INCREASED DIHYDROFOLATE REDUCTASE SYNTHESIS IN
DIPLOCOCCUS PNEUMONIAE FOLLOWING TRANSLATABLE
ALTERATION OF THE STRUCTURAL GENE. I. GENOTYPE
DERIVATION AND RECOMBINATIONAL ANALYSES

F. M. SIROTNAK AND SANDRA L. HACHTEL

*Division of Drug Resistance, Sloan-Kettering Institute for Cancer Research and Sloan-Kettering
Division, Cornell University Medical College, New York, N.Y. 10021*

Received May 31, 1968

MOST structural gene mutations, which are not of the "nonsense" or "frameshift" type, are recognized solely by effects on some property of the corresponding gene product or enzyme. A number of these mutations in a gene of the L-arabinose operon in *Escherichia coli* have also been found (ENGLESBERG 1961) to alter the rate of synthesis of the enzyme as well. This form of dual biochemical effect could be distinguished from effects on enzyme synthesis determined by "nonsense" or "frameshift" mutations (NEWTON, BECKWITH, ZIPSER and BRENNER 1965; MARTIN, SILBERT, SMITH and WHITFIELD 1966; and YANOFSKY and ITO 1966) in that the gene product, although inactive was still made (CRM^+) and the normal coordinate relationship in the synthesis of all three enzymes in the operon was usually maintained, i.e. there was no polarity (LEE and ENGLESBERG 1963). Moreover, these mutations sometimes determined a coordinate increase as well as decrease in the rate of enzyme synthesis. It was suggested (LEE and ENGLESBERG 1963) that the inability to demonstrate this form of dual-effect in other systems might be related to individual biochemical properties peculiar to each. Evidence for a similar effect of mutation in the dihydrofolate reductase gene of *Diplococcus pneumoniae* is reported now. Of interest was the fact that these mutations, unlike those found in the L-arabinose operon of *E. coli*, determined only increased rates of enzyme synthesis and at the same time had no grossly adverse effect on catalytic properties.

Mutant strains of *D. pneumoniae* with elevated levels of dihydrofolate reductase can be isolated by selecting for resistance to the antifolate, amethopterin. These effects on enzyme synthesis occur exclusively as a result of mutation in the previously identified *AME I* locus (SIROTNAK, LUNT and HUTCHISON 1964). The current studies revealed that almost all of these mutations are within the structural gene for the enzyme itself. Certain genetic properties and biochemical effects of a few of these mutations have been described earlier (SIROTNAK, LUNT and HUTCHISON 1964; SIROTNAK, DONATI and HUTCHISON 1964a, 1964b, 1964c; SIROTNAK and HUTCHISON 1965, 1966). The results of a more comprehensive genetic study of this class of mutation are presented here. Other findings related to the question of recombination during transformation in *D. pneumoniae*, which

were revealed during the course of these studies, are also discussed. A variety of effects on the properties and the rate of synthesis of dihydrofolate reductase, which are associated with the various mutant genotypes, are described in the accompanying report (SIROTNAK, HACHTEL and WILLIAMS 1969).

MATERIALS AND METHODS

General: Many of the procedures used in the present study have already been described in detail. Medium used for the isolation of mutant strains in the presence of amethopterin and for transformation experiments was a modification (SIROTNAK, LUNT and HUTCHISON 1960b) of the casein hydrolysate medium of ADAMS and ROE (1945) containing 0.2% bovine albumin (Armour Fraction V). A semidefined enzymatic casein hydrolysate medium (SIROTNAK, LUNT and HUTCHISON 1960a) was used for large scale culture production. Crude transforming DNA preparations were made from lysates of sodium desoxycholate treated cells in 0.14 M NaCl + 0.01 M sodium citrate (pH 7.0). Partial deproteinization was carried out by heat denaturation at 60°C in half saturated sodium acetate (MAYERS and SPIZIZEN 1956). Amethopterin (Methotrexate) was supplied by Dr. JAMES RUGESEGGER, Lederle Laboratories.

Transformation procedure: Competent recipient cultures were prepared by allowing growth to a cell density of 5×10^7 cells per ml in transformation medium containing an additional 0.6% albumin. Cultures were used immediately or after storage at -20°C in 10% glycerol. Cells were exposed to DNA (1 to 5 µg/ml) for 60 min at 30°C following 1:50 dilution in fresh transformation medium. Deoxyribonuclease (2.5 µg/ml) was then added, the mixture divided into two equal aliquots, placed in separate tubes and then reincubated at 37°C for phenotypic expression. One tube was removed after 25 min (amethopterin resistant transformants) and the second tube after 75 min (streptomycin resistant transformants). Cell suspensions were diluted in casein hydrolysate medium containing specific agglutinating antiserum and either amethopterin or streptomycin sulfate (Squibb), and transformants counted as colonies following overnight incubation at 37°C. The level of amethopterin resistance of mutants or transformants was determined by colony size comparison following dilution in antiserum medium containing varying concentrations of drug (SIROTNAK, LUNT and HUTCHISON 1960b and 1964).

Derivation of mutant strains: Resistant strains were initially derived from a rough variant of *D. pneumoniae* (wild type R6) by selection in casein hydrolysate medium containing amethopterin (SIROTNAK, LUNT and HUTCHISON 1964). However, most of these strains were resistant as a result of mutation at the *AME II* locus (SIROTNAK, LUNT and HUTCHISON 1964) and exhibited an impaired capacity to transport the drug. A more rapid isolation of mutants altered with respect to dihydrofolate reductase activity (mutation at the *AME I* locus) was obtained by a "second-step" selection following transfer of a suitable transport mutant in inhibitory concentrations of amethopterin. This was possible, since maximum resistance due to altered transport was reached when total uptake of drug was reduced by mutation to that level sustained by diffusion alone (SIROTNAK, SARGENT and HUTCHISON 1967a; 1967b). Further increases in resistance in such a strain must necessarily occur by mutation which affects some property other than transport. In actual practice, "second-step" mutants were derived both spontaneously and by induction with ultraviolet (UV) or ethylmethane sulfonate (EMS). Parallel cultures (10^8 cells per ml) of the R6 *ame^r-2* strain (100-fold resistant to amethopterin) were prepared with an inoculum of less than 100 cells in medium without drug. Cells were irradiated with UV to a survival of 10^{-3} or allowed to divide 4 to 6 times after dilution in medium with 0.5% EMS. An aliquot of each culture, after exposure to mutagen, was diluted in fresh medium and incubated until the cell concentration was again 10^8 cells per ml. Using a single portion from each treated or untreated culture, pour plates were prepared in enzymatic casein hydrolysate medium (SIROTNAK, LUNT and HUTCHISON 1960a) containing 1.5% agar (Difco) and at least 10 µg/ml amethopterin. Plates were incubated at 37°C under 10% CO₂ until colonies were visible. The number of mutant colonies derived from EMS treated cultures was usually greater than 100-fold that found in untreated cultures. Under these same conditions UV was a weak mutagen: induced mutants were

only 10- to 20-fold greater in number than the spontaneous mutants. Only one mutant colony was selected from each plate for study.

