THE ADDITION OF SULFHYDRYL COMPOUNDS TO DIPHOSPHOPYRIDINE NUCLEOTIDE AND ITS ANALOGUES*

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A number of reagents are known which react chemically with DPN. These include alkali (1), cyanide ions (2, 3), bisulfite ions (2, 3), dihydroxyacetone (4), dithionite ions (5), and hydroxylamine (6).

All these reagents are nucleophilic agents, which react on a positive center. It is generally assumed that these reactions involve the addition of the nucleophilic agent to the position of the nicotinamide moiety at which the oxidation-reduction transformation is localized, i.e. the par position (7). Addition at the para position has recently been shown for cyanide (8) and dithionite (5).

The present paper will report a new addition reaction of this type: the reaction of sulfhydryl groups with DPN. The over-all reaction can be described as

\[ \text{DPN}^+ + R-SH \rightarrow \text{DPN}-S-R + H^+ \]

Materials and Methods

All sulfhydryl compounds were commercial preparations. Reduced glutathione and cysteine were obtained from the Schwarz Laboratories, Inc., ergothioneine hydrochloride from the Mann Biochemical Corporation, phenylethyl mercaptan from the Olin Mathieson Chemical Corporation, octyl and decyl mercaptans from the Aldrich Chemical Company, and all other mercaptans from the Eastman Kodak Company.

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The abbreviations used in this paper are as follows: DPN and DPNH, oxidized and reduced diphosphopyridine nucleotide, respectively; TPN and TPNH, oxidized and reduced triphosphopyridine nucleotide, respectively; acetylpyridine-DPN, pyridine aldehyde-DPN, and ethyl nicotinate-DPN, the analogues of DPN of acetylpyridine, pyridine-3-aldehyde, and ethyl nicotinate, respectively.
DPN and TPN were obtained from the Pabst Laboratories. The analogues of DPN were prepared from DPN by the action of pig brain DPNase in the presence of the appropriate pyridine derivatives, as described for the isonicotinic acid hydrazide (9, 10) and acetylpyridine (10, 11) analogues of DPN. The deaminated analogues were prepared from the corresponding adenine-containing analogues as described for deaminodPN (12). The α isomer of DPN was obtained as described previously (10, 13). The mononucleotides of the pyridine bases were obtained by the action of snake venom pyrophosphatase on the appropriate dinucleotide (10). The riboside was obtained from the mononucleotide by the action of prostate phosphatase (10). The methylated pyridine derivatives were obtained from the free bases by the reaction with methyl iodide in the standard manner.

Concentrations of the DPN and its analogues were determined with yeast alcohol dehydrogenase whenever the coenzyme could serve as a hydrogen acceptor. In all other instances the cyanide addition reaction was employed (3).

When necessary, the solutions of the mercaptans were adjusted to the pH required for the particular experiment.

**Results**

**Effect of Sodium Sulfide on DPN and DPN Analogues**—The simplest sulphydryl derivative showing a reaction with DPN or DPN analogues is sodium sulfide. The reaction is characterized by an increase in absorption in the region 320 to 350 m\(\mu\), when the coenzyme is added to a solution of sodium sulfide. The actual absorption maxima for DPN and for two of the DPN analogues are given in Table I. Even in molar sodium sulfide the reaction is not instantaneous, as shown in Fig. 1. Both the extent and rate of addition are more favorable with the acetylpyridine-DPN. The pyridine aldehyde analogue is also more favorable in its reaction with SH compounds when compared to DPN.

The extreme alkalinity of the sodium sulfide solution has a marked destructive effect on DPN (1, 14). Thus the equilibrium is never reached, and the absorption of the complex disappears again in time (Fig. 1). However, under these conditions the addition with the acetylpyridine-DPN proceeds virtually to completion. The addition complex shows a stability to alkali similar to that of reduced pyridine nucleotides. Solutions of less alkalinity still show the measurable rate of addition. This high alkalinity required for the reaction between DPN and sulfide and the consequent destruction of the coenzyme are probably the explanation why Tereyama and Vestling failed to observe the non-enzymatic DPN-sulfide reaction (15).

