Reaction between Reduced Diphosphopyridine Nucleotide and Glutamate Oxalacetate Transaminases*

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SUMMARY

DPNH can be bound to pig heart cytoplasmic and bovine liver mitochondrial glutamate oxalacetate transaminases. The physiological function of this binding is not known, but DPNH does protect the mitochondrial enzyme from inactivation. Not only can DPNH be bound, but in the presence of ammonium ions it can be oxidized to DPN. This reaction is inhibited by phenylhydrazine. Consequently, it is believed that in this reaction a keto group which is tightly bound to the transaminase is reductively aminated. The product of the reaction other than DPN has not yet been identified. If DPNH with tritium on the B-side is incubated, then tritium is transferred from DPNH to water. While some pyridoxamine phosphate can be produced, it is produced in considerably lesser amounts than DPN. These reactions are apparently not the result of an artifact induced during the purification of the enzymes; they are quite slow and apparently take place independent of reactions between glutamate dehydrogenase and transaminase-bound pyridoxal or pyridoxamine phosphate.

Additional evidence is presented which suggests that glutamate dehydrogenase can form an enzyme-enzyme complex with the transaminase, and in the presence of DPNH (or TPNH) plus ammonium ions, glutamate dehydrogenase reacts with transaminase-bound pyridoxal phosphate to produce equal amounts of DPN (or TPN) and pyridoxamine phosphate. In the presence of DPN (or TPN), glutamate dehydrogenase reacts with transaminase-bound pyridoxamine phosphate to produce equal amounts of DPNH (or TPNH) plus pyridoxal phosphate. In these reactions glutamate dehydrogenase is apparently reacting directly with pyridoxal or pyridoxamine phosphate. That is, these reactions are apparently not mediated by some keto or amino acid. Glutamate dehydrogenase must have direct physical contact with the transaminase for there to be interaction between the two enzymes. In the enzyme-enzyme complex, glutamate dehydrogenase is the actual catalyst and transaminase-bound pyridoxal or pyridoxamine phosphate is the substrate.

It has been previously demonstrated that in the presence of TPNH, NH4Cl, transaminases and glutamate dehydrogenase (L-glutamate NAD(P) oxido-reductase EC 1.4.1.3), TPNH is oxidized to TPN and the transaminase is converted from the pyridoxal to pyridoxamine form. If some keto acids are added to this enzyme-enzyme system, then the rate of TPNH oxidation is increased, pyridoxamine phosphate does not accumulate and the corresponding amino acid is produced. These results suggested that in this enzyme-enzyme system the added keto acid is bound to, and reacts with, the pyridoxamine phosphate form of the transaminase (1, 2).

This communication describes a reaction between DPNH, ammonium ions and glutamate oxalacetate transaminase (L-aspartate-2 oxoglutarate amino transferase EC 2.6.1.11). In this reaction DPNH is converted to DPN even in the absence of glutamate dehydrogenase. TPNH cannot replace DPNH and even the transaminase apoenzyme reacts. Added keto acids have no effect on the initial rate of this reaction. In addition reactions with DPN, glutamate dehydrogenase, and the pyridoxamine phosphate form of the transaminase are described.

MATERIALS AND METHODS

Enzymes and Reagents—The bovine liver mitochondrial glutamate dehydrogenase and glutamate oxalacetate transaminase used in these experiments were prepared and crystallized with previously described methods (3-7). The pig heart glutamate oxalacetate and glutamate pyruvate transaminases were obtained from Boehringer Mannheim Corp. The pig heart malate dehydrogenases were obtained from P. L. Laboratories. Tritiated DPN [43H]DPN and 14C amino acids were obtained from New England Nuclear. Reagents for radioassays were obtained from Research Products International. Other substrates, coenzymes, nucleotides, and enzymes were obtained from Sigma Chemical Co. Stock solutions of all reagents were adjusted to the pH of the assays and prepared as sodium salts. Solutions of coenzymes were prepared fresh daily.

The enzymes were dialyzed two times versus 2 liters of 0.025 M sodium arsenate, 0.1 mM EDTA, pH 7.8 at 4° for 14 hours prior to use in these experiments. Unless otherwise specified, solutions referred to as dialyzed enzyme have been treated in this manner.

The transaminase was resolved and treated with phenylhydrazine according to previously described methods (8-10). After these treatments, the enzyme was dialyzed versus arsenate buffer. Some of the enzyme treated with phenylhydrazine became in-
soluble during dialysis. Therefore, this solution was clarified by centrifugation, and experiments were performed with the supernatant solution.

The pyridoxamine form of the transaminase was prepared by adding glutamate (200 mM) and dialyzing or chromatographing on Sephadex G-25.

**Enzyme, Pyridoxal, and Pyridoxamine Concentration**—Estimates of the transaminase concentration in either the pyridoxal or pyridoxamine form were calculated from the absorption spectrum of the transaminase using previously determined values of molar absorbances and molecular weights and two for the moles of pyridoxal phosphate bound per mole of transaminase (2, 5-15). The concentrations of glutamate, malate, and lactate dehydrogenase were measured spectrophotometrically using previously determined values of the extinction coefficients of these enzymes (16-18).

**Measurements of Products**—The concentration of DPN produced in the presence of DPNH and enzymes was calculated from the change in absorbance at 340 and 360 nm (1, 2). The validity of these calculations was confirmed by measuring the amount of DPN fluorescence after deproteinization and destruction of DPNH (19). Also, in some cases the incubation mixture was chromatographed on a column of DEAE-Sephadex A-25 of sufficient size to separate DPN from DPNH (20) (see below). The fractions from this column were assayed for DPN fluorometrically and by measuring the increase in optical density at 340 nm, following the addition of aliquots from this column to solutions of 0.028 mg per ml of glutamate dehydrogenase, 40 mM glutamate and 0.06 M Tris acetate, pH 8.8. It was found that all three assays (fluorescence, spectrophotometry, and enzymatic) agreed with respect to the amount of DPN produced.

The concentration of transaminase-bound pyridoxamine phosphate was measured with previously described methods (1, 2).

**Standard Enzyme Assays**—Glutamate dehydrogenase activity was measured in the presence of 2 mM α-ketoglutarate, 50 mM NH₄Cl, and 100 μM DPNH (3). Glutamate oxaloacetate transaminase activity was measured in the presence of 1 mM oxaloacetate and 40 mM glutamate (4). Transaminase dehydrogenase activity (conversion of the transaminase from the pyridoxal to pyridoxamine form) was measured in the presence of 100 μM TPNH, 50 mM NH₄Cl and 0.02 to 0.08 mg per ml of glutamate dehydrogenase (1, 2). The effect of transaminase on the reaction between glutamate dehydrogenase and pyruvate was measured in the presence of 100 μM TPNH, 50 mM NH₄Cl, 0.9 mM pyruvate and 0.5 mg per ml of glutamate dehydrogenase (1, 2). The assay used to measure the rate of oxidation of DPNH in the presence of transaminase and absence of glutamate dehydrogenase or keto acids (unless specified to the contrary) was 100 μM DPNH and 50 mM NH₄Cl. All of these assays were performed in 0.025 M sodium arsenate, 0.1 mM EDTA, pH 7.8 at 25°. In some cases (when radioactive compounds were used) 0.02 M Tris chloride was present in the assays for DPNH oxidizing activity of the transaminase. The addition of Tris chloride to this assay did not alter results, and similar results were obtained if only Tris buffer was used. All assays were performed with a Gilford model 2000 recorder and a Beckman DU monochromator as previously described (1-3).

**Ultracentrifugation**—These experiments were performed with a Beckman Spinco model E analytical ultracentrifuge at 20° in 0.025 M sodium arsenate, 0.1 mM EDTA, pH 7.8.

