THE DISTRIBUTION OF MUTATIONAL SITES AFFECTING RESISTANCE TO AMETHOPTERIN IN *DIPLOCOCCUS PNEUMONIAE*¹

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Received September 27, 1963

GENETIC analyses, utilizing transformation procedures to determine the relative position of mutationally altered regions of a DNA molecule, have been done in *Diplococcus pneumoniae* (HOTCHKISS and EVANS 1958; LACKS and HOTCHKISS 1960; ROTHEIM and RAVIN 1961; IYER and RAVIN 1962) and in *Bacillus subtilis* (EPHRATI-ELIZUR, SRINIVASAN and ZAMENHOF 1961; ANAGNOSTOPOULUS and CRAWFORD 1961; NESTER, SCHAFER and LEDERBERG 1963).

During transformation, genetic information is unidirectionally transferred to a suitable recipient cell by means of free DNA. Consequently, the total region which can be mapped is limited to one molecule. Furthermore, linkages between determinants affecting different biochemical steps, which have been revealed in this way, are still relatively rare. This is interpreted to mean that the amount of genetic information carried by a single molecule of DNA is very small in comparison to the total genomic complement of the cell. The total number of genetic loci found so far on a molecule of DNA has recently been extended to 13 (NESTER, SCHAFER and LEDERBERG 1963). On the other hand, transformation provides a means for recombination analyses at a molecular level, a valuable tool for studies eventually relating DNA structure to genetic maps (see review by RAVIN 1961, for comprehensive discussion of the genetics of transformation). The present work concerns the study of a group of spontaneous mutations inducing resistance to amethopterin in *D. pneumoniae*. Strains bearing these mutations were analyzed by transformation procedures in order to determine their position relative to one another and possibly to establish the number of sites, as well as loci, involved. Preliminary studies concerning a number of amethopterin resistant strains bearing some of these mutations have been reported previously (SIROTNAK, LUNT and HUTCHISON 1960a).

METHODS AND MATERIALS

Many of the procedures used during the course of this work were described in detail in previous reports (SIROTNAK, LUNT and HUTCHISON 1960a, 1963). Mutants were derived from a rough variant of *D. pneumoniae* (R6). Unless otherwise indicated the growth medium used was

¹ This work was supported in part by Public Health Service grant CA 03192-07 from the National Cancer Institute, and by grant T-107 from the American Cancer Society.

the semi-defined casein hydrolysate medium of ADAMS and ROE (1945), modified by omitting adenine, uracil and glutamine and adding fresh yeast extract (3 percent) and Armour fraction V bovine albumin (.05 percent).

Crude preparations of transforming DNA 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 1954). The crude nucleic acid solutions were stored in 2 M NaCl at 4°C.

Recipient cultures were prepared by allowing growth to a cell density equivalent to a viable count of 10×10^7 colony-forming units in casein hydrolysate medium containing an additional 0.16 percent albumin (transformation medium), and testing for the level of competence. Ten percent glycerol was added and each culture was frozen and stored at -20°C. For transformation, an aliquot of each culture was diluted at least 1:20 in fresh medium and crude nucleic acid extract was added to give a final DNA concentration between 1 and 5 µg/ml (saturating concentration). Following incubation for 30 min at 30°C, deoxyribonuclease (2.5 µg/ml) was added and the mixture was reincubated at 37°C for the time required for phenotypic expression of specific mutations.

Viable-cell number was determined by diluting cultures in medium containing anti-R agglutinating antibodies and counting colonies on the bottom of culture tubes following 17 hours incubation at 37°C. Medium for scoring drug resistant transformants was identical, but rendered selective by adding amethopterin (Lederle) or streptomycin (Squibb). The level of resistance of mutants or transformants to amethopterin was determined by scoring in anti-R medium containing varying amounts of drug and comparing colony size at each concentration. Levels of resistance of various mutant strains to other drugs, were determined by tube assay, using a liquid enzymatically hydrolyzed casein medium (SIROTNAK, LUNT and HUTCHISON 1960b). Growth in varying amounts of drug was optically determined in a Model B Beckman Spectrophotometer at 600 mµ.

