THE UNIVERSITY OF OKLAHOMA

GRADUATE COLLEGE

PROTEIN METABOLISM IN THE COTYLEDONS OF *Pisum sativum* L. DURING SEED DEVELOPMENT AND GERMINATION

A DISSERTATION

SUBMITTED TO THE GRADUATE FACULTY

in partial fulfillment of the requirements for the

degree of

DOCTOR OF PHILOSOPHY

BY

SHAIK MADANAPALLI MAHABOOB BASHA

Norman, Oklahoma

1974

PROTEIN METABOLISM IN THE COTYLEDONS OF Pisum sativum L.

DURING SEED DEVELOPMENT AND GERMINATION

APPROVED BY

Ronald Bressan.

Gene J. Goodman

Norman D. Bole

Elroy L. Kipe

Eddie Carol Smith

DISSERTATION COMMITTEE

ACKNOWLEDGMENTS

I express my deep sense of gratitude and sincere thanks to Dr. Leonard Beevers for his advice, encouragement, effective criticism during the course of this investigation and in preparation of this manuscript.

My thanks are also due to Dr. Gary N. Wells for his technical assistance and discussions about the experiments.

I am also thankful to Mr. Larry A. Kapustka for his help in photographic work.

Appreciation is also expressed to the members of my advisory committee: Dr. Elroy L. Rice, Dr. Norman H. Boke, Dr. George J. Goodman, and Dr. Eddie C. Smith for their advice and time.

The author is also grateful to the Department of Botany and Microbiology for the financial support provided in the form of a teaching assistantship during my studies at the University of Oklahoma.

TABLE OF CONTENTS

IV. RESULTS AND DISCUSSION (Cont.)

LIST OF TABLES

LIST OF ILLUSTRATIONS

ABSTRACT

Protein from pea cotyledons was isolated and fractionated into albumin, globulin, legumin and vicilin based on their solubility. On gel electrophoresis albumin showed numerous bands, while legumin and vicilin gave a diffuse band, suggesting that they are complex and heterogeneous. On SDS gel electrophoresis legumin showed two minor and three major subunits and vicilin showed five major subunits.

Overall changes in protein content during seed development revealed that albumin synthesis stopped earlier than globulin synthesis. However, during seed germination globulin depletion occurred earlier than albumin depletion. Deposition and utilization of legumin and vicilin were independent of each other. Investigation of these fractions during seed development and germination indicated that considerable changes occur in the composition of albumin and subunit ratio of legumin and vicilin, suggesting that they are not deposited or utilized as a single unit.

The pea proteins were found to be glycopeptides composed of both neutral (mannose and glucose) and amino sugars (glucosamine). The carbohydrate content of protein changed during seed development and germination. Legumin was rich in neutral sugars and vicilin in amino sugars. Labelling studies with ^{14}C-glucosamine during different days of seed development indicated that in legumin most of the label

incorporated into subunit V, while in vicilin incorporation occurred into all the subunits. Investigations concerned with determination of the time of attachment of carbohydrate units to the peptide showed that these units might be attached after the peptide synthesis.

Attempts were also made to characterize the pea protease by using native substrates viz., albumin, legumin and vicilin and to associate the proteolytic activity with the protein hydrolysis during seed germination. It was found that the pea protease is capable of hydrolysing all the three native substrates at an optimum pH of 5.0 and the proteolytic activity was in agreement with the observed protein hydrolysis during different days of seed germination.

PROTEIN METABOLISM IN THE COTYLEDONS OF *Pisum sativum* L. DURING SEED DEVELOPMENT AND GERMINATION

CHAPTER I

INTRODUCTION

Many mature dicotyledonous seeds contain an embryo and stored food enclosed in one or two seed coats. The embryo is the potential plant and develops from the fertilized egg, while the seed coat is part of the parent. The embryo cannot synthesize its own food; thus, initially it is dependent upon nutrients supplied by the parent. Subsequently, growth is arrested at seed maturity; thereafter growth of the embryo is resumed during germination. During germination the embryo depends on the nutrients supplied from the stored reserves. Until it becomes autotrophic the young seedling depends initially upon the food reserves.

In many dicotyledons the food reserves are deposited in the cotyledons after the termination of meristematic activity. As the seed matures, the metabolic activities and reserve deposition declines until a quiescent mature seed is produced. Metabolism is renewed during germination and activity is directed toward reserve hydrolysis.

The composition of seed reserves differs from seed to seed. In the pea, *Pisum sativum*, it is principally starch and protein. Plant proteins have been classified into water-and saline-soluble globulins,

alcohol-soluble prolamines, and dilute acid-or base-soluble glutelins. Pea protein is predominantly composed of albumins and globulins. The globulins are further subdivided on the basis of isoelectric precipitation into legumin and vicilin.

The amino acid composition of reserve proteins is entirely different than the proteins present in leaves. Legumin and vicilin also occur in other leguminosae seeds. These proteins have been well characterized and are assigned a molecular weight of 386,000 and 180,000 for legumin and vicilin respectively. When legumin and vicilin are subjected to gel electrophoresis legumin gives one major band, whereas vicilin gives a diffuse band, suggesting that they are heterogeneous and very complex. When legumin and vicilin are dissociated with sodium dodecyl sulfate and dithiothreotol and subjected to SDS gel electrophoresis, legumin shows three major and two minor bands while vicilin shows five major bands. Hence, legumin and vicilin are not simple proteins but are made up of subunits.

Since it has been shown that legumin and vicilin are not simple proteins but are made up of subunits, it was of interest to determine if there might be variation in the rate and time of synthesis or breakdown of these subunits during different phases of metabolism. For this purpose the pea proteins were fractionated and changes in the pea storage protein, followed during reserve deposition in developing seed and during reserve depletion in germinating seed. The composition and subunit structure of legumin and vicilin were studied during seed development and germination.

Attempts were also made to characterize the enzymes responsible for protein hydrolysis by using native substrates, viz., albumin,

legumin, and vicilin, and to associate the measured proteolytic activity with protein loss during germination.

Additionally, some legume seeds contain glycoproteins. Glycoproteins may be regarded as proteins that contain carbohydrate attached to the peptide portion by covalent linkage. Investigations were made in an attempt to study the glycopeptide nature of pea storage proteins and to isolate and identify the carbohydrates associated with the protein and to follow the changes in this carbohydrate content, during seed development and germination. Studies have been made to determine the subunit and sugar association and the time of attachment of carbohydrate to the peptide, i.e., during or after peptide synthesis.

CHAPTER II

LITERATURE REVIEW

Reserve Protein Characterization

As early as 1880 Emmerling studied protein synthesis in ripening seeds of broad bean, Vicia faba major. He showed that nitrogenous products were transported from different parts of the plant to the seed pod where they were stored before being transported to the ripening seeds. Emmerling further stated that seed proteins are synthesized from organic nitrogenous compounds (amides and amino acids) synthesized in leaves. Schulze and Winterstein (1910) found that the seed pod from the pea contained much asparagine and little arginine, while in the seeds the distribution was reversed. They also showed that the amount of protein nitrogen increased and the amount of nonprotein nitrogen decreased during the ripening process.

Danielson (1949), using Osborn's (1924) fractionation procedure, indicated that pea proteins consist of water-soluble albumins and saline-soluble globulins. The globulins could be further subdivided on the basis of isoelectric precipitation at pH 4.5, where vicilin is soluble and legumin is not. In mature pea seed, globulins constitute 60% and albumins 40% of the total protein. This composition varies from variety to variety (Silano and Pocchiari, 1969; McLeester et al. 1973). Within the globulin the legumin predominates over vicilin. The occurrence

of two components in the reserve globulins appears to be characteristic of most legume seeds (Altschul et al., 1966).

It has been suggested (Danielson, 1956) that the albumins contain enzymatic components, whereas globulins are pure reserve proteins. In contrast, Kretovich et al. (1954) reported the occurrence of enzymatic activity with the globulin fraction isolated from pea seeds, which Danielson (1956) attributed to the contamination of globulins with albumins.

Ultrastructural analyses have indicated that in mature cotyledons much of the protein is confined to subcellular organelles termed protein bodies (Varner and Schidlosky, 1963; Bain and Mercer, 1966) and are rich in globulins (Varner and Schidlosky, 1963). Recently it was observed in peanut (Daussant et al., 1969) and soybean (Tombs, 1967) that protein bodies contain only one of the globulin components, suggesting that not all of the reserve protein is confined to the protein body. However, immunological studies of Graham and Gunning (1970) indicate that legumin and vicilin occur in protein bodies of beans. Ory and Hennigsen (1969) has recently demonstrated that there may be enzymatic activity associated with the protein body.

Various studies on globulin fractions have revealed them to be highly complex, with high molecular weights. A molecular weight of 331,000 for legumin and 186,000 for vicilin from pea seeds has been reported (Danielson, 1950). Brand and Johnson (1956) and Johnson and Richards (1962) reported a molecular weight of 400,000 and 398,000 $\pm$ 15,000 for pea legumin. Similar high molecular weights (370,000 and 320,000) for Vicia faba legumin have been assigned (Shutov and Vaintraub, 1966; Bailey and Boulter, 1970).

The globulin fractions have a characteristic amino acid composition, being enriched in acidic amino acids (aspartic acid and glutamic acid) and are very low in sulphur-containing amino acids like cysteine and methionine (Grant and Lawrence, 1964; Bailey and Boulter, 1970).

Since Osborn's method of protein fractionation is based on solubility, it might be expected that the protein isolated by this technique would be heterogeneous. Wetter and McCalla (1949) attempted to investigate the pea proteins and reported to have obtained four components when different protein fractions were analysed by gel electrophoresis. In contrast, several workers (Danielson, 1950; Reznichenko, 1954; Kretovich et al., 1954) reported that these globulins are homogeneous when investigated by electrophoresis. However, Vaintraub et al., (1962) reported three components by electrophoresis, one of which was selectively bound with a pigment.

N-terminal analysis by the Vaintraub's group has suggested that legumin contains 12 chains with an average weight of 33,000 in Pisum sativum (Vaintraub and Gofman, 1961) and 40,000 in Vicia sativa (Vaintraub et al., 1962). They identified the N-terminal amino acids of legumin as glycine, leucine, and threonine. Similar N-terminal amino acids were found by Bailey and Boulter (1970) in Vicia faba seed.

The relatively high molecular weights for various seeds of legumes investigated in conjunction with other evidence described in the literature (Brand and Johnson, 1958; Gofman and Vaintraub, 1960; Johnson and Richards, 1962) indicated that each of these proteins may be composed of several peptide chains. It is possible that the vicilin

and legumin entities may be dissociated into subunits, i.e., the peptide chains of each may be held together by forces other than primary chemical bonds.

It has been shown by ultracentrifugation that the legumin particle may be dissociated by acid (Brand and Johnson, 1958), sodium dodecyl sulfate (Brand and Johnson, 1956; Grant and Lawrence, 1964), guanidium hydrochloride (Vaintraub and Nguen, 1971) or urea (Grant and Lawrence, 1964) into particles with an average weight of 30,000 and 60,000. At pH 10 vicilin undergoes a reversible dissociation, while legumin is unaffected up to pH 10.5. Urea or foramide solutions of pea (Pisum sativum) globulins cause the appearance of six protein bands on gel electrophoresis, four arising from legumin and two from vicilin. Whereas SDS treatment has been reported to result in 12 well-defined bands, four of these arose from vicilin, six others from legumin and two were of uncertain origin. Shutov and Vaintraub (1966) reported that legumin and vicilin of vetch seed are incapable of association and dissociation during a change in the ionic strength in the interval between pH 7 to 9. In the acid region legumin dissociate irreversibly and in stages into 8S and 2S subunits, whereas vicilin dissociate into 3S subunits. Bailey and Boulter (1970) assigned 3 subunits for legumin and reported a molecular weight of 56,000, 42,000, and 23,000, respectively. Similar increase in number of globulin components upon dissociation has been reported (Koshiyama, 1970; Hobday and Giles, 1973; Wright and Boulter, 1973; McLeester et al., 1973).

Grant and Lawrence (1964) noticed that the amino acid composition of all the fractions resulting from dissociation are similar and

had two or more different N-terminal amino acids. Difference in end-group composition occurred among the fractions, but some N-terminal amino acids were common to several fractions.

Glycopeptide

In 1964 Pusztai presented evidence that glucosamine is a normal constituent of seeds of higher plants. Since that time there have been several reports of the occurrence of glycoproteins in plants. Several workers have reported on the presence of glycopeptides in seeds. Pusztai (1965) confirmed the presence of glycoproteins in seeds by demonstrating the occurrence of various amounts of neutral and amino sugars in protein extracts from kidney beans (Phaseolus vulgaris). After isolating two proteins, glycoprotein I and trypsin inhibitor from kidney beans (Phaseolus vulgaris), he showed that the carbohydrate part was mainly composed of D-mannose and D-glucosamine together with smaller amounts of arabinose, xylose, and fucose (Pusztai, 1966). The presence of mannose (4%) and glucosamine (1.2%) in 7S protein of soybean (Glycine max) casein fraction was reported by Koshiyama (1966).

Recently Pusztai and Watt (1970) isolated a glycoprotein II having antigenic and non-haemagglutinin activity containing D-mannose and D-glucosamine but no uronic acids. Similar findings were reported with storage protein of Phaseolus vulgaris (Racusen and Foote, 1971; Bianco and Bellando, 1971), Phaseolus aureus (Ericson and Chrispeels, 1973).

Seed Development

As early as 1910, Schulze and Winterstein analysed the ripening

pea seeds at four different stages and they were able to show that the amount of protein nitrogen increased and the amount of nonprotein nitrogen decreased during the ripening process. After studying the nitrogen distribution in developing pea seeds, Danielson (1952) reported that globulin-N increased at a constant rate. Globulins and albumins are synthesized independently. He further observed that vicilin and legumin are synthesized at different rates and the concentration ratio of vicilin/legumin diminished as ripening proceeds.

It was observed (Tkachenko and Klimenko, 1971; Klimenko, 1972) in peas that biosynthesis of globulins occurred in the early stages of maturity while albumin levels did not depend on the stage of ripeness. Millered et al., (1971) reported that legumin was detected in the young cotyledons of Vicia faba, and when the cotyledons were about 10 mm long, cell division was essentially complete, and there was a sharp increase in the rate of legumin accumulation. In contrast, Wright and Boulter (1972) found that vicilin was formed prior to legumin in Vicia faba during seed development, although the rate of synthesis of legumin was faster so that in the mature seed the ratio of legumin to vicilin was about 4:1 by weight. Further they observed that the subunit structure of vicilin changed during development, whereas that of legumin did not; and thus they concluded that vicilin is not a single protein.

Ultrastructural changes during seed development revealed an increase in membrane-bound ribosomes which corresponds with the onset of storage protein deposition. It has been shown (Briarty et al., 1969) that the build up of storage protein occurs in the cytoplasm within

membrane-bound vacuoles which subsequently become the protein bodies of the mature seed, retaining the original tonoplast as the bounding membrane of the protein body. Later, Bailey et al., (1970), using ^{3}H-leucine, showed that the radioactivity is associated initially with the endoplasmic reticulum then moved to the protein bodies, revealing the intracellular transport of storage proteins.

Seed Germination

When seeds are placed in an environment favorable to germination, the rate of metabolism is markedly accelerated. Protein is broken down during germination, with a concomitant rise in amino acids and amides followed by protein synthesis de novo in the growing parts of the embryo, suggesting that the cotyledonary reserve proteins are hydrolysed to amino acids which are then transported to the developing axis and incorporated into proteins (Oota et al., 1953; Larson and Beevers, 1965; Beevers,and Guernsey, 1966). The appearance of a new amino acid during germination which is absent from the dry seeds was shown by Virtanen et al., (1953), and the actual synthesis of new protein was also reported (Young and Varner, 1959; Young et al., 1960; Miege, 1970).

Danielson (1951) observed in peas that during germination there was a pronounced breakdown of globulins between 5 to 10 days, which is the period when the new plant begins to form leaves. Vicilin and legumin are broken down with the same speed, whereas albumin breakdown was slow and occurred at a constant rate. The work of Buzila (1969) with pea vicilin indicated that during seed germination the vicilin is split, suggesting that in the early stages of hydrolysis the reserve

protein is broken down to small fragments which subsequently split up into peptides or amino acids. In a more detailed study Juo and Stotzsky (1970) also reported that in kidney beans (Phaseolus vulgaris) the globulins and basic proteins were hydrolysed more rapidly than albumins. They also noticed the disappearance of a number of components of albumin fraction during early stages of germination, but several new components were detected about 8 days after germination, suggesting the changes in the synthesis of various enzymes during germination.

Catsimpoolas et al., (1968) and Daussant et al., (1969) have studied the metabolism of soybean and peanut globulins, respectively. They found that the components of the globulin fraction were degraded at different rates during germination. Further, Catsimpoolas et al., (1968) reported that although the electrophoretic mobility of the 11S globulin fraction changed during germination, there was no appearance of new immunological components, suggesting that no new protein subunits were produced during the reserve protein degradation.

Racusen and Foote (1971) studied glycoprotein breakdown in bean seed and found that germination for up to 114 hours had little effect on the glycoprotein or on the total soluble protein. Later (1973), the same authors reported that glycoprotein II of bean seeds decreased most rapidly between 7 and 10 days. Very recently, Ericson and Chrispeels (1973) reported that in the cotyledons of Phaseolus aureus glucosamine is bound to specific storage protein (legumin and vicilin) and are metabolised during germination.

The biochemical analysis indicating the depletion of protein during germination is confirmed by ultrastructural studies. Bain and

Mercer (1966) demonstrated that the protein content of the protein bodies declined during germination. Smith and Flinn (1967) indicated that during protein depletion the protein bodies originally swell then coalesce before finally fragmenting to produce a vacuole-like structure. A similar situation has been observed in peanuts (Bagley et al., 1963) and beans (Opik, 1966; Briarty et al., 1970). Ericson and Chrispeels (1973) reported that the glucosamine-containing glycoproteins are associated with protein bodies.

Proteolytic Enzymes

During germination the protein content of the cotyledons declines, and there was an increase in alcohol-soluble alpha amino nitrogen. At the same time the protein and alpha amino nitrogen content of the axis tissue increase. These observations suggest that during germination the reserve protein of the cotyledons were hydrolysed to amino acids, which are then incorporated into protein during axis growth. It is generally considered that the reserve protein in the cotyledons is hydrolysed by proteolytic enzymes. However, although there are many reports of proteolytic enzymes in seeds, the role of such enzymes in reserve protein metabolism has not been completely established.

Danielson (1951) demonstrated the presence of enzyme in extracts of peas which hydrolysed gelatin. However, it was indicated that the assayed proteolytic activity was greatest in extracts prepared from unripe peas. Soedigo and Gruber (1960) purified a protease from dry seeds which hydrolysed casein at pH 8.0; however, no physiological role was assigned to this enzyme. Young and Varner (1959) and Henshall

and Goodwin (1964) indicated that proteolytic activity did not increase during the period of germination, when protein was being depleted. However, Beevers and Splittstoesser (1968) and Beevers (1968) demonstrated that while peptidase activity is high in early germination, the proteolytic activity develops only in latter phases of germination.

