Some Stereospecificity Studies with Tritiated Pyridine Nucleotides*

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The stereospecificity of hydrogen transfer for the pyridine nucleotide has been ascertained for a number of hitherto uninvestigated dehydrogenase reactions. These determinations have been made with the help of appropriate reference enzymes, and of DPN and TPN labeled with tritium at the 4 position of the nicotinamide ring. Evidence is presented that the following enzymes use the same position (i.e., *A* or α) as yeast alcohol dehydrogenase: DPN-linked formic dehydrogenase from pea seeds, DPN-linked glyoxylic reductase from spinach leaves, TPN-linked glyoxylic reductase from pea seeds, TPN-linked dihydroorotic dehydrogenase from an aerobic bacterium, and TPN-linked malic enzyme from liver. The opposite stereospecificity (*B* or β) is demonstrated for the DPN-linked liver dehydrogenase which oxidizes uridine diphosphoglucose to uridine diphosphoglucuronic acid.

The enzyme-catalyzed transfer of hydrogen to and from the nicotinamide ring of pyridine nucleotides has been shown to be sterically specific, in the sense that a particular enzyme operates only with one of the two available positions on the reduced carbon atom of the nicotinamide moiety of the pyridine nucleotides (Westheimer *et al.*, 1951; Vennesland and Westheimer, 1954; Vennesland, 1958; Levy *et al.*, 1962). The absolute configurations of the two hydrogen atoms at the reduced position have more recently been determined (Cornforth *et al.*, 1962).

In the earlier stereospecificity studies with the pyridine nucleotides, deuterium was employed. This isotope has advantages over tritium when high accuracy is desired; but for rapid estimation of stereospecificity, tritium offers advantages over deuterium because of the greater ease of estimation of the radioactive isotope. This paper describes the preparation of tritiated DPN and TPN of high specific activity and their use for determinations of the stereospecificity of the following enzymes: formic dehydrogenase, UDPG dehydrogenase, TPN-linked malic enzyme, TPN-linked dihydroorotic dehydrogenase, and DPN- and TPN-linked glyoxylic reductase.[1]

RESULTS AND DISCUSSION

General.—The procedure employed in most of these experiments was to reduce $DPN(T)^+$ or $TPN(T)^+$ with one enzyme system and then to reoxidize it with another enzyme system after heat inactivation of the first enzyme. In every instance, one of these reactions was carried out with the enzyme whose stereospecificity was to be determined, and the other reaction was carried out with an enzyme of known sterospecificity. After reduction and reoxidation, the pyridine nucleotide was hydrolyzed to nicotinamide, which was isolated with a suitable amount of unlabeled carrier nicotinamide and counted. The tritium content of the nicotinamide thus obtained was compared with the tritium content of the nicotinamide from the labeled pyridine nucleotide prior to reduction and reoxidation. Retention of all the tritium shows that the unknown enzyme has the same stereospecificity for pyridine nucleotide as the reference system. Removal of most of the tritium shows that the unknown enzyme has the opposite stereospecificity.

The objective of the present studies was not high accuracy, which is more easily achieved with deuterium, but rather the development of an economical procedure which can easily be applied in any laboratory, especially in view of the commercial availability of an adequate variety of reference enzymes of demonstrated stereospecificity.

Enzymes were selected for study with two purposes in mind: to test several generalizations which may apply to the pyridine nucleotide stereospecificity, and to test the general applicability of the procedure to a variety of crude enzyme preparations. Enzyme sources included bacteria as well as animal and plant tissues. No serious obstacle to stereospecificity determinations was encountered with any of the preparations examined. It should be noted, however, that all enzymes tested were soluble, that the reaction catalyzed by the dehydrogenase under examination was well defined, and that preparations showing appreciable pyridine nucleotide oxidase activity were avoided.

The procedures employed with the various enzymes, and the conclusions, have been summarized in Tables I and II. These conclusions were in each case supported by at least three experiments often done in different ways, but for economy of presentation the tables give only one representative result for each procedure. The results have been expressed numerically as the per cent of tritium removed from the nicotinamide moiety on reoxidation of the reduced pyridine nucleotide. The first experiment in Table I was performed as a control on the procedure, and represents essentially a repetition of an experiment previously carried out with deuterium (Levy and Vennesland 1957). Labeled DPN was reduced by ethanol in the presence of alcohol dehydrogenase (equation 1), and

