NUTRITIONAL REQUIREMENTS FOR THE REPRODUCTION OF COLIPHAGE T2 IN A STRAIN OF ESCHERICHIA COLI[1]

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The discovery of nutritional requirements for the reproduction of bacteriophage is not novel. Adams (1949) has clearly demonstrated a calcium requirement for T5 reproduction in *Escherichia coli*, strain B. Furthermore, Fowler and Cohen (1948) for T2 in B and more recently Gots and Hunt (1953) for λ in irradiated *E. coli*, strain K12, have demonstrated the growth stimulating effect of certain amino acids. However, in both cases the stimulation of the growth of T2 and λ was measured as an increase in burst size over the small increase consistently obtained in unsupplemented synthetic medium.

In a previous note (Gross, 1952) it was reported that T2r1 will not reproduce in washed and starved *E. coli*, strain K12, unless rather complex nutrients are added to the basal synthetic medium. In this case the effect is all or none in the sense that infected bacteria will not support any phage synthesis without the addition of essential growth factors. This conclusion is based upon the experiments described below which also indicate that certain amino acids are required for phage synthesis.

MATERIALS AND METHODS

T2r1 was used throughout these experiments and will be referred to simply as T2. The plating technique, method of purification of phage suspensions, and the composition of the nutrient broth, buffer, and synthetic growth medium (M9) have been described previously (Gross, 1954).

E. coli, strain K12, was used as the host. Unless otherwise noted, cultures of bacteria were prepared as is indicated in the general experimental protocol outlined in table 1.

As described previously (Gross, 1954), suspensions were plated in the presence of *E. coli*, strain B, to determine the total number of infectious centers (infected bacteria and free phage).

RESULTS

The growth factor requirement. Ordinarily, washed and starved cultures of *E. coli*, strain K12, when infected with phage T2 in buffer and subsequently incubated in nutrient broth, will yield 15 to 35 phage particles per infected bacterium. If the synthetic medium M9 is substituted for the broth, instead of an increase in number of infectious centers, which would be expected if normal phage reproduction took place, a decrease was consistently observed. It was found that yeast extract, and to some extent a tryptic digest of casein, could, when added to M9, replace nutrient broth as a stimulator of phage reproduction. In table 2 some data are presented illustrating this.

These data indicate that yeast extract, when added to M9, is as efficient in supporting phage reproduction as nutrient broth and that the limiting concentration is somewhere in the vicinity of 50 μg per ml. Concentrations of yeast extract between 50 and 5 μg per ml gave highly variable results. The data obtained with the tryptic digest of casein indicate that its activity, though less than that of yeast extract, remains fairly constant at concentrations of 5,000 and 500 μg per ml but decreases appreciably at 50 μg per ml. In this experiment, as in all to be presented, the number of recoverable infectious centers at the end of the incubation in M9 was less than the input.

Virus liberation from infected bacteria in M9, buffer, and yeast extract. In the experiment reported in figure 1 the fate of the infected bacterium was followed in M9, M9 supplemented

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TABLE 1

Protocol of growth experiments

TIME IN MINUTES	OPERATION
−110	An aerated, culture of *Escherichia coli*, strain K12 (concentration approximately 2.5 × 10^8 per ml) actively growing in M9, was centrifuged three times at 3,000 rpm in an International size 1 centrifuge for 10 minutes. The cells were suspended in buffer after each centrifugation.
−70	Washed cells were aerated for 60 minutes at 37 C.
−10	The cells were centrifuged once and resuspended in enough buffer to bring the final concentration of bacteria to 10^9 per ml.
0	0.1 ml of a stock suspension of T2 (concentration about 10^8 per ml) was added to 0.9 ml of the bacterial suspension.
+7	0.1 ml of the adsorption mixture was diluted in iced buffer (A) 1,000 and (B) 10,000-fold as quickly as possible.
+8	0.1 ml of dilution A was added to each of the growth tubes a–x containing 1.9 ml medium at 37 C as quickly as possible.
+12, +15	(Time depending on the number of growth tubes employed in the experiment.) 1.5 ml of dilution B was centrifuged at 4,000 rpm for 5 minutes and 0.1 ml samples plated for free phage determinations. During the centrifugation process 0.1 ml samples of the remainder of suspension B were plated for the total number of infectious centers (free phage plus infected bacteria). The maximum time required for these operations is 8 to 9 minutes.
+85	The growth tubes were removed and placed in ice bath. 0.1 ml aliquots from each growth tube were plated for the total number of infectious centers.