Segregation of newly derived mutations from the transport mutation (*ame^r-2*) was easily achieved by isolation of transformant colonies bearing one mutation following transformation of the wild type using DNA prepared from "second-step" mutant clones carrying *ame^r-2* and at least one more mutation. Transformants bearing individual mutations are identified by scoring at varying drug concentrations. The transfer of newly derived mutations in this way to the same wild-type recipient strain also insures that each exists in the same genetic background. The presence of more than one mutation in each transformant, although an unlikely possibility, can be determined during successive rounds of transformation using DNA derived from each transformant. A total of 47 mutations were obtained for study by these procedures.

Integration efficiency of individual mutations: The procedure for determining the efficiency with which amethopterin resistance (*ame^r*) mutations are transformed has been described (SIROTNAK, LUNT and HUTCHISON 1964). A relative evaluation, similar to that originally described by LACKS and HOTCHKISS (1960) was made by comparing the frequency of each *ame^r* mutation transformation to the frequency of transformation of a standard reference mutation (an *str^r* mutation determining a high level of resistance to streptomycin which was obtained from Dr. R. D. HOTCHKISS) carried on each *ame^r* donor strain. In this cross, *ame^r* transformants are scored in 0.1 to 0.2 $\mu\text{g/ml}$ and *str^r* transformants at 150 $\mu\text{g/ml}$ of the appropriate drug.

Recombination analyses: Strains bearing individual *ame^r* mutations or recombinant strains bearing more than one mutation are readily distinguishable because of resistance characteristics unique to each (SIROTNAK, LUNT and HUTCHISON, 1960b; 1964; see also companion paper by SIROTNAK, HACHTEL and WILLIAMS 1969). The detection of the various resistance genotypes during recombination experiments, then, was achieved by scoring for transformants over an appropriate concentration range.

The relative chromosomal position of two *ame^r* mutations was determined in at least one of three ways: (A) In a cross between the two mutant strains, the recipient bearing one mutation and the DNA donor bearing a second, the frequency of recombination is determined by comparing the number of recombinant transformants (bearing two mutations) to the total number of transformants which bear the donor mutation. When the resistance level determined by the mutation in the donor is greater than that determined by the mutation in the recipient, a direct comparison of recombinant and single (donor mutation) transformant classes is possible within a single cross. The calculation for recombination frequency (percent), where A^rB^r are the resistant alleles and A^sB^s are the sensitive alleles, is as follows (the symbol $\times$ indicates the direction of the cross, recipient $\times$ donor):

$$A^rB^s \times A^sB^r = A^rB^r / (A^rB^r + A^sB^r) \times 100 = \text{percent}$$

(B) When the resistance level determined by the donor mutation is equal to or less than the resistance level of the recipient strain, only the recombinant transformants can be detected in the mutant cross. A determination for donor mutation transformation must be done by a separate wild-type cross using the same DNA. Frequencies of both recombinant and donor mutation transformants in each cross are related and normalized with respect to the *str^r* reference mutation transformation (C^r = resistant allele, C^s = sensitive allele) in the following way:

$$\frac{A^rB^sC^s \times A^sB^rC^r}{A^sB^sC^s \times A^sB^rC^r} = \frac{A^rB^r/C^r}{A^sB^r/C^r} \times 100 = \text{percent (Recombination Index)}$$

This procedure and the numerical expression, Recombination Index (R.I.), was originally devised by LACKS and HOTCHKISS (1960) and has been used in recombination analyses of at least three genetic systems (EPHRATI-ELIZUR, SRINIVASAN and ZAMENHOF 1961; SIROTNAK, LUNT and HUTCHISON 1964; and LACKS 1966). Values derived in this two-point mutant cross by either procedure A or B are considered a measure of relative distance between two mutations. (C) In a cross between the wild-type strain and a donor of DNA bearing two mutations, the frequency of cotransfer is determined by comparing the number of recombinant transformants to the total

transformants, which also includes single transformants bearing each of the two mutations. The calculation for the frequency of double transformants (Cotransfer Index = C.I.) is as follows:

$$A^sB^s \times A^rB^r = A^rB^r / (A^rB^r + A^rB^s + A^sB^r) \times 100 = \text{percent}$$

The relative map distance by this procedure is defined as 1-Cotransfer Index. Quantitative mapping by this procedure has been carried out by NESTER, SCHAFER and LEDERBERG 1963.

Values for percent recombination derived by either method A or B appear to be equivalent. This was shown in two ways. In many crosses, involving a suitable mutant donor and recipient, the recombination value was obtained by both methods and always found to be the same. Secondly, the relationship between recombination data and cotransfer data is the same regardless of the method of recombination used.

RESULTS

Integration efficiencies of ame^r mutations: The efficiency of genomic integration during transformation is considered to be a characteristic property of individual mutations and can be used to distinguish among them (LACKS and HOTCHKISS 1960; IYER and RAVIN 1962; SIROTNAK, LUNT and HUTCHISON 1964; EPHRUSSI-TAYLOR, SICARD and KAMEN 1965 and LACKS 1966). Integration efficiency, as used here, is calculated from the ratio of ame^r mutation transformation to the str^r reference mutation transformation. The relative integration efficiencies for 47 ame^r mutations are given in Table 1 and Figure 1. Mean values varied

TABLE 1
Properties of various ame^r mutations at the AME I locus

Mutation (ame^r)	Derivation	Integration efficiency*		Resistance level†
		Mean‡	Standard Deviation	
44	EMS	0.037	± 0.006	6
47	EMS	0.048	± 0.010	6
46	EMS	0.051	± 0.014	6
45	EMS	0.054	± 0.008	6
48	EMS	0.058	± 0.012	6
49	EMS	0.058	± 0.015	6
39	EMS	0.066	± 0.018	6
37	EMS	0.069	± 0.005	6
31	EMS	0.074	± 0.025	6
40	EMS	0.071	± 0.024	6
34	EMS	0.076	± 0.004	6
33	EMS	0.076	± 0.021	6
36	EMS	0.079	± 0.015	6
11	UV	0.079	± 0.021	4
15	UV	0.079	± 0.020	4
38	EMS	0.080	± 0.011	6
41	EMS	0.101	± 0.022	6
12	EMS	0.102	± 0.028	4
5	Spontaneous	0.105	± 0.012	40
16	UV	0.112	± 0.014	2
58	UV	0.113	± 0.020	3
20	UV	0.295	± 0.061	3
17	UV	0.304	± 0.060	2.5

Mutation (<i>ame^r</i>)	Derivation	Integration efficiency*		Resistance level†
		Mean‡	Standard Deviation	
54	UV	0.310	± 0.072	3
13	UV	0.320	± 0.084	3
29	UV	0.500	± 0.114	8
56	UV	0.507	± 0.081	6
32	EMS	0.510	± 0.100	6
22	UV	0.520	± 0.072	8
23	UV	0.528	± 0.183	8
19	Spontaneous	0.530	± 0.110	8
55	UV	0.530	± 0.100	6
35	EMS	0.550	± 0.080	6
21	UV	0.550	± 0.160	8
27	UV	0.560	± 0.127	8
28	UV	0.572	± 0.120	8
26	UV	0.595	± 0.085	8
52	UV	0.730	± 0.090	6
51	UV	0.740	± 0.080	10
43	EMS	0.750	± 0.105	10
14	UV	0.770	± 0.083	10
6	Spontaneous	0.780	± 0.155	8
8	Spontaneous	0.800	± 0.170	8
57	UV	0.800	± 0.108	6
53	UV	0.810	± 0.124	6
3	Spontaneous	0.840	± 0.115	100
18	UV	1.090	± 0.181	4

* Ratio of *ame^r* transformants to *str^r* transformants.

