Weber, A., and Winicur, S. (1961), *J. Biol. Chem. 236*, 3198.
Weber, H. H., and Portzehl, H. (1962), *Ergeb. Physiol. Biol. Chem. Exptl. Pharmakol. 47*, 24.
Willis, J. B. (1960), *Spectrochim. Acta 16*, 551.

Effect of *N*-Bromosuccinimide on Dihydrofolate Reductase*

J. H. Freisheim† and F. M. Huennekens

ABSTRACT: Dihydrofolate reductase from chicken liver contains four tryptophan residues per mole (mol wt 22,000), as determined by: (a) the absorbancy ratio at 280 and 288 mμ at pH 6.5 in the presence of 6 M guanidinium chloride; or (b) the decrease in absorbancy at 278 mμ after treatment of the enzyme with excess *N*-bromosuccinimide at pH 4.0 in the presence of 5.3 M urea. Between pH 6 and 8, and in the absence of urea, the action of *N*-bromosuccinimide on the enzyme occurs in two stages. Relatively low levels of *N*-bromosuccinimide (up to 4 moles/mole of enzyme) cause a two- to threefold *increase* in catalytic activity without any appreciable destruction of the tryptophans. This activation is probably due to oxidation of a sulfhydryl group on the protein since it can be reversed by dithiothreitol. Higher concentrations of *N*-bromosuccinimide (*ca.* 10 moles/mole of enzyme) result in tryptophan oxidation with a concomitant *decrease* in enzymatic activity.

Complete loss of activity, however, corresponds to the oxidation of only one tryptophan residue. Incubation of the enzyme with either reduced triphosphopyridine nucleotide or dihydrofolate affords protection against the effects of *N*-bromosuccinimide. These results indicate that at least one tryptophan residue, and a sulfhydryl group as well, are located at or near the substrate binding sites of the enzyme, and that oxidation of these moieties by *N*-bromosuccinimide leads to conformational changes in the protein, either at the active site or at some other location which, in turn, can affect the active site.

Recent studies from this laboratory[1] have shown that the binding of substrates (TPNH and dihydrofolate) or inhibitors (aminopterin and amethopterin) to highly purified chicken liver dihydrofolate reductase is accompanied by a decrease in fluorescence of the protein. Tryptophan residues[2] are believed to be responsible for the observed fluorescence. Similar observations and interpretations have been made for dihydrofolate reductase from L1210 cells (Perkins and Bertino, 1966; Hillcoat *et al.*, 1967), as well as for a number of other pyridine nucleotide dependent enzymes (Boyer and Theorell, 1956; Velick, 1958; Winer and Schwert, 1959; Theorell and McKinley-McKee, 1961; McKay and Kaplan, 1964).

The present study was undertaken in order to investigate more directly the role of tryptophan residues in substrate binding and in the catalytic mechanism of the chicken liver enzyme. *N*-Bromosuccinimide, a reagent which attacks tryptophyl residues[3] in proteins (Viswanatha *et al.*, 1960; Viswanatha and Lawson, 1961; Okada *et al.*, 1963; Green, 1963; Hayashi *et al.*, 1965; Davidson and Westley, 1965; Steiner, 1966; Spande *et al.*, 1966), was allowed to react with the enzyme under various conditions. Changes in catalytic activity and loss of tryptophan residues (*i.e.*, conversion of the indole ring into an oxindole derivative) were followed as a function of the molar ratio of *N*-bromosuccinimide to enzyme.

Experimental Section

Materials. Dihydrofolate reductase was isolated from chicken liver according to the procedure of Mathews and Huennekens (1963), as modified by Mell *et al.* (1966). All preparations used in these studies had specific activities of 6–8 μmoles of dihydrofolate reduced per min per mg of protein. As discussed elsewhere (Kaufman and Gardiner, 1966[2]), preparations having a specific activity in this range are essentially pure (*i.e.*, free from extraneous proteins), although the enzyme may be resolved electrophoretically into multiple forms (Mell *et al.*, 1968). The molecular weight of the enzyme, as determined by passage through a standardized column of Sephadex G-100 (Mell *et al.*, 1966) or by sedimentation equilibrium analysis,[2] is 22,000. A 0.1% solution of the enzyme at pH 7.0 has an absorbance of 1.55 at 278 mμ.

