Studies on Transformations of *Hemophilus influenzae*

IV. Linked and unlinked transformations

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ABSTRACT Unlinked transformations were demonstrated to occur by varying the multiplicity of DNA molecules taken up by competent cells. The number of doubles was directly proportional to the product of the frequency of singles for varying concentrations of cells. The kinetics of transformation to doubles and the effect of DNA concentration on double transformations were consistent with the concept that the cell must take up two molecules of DNA in order to be doubly transformed. Linked markers, on the other hand, were a constant fraction of the single transformation for variations in DNA or cell concentration, or time. The kinetics of transformation of linked markers was the same as for the kinetics of single transforming factors. It was, therefore, concluded that linked transformations involve interaction between the cell and a molecule of DNA carrying both markers. The frequency of transformation was found to be the same from resistance to sensitivity as from sensitivity to resistance for the markers streptomycin (S) and cathomycin (C). Purified DNAs, in general, show lower levels of linkage than crude DNA preparations, and for some crude preparations all the S markers were linked to C, suggesting that some dispersion, at least, was a result of DNA preparation. The inactivation of linked markers by heat, ultraviolet, and DNAase was studied.

INTRODUCTION

In the early studies on the genetics of almost all microorganisms, transfers of hereditary factors were described as independent of one another, and it was only after a number of factors had been described and the organisms more intensely studied that non-random associations were found. Indeed, it was somewhat surprising then that linked transformations first observed for ab antigens in *Hemophilus influenzae* (1) and mannitol to streptomycin linkage in pneumococcus (2) should have been observed with so few characteristics studied. More recently, linkage has been discovered for cathomycin and

streptomycin loci in *Hemophilus* (3) and for three sulfanilamide loci in pneumococcus (4). Therefore, it was reasonable to question whether or not linkage exists among all markers in the genetic apparatus of a cell and what criteria should be used in analyzing linked markers. As a corollary to this, it was questioned whether unlinked markers actually existed in a solution of transforming DNA.

There are several lines of evidence which establish that transformations in their simplest form result from the interaction of single cells and single DNA molecules. In the experiments to be described below, it will be demonstrated that *linked* transformations resulted from interactions between single cells and single DNA molecules and, on the other hand, *unlinked* transformations to multiple markers resulted from the interaction between single cells and more than one molecule of DNA.

MATERIALS AND METHODS

The preparation of DNA, competent cells, media, and the diluents has been described previously (5) together with most of the laboratory techniques. The medium Elev is a 50-50 mixture of Levinthal broth and Eugonbroth.

The DNAase used was the once crystallized pancreatic preparation obtained from Worthington Biochemicals Corporation. The ultraviolet inactivation was performed by using a G.E. 15 watt steri-lamp with an output of approximately 33 ergs per mm^2 per sec. at 30 cm from the sample.

In testing for the composition of transformed clones the Lederberg replica plating technique (6) was used except that a fine mohair was substituted for the usual velveteen material. Since we employed an agar overlay technique in some of our experiments we have used the mohair material even though the efficiency of transfer from clones was of the order of 80 to 85 per cent compared to very close to 100 per cent with velveteen, but it had the advantage of enabling us to transfer colonies which were below the surface of the thin agar overlay.

Unless otherwise specified with linked transforming factors transformations for a specific marker include all the classes of this marker. In transforming $C^{sen}S^{sen}$ cells (Rd) by $C^{res}S^{res}$ DNA the streptomycin transformants include the $C^{sen}S^{res}$ and the $C^{res}S^{res}$ class. Separate classes are designated as $C^{res}S^{res}$ or $C^{sen}S^{res}$, etc.

EXPERIMENTAL RESULTS

Evidence that Transformations Result from Interaction between Single Cells and DNA Molecules

In agreement with the work of other investigators (7–9) it was found that a linear relationship existed between the number of transformations produced and the concentration of DNA in solution below the concentration of 0.02 μg per ml. With higher concentrations the number of transformations in-

creased more slowly and reached a plateau value above which the number of transformations did not increase. This linearity between DNA concentration and number of transformations suggested that an interaction between a single physical unit of DNA (a molecule) and a cell was sufficient to produce

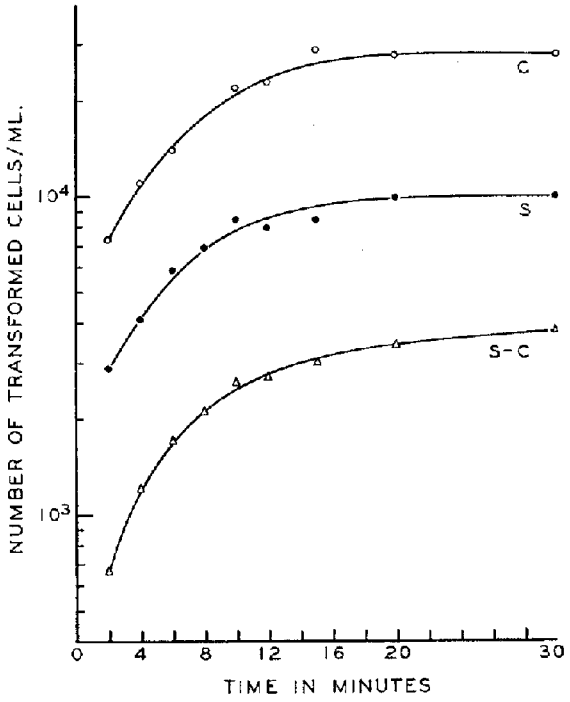

FIGURE 1. Kinetics of transformation. 0.1 ml Rd cells made competent by the usual aerobic-anaerobic procedure (5) was added to 2.8 ml of broth (final cell concentration = 3×10^7 per ml) and incubated for 10 minutes at 36°C at which time 0.1 ml of DNA was added (final concentration = 0.03 μg per ml). At the times indicated the samples were diluted at least 100-fold into broth and plated immediately by the usual pour plate procedure, incubated 2 hours at 37°C, and then layered with 500 μg per ml streptomycin, or 5 μg per ml cathomycin (novobiocin), or both. The plates were counted after 36 hours' incubation.

transformation. In addition, it was possible to demonstrate single interaction between cells and DNA kinetically. In Fig. 1 is plotted the number of transformations produced against time after introduction of transforming DNA into a culture of competent cells. The cells were incubated for 10 minutes before the addition of DNA and it was observed that the maximum level of transformation was reached after 25 to 30 minutes. The number of transformations (N_t) at any time, t, was taken as a fraction of the total maximum level (N_f). When the number to be transformed, *i.e.* one minus the fraction

transformed ($1 - N_t/N_f$), as a function of time, was plotted as shown in Fig. 2, a straight line relationship was obtained. Such a result is expected in cases of first or pseudo-first order kinetics and supports the notion that transformation involves an interaction between single molecules and cells.

