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Physical Properties and Subunit Structure of Butyrylcholinesterase from Horse Serum†

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ABSTRACT: Horse serum butyrylcholinesterase (ButChE) has been characterized by several physicochemical techniques. The results indicate that ButChE is a stable enzyme and exists as a single component, a conclusion supported by linear relationships of ln c *vs.* r^2 from sedimentation equilibrium studies. A molecular weight of $(4.4 \pm 0.4) \times 10^5$ is obtained from both high- and low-speed sedimentation equilibrium experiments with a measured value for $\bar{v} = 0.78$. The intrinsic viscosity, $[\eta]$, of ButChE is 6.6 ± 0.6 ml/g. By combining the basic hydrodynamic parameters, $s^0_{20,w}$, $[\eta]$, and molecular weight, it is concluded that ButChE may best be represented by a solvated and slightly asymmetric molecule. Sedimentation studies indicate that the quaternary structure of ButChE remains intact in the presence of (a) NaCl in concentrations as high as 2.5 M, (b) an organophosphate inhibitor, (c) a substrate analog, and (d) Mg^{2+} and Ca^{2+}. Changes in the sedimentation properties of ButChE observed in the presence of NaCl are due to preferential solvation of the protein.

Additional measurements indicate that ButChE is dissociated into its smallest subunits at pH 11.5 and in 6 M guanidine hydrochloride. The weight-average molecular weight of the subunits has been measured by sedimentation equilibrium and is $(1.2 \pm 0.1) \times 10^5$ and $(1.0 \pm 0.1) \times 10^5$ at pH 11.5 and in 6 M guanidine hydrochloride, respectively. Identical molecular weights are obtained for ButChE in guanidine hydrochloride with or without reduction and alkylation; hence, there is no interchain disulfide linkage. The available data indicate that native ButChE most likely has a tetrameric structure with subunits of equal size.

During the past decade several studies on butyrylcholinesterase (acylcholine acyl-hydrolase, EC 3.1.1.8) from horse serum have been reported. Most of these reports indicated that the enzyme had multiple molecular forms. A study of the rates of inhibition of ButChE[1] by organophosphates showed that the kinetic data could be resolved into at least two components, which were thought to reflect a rapid aggregation or dissociation of active components of the protein (Main, 1969). In addition there are several reports of isozymes (Oki *et al.*, 1965) and undefined multiple forms of the horse serum enzyme (Heilbronn, 1962; Reiner *et al.*, 1965; Zech and Engelhard, 1965). These studies were performed on serum or crude preparations of the enzyme and do not provide enough information to specify the molecular nature of the multiple forms.

Since the available data implied that ButChE consists of subunits, efforts have been made in this laboratory to purify and characterize the enzyme and determine its structure. In contrast to the earlier observations, it has been found that ButChE may be obtained in a purified form, which is homogeneous and stable. The protein does not undergo association, or dissociation in 0.01 M phosphate buffer (pH 7.0) (Lee and Harpst, 1971). However, it is possible that the enzyme behaves differently in the presence of metabolites which may interact with it to produce aggregation or dissociation, as demonstrated for glutamate dehydrogenase (Frieden, 1963) and cytosine triphosphate synthetase (Levitzki and Koshland, 1972). The present study has been undertaken in an effort to determine the subunit structure of ButChE and to begin resolving the apparent discrepancies between earlier observations and the work in this laboratory.

In this report the molecular weight of native ButChE has been measured by sedimentation equilibrium and light scattering techniques and the intrinsic viscosity determined. The data establish that native ButChE is a macromolecule with a molecular weight of 4.4×10^5 and, under the conditions studied, it does not dissociate or aggregate, even in the presence of the metabolites tested. Furthermore, it is shown that the native enzyme consists of four subunits which are identical or at least similar in size. Sedimentation data for ButChE in NaCl, which might have been interpreted as aggregation or dissociation, are explained in terms of preferential solvation.

Materials

D_2O (99.8%) was purchased from Stohler Isotope Chemicals. Acetyl-β-methylcholine bromide was purchased from Nutritional Biochemical Co. $(CH_2CH_2)_4PP$ was obtained from K & K Laboratories, Inc., and sucrose was from Mallinckrodt Chemical Works. Guanidine hydrochloride (Ultra Pure) was purchased from Mann Research Laboratories and β-mercaptoethanol was from E. H. Sargent and Co. Triethylamine was obtained from Aldrich Chemical Co. and iodoacetic acid was from Eastman Kodak Co. Other chemicals were of standard reagent grade. All materials were used without further purification.

Methods

Enzyme Preparation and Assay. ButChE was isolated and the activity measured by the methods previously described (Lee and Harpst, 1971). The stability of ButChE as a function

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[1] Abbreviations used are: ButChE, butyrylcholinesterase; Gdn·HCl, guanidine hydrochloride; $(CH_2CH_2)_4PP$, tetraethylene pyrophosphate.

of pH was measured by exposing the enzyme to a solution at the desired pH for 24 hr at 5° and measuring the remaining activity under standard assay conditions. All pH measurements were made at 25°.

Protein Concentrations. The extinction coefficient, $E^{1.0\,cm}_{280\,nm}$, of purified, native ButChE was determined by measuring refractive index increments with a Brice-Phoenix differential refractometer (Armstrong *et al.*, 1947; Brice and Halwer, 1951). Temperature was regulated at 20.00 ± 0.02° by a Tamson circulator and a Neslab portable cooling unit. The refractometer was calibrated against sucrose solutions (Brice and Halwer, 1951). A value of 0.187 ml/g (Armstrong *et al.*, 1947) was used as the standard refractive index increment at a wavelength of 546 nm. Multiple determinations gave a value of 1.10 ± 0.02 cm²/mg for $E^{1.0\,cm}_{280\,nm}$ of ButChE in 0.01 M potassium phosphate (pH 7.0). The extinction coefficient was substantiated by results obtained by the method of Waddell (1956). All reported concentrations were calculated from $A_{280\,nm}$ and the measured extinction coefficient.

The concentration of ButChE in 5 M Gdn·HCl was determined by comparing the absorbance at 280 nm of two portions of a ButChE solution to which the same volume of phosphate buffer or concentrated Gdn·HCl solution was added. The maximum absorbance for the native enzyme was at 278 nm but shifted to 276 nm when ButChE was denatured by Gdn·HCl. The absorbance at 280 nm decreased simultaneously. The ratio of absorbance of ButChE in Gdn·HCl at 280 nm to that in phosphate buffer was 0.86 ± 0.02 (average deviation). Since an extinction coefficient of 1.10 cm²/mg was chosen for native ButChE, a value of 0.95 cm²/mg was estimated for ButChE in 5 M Gdn·HCl. In this report it is assumed that the extinction coefficient of ButChE in 6 M Gdn·HCl is the same as in 5 M Gdn·HCl.

