Nicotinamide Adenine Dinucleotide-specific Glutamate Dehydrogenase of Neurospora

I. PURIFICATION AND MOLECULAR PROPERTIES*

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SUMMARY

Improved growth conditions were developed for normal strain 1A of Neurospora crassa in order to induce a high level of the NAD-specific glutamate dehydrogenase. A simplified purification procedure is described in order to obtain the enzyme in high yield and specific activity. The homogeneous enzyme has a molecular weight of 480,000 ± 27,000 by sedimentation equilibrium analysis, and is dissociable into four identical subunits of 116,000 ± 5,400 molecular weight. The amino acid composition of the enzyme was determined. Isophthalate was found to be a strong competitive inhibitor and an effective stabilizer of the enzyme. The molecular properties indicate that the subunit structure of this enzyme differs substantially from those of the glutamate dehydrogenases from vertebrates and of the NADP-specific enzyme from Neurospora.

Glutamate dehydrogenases occupy an important position in metabolism, since they catalyze the interconversion of α-amino groups and ammonia, and thus can serve in the incorporation or disposal of nitrogen. The kinetic and molecular characteristics of vertebrate glutamate dehydrogenases, particularly the bovine liver enzyme, have been studied extensively (for a review see Ref. 1). Glutamate dehydrogenases from microorganisms and plants usually have a specific requirement for either NAD or NADP, unlike the vertebrate enzymes which can use both coenzymes.

Neurospora crassa contains two glutamate dehydrogenases, each specific for one of the coenzymes. It has been suggested that the NAD-specific enzyme acts primarily as a catabolic catalyst whereas the NADP-specific dehydrogenase appears to have a synthetic function. It was of interest to examine the chemical and structural properties of these two enzymes, since comparison of such features may provide an understanding of evolutionary relationships not only of these two enzymes but also of other glutamate dehydrogenases from higher organisms.

Further, such studies should yield additional information on regulation and mechanism of action of dehydrogenases of this kind. Some of the molecular and chemical properties of the NADP-specific enzyme have recently been reported from this laboratory (2). In this paper we present an improved method of purification of the NAD-specific enzyme and some of its molecular properties. The accompanying papers (3, 4) describe reactions of the enzyme with group-specific chemical reagents and the amino acid sequences at regions of selectively labeled residues. The three reports indicate distinct structural dissimilarities between the NAD-specific enzyme and other glutamate dehydrogenases.

EXPERIMENTAL PROCEDURES

Materials—Glutamic acid, α-ketoglutaric acid, NAD, NADH, dithiothreitol (DTT), and protamine sulfate were purchased from Calbiochem. Isophthalic acid was obtained from Eastman. Bio-Gel A-1.5m was the product of Bio-Rad Laboratories, and Whatman was the source of DEAE-cellulose (DE52). TPCK-treated trypsin and diisopropyl fluoromtosinylated carboxypeptidase A were obtained from Worthington. The exopeptidase was recrystallized from 10% LiCl by the procedure of Neurath (5).

Growth of Neurospora Culture—Normal strain 1A of N. crassa was grown by a two-step procedure, based on previous studies that had shown that the specific activity of the NAD-glutamate dehydrogenase is increased by the presence in the medium of the amino acids glutamate (6, 7) and by limiting glucose or sucrose as a carbon source (8). Stock cultures used to obtain conidia for the initial inoculum were surface-grown for 7 days at 27° in 250-ml Erlenmeyer flasks on 50 ml of Vogel's minimal medium (9) containing 1.5% agar. The total aqueous conidial suspension derived from

