THE EVOLUTION OF EPISTASIS AND THE ADVANTAGE OF RECOMBINATION IN POPULATIONS OF BACTERIOPHAGE T4[1]

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ABSTRACT

Experiments reported here test two hypotheses about the evolution of recombination: first, the Fisher-Muller concept that sexual organisms respond to selection more rapidly than do asexual ones, and second, that epistasis is more likely to evolve in the absence of recombination. Populations of bacteriophage T4 were selected by the drug proflavine in discrete generations and the change in mean population fitness was monitored. Three separate selection series yielded results supporting the Fisher-Muller hypothesis. The amount of epistasis evolved was measured by partitioning the T4 map into regions and comparing the sum of the proflavine resistances of each region with the resistance of the whole. Significantly more interactions were found in phage isolated from the populations with lower total recombination than in those from populations with higher recombination. The degree to which these experiments fit preconceived notions about natural selection suggests that microorganisms may be advantageously used in other population genetics experiments.

IN recent years many aspects of the evolution of recombination have been discussed theoretically (see FELSENSTEIN 1974 for a review). This paper examines experimentally two hypotheses concerned with this problem in population genetics: first, the Fisher-Muller idea that newly arising beneficial mutations are incorporated more rapidly in a population with recombination than in one without recombination, and second that in the absence of recombination epistatic interactions are expected to arise; whereas, with free recombination additive genes are more likely to be incorporated.

The experimental system developed was designed to take advantage of special features of bacteriophage biology that make it suitable for evolutionary studies. These include control over the amount of recombination in a generation, ease of handling of large numbers, rapid generation time and the hope of being able to find molecular explanations for the events occurring.

In addition to testing the two specific hypotheses these experiments had the general goal of demonstrating the utility of microorganisms as a population genetics tool.

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MATERIALS

Media: M9CAA, a minimal medium supplemented with 0.1% casamino acids, was used as growth medium for most experiments. Hershey broth was used as a growth medium for stocks and in crosses where a rich medium was desirable. Tryptone broth was the all-purpose dilution medium for titrations. EHA top and bottom agar and M9CAA top and bottom agar were the rich and minimal media, respectively, for assays on petri dishes. Edgar slant medium was used for the making of bacterial storage slants. Phage pellets were resuspended in lambda buffer. The compositions of these media are those given by MALMBERG (1976).

Bacteria: *Escherichia coli* strain B/5 was the normal host bacterium. Strain S/6/5 was the normal indicator bacterium, except that B/5 was used whenever the phage being plated had just been exposed to an acridine dye. Strain CR63 (λ)/5, a suppressor+ strain, was used as a host and indicator for amber stocks or whenever an *rII* mutant was to be denied expression.

Bacteriophage: T4D was the normal wild-type virus. Two classes of mutants were used in this study: acridine-resistant mutants were involved in studying the selection by proflavine, and temperature sensitive mutants were used in mapping experiments. Mutants from the collection of DR. M. SUSMAN were: the acridine-resistant double mutant *ac-q*, the *r* mutants *rI48* and *rII*uv58, the amber *B25* (gene 34), the temperature-sensitive mutants *N49* (gene 13), *L65*(23), *A44*(34), *A14*(41). DR. W. WOOD of the California Institute of Technology kindly donated the temperature sensitive mutants *A2*(3), *B20*(30), *L93*(37) and *C9*(49). These temperature-sensitive mutants were stitched together into a single stock encumbered with all eight of the *ts* mutants listed above, referred to hereafter as 8TS. The positions of the mutants on the T4 map are shown in Figure 1.

Chemicals: 9-aminoacridine and proflavine were purchased from Mann Research Laboratories. They were dissolved in sterile distilled water at 50° and stored in a refrigerator. Fresh solutions of 9AA were made at least every month and of proflavine at least every week.

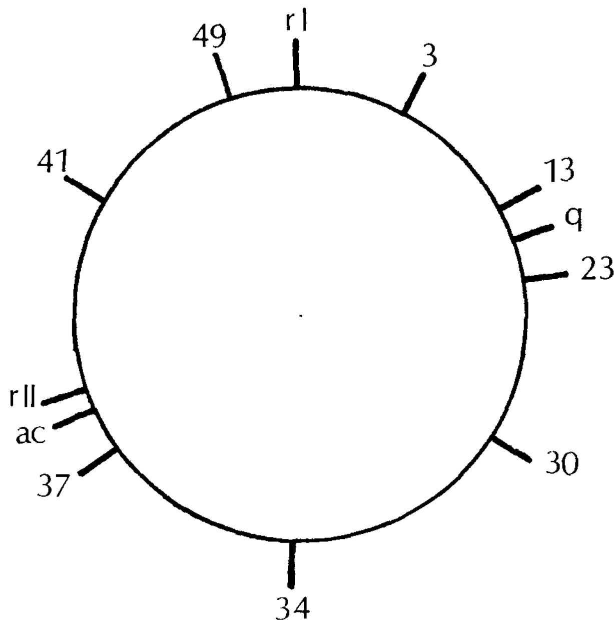

FIGURE 1.—The map positions of the T4 mutations used. The 8 temperature sensitive mutations used to construct the stock 8TS are indicated by the number of the gene in which they are located. The positions are from MOSIG 1970.