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† Postdoctoral fellow of the American Cancer Society.

[1] G. P. Mell and F. M. Huennekens, in preparation. See also Huennekens *et al.* (1967).

[2] G. P. Mell, J. H. Freisheim, F. M. Huennekens, and K. Dus, in preparation.

[3] Although *N*-bromosuccinimide is generally considered to be selective for tryptophan residues, effects on other functional groups have also been observed (Ramachandran and Witkop, 1959; Schmir and Cohen, 1961; Ramachandran, 1962).

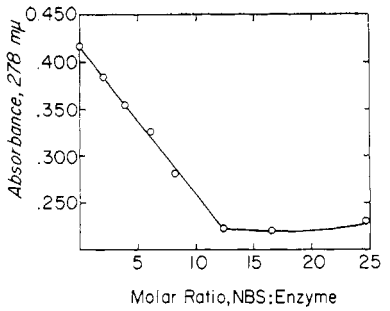

FIGURE 1: Reaction of tryptophan residues of dihydrofolate reductase with *N*-bromosuccinimide. Enzyme concentration was 1.22×10^{-5} M in 0.13 M sodium acetate–formate (pH 4.0) containing 5.3 M urea. Increments of *N*-bromosuccinimide were added as indicated.

TPNH and *p*-mercuribenzoate were obtained from the Sigma Chemical Co. *N*-Bromosuccinimide, supplied by Matheson Coleman and Bell, was recrystallized from glacial acetic acid. Dihydrofolic acid was prepared by the reduction of folic acid (Calbiochem) with sodium dithionite according to the method of Futterman (1957), as modified by Blakley (1960). The product was washed repeatedly with 5×10^{-3} N HCl, lyophilized, and stored in evacuated, sealed tubes. Urea was recrystallized from 70% ethanol. Guanidinium chloride (6 M) was stirred with Norit A for 2 hr at 45° and filtered to remove spectral impurities. All other chemicals were of reagent or analytical grade.

Methods. In general, the experimental procedure consisted of adding increasing amounts of *N*-bromosuccinimide to a known amount of dihydrofolate reductase and measuring one or both of the following parameters: (a) destruction of tryptophan residues and (b) changes in enzymatic activity. In the latter case, controls without *N*-bromosuccinimide were used to ensure that no time-dependent loss of enzymatic activity had occurred.

Destruction of tryptophan residues was measured by the following procedure. Dihydrofolate reductase (2 ml; *ca.* 1×10^{-5} M in 0.10 M phosphate or acetate buffer of the desired pH) was placed in a quartz cuvet having a light path of 1 cm. The blank cuvet contained only buffer. Successive 10-μl aliquots of aqueous 10^{-3} M *N*-bromosuccinimide were added with magnetic stirring to each cuvet and the absorbance at 278 mμ was measured in a Cary recording spectrophotometer, Model 14, at 25°. In some experiments, which were carried out at pH values between 6.0 and 8.0, faint turbidities were observed at high concentrations of *N*-bromosuccinimide. In such cases, absorbance readings at 278 mμ were corrected for the contribution due to light scattering by extrapolating the base line from the 380- to 320-mμ region (Spande *et al.*, 1966). As increasing amounts of *N*-bromosuccinimide were added to the enzyme, there was initially a linear drop in absorbance at 278 mμ followed by a flat minimum and then a gradual rise in absorbance; this final effect is probably due to side reactions at high *N*-bromosuccinimide concentrations (Witkop, 1961; Green and Witkop, 1964). Following each addition during the linear phase of the *N*-bromosuccinimide titration, the number of tryptophan residues oxidized per mole of enzyme was calculated from eq 1 (Witkop, 1961; Green and Witkop, 1964),

$$\Delta n = \frac{1.31 \times \Delta A_{278}}{5500 \times \text{molarity of enzyme}} \quad (1)$$

TABLE I: Oxidation of Dihydrofolate Reductase with *N*-Bromosuccinimide.

