Limited tryptic hydrolysis of the NAD-specific glutamate dehydrogenase of *Neurospora crassa* (EC 1.4.1.2) does not result in dissociation of the intact enzyme as shown by measurements of sedimentation velocity: $s_{20,w} = 14.1$ S for the intact, and 13.8 S for the nicked enzyme. Sodium dodecyl sulfate and thiol treatment of the trypsin-treated enzyme revealed two major peptides: Peptides T-A ($M_r = 47,300$) and T-B ($M_r = 63,100$), which are produced simultaneously in equivalent amounts, as the intact subunit ($M_r = 120,000$) disappears. Preliminary separation of Peptides T-A and T-B was aided by the insolubility of Peptide T-A in 8 M urea, pH 6. This was followed by preparative electrophoresis of each fraction on polyacrylamide gels. Sequence studies and isolation of some cyanogen bromide and maleylated tryptic peptides permitted identification of Peptide T-B as derived from the COOH-terminal 598 residues of the known sequence of the enzyme. Peptide T-A originates from the portion of the subunit NH$_2$-terminal to Peptide T-B. Peptide T-A contains approximately 419 residues and includes approximately 38 residues in unknown sequence. The portion of the enzyme subunit NH$_2$-terminal to Peptide T-A was presumably degraded by trypsin to very small fragments that were not recovered.

It is postulated that the dehydrogenase is comprised of two distinct domains represented by Peptides T-A and T-B that are linked by a random coil region which is accessible to action by trypsin and other proteinases. Domain A includes the regions of the enzyme containing residues homologous to functional residues in other glutamate dehydrogenases. Domain A shows no homology in sequence to other glutamate dehydrogenases or to Domain B. Presumably, the presence of these two distinct domains in the enzyme subunit is the result of fusion of two distinct genes or by the inclusion with Domain B of a large, previously noncoding segment of DNA.

For some years, work in this laboratory has been devoted to investigating the properties and structures of the glutamate dehydrogenase of eukaryotes (1). The amino acid sequences of the enzymes of bovine (2-4), chicken (3, 5), and human (4) liver have been described, as well as that of the NAD-specific glutamate dehydrogenase of *Neurospora crassa* (EC 1.4.1.2). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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The abbreviations used are: GDH, glutamate dehydrogenase; NAD-GDH, NAD-specific glutamate dehydrogenase of *Neurospora crassa*; NADP-GDH, NADP-specific glutamate dehydrogenase of *Neurospora crassa*, CNBr, cyanogen bromide; SDS, sodium dodecyl sulfate; Cys(Cmt), S-carboxymethylcysteine; CN, peptides derived from the cyanogen bromide digest; TM, peptides derived from the tryptic digest of the maleylated protein; PTH, phenylthiohydantoin; in identification of PTH-derivatives, G, gas-liquid chromatography; T, thin layer chromatography; R, regeneration of free amino acid; H, spot test on aqueous acid phase for arginine; DNAS or dansyl, 5-dimethylaminonaphthalene-1-sulfonyl. Analytical values for homoserine include values for the lactone also.

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Two Domains in NAD-specific Glutamate Dehydrogenase

proteolysis. For preparative production of peptides, the enzyme (5.2 amol) was hydrolyzed with trypsin treated with tosylphenylalanyl-ninenechloromethylketone (Worthington; 0.2%, w/w) in Buffer A (165 ml), pH 7.9, for 25 min at 26.5°C. Hydrolysis was terminated by addition of 1% diisopropylfluorophosphate (20 ml) and the solution was adjusted to pH 3.6 with 30 ml of glacial acetic acid and lyophilized.

Carboxymethylation—The fragments resulting from limited tryptic action were carboxymethylated (20) with iodo[1-14C]acetic acid (Amersham; 42 mg) with a specific radioactivity of 0.51 mCi/mmol.

Cleavage by Cyanogen Bromide—The procedures described by Groe (21) were utilized. The 3% sucrose gel protein (20) was dissolved in 0.1 M formic acid (Tridom/Fluka) and diluted to 15 to 20 mg/ml with the desired solvent. The solution was stirred under nitrogen with a 100-fold molar excess of solid CNBr over methionine for 16 to 24 h, diluted 5-fold with water, and dried by rotary evaporation under reduced pressure.