METHODS

General procedures were those described by ADAMS (1959), except where noted below.

Bacterial storage: S/6/5 and CR63 (λ)/5 were maintained by monthly serial subculture on slants. B/5 was grown to stationary phase in M9CAA, diluted and plated on M9CAA agar; from this a single colony was picked and grown in M9CAA to stationary phase. Sterile glycerol was mixed with the bacteria to a final solution of 25% glycerol. The mixture was dropped into the wells on a Falcon plastic Microtest II tissue culture plate, covered and frozen in an ordinary refrigerator freezer. After a month of decline, survival of the frozen bacteria leveled off at 20%; however, after two years of use, bacteria in some of the wells completely died. This method of B/5 storage helps ensure that the bacteria will be genetically identical during the course of a long experiment.

Bacterial preparation: Overnight cultures were obtained by growing samples of bacteria in the appropriate medium for at least 16 hours so that stationary phase was reached.

Host cells were prepared by making a 100× dilution of a M9CAA overnight into fresh M9CAA. The cells were grown into early log phase by aeration for 2½ hours at 30°. They were then spun in a refrigerated centrifuge for 20 minutes at approximately 4000g; the pellet was resuspended in fresh chilled medium and stored on ice or in a refrigerator until needed. Resuspension in a volume of medium one third the amount in which the culture was grown usually produced a titer of 2.5×10^8 cells per ml.

Indicator bacteria were prepared by making a 100× dilution of the appropriate overnight, normally into Hershey broth, then growing the cells into log phase by aeration for 2½ hours at 30°. The cells were centrifuged for 20 minutes at about 4000g and resuspended in 15 ml of Hershey broth, yielding about 2×10^9 cells per ml.

Phage stocks: In general, phage stocks were grown by picking a plaque that had been growing about four hours, or just long enough to be visible, resuspending the plaque in 1 ml medium, then mixing 0.1 ml of this with 1 ml host cells and 10 ml of medium treated with Dow Corning Antifoam A Spray. The mixtures were grown about 16 hours, lysed with chloroform, centrifuged to remove bacterial debris and stored over chloroform in a refrigerator, yielding a titer of about 10^{11} phage per ml for most wild type stocks. A wild-type stock was grown in M9CAA for experiments in which broth was not to be used.

Temperature-sensitive mutants produced small plaques, and hence they were allowed to grow on plates overnight before being picked.

Construction of 8TS: 8TS was made by standard crosses at room temperature using the spot test procedure described later to genotype the progeny. When offspring were found to be of a desired type, their master spots were picked, resuspended and replated. Several plaques from the replate were then picked and grown into stocks; each stock was genotyped and the one with the lowest reversion frequency was chosen for use in the subsequent cross. This procedure usually yielded stocks with a titer of 5×10^{10} phage per ml. About 100 phage of the stock 8TS were tested and each was found to contain all eight of the mutations.

Temperature-sensitive mutants have delayed lysis, as well as a lowered burst size. The lysis time for a room temperature cross can be estimated by the empirical formula:

$$T = (0.5 \times N) + 2$$

where T is time in hours and N is the mean number of *ts* mutations per infecting parental phage. For example, a cross between a stock with one mutation per genome and a stock with six will lyse at about 3¾ hours after infection. If the multiplicity is such that there are many cells infected with only one of the parental types, then lysis will be timed separately for the different populations of infective centers.

Mutation rate: Mutation rates induced by frameshift mutagens were measured by reversion of *rII*uv58 to $r+$.

Fitness component measurement: The fitness of a phage can be broken into the components of adsorption, burst size, lysis time and cold storability. Each of these was measured in a standard way as part of a one-step growth experiment, except for burst size. Whenever possible,

burst size was measured by computing the ratio of burst size of the phage to be tested to the burst size of a plaque morphology mutant, usually *rI*48. This was done as a variation of a one-step growth experiment as follows: (1) cells are infected at about the same time with the test phage and *rI*48 in separate adsorption tubes; (2) the infected cells are mixed in the growth tube; (3) at the appropriate times, samples are plated to determine infective center count and final titers; (4) the burst sizes of the test phage and the *rI*48 are determined by scoring both the infective center and lysate plates for *r* and *r*+ plaques separately, then dividing the final titer by the infective center count as usual; (5) the ratio of the test phage burst size to the *rI*48 burst size is taken and then multiplied by an independently determined mean value for the *rI* 48 burst size. The result is a standardized burst size that is less sensitive to the effects of day-to-day variables, such as humidity and random inexact pipetting. In practice the standardized burst size had a variance about one-seventh the variance of a comparable normal burst size determination. This technique can be used only when the burst sizes of the test phage and the morphology mutant are known to be similar.

