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AN AMINOPEPTIDASE FROM *AGAVE AMERICANA*, CHEMICAL PROPERTIES OF THE ENZYME*

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Key Word Index—*Agave americana*; Amaryllidaceae; proteolytic enzyme; aminopeptidase; properties.

Abstract—*Agave* aminopeptidase, a new enzyme obtained from the plant *Agave americana* displayed activity towards a variety of substrates. A free alphaamino group on these substrates was essential, but the enzyme did not need any metal ions for optimal activity. Aliphatic, aromatic and basic amino acids situated at the amino terminal end of substrates could be hydrolysed by the enzyme. The enzyme had no endopeptidase or other proteolytic activity. Values of the apparent Michaelis constants for different amino acid substrates, all in the range from 0.1 to 0.6 × 10^{-3} M, suggested a relative wide specificity. The pK-values of the two dissociating groups on the enzyme taking part in the catalytic process were pH 6.3 to 6.8 and pH 7.5 to 7.8. These and other studies suggested that histidine plays an active role in the catalytic process. The enzyme was inhibited competitively by free amino acids and this, together with other results, implied a compulsory order of product release.

INTRODUCTION

Although the proteolytic enzymes as a group are now being actively investigated, the same does not apply to the subgroup of aminopeptidases. These enzymes are more difficult to obtain, and usually have large molecular structures, making it difficult to conduct mechanistic studies on them. Leucine aminopeptidase has been studied to the largest extent of this group of enzymes [1–6].

Aminopeptidases have already been obtained from a variety of sources, including higher animals [7–10] and microorganisms [11, 12] but no such enzyme from a plant source has yet been characterized and studied. The aminopeptidase from *Agave americana* has certain unique properties distinguishing it from any other single aminopeptidase, although there are aspects in which this enzyme resembles certain other aminopeptidases. The object of the studies with this aminopeptidase was to find an aminopeptidase with a relative wide specificity to be used in practical research studies. The enzyme was isolated and characterized physically [13], and used in studies towards determination of the primary structures of peptides [14]. In this paper some chemical properties of the purified enzyme are reported.

RESULTS

Purity of enzyme

The purity of *Agave* aminopeptidase was determined by various procedures as described elsewhere [13]. The sp. act. of the enzyme was 0.621 units per mg protein. (A unit of enzyme activity is the amount of enzyme that hydrolyses 1 μmole lysine-*p*-nitroanilide in 1 min).

Specificity of enzyme

The primary specificity of the enzyme was determined by following the appearance of amino acids released from peptides by the enzyme [8, 15]. The amino terminal amino acid was liberated first, followed by the next and following amino acids. Results reported elsewhere [14] supported these findings. Various substrates were used by incubating them in the presence of enzyme in buffer at 45° for 30 to 60 min. All reaction mixtures had a final volume of 1 ml, and substrates were tested at a

Table 1. Substrate specificity of *Agave* aminopeptidase. Hydrolysis of different substrates at a concentration of 0.25 mM in 0.5 M Na-phosphate, pH 7.5 at 45° for 60 min

Substrate	Degree of hydrolysis (μmol/ml/hr)
Leu-*p*-*N*	0.25
Leu–Gly	0.107
Leu–Gly–Gly	0.2
Gly–Gly–Leu	0.214
Leu–Gly–Phe	0.21
Z-Gly–Phe	0.012
Z-Gly–Leu	0
ZTNE	0–0.024
BAPN	0.056
BAA	0.048
Albumin	0.02
Casein	0.042
Glucagon	0.019
BAEE	0
ATEE	0

*Part 2 of a series of papers describing the work reported in the PhD thesis submitted to the Rand Afrikaans University by the senior author. For part 1 see du Toit, P. J., Schabort, J. C., Kempff, P. G. and Laubscher, D. S. A. (1978) *Phytochemistry* **17**, 365.