Mergentime and Wiegand (1946) observed caseolytic activity in pea extracts at an optimum pH of 5.5, while Matile (1968) observed this at pH 4.2. However, Beevers (1968) demonstrated caseolytic activity in pea extracts with opitmum activity at pH 5.5 and 7.0. Recently Pusztai and Duncan (1971a) reported that the proteolytic and auto digesting activities in kidney bean extracts showed an optimum between 5.0 and 5.5.

Most of the characterization of proteases has been carried out with casein (Irving and Fontane, 1945; Mergentime and Wiegand, 1946; Young and Varner, 1959; Soedigo and Gruber, 1960; Beevers, 1968; Nakano and Asahi, 1974), gelatin (Danielson, 1951), bovine serum albumin (Garg and Virupaksha, 1970; Harvey and Oaks, 1974) p-nitrophenyl phosphate (Nakano and Asahi, 1974) and N, N-dimethyl albumin (Garg and Virupaksha, 1970).

Similar increases in proteolytic activity during germination have been reported in peanuts (Oota et al., 1953) beans (Pusztai and Duncan 1971) and maize (Harvey and Oaks, 1974). However, in peas the most rapid increase in caseolytic activity occurred after the onset of rapid depletion of cotyledonary protein (Beevers, 1968).

More recently, Nakano and Asahi (1972, 1973, 1974) reported that the pea cotyledons contain a membrane-bound protease which is

recovered in the microsomal fraction after differential centrifugation of whole homogenate in addition to cytosol protease and that membrane-bound protease activity increases during seed germination although cytosol protease activity does not.

In addition to the observed caseolytic activity which was demonstrated to increase during germination, Beevers (1968) reported that there were also enzymes present in the cotyledons which were capable of hydrolysing the synthetic peptides L-leucine paranitroamilide and alpha-benzoyl-DL-arginine paranitroanilide. The peptidases were present in the dry seed and their activity showed very little changes during the course of germination. Similar peptidase activity has been reported in barley seeds and they likewise show little change in activity as the seedling develops (Burger and Siegelman, 1966). In contrast Penner and Ashton (1966) reported that peptidase activity increased in squash cotyledons up to the third day following germination and then declined.

In spite of a lack of definitive information regarding the true role of hydrolytic enzymes during germination studies have been made regarding their origin. The work of Varner (1965) and Juliano and Varner (1969) demonstrated that in the barley endosperm system the hydrolytic enzymes ribonuclease, protease, and amylase are synthesized de novo following gibberellin application. In contrast to this evidence Shain and Mayer (1968) reported that other enzymes arise as a result of hydration or activation of inactive precursors.

In considering the synthesis of new enzymes during germination, it is apparent that the regulation of protein production can be

controlled at many loci. The recent evidence of Ihle and Dure (1969) indicates that the mRNA coding for protease production does not occur until imbibition. On the other hand, the inhibition of production of some enzymes by actinomycine D suggests that synthesis of these enzymes is dependent upon mRNA production.

CHAPTER III

MATERIALS AND METHODS

Plant Material

Pea seeds (Pisum sativum L., var. Burpeeana) were purchased from W. Atlee Burpee Company. Plants were grown from these seeds in a growth chamber in sterile vermiculite (Terralite) at a day temperature of 24°C, a night temperature of 13°C, a 12-hour day length, and a light intensity of 2500 ft-c.

Eight seeds were sowed per pot. The plants were irrigated every day with Hoagland's nutrient solution and were later thinned to four. Twenty days after germination they were staked and tagged. Flowering dates were noted daily. Pea pods were obtained from these plants during different periods of development. After harvesting, the testa and embryonic axis were removed from the seed. The cotyledons were homogenized and the homogenate was used for protein fractionation. For germination and enzyme studies the seedlings were grown in the dark in sterile vermiculite and watered with deionized water. The cotyledons were obtained from these seedlings during different days of germination.

Analytical Methods

Protein fractionation. Proteins were isolated and fractionated from the cotyledons by a procedure modified from Danielson (1949) Fig. 1.

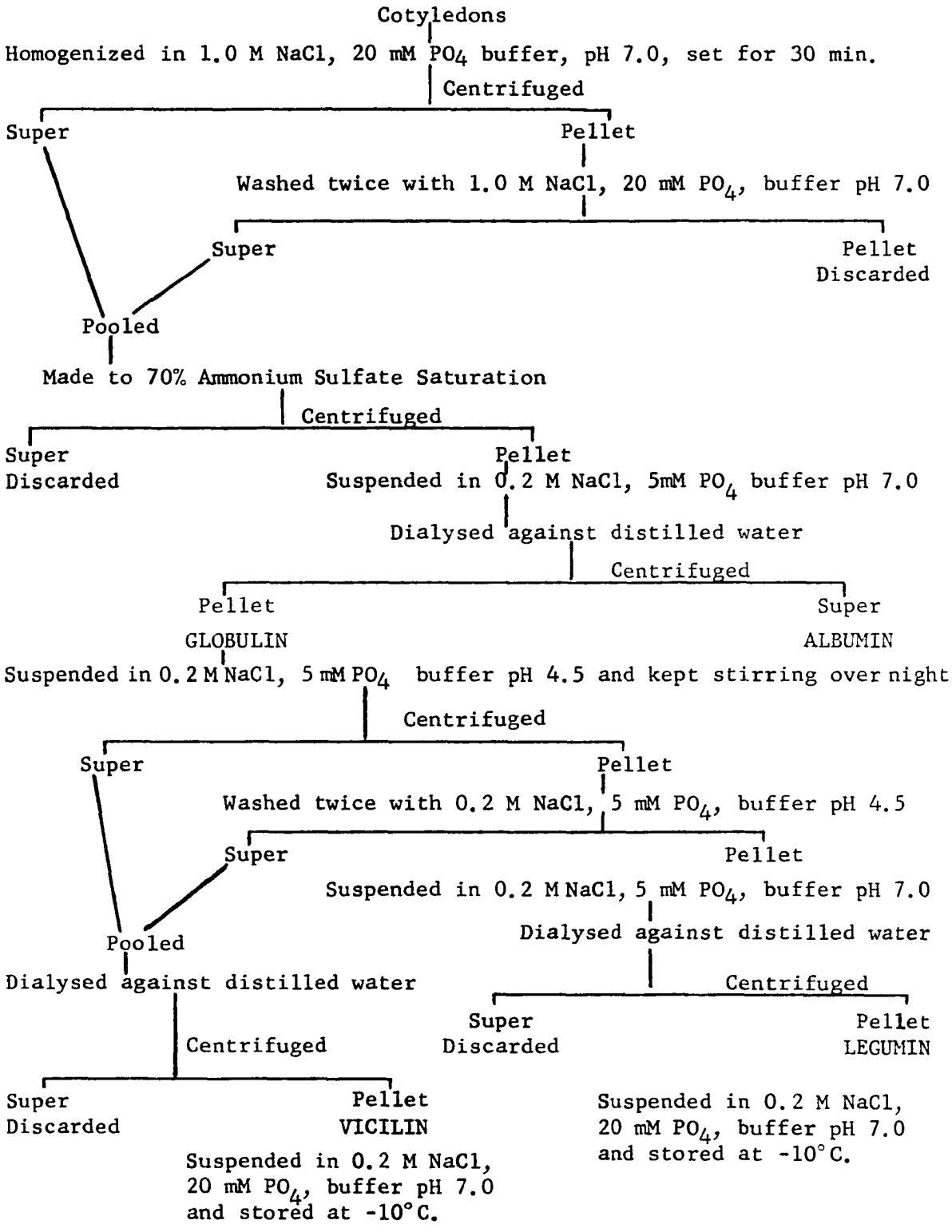


Fig. 1. Flow sheet of protein extraction and fractionation.

The cotyledons were homogenized in cold 1.0 M NaCl, 20 mM phosphate buffer, pH 7.0 with a VirTis tissue homogenizer at high speed for two minutes and at low speed for eight minutes. The homogenate was stirred for 30 minutes and then centrifuged at 20,000 x g for 15 minutes. The resulting pellet was extracted twice with the same buffer. The three supernatants were pooled and made to 70% saturation by adding solid ammonium sulfate $[(NH_4)_2SO_4]$ and kept at 4°C. After one hour the mixture was centrifuged at 30,000 x g for 20 minutes. The supernatant was discarded and the pellet suspended in 0.2 M NaCl, 5 mM phosphate buffer pH 7.0 and dialysed against distilled water for 2 days.

The dialysates were centrifuged at 20,000 x g for 15 minutes. The supernatant fraction was designated as albumin and the pellet as globulin. The globulin fraction was further suspended in 0.2 M NaCl, 5 mM phosphate buffer pH 4.5 and kept stirring overnight at 6°C. The globulin suspension was centrifuged at 3,000 x g for 10 minutes. The pellet was washed twice with the same suspension buffer and centrifuged. The three supernatants were pooled and dialysed against distilled water for two days. The material precipitated during dialysis is vicilin. The remaining pellet from the globulin fraction was suspended in 0.2 M NaCl, 5 mM phosphate buffer, pH 7.0 and dialysed against distilled water for two days. The dialysate was centrifuged at 20,000 x g for 15 minutes. This fraction is legumin. Legumin and vicilin were stored at -10°C or lyophilized and stored at -10°C, as necessary. The resulting three fractions, viz., albumin, legumin, and vicilin were used for further characterization. The temperature was held at 4°C during all the protein isolation steps.

Protein determination. Protein content in different fractions (Total protein, albumin, globulin, legumin and vicilin) was determined by the method of Lowry et al., (1951) with Bovine Serum Albumin as a standard.

Gel electrophoresis. Gel electrophoresis was performed according to the method of Davis (1964). The 7.5% gels (6 x 90 mm) were prepared by mixing 4 ml of Tris-HCl buffer (3.63% Tris, 4.8% 0.1 N HCl, 0.046% N, N, N', N'-tetramethyl ethylenediamine, pH 8.9), 8 ml of acrylamide solution (3.0% acrylamide, 0.08% N, N'-methylene bisacrylamide, 0.0015% potassium ferricyanide $[K_3 Fe(CN)_6]$, 16 ml freshly prepared ammonium persulfate (0.14%) and 4 ml of deionized water. The mixture was deaerated and pipetted into plexiglass tubes and allowed to polymerize. The upper and lower chambers of the electrophoresis apparatus were filled with reservoir buffer (0.576% glycine, 0.12% Tris, pH 8.3). Two to three drops of bromophenol blue were added to the upper reservoir buffer. The cathode was connected to the upper chamber and the anode to the lower chamber.

The protein samples suspended in 0.2 M NaCl, 20 mM phosphate buffer, pH 7.0 were made to 5% sucrose concentration and 100 to 200 μg of protein applied per gel. Electrophoresis was run at a constant current of 4 m.A/tube for 2 hours or until the tracking dye reached approximately one centimeter above the bottom of gel. After electrophoresis the gels were stained with 1% Amido black stain in 7.5% acetic acid for one hour. The gels were destained electrophoretically with 7.5% acetic acid. The gels were scanned in a Gilford instrument linear transport system at 620 nm.

Sodium dodecyl sulfate (SDS) gel electrophoresis. The proteins were dissociated essentially by the method of Palmiter et al., (1971). The protein was precipitated from solution by the addition of an equal volume of 10% trichloroacetic acid. The protein precipitate was collected by centrifugation and washed twice with 5% tichloroacetic acid. The TCA precipitated protein was dissociated by mixing the sample with a glass rod for 1 to 5 minutes, while heating in a boiling water bath, in a freshly made solution containing 1.2% of Tris (pH unadjusted), 1.5% of dithiothreotol, 1.0% sodium dodecylsulfate and 20% glycerol to give a final protein concentration of 2 mg/ml.

The sample was applied to the top of an acrylamide gel (6 x 90 mm) made by the procedure of Weber and Osborn (1969). The gel solution was made by mixing 15 ml of reservoir buffer (3.86% Na_2HPO_4, 0.78% $NaHPO_4$, 0.2% sodium dodecyl sulfate pH 7.1), 10 ml of acrylamide solution (11.1% acrylamide, 0.3% N, N'-methylene bisacrylamide), 0.025 ml of N, N, N', N'-tetramethylenediamine and 1.5 ml of freshly made ammonium persulfate (15 mg/ml).

The reservoir chambers were filled with reservoir buffer after diluting 1:1 with deionized water and the sample was laid over the gels and electrophoresed in a Quickfit-Instruments electrophoresis apparatus. The cathode was connected to the upper chamber and the anode to the lower chamber. A voltage of 2 mA/tube were applied for the first 20 minutes, then the amperage was increased to 10 mA/tube. Total running time was about 4 hours. The gels were removed from the plexiglass and fixed in 20% sulfosalicylic acid for 16 to 18 hours at 40°C and stained with 0.25% Coomasie blue in water for 3 hours. The gels were destained with repeated washings of 7.5% acetic acid, at 40°C for 2 days and

scanned in a Gilford linear transport system at 620 nm.

Neutral and amino sugars. Sixty milligrams of protein were hydrolysed with 1 N HCl for 1 hour, in sealed tubes in an autoclave at 124°C. After hydrolysis the samples were evaporated in a rotary evaporator in vacuo and the residues were taken up in 2 ml of 0.3 N HCl and applied to a column (8 x 70 mm) of Dowex 50 (200, H^+ form) resin. The sugars were eluted with a total volume of 20 ml, of 0.3 N HCl in 2 ml fractions (Gardel, 1953).

The neutral sugar content in the initial 3 fractions was determined by the Anthrone-Sulfuric acid method of Yemm and Willis (1954) The remaining fractions were used for amino sugar determination. The amino sugars were estimated by the modified Rondole-Morgan method (1955). The eluates were neutralised to the phenolpthalein end point. Then 1 ml of acetylacetone reagent (2% acetyl acetone in 0.5 N Na_2CO_3) was added. The samples were heated in boiling water for 20 minutes and then cooled to room temperature. One ml of Ehrlich's reagent (0.8 gm recrystalised p-dimethylaminobenzaldehyde in 30 ml ethanol and 30 ml concentrated HCl) was added and heated for 10 minutes at 60 to 65°C; cooled and the optical density was read at 530 nm.

Identification of sugars. The proteins were hydrolysed as described earlier and eluted through the column. The eluants were pooled and concentrated. One hundred microliters of concentrated samples were spotted on Whatman No. 1 chromatogram paper, 2.5 inches apart and separated by single dimensional descending chromatography using the solvent system n-propanol-ethyl acetate-water (7:4:2) as

recommended by Koshiyama (1966). After 18 hours the paper was dried and chromatographed once again in the same direction. The papers were dried at 90°C for 5 minutes and sprayed with alkaline acetyl acetone reagent [0.5 ml of solution B (5 ml 50% aquous KOH in 20 ml ethanol) in 10 ml solution A (1% acetyl-acetone in butanol)] and dried at 105°C for 5 minutes. The papers were again sprayed with Ehrlich's reagent (1 g p-dimethylaminobenzaldehyde in 30 ml ethanol and 30 ml concentrated HCl and diluted with 180 ml butanol before use). The Rf values were calculated and compared with the standard sugars (Patridge, 1948).

Characterization of ^{14}C-glucosamine labelled protein. This study was aimed to determine the sugar content of legumin and vicilin subunits. Five microliters (1 μc) of ^{14}C-glucosamine (specific activity 58 mci/m mole) were injected into each cotyledon of peas still in the pod attached to the plant and incubated for 6 hours in the light. The cotyledons were collected and homogenized, and protein isolated and fractionated as described earlier.

^{14}C-glucosamine labelled legumin and vicilin were dissociated with sodium dodecyl sulfate (SDS) and dithiothreotol, and subjected to SDS gel electrophoresis as described earlier. After optical scanning the gels were frozen with dry ice and the gels were transversely sectioned into 1 mm thick slices by means of a Mickel gel slicer. The slices were placed in the scintillation vials and 1 ml of solution (containing 10% piperidine, 1 mM EDTA in distilled water) was added and dried in an oven at 60°C. After 18 hours or after complete evaporation, 0.5 ml of water was added to each vial and the gel slices were allowed

to hydrate. After one hour 12 ml of scintillation fluid (8 g PPO in 1 liter Triton x-100 and 2 liters scintillation toluene) were added. Subsequently, the radioactivity in the slices was determined on a Beckman LS-100 liquid scintillation counter.

The presence of radioactivity in glucosamine of the protein was confirmed by hydrolysing ^{14}C-glucosamine labelled legumin and vicilin with 1N HCl. The hydrolysates were passed through the Dowex 50 (200, H^+ form) resin and the sugars were separated by paper chromatography as described earlier. The chromatographed strips were cut into one inch long pieces and each piece was further cut into smaller pieces and placed in scintillation vials. The cut pieces were eluted with one ml of water and the associated radioactivity was measured. The radioactivity was compared with the Rf values of standard and the sugars with which the radioactivity was associated were determined.

The influence of cycloheximide on incorporation of amino sugars and neutral sugars into protein. Pea pods were chilled immediately after harvest. Cotyledons were sliced to 1 to 2 mm thickness and kept at 4°C. The cotyledon slices were rinsed twice with 0.02 M citrate buffer, pH 5.5. Sixteen cotyledon slices were transferred into 5 ml incubation medium containing ^{14}C-glucosamine or ^{14}C-mannose and ^{3}H-amino acids with and without cycloheximide (50 μg/ml) in 0.02 M citrate buffer, pH 5.5.

The cotyledon slices were incubated in 25 ml flasks, in a shaker bath at 30°C for 4 hours. After 4 hour incubation, the slices were rinsed thoroughly with cold deionized water and homogenized. The protein extraction and fractionation was carried out as described

earlier. The experiment was performed in two replications. Aliquots were taken from protein fractions and counted for radioactivity in a Beckman LS-100 Scintillation counter.

Preparation of crude enzyme extract. Pea seeds were grown in sterile vemiculite in the dark. The cotyledons were obtained from the seedlings on different days after germination. After removing the seed coat and embryo-axis, enzyme extracts were obtained by homogenizing the cotyledons in a VirTis homogenizer, with 0.02 M sodium phosphate buffer, 5 mM 2-mercaptoethanol, pH 7.2 or 0.02 M sodium phosphate buffer, pH 7.2 using a tissue to buffer ratio of 1:4 (W/V). The homogenates were squeezed through a layer of cheese cloth and miracloth (Calbiochem) and centrifuged at 20,000 x g for 20 minutes. Protease activity in the supernatant was determined as described below.