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[1] The abbreviations used are: UDPG, uridine diphosphoglucose; UDPGA, uridine diphosphoglucuronic acid. The various forms of the oxidized, reduced, and tritium-labeled pyridine nucleotides are represented as follows: DPN and TPN, diphosphopyridine nucleotide and triphosphopyridine nucleotide, respectively, in a general sense; DPN^+ and TPN^+, the oxidized forms of DPN and TPN; DPNH and TPNH, the reduced forms of DPN and TPN; $DPN(T)^+$ and $TPN(T)^+$, the oxidized forms of DPN and TPN labeled with tritium specifically at C-4 of the nicotinamide ring. To indicate the position of the tritium on the methylene group in the reduced nicotinamide ring, the prefixes *A* and *B* are here used instead of the terms α and β, as explained in a recent review (Levy *et al.*, 1962). The *A* position is the position to which hydrogen is transferred by the yeast alcohol dehydrogenase reaction. *B* represents the other position. For the absolute configuration of *A*- and *B*-DPNT (and TPNT), see Cornforth *et al.* (1962).

TABLE I

STEREOSPECIFICITY FOR DPN

The reference enzyme system, with its previously determined stereospecificity, is given in italics. Note that reduction of DPN(T)$^+$ by an enzyme of *A*-stereospecificity gives *B*-DPNT, whereas reduction of DPN(T)$^+$ by an enzyme of *B*-stereospecificity gives *A*-DPNT.

Reducing Enzyme and Substrate	Oxidizing Enzyme and Substrate	Tritium Removed (%)	Conclusion
Alcohol dehydrogenase (yeast), ethanol, A	*Glutamic dehydrogenase, α-ketoglutarate and NH_3, B*	82	Control
Formic dehydrogenase, formate	*Lactic dehydrogenase, pyruvate, A*	4	Formic dehydrogenase, *A*
Formic dehydrogenase, formate	*Glutamic dehydrogenase, α-ketoglutarate and NH_3, B*	73	Formic dehydrogenase, *A*
UDPG dehydrogenase UDPG	*Lactic dehydrogenase, pyruvate, A*	83	UDPG dehydrogenase, *B*
Alcohol dehydrogenase (yeast), ethanol, A	Glyoxylic reductase (spinach), glyoxylate	5	Glyoxylic reductase, *A*
Glucose dehydrogenase, glucose, B	Glyoxylic reductase (spinach), glyoxylate	97	Glyoxylic reductase, *A*

TABLE II

STEREOSPECIFICITY FOR TPN

The reference enzyme system, with its previously determined stereospecificity, is given in italics. Note that reduction of TPN(T)$^+$ by an enzyme of *A*-stereospecificity gives *B*-TPNT, whereas reduction of TPN(T)$^+$ by an enzyme of *B*-stereospecificity gives *A*-TPNT.

Reducing Enzyme and Substrate	Oxidizing Enzyme and Substrate	Tritium Removed (%)	Conclusion
Glucose-6-phosphate dehydrogenase, glucose-6-phosphate, B	Dihydroorotic dehydrogenase, orotate	80	Dihydroorotic dehydrogenase, *A*
Malic enzyme, L-malate	*Glutamic dehydrogenase, α-ketoglutarate, and NH_3, B*	93	Malic enzyme, *A*
Glucose-6-phosphate dehydrogenase, glucose-6-phosphate, B	Malic enzyme, pyruvate, and CO_2	77	Malic enzyme, *A*
Glucose-6-phosphate dehydrogenase, glucose-6-phosphate, B	Glyoxylic reductase (peas), glyoxylate	76	Glyoxylic reductase *A*

reoxidized by α-ketoglutarate and ammonia in the presence of glutamic dehydrogenase (equation 2).

$$CH_3CH_2OH + DPN(T)^+ \rightarrow CH_3CHO + B\text{-DPNT} + H^+ \quad (1)$$

$$\alpha\text{-Ketoglutarate} + NH_4^+ + B\text{-DPNT} \rightarrow \text{glutamate} + DPN^+ \quad (2)$$

