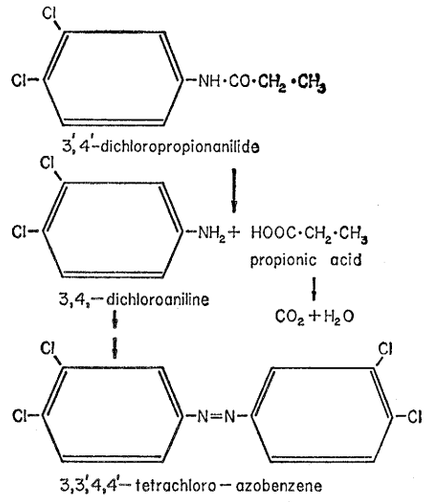

Fig. 1. Scheme of transformation of 3',4'-dichloropropionanilide in soil.

recrystallization as orange needles from absolute ethanol and from hexane. The mass spectrum indicated that the compound had a molecular weight of 322 and an empirical formula of $C_{12}H_6N_2Cl_4$. Authentic TCAB was synthesized by the use of $LiAlH_4$ for the reductive condensation of 3,4-dichloronitrobenzene (*2*). The identity of the synthetic TCAB and of the pesticide transformation product isolated from soil was established by comparing their movements on thin-layer plates, retention times in a gas chromatograph, infrared spectra, and melting points (157°C).

No DCA or TCAB could be isolated, from soil that had not received pesticide or from soil that was sterilized, treated with filter-sterilized herbicide solutions, and incubated at 28°C for 14 days before analysis. Moreover, tests were performed to exclude the possibility that DCA and TCAB were produced chemically during extraction or isolation procedures.

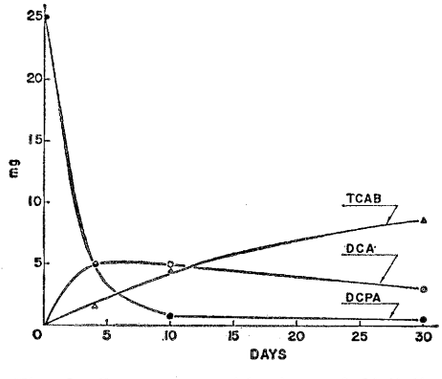

Fig. 2. Conversion with time of 3',4'-dichloropropionanilide (DCPA) to 3,4-dichloroaniline (DCA) and 3,3',4,4'-tetrachloroazobenzene (TCAB) in soil treated with 500 parts per million of the herbicide.

Apparently, in natural soil an acylamidase of microbial origin catalyzes the cleavage of the herbicide molecule DCPA into DCA and propionic acid. The latter compound is utilized as a source of carbon and energy by soil microorganisms and transformed to carbon dioxide, water, and cell substance. The condensation that produces TCAB from DCA may be a direct oxidative condensation of two molecules of DCA, or DCA may be first transformed in part to 3,4-dichloronitrosobenzene, after which a spontaneous condensation of one molecule of the aniline compound with one molecule of the nitroso compound may then occur. In this reaction the nitroso compound would be produced by the biological oxidation of the aniline compound, but coupling would be accomplished chemically rather than biochemically (Fig. 1).

The conversion of DCPA to DCA and ultimately to the azo compound during 30 days in the soil is illustrated in Fig. 2. Quantitative analysis of the pesticide and of its metabolites was performed by gas chromatography. The concentration of DCA reached a maximum within 10 days and then declined, but TCAB continued to accumulate for the duration of the experiment. In 30 days, 46 percent of the aromatic moiety of the added DCPA was recovered as the azo compound, but this value may be low because some TCAB may have been retained by the soil.

The TCAB was formed in soil that received DCPA or DCA only; this supports the conclusion that DCA is an intermediate in the transformation of the herbicide to TCAB. In view of the fact that anilines are products of the degradation of various phenylurea and phenylcarbamate herbicides (*3*), the possibility of condensation reactions and the formation of TCAB or other azo compounds in soil should be explored. The biological activity of TCAB has not been investigated; however, the fact that certain azo compounds are known to be carcinogenic (*4*) necessitates further study of the production and fate of these compounds during pesticide decomposition in soil.

RICHARD BARTHA
DAVID PRAMER
Department of Biochemistry and Microbiology, Bureau of Conservation and Environmental Science, Rutgers University, New Brunswick, New Jersey

References and Notes

1. R. Bartha, R. P. Lanzilotta, D. Pramer, *J. Appl. Microbiol.* **15**, 67 (1967).
2. J. F. Corbett and P. F. Holt, *J. Chem. Soc.* **1963**, 2385 (1963).
3. P. C. Kearney, *Advances Chem. Ser. No. 60*, 250 (1966).
4. V. J. P. Neish, H. M. Davies, P. M. Reeve, *Biochem. Pharmacol.* **13**, 1291 (1964).
5. Supported in part by PHS reserach grant ES-00016-03. We thank Dr. S. E. Katz for assistance and advice.

