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PHYSIOLOGICAL, BIOCHEMICAL, AND SURFACE STRUCTURAL STUDIES OF LETTUCE SEED GERMINATION

by

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A dissertation submitted to the Graduate Faculty in Biology in partial fulfillment of the requirements for the degree of Doctor of Philosophy, The City University of New York.

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Abstract

PHYSIOLOGICAL, BIOCHEMICAL, AND SURFACE STRUCTURAL STUDIES OF LETTUCE SEED GERMINATION

by

Alexander Dimitrij Pavlista

Advisor: Professor Jack G. Valdovinos

These investigations attempt to correlate a cell wall degrading enzyme--endoglucanase--and endosperm weakening to the germination of lettuce seeds.

Lettuce seeds (achenes), Lactuca sativa L., were imbibed in water at optimal conditions. Germination was observed after 13 hr from the start of imbibition and the rate of germination was maximum after 16 hr. Fresh weight increased rapidly during the first 3 hr to 173% of that of unwetted seeds. Between 3 and 18 hr from the start of imbibition, fresh weight remained constant, except for a slight rise at 13-14 hr. Dry weight remained constant throughout the first 18 hr from the start of imbibition.

Endoglucanase activity was determined in extracts from 0 to 12 hr imbibed seeds with low salt containing buffer (low salt extract) and with this buffer fortified with 1 M NaCl (high salt extract). The enzyme activity in high salt extracts remained constant throughout the experimental period. However, the activity in low salt extracts

fluctuated in time, giving a minimum after 6 hr and a peak after 9 to 10 hr from the start of imbibition prior to the onset of germination. These activities were found to arise from the seeds and not from microbial contamination. Unlike endoglucanase activity, protein content in low salt extract did not fluctuate but remained relatively constant. The relative amounts of endoglucanase(s) in the low salt extracts were determined. Optimal assay conditions for the low salt extracted activity from 0, 6, and 9 hr imbibed seeds were determined to be temperatures between 40 and 50°C and pHs between 5.2 and 6.1. It was also determined that the 6 hr minimum in low salt extracted activity was probably not due to the presence of an enzyme inhibitor(s) but might be explained by a leaching of the enzymes out of the seeds into the sowing medium. Endoglucanase activity was measured in the medium of 6 and 9 hr wetted seeds.

The effects of ribonucleic acid synthesis inhibitors, i.e., 6-methyl purine and actinomycin D, and a protein synthesis inhibitor, cycloheximide, were determined on intact and punctured seeds. The results suggest that, in order to obtain low salt extractable endoglucanase activity, ribonucleic acid synthesis is required between 4½ to 7 hr of imbibition and protein synthesis is required between 5½ to 9 hr of imbibition. High salt extractable endoglucanase activity does not seem to depend on either ribonucleic acid or protein synthesis.

Extracts, both low salt and high salt, were chromato-

graphed on columns containing either Sephadex G-100 or G-200 gels. Low salt extracts of 0 and 9 hr imbibed seeds had 3 enzyme peaks (E1, E2, E3) while those of 6 and 12 hr imbibed seeds each had a predominant peak (E1) and a relatively small peak (E2). High salt extracts of 0, 6, 9 an d 12 hr imbibed seeds had four peaks (E1, E2, E4, E5). The medium (leachate) of 9 hr imbibed seeds had two predominant peaks (E1, E3) and a relatively small third peak (E2); relatively little protein leached from these seeds. The molecular weights (in Daltons) of these enzymes were determined to be: E1 - 480,000, E2 - 280,000, E3 - 40,000, E4 - 5,000 and E5 - 1,800. Therefore, E3 enzyme may be associated with the seeds' preparation(s) for germination.

To further characterize these enzymes (E1, E2 and E3), their possible subunit composition and isoelectric points were estimated. Separate fractions corresponding to the E1, E2 and E3 peaks were pooled, treated with sodium dodecyl sulfate plus mercaptoethanol and rechromatographed. E1 fractions had the following subunit peaks: 280,000 (E2), 200,000 (S2), 90,000 (S3), 55,000 (S4), 40,000 (E3), 18,000 (S6) and 11,000 (S7); E2 had: 200,000 (S2), 90,000 (S3), and 55,000 (S4); E3 had: 18,000 (S6) and 11,000 (S7). One interpretation of these results suggests that E1 is composed of 1 E2 and 5 E3, E2 is composed of 2 S3 and 2 S4 and E3 is composed of 1 S6 and 2 S7. The isoelectric points were determined by gel electrophoresis using buffers at different pHs and measuring activities at the origin and moving sections of each gel. By this method, the isoelectric point for E1 was

estimated to be between pH 5.0 and 5.1, for E2 between 4.4 and 4.6 and for E3 between 4.0 and 4.2.

Bulk low salt and high salt extracted endoglucanase activities as well as molecular weight-differentiated endoglucanase activities were determined on surgically separated parts of 0-1 and 9-10 hr imbibed seeds. Low salt extracted endoglucanases in 0-1 imbibed seeds were found in the radicle end (E1, E2, E3) and cotyledon end (E1, E2). The enzymes in the radicle end were all found in the embryonic axis and in the endosperm present at the radicle end (surrounding the embryonic axis). In 9-10 hr imbibed seeds, the endoglucanase distribution is substantially different. All low salt extracted endoglucanases (E1, E2, E3) in these seeds were found to be in the endosperm in the radicle end (surrounding the embryonic axis) and not in either the embryonic axis or the cotyledon end. The endoglucanase activity peak measured prior to the onset of germination seems to be due to the synthesis of new endoglucanases in the endosperm of the radicle end. High salt extracted endoglucanases were found to be predominantly in the radicle end of 0-1 and 9-10 hr imbibed seeds.

Low and high salt extracted endoglucanase activities of 9 hr imbibed seeds were determined in seeds sown in different light and different temperature treatments. High salt extracted activities were not affected by either far red light treatment or continuous darkness. In contrast, low salt extracted endoglucanase activities were lowered by exposure to continuous darkness, short exposure to far red light and higher

temperatures. The changes in endoglucanase activities in 9 hr imbibed seeds correspond with the changes in germination as measured after 24 hr. At 35°C, activity, although less compared to 20 and 24°C, was still extracted (low salt) from light treated seeds but activity was not measured in extracts from dark treated seeds. These results suggest that low salt extracted endoglucanase activities in correlation with germination is at least partially regulated by light and temperature treatments.

In order to correlate endoglucanase activities to endosperm weakening, changes in the appearance of the endosperm at the radicle end were determined using a scanning electron microscope (SEM). Ungerminated seeds imbibed for 0-20 hr were decoated and the embryos with their surrounding endosperms were prepared for observation with an SEM. The surface appearance of the endosperm of dry seeds and 6 hr imbibed seeds was smooth, without disruptions. With increasing imbibition time from 8 to 12 hr, there was an increasing degree of disruption or degradation of the endosperm in the micropylar region. Cracks and pits were evident and the tip of the micropylar region of some seeds had a large break or an opening. The number of seeds with these openings increased to a peak after 12 hr. The size of these openings in ungerminated seeds increased with increasing imbibition time. At the 10th hr, three-fourths of all seeds were surrounded by an endosperm characterized as being weakened at the micropylar region. The percentage of seeds with cracks or with openings declined after 12 hr due to germination. The percent germination after 24 hr

was the same as the percentage of seeds displaying endosperm degradation. The peak of SEM-observable endosperm degradation, which is preceded by a peak in endoglucanase activity, is correlated with the possible role of the endosperm as a mechanical restraint on embryo expansion.

SEM-observable endosperm degradation was also determined in 12 hr seeds imbibed in far red light, darkness and at temperatures of 24 or 35°C. Far red light greatly inhibited both endosperm degradation (12 hr) and germination (24 hr); darkness was less effective. Imbibition of seeds in darkness at 24°C further decreased germination and endosperm weakening as compared to light-treated seeds at 24°C. Endosperm weakening was not observed in thermodormant seeds (0% germination) sown in darkness. Endosperm weakening was observed in thermodormant seeds sown in white light, although the percentage of seeds with weakened endosperms was reduced compared to seeds sown at lower temperatures. This suggests that light and temperature may play a role in endosperm weakening in addition to endoglucanase activity. A correlation between endosperm weakening after 12 hr and endoglucanase activities after 9 hr with germination after 24 hr is suggested.

Endoglucanase activity and endosperm weakening may have a role in facilitating the germination of lettuce seeds. It was determined that a separation of embryo elongation and endosperm rupture was possible with isocyanurate treatment of seeds. About half of the seeds sown in 5 mg/ml of isocyan-

urate indicated that the embryonic axis elongated without rupturing through the endosperm. (These seeds are termed as "buckled" seeds.) However in some cases, atypical germination, i.e., rupturing of endosperm at either cotyledon end or mid-seed, was observed. Isocyanurate-treated seeds did not have either endoglucanase activity or SEM-observable endosperm weakening. These results suggest that some isocyanurate concentrations inhibit the possible endoglucanase-promoted weakening of the endosperm at the radicle (micropylar) end.

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INTRODUCTION

The embryo in the lettuce achene is surrounded by an endosperm, predominantly two cell layers thick and up to four cell layers thick in the radicle end (micropylar region) (Borthwick and Robbins, 1928; Foard and Haber, 1966; Jones, 1974). When the endosperm is surgically removed or punctured, there is an increase in germination (Evenari and Neumann, 1952; Ikuma and Thimann, 1959, 1963a; Speer, 1974). Damage of the endosperm by deuteron radiation also causes increased germination (Klein and Preiss, 1958a; Preiss and Klein, 1958). This finding has been widely interpreted as indicating that the endosperm (but not the integumentary epidermis (Globerson et al., 1974)) acts as a mechanical restraint (Koller et al., 1962; Amen, 1968) and may be a site of phytochrome action (Ikuma and Thimann, 1963a; Ikuma, 1964), and possibly gibberellic acid (Bewley and Fountain, 1972) actions in addition to the embryo (Klein and Preiss, 1958b; Ikuma and Thimann, 1959; Ikuma, 1964). These interpretations are not universally accepted because light requirement for germination is reinstated in half seeds and isolated embryos sown in growth-inhibiting concentrations of osmotica--substances which exert an osmotic pressure external to the seeds--(Scheibe and Lang, 1965, 1967; Nabors and Lang, 1971a, b). However, the

osmotica, in addition to the cutting of seeds, may not be inert and may cause damage. There is also the possibility that these substances may alter various systems (Khan, 1960; Karssen, 1970a; Berrie et al., 1974; Speer, 1974; Reynolds, 1975). Lang and his colleagues interpreted their results as an indication that light and gibberellins affect the embryo. But light and some growth regulators may affect both endosperm weakening and embryo expansion; high external osmotic pressures may inhibit only the latter. The mechanical restraint of the lettuce endosperm may be overcome by two mechanisms: (1) mechanical force of the growing embryo pushing against the endosperm (Nabors and Lang, 1971a, b) and (2) a chemical (enzymatic) weakening of the endosperm, especially at the radicle end (Ikuma and Thimann, 1963a; Jones, 1974). Similar suggestions have been made for other seeds, dark-inhibited and dark-promoted (Chen and Thimann, 1964; Chen, 1968, 1970; Baskin and Quarterman, 1969; Oegema and Fletcher, 1972). The endosperm of lettuce seeds sown in isocyanuric acid seems to be capable of resisting the elongation of the embryo, resulting in embryo expansion without endosperm rupture ("buckling phenomenon") and in protrusion at the cotyledonary end or along the side ("atypical germination") (Pavlista and Haber, 1970). Therefore, it was of interest whether enzymes which may partially degrade the endosperm cell walls occur prior to embryo expansion--the pregermination period.

Weakening of the endosperm would involve a partial degradation of the cell walls and cell separation. A similar situation has been observed with abscission zones (Rasmussen and Bukovac, 1969; Valdovinos and Jensen, 1968; Valdovinos et al., 1974; Webster, 1968, 1970, 1973). Endoglucanase (endocellulase, carboxymethylcellulase) which is a true cellulase capable of acting on plant cell walls (Lewis et al., 1974; Sobotka and Stelzig, 1974; Fincher et al., 1976), has been found to play a role in the cell loosening or separation process in abscission zones (Horton and Osborne, 1967; Abeles, 1969; Lewis and Varner, 1970; Linkins et al., 1973; Reid et al., 1974; Ratner et al., 1969; Greenberg et al., 1975; Goren and Huberman, 1976). Endoglucanase has also been demonstrated to have a role in cell expansion of shoot cells (Maclachlan and Perrault, 1964; Fan and Maclachlan, 1966; Ridge and Osborne, 1969; Ferrari and Arnison, 1974; Bryne et al., 1975; Bal et al., 1976). Therefore, this enzyme may play a role in seed germination, either in the weakening of endosperm, in cell expansion, or in both. Activity of this enzyme has been reported in cotyledons of kidney beans (Lew and Lewis, 1974) and in a preliminary study on lettuce (Ikuma and Thimann, 1963a). In the latter study, cellulases were investigated only after germination and radicle growth occurs.

The formation of the seed and fruit coats during fruit development has been reported (Davis, 1924; Borthwick and Robbins, 1928). Cytological changes occurring in embryonic axes and cotyledon cells during germination and radicle growth

have been described (Paulson and Srivastava, 1968; Srivastava and Paulson, 1968; Das, 1973). Jones (1974) reported an ultrastructural study of endosperm cells and he reported a light microscopy study of the changes occurring in the thickness of endosperm cell walls during germination. The cross-sectional structure of the endosperm was correlated with its restrictive role.

A physiological and biochemical study on a cell wall degrading enzyme such as endoglucanase (carboxymethylcellulase) during the period prior to germination seemed appropriate. Due to the limitations of light and transmission electron microscopies, a time course study, using scanning electron microscopy, on the surface appearance of the endosperm would complement the enzymatic studies. An objective of this work is to further clarify the role of the endosperm as a mechanical restraint and the removal of this restraint during lettuce seed germination.

LITERATURE REVIEW

This was the goal of the leaf and the root.
For this did the blossom burn its hour.
This little grain is the ultimate fruit.
This is the awesome vessel of power.

For this is the source of the root and the bud....
World unto world unto world remolded.
This is the seed, compact of God.
Wherein all mystery is enfolded.
Georgie Starbuck Galbraith (1960)

Improved seed germination, seed vigor, emergence and early seedling growth is of major importance in the development of agriculture both nationally (U.S. Department of Agriculture, 1961) and internationally (International Seed Testing Association and Association of Official Seed Analysts) (Woodstock, 1973). For a general discussion of these topics, the reader may wish to refer to the many excellent books (Mayer and Poljakoff-Mayber, 1963; Kozlowski, 1972a, b, c; Heydecker, 1973) and recent review articles (Mayer and Shain, 1974; Amen, 1974; Mayer, 1973; Chen and Varner, 1973; Wareing and Saunders, 1971; Black, 1969; Amen, 1968; Wareing, 1966; 1965a; b; Vegis, 1964; Koller et al., 1962 and Toole et al., 1956). This literature review deals with the major mechanisms for seed germination and, conversely, seed dormancy, as these

apply to lettuce seeds. In order to discuss germination, it is important to interpret how germination is defined. A seed is commonly considered to have germinated when the embryonic axis has protruded through the outer coverings. However, such a scoring method inherently implies and assumes that growth processes and developmental processes are synonymous in seed germination (Ikuma, 1962; Evenari, 1965; Haber, 1967; Ketring, 1973; Thevenot and Côme, 1973). Germination has also been divided into different phases, variously named according to the points of view of the authors (e.g., Ikuma, 1962; Amen, 1968; Ching, 1973; Katoh and Esashi, 1975; Tissaoui and Côme, 1975). With our increasing knowledge of the germination phenomena, it is becoming more clear that seed germination and active growth of the embryonic axis are probably different and a seed is primed or made ready for embryo growth. In lettuce, this may suggest an early hydration phase, a triggering phase for developmental processes, a developmental or germination phase, a triggering phase for growth processes and an axis growth phase.

The separation of lettuce seed germination into two processes, developmental or germination and growth of the embryonic axis, has been increasingly favored (Evenari, 1965; Haber, 1967). On the grounds of respirational studies, Evenari (1965) reported an early rise in respiration in seeds due to hydration (Phase I), after which respiration is level from about the fourth to sixteenth hour from the

start of imbibition (Phase II). Up to this point, there is no discernable difference in respiration between seeds which will or will not germinate. During this period of time, Evenari suggests that developmental or germination processes are functioning within the seed which will germinate and that these processes will result in a respiratory switch and elongation of the radicle (Phase III). This interpretation was supported by the use of such inhibitors as arsenate (Speer, 1973). Haber (1967), after studies on the tricarboxylic acid cycle and other biochemical events (Haber and Tolbert, 1959a) and cytological changes (Haber and Luippold, 1960a; b) similarly suggested a separation of developmental and growth processes. This suggestion has also been proposed for other seeds, e.g., apple (Pirus malus L.) (Thévenot and Côme, 1973; Tissaoui and Côme, 1975). Ikuma (1964) divided lettuce seed germination into four phases based on his studies on the light requirement for germination: 1) Pre-induction involved hydration of the seed. 2) Induction is defined as the time of red/far red light sensitivity based on the hypothesis that the phytochrome system is the trigger mechanism for germination. (It should be noted, however, that other triggering mechanism, e.g., a temperature-dependent system (Takeba and Matsubara, 1976) and a non-phytochrome, pigment system (Negbi et al., 1968) may act in addition and may also supercede the phytochrome system--refer to later discussions.) 3) Post-induction is described by Ikuma as the dark process related to the escape from light control. (However, this phase probably comprises

all the developmental processes that control dormancy, allow the seed to prepare for the embryo's growth and protrusion to the outside world and may include a system(s) triggering the next phase.) and 4) Visible germination is the protrusion of the radicle through the seed coats and is the growth phase.

I. Anatomy of Lettuce Seeds

In order to analyze lettuce seed germination, it would be helpful to begin with a description of the seed's structure. In a strict sense, what is commonly called the lettuce seed is actually an achene (Hayward, 1938; Esau, 1953). The structure of the lettuce seed or "achene" has been depicted by a number of authors (Borthwick and Robbins, 1928; Evenari and Neumann, 1952; Esau, 1965). Morphologically, the lettuce embryo, a dicotyledonous plant, is surrounded by three layers in the mature seed (Borthwick and Robbins, 1928): 1) The outermost is the pericarp; 2) The middle morphological layer is the integument which consists of outer epidermis with the remnants of crushed disorganized cells and a conspicuous suberized layer which may be semipermeable and belongs to the wall of the inner epidermis; and 3) The layer closest to the embryo is the endosperm (refer to Figure 1 in Materials and Methods section). The endosperm consists of thick walled cells,

possibly analogous to the aleurone layer in grains and it is predominantly two cell layers thick (Borthwick and Robbins, 1928; Jones, 1974). However, at the radicle end the endosperm may be three and four cells thick (Borthwick and Robbins, 1928; Foard and Haber, 1966). Physiologically, however, the embryo may be considered to be surrounded by two layers: 1) the pericarp plus the outer epidermis and remnants of disorganized cells of the integument and some of the walls of the inner epidermis, and 2) the endosperm plus possibly some of the walls of the inner epidermis of the integument (Borthwick and Robbins, 1928; Ikuma and Thimann, 1963a; personal observations). Evidence suggests that the endosperm and not the pericarp or the integumentary remnants may have a role in germination (Evenari and Neumann, 1952; Globerson *et al.*, 1974). Although the pericarp and the integument do not play a role in the germination of the mature lettuce seed, they do play a role in the development of the seed; furthermore, the endosperm in the immature seed is composed of many cells which are digested and absorbed by the embryo (Jones, 1927; Borthwick and Hendricks, 1928; Esau, 1965; Fahr, 1967). This general structure is found in plants in many families (Hayward, 1938) and some of these seeds have been studied in a similar way as lettuce with respect to the role of the endosperm (Compositae (Cichorieae), *Lactuca sativa*) (*e.g.*, Hydrophyllaceae, *Phacelia tanacetifolia* and *Nemophila insignis*; Compositae (Helianthese), *Helianthus annus*;

Scrophulariaceae, Striga lutea; Labiata, Stachys alpina).

II. Environmental Factors Affecting Lettuce Seed Germination

A. Light Effects on Lettuce Seed Germination:

Borthwick and Robbins (1928) observed that puncturing or cutting the seed coat of lettuce seeds increases the percentage and rate of germination. Other investigators (Böhmer, 1928; refer to Ikuma, 1962; Chen and Thimann, 1964; Chen, 1970) found similar results with Phacelia tanacetifolia' seeds. In fact, removal of the endosperm--resulting in a naked embryo--allows the embryo to grow at higher temperatures and to even escape phytochrome regulation (Evenari and Neumann, 1952; 1953a; Ikuma and Thimann, 1964). Although this suggests a photosensitive site in the endosperm, this may not be the case (Ikuma and Thimann, 1959; Ikuma, 1964; Scheibe and Lang, 1965). In his study on the timing of germination and growth and the effects of light, Ikuma (1962; 1964) reported that the first sign of radicle elongation in naked embryos corresponds exactly with the time of visible germination. Using 25°C, the optimal temperature for separating red light-promoted from far red light-inhibited germination, Ikuma measured the growth of radicles, and did not find any difference in elongation with respect to light treatment. This finding was also true of growth at other temperatures. However, Ikuma (1964) did note that

higher temperatures (30 and 35°C) promote growth of radicles in germinating seeds, but inhibit the number of seeds which germinate. At lower temperatures (below 25°C) radicle growth rate of nakedeembryos is decreased but dark germination is increased. Furthermore, red/far red effect on germination becomes less pronounced at the lower temperatures. At all of these temperatures, growth of radicles is unaffected by light treatment (Ikuma, 1962; 1964). Therefore, Ikuma suggested that germination/dormancy phenomenon and growth phenomenon are dissimilar and that phytochrome triggers developmental processes which overcome dormancy mechanisms and prepares the seed for ultimate embryo expansion (refer to following section on mechanical restraint). Furthermore, Ikuma and Thimann (1964) reported that oxygen is required for the reaction of P_{fr} with some reactant which is necessary for germination. They found that nitrogen atmosphere blocks this reaction and, in addition, facilitates a dark conversion of P_{fr} to P_r (Appendix B, Schedule I). Mancinelli and Borthwick (1964) suggested a similar model.

Although it is well established that naked lettuce embryos escape phytochrome regulation, Scheibe and Lang (1965) found a phytochrome control operating in half seeds osmotically-inhibited with mannitol. However, in this work, the authors measured "germination" by the appearance of geotropic curvature, which itself may be under some phytochrome regulation (Wilkins and Goldsmith, 1964). Secondly,

the osmoticum, mannitol, may not be inert and may, along with the cutting of seeds, cause damage to the half seeds. There is also the possibility that the substance may alter various systems (Khan, 1960; Karssen, 1970a; b; Berrie et al., 1974). Scheibe and Lang (1967), aware of a possible phytochrome effect on geotropic curvature, reinvestigated their finding by measuring straight growth of radicles in the same osmoticum. They noted that their batches differed markedly with respect to germination rates in the dark at 20°C. Far red light, although inhibitory, did not prevent germination. In this study by Scheibe and Lang, there was no difference in the growth rate with respect to light treatment once the length of the radicle increased to more than 0.27mm. However, radicles, greater than 0.20mm in length, do show a red/far red effect on percentage of radicles growing and on growth rate. But it should be noted that: a) There is a high percentage of embryos growing after far red exposure. b) Differences in embryo growth due to light treatment appear after more than 27 hr at which time most seeds would have already germinated. c) Significant differences due to light do not appear except when radicles have elongated to 0.20mm. d) The graphically-presented growth rate of embryos appears to be different, but the significance of the differences is unknown. The growth rate may be influenced by light in a narrow growth range which occurs after the onset of growth and prior to later seedling growth (Scheibe and Lang, 1967).

Lang, Nabors and their colleagues interpreted their results as indicative of a light influence on initial growth in contradiction to the data of Ikuma (1962; 1964). The significance of Scheibe and Lang's data may actually be with respect to early penetration through soil, or as an interim for light to trigger the start of later seedling growth through soil or in air as a precaution against growth at, for instance, night time (emergence). This latter hypothesis agrees with the studies on the breakdown of food reserves for seedling growth (refer to section on food reserves).

In further studies on the phytochrome system in lettuce seeds, it was observed that prolonged far red light, regardless of intensity, has a second inhibitory effect (Mancinelli and Borthwick, 1964; Negbi et al., 1968; Blaauw-Jansen and Blaauw, 1976). Through studies on the interactions of gibberellins, prolonged far red light and dark storage (Ikuma and Thimann, 1960; Negbi et al., 1968; Bewley et al., 1968; Burdett, 1972a; Vidaver and Hsiao, 1974), a model (Appendix B, Schedule II) may be devised which is based on a two pigment system (Negbi et al., 1968; Vidaver and Hsiao, 1974), one of which is phytochrome and the other a dark-active pigment, appearing after 6 hr which may interact with the former at a pathway junction and which may be thermosensitive (Berrie, 1966). Superimposed on this model are suggestions on the incorporation of some hormonal effects and the model of Ikuma (1964) (Appendix B, Schedule I).

B. Temperature Effects on Lettuce Seed Germination:

The greater number of investigations on environmental factors affecting germination has been with light, specifically phytochrome-related effects. Most of the studies on phytochrome have been done with dark grown plants, which do not have any obvious use for the pigment and dark growing is not a common phenomenon (Bünning, 1960; Holdsworth, 1972). With lettuce seed germination specifically, lettuce growers and seedmen in southwest U.S.A. and other areas are not concerned with light/dark reactions as most investigators are. But these growers are having difficulty getting lettuce seeds to germinate at high temperatures (Koostra, 1973; Gray, 1975; Heydecker and Joshua, 1976). Lettuce seeds are planted normally on or near the surface of the soil where light may not be a major agricultural factor; however, reviews of temperature effects on seed germination are rare and are usually incorporated within reviews on light effects (Toole et al., 1956; Toole et al., 1957; Koller et al., 1962; Toole, 1973; Mayer, 1973).

Among the first studies on the effects of temperature on seeds was on cocklebur, in which Crocker (1906) demonstrated that the upper and lower seeds in the bur germinate in different temperature ranges. High temperature inhibition of germination of lettuce seeds (thermodormancy) and the loss of phytochrome control and variable increases in dark germination of different batches and varieties of

lettuce seeds (thermosensitivity) has been reported by many authors (Davis, 1924; Borthwick and Robbins, 1928; Shuck, 1935; Evenari, 1952; Evenari and Neumann, 1953b; Toole et al., 1957). Photosensitivity seems to be a function of temperature or a supplement to temperature (Evenari and Neumann, 1953a; Berrie, 1966; Vidaver and Hsiao, 1975; Carpita and Nabors, 1976a).

As has been noted earlier, lowering the temperature at which seeds or naked embryos are sown decreases the rate of growth while increasing the percentage of germination, and light exposure has no effect (Ikuma, 1962; 1964). Ikuma concluded that light does not affect radicle growth at lower temperatures but that these lower temperatures may alter the endosperm, the presence of which is required for light induction of seeds sown in water (cf. Evenari and Neumann, 1952). Thus, germination may be facilitated, possibly by enzymatic weakening to break a mechanical restraint (Ikuma and Thimann, 1963a). He further suggested (Ikuma, 1962; 1964; Ikuma and Thimann, 1960; 1963b, 1964) that the two phenomena--germination and radicle growth--are different, differing in their responses to the environment and to growth regulators. Evenari (1961) wrote: "Germination is a sequential chain of physiological events leading to the beginning of growth. When growth starts, the germination process is over."

Gibberellins were reported to by-pass any light

requirement for germination and to overcome some of the temperature blocks to germination (Toole and Cathey, 1961; Ikuma and Thimann, 1960). The presence of gibberellins during heat treatment inhibits the development of full thermodormancy; in fact, when red light and gibberellin treatments are combined prior to a lowering of temperature, the treatments' effect is additive (Poljakoff-Mayber et al., 1958). Studies on the effects of cytokinins, specifically kinetin, and gibberellin, with respect to differing temperatures, indicate that these growth regulators promote germination in different ranges of temperatures (Haber and Tolbert, 1959b). These findings suggest an interdependency between effects of growth regulators and light exposures and the temperatures at which these effects are studied. Reynolds and Thompson (1971), using thermogradient bars, determined low and high temperature inhibition of germination and time of imbibition. From these studies, the authors suggested two parameters--low temperature and high temperature cut-off points. Different concentrations of kinetin and abscisic acid alter these two parameters. These data were further extended and elucidated for kinetin, gibberellins, abscisic acid, various regimes of light and their interactions (Reynolds and Thompson, 1973) (Appendix B, Schedule III). Although most of this work has dealt with only Arctic King and Grand Rapids cultivars of lettuce and seed germination, Gray (1975) determined the same findings

with respect to high temperature cut-off points for both germination and early emergence for a large number of lettuce varieties. Reynolds (1975) recently reported that different osmotica concentrations in which seeds are sown also alter the high temperature cut-off point in light and dark. Inversely, the osmotic potential of the external medium required to inhibit germination is changed by the temperature at which the study is done. This latter finding suggests another parameter to be considered in interpreting growth rate of lettuce embryos in osmotic-inhibition studies (Scheibe and Lang, 1967; Nabors and Lang, 1971a) in which growth rates of embryos are observed to be different with planting temperatures (Ikuma, 1964; Scheibe and Lang, 1967). The data (Reynolds and Thompson, 1971; 1973; Reynolds, 1975) indicate that the temperature of 25°C which most investigators use in their studies of light effects on lettuce seed germination is near the high temperature cut-off point. Lettuce seeds germinated at approximately 25°C seem to be particularly susceptible to promotion and inhibition resulting in potentially erroneous interpretations.

C. Temperature-Light Interactions:

The abrupt change in germination (full to none) over a short temperature range suggests a switch or a triggering mechansim influenced by temperature (Reynolds, 1973). Evenari (1961) suggested that there may be at least two environment-sensitive blocks to germination: one is

removed by photomechanism(s) and the other by thermomechanism(s). In their germination studies on lettuce seeds, especially New York as well as other cultivars, Takeba and Matsubara (1976) suggested that temperature affects a thermo-labile process which is regulated by a thermo-labile factor. They hypothesized that this factor may undergo thermo-reversibility with temperatures of 20 and 30°C and may be involved with, for example, inactivation of inhibitory processes (Berrie, 1966) or membrane permeability (Evenari, 1961) and may interrelate with the phytochrome system in a number of ways (Takeba and Matsubara, 1976). Berrie (1966), based on his studies with temperature and light, reported that seeds which germinate at low temperatures are not affected by light, but that seeds which do not respond to low temperature do respond to the classical light effect. Temperature effects were found to influence the early stages of germination. From his studies and those of others, Berrie (1966) suggested that for lettuce, photoblasticity "is in fact an artifact and though it does simulate some naturally photoblastic seeds in its behavior, the real benefit derived from studies with such seeds is that possibly a mechanism of germination can be formulated." It is noted again that dark germination does occur. The development of lines of Grand Rapids lettuce seeds by genetic inbreeding has resulted in increased dark germination, even at 25°C (Globerson et al., 1973); high dark

germination has become more and more common in agriculture. Berrie (1966) therefore proposed a scheme (Appendix B, Schedule IV) suggesting two paths: one photosensitive and the other thermosensitive. In this scheme, the phytochrome system would be evoked as a special response to higher temperatures. Berrie suggested an inhibitor accumulation as a process in this model. The thermolabile system may be related to a second pigment system (Negbi et al., 1968) (Appendix B, Schedule II). This model may also partially account for the ethylene effects and might explain why puncturing alone of seeds may increase dark germination (Speer, 1974), possibly by releasing ethylene through a wounding response. The suggested effects of acetone, acetone-kinetin and dichloromethane permeation of lettuce seeds are also consistent with this model (Rao et al., 1976). Further support for a non-phytochrome-related, temperature-controlled system was presented through time course studies on seeds exposed to fluctuating temperatures (20 and 35°C) and light treatments (Carpita and Nabors, 1976a).

Evenari (1961) attempting to calculate enthalpy and entrophy values for the high temperature-inhibition of germination (Cohen, 1958), suggested that temperature may affect membrane permeability. Koostra (1973) reported on similarities between liquid crystals, seed germination and in turn membrane structure with respect to response to light,

temperature and mechanical stress. He suggested that these and other similarities indicate an environmental effect (and maybe hormonal effect) on cell membrane permeability. Studies on the leakage of endogenous amino acids and perhaps an inhibitor from lettuce (Wareing and Foda, 1956) suggest that the germination-inhibiting effect of high temperature (30 to 35°C) in darkness may involve membrane integrity (Hendricks and Taylorson, 1976). As temperature increases, leakage of amino acids increase, implying a loss of membrane integrity. Because these authors did not test red/far red light effects on leakage, one may only speculate about the possibility that red light may increase or further stabilize the integrity of the membrane and may counteract or inhibit the high temperature-promoted membrane disintegration (high temperature-inhibited germination?). Therefore, red light promotion of germination at 25°C (near the high temperature cut-off point, Reynolds and Thompson, 1973--Appendix B, Schedule III) may be due to this mechanism and would not be in conflict with Berrie's model (1966) (Appendix B, Schedule IV) and his suggestion that phytochrome effects are artifactual responses to temperature. There is some support for the suggestion of a phytochrome effect on membrane permeability from other studies on lettuce seeds (Bewley et al., 1968; Burdett, 1972a) and on other systems (Hendricks and Borthwick, 1967; Haupt, 1968; Haupt et al., 1969; Tanada, 1968; Jaffe, 1968). In summary, the germination and growth

responses of lettuce seeds and embryos to light, temperature and osmotica are very complex and are interrelated with each other as well as with hormones--the controls are still unclear.

