Coenzyme Binding by X-Irradiated Glutamate and Lactate Dehydrogenase*

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ABSTRACT: The radiolysis of glutamate dehydrogenase produces an enzyme with a reduced ability to enhance the fluorescence of reduced diphosphopyridine nucleotide (DPNH). This modification is not accompanied by a decrease in the fluorescence yield per binding site nor by a reduction in the site association constant, and is consistent with an absolute deficiency in the power of the protein to bind coenzyme. An inability to bind coenzyme can also be shown by ultracentrifuge separation. With this method 31 binding sites are found per mole while 62.7 sulfhydryl groups are titrated with 4 M urea at *p*H 4.6. Radiolysis leads to a linear relation between the loss of titratable sulfhydryl groups and coenzyme binding which suggests that specifically one of two sulfhydryl groups may be required for each DPNH molecule bound. The radiolysis of lactate dehydrogenase also results in a concomitant loss of coenzyme fluorescence enhancement and titratable sulfhydryl groups. The relation of both of these to activity suggests that one specific sulfhydryl is required for coenzyme binding while two groups are required for activity. Under this circumstance, the radiation inactivation of this enzyme can apparently be accounted for by sulfhydryl modification alone.

The susceptibility of sulfhydryl-containing pyridino proteins to radiation inactivation in aqueous solution was first emphasized in 1949 (Barron *et al.*, 1949) and has most recently been reevaluated with regard to several of these enzymes (Romani and Tappel, 1959; Lange and Pihl, 1960; Tanaka *et al.*, 1960). The radiolysis of one of these, glutamate dehydrogenase, results in a protein whose ability to enhance the fluorescence of DPNH, when mixtures of the enzyme and coenzyme are prepared, is greatly reduced (Adelstein and Mee, 1961). Loss of this property suggests that either the coenzyme binding by the protein has been modified or that its structure has been altered so as to change the fluorescent properties of the enzyme-coenzyme complex. In this study the binding of DPNH by X-irradiated solutions of glutamate and lactate dehydrogenase has been examined and the relation of the binding to titratable sulfhydryl groups is explored.

Experimental Procedures

Reagents. Beef liver glutamate dehydrogenase was purchased from C. F. Boehringer and Son. Before use the crystals were centrifuged from the ammonium sulfate, dissolved in 0.08 M potassium phosphate, *p*H 7.6, and dialyzed with three changes. Beef heart lactate dehydrogenase was purchased from Sigma Chemical Co. and treated in the same manner. Reduced and oxidized diphosphopyridine nucleotide, and *p*-mercuribenzoate were obtained from Sigma and used without further purification.

The concentrations of native glutamate dehydrogenase and lactate dehydrogenase were determined from their absorbancy at 279 mμ (Olson and Anfinsen, 1952) and 280 mμ (Takenaka and Schwert, 1956), respectively. The concentration of *p*-mercuribenzoate was determined at 234 mμ (Boyer, 1954) and that of DPNH at 340 mμ (Horecker and Kornberg, 1948).

Enzyme Assays. Glutamate dehydrogenase activity was determined by measuring the 5-minute increase in 340-mμ absorbancy on the addition of enzyme solution to a cuvet containing 13.2 μmoles of sodium glutamate, 10 μmoles of DPN, and 45 μmoles of potassium phosphate, *p*H 7.6. At the enzyme concentrations employed there was a linear relation between the amount of enzyme and the optical absorbancy difference. Lactate dehydrogenase activity was determined by the method of Millar and Schwert (1963).

Irradiations. Solutions were irradiated in glass beakers surrounded by ice water and covered with paraffin film. A 250-kvp constant-potential unit was employed with an unfiltered dose rate of about 2000 rads/minute. Dosimeters of identical geometry containing 0.8 mM ferrous ammonium sulfate in 0.1 N sulfuric acid were used; a *G* value[1] of 15 and a 304-mμ extinction coeffi-

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[1] Abbreviations used in this work: *G*, molecules converted per 100 ev of energy absorbed; D37, X-ray dose required to reduce activity or concentration to 37% of its initial value.

cient of 2189 at 25° for the ferric ion was assumed in the dosage calculations (Swallow, 1960). The radiation exposures were fractionated so that no dosimeter received more than 40,000 rads.