9 May 1967

Azure Mutants: A Type of Host-Dependent Mutant of the Bacteriophage f2

Abstract. *A new type of host-dependent mutant,* azure *mutant, of bacteriophage f2 has been isolated. Growth of these mutants was restricted specifically by* amber *suppressor genes in the host bacteria. Restriction of the formation of infective centers by different bacterial suppressor genes was 98 percent, 90 percent, and 70 percent with Su-3, Su-1, and Su-2 genes, respectively. Restriction, like suppression, was the dominant phenotype. The block in growth of the mutants occurred in an early stage of the infection cycle. Once infection was established, however, an infected cell produced approximately the same number of progeny phage as a cell without the suppressor genes did. It is proposed that the* azure *codon is the same as the* amber *codon (uracil, adenine, guanine) and that restriction results from improper termination of protein chains of the phage RNA polymerase. Similar mutants may exist in other systems.*

Amber mutants have been isolated from various bacteriophages, such as T4 (*1–4*) and RNA phage f2 (*5*). They are defined as mutants that grow in bacteria that have certain suppressor genes (Su+) but not in bacteria lacking these genes (Su−). We report here the isolation and preliminary characterization of a set of mutants of f2, the growth of which, contrary to *amber* mutants, is restricted by *amber* suppressor genes in the bacteria. Edgar (*6*) isolated a similar set of mutants of T4 that grow in *Escherichia coli* B but not in *E. coli* K12 harboring a suppressor gene and named them *azure* mutants. However,

Table 1. Plating properties of *azure* mutants and their derivatives. Plaques on K190 lawns were very turbid and more variable in size than those on the other indicators. This results from the spontaneous segregation from the organisms of Su-bacteria upon which the phage can grow. Values are expressed as plating efficiency relative to that of strain Su-. Letters in parentheses indicate sizes of the plaques; L, large; M, medium; S, small; and VS, very small.

Phage	Su⁻ (K38)		Su-1⁺ (K37)		Su-3⁺ (K110)		Su-$^{3+}$/Su⁻ (K190)
	35°C	42°C	35°C	42°C	35°C	42°C	35°C
f2	1 (L)	1.1 (L)	0.8 (L–M)	0.9 (L–M)	0.4 (L)	0.8 (L)	
az-1	1 (L)	0.8 (VS)	0.35 (S) 10^{-3} (L)	2×10^{-3} (S–VS)	3×10^{-2} (VS) 3×10^{-3} (M–S) 5×10^{-4} (L)	3×10^{-4} (S–VS)	2×10^{-2} (L–S)
az-1A	1 (L)	0.6 (S–VS)	0.7 (M)	0.6 (VS)	0.4 (L)	0.4 (S–VS)	
az-4	1 (L)	4×10^{-2} (L)	2×10^{-2} (S–VS) 4×10^{-4} (L–M)	7×10^{-4} (VS)	5×10^{-4} (L)	$<10^{-5}$	
az-4A	1 (L)	5×10^{-2} (VS) 1.6×10^{-2} (L)	0.8 (L–M)	1.3×10^{-2} (VS) 1.3×10^{-2} (L–M)	0.5 (L)	10^{-2} (S–VS) 10^{-2} (L–M)	
az-4B	1 (L)	1.2 (L)	0.3 (S) 2×10^{-3} (L–M)	0.1 (S) 2×10^{-3} (L-M)	3×10^{-3} (VS) 1×10^{-3} (L–S)	2×10^{-3} (VS) 1×10^{-3} (L–S)	2×10^{-2} (L–S)

the T4 mutants do not grow on any *E. coli* K strains. Our *azure* mutants of f2 grow normally in Su⁻ strains of *E. coli* K12 but are restricted in growth in isogenic bacterial strains of the Su+ type.

Benzer and Champe (*1*) and Garen and Siddiqi (*7*) have shown that some of the suppressible mutations in phage T4 and in *E. coli* are nonsense mutations. It is now well known that there are at least two types of suppressible nonsense mutations, *amber* and *ochre* (*8, 9*), and that the *amber* and *ochre* codons are uracil, adenine, guanine (UAG) and uracil, adenine, adenine (UAA), respectively (*10*). In Su⁻ bacteria the *amber* codon (and probably *ochre* codon, too) acts as nonsense and causes premature chain-termination, producing amino-terminal fragments of polypeptide chains (*3, 11*). This suggests that *amber* or *ochre* codons are the natural chain-terminating codons. In Su+ bacteria, the nonsense codons are read and this results in the insertion of an amino acid specific for each suppressor gene. Three different *amber* suppressor genes are known, Su-1, Su-2, and Su-3, and the amino acids inserted at the site of the *amber* mutation are serine, glutamine, and tyrosine, respectively (*12*).

In this study we used two sets of bacterial strains: K38 (Su⁻), K37 (Su-1+), K146 (Su-2+), and K110 (Su-3+) which are S26, S26R1e, S26R1d, and H12R8a, respectively (*13*), from Garen; and K140 (Su⁻), K141 (Su-1+), K142 (Su-2+), and K143 (Su-3+) which are D21, D30, D31, and D32, respectively, from Brenner (see Fig. 1). We also obtained from Brenner a diploid strain of genotype ϕ80dSu^{3}+/Su⁻ (K190).