Protease assay. Proteolytic activity was assayed using ^{3}H-albumin or ^{3}H-legumin or ^{3}H-vicilin of pea cotyledons as substrate. One ml of crude enzyme extracts were incubated with 0.2 ml ^{3}H-legumin (4.4×10^{-5}cpm/ml) or 0.2 ml ^{3}H-vicilin (2.7×10^{-5} cpm/ml) or 0.5 ml ^{3}H-albumin (1.1×10^{-5}cpm/ml) and 1.0 ml of 0.2 M sodium citrate buffer, pH 5.0, in a shaker bath at 40°C for 3 hours. The reaction was terminated by the addition of 0.8 ml (in ^{3}H-legumin and ^{3}H-vicilin) and 0.5 ml (in ^{3}H-albumin) of 1% casein, pH 7.0, followed by 1.0 ml of 20% trichloroacetic acid, to give a final volume of 4.0 ml. The reaction which was stopped with 20% TCA immediately after the addition of enzyme, served as the zero time control. After addition of TCA, the samples were kept overnight at 6°C and the TCA insoluble material was removed

by centrifugation. One ml aliquot was taken from the TCA soluble supernatant and the radioactivity was measured.

Effect of pH. The effect of various pH's was determined by incubating the enzyme as described earlier, except that reaction buffers of different pH's were used. For reaction buffer from pH 3.0 to 6.0, 0.2 M sodium citrate buffer was used, while from 6.5 to 9.0, 0.2 M sodium phosphate buffer was used.

CHAPTER IV

RESULTS AND DISCUSSION

Characterization of Seed Proteins

Extraction of mature dry peas with 1 M NaCl and precipitation of the solubilized protein, with ammonium sulfate indicated that there were 14.2 mg of protein per cotyledon. Other analyses in which the total protein content of the pea cotyledons was measured, indicated that the protein content was 15.7 mg per cotyledon. Thus saline extraction and ammonium sulfate precipitation effectively recovered over 90% of the protein in peas. Fractionation of the protein indicated that 40% was water-soluble albumin with the remainder being saline-soluble globulin. The globulin fraction was further subdivided into legumin and vicilin. The legumin, a globulin fraction, which is insoluble in 0.2 M NaCl at pH 4.5, was the major component. Globulin fractionation indicated that in the mature seed the ratio of legumin to vicilin was about 3:1 by weight (Table I). It was consistently found that during the globulin fractionation, the sum of the vicilin and legumin component did not total the globulin fraction. The reason for this discrepancy is not known, but may be due to the incomplete precipitation of the vicilin, following dialysis of the relatively dilute solution. During the fractionation procedure the legumin fraction became less soluble in dilute saline. The final legumin fraction was characteristically light green in color whereas vicilin was white.

TABLE I

Protein content of different fractions of cotyledon extracts from mature peas.

	mg per cotyledon			
TOTAL PROTEIN	ALBUMIN	GLOBULIN	LEGUMIN	VICILIN
14.20 mg	4.5 mg	9.20 mg	6.70 mg	2.09 mg

Gel Electrophoresis

When the albumin fraction was subjected to acrylamide gel electrophoresis at pH 8.3, it was found to be composed of at least 13 components (Fig. 2). This is expected, since it has been indicated (Danielson, 1956) that the albumin fraction contains the enzymatic proteins. Others have demonstrated, the presence of many components in the albumin fraction, of bean (Sayonova et al., 1971) and field pea (Pisum arvense) (Kretovich et al., 1954).

The legumin component of globulin produced one slow migrating band following gel electrophoresis. In contrast to the distinct protein bands produced by electrophoresis of the albumin fraction, the legumin band was diffuse suggesting some heterogeneity (Fig. 2). Others (Danielson, 1950; Brand and Johnson, 1956; Johnson and Richards, 1962) have estimated the molecular weight of legumin from peas to be 331,000 to 400,000. Vicilin migrated more rapidly than legumin, during gel electrophoresis and produced a much more diffuse band. The molecular weight of vicilin from peas has been estimated to be 186,000 (Danielson, 1950).

These high molecular weights and diffuse banding of the proteins after electrophoresis suggest that the reserve proteins may be heterogeneous or composed of subunits. The subunit components of the proteins was demonstrated by electrophoresis in SDS gels, of the products arising from treatment of the proteins with sodium dodecyl sulfate (SDS) and dithiothreotol. Legumin was found to produce 3 major bands and two minor bands, while vicilin yielded 5 major bands (Fig. 3). Grant and Lawrence (1964) indicated that the legumin and vicilin

ALBUMIN LEGUMIN VICILIN

Fig. 2. Gel electrophoresis of albumin, legumin and vicilin from mature pea cotyledons.

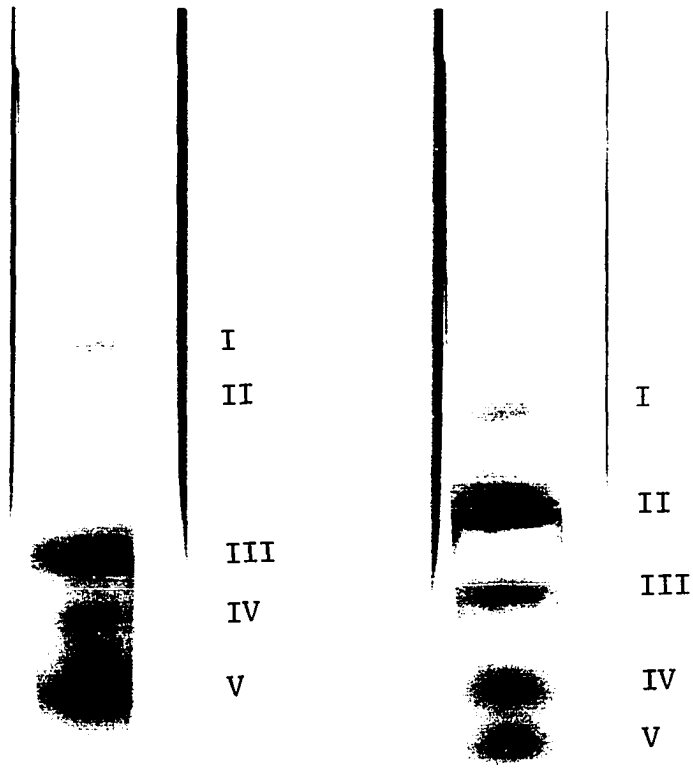


Fig. 3. Sodium dodecyl sulfate gel electrophoresis of legumin and vicilin from mature pea cotyledons.

fractions from peas, could be dissociated into 6 and 4 subunits respectively. The legumin from peas appears to be similar to that derived from Vicia faba, which also dissociated into three major and two minor subunits. However, Bailey and Boulter (1970) consider that the two minor subunits are impurities or by-products of disulphide coupling reactions between the subunits, due to the absence of sulfhydryl blocking agents. However, the evidence from the current study suggests that the two minor components might be real subunits. If these two minor subunits were artifacts, they would be expected to occur in all of the preparations made in the same manner. However, as is indicated later, the two minor subunits which migrate slowly during SDS gel electrophoresis, are not present in legumin preparations from young developing cotyledons and are broken down independently during germination. The nature of linkage between the component subunits has not been established. In some instances sulfhydryl moieties of cysteine residues, in component polypeptide chains are believed to interact and form disulphide -S-S- bridges between the component subunits. However, the low cysteine content of the globulins (Grant and Lawrence, 1964) tends to eliminate the possibility of cross linking in this manner. Similarly, the low cysteine content and the dissociation of the protein in 1.5% dithiothreotol would restrict the possibility of cross linkages between subunits, making it unlikely that the minor subunits are the products of recombination.

Vicilin produced five major subunits after dissociation with SDS and DTT. Among the five subunits, component II predominates, while subunit V is present in lowest amounts. However, in contrast, Wright

and Boulter (1972) and Ericson and Chrispeels (1973) reported that the vicilin from Vicia faba and Phaseolus aureus contains four subunits.

Glycopeptide

Analysis of the material resulting from hydrolysis of legumin and vicilin in 1 N HCl demonstrated the presence of amino sugars and neutral sugars. It was found (Table II) that legumin contained 1.25% neutral sugars and 0.1% amino sugars, while vicilin contained 0.3% neutral sugars and 0.2% amino sugars. The levels are considerably lower than those usually encountered in animal tissue and are less than that reported for the glycoprotein component of Phaseolus vulgaris (Pusztai and Watt, 1970; Racusen and Foote, 1971). However, they more nearly approach those reported by Ericson and Chrispeels (1973) in Phaseolus aureus, and Pusztai (1964) originally indicated that peas were lower in glucosamine content than many other seeds. In many glycopeptides the attachment of the sugars to the peptide occurs through acetyl glucosamine and asparagine residues, with the neutral sugar components attached as oligosaccharides to the glucosamine (Spiro, 1970). Pusztai (1966) has indicated that the glycoprotein from Phaseolus vulgaris was composed of D-glucosamine and D-mannose with small amounts of arabinose, xylose and fucose. Koshiyama (1966), Racusen and Foote (1971) and Ericson and Chrispeels (1973) have similarly detected the occurrence of glucosamine and mannose in glycopeptides from soybeans (Glycine max), beans (Phaseolus vulgaris) and Phaseolus aureus respectively. The glucosamine is in all probability present in the glycopeptides as acetyl glucosamine, in which the acetyl group is released during acid hydrolysis. In the present study it was found that the

TABLE II

Percent sugar content of protein
from mature pea cotyledons

LEGUMIN:

Neutral Sugars	Amino Sugars
1.25%	0.10%

VICILIN:

Neutral Sugars	Amino Sugars
0.32%	0.25%

hydrolysates of legumin contained glucose, mannose and glucosamine, while vicilin hydrolysates contained mannose and glucosamine (Table III). Thus the reserve components from peas appear to be similar but not identical to those from other legumes.

On the basis of the neutral sugar and amino sugar analysis, it appears that vicilin has several glucosamine residues with relatively short chain oligosaccarides attached. In contrast, the lower amino sugar and greater neutral sugar content of legumin suggest that this glycopeptide has fewer glucosamine residues with longer chain oligosaccharides attached.

Characterization of ^{14}C-Glucosamine Labelled Protein

The low amino sugar and neutral sugar content of these reserve proteins compared to those reported in glycopeptides of animal tissues, might be due to the fact that not all of the subunits in the complex reserve proteins are glycopeptides. To investigate this possibility the pea proteins during different days of seed development were labelled with ^{14}C-glucosamine and recovered legumin and vicilin were dissociated with SDS and dithiothreotol and separated by SDS gel-electrophoresis. The gels were sliced and the distribution of radioactivity determined. The results (Fig. 4) show that most of the radioactivity in legumin from 18 day peas was associated with the subunit V, suggesting that in early stages of development most of the glucosamine is incorporated in the subunit V. At later stages of seed development the incorporation of glucosamine was still predominantly occurred in the subunit V.

In contrast, the radioactivity from glucosamine was incorporated

TABLE III

Sugar composition of legumin and vicilin

I. LEGUMIN:

Neutral Sugars:

Glucose

Mannose

Amino Sugars:

Glucosamine

II. VICILIN:

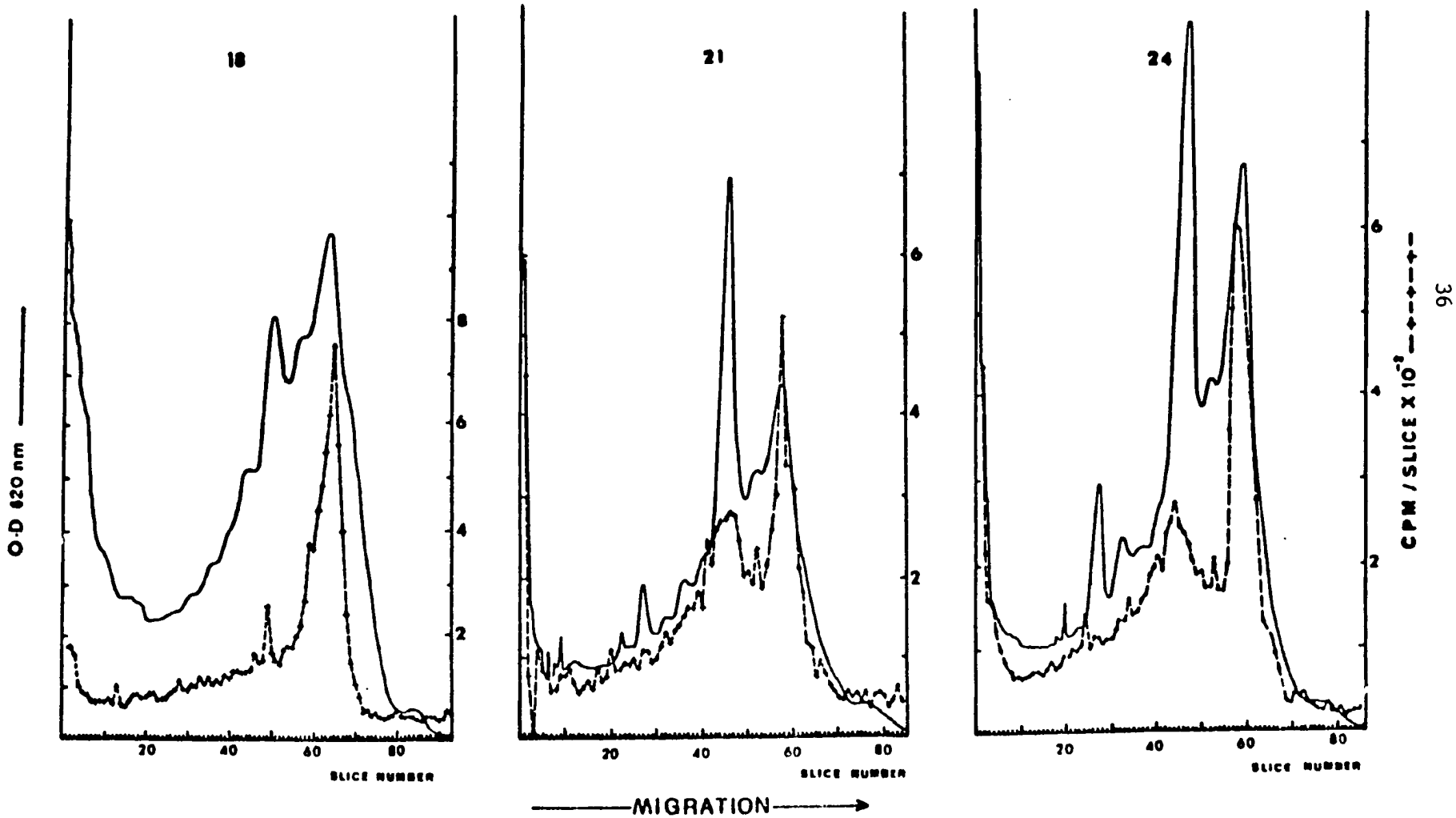
Neutral Sugars:

Mannose

Amino Sugars:

Glucosamine

Fig. 4. The distribution of radioactivity following SDS gel electrophoresis of legumin from cotyledons, injected with ^{14}C-glucosamine during different days of seed development (18, 21, and 24 day).



into all the subunits of vicilin (Fig. 5), from different days of seed development suggesting that all the subunits are glycopeptides and glucosamine is incorporated in all the subunits from the early stages of development. However, subunits I and II appear to incorporate the greatest amount of radioactivity. This is in agreement with the analytical data which indicated more glucosamine in vicilin than legumin.

That the radioactivity associated with the legumin and vicilin components was due to glucosamine and not products arising from its metabolism, was confirmed by hydrolysing the proteins with 1 N HCl. The hydrolysates were passed through Dowex 50 resin and the eluates were concentrated and the sugars were separated by paper chromatography as described in Methods, and compared with the standards to identify the sugars with which the radioactivity was associated. Such experiments indicated that 70% of the radioactivity in the labelled protein was associated with glucosamine.

Time of Attachment of Carbohydrate Units to the Peptide

Since it has been established that pea reserve proteins are glycoproteins and that all the subunits are not enriched with carbohydrates, the next question which arose was whether the carbohydrates were attached during the peptide synthesis or after the peptide synthesis.

The pea cotyledon slices were incubated in a medium containing ^{14}C-glucosamine or ^{14}C-mannose and ^{3}H-amino acids with and without cycloheximide. Cycloheximide inhibits protein synthesis on 80S ribosomes. It was observed (Table IV) that amino acid incorporation into legumin was inhibited by 95 to 100% by cycloheximide whereas

Fig. 5. The distribution of radioactivity following SDS gel electrophoresis of vicilin from cotyledons injected with ^{14}C-glucosamine during different days of seed development (18, 21, and 24 day).

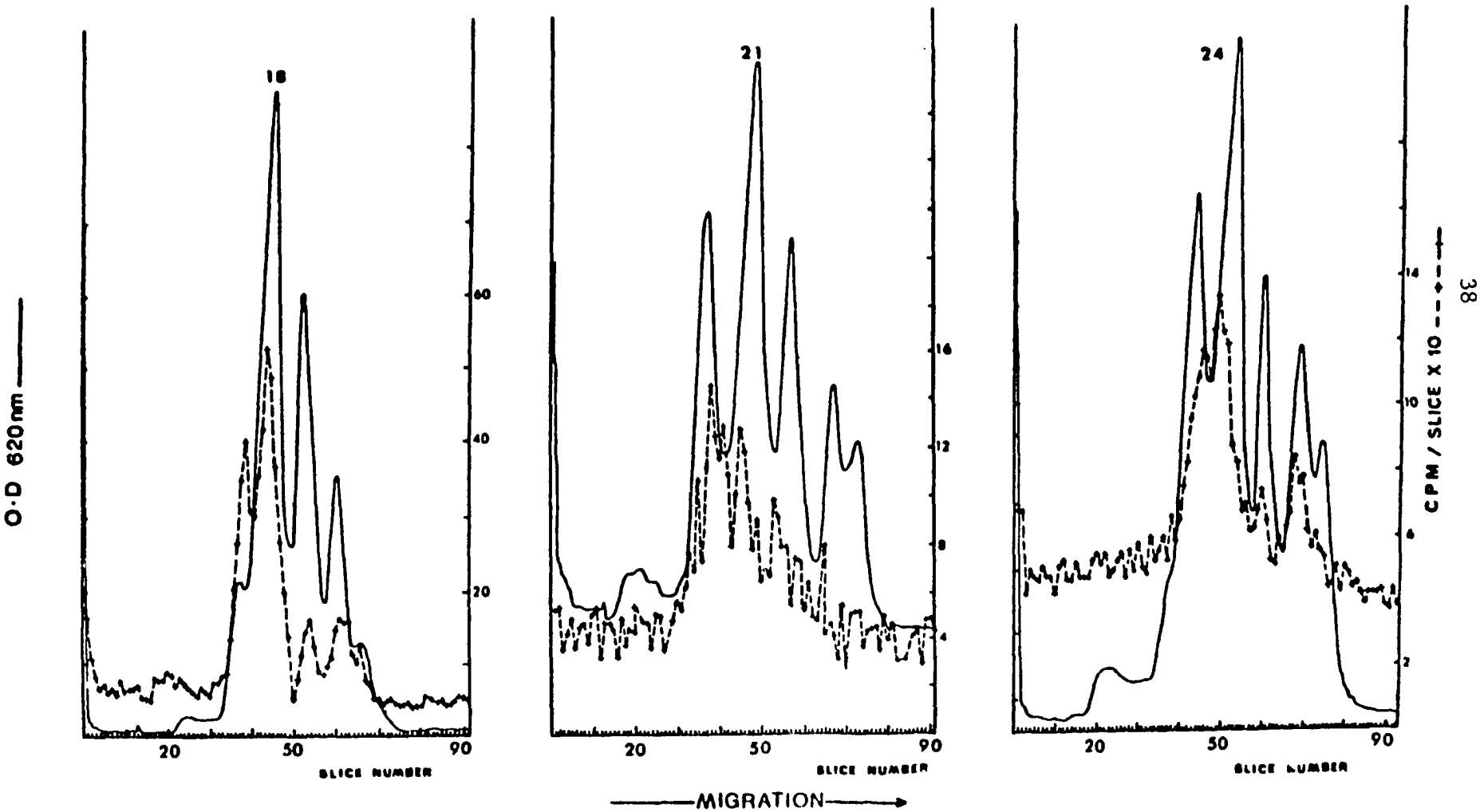


TABLE IV

Influence of cycloheximide on incorporation of ^{3}H-amino acids and ^{14}C-glucosamine or ^{14}C-mannose into protein

	CPM			
	R I		R I I	
	^{14}C	^{3}H	^{14}C	^{3}H
LEGUMIN:				
^{3}H-amino acids + ^{14}C-glucosamine	54460	23730	33600	17430
^{3}H-amino acids + ^{14}C-glucosamine + cycloheximide (50 µg/ml)	35700 (35%)	----- (100%)	29960 (11%)	840 (95%)
^{3}H-amino acids + ^{14}C-mannose	13580	20650	10360	9233
^{3}H-amino acids + ^{14}C-mannose + cycloheximide (50 µg/ml)	8680 (36%)	1610 (92%)	6160 (40%)	828 (91%)
VICILIN:				
^{3}H-amino acids + ^{14}C-glucosamine	3240	1822	2100	1037
^{3}H-amino acids + ^{14}C-glucosamine + cycloheximide (50 µg/ml)	840 (74%)	106 (94%)	720 (66%)	103 (90%)
^{3}H-amino acids + ^{14}C-mannose	1440	1530	1122	1319
^{3}H-amino acids + ^{14}C-mannose + cycloheximide (50 µg/ml)	600 (58%)	94 (94%)	602 (46%)	104 (92%)

Figures in parenthesis indicate percent inhibition over the control.

glucosamine incorporation was inhibited by only 11 to 35%. Thus glucosamine incorporation was less sensitive to inhibition by cycloheximide than amino acid incorporation. Similarly amino acid incorporation was inhibited by 91 to 92% by cycloheximide; however, mannose incorporation was inhibited by only 36 to 40%.

