AMINO ACID REQUIREMENTS FOR THE MATURATION OF BACTERIOPHAGE IN LYSOGENIC ESCHERICHIA COLI[1]

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The production of active phage in lysogenic bacteria is influenced not only by the induction effect, but also by various nutritional exposures of the bacterial host before and after induction (Lwoff *et al.*, 1950). Aptitude or the ability to be induced by ultraviolet, for example, depends upon the nutritional history of the organism prior to irradiation, i.e., the cells must be in a competent state before induction can occur. Following induction of such competent cells, phage development or "maturation" will not ensue unless, again, a proper nutritional environment is provided. These various nutritional effects have been demonstrated in *Bacillus megaterium* by Lwoff (1951), *Pseudomonas* strains by Jacob (1952), and *Escherichia coli*, strain K-12, by Borek (1952).

This paper deals primarily with the nature of the nutritional environment required for the maturation of *"lambda"* phage in the K-12 strain of *E. coli* after ultraviolet induction of competent cells. It will be shown that phage development cannot occur in the absence of amino acids and that leucine, valine, isoleucine, and threonine play critical roles. Lwoff (1951) previously has implicated amino acids as being necessary for aptitude in *B. megaterium*, but no specific effects were designated.

MATERIALS AND METHODS

Organisms. Analysis was made with the lysogenic *Escherichia coli*, strain K-12, and its carried bacteriophage, *lambda* (Lederberg and Lederberg, 1953). Assay of *lambda* was performed with the sensitive indicator strain, W-1485, a nonlysogenic derivative of K-12 obtained through the courtesy of E. M. Lederberg.

Since the wild type K-12 is known to be acutely sensitive to an inhibitory action of valine (Tatum, 1946), a property peculiar to this strain (Rowley, 1953), it became necessary, for certain experiments, to use a valine resistant mutant of strain K-12. This mutant, designated as *"K/val"* was isolated readily by plating large populations (more than 10^8) of the parent strain K-12 on minimal agar media containing 100 μg of valine per ml. Whereas the growth of the parent strain K-12 was inhibited completely by 1 μg of valine per ml, the resistant *K/val* was unaffected by 200 μg per ml. It was equally resistant to α-ketoisovaleric acid,[2] the deaminated analogue of valine, which like valine inhibits the growth of the parent K-12 organism. Growth inhibition by L-valine or α-ketoisovaleric acid can be prevented by either L-isoleucine or its deaminated analogue, α-keto-β-methyl-*n*-valeric acid (Umbarger and Mueller, 1951), but not by D-isoleucine. In addition, we have found that D-valine is also, but less efficiently, inhibitory, and either D- L- or ketoisoleucine can relieve this inhibition.

Media. The complex medium was a tryptone-yeast extract-glucose broth described by Morton and Engley (1945). In addition to this, nutrient broth and tryptone broth also were employed. The chemically defined medium consisted of inorganic salts with ammonium chloride (0.1 per cent) as nitrogen source and glucose (0.2 per cent) as carbon and energy source (Gots and Chu, 1952). Amino acid supplementation was facilitated by the use of five amino acid pools as follows:

Pool no. 1: DL-methionine, L-cystine, L-arginine, L-lysine.

Pool no. 2: DL-leucine, DL-isoleucine, and DL-valine.

Pool no. 3: DL-tryptophan, DL-phenylalanine, and L-tyrosine.

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Pool no. 4: DL-threonine, L-histidine, L-proline, and L-glutamic acid.

Pool no. 5: glycine, DL-serine, DL-alanine, and DL-aspartic acid.

The pools and solutions of their individual components were used in the media at a final concentration of 0.1 mg per ml (except tyrosine, 0.025 mg).

Measurement of phage multiplication. Except for preliminary analyses by mass lysis techniques, the maturation of *lambda* was determined routinely by the one step growth curve technique as described by Weigle and Delbrück (1951). *E. coli*, strain K-12, was harvested in the log phase of growth when an aerated culture in Morton and Engley broth, inoculated from a 24 hour culture, had reached an approximate concentration of 3×10^8 bacteria per ml. After two washings by centrifugation in phosphate buffer (Weigle and Delbrück, 1951), the bacteria were resuspended and diluted in the buffer to a concentration of 1×10^6 per ml and irradiated as described below. A 1/500 dilution of the irradiated bacteria in the respective medium under examination constituted the first growth tube. The second growth tube consisted of an additional 1/100 dilution in the same medium. The two growth tubes were incubated at 37 C, and samples were plated at prescribed intervals at least up to two hours. Time zero was taken at the time the organisms were diluted in the respective media in the growth tubes. Platings were made by the soft agar layer technique using a 24 hour broth culture of W-1485 for seeding.

