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Reaction of *N*-Bromosuccinimide with Reduced Ring Systems. Apparent Protection of Glutamate Dehydrogenase by Reduced Nicotinamide–Adenine Dinucleotide†

Murray R. Summers

ABSTRACT: In response to the use of reduced molecules to protect a protein from oxidation by *N*-bromosuccinimide, this reagent can be shown to react stoichiometrically with NADH, dihydrofolic acid, and 3,5-dimethyldihydropteridine. In each case, spectra characteristic of the oxidized molecule are generated. The reaction of *N*-bromosuccinimide with NADH is shown to occur even in the presence of a competing protein molecule (glutamate dehydrogenase). In this system, the NADH is oxidized preferentially to the protein; only when all NADH is reacted, does the *N*-bromosuccinimide begin to modify protein molecules, as determined by decreasing enzyme activity. This reaction of *N*-bromosuccinimide should be given consideration when one is "protecting" a protein from modification by adding reduced cofactors.

Dihydrofolate reductase has been shown to be irreversibly inhibited by oxidation with *N*-bromosuccinimide (Freisheim and Huennekens, 1969). The same investigators found that this inhibition was markedly delayed when the *N*-bromosuccinimide reaction was carried out in the presence of NADPH or dihydrofolic acid, both of which molecules are substrates for the enzyme. While evidence of this nature is suggestive of a modification in the active site, one must be certain that the modifying reagent does not react with the substrates themselves. This control was never done in the dihydrofolate reductase study. Experiments reported in this communication show that as expected *a priori* (Filler, 1963), a rapid reaction does occur between *N*-bromosuccinimide and reduced molecules, even in the presence of a competing protein molecule. The extent of protection, if any, by these reduced substrates is therefore difficult to judge, when molecules such as these are used to protect proteins from oxidation by reactive chemicals.

Experimental Section

Materials. *N*-Bromosuccinimide was purchased from Eastman Chemical Co. and was recrystallized once from H_2O before use. Both glutamate dehydrogenase (Type II) and NADH (Grade III) were obtained from Sigma Chemical Co. and were used with no further purification. The H_2folate and

† From the National Institute of Arthritis and Metabolic Diseases, National Institutes of Health, Bethesda, Maryland. *Received March 20, 1972.*

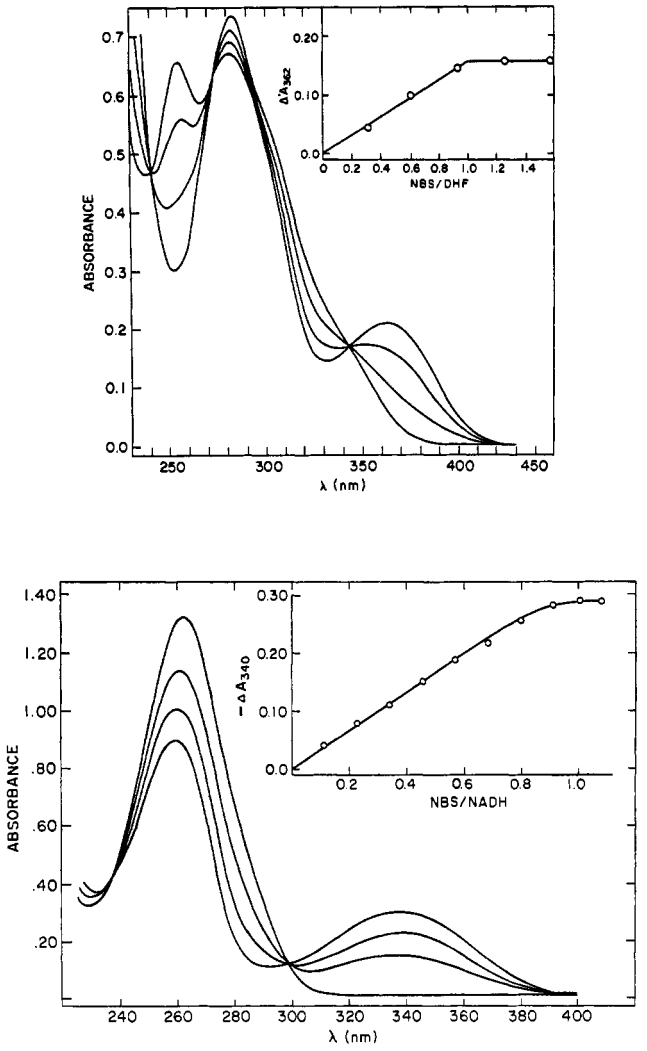

FIGURE 1: Spectrophotometric titration of dihydrofolic acid (DHF) (upper figure) or NADH (lower figure) with *N*-bromosuccinimide (NBS). DHF: the spectra shown aret hose obtained in the presence of 0, 8.2×10^{-6}, 1.6×10^{-5}, and 4.1×10^{-5} M NBS, respectively. NADH: the spectra shown are those obtained in the presence of 0, 1.7×10^{-5}, 3.5×10^{-5}, and 7×10^{-5} M NBS, respectively. Other spectra are omitted in both titrations for clarity, but all experimental points are shown on the inset graphs, which give the stoichiometry for both reactions.

