were destroyed. In another experiment, the conditions of Hershey & Chase (1952) were followed except that the tyrosine mutant was used and washed in buffered saline before infecting with phage. After treatment in the Blendor for 8 min., 90 % of the original number of infective centres were sedimentable at 2000 g in 10 min. and 7 % remained in the supernatant. The infective centres therefore consisted of infected bacteria and not of free phage.

The shearing forces produced in the Blendor remove the bulk of the phage protein from infected cells within a few minutes, so that, if the infective portion of the phage had not entered the cell it would have been removed with the phage protein. This would have caused a loss of sedimentable infective centres.

In another experiment, which essentially followed the procedure of Anderson (1953), it was shown that, after infecting the histidine-requiring *Esch. coli*, 80 % of the infective centres became resistant to osmotic shock in 5 min., independent of the presence of histidine. In this experiment, the washed bacteria (7×10^8 cells/ml.) were suspended in a mixture of 4 vol. mannitol medium and 1 vol. glycerol before infecting with T2r (0.04 phage per bacterium) which had previously been incubated in the glycerol-containing medium for 30 min. at 37° . Samples were subjected to osmotic shock by rapid dilution into 100 vol. of water. At least 99 % of the free phage are inactivated by this treatment and

the resistance which develops in the presence of the bacteria is presumably an indication of the entry of the DNA of the infecting bacteriophage into the host cell.

Effects of purine deficiency

Delayed addition (Figs. 5, 6) and ultraviolet sensitivity experiments with the purine-requiring mutants (Fig. 7) showed that an external source of purine was not required for the early stages of development. In some experiments, particularly with strain B-96, there was a small yield of phage (average of 2-4 per cell) in the absence of a purine or available derivative.

Fig. 5. Phage liberation in one-step growth experiment from purine-requiring *Esch. coli* (B-96) infected with phage T6r⁺ in the absence of purines. x, Adenine (7 mg./l.) added at time of diluting into growth medium, (0 min.); O, adenine added at 11.5 min.; Δ, no adenine. Lactate medium.

With strain B-96 in mannitol medium, the amounts of RNA and DNA were found to remain constant in the absence of purines whether the bacteria were infected with phage or not. The results with strain B-38 (adenine⁻) were somewhat variable, but in all cases they indicated an appreciable development in the absence of a source of adenine. However, sometimes, as in Fig. 7, the development appeared to be slower in the absence of adenine, while in other experiments the increase in the resistance to ultraviolet light was similar to that obtained in the presence of adenine.

Fig. 6. Phage liberation from adenine-requiring *Esch. coli* (B-38) infected with phage T2r in the absence of adenine. x, Adenine (7 mg./l.) added at time of diluting into growth medium (0 min.); Δ, adenine added at 10 min.; O, no adenine. Lactate medium.

Fig. 7. Effect of ultraviolet light on T2r-infected cells of purine-requiring *Esch. coli* following different periods of development in lactate medium after infection. Δ, Strain B-96, development in the presence of adenine (7 mg./l.); O, B-96, no adenine; □, B-38 with adenine; x, B-38, no adenine. For procedure see text under 'Materials and Methods'.

Metabolism of bacteria infected with bacteriophage

In studying the chemical changes in phage-infected bacteria it is desirable that practically all the cells should be infected with bacteriophage; this is not necessary, and indeed is often not desirable, in experiments such as those described above where the development of infected cells is studied by techniques which are not interfered with by the presence of uninfected cells. The following experiments were therefore performed with multiplicities of infection (ratio of infecting phage particles to bacterial cells) of between 5 and 10 and viable counts were routinely performed before and after adding the phage to check that at least 98 % of the cells were killed by the bacteriophage. Plaque counts were also performed when adsorption was complete and the number of infective centres were usually found to be within 10 % of the original number of viable bacterial cells.