TABLE 1

Maturation of lambda phage in various media

MEDIA	RELATIVE INCREASE IN INFECTIOUS CENTERS					
	Experiment 1			Experiment 2		
	70 min	90 min	120 min	70 min	90 min	120 min
S-G*	1.7	1.2	5.2	1.5	1.6	3.6
S-G + YE	3.6	18.9	103.0			
S-G + VM				0.9	1.9	7.0
S-G + CH	2.8	5.9	250.0	3.1	30.2	319.0
S-G + CH + YE				7.7	105.0	334.0
S-G + CH + VM				2.2	15.6	175.0
Tryptone broth	8.4	84.0	231.0			
Morton and Engley broth	28.4	54.0	370.0			

YE, Difco yeast extract (0.1%); VM, mixture of B vitamins; CH, casein hydrolyzate (0.1%).

*S-G, chemically defined salt-glucose media

Ultraviolet irradiation. Ten ml of the washed organisms were irradiated for 40 seconds in an open petri dish with continuous agitation. A 15 watt GE germicidal ultraviolet lamp at a distance of 47.5 cm was used. In order to minimize photoreactivation effects, the entire experiment from the time of irradiation until the last plating was carried out under subdued light. By this method, 80 to 95 per cent of the total irradiated population were converted to plaque formers; from 0 to 5 per cent could be recovered as colony formers; the remainder represented bacteria lost by the lethal action of the ultraviolet. Thus, on the basis of accountability as either a plaque former or colony former, induction occurred in 90 to 100 per cent of the accountable population.

RESULTS

When the irradiated competent cells were placed into complex media such as Morton and Engley, tryptone, or nutrient broth, virus production followed a one step growth curve similar to that shown by Weigle and Delbrück (1951). The average latent period was 70 minutes with a variable total increase in infectious centers (average burst size) ranging from 70 to more than 300. Maximum yield was obtained after a period of from 90 to 120 minutes. Readings beyond 3 hours often showed a decrease which was most marked in the supplemented synthetic media. In the chemically defined medium the total increase was rarely more than fivefold after two and even four hours from the time of irradiation. By supplementing the synthetic medium with various additions, it became evident immediately that amino acids were the most limiting factors (table 1). Except for a lengthened rise period, casein hydrolyzate was similar to the complete media. A mixture of B vitamins had no effect.

Addition of all 18 amino acids contained in the five amino acid pools could not only substitute for casein hydrolyzate but consistently allowed, on the average, a 34 per cent greater yield. Since single additions of the amino acid pools to the unsupplemented chemically defined medium had no effect, analysis was performed by depletion techniques. By single depletion of each pool from

the total mixture, it was at once apparent that the presence of isoleucine, leucine, and valine (amino acid pool no. 2) was essential for optimal phage maturation. The absence of these amino acids consistently reduced the activity of the total amino acid medium to that of the unsupplemented synthetic medium. This and the effects of depletion of the other amino acid pools are shown in figure 1. Omission of amino acid pools no. 3 or no. 5 resulted in only a relative and somewhat variable limiting of maturation ranging

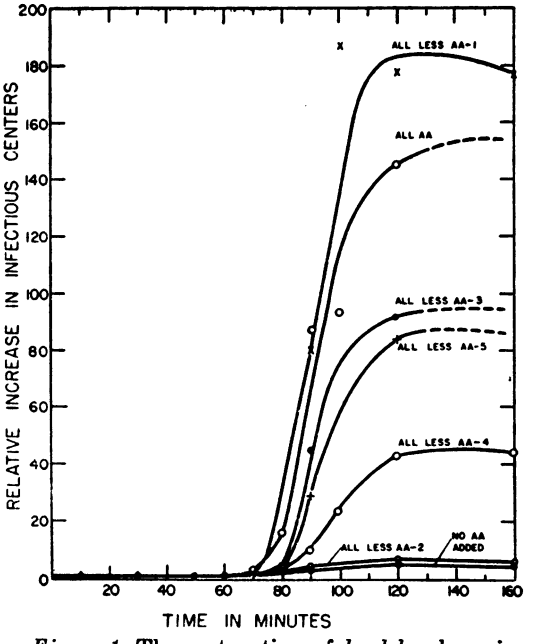

Figure 1. The maturation of *lambda* phage in amino acid containing media. This shows effects of depletion of the various amino acid (AA) pools from the total amino acid mixture.

