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ALCOHOL DEHYDROGENASE ISOENZYMES IN CHICKPEA COTYLEDONS*

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Key Word Index—*Cicer arietinum*; Leguminosae; chickpea; germination; ethanol; alcohol dehydrogenase; isoenzymes; properties.

Abstract—Alcohol dehydrogenase (ADH) (EC 1.1.1.1) in the cotyledons of chickpea consists of three isoenzymes, ADH-1, ADH-2 and ADH-3, in order of decreasing electrophoretic mobility. Isolation in different extraction media did not cause interconversion of these isoenzymes, but caused changes in their relative proportions and stabilities. Crude isoenzyme extract in Tris–HCl buffer containing mercaptoethanol and sucrose remained stable for several months. The isoenzymes were more stable to acid treatment and relative stability of the isoenzymes differed depending on pH. ADH-1, which is present in largest amount in dry seeds, was most heat-stable while ADH-3, which is present in the smallest amount, was most heat-labile. Thermal stability was not altered by NAD^+. Variations in the substrate and inhibitor specificities were observed between the isoenzymes. During germination the levels of ADH and ethanol reached their peaks after 24 hr and then declined. The relative amounts of the isoenzymes also changed during the same period. Activity of ADH-3 showed maximum increase while activity of ADH-1 showed minimum increase. ADH-3 remained active for the shortest time whereas ADH-1 remained active for the longest time after germination. ADH-2 showed intermediate *in vitro* and *in vivo* activity and stability.

INTRODUCTION

Alcohol dehydrogenase isoenzymes occur widely in higher plants [1–3]. The most detailed investigations in relation to genetics have been made with maize and several other higher plants, reviewed in refs. [1–3]. Studies on ADH isoenzyme structure and properties have been published for maize [4–8], wheat [9] and soybean [10]. Changes in ADH activity and its isoenzyme patterns are associated with many stages of development, including germination and differentiation of maize [11], wheat, rye and rice [12], and other plants [1–3]. Regulatory mechanisms governing ADH activity during development are not clearly understood. Scandalios *et al.* [13] proposed that ADH isoenzymes are irreversibly and sequentially inactivated after germination due to generation of an ADH specific inhibitor, while alternative mechanisms, including a two-factor inhibitor system, have been proposed by Schwartz *et al.* [14, 15] to explain regulation of ADH activity in maize.

Developmental changes of ADH activity [16] and ethanol [17] have been reported in germinating chickpea. However, developmental changes and properties of ADH isoenzymes in chickpea have not been studied. In this paper we report on some properties of three ADH isoenzymes in chickpea and the quantitative and qualitative variations of these isoenzymes during germination of chickpea seedlings, utilizing the techniques of polyacrylamide gel electrophoresis (PAGE) and densitometric tracings of the histochemically stained gels. In addition, we tried to correlate the *in vivo* and *in vitro* activities and stabilities of these isoenzymes with their possible physiological functions in germinating chickpea.

RESULTS AND DISCUSSION

PAGE electrophoresis of the isoenzymes

Comparative analyses by PAGE at pH 8.5 showed the presence of three distinct ADH isoenzymes called ADH-1, ADH-2 and ADH-3, in order of decreasing electrophoretic mobility, in the crude extracts of dry or germinated chickpea. ADH-3, last to appear during incubation of the gels, is always narrow and lightly stained, while ADH-2, second to appear, is clearer and more intensely stained. ADH-1, most intensely stained and clearest, is the first to appear.

We have tried to determine whether ADH isoenzymes are artifacts of the extraction procedure and ionic interactions. For comparison, the following extraction media were used: distilled water, potassium phosphate buffer (pH 6.0 and 7.6), 20% sucrose

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solution, Tris–HCl buffer (pH 7.6) with and without 10 mM mercaptoethanol and 12.5% sucrose. In no case could any *in vitro* conversion, change in mobility and number of isoenzymes be detected by electrophoresis. However, the relative proportions of the isoenzymes varied with respect to different solvent preparations. Extractability and stability of the isoenzymes were in the order: Tris–HCl buffer with mercaptoethanol and sucrose > 20% sucrose > Tris–HCl buffer > potassium phosphate buffer > distilled water. As several possibilities, e.g. endogenous or bound substrates, have been reported to be responsible for 'nothing dehydrogenase' activity [18], gels were stained in a solution containing all the reagents except ethanol. No reduction of tetrazolium salt occurred at any position on the gels, suggesting the absence of a 'nothing dehydrogenase' effect.

