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

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A unique class of mutations with dual biochemical effects has been found in a structural gene of the L-arabinose operon of *Escherichia coli* (ENGLESBERG 1961; and LEE and ENGLESBERG 1963). These mutations are clearly different from the "nonsense" or "frameshift" type (NEWTON, BECKWITH, ZIPSER and BRENNER 1965; MARTIN, SILBERT, SMITH and WHITFIELD 1966; YANOFSKY and ITO 1966 and WHITFIELD, MARTIN and AMES 1966) in that a gene product is detectable and both coordinate increases as well as decreases in the rate of synthesis of all the enzymes in the operon may be determined. We now report a similar multiple effect of mutation within the gene for dihydrofolate reductase in *Diplococcus pneumoniae*. Strains bearing these mutations (*ame*^r) were selected for their resistance to amethopterin. Of the mutations which were found to determine increased rates of synthesis of dihydrofolate reductase, thirty determine translatable alterations in the structural gene and approximately half of these appear to be associated with altered properties of the enzyme. Genetic characteristics of the mutations and other data on the corresponding linkage relationships have been given in the companion report (SIROTNAK and HACHTEL 1969). The present studies are an extension of other work reported earlier (SIROTNAK, LUNT and HUTCHISON 1964; SIROTNAK, DONATI and HUTCHISON 1964a, 1964b, 1964c; SIROTNAK and HUTCHISON, 1965, 1966).

MATERIALS AND METHODS

The derivation of the various *ame*^r mutations utilized in the present study has been described (SIROTNAK and HACHTEL 1969). Cultural and genetic procedures, drug inhibition assays and methods of recombinational analysis have also been given (SIROTNAK, LUNT and HUTCHISON 1960, 1964; SIROTNAK and HACHTEL 1969).

Cell-free extracts for enzyme assays were prepared by sonication from log-phase cultures as described earlier (SIROTNAK, DONATI and HUTCHISON 1964b 1964c). Protein concentration was determined by the method of LOWRY, ROSEBROUGH, FARR and RANDALL (1951). H₂-folate was prepared by the procedure of BLAKLEY (1960). Dihydrofolate reductase activity was determined by a method (SIROTNAK, DONATI and HUTCHISON 1964b) similar to that of OSBORN and HUEN-

NEKENS (1958) by measuring the decrease in absorbance (340 $m\mu$) at 25°C when 0.065 mM H_2 -folate and 0.065 mM TPNH were combined with enzyme extract in 0.05 M potassium phosphate buffer (pH 7.3) with 12.8 mM 2-mercaptoethanol. One unit of enzyme activity represents a change in absorbance of 0.1 per min. Determinations for enzyme concentration were performed by direct stoichiometric titration with amethopterin (SIROTNAK, DONATI and HUTCHISON, 1964b, 1964c). Two procedures were employed. In the method described by ACKERMANN and POTTER (1949), enzyme activity in varying amounts of cell-free extract was titrated with a constant amount of drug. The other procedure, described by WERKHEISER (1961), measures enzyme activity in the same amount of extract tested against varying drug concentrations. In both cases, the titration end-point in molar equivalents of drug represents the enzyme concentration.

RESULTS

Phenotypic consequence of mutation at the AME I locus: the effect on dihydrofolate reductase synthesis: Thirty-two mutations have been mapped by recombinational analysis at the *AME I* locus on the *D. pneumoniae* chromosome (SIROTNAK and HACHTEL 1969). The results of earlier biochemical studies (SIROTNAK, DONATI and HUTCHISON 1964a, 1964b, 1964c) with four of these mutations (*ame^r-3*, *ame^r-5*, *ame^r-6*, *ame^r-8*) have associated this locus with dihydrofolate reductase activity as measured by the TPNH reduction of H_2 -folate to H_4 -folate. The remainder of these mutations were also examined for effects on this enzyme activity. The results are shown in Table 1. Thirty-one of the mutations determined an increase in dihydrofolate reductase activity. Specific enzyme activities varied from 2- to 100-fold when compared to the wild-type level.

