Nucleoside Triphosphate-Nucleoside Diphosphate Transphosphorylase (Nucleoside Diphosphokinase)

III. SUBUNIT STRUCTURE OF THE CRISTALLINE ENZYME FROM BREWERS' YEAST

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SUMMARY

Previous physical studies from this laboratory by sedimentation velocity and diffusion and by sedimentation equilibrium (Yue, R. H., Ratliff, R. L., and Kuby, S. A. (1967) Biochemistry 6, 2923-2932) have permitted an assignment of the kinetic molecular weight of the crystalline brewers' yeast nucleoside diphosphokinase, namely 102,000 (±2,000) at infinite dilution. The observed concentration dependence of its weight average molecular weight value (Yue et al.) was likely, in part, the result of a complex set of association-dissociation equilibria. Evidence presented here and derived (a) from the amino acid analyses, tryptic peptides, and terminal group analyses; (b) from sedimentation equilibrium studies on the native enzyme and on its S-carboxymethylated derivative, in the presence of disruptive agents; (c) from polyacrylamide gel electrophoresis in 0.2% dodecyl sulfate; and (d) from gel filtration through 8% agarose in 6 M guanidinium chloride, all indicate that the protein molecule is composed of six similar, if not identical, subunits. The best value for the individual subunit molecular weight (of this hexameric structure) may be selected from its amino acid composition, 17,300 ± 500. The NTP-NDP transphosphorylase appears to be unique among the other ATP-transphosphorylases studied in this laboratory, in that it possesses a single, shielded sulfhydryl group per subunit polypeptide chain, and in that it displays unusual resistance to inactivation by guanidinium chloride.

In the first paper of this series (1), the isolation of the crystalline enzyme, nucleoside diphosphokinase, from brewers' yeast had been reported. A detailed description of the enzyme protein's physicochemical properties and a systematic study on its homogeneity followed in the second paper (2).

Since that time, a number of preparations have been reported from a variety of sources other than brewers' yeast, e.g. from human erythrocytes (3, 4), calf thymus (5), beef heart or liver mitochondria (6, 7 or 8, 9) and cytochrome (10), Bacillus subtilis (11), pea seeds (12), and a crystalline preparation has been obtained from bakers' yeast (13). However, only a few physical properties of this enzyme from other sources appear to have been determined, and in most cases only an approximate estimate of its molecular weight, approximately 10³, a number which appears to be in rough agreement with the best value assigned (102,000 ± 2000 at zero protein concentration) for the brewers' yeast enzyme (2).

During the investigation of the physicochemical properties of the crystalline brewers' yeast enzyme (2), no evidence for any gross heterogeneity in the protein preparation could be found by the following techniques; sedimentation velocity, sedimentation equilibrium, translational diffusion, and by electrophoresis (moving boundary). However, the measured weight-average molecular weights obtained by sedimentation velocity and diffusion, by "approach to equilibrium," and by true sedimentation equilibrium techniques, all appeared to be concentration-dependent. By suitable extrapolations to infinite dilution, satisfactory agreement in molecular weights (i.e. in $M_w$ and in $M_{ap}$) were obtained, and a "best" value thereby assigned for the kinetic unit of this enzyme. From plots of $1/M_w$ vs. $C_p$ a positive second virial coefficient could be estimated (5.0 x 10⁻⁴ molel per g²), and a hydrodynamic model proposed, namely that of a hydrated (0.5 g per g of hydration) and expanded sphere of 37 Å radius, with a $V_{mol}$, hydrated of 126 liters per mole. The slightly higher value of $B_M$ (5.0 cm⁴ per g), the concentration dependency in $M_w$, and an unusual ionic strength dependency of its isoelectric point (pI₀ = 8.0, at zero ionic strength), were considered to be all the likely result of departures from thermo...
dynamic ideality (i.e., an effect on the activity coefficients, or possibly an alteration in the frictional coefficient with concentration, during the hydrodynamic and transport measurements, as a result of interactions or preferential binding of solvent components), as well as, and especially in the high protein concentration region, the result of a complex and rapidly adjusting set of association-dissociation equilibria. These data, together with an estimate of its third virial coefficient, which appeared to be larger than expected according to any solution theory (cf. Hill (14)), and a preliminary estimation at that time of its amino acid composition, all pointed to the likely existence of an associated n-mer. The above studies, therefore, led to an estimation of its subunit structure, which is accordingly the subject of the present communication, and to a rather remarkable conclusion, namely that the enzyme protein at infinite dilution would exist as one of the rare hexamers, in solution (e.g. compare Refs. 15 and 16).

Preliminary reports (Palmieri et al. (17, 18)) have been presented elsewhere.

**EXPERIMENTAL PROCEDURE**

**Materials**

Crystalline nucleoside triphosphate-nucleoside diphosphate transphosphorylase was isolated from brewers' yeast by the procedure described by Ratliff et al. (1). All reagents used for buffers were the best available analytical grade commercial products. 2-Mercaptoethanol was redistilled in vacuo; guanidine hydrochloride (Eastman) was purified by the procedure of Nozaki and Tanford (19) and in later studies obtained from Mann ("Ultra Pure"). Urea was deionized and crystallized from saturated aqueous solutions according to Benesch et al. (20, 21), and, also in later studies was obtained from Mann ("Ultra Pure"). Sodium lauryl sulfate samples were obtained from Sigma.

Iodoacetic acid (Matheson, Coleman and Bell) was recrystallized from ether-hexane just prior to use, as described earlier (22). Pyridine was refluxed with ninhydrin and redistilled. Butanol, isooamyl alcohol, and all other solvents used were redistilled from reagent grade solvents. Redistilled constant boiling HCl was employed for all amino acid analyses. Twice distilled, deionized pyridine was used in the preparation of all solutions for the physical methods (except, see text) with the technique of sedimentation equilibrium in dynamic ideality (i.e. an effect on the activity coefficients, or possibly an alteration in the frictional coefficient with concentration, during the hydrodynamic and transport measurements, as a result of interactions or preferential binding of solvent components), as well as, and especially in the high protein concentration region, the result of a complex and rapidly adjusting set of association-dissociation equilibria. These data, together with an estimate of its third virial coefficient, which appeared to be larger than expected according to any solution theory (cf. Hill (14)), and a preliminary estimation at that time of its amino acid composition, all pointed to the likely existence of an associated n-mer. The above studies, therefore, led to an estimation of its subunit structure, which is accordingly the subject of the present communication, and to a rather remarkable conclusion, namely that the enzyme protein at infinite dilution would exist as one of the rare hexamers, in solution (e.g. compare Refs. 15 and 16).

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A gift from Eli-Lilly & Co. (Lot No. 3KE, 30A).