1 The abbreviations used are: TPCK, L-1-tosylamido-2-phenyl-ethyl chloromethyl ketone; DTT, dithiothreitol; DTNB, 5,5'-dithiobis(2-nitrobenzoic acid); dansyl, 5-dimethylaminonaphthalene-1-sulfonoyl.
each stock culture was used to inoculate a 4-liter culture of urea-containing medium for the first stage of growth. This medium was similar to that used previously (6) and had the following composition: sodium citrate, 3.0 g; KH₂PO₄, 6.25 g; MgSO₄, 0.2 g; CaCl₂, 0.1 g; biotin, 10 µg; sucrose, 10 g; urea, 3.0 g; streptomycin sulfate, 25 mg; water was added to 1 liter. In addition, it contained the trace elements used in Vogel’s medium and NaOH to bring the pH to 5.6. The mycelium was transferred into 4-liter Erlenmeyer flasks by autoclaving 40 min at 121°C. The urea was added as a filter-sterilized supplement. After inoculation with conidia, the cultures were grown for 48 hours at 27°C on a rotary shaker at 220 rpm. Loose plugs of nonabsorbent cotton were used to allow adequate aeration.

All cultures were based on extensive, preliminary studies involving variations in the growth conditions and the composition of the culture media. Many variables were studied--feeding schedules, air changes, amounts of the dehydrogenase. The 50-min autoclaving of the final cultures was similar to that used previously (6) and had the following composition: sodium citrate, 3.0 g; KH₂PO₄, 6.25 g; MgSO₄, 0.2 g; CaCl₂, 0.1 g; NH₄NO₃, 2.0 g; biotin, 10 µg; urea, 3.0 g; streptomycin sulfate, 10 mg; L-glutamic acid, 5.0 g; water was added to 1 liter with the trace elements used in Vogel’s medium and NaOH to bring the pH to 5.6. Ten liters of the medium in each culture were autoclaved for 50 min at 121°C. After transferring the growing 4-liter culture into this medium, growth was allowed to proceed 24 hours at 27°C under forced aeration with 3.5 liters per min of air. The mycelium harvested by filtration from a culture had a dry weight of 15 to 18 g and a specific activity of 25 to 30 units per mg with respect to the dehydrogenase. The final medium, containing all ingredients except urea, was sterilized in a 6-liter Erlenmeyer flask by autoclaving 40 min at 121°C. The urea was added as a filter-sterilized supplement. After inoculation with conidia, the cultures were grown for 48 hours at 27°C on a rotary shaker at 220 rpm. Loose plugs of nonabsorbent cotton were used to allow adequate aeration.

**Protein Estimation**—For crude preparations of the enzyme protein concentration was estimated spectrophotometrically (10). For determining concentration of purified enzyme the value ε₂₅₀ = 0.97 was used.

**Gal Electrolysis**—Sodium dodecyl sulfate polyacrylamide gel electrophoresis was performed by the procedure of Weber and Osborn (11).

**S-Cardoxymethylated Protein**—Protein was dissolved in 0.3 M Tris buffer at pH 8.6 containing 6 M guanidinium chloride and 0.2% 2-mercaptoethanol. Solid recrystallized iodoacetate acid was added in 1.2-fold molar excess over mercaptoethanol, together with an equal weight of solid Triton X-100. The reaction was followed by mixing being: DTNB test paper and was completed within 20 min. After addition of 2-mercaptoethanol in slight excess over the residual iodoacetate, the S-carboxymethylated protein was dialyzed against water.

**Amino Acid Analysis**—Analyses were performed with a Beckman model 121 automatic amino acid analyzer by the general procedures of Spackman et al. (12). Samples were hydrolyzed with 6 N HCl containing 15% mercaptoethanol and 0.05% phenol, in evacuated sealed tubes, for 22 to 72 hours at 110°C.