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2 We are grateful to Mr. M. R. Lamborg for supplying these compounds to us.
Effect of Mercaptans—As with sulfide, similar increases in absorption are observed when a mercaptan is added to a solution of DPN or its analogues. The rate of reaction, even at low concentrations of mercaptan or coenzyme, is extremely rapid. Hence the observed spectral changes reflect essentially the equilibrium point. The acetylpyridine and pyridine aldehyde-DPN again show a greater extent of reaction than does DPN. For this reason most data pertain to either one of these analogues. The limited water solubility of most mercaptans often did not allow DPN-mercaptan complex to appear in sufficiently high concentrations to obtain accurate measurements.

The increase in absorption is proportional to the concentration of the pyridine nucleotide, the mercaptan, and the hydrogen ion. Figs. 2 and 3 illustrate these points for the case of pyridine aldehyde-DPN and ethyl mercaptan. Even though the curve in Fig. 3 resembles strongly a titration curve, the extent of the reaction does not coincide with the concentration of ethyl mercaptide ion at the given pH values. The pK of ethyl mercaptan was determined by electrometric titration, and it was found to be 6.6.3 It is possible, however, that the curve represents the dissociation of the para position of the pyridine ring.

3 This value is different from the value of 10.0, as estimated by Calvin (16). This author determined the pK of mercaptoethanol at 9.5. However, the pK1 of sulfide is given as 7.0. Since sulfide addition shows a pH dependence similar to that of ethyl mercaptan addition, again the concentration of HS− ion cannot be responsible for the shape of the pH curve.
SULFHYDRYL COMPOUNDS AND DPN

Fig. 2. A, the effect of pyridine nucleotide concentration. Ethyl mercaptan 0.045 M, in 0.1 M phosphate buffer, pH 10.2; total volume, 3 ml. The nucleotide used was pyridine aldehyde-DPN. The increase in optical density was determined from the difference between a blank cuvette containing only the nucleotide in buffer and an experimental cuvette containing all three components. B, the effect of mercaptan concentration. Pyridine aldehyde-DPN 0.26 x 10^-3 M, in 0.1 M phosphate buffer, pH 9.3. The experiment was performed as described in A.

Fig. 3. The effect of hydrogen ion concentration. Ethyl mercaptan 0.045 M, pyridine aldehyde-DPN 0.26 x 10^-3 M, in 0.1 M phosphate buffer of the pH indicated; total volume, 3 ml. All values are corrected for the reading of pyridine aldehyde-DPN in buffer alone.

Specificity of Reaction—All sulfhydryl compounds tested to date will yield this addition reaction, regardless of the structure of the mercaptan. Also those DPN analogues which add cyanide will yield the addition
complex. Positive results were obtained with DPN, TPN, acetylpyridine-DPN, pyridine aldehyde-DPN, ethyl nicotinate-DPN, and the α isomer of DPN. The deaminated derivatives of DPN, acetylpyridine-DPN, and

**Table I**

*Absorption Maxima for Pyridine Nucleotide-Mercaptan Addition Complexes*

The maxima were estimated from difference spectra between the coenzyme and the coenzyme mixed with mercaptan. The spectra were corrected for any self-absorption the mercaptan had in the region examined. All reaction mixtures, with the exception of those which contained the ergothioneine, were examined at pH 10.2, phosphate buffer, 0.1 M.