Spot tests: Spot tests were done using the techniques developed by DOERMANN and BOEHNER (1970) as modified for temperature sensitive mutants and some acridine resistance mutants by MALMBERG (1976). The general principle is that the phage to be genotyped are transferred in a standard pattern by means of glass pyrex rods onto a master plate that allows all genotypes to grow. The next day the phage are transferred onto test plates that are selective and allow separation of genotypes.

Protocol for the generations: In general, a population was maintained by diluting the output of the previous generation so that a constant parental size of 3×10^7 phage was maintained. This was mixed with appropriate amounts of bacteria and proflavine then grown at 30° with aeration. Exact conditions are given in Table 1 and Figure 2 where the life cycle of a phage under this regime is summarized.

Measurement of epistasis: Individual phage were isolated from the final generation of series II and crossed with the 8TS stock. Progeny were tested for both their *ts* genotype and their proflavine resistance by the spot-test method previously described. This involved 18 test plates for each phage, eight for the genotype and 10 with different concentrations of proflavine in the bottom layer agar, ranging from 0.5 μg/ml to 5.0 μg/ml in increments of 0.5. Two identical master plates were used to effect the transfer in order to prevent maceration of any one master.

Regression analysis: The Madison Academic Computing Center mathematical subroutine NREG was used to obtain least squares fits by Marquardt's method to logistic curves.

TABLE 1

Conditions for the three series of populations PS I, II, and III

High recombination	Low recombination
Parental size = 3×10^7	Parental size = 3×10^7
Proflavine = 0.01 (I), 0.25 (II,III)	Proflavine = 0.12 (I). 0.25 (II,III)
Volume = 1.5 ml	Volume = 22.5 ml
Host = 10^8 B/5 per ml	Host = 10^8 B/5 per ml
MOI = 0.200	MOI = 0.013
Medium = M9CAA	Medium = M9CAA
Population structure:	Population Structure:
I. A = control, no proflavine	I. E = control, no proflavine
B, C, D = experimentals	F, G, H = experimentals
II, III. A, B = controls	II, III. F, G = controls
C, D, E = experimentals	H, I, J = experimentals

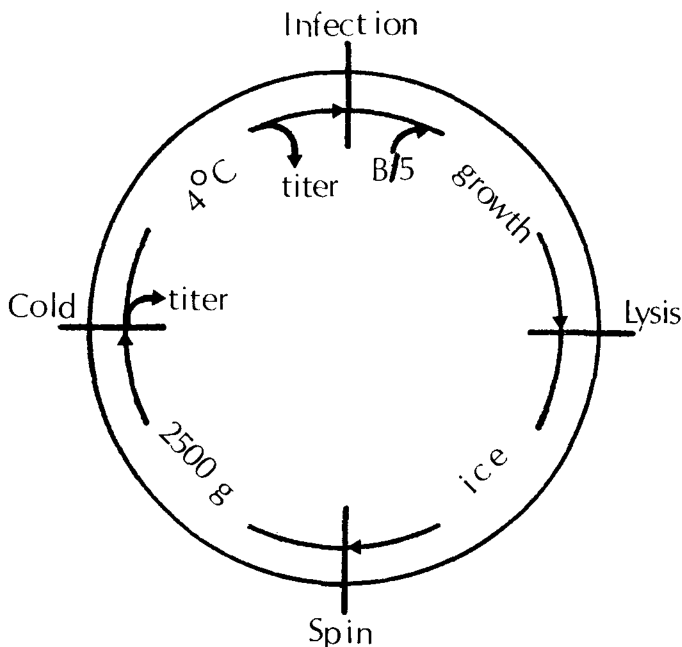

FIGURE 2.—The life cycle of a T4 during the selection series. B/5, proflavine, and 3×10^7 phage from the previous generation were mixed and incubated at 30° with aeration for 45 minutes. Lysis with chloroform was followed by 30 minutes storage on ice to reduce readsorption to remaining bacteria. The debris was pelleted by 30 minutes centrifugation in a room temperature centrifuge at about 2500g. The populations were titrated and refrigerated, the titer being used to determine the appropriate dilution for the next generation to ensure a parental size of 3×10^7. Just prior to the onset of the next growth cycle the population was titrated to determine the output for subsequent calculation of the mean fitness in each generation.

RESULTS

The general plan was to control the recombination within any generation of the three selection series, PS I, II, and III, by varying the ratio of infecting phage to bacteria (multiplicity of infection, or MOI). In order to vary the multiplicity while keeping the total population size constant, I decided to vary the total volume in which the generations grew and to keep the initial bacterial concentration constant. The only variables not satisfactorily controlled by this procedure are the concentration of infected cells, which must be higher in the high multiplicity populations, and its inverse, the concentration of uninfected cells, which is correspondingly higher in the low multiplicity lines. As long as there are no differences in the cell to cell competitive effects of infected and uninfected bacteria, this uncontrolled variable should be unimportant.