The *azure* mutants of f2 can be obtained as follows. Lysates of wild type f2 are treated with nitrous acid according to the procedure described by Zinder and Cooper (*5*). The treated sample is plated on the Su⁻ strain (K38), and the single plaques obtained are analyzed by spot-tests on plates seeded with either Su⁻ (K38) or Su-3+ (K110) cells. The spots showing lysis on Su⁻ but not on Su-3+ are purified by isolation of single plaques. When treated with nitrous acid to a survival of 10^{-6}, 0.5 to 1 percent of the plaques on Su⁻ were found to be *azure* mutants. So far we have isolated 11 *azure* mutants (az-1 to az-11), most of which are also temperature-sensitive. However, the sensitivity to temperature does not seem to be directly related to the *azure* mutation, for when revertants of the temperature-sensitive *azure* mutants were obtained on plates seeded with Su-3+ cells and incubated at 35°C, they were still temperature-sensitive. We probably isolated phage with two independent mutations, since at least 60 percent of the phage in treated stock are sensitive to temperature.

All of the *azure* mutants of f2 that we tested showed similar plating properties at 35°C. On Su⁻ strains they formed plaques as large as those of the wild type, and the plating efficiency (EOP) was defined as 1. On Su-2+ the EOP was 0.5 to 0.8, and the plaques were somewhat smaller than those on Su⁻; on Su-1+ the EOP was about 0.3, and the plaques were small and turbid. The plates contained large clear plaques that looked like wild type at an EOP of 0.5 to 1×10^{-3}. On Su-3+ the mutants formed very small and turbid plaques with an EOP of less than 5×10^{-2}, and large clear plaques of revertants at an EOP of 0.5 to 1×10^{-3}. Both Garen's and Brenner's strains have been used as indicators, and there were no obvious differences between the two sets. On *ochre* suppressor strains K178 (Su-4+) and K133 (Su-5+) (*9*) (obtained from Garen), the

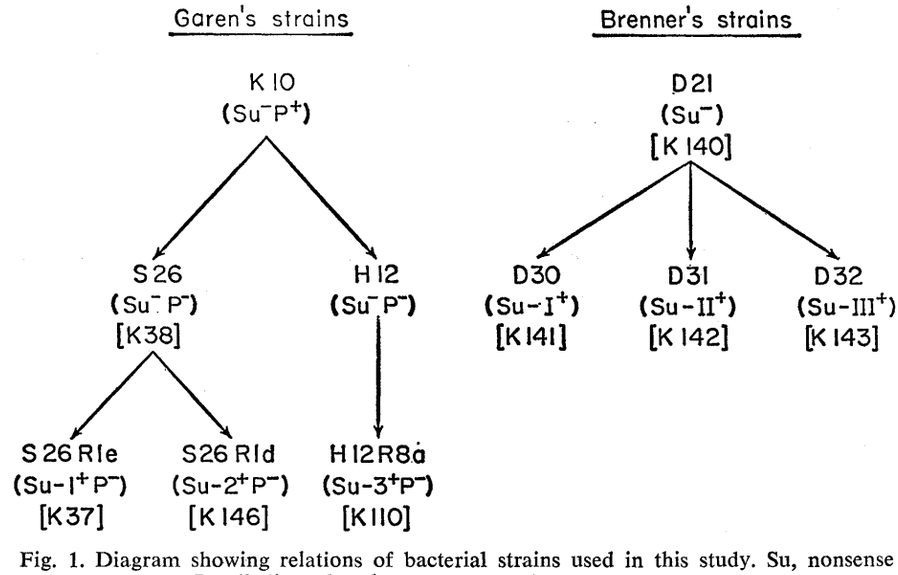

Fig. 1. Diagram showing relations of bacterial strains used in this study. Su, nonsense suppressor gene; P, alkaline phosphatase structural gene.

EOP and plaque morphology of *azure* mutants were exactly the same as on Su$^-$ strains. Table 1 gives the plating efficiency and plaque morphology of lysates of phages az-1, az-4, and their derivatives under various plating conditions. We obtained az-1A by picking a large plaque of az-1 from a plate seeded with Su-3+ (K110) and incubating it at 35°C. It is no longer *azure* but it is still temperature-sensitive. The sensitivity to temperature is genetically so stable that we have been unable to obtain revertants that are not temperature-sensitive from either az-1 or az-1A. Changes in burst size of az-1 upon shifts in temperature during the infection cycle were measured as described earlier (*14*) and showed that the sensitivity to temperature affects a late phage function. We obtained az-4A, a temperature-sensitive non-*azure* revertant, from az-4 in the same way that we obtained az-1A, but its sensitivity to temperature is highly revertible (5×10^{-2}). Phage az-4B was obtained by picking a large plaque of az-4 on a plate seeded with Su$^-$ (K38) and incubated at 42°C; it is not temperature-sensitive but is still *azure*. From either az-4A or az-4B it is easy to isolate revertants that are indistinguishable from wild type f2.