Buffer	No. of Tryptophans Oxidized	% Oxidation[a]
Phosphate (0.10), pH 6.0	2.0	50
Acetate (0.10), pH 4.0	2.7	68
Acetate–formate (0.13), pH 4.0, containing 5.3 M urea	3.8	95

[a] Assuming a total content of four tryptophans per molecule of enzyme. Enzyme concentration, 1.82×10^{-5} M. The molar ratio of *N*-Bromosuccinimide to enzyme was 15.3 in the first and second experiments and 12.3 in the third.

where Δn is the number of tryptophans oxidized, ΔA_{278} is the maximum decrease in absorbance at 278 mμ, 1.31 is an empirical factor based upon oxidation of model tryptophan peptides by *N*-bromosuccinimide (Patchornik *et al.*, 1958), and 5500 is the molar extinction coefficient for tryptophan at 278 mμ.

Enzymatic activity was measured spectrophotometrically (Mathews and Huennekens, 1963) by following the decrease in absorbance at 340 mμ using a Beckman Model DUR recording spectrophotometer equipped with a 2000 multiple-sample absorbance recorder and a Gilford Model 208 auxiliary offset control unit. The assay mixture in a volume of 1.25 ml contained 50 mmoles of potassium phosphate buffer (pH 7.5), 0.17 μmole of TPNH, 0.11 μmole of dihydrofolate, and suitable aliquots of the enzyme ($0.5–1.4 \times 10^{-7}$ M). The final concentration of *N*-bromosuccinimide in the assay mixtures ($<8 \times 10^{-6}$ M) did not affect the enzymatic activity.

Any variations from the above protocols are given in the legends for tables and figures.

The total tryptophan content of the enzyme was estimated by titration of the enzyme with *N*-bromosuccinimide in 0.13 M sodium acetate–formic acid containing 5.3 M urea (pH 4.0) as described above, or from absorbance measurements (280 and 288 mμ) of the enzyme at pH 6.5 in 6.0 M guanidinium chloride according to Edelhoch (1967).

Results and Discussion

Tryptophan Content of the Enzyme. Prior to investigating the effect of *N*-bromosuccinimide upon catalytic activity, it was necessary to determine the total number of tryptophan residues per molecule of enzyme. Edelhoch's (1967) procedure, which is based upon the absorbance ratio of the protein at 280 and 288 mμ in 0.02 M potassium phosphate (pH 6.5) containing 6.0 M guanidinium chloride, gave a value of 4.1 tryptophans/molecule of enzyme. This may be compared with the value obtained by an alternate method, based upon the change in absorbance at 278 mμ that occurred when the enzyme was treated with *N*-bromosuccinimide under acidic conditions and in the presence of urea. A typical *N*-bromosuccinimide titration of the enzyme, according to this latter procedure, is

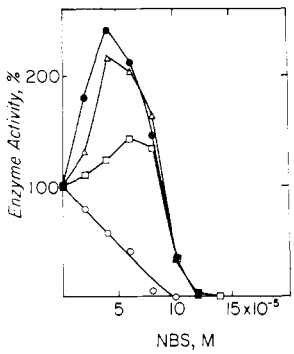

FIGURE 2: Effect of pH on the *N*-bromosuccinimide-mediated activation of dihydrofolate reductase. Enzyme concentration was 1.82×10^{-5} M in the following buffers (each at 0.1 M): sodium acetate, pH 5.0, ○; potassium phosphate, pH 6.0, □; pH 7.0, △; and pH 8.0, ●. Enzyme activity is expressed as per cent of untreated control.

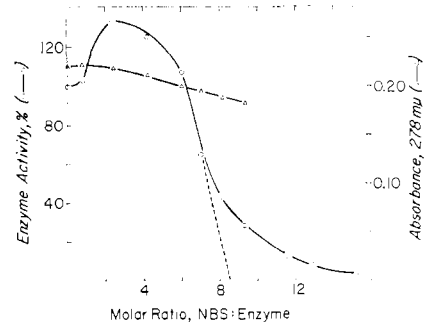

FIGURE 3: Activity and absorbance changes of dihydrofolate reductase as a function of molar excess of *N*-bromosuccinimide. Enzyme concentration was 0.64×10^{-5} M in 0.10 M potassium phosphate (pH 6.0). Enzyme activity is expressed as in Figure 2.

illustrated in Figure 1. In this experiment the maximum decrease in absorbance at 278 mμ, obtained from the initial, linear portion of the curve, was 0.196 and the enzyme concentration was 1.22×10^{-5} M. These terms, when substituted into eq 1, lead to a calculated value of 3.8 tryptophans oxidized. Similar determinations made on four different preparations of enzyme yielded an average content of 4.0 tryptophan residues/molecule of protein.

