INTEGRATION EFFICIENCY AND GENETIC RECOMBINATION IN PNEUMOCOCCAL TRANSFORMATION[1]

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Received August 30, 1965

CONSIDERABLE progress has been made toward an understanding of the molecular basis of transformation in pneumococcus. DNA particles outside the cell compete for entry (HOTCHKISS 1957a). Competent cells indiscriminately take up this DNA at a steady rate to the extent of as much as 5% of their own genetic material (FOX and HOTCHKISS 1957). Immediately following uptake, donor DNA loses its ability to transform other cells (FOX 1960). This appears to result from the conversion of native donor DNA to single strands upon uptake (LACKS 1962). Within one quarter of a generation donor material recovers its activity, apparently as a consequence of insertion of single-stranded segments into native DNA of the host (LACKS 1962; FOX and ALLEN 1964). Simultaneous with this recovery, genetically recombinant DNA appears (FOX 1960).

The mechanism of donor marker integration and recombination is less well understood. A prior analysis of several of the mutations reported here indicated that integration efficiencies for different markers may vary considerably and that recombination frequencies depend on integration efficiencies as well as on the physical distance between markers (LACKS and HOTCHKISS 1960a). EPHRUSSI-TAYLOR, SICARD and KAMEN (1965) have recently examined the problem of integration efficiency with a series of mutations in the *amiA* locus of pneumococcus.

The present report consists of an analysis of mutations in the amylomaltase locus of pneumococcus with respect to (a) specific mutagenic origin and reversal, (b) integration frequencies, (c) recombination frequencies between pairs of markers, and (d) relationship of recombination to genetic location. Integration frequencies are interpreted as reflecting specific base differences in donor and recipient DNA. A tentative molecular model is proposed to explain variation in integration efficiency and the relationship of recombination frequencies to integration efficiencies and to distance of separation in DNA.

MATERIALS AND METHODS

Bacterial strains: The wild-type strain of pneumococcus, R6, as well as derivatives of this strain bearing markers *sulf-d* and *str-r* were obtained from DR. R. D. HOTCHKISS. *sulf-d* corresponds to the *d* marker for sulfonamide resistance of HOTCHKISS and EVANS (1958). It was introduced into various mutant strains for purposes of reference. The streptomycin-resistance marker, *str-r*, has been designated *str-r41* by ROTHEIM and RAVIN (1964).

Media: The medium used for growth, transformation, and selection was based on that of

[1] Research carried out at Brookhaven National Laboratory under the auspices of the U.S. Atomic Energy Commission.

ADAMS and ROE (1945). It contains, per liter, 5 g acid-hydrolyzed casein (Difco), 1 g enzymatic casein hydrolysate (Nutritional Biochemicals), 40 mg L-cysteine·HCl, 6 mg L-tryptophan, 50 mg L-asparagine, 10 mg L-glutamine, 5 mg adenine, 5 mg choline chloride, 1.2 mg calcium pantothenate, 0.3 mg nicotinic acid, 0.3 mg pyridoxine·HCl, 0.3 mg thiamine·HCl, 0.14 mg riboflavine, 0.6 μg biotin, 8.5 g K_2HPO_4, 2 g $NaC_2H_3O_2$, 0.4 g $NaHCO_3$, 0.5 g $MgCl_2 \cdot 6H_2O$, 6 mg $CaCl_2$, 0.5 mg $FeSO_4 \cdot 7H_2O$, 0.5 mg $CuSO_4 \cdot 5H_2O$, 0.5 mg $ZnSO_4 \cdot 7H_2O$, 0.2 mg $MnSO_4 \cdot 4H_2O$, 0.5 g bovine albumin (Fraction V, Armour), and 3000 units catalase (crystalline, Worthington Biochemical). For routine growth this medium was supplemented with fresh yeast extract and 0.2% glucose. Selection for maltose utilization, sulfonamide resistance, and streptomycin resistance involved supplements of 0.2% maltose, 100 μg/ml sulfanilamide and 0.2% sucrose, and 100 μg/ml streptomycin and 0.2% sucrose, respectively. Selection of maltose-negative cells in the presence of wild-type on the basis of colony size was accomplished by supplementing the maltose medium with 0.02% sucrose, a limiting concentration.

For large-scale preparations of DNA, cultures were grown to maximum turbidity in a medium composed, per liter, of 2 g K_2HPO_4, 7 g casamino acids (Difco), 7 g tryptone (Difco), 7 g yeast extract (Difco), 7 g brain heart infusion (Difco), and 3 g sucrose, adjusted to pH 7.6 with NaOH.

DNA preparation: DNA was generally prepared according to the procedure of HOTCHKISS (1957b). However, DNA used in the study of inactivation by deoxyribonuclease was prepared from cells lysed with 1% sodium dodecyl sulfate instead of sodium deoxycholate. In the experiment depicted in Table 3, crude lysates were used without further purification. These lysates were prepared by incubating 2×10^{10} cells per ml solution containing 0.1 M sodium citrate, 0.15 M NaCl, and 0.1% sodium deoxycholate for 5 min at 37°C, followed by 20-fold dilution with 0.15 M NaCl and storage at —20°C.

Transformation procedure: Fresh cultures were grown to a concentration of about 10^8 colony-forming units (c.f.u.) per ml. Samples were incubated with DNA at a concentration of 2 μg/ml for 30 min at 30°C. Addition of deoxyribonuclease (pancreatic, Worthington Biochemical) to 1 μg/ml terminated entry. Incubation was continued for 30 min at 37°C. After appropriate dilution, samples were added to selective media containing 1% agar at 40°C and poured into plates to give 100 to 500 colonies per plate. Colonies were counted after 40 hr incubation at 37°C. Unless otherwise indicated all transformation frequencies are expressed as the ratio of transformants of the type in question to transformants receiving the unlinked reference marker, *sulf-d*, present in the donor DNA. Recombinant frequencies, in particular, refer to ratios of transformants containing only one of two particular donor markers to transformants containing the reference marker.

Maltose-negative mutations: Mutations to inability to use maltose were obtained after treatment of wild-type cells with ultraviolet light (UV) or proflavine (PRO) and after treatment of wild-type DNA with nitrous acid (HNO_2), hydroxylamine (NH_2OH), ethyl methanesulfonate (EMS), hydrogen peroxide (H_2O_2), or triethylene melamine (TEM). Although control measurements of spontaneous mutations were not made, the impression from the appreciable frequency of mutations following UV and HNO_2 treatment was that the mutations must have been elicited by these agents; mutations induced by the other agents were less frequent.

Conditions of exposure of cells or DNA were as follows. Cells: UV irradiation to 10^{-3} survival; growth in medium containing proflavine at 1.5 μg/ml, a concentration which doubles the normal generation time of 40 min, for 24 hr. (No attempt was made to exclude light from the proflavine-treated cultures.) DNA (at 20 to 200 μg/ml): 0.2 M $NaNO_2$ in 0.1 M sodium acetate buffer, pH 4.3, at 25°C for 10 min, followed by addition of K_2HPO_4 to 0.2 M; 0.05 M NH_2OH, pH 7.0, in 0.05 M NaCl at 25°C for 20 min, followed by addition of acetone to 10% and tenfold dilution in 0.15 M NaCl; 0.15 M EMS in 0.2 M sodium phosphate buffer, pH 7.5 at 37°C for 10 hr followed by addition of $Na_2S_2O_3$ to 0.15 M; 0.01 M H_2O_2 at 25°C for 1 hr followed by addition of 100 units/ml catalase (crystalline, Worthington Biochemical); 2.5 mM TEM at 25°C for 1 hr followed by tenfold dilution in 0.15 M NaCl. The transforming activity of the *str-r* marker contained in the above-treated DNA was reduced to about 30% by each of the treatments. Following treatment the DNA was used to transform wild-type cells.

Cultures of cells treated directly or subjected to treated DNA were grown for several genera-

tions, then exposed to penicillin selection (LEDERBERG and DAVIS 1950) in maltose medium, and finally plated in maltose medium containing limiting sucrose. Maltose-negative mutants from independently treated cultures were picked and purified by isolation of single clones.

Amylomaltase activity: The procedure for measuring the enzyme in cell extracts has been described (LACKS and HOTCHKISS 1960b).

Spontaneous reversion: The frequency of maltose-utilizing cells in populations of the order of 10^{10} c.f.u. was determined by plating in maltose medium. For mutations showing very high reversion frequency, populations of about 10^6 c.f.u. were examined. In all cases the populations were grown either from single cells or from inocula sufficiently small to assure that no maltose-positive cells were originally present.

HNO_2-induced reversion: DNA from a mutant strain carrying the *sulf-d* marker was treated for various times under the conditions given above for HNO_2 induction of negative mutations. The 0-time control consisted of a sample to which the stopping reagent was added prior to the HNO_2. The DNA samples were used to transform a negative strain carrying the same mutation as the DNA donor. Counts were made of maltose-positive and sulfonamide-resistant transformants as well as total c.f.u. in the treated cultures.

Deoxyribonuclease inactivation: DNA from wild-type cells carrying *sulf-d*, at a concentration of 40 μg/ml, was treated with 0.01 μg/ml deoxyribonuclease (pancreatic, Worthington Biochemical) in the presence of 10^{-3} M $MgCl_2$ at 25°C for various times. The reaction was terminated by addition of sodium citrate to 0.04 M and heating at 70°C for 15 min. Samples were then tested for transforming activity.

Double mutants: For unknown reasons, all of the maltose-negative strains are inhibited by maltose from growing on glucose. The impairment is less pronounced in the case of N1 and T5. This difference made possible the selection of cells carrying two different mutations following transformation of cultures of N1 or T5 by DNA from another mutant strain. Penicillin selection was carried out in medium containing 0.1% maltose and 0.1% glucose. Survivors carried either both N1 (or T5) and the donor mutation, or only the latter. These were distinguished by testing with DNA from the appropriate mutants, for when the same mutation is present in both donor and recipient, no maltose-positive recombinants are formed.