**Electrophoresis**—Electrophoresis on cellulose polyacetate strips with a Celman electrophoresis chamber was performed according to previously described methods (3).

**Synthesis of Radioactive Compounds**—Solutions of tritiated DPNH [45°H]DPNH with a specific activity of 1.24 Ci per mmole were diluted with nonradioactive DPNH. The B-form of tritiated DPNH was prepared by incubating ethanol (0.6 M), DPNH (0.2 M with a specific activity of 0.022 Ci per mmole) and yeast alcohol dehydrogenase (0.5 mg per ml for 60 min in 0.02 M Tris chloride, pH 8.5 at 24°). The A-form of tritiated DPNH was prepared by incubating DPNH (0.4 M with a specific activity of 0.011 Ci per mmole), gyceraldehyde 3-phosphate (0.5 mM), sodium arsenate (0.02 M), and rabbit muscle gyceraldehyde 3-phosphate dehydrogenase (1.0 mg per ml) in 0.07 M Tris chloride, 1.0 mM EDTA, pH 8.5 at 24°. The DPNH produced was purified by chromatographing on DEAE-Sephadex (see below). Freshly prepared samples were used in experiments. TheDPNH was not stored frozen.

Glutamate with tritium on the α-carbon was prepared by incubating the purified tritiated B-form of DPNH (100 μM) with pyruvate (1.0 mM), NH₄Cl (50 mM), and glutamate dehydrogenase (0.5 mg per ml) in 0.02 M Tris chloride, pH 7.5 for 6 hours at 24°.

Glutamate with tritium on the α-carbon was prepared as described above for alanine with α-ketoglutarate (10 mM) being used instead of pyruvate. Alternatively, tritiated glutamate was prepared by incubating α-ketoglutarate (10 mM), ethanol (0.6 M), NH₄Cl (50 mM), DPNH (0.5 mM and 0.011 Ci per mmole), yeast alcohol dehydrogenase (0.5 mg per ml), and glutamate dehydrogenase (0.1 mg per ml) 1 hour at 24° in 0.02 M Tris chloride, pH 8.5. The reaction was stopped by heating at 100° and the solution was clarified by centrifugation. Acid washed Norit (0.1 g per ml) was added to the supernatant solution (to remove coenzymes) and the mixture was then filtered. Glutamate was made with either method gave identical experimental results.

**Column Chromatography**—The tritiated DPNH, glutamate, and alanine synthesized in the above reactions were purified by chromatographing on a column of DEAE-Sephadex A-25 (1.5 × 15 cm) which had been equilibrated with 0.02 M Tris chloride, pH 7.5. The column was developed with a linear KC1 gradient consisting of 200 ml of 0.02 M Tris chloride, pH 7.5, in the mixing vessel and reservoir. The reservoir also contained 0.8 M KC1. Fractions with a volume of 2.8 ml were collected from the column. The entire procedure was performed at 24°. Only fractions of the highest purity and concentration (peak fractions) were used in subsequent experiments.

Reaction mixtures of DPNH, NH₄Cl, transaminase plus other additions, as well as samples of transaminase or glutamate dehydrogenase alone were chromatographed on an identical type of DEAE column. Chromatography of tritiated water on Dowex columns was performed as described previously (21). Samples of the tritiated glutamate and alanine which were synthesized were applied to amino acid analyzer. It was found that these compounds were eluted as the standard amino acids. Radioactivity and ninhydrin peaks from the analyzer were identical. Only freshly prepared tritiated amino acids were used in these experiments.

**Amino Acid Analysis**—Amino acid analyses were performed with a Beckman model 120C amino acid analyzer. Solutions containing enzymes were deproteinized (by heating at 100° for 1 to 3 min), clarified by centrifugation, and brought to pH 2.0 (with HCl) before applying to the analyzer. Standard solutions of amino acids were added to constituents of reaction mixtures and treated in a similar manner. It was found that these constituents (Tris, coenzyme, etc.) and this treatment had no sig-
significant effect on the elution time of the amino acids studied. In some experiments the eluate from the analyzer (with or without ninhydrin) was collected at 2-min intervals and assayed for radioactivity.

Assay for Radioactivity—These assays were performed with a Packard Tri-Carb (model 3310) liquid scintillation spectrophotometer at 42°. Aliquote (100 µl) of complex was added to 10 ml of either Bmy's solution (22) or another previously described standard scintillation solution (23). Both solutions gave comparable results with respect to the distribution of tritium in various compounds. Solutions to be assayed were incubated for 15 min at 4° prior to assaying.

RESULTS

Soluble Glutamate Oxalacetate Transaminase

There is no oxidation of TPNH in the absence of glutamate dehydrogenase and presence of TPNH, NH₄⁺, and transaminase (1, 2). However, if DPNH, NH₄⁺, and transaminase are incubated, DPNH is oxidized to DPN in the absence of glutamate dehydrogenase. The following compounds were found to have no effect on the initial rate of this reaction: α-ketoglutarate (10 mM), oxalacetae (0.1 mM), pyruvate (2 mM), hydroxypropionate (1 mM), glyoxalate (1 mM), glutamate (10 mM), glutamine (10 mM), scizinc (10 mM), alanine (10 mM), glycine (10 mM), phosphoserine (0.1-20 mM), histidine (1 mM), ADP (80 mM), ATP (80 mM) and GTP (80 mM). The reaction is dependent upon ammonium ions, and glutamine (10 mM) will not substitute for ammonium ions. Dialyzing the enzyme versus diethioerythritol (0.8 mM) or adding this reagent (0.8 mM) had no effect on initial velocity. The initial velocity was the same if 0.02 M Tris chloride, 0.1 mM EDTA, pH 7.8, was used instead of arsenate buffer.

In these reactions pyridoxamine phosphate is not produced and the specific activity (initial rate of DPNH oxidation per transaminase peptide chain) of the pyridoxamine phosphate form of the transaminase (prepared by adding glutamate and dialyzing (2)) was about 1.5-fold faster than that of the pyridoxal phosphate form. The specific activity of the resolved transaminase was essentially the same as that of the holoenzyme. The phenylhydrazine-treated enzyme did not oxidize DPNH at a measurable initial rate. The above results are summarized in Table I.

The transaminase preparation was chromatographed on a column of Sephadex G-200 (1). The fractions from this column containing the peak transaminase activity were concentrated by dialyzing versus a solution 80% saturated with respect to ammonium sulfate, pH 7.0. The ammonium sulfate suspension was centrifuged, the precipitate solubilized in 0.025 M sodium arsenate, 0.1 mM EDTA, pH 7.8, and dialyzed two times versus 2 liters of arsenate buffer. It was found that the above treatments of the transaminase preparation (chromatography, concentrating with ammonium sulfate and dialysis) did not alter the specific activity of DPNH oxidation by the transaminase. This transaminase was then chromatographed on DEAE-Sephadex. Three widely separated peaks of transaminase activity were eluted. On the basis of the absorption spectrum and elution profile of these three peaks it was concluded that the two most anionic peaks were the previously described β- and γ-subforms of the transaminase (24). The specific activity of DPNH oxidation with the β-subform and the least anionic fraction (which was mainly the α-subform) were, respectively, 2-fold lower and 1.7-fold higher than that of the original prepara-

| Form of transaminase | Initial activity (µM DPNH/µM transaminase) | Initial activity (µM DPNH/µM transaminase) | Initial activity (µM DPNH/µM transaminase) |
|----------------------|--------------------------------------------|--------------------------------------------|--------------------------------------------|
| Pyridoxal phosphate   | 0.12                                       | 4                                          |                                            |
| Pyridoxamine phosphate| 0.18                                       | 4                                          |                                            |
| Transaminase + α-ketoglutarate (10 mM) | 0.12                                       | 4                                          |                                            |
| Transaminase + glutamine (10 mM)   | 0.12                                       | 4                                          |                                            |
| Apoenzyme              | 0.14                                       | 2                                          |                                            |
| Phenylhydrazine-treated | 0.20                                       | 4                                          |                                            |
| α-Subform              | 0.04                                       | 3                                          |                                            |
| β-Subform              | 0.04                                       | 3                                          |                                            |
| γ-Subform              | 0.04                                       | 3                                          |                                            |

a The initial velocity per chain refers to the micromolar concentration of DPN produced per min per micromolar concentration (with respect to peptide chains) of transaminase incubated.

b DPN produced per chain refers to the micromolar concentration of DPN produced after 4 hours incubation per micromolar concentration (with respect to peptide chains) of transaminase incubated.