Respiration. The O_2 consumption of the tyrosine-requiring mutant in the mannitol medium was followed for 30 min. after the infection with phage T2r and was found to be independent of the presence of tyrosine. Uninfected cells in the

Table 1. Tyrosine requirement for synthesis of deoxyribonucleic acid by phage-infected tyrosine-requiring *Esch. coli*

Washed cells (5×10^8 /ml.) in mannitol medium infected with 5×10^9 T2r/ml. at 0 min.

Time (min.)	DNA (μmoles nucleotide/100 ml.)	
	No tyrosine	L-Tyrosine (40 mg./l.)
0	3.68	3.23
5	3.34	3.35
8	—	3.87
12	3.68	4.40
17	—	4.93
22	3.34	6.10
40	3.80	8.71

Table 2. Tryptophan requirement for synthesis of deoxyribonucleic acid by phage-infected tryptophan-requiring *Esch. coli*

Washed cells (4×10^8 /ml.) in fortified mannitol medium infected with 3×10^9 T2r/ml. at 0 min.

Time (min.)	DNA (μmoles nucleotide/100 ml.)	
	No tryptophan	L-Tryptophan (10 mg./l.)
0	2.47	2.57
5	2.64	2.55
15	2.55	4.51
35	2.53	6.82
45	2.61	8.9
55	2.68	—

absence of tyrosine respired at the same rate as a similar culture immediately after the addition of tyrosine. However, in the presence of tyrosine there was of course an increase in the rate of O_2 consumption in parallel with the cell multiplication. As shown by Cohen & Anderson (1946), cells infected with bacteriophage T2 respire at the same rate as uninfected cells; the respiration of infected cells is constant until the time of lysis.

Nucleic acid synthesis. Cohen (1948) showed that there is no net synthesis of RNA in *Esch. coli* infected with bacteriophage T2 and that the net synthesis of DNA, although suspended for about the first 8 min., soon occurs at a faster rate than the synthesis of DNA in comparable uninfected cultures. As shown in Tables 1 and 2, T2-infected cultures of the mutants requiring tyrosine or tryptophan did not synthesize DNA unless the appropriate amino acid was present. In these and in most similar experiments, the amount of RNA remained constant until the time of lysis. In some cultures, however, the RNA decreased by 20–30 % of the initial content, and the decrease in turbidity indicated that premature lysis had occurred.

In contrast to the behaviour of infected cells, uninfected cells continued to synthesize both RNA and DNA in the absence of a required amino acid, but at reduced rates (Table 3). Sands & Roberts (1952) studied the incorporation of ^{32}P into the

which mature intracellular phage normally appears. The following experiments show that the synthesis of DNA does not require tyrosine or tryptophan during the later stages of the infective cycle.

The mutant strains of bacteria were infected with T2r in the presence of the required amino acid, which was removed by centrifuging at about 5 min. after the infection. The synthesis of DNA continued at almost the same rate in the absence of the amino acid as when the amino acid was supplied (Table 4). However, as expected from experiments with singly infected cells, the amino acid was essential for the appearance of mature phage. The values for the infective centres shown in Table 4 were obtained both in the usual way after diluting in buffered saline and also after cyanide treatment. This involved dilution in buffered saline containing 0.01 M-NaCN to about 1000 infected cells per ml. and heating at 45° for 5 min. before taking samples for plaque counting. The cyanide treatment does not affect free phage but causes a large proportion of the infected cells to lyse and liberate any mature phage they may contain. An increase in the infective centres due to the cyanide treatment therefore indicates the presence of infected cells containing more than one phage particle per cell. Infected cells which do not contain phage will not give rise to plaques if they are lysed by the cyanide treatment; a fall in the infective centres due to the

Table 3. *Tyrosine and the synthesis of nucleic acids in tyrosine-requiring Esch. coli*

Washed bacteria were suspended in fresh mannitol medium (8×10^8 cells/ml.) at 0 min.

