

BIOCHEMICAL STUDIES OF SOME HYDROLASES AND
DEHYDROGENASES IN COTYLEDONS OF GERMINATING PSOPHOCARPUS
TETRAGONOLOBUS L. AND VICIA FABA L.

by

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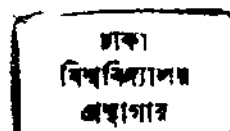
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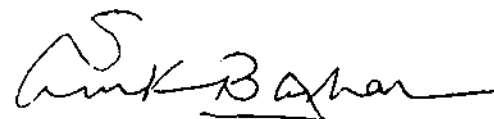
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CERTIFICATE

This is to certify that the thesis entitled "Biochemical Studies of Some Hydrolases and Dehydrogenases in Cotyledons of Germinating Psophocarpus tetragonolobus L. and Vicia faba L." is an original work and has been completed under our supervision towards the fulfilment of the requirements for Ph. D. degree.



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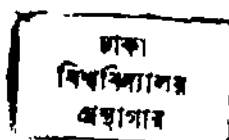


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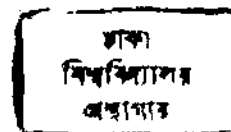
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Abbreviations and sources of chemicals used

<u>Chemicals used</u>	<u>Abbreviations</u>	<u>Sources</u>
Tris (hydroxymethyl)methylamine	Tris	BDH
β -mercaptoethanol	BME	Serva
Ethylene diamine tetraacetic acid, Na Salt	EDTA	BDH
Nicotinamide adenine dinucleotide	NAD	Sigma
Dithiothreitol	DTT	Serva
Bovine serum albumen	BSA	Sigma
Trichloroacetic acid	TCA	E.Merck
Dimethylsulfoxide	DMSO	BDH
Fast blue RR salt		Serva
N-N methylene bisacrylamide		Serva
Acrylamide		Serva
β -dimethyl aminopropionitrile		BDH
Potassium ferricyanide		BDH
Ammonium persulfate		BDH
Bromophenol blue		Serva
Dinitrosalicylic acid	DNSA	E.Merck
3-(4-Iodophenyl)-2-(4-nitrophenyl) 5-phenyl-2H-tetrazolium chloride	INT	E.Merck
Phenazine Methosulphate	PMS	E.Merck
Benzoyl-DL-arginine-p-nitro-anilide	BAPNA	BDH

β -naphthyl acetate	E. Merck
Ethanol, methanol, n-propanol, n-butanol, n-pentanol, allyl alcohol, <u>iso</u> -butanol, <u>sec</u> -butanol, <u>iso</u> -propanol, <u>tert</u> -amyl alcohol cyclohexanol and decanol.	BDH
Sodium hydrogen malate	Serva
Sucrose	E. Merck
Starch	E. Merck
Haemoglobin	Serva
Iodoacetamide, Eserine, O-phenanthroline, Cu So ₄	E. Merck

The object of the present investigation

The aims of the present investigation are :

- a. To study the changes in the activities of alcohol dehydrogenase, malate dehydrogenase, esterase, protease and amylase in germinating seeds of winged bean and kalomator in order to elucidate their roles in the mobilization of reserve materials.
- b. To study the changes of isoenzyme patterns of alcohol dehydrogenase, malate dehydrogenase and esterase during germination of winged bean and kalomator with a view to understanding the possible physiological functions of the isoenzymes during germination.
- c. To study the effects of aerobic and anaerobic conditions, different salt solutions, and light and dark on alcohol dehydrogenase activity and its isoenzyme patterns as these are known to have influences on enzyme activities.
- d. To characterize crude alcohol dehydrogenase in winged bean and kalomator with respect to K_m values, pH optima, pH stabilities, storage and thermostabilities, and substrate and inhibitor specificities.
- e. To characterize the alcohol dehydrogenase isoenzymes of winged bean with respect to pH and thermostability, and substrate and inhibitor specificities.

ABSTRACT

Alcohol dehydrogenase (ADH) (E.C.I.I.I.I.) extracted from germinated seeds of winged bean (Psophocarpus tetragonolobus L.) and kalomator (Vicia faba L.) in Tris-HCl buffer (pH 7.6) containing β -mercaptoethanol and sucrose remained stable for several months at $-20^{\circ}C$. β -mercaptoethanol and sucrose protected the enzyme activities. pH optimum for the oxidation of ethanol was in the alkaline region i.e. 8.8 for both ADHs. The Michaelis constants for ethanol were $1.4 \times 10^{-2}M$ and $0.6 \times 10^{-2}M$ for winged bean and kalomator ADHs respectively while K_m values for NAD ($2 \times 10^{-2}M$) and for allyl alcohol ($0.5 \times 10^{-2}M$) were identical for both plant ADHs. The substrate specificities were found to vary with different alcohols. Iodoacetamide, eserine, dimethyl sulphoxide, O-phenanthroline, $CuSO_4$ and EDTA inhibited enzyme activities differentially. Enzyme activities increased rapidly during the initial stage of germination to reach their peaks at 48 hours of germination and then declined in both the species.

In the cotyledons of winged bean and kalomator each ADH consists of three isoenzymes, ADH-1, ADH-2 and ADH-3, in order of increasing electrophoretic mobility. Quantitative densitometric analysis of the histochemically developed isoenzymes in dry seeds of winged bean showed that ADH-1, ADH-2 and ADH-3 accounted for 13.6, 51.1 and 35.2% of the total activity respectively. ADH-3 was most heat stable while ADH-1 was most heat liable. Variations in the substrate and inhibitor specificities were observed between the isoenzymes of winged bean.

During germination the levels of ADH and ethanol reached their peaks after 48 hours and then declined. The relative amounts of the isoenzymes also changed during the same period. Activity of ADH-1

showed maximum increase while activity of ADH-3 showed minimum increase at day 2. ADH-1 remained active for the shortest time whereas ADH-3 remained active for the longest time after germination. ADH-2 showed slightly less in vivo activity than ADH-3.

Activities of the three ADH isoenzymes in kalomator increased with germination. At day 2 the amounts of the isoenzymes reached peaks. At this stage ADH-2 and ADH-3 stained intensely and ADH-1 appeared fairly stained. At day 15 ADH-1 disappeared and the other two isoenzymes appeared fairly stained.

Complete anaerobiosis increased the ADH activity and the amount of each isoenzyme but no new bands appeared. At the peak ADH activity of dark grown seedlings of winged bean was 50% higher than in seeds grown in light. Germination of winged bean in presence of different salt solutions (20 mM) caused differential increases in ADH activity and the amount of each isoenzyme upto day 2, followed by decline.

Malate dehydrogenase (MDH) (E.C.I.I.I.37) activities in winged bean and kalomator increased at the beginning of germination, reached peaks at day 1 and decreased slowly with the progress of germination. In both the species MDH activities were resolved into four isoenzymes which differed in intensity and mobilities within and between the species. The fluctuations of MDH isoenzymes in winged bean and kalomator corroborated with the fluctuations of their respective total activities at different stages of germination.

Esterase activities in dry winged bean and kalomator seeds were low, increased slowly with the progress of germination and reached

peaks at day 11 and 13 respectively. Eight and two esterase bands were detected in dry seeds of winged bean and kalomotor respectively. With the onset of germination two new bands appeared in winged bean, while some old bands disappeared or some bands became stronger or some became weak. To the contrast, two esterase bands in germinating kalomotor showed changes in intensities only.

Protease activities as measured towards benzoyl-DL-arginine -p-nitroanilide (BAPNA) were quite high in resting cotyledons of winged bean and kalomotor, increased slightly with germination and then declined rapidly. To the contrary, protease activities towards haemoglobin were relatively low in resting seeds of winged bean and kalomotor, increased steadily with the onset of germination and reached peaks at days 11-13 and then declined.

Amylase activities in dry seed extracts of winged bean and kalomotor were low. These activities increased with the onset of germination and reached peaks at day 13 and 11 in winged bean and kalomotor respectively. The increases were 471 and 900% higher than those of dry seed extracts. The activities then gradually decreased.

CHAPTER 1

GENERAL INTRODUCTION

Plants synthesize various organic compounds. All these compounds are derived from the products of photosynthesis. The substances produced in greatest abundance are various carbohydrates, lipids (fats) and proteins. They are oxidized by the process of respiration releasing energy essential to other metabolic steps.

A large number of plants both monocotyledonous and dicotyledonous accumulate carbohydrate (maize, barley^e, oat, wheat, corn, rye),^{e/} proteins (bean, lentil, soybean) and lipids (rape, sunflower, castor bean, flax) as reserve substances in seeds. These substances are essential in the various metabolic activities. During germination of seeds, a massive breakdown of the reserve substances begins with the help of amylolytic, proteolytic and lipolytic enzymes and the products are transported to the growing seedlings for their development.

A significant portion of research on the control of germination has been devoted to elucidate the mechanism of synthesis and activation of enzymes and proteins during the early hours of germination. Dry seeds contain many enzymes including those responsible for the metabolic events that are still being conducted at an appreciable rate as the seeds are finally matured and dried out. Most of these enzymes are resistant to desiccation for they become active once more as soon as imbibition starts. A few enzymes, already present in the seed, undergo an activation process at some stage during germination, becoming fully active as a result of proteolysis or some conformational changes (Mayer and Marbach 1981). The progress of respiration in imbibing seeds is generally triphasic.

The rate rises rapidly in the first hours of imbibition, reaches a plateau value of variable duration and then increases further. This further rise is associated with cell division in the tissues of the root-shoot axis, but is present also on a more modest scale in the cotyledons of pea and bean in which there is no cell division.

The substrates consumed in respiratory metabolism during the early period of germination are sugars or oligosaccharides (Hsu et al. 1973, Mayer and Shain 1974). Several types of evidence suggest that the pathway of sugar breakdown in the first few hours of imbibition differs from that which is adopted in the later phases of germination and seedling growth. This conclusion emerges from inhibitor studies, work with labelled substrates and what is known about the activity of mitochondria. Using labelled glucose in mung bean (Vigna radiata), Morohashi and Shimokoriyama (1975) have observed that during the first 5.5 hours of imbibition glucose was converted predominantly to ethanol rather than to Krebs Cycle acids, while the opposite was true in the following 3 hours suggesting a shift from fermentation to respiration.

In fat containing seeds (e.g. castor bean), fatty acids are metabolized in special organelles, the glyoxysomes (Beevers 1979, 1980, Tolbert 1980). During seed germination, fatty acids are nearly quantitatively^e converted into carbohydrates by the process of gluconeogenesis which requires the contributions of glyoxysomes, mitochondria and the cytosol.

The large body of literature on protein synthesis during imbibition and germination has recently been reviewed (Bray 1979, Mayer

and Marbach 1981, Bewley and Black 1978, Bewley 1982). The synthesis of protein and RNA is resumed as soon as seed tissues begin to imbibe, but DNA synthesis follows later. It is further reported that stored mRNA have little role once imbibition commences and that it is liable to be degraded within a few hours (Simon 1980). The alternative view is that some of the stored mRNA has a role ~~at~~ / ~~a~~ much latter, when germinating seeds mobilize most of their reserves. It is further suggested that desiccation and rehydration terminate the synthesis of proteins normally accumulated during development and initiate the synthesis of different proteins: a 'germination pattern' (Dasgupta and Bewley 1982).

Cell division follows only some hours after the start of DNA synthesis, at about the time of germination. The lengthening of the radicle that is entailed in germination is normally accomplished by cell elongation alone, mitosis contributing little or nothing. Germination can indeed occur in the complete absence of cell division, as when lettuce seeds are treated with X-rays or germinated at low temperature (Haber and Luippold 1960).

It is apparent, therefore, that protein synthesis and RNA synthesis occur quite early in germination and that DNA replication is rather late. It is possible that DNA replication has occurred during seed ~~formation~~ / ~~to~~ a sufficient extent to permit the initial stages of germination. On the other hand, some proteins are activated during germination by mechanisms which are still not sufficiently clear. Unfortunately, the accuracy of determining the time of development of protein activation as compared to protein synthesis is also quite inadequate. Consequently, it is impossible to decide

which of the two processes controls and regulates germination. It seems quite possible that no general answer exists to this question and that in different tissues, the control mechanism differs enormously (Mayer and Shain 1974).

Different enzymes play significant roles in various ways at different stages of seed germination. Some of the major enzymes that are active during germination of seed are described briefly under two headings :

1.1 Dehydrogenases

1.2 Hydrolases

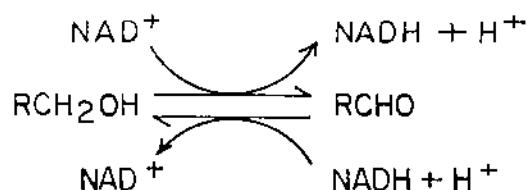
1.1 Dehydrogenases

This group of oxidation reduction enzymes participates in the transfer of hydrogen from one substance to another. Both donor and acceptor can be and usually are quite specific. This group of enzymes is concerned with biological oxidation and reduction, and therefore with respiration and fermentation processes. They catalyze oxidoreduction reactions. The substrate which is oxidized is regarded as hydrogen donor, and the systematic name is donor-acceptor oxidoreductase. The number of known dehydrogenases is rather large, and many of them have been intensively studied. The dehydrogenases are essential to the functioning of all metabolizing cells. Alcohol dehydrogenase (ADH) and malate dehydrogenase (MDH) are two important members of this large group of dehydrogenases.

1.1.1 Alcohol dehydrogenase

Alcohol dehydrogenase (EC 1.1.1.1.) (ADH) is an enzyme which reversibly catalyzes the conversion of alcohols to the corresponding

aldehyde coupled with the reversible oxidation reduction of nicotinamide adenine dinucleotide.



1.1.2 Plant alcohol dehydrogenase

Alcohol dehydrogenase is universally distributed in mammals, plants and bacteria. But in comparison to animal and yeast ADHs, much less is known about the higher plant ADHs because of instability of plant ADHs (Cossins et al. 1968). Adler and coworkers (1937) first reported isolation of wheat germ ADH which was found to be NAD^+ specific. Properties of ADHs were studied in peas (Cossins et al. 1968, Eriksson 1968, Leblova et al. 1977, Cerevska and Leblova 1981) in bean, wheat, rape, sunflower, rice and broad bean (Leblova and Stiborova 1975, Leblova et al. 1977) in cucumbers (Leblova and Perglerova 1977), in peanut (Pattee and Swaisgood 1968), in tea (Hatanaka et al. 1974), in potato (Davies et al. 1973), in chickpea (Gomes et al. 1982, Leblova and EL Ahmad 1985), in corn (Belszerova and Plotnikova 1984) in Nicotiana (Kul and Evans 1984) and in sorghum (Ellstrand et al. 1983).

Plant alcohol dehydrogenases show a very broad substrate specificity. Regarding substrate specificity, mostly quantitative rather than qualitative differences are found between individual plant ADHs. Unsaturated alcohols such as 2-propen-1-ol (allyl alcohol), trans-2-hexen-1-ol and trans-2-buten-1-ol are more rapidly

oxidized than their saturated analogues and ethanols (Eriksson 1968, Leblova et al. 1977). For other unsaturated alcohols, plant ADHs show much less affinity or do not oxidize at all. The initial rates of oxidation of alcohols decrease as the chain length increase from ethanol to hexanol. Other types of alcohols are oxidized very slowly or not at all (Eriksson 1968, Leblova and Stivorova 1975, Leblova and Perglerova 1977).

K_m values for plant ADHs vary in the range from $5 \times 10^{-2} M$ to $1 \times 10^{-3} M$ for ethanol and allyl alcohol and from 5×10^{-3} to 1×10^{-5} for NAD^+ (Leblova et al. 1977).

pH optima for plant ADH were found in the alkaline region i.e. from 8.2 to 8.8 for ethanol and allyl alcohol. The co-enzyme for plant ADH was found to be NAD^+ (Leblova et al. 1977).

In view of sensitivity to sulphhydryl poisons and chelating agents, plant ADHs were considered to be metallo-enzymes with SH group in the active site (Cossins et al. 1968, Leblova et al. 1977). According to Fisher and Schwartz (1973) this metal is zinc.

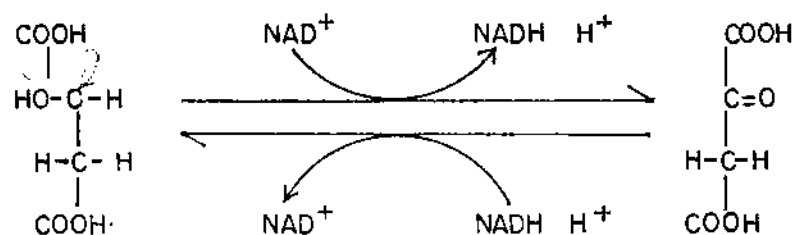
Molecular weights of plant ADH vary from 53,000 to 80,000 (Cossins et al. 1968, Eriksson 1968 and Leblova et al. 1977). However, molecular weight of peanut and tea ADHs were found to be twice as large (Pattee and Swaisgood 1968, Hatanaka et al. 1974).

The physiological function of higher plant ADH is similar to that of yeast. During the early hour of germination (natural anaerobiosis), pyruvate is converted to lactate which lowers the cell pH to slightly acidic region. At this pH , pyruvate ceases to be reduced and pyruvate decarboxylase is activated for

decarboxylating pyruvate to acetaldehyde. This acetaldehyde, perhaps, acts as substrate and induces the increase of ADH activity. Then acetaldehyde is reduced to ethanol by ADH coupled with oxidation of NADH which is, perhaps, obtained from the oxidation of 3-phosphoglyceraldehyde to 1,3²diphosphoglycerate. During aerobic condition (after the breakage of seed coat), the same enzyme oxidizes ethanol to acetate via acetaldehyde (Scandalios and Felder 1971, Davies *et al.* 1974, and Leblova *et al.* 1976).

1.1.3 Malate dehydrogenase

Malate dehydrogenase (EC 1.1.1.37) (MDH) is an enzyme which catalyzes the interconversion of malate and oxaloacetate in the presence of NAD^+ .



The reaction catalyzed by malate dehydrogenase is freely reversible, but the equilibrium favours the formation of malate and oxidation of ADH. Because of this, it is generally considered that any regulatory role of malate dehydrogenase would be due to the presence of oxaloacetate, inhibiting or preventing malate oxidation (Wiskich 1980).

1.1.4 Plant malate dehydrogenase

A variety of vascular plants ranging from primitive rootless form to advanced monocots and dicots have been surveyed and all

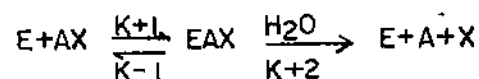
tissues have malate dehydrogenase suggestive of mitochondrial and soluble forms (Ting *et al.* 1975). Price and Thimann(1954) first showed MDH activity from mitochondrial and soluble fractions of plant tissues.

Three forms of MDH occur in plant tissues with distinct intracellular distribution (reviewed by Scandalios 1974) viz. mitochondrial form, cytoplasmic form and microbody form (glyoxysomes or peroxysomes). Mitochondrial form is a component of the Krebs cycle (Price and Thimann 1954, Leech and Ellis 1961). The cytoplasmic form was proposed to participate in interorganelle shuttling of electrons and other metabolic pathways (Price and Thimann 1954, Ting *et al.* 1966, Douce and Bonner 1972). Functionally, glyoxysomes and peroxysomes are very different. Peroxysomes play an important role in the process of photorespiration, while glyoxysomes are involved in certain phases of fat metabolism (Yamazaki and Tolbert 1969, Rocha and Ting 1970, Meyer *et al.* 1973).

Compared with other plant dehydrogenases this enzyme is relatively stable (Chilson *et al.* 1965). For this reason it has probably been extensively investigated in plants (Chen *et al.* 1970, O'Sullivan and Wedding 1972, Yang and Scandalios 1974, Gerhardt 1983).

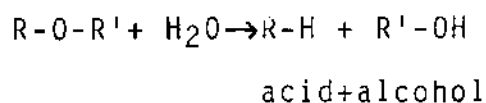
1.2 Hydrolases or Hydrolytic enzymes

The hydrolases are a large group of enzymes which have in common the involvement of water in formation of products. Because, one of the substrates for the hydrolytic reaction is water, the reaction catalyzed can be represented by the general equation.



1.2.1 Esterases

Esterases are involved in the splitting of ester linkages by the addition of water:

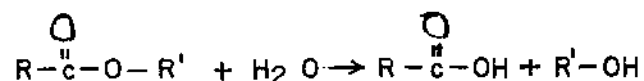


The alcohols may be monohydric or polyhydric, aliphatic or aromatic. The acid may be an organic or an inorganic acid. Esterases is specific for either the acid or the alcohol moiety but not for both.

The esterases have been subdivided into several groups primarily on the basis of specificity for the acid involved in ester substrates. These are:

(a) carboxylic ester hydrolases, (b) thiolester hydrolases, (c) phosphoric monoester hydrolases, (d) phosphoric diester hydrolases, (e) triphosphoric monoester hydrolases, (f) sulfuric ester

The carboxylic ester hydrolases (EC 3.1.1.) are a diverse group of enzyme which have in common the splitting of carboxylic acid esters by the addition of water:



The carboxylic ester hydrolases can be divided into two groups:

(a) the non-specific carboxylic ester hydrolases and (b) the specific carboxylic ester hydrolases.

Non-specific carboxylic ester hydrolases have been distinguished into 3 different types (carboxylic-ester hydrolases, aryl-ester hydrolases and acetic-ester hydrolases) on the basis of their substrate specificities, inhibition and activation. Non-specific esterases act on soluble and short chain fatty acid esters while lipases act only on triglycerides at water-lipid interface to liberate glycerol, monoglycerides, and fatty acids.

Specific carboxylic ester hydrolases have further been subdivided into several types such as phospholipase, chlorophyllase, pectinesterase and acetyl cholinesterases.

1.2.2 Esterases in Plants

Plant esterases had not been investigated as thoroughly as animal esterases (Hunter and Markert 1957, Markert and Hunter 1959). An important contribution to the study of this group of enzyme had been made by the use of electrophoretic methods followed by characterization of isolated enzymes based on their reactions with various substrates and inhibitors (Augustinsson 1958, 1959).

Plant tissues contain complex systems of esterases (Norgard and Montgomery 1968, Putnam and Montgomery 1968, Carino and Montgomery 1968). Studies during germination and development of plants have been made in pea (Safonova et al. 1970), in wheat (Daussant and Abbot 1969), in flax (Xanthium) (Chen et al. 1970) in bean and

potato (Jaaska et al. 1972) and in several legumes (Gomes 1978). Recently esterases have been studied in-hybrid rice (Yingguo and Weijian 1983) and oat (Xu and Chin 1985).

Specific physiological and biochemical functions of plant esterases have not yet been identified. However, Van Fleet(1961) proposed that these enzymes^{arc} involved in fat metabolism and suberin formation. In addition, De Duve and Wattiaux (1966) proposed that acid hydrolases, including non-specific esterases take part in controlled cell autolysis during the process of cell differentiation and senescence. Other possible functions of plant esterases might be their involvement in various metabolic systems which affect the flavour, texture and colour of food substances. Esterases could detoxify insecticides which otherwise would be harmful to the human. By inhibiting esterases, insecticides might affect the metabolism of plants and cause undesirable changes in the flavour, texture and colour of these plant parts used for food.

1.2.3 Proteases

Proteases play a central role in the biochemical mechanism of germination. The common reaction catalyzed by the proteolytic enzymes is the hydrolysis of peptide bonds of a protein. Most of the work on the biochemistry and the mechanism of action of proteolytic enzymes^{was} was carried out on animal and micro-organism.

Proteolytic enzymes may be broadly divided into two groups e.g. (1) endo-peptidases that cleave internal peptide bonds and (11) exopeptidases that cleave single amino acids from either the C - or N-terminal end of the polypeptide chain. A recent proposal

concerning the nomenclature of proteolytic enzymes^{was} that the term / protease be used to describe all enzymes that break peptide bonds, that endo^opeptidases be termed "proteinases" and that exopeptidases be termed "peptidases"(Barrett 1980).Based on mechanistic consideration, six sets of proteolytic enzymes were identified (Hartley 1960, Walsh 1975, Ryan and Simmons 1980).

1. Sulfhydryl endopeptidase e.g. papain, ficin etc.
2. Serine endopeptidase, e.g. trypsin, chymotrypsin etc.
3. Acid endopeptidase, e.g. pepsin, cathepsin B etc.
4. Metal endopeptidase, e.g. thermolysin, neutral proteinases etc.
5. Serine exopeptidase, e.g. carboxypeptidase C and Y.
6. Metal exopeptidase e.g. carboxypeptidase A and B.

1.2.4 Proteases in Plants

Reports on the roles of specific proteases in plants are relatively less compared to that of animals and micro organism. Several types of proteolytic enzymes work collectively to cleave proteins producing small peptides and amino acids. These products are utilized by cells or transported to other tissue.

Proteolytic activities during germination^{wt} was studied in peas (Beevers 1968, Yomo and Varner 1973, Basha and Beevers 1975), Kidney bean (Pusztai and Duncan 1971, Yomo and Srinivasan 1973, Mikkonen 1986), field beans (Lichtenfeld et al. 1979, in mung bean (Chrispeels and Boulter 1975), in pea nut (Basha and Cherry 1978), in vetch (Korolyova et al.1973) in Cucumis sativus (Grazyna 1980), in legume seeds (Lichtenfeld et al. 1981, Klaus et al. 1985), in lentil

(Alvarez and Guerra 1985) in black gram (Morohashi 1982), in finger millet (Vidyavathi et al 1983) and in wheat (Karakeeva et al. 1983) in Vigna mungo (Mitsunashi et al. 1986).

An increase in proteolytic activity during seed germination in many seeds has long been recognized (Ryan 1973, Ashton 1976). It was suggested that in germinating seeds and growing seedlings, sulfhydryl, acid and serine endo-peptidases are most responsible for the proteolytic activity (Rayan and Simmons 1980). Numerous accounts involving a variety of plant genera suggest that sulfhydryl endopeptidases are synthesized as one of the initial events in protein mobilization. Studies on the synthesis of an endopeptidase following the germination of mung beans and its localization within protein bodies have provided interesting insights into the early events of protein mobilization. More than 95% of the endopeptidase activity after 5 days of germination is accounted for by one sulfhydryl endopeptidase. At the time of synthesis of the enzyme (3 days), the protein bodies begin to undergo proteolysis (Chrispeels et al. 1976). In contrast, protein bodies isolated from Phaseolus vulgaris cotyledons, upto 4 days after initiating germination, showed no proteolytic activity at all (Pusztai et al. 1977). Recently Mikkonen (1986) suggested that acid proteinases and carboxypeptidases initiate the breakdown of storage proteins in germinating kidney bean while residual alkaline peptidases, dipeptidases and naphthylamidase complete the proteolytic breakdown of protein at later stage of germination.

1.2.5 Amylases

Amylases are among the earliest known enzymes which are almost universally distributed throughout the animal and plant kingdom.

Payen and Persoz (1833) isolated amylases from malt extract which converted starch into sugar. This substance which is now called amylase was named by them as "diastase". The amylase catalyzed hydrolysis of an α 1-4 linked glucose polymer is achieved by the transfer of a glucosyl residue to water. Amylases are either referred to as α or β -amylases or as endo or exoamylases. α -Amylases, α (1 $\rightarrow$ 4) glucan 4-glucano-hydrolase EC 3.2.1.1 possess the α configuration. α -Amylases attack large linear substrates at most internal bonds and are properly classified as endoenzymes. Later, it was discovered that another amylase hydrolyzes starch to yield a single product called β -maltose. β (1 $\rightarrow$ 4) glucan maltohydrolase, EC 3.2.1.2 are exoenzymes and attack alternate linkages from the non-reducing end of a substrate. Recently, a third class of amylases has been discovered which are poorly characterized and commonly called glucoamylases α (1 $\rightarrow$ 4) glucan glucohydrolase (EC 3.2.1.3). This amylase like β -amylase is exoenzyme but removes a single glucose unit from the non-reducing end of its substrate with inversion of configuration.

1.2.6 Amylases in plants

α -amylase is wide spread in plant and animal kingdoms. In contrast to α -amylase, β -amylase is restricted mainly to seeds and roots of higher plants (Thoma et al. 1971) and in pea seedlings (Swain and Dekker 1966b). The properties of α - and β -amylases have been well reviewed (Fischer and Stein 1961 and Thoma et al. 1971, French 1961). α and β -amylases from barley, wheat, maize^e, rice, rye, soybeans and lentils were extensively studied (Whitaker 1971,

Okamoto and Akazawa 1979, 1978, 1980, Jacobsen and Higgins 1982, Koshiha and Minamikawa 1983 and Morohashi 1982).

How these two hydrolases differing in their mode of action are involved in the breakdown of the reserve starch molecules is still not fully understood. Although there is no direct evidence to show that β -amylase is truly involved in the starch hydrolysis during germination of starchy seeds, the co-ordinated interaction of α -and β -amylases in the complete breakdown of starch has been postulated (Paleg 1960). Walker and Hope (1963) reported that β -amylase, unlike α -amylase could not attack the native starch granules. Based on the results of model experiments, it has been postulated ^{that} β -amylase may be involved in the secondary step of starch hydrolysis following the initial action exerted by α -amylase (Dunn 1974). It is generally agreed that the inactive latent form of β -amylase molecules associated with other proteins in cereal seeds can be cleaved either enzymically or by reducing agents, and eventually an active enzyme is released (Rowell and Goad 1962, Tronier and Ory 1970).

1.3 Enzymes and Their Multimolecular Forms

It is well established that many enzymes exist in multiple molecular forms. This may be due to the occurrence ^{of} multiple gene loci coding for distinct version of the enzyme protein or to the existence of multiple alleles at a single gene locus (Harris 1975). Such variation is a major explanation of the origin of isoenzymes, the name given to the multiple forms of an enzyme occurring ⁱⁿ a single species (Markert and Moller 1959). Prior to that time, molecular heterogeneity had been noted by Meister (1950) in beef heart

lactate dehydrogenase (LDH), but such heterogeneity was viewed as an artifact by biochemists. Subsequently, paper electrophoresis of LDH (Neilands 1952a, Wieland and Pfleiderer 1957) suggested that this heterogeneity might not all be artifactual but indeed reflect reality within the cells. In 1957, Vesell and Bearn showed by starch block electrophoresis that the highly reproducible pattern of multiple forms of LDH in normal human serum changed in myocardial infarction and acute myelogenous leukemia. Zone electrophoresis used by these investigators were laborious and had poor resolving power for separating the multiple forms of enzymes. As a result, these reports had little impact on investigators until starch gel electrophoresis (Smithies 1955) was coupled with histochemical staining procedure by Hunter and Markert (1957) to visualize separated isoenzymes.

Introduction of starch-gel and later polyacrylamide-gel electrophoresis (Raymond and Weintraub 1959, Ornstein and Davies 1959) as an electrophoretic medium made it possible to separate proteins in crude homogenates. With the advent of new and more refined separation techniques such as electrofocussing, chromatofocussing etc. in recent years, there has been an increasing awareness that enzyme multiplicity is the rule rather than exception.

1.3.1 Classification of Isoenzymes

Markert (1975) proposed that the general term isoenzymes can be modified by such terms as allelic, non allelic, homopolymeric, heteropolymeric, epigenetic conjugated, conformational and so forth. Shaw (1969) in his review, classified isoenzymes into two major categories: (a) those that are distinctly different molecules and are

presumably produced from different genetic sites and (b) those which result from secondary alterations on the structure of a single polypeptide species and may in many cases, be in vitro artifacts.

The IUPAC-IUB Commission on Biochemical Nomenclature (1977) suggested a classification of multiple forms, employing seven groups. These are:

1. Genetically independent proteins.
2. Heteropolymers (hybrids) of two or more polypeptide chains, non-covalently bound.
3. Allelic genetic variants.
- 4a. Proteins conjugated with other groups.
- 4b. Proteins derived from one polypeptide chain.
5. Polymers of a single sub-unit.
6. Conformationally different forms.

Group 1,2 and 3 are the true isoenzymes, whose synthesis is controlled by distinct stretches of DNA, whereas groups 4,5 and 6 are the secondary isoenzymes derived by post-synthetic modification of a single gene product.

1.3.2 Numbering of Isoenzymes

Wieland and Pfeleiderer (1957) first proposed the system of numbering I to the isoenzymes with the greatest anodal mobility. Most of the European investigators have been using this system, while majority of the American workers have described the slowest component as isoenzyme - I (Plagemann et al. 1960). To solve this numbering problem, the Standing Committee on Enzymes of the International Union of Biochemistry, has made a temporary rule to assigning number 1 to the isoenzyme having highest negative charge

i.e. with the greatest anodal mobility (Webb 1964).

1.3.3 Characteristics of Isoenzymes

Characterization of structure and function relationships of isozymes follow the general procedure long applied to the characterization of any enzyme. These include determinations of molecular weight, various kinetic parameters such as substrate affinity (k_m), pH optima, heat stability, substrate specificity, inhibitor effects etc. for comparing the similarities and dissimilarities in primary structures. Finally amino acid analysis, peptide finger printing, chromatography of proteolytic digest and cyanogen bromide cleavage products leading to determinations of amino acid sequences, and crystallographic analysis provide fine details of genetic and evolutionary relationship.

Most studies with isoenzymes have been purely descriptive in nature, merely noting the presence or absence of isoenzymes in various tissues during ontogeny and pathological conditions of an organism. Therefore, there is little data available on the relationship between the genetic and biochemical differences of isoenzymes. However, most of the available data have come from the work on LDH (Everse and Kaplan 1975, Nadal Ginard and Markert 1975, Markert et al. 1975 and many others). Creatine Kinase (Dawson et al. 1968), aldolase (Penhoet et al. 1966, Horecker 1975), Phosphorylase (Krebs and Fisher 1962) and alcohol dehydrogenase (Pietruszko 1975). The data conform to the well established relationship between the isoenzymes and genomes (DNA). And this relationship between LDH isoenzymes and genomes has well been reviewed by Winterhalter (1975).

