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pH Dependency of the nonenzymic oxidation of dihydroflavin by *p*-nitrobenzoic acid and its methyl ester

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RIBOFLAVIN, its derivatives, and flavin-containing enzymes are recognized for their ability to reduce nitro compounds. The fact that a flavo-enzyme could nonspecifically reduce nitro compounds was established over 25 years ago.[1] Since that time, more specific enzyme systems have been isolated and labeled nitro reductases. Fouts and Brodie[2] isolated a nitro reductase activity which is markedly stimulated by the addition of flavin derivatives. Later, Kamm and Gillette[3] demonstrated that one of the nitroreductase activities is associated with the microsomal NADH–cytochrome *c* reductase, that FAD is reduced, and that the incipient $FADH_2$ reacts nonenzymically with *p*-nitrobenzoic acid (NBA). This system was further characterized by Kato *et al.*,[4] who demonstrated that *p*-hydroxyaminobenzoate (HABA) is an intermediate, and therefore, that the reduction of NBA to *p*-aminobenzoate (ABA) proceeds stepwise.

In a completely nonenzymic system, Juchau *et al.*[5] demonstrated that sulfhydryl compounds and reduced pyridine nucleotides can supply the electrons, via flavin, to NBA. Upon the addition of boiled, dialyzed placental supernatant, the amount of ABA formed increased by 10-fold. A conclusion of these studies was that there is a component present in the suspension which catalyzes the production of ABA. Whether or not this enhancement reflects only the conversion from HABA to ABA was not determined.

The systems discussed above are still rather complex systems which have not clearly delineated the actual role of dihydroflavins. Consequently, we set out to examine the oxidation of dihydroflavin mononucleotide by NBA and its derivatives in a chemically well defined system. In our initial studies, we were struck by the relatively poor reactivity of NBA when compared with its methyl ester, and therefore, wish to present our findings on the relative rates of reaction for NBA and its methyl ester, and the influence of pH upon these rates.

NBA was purchased from Eastman Organic Chemicals. The methyl ester, prepared from an HCl–methanolic solution of NBA and recrystallized twice, melted at 96–97°, in agreement with the literature.[6] The compound gave ultraviolet and infrared spectra consistent with its structure. *p*-Hydroxyaminobenzoic acid was prepared by the method of Bauer and Rosenthal.[7] The 5% platinum on asbestos was obtained from Fisher Scientific Company. Riboflavin-5′-phosphate (FMN) was supplied *gratis* by Sigma Chemical Company.

The anaerobic system used for these studies is illustrated in Fig. 1. FMN (0·15 μmole), buffer as indicated in the legend to Fig. 2 (0·60 nmole), water to a total volume of 2·6 ml, approximately 10 mg platinum on asbestos, and a magnetic stirring bar were placed in the reduction flask (A). The nitro compound (0·8 to 3·0 μmoles NBA or 0·2 to 0·4 μmole methyl ester) and an appropriate amount of water for a final volume of 1·4 ml were placed in the sidearm (B). The system was evacuated and filled with hydrogen a number of times while being stirred. When the solution became almost colorless due to the reduction of the FMN, the system was evacuated and inverted. Introduction of either nitrogen or hydrogen allowed the solution of dihydroflavin to be filtered through paper and porous glass (C) into the spectrophotometer cell (D). The apparatus was then placed in a Cary model 14 recording spectrophotometer.

An initial reading of the absorbance at 445 nm was taken to insure that the FMN was fully reduced. The system was then tipped, mixing the dihydro-FMN with the nitro compound in the sidearm, and the increasing absorbance at 445 nm was continuously recorded. After an appropriate period of time, the apparatus was opened to allow any remaining dihydro-FMN to be oxidized by air and the final absorbance (A_f) was recorded. The absorbance at 0·25 min was arbitrarily designated as the initial absorbance (A_i). The fraction of dihydro-FMN remaining at any time was calculated from the absorbance at that time (A_t) by the relationship: fraction remaining equals $(A_f - A_t)/(A_f - A_i)$. When the reciprocal of the fraction remaining was plotted against time, a straight line was obtained until 15–20 per cent of the nitro compound had reacted. The slope divided by the initial concentration of dihydro-FMN results in an apparent second-order rate constant. When similar studies were done at different initial concentrations of the nitro compound and the apparent second-order rate constants were plotted against the initial concentration of the nitro-compound, a third-order rate constant could be calculated from the slope. The detailed kinetic analysis of these reactions will be presented at a later time.

Stoichiometric experiments confirmed that 2 moles dihydro-FMN is used for each mole of NBA-methyl ester added. These experiments were performed as described above, except that the dihydro-FMN was in excess, i.e. 0·30 mole dihydro-FMN and 0·10 mole of the ester; the pH was 7. The increment in absorbance was monitored until there was none for 30 min. Calculations revealed that the ratio of dihydro-FMN used per NBA-methyl ester added was 2. Therefore, the reaction can best be depicted by the equation:

$$2\ FMNH_2 + R{-}NO_2 \rightarrow 2\ FMN + R{-}NHOH + H_2O$$

where R is 4-carbomethoxyphenyl.