In the deuterium experiments, the isotope content of the glutamate formed in the glutamic dehydrogenase reaction was shown to be equal to the isotope content of the DPN$^+$ reduced by the alcohol dehydrogenase reaction. In the present experiments with tritium, only the isotope content of the DPN$^+$ formed in reaction (2) was determined and compared with the isotope content of the DPN(T)$^+$ used in reaction (1). The results showed 82% removal of tritium. There was 95–100% retention of label observed when the *B*-DPNT formed in reaction (1) was reoxidized by acetaldehyde in a reversal of reaction (1). The expected theoretical result after reoxidation by reaction (2) would be 100% removal of tritium, but values of 70–90% have been obtained in the present series of experiments. We regard such results as adequate to indicate the stereospecificity of the enzyme under investigation. The retention of some of the label does not represent absence of complete stereospecificity, but reflects the fact that the reduction and reoxidation of the pyridine nucleotide are never complete, but only approach completion, and the fact that the procedure of heat inactivation of the reducing enzyme is almost always accompanied by the nonenzymic reoxidation of the reduced pyridine nucleotide to a variable though limited extent. The amount of this reoxidation could be estimated from the data, and suitable corrections could be applied to the results to give values closer to the theoretical, but no such corrections have been applied to the data presented here. The retention of tritium is generally larger than similar retention of deuterium in previous experiments. This probably reflects the larger discrimination effects expected with the heavier isotope, as previously explained (Krakow *et al.*, 1962).

Formic Dehydrogenase.—The reaction catalyzed by formic dehydrogenase is shown in equation (3). It has previously been suggested that enzymes with *A* stereospecificity have smaller substrates than enzymes with *B* stereospecificity. Formic dehydrogenase has a very

$$HCOOH + DPN(T)^+ \rightarrow CO_2 + B\text{-DPNT} + H^+ \quad (3)$$

small substrate. The demonstration that the enzyme has *A*-stereospecificity (Table I) would support this generalization. We must caution, however, that there is an overlap in size and complexity of substrates with *A* and *B* stereospecificity, so that the generalization is at best highly approximate.

UDPG Dehydrogenase.—UDPG dehydrogenase catalyzes the oxidation of UDPG to UDPGA, as shown in equation (4). This is a four-electron oxidation. No

$$UDPG + 2\ DPN(T)^+ \rightarrow UDPGA + 2\ A\text{-DPNT} + 2\ H^+ \quad (4)$$

intermediate has been detected, though it seems reasonable to assume that the reaction occurs in two steps (Strominger *et al.*, 1957). It therefore seemed possible that the enzyme might act on both sides of the pyridine nucleotide; but results (Table I) show that the hydrogen transfer occurs primarily to the *B*-position of the nicotinamide ring of the pyridine nucleotide. Thus,

there is no indication that there are two reactions with different stereospecificities, and there is to date no exception to the generalization that enzymes which act on carbohydrates or carbohydrate derivatives have *B*-stereospecificity (except for those derivatives where an α-hydroxy acid is oxidized at the α position).

In the present experiments the DPN(T)$^+$ reduced by UDPG dehydrogenase was reoxidized by pyruvate in the presence of lactic dehydrogenase, an enzyme of *A*-stereospecificity (Loewus *et al.*, 1953). These experiments included a demonstration that the tritium of the *A*-DPNT formed in reaction (4) was transferred quantitatively to lactate in the lactic dehydrogenase reaction.

Experiments with Dihydroorotic Dehydrogenase.—Another generalization noted previously (Vennesland, 1961) is that the stereospecificity for the pyridine nucleotide is the same for a particular substrate, whether the reaction occurs with DPN or TPN. This is supported by the present studies (Table II) with a TPN-specific dihydroorotic dehydrogenase from an obligate aerobe adapted to grow on orotate (Udaka and Vennesland, 1962). The dihydroorotic dehydrogenase previously examined was a DPN-specific enzyme formed by an adapted anaerobe (Graves and Vennesland, 1957). Both enzymes have *A*-stereospecificity. In the present studies, *A*-TPNT was formed by reduction of TPN(T)$^+$ with glucose-6-phosphate (Stern and Vennesland, 1960) as shown in equation (5). When

$$\text{glucose-6-phosphate} + \text{TPN(T)}^+ \rightarrow \text{6-phosphogluconic acid} + A\text{-TPNT} + \text{H}^+ \quad (5)$$

the *A*-TPNT was reoxidized by orotate as shown in equation (6), the tritium appeared in the water of the

$$A\text{-TPNT} + \text{orotate} + \text{H}^+ \rightarrow \text{TPN}^+ + \text{dihydroorotate} \quad (6)$$

medium and not in the dihydroorotate, just as was previously shown for the analogous reaction with the DPN-linked enzyme (Graves and Vennesland, 1957).