**Sedimentation Equilibrium**—Weight average molecular weights were determined at 20° by the meniscus depletion method of Yphantis (13) in a Spinco model E ultracentrifuge equipped with Rayleigh interference optics and a temperature control unit. A swinging bucket rotor (model E model E, 100 volts input) was employed. Three different protein concentrations between 0.2 and 1.2 mg per ml were used in each cell, at speeds of 8,000 or 8,225 rpm for the native enzyme and at 21,740 to 24,000 rpm for the samples in guanidinium chloride. The interference patterns were recorded on Kodak metallicographic plates with an exposure of 35 s and the plates were read on a Gaertner microcomparator. Plates were analyzed at 24 and 48 hours to insure that equilibrium was complete. The x coordinate at each half-fringe was measured and the computations were performed with the aid of a Monroe Epic 3000 programable calculator. Weight average molecular weights were determined from the plotted slopes of ln c versus x², from Equation 1 where x is the distance from the axis of rotation to the position of the half-fringe of c (concentration).

\[
\overline{M} = \frac{2RT}{\left(1 - \frac{V_p}{V_r}\right) \alpha} \frac{d}{dx}^2
\]

R is the gas constant, T is the absolute temperature, \(\overline{V}\) is the partial specific volume, \(\alpha\) is the density of the solvent, and \(\alpha\) is the angular velocity of the rotor.

The solutions were dialyzed for 48 hours at 4°C. The density of the solutions was determined by pycnometer. Partial specific volumes were calculated from the amino acid composition and the values of \(\overline{V}\) for each amino acid residue given by Cohn and Edsall (14).

**Enzyme Methods**—Tryptic digestion of S-carboxymethylated protein was performed in 0.1 N NH₄HCO₃ at pH 8.0 and 40°C for 6 hours. The amount of enzyme was 5% by weight of the protein. The digest was acidified with acetic acid and was lyophilized repeatedly to remove the volatile salts.

For hydrolysis with carboxypeptidase A, the procedure described by Light (15) was followed, except for the use of sodium dodecyl sulfate in pH 8.0 NH₄HCO₃ buffer instead of sodium lauryl sulfate in Veronal buffer.

**Peptide Mapping**—Two-dimensional peptide maps were made on tryptic digests from approximately 1 mg of S-carboxymethylated protein. Electrophoresis at pH 1.9 (formic-acetic acid, 3000 volts) for 30 min was used for the first dimension, followed by descending chromatography for 16 hours in butanol-pyridine-acetic acid-water (15:10:3:12) for the second dimension, on What-

**Protein Electrophoresis**—For crude preparations of the enzyme protein concentration was estimated spectrophotometrically (10). For determining concentration of purified enzyme the value ε₂₅₀ = 0.97 was used.
man No. 3MM paper. Peptides were located by dipping the paper in ninhydrin-collidine reagent (16), or by spraying with amino acid-specific reagents as indicated.

**Other Methods**—Performic acid oxidation (17), N-succinylation (18), NH₂-terminal analyses by the cyanate (19), and the dansyl (20) methods, were performed by previously described procedures.

**RESULTS**

**Purification of NAD-specific Glutamate Dehydrogenase**

A representative procedure is described below for the supernatant solution obtained from 200 g (dry weight) of the mold. All operations were performed below 6°. All buffers contained 1 mM EDTA, 2.5 mM glutamate, and 0.1 mM DTT, unless otherwise specified.

**Step 1. Protamine Precipitation of Nucleic Acids**—Protamine sulfate was dissolved in the centrifuged extract at a concentration of 1.1 g per liter. After 20 min, the precipitate was removed by centrifugation for 30 min at 27,000 x g.

**Step 2. Ammonium Sulfate Fractionation**—The solution was brought to 27% saturation by slow addition of solid (NH₄)₂SO₄ with continuous stirring. After 30 min the suspension was centrifuged and the supernatant solution was taken to 45% saturation in (NH₄)₂SO₄ with NH₄OH, and after 30 min was centrifuged as above. The pellet contained most of the enzyme.

**Step 3. Dialysis**—The pellet was dissolved in 200 ml of 0.005 M ammonium phosphate at pH 8.0 and dialyzed for 1.5 hours against 8 liters of the same buffer, in the presence of ice, with four changes of buffer at 60-min intervals. The dialysis tubing used for this purpose (3% inch flat diameter with 0.002 inch thickness) was stretched for a few seconds under a hydrostatic pressure of 20 pounds per square inch to increase its permeability. The conductivity of the dialyzed solution was measured to ascertain that its ionic strength did not exceed that of 0.01 M ammonium phosphate buffer used as an eluent in the next step. When necessary, the conductivity was lowered by appropriate dilution with 2.5 mM isophthalate solution at pH 8.0.

