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[1] McLean, J. R., G. L. Cohn, I. K. Brandt, and M. V. Simpson, *J. Biol. Chem.*, **233**, 657 (1958).
[2] Roodyn, D. B., P. J. Reis, and T. S. Work, *Biochem. J.*, **80**, 9 (1961).
[3] Truman, D. E. S., and A. Korner, *Biochem. J.*, **83**, 588 (1962).
[4] Bronk, J. R., *Biochem. J.*, in press.
[5] Kielley, W. W., and R. K. Kielley, *J. Biol. Chem.*, **191**, 485 (1951).
[6] Lardy, H. A., D. Johnson, and W. C. McMurray, *Arch. Biochem. Biophys.*, **78**, 587 (1958).
[7] Kulka, R. G., and C. Cooper, *J. Biol. Chem.*, **237**, 936 (1962).
[8] Brierley, G. P., E. Murer, and D. E. Green, *Science*, **140**, 60 (1962).
[9] Bronk, J. R., *Ann. N. Y. Acad. Sci.*, **86**, 494 (1960).
[10] Bronk, J. R., *Biochim. Biophys. Acta*, **69**, 375 (1963).
[11] Berg, P., *Ann. Rev. Biochem.*, **30**, 293 (1961).
[12] Truman, D. E. S., and A. Korner, *Biochem. J.*, **85**, 154 (1962).

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*THYMIDINE-REQUIRING MUTANTS OF PHAGE T4**,†

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Infection with phage T2 can overcome the thymine requirement of a mutant of *Escherichia coli.*[1] Specifically, infection induces a thymidylate synthetase that is physically[2] and enzymatically[3] distinguishable from a normal cell's thymidylate synthetase. The new enzyme appears in cells that have their own enzyme as well as in cells, such as *E. coli* $15T^-$ or B3, that cannot make their own thymidylate synthetase. The thymidylate synthetases induced by T2 and T6 are different from each other.[3]

To understand the nature of the phage's genetic control over the new enzyme, we have isolated and studied T4 mutants that have lost the ability to grow in a thymineless strain of *E. coli* without exogenous thymidine. The location of the phage mutants on the genetic map has critical implications regarding the timing mechanism for gene expression.

Materials and Methods.—Tris-glucose: NaCl, 5.8 gm; KCl, 3.7 gm; $CaCl_2 \cdot 2H_2O$, 0.15 gm $MgCl_2 \cdot 6H_2O$, 0.10 gm; NH_4Cl, 1.1 gm; $FeCl_3$, 2.7×10^{-4} gm; Na_2SO_4, 0.142 gm; KH_2PO_4, 0.272 gm; 50% glucose (w/v), 10 ml; tris(hydroxymethyl)aminomethane, 12.1 gm; H_2O, to make 1 liter; pH adjusted to 7.4 with 12 *N* HCl.

Tris-glucose-thymidine: Tris-glucose plus 10 μg/ml thymidine.

Glycerophosphate tris-glucose agar plates: These synthetic plates were made with the tris-glucose ingredients with the addition per liter of 0.2 mg thymidine, 10 ml "vitamin-free" casein hydrolysate (acid) (Nutritional Biochemicals Corporation), 0.02 gm L-tryptophan, and 0.10 gm β-glycerophosphoric acid disodium salt (Eastman) replacing the KH_2PO_4. In addition, the bottom agar contained 12.0 gm Difco agar per liter, the top agar 7.0 gm. The plates were poured with approximately 30 ml of bottom agar per plate. The glucose for the bottom agar was autoclaved separately.

Tryptone agar plates: These plates were made with 10 gm Difco tryptone and 5 gm NaCl per liter of H_2O to which was added 12 gm Difco agar for the bottom layer and 7 gm Difco agar for the top layer.

Bacterial strains: E. coli B3, a thymine-requiring strain of *E. coli* deficient in thymidylate syn-

thetase activity, was originally isolated by Dr. S. Brenner;[4] *E. coli* BR2 was used for most of the genetic crosses; *E. coli* B and *E. coli* BB were used for growth studies.

Phage strains: $T4BO_1$, osmotic shock-resistant,[5] was the standard phage type from which mutants were isolated. The T4D mutants used for mapping were obtained as follows: *r*47, *r*48, *r*67, *tu*41 from Dr. A. H. Doermann; *am*81, *am*130, *am*134 from Dr. R. H. Epstein; *am*116 from Dr. R. S. Edgar.