Transfer of negative markers: In transformations of wild-type cells by DNA bearing maltose-negative mutations, negative transformants can be distinguished as minute colonies on plates containing maltose medium with limiting concentrations of sucrose. Following DNA treatment such transformed cultures were grown for five generations in order to allow segregation of maltose-negative c.f.u. prior to plating. Small colonies on the plates were replicated on blood agar plates to exclude non-pneumococcal contaminants and in maltose medium to confirm the inability to use maltose. Contaminants and maltose-positive types generally constituted less than 10% of the small colonies observed. When two negative mutations were present in the donor DNA, the genotypes of negative transformants were determined by testing with the appropriate DNA's. On account of the labor involved in the procedure, counts of negative transformants were usually limited to < 100, so that frequencies of such transformants are generally less accurate than those obtained from maltose-positive marker transfers.

RESULTS

Mapping by overlapping of multi-site mutations: Eighty maltose-negative mutations were obtained. Four of the mutants show very high spontaneous revertant frequencies: 45, 670, 770 and 2000 per 10^6 c.f.u., respectively. With these revertible mutants as recipients it is, nevertheless, possible to demonstrate considerably increased frequencies of maltose-positive cells on transformation by wild-type DNA but not by DNA from Me, which indicates that the mutations involved, as well as the remaining 76 which were more fully investigated, are located in the amylomaltase locus.

Mutants were tested in pairs, as both DNA donors and recipients, for recombination to give wild type. Recombination frequencies were measured as the ratio of maltose-utilizing transformants to sulfonamide-resistant transformants. Failure to recombine is taken to indicate that the two mutations under examination involved alteration of at least one identical component of the wild-type DNA. Nineteen of the mutations are multi-site as indicated by failure to recombine with two or more other mutations which recombine with each other; 57 mutations appear to be at single sites on the basis of this criterion. However, the possibility that some of these are short multi-site mutations cannot be ruled out. The lower limit for detecting recombination was generally a frequency of 2×10^{-7}, although when one member of the pair showed an appreciable revertant frequency ($>20 \times 10^{-10}$) this limit was higher. The lowest recombination frequency actually observed was 2×10^{-6} (T6 → N3) and the next lowest was 1×10^{-5} (V4 → E1).

Overlapping of the multi-site mutations places all the affected single sites into a linear array of 15 segments (Figure 1). Of 5,700 possible crosses, 1,271 have been tested and all the data are consistent with this scheme. Evidently, all the mutations occurred at the same genetic locus. Furthermore, the order of segments allows a qualitative determination of distance between sites.

Amylomaltase activity in mutants: It is evident from Table 1 that the locus under study contains a gene which affects amylomaltase production since the activity of this enzyme is less than 3% of the wild-type in all of the mutant strains, with the exception of V11 where it is 40%. It is conceivable that V11, which lies at one end of the array, is a mutation in a different, adjacent gene which is also involved in maltose utilization. None of the multi-site mutants show detectible activity. The limit of detection is $< 0.1\%$ of the activity of a wild-type extract which forms 0.2 μmoles glucose/min/mg protein under the assay conditions.

If it is assumed that the locus represents the structural gene (or genes) for amylomaltase, an estimate of its length in nucleotides can be obtained from the molecular weight of the enzyme. The latter has been roughly determined from

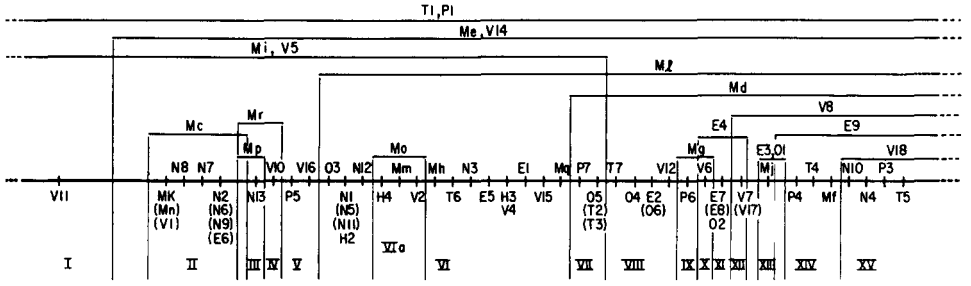

FIGURE 1.—Map of amylomaltase locus based on overlapping of multisite mutations. Genetic structure is represented by the heavy line with vertical bars indicating single sites. Multi-site mutations are represented as linear segments above this line. Their termini divide the locus into segments indicated by Roman numerals. Repeat mutations are enclosed by parentheses.

the sedimentation rate in a sucrose gradient, according to the procedure of MARTIN and AMES (1961), to be 90,000. However, since many proteins of such high molecular weight appear to be dimers (cf. *Brookhaven Symp. Biol.* **17,** 1964), it is more reasonable to assume a molecular weight for the distinct polypeptide(s) of 45,000. On the basis of a triplet code, this corresponds to a length of about 900 nucleotides.

TABLE 1

Properties of mutations at the amylomaltase locus

Mutation*	Inducing agent†	Integration efficiency‡	Amylomaltase activity§	Revertant frequency‖	HNO_2-induced reversion¶
(a) Single-site mutations					
N7	HNO_2	0.026	0.0	1.	0
N3	HNO_2	.027	0.0	9.	0
H2	NH_2OH	.030	0.1	3700.	0
N1	HNO_2	.037	1.8	5100.	0
N5	HNO_2	.038	1.8	4700.	.
N11	HNO_2	.040	1.9	3600.	.
N8	HNO_2	.039	0.0	1.	0
N9	HNO_2	.035	0.8	370.	0
N6	HNO_2	.037	0.6	20.	.
N2	HNO_2	.038	0.4	50.	0
E6	EMS	.045	0.3	120.	0
H4	NH_2OH	.036	0.0	16.	0
T2	TEM	.036	0.0	6.	0
O5	H_2O_2	.040	0.0	20.	0
T3	TEM	.043	0.0	10.	0
N13	HNO_2	.037	0.0	8.	0
V2	UV	.040	1.2	900.	0
N12	HNO_2	.042	1.1	1800.	0
H3	NH_2OH	.042	0.0	6.	0
Mj	UV	.042	0.0	20.	+
O4	H_2O_2	.043	0.0	30.	0
E7	EMS	.043	0.0	10.	0
E8	EMS	.051	. .	5.	.
P5	PRO	.043	0.4	20.	0
O3	H_2O_2	.044	0.0	90.	0
V17	UV	.045	0.0	2.	0
V7	UV	.049	0.0	3.	0
V12	UV	.048	0.4	40.	+
T7	TEM	.049	0.2	13.	+
N10	HNO_2	.050	0.5	14.	0
N4	HNO_2	.052	0.7	90.	0
P6	PRO	.057	0.0	<0.4	0
E1	EMS	.061	0.0	40.	0
V13	UV	.066	2.1	40.	0
T5	TEM	.068	1.7	45.	0
P7	PRO	.085	0.0	2.	0

TABLE 1—Continued

Properties of mutations at the amylomaltase locus

Mutation*	Inducing agent†	Integration efficiency‡	Amylomaltase activity§	Revertant frequency‖	HNO_2-induced reversion¶
Mm	UV	.17	0.4	0.8	0
V6	UV	.18	0.0	0.4	0
Mf	UV	.21	0.0	0.6	0
Mh	UV	.22	0.0	10.	0
V16	UV	.26	1.3	8.	0
Mq	UV	.49	0.1	2.	0
T6	TEM	.49	0.0	<0.5	0
O2	H_2O_2	.50	0.9	40.	+**
E5	EMS	.54	0.1	8.	0
P4	PRO	.54	0.0	2.	0
V10	UV	.59	0.1	<0.3	0
V11	UV	.62	40.0	. .	0
P3	PRO	.64	0.0	0.6	0
V4	UV	.86	0.0	<0.4	0
[E2	EMS	.91	0.0	<0.5	0
[O6	H_2O_2	.94	0.0	0.8	0
V15	UV	.95	0.0	5800.	.
[Mk	UV	.96	0.0	<0.3	0
[Mn	UV	.96	0.0	. .	.
[V1	UV	1.06	0.0	<0.4	.
T4	TEM	.98	0.0	10.	0
(b) Multi-site mutations					
T1	TEM	.005	0.0	<0.6	.
E9	EMS	.009	0.0	<0.5	.
P1	PRO	.018	0.0	<0.6	.
Me	UV	.035	0.0	<0.5	.
V14	UV	.16	0.0	<0.4	.
V5	UV	.17	0.0	<0.6	.
Mi	UV	.23	0.0	<0.3	.
Ml	UV	.24	0.0	<0.5	.
Md	UV	.30	0.0	<0.8	.
Mp	UV	.39	0.0	<0.4	.
O1	H_2O_2	.39	0.0	<0.5	.
V8	UV	.39	0.0	<0.5	.
Mr	UV	.58	0.0	<0.6	.
Mg	UV	.63	0.0	<0.4	.
Mc	UV	.65	0.0	<0.4	.
E3	EMS	.71	0.0	<0.3	.
V18	UV	.77	0.0	<0.5	.
Mo	UV	.80	0.0	<0.3	.
E4	EMS	.83	0.0	2.	.

* Brackets enclose apparently identical mutations.
† EMS, ethyl methane sulfonate; TEM, triethylene melamine; UV, ultraviolet light; PRO, proflavine.
‡ Ratio of *mal*+ transformants to *sulf-d* transformants in transformation with wild-type DNA containing *sulf-d* marker.
§ Specific activity expressed as percent of wild-type.
‖ Number of spontaneous maltose-positive cells per 10^{10} c.f.u.
¶ Inducibility >0.002 indicated as +, <0.0001 indicated as 0.
** Revertants shown not to be wild-type (see section on mutation at the E7.O2 site).

Spontaneous reversion: From Table 1 and the data given above, it can be seen that the frequency of revertants to the wild-type phenotype for different single-site mutants varies from $< 0.5 \times 10^{-10}$ to 2×10^{-3}, a range of seven orders of magnitude. There appears to be no relationship between reversion frequency and map position or amylomaltase activity. With regard to integration efficiency there is no strict correlation, but members of the lowest efficiency class generally show higher reversion rates.

Mutants containing multi-site mutations show no detectible reversions with the exception of E4. In this case, however, DNA prepared from the revertants, when tested on several receipients, did not give the same integration efficiencies for the maltose-positive allele as did wild-type DNA. Hence, the reversions of E4 to maltose utilization are not true back mutations. Such false reversions may account for unknown proportions of the reversion frequencies listed in Table 1, since few of the revertants have been subjected to the criterion of identity to the wild type with respect to integration efficiency.