Dissociation and Denaturation of ButChE at High pH and in Gdn·HCl. ButChE solutions with pH values of 8.0–10.5 were prepared by dialyzing at 5° for 12–16 hr against 1 l. of 0.011 M triethylamine–carbon dioxide buffers, which were adjusted to the desired pH by bubbling carbon dioxide through the solution at room temperature. For studies at a pH greater than 10.5, a solution of 0.011 M triethylamine was adjusted to the desired pH by adding 4 N NaOH at 25°. Small differences in pH of the buffers at 5 and 25° have been neglected; values determined only at 25° are used in this paper.

Reconstitution of pH-dissociated ButChE was carried out by dialyzing the enzyme solution for 8–12 hr in the cold against 0.01 M potassium phosphate (pH 7.0) buffer, after 24–36 hr at the high pH.

Denaturation of ButChE by 6 M Gdn·HCl was achieved by dialyzing 1.0 ml of enzyme solution (approximately 500 μg/ml) for at least 48 hr against two 50-ml changes of 6 M Gdn·HCl. For reduction of disulfide bonds (Crestfield *et al.*, 1963), a solution which had been dialyzed against 6 M Gdn·HCl was adjusted to pH 8.0 with 0.5 N NaOH and then dialyzed against 50 ml of 6 M Gdn·HCl–0.1 M β-mercaptoethanol at pH 8.0 for an additional 12–24 hr. Solid iodoacetic acid was added to the dialysate to make the final solution 0.2 M in iodoacetic acid and the reaction allowed to proceed for 6 hr. The alkylated ButChE was finally dialyzed against two 50-ml changes of fresh 6 M Gdn·HCl for 12–16 hr. All dialysis and alkylation procedures associated with Gdn·HCl denaturation were carried out at room temperature.

Density and Viscosity Measurements. Densities of solvents were determined by standard procedures in a 4.5-ml Lipkin pycnometer (Bauer, 1949). Volumes were measured in a constant-temperature bath at 20.00 ± 0.02° and weights included *in vacuo* corrections.

TABLE I: Measured Densities and Viscosities of Solvents at 20.00 ● 0.02°.

Solution	ρ_0	η_{rel}
0.011 M Triethylamine–carbon dioxide	0.9987	1.010
0.5 N NaCl–0.01 M potassium phosphate (pH 7.0)	1.0193	1.032
1.5 N NaCl–0.01 M potassium phosphate (pH 7.0)	1.0578	1.141
2.5 N NaCl–0.01 M potassium phosphate (pH 7.0)	1.0953	1.263
6 M Gdn·HCl	1.1550	1.630
0.01 M Potassium phosphate in 99.8% D_2O (pD 7.0)	1.0958	
0.01 M Potassium phosphate in 49.9% D_2O (pD 7.0)	1.0479	
0.01 M Potassium phosphate in H_2O (pH 7.0)	0.9988	

The viscosities of solvents relative to water were obtained at 20.00 ± 0.02° in a 5-ml Ostwald viscometer with a flow time of 82.0 sec for water. Standard relations (Yang, 1961) and measured values of the densities of solutions were used in calculations of relative viscosities for all solvents. Relative viscosities of protein solutions were determined with a 0.5-ml Ostwald-Cannon viscometer with a flow time of 288 sec for water at 25.00 ± 0.02°. However, densities were not measured for these solutions, so the experimental intrinsic viscosity was actually an intrinsic kinematic viscosity, $[\gamma]$, as defined by

$$[\gamma] = \lim_{C\to 0} (t - t_0)/t_0C$$

where t is the capillary flow time of solution, t_0 the flow time of solvent, and C the concentration in grams per milliliter. Values of $[\gamma]$ for native ButChE were obtained from the intercept of a plot of $(t - t_0)/t_0C$ *vs.* concentration (Tanford, 1955). Tanford (1955) has shown that the intrinsic viscosity, $[\eta]$, is related to the intrinsic kinematic viscosity as follows:

$$[\eta] = [\gamma] + (1 - \bar{v})/\rho_0$$

Therefore, the intrinsic viscosity of ButChE was obtained from the above equation and appropriate values for the partial specific volume, $\bar{v}$, and solvent density, ρ_0. Units of $[\eta]$ are milliliters per gram.

Measured densities and viscosities of various solvents used in this investigation are summarized in Table I.

Sedimentation Velocity. Measurements of sedimentation velocity were made in a Beckman-Spinco Model E analytical ultracentrifuge equipped with a photoelectric scanner and multiplexer. All measurements were made at 5° and 60,000 rpm, except when the multiple-cell rotor (An-F) was used. In the latter case the speed was 52,000 rpm. Monochromatic light of wavelength 280 nm was selected and sedimentation velocities were determined as described by Lee and Harpst (1971). Measured values for the viscosities and densities of

buffers were used to correct all sedimentation coefficients to standard conditions (Schachman, 1959).

Partial Specific Volume of ButChE. The partial specific volume was measured by the D_2O method of Edelstein and Schachman (1966). This method facilitates simultaneous measurement of partial specific volume and apparent weight-average molecular weight, $\bar{M}_{app}$, of a protein by sedimentation equilibrium experiments in H_2O and D_2O solutions. Approximately 0.5 ml of ButChE solution was dialyzed for 24 hr at 5° against two 50-ml portions of 0.01 M potassium phosphate (pD 7.0) buffer made up to 99.8, 49.9, or 0% (v/v) D_2O. pD was determined as pH measured with the glass electrode plus 0.4 (Glasoe and Long, 1960). High-speed sedimentation equilibrium experiments (see Sedimentation Equilibrium) were carried out simultaneously with samples of ButChE in H_2O and D_2O buffers with the six-channel Yphantis cell (Yphantis, 1964). The value of $\bar{v}$ was obtained with the following equation (Edelstein and Schachman, 1966)

$$\bar{v} = \frac{k - [(\mathrm{d} \ln c/\mathrm{d}r^2)_{D_2O}/(\mathrm{d} \ln c/\mathrm{d}r^2)_{H_2O}]}{\rho_{D_2O} - \rho_{H_2O}[(\mathrm{d} \ln c/\mathrm{d}r^2)_{D_2O}/(\mathrm{d} \ln c/\mathrm{d}r^2)_{H_2O}]}$$

where c is the concentration at r, the radial distance, ρ is the density of solvent indicated by the subscript, and the values of k are 1.0155 and 1.008 for measurements in 100 and 50% D_2O solutions, respectively (Edelstein and Schachman, 1966).