Although the above analyses were performed under acidic conditions in order to detect the maximum number of tryptophans, oxidation of these residues is usually carried out at slightly higher pH values in order to avoid cleavage of peptide bonds and to take advantage of the different reactivities of the individual residues (Viswanatha and Lawson, 1961; Okada *et al.*, 1963; Spande *et al.*, 1966). It was therefore of interest to ascertain the number of tryptophans in dihydrofolate reductase that could be titrated by *N*-bromosuccinimide at higher pH values and in the absence of urea. Representative results bearing on this point are summarized in Table I. At pH 6.0, only two of the four tryptophans were oxidized. At pH 4.0, a value approaching three was obtained and, as reported above, all four residues were accessible when the latter determination was repeated in the presence of 5.3 M urea. These results suggest that not all of the tryptophans in dihydrofolate reductase are exposed at physiological pH and that more acidic conditions produce a degree of unfolding in the protein. The latter conclusion is consistent with the observation[2] that lowering the pH also increases the susceptibility of the protein to tryptic digestion.

Effect of N-bromosuccinimide on Enzyme Activity. In view of the above results, the effect of *N*-bromosuccinimide upon the catalytic activity of dihydrofolate reductase was examined as a function of pH. The results are shown in Figure 2. At pH 7 and 8, *N*-bromosuccinimide initially *activates* the enzyme (over twofold), but this is eventually followed by inhibition. The same behavior (*i.e.*, activation prior to inhibition) has been observed previously when various dihydrofolate reductases are treated with chaotropic agents such as urea or ions (Bertino, 1962; Kaufman, 1963; Perkins and Bertino, 1964, 1965; Reyes and Huennekens, 1967a). At pH 6, the activation phase is less pronounced, while at pH 5, *N*-bromosuccinimide produces an almost linear inactivation of the enzyme.

It appeared from these experiments that the activation phase might involve an attack of *N*-bromosuccinimide on some group other than the indolyl moiety of tryptophan. This was investigated by treating the enzyme with increasing amounts of *N*-bromosuccinimide at pH 6.0 and following the decrease in absorbance at 278 mμ concomitantly with the changes in enzyme activity (Figure 3). The plot of activity as a function of added *N*-bromosuccinimide closely resembled that seen previously (*cf.* open squares in Figure 2). Measurements of the enzyme absorbance revealed that there was relatively little loss of tryptophan during the initial activation phase. During the later stages, however, the absorbance at 278 mμ began to decrease and this coincided with the loss of catalytic activity.

It will be noted that, during the inactivation phase shown in Figure 3, the curve is concave rather than linear, indicating that *N*-bromosuccinimide becomes less effective as complete loss of activity is approached. This result could be explained if oxidation of a critical tryptophan residue led to a conformational change that rendered the remaining tryptophans more accessible to, and competitive for, the available oxidant. Extrapolation of the initial slope of the inactivation portion of the curve in Figure 3 indicated that, in the absence of such secondary reactions, about 8.6 moles of *N*-bromosuccinimide would be required for complete inactivation of each mole of enzyme. At this point, the absorbance change in the protein was 0.027 which corresponded, according to eq 1, to the destruction of approximately one tryptophan per molecule.