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Table 2. Substrate specificity of *Agave* aminopeptidase. Relative rate of hydrolysis of different amino acid-*p*-nitroanilides and amino acid-β-naphtylamides in 0.18 M Tris–maleate, pH 7.2 at 30°

Substrate, 0.214 mM	Degree of hydrolysis (units/mg protein)	Relative rate of hydrolysis
Gly-*p*-N	0.057	9
Leu-*p*-N	0.291	48
Lys-*p*-N	0.621	100
Ala-β-N	0.355	100
Arg-β-N	0.296	84
Cys-diβ-N	0	0
Gly-β-N	0.026	7
Ile-β-N	0.179	50
Leu-β-N	0.285	80
Lys-β-N	0.348	98
Phe-β-N	0.171	48
Pro-β-N	0.089	25
Ser-β-N	0.023	6
Try-β-N	0.105	30
Tyr-β-N	0.159	45
Val-β-N	0.189	53

concentration of 0.25 mM. These results are summarized in Tables 1 and 2. With the exception of glucagon all protein substrates were denatured by heat treatment before use. Reactions were followed as indicated in the experimental section.

Kinetic constants

The kinetic constants of the enzyme were determined using different substrates, as described in the Experimental. The values obtained for some substrates are summarized in Table 3.

Effects of metal ions and other reagents and conditions

The extent to which the enzyme was affected by different compounds are indicated in Table 4. These results,

Table 3. Kinetic studies with *Agave* aminopeptidase. Kinetic constants of the enzyme using different substrates

Substrate	Buffer	Addition	K_m ($\times 10^3$ M)	V (units/mg protein)
Lys-*p*-N	0.05 M Na-phosphate pH 7.5	None	0.121	0.606
Lys-*p*-N	0.05 M Na-phosphate pH 7.5	5% (v/v) methanol	0.121	0.571
Leu-*p*-N	0.05 M Na-phosphate pH 7.5	5% (v/v) methanol	0.103	0.358
Gly-*p*-N	0.05 M Na-phosphate pH 7.5	5% (v/v) methanol	0.330	0.077
Lys-*p*-N	0.18 M Tris–maleate, pH 7.2	None	0.098	0.620
Ala-β-N	0.18 M Tris–maleate, pH 7.2	None	0.160	1.156
Phe-β-N	0.18 M Tris–maleate, pH 7.2	5% (v/v) methanol	0.450	0.210
Pro-β-N	0.18 M Tris–maleate, pH 7.2	5% (v/v) methanol	0.560	0.046

Table 4. Effects of different reagents on the catalytic activity of *Agave* aminopeptidase in the presence of 0.5 M Na-phosphate pH 7.5, with 0.05 mM leucine-*p*-nitroanilide as substrate at 25°

Addition		Reaction rate, (V), (units/mg protein)
None		0.621
Ascorbic acid	0.05 M	0.136
$CaCl_2$	0.02 M	0.621
$CoCl_2$	0.02 M	0.557
$MgCl_2$	0.02 M	0.579
$MnCl_2$	0.02 M	0.557
$NiCl_2$	0.02 M	0.316
$ZnCl_2$	0.02 M	0.581
EDTA	0.02 M	0.557
Dithiothreitol	0.005 M	0.463
Cysteine	0.02 M	0.505

as well as results of studies with different metal ions, EDTA and *o*-phenanthroline, led to the conclusion that this aminopeptidase do no not need metal ions for optimal activity. The enzyme displayed optimal activity in the presence of buffer concentrations of 0.18 to 0.2 M.

Results of pH-studies

During studies where different buffers were used, the same optimum pH (about pH 7.2), was observed with both lysine-*p*-nitroanilide and leucine-*p*-nitroanilide as substrates. Results with leucine-*p*-nitroanilide as substrate, as well as the buffers used, are depicted in Fig. 1. In 0.18 M Tris–maleate buffer, similar results were obtained with both lysine-*p*-nitroanilide and alanyl-β-naphthylamide as substrates.