For sedimentation velocity and equilibrium measurements at high pH, it was assumed that there was no change in $\bar{v}$ from that of native ButChE. The partial specific volume of ButChE in Gdn·HCl was assumed to be 0.02 ml/g less than for the native enzyme, as demonstrated for several proteins (Kielly and Harrington, 1960; Noelken and Timasheff, 1967; Hade and Tanford, 1967).

Sedimentation Equilibrium. Molecular weights were determined by sedimentation equilibrium measurements in the Model E analytical ultracentrifuge. Both high-speed (Yphantis, 1964) and low-speed (Van Holde and Baldwin, 1958) experiments were performed with a 12-mm, double-sector, aluminum-filled Epon centerpiece or a six-channel Yphantis centerpiece (Yphantis, 1964) at 4–6°. Procedures of testing for attainment of equilibrium and base-line corrections were the same as described by Schachman and Edelstein (1966).

The apparent weight-average molecular weights were calculated with the following equation (Tanford, 1961)

$$\bar{M}_{app} = \frac{2RT}{(1 - \bar{v}\rho)\omega^2} \frac{\mathrm{d} \ln c}{\mathrm{d}r^2}$$

where ω is the angular velocity and other symbols are standard or the same as used above. Concentrations of solute were determined from a calibration curve relating pen deflection of the photoelectric scanner to the absorbance (280 nm) of the solute. The weight-average molecular weight, $\bar{M}_w$, was obtained from a linear plot expressed by the following equation (Tanford, 1961)

$$\frac{1}{\bar{M}_{app}} = \frac{1}{\bar{M}_w} + 2A_2\bar{c} + \ldots$$

where A_2 is the second virial coefficient, and $\bar{c}$ is the mean concentration, determined by taking the arithmetic mean of the concentrations at the maximum and minimum values of r used for the ln c *vs.* r^2 plots.

Light Scattering. CLARIFICATION OF SOLUTIONS. All solutions were clarified by filtering slowly through Millipore filters at a pressure corresponding to the head of the solution itself (approximately 100 mm of H_2O). Buffer and protein solutions were filtered at least three times through a 0.45 μ pore size, type HA filter. The clarity of solutions was checked by visual obervation at low angles in the high-intensity beam of a low-angle light-scattering photometer (Harpst *et al.*, 1968).

The activities and sedimentation coefficients of a few randomly chosen enzyme solutions were checked before and after filtration. There was no loss in specific activity or detectable change in sedimentation behavior after filtration, although as much as 30–50% of protein was retained by the filter.

MEASUREMENTS. All measurements were taken with a Brice-Phoenix light-scattering photometer, dual photomultiplier type, at room temperature and with unpolarized light at a wavelength of 546 nm. A cylindrical, clear, C-105 Pyrex cell was used. Sample solutions were filtered directly into the cell. The scattering intensities at 90° were measured and the Rayleigh ratios were calculated by the method recommended by Tomimatsu and Palmer (1963). The weight-average molecular weight was determined by the following equation (Tanford, 1961)

$$\frac{KC}{R_{90}} = \frac{1}{\bar{M}_{app}} = \frac{1}{\bar{M}_w} + 2A_2C + \ldots$$

where R_{90} is the Rayleigh ratio at 90°, K is the optical constant, and other symbols are as defined above. The intercept of a plot of KC/R_{90} *vs.* C gives the value of $1/\bar{M}_w$ and the limiting slope is proportional to A_2.

Results

Physical Properties of Native ButChE

Intrinsic Viscosity. Measurements of intrinsic viscosity, together with sedimentation coefficients and molecular weights, can yield information which is useful in defining the shape of the ButChE molecule (Tanford, 1961). The intrinsic viscosity of native ButChE was determined from the data in Figure 1. A value of $[\eta] = 6.6 \pm 0.6$ ml/g (average deviation) at 25° was obtained by extrapolating to zero protein concentration and converting the intrinsic kinematic viscosity to intrinsic viscosity with the method described above and $\bar{v}$ obtained in the following section. Since an intrinsic viscosity greater than 4–5 ml/g is an indication of deviation from typical behavior of globular proteins ranging in molecular weight from 14,000 to 3.5×10^6 (Schachman, 1963), native ButChE may be a somewhat asymmetric protein.

Partial Specific Volume. The partial specific volume of ButChE was determined as described in Methods. Average values of 0.772 from four determinations and 0.785 from seven determinations were obtained for the $\bar{v}$ of ButChE in 50 and 100% D_2O, respectively. Hence, an average value of 0.78 ± 0.01 was adopted. This is higher than $\bar{v}$ usually observed (0.75) for other proteins (Van Holde, 1967). Further comments are included in the Discussion.

Molecular Weight. SEDIMENTATION EQUILIBRIUM. Within experimental uncertainty, there is no sharp or uniform deviation from linearity throughout the liquid column in plots of ln c *vs.* r^2 for ButChE in 0.01 M potassium phosphate, pH 7.0, 12,000 rpm. Linear plots were obtained also from low-speed (5200 rpm) experiments. The apparent weight-average molecular weights calculated from both the high- and

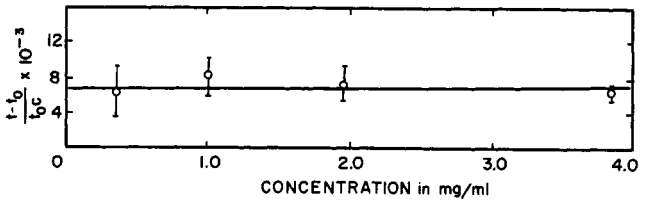

FIGURE 1: Reduced kinematic viscosity of native ButChE as a function of protein concentration. Experiments were performed with ButChE in 0.01 M potassium phosphate, pH 7.0 and at 25°. The bars indicate the maximum and minimum deviations observed in multiple determinations.