‡ Based on values from 8 to 16 replicate determinations. Standard deviation, as used here, represents the average difference between individual replicate values and the mean.

† Ratio of mutant to wild-type resistance level. The wild type is inhibited by amethopterin concentrations above 0.02 µg/ml.

from 0.037 to 1.09. The distribution of individual integration efficiencies appears to be nonrandom with at least four classes discernible: low (0.037–0.112), moderately low (0.295–0.32), intermediate (0.50–0.595), high (0.77–0.84) and one mutation with an integration efficiency of 1.09. Both mutagens employed here induced mutations in almost every class. However, in view of the fact that the mutant frequency in UV treated cultures was not excessively higher than that of untreated cultures, some of these mutations could have originated spontaneously. Even so, it would appear that most EMS induced mutations have low integration frequencies and most UV induced mutations are in the intermediate range. Reproducibility of values for integration efficiency for the entire group of mutations was generally good. Deviations from mean values, with a few exceptions, were slightly below 20% for mutations in the intermediate (IE) and high efficiency (HE) ranges. Deviations among values in the lower efficiency ranges (LE and MLE) in most instances were somewhat higher than 20%.

*Resistance to amethopterin associated with individual *ame^r* mutations:* The *ame^r* mutations determine a variety of quantitatively distinct resistance levels

FIGURE 1.—Distribution of *ame*^r mutations with respect to integration efficiency. Each bar represents the number of mutations with integration efficiencies within an interval of 0.01. The inserted numbers are the total number of mutations in each group. On the assumption that at least 4 groups are distinct, the probability (P) of randomness as determined by *F* test from analysis of variance is less than 0.001. When the opposite assumption is made, the observed distribution was found by the same test to be sufficiently different from random so that it must be discarded ($P < 0.001$).

which are readily distinguishable, thus permitting a convenient phenotypic classification. The relative resistance level associated with each mutation is listed in Table 1. Increases in resistance vary over a wide range when compared to the wild-type level. Individual levels discernible are 2-, 2.5-, 3-, 4-, 6-, 8-, 10-, 40- and 100-fold. Biochemical data presented in the companion report (SIROTNAK, HACHTEL and WILLIAMS 1969) relate these increases, in all but one case (*ame*^r-5), to corresponding increases in the dihydrofolate reductase level. The *ame*^r-5 mutation, instead of affecting the level of this enzyme, determines a reduced affinity of the enzyme for amethopterin (SIROTNAK, DONATI and HUTCHISON 1964c).

The problem of recipient culture variability during recombinational analyses: The question of recipient culture variability is of some importance to the recombination studies, since the map order of individual mutations was determined from relative values in a large number of two-point crosses involving many different mutant recipients. Data bearing on this question are given in Table 2. The degree of variability among values for percentage recombination (Method A described in MATERIALS and METHODS) derived in a series of replicate determinations with a single recipient culture was compared to variability among values from a second series of determinations using several different recipient cultures prepared on different days. The crosses involve mutational sites that are closely linked (*ame*^r-17 $\times$ *ame*^r-8 and *ame*^r-20 $\times$ *ame*^r-8) and only moderately linked (*ame*^r-13 $\times$ *ame*^r-22). In each case, variability, as indicated by the Stand-

TABLE 2

Recipient culture variability during two-point crosses between ame^r mutant strains

Recipient	Cross Donor	Competent culture	Transformants ($n \times 10^3$)			Mean	Standard deviation†
			Total	Recombinants	Percent		
13	22	A	29.01	6.30	21.3	...	...
			31.70	7.04	22.0		
			31.30	6.06	19.4		
			43.49	8.23	18.9		
			39.50	7.45	18.8		
		B	19.62	4.23	21.6	20.3	± 1.3
			32.40	6.57	20.3		
			5.40	1.00	18.5		
			68.00	12.60	18.5		
			18.79	3.90	20.8		
			10.20	2.22	22.0		
			9.00	1.55	17.2		
			6.68	1.33	19.9		
			3.60	0.58	16.1		
		I				19.6	± 1.17
20	8	A	20.20	2.45	12.1		
			18.90	2.39	12.7		
			20.46	2.43	11.8		
			21.00	2.47	11.7		
			19.90	2.55	12.8		
		B	19.50	2.87	14.7	12.3	± 0.77
			18.48	2.33	12.3		
			22.70	3.26	14.3		
			121.34	14.54	12.0		
			34.60	3.66	10.6		
			95.00	9.90	10.4		
			31.70	3.40	10.7		
			23.00	3.06	13.3		
		H				11.9	± 1.00
17	8	A	38.90	1.26	3.24		
			38.95	1.52	3.90		
			37.10	1.29	3.48		
			45.42	1.13	2.49		
			21.70	1.09	5.03		
		B	31.90	1.29	4.05	3.67	± 0.46
			35.52	1.31	3.67		
			11.80	0.40	3.39		
			8.80	0.31	3.52		
			6.85	0.22	3.24		
			26.60	1.10	4.14		
			15.50	0.60	3.90		
		G				3.66	± 0.26

* Each letter designates a separate culture prepared on different days.

† Standard deviation is calculated as the average difference between the individual values and the mean.

ard Deviation, was about the same for each group. This is not in agreement with the experience of IYER and RAVIN (1962) or of SICARD and EPHRUSSI-TAYLOR (1965) who have reported considerably more variation in replicate crosses in-

volving more than one recipient. Differences between the results obtained could be related to the cultural conditions used in each case. The present authors have attempted to standardize as rigorously as possible the conditions for culture preparation. The occasional discrepancy which has been observed was usually traced to some nutritional variation in the medium. To further insure constancy of results and to detect atypical recipients, most cultures were pretested by a standard cross. This precaution had been suggested by SICARD and EPHRUSSI-TAYLOR (1965) as a means for reducing the amount of variability among different recipient cultures.

Results of two-point crosses: The relative linear order of individual mutational sites was sought from two-point crosses involving a different mutation in the recipient culture and the donor DNA. The frequency of recombinant transformants bearing the two mutations was related to the total number of transformants bearing the donor mutation, either directly (Method A described in the MATERIALS and METHODS) or by use of the Recombination Index (Method B described in the MATERIALS and METHODS). The relative frequency, in percent, which can be derived in each case, has been used as a measure of distance between two mutational sites (LACKS and HOTCHKISS 1960; EPHRATI-ELIZUR, SRINIVASAN and ZAMENHOF 1961; SIROTNAK, LUNT and HUTCHISON 1964; and LACKS 1966). The validity of this measurement has been discussed in detail by LACKS (1966).