**Table I**

<table>
<thead>
<tr>
<th>Protein</th>
<th>Source</th>
<th>Assigned mol wt</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bovine serum albumin</td>
<td>Armour (crystallized) Lot No. E 71503</td>
<td>69,000</td>
<td>24</td>
</tr>
<tr>
<td>Ovalbumin</td>
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<td>43,000</td>
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<tr>
<td>Pepsin</td>
<td>Worthington (twice crystallized) Lot No. PM 7DB</td>
<td>35,000</td>
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<tr>
<td>Chymotrypsinogen A</td>
<td>Worthington (five times crystallized) Lot No. CGC 7CC</td>
<td>25,700</td>
<td>24</td>
</tr>
<tr>
<td>Tryptsin</td>
<td>Worthington (three times crystallized) Lot No. 6613</td>
<td>23,800</td>
<td>27</td>
</tr>
<tr>
<td>(\beta)-Lactoglobulin</td>
<td>Mann (three times crystallized) Lot No. U 1867</td>
<td>18,400</td>
<td>24</td>
</tr>
<tr>
<td>Bovine hemoglobin</td>
<td>Sigma type I (twice crystallized) Lot No. 106B 8441</td>
<td>15,500</td>
<td>28</td>
</tr>
<tr>
<td>Bovine pancreatic ribonuclease A ((S)-carboxymethylated)</td>
<td>Sigma (type XIII-A) Lot No. 25 B 8610 (alkylated with iodoacetate, see text)</td>
<td>14,064 (after crystallization)</td>
<td>28</td>
</tr>
<tr>
<td>Horse heart cytochrome c</td>
<td>Sigma type VI Lot No. 105B-7640</td>
<td>12,270</td>
<td>28</td>
</tr>
<tr>
<td>Ribonuclease T1 (Aspergillus oryzae)</td>
<td>Worthington Lot No. RI 0BA</td>
<td>11,100</td>
<td>29</td>
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<tr>
<td>Bovine (\beta) chain of insulin (oxidized)</td>
<td>Mann Lot No. S 3884</td>
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<td>Porcine glucagon</td>
<td>A gift from Eli-Lilly &amp; Co. (Lot No. 3KE, 30A)</td>
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<tr>
<td>Bacitracin A</td>
<td>Mann Lot No. U 4086</td>
<td>1,411</td>
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</table>

**Physical Methods**

Sedimentation Equilibrium—Sedimentation equilibrium experiments at 3 or 20° were made in a Spinco model E ultracentrifuge equipped with an RTIC temperature control system and a rotatable source. Use was made of Rayleigh interferometric optics (31) with the technique of sedimentation equilibrium in conditions: 8.0 M urea, 0.36 M Tris (Cl\(^-\)), pH 8.6, 4.82 \(\times\) 10\(^{-3}\) M EDTA, pH 8.6, 0.105 M 2 mercaptoethanol (calc. equil.), 0.101 M iodoacetic acid (purified above), and 2.55 mg per ml of nucleoside diphosphokinase (or 2.7 mg per ml of ribonuclease) in a total additive volume of 1.667 ml. The samples were then exhaustively dialyzed at 3° versus 0.01 M KCl followed by redistilled, deionized water (boiled) over a 24-hour period, and then lyophilized. Amino acid analyses revealed quantitative \(S\)-carboxymethylation of both ribonuclease and of nucleoside diphosphokinase (to be presented below).

The following proteins, and their sources, used as calibration standards, in the gel filtration and polyacrylamide gel electrophoresis experiments are described in Table I, together with their assumed molecular weights (and references). Only the molecular weight of ribonuclease (\(S\)-carboxymethylated) was checked by sedimentation equilibrium.
highly dilute protein solutions at relatively high speeds, according to the method of Yphantis (32), i.e. by the meniscus depletion technique. The precautions and experimental technique followed that of Yphantis (32) as described earlier (Yue et al. (33)). In the experiments with protein solutions containing 4 M guanidinium chloride at 20°, the preparation of the protein solutions were also as given earlier (33). Because of the relatively high speeds which proved necessary in this case with the meniscus depletion technique (as a result of the comparatively small size for this polypeptide chain), greater care than usual had to be exercised in the alignment of the optical system (34), and in the “blank” runs (32), to correct for optical inequalities and distortion between solvent and solution channels (of the 12-mm filled Epon double sector cell with sapphire windows). “Blank” runs were run immediately after each experiment in the same cell, cleaned without dismantling. Straight and parallel “air fringes” were used as a qualitative index of cleanliness of the lenses, and care exercised to insure that oil and dust did not collect on the collimating lenses. An evaluation of molecular weight averages (\(M_\theta, M_w, M_a\), and \(M_v\)) was also made according to the procedure of Yphantis (32) with the use of the measured values of \(\bar{v}\) at 20 or 3° for the native enzyme, or \(\bar{v}\) calculated (see below) from the amino acid composition for the case of the protein dissolved in 4 M guanidine solutions, at 20°. The selection of the appropriate value of the apparent partial specific volume for the disrupted polypeptide chains has its experimental justification in the consistent set of \(M_v\) values obtained earlier by Yue et al. (33) for a variety of protein sub-units, in 4 M guanidine HCl, when evaluated in this manner. It may be noted that the \(\bar{v}\) calculated from its amino acid composition was found to be usually lower by approximately 0.01 cm³/g, than the measured value for the native structure at 20°, for the several proteins investigated in this laboratory, and this difference (i.e. decrease in value) in guanidinium solutions may well account for these consistent data (cf. also Kawahara and Tanford (35) for a discussion of this point). Nevertheless, the largest source of error in the assignment of the molecular weight by sedimentation equilibrium of this polypeptide chain will hinge on selection of a value for its partial specific volume; and to distinguish between a hexameric and tetrameric structure in the final quaternary structure will of necessity demand measurements of a high degree of precision and accuracy. This point will be dealt with further, below; but for this main reason two other methods were employed (see below) which did not require a prior assignment of its apparent partial specific volume.

The unavoidable optical aberrations at the higher speeds employed in the Yphantis procedure (32) led to larger degrees of uncertainties in the molecular weight averages (i.e in \(M_v\)) than by the more conventional low speed sedimentation equilibrium procedure (see also Yue et al. (2)). Also, some unavoidable problems of aggregation were observed at the bottom of the cell in the absence of a reducing agent, but in 4 M guanidinium chloride solutions (see below). This difficulty could be largely obviated by the use of 0.1 M \(\beta\)-mercaptoethanol, but its use, in turn, led to a small uncertainty in balancing the refractive indices of the two sectors, if any oxidation had taken place in the solution sector. This final difficulty was eventually overcome with the use of an \(S\)-carboxymethylated sample of the enzyme, in solutions of 4 M guanidinium chloride, and in absence of \(\beta\)-mercaptoethanol.

**Gel Filtration in 6 M Guanidinium Chloride**—Gel filtration was performed with an experimental procedure based on that of Davison (36) and on that of Fish et al. (37). Because of the relatively small size of the polypeptide chain of nucleoside diphosphokinase compared to those studied by the above investigators with use of the Bio-Gel A-5M (6% agarose content), that gel which best appeared to resolve polypeptide chains in the neighborhood of the size of the nucleoside diphosphokinase proved to be Bio-Gel A-1.5M (with a nominal agarose content of 8%). Interestingly, as will be shown, a linear plot of log (mol wt) versus \(K_d\) resulted over the useful range in molecular weights (from about 7 × 10⁴ down to almost 1.5 × 10⁹), and with an experimental departure from the least squares plot of approximately ±3% (S.D.), which may be taken as the experimental error in estimation of \(K_d\) with the use of the assigned molecular weights (see Table I).