Nearly stoichiometric (irreversible) binding of amethopterin by dihydrofolate reductase from *D. pneumoniae* (SIROTNAK, DONATI and HUTCHISON 1964b, 1964c) permitted a direct titration for enzyme concentration in each of the mutant and wild-type preparations. The results, obtained by the ACKERMANN and POTTER (1949) procedure, are also given in Table 1. The data show an increase in drug binding sites in the mutant preparations, which in every case closely parallels the increase in specific activity. In a number of control experiments, mutant and wild-type extracts were mixed and tested for enzyme activity and drug binding. Values obtained with the mixture were always equal to the sum of the individual values obtained separately for each extract. The data, therefore, define the mutationally determined increase in dihydrofolate reductase activity as an actual increase in the concentration of the enzyme, rather than an increase in turnover rate. Assuming one binding site per molecule of dihydrofolate reductase, an absolute value for turnover per molecule can be calculated from the data presented.

In every case but one (*ame^r-5*), the extent of the alteration in the enzyme level agreed closely with the level of resistance to amethopterin mediated by each mutation (Table 1). The *ame^r-5* mutation has no effect on the level of this enzyme, but determines a drastic decrease in the affinity of the enzyme for drug, in addition to altering both catalytic and physical properties (SIROTNAK, DONATI and HUTCHISON 1964c). Otherwise, it would appear that individual levels of resistance are solely a quantitative phenotypic expression of increased rates of "target" enzyme synthesis. The relationship among the three parameters dis-

TABLE 1

The effect of various ame^r mutations on dihydrofolate reductase synthesis

Strains	Relative* resistance levels	Specific enzyme activity units [†]		Drug binding sites moles $\times 10^{-12}$	
		Per mg protein	Relative*	Per mg protein	Relative*
wild type	1	1.6	1.0	3.6	1.0
<i>ame^r-3</i>	100	174.0	109.0	403.0	112.0
<i>ame^r-5</i>	40	1.9	1.2	..‡	..‡
<i>ame^r-6</i>	8	10.9	6.8	28.4	7.9
<i>ame^r-8</i>	8	12.0	7.5	32.8	9.1
<i>ame^r-11</i>	4	6.7	4.2	15.8	4.4
<i>ame^r-12</i>	4	6.9	4.3	13.7	3.8
<i>ame^r-13</i>	3	5.4	3.4	9.7	2.7
<i>ame^r-14</i>	10	16.8	10.5	33.8	9.4
<i>ame^r-15</i>	4	8.8	5.6	16.9	4.7
<i>ame^r-16</i>	2	3.1	2.0	7.2	2.0
<i>ame^r-17</i>	2.5	4.2	2.6	7.3	2.1
<i>ame^r-18</i>	4	7.1	4.4	18.7	5.2
<i>ame^r-19</i>	8	17.4	10.9	25.6	7.1
<i>ame^r-20</i>	3	5.3	3.3	9.4	2.6
<i>ame^r-21</i>	8	14.4	9.0	25.2	7.0
<i>ame^r-22</i>	8	11.2	7.0	32.8	9.1
<i>ame^r-23</i>	8	14.9	9.3	36.0	10.0
<i>ame^r-26</i>	8	14.9	9.3	37.8	10.5
<i>ame^r-27</i>	8	12.3	7.7	37.8	10.5
<i>ame^r-28</i>	8	11.8	7.4	28.4	7.9
<i>ame^r-29</i>	8	11.4	7.1	30.6	8.5
<i>ame^r-31</i>	6	10.9	6.8	21.6	6.0
<i>ame^r-32</i>	6	11.2	7.0	25.5	7.1
<i>ame^r-33</i>	6	10.9	6.8	25.9	7.2
<i>ame^r-34</i>	6	10.3	6.4	26.6	7.4
<i>ame^r-35</i>	6	10.1	6.3	20.5	5.7
<i>ame^r-36</i>	6	10.3	6.4	22.0	6.1
<i>ame^r-37</i>	6	10.1	6.3	23.4	6.5
<i>ame^r-38</i>	6	11.2	7.0	25.6	7.1
<i>ame^r-39</i>	6	12.3	7.7	22.3	6.2
<i>ame^r-40</i>	6	10.3	6.4	21.2	5.9
<i>ame^r-41</i>	2.5	4.5	2.8	9.4	2.6

* Ratio of mutant/wild type.