When efficiency of the formation of infective centers and the burst size of *azure* mutants were measured on various bacterial strains, it was found that formation of infective centers by *azure* mutants was depressed in Su+ strains. However, there was no clear difference in the yield per infective center among the bacterial strains. Table 2 shows the kinds of results obtained at multiplicities of infection less than 1. When infection occurred at 35°C, *azure* mutants gave approximately the same number of infective centers as wild type f2 in Su$^-$ (K38) cells, but only 10, 27, and 3 percent of the mutant phage added gave infective centers in Su-1+ (K37), Su-2+ (K146), and Su-3+ (K110), respectively, when compared with Su$^-$ cells. When infection occurred at 42°C, the number of infective centers formed was fewer than at 35°C in Su+ cells but not in Su$^-$ cells. On the other hand, there were no similar large differences in the yield from an established infective center. These progeny phages were plated on both Su$^-$ and Su-3+ cells to be certain that they were still *azure*. The fact that an infective center, once formed in an Su+ cell, produces a normal phage yield was also confirmed by single-burst experiments. Thus, the restriction of these *azure* mutants by Su+ cells is an all-or-none phenomenon—they either do not grow at all or grow normally. When Brenner's set of strains was used instead of Garen's, or when other *azure* mutants were tested, essentially the same results were obtained.

In order to determine whether the few infective centers formed in Su+ strains were the result of some heterogeneity in the population of Su+ cells, the same experiment was carried out at higher multiplicities. The results in Table 3, when compared with those in Table 2, show that larger fractions of the Su+ cell population formed infective centers at higher multiplicities than at multiplicities less than 1. For infection of Su-3+ cells with az-4B, the response was a linear function of multiplicity of infection up to multiplicities of about 10. Thus there is no evidence for the existence of self-complementation. Attempts to complement the different *azure* mutants or to rescue *azure* mutants by mixed infections of Su+ cells with either wild type or *amber* mutants of f2 have also failed. These results suggest that when Su+ cells are infected with *azure* mutants, there is a certain probability of formation of infective centers per phage

Table 2. Yield of infective centers by *azure* mutants at low multiplicity. Cells were grown in broth at 37°C to about 2×10^8 cells per milliliter and were transferred to the indicated temperature. Five minutes later phage was added at multiplicity of 0.1 to 0.3. At 8 minutes after infection, antiserum to f2 was added (final K, 10 min^{-1}), and 5 minutes after this the culture was diluted. A portion was plated immediately on K38 (Su$^-$) for detection of infective centers. Another portion was incubated in broth after 10^4-fold dilution for 60 minutes at 35° or 42°C, lysed with chloroform and lysozyme, and plated for phage yield. Both K38 (Su$^-$) and K110 (Su-3+) were used as indicators to prove that the proper phage was produced.

Phage	Bacteria	Temperature (°C) (0 to 13 min)	Infective centers per phage added (%)	Burst size at 35°C (13 to 73 min)	Burst size at 42°C (13 to 73 min)
az-1	Su$^-$ (K38)	35	71	670	8.5
		42	42	1100	14
	Su-1+ (K37)	35	9.6	390	1.1
		42	1.7	500	1
	Su-2+ (K146)	35	17	890	6.7
		42	8.7	540	6.4
	Su-3+ (K110)	35	3.8	260	2.6
		42	0.8	220	1
az-4B	Su$^-$ (K38)	35	60	1300	900
		42	79	650	410
	Su-1+ (K37)	35	3.7	630	110
		42	1.3	530	90
	Su-2+ (K146)	35	18	480	380
		42	30	430	200
	Su-3+ (K110)	35	0.9	520	260
		42	0.2	430	210
f2	Su$^-$ (K38)	35	75	1000	550
		42	81	830	230
	Su-1+ (K37)	35	74	820	310
		42	87	1300	380
	Su-2+ (K146)	35	53	680	700
		42	62	310	180
	Su-3+ (K110)	35	74	510	460
		42	78	810	320

Table 3. Infective center yield of *azure* mutants at higher multiplicities. The experimental procedure was the same as in Table 2, except that the multiplicities of infection (MOI) indicated were used. The number of bacteria was determined by plating for colony formers at 5 minutes before phage addition. The number of surviving cells was determined by plating at the same time as the plating for infective centers. NT, not tested.

Bacteria	Temperature (°C)	MOI	Infective centers (%)	Surviving cells (%)
	Phage az-1			
Su$^-$ (K38)	35	2.5	100	NT
	42	2.5	91	NT
Su-1+ (K37)	35	9	45	NT
	42	9	10	NT
Su-2+ (K146)	35	2.5	78	NT
	42	2.5	23	NT
Su-3+ (K110)	35	6	16	NT
	Phage az-4B			
Su$^-$ (K38)	35	9	142*	17.5
	42	9	150*	18.2
Su-1+ (K37)	35	6	55	132*
	42	6	23	144*
Su-3+ (K110)	35	5	4.6	147*
	42	5	1.3	138*

* Values higher than 100 percent are due to growth of cells during the experiment.

particle, not per cell, and that this probability depends on the particular Su+ gene present.