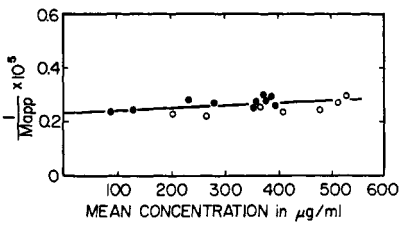

FIGURE 2: Relationship between $1/\bar{M}_{app}$ and mean concentration of ButChE in 0.01 M potassium phosphate (pH 7.0). The equilibrium experiments were performed at 12,000 (●) and 5200 (○) rpm.

low-speed experiments with $\bar{v} = 0.78$ ml/g were identical within experimental error, as indicated in Figure 2. A normal linear dependence of $1/\bar{M}_{app}$ on mean protein concentration was observed over a sixfold range (85–525 μg/ml). The second virial coefficient calculated from the slope of the plot is 1.1×10^{-3} mol cm³/g². The weight-average molecular weight of ButChE, obtained from the extrapolation to zero concentration, is $(4.4 \pm 0.4) \times 10^5$. The range of uncertainty expressed here and in following sections is the average deviation for multiple determinations, except where noted otherwise. The close correlation between $\bar{M}_{app}$ from high- and low-speed experiments, in addition to the fact that all plots of ln c *vs.* r^2 are linear, indicates that the purified ButChE is homogeneous and undergoes no detectable dissociation or aggregation under the conditions of the experiments. This conclusion is in agreement with that derived from earlier sedimentation velocity and disc gel electrophoresis measurements on ButChE (Lee and Harpst, 1971).

LIGHT SCATTERING. Although the molecular weight of ButChE was measured by sedimentation equilibrium, uncertainties in the determination of $\bar{v}$ made it desirable to measure $\bar{M}_w$ by at least one additional independent method, light scattering. A brief outline of the procedures used has been given in Methods. Figure 3 is a composite plot of KC/R_{90} *vs.* concentration of ButChE from two series of measurements on different preparations. Only the scattering intensities at an angle of 90° are reported, because no significant angular dependence was expected (Lee, 1971). A molecular weight of $(4.4 \pm 0.6) \times 10^5$ was obtained by extrapolation to zero concentration and the virial coefficient, A_2, from the slope is 1.7×10^{-2} mol cm³/g². The difference between A_2 from sedimentation equilibrium and light scattering is discussed below.

Various physical parameters of purified ButChE are in Table II.

TABLE II: Properties of Native ButChE in 0.01 M Potassium Phosphate.

Parameter	Av Value
Sedimentation coefficient, $s^0_{20,w}$	11.5
Partial specific volume, $\bar{v}$	0.78
Molecular weight, $\bar{M}_w$, from both sedimentation equilibrium and light scattering	4.4×10^5
Intrinsic viscosity, [η], at 25°, ml/g	6.6
f/f_0	1.46

Effects of Small Ions, a Substrate Analog and an Inhibitor

Behavior of ButChE in Sodium Chloride. The activity of ButChE has been observed to decrease at high concentrations of sodium chloride (Lee and Harpst, 1971). Since the loss of activity might indicate that the protein dissociates into inactive subunits, the sedimentation behavior of the enzyme at NaCl concentrations as high as 2.5 M was studied. At this concentration ButChE has no activity.

The concentration dependence of the sedimentation coefficients of ButChE (three to five concentrations, 140–1380 μg/ml) was studied at various constant concentrations of NaCl. In all cases a normal linear dependence of $s_{20,w}$ on protein concentration was observed. There was no sharp deviation from a linear relationship even at the lowest concentrations. Therefore, it was concluded that no dissociation or aggregation of ButChE occurred up to 2.5 M NaCl. The values of $s^0_{20,w}$, obtained by extrapolating to zero protein concentrations, were plotted against the concentration of NaCl and a linear relationship was obtained, as shown in Figure 4A.

The absence of curvature or a sharp deviation from linearity in Figure 4A makes it unlikely that the loss of enzyme activity is due to dissociation or aggregation of ButChE. However, since $s^0_{20,w}$ does change, sedimentation equilibrium measurements were made to test for any change in $\bar{M}_w$. For the three NaCl concentrations (0.5, 1.0, and 2.5 M) at which sedimentation equilibrium experiments were made, plots of ln c *vs.* r^2 were linear. Plots of $\bar{M}_{app}$ (r_i), *i.e.*, apparent weight-average molecular weight at radial distance, r_i, *vs.* $c(r_i)$, the concentration at r_i (Van Holde, 1967), were linear and the values of $\bar{M}_w$, obtained by extrapolating to zero concentration, were 4.0×10^5, 4.4×10^5, and 4.9×10^5 in the presence of 0.5, 1.0, and 2.5 M NaCl, respectively. For these calculations, it was assumed that the value of $\bar{v}$ was 0.78, regardless of NaCl concentration. The linearity of the plots and the small variations in $\bar{M}_w$ confirm the conclusions from measurements of $s^0_{20,w}$. Further analysis of these data is included in the discussion.

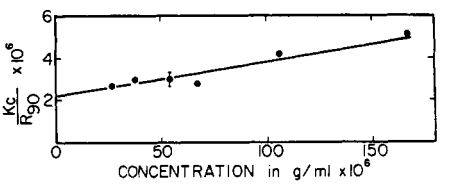

FIGURE 3: Graph of KC/R_{90} as a function of ButChE concentration in the same buffer indicated for Figure 2. The bar shows the maximum and minimum values for three measurements at the same concentration and approximate deviations observed at other concentrations. Measurements were made at room temperature.

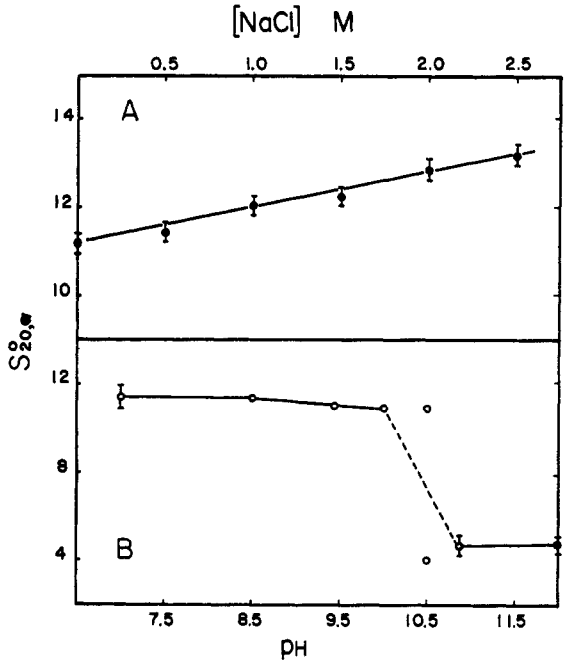

FIGURE 4: (A) Relationship between values of $s^0_{20,w}$ of ButChE as a function of sodium chloride concentration. Experimental conditions were: ButChE in 0.01 M potassium phosphate, with added salt as indicated, pH 7.0, and rotor speed of 52,000 rpm; temperature, 5°. (B) Values for $s^0_{20,w}$ of ButChE as a function of pH. The points at pH 10.5 represent the values obtained for the two sedimenting components observed. The bars indicate maximum experimental deviation. The buffer was 0.011 M triethylamine–CO_2 as described in Methods, except for pH 7.0, at which 0.01 M potassium phosphate was used.

