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REDOX REACTIONS OF ISOALLOXAZINES WITH 2-HYDROXY-1,3-PROPANEDITHIOL AND NADH; THE ROLES OF THE 4a- AND 5-POSITIONS.

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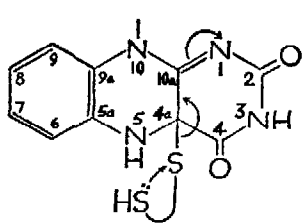
Summary: Kinetic evidence eliminates the 6- and 8-positions as essential centres for dithiol addition in reduction of some new isoalloxazines; and suggests that specific orientation effects of NADH within its complexes with the isoalloxazines may be responsible for the abnormal order of relative rates of isoalloxazine reduction observed.

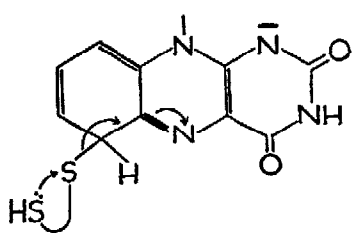
Since the initial suggestion by Hamilton[1] that in flavin reductions by dithiols, electron transfer might occur through the formation and breakdown of a covalent adduct at the 4a-position(I), consideration has been given to addition at alternative sites on the flavin molecule. One kinetic study[2] has eliminated the 10a-position as a centre for such a reaction, and another provides kinetic evidence for formation of a covalent adduct and implicates the 4a-position as a more likely centre for adduct formation than the 5-position[3]. The possible roles of the 6-position (e.g. as in II) and the 8- and 9a-positions have received little attention, although the latter is eliminated by the study[2] which excluded the 10a-position.

To determine whether free 6- or 8-positions are essential for flavin reduction by dithiols, and at the same time to study the effect of steric crowding in the region of the 5-position, we synthesised[4] isoalloxazines A, B and C, and compared their rates of anaerobic, dark reduction[5] by 2-hydroxy-1,3-propanedithiol.

Addition at the 6-position of A or the 8-position of B would involve



I



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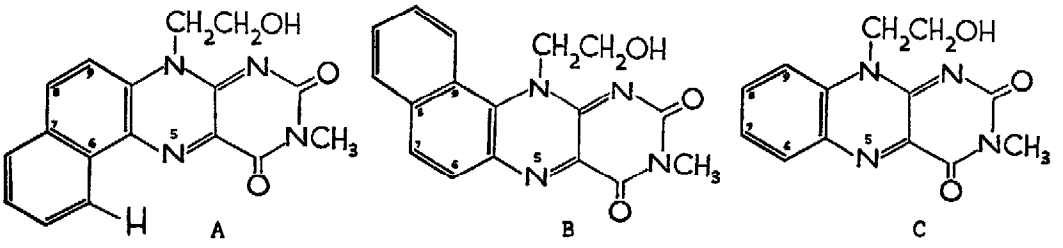


total loss of aromaticity of the naphthalene moiety and would be expected to be particularly unfavourable as compared with addition at the 6- or 8-positions of C. Furthermore, in A position 5 suffers in-plane crowding by hydrogen similar to the crowding of hydrogen atoms at positions 1 and 8 of naphthalene[6]. There is no such crowding in B or C.

We anticipated that dithiol oxidation involving essential adduct formation at the 5- or 6-positions (A) or the 8-position (B) might result in an abnormally low rate of reaction for the isoalloxazine concerned. As Figure 1 shows, however, a linear relationship exists between log k_2 for dithiol oxidation and the polarographic half-wave potentials measured[7] for the isoalloxazines. The gradient is $25V^{-1}$ which may be compared to a value of $32V^{-1}$ for the reaction of 1,4-butanedithiol with a different series of isoalloxazines[2]. Clearly, rates of isoalloxazine reduction by 2-hydroxy-1,3-propanedithiol are not depressed by reduced reactivity of (in A) the 6-position and (in B) the 8-position and we conclude that *these are not essential centres for adduct formation*.