Since data in Tables 2 and 3 show that the probability of the formation of infective centers in Su+ cells infected with *azure* mutants is dependent on the temperature at the time of the infection, we studied the effect of temperature shift on the formation of these centers. Results show that the time at which sensitivity to temperature occurs is between 0 to 8 minutes after infection (Fig. 2). Since the effect of temperature on the yield of infective centers is observed only in Su+ cells, it is highly probable that the infection of these cells with an *azure* mutant is blocked during the period between 0 and 8 minutes after infection. What is being affected by the temperature seems to be the probability of the formation of infective centers. Data in Table 3 also show that Su+ cells that did not form infective centers were not killed; this was true for all other *azure* mutants. These results suggest that *azure* mutants may only poorly adsorb to or inject their RNA into Su+ cells.

These possibilities were tested in the following experiments with az-1. (i) Phage az-1 was added to Su− (K38) or Su-1+ (K37) cells under the same conditions as those used for the assay of infective centers, and the unadsorbed phage was measured after the cells were killed with chloroform. This phage adsorbed to Su-1+ as well as to Su− cells. (ii) RNA was extracted from purified phage samples of az-1 and wild type f2, added to protoplasts of Su− and Su-1+ cells, and the yields of infective centers and progeny phages were measured. The efficiency of transformation with az-1 RNA in Su-1+ protoplasts was only 5 percent of that in Su− protoplasts, while with wild type f2 RNA the ratio was about 50 percent. This latter difference varied with different batches of protoplasts. Thus, phage RNA was as restricted in its infection as phage particles were. Therefore, the restriction of growth of *azure* mutants in Su+ cells cannot be the result of either poor adsorption of the phage or improper release of RNA from phage particles.

To determine whether any progeny phage RNA is synthesized in Su+ cells infected with *azure* mutants, assays for infectious RNA were done. The results in Fig. 3 show that the Su-3+ (K143) cells infected with az-4B formed less than 1 percent of the infectious RNA produced by Su− (K140) cells infected with the same mutant. When infected with wild type f2, both bacterial strains formed essentially the same amount of infectious RNA. Since Su-3+ cells infected with az-4B formed 1 percent of the number of infective centers produced by Su− cells infected with the same phage, there is no evidence for any synthesis of progeny RNA in the abortively infected Su-3+ cells. A similar experiment was carried out with az-1. The multiplicity of infection was 0.3 for Su− (K140) and 0.6 for Su-3+ (K143). In 8 minutes 68 percent of the phage that was added formed infective centers in Su−, while 2.2 percent did in Su-3+. At 60 minutes after infection the amount of infectious RNA in the Su-3+ culture was less than 6 percent of that in the Su− culture. Since the number of viable phage particles in the Su-3+ culture at 60 minutes was 3 percent of that in the Su− culture, it is clear that the Su-3+ cells, abortively infected with az-1, did not form more than 3 percent of the normal amount of infectious RNA. Thus, neither az-1 nor az-4B synthesizes any detectable amount of infectious RNA when the infection is abortive.

Table 4 shows results of the assay of phage antigen produced in cells infected with the *azure* mutants. For az-4B, the amount of antigen produced in the culture is proportional to the yields of both infective centers and progeny phages. For az-1, which is temperature-sensitive, the amount of antigen was again determined mainly by the number of infective centers. When the temperature was shifted from 35° to 42°C at 20 minutes after infection, the production of antigen decreased only by a factor of 2 compared with the control kept at 35°C, while the yield of viable phage decreased by a factor of 50, which indicates that the temperature-sensitivity of az-1 in Su− cells is due to a mutation in gene 2, the particle assembly gene [see Horiuchi *et al.* (*14*) and Lodish *et al.* (*15*)].

In summary, these results show that the *azure* mutants grow normally in Su− strains and in *ochre* suppressor strains, but their formation of infective centers is restricted in *amber* suppressor strains. The degree of restriction is highest in Su-3+ and lowest in Su−.

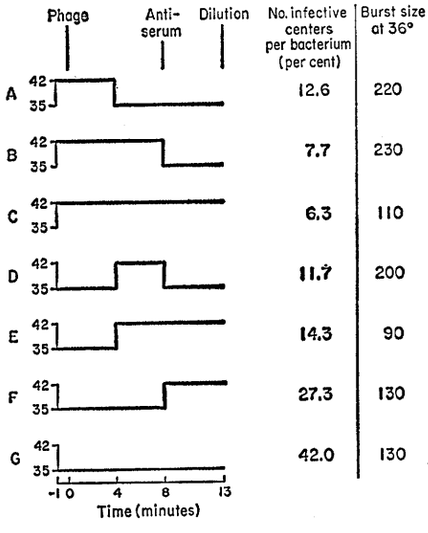

Fig. 2. Effect of 42°C pulse on infective center formation of az-1 in Su+ strain. Su-1+ bacteria (K37) were grown at 37°C in broth to 2 × 10^8 cells per milliliter. One minute before addition of phage, cells were transferred to 36° or 42°C as indicated, and az-1 was added at a multiplicity of 8 (0 times). At 4 and 8 minutes the temperature was shifted as indicated. At 8 minutes antiserum to f2 was added (final K, 10 min^{-1}), and 5 minutes later the cultures were diluted in saline. A portion was immediately plated on Su− (K38) for detection of infective centers. Another portion was diluted in broth and incubated at 36°C for 60 minutes for assay of the yield of progeny phage.