† Change in absorbancy of 0.01 at 340 m μ per min.

‡ This enzyme has a reduced affinity for amethopterin (SIROTNAK, DONATI and HUTCHISON 1964c). See text for explanation.

cussed is illustrated in Figure 1. Average relative values for enzyme activity and enzyme concentration are compared for each resistance class. Most significant is the fact that the 31 mutations are expressed as only eight quantitatively distinct effects on enzyme synthesis. Fifteen additional mutations, which have not been mapped as yet, were evaluated more recently (SIROTNAK and WILLIAMS, unpublished). These were also found to mediate biochemical effects which were quantitatively identical to those already described.

Joint effects on enzyme synthesis of two ame^r mutations in recombinant

FIGURE 1.—Concordance between mutationally determined increases in enzyme activity and enzyme concentration in various phenotypic classes. Values plotted represent averages within each group for specific enzyme activity and enzyme concentration as related to the wild type.

genomes: Individual *ame^r* mutations, 46 in all, determine one of eight different quantitative increases in dihydrofolate reductase synthesis. What effects on enzyme synthesis are jointly expressed when two mutations exist in the same genome? Most crosses involving two mutant strains did not yield phenotypically distinguishable recombinant strains (SIROTNAK and HACHTEL 1969). The resistance level, which is presumably an expression of the enzyme levels in these recombinant strains, appears to be no greater than the highest resistance level determined by the individual mutations themselves. Thus, when each of the two mutations involved in the cross determine equal levels of resistance, recombinants are not detectable. When resistance levels determined by each mutation are different, recombinants are detectable if the cross is made in the direction of the lower resistant recipient.

In those cases where crosses between two mutant strains yielded a phenotypically distinguishable recombinant, isolations were made and the corresponding strains tested for resistance. The results obtained with approximately 100 strains are listed in Table 2. Without exception, the resistance level of these strains was factorially related to resistance levels determined by the individual mutations (recombinant resistance levels equalled the product of the individual resistance levels). This would imply an independence of effect for each mutation when born jointly in the same genome. Again, since all of the individual mutations involved determine an increase in enzyme synthesis proportional to the increase in resistance, a corresponding increase in enzyme synthesis is presumed to exist

TABLE 2

Relative resistance level of various recombinant strains bearing two ame^r mutations

First mutation	<i>ame^r-13</i>	Relative resistance level*		<i>ame^r-20</i>
		Second mutation <i>ame^r-16</i>	<i>ame^r-17</i>	
<i>ame^r-6</i>	20	19	20(20)†	26(25)
<i>ame^r-8</i>	25	..	19(21)	25(24)
<i>ame^r-11</i>	12	..	10	12
<i>ame^r-12</i>	13	10	..	12
<i>ame^r-13</i>	..	6(5.6)	8	10(9.0)
<i>ame^r-15</i>	13	9	..	13
<i>ame^r-18</i>	12	8	10(10)	12(12.5)
<i>ame^r-19</i>	23	17	..	25
<i>ame^r-21</i>	22	..	18(20)	20(25)
<i>ame^r-22</i>	22	..	..	..
<i>ame^r-23</i>	20	18	18	20(24)
<i>ame^r-26</i>	25	17	19(20)	25(24)
<i>ame^r-27</i>	25	19	18	25
<i>ame^r-28</i>	25	19	21	23
<i>ame^r-29</i>	25	17	18(17)	25(26)
<i>ame^r-31</i>	18	12	15	18
<i>ame^r-32</i>	20	13	15	19
<i>ame^r-33</i>	21	12	14	19
<i>ame^r-34</i>	18	12	15	17
<i>ame^r-35</i>	20	13	16	18
<i>ame^r-36</i>	19	12	..	16
<i>ame^r-37</i>	20	12	15	17
<i>ame^r-38</i>	18	13	14	18
<i>ame^r-39</i>	20	12	15	18(17)
<i>ame^r-40</i>	18	11	16	18
<i>ame^r-41</i>	7	..	6	8

* Ratio of recombinant/wild type.