Isolation of td mutants: Mutagenic enrichment of phage with thymidineless (*td*) mutants was the key step in the isolation procedure. Phage were treated in 0.2 *M* hydroxylamine and 0.14 *M* sodium phosphate buffer at pH 6.0 for 10 hr at 37°C. The reaction was quenched by diluting 50-fold into broth containing 2% acetone.[6] The surviving fraction was approximately 10^{-3}. To eliminate chemically induced heterozygotes,[7, 8] the treated phage were grown for one step in *E. coli* B, yielding the final mutagenized phage stock. By visual inspection, at least 4% of the phage from the mutagenized stock made plaques that appeared mutant, about 2% being *r* mutants.

The mutagenized stock was plated on tryptone agar with an additional 200 μg thymidine, using *E. coli* B3 as indicator. From these plates an assortment of 200 plaques were picked and suspended in 2 ml of *E. coli* B3. The B3 suspension was made by growing the cells in tris-glucose-thymidine to about 5×10^8/ml, centrifuging and washing two times in tris-glucose, and finally resuspending in tris-glucose at 1×10^8/ml. The phage were incubated without aeration at 37°C for 3 hr and then chloroformed. Any tube that failed to clear was suspected of having been inoculated with *td* mutants. Controls showed that standard T4 phage will cause clearing within two to three hr.

Phage from 15 of the 200 plaques failed to clear the cell suspension. Of the 15, the 7 making the largest plaques were screened further by comparing their burst sizes in *E. coli* B3 in tris-glucose-thymidine and tris-glucose. Phage from two plaques, labeled *td*8 and *td*15, showed about a 20-fold lower burst of phage when thymidine was omitted. Detailed growth studies are described in a later section. The significant reduction in burst size when thymidine is omitted from the B3 culture is the property that defines a *td* mutant.

To help eliminate other mutations that might have accumulated during the hydroxylamine treatment, *td*8 was backcrossed five times with the original $T4BO_1$, and *td*15 was backcrossed once. All studies were done with these backcrossed strains.

Plaque morphology and simple tests for td mutants: Plaques of *td* mutants are indistinguishable from td^+ on tryptone agar; but the mutants can be distinguished on tris-glucose plates. The procedure is to preadsorb the phage for 15 min to B3 that was grown to about 5×10^8/ml in tris-glucose-thymidine. The infected cells are then plated on tris-glucose plates using 2 ml top agar containing 4 μg/ml thymidine and 2 drops of a culture of B3 grown overnight in tris-glucose-thymidine. The *td* mutants show appreciably smaller plaques than td^+ any time after 8 hr incubation at 37°C. The plaques are shown in Figure 1*a*. Without preadsorption the efficiency of plating of *td* phage is reduced.

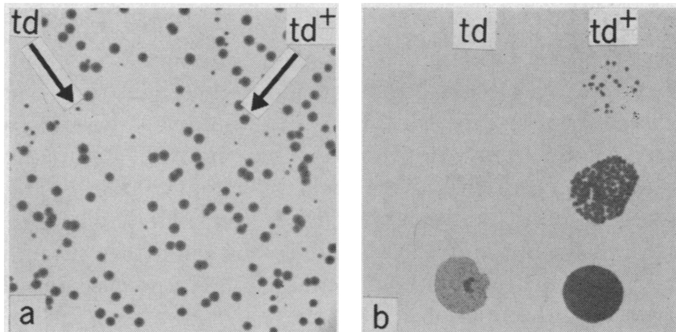

FIG. 1.—(*a*) Comparative morphology of *td* and td^+ plaques on glycerophosphate tris-glucose agar plates with B3 indicator, photographed after 14 hr incubation at 37°C.

(*b*) Spots of *td* and td^+ phage on glycerophosphate tris-glucose agar plates with B3 indicator photographed after 8 hr incubation at 37°C. The number of phages deposited in each spot varies from about 20 in the top row to about 10^4 in the bottom row.

A simple spot test was developed that distinguishes the *td* mutants from *td*$^+$. Using the same type of synthetic plates but now seeded with 5 drops of B3 in the overlay agar, roughly 0.002 ml of phage are deposited on the hardened overlay agar with a melting point capillary tube. The results are shown in Figure 1*b*. The test is not reliable when more than 10^4 phage are deposited in a spot.

