PYRIDINE NUCLEOTIDE TRANSHYDROGENASE

VI. MECHANISM AND STEREOSPECIFICITY OF THE REACTION
IN PSEUDOMONAS FLUORESCENS*

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It has been reported previously (1) that the enzyme, transhydrogenase,
of Pseudomonas fluorescens catalyzes a reaction between reduced and oxidized pyridine nucleotides. Studies of the nature of the products from
the reaction between TPNH and DPN labeled with C14-nicotinamide,
which was shown by Kaplan et al. (2) to proceed as follows

\[ \text{TPNH} + \text{C}^{14}\text{-DPN} \rightarrow \text{TPN} + \text{C}^{14}\text{-DPNH} \]

established unequivocally that either a hydrogen or electron transfer was involved in this reaction. The possibility of a transfer of the monoester phosphate group or an exchange of reduced and oxidized nicotinamide-containing moieties (nucleoside, nucleotide, etc.) was ruled out by this and other experiments. There still remained, however, the problem of distinguishing experimentally between the hydrogen and electron transfer mechanisms.

An approach to this problem was suggested by the work of Westheimer
et al. (3) in which they demonstrated, with deuterium as a tracer, that the
oxidation of ethanol by DPN, in the presence of yeast ADH, occurred by
a direct transfer of hydrogen from alcohol to DPN. The same group later
extended these studies and were able to show that the reaction was steri-
cally specific with respect to both DPN (4) and ethanol (5). We have used
this labeling technique to study the mechanism and stereospecificity of the
bacterial transhydrogenase reaction.

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1 The following abbreviations will be used: DPN, diphosphopyridine nucleo-
tide; TPN, triphosphopyridine nucleotide; DPNH and TPNH, the reduced forms;
DPN(D) and TPN(D), oxidized deuterio-DPN and oxidized deuterio-TPN; DPND
and TPND, reduced deuterio-DPN and reduced deuterio-TPN; Tris, tris(hydroxy-
methyl)aminomethane; ADH, alcohol dehydrogenase.
One of the reactions studied with the bacterial enzyme was the oxidation of chemically reduced deuterio-TPN by DPN. The results of these studies were consistent with the view that the reaction catalyzed by transhydrogenase occurred by a direct hydrogen transfer between reduced and oxidized nucleotides. Studies on the stereochemical configuration of the reduced deuterio-DPN showed that this product was different from that formed in the yeast ADH system, with respect to its behavior upon enzymatic oxidation with yeast ADH and acetaldehyde, as well as upon chemical oxidation with neutral ferricyanide. The transhydrogenase product, in contrast to the yeast ADH product, did not lose all of its deuterium upon enzymatic oxidation. Furthermore, the transhydrogenase product showed the same retention of deuterium upon enzymatic or chemical oxidation.

Fig. 1. Stereoisomers of reduced deuteriopyridine nucleotides. As defined here, Form A, containing deuterium on Side 1 of the pyridine ring, corresponds to the product of the reaction with yeast ADH and 1,1-dideuterioethanol. Form B is the opposite stereoisomer.

On the basis of this latter finding, we expressed the view that the transhydrogenase appeared to exhibit a lack of stereospecificity (6); that is, that the reduced deuterio-DPN formed in this reaction consisted of an equal mixture of two stereoisomers. One of these (Form A) would be identical to that formed by yeast ADH and 1,1-dideuterioethanol; the other (Form B) would have the opposite configuration (Fig. 1). This conclusion was based on the assumption of a lack of stereospecificity in the chemical oxidation process. Subsequent experiments on the stereochemistry of the chemical oxidation, presented below, revealed that whereas Form A retains about 50 per cent of its deuterium, Form B retains essentially all of its deuterium upon chemical oxidation. This finding suggested that the transhydrogenase product was not an equal mixture of Forms A and B, but rather consisted solely of Form B. These results, therefore, indicated that the transhydrogenase reaction had occurred in a stereospecific manner, but that the stereospecificity exhibited by this enzyme was opposite to that of yeast ADH. Additional experimental evidence in support of this
latter hypothesis has been obtained independently of the above considerations and will be presented below. In this paper, all of the results have been calculated on the basis that the stereospecificity exhibited by transhydrogenase is opposite to that of yeast ADH.