Malic Enzyme.—The malic enzyme catalyzes the reaction shown in equation (7). The stereospecificity of

$$\text{malate} + \text{TPN(T)}^+ \rightarrow \text{CO}_2 + \text{pyruvate} + B\text{-TPNT} \quad (7)$$

the reaction was shown to be *A*, by demonstrating that most of the tritium in the TPNT formed in reaction (7) was removed (Table II) by oxidation with α-ketoglutarate and NH_3 in the TPN-linked glutamic dehydrogenase reaction [analogous to that shown in equation (2) (Nakamoto and Vennesland, 1960)]. In another experiment, *A*-TPNT prepared by reaction (5) was reoxidized by pyruvate and CO_2 (reverse of reaction 7), and it was shown that the tritium was transferred from the *A*-TPNT to the malate. The malic enzyme reaction involves the oxidation of an alcohol carbon atom adjacent to a carboxyl group. All such α-hydroxy acid oxidations hitherto investigated have been found to have *A*-stereospecificity.

Glyoxylic Reductase.—This enzyme catalyzes the reduction of glyoxylate to glycolate, as shown in equation (8). The glyoxylic reductase prepared from

$$\text{glyoxylate} + \text{DPNH (or TPNH)} + \text{H}^+ \rightarrow \text{glycolate} + \text{DPN}^+ \text{ (or TPN}^+\text{)} \quad (8)$$

spinach as described by Zelitch (1955) is DPN-specific, but crude preparations of this enzyme were reported to have glyoxylic reductase activity with TPN as well as with DPN. Bacterial glyoxylic-reductase preparations with a marked preference for TPN have been previously described (Katagiri and Tochikura, 1960; Hassall and Hullin, 1962). In the present studies, use was made of the DPN-linked glyoxylic reductase from spinach, and of a glyoxylic reductase from pea seeds, not previously described. Crude preparations of the latter also react with both DPN and TPN, but it was possible to separate a TPN-specific enzyme for the stereospecificity determinations.

The studies with the spinach enzyme were carried out by oxidizing *B*-DPNT and *A*-DPNT with glyoxylate. The *B*-DPNT was prepared by reduction of DPN(T)$^+$ with alcohol dehydrogenase (equation 1); and the *A*-DPNT was prepared by reduction of DPN(T)$^+$ with liver glucose dehydrogenase (equation 9),

$$\text{glucose} + \text{DPN(T)}^+ \rightarrow \text{gluconolactone} + A\text{-DPNT} + \text{H}^+ \quad (9)$$

an enzyme with *B*-stereospecificity (Levy *et al.*, 1956). On enzymic oxidation by glyoxylate, the tritium of *B*-DPNT was retained, and the tritium of *A*-DPNT was removed (Table I). In the latter experiment, the nicotinamide was not isolated, and the removal of T was calculated from the specific activity of the recovered glycolate, not from the total radioactivity in glycolate. The high removal of tritium calculated in this way is attributable to the fact that some of the errors due to incomplete reduction and reoxidation of the pyridine nucleotide do not enter into such a calculation. The recovery of the tritium in the glycolate demonstrated the occurrence of direct hydrogen transfer, as expected. In addition, the steric position of the hydrogen transferred to glyoxylate was shown to be equivalent to that of the hydrogen in D-lactate, as will be described elsewhere (Krakow, and Vennesland, 1963).

The TPN-specific glyoxylic reductase from pea seeds was used to reoxidize *A*-TPNT prepared by reduction with glucose-6-phosphate dehydrogenase (reaction 5), and the removal of tritium (Table II) showed that the TPN-linked enzyme had the same *A*-stereospecificity for the pyridine nucleotide as the DPN-linked enzyme.

The results with glyoxylic reductase support the generalizations that the stereospecificity for the pyridine nucleotide is the same for a particular substrate, whether the enzyme is specific for DPN or for TPN, and that reactions involving the oxidation of alcohol carbon adjacent to carboxyl always have *A* stereospecificity for the pyridine nucleotide, regardless of the stereospecificity for the alcohol.

Experimental

Preparation of Tritiated Pyridine Nucleotides.—The procedure of San Pietro (1955) was used for incorporation of tritium into position 4 of the nicotinamide ring of DPN and TPN. The method involves the incubation of the pyridine nucleotide with alkaline cyanide in a medium of THO. In order to utilize a given amount of tritium most efficiently, the volume of THO employed should be small. The labeling procedure was therefore carried out in more concentrated solutions than were formerly employed with D_2O. For purification of the pyridine nucleotide from a solution of high salt content, charcoal-Celite was convenient. The following procedure gave preparations of high specific activity in sufficient amount to perform many experiments.