RESULTS

Derivation of mutations inducing amethopterin resistance: Described in this report are spontaneous mutations (*ame^r*) which have occurred in strains independently isolated following transfer of the parent R6 organism in drug medium. Strains bearing individual mutations were obtained by testing the original mutant strains for their level of resistance and analyzing them genetically by using them as donors of DNA for transforming the drug sensitive parent strain. Only one class of transformants (equal in resistance level to the donor strain) is obtained when the mutant donor strain bears a single mutation. When the mutant strain used as a donor of DNA possesses two or more mutations, more than one class of transformants is obtained. In this case, strains bearing only one mutation were obtained by retesting clones from the population of single transformants. These were distinguished from transformants receiving more than one mutation by their low level of resistance in comparison to the original donor strain, and by the relatively high frequency with which they occurred.

Twenty-three mutations conferring amethopterin resistance were derived during the course of this work (see Figure 1). Among them, ten different types could be distinguished, differing either in respect to the resistance level they induced or the frequency with which they were transferred during transformation of the parent organism. Since the results of the various recombination experiments to be described here, also, were essentially the same for all mutations of the same type, only one mutation representative of each type was selected for

FIGURE 1.—An approximate arrangement of the amethopterin resistance mutational sites. The figures in parentheses refer to the number of independently derived but indistinguishable mutations of each type which appear to be at the same site.

discussion. In doing so, it is not implied that all mutations of each type are identical, since each arose by an independent structural alteration. However, until means are available for elucidating any existing differences, it seemed unnecessary to discuss each individually. The designation chosen for each mutant allele identified a specific mutation, but also can be considered to refer to one class of mutations which so far are indistinguishable.

Resistance levels induced by different ame^r mutations: The level of resistance of strains bearing individual ame^r mutations is compared to that of the parent strain in Table 1. The values given are the highest concentration of drug permitting maximum colony size development in the casein hydrolysate medium ($0.02 \mu\text{g/ml}$ for the parent R6 strain). The increase in resistance over that exhibited by the drug sensitive parent strain (the most definitive and readily comparable parameter of drug resistance) was often, but not always, character-

TABLE 1
Resistance level of strains bearing different ame^r mutations

Mutation	Resistance level* (μg amethopterin per ml)
Parent (sensitive)	0.02
ame^r -1	0.2
ame^r -2	2.0
ame^r -3	2.0
ame^r -4	10.0
ame^r -5	0.8
ame^r -6	0.3
ame^r -7	0.6
ame^r -8	0.4
ame^r -9	2.0
ame^r -10	2.0

* Highest concentration of amethopterin permitting maximum colony development in the casein hydrolysate medium described in the text. Values obtained from four to eight replicate experiments never varied by more than 25 percent, even under a variety of nutritional conditions. It has subsequently been found that folic acid, N^5 -formyl folate- H_2 , purines, thymine and certain amino acids, which are capable of reversing amethopterin inhibition of other organisms and occur in varying amounts in nutritionally undefined media, are without effect here (SIROTNAK and HUTCHISON, unpublished results).

istically different for each mutation, varying considerably (10- to 500-fold) among the groups examined. As shown for other mutations of drug resistance (HOTCHKISS and EVANS 1958; RAVIN and IYER 1962; BRYAN 1961) and for some amethopterin resistance mutations discussed previously (SIROTNAK, LUNT and HUTCHISON 1960a), the *ame^r* mutations discussed here can not only be distinguished by the resistance level they induce individually, but also by the characteristic level of resistance (usually much higher than the sum of the resistance of both mutations individually) they induce when borne in combination. For example, the addition of *ame^r-5*, *ame^r-1*, *ame^r-7*, and *ame^r-2* to the genome of R6 *ame^r-3* results in, respectively, a 5-, 10-, 25- and 75-fold increase in resistance. In the absence of precise biochemical information, these differences in resistance provide a useful means of phenotypically recognizing mutants which are genetically different, and as indicated previously (HOTCHKISS and EVANS 1958; RAVIN and IYER 1962) offers immediate advantage in studies of the type described here.

