Biochimica et Biophysica Acta, 327 (1973) 157–165

BBA 67047

EFFECT OF MANGANESE AND AMINO ACIDS ON PROTEOLYTIC INACTIVATION OF BEEF LIVER ARGINASE

JUDITH S. BOND

Department of Biochemistry, Medical College of Virginia, Health Sciences Division, Virginia Commonwealth University, Richmond, Va. 23298 (U.S.A.)

(Received May 23rd, 1973)

SUMMARY

When beef liver extracts were incubated with Mn^{2+} or Co^{2+}, arginase (L-arginine amidinohydrolase, EC 3.5.3.1) was activated and its susceptibility to proteolytic inactivation was increased. Mg^{2+}, which did not activate, did not affect proteolytic susceptibility. Increasing concentrations of Mn^{2+} from 1 to 50 mM increased the extent of both activation and tryptic susceptibility. Trypsin and chymotrypsin were much more effective than pronase or subtilisin in inactivating Mn^{2+}-treated arginase. The chromatographic behavior of arginase on Sephadex G-200 before and after activation with Mn^{2+} was the same. Amino acids protected purified preparations of arginase against tryptic inactivation. The most effective protection was afforded by cysteine, isoleucine, leucine, valine and alanine. Isoleucine protected at concentrations as low as 0.38 mM, which is in the physiological range.

INTRODUCTION

Liver arginase (L-arginine amidinohydrolase, EC 3.5.3.1) from a wide variety of species is activated by Mn^{2+} (ref. 1). This cation is thought to be an essential component of arginase[2] although there is some question as to whether other divalent cations (*e.g.* Co^{2+} or Fe^{2+}) are involved *in vivo*[3]. In Mn^{2+}-deficient animals the enzyme is significantly reduced in activity[4,5]. The stability of the enzyme is increased by Mn^{2+} *in vitro*[1], and stabilization of the enzyme in cultured cells has also been reported[6].

This paper reports that Mn^{2+} increases the proteolytic susceptibility of beef liver arginase and that certain amino acids (isoleucine, leucine, valine, arginine, alanine and cysteine) protect the Mn^{2+}-activated enzyme from proteolytic inactivation. Because the proteolytic susceptibility of soluble enzymes appears to be an important determinant of their *in vivo* half-lives, factors that alter this property may be important physiologically[7].

METHODS

Beef livers were obtained from a slaughter house directly after the animals were killed. Livers were kept on ice for approximately 1 h before homogenization or stored at −20 °C. Thawed livers were disrupted in a Waring blender for 15 s with 0.15 M KCl (1 g liver + 3 ml KCl), and homogenized in a glass tube fitted with a Teflon pestle. Homogenates were centrifuged at 50 000 × *g* for 80 min, and the resulting supernatant fraction was adjusted to pH 7 with NaOH. These extracts were either used immediately or stored at −20 °C.

Arginase was purified from bovine liver through the heat-denaturation and dialysis step of the Greenberg method[8]. These preparations had a spec. act. of 75–100 units per mg protein and represented approximately a 50-fold purification.

Arginase activity was assayed by the method of Van Slyke and Archibald[9] which estimates urea produced utilizing α-isonitrosopropiophenone. Arginase activity is expressed as μmoles urea produced/min per ml of the reaction mixture. Addition of $MnCl_2$ (50 mM) or EDTA (2 mM) to the assay mixture did not affect arginase activities.

Arginase in beef liver extracts was pre-activated with 50 mM $MnCl_2$ in Tris–HCl buffer (25 mM, pH 7.3) at 50 °C for 10 min.

Trypsin (Type III, 2 times crystallized from bovine pancreas), chymotrypsin (Type II, 3 times crystallized from bovine pancreas), subtilisin (bacterial protease from *Bacillus subtilis*), and pronase (Type VI, fungal) were obtained from the Sigma Chemical Company, St. Louis, Mo. Their proteolytic activity was assessed by the method of Kunitz[10] using denatured casein, acid-denatured albumin[11], or dialyzed beef liver extracts as substrate. We measured either the increase in absorbance at 280 nm in trichloroacetic acid extracts or ninhydrin-positive material formed during incubation[12].

Protein was determined by the method of Lowry *et al.*[13] with crystalline bovine albumin as a standard. Since Mn^{2+} interferes with the assay, samples containing Mn^{2+} were dialyzed prior to protein determination.