The difference in plaque morphology between *td*$^+$ and *td* has been used to simplify subsequent isolations of *td* mutants. The mutagenized phage stock is plated on tris-glucose agar seeded with *E. coli* B3. The distinctly smaller plaques are picked and the phage tested for plaque size on two kinds of plates: tris-glucose agar seeded with B3 and tryptone agar seeded with B. We select phage which, compared with *td*$^+$, give standard-size plaques on B and small plaques on B3. Two mutants fitting this description were chosen and both gave a 20-fold lower burst size in B3 when grown in tris-glucose, compared with growth in tris-glucose-thymidine. These mutants were labeled *td*9 and *td*10.

D. L. Wulff and K. Metzger (*Virology*, 1963, in press) have independently isolated a thymine-requiring mutant of phage T4.

TABLE 1

BURST SIZE OF T4 MUTANTS IN THYMIDINE-DEPENDENT HOST *E. coli* B3 AND THYMIDINE-INDEPENDENT HOSTS *E. coli* B AND BB

	Phage Yield/Infected Cell			
	B3		B	BB
Phage	10 μg/ml thymidine	No thymidine	No thymidine	No thymidine
td$^+$	140	95	58	43
*td*8	100	7	60	46
*td*9	72	4	52	40
*td*10	120	6	42	41
*td*15	150	8	...	...

The cells were grown to 5×10^8/ml in tris-glucose-thymidine, washed two times in tris-glucose by centrifuging and finally resuspended at a concentration of 10^8/ml in tris-glucose supplemented with 0.002 *M* KCN and 20 μg/ml L-tryptophan. 0.1 to 0.2 phage per bacterium were added, allowed to adsorb for 6 min at 37°C without aeration, and then diluted 10^4-fold into tris-glucose with thymidine added as shown in the table. The cells were incubated at 37°C with gentle aeration for 100 min, lysed with chloroform, and assayed.

Results.—Evidence that td mutations affect a genetic region controlling the appearance of thymidylate synthetase activity: Table 1 shows the growth of *td* mutants in the presence and absence of added thymidine. *E. coli* B3 grown at 37 °C requires thymidine for growth in tris-glucose because it has a very low level of thymidylate synthetase activity.[4] Since *td* mutants can grow normally on B and BB, and cannot grow well in B3 without added thymidine, we conclude that the mutations affect a phage genetic region that controls the appearance of thymidylate synthetase activity.

One-step growth: The pattern of early phage synthesis in *td*8-infected B3 is shown in Figure 2*a*. Synthesis begins at about the same time whether or not thymidine is added, but the rate is less without thymidine. Results from the same experiment, but at later times after infection, are shown in Figure 2*b*. The addition of thymidine increases the phage yield by about 20-fold. The addition of FUDR is known to inhibit thymidylate synthetase.[3] FUDR plus uridine (Fig. 2*b*) consistently reduced the phage yield. Uridine alone had little or no effect. Similar results have been obtained with *td*9, *td*10, and *td*15. In no case does FUDR completely eliminate phage production.

It might be expected that the loss of a thymidine-synthesizing system would be a handicap for the phage even in the thymidine-independent host. Yet *td* phages seem to grow more or less normally on B or BB and make plaques which are indistinguishable from those of wild type on these hosts. Possibly a disadvantage would be clearly apparent if phage yields were high. This possibility was