III. Mechanical Restraint

The anatomy of lettuce seeds (refer to the earlier sections) indicates that the endosperm envelopes the embryo as a sack-like structure. This observation suggests that the endosperm may act as a resistive force to embryo expansion as well as a barrier (refer to earlier sections) (Toole et al., 1956; Koller et al., 1962). With respect to the phases of germination proposed by Ikuma (1962; 1964), the removal of the mechanical restraint may occur during the postinductive phase if it is a prerequisite for germination, but would occur during the visible germination phase if it is a result of embryo growth. Evenari et al. (1957) reported that mitosis, the start of cellular elongation and radicle protrusion, occurs together during germination after 13 hr from the start of imbibition. There is not any significant difference in length of radicles between 0 and 12 hr. However, the lengths of radicles after 12, 13, and 14 hr from the start of imbibition, respectively, are significantly different from each other. The lack of radicle elongation prior to visible germination was also observed by others (Haber and Luippold, 1960a; Ikuma, 1962;

1964). If the endosperm acts as a mechanical restraint, then this restraint may be removed by a chemical weakening (Ikuma and Thimann, 1963a) or by an increased expansion force (Scheibe and Lang, 1967) or by both means (Koller et al., 1962; Chen and Thimann, 1966; Wareing and Saunders, 1971).

A. Endosperm Disruption:

As early as 1906, Crocker reported that cocklebur and iris among others, are induced to germinate after the seed coats are disrupted by chipping or cutting. Randolph and Cox (1943) confirmed these results with iris. Both laboratories observed that germination is most successful when it is done in the hilar region of seeds exposing the radicle tip. The stimulation of germination of various seeds by pricking, exposing the tip of the radicle or scarification, has been observed in many seeds (Toole et al., 1956; Koller et al., 1962; Ikuma, 1962; Ballard, 1973), including Phacelia tanacetifolia (Bohmer, 1928), Arachis hypogaea (Toole et al., 1964), Oryzopsis hymenoides (Barton et al., 1971), Asclepias syriaea (Oegema and Fletcher, 1972), Anemone coronaria (Bullowa et al., 1975), and Taxodium distichum (Murphy and Stanley, 1975). Many authors interpreted these studies as indicative of a mechanical restraint imposed by the coats, e.g., Phacelia tanacetifolia (Chen and Thimann, 1964; 1966; Chen, 1970), Nemophila insignis (Chen, 1968), Astragalus tennesseensis (Baskin and Quarterman, 1969), Stachys alpina (Pinfield et al., 1972) and Striga lutea (Egley, 1972).

Borthwick and Robbins (1928) observed a stimulation of germination with lettuce seeds when seeds were punctured and this has been confirmed by others (e.g., Evenari and Neumann, 1952). Ikuma and Thimann (1963a) using dissected lettuce seeds concluded that the endosperm does act as a mechanical restraint and that it is weakened by enzymatic reactions (discussed later in this section).

B. Endosperm as Site of Action: Chemical Weakening of Restraint:

Evenari and Neumann (1953b) observed water penetration of the fruit and seed coats within 3 min after wetting. Photosensitivity manifests itself in 5 to 8 min after wetting. These authors suggested that photosensitivity is localized in the outermost living tissue, endosperm or the outer cell layers of the embryo. When seeds are exposed to deuteron radiation of different intensity and penetrability, dark germination increases if the deuteron radiation disrupts the endosperm without damaging the embryo (Klein and Preiss, 1958a). Irradiation of the fruit coat alone is not sufficient to increase germination, while irradiation of the embryo moderates the effect and too much is lethal. The deuteron radiation effect on the endosperm is not reversed by far red light (Klein and Preiss, 1958a). Measuring the energy losses, Preiss and Klein (1958) determined that irradiation of the embryo is not required to overcome the dark dormancy, but that ionizations occurring in the

endosperm may be necessary and sufficient for increasing dark germination. Klein and Preiss (1958b) also studied light exposure. Seeds irradiated on one side with red light show red/far red reversibility if exposed to far red light on the other side shortly thereafter. These authors suggested that the photoreceptors (phytochrome forms) are probably at the tip at the radicle end of either embryonic axis or of the endosperm, since these would be the only places that could have been exposed to both lights.

Taking a genetic approach, Globerson et al. (1974), studying three pure lines of lettuce and crossing them, determined that factors controlling germination segregated in a normal Mendelian manner. From these studies, the authors concluded that the mechanical restriction is imposed only by the endosperm, a conclusion which is in agreement with that of Ikuma and Thimann (1963a). In this view, although possibly acting as a permeability barrier (Klein et al., 1971), the integumentary epidermis on the outer surface of the endosperm does not play a role in germination.

Seeds sown after gamma-irradiation at 26°C or sown at 10°C germinate without mitosis being evident. That is, radicle elongation occurs by cell expansion without cell division (Haber and Luippold, 1960a). Mannitol treatment at 26°C prevents cellular expansion by interfering with water uptake (Cleland and Bonner, 1956). Likewise, it prevents germination and cell expansion but mannitol does not prevent

the occurrence of mitosis in lettuce seeds. Therefore, Haber and Luippold concluded that radicle protrusion (embryo expansion) results from cellular expansion alone. This investigation was extended further through studies by Foard and Haber (1966). In a histological study of thermodormant lettuce seeds, they demonstrated that localized cell expansion occurs even though germination does not occur under these conditions. These authors also noted that cellular expansion in some parts of the radicle is accompanied by localized cellular compression. Foard and Haber interpreted these results as indicating that the endosperm,acting as a mechanical restraint, could account for the prevention of overall expansion and compression. In a preliminary study, Pavlista and Haber (1970) extended this work by studying the affects of an s-triazine herbicide, sodium 2,4 dichloroisocyanuric acid. The embryo axis of some treated seeds elongates as much as three fold without rupturing the endosperm. This suggests that the endosperm does act as a mechanical restraint in at least some circumstances. Furthermore, with a small percentage of seeds, the "buckled" embryos are finally able to develop enough force to protrude through the endosperm. But when this occurs, endosperm rupture develops either through the cotyledon end or sideways ("atypical" germination, refer below). It was also noted that few of the "buckled" and "atypically" germinated seeds are green, indicating chlorophyll synthesis may occur. These data, along with that of

Haber and Luippold (1960a) and Foard and Haber (1966) is interpreted as suggesting the requirement of a chemical weakening of the endosperm. However, short of a correlation of seed germination with an enzyme system(s) capable of weakening the endosperm, this hypothesis remains speculative.

Ikuma and Thimann (1963a) dissected seeds in various ways. As investigators before them, they observed that removal of the restraining force of the endosperm is sufficient to induce nearly total germination, irrespective of light treatments (Ikuma and Thimann, 1959). Since light treatments do not affect the elongation of the radicle in decoated seeds, whereas these treatments affect germination at higher temperatures, radicle elongation itself is probably not limiting in the early stages of germination. Ikuma and Thimann deduced that light effects the mechanical properties of the endosperm layer. In observing the structure of the lettuce seed (Figure 1, Materials and Methods), the radicle end (micropylar region) can be noted as being considerably narrower than the cotyledon end. Therefore, when the embryo grows--more specifically, when the radicle elongates--the amount of force or pressure is greatest against the endosperm at the radicle end. However, to partially counteract this pressure, the endosperm is also thickest at the very tip. When Ikuma and Thimann (1963a) cut seeds far back in the cotyledon end, they found that dark germination is little affected and the seeds show red/far red light reversibility.

But when seeds are cut in half or longitudinally, complete (90%) germination occurs in darkness and these seeds are unaffected by red or far red light. Most germination occurs "atypically" (a phenomenon first observed by Crocker, 1906 with Xanthium). Atypical germination is observed by the radicle elongating--pushing the endosperm in front of it (cotyledon protrusion or back-end germination). Interestingly, although these seeds are scored as atypically germinated, some radicles penetrate through the endosperm as well as push them forward (Appendix B, Schedule V). This is significant since the embryo does not have a "spring board" from which it can exert a mechanical force. Therefore, the protrusion of the radicle through the endosperm cannot be accomplished by mechanical expansion force caused by the embryo, but probably by a chemically weakened or degraded endosperm which offers little or no resistance to the radicle. Similar results have been reported for light-inhibited Phacelia tanacetifolia (honeybee plant) (Chen and Thimann, 1964) and for cold-requiring Syringa spp. (Juntilla, 1973a). Speer (1974) observed that puncturing alone of lettuce seeds is able to raise the level of dark germination while still letting the seeds have red/far red reversibility effects. It was suggested by this author that at least two mechanisms are involved independently in germination, one of which is activated by puncturing alone and another by light. These same observations were made with seeds sown under osmotic

stress (Khan, 1960). Kinetin seems to act on cotyledon expansion. Ikuma and Thimann (1963b) determined that some dark germination (at 25°C) may be brought about by the kinetin-induced expanding cotyledon's rupturing of the endosperm. Under these conditions, some seeds germinate atypically. Therefore it seems that those seeds whose endosperms are weak may be induced to germinate atypically in the dark by kinetin. However, an important comment about all the surgical studies is that the endosperm is disrupted or broken and this act alone may--and does--induce germination. It is possible that endosperm disturbances may release a substance which triggers germination and radicle elongation, speculatively, ethylene, which is known to be released by damaged cells (refer to reviews on abscission and fruit ripening) and which may play a role in endogenous regulation. This possibility may be speculated from studies on other seeds, e.g., species of Syringa (Juntilla, 1973a) and Striga lutea (witchweed) (Egley, 1972). (Refer to Discussion for a proposed model of lettuce seed germination).

While the scanning electron microscope observations of the endosperm of lettuce seeds reported in this thesis were being studied, Jones (1974) investigated the endosperms using transmission electron and light microscopes. He observed cell wall protruberances and thick cell walls which might account for the structural rigidity of the tissue and for its ability to act as a mechanical restraint.

With light microscopy, using periodic acid-Schiff staining technique (Jensen, 1962), Jones observed the onset of degradation as early as 8 hr from the start of imbibition (Park and Chen, 1974) and extensive digestion, as measured by loss of stain, of the endosperm's cell walls after 12-15 hr from the start of imbibition in light. After 14 hr from the start of imbibition, germination, as measured by radicle protrusion, began. Jones also observed that endosperm tissue removed from seeds imbibed for 24 hr possesses very little mechanical rigidity as measured by tearing with forceps while tissue removed from 1 hr imbibed seeds offers considerable resistance to such tearing. This observation clearly indicates that endosperm degradation occurs, at least, just prior to radicle protrusion. Furthermore, in this study Jones noticed that the cell walls are degraded from within outwardly; thereby suggesting that degradation is a result of enzymes formed within the endosperm cells. This study is further discussed in the Discussion of this thesis. Cell wall breakdown, therefore, is correlated with radicle protrusion and causal relationship with germination is possible.

C. Embryo as Site of Action: Expansion Force of the Embryo:

As was discussed in an earlier section ("Light effects on lettuce seed germination"), decoated lettuce seeds sown in water are not affected by light treatments. That is,

radicle elongation occurs after dark, red light or far red light treatments at the same rate and to the same extent (Ikuma, 1964 and others listed earlier). However, as noted earlier, imbibition of half seeds under high osmotic pressure reinstates the light control, that is, regulation of radicle elongation of lettuce (Scheibe and Lang, 1965, 1967) as with other seeds, e.g., honeybee plant (Chen, 1970). In an earlier section, osmotic effects were briefly reviewed and commented upon. This information is also relevant here since the work on expansion force of the embryo is based on results obtained from osmotic treatment. Nabors and Lang (1971a) sowed naked embryos in varying concentrations of mannitol, galactose and polyethylene glycol 4000 (PEG 4000). Contrary to Ikuma (1964), Nabors and Lang observed a difference in water uptake with respect to light treatment. Using the osmotic technique, they calculated that red-light treated embryos have a lower water potential (greater osmotic potential) than dark treated embryos; the difference being equivalent to the potential of 0.30 molal mannitol solution. These authors also removed the endosperm of 18 to 20 hr imbibed dark-sown seeds and, inserting into the endosperm half a glass rod with its distal end resting on a Mettler balance, they pulled the endosperm over the rod and recorded the force required to break through the endosperm. This force is equivalent to the osmotic potential of mannitol solutions between 0.16 to 0.38 molal. These authors

concluded that red light induces a decrease in water potential within the radicle which equals that required to break the endosperm's restraint. Dark sown seeds, on the other hand, do not have enough growth force to penetrate the endosperm. In dark dormancy, the endosperm is capable of restraining the expansion force of the embryo. Note any differences in mechanical restraints of endosperm in dark and light sown seeds were not measured. Changes in mechanical restraint of the endosperm which may occur are not known with respect to imbibition time nor on light treated seeds. There are a number of comments, in addition to those on osmotica referred to above, with respect to this work: a) The difference in potential between red light exposed and dark sown embryos does not develop until one hour after the radicle has begun to elongate. This seems to agree with earlier work (Scheibe and Lang, 1967) commented on in the earlier section. Furthermore, Nabors and Lang reported that the full growth potential is reached after 15 hr, yet germination, in terms of radicle growth, is 80 to 90% by 16 hr. b) The increased potential, reportedly equivalent to 0.30 molal mannitol, may not be enough to overcome the endosperm whose "strength" is reportedly within a range equivalent to 0.16 to 0.38 molal mannitol. c) The method of pulling the endosperm upon the rod was not described, but this act may cause assorted other forces to act on the isolated endosperm, e.g., shearing and pull forces.

Furthermore, the cutting of the endosperm may have caused changes to occur within the endosperm cells (as indicated by the puncturing experiments of Khan, 1960 and Speer, 1974a). This comment also applies to half seeds. Also, there may have been drying of the endosperm cells during preparation for the resistance measurements. d) Surgical damage may have occurred during embryo isolation. The osmotica may also damage embryo cells and may penetrate within. e) The osmotica may cause plasmolysis of cells, which would alter their properties. f) If the endosperm's mechanical restraint is the reason for dark dormancy because of its ability to withstand the growth potential of the embryo, then one might expect to see a "buckling" phenomenon to some extent (Pavlista and Haber, 1970). The endosperm's mechanical restraint is not necessary when the radicle isn't producing a countering force since it is not elongating (Ikuma 1962; 1964). However, the restraint's weakening may result in a "triggering" mechanism for radicle growth.

In a companion paper (Nabors and Lang, 1971b), isolated embryos were sown in water and it was reported that red light treated embryos grow more rapidly than dark sown embryos. This was contrary to the findings of Ikuma (1962; 1964). Light-treated embryos were placed into osmotica (mannitol or PEG 4000) after 15.5 hr to measure their water potential; dark sown embryos were handled likewise after 18.5 hr. The water potential of both water imbibed embryos is the same

and is zero to 0.10 molal mannitol. It should be noted that in both cases the radicles of approximately 80% of the embryos have already begun to elongate and therefore are past germination (refer to earlier discussions). Nabors and Lang (1971b) concluded that, since the water potential of growing radicles is the same for light and dark treated embryos, and since the red light treated embryos grow faster than dark treated ones, the growth potential due to red light treatment is not allowed to accumulate but is converted into growth. A red light induced increase in the radicle's osmotic potential would be due to degradation of storage substances as proteins and lipids. Nabors et al. (1974) observed such degradation, but noted that it does not occur until after 24 hr from the start of imbibition when most seeds have already germinated. Furthermore, the breakdown of sucrose and appearance of reducing sugar seems to be major differences between red, far red and dark treated seeds, and this can not account for any changes in osmotic pressures and therby growth potentials (Carpita and Nabors, 1976b). (The use of food reserves during lettuce seed germination is discussed in the next section.) In contrast to the data reported by Nabors and Lang (1971a), Loercher (1974), although inducing red light sensitivity in Grand Rapids lettuce with 0.3 M mannitol (as Nabors and Lang), reported that germination is only possible if, after irradiation in this osmoticum, seeds are transferred to solutions of lower osmotic potential. Far red reversibility is

evident in these seeds and declines, with respect to time between exposure, at a slower rate than seeds sown in water; i.e., many seeds sown in 0.3 M mannitol respond to far red light exposure even when seeds sown in water do not, while all seeds sown in 0.6 M mannitol still respond to far red reversibility. Loercher (1974) related these effects to cell water content required for phytochrome reaction and for radicle elongation. However these results are in direct conflict with those of Nabors and Lang (1971a). Great Lakes variety of lettuce, considered to be photo-insensitive, can be induced to be photosensitive when naked embryos are sown in osmotica as mannitol (Negm et al., 1973). Under this condition, however, the radicle may be induced to elongate not only by red light, but also by ethylene or ethylene plus carbon dioxide even after 7 days incubation at 25°C. Whether this stimulation occurs prior to radicle elongation or after its onset and growth rate measurements were not reported. However, this suggests, at least, that phytochrome is not the sole regulator or influence in countering osmotic effects.

Besides the above studies on lettuce seeds, two other groups of seeds have been studied similarily: Xanthium pennsylvanicum (Esashi and Leopold, 1968) and Syringa spp. reflexa, vulgaris and josikaea (Juntilla, 1973a, b). Esashi and Leopold compared the growth potential of the smaller, upper dormant cocklebur seeds and the larger, lower nondormant seeds. The initial percent increase in fresh weight

due to hydration is the same for both seeds. They measured growth potential at 25°C in darkness for 22 to 24 hr, not by osmoticum-treatment, but by a mechanical apparatus using a piston, mercury and a red dye which enters a vertical calibrated column. Strength of the testa was measured by adding weight (mercury) into a container placed atop of a piece of soft pencil lead acting as a simulated axis; the pencil lead was inserted into the testa, which was clamped by its edges by lucite blocks. Esashi and Leopold (1968) concluded that the nondormant seeds developed twice as much thrust as the dormant seeds as measured by push force against mercury, and that this increased force is capable of rupturing the testa. It is noted that light is not a factor in these experiments. Since osmotica were not used, any influences of these substances do not apply. However, this work does involve problems with respect to mechanical technique similar to those discussed earlier. Furthermore, the seeds are different in other ways than their dormancy state as size and the conditions under which these measurements were obtained are far from natural.

Juntilla (1973a; b), studying Syringa spp., not only did a surgical approach on the role of the endosperm as Ikuma and Thimann (1963a) and Chen and Thimann (1964), but also measured growth potential of the embryo and mechanical resistance of the endosperm. Growth potential was measured using osmotica and the resistance of the endosperm was also

measured by a similar method to Nabors and Lang (1971a; b); therefore, these measurements are subject to the same comments given earlier. Juntilla in his study determined that the growth potential of nondormant seeds was greater than dormant embryos and that chilling, gibberellin (GA_3) and high temperature treatments favoring germination, increase the growth potential. Also, the growth potential of the less dormant species, josikaea and vulgaris, is generally higher than for the more dormant species, reflexa. However, in a time course study on the mechanical restraint of the endosperm, Juntilla measured a rapid decrease in this restraint occuring prior to visible germination of nondormant seeds, especially if the axis is present in the cap, the latter suggesting an embryo/endosperm interaction. This result does not occur with dormant seeds. Generally, the endosperm's strength is also weaker in the less dormant species, as opposed to the more dormant species. Juntilla (1973a) concluded that these results with Syringa spp. indicate that the weakening of the endosperm's mechanical resistance, probably by enzymatic means, is an important part of the germination process.

By applying a slight pressure on Striga lutea seeds with a blunt dissecting needle after different imbibition times, Egley (1972) estimated the relative resistance of the endosperm (aleurone) to embryo growth. In seeds stimulated to germinate, there is an increase in the percentage of seeds with weakened endosperm. This increase begins 2 hr

prior to the onset of germination. The maximum percentage of seeds with weakened endosperms (95%) is reached 1 hr prior to the first detection of germination. The weakening of the endosperm only occurs at the radicle end.

D. Endosperm Weakening and Embryo Expansion in Relation to Light and Temperature:

The endosperm of lettuce seeds evidently does act as a mechanical restraint and this is now widely accepted. Whether this restraint is removed by chemical (enzymatic) weakening or is ruptured by a stronger mechanical force exerted by an expanding embryo is still rather controversial. The finding of an enzyme system which acts prior to the onset of germination or radicle elongation would support the former suggestion. A precise time course study on partial endosperm weakening following the enzymatic activity but preceeding radicle protrusion should be demonstrated. To measure the expansion force and relate this to endosperm resistance without interpretive disagreements may be quite difficult. One problem in the work on measuring the resistance of the endosperm (Nabors and Lang, 1971a) is that the measurement was performed only on endosperms of seeds imbibed in the dark for 18-20 hr. This assumes that the endosperms have not already been partially weakened at least in some seeds, and, therefore, it is a weakened endosperm that is measured. A time course study in both light and dark-treated seeds similar to that performed on Syringa

spp. (Juntilla, 1973a) would have been more informative. These areas of study may not have been investigated by Nabors and Lang (1971a) because of an assumption that light is essential and is the only "triggering" mechanism for lettuce seed germination (Lang, personal communication; Carpita, personal communication). However, there is ample evidence to demonstrate that phytochrome is not the sole system involved in germination but may in fact be a supplementary system activated due to high temperature stress (Berrie, 1966; Globerson et al., 1974). As examples; Speer (1974) demonstrated that simply puncturing the endosperm is sufficient to induce more than 60% germination in 24 hr at 26°C and he suggested that this puncturing activates a system apart from light (ethylene due to wounding? Cooper et al., 1969; Rasmussen, 1973). Bewley and Fountain (1972) demonstrated that gibberellic acid must be added along with cytokinins to dark-imbibed intact seeds in order to reverse the inhibitory effect of abscisic acid, but with naked embryos (endosperm removed), gibberellic acid is not required. Negm et al. (1973) showed that the phytochrome system is functional and stable at 35°C and therefore thermodormancy is not due to a direct inactivation of the phytochrome system itself. Furthermore, ethylene in the presence of carbon dioxide acts on the high temperature-induced block(s) as separate from phytochrome. Rao et al. (1976) suggested that organic acids used as nonaqueous penetrants, as dichloromethane and acetone,

act primarily in weakening of the endosperm as opposed to kinetin which acts on the embryo in alleviating the effects of inhibitors. Therefore, even when a red/far red control or dark-inhibition may not be demonstratable on a particular system(s), e.g., enzyme activity, it is not precluded that the system(s) is not playing a role and is not necessary for lettuce seed germination.

In referring again to Nabors and Lang (1971a), even if the expansion force of a nondormant embryo is greater than the resistance of the endosperm, during the mechanical rupturing, the radicle tip may be severly damaged, which would lead to poor development of the seedling and may even be fatal. Such damage has been reported by Ikuma (1962; and Thimann, 1963a) when he injected seeds with water and other solutions, indicating a considerable degree of fragility. Therefore, it may still be maintained at the very least that endosperm weakening is necessary, although not sufficient, in order to facilitate protrusion and thereby avoiding damage to the radicle tip and assisting the result of a growing, healthy and successful seedling.

E. Food Reserves:

Fats and oils make up most of the food reserves in lettuce seeds, although proteins may also be an important reserve (Paulson and Srivastava, 1968). Fats are stored as globules in the cotyledons and, to a smaller extent, in the hypocotyl and undeveloped plumule. Griffiths (1938) did not

detect any change in fat globules prior to the onset of germination at about 12 hr. After the onset of germination, the globules begin to break up, becoming smaller and ultimately disappearing by the end of the second day. Fat hydrolysis is most rapid between 32 and 64 hr from the start of imbibition; during this same period, the greatest increase in reducing sugar content occurs as well. Lipase activity was measured prior to these determinations; most activity in lettuce is found between 24 and 48 hr. The presence of two lipases, an acid and neutral pH activity peaks, was determined (Rimon, 1957). The neutral lipase activity (the lesser of the two) increases to a peak after 72 hr. Although the activity in light treated seeds starts earlier, the neutral lipase activity peaks to the same extent regardless of light or dark treatment. Acid lipase activity (the greater of the two), on the other hand, decreases during the first 24 hr and increases only after 48 hr regardless of light or dark treatment (Rimon, 1957). Nabors et al. (1974) reported observing a moderate degradation of lipid and an extensive one of protein in embryos, but, as with Griffiths, not until after 24 hr from the start of imbibition. Therefore, the degradation is associated with radicle growth after germination. This interpretation agrees with that of Srivastava and Paulson (1968) who observed considerable degradation of lipid and protein in the embryo after 36 hr. Besides fat reserves, lettuce seeds also contain reserves of sucrose.

By measuring sucrose during the first 24 hr, Poljakoff-Mayber (1952) suggested that the initial energy for germination is not from fat hydrolysis, but from the oxidation of nonreducing sugar reserves, sucrose. Since acid invertase is known to hydrolyze sucrose, Eldan and Mayber (1974) measured its activity during germination. This enzyme is found to be synthesized de novo, but only after more than 10 hr and is significantly measured after 15 hr. Therefore, the enzyme activity seems to be associated mainly with germination (radicle protrusion) and not with the pregermination period. This is consistent with the findings that sucrose seems to be utilized prior to the enzyme's appearance. Since proteins may also be a reserve, Poljakoff-Mayber (1953a) measured protease activity. Activity was found to be present in dry seeds and to increase steadily to about 24 hr. Protease activity then levels and decreases after 48 hr. Shain and Mayer (1965) determined the presence of three distinct types of proteolytic activity, pHs 4.8, 5.6 and 6.8. The acid-active proteases do not change significantly during the first 24 hr from the start of imbibition, but the near neutral (pH 6.8) protease activity decreases steadily to a minimum after 24 hr. After 24 hr, pH 6.8-activity increases again and was reported to be trypsin-like, inhibited during the first 24 hr by a trypsin inhibitor, and is activated, not synthesized de novo, after the inhibitor disappears (Shain and Mayer, 1965, 1968). Starch and amylase, a starch-

degrading enzyme, have been found commonly in some seeds (Marcus, 1971). In lettuce, however, starch is not present and does not act as a food reserve (Griffiths, 1938; Poljakoff-Mayber, 1953b) and amylase activity likewise has not been found (Poljakoff-Mayber, 1953b; Chen and Chang, 1972). Furthermore, although a phosphorous source (Gesundheit and Poljakoff-Mayber, 1962), phytin could not be demonstrated to be an energy source; in addition, the presence of a phytin transphosphorylation (phytin-nucleotide diphosphate phospho transferase) could not be demonstrated (Mayer, 1973). Phytase activity, although occuring, does not develop until after the pregermination period is over (Meyer et al., 1971). There are a number of recent reviews which deal with stored reserves and their associated degradation enzymes in seeds (Mayer and Shain, 1974; protein and peptidases--Altschul et al., 1966; Ryan, 1973; Ashton, 1976; lipids and lipases--Mazliak, 1973; Mayer and Shain, 1974; Mudd, 1967).

The above discussion deals with degradation occuring primarily in the embryo; but the endosperm may also act as a reserve-containing tissue. Its degradation as observed by light microscopy, has been mentioned above (Jones, 1974). The dense cytoplasm of endosperm cells contains protein bodies and lipid-containing spherosomes and the cell walls form many peg-like projections into the cytoplasm. By 12-15 hr from the start of imbibition these cells are considerably degraded; also, there is an early decrease in amiline

blue-black staining indicating a gradual loss of protein bodies. Park and Chen (1974), measuring the dry weight changes of the endosperm over a 3 day period, observed a gradual loss in this parameter. The fats, which are the substrates for respiration, are metabolized to glucose which is transported as sucrose to the embryo. Isolated embryos, although growing normally, do not grow as well as when the endosperm is present. This clearly suggests that the endosperm can be used as a food source but as a food source is primarily involved in the early seedling growth, postgermination period, and not in the germination processes itself. It was noted also that starch is not present in the endosperm. Therefore, most carbohydrates probably come from the cell wall polysaccharides (Park and Chen, 1974). Halmer et al. (1975) determined the sugar composition of the endosperm cell walls which accounts for two-thirds of the total seed cell wall polysaccharides. Endosperm cell walls have a marked difference in composition compared to those of the embryo, radicle and cotyledon. The endosperm walls contain predominantly mannose, 60% of the sugar composition and also glucose, galactose, arabinose and uronic acids, each accounting for 10% of the sugar composition. Halmer et al. (1975; 1976) reported the slight presence of endo-β-mannanase activity in dry lettuce, which does not change until 14 hr from the start of imbibition, at which time activity increases. This increase occurs three hours after radicle

protrusion. This enzyme's appearance is correlated with germination of dark-sown and gibberellin-treated seeds. However, the increase in activity was not measured prior to radicle protrusion in any experiment. The low persistent level of activity during the pregermination period may play a role in endosperm weakening (Halmer, personal communication). Similar results have been obtained with studies on galacto-mannanases, galactosidase, mannanases and mannosidases in legume seeds (Hylin and Sawai, 1964; Reid, 1971; Reid and Meier, 1972; McCleary and Matheson, 1974; 1975; 1976; McClendon et al., 1976; Villarroya and Petek, 1976). Carboxymethylcellulases (refer below) have been found in cotyledons of Phaseolus vulgaris during germination (Lew and Lewis, 1974). Ikuma and Thimann (1963a) attempted to measure pectinase and carboxymethylcellulase activities in both radicle and cotyledon ends of seeds. They did not detect any activity in the radicle end, but did detect a little activity in the cotyledon end. However, the seeds were extracted shortly after the onset of germination or radicle protrusion and growth; the techniques used are relatively insensitive compared to viscometric assays. Injection of commercially obtained enzymes (pectinase, pentosanase and cellulase) into the radicle end under the endosperm without puncturing the embryo significantly stimulates germination of dark sown seeds compared to water-injected controls. However, injection of water alone

stimulates some germination compared to uninjected controls; this is probably due to just puncturing of the endosperm (Speer, 1974). Although this suggests that these enzymes might play a role as a preparatory system for radicle protrusion and lettuce seed germination, this suggestion remains speculative from these data.

IV. Transcription and Translation Requirements for Lettuce Seed Germination

Early studies on the requirement of RNA synthesis for germination were done using uracil derivatives, e.g., 2-thiouracil, 5-fluorouracil, 5-bromouracil, as inhibitors. Since some of these antimetabolites of nucleic acids inhibited and even prevented light- and gibberellin-stimulated germination, it was concluded that RNA synthesis is a requirement (Smith and Frankland, 1966; Khan, 1966; 1967a). As an aside, Smith and Frankland (1966) reported that a greater concentration of uracil derivatives is required to decrease the percentage of germination of dark-imbibed half seeds than light-treated intact seeds, further indicating that cutting the endosperm is sufficient to cause gross changes in the systems. The conclusion that RNA synthesis is a requirement was further supported with the use of other synthesis inhibitors as 6-azauracil, 8-azaguanine (Khan, 1967a; b), 6-methyl purine (Frankland et al., 1971) and cordycepin (Tao and Khan, 1976). Khan (1967b), however, reported that

actinomycin D (AD) and puromycin were ineffective in preventing seed germination or radicle elongation. These results were confirmed (Black and Richardson, 1968). These authors suggested that seed germination in lettuce may involve an AD-resistant RNA synthesis. This possibility seems less likely due to later studies on the penetration of labeled AD (Tao and Khan, 1976). When seeds are "clipped" at the cotyledonary end and sown in AD, RNA synthesis and germination are inhibited. By measuring the distribution of labeled AD, AD was found not to enter intact seeds significantly. Therefore, it was concluded that RNA synthesis is a requirement and that earlier results with AD were due to the lack of AD penetration into the seeds. Frankland et al. (1971) measured labeled-phosphate incorporation into RNA with time in seeds sown in light or dark at 27°C. RNA synthesis occurs in the light-treated and dark-sown seeds and the rate of synthesis is the same in both until after 12 hr, at which time the radicles begin to appear. The above findings were confirmed with studies using labeled uracil incorporation; there is no difference in incorporation into RNA between seeds treated with red light or far red light at 20°C (Hecker and Köhler, 1975).