Sephadex G-200, purchased from Pharmacia Fine Chemicals, Inc., Piscataway, N. J., was suspended (7 g/400 ml) in 0.15 M KCl containing 1 mM Tris buffer (pH 7.3) and boiled for 5 h. After cooling at room temperature (25 °C), it was mixed with 6-mm glass beads that were pre-treated with dichlorodimethylsilane[14]. Columns (2.5 cm × 45 cm) were packed by gravity and washed overnight with the KCl solution before application of a sample. Chromatography was carried out at 6 °C. Samples were added in 0.3 M KCl and eluted with 0.15 M KCl at a flow rate of 12 ml/h. Fractions (4.4 ml) were collected and assayed for arginase activity or absorbance at 280 nm. Blue dextran, mol. wt 2 000 000, horse heart cytochrome *c*, mol. wt 12 400 and beef heart lactic dehydrogenase, mol. wt 138 000 were used as standards.

RESULTS

Arginase activity in beef liver extracts can be activated by Mn^{2+}, and under these conditions it was susceptible to tryptic inactivation; the unactivated enzyme was not affected by trypsin (Fig. 1). The extent of inactivation by trypsin varied

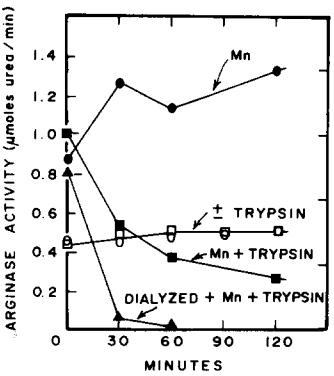


Fig. 1. Effect of Mn^{2+} on tryptic inactivation of arginase. Beef liver extracts were pre-incubated at 50 °C for 10 min with Tris buffer (28 mM, pH 8.3) in the presence (solid symbols) or absence (open symbols) of $MnCl_2$ (50 mM). These samples (0.9 ml) were then mixed either with 0.1 ml trypsin (□, ■, ▲) (2 mg/ml Tris buffer, 0.25 M, pH 8.3) or 0.1 ml of the Tris buffer alone (○, ●) and incubated at 37 °C. The protein concentration in the final incubation mixture was 7.5 mg/ml. One sample (▲) was dialyzed for 48 h at 4 °C against 0.15 M KCl containing 1 mM Tris–HCl buffer, pH 7.3, before Mn^{2+} activation and incubation with trypsin. Portions of the incubation mixtures (40 μl) were removed with time and assayed for arginase activity.

considerably with different beef liver extracts. In some instances there was only 30% inactivation after 2 h while in others up to 85%. In extracts dialyzed against 0.15 M KCl before Mn^{2+} activation, arginase activity consistently decayed more dramatically in comparison to undialyzed controls (Fig. 1). No loss of arginase activity occurred if trypsin was mixed with soy bean trypsin inhibitor (in a 1:2 ratio), before addition to liver extracts, indicating that an active trypsin molecule was necessary for the decay of arginase activity.

In control experiments Mn^{2+} had no effect on the rate of production of ninhydrin-positive material when beef liver extracts or albumin were used as substrates

TABLE I

EFFECT OF Mn^{2+} ON TRYPSIN ACTIVITY

Dialyzed beef liver extracts were pre-activated with 50 mM $MnCl_2$ in Tris buffer (25 mM) or pre-incubated with Tris buffer alone. These samples were then incubated at 37 °C with trypsin (200 μg per ml of final incubation mixture) at pH 8.3. Samples were removed with time, diluted 6-fold with 7% trichloroacetic acid, centrifuged and the trichloroacetic acid extracts were assayed for ninhydrin-positive material[12]. Activity is expressed as μmoles of leucine equivalents formed per ml of the trichloroacetic acid extract. Dialyzed acid-denatured albumin (22.5 mg/ml) was incubated at 37 °C with trypsin (200 μg/ml) at pH 8.3 in 25 mM Tris buffer, in the presence or absence of 50 mM $MnCl_2$. Samples were removed with time, the reaction stopped with 2 vol. of trichloroacetic acid (7%). After centrifugation, trichloroacetic acid extracts were assayed for ninhydrin-positive material. Activity is expressed as stated above.

Substrate	*Time incubated (min)*	*Trypsin activity*	
		No Mn^{2+}	*+Mn^{2+}*
Beef liver extract	30	1.8	1.7
	60	3.3	2.9
Denatured albumin	10	0.69	0.58
	20	0.96	0.88
	40	1.17	1.27

for trypsin (Table I). Thus activation of trypsin cannot explain the increased susceptibility of arginase in the presence of Mn^{2+}.