Amino acid incorporation in vicilin was greatly inhibited (90 to 94%) by cycloheximide. However, the glucosamine and mannose incorporation was inhibited by 66 to 74% and 40 to 58% respectively. Here again, it is apparent that amino acid incorporation is more sensitive to inhibition by cycloheximide than is the incorporation of glucosamine and mannose.

If carbohydrate attachment occurred during peptide synthesis, inhibition of peptide production should prevent carbohydrate incorporation into the proteins. However, the data indicate that this is not the case and it is concluded that the carbohydrate residues are attached after the complete peptide was synthesized. Significantly, the incorporation of glucosamine and mannose into vicilin was inhibited by cycloheximide to a greater extent than was the incorporation of carbohydrates into legumin. Since the incorporation of sugars into the protein depends on the availability of acceptor sites, it is clear that the amount of sugar incorporated will depend on the rate of protein synthesis. In this regard it was observed (Table IV) that the amino acid incorporation into legumin was ten times higher than that into vicilin, suggesting that legumin synthesis exceeds that of vicilin. Thus, a low rate of synthesis of vicilin will reduce the acceptor sites present for glycosylation and this inhibition of protein synthesis will

effectively reduce the glycosylation. The observation that the proteins are glycosylated after peptide chain synthesis is consistent with the observation by Lew and Shannon (1973) indicating that in horse radish peroxidase the carbohydrate units are attached after peptide synthesis.

Seed Development

Changes in protein content. Cotyledons of different ages were obtained from developing pods, homogenized and the protein was fractionated as described in Methods. The findings (Fig. 6) show that a small amount of protein was present (0.32 mg/cotyledon) at early stages (12 days after flowering) of seed development. However, maximum protein deposition occurred between day 18 and 27 after flowering. When the protein was fractionated and individual components studied, it was observed (Fig. 6) that albumin synthesis occurred up to day 24 and after day 24 albumin deposition declined. However, globulin deposition was continued up to day 27. It appears that legumin and vicilin were synthesized independently with legumin deposition predominating the vicilin deposition. This contrasts with Phaseolus aureus where vicilin is the principal globulin (Ericson and Chrispeels, 1973). Deposition of legumin and vicilin occurred up to day 27.

Similar increases in albumin and globulin fractions during seed development were recorded by Danielson (1952) in peas, Bailey et al., (1970) in Vicia faba, and Klimenko (1972) in peas, lentil and Cicer arietinum. Millered et al., (1971) found that in Vicia faba, legumin was detected in young cotyledons and there was a sharp increase in legumin accumulation during cotyledon development. Wright and Boulter (1972) reported that vicilin was formed prior to legumin in

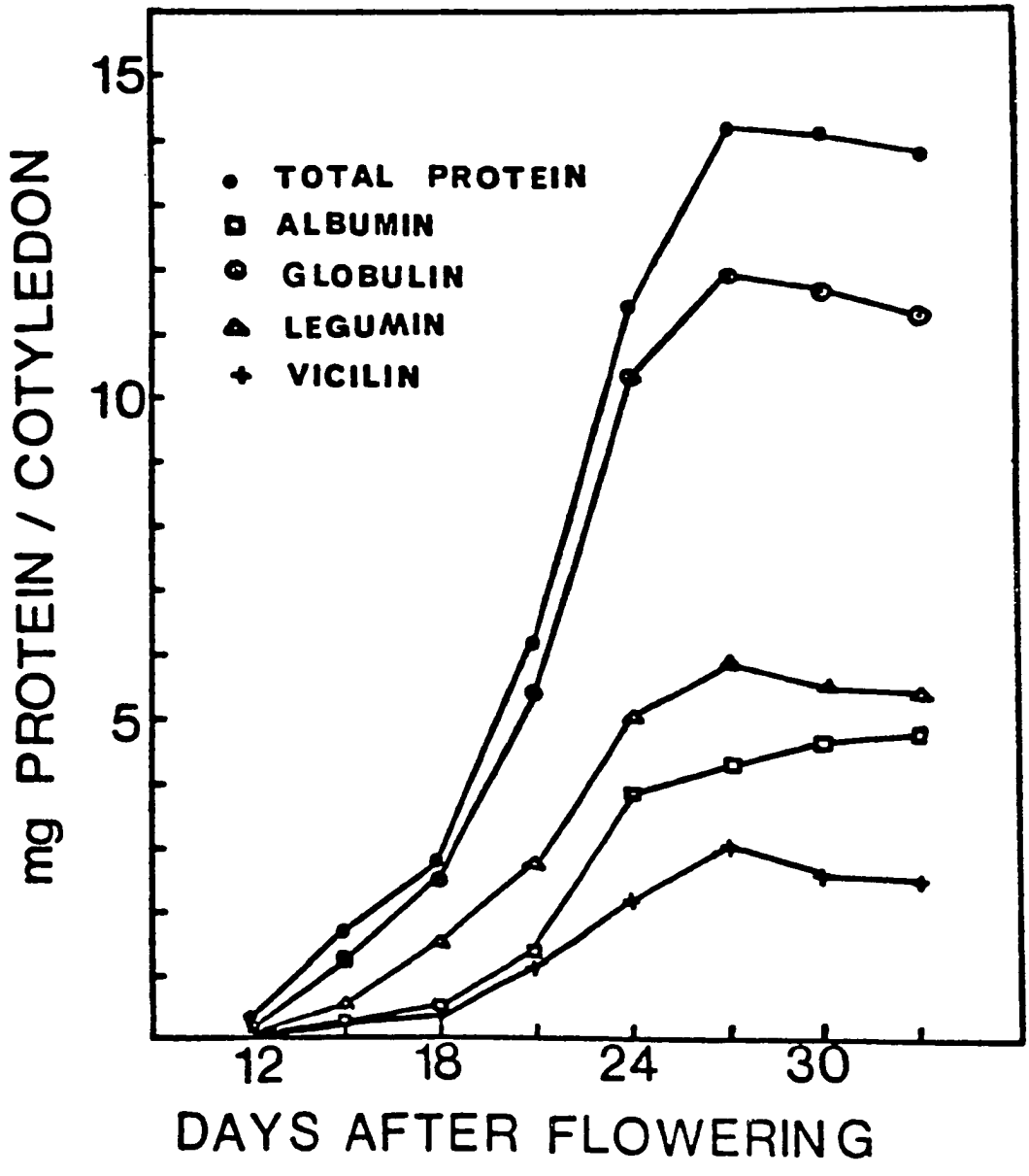


Fig. 6. Changes in protein content of various fractions from pea cotyledons during seed development.

Vicia faba although the rate of legumin synthesis was faster, so that in the mature seed the ratio of legumin to vicilin was about 4:1 by weight.

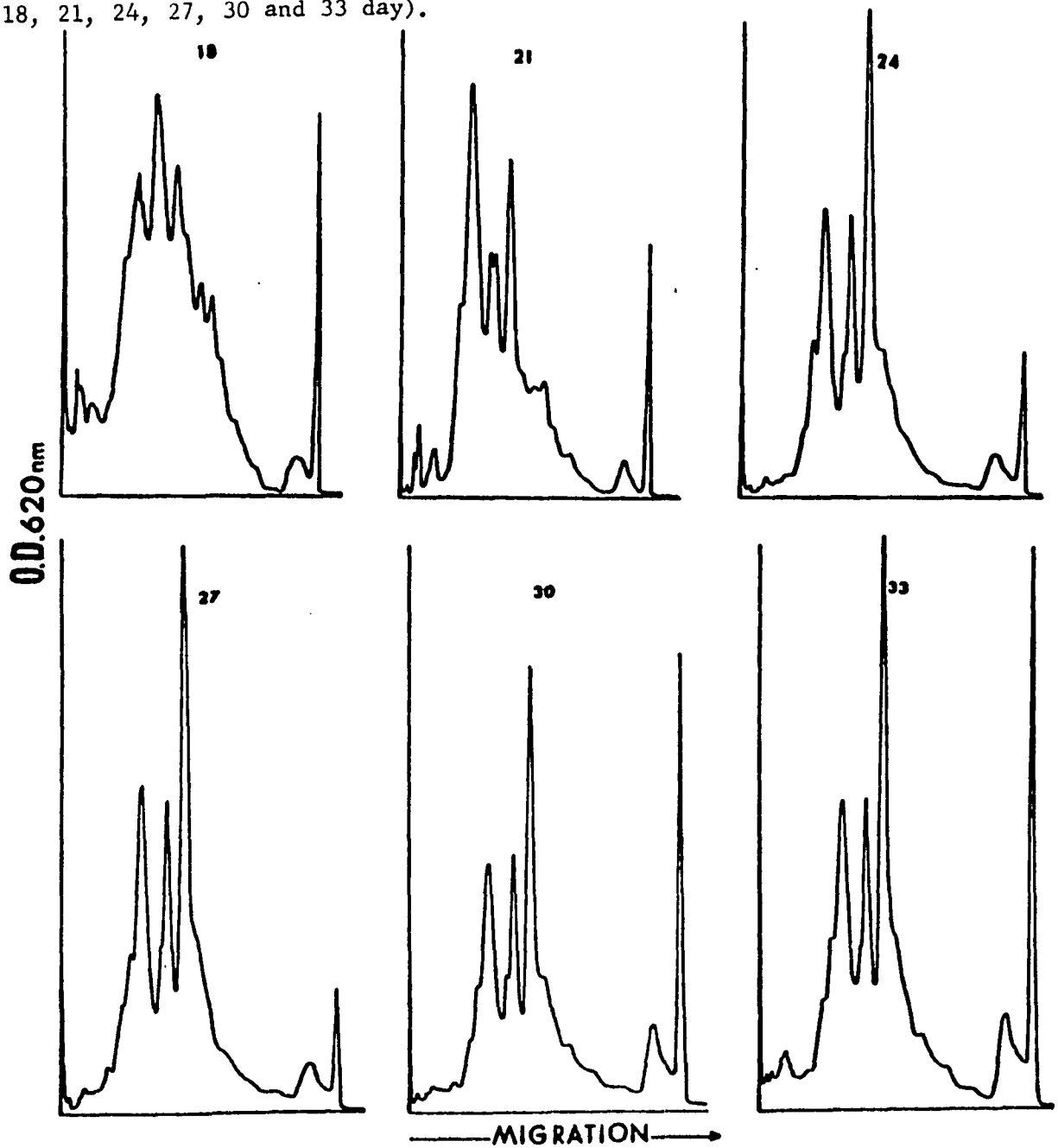
The present observation that globulin content exceeded the albumin content in the young cotyledons, is similar to that reported by Danielson (1952). However, Beevers and Poulson (1972) reported that initially the albumin content was greater than that of globulin. These discrepancies may be attributed to a more rapid maturation of the peas in Oklahoma compared to Illinois and a modification in the extraction procedure. By using 1 M NaCl in comparison to the 0.5 M NaCl, used Beevers and Poulson (1972) the fractionation of albumin and globulin by dialysis against distilled water was much more efficient.

The albumin, legumin and vicilin fractions were further investigated by gel electrophoresis, to determine if there are any changes in the composition of protein fractions during seed development.

Gel electrophoresis of albumin. When the albumin prepared from cotyledons of different developmental age were subjected to gel electrophoresis, considerable variation in the albumin composition was found (Fig. 7). Some protein bands became less prominant, while other new protein bands appeared. Changes in the albumin composition occurred up to day 24, during which period albumin deposition was active. After day 24 no great alterations in composition were noticed.

Since it was postulated that the albumin fraction contains the enzyme proteins, changes in composition are to be expected (Danielson, 1956), reflecting changes in enzyme composition associated with changes in metabolism of the developing cotyledon.

Fig. 7. Changes in the albumin composition during different days of seed development (18, 21, 24, 27, 30 and 33 day).



SDS gel electrophoresis of legumin and vicilin. When legumin from developing cotyledons was dissociated by SDS and DTT and investigated by SDS gel electrophoresis, it was found (Fig. 8) that the subunit composition of legumin changed during seed development. It appears that among the five subunits of legumin, subunit V was produced earlier followed by subunit III. By day 24 all the five subunits that are found in mature cotyledons were present. After day 27 there was not much change in the subunit ratios. However, Wright and Boulter (1972) reported that in Vicia faba subunit structure of legumin did not change during development.

Like legumin, vicilin also showed changes in the subunit structure during development (Fig. 9). It was found that subunit II, III and IV were present in vicilin preparations from young cotyledons. All the five subunits found in vicilin from mature cotyledons were present by day 24, again after day 27 there was not much change in the subunit composition. These findings are similar to those of Wright and Boulter (1972), who found that the subunit structure of Vicia faba vicilin changed during development. However, as indicated previously vicilin from Vicia faba contains only 4 subunits.

Changes in sugar content of protein during seed development. The sugar content of legumin and vicilin from developing pea seeds was studied, after hydrolysing the protein with 1 N HCl. The study revealed (Table V) that legumin from young cotyledons (15 day) had a lower neutral sugar content (0.85%) than from mature cotyledons (1.2%), suggesting an increase in neutral sugar content during development. However, very small changes were noticed in amino sugar content

Fig. 8. Changes in subunit composition of legumin during different days of seed development (18, 21, 24, 27, 30, and 33 day).

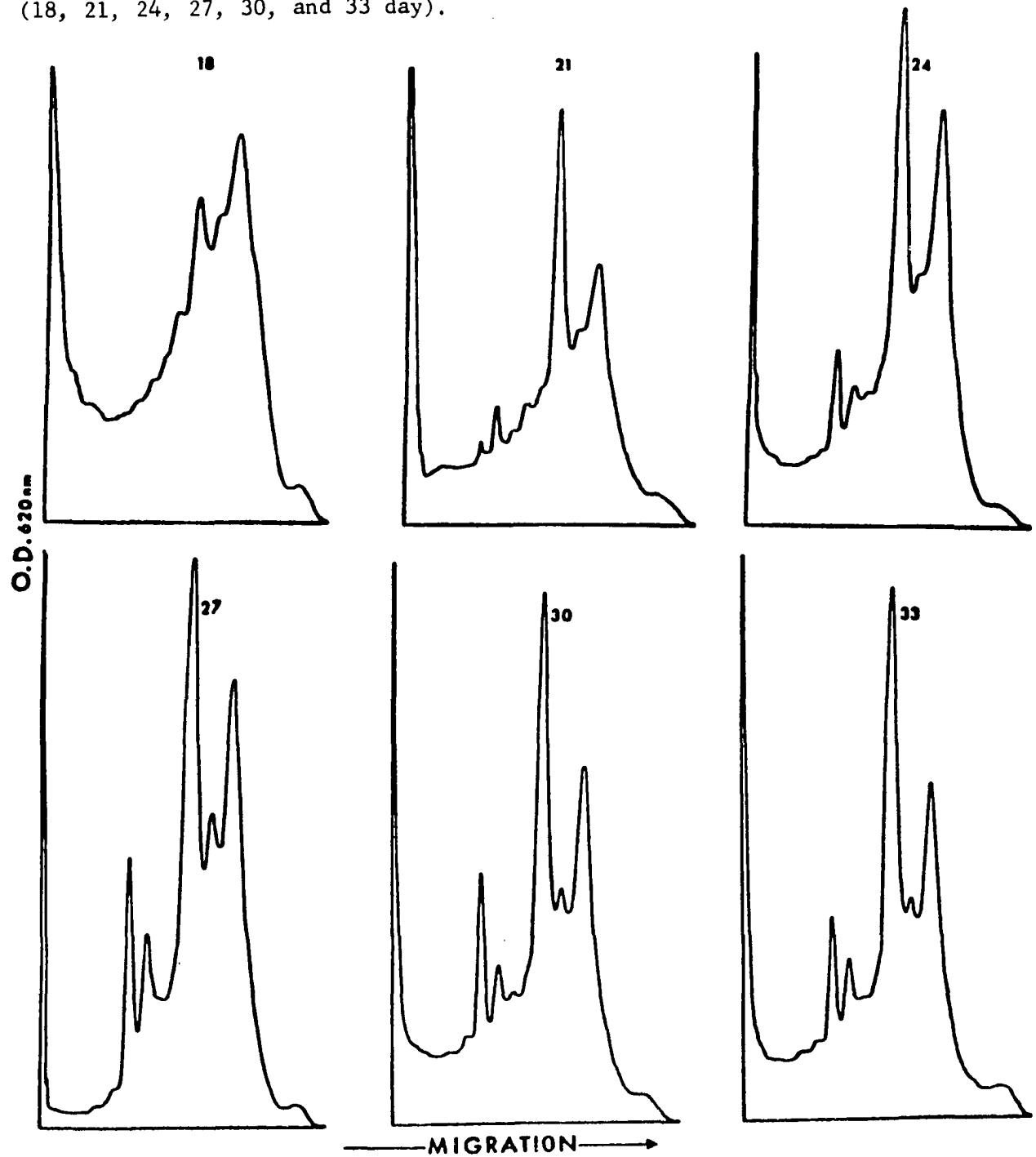


Fig. 9. Changes in subunit composition of vicilin during different days of seed development (18, 21, 24, 27, 30, and 33 day).