Five ml of T_2O containing about 1.5 curies of T was added to 325 mg of KCN and 700 mg of DPN or TPN; and 0.2 ml of 5 M NaOH was added to bring the *p*H to 11.4. The solution was kept at room temperature for 2 hours, after which the *p*H was adjusted to 6.7 by addition of 0.3 ml concentrated H_3PO_4, with care to avoid excess local acidity. After 0.5 hour,

the solution was poured on to a column of Darco-Celite. For preparation of the column, Darco G-60 was boiled with 40% ethanol for a few minutes, washed several times with water, and dried at 100°. A slurry containing 4 g of Darco G-60, 2 g of Celite, and 30 ml of H_2O was poured into a 2.5 × 15-cm glass column and packed loosely. After adsorption of the tritiated pyridine nucleotide, the column was washed with water until phosphate-free (900–1000 ml required). The pyridine nucleotide was eluted with 400–500 ml 2% pyridine in 40% ethanol. The eluate was filtered and dried *in vacuo* at 50–60°. The residue was dissolved in 10 ml of H_2O, and the pyridine nucleotide was precipitated by addition of 100 ml of a mixture of equal volumes of alcohol and acetone containing 0.05 ml concentrated HCl. The precipitate was recovered by centrifugation, washed with acetone, then with ether, and dried *in vacuo* at room temperature. The yield of DPN(T)$^+$ from the above procedure averaged 60%; of TPN(T)$^+$, 30%. The level of radioactivity achieved was about 2–3 × 10^6 cpm/min/μmole (after correction for counting efficiency of the instrument). This activity was sufficiently high to permit experiments to be performed with a few tenths μmole of pyridine nucleotide in a 3-ml volume under conditions generally employed for enzyme assay. Lower levels of labeling are adequate for most purposes, and the pyridine nucleotides could be diluted with unlabeled carrier before use. The experiments with the malic enzyme were carried out with TPN(T)$^+$ prepared by treatment with 45 mc T_2O approximately as described above, but purified on a Dowex-formate column (Kornberg and Pricer, 1953).

To confirm the location of the tritium in the DPN(T)$^+$ and TPN(T)$^+$, samples were hydrolyzed to nicotinamide, which was converted to N^1-methylnicotinamide iodide as described by Pullman *et al.* (1954). The latter compound was oxidized to the 2- and the 6-pyridones by alkaline ferricyanide, as described by Pullman and Colowick (1954). The light absorption and melting points of the separated pyridones coincided with those already described. The specific activity of the nicotinamide was compared with that of the 2- and 6-pyridones, and agreement was observed within 4% for the products from DPN(T)$^+$ and within 3% for the products from TPN(T)$^+$. For these measurements, about 1.5 mg of the pyridine nucleotides were employed, and sufficient unlabeled carrier nicotinamide was added to dilute the labeled nicotinamide about 1700-fold. The counting procedure was the same as previously described (Krakow *et al.*, 1962). The quenching effect of N^1-methylnicotinamide iodide on the scintillation count was high and no effort was made to obtain accurate specific activity measurements for this derivative, but the other compounds could be counted in the range of 1–5 mg per sample with only a small correction for quenching by the 2-pyridone. The fact that tritium was not removed on oxidation of the 2 and 6 position of the N^1-methylnicotinamide confirmed the previous demonstration that the hydrogen label introduced from water by alkaline cyanide treatment of the pyridine nucleotide is located at position 4 of the nicotinamide ring (Pullman and Colowick, 1954).

General Procedure.—The reactions were carried out usually in 1-cm Beckman cuvets, and the reduction and reoxidation of the pyridine nucleotide were determined directly from absorbancy readings at 340 mμ. An extinction coefficient of 6.22 × 10^6 cm^2 mole^{-1} was employed for calculations with both pyridine nucleotides. The experiment involving reduction of DPN(T)$^+$ by yeast alcohol dehydrogenase and the reoxidation by glutamic dehydrogenase is described below for illustration of the procedure in detail.

