Human Kidney Alanine Aminopeptidase: Physical and Kinetic Properties of a Sialic Acid Containing Glycoprotein†

Y. John Kao, W. L. Starnes, and F. J. Behal*

ABSTRACT: Human kidney alanine aminopeptidase has been purified to apparent homogeneity as judged by electrophoresis and sedimentation in the analytical ultracentrifuge. Amino acid analyses indicate that the enzyme is high in tryptophan content and low in cysteine content. The enzyme contains sialic acid, hexoses, and glucosamine, which make up 21% of its dry weight. In dilute buffer, the enzyme exhibits a molecular weight near 236 000, but in denaturing solvents the enzyme exhibits a molecular weight near 119 500. Zinc analyses by atomic absorption demonstrate 1 mol of zinc for 113 500 ± 6900 g of protein. The zinc is firmly bound, since exhaustive dialysis against chelating agents does not remove the zinc or inactivate the enzyme. The enzyme is stimulated by Co^{2+} 1.65-fold, but, in contrast to the enzyme–zinc complex, the enzyme–cobalt complex dissociates upon dialysis. Kinetic studies with a series of aminoacyl-β-naphthylamides indicated that the highest k_{cat} value was obtained for L-alanyl-β-naphthylamide (8.43×10^3 s^{-1}), whereas the lowest K_m value was obtained for L-methionyl-β-naphthylamide (1.4×10^{-5} M).

There are several alanine aminopeptidases which occur in human blood which are distinguishable on the basis of their electrophoretic or chromatographic behavior (Behal et al., 1964, 1965; Smith and Rutenburg, 1966; Panveliwalla and Moss, 1966). Workers in this field first thought that the activity exhibited by this group of human alanine aminopeptidases was a single blood enzyme of the leucine aminopeptidase type (Nachlas et al., 1957; Goldbarg and Rutenburg, 1958; Rutenburg et al., 1958); routine assay usually involved chromogenic substrates such as L-leucyl-β-naphthylamide. Subsequent studies in our laboratory showed that this "single" activity actually was a group of at least five closely related alanine aminopeptidases (Behal et al., 1966). The tissue origin(s) of some of these aminopeptidases has been established: one is from liver, one is from kidney, one is from duodenum, and two are from pancreas (Behal et al., 1965). These alanine aminopeptidases have been selected for study in our laboratory. We first studied human liver alanine aminopeptidase; the liver enzyme has been purified to homogeneity, after which its composition, subunit structure, and its stoichiometric binding of zinc were determined (Garner and Behal, 1974; Starnes and Behal, 1974; Garner and Behal, 1975a,b). Subsequently, we have evaluated the topography of the active site of the liver enzyme (Garner and Behal, 1975a,b, 1977). In this communication, we report some of the properties of human kidney alanine aminopeptidase, one of the enzymes in this group; the kidney enzyme has been purified to homogeneity, and we compare its properties with those of the liver enzyme.

Experimental Procedure

Materials. Except as may be otherwise indicated, all chemicals used were as specified previously by Starnes and Behal (1974).

Aminopeptidase Assay. A colorimetric assay for the hydrolysis of aminoacyl-β-naphthylamides specifically developed by Behal et al. (1966) for this enzyme was used in order to assay for activity during the purification procedure.

A spectrophotometric assay was used for some of the kinetic studies on this enzyme. One of the products of the reaction, β-naphthylamine, absorbs at 340 nm (ε 1780, Lee et al., 1971) but substrate does not. In a total volume of 1.0 mL, the components of the reaction mixture were 10 μmol of phosphate buffer (pH 6.8), 1 μmol of L-alanyl-β-naphthylamide, and enzyme as required. A 1.0-mL cuvette with a 1.0-cm light path was used.

A fluorometric β-naphthylamine assay (Roth, 1965) was used for most of the kinetic studies on this enzyme. In a total volume of 1.0 mL, the components of the reaction mixture were 10 μmol of phosphate buffer (pH 6.8) and L-aminoacyl-β-naphthylamides and enzyme as required. The concentrations of substrate used ranged from one-fifth to five times the K_m value of each specific substrate. The final concentration of enzyme ranged from 0.4 to 4 nM.