**Sodium Dodecyl Sulfate Acrylamide Gel Electrophoresis**—Gel electrophoresis for estimation of subunit size was conducted according to the method of Weber and Osborn (38), except for the preparation of the disrupted protein solutions and their application. The proteins (at concentrations of 2 to 3 mg per ml) were disaggregated in a solution containing 2% (w/v) sodium dodecyl sulfate, 4 M urea, 3% (v/v) \(\beta\)-mercaptoethanol, 10⁻⁴ M EDTA, 0.01 M sodium phosphate, pH 7.0, for 2 hours at 40°. 10% (w/v) acrylamide-0.27% (w/v) methylenebisacrylamide was polymerized with persulfate in Pyrex tubes, 10 × 0.6 cm (inside diameter), in 0.10 M sodium phosphate-0.1% sodium dodecyl sulfate according to the description of Weber and Osborn (38). Each protein sample in a micro test tube was prepared as follows: to 10 μl of disrupted protein solution, 25 μl of the above disrupting solution, diluted 1:10, was added and mixed with 25 μl of 0.005% bromphenol blue (in water), 5 μl of \(\beta\)-mercaptoethanol, and 1 drop of glycerol. These mixed samples were transferred to the gels and carefully overlayered with the 0.10 M sodium phosphate-0.1% sodium dodecyl sulfate electrode buffer (as described in Ref. 38). Electrophoresis was conducted at room temperature (approximately 20-25°) at a constant current of 8 ma per gel; the gels were stained with 0.5% Coomassie brilliant blue in 50% methanol-acetic acid (454 + 46 ml) for 4 to 5 hours, and destained and stored, as given in (38). The mobility was calculated relative to the positions of bromphenol blue, after correction for the swelling of the gel (38); semilog plots of molecular weight versus mobility were employed to facilitate the estimation of the molecular weights. Molecular weight markers introduced simultaneously with the nucleoside diphosphokinase on the same gel, also served to increase the reliability of the estimation; however, departures from linearity in the semilog plots below approximately 11,000 to 14,000 in molecular weight was observed for 10% gels with cross-linker ratios of 1:37 (bis to acrylamide) as noted also by Swank and Munkres (39). Therefore, a much larger error in estimation of the small polypeptide chain of nucleoside diphosphokinase was found by this technique than estimated by Weber and Osborn (38) for the molecular weight range of 30,000 to 60,000 (about ±10%). Nevertheless, this technique possesses the major advantage of permitting a qualitative detection of polypeptide chains with dissimilar size, if present. In the present case, the nucleoside diphosphokinase appeared to be present as a single polypeptide component, under all the conditions employed, including a change in concentration applied to the gel, and also after alkylation of the protein.

4 To conserve space, details of the chromatography and preparation of the samples prior to gel filtration have been omitted, but are available on request.
Chemical Methods

Amino Acid Analyses—The procedure for amino acid analyses, calibration of the analyzer, and the preparation of the samples for acid hydrolysis with the precautions indicated to exclude oxygen during hydrolysis, followed that described earlier for rabbit muscle myokinase and ATP-creatine transphosphorylase (22, 40). Samples in sealed, evacuated tubes (Pyrex) were hydrolyzed for periods of 20, 40, 70, and 140 hours in redistilled constant boiling HCl. Analyses were conducted with a Spinox 120 B amino acid analyzer modified to enhance the sensitivity to the nanomole range. Thus, a 6.6-mm optical path cuvette in combination with a 1 mv span across the multispot recorder, permitted at least a 15-fold increase in sensitivity over the previous analyses (cf. Ref. 22).

The samples after hydrolysis were rapidly taken to dryness in the glass tubes on a rotary evaporator; the residues dissolved in the pH 2.2 sodium citrate buffer (41) and analyzed with the two-column system of Spackman et al. (41) at accelerated flow conditions (Spackman (42)) of 70 ml per hour, or in some cases 90 ml per hour with a 200-foot reaction coil to provide maximum color yield. A few pieces of data had been obtained very early, with the original 150- and 10-cm columns, and 30 ml per hour buffer flow system of Moore et al. (43). These results were aged into the more recently gathered data. A column, 10 to 8 × 0.9 cm, of Beckman grade sullenic acid resin, 15-A blend (25 to 30 μ, 8% cross-linkage) was used to separate the basic amino acids, and a spherical beaded resin, Aminex A-4 (Bio-Rad, 16 to 24 μ, 8% cross-linkage), in columns, 55 to 60 × 0.9 cm, were used for separation of the acidic and neutral amino acids, when operated at 55° with 4% (v/v) methanol added to the 0.2 residue per subunit (see below).

Under these high sensitivity and accelerated flow operating conditions, there appears to be some sacrifice in accuracy and precision and the present average precision would appear to be about ±3% (in the 0.01 to 0.02 μmole range) compared to the ±1% for the former analyses (in the 0.25 to 0.500 μmole range). However, from the former sensitivity of about ±0.003 μmole, the present sensitivity of the analyses has been decreased, on an average, to approximately ±0.0001 μmole (or about ±0.1 nmole); although there is some loss in accuracy, a complete amino acid analysis under standard conditions for a single analysis required less than 0.1 mg of protein, an important consideration for the more difficultly obtainable brewers' yeast nucleoside diphosphokinase (Ratif et al. (1)) compared to the +1% for the former analyses (in the 0.25 to 0.500 μmole range).

Tryptophan Analyses—Tryptophan analyses were performed after hydrolysis with 4 × Ba(OH)2 for periods of 50 to 70 hours at 110° in sealed evacuated Vycor glass tubes, as described earlier (Noltmann et al. (22)). Analyses were conducted on 20- to 16-cm columns of Spinox crushed resin 15-A (IR-120; approximately 25 to 30 μ). The 20-cm column, as indicated before (22), proved necessary to completely resolve tryptophan from the huge excess of lysine + ornithine, when eluted at 55° at 60 ml per hour with 0.35 M sodium citrate, pH 5.38. It had also been noted earlier (22) that an unknown alkaline hydrolysis degradation product might emerge at the tail end of the tryptophan peak on this column, and careful integration of the tryptophan area should take this component in mind. During the course of the following years, several spherical resins have been tried to better resolve tryptophan from either lysine plus ornithine or from this alkaline degradation peak. Thus, Bio-Rad's spherical resins Q-15 S, Aminex A-4, and Spinox's spherical resin AA-27, proved inferior to the crushed resin 15-A, in the sense, that the tryptophan peak was invariably retarded by the greater than usual affinity of the spherical resin for tryptophan and for aromatic amino acids. Therefore, the crushed resin 15-A, with at least twice the length normally used (i.e. approximately 20 cm) was employed for these analyses, as it had been earlier. In the recent report of Hugli and Moore (45), this alkaline degradation product has apparently been identified as N'-[N-2-amino-2-carboxyethyl]-L-lysine, and to resolve this component on a Beckman PA 35 spherical resin at 55°C, they had to decrease the sodium ion concentration to 0.21 M, at pH 5.4. Accordingly, aliquots of the Ba(OH)2 hydrolysates (after removal of the Ba++) were checked for the presence of lysinoalanine by chromatography on 15-A resin, but with the developing system of Hugli and Moore (45). A comparison was made with an authentic sample of lysinoalanine, prepared from ribonuclese, by a 1-hour treatment with 0.2 N NaOH at 40°, followed by acid hydrolysis, according to the description of Bohak (46). The position of lysinoalanine on a 16-cm column of 15-A resin when developed at 55°, with 0.21 N NaOH (citrate), pH 5.35 (measured at 25°), at 60 ml per hour appeared at 75 min, compared to 46 min for tryptophan, and 83 min for lysine + ornithine. Under these conditions, only traces of lysinoalanine were found in the 50- and 70-hour Ba(OH)2 hydrolysates, in amounts less than 0.1 to 0.2 residue per subunit (see below).

Nevertheless, the tryptophan analyses were corrected for their presence. Thus, in agreement with the observations of Bohak (46), lysinoalanine, in the case of some proteins, may not be formed in significant amounts from lysine and cystine (and, or, serine) after alkaline hydrolysis. Of interest here, is the unusually high content of tryptophan and low content of cysteine (see below).

Half-cystine Determinations—Half-cystine was determined either (a) as S-carboxymethylcysteine (after reduction with β-mercaptoethanol and alkylation in 8 M urea with iodoacetate acid, as described above under "Materials"), or (b) as cysteic acid, after performic acid oxidation by the procedure described by Moore (47). Both amino acids after acid hydrolysis were referred to the measured value of leucine, or to norleucine as an internal standard. It has been found, at the microscale employed, that the yields of methionine sulfone (formed from methionine) under these oxidizing conditions (47), appear to be quantitative, and therefore, the extent of conversion from methionine to its sulfone (as compared to the previously measured value of methionine) could be used to correct, if necessary, for any incomplete oxidation of half-cystine to cysteic acid.

