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*J Clin Invest.* 1963;[42\(12\)](#):1908-1912. <https://doi.org/10.1172/JCI104876>.

Research Article

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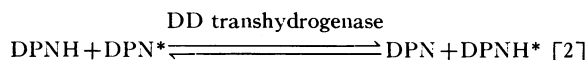
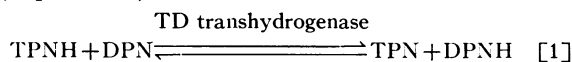
## STUDIES ON NORMAL AND LEUKEMIC LEUKOCYTES. V. PYRIDINE NUCLEOTIDE TRANSHYDROGENASES \*

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(Submitted for publication May 31, 1962; accepted August 16, 1963)

Enzymatic transhydrogenation reactions involving pyridine nucleotides have been described in bacteria (2), beef heart (3), rat and rabbit tissues (4), human placenta (5), and spinach (6). TD transhydrogenase<sup>1</sup> catalyzes a reaction between TPNH and DPN, as shown in Equation 1, and DD transhydrogenase catalyzes a similar reaction between the oxidized and reduced forms of DPN<sup>2</sup> (Equation 2).



The function of these enzymes *in vivo* is not yet entirely clear, but the TD transhydrogenase may link TPNH to the mitochondrial electron transport system that oxidizes DPNH via molecular oxygen. The DD enzyme, alternatively, may function in the equilibration of free and mitochondrial-bound DPN (3). Hormonal influence on transhydrogenase reactions has been noted by Villee (5), who described the stimulation of a placental TD transhydrogenase by 17- $\beta$ -estradiol, and by Ball and Cooper (7), who demonstrated the inhibition of a TD transhydrogenase by thyroid hormones.

\* This investigation was supported by a grant (P-203) from the American Cancer Society. A preliminary report on this work has appeared elsewhere (1).

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<sup>1</sup> Abbreviations: TD and DD, TPNH-DPN transhydrogenase and DPNH-DPN transhydrogenase; DPN and TPN, di- and triphosphopyridine nucleotide; DPNH and TPNH, reduced DPN and TPN; *p*CMB, *p*-chloromercuribenzoate; and EDTA, ethylenediaminetetraacetate.

<sup>2</sup> The asterisk in Equation 2 indicates a labeled DPN molecule or a DPN analogue to distinguish between the two components.

A previous study from this laboratory (8) on the pyridine nucleotides of human leukocytes revealed a marked elevation of the DPN content of leukemic cells. Because of this finding, a further examination has been made of various enzymes responsible for the interconversion of pyridine nucleotides. The present report describes the identification, characterization, and levels of the two pyridine nucleotide transhydrogenases in the leukocytes of normal subjects and various leukemia patients.

### METHODS

The following materials were used: DPNH, TPNH, and protamine sulfate;<sup>3</sup> 3-acetylpyridine-DPN, 3-pyridine aldehyde-DPN, 3-acetylpyridine-deamino-DPN, and thionicotinamide-DPN;<sup>4</sup> EDTA;<sup>5</sup> thyroxine and 3'-triiodothyronine;<sup>6</sup> 17- $\beta$ -estradiol, diethylstilbesterol, and androsterone;<sup>7</sup> and 3-acetylpyridine-TPN.<sup>8</sup>

Clinical material for this study was selected according to the criteria used in the preceding paper (9). In general, cells from leukemia patients were obtained before treatment, except that two of the chronic myelocytic leukemia patients were being treated with busulfan and four of the chronic lymphocytic leukemia patients were receiving chlorambucil at the time of sampling. The leukocyte counts and enzyme levels of these treated patients, however, were comparable to those of untreated patients in the same clinical categories, and the data are included, therefore, in this paper. Leukocytes were isolated from whole blood by the procedure described previously (8, 9). Enzyme assays were performed on cell lysates prepared as follows: The isolated leukocytes were suspended in 0.08 M phosphate buffer, pH 7.5, and homogenized for 3 minutes at 45,000 rpm in a Virtis "45" homogenizer (4- to 15-ml cup). The homogenate was centrifuged for 15 minutes at 10,900  $\times g$  in an International refrigerated centrifuge, model PR-2, and the

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<sup>7</sup> Mann Research Laboratories, Inc., New York, N. Y.