Activity with each assay was determined by comparison to standard curves obtained under conditions identical to those for the assay. The units of specific activity are μmol min^{-1} (mg of protein)$^{-1}$.

Kinetic Analysis. The K_m and V_{max} values were obtained using a least-squares linear-regression of the plots of v vs. $v/[S]$, and $k_{cat} = V_{max}/E$, where V_{max} is the maximal velocity in μmol hydrolyzed mL^{-1} min^{-1}, and E is μmol of enzyme mL^{-1} in the assay calculated on the basis that the extinction coefficient is 1.56 mL mg^{-1} cm^{-1} and the molecular weight is 236 000. Three separate series of from five to eight substrate concentrations were used in the calculations for each aminoacyl-β-naphthylamide.

Protein Assays. Protein was assayed during the purification procedure using the optical density ratio method (Warburg and Christian, 1942). The extinction coefficient (see below) was used for all other experiments to estimate protein concentration.

Purification Procedure. Human kidneys were obtained from autopsies performed within 6 h after death, when no primary renal disease was apparent. Kidneys were stored at −20 °C. One kilogram of tissue was homogenized in 3 L of 0.01 M phosphate buffer (pH 6.8). The kidney enzyme was then solubilized by autolysis as described by Matheson et al. (1963)

† From the Department of Biochemistry, Texas Tech University School of Medicine, Lubbock, Texas 79409. *Received October 10, 1977; revised manuscript received April 25, 1978.* Supported in part by Grant D-529 from the Robert A. Welch Foundation and Biomedical Research Support Grant 5 S07 RR05773-03 from the United States Public Health Service, Department of Health, Education, and Welfare.

0006-2960/78/0417-2990$01.00/0

and as modified by Behal et al. (1969). Autolysis was carried out for 48 h at 37 °C. After autolysis, a clarified extract was prepared, and from this point on the enzyme was purified as described by Garner and Behal (1975a).

Amino Acid Analyses. The amino acid analyses were performed according to the method of Simpson et al. (1976). A Beckman 121-HP automatic amino acid analyzer equipped with appropriate Beckman resins was used. Since the kidney enzyme is a glycoprotein, alkaline hydrolyses (Hugli and Moore, 1972) were also carried out in order to obtain additional values for tryptophan.

Carbohydrate Analysis. Sialic acid was assayed by the thiobarbituric acid reaction (Warren, 1959; Aminoff, 1961; Spiro, 1966) after the enzyme had been incubated with neuraminidase (1 h, 37 °C, 5 mM potassium phosphate, pH 7.0). Protein-bound hexose(s) was determined by the tryptophan method (Shetlar et al., 1948), and glucosamine was determined during amino acid analysis of the protein.

Zinc Analysis. The zinc content of the kidney enzyme was determined by atomic absorption using a Perkin-Elmer atomic absorption spectrophotometer, Model 303. Enzyme samples were dialyzed for 2 days against 0.1 mM EDTA[1] at 4 °C, followed by dialysis for 4 days against six changes of deionized water, the zinc content of which was below detectable limits. Analyses were performed on pure enzyme solutions of 0.4–0.5 mg/mL. $ZnCl_2$ was used as a standard.

Determination of the Extinction Coefficient of the Native Enzyme. The extinction coefficient of the enzyme was calculated on the basis of the dry weight of the enzyme. Three aliquots of a solution of the enzyme were lyophilized and then dried at 110 °C to constant weight (Schachman, 1957). As a secondary and supporting measure, as well as for comparative purposes, the average fringe number for globular proteins, 4.1 fringes mL^{-1} mg^{-1} (Babul and Stellwagen, 1969), was used to calculate a second value for the dry weight. A Beckman analytical ultracentrifuge, with an enzyme containing synthetic boundary cell in place, was used as a differential refractometer for this purpose.