Time (min.)	DNA (μ moles nucleotide/100 ml.)		RNA (μ moles nucleotide/100 ml.)	
	No tyrosine	L-Tyrosine (0.1 g./l.)	No tyrosine	L-Tyrosine (0.1 g./l.)
0	3.6	3.6	10.5	10.3
20	4.1	4.6	11.8	13.2
30	4.6	5.3	13.2	14.0
40	5.0	5.7	14.2	15.8

nucleic acids of a mutant of *Esch. coli* which required both histidine and tryptophan. In qualitative agreement with the results of Table 3, Sands & Roberts found that, in the absence of one or both of the required amino acids, the incorporation of ^{32}P into the RNA and the DNA fractions was reduced but not completely abolished.

In the previous section of this paper, it was shown that tyrosine was necessary for the development of bacteriophage not only in the first few minutes after infection but also at later times. Cohen & Fowler's (1947) experiments with 5-methyltryptophan also indicate that amino acids are essential throughout the period of bacteriophage development, even as late as the time at

cyanide treatment therefore indicates that the infected cells contain an average of less than one phage per cell. Cells which do not contain phage are not so readily lysed in the cyanide as those which do contain phage.

Results analogous to those in Table 4 were obtained by adding 5-methyltryptophan at various times after infecting the wild-type strain with bacteriophage (Fig. 8). The rate of DNA synthesis with 5-methyltryptophan added 7 min. after the infection was 50–60 % of the rate of synthesis observed in the absence of the analogue. In the presence of 5-methyltryptophan, lysis did not occur within the experimental period of 2 hr. and no liberation of mature phage was detected. As

Table 4. *Deoxyribonucleic acid synthesis and bacteriophage formation when amino acids required by host bacteria are withdrawn from phage-infected cells*

Expt. 1. Tyrosine-requiring *Esch. coli* (9×10^8 cells/ml.) washed and infected at 37° with 4×10^8 T2r/ml. in aerated mannitol medium containing 3 mg./l. L-tyrosine. After 5 min. centrifuged at 4° and re-suspended in fresh medium. A with L-tyrosine (40 mg./l.); B no tyrosine.

Expt. 2. Tryptophan-requiring *Esch. coli* (9×10^8 /ml.) washed and infected with 5×10^8 T2r/ml. in aerated fortified mannitol medium containing 1 mg./l. L-tryptophan. After 3.5 min. centrifuged and re-suspended in fresh medium. A with L-tryptophan (10 mg./l.); B no tryptophan.

Time after resuspending (min.)	DNA (μ moles nucleotide/100 ml.)		Bacteriophage plaque count (infective centres/ 10^{-11} l.)			
			No CN ⁻		Treated CN ⁻	
	A	B	A	B	A	B
	Experiment 1					
0	2.60	2.56	—	2.94	—	1.8
3	3.15	2.80	—	—	—	—
6	3.23	3.20	—	—	3.5	1.5
10	3.69	3.60	—	—	—	—
12	—	—	—	—	25	1.4
16	4.74	4.31	—	—	—	—
20	—	—	9.2	2.6	—	0.55
25	6.66	5.31	—	—	—	—
35	6.94	5.74	20	2.8	28	0.72
Experiment 2						
0	2.96	3.13	2.9	2.9	2.2	2.2
3.5	3.45	3.17	—	—	—	—
6.5	4.87	3.91	—	—	—	—
9	—	—	3.3	3.4	2.6	2.2
10.5	5.72	4.17	—	—	—	—
14.5	7.1	4.79	24	2.8	120	0.3
21	—	—	—	2.1	150	0.63
26	8.5	5.16	—	—	—	—
28	—	—	52	3.4	55	—
35	9.3	6.4	40	3.4	50	0.76

Fig. 8. Effect of 4×10^{-4} M DL-5-methyltryptophan on the synthesis of deoxyribonucleic acid in phage-infected *Esch. coli* B. 4×10^8 cells/ml. in mannitol medium, infected with 1.5×10^8 T2r/ml. at 0 min. O, No 5-methyltryptophan; 5-methyltryptophan added 3 min. (Δ) before phage, 1 min. ($\square$) after phage and 7 min. ($\times$) after phage.

previously shown by Cohen (1948), when the inhibitor was added before the infecting phage there was no net synthesis of DNA.