<sup>8</sup> Generously supplied by Dr. N. O. Kaplan.

supernatant fraction was dialyzed for 16 hours at 4° C against 0.05 M potassium phosphate buffer, pH 7.5, containing 0.005 M EDTA. Any precipitate resulting from dialysis was removed by centrifugation at  $10,900 \times g$  for 15 minutes. Protein concentration in the dialyzed supernatant fraction was determined with the biuret reagent.

**Enzyme assays.** Transhydrogenase reactions were carried out at 25° C according to the procedures of Stein, Kaplan, and Ciotti (4). For the assay of DD transhydrogenase, the reaction mixture contained 100  $\mu$ moles of potassium phosphate buffer, pH 7.5, 1.5  $\mu$ moles of sodium cyanide, 0.2  $\mu$ mole of DPNH, 0.2  $\mu$ mole of 3-acetylpyridine-DPN, and the enzyme (1 to 3 mg of protein) in a total volume of 1.0 ml. For the assay of TD transhydrogenase, the components were the same except that pH 6.5 phosphate buffer was used, TPNH was substituted for DPNH, and 10  $\mu$ moles of EDTA was added. In both reactions, the increase in absorbancy at 375 m $\mu$  was determined over a 5-minute period and corrected for blank reactions in which *a*) the pyridine nucleotide analogue was omitted and *b*) the reduced pyridine nucleotide was omitted. The control reactions were less than 10% of the complete reaction. A millimolar extinction coefficient of 5.1 was used to calculate concentration changes in the above reactions (4).

**Purification of leukocyte DD transhydrogenase.** A leukocyte homogenate was dialyzed for 16 hours against 0.05 M potassium phosphate, pH 7.5, containing 0.005 M EDTA, to remove endogenous substrates. One vol of 2% protamine sulfate was added to the dialyzed homogenate, and the precipitate was removed by centrifugation. The supernatant fluid was fractionated with ammonium sulfate at 4° C by the addition of 24.3 g of ammonium sulfate per 100 ml of solution. After centrifugation, the precipitate (0 to 40% fraction) was discarded, and additional ammonium sulfate (24.5 g per 100 ml of solution)

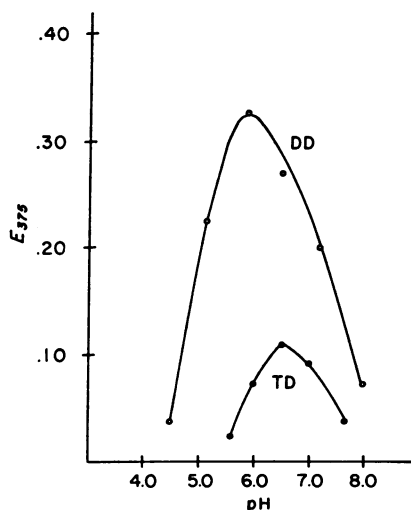


FIG. 1. pH-ACTIVITY CURVES FOR LEUKOCYTE DPNH-DPN (DD) AND TPNH-DPN (TD) TRANSHYDROGENASES.

TABLE I

*Effect of mono- and dithiol compounds on the inhibition of transhydrogenases by Cd<sup>++</sup> and p-chloromercuribenzoate (pCMB)\**

System	Activity ( $\Delta E_{375}$ )	
	DD	TD
Control	0.210	0.100
Cd <sup>++</sup> ( $5 \times 10^{-4}$ M)	0.080	0.100
Cd <sup>++</sup> followed by 2,3-dimercapto-1-propanol ( $1 \times 10^{-3}$ M)	0.200	
Cd <sup>++</sup> followed by glutathione ( $5 \times 10^{-3}$ M)	0.105	
Cd <sup>++</sup> followed by cysteine ( $2 \times 10^{-3}$ M)	0.095	
pCMB ( $5 \times 10^{-4}$ M)	0.100	0.015
pCMB followed by cysteine ( $2 \times 10^{-3}$ M)	0.180	0.090