Partial Specific Volume. The partial specific volume, $\bar{v}$, of the kidney enzyme was estimated from the amino acid and carbohydrate content of the enzyme (Cohn and Edsall, 1943; McMeekiof Munk and Cox (1972). The value for 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). Experiments were done at several 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 due to mechanical strain on the cell were made from photographs of interference patterns generated by light passing through the water-filled cell.

Preparation of the Native and Dissociated Enzyme and Sedimentation Experiments. The homogeneity of the kidney enzyme for sedimentation equilibrium experiments was confirmed by electrophoresis in the presence and absence of denaturants (sodium dodecyl sulfate, as outlined below). A solution of the enzyme (usually 0.5 to 1 mL of the solution with 1–3 mg/mL protein, as measured by the extinction coefficient of the protein at 280 nm) was dialyzed against buffer A (Starnes and Behal, 1974) at 5 °C for 24 h and diluted appropriately with the dialysis buffer to achieve a protein concentration of about 0.10 to 0.25 mg/mL. A portion of this diluted sample was then separated and utilized for equilibrium experiments on the enzyme in diluted salt solution, and the remainder was dialyzed against buffer B (Starnes and Behal, 1974) for 4 days at 5 °C. Although the volume of the solution decreased with dialysis, the approximate original volume of the enzyme solution was restored after dialysis by adding the appropriate quantity of the dialysis buffer. The protein solution at this stage was used for sedimentation equilibrium experiments in the presence of guanidinium chloride.

[1] Abbreviation used: EDTA, (ethylenedinitrilo)tetraacetic acid.

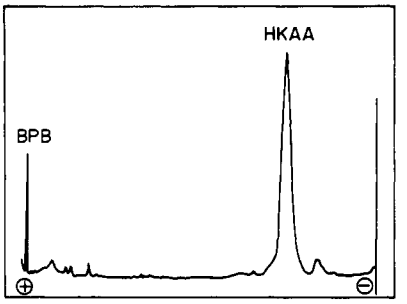

FIGURE 1: Polyacrylamide gel electrophoresis of human kidney alanine aminopeptidase. Spectrophotometric scan (at 580 nm) of the enzyme (~20 μg) on polyacrylamide gel with bromophenol blue (BPB) as marker. Coomassie brilliant blue was the stain.

Acrylamide Gels in the Presence of Sodium Dodecyl Sulfate. The sodium dodecyl sulfate–polyacrylamide gel experiments were performed as indicated by Weber and Osborn (1969) with the exception that the dialysis step was omitted. The molecular weights for the standard proteins were taken from Klotz and Darnall (1969) or they were supplied with the standard by the vendor.

Results

Purification and Homogeneity of the Native Enzyme. The kidney enzyme preparations obtained were greater than 96% pure, as indicated by disc gels (Figure 1) in nondenaturing solvents. 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 purification is summarized in Table I.

Extinction Coefficient of the Enzyme. The extinction coefficient of the enzyme at 280 nm in a 1.0-cm light path by the dry weight method is 1.56 mL mg^{-1} cm^{-1}. The value calculated from the tryptophan and tyrosine content of the enzyme is slightly lower. Another value, 1.57 mL mg^{-1} cm^{-1}, was obtained by the differential refractometric method for the estimation of protein concentration. Although the kidney enzyme may have a fringe number somewhat different from that for globular proteins, close agreement resulted. No suitable highly purified glycoprotein standards were available in sufficient quantity for direct comparison.

Amino Acid, Carbohydrate, and Zinc Content of the Enzyme. Table II shows the average amino acid and carbohydrate composition of four samples of the enzyme isolated from human kidney. Two additional samples were hydrolyzed under alkaline conditions; therefore, the tryptophan value is the average of six determinations. The nearest-integer composition computations assume that the metal ion and any as yet unidentified conjugated material, if any, make negligible contributions to the molecular weight of the complex. One major peak does not elute with the standard amino acids but rather elutes coincidentally with the glucosamine standard, and the 570- to 440-nm optical density ratio of this peak is identical with that for the glucosamine standard. The other carbohydrates shown were obtained by assays which are specific for

TABLE I: Purification of Human Kidney Alanine Aminopeptidase.