In Figs 2 and 3 the results of kinetic studies at different pH-values, using both 0.18 M Tris–maleate and 0.5 M Na-phosphate buffers, are depicted. The curve for the value of V_{max} as plotted against pH, moves through a maximum, whilst the line indicating the variation of K_m with pH variation, moves through a minimum value. From these results the dissociation constants of the groups on the enzyme, taking part in the catalytic process, were calculated according to the procedures of Laidler [16]. Two pK-values were obtained, one in the region from pH 6.3–6.8 and the other from pH 7.5–7.8. It was also observed that $K_a' < K_a$ and $K_b' > K_b$, although the differences in these values were small. When V^0_{max}/V^0 is plotted against either $[H^+]$ or $[^1/H^+]$ straight lines were obtained, of which the slopes were dependent upon substrate concentration.

Effect of diethyl pyrocarbonate

Incubation of the enzyme in the presence of twice its molar concentration of diethyl pyrocarbonate, resulted in a 83.4% loss of catalytic activity. During subsequent treatment with hydroxylamine and dialysis, the enzyme was reactivated to the extent that 80.7% of the original activity was again obtained. According to A measurements at 250 nm it was deduced that two histidine residues were affected. Results of these studies are shown in Table 5. These results supported the pH-studies in the deduction that histidine serves a function during catalytic functioning of the enzyme, and do exclude the

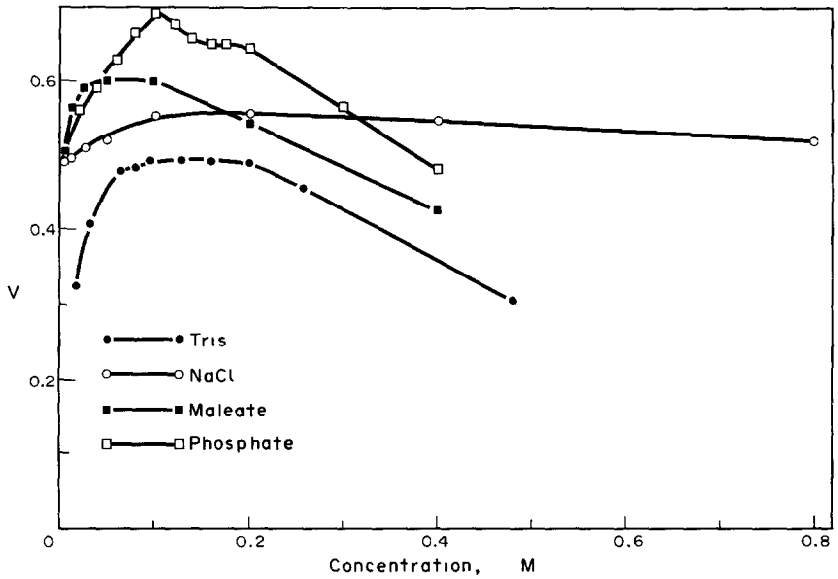

Fig. 1. Effects of different buffers as well as an increase in ionic strength on *Agave aminopeptidase*. V = aminopeptidase activity, units/min/mg protein, 2 mM leucine-*p*-nitroanilide as substrate. NaCl, Na-maleate and Na-phosphate were all added to 0.08 M Tris, pH 7.5, 5.1 μg enzyme used per assay.

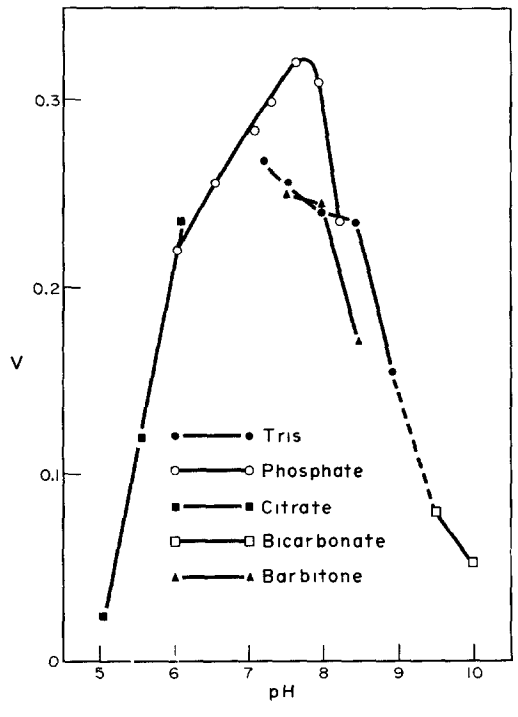

Fig. 2. Effect of pH-variation on *Agave* aminopeptidase. Results obtained with 2 mM leucine-*p*-nitroanilide as substrate in the presence of the different buffers as shown. V = aminopeptidase activity, units/min/mg protein, 0.0102 mg enzyme used per assay.