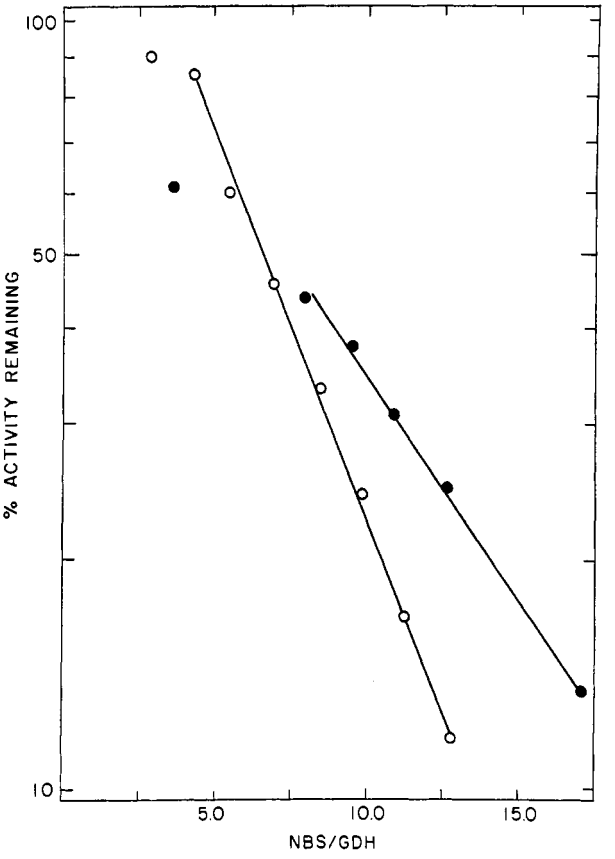

FIGURE 2: The loss of glutamate dehydrogenase (GDH) activity as a function of NBS concentration. (○) Addition of NBS to a solution containing only GDH; (●) corrected points (as described in the text) for the addition of NBS to a solution containing GDH and 16×10^{-5} M NADH.

3,5-dimethyldihydropteridine were gifts of Drs. Bernard T. Kaufman and Gordon Guroff, respectively.

Methods. N-BROMOSUCCINIMIDE REACTION WITH NADH. A spectrophotometer cell was filled with 2.5 ml of a solution of NADH (5×10^{-5} M) in 0.02 M phosphate buffer (pH 7) containing 0.1 M sodium acetate. The spectrum of this solution was then determined in a Cary 15 spectrophotometer. Aliquots of several microliters of 7 mM aqueous *N*-bromosuccinimide were added to the cuvet and the contents were mixed by inverting. The spectrum was again recorded. This procedure was repeated until the titration was complete.

N-BROMOSUCCINIMIDE REACTION WITH H_2FOLATE OR 3,5-DIMETHYLDIHYDROPTERIDINE. These procedures were identical to that for NADH except that they were carried out in 20 mM Tris-HCl (pH 8).

N-BROMOSUCCINIMIDE REACTION WITH GLUTAMATE DEHYDROGENASE (±NADH). Ten milliliters of glutamate dehydrogenase (0.5 mg/ml) (±16×10^{-5} M NADH) was prepared in 0.02 M phosphate buffer (pH 7.0) containing 0.1 M sodium acetate. Two aliquots of this solution were withdrawn. One (20 μl) was used to assay for glutamate dehydrogenase activity. The other (1.5 ml) was placed in a spectrophotometer cell, scanned in the ultraviolet region, and then returned to the reaction mix. Aqueous *N*-bromosuccinimide (7 mM) was added and the solution was well mixed using a magnetic stirrer. Two aliquots were again withdrawn, etc.

GLUTAMATE DEHYDROGENASE ACTIVITY ASSAY. The 20-μl aliquot of glutamate dehydrogenase was added to a cuvet containing 10^{-4} M NADH, 10^{-3} M α-ketoglutarate, 0.1 M NH_4Cl, and 10^{-3} M EDTA in 2.5 ml of the same phosphate buffer as above. The contents were mixed by inverting and the rate of disappearance of NADH was followed at 340 nm.