Sedimentation of ButChE in the Presence of a Substrate Analog, an Inhibitor and Other Small Ions. Binding of metabolites and small inorganic or organic ions has been shown to dissociate some enzymes into smaller structural units (Klotz *et al.*, 1970). In an effort to define conditions under which ButChE may be dissociated, its sedimentation behavior in the presence of a substrate analog was studied. Previous experience showed that acetyl-β-methylcholine acts as a competitive inhibitor and is not hydrolyzed by ButChE (Lee, 1971). Therefore, the sedimentation behavior of the enzyme was studied in the presence of 0.2% (w/v) acetyl-β-methylcholine. In some cases the sedimentation coefficient of an identical ButChE solution without the analog was measured in the same experiment by the method of differential sedimentation described by Gerhart and Schachman (1968). The only differences between the method described and that used in the present study were the utilization of the photoelectric scanner, an AN-F rotor, and much lower protein concentrations. There was no detectable difference outside of experimental error (±0.5%) between the sedimentation behavior of ButChE in the presence or absence of the inhibitor. Similar results were obtained from sedimentation velocity experiments with ButChE in the presence of 1×10^{-4} M Ca^{2+}, 4×10^{-2} M Mg^{2+}, and 4×10^{-5} M $(CH_2CH_2)_4PP$, a specific inhibitor for cholinesterases. The data show that ButChE does not dissociate into subunits, or undergo other detectable changes in quaternary structure, in the presence of substrate analog, Ca^{2+}, Mg^{2+}, and $(CH_2CH_2)_4PP$, at least at concentrations of reagents which significantly affect the reaction kinetics of the enzyme (Lee and Harpst, 1971; Lee, 1971).

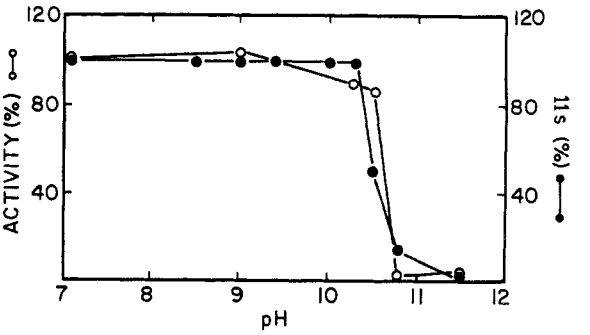

FIGURE 5: Stability of ButChE as a function of pH. Stability is expressed as per cent remaining activity and also as the per cent of 11S ButChE remaining after exposure to the indicated pH for at least 20 hr at 5°.

Dissociation and Denaturation of ButChE

Effects of Alkali. The sedimentation behavior of ButChE has been studied as a function of pH. Preliminary work indicated that the purified enzyme was stable at least for 4 hr and showed no change in $s_{20,w}$ (11 S) at pH values as low as 1.4. When the same solution was tested after standing 24 hr at pH 1.4, no distinct sedimenting boundary could be seen upon ultracentrifugation. However, after extended treatment at pH 11.5 a single 4S component was observed. Since these results indicated that ButChE was stable only for short times at low pH, but could be dissociated to a stable form at high pH, a systematic investigation was undertaken in the pH range, 7.0–11.5. At each pH value chosen sedimentation coefficients were measured as a function of protein concentration (three to five different concentrations from 100 to 1300 μg per ml). The values of $s^0_{20,w}$ obtained at each pH by extrapolating data to zero concentration were plotted against pH, as shown in Figure 4B. The sharp decrease in $s^0_{20,w}$ at pH 10.3–10.8 implies that a gross change, probably dissociation, occurs in the structure of ButChE.

A normal linear concentration dependence for the sedimentation coefficient at pH values of 7.5–10.0 and above 11.5 indicates that no molecular aggregation or dissociation of the protein occurs under these conditions. However, there were two distinct sedimenting boundaries, corresponding to an 11S specie and a slower 4S component, at pH 10.5 (Figure 4B). Within experimental error and within the concentration range studied the relative amount of the 11S to the 4S component was not significantly affected by initial protein concentration, but depended on the pH, as shown in Figure 5. Dissociation of ButChE begins at approximately pH 10.3, and is complete at approximately pH 11.5, at which the only sedimenting component is the 4S specie.

Reversibility of the dissociation of ButChE at high pH was tested by comparing the sedimentation coefficients of the enzyme before, during, and after exposure to pH 10.5 for 24 hr at 5° (Figure 4B). Upon dialyzing the alkaline solution, which contained two sedimenting components, against 0.01 M phosphate buffer (pH 7.0) for 12 hr at 5°, at least 95% of the ButChE sedimented in the 11S form, as determined from photoelectric scanner tracings. If ButChE were exposed to pH 10.5 for periods much longer than 24 hr or to a higher pH, the 4S component would not return to the 11S form. Therefore, the dissociation of ButChE was reversible under the conditions described. The stability of ButChE was also monitored by measuring enzyme activity as described in Methods.

TABLE III: Molecular Weight and Number of Subunits of ButChE in Two Solvents.

Experiment and Conditions	Mol Wt[a] ($\times 10^{-5}$)	No. of Subunits[b]
6 M Gdn·HCl		
Sedimentation equilibrium for samples both with and without reduction and alkylation	1.0 ± 0.1	4.4 ± 0.9
Sedimentation coefficients (s^0_{25}) for samples both with and without reduction and alkylation	1.3 ± 0.4	3.4 ± 1.0
Intrinsic viscosity for samples reduced and alkylated	1.1 ± 0.2	4.0 ± 1.2
pH 11.5		
Sedimentation equilibrium	1.2 ± 0.1	3.7 ± 0.7

[a] Measured or estimated as described in the text. [b] The number of subunits was calculated with a molecular weight of (4.4 ± 0.4) $\times 10^5$ for native ButChE (Table II). The deviations indicated in the subunit number are the maximum values expected.