Fig. 1. Plot of initial velocity of DPNH oxidation (in units of µmolar concentration of DPN produced per min) as a function of the concentration of cytoplasmic glutamate-oxalacetate transaminase incubated. All of the above experiments were performed with the least anionic (predominantly α-subform) fraction from the DEAE-Sephadex column. A plot of the rate of DPNH oxidation as a function of transaminase concentration is shown in Fig. 1. It can be seen that this is essentially a linear relationship. Fig. 2 shows a plot of the specific activity of DPNH oxidation as a function of DPNH concentration in the presence of two different concentrations of the transaminase. In both cases the solid lines have been calculated by assuming that 1 mole of DPNH is bound per mole of transaminase peptide chain, the dissociation or apparent Michaelis constant of DPNH is equal to 10 µM and
The concentration of transaminase peptide chains added was 5.0 μM in Curve A and 9.0 μM in Curve B. The solid lines have been calculated by assuming that 1 mole of DPNH is bound per peptide chain, the dissociation or apparent Michaelis constant of transaminase (with respect to peptide chains) is 0.22 milli-μM. Remaining experimental conditions are given in the legend to Fig. 1.

The results obtained in the presence of 50 μM DPN and either the pyridoxal or pyridoxamine form of the transaminase are shown in Curve C. Remaining experimental conditions are given in the legend to Fig. 1.

**Time Course of DPN Production**—As shown in Fig. 4, about 2 moles of DPN are produced per mole of incubated transaminase peptide chain in 40 min when transaminase (22 μM with respect to peptide chains), DPNH (100 μM), and NH₄Cl (50 mM) are incubated. An additional 2 moles of DPN per mole of peptide chain are produced slowly over the next 2 hours. In similar experiments it was found that the addition of the previously mentioned (see section on initial rate) keto acids, amino acids, or purine nucleotides did not alter the time course of DPN production shown in Fig. 4. Similarly, dialyzing the transaminase versus adding dithioerythritol (0.8 mM) had no effect on these results. Also, substituting 0.02 M Tris chloride, 0.1 mM EDTA, pH 7.8, for arsenate buffer did not alter the time course of DPN production. The time course of DPN production was similar if either the pyridoxal or pyridoxamine form of the transaminase was incubated (Fig. 4). The addition of DPN (but not TPN) decreased the amount of DPN produced (Fig. 4). As shown in Table I, less DPN is produced in 4 hours if the β-subform of the transaminase is incubated and the γ-subform does not oxidize DPN. While the initial velocity is faster with the predominantly α-subform than with the mixture of subforms, the amount of DPN produced after 4 hours incubation is the same in both cases (Table I). If a low concentration of transaminase is incubated with 100 μM DPNH and 50 mM NH₄Cl for 3 hours, then as many as 15 moles of DPN rather than 3 to 4 are produced per peptide chain (Fig. 5). Thus, the amount of DPN produced during 3 hours incubation is apparently related to the initial velocity of DPNH oxidation (which is directly proportional to the concentration of transaminase incubated (Fig. 1)), the amount of DPN produced (which inhibits DPNH oxidation (Figs. 3 and 4), the initial ratio of DPNH per transaminase peptide chain and the amount of DPN remaining at any given time. That is, DPNH oxidation does not stop when a certain amount of DPN per incubated peptide chain has been produced but apparently proceeds until the ratio of DPN to DPNH concentration is quite high. This is also illustrated by the results shown in Table II. If a high concentration of transaminase (40 μM with respect to peptide chains) is incubated with a high concentration of DPNH (1 mM) and 50 mM NH₄Cl for 20 hours, then at the end of this time about 20 moles of DPN are produced per

![Fig. 2. Plot of specific activity of DPN production in units of molar concentration of DPN produced per min per molar concentration of cytoplasmic transaminase peptide chains (predominantly α-subform) incubated versus the concentration of DPNH added. The concentration of transaminase peptide chains added was 5.0 μM in Curve A and 9.0 μM in Curve B. The solid lines have been calculated by assuming that 1 mole of DPNH is bound per peptide chain, the dissociation or apparent Michaelis constant of transaminase (with respect to peptide chains) is 0.22 milli-μM. Remaining experimental conditions are given in the legend to Fig. 1.](image1)

![Fig. 3. Plot of specific activity of DPN production (same units as Fig. 2) versus the concentration of either DPN or TPN added. Curves A and B show the results obtained in the presence of 22 μM transaminase (with respect to peptide chains) of the pyridoxamine phosphate form of the cytoplasmic enzyme and TPN (Curve A) or DPN (Curve B). Curve C shows the results obtained in the presence of 23 μM transaminase (with respect to peptide chains) of the pyridoxal phosphate form and DPN. These experiments were performed with the predominantly α-subform in the presence of 100 μM DPNH and 50 mM NH₄Cl. Remaining experimental conditions are given in the legend to Fig. 1.](image2)

![Fig. 4. Plot of moles of DPN produced per mole transaminase peptide chain incubated versus time. These experiments were performed in the presence of 22 μM cytoplasmic transaminase peptide chain (predominantly α-subform), 100 μM DPNH and 50 mM NH₄Cl. The enzyme was in the pyridoxal form in Curves B and C (open circles) and the pyridoxamine form in Curves A and C (closed circles). Curves A and B show the results obtained in the absence of DPN. The results obtained in the presence of 50 μM DPN and either the pyridoxal or pyridoxamine form of the transaminase are shown in Curve C. Remaining experimental conditions are given in the legend to Fig. 1.](image3)
Fig. 5. Plot of moles DPN produced (after 3 hours) per mole of transaminase peptide chain versus concentration of cytoplasmic transaminase peptide chain (predominantly a subform) incubated. These experiments were performed in the presence of 100 μM DPNH and 50 mM NH₄Cl. Remaining experimental conditions are given in the legend to Fig. 1.