Mechanism of Activation by N-Bromosuccinimide. It has been shown that chicken liver dihydrofolate reductase contains two cysteine residues per mole, only one of which is accessible to titration by mercurials.[2] The catalytic activity of the enzyme is increased severalfold when this group is modified by: alkylation with mercurials (Kaufman, 1964; Perkins and Bertino, 1965), oxidation with I_2 (Kaufman, 1966), or formation of a mixed disulfide with 5,5′-dithiobis(2-nitrobenzoic acid) (Reyes and Huennekens, 1967a). It seemed likely, therefore, that the activation observed with *N*-bromosuccinimide was also due to some modification of this sulfhydryl group. *N*-Bromosuccinimide is known to oxidize cysteine in proteins (Witkop, 1961) and, moreover, in the case of bovine serum albumin and hemoglobin A (which contain one and eight cysteines per molecule, respectively), 5 and 20–30 moles of *N*-bromosuccinimide are consumed before any decrease in absorbancy at 280 mμ is detected (Hughes, 1950; Ingram, 1955; Benesch *et al.*, 1955).

More direct evidence on this point was obtained, however,

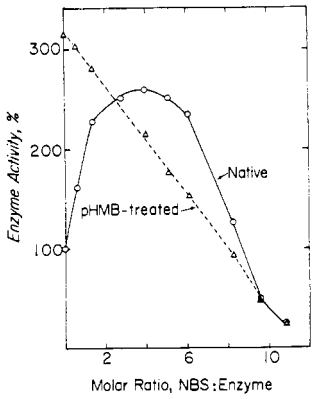

FIGURE 4: Effect of *N*-bromosuccinimide on mercurial-treated dihydrofolate reductase. Enzyme (0.88×10^{-5} M in 2.0 ml of 0.10 M potassium phosphate, pH 7.5) was treated with *p*-mercuribenzoate (5×10^{-5} M in 2.0 ml). Aliquots (10 μl) from the treated enzyme and the control were assayed following *N*-bromosuccinimide additions as indicated. Native enzyme, O; *p*-mercuribenzoate–enzyme, Δ. Enzyme activity is expressed as in Figure 2.

by an experiment in which the enzyme was treated with a mercurial prior to reaction with *N*-bromosuccinimide. As can be seen in Figure 4, addition of *N*-bromosuccinimide to the *p*-mercuribenzoate-activated enzyme produced only a linear inactivation, while the control (*i.e.*, untreated enzyme) showed the usual biphasic response to *N*-bromosuccinimide (*cf.* Figures 2 and 3). Thus, both the mercurial and *N*-bromosuccinimide appear to react with the same functional group during activation of the enzyme.

As part of the same experiment recorded in Figure 4, enzyme activity in the mercurial-treated enzyme was also followed as a function of the number of tryptophans oxidized (Figure 5). It can be seen that the curve showing activity of the *p*-mercuribenzoate-modified enzyme as a function of tryptophans oxidized remained linear for about 80% of its possible range. Extrapolation of the initial slope to complete inactivation again verified that the loss of only one tryptophan residue corresponds to the abolition of catalytic function.

Further supporting the premise that the *N*-bromosuccinimide activation of the enzyme involves oxidation of a sulfhydryl group, it was observed that dithiothreitol could completely reverse the activation process (expt A, Table II). However, incubation of the *N*-bromosuccinimide-treated enzyme with either of the substrates (TPNH or dihydrofolate), prior to addition of the dithiol, largely prevented reversal of the activation (expt B, Table II). The fact that the relatively small dithiothreitol molecule is unable to react with the modified cysteine in the presence of either substrate indicates that the cysteine residue susceptible to *N*-bromosuccinimide attack is close to the binding sites for TPNH and dihydrofolate on the enzyme. This is also consistent with the observation, made previously with the L1210 dihydrofolate reductase (Reyes and Huennekens, 1967b), that both substrates also prevent deactivation by thiols of the 5,5′-dithiobis(2-nitrobenzoic acid)-activated enzyme.

The chemical nature of the *N*-bromosuccinimide-modified cysteine residue in dihydrofolate reductase has not yet been established. Since the second cysteine residue in the molecule is not readily accessible,[2] it does not appear likely that a disulfide bridge is being formed, either inter- or intramolecularly. Alternatively, the sulfhydryl group might have undergone oxidation to the level of a sulfenic acid.[4] This would be consistent

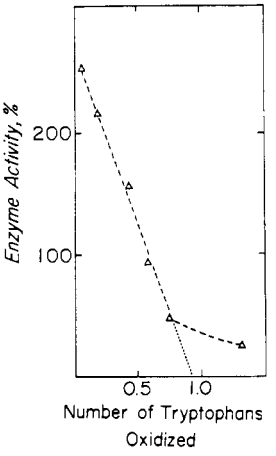

FIGURE 5: Activity of the *p*-mercuribenzoate-treated (Δ) dihydrofolate reductase as a function of the number of tryptophans oxidized. Experimental conditions as in Figure 4.