More than 900 crosses involving 32 of the *ame*^r mutations have been completed. Whenever possible each mutation was used alternately in both the recipient and donor strains in reciprocal transformations. The results are shown in Figure 2. Approximately 100 crosses with mutations 3, 5 and 16 could not be done under conditions which would favor the selection of recombinants (see explanation in legend of Figure 2). Recombinants bearing two mutations, selected on the basis of a higher resistance level, were detected in about 25% of the crosses done. Strains bearing mutations 13, 16, 17 and 20 yielded phenotypically distinguishable recombinants in crosses involving strains bearing almost all of the *ame*^r mutations tested. Strains bearing any one of the other mutations, except 3 and 14, yielded distinguishable recombinants when crossed only with strains bearing 13, 16, 17 and 20. Strains bearing 3 and 14 yielded distinguishable recombinants only when crossed with strains bearing 5.

The numerical values given in Figure 2, which vary from less than 2% to 100%, are averages of four to six crosses. Standard deviations in a few cases,

FIGURE 2.—The results of reciprocal two-point crosses between *ame*^r mutant strains. Numerical values represent average percentage recombination obtained in 4 to 6 replicate crosses. The underlined values are averages for a group of replicate crosses carried out either exclusively or at least partially by method A (see METHODS section of the text). Values that are not underlined were obtained exclusively by method B. When resistance levels of recipient cultures are appreciably higher than donor strains, it is not always possible to detect recombinant transformants. In these cases no value is given and the cross is designated by a dashed line (—). Under optimum conditions for recombinant detection a negative value (0) may mean either the absence of recombination or the inability of recombinants to express higher resistance levels (see pertinent section of the text).

involving low efficiency integrating mutations, were near $\pm 30\%$. However, for most crosses the standard deviation varied from below $\pm 10\%$ (direct method) to about $\pm 20\%$ (Recombination Index procedure). Because of a relatively high mutant background in recipient cultures, the limit of recombination detectable varied from 0.1% for high efficiency donor mutations to 1.0% for low efficiency donor mutations. This is an absolute transformation frequency of 0.00002 to 0.0001 depending on the level of competence.

The inability to detect recombinants following crosses between many strains bearing mutations, which do not appear to be at overlapping or adjacent sites was puzzling (see Figure 3 and the appropriate section of the text). Does recombination actually occur in these cases? If it does, then the absence of recombinants could be related to a lack of any increase in resistance. A similar inability of joint expression of certain mutations at an erythromycin resistance locus has been described by RAVIN and IYER (1961). Information bearing on this question was obtained by an analysis of mutant crosses involving a donor strain which is more resistant than the recipient. In such a cross, only one transformant class, which is identical to the donor type, can be distinguished. These transformants occur at the same relative frequency (compared to *str^r* reference mutation transformation) obtained in the corresponding wild-type cross. Since some of these transformants, therefore, would be expected to possess both recipient and donor mutations, this would suggest that the recombinant form has the same phenotypic characteristics as the donor type. More conclusive evidence of recombination was obtained in the following way. A suitable recipient was crossed with DNA from a more resistant donor strain. A large number of donor type transformants are then selected, simultaneously, at drug concentrations which eliminate the recipient cells. When DNA was prepared from a mixture of these transformants and used in a cross with the wild type, most of the transformants found were "donor" types. A few transformants, however, were the "recipient" type. The identity of this transformant class could be further tested by crosses involving other donors. Under these conditions, the "recipient" mutation in this transformant class must have been derived from a recombinant cell. "Mutation recovery" experiments of this type were successfully carried out in the following crosses, *ame^r-13* $\times$ *ame^r-19*, *ame^r-13* $\times$ *ame^r-3*, *ame^r-18* $\times$ *ame^r-3*, *ame^r-18* $\times$ *ame^r-14*, *ame^r-18* $\times$ *ame^r-22*, *ame^r-18* $\times$ *ame^r-5* and *ame^r-32* $\times$ *ame^r-14*. It would seem, that at least in these cases and probably in others, both mutations can exist in the same genome simultaneously and that the inability to detect recombinants in these cases is not related to any genetic incompatibility. Rather, the results appear to suggest an inability for joint biochemical expression of the mutations in these recombinant genomes. Further discussion of this question will be presented in the companion report (SIROTNAK, HACHTEL and WILLIAMS 1969).

"Asymmetry" between recombination values obtained in reciprocal two-point crosses: The results in Figure 2 reveal considerable discrepancy between values for percentage recombination derived in many reciprocal crosses. Similar discrepancies ("asymmetry") have been reported by SICARD and EPHRUSSI-TAYLOR (1965). These workers found values for recombination frequency were lower

when the cross was made in the direction of an LE recipient. In comparison, the data in Figure 2 derived with the *ame^r* mutations are characterized by a general lack of correlation in respect to the efficiency classes of the mutations involved in "asymmetric" crosses. For instance, lower recombination values were obtained in the direction of an LE recipient in reciprocal crosses involving LE and either MLE or HE mutations. In other reciprocal crosses, involving MLE and IE mutations or MLE and HE mutations, lower recombination values were obtained in the direction of the IE and HE recipients. Moreover, discrepancies in recombination values were also seen in reciprocal crosses involving *ame^r* mutations that were both LE transforming.

There appears to be some correlation, however, between the degree of "asymmetry" obtained in reciprocal crosses and the linkage relationship of the two *ame^r* mutations involved. This can be seen in Table 3 where numerical relationships between a series of reciprocal crosses involving mutations in different efficiency classes are summarized. Each value is the average ratio between recombination frequencies derived in reciprocal crosses involving the three MLE mutations (*ame^r-13*, *ame^r-17* and *ame^r-20*) and most of the mutations in the HE, IE and LE classes. The ratios obtained with representative mutations in each class are given in the legend of Table 3. Close linkage of all mutations in these three classes to *ame^r-17* and *ame^r-20*, and moderate linkage to *ame^r-13*, can be inferred from the data in Figure 2 regardless of the direction of each cross. The map assignments for the HE, IE and LE mutations (Figure 3), based on the results

TABLE 3

*Average numerical ratios between recombination frequencies derived in reciprocal crosses involving mutations in various efficiency classes**

Reciprocal cross†	Site of MLE mutation		
	<i>ame^r-17</i>	<i>ame^r-20</i>	<i>ame^r-13</i>
MLE $\times$ HE =	1.05	0.88	1.52
HE $\times$ MLE			
MLE $\times$ IE =	1.00	1.00	2.00
IE $\times$ MLE			
MLE $\times$ LE =	1.80	2.00	4.00
LE $\times$ MLE			

* HE, high efficiency; IE, intermediate efficiency; MLE, moderately low efficiency; LE, low efficiency.