<table>
<thead>
<tr>
<th>Mercaptan</th>
<th>Pyridine nucleotide-mercaptan absorption maximum</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>DPN</td>
</tr>
<tr>
<td>Cysteine</td>
<td>335</td>
</tr>
<tr>
<td>Glutathione</td>
<td>335</td>
</tr>
<tr>
<td>Ethanethiol</td>
<td>330</td>
</tr>
<tr>
<td>Propanethiol</td>
<td>330</td>
</tr>
<tr>
<td>Butanethiol</td>
<td>335</td>
</tr>
<tr>
<td>Pentanethiol</td>
<td>335</td>
</tr>
<tr>
<td>Hexanethiol</td>
<td>*</td>
</tr>
<tr>
<td>Heptanethiol</td>
<td>*</td>
</tr>
<tr>
<td>Octanethiol</td>
<td>*</td>
</tr>
<tr>
<td>α-Toluenethiol</td>
<td>*</td>
</tr>
<tr>
<td>Mercaptosuccinic acid</td>
<td>330</td>
</tr>
<tr>
<td>Mercaptoacetic acid</td>
<td>330</td>
</tr>
<tr>
<td>2,3-Dithioopropanol†</td>
<td>330</td>
</tr>
<tr>
<td>Thioethanol</td>
<td>330</td>
</tr>
<tr>
<td>Sulfide</td>
<td>335</td>
</tr>
<tr>
<td>Ergothioneine†</td>
<td>$</td>
</tr>
</tbody>
</table>

* The sulfhydryl compound is too insoluble in water to allow a measure of the maximum.
† British antilewisite (BAL).
‡ Maximum obtained in methanol as solvent.
§ The pyridine nucleotide is too insoluble in methanol to allow an estimate of the absorption maximum.

Pyridine aldehyde-DPN will also react. In some cases the reaction did not reach completion, owing to the limited water solubility of the mercaptans, but spectral evidence for addition is always obtainable. In other cases the mercaptan is alkali-labile, and therefore the reaction cannot be executed practically in an aqueous medium. For this reason the spectrum of ergothioneine-pyridine aldehyde-DPN was observed with methanol as a solvent. It has been shown previously for the N²-methyl-
pyridinium compounds, when the cyanide addition reaction is studied, that the addition is favored by a solvent of low dielectric constant (17).

In Table I, which illustrates the scope of the reaction, the absorption maxima of the addition complexes are reported for DPN and two analogues of DPN. As mentioned above, the deaminated analogues show the same maxima as the parent nucleotides. A typical spectrum is illustrated in Fig. 4 with acetylpyridine-DPN and mercaptosuccinic acid.

Requirement for Quaternary Nitrogen—Not only the intact coenzymes will give the addition reaction with mercaptans, but also all derivatives which contain a quaternary nitrogen in the pyridine ring. Thus, apart from DPN and TPN, nicotinamide mononucleotide, nicotinamide riboside, and $N^1$-methylnicotinamide show spectral evidence for an addition complex. However, nicotinamide itself fails to show any complex, even at very high concentrations of the base. The same holds true for the acetylpyridine series. All acetylpyridine derivatives tested which contain a quaternary nitrogen show the addition reaction, while free acetylpyridine does not. These points are illustrated in Table II. It is of interest that the complexes of the $N^1$-methyl derivatives have their absorption maxima about 10 mμ towards longer wave lengths when compared to the ribosides. This is similar to their behavior in the cyanide addition reaction (3). Furthermore, the $N^1$-methyl derivatives appear to have a much lower tendency to form complexes, as compared to the dinucleotide.

Equilibrium Constants for Reaction—If $C$ represents the concentration

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**Fig. 4.** The spectrum of the acetylpyridine-mercaptosuccinic acid complex. The spectrum was taken as a difference spectrum. The experimental cuvette contained mercaptosuccinic acid $0.017 \text{ m}$, acetylpyridine-DPN $0.8 \times 10^{-5} \text{ m}$, in $0.1 \text{ m}$ phosphate buffer, pH 10.2. The blank cuvette contained no mercaptosuccinic acid.
TABLE II
Comparison of Mercaptan Addition Reaction of Different Derivatives of Nicotinamide and Acetylpyridine

All spectra were observed in 0.1 M phosphate buffer, pH 11.0. Ethyl mercaptan was used in a final concentration of 0.045 M. 2.8 pmoles of the derivatives in the nicotinamide series were added; 0.6 pmole was added in the acetylpyridine series. The final volume was 3.0 ml.