Integration efficiency: In the ensuing, the term marker will be used to designate the localized difference between donor and recipient DNA which corresponds to a single mutation. *mal*$^-$ marker will refer to the alteration in the mutant DNA; *mal*$^+$ marker to its counterpart in the wild-type DNA. *Integration frequency* will be used as a general term to describe the ratio of transformants containing a donor marker or set of markers to transformants containing the reference marker, *sulf-d*, in situations where donor and recipient may differ by one or more markers in the amylomaltase locus. *Integration efficiency* is defined as the ratio between transformants containing the donor marker and those containing the reference marker when there is only a single difference in the locus between donor and recipient. Thus defined, the *mal*$^+$ and *mal*$^-$ integration efficiencies can be considered as properties of the mutations.

mal$^+$ integration efficiencies are listed in Table 1. Values given represent the mean of two determinations on separate cultures treated with the same wild-type DNA preparation. Deviations from the mean of 35% and 31% were observed with N8 and P4, respectively. In 14 cases the deviation fell between 10% and 20%, while in the remainder it was $< 10\%$, which is within the expected experimental error. Such reproducibility is not, however, always observed. Low-efficiency markers, particularly, occasionally give values as low as half or as high as double those listed. On the one hand, these variations may reflect differences in recipient cultures resulting from subtle variations in culture growth or medium, or, on the other hand, they may possibly arise from differences inherent in different DNA preparations.

Repeat mutations: A number of independently isolated single-site mutations show similar properties in that site of mutation, reversion frequency, integration efficiency, and amylomaltase activity are virtually identical. These sets of apparently identical mutations are indicated in Table 1. Except for amylomaltase activity, H2 appears to be identical to N1, N5 and N11. However, due to relatively high reversion rates, recombination frequencies among this group could

only be ascertained to be $< 10^{-5}$, so it is possible that two different sites are involved. In two other instances of identical or overlapping locations, E7 with O2 and H3 with V4, other properties of the mutations clearly distinguished them. The single-site group, therefore, includes 44 distinct mutations at 41 or 42 different sites.

Deoxyribonuclease inactivation: Evidence other than genetic that multi-site mutations involve extensive regions of DNA comes from studies on the inactivation of *mal*$^+$ markers by treatment of wild-type DNA with deoxyribonuclease. The results, presented in Figure 2, are similar to those previously obtained with subcritical temperature inactivation (LACKS and HOTCHKISS 1960a).

Single-site markers all show relatively slow rates of inactivation and there is no correlation between inactivation rate and integration efficiency. Small multi-site markers are also inactivated at the same slow rate. Larger multi-site markers

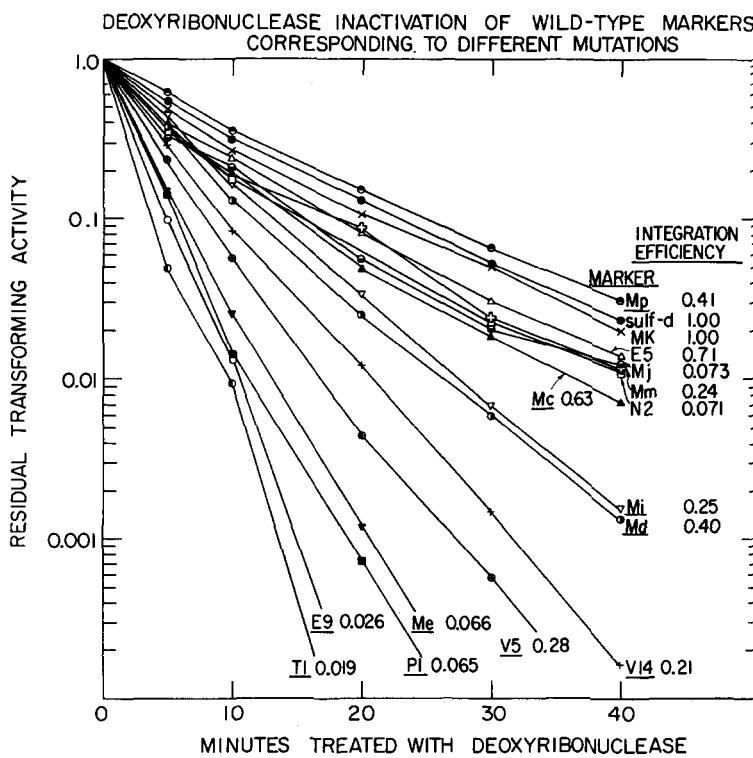

FIGURE 2.—Deoxyribonuclease inactivation of markers corresponding to different mutations. Wild-type DNA at 40 μg/ml was treated with pancreatic deoxyribonuclease at 0.01 μg/ml. The treated DNA was used to transform various *mal*$^-$ recipients as indicated in the figure. Recipients bearing multisite mutations are underlined. The curve labeled *sulf-d* represents inactivation of the *sulf-d* marker in the treated DNA as tested with a sulfonamide-sensitive recipient. Integration efficiencies determined in this experiment are somewhat higher than those listed in Table 1; this may reflect differences in the method used for preparation of the DNA. Experimental points not shown in the figure: T1, 20′, <0.00003; E9, 20′, <0.0003; P1, 30′, 0.00008; Me, 30′, 0.00005; V5, 40′, 0.00002.

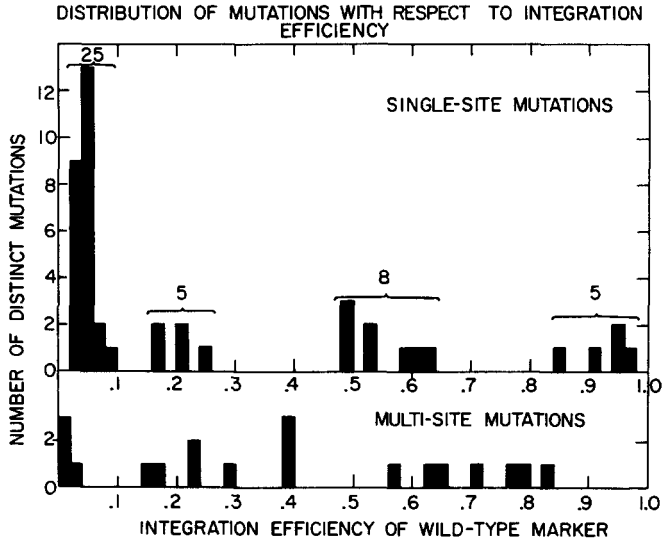

FIGURE 3.—Distribution of mutations with respect to integration efficiency. Each bar represents the number of mutations, excluding repeats, giving rise to *mal*+ marker integration efficiencies within an interval of 0.02. Brackets indicate the classes of single-site mutations and the number of distinct mutations in each.

are inactivated more rapidly, and the rate of inactivation appears to be correlated with the size of the alteration estimated from the genetic data. Integration efficiency, at least for the larger markers, is inversely related to inactivation rate, and hence, presumably, to marker size. This is not always true for smaller multi-site markers such as *mal*-Mp$^+$ which, although slowly inactivated and genetically of limited extent, nevertheless gives an integration efficiency of only 0.41.

Distribution of integration efficiencies: Integration efficiencies of single-site markers vary over a range from 0.026 to 1.06 (Table 1). Multi-site markers also show variation over a wide range, from 0.005 to 0.83. In the latter case there appears to be a continuous distribution over the range (Figure 3). This presumably reflects a continuous distribution of marker lengths, which for multi-site markers appear to be of principal importance in determining integration efficiency.

Integration efficiencies of single-site markers, on the other hand, fall into several classes (Figure 3): low (0.026–0.085), moderately low (0.17–0.26), intermediate (0.49–0.64), and high (0.86–1.06). HNO_2 and NH_2OH only induced single-site mutations in the low efficiency class. Mutations in the moderately low class were induced only by UV. All the other mutagens and UV, as well, elicited single-site mutations in all classes other than this one (except no proflavine mutations fell in the high class). All the mutagens with the exception of HNO_2 and NH_2OH also elicited multi-site mutations.

Reversion by nitrous acid: Table 2 lists representative data for transformations of mutants by HNO_2-treated DNA carrying the same *mal*$^-$ mutation as well as the *sulf-d* reference marker. There are clear-cut differences in the ability of

TABLE 2

Induction of back-mutations by nitrous acid

Recipient strain	DNA treatment (min)	c.f.u. per ml transformed culture			Survival (A/A_0)*	Inducibility $\left(\frac{kB - B'_0}{kA \ln(A_0/A)}\right)$*
		mal+	*sulf-d*	Total		
Mj	0	3	1.64×10^7	2.75×10^8	(1.00)	....
(k = .042)	1	96	1.64×10^7		1.00	....
	2	182	1.44×10^7		.88	0.0023
	5	372	1.37×10^7		.83	.0035
	10	363	1.27×10^7	3.20×10^8	.77	.0027
	20	544	9.59×10^6		.58	.0025
	40	570	4.86×10^6		.30	.0023
	60	414	2.21×10^6	2.86×10^8	.135	.0022
	120	230	7.60×10^5		.046	.0023
V12	0	6	3.06×10^6	1.60×10^8	(1.00)	....
(k = .048)	5	246	2.38×10^6	1.92×10^8	.78	0.0083
	10	371	1.69×10^6	1.52×10^8	.55	.0076
	20	344	8.40×10^5	1.60×10^8	.27	.0065
	60	179	2.62×10^5	1.92×10^8	.086	.0056
	100	180	2.48×10^5	1.44×10^8	.081	.0058
T7	0	23	7.50×10^6	3.41×10^8	(1.00)	
(k = .049)	5	700	5.94×10^6	3.42×10^8	.79	0.0102
	10	1250	4.21×10^6	3.40×10^8	.56	.0103
	20	1350	3.01×10^6	3.28×10^8	.40	.0098
	60	1130	1.07×10^6	2.91×10^8	.143	.0108
	100	960	6.45×10^5	3.10×10^8	.086	.0121
N4	0	11	1.17×10^7	3.06×10^8	(1.00)	....
(k = .052)	10	12	4.75×10^6		.41	0.000005
	60	8	1.22×10^6		.10	<.000008
N13	0	1	2.43×10^6	2.79×10^8	(1.00)	....
(k = .037)	10	5	1.10×10^6		.45	0.00012
	60	2	2.88×10^5		.12	.00004
V6	0	0	1.36×10^5	2.62×10^8	(1.00)	....
(k = .18)	10	0	9.24×10^4		.68	<.00016
	60	0	3.00×10^4		.22	<.00012
E2	0	1	9.1×10^6	3.17×10^8	(1.00)	....
(k = .91)	10	1	6.7×10^6		.74	<.0000006
	60	1	1.5×10^6		.17	<.0000004

* Symbols explained in text.

