Effects of Specific Divalent Cations on Some Physical and Chemical Properties of Glutamine Synthetase from *Escherichia coli*. Taut and Relaxed Enzyme Forms*

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ABSTRACT: Some chemical and physical properties of taut (native) and relaxed (divalent cation-free) preparations of glutamine synthetase from *Escherichia coli* have been determined. At pH 7, the molecular weight of either the taut or relaxed enzyme is about 592,000. The taut form behaves as a compact, nearly spherical particle in solution, whereas the relaxed form appears to have a less compact and somewhat asymmetric structure. This is shown by the lower sedimentation coefficient, the higher viscosity, and the sometimes higher apparent specific volume of the relaxed (manganous ion-free) preparations. In addition, the removal of Mn^{2+} from native glutamine synthetase by EDTA and gel filtration treatments produces a more labile species, which then is susceptible to reaction with and inactivation by sulfhydryl reagents and also to disaggregation by the action of mild denaturants or alkaline pH at low ionic strength. Difference spectra between the taut and relaxed enzyme preparations at pH 7 show that during the relaxation process approximately 12–24 residues of both tyrosine and tryptophan are transferred from a nonpolar to a more polar environment. The exposure of aromatic residues is reversed by the addition of those divalent cations (Mg^{2+}, Mn^{2+}, or Ca^{2+}) which reactivate the inactive relaxed enzyme. Other cations (Ba^{2+}, Co^{2+}, Sr^{2+}, Ni^{2+}, or spermidine) are ineffective. The kinetics of the spectral change that occurs upon addition of Mn^{2+} to relaxed enzyme preparations indicate that the tyrosine and tryptophan perturbations are associated with a slow conformational change. This results in a tightened enzyme form that appears to differ from the taut glutamine synthetase only in its tendency to crystallize out of solutions of low ionic strength. Three different preparations of glutamine synthetase isolated with varying average amounts of 5′-adenylic acid covalently bound to the enzyme have been studied. Of the different properties examined, the only one that differed significantly from one preparation of taut enzyme to another was the apparent specific volume. However, heterogeneity with respect to the molecular distribution of the covalently bound adenosine monophosphate on the enzyme appears to mask slight differences in the hydrodynamic properties of the taut forms.

Essentially no differences were detected between relaxed inactive preparations of different states of adenylylation. These findings suggest that the dramatic differences between the different enzyme preparations in their responses to feedback inhibitors and other catalytic properties are effected by only subtle structural changes in the enzymes induced by the covalent attachment of 5′-adenylyl groups.

Previous investigations have demonstrated that the glutamine synthetase isolated from *Escherichia coli* exists as a dodecameric aggregate of identical subunits (Woolfolk *et al.*, 1966; Shapiro *et al.*, 1967b) which are shown by electron microscopy to be arranged in two superimposed hexagonal layers (see Valentine *et al.*, 1968). As isolated, the enzyme contains variable amounts of Mn^{2+} associated with it (Woolfolk *et al.*, 1966; Shapiro and Stadtman, 1967). Such an enzyme preparation is fully active and is resistant to mild denaturants (1.0 M urea at pH 8.0; Woolfolk and Stadtman, 1967) and the sulfhydryl groups do not react with alkylating agents or with organic mercurials (Shapiro and Stadtman, 1967). This form of the enzyme is referred to as *taut* enzyme. Removal of divalent cations by treatment with 0.01 M EDTA converts the enzyme into a *relaxed* form which is catalytically inactive (see Kingdon *et al.*, 1968) and is susceptible to disaggregation by the action of alkaline pH or mild denaturants (Woolfolk and Stadtman, 1967). In addition, the sulfhydryl groups of the relaxed form react readily with iodoacetate or organic mercurials, with attendant disaggregation of the subunits (Shapiro and Stadtman, 1967). When divalent cations are added back to the relaxed enzyme, it is converted into a *tightened* form that is indistinguishable from the original, taut enzyme on the basis of its catalytic properties and resistance to denaturants or to reaction with sulfhydryl group reagents. However, under certain conditions the tightened form does differ from the taut form in being much less soluble in dilute buffer solution (Valentine *et al.*, 1968).

The present paper deals with the nature of the structural alterations undergone by the enzyme during relaxation (removal of Mn^{2+}) and tightening (addition of divalent cations). Further evidence for the lability of glutamine synthetase in the relaxed state also is pre-

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sented. An accompanying paper (Kingdon *et al.*, 1968) is concerned with catalytic effects of the relaxation process. The accumulated evidence indicates that the taut enzyme form is a catalytically active, fairly symmetric, globular protein with buried sulfhydryl and aromatic residues. Previous results had indicated that glutamine synthetase had a molecular weight of 680,000 with probably identical subunits of 48,500 molecular weight (Woolfolk *et al.*, 1966). The molecular weight of the native protein reported here is somewhat lower (592,000). Upon relaxation at pH 7, the enzyme is converted into a catalytically inactive species of the same size, that is rather more asymmetric than the taut form. Sulfhydryl, tryptophanyl, and tyrosyl residues are exposed in the relaxed state. The exposed aromatic amino acid residues are "reburied" by the addition of specific divalent cations with the same kinetics and specificity as that found for the reactivation of the relaxed enzyme by specific divalent cations (Kingdon *et al.*, 1968).

While studies on relaxed and taut forms of glutamine synthetase were being completed, another variation between different preparations of glutamine synthetase was discovered. It was found that purified enzyme preparations had varying amounts of covalently bound 5′-adenylic acid (Shapiro *et al.*, 1967a) depending on the conditions of growth of *E. coli* (Kingdon and Stadtman, 1967), and that the protein-bound AMP led to dramatic alterations in the divalent ion requirement for activity and the nature of the response to feedback effectors (Kingdon *et al.*, 1967). The present paper contains physical and chemical measurements on relaxed and taut forms of enzyme preparations of different AMP content and suggests that the amount of covalently bound AMP does not interfere with the interconversion of relaxed and taut enzyme forms effected by divalent cation.

Materials and Methods

Glutamine Synthetase Preparations. Glutamine synthetase was prepared from *E. coli* as described by Woolfolk *et al.* (1966). The different enzyme preparations are isolated with varying equivalents of covalently bound AMP to the protein.[1] The enzymes $E_{\overline{9.0}}$ and $E_{\overline{1.2}}$ have been described by Shapiro *et al.* (1967a), the $E_{\overline{1.2}}$ preparation being a generous gift of Dr. Henry S. Kingdon. The preparation $E_{\overline{2.3}}$ was a gift from Dr. M. D. Denton of this laboratory.[2] Of these preparations, only $E_{\overline{9.0}}$ is stored as a crystalline suspension in half-saturated ammonium sulfate; $E_{\overline{1.2}}$ and $E_{\overline{2.3}}$ are ammonium sulfate fractions which show >95% homogeneity by disc gel electrophoresis and ultracentrifugation. Protein concentrations were obtained from absorbancy measurements at 280 mμ, corrected for any turbidity in the solutions by linear extrapolations of A_{340}–A_{310} to 280 mμ, or fourth power extrapolations to 280 mμ of the 340-mμ absorbance. (Absorbancy constants at 280 mμ are given in Table I; see Results.)

The removal of divalent cations from the enzyme preparations was accomplished by treatment of protein solutions at low ionic strength and at pH 7 with a tenfold excess of EDTA (see Results), followed by either gel filtration of the solution through Sephadex G-25 equilibrated with 0.05 M imidazole chloride–0.1 M NaCl at pH 7, or exhaustive dialysis against the same buffer containing 0.01 M EDTA.

Glutamine Synthetase Assays. Enzyme activities were measured routinely by the γ-glutamyl transfer reaction or the phosphate biosynthetic assay at 37° (Woolfolk *et al.*, 1966). To measure the initial catalytic rates of relaxed *vs.* taut preparations, ADP produced in the biosynthetic reaction was followed spectrophotometrically in a coupled assay system developed by Kingdon *et al.* (1968). The sodium salt of phosphoenolpyruvate and crystalline suspensions of lactic dehydrogenase and pyruvate kinase from rabbit muscle (essentially free of myokinase activity) were obtained from the Boehringer Mannheim Corp. Crystalline bovine serum albumin (A grade; <0.05% carbohydrate) was supplied by the Pentex Corp. The sodium salts of chemically pure ATP and ADP, PMPS[3] and γ-glutamyl hydroxamate, and also *N*-acetyl-L-tryptophanomide and *N*-acetyl-L-tyrosinamide were obtained from the Sigma Chemical Corp.; the disodium salt of DPNH was supplied by the California Corp. for Biochemical Research.

Deionized water with a conductivity of $\leq 1.7 \times 10^{-6}$ ohm^{-1} was obtained from a water deionizing unit (Model DJ-128) of the Crystal Research Laboratory Inc., and this water was used exclusively for the preparation of samples for the hydrodynamic studies. Solutions of imidazole (Eastman Organic Chemicals) were lightly treated with charcoal and filtered to remove any yellow color before use. Routinely, protein solutions were exhaustively dialyzed over a 2–3-day period, with two to three dialysate changes of imidazole buffers at pH 7.0 containing at least 10^{-3} M Mn^{2+} or Mg^{2+} for stabilization of the enzyme. For the hydrodynamic studies with relaxed preparations, 0.01 M EDTA was subtituted for the divalent cation. (Prolonged dialysis

[1] For simplicity, $E_0, E_{\overline{1}}, E_{\overline{2}}, \ldots E_{\overline{12}}$ refer to different preparations of purified glutamine synthetase (E) with the same amino acid composition, which contain the average (denoted by the bar) amount of covalently bound AMP (designated by the subscript) per molecule of glutamine synthetase of 600,000 molecular weight (Shapiro *et al.*, 1967a).

The extent of adenylylation in each case is determined by phosphate and spectral analyses of the enzyme preparation (Kingdon *et al.*, 1967). Consequently, this reflects the average state of adenylylation of the entire enzyme population since the extent and loci of adenylylation of any one molecule are not known at the present time; *i.e.*, there might exist heterogeneity within a population. The different preparations of glutamine synthetase ($E_{\overline{1.2}}$, $E_{\overline{2.3}}$, and $E_{\overline{9.0}}$) do show quite different kinetic properties, both with respect to metal ion activation and sensitivity to feedback inhibitors.

[2] The description of this enzyme preparation (designated as $E_{\overline{2.3}}$) together with studies on the equilibrium binding of Mn^{2+} to glutamine synthetase will be reported soon (M. D. Denton and A. Ginsburg, manuscript in preparation).