Sulphydryl Group Determinations—Determinations were conducted with 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) (48) as described by Okabe et al. (49), in the presence of several varying concentrations of guanidinium chloride. In one experiment, alkylation with [2-3H]iodoacetate was used to locate by radioautography the position of the —SH group-containing peptide (or peptides) on the peptide map, conducted on thin layer sheets of cellulose (see below).

Hydrazinolysis—Hydrazinolysis for COOH-terminal amino acids was conducted quantitatively at 85° according to the detailed procedure of Yue et al. (33), with careful introduction of glycine controls for its degradation during the hydrazinolysis. Essentially only glycine appeared to be liberated from the protein in near-stoichiometric amounts, but trace amounts of other amino acids could be detected chromatographically, as described earlier (33).
Tryptic Digestion—Tryptic digestion of the S-carboxymethylated nucleoside diphosphokinase protein (alkylated in 8 M urea, rendered salt-free and lyophilized; see above, “Materials” were conducted on weighed samples in small screw-capped vials. The final conditions (at 30°C) were as follows: 7.7 mg per ml of protein substrate concentration in 0.1 M NH₄HCO₃ (pH 8.5 at 25°C, freshly prepared), 0.9% (w/w) trypsin (Worthington, treated with L-1-tosylamide-2-phenylthyl chloromethyl ketone to inhibit contaminant chymotryptic activity (50)) added at 0, 6, and 17 hours; and the digestion continued at 30°C for a total time of 24 hours, after which time, it was shell-frozen and lyophi-

lized directly within the vial; just prior to peptide mapping on thin cellulose layers, the tryptic digests were redissolved (at pH 8.2).

Peptide mapping on a microscale was conducted on Eastman cellulose chromatogram sheets No. 6062 (20 × 20 cm) (160 μ thick, without fluorescent indicator, and without binder, on inert polyethylene terephthalate backing). These sheets were prepared for high voltage electrophoresis in a Varsol-cooled Savant electrophoresis tank (model LT-36); and after application of the sample (equivalent to approximately 0.1 mg of protein), located off center, electrophoresis at pH 6.5 (pH of electrode buffer, measured at 25°C, and of composition-pyridine-acetic acid-water, 25:1.0:500) was initiated at 12–15°C, for 40 min at 2000 volts. Buffer and Varsol were rapidly removed, at first, with a stream of warm air and finally, the sheets completely dried overnight at 30°C in a forced draft chromatography oven. Ascending chromato-

graphy (at 25°C, 4 to 5 hours) was then conducted at right angles to the direction of the electrophoresis, with the use of a solvent system suggested by Burns and Turner (51), isooctyl alcohol-ethanol-pyridine-water-acetic acid (70:20.70:80:5), until the DNP-leucine (applied as a marker along the line of the electrophoretically separated peptides) had migrated approxi-

mately 18 cm. The solvent was removed by drying as before at 30–40°C, and the peptides visualized by dipping the sheet in ninhydrin (0.1% in acetone), and developed at 70°C for 30 to 45 min. Other specific stains, as summarized in Dawson et al. (32), were used as dipping reagents to detect the following amino acids: arginine, histidine + tyrosine, tryptophan, and methionine + S-carboxymethylcysteine. The multiple staining technique of Zweig and Whitaker (53) was employed, since it aided in the orientation of the peptides and conserved the amount of sample used for these studies.

RESULTS AND DISCUSSION

Physical Studies on the Protein and on Its Polypeptide Chains

Sedimentation Equilibrium Studies—The previous studies (Yue et al. (2)) on the native enzyme had made use of true sedimentation equilibrium runs with Rayleigh interferometric optics (as described in Ref. 54), as well as Ehrenberg’s (55) adaptation of the Archibald “approach to sedimentation equilibrium” technique. Although these combined studies permitted a description of the apparent weight-average molecular weight as a function of protein concentration over a relatively wide concentration range (approximately 1 to 15 mg per ml), the assignment of the extrapolated value to infinite dilution necessarily suffered from the quadratic nature of the expression (in concentration) required to define the over-all and unusual dependency. To confirm, therefore, the extrapolated value for \( M_w \), the interferometric technique of sedimentation equilibrium in highly dilute protein solutions and at relatively high speeds according to the method of Yphantis (32) was also utilized in this study. Previous studies conducted at 3°C had shown that the extrapolated values for \( M_w \), by sedimentation equilibrium, or of \( M_L \), by sedimentation velocity and translational diffusion, were essentially independent of pH. However, the limiting slope of a \( 1/M_w \) versus \( C_m \) plot appeared to double from \( p \) 5.6 to 7.9, indicative of the effect of charge on the second virial coefficient, in a manner similar to that described by Sechatard et al. (56) and by Eddshall et al. (57) for serum albumin. Moreover, an unusual dependency of isoelectric point on ionic strength was observed, which extrapolated from a \( p \) 7 of 5.6 (3°C) and 0.15 ionic strength to a \( p \) 7 of 8.0 (3°C) at zero ionic strength. To fully define the system this had necessitated both hydrodynamic and thermod-

dynamic measurements on the native enzyme at several pH values, but at 3°C; and this point will be dealt with again, below. For the present studies on the native enzyme, a pH of about 7.8 was employed, but at two temperatures, since disruptive experiments were contemplated for 20°C, and it was also of interest to compare the molecular weight of the associated species of the native enzyme at both 3 and 20°C.

Thus, in Fig. 1 typical data are presented which were obtained by the high speed meniscus depletion technique of Yphantis (32), for the native enzyme at 0.16 ionic strength, pH 7.8, at both 3 and 20°C. Since the initial protein concentration in both cases was only approximately 0.22 mg per ml, the \( M_w \) values obtained should approach those found earlier by extrapolation in the
An apparent partial specific volume is defined here, i.e. $\bar{\nu}_{\text{app},2}$ for Component 2, in the sense that for a two-component system the true partial specific volume at infinite dilution of Component 2 would be obtained by extrapolation of Component 2 (82).
such as 4 several proteins tested, when measured in a disruptive solvent of v &pp,2 by +* in the molecular weight determination for the movement is that it appears to accomplish the same as substitution component 2 of any small molecular weight solutes. Thus, experimentally, as noted above (see Yue et al. (33)) the justification for use of a $p$ calculated (see below) from the amino acid composition is that it appears to accomplish the same as substitution of $v_{app,2}$ by $\phi^*$ in the molecular weight determination for the several proteins tested, when measured in a disruptive solvent such as 4 guanidinium chloride-0.001 M EDTA, provided the preferential binding is only very slight.