Appearance of 5-hydroxymethylcytosine in the DNA

The DNA of T2 bacteriophage contains 5-hydroxymethylcytosine (Wyatt & Cohen, 1953) and no cytosine, whereas the bacterial DNA, like all other DNA samples which have been examined, contains cytosine but no 5-hydroxymethylcytosine. It was therefore of interest to estimate the 5-hydroxymethylcytosine in the DNA fraction of infected cells under the various conditions (Table 5). It was found that, if tryptophan had been removed from strain B/1, *t*, or if 5-methyltryptophan had been added to the wild-type strain before infection, there was virtually no appearance of 5-hydroxymethylcytosine, and as shown in the above experiments there was no DNA synthesis. When development was allowed to continue normally for some minutes after phage infection before interfering with the availability of tryptophan, the DNA which was synthesized contained 5-hydroxymethylcytosine, and the amount of cytosine

Table 5. *Synthesis of deoxyribonucleic acid containing 5-hydroxymethylcytosine*

	Time (min.)	DNA (μ moles nucleotide/ 100 ml. culture)		Bases in DNA fraction after hydrolysis (μ moles/100 ml. culture)					
		Total	In DNA- fraction	Adenine	Cytosine	Guanine	5-Hydroxy- methyl- cytosine	Thymine	Total
Experiment 1									
Uninfected bacteria	—	2.38	2.26	0.40	0.38	0.49	0.00	0.44	1.71
No 5-methyltryptophan	8.5	3.98	3.90	0.55	0.23	0.56	0.08	0.72	2.14
No 5-methyltryptophan	45	11.6	10.5	2.27	0.06	1.68	1.05	2.55	7.61
5-Methyltryptophan added 4 min. before bacteriophage	45	2.90	2.78	0.39	0.30	0.45	0.05	0.42	1.61
5-Methyltryptophan added 9 min. after bacteriophage	45	7.6	7.2	1.46	0.04	1.13	0.66	1.76	5.05
Experiment 2									
Infected in original growth medium containing tryptophan	45	8.9	8.1	2.01	0.09	0.79	0.97	2.14	6.00
Infected in original medium, centrifuged 5 min. later, washed and resuspended. No tryptophan	38 after resuspending	8.4	7.42	1.71	0.11	0.71	0.78	2.11	5.42
Washed before infecting. No tryptophan	45	3.8	3.17	0.51	0.52	0.39	0.00	0.64	2.06

decreased. It will be seen from Table 5 that the recoveries of the DNA bases on hydrolysis (55–75%) were lower than those of about 80% obtained by Hershey *et al.* (1953). The causes of the larger losses in the present experiments are not known.

DISCUSSION

Whilst Barner & Cohen (1954) found that bacteriophage T2 induces the synthesis of thymine in a thymine-requiring mutant of *Esch. coli*, the evidence in the present work is that the phage does not induce the synthesis of the amino acids and purines required by the six mutants which have been studied. Each of the four amino acid-requiring mutants has been found to require the appropriate amino acid (tyrosine, tryptophan, leucine or histidine) for an early stage in the development of bacteriophage following the infection of the host cell. Although the purine-requiring mutants require purines for the net synthesis of nucleic acids they do not require purines to support the early stages of bacteriophage development. Presumably, as in the normal infective cycle, the adenine and guanine in the nucleic acids of the host cell are utilized for the formation of bacteriophage nucleic acid (Koch, Putnam & Evans, 1952).