Origin of Double Transformations

Double transformations could be obtained by using mixtures of differently marked transforming DNAs or by using single multiply marked stocks. When

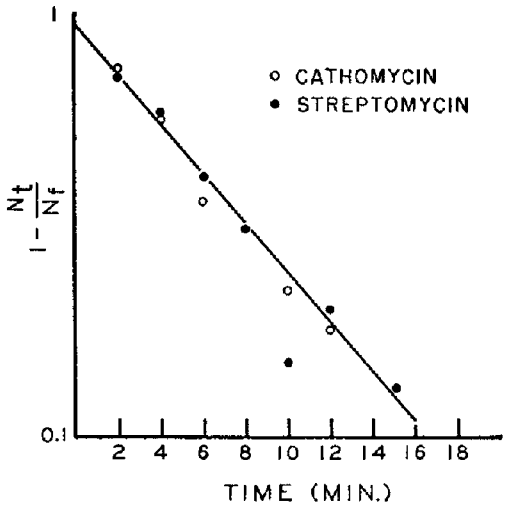

FIGURE 2. Kinetics of transformation for single factors. The data of Fig. 1 were used to calculate the value $1 - N_t/N_f$, where N_t is the number of transformations at time t, and N_f is the final plateau number of transformations = the number at 30 minutes.

mixtures of variant DNAs were used, the frequency of double transformations was low relative to the frequency of single transformations, and indeed, could be precisely described as a function of the frequency of single transformations (5). In the case in which DNA from multiply marked cells was used to obtain double transformations, there were two classes of markers, those markers which gave the same relative frequency of double transformations as a mixture of DNAs produced, and those which gave a frequency of doubles which was far in excess of the frequency to be expected on the basis of single transformations.

From the evidence to be presented below we conclude that the first class of markers is unlinked and represents markers on different DNA molecules. The second class represents markers which are linked and are present on the same DNA molecule (for example, see Table I).

Evidence for Unlinked Transformations

Unlinked transformations are defined as transformations for two or more factors which are not interdependent, *i.e.*, the probability of transformation to one characteristic does not influence the probability of transformation of another. If two transforming factors are on different DNA particles in solution, then it is clear that two interactions between a competent cell and the

TABLE I

TRANSFORMATIONS FOR LINKED AND UNLINKED MARKERS

0.1 ml of Rd cells (to give 5×10^7 per ml final) was diluted into 2.9 ml of Elev plus 0.1 to 1.0 μg per ml of DNA containing the markers indicated. The number of transformations was determined by the usual assay procedure. DNA denoted as S + C was a mixture of DNAs from two cell stocks each of which was resistant to one of these antibiotics, whereas SC came from a single stock that was resistant to both antibiotics. A similar procedure was followed with the other markers.

	No. of transformations to the indicated antibiotic resistances						
DNA	S	C	SC	E	SE	V	CV
S + C	1.9×10^5	8.6×10^5	8.2×10^2				
SC	1.0×10^5	2.8×10^5	3.8×10^4				
E + S	5.0×10^5			2.6×10^5	1.2×10^3		
ES	3.6×10^5			6.6×10^5	2.2×10^3		
CV		6.3×10^6				4.2×10^6	1.2×10^5
E + S	1.8×10^6			2.4×10^6	2.1×10^4		

particles are necessary in order to obtain a double transformation. This may be visualized as:

$$\mathrm{Rd} \xrightarrow{A} \mathrm{Rd_{Ap}} \xrightarrow{B} \mathrm{Rd_{ApBp}},$$

or

$$\mathrm{Rd} \xrightarrow{B} \mathrm{Rd_{Bp}} \xrightarrow{A} \mathrm{Rd_{BpAp}}$$

where *A* is a molecule of DNA which will produce a potential transformation in Rd cells to $\mathrm{Rd_{Ap}}$, etc.

Starting with a given number of DNA molecules and removing all or almost all these molecules from solution with different concentrations of cells, it was found that *the number of double transformations was a function of the frequency of transformation to singles* and not a function of the amount of transforming activity removed from the solution. Consequently, it was concluded that these markers were on separate molecules. A typical experiment is shown in Table II.

With the hypothesis that the markers were unlinked it is to expected that the frequency or the number of double transformations would fall off much more rapidly than the number of single transformations as a function of decreasing DNA concentration. These results are illustrated in Fig. 3. It may be noted that the singles decrease directly with DNA concentration, *i.e.* a slope of 1, whereas the double transformations decreased with a slope of about 2. In a manner similar to that discussed above for single transformation, one could ask whether the kinetics of double transformations is compatible with single interactions or multiple interactions between the cells

TABLE II

EFFECT OF CELL CONCENTRATION ON THE TRANSFORMATION TO RESISTANCE TO STREPTOMYCIN AND ERYTHROMYCIN

Rd cells were diluted with 0.125 M saline, to obtain 3 ml of the indicated final concentrations, +0.005 μg per ml of DNA carrying both streptomycin and high level erythromycin (E-50) resistance. After incubation for 30 minutes, cells were diluted and plated with the indicated antibiotics.

No. of viable cells/ml	No. of transformations/ml			No. of competent cells calculated from No. of doubles*
	SE	S	E	
2.5×10^7	4×10^3	4.3×10^5	6.0×10^5	6.5×10^7
5×10^7	4.4×10^3	6.2×10^5	7.7×10^5	1.1×10^8
1×10^8	2.9×10^3	7.3×10^5	9.3×10^5	2.3×10^8
2×10^8	1.4×10^3	7.7×10^5	8.6×10^5	4.7×10^8
5×10^8	0.75×10^3	9.2×10^5	9.6×10^5	1.2×10^9
7.5×10^8	0.64×10^3	9.4×10^5	11.0×10^5	1.6×10^9

* The significance of the constant discrepancy between the figures in this column and those in the first column has been discussed elsewhere (5).

and DNA molecules. Transformation to double resistance, *i.e.* cathomycin and streptomycin using a mixture of DNAs from single resistant stocks, is shown in Fig. 4. The fraction of cells not yet transformed $(1 - N_t/N_f)$ is plotted on a logarithmic scale against time of interaction to bring out the mechanism of the interaction. The individual or single transformations are included for comparison. It is clear that in contrast to the singles, the double transformations (SC) are not single-hit interactions, for the experimental points do not fall on a straight line passing through the origin. This SC curve is in agreement with the idea that double interactions are necessary.