Finally, it is necessary to discuss the magnitude of an additional source of error which may be inherent in the above measurements, namely the use of the limiting and ideal expression for sedimentation equilibrium by the Yphantis approach. Thus,

$$
\bar{M}_{w,app} = \frac{2RT}{\omega(1 - \bar{v}_{app,p})} \frac{d\ln \mu}{d(X')} 
$$

does not correct for the effect of charge, if the protein were removed from its isoelectric state in the above three-component system, and the resulting Donnan effect could tend to affect the gradient in protein, at sedimentation equilibrium. To estimate this source of error, the equation derived originally by Scatchard and co-workers (63; also see 64-67) may be applied in limiting form to the meniscus depletion technique.

$$
\bar{M}_{w,app} = \frac{2RT}{\omega(1 - \bar{v}_{pp,app})} \frac{d\ln \mu}{d(X')} + \frac{1}{2} Z_{eq} M_s \frac{(1 - \bar{v}_{pp})}{(1 - \bar{v}_{pp})}
$$

where $Z_{eq}$ is the net proton charge on the protein, $M_s$ the molecular weight of Component 3 (guanidinium chloride) with an apparent partial specific volume $\bar{v}_{pp,app}$. At pH 6.4 (see below), the maximum theoretical proton charge calculated from the amino acid composition assuming that electrostatic interactions between charged groups within the molecule may be neglected, as e.g.-in 4 M guanidinium chloride, and that there is one COOH group added per subunit through S-carboxymethylation, see below-is 17 - 1 = +16 for the S-carboxymethylated subunit. With the use of 0.760 for $\bar{v}_{pp,app}$ at 1 M guanidinium chloride (50), $\bar{v}_{pp}$ = 0.741 and $\bar{p}_{pp}$ = 1.0886 (as measured at 20°) for Fig. 2C, leads to a calculated $\bar{M}_{w,app} = 17,860 + 686$. Thus, the value is increased by only about 3.8%; and since the maximum proton charge may be slightly less, if there are any residual electrostatic effects, this increment would tend to further decrease.

In conclusion, therefore, it is likely that the measured weight-average molecular weight of Fig. 2C reflects that of a subunit one-sixth the size of the native molecule, and that all six subunits would appear to be very similar if not identical in size; moreover, since interchain disulfides have been ruled out, the six subunits

![Fig. 3. Molecular weight estimation of the subunit polypeptide chains of nucleoside diphosphokinase by gel filtration through 6% agarose (6 x 0.9 cm) in 6 M guanidinium chloride, 0.10 M EDTA, 0.01 M 2-mercaptoethanol (pH 6.5) at 25°. The data (also see Table 1) are expressed in the form of a semilog plot: log (molecular weight) versus K'd, which may be fitted (within ±10%, S.D.) to the expression log (mol wt) = 5.148 - 2.029 K'd, where K'd (68) is defined as K'd = (V_a - V_b)/(V_c - V_b), and V_a is the elution volume of solvent at the peak concentration of the eluting solute, V_b is the exclusion or void volume as indicated by blue dextran, and V_c is the internal volume as indicated by dimethylphenyl alanine. Only a single component was observed for nucleoside diphosphokinase and the K'd value (0.444) for S-carboxymethylated NTP-NDP transphosphorylase measured is indicated by the solid black point, which results in a calculated (by the above expression) molecular weight of 17,620 (±1,000).](http://www.jbc.org/)
molecular weight size of the nucleoside diphosphokinase by this technique, which apparently depends on polypeptide chain length, hinges on the appropriate and accurate assignments of the subunit molecular weights used for standardizing the gel chromatography (see Table I), and on the basic assumption that all the standards adopt the same conformation in 6 M guanidinium (that of a linear random coil, if all disulfides are broken, Tanford et al. (64) and Tanford (24)) as that of the unknown polypeptide chain. If one assumes that the ±3% (S.D.) in experimental departure from the least squares plot reflects the error in estimation of the K'a with the use of the assigned molecular weights (Table I), then the measured K'a = 0.444 for the S-carboxymethylated nucleoside diphosphokinase results in an estimated (by the expression above) molecular weight of 17,620 ± 1,090. The agreement by this hydrodynamic technique with the data obtained above from thermodynamic means, i.e. by sedimentation equilibrium, is all the more remarkable when one considers all the errors and assumptions involved. In addition, and most notably, only a single eluted component was discernible from the gel exclusion studies, again pointing to only a single sized polypeptide component present in the nucleoside diphosphokinase hexameric molecule.

Dodecyl Sulfate Polyacrylamide Gel Electrophoresis—Gel electrophoresis (Weber and Osborn (38)) also revealed only a single component and an estimation of the subunit size in the neighborhood of 17,000 (Fig. 4). A relatively large error (approximately ±2000) was found in the estimation of this relatively small subunit size by this technique, as a result of the departure from linearity in the semilog plot of log (mol wt) versus electrophoretic mobility below about 11,000 to 14,000 in molecular weight, for the 10% gels employed with cross-linker ratios of 1:37 (see “Experimental Procedure,” and also Ref. 39). Nevertheless, the semiquantitative agreement with the other methods, by this technique which depends on still another manifestation of polypeptide chain length, namely the ability of dodecyl sulfate to convert both the marker proteins and the nucleoside diphosphokinase into similar rod-like structures (68), supports the thermodynamically assigned six-subunit, noneovalently linked structure. Also, since qualitatively no additional components were observed, this technique further enhances the view that each of the subunits are similar, if not identical, in size.

Chemical Studies on the Protein and Its Polypeptide Chains

Amino Acid Analyses—The amino acid analyses are summarized in Table II in terms of grams of amino acid residue per 100 g of protein (Column 2). Recalculation of these data (cf. 22 and 40) in terms of moles of amino acid residue per 1,000 g of protein (Column 4), i.e. per hypothetical one-sixth molecule derived from the physical data, followed by calculation of the product (Column 9) of nearest integral number of residues (Column 5) times their respective minimal molecular weights (Column 3), leads to an average value for the molecular weight of this hypothetical one-sixth molecule, viz. 17,294 ± 477. In Column 8, are presented the nearest integral numbers of amino acid residues for either 17,263 or 17,294 g of protein, with the latter number taken as apparently the most consistent estimate for the subunit molecular weight from the amino acid data alone. The iterative process of taking the sum (Column 6) of the products of nearest integral number of residues (Column 5) times their respective residue molecular weights leads to the second approximation of 17,263 g per mole (Column 6), a value which when employed in the calculations shown in Columns 7 and 8, yields those integral numbers of residues (Column 8) which seem to fit either 17,263 or 17,294 (i.e. the third approximation) as the best assigned value for the subunit molecular weight. One is inclined, however, toward 17,294 (±477) as the most consistent value for the one-sixth molecule, since the convergence to this value by the iterative process seems too “slow” and the value may be arrived at more directly by the procedure outlined above from the measured weight percentages (Column 2); nevertheless, there would seem to be no significant difference between 17,263 and 17,294 of polypeptide chain, since the experimental error of these analyses (see above, “Experimental Procedure”) is too large to allow a distinction between these two values, or that derived from the physical data for the subunit size (namely (102,000 /6) = 17,000).

Column 2 of Table II reveals some interesting data which may be compared against two other ATP-transphosphorylases studied in this laboratory. Thus, in comparison with the rabbit muscle ATP-creatine transphosphorylases (Noltmann et al. (22)), the most notable difference appears to be in the surprisingly high concentration of tryptophan (4.79 g of residue per 100 g of nucleoside diphosphokinase versus 1.60 g of residue per 100 g); the lowered content of histidine (2.22 g of residue per 100 g versus 5.65); and the very much lowered content of methionine (1.38 g of residue per 100 g versus 3.08) and half-cystine (0.56 g/100 g versus 5.65) of proteins of these analyses (see above, “Experimental Procedure”) is too large to allow a distinction between these two values, or that derived from the physical data for the subunit size (namely (102,000 /6) = 17,000).
buffering region in the vicinity of the imidazole ionization to be
and (lysine + histidine + arginine) is five basic groups in nucleo-
side diphosphokinase versus 7 in myokinase (and 16 per subunit
studies presented here (see above).
assumed unusual importance in the sedimentation equilibrium
property, that of its partial
ases (Noltmann et al. (22) ; Mahowald et al. (40)) ; and, in addi-
tions had been calculated for the other two ATP-transphosphory-
for NTP-NDP transphosphorylase from the amino acid data,
physical studies), to reconstruct the theoretical titration curve
calculations which may be made for still another physicochemical
germane to this discussion (as well as the prior discussion, under
slightly alkaline isoionic point for the nucleoside diphosphokinase,
and one would predict a
and similar to the myokinase, the absence of the strong
buffering region in the vicinity of the imidazole ionization to be
found in the ATP-creatine transphosphorylase. It is therefore
germane to this discussion (as well as the prior discussion, under
physical studies), to reconstruct the theoretical titration curve
for NTP-NDP transphosphorylase from the amino acid data,
as had been calculated for the other two ATP-transphosphoryl-
ases (Noltmann et al. (22); Mahowald et al. (40)); and, in addi-
tion, to present from these amino acid data, the theoretical cal-
culations which may be made for still another physicochemical
property, that of its partial specific volume, a value which has
assumed unusual importance in the sedimentation equilibrium
studies presented here (see above).
In Table III, therefore, the partial specific volume of the
NTP-NDP transphosphorylase is calculated from the weight
percentages of the amino acid residues. The value of 0.741 cm³
per g may be compared with the measured values of \( \bar{\varepsilon}_{\text{app}} \approx 0.736 \) (for \( e_2 = 1.8\% \)), and \( \bar{\varepsilon}_{\text{app,pr}} = 0.761 \) (for \( e_3 = 1.8\% \)), both measured values not having been extrapolated to zero protein
concentration. It is curious that the value for the theoretical partial specific volume calculated from the amino acid composition
is identical to that calculated for myokinase (Mahowald et al. (40)) and is only slightly higher than the value calculated
for the rabbit muscle ATP-creatine transphosphorylase, i.e. 0.735 cm³ per g (Noltmann et al. (22)).