Properties of the isoenzymes

Crude isoenzyme extracts stored at −20° in Tris–HCl buffer (pH 7.6) containing mercaptoethanol and sucrose appeared to show only 10–20% loss of activity over a period of several months, whereas storage at 0–4° resulted in a complete inactivation within 1 week. The isoenzymes were completely inactivated within 24 hr during storage at room temperature. Dilution resulted in rapid loss of enzyme activity. Storage under different conditions and dilutions did not indicate interconversion or alteration in the mobility of the isoenzymes.

The effect of heating the isoenzymes is shown in Table 1. *Ca* 95% of ADH-3 was inactivated after 30 min at 52° while 63.7% of ADH-1 was inactivated. ADH-2 showed intermediate heat stability. Change in electrophoretic mobility as well as conversion of a thermolabile isoenzyme to a thermostable one by binding of NAD^+ [19] has been demonstrated for *Drosophila* ADH isoenzymes. To investigate this, crude enzyme extracts were either dialysed for 15 hr at 4° against 500 volumes of potassium phosphate buffer (pH 6.0) containing various concentrations of NAD^+ *ca* 1–10 mM or preincubated with 5–20 mM NAD^+ for 2–3 hr at 4° and then electrophoresed. Our results showed no effect on electrophoretic mobility or heat stability of the isoenzymes. Thus variation in their heat stability may be a result of differing amino acid composition, as suggested for two ADH isoenzymes, coded by distinct loci in maize [6].

The fact that ADH-1 is the most heat stable and active may raise questions regarding the stability of the isoenzymes to different pH values. Therefore, pH stability of the isoenzymes was investigated. As shown in Table 2 the isoenzymes were most stable at pH 6.0 and totally inactivated at pH 9.8 when crude enzyme extract was dialysed against buffers at the appropriate pH at 4°. In addition, the relative stability of the isoenzymes differed, ADH-1 being most stable and ADH-3 being least stable. Previous reports have also shown that maize ADH and its isoenzymes are more stable at low pH [5, 20]. ADH in crude extract showed maximum activity at pH 8.8 and half maximum activity at *ca* pH 7.8 and 9.5. This pH optimum is similar or close to those already reported for other higher plant ADHs [21–23].

Chickpea ADH isoenzymes oxidized different alcohols (Table 3). However, variations in the degree of substrate specificity between the individual isoenzymes were observed. ADH-1 always appeared to be catalytically most active with different alcohols. In contrast, ADH-3 showed the least activity. Moreover, differences in specificity of the isoenzymes towards different substrates were observed. As far as substrate specificity is concerned, chickpea ADH isoenzymes showed a low degree of substrate specificity and resemble ADHs in maize [4, 5], wheat [9], various legumes [21–23] and potato [24].

Inhibition of the activity of the isoenzymes by several inhibitors is summarized in Table 4. They are differentially inhibited by sulphydryl reagents and chelating agents which are known to inhibit plant ADHs [21–23]. Regardless of the inhibitor used,

Table 1. Thermal inactivation of alcohol dehydrogenase isoenzymes in chickpea*

Isoenzymes	%Enzyme activity inhibited (control = 100)† Minutes of heating			
	0	10	20	30
ADH-1	0	46.0	53.4	63.7
ADH-2	0	53.4	60.0	68.1
ADH-3	0	86.7	90.1	93.4

*Fresh crude enzyme extracts either preincubated at 52° for the indicated times and then electrophoresed or, after electrophoresis the gels were incubated at 52° in Tris–glycine buffer (pH 8.5) for the indicated times. Control gels in buffer or enzyme extracts were stored at 4°. The isoenzymes were stained and quantitated as described in the Experimental.