Results

Reaction of N-Bromosuccinimide with Reduced Molecules. Figure 1 shows the titration of two reduced ring systems with *N*-bromosuccinimide in the absence of protein. The changes in absorbance were stoichiometric to the amount of *N*-bromosuccinimide added, as shown in the inset figures. For NADH, zero absorbance at 340 nm is reached when the *N*-bromosuccinimide:NADH molar ratio is approximately unity. The remaining spectrum is typical of oxidized NADH, although the peak at 260 nm has undergone a slight red shift. For H_2folate, the peak absorbance at 362 nm, a characteristic

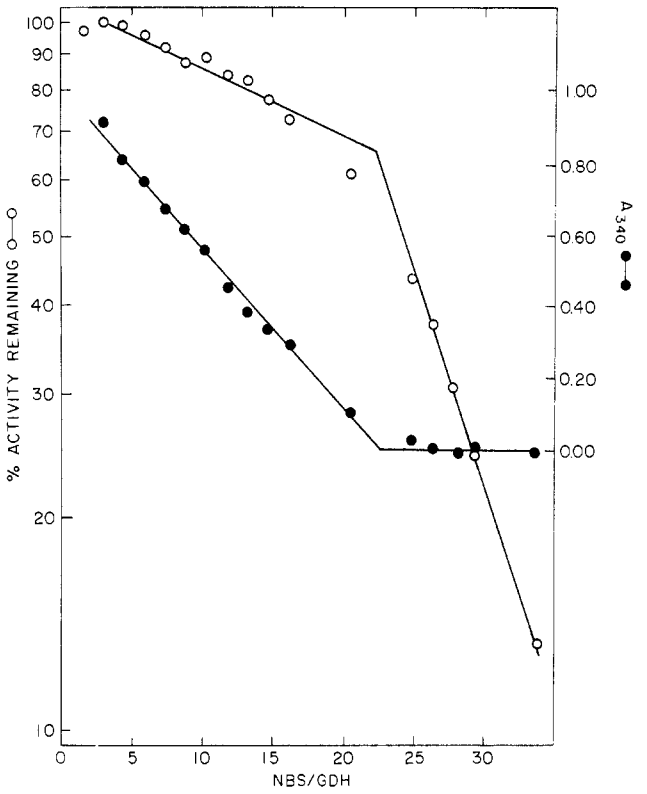

FIGURE 3: The reactions of NBS in the presence of GDH and NADH. The loss of GDH activity in the presence of 16×10^{-5} M NADH is shown by (○). The corrected A_{340} of the inactivation mix at the same NBS concentrations is shown by (●).

of oxidized folates,[1] reaches a maximum value when *N*-bromosuccinimide $\cong$ H_2folate. Completely analogous results were also obtained with 3,5-dimethyldihydripteridine.

Reaction of N-bromosuccinimide with Glutamate Dehydrogenase (±NADH). In order to examine how this reaction of *N*-bromosuccinimide might interfere with the interpretation of "protection" experiments, the modification of glutamate dehydrogenase by *N*-bromosuccinimide in the absence of NADH was studied. These data are shown by the open circles, in Figure 2. Below a 5 molar excess of *N*-bromosuccinimide there is evidence that a sulfhydryl group in glutamate dehydrogenase is consuming modifying reagent.[2] At *N*-bromosuccinimide concentrations higher than this, there is a definite loss of enzyme activity. Less than 12% of the original activity remains after the enzyme is treated with a 13-fold excess of *N*-bromosuccinimide. When the same inactivation experiment is carried out in the presence of NADH (Figure 3), the loss of catalytic activity is biphasic and much delayed with respect to the control curve in Figure 2. There would appear to be an efficient protection of glutamate dehydrogenase activity by the presence of NADH. If, however, the effect of NADH is simply to consume added *N*-bromosuccinimide before it has an opportunity to react with glutamate dehydrogenase thereby delaying the onset of loss of glutamate dehydrogenase activity, this oxidation of NADH should be apparent from the A_{340} of the reaction mixture. This is the case, and the experimental points are shown by the filled circles in Figure 3. These absorbance values have been corrected for protein scattering. Only when A_{340} has reached zero does the loss of glutamate dehydrogenase activity approach an *N*-bromosuccinimide dependence equivalent to the control. Thus, the apparent protection does not reflect an altered reactivity of the glutamate dehydrogenase–NADH complex; rather, it shows the progressive consumption of modifying reagent as long as any NADH is present.

Discussion

These data show that *N*-bromosuccinimide reacts with NADH even in the presence of a competing protein. The reaction of *N*-bromosuccinimide with the protein does not occur at a normal pace until all of the NADH has been consumed. Subtraction of the excess *N*-bromosuccinimide required by the reaction with NADH from the total *N*-bromosuccinimide concentrations shown in Figure 3 generates the filled circles shown in Figure 2. The difference in slope between the corrected inactivation and the control inactivation curves almost certainly does not reflect a protection of the enzyme by NADH, since the cofactor has already been consumed. These reactions of *N*-bromosuccinimide with reduced ring systems as well as the character of the reaction with glutamate dehydrogenase in the presence and absence of NADH demonstrate the difficulty of interpretation of protection-type experiments with these compounds.

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[1] This reaction may be of some interest as a means of preparing oxidized folates. It also appears to convert N^5-formyltetrahydrofolate to an oxidized form in good yield.

[2] Manuscript in preparation.