A theoretical titration curve is shown in Fig. 5, for a total of
the 300 ionizable groups to be found in the assumed hexameric
protein molecule (or 50 charged groups per subunit). Calcula-
tions had been made with the assumption that there is no electro-
static interaction between ionizable groups and that each
member of each species is identical; moreover, it was assumed
that in the hexameric molecule association of subunits did not
alter the intrinsic pK values selected. For this hypothetical
case, as may be seen in Fig. 5, a theoretical isoionic point of 8.80
is obtained. This value is in surprisingly good agreement with
the measured pI, extrapolated to zero ionic strength, namely
8.0 (Yue et al. (2)). Thus, one may conclude that any electro-
static influences during free boundary electrophoretic measure-
The 10 amide groups have been distributed according to the ratio found for glutamic acid to aspartic acid to give 6 glutamine and 4 asparagine residues. Values for $\bar{r}$ for the amino acid residues were taken from Cohn and Edsall (70).

<table>
<thead>
<tr>
<th>Amino acid residue</th>
<th>No. of residues per 17,294 g protein</th>
<th>No. of residues for hexameric protein of 103,764 g</th>
<th>$\bar{r}$/100 g protein</th>
<th>$\bar{r}$</th>
<th>$\bar{r}$ x wt %</th>
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<tbody>
<tr>
<td>Aspartic acid</td>
<td>9</td>
<td>54</td>
<td>6.02</td>
<td>0.60</td>
<td>3.61</td>
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<tr>
<td>Asparagine</td>
<td>6</td>
<td>24</td>
<td>2.67</td>
<td>0.02</td>
<td>1.06</td>
</tr>
<tr>
<td>Threonine</td>
<td>6</td>
<td>36</td>
<td>3.54</td>
<td>0.70</td>
<td>2.48</td>
</tr>
<tr>
<td>Serine</td>
<td>12</td>
<td>78</td>
<td>6.61</td>
<td>0.62</td>
<td>4.16</td>
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<tr>
<td>Glutamic acid</td>
<td>11</td>
<td>66</td>
<td>8.33</td>
<td>0.66</td>
<td>5.50</td>
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<tr>
<td>Glutamine</td>
<td>6</td>
<td>30</td>
<td>4.55</td>
<td>0.67</td>
<td>3.05</td>
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<tr>
<td>Proline</td>
<td>9</td>
<td>30</td>
<td>3.46</td>
<td>0.76</td>
<td>2.63</td>
</tr>
<tr>
<td>Glycine</td>
<td>13</td>
<td>78</td>
<td>4.35</td>
<td>0.64</td>
<td>2.70</td>
</tr>
<tr>
<td>Alanine</td>
<td>10</td>
<td>60</td>
<td>4.26</td>
<td>0.74</td>
<td>3.15</td>
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<tr>
<td>Valine</td>
<td>13</td>
<td>78</td>
<td>7.35</td>
<td>0.86</td>
<td>6.32</td>
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<tr>
<td>Methionine</td>
<td>2</td>
<td>12</td>
<td>1.38</td>
<td>0.75</td>
<td>1.04</td>
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<tr>
<td>Isoleucine</td>
<td>9</td>
<td>54</td>
<td>5.94</td>
<td>0.90</td>
<td>5.35</td>
</tr>
<tr>
<td>Leucine</td>
<td>12</td>
<td>72</td>
<td>8.12</td>
<td>0.90</td>
<td>7.31</td>
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<tr>
<td>Tyrosine</td>
<td>3</td>
<td>18</td>
<td>2.82</td>
<td>0.71</td>
<td>2.00</td>
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<tr>
<td>Phenylalanine</td>
<td>7</td>
<td>42</td>
<td>5.77</td>
<td>0.77</td>
<td>4.44</td>
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<tr>
<td>Lysine</td>
<td>14</td>
<td>84</td>
<td>10.14</td>
<td>0.82</td>
<td>8.31</td>
</tr>
<tr>
<td>Histidine</td>
<td>3</td>
<td>18</td>
<td>2.22</td>
<td>0.67</td>
<td>1.49</td>
</tr>
<tr>
<td>Arginine</td>
<td>8</td>
<td>48</td>
<td>7.32</td>
<td>0.70</td>
<td>5.12</td>
</tr>
<tr>
<td>Tryptophan</td>
<td>5</td>
<td>30</td>
<td>4.79</td>
<td>0.74</td>
<td>3.54</td>
</tr>
<tr>
<td>Cysteine</td>
<td>1</td>
<td>6</td>
<td>0.56</td>
<td>0.61</td>
<td>0.34</td>
</tr>
<tr>
<td>Total</td>
<td>155</td>
<td>930</td>
<td>100.21</td>
<td></td>
<td>74.2909*</td>
</tr>
</tbody>
</table>

* The partial specific volume of nucleoside diphosphokinase was calculated as follows: $\bar{r} = 74.2909/100.21 = 0.7414 \text{cm}^3/\text{g} \approx 0.741$.

At the microlevel employed for these analyses, the results are very satisfactory and point to 1.0 COOH-terminal glycine per 17,000 g of protein, in confirmation of the assigned subunit size, and in support of the conclusion that all subunits are chemically similar.

**NH$_2$-terminal Group Analysis**—Analysis failed to reveal any NH$_2$-terminal amino acid in significant amounts, by either reaction with 2,4-dinitrofluorobenzene or by the Edman reaction with phenylisothiocyanate (followed by alkaline conversion of any resulting phenylthiohydantoin to its amino acid). Therefore, similar to the rabbit muscle ATP-creatine transphosphorylase (22) and to myokinase (40), the yeast nucleoside diphosphokinase does not appear to possess any free NH$_2$-terminal group.