From these data it is concluded that DNA markers which were derived from a multiply marked stock were unlinked for the markers erythromycin, viomycin, and streptomycin, and cathomycin was not linked to erythromycin or viomycin.

Evidence for Linked Transformations

Using the DNA from a cell stock which was resistant to both S and C, the number of double transformations obtained was a constant proportion of the number of single transformations. When the number of double transformations was determined as the function of DNA concentration, the number of doubles (SC) was at all points a constant fraction of the number of single

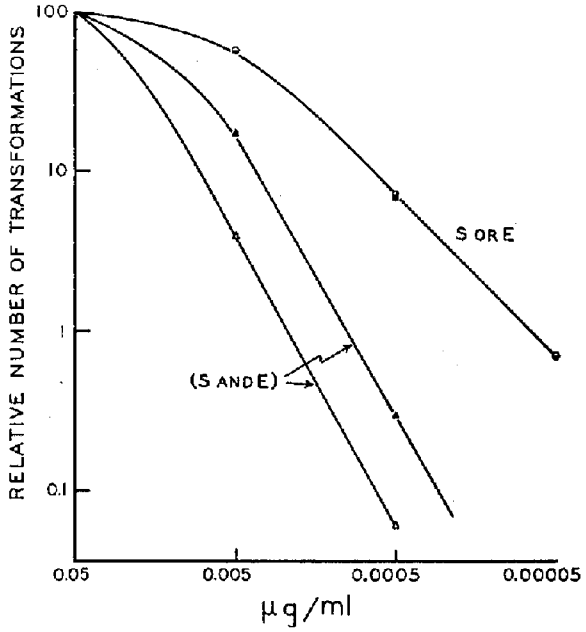

FIGURE 3. The effect of DNA concentration on the relative number of double transformations. The procedure was similar to that described in Table III except that the cell concentration and the DNA concentration were both varied as indicated. ○, relative number of transformations to streptomycin, ●, relative number of transformations to erythromycin for concentration of viable cells = 5×10^7. The same curves fit the data for concentrations of 1×10^8 and 5×10^8 cells. ▲, relative number of SE at a cell concentration of 5×10^7 per ml, △, relative number of SE at a cell concentration of 1×10^8 per ml.

transformations. This is shown in Fig. 5. If the cell concentration was varied, the number of double transformations was still a function of the number of single transformations and agreed with the kinetic analysis for single transformations. If one plotted the function, $1 - N_t/N_f$, *versus* t a straight line through the origin was obtained. These results are shown in Table III and Figs. 1 and 6. If, on the other hand, one used a mixture of DNAs from a stock resistant to S and another resistant to C, then the above relationships

were not obtained. Instead they were consistent with the relationship established for unlinked markers. It is necessary, therefore, to conclude that in the DNA preparations from cells resistant to both streptomycin and cathomycin these two markers were linked on the same molecules, at least in 20 per cent or more of the molecules carrying the streptomycin or cathomycin marker.

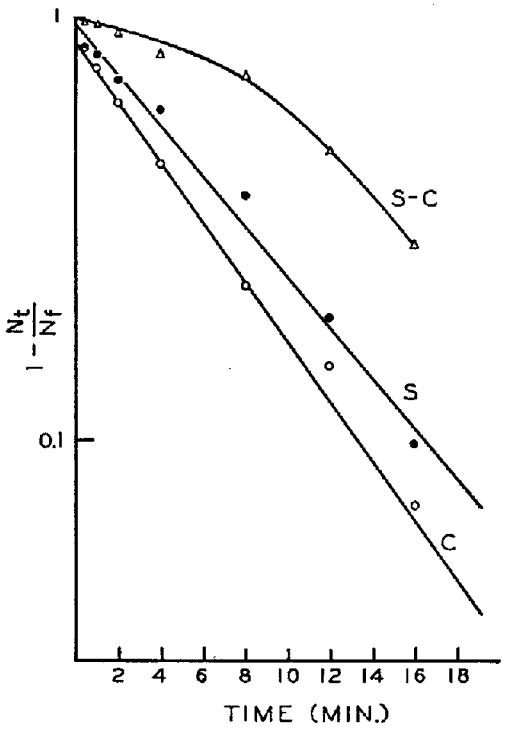

FIGURE 4. The kinetics of unlinked double transformation. Rd cells were diluted into Elev broth (3.5×10^7 per ml) containing a mixture of DNAs marked with cathomycin and streptomycin resistance, 0.12 μg per ml each, and at the time intervals indicated were diluted in broth containing DNAase, 1.0 μg per ml, and 0.003 M Mg^{++}. The plateau number of transformations (N_f) for SC was 4.2×10^2 transformations per ml. The usual assay procedure after dilution and plating was followed. The log of $(1 - N_t/N_f)$ is plotted as a function of time where N_t is the number of transformations at time t.

Origin of Single Transformants from Donor Cells Containing Multiply Linked Markers

When DNA from a population of doubly marked SC cells was used to transform host cells to resistant characteristics, all three kinds of cells were found; *i.e.*, streptomycin-resistant alone, cathomycin-resistant alone, and streptomycin–cathomycin-resistant. In view of the fact that streptomycin and cathomycin were linked, the origin of single S and C transformations required an explanation. Indeed, it was found that when fresh crude lysates