The practical lower bound for the total volume was 1.5 ml, since below that the combination of loss through evaporation and the several samples that had to be taken sometimes decreased the liquid to the extent that it was difficult to start the next generation. The practical upper bound was 25 ml because of physical limitations. Thus, I limited myself to the 15× difference between 1.5 ml

and 22.5 ml and was correspondingly restricted to a 15× difference in multiplicity of infection. I decided to use the multiplicities of 0.200 and 0.013, since the 15× difference at these low MOI results in a greater expected difference in recombination frequencies than would a higher pair of multiplicities. Within any one generation, these multiplicities result in a large variance in the amount of recombination between infected cells. In other words, most cells are infected with only one phage, and the mean recombination comes from the rare multiply infected cells where recombinational orgies are taking place. The difference between the high and low lines is that the former has more of these than the latter.

In order to establish the proper amount of proflavine to be used, simulations of the first generations were done at various concentrations; the data are displayed in Table 2. The fitness of a population was defined to be the mean number of offspring per parental phage, a quantity similar to burst size, but taking into account adsorption and the other stages of the phage life cycle. It should be noted that although other work on acridines has used timed pulses of the chemical, diluting into and out of the drug at appropriate times, all of my experiments are performed with acridine present the entire time.

In comparing the data for the series, it is important to keep in mind the relationship between the T4 stocks used to initiate these experiments. The stock for PS I was a homogenous stock grown in a conventional manner in M9CAA; the starter for PS II was generation twenty of population E of PS I, the low multiplicity control which had evolved in the absence of proflavine. The stock that began PS II had already undergone twenty generations of adaptation to the imposed generation scheme and could thus be expected to withstand more severe selection by proflavine than the starter for PS I, which would have to adapt to both proflavine and the life cycle. The stock for PS III was a homogeneous phage stock grown from a single plaque of population A of PS II, the high multiplicity

TABLE 2

The fitnesses of the starter populations in various concentrations of proflavine

Proflavine μg/ml	PS I		PS II		PS III	
	High	Low	High	Low	High	Low
0.00	58(1.00)	16(1.00)	210(1.00)	151(1.00)	158(1.00)	165(1.00)
0.03	41(0.71)	14(0.88)				
0.06	37(0.64)	16(1.00)				
0.09	10(0.17)	16(1.00)				
0.10					127(0.80)	147(0.89)
0.12	28(0.48)	14(0.88)				
0.15	18(0.31)	7(0.44)	122(0.60)	113(0.75)	64(0.41)	34(0.21)
0.20			90(0.43)	68(0.45)	76(0.48)	22(0.13)
0.25			52(0.25)	36(0.24)	40(0.25)	2(0.01)
0.30					44(0.28)	2(0.01)

Fitness is defined as the mean progeny per parent phage. The figures in the parentheses are the normalized fitnesses, from which the concentrations used were chosen.

control. Thus, it was adapted by 35 generations of selection and should be similar to PS II.

The concentrations chosen were 0.01 and 0.12 μg per ml for the high and low lines of PS I, respectively, and 0.25 μg per ml for both lines of PS II and PS III. These values were chosen so that the relative reductions in fitnesses from their controls were the same.

A related preliminary experiment involved the measurement of forward mutation of wild type to phage resistant to 1.5 μg per ml proflavine in the bottom agar of plates. I was unable to detect any increase in mutant frequency over the spontaneous rate, and hence erroneously concluded that 0.01 and 0.12 were equivalent in this way, as well as in their fitness effects. After a peculiar feature of PS I suggested that there really was a difference, I used the more sensitive test of reversion of *rII*uv58. As shown in Table 3, it turns out there was a higher rate for the low line. The concentrations used in PS II and III showed no difference.

The first generations of PS I and PS II were simulated, and the amount of recombination actually occurring was measured. This was done by crossing the double resistance mutant *ac-q* to the started phage, aiming for a total multiplicity of 0.200 and 0.013, equally divided among the parental types. The offspring were scored for genotypes by a spot-test procedure; the results are revealed in Table 4. As was to be expected in a cross in the presence of proflavine, there was substantial selection for the resistance mutations. The *ac-q* phenotype appeared nearly twice as often among the offspring as the wild phenotype.

Rate of response to selection: In order to test the Fisher-Muller hypothesis that the evolutionary advantage of recombination is that it increases the rate of response to selection in a new environment, it was necessary to have a measure of the mean number of progeny per parent in a given generation, the same quantity used in some of the preliminary experiments. This was determined by titrating the output of a generation just prior to the dilution-random sampling step at the beginning of the next generation to bring the parental size close to 3×10^7 phage. Knowledge of this titer allows calculation of both the output of genera-

TABLE 3

Reversion of rII*uv58 by various concentrations of proflavine*

MOI	Proflavine	Increase in frequency of r^+
0.200	0.00	6.9×10^{-4}
0.200	0.01	7.0×10^{-4}
0.200	0.25	3.5×10^{-3}
0.013	0.00	7.8×10^{-4}
0.013	0.12	1.8×10^{-3}
0.013	0.25	3.2×10^{-3}

These experiments were performed in exactly the same manner as the selection series except that the cells were infected with *rII*uv58. The frequency of revertants was measured by plating on a lambda lysogen of CR63/5. The figure shown is the frequency at the end of the experiment minus the input frequency.