Polyacrylamide Gel Electrophoresis—For analytical studies, samples were run in System I, 7.5% acrylamide gels (0.6 X 9 cm), 37.1 cm cross-linkage, in 0.1% SDS, 0.1 M sodium phosphate, pH 7.2 (22); or in System II, 12.5% acrylamide gels, 10.1 cm cross-linkage, in 0.1% SDS, 8 M urea, Tris/phosphate (0.025 M in Tris base), pH 6.8 (23). A stacking gel of 2.7% acrylamide was utilized in both systems. Gels stained with 0.25% Coomassie Blue (Canaclon) were scanned at 500 nm (Gilford linear scanner).

For preparative purposes, the Poly-Prep 200 (Buchler) apparatus with constant power supply (Isco) and temperature control (Haake) was utilized. A separating gel (9 cm, 180 ml) of 0.3 mm tubing against water at pH 8 containing a porous bag of Dowex AG may (Amersham; 42 mg) with a specific radioactivity of 0.51 mCi/mmol was overlaid with a stacking gel (1 cm, 20 ml) of 3% acrylamide of the same cross-linkage and buffer (24). An upper gel buffer of 0.05 M Tris/glycine and 0.1% SDS, pH 8.9, and lower gel eluting buffers of 0.1 M Tris and 0.1% SDS, pH 8.1 (22), were circulated at 60 ml/h with a multi-channel pump (Gibson). Temperature was maintained at 30°C during polymerization and thereafter. After pre-electrophoresis for 16 to 24 h at 1.5 mA/cm², the sample (30 to 100 mg protein) was applied in upper gel buffer (5 ml) containing 7% SDS, 0.25% bromophenol blue, and 5% sucrose. Initial electrophoresis was conducted at 1.5 mA/cm² until the sample had fully entered the separating gel, and then at 4 mA/cm². Collection of effluent was begun just prior to elution of the dye front. Pooled fractions were dialyzed in Spectrapor tubing against water at pH 4 for a molecular weight of 113,500, only slightly smaller than the previously estimated value of 116,000 for the subunit (19).

Other Materials and Methods—Procedures for the isolation, characterization, and analysis of peptides, as well as for sequence determinations, have been reported elsewhere (11, 14).

RESULTS

Prior to the use of serine protease inhibitor (11) in the purification of the NAD-GDH, it was noted that the specific activity of certain preparations decreased during storage at 4°C. Analysis by polyacrylamide gel electrophoresis (System I) showed that the enzyme subunit had been partially degraded to several smaller, discrete fragments. Presumably, portions of this paper (including Figs. 3 to 5 and 8 to 11 and Table 1 to IV) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are available from the Journal of Biological Chemistry, 9650 Rockville Pike, Bethesda, Md. 20014. Request Document No. 80 M-312, cite authors, and include a check or money order for $1.65 per set of photocopies. Full size photocopies are also included in the microfilm edition of the Journal that is available from Waverly Press.
Two Domains in NAD-specific Glutamate Dehydrogenase

FIG. 1. Proteolytic fragmentation pattern of NAD-GDH. Native enzyme was incubated with chymotrypsin (C), 0.2% (w/w); thermolysin (L), 0.07% (w/w); or staphylococcal proteinase (S), 0.2% (w/w) in Buffer A, pH 8.1, at 26.5°C. At the times indicated, aliquots were withdrawn and analyzed by electrophoresis in SDS-polyacrylamide gels (System I) as described under "Experimental Procedures." Standard proteins and their molecular weights, respectively, included for comparison were phosphorylase a, 94,000 (Ο); bovine serum albumin, 68,000 (Δ); ovalbumin, 43,000 (●); chymotrypsinogen, 25,700 (▲); and cytochrome c, 11,700 (■).

FIG. 2. Hydrolysis of native NAD-GDH with trypsin. The enzyme was incubated with trypsin (0.2%, w/w) in Buffer A, pH 7.6, at 26.5°C. Aliquots were withdrawn at the times indicated and analyzed by electrophoresis as described in the legend to Fig. 1.