**Step 4. Chromatography on DEAE-cellulose**—The dialyzed solution was made 1.0 M in NaCl and applied to a column (5 x 25 cm) of DEAE-cellulose (Whatman microgranular DE52) which had been washed previously by alternate treatments with 0.5 M HCl and 0.5 M NaOH, converted to the phosphate form with 0.15 M ammonium phosphate buffer (without supplements) at pH 8.0, and equilibrated with 0.01 M ammonium phosphate buffer containing 2.0 M EDTA, 0.1 mM DTT, and 2.5 mM isophthalate, at pH 8. After washing the column with 350 ml of the equilibrating buffer, the enzyme was eluted with a linear gradient of ammonium phosphate, formed by placing 3 liters of 0.01 M buffer at pH 8.0 in the mixing chamber and 3 liters of 0.15 M buffer at the same pH in the reservoir. The concentrations of EDTA, DTT, and isophthalate in the eluting buffer were the same as those in the equilibrating buffer. Fractions of 20 ml were collected at a flow rate of 35 ml per hour. The enzyme was eluted at approximately 0.015 M ammonium phosphate. A typical elution profile is shown in Fig. 1. Fractions of highest specific activity were pooled and the enzyme was precipitated by adding an equal volume of a saturated (NH₄)₂SO₄ solution at pH 8.0. The precipitate was collected by centrifugation.

**Step 5. Gel Filtration**—The precipitate was dissolved in 10 ml of 0.1 M ammonium phosphate at pH 8.0, and applied to a column (5 x 95 cm) of Bio-Gel A-1.5m (200 to 400 mesh) previously equilibrated with the same buffer. The column was eluted at a flow rate of 35 ml per hour and fractions of 10 ml were collected as shown in Fig. 2.

The purity of the enzyme was indicated by the constant specific activity of fractions within the activity peak. The preparation gave a single sharp band on gel electrophoresis in the presence of sodium dodecyl sulfate, as shown in Fig. 3b.

The purification procedure is summarized in Table I. The specific activity of the pure enzyme was approximately 8000 units per mg, corresponding to an 80-fold purification with respect to the initial extract. The over-all recovery was 38%. It is evident from Table I that the main losses of activity occurred during dialysis, possibly owing to the action of proteases present in the solution. It was essential, therefore, to keep the time of dialysis to a minimum, and the temperature at 0°, in order to avoid greater losses of activity.

**Properties of Enzyme**

The enzyme was stable for months when stored as a suspension in 50% saturated ammonium sulfate at 4° in the presence of 0.1 mM DTT and 25 mM glutamate. When kept as a frozen
Fig. 3. Sodium dodecyl sulfate polyacrylamide gel electrophoresis of glutamate dehydrogenases. a, chicken liver glutamate dehydrogenase; b, NAD-specific glutamate dehydrogenase of Neurospora crassa; c, bovine liver glutamate dehydrogenase. The direction of migration is from top to bottom. Electrophoresis was run for 8 hours at 8 mA per tube.

