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A Human Liver Aminopeptidase. The Amino Acid and Carbohydrate Content, and Some Physical Properties of a Sialic Acid Containing Glycoprotein†

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ABSTRACT: An aminopeptidase has been purified to homogeneity from human liver. The amino acid analyses indicate that this enzyme is quite high in tryptophan content and that cysteine and cystine content are below the level of detection. Carbohydrate analyses show that the peptide portion of the enzyme is conjugated with sialic acid, neutral hexoses, and glucosamine, and that these residues make up about 17.5% of the dry weight of the purified enzyme. The sialic acid content, 4.14%, is unusually high for a carbohydrate-containing enzyme. In denaturing solvents, the purified enzyme exhibits a molecular weight near 118,000 (polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate as well as in the presence of sodium dodecyl sulfate combined with urea, and sedimentation equilibrium in the presence of concentrated guanidinium chloride), and appears to behave ideally. In dilute salt solution, an equilibrium exists apparently between this species and a second species with a molecular weight near 235,000, presumably a dimer of the first species.

The aminopeptidases and particularly those enzymes of this group that catalyze the rapid hydrolysis of aminoacyl-β-naphthylamides have been the subject of continuing intensive study in our laboratory. Several enzymes belonging to this group of aminopeptidases have been identified in human tissues, and at least five distinct species separable by ion exchange chromatography are known to appear in blood (Behal *et al.*, 1964, 1965; Smith and Rutenburg, 1966; Panveliwalla and Moss, 1966). These five activities originate in specific organs, *i.e.*, one in the kidney, one in the duodenum, one in the liver, and two in the pancreas (Behal *et al.*, 1965).

This particular group of aminopeptidases is most active when Co^{2+} is present in the assay mixture (Thompson and Schwartz, 1959; Behal *et al.*, 1965, 1966, 1968; Behal and Little, 1968; and Behal and Story, 1969), although Co^{2+} is not the ion bound to the enzyme isolated from human liver (Garner and Behal, 1974). A broad range of substrate specificity is characteristic of this group of aminopeptidases, but aminoacyl-β-naphthylamides with nonpolar aminoacyl residues are preferred. L-Alanyl-β-naphthylamide is the most rapidly hydrolyzed substrate, and the enzyme has very low activity with acidic (*e.g.*, L-aspartyl-) and β-branched (*e.g.*, L-valyl-) β-naphthylamides. In addition, D-aminoacyl-β-naphthylamides are not hydrolyzed, a free α-amino group is required for enzymatic activity, and peptides are sequentially hydrolyzed from the N-terminus (Behal *et al.*, 1968). These features provide a unique opportunity to study a variety of enzyme-effector interactions.

While considerable information has been obtained about the use of human aminopeptidases in diagnosis of disease, little study has been directed toward defining the chemical and physical properties of these aminopeptidases.

This report describes some of the physical properties and the amino acid and carbohydrate content of an aminopeptidase isolated from human liver. A companion paper (Garner and Behal, 1974) describes the metal ion composition and role of metal ions in the activity of the enzyme.

Experimental Section

Materials

Except as otherwise indicated, all inorganic chemicals used were either Fisher, Baker and Adamson, J. T. Baker, or Mallinkrodt reagent grades. No differences resulted from the substitution of one brand for another. Fisher Chemical Co. was the source of β-naphthylamine hydrochloride, tris(hydroxymethyl)aminomethane (THAM, Tris), and *N*-(1-naphthyl)ethylenediamine dihydrochloride. Protein standards for gel electrophoresis and Sephadex G-200 chromatography were obtained from Worthington, Sigma Chemical Company, P-L Biochemicals, Inc., and Schwarz/Mann. Guanidinium chloride, *N,N,N′,N′*-tetramethylethylenediamine, Amido Schwarz, Coomassie Brilliant Blue, thioglycolic acid, dithiothreitol, neuraminidase, 2-mercaptoethanol, and sodium dodecyl sulfate

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TABLE I: Summary of Purification.

Fraction	Protein (mg)	Activity (μmol/min)	% Yield	Specific Activity (μmol min^{-1} mg^{-1})	Purification
I. Autolyzed and clarified homogenate	103,000	1990	100	0.0193	1
II. Bio-Glas, $(NH_4)_2SO_4$	4,720	2590	130	0.521	27
III. Sephadex G-200	578	2560	129	4.43	229
IV. DEAE-cellulose	63.7	1090	55	17.1	887
V. Hydroxylapatite[c]	18.0 (11.5)[a]	824	41	45.7 (71.7)[a,b]	2370

[a] These figures are based on the extinction coefficient of the purified protein. [b] The specific activity of the native protein is usually 30–50% greater in the presence of Co^{2+} ion although this stimulation is variable. [c] This step may be omitted from the purification if the Bio-Glas step is done twice. No alteration in the quality of the preparation results.

were purchased from Sigma Chemical Co., and substrates for the activity assay were purchased from Schwarz/Mann. Acrylamide and *N,N'*-methylenebisacrylamide were the products of Eastman Chemical Co. and Canalco, Inc., respectively. Bio-Glas 200 and DEAE-cellulose were obtained from Bio-Rad. Sephadex G-200 was the product of Pharmacia Fine Chemicals, and modified hydroxylapatite (Hypatite-C) was obtained from Clarkson Chemical Co. Glass-distilled water was used for all solutions.