Chloramphenicol was found either to have no effect on or to stimulate germination in dark sown seeds (Black and Richardson, 1965; 1967; 1968; Khan, 1967b). This suggests that protein synthesis inhibitor prevents

the synthesis of a germination inhibitor. It has also been noted through autoradiographic studies with labeled leucine that the endosperm is the site of protein synthesis but the endosperm has been shown to be a barrier to leucine uptake (Klein et al., 1971). Cycloheximide, on the other hand, prevents germination and inhibits protein synthesis (Khan, 1967b; Black and Richardson, 1968; Bewley and Black, 1972). Hecker and Köhler (1975) demonstrated, measuring labeled leucine uptake and incorporation, that protein synthesis begins as early as 5 hr and increases to 15 hr after start of imbibition. Similarly to RNA synthesis, these authors did not detect any difference between seeds sown in dark at 20 or 30°C, or treated with red or far red light. The formation of polysomes is another indication of the occurrence of protein synthesis. Polysome formation increases up to the time of radicle protrusion in light-treated seeds; in dark-sown seeds, there is no increase in polysomes (Mitchell and Villiers, 1972). However, a later study indicated that in dark-sown seeds, polysome formation and protein synthesis occur early during imbibition. The polysomes, still active, persist after 24 and 48 hr (Fountain and Bewley, 1973). This conflict remains unresolved. Polysome formation and protein synthesis, however, may be affected by growth regulators corresponding with their effects on germination (Rao et al., 1975; Fountain and Bewley, 1976).

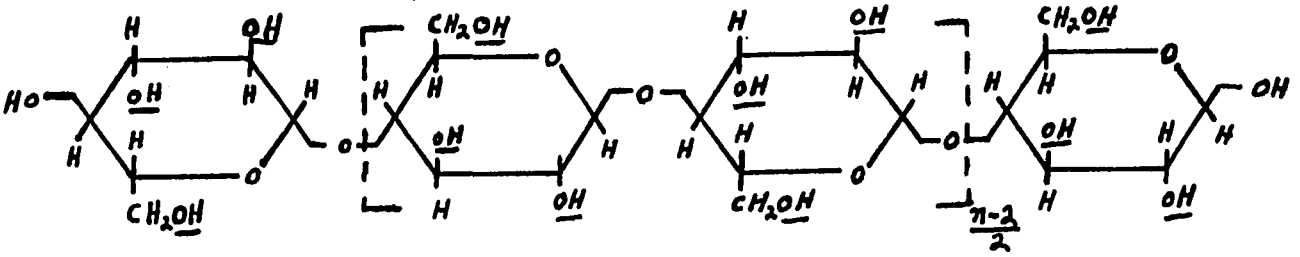
A note is added about work on synthesis inhibitors in

that, although the respective inhibitors may act on inhibiting synthesis, they may also act on other systems (e.g., cycloheximide, McMahon, 1975). In addition, synthesis occurrence and inhibition are measured quantitatively and therefore aren't indicative of qualitative differences, for instance, between light, dark or high temperature treated seeds (Bewley and Black, 1972; Hecker and Köhler, 1975). In lettuce seeds, enzymes have been reported to arise from synthesis de novo, invertase (Eldan and Mayer, 1974), endo-β-mannanase (Halmer et al., 1976) and endoglucanase herein presented. However, enzymes, adenosine triphosphatase (Meyer et al., 1972), have been reported not to be synthesized de novo but to arise by activation, even showing a wet-dry reversibility.

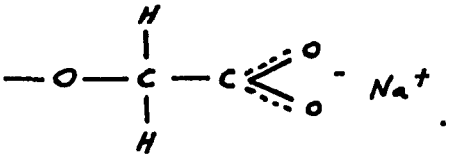
V. Endo $\beta(1\rightarrow4)$ Glucanase (Carboxymethylcellulase)

Cellulase is a general term used to describe a group of enzymes which degrade cellulose, consisting of: C_1, which is an enzyme whose action is unspecified and obscure, C_x, which is a multi-component enzyme and is considered to be synonymous with the term $\beta(1\rightarrow4)$ glucanase, and β-glucosidases, including cellobiase, which degrades oligosaccharides, cellobiose to cellohexose (King and Vessal, 1969). Carboxymethylcellulase, which degrades cellulose substituted with

carboxymethyl groups to varying degrees of substitution (DS) is considered to be an endo (1 4) glucanase (a C_X-type of cellulase) (Halliwell, 1961; Klop and Kooiman, 1965; Wirik, 1968; Selby and Maitland, 1967). The structure of sodium carboxymethylcellulose is: cellulose with the



hydroxy groups (OH) (underlined) substituted with sodium carboxymethyl groups



The DS is the number of OH groups substituted per anhydrous glucose units; therefore, the maximum DS is three. The CMC used in most studies in higher plants has a DS of 0.7; in other words, there are seven OH groups substituted in every 10 glucose units or 30 possible substitution points. The higher the DS, the more resistant is the CMC to enzymatic degradation, DS of 1 being rather resistant. The lower the DS, the less soluble is CMC. Carboxymethylcellulase or endo $\beta(1\rightarrow4)$ glucanase attacks the molecule between two unsubstituted anhydrous glucose and only at sequences of two or more unsubstituted anhydrous molecules (Hercules Inc., 1971; Isherwood, 1970; Klop and Kooiman, 1965; Wirick, 1968). Books presenting general surveys of cellulase enzymes have been published (Gould, 1969; Reese, 1963). There has been

some controversy as to the biological significance of this enzyme because it attacks solubilized cellulose. However, there is growing evidence that this enzyme acts in conjunction with C_1 enzymes, will attack insoluble cellulose given longer incubation time and is a true cellulase (Selby and Maitland, 1967; Hartley et al., 1973; Sobotka and Stelzig, 1974; Vose, 1974; Lewis et al., 1974 Kanda et al., Fincher et al., 1976). Endo $\beta(1\rightarrow4)$ glucanase has been implicated in a few higher plant processes--fruit ripening, abscission and cell elongation and has been related to the role of some growth regulators (Hulme, 1970; Hobson, 1968; Crane, 1964; Marcus, 1971; Fan and Maclachlan, 1966; Byrne et al., 1975; Abeles, 1969; Linkins et al., 1973; Reid et al., 1974; Greenberg et al., 1975). A comparison between the findings on endo $\beta(1\rightarrow4)$ glucanases in these studies and that reported in this thesis will be discussed later in the thesis ("Discussion" section).

MATERIALS AND METHODS

Seed Source and Storage

Lettuce seeds or achenes (Lactuca sativa L. cultivar Grand Rapids) from 1971 harvest were obtained from Vaughan's Seed Co., Downer's Grove, Ill. Seeds were placed in brown bottles enwrapped with aluminum foil and stored in a dessicator maintained at 4°C in a refrigerator. Prior to use, seeds were allowed to acclimate for one half to 15 hours at room temperature.

Surface Sterilization

In all experiments, one gram of seeds was placed in a fritted funnel, 150 ml volume and 6.5 cm in diameter, which was connected to a vacuum line. To the seeds, 10 ml of 5% chlorox dissolved in 5% ethanol was added. After 1 min, the vacuum line was opened to remove the solution and then closed. Ten ml of 70% ethanol was then added for 1 min, after which this solution was also removed by vacuum. Following this surface sterilization, seeds were washed four times with sterile distilled water, each was being removed by vacuum. Seeds were allowed to dry under vacuum

for 30 min. During seed exposure to each solution, the funnel was agitated to ensure that all seeds were surface sterilized.

Table 1 demonstrates the relative effectiveness of 70% ethanol as compared to other concentrations.

Seed Germination Conditions

One gram of seeds was sown in five sterile Petri dishes, 15 X 90 mm, each containing 10 ml of sterile distilled water or an experimental solution. Petri dishes were placed in a Sherer-Gillette controlled environment chamber, model CEL 255-6, set at 20°C. Standard light exposure for germination was continuous white light, a mixture of fluorescent and incandescent bulbs, approximately 750 ft-cdl at seed level. In some experiments, seeds were sown in darkness plus a 30 min exposure to white light, approximately 375 ft-cdl, given after 3 hr of darkness. In other experiments, seeds sown and kept in darkness. In addition, some seeds received a 1 hr exposure to far red light, furnished by two incandescent 150 watt lamps underlaid with a 3 mm thick slab of Rohm-Haas 2025 black plexiglas and 3 cm of water, given also after 3 hr of darkness. In order to observe the effects of higher temperatures, seeds were also sown at 24 and 35°C.

Germination was scored at 10 to 30X and was indicated by the slight appearance of the radicle's tip.

Table 1: Relative effectiveness of different ethanol concentrations as seed surface sterilization agents.

a) Seeds were sown at 35°C in continuous darkness. No germination was observed during this time.

b) Relative visible contamination represented by:
- (no contamination), -/+ (little contamination--1 or 2 seeds), + (mild contamination--a number of seeds or areas), ++ (excessive contamination)

TABLE 1

days at 35°C[a]	ethanol concentrations, %						
	0	10	25	50	70	85	95
2	+[b]	-	-	-	-	-/+	-
5	++	+	+	+	-	+	+
7	++	+	+	+	-	++	+
9		++	++	+	-	++	++
11					+		

Weight Determinations

Seeds were removed from Petri dishes after 15 min to 18 hr of imbibition. They were blotted dry and placed on a previously dried and weighed weighing paper and weighed on a Mettler H10 balance. These weights after subtracting for weight of paper and dividing by number of seeds comprised the fresh weight/seed. These seeds plus the weighing paper were placed in an oven set at 105°C for 18 hr and were weighed again. These weights after the above adjustments comprised the dry weight/seed. Fresh and dry weights were also determined on dry, 0 hr imbibed seeds.

Low Salt Extraction of Endoglucanases

One gram of seeds was ground with a pestle in an iced mortar containing 5 ml of cold homogenizing solution (Appendix Schedule I). The ground seeds were transferred to a cooled grinding vessel. Five ml of cold homogenizing solution was added to the mortar and the residue was washed into the vessel. The ground seeds in the 10 ml of solution were further homogenized using a "drill press" homogenizer. After homogenization, the homogenate plus 5 ml of cold homogenizing solution, which was used to wash the tissue grinder, were transferred to a centrifuge tube and centrifuged in a Sorvall ultracentrifuge, model RD-2B, at 10,000 G for 10 min at 4°C. The pellet was used to make high salt extract.

The supernatant was transferred to another tube and recentrifuged at 37,000 g for 20 min at 4°C. The resulting supernatant was aspirated through a millipore filter, 0.22 or 0.45 um pore size. This filtrate is termed the "Low Salt" extract.

In early extractions, 20 mg polyvinylpyrrilidone (PVP) GAF was added per ml filtrate. This mixture was kept in an ice bath for 30 min and agitated every 5 min. PVP was filtered out by passing the mixture through another millipore filter. Later extractions indicated that the use of PVP was not necessary during acidic extractions (below pH 6.5), and therefore this step was eliminated.

The enzyme activity did not diminish when extracts were frozen and stored in a freezer for two weeks.

High Salt Extractions of Endoglucanases

"Complete High Salt" extract was obtained by extracting seeds with homogenizing solution fortified with NaCl salt. "High Salt" extract was obtained by rehomogenizing the pellet, resulting from first (10,000 g) centrifugation, in a grinding vessel with 15 ml homogenizing solution fortified with salt then centrifuged at 37,000 g for 20 min at 4°C and passed through a millipore.

Assay of Endoglucanases

Equal quantities of extracts and preincubated (15 to

30 min) substrate solution (Appendix, Schedule I) were conbined and mixed for 10 sec using a vortex-genie. These assay solutions, having a pH of 5.8 ± 0.1, were incubated in a water bath set at 41 ± 1°C. After 0, 0.25, 0.5, 1, 2, 3, 5 and/or 8 hr, 1 ml aliquots were removed from the assay solutions and added to the measuring chamber (stainless steel chamber and spindle), kept at 41 ± 1°C via connection to pump in the water bath, of a Wells-Brookfield Rotary Spindle Microviscometer and viscosity was measured. Buffer blanks and/or boiled extracts were used as assay controls. Other pHs and temperatures were used to determine optimal values.

Endoglucanase activity was expressed as a percent decrease in viscosity of enzyme plus substrate solutions after different incubation times. Boiled enzyme plus substrate (1 part extract: 1 part 1.5% CMC) and diluted substrate solutions (0.75% CMC) were used as controls.

Protein Determination from Extracts

Protein contents of 1 ml solutions of 1 part extract to 1 part buffer were measured using the microbiuret method (Goa, 1953). Bovine serum albumin was used as a standard (Appendix Schedule II).

Concentration of Media

The sterile distilled water in which seeds were sown for different time periods was removed from 5 Petri dishes, pooled (50 ml) and placed into an Amicon Ultrafiltration cell, Model 202, with an Amicon Diaflo Ultrafiltration membrane, UM2. Using 3 atm N_2 pressure, the media were concentrated from 100 ml to less than 15 ml, then removed from apparatus and diluted to 15 ml. Aliquots of these concentrates were assayed for endoglucanase activity.

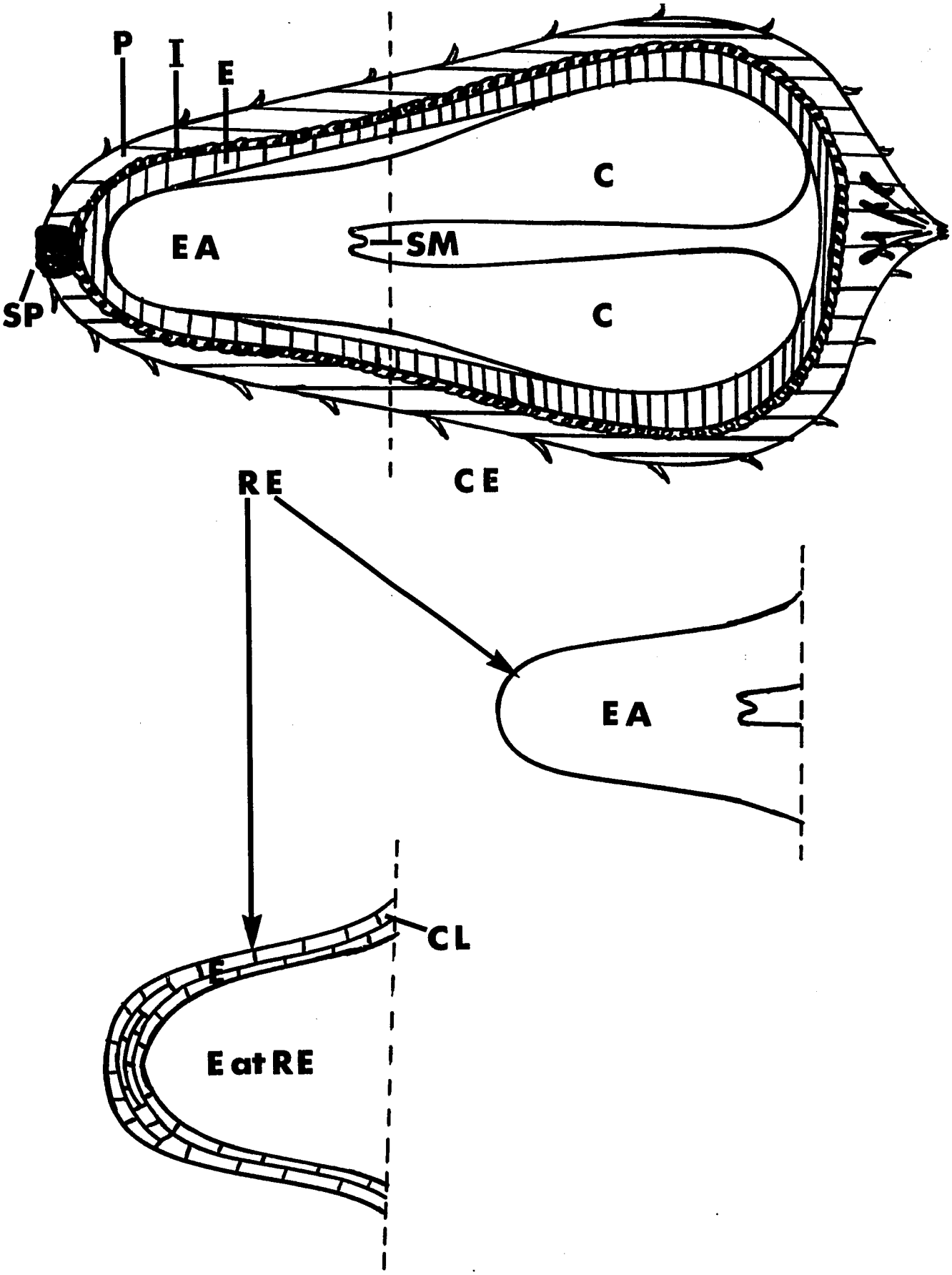
Seed Dissections

The major anatomical parts of a lettuce seed (achene) are represented in Figure 1. The different dissections were as follows: i) 0.6 gm seeds were sown for 1 hr, after which the media were removed. The pericarp of each seed was removed with a forcept and dissecting needle; these depericarped seeds were then resown in sterile distilled water for 8 hr before extraction in 9 ml of homogenizing solution. Removal of pericarp from dry seeds and from dry seeds wetted for 5 min with water was poor, often resulting in damage to seeds. ii) Dry seeds (slightly wetted) and 9 hr imbibed seeds were cut transversely with a scalpel and separated into a micropylar end (1/3 length of seed) and cotyledonary end (2/3 length of seed). These ends were extracted separately. iii) Micropylar ends were

Figure 1. Schematic diagram of lettuce seeds and seed parts resulting from dissection (dashed lines). Enzymes were extracted from whole seeds, cotyledon ends, radicle ends, embryonic axes, endosperm from radicle ends and depericarped seeds.

C - cotyledon, CE - cotyledon end, CL - cell layer, E - endosperm, EA - embryonic axis, I - integumentary remains, P - pericarp, RE - radicle end, SM - shoot meristem, SP - seed plug.

Figure 1



removed from 1 and 9 hr imbibed seeds and were dissected into embryonic axis and endosperm (part surrounding embryonic axis). These parts were extracted for endo-glucanase enzymes. The separation of cotyledons from their surrounding endosperm of 1 hr imbibed seeds often resulted in damage to both and cotyledon debris could not be completely removed from within the endosperm sack.

Inhibitors

Seeds were sown for different time periods up to 9 hr in solutions of (CH) cycloheximide (100 mg/L), (Sigma), (AD) actinomycin D (100 mg/L) (Merch, Sharp & Dohme) or (MP) 6-methyl purine (67 mg/L) (Sigma). In the case of AD and MP treatments some seeds were punctured with a 22 G 1 syringe needle (B-D) in the cotyledonary end. Seeds were first placed in 125 ml Erlenmeyer flasks and rinsed with the appropriate sowing media; the rinsing solutions were removed and the seeds were sown in fresh media. Seeds, transferred from one sowing medium to another, were rinsed with the new medium before resowing the seeds with fresh medium.

Extraction for Column Chromatographic Separation

Three grams of seeds were ground with a pestle in a cooled mortar containing 4 ml of homogenizing solution (Appendix Schedule I). The resulting slurry was transferred

to an iced grinding vessel and the mortar was washed with 3 ml of homogenizing solution which was then added to the grinding vessel with slurry. The ground seeds were homogenized in a "drill press" homogenizer and transferred afterwards to a centrifuge tube. The tissue grinder was washed with 2 ml of homogenizing solution which was then added to homogenate. The homogenate was centrifuged in a Sorvall ultracentrifuge at 10,000 g for 10 min at 4°C. The resulting supernatant was recentrifuged in another tube at 37,000 g for 20 min at 4°C; afterwhich the centrifugate was passed through a millipore filter, 0.22 or 0.45 um. This centrifugate comprised the "low salt" extract.

The pellet, formed as a result of the first (10,000 g) centrifugation was resuspended in salt fortified homogenizing solution (Appendix, Schedule I), and ground and homogenized as ground seeds. The resulting homogenate was centrifuged only at 37,000 g for 20 min at 4°C and passed through a millipore filter, 0.22 or 0.45 um. This centrifugate comprised the "high salt" extract.

The medium in which 9 hr imbibed seeds were sown was collected. With the use of an Amicon Ultrafiltration Cell (refer above), 150 ml of medium was concentrated to 9 ml. In some separations, sucrose was added to concentrate to form a 10% sucrose solution in order to give density to the concentrate, "medium" extract.

Cotyledon ends and micropylar ends were dissected from 2 gm seeds and were homogenized as above in 6 ml of

homogenizing solution, 2 ml of this homogenate was added to the column.

Embryonic axes and endosperm tissue from micropylar ends from 1 gm seeds were homogenized as above in 3 ml of homogenizing solution. Two ml of this homogenate was added to the column.

Column Chromatography

Two ml of extract was passed through a column (Pharmecia Fine Chemicals K25/45) maintained at 8°C in a cold room and packed with either Sephadex G-100 or G-200 (Pharmecia) swelled in buffer, pH 5.9 plus an anti-microbial agent (Appendix, Schedule III). In early runs, the column was fitted with a sample applicator but later a flow-adaptor and a 4-way valve was used (Pharmecia A-25 and LV-4). In the latter case, 2 ml of extract followed by 1 ml of 10% sucrose was injected through valve and adaptor. Fractions of 60 or 120 drops were collected by tubes placed in a Fractomette 200 fraction collector. Each fraction was assayed for endoglucanase activity as described above, using an assay incubation time of 24 hr. Column dimensions, bed height and volume, flow rate, operating pressure, void volume, gel swelling procedure and column packing procedure are described in Appendix, Schedule III.

Molecular Weight Determinations

Molecular weight of endoglucanase peaks was determined using both gels, Sephadex G-100 and G-200. Protein standards (Pharmacia-Calibration Kit) were aldolase, ovalbumin, chymotrypsinogen A, ribonuclease A and Blue Dextran 2000; bovine serum albumin was also used. The partition coefficients (Kav) were calculated and plotted against the logarithm (base 10) of the respective molecular weights. Kav for the endoglucanase peaks was calculated and the molecular weights were determined from graphs (Figures 33 and 39). Protein standard solutions and Kav calculations are described in Appendix, Schedule IV.

Protein Determination of Fractions

The optical density of 120 drop/tube fractions (or two pooled 60 drop/tube fractions) were measured at wave lengths of 260 and 280 nm with a Beckman spectrophotometer, Model 25. Protein contents were calculated using the formula mg protein/ml equals 1.45 absorption coefficient at 280 nm minus 0.74 absorption coefficient at 260 nm (mg protein/ml = 1.45 E280 - 0.74 E260) (Warburg and Christian, 1942; Kalckar, 1947; Bailey, 1967).

Subunit Determinations

Extracts were fractionated by chromatography in a column (refer above) packed with Sephadex G-200. Appropriate tubes were pooled and concentrated to 0.2 ml using Centriflo membrane cones, type CF25 (Amicon). The concentrate was diluted to 2 ml with SDS solution (Appendix Schedule I) and incubated at 40°C. After 4 hr of incubation, tubes were transferred to 4°C, and after 30 min at 4°C, the concentrate/SDS solution was clarified (centrifuged at about 1,000 g for 1 min). Two ml of supernatant was added to the column and refractionated. Fractions were assayed for endoglucanase activity as described above using an assay incubation time of 24 hr.

Isoelectric Point Determination and Gel Electrophoresis

Extracts were fractionated by chromatography in a column (refer above) packed sith Sephadex G-200. Appropriate tubes were pooled and concentrated to less than 1 ml using centrifuge cones (refer above). The concentrate was diluted to 1 ml with distilled water. 0.1 ml of extract was added to each polyacrylamide gel. Tubes were loaded with separating gel (ammonium persulfate as a catalyst) and stacking gel (riboflavin as a catalyst) and were placed in a vertical polyacrylamide gel electrophoresis apparatus (Canalco) kept in an ice bath (Appendix Schedule V). The gels were run in

buffers with different pHs (4.0 to 9.0) (Appendix Schedule V) for 1 hr with a current of 3 milliamps/tube. Bromcresol green and bromphenol blue were used as tracking dyes. After an hour, the tracking dye moved 1.5 cm; the gels were divided into an origin (from origin to 3 mm) and moving section (from 3 to 15 mm). Each section was cut, placed into a grinding vessel containing 2 ml of homogenizing solution (Appendix Schedule I), homogenized with a "drill press" homogenizer and then centrifuged (Sorvall Ultracentrifuge RC-2B) at 10,000 g for 5 min at 4°C. The supernatant was assayed for endoglucanase activity as described above using an assay-incubation time of 48 hr. Isoelectric points were the pHs at which there was a loss of activity from the moving sections and the simultaneous appearance of activity in the origin section. (Gel solutions, gel preparation, tracking dye solutions, dye application, and buffer solutions are described in Appendix Schedule V).

Preparation for Scanning Electron Microscopy

After different times of imbibition, seeds were fixed in 3% gluteraldehyde in 0.5M sodium cacodylate buffer, pH 7.2, for 2 days at 4°C (Appendix Schedule VI). The seeds were then rinsed three times in buffer. The pericarp and remnants of the integument including the inner epidermis were removed with dissecting forcepts. A small puncture was made through the cotyledonary end of some seeds with a

dissecting needle to facilitate dehydration. The embryos with their surrounding endosperms were dehydrated in ethanol and freon series (Appendix Schedule VI) and dried in a critical point drier using freon-13. The dissected seeds were mounted on studs an d coated with gold in a Technic's Hummer and examined in a scanning electron microscope (JEOLCO JSM-U3) using accelerating voltages of 20-25KV.

Isocyanurate Treatment of Seeds

Seeds were sown in sodium 2,4 dichloroisocyanuric acid (iC) (Sears) concentrations of 1, 2.5, 5.0 and 10.0 mg/ml for various time periods. Germination, embryo "buckling" and "atypical" germination (refer to text for description) were observed under 10 to 30X power with a dissecting scope (Bausch-Lomb HC-PHI). Seeds treated for 9 hr in iC were extracted and assayed for endoglucanase activity as described above. Seeds treated with iC were also prepared for observation with a scanning electron microscope as described above.

RESULTS AND OBSERVATIONS

Germination and Weights

Germination was scored in seeds observed with a dissecting microscope at a magnification of 10 to 30. Germination was indicated by the slight appearance of the radicle's tip. No germination was observed during the first 12 hr (Figure 2). The rate of germination peaked during the 15th and 16th hr from the start of imbibition (Figure 51, Curve C). After 18 and 24 hr, percents germination were 74 and 78 respectively. (Percent germination continued to rise but at a much slower rate--84% after 48 hr, 95% after 96 hr.)

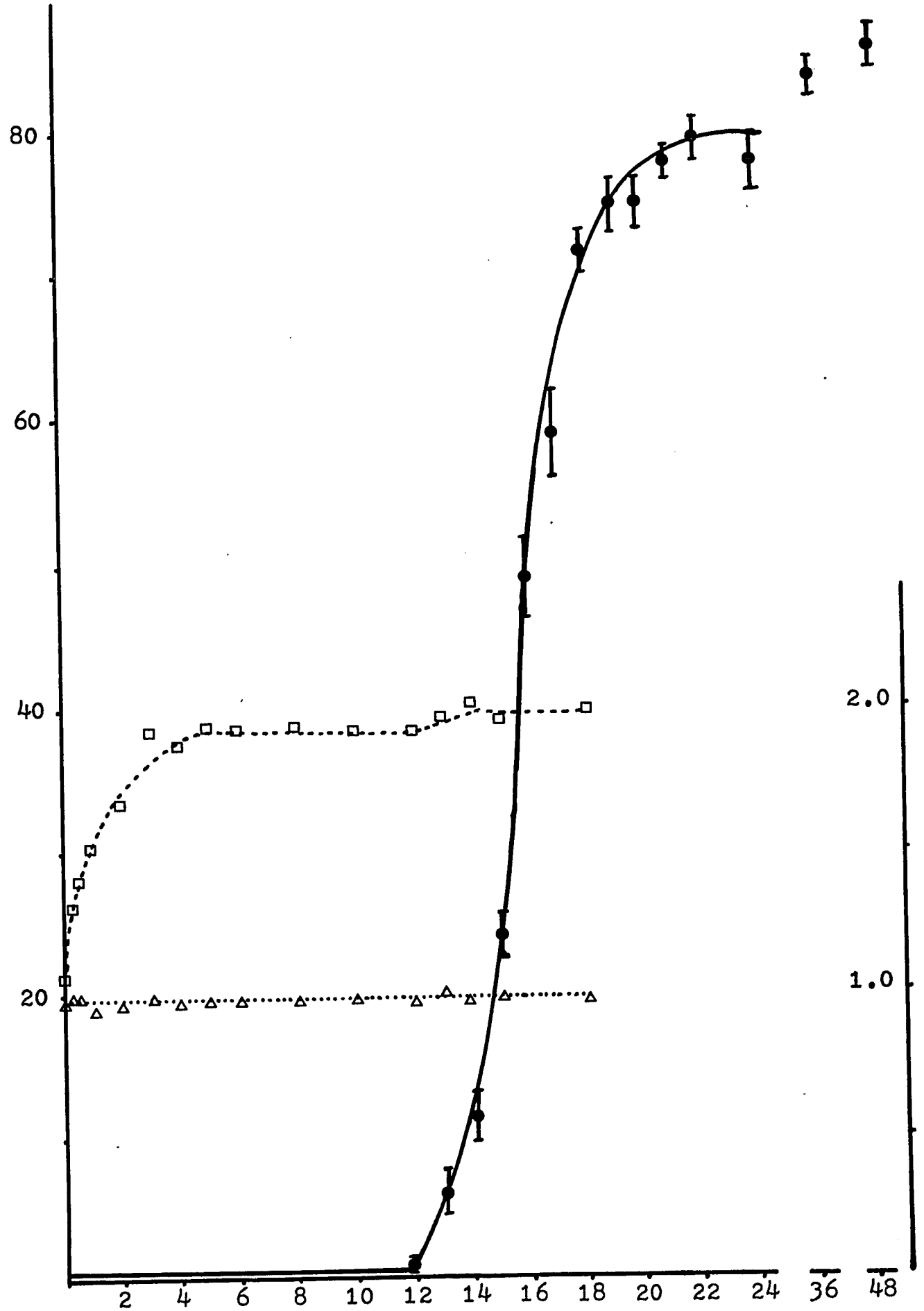
There was rapid uptake of water in the first 15 min as indicated by an increase in fresh weight from 1.1 to 1.4 mg/seed (27% increase) (Figure 2). After 3 hr of imbibition, fresh weight levels were at 1.9 mg/seed (73% increase with respect to unsown seed). The fresh weight rise, which appeared between the 13th and the 14th hr, corresponded with the onset of germination. During the 18 hr period of imbibition and germination, dry weight remained constant at 0.98 to 1.0 mg/seed (Figure 2).

Figure 2. Germination, fresh weight and dry weight of lettuce seeds, cultivar Grand Rapids, sown in 20°C, continuous light.

abscissa = hours of imbibition
primary ordinate = % germination
secondary ordinate = mg/seed

germination (closed circles)
fresh weight (open squares)
dry weight (open triangles)
(bars = standard error)

FIGURE 2



Endoglucanase Activity Prior to Germination

Seeds imbibed for 0, 2, 4, 6, 8, 9, 10 and 12 hr were extracted for endoglucanase activity. Extractions consisted of three types: low salt (no added salt--unfortified), high salt (1 M NaCl fortified buffer--extracted from residue after low salt extraction) and "complete" high salt (1 M NaCl fortified buffer--seeds were not extracted previously). Both high salt and "complete" high salt extracted endoglucanase activities were constant throughout the 12 hr imbibition period (period prior to the onset of germination) (Figure 3). The high salt extracted enzymes may not have a role in the germination process. In contrast, low salt extracted endoglucanase activity fluctuated during this period. Activity was present in dry (0 hr imbibed) seeds, decreased to a minimum at 6 hr and thereafter increased to a peak (maximum) after 9 to 10 hr of imbibition. After the activity peaked, it decreased somewhat before the onset of germination (Figure 3). Protein content of the low salt extracts of 0, 6, 9 and 12 hr imbibed seeds was measured using the microbiuret method (Goa, 1951). Protein content, expressed as mg protein per ml extract (11--12 ml per extract), increased from approximately 1.6 to 2.1 for 0 and 6 hr imbibed seeds respectively; there was no significant difference in protein content between 6, 9 and 12 hr imbibed seeds (Figure 3).