† Some individual values for this ratio are as follows: Those derived with the HE mutations, *ame^r-6* were 0.8 ($\times$ *ame^r-17*), 1.1 ($\times$ *ame^r-20*) and 2.6 ($\times$ *ame^r-13*) and *ame^r-8* were 1.2 ($\times$ *ame^r-17*), 1.0 ($\times$ *ame^r-20*) and 1.7 ($\times$ *ame^r-13*). Those derived with the IE mutations, *ame^r-23* were 1.0 ($\times$ *ame^r-17*), 1.2 ($\times$ *ame^r-20*) and 1.9 ($\times$ *ame^r-13*) and *ame^r-35* were 1.0 ($\times$ *ame^r-17*), 1.1 ($\times$ *ame^r-20*) and 2.1 ($\times$ *ame^r-13*). Those derived with the LE mutations, *ame^r-12* were 2.0 ($\times$ *ame^r-17*), 2.5 ($\times$ *ame^r-20*) and 4.7 ($\times$ *ame^r-13*) and *ame^r-31* were 1.8 ($\times$ *ame^r-17*), 1.7 ($\times$ *ame^r-20*) and 4.9 ($\times$ *ame^r-13*).

of both reciprocal recombination and cotransfer experiments, is in a relatively small segment of the chromosome to the side of *ame^r-17* and *ame^r-20* opposite from *ame^r-13*. Justification for individual map assignments will be presented in the next two sections. It need only be assumed now that sites of the HE, IE and LE mutations are much closer to *ame^r-17* and *ame^r-20* than to *ame^r-13*. Values for recombination frequency (Figure 2) derived in reciprocal crosses between *ame^r-13* and *ame^r-20* agree quite well. Values for reciprocal crosses between *ame^r-13* and *ame^r-17* are somewhat discordant. No "asymmetry" is apparent (ratio in Table 3 is nearly 1.0) in crosses involving either HE or IE mutations, and both of the MLE mutations, *ame^r-17* or *ame^r-20*. A one and one half- to two-fold difference, on the other hand, is evident in reciprocal crosses involving all but one mutation (*ame^r-22*) in both these efficiency classes and *ame^r-13*, which is at a more distal site. Crosses involving LE mutations and all three of the MLE mutations were "asymmetric." However, the degree of "asymmetry" varied from nearly 2.0- to 4.0-fold depending upon the proximity of the LE mutation to the MLE site. It is noted that a lower recombination value was always obtained when the cross was made with the MLE mutation on the donor, regardless of the efficiency class of the mutation in the recipient.

Mapping of the ame^r mutations by two point crosses: Ordering of most of the 32 *ame^r* mutations at individual chromosomal sites is possible from the results of the various crosses involving mutations 13, 16, 17 and 20 and to a limited extent with *ame^r-5*. The "asymmetry" of many reciprocal crosses presented considerable difficulty in regard to the initial choice of individual recombination values. However, a map order based on the average of both values obtained in each reciprocal cross, even though differences were as great as 4-fold, made by far the most sense. Subsequent analysis for additivity of individual map intervals and ultimate confirmation of most position assignments by cotransfer experiments, which are not subject to "asymmetric" effects, suggest that this was probably the correct choice.

The relative map position of the 32 *ame^r* mutations is given in Figure 3. Assignment in numerical recombination units was made primarily on the basis of crosses

FIGURE 3.—Map of the *AME I* locus based on the average of reciprocal recombination values. Each position is based on the average recombination in percent obtained in reciprocal crosses.

with *ame^r-13* and *ame^r-20*. Only an approximate localization of individual mutational sites could be made on the basis of crosses involving *ame^r-16*. The sites of *ame^r-3*, *ame^r-5* and *ame^r-14* could not be established from the recombination data, since an adequate number of values from reciprocal crosses could not be obtained. However, the position of *ame^r-5*, to the right of *ame^r-20*, was determined by cotransfer data (see Table 4 and Figure 3). Although *ame^r-3* and *ame^r-14* are shown as existing to the left of *ame^r-5* in Figure 3, both mutations could very well be the same distance to the right. The position of *ame^r-16* to the left of *ame^r-13* was also established from cotransfer data, since a usable numerical value was only obtained in crosses using *ame^r-16* as a donor.

Recombination values derived from crosses with *ame^r-17* exhibited poor additivity with respect to crosses with *ame^r-13* and were not used for position assignments. However, the relative order, established on the basis of these crosses, agreed quite well with the final map order. The site of *ame^r-17* was assigned on the basis of both reciprocal recombination values and cotransfer data (Table 4). The lack of additivity in crosses involving *ame^r-17* suggests the possibility that this mutation is multisite in nature. The total dimension of the map is approximately 70 recombination units (1 unit = 1% recombination). All but two mutations, *ame^r-13* and *ame^r-16*, are at sites within a segment of about 15 units in length. Distribution of the other 30 mutations within this segment appears to be random. Additivity, between map positions within the segment assigned on the basis of the *ame^r-13* cross, and the sum of the *ame^r-20* cross and the *ame^r-3* ×

TABLE 4

Cotransfer of two ame^r mutations in wild-type crosses using various recombinant strains as the donor

Donor Recombinant	Cotransfer index* Percent	Donor Recombinant	Cotransfer index* Percent
<i>ame^r-3-5</i>	90.0	<i>ame^r-20-39</i>	85.0
<i>ame^r-8-13</i>	31.9	<i>ame^r-11-13</i>	33.0
<i>ame^r-12-13</i>	31.0	<i>ame^r-12-20</i>	78.5
<i>ame^r-13-18</i>	27.8	<i>ame^r-13-15</i>	25.0
<i>ame^r-13-20</i>	55.8	<i>ame^r-13-16</i>	1.8
<i>ame^r-13-22</i>	50.3	<i>ame^r-16-18</i>	0
<i>ame^r-13-26</i>	25.1	<i>ame^r-16-19</i>	0
<i>ame^r-13-19</i>	37.2	<i>ame^r-5-13</i>	38.0
<i>ame^r-17-26</i>	80.0	<i>ame^r-5-14</i>	89.0
<i>ame^r-8-20</i>	72.5	<i>ame^r-13-34</i>	44.0
<i>ame^r-18-20</i>	76.0	<i>ame^r-13-37</i>	43.0
<i>ame^r-20-26</i>	67.0	<i>ame^r-20-27</i>	67.0
<i>ame^r-19-20</i>	82.0	<i>ame^r-13-32</i>	71.0
<i>ame^r-11-20</i>	79.0	<i>ame^r-20-35</i>	73.0
<i>ame^r-13-39</i>	29.4	<i>ame^r-16-26</i>	0
<i>ame^r-16-32</i>	0	<i>ame^r-13-31</i>	41.1
<i>ame^r-13-17</i>	55.0		

* Calculated from a comparison of double to single transformants as indicated in the MATERIALS AND METHODS.

ame^r-20 cross was exceptionally good. Differences averaged ± 2.9 units for all of the crosses involving LE mutations, which were highly "asymmetric," and ± 1.4 units for all other crosses.