<table>
<thead>
<tr>
<th>Derivative</th>
<th>Nicotinamide</th>
<th>Acetylpyridine</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$\Delta E_{\text{max}}$</td>
<td>$\lambda_{\text{max}}$</td>
</tr>
<tr>
<td>Diphosphodinucleotide</td>
<td>0.225</td>
<td>330</td>
</tr>
<tr>
<td>Triphosphodinucleotide</td>
<td>0.102</td>
<td>330</td>
</tr>
<tr>
<td>Mononucleotide</td>
<td>0.210</td>
<td>330</td>
</tr>
<tr>
<td>Riboside</td>
<td>0.135</td>
<td>330</td>
</tr>
<tr>
<td>N$^1$-Methyl derivative</td>
<td>0.040</td>
<td>340</td>
</tr>
<tr>
<td>Free base</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

TABLE III
Equilibrium Constants for Pyridine Aldehyde-DPN-Mercaptan Complex Formation

Pyridine aldehyde-DPN and mercaptan were mixed in 0.1 M phosphate buffer; the mercaptan concentration was assumed to be constant. The complex was estimated by the increase in optical density at the appropriate maximum by using a molar extinction coefficient of $7.0 \times 10^3$. By subtraction of the concentration of the complex, the concentration of free pyridine aldehyde-DPN could be determined. The constants are expressed as $K = ((\text{pyridine aldehyde-DPN-SR})(\text{H}^+))/((\text{pyridine aldehyde-DPN})(\text{R-SH}))$ and are uncorrected for activity.

<table>
<thead>
<tr>
<th>Mercaptan</th>
<th>Equilibrium constant*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glutathione</td>
<td>$1.6 \times 10^{-9}$ (±0.63 $\times 10^{-9}$)</td>
</tr>
<tr>
<td>Cysteine</td>
<td>$2.4 \times 10^{-9}$ (±0.48 $\times 10^{-9}$)</td>
</tr>
<tr>
<td>Thioethanol</td>
<td>$5.1 \times 10^{-10}$ (±1.10 $\times 10^{-10}$)</td>
</tr>
<tr>
<td>Mercapto succinic acid</td>
<td>$1.2 \times 10^{-9}$ (±0.20 $\times 10^{-9}$)</td>
</tr>
<tr>
<td>Ethanethiol</td>
<td>$1.0 \times 10^{-9}$ (±0.60 $\times 10^{-9}$)</td>
</tr>
<tr>
<td>Propanethiol†</td>
<td>$2.0 \times 10^{-10}$ (±0.68 $\times 10^{-10}$)</td>
</tr>
<tr>
<td>Butanethiol</td>
<td>$7.2 \times 10^{-10}$ (±1.90 $\times 10^{-10}$)</td>
</tr>
<tr>
<td>Pentanethiol</td>
<td>$5.9 \times 10^{-10}$ (±2.10 $\times 10^{-10}$)</td>
</tr>
<tr>
<td>Hexanethiol</td>
<td>$3.3 \times 10^{-10}$ (±0.66 $\times 10^{-10}$)</td>
</tr>
</tbody>
</table>

* The values in parentheses represent the standard deviation.
† The purity of propanethiol was doubtful.

of a pyridine nucleotide, $(SH)$ the concentration of a mercaptan, and $(C-S)$ the concentration of the complex formed, the apparent equilibrium constant of the reaction is given by

$$K = \frac{(C-S) \times (H^+)}{(C) \times (SH)}$$
Some variation in the determination of equilibrium constants was encountered, since the autoxidation of the mercaptans introduced a serious uncertainty in the concentration of these substances. Representative equilibrium constants for pyridine aldehyde-DPN and mercaptans are given in Table III. In all cases the equilibrium constants for DPN and acetylpyridine-DPN, when obtainable, were in the order of $10^{-2}$ and $10^{-1}$ times the constant for pyridine aldehyde-DPN, respectively. For example the constants for the ethyl mercaptan complexes were as follows: DPN, $2.4 \pm 0.48 \times 10^{-11}$; acetylpyridine-DPN, $4.5 \pm 0.53 \times 10^{-10}$; and pyridine aldehyde-DPN, $1.0 \pm 0.60 \times 10^{-9}$. This is in line with the equilibrium constants for the cyanide addition reaction (11) and the oxidation-reduction potentials (11, 18) of the analogues.