Buffers used in the preparation of the purified protein are listed elsewhere (Little, 1970). Buffers used in the ultracentrifugation experiments described here are as follows: buffer A, 0.005 M potassium phosphate (pH 7.0)–0.1 M potassium chloride; buffer B, 0.005 M potassium phosphate (pH 7.0)–6 M guanidinium chloride–0.05 M dithiothreitol.

Methods

Aminopeptidase Assay. A colorimetric assay developed for this enzyme was used to assay for activity during the purification (Little, 1970). In a total volume of 1.5 ml, the components of the reaction mixture (pH 6.8) were Tris (3 mM), L-alanyl-β-naphthylamide (1 mM), cobaltous chloride (5 mM), manganous chloride (5 mM), magnesium chloride (5 mM), and rate-limiting enzyme. After incubation (37°, 30 min) the reaction was stopped by addition of 0.5 ml of 2.5 M trichloroacetic acid. The liberated β-naphthylamine was measured as follows: sodium nitrite (1.0 ml, 15 mM) was added to the reaction mixture, and after 3 min, the excess was decomposed by addition of ammonium sulfamate (1.0 ml, 45 mM). *N*-(1-Naphthyl)ethylenediamine dihydrochloride (2.0 ml, 2 mM in 95% ethanol) was added, and after 30 min the optical density of the blue solution was determined at 580 nm. The activity was calculated from a standard curve and the units of specific activity are μmol min^{-1} mg of $protein^{-1}$.

Protein Assays. Protein was assayed throughout the purification using the optical density ratio method (Warburg and Christian, 1942), and the specific activities reported in Table I are calculated using this assay. The amount of the purified protein and the specific activity based on the extinction coefficient of the protein are also reported in Table I for the purified fraction only. Subsequent estimates of protein concentrations are based on the extinction coefficient of the protein.

Purification Procedures. The purification procedures used here are essentially identical with those reported previously (Little, 1970) with the exception that the Bio-Glas procedure was changed to a batch process and was utilized twice to improve the efficiency of later steps. The Sephadex G-200 column was calibrated prior to use in the purification, and the elution volume of the activity was used to estimate a molecular weight (Andrews, 1965). The last sample prepared for this study omitted the hydroxylapatite column, since experience indicated that the second Bio-Glas column and the hydroxylapatite column removed essentially the same impurities, and the purified enzyme loses considerable activity on the hydroxylapatite column.

Analytical Acrylamide Gels. The procedure used here for analytical acrylamide gels has been previously described (Starnes *et al.*, 1972) and is essentially the procedure of Davis (1964). Gels were scanned spectrophotometrically with a Beckman Acta III spectrophotometer equipped with a gel scanning device.

Amino Acid Analyses. The amino acid analyses on both oxidized and nonoxidized samples of the protein were done here as outlined for another protein elsewhere (Starnes *et al.*, 1972). On a standard program for analysis of amino acids, glucosamine and tyrosine are not resolved. Therefore, tyrosine and glucosamine were resolved separately (27.5 × 1.0 cm column, pH 5.25, 0.2 N citrate, 55°). A Beckman 121-HP automatic amino acid analyzer with the appropriate Beckman resins was used here.

Analysis of the Conjugated Carbohydrates. Sialic acid(s) was assayed by two different methods. The resorcinol reaction (Svennerholm, 1957; Spiro, 1966) was used to assay for sialic acid(s) which remained bound to protein, and the thiobarbituric acid reaction (Warren, 1959; Aminoff, 1961; Spiro, 1966) was used to assay for sialic acid(s) removed from the protein by action of neuraminidase (1 hr, 37°, pH 7.0, 0.005 M phosphate).

Total hexoses were assayed by a tryptophan–sulfuric acid reaction (Shetlar *et al.*, 1948), and amino sugars were assayed during amino acid analysis of the protein.

Calculation of the Partial Specific Volume. The partial specific volume, $\bar{v}$, of the enzyme, 0.730 cm^3/g, was estimated from the amino acid and carbohydrate content of the protein (Cohn and Edsall, 1943; McMeekin and Marshall, 1952). The effective specific volume, $\bar{v}^*$, in 6 M guanidinium chloride was assumed to be 0.720 cm^3/g. (See Reisler and Eisenberg (1969) for the considerations which justify this reduction.) For this computation, the volume of the carbohydrate residues was considered to be that of an average amino acid residue.