Co^{2+} and Mn^{2+} activated the enzyme at pH 7.3 and both promoted inactivation in the presence of trypsin at this pH (Table II). Mg^{2+} did not activate nor did it affect the proteolytic susceptibility.

TABLE II

THE EFFECTS OF DIVALENT CATIONS ON ACTIVATION AND PROTEOLYTIC INACTIVATION OF ARGINASE ACTIVITY

Dialyzed beef liver extracts were pre-incubated (50 °C, 10 min) with Tris buffer alone (25 mM, pH 7.3) or with one of the divalent cations (as chloride salt) dissolved in Tris buffer. These samples were assayed for arginase activity at pH 7.3. Pre-incubated samples were subsequently incubated with trypsin at 37 °C and arginase activity measured initially and after 2 h. The incubation mixture consisted of 0.9 ml of the pre-incubated beef liver extract [containing approximately 8 mg protein and 0.1 ml buffer or salt (50 mM in Tris buffer)] and 0.1 ml of trypsin (2 mg/ml water).

Salts	*Arginase activity (μmoles urea/min per ml)*	*Percent activity remaining after 2 h incubation with trypsin*
None	0.087	98
$MnCl_2$	0.200	40
$CoCl_2$	0.233	25
$MgCl_2$	0.093	94

Increasing concentrations of Mn^{2+} from 1 to 50 mM increased both the extent of activation and tryptic susceptibility of arginase (Figs 2 and 3). There was not, however, an exact correlation between the extent of these two effects. At 10 mM Mn^{2+}, for example, the enzyme appeared to be 85% activated and yet the rate of proteolytic inactivation was much slower at this concentration than at 50 mM Mn^{2+}.

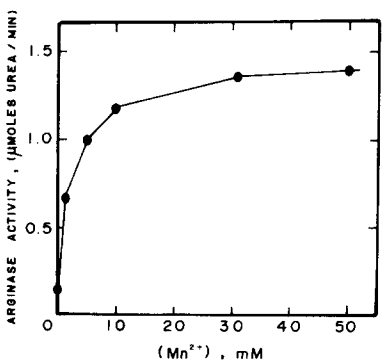


Fig. 2. Effect of Mn^{2+} concentration on arginase activation. Dialyzed beef liver extracts were pre-incubated in the presence of varying concentrations of Mn^{2+} at 50 °C for 10 min. They were then assayed for arginase activity to determine the extent of activation.

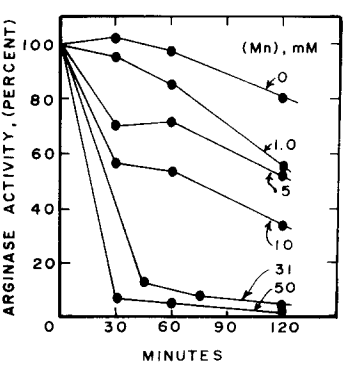


Fig. 3. Effect of Mn^{2+} concentrations on tryptic inactivation of arginase. Dialyzed beef liver extracts, pre-activated with varying concentrations of Mn^{2+}, as in Fig. 2, were subsequently incubated with trypsin (200 μg per ml) at 37 °C. Arginase activity was measured with time of incubation. Results are expressed as percent of initial activity.

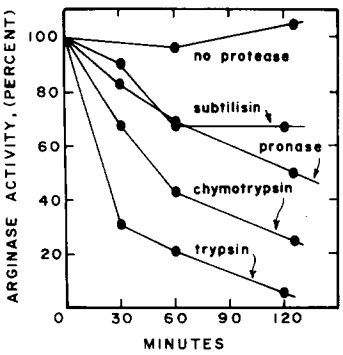


Fig. 4. Effect of proteases on dialyzed, Mn^{2+}-activated beef liver arginase. Dialyzed beef liver extracts were pre-incubated with 50 mM $MnCl_2$ in 25 mM Tris buffer, pH 8.3, for 10 min at 50 °C and then cooled on ice. Proteases were then added (at a final concentration of 167 μg per ml incubation mixture) and incubation resumed at 37 °C. Samples (40 μl) were removed with time and assayed for arginase activity. Results are expressed as percent of initial activity. Initial activity was not affected by any of the proteases.

Proteases other than trypsin also inactivated Mn^{2+}-treated arginase (Fig. 4). Trypsin appeared to be the most potent inactivator followed by chymotrypsin, pronase and subtilisin. This order of potency did not correspond to the order of hydrolytic activity of the proteases tested; for example, subtilisin produced 9.8 μmoles of leucine equivalents per ml in 30 min compared to 3.8 for pronase and 1.5 for trypsin or chymotrypsin when these proteases were incubated with dialyzed, Mn^{2+}-treated beef liver extracts.