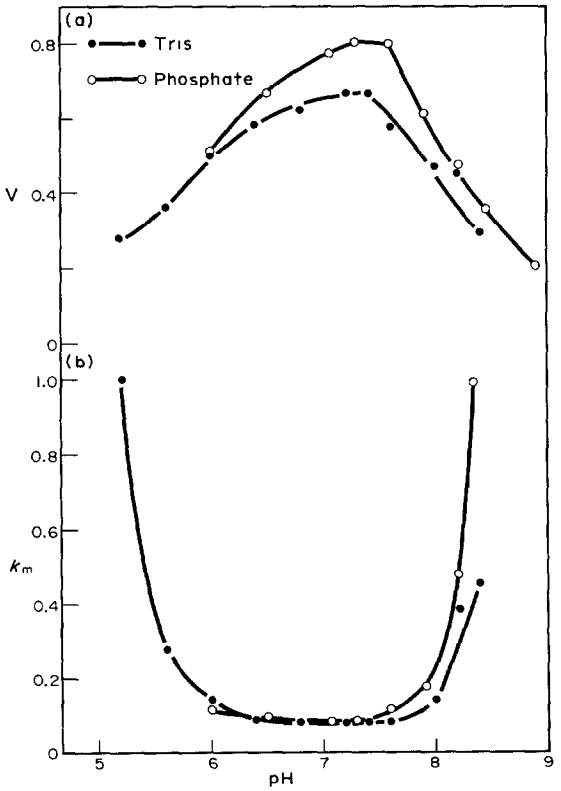

Fig. 3. Variation of K_m and V_{max} with pH as determined in 0.05 M Na-phosphate and 0.18 M Tris–maleate buffers. $V = V_{max}$ = aminopeptidase activity, units/min/mg protein. K_m = indicated value × 10^3 M.

Table 5. Effect of diethyl pyrocarbonate on *Agave* aminopeptidase

Time after addition	Addition, treatment	Activity (units/mg P)	% of initial activity
0	Diethylpyrocarbonate	0.684	100
10 sec		0.465	68
2 min		0.234	34.2
4 min		0.204	29.9
7 min		0.156	22.8
10 min		0.156	22.8
15 min		0.126	18.4
20 min	NH_4OH	0.108	15.8
4 hr	Dialysis Na-phosphate buffer	0.156	22.8
16 hr		0.551	81

possible participation of an α-amino group to some extent.

Inhibition studies

Lysine-*p*-nitroanilide was used as substrate during product inhibition studies. Concentration of the substrate was varied from 0.025 to 1.35 mM, and the concentration of lysine, as inhibitor, from 0.544 to 8.704 mM. Linear plots were obtained and the enzyme was inhibited competitively by lysine with an apparent K_i-value of 4.3 to 4.5 × 10^{-3} M. Under similar conditions with respect to substrate concentration as well as buffer, 0.18 M Tris–maleate buffer, pH 7.2, the concentration of *p*-nitroaniline, as inhibitor, was varied from 0.0034 to 0.128 mM. Linear plots were also obtained indicating pure non-competitive inhibition with an apparent K_i-value of 0.11 × 10^{-3} M. The K_m-value of the enzyme for lysine-*p*-nitroanilide under these conditions was 0.098 × 10^{-3} M.

DISCUSSION

The catalytic activity of *Agave* aminopeptidase was not influenced by the presence of divalent metal ions. This is illustrated by the result of different studies with metal ions as well as chelating agents. This aminopeptidases differs consequently from most other known aminopeptidases.