† Sixteen strains were tested for specific enzyme activity. The corresponding values for relative enzyme activity are given in parenthesis.

in each recombinant. In sixteen strains, which were actually tested (see Table 2), this was in fact the case.

Phenotypic consequence of mutation at the AME I locus: effects on the properties of dihydrofolate reductase: The effect of increasing amounts of amethopterin on the growth of *D. pneumoniae* is characteristically abrupt once the inhibitory concentration range is reached. For instance, the concentration required for complete inhibition of the wild type is only about 3.5-fold greater than the highest concentration which will permit maximum growth. Such a small concentration difference (inhibition multiple) is attributable to the nearly stoichiometric nature of the association between this drug and the "target" enzyme, dihydrofolate reductase (SIROTNAK, DONATI and HUTCHISON 1964b). When the *in vivo* inhibitory response of the various mutant and recombinant forms to amethopterin was similarly evaluated, a number of differences from the wild type were evident. These can be seen from the data shown in Table 3.

TABLE 3

*Inhibition characteristics of mutant strains bearing a single ame^r mutation and recombinant strains bearing two mutations**

Mutations	Single mutant strains	With ame ^r -13	Double recombinant strains With ame ^r -16	With ame ^r -17	With ame ^r -20
ame ^r -6	3.8	3.5	3.5	6.7	6.2
ame ^r -8	3.3	3.6	..	6.7	6.2
ame ^r -11	3.4	3.3	3.0	..	3.4
ame ^r -12	4.2	4.1	4.0	4.2	4.0
ame ^r -13	3.5	..	3.4	3.2	3.0
ame ^r -14	10.0	..	..	..	..
ame ^r -15	3.3	3.3	3.3	3.3	3.4
ame ^r -16	3.4	..	..	..	..
ame ^r -17	3.2	..	..	..	..
ame ^r -18	3.3	3.3	3.5	5.0	4.7
ame ^r -19	2.8	2.6	..	..	3.0
ame ^r -20	3.3	..	..	..	..
ame ^r -21	3.0	2.7	..	6.2	5.6
ame ^r -22	12.0	12.0	..	..	..
ame ^r -23	6.7	6.3	6.5	6.5	6.7
ame ^r -26	3.3	2.6	2.9	6.0	5.7
ame ^r -27	6.0	6.0	6.2	6.0	6.0
ame ^r -28	3.6	3.0	3.8	3.1	3.3
ame ^r -29	3.3	3.0	3.4	7.2	5.7
ame ^r -31	3.3	3.4	3.5	3.2	3.1
ame ^r -32	3.5	3.3	3.5	3.1	3.2
ame ^r -33	3.4	3.3	3.5	3.5	3.4
ame ^r -34	3.6	3.3	3.5	3.5	3.2
ame ^r -35	3.3	3.3	3.5	3.2	3.2
ame ^r -36	3.5	3.3	3.5	..	3.5
ame ^r -37	3.3	3.4	3.5	3.6	3.2
ame ^r -38	3.4	3.4	3.5	3.3	3.0
ame ^r -39	3.5	3.4	3.5	3.8	3.2
ame ^r -40	3.4	3.7	3.5	3.3	3.2
ame ^r -41	3.6	3.3	..	3.2	2.7

* Values given represent ratio of drug concentration required for complete inhibition/highest drug concentration allowing maximum colony size development. The wild-type value = 3.5.