Methods and Materials

DPN was purchased from either the Sigma Chemical Company or the Pabst Laboratories and TPN from the Sigma Chemical Company. dl-Isocitrate (as the trisodium salt) was purchased from the H. M. Chemical Company. Oxidized glutathione and 2'-adenylic acid were obtained from the Schwarz Laboratories.

Chemically reduced deuterio-DPN and deuterio-TPN were prepared by reduction of the oxidized nucleotide with sodium hydrosulfite in 99.5 per cent deuterium oxide, as described by Pullman et al. (7). Enzymatically reduced deuterio-DPN was prepared by means of yeast ADH and 1,1-dideuterioethanol. Crystalline yeast ADH was prepared by the method of Racker (8).

The transhydrogenase was purified as described previously (9). The DPNase was a purified enzyme which was obtained from zinc-deficient Neurospora according to the method of Kaplan et al. (10). The TPN-specific isocitric dehydrogenase was obtained from an acetone powder of pig heart and was fractionated essentially as described by Grafflin and Ochoa (11). A glutathione reductase from peas specific for reduced TPN has been described by Mapson and Goddard (12). It was partially purified as described previously (13).

The general experimental procedures used in these studies have been described in detail by Pullman et al. (7). All deuterium analyses were carried out on crystalline samples of nicotinamide derived from cleavage of the oxidized nucleotides with DPNase. The separation of DPN and TPN by means of column chromatography was based on the method of Kornberg and Horcoker (14).

Results

Stereochemistry of Chemical Oxidation of Reduced Deuterio-DPN—The method employed here for analysis for deuterium in reduced pyridine

2 The heavy water used in these experiments was obtained on allocation from the United States Atomic Energy Commission.

3 The authors are indebted to Dr. Frank H. Westheimer, Dr. Frank A. Loewus, and Dr. Birgit Vennesand for making their laboratory facilities available to us for the preparation of the 1,1-dideuterioethanol. It was prepared according to the procedure of Fisher et al. (4).

4 These experiments were carried out in collaboration with Dr. Maynard E. Pullman.
nucleotides consists in chemical or enzymatic oxidation of the latter, followed by cleavage of the oxidized nucleotides with DPNase and analysis of the resulting nicotinamide for deuterium (7). While the stereospecificity of enzymatic oxidation with acetaldehyde and yeast ADH is well established (4, 7), an investigation of the stereochemistry of chemical oxidation was essential in order to permit interpretation of the analytical data.

Reduced deuterio-DPN (Form A), prepared by reduction of DPN with 1,1-dideuterioethanol and yeast ADH, retains about 50 per cent of its deuterium after chemical oxidation to DPN with neutral ferricyanide

<table>
<thead>
<tr>
<th>Experiment No.</th>
<th>Reduced deuterio-DPN Form</th>
<th>Mode of oxidation</th>
<th>Per cent deuterium remaining</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>A*</td>
<td>Chemical</td>
<td>67†</td>
</tr>
<tr>
<td>2</td>
<td>A†</td>
<td>Enzymatic</td>
<td>3†</td>
</tr>
<tr>
<td>3</td>
<td>A*</td>
<td>Chemical</td>
<td>49</td>
</tr>
<tr>
<td>4</td>
<td>B§</td>
<td>&quot;</td>
<td>90</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Enzymatic</td>
<td>100</td>
</tr>
</tbody>
</table>

* Assumed to contain 1 atom of deuterium per molecule.
† Data from Pullman et al. (7).
‡ Contained 0.67 atom of deuterium per molecule by direct analysis as described by Fisher et al. (4).
§ Prepared by enzymatic reduction of the oxidized deuterio-DPN formed in Experiment 3; contained 0.49 atom of deuterium per molecule, assuming that no deuterium was lost on enzymatic oxidation.