\* Enzyme and pyridine nucleotide were preincubated at 25° with Cd<sup>++</sup> or pCMB for 5 minutes. The thiol compounds were then added, and after a second incubation period of 5 minutes, the reaction was started by the addition of 3-acetylpyridine-DPN.  $\Delta E_{375}$  refers to the change in absorbancy at 375 m $\mu$  over a 5-minute period. DD = DPNH-DPN transhydrogenase, and TD = TPNH-DPN transhydrogenase.

was added to the supernatant fluid. The resulting precipitate (40 to 75% fraction) contained the DD transhydrogenase and was resuspended in 0.05 M phosphate buffer, pH 7.5. The mixture was dialyzed according to the above procedure to remove ammonium sulfate, and an acetone fractionation was carried out at -20° C (6). The fraction that precipitated between 35 to 75% acetone was resuspended in 0.05 M phosphate buffer, pH 7.5, and dialyzed. The above procedure results in a DD transhydrogenase purified four times relative to the original homogenate and free of TD transhydrogenase. Studies on the latter enzyme were performed with the initial homogenate.

## RESULTS

**Characterization of DD and TD transhydrogenases.** The effect of pH on the activities of both transhydrogenases is shown in Figure 1. The pH optima for the two enzymes are quite similar, i.e., pH 5.8 for the DD transhydrogenase and pH 6.3 for the TD enzyme, but the relative activity of the DD enzyme is about three times greater at the optimum.

The Michaelis constants for the substrates of the leukocyte transhydrogenases were determined by the conventional Lineweaver-Burk plots—for the DD enzyme,  $K_m = 5 \times 10^{-5}$  M and  $7 \times 10^{-5}$  M, respectively, for DPNH and 3-acetylpyridine-DPN; for the TD enzyme,  $K_m = 2 \times 10^{-4}$  M and

TABLE II  
Reactivity of transhydrogenase enzymes  
with pyridine nucleotide analogues

Analogue	Relative rate*	
	DD	TD
3-Acetylpyridine-DPN	100	100
3-Acetylpyridine-deamino-DPN	10	15
3-Pyridinealdehyde-DPN	43	20
3-Acetylpyridine-TPN	0	0
Thionicotinamide-DPN	140	5

\* The rates of DD and TD transhydrogenase reactions with 3-acetylpyridine-DPN were set arbitrarily at 100. The following wavelengths were used to follow the reactions: 375 m $\mu$  for 3-acetylpyridine-DPN, 3-acetylpyridine-deamino-DPN, and 3-acetylpyridine-TPN; 365 m $\mu$  for 3-pyridinealdehyde-DPN; and 400 m $\mu$  for thionicotinamide-DPN.

$4 \times 10^{-4}$  M, respectively, for TPNH and 3-acetylpyridine-DPN.

A study of the effect of metal ions (at  $5 \times 10^{-4}$  M) on the two transhydrogenases indicated that, although  $\text{Ca}^{++}$ ,  $\text{Mg}^{++}$ ,  $\text{Mn}^{++}$ , and  $\text{Ba}^{++}$  do not alter the activity of either enzyme,  $\text{Hg}^{++}$  inhibits the DD and TD transhydrogenases 30 and 50%, respectively. At  $5 \times 10^{-4}$  M,  $\text{Cd}^{++}$  shows selective inhibition (62%) of DD transhydrogenase, but has no appreciable effect on the TD enzyme (Table I). The inhibition of DD transhydrogenase by  $\text{Cd}^{++}$  was reversed by the dithiol, 2,3-dimercapto-1-propanol, whereas the monothiols, cysteine and glutathione, were ineffective even when present at a concentration five times greater than that of the dithiol. Both the DD and TD enzymes were inhibited about 50% by pCMB at  $5 \times 10^{-4}$  M, and in both cases, the inhibition could be reversed by the addition of cysteine.