### Table I

**Purification of Neurospora crassa NAD-specific glutamate dehydrogenase**

<table>
<thead>
<tr>
<th>Step</th>
<th>Total protein</th>
<th>Total enzyme</th>
<th>Specific activity</th>
<th>Yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude extract</td>
<td>34,600 mg</td>
<td>3.4 x 10^6 units</td>
<td></td>
<td>100</td>
</tr>
<tr>
<td>1. Supernatant after protamine</td>
<td>34,600 mg</td>
<td>3.4 x 10^6 units</td>
<td></td>
<td>100</td>
</tr>
<tr>
<td>2. Supernatant after 27% ammonium</td>
<td>21,600 mg</td>
<td>3.3 x 10^6 units</td>
<td>157</td>
<td>97</td>
</tr>
<tr>
<td>3. Ammonium sulfate precipitate</td>
<td>20,000 mg</td>
<td>1.7 x 10^6 units</td>
<td>85</td>
<td>50</td>
</tr>
<tr>
<td>4. DEAE-cellulose effluent</td>
<td>500 mg</td>
<td>1.5 x 10^6 units</td>
<td>3000</td>
<td>44</td>
</tr>
<tr>
<td>5. Bio-Gel A-1.5m effluent</td>
<td>180 mg</td>
<td>1.3 x 10^6 units</td>
<td>8100</td>
<td>38</td>
</tr>
</tbody>
</table>

### Table II

**Composition of NAD-specific glutamate dehydrogenase**

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Residues per subunit</th>
<th>Nearest integer residues per subunit</th>
<th>Strain 74-OR8-1s (residues per subunit)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lysine</td>
<td>65.0 ± 2.5</td>
<td>65</td>
<td>66</td>
</tr>
<tr>
<td>Histidine</td>
<td>24.8 ± 1.0</td>
<td>25</td>
<td>25</td>
</tr>
<tr>
<td>Arginine</td>
<td>63.4 ± 1.5</td>
<td>63</td>
<td>62</td>
</tr>
<tr>
<td>Aspartic acid</td>
<td>114.0 ± 1.1</td>
<td>114</td>
<td>117</td>
</tr>
<tr>
<td>Threonine</td>
<td>48.3 ± 3.6</td>
<td>48</td>
<td>50</td>
</tr>
<tr>
<td>Serine</td>
<td>73.5 ± 5.8</td>
<td>74</td>
<td>76</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>103.0 ± 1.0</td>
<td>103</td>
<td>108</td>
</tr>
<tr>
<td>Proline</td>
<td>49.2 ± 3.9</td>
<td>49</td>
<td>46</td>
</tr>
<tr>
<td>Glycine</td>
<td>66.3 ± 2.6</td>
<td>66</td>
<td>66</td>
</tr>
<tr>
<td>Alanine</td>
<td>70.5 ± 1.0</td>
<td>71</td>
<td>73</td>
</tr>
<tr>
<td>Half-cystine</td>
<td>9.1 ± 0.5</td>
<td>9</td>
<td>9</td>
</tr>
<tr>
<td>Valine</td>
<td>68.7 ± 3.8</td>
<td>69</td>
<td>62</td>
</tr>
<tr>
<td>Methionine</td>
<td>20.3 ± 2.4</td>
<td>20</td>
<td>21</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>66.7 ± 2.5</td>
<td>67</td>
<td>67</td>
</tr>
<tr>
<td>Leucine</td>
<td>92.3 ± 2.0</td>
<td>92</td>
<td>92</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>40.8 ± 1.1</td>
<td>41</td>
<td>41</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>46.8 ± 0.8</td>
<td>47</td>
<td>50</td>
</tr>
<tr>
<td>Tryptophan</td>
<td>7.2</td>
<td>7</td>
<td>2</td>
</tr>
<tr>
<td>Total residues</td>
<td>1030</td>
<td>1030</td>
<td></td>
</tr>
</tbody>
</table>

| Average of values obtained from hydrolysates of four different enzyme preparations, calculated for a molecular weight of 116,000. |
| Data of Strickland et al. (24), recalculated to the nearest integer for a subunit of molecular weight 116,000. |
| Corrected for decomposition by extrapolation of values obtained from 24- and 72-hour hydrolysates. |
| Determined as cysteic acid after performic acid oxidation. An average of 9.0 ± 0.2 residues was obtained on the carboxymethylated protein and of 9.3 residues by titration with DTNB in guanidinium chloride. |
| * Values obtained from 72-hour hydrolysate. |
| / Determined by amino acid analysis after hydrolysis with p-toluene sulfonic acid (21). The same value was obtained spectrophotometrically. |

Values given for half-cystine are those obtained as cysteic acid from analyses of samples that had been oxidized with performic acid. The content of methionine sulfone in these samples was similar to that of methionine found in nonoxidized preparations, namely 20 to 21 residues per subunit.