Transformation of the drug sensitive parent strain by DNA obtained from strains bearing different ame^r mutations: A measure of the relative ability of each *ame^r* mutation for genomic integration during transformation of the parent R6 strain was obtained by comparing the frequency of transformation of each to the frequency of transformation of an unrelated and unlinked "reference" mutation. This technique, used originally by LACKS and HOTCHKISS (1960), and employed later for distinguishing between erythromycin resistance mutations (IYER and RAVIN 1962), provides a ratio of individual transformation frequencies which is independent of factors affecting the overall level of transformation, such as recipient culture variability, and quality or concentration of DNA. For this purpose the same streptomycin resistance (*str^r*) mutation was added by transformation to the genome of each of the strains bearing a different *ame^r* mutation. The DNA prepared from each was then used to transform the parent strain. The incubation period at 37° C required for maximum phenotypic expression was 20 min for transformants integrating each of the *ame^r* mutations, and 70 min for the *str^r* transformants. The period of growth required for the expression of the *ame^r* transformants is only apparently shorter because of the lack of an immediate inhibitory effect on growth by amethopterin. In this case, inhibition is reversed by folic acid coenzymes accumulated intracellularly in excess amounts (SIROTNAK, DONATI and HUTCHISON 1963). The necessary depletion of these coenzymes permits several cell divisions in the presence of drug.

Table 2 shows the variability in relative transformation frequency (*ame^r/str^r*) for two *ame^r* determinants obtained in replicate experiments and in experiments using different DNA preparations and recipient cultures. As determined by a comparison of values for standard deviation obtained in each case, there was no significant difference in variability. The ratio of each *ame^r* transformation to *str^r* transformation is shown in Table 3. Although relative frequencies were similar in some cases, others were characteristically different, varying over an 18-fold range.

Recombination between ame^r mutations in mutant strains: When strains bearing individual *ame^r* mutations were exposed to DNA from strains bearing other

TABLE 2

The transformation of ame^r-3 and ame^r-5 relative to str^r transformation, using different DNA preparations and recipient cultures

Mutation	DNA* preparation	Recipient culture	Transformants $\times 10^3$ /ml		Ratio ame^r/str^r		Standard deviation
			ame^r	str^r			
ame^r-3	A	(a)	13.8	15.6	0.88		
			31.2	30.5	1.02		
			19.9	28.1	0.71		
			27.0	33.2	0.81		
			25.0	28.6	0.87	Average 0.86	0.113
	A	(a)	23.3	28.0	0.83		
		(b)	2.1	3.1	0.68		
		(c)	16.6	19.7	0.84		
		(d)	12.5	15.6	0.80		
		(e)	5.6	5.8	0.97	Average 0.82	0.104
	A	(e)	3.2	4.0	0.79		
	B		26.8	34.6	0.79		
	C		1.9	2.2	0.87		
	D		3.5	3.3	1.06		
	E		6.2	8.3	0.75	Average 0.85	0.126
	A	(a)	2.7	27.2	0.1		
			1.9	20.0	0.095		
			3.3	25.1	0.132		
			3.0	35.4	0.086		
			2.1	24.0	0.088	Average 0.1	0.0186
	A	(a)	1.9	18.1	0.108		
		(b)	0.09	1.04	0.087		
		(c)	0.16	1.4	0.110		
		(d)	1.80	25.1	0.072		
		(e)	2.08	18.0	0.120	Average 0.099	0.0195
	A	(e)	1.2	12.0	0.1		
	B		1.66	12.8	0.13		
	C		0.17	1.9	0.89		
	D		1.01	8.7	0.12		
	E		0.044	0.44	0.10	Average 0.108	0.0167

* Each DNA carries the str^r mutation and either ame^r-3 or ame^r-5 .

ame^r mutations, transformants to higher levels of resistance were often, but not always, obtained. That these transformants were recombinants bearing both mutations, was shown by using them as donors of DNA for the transformation of the R6 parent strain. Obviously, the presence of two mutations in the same genome is possible only if each is the result of an alteration at two different sites. These may occur either at two different loci or within the same locus (non-overlapping "nonidentical" alleles; DEMEREC 1956). Since the frequency with which recombination can occur bears some relation to the distance between the sites occupied by each mutation, at least an approximate arrangement of the various mutational sites is possible. For this purpose, reciprocal transformation