Although the inhibitory concentration range is shifted upward because of the increased "target" enzyme content, inhibition multiples which were obtained with most of these strains were close to the wild-type value. However, values derived with a number of the strains were significantly higher. With five mutant strains bearing *ame^r-12*, *ame^r-14*, *ame^r-22*, *ame^r-23* or *ame^r-27*, inhibition multiples varied from 4.2 to 12.0. Other values derived with twelve related recombinant strains bearing one of these mutations were correspondingly higher as well. In addition, values derived for twelve other recombinant strains (6-17, 8-17, 17-18, 17-21, 17-26, 17-29, 6-20, 8-20, 18-20, 20-21, 20-26, 20-29) were also appreciably higher. The latter was of considerable interest, since values derived

with strains bearing each of these mutations alone were no different than for the wild type.

It seemed unlikely that these *in vivo* effects, i.e. differences in inhibitory response, were simply an indirect result of the higher enzyme level in cells of these five mutant and twenty-two recombinant strains, since the majority of strains with similarly high enzyme levels did not exhibit the same response. Rather, the higher inhibition multiples obtained in these cases appeared to be suggestive of some alteration of the enzymes themselves. An effect of the *ame^r-5* mutation on the enzyme-drug association has already been described (SIROTNAK, DONATI and HUTCHISON 1964c). This mutation determines a drastic reduction in affinity of dihydrofolate reductase for amethopterin. A similar alteration of this enzyme in these strains would be expected to be small, since ACKERMANN-POTTER (1949) titration of the enzyme with amethopterin was still possible. Using the procedure of WERKHEISER (1961), a titration curve illustrating the binding by wild-type dihydrofolate reductase of varying amounts of amethopterin is shown in Figure 2. A linear representation is obtained until the drug concentration approaches a concentration equal to that of the enzyme (molar equivalent concentration).

FIGURE 2.—Amethopterin titration of dihydrofolate reductase in wild-type and mutant *D. pneumoniae*. Inhibition of enzyme activity is plotted against the total amount of drug used in the enzyme reaction mixture. Additional information is given in the MATERIALS AND METHODS.

The actual value is obtained by extrapolation and represents the stoichiometric titration end-point, i.e. the theoretical amount of drug required for saturation of all the binding sites present in the reaction mixture. Binding of amethopterin by dihydrofolate reductase from *D. pneumoniae* is not completely stoichiometric (SIROTNAK, DONATI and HUTCHISON 1964b, 1964c), hence the deviation from linearity seen at high drug concentrations. This is attributed to an increased tendency for dissociation of the enzyme-drug complex in this concentration range.

Enzyme from the 5 mutant and 12 genetically non-identical recombinant strains, which exhibited high inhibition multiples during the *in vivo* assays were examined by the same titration procedure. Some of the results are given in Figures 3 and 4. The enzyme levels in the five recombinants selected for comparison in Figure 4 are the same. The results obtained with enzymes from the other recombinant strains are almost identical to those shown. The titration curves in every case approximate that obtained with the wild-type enzyme. However, a linear representation was obtained over a narrower concentration range. In addition, the deviation from linearity observed at higher drug concentrations was considerably more pronounced, especially for four of the five mutant enzymes (Figure 3). In every case, complete inhibition was not obtained within the concentration range shown, whereas complete inhibition of the wild-type enzyme occurred at a concentration above 1×10^{-12} moles per ml. Inhibition of the mutant enzymes was always compared to inhibition of the wild-type enzyme within the

FIGURE 3.—Altered amethopterin titration of mutant dihydrofolate reductase. See legend in Figure 2 and Table 4 for other details.