Fluorometric Analyses. In working with glutamate dehydrogenase a Turner fluorometer was employed. The primary filter was a Corning 7-60 having a peak at 360 mμ, the secondary filter was a Wratten 2A permitting the passage of wavelengths greater than 415 mμ. At the slit widths used a Kodak 96 neutral density filter transmitting 1% of the incident light was fitted over the secondary filter. This allowed a linear relation between the DPNH concentration and the measured fluorescence.

Fluorometric measurements on lactate dehydrogenase were made with a Farrand single-beam spectrofluorometer. The temperature was 25°, the activation wavelength was 345 mμ, and the emission wavelength was 453 mμ. The cuvets contained 1 mg of lactate dehydrogenase, 0.47 μmole of DPNH, and 250 μmoles of potassium phosphate, *p*H 7.6, in a total volume of 2.5 ml. The protein blank reading was made before the addition of DPNH since irradiation of the enzyme solutions increased their light scattering. Under these conditions a linear relation could be demonstrated between the amount of unirradiated lactate dehydrogenase added to the cuvet and the fluorescence measured, corrected for the DPNH blank. The corrected fluorescence could thus be used as a direct measure of the coenzyme binding.

Sulfhydryl Determinations. The interaction of *p*-mercuribenzoate with the sulfhydryl groups of glutamate dehydrogenase was measured spectrophotometrically at 255 mμ (Boyer, 1954). Twenty-μl quantities of 6.5×10^{-4} M *p*-mercuribenzoate were added to a 1-cm path length cuvet containing 2.5 mg of glutamate dehydrogenase, 10 mmoles of urea, and 400 μmoles of sodium acetate, *p*H 4.6, in a total volume of 2.5 ml. The absorbance was measured after each addition and the end point was estimated graphically. The sulfhydryl content of lactate dehydrogenase was estimated by the method of Millar and Schwert (1963).

Ultracentrifuge Separation. The method of Velick *et al.* (1953) was modified in the following manner. Glutamate dehydrogenase and DPNH were mixed together in the presence of 0.08 M potassium phosphate, *p*H 7.6. A 4.5-ml portion of the mixtures was centrifuged for 5 hours at 125,000 × *g* in a SW39L swinging bucket rotor of a Spinco preparative ultracentrifuge. The centrifuge was stopped without braking and the tubes were removed with extreme care; a small pellet of sedimented protein was visible in each tube. The top 2.5 ml of supernatant fluid was removed cautiously using a bent capillary pipet. The absorbance was measured at 340 and 279 mμ, the latter to ensure that all the enzyme had been precipitated. The 340-mμ absorbancy was a measure of free coenzyme concentration and this was subtracted from the total coenzyme concentration to yield the bound coenzyme. The gel-like pellet of protein found at the bottom of the tube had a different appearance when the enzyme had been irradiated. Native glutamate dehydrogenase appeared transluscent and colorless while irradiated enzyme appeared cloudy and tinged with yellow-brown.

Results

Glutamate Dehydrogenase. Initial observations (Adelstein and Mee, 1961) demonstrated that the radiation inactivation of glutamate dehydrogenase solutions was accompanied by a decrease in their ability to enhance the fluorescence of added DPNH. In general, the enhanced fluorescence observed upon the mixing of native apo-pyridino proteins with reduced coenzyme is caused by the formation of a stereospecific complex which increases the fluorescence yield of the latter (Udenfriend, 1962). (In some instances, e.g., glyceraldehyde-phosphate dehydrogenase, the formation of the enzyme-coenzyme complex is accompanied by a decrease rather than an increase of the DPNH fluorescence yield.) The loss of fluorescence enhancement observed on irradiation of glutamate dehydrogenase can thus be ascribed to one or more of three possibilities: First, the number of sites capable of binding coenzyme has been reduced. Second, the coenzyme affinity of certain sites, but not their number, has been decreased. Third, the fluorescence yield of the enzyme-coenzyme complex has been diminished. The three possibilities were explored.

