REPLACEMENT OF 5-METHYLURACIL (THYMINE) BY 5-ETHYLURACIL IN BACTERIAL DNA

Mirosława Piechowska & D. Shugar

(Department of Biophysics, Institute of Biochemistry & Biophysics, Academy of Sciences, Warsaw).

Received August 20, 1965

The various anti-metabolic 5-substituted uracil analogues capable of undergoing incorporation into DNA in place of thymine are normally those containing a 5-substituent (Cl, Br, I, CF_3, NH_2) which appreciably modifies both the electronic and hydrogen bonding properties of the pyrimidine ring. We report here preliminary findings on the incorporation into DNA of a thymine analogue containing a higher alkyl group (5-ethyluracil, 5EtU) which leaves essentially unmodified the hydrogen bonding properties of the pyrimidine ring, and differs from thymine (5MeU) principally by the larger van der Waals radius of the 5-substituent.

Our interest in this problem stemmed initially from the finding that poly-ribosethymidylic acid (poly-5MeU or poly-rT) forms a highly ordered structure under conditions where poly-U is a random coil; while the twin-stranded complex poly-(A + 5MeU) exhibits a T_m about 20° higher than the corresponding poly-(A + U) (Shugar & Szer, 1962). The enhanced helix stability due to the 5-methyl substituent cannot be due to modifications of hydrogen bonding properties; but may be accounted for, amongst others, by the hydrophobic properties of the apolar methyl group as in the case of polypeptides (Swierkowski, Szer & Shugar, 1965). It seemd desirable therefore to prepare poly-5-ethyl-uridylic acid (poly-5EtU) and poly-(A + 5EtU). The resulting availability

of 5-ethyluracil and 5-ethyluridine (Burchalter & Scarborough, 1955; Shapira, 1962; Swierkowski & Shugar, 1965) led to an examination of their effects on various microorganisms.

It had earlier been observed by Zamenhof & Griboff (1954) that 5-ethyluracil cannot replace thymine as a growth factor for E.coli T^- and that it suppresses growth in the presence of thymine. The growth of a prototrophic strain such as E.coli B, determined nephelometrically, has been claimed to be unaffected by 5-ethyluracil (Gut et al., 1959). While 5-ethyluracil, 5-butyluracil and 5-hexyluracil do not appear to affect the replication of animal viruses (Muraoka et al., 1962) all three have been reported to inhibit growth of E.coli T^-, estimated turbidimetrically (Shapira, Lowder & Hale, 1962).

We have found that 5-ethyluracil competitively antagonizes the utilization of thymine in cultures of the two thymine-dependent E.coli $15T^-$ and CR-34 strains. As the ratio of 5-EtU to 5-MeU is increased, there is initially inhibition of cell division with non-impairment of growth of cell mass; higher 5-EtU/5-MeU ratios result in suppression of cell growth and death of the cells. This is illustrated in Figs. 1 and 2, which present growth curves for E.coli $15T^-$ on the synthetic medium of Roberts et al. (1955) in the presence of 1 µg thymine per ml. (minimal concentration for normal growth) and increasing concentrations (10 to 100 µg/ml.) of 5-ethyluracil. It should, of course, be noted that measurements of colony formation are purely orientative and do not quantitatively correspond to the actual number of viable cells. This is due to the fact that the presence of 5-ethyluracil provokes the appearance of numerous elongated forms which attain up to 15 times the length of normal cells, as well as filaments consisting of up to a dozen or more organisms non-separated from each other following cell division. These are the usual manifestations of "thymineless death" (Barner & Cohen, 1954),