HNO_2 to induce reversions when different mutations are examined. A quantitative expression of this inductive effect is the object of the following analysis.

It is assumed that the numbers of *mal*$^-$ and *sulf-d* genes initially present are equal and that they are inactivated at the same rate. (In the case of *mal*$^-$ it is its potential to become *mal*$^+$ which is inactivated.) It is also assumed that the number of mutagenic events is proportional to the number of inactivation events. Let

A = number of surviving *mal*$^-$ or *sulf-d* genes, B = number of *mal* genes mutated to wild-type and not inactivated, h = number of HNO_2-elicited events causing inactivation, and i = inducibility, or the ratio of mutagenic events to inactivation events. The relationships among these quantities are expressed by the differential equations: $dA/dh = -A$ and $dB/dh = iA - B$. Solution of the equations gives $i = \frac{B - B_0(A/A_0)}{A \ln(A_0/A)}$, where A_0 and B_0 represent quantities initially present. This treatment is essentially similar to that of FREESE (1963) relating induced mutations to inactivation of bacteriophage.

In the transformation system *mal*$^+$ types present in cultures transformed by untreated DNA represent, principally, spontaneous reversions of recipient cells. Their number is designated as B'_0. Compared to them, the number of *mal*$^+$ types due to transformation by spontaneously revertant DNA, that is, B_0, is negligible. Furthermore, since the integration efficiency of the maltose-positive allele may not be the same as for *sulf-d*, a factor, k, representing the ratio of these efficiencies must be introduced in order to express i in terms of observable quantities, so $i = \frac{kB - B'_0}{kA \ln(A_0/A)}$ where kB is the number of *mal*$^+$ types observed with treated DNA. It will be assumed that inductions giving rise to phenotypically wild-type cells represent true back mutations. Even with this assumption there is uncertainty as to the value to assign to k since the HNO_2-mutated allele would contain an abnormal base instead of the corresponding normal DNA base and so would not be truly wild type. The values of i in Table 2, however, have been computed on the assumption that k for the mutated allele is equivalent to that for the wild type.

The observations that *mal*$^+$ reaches a maximum at about 37% survival of *sulf-d* and that i is relatively constant support the assumptions made above with regard to mutagenic and inactivation events.

Table 1 lists the nitrous acid response for almost all of the mutants. Back mutation appears to occur in only three cases, corresponding to the UV-induced mutations, Mj and V12, and the TEM mutation, T7. All of these are in the low efficiency class. The response given by O2 represents a mutation which only partially restores the ability to use maltose; this will be discussed in the following section.

Mutations at the E7, O2 site: Mutations E7 and O2 appear to have occurred at the same site since there is no detectible recombination between them, yet they show distinctly different efficiencies of integration of the wild-type marker. All indications suggest that both are truly single-site mutations and that they represent different base changes at the same site.

Nitrous acid treatment of E7 DNA gives no reversions, but treated O2 DNA on interaction with O2 cells does give rise to transformants which can grow in maltose medium, but which form smaller colonies than the wild type. The yield of such revertants approximates that found for other HNO_2 inductions. Revertant DNA failed to transfer the original O2 mutation to wild-type cells so as to give maltose-negative transformants (frequency <0.04% of O2 DNA control), which

indicates that the reverting mutations occurred either at or very near the E7,O2 site. Sixteen colonies were picked at random from a plate and examined with respect to growth rate with maltose and behavior as a maltose-positive donor in transformation.

Thirteen of the 16 revertant clones showed a generation time in maltose medium of 60 min as compared to 40 min for the wild-type. One clone grew much more slowly; two others grew at the wild-type rate. The latter, however, did not appear to be wild-type since the behavior of their alleles as donor did not correspond to the wild-type when tested on O2, E7, V7 and E4 as recipients; they gave, instead, moderately low efficiencies with all. The nature of the three atypical clones has not been investigated further.

DNA from the 13 partially maltose-positive clones representing the majority type was also tested with O2, E7, V7 and E4 as recipients. The results are shown in Table 3. Whereas the wild-type allele gives intermediate efficiency with O2, the new allele gives low efficiency. Contrariwise, the new allele transforms E7 with moderately low efficiency as opposed to the low efficiency of the wild-type. Both alleles give low efficiency with V7 which bears a mutation at a site different from, but close to, E7 and O2. Both alleles show the same high efficiency in transforming E4 which bears a multi-site mutation containing the E7,O2 and V7 sites. There are no significant differences among the 13 clones tested. The results can be interpreted as evidence that yet a third base change was induced at the E7,O2 site by nitrous acid.

Reciprocal integration efficiencies: DNA from a number of mutant strains which also carried the *sulf-d* reference marker was used to transform wild-type cells in order to determine the integration efficiencies of different *mal*$^-$ markers. These efficiencies are compared to the corresponding *mal*$^+$ marker efficiencies in Figure 4. It is seen that for single-site markers there is a fairly good correlation between positive and negative integration efficiencies. Such a correlation has also been observed for mutations in the *amiA* locus by EPHRUSSI-TAYLOR, SICARD and KAMEN (1965).

Lengthy multi-site mutations (such as T1, E9 and Me) in the donor appear to be integrated more readily than their wild-type counterparts. This is not a result

TABLE 3

Integration efficiency of maltose-positive alleles from HNO_2-induced revertants of O2 compared with wild-type

Recipient	Integration efficiency		Variation among O2 revertants	
	Wild-type DNA	O2 revertant DNA	Standard deviation*	Number tested
O2	0.50	0.060	±.013	13
E7	.043	.21	±.03	13
V7	.049	.047	±.007	7
E4	.83	.68	±.07	13

* Root-mean-square deviation; each revertant tested once.

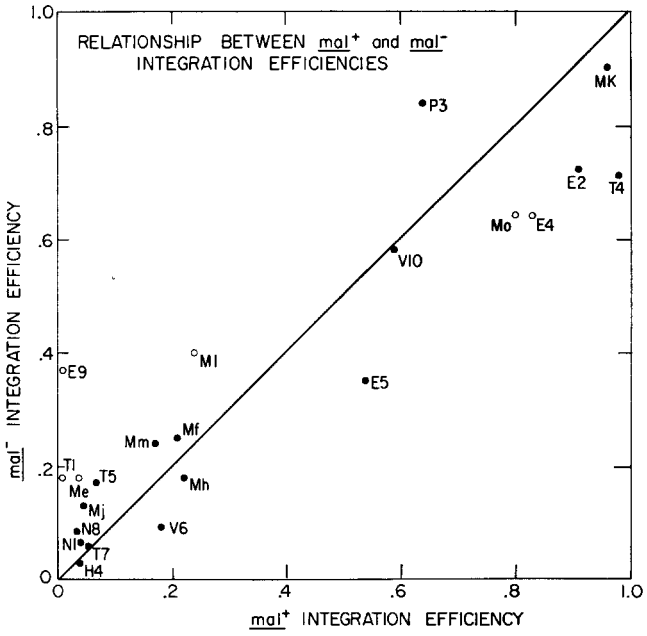

FIGURE 4.—Relationship between integration efficiencies of *mal*+ and *mal*− markers. Wild-type DNA was used to transform mutant recipients; mutant DNA was used to transform wild-type recipients. Markers correspond to single-site (●) or multi-site (○) mutations.

of dispersive transfer of parts of the multi-site mutation since testing of the negative transformants shows them all to possess the mutation in its entire extent.

Integration in double mutants: Wild-type DNA was used to transform recipient cells containing two mutations in the amylomaltase locus in order to investigate the frequency of simultaneous integration of two closely linked markers which individually show different integration efficiencies. Table 4 compares the inte-

TABLE 4

Integration frequencies for doubly-marked donor DNA

Recipient		DNA markers a	DNA markers b	Double marker integration frequency	Single marker integration efficiency a	Single marker integration efficiency b
Mk	N1	Mk+	N1+	0.072	0.96	0.037
E2	N1	E2+	N1+	.034	.91	.037
P4	N1	P4+	N1+	.033	.54	.037
Mh	N1	Mh+	N1+	.035	.22	.037
Mf	N1	Mf+	N1+	.032	.21	.037
N8	N1	N8+	N1+	.021	.031	.037
T4	T5	T4+	T5+	.067	.98	.068
E2	T5	E2+	T5+	.055	.91	.068
Mo	T5	Mo+	T5+	.060	.80	.068
Mk	T5	Mk+	T5+	.047	.96	.068

gration frequencies observed with the integration efficiencies of the component markers. It appears that the double marker integration frequency is never greater than the lower integration efficiency of its two components. (The case of MkN1 as recipient may constitute a significant exception; however, the value observed, 0.072, falls within the range of variation of markers in the low efficiency class.) Thus, the presence of a high-efficiency marker does not enhance the probability of inclusion of an adjacent low-efficiency marker. Furthermore, the double marker frequency is independent of the integration efficiency of the component with the higher efficiency (at least in the cases examined, where one marker was always of the lowest efficiency class). This means that there is no appreciable compounding of inability to integrate. For example, in the case of N8N1, although both *mal*$^+$ markers in the donor individually show low frequencies of integration, the integration of both markers together in the N8N1 recipient is only slightly less frequent, and the reduction is nowhere near as great as it would be if the combined frequency were the arithmetic product of the component efficiencies.