T-A (Fig. 4) and Peptide T-B (Fig. 5) was achieved separately by preparative gel electrophoresis.

As shown in Fig. 6, each purified fragment gave a single, major band by gel electrophoresis in System I. The resolving power of this procedure is readily seen by comparison to the samples of precipitate and supernatant that were utilized for the purification of Peptides T-A and T-B, respectively (Fig. 6).

Attempts by various procedures to isolate Peptides T-A and T-B from the original hydrolysate by using either gel filtration or anion exchange chromatography gave unsatisfactory resolution of the large fragments.

Identification of Peptides T-A and T-B—As described in the following sections, information obtained from the study of the isolated Peptides T-A and T-B has permitted identification of the location of each within the sequence of the dehydrogenase subunit. The relevant areas of primary structure relating to the origin of Peptide T-A from the NH2-terminal portion, and of Peptide T-B from the COOH-terminal region of the subunit, are shown in Fig. 7. The detailed sequences of peptides contained within each fragment have been reported previously (9, 15).

Characterization of Peptide T-A—Peptide T-A contained a single component of \( M_r = 47,300 \pm 1600 \), as judged by sedimentation equilibrium studies (Fig. 8). The partial specific volume was calculated to be 0.735 from the amino acid composition (see "Discussion").

Automated Edman degradation of Peptide T-A (Table I) permitted identification of the first 24 residues at its NH2-terminal end. This sequence is identical with that of fragment II of the NAD-GDH (15). This fragment is preceded by 1 arginine residue, based on studies of Peptide TM1-C (13), and its formation is consistent with the known specificity of trypsin in hydrolyzing an Arg-Ser bond. No other PTH-derivatives in significant quantity were observed during determination of the sequence, suggesting that specific hydrolysis by trypsin had produced Peptide T-A. This was confirmed by identification of dansyl-serine as the only NH2-terminal residue of Peptide T-A.

From its size and the identification of its NH2-terminal sequence, Peptide T-A should contain, in addition to all of fragment II (15), gap A and a portion of the NH2-terminal part of the previously established 669-residue COOH-terminal region (9). To confirm this identification, Peptide T-A (1.5 \( \mu \)mol) was fragmented with CNBr. After successive treatments in 70% formic acid (16 h), 99% formic acid (3.5 h), 95% formic acid (16 h), and 0.1 N HCl (24 h), the conversion of methionine to homoserine and homoserine lactone was only 37%, as judged by amino acid analysis after hydrolysis in 6 M HCl for 24 h. Incubation of Peptide T-A with mercaptoacetic acid (5-
Purified from the supernatant fraction (Gel A) and from the insoluble fraction (Gel B), 7996 peptides had been produced. Accordingly, the CNBr digest was purified from the supernatant fraction (Gel A) and from the insoluble fraction (Gel B), 7996 peptides had been produced. Accordingly, the CNBr digest was purified by gel filtration (Fig. 9).

CNBr to reduce methionine residues did not improve the yield. It seems that during the isolation of Peptide T-A, chemical modifications of methionine residues had occurred which prevented more complete cleavage. Gel electrophoresis (System II) indicated that, although the scission by CNBr had been incomplete, several peptides smaller than 100 residues had been produced. Accordingly, the CNBr digest was fractionated by gel filtration (Fig. 9).

Peptide T-A-CN-D7, purified to homogeneity (System II) by ion exchange chromatography (Fig. 10) of Fraction D (Fig. 9), was identified by automated sequence determination (Table II) as the NH₂-terminal 45 residues of Peptide CN22 (14). The final residue of Peptide T-A-CN-D7, Arg-C599, of the sequence of the NAD-GDH (9), must represent the site of tryptic hydrolysis at the COOH terminus of Peptide T-A, inasmuch as CNBr cleavage would not yield a peptide terminating in an arginine residue.

Automated Edman degradation of Fraction D5 (Fig. 10) showed that Peptides CN31 (15) and T-A-CN-D7 were present (Table III). The finding that Peptide CN31 is included in Peptide T-A supports the previous identification of its location in the sequence of the NAD-GDH (15).