[3] Abbreviations used are that are not listed in *Biochemistry* 5, 1445 (1966), are: SDS, sodium dodecyl sulfate; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); PMPS, *p*-mercuriphenylsulfonate.

against buffers at low ionic strength with $<10^{-3}$ M $MnCl_2$ or $MgCl_2$ and no EDTA results in irreversible activity loss.) After dialysis, protein solutions were clarified by centrifugation before use. Dialyzed enzyme solutions are stable at 4° for several months, but usually were used immediately.

Organic Mercurial Inactivation. Glutamine synthetase (25 μg ± Mn^{2+}) was incubated at 0° (unless otherwise specified) in a mixture of 10^{-4} M PMPS in 0.1 M Tris buffer at pH 7.9–8.0. Aliquots were removed at different times for testing for enzyme activity by the transfer assay.

Sedimentation Studies. The Spinco Model E ultracentrifuge equipped with the RTIC and phase plate was used for all sedimentation experiments. Rayleigh interference optics were used for the equilibrium sedimentation experiments. The meniscus depletion method of Yphantis (1964), using short columns and the six-hole carbon-filled centerpiece designed by Yphantis, was used for the molecular weight determinations at three concentrations of each protein. Perfluorotributylamine (FC-43, Minnesota Mining and Manufacturing Co.) was employed as a base for the liquid columns used in the equilibrium studies (Ginsburg *et al.*, 1956; Yphantis, 1960). Interference patterns were photographed on type II G emulsion spectroscopic plates (Eastman Kodak), and schlieren patterns from sedimentation velocity experiments were recorded on metallographic plates.

Kel-F-coated metal centerpieces were utilized in all sedimentation studies. In general, separate runs at each protein concentration were made. However, in an early single experiment, the wedge window was utilized in order to sediment simultaneously both the taut and relaxed forms of $E_{9.0}$[1] at 2.3-mg/ml concentration. Since a difference of about 1 S in the sedimentation coefficients of these forms was observed, separate sedimentation experiments were made thereafter to decrease the inaccuracy caused by the optical uncertainty of the position of the air-reference hole in a two-cell experiment. Since the largest error in the calculations of the sedimentation coefficients arises from the uncertainty in the temperature, the AnD rotor in water and temperature control unit were calibrated against a thermometer which in turn had been calibrated against a National Bureau of Standards certified thermometer. A Teflon-coated thermistor probe (Yellow Springs Instrument Co., no. 44103) which was bonded into a Teflon stopper (Ginsburg and Carroll, 1965) verified that the RTIC unit correctly gave the temperature of the rotor inside or outside of the chamber at ambient temperature. A Kel-F-coated double-sector cell and a titanium rotor were employed only for the solutions containing urea. The observed sedimentation coefficients are corrected to values corresponding to a solvent with a viscosity and density of water at 20° ($s_{20,w}$). All sedimentation velocity experiments were conducted near 20°, whereas the sedimentation equilibrium experiments were at about 7°.

Density Measurements. All solvent densities were measured at 25 ± 0.002° in 10- or 25-ml pycnometers calibrated to 0.0002 ml in the capillary stem. Values for solvent densities at other than 25° were extrapolated from relative water densities.

Viscosity measurements were made in an Ostwald viscometer with an average shear gradient of 212 sec^{-1} and an outflow time of 55.58 ± 0.03 sec for 1 ml of water at 25.0°. Solution densities used in viscosity calculations were in turn calculated from the measured solvent densities and from the weight fraction and the partial specific volume of the protein.

The Determination of Apparent Specific Volumes. A 20-cm density gradient tube as described by Linderstrøm-Lang and his associates (Linderstrøm-Lang and Lanz, 1938; Hvidt *et al.*, 1954) was used in a 25 ± 0.002° water bath. The apparatus is capable of a sensitivity of one part in 10^{-6} g/ml. The gradient was prepared from two *o*-dichlorobenzene–dodecane mixtures which had been previously saturated with 0.05 M imidazole and 0.10 M NaCl buffer at pH 7, and differed in density by about 0.018.

Absolute densities were obtained by framing each series of drops (1 μl/drop) between standard KCl drops of the same size. The standard KCl solutions were prepared from oven-dried and weighed analytical grade KCl[4] diluted with water to 100 ml in volumetric flasks. Calculations of the density at 25° (ρ^{25}) of each KCl standard based on weights or volumes (taking into account the volume of KCl from density values in the International Critical Tables and the temperature) agreed within at least 0.00001 g/ml using the formula of Harrington and Schellman (1956) corrected to the density at 25°; $\rho^{25} = 0.99881(0.99823 + 0.00640w)$, where w is per cent by weight of the KCl in water. The volume calculation of w was used and w values for the standards ranged from 0.7 to 2.5% KCl. Groups of five to eight 1-μl drops were used for standard and unknown solutions. Equilibrium positions were attained within 1 hr. A Gaertner cathetometer was used for reading the drop positions over a period of 4 hr. Spectrophotometric determinations of solute concentrations (c_2) were made carefully in duplicate using a Cary 15 recording spectrophotometer. All enzyme samples had prior exhaustive dialysis against the described buffer. The appropriate equations are given with the reported values for the apparent specific volumes (ϕ'_2) of the different solutes.

Difference Spectral Measurements. All spectra were recorded in a Cary Model 15 recording spectrophotometer equipped with the 0–0.1 and 0–1 slidewires and thermostated sample and reference compartments. The cell compartments were controlled at 18° by a constant-temperature water circulator for the experiments in which divalent cation was added and the spectral changes followed as a function of time. Matched cuvets (1 ml) were used for all measurements. Wavelength scans were made of identical samples before the addition of any modifier to normalize any slight differences in turbidity between solutions. All enzyme solutions and

[4] The authors are indebted to Dr. William C. Alford of the Microanalytical Laboratory (National Institute of Arthritis and Metabolic Diseases) for providing accurately weighted (±5 μg) samples of dried (15 hr at 110°) KCl.

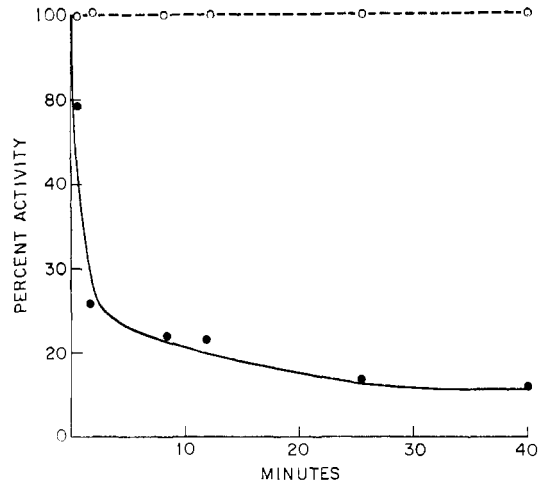

FIGURE 1: Time course of the inactivation of two different preparations of glutamine synthetase by SDS. (O- - -O) Taut enzyme containing 40 moles of Mn^{2+}/mole; (●—●) partially relaxed enzyme containing 2 moles of Mn^{2+}/mole of enzyme. Samples were taken at the times shown from mixtures containing 1% sodium dodecyl sulfate (pH 7.0) in 0.01 M imidazole (20°) and assayed for residual activity by the standard transfer assay (see Methods).

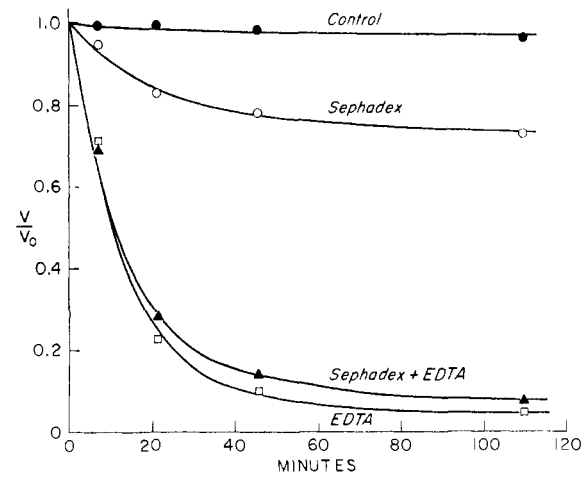

FIGURE 2: Efficiency of different methods of relaxing taut enzyme preparations where final V/V_0 is proportional to the amount of taut enzyme present (V is the activity in a mercurial mixture at the time shown and V_0 is the initial specific activity). Control is taut enzyme; Sephadex is enzyme which has been placed over a Sephadex G-25 column equilibrated with 0.01 M imidazole (pH 7.0) (20°) at a protein concentration of 8.6 mg/ml, and eluted with the same buffer; EDTA is enzyme preincubated in 0.01 M EDTA and 0.01 M imidazole (pH 7.0) (O°) for 3 hr at a concentration of 8.6 mg/ml; EDTA Sephadex is enzyme treated as in EDTA, then passed over Sephadex G-25 as in Sephadex. The samples thus obtained were diluted to a concentration of 20 μg/ml into 0.1 M Tris (pH 8.0) (O°)–10^{-4} M PMPS mixture in ice. Samples were taken at the times shown and assayed for activity in the standard transfer assay.

reagents were filtered through Millipore filters (0.45 μ) before examination. When EDTA was added, cells in a tandem arrangement were used to correct for EDTA absorbance. No similar precaution was needed with the divalent cation additions.

Light Scattering. Light-scattering determinations were made in Brice-Phoenix light-scattering photometer, equipped with a ratio recorder and a thermostated cell compartment as previously described (Shapiro and Stadtman, 1967).

Results

Taut and Relaxed Enzyme Forms. The remarkable protection against the action of denaturants or mercurial inactivation afforded glutamine synthetase molecules by the presence of manganese cations is illustrated in Figures 1 and 2, respectively. The taut and partially relaxed enzyme preparations of Figure 1 were characterized as containing 40 and 2.3 moles of Mn^{2+} bound per mole of glutamine synthetase, respectively, by atomic absorption spectroscopy measurements (Shapiro and Stadtman, 1967). While the taut form is completely resistant to the action of 1% sodium dodecyl sulfate (SDS) with respect to either activity (Figure 1) or sulfhydryl group exposure, treatment of this enzyme preparation with 4 M guanidine-HCl did lead to a complete exposure of the sulfhydryl groups for reaction with DTNB (Shapiro and Stadtman, 1967). The partially relaxed enzyme of Figure 1 was isolated as such from a smaller batch of *E. coli* cells using lower Mn^{2+} concentrations in the purification steps of Woolfolk *et al.* (1966). The inactivation of this enzyme form with 1% SDS is accompanied by disaggregation. In contrast, ultracentrifugation of the taut or active enzyme form in 1% SDS showed that it sedimented as native dodecameric aggregates.