Regarding the specificity of the enzyme, it was observed that a free α-amino group on the substrate was essential for activity. The most suitable substrates for the enzyme were according to results, peptide structures with more than 3 amino acid residues but less residues than that of a structure like glucagon. The aminopeptidase displayed no esterolytic or carboxypeptidase and neglectably low proteolytic activity. Aliphatic-, aromatic- and basic amino acids situated at the amino terminal end of a peptide could be hydrolysed by the enzyme. Proline situated at this position could also be removed by the enzyme under certain circumstances. Results of studies reported elsewhere [14], indicated that amino terminal aspartic acid and glutamic acid could also be removed by the enzyme. When proline was situated at the second position on the substrate, this aminopeptidase, like all known aminopeptidases, except aminopeptidase P [17], could not always remove the amino terminal amino acid. According to these and other observations, it seems as if the side chains of amino acids in the substrate, in close vicinity of the amino terminal amino acid, had an effect upon the rate of removal of the said amino terminal residue.

According to the results of the kinetic studies at different pH values, there were two main ionizing groups on the enzyme taking part in the catalytic process. These groups had pK-values of 6.3–6.8 and 7.5–7.8 respectively. Possible groups that could thus be operative are the imidazole group of histidine or an α-amino group. The imidazole group of histidine seem to be the group active in the catalytic process of the enzyme, as shown by the effects of TPCK and diethylpyrocarbonate. The aminopeptidase was not influenced by either sulphydryl activating or inhibiting reagents which excludes the possible participation of a free sulphydryl group. Even using different substrates, the optimum pH of the enzyme was always pH 7.2. From the above-mentioned studies further conclusions could also be made. During combination of the enzyme and substrate, the substrate seems to complex with both ionizable groups on the enzyme. The enzyme thus falls in group I as classified by Laidler [18]. There was, however, one aspect in which the conduct of the enzyme differed, that was the observation that reaction rate was still dependent upon pH even at very high substrate concentrations. One should, however, keep in mind that the substrate used during these studies is not the natural substrate for the enzyme, which could be the cause of the deviation in the general expected pattern.

The apparent Michaelis constants of the enzyme for different substrates as used were all of the same order of magnitude. The primary interaction between enzyme and substrate thus seems to be based on ionic interactions rather than on other non-polar interactions. This observation is in accordance with the observed ionic strength effects. These conclusions are further substantiated by results reported elsewhere [19].

Agave aminopeptidase was inhibited competitively by lysine and non competitively by *p*-nitroaniline when lysine-*p*-nitroanilide was used as substrate. The free amino acid thus competes with the substrate for binding sites on the enzyme. This observation is similar to results reported for other aminopeptidases and fits into the general pattern of catalysis for *Agave* aminopeptidase.

The main obstacle in obtaining further information on the properties of the enzyme are the difficulties in obtaining large quantities of this aminopeptidase. Further studies carried out are reported elsewhere [19], and the enzyme was also used in studies for the determination of the primary structure of peptides [14].

EXPERIMENTAL

Aminopeptidase was isolated from *Agave americana* L. Am. trop. and the purity of the enzyme was determined by electrophoretic studies, gel filtration studies as well as ultracentrifugal analysis [13]. Substrates used during studies included the tripeptides Gly–Leu–Tyr, Gly–Gly–Leu and Leu–Gly–Gly, some dipeptides, the *p*-nitroanilides of glycine, leucine and lysine and the β-naphthylamides of most naturally occurring amino acids. Other substrates used were Hammersten casein, BSA, glucagon and various substrates for determination of other specific proteolytic activities, which will be mentioned in the section regarding activity determinations. All other chemicals and biochemicals used were also analytically pure.

Enzyme assays, enzyme specificity. Tripeptide method. Aminopeptidase activity was assayed by measuring the hydrolysis of the tripeptides to their constituent amino acids [8]. Solns of tripeptides (0·001–0·03 M) in 0·05–0·18 M Tris–maleate buffer, pH 7.2, were incubated with the enzyme and aliquots (2–5 μl) were withdrawn for TLC on Si gel. The solvent was BuOH–HoAc–H_2O (4:1:1) and amino acids were visualized by a ninhydrin spray reagent using conventional colour developing procedures. Protein substrates were also tested under these conditions. Hydrolysis rates were also determined as described in refs [1, 7].