(Table I, Experiments 1 to 3). This figure is approximate; three separate determinations have given values of 67 per cent (7), 49 per cent, and 46 per cent. Assuming the probable existence of an isotope effect (15, 16) in this oxidation, i.e. that the C—D bond is less susceptible to oxidation than the C—H bond, one would have expected a preferential retention of deuterium. It appeared, therefore, that there had been a preferential attack of ferricyanide on that side of the pyridine ring having the C—D bond, i.e. Side 1. A preference for Side 1 opposed by an isotope effect of equal magnitude in favor of Side 2 would account for the observed results.

In order to confirm this conclusion, oxidized DPN containing deuterium (7) was reduced with yeast ADH and unlabeled alcohol, thereby yielding reduced DPN (Form B) in which the deuterium was on the opposite side of the pyridine ring, i.e. Side 2. If chemical oxidation were now to show a
preference for Side 1, this combined with an isotope effect favoring Side 1 should lead to a retention of essentially all of the deuterium.\(^5\) This result would be practically indistinguishable from that expected upon enzymatic oxidation with acetaldehyde and yeast ADH, which has been shown to be completely specific for Side 1 and would therefore lead to 100 per cent retention of deuterium.

The results of such an experiment are reported in Table I, Experiment 4. The DPN samples resulting from chemical and enzymatic oxidation were practically identical in deuterium content. One may conclude that, when reduced pyridine nucleotides contain deuterium on Side 2, both chemical and enzymatic oxidation will lead to essentially complete retention of deuterium, whereas, when they contain deuterium on Side 1, chemical oxidation will lead to about 50 per cent retention, while enzymatic oxidation will of course result in zero retention of deuterium. These factors were therefore used in calculating the results of the experiments which follow. It is of some interest to note that the preference of chemical oxidation for Side 1 is in line with the fact that chemical reduction with dithionite also shows some preference for Side 1 (4, 7).

Transhydrogenase-Catalyzed Reaction between Chemically Reduced Deutério-TPN and Oxidized DPN—Analyses of the reduced DPN and oxidized TPN formed in this reaction are presented in Table II. The DPN samples obtained after chemical or enzymatic oxidation of the reduced DPN were essentially equal in deuterium content. From the considerations in the previous section one may conclude that the reduced deutério-DPN formed in the transhydrogenase reaction contained deuterium on Side 2 only. If an appreciable amount of deuterium had been on Side 1, the value after enzymatic oxidation would have been less than that after chemical oxidation. Therefore, the results not only demonstrate that the transhydrogenase reaction involves a direct hydrogen transfer, but also support the view that the enzyme is stereospecific for Side 2, in contrast to certain dehydrogenases (4, 17) which are specific for Side 1. The earlier conclusion from these data, namely that the transhydrogenase exhibits a lack of stereospecificity (6), was based on a misconception of the stereochemistry of the chemical oxidation process, as pointed out above.

Some of the quantitative aspects of the experiment in Table II are worth noting. The chemically reduced deutério-TPN would be expected, by analogy with chemically reduced DPN (7), to be about 70 per cent in the form containing deuterium on Side 1 and about 30 per cent in the form containing deuterium on Side 2. After the transhydrogenase reaction had proceeded to equilibrium by a reaction completely specific for Side 2,
The oxidized TPN should consist of 70 per cent deuterio-TPN and 30 per cent unlabeled TPN, while the reduced DPN should consist of 30 per cent DPND and 70 per cent DPNH. The results in the last column of Table II are in good agreement with this expectation. The reduced DPN, ana-

**Table II**

_Reaction between Chemically Reduced Deuterio-TPN and Oxidized DPN_

TPND + DPN → DPND + TPN(D)