The absorption spectrum of the native enzyme was determined on a solution of the enzyme in a 1-cm cell in an Acta III uv–visible spectrophotometer.

Determination of the Extinction coefficient of the Native Enzyme. The extinction coefficient of the enzyme was obtained by each of three methods which differed by the manner in which the dry weight of the enzyme was determined. Dry weights were determined as follows: (1) the dry weight of a ly-

ophilized sample was directly determined; (2) the fringe count obtained from a photograph of an artificial boundary between solvent and solution in the ultracentrifuge was corrected to units of concentration using an average refractive increment of 4.1 fringes mg^{-1} ml^{-1} (Babul and Stellwagen, 1969); and (3) the dry weight was determined by summation of the weights of the aminoacyl and carbohydrate residues obtained from amino acid and carbohydrate analysis of a quantity of the enzyme, the absorbance of a solution of which had been measured prior to hydrolysis and analysis. The extinction coefficient has also been estimated from the tryptophan and tyrosine content of the enzyme. Here the extinction coefficients of tyrosine and tryptophan in 6 M guanidinium chloride (Edelhoch, 1967) were used, and the ratio of absorbance of the enzyme in buffer B to absorbance of the same amount of enzyme in dilute buffer was used to correct for the shift of the spectrum due to denaturation of the enzyme.

Acrylamide Gels in the Presence of Sodium Dodecyl Sulfate. The sodium dodecyl sulfate polyacrylamide gel experiments were performed exactly as indicated by Weber and Osborn (1969) with the exception that the dialysis step was omitted in experiments not requiring it. Experiments were performed both in the presence and absence of urea without alteration of the results. In addition, enzyme treated with excess sulfhydryl reagent or with 6 M guanidinium chloride followed by dialysis into urea and sodium dodecyl sulfate before electrophoresis in the presence of urea did not produce significant differences in the results. The molecular weights for the standard proteins were taken from Klotz and Darnall (1969), or they were supplied with the standard by the manufacturer.

Cellulose Acetate Electrophoresis. Cellulose acetate electrophoresis was done in a Beckman Microzone apparatus with Beckman equipment. Staining with Ponceau S for protein and with fuchsin-sulfite reagent for bound carbohydrate was done according to a manual on electrophoretic procedures supplied by Beckman Instruments. The densitometric traces were obtained with a Densicord densitometer.

Preparation of the Native and Dissociated Enzyme for Equilibrium Experiments. The homogeneity of the samples for sedimentation equilibrium was confirmed by electrophoresis on acrylamide gels in the presence and absence of denaturants (sodium dodecyl sulfate and urea). A solution of the enzyme (usually 1-2 ml of the solution with about 3-6 mg/ml of enzyme) was dialyzed at least overnight against 250 ml of buffer A. The optical density of the solution was determined on an aliquot, and the concentration in mg/ml was estima A portion was then separated for the equilibrium experiments on the enzyme in dilute salt solution, and the remaining portion was utilized to prepare a sample of the enzyme in denaturing solvents.

About 1 ml of the solution of the enzyme in buffer B was dialyzed at least 4 days at 5° against buffer B. The volume of the protein solution decreased with time of dialysis, but the approximate original volume was restored by addition of dialysate at the end of the dialysis time.

Ultracentrifuge Experiments. All ultracentrifugal experiments performed here were done by the high-speed meniscus depletion technique as described elsewhere (Starnes *et al.*, 1972; Munk and Cox, 1972). The evaluation of the experimental data taking into account the possibility of nonideality is essentially the treatment of Munk and Cox (1972). The value of the apparent weight average molecular weight of the enzyme as a function of concentration at a radius r in the centrifuge cell was estimated essentially according to the method of Yphantis (1964), and Figure 3 is plotted as in Teller *et al.* (1969). Experiments in nondenaturing solvents were done at several