The half-cystine content was also determined by two other methods. S-Carboxymethylation of the enzyme in 6 M guanidinium chloride in the presence of 2-mercaptoethanol, followed by acid hydrolysis and amino acid analysis yielded 9.0 ± 0.2 S-carboxymethylcysteine residues per subunit. By spectrophotometric titration of a sample of a DTT-free, fully active enzyme with DTNB in 6 M guanidinium chloride at pH 8.0, 9.3 sulfhydrol groups were found per subunit (3). This last result showed that all 9 half-cystines are present as cysteine residues.

Tryptophan was determined after hydrolysis with p-toluene-sulfonic acid, as described by Liu and Chang (21). Tryptophan was also estimated spectrophotometrically as follows. The absorbance of an enzyme solution in 0.1 N NaOH in 6 M guanidinium chloride at 280.0 and 294.4 nm was 0.67 and 0.59, respectively. From the equation of Goodwin and Morton (22), as modified by Beaven and Holliday (23), the value of 5.84 was obtained for the molar ratio of tyrosine to tryptophan. This corresponds to a ratio of 41 to 7.0, in excellent agreement with the value obtained after acid hydrolysis.

Included in Table II is the amino acid composition of the NAD-
performed at 20° at a rotor speed of 24,000 rpm. B, sedimentation equilibrium of the enzyme in 6 M guanidinium hydrochloride containing 0.5\% mercaptoethanol at pH 7.0. Centrifugation was performed at 20° at a rotor speed of 24,000 rpm.

The enzyme isolated from the \textit{N. crassa} strain 74-OR\textasciitilde, that was recalculated for a subunit of 116,000 molecular weight from the data reported by Strickland \textit{et al.} (24). The results are in good agreement with ours except for the values for tryptophan, valine, and isoleucine. The discrepancy in the value for tryptophan is probably due to differences in the methods used for analysis, whereas the variance in the values for the other two amino acids is undoubtedly due to the difference in time of hydrolysis. The present results were obtained from 72-hour hydrolysates, whereas the previously reported data were obtained from 24- to 48-hour hydrolysates. Valine and isoleucine are generally not released completely in less than 72 hours. Despite these differences it does not appear that the enzyme differs significantly in the two strains.

\textit{Molecular Weight}—Native enzyme was dialyzed at 4° against 0.1 M ammonium phosphate containing 0.1 mM EDTA and 2.5 mM glutamic acid at pH 7.8 for 48 hours before each run. Plots of log \(c \) versus \(x^2\) (13) conformed to a straight line indicating homogeneity in all cases. Fig. 4A shows a representative example of such a determination. Although there was a slight decrease in molecular weight in the presence of added glutamic acid, this change was within the experimental error of the method. Eighteen independent values were determined on different preparations of the native enzyme, yielding a molecular weight of 480,000 \(\pm 27,000\). The calculated partial specific volume (\(\bar{v}\)) from the amino acid composition was 0.737.

Subunit size was investigated on samples containing 6 M guanidine HCl and 0.5\% 2-mercaptoethanol at pH 7.1, after dialysis for 48 hours at 4°. Complete homogeneity of each sample was observed; a representative run is shown in Fig. 4B.

Thirteen values from three different enzyme preparations gave a molecular weight for the subunit of 116,000 \(\pm 5,400\), indicating that the native enzyme possesses four subunits per molecule. Similar values for the size of the subunit were obtained for enzyme samples that had previously been oxidized with performic acid or that had been N-succinylated before estimation in 6 M guanidinium chloride: 110,000 \(\pm 3,000\) and 111,000 \(\pm 5,000\), respectively.