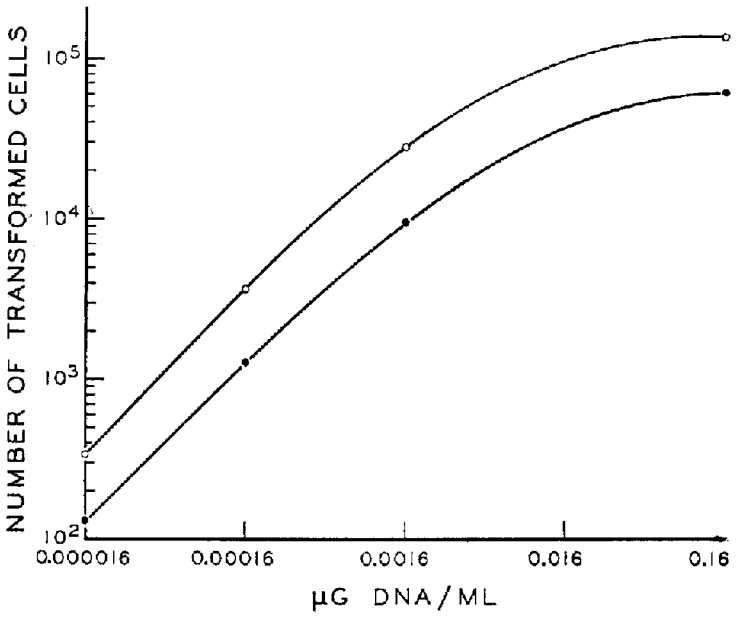

FIGURE 5. Constant relationship between the number of SC and S transformations with varying DNA concentration. ○, transformations to S (including SC class). ●, transformations to SC.

were used the amount of linkage between S and C was considerably higher, particularly when the transformation assay was done in 0.125 M saline in place of broth. These results are given in Tables IV and V. In one experiment (5 of Table IV) all those colonies which were S also carried C, thus demonstrating that at least in this case all the S transformations were linked to C.

TABLE III

THE CONSTANCY OF LINKED DOUBLE TRANSFORMATIONS (S-C) TO SINGLE TRANSFORMATIONS WITH DIFFERENT CONCENTRATIONS OF CELLS

Rd cells made competent by the aerobic-anaerobic technique were diluted into Elev broth containing 0.01 μg per ml of DNA marked with streptomycin and cathomycin. The final volume was 3 ml. The S and C classes include the S-C class.

No. of cells	No. of transformations × 10^{-4}			Ratio SC/S
	S	C	SC	
4×10^7	3.2	6.3	0.8	0.25
8×10^7	5.8	13	1.5	0.26
2×10^8	13	30	3.5	0.27
4×10^8	19	43	3.3	0.18

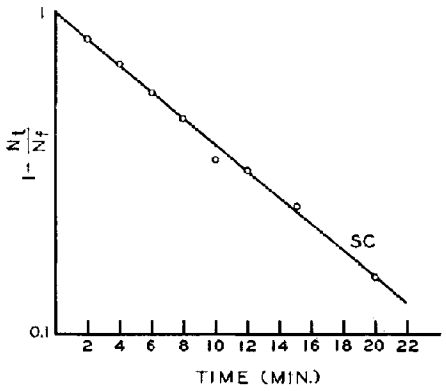

FIGURE 6. Kinetics of transformation to SC doubles. The data of Fig. 1 for SC doubles are plotted as a function of the final plateau number of transformations. As in Fig. 2, the plot on semilog paper shows $(1 - N_t/N_f)$ *vs.* time, where N_t is the number of transformations at time t, and N_f is the number of transformations at 30 minutes.

As a result of these experiments it seemed likely that single transformations from linked markers resulted either from recombination and elimination of some of the markers during incorporation or the prior inactivation of the other linked marker.

TABLE IV

DEGREE OF LINKAGE OF S AND C FOR SEVERAL DNA PREPARATIONS

Competent Rd cells were diluted into 2.9 ml Elev broth or 0.125 M saline (for experiments 3 and 5) containing the concentration of DNA indicated and assayed by the routine assay procedure. Different batches of Rd cells at a concentration of 5×10^7 per ml were used in each experiment.

Experiment	DNA concentration	No. of transformations			Ratio	
		S	C	SC	SC/S	SC/C
	μg/ml					
1. Purified SC	0.1	9.9×10^5	2.2×10^6	2.9×10^5	0.29	0.13
	0.01	2.9×10^5	6.5×10^5	9.4×10^4	0.32	0.14
2. Purified SC + Rd	0.03 + 0.006	3.2×10^5	8.2×10^5	8.2×10^4	0.26	0.10
	0.03 + 0.6	3.6×10^4	7.3×10^4	1×10^4	0.28	0.14
3. Crude	1.0	9.6×10^5	1.7×10^6	5.8×10^5	0.59	0.33
	0.01	4.0×10^5	8.4×10^5	2.6×10^5	0.65	0.31
4. Crude SC	0.1	1.7×10^5	4.1×10^5	7.9×10^4	0.46	0.19
	0.1	1.6×10^5	4.2×10^5	6.6×10^4	0.41	0.16
5. Crude SC	0.2	5.1×10^5	1.1×10^6	5.5×10^5	1.1	0.50

Since in no experiment were all the cathomycin markers found to be linked to streptomycin and since a small fraction of the markers could still involve another mechanism, a series of experiments designed to determine the origin of the single transformants is presented below.

The possibility that the SC population used to make the transforming DNA contained an appreciable number of S and C cells was ruled out by growing the cells in the presence and absence of the antibiotics, streptomycin and cathomycin, and looking for the presence of singly marked cells by the replicate plating technique. DNA from SC cells grown in the presence and absence of the antibiotic gave the same variations in S, C, and SC transformations. This suggested that the SC cells did not produce an appreciable

TABLE V

INCREASE OF LINKED TRANSFORMATIONS IN SALINE COMPARED TO ELEV BROTH

Rd (recipient cells) were diluted (0.1 per 2.9 ml) from a stock of competent cells to give 5×10^7 per ml in 3 ml of Elev broth or saline 0.125 M containing 0.6 μg per ml (experiment 1) and 0.001 μg per ml (experiment 2) of crude transforming factor marked with streptomycin and cathomycin resistance.

	No. of transformations			
	Experiment 1		Experiment 2	
Marker	Elev	Saline	Elev	Saline
S	1.1×10^8	1.6×10^6	5.5×10^4	8.1×10^4
C	2.4×10^6	2.6×10^6	1.1×10^5	1.3×10^5
SC	4.6×10^5	1.2×10^6	2.4×10^4	6.0×10^4
Ratio SC/S	0.42	0.76	0.44	0.74
Ratio SC/C	0.19	0.46	0.22	0.46

fraction of molecules carrying single markers. In addition, it was found that SC cells obtained by double transformation in one step gave rise to transforming factor with the same capacity to produce single transformations as the original cells.