Figure 2 illustrates some different methods of converting the taut into the relaxed enzyme form. The effectiveness of this conversion is conveniently estimated by measuring the inactivation produced by treatment of the enzyme with an excess of PMPS at pH 8 (Shapiro and Stadtman, 1967). Atomic absorption spectroscopy showed quantitatively that preparations which are fully inactivated by the mercurial reagent contained less than 0.5 equiv of Mn^{2+}/molecule of glutamine synthetase in contrast to the 40 equiv of Mn^{2+} found in the control sample (Figure 2). Complete relaxation of glutamine synthetase can be effected by treatment of the taut form with EDTA. Subsequent gel filtration may be used to obtain the relaxed enzyme form free of the EDTA–Mn^{2+} complex. Gel filtration alone is only partially effective in removing the divalent cations, due to the high affinity of glutamine synthetase for Mn^{2+} ions.[2] The addition of divalent cations to the relaxed enzyme form reverses the susceptibility to disaggregation and inactivation illustrated in Figures 1 and 2 (Shapiro and Stadtman, 1967).

The interconversion of taut and relaxed enzyme forms appears to be independent of the AMP content of different purified preparations of glutamine synthetase.[1] The enzymes, $E_{\overline{1.2}}$, $E_{\overline{2.3}}$, or $E_{\overline{9.0}}$, all had PMPS inactivation curves as shown for $E_{\overline{9.0}}$ in Figure 2.

Kinetics of the Relaxation Process. When glutamine synthetase is placed in 0.01 M EDTA at pH 7.0 (0.02–0.05 M imidazole buffer and ≤ 3 mM $MnCl_2$) at 20° the enzyme becomes susceptible to complete inactivation

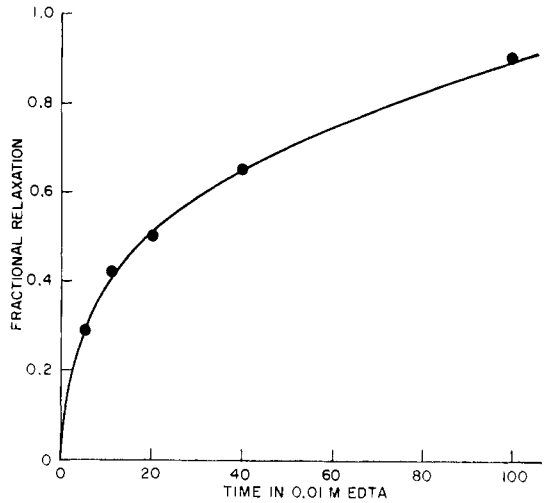

FIGURE 3: Kinetics of relaxation of glutamine synthetase at 0°. Taut enzyme at 7.4 mg/ml was incubated in an iced mixture of 0.01 M imidazole and 0.01 M EDTA (pH 7.2). Samples were taken at the indicated times and placed in the 0.1 M Tris (pH 8)–10^{-4} M PMPS mixture and assayed as described in the legend to Figure 2. Fractional relaxation (see text) as a function of the minutes in 0.01 M EDTA at 0° is shown.

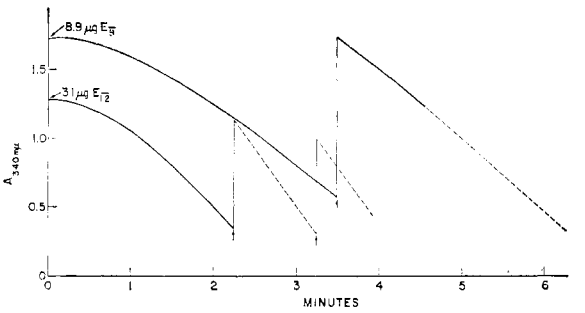

FIGURE 4: The absorbancy change at 340 mμ of assay mixtures at 24° to which two different preparations of relaxed glutamine synthetase ($E_{\overline{1.2}}$ and $E_{\overline{9.0}}$) in 0.01 M EDTA at pH 7 and 0.15 ionic strength were added at zero time. (The preparations E_0 and $E_{\overline{1.2}}$ are enzymes with an average of 9 and 1.2 equiv of covalently bound AMP per 600,000 g of enzyme, respectively.) The 1-ml assay mixtures (pH 7) contained 50 μmoles of imidazole buffer (pH 7), 1 μmole of phosphoenolpyruvate, 50 μmoles of $MgCl_2$, 90 μmoles of KCl, 100 μmoles of sodium glutamate, 50 μmoles of NH_4Cl, 3.75 μmoles of ATP, 100 μg of lactic dehydrogenase, 25 μg of pyruvate kinase, and 0.3–0.5 μmole of DPNH. Any ADP in the ATP (usually 1–3%) was converted into ATP prior to initiating the reaction by the addition of glutamine synthetase. The dashed linear portions represent the attainment of the theoretical rate of the respective taut enzyme forms, and the arrows show sequential DPNH additions. The absorbancy at 340 mμ was corrected to the initial 1-ml volume.

by PMPS after only 2 min. Longer incubation at 20° did not influence the extent of inactivation of either $E_{\overline{1.2}}$ or $E_{\overline{9.0}}$.[1] However, if comparable incubations are made at 0° the relaxation process is sufficiently slow to follow as a function of time. To measure the amount of relaxed enzyme that is present after various periods of time in EDTA at 0°, aliquots of the above incubation mixtures were incubated for 90 min with 10^{-4} M organic mercurial at pH 8.0. This results in nearly complete inactivation of relaxed enzyme (see Figure 2 and Shapiro and Stadtman, 1967). The catalytic activity that remains after 90 min in the mercurial mixture therefore is defined as a measure of the taut enzyme present. Figure 3 illustrates the fractional relaxation (1.00 minus the fractional residual activity) as a function of the time of preincubation of the enzyme in 0.01 M EDTA at 0°. Complete relaxation requires over 100 min to occur, in contrast with the situation at 20°. For this reason, the relaxed glutamine synthetase used in the subsequent experiments was prepared by incubating the protein, which was initially 1–10 mM in $MnCl_2$, at 0° in a fourfold excess (v/v) of 0.01 M EDTA and 0.01–0.05 M imidazole buffer at pH 7 (and at relatively low ionic strength) for a minimum of 3 hr. Each preparation of relaxed enzyme was 90% inactivated in 100 min by incubation with PMPS at 0°. There was no irreversible loss in enzyme activity with protein which had been incubated in 0.01 M EDTA under these conditions for several days. By adjusting the solutions to 0.15–0.2 ionic strength, enzyme integrity in 0.01 M EDTA is maintained for weeks.

Activity of Relaxed and Taut Preparations. As is demonstrated in the accompanying paper (Kingdon *et al.*, 1968), relaxed glutamine synthetase preparations show a lag before achieving full biosynthetic activity in a Mg^{2+} assay system at 20° which measures ADP production in a pyruvate kinase and lactic dehydrogenase coupling system. The lag in the rate of DPNH oxidation is interpreted to mean that the relaxed enzyme is fully inactive, but becomes active in the reaction mixture which contains divalent cations. Indeed, preincubation of relaxed enzyme preparations with Mn^{2+} or Mg^{2+} for about 20 min completely eliminates the lag, the subsequent DPNH oxidation being linear with time (Kingdon *et al.*, 1968). However, as shown in Figure 4, with all components of the forward assay present at saturating concentrations, the full reactivation of the completely relaxed enzyme at 24° (with sequential additions of DPNH) occurs within a few minutes. Note that the time of reactivation is dependent on the potential maximal activity of the reactivated form in the Mg^{2+} forward assay rather than on the total micrograms of relaxed enzyme added. Even though the specific activities of $E_{\overline{1.2}}$ and $E_{\overline{9.0}}$ are quite different, both relaxed forms attain the theoretical velocity of the respective taut forms. The relationship between the time required to achieve full activity and potential maximal specific activity can be related to the finding of Kingdon *et al.* (1967) that enzymes with varying amounts of covalently bound AMP[1] vary proportionately in their divalent cation specificity in the forward assay. The two extreme cases would be E_0 and E_{12}[1] which would have an almost absolute requirement for Mg^{2+} or Mn^{2+}, respectively. Using data from the reactivation of 1–11-μg quantities of different relaxed enzyme preparations, of which those in Figure 4 are representative, it could be calculated that the reactivation of the relaxed enzyme forms is first order with respect to the concentration of nonadenylylated enzyme sites in the active polymer, which are equated to catalytic sites acting in the Mg^{2+} assay. (It is assumed in this calculation that each sub-

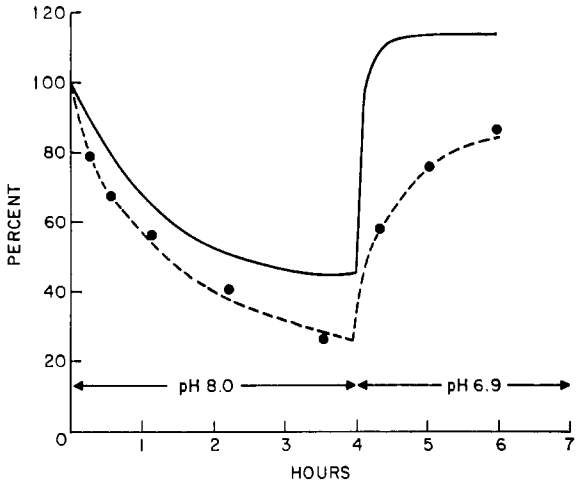

FIGURE 5: Effect of pH on the size and activity of relaxed enzyme. Glutamine synthetase ($E_{\bar{9}}$) at a concentration of 0.685 mg/ml in 2.6 ml of 0.01 M EDTA–0.01 M imidazole at pH 7.1 (0°) was filtered through a Millipore filter (0.45 μ) into a 3.0-ml cuvet kept in a thermostated cell holder at 4° in the light-scattering photometer. There was no change in the activity or light-scattering properties for 2 hr before the experiment was started. At zero time, the pH was adjusted from 7.1 to 8.0 by the addition of 20 μl of 1.0 M Tris base (pH 10.8), and light-scattering (——) and activity (●- - -●) changes were followed as shown. After 4 hr, the pH was adjusted to 6.9 (0°) by the addition of 70 μl of 1.0 M potassium acetate (pH 5.5). Activity was determined on aliquots of the enzyme mixture in the standard transfer assay.