*Concordance between the cotransfer and map positions of various *ame^r* mutations:* The tendency for joint transfer of two mutations during transformation is related to the linkage between them and has been used as a measure of distance between the corresponding sites on the DNA (NESTER, SCHAFER and LEDERBERG 1963). Mapping of mutational sites by this method has an advantage over the two point recombinational procedure in that the problem of "asymmetry" observed during reciprocal crosses is avoided. Quantitation requires a comparison of joint transformation of two mutations to individual mutation transformation (see method C in MATERIALS and METHODS). Differences in integration efficiency between any two mutations do not seriously influence the results when closely linked sites are involved. It has been found (LACKS 1966; GRAY and EPHRUSSI-TAYLOR 1967) that during this type of cross, both mutations, either individually or jointly, are integrated at approximately the same frequency as the low efficiency mutation. Similar observations have been made during the course of the present studies (SIROTNAK and HACHTEL, unpublished). The depressive effect on the integration frequency of the high efficiency mutation is somewhat diminished with loosely linked mutations (LACKS 1966), so that cotransfer indices in the case of mutations with widely different integration efficiencies could be appreciably distorted. However, in the present experiments, relative map intervals were estimated by cotransfer of mutations with no more than 4-fold difference in integration efficiency and which were at least moderately linked.

DNAs from a total of 33 recombinant strains were tested in a cross with the wild-type strain to determine the frequency of cotransfer of the two mutations.

FIGURE 4.—The cotransfer of paired *ame^r* mutations as compared to the corresponding map position in recombination units.

The recombinants selected bear mutations which were found in recombination experiments to exhibit varying degrees of linkage. Certain recombinants were also selected because the corresponding reciprocal crosses involving the two mutations were "asymmetric." Cotransfer indices shown in Table 4 vary from about 20% (loosely linked mutations) to 90% (closely linked mutations). The cotransfer data (expressed as 100-cotransfer index) are related to the corresponding map interval derived from reciprocal recombination experiments in Figure 4. Good agreement, as evinced from the linear relationship over a 10-fold range in recombination units, was obtained between both sets of data. These results would seem to confirm the relative position assignments (Figure 3) for the nearly 2 dozen mutations involved. Confirmation for the relative map positions of the remaining mutations is inferred, since the concordance observed also serves to establish the validity of the calculations employed in determining map order.

DISCUSSION

The separation of the *ame*^r mutations into a number of distinct classes on the basis of the efficiency of integration during transformation confirms the earlier observation in *D. pneumoniae* of LACKS (1966) for a large number of mutations in the amylomaltase locus and of CHEN and RAVIN (1966) for mutations at a streptomycin resistance locus. Findings in all three cases differ with the results of similar studies with a group of aminopterin resistance mutations by EPHRUSSI-TAYLOR, SICARD and KAMEN (1965). These workers reported only two broadly distributed, but non-overlapping efficiency classes. At least 4 classes are discernible among the *ame*^r mutations. The *ame*^r-18 mutation, as well, may belong to a separate efficiency class. It would be difficult to speculate at this time as to why the data of EPHRUSSI-TAYLOR, SICARD and KAMEN (1965) differ from other findings, since additional experimentation will be necessary. However, differences could be related to locus dependent effects or to the methods of selection used in each case.

A more direct comparison of the data obtained with the *ame*^r mutations and those of LACKS (1966) was recently made possible by relating the corresponding reference mutation transformations. It was found (LACKS, personal communication) that the ratio of *str*^r reference mutation transformation to *sulf*-d reference mutation transformation used by LACKS (1966) was 0.61. Using this factor to adjust the data given here, it appears that three efficiency classes are the same in both cases. These are equivalent to the LE, MLE and HE classes described here. One additional class was also observed in each case. They are the IE class observed here with the *ame*^r mutations and a very high efficiency class observed by LACKS (1966). This brings the total number of efficiency classes observed to five.

Both EPHRUSSI-TAYLOR, SICARD and KAMEN (1965) and LACKS (1966) have suggested a possible association between integration efficiency and base pair differences between donor and recipient DNA. LACKS (1966) has attempted to correlate specific base pair changes with a particular efficiency class by employing

a variety of agents to induce the mutations. Since a total of 6 base pair changes are possible, the findings of LACKS (1966) and those presented here, which suggest the existence of at least 5 efficiency classes, are of interest. All that can be said of the *ame^r* mutations in the former regard is that low efficiency mutations appear to be more common when EMS is used. The same observation was made by EPHRUSSI-TAYLOR, SICARD and KAMEN (1965). With UV, on the other hand, very few low efficiency mutations were produced. Of additional interest is the finding that *ame^r* mutation could not be induced following treatment of DNA with HNO₂ (SIROTNAK and HACHTEL, unpublished). The general significance of these results is unclear, since they involve only two mutagens and may also reflect to a great extent the basis of selection employed. The possibility that some of the *ame^r* mutations are multisite must also be considered, since LACKS (1966) has shown that linear dimension is also of some importance in determining integration efficiency. However, this seems unlikely for two reasons. First, a far more random or continuous distribution of integration efficiency would be expected (LACKS 1966). Secondly, since almost all of the mutations are in the structural gene for dihydrofolate reductase (SIROTNAK, HACHTEL and WILLIAMS 1969), any alteration covering an extended nucleotide sequence would probably have inactivated the enzyme and would be lethal under the conditions of selection. It is also interesting to note, that the distribution of the 32 *ame^r* mutations already mapped exhibits no correlation with respect to efficiency class. A similar lack of any correlation with specific sites on the DNA was reported by EPHRUSSI-TAYLOR, SICARD and KAMEN (1965) and LACKS (1966).

A genetic study of the *ame^r* mutations in *D. pneumoniae* was initiated primarily to establish the map order of the corresponding chromosomal sites. This information was deemed necessary for meaningful interpretation of the biochemical effects associated with these mutations (see companion report by SIROTNAK, HACHTEL and WILLIAMS 1969). Both recipient culture variability and a lack of numerical agreement between the results of reciprocal two point crosses have been discussed (SICARD and EPHRUSSI-TAYLOR 1965; LACKS 1966) as possible factors in arriving at a correct map order by recombinational analysis based on a DNA-mediated transformation. The results presented here, which are in some disagreement with the experience of SICARD and EPHRUSSI-TAYLOR (1965), would appear to suggest that recipient culture variability need not be of major concern when competent cultures are prepared under well standardized conditions. Furthermore, utilization of a standard cross to test recipient cultures seems to be a practical solution for insuring reproducibility.

The question of the "asymmetry" observed during reciprocal crosses between strains bearing different *ame^r* mutations is of critical importance in respect to the mapping of mutational sites by this type of recombinational analysis. In a study of mutation in the amylomaltase gene, LACKS (1966) did not observe this type of discrepancy except in crosses involving mutational sites which were less than two recombination units (2%) apart. This is in sharp contrast, not only to findings discussed here, but to others reported earlier by SICARD and EPHRUSSI-TAYLOR (1965).