Table II

Amount of DPN produced by various forms of cytoplasmic transaminase

All of these experiments were performed in the presence of 50 mM NH₄Cl, 0.025 mM sodium arsenate, 0.1 mM EDTA, pH 7.8 at 25°, except the last experiment. In this experiment NH₄Cl was not added, but glutamate dehydrogenase (0.17 mg per ml) and α-ketoglutarate (1 mM) was added.

| Form of incubated transaminase | Conditions of incubation | DPN produced per peptide chain |
|-------------------------------|---------------------------|-------------------|
|                               | DPNH | Time | μM | hrs | moles |
| Pyridoxal phosphate (7.6 μM)  | 0.1  | 4    | 7  |
| Apoenzyme (7.6 μM)            | 0.1  | 4    | 3  |
| Pyridoxal phosphate (40 μM)   | 0.2  | 4    | 5  |
| Apoenzyme (40 μM)             | 0.2  | 4    | 3  |
| Pyridoxal phosphate (50 μM)   | 1.0  | 20   | 20 |
| Apoenzyme (40 μM)             | 1.0  | 20   | 6  |
| Pyridoxal phosphate (16 μM)   | 0.5  | 20   | 18 |
| Pyridoxamine phosphate (16 μM)| 0.5  | 20   | 8  |
| Pyridoxal phosphate (36 μM)   | 0.5  | 20   | 12 |
| Pyridoxal phosphate no added NH₄⁺ (36 μM) | 0.5 | 20 | 2 |

mole of transaminase peptide chain. Therefore, again in the presence of a high initial ratio of DPNH to transaminase the reaction slowly proceeds until the ratio of DPN to DPNH concentration is quite high or most of the incubated DPNH has been converted to DPN. This is not the case if the apoenzyme or the pyridoxamine phosphate form of the transaminase is incubated (Table II) in the presence of a considerable excess of DPNH. In these latter cases only 6 or 8 moles, respectively, of DPN are produced per mole of incubated peptide chain even after 20 hours incubation. Similarly, if the apoenzyme is incubated with 0.1 or 0.2 mM DPNH, then after 4 hours 2 to 3, rather than 4 to 5, moles of DPN are produced per incubated peptide chain (Table II).

Effect of Dehydrogenases—The addition of glutamate, lactate, or malate dehydrogenase to DPNH (100 μM), NH₄Cl (50 mM) and transaminase (20 μM with respect to peptide chains) had no effect on the initial rate of DPNH oxidation or the time course of DPN production in 3 to 4 hours. The levels of these dehydrogenases used in these experiments were sufficiently high to react with low levels (20 to 100 μM) of their keto acid substrates. When the level of glutamate dehydrogenase is higher (0.01 mg per ml), then this enzyme increases the initial rate of DPNH oxidation until a plateau is reached when the level of this enzyme is about 0.1 mg per ml. At this concentration glutamate dehydrogenase increases the initial rate of DPNH oxidation about 1.4-fold. The addition of glutamate dehydrogenase not only increases DPN production but now pyridoxamine phosphate is rapidly produced.

Fig. 6 shows a comparison between the reactivity of DPNH versus TPNH in the presence of low levels of glutamate dehydrogenase. It can be seen that in the presence of low levels of glutamate dehydrogenase, DPNH is produced in excess of pyridoxamine phosphate. In the presence of any level of glutamate dehydrogenase, pyridoxamine phosphate and TPN are produced in equal amounts. In these experiments the initial rate of oxidized pyridine nucleotide production is about 5-fold faster when DPNH rather than TPNH is the coenzyme. However, the initial rate and amount of pyridoxamine phosphate produced over the entire incubation period is essentially the same with either coenzyme.

Results obtained in the presence of DPNH, NH₄Cl, transaminase, and a higher “optimal” concentration of glutamate dehydrogenase (0.11 mg per ml) are shown in Fig. 7. It can be seen that now DPN and pyridoxamine phosphate production are initially essentially equal. With time DPN production
The presence of reduced pyridine nucleotides, NH₄⁺ and glutamate dehydrogenase is still slowly produced. The amount of product produced exceeds that of pyridoxamine phosphate. Even when the transaminase is completely in the pyridoxamine phosphate form, glutamate dehydrogenase could leave the transaminase, bind to glutamate dehydrogenase, and be converted to amino acids. These amino acids would then leave glutamate dehydrogenase, react with transaminase-bound pyridoxal phosphate to produce the pyridoxamine phosphate form of the transaminase. The rejuvenated keto acid would be too tightly bound to this latter form to leave the transaminase and react again with glutamate dehydrogenase. Thus, there would not be an enzyme-enzyme complex but merely a transfer of keto acids from the transaminase to glutamate dehydrogenase. To test this possibility, a 1-ml solution of transaminase (48 μM with respect to peptide chains), NH₄Cl (50 mM) and TPNH (100 μM) was placed into a dialysis sac and dialyzed versus a 5-ml solution of TPNH (100 μM), NH₄Cl (50 mM) and glutamate dehydrogenase (0.6 mg per ml). Both the inside and outside compartments of the system contained 0.025 M sodium arsenate, 0.1 mM EDTA, pH 7.8. The sample was then dialyzed for 6 hours at 25°C. At this time the dialysis sac was removed and the sample dialyzed versus 2 liters of the arsenate buffer. The dialyzed transaminase was in the pyridoxal phosphate form. Adding glutamate converted the enzyme to the pyridoxamine phosphate form. In a control experiment the same amount of transaminase not enclosed in a dialysis sac was incubated within an identical 5-ml solution of TPNH, NH₄Cl, and glutamate dehydrogenase. In these control experiments the transaminase is rapidly converted to the pyridoxamine phosphate form and remains in this form during subsequent dialysis. Therefore, it seems likely that the two enzymes must have contact with each other if the transaminase is to be converted from the pyridoxal to the pyridoxamine phosphate form.

Effect of Ammonium Ions—The endogenous concentration of ammonium ions in a solution was decreased by adding glutamate dehydrogenase (0.17 mg per ml) and α-ketoglutarate (1 mM) to transaminase (36 μM with respect to peptide chains) and DPNH (500 μM). If this solution was incubated for as long as 20 hours, then at this time the concentration of DPN was only 114 μM. However, 50 μM DPN was produced almost instantaneously and this was found to be the level of endogenous ammonium ions in the incubated solution (as measured by assaying the solutions of glutamate dehydrogenase and transaminase used independently for ammonium ions (25)). Therefore, in the presence of α-ketoglutarate, glutamate dehydrogenase transaminase, and no added ammonium ions, very little DPNH is oxidized (Table II).

If corrections are made for the endogenous level of ammonium ions, then only 64 μM DPN or about 2 moles per mole of incubated transaminase peptide chain was produced in 20 hours in the absence of ammonium ions. Neither α-ketoglutarate nor these low levels of glutamate dehydrogenase alone can prevent large amounts of DPNH from being oxidized in the presence of transaminase and 50 mM NH₄Cl.

Properties of Incubated Enzyme—Transaminase (24 μM with respect to peptide chains) was incubated with DPNH (500 μM) plus NH₄Cl (50 mM) for 24 hours. At this time about 15 moles of DPN were produced per incubated peptide chain. The incubated enzyme solution was then dialyzed. A quantitative ninhydrin (26) performed on a 0.1-ml aliquot of the incubated dialyzed enzyme revealed that there were only two to three more amine groups per peptide chain on the incubated enzyme than a control which was not incubated with DPNH. If the incubated enzyme was deproteinized and applied to the amino acid analyzer, several different amino acids were found. However, the total amount of amino acids found was 2 to 3 per incubated peptide chain. Of these the most abundant were serine and
Table III
Release of serine and glycine from deproteinized transaminase after various incubations

These experiments were performed with extensively dialyzed solutions of transaminase. Incubations of the dialyzed transaminase (24 µM with respect to peptide chains) were performed in 50 mM NH₄Cl, 500 µM DPNH (where indicated), 0.025 M sodium arsenate, 0.1 mM EDTA, pH 7.8 at 25°C. Enzyme was dialyzed after incubation (where indicated by dialysis) for 24 hours versus 2 liters of 0.025 M sodium arsenate, 0.1 mM EDTA, pH 7.8 at 4°C (two buffer changes). The amount of amino acids released from the transaminase after deproteinization with heat (see “Materials and Methods”) was measured with the amino acid analyzer. Various concentrations of transaminase (20 to 40 µM with respect to peptide chains) were used in other experiments of this type. Results when expressed as moles of amino acids per mole of peptide chain were quite reproducible.