	prep	protein (mg)	act. (units)	yield (%)	sp act. (units/mg)	purif
I	Autoloyzed	430 000	36 800	100	0.086	1
II	Bio-Glass	2 010	8 320	23	4.14	49
III	Sephadex G-200	225	6 860	19	30.5	359
IV	DEAE-agarose[a]	15	1 150	3.1	76.6	901

[a] This step was as described by Garner and Behal (1975a), except that a second DEAE-agarose step was added. The activity eluted from the first DEAE-agarose column was rechromatographed on a 0.5 × 40 cm column equilibrated with 0.05 M imidazole buffer, pH 6.8. The enzyme was eluted with a shallower NaCl gradient (0.02–0.08 M, pH 6.8) to remove the last minor contaminants.

TABLE II: Amino Acid, Carbohydrate, and Zinc Content of a Human Kidney Alanine Aminopeptidase.

residue	g/100 g of glycoprotein	± SD	nearest integer per 119 000 daltons
Lys	4.44	0.29	41
His	1.98	0.25	17
NH_3	1.15		85
Arg	4.73	0.50	36
Asp	8.78	0.57	90
Thr	4.23		49
Ser	3.82		52
Glu	10.12	0.69	92
Pro	3.22	0.61	39
Gly	2.10	0.17	43
Ala	3.63	0.15	60
½-Cys[a]	0.46		5
Val	4.49	0.54	53
Met	1.69	0.11	15
Ile	3.65	0.34	38
Leu	8.24	0.68	86
Tyr	4.95	0.65	36
Phe	4.80	0.44	38
Trp[b]	2.79		18
total % amino acids	79.27	total residues amino acids	893
Glc-NH_2	7.44	0.52	49
hex[c]	9.18	0.24	67
SA	4.05	0.25	16
total % carbohydr	20.67	total residues carbohydr	132
Zn	0.0576	0.0035	1

[a] Determined as cystine. The standards were treated with 4 N methanesulfonic acid and 0.2% 3-(2-aminoethyl)indole just as the protein samples were during hydrolysis. No cysteic acid peaks were observed, indicating the absence of oxidation of this residue. [b] Tryptophan contents by acid hydrolysis (4 samples) were 3.15 g/100 g of protein, and tryptophan content by alkaline hydrolysis was 2.08 g/100 g of protein. [c] Hex, hexose; SA, sialic acid.

the residues indicated in Table II. The total carbohydrate content is 20.67%. Zinc was determined on four different samples. The zinc content of 0.0576 ± 0.0035 g/100 g corresponds to a minimum molecular weight of 113 500 ± 6900. The enzyme activity of the sample taken for zinc analysis remained constant during the course of the extensive dialysis period employed.

Molecular Weight of the Native Enzyme. Plots of ln c vs. r^2, $(1/r)\,dc/dr$ vs. c, and M_{app}^{-1} vs. c, from which, respectively, were obtained estimates of $\overline{M}_w$*, $\overline{M}_z$*, and M_{app}, were all linear throughout the entire solution column in the ultracentrifuge cell. The M_{app}^{-1}-type plot yields an estimate of the second virial coefficient, B, if the solute behaves nonideally (Munk and Cox, 1972). The native enzyme behaved ideally and the average molecular weight for the native enzyme was 236 000 ± 9400.

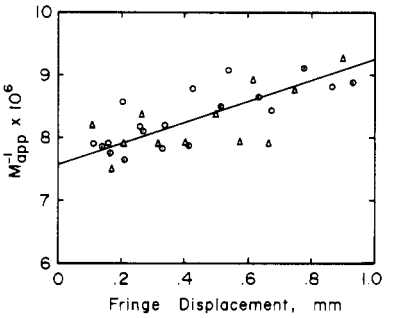

FIGURE 2: M_{app}^{-1} vs. fringe displacement for human kidney alanine aminopeptidase (Munk and Cox, 1972). M at zero fringe displacement (extrapolated) for this plot is 126 000 ± 7200.

Molecular Weight of the Aminopeptidase in Denaturing Solvents. Two different enzyme preparations were examined by acrylamide gel electrophoresis in the presence of sodium dodecyl sulfate. The result was nearly identical for both preparations, and the average of all the experiments was 119 000 ± 9500.