It is difficult to compare the data presented here to those of SICARD and

EPHRUSSI-TAYLOR (1965), since the number of efficiency classes observed differs in each case. Moreover, crosses involving many *ame^r* mutations do not yield phenotypically distinguishable recombinant types. Even so, the pattern of "asymmetry" observed in the present case is clearly more complex. Unlike the results of SICARD and EPHRUSSI-TAYLOR (1965), there appears to be no simple correlation of "asymmetry" with the efficiency class of either the donor or recipient mutation. However, discrepancies between reciprocal crosses are usually larger when an LE mutation is involved. In addition, discrepancies are greater in crosses involving *ame^r* mutations at distally located sites. Since these comparisons could only be made on the basis of crosses with *ame^r-13*, *ame^r-17* and *ame^r-20*, the results might only be peculiar to crosses involving these three sites and not necessarily a general reflection of the linkage relationships which exist.

The "asymmetry" described for reciprocal crosses between mutations in the *ami A* locus (SICARD and EPHRUSSI-TAYLOR 1965) appears to result from a distortion of the recombination frequency in one direction, i.e. the frequency is lower when the LE mutation is used as a recipient. In contrast, the "asymmetry" between reciprocal crosses observed in the present case results from a distortion in both directions of the cross. When recombination is depressed in one direction, it is equally enhanced in the other direction. This conclusion is derived from the fact that the order of mutational sites based on the average recombination frequency of reciprocal "asymmetric" crosses is in very good agreement with the order based on cotransfer data. As a specific example, consider crosses involving the LE mutation, *ame^r-39* and the HE mutation, *ame^r-18*. From cotransfer data (Table 4), which represent a measure of map distance independent from that derived by reciprocal recombination experiments, it would appear that both mutations are about the same distance from the MLE mutation, *ame^r-13*. With *ame^r-13* as the recipient, crosses with *ame^r-18* and *ame^r-38* yield, respectively, 34.2 and 49.7% recombination. With *ame^r-13* as the donor, the corresponding reciprocal crosses occur with 24.2 and 9.3% recombination, respectively. The average for both reciprocal crosses is just slightly above 29%. Other examples of this type of relationship are numerous.

Further support for this conclusion is derived from the fact that overall additivity of individual map intervals was only observed when map assignments were made on the basis of the average of the reciprocal recombination values. Many of these values are also derived from reciprocal crosses that were not "asymmetric." Additivity only among average values is understandable in view of findings which appear to suggest that the degree of discrepancy for reciprocal crosses is unique to each pair of mutations involved, being related to both integration efficiency of each and their proximity to each other.

It was the authors' intention to examine the discrepancies between recombination frequencies obtained in reciprocal crosses only insofar as was necessary to be able to arrive at correct relative values. Therefore, no satisfactory explanation of the "asymmetry" observed can be devised at this time. These effects might be related to actual nucleotide differences determined by each mutant or possibly to the position of each site within a particular nucleotide sequence (RAVIN and IYER 1962) or in respect to the end of the DNA molecule. In either case, some influence

on the "pairing" process during synapsis might result (RAVIN and CHEN 1967). Interpretation of these results in respect to models for recombination proposed by LACKS (1966) or GRAY and EPHRUSSI-TAYLOR (1967) is difficult without further experimentation in view of the differences seen among the three sets of data.

A total of 32 *ame^r* mutations have been mapped so far at the *AME I* locus in *D. pneumoniae*. Although recombinational analysis was hampered by a scarcity of crosses which gave distinguishable recombinants, good additivity of the distances so determined suggests some degree of validity for the general linear order shown in Figure 3. Additional support for the individual map assignments was obtained by cotransfer experiments which confirmed the relative order of sites for most of the mutations. A more precise ordering of mutations which are at neighboring sites (< 1 map unit apart) will require additional crosses involving closely linked mutations. Nevertheless, in agreement with the experience of LACKS (1966), it would seem that percentage recombination as determined here is a reliable measure of relative distance. The varying pattern of "asymmetry" observed among the recombination data points to the necessity of carrying out crosses in both directions, since it is the only way of determining if such an effect occurs in the first place.

In addition to determining increased rates of dihydrofolate reductase synthesis, fourteen of the *ame^r* mutations (3, 6, 8, 12, 14, 17, 18, 20, 21, 22, 23, 26, 27, 29) were found to have an effect on the properties of the enzyme itself (SIROTNAK, HACHTEL and WILLIAMS 1969). These mutations, along with *ame^r-5*, shown earlier to alter the properties of this enzyme (SIROTNAK, DONATI and HUTCHISON 1964c), are randomly distributed within the dense cluster of sites at the right side of the genetic map (Figure 3). This region, encompassing less than one fourth (15 recombination units) of the total map, would seem to represent at least a sizable portion of the gene for dihydrofolate reductase. The molecular weight of the enzyme in *D. pneumoniae* has already been found to be in the neighborhood of 20,000 (SIROTNAK and HUTCHISON 1965; 1966). Since most proteins of this size do not consist of subunits and if we only assume that the corresponding segment of the genetic map delineates the majority of the gene, then on the basis of the triplet code this region involves a segment of the DNA of somewhat less than 450 nucleotides in length. This estimate agrees very well with a value of 900 nucleotides suggested by LACKS (1966) for the absolute dimensions of the amylomaltase gene with a map length of 40 units, based on a molecular weight of 45,000 for two subunits of amylomaltase.

The encouragement and helpful advice of DR. DORRIS J. HUTCHISON during the course of these studies and in the preparation of the manuscript is gratefully acknowledged. The statistical evaluation of the data on integration efficiency was prepared by DR. ISABEL M. MOUNTAIN. This investigation was supported in part by USPHS Grant CA-08748 from the National Cancer Institute, and by Grant T-107 from the American Cancer Society.

SUMMARY

Forty seven *ame^r* mutations in *D. pneumoniae* which affect the properties and/or rate of synthesis of dihydrofolate reductase, have been examined with

respect to mutagenic origin, integration during transformation and amethopterin resistance level associated with each. Thirty mutations, of a total of 32 which were mapped by reciprocal two-point crosses at the *AME I* locus, are in the structural gene for dihydrofolate reductase. Map order was confirmed by the extent of two-point cotransfer in crosses between wild-type and recombinant DNA donor strains.—Efficiencies of integration of individual *ame*^r mutations were distributed in at least 4 nonoverlapping classes with relative values of 0.1:0.4:0.7:1.0. Some correlation could be made between individual efficiency classes and the mutagen employed. The data on integration efficiency agree closely with those reported by LACKS (1966) for mutations in the amylomaltase locus. Three efficiency classes were identical and one additional (non-identical) class was peculiar to each system: the total number of efficiency classes delineated between the two systems was 5.—Both recipient culture variability and a lack of numerical agreement between the results of reciprocal two-point crosses were considered experimentally as possible factors in arriving at a correct map order by recombinational analysis based on a DNA-mediated transformation. Recipient culture variability was found not to be of major concern when competent cultures were prepared under well standardized conditions. Discrepancies observed in reciprocal crosses, on the other hand, were of critical importance. The degree of such “asymmetry” observed between reciprocal crosses varied to some extent with the efficiency class and the linkage relationships of the mutations involved, but no strict correlation was discernable. Moreover, not all reciprocal crosses were “asymmetric.” Distortions in values for recombination frequency in “asymmetric” crosses were found to occur in both directions of the cross: when recombination was depressed in one direction, it was equally enhanced in the other direction.—Validity for the general linear order of the mutations at the *AME I* locus was suggested by the additivity of individual map intervals and confirmed by cotransfer experiments. Percentage recombination as determined in this study appeared to be a reliable measure of relative distance on the DNA molecule, at least for segments of the map which did not appreciably exceed 30% recombination.