But with regard to the sterically crowded 5-position in A, can the lack of rate depression for A be taken to eliminate the possibility of adduct formation here? If, as Hemmerich states[8], "5-addition involves strictly in-plane attack" with respect to the planar flavin molecule, then steric hindrance of 5-addition of dithiol would be expected for A and our results would eliminate

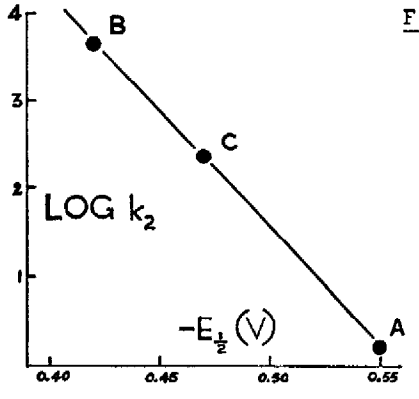
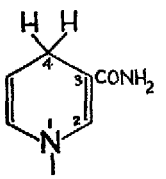


Figure 1: Log k_2 for reduction of A, B and C by 2-hydroxy-1,3-propanethiol vs. polarographic half-wave potentials. k_2 values ($M^{-1}min^{-1}$) were determined at 30° and pH 9.20 (0.1M borate).

that position as an essential one for thiol addition. However, we see no objection to attack at position 5 from above the plane of the isoalloxazine and even though such attack might still be hindered in A, we consider that the linear relationship of Figure 1 is *insufficient evidence to exclude conclusively a 5-adduct as an intermediate in the redox reaction.*

The direct transfer of hydrogen from the 4-position of the 1,4-dihydronicotinamide moiety (III) of NADH to the 5-position of 5-deazaflavin[9] along with evidence[2] that a face-to-face molecular complex of flavin and NADH is formed prior to hydrogen transfer, together suggest that hydrogen transfer occurs to the 5-position of flavin from within such a complex. It might therefore be expected that the relative orientation of the 4-position of NADH and the 5-position of flavin in such a complex would in part determine the overall rate of flavin reduction.

The second order rate constants for reduction by NADH of isoalloxazines A, B and C were determined[5] as 58, 19 and 50$M^{-1}min^{-1}$ respectively (pH 6.90; 0.05M phosphate; 30°). This unusual order of flavin reactivity (A>C>B) is the reverse of that expected from $E_{\frac{1}{2}}$ values and from dithiol oxidation rates (see Figure 1); in particular, A is reduced three times more rapidly than B by NADH whereas in the reaction with 2-hydroxy-1,3-propanedithiol B is reduced nearly three thousand times faster than A. Previous studies[10] have shown that rates of reduction of flavins by NADH parallel the flavin $E_{\frac{1}{2}}$ values and rates of dithiol oxidation provided[2,10] there are no bulky out-of-plane substituents which would sterically hinder complexing. In the absence of any such substituents in isoalloxazines A, B and C, we are forced to the conclusion that the exceptional order of reactivity of these isoalloxazines results from *specific differences in orientation of NADH with respect to isoalloxazine within its complexes with A, B and C.* Thus, the data are consistent with the interpretation that an additional benzo-substituent on the isoalloxazine can, depending on its orientation, promote complexing of NADH either closer to the 5-position of isoalloxazine (A; 6,7-benzo substituent) or further away from the 5-position (B; 8,9-benzo substituent); such complexing thus becomes more (A) or less (B) productive in terms of subsequent hydrogen transfer from the 4-position of NADH to the 5-position of isoalloxazine, this difference being strongly reflected in the overall rates of the redox reactions.



III

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4. Satisfactory elemental analyses and mass spectra have been obtained for A, B and C.

5. Rates were determined on a Cary 17 spectrophotometer by following the disappearance of the visible absorption band of isoalloxazine. Addition of air after completion of reaction gave immediate quantitative formation of reoxidised isoalloxazine.

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7. $E_{\frac{1}{2}}$ values were measured anaerobically at pH 8.9 in 0.1M borate buffer, using a saturated calomel reference electrode.

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