The possibility that transformed cells actually represented a kind of heterogenote was also examined (10) by looking for the presence of segregants from transformed cells and by testing the DNA of transformed cells for both recipient and donor alleles. No evidence was found either for the segregation of markers from *selected* clones, or the presence of more than one allele in the transformed cell as tested by transformation. This latter experiment was performed in the following way. Recipient cells $C^{sens}S^{sens}$ were transformed to $C^{res}S^{res}$ by a mixture of DNAs, $C^{sens}S^{res}$ and $C^{res}S^{sens}$. If the resulting $C^{res}S^{res}$ transformed cells carried a sensitive marker, $C^{res}S^{sens}$ for example,

when one transformed a second recipient $C^{sens}S^{res}$ some of the cells should also be transformed to S^{sens}.

Of 810 transformed colonies from the second recipient tested by selecting first on the single antibiotic and replicating to a mixture of streptomycin and cathomycin, no colonies were found which did not replicate on both strepto-

TABLE VI

INDEPENDENCE OF TRANSFORMATION: SINGLE TYPES PRODUCED IN EACH CLONE

Antibiotic sensitive Rd cells were made competent by the aerobic-anaerobic method (5), (concentration = 3.3×10^9 per ml) and diluted 0.1 to 2.9 ml of broth containing 18 μg per ml of crude DNA with the linked markers for streptomycin and cathomycin (SC). Samples were taken at 30, 60, 90, and 120 minutes, and diluted into broth containing 1 μg per ml DNAase with 0.003 M Mg^{++}. At each time noted, one sample was assayed by the agar overlay technique while other samples were spread on the surface of plain Elev agar with 2 ml soft agar (.75 per cent) overlay. The surface plates were then replica-plated to plates containing streptomycin or cathomycin, or both antibiotics.

Time	No. of colonies on replica plates			Coincidence of colonies on different replica plates
	S	C	SC	
min.				
30	23	51	11	11 SC also S and C
60	41	83	24	24 SC also S and C
90	26	56	15	15 SC also S and C
120	32	64	17	17 SC also S and C
Totals	122	254	67	

Marker	Comparison of frequency of transformation obtained by non-selective (surface colonies) and selective (layer plate) techniques			
	Time	No. of transformations	Layer technique frequency	Surface colonies frequency (4×10^4 cells tested)
	min.			
Streptomycin	30	1.1×10^5	0.69×10^{-3}	0.6×10^{-3}
Cathomycin	30	2.5×10^5	1.6×10^{-3}	1.3×10^{-3}
SC	30	5.1×10^4	0.32×10^{-3}	0.3×10^{-3}

mycin and cathomycin, demonstrating that the SC DNA carried no detectable fraction of duplicate (allelic) markers.

The possibility that antibiotic selection procedures used in the experiments could account for the different frequency of transformation of markers was ruled out by comparing the frequency of transformation obtained in the absence of selecting agents with the number obtained by direct selection. These results are shown in the second part of Table VI.

From an analysis of the bacteria in clones derived from transformed cells it can be shown that independent transformations occur in the large majority of cases in different cells (19). Similar results were obtained for preparations of linked markers. As noted above, Rd cells ($S^{sens}C^{sens}$) transformed with DNA from cells which were streptomycin- and cathomycin-resistant produce three kinds of transformants, those resistant to S alone, C alone, and both S and C. When clones which produce these transformants were permitted to develop in a non-selecting media and then tested for antibiotic resistance by replica plating to a series of plates containing streptomycin, or cathomycin, or both, none of the transformants to S or C occupied the same position on the replica plates. The SC transformant, of course, replicated on all three plates (Table VI). In addition, 30 of the non-selected clones which contained SC transformants were resuspended in broth and shown to contain no cells which were resistant to streptomycin or cathomycin alone. (These clones did contain an eight- to tenfold excess of the recipient Rd cells.) Therefore, it was concluded that no segregation of S resistant cells from C resistant cells had taken place to account for the origin of single transformants from DNA containing linked markers. This left only recombination and elimination of a portion of the markers during incorporation, or the inactivation or separation of single markers prior to uptake by the cell, to account for the presence of single transformations.

The Frequency of Transformation from Resistance to Sensitivity

If DNA preparations were made in the same way, the same *frequency* of linked transformations could be demonstrated to occur in either direction; *i.e.*, from resistance to sensitivity as well as from sensitivity to resistance. For example, streptomycin-resistant cells could be transformed to streptomycin sensitivity by using the DNA from cells sensitive to streptomycin, but resistant to cathomycin, and plating in the presence of cathomycin, and both antibiotics. In the same way transformation to cathomycin sensitivity could be determined by using cathomycin as a non-selective marker; *i.e.*, plating with streptomycin and both S and C. The results are given in Table VII and demonstrate that the degree of linkage of streptomycin-cathomycin was the same for both the sensitive marker and the resistant marker; the same relation held for cathomycin sensitivity and cathomycin resistance. The degree of linkage of streptomycin to cathomycin sensitivity and streptomycin sensitivity to cathomycin explained an apparently anomalous result obtained when the frequency of transformations to double SC was measured using a mixture of streptomycin and cathomycin DNA. In this case the frequency of doubles was about one-half of that expected on the basis of random interaction using streptomycin and erythromycin markers or other unlinked

TABLE VII

FREQUENCY OF TRANSFORMATION FROM SENSITIVITY TO RESISTANCE AND RESISTANCE TO SENSITIVITY FOR CATHOMYCIN AND STREPTOMYCIN LOCI

Crude DNAs were prepared according to the technique outlined previously and the cells were made competent by the aerobic-anaerobic procedure. 0.1 ml of Rd cells was diluted into 2.9 ml of 0.125 M saline (5×10^7 per ml) containing 0.1 μg per ml of crude DNA.

Recipient cells	Donor DNA	No. of transformations			Proportion of doubles	
		S	C	SC	SC/C or $S^{sen}C/C$	SC/S or SC^{sen}/S
Rd $C^{sen}S^{sen}$	SC	4×10^5	8.4×10^5	2.6×10^5	0.31	0.65
$C^{sen}S$	CS^{sen}		6.5×10^5	4.4×10^5	0.32	
CS^{sen}	$C^{sen}S$	2.4×10^5		1.3×10^4		0.54

The number of doubles for the markers for sensitivity was calculated by subtracting the resistance class of doubles from the singles; *i.e.*, C − CS = CS^{sen}. The frequency of linkage = CS^{sen}/C or in the cross CS^{sen} by S DNA, S − CS = $C^{sen}S$, etc.

markers. If, however, one corrected for the classes of double transformations, which include the sensitive markers, then the observed number of SC transformations was obtained. Cells (C^-S^{sens}) transformed to streptomycin resistance (C^-S^{res}) produce two classes of transformants, that in which the

TABLE VIII

THE FREQUENCY OF DOUBLE TRANSFORMATION TO SC WITH A MIXTURE OF S AND C DNAs COMPARED TO THE FREQUENCY OF DOUBLES FOR OTHER UNLINKED MARKERS

Rd cells were diluted 1/10 into 0.125 M saline to give a final concentration of 2.4×10^8 per ml. DNA at a final concentration of 0.1 μg per ml was a mixture from cells resistant to streptomycin and erythromycin with that from cells resistant to cathomycin and viomycin in a final concentration of 0.1 μg per ml each. From a previous experiment we calculated 1.9×10^8 competent cells per ml.