A reaction mixture was prepared to contain about 0.5 μmole of DPN(T)$^+$, 200 μmoles of K_2HPO_4, 0.05 ml of ethanol, and 0.1 mg of yeast alcohol dehydrogenase (Sigma), in a volume of 4 ml. The *p*H was 8.8. After the reduction was almost complete (0.480 μmole DPNT formed), the sample was held in a boiling water bath for 2 minutes, cooled, and centrifuged if necessary. A 3-ml aliquot was transferred to a fresh cuvet and the *p*H was adjusted to 7–7.4 with dilute sulfuric acid, with care to avoid excess local acidity. Then 0.6 μmole of α-ketoglutaric acid and 0.2 mg of glutamic dehydrogenase (in 0.1 ml of 1:10 dilution of a stock suspension from Boehringer) were added, bringing the total reaction volume to 3.5 ml. Since the stock enzyme suspensions were in $(NH_4)_2SO_4$ solution, no addition of ammonia was required. The change in absorbancy showed that 0.34 μmole of DPNT had been reoxidized in the glutamic dehydrogenase reaction. Fifty mg of diluent nicotinamide and 3 ml of 0.6 M sodium phosphate buffer of *p*H 9.6 were added to a 3-ml aliquot of the reaction mixture. (If necessary, the pH is adjusted to 9.5 by further addition of NaOH.) The sample was placed in a boiling water bath to hydrolyze the DPN$^+$ completely to nicotinamide (Marcus *et al.*, 1958). After readjustment of the *p*H to 7–8 with dilute sulfuric acid, the sample was extracted continuously with ether until most of the nicotinamide could be recovered from the ether phase. The ether was removed by evaporation, and the nicotinamide was recrystallized twice from benzene, mp 130–131°. Two samples of nicotinamide (about 2 and 4 mg) were weighed out and counted in a scintillation counter as previously described (Krakow *et al.* 1962). For comparison, the tritium content of the DPN(T)$^+$ prior to reduction and reoxidation was determined similarly after dilution with unlabeled nicotinamide. The control sample was carried through the identical procedure as the experimental sample except that the enzymes or the substrates were omitted.

Unless otherwise indicated, the following experiments were carried out in the manner described above. The possibility that the various enzymes were active without added substrate was checked and precluded in every instance. Where amounts of enzyme are not given, the quantities taken were sufficient to give a complete reaction in less than 0.5 hour. The units of enzyme activity employed are those defined in connection with the various preparative procedures used, except for the malic enzyme preparation, where information was required regarding the relative activities of other enzymes which might affect the results.

Experiments with Formic Dehydrogenase.—The enzyme was fractionated from pea seeds as described by Mathews and Vennesland (1950). The enzyme preparation employed had a specific activity W of 4.35, where $W = K'/g$ protein and K' is the first-order rate constant with respect to DPN, determined as previously described (Mathews and Vennesland, 1950). The initial reaction mixture contained 750 μmoles of phosphate buffer of *p*H 8.0, 200 μmoles of sodium formate, 7 mg of enzyme protein, and about 0.5 μmole DPN(T)$^+$, in a volume of 4.0 ml. The reduction of DPN$^+$ is irreversible in this reaction, formate being converted to CO_2. After readjustment of the *p*H to about 7.5, the DPNT was reoxidized by a small (about 30%) excess of pyruvate in the presence of muscle lactic dehydrogenase (Nutritional Biochemicals), or by α-ketoglutarate and ammonia in the presence of glutamic dehydrogenase; the nicotinamide was isolated after dilution as previously described.

Experiments with UDPG Dehydrogenase.—UDPG dehydrogenase Type III and the sodium salt of UDPG were obtained from Sigma Chemical Company. The reaction mixture of a typical experiment contained 400 μmoles of glycine buffer of *p*H 8.7, 6 μmoles of UDPG, about 0.3 μmole of DPN(T)$^+$, and 1000 units of UDPG dehydrogenase (Strominger *et al.*, 1957) in a volume of 3.0 ml. After adjustment of the *p*H to 7.5, the reoxidation of DPNT was carried out by addition of lactic dehydrogenase and a small excess of sodium pyruvate. In these experiments, an aliquot of the final reaction mixture was used for isolation of nicotinamide, as previously described. Another aliquot was used for isolation of lactate as the phenacyl derivative, after addition of carrier lactate and extraction of the lactic acid with ether from an acidified solution (Loewus *et al.*, 1953). Radioactivity of phenacyl lactate was counted by the same procedure as described for nicotinamide by Krakow *et al.* (1962). The results of two separate experiments agreed within 10%, and showed that 76% of the tritium was removed from the nicotinamide and transferred to pyruvate to form lactate in the reoxidation reaction.