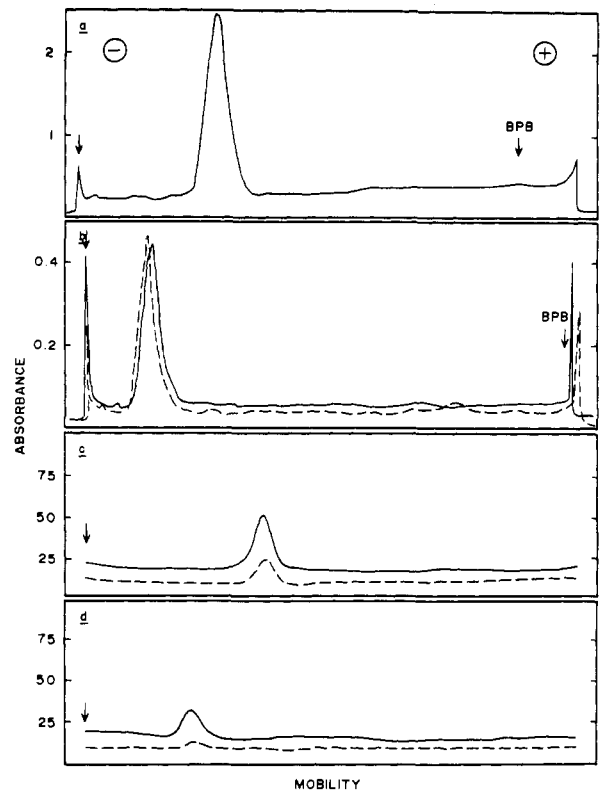

FIGURE 1: Acrylamide gel and cellulose acetate electrophoresis of human liver aminopeptidase. (a) Spectrophotometric scan (600 nm) of human liver aminopeptidase (about 200 μg) on analytical disc gels stained with Amido Schwarz. (b) Spectrophotometric scan (580 nm) of human liver aminopeptidase (about 50 μg) on sodium dodecyl sulfate polyacrylamide gels in the presence of urea (- - -) and the absence of urea (—). Coomassie Brilliant Blue was the stain here. (c) Densitometric scan of human liver aminopeptidase (about 30 μg) on cellulose acetate membranes. The stain for protein (—) was Ponceau S, and the stain for carbohydrate was fuschin-sulfite (- - -). (d) The details and stains for protein (—) and carbohydrate (- - -) are the same as in (c) except that here the enzyme was treated with neuraminidase prior to electrophoresis. Bromophenol Blue (BPB) was the reference marker for (a) and (b). Absorbance for (c) and (d) is given in chart units, and mobilities have been standardized graphically to permit the presentation of this data in one figure.

temperatures and speeds. As is the custom (Yphantis, 1964; Munk and Cox, 1972), only fringe displacements greater than 0.1 mm were used in the evaluation of the data. Checks and corrections for nonzero meniscus concentrations have been routinely made by the computer program used to calculate the results of the experiments. Corrections for optical distortions were made from photographs of interference patterns generated by light passing through a water-filled cell.

Results

Purification and Homogeneity of the Native Enzyme. The preparation of the enzyme results in an apparently homogeneous protein as indicated by disc gels (Figures 1a and 1b) in nondenaturing as well as denaturing solvents. The specific activity of the enzyme purified here (Table I) is slightly higher than the specific activity previously reported, but the difference is not significant (Little, 1970). in addition, specific activity of the enzyme varies according to the length of preincubation of the enzyme at elevated temperatures or according to length of preincubation with Co^{2+} (Garner and Behal, 1974). The ultraviolet spectrum (not shown here) of the enzymatic preparation is a typical protein spectrum, and chromophores which absorb in the visible region are absent. The preparation of the enzyme

TABLE II: Amino Acid and Carbohydrate Content of a Human Liver Aminopeptidase.

Residue	g/100 g of glycoprotein	± SD	Nearest Integer per 118,000 daltons
Amino acids			
Lysine	4.41	0.21	41
Histidine	2.18	0.36	19
Arginine	4.66	0.70	35
Tryptophan[b]	4.91		31
Aspartic acid[a]	10.19	0.23	105
Threonine[b]	4.59		54
Serine[b]	4.23		57
Glutamic acid[a]	10.52	0.66	96
Proline	3.65	0.38	44
Glycine	2.06	0.09	43
Alanine	3.46	0.09	58
Cysteine[d]	Trace		
Valine[c]	4.48		53
Methionine[b]	1.80		16
Isoleucine[c]	3.34		35
Leucine	7.75	0.26	81
Tyrosine	5.81	0.33	42
Phenylalanine	4.50	0.17	36
Total % amino acids	82.54	Total residues · amino acids	846
Carbohydrates			
Glucosamine[b]	4.32		29
Sialic acid(s)	4.14		16
Hexoses	9.00		62
Total % carbohydrates	17.46	Total residues carbohydrates	107

[a] Free acid plus amide. [b] Corrected to zero hydrolysis time. [c] Corrected to infinite hydrolysis time. [d] As cysteic acid.

involves a gel chromatography step on Sephadex G-200. The elution volume of the activity is characteristic of a globular protein with a molecular weight of 242,000 ± 21,000 (Andrews, 1965).

Extinction Coefficient of the Enzyme. The extinction coefficient of the enzyme as estimated by the techniques outlined in methods is 1.75 ± 0.18 mg^{-1} ml^{-1}. The value calculated from the tryptophand tyrosine content of thenzyme is slightly higher and the value obtained from the dry weight of the enzyme is somewhat less. The most consistent value is obtained by the differential refractometric method of Babul and Stellwagen (1969) for estimation of protein concentration.