Factors	No. of transformations	Frequency (observed)	Frequency of doubles expected from singles	SC corrected for linkage
S	1.25×10^6	6.6×10^{-3}		$SC^{sen} = 0.60$
E	1.8×10^6	9.5×10^{-3}		
C	5.6×10^6	3.0×10^{-2}		$S^{sen}C = 0.32$
V	3.8×10^6	2.0×10^{-2}		
SE	1.3×10^4	6.8×10^{-5}	6.3×10^{-5}	
SV	2.6×10^4	1.4×10^{-4}	1.3×10^{-4}	
SC	1.9×10^4	1.0×10^{-4}	2×10^{-4}	1.0×10^{-4}
EV	4.2×10^4	2.4×10^{-4}	2×10^{-4}	
EC	4.3×10^4	2.3×10^{-4}	2.9×10^{-4}	
VC	9.9×10^4	5.2×10^{-4}	6×10^{-4}	

C^- marker is contributed by the recipient and that in which the C^- marker is transformed in with the S^{res} marker. The same applies to the reciprocal $C^{res}S^-$ transformation. Therefore, in transforming $C^{sens}S^{sens}$ (the argument, of course, is applicable to any combination of sensitive and resistant markers) by a mixture of $C^{sens}S^{res}$ plus $C^{res}S^{sens}$ DNA, the calculation of the expected number of doubles $C^{res}S^{res}$ requires that only that class of transformants be used in which the single resistance marker was contributed by the donor

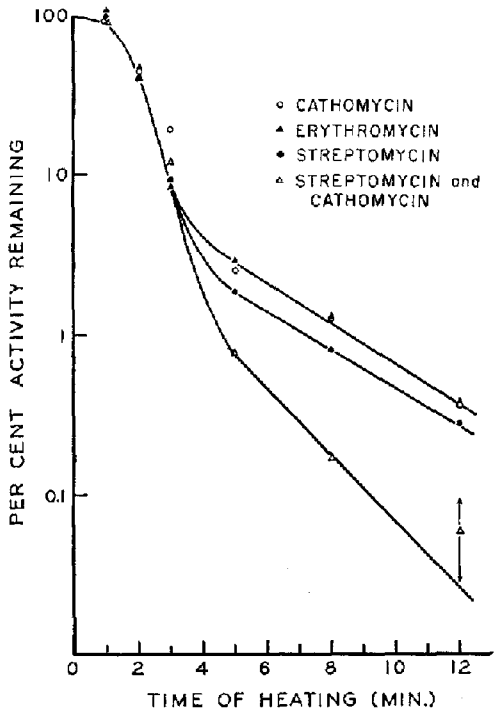

FIGURE 7. Effect of heat on various markers. To 4.0 ml of Elev broth was added 0.8 ml of DNA (= 0.8 μg) marked with streptomycin, cathomycin, and erythromycin resistance. The mixture in a 13 × 100 mm test tube was immersed in a boiling water bath and aliquots removed at the times indicated and assayed. The plot gives the residual activity as a function of time.

DNA. Correcting for the classes of double transformants $C^{sens}S^{res}$ as the product of the frequency of the singles $C^{res} \times S^{res}$ gave the observed number of double transformations. These results are given in Table VIII.

Effects of Physical and Chemical Agents on Linkage

There have been a number of reports on the effects of physical and chemical agents on the inactivation of various transforming factors (11, 12). The ex-

periments reported here are concerned with some of the quantitative aspects of the inactivation of transforming activity by a number of agents, but particularly with the effects on linkage between streptomycin and cathomycin.

Inactivation by Heat

Reports by Zamenhof, Alexander, and Leidy (13) and Lerman and Tolmach (12) have indicated that transforming activity can be destroyed by temperatures above 80°C, and Doty and collaborators (14) have shown that the

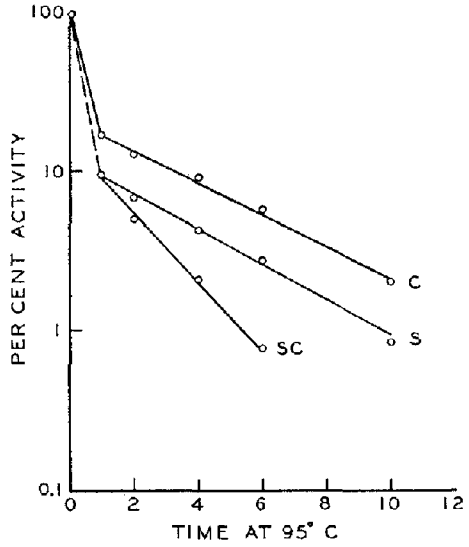

FIGURE 8. The effect of heat on linked transformation. To 4 ml of Elev broth kept for 5 minutes at 95°C was added 2 μg of DNA in 0.2 ml of citrate saline buffer (0.014 citrate and 0.15 M saline). At the times indicated 0.1 ml samples were transferred to 2.8 ml of broth at room temperature. After all samples had been taken, 0.1 ml of Rd cells (5×10^7 per ml) was added and the samples assayed for transformations. The plot gives the per cent of residual activity as a function of time of heating.

inactivation of DNA involved a collapse in the molecule, and was dependent upon ionic and other conditions. In the present experiments heat was used to determine whether the inactivation of linked and unlinked transforming factors was due to the destruction of the entire molecular unit or to the inactivation of the subunits. Heating at 95 or 96°C destroyed transforming activity of both linked and unlinked factors as shown in Figs. 7 and 8. The initial rate of inactivation for linked and unlinked markers was the same and suggested that the process was a general one and involved complete molecular units. However, after the first few minutes of heating the rate of inactivation was logarithmic with time. During this portion of the curve the rate of inac-

tivation of the linked markers was the sum of the rates of inactivation of the single markers. This suggested that the linked factors were being inactivated independently and involved a "single hit" process. The specific mechanism of this process is not understood. The heat-inactivated DNA contained residual activity which was different from the original material in that the ultraviolet sensitivity of the surviving transforming activity was slightly more resistant to ultraviolet radiation than the original material. This is shown in Fig. 9.