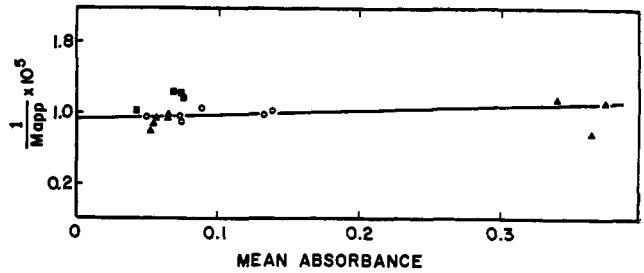

FIGURE 6: Relationship between $1/\bar{M}_{app}$ and mean absorbance at 280 nm of ButChE in 6 M Gdn·HCl. The symbols utilized are: (○), without reduction and (▲,■), with reduction and alkylation. Sedimentation equilibrium experiments were carried out at (○) 20,000, (▲) 28,000, and (■) 34,000 rpm. The line was drawn by eye through (▲) and (○) only.

As shown in Figure 5, the activity of ButChE after exposure to various values of pH corresponds closely with the relative amount of the 11S component. Reversibility of activity corresponds to that observed in the sedimentation studies.

The weight-average molecular weight of the 4S component of ButChE was determined by high-speed sedimentation equilibrium at pH 11.5 in the cold. In all runs the graph of ln c *vs.* r^2 was linear. This observation implies that the alkali-treated protein is homogeneous. With the assumed value of 0.78 for $\bar{v}$, $\bar{M}_w$ is (1.2 ± 0.1) $\times 10^5$, obtained by extrapolating $1/\bar{M}_{app}$ to zero concentration as described in Methods and illustrated by Figure 3. The second virial coefficient, A_2, from a least-squares plot was 4.3 $\times 10^{-3}$ mol cm^3/g^2. The much lower value of $\bar{M}_w$ for ButChE at pH 11.5 demonstrates that the protein is dissociated.

Denaturation by Gdn·HCl. Although ButChE is dissociated into subunits at high pH, these subunits may not represent the smallest components. Hence, the sedimentation behavior of ButChE in 6 M Gdn·HCl, with and without reduction of disulfide bonds, was studied. There is no significant difference in the sedimentation behavior of ButChE with or without reduction and alkylation of the disulfide bonds. A value of 2.6 S was obtained for $s^0_{20,w}$. This small sedimentation coefficient of the protein in 6 M Gdn·HCl led to decreased precision in the measurements, resulting in relatively high scatter in the data.

The $\bar{M}_w$ for ButChE in 6 M Gdn·HCl was determined by sedimentation equilibrium. Measurements were made on samples with and without reduction of disulfide bonds. Plots of ln c *vs.* r^2 for ButChE with or without disulfide bonds reduced were linear. The linear plots indicate that the subunits of ButChE are homogeneous within experimental uncertainty. Molecular weights were calculated from the equilibrium data and from a value of 0.76 for $\bar{v}$, estimated as described in Methods. The $\bar{M}_w$ was identical for ButChE with or without reduction of the disulfide bonds (Table III). A molecular weight of (1.0 ± 0.1) $\times 10^5$ was obtained by extrapolating appropriate plots of $1/\bar{M}_{app}$ *vs.* mean absorbance to zero concentration as shown in Figure 6. The second virial coefficient, A_2, is 1.6 $\times 10^{-3}$ mol cm^3/g^2. The same figure demonstrates that identical values of $\bar{M}_w$ were derived from measurements at various rotor speeds. Along with the linearity in the plots of ln c *vs.* r^2, this indicates that the ButChE subunits are identical in size within the limits of experimental uncertainty. The fact that $\bar{M}_w$ of ButChE in Gdn·HCl is identical with or without reducing agent indicates that there is no disulfide linkage between polypeptide chains and that the smallest single chains are obtained (Tanford *et al.*, 1967).

The possibility for existence of intrachain disulfide bonds was tested with reduced viscosity measurements of ButChE in 6 M Gdn·HCl. Owing to the lack of precision in measurement and difficulty in making concentration extrapolations, only average values of η_{sp}/c, the reduced viscosity (Yang, 1961), in 6 M Gdn·HCl could be obtained. The viscosity measurements were made with protein concentrations between 140 and 360 μg per ml for the reduced samples and 120 and 1360 μg per ml for the nonreduced samples. Corrections necessary for conversion of measured kinematic viscosities to reduced viscosities were negligible. The value of η_{sp}/c for the sample which was reduced and alkylated was 70 ± 6 ml/g, compared to 40 ± 5 ml/g for the untreated samples. The lower reduced viscosity for the untreated protein indicates that there may be intrachain disulfide bonds which maintain the subunits of ButChE in a more compact configuration (Tanford *et al.*, 1967). With the cleavage of such bonds the individual polypeptides may become true random coils, as implied by the increase in reduced viscosity.

Discussion

Previous studies have shown that purified horse serum ButChE is a homogeneous, stable enzyme which undergoes no self-association or dissociation in 0.01 M potassium phosphate, pH 7.0 (Lee and Harpst, 1971). These conclusions are supported by additional observations in this paper, which include: (a) the linear dependence of protein concentration on radial distance, observed in sedimentation equilibrium, and (b) identical values for $\bar{M}_w$ obtained from high- and low-speed equilibrium studies.

The $\bar{M}_w$ of ButChE determined both by sedimentation equilibrium and independently by light scattering is 440,000 ± 10% (Table II). Since the sedimentation coefficient and intrinsic viscosity of the protein have been determined, the molecular weight can be estimated from the Scheraga–Mandelkern–Flory equation (Mandelkern and Flory, 1952; Scheraga and Mandelkern, 1953) which relates the hydrody-

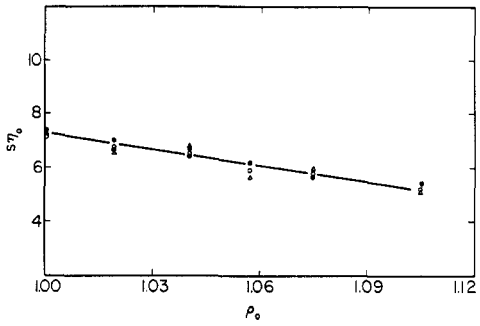

FIGURE 7: Relationship of $s\eta_0$ to ρ_0 at 20° in the presence of sodium chloride. Sedimentation behavior of ButChE was measured at three different protein concentrations. The symbols and concentrations are: (△) 1410 μg/ml; (●) 287 μg/ml; (○) 145 μg/ml. The line was obtained by least-squares analysis.

namic parameters to the molecular weight and to the shape factor, β. A molecular weight of 4.5×10^5 is obtained from the experimentally determined parameters, $[\eta] = 0.066$ dl/g, $s^0_{20,w} = 11.5$ S, $\bar{v} = 0.78$, appropriate solvent density and viscosity, and a value for β of 2.16×10^6, which is typical for globular proteins. This estimate of the molecular weight from hydrodynamic parameters agrees closely with the values from sedimentation equilibrium and light-scattering measurements.