Carbohydrate Conjugation with the Protein. Proof that the carbohydrate residues are conjugated to the peptide portion of the purified enzyme is shown in Figure 1c and d. Specific staining for protein and for carbohydrate demonstrates that both types of residues comigrate (Figure 1c). When the terminal sialic acid residues are removed by neuraminidase, the mobility of the two types of residues is reduced, but both types of residues again comigrate (Figure 1d).

Amino Acid and Carbohydrate Content of the Complex. Table II shows the average amino acid and carbohydrate composition of four samples of the enzyme isolated from human liver. The protein is quite high in tryptophan, which is consistent with the relatively high extinction coefficient of the enzyme, and cysteine and cystine are below detectable limits. The nearest integer composition computation assumes that the metal ion (Garner and Behal, 1974) and any as yet unidentified conjugated material, if any, make negligible contributions to the molecular weight of the complex. One major peak in the analysis does not elute with the standard amino acids in the nonoxidized samples. This peak elutes coincidentally with glucosamine, and the 570 to 440 nm optical density ratio for the unknown is identical with that for glucosamine. The proportion of glucosamine decreases slowly with time of hydrolysis. Therefore, it is likely that this residue is present largely as the *N*-acetyl derivative, since free glucosamine should be readily destroyed by these hydrolysis conditions (Spiro, 1966). The other carbohydrates shown were obtained by assays which are specific for the residues indicated in the table, but the results are less precise. The total carbohydrate content is 17.46%.

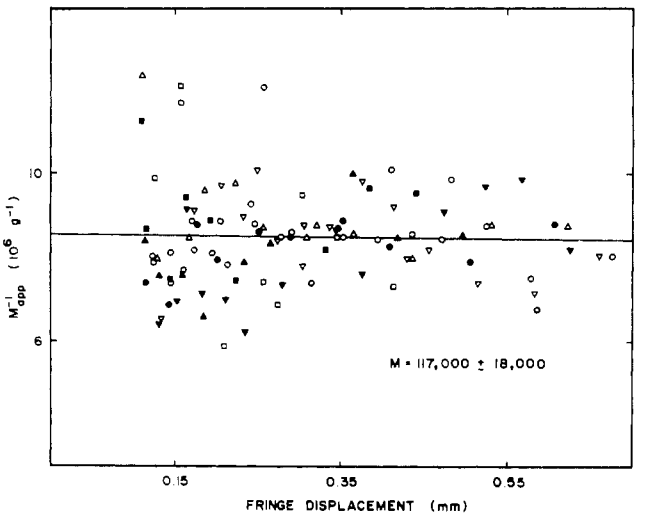

FIGURE 2: The M_{app}^{-1}-type plot (Munk and Cox, 1972) for human liver aminopeptidase in 6 M guanidinium chloride. Two different experiments are shown. Experiment 1: Sedimentation equilibrium was obtained in buffer B (c_i = 0.18 mg/ml) after about 24 (△), 48 (○), 72 (●), and 96 (▲) hr at 24,000 rpm. Experiment 2: Sedimentation equilibrium was obtained in buffer B (c_i = 0.21 mg/ml) after 24 (□), 48 (■), 72 (▽), and 96 (▼) at 26,000 rpm.

Molecular Weight of the Aminopeptidase in Denaturing Solvents. Five different enzyme preparations were examined by acrylamide gel electrophoresis in the presence of sodium dodecyl sulfate. The result was nearly identical from preparation to preparation, and the average of all experiments was 120,000 ± 12,000. The relative mobility of the protein on these gels is not significantly altered by the presence of urea (Figure 1b), by an increase or decrease in the concentration of the gels, or by an increased concentration of sulfhydryl reagent. Furthermore, extensive dialysis against 6 M guanidinium chloride followed by dialysis into urea and then by electrophoresis in the presence of urea did not alter the results.

In spite of the uncertainty in the estimation of the partial specific volume of the enzyme, and, thus, the uncertainty in the apparent specific volume, the molecular weight of the protein as determined by sedimentation equilibrium experiments in 6 M guanidinium chloride is essentially the same value as that observed in the polyacrylamide gel experiments in the presence of sodium dodecyl sulfate. M_w-type plots, ln c *vs.* r^2, and M_z-type plots, $(1/r)(dc/dr)$ *vs.* c, are linear throughout the solution column of the centrifuge cell. Figure 2, an M_{app}^{-1}-type plot derived from a typical experiment in which $\bar{M}_w^*$ = 116,000 ± 2000 and $\bar{M}_z^*$ = 123,000 ± 5000, indicates that, in spite of the relatively large molecular weight, the denatured protein behaves ideally. (Nonideality would be demonstrated