Cysteine and Total Half-cystine Analyses—Analyses are summarized in Table V. As may be seen, by S-carboxymethylation with iodoacetate in 8 M urea after reduction with β-mercaptoethanol, or after performic acid oxidation, a total of only 1 residue of half-cystine may be measured for 17,200 g of protein. Moreover, after reaction with DTNB, in approximately 3.4 M guanidinium chloride, approximately 0.9 to 1 sulfhydryl group per 17,200 g may be observed; whereas, in the absence of a disrupting agent, almost no —SH groups (about 0.02 mole per mole) are exposed for reaction, even with a large excess of DTNB. Thus, the enzyme contains a shielded or unreactive —SH group toward DTNB in the native state, but this single —SH group is readily exposed for reaction in guanidinium chloride. Moreover, there do not appear to be any disulfides present, either as inter- or intramolecular disulfides; and this observation, therefore, confirms the sedimentation equilibrium studies conducted with and without β-mercaptoethanol, in 4 M guanidinium chloride. The single —SH group per subunit seems somewhat unique to the ATP-creatine phosphorylase; moreover, its shielded nature in the native enzymic structure is of definite interest. By way of contrast, the rabbit muscle myokinase contains a total of two reactive —SH groups per mole (75), and the calf muscle and calf brain ATP-creatine transphosphorylases each contain one reactive —SH group per subunit plus an additional three and four —SH groups, respectively, per polypeptide chain which appear to be shielded or unreactive to DTNB (Okabe et al. (49)).

It was of interest, therefore, to determine the concentration range of this disruptive agent, guanidinium chloride, which was required to “unfold” and to expose the single —SH for reaction with DTNB; moreover, and coincident with these studies, the first preliminary and exploratory studies were conducted on the relationship, if any, between this single sulfhydryl group and the enzymatic activity. These data are summarized in Fig. 6. It is evident that a progressive exposure of the —SH group to reaction with DTNB occurs with increasing guanidinium chloride concentrations, in what appears to be a sigmoidal fashion. A concentration of guanidinium chloride equal to 0.7 to 0.8 M is required to expose for reaction with DTNB approximately 50% of the sulfhydryl group. Presumably, this concentration of guanidinium chloride would also be that required for 50% dissociation, with or without unfolding, if intermolecular interactions were the cause of the shielded nature of this —SH group, but this will require confirmation by sedimentation analysis, and will be an interesting topic for further studies. The enzyme shows unusual resistance to the irreversible denaturing effects of guanidinium hydrochloride, since it retains more than 90% of its enzymatic activity even after exposure for almost 1 1/2 hours at 30° in 0.8 to 1.0 M guanidinium chloride. Between 0 to about 0.9 M guanidinium chloride, in the presence of initially approximately a 100-fold excess of DTNB, the loss in activity very roughly parallels the extent of reaction of DTNB with the single —SH group; but beyond this point, slight precipitation and

![Graph showing the relationship between guanidine - HCl (Molarity) and enzyme activity](http://www.jbc.org/

**Table V**

<table>
<thead>
<tr>
<th>Method</th>
<th>Sulfhydryl groups per 17,200 g of protein</th>
<th>Total half-cystine</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reaction with DTNB (0.797 × 10⁻³ M) in 0.05 M Tris (acetate), 0.005 M EDTA, pH 7.9 at 30°</td>
<td>0.02</td>
<td>mole/subunit</td>
</tr>
<tr>
<td>Reaction with DTNB (0.793 × 10⁻³ M) in 3.36 M guanidine HCl, in 0.05 M Tris (acetate), 0.005 M EDTA, pH 7.9 at 30°, DTNB₂⋅P₄ ≅ 85:1</td>
<td>0.91*</td>
<td>mole/subunit</td>
</tr>
<tr>
<td>Reaction with DTNB (0.793 × 10⁻³ M), as above, but in 1.38 M guanidine HCl</td>
<td>0.97</td>
<td>mole/subunit</td>
</tr>
<tr>
<td>Conversion of iodoacetate to S-carboxymethylcysteine (in 8 M urea, after reduction with 0.175 M β-mercaptoethanol)</td>
<td>1.12</td>
<td>mole/subunit</td>
</tr>
<tr>
<td>Cysteic acid, measured after performic acid oxidation</td>
<td>0.95</td>
<td>mole/subunit</td>
</tr>
</tbody>
</table>

* Taken at the maximum 0.142 reached within 5 min after addition of enzyme to reaction mixture. Thereafter, a small decline was observed similar to that observed in the studies on the calf brain and muscle ATP-creatine transphosphorylase by Okabe et al. (40). In 1.38 M guanidine HCl, the decline in A₄₁₂ is less noticeable, but still evident.
Fig. 7. Tryptic peptide map of S-carboxymethylated nucleoside triphosphate-nucleoside diphosphate transphosphorylase on thin layer sheets (20 X 20 cm) of cellulose (see "Experimental Procedure"). Vertical direction: ascending chromatography at 25° with isoamyl alcohol-ethyl-pyridine-water-acetic acid (70:20:70:60:5) for 4 to 5 hours; horizontal direction: electrophoresis at 2000 volts for 40 min, at 20-15°, in pyridine-acetic acid (pH 6.5). The map represents a composite drawing of several runs, conducted on a microscale, of tryptic digests. DNP-leucine was added as a marker for the chromatography on either side of the "map." The physical and chemical evidence presented here, together with those data obtained earlier in this laboratory (Yue et al. (2)) on the native structure are now combined and summarized in Table VII (for those data which may be conveniently tabulated here, together with those data obtained earlier in this laboratory (Yue et al. (2)) on the native structure are now combined and summarized in Table VII). The results obtained were in such low yield as to be too faint to be detected by ninhydrin, but were detected by the other reagents.

<table>
<thead>
<tr>
<th>Amino acid residue</th>
<th>Specific reagent</th>
<th>Peptides found</th>
<th>No. of peptides expected for six identical subunits of 17,000 g (see Table II)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lysine + arginine + COOH terminus</td>
<td>Ninhydrin</td>
<td>23^a</td>
<td>21-24 + 1^c = 23</td>
</tr>
<tr>
<td>Arginine</td>
<td>Sakaguchi</td>
<td>12</td>
<td>8-13</td>
</tr>
<tr>
<td>Histidine + tyrosine</td>
<td>Pauly</td>
<td>6</td>
<td>6-8</td>
</tr>
<tr>
<td>Tryptophan</td>
<td>Ehrlich</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>Methionine + S-carboxymethylcysteine</td>
<td>Platinic iodine</td>
<td>3</td>
<td>3</td>
</tr>
</tbody>
</table>

* The number of peptides in the summary represent the most reliable results from several separate maps, run under the same conditions.

† The total of 23 ninhydrin spots includes two peptides present in such very low yield that they were too faint to be detected by ninhydrin, but were detected by the other reagents.

‡ Includes the 14 lysine-containing plus 8 arginine-containing peptides, and the single COOH-terminal peptide ending in glycine.

in glycine) expected for a single polypeptide chain of 102,000 g, may be found on this composite peptide map; furthermore, if all six subunit polypeptide chains of approximately 17,000 differed significantly, the results also are much less than the theoretical expected number of 132 + 6 COOH-terminal glycine peptides. In fact, only about one-sixth of this number, or approximately 23 peptides, are obtained, with two peptides apparently in very low yield. The results of Table VI would support the contention that each of the six subunit polypeptides is likely identical; thus, of the three S-carboxymethylcysteine- plus methionine-containing peptides, of the six histidine- plus tyrosine-containing peptides, and of the eight arginine-containing peptides to be expected for six identical subunits of 17,300 g, close to the theoretical number of areas was found, namely 3, 5, 6 to 8, and 8 to 13, respectively. The possibility of traces of undigested material make it important to quote these values as ranges; with the most likely values, in terms of yields, given in Column 3 of Table VI. From the results of a single experiment with radioactive [14C]iodoacetate, it seems that Area 5 in Fig. 7, contains the S-carboxymethylcysteine-peptide, and efforts to deduce its sequence are to be anticipated. Therefore, these analyses of the tryptic peptides would point heavily to an identity of the six polypeptide chains. However, only a complete determination of the amino acid sequence of all the isolated tryptic peptides will establish this point. There is, of course, the inherent danger in such preliminary and qualitative analyses that the six chains may be very similar, but differ only slightly in the sequence of a few amino acid residues; and therefore, only an evaluation of the total amino acid sequence of the protein will ultimately establish the identity or nonidentity of these six polypeptide chains.