from 30 to 60 per cent decrease in the total virus yield, with a delay in the time of liberation. Pool no. 4 was more severely limiting in that its absence resulted in only 10 to 30 per cent of the total yield with a significant increase in latent period. The depletion of pool no. 1 consistently allowed a greater increase in virus production indicating that its presence exerted an inhibitory effect.

Effects of isoleucine, leucine, and valine. It can be seen from table 2 that all of the components of amino acid pool no. 2, isoleucine, leucine, and valine, must be present before optimal phage production can occur. The omission of any one of these three amino acids reduced the capacity of the amino acid medium for supporting virus synthesis to a level equal to, and sometimes less than, the unsupplemented chemically defined medium. Restoration of activity could be obtained by the addition of all three amino acids to the deficient medium as long as one hour after irradiation. In the experiment depicted in figure 2, when these amino acids were present in the medium immediately receiving the irradiated organisms, virus liberation occurred at 76 minutes with a total increase of 150 (curve A). When pool no. 2 was not added (curve E), a maximum increase of

TABLE 2

Effect of components of amino acid pool no. 2 on phage development

ISOLEUCINE	LEUCINE	VALINE	RELATIVE INCREASE IN INFECTIOUS CENTERS (120 MINUTES)
+	+	+	236
−	−	−	2.2
−	+	+	6.3
+	−	+	1.6
+	+	−	4.0
+	−	−	0.8
−	+	−	1.9
−	−	+	2.5
Chemically defined media only (no amino acids)			5.2

Depletions (−) and additions (+) as indicated above were made in presence of all other amino acid pools (1, 3, 4, and 5).

only 5 could be detected after 120 minutes. When pool no. 2 was added at 20, 35, and 60 minutes after irradiation (curves B, C, and D), virus liberation ensued at 64, 61, and 62 minutes after these respective additions with variable, but comparable, total yields. Thus, the irradiated organisms could be kept in a resting state with respect to virus production by the depletion of isoleucine, leucine, and valine for at least one hour. Furthermore, the average 13 minute decrease in latent period given by the systems in which the addition of the essential amino acids was delayed would indicate that these amino acids are not required for the first 13 minutes after irradiation. It appears then that the events leading to phage maturation can proceed for 13

minutes independent of the presence of isoleucine, leucine, and valine, but development beyond this ceases until these amino acids are provided.

Throughout these experiments, concomitant examination of the effects of the particular environments on the growth of the normal noninduced bacteria also has been followed. The environments which limit phage production as depicted in figures 1 and 2 have no effect on the normal growth of the noninduced bacteria. However, wherever valine is present in the absence of isoleucine, as occurs in two of the combinations

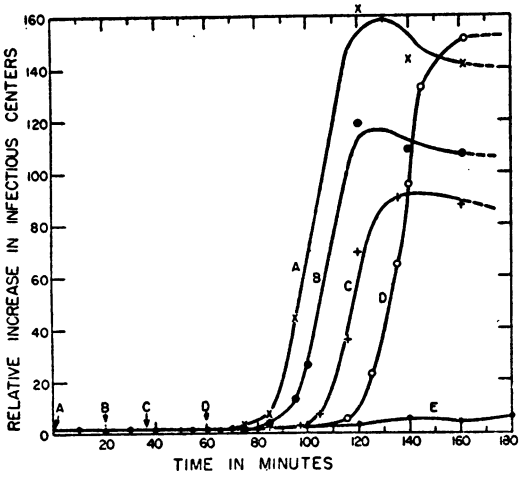

Figure 2. Effects on *lambda* maturation by depletion (curve E) and the addition of isoleucine, leucine, and valine at various times. Indicated by A (0 minutes), B (20 minutes), C (35 minutes), and D (60 minutes). All other amino acids (pools 1, 3, 4, and 5) are present.

in table 2, the growth of the noninduced bacteria is inhibited severely.