TABLE 4

The frequency of recombination in the initial generations of PS I and II

MOI	Proflavine	PS I	PS II
0.200	0.00	0.041	0.032
0.200	0.01	0.071	
0.200	0.25		0.079
0.013	0.00	0.000	0.004
0.013	0.12	0.001	
0.013	0.25		0.001

The initial generations were simulated by infecting with half each of the resistant mutant ac-q and the appropriate starter phage stock, so that the final total MOI was that shown. The rest of the simulation was performed in the same manner as the real first generation. The frequency of recombination was measured by scoring the genotypes of 760 progeny in each of the crosses shown above by a spot test procedure.

tion k, and, on the basis of the dilutions used, estimation of the exact input of generation $(k+1)$. Thus, the mean number of progeny per parental phage in all generations could be determined by the succession of these titers. It should be noted that whereas some estimates of fitness used in experimental population genetics are extrinsic, defined by putting the organism in an optimal environment apart from the experiment itself, this measure is intrinsic, derived directly from the main sequence. The fitness data are given in Tables 5, 6 and 7.

TABLE 5

The fitnesses of the eight populations of PS I

	High recombination				Low recombination			
	No P		Proflavine		No P		Proflavine	
Generation	A	B	C	D	E	F	G	H
1	24	27	43	28	2	5	1	3
2	43	20	13	23	4	3	1	3
3	59	30	15	41	22	11	30	23
4	83	27	184	37	32	15	29	23
5	81	58	70	58	34	20	31	4
6	16	8	11	27	57	53	52	53
7	11	2	14	21	89	55	58	60
8	17	20	20	23	114	45	56	54
9	24	32	43	23	90	63	55	74
10	27	31	29	21	226	172	147	185
11	83	88	84	87	162	70	79	96
12	115	76	94	117	166	114	90	86
13	143	148	150	153	143	122	92	86
14	134	126	156	125	155	110	135	135
15	134	146	106	128	159	127	112	97
16	87	87	116	97	139	150	159	94
17	127	177	159	119	127	65	92	124
18	117	130	139	142	117	84	137	86
19	99	110	105	100	179	150	116	121
20	124	143	163	73	146	114	72	119

TABLE 6

The fitnesses of the ten populations of PS II

	High recombination					Low recombination				
	No P		Proflavine			No P		Proflavine		
Generation	A	B	C	D	E	F	G	H	I	J
1	270	106	4	5	12	124	159	6	4	4
2	34	83	25	20	26	186	189	22	34	34
3	77	78	42	8	3	157	123	4	4	4
4	55	61	15	41	68	78	117	21	34	43
5	107	126	50	58	83	169	169	43	52	60
6	36	84	63	100	118	94	59	63	104	72
7	86	52	39	62	60	247	231	90	119	119
8	116	79	152	124	110	123	58	122	58	75
9	120	96	85	126	100	175	208	117	218	143
10	59	79	66	63	76	171	243	247	171	123
11	69	142	107	119	129	105	160	129	203	118
12	74	76	69	80	56	212	216	160	209	170
13	53	59	63	88	53	177	89	172	222	137
14	93	205	110	137	119	216	190	153	140	166
15	106	56	44	72	70	260	289	174	142	272

After the first series was initiated and had been maintained for several generations, I noticed a day-to-day effect in which the fitnesses of all four of a group of populations fluctuated together, in the same direction in any one generation, but randomly across generations. It was as if some variable such as the daily humidity was having a larger effect on the fitness of the population than the

TABLE 7

The fitnesses of the ten populations of PS III

	High recombination					Low recombination				
	No P		Proflavine			No P		Proflavine		
Generation	A	B	C	D	E	F	G	H	I	J
1	4	4	3	3	1	37	50	21	18	23
2	64	55	13	44	44	273	236	55	58	18
3	120	148	40	19	13	259	60	42	49	13
4	41	49	27	164	62	174	159	40	33	67
5	85	100	121	88	100	151	196	93	117	99
6	95	150	17	128	81	107	118	47	49	74
7	126	126	76	77	105	71	154	71	77	48
8	83	94	511	53	77	62	68	170	105	109
9	87	158	87	50	87	60	68	65	88	88
10	122	106	145	100	148	188	47	29	268	103
11	144	122	136	96	123	67	51	120	26	31
12	200	143	188	191	204	93	76	88	78	113
13	111	150	141	85	133	163	121	187	95	108
14	177	136	144	120	92	154	145	171	92	115
15	112	280	150	195	189	141	122	136	83	144
16	174	121	188	102	112	173	104	145	144	147
17	270	62	78	228	96	203	174	251	96	208

genetic increases I hoped to measure. This noise was eliminated by transforming the data by dividing the fitness of an experimental population by the fitness of its control in that generation; this ratio was then multiplied by the mean of the two controls to obtain a normalized value of the same scale and units as the untransformed data. The result is that information from eight populations was rearranged into information on six populations in which the two controls act as monitors of the day-to-day effect to reduce the variance of the experimentals. The necessity of using this ratio of fitness made the controls more important than I had anticipated; for this reason in PS II and III the number of controls for each of the high and low recombination groups was increased to two. The fitness of a given PS II or III population was transformed by dividing by the mean of its controls and then rescaled by multiplying by the mean of all four controls. The averages of the transformed data are graphed in Figure 3.