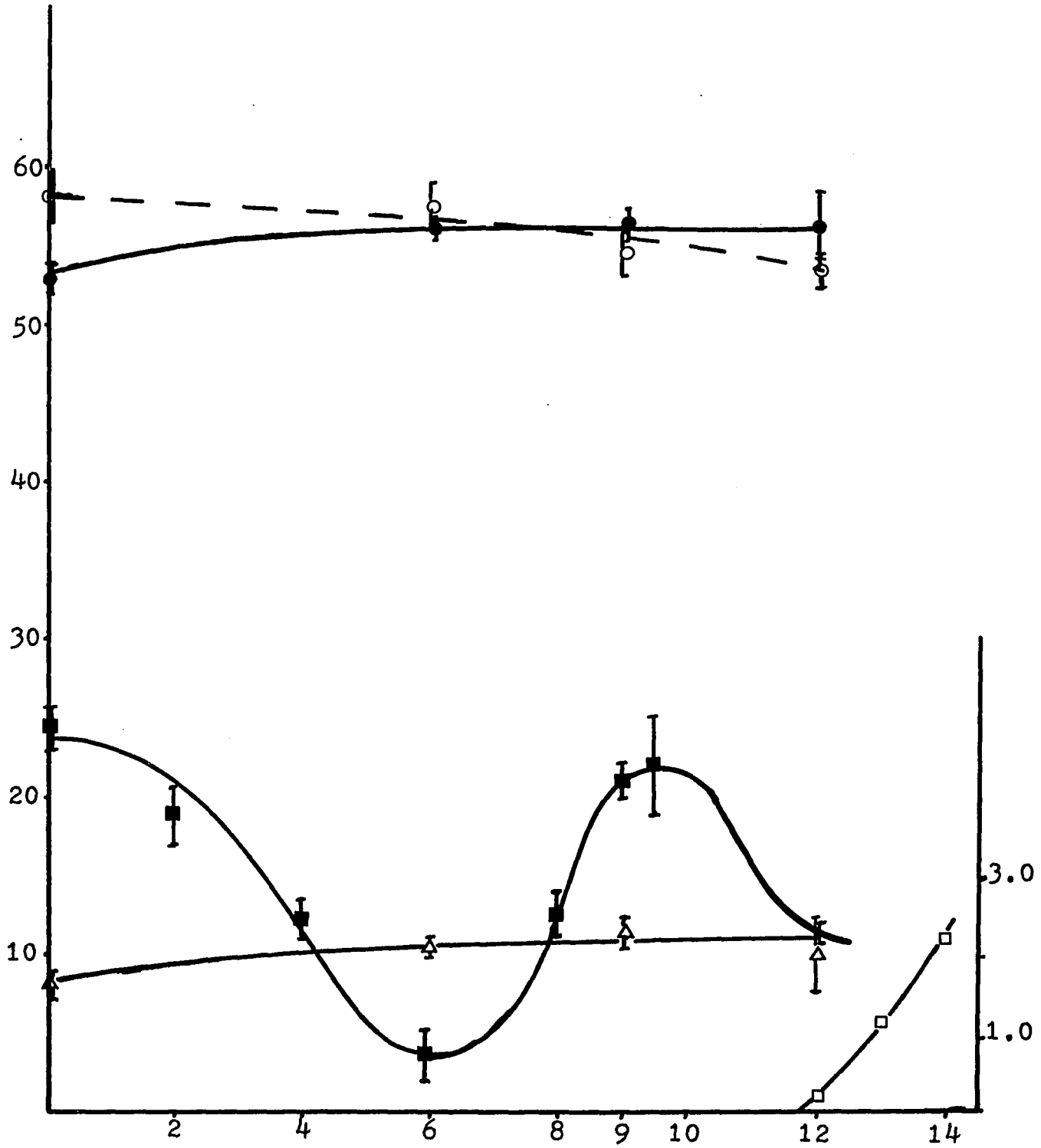
Endoglucanase activities were also assayed in the water (the medium in which seeds were sown) in which seeds were

Figure 3. Endoglucanase activities of salt-fortified and unfortified extracts and protein content of unfortified extracts.

abscissa = hr of imbibition
primary ordinate = % degrease in viscosity of 0.75% CMC after 2 hr (% $\Delta\eta$2 hr) (or % germination)
secondary ordinate + mg protein/ml extract

endoglucanase activity in low salt extracts (closed squares)
high salt extracts (closed circles)
"complete" high salt extracts (open circles)
protein content in low salt extracts (open triangle)
germination (open squares)

FIGURE 3



imbibed for 4, 6, 9 or 12 hr. The media were assayed in two different ways: 1--media were added directly to substrate solutions and incubated for 48 hr (Figure 4) or 2--media were concentrated 3.3 fold, then added to substrate solutions and incubated for 2 hr (Figure 5). By both procedures, endoglucanase activity appeared in the media of 6 and 9 hr imbibed seeds but did not appear to be significant in the media of 4 or 12 hr imbibed seeds. The enzymes, therefore, leached out of the seeds and may account, at lease partially, for the minimum in activity after 6 hr of imbibition. The enzyme may also be partially unstable in the media after 9 hr, therefore accounting for the decrease in activity contained in the medium of 12 hr imbibed seeds.

A partial study on the kinetics of the activities extracted from 0, 6, 9 and 12 hr imbibed seeds was conducted in order to determine the relative quantities of endoglucanases in these extracts. Figure 6 represents the decrease in viscosity of 0.75% CMC by low salt extracts from 0, 6, 9 and 12 hr imbibed seeds after incubation times of 15 min, 1, 2, 3, 5 and 8 hr. Figure 7 represents the decrease in viscosity of 0.75% CMC by different dilutions (1/1, 1/2, 1/4, 1/8 and 1/16) of low salt extracts from 0 hr imbibed (dry) seeds after incubation times of 15 min, 1, 2, 3, 5 and 8 hr. Data from Figure 6 and Figure 7 were combined to plot viscosity decrease of CMC against dilutions of "0 hr extract" for different times and matching these activities with those obtained from 6, 9 and 12 hr imbibed seeds (Figure 8).

Figure 4. Endoglucanase activities of unconcentrated leachates of 4, 6, 9 and 12 hr imbibed seeds.

abscissa = hr of imbibition
ordinate = $\%\Delta\eta$ 48hr

Figure 5. Endoglucanase activities of concentrated leachates of 4, 6, 9 and 12 hr imbibed seeds.

abscissa = hr of imbibition
ordinate = $\%\Delta\eta$ 2 hr

FIGURE 4

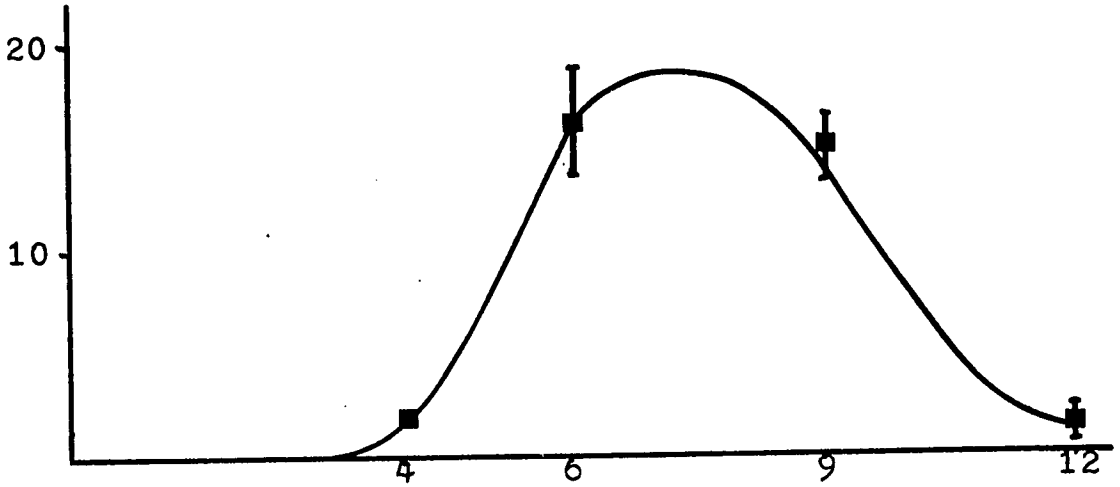


FIGURE 5

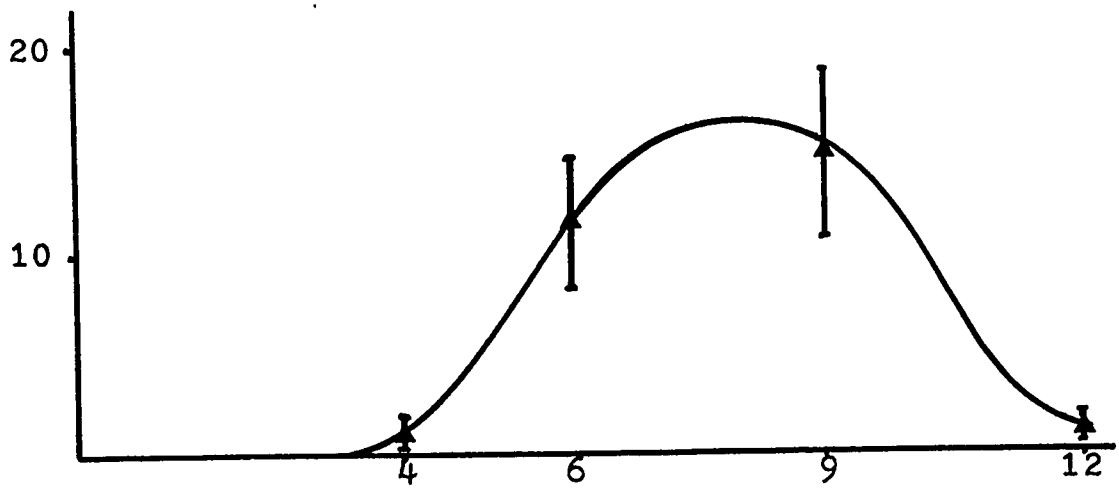


Figure 6. Endoglucanase activities of low salt extracts of 0, 6, 9 and 12 hr imbibed seeds with respect to assay time.

abscissa = hr of assay incubation
ordinate = $\% \Delta \eta$

0 hr (closed squares)
6 hr (open circles)
9 hr (closed circles)
12 hr (open squares)

Figure 7. Endoglucanase activities of different dilutions of low salt extracts from dry seeds with respect to assay time.

abscissa = hr of assay incubation
ordinate = $\% \Delta \eta$

0 hr (closed triangles)
0 hr/2 (closed circles)
0 hr/4 (closed squares)
0 hr/8 (open squares)
0 hr/16 (open triangles)

FIGURE 6

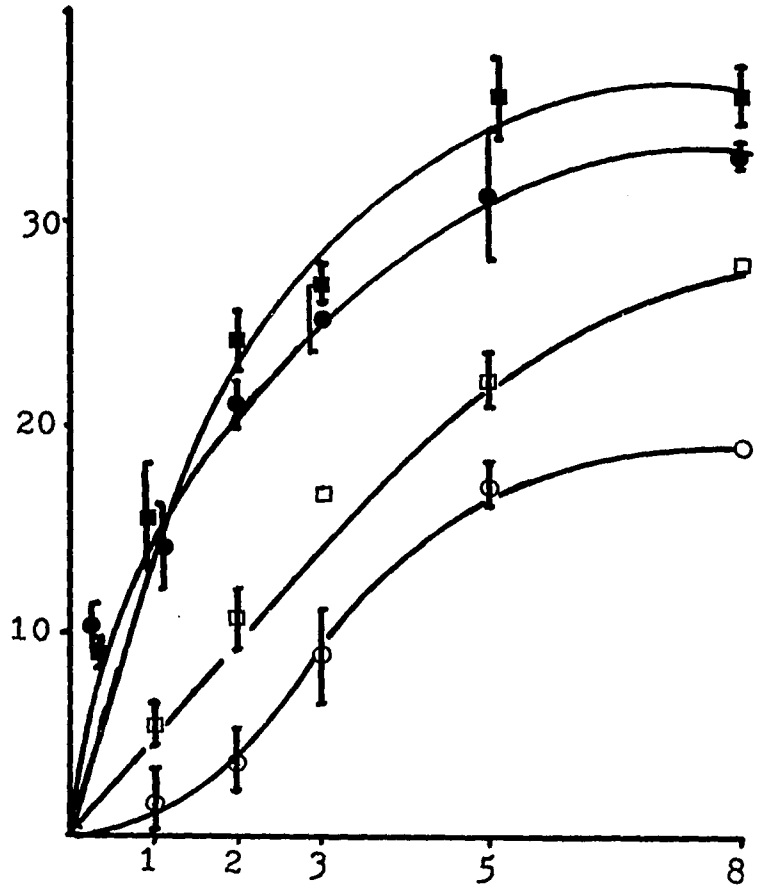


FIGURE 7

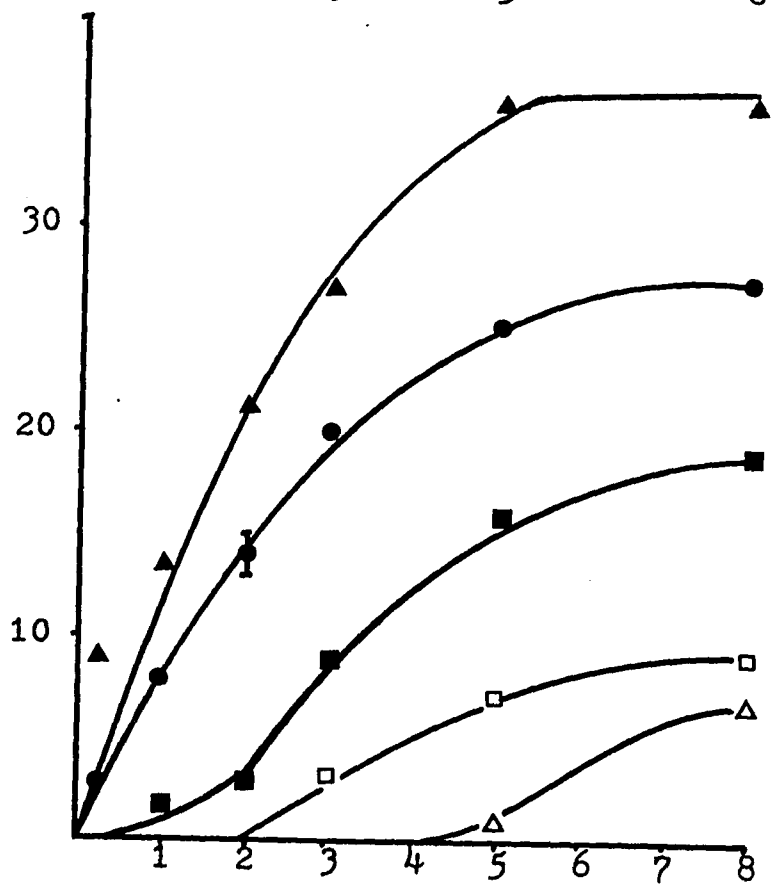


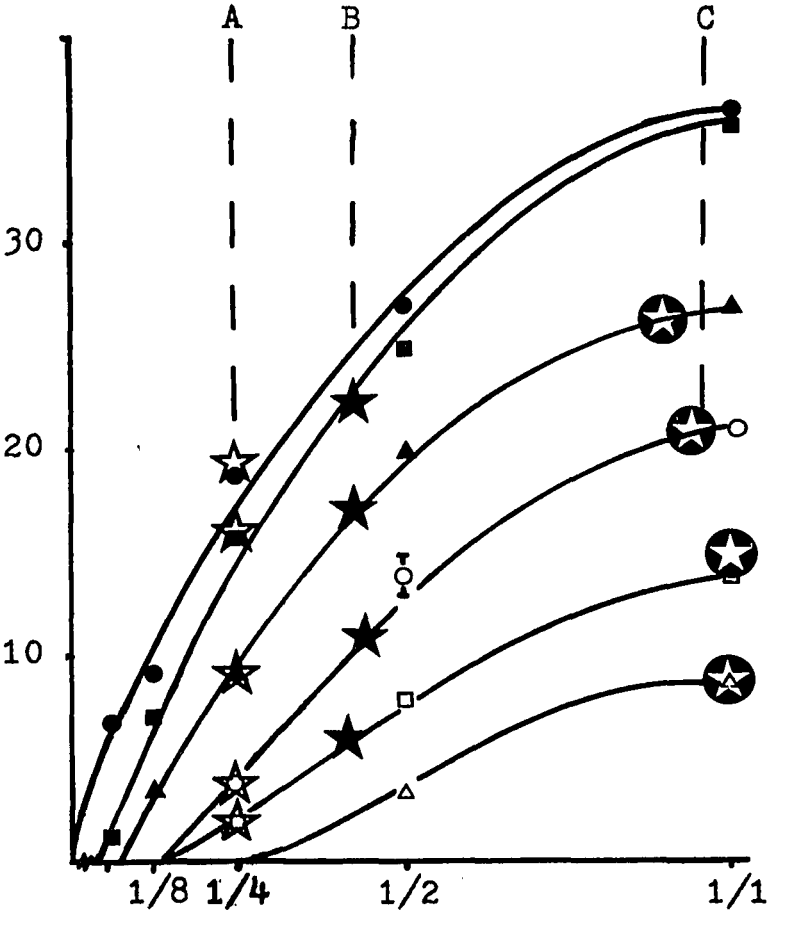
Figure 8. Relative amounts of endoglucanase in 6, 9 and 12 hr imbibed seeds compared to different dilutions of dry seed extracts.

abscissa = fractional dilution of low salt extracts from 0 hr imbibed seeds
ordinate = $\% \Delta \eta$

assay incubation of:
8 hr (closed circles)
5 hr (closed squares)
3 hr (closed triangles)
2 hr (open circles)
1 hr (open squares)
15 min (open triangles

A = 6 hr low salt extract (open star)
B = 12 hr low salt extract (closed star)
C = 9 hr low salt extract (encircled star)

FIGURE 8



According to this method (Fan and Maclachlan, 1966), the relative quantities of endoglucanases (using the activity in 0 hr imbibed seeds as equal to 100%) were 85 to 100% in 9 hr imbibed seeds, 40% in 12 hr imbibed seeds and 25% in 6 hr imbibed seeds (Figure 8).

Since the difference in activities of the different low salt extracts may be due to enzymes which are active under different conditions and to determine whether the activities measured are the highest with respect to assay conditions, optimal assay temperatures (Figure 9) and assay/extraction pHs (Figure 10) were determined. Low salt extracts from 0, 6 and 9 hr imbibed seeds were assayed at incubation temperatures ranging from 30 to 60°C and at a pH of 5.8 (approx.). There was no increase in activity extracted from 6 hr imbibed seeds compared to the activity assayed at 41 ± 1°C (standard condition). Optimal temperature for assay was between 40 and 50°C for activity from both 0 and 9 hr imbibed seeds (Figure 9). Low salt extracts from 0, 6 and 9 hr imbibed seeds were extracted and assayed at pHs ranging from 4.8 to 7.8 and assayed at 41°C. (Note, PVP was used in extraction procedures). Activity in low salt extracts of 6 hr imbibed seeds was not increased by pHs other than 5.8 ± 0.1 (standard condition). Optimal pH for combined extraction and assay was between 5.2 and 6.2 (Figure 10).

Figure 9. Optimal assay temperature for endoglucanase activities of unfortified extracts.

abscissa = assay temperature
ordinate = $\% \Delta \eta$ 2hr

0 hr (closed circles)
6 hr (closed triangles)
9 hr (closed squares)

Figure 10. Optimal extraction and assay pH for endoglucanase activities of unfortified extracts.

abscissa = extraction/assay pH
ordinate = $\% \Delta \eta$ 2hr

0 hr (closed circles)
6 hr (closed triangles)
9 hr (closed squares)

FIGURE 9

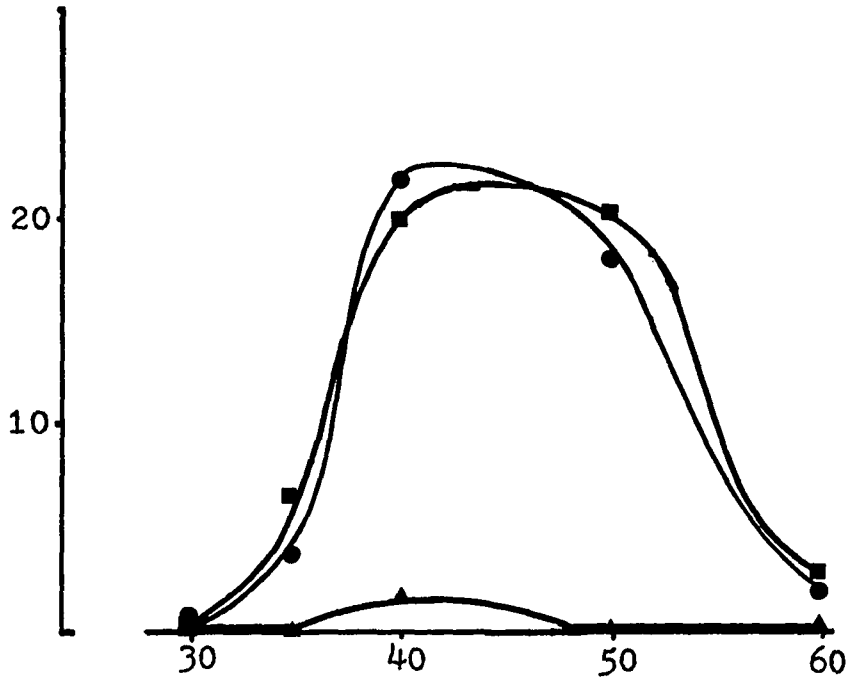
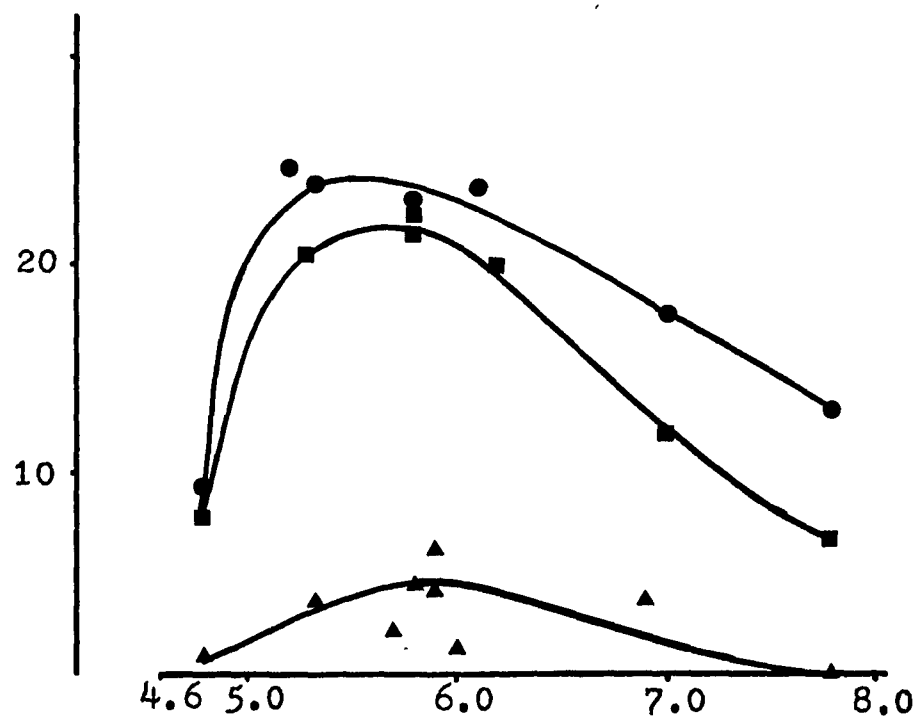


FIGURE 10



Endogenous Endoglucanase Inhibitors

Although endoglucanase activity was found to leach out of seeds during imbibition (Figures 4; 5), the possible existence of endoglucanase inhibitors in the low salt extracts from 6 hr imbibed seeds may also account for the minimum in activity measured in 6 hr imbibed seeds. Mixing low salt extracts of 6 hr imbibed seeds with low and high salt extracts of 0 and 9 hr imbibed seeds and comparing these to 50% dilutions of low and high salt extracts of 0 and 9 hr imbibed seeds did not support the possibility of endogenous endoglucanase inhibitors in "6 hr low salt extracts" (Table 2). This finding is further supported by work on the fractionation of these extracts (refer below).

Microbial-Origin of Endoglucanase

Endoglucanases have also been found in microbial organisms, e.g., bacteria, fungi. Therefore, an attempt to determine whether or not the seed-extracted endoglucanase activities were from contaminating microbes was made. Table 3 depicts the endoglucanase activities measured from low salt extracts of 9 hr imbibed seeds which were either surface sterilized, not surface sterilized or depericarped. In addition, activities were measured in the unconcentrated media in which 9 hr imbibed seeds, either surface sterilized

Table 2. Determination of the presence of inhibitors of endoglucanase activity in 6 hr imbibed seeds.

Table 3. Determination of contamination-related endoglucanase activity.

TABLE 2

imbibition time, hr	extraction	DILUTED WITH			
		----	buffer	6hr, low salt	6hr, low salt (2-fold)
0	low salt	23±1*	13±3	11±4	28
9	low salt	20±1	9±1	7±1	
0	high salt	53±1	34	34	
9	high salt	56±1	35	33	

* % decrease in viscosity of 0.75% CMC after 2hr

TABLE 3

imbibition time & extraction	SEED CONDITION		
	surface sterilized	surface unsterilized	depericarped
9hr, low salt	20*	19	20
9hr, medium (unconcentrated)	20+	65	

* % decrease in viscosity of 0.75% CMC after 2hr

+ % decrease in viscosity of 0.75% CMC after 48hr

or unsterilized, were sown. The activity was the same in all three extracts suggesting that activity did not have a microbial origin. However, there was substantially more activity in the medium of unsterilized seeds compared to that of surface sterilized seeds. This indicated further that surface sterilization with chlorox and ethanol was significantly effective. Other support that the endoglucanase activities did not have a microbial origin, comes from studies on antibiotics (refer below).

Antibiotic Effects

To determine a requirement of RNA or protein synthesis for the endoglucanase activity peak measured in low salt extracts of 9 and 10 hr imbibed seeds, seeds were sown in the presence of inhibitors of RNA and protein synthesis. Intact seeds were sown in either actinomycin D (Figure 12), 6-methyl purine (Figure 13), both RNA synthesis inhibitors or cycloheximide (Figure 11), a protein synthesis inhibitor.

Cycloheximide (CH) treatment of intact seeds had no effect on high salt extracted endoglucanase activities from 6, 9 and 12 hr imbibed seeds. In contrast, endoglucanase activities in low salt extracts did not increase after 6 hr of imbibition and form a peak after 9 hr of imbibition as obtained from untreated seeds (Figure 11). Germination was also drastically inhibited. Since these data suggest that

Figure 11. Endoglucanase activity of salt fortified and unfortified extracts from seeds treated with cycloheximide. Protein content of unfortified extracts.

abscissa = hr of imbibition
primary ordinate = % $\Delta\eta$ 2hr (or % germination)
secondary ordinate = mg protein/ml extract

endoglucanase activity in:
low salt extracts (closed circles)
high salt extracts (closed triangles)
protein content in low salt extracts (closed squares)
germination (open circles)

FIGURE 11

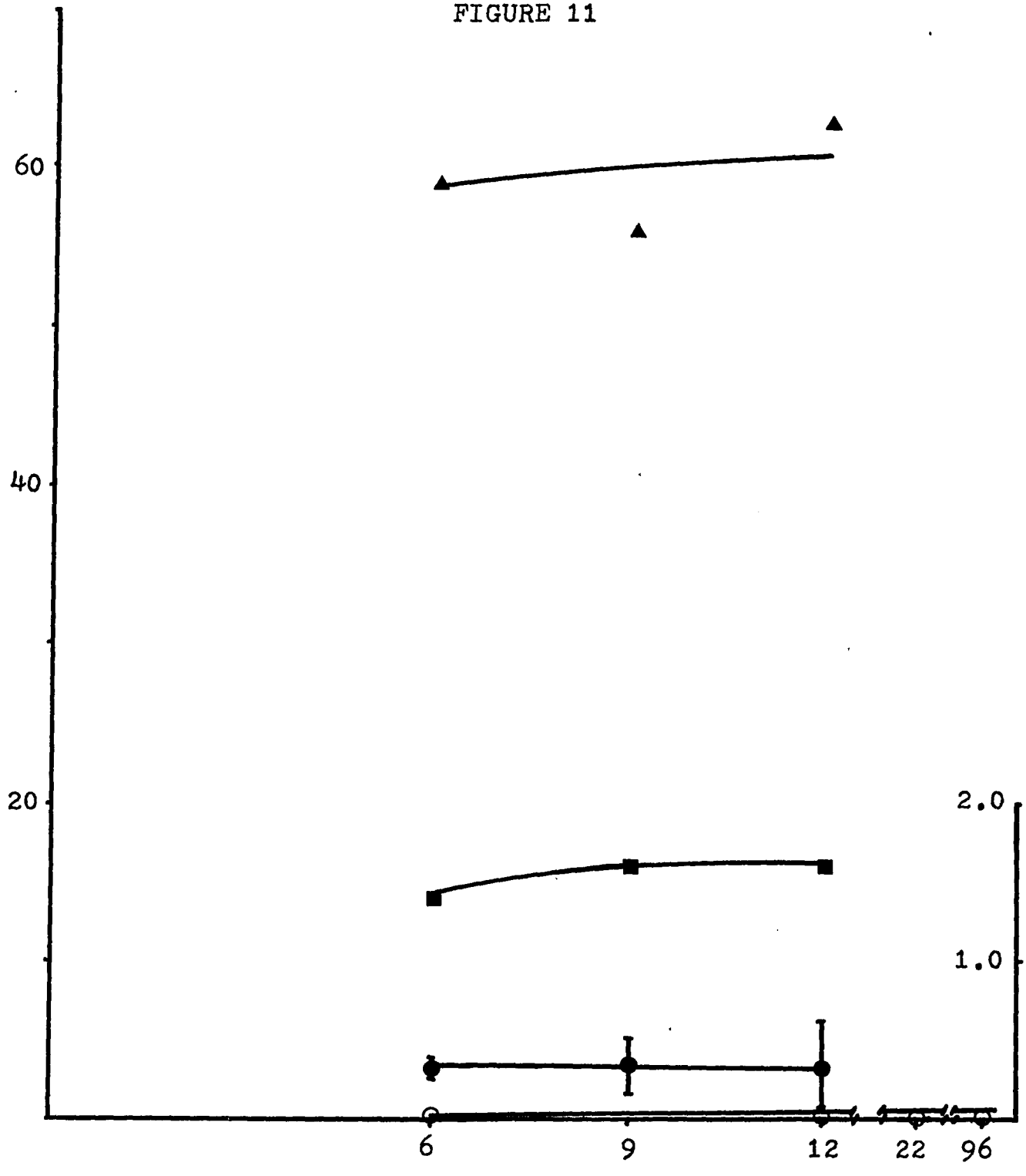


Figure 12. Endoglucanase activity of salt fortified and unfortified extracts from seeds treated with actinomycin D. Protein content of unfortified extracts.

abscissa = hr of imbibition
primary ordinate = $\%\Delta\eta$ 2hr (or % germination)
secondary ordinate = mg protein/ml extract

endoglucanase activity in:
low salt extracts (closed circles)
high salt extracts (closed triangles)
protein content in low salt extracts (closed squares)
germination (open circles)