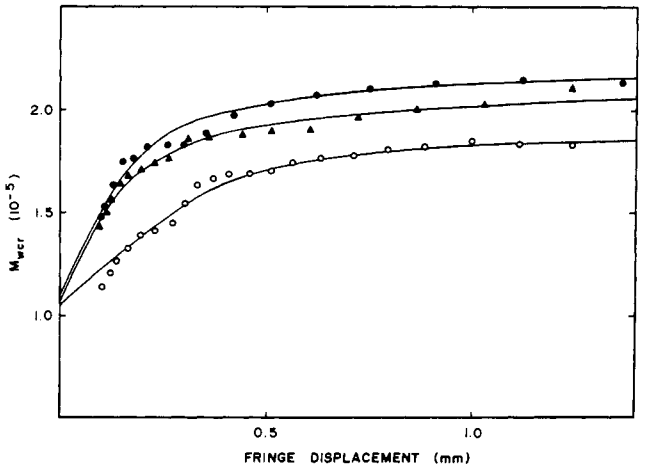

FIGURE 3: The apparent weight average molecular weight (M_{wcr}) of human liver aminopeptidase *vs.* the concentration at radius r and at three temperatures. Sedimentation equilibrium was established (buffer A) after 72 hr at each of three conditions of temperature: 5.82°, 15,000 rpm (●); 20.00°, 15,000 rpm (△); and 29.81°, 18,000 rpm (○). The initial concentration, c_i = 0.21 mg/ml, was guaranteed by using the same cell at all conditions. The lines drawn are empirical and are constrained to pass through the same intercept (see text).

by a positive slope in Figure 2 and by a nonzero intercept in an M_z-type plot according to Munk and Cox (1972). This type of plot is very sensitive to heterogeneity, but in spite of the inherent imprecision, the molecular weight, 117,000 ± 18,000, demonstrates, when taken with the other equilibrium data, that the denatured enzyme is homogeneous. The average molecular weight obtained from all equilibrium experiments in denaturing solvents is 118,000 ± 5000.

Sedimentation Equilibrium in Nondenaturing Solvents. Figure 3 shows the results of sedimentation equilibrium experiments in nondenaturing solvents. Here M_{wcr} is plotted *vs.* concentration. (If M_{zcr} is used in the plot, the result is the same but is less precise. Plots of ln c *vs.* r^2 and $(1/r)(dc/dr)$ *vs.* c, M_w-type and M_{rz}-type plots, respectively, show upward curvature characteristic of a heterogeneous solute.) Apparently the predominant form of the enzyme as isolated from liver exhibits a molecular weight approximately twice the molecular weight of the species obtained in denaturing solvents. This result appears to confirm the result obtained from sedimentation and diffusion experiments (at higher concentrations, mol wt 235,000 ± 10,000, Little and Behal (1971)) and from gel chromatography. However, near the meniscus in the region of very low solute concentration, the weight average molecular weight is near the molecular weight of the species found in denaturing solvents. Here the average intercept at zero concentration as estimated by linear regression of all points between 0.1 and 0.2-mm fringe displacement is 106,000 ± 19,000. For theoretical reasons, the curves drawn in Figure 3 have been constrained to pass through this point, but the intercepts of each curve were estimated separately. The curve at 30° indicates that a species somewhat smaller than 106,000 may be present in significant amounts at higher temperatures. However, the potential for systematic error is very high in this region of the plot. If the material maintained at equilibrium at 5.82° and at 29.81° is returned to equilibrium at 20°, the curve for 20° is reproduced. Therefore, there exists a dynamic equilibrium between the various species prnt in the cell. Presumably, equilibrium is between the species obtained in denaturing solvents and the species observed at higher concentrations. The experimental asymptote[1] for all experiments like that in Figure 3 averaged 223,000 ± 17,000.

TABLE III: Summary of Properties of Human Liver Aminopeptidase.

Property	Method	Value obtained
Molecular weight of the enzyme in dilute buffers	Estimated from high speed sedimentation equilibrium	223,000 ± 17,000
	Svedberg method[a]	235,000 ± 10,000
	Gel filtration	242,000 ± 21,000
Molecular weight of the enzyme in denaturing solvents	Sedimentation equilibrium in guanidinium chloride	118,000 ± 5,000
	Sodium dodecyl sulfate gel electrophoresis	120,000 ± 12,000
Sedimentation coefficient ($s_{20,w}°$)	Sedimentation velocity[a]	10.1×10^{-13} sec^{-1}
Diffusion coefficient ($D_{20,w}°$)	Synthetic boundary diffusion[a]	4.02×10^{-7} cm^2/sec
Partial specific volume ($\bar{v}$)	From amino acid content	0.730 cm^3/g
Extinction coefficient ($E_{280\ nm}^{0.1\%}$)	Uv spectrum and dry weight	1.75 mg^{-1} ml^{-1}

[a] Little and Behal (1971).