As previously discussed, this phenomenon of valine inhibition which is reversible by isoleucine is an unexplained peculiarity of this strain. Thus, the impairment of phage maturation resulting from the single depletion of isoleucine may be merely an expression of an inhibited host metabolism in an environment where valine may be allowed to exert its inhibition. In order to elucidate the exact role of isoleucine it became necessary to test the effects of its depletion with the valine resistant strain, *K/val*. *Lambda* production following the irradiation of *K/val* was identical with that of the wild type and showed the same requirement for pool no. 2 in the amino acid media. The comparative effects of single depletion of the components of pool no. 2 are recorded in table 3. The absolute requirement for leucine was the same for both organisms. The requirement for valine remained critical for *lambda* production in *K/val* but was less marked than in strain K-12. Isoleucine, however, was reduced markedly from an absolute requirement for the K-12 system to only a relative one for the *K/val* system. It appears then that the presence of isoleucine for phage maturation in the K-12 wild type is required primarily to prevent the inhibition of the host by valine. In the *K/val* strain, where this inhibition does not exist, isoleucine is necessary for only a relative (twofold) increase in the yield of total virus.

TABLE 3

Comparison of leucine, isoleucine (or ketoisoleucine), and valine (or ketovaline) requirements for phage production in Escherichia coli, strain K-12, and its valine resistant mutant, K/val

LEUCINE	VALINE	ISO-LEUCINE	KETO-VALINE	KETOISO-LEUCINE	PERCENTAGE OF MAXIMUM PHAGE YIELD	
					K-12	*K/val*
+	+	+	−	−	100	100
−	−	−	−	−	3.3	8.0
−	+	+	−	−	1.6	2.5
+	−	+	−	−	3.0	13.6
+	−	+	+	−	83.0	67.5
+	+	−	−	−	4.0	50.0
+	+	−	−	+	57.2	70.5

Depletions (−) and additions (+) as indicated were made in the presence of amino acid pools 3, 4, and 5.

Table 3 also shows that the α-keto acid analogues of valine (α-ketoisovaleric acid) or isoleucine (α-keto-β-methyl-*n*-valeric acid) can almost, but not completely, replace the requirements for their respective amino acids. Again, the ability of ketoisoleucine to replace isoleucine can be ascribed to a similar relief of the valine inhibition. The keto analogue of leucine was not available for testing.

An analysis of the respective D and L isomers of isoleucine and valine showed that their activities were entirely dependent upon the L form. The D forms were entirely inactive. With leucine, however, either the D or L form could restore complete activity to a leucine deficient system.

Threonine requirement and its relation to cyst(e)ine inhibition. With isoleucine, leucine, and valine provided, the next most limiting supplement was amino acid pool no. 4 (figure 1), the activity of which could be assigned completely to threonine (table 4). Table 4 also shows the partial ability of homoserine and inability of α-aminobutyric acid to substitute for threonine. In subsequent experiments where pool no. 1 was omitted because of its apparent inhibitory action, phage maturation was found to be less dependent on the presence of threonine. Instead of the 70 to 90 per cent decrease in virus yield obtained by the omission of pool no. 4 (or threonine) in the presence of pool no. 1, only a 20 to 50 per cent decrease was obtained when the latter was absent.

TABLE 4

Analysis of the effect of the components of pool no. 4 on phage production

COMPONENT (DEPLETED FROM A OR ADDED TO B)	PERCENTAGE OF MAXIMUM PHAGE YIELD	
	A All amino acids	B All minus pool no. 4
None	100	12.2
Histidine	108	15.1
Proline	107	19.3
Glutamic acid	109	12.5
Threonine	11.2	95.0
Homoserine		56.0
α-Aminobutyric acid		15.3

In either case, the same increase in latent period was obtained. This increased requirement for threonine in the presence of pool no. 1 may be expressed also as an increased inhibition of pool no. 1 in the absence of threonine. The inhibitory action was due entirely to the cystine content. The curves in figure 3 show the inhibitory action of cystine and the ability of both threonine and homoserine to overcome this inhibition.