FIGURE 12

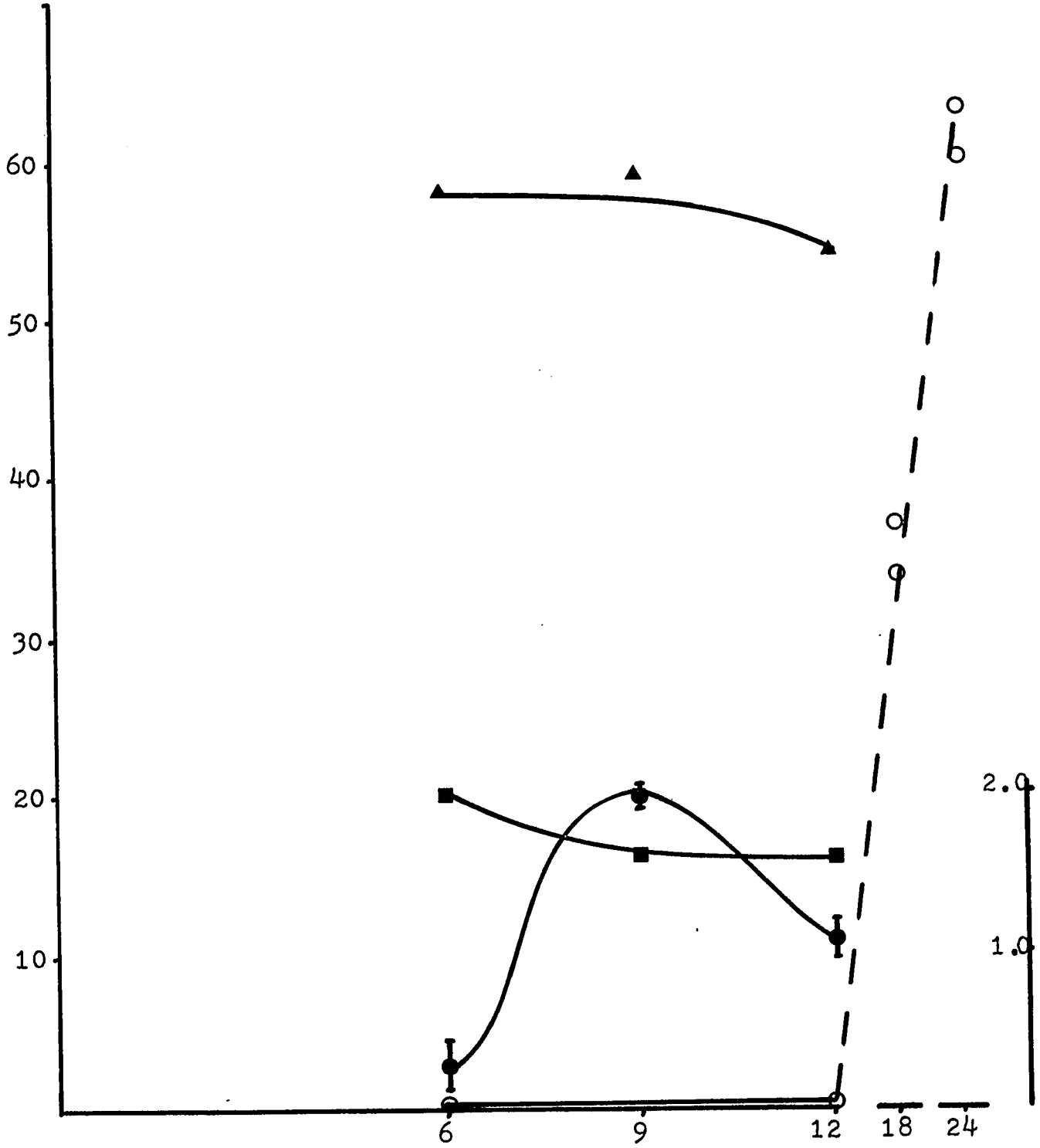
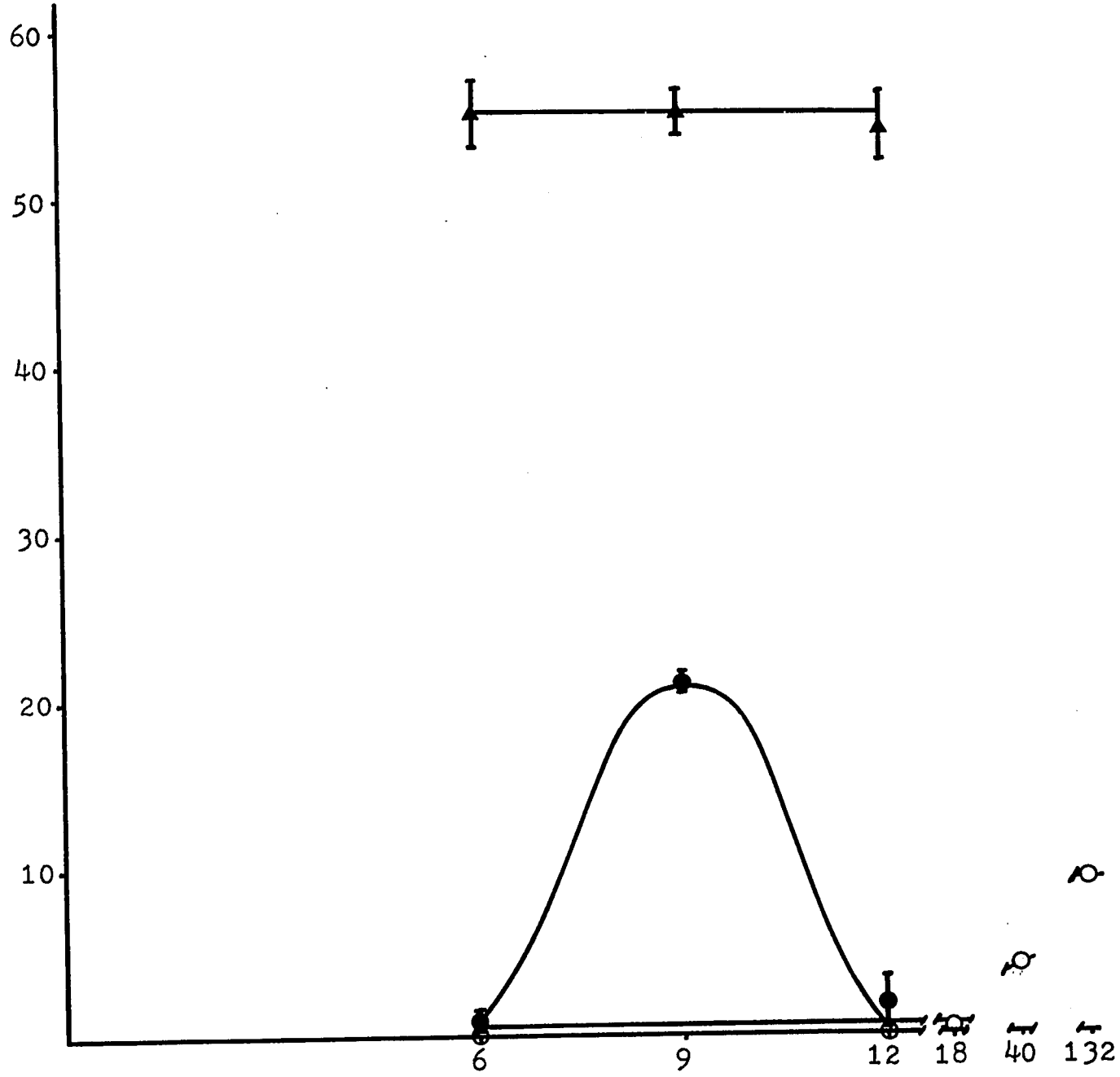


Figure 13. Endoglucanase activity of salt fortified and unfortified extracts from seeds treated with 6-methyl purine.

abscissa = hr of imbibition
ordinate = $\%\Delta\eta$ 2hr (% germination)

endoglucanase activity in:
low salt extracts (closed circles)
high salt extracts (closed triangles)
germination (open circles)

FIGURE 13



protein synthesis is a requirement for the low salt extracted endoglucanase activity peak at 9-10 hr of imbibition, seeds were sown in CH for different periods of time: I. between 0 and 5½ hr, II. between 5½ and 9 hr, and III. between 5½ and 9 hr with an extraction after 12½ hr of imbibition (Table 4). Seeds were washed at each transfer and prior to extraction. Treatment I had no effect on the development of endoglucanase activity in low salt extracts of 9 hr imbibed seeds. Germination did not occur for three days in seeds undergoing treatment I. However, after 3 days, germination increased rapidly until the 4th day, reaching almost the same level as control seeds after 1 day. Treatment II inhibited the development of the endoglucanase activity peak; activity was the same as that measured in 6 hr imbibed seeds. Germination of seeds receiving treatment II, however, was not observed even after 4 days (note seeds remained in CH continuously). To determine whether CH inhibition of the development of the endoglucanase activity peak (treatment II) was reversed by a short (3½ hr) water post treatment, seeds were extracted 3½ hr after CH treatment (treatment III) (Table 4). Endoglucanase activity was almost completely returned by this treatment (III); this indicates that most of the RNA, coding for the endoglucanases, remained stable during CH treatment. It may be interpreted that protein synthesis, required for the endoglucanase activity peak at 9-10 hr of imbibition, occurs between 5½ and 9 hr of imbibition and, in turn, suggests that the low salt extracted

endoglucanase activities from 9 and 10 hr imbibed seeds are synthesized de novo and does not appear by activation of these enzymes.

Low salt extracted and high salt extracted endoglucanase activities from 6, 9 and 12 hr imbibed intact seeds as well as germination were not significantly affected by actinomycin D (AD) treatment (Figure 12). 6-methyl purine (MP) treatment did drastically inhibit germination of intact seeds. This treatment did not inhibit endoglucanase activity in high salt extracts; except for the rapid decrease in endoglucanases after 9 hr (Figure 13), the activity in low salt extracts peaked after 9 hr imbibition as in untreated seeds. Since the lack of inhibition may be due to a lack or low penetration of these chemicals, seeds were punctured through the cotyledon end in order to facilitate penetration and these seeds were sown in AD or MP. Puncturing of seeds may have slightly decreased germination and endoglucanase activity as determined by comparing water controls (Table 4). Both AD and MP eliminate the endoglucanase activity peak and drastically inhibit the germination of punctured seeds (Table 4). Since these data suggest that RNA synthesis in addition to protein synthesis is required for the development of the endoglucanase activity peak, punctured seeds were sown in MP for different periods of time. In this way, the time period during which the RNA, coding for the endoglucanases, is synthesized, may be estimated. Time periods were: I. between

Table 4. Germination and endoglucanase activities of extracts from seeds treated with RNA or protein synthesis inhibitors for different periods of imbibition time.

W = water
AD = 100 mg/ml Actinomycin D
MP = 67 mg/ml 6-Methyl Purine
CH = 100 mg/ml Cycloheximide
E = extracts after 9 or 12½ hr of imbibition
P = seeds punctured at cotyledon end

TABLE 4

times of treatment, hr		endoglucanase activity* low salt	germination, % after+ 24hr	48hr	3dy	4dy
0 W 9	E	20	75	84	--	95
CH	E	3	0	0	0	0
CH W (5½)	E	20	0	0	18	72(1)
W CH (5½)	E	2	0	0	0	0
W CH W (5½ 9 12½)	E	16	--	--	--	--
P W	E	18±1.5	65	83	--	91
AD	E	19±0.4	56	80	--	94
P AD	E	4±1.2	0	10(5)	13(7)	25(14)
MP	E	21±0.3	1	5	--	9
P MP	E	3±0.7	0	0	0	0
P MP W (5½)	E	5±2.1	7(1)	39(6)	--	55(11)
P W MP (5½)	E	9±1.5	6	8(3)	--	9(3)
P W MP W (4½ 7)	E	2±1.2	--	--	--	--
P MP W MP (4½ 9)	E	18	17(2)	49(2)	--	64(4)

* % decrease in viscosity of 0.75% CMC after 2hr

+seeds remain in the last solution in which they are sown

(#) % atypical germination

0 and 5½ hr, II. between 5½ and 9 hr, III. between 4½ and 7 hr and IV. between 0 and 4½ plus between 7 and 9 hr (Table 4). Seeds were washed at each transfer and prior to extraction. The endoglucanase activities in low salt extracts from 9 hr imbibed punctured seeds were decreased a half or less of controls by treatments I and II. Unlike treatments I and II, treatment III inhibited the development of the activity peak; the activity was the same as that measured in 6 hr imbibed seeds. Treatment IV had no significant effect on endoglucanase activity. This indicates that RNA synthesis, required for the endoglucanase activity peak at 9-10 hr of imbibition, occurs between 4½ and 7 hr of imbibition. Germination of punctured seeds was inhibited by all treatments, however, to varying degrees. Atypical germination was also observed, suggesting that in some seeds the endosperm was not weakened at the radicle end although the embryo elongated (Table 4).

Effects of Light and Temperature--
Endoglucanase Activity

Endoglucanase activities were measured in extracts (low salt, high salt and medium) from 9 hr imbibed seeds sown in different light (Table 5; 6) and temperature (Table 6) treatments. Seeds sown at 20°C (standard condition) were subjected to either continuous white light (750 ft-cdl)

Figure 14. Germination of lettuce seeds, cultivar Grand Rapids, 1971 Harvest, sown in darkness or in white light at different temperatures. Germination of seeds exposed to far red light for 1 hr (between 3rd and 4th hr of imbibition) at 20°C.

abscissa = temperature (°C)
ordinate = % germination

light-sown (closed inverted triangles)
dark-sown (closed triangles)
far red-treated (open star)

FIGURE 14

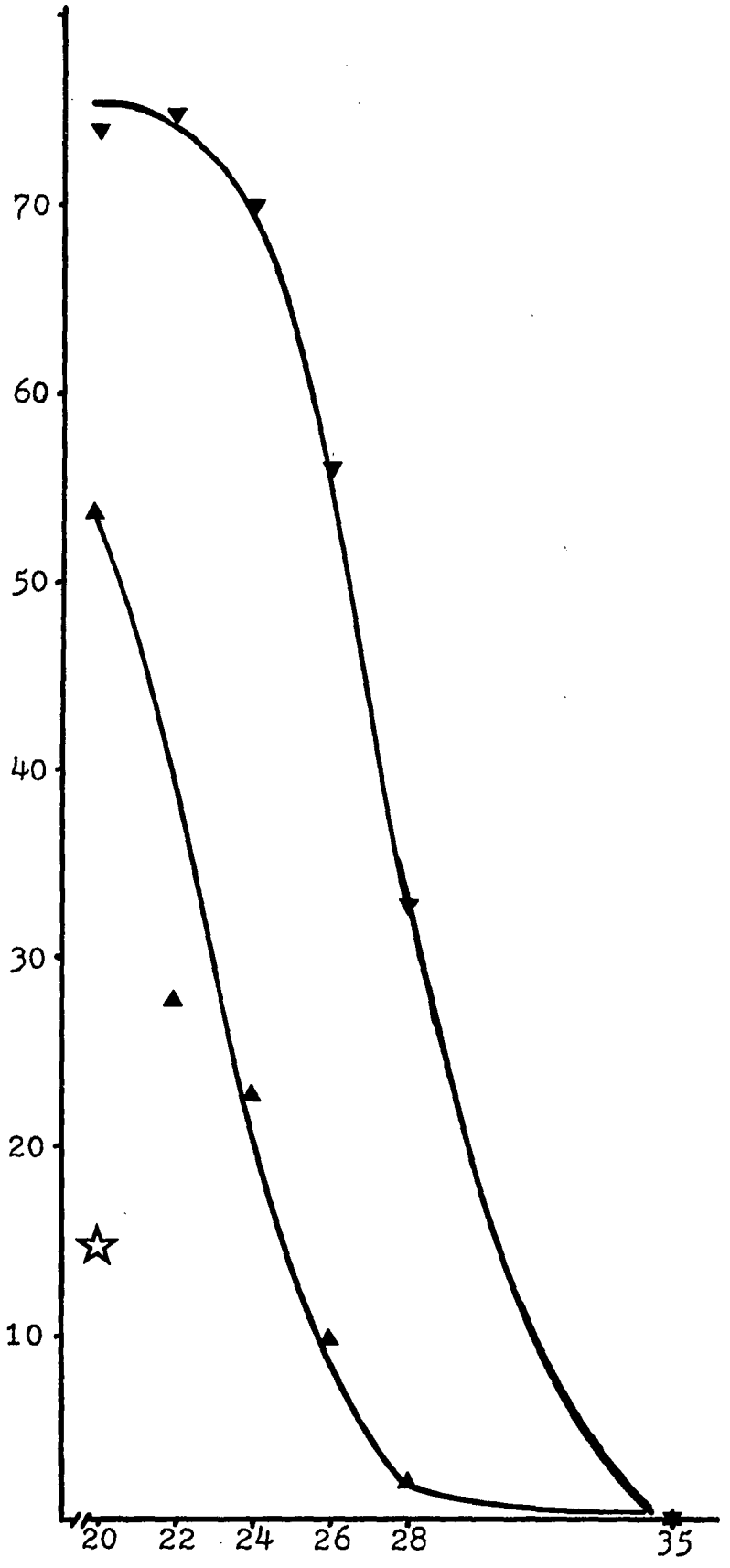


Table 5. Germination and endoglucanase activity of extracts from seeds sown in darkness, white light or far red light at 20°C.

a = continuous white (750 ft-cdl)
b = 1 hr far red exposure after 3 hr of dark imbibition
c = 15 min white (750 ft-cdl) exposure after $3\frac{1}{2}$ hr of dark imbibition
d = 30 min white (375 ft-ddl) exposure after 3 hr of dark imbibition

TABLE 5

lighting condition	germination after 24hr, %	extraction 9hr	endoglucanase activity**
[a]white	78	low salt	20
"	"	high salt	56
"	"	medium (unconcentrated)	20++
darkness	48	low salt	10
[b]dark-far red-dark	15	low salt	2
"	"	high salt	48
"	"	medium (unconcentrated)	19++
[c]dark-white-dark	74	low salt	21
[d]dark-white-dark	76	low salt	20

** % decrease in viscosity of 0.75% CMC after 2hr

++ % decrease in viscosity of 0.75% CMC after 48hr

Table 6. Germination and endoglucanase activity in salt fortified and unfortified extracts of seeds sown in darkness and white light at 24 and 35°C.

a = continuous white (750 ft-cdl)

TABLE 6

temperature, °C	lighting condition	germination after 24hr, %	extraction 9hr	endoglucanase activity**
24	[a]white	70	low salt	22
24	[a]white	"	high salt	50
24	darkness	23	low salt	4
24	darkness	"	high salt	50
35	[a]white	0	low salt	11
35	darkness	0	low salt	1

** % decrease in viscosity of 0.75% CMC after 2hr

(standard condition), continuous darkness, darkness plus 1 hr exposure to far red light at 3rd hr of imbibition, darkness plus 15 min of white light (750 ft-cdl) at 3½ hr and darkness plus 30 min of white light (375 ft-cdl) at 3 hr (Table 5). Imbibition in continuous darkness inhibited about half the development of endoglucanase activity in "9 hr low salt" extract and germination after 24 hr of imbibition. Germination after 24 hr was further inhibited by far red light treatment (Figure 14); this treatment also inhibited the development of the low salt extracted endoglucanase activity peak at 9-10 hr of imbibition. High salt extracted activity from 9 hr imbibed seeds and activity in the imbibition medium in which seeds were sown for 9 hr was not changed by far red light exposure of seeds. Unlike dark and far red treatments, lowered white light exposure did not effect either germination (24 hr) or low salt extracted endoglucanase activity (9 hr). At 24°C (imbibition temperature), the difference in germination between light (white) and dark exposure was more pronounced (Figure 14). In conjunction with germination inhibition, the low salt extracted endoglucanase activity peak was inhibited (Table 6). High salt extracted endoglucanase activity peak was affected. At 35°C (imbibition temperature), germination was not observed for as long as 6 days regardless of light or dark exposure (Figure 14). Low salt extracts of 9 hr dark imbibed seeds did not indicate the occurance of an endoglucanase activity peak; in contrast, low salt extracts of 9 hr light

imbibed seeds contained about half the endoglucanase activity normally obtained from seeds imbibed at lower temperatures for 9 hr (Table 6). These results of different light and temperature treatments suggest that endoglucanase activity's appearance in 9 and 10 hr imbibed seeds was partially controlled by light, that far red light inhibited this appearance, and that the enzymes' appearance was not due to hydration alone.

Fractionations of Endoglucanases--
Molecular Weight Determinations

Since the above extractions were made directly from seeds, the endoglucanase activity therein represented "bulk" activity. Therefore, the activity curves do not yeild any indication on the number of endoglucanases which may have contributed to the activity. Extracts, both low salt and high salt, from 0, 6, 9 and 12 hr imbibed seeds and the medium in which seeds were imbibed for 9 hr, were fractionated using a chromatography column packed with Sephadex G-100 or G-200 (Appendix, Schedule III). Fractionation (120 drop/tube) of low salt extracts from 0 (Figure 15) and 9 hr (Figure 17) imbibed seeds yields three peaks of activity, E1, E2 and E3. Low salt extracts of 6 hr imbibed seeds yielded peak E1 with possible traces of E2 and E3 (Figure 16); low salt extracts of 12 hr imbibed seeds yielded E1 predominantly, some E2 and possibly a trace of E3 (Figure 18). Protein profiles for

Figure 15. Endoglucanase activity and protein content per 120 drop fractions of Sephadex G-100 fractionated unfortified extracts of dry seeds.

abscissa = fractions
primary ordinate = $\%\Delta\eta$ 24hr
secondary ordinate = mg protein/fraction

endoglucanase activity (solid line)
protein content (dashed line)

Figure 16. Endoglucanase activity and protein content per 120 drop fractions of Sephadex G-100 fractionated unfortified extracts of 6 hr imbibed seeds.

abscissa = fractions
primary ordinate = $\%\Delta\eta$ 24hr
secondary ordinate = mg protein/fraction

endoglucanase activity (solid line)
protein content (dashed line)

FIGURE 15

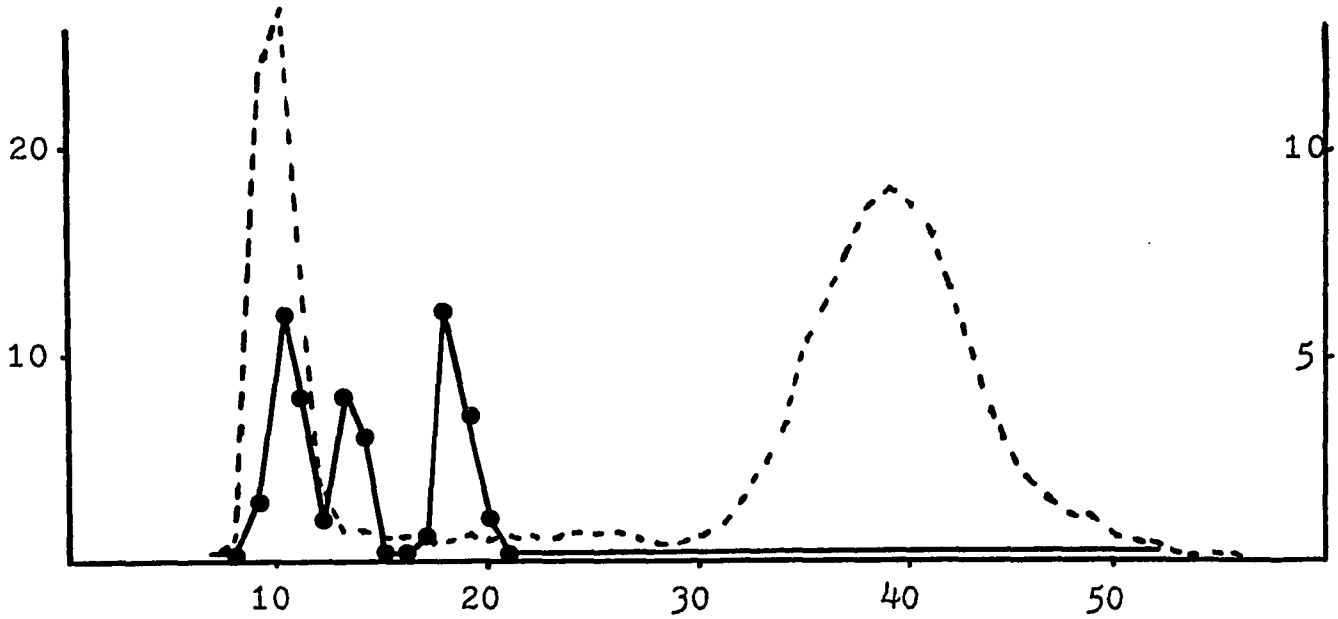


FIGURE 16

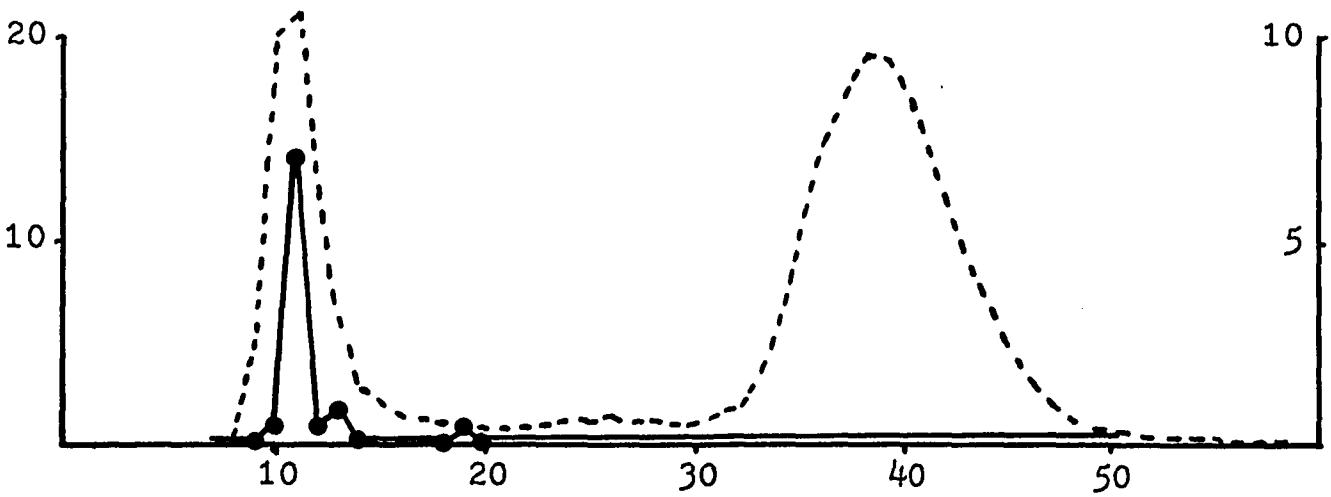


Figure 17. Endoglucanase activity and protein content per 120 drop fractions of Sephadex G-100 fractionated unfortified extracts of 9 hr imbibed seeds.

abscissa = fractions
primary ordinate = $\%\Delta\eta$ 24 hr
secondary ordinate = mg protein/fraction

endoglucanase activity (solid line)
protein content (dashed line)

Figure 18. Endoglucanase activity and protein content per 120 drop fractions of Sephadex G-100 fractionated unfortified extracts of 12 hr imbibed seeds.

abscissa = fractions
primary ordinate = $\%\Delta\eta$ 24 hr
secondary ordinate = mg protein/fraction

endoglucanase activity (solid line)
protein content (dashed line)

FIGURE 17

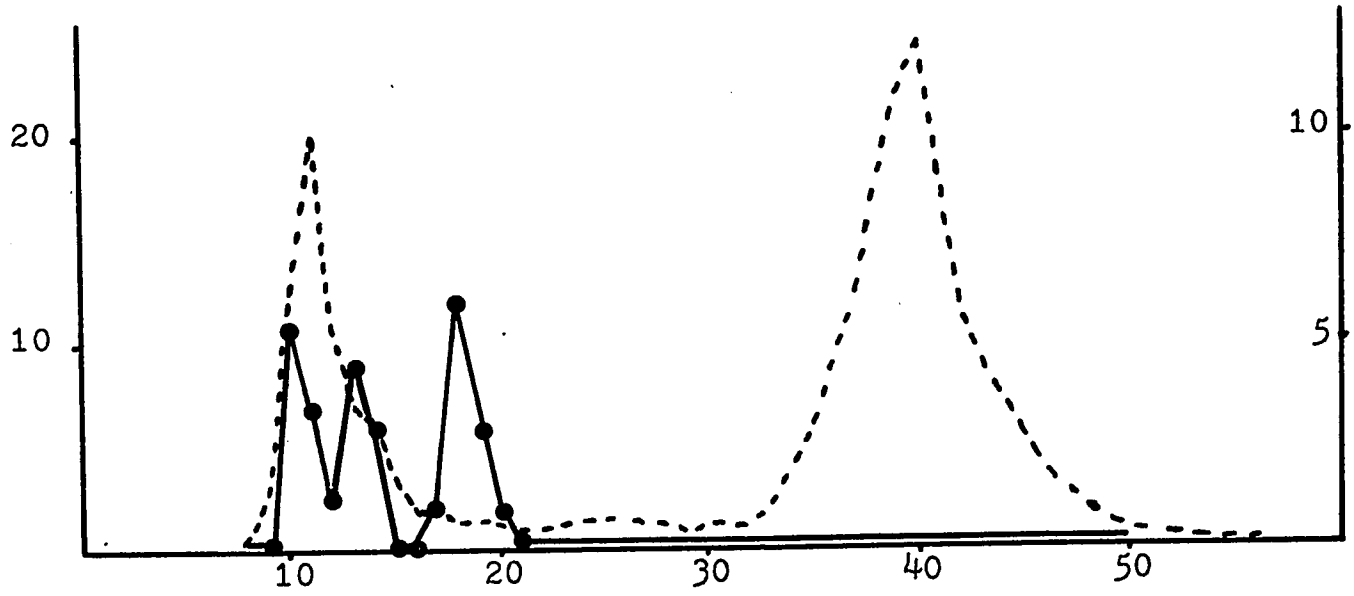


FIGURE 18

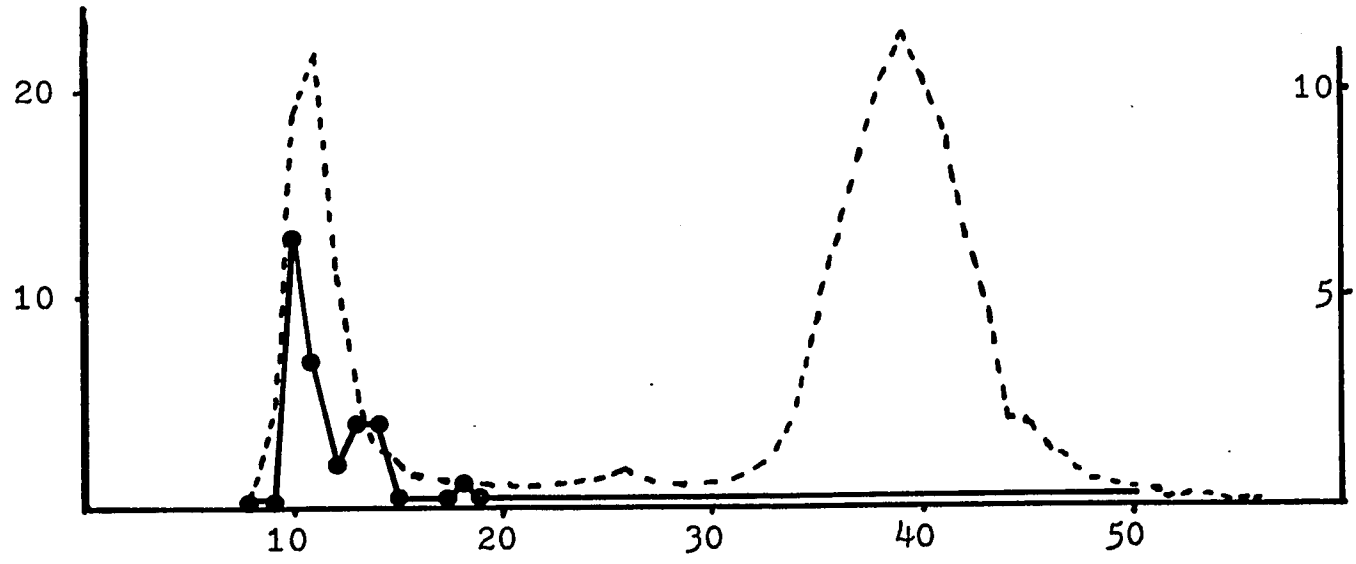


Figure 19. Endoglucanase activities and protein content per 120 drop fraction of Sephadex G-100 fractionated, concentrated leachates of 9 hr imbibed seeds.

abscissa = fractions
primary ordinate = $\% \Delta \eta$ 24hr
secondar ordinate = mg protein/fraction

endoglucanase activity (solid line)
protein content (dashed line)

FIGURE 19

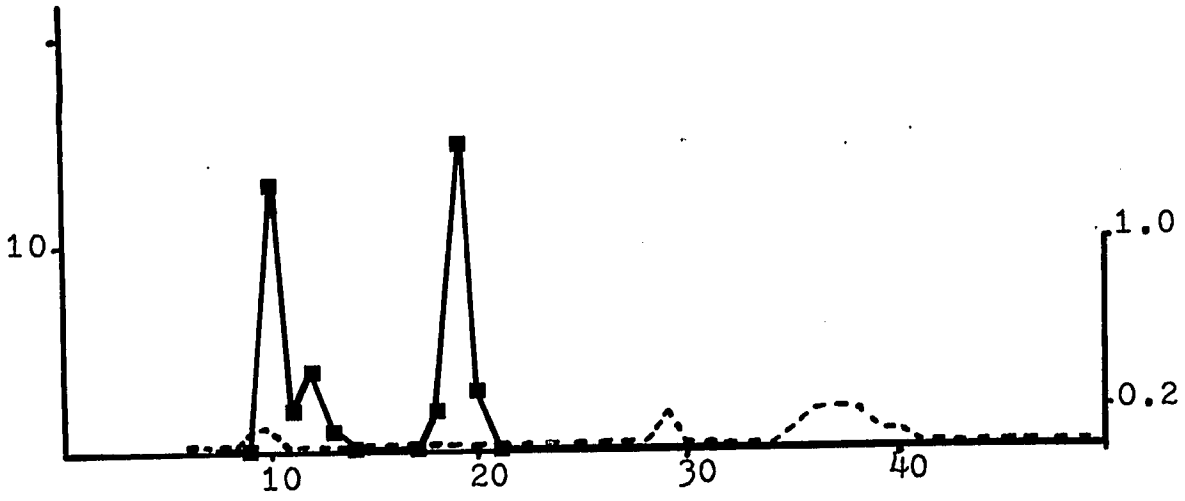


Figure 20. Endoglucanase activity and protein content per 120 drop fractions of Sephadex G-100 fractionated salt fortified extracts of dry seeds.

abscissa = fractions
primary ordinate = $\% \Delta \eta$ 24hr
secondary ordinate = mg protein/fraction

endoglucanase activity (solid line)
protein content (dashed line)

Figure 21. Endoglucanase activity and protein content per 120 drop fractions of Sephadex G-100 fractionated salt fortified extracts of 6 hr imbibed seeds.