Exclusion effect: The use of a double mutant DNA as donor in transformation of a wild-type recipient allows determination not only of the double marker integration frequency, but also of the integration frequencies of the component negative markers. Results from this type of experiment are presented in Table 5. As was found in the preceding section, the double marker integration frequency corresponds to the lower efficiency of its components. What is perhaps more significant is that the integration frequency of a high efficiency marker is considerably reduced by the presence of a linked low efficiency marker. It appears that the exclusion of a donor marker results in the exclusion of adjacent portions of the DNA as well. The extent of exclusion depends inversely, and the escape from exclusion depends directly, on the distance between markers, as inferred from map segment location. This exclusion phenomenon which underlies the variation in integration efficiency among single-site markers must be considered in the analysis of genetic recombination in Pneumococcus.

TABLE 5

Exclusion of donor markers linked to incompatible markers

Donor markers		Transformation frequency			Integration frequency				Exclusion effect (%)	Escape from exclusion (%)	Map segment location	
		Donor type	Recombinants		Double donor		Single donor					
a	*b*	a^-b^-	a^-b^+	a^+b^-	a_2	b_2	a_1	b_1			*a*	*b*
E2	N1	0.117	0.036	0.003	0.153	0.120	0.72	0.062	79	5	VIII	VI
N8	N1	.074	.002	.004	.076	.078	.085	.062	11	.	II	VI
T4	T5	.110	.014	.0012	.124	.111	.71	.17	83	2	XIV	XV
E2	T5	.133	.058	.0075	.191	.140	.72	.17	74	9	VIII	XV
Mo	T5	.146	.090	.016	.236	.162	.64	.17	63	15	VI	XV
Mk	T5	.142	.193	.028	.335	.170	.90	.17	62	23	II	XV

Wild-type cells were transformed by DNA containing one (single donor) or two (double donor) *mal*$^-$ markers, designated *a* and *b*. Integration frequencies a_1 and b_1 were obtained from transformations by single donors. Double donors yield three classes of transformants and the frequency of each is listed. Integration frequencies a_2 and b_2 for markers in the double donor represent the total of donor and recombinant types carrying the marker. Percent exclusion $= [(a_1-a_2)/a_1] \times 100$. Percent escape from exclusion $= [(a_2-b_2)/(a_1-b_2)] \times 100$.

TABLE 6

Comparison of recombination indices computed from independent data

Parental genotypes		Recombinants		Inserted donor marker integration efficiency	Recombination index	Map segment interval
Donor	Recipient	Genotype	Frequency			
T4— T5+	T4+ T5—	T4+ T5+	0.0015	0.0556	0.027	XIV–XV
T4+ T5—	T4— T5+	T4+ T5+	.0166	1.03	.016	
T4— T5—	T4+ T5+	T4— T5+	.014	.71	.020	
T4— T5—	T4+ T5+	T4+ T5—	.0012	.17	.007	
E2— T5+	E2+ T5—	E2+ T5+	.0067	.0556	.125	VIII–XV
E2+ T5—	E2— T5+	E2+ T5+	.086	.760	.113	
E2— T5—	E2+ T5+	E2— T5+	.058	.72	.081	
E2— T5—	E2+ T5+	E2+ T5—	.0075	.17	.044	
Mo— T5+	Mo+ T5—	Mo+ T5+	.0142	.0556	.255	VI–XV
Mo+ T5—	Mo— T5+	Mo+ T5+	.178	.756	.235	
Mo— T5—	Mo+ T5+	Mo— T5+	.090	.64	.14	
Mo— T5—	Mo+ T5+	Mo+ T5—	.016	.17	.10	
Mk— T5+	Mk+ T5—	Mk+ T5+	.0211	.0556	.379	II–XV
Mk+ T5—	Mk— T5+	Mk+ T5+	.348	1.13	.308	
Mk— T5—	Mk+ T5+	Mk— T5+	.193	.90	.21	
Mk— T5—	Mk+ T5+	Mk+ T5—	.028	.17	.17	

Recombination index and distance: The *recombination index,* defined as the ratio of the recombinant frequency to the inserted donor marker integration efficiency, has been used as a measure of distance between marker sites (LACKS and HOTCHKISS 1960a; EPHRATI-ELIZUR, SRINIVASAN and ZAMENHOF 1961; SIROTNAK, LUNT and HUTCHISON 1964). Theoretical justification for its use is presented in the DISCUSSION.

Table 6 presents data for recombinant frequencies for different sets of donor markers located at pairs of sites. The recombination indices are computed from the independent sets of data and compared with each other and with the relative distance between the sites as determined from the map segment interval. Recombination indices calculated from the reciprocal crosses in which wild-type recombinants were selected are in excellent agreement, despite differences in donor marker integration efficiencies as great as 20-fold. Results from the other two crosses in each group are in qualitative agreement, but the values obtained tend to be somewhat lower. If the difference is significant, it could mean that recombination is less frequent when mutant markers are present at both sites in the donor than when one donor marker is wild-type; however, recombination indices calculated from crosses in which negative recombinants were selected may be expected to be less accurate since fewer colonies were counted. This was particularly true in the case of recombinants including a low-efficiency marker (fourth member of each group in Table 6) where only one to seven recombinants were actually counted. In any event, there is in every case a clear correlation between recombination index and distance determined by the ordering of map segments.

Recombination index mapping: An attempt was made to construct a map of the amylomaltase locus on the basis of recombination indices. Nine mutant strains which show efficiencies of integration of the wild-type marker ranging from 0.031 to 0.96 were used as recipients. They were transformed with donor DNA from 24 to 34 different mutants which also represent a wide range of integration efficiencies. All the donor and recipient markers involved are either single-site or short multi-site. They are scattered throughout the various map segments, but certain groups of closely linked or overlapping markers were included for purposes of comparison. Duplicate determinations were made for each wild-type recombinant frequency. The values obtained, and the recombination indices derived from them, showed variable reproducibility which appeared to reflect a variable property of the recipient culture. For example, two cultures of Mj both gave values about 60% greater than a third culture grown on a different day. Deviations of ±30% from the mean were observed with Mj and N8 as recipients, while the others all showed deviations generally less than 10%.

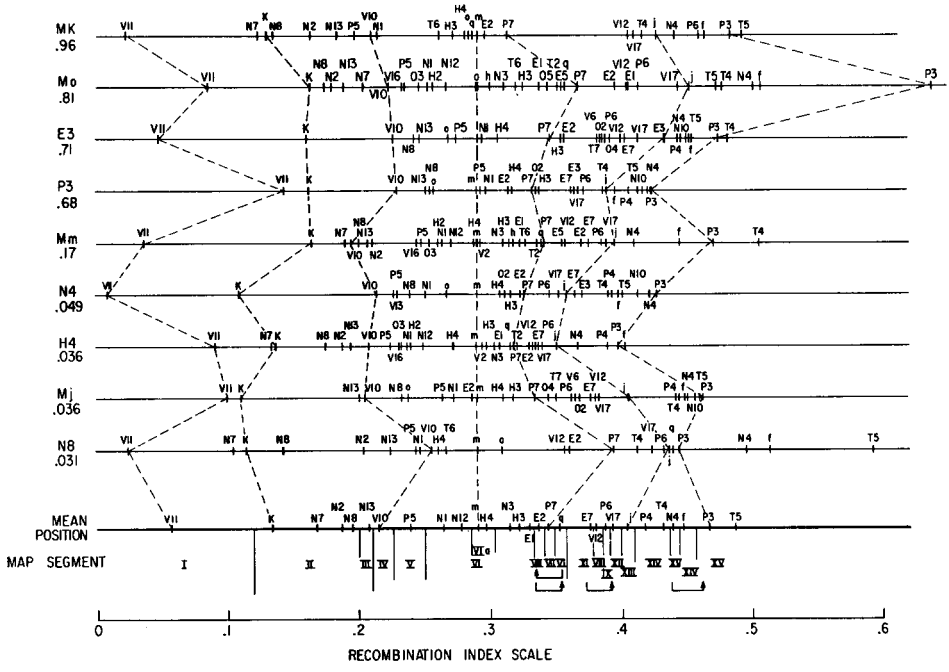

FIGURE 5.—Recombination index mapping. DNA bearing various mutant markers was used to transform mutant recipients indicated by symbols at the left of each line. The number below the recipient symbol represents the integration efficiency for the wild-type marker. Recipient marker sites are indicated by heavy vertical bars. Lighter vertical bars represent donor marker sites; recombination indices obtained in the crosses determine their distance of separation from the recipient site. Maps corresponding to different recipients are aligned at the position of Mm (Mx symbols are abbreviated as x). Dashed lines connect single marker sites in the various maps. Their mean positions are shown on the heavy line at the bottom of the figure. Roman numerals define segments of the "deletion" map (Figure 1). Arrows indicate deviations of recombination index map order from "deletion" map order.

The data are presented vectorially in Figure 5. Each recombination index is represented by a linear distance according to the indicated scale. The direction of the vector was assigned on the basis of segment location, preliminary results of three-factor crosses, or arbitrary assumption. Maps corresponding to different recipients are aligned at the site of Mm.

It is apparent that there is a general concordance of the data from different crosses in determining the position of a site. Furthermore, the over-all span of the locus determined by different recipients varies by less than a factor of two even though the observed recombinant frequencies between most distant marker pairs varied by as much as 40-fold. There is considerable variation in the position of a site determined in different crosses—this is demonstrated by the dashed line in Figure 5. A mean position calculated by averaging all values (except the extreme position of P3 given with Mo as recipient) is also represented graphically. There is a clear correspondence of these mean positions with the map order determined by overlapping of multi-site mutations.

It appears that the recombination index does represent distance at least to an accuracy of ± 0.04 units within a locus which is 0.45 units in extent. Within this accuracy the distances so determined appear to be additive. It should be possible by this method alone to order markers at fairly well separated sites. Since the accuracy in absolute terms is greater for more closely linked markers, it may even be possible to order sites 0.02 units apart. Closer than this the equivalence of the recombination index in reciprocal crosses does not appear to hold. For example: Mk → N8 gives 0.025 while N8 → Mk gives 0.0043; P3 → N4 gives 0.0054 while N4 → P3 gives 0.00017; Mm → H4 gives 0.018 while H4 → Mm gives 0.0051. In these cases the recombinant frequency is perhaps determined by either the lower or the higher donor marker integration efficiency, irrespective of which marker is inserted in the recombinant. This would contrast with the situation generally observed for markers more than 0.02 units apart where the frequency of recombinants carrying one donor marker is not influenced by the integration efficiency (or exclusion effect) of the donor marker which is not inserted.