Winterhalter (1975) has shown that one molecular form of LDH exists in Lampetra planeri which is phylogenetically lowest among the investigated vertebrates and belongs to Cylostomata. She proposed the existence of only one LDH locus for the synthesis of LDH polypeptides. Similar finding has been reported by Markert et al. (1975).

In human and other vertebrates LDHs were found to exist in five molecular forms, which are formed by tetrameric arrangement of two different subunits co-ordinated by distinct genetic loci. Existence of two genetic loci have been shown to be situated on specific pair of chromosomes, characteristics of diploid species. Comparison of enzymograms with known karyotype of most of the examined species also indicated the diploid status (Winterhalter 1975). Increased number of genetic loci (Winterhalter 1975) in fish confirmed the polyploid (tetraploid) status.

Through studies dealing with LDH isoenzymes in fish (Markert et al. 1975) and haemoglobin (Jukes 1966), it has been proposed that enzymes and proteins with similar activities, but differing by various physical and chemical criteria, are the result of divergent evolution (duplication and mutation) of "an ancestral gene".

Allelic isoenzymes can differ significantly or they can be quite similar to each other with regard to their physical and catalytic properties. Two variants, A and B of glucose 6-phosphate dehydrogenase found in Drosophila melanogaster (Steel et al. 1968) differ from each other with regard to pH optima, heat stability, substrate specificity and immunological properties, although the two variants appear to be inherited as alleles at a single locus.

There is much similarity between this allelic system and the two gene LDH system. On the other hand, only slight differences are found between the allelic isoenzymes of human glucose-6-phosphate dehydrogenase (Yoshida 1968).

1.3.4 Isoenzymes in Development and Differentiation

It is now well documented that as cellular differentiation progresses to bring about morphological development and functional specialization there is change of isoenzyme pattern because of continuous synthesis and or degradation of specific enzymes and structural proteins. The exact mechanisms responsible for change in isoenzyme pattern in higher multicellular organisms during development cycle are not known. It has been proposed that isoenzyme alternation is the result of change at the level of gene transcription (Whitt 1975). The specific m-RNA for an isoenzyme may have regulated exit from the nucleus to the cytoplasm and even it does enter the cytoplasm, the translational process may be controlled. After translation the assembly of the polypeptide subunits and expression of enzymatic activity can be modified by epigenetic events. Finally, it has been proposed that the final gene product, the isoenzymes, can be drastically changed in its cellular levels by changing rate of degradation, or isoenzyme leakage in some diseases (whitt 1975).

1.3.5 Physiological Function of Isoenzymes

The most important physiological function of isozymes is their participation in regulating the cell metabolism both in aerobic and anaerobic conditions. Beside this, several distinct functions of isoenzymes have been identified in intracellular biochemical

reactions. Firstly, it is known that the subcellular particles possess different electrical charges. So, the different forms of an enzyme, each having a different charge, will bind with different particles and, hence, have functions on different particles. Such a situation has been described in LDH (Allen 1961) and MDH (Munkres 1968).

The second possible regulatory function of isoenzymes involves reversible reactions in which one isoenzyme catalyzes the forward reaction and the other serves as a better catalyst for the backward reaction. For example, Aldolase A favours cleavage of fructose 1, 6-diphosphate, and, therefore, glycolysis. On the other hand, aldolase B is better suited to fructose/ 1,6-diphosphate synthesis and, thus, ~~and~~ gluconeogenesis (Rutter et al. 1968).

A third regulatory function of isoenzymes resides in regulating the rates of metabolite flow at branched pathways in bacteria involving differential feedback inhibition mechanism (Stadtman 1968). Such a mechanism has been observed in E. coli where aspartyl phosphate is an intermediate in the biosynthesis of methionine, threonine and lysine. Finally, it has been observed that certain intracellular metabolites (e.g. peptides, urea and oxalate) differentially degrade different forms of an enzyme (Schoenenberger and Wacker 1966, Vesell and Yielding 1968) while some metabolites differentially protect some isoenzymes from degradation (Vesell and Yielding 1968).

Large differences exist among tissues in isoenzyme distribution and this can arise from differences in rates of degradation and protection of different forms in different tissues (Fritz et al. 1973). Accordingly, differences in degradation might suggest that

some isoenzyme might have arisen from the biochemical necessity of maintaining the activity of critical enzymes in the face of degradation in certain physiochemical conditions and in concentration of various metabolites.

1.3.6 Application of Isoenzyme

Isoenzymes are being used effectively as markers for solving and answering a large number of questions. Plant isozymes have proved to be useful to biologists, botanists, systematists, plant breeders and others as markers for selecting, identifying and registering cultivars^{to} of different crops. Plant breeder^e supplement their morphological, cytological and reproductive data with the help of isoenzyme (Brewer 1970).

Since the isoenzymes are presumed to be direct products of the gene, they may serve as molecular markers in analyzing the phylogenetic similarities or dissimilarities between species, genera and phyla. Accordingly, band derived from two different species, but migrating the same distance in electrophoretic medium, are likely to be produced by a gene common to both species. On the other hand, bands that migrate differentially are likely to be controlled by different genes or different alleles of a locus. Therefore, the variability of zymogram within and between living organisms is of special importance from the genetical and evolutionary point of view (Boulter et al. 1966, Scandalios 1969). Because of these and various other reasons, zymogram could serve only as one of the markers of taxonomical relationships together with a series of other data.

The classification or phylogeny of various genera which has been worked out on cytological, morphological and reproductive grounds has been supplemented by using zymogram methods (Hart 1970, Mitra et al. 1970, Wall and Wall 1975, Karataglis 1975).

A lot of work in this field has been done in animals (Wilson et al. 1964, Jadric et al. 1971b, Winterhalter 1975), while in plants using this zymogram method, taxonomic studies have been made in the genus Nicotiana (Smith et al. 1970), Datura (Conklin and Smith 1971), Hordium (Macdaniel 1970, Mitra et al. 1970), Triticum (Mitra and Bhatia 1971), Gossypium (Johnson and Thein 1970), Aegilops (Karataglis 1975) and Leguminosae family (Gomes et al. 1977a, 1977b).

In many cases, plant isozymes offer advantages over animals for studying specific problems. In relatively little space, plants can be raised cheaply and ontogenetic and organ studies can be made. Besides this, plant isoenzymes provide unique material for studying evolution of enzymes, species etc. (Lewontin and Hubby 1966, Brewer 1970, Marshall and Allard 1970, Scandalios 1974, Wall and Wall 1975).

Isoenzymes have wide applications in clinical biochemistry and medicine where they are successfully being used in diagnosis of several diseases such as myocardial infarction, malignant and neurological diseases, diseases of liver, muscles, joints, and kidney etc. Isoenzymes are used by geneticist for estimating degree of genetic polymorphism present in populations, genetic difference between species, differential gene action and expression during development. Isoenzymes provide unparalleled opportunity to conveniently study the range of substrate specificities and to detect cross specificities

of apparently unrelated enzymes (Dawson et al. 1968, Brewer 1970, Pietruszko 1975, Everse and Kaplan 1975, Horecker 1975, Cerovska and Leblova 1981, Gomes et al. 1982).

1.3.7 Isoenzymes of ADH, MDH and esterase in plants

The occurrence of ADH isoenzymes among plants is widespread. Among higher plants, isoenzymes of ADH have been studied for maize (Scandalios 1967, 1969, Schwartz and Endo 1966, Scandalios and Felder 1971), wheat (Hart 1970), bean, pea, rape, sunflower and rice (Leblova et al. 1977, Cerovska and Leblova 1981) and chickpea (Gomes et al. 1982) with respect to genetic and biochemical regulation, physiological properties, ontogeny and specificities and physiological roles.

Most of the ADH isoenzymes which have been studied in maize are the results of polymorphism. Schwartz and Endo (1966) described four alleles for ADH^H gene which codes for four ADH isoenzymes e.g. SS (slowest), C^tC^t (fastest), FF (intermediate) and C^mC^m (identical mobility with C^tC^t but has low activity). In heterozygous maize for any two alleles, hybrid ADH isoenzyme bands in addition to the two parental bands are observed. The migration rate of the hybrid band is intermediate between the two parental bands. To the contrary, Scandalios (1967, 1969) postulated that of the five ADH isoenzymes in maize, ADH-1 and ADH-2 are coded by two closely linked genes and each locus has two alleles which code for either a slow or a fast migrating isoenzyme in a given inbred line. Codominant alleles coding for ADH monomeric and dimeric structure of the allozymes have been reported for sunflower (Torres 1974), wild, cultivated

and feral populations of the Mexican Ph. vulgaris Ph. coccineus (Wall and Wall 1975) and wheat (Hart 1971). The hypothesis of dimeric structure of ADH and increase in band number with increase in ploidy level was confirmed by dissociation and reassociation technique (Hart 1971). Gene duplication for the dimeric ADH isoenzyme in all individuals of the diploid plant Clarkia franciscana has been reported (Gottlieb 1974). Consequently, the duplication provides enzyme variability in the absence of genetic variability.

Qualitative and quantitative fluctuations in ADH isoenzyme patterns were found during the course of development and differentiation of maize by Scandalios and Felder (1971) who detected 5 distinct ADH isoenzymes in maize. During the early stages of kernel development in maize, ADH-1 (fastest) and ADH-2 (coded by two closely linked genes) are formed and are the most common in all tissues (e.g. scutellum, endosperm, root, shoot, leaf and young plant). ADH-3 (slightly faster than ADH-2) and ADH-4 (slower than ADH-2) are present in the scutella of mature kernels and during the early stage of germination. ADH-T, being present only in the pericarp of 19-40 day old kernels, is controlled spatially and temporally. ADH-1, ADH-3 and ADH-4 usually disappear by the 4th day of germination while ADH-2 remains up to 10 days of germination. Since mutation affected the electrophoretic mobility of ADH-2 but not of ADH-3 or ADH-4 it was suggested that these isoenzymes are controlled by separate structural genes (Scandalios and Felder 1971). The relative amounts of the three ADH isoenzymes were found to change during the germination (Gomes et al. 1982). Activity of ADH-3 (slowest) showed maximum increase while activity of ADH-1 (fastest) showed minimum

increase during the initial stage of germination. 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. All the three isoenzymes could be resolved in the root of 5-day-old chickpea seedling while a small amount of ADH-1 and a trace ADH-2 were observed in the shoot. Misharin et al. (1971) reported differences between isoenzyme patterns of resting and developing bulbs of tulip.

The biochemical properties of ADH isoenzymes are dependent on the genetic background. Examination of the properties like heat stability, substrate specificity, K_m values, etc. of the ADH-1 and ADH-2 isolated from homozygous and heterozygous maize showed differences (Felder and Scandalios 1971). Gomes et al. (1982) observed differences in the heat and pH stabilities and substrate and inhibitor specificities of the three ADH isoenzymes in chickpea. Similar amino acid composition of two variant forms of ADH-2 (ADH-2S and ADH-2F) led Felder and Scandalios (1971) to assume that the difference in the properties of ADH isoenzymes ^{is} are most probably due to / only one or few amino acid substitution which differentiate the isoenzymes in respect of tertiary and quaternary organization of ADH isoenzymes.

Lai and Scandalios (1977) proposed that ADH isoenzymes are irreversibly and sequentially inactivated after germination due to the generation of an ADH specific inhibitor.

Isoenzymes of MDH are known to occur in a variety of plant tissue. Plants studied include maize (Longo and Scandalios 1969, Leskovac et al. 1975), barley (Duffus 1968), cucumber (Kitto et al. 1966) and spinach (Zschoche and Ting 1973b, Rocha and Ting 1971).

Relatively little is known about the genetic control of MDH isoenzymes which have been found in association with a wide array of subcellular organelles i.e. microbody (gloxyosome), mitochondria, chloroplast and cytoplasm (Rocha and Ting 1970, Longo and Scandalios 1969). Since MDH isoenzymes are associated with different subcellular organelles there is dramatic difference in the regulation of these isoenzyme activities at various subcellular sites (Scandalios 1974). After observing Mendelian inheritance pattern of MDHs and dosage effects in triploid tissues of maize, Longo and Scandalios (1969) postulated that the structural genes for the synthesis or control of mitochondrial MDH isoenzymes are located in the nuclear rather than mitochondrial genome and it has been further proposed that the isoenzymes are under the control of two groups of loci located on two different chromosomes. Evidence of nuclear gene control has been obtained by other investigators (Scandalios 1974).

Changes in the complements of MDH isoenzymes during plant development have been reported for a number of plants. The number and position of different MDH isoenzymes remain constant at various states of development of maize (Yang and Scandalios 1974), but the activities of different isoenzymes increase or decrease differentially at different time. Moreover, the number of MDH isoenzymes are same in all organs of young maize plant (Longo and Scandalios 1969).

The increase in MDH activity during the early maize development has been attributed to the de novo synthesis of the enzyme (Longo and Scandalios 1969). The soluble (cytoplasmic) forms make up a substantial portion of MDH activity in maize (Longo and Scandalios 1969). Studies indicated that mitochondrial MDH isoenzymes are synthesised in cytoplasmic ribosomes and subsequently incorporated into the mitochondria (Yang and Scandalios 1974). After studying MDH activity in maize during different stages of germination, Leskovac et al. (1975) concluded that the levels of MDH do not follow the regularities observed for other enzymes and suggested that MDH in each compartment probably participates in a specific metabolic pathway. They further suggested that microbody MDH is synthesized in large quantities after the onset of germination.

Although substantial work has been done on the structure of mammalian MDHs very little is known about the structure and property of plant MDHs. Molecular weights of all the plant MDH forms have been found to vary from 66,000 to 70,000 (Rocha and Ting 1971). Properties such as K_m values, heat stability, sensitivity to inhibitors, substrate inhibition, etc. of the isoenzymes are not significantly different and all the forms favour the formation of malate (Rocha and Ting 1971, Zschoche and Ting 1973b, Ting et al. 1975). Relatively little is known regarding the in vivo metabolic regulation of plant MDHs.

Multiple forms of esterases are widespread in plants (Shannon 1968, Scandalios 1974, Nelson and Burr 1973). Genetic control of the nature of esterase isoenzyme in maize kernel has been reported by Schwartz (1960). Esterases are under the control of three allelic

genes in maize and homozygotes show either a fast or slow moving enzyme and the heterozygotes show a hybrid enzyme of intermediate mobility as well as the two parental types (Schwartz 1960). Esterase isoenzymes have been studied by many other authors mostly with relation to genetics, evolution, phylogenetic^e relationship, polymorphism, etc. (reviewed by Nelson and Burr 1973, Shannon 1968, Scandalios 1974).

Evidence for molecular structure of esterase is not easily obtainable because they show broad substrate specificity. However, based on genetic studies only Schwartz et al. (1965) proposed that esterases in maize are dimers (hetero or homo). The isoenzymes, coded by 7 alleles, can occupy 28 possible positions in homozygous and heterozygous combinations and there are 21 different hybrid or allodimer enzymes which can be formed (Schwartz et al. 1965). On the other hand, the occurrence^e of 15 esterase isoenzymes in potato was postulated to be based on an active isoenzyme of a tetramer of one, two or three types of monomers (Desborough and Peloquin 1967).

Differences in the substrate and inhibitor specificities indicated differences among the esterase isoenzymes in peas and bean (Veerabhadrapa and Montgomery 1971), broad bean (Sahulka and Benes 1969) and green gram and black gram (Gomes 1978), and based on these studies esterase isoenzymes were classified into several groups e.g. carboxylesterases, arylesterases and acetylerases.

Changes in the esterase isoenzyme patterns during development and differentiation have been studied in a large number of plants

e.g. onion (Makinen 1968), barley (Upadhyia and Yee 1968), wheat (Bhatia and Nilson 1969), Saguaro cactus (Keswani and Upadhyia 1969), peanut (Cherry and Ory 1973), Aegilops (Karataglis 1975), pea (Safonova et al. 1970), green gram and black gram (Gomes 1978) and many other plants (Scandalios 1974). The number and intensities of isoenzyme bands were found different for different dry seed species. With the onset of germination some new bands appeared, some old bands disappeared, some bands became more active and some bands became weaker indicating qualitative and quantitative changes during germination. The mechanism of regulation of esterase isoenzyme action at different stages of germination is not yet clear.

CHAPTER 2

MATERIALS AND METHODS

2.1 Plant materials used

Psophocarpus tetragonolabus L.cv. "Sylhet" (Winged bean) and Vicia faba L.cv. Kalomotor, seeds of the family Fabaceae were used in the present experiment. Kalomotor seeds were obtained from a village market in Dhaka District and winged bean seeds were obtained from Bangladesh Agricultural Research Institute, Joydevpur.

2.2 Cultivation of plants

Seeds of uniform size were soaked for 24 hours in distilled water and washed extensively with distilled water to remove surface contaminants. The seeds were then germinated in a dark thermostatic chamber at 27-28°C in separate Petridishes (diameter 14-16cm) on a layer of filter paper moistened with distilled water (about 5 ml) for 24 hours. Then the seeds were again washed and planted in glass or porcelain trays on layer (1-2 cm) of sterile sand moistened with distilled water for 0-15 days. This process of germination was followed throughout the whole experiment, except where otherwise described.

2.3 Crude enzyme preparation2.3.1 Alcohol and malate dehydrogenases

Only cotyledons were removed from the seedlings of various ages, washed with distilled water and gently blotted dry with filter papers. Extraction was done by homogenizing 1 gm tissues for 5 minutes in a chilled mortar with 5 ml 0.05 M Tris-HCl buffer (pH = 7.6) containing 10 mM β -mercaptoethanol (BME) and 12.5% sucrose. The slurry was centrifuged at 10,000g for 30 minutes at 4°C.

The supernatant was filtered through Whatman No-1 filter paper. This filtration removed most of the lipid material found floating on top of the supernatant. The resultant preparation was used directly for protein estimation, assay of enzyme activity and polyacrylamide gel electrophoresis. Generally, fresh extracts were used. However, sometimes the extract was stored at 4°C in a stoppered test tube for 24 hours. This storage caused precipitation of some of the carbohydrate material which was then centrifuged off and the clear supernatant was used. But there was always some drop in enzyme activity due to storage.

2.3.2 Esterases

The grinding medium for extraction of esterase consisted of 0.05 M Tris-HCl buffer ($pH=7.5$) containing 0.001M dithiothreitol, 0.001 M EDTA, 0.01 M KCl and 0.001 M $MgCl_2$. 1 gm tissue was extracted with 5ml buffer, centrifuged and filtered as described for dehydrogenases.

2.3.3 Proteases

Acid protease was extracted by homogenizing 1 gm tissue with 5ml McIlvaine buffer ($pH=5.5$), centrifuged and filtered as described for dehydrogenases. Alkaline protease was extracted by grinding 1gm tissue with Tris-HCl buffer, $pH=7.6$.

2.3.4 Amylases

Amylase was extracted by grinding 1gm tissue with 6 mM $CaCl_2$, centrifuged and filtered as described for dehydrogenases.

2.4 Measurement of enzyme activity

2.4.1 ADH and MDH Assays

ADH activity was measured according to the method of Bonnichsen and Brink (1955) with slight modification. The enzyme reaction mixture consisted of 2.75 ml of 0.05 M Tris-HCl buffer ($pH=8.8$), 0.1 ml of 1.13 M ethanol and 0.05 ml of the enzyme extract. This solution was then used to set the machine at zero. Absorbance was measured at 340 nm on a LKB digital spectrophotometer (Model, Ultrospec. 4050). The increase in absorbance of the reduced form of NAD^+ was recorded continuously for 5 minutes at 30 seconds intervals after the addition of 0.1 ml of 0.4 mM NAD^+ . One enzyme unit is defined as the amount of enzyme necessary to produce a change of 0.001 O.D per minute per mg of protein. The change in O.D was linear with time for at least one minute and the velocity was proportional to the amount of enzyme added over the range of enzyme activity used in these experiments. Whenever necessary the enzyme extract was diluted suitably immediately before activity measurement so as to keep the O.D change within 0.05 to 0.07 per minute.

The method for MDH assay was similar to that of ADH except the substrate used. In case of MDH 0.1 ml of 1.0 M sodium L-malate was used.

2.4.2 Esterases

The total esterase activity was determined according to the method of Vecerek et al. (1967), further modified by Miholjicic et al. (1974) and us.

The reaction mixture consisted of 3.3 ml Veronal buffer ($pH=7.5$), 0.1 ml freshly prepared 0.30% β -naphthyl acetate in 99.8% ethanol, 20 mg bovine serum albumen in 0.1 ml H_2O , 0.1 ml freshly prepared fast blue RR salt and 0.05 ml enzyme extract. The reaction mixture was incubated at $37^{\circ}C$ for 15 minutes. The enzyme activity was stopped by adding 0.5 ml of 1N HCl and diluted to 8 ml with H_2O . Simultaneously a control experiment was taken for each sample by inactivating the enzyme using 0.5 ml 1N HCl prior to incubation. A blank was prepared with all the reactants except enzyme extract. O.D. of the azo dye was measured by reading absorbance at 550 nm in a spectrophotometer. Absorbance of the control was subtracted from the sample to obtain the amount of β -naphthol liberated only due to enzyme action. The amount of β -naphthol liberated was estimated by drawing a standard curve using β -naphthol. Esterase activity was expressed in terms of μ -mole β -naphthol liberated / mg protein/ minute.

2.4.3 Proteases

Acid protease (haemoglobinase) activity of cotyledons of different ages was measured according to the method of Reimerdes and Meyer (1976) with some modification. The reaction mixture consisted of 3 ml McIlvaine buffer ($pH=5.5$) containing 0.1% haemoglobin as substrate and 0.25 ml enzyme extract. The reaction mixture was incubated at $37^{\circ}C$ for 2 hours. After incubation 3 ml of 5% trichloroacetic acid (TCA) was added to terminate the reaction. Simultaneously, a control experiment was taken for each sample in which 3 ml of 5% TCA was added immediately after the addition of enzyme extract.

The reaction mixture was then centrifuged or filtered to obtain a clear supernatant .

From this supernatant 0.5 ml aliquot was taken from each tube and then 0.5 ml H_2O and 1 ml ninhydrin solution was mixed and heated in a boiling water bath for 20 minutes. After cooling the tubes 5 ml diluent (1:1= H_2O :n-propanol) was added to each tube and O.D. was recorded at 570 nm after 15 minutes. The free amino acid produced by the proteolytic action on haemoglobin was estimated against a standard curve prepared with leucine. Acid protease activity was expressed in terms of μ gm leucine equivalent produced per mg protein per minute.

Alkaline protease (amidase) activity was determined according to the method of Erlanger et al. (1961). The reaction mixture consisted of 0.1-0.2 ml enzyme extract, 0.8-0.9 ml water, 5 ml substrate solution (43.5 mg benzoyl - DL - arginine - p - nitroanilide hydrochloride dissolved in 1 ml dimethylsulfoxide and diluted to 100 ml with Tris-HCl buffer (pH 7.6)).

The reaction mixture was incubated at 25 or 37°C for 25 minutes and the reaction was terminated by the addition of 1 ml of 30% acetic acid. The quantity of the liberated p -nitroaniline was estimated spectrophotometrically at 410 nm. Control tubes contained all the reagents except enzyme extract.

The amount of substrate hydrolysed by the enzyme was calculated according to the molar extinction of p -nitroaniline at 410 nm ($E=8800$). One unit of benzoyl - DL - arginine - p - nitroanilide hydrochloridease activity was the amount of enzyme which hydrolyzed 1 μ -mole of substrate per minute.

2.4.4 Amylases

Amylase activity was measured according to the method of Peter Bernfeld (1955) with modification. The reaction mixture consisted of 0.4 ml sodium acetate buffer ($pH=5.4$), 0.5 ml soluble starch as substrate (10 mg/ml) and 0.1 ml enzyme extract. The mixture was then incubated at $30^{\circ}C$ water bath for 20 minutes. After incubation 1 ml DNSA reagent was added and boiled for 10 minutes in a boiling water bath and then diluted with 10 ml H_2O . Finally O.D was recorded at 540 nm. The enzyme activity in the control was inactivated by adding 1 ml DNSA reagent prior to incubation. Simultaneously, a blank was made without extract. The reducing sugar released by action of amylases was estimated against a standard curve prepared with maltose. Amylase activity was expressed in terms of micrograms of maltose liberated/mg protein/minute.

2.5 Determination of protein

The protein concentration of crude extracts was determined according to the method of Lowry et al. (1951) using bovine serum albumen as standard. Since the fresh weight of the seeds changed during germination the estimation of protein concentration in extracts from seeds of different ages was very necessary for comparative study.

2.6 Ethanol estimation

Cotyledons (1 gm) 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. Ethanol in the distillate was determined

spectrophotometrically by the method of Sunshine and Appleton (1961).

2.7 Electrophoretic procedure

Protein separation was carried out by polyacrylamide disc electrophoresis according to the method of Bloemendaal (1963). Power supply and electrophoretic set, manufactured by Sargent Welch, USA., were used.

2.7.1 Preparation of stock Tris-glycine buffer

3 gm , of Tris (hydroxymethyl) methylamine (0.247 M) and 14.4 gms of glycine (0.191 M) were dissolved in 100 ml redistilled water.

2.7.2 Working buffer

The stock buffer solution was diluted ten times with redistilled water before use. The p_H of this buffer was 8.5. For each electrophoretic run 800 ml of this buffer was needed. 600 ml for the lower and 200 ml for the upper buffer reservoirs.

2.7.3 Preparation of stock gel solutions

- a. 30 gm of acrylamide and 0.8 gms of N-N methylene bisacrylamide were dissolved in 100 ml redistilled water and filtered through Whatman No-1 filter paper.
- b. 1.6 gm of beta dimethyl aminopropionitrile was added to 40 ml stock Tris-glycine buffer which was then diluted 2.5 times to make the final volume 100 ml (for polymerization).
- c. 30 mg% potassium ferricyanide solution was made in redistilled water (for delaying polymerization).
- d. 480 mg% ammonium persulfate solution was made in redistilled water (for enhancing polymerization).

The above solutions remained stable for at least one month if stored in a brown bottle at 4⁰C.

2.7.4 Polymerization of gels

For polymerization of gels and electrophoresis, 18 glass tubes, 8 cm long and inside diameter 5 mm, were placed in a vertical position on a plastic rack with suitable holes and screws. The lower end of the tube was closed by rubber caps. 10 ml each of a,b,c,d (Section 2.7.3) were pipetted sequentially in a conical flask. The gel mixture was immediately poured into gel tubes upto 5 mm from the upper rim. The gels were allowed to polymerize at room temperature. The polymerization occurred within 40-50 minutes to form a colourless gel. The completion of polymerization was indicated by a sharp boundary line between the polymerized and unpolymerized portions. The unpolymerized solution was discarded and the gel surface was washed with the working buffer.

2.7.5 Pre-electrophoresis

The tubes with polymerized gels were removed from the plastic rack and the rubber caps were removed carefully. Immediately the tubes were inserted into the rubber grommet-lined bores of the upper electrode buffer reservoir so that the rims projected about 2 cm above the bores. Pre-cooled (4⁰C) electrode buffer (Tris-glycine working buffer,) was slowly poured into the upper and lower electrode reservoirs and special care was taken to prevent the formation of air bubbles at the tube rims. The electrodes were connected with the power supply and pre-electrophoresis was carried out for 15-20 minutes at 100 V i.e. 2-3 mA/gel to purify the gels from unreacted

materials of polymerization process and ionic impurities of the gel components. In addition to this, pre-electrophoresis set up a constant resistance inside the gel.

2.7.6 Application of sample

After pre-electrophoresis the upper reservoir was removed and seed extracts containing equal amounts of proteins were applied on top of individual gel to form a layer under the buffer layer. 0.05 ml 20% sucrose solution was mixed with each individual seed extract before addition to increase the density of the extract. For comparative studies, the addition of same amount of proteins was essential.

In most analysis, small amount of 0.001% bromophenol blue with 10 μ l of 20% bovine serum albumin was added on top of one gel without sample. Bromophenol blue formed complex with albumin and the dye served as a marker of the migrating boundary during electrophoresis.

After application of the samples, the upper reservoir was replaced on the lower reservoir and the apparatus was connected to the power supply. Electrophoresis was performed at 100 V (2.5-3.0 mA/gel) for 15 minutes and then for 50-60 minutes at 200 V (3-4 mA/gel) for satisfactory separation of the bands. Electrophoresis was always performed at room temperature under continuous water circulation around the buffer reservoirs.

2.7.7. Treatment of gels and detection of isoenzymes

Immediately after electrophoresis the gels were removed from the tubes with the help of ejecting water from a hypodermic needle attached to a syringe and washed in distilled water. The enzyme bands were located by incubating the gels in appropriate enzyme substrate

mixture described below. A control gel for each individual enzyme was incubated in the reaction mixtures without the substrate. In control gels no band was detected.

2.7.8. Staining method for dehydrogenases

The method for staining dehydrogenases (NAD-dependent) was developed by us based on the technique developed for quantitative determination of LDH activity by Babson and Phillips (1965). The staining method is based on the fact that the protons transferred from the substrates (proton donors) to nicotinamide adenine dinucleotide (NAD) by dehydrogenases to form NADH_2 is taken up by phenazine methosulfate (PMS) and then transferred to 3-(4-Iodophenyl)-2-(4-nitrophenyl)-5-phenyl-2H-tetrazolium chloride (INT) which is thereby reduced to an insoluble formazan which becomes visible as a purple red colour.

The colour reagent for visualizing dehydrogenases was prepared by dissolving INT (200mg), PMS (50mg) and NAD (200mg) in 40 ml Tris-glycine buffer pH 8.8. The reagent was prepared freshly just before use.

2.7.9 Staining mixture for ADH

For each gel the reaction mixture consisted of 3.7 ml Tris-glycine buffer, pH 8.5, 0.1 ml 99.8% ethanol and 0.3 ml colour reagent. The zymograms were allowed to stain in a water bath at 37°C in the dark for 15-25 minutes until the bands had reached a desired intensity.

2.7.10 Staining mixture for MDH

For each gel the staining mixture consisted of 3.7 ml Tris-

glycine buffer, pH 8.5, 20 mg Na-malate in 1 ml Tris-glycine buffer, pH 8.5 and 0.3 ml colour reagent. The gels were incubated for 15-25 minutes in a water bath at $37^{\circ}C$ in darkness.

2.7.11 Staining mixture for esterase

Esterases were detected by the modified method of Markert and Hunter (1959). The procedure included presoaking the gels in Tris-HCl buffer pH -7.5 for 15 minutes at room temperature. Then the gels were transferred to a solution containing 95 ml Tris-HCl buffer, pH -7.5, 40 mg β -naphthyl acetate dissolved in 2 ml 99.8% ethanol and 2 ml water, and 70 mg fast blue RR salt. Individual gel was incubated in 8 to 10 ml of the staining mixture for 20-25 minutes at $37^{\circ}C$ in darkness.

2.7.12 Storage of stained gels

Following staining the gels were washed with distilled water and placed in stoppered test tubes in 2% acetic acid. In this solution the separated fractions of isoenzymes remained stable without loss of intensity for several months if stored in the dark at $4^{\circ}C$.

2.7.13 Quantitative densitometric analysis

In some cases densitometric tracings of the stained gels were obtained on a densitometer at 540 nm for the quantitative estimation of each isoenzyme. The total peak area under each isoenzyme was calculated applying the formula: $A = \frac{h \times l}{2}$, where h and l represent height and length of the peak respectively.

2.7.14 Processing and representation of electrophoretic results

The distance of each zone of enzyme activity on the gel was

measured from the starting line (line of application of sample) and on the basis of this measurement the mobilities of isoenzymes have been expressed.

The isoenzymes in the investigated species have been shown schematically and whenever possible original photographs of zymograms have been presented. In each schematic representation positive and negative poles have been marked by (+) and (-) signs respectively. The line of application of sample is always shown on the upper end of the gel. The electrophoretically separated bands have been numbered according to the recommendation of Standard Committee of Enzymes (Webb 1964).

CHAPTER 3

RESULTS AND DISCUSSION3.1 Alcohol dehydrogenases and their properties in winged bean and kalomator cotyledons.3.1.1 Extractability and stability

Crude enzyme extracts were prepared from 48 hours' germinated winged bean and kalomator cotyledons in extraction medium of increasing pH. Phosphate buffer having pH range from 5.0 to 8.0 and Tris-HCl buffer having pH range from 7.0 to 9.0 were used in order to take account of possible ion effects of the buffer on the stability and activity of the enzyme.

Although substantial amounts of total ADH activities were found in winged bean and kalomator extracts with both Tris-HCl and phosphate buffers at different pH values, yet much higher specific activities of the enzymes in fresh phosphate buffer extracts suggest that less total soluble protein and more enzyme protein were extracted in this buffer (Table 1 and 2). However, the enzymes extracted in Tris-HCl buffer remained much more stable during storage at -20°C , maximum stability being at pH 7.6. Much less stability of the enzymes in phosphate buffer may be due to inadequacy of the ionic strength and buffering action. The inactivation rate of the enzymes was more rapid below or above pH 7.6 most probably due to denaturation of the enzymes. Since Tris-HCl buffer, pH 7.6 showed better buffering and protective capacities it was selected for subsequent studies.