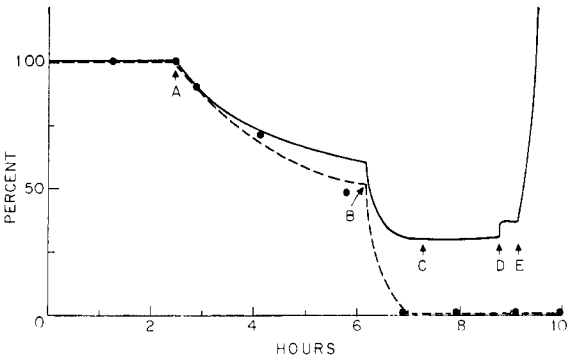

FIGURE 6: Effect of urea and alkaline pH on the activity and size of relaxed glutamine synthetase. Glutamine synthetase ($E_{\bar{0}}$) was relaxed as described in Methods, and the Mn^{2+}–EDTA complex was removed by Sephadex gel filtration. The resultant enzyme preparation was diluted to 0.545 mg/ml into 2.0 ml of 0.01 M imidazole at pH 7.2 (0°), filtered, and placed in the light-scattering apparatus at 4° (see legend to Figure 5). At A, the pH was adjusted to 8.0 by the addition of 20 μl of Tris base (1.0 M), and the decrement in scattered light (——) and activity (●- - -●) were followed as shown. At B, 0.3 ml of 8 M urea was added to the cell and the further decrement of light scattering and activity was followed. At C, the pH was adjusted to 6.9 with 70 μl of 1.0 M potassium acetate (pH 5.5). At D, Mn^{2+} was added to a concentration of 0.45 mM. At E, the Mn^{2+} was increased to 4.8 mM.

unit of the enzyme has an independent catalytic site of which the divalent cation specificity is determined by the presence or absence of bound AMP to the subunit.) On this basis, the effective amounts of $E_{\bar{9}}$ and $E_{\overline{1\cdot2}}$ in Figure 4 correspond to 2.2 and 2.8 μg of Mg^{2+} specific activity (E_0), respectively. The half-time of reactivation of 1 μg of the Mg^{2+} enzyme form (E_0) is 5.6 min, or only 1.9 min for 3 μg of E_0. If reassociation of inactive subunits was involved during the reactivation of the relaxed enzyme, the reaction would be of a higher order than one. Since the reactivation of the *intact* enzyme should be first order in the presence of saturating substrates, the finding that this is so rather supports the assumption leading to this result, *i.e.*, nonadenylylated sites do approximately equal independent catalytic sites specific for Mg^{2+}. Glutamate has some effect on the reactivation process, for a 30-min preincubation of the relaxed enzyme in an assay mixture without glutamate results in approximately a 50% irreversible activity loss. It has been observed previously that ATP destabilizes the relaxed enzyme with respect to organic mercurial inactivation (Shapiro and Stadtman, 1967), and Kingdon *et al.* (1968) find that the reactivation by divalent cations alone is relatively slow.

Although the lag phenomenon is rather pronounced at 24°, at 37° the lag is reversed so rapidly that under the conditions of either the transfer or phosphate biosynthetic assay (see Methods) the specific activities of corresponding taut and relaxed enzyme forms are the same within 1–2%. Consequently, the testing for any losses in enzyme integrity by activity assay is most conveniently performed at 37°, as was the routine practice in the experiments described here; such activity is termed *latent* activity.

Light-Scattering Studies of the Dissociation of the Relaxed Enzymes. Figure 5 demonstrates the effect of alkaline pH on the molecular weight and catalytic activity of a relaxed enzyme preparation. The pH was kept at 7.0 and 0° to allow complete relaxation to occur; after 3 hr the experiment was begun by adjusting the pH to 8.0. The weight-average molecular weight, M_w, of the enzyme decreased and this was accompanied by a loss of latent activity which was somewhat more than would be predicted from the light-scattering changes alone. This could indicate that under these conditions partially disaggregated forms of the enzyme are produced and cannot be reactivated at 37° under the assay conditions. Upon adjustment of the pH to 6.9, the enzyme rapidly regained its aggregated state, with some evidence of superaggregate formation since the scattering ratio became greater than the original. The enzyme activity of this reaggregated form at 37° in assay mixtures increased somewhat more slowly than the changes in light scattering. It is of interest that there was no need to add manganese back to the enzyme preparation to restore latent activity and molecular size, but the disaggregation reaction was reversed merely upon readjusting the pH. In other experiments of this kind, it was found that increasing disaggregation ensued as the pH was increased, but that this was partially offset by the concomitant increases in ionic strength which protects the enzyme from disaggregation. As the pH is raised above 8.5 irreversible changes occur so that a smaller fraction of the catalytic activity was restored upon readjustment of the pH to 7.0. When an experiment similar to that of Figure 5 was performed with 0.1 M NaCl in addition to the other re-

agents, much less disaggregation and loss in activity occurred, indicating that the higher ionic strength protected enzyme integrity. In the ultracentrifuge, these solutions at pH 8.0 and 0.15 ionic strength had 90% of the material sedimenting as the dodecameric aggregate with the other 10% visible near the meniscus as much smaller species.

Woolfolk and Stadtman (1967) have shown that disaggregation of glutamine synthetase was obtained in the presence of alkaline pH, 0.01 M EDTA, and 1.0 M urea. It was not clear from their results whether EDTA and urea behaved in a concerted fashion, or whether EDTA alone would make glutamine synthetase more labile, permitting disaggregation by many mild procedures, among which is 1.0 M urea at pH 8.0. To investigate whether the urea-induced disaggregation would occur with relaxed enzyme in the absence of EDTA, the experiment shown in Figure 6 was performed. Here enzyme was relaxed in 0.01 M EDTA for 3 hr (0°) at pH 7 and was then filtered through Sephadex G-25 to remove the EDTA and metal chelate derivatives. The relaxed enzyme was then placed in a scattering cell where the activity and light-scattering properties were observed for 2.5 hr. At the end of that time, during which there was no change in the measured variables, the pH was adjusted to 8.0 with Tris buffer and the decrement in scatter and latent activity was followed, as in Figure 5. When the decrease in light scatter had reached a plateau, the solution was made 1.0 M in urea and a further decrement in light scattering occurred with a complete loss of latent activity. The observation that adjustment of pH to 8.0 did not cause a decrease in molecular size of similar magnitude to that seen in Figure 5 may reflect the presence of low levels of divalent cations contaminating the buffers used; such cations could protect the enzyme in this experiment in which there was no EDTA[5]. Similarly, the finding that urea did not reduce the molecular weight to a value more in accord with the subunit size (0.083 that of the native enzyme), as was seen by Woolfolk and Stadtman, may also be due to divalent ion contaminants stabilizing higher molecular weight inactive species. Alternatively, the higher ionic strength of these solutions perhaps alone can account for this discrepancy (see below). However, the results do demonstrate that the dissociation of glutamine synthetase does not require a concerted action of EDTA and urea, but rather that the action of urea alone can dissociate the relaxed enzyme form at pH 8.

With urea present, adjustment of the pH to 6.9 did not produce reassociation of the diasggregated protein, nor was there reaggregation when the manganese concentration was increased to as high as 0.45 mM. However, when much higher manganese levels were added (4.8 mM), the enzyme rapidly aggregated, as seen by the enormous increase in scattered light, and soon began to crystallize out of solution, as demonstrated by flow birefringence and phase-contrast microscopy. Macroaggregates of glutamine synthetase were seen by Woolfolk and Stadtman (1967) in their similar reversible disaggregation experiments performed in the presence of EDTA, but the aggregates were not noted to crystallize out of solution. The relaxed form of glutamine synthetase in the absence of EDTA and at low ionic strength and ambient temperatures has been observed to crystallize upon the addition of several divalent cations, and electron micrographs of this phenomenon together with the dissociation of the dodecamer are being presented in an accompanying paper (Valentine *et al.*, 1968). No catalytic activity was restored upon addition of manganese despite aggregation to higher molecular weight states (Figure 6). This is not too surprising in light of the previous finding that reaggregation of glutamine synthetase from its subunits is a delicate phenomenon, leading to transient reactivation at best, and the amount of activity regained is related to both the conditions of disaggregation and reaggregation (Woolfolk and Stadtman, 1967).

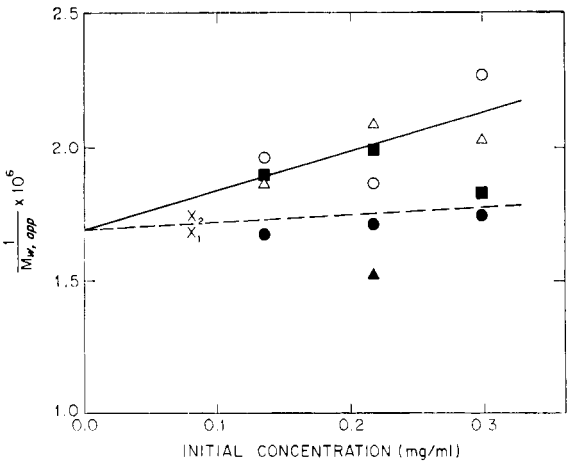

FIGURE 7: Concentration dependence of the reciprocal of apparent weight-average molecular weights, $1/M_{w,app}$, of relaxed (—) and taut (- - -) forms of glutamine synthetase ($E_{\overline{9.0}}$ preparation). The relaxed enzyme form (△, ○) was in 0.05 M imidazole chloride and 0.1 M NaCl (pH 7) after gel filtration through G-25 Sephadex. (△ represent values after equilibrium at 6166 rpm for 29.5 hr; and ○ after a further 20-hr equilibration at 7928 rpm.) The taut enzyme form (●, ■, ▲) was in 0.05 M imidazole chloride, 0.1 M NaCl, and 0.01 M $MnCl_2$ at pH 7 (● and ■ represent values after equilibration at 7928 rpm for 30 and 47 hr, respectively; ▲ is a value obtained after the speed had been reduced to and operated at 6166 rpm for 27 hr), the points X are comparable reciprocal apparent weight-average molecular weights at 6995 rpm for 23 hr from the data of Woolfolk *et al.* (1966) for native (taut) glutamine synthetase for $\phi'_2 = 0.70$ ml/g (X_1) and $\phi'_2 = 0.69$ ml/g (X_2).