Peptide T-A-CN-E1, purified by paper electrophoresis at pH 1.9 of Fraction E (Fig. 9), was identical in sequence and composition (Table I) with the NH₂-terminal 18 residues of T-A-CN-D7. This corresponds to the NH₂-terminal sequence of the intact Peptide T-A given in Table I.

Peptide T-A-CN-E2, purified in the same manner as Peptide T-A-CN-E1, was identified by composition (Table II) and R_{s/v} value as Peptide CN4 (11). This was confirmed by identification of dansyl-proline as the NH₂-terminal residue. Peptide CN4 immediately precedes Peptide CN22 in the sequence of the dehydrogenase (9).

Sequenator studies of Fraction D (3 + 4) (Fig. 10) and Fraction C (Fig. 9) indicated a mixture of three peptides in each fraction. The results were consistent with the presence of Peptides CN6 (11), CN30, and CN31 (15) in Fraction D (3 + 4), and of Peptides CN23 (14), CN30, and CN31 (15) in Fraction C. The identification of all of these peptides in Peptide T-A is consonant with the view that Peptide T-A comprises a large part of the NH₂-terminal portion of the dehydrogenase (14, 15). However, since there was insufficient material for isolation of individual components, confirmation of the identity of these fragments could not be performed.

Fractions A and B (Fig. 9), containing large fragments of Peptide T-A (80 mg), were combined, maleylated, and hydrolyzed with trypsin (5%, w/w). After chromatography (Fig. 11), only two peptides were isolated in sufficient yield to permit identification. Peptides T-A-CN-A-TM61 and T-A-CN-A-TM62, purified from Fraction 6 (Fig. 11) by preparative electrophoresis at pH 1.9, were identical in composition and R_{s/v} values (Table II) with Peptides CN27-TM2 and CN27-TM4 (14), respectively. Identification of these peptides is also consonant with the proposed location of Peptide CN27 and hence of Peptide T-A within the NH₂-terminal half of the enzyme (15).

Characterization of Peptide T-B—Sedimentation equilibrium measurements on Peptide T-B (Fig. 8) gave M, = 63,000 ± 2600 and showed that the material was homogeneous in size. The partial specific volume calculated from its amino acid composition was 0.736. The NH₂-terminal residue was identified as dansyl-histidine. Automated Edman degradation gave only trace amounts of materials. This suggested that the large size of Peptide T-B prevented analysis by automated Edman degradation.

Peptide T-B (0.55 µmol) was treated with CNBr in 70% formic acid (24 h). The resulting peptides, that were soluble

![Figure 6](image1.png)

**FIG. 6.** Analytical gel electrophoresis of isolated Peptides T-A and T-B. As described in the text, Peptide T-A (Gel 1) was purified from the insoluble fraction (Gel 1), and Peptide T-B (Gel 4) was purified from the supernatant fraction (Gel 2) by using preparative polyacrylamide gel electrophoresis.

![Figure 7](image2.png)

**FIG. 7.** Diagrammatic representation of the location of Peptides T-A and T-B in the overall sequence of the subunit of the NAD-GDH. The bonds hydrolyzed by trypsin (T) to produce Peptides T-A and T-B are indicated by vertical arrows. The sequence of Peptide T-B from residues C598 through Cl is known (9). The sequence of Peptide T-A contains fragment II (313 residues) (15), an unknown region of 38 residues, and residues C669 through C599 (9).
in formic acid/acetic acid/water (2:3:5), were isolated by preparative paper electrophoresis at pH 1.9. The results are summarized in Table IV.

Peptide T-B-CN1 corresponds in composition and $R_{\text{sep}}$ value to the NH$_2$-terminal nonapeptide of Peptide T-B and, namely the previously known Peptide CN2-TM16 (14), representing residues C585 through C596 of the sequence of the enzyme (9). The preceding residue of Peptide CN2, Arg-C589, represents the COOH-terminal residue of Peptide T-A. Based on the isolation of Peptide T-B-CN1, the NH$_2$-terminal residue of Peptide T-B is His-C598. Thus, in the intact native enzyme, trypsin hydrolyzed an Arg-His bond.

Peptide T-B-CN2 corresponds to Peptide CN2 (11), the known COOH-terminal sequence of the subunit of the dehydrogenase (9). Thus, the isolation of Peptides T-B-CN1 and T-B-CN2 indicates that residues C598 through C1 are included in Peptide T-B, or at least in the bulk of the peptide material comprising Peptide T-B.