3.1.2 Effects of BME and sucrose

Effects of BME and sucrose on ADH activity were determined after 2 and 24 hours of extraction of the sample using Tris-HCl buffer (pH 7.6). The following extraction media were used: Tris-HCl buffer, Tris-HCl buffer with 10 mM BME, Tris-HCl buffer with 12.5% sucrose and

TABLE 1. EXTRACTION AND STABILITY OF ALCOHOL DEHYDROGENASE IN WINGED BEAN AT DIFFERENT pH . 44

Extraction Conditions	Total Soluble Protein mg/ml	Total Activity units/ml after 2 hrs.	Specific Activity units/mg prot./min after 2 hrs	Total Activity units/ml after 22 hrs.	Specific Activity units/mg prot./min after 22 hrs.	% inactivated after 24 hours.
Tris-HCl						
pH 7.0	22.00	7000.0	318.18	6700.0	304.54	4.20
7.6	23.00	8000.0	347.82	7400.0	321.73	2.64
8.0	30.40	7600.0	250.00	6800.0	223.68	9.34
9.0	35.60	6200.0	174.15	4800.0	137.14	21.26
Phosphate buffer.						
pH 5.0	11.20	7000.0	625.00	1500.0	133.92	78.58
6.0	16.00	7800.0	487.50	2600.0	162.50	66.66
7.0	22.00	7000.0	318.18	2900.0	131.81	58.58
7.6	23.00	7800.0	339.13	4600.0	200.00	41.03
8.0	24.00	6800.0	283.33	3500.0	145.83	48.83

Values are means of triplicate experiments.

TABLE 2. EXTRACTION AND STABILITY OF ALCOHOL DEHYDROGENASE IN KALOMOTOR AT DIFFERENT pH 45

Extraction Conditions	Total Soluble Protein mg/ml	Total Activity units/ml after 2 hours.	Specific Activity units/mg prot./min. after 2 hrs. hours.	Total Activity units/ml after 22 hours.	Specific Activity units/mg prot./min after 22 hours.	% inactivated after 24 hours.
Tris-HCl						
pH 7.0	10.0	4500.0	450.0	3800.0	380.0	15.6
7.6	18.8	7332.0	390.0	7238.0	385.0	1.3
8.0	31.2	6800.0	217.9	6193.0	198.5	9.0
9.0	38.0	6600.0	173.7	5200.0	136.8	21.2
Phosphate Buffer						
pH 5.0	6.00	4500.0	750.00	2000.0	333.30	55.60
6.0	8.80	6400.0	727.30	3000.0	341.00	53.10
7.0	14.80	8600.0	581.10	4900.0	331.00	43.00
7.6	17.40	7000.0	402.29	5200.0	298.85	25.72
8.0	19.20	7400.0	385.40	5100.0	265.00	31.10

Values are means of triplicate experiments.

Tris-HCl buffer with 10 mM and 12.5% sucrose. The highest enzyme activity was retained in presence of BME and sucrose both for winged bean and kalomator after 2 and 24 hours of extraction (Table 3). Since the enzyme is protected by BME it is suggested that the enzyme has sulphhydryl groups which is essential for maintaining the three dimensional structure of the protein molecule and the formation of quaternary enzyme co-enzyme-substrate complex (Suzuki and Kyuwa 1972, Whitehead and Rabin 1964). Although the protective action of sucrose on ADH activity was reported (Gomes *et al.* 1982), its mechanism of action is not yet clear.

3.1.3 Storage stability at different temperatures for different periods

After assaying the initial activities of the crude enzyme extracts in Tris-HCl buffer (pH 7.6) containing BME and sucrose, the extracts were stored in separate stoppered test tubes at -20°C , 4°C and room temperatures ($25-30^{\circ}\text{C}$) for different periods of time. After storage, the samples were brought to 4°C before assaying the residual enzyme activities at 30°C . Table 4 shows that 82.99 and 75.1% ADH activities were retained in kalomator and winged bean extracts respectively after storage for 5 months at -20°C whereas storage at 4°C for a week resulted in a retention of only 52.87 and 50.57% enzyme activities in kalomator and winged bean extracts respectively. The enzyme activity for both the species was found to be completely inactivated within 24 hours' storage at room temperature. The slow inactivation of ADH at -20°C over a period of 5 months may be due to the presence of endogenous inhibitors in the crude extracts. Assays were made mostly in fresh extracts and sometimes on stored extracts (within one month of storage at -20°C).

3.1.4 pH optima

Seeds of winged bean and kalomator germinated for 48 hours contained maximum levels of ADH (details given in the developmental

TABLE 3. EFFECT OF BME AND SUCROSE ON ADH ACTIVITY
IN WINGED BEAN AND KALOMOTOR COTYLEDONS

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* Sample and assay time after extraction	Specific ADH activity (units/mg prot./min)		
	Tris-HCl buffer (pH-7.6)	Tris-HCl+Sucrose (pH-7.6)	Tris-HCl+BME (pH-7.6) Tris-HCl+Sucrose+BME (pH-7.6)
Winged bean			
After 2 hours	318.3	332.2	348.2 363.5
After 24 hours	310.7	329.4	345.1 362.0
Kalomotor			
After 2 hours	385.6	395.0	405.44 428.00
After 24 hours	380	392.5	403.00 427.31

* Samples were kept at 0°C for 24 hrs.

Values are means of triplicate experiments.

TABLE 4. STABILITY OF KALOMOTOR AND WINGED BEAN ADHS
AT DIFFERENT TEMPERATURES FOR DIFFERENT PERIODS.

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Sample	Temperature	Specific activity units/mg protein/min for different storage periods.			
		after 2 hours	after 24 hours	after one week	after 5 months
Kalomotor	-20°	436.55	435.55	425.00	362.33
	4°C	436.55	430.00	230.84	0
	25-30°C	436.00	0	0	0
Winged bean	-20°C	362.10	362.00	355.60	271.96
	4°C	362.10	358.00	179.00	0
	25-30°C	362.10	0	0	0

Values are means of triplicate experiments.

studies of ADH) Therefore, 48 hour old seedlings extract was used as a source of the enzyme in all subsequent experiments unless otherwise stated.

Since optimum pH for ADH activity is different for different plant ADHs it is necessary to determine the optimum pH to be used for assaying ADH activities in winged bean and kalomotor. The rate of ethanol oxidation was determined between pH 6.0 and 9.8 using Tris-HCl (0.05 M), glycine-NaOH (0.1 M) and phosphate (0.06 M) buffers. The result is shown graphically. It can be seen from Figs 1 and 2 that optimum pH for winged bean and kalomotor was 8.8, that is in the alkaline region. This is very similar to the pH optima of other plant ADHs reported earlier. pH optima for other plants have been reported as 8.2 for Phaseolus vulgaris, 8.8 for Brassica napus, 8.5 for Oryza sativa, 8.7 for Pisum sativum, Vicia faba (broad bean), and Zea mays (Leblova and Stiborova 1975, Leblova et al. 1976, 1977), 8.8 for pea (Cossins et al. 1968) and 8.3 for barley ADH (Duffus 1968). However our results contrast the pH optimum of 7.1 for soybean ADH (Leblova and Perglerova 1976).

3.1.5 K_m values

Michaelis constants (K_m) were estimated with crude enzyme preparation from winged bean and kalomotor at 30°C. In the direction of ethanol and allyl alcohol oxidation to corresponding aldehydes, K_m values were estimated at pH 8.8 in Tris-HCl buffer. With ethanol and allyl alcohol as the variable substrates, NAD^+ was held constant at 0.4 mM and with NAD^+ as the variable, ethanol and allyl alcohol were constant at 40 mM. The data were fitted to the double

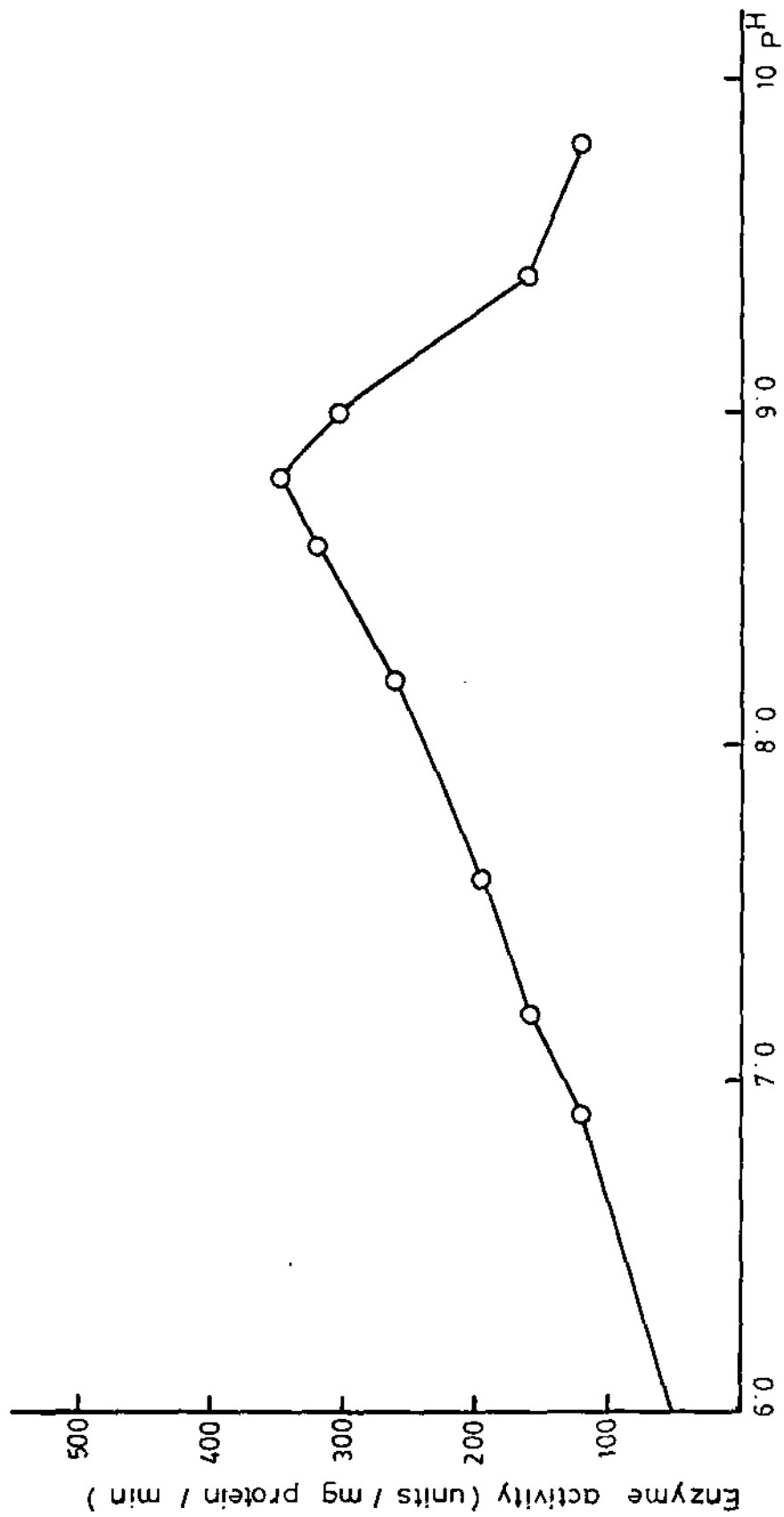


Fig. 1. Effect of increasing p^H on the activity of ADH from 48 hr germinated seeds of winged bean.

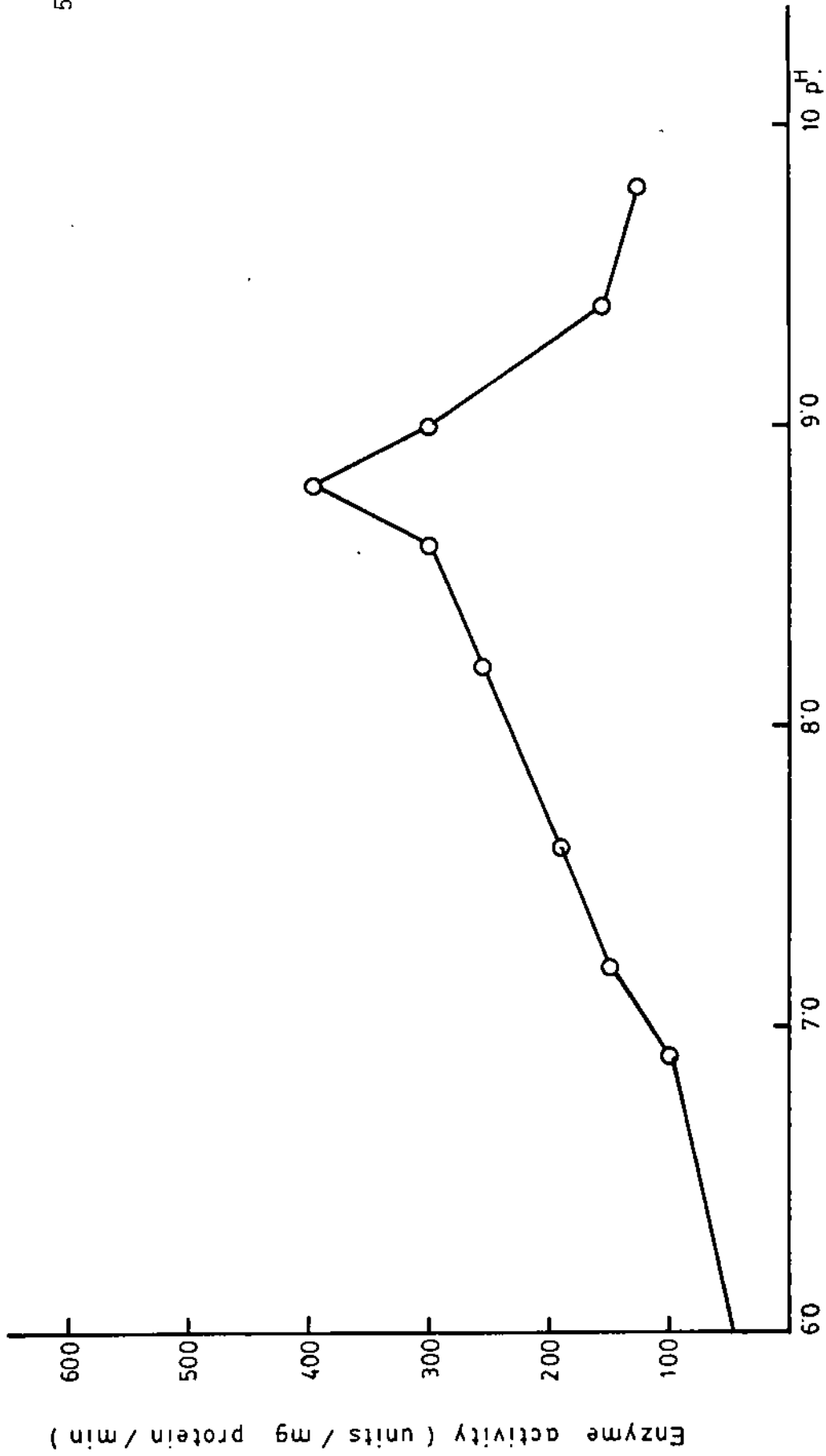


Fig. 2. Effect of increasing p^H on the activity of ADH from 48 hr. germinated seeds of kalomator

reciprocal plots of initial rates versus variable substrate concentrations for estimation of the K_m values. The results are shown graphically (Figs. 3-8). K_m value ($2 \times 10^{-2}M$) for NAD was identical for winged bean and kalomator ADHs (Figs. 3 & 4). K_m values for ethanol were $1.4 \times 10^{-2}M$ and $0.6 \times 10^{-2}M$ for winged bean and kalomator ADHs respectively (Figs. 5 & 6) while K_m value ($0.5 \times 10^{-2}M$) for allyl alcohol was identical for both plant ADHs (Figs. 7 & 8). About twice higher K_m value of winged bean ADH for ethanol than that of kalomator ADH indicates lower and higher affinity for ethanol of winged bean and kalomator ADHs respectively. These results obtained for winged bean and kalomator ADHs are quite comparable with the results of other authors (Leblova et al. 1971, 1977 Leblova and Stiborova 1975) for different plant ADHs (Table 5).

The K_m values of winged bean and kalomator ADHs for the two substrates seem to be slightly lower and for NAD slightly higher than those of related plants (Table 5). These may be either due to the use of crude enzyme preparations or due to real difference in the catalytic properties of these enzymes.

3.1.6 Thermostability

Heat stabilities of winged bean and kalomator ADHs from 48 hours' germinated seeds were studied. Duplicate samples of crude extracts (0.05 ml) were heated in a water bath at 45, 55 and 65°C for a time interval of 10, 20 and 30 minutes, in each case, using stoppered test tubes. After heating, the samples were withdrawn and cooled immediately in ice cold water bath. Then the residual activity was determined at 30°C according to the method described

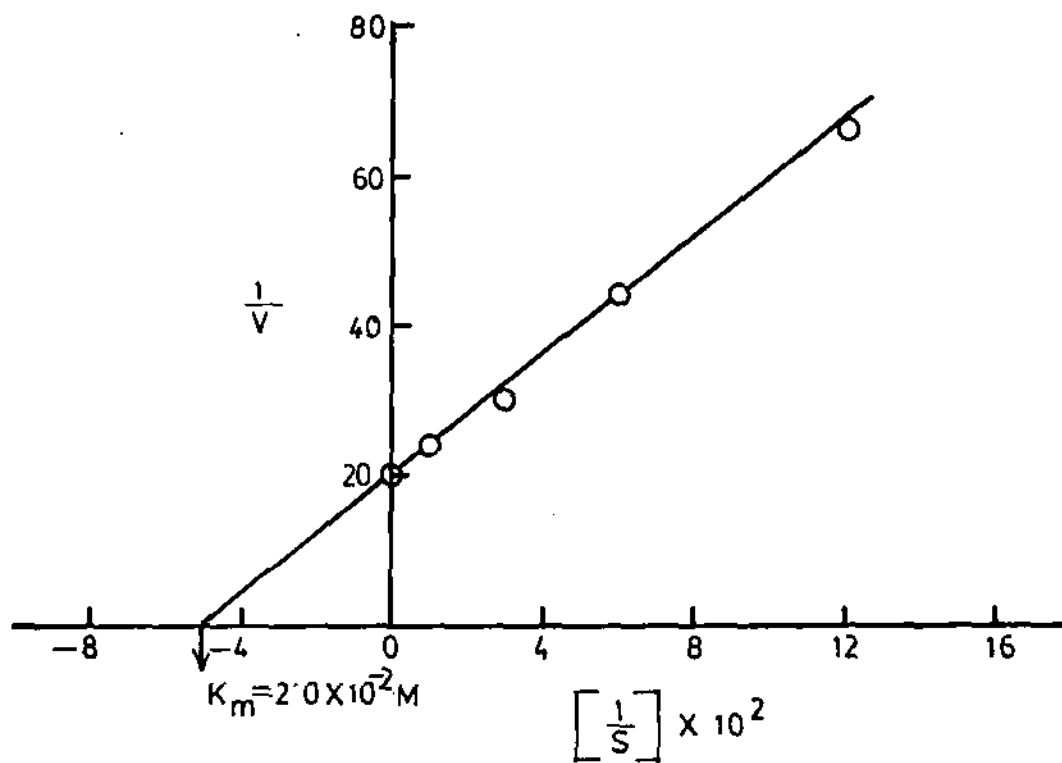


Fig 3. Lineweaver - Burk reciprocal plot of velocity versus substrate concentration to determine the K_m for winged bean ADH for NAD.

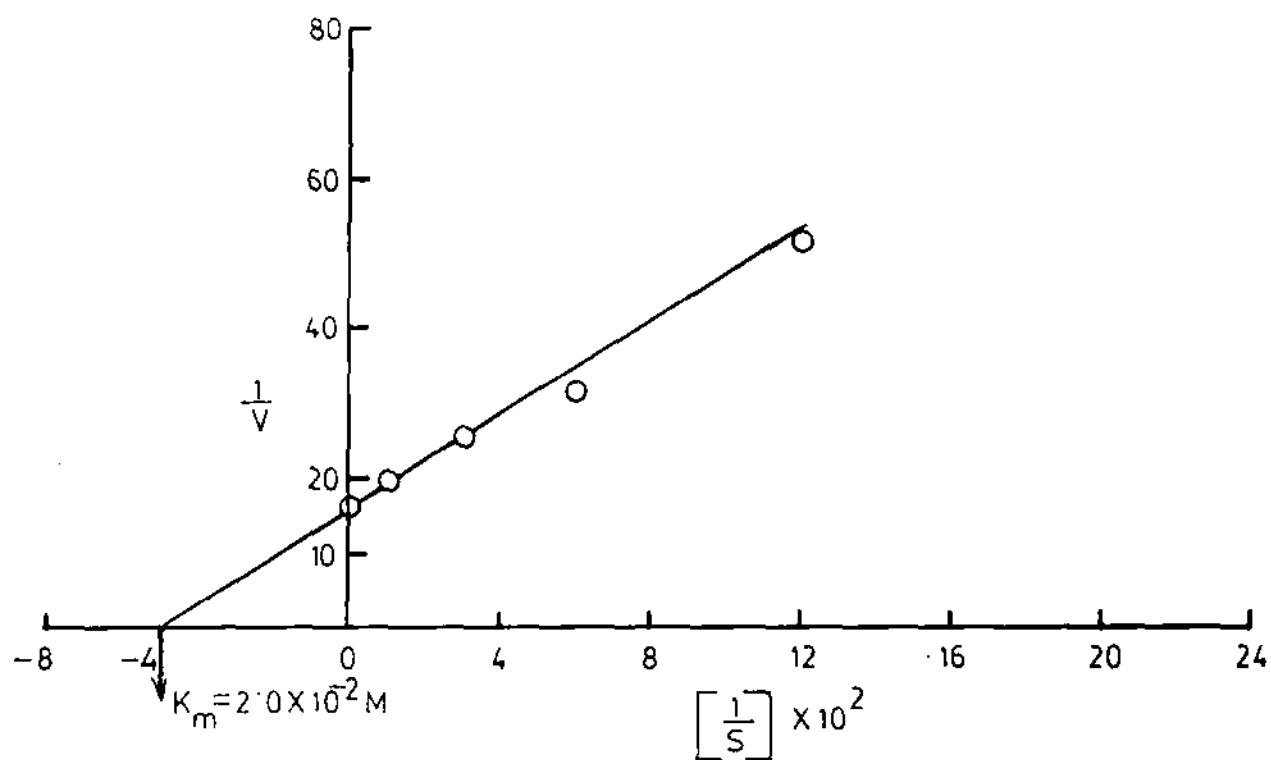


Fig. 4. Lineweaver - Burk reciprocal plot of velocity versus substrate concentration to determine K_m for kalomotor ADH for NAD.

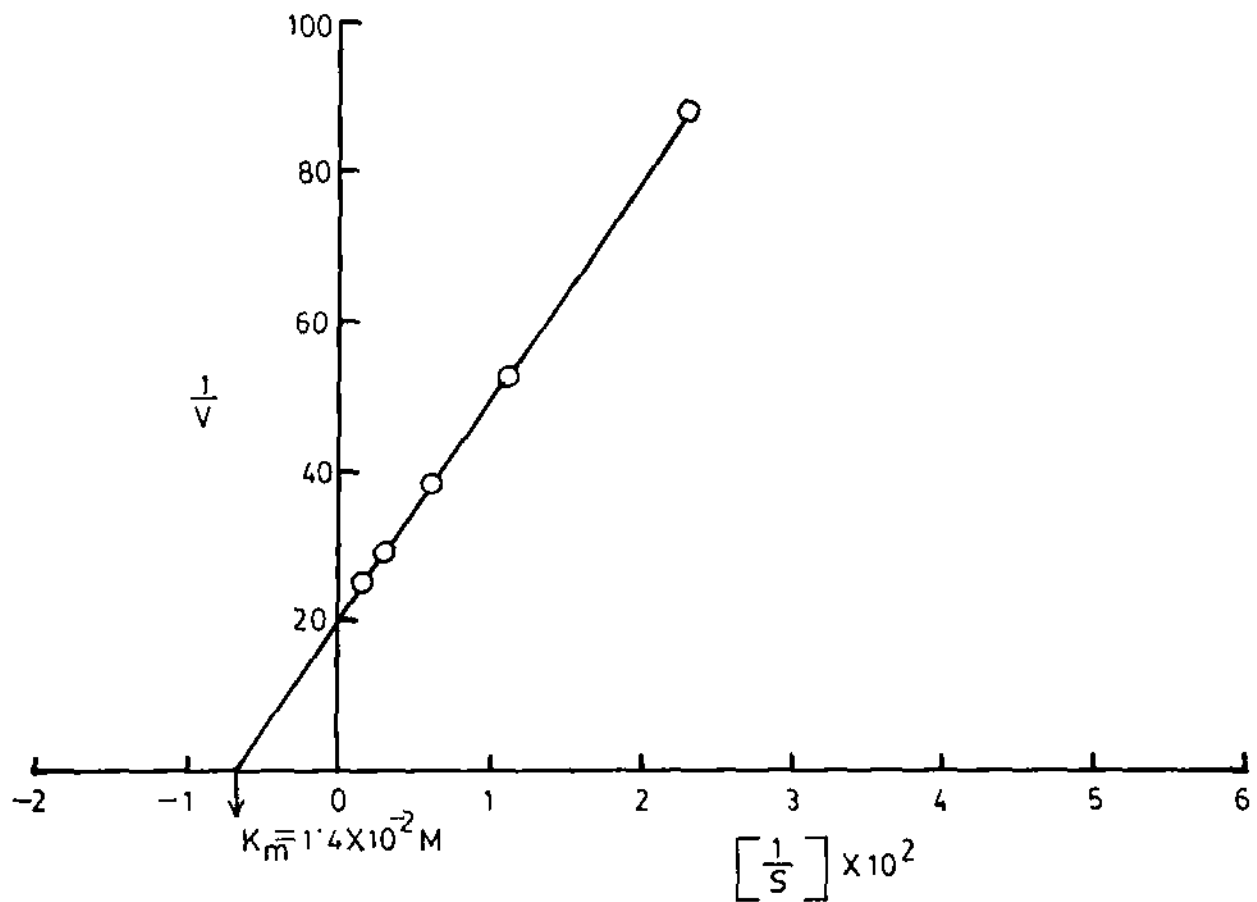


Fig 5. Lineweaver - Burk reciprocal plot of velocity versus substrate concentration to determine the K_m for ethanol of winged bean ADH.

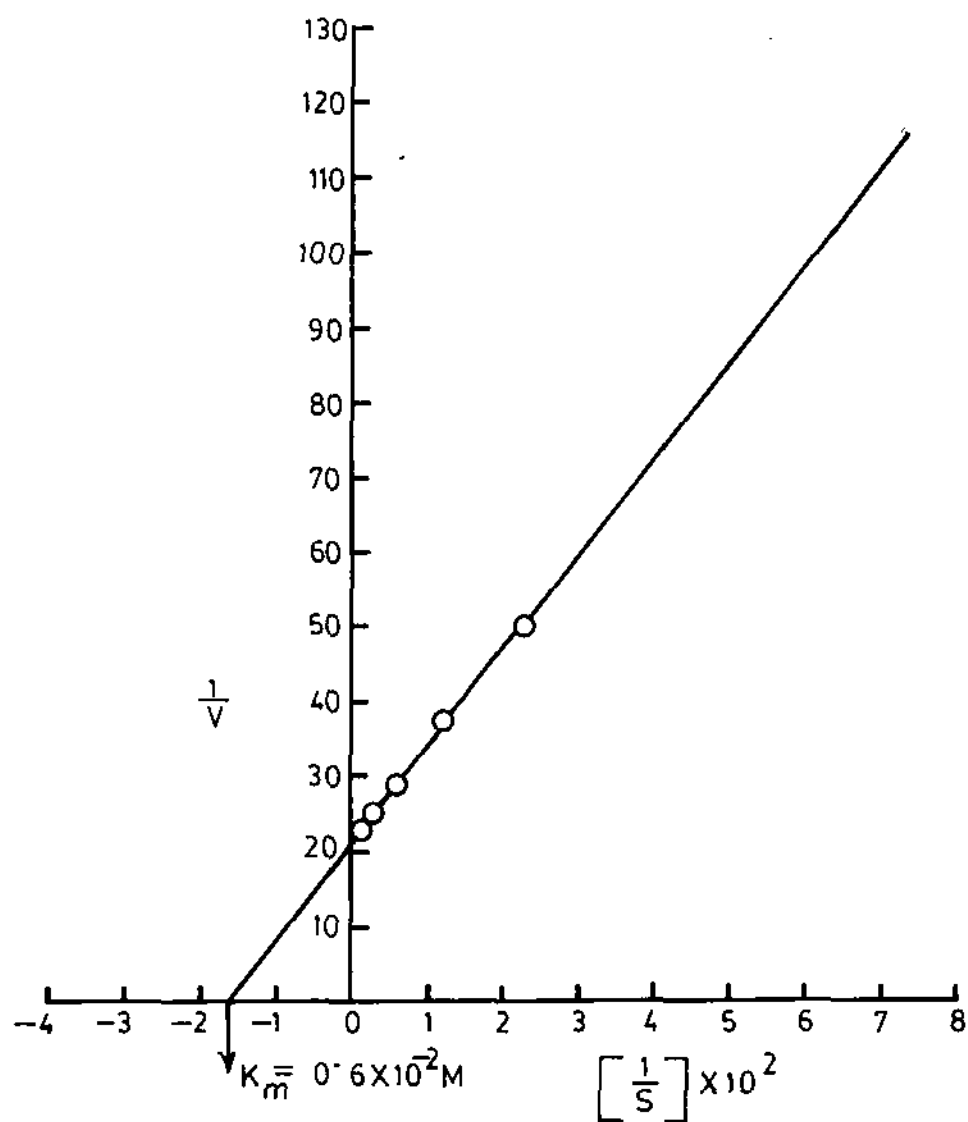


Fig. 6. Lineweaver - Burk reciprocal plot of velocity versus substrate concentration to determine K_m for ethanol of kalomotor A D H.

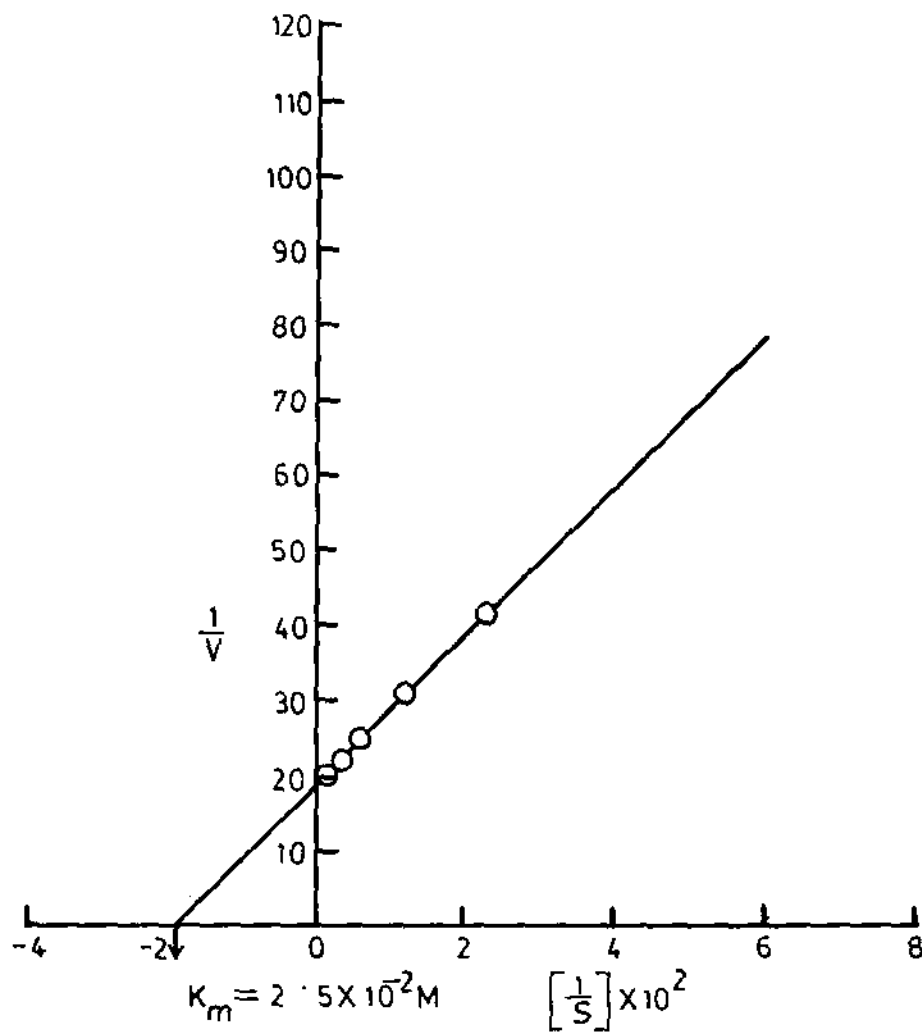


Fig . 7. Lineweaver - Burk reciprocal plot of velocity versus substrate concentration to determine k_m for allyl alcohol of winged bean ADH.

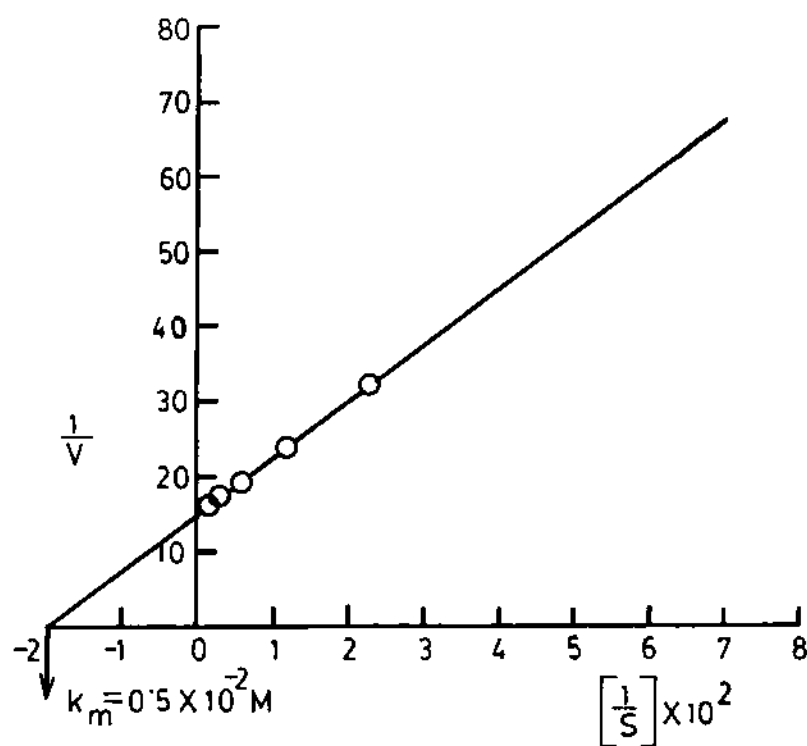


Fig. 8. Lineweaver – Burk reciprocal plot of velocity versus substrate concentration to determine k_m for allyl alcohol of kalomator.