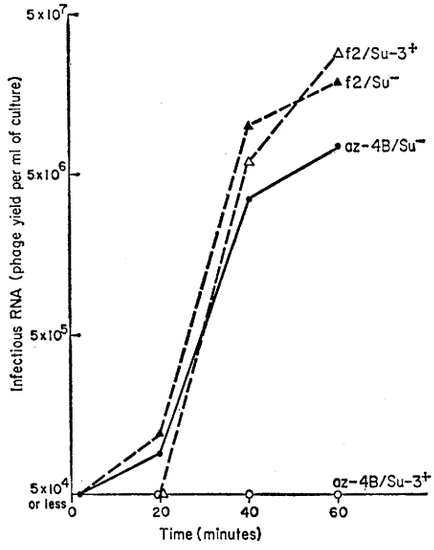

Fig. 3. Infectious progeny RNA in cells infected with az-4B. Su− (K140) and Su-3+ (K143) bacteria were grown in broth to about 2 × 10^8 cells per milliliter. Phage was added at a multiplicity of 0.5 and the cultures were shaken at 35°C. At 8 minutes a sample was taken, and the yield of infective centers was measured after treatment with antiserum to f2. The number of infective centers formed per phage added was 1 percent for Su-3+ infected with az-4B and 100 percent for the other three samples. At 2, 20, 40, and 60 minutes samples were taken for the extraction of RNA, and the infectivity of the RNA was assayed with K56 (Su−, f2-resistant) protoplasts at 36°C as described previously (*14*).

Table 4. Production of phage antigen in cells infected with *azure* mutants. Cells were grown in broth at 37°C to about 2×10^8 cells per milliliter and a sample was plated to determine the number of bacteria. After 5 minutes of incubation at the indicated temperature, phage was added. At 8 minutes a sample was taken and the number of infective centers was measured after treatment with antiserum to f2. After 70 minutes the culture was lysed with chloroform and lysozyme, and a sample was plated for the assay of viable phage, while the remainder was used for the assay of antigen by a procedure already described (*14*). MOI, multiplicities of infection; NT, not tested.

Phage	Bacteria	Temperature (°C)	MOI	Infective centers (%)	At 70 minutes	
					Plaque-forming units (per ml)	Antigen (μg/ml)
az-4B	Su$^-$ (K38)	35	9	89	6×10^{11}	10
		42	9	89	8×10^{11}	10
	Su-1$^+$ (K37)	35	6	29	2×10^{11}	2.5
		42	6	14	7×10^{10}	1.2
	Su-3$^+$ (K110)	35	5	3	8.5×10^9	< 0.5
		42	5	1	6.5×10^8	$< .5$
az-1	Su$^-$ (K38)	35	6	143	4.6×10^{11}	12
		Shift up*	6	143	1.2×10^{10}	6
		42	6	187	1.5×10^{10}	6
	Su-1$^+$ (K37)	35	9	43	6.3×10^{10}	2
		Shift up*	9	43	1.0×10^9	1
		42	9	18	3.6×10^8	< 0.5
f2	Su-1$^+$ (K37)	35	3	NT	1.3×10^{12}	15
		Shift up*	3	NT	8×10^{11}	15
		42	3	NT	6×10^{11}	15

* Shifted up from 35° to 42°C at 20 minutes after infection.

Adsorption of *azure* mutants to the Su$^+$ cell is normal, and the restriction of productive infection by *amber* suppressor genes is demonstrable upon infection of protoplasts with mutant RNA. Once an infective center is formed, the infected cell produces a normal amount of viable mutant phage; in other words, the restriction is all or none. The few infective centers formed in Su$^+$ cells are not due to a special fraction of the cell population but reflect some intrinsic probability of the formation of infective centers for each phage particle. When the infection is abortive, it is aborted at an early stage of infection—neither infectious RNA nor phage-specific antigen is formed, and the cells are not killed. In addition, abortion of the infection cannot be prevented or reversed by simultaneous infection with wild-type phage.

The nature of the *azure* mutation and the reason its presence restricts phage growth are still uncertain. It is not even certain that there is a class of *azure* mutants, in the sense that there is a class of *amber* and *ochre* mutants, or whether these *azures* are merely a manifestation of some singular attribute of RNA phage physiology, as the T4 *azure* mutants seem to be related to some special biology of T4 infection. We have isolated similar mutants from other RNA phages, but so far we have failed to isolate them from such DNA phages as f1 and T4. At this stage in the analysis it is difficult to differentiate the supposed "class properties" of *azure* mutants from the properties of the particular mutants that we have isolated. The class property we believe to be relevant is the gradient of restriction among different restricting host strains which more or less parallels the gradient of suppression. One cogent point is that, as suppression is the dominant phenotype for *amber* mutants (*16*), restriction is the dominant phenotype for *azure* mutants (see Table 1). This eliminates the hypotheses in which restriction is based on the loss of some relevant substance in going from Su$^-$ to Su$^+$.