The complete system contained 14.2 μmoles of TPND, 45.5 μmoles of DPN, about 1000 units of transhydrogenase, and 0.067 M Tris buffer, pH 7.5, in a total volume of 7.5 ml. Incubation for 60 minutes at 37°. After incubation, the reaction mixture, which contained 10.8 μmoles of reduced DPN, was divided into two approximately equal fractions. One fraction was treated with neutral ferricyanide to oxidize the reduced nucleotides chemically, whereas the reduced nucleotides in the second fraction were oxidized enzymatically with yeast ADH and acetaldehyde. In each case, the total oxidized nucleotide fraction was precipitated with acid-acetone and collected by centrifugation. An aqueous solution of the oxidized nucleotides was placed on a Dowex 1 formate column and the DPN and TPN fractions separated by elution with 0.1 M formic acid-sodium formate buffer and 0.1 M HNO₃, respectively. Each nucleotide fraction was then precipitated with acid-acetone either directly or after a known dilution with unlabeled oxidized nucleotide. The precipitates were dissolved in 0.05 M sodium acetate, cleaved with Neurospora DPNase, and the resulting nicotinamide was diluted by a known factor with unlabeled nicotinamide. The nicotinamide was isolated by crystallization from benzene and analyzed for deuterium in the usual manner.

The deuterium content of the nicotinamide, expressed as atoms of deuterium per molecule, is identical to the deuterium content, expressed similarly, of the oxidized nucleotide from which it was derived (7). For nicotinamide, a deuterium content of 1 atom of deuterium per molecule corresponds to a value of 16.7 atoms per cent excess.

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<table>
<thead>
<tr>
<th>Nucleotide formed</th>
<th>Mode of oxidation</th>
<th>Dilution factor</th>
<th>Deuterium content</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Atom per cent</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>excess*</td>
</tr>
<tr>
<td>Reduced DPN</td>
<td>Enzymatic</td>
<td>4.6</td>
<td>0.26</td>
</tr>
<tr>
<td>“</td>
<td>Chemical</td>
<td>5.6</td>
<td>0.17</td>
</tr>
<tr>
<td>Oxidized TPN</td>
<td>Enzymatic</td>
<td>32.4</td>
<td>0.33</td>
</tr>
</tbody>
</table>

* Experimental values.
† Values corrected for dilution (see the text).

lyzed after enzymatic oxidation with acetaldehyde and yeast ADH (which should remove no deuterium), contained 0.3 atom of deuterium per molecule, while the deuterium content of oxidized TPN was 0.64 atom per molecule.

A few comments are necessary to explain dilution factors employed in calculating the results. The dilution factors listed in Table II refer to
the extent of dilution of the oxidized nucleotide fractions by added unlabeled nucleotide or nicotinamide in the course of isolation. An additional dilution factor, not shown in Table II, was used to arrive at the final values for reduced DPN in the last column. This factor, used to correct for dilution by oxidized DPN remaining in the transhydrogenase reaction mixture, was 45.5/10.8 or 4.2 in this case. It is of course assumed, in applying this factor, that any oxidized DPN remaining in the reaction mixture will be unlabeled, and this assumption is valid for a system exhibiting strictly stereospecific hydrogen transfer.

In the case of the value for oxidized TPN, there was no need to apply this kind of additional dilution factor. The only oxidized TPN present after "enzymatic oxidation" was that formed via the transhydrogenase reaction, since the acetaldehyde-yeast ADH system used for enzymatic oxidation is DPN-specific and does not oxidize reduced TPN detectably under the conditions employed.

Transhydrogenase-Catalyzed Reaction between Chemically Reduced Deuterio-DPN and Oxidized TPN—Analyses of the oxidized DPN and reduced TPN formed in this reaction are presented in Table III. The observed values of 0.63 and 0.38 atoms of deuterium per molecule, respectively, are in fairly good agreement with the values expected for a direct transfer of hydrogen specific for Side 2, when the starting material consists of two kinds of reduced deuterio-DPN, about 70 per cent having the deuterium on Side 1 and about 30 per cent having the deuterium on Side 2.

It should be noted that in this experiment the oxidized DPN was not separated from oxidized TPN present in the reaction mixture prior to analysis. Instead, a dilution factor, not reported in Table III, of 51/19 or 2.7, was applied to correct for the degree of dilution of labeled oxidized DPN by unlabeled oxidized TPN. The assumption that any oxidized TPN present in the reaction mixture will be unlabeled is valid, provided that the enzymatic reaction is stereospecific.

