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A Suggested Mechanism for the Selective Procedure for Isolating Thymine-Requiring Mutants of *Escherichia coli*

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Aminopterin, an analogue of folic acid, binds very strongly to the enzyme dihydrofolate reductase, preventing the generation of tetrahydrofolate, the cofactor of a number of C_1 transfers (Huennekens, 1963). The synthesis of thymine, the purines, serine and methionine is thereby prevented and the inhibition of bacterial growth by aminopterin can only be overcome by supplying these metabolites. Ryan, Yanagisawa & Okada (1961) made the surprising observation with *Escherichia coli* that among the survivors of a prolonged incubation in the presence of high concentrations (400 μg./ml.) of aminopterin, in media supplemented with these metabolites, there were many cells that had lost the ability to synthesize their own supply of thymidylate. Incubation with aminopterin therefore provides a selective technique for the isolation of *thy*$^-$ mutants, which are otherwise but rarely found. It was later shown that this technique is much more powerful if a minimal medium supplemented only with thymine is used (Okada, Homma & Sonnohara, 1962; Stacey & Simson, 1965). Another inhibitor of dihydrofolate reductase, trimethoprim [2,4-diamino-5-(3,4,5-trimethoxybenzyl)pyrimidine] (Roth, Falco & Hitchings, 1962), because it penetrates *E. coli* cells more easily is an even more efficient selective agent (Stacey & Simson, 1965). With this drug it was possible to show that the mutation to thymine-dependence carries with it a genuine and substantial increase in resistance to the inhibitor (which is also shown by mutants selected by aminopterin). Moreover, *thy*$^-$ mutants isolated by other methods (e.g. *E. coli* 15T$^-$) are already very resistant to trimethoprim.

In *Diplococcus pneumoniae* two kinds of mutational change that give resistance to aminopterin are already known: one that leads to a higher dihydrofolate-reductase activity and another in which this enzyme is so altered as to be much less sensitive to the inhibitor (Sirotnak, Donati & Hutchison, 1964*a*,*b*; Albrecht, Palmer & Hutchison, 1966).

It was therefore decided to test these possibilities with the *thy*$^-$ mutants of *E. coli* (Hfr Cavalli) isolated with trimethoprim. No evidence for an altered dihydrofolate reductase could be adduced. Thus, when extracts of the wild-type *thy*$^+$ and of a *thy*$^-$ mutant were examined, K_m values for the reductase were similar (7×10^{-6}M at pH 7·0) and inhibition by trimethoprim was similar (Fig. 1), as were the effects of KCl, $CaCl_2$ and organic mercurials (Table 1). The dihydrofolate-reductase activity in the *thy*$^-$ mutant was the same as that of its *thy*$^+$ parent; the activities of other enzymes of C_1 metabolism found to be unchanged were serine hydroxymethylase and N^5N^{10}-methylene dehydrogenase. N^{10}-Formyltetrahydrofolate-synthetase activity could not be detected in extracts of either strain. Thus neither of the mechanisms of resistance observed in *Diplococcus* seems to be applicable to the *thy*$^-$ mutants of *E. coli*.

It would seem that the correlation between the loss of thymidylate synthetase and resistance to inhibitors of dihydrofolate reductase may be due to the one striking difference between thymidylate synthetase, which oxidizes tetrahydrofolate during methylation, and all the other biosynthetic enzymes, which transfer C_1 groups without oxidation. Thus tetrahydrofolate is consumed in substrate amounts

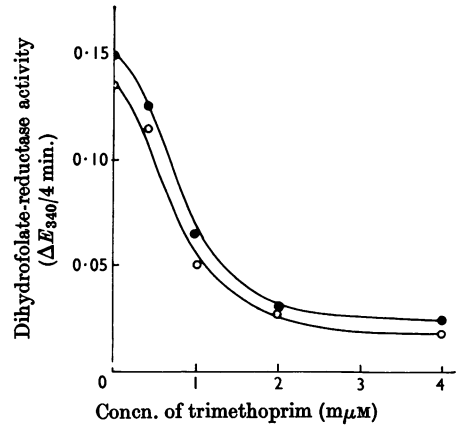

Fig. 1. Inhibition by trimethoprim of the dihydrofolate-reductase activity in extracts of wild-type (T$^+$) (●) and mutant (T$^-$) (○) strains of *E. coli*. The conditions of the experiments are detailed in Table 1.