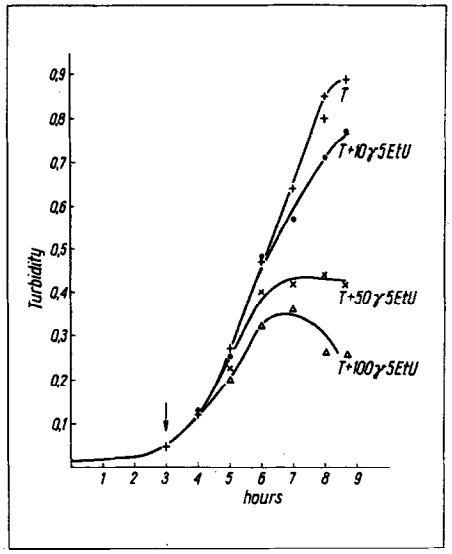

Fig. 1

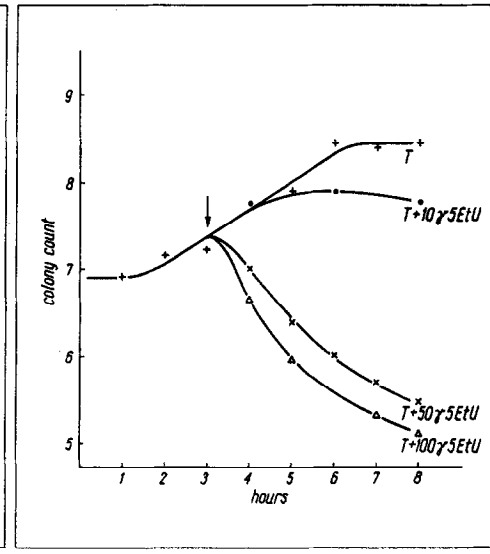

Fig. 2

Influence of various concentrations of 5-ethyluracil in the presence of 1 μg thymine (per ml.) on growth of E.coli 15T⁻ measured turbidimetrically (Fig. 1) and by colony count (Fig. 2).

When cultures of E.coli 15T⁻ or CR-34 were passaged from a thymine-containing medium to another in which thymine was completely replaced by 5-ethyluracil, loss of viability resulted with the same rapidity as in the absence of either thymine or 5-ethyluracil (furthermore, it did not prove possible under these conditions, to inhibit cell death by addition of other 5-alkyluracils such as 5-butyluracil and 5-hexyluracil). Measurements of the DNA content per unit mass of dried cells demonstrated inhibition of DNA synthesis in such cultures. By contrast, the addition of 5-bromouracil can, under identical conditions, maintain cell growth and DNA replication for short periods of time (Cohen & Barner, 1956).

Since turbidity is hardly the best criterion of cell growth under potentially abnormal conditions, the experiments of Gut et al. (1959), referred to above, were repeated. The prototrophic strain E.coli B was cultured on a synthetic medium in the presence of up to 1000 μg/ml. 5-ethyluracil. Measurements of the number of viable cells demonstrated the complete absence of any effect on growth and viability. In view of the absence of any influence of 5-ethyluracil on the prototrophic strain, it seems reasonable to conclude that the inhibitory effect on the auxotrophic strain is due exclusively to competition with thymine.

Incorporation of 5-ethyluracil into the DNA of E.coli $15T^-$ was examined in a culture medium containing the analogue in the presence of sub-optimal concentrations of thymine. The medium employed was that of Roberts et al. (1955) supplemented with 0.4 μg/ml. thymine (optimal requirement 1 - 2 μg/ml.). To a strongly aerated culture in the initial logarithmic growth phase (about 1.5×10^7 cells/ml.) was added 20 μg/ml. 5-ethyluracil and the culture maintained for 5 hours. Under these conditions there was a four-fold increase in cell mass; at the same time the number of colony-forming units remained unchanged or, in some instances, decreased at most 2 to 3-fold. The cells were harvested by centrifugation, washed with culture medium free of 5-ethyluracil, and the DNA then isolated according to the procedure of Marmur (1961). The purified DNA was hydrolyzed in formic acid (Wyatt, 1955) and the hydrolyzate chromatographed on Whatman 1 paper (ascending) with the solvent system isopropanol:HCl:water, 42.5:10.25:9.8, v/v/v. The spot corresponding to 5-ethyluracil was identified by comparison with a standard, as well as by elution and spectrophotometry, and repeated chromatography in water-saturated butanol. In the isopropanol-HCl-water solvent system the R_f values of 5-ethyluracil and thymine were 0.9 and 0.8 respectively. In water-saturated butanol the corresponding values were 0.67 and 0.48. The amount of 5-ethyluracil was quantitatively

estimated by spectral methods (Swierkowski & Shugar, 1965). Under the foregoing conditions it was found that up to 15% of the thymine in E.coli 15T⁻ was replaced by 5-ethyluracil.