| Incubation                              | DPN produced/chain | Amino acid eluted/chain |
|-----------------------------------------|--------------------|------------------------|
|                                        | Serine  | Glycine   |
| DPNH plus transaminase, 24 hrs, no     | 15      | 0.3       | 0.2  |
| dialysis                                |         |           |      |
| DPNH plus transaminase, 24 hrs, dialyze | 15      | 1.6       | 1.2  |
| Transaminase, 24 hrs, dialyze           | 0       | 0.3       | 0.2  |

Table IV
Reaction between pyridine nucleotides, glutamate dehydrogenase and cytoplasmic glutamate oxalacetate transaminase

These incubations were performed in 0.025 M sodium arsenate, 0.1 mM EDTA, pH 7.8 at 25°C. The concentrations used in either first or second incubations were transaminase (20 to 40 µM with respect to peptide chains), reduced pyridine nucleotides (100 µM, except in the first four incubations), oxidized pyridine nucleotides (1 to 2 mM), glutamate dehydrogenase (0.2 to 0.3 mg per ml) and NH₄Cl (50 mM). First incubations were for at least 4 hours, except in the first four incubations. In second incubations with DPN the reaction was complete in about 10 min and products were stable for at least 1 hour. The second incubations with DPNH were for 20 hours. The second incubation with TPNH was followed for 3 hours but the reaction was complete in 30 min. The abbreviations used are: PMP, pyridoxamine phosphate; PLP, pyridoxal phosphate; GDH, glutamate dehydrogenase; and GOT, glutamate oxalacetate transaminase. After the first incubations, the solutions were either extensively dialyzed or chromatographed on Sephadex G-25 (see “Materials and Methods”).

| First incubation | Products/chain | Dialyzed or chromatographed | GOT or GTP from first incubation plus: |
|------------------|----------------|-----------------------------|----------------------------------------|
|                  | TPN or DPN | PMP                         | DPNH                                  | PLP | DPN or TPN | PMP |
| Transaminase, NH₄⁺ plus: |            |                             |                                        |     |            |     |
| DPNH (1.0 mM), 20 hrs | 20   | 0                           | DPN                                    | 0   | 0          | 0   |
| DPNH (1.0 mM), 20 hrs | 20   | 0                           | DPN plus GDH                           | 0.3 | 0.3        | 0   |
| DPNH (1.0 mM), 20 hrs | 30   | 0                           | DPNH plus NH₄⁺, 20 hrs                 | 0.7 | 0.7        |     |
| DPNH (1.0 mM), 20 hrs | 30   | 0                           | DPNH, NH₄⁺ plus GDH                    |     |            |     |
| TPNH plus GDH, 4 hrs | 1.0  | 1.0                         | DPN                                    | 1.0 | 1.0        |     |
| DPNH plus GDH, 4 hrs | 1.0  | 1.0                         | DPN                                    | 1.0 | 1.0        |     |
| Glutamate (0.1 M)  | 0.4  | 0.4                         | DPN                                    | 0.4 | 0.4        |     |
| Glutamate (0.1 M)  | 0.4  | 0.4                         | DPN                                    | 0.4 | 0.4        |     |
| None              | 0    | 0                           | DPN                                    | 0   | 0          |     |

While there is no evidence that pyridoxamine phosphate is produced when low levels of DPNH are incubated with transaminase for 3 to 4 hours, the absorption spectrum of the above described incubated dialyzed enzyme (incubated with high levels of DPNH for a long period of time) suggests that now the transaminase is at least partially in the pyridoxamine phosphate form. This conclusion is supported by the fact that if α-ketoglutarate is added to this incubated dialyzed enzyme, the absorption spectrum slowly shifts to that of the pyridoxid phosphate form of the transaminase (Fig. 9).

The above results reveal that after incubation and dialysis, some amines or amino acids are found. However, the total amount of amines or amino acids found is considerably less than the amount of DPNH oxidized. If the enzyme is incubated for 24 hours (as described above), not dialyzed, but deproteinized, and applied to the amino acid analyzer, then even fewer amino acids are found than in the dialyzed sample (Table III).

Since serine and glycine were the most abundant amino acids found, the transaminase (30 µM) was incubated with DPNH (10 to 100 µM) plus glyoxalate reductase (0.05 mg per ml) for as long as 48 hours in the absence of NH₄⁺. Incubating with glyoxalate reductase did not result in any more DPN production than the small amount observed in the absence of NH₄⁺. At the end of the 48-hour incubation NH₄Cl (50 mM) was added to the transaminase glyoxalate solution and a control solution which

glycine (Table III).1 The incubated dialyzed enzyme was no different with respect to electrophoretic mobility, transaminase activity, or sedimentation coefficient than a control which was not incubated with DPNH. If the incubated dialyzed enzyme was again incubated with DPNH plus NH₄Cl for 24 hours, then again 20 moles of DPN were produced per peptide chain (Table IV).

1 Table III does not show the amount of aspartate, threonine, glutamate, alanine, valine, methionine, leucine, or isoleucine eluted. These amino acids were always quite low.

Since several different amino acids were eluted when the incubated transaminase was applied to the amino acid analyzer, the transaminase (40 µM) was assayed for peptidase activity with denatured casein (27). No peptidase activity was found.

Reaction with DPN—The reaction with DPNH, NH₄⁺, and the pyridoxid or pyridoxamine phosphate form of the transaminase is quite complicated. Therefore, the reaction with DPN plus the pyridoxid or pyridoxamine phosphate form was studied.
Transaminase-bound pyridoxamine phosphate was prepared by incubating the pyridoxal phosphate form with glutamate; DPNH plus NH₄⁺, with or without glutamate dehydrogenase, and TPNH, NH₄⁺ plus glutamate dehydrogenase (Table IV). These incubated solutions were then dialyzed or chromatographed on Sephadex G-25. In the experiments with glutamate sufficient amounts of ¹³C-labeled glutamate were added so that even trace amounts of glutamate or α-ketoglutarate associated with the transaminase could be detected (12). No radioactivity was detected in the transaminase peaks from the Sephadex G-25 column.

After dialysis or chromatography the transaminase was predominantly in the pyridoxamine phosphate form if this form was prepared by incubating with glutamate or TPNH, NH₄⁺ plus glutamate dehydrogenase. If this form was prepared by incubating with DPNH plus NH₄⁺, with or without glutamate dehydrogenase, then only about 0.3 to 0.4 mole of pyridoxamine phosphate per transaminase peptide chain was present after dialysis (Table IV). This is in spite of the fact that incubating with DPNH, NH₄⁺ plus glutamate dehydrogenase converts the transaminase completely into the pyridoxamine phosphate form.

In the presence of DPN and glutamate dehydrogenase the dialyzed transaminase-bound pyridoxamine phosphate (prepared by any of the above described methods) is rapidly converted to pyridoxal phosphate plus DPNH. Pyridoxal phosphate and DPNH were produced in equal amounts throughout the incubation. These reactions were complete in at least 8 min and additional incubation did not alter the amount of product formed.

If DPN alone was added to transaminase-bound pyridoxamine phosphate, there was no reaction even if the pH of the solution was increased to 8.8 (by adding Tris). When the enzyme is completely in the pyridoxal phosphate form, it does not react with DPN, with or without glutamate dehydrogenase (Table IV). Therefore, these results demonstrate that pyridoxamine phosphate is the only amine on the transaminase which can react with DPN and glutamate dehydrogenase is required for this reaction.