TABLE II: Reversal of *N*-Bromosuccinimide Activation of Dihydrofolate Reductase by Treatment with Dithiothreitol and Prevention of Reversal by Substrates.

Expt	Conditions	Enzyme Act. (ΔA_{340}/min)
A	Native enzyme	0.052
	N-Bromosuccinimide-treated enzyme	0.170
	N-Bromosuccinimide-treated enzyme + dithiothreitol	0.054
	Native enzyme + dithiothreitol	0.052
B	Native enzyme + TPNH + dithiothreitol	0.054
	N-Bromosuccinimide-treated enzyme + TPNH + dithiothreitol	0.132
	Native enzyme + dihydrofolate + dithiothreitol	0.055
	N-Bromosuccinimide-treated enzyme + dihydrofolate + dithiothreitol	0.140

[a] To 0.50 ml of enzyme (0.09 mg/ml) in 0.05 M potassium phosphate (pH 7.5) was added 10 μl of 10^{-3} M *N*-bromosuccinimide. A 10-μl aliquot of the *N*-bromosuccinimide-modified (or native) enzyme was then placed in a cuvet and treated with dithiothreitol (5 μmoles), TPNH (0.17 mmole), or dihydrofolate (0.11 mmole) in the order indicated in the table (the solution was allowed to stand for 2 min at 24° after each addition). After all additions had been made, any necessary remaining components were added and the enzyme activity was determined in the usual manner (see Experimental Section).

[4] It is also conceivable that *N*-bromosuccinimide forms an adduct with the sulfhydryl group.

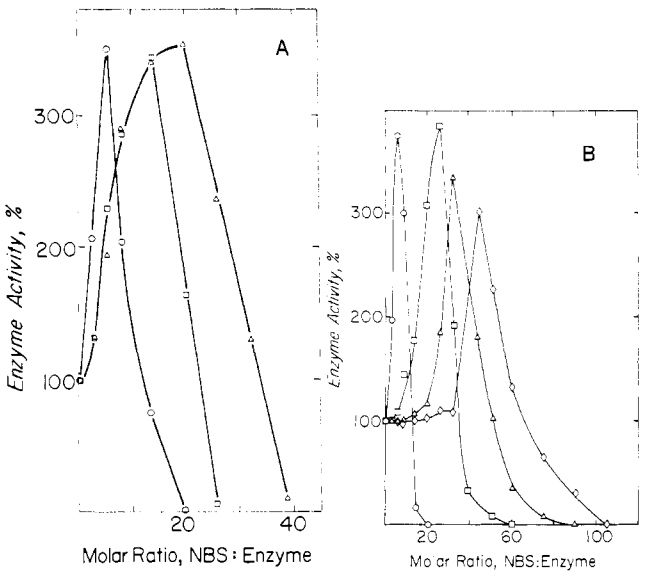

FIGURE 6: Effect of substrates upon the action of *N*-bromosuccinimide on dihydrofolate reductase. The enzyme (0.68×10^{-5} M in 0.10 M potassium phosphate, pH 7.5) was incubated at 24° with either TPNH or dihydrofolate (total volume, 0.58 ml) for 5 min prior to additions of *N*-bromosuccinimide. Aliquots of 10 μl were taken for assay. Enzyme activity is expressed as in Figure 2. In panel A, the molar ratios of TPNH to enzyme were: 0:1, ○; 12:1, □; and 37:1, △. In panel B, the molar ratios of dihydrofolate to enzyme were: 0:1, ○; 8:1, □; 17:1, △; and 25:1, ◊.