Discussion

The results reported here and summarized in Table III (along with information from another source, Little and Behal (1971)) show that this aminopeptidase has a well-defined molecular weight in denaturing solvents near 118,000 and suggest that this species, in dilute salt solution near neutral conditions, exists in equilibrium with another species, presumably a dimer, with a molecular weight near 235,000 (Little and Behal, 1971). The molecular weight obtained here by extrapolation to an experimental asymptote at infinite concentrations, mol wt 223,000 ± 17,000, approaches the value previously obtained, but theoretical and practical considerations limit the precision and accuracy of this extrapolation.[1] As the equilibrium is studied further and as the partial specific volume of the purified enzyme becomes more accurately known, some refinements of these numbers will undoubtedly be necessary. The species with a molecular weight near 118,000 appears homogeneous in sedimentation equilibrium experiments in guanidinium chloride and in polyacrylamide gel experiments done in the presence of sodium dodecyl sulfate. Therefore, the dimeric species is apparently composed of subunits of identical molecular weight.

During the preparation of the enzyme, the molecular weight of the soluble activity has been estimated by gel chromatography to be 242,000 ± 21,000. The chromatographic step in the purification is at a relatively high protein concentration and is, relative to sedimentation equilibrium, a more rapid process. Furthermore, low concentrations of active enzyme at different

[1] M_{wcr} will approach an asymptote which will be near the molecular weight of the n-mer in an ideal system at practical experimental concentrations if the equilibrium constant for the monomer-n-mer association is sufficiently large. Nonideality and experimental imprecision near the solution-bottom fluid interface make an extrapolation of M_{wcr} to infinite concentrations uncertain, but the result is informative.

elution volumes would not be observed under the conditions of these experiments, and inactive material would be undetectable in the presence of contaminating proteins. Likewise, the previous estimate of the molecular weight of what appears to be a dimeric species was made at relatively high concentrations with respect to the experiments performed here by sedimentation equilibrium. A significant amount of the species with mol wt 118,000 is observed only at protein concentrations much lower than 1 mg/ml. Nevertheless, sedimentation equilibrium measurements of the apparent weight average and apparent "z"-average molecular weights show that the presence of the dissociated material produces significant curvature in the appropriate plots up to initial loading concentrations of at least 2 mg/ml (W. L. Starnes and F. J. Behal, unpublished results).

Some glycoproteins have been observed to behave differently from other soluble proteins in polyacrylamide gel electrophoresis done in the presence of sodium dodecyl sulfate (Segrest and Jackson, 1971). For this reason, cauon inn the interpretation of the results reported here has been exercised. Guanidinium chloride has been shown in many cases to reduce ordered three-dimensional structures of proteins to random coils. Ideal behavior of a random coil in a concentrated salt solution is not routinely expected (Munk and Cox, 1972). If large molecules exhibit ideal behavior under these conditions, it may be that the solute remains in a fairly compact three-dimensional structure. Since this protein exhibits ideal behavior here, there must be concern that the species reported by Little and Behal (1971) with a molecular weight near 38,000 might actually be present here as a trimer. Although there is a suggestion from the equilibrium work that a species smaller than 118,000 might be present particularly at higher temperatures, efforts to obtain a more highly dissociated species have so far failed.

Following the homogenization in the preparation of the enzyme, most of this activity remains in an insoluble fraction, presumably bound to membranes. Autolysis for approximately 24 hr at 37° (longer at reduced temperatures) is required to release this activity into the soluble fraction. Therefore, any proposed structural relationship between the insoluble and soluble forms of the activity is tenuous. Nevertheless, this particular activity is found in blood, and the purified enzyme should be similar to the circulating species.

The carbohydrate content of the purified enzyme is similar to the carbohydrate content of the haptoglobins, both in the total and relative amounts of each type of residue (Schultze *et al.*, 1963). Relatively few proteins with enzymatic activity are also described as glycoproteins, and, of those, only a very few have significant amounts of sialic acid associated with the conjugated carbohydrate (*e.g.*, Montgomery, 1970). Recently, two aminopeptidases have been purified from pig kidney (Lehky *et al.*, 1973) and pig intestine (Maroux *et al.*, 1973), respectively. Both are glycoproteins containing 20–23% carbohydrate, both are zinc metalloenzymes, both have a molecular weight near 280,000, and both are complex enzymes with subunit structure. Comparatively, the major differences in carbohydrate content between the human liver enzyme reported here and these two porcine activities are that both porcine enzymes are quite high in the neutral hexoses and the amino sugars and quite low in sialic acid. Ribonuclease B (Plummer and Hirs, 1963) and glucose oxidase (Pazur *et al.*, 1965) have similar amounts of neutral hexoses and hexosamines (*N*-acetyl derivatives), but are, respectively, low and lacking in sialic acids.