procedures similar to those described by LACKS and HOTCHKISS (1960) and EPHRATI-ELIZUR, SRINIVASAN and ZAMENHOF (1961) were employed, in which each strain bearing a different *ame^r* mutation was used, alternately, as a recipient and donor of DNA in various "crosses." Recombinants bearing both mutations were scored at drug concentrations just high enough to inhibit completely, both the untransformed recipient cells and transformants possessing only the *ame^r* mutation from the donor (these occur during integration of the mutation acquired exogenously from donor DNA by a simultaneous replacement of an endogenous segment including the recipient mutation). Strains used as DNA donors also carried the *str^r* reference mutation. In any particular "cross," the frequency of transformation of the mutant recipient strain to a higher level of resistance, relative to the *str^r* mutation transformation, was compared to a similar ratio of *ame^r* mutation to *str^r* mutation transformation of the wild strain by the same DNA (estimates the characteristic ability for integration of the mutation in the donor for each "cross"). In actual practice, competent recipient cultures of both the parent R6 and the mutant strains were harvested at the same phase of growth (at the experimentally determined peak of competence) in aliquots of the same transformation medium, and were then exposed simultaneously to the same transforming DNA. A quantitative estimate of the frequency of recombination between any two mutations could then be calculated in the following manner:

$$\text{Percent recombination} = \frac{\text{ame}^r \text{ (double)}/\text{str}^r \text{ (mutant recipient)}}{\text{ame}^r \text{ (single)}/\text{str}^r \text{ (parent recipient)}} \times 100$$

The results of "crosses" between all of the mutant strains are shown in Table 4. With only two exceptions, recombinants bearing both mutations occurred either with similarly high frequency or were not observed at all. Recombinants occurred with high frequency (at or close to 100 percent) in "crosses" between one group of mutations consisting of *ame^r*-1, *ame^r*-2, *ame^r*-7, *ame^r*-9 and *ame^r*-10 and another, consisting of *ame^r*-3, *ame^r*-4, *ame^r*-5 and *ame^r*-6. Recombination of sig-

TABLE 3

Relative frequency of transformation of various ame^r mutations

Mutation	Relative frequency* <i>ame^r</i> / <i>str^r</i> transformants	Standard deviation
<i>ame^r</i> -1	1.2	0.120
<i>ame^r</i> -2	0.095	0.027
<i>ame^r</i> -3	0.084	0.114
<i>ame^r</i> -4	0.093	0.021
<i>ame^r</i> -5	0.108	0.018
<i>ame^r</i> -6	0.75	0.110
<i>ame^r</i> -7	0.69	0.130
<i>ame^r</i> -8	0.70	0.140
<i>ame^r</i> -9	0.95	0.115
<i>ame^r</i> -10	1.75	0.240

* Mean values of the ratio of amethopterin-resistant to streptomycin-resistant transformants obtained in 15 to 20 experiments, using DNA bearing *ame^r* and *str^r* mutations.

TABLE 4

Summary of reciprocal transformations determining the relative frequency of transformation of mutant strains to increased levels of amethopterin resistance as compared to the transformation of the parent strain by the same DNA

Recipient strain	DNA donor strain*									
	<i>ame^r-1</i>	<i>ame^r-2</i>	<i>ame^r-3</i>	<i>ame^r-4</i>	<i>ame^r-5</i>	<i>ame^r-6</i>	<i>ame^r-7</i>	<i>ame^r-8</i>	<i>ame^r-9</i>	<i>ame^r-10</i>
<i>ame^r-1</i>	0	0	73	66	63	100	40	100	0	0
<i>ame^r-2</i>	0	0	86	65	68	90	0	100	0	0
<i>ame^r-3</i>	95	61	0	0	3.3	0	85	0	100	100
<i>ame^r-4</i>	100	89	0	0	0	0	80	0	..†	..
<i>ame^r-5</i>	81	74	2.6	0	0	0	100	0	90	100
<i>ame^r-6</i>	90	100	0	0	0	0	100	0	..	..
<i>ame^r-7</i>	36	0	100	100	100	90	0	100	0	0
<i>ame^r-8</i>	100	88	0	0	0	0	100	0	80	..
<i>ame^r-9</i>	0	0	100	..†	100	100	0	100	0	0
<i>ame^r-10</i>	0	0	100	..	..	100	0	100	0	0

Numbers in the body of the table are mean values in percent, of five to seven experiments with standard deviation below 30 percent. Recombinants occurring below a frequency of 0.1 percent would not be detected.

