THE REDUCTION OF FOLATE BY BOROHYDRIDE AND BY DITHIONITE

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Dihydrofolate (DHF) prepared by dithionite reduction of folate (Blakley, 1960) or by catalytic hydrogenation is the substrate for bacterial and mammalian tetrahydrofolate dehydrogenase and has been considered to have the 7,8-dihydro structure (O'Dell <u>et al.</u>, 1947; Blakley and McDougall, 1962). Recently however, Smith <u>et al.</u> (1963) claimed to have prepared a new dihydrofolate isomer by borohydride reduction of folate. From a study of the chemical properties of this material they concluded that it was 7,8-DHF, and that, by exclusion, the product of dithionite reduction of folate must be 5,8-DHF. We present here the results of an investigation of the products of the action of borohydride and of dithionite on folate.

Folate was reduced by potassium borohydride as described by Smith <u>et al.</u> (1963) but without precipitation of the products at acid pH. After high voltage paper electrophoresis of the product several bands were visible under ultra-violet light, and the positions and appearance

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of these corresponded to reference samples of folate, DHF and to pteridines formed by breakdown of folate compounds.

To further investigate these products, 119 μmoles of folate was reduced with borohydride (Smith _et al._, 1963) and when the reaction was complete formaldehyde and 2-mercaptoethanol were added. Enzymic assay with THF dehydrogenase showed that 3% of the starting material had been converted to the "dithionite DHF". The mixture was chromatographed on a column of TEAE-cellulose (60 x 2.8 cm.) and eluted with a linear gradient obtained by the use of 2.5 l. each of 0.01 M and 1.0 M potassium phosphate buffers, pH 7.3, containing 50 mM 2-mercaptoethanol and 50 mM formaldehyde. The _l_,L- and _d_,L-methylene-THF were eluted as separate peaks (Ramasastri and Blakley, 1963) and the total amount of THF (from absorbancy) accounted for 20% of the original folate. A third peak was eluted at higher phosphate concentration and this material, when isolated, showed spectra identical to those of folate at acid, alkaline and neutral pH values. However, as it was chromatographically and electrophoretically distinct from folate and was much more soluble than the latter in organic solvents, it was at first thought to be the new dihydrofolate isomer which Smith _et al._ (1963) claimed to have synthesised. Assuming a similar molar extinction to that of folate, this material accounted for approximately 60% of the original folate.

When the products from a similar reduction were chromatographed on TEAE-cellulose without addition of 2-mercaptoethanol and formaldehyde to the reaction mixture or to the eluting buffers, the methylene-THF peaks were eluted from the column as previously, but the material obtained from the third peak was in this case identical with folate in all respects, i.e. solubility, absorption spectra and behaviour on paper chromatography and electrophoresis. This suggested that the new compound obtained in the original experiment had been formed by the reaction of folate with 2-mercaptoethanol and formaldehyde during evaporation of the fractions from the column. Subsequent experiments confirmed that folate does react with mercaptoethanol and formaldehyde under certain conditions to produce the new compound which is under investigation.

The large amount of unreacted folate recovered from the reaction mixture suggested that insufficient borohydride might have been used. The procedure of Smith et al. (1963) was therefore repeated four times on a single solution of folate with readjustment of the mixture to pH 5.0 before each addition of borohydride. Paper electrophoresis showed that although repeated borohydride treatment increased the breakdown of folate compounds, the yield of THF increased to 50% of the original material (methylene-THF dehydrogenase assay), and residual unreacted folate was decreased in amount but not eliminated. An increase of the yield of THF to 47% and a decrease in unreacted folate to 30% was obtained when a single reduction by the method of Smith et al. (1963) was modified by using a solution of folate eight times more concentrated.

As it appears likely that the chemical studies which Smith et al. (1963) report were carried out on a mixture of products similar to those which we obtained, the evidence they present regarding the 7,8-DHF structure of their product cannot be accepted as valid. We found no evidence of the new compound to which Smith et al. (1963) refer.

In contrast to the complex mixture of products obtained with borohydride, we found dithionite reduction of folate (Blakley, 1960) produces no THF and few other contaminants of the major product. It was observed that solutions of this material were relatively stable in the absence of 2-mercaptoethanol at pH 9.5, i.e. when the DHF was present as the trisodium salt. High voltage paper electrophoresis at this pH revealed the presence of a small amount of an orange and a blue fluorescing contaminant. The nuclear magnetic resonance (NMR) spectrum of the trisodium salt in D_2O was examined by Dr. A.V. Robertson of the University of Sydney, and indicated that the major constituent was 7,8-DHF. While this work was in progress, Pastore et al. (1963) reported an NMR spectrum almost identical with that obtained by Dr. Robertson. As reported by others (Pastore et al., 1963; Zakrzewski, 1963) we found no significant incorporation of tritium into DHF to occur when folate was reduced by dithionite in tritiated water, but incorporation of approximately 1 atom of deuterium per molecule of DHF occurred when reduction took place in D_2O. This result with D_2O is consistent with a 7,8-dihydro structure for the product of dithionite reduction of folate and the absence of tritium incorporation into this product from tritiated water must be due to an extraordinarily high isotope selection effect.

The finding that borohydride action on folate produces THF and a small amount of 7,8-DHF, while the latter is the major product of dithionite action, indicates that the two agents reduce folate in a similar manner, differing mainly in the fact that borohydride is able to carry on the reduction of 7,8-DHF to THF. It is noteworthy that in the only case in which there appeared to be definite evidence for the chemical reduction of a pteridine to a 5,8-dihydropteridine (Pfleiderer and Taylor, 1960) further studies have indicated that the reduced product does not have a 5,8-dihydro structure (Taylor, 1963). It therefore seems unlikely that 5,8-dihydrofolate would be formed by the action of dithionite on folate.

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