Cysteine was not only as active as cystine but, in addition, its presence in the absence of threonine brought about an abortion of maturation as evidenced by a rapid and complete logarithmic loss of infectious centers. This means that the potential plaque forming units which were maintained ordinarily at a uniform level during the latent period were lost when cysteine was added. Only the initial and early developing units were sensitive to this inhibition with resistance progressing through the latent period. This is shown by the addition of cysteine at various periods after the onset of the latent period. As is shown in figure 4, cysteine added 10 minutes after irradiation did not bring about the rapid drop which occurred when cysteine was present from the beginning. Instead, an initial 40 per cent drop occurred with a maintenance of the surviving

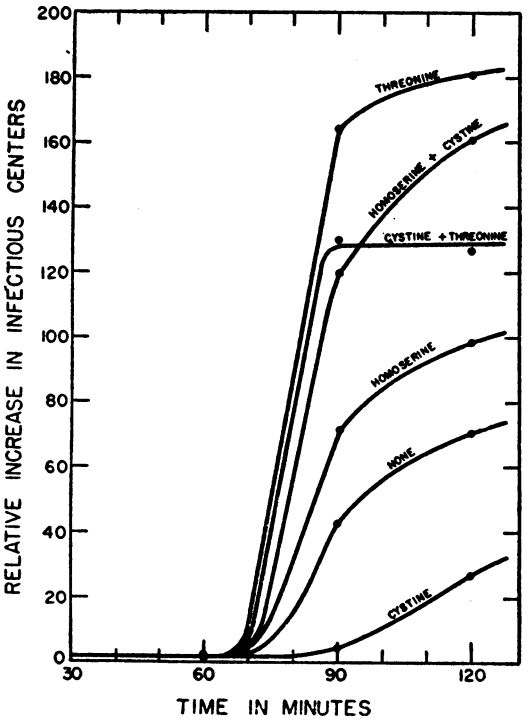

Figure 3. Interrelationships of threonine or homoserine requirement and cystine inhibition. All additions as shown are made to a control ("none") containing all amino acids except amino acid pool no. 1 and threonine.

infectious centers through a normal latent period with an eventual burst equal to the cysteine-free control. The initial drop became progressively less with the addition of cysteine at intervals up to 30 minutes. After this, addition of cysteine had no significant effect on the shape of the one step growth curve.

The units lost as plaque formers by the initial action of cysteine could not be recovered as colony formers. This would indicate that the cysteine effect was not due to a chemoreactivation of the ultraviolet action. Cysteine had no effect

on the growth of the normal noninduced organisms.

The action of cysteine could not be ascribed to a general nonspecific action of sulfhydryl compounds since neither thioglycolate nor homocysteine had any effect on the course of phage development. Furthermore, a mixture of salts of bivalent metals (Mn, Cu, Zn, Fe, and Co) did not disturb the events brought about by cysteine.

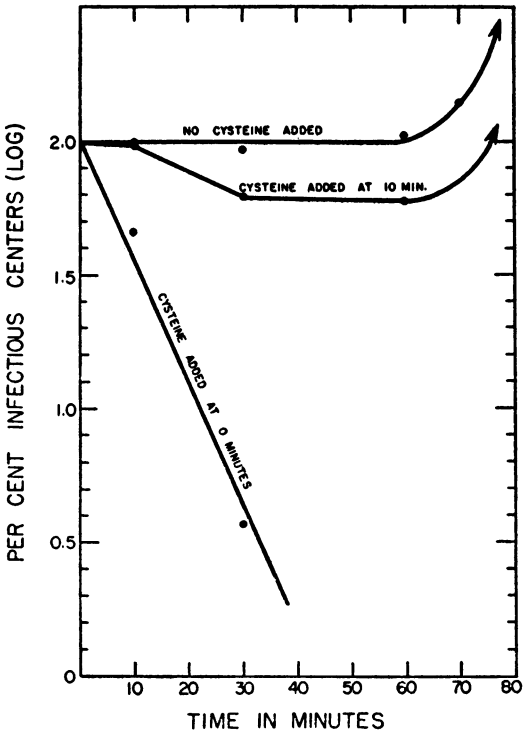

Figure 4. Effect of cysteine on initial infectious centers. All amino acids present except pool no. 1 and threonine.