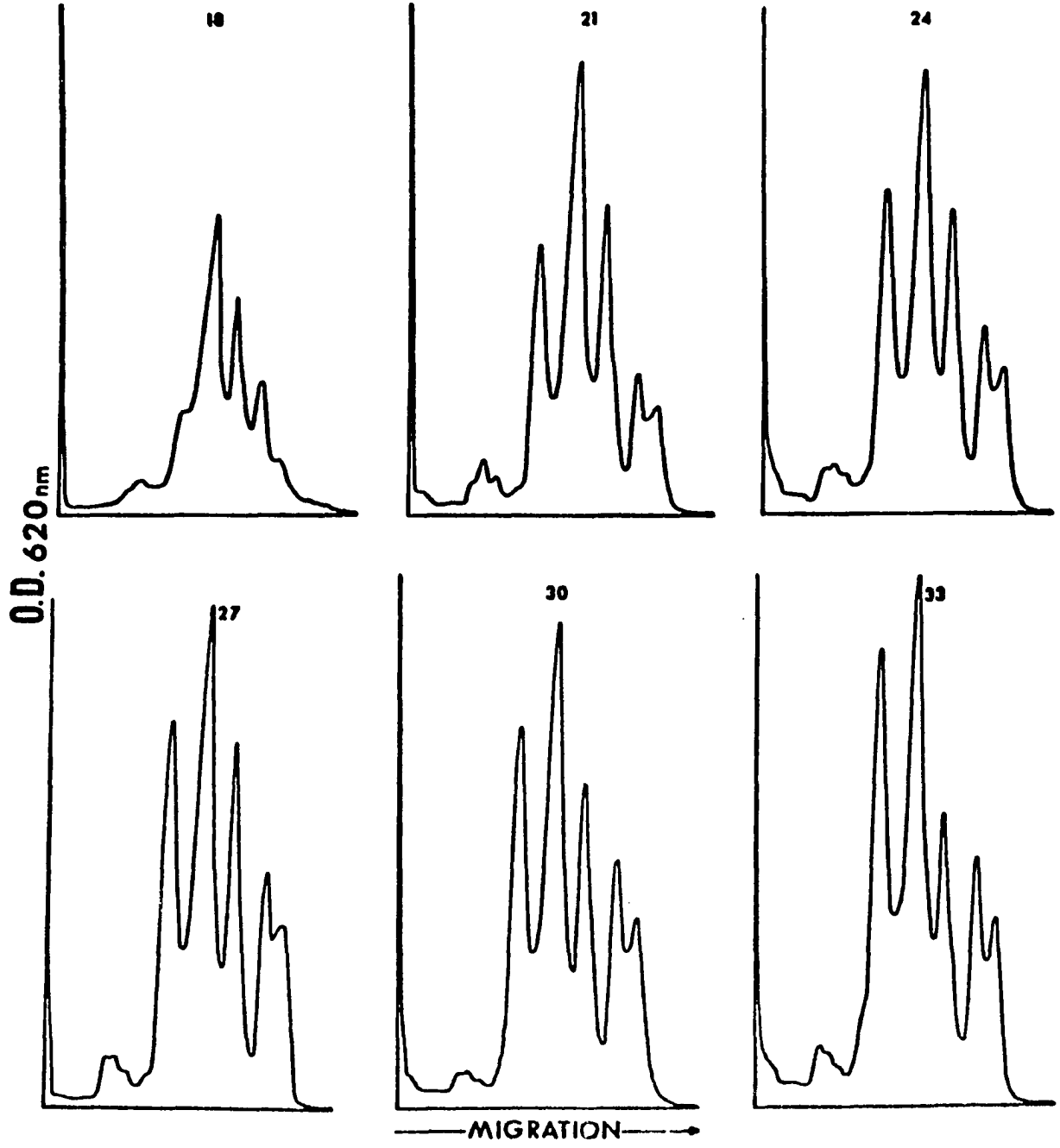


TABLE V

Changes in percent sugar content of protein during pea seed development

DAYS	LEGUMIN		VICILIN	
	Neutral Sugars	Amino Sugars	Neutral Sugars	Amino Sugars
15	0.856	0.100	1.020	0.180
18	1.326	0.082	0.930	0.250
21	1.410	0.078	0.601	0.270
24	1.360	0.056	0.260	0.177
27	1.440	0.066	0.250	0.184
30	1.070	0.066	0.290	0.150
33	1.250	0.073	0.320	0.254

(0.1% to 0.07%) of legumin during development, revealing that there is not much change in amino sugar content.

In contrast to legumin, the neutral sugar content of vicilin decreased (from 1.02% to 0.32%) and amino sugar content increased from 0.18% to 0.25% during seed development. These results show that during seed maturation incorporation of neutral and amino sugars occurs at different rates. An increase in neutral sugar content might be attributed to the elongation of oligosaccharide chains on the protein, while a decrease might be due to breakdown of oligosaccharide chains. It appears that in legumin elongation of oligosaccharide chains occurs during seed development, since there was an increase in neutral sugar content, while the amino sugar content remains more or less constant. However, in vicilin there is an increase in amino sugar content. This may be due to the addition of shorter chain oligosaccharides to the peptides during seed development and a shortening of the oligosaccharide chains initially associated with the proteins. Alternatively, the oligosaccharides associated with subunit I and IV (i.e., the subunits produced later during seed development) may have a different composition (i.e., high glucosamine to neutral sugar content) than that of subunits II, III and V developed early in seed development.

Seed Germination

Changes in protein content. The pattern of protein breakdown during seed germination was investigated, by following the changes in protein composition in cotyledons of germinating seeds. It was found (Fig. 10) that protein breakdown was slow (17%) during the first 4 days and from the 5th day onward rapid protein breakdown occurred. By day

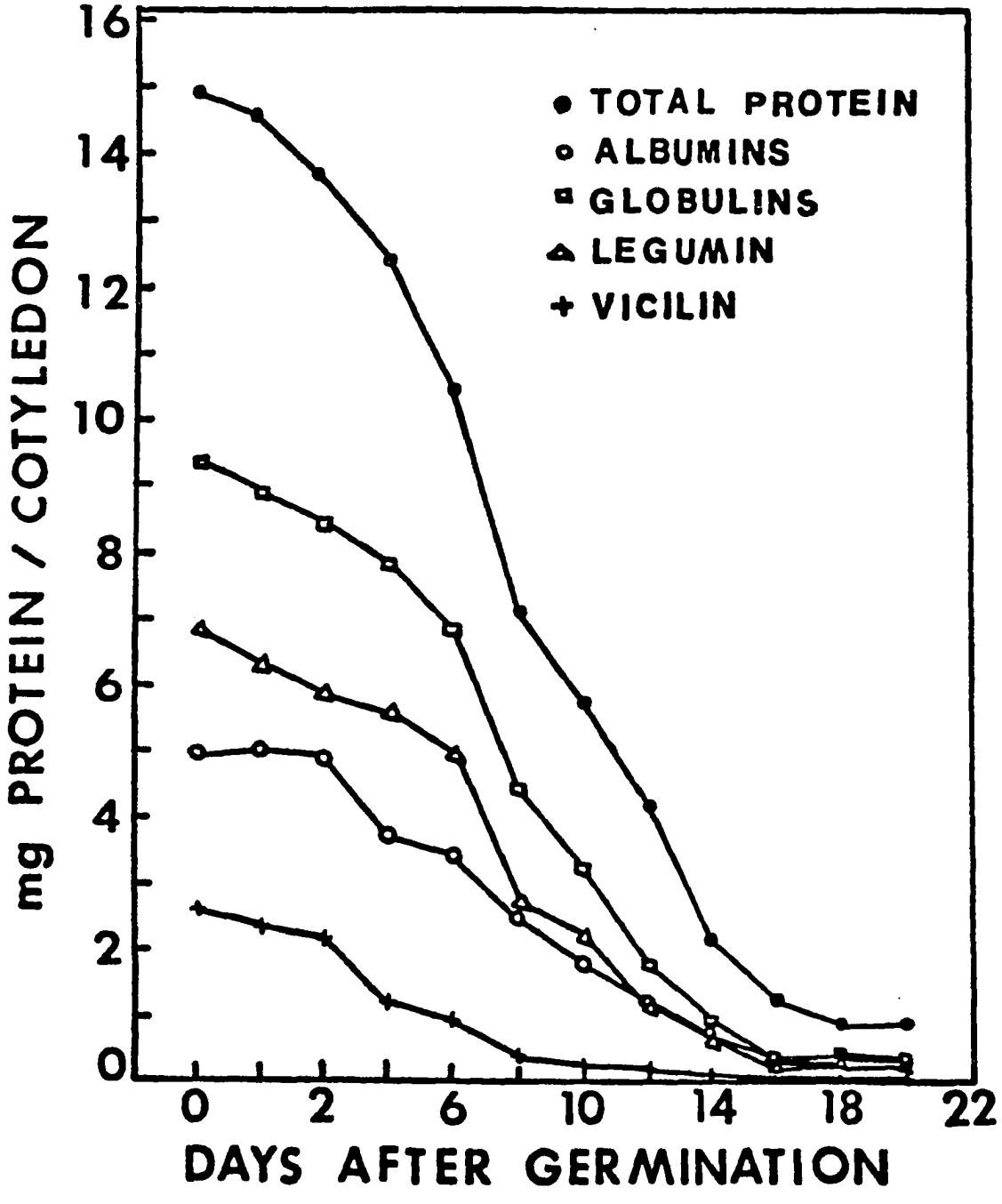


Fig. 10. Changes in protein content of different fractions during seed germination.

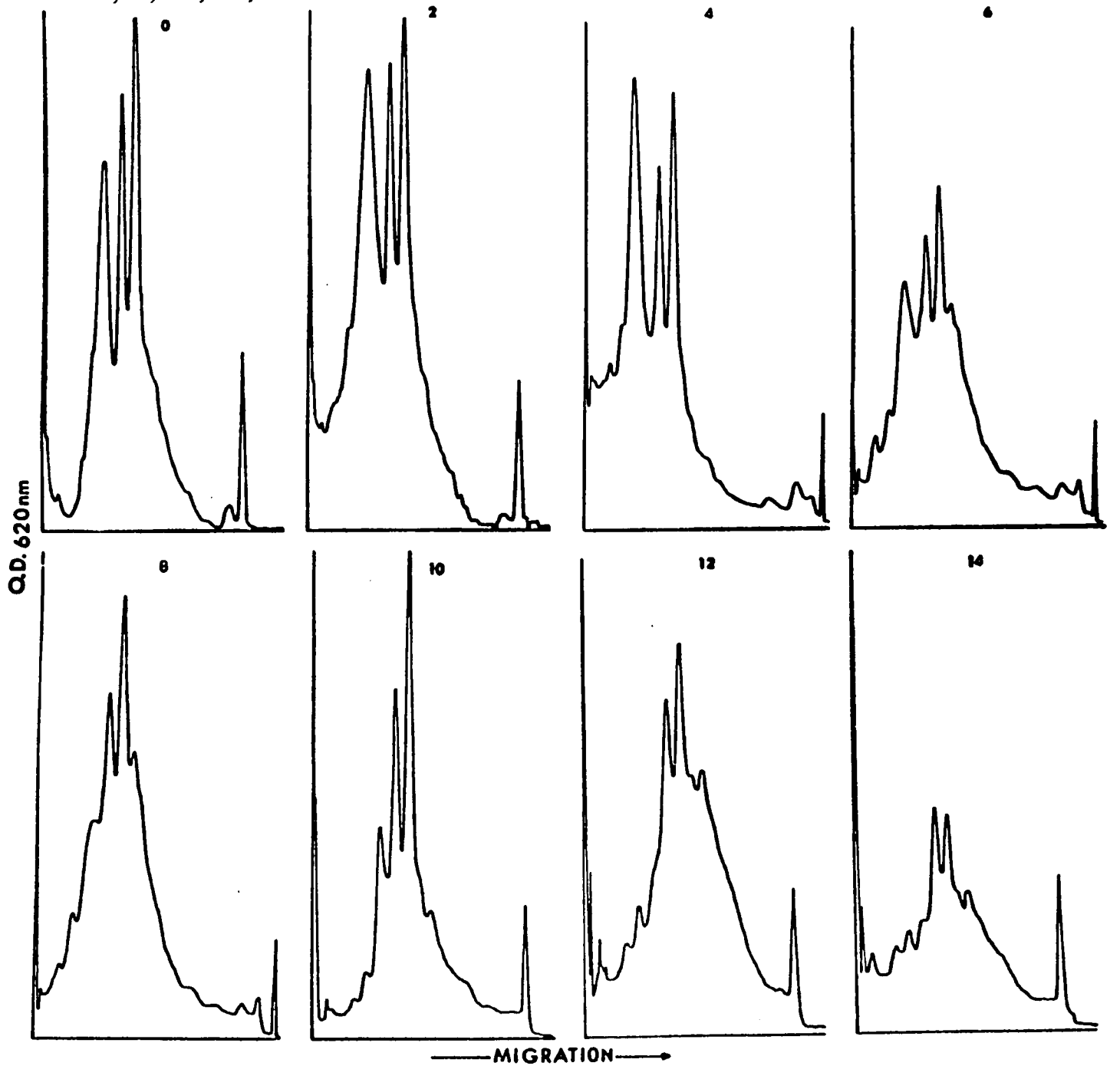
16, 92% of the protein was utilized indicating that rapid protein hydrolysis occurred between day 5 and 16.

Among the various fractions globulin depletion occurred earlier than albumin depletion. During the first four days of germination, globulin hydrolysis was slow (16%). However, after 5 to 6 days the globulin breakdown increased rapidly and by the 16th day most of the globulin (97%) was utilized. Legumin and vicilin were broken down at a similar rate and both reached a low level (0.22 and 0.05 mg per cotyledon respectively) by day 16. The albumin content decreased more slowly than globulin during early stages of germination, but by day 16 most of the albumin (93%) had been disappeared.

These results confirm the findings of Danielson (1951) who indicated that during pea seed germination, there was a pronounced breakdown of globulins between days 5 to 10. Vicilin and legumin were broken down at the same rate, while the decrease in albumin was slow and constant. Similarly Juo and Stotzsky (1970) reported that in kidney beans (Phaseolus vulgaris), the globulin and basic proteins were hydrolysed more rapidly than albumins.

Gel electrophoresis of albumin. The breakdown of protein fractions during germination was further investigated by gel electrophoresis. The albumin composition starts to change after day 2 with the most dramatic changes occurring after day 4 (Fig. 11). Some components decreased during germination, and in addition new peaks were produced. These changes in albumin composition might be expected because of the enzymatic nature of albumins. The enzyme components will change with the altered metabolic activity of the seed during

Fig. 11. Changes in albumin composition during different days of seed germination (0, 2, 4, 6, 8, 10, 12, and 14 day).



germination. Juo and Stotzsky (1970) also found the disappearance of a number of components of albumin fraction in beans, during early stages of germination with the appearance of new components after 8 days of germination.

Gel electrophoresis of legumin and vicilin. On gel electrophoresis, legumin shows one major band. It was observed (Fig. 12) that there is a change in the electrophoretic mobility of legumin fractions, prepared from peas at successive stages of germination. In legumin there is increased movement toward the anode (positive pole). This observation suggests that it is becoming more negatively charged during germination. This increase in negative charge might be due to the loss of amino groups from the legumin by the action of deaminases or peptidases.

After electrophoresis vicilin produces a broad diffuse band and it is relatively difficult to assess the electrophoretic mobility. However, like legumin, vicilin also showed (Fig. 13) changes in the electrophoretic mobility during germination. Unlike legumin, the migration of vicilin toward the cathode (negative-pole) decreased during germination, revealing that the protein is becoming more positively charged. This could be attributed to the loss of carboxyl groups by the action of decarboxylases or peptidases giving a more positive charge to the protein. Catsimpoolas et al., (1968) also found changes in electrophoretic mobility of 11S and 7S components of soybean (Glycine max) reserve protein during germination.

SDS gel electrophoresis of legumin. It was found (Fig. 14) that

Fig. 12. Changes in the electrophoretic behavior of legumin during different days of seed germination.