In spite of the uncertainty inherent in estimating 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 with correction for nonideal behavior (Munk and Cox, 1972) was essentially the same as that observed in the polyacrylamide gel experiments in the presence of sodium dodecyl sulfate. Figure 2, an M_{app}^{-1}-type plot derived from a typical experiment in which $\overline{M}_w$* = 115 000 ± 3800 and $\overline{M}_z$* = 114 000 ± 5700, illustrates the method of the correction for nonideal behavior. The estimate of the molecular weight obtained from all experiments in the presence of 6 M guanidinium chloride was 126 000 ± 7200. The average value of the second virial coefficient obtained in this way was 1.49 ± 0.09 10^{-9} mol cm^3 g^{-2}. The physical properties are summarized and compared with properties of the liver enzyme in Table III.

Stimulation of Kidney Enzyme by Co^{2+}. Human kidney alanine aminopeptidase was stimulated 1.65-fold by Co^{2+} as shown in Figure 3. The kidney enzyme sample taken for the Co^{2+}-stimulation study was dialyzed in the same manner as the sample taken for zinc analysis. The Co^{2+} stimulation obtained could be reversed by dialysis of the stimulated kidney enzyme sample against distilled water for 12 h. The original level of kidney enzyme activity was restored, thus indicating that Co^{2+} is very loosely bound to kidney enzyme whereas Zn^{2+} is very tightly bound. When Zn^{2+} or Mg^{2+} rather than

TABLE III: Comparison of Physical Properties of Human Liver Alanine Aminopeptidase and Human Kidney Alanine Aminopeptidase.

property	method	value obtained: kidney enz	value obtained: liver enz
mol wt, native enz	sediment. equilibr dil buffers, high-speed meth	236 000 ± 9 400	233 000 ± 10 000[a]
mol wt, denatured enz	high-speed sediment. equilibr in 6 M Gdm-Cl	126 000 ± 7 200	118 000 ± 5 000[b]
	$NaDodSO_4$–acrylamide gels	119 000 ± 9 500	120 000 ± 12 000[c]
mol wt, min	Zn content	113 500 ± 6 900	122 000 ± 20 000[c]
extinction coeff	by dry wt	1.56 ± 0.02	1.75 ± 0.03[b]

[a] Average for value determined by high-speed sedimentation equilibrium, Svedberg method, and gel filtration as reported by Starnes and Behal (1974). [b] Starnes and Behal (1974). [c] Garner and Behal (1974).

TABLE IV: Kinetic Data for Human Kidney Alanine Aminopeptidase.

amino acid β-naphthylamide	$K_m \times 10^4$	$k_{cat} \times 10^{-3}$	$k_{cat}/K_m \times 10^{-7}$
Ala	1.23 ± 0.12	8.43 ± 2.71	6.85
Leu	0.71 ± 0.03	2.97 ± 0.21	4.18
Phe	1.42 ± 0.32	4.47 ± 0.99	3.13
Met	0.14 ± 0.01	5.73 ± 1.46	40.9
Val	0.42 ± 0.06	0.23 ± 0.05	0.55
Lys	0.40 ± 0.06	0.68 ± 0.09	1.70
Ile	0.48 ± 0.06	0.38 ± 0.03	0.79
Arg	0.54 ± 0.08	1.33 ± 0.15	2.46

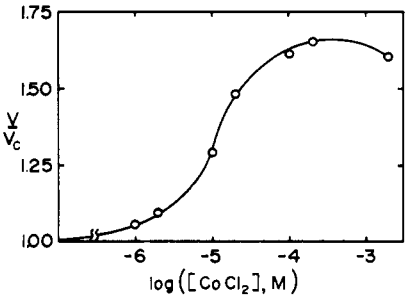

FIGURE 3: Stimulation of aminopeptidase by Co^{2+}. The amount of β-naphthylamine formed in the presence of varying amounts of $CoCl_2$ was determined by colorimetric assay described in the Experiment Procedure. v/v_c is the relative activity of the enzymes with and without $CoCl_2$.