†The amount of each isoenzyme is expressed as a percentage of the control. The amounts of the control were (peak area, mm^2) ADH-1, 329.9; ADH-2, 255.2; ADH-3, 172.7. Each value represents the average value of four independent experiments with cotyledons of seeds germinated for 24 hr.

Table 2. pH stability of alcohol dehydrogenase isoenzymes in chickpea*

Isoenzymes	%Enzyme activity inhibited (control = 100)† pH values		
	6.0	8.0	9.8
ADH-1	22.5	87.6	100
ADH-2	51.8	89.4	100
ADH-3	64.0	95.0	100

*Aliquots (1 ml) of crude enzyme extract were dialysed separately against 500 volumes of 0.1 M KPi (pH 6.0 and 8.0) and glycine–NaOH buffers (pH 9.8) at 4° for 15 hr. An undialysed extract stored at 4° served as control. Equal amounts of protein from the dialysate and the control extract were electrophoresed. Histochemically stained isoenzymes were quantitated as described in the Experimental.

†The amount of each isoenzyme is expressed as a percentage of the control. The amounts of the control were (peak area, mm^2) ADH-1, 329.9; ADH-2, 255.2; ADH-3, 172.7. The values are the means of four sets of experiments with cotyledons of seeds germinated for 24 hr.

Table 3. Substrate specificity of alcohol dehydrogenase isoenzymes in chickpea*

Substrates	Amount of isoenzyme relative to control = 100†		
	ADH-1	ADH-2	ADH-3
Methanol	2.68	0.00	0.00
Ethanol	100.00	100.00	100.00
n-Propanol	58.93	22.00	13.00
iso-Propanol	3.20	0.00	0.00
n-Butanol	75.00	26.83	19.00
iso-Butanol	12.50	3.00	0.00
n-Pentanol	43.50	16.50	9.70
n-Hexanol	26.00	10.00	5.00

*After the electrophoretic run, the gels were incubated in a staining solution containing substrate at a final concentration of 0.43 M. Staining, incubation and densitometric tracings were determined as described in the Experimental.

†The amount of each isoenzyme is expressed as a percentage of ethanol oxidation. The amounts of the control were (peak area, mm^2) ADH-1, 329.9; ADH-2, 255.2; ADH-3, 172.7. The values are the means of four sets of experiments, with the cotyledons of seeds germinated for 24 hr; the variability of different experiments was less than 10%.

ADH-1 was least affected and ADH-3 was most affected, while ADH-2 showed intermediate sensitivity towards the inhibitors. The inhibitory effects of sulphydryl reagents and metal chelators indicate that chickpea ADH is a metalloenzyme with a sulphydryl group in the active centre.

It can be concluded that chickpea ADH exists as three isoenzymes.

Ethanol, ADH and its isoenzymes in germinating chickpea

The changes in the levels of ethanol and ADH during the germination of chickpea are shown in Table 5. The results show that although no ethanol could be detected in dry seeds, there was considerable ADH activity in the same seeds. ADH activity increased during the initial hours of germination when ethanol made its appearance. However, the maximal enzyme activity coincided with the maximal ethanol concentration after 24 hr and subsequently decreased enzyme activity was associated with the depletion of the ethanol level. At day 15 only 5.27% of the peak activity could be detected. No residual activity could be detected at day 20. Similar fluctuations in the levels of ADH and ethanol have been reported in maize [11], chickpea [16, 17] and many other higher plants [22].