* Donors of DNA also carried the *str^r* determinant.

† "Crosses" indicated with dots were not performed.

nificantly lower frequency occurred between *ame^r-1* and *ame^r-7*. Mutations *ame^r-3* and *ame^r-5* recombined with the lowest frequency. Recombinants were not detected in "crosses" between any of the other mutations. The inability of certain mutations to recombine to form highly resistant transformants could indicate that they are at the same site, or at overlapping sites, or at adjacent sites but so close that recombinants can not be detected. In each case, one would expect during reciprocal transformation, a replacement of the mutation in the recipient by that in the donor. This can be shown by simultaneously testing for transformants bearing only the donor mutation (possible only in crosses involving a donor mutation inducing a higher level of resistance than the mutation in the recipient). Transformants that were identical to strains bearing donor mutations were observed in the following crosses where no recombination could be detected:

Donor DNA	Recipient cell
<i>ame^r-2, ame^r-9, ame^r-10</i>	<i>ame^r-1, ame^r-7</i>
<i>ame^r-3</i>	<i>ame^r-6, ame^r-8</i>
<i>ame^r-4</i>	<i>ame^r-3, ame^r-5, ame^r-6, ame^r-8</i>
<i>ame^r-5</i>	<i>ame^r-6</i>

Thus, in every reciprocal "cross," either recombinants bearing both donor and recipient mutations did not occur, or when they did occur, the recombinants always showed a level of resistance higher than that induced individually by each mutation.

Transformation of the sensitive strain by DNA from strains bearing two ame^r mutations: The results of reciprocal transformation experiments suggested close linkage between sites of the *ame^r-3* and *ame^r-5* mutations and to a lesser extent

between *ame^r-1* and *ame^r-7* (the mutational sites were close enough so that, more often than not during transformation, a segment adjacent to the mutation in the donor DNA replaced the mutation in the recipient). If this is so, when recombinants, obtained from each "cross," are used as donors of DNA during transformation of the sensitive strain, both mutations should be transferred simultaneously with a frequency higher than might be expected if such a "double transformant" was the result of two independent recombination events. The sensitive strain was exposed in the usual manner to DNA from donors bearing two mutations, and then scored in varying concentrations of drug to distinguish between the "single" transformants having a low level of resistance and the highly resistant "double" transformants. The results are given in Table 5. When DNA from the *ame^r-1-7* recombinant was used, the proportion of double transformants was very high. Important also was the fact that, despite variation in conditions which altered the overall transformation frequency (*viz.* DNA concentration), the proportion of double to single transformants remained constant. No double transformants were obtained when the same recipient culture was exposed to a mixture of DNA from strains bearing each mutation separately. In experiments using DNA from an *ame^r-3-5* recombinant, only "double" transformants could be found. Again, no double transformants were obtained when a mixture consisting of DNA bearing each mutation separately was used. In view of the rather surprising result obtained with DNA from this *ame^r-3-5* recombinant, a number of other recombinants were prepared from both *ame^r-5* $\times$ *ame^r-3* and *ame^r-3* $\times$ *ame^r-5* "crosses" and isolated at a level of transformation well above the mutational background. When these were tested the results were always the same.

Recombinants occurring with very high frequency during reciprocal trans-

TABLE 5

Transformation of the parent strain with DNA bearing two different ame^r mutations

Donor	<i>str^r</i> transformants $\times 10^8$ (a)	<i>ame^r</i> transformants			Ratio	
		total $\times 10^3$ (b)	double $\times 10^3$ (c)	double percent	(c)/(a)	(b)/(a)
<i>ame^r-1-7 str^r</i>	21.0	28.6	5.1	17.8	0.24†	...
<i>ame^r-1 str^r + ame^r-7 str^r</i>	...	31.0	0	0	...	...
<i>ame^r-2 str^r</i>	24.4	5.4	...	...	...	0.23†
<i>ame^r-3-5 str^r</i>	58.0	6.2	6.2	100	0.1	...
<i>ame^r-3 str^r + ame^r-5 str^r</i>	...	15.0	0	0	...	...
<i>ame^r-5 str^r</i>	29.0	3.2	...	...	...	0.11
<i>ame^r-1-5 str^r</i>	...	27.8	0.01	0.036	...	...
<i>ame^r-1 str^r + ame^r-5 str^r</i>	...	13.6	0.02	0.147	...	...
<i>ame^r-1-8 str^r</i>	...	21.3	0	0	...	...
<i>ame^r-1 str^r + ame^r-8 str^r</i>	...	10.6	0	0	...	...