It is obvious that the initial transformation which 5-ethyluracil must undergo, for incorporation into DNA, is to the corresponding deoxyriboside. An examination was therefore made of the behaviour of this analogue towards thymidine phosphorylase, using the E.coli B enzyme purified as described by Razzell & Khorana (1958). Under conditions of enzymatic exchange, where either uracil or 5-bromouracil underwent 50% transformation to the deoxyribosides in the presence of thymine, the extent of conversion of 5-ethyluracil was found to be only 6%. The low affinity of this analogue for thymidine phosphorylase was further estimated from the extent to which it inhibited the arsenolysis of thymidine. Under conditions where 0.25 mole equivalents (with respect to thymidine) of thymine gave 50% inhibition of arsenolysis, a similar concentration of 5-ethyluracil gave only 4% inhibition.

Consequently, in view of the up to 15% observed incorporation of 5-ethyluracil into the bacterial DNA, it may be concluded that a major obstacle to more extensive incorporation is the small extent to which it is transformed by the bacterial phosphorylase to 5-ethyluracil deoxyriboside. One cannot, of course, a priori exclude the possibility that a further block exists in the transformation of the deoxyriboside to the triphosphate. Attempts are therefore under way to prepare 5-ethyldeoxyuridine and to investigate both its degree of incorporation into bacterial DNA as well as its susceptibility to the various kinases involved in the formation of the triphosphate.

An examination has also been made of the utilization of 5EtU by two strains from another systemic group, Streptococcus faecalis 8043 and 9790, which are thymine-dependent in the absence of folic acid. For both of these 5EtU was unable to substitute for thymine, while addition

of 5EtU to a medium containing 1 μg thymine per ml. (optimal for normal growth) inhibited growth only at a concentration of about 500 μg/ml, hence much greater than for E.coli (see Figs. 1 and 2).

Finally, 5-ethyluridine was found to inhibit cell division of the thymine dependent E.coli and S. faecalis strains only at concentrations of the order of 200-500 μg/ml. By contrast that of the prototrophic E.coli strain was unaffected under analogous conditions; in fact the growth of E.coli B could be readily maintained by 5-ethyluridine on a glucose-deficient medium, indicating that the nucleoside serves as a source of sugar, which the organisms are apparently capable of liberating from the nucleoside.

Acknowledgments: We are indebted to Dr. W.H. Prusoff for bacterial strains; to Parke, Davis & Company for samples of 5-butyluracil and 5-hexyluracil; and to the World Health Organization for financial support for this work.

REFERENCES

1. Burchalter J.H. & Scarborough H.C., J.Am.Pharm.Assoc., 44, 545 (1955)
2. Cohen S.S. & Barner H.D., Proc.Nat.Acad.Sci., 40, 885 (1954)
3. Cohen S.S. & Barner H.D., J.Bacteriol., 71, 558 (1956)
4. Gut G., Morávek J., Párkányi C., Prystaš M., Škoda J. & Šorm F., Coll.Czech.Chem.Comm., 24, 3154 (1959)
5. Marmur J., J.Molec.Biol., 3, 208 (1961)
6. Muraoka M., Takada A. & Neda T., Keio J.Med., 11, 95 (1962)
7. Razzell W.E. & Khorana H.G., Biochim.Biophys.Acta, 28, 562 (1958)
8. Roberts R.B., Cowie D.B., Abelson P.H., Bolton E.T. & Britten R.J., "Studies of Biosynthesis in Escherichia Coli", Carnegie Institute of Washington Public., p. 5 (1955)
9. Shapira J., J.Org.Chem., 27, 1918 (1962)
10. Shapira J., Lowden L. & Hale R., J.Bacteriol., 83, 919 (1962)
11. Shugar D. & Szer W., J.Mol.Biol., 5, 580 (1962)
12. Swierkowski M., Szer W. & Shugar D., Biochem.Zeit., in press (1965)
13. Swierkowski M. & Shugar D., in preparation
14. Wyatt G.R., in THE NUCLEIC ACIDS (E. Chargaff & J.N. Davidson eds.), Vol. I, p. 243, Academic Press, New York (1955)
15. Zamenhof S. & Groboff G., Nature, 174, 306 (1954)