The above measurements were confirmed by electrophoresis of the enzyme on polyacrylamide gels in the presence of sodium dodecyl sulfate (Fig. 3). In accord with an estimated molecular weight of 116,000, the migration of the subunit of the \textit{Neurospora} enzyme (Fig. 3b) was about half as much as that of the subunits of either the chicken or bovine liver glutamate dehydrogenases (Fig. 3, a and c, respectively). The subunits of these two enzymes had been previously shown to have a molecular weight of approximately 55,000 both by sedimentation equilibrium measurements (25-27) and by determination of the amino acid sequences (27-29). From the calibration curves of Weber and Osborn (11), the electrophoretic mobility in sodium dodecyl sulfate polyacrylamide gel is almost linearly inversely proportional to the molecular weight in this range.

**Peptide Maps**—Peptide mapping performed on a tryptic digest of \(S\)-carboxymethylated enzyme showed 65 to 70 ninhydrin-positive spots of varying intensity. Pauly’s reagent spray revealed 11 histidine-positive spots and 10 tyrosine-positive spots. Three definite spots and another very faint spot were positive for tryptophan with Ehrlich’s reagent.

**Attempts to Identify Terminal Residues**—Neither NH\textsubscript{2}-terminal nor COOH-terminal amino acid residues could be identified. With the cyanate method for NH\textsubscript{2}-terminal analysis (19), only trace amounts of amino acids were found at levels that were not significantly different from those found in control samples lacking either cyanate or enzyme. Similarly, no identifiable \(N\)-dansyl amino acid could be detected in the acid hydrolysates of the dansylated protein (20, 30). Subjecting the protein to 12 cycles of Edman degradation with a Beckman sequenator produced no detectable phenylthiohydantoins on gas chromatography.

Treatment of the protein for 24 hours with recrystallized carboxypeptidase A in 0.1 M ammonium bicarbonate at pH 8.0, containing 1.2\% sodium dodecyl sulfate, revealed no free amino acid other than those present at impurity levels in control samples lacking either the protein-substrate or the exopeptidase. Under identical conditions, bovine glutamate dehydrogenase released threonine and phenylalanine, as expected from the known sequence of the enzyme (28, 29).

**Competitive Inhibition**—Isophthalic acid was shown by Caughey \textit{et al.} (31) to be a strong competitive inhibitor of bovine glutamate dehydrogenase. The powerful inhibition has been attributed to the rigidity of the extended 1,3-dicarboxylate structure of the aromatic compound. The \(N\)-dependent glutamic dehydrogenase of \textit{Neurospora} is inhibited by isophthalic acid even more strongly than is the bovine enzyme. This is shown in Fig. 5, which presents the residual activities of the two enzymes at various inhibitor-to-substrate molar ratios. Thus, whereas the inhibition of the \textit{Neurospora} enzyme is competitive is shown in Fig. 6, which presents Lineweaver-Burk plots of the dependence of the activity on the substrate concentration in the absence and presence of 6.0 \(\times 10^{-4}\) M isophthalate. From the slopes of these plots, \(K_M = 2.0 \times 10^{-4}\) and \(K_I = 6.1 \times 10^{-4}\). In a parallel experiment, the \(K_I\) was 6.2 \(\times 10^{-4}\) for the isophthalate inhibition of the bovine enzyme, in good agreement with the value 5.6 \(\times 10^{-4}\), reported by Caughey \textit{et al.} (31). Isophthalic acid is thus approximately 10 times stronger as a competitive inhibitor for the \textit{Neurospora} enzyme than for the bovine enzyme.

Isophthalate-linked agarose has recently been used for affinity chromatography of bovine glutamate dehydrogenase.\textsuperscript{1} The

\textsuperscript{1}M. Wilchek, personal communication.
molar ratios indicated. 