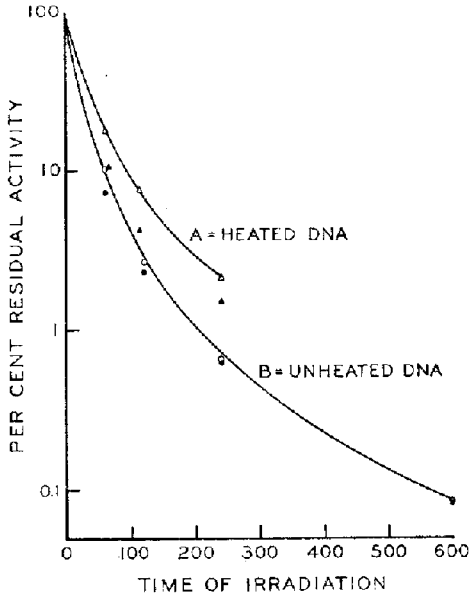

FIGURE 9. Ultraviolet inactivation of heated and unheated DNA. DNA in 0.15 M saline and 0.014 M citrate saline was diluted 1/10 in Elev broth to give a concentration of 3.6 μg per ml. 1 ml sample was heated 3 minutes in a boiling water bath which reduced its transforming activity for streptomycin by a factor of 5×10^2. The heated (△, ▲) and unheated (○, ●) material was then diluted (0.15 ml to 4.35 ml of citrate saline) and irradiated with ultraviolet light. Small aliquots were removed at times indicated and measured for transforming activity. The curve shows the fraction of activity remaining as a function of dose of ultraviolet (dose rate = 30 ergs per mm^2 per sec.).

It is most significant that the ultraviolet sensitivity was not greater than that of the original material which might be expected if the heated DNA were single stranded.

Inactivation by DNAase

DNA prepared from the multiply marked SC cell was inactivated by deoxyribonuclease (DNAase) by first order kinetics. The results of a typical experi-

ment are given in Fig. 10. The C marker in this case was inactivated at a slightly slower rate than the S marker and SC double transformations were inactivated as the sum of the inactivation rates of the single markers. This suggested that the inactivation of transforming activity by DNAase involved the inactivation of the marker directly and that the markers were inactivated independently. It should be noted that the amount of inactivation produced

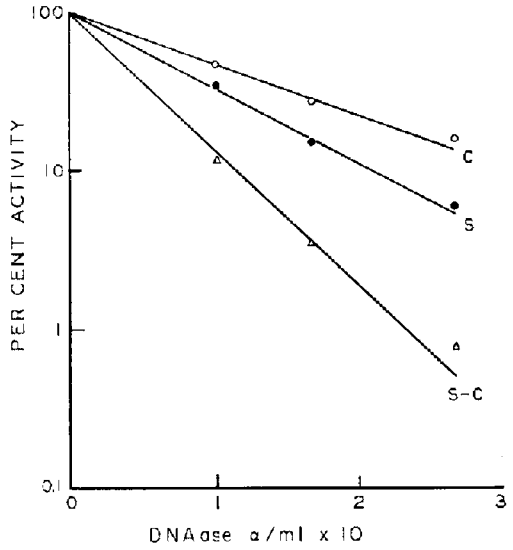

FIGURE 10. Effect of DNAase concentration on the activity of S, C, and SC. A solution of Worthington crystalline DNAase was diluted from a stock of 1 mg per ml (to give the concentrations indicated) into a mixture of 0.03 μg per ml DNA marked with streptomycin, and cathomycin resistance in Elev broth containing 0.003 μg per ml Mg^{++}. The mixture (2.9 ml final volume) was precooled to 15°C and the reaction permitted to run for 2 minutes at which time the tubes were immersed in a 90°C water bath for 1 minute. which inactivated the DNAase. 0.1 ml of Rd was added to the broth tubes after cooling and the number of transformations determined. Similar curves were obtained if the DNA–DNAase mixture was diluted without heating before transformation.

in these experiments was accompanied by only a very small drop in viscosity of the DNA solution.

Inactivation by Ultraviolet Light

Although the mechanism of ultraviolet light inactivation is not known, it was a particularly interesting agent to use because of its mutagenic, carcinogenic, prophage-inducing, and other special properties and because its photochemical energy is too low to break most primary covalent bonds. Ultraviolet light does not markedly affect the viscosity of DNA solutions except at extremely

high doses. In addition, ultraviolet light is known to produce chromatid breaks but does not produce chromosome breaks (15).

Exposing doubly marked SC transforming DNA to ultraviolet radiation from a 15 watt steri-lamp for various periods of time led to the curves in Fig. 11. These semilogarithmic curves showed that the S and C markers were inactivated at different rates and that the SC unit was inactivated at a rate which was somewhat faster than the sum of the two markers. In the curves

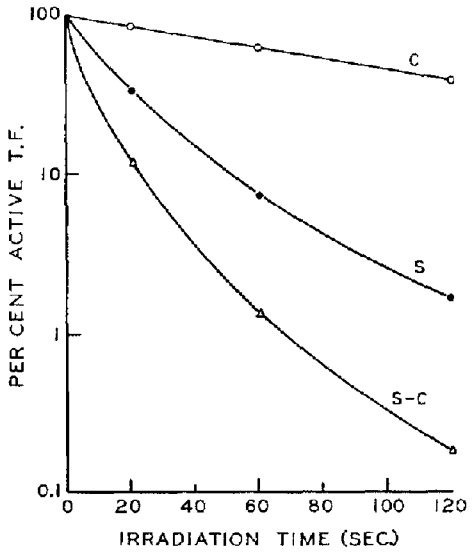

FIGURE 11. The ultraviolet inactivation of linked transforming factors. DNA marked with streptomycin and cathomycin at 1 μg per ml was irradiated in an open Petri dish mounted on a rotary shaker at 14 inches from a 15 watt G.E. steri-lamp. At the times indicated 0.1 ml was sampled into 2.7 ml of broth and the transformation assay performed in the usual manner except that the total volume in the transformation tube was 1.5 ml. The plot gives the residual activity as a function of ultraviolet dose (1 second = 32 ergs per mm^2).

shown it was clear that there was no portion of the curve which showed a logarithmic drop in activity with time.