The fluorescence yield per binding site of the coenzyme-enzyme complex can be obtained by measuring the fluorescence of coenzyme-enzyme mixtures at varying dehydrogenase concentrations and extrapolating to infinite enzyme concentration (Tomkins *et al.*, 1962). This has been done for both native and irradiated glutamate dehydrogenase as shown in Figure 1, where the reciprocal of fluorescence is plotted against the reciprocal of the enzyme concentration. If the average fluorescence yield per site were different for the native and irradiated enzyme then the ordinate intercept should be different, since reciprocal glutamate dehydrogenase concentration equals zero at this point. The common intercept observed demonstrates that the yields are identical and precludes a difference in these as a cause of the fluorescence loss.

The affinity of enzyme for coenzyme as measured by the site association constant can be obtained also. Let K = site association constant, C = free coenzyme concentration, C_o = total coenzyme concentration, E = free enzyme site concentration, E_o = total enzyme site concentration, EC = enzyme-coenzyme complex concentration. Then,

$$K = \frac{(E)(C)}{(EC)} = \frac{(E_o - EC)(C)}{(EC)} \qquad (1)$$

Rearranging

$$\frac{(E_o)}{(EC)} = \frac{K}{(C)} + 1 \qquad (2)$$

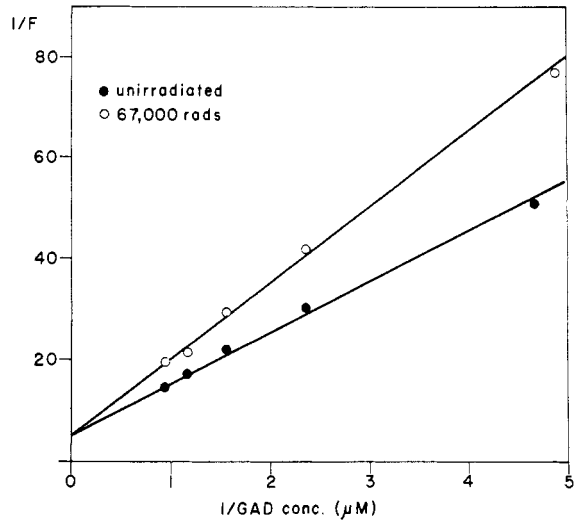

FIGURE 1: Reciprocal fluorescence versus reciprocal glutamate dehydrogenase (GAD) concentration for native and 67,000 rad–irradiated glutamate dehydrogenase. Protein concentration on irradiation 3.77 mg/ml; DPNH concentration 0.02 mM.

Let D_o = fluorometer deflection for total free coenzyme concentration C_o, D = fluorometer deflection for bound coenzyme concentration EC plus free coenzyme concentration C. Then,

$$D_o = \beta_o(C_o)$$

$$D = \beta_o(C) + \beta(EC)$$

Let

$$F = D - D_o = \beta(EC) - \beta_o(C_o - C)$$
$$= (\beta - \beta_o)(EC) \qquad (3)$$

Combining equations (2) and (3):

$$\frac{(\beta - \beta_o)(E_o)}{F} = \frac{K}{(C)} + 1 \qquad (4)$$

A plot of $1/F$ versus the reciprocal of coenzyme concentration should then yield a straight line with abscissal intercept ($1/F = 0$) equal to minus $1/K$. This treatment assumes that the sites on the native enzyme are all equivalent and do not interact. If irradiation changes the site association constant K of certain sites then plots of $1/F$ versus reciprocal coenzyme concentration should not be linear and the abscissal intercept should deviate from the native enzyme. Figure 2 shows that for both native and irradiated enzyme $1/F$ is a linear function of reciprocal DPNH concentration having a common abscissal intercept corresponding to $K = 1.7 \times 10^{-5}$ M. This indicates that the affinity of these sites capable of binding coenzyme has not been modified as a result of the irradiation.