Some Hydrodynamic Properties of the Taut and Relaxed Forms of Glutamine Synthetase. Figure 7 shows the results of two equilibrium sedimentation experiments utilizing dilute solutions of the enzyme $E_{\overline{9.0}}$, 0.3-cm columns, and the meniscus depletion method of Yphantis (1964). Fairly linear plots of the log fringe displacement *vs.* the square of the radius from the center of rotation were obtained, although there was slight upward curvature at the ends of the gradient in some cases;

[5] The affinity of glutamine synthetase for Mn^{2+} increases approximately one order of magnitude from pH 7 to 8 (unpublished data of M. D. Denton and A. Ginsburg; see footnote 2).

TABLE I: Apparent or Partial Specific Volumes at 25°.

Glutamine Synthetase Prepn[a]	$A^{0.1\%}_{280\ m\mu}$ (assumed)[b]	c_2 (g/ml × 10^3)	ϕ'_2[c] (ml/g)	$\bar{V}$[d] (ϕ'_2)
Relaxed forms[e]				
$E_{2.3}$	0.734	34.90	0.710	
		26.66	0.711	0.709
		13.35	0.703	
$E_{1.2}$	0.726	14.20	0.714	
		9.46	0.714	0.713
		7.10	0.711	
$E_{9.0}$	0.756	29.98	0.709	
		15.29	0.707	0.707
		7.67	0.697	
Taut forms[f]				
$E_{2.3}$	0.748	41.09	0.707	
		30.56	0.709	0.707
		15.37	0.702	
$E_{1.2}$	0.740	15.17	0.697	
		10.12	0.698	0.698
$E_{9.0}$	0.77	31.01	0.695	
		14.24	0.682	0.690
		7.77	0.685	
Adensoine 5′-monophosphate[g]				
pH 7, sodium salt in buffer[e]		7.19		
		3.47		0.425
		0.72		
pH 5, buffer[h]		3.49		
		1.76		0.445
		1.17		
Bovine serum albumin				
In buffer[e]	0.659[i]	44.80	0.734	
		37.06	0.734	0.734
		29.14	0.734	
In buffer[f]		55.42	0.734	
		44.49	0.735	0.734
		23.24	0.721	

[a] See footnote 1 of this paper. [b] The specific absorbancy at 280 mμ (1 cm) for 0.1% solutions of the different enzyme preparations is based on the dry weight determination of this spectrophotometric constant for taut E_3^5 (Shapiro and Stadtman, 1967) and calculation of the AMP contribution to the absorbance at 280 mμ (Shapiro *et al.*, 1967a). The differences between $A^{0.1\%}_{280\ m\mu}$ of respective taut and relaxed forms is shown in Figure 11. [c] The apparent relative specific volumes, ϕ'_2, are calculated from: $\phi'_2 = 1/\rho_s(1 - \Delta\rho/c_2)$, where ρ_s is the density of the solvent, $\Delta\rho$ is the difference in density between solution and solvent at a concentration of solute c_2 in grams per milliliter (determined from the absorbance at 280 mμ corrected by fourth power extrapolations of the 340-mμ absorption, which is an approximate 5% correction in each case). [d] ϕ'_2, with no concentration dependence, is assumed to be equal to the partial specific volume, $\bar{V}$, *only* if the mass of preferentially bound ions and/or water is assumed to be negligible. The values in this column were calculated from $d\rho/dc_2$ and ρ_s using a least-squares analysis, where $d\rho/dc_2 = (1 - \phi'_2\rho_s)$, since the error of the measurements is reduced at the higher concentrations of solute. [e] Dialysate is 0.05 M imidazole chloride, 0.10 M NaCl, and 0.01 M EDTA (pH 7.15) ($\rho^{25} = 1.00426$). Enzymes were dialyzed simultaneously against three-1000× volumes of the same buffer to make free electrolyte concentrations equivalent. [f] Dialysate is 0.05 M imidazole chloride, 0.10 M NaCl, and 0.01 M $MnCl_2$ (pH 7.09) ($\rho^{25} = 1.00330$). Enzymes dialyzed as in *e* to give equivalent free electrolyte concentrations. [g] The values used were ϵ_m 15.4 × 10^3 M^{-1} cm^{-1} at 259 mμ (Morell and Bock, 1954) with molecular weight values of 369.23 for the sodium salt and 347.23 for ionized AMP. [h] Sodium acetate–KCl buffer at 0.12 ionic strength. [i] Based on the constant $A^{0.1\%}_{1\ cm}$ 0.667 at 279 mμ determined by Foster and Sterman (1956) for the isoionic protein. The absorbancy at 340 mμ (about 1.5% of $A_{280\ m\mu}$) is not used to correct that measured at 280 mμ (personal communication of Dr. J. F. Foster).

weight-average molecular weights were calculated from least-squares analyses of these data.[6] The reciprocal of weight-average molecular weights *vs.* the initial concentrations of the enzyme (before depleting the meniscus) are plotted in Figure 7. The solid line of Figure 7 is a least-squares fit of the data (±7%) for the relaxed enzyme and is described by $1/M_{w,app} = 1.69 \times 10^{-6} + 1.47 \times 10^{-3}c$, where c is the initial concentration in grams per milliliter. The weight-average molecular weight at infinite dilution from this equation is 592,000. There is considerably more scatter in the data for the taut enzyme in Figure 7. It is suspected that some aggregation occurred in these solutions during the longer periods of time at speed since a subsequent reduction in speed gave a higher value for $M_{w,app}$. However, the data could be fitted approximately by $1/M_{w,app} = 1.69 \times 10^{-6} + 0.27 \times 10^{-3}c$ (dashed line of Figure 7). The $M_{w,app}$ values for the taut enzyme even at the earliest time appear to be slightly high, and this could be a consequence of the presence in these solutions of a small percentage of rods seen occasionally by electron microscopy. The molecular weights for $E_{9.0}^{-}$ (Figure 7) were calculated using the values shown in Table I for the apparent specific volumes of the relaxed and taut forms.

A value of 680,000 for $M_{w,app}$, which was obtained by Woolfolk *et al.* (1966) for 0.008% of a different preparation of native glutamine synthetase, was based on a calculated value for $\bar{V}$ of 0.737 ml/g from an early amino acid analysis. This preparation now can be estimated to have contained 5.1 equiv of covalently bound AMP from the published spectra of the enzyme (Woolfolk *et al.*, 1966). Two more reasonable values for ϕ'_2 may be assumed (legend to Figure 7) for correcting the 680,000 value (to M_w = 575,000 or 594,000 with ϕ'_2 = 0.69 or 0.70 ml/g, respectively), based on the measured ϕ'_2 values for the taut $E_{1.2}^{-}$, $E_{2.3}^{-}$, and $E_{9.0}^{-}$, (Table I). The reciprocals of the corrected published value are shown in Figure 7.

Since the protein is concentrated at least threefold over the initial concentration in the equilibrium experiments, the slopes of the lines in Figure 7 correctly should be decreased accordingly. Even so, the apparent concentration dependence of $1/M_{w,app}$ of the relaxed form appears to be too great (see below). However, the data of Figure 7 do indicate that the weight-average molecular weights of the relaxed and taut forms of glutamine synthetase are very nearly the same, and that the relaxed form exhibits a greater concentration dependence in this parameter than does the taut form.

Measured values of the apparent specific volumes, ϕ'_2, for the relaxed and taut forms of some different preparations of glutamine synthetase are given in Table I. Whereas the relaxed enzyme forms all have about the same ϕ'_2, the taut forms of different AMP content do have different ϕ'_2 values. The relative differences between relaxed and taut enzyme forms therefore are not constant. Further, the values of ϕ'_2 for the taut enzymes are not a simple function of the extent of adenylylation.

Even discounting the results on the relative specific volumes of the relaxed enzyme forms, the differences between the native forms cannot be accounted for by averaging in the AMP content[1] of each enzyme preparation with the known amino acid composition (Shapiro *et al.*, 1967a)[2] by the procedure of Cohn and Edsall (1943). Estimates of the thermodynamic $\bar{V}$ for $E_{1.2}^{-}$ and $E_{9.0}^{-}$, from the amino acid analyses (amide group number and distribution approximated) and from the value of ϕ'_2 for AMP at pH 5 (Table I) are 0.726 and 0.725 ml/g, respectively. These values are closer to the ϕ'_2 values measured for the relaxed enzyme forms.

It is known[2] that all of the enzyme preparations have high affinities for Mn^{2+}. Even with 40–100 moles of Mn^{2+} bound/600,000 g of enzyme the calculated change is $\bar{V} \cong 0.002$–0.006, which is much less than ϕ'_2 for $E_{9.0}^{-}$ (taut *vs.* relaxed forms). Thus, the differences in ϕ'_2 of the taut series or taut *vs.* relaxed do not simply reflect the preferential binding of Mn^{2+}—possibly water and the covalently bound AMP are factors in addition to Mn^{+2} binding influencing ϕ'_2. Specific binding of the organic phase of the density column cannot be discounted, but then one would have to speculate that mainly the taut enzyme preparations $E_{2.3}^{-}$ and $E_{1.2}^{-}$ and the relaxed $E_{1.2}^{-}$ and $E_{9.0}^{-}$ enzymes selectively show this property. Whatever the reasons for the different values obtained for the apparent specific volumes, ϕ'_2, of the glutamine synthetase enzyme forms, the differences must reflect some basic structural variations of the proteins in these solvent systems.

Bovine serum albumin was used to test our procedure using the same density gradient column. Measurements of the apparent relative specific volume of bovine serum albumin in the buffers with or without divalent cation (plus EDTA) at pH 7 gave a value of 0.734 ml/g in both cases. This value is the same as that very accurately measured for this protein in water by Dayhoff *et al.* (1952). It is of some interest that pH 7 is an approximate minimum between anion and cation binding by serum albumin (Saroff, 1957; Saroff and Lewis, 1963). Perhaps this is the reason that ϕ'_2 which only includes the selective binding of water in the measurements of Dayhoff *et al.* (1952) is the same as that measured here at pH 7.

The values for the relative specific volumes of the different preparations of glutamine synthetase in Table I are quite dependent upon the specific absorbancy constants assumed in determining the concentration of each protein. For this reason, the latter values have been listed also in Table I. However, this will not influence the relative differences observed in the ϕ'_2 values. In an extrapolation of $A_{280\,m\mu}^{0.1\%}$ of E_9^- (0.77) to E_0 (0.73), the latter value, when further corrected to that of the relaxed form, is in fair agreement with the constant determined by Foster and Sterman (1956) for bovine serum albumin, when the tyrosyl and tryptophanyl residues of the two proteins are normalized (Wetlaufer, 1962).