TABLE 5. COMPARATIVE K_m VALUES AND p^H OPTIMA OF DIFFERENT PLANT ADHS. 59

ADH From	p^H optimum	K_m ethanol (M)	K_m allyl alcohol (M)	K_m NAD (M)
Bean	8.2	3.3×10^{-2}	0.8×10^{-2}	1.0×10^{-4}
,,	8.5	3.3×10^{-2}	x	x
Pea	8.7	1.8×10^{-2}	1.0×10^{-2}	0.7×10^{-4}
,,	8.0	1.7×10^{-5}	x	x
Broad bean	8.5	2.0×10^{-2}	x	x
,,	8.7	1×10^{-2}	x	x
Sun flower	8.8	1.5×10^{-5}	1.0×10^{-2}	0.8×10^{-4}
,,	8.8	1.2×10^{-5}	x	x
Rape	8.5	2.5×10^{-2}	x	x
,,	8.8	2.5×10^{-2}	2.0×10^{-2}	1.0×10^{-4}
Maize	8.7	1.8×10^{-2}	x	x
Wheat	8.5	2.2×10^{-2}	x	x
Rice	8.5	0.8×10^{-2}	0.7×10^{-2}	1.5×10^{-4}
Cucumber	8.5	1.1×10^{-2}	0.9×10^{-2}	1.1×10^{-4}

Values are means of triplicate experiments.

in section 2.4.1 in Materials and Methods. A control for each sample was kept in ice cold water bath during the course of heating and the loss of activity in heated sample was compared with the activity in control sample.

Fig 9 shows that 27, 33 and 54% of winged bean ADH activity was inactivated after 10 minutes heating at 45, 55 and 65°C respectively while kalomator ADH lost 28, 34 and 58% enzyme activity respectively at above said respective temperatures (fig 10). The rate of inactivation was rather slow during the following 20 minutes heating at these temperatures. After 30 minutes heating at the above temperatures winged bean ADH lost 21, 42 and 77% activity respectively and kalomator ADH lost 41, 47 and 78% activity respectively.

These results indicate that thermostability of winged bean and kalomator ADHs was more or less similar. The heat stability of winged bean and kalomator ADHs was found to be slightly more than that of other plant ADHs (Gomes *et al.* 1982, Leblova *et al.* 1977). This may be due to the difference in amino acid composition or tertiary or quaternary structure of ADHs. Moreover, the presence of endogenous protectors and proteins, and added sucrose might have influenced heat stability. The protective action of sugars on thermodenaturation of serum proteins was demonstrated (Kerekes 1963).

3.1.7 Substrate specificity

Enzyme catalysis is dependent upon the enzyme's ability to bind a substrate molecule through a particular site on the enzyme i.e. the active site, thus, forming an active enzyme-substrate (ES) complex. This site is quite selective and discriminating conferring

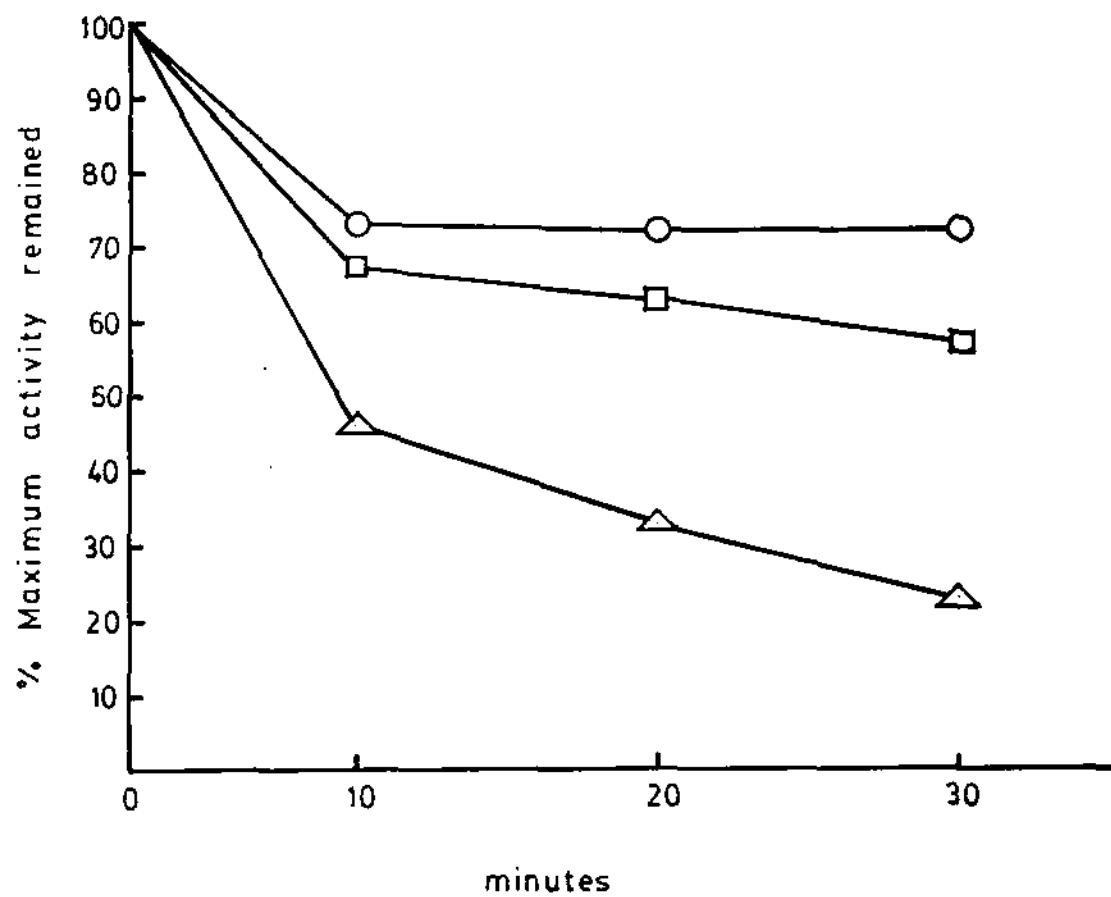


Fig. 9. Thermostability of 48 hr. germinated winged bean alcohol dehydrogenase activity.

○—○—○ = 45°C □—□—□ = 55°C △—△—△ = 65°C .

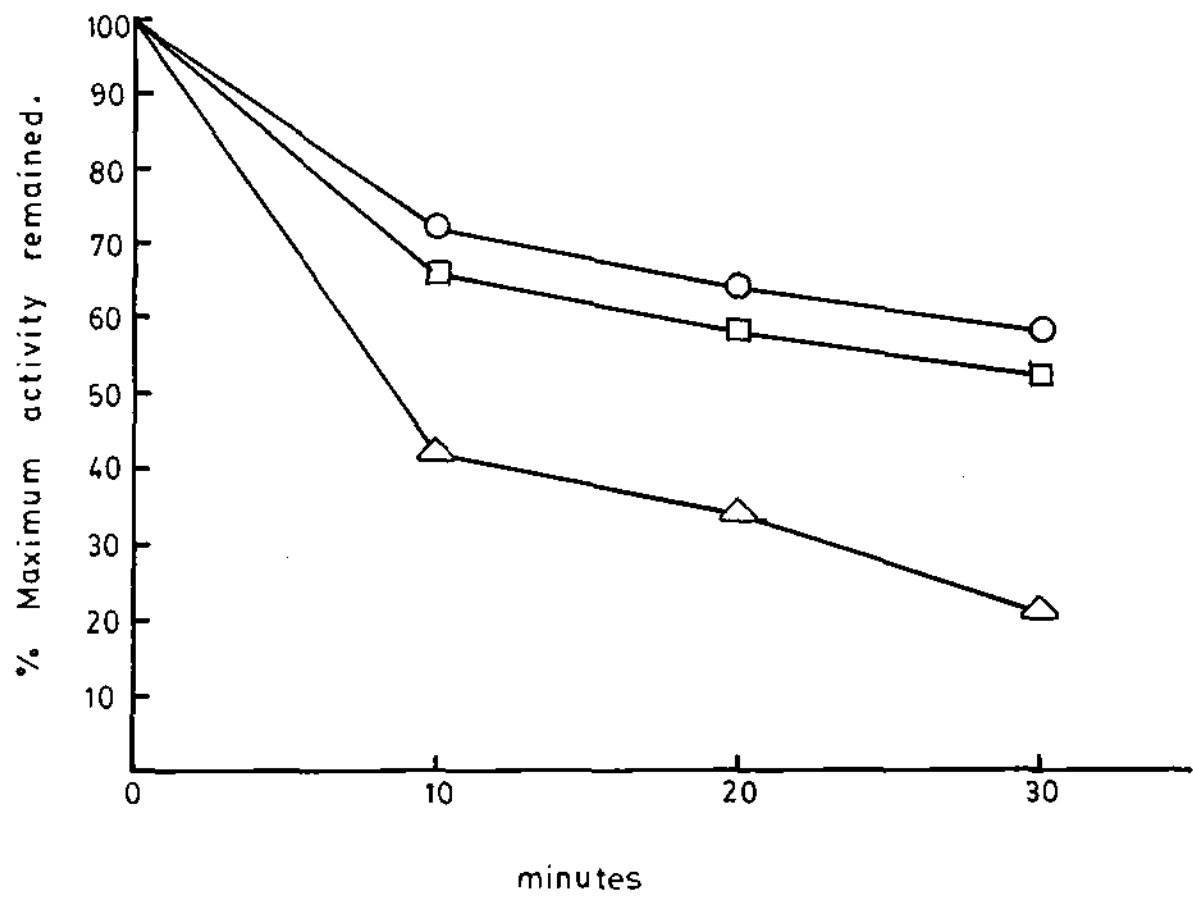


Fig. 10. Thermostability of 48 hr germinated kalamotor alcohol dehydrogenase activity.

○—○—○ = 45°C □—□—□ = 55°C △—△—△ = 65°C

high substrate specificity to enzymes. A study to establish the range of this specificity can reveal information about the active site of the enzymes, since their specificity represents a type of chemical "impression" of the enzyme surface.

Winged bean and kalomator ADHs catalyzed the oxidation of a number of alcohols (Table 6). Aqueous solutions of the alcohols at the concentration of $3 \times 10^{-2} \text{M}$ were used. Saturated solutions of weakly soluble substrates were used. Quantitative differences existed in the rates of oxidation among the alcohols utilized by the winged bean and kalomator ADHs. Allyl alcohol was oxidized most rapidly followed by ethanol. n-Propanol, n-butanol and n-pentanol were utilized more slowly than ethanol. Iso-butanol and sec-butanol were slightly oxidized. Methanol, isopropanol, tertiary-amyl alcohol, cyclohexanol and decanol were not oxidized at all by ADHs of any of these two species. However, oxidation rate of kalomator ADH was higher with all the utilized substrates (Table 6). Unsaturated alcohols such as 2-propen-1-ol (allyl alcohol), trans-2-hexen-1-ol and trans-2-buten-1-ol are more rapidly oxidized than their saturated analogues and ethanol (Eriksson 1968, Leblova et al 1977). For other unsaturated alcohols plant ADHs show much less affinity or do not oxidize at all. As a general rule the initial rate of alcohol oxidation decreases with the increasing number of carbon atoms in the substrate molecules. Other types of alcohols such as diols, terpene alcohol, cyclic and aromatic alcohols, with the exception of cinnamyl and phenyl alcohols, are oxidized very slowly or not at all (Eriksson 1968, Leblova and Stiborova 1975, Leblova and Perglerova 1977).

TABLE 6.

SUBSTRATE SPECIFICITY OF WINGED BEAN AND KALOMOTOR ADHs.

Substrate	Concentration used (M)	Relative rate of oxidation ^a	
		WINGED BEAN	KALOMOTOR
Methanol	3×10^{-2}	0	0
Ethanol	,,	100	100
n-Propanol	,,	62	40
Iso-Propanol	,,	0	0
Allyl alcohol	,,	120	118
n-butanol	,,	56	35
Iso-butanol	,,	6	5
Sec-butanol	,,	4	3
n-Pentanol	,,	51	32
Ter-amyl alcohol	,,	0	0
Cyclohexanol	,,	0	0
Decanol	,,	0	0

a. The velocity of ethanol oxidation was taken 100.

Values are means of triplicate determinations.

It is evident that winged bean and kalomator ADHs showed broad or low substrate specificity. Such a low degree of substrate specificity of alcohol dehydrogenase from various plant sources e.g. pea, broad bean, kidney bean, wheat, maize, cucumber, chickpea, etc. has been reported (Eriksson 1968, Leblova and Mancal 1975, Leblova and Stiborova 1975, Leblova and Perglerova 1977, Gomes et al. 1982). This low degree of substrate specificity of winged bean and kalomator ADHs suggests multiple metabolic functions of these enzymes.

3.1.8 Inhibitor specificity

Concerning the function of the secondary and tertiary structure of proteins or enzymes, inhibition studies are very informative. Depending on the nature of inhibitors various inhibition has been divided into three broad classes. These are reversible inhibition where a substrate like molecule competes with the substrate (e.g. malonic acid competes with succinic acid) for the effective site on the enzyme surface. This types of inhibition is also called competitive inhibition. This inhibition can be reversed by raising the level of substrate. The second class of inhibition is not reversible by raising the level of substrate. Materials such as heavy metal ions, reagents that react covalently with the enzyme, or other reagents that lead to denaturation of enzyme structure, are placed in this group.

Metabolite effectors of regulatory enzymes are not isosteric but rather allosteric (i.e. no structural resemblance with the substrate) with respect to the structure of the normal substrates of the enzyme (Stadtman 1970) the regulatory enzymes probably contain separate binding sites for substrate and effector substances.

Feedback inhibitors do not compete with the substrate for the active site of the enzyme. Instead they may react at an allosteric site and thereby stabilize or induce conformational changes of the enzymes that decrease the intrinsic affinity of the enzyme for substrates at the catalytic site.

These studies are normally done with purified enzyme under rigidly controlled conditions. Such studies are not within the scope of this investigation.

Crude enzyme extracts (0.05 ml) and inhibitors at different concentrations were pre-incubated at room temperatures for 30 minutes. The residual activity was measured by the usual technique and compared with the control. Table 7 shows that different inhibitors used, differentially inhibited ADH activities of winged bean and kalomotor.

Iodoacetamide exerted maximum (98 and 92%) inhibitory effect, and EDTA showed least (55 and 55%) inhibitory effect (Table 7) on winged bean and kalomotor ADHs respectively. Iodoacetamide irreversibly inhibits enzyme by its ability to alkylate sulphydryl and amino groups (Dixon and Webb 1969). EDTA is a metal chelator. The loss of activity brought about by the presence of this agent is the result of its binding to metal ions which may be a component of the active site. These indicate the presence of sulphydryl groups and metal in the active centre of the enzymes. This metal may be zinc (Cossins et al. 1968, Leblova et al. 1977). Zinc has been found to be a component in some NAD linked dehydrogenases (Valle and Hoch 1955) including liver and yeast ADHs. Eklund et al. (1974) found that horse

TABLE 7. EFFECTS OF INHIBITORS ON ADH ACTIVITY OF
WINGED BEAN AND KALOMOTOR.

Inhibitors	Concentration mM	Inhibition percentage ^a	
		Winged Bean	Kalomotor
Iodoacetamide	1	98	92
Eserine	1	67	60
O-Phenanthroline	1	72	68
Dimethyl sulphoxide	10	74	76
Copper sulphate	1	86	89
Ethylene-diaminotetra acetic acid disodium salt.	1	55	55

a. Percent of the maximum activity in control sample.

Values are the means of triplicate determinations.

liver ADH contains four zinc atoms per molecule, contributing to the stabilization of the enzyme. The presence of zinc ions which play an important role in the active centre of the enzyme has also been proved in pea (Suzuki 1966, Cossins et al. 1968) in bean, rape and wheat (Leblova and Stiborova 1975), in kidney bean (Gomes et al. 1982). Functions of -SH groups and zinc ions have been discussed in detail with models of active sites of liver ADH (Eklund et al. 1974).

Because of the sensitivity of winged bean and kalomator ADHs to sulphydryl poisons, chelating agents and metal ion, it may be presumed that the enzyme is a metalloenzyme with sulphydryl groups in the active centre.

3.2 Alcohol dehydrogenase isoenzymes in winged bean and their properties

3.2.1 Electrophoretic detection and quantitative estimation of ADH isoenzymes

Electrophoretic studies revealed that ADHs exist as three distinct molecular forms, ADH-1, ADH-2 and ADH-3, in order of increasing electrophoretic mobility in crude extracts of dry or germinated seeds of winged bean. The patterns are shown in Plate No. 1. ADH-1, last to appear during incubation of the gels, is always narrow and lightly stained, while ADH-3, second to appear, is clearer and intensely stained. ADH-2, most intensely stained and clearest, is the first to appear. Quantitative densitometric analysis of the histochemically developed ADH in dry seeds of winged bean showed that ADH-1, ADH-2 and ADH-3 accounted for 13.6, 51.1, and 35.2% of the total activity respectively (discussed in detail at page 91).

3.2.2 Thermostability of ADH isoenzymes

In the present investigation equal amounts of proteins were electrophoresed. After electrophoresis the gels were incubated at 65°C in a water bath for varying periods of time and then cooled in an ice bath. The gels were then stained for ADH activity. Simultaneously an unheated gel was considered as control. Histochemically developed bands were densitometrically estimated to determine the loss of activity in the heated sample as compared to the control sample. The heat stability of the three ADH isoenzymes was significantly different (Plate No 2). After 10 minutes of incubation at 65°C ADH-1 was completely absent. To the contrary, ADH-3 and ADH-2 retained (Table 8)

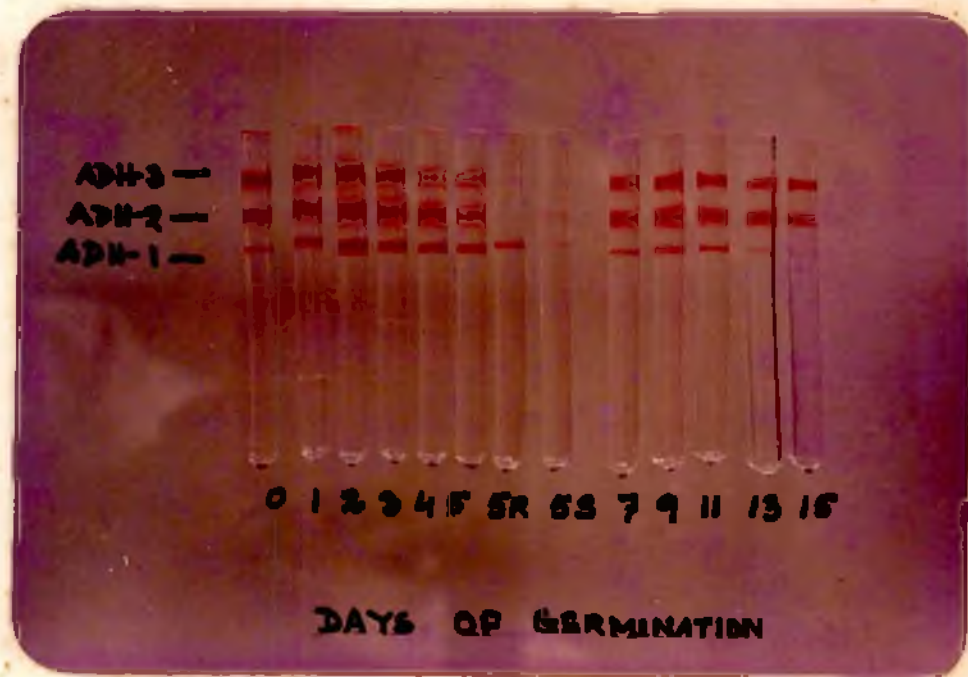


Plate No 1. Zymogram showing the electrophoretic patterns of ADH isoenzymes in germinating winged bean. 0-15 = cotyledon extract of 0-15 days of germination. 5 R = root extract of 5 day old seedlings. 5 S = shoot extract of 5 day old seedlings.

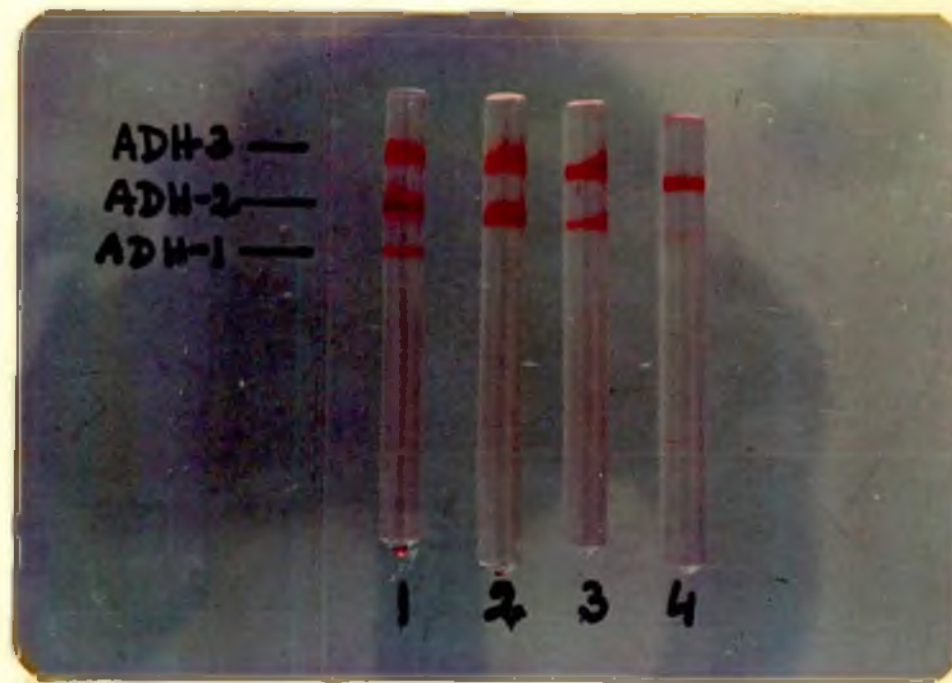


Plate No 2. Zymogram showing the thermostability of ADH isoenzymes in winged bean. 1 - control ; 2 - sample heated for 10 min. after electrophoresis ; 3 - sample heated for 20 min. after electrophoresis ; 4 - sample heated for 30 min. after electrophoresis.

TABLE 8. THERMAL INACTIVATION OF ALCOHOL DEHYDROGENASE
ISOENZYMES IN WINGED BEAN.

Isoenzyme	% Enzyme activity inhibited (control=100) ⁺ Minutes of heating.			
	0	10	20	30
ADH - 1	0	100	100	100
ADH - 2	0	49.8	76.11	93.78
ADH - 3	0	25.22	32.72	42.81

⁺ The amount of each isoenzyme is expressed as a percentage of the control. The amounts of the control were (peak area, cm²)
ADH - 1, 2.6, ADH - 2, 9.9, ADH - 3, 6.8. Each value represents
the average value of three replicates of dry seed extract.

58.00 and 6.22 % of their zero-time activities respectively even after 30 minutes' heating. Differences in the stabilities of the isoenzymes can be correlated with charge that is the fastest isoenzyme (ADH-1) (most negatively charged) showed lowest thermostability while the slowest one (ADH-3) showed highest thermostability. Perhaps such large difference indicate differences in the tertiary structure of the proteins as a result of difference in the primary structure (amino acid composition and sequence) of the isoenzymes. Difference in primary structure may again be consistent with the isoenzymes being different gene products. ADH isolated from maize (Laughner, 1970) and chickpea (Gomes et al. 1982) also showed different thermal stabilities. The correlation of thermal stabilities of maize and chickpea isoenzymes with charge was the reverse from that reported here. Binding of NAD to the isoenzymes could result in the variation of heat stabilities of the isoenzymes. Change in the thermostability by binding of NAD to the Drosophila ADH isoenzymes has been demonstrated (Jacobson 1968).

3.2.3 p_H stability of ADH isoenzymes

Foregoing studies showed that ADH-2 and ADH-3 was^{re} the most heat stable and showed maximal activity with ethanol. This may raise questions regarding the stability of the isoenzymes at different p_H values. Therefore p_H satability of winged bean ADH isoenzymes was investigated.

Crude enzyme extract was prepared in Tris-HCl buffer, p_H 7.6. Then equal amounts of protein were subjected to electrophoresis. After electrophoresis gels were kept in test tubes containing

phosphate (pH 5.0 and 8.0) and Tris-glycine (pH 8.5 and 9.5) buffers for half an hour at room temperature. The control gels were kept in extraction buffer (Tris-HCl, pH 7.6). Then the gels were washed with distilled water and stained for ADH activity as described in section 2.7.8 of Methods and Materials. The activity of each isoenzyme (Plate No 3) was then estimated densitometrically. Table 9 shows distinct differences in the pH stabilities of the ADH isoenzyme. The isoenzymes were most stable at pH 8.0 where ADH-3, ADH-2 ADH-1 retained 95.1, 97.5 and 53.8 % of their control activities respectively. At pH 5.0 ADH-3, ADH-2 and ADH-1 retained 79.81, 67.2 and 41.7 % of their initial activities respectively while at pH 9.5 these isoenzymes retained 67.3, 27.0 and 0 % activities respectively. These indicate more stabilities of the isoenzymes in the slightly acidic region than in the highly alkaline region where the isoenzymes were most probably denatured more quickly. Considering the relative stabilities of the isoenzymes at the different pH values tested it can be concluded that ADH-3 was the most stable and ADH-1 was the least stable. Differences in the pH stabilities can also be correlated with charge of the isoenzymes i.e. the slowest one being most stable and the fastest one being least stable. These results reflect the structural differences between the isoenzymes. These results support our results of extractability and stability of winged bean ADH at different pH values. Previous reports have shown that maize (Laughner 1970) and chickpea (Gomes *et al.* 1982) ADHs and their isoenzymes are more stable between pH 6 and 7.

3.2.4 Inhibitor study of ADH isoenzymes

Inhibitor studies reported here are mainly concerned with the relative inhibition of the three isoenzymes by some specific

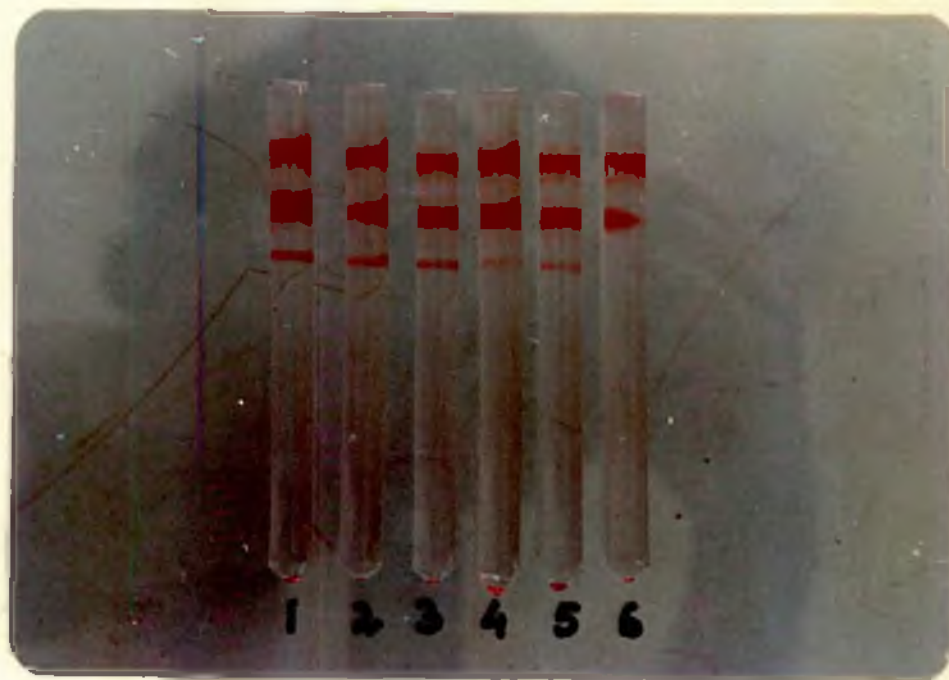


Plate No 3. Zymogram showing the pH stability of ADH isoenzymes in winged bean. 1 - control (extraction buffer pH 7.6) ; 2 - pH 5.0 ; 3 - pH 7.0 ; 4 - pH 8.0 ; 5 - pH 8.5 ; 6 - pH 9.5.

TABLE 9. p^H STABILITY OF ADH ISOENZYMES IN WINGED BEAN.

Isoenzymes	% Enzyme activity inhibited (control = 100) ⁺ p^H values.				
	5	7	8	8.5	9.5
ADH - 1	58.3	47.1	46.2	53.9	100.0
ADH - 2	32.8	17.20	2.5	5.5	73.0
ADH - 3	20.19	16.38	4.9	10.9	32.7

⁺ The amount of each isoenzyme was expressed as a percentage of the control. The amounts of the control were (peak area cm^2)

ADH - 1, 2.6; ADH - 2, 9.6; ADH - 3, 6.8. Values are the means of 3 sets of experiments with dry seed extract.

inhibitors. Equal amounts of protein (200-400 μg in 40 μl) were electrophoresed after which gels were incubated for 30 minutes / separately in Tris-HCl buffer (pH 8.5) containing the inhibitors and washed thoroughly. Gels were then stained and quantitated densitometrically as described in section 2.7.13 of Materials and Methods. Control consisted of duplicate gels stained with ethanol in the absence of inhibitors.

Differential inhibition of the activity of the isoenzymes by the inhibitors used are shown in Plate No 4 and Table 10. Iodoacetamide and CuSO_4 completely inhibited all the three isoenzymes while EDTA showed least inhibitory effect on the isoenzymes. Regardless of the inhibitor used, ADH-2 was least affected and ADH-1 was most affected and ADH-3 showed more sensitivity towards the inhibitors than ADH-2. As discussed earlier in section 3.1.8, the irreversible inhibitory effect of iodoacetamide indicates the presence of sulphhydryl groups in the active centre and inhibition by EDTA, CuSO_4 and DMSO indicates the presence of metal in the enzyme molecule. Differential inhibition of the isoenzymes by different inhibitors suggests structural differences between the isoenzymes. Similar findings have been reported for chickpea (Gomes *et al.* 1982)

3.2.5 Substrate specificity of ADH isoenzymes

Winged bean ADH isoenzymes were studied to find out any difference between the three isoenzymes in their ability to oxidize different alcohols.

Isoenzymes in crude extracts were separated using electrophoresis and development of the bands were carried out using separate

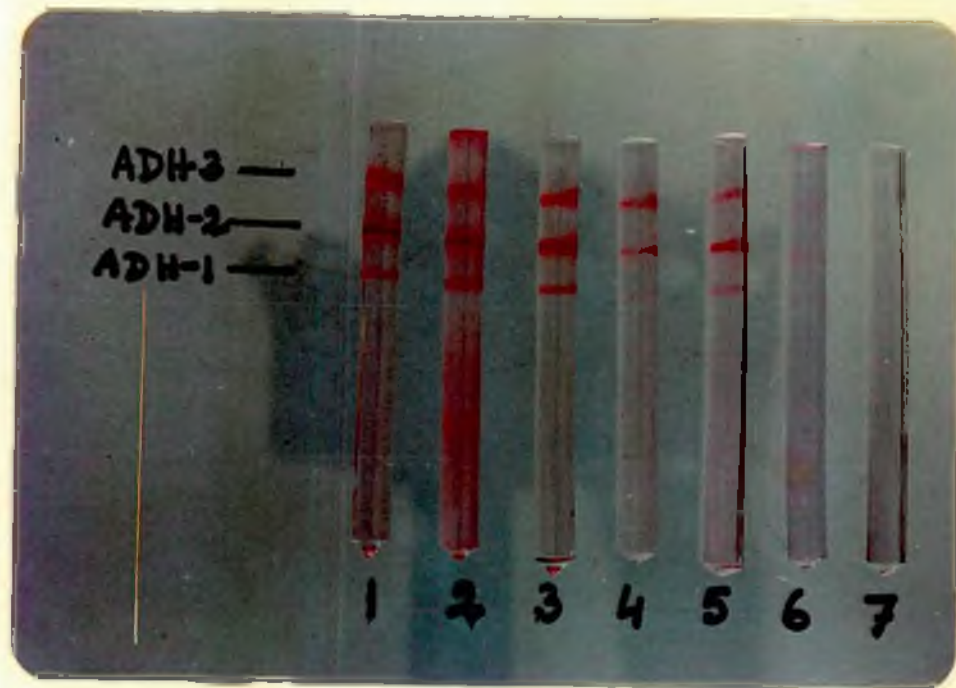


Plate No 4. Zymogram showing the inhibition of ADH isoenzymes in winged bean by different inhibitors.
1 - control ; 2 - eserine ; 3 - EDTA ; 4 - DMSO ;
5 - 0 - phenanthroline ; 6 - CuSO₄ ; 7 - iodoacetamide.

TABLE 10. EFFECT OF INHIBITORS ON ALCOHOL DEHYDROGENASE ISOENZYMES IN WINGED BEAN.