The role of sialic acids and other carbohydrates in the function of proteins is not established. It has been suggested that high sialic acid content is a property only of proteins to be released into circulation and that the same proteins would contain little or no sialic acid in the cell. Since this aminopeptidase is apparently membrane bound, this explanation is not appropriate. It may be that conjugated carbohydrates confer a degree of immunologic specificity on the enzyme, or protect the protein from itself and other peptidases.

No attempt has been made to determine whether the glucosamine residues in this aminopeptidase are present as the acylated derivatives, but most glucosamine in glycoproteins is present in the N-acylated form (Montgomery, 1970). The type of sialic acid (or acids) has not been determined, although the neuraminidase preparation used prefers a terminal *N*-acetylneuraminic acid as its substrate. The sialic acid is located on the terminus as the amount released by neuraminidase is identical with the amount found by analysis of the total conjugated sialic acids. The various hexoses present have yet to be identified, and this introduces some uncertainty into the determination of total hexose content, and more than usual uncertainty into the computation of the partial specific volume.

The potential for different isozymes with respect to the structure of the peptide portion of the molecule appears limited. However, there remains considerable potential for variation in the carbohydrate portions of the molecule. The other organ specific activities found in circulation may be different simply in the amount and type of carbohydrate conjugated with the peptide. In fact, the separation of the five activities found in human blood was achieved by ion exchange chromatography (Behal *et al.*, 1965), a method based largely upon charge differences between molecular species. The loss or addition of sialic acid residues provides a way in which charge differences might arise between otherwise quite similar glycoproteins. Differences in sialic acid content lead to different relative mobilities in electrophoresis and, in fact, one such electrophoretic variant which contained less than normal sialic acid was obtained from the purification of the liver enzyme (W. L. Starnes and F. J. Behal, unpublished results).

Because of the lability of the sialic acid residues consideration has been given to alternate purification procedures which would permit the deletion of the autolysis process, since it is probable that this is the step in which uncontrollable degradation would occur. Detergent extraction of the particulate matter after homogenization of the liver has not been successful so far (Little, 1970), but efforts are continuing to find a suitable means of extraction of the activity without autolysis.

The physiological role of aminopeptidases within cells and in circulation is not well established. Many suggestions of possible functions in the biochemical processes or organisms have been made. Because the enzymes have such broad substrate specificities with respect to the ultimate and penultimate amino acid residues of peptides (*e.g.*, Behal *et al.*, 1968), it appears likely that no single specific role exists for th aactivity. The enzymes could be responsible for turnover of several peptide hormones, for hydrolysis of peptides absorbed from the intestine, or any of a multitude of different processes. Since no ability to hydrolyze native proteins has been established, it is unlikely that this particular activity has a role in "general protein turnover." Sialic acid containing glycoproteins have been implicated in the processes of cellular and circulating immune responses and particularly in antibody recognition of specific cell surface antigens. A role could exist for this and other similar circulating and membrane bound aminopeptidases in the defense mechanisms against invasion of an organism or cell by foreign materials.

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Human Liver Aminopeptidase. Role of Metal Ions in Mechanism of Action†

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ABSTRACT: Human liver aminopeptidase is activated 2.4-fold by Co^{2+} which binds in the uncompetitive or coupling fashion. The large dissociation constant (K_a = 50 μM) indicates that the enzyme-Co^{2+} complex dissociates readily, thus preventing the isolation of the intact complex. The enzyme also contains 8.3 ± 1.5 nmol of zinc/mg of protein (*i.e.*, 1 mol of Zn/122,000 ± 20,000 g of protein) and is inhibited by several chelators and complexing agents. Aminopeptidase is inhibited by ethylene glycol bis(β-aminoethyl ether)-*N,N'*-tetraacetic acid (an EDTA analog) and by 1,10-phenanthroline each in the competitive manner with K_i = 0.012 and 0.028 mM, respectively. Sulfide also inhibits but with kinetics of the mixed type, K_i = 0.076 mM. Inhibition by these and other agents is reversible by removal of the agent through dialysis or gel filtration, by dilution, or by titration with divalet metal ions. The presence of divalent transition metal ions also prevents chelator inhibition. When aminopeptidase is treated at 60° with EDTA, the inhibition is no longer reversible and there is a concomitant loss of zinc. The data suggest that the enzyme forms a cobalt metalenzyme complex and is a zinc metalloenzyme, zinc being located near the substrate binding site.

Enzymes which catalyze the hydrolysis of aminoacyl-β-naphthylamides have been found in numerous human tissues (Behal *et al.*, 1965; Panveliwalla and Moss, 1966; Hopsu-Havu and Makinen, 1967; Marks *et al.*, 1968) and in serum, especially during hepatobiliary disease (Goldbarg and Rutenburg, 1958). Many of the aminopeptidases have been called "arylamidases" after the suggestion of Patterson *et al.* (1963), but amino acids are cleaved from the N-terminal end of numerous substrates having no aryl group, including simple dipeptides.

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