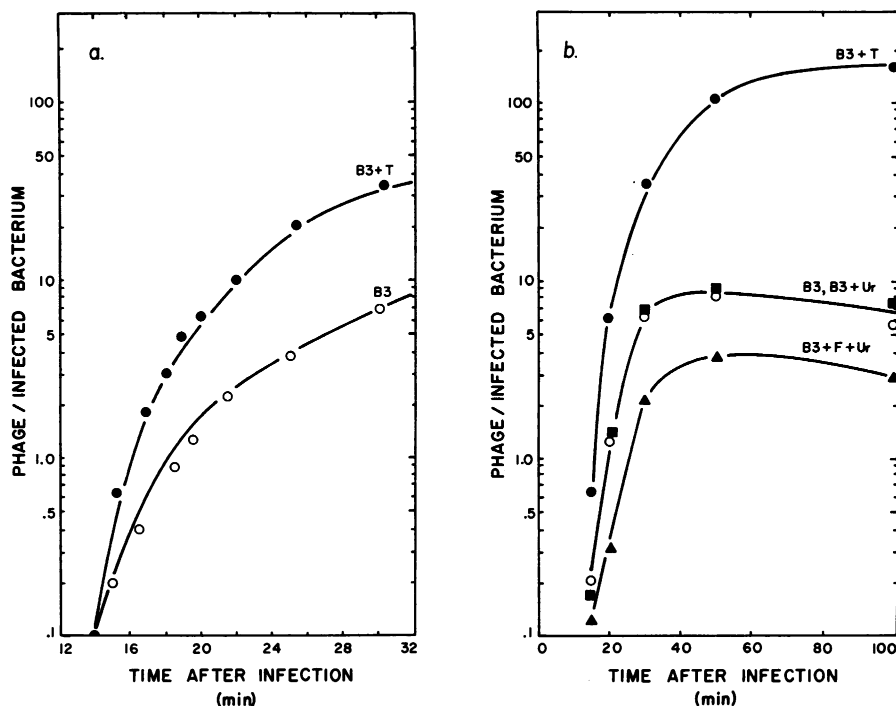

FIG. 2.—(*a*) Production of phage *td*8 in *E. coli* B3 at early times after infection in the presence and absence of thymidine. The conditions of infection are described in the legend to Table 1. When samples were taken, 1 ml of the culture was added to 1 ml nutrient broth, and lysed with chloroform. The growth media are indicated on the drawing. B3 = control containing tris-glucose; T = 10 μg/ml thymidine.

(*b*) Same experiment as in 2*a*, showing the effect of various additives over the entire course of the infection. The growth media are indicated on the drawing. B3 = control containing tris-glucose; T = 10 μg/ml thymidine; F = 5 μg/ml fluorodeoxyuridine; Ur = 20 μg/ml uridine.

tested by infecting BB with td^+ and *td*8 and inhibiting lysis by superinfecting at later times[9] as described in Figure 3. Even though the burst sizes exceeded 500 phage per bacterium there was no significant difference between *td*8- and td^+-infected cells, and addition of thymidine had no noticeable effect.

Complementation: Complementation was measured by the growth of phage in mixedly infected B3, without thymidine. Table 2 shows the burst sizes from B3 infected with all possible pairs of mutants. The first row of numbers shows that the effect of the td^+ gene together with any of the *td* mutants is to allow a normal burst, the burst containing roughly equal numbers of parental types. But growth of any pair of mutants was not measurably better than the growth of either mutant alone. Therefore, the four *td* mutants isolated show no complementation as measured by the burst size. We use the lack of complementation in the *trans* configuration as the criterion for tentatively placing the four mutants in the same cistron.

Genetic mapping of the td mutants: Each *td* mutant recombines with the other three, the frequencies ranging from 1 to 4 per cent.

To map the *td* mutants on the circular map of T4,[10] *td*8 which had been backcrossed five times to $T4BO_1$ and then backcrossed four times to T4D was used.

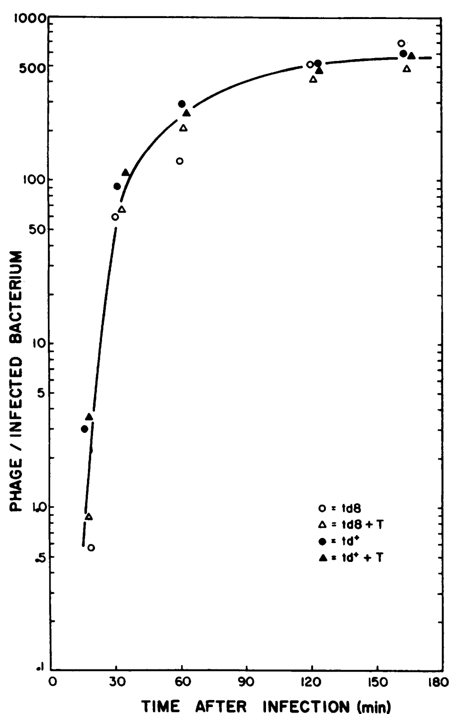

FIG. 3.—Comparison of growth rates of td^+ and *td*8 under conditions of lysis inhibition. *E. coli* BB was grown to 5×10^8/ml in tris-glucose, and supplemented with tryptophan immediately before infection. The bacteria were infected with either td^+ or *td*8 at a multiplicity of 3–5 phage per bacterium and superinfected at times 13, 32, 62, and 122 min after infection with seven phage of the same type per bacterium. Indicated cultures were supplemented with 10 μg/ml thymidine. Cells were diluted 100-fold into broth at the given times and lysed with chloroform before assaying.