Isoenzymes	Incubation time (min)	% Enzyme activity inhibited (control=100) ⁺ Inhibitors and Concentrations					
		EDTA (0.3M)	Eserine (0.3M)	O-phenanthroline (0.3M)	DMSO (3M)	Cuso ₄ (0.3M)	Iodoacetamide (0.3M)
ADH - 1	30	30.1	33.0	34.1	100	100	100
ADH - 2	30	19.0	20.12	24.8	25.0	100	100
ADH - 3	30	27.0	28.4	35.9	33.3	100	100

⁺ The amount of each isoenzyme was expressed as a percentage of the control. The amounts of control were (peak area cm²) ADH - 1, 2.6; ADH - 2, 9.9; ADH - 3, 6.8. The values are the means of triplicate experiments with dry seed extract.

staining mixture containing individual alcohol at a final concentration of 0.2 M, except pentanol which was 0.1 M. Always equal amounts of protein extracts from the same lot was used for making a comparative study of substrate specificity. The amount of each isoenzyme was determined by densitometric tracings of the histochemically stained gels and the amount of each isoenzyme has been expressed as a percentage of ethanol oxidation.

Winged bean ADH isoenzymes oxidized different alcohols differentially (Plate No. 5 and Table 11). Each isoenzyme oxidized allyl alcohol maximally and most rapidly followed by ethanol, n-propanol, n-butanol, n-pentanol and iso-butanol. ADH-3 isoenzyme activities relative to the control (ethanol oxidation = 100) were 117.6, 66.2, 61.3, 12.2 and 6.1 % for allyl alcohol, n-propanol, n-butanol, n-pentanol and iso-butanol respectively. ADH-2 activities relative to the control were 128.6, 69.3, 71.3, 20.1, 11.3 and 1.1 % for allyl alcohol, n-propanol, n-butanol, n-pentanol, iso-butanol and sec-butanol respectively. Relative ADH-1 activities were 108.0, 33.8, 34.1, 14.7, and 5.3 % for allyl alcohol, n-propanol, n-butanol, n-pentanol and iso-butanol respectively. None of the isoenzymes showed any reactivity with tert-butanol, iso-propanol, methanol and tert-amyl alcohol. Only ADH-2 oxidized sec-butanol.

ADH-2 always appeared to be catalytically most active with different alcohols and ADH-3 appeared little less active than ADH-2. To the contrary, ADH-1 showed little reactivity with the alcohols oxidized. These variations observed in the degree of substrate specificity between the isoenzymes would reflect the structural

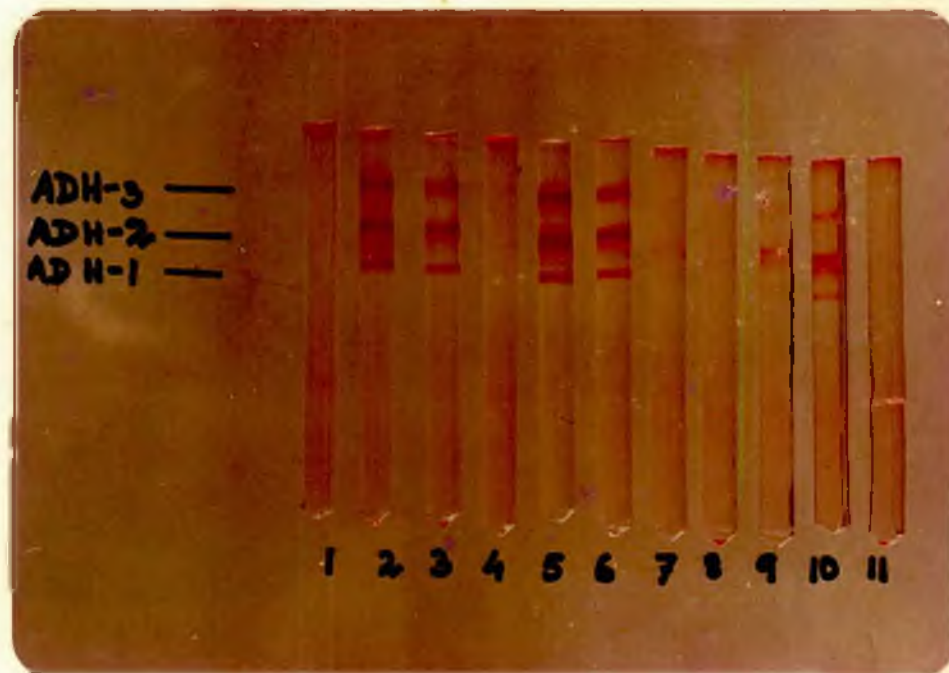


Plate No 5. Zymogram showing the substrate specificity of ADH isoenzymes in winged bean. 1 - methanol ; 2 - ethanol ; 3 - n-propanol ; 4 - iso-propanol ; 5 - allyl alcohol ; 6 - n-butanol ; 7 - sec-butanol ; 8 - tert-butanol ; 9 - iso-butanol ; 10 - pentanol ; 11 - tert-amyl alcohol.

TABLE 11. SUBSTRATE SPECIFICITY OF ALCOHOL DEHYDROGENASE
ISOENZYMES IN WINGED BEAN.

Substrates	Amount of isoenzyme relative to control=100 ⁺		
	ADH - 1	ADH - 2	ADH - 3
Methanol	0	0	0
Ethanol	100	100	100
n-propanol	33.8	69.3	61.3
Iso-propanol	0	0	0
Allyl alcohol	108.0	128.6	117.6
n-butanol	34.1	71.3	66.2
Iso-butanol	5.3	11.3	6.1
Sec-butanol	0	1.10	0
Ter-butanol	0	0	0
n-pentanol [*]	14.7	20.1	12.2
Ter-amyl alcohol	0	0	0

⁺ The amount of each isoenzyme is expressed as a percentage of ethanol oxidation. The amounts of the control were (peak area, cm^2)
ADH - 1, 2.6; ADH - 2, 9.9; ADH - 3, 6.8. Values are the means
of 4 sets of experiments of dry seed extract.

^{*} Final cocentration of the substrates is 0.2M, except pentanol which
is 0.1M.

differences between the isoenzyme molecules. Moreover, differences in the specificity of the isoenzymes towards different alcohols may be due to differences in the formation of enzyme-substrate complex which is greatly influenced by the structure of the substrates. Similar differences in the substrate specificity of ADH isoenzymes have been reported for maize (Longo and Scandalios 1968, Laughner 1970), chickpea (Gomes et al. 1982) and in pea (Cеровска and Leblova 1981). The low degree of substrate specificity of winged bean ADH isoenzymes suggests that they have different metabolic functions in winged bean under different physiological conditions.

3.2.6 Total ADH activity in germinating winged bean and kalomotor cotyledons during germination

Seeds germinated for 0-15 days were extracted with Tris-HCl buffer (pH 7.6) containing BME and sucrose and ADH activities were measured (section 2.4.1 Chap. 2). The fresh weight and protein content of the seedlings changed considerably during the 15 days of germination. Therefore, the protein concentration in the extracts from the cotyledons of different ages was measured and enzyme activity has been expressed per mg of protein.

Considerably high ADH activity was observed in dry cotyledons of winged bean and kalomotor (Fig. 11). The activity was higher in kalomotor. The specific ADH activity increased sharply and reached their peaks after 24 hours' soaking and 48 hours' germination in both winged bean and kalomotor. The activity in winged bean cotyledons at its maximum was 134.42 % higher than in the dry seeds. In the following two days the activity underwent a rapid decline

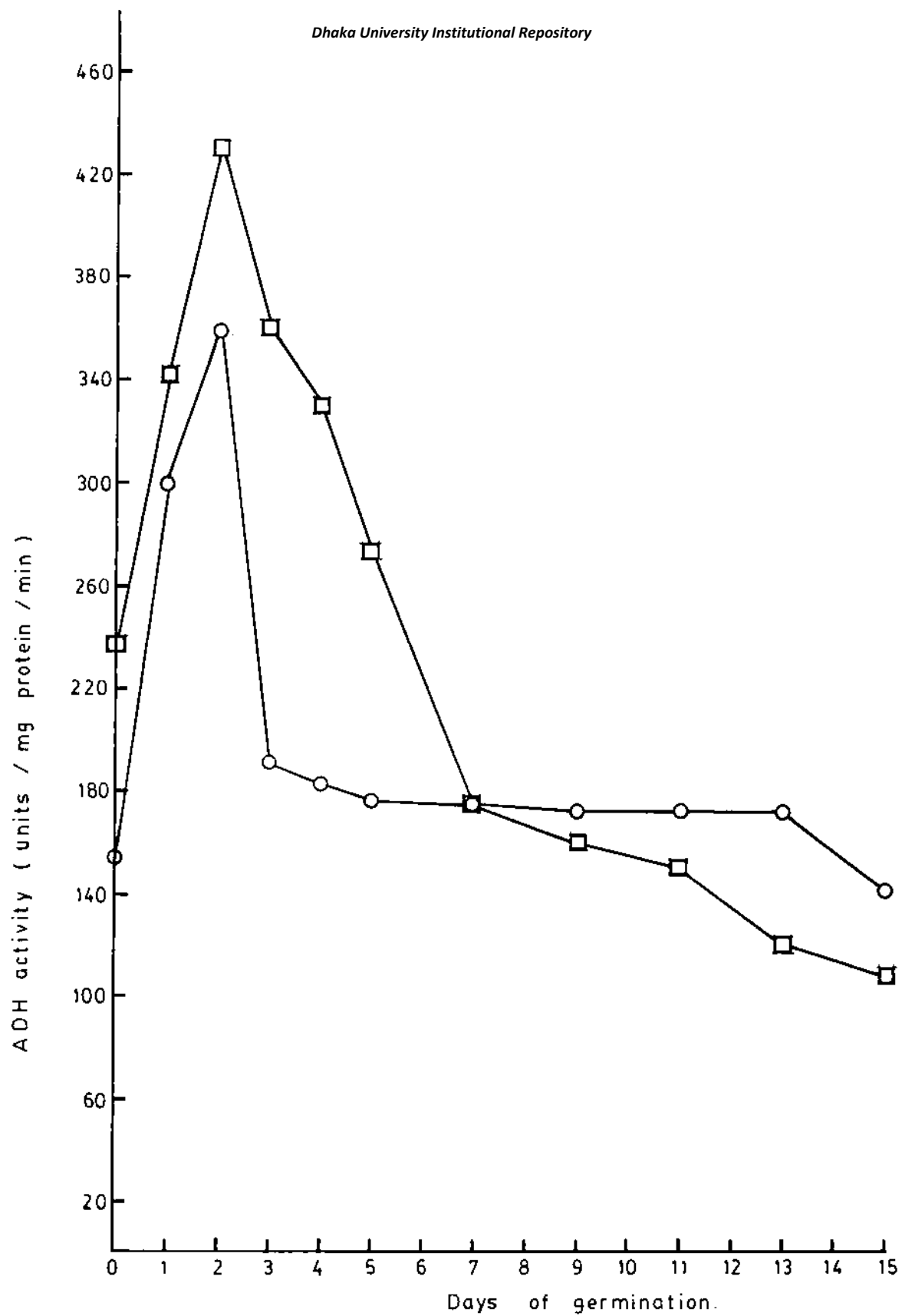


Fig. 11. Total alcohol dehydrogenase activity change during germination of winged bean and kalomator cotyledons.

□—□—□ = Kalomator ○—○—○ = Winged bean.

and during the 5th-13th day of germination the activity remained fairly constant. The activity again declined gradually after day 13 and 39.3% of the peak activity remained in the seeds at day 15. ADH activity in kalomator showed almost similar fluctuations, but the increase in activity was less pronounced. The activity increased by about 79 % after 48 hours' germination and then declined rapidly, reaching the level of the dry seeds within 6 days of germination. There was further decrease during 6th-15th days and by the 15th day only 25.2% of the peak activity was retained.

Although high ADH activity was present in the dry winged bean seeds, ethanol, the substrate of ADH, could not be detected in these seeds (Table 12). Ethanol made its appearance only during imbibition and its concentration rapidly increased and reached a maximum (12.0 $\mu\text{mol/gm fr. wt.}$) after 48 hours of germination when the activity of ADH was at its peak. In the following days ethanol concentration decreased rapidly and by day 15th only 1.0 μmol was detected. Similar fluctuations in the levels of ADH and ethanol have been reported in maize (Scandalios and Felder 1971), chickpea (Gomes et al. 1982, Aldasoro and Nicolas 1980) and in many other higher plants (Leblova et al. 1971, Leblova 1975).

Increases in ADH and ethanol during the initial hours of germination have been observed in many plants, but the biochemical mechanism is not entirely clear. It has been proposed that lactate and malate accumulate in anaerobically respiring tissues (e.g. germinating seeds with unruptured seed coat) and cause a fall in pH (Davies et al. 1974). Acidic pH restricts further reduction of

TABLE 12. CHANGES IN ADH ACTIVITY AND ETHANOL CONCENTRATION
DURING GERMINATION OF WINGED BEAN SEEDS.

Days of germination	Sp. act. of ADH (units/mg protein)	Ethanol conc. μ mol/gm.fr.wt.
0	153.57	0
1	300.00	10.2
2	360.00	12.0
3	191.78	8.0
4	184.61	6.5
5	176.61	5.0
7	175.43	3.8
9	172.41	2.6
11	172.72	2.5
13	172.70	1.8
15	141.50	1.0

Values are means of triplicate experiments.

pyruvate by LDH to lactate, but favours the decarboxylation of pyruvate to acetaldehyde (Davies et al. 1974, Leblova 1975). Acetaldehyde acts as a substrate of ADH and induces it. The ability of acetaldehyde to induce ADH has been reported (McManmon and Crawford 1971). Acetaldehyde, being toxic to plants, is immediately reduced to ethanol by ADH (Davies et al. 1974). In the presence of oxygen, ethanol is again oxidized by ADH to acetaldehyde which is further metabolized via the TCA cycle (Cameron and Cossins 1967). However, other mechanism for the degradation of ethanol has been proposed. Wagner (1959) and Kenefick (1962) proposed that ethanol is removed by respiration while Davis (1958) proposed that ethanol, lacking any charge, is released from the cell to the surroundings thereby protecting the cells from toxicity of ethanol.

The absence of ethanol in dry winged bean seeds, but its formation and rapid rise during imbibition and 48 hours' germination respectively, and coincidence of the peak ADH activity with the appearance of ethanol suggest that ADH may be induced by acetaldehyde prior to the formation of ethanol (McManmon and Crawford 1971). The reduction of acetaldehyde is then, perhaps, catalyzed by the induced or activated ADH to produce ethanol during the initial hours of germination (anaerobic respiration). The rapid decline of ADH and ethanol after 2nd day of germination and the gradual disappearance of ADH during 5th-15th day in aerobically respiring seeds (e.g. germinating seeds with ruptured seed coat) suggest that ADH perhaps participates in the oxidation of ethanol formed during aerobic respiration, to acetaldehyde.

The initial rise in the activity of ADH in germinating winged bean and kalomator cotyledons may be due to several reasons such as (i) activation or induction of the pre-existing enzyme molecules by various factors, (ii) de novo synthesis of ADH protein, (iii) inhibition or removal of ADH specific inhibitors during initial stage of germination, (iv) stage of thiol radical in the ADH molecules, and (v) influence of plant hormones or growth substances.

The presence of high ADH activity in winged bean and kalomator suggests that like many other enzymes ADH molecules were formed during development and ripening of the seeds and remained both in active and inactive forms. Activation of these pre-existing enzyme molecules by imbibition in water or reduced oxygen supply during initial stage of germination i.e. prior to the rupture of seed coat may cause the rise in ADH activity. Activation of the pre-existing forms of enzymes by imbibition was reported in pea (Flinn and Smith 1967, Barker et al. 1971, Suzuki and Kyuwa 1972), maize (Ho-David and Scandalios 1975) and many cereals and legumes (Leblova 1975, Gomes et al. 1982). Activation or induction of ADH by acetaldehyde may also cause rise in ADH activity in winged bean and kalomator. Such induction of ADH by acetaldehyde has been proposed by McManmon and Crawford (1971), Scandalios and Felder (1971).

De novo synthesis of ADH in germinating winged bean and kalomator may account for the rise in the activity. Using density labelling experiments Lai and Scandalios (1977) found that the increase in ADH activity in anaerobically respiring maize was due to de novo synthesis of ADH protein. To the contrary, Ho-David and Scandalios

(1975) suggested that the rapid rise in ADH activity in germinating maize was not due to denovo synthesis and the activity was not dependent on transcription and translation since it was not possible to prevent the increase of ADH by known inhibitors of DNA, RNA and protein syntheses and ADH did not become density labelled during germination of maize in D_2O and $^{15}NH_4Cl$.

The rise in ADH activity may be due to the inhibition of endogenous ADH specific inhibitors by some unknown factors during the early hours of germination. Majumder and Biswas (1973) reported that the increase in enzyme activity during germination was due to the removal of endogenous inhibitors. The stage of thiol radical in ADH in germinating seeds may be responsible for the increased activity of the enzyme. Since BME activated ADH enzyme in germinating pea, Suzuki and Kyuwa (1972) suggested that the disulphide bridge in the ADH molecule may be converted to dithiol groups by BME leading to the activation of the enzyme.

Besides the above mentioned factors, endogenous plant hormones and growth substances may play important roles for the increase of ADH activity. A large number of investigators have demonstrated that plant hormones and growth substances in some way control many enzyme activities (reviewed by Akazawa and Miyata 1982, Laidman 1982, Mayer and Shain 1974).

The observed decrease in ADH activity during later stage of germination could be due to the accumulation of long chain fatty acids that inactivate alcohol dehydrogenase. Kolloffel (1970) showed that the extracts from the cotyledons in the later stage of germination contain long chain fatty acids such as linoleic acid and oleic

acid which are able to inactivate ADH. It was reported that mixing of cotyledon extracts from early and later stage of germination cause decrease in ADH activity, suggesting that inhibitor or inhibitors are generated in the later stage of germination (Kolloffel 1970, Suzuki and Kyuwa 1972, Ho-David and Scandalios 1975). The decrease in ADH activity in germinating pea was attributed to increasing aerobic environment of the germinating seeds (Kolloffel 1968).

The progressive inhibition of ADH in germinating seed extracts can be prevented by BME and dithio-threitol (DTT) suggesting that the inhibitor is not a protease since no protease is inhibited by BME and DTT (Ho-David and Scandalios 1975). Moreover, treatments of pea ADH with proteolytic enzymes e.g. peptidase, pepsin, trypsin and α -chymotrypsin did neither inhibit nor activate the enzyme (Suzuki and Kyuwa 1972). The facts that BME or DTT can prevent the inhibition of ADH in germinating maize, no proteolytic enzymes are involved in the inactivation of ADH. As the amount of inhibition rose steadily after 24 hours of germination, Ho-David and Scandalios (1975) suggested that the structural genes for ADH are probably repressed in the cotyledons by some mechanisms during the onset of germination, and that the decline in ADH activity is due to the increase in ADH specific inhibitor. Suzuki and Kyuwa (1972) reported that protein synthesis is necessary for the inactivation of alcohol dehydrogenase because inhibitors of protein synthesis failed to block the increase in ADH activity but prevented its decline.

3.2.7 ADH isoenzyme patterns in winged bean and kalomator cotyledons during germination

Electrophoretic techniques, coupled with zymogram method, have facilitated the study of the developmental processes at molecular level. The differentiation of protein pattern is under the control of ontogenetic changes and difference of tissues. So, the isoenzyme pattern of a tissue may be indicative of its stage of development and physiological functions. Therefore, both qualitative and quantitative changes of ADH isoenzyme patterns were studied during germination of winged bean, and only qualitative changes of ADH isoenzymes were studied during germination of kalomator. Equal amounts of proteins from seedlings of different ages were electrophoretically separated and ADH activity was developed histochemically as described in section 2.7 of Materials and Methods. The changes in the activity of each individual isoenzyme band in winged bean were followed by direct densitometry of the stained gels throughout the period of germination of winged bean. The changes in the intensity of kalomator ADH isoenzymes were followed by visual assessment only at different stages of germination as these isoenzymes separated so closely that it was not possible to quantify them densitometrically.

Plate No 1 shows that winged bean seed extracts contained three distinct and well separated isoenzymes designated as ADH-1, ADH-2 and ADH-3. The changes in the amounts of each isoenzyme during the course of germination are presented in Table 13. In the cotyledons of dry seeds ADH-1, ADH-2 and ADH-3 accounted for 13.6, 51.1 and 35.2% of the total activities respectively. As germination

TABLE 13. CHANGES IN THE AMOUNTS OF THREE ADH ISOENZYMES IN THE COTYLEDONS OF WINGED BEAN DURING GERMINATION.

		Amount of isoenzyme expressed as a percentage of that at day 2 [†] Days of germination												
		0	1	2	3	4	5	7	9	11	13	15		
ADH - 1	28.9	41.3	100	36.8	31.3	21.2	9.7	8.15	7.8	5.3	1.52			
ADH - 2	73.3	73.8	100	66.7	65.16	62.73	46.98	44.9	41.7	37.4	33.8			
ADH - 3	64.5	70.4	100	54.5	42.06	44.31	42.8	42.4	37.08	32.11	29.22			

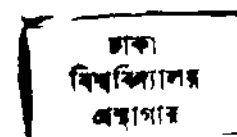
[†]The amounts of isoenzymes at day 2 were (peak area cm²)

ADH - 1, 9.2; ADH - 2, 13.56; ADH - 3, 10.65. The values are the means of three sets of experiments.

proceeded in darkness, the amount of ADH-1 increased at a faster rate than those of other isoenzymes, but they all peaked together at day 2. Of the total activity at this stage, 27.53% was represented by ADH-1, 40.58% by ADH-2, and 31.89% by ADH-3. There were increases of about 245.0, 36.34 and 35.5% in ADH-1, ADH-2 and ADH-3 respectively. After day 2, the amount of each isoenzyme declined at different rates. However, the ADH-1 amount remained at higher level than that in dry seeds up to day 3 whereas the amounts of ADH-2 and ADH-3 dropped below the levels of ADH-2 and ADH-3 in dry seeds at this stage. At day 3, activities of ADH-1, ADH-2 and ADH-3 dropped by 63.2, 33.2 and 45.54% of their peak activities respectively and continued to decrease further with the progress of germination. At day 15, the amount of ADH-1 dropped by 98.48% of its peak activity whereas those of ADH-2 and ADH-3 dropped by 66.08 and 70.71% of their peak activities respectively. At this stage the relative amounts of ADH-1, ADH-2 and ADH-3 were 1.52, 33.87 and 29.22% respectively. They collectively retained only 23.52% of the sum of peak activities at day 15.

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From the root and shoot extracts of 5-day-old winged bean, all the three ADH isoenzymes could be resolved but they stained faintly (Plate No 1 where all the isoenzymes cannot be seen because they stained faintly). In contrast to the seed extracts, ADH-1 appeared most active in the root extracts while ADH-2 and ADH-3 appeared in trace amounts. In the shoot extracts ADH-2 and ADH-3 appeared much more active than ADH-1. In leaf, stem and root extracts of older plants (20-21 days) no ADH band could be detected (Results not shown).



Dry seed extracts of kalomator also contained three ADH isoenzymes which were not distinctly and well separated (Fig. 12). The activities of the three isoenzymes increased with the onset of germination. At day 2 of germination, the amounts of the isoenzymes reached peaks. At this stage ADH-2 and ADH-3 stained intensely and ADH-1 appeared fairly stained. Between day 2 and 5 the activities of the isoenzymes slightly declined. From day 5, the activity of ADH-1 decreased drastically whereas those of ADH-2 and ADH-3 decreased at slower rates. At day 15, ADH-1 could not be detected whereas ADH-2 and ADH-3 appeared faintly stained and weak. However, the time course of disappearance of ADH isoenzymes in both winged bean and kalomator 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 activities during germination of winged bean and kalomator. Changes in ADH isoenzymes patterns during germination have been described for maize (Scandalios and Felden 1971), wheat, rye and rice (Jaaska 1976), chickpea (Gomes et al. 1982) and many other plants (Shannon 1968, Scandalios 1974, 1969).

At this point the functional significance of ADH isoenzymes in winged bean and kalomator can only be theorized. The fact that the fastest isoenzyme ADH-1 in winged bean exhibited maximum rise (about 245.0% of the initial activity after 48 hours) and showed most pronounced decay rate, it is suggested that it functions only in storage cotyledons participating in the reduction of acetaldehyde during the initial hours of germination (anaerobic respiration). Similar may be the function of ADH-1 in kalomator. This suggestion is supported by the previous studies with maize by Scandalios (1974)

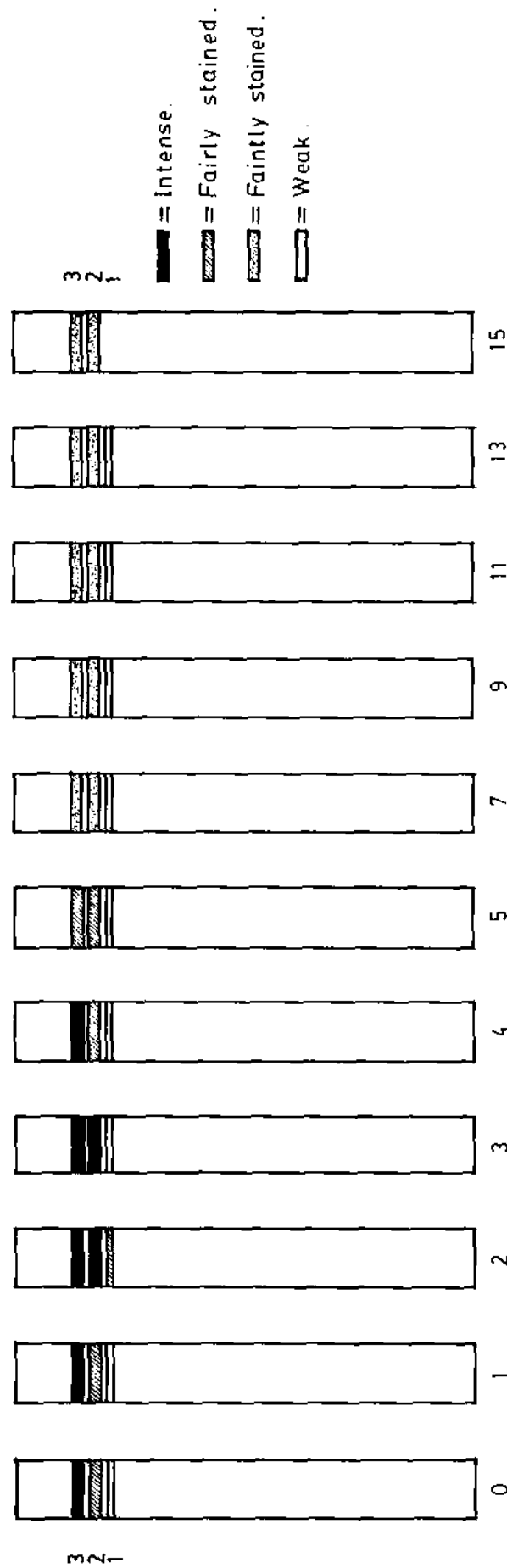


Fig. 12. Schematic representation of alcohol dehydrogenase zymogram of germinating xalomotor seeds.

who has shown that the fastest ADH isoenzyme increased most significantly under anaerobic conditions as compared to other isoenzymes and it seemed to favour the reduction of acetaldehyde. The present results differ from the results of Gomes et al. (1982) who found that the slowest ADH isoenzyme in chickpea showed most significant increase during the anaerobic phase of germination. The much less and almost identical increases (35.5 - 36.3%) occurring in ADH-2 and ADH-3 activities and their gradual and simultaneous decline to very low levels by day 15 in aerobically respiring winged bean seeds (e.g. germinated seeds with ruptured seed coat) suggest that they perhaps participate in the oxidation of ethanol formed during anaerobic respiration. The same suggestion may be true for ADH-2 and ADH-3 in kalomator. These interpretations are supported by the findings that ADH-1 existed in maximum amount in root extracts of winged bean seedlings while the quantities of ADH-2 and ADH-3 in shoot extracts were much more than that of ADH-1.

The reasons for the increases and decreases of each ADH isoenzyme in germinating winged bean and kalomator may be similar to those already described in section 3.2.6 of results and discussions. However, the differential increases and decreases in ADH isoenzyme activities at different stages of germination of winged bean and kalomator may be due to differential genetic control, transcription, activation, accumulation, degradation and inhibition of the isoenzyme molecules (Scandalios 1974).

Since the three ADH isoenzymes, common in the three tissues (root, shoot and cotyledon) of 5-day-old seedlings showed differences in activities, it is suggested that the gene or genes or pair

or pairs of alleles, which are responsible for the formation of ADH isoenzymes, are at different stages of activation in different tissues. Alternatively, the cellular environment (e.g. pH , oxygen supply, presence of substrates or inhibitors) in different tissues may be responsible for the different amounts of different ADH isoenzymes.

Differences in in vivo activation and inactivation, activity, stability (pH , heat and storage), and substrate and inhibitor specificities between the ADH isoenzymes in winged bean suggest that they are coded by distinct gene loci. Schwartz and Endo (1966) suggested that a compound locus composed of two genes direct the synthesis of ADH isoenzymes in maize while Scandalios (1967) postulated that the maize ADH isoenzymes are coded by two closely linked genes each having two alleles. Codominant alleles coding for ADH isoenzymes have been reported in sunflower (Torres 1974), Phaseolus vulgaris - Ph. Coccineus complex (Wall and Wall 1975) and wheat (Hart 1970). Duplicated genes fixed for alternative alleles have been shown to produce ADH isoenzymes in Clarkia franciscana (Gottlieb 1974). Most of these authors have found that ADH molecules are dimeric while some reported monomers of ADH.

It is interesting to note that remarkable differences existed between the ADH isoenzyme patterns of winged bean (Genus: Psophocarpus) and kalomator (Genus: Vicia). Three ADH isoenzymes in winged bean were quite distinctly separated on the gels while those in kalomator migrated almost to the same position on the gels and only after prolonged electrophoresis they were very closely separated. Moreover, there were differences in intensities between the two

sets of ADH isoenzymes. Differences in mobilities and intensities between the isoenzymes derived from two different species suggest that the isoenzymes are controlled by different genes or different alleles of a locus specific for each plant species. In fact the dissimilarity between the ADH patterns suggest that the two species (Vicia faba and Psophocarpus tetragonolobus) display a distant phylogenetic relationship. These results further show that ADH isoenzymes may serve as good genetic markers (Nelson and Burr 1973).

3.2.8 Effects of complete and partial anaerobioses on winged bean ADH and its isoenzymes

Winged bean seeds were germinated under three different conditions: (a) Complete anaerobic condition — twenty seeds were soaked in 50 ml distilled water in a beaker (cap. 100 ml) for 4 hours, transferred to a conical flask (cap. 250 ml) on a filter paper moistened with distilled water and germinated under continuous supply of N_2 for 44 hours, (b) Partial anaerobic condition — twenty seeds were soaked and germinated in 50 ml distilled water in a beaker (cap. 100 ml) for 48 hours, and (c) Aerobic condition (control) — twenty seeds were soaked in 50 ml water for 4 hours and germinated for 44 hours on distilled water-moistened filter paper in a beaker (cap. 100 ml) covered with a watch glass. Germination was allowed to proceed at 28-30°C in a dark thermostatic chamber. ADH extraction, assay and electrophoretic procedures were the same as described in section 2.4.1 of Materials and Methods.

Effects of complete and partial anaerobioses on ADH activity are shown in Fig 13. ADH activity in winged bean increased under the

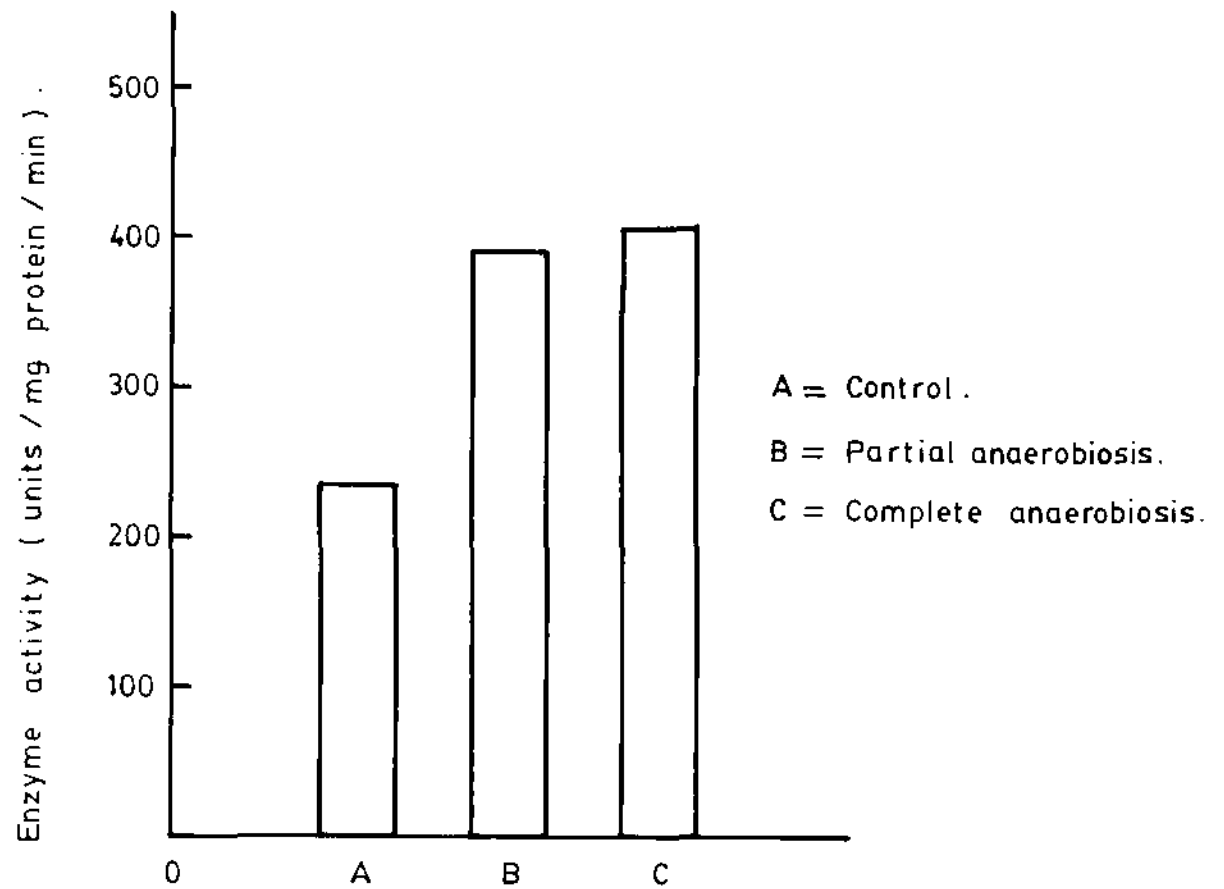


Fig. 13. Effect of aerobic and anaerobic condition on ADH activity of 48 hr. germinated winged bean seeds.

two different conditions, but the increase was most rapid in complete anaerobically grown seedlings and least rapid in aerobically grown seedlings. Enzyme activities in the seedlings grown under complete and partial anaerobic conditions were 75.4 and 65.5% higher respectively than that in the aerobically grown seedlings. Similar results were obtained for various kinds of seeds (Leblova 1975, Leblova et al. 1976). Complete or partial anaerobioses caused differential increases only in the intensities of isoenzyme bands, but no new bands appeared. Maximum increase in band intensities occurred in seeds germinated under complete anaerobic condition. These results suggest that perhaps activation of the pre-existing isoenzyme molecules took place instead of de novo synthesis. Nielsen and Scandalios (1971) reported that increase in ADH activity in anaerobically grown maize was due to activation of pre-existing ADH molecules while Lai and Scandalios (1977) reported that de novo synthesis of ADH molecule caused increase in ADH activity in maize. To the contrary, Jaaska (1976) found that anaerobiosis caused synthesis of several faster migrating ADH isoenzymes in 1-day-old germinating rye and wheat. The mechanisms of increase in ADH activity under anaerobic conditions may be similar to those discussed earlier in section 3.2.6 of results and discussions.