* The same recipient culture was used in each experiment, and each experiment was performed at least twice.

† Due to an undetermined physiological deficiency of the double *ame^r-1-7* transformant, a 120 min incubation period at 37°C was used for the expression of the *ame^r* transformants in these experiments, giving a higher ratio of *ame^r* transformants/*str^r* transformants.

formation were also tested in simultaneous transformation experiments. Only the results obtained when recombinants *ame*^r-1-5 and *ame*^r-1-8 were used as donors are given in Table 5, since these data are representative of those obtained when the other recombinants, i.e. those occurring with similarly high frequencies, were used. In every case, either "double" transformants could not be detected, or they occurred at frequencies far below what might be expected if linked sites were involved.

Cross-resistance studies: The results of genetic experiments suggest that the *ame*^r mutations occur at sites in two separate regions of the DNA. Supporting biochemical evidence of these findings, although indirect, was obtained when strains bearing different *ame*^r mutations were examined for their resistance to various other antimetabolites (compounds selected because of their effect on folic acid synthesis or on enzymatic reactions dependent upon prior folic acid synthesis, including p-amino-benzenesulfonamide, 6-mercaptopurine, 5-fluorouracil, 8-azaguanine, azathymine, ethionine, 6-diazo-5-oxo-L-norleucine). Variation in response among the different strains was obtained only with p-amino-benzenesulfonamide, ethionine and azathymine. Differences were quite appreciable and consistent, although the latter two compounds were weak inhibitors (at least 0.003 M required for 50 percent inhibition) under the conditions used. Data for half-maximum inhibition obtained with each strain are related to similar data obtained with the parent strain in Table 6. On the basis of their inhibition by each drug, the mutants fall into two groups. A striking correlation with genetic data is immediately apparent, since mutant strains in each group bear mutations which by genetic experiments have been shown to cluster in the same region. Mutants bearing mutations *ame*^r-3, *ame*^r-4, *ame*^r-5, *ame*^r-6, *ame*^r-8 in Region I exhibit increased sensitivity to p-amino-benzenesulfonamide and azathymine, but are like the wild strain in their inhibition by ethionine. Mutants bearing mutations *ame*^r-1, *ame*^r-2, *ame*^r-7, *ame*^r-9, *ame*^r-10 in Region II exhibit increased

TABLE 6

Inhibition by selected antimetabolites of strains bearing different ame^r mutations

Region	Mutation	Resistance level (mutant/parent)*		
		p-amino-benzenesulfonamide	Ethionine	Azathymine
I	<i>ame</i> ^r -3	0.32	1.0	<0.62
	<i>ame</i> ^r -4	0.48	1.0	<0.68
	<i>ame</i> ^r -5	0.29	1.0	<0.65
	<i>ame</i> ^r -6	0.36	1.0	<0.57
	<i>ame</i> ^r -8	0.48	1.0	<0.5
II	<i>ame</i> ^r -1	2.5	0.14	1.0
	<i>ame</i> ^r -2	1.8	0.11	1.0
	<i>ame</i> ^r -7	2.8	0.15	1.0
	<i>ame</i> ^r -9	1.8	0.14	1.0
	<i>ame</i> ^r -10	1.7	0.14	1.0

* Ratio calculated from half-maximum inhibition data obtained from two to four tube-assay experiments described in text.

resistance to p-amino-benzenesulfonamide, increased sensitivity to ethionine, and are like the wild strain in their inhibition by azathymine.

DISCUSSION

Genetic experiments have shown that the spontaneous mutations described above (determinants of amethopterin resistance representing ten distinguishable classes) occur at overlapping or nearly adjacent sites located at two distinct regions of the DNA of *D. pneumoniae*. An approximate arrangement of the various mutational sites is given in Figure 1.