The most attractive hypotheses are based on suppression itself being the restrictive element. However, in this particular instance, we are faced with the striking fact that restriction is more efficient than suppression. The efficiency of restoration of phenotype by *amber* suppressors has been reported as 55, 28, and 14 percent for Su-3+, Su-1+, and Su-2+, respectively, by Garen *et al.* (*13*), and as 63, 51, and 30 percent for Su-1+, Su-3+, and Su-2+, respectively, by Kaplan *et al.* (*12*). The efficiency of restriction of infective center formation is 98, 90, and 70 percent per phage particle with Su-3+, Su-1+, and Su-2+, respectively. If we assume that suppression and restriction are caused by the same kind of mechanism, our results are in agreement with Garen *et al.* as far as the relative order of effectiveness of suppressor genes is concerned. There is, however, a quantitative difference. If *azure* mutants had been maximally restricted to 65 percent, it is improbable that we would have isolated the mutants by the procedures we employed. Efficiency of suppression is assumed to be the efficiency by which the *amber* codon is read as propagation of protein chains as opposed to termination of protein chains (*12*, *13*). If chain propagation is the restrictive element, its efficiency must be amplified to explain our results.

It is known that Su+ bacteria have acquired, by mutation, special soluble RNA (sRNA) molecules for the translation of *amber* codons (*11*, *16*). In this discussion we will assume that the suppressor genes are the structural genes for these molecules, a hypothesis for which there is now some preliminary evidence (*17*). If it is true, we can eliminate all hypotheses based on enzymatic modification by the suppressor genes of other sRNA molecules, or of phage RNA itself, that is, a pleiotropic effect of the postulated suppressor-sRNA modifying enzyme.

Restriction by chain propagation could occur by either a direct or indirect route. The indirect route would imply that there exists in our bacterial strains an *amber* mutant in some gene. The product of this gene recognizes the *azure* codon in such a way as to prevent the initiation of RNA phage replication. If the phage replicates once, then it is safe from further intervention. The virtue of this hypothesis is that it does not necessarily restrict the number of *azure* codons or their position on the phage RNA; the difficulty is that it predicts breakdown of the correlation of suppression and restriction in some hosts. We have tested strains as diverse as *E. coli* B and *E. coli* C, and the correlate remains.

Chain propagation could interfere directly with the replication of RNA phage in the following manner. There is a codon that normally results in the termination of protein chains. The codon UAA is a good candidate. It has mutated to the *amber* codon UAG; the *azure* codon would then be the same as the *amber* codon. Such mutations should occur regardless of whether the particular f2 mutants described here are of this kind. Suppression of these codons should result in either the coupling of proteins or the addition of an amino acid to the C-terminus of a protein, depending upon the nature of intercistronic punctuation. However, in most instances, such

modifications would probably be without effect. Fifty-percent reductions in the amounts of most proteins would scarcely be lethal unless the modified protein chains themselves were the lethal agent (a dominant effect).

There are three known genes (*14*) in RNA phages, and it was expected that several types of *azure* mutants would be found. However, all mutants tested so far appear to be similar. Either there is a mutational "hot spot," which is especially sensitive to nitrous acid, or mutants in the other functions are so "leaky" as to be undetectable.

The *azure* mutants that we isolated are restricted early in their growth, but when they escape this early restriction, they proceed to grow normally. Putting together what is known about RNA phage physiology (*18*) with the properties of these mutants, we offer the following interpretation of the results. The f2 *azure* mutations are at the codon that normally results in termination of the chains of the phage RNA polymerase. The polymerase itself is a multimeric protein. Each monomer that is synthesized has a probability of being properly terminated equal to the frequency of chain-termination in the different host bacteria. However, the improperly terminated monomers might still participate in forming the multimeric enzyme. Should any of the monomers be abnormal, an irreversible block in replication of the input-RNA occurs. Alternatively, the proper number of monomers must assemble by a fixed time or the RNA is inactivated. These kinds of models provide the necessary amplification of suppression. Were the probability of proper chain-termination 0.5 each time a protein chain was synthesized, then in only 6 percent of the bacteria would there be a tetramer, for example, with all four chains properly terminated. Such a hypothesis also aids in explaining why efficiency of the formation of infective centers is a function of the number of input-phage particles and why the *azure* mutants cannot be rescued. It is the protein molecules synthesized directly from the input-RNA that are involved.