**DISCUSSION**

Limited hydrolysis of the native NAD-GDH by trypsin appears to have little effect on the structural integrity of the enzyme, as judged by the small change in the sedimentation constant. Similar results have been obtained for the action of other endopeptidases on a variety of enzymes, e.g. ribonuclease A (27), $\beta$-galactosidase (28), and glutamine synthetase (29, 30).

Dissociation of the trypsin-treated dehydrogenase with sodium dodecyl sulfate and thiol revealed that two major fragments were produced in similar amounts with a concomitant decrease in the amount of intact subunit (Fig. 3). Peptide T-A, $M_r = 47,300$, arises from the NH$_2$-terminal region, and Peptide T-B, $M_r = 63,100$, arises from the COOH terminus of the subunit. The sum of the molecular weights of these fragments, 110,400, accounts for 95% of the apparent mass of the subunit, $M_r = 116,000$ (19). The NH$_2$-terminal portion of the sequence preceding Peptide T-A, which is still partially unknown (15), accounts for the remainder of the subunit and presumably was lost because of degradation to very small fragments.

Peptide T-A is formed by trypsin by scission at two discrete bonds, Arg-Ser and Arg-His, resulting in a fragment (Fig. 7) extending from the NH$_2$ terminus of fragment II (14, 15) through Arg-C599 (9). All of the isolated peptides from Peptide T-A, derived by secondary cleavage, have been identified as arising from either the known sequence of fragment II (15) or the initial portion of the 669-residue COOH-terminal portion of the enzyme predicted Arg-C599 (9). Comparison of the amino acid composition of Peptide T-A to that of the sequence known to be included in the peptide (Table V) suggests that approximately 38 residues are present in the single unsequenced gap between fragment I (15) and the 669-residue COOH-terminal sequence (9). This region includes the only 1 of the 9 cysteine residues of the protein that has not been placed in sequence.

Peptide T-B includes residues C598 through C1 of the COOH-terminal portion of the subunit (9). Evidence supporting this identification includes the isolation of Peptides T-B-CN1 and T-B-CN2, which correspond in composition to residues C588 through C590 and C7 through C1, respectively. The good agreement between the measured molecular weight and amino acid composition of Peptide T-B and that derived from the sequence of residues C598 through C1 (Table V) aids in establishing this identification.

The formulations developed and refined by Chou and Fasman (31) have been used to calculate the most probable secondary structure formed by the known parts of the sequence of the protein (32). The region at the bond susceptible to hydrolysis by trypsin between Peptides T-A and T-B is predicted to form a random structure, which is flanked by two tetrapeptide sequences in $\beta$-turn conformation. Thus, there appear to be two distinct domains linked by residues C600 through C595, and it is this region which is accessible to hydrolysis by trypsin in each of the four identical subunits of the enzyme.

The selective hydrolysis of the native, tetrameric NAD-GDH by trypsin suggests that the tertiary structure of the subunit is organized in two major domains. Comparison (9) of the 669-residue COOH-terminal sequence of the subunit to the known sequences of the animal enzymes and the NADP-GDH showed that homology is restricted to small areas of sequence around certain reactive residues. Peptide T-B includes all of these known limited areas of homology, and thus appears to represent the portion of the subunit primarily involved in glutamate dehydrogenase function.

Peptide T-A, representing most of the NH$_2$-terminal domain, shows no obvious homology to either the COOH-terminal domain or to other glutamate dehydrogenases of established sequence (15). This domain could be either catalytic or regulatory in function, or perhaps represent transcription of previously noncoding DNA of the eukaryote (33). Since synthesis of the NAD-GDH is induced by an external source of nitrogen (16, 17) in the presence of low concentrations of ammonium salts.