Co^{2+} was added, as a control for the stimulation effect, there was no change in the "baseline" activity level of the kidney enzyme sample.

Determination of K_m and k_{cat} for Kidney Enzyme. K_m and k_{cat} values were determined for kidney enzyme with L-alanyl-, L-leucyl-, L-methionyl-, L-valyl-, L-isoleucyl-, L-lysyl-, L-arginyl-, and L-phenylalanyl-β-naphthylamide. These values are shown in Table IV. Hydrolytic coefficient values (k_{cat}/K_m) were calculated for each substrate.

Discussion

This report is concerned with our continuing study of the comparative biochemistry of human alanine aminopeptidases. The liver enzyme has been characterized in our laboratory (Starnes and Behal, 1974; Garner and Behal, 1974, 1975a,b). The liver enzyme has an apparent native molecular weight of 233 000 ± 17 000; it is composed of two subunits of equal molecular weights (119 500) with one atom of firmly bound zinc per enzyme subunit. The liver enzyme is a sialic acid containing glycoprotein with a total carbohydrate content of 17.5%. An active center for the liver enzyme with three subsites has been proposed on the basis of kinetic and inhibition studies. Preliminary evidence (Behal et al., 1965) suggested a similarity between the liver enzyme, the pancreas enzyme, and the alanine aminopeptidase from human small intestine. All three of these enzymes have catalytic properties similar to those of the kidney enzyme when K_m and V_{max} (or k_{cat}) are compared for a substrate series of amino acid β-naphthylamides when the amino acids are alanine, leucine, phenylalanine, valine, isoleucine, methionine, arginine, and lysine. Recent reports by Gray and Santiago (1977) and Kania et al. (1977) indicate that rat intestinal aminooligopeptidase, with kinetic properties very similar to those of the liver enzyme and the kidney enzyme, occurs in two forms, one with a molecular weight of 320 000 and another with a molecular weight of 180 000.

The results reported here and summarized in Table III show that the kidney enzyme has a well-defined molecular weight in denaturing solvents near 119 500 and suggest that this species, in dilute salt solution near neutral pH, exists as a dimer, with a molecular weight near 236 000. The species with a molecular weight very near one-half of the native molecular weight appears homogeneous in sedimentation equilibrium experiments in guanidinium chloride and in polyacrylamide gel electrophoresis experiments done in the presence of sodium dodecyl sulfate. The minimum molecular weight based on zinc content is 113 500 ± 6900. The average value for the monomer by all three methods is 119 500 ± 7300. Therefore, as is the case for the liver enzyme, the kidney enzyme appears to be a dimeric species composed of two subunits of identical molecular weight.

The K_m and k_{cat} values for kidney enzyme follow the general pattern for alanine aminopeptidase of human liver. The mechanism for the human liver and kidney alanine aminopeptidases may be quite similar because the K_m values of kidney enzyme for β-branched and the γ-branched amino acid β-naphthylamides are similar, but of these only the γ-branched compound, L-leucyl-β-naphthylamide, is hydrolyzed at a significant rate. We have previously attributed this to steric hindrance by substrates with β-branching in the case of the liver enzyme (Behal et al., 1965). The data now being reported indicate that the same factors, vis-a-vis γ-branching being favored over β-branching, also are the case for the kidney enzyme.

The enzyme is a zinc metalloenzyme having its zinc ion firmly bound into its structure. EDTA absolishes activity, although dialysis restores activity fully, similar to the liver enzyme (Garner and Behal, 1974). In contrast, the pig kidney particulate aminopeptidase described by Lehky et al. (1973) and Wacker et al. (1976) loses its zinc upon treatment with chelating agents.

Acknowledgments

The authors express their appreciation to Mr. Scott Shannon and Mr. Harvey Olney for their technical assistance and to

Mrs. Michelle Turpin for assistance in preparing this paper.