Experiments with Dihydroorotic Dehydrogenase.—The dihydroorotic dehydrogenase was a TPN-specific enzyme fractionated from an aerobic bacterium adapted to grow on orotate (Udaka and Vennesland, 1962). The TPN(T)$^+$ was reduced first with glucose-6-phosphate (Sigma) in the presence of glucose-6-phosphate dehydrogenase (Sigma). The reaction mixture contained 200 μmoles of potassium phosphate buffer of *p*H 7.8, 0.3 μmole of TPN(T)$^+$, 5 μmoles of glucose-6-phosphate, and 0.2 mg of glucose-6-phosphate dehydrogenase, in a total volume of 3.0 ml. After reduction of the TPN(T)$^+$ to *A*-TPNT (Stern and Vennesland, 1960), and heat inactivation of the enzyme, reoxidation was carried out by addition of 1 μmole of sodium orotate and about 400 units of dihydroorotic dehydrogenase (enzyme from both step II and step III of the purification was employed in different experiments). After reoxidation, nicotinamide was isolated from one aliquot of the reaction mixture. Dihydroorotic acid was isolated from another aliquot after addition of carrier, adjustment of the *p*H to 3.3 with 2 N phosphoric acid, and removal of the water by lyophilization. A sample of dihydroorotic acid was recovered by sublimation and found to contain no detectable tritium. An aliquot of the water recovered by lyophilization was also counted for tritium (Krakow *et al.*, 1962). In two experiments, 76 and 81% of the tritium in the TPN(T)$^+$ was removed from the nicotinamide after reduction and reoxidation. In these experiments the tritium content of the recovered water accounted for 80% and 83%, respectively, of the tritium removed from the nicotinamide. Separate measurements showed that there was no detectable loss of tritium from TPNT(T)$^+$ incubated with enzyme alone in the absence of substrate.

Experiments with the Malic Enzyme.—Malic enzyme was prepared from liver through stage 3 of the procedure described by Rutter and Lardy (1958). The preparation contained 0.49 unit/ml, where a unit is defined as the amount of enzyme catalyzing TPN reduction at an initial rate of 1 μmole/min under the conditions of the stereospecificity experiments. Other enzyme activities present were: lactic dehydrogenase, 0.07 unit/ml; glucose-6-phosphate dehydrogenase, 0.1 unit/ml; and 6-phosphogluconic dehydrogenase, 0.004 unit/ml.

The reduction of TPN(T)$^+$ by malate was carried out in 10 ml of reaction mixture containing 800 μmoles of glycylglycine buffer of *p*H 7.6, 13.8 μmoles of TPN(T)$^+$, 10 μmoles of $MgCl_2$, 125 μmoles of L-malate, and 0.4 unit of malic enzyme. After reduction of the TPN and inactivation of the malic enzyme, an aliquot containing 7.9 μmoles of TPNT was supplemented with 125 μmoles of α-ketoglutarate and 0.1 ml of glutamic dehydrogenase suspension in $(NH_4)_2SO_4$, to reoxidize the reduced pyridine nucleotide. Nicotinamide isolated after addition of diluent and hydrolysis, as described elsewhere, contained 7% as much T as the nicotinamide isolated from the original TPN(T)$^+$.

The reduction of TPN(T)$^+$ by glucose-6-phosphate was carried out in 10 ml of reaction mixture containing 700 μmoles of glycylglycine buffer of *p*H 7.6, 18.7 μmoles of TPN(T)$^+$, 10 μmoles of $MgCl_2$, 40 μmoles of glucose-6-phosphate, and about 1 unit of glucose-6-phosphate dehydrogenase. For the reoxidation of the TPNT by the malic enzyme reaction, the solution was heated to inactivate the glucose-6-phosphate dehydrogenase, and an aliquot containing 13.4 μmoles of TPNT was saturated with sodium bicarbonate and supplemented with 55 μmoles of sodium pyruvate and 0.4 unit of malic enzyme. After hydrolysis of the reoxidized TPN$^+$ and recovery of a sample of diluted nicotinamide by ether extraction, as described elsewhere, the neutral solution from which the nicotinamide had been extracted was adjusted to about *p*H 1 with sulfuric acid, and the L-malic acid was recovered by further continuous ether extraction. Carrier was added and the malic acid was converted to diphenacyl-L-malate by treatment with phenacyl bromide as previously described (Loewus *et al.*, 1955). The diphenacyl-malate was counted in the same scintillation mixture as the nicotinamide (Krakow *et al.*, 1962). After correction for dilution, the counts per minute per μmole, expressed as % of the specific activity of the nicotinamide from the TPN(T)$^+$ originally employed, were 104% for the diphenacyl-L-malate, and 23% for the nicotinamide from the reoxidized TPN. In other words, 77% of the tritium had been removed from the *A*-TPNT by reoxidation with the malic enzyme reaction, and all this tritium appeared in the malic acid.