The molecular weights reported in this study might be questioned because of the relatively high values obtained for A_2, when compared to an expected value around 10^{-4} mol cm^3/g^2 (Tanford, 1961), and because of some apparent inconsistencies in the data. It must be remembered, however, that the higher A_2 of 1.1×10^{-3} mol cm^3/g^2 was obtained from sedimentation equilibrium measurements in 0.01 M phosphate buffer, one of low ionic strength. In such a buffer, the net charge on the protein should be large enough for a net repulsion of solute molecules to be reflected in a large, positive second virial coefficient. If the net repulsion is screened by the addition of neutral salt, the values of A_2 should approach the range expected. This expectation is substantiated by our observation of a value of A_2 equal to 1.3×10^{-4} mol cm^3/g^2 from sedimentation equilibrium data for ButChE in 0.5 M NaCl–0.01 M potassium phosphate buffer (pH 7.0). Very nearly the same virial coefficient is obtained in 1.0 and 2.5 M NaCl. An inconsistency in the results appears on comparison of the high value of A_2 from light scattering (1.7×10^{-2} mol cm^3/g^2) with that from sedimentation equilibrium. However, the light scattering was performed at room temperature, while sedimentation was done near 5°. Clearly the results are consistent with the large increase in A_2 which is observed with increasing temperature in poor solvents (Tanford, 1961). Therefore, the high values of A_2 are reasonable, when solvent conditions and temperature of the measurements are considered, and the derived molecular weights should be reliable.

A second problem with the numerical data is the high value of $\bar{v}$, as mentioned in Results, and its effect on the molecular weights from sedimentation equilibrium. It may be argued that the result is too high. However, it is substantiated in this paper by at least two observations: (a) the agreement between molecular weights from sedimentation equilibrium and from light scattering, an independent method, and (b) the agreement between values of $\bar{M}_w$ for dissociated subunits from sedimentation equilibrium and from measurements of $s^0_{20,w}$ and $[\eta]$ (see below and Table III). Even with the indicated uncertainties in the measurements reported here, it is highly unlikely that the observed agreement in results from the three different types of molecular weight estimations is fortuitous. Therefore, the experimentally determined value of $\bar{v}$ has been used in this paper, rather than a less controversial, but assumed one. It should be noted that the major conclusions of this paper regarding the structure of ButChE (below) would not be affected seriously by a revision of $\bar{v}$.

Because of the uncertainty in $\bar{v}$, conclusions from a comparison of the molecular weight of native ButChE in this paper with other values in the literature are tentative. The molecular weight of 440,000 is significantly higher than the value of 315,000 reported recently (Main *et al.*, 1972) for horse serum ButChE, and is higher than the value of 368,000 obtained for porcine parotid cholinesterase (Tucci and Seifter, 1969). However, if an assumed value of $\bar{v} = 0.73$ is used, as was done by Tucci and Seifter, $\bar{M}_w$ from the sedimentation equilibrium results in this paper drops to 352,000. Although it is highly unlikely that the lower value is correct, further comparisons of molecular weights and drawing conclusions from them should await the confirmation of values for $\bar{v}$ or $\bar{M}_w$ by other techniques.

The general shape or physical classification of ButChE has been estimated from the measured hydrodynamic parameters. The ratio of frictional coefficients, f/f_0, is related to the asymmetry of a macromolecule (Tanford, 1961; Schachman, 1959), where f is the actual frictional coefficient of the molecule and f_0 is that for a rigid sphere with equivalent mass. Typical globular proteins have values of 1.14–1.31 for f/f_0 (Tanford, 1961). The value for native ButChE is 1.46, which represents the maximum frictional ratio including both shape and hydration factors. A value for maximum hydration (w) can be estimated by assuming that the particle is spherical. In this case the maximum hydration of ButChE is 1.64 g of H_2O/g of protein. Both values, 1.46 for f/f_0 and 1.64 g of H_2O/g of protein for w, are higher than those obtained for many globular proteins (Tanford, 1961). However, if w is 0.2 g of H_2O/g of protein (a plausible value which is often assumed in such calculations) the contribution of hydration to f/f_0 becomes 1.26 and the shape factor is reduced to 1.16. Hence, on the basis of frictional ratios alone ButChE may be considered to be substantially hydrated or, more likely, it is slightly asymmetric and hydrated.

The sedimentation behavior of ButChE in sodium chloride indicates that high ionic strengths neither dissociate the enzyme nor cause it to aggregate, even though ButChE does lose its activity. The linear increase in $s^0_{20,w}$ as a function of sodium chloride concentration (Figure 4A) may be caused by a shielding of charges in the native ButChE molecule so that the protein becomes more compact or by an alteration in effective hydrodynamic volume due to a change in hydration. Preferential hydration in such a system can be evaluated as recommended by Cox and Schumaker (1961). Figure 7 is a plot of $s\eta_0$ *vs.* ρ_0, where s is the observed sedimentation coefficient corrected to 20° and η_0 is the viscosity of the solvent at the same temperature. A linear relationship is obtained under the conditions studied. Extrapolation of the line to $s\eta_0 = 0$ gives an intercept at $\rho_0 = \rho_h$ (Cox and Schumaker, 1961), where there is no sedimentation and the system is in chemical equilibrium. The data in Figure 7 yield a value of 1.360 for ρ_h and 0.735 for $\bar{v}_h$, which is equivalent to the partial specific volume of the protein obtained by comparing the density of the protein solution and its dialysate at high salt concentrations. A value of −0.17 g of H_2O/g of protein is obtained for

the preferential hydration of ButChE in sodium chloride solutions. The negative sign implies that preferential binding of NaCl occurs (Cox and Schumaker, 1961). This analysis indicates that the effects of NaCl on sedimentation coefficients of ButChE are due to changes in hydration.