The concentration dependence of the sedimentation coefficients of the relaxed and taut enzyme forms is shown in Figure 8. The data for the taut enzyme forms

[6] The FORTRAN program (PAS 001C) of Small and Resnick (1965) was used for analysis of the data. The authors are grateful to Mr. George Atta of the Computation and Data Processing Branch, National Institutes of Health, for converting this program from the Minneapolis-Honewell 800 into the IBM 360 computer.

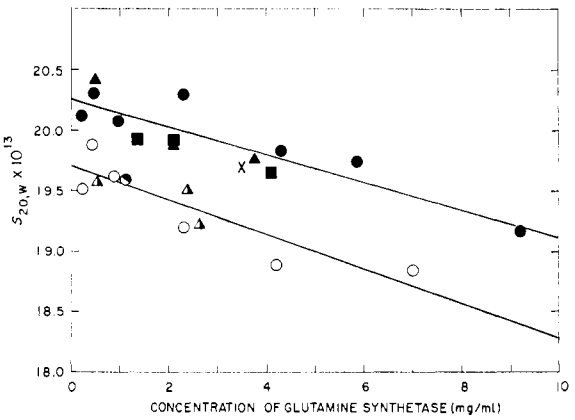

FIGURE 8: The concentration dependence of the sedimentation coefficient of taut (closed symbols) or of relaxed (open or half-open symbols) forms of glutamine synthetase. The taut enzyme forms were in 0.05 M imidazole chloride, 0.1 M NaCl, and 0.01 M $MnCl_2$ (pH 7); the enzyme buffer was 0.05 M imidazole chloride and 0.1 M NaCl (pH 7) for the gel-filtered relaxed forms (open symbols) or the same buffer containing also 0.01 M EDTA at pH 7 for dialyzed relaxed enzyme forms (half-open symbols). The symbol X represents a single experiment with AMP (0.01 M) included in the buffer: 0.02 M imidazole, 0.1 M KCl, and 1 mM $MnCl_2$ (pH 7) of the $E_{\overline{9.0}}$ preparation. The different enzyme preparations have the following symbols: $E_{\overline{9.0}}$ (●, ◑, or ○), $E_{\overline{2.3}}$ (▲, or △), and $E_{1.2}$ (■). Speeds of 42,040 or 59,780 rpm were used.

in 0.01 M $MnCl_2$ at pH 7 could be fitted by a least-squares analysis yielding the equation $s_{20,w}(\pm 0.2) = 20.3(1-0.006c)$, where c is the concentration in milligrams per milliliter. Likewise, the data for the relaxed forms in the absence of divalent cation, with or without EDTA, yielded the equation of $s_{20,w}$ (± 0.2) = $19.7(1-0.007c)$. Thus, the relaxed enzyme has a sedimentation coefficient which is only slightly more concentration dependent than that of the taut enzyme.

The different taut enzyme preparations with varying amounts of covalently bound AMP and particle densities (Table I) had the same sedimentation properties (Figure 8; see Discussion below). Similarly, the different relaxed enzyme forms with EDTA all had the same sedimentation properties, which were also the same as those of the gel-filtered enzyme preparation containing no divalent cations. The addition of AMP (0.01 M) to the native enzyme ($E_{\overline{9}}$) appeared to have no effect on the observed sedimentation coefficient, despite the observations that AMP is a potent inhibitor of the enzyme and binds to the extent of 1 equiv/subunit under these conditions.[7]

The $s^0_{20,w}$ values (Table II), which represent values extrapolated to infinite dilution for the taut *vs.* the relaxed enzyme, differ by 0.6S units. In a single experiment, simultaneous sedimentation of the taut and relaxed forms of $E_{\overline{9.0}}$, at concentrations of 2.3 mg/ml each, yielded Δs = 1.0S which is comparable with that shown by the difference between the curves of Figure 8 at that concentration (see Methods).

The reduced viscosity measurements on the samples used in the sedimentation velocity experiments of Figure 8 are shown in Figure 9. There is some trend in the data of Figure 9 for the taut forms to suggest that the different preparations of native enzyme may have different intrinsic viscosity values. However, there was sufficient scatter in the data to justify fitting all of the values for the taut enzymes by least-square analyses $\eta_{sp}/c = 4.9-0.024c$.

TABLE II: Summary of the Physical and Chemical Properties of the Taut and Relaxed Forms of Glutamine Synthetase.[a]

	Taut Enzyme	Relaxed Enzyme
$s^0_{20,w}$ (S)	20.3	19.7
$[\eta]$ (ml/g)	4.9	6.7 (?)
M^0_w	592,000	592,000
ϕ'_2 (ml/g)	0.690	0.707
Sulfhydryl reactivity	−	+
Tryptophan and tyrosine exposure	−	+
Enzymatic activity[b]	+	−

[a] Preparation $E_{\overline{9.0}}$. [b] In the biosynthetic spectrophotometric assay.

As illustrated in Figure 9, the viscosity of the relaxed enzyme forms showed a striking concentration dependence at the low concentrations of protein. $\eta_{sp}/c = 6.7-0.37c$, where c is the concentration in milligram per milliliter (0–5 mg/ml). The viscosities of these solutions are difficult to measure because visible aggregation can be caused by mechanical shearing. However, all solutions were clarified by centrifugation and appeared clear during the viscosity determinations. Also, the reduced viscosity values were fairly reproducible and include measurements on samples containing 0.01 M EDTA which appear less susceptible to aggregation. This physical behavior of the relaxed enzymes forms is perhaps related to the Ostwald-type capillary viscometer used (see Methods). If fiber-like formation is induced by alignment of the particles along the capillary walls (with no visible aggregation), this only occurs in the case of the relaxed enzyme preparations, and in this case only at the low concentrations.

Since viscosity measurements are quite sensitive to the presence of any aggregates in the protein solutions, the intrinsic viscosity values of Table II are presented with some reservation. However, the difference between the reduced viscosity values for the taut and relaxed forms at moderate concentrations suggest that Mn^{2+} removal does produce a slight increase in the hydrodynamic volume of the particles.

There is no evidence of dissociation of the relaxed or taut enzyme forms in the sedimentation or viscosity studies shown in Figures 8 and 9. In addition, a comparison of the light scattering of the taut and relaxed enzyme forms ($E_{\overline{9}}$) at identical concentrations (at pH 7) showed a scatter ratio of 1.0. This is further support for

[7] A. Ginsburg (manuscript in preparation).

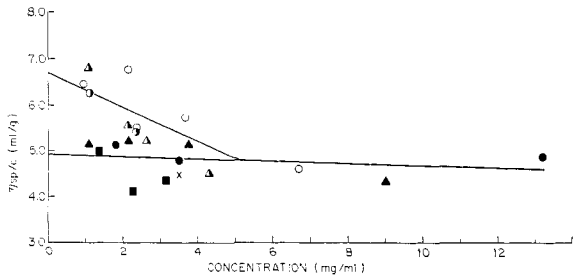

FIGURE 9: The concentration dependence of the reduced viscosity of taut (closed symbols) or of relaxed (open or half-open symbols) forms of glutamine synthetase. The symbols are the same as described in the legend to Figure 8.

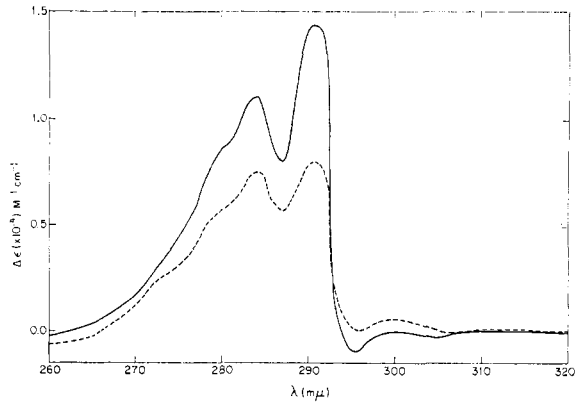

FIGURE 10: Difference spectra between taut (or tightened) and relaxed (solid curve), or between relaxed and partially tightened (dashed curve) glutamine synthetase ($E_{\bar{9}}$). The solid curve represents difference spectra obtained in either of two ways. (a) The base line from 340 to 240 mμ was determined with two solutions of taut enzyme (2.4 mg/ml, 0.8 ml, buffer as in Figures 7–9). Then, 10 μmoles of EDTA (pH 7) was added to the taut enzyme solution in the reference compartment, with the same volume of water added to the enzyme solution in the sample compartment, with the appropriate controls in a tandem arrangement in both compartments (see Methods). The difference spectrum was recorded repeatedly over a 36-min period, with the spectral changes (solid curve) being constant from 2 to 36 min; (b) the base line was determined as in part a with two solutions of relaxed glutamine synthetase (2.4 mg/ml) which had been freed of the EDTA–Mn^{2+} complex by gel filtration through a column of Sephadex G-25 equilibrated with 0.01 M imidazole chloride–0.1 M NaCl (pH 7.05) (see Methods). Then $MnCl_2$ was added to the enzyme solution in the sample compartment to a final concentration of 1.2 mM $MnCl_2$. An equivalent volume of deionized water was added to the enzyme solution in the reference compartment. The spectrum recorded (solid line) is that obtained after 30 min, at which time there was no further spectral change observed (see Figure 11). The dashed difference spectrum was obtained in a manner identical with part b except that the final $MnCl_2$ concentration was only 0.018 mM.

the view that the taut and relaxed forms of glutamine synthetase at pH 7 have the same weight-average molecular weight.

Sedimentation measurements on relaxed glutamine synthetase treated with 1 M urea at pH 8 in the presence of EDTA at 0.25 ionic strength showed that high salt concentrations protected the enzyme against disaggregation (see above). Under these conditions it was estimated that only about 10% disaggregation to the monomer state occurred. Instead, appreciable amounts of 31S and 21S material (about 3:2) were present.