Experiments with DPN-linked Glyoxylic Reductase.—Glyoxylic reductase was prepared from spinach leaves by the procedure described by Zelitch (1955) for purification of the enzyme from tobacco leaves. The purification was carried only through step 4. The specific activities of such enzyme preparations were comparable to those reported by Zelitch.

B-DPNT was prepared by reduction of DPN(T)$^+$ with ethanol and yeast alcohol dehydrogenase, and was isolated as the salt of tris(hydroxymethyl)aminomethane (Fisher *et al.*, 1953). For the reoxidation of the *B*-DPNT, the reaction mixture of 3 ml was made up to contain 100 μmoles of potassium phosphate buffer of *p*H 6.4, 0.3 μmole of *B*-DPNT, 50 μmoles of glyoxylate (the monohydrate of sodium glyoxylate was purchased from Sigma Chemical Company), and enough enzyme to give complete oxidation in a few minutes. After completion of the reaction, inactivation of enzyme, addition of carrier, and hydrolysis of the DPN$^+$ to nicotinamide, a sample of nicotinamide was isolated and counted, all as described elsewhere. The specific activity of the tritium in the isolated nicotinamide was 95% of that in the nicotinamide obtained from the original DPN(T)$^+$.

A-DPNT was prepared by reduction of DPN(T)$^+$ with glucose in the presence of glucose dehydrogenase (Levy *et al.*, 1956). The reaction mixture of 3 ml contained 125 μmoles of potassium phosphate buffer of *p*H 7.6, 0.35 μmole of DPN(T)$^+$, 400 μmoles of glucose, and enzyme. After reduction, heat inactivation of the enzyme, and clarification by centrifugation, an

aliquot containing 0.25 μmole of *A*-DPNT was reoxidized by glyoxylate as described above for the reoxidation of *B*-DPNT. When the reoxidation was complete, the protein was removed by heat-precipitation, and the glycolic acid was separated on a Dowex-1 acetate column as described by Zelitch (1958). The glycolic acid content of the eluate was measured by the method of Calkins (1953), and the tritium content was determined by counting small aliquots of the aqueous solution. There was a 1:1 correlation of tritium content and glycolic acid content over the range where glycolic acid was eluted. The specific activity of the glycolic acid (cpm/μmole) was 97% of the specific activity of the nicotinamide in the original DPN(T)$^+$.

Experiments with TPN-linked Glyoxylic Reductase.—A glyoxylic reductase specific for TPN was prepared from Paxton's Progress pea seeds (Vaughan's Seed Store, Chicago). One hundred g of peas were soaked in cold water for 15 hours, drained, and ground in a Waring Blendor with 200 ml of 0.1 M Na_2HPO_4 solution for 1.5 minutes. This material was squeezed through cheesecloth and centrifuged at 30,000 × g for 0.5 hour. The supernatant fluid was filtered through fluted paper to remove fatty material, and dialyzed overnight against deionized water. After clarification by centrifugation, 20 g $(NH_4)_2SO_4$ was added for each 100 ml of extract, with stirring for 0.5 hour. The precipitate was discarded. An additional 35 g of $(NH_4)_2SO_4$ was added for each original 100 ml of extract, with stirring for 0.5 hour, and the precipitate was collected by centrifugation. The precipitate was then extracted in stepwise fashion with 2-ml aliquots of a 0.2 M sodium–potassium phosphate buffer of *p*H 7 containing 0.05 M ethylenediaminetetraacetate. Each extract was dialyzed against deionized water and tested for glyoxylic reductase activity with DPNH and TPNH. The TPN-specific enzyme was found in fraction 6 or 7. When the assay was carried out with TPNH and 10 μmoles of glyoxylate, but otherwise as described by Zelitch, the specific activity was 9 units/mg protein, where a unit is defined as the amount of enzyme that brings about a decrease in optical density of 0.01/min at 340 mμ (Zelitch, 1955).

A-TPNT was prepared by reduction of TPN(T)$^+$ with glucose-6-phosphate in the presence of glucose-6-phosphate dehydrogenase. Three ml of reaction mixture contained 280 μmoles of sodium maleate buffer of *p*H 6.4, 0.3 μmole of TPN(T)$^+$, 10 μmoles of glucose-6-phosphate, and enzyme. After completion of the reduction, and enzyme inactivation, the reoxidation was carried out by addition of 10 μmoles of glyoxylate and the pea enzyme to an aliquot containing 0.24 μmole of *A*-TPNT. Nicotinamide was isolated as described above, and counting showed that 76% of the tritium had been removed by the reoxidation after the reduction.

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