3.2.9 Effects of different salt solutions on winged bean ADH and its isoenzymes

Sets of twenty winged bean seeds were soaked separately in 20 mM salt solutions (50 ml in 100 ml beaker) for 24 hour and then germinated for 24 hours in the dark thermostat at 28-30°C

in Petri-dishes on filter papers moistened with the respective salt solutions. The control sample was soaked and germinated in presence of distilled water keeping the other conditions identical. Extraction, assay and electrophoretic procedures were the same as described in section 2.4.1 of Materials and Methods.

It can be seen from Table 14 that in presence of all the salt solutions and water ADH activity increased differentially in germinating winged bean seedlings. However, enzyme activities were 15.6 -- 24.3% higher for the seeds germinated in presence of different salt solutions than for the control seeds. The intensity of each isoenzyme increased differentially in presence of different salt solutions and water but no new isoenzyme band appeared. It was also noted that winged bean seeds showed poor signs of germination in presence of the salt solutions indicating most probably toxic effects of the salts at the concentration (20 mM) used.

The cause of more increase in ADH activity in seeds grown under different salt solutions may be due to activation of pre-existing enzyme molecules by different ions. Alternatively, lower supply of oxygen in the soaking water and the toxic effect of salts may be responsible for the increase in ADH activity. Arredondo-Vega and Arredondo-Vega (1986) reported that less saline environment caused decrease in ADH activity in germinating sunflower whereas more saline environment caused ADH activity to persist or increase, but without signs of germination. On the other ^{hand}, Jaaska (1976) observed

TABLE 14. EFFECT OF DIFFERENT SALT MEDIA ON ADH ACTIVITY OF
48 HOURS GERMINATED SEEDS OF WINGED BEAN

Name of the salts	Concentration mM	Specific activity units/mg prot/min
Control (H_2O)		352.18
NH_4Cl	20	437.68
KH_2PO_4	20	432.58
Na_2HPO_4	20	431.70
$MgSO_4$	20	415.46
$Ca(NO_3)_2$	20	407.12

Values are means of triplicate experiments.

higher ADH activity and induction of several faster migrating ADH isoenzymes in 1-day-old germinating rye and wheat as a response to partial anaerobiosis by immersion for 24 hours in 20 mM solutions of NH_4Cl , $\text{Ca}(\text{NO}_3)_2$ and Na_2HPO_4 .

Since poor signs of germination and higher ADH activities were observed in winged bean germinated in presence of 20 mM salt solutions it is suggested that this salt concentration (20 mM) appeared toxic to the germinating seed. However, more intensive investigations are needed using different concentrations of salts for understanding their mode of influence on germination and enzyme activities of different legumes.

3.2.10 Effect of light and dark on winged bean ADH activity and its isoenzymes

Winged bean seeds were germinated under two different conditions: (a) One set of seeds (50) was first soaked for 4 hours in distilled water and then germinated for 0-8 days on distilled water-moistened filter paper in Petri-dish under normal day light (day length 13 hours) and dark (night length 11 hours) at ambient temperature (b) Another set of seeds (50) was germinated in complete darkness for 0-8 days keeping the other conditions identical with the above experiment. Five seeds were collected from each of the two sets of experiments of alternate days (0, 2, 4, 6 and 8) and ADH was extracted, assayed and electrophoretically separated according to the methods described in section 2.4.1 of materials and methods.

The specific ADH activity increased in dark- and light-grown seedlings of winged bean and reached peaks at 48 hour of germination Fig. 14. However, at peaks the activity of ADH was 50% higher

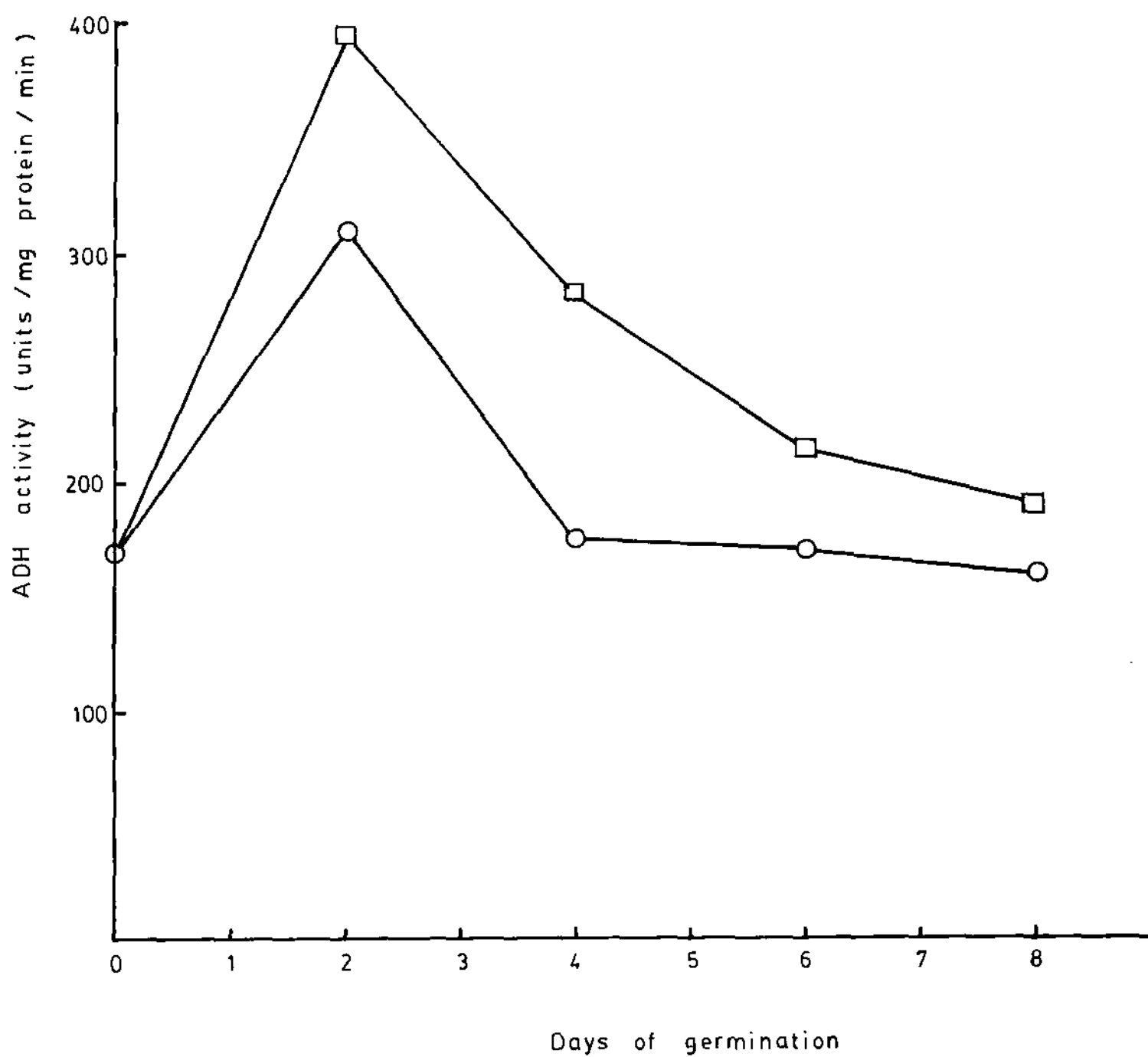


Fig. 14. Effect of light and dark on alcohol dehydrogenase activity of germinated winged bean

○—○—○ = Light grown, □—□—□ = Dark grown.

in dark-grown seedlings than in light-grown seedlings. Between days 2 and 4 of germination the enzyme activity declined rapidly in light-grown seedlings, followed by very slow decline while in dark-grown seedlings ADH activity declined rapidly between days 2 and 6, followed by gradual decline. At day 8 the activity decreased by about 51.9 and 49% of the peak activity in dark- and light-grown seedlings respectively. Light and dark did not show any significant effect on the ADH isoenzyme patterns of winged bean. Only the amounts of the three isoenzymes in dark-grown seedlings increased slightly more than those of the isoenzymes in light-grown seedlings but they all peaked together at day 2. After day 2, the amount of each individual isoenzyme declined. Less increase in ADH activity in light-grown seedlings may be due to the appearance of chlorophyll which might have inhibitory effect on ADH activity. These results disagree with the results of Leblova et al. (1971) who found higher ADH activity in light-grown plants than in dark-grown plants. The drastic inhibitory effect of light on the cotyledons of cucumber was demonstrated by the rapid decline of MDH activity and disappearance of some isoenzymes (Liu and Huang 1976).

3.3 Malate dehydrogenase (MDH) activities and isoenzymes in the cotyledons of germinating winged bean and kalomotor

The changes in the activities of MDH and its isoenzymes during different stages (0-15 days) of germination of winged bean and kalomotor were investigated. Germination of seeds, extraction, assay and electrophoresis of MDH were done according to the procedures described in sections 2.3.1, 2.4.1 and 2.7 of Materials and Methods.

MDH activities, which were observed in good amounts in dry seeds of both the species, showed similar pattern of changes during germination (Fig. 15). Enzyme activities increased rapidly at the beginning of germination and reached peaks on the 2nd day of germination of both the species. At the peaks, the activities were 250.0 and 110.0 % higher in winged bean and kalomotor respectively than those of the dry seeds indicating that the increase was more pronounced in winged bean. After day 2, MDH activities declined gradually and fell about 46.4 and 23.8 % of the peak activities in winged bean and kalomotor respectively after 15 days of germination. At day 15 enzyme activities of both winged bean and kalomotor remained at much higher levels than those of the dry seeds. Similar to the present findings, MDH activities in maize (Longo and Scandalios 1969, Yang and Scandalios 1974), cucumber (Trelease et al. 1971, Liu and Huang 1976), and watermelon (Hock 1972, 1973; Kagawa and Beevers 1975) were found to rise to the peaks between days 1 and 3 of germination followed by gradual decline.

Polyacrylamide gel electrophoresis of the cotyledon extracts from winged bean and kalomotor seedlings, grown in darkness for

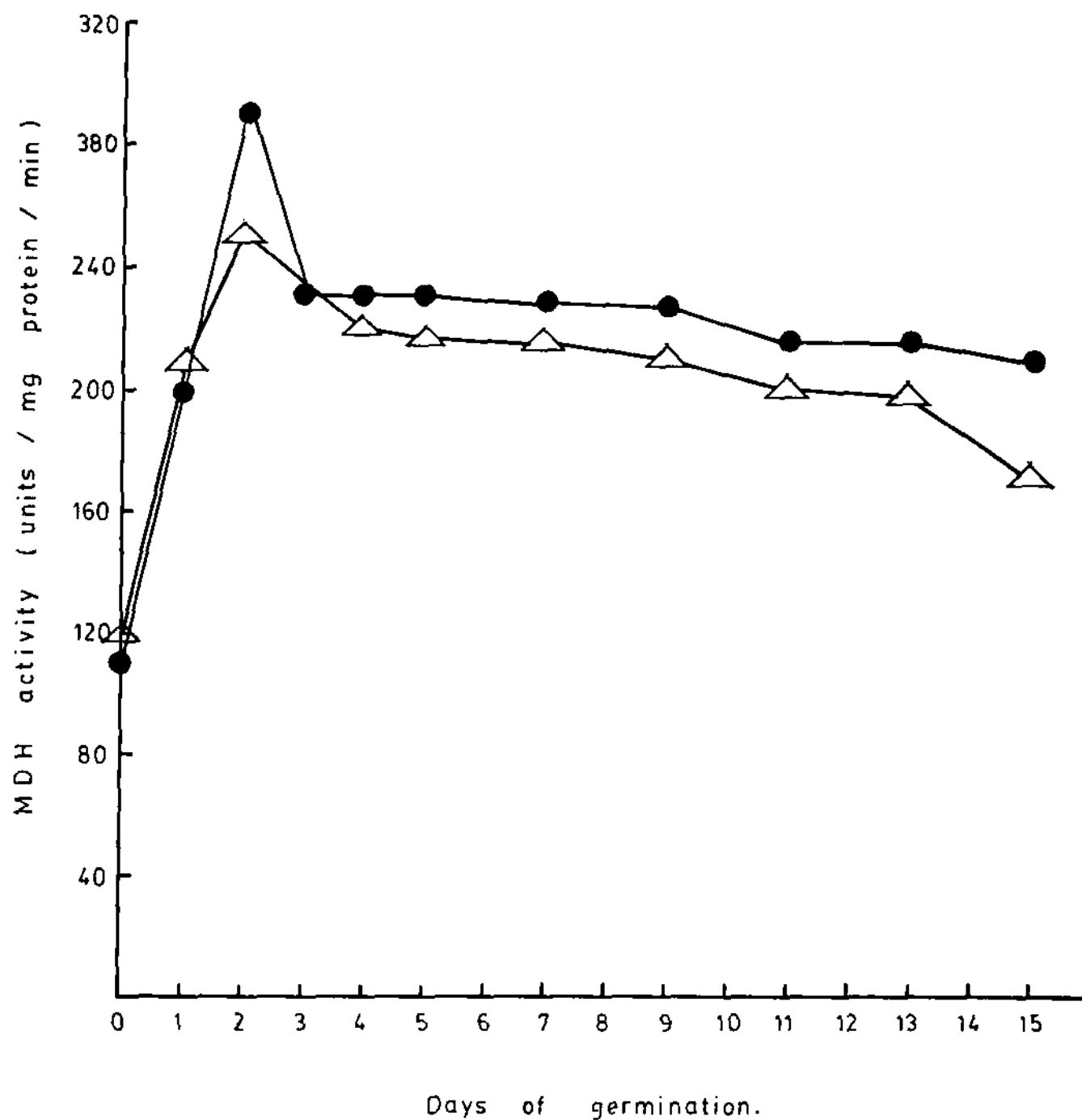


Fig. 15. Changes of malate dehydrogenase activity in winged bean and kalomotor during germination from 0-15 days.

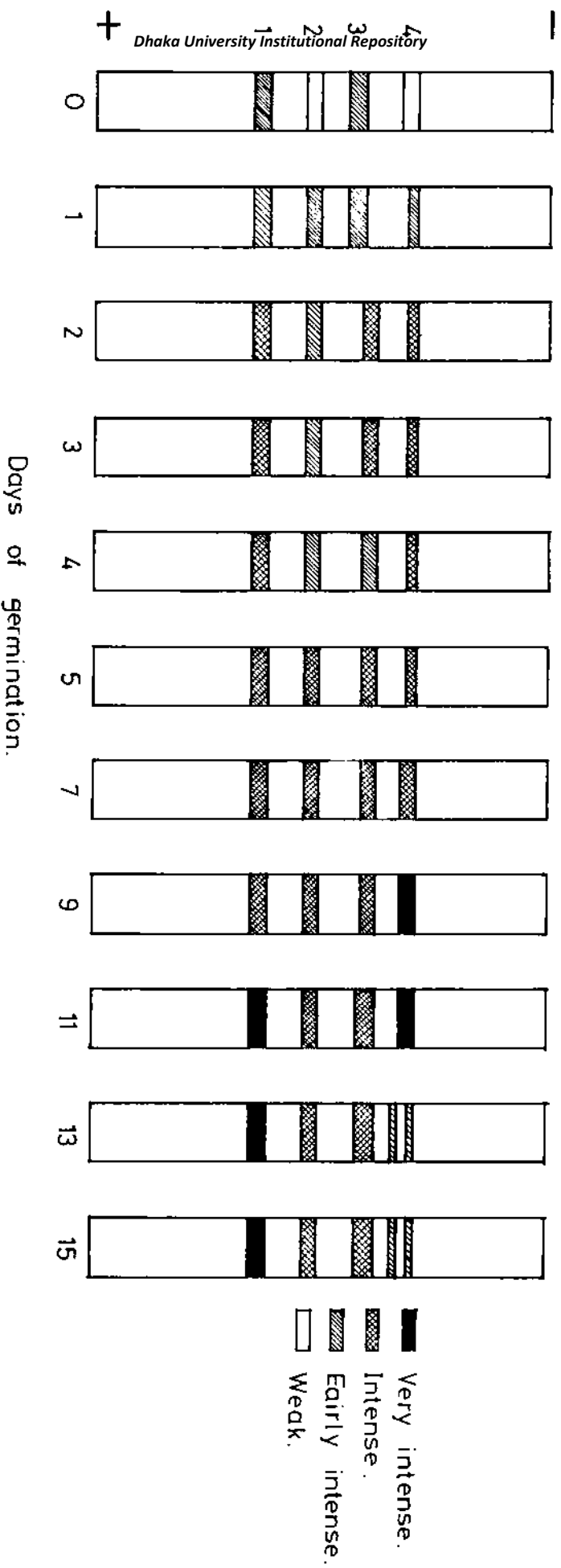
△-△-△ = Kalomotor, ●-●-● = Winged bean.

0-15 days, was performed for detecting multiple molecular forms of MDH and for comparison of the development of each individual isoenzyme during germination.

MDH activities in each of the extracts of winged bean and kalomator of different ages were resolved into four isoenzymes (Figs. 16 and 17) which differed in intensities and mobilities within and between the species.

In dry seed extracts of winged bean MDH isoenzymes 1 and 3 appeared most active and fairly intense while isoenzymes 2 and 4 appeared weak. As germination proceeded, the amount of isoenzyme 1 increased significantly and appeared very intense and broader at day 11 where from its activity remained fairly constant. The activities of isoenzymes 2 and 3 showed increase upto day 2 and there after remained some what unchanged except the fact that isoenzyme 3 became narrower at the later stage of germination. With the onset of germination the amount of isoenzyme 4 increased slightly and at day 11 it dissociated into two narrow bands.

In dry cotyledon extracts of kalomator isoenzyme 1 appeared intense while isoenzymes 2,3 and 4 appeared fairly intense. With the onset of germination the activity of isoenzyme 1 gradually decreased and it became very weak at day 15. On the other hand, the amounts of isoenzyme 2 and 4 increased considerably and they appeared as very intense and broad bands between days 2 and 15. Isoenzyme 3 showed little change.



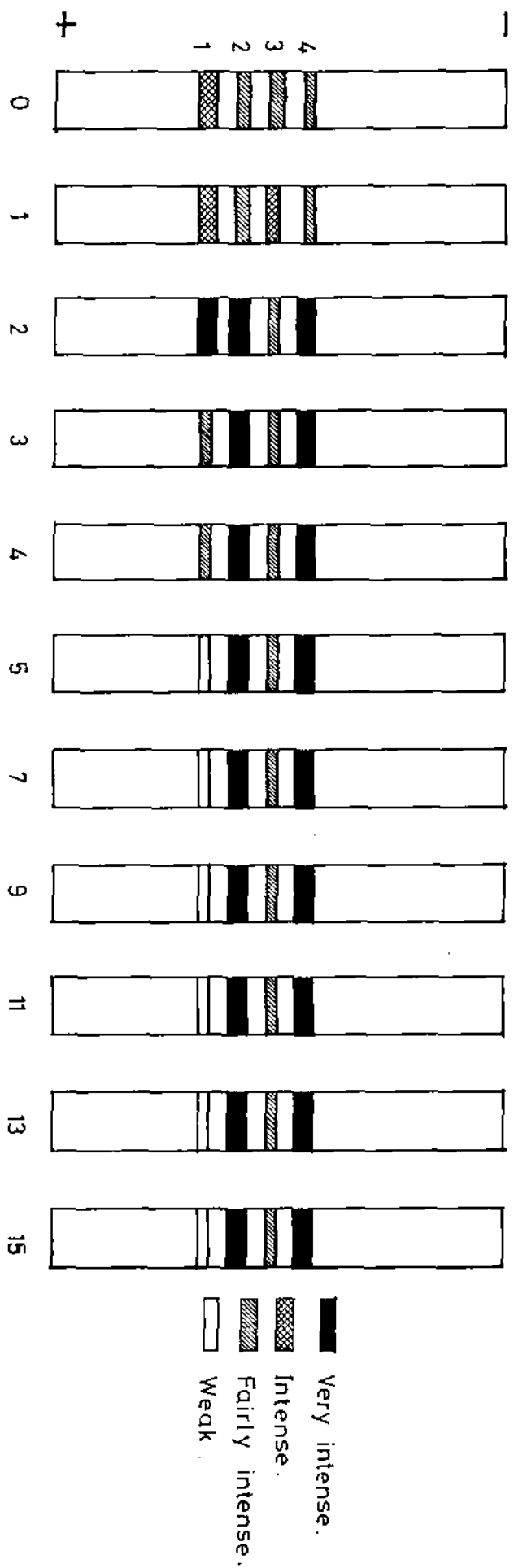


Fig. 17. Schematic representation of malate dehydrogenase zymogram of germinating kalomator seeds.

The fluctuations observed in the activities of MDH isoenzymes in winged bean and kalomator appeared to corroborate with the fluctuations of their respective total MDH activities at different stages of germination. It can be concluded that the number of MDH isoenzymes in both the species remained constant throughout the whole period of germination, but the activities of different isoenzymes increased or decreased differentially at different stages of germination. The constancy in the number of MDH isoenzymes but changes in their intensities at various stages of germination have been reported for maize (Longo and Scandalios 1969, Yang and Scandalios 1974), cucumber (Trelease et al. 1971, Liu and Huang 1976) and Watermelon (Kagawa and Beevers 1975).

Although it is well established that MDH isoenzymes in plants are associated with different subcellular fractions e.g. cytoplasm, mitochondria, microbody and chloroplast (Weimberg 1968, Mukerji and Ting 1969, Rocha and Ting 1970, 1971, Ting et al. 1975, Yang 1975, Kagawa and Beevers 1975, Liu and Huang 1976) assumptions have been made regarding the association of MDH isoenzymes with different subcellular fractions in winged bean and kalomator. On the basis of electrophoretic mobilities and activity changes of MDH isoenzymes it is suggested that isoenzymes 1 and 2 in winged bean and kalomator may be cytoplasmic, isoenzymes 3 may be mitochondrial and isoenzyme 4 of microbody origin. Previous reports showed that cytoplasmic MDH isoenzymes in different tissues generally appear intensely stained and migrate furthest towards the anode (Weimberg 1968, Mukerji and

Ting 1969, Rocha and Ting 1970, 1971, Ting et al. 1975, Yang 1975, Liu and Huang 1976). Bands, slightly slower than cytoplasmic MDH and less intense, were identified as mitochondrial forms while the slowest and fairly active isoenzymes were shown to correspond to microbody forms (Weimberg 1968, Mukerji and Ting 1969, Rocha and Ting 1970, 1971; Ting et al. 1975, Yang 1975, Liu and Huang 1976). Furthermore, it has been reported that microbody MDH is synthesized in large quantities after the onset of germination (Leskovac et al. 1975). The increase in the quantity of MDH 4 in both winged bean and kalomator suggest that it may be of microbody origin.

The initial rises in the specific activities of MDHs and in the activities of most of the MDH isoenzymes in both winged bean and kalomator may be mainly due to in vivo synthesis of the enzymes during the initial stage of germination. The gradual fall in the specific activities and the amounts of some of the MDH isoenzymes after 2nd day of germination may be attributed to faster degradation of enzymes than their synthesis. Using density labelling experiments, Yang and Scandalios (1974) showed that accumulation of MDH isoenzymes is probably controlled by in vivo synthesis as well as degradation instead of activation and inactivation. In vivo synthesis and degradation of different MDH isoenzymes would again be under genetic control. Thus, the occurrence of MDH isoenzymes in different amounts in dry winged bean and kalomator seeds and different rates of appearance or disappearance of these isoenzymes during germination suggest that they may be under different control mechanisms of gene expressions. Moreover, dispersal of MDH isoenzymes in several cell compartments may be responsible for the differential appearance or disappearance

pearance of these isoenzymes. Such view has been put forward by Leskovac et al. (1975) and Scandalios (1974). Differences in the expression of MDH isoenzymes between the two plant species (winged bean and kalomator) indicate that each plant synthesizes a complement of MDH isoenzyme which is distinctive for a particular plant as suggested by Weimberg (1968). On the other hand, similar MDH isoenzyme patterns for the related plant genera e.g. Vicia and Psophocarpus indicate that MDH isoenzymes act as poor genetical marker as reported by Ganapathy and Scandalios (1973) and Nelson and Burr (1973) for different related plant species.

Since all the four isoenzymes were present in different amounts in dry and germinating winged bean and kalomator seeds, it is suggested that they all participate in the basic cellular metabolism in different manners at different sites of the cell. The gradual disappearance of isoenzyme 1 (presumed to be cytosolic) with the progress of germination of kalomator suggest that it perhaps functions only in the storage cotyledons, and not in greening cotyledons, in food mobilization during germination. Such a view was put forward for the two disappearing cytosolic MDH isoenzymes in cucumber by Liu and Huang (1976). But this view is not supported by the time course of winged bean MDH-1 which showed increase during germination.

MDH isoenzymes associated with cytoplasm, mitochondria and microbody are specific for NAD (Rocha and Ting 1971, Ting et al. 1975) while chloroplastic MDH is specific for NADP (Rocha and Ting 1970). The distribution of MDH isoenzymes related to malate oxidation has two important sequences in the major cell compartments:

(a) reducing equivalents can be moved from one cellular compartment to another by a variety of shuttle mechanism involving malate and oxaloacetic acid; (b) by reducing appropriate NAD or NADP specific MDH isoenzymes NADH and NADPH can be easily interconverted thus amounting to a transdehydrogenase reaction (Lance and Rustin 1984). Shuttle mechanisms are used for the oxidation of reduced equivalents in cytosolic fatty acid α -oxidation (Wu and Laties 1983), in glyoxysomal fatty acid β -oxidation (Mettler and Beevers 1980), in the reduction of nitrate (Woo et al. 1980), etc. In these exchanges of reducing equivalents malate plays a prominent role. However, plant mitochondria do not need a shuttle mechanism for the oxidation of cytosolic NADH or NADPH since they possess appropriate NADH and NADPH dehydrogenases (e.g. malate dehydrogenase, malic enzyme) on the outer face of the inner membrane (Palmer and Moller 1982). Such shuttle mechanism was shown to involve in the export of reduced equivalents out of the mitochondria (Journet et al. 1981). Moreover, plant mitochondria have a very efficient transport system for oxaloacetic acid which readily moves in and out of the mitochondria, thereby regulating the activity of MDH and of all other NAD-linked dehydrogenases (Day and Wiskich 1984).

Recently it has been proposed that in plant cells 2 moles of malate, instead of pyruvate, could be the major terminal product of glycolysis by appropriate combination of enzyme action including cytosolic MDH and this malate could be the primary substrate for the TCA cycle (ap Rees et al. 1983, Lance and Rustin 1984). By the combine action of mitochondrial MDH and malic enzyme 2 moles of malate can generate one mole of citrate while releasing 2CO_2 and

6 reducing equivalents. Furthermore, the high probability of concurrent participation of the "pyruvate pathway" and the "malate pathway" in the metabolism of most plant tissues has been stressed (Lance and Rustin 1984). It has also been shown that like lactate or ethanol, malate (malate fermentation) can be a regular end product of the anaerobic carbohydrate breakdown in plant tissues (Davies 1978).

Lance and Rustin (1984) concluded that plant mitochondria do not oxidize fatty acids. In fat-storing seeds, fatty acids are metabolized in glyoxysomes (Beevers 1979). During germination, fatty acids are quantitatively converted into carbohydrates by the process of gluconeogenesis which requires the contribution of glyoxysomes, mitochondria and cytosol. In non-fatty acid plant tissues, fatty acids are broken down through β -oxidation in the peroxisomes (Macey and Stumpf 1982, Gerhardt 1983) leading to the production of acetyl-CoA which is perhaps reoxidized in the mitochondria using cytosolic transport system (Lance and Rustin 1984).

Since mitochondria are endowed with two malate oxidizing systems (malate dehydrogenase and malic enzyme) they produce their own pyruvate and initiate the TCA cycle reactions from malate only (Palmer 1976, 1984). The functioning of these enzymes is tightly controlled by the size of the endogenous pool of NAD (Neuburger et al. 1984), the addition of exogenous NAD (Palmer and Arron 1976), the internal p^H (Macrae 1971) and the inhibition of cytochrome oxidase by cyanide (Rustin et al. 1980).

3.4 Changes in esterase activities and their isoenzyme patterns in winged bean and kalomator during germination.

Since esterases mediate the hydrolysis of ester linkages and mobilization of fatty acids, it was of interest to determine the changes in the total activities of esterases and their isoenzymes during germination of winged bean and kalomator. For these, the seeds were germinated for 0-15 days in the dark and esterases were extracted from the seedlings of different ages with Tris-HCl buffer (pH 7.5) containing DTT (0.00IM), EDTA (0.00IM), KCl (0.0IM) and $MgCl_2$ (0.00IM). Esterase activities were assayed in Veronal buffer (pH 7.5) according to the method described in section 2.4.2 of Materials and Methods. Since the fresh weight of the seedlings changed considerably during germination, esterase activities have been expressed per mg of protein which was determined in each extract. The same enzyme extracts were used for electrophoretic analysis of esterases.

Fig. 18 shows the changes in specific activities of esterase in winged bean and kalomator during germination. The changes could be divided into two phases i.e. between 0 and 5th days and between 6th and 15th days. Esterase activities were low in resting seeds of both the species. After the initiation of germination esterase activities increased and exhibited 6- and 3.2 - fold increases in activities in winged bean and kalomator respectively at day 4. The activities declined slightly in both the species at day 5. Again the enzyme activities began to increase from the 6th day and showed 4-fold increase of the initial activity in kalomator at day 11 and 24.4-fold increase in winged bean at day 13. At day 15, enzyme activities dropped by

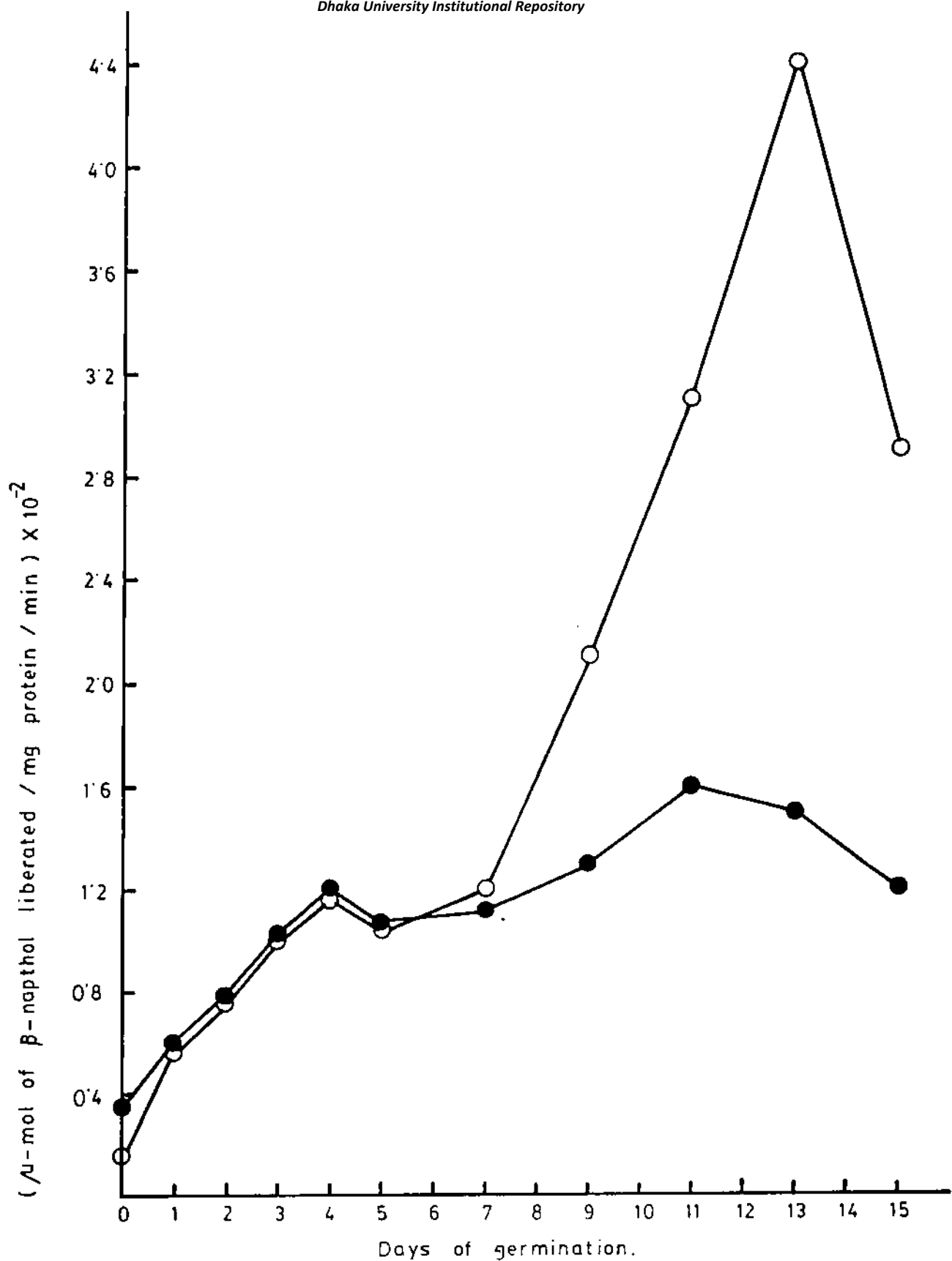


Fig. 18. Time courses of non-specific esterase activities in germinating winged bean and kalomator cotyledons

○—○—○ = Winged bean. — — = Kalomator.

about 33.5 and 30.7% of the peak activities in winged bean and kalomator respectively. Fluctuations in esterolytic activities were observed in germinating rice (Palmiano and Juliano 1973), Aegilops (Karataglis 1975), blackgram and lentil (Gomes 1978), oat (Xu and Chen 1985), maize (Everett et al. 1985) and pearl millet (Chanda et al. 1986).