Role of other amino acids. Though isoleucine, leucine, valine, and threonine were the most critical as demonstrated by the depletion experiments, they could not account for the total activity of the entire amino acid mixture. Their addition to the unsupplemented synthetic medium resulted in only a 3 to 4-fold increase in yield representing, at the most, 30 per cent of the yield provided by the total amino acid mixture. It is obvious that other amino acids are necessary, in a cooperative manner, for the manifestation of full activity. Except for the amino acids in pool no. 1 none of the others could be dispensed with entirely. In the absence of pool no. 1, the aromatic amino acids of pool no. 3 were the least necessary; their absence led to a 20 to 30 per cent depression of activity. Though none of the individual components of this supplement could replace entirely the activity of the whole, tryptophan appeared to be the most critical.

The best minimal medium so far obtained contains the 11 amino acids found in pools no. 2, 4, and 5. This mixture can account for approximately 75 per cent of the total activity. Under these conditions the activity of pool no. 4 was still dependent upon the presence of threonine, but this alone, as in the case (table 4) where cysteine was present, could not account for the full activity of the supplement. The presence of all of the other components of pool no. 4 was necessary for total activity. The major activity of pool no. 5 could be accounted for by the presence of both serine and aspartic acid. Glycine was not only dispensable, but in some cases, its presence exerted a slight inhibition.

DISCUSSION

The results presented here show that in order for *lambda* phage to mature in induced *E. coli*, strain K-12, amino acids must be provided to the environment. At least leucine and valine are absolutely required. Amino acids have been implicated previously as environmental factors necessary for the optimum synthesis of some lytic phages (Fowler and Cohen, 1948; Cohen and Fowler, 1948; Spizizen, 1943) as well as some temperate phages carried by lysogens (Lwoff 1951). None of these studies, however, could demonstrate the specific all-or-none effects which are evident in the system reported here. Cohen and Fowler (1948) did reveal that leucine and valine, which are absolutely essential for optimal *lambda* phage production, were also among those amino acids most necessary for the maintenance of maximum T_2 coliphage synthesis in *E. coli*, strain B.

The all-or-none effect exhibited by isoleucine could be demonstrated only under conditions where the noninduced bacterial host itself manifested a growth requirement for isoleucine. In the valine resistant mutant, where the isoleucine requirement created by valine does not exist, isoleucine had only a relative effect on *lambda* maturation. This serves only to emphasize that bacterial viruses are products of their hosts'

metabolism. This may be exemplified further, as determined independently by us and Borek (1952), that auxotrophic mutants of lysogenic hosts are unable to support phage synthesis in the absence of their particular required metabolite, even though this metabolite is not required for phage maturation in the prototrophic lysogen. Since the amino acid contents of bacteriophage and host proteins are essentially qualitatively similar (Polson and Wyckoff, 1948), it would be expected that a bacterial host which is impaired with respect to bacterial protein synthesis would remain impaired when called upon to synthesize phage protein. Though this would appear to be an obvious concept, several published studies indicate that it has not always been completely appreciated.

The K-12 strain of *E. coli* is one which can satisfy readily its nitrogen requirements for bacterial growth by nothing more complicated than inorganic ammonium salts. Why then, when this organism is forced into the synthesis of bacterial viruses from the latent prophage form by means of ultraviolet action, must more complex nitrogenous substances be provided? Two possible explanations come to mind. Either the synthetic capacities of the infected cell have been impaired with respect to the essential amino acids involved, or the demand for these amino acids for virus synthesis has become much greater than can be supplied endogenously. It is possible also, based on analogies drawn from many patterns of bacterial nutrition, that the substances required are not in themselves utilizable but are necessary for the depression of another deleterious reaction, one that might have been initiated by the ultraviolet treatment.