Washed cells of a nicotinamide-requiring mutant of *Esch. coli* (Gots' B-159) have been found to be capable of supporting phage formation in the mannitol medium containing no nicotinamide (K. Burton, unpublished). The latent period and burst size were similar to those obtained in the presence of nicotinamide or with the wild-type strain. This is consistent with the absence of any increase of the respiration rate following the infection with phage (Cohen & Anderson, 1946). It also indicates that the requirement for tryptophan cannot be due to its possible role as a precursor of nicotinamide.

The amino-acid requirements for phage development and also the inhibition by 5-methyltryptophan (Cohen & Fowler, 1947) show that the proteins of the host cell cannot be degraded to provide any appreciable amount of the required amino acids under conditions of amino acid deficiency. This is in agreement with the findings that protein synthesis and amino acid utilization continue during normal phage development until shortly before lysis (Cohen, 1948; Raff & Cohen, 1950) and that almost all the bacteriophage protein is synthesized from the constituents of the medium after infection (Siddiqi, Kozloff, Putnam & Evans, 1952).

If it is correct that the amino acid requirements studied in the present work are due to the dependence of bacteriophage development on the

synthesis of protein, it would follow that the first stage of the infective cycle necessitating protein synthesis occurs after the introduction of the DNA of the infecting phage into the bacterial cell. It precedes the decrease in the ultraviolet sensitivity, the conversion of bacterial DNA into DNA containing the characteristic viral pyrimidine (5-hydroxymethylcytosine) and the net synthesis of DNA. If no tyrosine or tryptophan is available during later stages of the phage development, the synthesis of bacteriophage DNA continues although no mature phage appear.

These phenomena suggest that the first proteins synthesized after infection include the enzymes and/or templates necessary for synthesizing the phage-specific DNA which differs from the bacterial DNA. Once the synthesis of this DNA has been initiated, it can continue independently of the synthesis of proteins containing tyrosine or tryptophan. This independence may be related to the fact that uninfected amino acid-requiring bacteria can synthesize nucleic acids in the absence of amino acids required for growth.

Recently, two groups of workers have been studying the action of chloramphenicol on T2-infected *Esch. coli* (Melechen; Rosenbaum, Halvorson, Preston & Levinthal, private communications). The drug appears to act similarly to 5-methyltryptophan in that it can, under suitable conditions, inhibit the synthesis of DNA if added before the infecting phage; if added 10 min. after the infection it allows DNA synthesis to continue but still prevents bacteriophage formation. In uninfected bacteria, chloramphenicol inhibits the synthesis of bacterial protein without inhibiting the synthesis of bacterial nucleic acid (Gale & Folkes, 1953; Wissemann, Smadel, Hahn & Hopps, 1954).

The conclusions of this paper are related to the findings of Hershey, Garen, Fraser & Hudis (1954), who studied the incorporation of ^{35}S from inorganic sulphate into the protein of infected cells and the subsequent appearance of the tracer in the phage protein. Their results show that only about 10% of the sulphur incorporated by the cells during the first 5 min. after infection appears in the mature phage, whereas at later times 50–60% of the incorporated sulphur appears in the phage. Hence it is likely that the proteins which are required to be synthesized in order to initiate the synthesis of DNA do not themselves form part of the structure of the bacteriophage.

SUMMARY

1. By the use of amino acid-requiring mutants of *Escherichia coli* B as host cells, histidine, leucine, tryptophan and tyrosine have been shown to be required for the multiplication of T2 bacteriophage

after the entry of the deoxyribonucleic acid (DNA) of the infecting phage into the host. Without the required amino acid the infected cells cannot develop the increased resistance to the action of ultraviolet light in suppressing the formation of bacteriophage.

2. The amino acid requirements for the synthesis of bacteriophage DNA were studied using two of the strains (tyrosine- or tryptophan-requiring). In the absence of the required amino acid at the time of infection, there was no net synthesis of DNA and no appearance of the characteristic T2 pyrimidine (5-hydroxymethylcytosine) in the DNA of the infected cells.