DISCUSSION

Base changes and integration efficiency: The distribution of integration efficiencies of single-site markers into four distinct classes suggests a relationship between integration efficiency and base pair change since there are only six possible changes which can occur: transitions AT → GC and GC → AT and transversions AT → TA, GC → CG, AT → CG and GC → TA (where A, T, G and C represent adenine, thymine, guanine and cytosine, respectively). Consequently, it is of interest to analyze the mutagenic origin of mutations in the different classes in the light of present knowledge of the specificity of the mutagens. Hydroxylamine is expected to produce only GC → AT transitions (FREESE, BAUTZ and BAUTZ-FREESE 1961). Nitrous acid can potentially cause both types of transitions (BAUTZ-FREESE and FREESE 1961; TESSMAN, PODDAR and KUMAR 1964), but with double-stranded DNA GC → AT changes predominate (FREESE 1963). This appears to

be true in the present study since none of the hydroxylamine or nitrous acid mutations are induced to back mutate by nitrous acid. Nitrous acid does cause back mutation of forward mutations induced by UV and TEM, both of which are capable of altering thymine (BEUKERS and BERENDS 1960; LORKIEWICZ and SZYBALSKI 1961). All of the mutations elicited by hydroxylamine or nitrous acid, as well as those induced to back mutate by nitrous acid, fall into the lowest efficiency class. EMS is supposed to attack guanine preferentially (BROOKES and LAWLEY 1961) and thereby to elicit both transitions and transversions of GC (BAUTZ and FREESE 1960). EMS-induced mutations fall into several efficiency classes, including the lowest one. Although there is no chemical evidence that hydrogen peroxide preferentially attacks guanine, its pattern of mutations resembles that of EMS. Mutations in the moderately low class were induced only by UV and hence may represent transversions at AT sites.

A distribution of mutations at the *amiA* locus of pneumococcus into two efficiency classes has been reported by EPHRUSSI-TAYLOR, SICARD and KAMEN (1965). They expressed efficiencies in terms of the *str-r* reference marker which itself shows an integration frequency of 0.58 relative to *sulf-d*, so their efficiencies must be multiplied by this factor in order to compare results. The low efficiency class they observed corresponds to the lowest class reported here; their high efficiency class appears to correspond to the classes here called intermediate and high. None of their mutations were induced by UV, and this may explain the absence of a moderately low integration efficiency class among their mutants.

The mode of action of nitrous acid deserves further consideration. Deamination of A and C gives rise to bases the tautomeric forms of which are analogous to G and T, respectively. Deamination of G gives rise to xanthine which has been supposed to remain in the same tautomeric form as G and not to give rise to a mutation (VIELMETTER and SCHUSTER 1960). TESSMAN, PODDAR and KUMAR (1964), however, present data indicating mutation of G → A following HNO_2 treatment of phage containing single-stranded DNA. These authors suggest that the xanthine residue is anionic at physiological pH and hence may pair with T. It should be recognized that the mutagenic specificity of HNO_2 depends not only on the nature of the products it can form but also on the susceptibility of different bases in DNA. SCHUSTER (1960) found that G, C and A in native (double-stranded) DNA were deaminated at pH 4.2 at rates in the ratio of about 5:2:1. LITMAN (1961) found that for pneumococcal DNA the ultimate rates of deamination of the bases were in this ratio, but that, initially, G was attacked much more rapidly and C much less rapidly. By the time of the earliest chemical determination when 10 to 15% of G, 1 to 2% of A, and no detectible C were deaminated, 99.9% of transforming activity had been destroyed. It would seem, therefore, that the *initial* rates of deamination are of greater biological significance. Certain G residues may be particularly susceptible on account of the nature of their nearest neighbors; deamination of members of this fraction could account for the majority of induced mutations. The observation that 6 out of 13 HNO_2-induced maltose-negative mutations occurred at two "hot spots" is consistent with the interpretation that highly mutable GC pairs occur at a limited number of sites

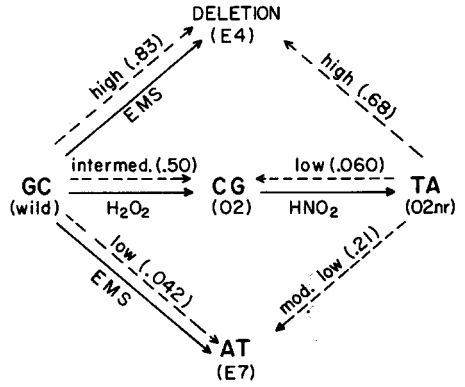

FIGURE 6.—Base changes at the E7,02 site. Strain designations are given in parentheses below the corresponding base pair configurations. Mutations are represented as solid arrows with the inducing agent below. Transformations are represented as dashed arrows (from donor to recipient) with integration efficiencies, in parentheses, above.

in the locus. Conversely, back-mutation induction may only be demonstrable for a fraction of forward mutations corresponding to AT → GC transitions.

A designation of base changes occurring at the E7,02 site is proposed in Figure 6. Since E7 was induced by EMS and is not revertible by HNO_2 it could represent the mutation GC → AT. Inasmuch as 02 occurs at the same site, it is reasonable that H_2O_2 by attacking G induced the transversion GC → CG, so that 02 would still contain G which could be susceptible to HNO_2. Reaction with this reagent causes the transition CG → TA, not to the wild type, but to the partial phenotypic revertant, 02nr. The efficiency of integration of the new allele into 02 is low, corresponding to the class for transitions, while with E7, which involves the transformation TA → AT, the efficiency is moderately low. Both wild-type and 02nr alleles are integrated with low efficiency by V7 since exclusion of the *mal*-$V7^+$ marker is maximal and independent of changes at an adjacent site. Both alleles give high efficiency of integration in the multi-site mutant E4; an explanation for this is offered in the following section.

The above observations support the hypothesis that integration efficiency is determined by the nature of the base pair in the donor as compared to the base pair at the corresponding site (with regard to both position and polarity) in the recipient DNA. A tentative assignment of base changes corresponding to mutations which fall into three of the efficiency classes can be made. Transitions AT → GC and GC → AT are in the lowest class; AT → TA transversions are in the moderately low class; GC → CG transversions are in the intermediate class. Transversions GC → TA and TA → GC are not yet classified; one or both may fall either into the high class or into one of the others. For the classes identified it is to be expected that reciprocal integration efficiencies should correspond since

both transitions fall into the same class and the two other classes each represent reciprocal transversions.

Base incompatibilities and exclusion: Although there is no direct evidence, it is reasonable to assume that multi-site maltose-negative mutations represent deletions of parts of the DNA sequence. Thus, the corresponding wild-type markers are truly multi-site in that they consist of chains of nucleotides which may be of considerable length, while the mutant markers resulting from deletions have no physical size. This distinction is important in considerations of integration efficiency or of marker inactivation.

Multi-site markers corresponding to short deletions generally show high integration efficiencies. This is true despite the fact that markers corresponding to mutations at single sites within their span may be integrated with low efficiency. It appears that neither the extent of alteration (when small) nor the overall composition of the altered region determines integration efficiency. The significant factor is the nature of the difference between donor and recipient DNA. Single-base differences arising from single-site mutations are often more detrimental to integration than even the absence of a sequence as much as 100 nucleotides in length (based on extents of mutations Mc and V18 relative to the entire locus as estimated from recombination index measurements between most distant included sites depicted in Figure 5). This suggests that low integration efficiency is due to an *incompatibility* between corresponding donor and recipient DNA bases. In transformation of a deletion, recipient bases are either identical to the donor or absent, so no incompatibility exists. Thus, integration of both wild-type and O2nr markers into E4 occurs readily and is independent of the base present at the O2 site since the latter is encompassed in the deletion corresponding to E4.

Multi-site markers corresponding to lengthy deletions do show low efficiencies of integration. This may be attributed to end effects, that is, termination of the donor DNA fragment within or near the marker region. If fragments are produced by random breaks during DNA extraction, lengthy markers will more frequently be found in such situations. Markers which span the entire amylomaltase locus, for example, must be over 900 nucleotides long and therefore would cover an appreciable fraction of the 12,000 nucleotides of a donor DNA particle [based on a molecular weight of 8×10^6 (LITT *et al.* 1958)]. The efficiency of integration of the deletion itself, however, should not be influenced by such effects; this may explain the differences observed in reciprocal integration efficiencies for markers corresponding to multi-site mutations. For example, *mal*-E9$^+$, which on the basis of inactivation data is a large multi-site marker, gives an integration efficiency of 0.009, while its counterpart, *mal*-E9$^-$, is integrated with an efficiency of 0.40.

Streptomycin-resistance markers which involve considerable genetic lengths (ROTHEIM and RAVIN 1964) also show high integration efficiencies. If the multi-site nature of these markers corresponds to a deletion, then the streptomycin-resistant marker, which arises by mutation from the wild-type, presumably represents the deletion, so that the transformation of streptomycin-sensitive recipients involves transmission of a deletion rather than the corresponding

multi-site segment. From this point of view, also, it is not surprising that the sensitivity of *str-r* to subcritical temperature corresponds to that of a single-site marker rather than a truly multi-site marker (LACKS and HOTCHKISS 1960a).

In considering a mechanism by which single-base differences affect integration efficiency it is evident from the double mutant experiments that these differences have only a negative, or excluding, effect. There is no enhancement of integration of a low-efficiency marker by an adjacent high-efficiency marker. Integration frequency is apparently maximal when donor bases are identical to the host, and base differences give rise to various degrees of exclusion. Since with DNA containing two low-efficiency markers the integration frequency of each is almost as great as when each marker is present alone, it would appear that exclusion effects are not compounded. Rather, the data indicate that extent of exclusion depends only on the most incompatible marker in the DNA. An alternative possibility is that exclusion effects are compounded but that a basal level of integration (corresponding, approximately, to the lowest class) always remains. Any mechanism to explain exclusion must account for the effect of a single base difference on material hundreds of nucleotides away. The notion that integration corresponds to a linear process proceeding along the DNA and that this process is blocked by the incompatible base is not acceptable, for such a mechanism could prevent at most only half the integration of another marker. Actually, exclusion may be as great as 95%. In order to explain the "action at a distance" and the magnitude of the effect, the model proposed below invokes a thermodynamic equilibrium involving entire tracts of DNA.