The esterase isoenzyme patterns in dry winged bean and kalomator seeds and their changes during germination are shown schematically in Figs. 19 and 20 respectively. Esterase bands in winged bean could be divided into three groups depending on their mobilities on the gels: (a) area of high mobility (band 1), (b) area of intermediate mobility (bands 2-4) and (c) area of low mobility (bands 5-10). Dry winged bean seeds contained 8 esterase bands of which bands 1 and 4-7 appeared fairly intense; bands 2 and 3 appeared weak and band 8 appeared very weak. With the onset of germination esterase band 1 in the fast moving area showed gradual decrease in intensity and by day 15 it became very weak. Esterase bands in the intermediate and fast moving areas showed changes both in numbers and intensities during germination. Bands 2 and 5-7 disappeared with the onset of germination while two new bands (9 and 10) appeared and became most active at day 13. Bands 3 and 4 became very weak and disappeared by day 5 whereas band 8 became very intense by day 11.

On the other hand, only two esterase bands of intermediate mobilities could be detected in kalomator cotyledons (Fig. 20). These bands stained as dark reddish areas of nearly same intensities

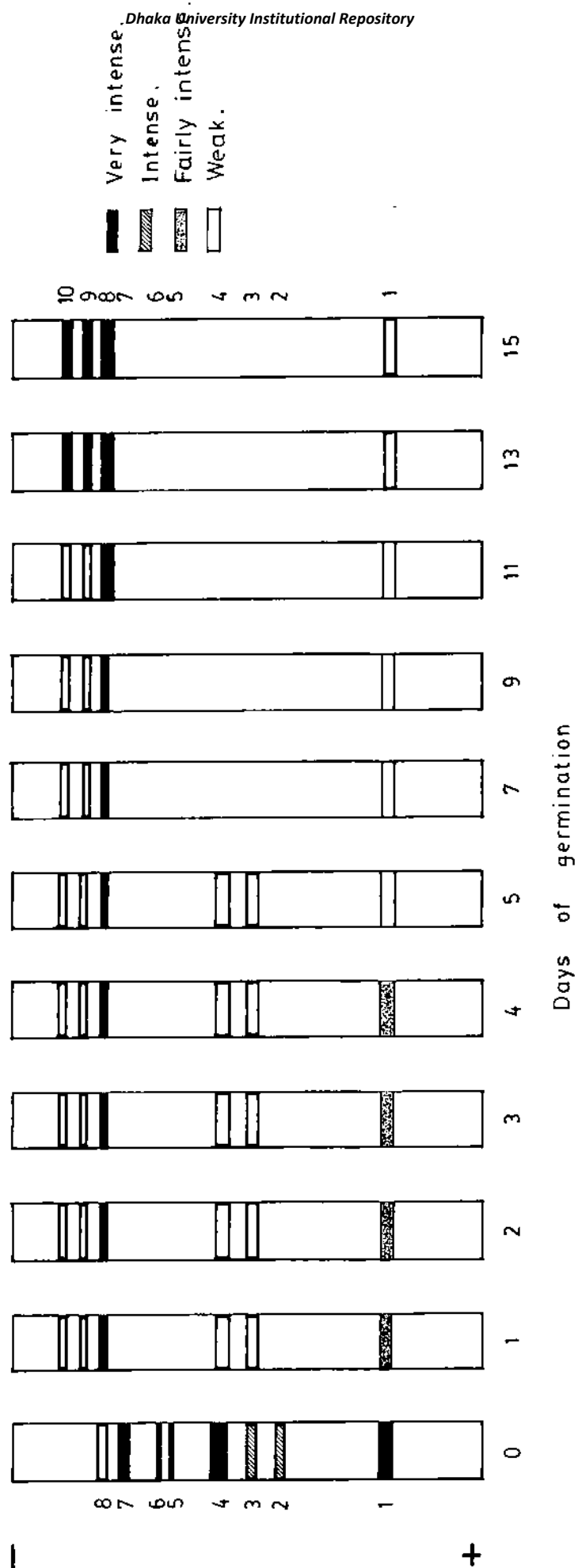


Fig. 19. Schematic representation of non-specific esterase zymogram of germinating winged bean seeds.

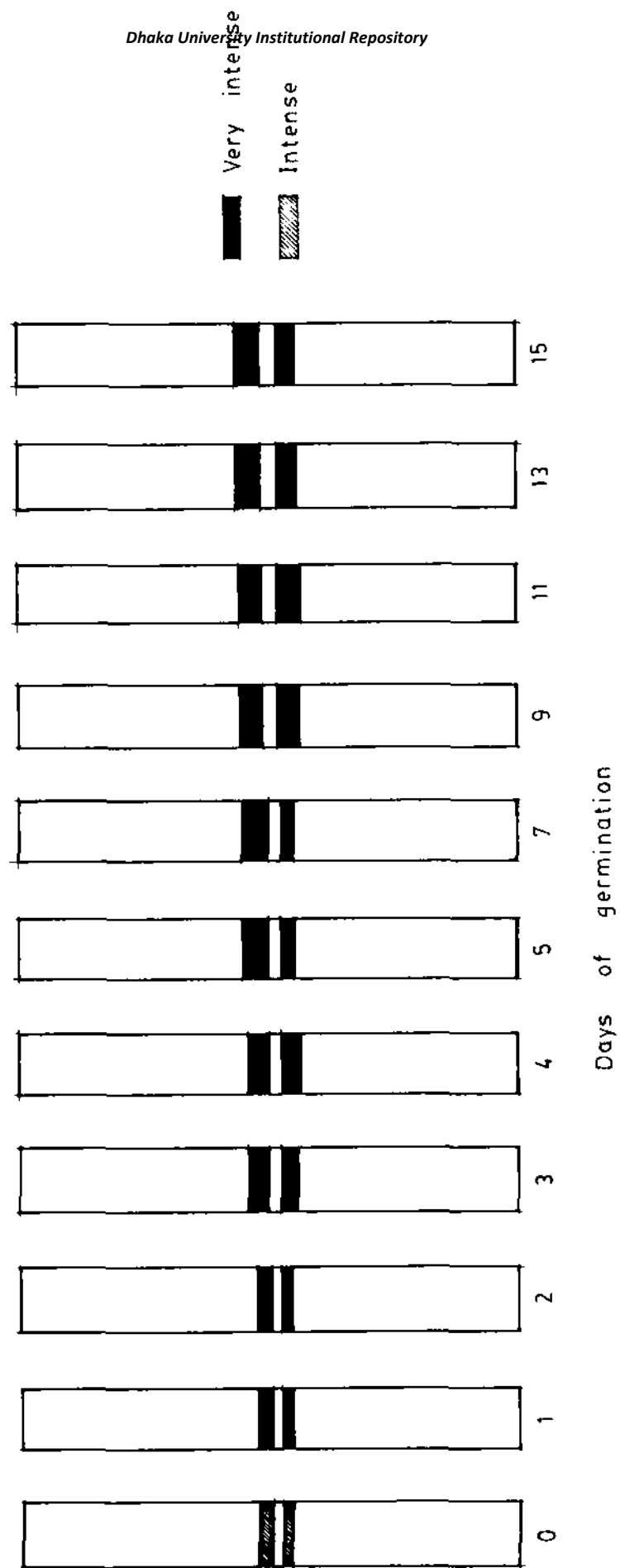


Fig. 20. Schematic representation of non-specific esterase zymogram of germinating katomator seeds.

and mobilities. Upto day 2 of germination activities of both the bands increased but their width remained unchanged. At days 3 and 4 they became slightly broader and between day 5 and 7 band 1 again became narrower. Maximum intensity was attained by these bands between days 9 and 11 after which they became slightly narrower keeping the band intensity same. Changes in esterase patterns and activities, and their relationship with development of plants have been shown in onion seedlings (Makinen 1968), Aegilops (Karataglis 1975), black gram and lentil (Gomes 1978), oat (Xu and Chen 1985), maize (Chauhan et al. 1985).

The presence of low esterase activities in dormant seeds and increases by 4 to 24.4-fold in the activities after the germination between 11 and 13 days would support the view that esterases are induced during imbibition and germination. Induction by various factors may require in vivo synthesis of new enzyme molecules or activation of the pre-existing ones leading to the increase in esterase activities (Mayer and Shain 1974). Other factors such as inactivation or removal of enzyme inhibitors, limited proteolysis of the inactive enzyme molecules to active forms and influence of plant hormones or growth substances may play important roles in the increase in esterase activities (Majumder and Biswas 1973, Mayer and Shain 1974). Mapelli et al. (1984) found that and gibberellic acid increases the esterase activity and abscisic acid maintained the activity at control level in barley seeds.

Gomes and co-workers (1982, 1984, 1985) found decreases in dry weight of the cotyledons and lipid contents in winged bean and

kalomotor with the progress of germination, while axis weight and free fatty acids were found to increase. Thus, decrease in lipid contents and dry cotyledon weights and increase in free fatty acid and axis weight could be related to the increase in esterase activity in germinating winged bean cotyledons between day 6 and 13 and greater number of esterase isoenzymes in winged bean might be related to the higher content of lipid (20%) which perhaps requires greater activities and various kinds of esterases and lipases for being hydrolyzed effectively and mobilized to the growing axis during germination. To the contrary, less lipid content (2%) in kalomotor would require less esterase activity of isoenzymes for being hydrolyzed and metabolized.

Variations in the number and intensity of esterase bands in winged bean at different stages of germination could be due to differential gene expressions, transcription, translation, activation, degradation, partial proteolysis, inhibition accumulation and organelle localization of esterases (Scandalios 1974, Mayer and Shain 1974). The consistent presence of few esterase bands at all stages of germination, appearance of some forms with the onset of germination and disappearance of some forms at later stages of germination suggest that these isoenzymes are controlled by multiple genes or different alleles of a locus which are activated or expressed at one stage or deactivated at another stage of germination. To the contrary, the constancy in the number but variation in the intensity of esterase bands in kalomotor may be attributed to the fact that the responsible gene is at different stages of activation (Mayer and Shain 1974,

Scandalios 1974). Furthermore, it was observed that when activities of the fast or intermediate moving bands in winged bean were decreasing, the activities of the slow moving bands were increasing (Fig 19). These led to suggest that the factor which activate^s the loci controlling the slow moving isoenzyme, most probably act^s as an inactivator of the gene loci controlling the disappearing bands of fast moving and intermediate moving groups.

Great difference between the esterase isoenzyme patterns (number, mobility and intensity) of winged bean and kalomator suggest the existence of distant phylogenetic relationship between these two species and further show that esterase isoenzyme patterns may serve as good genetic markers as reported by others (Upadhya et al. 1985, Cooke 1985).

3.5 Changes in proteolytic activities in winged bean and kalomotor during different stages of germination

Proteolytic enzymes in germinating seeds of winged bean and kalomotor were extracted in McIlvaine buffer (pH 5.5) and in Tris-HCl buffer (pH 7.6) and assayed as described in section 2.3.3 and 2.3.4 of chapter 2 with a view to correlating their roles in the mobilization of protein reserves during germination of the cotyledons.

Protease activities, towards haemoglobin and the synthetic substrate BAPNA, of winged bean and kalomotor extracts were measured at various stages during germination (Fig. 21). Trypsin like activities (TLA) as measured towards BAPNA, were quite high in resting cotyledons of winged bean and kalomotor, increased slightly during the first day of germination and then declined rapidly. However, the decay rate of TLA in kalomotor was much slower than that in winged bean. These observations agree with the results of Mikkonen (1986) and Crump and Murray (1979) who reported high protease activity towards BAPNA in dry kidney bean and decrease in this activity during germination, but disagree with the results of Nielsen and Liener (1984) who found increase and Pusztai and Duncan (1971) who found little change in TLA activity in germinating kidney bean.

In contrast, protease activities towards haemoglobin (haemoglobinase, acid protease-type) were relatively low in resting seeds of winged bean and kalomotor but increased steadily with the onset of germination, reached peaks at days 11-13 and then declined

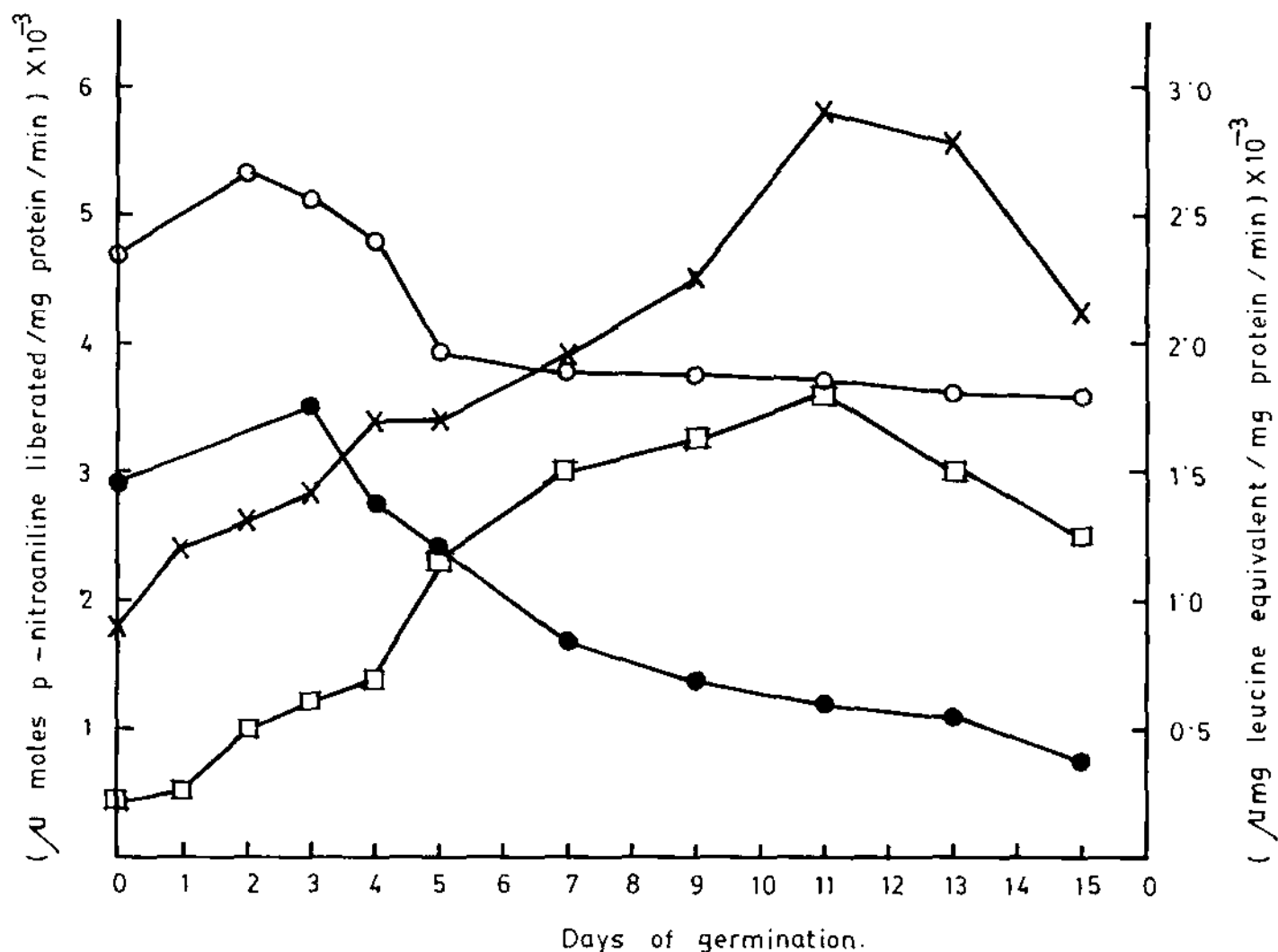


Fig. 21. Proteolytic activity changes during different stages of germination of winged bean and kalomator cotyledons.

- kalomator with BAPNA.
- winged bean with BAPNA.
- X—X kalomator with Haemoglobin.
- winged bean with Haemoglobin.

gradually. These results agree with the results of others (Mikkonen 1986, Alvarez and Guerra 1985, Nielsen and Liener 1984, Feller 1979, Basha and Cherry 1978, Tully and Beevers 1978, Yomo and Srinivasan 1973) who reported that protease activities of seed (e.g. kidney bean, castor bean, wheat, lentil) extracts on casein or azocasein or haemoglobin were low at day 0, increased steadily during germination, reached peaks between days 4 and 10 and then declined.

Changes in proteolytic activities in germinating winged bean and kalomator could be correlated with the changes in protein nitrogen, total nitrogen, non-protein nitrogen and free amino acids (Gomes et al. 1982, 1984, 1985). They found that protein nitrogen in the cotyledons of winged bean increased slightly (2%) up to day 3 and then declined gradually by about 18.4 % at day 15 whereas total nitrogen increased by about 14.6 % up to day 6 and then declined gradually. To the contrary, protein and total nitrogen contents were found to decline continuously in kalomator after the onset of germination (Gomes et al. 1984). Non-protein nitrogen and free amino acids in winged bean were found to increase by 3- and 5-fold respectively between days 9 and 12 of germination (Gomes et al. 1982, 1985). Rate of increase in non-protein nitrogen and free amino acids was rather slow in kalomator and peaks were attained between day 6 and 9 (Gomes et al. 1984). Since protein nitrogen increased slightly up to day 3 with concomitant slow increase in non-protein nitrogen, free amino acids and protease activities in winged bean, it is suggested that in vivo synthesis of protein took place at a faster rate than proteolytic breakdown of the reserve protein materials up to day 3. The gradual decrease in protein nitrogen after day 3 and rapid

increase in non-protein nitrogen, free amino acids and protease (haemoglobinase) activities indicate faster proteolytic breakdown of protein than in vivo synthesis of protein. In kalomator the proteolytic breakdown of protein was faster than possible in vivo synthesis from the very beginning of germination resulting in continuous decrease in protein nitrogen and increases in non-protein nitrogen and free amino acids. As the free amino acids and non-protein nitrogen levels increased little after day 9 or 12 of germination of both the species (Gomes et al. 1984, 1985), it is suggested that perhaps amino acids were used at the same rate at which they were released by declining proteolytic activities. The degradation of reserve protein to small peptides and free amino acids during germination of winged bean and kalomator together with the increase in proteolytic activity is consistent with the reports of Mikkonen (1986), Alvarez and Guerra (1985), Nielsen and Liener (1984), Morohashi (1982), Basha and Cherry (1978), Kern and Chrispeels (1978) and Jacobsen et al. (1970) who found coincidence of increased proteolytic activity with the decreased protein nitrogen and various protein fractions and increases in free amino acids.

Since proteases in winged bean and kalomator showed different time course of development during germination and one (haemoglobinase) was active at pH 5.5 and the other (TLA) was active at pH 7.6, it is tentatively concluded that TLA and haemoglobinase were in fact two different enzymes. However, further studies are required to characterize these enzymes. Distinct proteases of these types were reported in kidney bean (Nielsen and Liener 1984, Mikkonen 1986, Pusztai and Duncan 1971) and castor bean (Tully and Beevers 1978). Further, it is

suggested that the breakdown of storage protein was initiated by carboxypeptidase and acid protease (e.g. haemoglobinase) which was low in resting seeds of winged bean and kalomator and reached peaks when the mobilization of nitrogen was highest (between days 9 and 12), and the remaining activities of alkaline peptidase, naphthylamidase (e.g. TLA) and acid protease (e.g. haemoglobinase) were enough for the completion of the proteolytic breakdown of protein. Similar suggestion was made by Mikkonen (1986) for the proteolytic enzymes in germinating kidney bean.

Several factors present in the seedlings of winged bean and kalomator may regulate protease formation (increase) and degradation (decrease). The presence of high TLA in the resting seeds of both the species and slight increase during the first day of germination may indicate activation of the enzyme rather than synthesis as suggested by Mayer and Shain (1974). To the contrary, low haemoglobinase (acid protease-type) activity in resting seeds and steady increase in activity of this enzyme with the progress of germination may represent an enhanced de novo synthesis and/or an activation of this enzyme. Nielsen and Liener (1984), Morohashi (1982), Klaus et al. (1985), Karakeeva et al. (1983), Yomo and Srinivasan (1973) and Varner (1965) reported de novo synthesis of proteases in germinating legumes and cereals. These authors concluded that continuous protein synthesis was necessary for the development of proteolytic activity.

The increased proteolytic activity of germinating winged bean and kalomator may also be due to breakdown or removal of stored inhi-

bitors of proteolytic enzymes following germination (Mayer and Shain 1974, Pusztai 1972). Shain and Mayer (1965) reported that the increased proteolytic activity was the result of gradual removal of inhibitor from the pre-formed and inactive enzyme-inhibitor complex during germination. On the other hand, Hobday et al. (1973) reported that legume cotyledons are rich sources of protease inhibitors, but these are thought to be localized outside the protein bodies and have never been shown to inhibit the endogenous protease or to be involved in the control of reserve protein.

Factors present in the embryonic axis of germinating seeds or mobilization of the breakdown products e.g. amino acids or small peptides to the growing axis may cause increase or decrease in the proteolytic activity. It has been reported that the presence of embryonic axis is required to promote the increase of protease activity in germinating leguminous seeds (Mitsuhashi et al. 1984, 1986; Morohashi 1982, Davies and Slack 1981, kern and Chrispeels 1978). On the other hand, protease formation in attached cotyledons of peas (Guardiola and Sutcliffe 1971, Yomo and Varner 1973) and Kidney beans (Yomo and Srinivasan 1973) was found to be inhibited. One possible explanation for these results was that accumulated free amino acids in beans and peas eventually inhibited protease activity.

Although the embryo is known to control hydrolases (protease and amylase) through hormones or growth regulators in cereals, no definite conclusion has been made on hormonal control of hydrolase development in legumes (Davies and Slack 1981). It is likely that hormones present in winged bean and kalomator have control over the protease.

Alternative mechanism of osmotic regulation of protease may be operative in winged bean and kalomator. Enough water supply to the attached cotyledons during imbibition and germination may enhance protease synthesis and water stress during later stage of germination may inhibit protease synthesis. Armstrong and Jones (1973) demonstrated that water stress inhibits hydrolase synthesis in germinating barley.

Another alternative mechanism is that accumulated end products of proteolytic enzymes in cotyledons may exert a feedback-like repressive action on the synthesis of the protease in winged bean and kalomator. Yomo and Varner (1973) suggested this possibility for the regulation of proteinase formation in pea cotyledons. On the other hand, Morohashi (1982) suggested that feedback-like repression was unlikely to be the mechanism by which hydrolase activity was regulated in black gram cotyledons. He found that decrease in end product contents did not always bring about a rise in hydroalase activities, nor was inhibition of hydrolase formation always correlated with an increase in end product contents.

Variable association between proteolytic enzymes and protein storage bodies, and autolysis of the protein bodies during imbibition and germination may lead to the increase in protease activity (Varner and Mense 1972). The location of proteinase enzymes within storing bodies has been reported for hemp, cotton, vetch, sunflower, lentil, etc. and in at least some of these species the protein bodies were found to assume autolytic role during imbibition, germination and protein reserve mobilization (Alvarez and Guerra

1985, Varner and Mense 1972). It was found that the mobilization of protein was accompanied by fusion of the protein bodies into a large central vacuole and the loss of protein was paralleled by an increase in the activity of an enzyme system in the protein bodies that hydrolyzed casein.

It appears from the above discussions that many contradictory reports are available regarding the development of protease activities, mobilization of reserve protein and control of protease activities. These contradictions may be due to genuine differences in the metabolism of the different seeds. Furthermore, differences in experimental techniques and the possibility of bacterial contamination may have also contributed to the lack of correlation among various reports as regards to the general role of proteolytic enzymes in the metabolism of germinating seeds.

Contradictions due to experimental techniques could be minimized to a minimum and a more general agreement between reports could be expected, if in addition to the measurement of proteolytic activities towards a number of small synthetic substrates, the true proteolytic or the autodigesting activity at or near physiological pH values were measured.

3.6 Changes of amylase activities in winged bean and kalomotor during different stages of germination

Amylase activities in germinating winged bean and kalomotor were extracted in water, containing 6mM CaCl_2 and assayed as described in section 2.4.4. of Materials and Methods with a view to comparing the changes in amylase activities with the changes in starch and soluble sugars during germination.

The changes of amylase activities in winged bean and kalomotor cotyledons during germination (0-15 days) are shown in Fig-22. In dry seed extracts of both the species amylase activities were low and almost similar. As germination proceeded the activity increased by 19 and 80% after 24 hour in winged bean and kalomotor respectively. The activities then gradually increased and reached peak at day 13 and 11 in winged bean and kalomotor respectively. The increases were 471 and 900% higher than those of dry seed extracts of winged bean and kalomotor respectively. The activities then gradually decreased. The decreases were 17 and 20% of the peak activities in winged bean and kalomotor. Similar results were obtained in peas (Yomo and Varner 1973, Monerri et al. 1986), where amylase activity reached peak between day 9-10.

Attempts have been made to correlate the amylase activity changes with the changes in starch and soluble sugars determined in kalomotor (Gomes et al. 1984) and winged bean (Gomes et al. 1985). It has been reported that soluble sugars decreased rapidly in winged bean after the onset of germination while starch content (0.5-0.9% in dry seed) increased (Gomes et al. 1985). On the other hand,

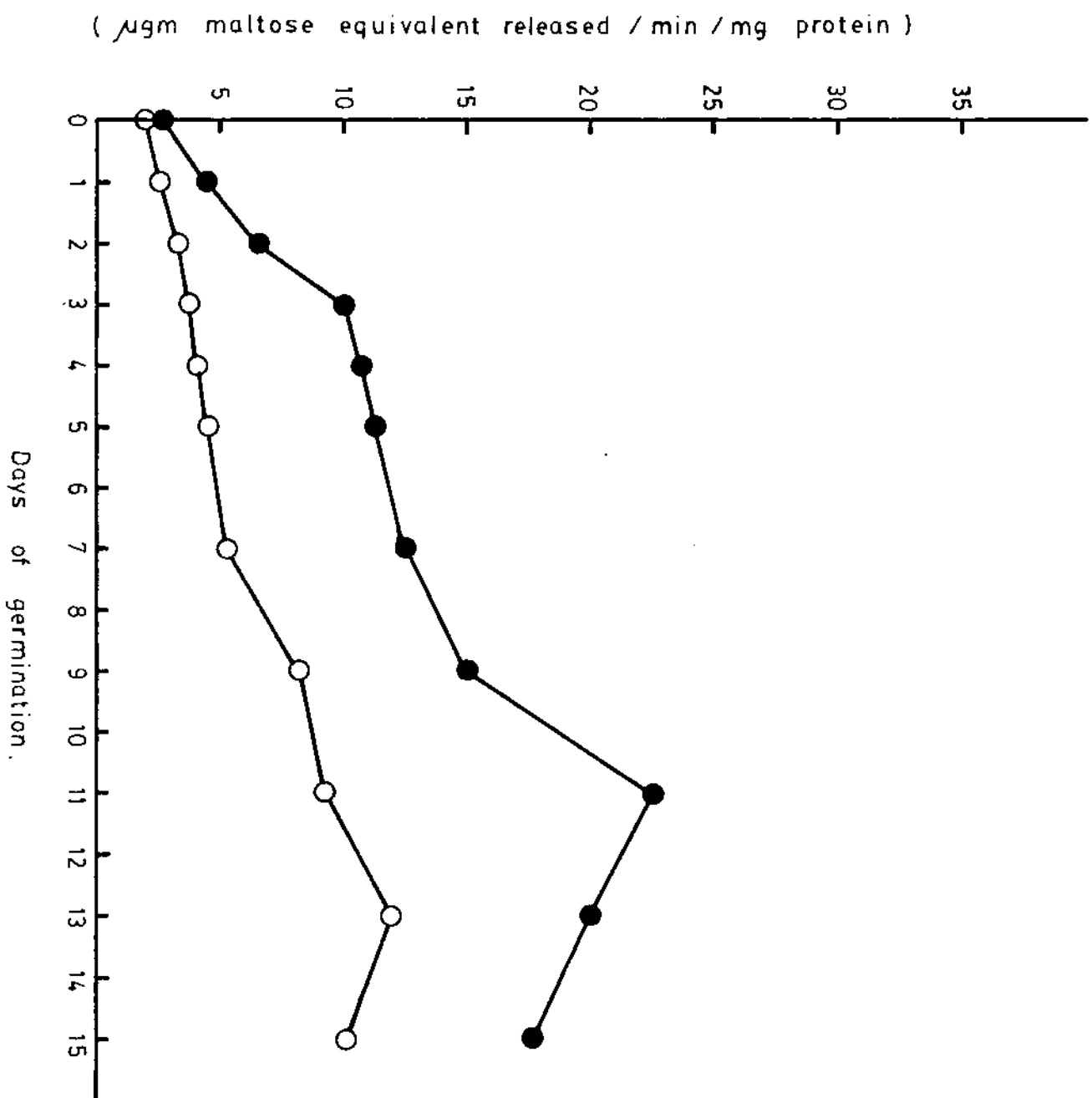


Fig. 22. Time courses of amylase activities in germinating winged bean and kalomator seeds.

soluble sugars increased in kalomator up to day 9 or 10 of germination with simultaneous decrease in starch content (35-38% in dry seed), (Gomes et al. 1985). The onset of starch hydrolysis and increase in soluble sugars in kalomator cotyledons coincided with the increase in amylase activity. Similar coincidence has been reported for Alaska pea (Monerri et al. 1986). On the other hand, the increase in amylase activity in winged bean cotyledons was associated with the decrease in soluble sugars content and increase in starch content during the progress of germination.

The rapid depletion of soluble sugars and increase in starch content along with increased amylase activity in winged bean may be attributed to the fact that resynthesis of starch was faster than its hydrolysis by amylase. Juliano and Varner (1969) have shown that starch synthesis occurred during rapid phase of starch degradation in peas. Alternatively, the rapid metabolism of soluble sugars by the germinating seedlings may result in the fast depletion of soluble sugars. The simultaneous increase in soluble sugars and amylase activity and decrease in starch content in kalomator may be due to faster breakdown of starch than its possible synthesis. It may also be that de novo synthesis of starch does not take place at all during germination of kalomator.

At the peaks amylase activity in kalomator cotyledons was 87.5% higher than in winged bean cotyledons. The faster increase in amylase activity, higher amount of amylase at the peak and continuous degradation of starch in the cotyledons of the kalomator may be due to higher amount of starch in this species or due to differences in the composition of the starch molecules.

Since amylase activities increased both during sugar depletion (in winged bean) and during sugar accumulation (in kalomator) it is suggested that amylase activity is independent of sugar accumulation. This suggestion is in agreement with Morohashi (1982, 1984) and Monerri et al. (1986). A regulation of the increase in amylase activity by the concentration of reducing sugars as suggested by Parys et al. (1983) seems unlikely from the present results.

Mobilization of breakdown products to the embryonic axis or occurrence of factors or chemical signals between the cotyledons and growing axis may regulate the development of amylase activities in winged bean and kalomator as suggested for amylase regulation in black gram (Morohashi 1982) and other legum^es (Davies and Slack 1981).

Endogenously produced hormones may influence for the slow activation or release of prepacked enzyme or de-novo synthesis of amylase. The role of gibberellic acid and other endogenously produced hormones in the formation and secretion of amylase has been most thoroughly studied (reviewed by Mayer and Shain 1974). Reports are available that gibberellin, kineten and abscisic acid were involved in amylase formation in germinating peas and beans (Varner et al. 1963, Dale 1969, Gepstain and Ilan 1970 and Yomo and Varner 1973).

CONCLUSION

The various changing features of enzymes in germinating seeds were mentioned in results and discussion. In this concluding chapter no attempt is made to report detailed properties of the enzymes studied and all the changes in enzyme activity of germinating seeds, but attention is focussed to co-ordinate results to obtain a picture of the role of some hydrolases and dehydrogenases during different stages of germination in winged bean and kalomator.