abscissa = fractions
primary ordinate = $\% \Delta \eta$ 24hr
secondary ordinate = mg protein/fraction

endoglucanase activity (solid line)
protein content (dashed line)

FIGURE 20

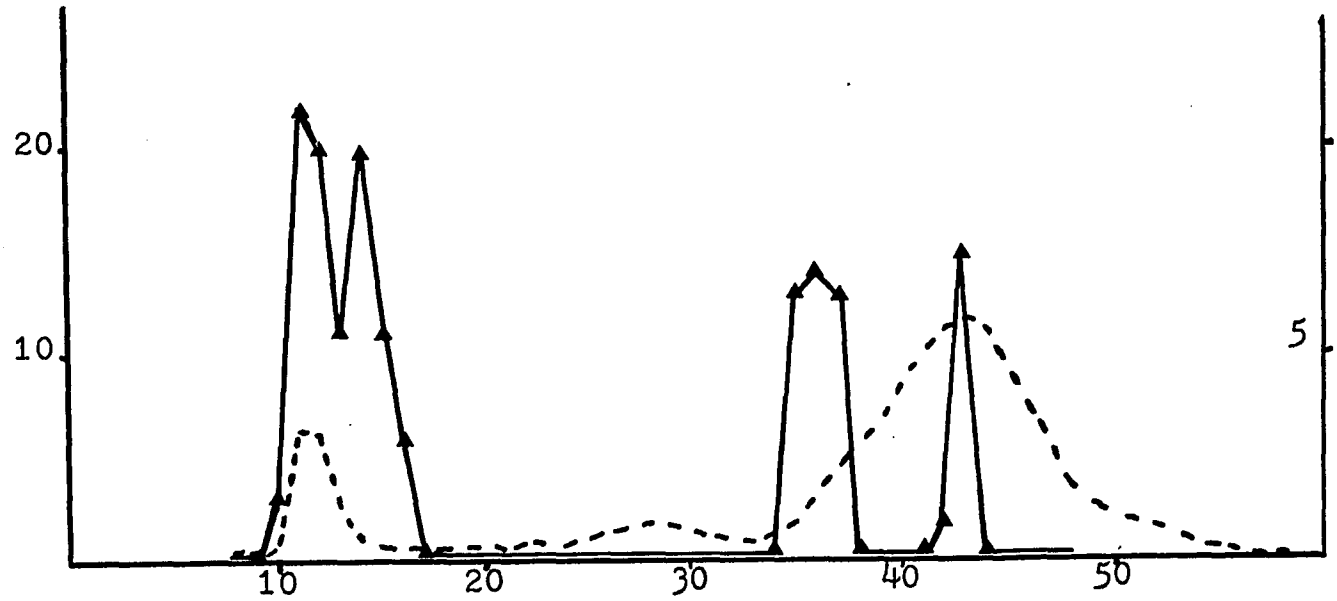


FIGURE 21

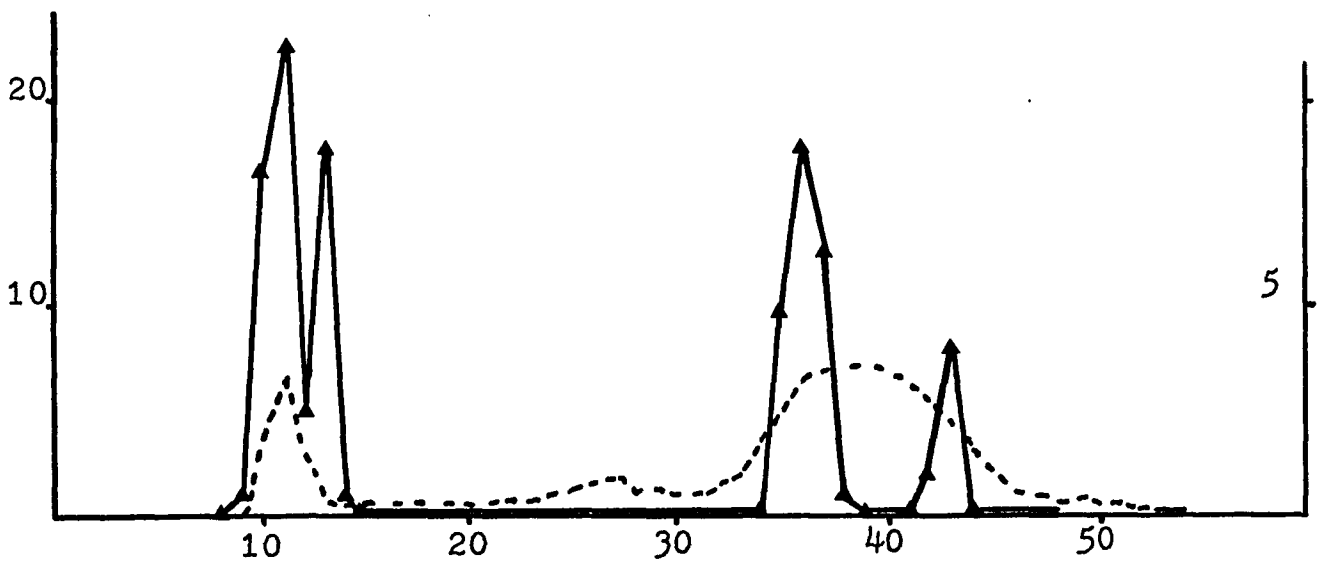


Figure 22. Endoglucanase activity and protein content per 120 drop fractions of Sephadex G-100 fractionated salt fortified extracts of 9 hr imbibed seeds.

abscissa = fractions
primary ordinate = $\% \Delta \eta$ 24 hr
secondary ordinate = mg protein/fraction

endoglucanase activity (solid line)
protein content (dashed line)

Figure 23. Endoglucanase activity and protein content per 120 drop fractions of Sephadex G-100 fractionated salt fortified extracts of 12 hr imbibed seeds.

abscissa = fractions
primary ordinate = $\% \Delta \eta$ 24 hr
secondary ordinate + mg protein/fraction

endoglucanase activity (solid line)
protein content (dashed line)

FIGURE 22

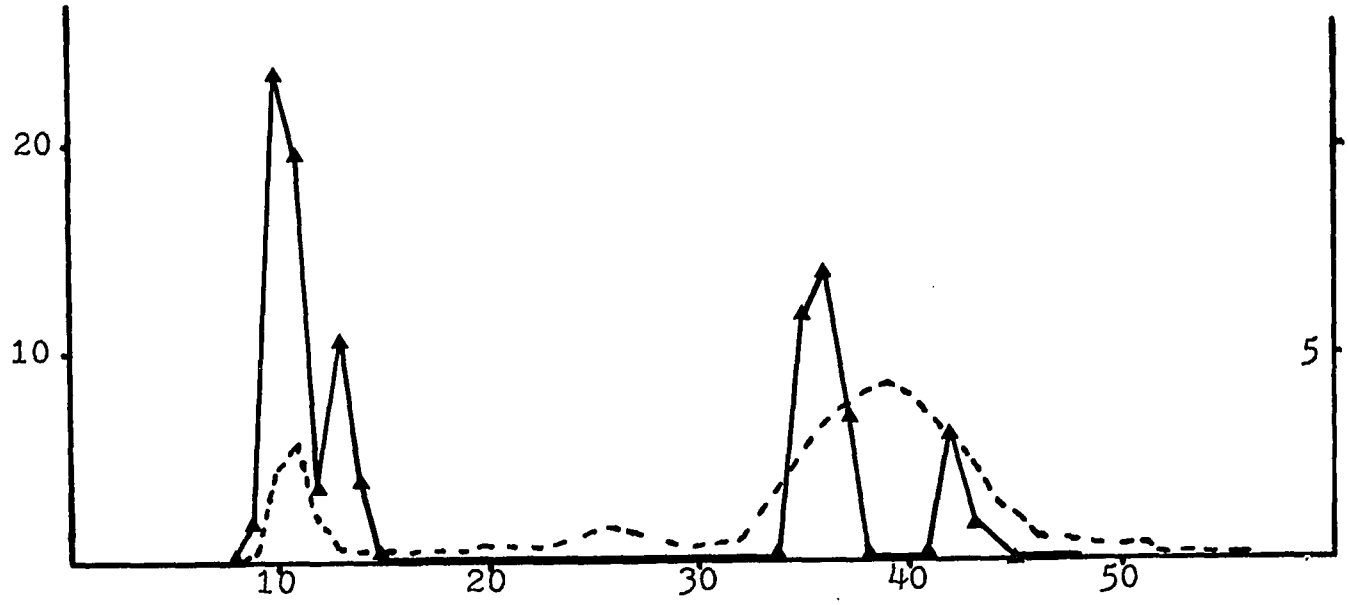
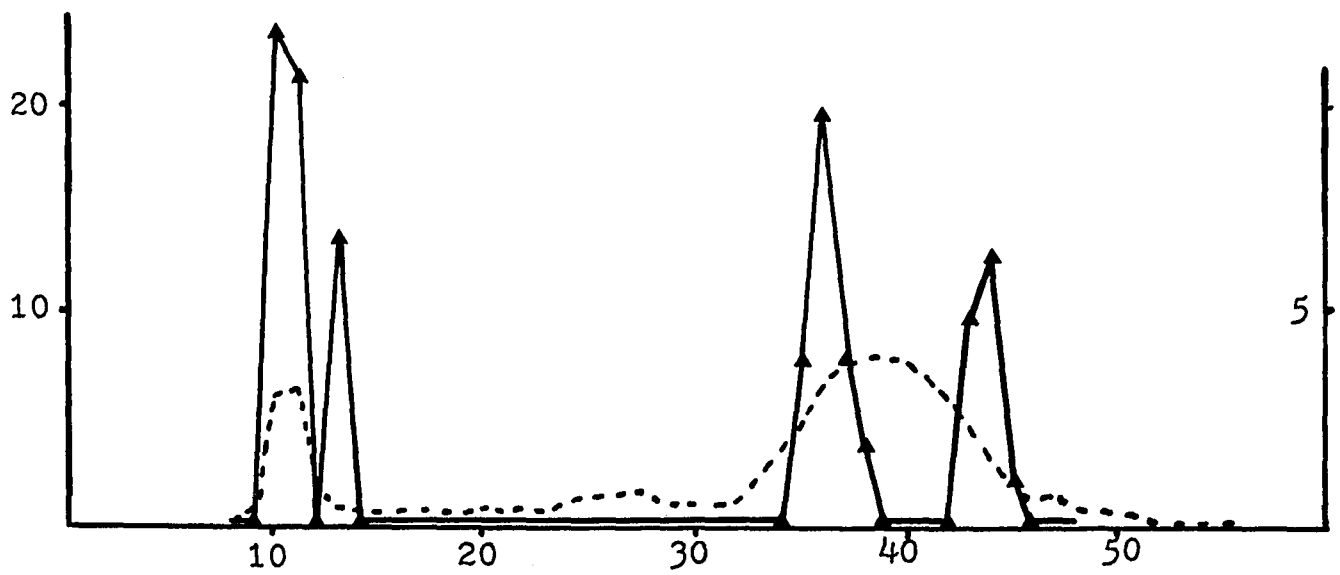


FIGURE 23



the above extracts were the same and indicate a large bulk of low molecular weight peptides and a large bulk eluting at or near the void volume. Figure 19 represents the G-100 fractionation profile (120 drops/tube) of 16-fold concentrated medium in which seeds were imbibed for 9 hr; peaks E1 and E3 plus some E2 were observed and little protein was indicated. In contrast, all high salt extracts (from 0, 6, 9 and 12 hr imbibed seeds) fractionated on Sephadex G-100 yielded the same four peaks, E1, E2, E4 and E5 (Figures 20; 21; 22; 23). Protein profiles were about the same for these extracts and indicated two major groups of peptides, at or near the void volume and a low molecular weight region, and possibly a small peptide peak in between. In order to further separate and narrow the peaks and to aid in the determination of the molecular weights, "0 hr low salt," "9 hr low salt" and "9 hr high salt" extracts were fractionated into 60 drops/tube (Figures 24; 25; 26). As found in the 120 drop/tube fractionation, the low salt extracts yielded E1, E2 and E3 and the high salt extracts yielded E1, E2, E4 and E5. The partition coefficients for each of these enzyme peaks were determined (Appendix Schedule IV). Protein standards (known molecular weights), aldolase, bovine serum albumin, ovalbumin, chymotrypsinogen A and ribonuclease A, were chromatographed on the column packed with Sephadex G-100 gel (Figure 27). The partition coefficients of these standards were determined. Figure 28 represents the plot of partition coefficient (Kav) versus log of molecular weights; these values for the standards were plotted. The molecular weights

Figure 24. Endoglucanase activity per 60 drop fractions of Sephadex G-100 fractionated unfortified extracts of dry seeds.

abscissa = fractions
ordinate = $\% \Delta \eta$ 24hr

endoglucanase activity (solid line)

Figure 25. Endoglucanase activity per 60 drop fractions of Sephadex G-100 fractionated unfortified extracts of 9 hr imbibed seeds.

abscissa = fractions
ordinate = $\% \Delta \eta$ 24hr

endoglucanase activity (solid line)

Figure 26. Endoglucanase activity per 60 drop fractions of Sephadex G-100 fractionated salt fortified extracts of 9 hr imbibed seeds.

abscissa = fractions
ordinate = $\% \Delta \eta$ 24hr

endoglucanase activity (solid line)

FIGURE 24

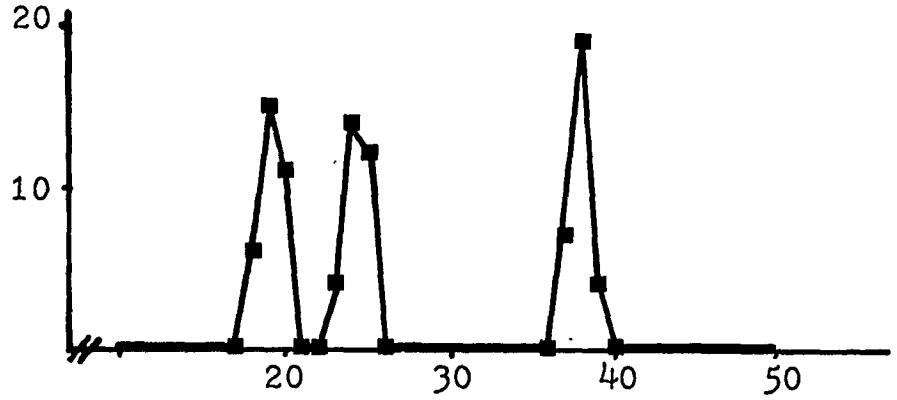


FIGURE 25

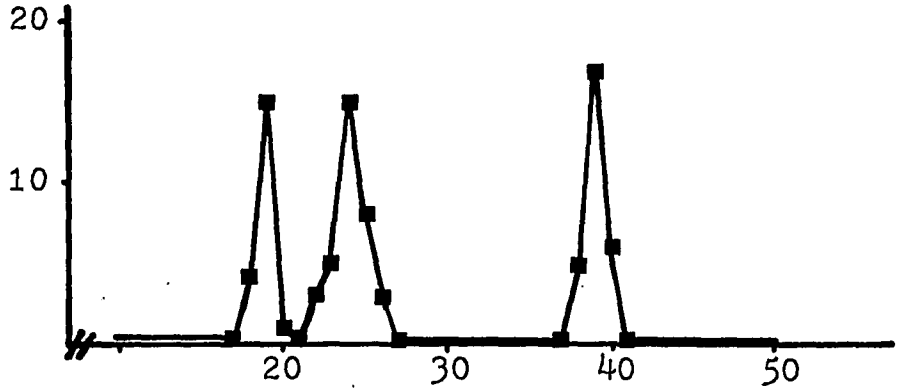


FIGURE 26

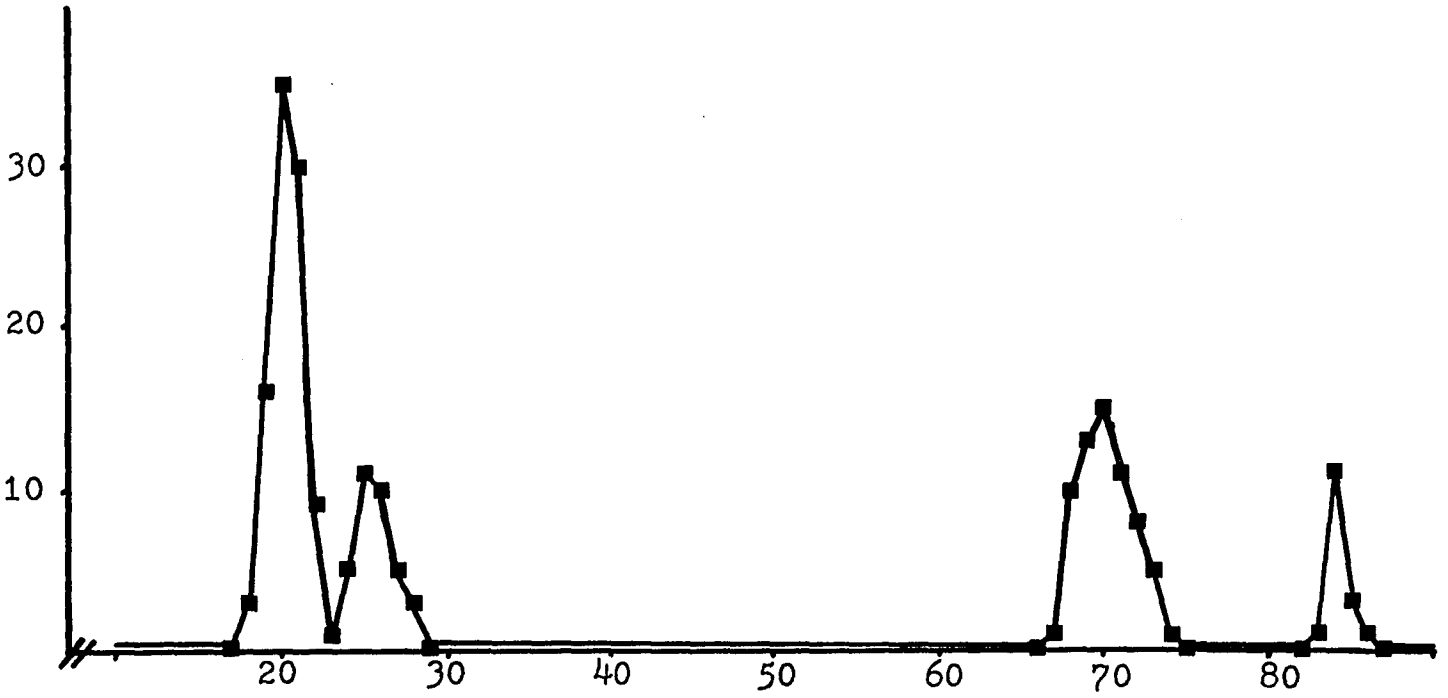


Figure 27. Fraction locations of molecular weight standards after Sephadex G-100 fractionation. Individual lines represent individual mixtures of standards.

abscissa = fractions
primary ordinate = mg protein/fraction
secondary ordinate = A_{620}

standards:
aldolase (open diamond)
bovine serum albumin (open inverted triangle)
ovalbumin (open triangle)
chymotrypsinogen A (open square)
ribonuclease A (open circle)
blue dextran 2000-void volume (solid line-refers to A_{620})

Figure 28. Molecular weight determinations of endoglucanase activity peaks obtained through Sephadex G-100 fractionation.

abscissa = molecular weights (daltons)
ordinate = Kav (partition coefficient)

standards represented as in Figure 27

endoglucanase peak fractions represented:
E3 (closed 6-point star)
E4 (closed square)
E5 (closed circle)

FIGURE 27

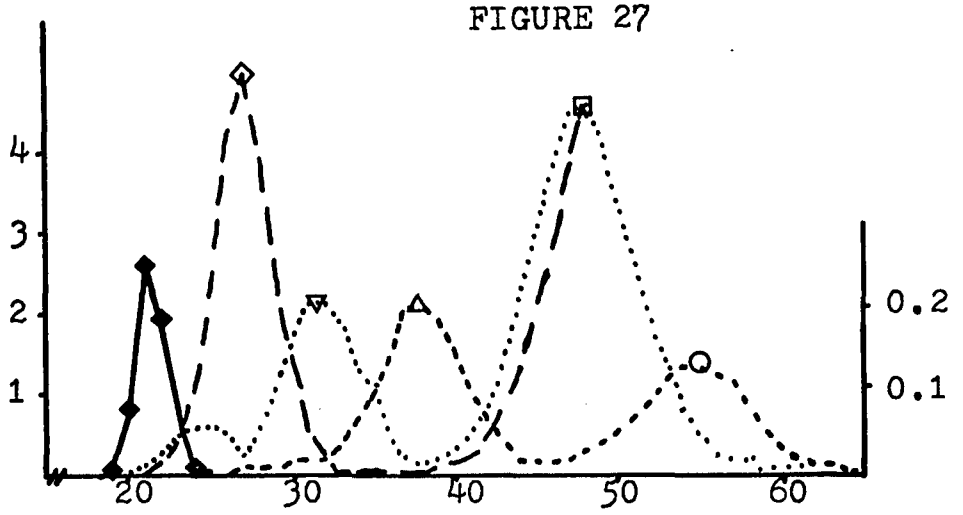


FIGURE 28

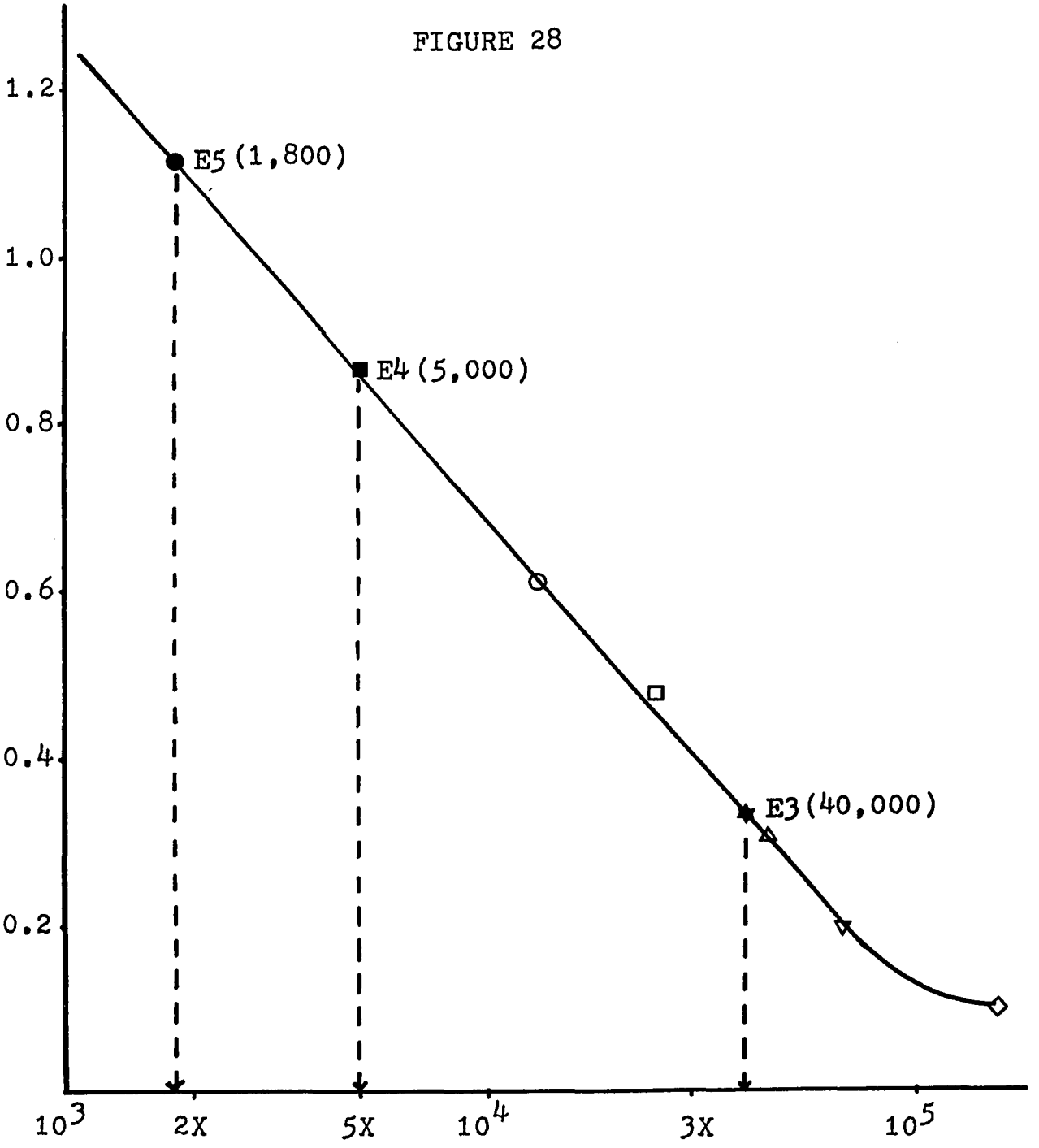


Figure 29. Endoglucanase activity and protein content per 60 drop fractions of Sephadex G-200 fractionated unfortified extracts of dry seeds.

abscissa = fractions
primary ordinate = $\%\Delta\eta$ 24hr
secondary ordinate = mg protein/2 combined fractions

endoglucanase activity (solid line)
protein content (dashed line)

Figure 30. Endoglucanase activity and protein content per 60 drop fractions of Sephadex G-200 fractionated unfortified extracts of 9 hr imbibed seeds.

abscissa = fractions
primary ordinate = $\%\Delta\eta$ 24hr
secondary ordinate = mg protein/2 combined fractions

endoglucanase activity (solid line)
protein content (dashed line)

FIGURE 29

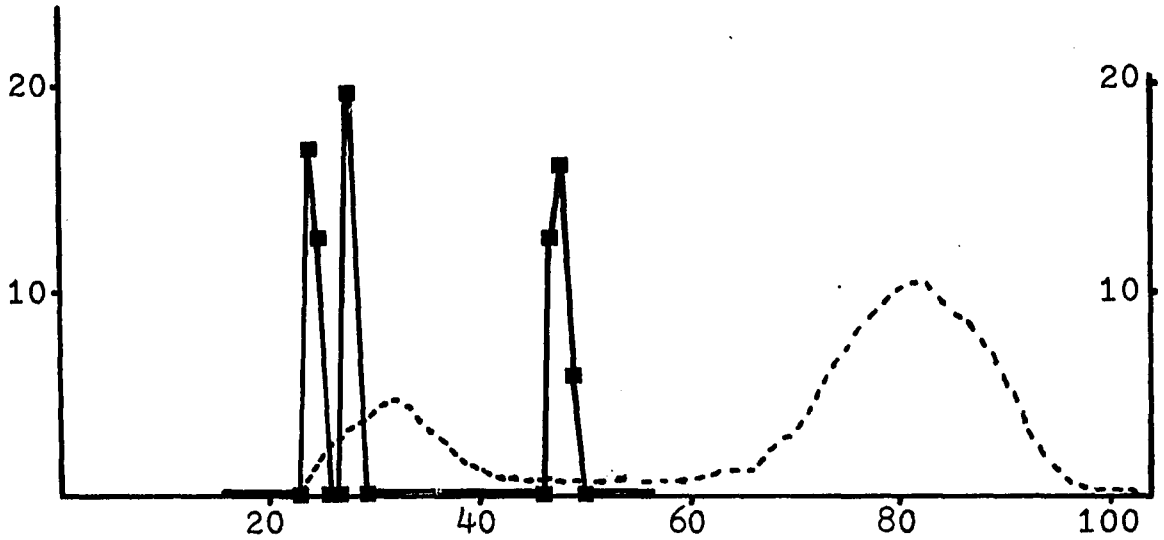


FIGURE 30

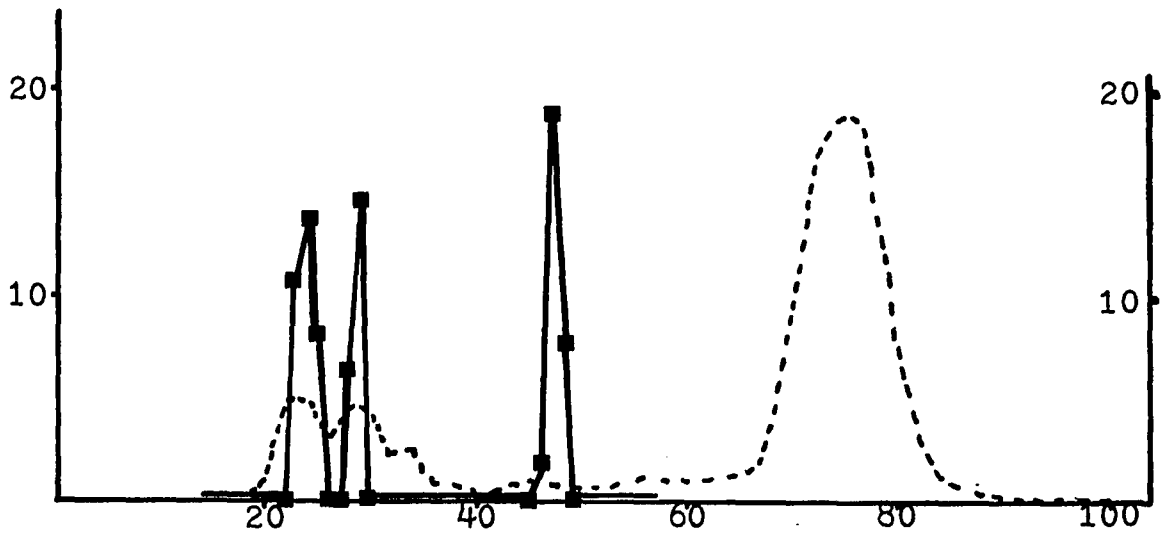


Figure 31. Fraction locations of molecular weight standards after Sephadex G-200 fractionation. Individual lines represent individual mixtures of standards.

abscissa = fractions
primary ordinate = mg protein/fraction
secondary ordinate = A_{620}

standards:
aldolase (open diamond)
bovine serum albumin (open inverted triangle)
ovalbumin (open triangle)
chymotrypsinogen A (open square)
ribonuclease A (open circle)
blue dextran 2000-void volume (solid line-refers to A_{620})

Figure 32. Molecular weight determinations of endoglucanase activity peaks and subunits obtained through Sephadex G-200 fractionation.

abscissa = fractions
primary ordinate = mg protein/fraction

standards:
aldolase (open diamond)
bovine serum albumin (open inverted triangle)
chymotrypsinogen A (open circle)
blue dextran 2000-void volume (solid line-refers to A_{620})

FIGURE 31

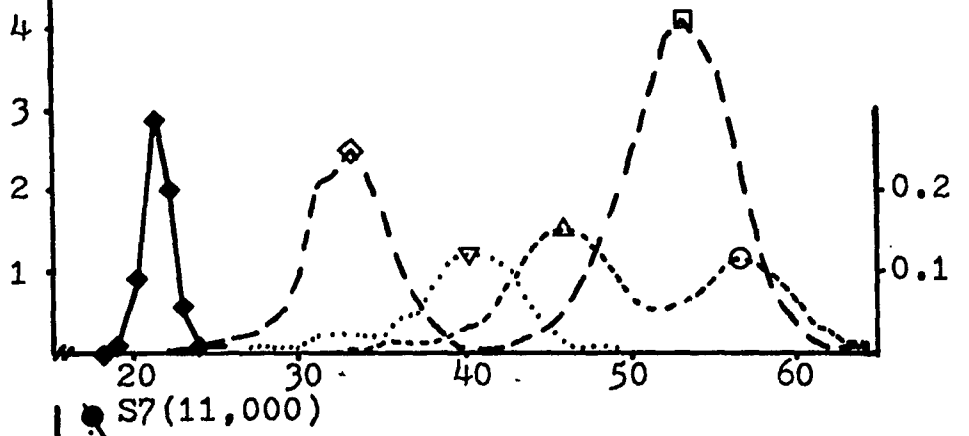
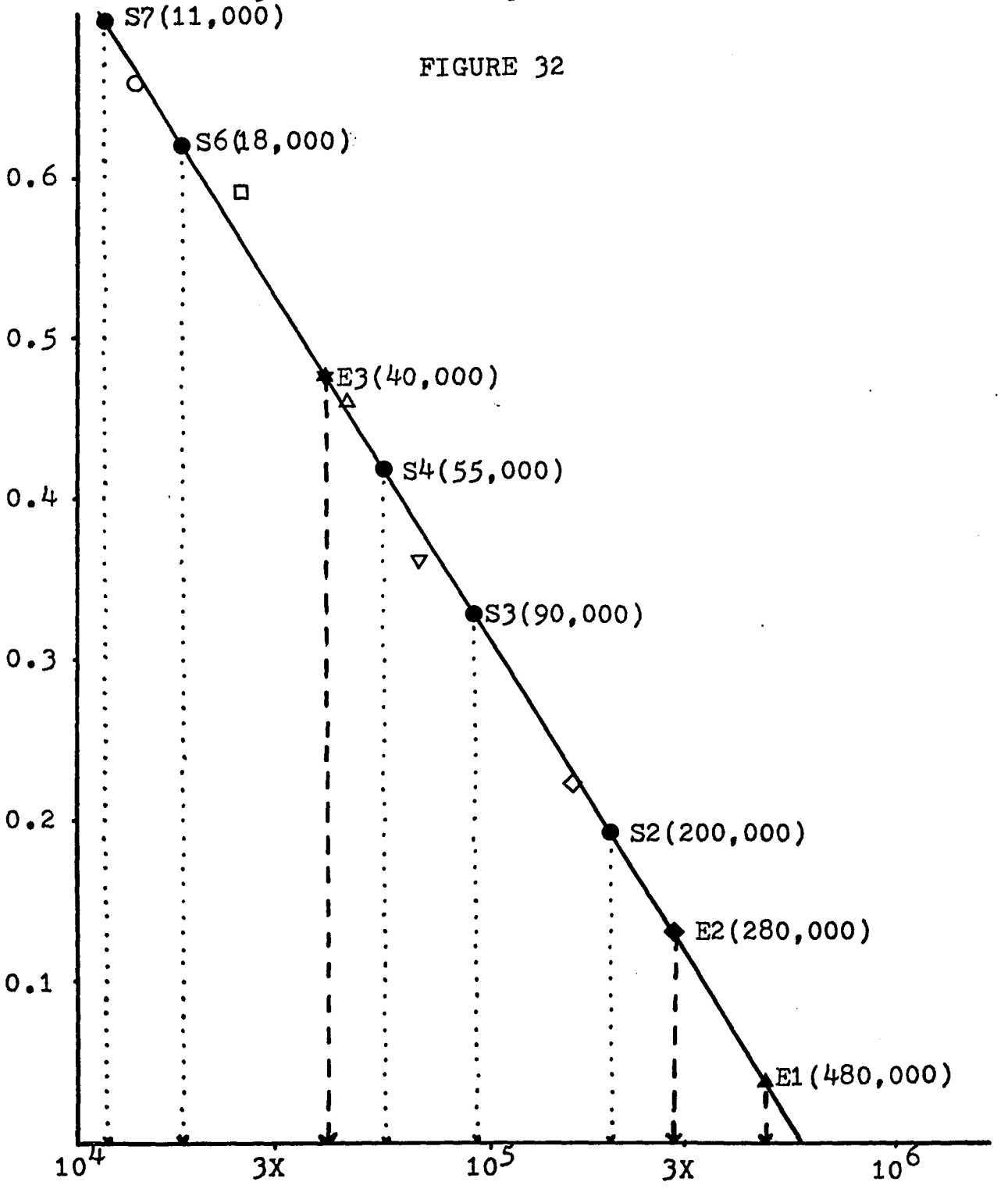


FIGURE 32



of the endoglucanase peaks were read off the graph using the peaks' Kav's. Molecular weight for E1 and E2 were too high for determinations on G-100; molecular weights (in Daltons) for E3 was 40,000, for E4 was 5,000 and for E5 was 1,800 (Figure 28). In order to determine the molecular weights of E1 and E2 and to further verify that of E3, "0 hr and 9 hr low salt" extracts were chromatographed on the column packed with Sephadex G-200 gel into 60 drop/tube fractions (Figures 29; 30). Protein standards (refer above) were also chromatographed (Figure 31). The Kav's of the standards and enzyme peaks were determined and, as above, the molecular weights of the endoglucanase peaks were read off the graph. The molecular weights (in Daltons) for E1 was about 480,000, for E2 was about 280,000 and for E3 was 40,000 (Figure 32).