The "depressor" factor found by GREEN (1959) to influence integration of a streptomycin-resistance marker in pneumococcus behaves just like a low-efficiency marker. A plausible interpretation is that it represents a mutation to give a base incompatibility which has no observable phenotypic effect other than to exclude integration of the closely-linked streptomycin-resistance marker.

Recombination analysis: There are four processes potentially capable of separating donor markers so that genetic recombinants are produced.

(1) Double-strand breaks caused by shearing during the preparation of DNA. When both markers are not present on the same particle, they cannot enter the cell together, let alone be integrated simultaneously. Such breaks appear to be responsible for most of the recombination between distant markers, since low levels of linkage (about 4%) have been reported (HOTCHKISS and EVANS 1958) even though at least 25% of high-efficiency DNA markers introduced into a cell are integrated (LACKS 1962).

(2) Single-strand breaks caused by action of nucleases in donor or recipient cells. In this case both markers would enter a cell, but on conversion to the single-stranded form they would be separated and therefore independently integrated. There is no evidence that this process is of importance.

(3) Recombination switches by an unknown mechanism following synapsis of donor DNA with host. Evidence for such short-range recombination comes from preliminary results of three-factor crosses with markers in the amylomaltase locus which show that short donor segments are not inserted less frequently than

would be predicted from two-factor crosses. It would be expected that small pieces produced by processes (1) or (2) would not be readily integrated since small pieces artificially produced by sonic treatment are ineffective in transformation (LITT, *et al.* 1958). KENT and HOTCHKISS (1964) have shown by kinetic methods that, in a three-factor cross involving drug-resistance markers, separately inserted donor markers are derived from a single donor particle; only processes (2) or (3) could account for this finding.

(4) Exclusion, due to incompatibility, of one donor marker but not of the other. Such differential exclusion is distance-dependent. Comparison of data in Table 5 and Figure 5 suggests that the probability of the exclusion effect terminating within a given distance is approximately the same as the probability of a recombination event between nonexcluding markers separated by that distance.

Mathematical expressions for the effects of these separatory processes on recombination can be obtained. Consider two markers n nucleotides apart. Assume that processes (1), (2), (3) and (4) act randomly within this span with the average number of events per nucleotide given by a, b, c and d, respectively, where $a < c \approx d$ and $b < c$. In the absence of exclusion effects, the recombinant frequency,

$$r = 1 - e^{-na}\left(\frac{1 + e^{-n(b+2c)}}{2}\right).$$

For small n the expression reduces to $1 - e^{-nc}$. In order to consider exclusion effects, let E_1 and E_2 indicate the integration efficiencies of the donor markers and r_{10} represent the frequency of recombinants containing only the first donor marker. Since the proportion excluded is not available for recombination, $r_{10} = E_1E_2\ (1 - e^{-nc}) + (E_1 - E_1E_2)\ (1 - e^{-nd})$. This will be true whatever value E_1E_2 assumes—whether it represents the product of E_1 and E_2, the lower of the two, or a basal level inherent to the system. If $c = d$, the expression reduces to $r_{10} = E_1(1 - e^{-nc})$. When nc is sufficiently small, $nc = r_{10}/E_1$, from which it is seen that n, the distance in nucleotides, is proportional to the recombination index.

If the amylomaltase gene locus contains 900 nucleotides (see section on amylomaltase activity in mutants) and is equivalent to 0.45 recombination index units in length (Figure 5), then the probability of a recombination event per nucleotide, c, is 0.0005. From this number the mean length of integrated donor strand segments can be calculated to be about 2000 nucleotides, or roughly two genes, in length. This value is comparable to the target sizes for marker inactivation calculated by various investigators from data on the inactivation of transforming activity: 900 to 3000 for P^{32} decay (Fox 1963), ~1700 for ultrasonic treatment (LITT, *et al.* 1958), ~1600 for various inactivating agents (LERMAN and TOLMACH 1959). FOX and ALLEN (1964) have, more directly, estimated the lengths of integrated donor strand segments to be in the range of 1700 to 3300 nucleotides

Molecular model: The following is an attempt to explain integration, recombination and exclusion phenomena in terms of plausible molecular structures and processes.

Donor DNA: Since the DNA of *E. coli* forms a single structure (CAIRNS 1963), it is assumed that this is true, also, in pneumococcus. During preparation shear-

ing forces fragment the donor DNA into particles which vary narrowly about a mean size which is determined by the maximum shear. The number of breaks is sufficient to randomize the position of a marker relative to the ends of the fragments containing it. On entry into a recipient cell, and perhaps as a consequence of the mechanism of entry (LACKS 1962), the double-stranded DNA is converted into a single strand. This may be accomplished by the action of an enzyme similar to Exonuclease III of *E. coli* (RICHARDSON, LEHMAN and KORNBERG 1964). Depending on which end of the duplex penetrates the cell first, either donor strand may remain intact and serve as the vehicle of genetic transfer. Relative to the polarity of the strands, markers on sister strands are reversed in order.

Synapsis: The donor strand moves about in the cell until a point on it makes contact with the homologous point on the recipient DNA duplex. The time required for initial contact may be rate-limiting; thus, single-stranded donor DNA can be isolated for several minutes following uptake. The specificity of contact and attachment is based on the formation of base triplets according to the scheme in Figure 7. Once contact is established, the structure zips up to give a three-stranded configuration with the donor strand twisted about the duplex in its large groove. The twist given to the donor strand during attachment corresponds to its ultimate twist following integration.

In Figures 7 and 8(a) the attached donor strand is in position III, its recipient homologue, which has the same polarity, is in position II, and the complementary recipient strand is in position I. Hydrogen bonds between the bases and Van der Waals attractions, which arise from the close approximation of bases made possible by the hydrogen bonds as well as from the vertical stacking of bases, presumably stabilize the three-stranded structure. Two of the triplet configurations (pyrimidines in position III) were proposed by STENT (1958) in a model for DNA replication. These and the triplet with adenine in position III may be criticized in that the hydrogen bonds make uncommonly large angles with the N-H bonds.

Double-stranded DNA probably exists in solution in a form similar to that proposed by LANGRIDGE *et al.* (1960) for the *B* form of crystalline DNA. This structure may be favored over the one originally proposed by CRICK and WATSON (1954) on account of maximal stacking of base pairs when they are closer to the helix axis. However, it does not appear to be possible to fit a third strand around the LANGRIDGE structure. Conceivably, attachment of the third strand shifts the duplex into the broader diameter structure of CRICK and WATSON which would now be stabilized by maximal stacking of base triplets. This shift, by moving the helix axis further away from the base pairs of the recipient duplex, enables it to come closer to the third strand which can then be wound around with its backbone in the configuration of the LANGRIDGE model. Such a three-stranded structure appears to be permissible, but it has not yet been determined whether it would satisfy the requirement of precise stereochemical fit. INMAN (1964) has reported the occurrence of a structure composed of two strands of poly-deoxyinosinate and one strand of poly-bromodeoxycytidylate; although the na-

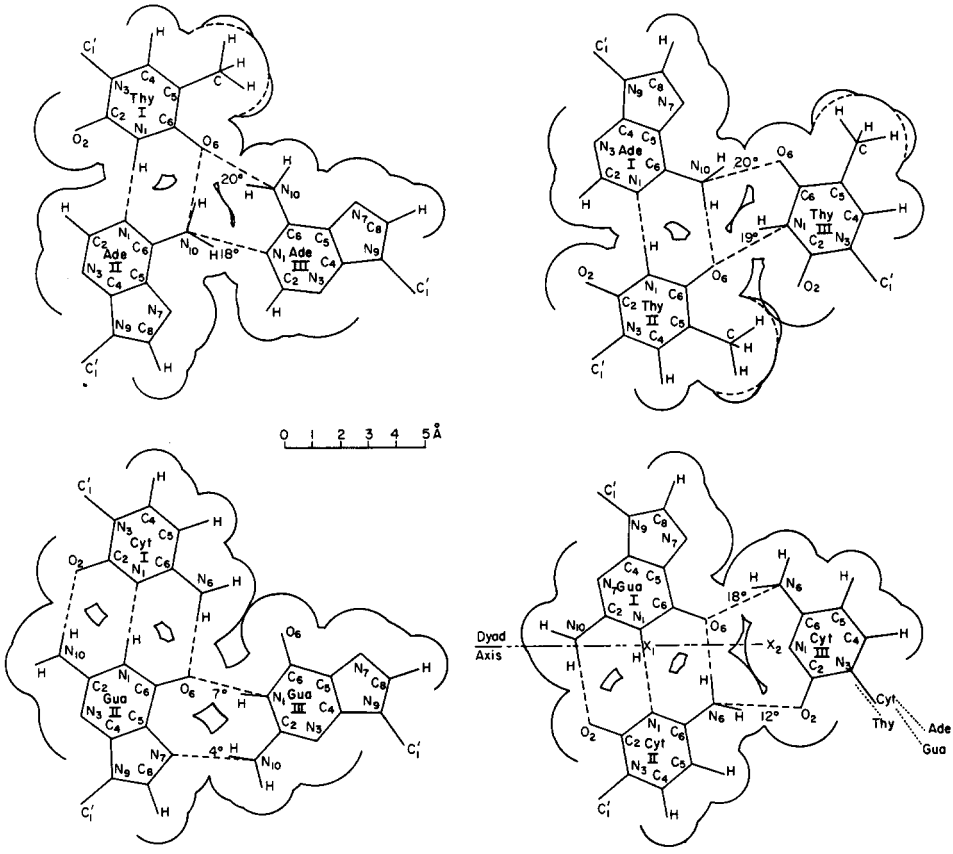

FIGURE 7.—Base triplet configurations proposed for synapsis of donor strand with host duplex. Roman numerals signify bases in the complementary host strand, homologous host strand, and donor strand, respectively. Hydrogen bonds are indicated by dashed lines. Angles between hydrogen bonds and the component N-H bonds are given when appreciable. Positions of the glycosidic bonds for thymine, adenine, and guanine in the III position are superimposed as dotted lines on the configuration for cytosine in the III position. X_1 and X_2 represent positions of the helix axis for the LANGRIDGE *et al.* (1960) and CRICK and WATSON (1954) structures, respectively. Bond lengths and angles are those estimated by SPENCER (1959). Van der Waals envelopes were constructed on the basis of radii given by PAULING (1960) with the exception of H which was taken as 1.0 Å. For the thymine methyl groups, the dashed line indicates minimal, the solid line maximal, extension of the envelope in the plane of the bases.

ture of this structure has not been elucidated, it may resemble the type postulated here.