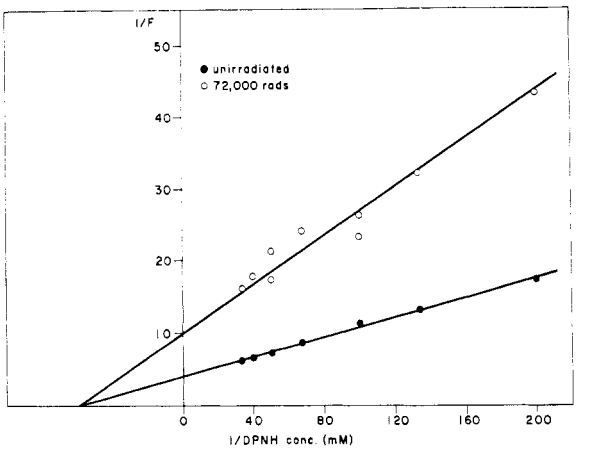

FIGURE 2: Reciprocal fluorescence versus reciprocal DPNH concentration for native and 72,000 rad–irradiated glutamate dehydrogenase. Protein concentration 0.9 mg/ml.

By exclusion, therefore, these experiments are consistent with the hypothesis that radiation destroys the ability of certain sites to bind coenzyme, that the remaining enzymatic sites have the same affinity for DPNH, and that the complexes formed have the same fluorescent yield as do those of the native protein.

As an alternative approach, the binding of coenzyme to native and irradiated glutamate dehydrogenase has also been measured by ultracentrifuge separation. In this method (Velick *et al.*, 1953), enzyme and coenzyme are mixed together and the large protein molecules are sedimented at high gravitational fields. Bound coenzyme sediments with the enzyme and this can be measured by subtracting the final from the starting concentration of coenzyme in the supernatant fluids. By varying the initial concentration of coenzyme, the total number of binding sites and their association constant for the coenzyme can be estimated (Adelstein, 1959). When this is done with native glutamate dehydrogenase 30 to 32 binding sites for DPNH are found per kinetic molecular unit of 1×10^6, with a site association constant of 3.9×10^{-5} M (S. J. Adelstein and B. L. Vallee, unpublished observations).

When glutamate dehydrogenase is irradiated, the number of DPNH molecules bound per gram of protein is less than that found for the native enzyme. Figure 3 shows the result from a typical experiment. For the native enzyme 20 moles of DPNH are bound per mole of 1×10^6 mw glutamate dehydrogenase at an initial DPNH concentration of 0.12 mM; enzyme irradiated with 62 krads binds 15 moles per mole, and enzyme irradiated with 124 krads binds 11 moles per mole.

Doses of irradiation that reduce the binding of DPNH to glutamate dehydrogenase also decrease the number of titratable sulfhydryl groups. When this enzyme is titrated with *p*-mercuribenzoate at *p*H 4.6 in 4 M urea 62.7 ± 4.2 free sulfhydryl groups are found, an average of 2 for each binding site measured by ultracentrifuge

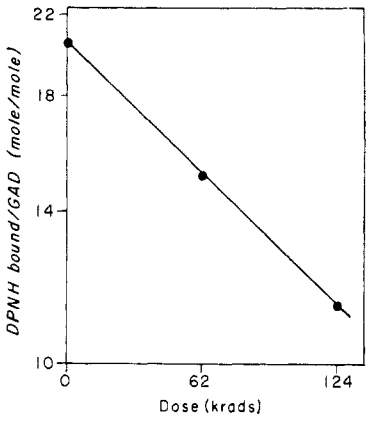

FIGURE 3: Moles DPNH bound per mole of glutamate dehydrogenase by ultracentrifuge separation versus X-ray dose. Protein concentration 2.3 mg/ml, DPNH 0.12 mM.