**Stereospecificity of Reaction**—If the A-form of tritiated DPNH was incubated with transaminase plus NH₄Cl and this solution was then chromatographed on DEAE-Sephadex, it was found that tritiated DPN was produced (Table V). If the B-form of tritiated DPNH was incubated and this solution was chromatographed on DEAE-Sephadex, then no radioactivity was found in the fractions containing DPN. In these experiments radioactivity was found in a peak which was eluted immediately after the major transaminase peak (Fig. 10). No radioactivity was found associated with the transaminase. If the radioactive compound eluted from the DEAE-Sephadex column was applied to the amino acid analyzer, it was eluted as a peak after 28 min. This compound was volatile, it did not react with ninhydrin, it would pass through a dialysis membrane, it did not absorb light, it was not absorbed to Norit, and it did not react as a keto acid. It was eluted from a Dowex 50 column and distilled like tritiated water (21). The same tritiated compound was produced (as judged by volatility, chromatography on Dowex 50, DEAE-Sephadex, and the amino acid analyzer) if either glutamate or alanine (with tritium on the α-carbon) were incubated with transaminase (Table VI). It is known that the tritiated product in these latter reactions is tritiated water (28, 20). Therefore, for this reason and since the tritiated compound had the properties described above, it was concluded that when the B-form of tritiated DPNH is incubated with transaminase and NH₄Cl, DPN is produced and in this reaction tritium is transferred from...
the B-side of DPNH to water. The results of several of these experiments are summarized in Table V. It can be seen that similar results were obtained if α-ketoglutarate or glutamate dehydrogenase was added to the incubation mixture. Also, tritiated water was eluted if the incubated solution was deproteinized (by heating) before it was added to the DEAE-Sephadex column. Even if the apoenzymes with the B-form of tritiated DPNH plus NH₄⁺, then tritium is transferred from the B-form to water. The elution profiles of the above incubations with the B-form of DPNH on DEAE-Sephadex were essentially all identical to that shown in Fig. 10. Tritium was not eluted in the transaminase peak in any of these experiments.

Effect of Purification on Reaction—The above experiments were performed with highly purified pig heart cytoplasmic glutamate oxalacetate transaminase. In one of the purification steps employed, the transaminase is heated in the presence of dicarboxylic acids (12). To test the possibility that this heat step in some way induces an alteration in the transaminase so that it is less reactive than DPNH. With the mitochondrial enzyme, only 0.6 to 0.7 mole of DPN is produced per incubated peptide chain (100 mM), NH₄⁺, glutamate dehydrogenase and transaminase were produced in equal amounts. These activities paralleled transaminase activity during additional purification procedures. Therefore, a heat step with dicarboxylic acids is not necessary to induce these activities. Furthermore, a heat step performed with α-ketoglutarate (12) on this purified enzyme did not alter these activities.

Mitochondrial Glutamate Oxaloacetate Transaminase

The initial velocity of DPNH oxidation with the mitochondrial transaminase is quite slow compared with the cytoplasmic and essentially too slow to measure accurately. Therefore, measurements were made of the amount of DPN produced during prolonged incubations (Fig. 11). This reaction, like the cytoplasmic, is dependent upon ammonium ions, and TPNH is much less reactive than DPNH. With the mitochondrial enzyme, only the presence of DPNH plus NH₄⁺. Also, in the presence of TPNH, NH₄⁺, glutamate dehydrogenase, and transaminase eluted from the DEAE-Sephadex column, TPN and pyridoxamine phosphate were produced in equal amounts. These activities paralleled transaminase activity during additional purification procedures. Therefore, a heat step with dicarboxylic acids is not necessary to induce these activities. Furthermore, a heat step performed with α-ketoglutarate (12) on this purified enzyme did not alter these activities.

| Table VI |
| Percentage of tritium recovered in water or amino acids after incubating amino acids with tritium on α-carbon with transaminases |
| **Incubation time** | **Percentage of tritium in Water** | **Amino acid** |
| --- | --- | --- |
| Glutamate-oxaloacetate (0.1 mg/ml) | Glutamate (1.0 μM) | 5 | 94 | 0 |
| Glutamate-oxaloacetate (0.1 mg/ml) | Alamine (1.0 μM) | 120 | 11 | 89 |
| Glutamate-oxaloacetate (0.25 mg/ml) | Glutamate (76 μM) | 230 | 100 | 0 |
| Glutamate-pyruvate (0.1 mg/ml) | Glutamate (1.0 μM) | 120 | 67 | 33 |
| Glutamate-pyruvate (0.1 mg/ml) | Alamine (1.0 μM) | 120 | 69 | 31 |
Fig. 11. Plot of molar concentration DPN or TPN produced per molar concentration of transaminase (with respect to peptide chains) incubated versus time. These experiments were performed with 18 μM (with respect to peptide chains) mitochondrial transaminase. Curve A shows the results obtained in the presence of glutamate dehydrogenase (53 μM per ml) with (Curve A) and without (Curve B) ADP (100 μM). The experiments were performed in the absence of glutamate dehydrogenase. Curve C shows the results obtained in the presence of glutamate dehydrogenase (53 μM per ml) and presence (Curve A) or absence of ADP (0 μM). In all experiments the NH₄Cl concentration was 50 mM. Additional experimental conditions are given in the legend to Fig. 1.

Fig. 12. Plot of molar concentration pyridoxamine phosphate produced per molar concentration transaminase (with respect to peptide chains) incubated versus time. These experiments were performed with 18 μM (with respect to peptide chains) mitochondrial glutamate oxalacetate transaminase. Curve A shows the results obtained in the presence of glutamate dehydrogenase (53 μM per ml) and presence (Δ) or absence of ADP (○) (100 μM). Curves B and C show the results obtained in the absence of glutamate dehydrogenase and presence (Curve B) and absence of ADP (Curve C). In all experiments the DPNH and NH₄Cl concentrations were, respectively, 100 μM and 50 mM. Additional experimental conditions are given in the legend to Fig. 1.

enzyme is not incubated (2). The glutamate oxalacetate transaminase activity (using glutamate and oxalacetate as substrate (1)) of the incubated dialyzed enzyme is the same as a nonincubated control.

Like the cytoplasmic transaminase, the addition of α-ketoglutarate (1 mM) to an incubation of DPNH (100 μM), NH₄Cl (50 mM), and mitochondrial transaminase (20 μM with respect to peptide chains) has no effect on DPN production. However, in the presence of α-ketoglutarate, pyridoxamine phosphate is not produced. The pyridoxamine phosphate form of the mitochondrial, unlike the cytoplasmic (prepared by adding glutamate and dialyzing (2)), does not oxidize DPNH in the presence of NH₄Cl.

If glutamate dehydrogenase is added to DPNH, NH₄Cl, and mitochondrial transaminase, then the initial rate of DPN and pyridoxamine phosphate production is increased considerably (Figs. 11 and 12). Initially DPN and pyridoxamine phosphate are produced in equal amounts. After about 10 min, when about half of the transaminase is in the pyridoxamine phosphate form, DPN exceeds pyridoxamine phosphate production. After prolonged incubation (100 min), the transaminase is completely in the pyridoxamine phosphate form and about 1.6-fold more DPN than pyridoxamine phosphate has been produced. Unlike experiments performed with the cytoplasmic transaminase, little additional DPN is produced after the mitochondrial transaminase is completely in the pyridoxamine phosphate form.

The initial velocity of reduced pyridine nucleotide oxidation is 3.5-fold greater when DPNH, rather than TPNH, is the coenzyme in the incubation of the mitochondrial transaminase and glutamate dehydrogenase (conditions identical with those described in the legends to Figs. 11 and 12). When TPNH (100 μM) is the coenzyme, TPN and pyridoxamine phosphate are produced in equal amounts throughout the course of the incubation (100 min).