No additional dilution factor was required for the reduced deuterio-TPN formed in the reaction mixture, since the reduced TPN and reduced DPN fractions were separated chromatographically after chemical oxidation. Finally, it should be noted that, while the value of 0.38 atom of deuterium per molecule is not a direct measure of deuterium in the reduced TPN, it is essentially that, since chemical oxidation of reduced TPN containing deuterium only on Side 2 would not be expected to remove an appreciable amount of deuterium.

Transhydrogenase-Catalyzed Reaction between Enzymatically Formed Reduced Deuterio-TPN and Oxidized DPN—This is essentially the same as the first reaction which was described with the exception that the reduced deuterio-TPN was formed enzymatically rather than chemically. A cata-
lytic amount of TPN was used for the enzymatic reduction of DPN by isocitrate in the following manner. Reduced deuterio-TPN, which was continuously generated by the isocitric dehydrogenase system from pig heart in heavy water, was oxidized with excess DPN and transhydrogenase. The deuterium content of the reduced DPN formed during the reaction was determined as described in Table IV. It can be seen that the labeled oxidized DPN, resulting from either chemical or enzymatic oxidation of the reduced DPN, contained the same amount of deuterium, namely 0.2 atom of deuterium per molecule. This finding is in support of the hypothesis that the transhydrogenase is stereospecific for Side 2. As pointed out above, the reduced DPN formed during the reaction would be expected to contain the same amount of deuterium as the oxidized DPN derived from it by chemical or enzymatic oxidation.

The value of 0.2 atom of deuterium per molecule is in agreement with

The mechanism whereby deuterium enters the reduced TPN in this reaction is currently being investigated. The possibility that the action of aconitase is responsible for this incorporation is under investigation.

### Table III

**Reaction between Chemically Reduced Deuterio-DPN and Oxidized TPN**

<table>
<thead>
<tr>
<th>Nucleotide formed</th>
<th>Mode of oxidation</th>
<th>Dilution factor</th>
<th>Deuterium content (Atom per cent excess)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oxidized DPN</td>
<td></td>
<td>6</td>
<td>0.65</td>
</tr>
<tr>
<td>Reduced TPN</td>
<td>Chemical</td>
<td>64</td>
<td>0.10</td>
</tr>
</tbody>
</table>

* Experimental values.
† Values corrected for dilution (see the text).
that expected for a reaction carried out in 50 per cent heavy water, assuming an isotope effect of about 4:1.

It should be noted that in this experiment the unlabeled oxidized DPN was not removed from the reaction mixture prior to oxidation of the reduced DPN. The unlabeled oxidized DPN served, therefore, to dilute by a known factor the labeled oxidized DPN. This dilution factor, which is not included in Table IV, was 148/76.5 or 1.94.

Transhydrogenase-Catalyzed Reaction between Enzymatically Reduced Deuterio-DPN (Form A) and Oxidized DPN—The results of the isotope measurements for this experiment are presented in Table V. It can be seen that the oxidized DPN contained 0.34 atom of deuterium per molecule, whereas the DPN resulting from enzymatic oxidation of the reduced DPN was practically devoid of deuterium. This latter result, that the reduced DPN lost all of its deuterium upon enzymatic oxidation, demonstrated that the reduced DPN maintained its original stereochemical configuration during the reaction. From the isotope distribution in the oxidized and reduced DPN, as well as the stereochemical configuration of the reduced DPN, one can deduce the nature of the stereospecificity exhibited by transhydrogenase. If the stereospecificity exhibited by transhydrogenase were identical to that of yeast ADH, the oxidized DPN would never have contained any deuterium. Had the transhydrogenase exhibited a lack of
stereospecificity, the reduced DPN would not have maintained its original stereochemical configuration. The experimental results clearly indicate that the transhydrogenase could not have exhibited either of these two
types of stereospecificity; that is, either a stereospecificity identical to that of yeast ADH or a lack of stereospecificity. These findings conclusively demonstrate that the stereospecificity exhibited by transhydrogenase is opposite to that of yeast ADH, as shown in Fig. 2, which illustrates the changes occurring at carbon 4 of the pyridine ring.