It is of interest to note that the equilibrium constants of the $n$-alkyl mercaptans follow the same series (with the exception of $n$-propyl mercaptan) as the activity of the corresponding alcohols as substrates for yeast alcohol dehydrogenase (19). Both effects are likely to be a reflection of the decreased nucleophilic character of the compounds, which arises from the increased length of the carbon chain.

**DISCUSSION**

The ubiquitous nature of sulfhydryl compounds in biological systems makes these addition reactions of particular interest. A second point of interest arises from the fact that the mercaptans can be regarded in essence as alcohol analogues.

The possibility that the addition complexes at the para position of DPN are intermediate in the oxidation of alcohols has been discussed extensively by Burton and Kaplan (4). Further treatment of this theory is presented in other publications. The implication can be drawn, however, from the generality of the addition reaction that not only alcohol dehydrogenase proceeds via such a mechanism, but also all dehydrogenases of the following type:

$$R_1 \quad R_1$$

$$\quad H-C-OH + DPN \rightleftharpoons C=O + DPNH + H^+$$

$$R_2 \quad R_2$$

In this class are alcohol dehydrogenases ($R_1 = \text{CH}_3\text{--}, \quad R_2 = \text{--H}$), lactic dehydrogenases ($R_1 = \text{CH}_3\text{--}, \quad R_2 = \text{--CO}_2\text{H}$), malic dehydrogenase, polyol dehydrogenases, etc. If the alcohol, indeed, adds to the para position of the coenzyme, the corresponding mercaptan would be expected

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4 A preliminary report has appeared (20). Further treatment of this theory will be forthcoming (J. van Eys, A. San Pietro, and N. O. Kaplan, in preparation).
to be a strong inhibitor of the oxidation of the alcohol. This is similar to the hydroxylamine effect on yeast and liver alcohol dehydrogenase (21, 22), the sulfide effect on rat liver lactic dehydrogenase (15), and the sulfite effect on heart muscle lactic dehydrogenase (23). Preliminary data confirming this postulate have been presented (24).

It is also of interest to consider a second group of enzymes, which are pyridine nucleotide-dependent. The reaction of this group of enzymes can be represented as follows:

$$R-S-S-R + DPNH \text{ or } TPNH + H^+ \to 2RSH + DPN^+ \text{ or } TPN^+$$

From the results described in this paper one might postulate the following mechanism:

$$R-S-S-R + TPNH + H^+ \to R-S-TPN + H^+ + R-SH + 2R-SH + TPN^+$$

In this scheme the coenzyme-thiol complex is intermediary in the reduction of disulfides. In this class of enzymes fall TPNH-glutathione reductase (25) and lipoic acid oxidase (26).

Racker and Krimsky have postulated a DPN-glutathione complex as an intermediate in the reaction of triosephosphate dehydrogenase (27). This postulation gains some support from the actual existence of the DPN-glutathione complex non-enzymatically described in the present work.

The SH addition reaction is assumed to be in the para position of the pyridine moiety of the coenzyme. This is based on the evidence that the absorption characteristics are very close to those of the previously reported addition complexes. For two of these complexes, namely the cyanide addition (8) and the dithionite addition (5), the reaction has been shown to take place at the para position. It has been postulated that these reactions are not necessarily actual addition reactions but rather "charge-transfer" complexes (28, 29). However, these addition complexes can be isolated and treated like chemical compounds. For example, the dihydroxyacetone complex has been obtained and could be oxidized by ferricyanide to an oxidized analogue of DPN (30). This behavior would not be expected from the labile junction of dihydroxyacetone and DPN as would result from a "charge-transfer" complex. Recently Marti et al. (31) have actually isolated in crystalline form the cyanide addition product of $N'$-methylnicotinamide. This crystalline complex still had the properties of the addition complex as observed in solution.

**SUMMARY**

The reaction between sulfhydryl compounds and pyridine nucleotides has been described. Products are obtained which have absorption maxima
in the region obtained for the complexes formed by previously described addition reactions.

The implications of the findings with regard to the mechanism of pyridine nucleotide-dependent enzymes are discussed.

BIBLIOGRAPHY

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