The results of experiments concerned with the effect of certain hormones on the leukocyte DD and TD transhydrogenases showed that the latter enzyme was inhibited about 30% by thyroid hor-

mones (l-thyroxine and 3',5'-triiodothyronine) at a concentration of  $10^{-4}$  M, whereas the activity of the DD enzyme was not altered under these conditions. On the other hand, neither of the enzymes was affected by estrogens or androgens, such as 17- $\beta$ -estradiol (3  $\mu\text{g}$  per ml), a commercial preparation (Premarin) of conjugated estrogens (30  $\mu\text{g}$  per ml), diethylstilbestrol ( $10^{-4}$  M), and androsterone ( $10^{-4}$  M).

The relative activities of the leukocyte DD and TD transhydrogenases were studied with several pyridine nucleotide analogues that replaced DPN and DPN,\* respectively, as the oxidants in Reactions 1 and 2. The analogues differed from DPN in terms of the substituent groups at the 3 position of the pyridine ring or at the 6 position of the adenine moiety. The data for the activities of these analogues with the two transhydrogenases are presented in Table II. When the activity with 3-acetylpyridine-DPN is set arbitrarily at 100, decreased rates in both enzyme systems were noted when the analogue was changed by 1) deamination of the adenine, 2) replacement of the acetyl group with an aldehyde, or 3) addition of a third phosphate to give 3-acetylpyridine-TPN. Replacement of the 3-acetyl group by a thioamide group, as in thionicotinamide-DPN, however, enhanced the rate of the DD enzyme, but caused a decrease in the activity of the TD enzyme.

*Levels of DD and TD transhydrogenases in normal and leukemic leukocytes.* The levels of both transhydrogenase enzymes were determined in 25 normal subjects and in 25 patients with leukemia (Table III).<sup>9</sup> The DD transhydrogenase

<sup>9</sup> Using a different assay than the one employed here, Evans and Karnovsky (10) were unable to detect the presence of TD transhydrogenase in polymorphonuclear leukocytes from peritoneal exudates of guinea pigs, although a pyridine nucleotide oxidase was found with a

TABLE III  
Levels of transhydrogenases in normal and leukemic leukocytes

Cell type	No. of subjects	Transhydrogenase activity	
		DD	TD
		m $\mu$ moles/hr/mg protein	
Normal	25	178 $\pm$ 12 (90-280)*	37 $\pm$ 6 (20-80)
Chronic myelocytic leukemia	8	286 $\pm$ 46 (150-430)	72 $\pm$ 20 (30-200)
Chronic lymphocytic leukemia	10	287 $\pm$ 46 (100-550)	161 $\pm$ 46 (59-290)
Acute leukemia	7	362 $\pm$ 98 (130-600)	150 $\pm$ 32 (49-220)

\* Mean  $\pm$  SE. The range for each group is indicated in parentheses.

activity is about 2 to 4 times higher than that of the TD enzyme in each of the cell types. The mean level of the DD transhydrogenase in leukemic cells is somewhat higher than in normal leukocytes, but there is considerable overlap between the ranges, and the difference between the means is of a relatively low degree of statistical significance ( $p = 0.05$  to  $0.1$ ). On the other hand, the mean level of TD transhydrogenase is 3 to 4 times greater than normal in the cells of chronic lymphocytic leukemia and acute leukemia; these differences are significant at the 1% level. Even the TD value for chronic myelocytic leukemia is increased above normal in a statistically significant manner ( $p < 0.05$ ). The pH curves and Michaelis constants of the transhydrogenases from leukemic cells were identical with those of normal leukocytes, thus validating the use of the same assay systems given in the methods section for determining the levels of these enzymes in both normal and leukemic cells.