Choromogenic substrates method. Specificity and other studies were conducted with a variety of substrates under varying conditions. Enzymatic activity with amino acid-*p*-nitroanilides as substrate was determined according to ref. [15] and that with the amino acid β-naphthylamides according to the procedure of ref. [20]. Buffers used during these studies included 0.05 M Na-Pi, pH 7.5, and 0.18 M Tris–maleate, pH 7.2. Substrate concns used were as indicated together with the results.

Other activity determinations. Protease activity was determined with a Kunitz procedure, incubating the enzyme in the presence of a buffered protein soln at 25–45° for 30–60 min. Non-hydrolysed protein was pptd with 10% TCA, filtered off, and the *A* increase of the filtrate was determined at 280 nm. Esterase activity was determined by using *N*-benzyloxycarbonyl-L-tyrosine *p*-nitrophenyl ester (CBz–Tyr–ONp) [21], benzoyl arginine ethyl ester (Bz–Arg–OEt) [22] and *N*-acetyl-L-tyrosine ethyl ester (Ac–Tyr–OEt) [23] as substrates. Amidase activity was determined with benzoyl-arginine amide (Bz–Arg–NH_2) [22] and benzoyl-arginine-*p*-nitroanilide (Bz–Arg–NAn) [24], whilst carboxypeptidase activity was determined with carbobenzoxyglycyl-phenylalanine [25], hippuryl-arginine [26] and hippuryl-L-phenylalanine [27]. In all these cases the conditions employed were similar to those already mentioned. Where possible, conditions were adapted to the optimum conditions for *Agave* aminopeptidase.

Kinetic studies. Determination of the constants K_m and V_{max} where conducted with different substrates under a variety of conditions at 25° as indicated in Table 3. Inhibition studies were conducted with lysine-*p*-nitroanilide and leucine-*p*-nitroanilide in 0.18 M Tris–maleate buffer, pH 7.2, with lysine or leucine (concn ranging from 0.5 to 8.0 mM) and *p*-nitroaniline (concn ranging from 0.003 to 0.128 mM). The numerical values of all constants were obtained by double reciprocal and single reciprocal plots. In all cases lines were fitted to the experimental data by the least squares method.

Effect of pH variation. Using different buffers and different substrates, as indicated in the results, the effect of pH variation on the activity of the aminopeptidase was determined. Kinetic studies, with lysine-*p*-nitroanilide and leucine-*p*-nitroanilide as substrates, were also conducted over the range pH 5–9 in 0.05 M Na-Pi and 0.18 M Tris–maleate buffers. Results of these were analyzed according to ref. [18].

Other determinations. Effects of increased salt concentrations as well as those of different buffers were determined using the *p*-nitroanilide assay procedure. Buffers used included Pi, Tris–maleate and Tris, and the effects of EDTA, 1,10-phenanthroline, $CaCl_2$, NaCl, ascorbic acid, dithiothreitol, $MgCl_2$, $MnCl_2$, $CoCl_2$, $NiCl_2$, $ZnCl_2$, cysteine, *p*-chloromercuribenzoate and TPCK were assessed at various concns as indicated in the results. *Agave* aminopeptidase was also incubated in EDTA and 1,10-phenanthroline separately and together up to concns of 0.01 M in 0.18 M Tris–maleate buffer, pH 7.2, at 37° for 30 min before activity was determined by the usual procedure. The effect of diethylpyrocarbonate on the enzyme was tested according to the procedure described in ref. [28].