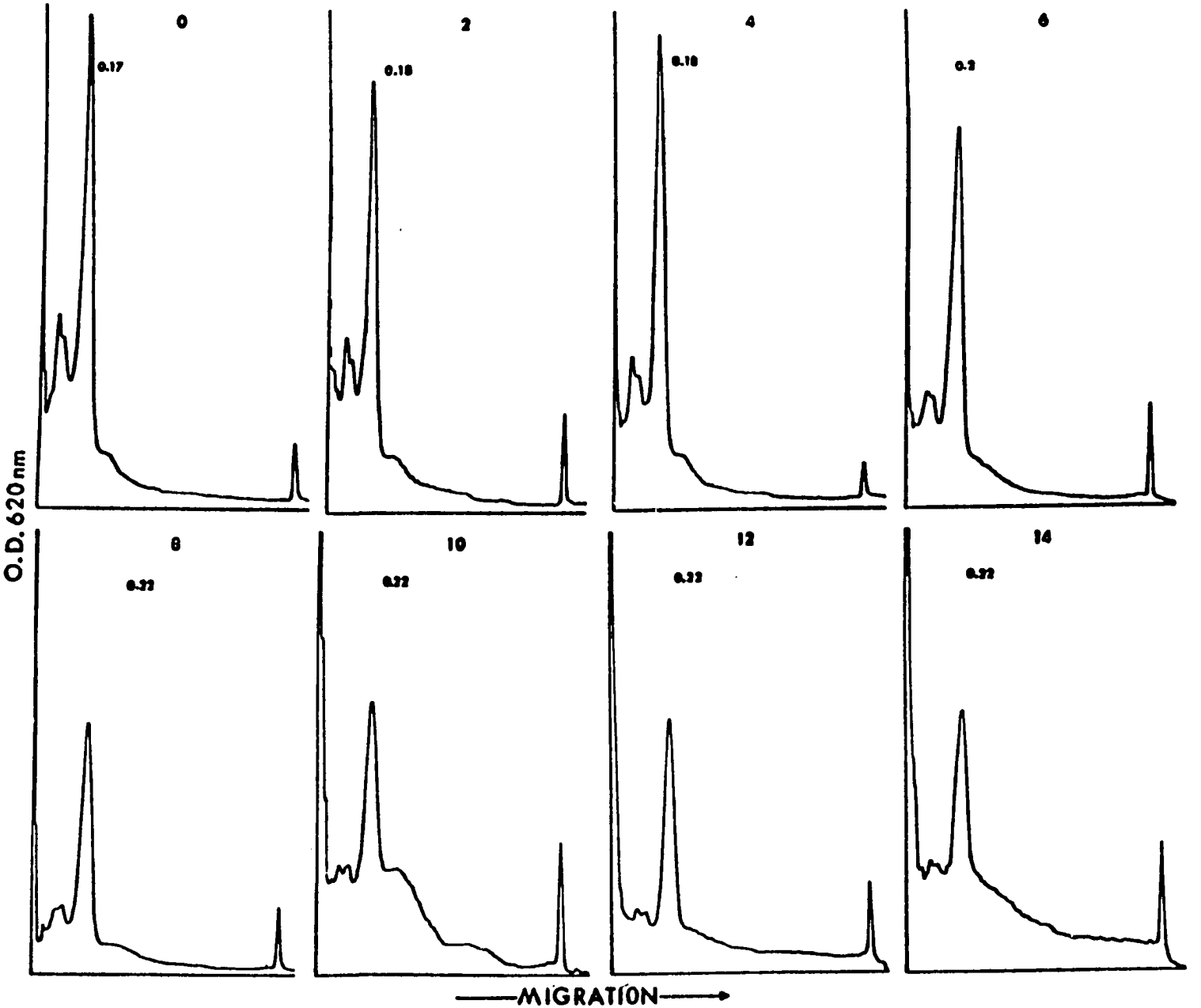
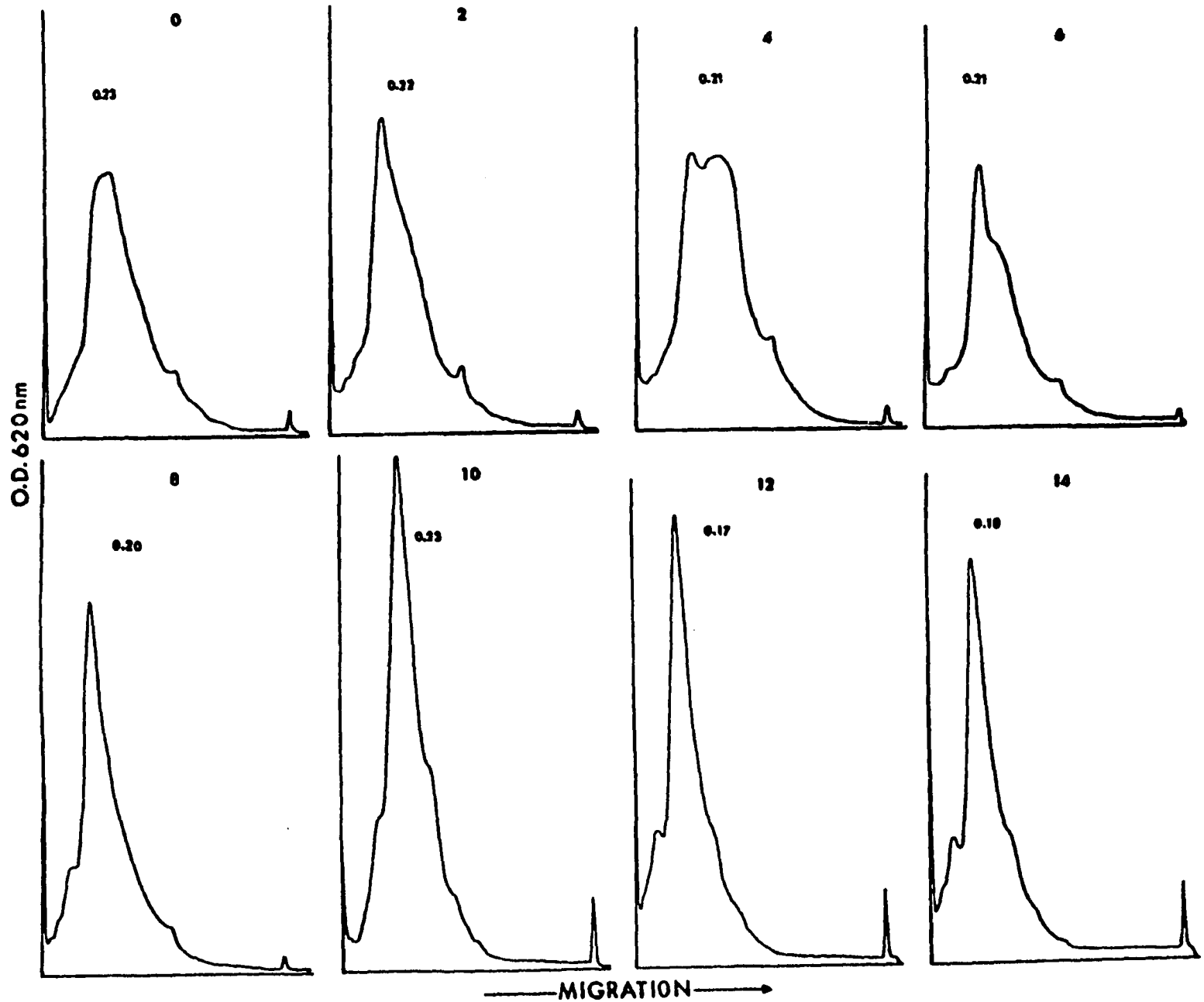


Fig. 13. Changes in the electrophoretic behavior of vicilin during different days of seed germination.



the subunit composition of legumin changed during germination. Subunit III disappeared early during germination, while subunit V was still present 16 days after germination. It appears that subunit III might be more susceptible to hydrolysis than subunit V. Significantly subunit V is the component which incorporated the greatest amount of ^{14}C-glucosamine.

SDS gel electrophoresis of vicilin. In case of vicilin major changes in the subunits occurred after day 4 of germination (Fig. 15). Among the subunits, IV and V were hydrolysed slowly and can be seen even in late stages of germination, while subunit I was utilized by day 6 followed by II and III. Appearance of a new minor subunit was observed on day 10, indicating the possibility of existence of high molecular weight intermediate products of hydrolysis.

Changes in carbohydrate content of legumin and vicilin during seed germination. The carbohydrate content of legumin and vicilin prepared from cotyledons at various stages of germination was determined and it was found (Table VI) that in legumin both neutral and amino sugar content increased (from 1.63% to 10.00% and 0.078% to 0.135% respectively) during germination. Similarly, in vicilin also neutral sugar content increased from 0.36% to 2.9% and amino sugar content from 0.24% to 0.96%. However, many fluctuations in carbohydrate content were noticed during different days of germination. The increase in both neutral and amino sugar content indicates that during germination non-glycopeptides are depleted more rapidly than glycopeptides. This preferential cleavage of non-glycoprotein could produce a glycoprotein

Fig. 14. Changes in the subunit composition of legumin during different days of seed germination.

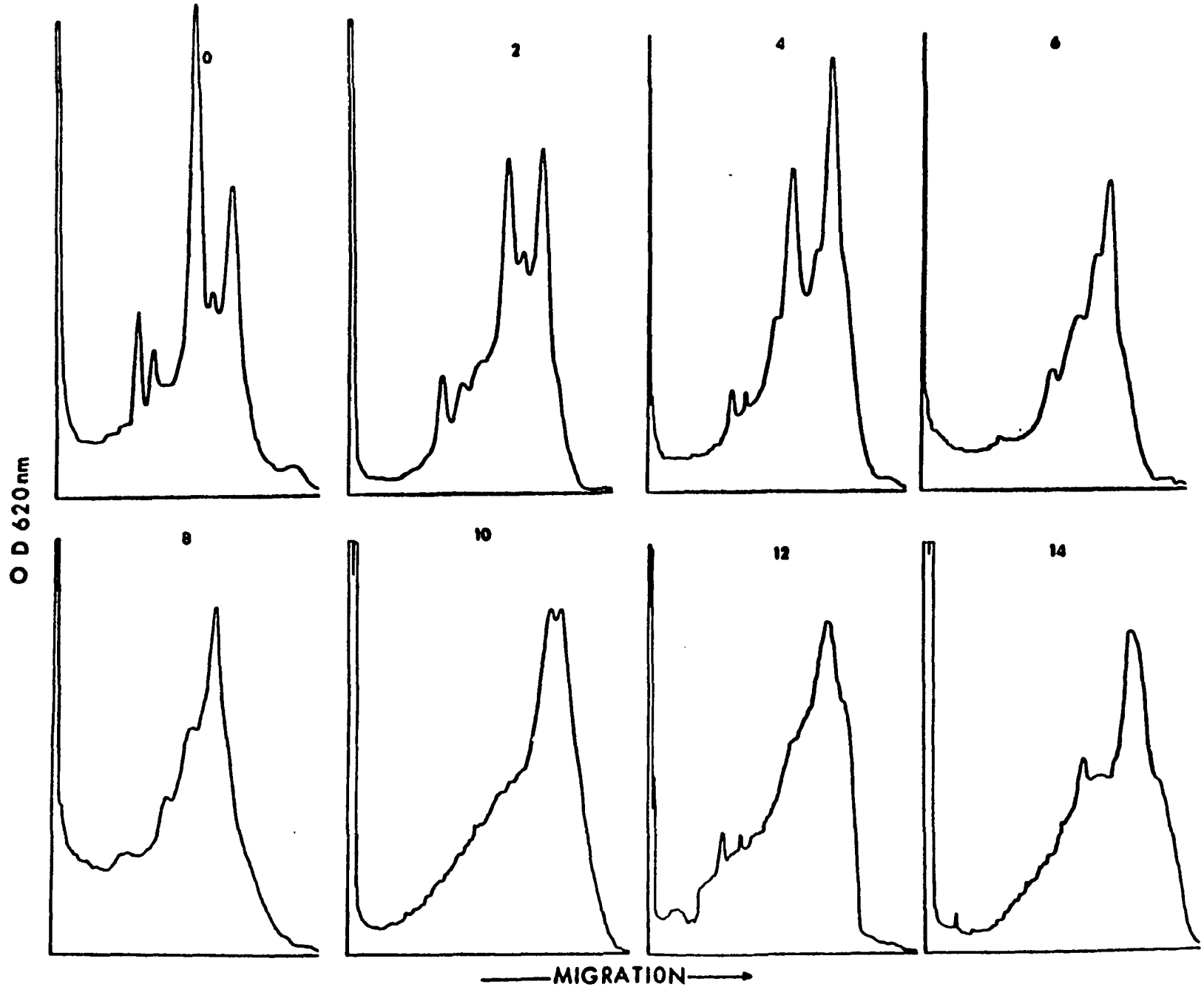


Fig. 15. Changes in the subunit composition of vicilin during different days of seed germination.

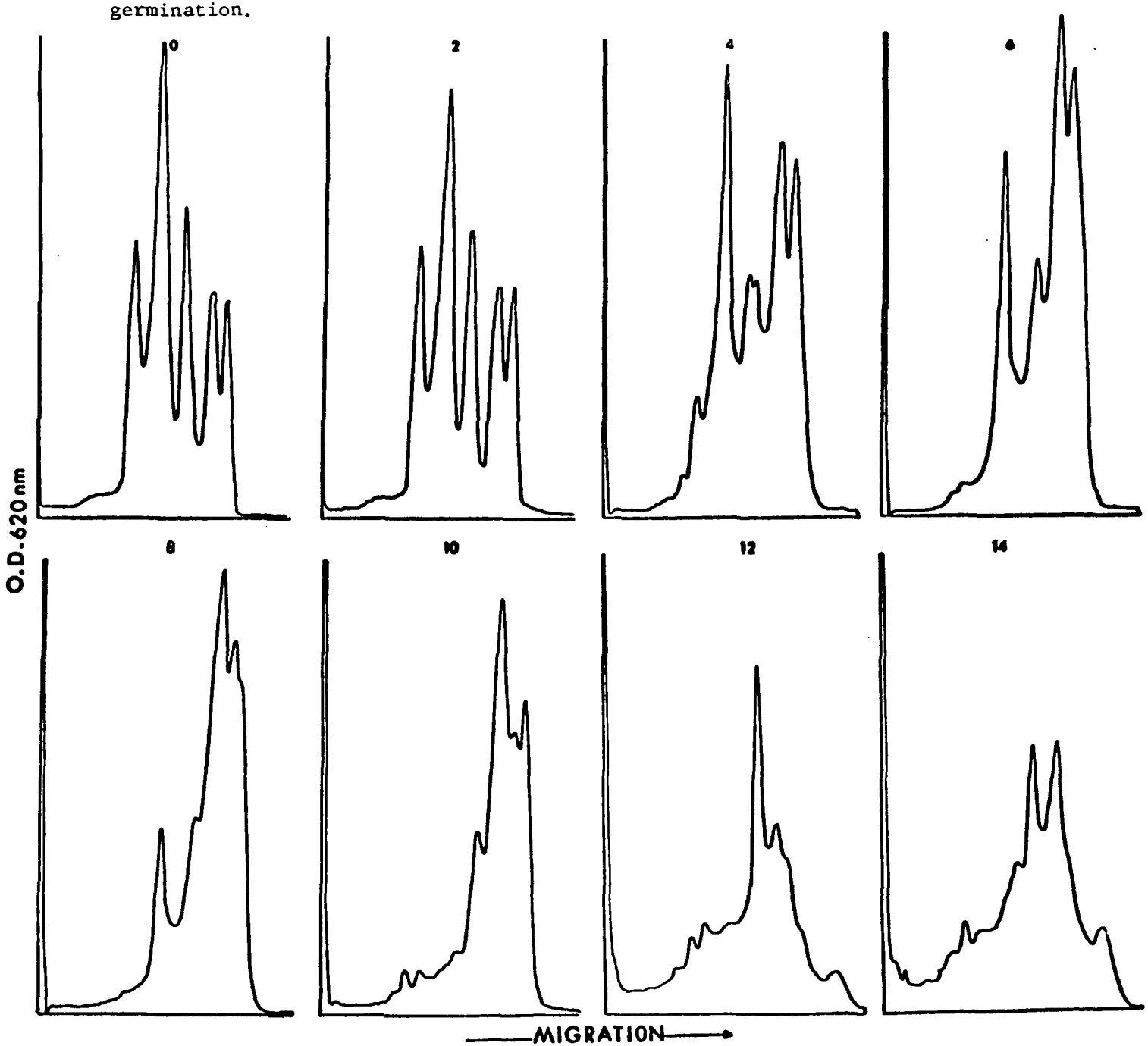


TABLE VI

Changes in percent sugar content of protein during pea seed germination

DAYS	LEGUMIN		VICILIN	
	Neutral Sugars	Amino Sugars	Neutral Sugars	Amino Sugars
0	1.630	0.078	0.360	0.241
2	1.430	0.080	0.710	0.230
4	2.860	0.083	1.112	0.180
6	1.410	0.091	2.060	0.200
8	1.456	0.110	2.270	1.015
10	2.516	0.116	2.080	0.600
12	2.330	0.125	2.970	0.960
14	10.153	0.135	1.080	0.280

rich protein and give rise to the observed increase in sugar content. It thus appears that glycopeptides are more resistant to hydrolysis than non-glycopeptides. Subunit V of legumin and subunit II, III, IV and V of vicilin which incorporated the greatest amount of ^{14}C-glucosamine were hydrolysed slowly during germination to support this idea.

Proteolytic Activity

It is generally believed that during germination the proteins are hydrolysed. The enzyme protease is believed to be responsible for protein breakdown. Most of the characterization of proteases from germinating seeds has been done with casein, a milk protein, and haemoglobin as a substrate. In this study attempts were made to use native substrates in order to better characterize pea proteases, which might be found in seed. The substrates used were pea ^{3}H-albumin, ^{3}H-legumin and ^{3}H-vicilin.

Enzyme extraction and assay procedure. Cotyledons from germinating seeds were used for enzyme extraction as described in Methods. By differential centrifugation it was found that most of the enzyme activity is associated with the 20,000 x g supernatant.

The assay conditions were similar to those reported by Beevers (1968) except, ^{3}H-albumin, ^{3}H-legumin, or ^{3}H-vicilin was substituted for casein. One-half to 0.8 ml of 1% casein was added to the reaction mix just before stopping the reaction with TCA, to facilitate precipitation of protein. The TCA soluble counts were measured and the enzyme activity expressed as CPM released per milliliter of enzyme per 3 hour reaction period.

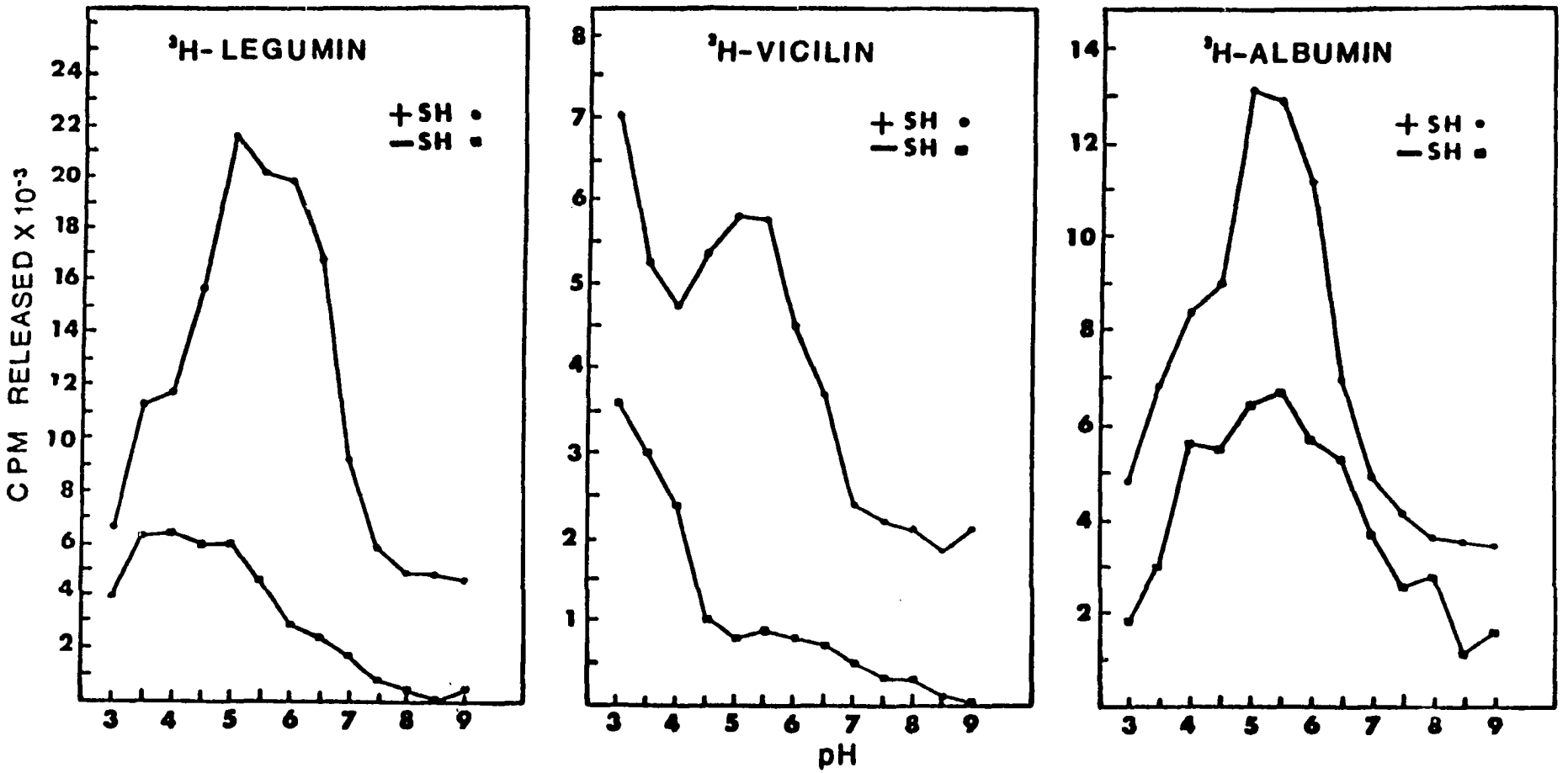
Assays were also performed by substituting the grinding buffer for enzyme, to ensure that the observed proteolytic activity was not due to bacteria or autodigestion. In addition, one drop of toluene was added to the reaction mix and test tubes were sealed with parafilm, to reduce any possible microbial contamination.