The entire experiment usually was done in duplicate using the same starting material.

with yeast extract, buffer at 37 C, and in iced buffer. The protocol of the experiment was essentially the same as that outlined in table 1 except that the concentration of infected bacteria in the M9 and buffer growth tubes was about ten times as great as previously employed, and samples were removed for assay at frequent intervals during the incubation period. In M9 the number of infected bacteria recoverable as plaque formers begins to decrease immediately. In the experiment reported here there is a sharp increase in the rate of inactivation at about the 40 minute mark. This change in rate has frequently, but not always, been found. In buffer at 37 C there is a similar decrease in infectious centers, but the rate and change in rate are much slower. In iced buffer the number of infectious centers remained constant throughout the experiment. In the presence of yeast extract there is no evidence of any decrease of infectious centers. After a minimum latent period of about 25 to 26 minutes, a 35 minute period of increase of infectious centers was observed leading to a final yield of about 20 phage particles per infected bacterium.

The results indicate that unless some exogenous source of phage growth stimulating substance is presented to the infected bacterium, the bacterium loses its ability to support phage synthesis and disappears as an infectious center. The temperature dependence of the reaction indicates that the loss of the ability to synthesize phage is a function of the endogenous metabolism of the infected cell.

Properties of the active substance or substances in yeast extract. The activity of yeast extract is destroyed completely by incineration but not by autoclaving. The active substance can be dialyzed. Attempts to isolate the active compound by adsorption on ion exchange resins and charcoals, by alcohol fractionation, and by paper chromatography were unsuccessful. In most fractions there was a correlation between the presence of amino acids and peptides and activity.

Tests of various compounds for their growth promoting ability. Most of the naturally occurring amino acids, purines, pyrimidines, nucleosides, nucleotides, and vitamins were tested by simple addition to the synthetic medium. All were ineffective including hydroxymethyl cytosine and its deoxyriboside kindly provided by Dr. S.

TABLE 2

The effect of different incubation media on the reproduction of T2 in washed and starved Escherichia coli, strain K12

SUBSTRATE	SOURCE	CONCENTRATION SUPPLEMENT μg PER ML M9	PLAQUE COUNTS CORRECTED FOR ORIGINAL FREE PHAGE
M9		—	18
M9 plus yeast extract	Difco	5,000	835
		500	810
		50	465
		5	34
M9 plus N-Z case	Sheffield	5,000	221
		500	203
		50	92
		5	28
Nutrient broth	Difco	—	823

In this experiment the total number of infectious centers at the dilution plated at the beginning of incubation was 63, of which 22 were free phage as measured after centrifugation. Therefore, 41 plaques represented the number of phage yielding infected bacteria. The average burst size measured after growth in nutrient broth is therefore 20.

The incubation time was 70 to 80 minutes.

Cohen. The author wishes to express his thanks to Dr. W. Cohn of the Oak Ridge National Laboratory for the deoxycytidylic acid, barium deoxyadenylate, barium thymidylate, and thymidine.

As Cohen (1949) and Gots and Hunt (1953) have observed, cysteine proved to be strongly inhibitory. This effect was reflected in an increased loss of infected bacteria as infectious centers at the end of the incubation period.

Further tests of casein hydrolyzates and amino acids. Two vitamin free hydrolyzates of casein were employed. One was a pancreatic digest and the other a constant boiling hydrochloric acid hydrolyzate in which most of the tryptophan had been destroyed.