with Kaufman's proposal that activation of the chicken liver dihydrofolate reductase by iodine involves the formation of a sulfenyl iodide on the protein (Kaufman, 1966). Sulfenyl iodides have also been postulated to occur when mercaptalbumin, β-lactoglobulin, and ovalalbumin are oxidized by iodine (Hughes and Straessle, 1950; Cunningham and Nuenke, 1959–1961). As shown by the data in Figures 2 and 4, maximum activation at pH 7–8 occurred when the molar ratio of *N*-bromosuccinimide to enzyme was between 2 and 4. Since oxidation to the sulfenic acid stage would require 2 moles of *N*-bromosuccinimide/mole of protein, it is not likely (in view of the fact that some *N*-bromosuccinimide is undoubtedly being consumed in side reactions) that any higher oxidation state is involved. The ability of dithiothreitol to reverse the activation likewise tends to rule out oxidation states above sulfenic acid.

Since both substrates prevented deactivation of the *N*-bromosuccinimide-treated enzyme, the converse process (*i.e.*, activation by *N*-bromosuccinimide in the presence of either TPNH or dihydrofolate) was examined in detail. The effect of TPNH on *N*-bromosuccinimide activation is shown in Figure 6A. It can be seen that, whereas about a 5-fold molar excess of the oxidant (open circles) was sufficient to produce maximum activation in the absence of TPNH, considerably larger amounts (about 14- and 20-fold, respectively) were necessary when the coenzyme was present.

The effect of dihydrofolate on *N*-bromosuccinimide activation of the enzyme (Figure 6B) was somewhat different from the effect of TPNH. Incubation of the enzyme with increasing concentrations of dihydrofolate prior to reaction with *N*-bromosuccinimide resulted again in corresponding increases in the level of *N*-bromosuccinimide needed to effect activation. When the molar ratios of dihydrofolate:enzyme were 8:1, 17:1, and 25:1, the molar excesses of *N*-bromosuccinimide to enzyme necessary to bring about maximum activation were about 26, 32, and 44, respectively. The extent of activation, however, was somewhat lower with increasing concentrations of dihydrofolate.

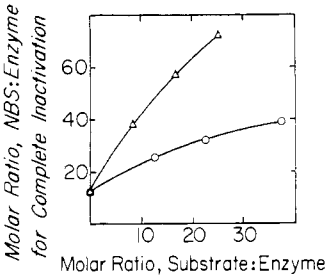

FIGURE 7: Effect of variation of the molar excess of substrates on the inactivation of dihydrofolate reductase. The linear portion of the inactivation curves (*cf.* Figure 6A,B) in the presence of TPNH (○) and dihydrofolate (△) were extrapolated to complete loss of enzymatic activity.

These data again suggest that either substrate partially shields a cysteine residue and a tryptophan residue from the action of *N*-bromosuccinimide. Due to the high absorbances of the substrates in the region of 278 mμ, the extent of tryptophan oxidation could not be followed directly under these conditions. However, if the linear portions of the inactivation curves are extrapolated to complete inhibition at each concentration of TPNH (Figure 6A) or dihydrofolate (Figure 6B), an estimate can be obtained of the relative protection against tryptophan oxidation afforded by each substrate. These data are shown in Figure 7. Over the concentration range studied (*i.e.*, a 10- to 25-fold molar excess of substrate to enzyme), dihydrofolate is seen to be about twice as effective as TPNH in protecting the enzyme against inactivation. A similar analysis of the activation phases of Figure 6A,B shows that dihydrofolate is also more effective than TPNH in protecting the enzyme against activation by *N*-bromosuccinimide.

References

Benesch, R. T., Lardy, H. A., and Benesch, R. (1955), *J Biol. Chem. 216*, 663.

Bertino, J. R. (1962), *Biochim. Biophys. Acta 58*, 377.

Blakley, R. L. (1960), *Nature 188*, 231.

Boyer, P. D., and Theorell, H. (1956), *Acta Chem. Scand. 10*, 447.

Cunningham, L. W., and Nuenke, B. J. (1959), *J. Biol. Chem. 234*, 1447.

Cunningham, L. W., and Nuenke, B. J. (1960), *J. Biol. Chem. 235*, 1711.

Cunningham, L. W., and Nuenke, B. J. (1961), *J. Biol. Chem. 236*, 1716.