Several qualitative conclusions are apparent immediately. The high recombination lines reached high fitness levels before the low lines, but in PS I this rate of difference is also accompanied by a higher final level; whereas, in the others the levels appear to be the same. A further difference between the series is that there is a delay in PS I before the fitnesses begin to climb substantially, but in PS II and III the rise begins quite soon after initiation.

On the Fisher-Muller theory, we would expect the rate of approach to the plateau to be greater for the high recombination lines, but not the final values. The difference should be in how fast the same mutations are incorporated, not in which mutations arise. This is the peculiar result that led me to reexamine the mutation rates as described earlier. Since the mutation rate in the low lines of PS I is greater, the lower plateau levels could be due to the equilibrium phenomenon of having a higher mutational genetic load. This does not invalidate the rate differences since, in a nonequilibrium situation, an increased mutation rate should lead to a more rapid adaptation; in other words, if there had been no mutational difference, the rate advantage of the high recombination lines would have been even greater.

To quantify these conclusions, nonlinear regressions were performed using the three parameter logistic equation as a model:

$$y = \frac{T}{1 + B\,e^{-Rx}}$$

where T is the upper asymptote, B equals $[(T-I)/I]$ where I is the initial value and R is an exponential rate constant. This model is appropriate not only because it specifies an S-shaped curve with easily interpretable parameters, but also because it is the standard equation for the frequency of a selected allele and thus the mean population fitness change as a function of time.

Examination of the residuals reveals no significant departures from normality assumptions and furthermore that autocorrelation, the bane of time-sequence analysis, does not exist to any important degree; thus normal statistical procedures could be used. An analysis of covariance was performed to determine whether the lines were significantly different from each other, and if so,

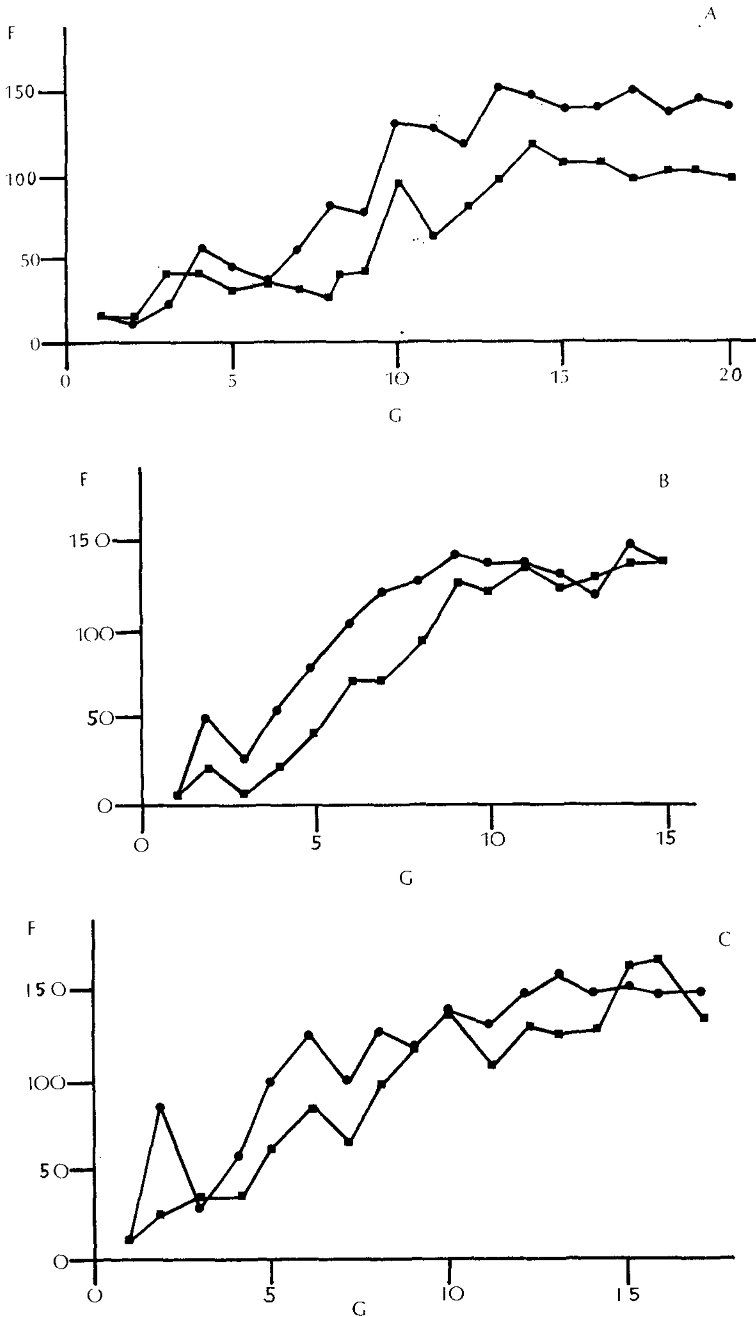

FIGURE 3.—The mean fitnesses of the high and low lines transformed data. ● represents the high moi lines, ■ represents the low moi lines; F is the fitness and G is the generation number. (A) PS I, (B) PS II, (C) PS III. From a nonlinear regression the probabilities under the null hypothesis that the data are best fit by a single curve are less than 0.005 for PS I, 0.025 for PS II, and 0.10 for PS III.