From data on both the frequency with which one mutation will combine with another in a "cross" between two mutants and the frequency of simultaneous transfer of two mutations to the parent strain, it can only be concluded that the distance between the sites of mutation at different regions is either large relative to the distance between other sites at the same region, or that each region exists on a different molecule of DNA. Evidence of linkage between the two regions, in the order shown, is only barely suggested by the frequencies of recombination observed in certain reciprocal "crosses," and is not confirmed by other data on simultaneous transformations involving the same mutations. Ultimate verification of linkage between the two regions, if it exists, will require the mapping of sites at each region in respect to other mutations, not necessarily affecting the same phenotypic property, but at sites between the two regions. This approach has been successfully used by NESTER, SCHAFER and LEDERBERG (1963) for verifying linkage between mutations at distant sites in DNA of *Bacillus subtilis*.

Discrepancies observed in certain reciprocal "crosses," where recombinants bearing two mutations occurred with very high frequency (i.e., between sites at different regions), are probably not the result of genetic factors, such as those discussed by EPHRUSSI-TAYLOR (1960, 1963). "Bisymmetry" due to differences in the size or position of each mutation in reference to the end of the DNA molecule would be expected to be characteristic of "crosses" involving mutations at fairly closely linked sites. Evidence for linkage between Regions I and II is meager at best. A more likely explanation concerns irregularities that have been observed in colony size of transformants of mutant recipients already bearing Group II mutations, notably *ame^r-1* and *ame^r-2*, which would affect scoring accuracy. In any event, no attempt has been made to relate quantitatively recombination which occurred with very high frequency in terms of "map" distances.

Significant, even though indirect, biochemical evidence consistent with genetic findings is provided by the similar patterns of cross-resistance and collateral sensitivity obtained with strains bearing mutations at sites within the same region during inhibition studies with p-amino-benzenesulfonamide, ethionine and azathymine. The metabolic basis for the differences observed, however, is not immediately apparent and will require more direct biochemical studies. Two related observations should be mentioned at this time. Mutants bearing determinants which map in Region II under certain nutritional conditions exhibit

lower growth rates in comparison to the wild strain and the other mutant strains. More important, evidence has recently been obtained in this laboratory (SINOTNAK, DONATI and HUTCHISON 1964) which indicates that mutation in Region I alters the enzyme dihydrofolate reductase.

The characteristic differences obtained here in the ability for integration of the various *ame^r* mutations during transformation of the wild strain is in agreement with observations made previously during the transformation of other mutations (LACKS and HOTCHKISS 1960; IYER and RAVIN 1962). That the relative transformation frequency for individual mutations is an experimentally reliable value is indicated by the data in Table 2. It has been suggested that the efficiency of integration is dependent, at least in part, on the size of the mutational segment being transformed and that the tendency for transformation of a large segment is less than for a small one. This is substantiated by observations on the low frequency of transformation of linked mutations in comparison to the transformation of single mutations (HOTCHKISS 1958; HOTCHKISS and EVANS 1958; GOODGAL 1961; NESTER and LEDERBERG 1961; EPHRAI-ELIZUR, SRINIVASAN and ZAMENHOF 1961; NESTER, SCHAFER and LEDERBERG 1963). This would require the integration of a larger segment extending over two adjacent or nearly adjacent sites. A similar case, cited here, was the low frequency of simultaneous transformation of the linked mutations, *ame^r-1* and *ame^r-7*, in comparison to the transformation of each individually. Also, LACKS and HOTCHKISS (1960) have reported that mutations which have been found genetically to be multisite (DEMEREK 1956), or by physical means to encompass relatively large regions, did in fact integrate with less efficiency. An additional factor to be considered in this regard is the specific nucleotide composition of each mutation. Although it has not been established experimentally, the possibility of this influencing the efficiency of integration has been suggested (HOTCHKISS 1958, IYER and RAVIN 1962).