FIGURE 4.—Altered amethopterin titration of recombinant forms of dihydrofolate reductase. See legend in Figure 2 and Table 4 for other details.

same experiment. Differences in the inhibitory properties of dihydrofolate reductase from these strains can also be quantitatively evaluated by comparing the inhibition obtained at the molar equivalent concentration. Results for the 5 mutant and the 12 recombinant enzymes, as well as the wild-type enzyme, are summarized in Table 4. Individual values were averages based on 2- to 3- replicate assays. While 92% of the wild-type enzyme activity is inhibited at the molar equivalent concentration, no more than 80–84% of the other enzyme activities are inhibited. By comparison, the markedly altered *ame^r-5* enzyme is only inhibited to the extent of 15%.

Enzymes prepared from several other mutant and recombinant strains which exhibited normal inhibition multiples during *in vivo* tests were also examined in this way. In each case, titration curves were indistinguishable from that obtained with the wild-type enzyme. Data for three of these enzymes are given in Figure 2. It is important to emphasize here that differences based on a comparison of inhibition at the molar equivalent concentration, although small, are verified by other features of the titration curves. Enzyme from the wild type and most mutant or recombinant strains is completely inhibited by drug concentrations of around 10^{-12} moles. Complete inhibition of enzyme from the 17 strains under discussion requires a concentration 2- to 3-fold higher. The results presented in Figures 3 and 4 are interpreted as an increase in the amount of dissociation, normally characteristic of enzyme-drug complexes, at high drug concentrations. The 14 mutations involved appear to determine subtle, but definite, differences in affinity of the enzyme for amethopterin.

DISCUSSION

The finding that thirteen of the *ame^r* mutations, which determine increased rates of enzyme synthesis, also appear to be related to effects on the properties of dihydrofolate reductase was fortunate, since it provides some direct evidence for concluding that these mutations are within the structural gene for this enzyme. All but two of the thirty-two *ame^r* mutations are densely clustered at one end of the genetic map of the *AME I* locus. (See companion paper by SIROTNAK and HACHTEL 1969.) The segment of the map involved is about 15 recombination units (per cent) in length. The relative positions of the mutational sites within this segment are shown in Figure 5. The mutations which have been as-

FIGURE 5.—Map of the dihydrofolate reductase gene in *D. pneumoniae*. Methods of recombination analyses and other details are given in the companion paper (SIROTNAK and HACHTEL 1969).

sociated with an effect on the properties of dihydrofolate reductase are designated in heavier print. In addition to those discussed here, *ame^r-3* and *ame^r-5* have also been shown to alter physical and catalytic properties of this enzyme (SIROTNAK, DONATI and HUTCHISON 1964; SIROTNAK and HUTCHISON 1965, 1966). Close linkage of these two mutations to the other thirteen, which were related to rather small differences in enzyme properties, is of considerable importance, since it lends further support to the conclusion that they are also within the structural gene. Since all fifteen mutations are at sites distributed throughout the entire segment, it would appear as if a sizable portion of the dihydrofolate reductase gene is delineated. The lack of an effect on enzyme properties by *ame^r-13* or *ame^r-16*, either individually or jointly (except where the second mutation has a demonstrable effect alone), is not unexpected in view of the respective map positions that are rather remotely situated from the other *ame^r* sites. It should be mentioned at this time, that the data by itself do not entirely exclude the possibility that the higher enzyme levels determined by the mutations within the dihydrofolate reductase structural gene are only apparent and are an indirect result of a more stable or less degradable enzyme being formed. However, this appears unlikely, since it would be difficult to account in this way for increases that are as high as 100-fold. It has been observed, in this connection, that mutant enzymes in cell-free preparations were no more stable than the wild-type enzyme (SIROTNAK, DONATI and HUTCHISON 1964c; SIROTNAK and HUTCHISON, unpublished). Of course, this does not mean that such differences do not exist within the cell. Additional studies bearing on this question are in progress.