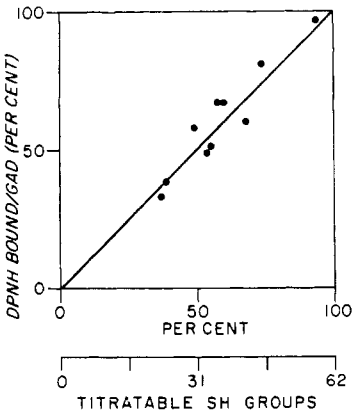

FIGURE 4: Moles DPNH bound per mole of glutamate dehydrogenase by ultracentrifuge separation versus per cent and number of titratable sulfhydryl groups. With this method there are 31 binding sites; the values shown have been corrected to infinite DPNH concentration.

separation. The relation of the number of titratable sulfhydryl groups to coenzyme binding measured with the ultracentrifuge was explored and the results are shown in Figure 4. The per cent of coenzyme molecules bound relative to the native protein is plotted as a function of titrable sulfhydryl groups. The 45-degree line relating per cent sulfhydryl to per cent DPNH binding is given for reference. There appears to be a one-to-one correspondence between the per cent loss in total binding sites and the per cent loss in titratable sulfhydryl groups. Since there are 31 binding sites, 2 sulfhydryl groups are rendered nonreactable with *p*-mercuribenzoate for each binding site destroyed.

Lactate Dehydrogenase. Beef heart lactate dehydrogenase, mw 72,000, has 2 DPNH binding sites and 4 titratable sulfhydryl groups in 4 M urea at *p*H 7.4 (Takenaka and Schwert, 1956; Millar and Schwert, 1963). The average number of sulfhydryl groups found in our enzyme preparations was 3.9, in good agreement with the reported results. When 1 mg/ml solutions of this enzyme are irradiated there is a logarithmic decrease in activity with a D37 of 28.5 krads (Figure 5).

As with glutamate dehydrogenase, the addition of lactate dehydrogenase to DPNH solutions enhances the fluorescence of the latter (Winer *et al.*, 1957). X-Irradiation of lactate dehydrogenase also reduced the ability of this enzyme to enhance the fluorescence of the reduced coenzyme. Concomitant fluorescence and sulfhydryl determinations show a coincident fall in both of these with increasing doses of radiation (Figure 5). When compared with the decrease in activity, the percentage loss in these parameters is less than that of activity with a D37 twice that of the activity loss. The calculated *G*(-SH) equals 0.93.

Discussion

It is apparent that the decrease in DPNH fluorescence enhancement observed on the radiation inactivation of apoglutamate dehydrogenase is caused by the loss of coenzyme binding sites participating in this phenomenon. The inference is indirect but is the only alternative consistent with the observed invariance of both the fluorescence yield and the coenzyme site association constant. The mechanism for the decrease in binding sites is not obvious; it could derive either from the destruction of an essential binding residue, or from a change in molecular conformation. It has been shown previously that the radiolysis of glutamate dehydrogenase results in a change in the sedimentation pattern consistent with disaggregation (Adelstein and Mee, 1961) and this might lead to a decrease in coenzyme binding. On the other hand, the addition of diethylstilbestrol to solutions of the enzyme has been reported to result in both disaggregation and in an increase in the number of binding sites measured fluorometrically (Tomkins *et al.*, 1962), so that the relation of protein conformation to coenzyme binding is not clear.

X-Irradiation also decreases the degree of DPNH binding as measured by ultracentrifuge separation. The number of binding sites estimated by this method (31) is substantially greater than those determined fluorometrically (10) in this study and by others (Tomkins *et al.*, 1962; Frieden, 1963a). It has been suggested that glutamate dehydrogenase contains more than one type of coenzyme binding site (Frieden, 1963b) and it may be that the discrepancy in numbers obtained by the two methods reflects this phenomenon. Silver titration of glutamate dehydrogenase in the absence of urea is reported to give 31 ± 1 sulfhydryl groups per mole (Grisolia *et al.*, 1962). The relation of these sulfhydryl groups to DPNH binding is not known except that 42 ± 1 sulfhydryl groups have been titrated in DPNH-inactivated enzyme.