The variation of each individual isoenzyme during germination was compared directly by applying equal amounts of protein (*ca* 200–300 μg in 20–30 μl) from seedlings of various ages to different gels for simultaneous electrophoresis. The changes in the intensity of each individual isoenzyme band was followed by direct densitometry of the stained gels throughout the period of germination. The data are presented in Table 6. In the cotyledons of dry seeds, ADH-1, ADH-2 and ADH-3 accounted for 52.6, 32.5 and 14.9% of the total activity respectively. As germination proceeded in darkness, the amount of ADH-3 increased at a faster rate than those of the other isoenzymes, but they all peaked together at 24 hr. Of the total activity at this stage, 43.5% was represented by ADH-1, 33.7% by ADH-2, and 22.8% by ADH-3. There were increases of *ca* 82.8, 130 and 237% in ADH-1, ADH-2 and ADH-3 respectively. After 24 hr, the amount of each individual isoenzyme declined at different rates. ADH-3 disappeared most rapidly by

Table 4. Effect of inhibitors on alcohol dehydrogenase isoenzymes in chickpea*

Inhibitor and concentration	Incubation time (min)	Amount of isoenzyme activity relative to control = 100†		
		ADH-1	ADH-2	ADH-3
Iodoacetamide (10 mM)	10	64	56	36
	20	51	43	26
	30	35	28	18
p-Chloromercuribenzoate (0.1 mM)	10	46	20	10
	20	13	0	0
	30	0	0	0
EDTA (1 mM)	10	63.3	59	45
	20	46.6	37	25
	30	28.9	15	5
$CuSO_4$ (0.1 mM)	10	40	30	15
	20	23.2	10	0
	30	0	0	0

*Inhibitors were pre-incubated with fresh enzyme extract (1.6 mg protein in 0.4 ml) for indicated times at room temperature and then aliquots (200–400 μg protein in 30–40 μl) were immediately electrophoresed. Alternatively, after electrophoresis gels were incubated for indicated times in Tris–glycine buffer (pH 8.5) containing inhibitors and washed thoroughly. Gels were then stained and quantitated as described in the Experimental. Control consisted of duplicate gels stained with ethanol in absence of inhibitors.

†The amount of each isoenzyme is expressed as a percentage of the control. The amounts of the control were (peak area, mm^2) ADH-1, 329.9; ADH-2, 255.2; ADH-3, 172.7. The values are the means of four sets of experiments with 24 hr germinated seeds.

Table 5. Changes in ADH activity and ethanol concentration during germination of chickpea

Days of germination	Sp. act. of ADH (units/mg protein)	Ethanol concentration (μ mol/g fr. wt)
D	342	0
S	415	5.0
$\frac{1}{2}$	580	10.2
1	760	20.1
2	590	9.0
3	540	4.5
4	446	2.2
5	355	1.8
7	230	0.5
11	149	0.0
13	69	0.0
15	40	0.0
20	0	0.0

ADH activity and ethanol concentration were determined as described in the Experimental.

D and S indicate cotyledon extracts from dry seeds and soaked seeds respectively.

day 7, followed by the disappearance of ADH-2 by day 13 and ADH-1 by day 20. At day 15 only 5.1% of the peak activity was retained by ADH-1 alone. At day 20 no residual activity could be detected electrophoretically. However, the time course of disappearance of these isoenzymes varied slightly, depending on the conditions of germination. Thus the changes in the activity of the individual isoenzyme agreed well with the changes in total ADH activity during germination. Changes in isoenzyme pattern during germination have been described for maize [11], wheat, rye and rice [12] and other plants [1–3].

From the root extracts of 5-day-old chickpea seedlings, all three isoenzymes could be resolved while a small amount of ADH-1 and a trace of ADH-2 were obtained from extracts of shoot. In leaf, stem and root extracts of older plants (20-day-old) no ADH band could be detected.

Increases in ADH and in ethanol during the initial hours of germination have been observed in many plants, but the biochemical mechanism is not entirely clean. Davies [25] has proposed that at first lactate and malate accumulate in anaerobically respiring tissues (e.g. germinating seeds with an unruptured seed coat) causing a fall in pH. Acidic pH restricts further reduction of pyruvate by lactate dehydrogenase (LDH), but favours the decarboxylation of pyruvate to acetaldehyde [25] which acts as a substrate and induces ADH. The ability of acetaldehyde to induce ADH has been reported [26]. Acetaldehyde is immediately reduced to ethanol by ADH [25]. In the presence of oxygen, ethanol is metabolized via the TCA cycle [27]. Besides these, ADH may in some way be connected with steroid [28] and metabolism of other substrates [24].