The data in table 3 indicate that there is essentially no difference between the activities of yeast extract and the enzymatic hydrolyzate of casein at the concentrations tested. Tryptophan must be added to the hydrochloric acid hydrolyzate, however, to raise its activity to the level of yeast extract. In this mixture, at least, it can be concluded that tryptophan is required for the maximum yield of phage. This finding is in agreement with that of Fowler and Cohen (1948) for the reproduction of T2 in strain B.

Gots and Hunt (1953) found that certain combinations of isoleucine, leucine, valine, threonine, serine, aspartic acid, histidine, phenylalanine, and glutamic acid increased the burst size of λ in strain K12.

Combinations of the DL amino acids mentioned above were tried with little or no effect. When only the L isomers were used, marked stimulation of growth resulted as is shown in table 4. Tryptophan also was added because of its previously observed stimulatory effect. Each amino acid was omitted singly from the mixture in order to test for a specific requirement. The results indicate that in the absence of isoleucine the mixture is totally devoid of activity. Tryptophan in such a mixture has no stimulatory effect, yet its effect in the acid hydrolyzate of casein

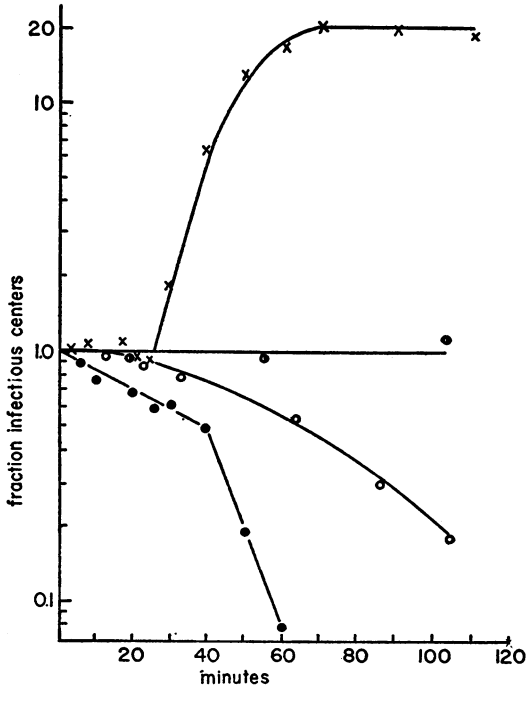

Figure 1. The fate of the infected bacterium. ●: Data obtained after incubation in M9. ○: Data obtained after incubation in buffer at 37 C. ◑: Data obtained after incubation in iced buffer. ×: Data obtained after incubation in M9 supplemented with 500 μg yeast extract per ml.

TABLE 3

The effect of the addition of casein hydrolyzates to M9 on the reproduction of T2

EXPERIMENT	SUBSTRATE	NUMBER OF INFECTED BACTERIA	PLAQUE COUNTS 70 TO 75 MINUTES AFTER INCUBATION	
			Concentration per ml M9	
			500 μg	50 μg
1	Casein hydrolyzate-enzymatic	32	452	380
		26	402	310
2		23	487	240
3		28	510	209
1	Casein hydrolyzate-HCl	32	249	171
		26	172	132
2		23	180	105
3		28	258	167
1	Casein hydrolyzate-HCl plus tryptophan	32	411	357
		26	292	370
2		23	417	263
3		28	—	—
1	Yeast extract	32	513	450
		26	401	360
2		23	591	219
3		28	446	127
1	M9	32	13	
		26	9	
2		23	6	
3		28	16	

Casein hydrolyzate—Enzymatic control no. 4164.

Casein hydrolyzate—HCl control no. 3393.

Tryptophan supplemented casein hydrolyzate contained 1 μg tryptophan per 50 μg casein hydrolyzate.

All numbers are averages of two plates. Duplicate trials using the same batch of cells were done in experiment 1.

was quite marked. Phenylalanine, on the other hand, acts as an inhibitor. In its absence the activity of the amino acid mixture approximates that of the casein hydrolyzate. This effect is surprising since one would expect 500 μg of casein to contain about 19.4 μg of phenylalanine (Cohn *et al.*, 1943). This is fairly close to the concentration of phenylalanine (25.2 μg per ml) used in this experiment. In another experiment the concentration of phenylalanine was reduced at 20 μg per ml; the inhibition was still in evidence. It would seem then that this inhibition might be due to the amino acid balance peculiar to the combination used.