Effect of pH. Pea protease from cotyledons of 13 day old seedlings was extracted with and without 2-mercaptoethanol (sulfhydryl, -SH) in the grinding medium and incubated in a reaction mix at pH 3.0 to 9.0. The results revealed that maximum protease activity occurred (Fig. 16) at pH 5.0 with all the three substrates, ^{3}H-albumin, ^{3}H-legumin and ^{3}H-vicilin. The enzyme extracted with -SH, showed higher activity than the one without -SH, suggesting that -SH is necessary for activation of the enzyme. Similar -SH dependence for enzyme stimulation with all the three substrates was observed. The enzyme showed very little activity at either pH extremes. However, when vicilin was used as the substrate, it was found that in addition to the optimum pH of 5.0 a peak of hydrolytic activity occurred at pH 2.5.

These results demonstrate that the pea protease is capable of hydrolysing all three native substrates found in the seed at an optimum pH of 5.0 and it is -SH dependent. A similar optimum pH for protease activity was noticed by other workers in peas (Mergentime and Wiegard, 1946; Beevers, 1968) and beans (Pusztai and Duncan, 1971).

Effect of addition and removal of -SH groups in the reaction mix. Since it was shown (Fig. 16) that the enzyme activity was greater in the cotyledon extracts prepared in the presence of 2-mercaptoethanol,

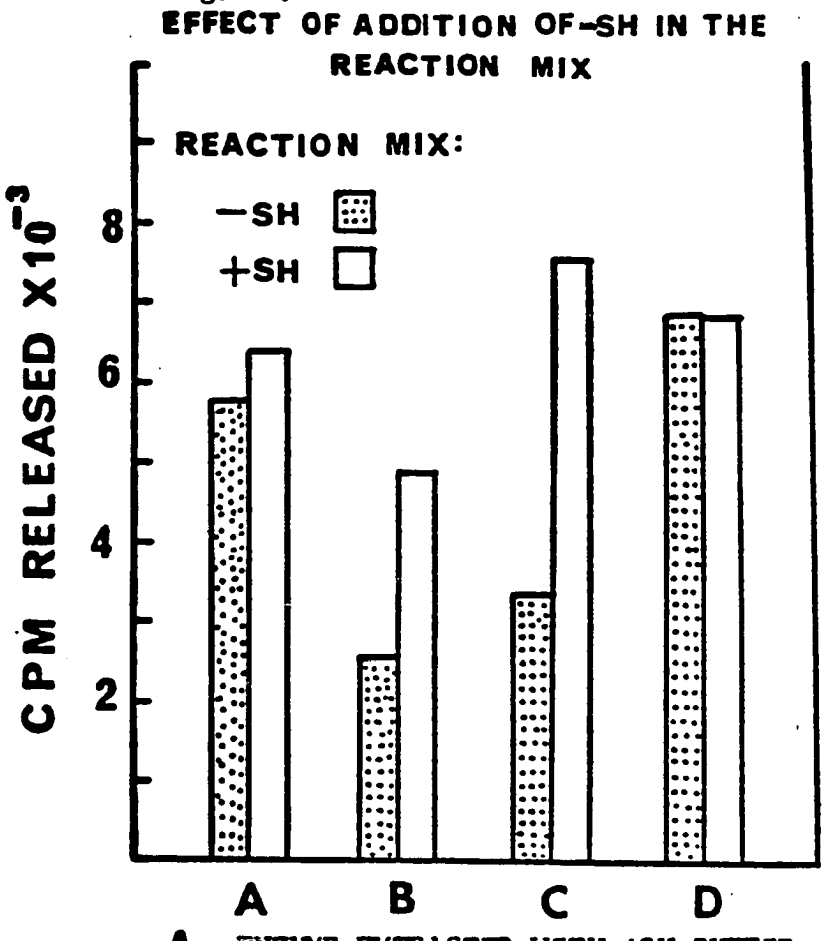
Fig. 16. Effect of pH on the proteolytic activity with different substrates.



further studies were made to determine whether the -SH requirement was limited only to the enzyme extraction or throughout the assay period. For this purpose four enzyme preparations were made and used for assay: (1) Enzyme A: enzyme extracts prepared with 2-mercaptoethanol in grinding medium; (2) Enzyme B: enzyme extracts prepared without 2-mercaptoethanol in grinding medium; (3) Enzyme C: enzyme extracts prepared with 2-mercaptoethanol in grinding medium, precipitated with ammonium sulfate and the precipitate suspended in buffer without 2-mercaptoethanol; (4) Enzyme D: enzyme extracts prepared without 2-mercaptoethanol in grinding medium, precipitated with ammonium sulfate and the precipitate suspended in buffer containing 2-mercaptoethanol.

It was found (Fig. 17) that there is not much increase in the activity of Enzyme A, with the addition of additional SH groups in the reaction mix, probably due to saturation. Enzyme B which was extracted without SH in the grinding medium, showed a greater stimulation than Enzyme A upon addition of -SH to the reaction mix. Enzyme C, which was prepared from Enzyme A after removing -SH groups from the suspension medium, showed maximum stimulation upon addition of -SH to the reaction mix. Without addition of -SH to the reaction mix, Enzyme C showed lower activity than Enzyme A, indicating that it lost its original activity due to removal of -SH from the medium. In contrast, Enzyme D which was prepared from Enzyme B, by addition of -SH in the suspension medium, showed higher activity than Enzyme B, suggesting that addition of -SH in the suspension medium stimulated the enzyme activity. However, no further increase in activity was noticed on further addition of more SH to the reaction mix indicating a saturation effect.

Fig. 17.



A - ENZYME EXTRACTED WITH +SH BUFFER
B - ENZYME EXTRACTED WITH -SH BUFFER
C - ENZYME EXTRACTED WITH +SH BUFFER - PPT. WITH AMMO. SULFATE AND - SUSPENDED IN -SH BUFFER.
D - ENZYME EXTRACTED WITH -SH BUFFER - PPT. WITH AMMO. SULFATE AND - SUSPENDED IN +SH BUFFER.

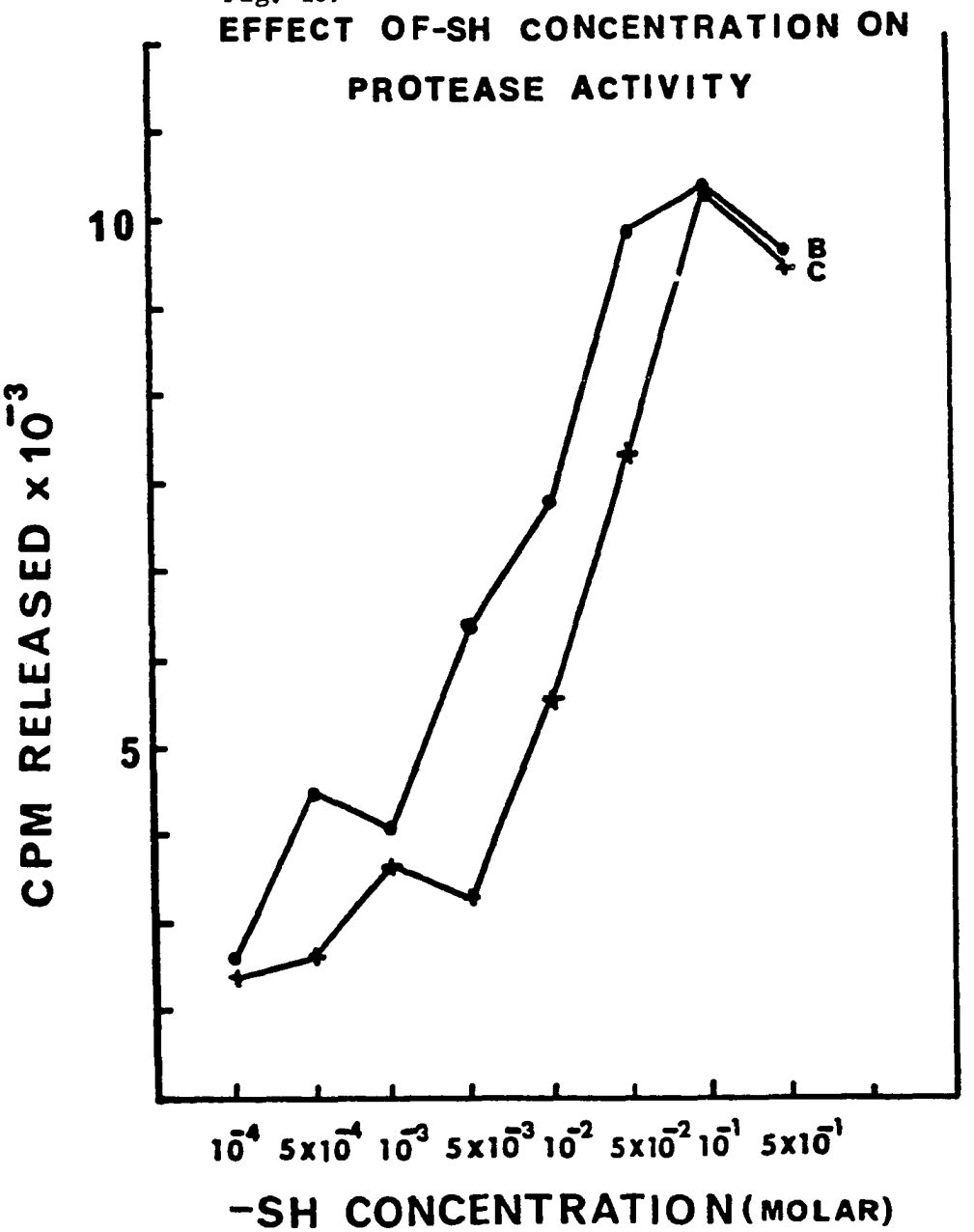
This experiment clearly indicated that the presence of -SH is not only protecting the enzyme during extraction, but is also required for proteolytic enzyme activity. On this basis the protease may be characterized as an acid -SH protease.

Effect of -SH concentration. Incubation of enzyme with various concentrations of 2-mercaptoethanol indicated (Fig. 18) that increasing -SH concentration up to 0.1 M stimulated the enzyme activity, suggesting that the enzyme had a rather high -SH requirement.

Proteolytic activity during germination. The cotyledons from germinating seeds of various ages were analysed for enzyme activity. The cotyledons showed (Fig. 19) very little proteolytic activity during early stages of germination and no increase in activity was noticed during the first two days of germination. However, after day 3 the enzyme activity increased very slowly up to day 5 and from day 5 there was an 8-fold increase in enzyme activity up to day 15, and then the activity declined.

When the proteolytic activity was measured at pH 7.5, no appreciable increase in enzyme activity was observed until day 9 and after day 9 a 14-fold increase in activity was observed. This activity remained more or less constant up to day 15 and then declined. There are two possible explanations for this behavior: first, the observed activity might be the residual activity of the same enzyme. If it is considered as residual activity, the pattern of enzyme activity changes which occurred at pH 5.0, should have been reflected at pH 7.5 also. In contrast, no similarities were noticed between the two pH's with

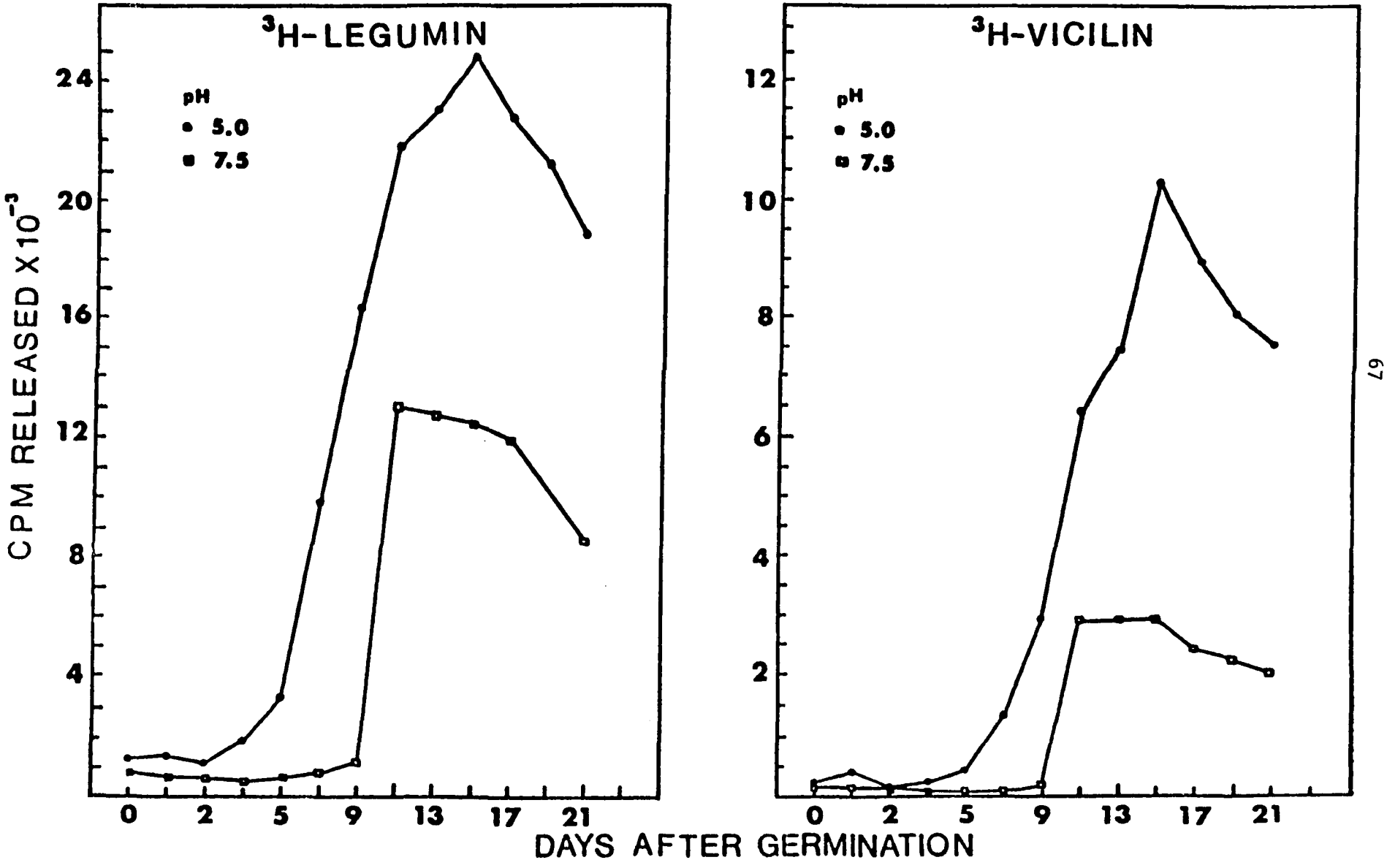
Fig. 18.



B - ENZYME EXTRACTED WITH -SH BUFFER.

C - ENZYME EXTRACTED WITH +SH BUFFER - PPT. WITH AMMO. SULFATE AND SUSPENDED - IN -SH BUFFER.

Fig. 19. Changes in the proteolytic activity during different days of pea seed germination.



both legumin and vicilin whose enzyme activity profiles were similar. The second possibility might be production of new enzyme during the later stages (day 9) of germination, having a higher pH optimum. The second possibility could be strongly supported since with both legumin and vicilin as substrates, very identical enzyme activity profiles at both pH 5.0 and 7.5 were observed.

Other studies (Beevers and Splittstoesser, 1968; Beevers, 1968) have indicated an increase in caseolytic activity during germination; however, Young and Varner (1959) indicated that proteolytic activity did not increase during the period of germination, when protein was being depleted. Beevers (1968) showed that caseolytic activity occurred after the onset of rapid depletion of cotyledonary protein and the peptidase activity was high in early germination.

The current study showed that very little protein was depleted from the seeds up to day 2 after germination, during which period low proteolytic activity was observed. However, the proteolytic activity slowly increased from day 3 to 5 accompanied by considerable protein degradation. Rapid increase in enzyme activity from day 5 to 15 was in agreement with the protein hydrolysis data which showed maximum protein breakdown between day 6 to 16. After day 16 very little protein was left in the cotyledon. Hence it can be suggested from this study that pea protease from germinating seeds has the capacity to utilize all the three native substrates, it is -SH dependent. The protein hydrolysis data during germination is in close agreement with the increase in enzyme activity.

CHAPTER V

CONCLUSIONS

Isolation and fractionation of pea proteins indicated that they are composed of two major fractions viz: albumin and globulin, the latter of which can be further subdivided into legumin and vicilin. Thus the pea proteins consist of characteristic components of legume seeds. Globulins constitute 60% of the total protein (Table I), and the reamining is albumin. In globulins legumin and vicilin distribution is 3:1. Further, investigation of the individual fractions by gel electrophoresis indicated (Fig. 2) the presence of numerous proteins in albumin, which might be due to its enzymatic nature. However, legumin and vicilin gave diffuse bands on electrophoresis (Fig. 2) suggesting that they are complex and heterogeneous. On dissociation with SDS and DTT, legumin and vicilin showed that they are not simple proteins but are made up of subunits (Fig. 3).

The studies directed toward determining the possible glycopeptide nature of reserve proteins indicated that in fact the pea proteins are glycopeptides, containing both neutral and amino sugars. However, it appears that legumin has a higher neutral sugar content than vicilin (TableII). It is possible that legumin has fewer glucosamine residues with longer chain oligosaccharides, while vicilin has several glucosamine residues with relatively short chain oligosaccharides attached.