### Table V

**Reaction between Enzymatically Reduced Deuterio-DPN (Form A) and Oxidized DPN**

<table>
<thead>
<tr>
<th>Nucleotide formed</th>
<th>Mode of oxidation</th>
<th>Dilution factor</th>
<th>Deuterium content</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Atom per cent</td>
</tr>
<tr>
<td>Oxidized DPN</td>
<td>Enzymatic</td>
<td>16.3</td>
<td>0.35</td>
</tr>
<tr>
<td>Reduced <strong>[II]</strong></td>
<td></td>
<td>15.9</td>
<td>0.03</td>
</tr>
</tbody>
</table>

* Experimental values.
† Values corrected for dilution.

![Chemical diagram](http://www.jbc.org/)

**Fig. 2. Stereospecificity of transhydrogenase**
DISCUSSION

It has been possible, with deuterium as a tracer, to demonstrate unequivocally that the mechanism of the reaction catalyzed by Pseudomonas transhydrogenase involves a direct hydrogen transfer rather than an electron transfer. This finding, however, does not provide any direct information concerning the intimate details of the mechanism or the nature of the chemical species which is actually transferred. It does, however, exclude from consideration all mechanisms which require or allow hydrogen to exchange with the medium. There are several possible mechanisms which might be compatible with this finding. One such possible mechanism would involve a reversible hydrogen transfer from nucleotide to the enzyme and then from the enzyme to a 2nd nucleotide molecule. This mechanism would involve a reduced enzyme as an intermediate. While it may be possible that the enzyme could be reduced in such a manner as to contain non-exchangeable deuterium, it does not appear to be very likely, since this mechanism appears to have been ruled out in the case of certain dehydrogenases (2, 18). A more probable mechanism would involve a direct interaction between two pyridine nucleotides which are simultaneously bound on the surface of the enzyme.

The spatial arrangement of the nucleotides on the enzyme surface would determine the stereospecificity exhibited by the enzyme. The various types of stereospecificity an enzyme might be expected to exhibit, relative to yeast ADH, can be classified as follows: (a) identical to yeast ADH; (b) opposite to yeast ADH; (c) a lack of stereospecificity. In the case of Pseudomonas transhydrogenase, it has been possible to eliminate possibilities (a) and (c) and demonstrate that possibility (b) is the correct one.

Thus far, the pyridine nucleotide-linked enzymes studied have fallen into categories (a) or (b). Lactic dehydrogenase from beef heart has been shown to exhibit the same stereospecificity as yeast ADH (17). Talalay, Loewus, and Vennesland found that a β-hydroxysteroid dehydrogenase from certain species of Pseudomonas, like the transhydrogenase investigated here, exhibits a stereospecificity opposite to that of yeast ADH (19).

The authors are indebted to Dr. David Rittenberg for the isotope analyses listed in Tables II and III, and to Dr. Theodore Enns and Dr. Susanne von Schuching, working under Veterans Administration contract No. V1001M-527, for their cooperation in obtaining the data presented in Tables I, IV, and V. We also wish to thank Mr. Francis E. Stolzenbach for the enzyme preparations used in this study.

SUMMARY

Experiments have been described, with deuterium as a tracer, which indicate that the reaction catalyzed by the pyridine nucleotide transhydro-
genase from *Pseudomonas fluorescens* involves a direct hydrogen transfer rather than electron transfer. In addition, the stereospecificity exhibited by this enzyme has been shown to be opposite to that of yeast alcohol dehydrogenase.

**BIBLIOGRAPHY**

PYRIDINE NUCLEOTIDE TRANSHYDROGENASE: VI. MECHANISM AND STEREOSELECTIVITY OF THE REACTION IN PSEUDOMONAS FLUORESCENS
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