There appears to be some small shape and/or volume change induced by the removal of divalent cations. The sedimentation studies on the $E_{\overline{2,3}}$ preparation give the most reliable data for this shape change (see Discussion below), since this analysis is seemingly less complicated by heterogeneity in densities and is relatively insensitive to the presence of small amounts of aggregates. The hydrodynamic data summarized in Table II for the $E_{\overline{9,0}}$ preparation suggest that native glutamine synthetase exists in solution as compact, essentially globular particles. The hydrodynamic data, when combined with the molecular weight from sedimentation equilibrium data, for the taut and relaxed enzyme ($E_{\overline{9,0}}$) forms give values of 2.05 and 2.33 × 10^6, respectively, for the parameter, β, which describes the shape of the kinetic units in solution (Scheraga and Mandelkern, 1953). These values may be compared with $\beta = 2.12 \times 10^6$ as the accepted value for a spherical particle. Even though β is an insensitive parameter of shape, the values of β do reflect all of the measurements and are in accord with the sedimentation data that indicate that the glutamine synthetase molecule in solution has a high degree of spherical symmetry. (Note that the β value for the relaxed form will be in error by the $[\eta]^{1/3}$ term.) Combining $s^0_{20,w}$, ϕ'_2, and M^0_w values of Table II, the calculated diffusion coefficients, $D^0_{20,w}$, are about 2.7 × 10^{-7} cm^2 sec^{-1} for the taut and relaxed $E_{\overline{9,0}}$ enzyme forms. The conversion of the taut into the relaxed form in this case thus seems to involve more of a density than an over-all shape change (see above) as the quarternary structure becomes loosened.

Difference Spectral Measurements. An investigation of the spectral characteristics of taut and relaxed enzyme forms was undertaken with the purpose of studying the modification of the environment of the aromatic amino acid residues upon interconversion between these two states. Figure 10 (solid line) shows a difference spectrum between a preparation of taut enzyme ($E_{\overline{9,0}}$) to which EDTA was added in the reference compartment and one to which a similar amount of water was added (see Methods). The peaks at 290.5 and 284 mμ illustrate a blue shift induced by EDTA, suggesting that there is exposure of tyrosine and tryptophan residues as a result of EDTA treatment. (The different taut enzyme preparations qualitatively show the same difference spectra upon the addition of EDTA.) The magnitude of change induced by EDTA alone is the same as the difference spectrum obtained by adding relatively high concentrations of $MnCl_2$ to relaxed, gel-filtered enzyme (solid curve, Figure 10). Figure 10 also illustrates difference spectra at two different levels of Mn^{2+} addition to relaxed glutamine synthetase. In these cases, the spectral peaks are in reference to the relaxed enzyme, indicating a red shift caused by the addition of divalent cation in the tightening process. The results shown in Figure 10 indicate that the chromophoric enivronmental change undergone during the relaxation process is reversed with tightening of the enzyme preparation by the addition of Mn^{2+}.

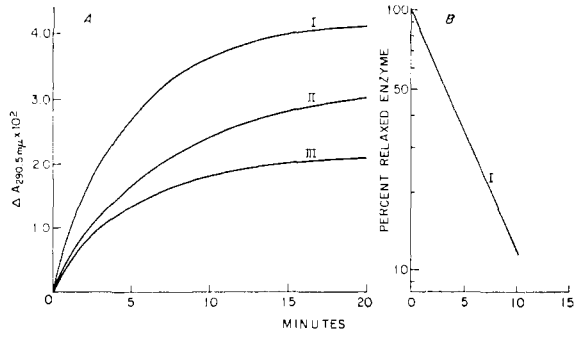

FIGURE 11: Kinetics of the appearance of the difference spectral change upon addition of Mn^{2+} to relaxed enzyme. The procedure is outlined in the legend to part b of Figure 10. The wavelength was set at 290.5 mμ (the maximum of the solid curve of Figure 10) and absorbancy changes were recorded as a function of time. In A, $MnCl_2$ was added to make the final concentrations of Mn^{2+} equal to 1.2 mM (I), 2.5 μM (II), and 1.8 μM (III). In B, the decrease in the per cent relaxed enzyme (calculated from relaxed enzyme = $\Delta A^{max}_{290.5\ m\mu} - \Delta A^{obsd.}_{290.5\ m\mu}$) is plotted to illustrate the first-order decay of a relaxed enzyme preparation upon addition of Mn^{2+} as in curve 1 of A. The first-order constants for the decrease in relaxed enzyme for the three experiments shown in Figure 12A are $k_I = 0.19\ min^{-1}$, $k_{II} = 0.17\ min^{-1}$, and $k_{III} = 0.22\ min^{-1}$.

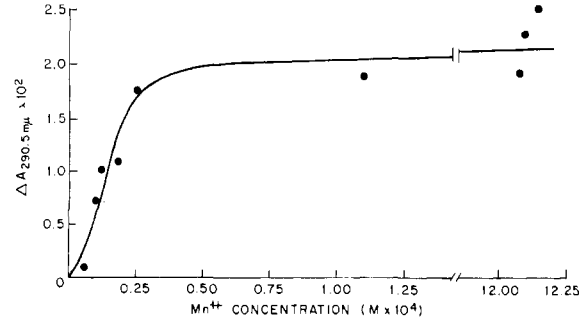

FIGURE 12: Effect of Mn^{2+} concentration upon the total observable difference spectral change. Glutamine synthetase (2.0 mg/ml) was relaxed and placed in a Cary 15 spectrophotometer as described in the legend b of Figure 10. Sequential additions of manganous ion were made, and the total spectral change at 290.5 mμ was recorded at the end of 30 min. The points represent experimental values for three separate experiments.

The relative contribution of tyrosine and tryptophan to the difference spectrum is difficult to assess, but these chromophores seem to be buried to different extents during the tightening process. When low levels of cation are added, as is shown in the dotted line of Figure 10, the 284-mμ peak is relatively more prominent, perhaps indicating that tyrosine residues are buried first upon the addition of metal. All of the tightening experiments here were done at high ionic strength (0.1 M NaCl) and at room temperature to avoid the aggregation and attendant crystallization of relaxed enzyme observed when divalent cation is added back at low ionic strengths (Valentine *et al.*, 1968). The presence of 0.1 M NaCl prevented nonspecific spectral changes due to indirect ionic strength effects on the tertiary or quaternary structure of the protein, including any brought about by the addition of divalent cation itself. The Mn^{2+}-induced difference spectrum was totally reversed by EDTA, further illustrating a reversible dependence of the spectral changes on divalent cation. No similar spectral phenomena were seen with Mn^{2+} addition to *N*-acetyltryptophanamide or *N*-acetyltryosinamide, suggesting that the spectral changes are due to exposure and burial of aromatic residues, rather than to direct aromatic residue-divalent cation interaction.

Additional evidence for the specificity of the divalent cation–glutamine synthetase interaction as examined spectrally is the finding that only Mn^{2+}, Mg^{2+}, and Ca^{2+} produce the spectral changes when added to the relaxed enzyme; Ba^{2+}, Co^{2+}, Sr^{2+}, Ni^{2+}, and spermidine were not effective. Mn^{2+}, Mg^{2+}, and Ca^{2+} are the same cations that are uniquely able to reverse the lag phenomenon when preincubated with relaxed glutamine synthetase (Kingdon *et al.*, 1968).

Relaxation of glutamine synthetase by EDTA occurs rapidly at room temperature, being completed within 2 min, when measured by SH group exposure (see above) or by difference spectral changes. A notable feature of the difference spectral change upon addition of divalent cation to glutamine synthetase is the time required for the reaction to go to completion. The effect of Mn^{2+} concentration on the change in absorbancy at 290.5 mμ as a function of time is illustrated in Figure 11A. Note that at each concentration of Mn^{2+} about 15–20 min is required to obtain the maximum change in absorbancy at 290.5 mμ.

Also, as is shown in Figure 11B, the reaction is first order in concentration of the relaxed enzyme which ultimately will be tightened (*i.e.*, 1.00 minus the fractional total change in absorbancy at 290.5 mμ). First-order rate constants for tightening at all three metal concentrations are essentially the same, as shown in Figure 11B, although the Mn^{2+} concentration varies 700-fold. These observations are consistent with the idea that the rate-limiting step in difference spectral generation is not cation–enzyme interaction, but rather some ensuing enzyme conformational change, since the rate of spectral alteration is essentially independent of divalent ion concentration.

Figure 12 illustrates the magnitude of the total difference spectral change at 290.5 mμ seen with addition of different amounts of Mn^{2+} to a relaxed preparation of glutamine synthetase, compared with a relaxed preparation to which an equivalent amount of water has been added. Each point was determined at the end of 20–30 min after addition of Mn^{2+}, when there was no further spectral change. It is unclear at present whether the apparent sigmoid nature of the change–concentration curve reflects cooperativity in the binding of Mn^{2+}. The validity of the individual points is difficult to assess since there is quite a bit of scatter in the experimental points associated with measuring such small spectral changes.

Discussion

It is clear from the results presented above (Figures 1–3, 5, and 6) and elsewhere (Shapiro and Stadtman, 1967; Woolfolk and Stadtman, 1967) that the removal of spe-

cific divalent cations from *E. coli* glutamine synthetase destabilizes the enzyme, allowing several rather mild procedures (pH 8.0 and mercurials or 1.0 M urea at low ionic strength) to effect inactivation and disaggregation of the enzyme. It is also clear that divalent cation, such as Mn^{2+}, protects the enzyme from the action of rather stronger denaturants (1% SDS). Thus, Mn^{2+} plays an active specific structural role in glutamine synthetase, as well as a catalytic one (Woolfolk *et al.*, 1966). There is also some evidence from our studies that high ionic strength can exert some protective effect on the enzyme, but at much higher concentrations than does divalent cation. Thus, 0.1 M NaCl permits only minimal dissociation of relaxed enzyme at pH 8.0 when examined either in the ultracentrifuge or by light scattering. Still higher ionic strength even protects the enzyme against the dissociating action of 1 M urea at pH 8. However, the effect of the manganous ion is a considerably more specific one, being exerted at less than 1 mM concentrations.

The molecular weight of taut or relaxed glutamine synthetase has been determined here to be 590,000. There are 12 subunits in the enzyme (Valentine *et al.*, 1968), which yield an average subunit weight of 49,200. This value is in excellent agreement with the minimum molecular weight of 49,000 calculated from amino acid analysis of the same enzyme preparation (Shapiro *et al.*, 1967a). Likewise, the former value obtained for the molecular weight, 680,000 (Woolfolk *et al.*, 1966), is changed to *ca.* 585,000 which is more in accord with present measurements (Figure 7) if a value for the apparent specific volume extrapolated from our direct measurements of this parameter is substituted in the calculation. In addition, electron microscopic measurements indicate that the subunit particle has a volume of about 56×10^3 Å^3 (Valentine *et al.*, 1967) which corresponds to a molecular weight of 48,200, assuming a partial specific volume of 0.70 ml/g. The previously reported subunit molecular weight from sedimentation equilibrium experiments in 4 M guanidine-HCl, assuming $\bar{V} = 0.737$ ml/g, was 48,500 (Woolfolk *et al.*, 1966), in agreement with these values.