#### DISCUSSION

In general, properties of the leukocyte transhydrogenases, such as the Michaelis constants, inhibition by  $Hg^{++}$  and  $Cd^{++}$ , and reactivity toward pyridine nucleotide analogues, are similar to those of the transhydrogenases from other sources. Since the  $Cd^{++}$  inhibition of DD transhydrogenase can be reversed by dithiols, but not by monothiols, it is likely that vicinal dithiol groups are involved in the mechanism of action of this enzyme, which thus resembles its counterpart in beef heart mitochondria (3). The leukocyte enzymes are unique, however, among transhydrogenases insofar as their pH optima are about 2 pH U lower than usual.

Evidence that two separate enzymes are involved in Reactions 1 and 2 is provided by the following observations: 1) TD activity is lost during the purification procedure for the DD activity; 2)  $Cd^{++}$  at  $5 \times 10^{-4}$  M inhibits the DD activity, but does not alter TD activity; 3) DD transhydrogenase is more active with thionicotinamide-DPN as a substrate than with 3-acetylpyridine-DPN, whereas the converse relationship obtains for the TD enzyme; and 4) thyroid hormones inhibit TD transhydrogenase, but not DD transhydrogenase.

similar pH optimum and  $K_M$  values like those of the transhydrogenase described above.

Although various transhydrogenases have been reported to be affected by thyroid hormones (7) and by certain steroid hormones (5), the leukocyte enzymes are relatively insensitive to these agents. The TD transhydrogenase is partially inhibited by thyroxine or triiodothyronine, but only when these hormones are present at  $10^{-4}$  M, and the *in vivo* significance of this finding is doubtful. None of the steroid hormones tested had any effect on either leukocyte transhydrogenase activity.

The finding that the level of TD transhydrogenase is markedly elevated in the leukemic leukocyte represents a second quantitative difference from normal cells encountered in the general area of pyridine nucleotide metabolism. Thus, there appears to be a general relationship in leukocytes between TD transhydrogenase levels and the DPN levels (8), inasmuch as the highest level of the enzyme is found in acute and chronic lymphocytic leukemic cells that also contain the highest concentration of DPN. Conversely, normal leukocytes contain the lowest level of both DPN and TD transhydrogenase, whereas cells from patients with chronic myelocytic leukemia are intermediate in both respects. Because of the possible relationship of DPN to the altered respiratory patterns of tumor tissue (11) and the suggested role of these coenzymes in the control of metabolic pathways, further investigation along this line seems indicated.

#### SUMMARY

1) Leukocytes contain a TD transhydrogenase ( $TPNH + DPN \rightleftharpoons TPN + DPNH$ ) and a DD transhydrogenase ( $DPNH + DPN^* \rightleftharpoons DPN + DPNH^*$ ).<sup>2</sup>

2) The pH optima for the DD and TD transhydrogenases are 5.8 and 6.3, respectively. At their optima, the relative activity of the DD enzyme is about 3 times that of the TD enzyme.

3) Both transhydrogenases are inhibited about 50% by *p*-chloromercuribenzoate at  $5 \times 10^{-4}$  M, and the inhibitions are reversed by the addition of thiols.  $Cd^{++}$  at  $5 \times 10^{-4}$  M inhibits the DD enzyme about 65%, but does not affect the TD enzyme; this inhibition is reversed by dithiols but not by monothiols.

4) Thyroxine and triiodothyronine at  $10^{-4}$  M inhibit the TD enzyme by about 30%, but do not

affect the DD enzyme. Steroid hormones do not affect either transhydrogenase.

5) Levels of the two transhydrogenases have been determined in 25 normal subjects and in 25 patients with leukemia. The level of the DD enzyme is 2 to 4 times higher than that of the TD enzyme in both normal and leukemic cells. The level of the TD transhydrogenase is 3 to 4 times higher than normal in acute and chronic lymphocytic leukemic cells and slightly higher than normal in chronic myelocytic cells. The level of the DD enzyme is elevated slightly above normal in the leukemic cells.

#### ACKNOWLEDGMENT

The authors are indebted to Drs. J. R. Czajkowski, D. H. Coleman, Q. B. DeMarsh, and P. A. Ragen for providing us with blood specimens.

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