References

Aminoff, D. (1961), *Biochem. J. 81,* 384.
Babul, J., and Stellwagen, E. (1969), *Anal. Biochem. 28,* 216.
Behal, F. J., Asserson, B., Dawson, F., and Harman, J. (1965), *Arch. Biochem. Biophys. 111,* 335.
Behal, F. J., Hamilton, R. D., Dawson, F., and Terrel, L. D. (1964), *Arch. Biochem. Biophys. 108,* 207.
Behal, F. J., Klein, R. A., and Dawson, F. B. (1966), *Arch. Biochem. Biophys. 115,* 545.
Behal, F. J., Little, G. H., and Klein, R. A. (1969), *Biochim. Biophys. Acta 178,* 118.
Cohn, E. J., and Edsall, J. T., Ed. (1943), Proteins, Amino Acids, and Peptides, New York, N.Y., Reinhold, p 370.
Garner, C. W., and Behal, F. J. (1974), *Biochemistry 13,* 3227.
Garner, C. W., and Behal, F. J. (1975a), *Biochemistry 14,* 3208.
Garner, C. W., and Behal, F. J. (1975b), *Biochemistry 14,* 5084.
Garner, C. W., and Behal, F. J. (1977), *Arch. Biochem. Biophys. 182,* 667.
Goldbarg, J. W., and Rutenburg, A. M. (1958), *Cancer 11,* 283.
Gray, G. M., and Santiago, N. A. (1977), *J. Biol. Chem. 252,* 4922.
Hugli, T. E., and Moore, S. (1972), *J. Biol. Chem. 247,* 2828.
Kania, R. K., Santiago, N. A., and Gray, G. M. (1977), *J. Biol. Chem. 252,* 4929.
Klotz, I. M., and Darnall, D. W. (1969), *Science 166,* 126.
Lee, H. J., LaRue, J. N., and Wilson, I. B. (1971) *Anal. Biochem. 41,* 397.
Lehky, P., Kosowski, J., Wolf, D. P., Wacker, H., and Stein, E. A. (1973), *Biochim. Biophys. Acta 321,* 274.
Matheson, A. T., Bjerre, S., and Hanes, C. S. (1963), *Can. J. Biochem. Physiol. 41,* 1741.
McMeekin, T. C., and Marshall, K. (1953), *Science 116,* 142.
Munk, P., and Cox, D. J. (1972), *Biochemistry 11,* 687.
Nachlas, M. M., Crawford, D. T., and Seligman, A. M. (1957), *J. Histochem. Cytochem. 5,* 264.
Panveliwalla, D. K., and Moss, D. W. (1966), *Biochem. J. 99,* 1966.
Reisler, E., and Eisenberg, H. (1969), *Biochemistry 8,* 4572.
Roth, M. (1965), Enzymes in Clinical Chemistry, Elsevier, Amsterdam, pp 10–18.
Rutenberg, A. M., Goldbarg, J. A., and Pineda, E. P. (1958), *N. Eng. J. Med. 259,* 469.
Schachman, H. K. (1957), *Methods Enzymol. 4,* 69.
Shetlar, M. R., Foster, J. V., and Everett, M. R. (1948), *Proc. Soc. Exp. Biol. Med. 67,* 125.
Simpson, R. J., Neuberger, M. R., and Liu, T. Y. (1976), *J. Biol. Chem. 251,* 1936.
Smith, E. E., and Rutenburg, A. M. (1966), *Science 152,* 1256.
Spiro, R. G. (1966), *Methods Enzymol. 8,* 3.
Starnes, W. L., and Behal, F. J. (1974), *Biochemistry 13,* 3221.
Starnes, W. L., Munk, P., Maul, S. B., Cunningham, G. N., Cox, D. J., and Shive, W. (1972), *Biochemistry 11,* 677.
Wacker, H., Kehky, P., Vanderhaege, F., and Stein, E. A. (1976), *Biochim. Biophys. Acta 429,* 546.
Warburg, O., and Christian, W. (1942), *Biochem. Z. 310,* 384.
Warren, L. (1959), *J. Biol. Chem. 234,* 1971.
Weber, K., and Osborn, M. (1969), *J. Biol. Chem. 244,* 4406.
Yphantis, D. (1964), *Biochemistry 3,* 297.