In any event, the binding of DPNH as measured by ultracentrifuge separation seems to relate to a single titratable sulfhydryl group. Under the conditions of the

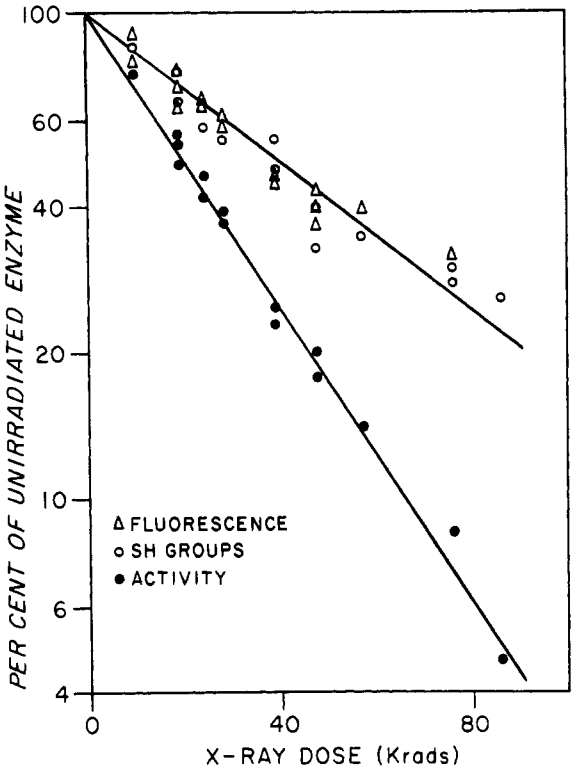

FIGURE 5: Activity, fluorescence, and titratable sulfhydryl groups of lactate dehydrogenase, in per cent of native enzyme, versus X-ray dose. Protein concentration on irradiation 1 mg/ml.

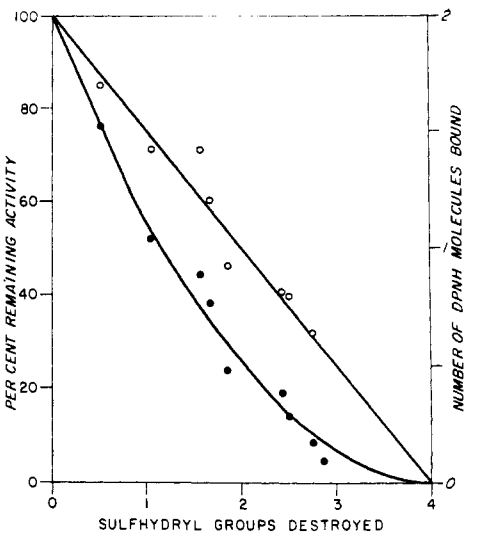

FIGURE 6: Remaining activity (●) and DPNH binding (O) from fluorescence measurements of X-irradiated lactate dehydrogenase versus decrease in the number of titratable sulfhydryl groups per mole. The lower curve represents theoretical activity assuming both of two sulfhydryl groups are required per active site; the diagonal line represents theoretical coenzyme binding assuming each bound DPNH molecule requires one specific sulfhydryl group.

sulfhydryl titration as carried out in this study there are 2 sulfhydryls found for each DPNH molecule bound but the coincident fall in per cent binding with per cent remaining sulfhydryl groups implies that only one, and a specific one, is required to form the enzyme-coenzyme complex. The radiolytic destruction of this sulfhydryl group would seem to be the proximate cause for the decrease in coenzyme binding, but this will require a more direct demonstration.

The situation with regard to lactate dehydrogenase appears more straightforward. The enzyme has 2 binding sites per mole measured fluorometrically, and sulfhydryl groups are apparently required for coenzyme binding since *p*-mercuribenzoate can block the formation of the coenzyme-enzyme complex as well as dissociate the complex when formed (Winer *et al.*, 1959).

The radiolysis of lactate dehydrogenase is accompanied by a decrease in DPNH fluorescence enhancement indicating a reduction in coenzyme binding. In addition, there is a concomitant reduction in the fraction of titratable sulfhydryl groups, implying a relationship between the latter and the binding of each DPNH molecule. On the other hand, the relation between titratable sulfhydryl groups and activity is more complex, the D37 for activity being half of that for sulfhydryl groups.