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REFERENCES

1. de Lange, R. J. and Smith, E. L. (1971) *The Enzymes* (Boyer, P. D. ed.) Vol. 3, pp. 81–119. Academic Press, New York.
2. Smith, E. L. and Hill, R. L. (1960) *The Enzymes* (Boyer, P. D., Lardy, K. and Mijrback, H. eds) Vol. 4, pp. 37–62. Academic Press, New York.
3. Light, A. (1970) *Methods in Enzymology* (Colowick, S. P. and Kaplan, N. O. eds) Vol. 11, pp. 426–436. Academic Press, New York.
4. Himmelhoch, S. R. (1970) *Methods in Enzymology* (Perlmann, G. E. and Lorand, L. eds) Vol. 19, pp. 508–513. Academic Press, New York.
5. Metrione, R. M. (1972) *Biochim. Biophys. Acta* **268**, 518.
6. Birch, P. L., el-Obeid, H. A. and Akhtar, M. (1972) *Arch. Biochem. Biophys.* **148**, 447.
7. Pfleiderer, G. (1970) *Methods in Enzymology* (Perlmann, G. E. and Lorand, L. eds) Vol. 19, pp. 514–521. Academic Press, New York.
8. Marks, N. and Lajtha, A. (1970) *Methods in Enzymology* (Perlmann, G. E. and Lorand, L. eds) Vol. 19, pp. 534–543. Academic Press, New York.
9. Dehm, P. and Nordwig, A. (1970) *European J. Biochem.* **17**, 364.
10. Jones, D. D., Williams, G. F. and Prochazka, B. (1972) *Enzymologia* **43**, 325, 339.
11. Roncari, G. and Zuber, H. (1970) *Methods in Enzymology* (Perlmann, G. E. and Lorand, L. eds) Vol. 19, pp 544–552. Academic Press, New York.
12. Chapuis, R. and Zuber, H. (1970) *Methods in Enzymology* (Perlmann, G. E. and Lorand, L. eds) Vol. 19, pp. 552–555. Academic Press, New York.
13. du Toit, P. J., Schabort, J. C., Kempff, P. G. and Laubscher, D. S. A. (1978) *Phytochemistry* **17**, 365.
14. du Toit, P. J. (1976) *Tydskr. vir Natuurwet.* **16**, 2.
15. Tuppy, H., Wiesbauer, U. and Wintersberger, E. (1962) *Z. Physiol. Chem.* **329**, 278.
16. Laidler, K. J. (1958) *The Chemical Kinetics of Enzyme Action* pp. 55–93. Clarendon Press, Oxford.
17. Dehm, P. and Nordwig, A. (1970) *European J. Biochem.* **17**, 364.
18. Laidler, K. J. (1958) *The Chemical Kinetics of Enzyme Action*, pp. 117–162. Clarendon Press, Oxford.
19. du Toit, P. J. and Schabort, J. C. (1978) *Phytochemistry* **17**, 377.
20. Lee, H. J., Larne, J. N. and Wilson, I. B (1970) *Anal. Biochem.* **41**, 397.
21. Walsh, K. A. and Wilcox, P. E. (1970) *Methods in Enzymology* Perlmann, G. E. and Lorand, L. eds) Vol. 19, pp. 31–41. Academic Press, New York.
22. Murachi, T. (1970) *Methods in Enzymology* (Perlmann, G. E. and Lorand, L. eds) Vol. 19, pp. 273–284. Academic Press, New York.
23. Wilcox, P. E. (1970) *Methods in Enzymology* (Perlmann, G. E. and Lorand, L. eds) Vol. 19, pp. 64–112. Academic Press, New York.
24. Arnon, R. (1970) *Methods in Enzymology* (Perlmann, G. E. and Lorand, L. eds) Vol. 19, pp. 226–244. Academic Press, New York.
25. Hoffman, K. and Bergmann, M. (1940) *J. Biol. Chem.* **134**, 225.
26. Folk, J. E. (1970) *Methods in Enzymology* (Perlmann, G. E. and Lorand, L. eds) Vol. 19, pp. 504–508. Academic Press, New York.
27. Snoke, J. E. and Neurath, H. (1949) *J. Biol. Chem.* **181**, 789.
28. Wallis, R. B. and Holbrook, J. J. (1973) *Biochem. J.* **133**, 183.