Figure 2 shows the pH dependency of the oxidation of dihydro-FMN by NBA and its methyl ester. With regard to the acid from pH 4 to pH 9, the rate of nonenzymic oxidation is low when compared to the rate at either end of the pH range employed. The higher rate of oxidation of the dihydro-FMN at the low pH range probably represents the protonation of the carboxylate, which then behaves somewhat like the ester. The higher rate of oxidation at high pH is presently under study and probably reflects a specific base catalysis not common to the low and mid-range pH values.

The pH dependency of the reaction with the methyl ester differs significantly from that of the free acid. The reaction rate changes appreciably in the range from 5 to 7, such that at pH 7 the ester oxidizes dihydro-FMN at a rate approximately 100 times that of the free acid. It should be noted that the calculated pK involved in the region is about 6·2 and may very well represent the ionization of the dihydro-FMN.

While others have used NBA extensively in studies on the nitro reductases, our studies have shown that, in nonenzymic systems at physiological pH, it is more appropriate to use the methyl ester of NBA rather than the acid. However, the same order of reactivity remains to be shown for enzymic systems. Since flavo-enzymes, e.g. D-amino acid oxidase,[8] are known to form complexes with benzoic acid, it is feasible that in an enzymic system the NBA present as a protein complex would behave as if it were protonated and would have a greater reactivity.

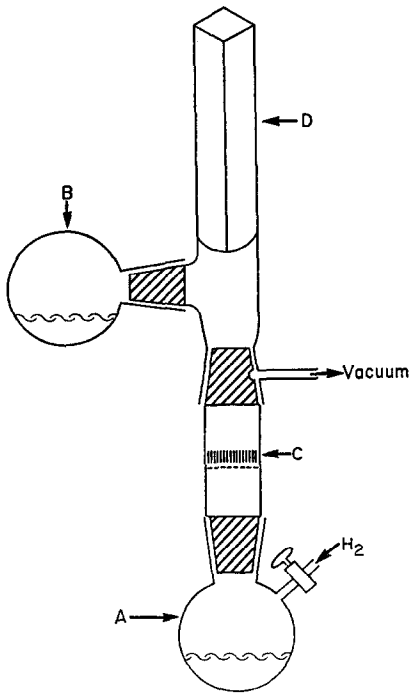

FIG. 1. The anaerobic apparatus shown in the reduction mode. The flasks (A and B) are 10-ml round bottom. The filter (C) is a medium porosity glass filter covered with Whatman No. 1 filter paper. The spectophotometer tube (D) is Pyrex. All glass joints (14/20) were sealed with A. H. Thomas Lubriseal high vacuum grease.

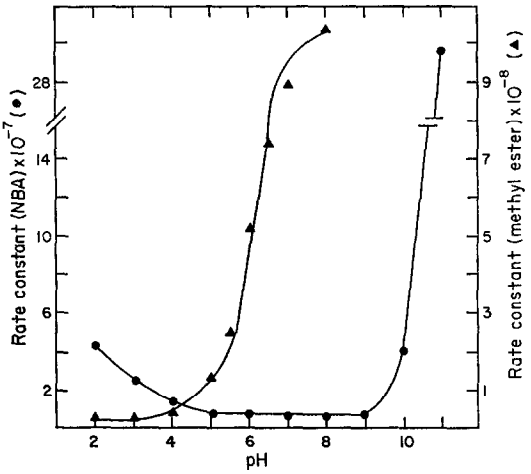

FIG. 2. Effect of pH on the third-order rate constant of oxidation of dihydro-FMN by NBA and its methyl ester. The rate constants have the dimensions of M^{-2} min^{-1}. The rate of oxidation of dihydro-FMN by NBA (●——●) and its methyl ester (▲——▲) are shown. Buffers: pH 2, 3, 6·0, 6·5, 7·0 and 11·0 were phosphate; pH 4, 5 and 5·5 were acetate; pH 9·0 and 10 carbonate; pH 8·5 was Tris.

The primary product of the nonenzymic reduction of nitro compounds by reduced flavins is the hydroxyamino compound and not the amino compound. This is based upon our observations that the reaction follows second-order kinetics with respect to the flavin and that only 2 moles dihydro-FMN is used for each mole of NBA-methyl ester added. The hydroxyamino compound is known to disproportionate, giving the nitroso and amino compounds[9] and, while this is a slow reaction with respect to the flavin-catalyzed reaction, it makes identification of the products by chromatography very equivocal. We have tried two thin-layer chromatography systems[4,10] and have found that both lead to the disproportionation of authentic *p*-hydroxyaminobenzoate. It therefore seems inappropriate to measure the activity of nonenzymic reduction of NBA with dihydroflavin by assaying for the formation of ABA, as has been done by others.[5]

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