**OVER-ALL DISCUSSION AND SUMMARY**

The physical and chemical evidence presented here, together with those data obtained earlier in this laboratory (Yue et al. (2)) on the native structure are now combined and summarized in Table VII (for those data which may be conveniently tabu-
lated). The combined evidence shows that the native, compact, and ordered enzyme molecule contains six polypeptide moieties, each of which appears to have a COOH-terminal glycine. Moreover, these six polypeptide components do not appear to be held together, within the ordered structure of the native molecule, by any covalent linkages; interpolypeptide disulfide linkages have been excluded. The crystalline brewers' yeast nucleoside diphosphokinase is even more remarkable than the several other ATP-transphosphorylases studied in the laboratory, in that, even at infinite dilution, the enzyme would appear to retain its hexameric structure; in that, it possesses a single and shielded sulfhydryl group per subunit polypeptide size of 17,300 (±500) (a value taken from the amino acid analysis and which would appear to be the most reliable estimate of its subunit size); in that, it contains a comparatively high content of tryptophan, and possibly as a result of this, unusually large and which would appear to be the most reliable estimate of its molecular weight by sedimentation equilibration. Furthermore, the information presented here will now lay a good foundation for studies on the primary sequence of this protein, a problem which is amenable to modern methods of sequencing and a problem which is now greatly simplified by the fact that the brewers' yeast NTP-NDP transphosphorylase protein molecule consists of six noncovalently linked polypeptide chains of similar molecular weight, 17,300 (±500). This value for the subunit molecular weight places it amongst the smallest in size of all the ATP-transphosphorylases currently being studied in this laboratory (e.g. Yue (38), Yue et al. (78), Mahowald et al. (40), Keutel et al. (79)); and the fact that it may be crystallized in relatively large paralleloiped (1) may make it amenable to study by x-ray crystallography.

### Acknowledgements
The authors wish to thank Anheuser-Busch, Inc. (Dr. R. Seeley and Mr. M. Dackshell), for generous quantities of their dried brewers' yeast. Also, they wish to thank Dr. D. Brodie of Sigma Chemical Co. for his kind assistance in the large scale, low temperature drying of one lot of this brewers' yeast. The technical assistance of the following individuals in the preparations is gratefully acknowledged: Mr. C. Richardson, P. Parham, S. Hofmeister, G. Fleming, and Dr. G. K. Chua.

### REFERENCES

### TABLE VII

#### A. Kinetic unit (native enzyme)

<table>
<thead>
<tr>
<th>Property</th>
<th>Value</th>
</tr>
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<tbody>
<tr>
<td>$s_{20,w}$ sedimentation coefficient ($c</td>
<td></td>
</tr>
</tbody>
</table><p>ightarrow o$, pH 7.9) | $5.82 	imes 10^{-12}$ s |
| $s_{20,w}$ sedimentation coefficient ($cightarrow o$, pH 6.8) | $5.58 	imes 10^{-12}$ s |
| $s_{20,w}$ sedimentation coefficient ($cightarrow o$, pH 5.6) | $5.67 	imes 10^{-12}$ s |
| $D_{20,w}$ diffusion coefficient ($cightarrow o$, pH 7.9) | $6.69 	imes 10^{-15}$ cm$^2$/s |
| $D_{20,w}$ diffusion coefficient ($cightarrow o$, pH 5.6) | $5.63 	imes 10^{-13}$ cm$^2$/s |
| $M_{s}$, molecular weight by sedimentation and diffusion ($cightarrow o$, pH 5.6, and at pH 7.9) | 102,000 ± 2,000 |
| $M_{s}$, weight-average molecular weight by sedimentation equilibrium ($cightarrow o$, pH 5.9, 3°C) | 100,000 ± 4,000 |
| $M_{w}$: $M_{s}$ (5.0 mg/ml, pH 5.9) | 1.03 |
| $M_{w}$, weight-average molecular weight (0.22 mg/ml, pH 7.8, 3°C) | 102,700 |
| $M_{s}$, weight-average molecular weight (0.22 mg/ml, pH 7.8, 20°C) | 108,200 |
| $\delta$, partial specific volume from amino acid composition | 0.741 cm$^3$/g |
| $\delta_{p}$, apparent partial specific volume | 0.786 cm$^3$/g |
| $\delta_{m}$, apparent partial specific volume | 0.761 cm$^3$/g |
| $f/f_0$, molar frictional ratio | 1.19 (a:b = 4.2 for anhydrous protein) |</p>
| $f/f_0$, molar frictional ratio | 5.0 × 10^{-5} mole-
| $f/f_0$, molar frictional ratio | ml/g$^2$ |
| $f/f_0$, molar frictional ratio | 1.93 × 10^{-2} (g/100
| $f/f_0$, molar frictional ratio | ml$^{-1}$ |
| $f/f_0$, molar frictional ratio | 6.66 |
| $\Delta c$, refractive index increment (546 | 8.0 |
| $\Delta c$, refractive index increment (546 | 8.8 |

#### B. Polypeptide chains (noncovalently linked)

<table>
<thead>
<tr>
<th>Property</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>$M_{w}$, weight-average molecular weight (4 M guanidine HCl-0.001 M EDTA-0.1 M RSH), pH 6.5, 0.25 mg/ml, 20°C</td>
<td>17,300 ($\delta = 0.741$)</td>
</tr>
<tr>
<td>$M_{w}$, weight-average molecular weight (4 M guanidine HCl-0.01 M EDTA-0.1 M RSH), pH 6.5, 0.22 mg/ml, 20°C</td>
<td>19,000 ($\delta = 0.741$)</td>
</tr>
<tr>
<td>$M_{s}$, weight-average molecular weight (4 M guanidine HCl-0.01 M EDTA-0.1 M RSH), pH 6.5, 0.22 mg/ml, 20°C</td>
<td>17,862 ($\delta = 0.741$)</td>
</tr>
<tr>
<td>$M_{s}$, weight-average molecular weight (4 M guanidine HCl-0.01 M EDTA-0.1 M RSH), pH 6.5, 0.22 mg/ml, 20°C</td>
<td>17,020 (±1000)</td>
</tr>
<tr>
<td>Molecular weight by gel filtration through S-agarose (4 M guanidine HCl-0.01 M EDTA-0.1 M RSH), pH 6.5, 0.22 mg/ml, 20°C</td>
<td>$M_{s}$, weight-average molecular weight (4 M guanidine HCl-0.01 M EDTA-0.1 M RSH), pH 6.5, 0.22 mg/ml, 20°C</td>
</tr>
<tr>
<td>Molecular weight by dodecyl sulfate polyacrylamide gel electrophoresis</td>
<td>17,000 (±2,000)</td>
</tr>
<tr>
<td>Molecular weight by dodecyl sulfate polyacrylamide gel electrophoresis</td>
<td>17,201 (±4.77)</td>
</tr>
<tr>
<td>Molecular weight by dodecyl sulfate polyacrylamide gel electrophoresis</td>
<td>Approximates theoretical number per 17,300 g</td>
</tr>
<tr>
<td>Molecular weight by dodecyl sulfate polyacrylamide gel electrophoresis</td>
<td>1.12/17,200 g</td>
</tr>
<tr>
<td>Molecular weight by dodecyl sulfate polyacrylamide gel electrophoresis</td>
<td>0.97/17,200 g</td>
</tr>
</tbody>
</table>

* RSH, 2-mercaptoethanol.

### TABLE VII—Continued

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