The relation between intact sulfhydryl groups, residual enzyme activity, and coenzyme binding is more clearly demonstrated by plotting the latter two variables against the first. This has been done in Figure 6 for the averaged values of Figure 5. Activity is shown as closed dots and coenzyme binding as open circles.

The drawn curve, concave upward, is the locus of expected values assuming that all 4 sulfhydryl groups are equally susceptible to radiation damage, that 2 are required for each active site, and that both must be intact for the site to be active. Under these conditions if P_1 is the probability that 1 of the 2 essential sulfhydryl groups is destroyed by a given dose of radiation, and P_2 is the probability that the other is destroyed, then $P_1 = P_2$ and the residual activity is $1 - (P_1 + P_2 - P_1P_2) = 1 - 2P_1 + P_1^2$, from which the curve has been constructed. The close fit between the predicted and observed activity values is apparent, and this is consistent with the hypothesis that at least 2 of the titratable sulfhydryl groups are required for enzymatic activity. A similar relation between activity and mercaptide formation has been obtained when lactate dehydrogenase is titrated with *p*-mercuribenzoate (Millar and Schwert, 1963).

On the other hand, the DPNH binding values are consistent with the model that *specifically* 1 of the 2 sulfhydryl groups titrated per binding site is required to bind DPNH. Under these circumstances DPNH binding equals $1 - P_1$. The diagonal straight line bisecting Figure 6 represents this expression. If DPNH binding required that *either* of the 2 sulfhydryls ti-

trated per binding site be intact then DPNH binding would equal $1 - P_1^2$, a theoretical curve convex upward that is obviously not consistent with the observed values.

Neither the radiolytic nor chemical data permit one to choose between two alternative models for this enzyme: (1) There are two active sites and two binding sites. Each active site contains 2 titratable sulfhydryl groups, both of which are required for activity, and one specifically is required for coenzyme binding. (2) There are one active site and two coenzyme binding sites per mole. The coenzyme molecules are bound by single specific sulfhydryl groups, both of which are required for activity; the remaining 2 sulfhydryl groups are not required for activity.

In either case, it appears that the radiation inactivation of lactate dehydrogenase is correlated with the disappearance of titratable sulfhydryl residues, and that this disappearance of sulfhydryl groups at coenzyme binding sites is responsible for at least part, if not all, of the activity loss. It is not clear whether the direct radiolytic destruction of sulfhydryl groups causes the decrease of titratable number or whether this is secondary to (1) a protein conformational change which makes the groups less available to *p*-mercuribenzoate titration, or (2) intermolecular or intramolecular disulfide formation which would also make fewer groups available. The G(-SH) obtained, 0.93, is considerably less than that which has been observed for more concentrated cysteine solutions (Swallow, 1952), and this implies either that the high values obtained for the free amino acid are owing to reactions not taking place in the protein molecule or that other sites on the protein are interacting with the aqueous radicals without affecting enzymatic activity. The latter hypothesis has been proposed for glyceraldehyde phosphate dehydrogenase where the 9 titratable sulfhydryl groups have a G(-SH) = 0.23, which is considerably less than that observed in this study (Lange and Pihl, 1960). It has been suggested for this latter enzyme that sulfhydryl group disappearance completely accounts for the loss of activity, as it appears to also for lactate dehydrogenase. This does not imply that the primary and sole action of the aqueous radicals is the destruction of sulfhydryl groups but rather that the disappearance of titratable groups correlates regularly and systematically with the loss of coenzyme binding and activity.

The results obtained with X-irradiation are in contrast with those obtained on the photooxidation of lactate dehydrogenase in the presence of methylene blue (Millar and Schwert, 1963). In the latter instance the initial inactivation does not seem to depend upon either the loss of binding sites for DPNH or the destruction of cysteine residues. However, the ability of the enzyme to form the highly fluorescent enzyme-DPNH-oxalate mixture decreases directly with loss of enzymatic activity. It would seem therefore that photooxidation interferes with the interaction of substrate with the enzyme-coenzyme complex while X-irradiation prevents the formation of the complex by modifying free sulfhydryl groups.

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