When extracts, which were treated at 50 °C for 10 min in the presence or absence of Mn^{2+}, were filtered through Sephadex G-200, Mn^{2+}-activated and unactivated arginase activity profiles were very similar (Fig. 5). Peak activity occurred at Tube 18 indicating a mol. wt of approximately 100 000 by comparison to standards. Thus the

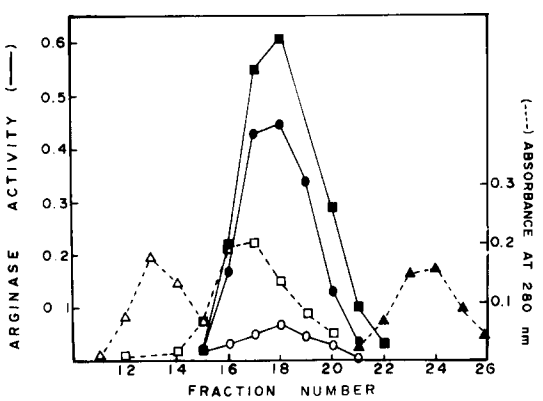
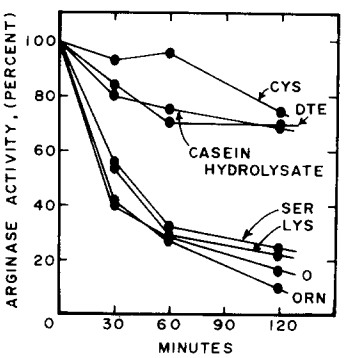


Fig. 5. Effect of activation of arginase on chromatographic behavior of the enzyme. Dialyzed beef liver extracts were pre-incubated (50 °C, 10 min) with Tris buffer (25 mM, pH 7.3) in the presence (●) or absence (○) of 50 mM $MnCl_2$. The samples were centrifuged and adjusted to 10 mg protein per ml KCl (0.3 M) before application to the column. Purified arginase, 1.25 mg protein, (■) was added in a 1.0-ml volume of 0.3 M KCl. Solid lines indicate arginase activity in units of μmoles urea formed per ml of the fraction collected. Standards applied to the column were: 2 mg blue dextran (△), 3 mg lactate dehydrogenase (□), and 2 mg cytochrome *c* (▲). The absorbance of these standards was monitored at 280 nm as indicated by dashed lines.

effect of Mn^{2+} on proteolytic susceptibility of arginase cannot be attributed to either dissociation of the enzyme into subunits or aggregation.

Purified preparations of arginase displayed identical chromatographic behavior to arginase in extracts (Fig. 5). In addition, arginase in extracts that were untreated (no dialysis, no 50 °C heat treatment, no purification) also had similar chromatographic profiles (data not shown).

Purified preparations of arginase were protected from trypsin inactivation by casein hydrolysates and certain amino acids (Figs 6 and 7). Of the amino acids tested for their ability to protect against tryptic inactivation, cysteine, isoleucine, valine and leucine were particularly effective. Alanine was moderately protective while glycine, serine, lysine and ornithine had little or no effect. Tryptophan, tyrosine, methionine, glutamic acid and cystine (not shown on Figs 6 and 7) were also tested



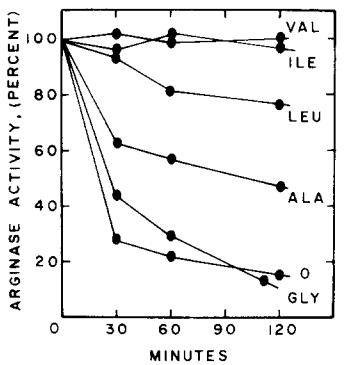


Fig. 6. Effect of casein hydrolysate, individual amino acids or dithioerythritol on tryptic inactivation of arginase. Purified arginase preparations, fully activated with Mn^{2+}, were incubated with trypsin at 37 °C. The incubation mixture contained: 0.5 ml arginase (containing 2 mg protein), 0.4 ml KCl (0.15 M) or 0.4 ml dithioerythritol, casein hydrolysate or an amino acid (all at 25 mg/ml KCl, adjusted to pH 7) and 0.1 ml trypsin (2 mg/ml Tris buffer, 0.25 M, pH 7.3). Amino acids were present in the incubation mixture at 75–130 mM. Samples (20 μl) were removed with time and assayed for arginase activity. Activity is expressed as percent of initial activity. None of the compounds affected the initial activity. DTE, dithioerythritol; Cys, cysteine; Ser, serine; Lys, lysine; Orn, ornithine.