The maximum rise in the specific activity of ADH and the peak activity of each individual isoenzyme coincide with the appearance of alcohol in chickpea. These suggest that ADH may be induced by acetaldehyde prior to the formation of ethanol [26]. The fact that ADH-3 exhibited the maximum rise (*ca* 237% of the initial activity after 24 hr) and showed the most pronounced decay rate, suggested that it functions only in storage cotyledons participating in the reduction of acetaldehyde during the initial hours of germination (anaerobic respiration). Previous studies with maize ADH isoenzymes [3] have shown that the fastest isoenzyme increased most significantly under anaerobic conditions as compared to other isoenzymes and it seemed to favour the reduction of acetaldehyde. The least increase (82.8%) occurring in ADH-1 activity and its gradual disappearance by day 20 in aerobically respiring seeds (e.g. germinating seeds with ruptured seed coat) suggest that it perhpas participates in the oxidation of ethanol formed during aerobic respiration, to acetaldehyde. These interpretations are supported by the finding that, from the root extract of 5-day-old seedlings, all three isoenzymes were resolved, while from extracts of shoot and leaves, only ADH-1 and ADH-2 were obtained. Intermediate *in vitro* enzymatic activity and stability, and *in vivo* inactivation of ADH-2 in germinating chickpea suggest that if may function in both directions.

Table 6. Changes in the amounts of three ADH isoenzymes in the cotyledons of chickpea during germination*

Isoenzyme	Amount of isoenzyme expressed as a percentage of that at day 1† Days of germination											
	D	S	$\frac{1}{2}$	1	2	3	4	5	7	11	13	15
ADH-1	54.7	76.9	94.2	100	81.8	77.2	71.1	63.2	48.3	30.1	20.6	12.2
ADH-2	43.5	65.9	92.2	100	74.1	62.0	55.3	45.1	27.8	14.9	0.0	0.0
ADH-3	29.7	64.5	87.2	100	72.7	59.1	47.1	22.1	0.0	0.0	0.0	0.0

*The total extracts of the cotyledons from dry seeds, soaked seeds and seedlings germinated from day 1 to day 15 were used. Since fr. wt and the protein contents of the cotyledons changed during germination, equal amounts of protein (200–400 μg in 20–40 μl) in the supernatant fraction of the cotyledons of different ages were used for electrophoresis. The gels were stained and quantitated as described in the Experimental.

†The amounts of isoenzymes at day 1 were (peak area, mm^2) ADH-1, 329.9; ADH-2, 255.2; ADH-3, 172.7. D and S indicate cotyledon extracts from dry seeds and soaked seeds respectively. The values are the means of three sets of experiments.

Since ADH in germinating maize remained unaffected by inhibitors of nucleic acid and protein synthesis, and density labelling techniques showed absence of turnover of ADH molecules during germination [29], the increase in ADH activity may simply represent an activation of existing enzyme protein rather than *de novo* synthesis of enzyme [26]. However, it has been reported that *de novo* synthesis of ADH isoenzymes can be induced by subjecting the seedlings to prolonged anaerobiosis [12, 13]. The decrease in ADH activity may be due to repression of the structural genes for ADH by some unknown mechanism during the onset of germination, resulting in the termination of polypeptide synthesis and selective elimination of ADH, mediated by some endogenous ADH specific inhibitor [13]. Since the inhibition could be prevented by mercaptoethanol, and maize protease and ADH are not affected by mercaptoethanol and polyvinylpyrrolidone respectively, it has been suggested that the inhibitor (inhibitors) is not a protease or phenolic substance [2, 3].