These data indicate that the endoglucanase fractionation peak, E3, was contained only in low salt extracts and that this peak and, to some extent, the E2 peak fluctuated during the 12 hr pregermination period. These suggest a physiological importance of endoglucanase-E3.

Subunit Structure

Endoglucanases E2 and E3 may be the result of a dissociation of the large E1 enzyme. This possiblity was elucidated by: 1. fractionating either "0 hr or 9 hr low salt" extracts on G-200 gels; 2. pooling and concentrating

Figure 33. Subunit composition of SDS-treated fractions 22 to 26 eluted from Sephadex G-200 column loaded with low extracts from 0 or 9 hr imbibed seeds. Endoglucanase activity per 60 drop fractions of Sephadex G-200, SDS-treated, concentrated fractions.

abscissa = fractions
ordinate = % $\Delta\eta$ 24hr

Figure 34. Subunit composition of SDS-treated fractions 27 to 30 eluted from Sephadex G-200 column loaded with low extracts from 0 or 9 hr imbibed seeds. Endoglucanase activity per 60 drop fractions of Sephadex G-200, SDS-treated, concentrated fractions.

abscissa = fractions
ordinate = % $\Delta\eta$ 24hr

Figure 35. Subunit composition of SDS-treated fractions 44 to 49 eluted from Sephadex G-200 column loaded with low extracts from 0 or 9 hr imbibed seeds. Endoglucanase activity per 60 drop fractions of Sephadex G-200, SDS-treated, concentrated fractions.

abscissa + fractions
ordinate = % $\Delta\eta$ 24hr

FIGURE 33

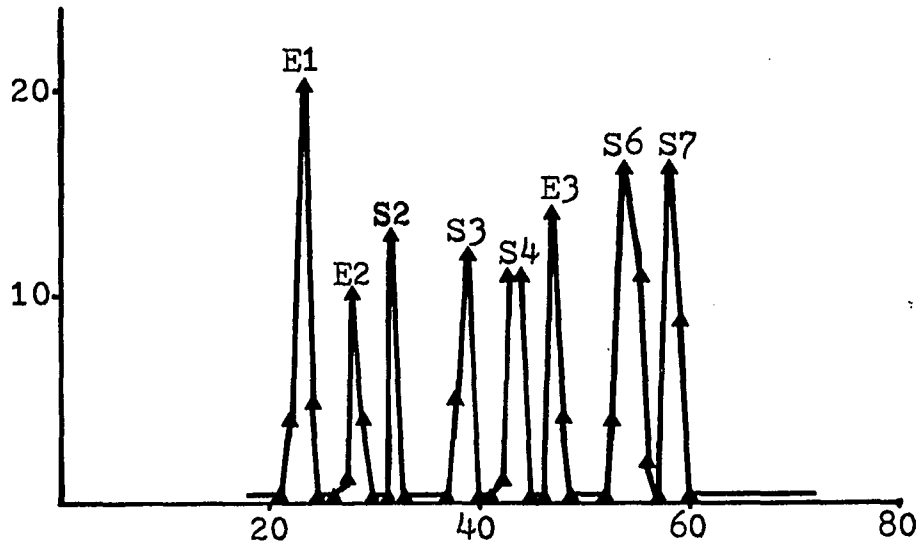


FIGURE 34

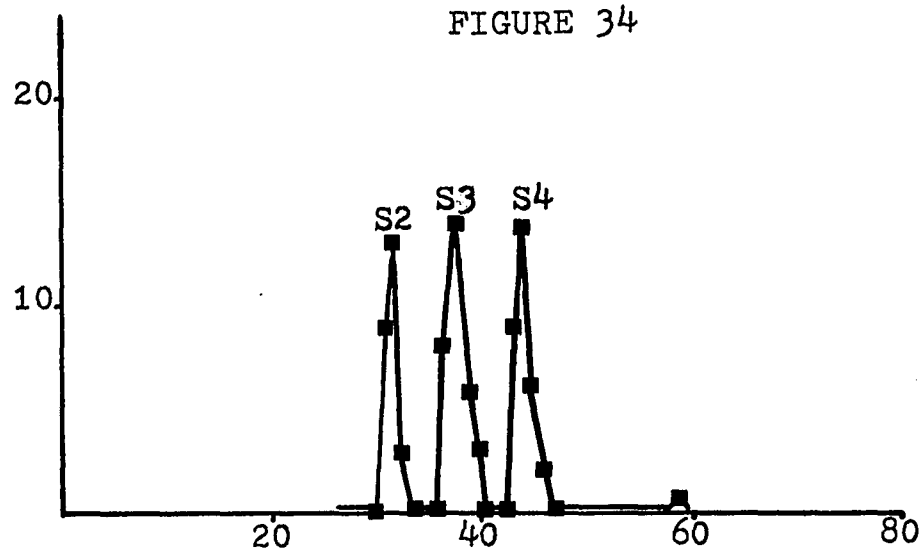
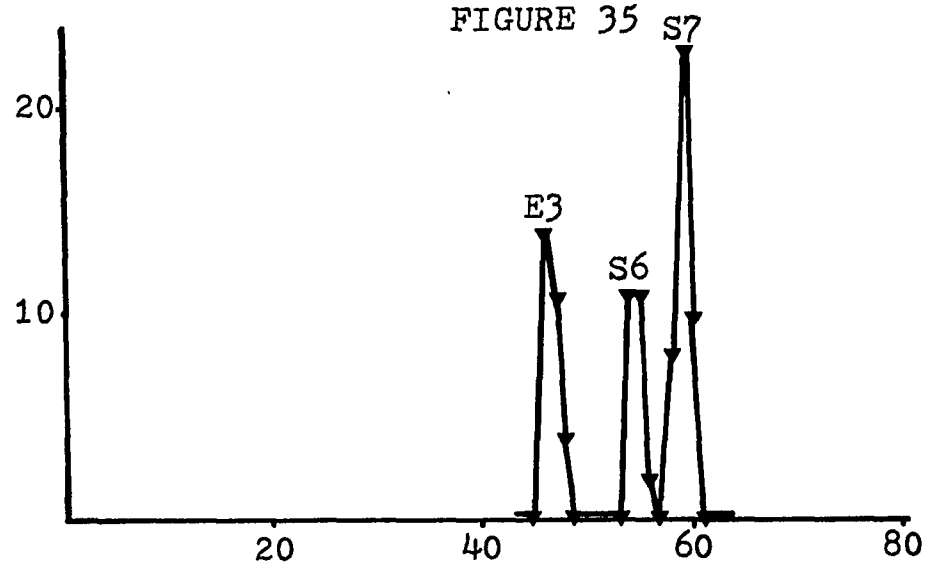


FIGURE 35



the fractions which correspond to either peaks E1, E2 or E3; 3. treating mildly these concentrates with sodium dodecyl sulfate (SDS) in the presence of mercaptoethanol; 4. after discarding the SDS from the treated concentrates by centrifugation, rechromatographing the treated concentrates on G-200 gels. The fractions which resulted from these rechromatography were assayed for endoglucanase activities. The "subunit" endoglucanase activities obtained from the E1 peak yielded 7 peaks; E2, S2, S3, S4, E3, S6 and S7 (Figure 33); from the E2 peak, 3 "subunit" peaks were yielded: S2, S3, S4 (Figure 34); from the E3 peak, 2 "subunit" peaks were yielded: S6 and S7 (Figure 35). The partition coefficients (Kav) were calculated (Appendix Schedule IV) for all of these "subunit" peaks and as above, their molecular weights were read off the graph (Figure 32). The respective molecular weights were estimated at: S2--200,000, S3--90,000, S4--55,000, S6--18,000 and S7--11,000. It may be interpreted from these data that E1 is composed of 1 E2 and 5 E3, that E2 is composed of 2 S3 and 2 S4 (S2 possibly being a trimer of 1 S3 and 2 S4) and that E3 is composed of 1 S6 and 2 S7 or 4 S7 if S6 is an S7 dimer.

Isoelectric Points

In order to further characterize the endoglucanase activity peaks obtained from "0 hr and 9 hr low salt" extracts,

Table 7. Determination of isoelectric point of endoglucanase activity peak in fractions 22 to 26 (E1) obtained through Sephadex G-200 fractionation of dry or 9 hr imbibed seeds.

TABLE 7

ENZYME 1

pH	ORIGIN	MOVING	% decrease in viscosity of 0.75% CMC after 48hr
9.0	0	30	
8.0	0	35	
7.0	0	24	
6.0	2	32	
5.2	0	27	
5.1	10	20	
5.0	11	18	
4.8	4	2	
4.6	0	5	
4.5	0	6	
4.4	0	0	
4.3	0	0	
4.2	0	0	
4.0	0	0	

ISOELECTRIC POINT -- 5.0-5.1

Table 8. Determination of isoelectric point of endo-glucanase activity peaks in fractions 27 to 30 (E2) obtained through Sephadex G-200 fractionation of dry or 9 hr imbibed seeds.

TABLE 8

	ENZYME 2		
pH	ORIGIN	MOVING	% decrease in viscosity of 0.75% CMC after 48hr
9.0	0	35	
8.0	2	26	
7.0	0	28	
6.0	0	24	
5.2	0	23	
5.1	0	27	
5.0	0	19	
4.8	0	19	
4.6	8	9	
4.5	8	9	
4.4	6	6	
4.3	0	0	
4.2	0	2	
4.0	0	0	

ISOELECTRIC POINT -- 4.4-4.6

Table 9. Determination of isoelectric point of endo-glucanase activity peaks in fractions 44 to 49 (E3) obtained through Sephadex G-200 fractionation of dry or 9 hr imbibed seeds.

TABLE 9

	ENZYME 3		
pH	ORIGIN	MOVING	% decrease in viscosity of 0.75% CMC after 48hr
9.0	0	30	
8.0	0	30	
7.0	0	24	
6.0	0	28	
5.2	-	24	
5.1	-	17	
5.0	0	19	
4.8	0	14	
4.6	4	12	
4.5	0	18	
4.4	4	14	
4.3	0	18	
4.2	14	4	
4.0	5	0	

ISOELECTRIC POINT -- 4.0-4.2

i.e., E1, E2 and E3, their respective isoelectric points were estimated. As with the "subunit" estimation above, fractions corresponding to peaks E1, E2 and E3 were pooled and concentrated. Aliquots (0.1 ml) from these concentrates were placed atop stacking and separating polyacrylamide gels in an electrophoretic apparatus filled with buffers of different pHs and subjected to 3 ma/gel (Appendix Schedule V). The buffered pHs were 9, 8, 7, 6, 5.2, 5.1, 5.0, 4.8, 4.6, 4.5, 4.4, 4.3, 4.2 and 4.0; the gels were divided into origin sections (from origin to 3 mm) and moving sections (from 3 mm to 15 mm). The appearance of endoglucanase activity in the origin sections and the decrease in endoglucanase activity in the moving sections were the criteria for isoelectric point determinations. The isoelectric points were determined to be: for E1--between 5.0 and 5.1 (Table 7), for E2--between 4.4 and 4.6 (Table 8) and for E3--between 4.0 and 4.2 (Table 9).

Localization of Endoglucanases in Seed Parts

Endoglucanase activities were measured in extracts obtained from whole seeds. However, the localization of the "bulk" activities and the Sephadex-fractionated activity peaks in different parts of the seed would aid in interpreting the role of the pregermination endoglucanase activity peak, obtained at 9-10 hr of imbibition, in the germination process in lettuce seeds. Seeds, after being imbibed for 0 or 9 hr,

were dissected into radicle and cotyledon ends and these ends were extracted, both low salt and high salt, and assayed. Endoglucanase activity in high salt extracts was predominantly measured in the radicle ends, although a substantial amount was also measured in the cotyledon ends. These observations occured with "high salt" extracts for both 0 (Table 10) and 9-10 hr (Table 11) imbibed seeds. Low salt extracts from 0 hr imbibed seeds indicated that endoglucanase activity was slightly higher from radicle ends than from cotyledon ends. Radicle ends from 45 to 75 min imbibed seeds were further dissected into embryonic axes and endosperms and each of these was extracted with unfortified (low salt) buffer and assayed for endoglucanase activity. Activities were measured in both axes and endosperms to about the same extent (Table 10). Low salt extracts of the above seed parts, radicle ends, cotyledon ends, embryonic axes and endosperms from radicle ends, were fractionated with Sephadex G-100 column chromatography (Appendix Schedule III) to determine the location of the endoglucanase peaks, E1, E2 and E3. Endoglucanases E1 and E2 were measured in both radicle ends (Figure 36) and cotyledon ends (Figure 39), but endoglucanase E3 was only measured in the radicle ends. Protein profiles (void volume and low molecular weight peaks) of the fractionations of these ends indicated that most of the seed protein was present in the cotyledon ends. To further localize the E3 peak, low salt extracts from embryonic axes and endosperms from radicle ends were also fractionated; no significant differences in

Table 10. Localization of endoglucanase activities in 0-1 hr imbibed seeds.

Table 11. Localization of endoglucanase activities in 9-10 hr imbibed seeds.

TABLE 10

seed part		extraction	endoglucanase activity*
radicle end	-----	low salt	14±1.9
	-----	high salt	45±6.0
	embryonic axis	low salt	8±1.5
	endosperm	low salt	14±1.0
cotyledon end	-----	low salt	11±1.0
	-----	high salt	15±3.5

* % decrease in viscosity of 0.75% CMC after 2hr

TABLE 11

seed part		extraction	endoglucanase activity*
radicle end	-----	low salt	20±1.2
	-----	high salt	50±1.0
	embryonic axis	low salt	1±1.0
	endosperm	low salt	21±3.0
cotyledon end	-----	low salt	1±0.7
	-----	high salt	12±2.0

* % decrease in viscosity of 0.75% CMC after 2hr

Figure 36. Endoglucanase activity and protein content per 120 drop fractions of Sephadex G-100 fractionated unfortified extracts of the radicle end of dry seeds.

abscissa = fractions primary ordinate = $\%\Delta\eta$ 24hr
secondary ordinate = mg protein/fraction

endoglucanase activity (solid line)
protein content (dashed line)

Figure 37. Endoglucanase activity in 120 drop fractions of Sephadex G-100 fractionated unfortified extracts of embryonic axis of 1 hr imbibed seeds.

abscissa = fractions ordinate = $\%\Delta\eta$ 24hr

Figure 38. Endoglucanase activity in 120 drop fractions of Sephadex G-100 fractionated unfortified extracts of the endosperm removed from radicle end of 1 hr imbibed seeds.

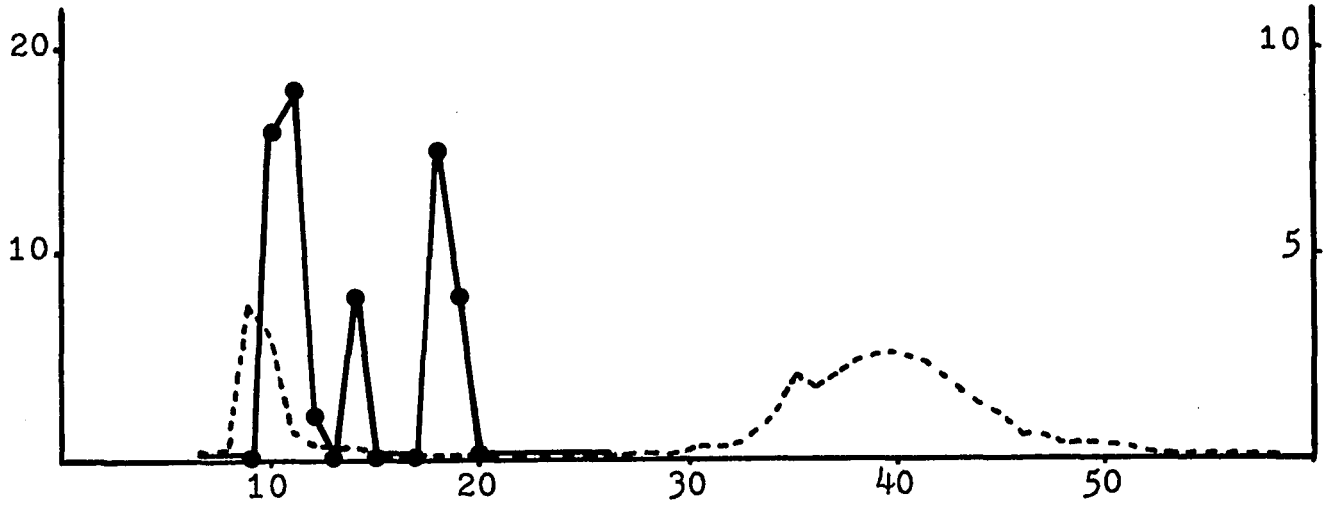
abscissa = fractions ordinate = $\%\Delta\eta$ 24hr

Figure 39. Endoglucanase activity and protein content per 120 drop fractions of Sephadex G-100 fractionated unfortified extracts of the cotyledon end of dry seeds.

abscissa = fractions primary ordinate = $\%\Delta\eta$ 24hr
secondary ordinate = mg protein/fraction

endoglucanase activity (solid line)
protein content (dashed line)

FIGURE 36



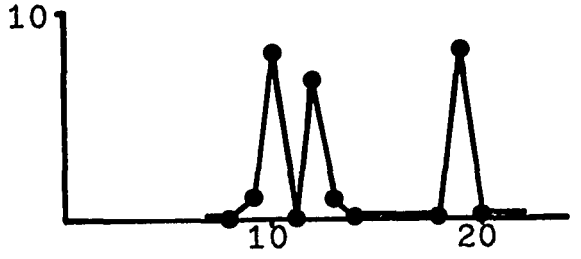


FIGURE 37

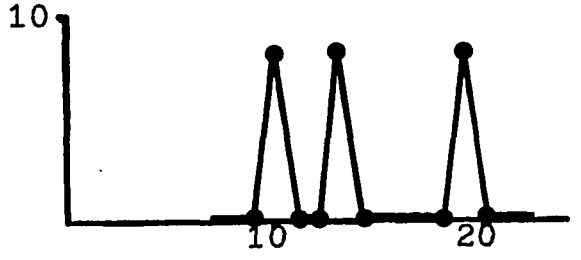
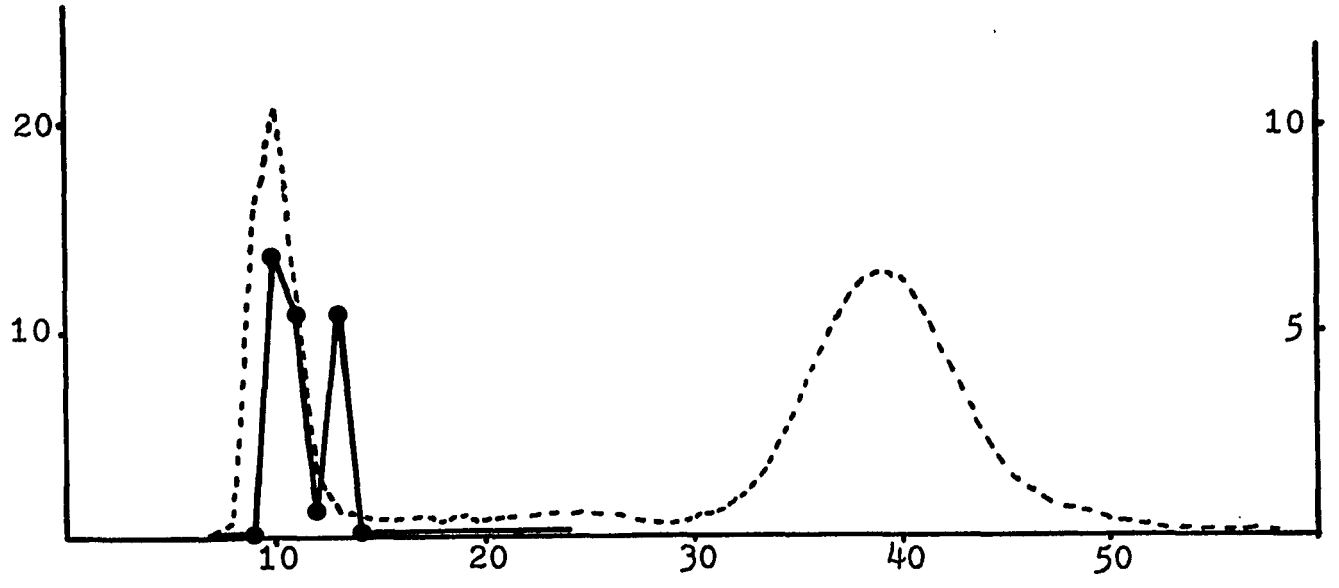


FIGURE 38

FIGURE 39



endoglucanases' activity, E1, E2 and E3, were measured between these two parts of the radicle ends (Figure 37; 38). Assays of low salt extracts from 9-10 hr imbibed seeds indicate that all of the pregermination endoglucanase activity peak was in the endosperms dissected from the radicle ends (Table 11).

These data indicate that low salt extracted endoglucanases change from being more or less diffuse throughout the 0 hr imbibed (dry) seed--with the exception of peak E3 which is diffuse through the radicle ends but is not present in the cotyledon ends--to being in only the endosperm of radicle ends of 9-10 hr imbibed seeds. These observations further suggest that the endoglucanases are synthesized de novo (Table 4) in the endosperm at the radicle (or micropylar) ends which would be the site for a chemical weakening to remove the mechanical restraint imposed by the endosperm or embryo expansion.

SEM Observations of Seeds
Imbibed for up to 6 Hours

Samples of 15 to 25 seeds were randomly picked after 6 to 20 hr after the start of imbibition at one hr intervals and the seeds were dissected and prepared for observation. Dry seeds and 3 hr imbibed seeds were also fixed, dissected and prepared. Theouter appearance of the endosperm in the micropylar region (radicle end) of 0 (Figure 40), 3 (Figure

41) and 6 hr (Figure 42) imbibed seeds was smooth without disruptions. The cell walls seemed slightly depressed, giving a rolling hill appearance. There were no gaps or pits in the endosperm and the cell walls did not have any cracks or breaks (Figures 40; 41; 42).

Cracks and Pits in Endosperm

Although 6 hr imbibed seeds showed no indication of degradation, cracks and pits began to appear in 8 hr (Figure 43) and slightly more extensively in 9 hr (Figure 44) and 10 hr (Figure 45) imbibed seeds. Small pits were present at the tip of the micropylar region (Figure 43a; c; d). Some pits and small cracks were evident through much of the region (Figure 43a; d); but areas without pits or cracks were also evident in 8 hr imbibed seeds (Figure 43a; b; d). In 9 hr imbibed seeds, pits and cracks in the endosperm were observed throughout the micropylar region (Figure 44). The tips of some 10 hr imbibed seeds showed a greater degree of degradation; breaks and net-like sections may be observed (Figure 45). In addition to many pits, the cell walls may have a pronounced wrinkled appearance (Figure 45b). The degree of degradation of the endosperm of ungerminated seeds increased with imbibition time. At 12 hr, large pits and cracks appeared at the tip of the micropylar region (Figures 46; 47). The tips of many 10 to 14 hr imbibed seeds were partially collapsed, and showed

the separation of cells from each other (Figure 46a, b). Large pits and cracks were evident (Figures 46a, c; 47a, b) as well as small pits and cracks between endosperm cells (Figures 46d; 47b, c). Part of the micropylar regions may have a net-like appearance as well (Figures 45; 47a). In contrast to the micropylar region, the cotyledon end and the mid-section do not indicate the occurrence of degradation; pits and cracks were not present (Figure 49d).

Openings in the Endosperm

In addition to seeds with pits and cracks appearing in the endosperm, some seeds after imbibition times of 10 hr or more had an opening or large break (fissure) in the endosperm at the tip of the micropylar region. Openings observed in 10, 11 and 12 hr seeds are shown in Figures 48 and 49. A large fissure was present in some seeds (Figures 48a, b; 49b), which may indicate that a part of the endosperm is about to fall out (Figure 48b). The 11 hr imbibed seeds (Figure 48c) had a greater degree of degradation than 10 hr imbibed seeds (Figure 48b); a few breaks were present and parts of the tips were collapsed. A fully digested endosperm is evident in Figures 48d and 49c. A net-like appearance at the tip is also present in these seeds. An indication of the stages in the formation of an opening is shown in Figure 49. Endosperm degradation was indicated by two or three large cracks and a collapsed area (Figure 49a). A large fissure

appeared, possibly cutting off part of the endosperm (Figure 49b), and finally a large opening was evident (Figure 49c). Parts of the endosperm may have a net-like appearance, may be collapsed and/or have large cracks. The inner side of the endosperm may be viewed with seeds in which an endosperm flap remained (Figure 50). Extensive degradation was observed. Inner and cross walls were usually not present; small sections of cross walls sometimes may be observed (Figure 50c, d); the breakup of intracellular structure may also be observed (Figure 50a, b). Grains measuring 2.0 to 4.0 um seemed to be embedded in the matrix or in the process of being released. These observations suggest the extensive degradation that may be occuring on the side of the endosperm facing the embryo.

Comparison of Endosperm Degradation with Germination

No degradation of the endosperm was visible (with a scanning electron microscope) in seeds which had been imbibed for up to 7 hr. After 8 hr of imbibition, 10-12% of all seeds have small pits and cracks in their endosperms (Figure 51, Curve A). After 9 hr, the micropylar region (radicle end) of a higher percentage of seeds showed more signs of degradation. Accompanying the increase in degradation of the endosperm of individual seeds with an increase in the time of imbibition is an increase in the total number of seeds undergoing the degradation. The percentage of seeds with pits and cracks, but without an opening or break, peaks after 12 hr of imbibition

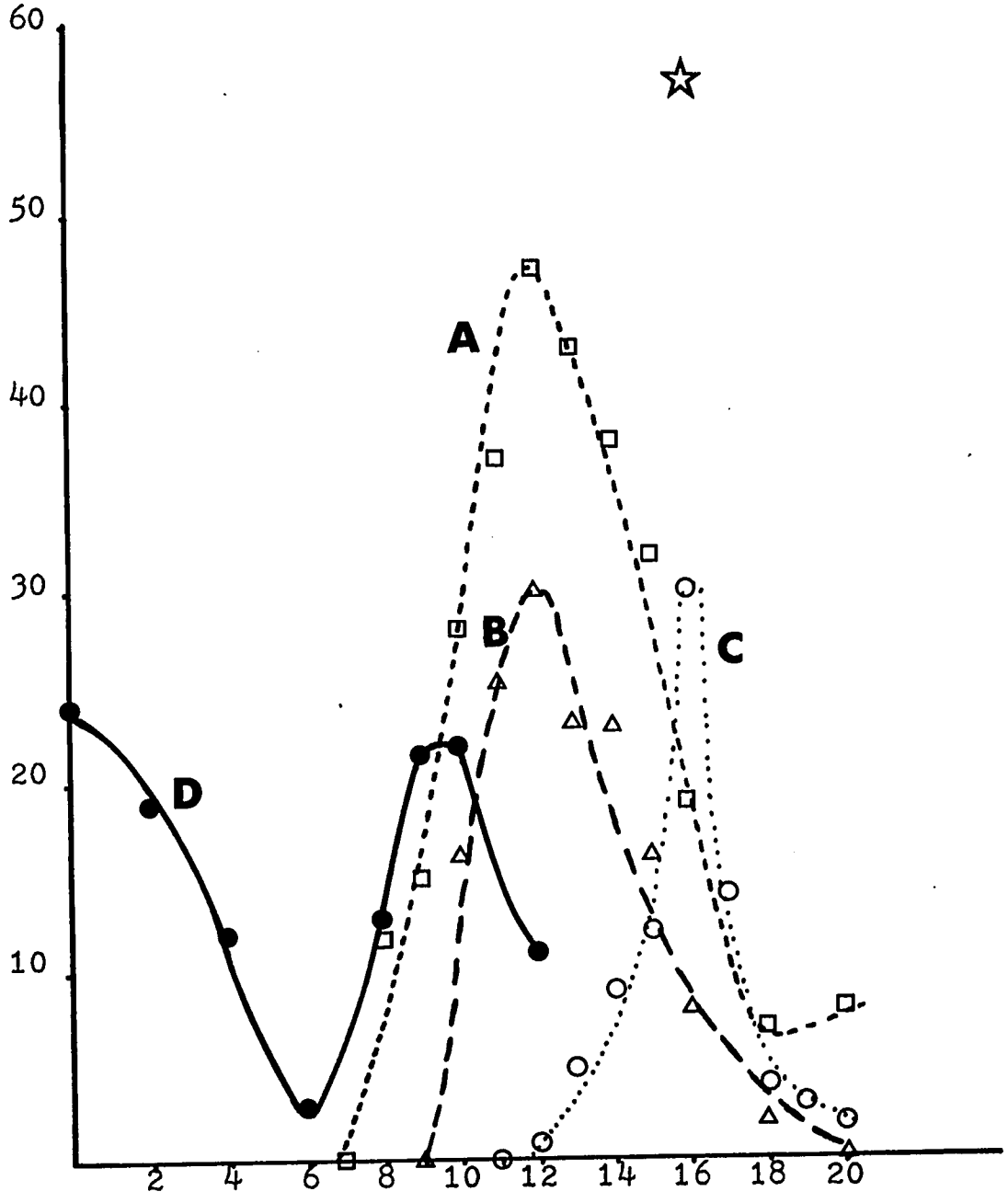
Figure 51. Endosperm degradation correlated with germination and endoglucanase activity during imbibition and germination of seeds.

abscissa = hr of imbibition
ordinate = % seeds (or $\%\Delta\eta$ 2hr)

A = cracks/pits in endosperm (short dashed line--open squares)
B = openings/breaks in endosperm (long dashed line--open triangles)
C = increase in germination above previous hour (dotted line--open circles)
D = endoglucanase activity of low salt extracts (solid line--closed circles)

% germination (open star)

FIGURE 51



and is approximately 47% (Figure 51, Curve A, Table 12). A similar observation is made of seeds with an opening or break. The first seeds (15%) with an open endosperm at the tip of the micropylar region (radicle end) are observed after 10 hr of imbibition (Figure 51, Curve B). With longer imbibition time (10-16 hr), the openings are larger and the degradation in the area around the openings is more extensive. The percentage of such seeds increases to a peak after 12 hr; nearly 30% of imbibed seeds have an opening (Figure 51, Curve B). The decrease in the percentages of seeds having endosperm degradation is probably due to the germination of seeds which begins after 12 hr. The rate of germination peaks between the 15th and 16th hr of imbibition, at which time about 55% of the seeds have germinated (Figure 51, Curve C). Note that the development of endosperm degradation, i.e., pits and openings, starts 5 and 3 hr respectively, prior to the onset of germination and the percentages of seeds with degradation peak 3-4 hr prior to the peak in the rate of germination (Figure 51). The total number of seeds with degradation after 12 hr of imbibition is approximately the same as the number of seeds germinating in the first day (Table 12). The activity of endoglucanases begins to increase prior to the appearance of endosperm degradation (Figures 3; 51, Curve D). As with endoglucanase activity, endosperm degradation also appears after water uptake by seeds had ceased (Figures 2; 51).