Four-factor crosses (Table 3) place *td*8 between *am*134 and *r*67 (Fig. 4), well removed from the so-called "early region," between *r*47 and *r*48.[11]

Discussion.—*E. coli* B3 lacks thymidylate synthetase activity when grown at 37°C.[4] From the comparative growth of *td* mutants on B, BB, and B3, with and without added thymidine, we conclude that the mutants are deficient in the ability to produce thymidylate synthetase activity. This has been confirmed by examination of the enzymatic activities in extracts from cells infected with each of the *td* mutants.[12] Thus, there is now direct evidence that T4 asserts genetic control over the production of thymidylate synthetase activity. The lack of complementation among the four *td* mutants suggests that these mutants affect the same protein.

B3 infected with *td* phage produces about 8 phage per bacterium in the absence of added thymidine, about 120 in its presence. Since about 50 phage DNA equivalents of thymidine are available via breakdown of bacterial DNA,[13] why is so little phage produced in the absence of added thymidine? An explanation is suggested by experiments which show that phage-precursor molecules mature after FUDR is added to a T4-infected culture, but that 20–30 phage-precursor molecules remain unmatured whatever the size of the pre-existing pool.[14] Hence, a minimum pool of this size may be necessary before there can be any phage maturation. Since only 50 per cent of the bac-

TABLE 2

COMPLEMENTATION OF *td*-MUTANTS

	td^+	*td*8	*td*9	*td*10	*td*15
td^+	70	60	120	90	120
*td*8		3	7	6	5
*td*9			4	5	9
*td*10				4	6
*td*15					5

A number within the matrix is the burst size of phage from B3 cells mixedly infected with the two phage types heading the corresponding row and column.

E. coli B3 was grown to 1 to 2×10^8/ml in tris-glucose-thymidine, washed two times by centrifuging, and concentrated to 5×10^8/ml, all in tris-glucose. Twenty μg/ml L-tryptophan was added for adsorption. One ml aliquots of the concentrated B3 were infected with the indicated pairs of phages, each phage at a multiplicity of infection of 4. The mixtures were aerated at 37°C for one hr, lysed with chloroform, and assayed. In the first row the numbers comprise roughly equal amounts of each parental type.

TABLE 3

RELATIVE NUMBERS OF *td* AND td^{+} AMONG RECOMBINANTS IN FOUR-FACTOR CROSSES

r67tu41td8 × *am134*			*tu41td8* × *r67am134*		
Recombinants	Exp. 1	Exp. 2	Recombinants	Exp. 1	Exp. 2
$r67am^{+}tu^{+}td$	22	67	$r^{+}am^{+}tu^{+}td$	18	33
$r67am^{+}tu^{+}td^{+}$	1	12	$r^{+}am^{+}tu^{+}td^{+}$	1	0
$r^{+}am^{+}tu41td$	18	45	$r67am^{+}tu41td$	14	26
$r^{+}am^{+}tu41td^{+}$	5	29	$r67am^{+}tu41td^{+}$	7	8

Recombinants	*r47r48td8* × *am116*	*r47r48td8* × *am81*	*r47r48td8* × *am130*
$r47am^{+}r^{+}td$	20	11	16
$r47am^{+}r^{+}td^{+}$	20	12	13
$r^{+}am^{+}r48td$	17	26	21
$r^{+}am^{+}r48td^{+}$	32	51	61

terial DNA appears in phage,[13] the low yield is not surprising. The partial sensitivity of the yield to FUDR shows that thymidylate synthetase activity is indeed the factor limiting burst size.

Why does the phage possess the td^{+} function? Flaks and Cohen[15] have suggested the reasonable explanation that a large increase in thymidylate synthetase activity is needed after infection to match the increased need for thymine. They suggested that thymidylate synthetase activity is a rate-limiting step in phage synthesis. We find no support for this explanation. In tris-glucose, *td* mutants grow approximately as well as td^{+} in *E. coli* B and BB, even under conditions of lysis inhibition where the burst size is as high as 500. Furthermore, DNA synthesis starts at the same time and proceeds at the same rate in cultures of BB infected with *td*8 or td^{+}.[16] Only under the conditions of extreme thymidine deprivation present in *E. coli* B3 do we find a significant distinction between *td* and td^{+}. The td^{+} genetic region might normally confer only a slight advantage to the phage. Possibly its true worth cannot be fully appreciated outside such environments as colons and sewers.