Mixtures of isoleucine, leucine, valine, threonine, serine, and aspartic acid at the concentrations used in the preceding experiment have given yields equivalent to about 50 per cent of the maximum obtained with tryptophan supplemented casein hydrolyzates. All of the amino acids tested singly were without any growth promoting effect. Mixtures of isoleucine, leucine, and valine likewise have been found to be devoid of activity.

Variations in the method of preparation of the host and the growth of T2 in cells incubated in "old" synthetic medium. The evidence presented

TABLE 4

Total phage yields in mixtures of amino acids

EXPERIMENT	1a	1b	11a	11b	111a	111b
Number infected bacteria	35	38	16	20	28	24
	Plaque counts after incubation minus original free phage					
All amino acids	164	107	78	96	222	224
All minus phenylalanine	320	423	222	186	382	400
All minus valine	228	233	124	84	248	208
All minus glutamic acid	181	105	88	76		
All minus histidine	140	122	60	54		
All minus serine	134	85	72	92		
All minus threonine	177	127	104	108		
All minus aspartic acid	122	99	64	40		
All minus leucine	144	141	108	78		
All minus tryptophan	148	105	116	102		
All minus isoleucine	17	15	12	2		
None added	19	9	3	6	18	10
500 μg casein hydrolyzate-acid plus 57 μg tryptophan per ml	318	290	240	256	452	616

The complete mixture of L amino acids contained isoleucine, 27.6 μg; phenylalanine, 25.2 μg; valine, 30.3 μg; threonine, 33.6 μg; glutamic acid, 37.7 μg; histidine monohydrochloride, 28.7 μg; serine, 36.6 μg; aspartic acid, 38.4 μg; tryptophan, 57.0 μg; leucine, 27.6 μg, per ml M9.

All data are averages of duplicate platings from each growth tube.

a and b refer to two independent determinations of activity using the same batch of starved cells.

The incubation time was 70 to 80 minutes.

TABLE 5

The growth of T2 with varying conditions of starvation and different incubation media

EXPERIMENT	TREATMENT OF CELLS	INCUBATION MEDIUM	A		B	
			Number of infected cells	Plaque count after incubation	Number of infected cells	Plaque count after incubation
1	none	fresh M9	22	210	25	169
		yeast extract	22	334	25	280
2	none	fresh M9	13	265	20	355
		yeast extract	13	265	20	365
3	none	fresh M9	30	396	35	378
		yeast extract	30	620	35	635
4	washed but not starved	fresh M9	10	16	13	22
		yeast extract	10	230	13	385
5	washed but not starved	fresh M9	43	71	31	36
		yeast extract	43	841	31	851
6	washed and starved	"old M9"	23	54	32	102
		yeast extract	23	410	32	395
7	washed and starved	"old M9"	14	428	8	40
		yeast extract	14	270	8	393

In experiments 1 through 3 the adsorption of T2 was allowed to proceed in the M9 in which the cells were growing.

"Old M9" refers to sterile filtrates of the M9 in which the cells used in the experiment were allowed to grow from 1×10^7 to 2.5×10^8 per ml.

Yeast extract refers to M9 supplemented with 500 μg yeast extract per ml.

A and B refer to two different determinations made with the same batch of cells.

The incubation time was 70 to 80 minutes.

indicates that T2 will not undergo reproduction in washed, starved cultures of strain K12. It was observed, however, that cells growing in the logarithmic phase in synthetic medium, when infected with T2, will lyse with a concomitant release of phage, provided that the infection and reproduction are allowed to proceed in the original growth medium. Since this observation was made on mass cultures, a study of the phenomenon was undertaken under conditions more closely resembling those maintained in the experiments on starved cells.

Table 5 contains the results of experiments in which cells were infected in the original growth medium without washing, then diluted and added to fresh M9 for incubation; in which cells were washed and incubated in fresh M9; and in which cells were washed, starved, and incubated in a sterile filtrate of the original growth medium (old M9). Except in the first case, adsorption of phage on bacteria was allowed to proceed in buffer.