**DISCUSSION**

Our simplified procedure for the purification of the NAD-specific glutamate dehydrogenase of *N. crassa* represents a significant improvement over earlier methods, both in specific activity and yield of the purified enzyme. The specific activity of our purified material was 8100 units per mg, about 20% higher than that reported by Strickland *et al.* (24), and 40% higher than that of Jacobson's preparation (32). The recovery of the enzyme was usually higher than 35%, compared to 26% obtained in the previous procedures.

The molecular weight of the native enzyme was found to be near 480,000, whereas that of the dissociated subunit was 116,000. The NAD-specific enzyme thus appears to be a tetramer, consisting of four identical subunits. The measured value of the molecular weight of the subunit as found by sedimentation-equilibrium, was supported by sodium dodecyl sulfate polyacrylamide gel electrophoresis. This value, 116,000, is approximately twice that of the subunits of bovine and chicken glutamate dehydrogenases, as well as that of the NADP-specific enzyme from *N. crassa* (2). The unusually large subunit survived such treatments as prolonged incubation in 6 M guanidinium chloride containing 2-mercaptoethanol, exhaustive N-succinylation, and performic acid oxidation, as well as treatment with sodium dodecyl sulfate. Such conditions would most probably have resulted in dissociation of polypeptide chains linked either noncovalently through hydrophobic, electrostatic, or hydrogen bonding, or covalently by disulfide bridges. However, the last possibility can be excluded since all 9 cysteine residues per subunit are titratable without reduction. It can be concluded, therefore, that the subunit consists of a single polypeptide chain of approximately 1050 residues lacking disulfide bonds.

In the earlier study of Strickland *et al.* (24), the molecular weight of the subunit of the NAD-specific enzyme was reported to be 51,523 on the basis of amino acid analysis and peptide mapping. Both the amino acid composition and the peptide maps obtained in our study were essentially similar to those found previously. The number of detectable spots in the peptide maps is indeed compatible with a subunit of only half the molecular weight found by the physical measurements. This seeming discrepancy might lead one to suggest a subunit structure consisting of two sequentially identical, covalently linked segments, that could have arisen from gene duplication. This possibility, however, is now considered unlikely in view of our presently incomplete sequence studies, currently in progress in this laboratory. From a tryptic digest of the enzyme, more than 80 peptides have been isolated thus far; these account for 64 unique lysine residues of the 65 estimated to be present in a subunit of molecular weight 116,000.

These results render questionable the significance of the number of spots detectable in peptide maps in a polypeptide chain as large as this one.

As already noted (24), some resemblance can be found between the compositions of the *Neurospora* NAD-enzyme and the bovine enzyme (assuming a similar molecular weight for the respective subunits), in contrast to the amino acid composition of the *Neurospora* NADP-enzyme. On the basis of this similarity in composition, and having postulated a hexameric structure for the NAD-enzyme, the previous investigators suggested the existence of an evolutionary homology between the bovine enzyme and the *Neurospora* NAD-glutamate dehydrogenase. In view of the large difference now found between the molecular weights of the respective subunits, as well as substantial dissimilarities observed in other chemical and structural features of the two enzymes, this possibility can be excluded.
dehydrogenases (3, 4), it is doubtful whether the resemblance in the compositions of these enzymes is more than coincidental.

No NH₂-terminal amino acid could be found by either the cyanate or the dansyl method. Nor did the protein undergo Edman degradation. This suggests that the α-amino group of the protein is blocked. The failure of carboxypeptidase A to release detectable amounts of amino acids can be explained in view of recent results obtained from sequence studies. From both the tryptic and the cyanogen bromide digests of the protein, peptides originating from the carboxyl end of the chain have been isolated. The sequences of these two peptides show that the COOH-terminal sequence of the protein is -Lys-Glu-OH. Glutamic acid, like other acidic amino acids, is known to be released by carboxypeptidase A at a very slow rate when compared with non-acidic COOH-terminal residues (33). It is noteworthy that the above tryptic and CNBr COOH-terminal peptides showed little or no tendency to be hydrolyzed by carboxypeptidase A or A + B under the usual conditions.

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