Fig. 7. Effect of amino acids with non-polar, aliphatic side chains on tryptic inactivation of arginase. The incubation mixture was similar to that descirbed in Fig. 6. The amino acids added were valine (Val), leucine (Leu), isoleucine (Ile), alanine (Ala) and glycine (Gly).

in this system and found to have no effect. Dithioerythritol had a protective effect (Fig. 6) indicating that the protection afforded by cysteine may be due to its sulfhydryl group.

Arginine was also able to protect the enzyme from inactivation by trypsin, but in this instance very high concentrations of arginine were necessary (185 mM); concentrations as high as 95 mM did not protect.

Control experiments for all of the above amino acids were conducted to determine if they affected trypsin activity. No inhibition of trypsin was found by measuring the rate of digestion of casein (Kunitz method).

In order to determine whether low concentrations of amino acids would protect, the isoleucine concentration was varied (Fig. 8). At a concentration as low as 0.38 mM, isoleucine protected slightly.

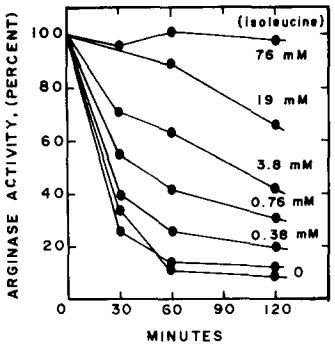


Fig. 8. Effect of isoleucine concentration on protection of arginase against tryptic inactivation. Purified arginase preparations were incubated with trypsin as described in Fig. 6 except that the concentration of isoleucine in the incubation mixture was varied. Isoleucine was present at 0.38 to 76 mM.

Amino acid analysis of a beef liver extract (Table III) indicated that isoleucine, leucine, valine, alanine and cysteine were present in concentrations of 0.1 to 0.7 mM. No arginine could be detected.

TABLE III

AMINO ACID CONCENTRATIONS IN A BEEF LIVER EXTRACT

The amino acid content of protein-free filtrates of beef liver extracts were determined by elution chromatography according to the procedure of Spackman *et al.*[15]. The amino acid concentrations are reported as μmoles per ml of beef liver extract and are taken from a single representative experiment.

Amino acid	*Extract concentration (mM)*
Isoleucine	0.14
Leucine	0.33
Valine	0.29
Alanine	0.72
Cysteine	0.50
Aspartic acid	0.45
Threonine	0.26
Serine	0.29
Glutamic acid	1.66
Glycine	3.04
Methionine	0.12
Tyrosine	0.12
Phenylalanine	0.14
Lysine	0.26
Histidine	0.13
Arginine	0

DISCUSSION

It is obvious that Mn^{2+} activates beef liver arginase and increases its susceptibility to proteolytic inactivation. Co^{2+} also does both, while Mg^{2+} does neither. This indicates that these activators induce a conformational change although this

does not involve subunit dissociation or aggregation. The change either exposes peptide bonds (to proteases) that were not accessible before activation or affects the active site of the enzyme so that the splitting of a specific peptide bond(s) alters catalytic activity of activated but not unactivated arginase.

The finding that Mn^{2+} increased the proteolytic susceptibility of beef liver arginase was unexpected because it had been previously reported that this cation protects rat liver arginase against inactivation by trypsin[16] and lysosomal enzymes[17]. This may be a species difference because arginase does differ substantially in different species[18–20]. The rat and rabbit enzymes, for instance, have recently been found to differ in: (a) charge, (b) affinity for Mn^{2+}, (c) antigenic behavior and (d) structural changes during activation. The beef enzyme resembles the rabbit enzyme in charge[1] while it behaves more like the rat enzyme with regard to Mn^{2+} activation. The increasing evidence of differences between arginases makes it important to be cautious of extrapolating data from one species to another.

Amino acids are known to interact with beef liver arginase. Hunter and Downs[21] found that ornithine and lysine were competitive inhibitors while cysteine, isoleucine, valine and leucine were non-competitive inhibitors, as were alanine, aspartic acid and proline to a lesser extent. Others (*e.g.* glycine, serine, histidine) showed little or no inhibition. In the present studies I found that only the amino acids which inhibit non-competitively protected against trypsin. This is consistent with the mechanism[22] for non-competitive inhibition, in which the inhibitor combines reversibly with the enzyme at a point different from the substrate. These ligands probably cause a conformational change which (a) impairs the turnover of the enzyme–substrate complex and (b) increases the susceptibility of the enzyme to proteolytic inactivation.