whether this difference could be assigned to any of the three parameters individually. I was most interested in seeing if the rate constants for the high and low lines were very different, since this would be the most direct consequence of the Fisher-Muller theory. The null hypotheses that the high and low lines were identical and that each of the three parameters individually were identical were tested against the alternative that all three constants were different. The results of this analysis show that we can reject the idea that the curves are identical, but not that any of the parameters are identical. We know a difference exists but can not assign it to a single cause on the basis of these data.

The conclusion we can draw is that in some sense the populations with more recombination responded faster to selection. Our confidence in the Fisher-Muller explanation of an advantage of sex can be increased, especially since the difference was consistent under three somewhat different conditions.

To help understand the biological basis of the increases in fitnesses. I performed experiments to apportion the change to the various components of the life cycle. This was done by taking samples from the initial and final generations of PS I and II and then running them through a growth cycle while titrating at many points in the manner of a one-step growth experiment. In a typical generation, when the cells are lysed and then placed on ice, two processes occur simultaneously: phage are released and some adsorption to debris occurs. In order to separate these, an additional experiment was performed measuring the cold adsorption of the phage in a nonlytic situation; the true burst size could be calculated from this and the samples taken during the typical generation. The results are displayed in Table 8 as fractions of the total change that occurred.

For PS I it is clear that selection has been operating on the burst size, the most logical place. For PS II there is substantial selection or burst size and on the cold related steps of ice nonadsorption and refrigerator storability. One possible explanation involves the phenomenon of phage being released together in clumps during lysis. These clumps will make only a single plaque; however, upon resting for a while the phage untangle themselves and will make many plaques. Perhaps

TABLE 8

The change in fitness in PS I and II divided into components

	I				II			
	High No P	High P	Low No P	Low P	High No P	High P	Low No P	Low P
Adsorption	0.03	0.02	0.00	0.00	0.06	0.01	0.01	0.01
Burst size	0.81	0.71	0.45	0.84	0.41	0.53	0.52	0.19
Ice nonadsorption	0.13	0.05	0.08	0.16	0.36	0.07	0.30	0.14
Nonsedimentation	0.00	0.22	0.46	0.00	0.00	0.00	0.00	0.22
Storability	0.03	0.00	0.01	0.00	0.17	0.39	0.17	0.44

Samples from each of the populations' initial and final generations were run through the generation protocol and sampled at various points to measure the quantities shown above. The figures in the table are the fractions of the total increase in fitness that may be attributable to each of the individual steps on the basis of this experiment.

the increase in cold fitness represents either a more rapid unsnarling of tail fibers or a developmental modification leading to fewer clumps being formed.

A final important difference between PS I and PS II was that the latter evolved phage in both the high and low lines that were substantially resistant to proflavine as measured by plating efficiency on plates with the drug in the bottom layer, whereas the former did not. Evidently, in PS I the adaptation to the imposed life cycle was a more important force than the low concentrations of proflavine used. In PS II the concentrations were substantially higher since the phage were already at home with their life and resistance could evolve. The selection intensities of PS I and PS II were equal, but the selection was on somewhat different polygenic traits.

The evolution of epistasis: The amount of epistasis (interaction) evolved in the highly proflavine resistant phage of PS II was determined by a mapping procedure: the T4 map was broken into eight segments; the resistance of each was compared to the original total of the complete phage to see if there were departures from additivity. This was accomplished by isolating individual phage from the high and low lines, then crossing them to the multiply marked 8TS. The progeny were analyzed by a spot testing procedure to determine both their *ts* genotype and their resistance on an incremented series of plates containing proflavine in the bottom agar. By averaging the resistance of offspring containing the resistant marker for a given one of the eight loci and seven *ts* alleles at the other loci, a measurement was obtained for each of the eight regions defined by 8TS.

In the absence of interference, we would expect that small insertions scattered around the map would lead to the resistance of a given region being overestimated, thereby biasing the results in the direction of not finding epistasis even if it exists. The weak negative interference found over large regions of the T4 map would tend to compensate for this overestimate. In any case, the frequencies of recombination were low enough that this was not a serious problem, a typical intergenic frequency being about 0.30.

Phage were isolated from populations C and I, high and low lines of PS II, by plating a sample of the population on a plate containing 2μg/ml proflavine. This ensured that the T4 to be studied were from the substantially resistant portion of the distributions within C and I. Five plaques were picked from each plate and samples were replated on normal plates to eliminate residual proflavine; from each of the ten plates a plaque was picked and grown into a stock. These were crossed to 8TS and approximately 370 offspring per cross were analyzed by spot tests. The mean increment of resistance above 8TS background was thus found for each of the map regions, although some intervals were represented by only a few offspring. The epistasis was quantified by summing the increments, substracting this from the total increment of the resistant parent above 8TS, and then dividing by the increment of the resistant parent so as to rescale to a fraction of the resistance evolved in such parental phage. The results are given in Table 9.