EXPERIMENTAL

Plant materials. Chickpea seeds (*Cicer arietinum*, variety unknown) were purchased from the local market and used in the present investigation. Seeds of uniform size were soaked for 6 hr in running tap H_2O and washed extensively with distilled H_2O after which they were germinated in a dark thermostatic chamber at 22–23°; in distilled H_2O-moistened filter paper in a Petri-dish (diam. 19 cm) for 24 hr. When plants older than 24 hr were desired, soaked and washed seeds were planted in plastic trays on a layer (1.5–2.0 cm) of sterilized quartz sand moistened with Knop's nutritive soln and then germinated as described above for 0–20 days. After removal of the seed coat the seeds were separated into cotyledons and embryos.

EtOH *determination.* Cotyledons (0.5 g) were quickly ground in a chilled mortar and pestle and the homogenate was immediately washed with 30 ml ice-cold H_2O directly into a ground joint flask which was then connected to the distillation set. EtOH in the distillate was determined colorimetrically by the method of ref. [30].

Extraction of ADH. Cotyledons (1 g) were ground in 4 ml 50 mM Tris–HCl buffer (pH 7.6) containing 10 mM mercaptoethanol and 12.5% sucrose using sand and a chilled mortar and pestle. 1 ml grinding medium was used per g fr. wt of other seedling organs. The slurry was centrifuged at 6000 *g* for 15 min and the supernatant filtered through Whatman No. 1 filter paper which removed most of the lipid material floating on top of the supernatant. The filtrate was centrifuged at 10 000 *g* for 15 min. The resultant preparation was used for protein and enzyme determinations and PAGE.

Enzyme assays and protein determination. ADH was determined in the direction of NAD^+ reduction as described in ref. [31]. The reaction mixture consisted of 2.7 ml 5 mM Tris–HCl buffer (pH 8.8), 0.1 ml 5 mg/ml NAD^+ and 0.05–0.1 ml enzyme extract. This soln was used to set the instrument at zero. Increase in *A* was measured at 30 sec intervals on a Calbiochem UV Enzymeter at 30° upon the addition of 0.1 ml 1.713 M EtOH. 1 unit of ADH activity is defined as the amount of enzyme necessary to produce a change in A_{340} of 0.001/min. Protein determinations were by the Biuret method both before and after TCA precipitation using BSA as standard [32].

PAGE. Electrophoresis was performed with 7.5% acrylamide gels (0.5 × 7 cm) according to the method of ref. [33]. Gel and reservoir buffer was 0.025 M Tris–0.192 M glycine, pH 8.5 and 0.001% bromophenol blue in 10 μl 20% BSA was the marker. The protein soln (200–400 μg in 20–40 μl) was inserted at the top of the gels to form a layer under the buffer. Electrophoresis was conducted initially for 30 min at 2.5 mA/tube (80 V) and then for 60–70 min at 3–4 mA/tube (160 V) at 4–8°. The staining mixture for locating ADH on the gels contained 4.7 ml Tris–glycine buffer (pH 8.5), 0.1 ml EtOH and 0.2 ml colour reagent (supplied with Test Kit, Lac-Dehystrate, for LDH assay; manufactured by Division of Warner-Lambert Co., Morris Plains, NJ, U.S.A.) containing 0.4 mg 2-*p*-iodophenyl-3-*p*-nitrophenyl-5-phenyl tetrazolium chloride, 0.1 mg phenazine methosulphate and 1 mg NAD^+. Since this preformulated colour reagent did not stain the gels at all it proved to be more satisfactory in staining ADH than our prepared staining mixture as described in ref. [34]. After stain development at 37° in the darkness, the gels were rinsed in H_2O and stored in 4% HOAc at 4° in the dark. Stained gels were scanned at 550 nm using a gel scanning apparatus for measuring the intensity of the stained ADH as peak height on the recorder tracing. Peak heights were determined by comparing tracings of gels incubated in complete assay mixtures with those of control gels where EtOH was omitted.

