EVIDENCE FOR A GENETIC ALTERATION OF DIHYDROFOLATE REDUCTASE ASSOCIATED WITH AMETHOPTERIN RESISTANCE IN DIPLOCOCCUS PNEUMONIAE

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Enzyme alteration as a consequence of mutation to antimetabolite resistance has been reported by Moyed and Friedman (1959) and more recently by Wolf and Hotchkiss (1963) and Kalle and Gots (1963). The modified protein in each case retained its specific catalytic properties, but could be recognized by a decreased ability for interaction with a specific inhibitor. Other instances of genetically induced enzyme alteration, which, in some cases, did not modify reactivity with the normal substrate, have been reviewed by Fincham (1960). This report deals with apparent differences in the dihydrofolate reductase in extracts from a wild-type strain of Diplococcus pneumoniae and a mutant, ame^{r}-4, bearing a single mutation inducing a 500-fold increase in resistance to amethopterin. The methods used to obtain resistant mutants of D. pneumoniae and to identify the mutation borne by each have been described elsewhere (Sirotnak, Lunt and Hutchison, 1960a, 1964).

After harvesting in late logarithmic phase of growth from an enzymatically hydrolyzed casein medium (Sirotnak, Lunt and Hutchison, 1960b), wild-type and mutant cells were washed once in 0.05 M tris buffer (pH 7.4), resuspended again in 1/100 of the original volume and disrupted by sonication for 90 sec. at 5°C in an ultrasonic disintegrator (Measuring and Scientific Equipment Co., Ltd., London, England).

Folate-H_2 was prepared by the Blakley (1960) modification of the method of Futterman (1957). Dihydrofolate reductase activity was determined in a manner similar to that of Osborn and Huennekens (1958) by measuring the rate of decrease in absorbance (340 mμ) at 25°C when folate-H_2 and TPNH were combined with enzyme extract in 0.05 M potassium phosphate buffer containing 12.8 mM 2-mercaptoethanol.

Enzyme in extracts from the wild-type strain reduced folate-H_2 at pH 7.0 under the above conditions to the extent of 5 mμmoles/min/mg total protein. Activity of the mutant extracts at the same pH were somewhat higher. Following the addition of wild-type or mutant extract to the reaction system, the ultraviolet absorption spectrum of folate-H_2 was progressively changed to a spectrum characteristic of folate-H_4 at the completion of the reaction. Salient features of both wild-type and mutant enzyme activities are given in Table 1.

Table 1. Some properties of dihydrofolate reductase in extracts from wild-type and mutant strains of D. pneumoniae.

Property	Strain	
	wild-type	mutant
optimum pH	7.3	6.7
K_m for folate-H_2	3.0×10^{-6}M	3.2×10^{-6}M
K_m for TPNH	2.3×10^{-5}M	2.8×10^{-5}M
conc. of amethopterin required for 50% inhibition at pH 7.3	6.5×10^{-10}M	5.5×10^{-8}M
pH 6.7	6.7×10^{-10}M	6.0×10^{-9}M
time required for 50% inactivation at 50°C	6.0 min	0.5 min

The pH for optimum activity of dihydrofolate reductase in the wild-type extract was 7.3 as compared to 6.7 for the enzyme in the mutant extract. Enzyme-substrate interaction, otherwise, was apparently unchanged. The K_m value for folate-H_2 obtained in Lineweaver and Burk (1934) plots was 3×10^{-6} for enzyme in the wild-type preparation and 3.2×10^{-6} for enzyme in the mutant. The K_m

value for TPNH was 2.3×10^{-5} for the wild-type and 2.8×10^{-5} for enzyme in the mutant extract.

The mutant enzyme appears to be severely affected in its ability to react with amethopterin. The concentration of drug required for 50 percent inhibition was approximately 80 times that required for equal inhibition of the reductase in the same amount of wild-type preparation at pH 7.3 (optimum for wild-type enzyme activity). The difference in drug concentration required for the same level of inhibition at pH 6.7 (optimum for mutant enzyme activity) was only 11-fold.

Heat inactivation experiments suggest a far greater temperature sensitivity for the mutant enzyme. Fifty percent inactivation, at 50°C, of the activity in the mutant extract was 12 times more rapid then that seen with the wild-type preparation. That these observations are due to differences in the enzymes themselves was strongly indicated by the results of conventional mixing experiments. The residual activity of a mixture of both mutant and wild-type extracts after heating was always equal to the sum of the individual residual activities over a 5-fold range in protein concentration. The enzyme in each extract still retained characteristic heat sensitivity in the presence of the other.

It is suggested that the differences observed in pH optima, amethopterin inhibition and heat inactivation of the folate-H_2 reducing activities in wild-type and mutant preparations can best be explained on the basis of a genetically determined difference in the dihydrofolate reductase.

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