LITERATURE CITED

- ADAMS, M. H., and A. S. ROE, 1945 A partially defined medium for cultivation of pneumococcus. *J. Bacteriol.* **49**: 401–409.
- CHEN, K., and A. W. RAVIN, 1966 Heterospecific transformation of pneumococcus and streptococcus. II. Dependence of relative efficiency of marker integration on host genome. *J. Mol. Biol.* **22**: 123–134.
- ENGLESBERG, E., 1961 Enzymatic characterization of 17 L-arabinose negative mutants of *Escherichia coli*. *J. Bacteriol.* **81**: 996–1006.
- EPHRATI-ELIZUR, E., P. R. SRINIVASAN, and S. ZAMENHOF, 1961 Genetic analyses, by means of transformation, of histidine linkage groups in *Bacillus subtilis*. *Proc. Natl. Acad. Sci. U.S.* **47**: 56–63.
- EPHRUSSI-TAYLOR, H., A. M. SICARD, and R. KAMEN, 1965 Genetic recombination in DNA-induced transformation of pneumococcus I. The problem of relative efficiency of transforming factors. *Genetics* **51**: 455–475.
- GRAY, T. C., and H. EPHRUSSI-TAYLOR, 1967 Genetic recombination in DNA-induced transfor-

- mation of pneumococcus V. The absence of interference and evidence for the selective elimination of certain donor sites from the final recombinants. *Genetics* **57**: 125-153.
- IYER, V. N., and A. W. RAVIN, 1962 Integration and expression of different lengths of DNA during the transformation of pneumococcus to erythromycin resistance. *Genetics* **47**: 1355-1368.
- LACKS, S., 1966 Integration efficiency and genetic recombination in pneumococcal transformation. *Genetics* **53**: 207-235.
- LACKS, S., and R. D. HOTCHKISS, 1960 A study of the genetic material determining an enzyme activity in pneumococcus. *Biochim. Biophys. Acta* **39**: 508-517.
- LEE, N., and E. ENGBERG, 1963 Coordinate variations in induced syntheses of enzymes associated with mutations in a structural gene. *Proc. Natl. Acad. Sci. U.S.A.* **50**: 696-702.
- MARTIN, R. G., D. F. SILBERT, D. W. E. SMITH, and H. J. WHITFIELD, JR., 1966 Polarity in the histidine operon. *J. Mol. Biol.* **21**: 357-369.
- MAYERS, V. L., and J. SPIZIZEN, 1954 The isolation of deoxyribonucleic acid from bacteriophages by an improved method. *J. Biol. Chem.* **210**: 877-884.
- NESTER, E. W., M. SCHAFER, and J. LEDERBERG, 1963 Gene linkage in DNA transfer: a cluster of genes concerned with aromatic biosynthesis in *Bacillus subtilis*. *Genetics* **48**: 529-551.
- NEWTON, W. A., J. R. BECKWITH, D. ZIPSER, and S. BRENNER, 1965 Nonsense mutants and polarity in the *lac* operon of *E. coli*. *J. Mol. Biol.* **14**: 290-295.
- RAVIN, A. W., and V. N. IYER, 1961 The genetic relationships and phenotypic expression of mutations endowing pneumococcus with resistance to erythromycin. *J. Gen. Microbiol.* **26**: 277-301. — 1962 Genetic mapping of DNA: influence of the mutation configuration on the frequency of recombination along the length of the molecule. *Genetics* **47**: 1369-1384.
- RAVIN, A. W., and K. CHEN, 1967 Heterospecific transformation of pneumococcus and streptococcus III. Reduction of linkage. *Genetics* **57**: 851-864.
- SICARD, A. M., and H. EPHRUSSI-TAYLOR, 1965 Genetic recombination in DNA-induced transformation of pneumococcus II. Mapping the *amiA* region. *Genetics* **52**: 1207-1227.
- SIROTNAK, F. M., G. J. DONATI, and D. J. HUTCHISON, 1964a Evidence for a genetic alteration of dihydrofolate reductase associated with amethopterin resistance in *Diplococcus pneumoniae*. *Biochem. Biophys. Res. Commun.* **14**: 292-295. — 1964b Dihydrofolate reductase in genotypically distinguishable amethopterin-resistant *Diplococcus pneumoniae*. *J. Biol. Chem.* **239**: 2677-2682. — 1964c Genetic modification of the structure and amount of dihydrofolate reductase in amethopterin-resistant *Diplococcus pneumoniae*. *J. Biol. Chem.* **239**: 4298-4302.
- SIROTNAK, F. M., S. L. HACHTEL, and W. A. WILLIAMS, 1969 Increased dihydrofolate reductase synthesis in *Diplococcus pneumoniae* following translatable alteration of the structural gene. II. Individual and dual effects on the properties and rate of synthesis of the enzyme. *Genetics* **61**: 313-326.
- SIROTNAK, F. M., and D. J. HUTCHISON, 1965 Gel filtration properties of mutant and wild-type dihydrofolate reductase from *Diplococcus pneumoniae*. *Biochem. Biophys. Res. Commun.* **19**: 734-738. — 1966 Purification and properties of mutant and wild-type dihydrofolate reductase from *Diplococcus pneumoniae*. *J. Biol. Chem.* **241**: 2900-2906.
- SIROTNAK, F. M., R. B. LUNT, and D. J. HUTCHISON, 1960a Transformation of resistance to 8-azaguanine in *Diplococcus pneumoniae*. *Nature* **187**: 800-801. — 1960b Genetic studies of amethopterin resistance in *Diplococcus pneumoniae*. *J. Bacteriol.* **80**: 648-653. — 1964 The distribution of mutational sites affecting resistance to amethopterin in *Diplococcus pneumoniae*. *Genetics* **49**: 439-452.
- SIROTNAK, F. M., M. G. SARGENT, and D. J. HUTCHISON, 1967a Genetically alterable transport of amethopterin in *Diplococcus pneumoniae* I. Physiological properties and kinetics of the wild-type system. *J. Bacteriol.* **93**: 309-314. — 1967b Genetically alterable transport of amethopterin in *Diplococcus pneumoniae* II. Impairment of the system associated with various mutant genotypes. *J. Bacteriol.* **93**: 315-319.
- YANOFSKY, C., and J. ITO, 1966 Nonsense codons and polarity in the tryptophan operon. *J. Mol. Biol.* **21**: 313-334.