Equilibrium: Since the strands in the II and III positions are virtually identical, they can compete on equal terms for attachment to the complementary strand in the I position. Counteracting the tendency toward randomization of donor strand and homologous recipient strand bases among the II and III positions is the need for looping of the III strand over the II strand in order that positions be exchanged. Such looping forms two points of junction or switching of strand

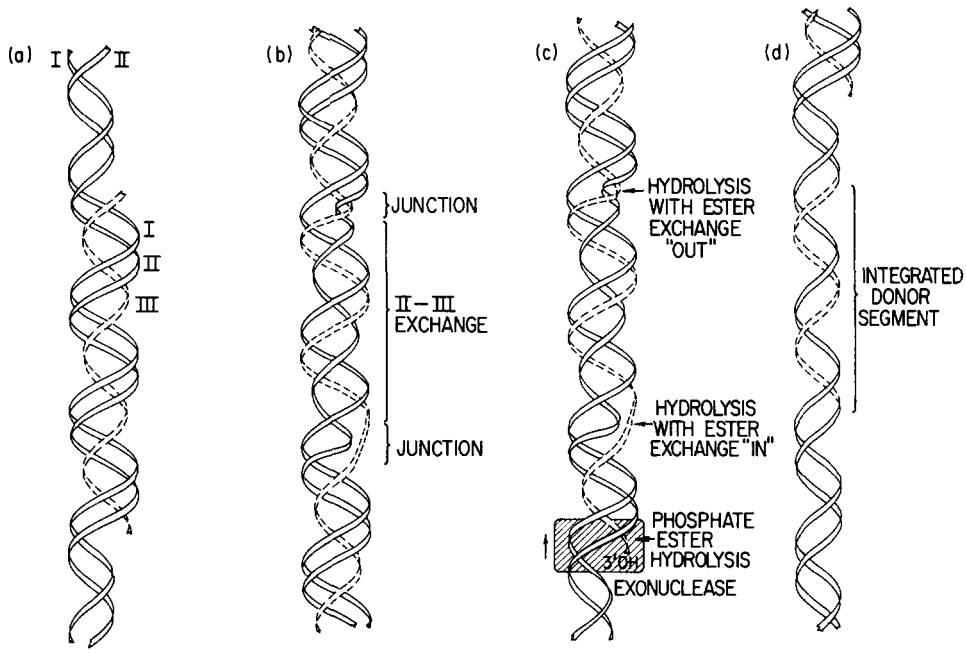

FIGURE 8.—Model for strand interactions and recombination in pneumococcal transformation. (a) Synapsis. (b) Equilibrium. (c) Recombination. (d) Product. Helical ribbons portray positions of C'_1 atoms of nucleotide residues in donor strands (dashed lines) and recipient duplex strands (solid lines). Roman numerals refer to the original positions (a) of complementary host strand, homologous host strand, and donor strand, respectively. Where the structure is three-stranded, strands in the I and II positions are in the CRICK and WATSON (1954) configuration; outside of such regions they are in the LANGRIDGE *et al.* (1960) configuration. The strand in position III is also in the latter configuration. At equilibrium (b) tracts of the donor strand exchange positions with corresponding tracts of the homologous host strand giving rise to junctions where the donor strand passes over its counterpart. An exonuclease (c) acts progressively, in the direction of the vertical arrrow, on the end of the strand in position III to release 5'-mononucleotides. At junction points the exonuclease catalyses phosphate ester exchanges between strands in positions II and III and thus gives rise to the recombinant product (d).

positions, as shown in Figure 8(b). In order to allow one strand to pass over the other at a junction point it appears that at least two hydrogen-bonded triplets must be disrupted. Such disruption could involve a ΔF of the order of 5 kcal. This will counter the tendency of the donor strand to randomly replace the recipient strand in the II position so that at thermodynamic equilibrium, which is presumed to be readily attained, lengthy tracts of the order of 2000 nucleotides of donor and recipient strands will alternate in the II position. It is proposed that this equilibrium situation, depicted in Figure 8(b), underlies integration and short-range recombination in pneumococcal transformation.

Exclusion: When a base in the donor strand is not identical to its recipient homologue, the triplet with the donor base in the II position may be expected to be generally less favored energetically than when it is in the III position since the former situation requires disruption of the original base pair with little com-

pensation in hydrogen bonding or goodness of stereochemical fit. Such differential stability of triplets will depend on the precise base change involved. Insertion of the incompatible base into the II position could conceivably correspond to a ΔF ranging from 0 to as much as 2 kcal. At equilibrium, a whole tract of the donor strand containing the incompatibility would be excluded from the II position to the degree corresponding to this ΔF, which would mean 0 to 95% exclusion. The size of the excluded tract would be comparable to the size of tracts responsible for recombination. Since markers may be carried by either donor strand, the effect of base differences must be averaged for the two base incompatibility situations involved in transformation by the different donor strands. The integration efficiency of a donor marker and the various classes observed should then be referable to the ΔF of the abnormal triplet combinations.

Donor strands containing multi-site markers corresponding to deletions in the recipient DNA could synapse so that a single-stranded loop protrudes from the three-stranded structure. Since the loop would not participate in base pairing, it should not interfere with II-III strand exchange. Thus, short multi-site markers would not appreciably diminish integration efficiency. Extensive multi-site markers which occupy an appreciable fraction of the donor particle will often be either broken or close to an end of the fragment since all particles are sheared to roughly the same size. Proximity to an end may lower the integration efficiency since little room would be available for synapsis and recombination. Deletion markers in the donor should not interfere with exchange since here, also, there is no competition between donor and recipient bases. However, difficulties in synapsing, which requires looping out of the recipient double helix, may account for the variable integration efficiency of such markers.

Recombination: Once the 3′-OH end of the donor strand is attached to the recipient duplex, an exonuclease of appropriate specificity acts on the strand in the III position to split off mononucleotides. This process is illustrated in Figure 8(c). Fragmented donor DNA may be terminated by 3′-phosphate groups, but if the enzyme has a phosphatase function similar to *E. coli* Exonuclease III (Richardson, Lehman and Kornberg 1964), the phosphate will be removed prior to removal of nucleotides. While the enzyme progressively eats away the III strand, junctions move back and forth over the triple-stranded region. When a junction is present at the site of exonuclease action, there is a high probability that the enzyme will catalyze a phosphate ester exchange to unite the strand formerly in the II position with the strand now entering the II position. This action eliminates the junction and transfers the free end to the other strand, which is now in the III position, so that the attack progresses on this strand. Loops or deletions in either strand do not affect passage of the enzyme and any excess material in the III position is hydrolyzed.

If the first junction begins the insertion of donor material, the next junction will reattach this donor material to the recipient strand so that continuity of the strand in the resulting duplex is preserved. Since junctions are formed in pairs, once a single switch occurs, the remaining junction will persist until switched "out" by the enzyme. This provides for an even number of switches in recom-

bination. If the probability of the enzyme causing a switch at a junction is less than 1.0, there may be leakage of otherwise excluded tracts into the recombinant. This could account for a basal level of integration which would explain the apparent absence of compounding of incompatible marker effects. When the exonuclease has completed its traversal of the synapsed structure, a double helix with one recombinant strand emerges as shown in Figure 8(d).

The proposed model is, of course, speculative. Certain of its features are attractive, others are not. Its novelty lies in the assumption of a dynamic equilibrium as a basis for random recombination events. Certain aspects of the model may have application in other systems, even those in which two-strand exchanges occur (MESELSON 1964) if single strands at the termini of exchanged segments are responsible for rejoining. WHITEHOUSE (1963) has proposed a model for such recombination in which single-strand breaks occur in complementary strands of parental DNA molecules and synthesis occurs in the vicinity of the breaks to give a situation in which each parental DNA is three-stranded over a section of its length. Following interaction of the single-stranded ends, recombination is presumed to involve elimination of duplicated sections concomitant with rejoining of the strands to give recombinant molecules. The situation is closely analagous to transformation where duplicate material is introduced rather than formed by partial replication. In both cases elimination of excess material and formation of new ester links are presumed to occur. It is thus possible that two-strand recombination follows a course of events similar to the one proposed for transformation.

I am grateful to MR. B. GREENBERG, MRS. A. LLACER, MR. A. ZEITLIN, MISS B. SCHULTHEIS, MR. B. PIVO and MRS. E. LACKS for their competent assistance in various phases of the work.

SUMMARY

Seventy-six mutations at the amylomaltase locus of pneumococcus were analyzed with respect to mutagenic origin, reversion to wild type, integration of both positive and negative markers in transformation, and frequencies of recombination between pairs of markers. Overlapping multi-site mutations established the linear order of sites in the locus.

Single-site mutations gave efficiences of integration of the wild-type marker which fell into four classes with values in the ratio of 0.05 : 0.2 : 0.5 : 1.0. Different mutations at a single site gave different efficiencies. Integration efficiencies of negative markers correlated with the reciprocal efficiencies. It was concluded that particular base changes are responsible for the observed integration efficiencies. Tentative assignments of base changes corresponding to the various classes have been made.

By the use of doubly marked DNA it was shown that incompatible base changes responsible for low integration efficiency also exclude adjacent donor markers. The relationship between exclusion and linkage is such that the recombination index, that is, the ratio of recombinant frequency to inserted donor marker integration efficiency, is an approximately valid measure of genetic distance, as deduced from the order of sites in the locus.

A model, based on synapsis of a donor single strand with the host cell DNA followed by a dynamic equilibrium between segments of donor and host strands in the resultant three-stranded structure, is proposed to account in molecular terms for the phenomena of exclusion and genetic recombination.

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