Crude enzyme preparation from 48 hours germinated seeds of winged bean and kalomator in extraction medium of increasing pH showed that ADHs remain most stable in Tris-HCl buffer at pH 7.6. The rate of inactivation is very rapid at pH below or above pH 7.6 most probably due to denaturation of the enzyme. Storage of the enzyme extract in Tris-HCl buffer with BME and sucrose remain most stable for several months at -20°C whereas storage at 4°C for a week results in a loss of 47.13 and 49.43% enzyme activity in kalomator and winged bean respectively. Storage at room temperature for 24 hours completely inactivates the ADH activities for both the species.

pH optimum of the winged bean and kalomator ADHs for oxidation of ethanol was 8.8. K_m values for NAD ($2 \times 10^{-2}\text{M}$) and allyl alcohol ($0.5 \times 10^{-2}\text{M}$) were identical for winged bean and kalomator ADHs, while K_m values for ethanol were $1.9 \times 10^{-2}\text{M}$ and $0.6 \times 10^{-2}\text{M}$ for winged bean and kalomator ADHs respectively. About twice higher K_m value for winged bean ADH for ethanol than that of kalomator ADH indicates lower affinity for ethanol of winged bean ADH. Heat stability study showed that the enzymes are inactivated differentially during heating at different temperatures for varying times. No remarkable differences exist between the two ADHs in respect of thermostability.

Substrate specificity study with different alcohols reveals that winged bean and kalomator ADHs oxidize unsaturated alcohol such as 2-propen-1-ol (allyl alcohol) most rapidly, followed by ethanol, n-propanol, n-butanol and n-pentanol. These ADHs are very little reactive with some branched chain alcohols (e.g. iso-butanol and sec-butanol), while other branched chain alcohols (e.g. iso-propanol and tert-amyl alcohol), cyclic alcohol (cyclohexanol) and methanol are not oxidized at all. However, the rate of oxidation of kalomator ADH is faster with all the substrates oxidized by it. These results led to conclude that both winged bean and kalomator ADHs show low substrate specificity like other plant ADHs.

Based on studies involving extractability, pH optimum, stability (at different pH values and temperatures for varying periods of time), sensitivity to inhibitors and reactivity with different alcohols, it can be concluded that winged bean and kalomator ADHs are more or less similar with some differences in respect of K_m values for ethanol and rate of oxidation of alcohols. Further, it is concluded that properties of winged bean and kalomator ADHs are similar to those of other plant ADHs.

Electrophoretic studies reveal that both winged bean and kalomator ADHs exist as three molecular forms. The isoenzymes of winged bean are distinct and well separated while those of kalomator ADH are very closely separated.

Study of physical and chemical properties such as thermal and pH stabilities, degree of inactivation by various inhibitors and reactivity with different alcohols provide evidence for difference between the ADH isoenzymes of winged bean.

Heat stability of the three isoenzymes is significantly different. The fastest isoenzyme (ADH-1) shows lowest thermostability while the slowest one (ADH-3) shows highest thermostability. Distinct differences exist in the pH stabilities of the ADH isoenzymes in winged bean and found that ADH-3 is the most stable and ADH-1 is the least stable at different pH values. All the isoenzymes are most stable at pH 8.0. Inhibition studies indicate that ADH isoenzymes are differentially inhibited by sulphydryl reagents, chelating agents and metal ion. Regardless of the inhibitor used, ADH-2 is least affected while ADH-1 is most affected. ADH-3 shows slightly less sensitivity than ADH-2 towards the inhibitors.

Winged bean ADH isoenzymes oxidize different alcohols differentially. Each isoenzyme oxidizes allyl alcohol most rapidly and maximally followed by ethanol. ADH-2 always appears to be catalytically most active with different alcohols while ADH-1 shows little reactivity with these alcohols. ADH-3 appears little less active than ADH-2.

Differences in stabilities (pH and heat), substrate and inhibitor specificities between the ADH isoenzymes in winged bean lead to assume that structural differences exist between the isoenzymes which are perhaps coded by distinct gene loci.

Although dry winged bean seed contains high ADH activity, it contains no ethanol which makes its appearance during imbibition. The absence of ethanol in dry seeds and the simultaneous increase in the levels of ADH and ethanol suggests that ADH may be induced by acetaldehyde prior to the formation of ethanol. The reduction of acetaldehyde is then, perhaps, catalyzed by the induced or activated ADH

to produce ethanol during the initial hours of germination (anaerobic respiration). The rapid decline of ADH and ethanol after the 2nd day of germination suggests that, perhaps, ADH participates in the oxidation of ethanol to acetaldehyde during later phase of germination.

Developmental study of the ADH isoenzymes in both winged bean and kalomator shows that the amount of the three isoenzymes increase at different rates with the onset of germination. Amount of ADH-1 increases maximally at 48 hour while ADH-2 and ADH-3 increase slightly. The decay rate of ADH-1 is most rapid while that of ADH-2 and ADH-3 is gradual.

Since ADH-1 shows most pronounced increase during initial stage of germination and declines most rapidly after 48 hours, it is assumed that it functions only in the storage cotyledons participating in the reduction of acetaldehyde during initial hours of germination. The much less and almost identical increase occurring in ADH-2 and ADH-3 isoenzymes and their gradual and simultaneous decline to low levels by day 15 suggest that they perhaps, participate in the oxidation of ethanol formed during aerobic respiration. Similar functional significance of ADH isoenzymes in germinating seeds of kalomator can only be theorized by visualizing the intensity and rate of increase or decrease of individual isoenzyme at different stages of germination.

Differences in mobilities and intensities between the ADH isoenzymes derived from two different plant species suggest that the isoenzymes are controlled by different genes or different

alleles of a locus specific for each plant species. In fact the dissimilarity between the ADH patterns suggest that the two species (Vicia faba and Psophocarpous tetragonolobus) display a distant phylogenetic relationship.

ADH activity in winged bean increases differentially under partial or complete anaerobic conditions. Enzyme activity increases maximally under complete anaerobic condition. The relative amounts of each isoenzyme also increase during anaerobiosis, but no new ADH band appears. Soaking of winged bean seeds in different salt solutions increase ADH activity slightly than that of control. It is suggested that more increase in ADH activity is due to the toxic effect of salt solutions used (20mM). The faster decrease in ADH activity in light grown seedlings leads to suggest that appearance of chlorophyll may have inhibitory effect on ADH activity. No significant variation takes place in ADH isoenzyme patterns of winged bean grown under light and dark.

MDH activities in winged bean and kalomator increase sharply upto day 2 of germination followed by a slow decline. MDH in each of these species exists as four isoenzymes which differ in intensities and mobilities within and between the species. During germination, only the relative amounts of the isoenzymes change.

On the basis of electrophoretic mobilities, it is presumed that isoenzyme 1 and 2 in winged bean and kalomator may be cytoplasmic, isoenzyme 3 may be mitochondrial and isoenzyme 4 of microbody origin. The occurrence of MDH isoenzymes in different amounts in winged bean and kalomator seeds and their rates of appearance

and disappearance during different periods of germination suggest that they may be under different control mechanisms of gene expressions.

Since all the four isoenzymes are present in different amounts in dry and germinating seeds of winged bean and kalomator it is suggestive that they all participate in the basic cellular metabolism in different manners at different sites of the cell.

Esterase activities in winged bean and kalomator change in two phases e.g. between 0-5th day and between 6th - 15th day. Activities are low in dry seeds, but increase gradually with the onset of germination and then decline slightly at day 5. Again the enzyme activities increase during the later part of germination. Ten esterase bands of different mobilities and two esterase bands of intermediate mobilities could be detected in winged bean and kalomator respectively. With the onset of germination esterase bands in the intermediate and fast moving areas show changes both in numbers and intensities during germination in winged bean, while the two bands in kalomator show changes in intensities only.

The consistent presence of a few esterase bands in winged bean at all stages of germination, appearance of some forms with the onset of germination and disappearance of some forms at later stage of germination lead to suggest that these isoenzymes are controlled by multiple genes or different alleles which are activated or deactivated at different stages of germination. Furthermore, disappearance of fast and intermediate moving bands and appearance of slow moving bands suggest that the factor which activates the gene loci controlling

the slow moving isoenzyme, probably act as an inactivator of the gene loci controlling the disappearing bands of fast and intermediate moving bands. To the contrary, the constancy in the number but variation in the intensity of esterase bands in kalomotor suggest that the responsible gene is at different stages of activation.

Trypsin like activities (TLA) are high in dry winged bean and kalomotor seeds, while haemoglobinase activities are low in these seeds. TLA activities increase slightly upto day 1, followed by rapid decline. To the contrary, haemoglobinase activities increase gradually during germination and reach peaks at day 11 or 13 followed by slow decline.

Since TLA and haemoglobinase show different time courses of development and haemoglobinase is active at pH 5.5 and TLA is active at pH 7.6, it is tentatively concluded that these are infact two different proteolytic enzymes. Further studies are required to characterize these enzymes.

In dry seed extracts of winged bean and kalomotor amylase activities are low. As germination proceeds the activity increase much faster in kalomotor than in winged bean and reaches peak between days 11 and 13 of germination in both the species.

The faster increase in amylase activity in kalomotor, higher amount of amylase at the peak and continuous degradation of starch in this species (reported by Gomes et al. 1984) may be due to the presence of higher starch content or due to differences in the composition of the starch molecules. Since amylase activities

increase both during sugar depletion (winged bean) and during sugar accumulation (kalomator), it is suggested that amylase activity is independent of sugar accumulation.

Quite clear differences have been observed in the present experiments between the two groups of enzymes as regards their various roles in germinating seeds of winged bean and kalomator. Dehydrogenases appear very active during the early hours of germination (anaerobic phase) while the hydrolytic enzyme activities increase significantly during the later part (9-15) of germination period.

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ADDENDUM

Psophocarpus tetragonolobus L. (Winged bean). (Family - Fabaceae).

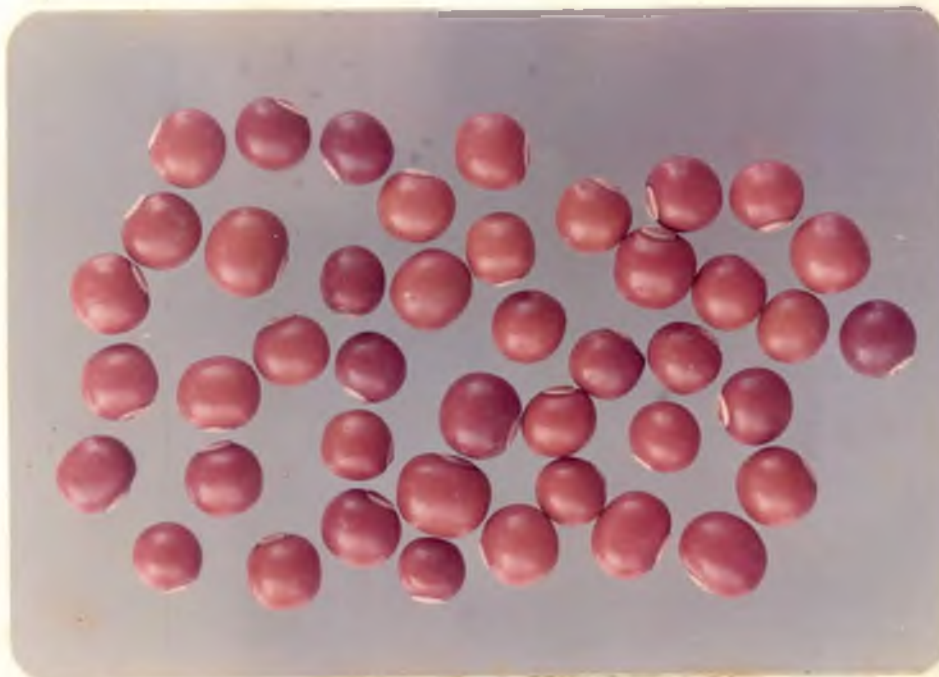
The winged bean is a perennial climber (Fig. I) with tuberous roots. Its stem is weak, twiner. Leaves 3-foliate with broad ovate leaflets, 7.5-15 cm long. Flowers large, light blue, in lax racemes. Pods 4-angled, 15-22 cm long, 2-3 cm broad, with each angle continued into a much crisped and toothed papery wing 3-6 mm broad. Seeds nearly globular, 6 mm across, smooth.

It is cultivated as a subsistence (backyard) crop in the Southern and South - Eastern Asia and Western Pacific. It remained a neglected legume despite the fact that it contains as much protein (40-45%) and oil (20-25%) in its seed as the soybeans. Its all parts (green leaves, pods, flowers, dry seeds and roots) are edible and it received a great deal of scientific attention. For the high contents of protein and oil in the seeds, it has been hailed as 'the possible soybean' for the tropics, where protein malnutrition is widespread.

Fig. 1



Wingedbean plant with flowers and fruits.



Wingedbean seeds.

Vicia faba L. (Kalomotor). (Family - Fabaceae).

Different types of Vicia faba are cultivated in different countries. Of them two main varieties are (i) the Broad beans proper grown as a vegetable for human consumption and (ii) the Field beans grown for stock feedings. The sample used in our experiment was identified as field bean. However, it differs from the field beans cultivated in European countries. It is an erect hardy annual, easily recognized by its four-ribbed stem, sparsely branched (Fig. II). The pinnately compound leaves are composed of a few large leaflets and bear large stipules at their base. The white black-blotched flowers are borne in axillary clusters. The pods are short containing about four, small, almost circular seeds.

The species, commonly known as vetches are extensively used as cover crops. They are edible and palatable to livestock. The main constituent of mature seeds are carbohydrate (24-4%) and protein (26-36%). In Bangladesh, the present variety is grown as a fodder for cattle and seeds are eaten by the poor people.

Fig. II



Kalomotor plant with flowers and fruits.



Kalomotor seeds.

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ALCOHOL DEHYDROGENASE IN GERMINATING *VICIA FABA* L. CV KALOMOTOR COTYLEDONS

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Key words : Dehydrogenase, Germinating, Vicia, Cotyledons

Abstract

Alcohol dehydrogenase (ADH) (EC 1.1.1.1), extracted from germinated seeds of *Vicia faba* cv Kalomotor was found to have K_m values of 6.0×10^{-3} M and 5.0×10^{-3} M for ethanol and allyl alcohol, respectively. pH optimum of the enzyme for oxidation of ethanol was 8.8. With Tris-HCl buffer the solubility and stability of the enzyme was highest at pH 7.6. Crude enzyme extract in Tris-HCl buffer containing β -mercaptoethanol and sucrose remained almost stable for several months at -20°C . The enzyme was inactivated differentially during heating at different temperatures for varying times. The substrate specificity of the enzyme was found to vary with different alcohols. β -mercaptoethanol and sucrose protected the enzyme while iodoacetamide, eserine, dimethyl sulphoxide, o-phenanthroline, CuSO_4 and EDTA inhibited it. The activity of the enzyme increased rapidly during the initial stage of germination to reach its peak at 48 hrs of germination and then declined gradually.

Introduction

Alcohol dehydrogenase (ADH) is widely distributed in mammals, plants, insects and microorganisms (Bonnichsen and Brink 1955; Hayes and Velick 1954; Moser *et al.* 1968; Scandalios 1974; Shannon 1968; Ursprung and Leone 1965) and catalyzes the oxidation of alcohols and reduction of aldehydes coupled with the reversible oxidation-reduction of nicotinamide adenine dinucleotide (NAD).

ADH is a metalloprotein with four atoms of zinc which take part in the binding of NAD and substrate, and contribute to the stabilization of protein molecule (Evans and Rabin 1968). Sulphydryl groups are also necessary for the activity of this enzyme (Witter 1960).

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In comparison to animal and yeast ADHs, much less is known about the structure and properties of higher plant ADH because of its instability (Cossins *et al.* 1968). ADHs have been characterized in peas (Cossins *et al.* 1968; Ericksson 1968; Leblova *et al.* 1977), bean, wheat rape, sunflower, rice and broad bean (Leblova and Stiborova 1975; Leblova *et al.* 1977), chick pea (Gomes *et al.* 1982) and in potato (Davies *et al.* 1973).

ADH activity and ethanol concentration increase in anaerobically respiring seeds i.e. in germinating seeds with unruptured testa (Cameron and Cossins 1967; Gomes *et al.* 1982; Leblova *et al.* 1971; Shannon 1968; Scandalios 1974). After the rupture of the testa, ethanol is lost either in the surroundings (Wagner 1959) or oxidized by ADH to acetaldehyde which is further metabolized via TCA cycle (Cameron and Cossins 1967; Cossins and Turner 1962; Peterson and Cossins 1966). With the disappearance of ethanol ADH activity declines rapidly (Cossins and Turner 1962; Gomes *et al.* 1982; Leblova *et al.* 1971).

The aim of the present work has been to characterize ADH present in *Vicia faba* cv Kalomotor and to determine its changes during germination with a view to correlating the activities and properties of the enzyme with its possible physiological function.

Material and Methods

Plant material : Seeds of *Vicia faba* cv Kalomotor were obtained from a village market in Dhaka District. Seeds of uniform size were soaked for 24 hrs in distilled water and washed thoroughly with fresh distilled water. The seeds were then germinated in a dark thermostatic chamber at 27 ± 1 C spread over a distilled water-moistened filter paper in a Petri dish for 5 days. When plants older than 5 days were desired, soaked and washed seeds were planted in glass or porcelain trays on a layer (1-3 cm) of sterile sand moistened with distilled water and then germinated for 0-15 days as described above.

Extraction of ADH : Only cotyledons were removed from the seedlings of various ages, washed with distilled water and gently blotted with filter paper. Extraction was done by homogenizing 1g tissue for 5 min in a chilled mortar with 5ml 0.05 M Tris-HCl buffer (pH 7.6) containing 10 mM-mercaptoethanol (BME) and 12.5% sucrose. The slurry was centrifuged at 10000 g for 30 min at 4 C. The supernatant was filtered through Whatman No. 1 filter paper. This filtration removed most of the lipid material found floating on top of the supernatant. The resultant preparation was used for determinations of ADH activity and protein content.

Enzyme assay : ADH activity was measured according to the method of Bonnichsen and Brink (1955) with slight modification. Absorbance was measured at 340 nm on a LKB digital spectrophotometer (Model, Ultrospec 4050). The increase in absorbance of the reduced form of NAD was recorded continuously for 5 min at 30 sec intervals after addition of 0.1 ml of 1.2 mM NAD. The enzyme reaction mixture consisted of 2.75 of 0.05 M Tris-HCl buffer (pH 8.8), 0.1 ml of 1.13 M ethanol and 0.05 ml of the enzyme extract. This solution was then used to set the machine at zero. One unit of ADH activity is defined as the amount of enzyme necessary to produce a change of 0.001 per minute per mg of protein. Whenever necessary the enzyme extract was diluted suitably immediately before activity measurement so as to keep the O.D. change from 0.05 to 0.07 per minute.

Protein determination : The protein concentration of crude extract was determined according to the method of Lowry *et al.* (1951) using bovine serum albumin as standard.

Results and Discussions

Extractability and stability of ADH : Crude enzyme extracts from 48 hrs germinating seeds were prepared in 0.06 M phosphate buffer (pH 6.3, 7.0, 7.6 and 8.0) and in 0.05 M Tris-HCl buffer (pH 7.0, 7.6, 8.0 and 9.0) for investigating extractability and stability of ADH. It can be seen from Table 1 that

Table 1. Extraction and stability of alcohol dehydrogenase in Kalomator.

Extraction condition	Total soluble protein (mg/ml)	Alcohol dehydrogenase activity				Per cent inactivated after 22 hr
		Total activity (units/ml) after 2 hr	Specific activity (units/mg prot., min) after 22 hr	Total activity (units/ml) after 22 hr	Specific activity (units/mg prot./min) after 22 hr	
Tris-HCl buffer						
pH 7.0	10.0	4500	450.0	3800	380.0	15.6
7.6	18.8	7332	390.0	7238	385.0	1.3
8.0	31.2	6800	217.9	6193	198.5	9.0
9.0	38.0	6600	173.7	5200	136.8	21.2
Phosphate buffer						
pH 6.0	8.8	6400	727.3	3000	341.0	53.1
7.0	14.8	8600	581.1	4900	331.0	43.0
7.6	17.4	7000	402.3	5200	298.9	25.7
8.0	19.2	7400	385.4	5100	265.0	31.1

a ADH activity in the extract was assayed in 0.05 M Tris-HCl buffer, pH 8.8. Values are means of triplicate determinations.

more total protein and less enzyme protein were solubilized with Tris-HCl buffer. When the enzyme extracts were stored for 22 hrs at 20 C ADH in phosphate buffer was inactivated at a faster rate indicating inadequacy of the ionic strength and buffering action of this buffer for maintaining the enzyme activity. The enzyme remained most stable with Tris-HCl buffer at pH 7.6 and lost only 1.3% activity after 24 hrs of storage. Below or above pH 7.0 - 8.0 the rate of inactivation of the enzyme increased most probably due to denaturation of the enzyme. Since Tris-HCl buffer having pH 7.6 showed better buffering and protective capacities it was selected for further extractions and studies.

Effect of BME and sucrose on ADH activity: For comparison of the effects of BME and sucrose on ADH activity the following extraction media were used: Tris-HCl buffer (pH 7.6), Tris-HCl buffer with 10 mM BME, Tris-HCl buffer with 12.5% sucrose, Tris-HCl buffer with 10 mM BME and 12.5% sucrose. Most enzyme activity was retained in the presence of BME and sucrose. The results are summarized in Table 2. Since the enzyme is protected by BME it

Table 2. Effect of BME and sucrose on ADH activity in Kalomator.

Extraction condition	Specific ADH activity ^a (units/mg prot./min)	
	After 2 hr	After 22 hr
Buffer alone ^b	385	380
Buffer with sucrose	395	393
Buffer with BME	405	403
Buffer with sucrose and BME	428	427

a ADH activity in the extract was assayed in 0.05 M Tris-HCl buffer, pH 8.8.

b 0.05 M Tris-HCl buffer, pH 7.6. Values are means of triplicate experiments.

is suggested that the enzyme has sulphhydryl group which is essential for maintaining the three dimensional structure of the protein molecule and the formation of a ternary enzyme-coenzyme-substrate complex (Suzuki and Kyuwa 1972; Whitehead and Rabin 1964). Although the protective action of sucrose has been shown (Gomes *et al.* 1982), its mechanism of action is not yet clear.

Storage stability of ADH at different temperatures: Crude enzyme extracts in Tris-HCl buffer (pH 7.6) containing BME and sucrose were stored at -20, 4 C and room temperature for different periods of time. About 80-85% ADH activity was retained after storage for months at -20 C whereas storage at 4 C

resulted in a loss of 50-55% of enzyme activity after a week. The enzyme was completely inactivated after 24 hrs storage at room temperature. The slow inactivation of ADH, even at -20°C , over a period of four months, may be due to the presence of endogenous inhibitors in the crude extract. Since the enzyme extract could be stored frozen for a few months and thawed with only 15-20% loss of enzyme activity, it was kept in a deep freeze at -20°C for future use. Assays were made mostly on fresh enzyme extracts, if not possible, on stored extracts.

pH optimum : The pH optimum for activity of ADH in the direction of ethanol oxidation was determined by using 0.05 M Tris-HCl buffer at varying pH values and room temperature. When assayed at 0.04 M ethanol and 0.4 mM NAD, the highest activity was found at pH 8.8. This pH is similar or close to those already reported for other higher plant ADHs (Cossins *et al.* 1961; Gomes *et al.* 1982; Leblova *et al.* 1971, 1977).

Temperature stability of ADH : Duplicate samples of crude extracts (0.5ml) were heated in a water bath at 45, 55 and 65°C for 10, 20, and 30 min, using stoppered test tubes. After heating, the samples were withdrawn, cooled immediately in ice cold water bath and the residual activity was assayed by the usual technique. A control sample was kept in ice cold water bath during the course of heating and the loss of activity in heated sample was compared with the activity in unheated samples. It can be seen from Table 3 that heating

Table 3. Effect of temperature on the stability of Kalomator ADH.

Time of heating ^a (min)	% ADH activity inactivated ^b temperature of heating (in $^{\circ}\text{C}$)		
	45	55	65
10	28	34	58
20	36	42	65
30	42	48	79

a Crude extracts were heated in stoppered test tubes on water bath, cooled immediately on ice cold water bath and activity was assayed.

b Per cent of the maximum activity in control sample stored on ice cold water bath. Values are means of triplicate experiments.

at different temperatures inactivated ADH at different rates. The heat stability of Kalomator ADH was found to be slightly more than other plant ADHs (Gomes *et al.* 1982; Leblova *et al.* 1977). This may be due to the difference in

amino acid composition or tertiary and quaternary structure of ADHs. Moreover, the presence of endogenous protectors and proteins, and added sucrose might have influenced the heat stability of Kalomator ADH. The protective action of sugars on thermodenaturation of serum proteins have been demonstrated (Kerekes 1963).

Michaelis constant: Apparent Michaelis constants (K_m) for ethanol and allyl alcohol were determined with a crude enzyme preparation at pH 2.8. For both the substrates, the kinetics were apparently hyperbolic with no indication of sigmoidicity or significant substrate inhibition. The data were fitted to double reciprocal plots of velocity vs concentration for estimation of K_m values. Graphical analyses of the two curves resulted in the following K_m estimations: ethanol = $6.0 \times 10^{-3}M$ and allyl alcohol = $5.0 \times 10^{-3}M$. The K_m values of Kalomator ADH for the two substrates seem to be slightly lower than those of related plant ADHs (Leblova *et al.* 1977). This may be due to the use of crude enzyme preparation.

Substrate specificity: Kalomator ADH catalyzes the oxidation of different alcohols (Table 4). Aqueous solution of the alcohols at the concentrations shown in the table were used. Saturated solutions of weakly soluble substrates were

Table 4. Substrate specificity of Kalomator ADH.

Substrate	Conc	Relative rate of oxidation ^a
Methanol	$3 \times 10^{-2}M$	0
Ethanol	"	100
<i>n</i> -Propanol	"	40
<i>iso</i> -Propanol	"	0
Allyl alcohol	"	118
<i>n</i> -Butanol	"	35
<i>iso</i> -Butanol	"	5
<i>sec</i> -Butanol	"	3
<i>tert</i> -Butanol	"	0
<i>n</i> -Pentanol	Saturated	0
Tert.-Amyl alcohol	"	0
Cyclohexanol	"	0
Decanol	"	0
Oleyl alcohol	"	0

^a The velocity of ethanol oxidation was taken 100. Values are means of triplicate determinations.

used. Quantitative differences in the rates of oxidation were observed. Of the alcohols which were utilized allyl alcohol was oxidized most rapidly followed by ethanol. n-propanol, n-butanol and n-pentanol were utilized more slowly than ethanol. As a general rule, the rate of alcohol oxidation decreases with the increasing number of carbon atoms in the substrate molecules. As far as substrate specificity is concerned, Kalomotor ADH showed a low degree of substrate specificity and resembles ADHs in pea, broad bean, kidney bean (Ericksson 1968; Leblova and Manca 1975) and chick pea (Gomes *et al.* 1982).

Inhibitors of ADH: The effects of some inhibitors on Kalomotor ADH are shown in Table 5. Crude enzyme extracts (0.5 ml) and inhibitors at different concentrations were pre-incubated at room temperature for 10 min. The residual activity was measured by the usual technique and compared with the control. Sulphydryl reagents and metal chelators differentially inhibited Kalomotor ADH. This indicates the presence of sulphydryl groups and metal in the active centre of the enzyme. The present results are in good agreement with those of Cossins *et al.* (1968), Gomes *et al.* (1982) and Leblova *et al.* (1977).

Table 5. Effect of inhibitors on alcohol dehydrogenase in Kalomotor.

Inhibitors	Conc (mM)	Inhibition percentage
Iodoacetamide	1	92.20
Eserine	1	60.53
o-phenanthroline	1	68.50
Dimethyl Sulfoxide	10	76.32
Copper sulphate	1	89.48
Ethylenediaminetetra-acetic acid disodium salt	1	55.30

a Per cent of the maximum activity in control sample. Values are the means of triplicate determinations.

Changes in the activity of ADH during germination: The changes in the specific activity of Kalomotor ADH are shown in Table 6. The results show that considerable ADH activity could be detected in dry seeds. The enzyme activity increased rapidly and reached peak at 48 hrs of germination after which the activity decreased gradually as the germination proceeded. At day 15 about 45.2% of the initial activity and 25.5% of the peak activity remained in the seeds. Very little activity could be detected at day 19 of germination. Similar fluctuations in ADH activity of germinating seeds have been reported in

other plants (Cossins and Turner 1962; Gomes *et al.* 1982; Leblova *et al.* 1971). It is known that activities of many enzymes in plants increase during the first few hours of germination (Mayer and Shain 1974). Increase in ADH activity and ethanol concentration during the initial hours of germination have been reported in many seeds (Cameron and Cossins 1967; Leblova *et al.* 1971; Davies *et al.* 1974; Gomes *et al.* 1982), but the biochemical mechanism is not entirely clear. Davies *et al.* (1974) have proposed that at first lactate and malate accumulate in anaerobically respiring tissues (e.g. germinating seeds with unruptured

Table 6 . Changes of ADH activity in Kalomator during germination.

Days of germination	Specific ADH activity (units/mg prot./min)
0	238.97
1	342.85
2	430.41
3	359.64
4	353.84
5	274.50
7	175.92
9	160.81
11	150.00
13	120.00
15	108.33

Values are the average of triplicate experiments.

seed coat) causing a fall in pH. Acidic pH restricts further reduction of pyruvate by lactate dehydrogenase, but favours the decarboxylation of pyruvate to acetaldehyde (Davies *et al.* 1974) which acts as a substrate of ADH and induces it. Acetaldehyde is immediately reduced to ethanol by ADH (Davies *et al.* 1974). In the presence of oxygen ethanol is metabolized via the TCA cycle (Cameron and Cossins 1967). Besides these, ADH may in some way be connected with the metabolism of other substrates (Davies *et al.* 1973).

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ABSTRACTS OF THE SCIENTIFIC PAPERS

SECTION—VI
BIOCHEMISTRY PHARMACY MEDICINE & VET. SCIENCE



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electrophoresis. The purification steps involve sulphate-mediated chromatography on Cibacron Blue-Sepharose 6B column. Preliminary studies on the properties of the enzyme suggest that it is the Nagao variant of the enzyme rather than the Regan variant found in many tumours.

18. Effect of Iron Supplementation on Antibody Formation in Malnourished Rats.
Faruk Ahmed and Syed Saleheen Qadri.

Malnourished rats, supplemented with 10 ppm Ferrous iron, showed increased antilody response, 1:64 as against 1:16 in the protein-deficient malnourished ones. These rats also showed increased values for RBC count, blood hemoglobin and serum iron. There were however, no significant differences in total serum protein, serum urea, serum TIBC, serum GOT, serum GPT and serum alkaline phosphatase. The amount of iron in the diet seems to influence the immune response in malnourished animals.

★ **19. Studies on some enzymes in germinating winged bean (*Psophocarpus tetragonolobus*) and horse bean (*Vicia faba*).—Nilufar Sadeque and Joseph Gomes**

Activities of alcohol, malate and lactate dehydrogenases, amylase, protease and esterase were measured in the extracts of the cotyledons of winged bean (kamranga seem) and horse bean (kalomotor) at various stages of germination up to 15 days. All the enzymes had relatively low activities in dry seeds, but the activities increased significantly during the initial stages of germination and then declined gradually. The effects of different concentrations of giberellic acid and abscic acid on the alcohol, malate and lactate dehydrogenases were studied. Giberellic acid caused increase in the enzyme activities and 10^{-4} M solution caused maximum increase. On the other hand abscic acid caused decrease in the enzyme activities and 10^{-4} M solution caused maximum decrease in enzyme activities.

- ★ 20. Alcohol dehydrogenase from germinated winged bean (*Psophocarpus tetragonolobus*) and horse bean (*Vicia faba*), Nilufar Sadeque and Joseps Comes.

20. Alcohol dehydrogenase (ADH) (EC 1.1.1.1) extracted from germinated winged bean (kamranga seem) and horse bean (kalomotor) had pH optima between 8.7 and 8.8. Some properties such as K_m for ethanol as substrate and NAD as coenzyme, substrate specificities towards different alcohols and the effect of some inhibitors on the activity of the two plant ADHs were studied. The specificity of both ADHs towards alcohols is relatively wide. Allyl alcohol is oxidised better than ethanol, while methanol, n-butanol, n-propanol, isobutanol and pentanol are oxidised slowly. Both ADHs do not oxidise isopropanol, tert. amylalcohol, cyclohexanol, decanol, oleylalcohol and benzylalcohol. Inhibition of both the plant ADHs by iodoacetamide, EDTA, eserine sulphate, o-phenanthroline, dimethylsulphoxide and CuSO_4 , indicated the presence of -SH groups and metal in the enzyme molecule. Storage and thermal stabilities of the ADHs were determined. Crude enzyme extracts stored at -20°C in Tris-HCl buffer (pH 7.6) containing betamercaptoethanol and sucrose appeared to show minimum loss of activity over a period of several weeks, whereas storage $0-4^\circ\text{C}$ resulted in a complete inactivation within 1 or 2 weeks. The enzymes were completely inactivated within 24 hrs during storage at room temperature. The enzymes lost most of their activity after being heated at 5°C and was totally inactivated at 70°C .

21. Effect of germination on winged bean (*Psophocarpus tetragonolobus*), Joseph Gomes, J. C. Debnath, Kabir Ahmed, L. A. Banu and Q. N. Haq.

The effect of germination on changes in low molecular weight carbohydrates starch, lipids, fatty acids, protein, free amino acids and ash were investigated in raw and germinated (3 & 6 days) winged bean. The total monosaccharides (galactose and glucose) in 80% ethanol extract constituted 0.81% of the dry matter and oligosaccharides (sucrose, raffinose and stachyose) amounted to 5.1%. The levels of both monosaccharides and oligosaccharides decreased with the progress of germination probably due to their utilization for easily available source of germination energy. To the contrary, starch content increased from 0.9% at day 0 to