Originally I was concerned about the possibility of substantial inherent resistance of 8TS or surprising interactions among the *ts* alleles and the resistance loci

TABLE 9

Epistasis among phage selected from each of a high and low recombination population of PS II

	High recombination phage					Low recombination phage				
	1	2	3	4	5	1	2	3	4	5
R Parent	3.83	3.06	2.54	3.08	1.63	2.19	2.68	3.21	2.82	3.10
8TS	0.13	0.17	0.13	0.14	0.10	0.14	0.12	0.10	0.16	0.12
3	0.22	0.60	1.00	0.63	0.00	0.50	0.00	0.55	0.64	0.56
13	0.75	0.58	0.00	0.19	0.50	0.72	0.00	0.50	0.92	0.25
23	0.25	0.25	0.50	0.20	0.50	0.50	0.00	0.17	0.50	0.00
30	0.64	1.03	1.10	0.87	0.50	0.45	0.36	0.75	0.57	0.56
34	1.08	0.69	0.80	0.72	0.30	0.67	0.29	0.79	0.50	0.75
37	0.45	0.42	0.00	0.50	0.00	0.00	0.25	0.00	0.10	0.17
41	0.69	0.83	0.00	0.40	0.50	0.00	0.10	0.00	0.17	0.00
49	0.50	0.00	0.00	0.33	0.00	0.00	0.25	0.00	0.33	0.50
Sum	4.58	4.40	3.40	3.84	2.30	2.84	1.25	2.76	3.73	2.79
Epistasis	0.16	—.15	—.12	0.22	0.03	0.33	2.27	1.13	0.21	1.15
Scaled	.04	—.05	—.05	.07	.02	.16	.89	.36	.08	.39

The left hand column refers to offspring of the cross R × 8TS containing the region around that gene from the resistant parent, and the other seven regions from 8TS.

being inserted. This possibility was checked by isolating five phage from each of the nonresistant control populations A and F, and then going through the same crossing procedure. In general, very little turned up that had any resistance at all, and nothing greater than 0.5 μg/ml, the smallest concentration I tested. Thus within the limits of my revolving power, the *ts* markers were satisfactory for use. Unfortunately, I cannot rule out the possibility that interactions might exist with the resistant, but not with the control phage. This seems unlikely in view of the consistent results I obtained, but it cannot be eliminated altogether.

Comparing the five values obtained for each of the high and low lines, it is clear that there are substantial differences in epistasis; the statistical significance was checked by a *t*-like test for distinguishing means with unequal variances. The *t* value of 2.72 with 4.26 degrees of freedom corresponds to a probability under the null hypothesis that the means are equal of less than 0.025. The results are completely consistent with the hypothesis that greater epistasis will evolve in asexual populations. The results were not confirmed in a separate experiment as were the rate of evolution differences; nonetheless, we can be fairly sure of the generality of the result because it fits in with observations from other organisms. In a similar experiment with Drosophila, CROW (1956) found approximately 95% additivity; whereas, CAVALLI and MACCACARO (1952) found that haploid *E. coli* evolved substantial interactions among the resistance loci. In a sense, these results, although useful because they confirm a concept within one experimental system, are more important from the standpoint of demonstrating that T4 is a satisfactory organism for evolutionary studies requiring the use of complex polygenic traits.

Between the function studies in which the fitness change was divided into its components and the mapping of proflavine resistance experiments, a partial char-

acterization of proflavine resistance has been completed. We can ask how this fits in with previous work on the effects of acridine on T4. From the mapping there appear to be two major centers of resistance in both recombination groups, one near gene 13 and one between genes 30 and 34. The first of these might be a reisolation of the *q* mutation located in gene 17 which affects phase assembly. This suggests that the allele isolated in this study might be involved in the rearrangement of morphogenesis postulated earlier to produce T4 that is released in fewer or smaller entangled bunches. It would be interesting to check the kinetics of phage release and cold storage of the original *q* mutant to see if they fit the pattern observed here.

No previous resistance has been reported between genes 30 and 34, making it possible that this is a multiple mutation within one gene which could not have been isolated by any procedure other than a long-term selection. There is too much diverse genetic material in this region to be able to make a deduction from the map position alone about the function affected by this mutation; one intriguing possibility is that it might be in the T4 unwinding protein, gene 32. This would make sense from the standpoint that proflavine intercalates into DNA and might interfere with the normal melting activity.

Scattered across the rest of the map are various small amounts of resistance. A T4 mutant can have a general effect on total burst size as well as a specific effect on one function; these small pockets of resistance are probably of the general type, increasing the burst size and corresponding to the change revealed during the fitness analysis in phage burst size. Both the high and the low lines discovered the two major resistance loci, so that the difference in epistasis is probably attributable to the areas of small effect. True polygenic selection occurred in these regions.

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