There seems to be no correlation between the overall distribution of the *ame^r* mutations within the dihydrofolate reductase gene and the magnitude of the quantitative effect each determines on the rate of enzyme synthesis. Individual

TABLE 4
*Amethopterin inhibition of dihydrofolate reductase from various ame^r
mutant and recombinant strains*

Enzyme source	Percent inhibition*	Enzyme source	Percent inhibition*
wild type	92.0	<i>ame^r-17-21</i>	83.0
<i>ame^r-5</i>	15.0	<i>ame^r-17-26</i>	83.0
<i>ame^r-12</i>	85.0	<i>ame^r-17-29</i>	79.0
<i>ame^r-14</i>	80.0	<i>ame^r-6-20</i>	84.0
<i>ame^r-22</i>	77.0	<i>ame^r-8-20</i>	83.0
<i>ame^r-23</i>	83.0	<i>ame^r-18-20</i>	83.0
<i>ame^r-27</i>	84.0	<i>ame^r-20-21</i>	82.0
<i>ame^r-6-17</i>	84.0	<i>ame^r-20-26</i>	83.0
<i>ame^r-8-17</i>	83.0	<i>ame^r-20-29</i>	81.0
<i>ame^r-17-18</i>	83.0		

* Inhibition at molar equivalent concentration of enzyme and drug. Each value is based on at least two and sometimes three individual assays which vary by approximately $\pm 5\%$. Any differences from the wild-type enzyme were established by a comparison of both wild-type and mutant enzyme within the same experiment.

effects, therefore, might be related to the specific codon alteration involved in each case, i.e. to the nature of the mutation rather than the position. It is also of interest to note that recombination involving two mutations within this segment, with the exception of crosses involving *ame^r-17* and *ame^r-20* which appear to be at one end, does not result in levels of enzyme synthesis higher than those already determined by the individual mutations. This was in contrast to the results of crosses with *ame^r-13*, *ame^r-16*, *ame^r-17* and *ame^r-20*, where enzyme levels in the recombinants were a factorial expression of individually determined effects. Joint effects on enzyme synthesis do not seem to be factorial when more than two mutations are involved, suggesting some limit to the effects which can be determined. As an example, *ame^r-13*, *ame^r-20* and *ame^r-6* determine 3- and 8-fold increases in enzyme synthesis, respectively. Recombinants bearing either *ame^r-6* and *ame^r-13* or *ame^r-6* and *ame^r-20* exhibit a 24-fold increase in enzyme synthesis. A recombinant bearing all three mutations, however, exhibits only a 50-fold increase, instead of the nearly 75-fold expected for a fully factorial joint expression (SIROTNAK, WILLIAMS and HUTCHISON, unpublished). Only *ame^r-3* and *ame^r-14* seem unable to determine joint effects on enzyme synthesis when existing in a chromosome bearing a second *ame^r* mutation determining a related effect. Both mutations, however, form a phenotypically distinguishable recombinant with *ame^r-5*. The effects on the level of enzyme synthesis are unique. No effect on enzyme synthesis is determined by *ame^r-5* alone, i.e. it is identical to the wild-type. While *ame^r-3* and *ame^r-14* determine 110- and 10-fold increases, respectively, the corresponding recombinant genomes, *ame^r-3-5* and *ame^r-5-14*, determine only 6- and 3-fold increases in enzyme synthesis (SIROTNAK, DONATI and HUTCHISON 1964c; SIROTNAK and WILLIAMS, unpublished). The effects of *ame^r-3* and *ame^r-14* are almost completely nullified by the presence of the *ame^r-5* mutation in the same genome. Isolation of these recombinants is only possible because of the net increase in resistance determined by *ame^r-5*.

It seems likely that the *ame^r* mutations in the dihydrofolate reductase gene are of the "missense" type. Both the increased rates of enzyme synthesis observed and the absence of a severely adverse effect on the enzyme itself, indicating a translatable codon alteration, are incompatible with the idea of a "nonsense" or "frameshift" alteration. The former is believed to result in a termination of synthesis of the corresponding polypeptide (SARABHAI, STRETTON, BRENNER and BOLLE 1964; STRETTON and BRENNER 1965), and the latter in the synthesis of an inactive polypeptide chain of undefined sequence (CRICK, BARNETT, BRENNER and WATTS-TOBIN 1961; AMES and WHITFIELD 1966; BRAMMER, BERGER and YANOFSKY 1967). Moreover, a deletion involving even a short nucleotide sequence within the gene would be expected to result in an inactive enzyme.