KENSUKE HORIUCHI*
NORTON D. ZINDER
Rockefeller University,
New York 10021

References and Notes

1. S. Benzer and S. Champe, *Proc. Nat. Acad. Sci. U.S.* **48**, 1114 (1962).
2. R. Epstein, A. Bolle, C. Steinberg, E. Kellenberger, E. Boy de la Tour, R. Chevalley, R. Edgar, M. Susman, G. Denhardt, A. Lielausis, *Cold Spring Harbor Symp. Quant. Biol.* **28**, 375 (1963).
3. A. Sarabhai, A. Stretton, S. Brenner, *Nature* **201**, 13 (1964).
4. A. Campbell, *Virology* **14**, 22 (1961).
5. N. Zinder and S. Cooper, *ibid.* **23**, 152 (1964).
6. R. Edgar, personal communication.
7. A. Garen and O. Siddiqi, *Proc. Nat. Acad. Sci. U.S.* **48**, 1121 (1962).
8. S. Brenner and J. Beckwith, *J. Mol. Biol.* **13**, 629 (1965).
9. E. Galluci and A. Garen, *ibid.* **15**, 193 (1966).
10. M. Weigert and A. Garen, *Nature* **206**, 992 (1965); S. Brenner, A. Stretton, S. Kaplan, *ibid.*, p. 994.
11. D. Engelhardt, R. Webster, R. Wilhelm, N. Zinder, *Proc. Nat. Acad. Sci. U.S.* **54**, 1791 (1965).
12. G. Notani, D. Engelhardt, W. Konigsberg, N. Zinder, *J. Mol. Biol.* **12**, 439 (1965); M. Weigert and A. Garen, *ibid.*, p. 448; A. Stretton and S. Brenner, *ibid.*, p. 456; M. Weigert, E. Lanka, A. Garen, *ibid.* **14**, 522 (1965); S. Kaplan, A. Stretton, S. Brenner, *ibid.*, p. 528.
13. A. Garen, S. Garen, R. Wilhelm, *ibid.* **14**, 167 (1965).
14. K. Horiuchi, H. Lodish, N. Zinder, *Virology* **28**, 438 (1966).
15. H. Lodish, K. Horiuchi, N. Zinder, *ibid.* **27**, 139 (1965).
16. M. Capecchi and G. Gussin, *Science* **149**, 417 (1965).
17. S. Brenner, personal communication.
18. N. Zinder, *Annu. Rev. Microbiol.* **19**, 455 (1965).
19. Supported in part by a grant from NSF. We thank Mrs. Linda Lyons for some of the rescue and complementation experiments. This report is dedicated to Professor Juro Ishida on the occasion of his 60th birthday.
* Present address: Institute for Infectious Diseases, University of Tokyo, Minato-ku, Tokyo, Japan.

20 March 1967

Stearic Acid as Plasma Replacement for Intracellular in vitro Culture of Plasmodium knowlesi

Abstract. *A chloroform extract of Cohn's fraction IV-4 of human plasma successfully replaced whole fraction IV-4 for the intracellular in vitro culture of* Plasmodium knowlesi. *We are now able to report the successful replacement of monkey plasma by stearic acid.*

Recently we reported (*1*) the successful replacement of monkey (*Macaca mulatta*) plasma by Cohn's fraction IV-4 (*2*) of human plasma for the intracellular in vitro cultivation of *Plasmodium knowlesi.* While this was significant progress toward our goal of attaining a truly chemically defined growth medium, it was emphasized that fraction IV-4 is a complex mixture of known and unknown materials (*3*). Experiments were conducted to identify the substance(s) which might replace fraction IV-4. By fractionation of fraction IV-4 it was found that a chloroform extract successfully replaced fraction IV-4, suggesting that the active material might be a lipid. Proceeding from this result we are now able to report the successful replacement of monkey plasma by stearic acid for the intracellular in vitro cultivation of *P. knowlesi.*

The techniques for maintaining, culturing, counting, and evaluating the malarial parasites were based on those previously described (*1, 4*). The synthetic medium originally described by Anfinsen *et al.* (*5*) was modified as described previously (*1*). The technique for the preparation of plasma-free host cells has also been reported previously (*1*).

In order to ascertain the nature of the active materials in fraction IV-4, a simple fractionation was employed. Two and one-quarter grams of Cohn's fraction IV-4 of human plasma (Nutritional Biochemicals Corp.) were suspended in glass-distilled water and made up to a final volume of 15 ml. The suspension was cooled to 4°C and, over a period of 2 hours, 10.6 g of "enzyme grade" ammonium sulfate (Nutritional Biochemicals Corp.) were added in small portions with continuous stirring. After the salt ceased to dissolve, the stirring was continued for an additional half hour. Undissolved crystals were removed by centrifugation at 2000 rev/min for 10 minutes. The suspension was drawn off and filtered through a microfiber glass disc (type AP20, Millipore Corp.) with suction. The filtrate was labeled fraction A and was dialyzed against glass-distilled water until a test for SO^{--}_4 in the external liquid was negative. The precipitate (fraction B) was suspended in 15 ml of water and dialyzed in the same manner as was fraction A.

Half of fraction A was extracted three times with equal volumes of chloroform. The interface material and the aqueous layer were drawn off and air was passed through the liquid until there was no longer an odor of chloroform. This was labeled fraction C. The combined chloroform layers were evaporated to dryness by warming to 60° to 70°C. The residue was dissolved in water (volume equal to that of fraction A originally extracted). Air was passed through to remove any residual chloroform. This solution was labeled fraction D. For addition to the cultures