DISCUSSION

Transformations result from interactions between cells and DNA (16, 17). The number and kinds of transformation produced are dependent upon the number of cells which are competent to interact with DNA, and the number and kinds of DNA molecules in solution. Our first conclusion regarding this interaction states that an interaction between a cell and a single DNA molecule is sufficient to produce a transformation. This conclusion is based on the

following facts. First, the number of transformations for any one specific character is a linear function of the amount of DNA which is taken up by the cells irreversibly. Consequently, at low concentrations of DNA, the amount of DNA taken up by the cells irreversibly is directly proportional to the DNA concentration in solution. In addition, the kinetics of production of transformed cells is consistent with the notion that an interaction between the cell and a single DNA molecule is sufficient for transformation.

The second conclusion states that a DNA molecule or particle can transform for two or more factors (linked) as a result of a single interaction between the particle and cell. It is not sufficient to conclude that markers are linked merely on the basis of the excess in the observed frequency of transformation compared to the expected number of transformations because the frequency of transformation is a function of the number of cells which are capable of taking up DNA as well as the number of molecules which react with these cells. Whenever the population of cells contained only a fraction of its number as competent cells, the number of double transformations calculated on the basis of the whole population would be considerably below the number which would be expected on the basis of the actual number of competent cells (5).

The evidence for linkage of streptomycin and cathomycin was based on the fact that the *ratio of the frequencies of double to single transformations was constant* with variations in cell and DNA concentration and the absolute number of transformations, in addition to the fact that the kinetics of transformation to produce doubles followed the same function as single transformations.

The third conclusion concerning interactions during transformation is that for unlinked markers double transformations result from multiple interactions between the cell and DNA molecules. It has been shown that for a number of *Hemophilus* markers, the frequency of double transformation was simply the product of the frequency of single transformations and this relationship was an exact one if the number of competent cells in the population was taken into account. This relationship held whether or not the DNAs came from a multiply marked stock or from a mixture of singly marked DNAs. The fact that the frequency of double transformations for a given cell or DNA concentration was a product of the frequency of the singles did not distinguish between the possibilities that the cells had interacted with several molecules of DNA or with a single molecule of DNA carrying two markers. Evidence that transformations result from multiple interactions was obtained from a study of the effect of varying cell and DNA concentrations on the number of double transformations. It was demonstrated that for a given amount of DNA removed from solution, the *number* of double transformations was not simply a function of the *number* of singly marked transformations obtained, but rather that the *frequency* of double transformations

was a function of the *frequency* of singles and was indeed a *product* of the two single *frequencies*.

A theoretical model to explain the relations found above is given in the following discussion. Experimentally it has been shown that the number (N) of molecules of DNA removed from solution is directly proportional to the concentration of DNA (D) at low concentration of DNA and to the cell concentration (C) where the fraction of DNA removed is small.

$$N = kDC$$

The number of transformations to a factor a (T_a) is directly proportional to the number of molecules removed

$$T_a = k_a N$$

then $T_a = k_a k \cdot D \cdot C = K_a DC$ and the probability of transformation (frequency) (P_a) is directly proportional to the DNA concentration

$$P_a = \frac{T_a}{C} = K_a D$$

In the same way

$$P_b = \frac{T_b}{C} = K_b D$$

and if a and b are independent events (unlinked markers) then,

$$P_{ab} = \frac{T_{ab}}{C} = P_a \times P_b = \frac{T_a}{C} \times \frac{T_b}{C} = K_a K_b \cdot D^2 = K_{ab} D^2$$

the frequency of transformation to the double ab is a function of the square of the DNA concentration as we have noted above. If all the molecules added to a solution are removed by the cells, then $N = D$ and

$$T_a = k_a N = k_a D$$

and

$$P_a = \frac{T_a}{C} = \frac{k_a D}{C} = \frac{k_{Da}}{C}$$

and in the same way

$$P_b = \frac{k_{Db}}{C}$$

and

$$P_{ab} = \frac{T_{ab}}{C} = \frac{k_{Da}}{C} \times \frac{k_{Db}}{C} = \frac{k_{Dab}}{C^2}$$

i.e., the frequency of unlinked double transformations *ab* is inversely proportional to the square of the cell concentration under conditions in which all the DNA molecules are removed from solution. The experimental data in Table II fit this relationship.

The inactivation of linked markers by DNAase, heat, and ultraviolet light is consistent with the hypothesis that linked markers are present in the same DNA particle.

Heat inactivation of transforming factor was clearly separated into two categories. First, there was a general inactivation of DNA molecules which was shown by the loss of transforming activity of streptomycin, cathomycin, erythromycin, and the double S-C at the same rate by treatment at high temperature. This initial loss in activity was accompanied by a loss in the interference property of DNA and agreed with the concept suggested by Rice and Doty (14), in which they visualized the effects of heat on DNA as resulting in a general collapse of the DNA molecule. After this initial rapid inactivation in the DNA, attributed to collapse of the molecule, there was a slower inactivation of the residual transforming activity. With continued heating the activity fell with a linear relationship for single markers while the linked double transformations fell as the sum of the rates of the single inactivations. With DNAase, also, the two individual markers were inactivated at almost the same rate (12), and the linked markers, S-C, were inactivated as the sum of the rates of the single inactivations. These results were obtained measuring residual activity as a function of both DNAase concentration acting for a specific period of time or for kinetics of inactivation at any single DNAase concentration. It is suggested, therefore, that the splitting of a critical phosphate ester bond was sufficient to produce an inactivation of a single marker. While heat and DNAase inactivation showed a linear response with dose, ultraviolet inactivation showed no such apparent relationship. At no place did the streptomycin or cathomycin markers or the double S-C markers show a logarithmic inactivation as a function of dose. The inactivation by ultraviolet light of streptomycin and cathomycin markers was clearly different and was qualitatively similar to inactivation of some markers obtained by Zamenhof *et al.* (18). The linked transformation showed a slightly greater inactivation than the sum of the rates of inactivation of the single markers. From the data presented, however, it is not possible to decide whether or not as a result of the inactivation of one marker on a molecule, the remaining markers may still be active. A more detailed discussion of the

effects of ultraviolet irradiation on transforming factor will be published elsewhere.

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