The absence of association or dissociation of ButChE in NaCl under the conditions studied is further substantiated by the results of sedimentation equilibrium experiments. A small increase in molecular weight with increasing NaCl concentrations has been shown (see Results). The higher values are those obtained when the effect of preferential solvation is ignored. To account for solvation the data have been analyzed as suggested by Aune and Timasheff (1970), who use measured values of s, η_0, ρ_0, and $\bar{v}$ in low salt concentrations to estimate ϕ', an apparent partial specific volume corrected for hydration, in 2.5 M NaCl. A value of 0.768 is obtained. The sedimentation equilibrium data in 2.5 M NaCl and ϕ' give $\bar{M}_w = 4.5 \times 10^5$, which is in good agreement with the molecular weight for native ButChE in low salt. Therefore, it must be concluded that ButChE neither associates nor dissociates in the presence of NaCl under the conditions studied, and all observed changes in sedimentation properties may be explained in terms of preferential solvation.

The sedimentation behavior of ButChE as a function of pH (Figure 4B) indicates that a drastic change in the quaternary structure of the protein occurs. The sedimentation behavior of ButChE in the pH range 10.3–10.8 (Figure 4B) gives a preliminary indication that the dissociation process follows an "all-or-none" pattern, represented by the equation $A_n \rightleftharpoons nA$. Over the limited concentration range studied, no intermediate sedimenting species have been detected, but at low initial protein concentrations (less than 0.5 mg/ml), the photoelectric scanner would not detect them.

Molecular weights of the ButChE subunits, derived from sedimentation equilibrium studies, are summarized in Table III. Confirmation of these results has been obtained from the values of $s^0_{20,w}$ and $[\eta]$ for the reduced and alkylated protein in 6 M Gdn·HCl, which have been used to estimate subunit molecular weights from the equations given by Tanford *et al.* (1967). The agreement of the calculated molecular weights (Table III) with those from sedimentation equilibrium measurements substantiates the assumption of a random coil structure for reduced and alkylated ButChE in 6 M Gdn·HCl.

All results summarized in Table III show that the average $\bar{M}_w$ for ButChE subunits is $(1.1 \pm 0.1) \times 10^5$ and the average number of subunits for native ButChE is 4.0 ± 0.7. On the basis of the somewhat more accurate equilibrium experiments, and known cases where tetrameric proteins exist, the most probable whole number of subunits is 4. This number is supported by appropriate values of $s^0_{20,w}$ and the relation proposed by Scheraga and Mandelkern (1953), which give the ratio of 4.1 between molecular weights for native and dissociated protein. However, it would be within experimental uncertainty to postulate a trimeric or pentameric structure. The estimate of four subunits agrees with recent reports by Main *et al.* (1972) on the horse serum enzyme, and by Scott and Powers (1972) on human serum cholinesterase.

Organophosphates, which are potent inhibitors of cholinesterases, are reported to act through phosphorylation of the serine present in the active sites of cholinesterases (Jansz *et al.*, 1959; Cohen *et al.*, 1959; Sanger, 1963). Titration of ButChE by $(CH_2CH_2)_4PP$ has indicated that there are four binding sites in each molecule of native enzyme (Lee, 1971). If it is assumed that each subunit possesses one binding site, then the inhibition study implies that there are four subunits in ButChE, which is consistent with the number of subunits determined by the physical methods.

In summary, the present work demonstrates that horse serum ButChE is a stable enzyme with a molecular weight of 4.4×10^5. Hydrodynamic properties indicate that native ButChE most likely is somewhat hydrated and slightly asymmetric. Under the conditions studied, the quaternary structure of ButChE is not changed in the presence of NaCl, a substrate analog, an organophosphate or activating divalent cations, even though the enzyme is affected kinetically by these reagents. Studies in Gdn·HCl and in alkali show that ButChE dissociates into four subunits, each with a molecular weight of 110,000. Present knowledge about ButChE is inadequate to explain satisfactorily the earlier reports of isozymes and other molecular forms of the enzyme (Heilbronn, 1962; Reiner *et al.*, 1965; Zech and Engelhard, 1965; Main, 1969). Further work is required to characterize more fully the subunits and their interactions, and to clarify the molecular basis for multimolecular forms of ButChE.

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Synthesis, Biological Activity, and ^{19}F Nuclear Magnetic Resonance Spectra of Angiotensin II Analogs Containing Fluorine†

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ABSTRACT: [Asp^1,*p*-fluoro-L-phenylalanine^4]-angiotensin II ([pFPhe^4]-AII) and [Asp^1,*p*-fluoro-L-phenylalanine^8]-angiotensin II ([pFPhe^8]-AII) were synthesized by the solid-phase procedure in order to study their biological activity and ^{19}F nuclear magnetic resonance (nmr) spectra. *p*-Fluoro-D,L-phenylalanine was resolved by enzymatic hydrolysis of *N*-trifluoroacetyl-*p*-fluoro-D,L-phenylalanine with carboxypeptidase A. [pFPhe^8]-AII is at least as potent as angiotensin II and [pFPhe^4]-AII is an antagonist of angiotensin II in the rat oxytocic, the rat blood pressure, and the prostaglandin release assay. The ^{19}F nmr spectra of [pFPhe^8]-AII show that the C-terminal carboxyl has a pK_a of 3.1, that above pH 7 two conformations exist, and that the rate of exchange between these two conformations is slow on the nmr time scale. A conformational change with a pK of 6.1 is the most likely cause of the chemical shift change seen in the ^{19}F nmr spectra of [pFPhe^4]-AII.

The replacement of hydrogen by fluorine has become a useful tool for pharmacological and structural analysis of organic compounds. The similarity of van der Waals' radii (H = 1.20 Å; F = 1.35 Å) and the dissimilarity of electronegativity (H = 2.1; F = 4.0) (Sheppard and Sharts, 1969) suggest that analogs with fluorine substituted for hydrogen might have interesting pharmacological activities. Fluorine replacement of hydrogen in corticosteroids, in pyrimidines (Goodman and Gilman, 1970), and in amino acids results in compounds with significantly altered properties. Fluorine amino acids can be either toxic or support the growth of bacterial auxotrophs (Loncrini, 1969). In particular pFPhe[1] is incorporated into *Escherichia coli* proteins and is bacterio-

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[1] Abbreviations used are: pFPhe, *p*-fluoro-L-phenylalanine; [pFPhe^4]-AII, [Asp^1,pFPhe^4]-angiotensin II; [pFPhe^8]-AII, [Asp^1,pFPhe^8]-angiotensin II; AII, [Asn^1,Val^5]-angiotensin II; A, 1-butanol–acetic acid–water (4:1:1); B, 2% acetic acid–acetone; C, 1-butanol–pyridine–acetic acid–water (15:10:3:2); D, 1-propanol–water (2:1); *t*-Boc, *tert*-butyloxycarbonyl.