3. When the amino acid was present for the first 5 min. after infection and then removed by washing the infected cells, no mature bacteriophage appeared, but DNA (containing 5-hydroxymethylcytosine) was synthesized at about 80% of the rate which occurred when the amino acid was present.

4. Analogous results were obtained with the wild-type strain of *Esch. coli* B and an antimetabolite (5-methyltryptophan) which prevents the biosynthesis of tryptophan. When added 3 min. before the infecting bacteriophage there was no net synthesis of DNA and little 5-hydroxymethylcytosine appeared in the DNA of the infected cells. When 5-methyltryptophan was added 7 min. after the infecting bacteriophage, no mature bacteriophage was formed, but DNA containing 5-hydroxymethylcytosine was synthesized at about 60% of the normal rate.

5. It is concluded that, after infection of the host cell, there must be a synthesis of protein before the synthesis of the characteristic bacteriophage DNA is initiated. When these proteins have been synthesized, the synthesis of bacteriophage DNA is largely independent of the synthesis of proteins containing tyrosine or tryptophan, although further protein synthesis is necessary for bacteriophage formation.

6. Some experiments with purine-requiring mutants as host bacteria are also described. Purines were not required to be present in the growth medium during the early stages of bacteriophage development. Presumably the DNA of the host cell is utilized for the bacteriophage development.

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Chromatographic Studies on Corticotropin

BY H. B. F. DIXON AND M. P. STACK-DUNNE*

Department of Biochemistry, University of Cambridge

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Adrenocorticotrophic hormone has been assayed and defined in several ways, but of late the term has been restricted to substances active in the ascorbic acid depletion test of Sayers, Sayers & Woodbury (1948). It is with factors active in this test by intravenous injection into hypophysectomized rats that this paper is concerned. Corticotropin A has been chemically characterized (White, 1953) after isolation from pig pituitaries and shown to possess activity. The designation A is to distinguish it from corticotropin B, isolated after treatment of concentrates with pepsin by Brink *et al.* (1952). As we, in agreement with Bell (1954), are reporting the presence of more than one active substance in such concentrates before hydrolysis, it is at present convenient to give these designations A₁, A₂ etc. The corticotropin B group may require similar subdivision.

Preliminary results (Dixon, Moore, Stack-Dunne & Young, 1951) suggested the use of ion-exchange chromatography with a carboxylic resin, because of the success of this method with the basic proteins ribonuclease and lysozyme (Hirs, Moore & Stein, 1953; Tallan & Stein, 1953). Some of our earlier results have been published, in particular the

separation of the active material from a preparation of the protein hormone which had been thought to be the active substance. Extension of the method to concentrates gave better results than with crude materials (Dixon, Stack-Dunne & Young, 1953). Many other workers have since used ion-exchange chromatography with ACTH, and the chief successes of the method have been the isolation of corticotropin A (White & Fierce, 1953) and α -corticotropin (Li *et al.* 1954).

METHODS

Materials

(1) *Pituitary powder.* Whole pig pituitaries were collected in acetone and dried with acetone after mincing. The pituitaries were removed within 1 hr. of death, and the final drying was within 10 days. Potency, 0.1–0.2 i.u./mg.

(2) *Crude corticotropin and oxycellulose-purified corticotropin (OPC preparation).* These were prepared from whole-pituitary powder by the methods of Payne, Raben & Astwood (1950) and Astwood, Raben, Payne & Grady (1951).

(3) *Fraction L (crude prolactin).* This was prepared by a modification of the method of Lyons (1937). The advantage of raising the acetone concentration during the extraction was retained by adding further acetone, before separating the solids, after extraction at a low acetone concentration. The preparation was carried out at 0–3°.

* Present address: Department of Physiology, Yale University School of Medicine, New Haven, Conn., U.S.A.