A series of experiments were performed with the mitochondrial transaminase, tritiated DPNH, and NH₄Cl. These experiments were performed essentially the same as those with the cytoplasmic transaminase. After incubating, the reaction mixture was assayed for DPN and pyridoxamine phosphate, chromatographed on DEAE-Sephadex, and the eluted products assayed. If either the A- or B-forms of tritiated DPNH were incubated with the mitochondrial transaminase in either the presence or absence of glutamate dehydrogenase, then both tritiated DPN and water were eluted from the DEAE-Sephadex columns. No tritium was found in the transaminase fractions even when the transaminase was eluted predominantly in the pyridoxamine phosphate form, i.e. in the presence of glutamate dehydrogenase. When the B-form of tritiated DPNH is incubated in the absence of glutamate dehydrogenase, then 2-fold more tritium is recovered in the DPN than tritiated water fractions from the column (Table VII). If the B-form of tritiated DPNH is incubated in the presence of glutamate dehydrogenase, then 2-fold more tritium is found in the water than DPN fractions eluted from the column. If the A-form of tritiated DPNH is incubated in the presence of glutamate dehydrogenase, then the reverse is the case, i.e. 2-fold more tritium is found in the DPN than water fractions (Table VII).

The above results suggest that there are two different groups on the mitochondrial transaminase (A and B) which in the presence of ammonium ions can react with DPNH. Group A removes hydrogen from the A-side and Group B from the B-side of DPNH. The tritiated product produced in the reaction with either Group A or B can then exchange tritium with water. In the above experiments the amount of DPN eluted from the column was equal to the amount of DPN produced in the incubations and loaded on the column. The total counts eluted from the column in the DPN plus water peaks divided by the amount of DPN produced in the incubation was equal to the specific activity of the starting DPNH incubated. Therefore, the total amount of DPN produced in the incubation and eluted from the column is apparently equal to the sum of the reaction with Group A plus Group B. If the B-form of tritiated DPNH is incubated, the ratio of tritium recovered in DPN to water is equal to the ratio of the reaction with Group A to Group B. A
shown in Figs. 11 and 12 were performed with the enzyme preparation before incubation in α-ketoglutarate and crystallization. The amount of tritium in the water and DPN peaks eluted from the column was measured. The values for the extent of the reaction with A and B were calculated as described in the text.

reciprocal relationship exists if the A-form of tritiated DPNH is incubated. Thus, by solving these simultaneous linear relationships, the amount of the reaction with either Group A or B can be calculated. The result of these calculations are summarized in Table VII. These calculations suggest that glutamate dehydrogenase increases the reaction with group B since the addition of this enzyme doubles the reaction with this group and has no effect on the reaction with Group A. Also, in either the presence or absence of glutamate dehydrogenase the amount of Group B which reacts is essentially equal to the amount of pyridoxamine phosphate produced.

In the absence of glutamate dehydrogenase, ADP has no effect on the reaction of the cytoplasmic transaminase with DPNH. This nucleotide, however, increases the amount of DPN and pyridoxamine phosphate produced with the mitochondrial transaminase (Figs. 11 and 12). In the presence of DPNH, NH₄Cl, mitochondrial transaminase, and glutamate dehydrogenase, ADP increases the initial rate of both DPNH oxidation and pyridoxamine phosphate production 2-fold. Initially, in both the presence and absence of ADP, DPN, and pyridoxamine phosphate production are equal. However, after 5 min incubation, about 1.8 fold more DPN than pyridoxamine phosphate is produced in the presence of ADP. At the end of the incubation (100 min) the system is apparently close to equilibrium, the transaminase is completely in the pyridoxamine phosphate form, and the same amount of DPN is produced in either the presence or absence of ADP (1.8 versus 1.6 moles per mole of transaminase peptide chain).

The above experiments with mitochondrial transaminase were performed with this enzyme after it had been crystallized in the presence of α-ketoglutarate (4-6). Before use in these experiments, the crystalline enzyme is extensively dialyzed, and it has been shown that interactions between this enzyme and glutamate dehydrogenase cannot be explained on the basis of trace amounts of free α-ketoglutarate (1, 2). Experiments similar to those shown in Figs. 11 and 12 were performed with the enzyme preparation before incubation in α-ketoglutarate and crystallization. It was found that this preparation oxidized DPNH in the absence of glutamate dehydrogenase at a 2-fold lower specific activity than the crystalline enzyme. This decrease in specific activity is apparently not related to impurities in the precrystalline preparation since the specific activity of the transaminase reaction does not increase markedly with crystallization, and the precrystalline preparation behaves as the crystalline when subjected to electrophoresis on cellulose polyacrylate (3). It was, however, found that the precrystalline enzyme was predominantly in the pyridoxamine phosphate form. If the precrystalline enzyme was incubated with α-ketoglutarate (3 mM) for 10 min and then dialyzed, then it oxidized DPNH in the absence of glutamate dehydrogenase like the crystalline enzyme.

Protection of Mitochondrial Transaminase by DPNH—To further confirm that DPNH can be bound to the transaminase, low concentrations of the mitochondrial enzyme (0.014 μm with respect to peptide chains) were incubated with DPNH. At various time intervals 0.1-ml aliquots of the incubated solutions were withdrawn and assayed for transaminase activity in a 1-ml solution of a coupled assay system with α-ketoglutarate, aspartate, DPNH, and malate dehydrogenase (4). It was ascertained that the small amounts of DPNH added to the assays from the incubated solution (the concentration of DPNH in the assays ranged only from 200 to 250 μm) had no effect on the initial velocity of the transaminase reaction. That is, the initial velocity of this reaction was the same after the transaminase had been incubated for 1 min, regardless of the concentration of DPNH in the incubation mixture. However, after longer periods of time, transaminase incubated in the absence of DPNH slowly lost activity while the addition of 400 μm DPNH completely protected the enzyme against inactivation. A plot of the log of the velocity of transaminase activity versus time was linear for 50 min. A plot of the slope of these plots (k) (the first order rate constant of inactivation) versus the concentration of DPNH incubated is shown in Fig. 13. Protection was specific for DPNH since glutamate (40 mM), α-ketoglutarate (1 mM), DPN (1.2 mM), TPNH (0.3 mM), ADP (5 mM), and ATP (7 mM) did not protect the transaminase against inactivation. In these experiments no NH₄⁺ is added to the incubation and the concentration of transaminase is quite low. Therefore, there is no DPNH oxidation during the incubation.

Glutamate Pyruvate Transaminase

While this enzyme interacts with glutamate dehydrogenase (1, 2), it does not oxidize DPNH in the presence of NH₄⁺ and absence of glutamate dehydrogenase.

**DISCUSSION**

These results indicate that glutamate-oxalacetic transaminases have at least one tightly bound group other than pyridoxal phosphate which in the presence of NH₄⁺ can react with DPNH. It is believed that this group is a keto group since the reaction is inhibited by phenylhydrazine. The group is apparently tightly bound to the transaminase because the enzymes can be extensively dialyzed and chromatographed without loss of this activity. No known keto acids are so tightly bound to the pyridoxal phosphate form of the transaminase that they would remain with it through these procedures. It is true that the γ-subform of the cytoplasmic enzyme does not have this activity; however, this could mean that this subform does not have this group, or it is on this subform but does not react. This does not necessarily mean that this group is loosely bound and removed by chromatography. Other arguments which support the concept that this group is tightly bound are that a plot of velocity versus...
the concentration of DPNH was incubated. Mitochondrial glutamate oxalacetate transaminase (0.014 μmol with respect to peptide chains) was incubated with different concentrations of DPNH in 0.025 M sodium arsenate, 0.1 mM EDTA, pH 7.8 at 25°. At various time intervals (up to 50 min) 0.1 ml aliquots were removed and assayed for transaminase activity in a 1-ml solution of α-ketoglutarate (10 mM), aspartate (40 mM), DPNH (100 μM) and excess malate dehydrogenase (4) in the same arsenate buffer. The first order rate constant of inactivation was measured from linear plots of the log of the velocity of the transaminase reaction versus time.

transaminase concentration is linear and the addition of several different keto acids does not increase activity. It is believed that a group other than pyridoxal phosphate can react because the amines are produced by the pyridoxamine phosphate form of the cytoplasmic enzyme which require initially when the cytoplasmic enzyme is incubated, and with both enzymes considerably more DPN than pyridoxamine phosphate is produced.