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Table 1. *Effect of salts and organic mercurials on the dihydrofolate-reductase activity of wild-type (T^+) and mutant (T^-) strains of* E. coli

The complete system contained, in a final volume of 1·0 ml.: 50 μmoles of tris–HCl buffer, pH 7·0, 0·06 μmole of NADPH, 0·1 ml. of enzyme [50–90%-saturated $(NH_4)_2SO_4$ fraction] and 0·06 μmole of dihydrofolate containing 1 μmole of 2-mercaptoethanol. The change in E_{340} was recorded at 15 sec. intervals with a Gilford Multisample Absorbance Recorder attached to a Beckman DU spectrophotometer. Appropriate corrections were made for the slight oxidation of NADPH that occurred in the absence of dihydrofolate.

Addition	Dihydrofolate-reductase activity (ΔE_{340}/4 min.) Strain T⁻	Strain T⁺
None	0·145	0·080
KCl (0·15 M)	0·050	0·040
$CaCl_2$ (0·1 M)	0·015	0·015
Chloromercuribenzoate (0·5 mM)	0·100	0·060
Methylmercuric bromide (0·1 mM)	0·150	0·085
Urea (4 M)	0·165	0·090

in the synthesis of thymidylate, though it acts only catalytically and is therefore conserved in most of its other functions. We propose that, under the conditions of blockade of dihydrofolate reductase, the remaining supply of tetrahydrofolates is depleted by the action of thymidylate synthetase even though an ample supply of exogenous thymine is available. When, owing to a mutational change, this enzyme is not functional, sufficient tetrahydrofolate survives to fulfil its catalytic functions in all the other biosyntheses. The selective advantage of *thy*⁻ mutants under conditions of limiting tetrahydrofolate therefore lies in the economical use of this essential cofactor, which would be greater in minimal media in which the whole of the cell's requirements of serine, methionine and the purines must be synthesized. In line with this hypothesis, which is not easily tested directly, there are two other observations. The activity of at least two of the enzymes of the tetrahydrofolate network were unaltered in activity in the *thy*⁻ mutant. Two *thy*⁻ mutants of *E. coli*, 15T and B3, both of which were isolated directly, differ in their resistance to trimethoprim, strain 15T⁻ being more resistant than strain B3. They differ also in that strain 15T⁻ has virtually no functional thymidylate synthetase (Barner & Cohen, 1959) whereas strain B3 can synthesize some 20% of its thymine requirement (Seno & Melechen, 1964).

The possibility that a by-pass mechanism of this sort should confer resistance has also been suggested by Hitchings & Burchall (1965). But they point out that the experiments of Winkler & de Haan (1948) suggest that inhibition of methionine synthesis is the major block in sulphanilamide-inhibited *E. coli*. These experiments do not contradict the hypothesis presented above because sulphanilamide blocks the synthesis and not the reduction of folic acid.

We have confirmed that trimethoprim selects preferentially *thy*⁻ mutants and the yield is decreased if hypoxanthine is added to the selection medium. Methionine appears to have little or no effect.

Although changes in permeability to trimethoprim have not been looked for it seems unlikely that the resistance to this drug arises from changes of this kind, for revertants to thymine-independence regain their sensitivity to trimethoprim.

Note added in proof. The hypothesis presented here has been cogently argued by Wilson, Farmer & Rothman to explain the resistance to aminopterin of thymine-requiring mutants of *Bacillus subtilis*.

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