The data indicate that multiplying strain K12, when infected in the original growth medium, is capable of supporting a great deal of phage synthesis. This capability is reduced greatly upon washing, but the ability to support some synthesis still persists. When cells were washed and starved as usual, and incubation of infected cells was allowed to proceed in "old M9", an increase in phage yield was observed. This increase was small, but it should be emphasized that, under these conditions, no increase has ever been observed with cells incubated in fresh M9.

It would seem, then, that the washing procedure employed deprives the cell of some phage promoting substances normally present in the untreated cell. These substances seem to leak into the culture medium of a normally dividing culture. Whether this leakage is due to lysis after λ reproduction in a small fraction of the bacterial population can only be conjectured.

The effect of starvation on the onset of bacterial multiplication. In order to test whether the phage growth factor requirement is related directly to an induced lag in bacterial multiplication, the

growth of starved cultures was followed densitimetrically as well as by colony counts. Starved cells show a 30 minute lag (as determined by colony counts) when growth is allowed to resume in M9. The lag is only slightly reduced in nutrient broth, and unchanged in the amino acid mixtures. When growth is followed densitimetrically, little or no lag can be observed even when growth is resumed in M9. It seems, therefore, that even though starvation delays the onset of cell division the cells can resume growth as soon as they are transferred to M9. A direct relationship between the lag in bacterial multiplication and the biochemical requirement for phage reproduction seems difficult to demonstrate.

DISCUSSION

It has been shown that T2 will not reproduce in washed and starved *E. coli*, strain K12, unless a complex of amino acids is added to the basal synthetic medium. In the absence of these amino acids there is a decrease, with time, in the number of recoverable infectious centers after incubation at 37 C in either buffer or synthetic growth medium. This phenomenon is quite different from "abortive infection" (Gross, 1954) in the sense that no complex medium tried has been found capable of preventing the initial loss of infected bacteria as plaque forming units after the adsorption of T2 on starved cells. In other words, there seems to be two distinct processes leading to the loss of the infecting phage particle. Abortive infection seems to involve the attachment of phage to cells that have lost completely the ability to support phage growth. The other process is reversible in the sense that it can be prevented by the addition of substances to the growth medium after infection. The two processes might be related, but a discussion of this point will be deferred until more direct evidence is obtained.

A rather complex requirement for phage synthesis has been demonstrated. In fact, there are strong indications that the activity of any single component of the amino acid mixtures employed depends upon the amino acids present and their concentrations. Tatum (1946) has shown that valine inhibits the growth of K12 and that isoleucine reverses this inhibition. In the experiments reported here the absolute requirement for isoleucine might have resulted from such an inhibition since isoleucine was omitted from combinations of amino acids containing valine. The inhibition caused by phenylalanine is as yet inexplicable.

The requirement for amino acids has been demonstrated only in starved cells. This raises the question as to whether starvation might alter the physiological state of the bacterium so that phage synthesis could not proceed unless amino acids were added. According to this view the requirement is a bacterial one and only incidental to phage multiplication. The evidence presented here does not preclude such an hypothesis. An answer might come from a study of the immediate fate of the amino acids fed to starved infected cells.

SUMMARY

Coliphage T2 does not undergo reproduction in washed and starved *Escherichia coli*, strain K12, unless yeast extract, nutrient broth, or casein hydrolyzate is added to the synthetic medium M9.

This growth factor requirement can be satisfied by a mixture of amino acids. An absolute requirement for isoleucine was demonstrated in mixtures of amino acids tested although it did not stimulate phage synthesis when tested alone. Phenylalanine was inhibitory in the mixtures used, and tryptophan was shown to have a stimulatory effect only in conjunction with a hydrochloric acid hydrolyzate of casein.

Bacteria not subjected to starvation were capable of supporting phage synthesis. Evidence is presented in support of the view that the washing and starvation procedures deprive the cell of some phage growth promoting substances.

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