Several results suggest that oxidation by DPNH is catalyzed by the transaminase and not some dehydrogenase impurity. Among these are that this activity remains with the transaminase through chromatography on DEAE-Sephadex and Sephadex G-200, and the addition of several enzymes which can react with keto acids does not increase activity. A plausible possibility is that the reaction is catalyzed by a trace amount of glutamate dehydrogenase. This is because both transaminases have at least some B-specificity with respect to DPNH and ammonium is required for the reaction. However, this enzyme is separated from the transaminase on Sephadex G-200. Also, unlike reactions catalyzed by glutamate dehydrogenase, neither transaminase reacts with TPNH. Furthermore, the addition of α-ketoglutarate to these transaminases in the presence of DPNH plus ammonium ions does not increase the rate of DPNH oxidation. The reaction with the mitochondrial transaminase has some A-specificity with respect to DPNH. There is some malate dehydrogenase in these preparations (which has A-specificity) (30). However, we have found that neither mitochondrial nor cytoplasmic malate dehydrogenase can convert α-ketoglutarate or oxalacetate to their corresponding amino acids in the presence of DPNH plus NH₄Cl. The products formed if the A-form of tritiated DPNH is incubated with these keto acids and malate dehydrogenase are their respective tritiated hydroxyacids. No tritiated water or tritiated DPN is found. No amino acids are found when this incubate is chromatographed on the amino acid analyzer. While it is still possible that some dehydrogenase impurity with A-specificity slowly reductively aminates a keto group on the mitochondrial transaminase, it is difficult to conceive of which dehydrogenase this might be. An impurity, however, would be only part of the reaction since the mitochondrial enzyme also has B-specificity with respect to DPNH, and as mentioned above there is no evidence of even a trace of glutamate dehydrogenase activity in these preparations.

The fact that DPNH protects the mitochondrial transaminase against inactivation is a strong argument that DPNH is actually bound to this enzyme.

The mitochondrial enzyme seems to have one tightly bound keto group other than pyridoxal phosphate which slowly reacts with DPNH. This reaction has A-specificity with respect to DPNH. DPNH plus NH₄⁺ also apparently reacts with pyridoxal phosphate to produce pyridoxamine phosphate. This latter reaction is slower and has B-specificity with respect to DPNH. The number of groups on the cytoplasmic enzyme cannot yet be even estimated.

A logical interpretation of the reaction with both enzymes is that an amine is produced by reductive amination. This amine can then exchange hydrogen with water perhaps by forming a Schiff base with a keto group or pyridoxal phosphate (28, 29). However, these were products of the reaction with DPNH and transaminase, it would be more difficult to explain why the reaction is not altered by adding dithioerythritol, the ammonia dependency, and the inhibition by phenylhydrazine. Also, if a reduced flavoprotein was produced in amounts equivalent to that of DPNH, one would be expected that this flavoprotein could be detected spectrophotometrically. This is not the case.

At this time this complicated reaction cannot be completely described. If amines are produced, their net production is considerably less than that of DPNH. A significant amount of amines are apparently not produced which remain bound to the cytoplasmic transaminase during dialysis and deproteinization. This is because a quantitative ninhydrin performed on the incubated dialyzed enzyme reveals only a few additional amines. Also, incubation plus dialysis does not alter the electrophoretic mobility of the transaminase. If any amino acids are produced which are loosely bound and leave the enzyme during deproteinization and dialysis. This is because only a few amino acids are found when the incubated enzyme is deproteinized and applied to the amino acid analyzer without intervening dialysis. Also, the incubated dialyzed cytoplasmic enzyme can again oxidize as many as 20 moles of DPNH per incubated peptide chain. One possibility is that an amine is produced which can be recycled back to the original keto group. This would be consistent with all present results. A logical possibility is that this proposed recycling results from amine oxidase activity. It is known that pyridoxal can catalyze amine oxidase reactions (32). A reaction of this type facilitated by
pyridoxal phosphate would be consistent with the observed exchange of hydrogen with water and the fact that less DPNH is oxidized if the apoenzyme or pyridoxamine phosphate form of the transaminase is incubated. However, our efforts to measure net oxygen uptake or H₂O₂ production have not been successful perhaps because the rate of the proposed reaction is too slow to be detected with the assay employed. Therefore, complete characterization of the reaction and identification of the group or groups on the transaminase which react requires more investigation.

The origin of the amino acids found is obscure. It is unlikely that serine and glycine are produced by reductive amination since glyoxylate reductase and lactate dehydrogenase do not react with the transaminase. Also, even after the cytoplasmic transaminase is incubated and dialyzed it can still oxidize as many as 30 moles of DPNH per incubated peptide chain. Since many different amino acids are found, it seems possible that these are released by a peptidase which is most reactive after the enzyme has been incubated with DPNH plus NH₄⁺.

The slow reaction between DPNH, NH₄⁺, and the transaminase is quite complicated, not completely explained, and has no obvious physiological significance. However, the fact that this reaction can take place before keto acids have been added to the enzyme in the purification procedure or a heat step has been performed suggests that this reaction does not result from an artifact produced by the purification procedure. Recently, several compounds similar to or identical with keto acids have been found on enzymes. In some cases these compounds play a role similar to that of pyridoxal phosphate or they interact with this coenzyme (33-39).

While there is no obvious physiological significance associated with oxidation of DP/NH by transaminase, the binding of DP/NH and DPN to these enzymes could be significant. Both DP/NH and DPN are apparently bound rather tightly. In the case of the mitochondrial transaminase this is of interest because of the known interaction between this enzyme and glutamate dehydrogenase (1, 2). With this transaminase the reaction with glutamate dehydrogenase is considerably more rapid than that in the absence of glutamate dehydrogenase.

Apparently glutamate dehydrogenase does not react with the proposed keto group on either transaminase but with pyridoxal phosphate. This conclusion is supported by the fact that the resolved apoenzyme oxidizes DP/NH in the absence of glutamate dehydrogenase but does not react with TPNH even in the presence of glutamate dehydrogenase (1). Therefore, resolution does not remove the group which reacts with pyridoxal phosphate. Therefore, there is no evidence that any of the amino acids reacted with glutamate dehydrogenase. This is not surprising since the amino acids found are poor substrates of glutamate dehydrogenase.

Apparently glutamate dehydrogenase must have direct contact with transaminase to react since pyridoxamine phosphate is not produced if the transaminase is dialyzed versus a small volume of TPNH, NH₄⁺, and glutamate dehydrogenase. Therefore, it seems unlikely that interaction between these two enzymes is mediated by a keto group which can readily leave the transaminase and pass through a dialysis membrane.

Several experiments suggest that in the presence of glutamate dehydrogenase and transaminase the reduced pyridine nucleotide is bound to and oxidized on glutamate dehydrogenase; that is, this enzyme is the actual catalyst of the reaction with pyridoxal phosphate. This would explain why under these conditions, unlike in the absence of glutamate dehydrogenase, both TPNH and DPNH react, pyridoxamine phosphate is rapidly produced, keto acids are activators of reduced pyridine nucleotide oxidation (since they react with the pyridoxamine phosphate produced), ADP is an activator of the cytoplasmic enzyme, GTP is an inhibitor (1, 2), and the addition of glutamate dehydrogenase to the mitochondrial transaminase increases the rate of the reaction with B-specificity with respect to DP/NH.

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