There is an apparent inconsistency between the sedimentation and specific volume (Table I) data when corrections for the effective mass or $M(1 - \phi'_2\rho)$ are applied to the $s^0_{20,w}$ values of Figure 8. These data for the $E_{2.3}^{-}$ preparation suggest, in contrast to the $E_{9.0}^{-}$ case, that a hydrodynamic volume or frictional coefficient change occurs upon metal removal. Also the difference in the effective mass between the taut $E_{2.3}^{-}$ and $E_{9.0}^{-}$ preparations is not reflected in the observed $s_{20,w}$ values. As noted,[1] the different preparations of glutamine synthetase possibly are heterogeneous with respect to the AMP distribution on the enzyme molecules. The particle density, and possibly also to a small degree the hydrodynamic frictional coefficient (f), are functions of the state of adenylylation. Heterogeneity with respect to ϕ'_2 and f within a given taut enzyme preparation could influence the observed sedimentation rate. Indeed, although all schlieren boundaries qualitatively were symmetrical, those of $E_{2.3}^{-}$ and $E_{9.0}^{-}$ preparations at the low concentrations appeared hypersharp. In addition, the reduced viscosities of the taut $E_{1.2}^{-}$ (Figure 9) were somewhat lower than those measured for the $E_{2.3}^{-}$ and $E_{9.0}^{-}$ preparations. Then, the hydrodynamic frictional coefficient change in the relaxation process (with roughly the same enzyme form produced in each case) may be a function also of the state of adenylylation and may be partially obscured by heterogeneity in the AMP distribution throughout the enzyme population. However, until homogeneous preparations of nonadenylylated and adenylylated glutamine synthetase are available, such subtle differences in particle density and shape only can be postulated as a possible interpretation of the data presented here.

In summary, the hydrodynamic data presented for the taut and relaxed enzyme forms at pH 7 and about 0.16 ionic strength, indicate that the removal of specific divalent cations is accompanied by a small shape and/or volume change, but not by any disaggregation. The relaxation process perhaps can best be described as a structural loosening of the glutamine synthetase molecule. The sedimentation and viscosity properties of the taut enzyme show that it behaves as a compact, nearly spherical particle in solution. The change induced by removing manganous ions appears to involve some fairly small shape alterations which may accompany the loosening of the quaternary structure. The apparent specific volumes measured for the relaxed enzyme forms of glutamine synthetase preparations of different states of adenylylation were nearly the same, which is of interest since these are the inactive enzyme forms with the same physical and chemical properties. However, the taut enzyme forms which can be differentiated on the basis of the extent of adenylylation[1] (Shapiro *et al.*, 1967a; Kingdon *et al.*, 1967) had measurably different apparent specific volumes. These differences cannot be accounted for simply on the basis of the AMP composition of the different enzyme preparations. Furthermore, the differences in particle densities (Table I) are not simple functions of the degree of adenylylation which strongly suggests that the native glutamine synthetase preparations[1] are not merely mixtures of fully adenylylated and nonadenylylated enzymes. The different enzyme preparations have been shown to differ markedly in their catalytic properties, with respect to both their divalent cation requirement for catalysis and their sensitivities toward the different feedback inhibitors of glutamine synthetase (Kingdon and Stadtman, 1967; Kingdon *et al.*, 1967). However, the sedimentation and viscosity behavior of the different taut preparations of glutamine synthetase were essentially the same, as was also the stability to sulfhydryl reagents and other disaggregating reagents. These results could well reflect that the adenylylation of glutamine synthetase which can occur to the extent of 1 equiv of AMP/subunit (Kingdon *et al.*, 1967) only produces subtle structural alterations in the taut dodecamer molecule which somehow influences the net density of the particles (uncoupled by relaxation) without affecting their gross hydrodynamic behavior in solution. In this respect, a potent feedback inhibitor of the $E_{9.0}^{-}$ preparation[1] is AMP and when this inhibitor was added to $E_{9.0}^{-}$ under conditions which saturate 12 binding sites/molecule of glutamine synthetase,[7] no change in

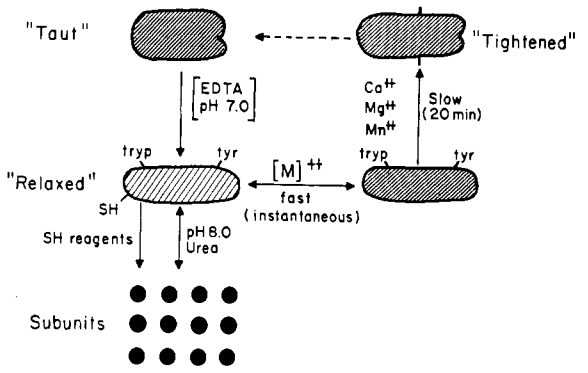

FIGURE 13: Scheme for the interconversion of taut, relaxed, and tightened enzyme forms. See Discussion for explanation of symbols.

the sedimentation or viscosity properties of the enzyme could be detected.

The exposure of tyrosine and tryptophan residues upon relaxation of the enzyme, as indicated by the ensuing blue shift with addition of EDTA, or the burial of the same residues with tightening of the enzyme, indicated by a red shift in the spectra (Figure 10), is in accord with the observations on resistance to inactivation, sulfhydryl group accessibility, and structural changes presented above. The net exposure of tyrosine and tryptophan residues in the relaxation process can be estimated to be one to two residues per subunit, or less than 10% of the total possible shift if all of the aromatic residues (sixteen tyrosine and four tryptophan per subunit; Woolfolk *et al.*, 1966), were to change from a nonpolar to a polar environment during relaxation (Wetlaufer, 1962).

The slow conformational change (Figure 11) occurring after the addition of specific divalent cations (Mn^{2+}, Mg^{2+}, and Ca^{2+}) to relaxed enzyme has the same time constant and divalent cation specificity as the recovery of activity which is demonstrated by Kingdon *et al.* (1968) in the accompanying paper. In addition, the concentration of Mn^{2+} which effected half of the maximal spectral change (1.5×10^{-5} M) is in good agreement with the concentration of Mn^{2+} which, when preincubated with relaxed enzyme, would half eliminate the lag (2.5×10^{-5} M; Kingdon *et al.*, 1968). (Actually, the amount of Mn^{2+} bound to the enzyme in the two experiments cited cannot assume to be negligible;[2] the values obtained may well correspond to the value ($K_D' = 4 \times 10^{-6}$ M) measured by M. D. Denton[2] for the equilibrium binding of Mn^{2+} by this enzyme preparation at pH 7.) Thus, the burial of aromatic amino acid residues is the one structural parameter which has been shown to directly parallel the activation of relaxed enzyme by specific divalent cations. Whether or not this is related to tyrosine/tryptophan residue participation at the active site of the enzyme, or merely secondary to a general conformational change, is not known. The activation of relaxed enzyme is accelerated about tenfold by the presence of all the substrates (glutamate, NH_3, ATP, and Mg^{2+}) at saturating concentrations (Figure 4).

Figure 13 is a scheme summarizing our data concerning relaxed, taut, and tightened forms of glutamine synthetase. Taut enzyme is that purified from *E. coli*, with Mn^{2+} bound to it, since this divalent ion was used in all purification buffers. There is no evidence for any bound Mg^{2+} or Ca^{2+} as determined by atomic absorption spectroscopy (Shapiro and Stadtman, 1967). The enzyme as isolated is catalytically active (the cleft on the side of the molecule in Figure 13 denotes the active site, as representative of the probable twelve active sites, or one per subunit). The sulfhydryl and aromatic residues are buried, and the enzyme is resistant to disaggregation. Treatment of the taut enzyme with EDTA in excess of the Mn^{2+} present with the enzyme converts glutamine synthetase into a relaxed form. This conversion is almost instantaneous at 20°, as shown by susceptibility of the enzyme to mercurial inactivation and the rapid occurrence of spectral changes, but requires about 2 hr to reach completion at 0°, as demonstrated by organic mercurial inactivation studies (Figure 3). The resultant relaxed enzyme preparation is inactive, as measured in a coupled assay which determines initial rates, but rapidly becomes active under the conditions of assay (Kingdon *et al.*, 1968; Figure 4). In addition, the sulfhydryl residues become titratable by DTNB and PMPS (Shapiro and Stadtman, 1967) and the tyrosine and tryptophan residues become exposed to the solvent. Hydrodynamic measurements (Figures 7–9) indicate that the relaxed enzyme is less symmetric than the taut form. The relaxed preparation is labile, being susceptible to disaggregation at pH 8, particularly at low ionic strengths. This is enhanced by organic mercurials (Figure 2; Shapiro and Stadtman, 1967) or urea (Figures 5 and 6; Woolfolk and Stadtman, 1967), or at neutral pH by sodium dodecyl sulfate (Figure 1), whereas the taut enzyme is resistant to all such treatments. Upon addition of divalent cation to relaxed enzyme, it becomes tightened. The first stage in this process is protection of SH groups, so that the enzyme is not inactivated by organic mercurials (Shapiro and Stadtman, 1967). This may be accomplished by many divalent cations, without apparent specificity, and may therefore reflect principally a protection against a dissociating action of the mercurial reagents. Another rapid change in the enzyme is the alteration in the fluorescence of a hydrophobic probe when divalent cations are added (Kingdon *et al.*, 1968). The second stage in the tightening process is brought about only if Mg^{2+}, Mn^{2+}, or Ca^{2+} are used as the divalent cations added to relaxed enzyme. There is a slow conformational change accompanied by aromatic residue burial, with an attendant activation of the enzyme as indicated by an elimination of the lag phenomenon seen with relaxed enzyme. The resulting tightened enzyme is so named because, even though it behaves catalytically and spectrally like taut enzyme at relatively high ionic strengths, it has the additional property of crystallizing from dilute buffer solutions (Shapiro *et al.*, 1967b; Valentine *et al.*, 1968). This has never been observed with taut enzyme upon addition of more divalent cations, so it seems to indicate a structural difference between taut and tightened glutamine synthetase preparations. This is the only difference which we have noted and it may well be obliterated in solutions of high ionic strength or of substrate mixtures. The dotted arrow from

tightened to taut in Figure 13 expresses our lack of information about the extent of nonidentity of these two forms. The scheme presented in Figure 13 is intended only to illustrate the interrelationships described in this and other papers; the drawings are no attempt to capture the reality of the protein structure and its alterations, nor are the arrows indicative of true chemical reactions, but rather only of directions of observed interconversions.

Acknowledgments

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