Tentatively, then, two types of "missense" changes, which increase rates of enzyme synthesis, can be distinguished. Approximately half of the *ame^r* mutations, because of the modification seen in the properties of this enzyme, would appear to determine an amino acid substitution. The other mutations have no demonstrable effect on the properties of this enzyme. In these cases, since the genetic code is degenerate, the new triplet formed by the mutation could code for

another transfer RNA carrying the same amino acid. Alternatively, a different amino acid might be substituted at that point on the polypeptide chain, but with no detectable effect on the properties of the enzyme.

How can a "missense" alteration in a structural gene affect upward limits of enzyme synthesis? An answer to this question will require further biochemical data, since effects at both transcriptional and translational levels could be involved in addition to possible effects on mRNA stability. None of these alternatives are mutually exclusive. Differences observed among the various *ame^r* mutations in the ability for joint expression, for instance, might very well be related to effects which are determined at different levels of protein synthesis. Considering the number of quantitative effects associated with the *ame^r* mutations, it is easier to visualize an alteration at the translational level. Differences in availability of specific transfer RNA could provide some basis for the kind and variety of effects observed. Indeed, a model of this sort has been proposed (STENT 1964) to explain how the nucleotide sequence at any point in a structural gene might influence the translational process. Both increases, as well as decreases, in rates of synthesis could occur as a result of mutation. "Dual-Effect" mutations leading to both these effects have already been reported in the L-arabinose system (LEE and ENGBERG 1963). The effect of the *ame^r*-5 mutation in modifying, downward, high rates of synthesis determined by *ame^r*-3 and *ame^r*-14 could also be explained by such a model. In any event, it seems apparent that limits of enzyme synthesis are set in some way by the nucleotide sequence of the corresponding structural gene itself and that these limits can be altered either upward or downward by new translatable sequences introduced by mutation.

The "modulation theory" was originally proposed by AMES and MARTIN (1964) to explain the polarity effects of "nonsense" mutations on rates of enzyme synthesis within an operon. It was later suggested (AMES, GOLDBERGER, HARTMAN, MARTIN and ROTH 1967) that modulating codons (nonsense triplets) may exist normally within structural genes of wild-type organisms as a fixed means by which the cell can synthesize unequal numbers of enzymes. The *ame^r*-3 mutation might very well have occurred at such a modulating codon. It seems to be near the end of the gene, which is where it would be most definitively expressed, and determines an extraordinarily large (>100-fold) increase in the rate of synthesis of dihydrofolate reductase. The inability for joint expression with the *ame^r* mutations could mean that the level determined by this mutation alone is maximal for the translational machinery of this cell.

The basis for the effects on dihydrofolate reductase synthesis determined by *ame^r*-13 and *ame^r*-16 are also of interest, since *ame^r*-16, and probably *ame^r*-13, appear to be outside the gene for this enzyme. The size of the dihydrofolate reductase gene was estimated as being somewhere around 20 recombination units (percent), in length, (SIROTNAK and HACHTEL 1969). Further studies with these mutations are now in progress.

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SUMMARY

Thirty-two mutations, which map at the *AME I* locus on the *D. pneumoniae* chromosome, determine increased rates of dihydrofolate reductase synthesis. Thirty of these mutations appear to be at sites within the dihydrofolate reductase structural gene. Approximately half of these mutations also determine effects on the properties of the enzyme, primarily an altered affinity for amethopterin, but in two cases catalytic and physical modifications.—The effects on rates of enzyme synthesis by the structural gene mutations are best explained as translatable “missense” alterations of the nucleotide sequence of the gene introducing new limits of synthesis. The data do not exclude *a priori* effects at the transcriptional or translational levels or on the stability of the corresponding mRNA. There was also some indication that these effects may not all be on the same level of protein synthesis.

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