The presence of amino acids in beef liver extracts may account for the increased susceptibility of arginase to trypsin in dialyzed compared to undialyzed extracts. In addition, the lack of effectiveness of subtilisin and pronase on the inactivation of arginase may be due to the amino acids that are produced by their activity.

Casein hydrolysates and individual amino acids (at 100 mM) also inhibit the inactivation of partially purified rat liver arginase by lysosomes[17]. The amino acid concentrations reported in lysosomes are in this range (60 mM) (ref. 23). In the present studies isoleucine concentrations as low as 0.38 mM protected. This is in the physiological range for amino acids in the cytoplasm as well, and indicates that amino acids interact with arginase *in vivo*. Accordingly fluctuations in amino acid pools due to diet or metabolic conditions may affect arginase structure and possibly the stability or half-life of the enzyme. Indeed, the half-life of arginase in rat liver has been found to change in response to dietary changes[24]. For example, after a change from a 70% protein diet to one containing 8% protein, there was an increase in the rate of degradation of arginase.

ACKNOWLEDGEMENTS

The author is grateful to Mrs Kathleen McKay for expert technical assistance and to Dr William Banks Jr who performed the amino acid analyses. I am indebted to Dr Guy H. Bond and Dr Kenneth S. Rogers for help during the course of the work and in preparation of the manuscript.

This work was supported by grant GB-31178 from the National Science Foundation.

REFERENCES

1 Greenberg, D. M. (1960) in *The Enzymes* (Boyer, P. D., Lardy, H. and Myrback, K., eds), 2nd edn, Vol. IV, pp. 257–267, Academic Press, New York
2 Bach, S. J. and Whitehouse, D. B. (1954) *Biochem. J.* 57, xxxi
3 Middelhoven, W. J. (1969) *Biochim. Biophys. Acta* 191, 110–121
4 Boyer, P. D., Shaw, J. H. and Phillips, P. H. (1942) *J. Biol. Chem.* 143, 417–425
5 Shils, M. E. and McCollum, E. V. (1943) *J. Nutr.* 26, 1–19
6 Schimke, R. T. (1964) *Natl. Cancer Inst. Monogr.* 13, 197–216
7 Bond, J. S. (1971) *Biochem. Biophys. Res. Commun.* 43, 333–339
8 Greenberg, D. M. (1955) in *Methods in Enzymology* (Colowick, S. P. and Kaplan, N. O., eds), Vol. II, pp. 368–374, Academic Press, New York
9 Van Slyke, D. D. and Archibald, R. M. (1946) *J. Biol. Chem.* 165, 293–309
10 Kunitz, M. (1947) *J. Gen. Physiol.* 30, 291–310
11 Coffey, J. W. and de Duve, C. (1968) *J. Biol. Chem.* 243, 3255–3263
12 Rosen, H. (1957) *Arch. Biochem. Biophys.* 67, 10–15
13 Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) *J. Biol. Chem.* 193, 265–275
14 Sachs, D. H. and Painter, E. (1972) *Science* 175, 781–782
15 Spackman, D. H., Stein, W. H. and Moore, S. (1958) *Anal. Chem.* 30, 1190–1206
16 Edlbacher, S. and Zeller, A. (1936) *Z. Physiol. Chem.* 245, 65–75
17 Haider, M. and Segal, H. L. (1972) *Arch. Biochem. Biophys.* 148, 228–237
18 Vielle-Breitburd, F. and Orth, G. (1972) *J. Biol. Chem.* 247, 1227–1235
19 Hirsch-Kolb, H., Kolb, H. J. and Greenberg, D. M. (1971) *J. Biol. Chem.* 246, 395–401
20 Bach, S. J. and Killip, J. D. (1961) *Biochim. Biophys. Acta* 47, 336–343
21 Hunter, A. and Downs, C. E. (1945) *J. Biol. Chem.* 157, 427–446
22 Dixon, M. and Webb, E. C. (1964) *Enzymes*, 2nd edn, p. 322, Academic Press, New York
23 Tappel, A. L., Shibko, S., Stein, M. and Susz, J. P. (1965) *J. Food Sci.* 30, 498–503
24 Schimke, R. T. (1964) *J. Biol. Chem.* 239, 3808–3817