## TABLE V

Characterization of Peptides T-A and T-B

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<thead>
<tr>
<th>Determination</th>
<th>Peptide T-A</th>
<th>Peptide T-B</th>
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<tr>
<td>Yield (%)</td>
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<td>19</td>
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<tr>
<td>SDS-electrophoresis (M&lt;sub&gt;r&lt;/sub&gt;)</td>
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<td>67,900</td>
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<td>Sedimentation equilibrium, (M&lt;sub&gt;r&lt;/sub&gt;)</td>
<td>47,300 ± 1600</td>
<td>63,100 ± 2600</td>
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<table>
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<tr>
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<td>Total residues</td>
<td>419</td>
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<td>38</td>
<td>599</td>
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<sup>a</sup> ND, not determined; parenthetical values are assumed.
<sup>b</sup> Values are from the specific radioactivity obtained for the intact peptides.
<sup>c</sup> Corrected for decomposition by extrapolation to zero time of values obtained from the 24- and 72-h hydrolysates.
<sup>d</sup> Values obtained from the 72-h hydrolysate.
carbohydrate (34), a second catalytic activity, if present in the intact subunit, might also be involved in utilization of compounds of nitrogen or carbohydrate metabolism. The identification of enzymes containing multiple enzymic activities on the same polypeptide chains in various eukaryotes, e.g. in Neurospora (35, 36) and yeast (37), as well as other organisms (38, 39), lends credence to this possibility.

Regulation of the tetrameric NAD-specific glutamate dehydrogenase of Candida utilis has been reported to involve rapid inactivation by in vivo phosphorylation of the enzyme (40, 41); a similar mode of autoregulation for the NAD-specific dehydrogenase of Neurospora, however, could not be demonstrated (40).

It is noteworthy that the NADP-specific dehydrogenases of the prokaryote Escherichia coli and the eukaryote Neurospora, as well as the glutamate dehydrogenases of vertebrates which can utilize either NAD or NADP, are hexameric in structure, with subunit molecular weights ranging from 44,000 (E. coli (42)) to 55,000 (human (4)). The primary structures of the eukaryote enzymes show marked homology, despite differences in coenzyme specificity and mode of regulation of catalytic activity (9). In contrast, the NAD-specific dehydrogenase of Neurospora is composed of four identical subunits of Mr = 116,000 (19). Thus, despite the differences among these enzymes, it can be assumed that the genes coding for these proteins with the same catalytic function are related in an evolutionary sense. The NAD-specific enzyme of Neurospora may have arisen by duplication of the NAD-specific dehydrogenase gene followed by fusion with an ancestrally distinct gene. The primary, tertiary, and quaternary structures, as well as the change in coenzyme specificity, may then reflect this genetic event. A proposal regarding the evolution of glutamate dehydrogenase in eukaryotes has recently been proposed (33, 43) which further defines and expands this concept.

It has been reported that the mitochondrial NAD-specific dehydrogenases of pea seeds (Pisum sativum) and duck weed (Lemma minor) are composed of four subunits of Mr = 58,000 (44). This finding suggests that the tetrameric structure and coenzyme specificity may have evolved prior to the proposed fusion to form the Neurospora NAD-specific enzyme. It is clear that more chemical information and other evidence will be needed to determine further the relationships of the hexameric and tetrameric glutamate dehydrogenases.

Acknowledgments—We are indebted to Douglas M. Brown for the sedimentation studies and the sequencer analyses, to Dorothy McNall for the amino acid analyses, and to Larry Tabata and Richard Ito for preparation of the enzyme.

REFERENCES

Two Domains in NAD-specific Glutamate Dehydrogenase

Table 1

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<td>1.7</td>
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</table>

* Peptide T-A and T-B were purified by chromatography on Teflon columns and gel filtration on Sephadex G-75. The purified peptides were lyophilized and stored at -20°C. The peptides were then lyophilized and stored at -20°C. The peptides were then lyophilized and stored at -20°C. The peptides were then lyophilized and stored at -20°C. The peptides were then lyophilized and stored at -20°C. The peptides were then lyophilized and stored at -20°C.

Table 2

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Fig. 1. Inhibition of Peptide T-A. Peptide T-A (100 nM) was incubated with NAD-specific glutamate dehydrogenase (100 nM) at pH 7.0 for 30 min. The reaction was then stopped by the addition of 100 mM Tris-Cl, pH 7.0, and the absorbance at 280 nm was determined. The data are expressed as the mean ± SEM of three experiments.