Davidson, B., and Westley, J. (1965), *J. Biol. Chem. 240*, 4463.

Edelhoch, H. (1967), *Biochemistry 6*, 1948.

Freisheim, J. (1968), *Federation Proc. 27*, 390.

Futterman, S. (1957), *J. Biol. Chem. 228*, 1031.

Green, N. M. (1963), *Biochem. J. 89*, 599.

Green, N. M., and Witkop, B. (1964), *Trans. N. Y. Acad. Sci. 26*, 659.

Hayashi, K., Imoto, T., Funatsu, G., and Funatsu, M. (1965), *J. Biochem. (Tokyo) 58*, 227.

Hillcoat, B. L., Perkins, J. P., and Bertino, J. R. (1967), *J. Biol. Chem. 242*, 4777.
Huennekens, F. M., Mell, G. P., Reyes, P., and Freisheim, J. H. (1967), *VII Intern. Congr. Biochem. 4*, 821.
Hughes, W. L., Jr. (1950), *Cold Spring Harbor Symp. Quant. Biol. 14*, 79.
Hughes, W. L., Jr., and Straessle, R. (1950), *J. Amer. Chem. Soc. 72*, 452.
Ingram, V. M. (1955), *Biochem. J. 59*, 653.
Kaufman, B. T. (1963), *Biochem. Biophys. Res. Commun. 10*, 449.
Kaufman, B. T. (1964), *J. Biol. Chem. 239*, PC669.
Kaufman, B. T. (1966), *Proc. Natl. Acad. Sci. U. S. 56*, 695.
Kaufman, B. T., and Gardiner, R. C. (1966), *J. Biol. Chem. 241*, 1319
Mathews, C. K., and Huennekens, F. M. (1963), *J. Biol. Chem. 238*, 3436.
McKay, R. H., and Kaplan, N. O. (1964), *Biochem. Biophys. Acta 79*, 273.
Mell, G. P., Martelli, M. F., Kirchner, J., and Huennekens, F. M. (1968), *Biochem. Biophys. Res. Commun. 33*, 74.
Mell, G. P., Schroeder, G. M., and Huennekens, F. M. (1966), *Federation Proc. 25*, 277.
Okada, Y., Onoue, K., Nakashima, S., and Yamamura, Y. (1963), *J. Biochem. (Tokyo) 54*, 477.
Patchornik, A., Lawson, W. B., and Witkop, B. (1958), *J. Amer. Chem. Soc. 80*, 4747.
Perkins, J. P., and Bertino, J. R. (1964), *Biochem. Biophys. Res. Commun. 15*, 121.
Perkins, J. P., and Bertino, J. R. (1965), *Biochemistry 4*, 847.
Perkins, J. P., and Bertino, J. R. (1966), *Biochemistry 5*, 1005.
Ramachandran, L. K. (1962), *J. Sci. Ind. Res. 21C*, 111.
Ramachandran, L. K., and Witkop, B. (1959), *J. Amer. Chem. Soc. 81*, 4028.
Reyes, P., and Huennekens, F. M. (1967a), *Biochemistry 6*, 3519.
Reyes, P., and Huennekens, F. M. (1967b), *Biochem. Biophys. Res. Commun. 28*, 833.
Schmir, G. L., and Cohen, L. A. (1961), *J. Amer. Chem. Soc. 83*, 723.
Spande, T. F., Green, N. M., and Witkop, B (1966), *Biochemistry 5*, 1926.
Steiner, R. F. (1966), *Biochemistry 5*, 1964.
Theorell, H., and McKinley-McKee, J. S. (1961), *Acta Chem. Scand. 15*, 1811.
Velick, S. F. (1958), *J. Biol. Chem. 233*, 1455.
Viswanatha, T., and Lawson, W. B. (1961), *Arch. Biochem. Biophys. 93*, 128.
Viswanatha, T., Lawson, W. B., and Witkop, B. (1960), *Biochim. Biophys. Acta 40*, 216.
Winer, A. P., and Schwert, G. W. (1959), *J. Biol. Chem. 234*, 1155.
Witkop, B. (1961), *Advan. Protein Chem. 16*, 221.