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REFERENCES

1. Shannon, L. M. (1968) *Annu. Rev. Plant Physiol.* **19**, 187.
2. Scandalios, J. G. (1978) in *Reg. Dev. Processes Plants, Proc. Conf.* (Schutle, H. R. and Gross, D., eds.) p. 36. Fisher, Jena.
3. Scandalios, J. G. (1974) *Annu. Rev. Plant Physiol.* **25**, 225.
4. Longo, G. P. and Scandalios, J. G. (1968) *Biochem. Genet.* **2**, 177.
5. Laughner, W. J. (1970) Ph.D. Thesis, Indiana University, U.S.A.
6. Felder, M. R. and Scandalios, J. G. (1971) *Mol. Gen. Genet.* **111**, 317.
7. Felder, M. R., Scandalios, J. G. and Liu, E. (1973) *Biochim. Biophys. Acta* **317**, 149.
8. Kelly, J. and Freeling, M. (1980) *Biochim. Biophys. Acta* **624**, 102.
9. Jaaska, V. and Jaaska, V. (1978) *Biokhimiya* **43**, 2011.
10. Beremand, M. N. (1979) *Diss. Abstr. Int. B* **40**, 1513.
11. Scandalios, J. G. and Felder, M. R. (1971) *Dev. Biol.* **25**, 641.
12. Jaaska, V. (1976) *Eesti NSV Tead. Akad. Toim. Biol.* **25**, 237.
13. Lai, Y. K. and Scandalios, J. G. (1977) *Differentiation* **9**, 111.
14. Freeling, M. and Schwartz, D. (1971) *Biochem. Genet.* **8**, 27.
15. Fisher, D. (1974) *Diss. Abstr. Int. B* **35**, 313.
16. De la Fuente P. and Nicolas, G. (1974) *Plant Sci. Letters* **3**, 143.
17. Aldarso, J. and Nicolas, G. (1980) *Phytochemistry* **19**, 3.
18. Ferguson, E. E. (1971) *Enzymologia* **40**, 81.
19. Jacobson, K. B. (1968) *Science* **159**, 324.
20. Schwartz, D. and Laughner, W. (1969) *Science* **166**, 626.
21. Cossins, E. A., Kopala, L. C., Blowacki, E. and Spronk, A. M. (1968) *Phytochemistry* **7**, 1125.

22. Leblová, S., Zimaková, I., Barthová, J. and Ehlichová, D. (1971) *Biol. Plant.* **13**, 33.
23. Leblova, S., Perglerová, E. and Hlochová, J. (1977). *Biol. Plant.* **19**, 88.
24. Davies, D. D., Patil, K. D., Ugochukwu, E. N. and Towers, G. H. N. (1973) *Phytochemistry* **12**, 523.
25. Davies, D. D. (1973) in *Biosynthesis and its Control in Higher Plants* (Milborrow, B. V., ed.) p. 1. Academic Press, New York.
26. Crawford, R. M. M. and MacMannon, M. (1968) *J. Exp. Botany* **19**, 435.
27. Cameron, D. S. and Cossins, E. A. (1967) *Biochem. J.* **105**, 323.
28. Jornvall, H. (1977) *Biochem. Soc. Trans.* **5**, 636.
29. Ho, D. T. H. and Scandalios, J. G. (1975) *Plant Physiol.* **56**, 59.
30. Sunshine, I. and Appleton, H. D. (1961) in *Standard Methods of Clinical Chemistry* (Seligman, D., ed.) Vol. 3, p. 1. Academic Press, New York.
31. Bonnichsen, R. F. and Brink, N. G. (1955) in *Methods in Enzymology* (Colowick S. P. and Kaplan, N. O., eds.) Vol. 1, p. 495. Academic Press, New York.
32. Gornall, A. G., Bardawill, C. J. and David, M. M. (1949) *J. Biol. Chem.* **177**, 751.
33. Bloemendaal, H. (1963) *Zone Electrophoresis in Blocks and Columns.* Elsevier, Amsterdam.
34. Markert, C. L. and Moller, F. (1959) *Proc. Natl. Acad. Sci. U.S.A.* **45**, 733.