The ability of α-ketoisovaleric acid to satisfy the valine requirement would indicate that at least the amination or transamination mechanisms necessary for the conversion of this keto acid to valine remain intact. The inability of D-valine and D-isoleucine to substitute for their respective L forms in the support of phage maturation is in keeping with the observations that the growth of *E. coli* auxotrophic mutants requiring these amino acids can be supported only by the L isomers (Umbarger and Mueller, 1951). Also, the isoleucine requirement created by L-valine in the K-12 strain cannot be satisfied by D-isoleucine. This would indicate that *E. coli* does not possess a functioning racemase for these amino acids. The data with leucine, however, are more disturbing. Either D- or L-leucine can function in the requirement for phage maturation, yet in similar studies with leucine requiring mutants of *E. coli* (including strain K-12), only L-leucine can support bacterial growth (Gots, *unpublished observations*). This implies that either the *lambda* producing cell can manifest a functioning leucine racemase which is absent in the noninduced cell or that D-leucine, *per se*, can be used for phage protein.

Cysteine has been incriminated previously as an inhibitor of the development of the lytic coliphages (Fowler and Cohen, 1948; Mutsaars, 1950). Where analyzed, such inhibition has been found to be associated primarily with its sulfhydryl action involving binding of essential metals (Spizizen *et al.*, 1951; Joklik, 1952). In the production of phage in lysogenic *B. megaterium*, cysteine as well as other sulfhydryl agents actually can bring about induction in a manner similar to the ultraviolet action (Lwoff and Siminovitch, 1952). The cysteine effect, as described here, could not be attributed to a nonspecific sulfhydryl action but involves a more specific action apparently associated with threonine metabolism.

For purposes of a working hypothesis we have formulated the following tentative interpretation which is compatible with the results obtained. In *E. coli*, as in other organisms (Teas *et al.*, 1948; Teas, 1950), it has been shown that homoserine has two functions (Gots and Koh, 1950): to act as (1) a threonine precursor and (2) a methionine precursor via condensation with cysteine to yield cystathionine. In a normally functioning cell the distribution of these two functions must be so regulated as to allow optimal production of both end products. It is conceivable that in a cell whose metabolism has been disrupted by ultraviolet shock towards the synthesis of viral substances, a shift in the normal apportionment may occur allowing one reaction to take preference over the other. Thus, if homoserine preferentially reacts with cysteine, less will be available for threonine synthesis. This would lead to a threonine requirement which could be replaced by additional homoserine; such is the case.

Why cysteine (but not cystine) should bring about an abortion or loss of potentially infective units and why this occurs primarily during the early period of development is less easily interpretable. At least with T_1 and T_2 lytic coliphages

the loss of effective infectious centers can be explained by the findings of Joklik (1952) that cysteine does not inhibit phage multiplication but prevents the liberation of the developing phage units.

Borek (1952) has reported the inability of *E. coli*, strain K-12, to be induced to form *lambda* phage in a synthetic medium when the organisms were "starved" of nitrogen or glucose for several hours prior to ultraviolet treatment. He interpreted this as a loss of aptitude brought about by the period of starvation. From the data which we have presented here, this can be explained more readily, not as a loss in aptitude, but merely as a lack of phage maturation in an unsupplemented synthetic medium. We have kept washed buffer suspensions of *E. coli*, strain K-12, corresponding to the starvation regime of Borek, not only for hours but for several days with no loss in the ability to produce *lambda* provided the proper amino acid environment is supplied *after* induction.

SUMMARY

For optimal maturation of *lambda* phage, to occur in the lysogenic *Escherichia coli*, strain K-12, amino acids must be present in the environment after the cells have been induced to produce phage by ultraviolet irradiation. Leucine and valine are essential. Isoleucine is also essential but only because its presence is required to prevent inhibition of the bacterial host by valine. In a valine resistant mutant of strain K-12, isoleucine shows only a relative requirement for the production of *lambda*. This is also true for the α-keto acid analogue of isoleucine. Valine requirement can be replaced by its corresponding α-keto acid analogue, α-ketoisovaleric acid. Only the L forms of valine and isoleucine are active, but either the D or L form of leucine can support *lambda* maturation. Threonine also is required for optimum production, but this requirement is associated with an inhibitory action exerted by cystine. Homoserine can replace partially the threonine requirement. Cysteine brings about an abortion of developing phage as shown by a rapid loss in infectious centers when added at the beginning of the latent period. This does not occur when cysteine addition is delayed. Delayed addition of isoleucine, leucine, and valine indicated that development during the first 13 minutes of the latent period does not require these amino acids. A number of other amino acids have a relative effect in stimulating maturation; of these, serine and aspartic acid are the most critical.

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