FIG. 4.—The location of the *td* locus on the genetic map of T4.[17]

The position of *td* on the T4 genetic map is relevant to theories of phage development. In a study of the physiology of T4 mutants, Edgar, Denhardt, and Epstein[17] have observed that phage mutants affecting functions which are expressed at about the same time are arranged in contiguous regions of the map. For example, the genetic region between *r*II and *r*I seems to contain only genes responsible for "early functions," controlling the formation of enzymes that appear early in phage development before DNA synthesis begins. In cells infected with T-even phages, thymidylate synthetase activity starts to increase approximately five minutes after infection.[15, 18, 19] In T6, dCMP hydroxymethylase activity appears immediately after infection, and lysozyme activity ten minutes after infection.[18] Therefore, *td* should fall in the "early region," possibly between *am*122, which seems to control dCMP hydroxymethylase,[19] and *e*,[20] which controls the phage lysozyme. Contrary to these expectations, the *td* locus is nowhere near this region,

but is well within a "late region" containing cistrons that control the formation and assembly of phage coat protein. Therefore, phage mutants affecting functions which are expressed at about the same time need not be arranged in contiguous regions of the map.

Summary.—Four mutants (*td*) of phage T4 have been isolated. They are deficient in their ability to grow in a thymine-requiring strain of *E. coli* without exogenous thymidine, but grow normally when thymidine is added. The mutants are all in the same cistron, which is responsible for the appearance of phage-controlled thymidylate synthetase activity. The *td* mutants map in a position far removed from a region controlling other functions that are also expressed early in phage development, showing that phage mutants affecting functions which are expressed at about the same time need not be arranged in contiguous regions of the map.

Note added in proof: Recent experiments have shown that thymine, as well as thymidine, is effective in supporting growth of *td* mutants in *E. coli* B3. Therefore, the *td* mutants are, strictly speaking, thymine-requiring.

* This investigation was supported in part by research grants GF-14053 and G-17611 from the National Science Foundation.

† The following abbreviations are used: FUDR, 5-fluorodeoxyuridine; dCMP, deoxycytidine-monophosphate.

[1] Barner, H. D., and S. S. Cohen, *J. Bacteriol.*, **68**, 80 (1954).

[2] Greenberg, G. R., R. L. Somerville, and S. De Wolf, these PROCEEDINGS, **48**, 242 (1962).

[3] Mathews, C. K., and S. S. Cohen, *J. Biol. Chem.*, **238**, 367 (1963).

[4] Barner, H. D., and S. S. Cohen, *J. Biol. Chem.*, **234**, 2987 (1959).

[5] Brenner, S., and L. Barnett, in *Structure and Function of Genetic Elements*, Brookhaven Symposia in Biology, No. 12 (1959), p. 86.

[6] Freese, E., E. Bautz-Freese, and E. Bautz, *J. Mol. Biol.*, **3**, 133 (1961).

[7] Tessman, I., *Virology*, **9**, 375 (1959).

[8] Vielmetter, W., and C. M. Wieder, *Z. Naturforsch.*, **146**, 312 (1959).

[9] Doermann, A. H., *J. Bacteriol.*, **55**, 257 (1948).

[10] Streisinger, G., R. S. Edgar, and G. H. Denhardt, personal communication.

[11] A peculiar aspect of the last set of crosses is that *td*8$^+$ appears more closely linked to *r*47$^+$ than *td*8 is to *r*47.

[12] Shapiro, D., J. Eigner, and G. R. Greenberg, personal communication.

[13] Hershey, A. D., and N. E. Melechen, *Virology*, **3**, 207 (1957).

[14] Simon, E. H., *Bact. Proc.*, p. 148 (1961), and unpublished data.

[15] Flaks, J. G., and S. S. Cohen, *J. Biol. Chem.*, **234**, 2981 (1959).

[16] Simon, E. H., unpublished data.

[17] Edgar, R. S., G. H. Denhardt, and R. H. Epstein, personal communication; *Biol. Ann. Rept. Calif. Inst. Tech.* (1962); see also Champe, S. P., *Ann. Rev. Microbiol.*, **17**, in press (1963); Luria, S. E., *Ann. Rev. Microbiol.*, **16**, 205 (1962).

[18] Cohen, S. S., M. Sekiguchi, J. L. Stern, and H. D. Barner, these PROCEEDINGS, **49**, 699 (1963).

[19] Wiberg, J. S., M. Dirksen, R. H. Epstein, S. E. Luria, and J. M. Buchanan, these PROCEEDINGS, **48**, 293 (1962).

[20] Streisinger, G., F. H. Mukai, F. Dreyer, B. Miller, and S. Horiuchi, in *Cellular Regulatory Mechanisms*, Cold Spring Harbor Symposia on Quantitative Biology, vol. 26 (1961), p. 25.