Both reciprocal and simultaneous transformation data indicate that the mutational sites of *ame^r-1* and *ame^r-7* in Region II are close, although probably not adjacent, to one another. The absence of recombination among *ame^r-2*, *ame^r-9* and *ame^r-10*, or between these mutations and *ame^r-1* and *ame^r-7*, suggests that they are alleles, not necessarily at identical sites, but since they do not recombine with *ame^r-1* or *ame^r-7*, certainly overlapping along an appreciable portion of the length between them. A more exact position of the sites of the three mutations can probably be shown by mapping them in respect to additional, but more remote, sites. Solely on the basis of these results, it can not be concluded that *ame^r-2*, *ame^r-9*, or *ame^r-10* are multisite mutations (DEMEREK 1956), since the inability to observe recombinants bearing these mutations and either *ame^r-1* or *ame^r-7*, could be due to the fact that adjacent sites are involved. However, at least in the case of mutation *ame^r-2*, observations on the unusually low frequency of its transformation (identical to that shown for the simultaneous transformation of *ame^r-1* and *ame^r-7* in Table 5) is not inconsistent with such a conclusion.

The sites of *ame^r-3* and *ame^r-5* as shown by reciprocal transformation experiments appear to be extremely close ("crosses" combining both of them occurred

with a frequency of less than 3 percent). That the length of the segment between the two mutational sites, and possibly of the *ame^r-3* mutation, is small particularly in relation to *ame^r-5* is also suggested by observations on the frequency of transformation of the segment including both *ame^r-3* and *ame^r-5*. This was not observably different from the frequency of transformation of *ame^r-5* alone (compare the ratio of double *ame^r-3-5/str^r* transformation with that for *ame^r-5/str^r* transformation in Table 5). Significant also, is the apparent absence of single *ame^r-3* or *ame^r-5* transformants in the same transformation (i.e. with DNA bearing both mutations). This would be expected, since the opportunity for "crossover" between adjacent or nearly adjacent sites would be severely limited. This was already shown to be less than 3 percent during reciprocal transformation experiments. In a similar case cited by RAVIN and IYER (1962), the transformation of two linked mutations was seen to occur more often than the transformation of one of them. Additional experiments bearing on these observations are now in progress. Even though they appear to be allelic, the sites of the *ame^r-4*, *ame^r-6* and *ame^r-8* mutations can barely be approximated since the region where overlapping must occur and still permit no recombination with *ame^r-3* or *ame^r-5* is apparently quite small (possibly much smaller than the total length of each mutation). Here again it will be necessary to map additional, but more remotely situated, sites to determine the position of these alleles and also to determine if any are multisite mutations.

It would be difficult at this point to assess adequately the significance of these findings in relation to other information concerning the nature of amethopterin resistance. However, a few remarks pertaining to possible genetic-biochemical relationships seem appropriate. Antimetabolites, amethopterin among them, inhibit cell growth by interfering with specific enzymatic activities at crucial physiological sites, including the synthesis of nucleic acids and certain amino acids. Resistance to these agents has often been shown to be a result of genetically induced alteration in enzyme structure or enzyme level, either of the inhibited enzyme itself, or in a compensating way, some enzyme at a related metabolic step (see reviews by JUKES and BROQUIST 1963; HUTCHISON 1963). The magnitude of resistance, therefore, would not only depend upon the number of mutational events which have occurred, but also on the nature of the enzymatic alteration evoked, i.e., the specific loci involved. For instance, mutational changes involving more than one locus (and presumably more than one enzymatic step), as might be expected, appear to result in markedly higher levels of resistance. In the present case, certain strains bearing mutations at both Region I and II exhibit resistance levels which are as much as 50,000 times higher than that of the wild strain. Not to be overlooked are other factors pertaining to the mutation itself, which conceivably would quantitatively modify a specific biochemical alteration. Among these are size (segment of nucleotides involved), nature of the nucleotide alteration, and its location within a locus, especially in respect to genetic regions specifying the active center of the enzyme.

SUMMARY

Utilizing transformation procedures in a genetic analysis of mutant strains of

Diplococcus pneumoniae, 23 spontaneous mutations determining resistance to amethopterin were independently derived. From a comparison of the resistance level each conferred and their transformation frequencies, the mutations could be placed in ten distinguishable categories. The relative frequency of combining two mutations in a "cross" between two mutants, and of the simultaneous transfer of two mutations to the parent strain, indicates that the mutations occurred at sites in two distinct regions of the DNA. Cross-resistance and collateral sensitivity patterns among the amethopterin resistant mutants obtained in inhibition studies with related drugs show a relationship between the sites of the individual mutations which is in complete agreement with genetic data.

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