The experiments in which cotyledons were injected with ^{14}C-glucosamine indicated that in legumin, subunit V showed the highest glucosamine incorporating capacity, even in the early stages (18 days after flowering) of development, while in other subunits the incorporation is low, suggesting that only subunit V of legumin is the major glycosylated component. In contrast, all the five subunits of vicilin showed glucosamine incorporation during all stages of development, suggesting that in vicilin all the subunits contain glucosamine and are glycosylated. Attempts to determine the time of attachment of carbohydrate units onto peptide showed that the carbohydrates are attached after the peptide synthesis.

Although seed proteins are composed of different components they are not synthesized concomitantly. Developmental studies have indicated (Fig. 6) that albumin deposition declined (24 days after flowering) earlier than globulin (27 day after flowering), while legumin and vicilin deposition occurred independently. Investigation of individual protein fractions by gel electrophoresis showed (Fig. 7) that the albumin composition changed during seed development. This was expected because of the enzymatic nature of albumins. Changes in sub-unit composition of legumin and vicilin suggest (Figs. 8 and 9) that these proteins are not synthesized as a single unit, but on a subunit basis. Studies of the carbohydrate composition of the protein during seed development (Table V) indicate that the glycopeptides may have varying carbohydrate content.

Studies of seed germination have demonstrated (Fig. 10) that globulin depletion occurred earlier than albumin depletion and the

reserve protein hydrolysis commenced soon after imbibition. Gel-electrophoretic investigation (Fig. 11) of albumin showed that its composition changed during germination, presumably associated with its enzymatic nature, which would change with the changed metabolic activity of the seed during germination. Gel electrophoretic investigation of legumin (Fig. 12) and vicilin (Fig. 13) indicated that during germination there was a change in electrophoretic mobility of the proteins. It appears that legumin becomes more negatively charged and vicilin more positively charged. This might be due to the action of deaminases or decarboxylases and/or peptidases.

Dissociation with SDS and DTT showed (Figs. 14 and 15) that in both legumin and vicilin some subunits were hydrolysed faster than others. Significantly the subunits which showed higher ^{14}C-glucosamine incorporation were depleted slower than the others. Hence, it might be possible that the subunits rich in carbohydrates might be resistant to hydrolysis. Further, the carbohydrate content of legumin and vicilin increased (Table VI) during germination, indicating a preferential cleavage of non-glycopeptides over glycopeptides.

Investigations dealing with proteolytic activity of the cotyledon extracts, from germinating seedlings indicated (Fig. 16) that the pea protease is capable of hydrolysing all the three native substrates, albumin, legumin and vicilin at an optimum pH of 5.0. It requires the presence of sulfhydryl for maximal activity. Hence the pea protease is an acid-SH-protease.

The enzyme had a low activity during early stages of germination (5 days), which increased rapidly by day 15 and then declined

(Fig. 19). This increase in enzyme activity was in close agreement with the protein depletion during germination, indicating that active protein breakdown synchronizes with the increased enzyme activity. An increase in proteolytic activity was noticed at pH 7.5 after 9 days of germination. It seems that synthesis of a new proteolytic enzyme complement, with a higher pH optimum may occur during later stages (after 9 days) of seed germination.

LITERATURE CITED

Altschul, A. M., Yatsu, L. Y., Ory, R. L., and Engleman, E. M. 1966. Seed proteins. Ann. Rev. Plant Physiol., 17, 113-136.

Bagley, B. W., Cherry, J. H., Rollins, M. L., and Altschul, A. M. 1963. A study of protein bodies during germination of peanut (Arachis hypogaea) seed. Amer. J. Bot. 50, 523-531.

Bailey, C. J., and Boulter, D. 1970. The structure of legumin a storage protein of broad bean (Vicia faba) seed. Eur. J. Biochem. 17, 460-466.

Bailey, C. J., Cobb, A., and Boulter, D. 1970. A cotyledon slice system for the synthesis and intracellular transport of the seed storage protein of Vicia faba. Planta, 95, 103-118.

Bain, J. M., and Mercer, F. V. 1966. Subcellular organization of the cotyledons in the germinating seeds and seedlings of Pisum sativum L. Aust. J. Biol. Sci. 19, 69-84.

Beevers, L. 1968. Protein degradation and proteolytic activity in the cotyledons of germinating pea seeds (Pisum sativum). Phytochemistry, 7, 1837-1844.

Beevers, L., and Guernsey, F. S. 1966. Changes in some nitrogenous components during the germination of pea seeds. Plant Physiol. 41, 1455-1458.

Beevers, L., and Poulson, R. 1972. Protein synthesis in cotyledons of Pisum sativum L. Plant Physiol. 49, 476-481.

Beevers, L., and Splittstoesser. 1968. Protein and nucleic acid metabolism in germinating pea. J. Expt. Bot. 19, 698-711.

Bianco, M. A., and Bellando, M. 1971. On the protein of Phaseolus vulgaris. III. Isolation of a water soluble protein from cotyledons in the resting stage. Atti. Accad. Sci. Torino. Cl. Sci. Fis. Mat. Natur. 105, 195-202.

Brand, B. P., and Johnson, P. 1956. On the interaction of sodium dodecyl sulfate with legumin. Trans. Faraday Soc. 52, 438-451.

Brand, B. P., and Johnson, P. 1958. A physico-chemical study of the protein legumin. Trans. Faraday Soc. 54, 1191-1921.

Briarty, L. G., Coult, D. A., and Boulter, D. 1969. Protein bodies of developing seeds of Vicia faba. J. Expt. Bot. 20, 358-372.

Briarty, L. G., Coult, D. A., and Boulter, D. 1970. Protein bodies of germinating seeds of Vicia faba. J. Exp. Bot. 21, 513-524.

Burger, W. C., Prentice, N., Kastenschmidt, J., and Huddle, J. D. 1966. Partial purification of proteinases from germinating barley. Cereal Chem. 43, 546-554.

Burger, W. C., and Siegelman, H. W. 1966. Location of a protease and its inhibitor in the barley kernel. Physiol. Plant. 19, 1089-1093.

Buzila, L. 1969. Breakdown of vicilin in pea seeds during germination. Stud. Cercet. Biochem. 12, 229-232.

Catsimpoolas, N., Campbell, T. G., and Mayer, E. W. 1968. Immuno-chemical study of changes in reserve proteins of germinating soybean seeds. Plant Physiol 43, 799-805.

Danielson, C. E. 1949. Seed globulins of the Graminaeae and Leguminosae. Biochem. J. 44, 387-400.

Danielson, C. E. 1950. An electrophoretic investigation of vicilin and legumin from seeds of peas. Acta Chem. Scand. 4, 762-771.

Danielson, C. E. 1951. The breakdown of the high molecular weight reserve protein of peas during germination. Acta. Chem. Scand. 5, 541-554.

Danielson, C. E. 1952. A contribution to the study of the synthesis of the reserve proteins in ripening pea seeds. Acta. Chem. Scand. 6, 149-159.

Danielson, C. E. 1956. Plant proteins. Ann. Rev. Plant Physiol. 7, 215-236.

Daussant, N., Neucere, N. J., and Yatsu, 1969. Immunological studies on Arachis hypogaea proteins with particular reference to reserve proteins. I. Characterization, distribution and properties of alpha arachin and alpha conarachin. Plant Physiol. 44, 471-479.

Davis, B. J. 1964. Disc electrophoresis. II. Method and application to human serum proteins. Ann. New York Acad. Sci. 121, 404-427.

Emmerling, A. 1881. Landaw verb. Stn. 24, 113.

Ericson, M. E., and Chrispeels, M. J. 1973. Isolation and characterization of glucosamine containing storage glycoproteins from the cotyledons of Phaseolus aureus. Plant Physiol. 52, 98-104.

Gardell, S. 1953. Separation on Dowex 50 ion exchange resin of glucosamine and galactosamine and their quantitative determination. Acta. Chem. Scand. 7, 207-215.

Garg, G., and Virupaksha, T. K. 1970. Acid protease from germinated sorghum. 1. Purification and characterization of the enzyme. Eur. J. Biochem. 17, 4-12.

Gofman, Yu. and Vaintraub, I. A., 1960. Paper electrophoresis study of plant proteins. Biochemistry (USSR), 25, 1049-1054.

Graham, T. A., Gunning, B. E. S., 1970. Localization of legumin and vicilin in bean cotyledon cells using fluorescent antibodies. Nature (London), 228, 81-82.

Grant, D. R., and Lawrence, J. M. 1964. Effects of sodium dodecylsulfate and other dissociating reagents on the globulins of peas. Arch. Biochem and Biophys. 108, 552-561.

Harvey, B. M. R., and Oaks, A. 1974. The hydrolysis of endosperm protein in Zea mays. Plant Physiol. 53, 453-457.

Henshall, J. D. and Goodwin, T. W. 1964. Amino acid-activating enzymes in germinating pea seedlings. Phytochemistry, 3, 677-691.

Hobday, S. M. and Giles, K. W. 1973. Globulin storage proteins of Glycine max. Phytochemistry, 12, 2623-2626.

Ihle, J. N. and Dure, L. 1969. Synthesis of protease in germinating cotton cotyledons catalyzed by mRNA synthesized during embryogenesis. Biochem. Biophys. Res. Comm. 36, 705-710.

Irving, G. W. and Fontaine, T. D. 1945. Purification and properties of arachin a newly discovered proteolytic enzyme of the peanut. Arch. Biochem. 6, 351-364.

Johnson, P. and Richards, E. G. 1962. The study of legumin by depolarization of fluorescence and other physicochemical methods. Arch. Biochem. and Biophys. 97, 260-276.

Juliano, B. O. and Varner, J. E. 1969. Enzymatic degradation of starch content in the cotyledons of germinating peas. Plant Physiol. 44, 886-892.

Juo, Pei-Show, and Stotzky, G. 1970. Changes in protein spectra of bean seed during germination. Can. J. Bot. 48, 1347-1350.

Klimenko, V. G. 1972. Comparative study of proteins of ripening and germinating pea seeds by chromatography on DEAE cellulose. Izv. Khim. Nauk. 2, 39-46.

Klimenko, V. G., and Azimov, B. A. 1972. A study of protein in seeds of leguminous plants grown in Tadzhikistan and Moldavia (USSR) by a gradient extraction column. Izv. Akad. Nauk. Moldssr. Ser. Biol. Khim. Nauk. 4, 13-21.

Koshiyama, I. 1966. Carbohydrate component in 7S protein of soybean casein fraction. Agr. Biol. Chem. 30, 646-650.

Koshiyama, I. 1970. Dissociation into subunits of a 7S protein in soybean globulins with urea and sodium dodecyl sulfate. Agr. Biol. Chem. 34, 1815-1820.

Kretovich, V. L., Bundel, A. A., Melik-Sarkisyan, S. S., and Stepanovich, K. M. 1954. The so called reserve proteins of seeds. Biochemistry (USSR), 19, 208-215.

Larson, L. A., and Beevers, H. 1965. Amino acid metabolism in young pea seedlings. Plant Physiol. 40, 424-432.

Lew, J. Y. and Shanon, L. M. 1973. Incorporation of carbohydrate residues into peroxidase isoenzymes in horse radish roots. Plant Physiol. 52, 462-465.

Lowry, O. H., Rosebrough, N. J., Fary, A. L., and Randall, R. J. 1951. Protein measurement with Folin-Phenol reagent. J. Biol. Chem. 193, 265-275.

Matile, P. 1968. Aleurone vacuoles as lyosomes. Z.Pflanzenphysiol. 58, 365-368.

McLeester, R. C., Hall, T. C., Sun, S. M., and Bliss, F. A. 1973. Comparison of globulin proteins from Phaseolus vulgaris with those from Vicia faba. Phytochemistry. 2, 85-93.

Mergentime, M. and Wiegand, E. H. 1946. Low temperature characteristics of a pea proteinase. Fruit Products J. 26, 72-80.

Miege, M. N. 1970. Study of seed proteins of legume: Lablab niger-Medik. Arch. Sci. (Geneva). 23, 75-149.

Millered, A., Simon, M., and Stern, H. 1971. Legumin synthesis in developing cotyledons of Vicia faba L. Plant Physiol. 48, 419-425.

Nakano, M., and Asahi, T. 1972. Subcellular distribution of hydrolase in germinating pea cotyledons. Plant and Cell Physiol. 13, 101-110.

Nakano, M., and Asahi, T. 1973. Membrane bound proteases in pea cotyledons. Plant and Cell Physiol.14, 1205-1208.

Nakano, M. and Asahi, T. 1974. Properties of membrane-bound protease in the cotyledons of germinating pea seeds. Agr. Biol. Chem. 38, 219-221.

Oota, Y., Fujii, R., and Osawa, S. 1953. Changes in chemical constituents during the germination stage of a bean, Vigna-sesquipedalis. J. Biochem. (Tokyo). 40, 649-661.

Opik, H. 1966. Changes in cell fine structure in the cotyledons of Phaseolus vulgaris L. during germination. J. Expt. Bot. 17, 427-439.

Ory, R. L. and Hennigsen, K. W. 1969. Enzymes associated with protein bodies isolated from ungerminated barley seeds. Plant Physiol. 44, 1488-1498.

Osborne, T. B. 1924. The vegetable proteins, Longmans Green, New York.

Palmiter, R. D., Oka, R. D. T., and Schimke, R. T. 1971. Modulation of ovalbumin synthesis by Estradio-17B and Actinomycine D as studied in explants of chick oviduct in culture. J. Biol. Chem. 246, 724-737.

Patridge, S. M. 1948. Filter-paper partition chromatography of sugars. Biochem. J. 42, 238-250.

Penner, D. and Ashton, F. M. 1966. Proteolytic enzyme control in squash cotyledons. Nature (London), 212, 935-936.

Pusztai, A. 1964. Hexosamine in the seeds of higher plants (spermatophytes). Nature (London), 201, 1328-1329.

Pusztai, A. 1965. A study on the glucosamine containing constituents of the seeds of kidney beans (Phaseolus vulgaris). Biochem. J. 94, 604-610.

Pusztai, A. 1966. The isolation of two proteins, Glycoprotein I and Trypsin inhibitor from the seeds of kidney beans (Phaseolus vulgaris). Biochem. J. 101, 379-384.

Pusztai, A., and Duncan, I. 1971. Glycoprotein I of Phaseolus vulgaris: Homogenicity and enzymic properties. Biochem. Biophys. Acta. 229, 785-794.

Pusztai, A., and Duncan, I. 1971a. Changes in proteolytic enzyme activities and transformation of nitrogenous compounds in the germinating seeds of kidney bean. Planta, 96, 317-325.

Pusztai, A. and Watt, W. B. 1970. Glycoprotein II. The isolation and characterization of a major antigenic and non-haemagglutinating glycoprotein from Phaseolus vulgaris. Biochem. and Biophys. Acta. 207, 413-431.

Racusen, D. and Foote, M. 1971. The major glycoprotein in germinating bean seeds. Can. J. Bot. 49, 2107-2111.

Racusen, D. and Foote, M. 1973. Glycoprotein II in developing and germinating bean seeds. Can. J. Bot. 51, 495-497.

Reznichenko, M. S. 1954. Studies on the S. S. Perov theory of the structure of proteins. Biochemistry (USSR). 19, 586-589.

Rondole, C. J. M. and Morgan, W. T. J. 1955. The determination of glucosamine and galactosamine. Biochem. J. 61, 586-

Sayanova, V. V., Slavnaya, T. S., and Sumenkova, V. V. 1971. Study of the albumin fraction of the seeds of some bean species. Fiziol. Biokhim. Kul't. Rast. 3, 202-206.

Schulze, E. and Winterstein, E. Z. 1910. Physiol. Chem. 65, 431.

Shain, Y. and Mayer, A. M. 1968. Activation of enzymes during germination. Trypsin-like enzyme in lettuce. Phytochemistry, 7, 1491-1498.

Shutov, A. D. and Vaintraub, I. A. 1966. Chromatographic isolation and some properties of the legumin and vicilin of vetch. Biochemistry (USSR). 31, 634-641.

Silano, V. V. de Cillis., and Pocchiari, F. 1969. Varietal differences in albumin fractions of Triticum aestivum and Triticum durum. J. Sci. Food Agr. 20, 260-261.

Smith, D. L., and Flinn, A. M. 1967. Histology and histochemistry of the cotyledons of Pisum arvense L. during germination. Planta. 74, 72-85.

Soedigo, R. and Gruber, M. 1960. Purification and some properties of a protease from pea seeds. Pisum sativum L.S. sp. arvense A. and G. Biochem. Biophys. Acta. 44, 315-323.

Spiro, R. G. 1970. Glycoproteins. Ann. Rev. Biochem. 39, 599-638.

Tkachenko, R. I. and Klimenko, V. G. 1971. A study of proteins of seeds of peas by gradient centrifugation in column and hydroxylapatite chromatography. Izv. Akad. Nauk. Mold. Ssrser. Biol. Khim. Nauk. 1, 28-33.

Tombs, M. P. 1967. Protein bodies in the soybean. Plant Physiol. 42, 797-813.

Vaintraub, I. A. and Nguen Tkhantven. 1971. The quaternary structure of vetch seed legumin. Mol. Biol. 5, 59-68.

Vaintraub, I.A., Shutov., and Klimenko, V. G. 1962. The globulins of vetch seeds. Biochemistry (USSR), 27, 293-305.

Vaintraub, I. A., and Gofman, Yu. Yu. 1961. N-terminal amino acids of pea legumin and vicilin. Biochemistry (USSR), 26, 13-18.

Varner, J. E., 1965. Seed development and germination. pp. 763-792. In plant biochemistry, des. Bonner and Varner. Acad. Press, N.Y.

Varner, J. E. and Schidlovsky, G. 1963. Intracellular distribution of proteins in pea cotyledons. Plant Physiol. 38, 139-143.

Virtanen, A. I., Berg, A. M., and Kari, S. 1953. Formation of homoserine in germinating pea seeds. Acta. Chem. Scand. 7, 1423-1424.

Weber, K. and Osborn, M. 1969. The reliability of molecular weight determinations by sodium dodecyl sulfate polyacrylamide gel electrophoresis. J. Biol. Chem. 244, 4406-4412.

Wetter, R. L. and McCalla, A. G. 1949. Electrophoretic properties of pea proteins. Can. J. Res. 27, 96-102.

Wright, D. J. and Boulter, D. 1972. The characterization of vicilin during seed development of Vicia faba L. Planta, 105, 60-65.

Wright, D. J. and Boulter, D. 1973. A comparison of acid extracted globulin fractions and vicilin and legumin of Vicia faba. Phytochemistry, 12, 79-84.

Yemm, E. W. and Willis, A. J. 1954. The estimation of carbohydrates in plant extracts by anthrone. Biochem. J. 57, 508-514.

Young, J. L., Huang, R. C., Vanecko, S., Marks, J. D., and Varner, J. E. 1960. Conditions affecting enzyme synthesis in cotyledons of germinating seeds. Plant Physiol. 35, 288-292.

Young, J. L. and Varner, J. E. 1959. Enzyme synthesis in the cotyledons of germinating seeds. Arch. Biochem. Biophys. 84, 71-78.