Effects of Light and Temperature--
Endosperm Degradation

Endosperm degradation (pits and cracks and openings) is as apparent in 12 hr imbibed lettuce seeds sown in the dark with a 30 min exposure to white light (375 ft-cdl) after 3 hr of imbibition as it is in 12 hr imbibed seeds sown in continuous white light (Table 12). Germination after 24 hr is approximately 76%, which corresponds to the percentages of seeds showing endosperm degradation. In contrast, germination after 24 hr (Figure 14, Table 12) and endosperm degradation after 12 hr (Table 12) are significantly reduced in seeds sown in continuous darkness. Reduction of germination (Table 12) and endosperm degradation (Figure 52) is more pronounced when seeds are sown in the dark with a 1 hr exposure to far red light after 3 hr.

Lettuce seeds sown in continuous light at 24°C begin germination earlier but germination is lower after 24 hr compared to seeds sown at 20°C (Figure 14; Table 12). Overall endosperm degradation is proportionately less than that of seeds sown at 20°C; however, the degradation is as extensive (Figure 53a, b). Germination of dark-sown seeds at 24°C is significantly reduced and endosperm degradation is correspondingly lowered (Figure 53d, e). Thermodormant seeds sown at 35°C do not germinate regardless of light or dark treatments (Figure 14; Table 12), although they maintain viability for several days.

Table 12. SEM-observable endosperm weakening and germination of lettuce seeds in white light, far red light, darkness and temperatures of 20, 24 and 35°C.

a = continuous white (750 ft-cdl)
b = 30 min white (375 ft-cdl) exposure after 3 hr of dark imbibition
c = 1 hr far red exposure after 3 hr of dark imbibition

TABLE 12

temperature °C	lighting condition	germination % 12hr	germination % 24hr	pits & cracks*	openings & breaks*
20	[a]white	1	78	47	30
20	[b]dark-white-dark	-	76	50	25
20	darkness	0	48	50	0
20	[c]dark-far red - dark	-	15	8	0
24	[a]white	10	70	51	15
24	darkness	0	23	9	0
35	[a]white	0	0	40	0
35	darkness	0	0	0	0

* % total seeds after 12hr having ....

However, endosperm weakening, i.e., pits, cracks and cell wall separations (Figure 54a, b, c), is evident in 40% of the thermodormant seeds sown in continuous white light (Table 12). In contrast, endosperms of thermodormant seeds sown in continuous darkness do not give indications of degradation (Figure 54d, e).

Effects of Isocyanurate

Lettuce seeds, cultivar New York, were sown in various concentrations of sodium dichloroisocyanuric acid (iC), also known as sodium dichloro-s-triazine trione, a herbicide of the s-triazine family (Figure 55). Concentrations of 2.5 and 5.0 mg/ml induced embryo expansion without protrusion, "buckling", (Figure 56a, b, e) in 6 and 29% of the seeds respectively (Table 13) after 4 dy of imbibition. "Atypical" germination (Figure 56c, d, f) was also observed in 3 and 1% of the seeds respectively (Table 13). Most of the "atypically" germinated seeds appear to have resulted from "buckled" seeds. The embryonic axes elongated from about 1 to approximately 2.5 mm which the cotyledons showed a relatively insignificant expansion. (In typical germination, no embryo growth was detected before protrusion.) These germination or embryo expansion patterns are in marked contrast to typical germinating and nongerminating seeds (Figure 57). No buckling was observed in seeds cut transversely through the cotyledons in such a way that the endosperm's mechanical restraints to

Table 13. Effects of 4 day treatments of sodium 2,4 dichloroisocyanurate on lettuce seeds, cultivar New York.

a = the embryonic axis elongated about 1.5 mm (initial length when fully imbibed was 0.7-1.3 mm). The cotyledons elongated about 0.4 mm (initial length when fully imbibed was 2.0-2.3 mm).

Table 14. Differential inhibition of embryo expansion and endosperm weakening proposed for sodium 2,4 dichloroisocyanurate--an interpretation derived from data presented in Table 13.

TABLE 13

sodium 2,4 dichloro-isocyanurate mg/ml	protrusion through radicle end* % seeds	embryo expansion-- no protrusion+ % seeds	protrusion not through radicle end# % seeds
0	100	0	0
1.0	100	0	0
2.5	90±1.5[a]	5.8±1.5[a]	3.2±0.1
5.0	69±1.0[a]	29±1.5[a]	1.1±0.2
10.0	0	9	0

*typically germinated

+embryo buckling

#atypically germinated

TABLE 14

sodium 2,4 dichloroisocyanurate mg/ml	1.0	2.5-5.0	10
embryo expansion	permitted (+)	permitted (+)	inhibited (-)
endosperm weakening	permitted (+)	inhibited (-)	inhibited (-)

expansion of the axes was removed (Figure 57c). Consequently, this compound does not cause the growth abnormality on embryos unless there is mechanical restraint by the endosperm. Lower concentrations of iC (e.g., 1 mg/ml) did not seem to significantly interfere with typical germination; higher concentrations (e.g., 10 mg.ml) prevented embryo growth (Table 13).

Since these observations are interpreted to indicate that, in some seeds, iC (between 2.5-5.0 mg/ml) partially inhibits a chemical weakening of the endosperm that would have facilitated radicle protrusion in typical germination (Table 14), it was of interest to determine possible endosperm degradation and endoglucanase activities. For these determinations, lettuce seeds of the cultivar Grand Rapids (1974 batch) were used. Endoglucanase activities in low salt extracts of this seed batch were the same as for the 1971 seed batch (Figure 3); however, activities in high salt extracts of the 1974 seed batch, although constant during the pregermination period, were substantially lower. 25% of these seeds sown in 5 mg/ml iC were buckled and 4% of the seeds sown in 2.5 mg/ml iC were buckled; 1 mg/ml iC had little effect and 10 mg/ml iC inhibited all growth. Seeds being both "buckled" and "greened" (suggesting chlorophyll synthesis) were observed in 1 to 2% of all seeds or in approximately 6% of the "buckled" seeds. Seeds imbibed for 9 hr in 0, 1.0, 2.5, 5.0 and 10.0 mg/ml were extracted with salt fortified (high salt) and unfortified (low salt) buffers. As seeds are sown in higher concentrations

Figure 55. Structure of sodium 2, 4 dichloroisocyanurate (sodium 2, 4 dichloro-5-triazine trione) (iC).

Figure 58. Endoglucanase activity, typical germination, buckling phenemenon and atypical germination in lettuce seeds, cultivar Grand Rapids, sown in either 0, 1, 2.5, 5 or 10 mg/ml sodium dichloroisocyanurate (iC)

abscissa = iC concentration (mg/ml)
primary ordinate = $\%\Delta\eta$ 2hr (% buckled or atypical germinated seeds)
secondary ordinate = % germination

endoglucanase activity in:
9 hr low salt extracts (closed circles)
9 hr high salt extracts (closed squares)
"buckled" seeds after 6 days (open circles)
"atypically" germinated seeds after 6 days (open squares)
typical germination after 27 hr (closed stars)
typical germination after 6 days (open triangles)

FIGURE 55

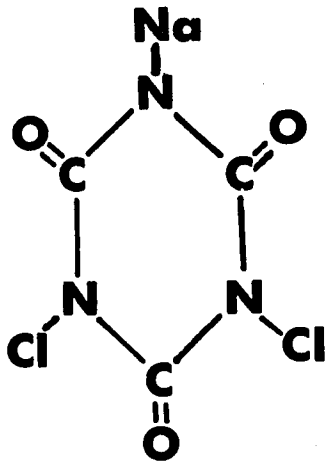
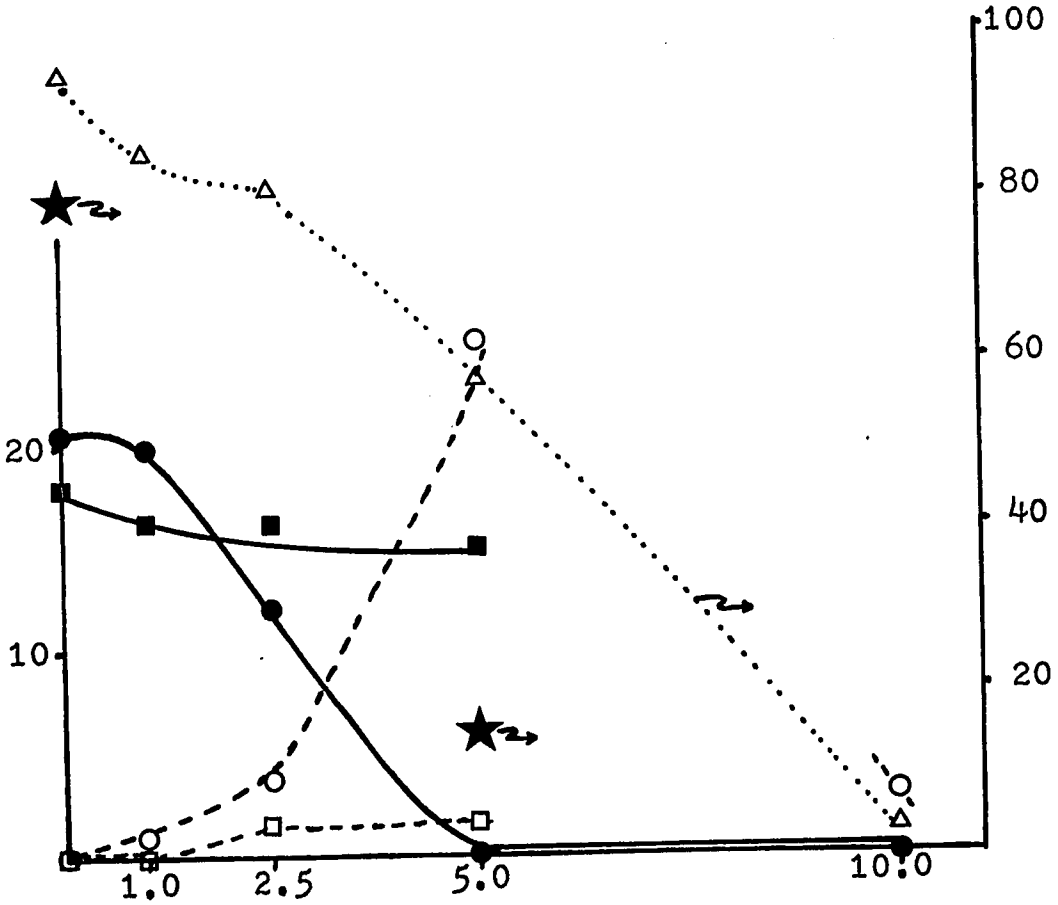


FIGURE 58



of iC, activity in low salt extracts of 9 hr imbibed seeds decreased (Figure 58). Activity (low salt extracts) of seeds sown in 1 mg/ml iC was approximately the same as water controls. Low salt extracts from seeds imbibed for 6 and 9 hr in 5 mg/ml iC had relatively little activity; activities were measured after 2 hr assay incubation but the presence of some enzyme in 6 hr and 9 hr seeds was indicated by % of 20 and 17 respectively after 4.5 dy assay incubation. No difference between 6 and 9 hr low salt extracts was detected. Activity in high salt extracts of seeds imbibed for 9 hr in these iC concentrations was not significantly altered by iC. These results suggest a differential effect of 5 mg/ml iC; this suggestion is also supported by the finding of some "buckled" seeds with green embryos. "Buckled" seeds began to appear and the percentage of these seeds increased with increasing iC concentration from 2.5 to 5.0 mg/ml; "atypical" germination was also observed (Figure 58). Percent germination measured after 27 hr and 6 dy decreased with higher iC concentrations. Further indirect support for the inhibition of endoglucanases by iC was obtained from SEM-observation of the endosperms of iC-treated (5 mg/ml) seeds. Scanning electron micrographs (Figure 59) of iC-treated seeds indicated an absence of endosperm degradation or weakening (even after 4 dy). Also, it was observed that many of the germinated seeds had radicles which looked pinched by only a partially weakened endosperm (Figure 59d). Therefore, iC not only inhibited the appearance of endo-

glucanase activity in seeds but also the occurrence of SEM-observable endosperm weakening.

DISCUSSION

The endosperm of lettuce seeds (achenes) has been considered to play a major role in germination (Klein and Preiss, 1958a; Koller et al., 1962; Globerson et al., 1974). Some authors have suggested that the endosperm acts as a mechanical restraint against the elongation of the embryonic axis (Evenari and Neumann, 1952; Pavlista and Haber, 1970). This mechanical restraint may be overcome by a chemical weakening of the endosperm (Ikuma and Thimann, 1963a; Jones, 1974). This report provides support that such a weakening occurs prior to the onset of germination (cf. Figure 51) and an enzyme, endoglucanase, which may be partially responsible for this weakening is present (cf. Figure 3), appearing specifically in both the radicle end and in the endosperm (cf. Table 10; 11). Figure 60 and Table 15 summarize the physiological and biochemical characteristics of this enzyme during lettuce seed germination. Of the enzymes separated by Sephadex column chromatography, E3 may have the main physiological importance (Figure 60).

The subunit structure of the endoglucanases were estimated by sodium dodecyl sulfate treatment. The subunits retained activity. This unusual finding may be due to the

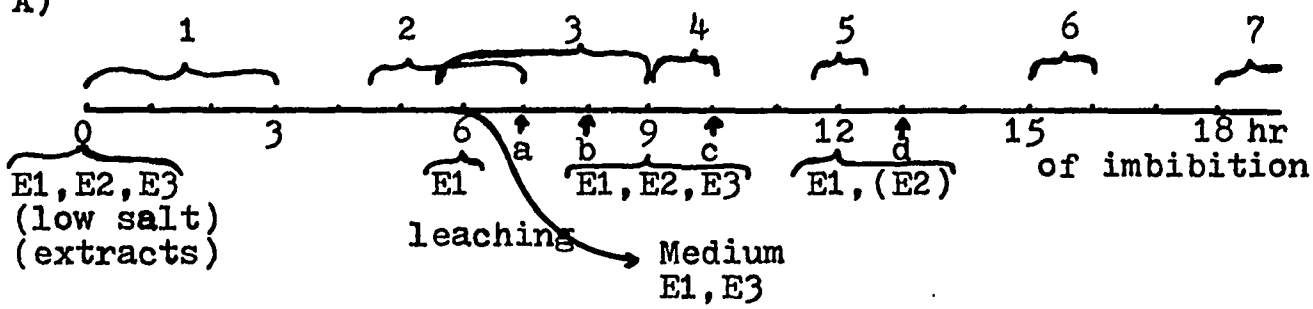
Figure 60. Summary of the physiological data:

A) time course relationships

B) a proposed scheme of sequential events

Figure 60

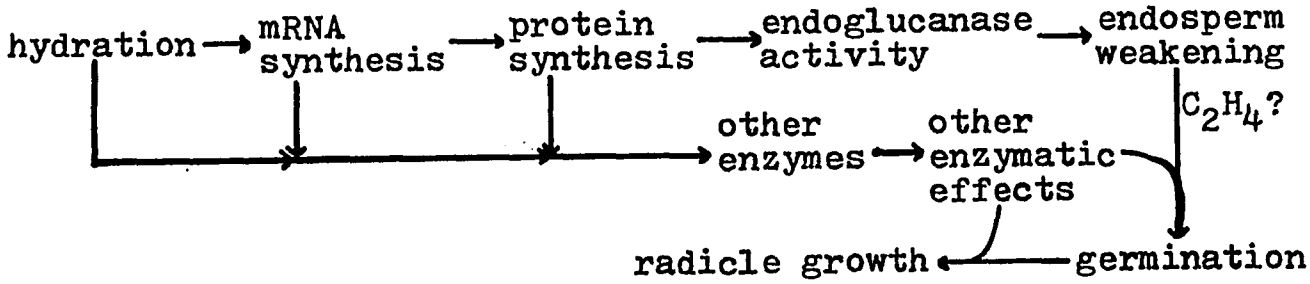
A)



1=rapid water uptake 2=synthesis of mRNA for endoglucanase
3=synthesis of endoglucanase polypeptides
4=endoglucanase activity peak 5=endosperm weakening peak
6=germination rate peak 7=slow increase in germination

a=first appearance of endoglucanase peak
b=first appearance of endosperm weakening (cracks/pits)
c=first appearance of breaks and openings in endosperm
d=first appearance of germination/radicle growth

B)



resilient nature of these enzymes. The SDS treatment was mild--treatment being at 40°C for 4 hr. SDS was partly removed by centrifugation at 4°C. The mildness of the treatment was evidenced by the presence of some enzyme aggregates even after treatment.

Endoglucanase activity leaches from seeds sown in water for 6 and 9 hr and these leachates contain E1 and E3 enzymes plus a trace amount of E2. These leachates may partially explain the loss of activity between 0 and 6 hr from the start of imbibition; enzyme inhibitors are not present in 6 hr extracts and therefore cannot explain it.

Endoglucanase activities (C_x cellulase, carboxymethylcellulase) have been reported to be involved in a number of processes in many plants, e.g., abscission and cell expansion. Optimal assay pHs have been reported between a pH of 5 and 7 and optimal assay temperatures anywhere between 20 and 60°C. In some systems, the molecular weights and isoelectric points have been determined for these enzymes and there is a wide range of reported characteristics. Enzymes have been extracted with salt fortified and unfortified buffers. A partial comparison of these enzymes from different systems and those reported here is presented in Table 16. Heavy endoglucanases as reported here have not been previously observed; however, endoglucanases composed of endoglucanase-active subunits have been previously reported (Linkins and Lewis, 1974; Byrne et al., 1975).

Table 15: Summary of the biochemical data on the endoglucanase activity peaks obtained through Sephadex fractionations.

TABLE 15

endoglucanase	E1	E2	E3	E4	E5
presence in low salt extracts of 0 & 9hr seeds and 9hr medium	++	+	++	-	-
presence in low salt extracts of 6 & 12hr seeds	+	+/-	-	-	-
presence in high salt extracts	++	+	-	++	+
molecular weight (Daltons)	480,000	280,000	40,000	5,000	1,800
subunits and their molecular weights	E2--280,000 E3-- 40,000	S2--200,000 S3-- 90,000 S4-- 55,000	S6--18,000 S7--11,000	?	?
possible subunit composition	E1--1E2 5E3	E2--2S3 2S4 S2--1S3 2S4	E3--1S6 2S7	?	?
isoelectric point	5.0--5.1	4.4--4.6	4.0--4.2	?	?

Table 16: Review of biochemical characteristics and assay conditions for endoglucanases extracted from various plant systems.

TABLE 16

	red kidney bean: stem, petiole, cotyledon, abscission zone			cotyledons: germinating seed		pea: expanding epicotyl		avocado: ripe fruit
extraction salt	high			low		low	high	high
assay conditions	23°C pH6.1			38-40°C pH6.1		35°C pH6.0	"	
molecular weight, $X10^3$	60-70	30-35	120	70	70	20	70	50-55
isoelectric point	4.5	9.5	6.1	4.5	4.8	5.2	6.9	4.7
optimal assay pH				5.7-6.2	4.8-5.6	5.5-6.0		
						5.5	7.0	
optimal assay temperature				45-60°C	35-43°C			
CMC concentration	0.8%			0.8%		0.72% & 0.8%		
assay times	1-2hr			30-60min		2hr		
comments	a	b	c			d	e	
						f	g	
references	Lewis et al.1974 Reid et al.1974 Linkins et al.1973 Linkins & Lewis 1974			Lew & Lewis 1974		Byrne et al. 1975 Ferrari & Arnison 1974		Lewis et al. 1974

a-increases with auxin treatment
b-increases with ethylene treatment, intercellular localization
c-parent form of a
d-dimerizes upon purification, increases with auxin treatment not affected by NaCl in assay
e-increases with auxin treatment, not affected by NaCl in assay
f-increases with auxin treatment, inhibited by NaCl in assay thermostable
g-increases with ethylene treatment, NaCl required for maximum activity, thermolabile

Protein and RNA synthesis inhibitors prevent the appearance of the 9-10 hr endoglucanase activity peak; this suggests that E3 is synthesized de novo. The E1 enzyme may likewise be synthesized de novo but since this enzyme is also present in 6 hr extracts, this cannot be resolved by using inhibitors. Time course studies of the application of inhibitors suggest that the mRNA and the polypeptides for E3 endoglucanase are synthesized between 4½ to 7 hr and 5½ to 9 hr, respectively (Table 4; Figure 60). RNA and protein synthesis have been demonstrated to be required for lettuce seed germination (Smith and Frankland, 1966; Khan, 1966; 1967a, 1967b; Black and Richardson, 1968; Bewley and Black, 1972). The data presented here are in agreement with the earlier reports. Seeds were found to be unaffected by actinomycin D and to some extent, by 6-methyl purine, unless the seeds were punctured. This is probably due to the lack of easy penetration of these substances through the seed coats (Tao and Khan, 1976).

Since it is hypothesized that endoglucanases are involved in a chemical weakening of the endosperm, it appeared worth while to attempt to localize these enzymes in the different parts of the seed. In dry seeds, low salt-extracted endoglucanases are present in the radicle and the cotyledon ends in about a 3:2 ratio (Table 10). Determination of the presence of the different peaks agreed with this finding; E1, E2 and E3 are present in the radicle end but

only E1 and E2 are in the cotyledon end (Figure 36; 39). Separate extractions of the enzyme from embryonic axes and endosperm regions of the radicle ends of 1 hr imbibed seeds indicate that all three buffer-soluble forms are present in both tissues (Figure 37; 38). In 0 to 10 hr imbibed seeds, the pattern is very different. Low salt extracted endoglucanases are found only in the radicle end and in this third of the seed nearly all of this activity is in the endosperm (Table 11). Therefore, the Sephadex-fractionated profile of low salt endoglucanases in 9-10 hr imbibed seeds represents the endoglucanases, E1, E2 and E3, found in the endosperm surrounding the embryonic axis. It is suggested that endoglucanases are leached out or destroyed from the seed during the first 6 hr of imbibition and these enzymes (especially E3) are synthesized de novo in the endosperm exclusively at the radicle (or micropylar) end. Therefore, endoglucanases are present in the location where endosperm weakening would be most likely in order to facilitate germination and trigger radicle growth. These results agree with those of Jones (1974) who reported observing a loss of cell wall material in the endosperm from the cytoplasm-facing side outward. Furthermore, Halmer et al. (1976) observed the appearance of an endomannanase within endosperm cells.

Ikuma (1962) and Ikuma and Thimann (1963a) attempted to detect cellulase as well as pectinase activity within the lettuce seeds. These authors detected a small amount of enzyme activity only in the cotyledon ends. This seems to

be in contrast to findings reported here. However, carboxymethylcellulase was extracted by Ikuma (1962) shortly after radicle protrusion and not during the pregermination period. Furthermore, the conditions of his extraction and assay are significantly different: low salt extract at pH 7, no homogenization, only grinding with a mortar and pestle, no high speed centrifugation (7,000 g was the only centrifugation) and an assay at 25°C. The assay technique used was KI_3 titration as described by De Stevens (1955) which is less sensitive than viscometric assays by a couple of orders of magnitude (Mussell and Morre, 1969). The viscometric assay system has been shown to be an accurate method for determining cellulase and pectinase activities (Sherwood and Kelman, 1964); a small decrease in potential reducing power in CMC corresponds to a large loss in solution viscosity (Maclachlan and Perrault, 1964; Byrne et al., 1975). The microviscometer using a cone plate detection method has further extended the use of this method by accurately measuring small quantities of solution (1 ml) (Wells et al., 1961; Abeles, 1969; Abeles and Leather, 1971; Rasmussen and Jones, 1971; Rasmussen, 1973).

Halmer et al (1975; 1976) reported the presence of an endomannanase in lettuce seeds. This level is low until the onset of germination at which time activity increases five fold, and is localized in the endosperm. Since the cell walls of the endosperm are composed predominantly of mannose (about 60%), this enzyme would be very important in the total

degradation of the endosperm as reported by Park and Chen (1974). The endosperm is also composed of glucose (10%) (Halmer et al., 1975). Weakening of this tissue would not require complete or even major degradation of the cell walls, but would require only a loosening or development of weak points (pits and cracks) in the radicle end. Therefore, it seemed appropriate to examine the surface appearance of the endosperm during seed germination.

The endosperm of dry seeds is a continuous, smooth-looking structure. Upon hydration, no changes can be discerned in the outer appearance of this tissue. Water uptake plateaus after 3 hr, which is well in advance of the beginning of endosperm degradation (Figure 3; 51). This suggests that hydration itself does not grossly alter the endosperm. After 6 hr of imbibition, the endosperm appears to be similar to the dry seed (Figure 42). At this time, endoglucanase activity is at a minimum. From 6 to 9 hr, endoglucanase activity increases and forms a peak after 9 to 10 hr of imbibition (Figure 3). Endosperm degradation appears 2 hr after the onset of endoglucanase activity and 4 to 5 hr prior to the onset of germination (Figure 4; 51). The peaks of endosperm degradation, i.e., pits and cracks (Figure 46; 47) and openings (Figure 48; 49) occur 3 hr after the peak in endoglucanase activity and 3 to 4 hr prior to the peak in germination rate (Figure 51). The time course appearances and interrelationships are depicted in Figure 60a; b.

Time course studies on endosperm weakening have been reported with Striga lutea (Egley, 1972) and Syringa spp. (Juntilla, 1973a). Although these authors used very different techniques in their studies, they concluded that endosperm weakening preceeded germination--by 2 or more hours for Striga lutea--by 3 or more days for the Syringa spp. Juntilla further related the strength of endosperms with the dormancy condition of different species of Syringa. Measuring swelling and growth of Striga lutea embryos, Egley concluded that the observed weakening is not due to radicle penetration. This agrees with the findings on lettuce (Evenari et al., 1957; Haber and Luippold, 1960a; Ikuma, 1962, 1964) that the onset of radicle growth corresponds within minutes to the onset of germination. Nabors and Lang (1971a) and Esashi and Leopold (1968) measured the strength of lettuce endosperms and cocklebur testa respectively of seeds maintained in adverse germination conditions. The authors compared this strength to the growth potential of growing, isolated embryos (as determined by osmotic or mechanical studies) and concluded that the expansion force of the growing embryo is equal to or greater than the mechanical resistance of the endosperm. However, the above authors did not do a time course study and the coats were surgically removed from seeds already sown for 18 or more hours in the dark. Therefore, the endosperms may have already been weakened somewhat.

When the endosperm of lettuce seeds at the micropylar region is open, sometimes there remains a flap of the endosperm (Figure 48; 49). This allows the inner side, i.e., side facing the embryo, to be observed. The endosperm's cell walls closest to the embryo seem to have been totally digested, therefore exposing the interior of the cells (Figure 50). No intact cross walls were observed; however, occasionally a piece of cross wall may still remain. The width of these is 1-2 um which is less than the 6-10 um width reported for nondegraded inner cell walls (Jones, 1974). Grains are present and these may be embedded in the cell wall matrix. These grains range in diameter from 2.0 to 4.0 um, which corresponds to the size reported for aleurone grains in barley (Jones, 1969) and wheat (Stevens, 1973). These grains may be the protein bodies which were reported by Jones (1974) in lettuce.

The above results were determined on lettuce seeds sown at 20°C in continuous white light at 750 ft-cdl. Seeds sown, when first received, in lower light intensity germinated at a slower rate, reaching 65% germination after 42 hr. Under stronger light, germination was more synchronized, therefore accounting for a sharp germination increase between 13 and 18 hr (Figure 2; 51). Note that 76% of the seeds, stored for three years in a dessicator in a refrigerator, germinate after 24 hr when sown in the dark with a brief exposure to light at a reduced intensity (Table 12). Similar findings have been previously reported (Borthwick

and Robbins, 1928). Endoglucanase activity and endosperm weakening occur to approximately the same extent as in seeds sown in continuous light. Exposure to continuous darkness or brief exposure to far red light at 20°C inhibits germination after 24 hr, endoglucanase activity at 9 hr and endosperm degradation after 12 hr to corresponding degrees (Table 5; 6; 12).

Germination of seeds sown at 24°C in continuous light occurs in less than 12 hr and is 70% after 24 hr. Endoglucanase activity extracted with unfortified buffer from seeds imbibed for 9 hr under this condition is the same as at 20°C, continuous light (Table 6). The percentage of seeds with open endosperm tips is less than that of seeds sown at 20°C, probably due to the shorter lag period before germination begins. However, endosperm degradation is observable in 66% of the seeds (Table 12). Seeds sown in the dark at this temperature have significantly reduced germination, low salt extracted endoglucanase activity and endosperm weakening.

These results are partially in contrast with those of Ikuma and Thimann (1963a) who did not observe any difference in cellulase or pectinase activities in the cotyledons of light and dark sown seeds; but agree with the observations on endomannanase activity (Halmer et al., 1976) and on the loss of cell wall material in the endosperm (Jones, 1974). High salt extracted endoglucanase activity

is unaffected by dark, far red or 24°C-treatment. Dark sown thermodormant seeds do not germinate nor contain low salt extractable endoglucanase activity nor exhibit endosperm weakening. Although not inducing germination of thermodormant seeds for up to six days, continuous light treatment does induce endosperm weakening in 40% of the seeds and the seeds contain about half the low salt extractable endoglucanase activity ($\%\Delta\eta$2 hr = 11) (Table 6; 12). These effects of different light and temperature treatments suggest that endoglucanase activity and endosperm weakening are partially controlled by light and temperature, are inhibited by far red light and are not due to hydration. The close correlation of endoglucanase activity, endosperm weakening and germination are further supported by these results (Figure 60).

Park and Chen (1974) have demonstrated that in lettuce seeds the endosperm's dry weight decreases with time. After 3 days of imbibition under conditions which allow germination, the dry weight of the endosperm is nearly zero. If the endosperms are removed, isolated embryos will germinate normally and grow, although they do not grow as well as intact seeds. This suggests that the endosperm is degraded and may be used as a food source for the development and growth of the newly germinated seedling. The endosperm does not store starch, but some carbohydrates are present and there is a transfer of sucrose from the endosperm

to the embryo (Park and Chen, 1974). Most of the carbohydrates may come from the degradation of the cell walls. This is supported by Jones (1974) and by data presented here. Jones (1974) presented light micrographs of sections through the endosperm depicting the degradation of the cell walls during germination. He noted that the periodic-acid-Schiff stain of the cell walls was lost from near the plasmalemma outwardly, indicating that the degrading enzymes are released by the endosperm cells. The stain was lost in all the walls except those adjacent to the integument. The latter observation is not in agreement with data presented here. This may be accounted for by three differences in the techniques: 1) Jones looked at cross sections of the endosperm while, with the SEM, the entire surface is able to be observed. 2) In this thesis, the micropylar region is specifically examined; Jones did not specify from which area of the seed his endosperm sections came. 3) The seeds used in this thesis germinated significantly more rapidly than the seeds which Jones observed. (Jones reported germination began after 14 hr and was 35-40% after 24 hr.)

Speer and Hsiao (1976) reported cellulose in the endosperm acting as an osmotic barrier. A loss of endosperm integrity occurred in 60% of seeds sown for 10 to 12 hr, paralleling radicle extrusion. Endoglucanase (carboxymethylcellulase) activity and endosperm weakening do not occur significantly prior to loss of endosperm osmotic integrity. This might be expected if endosperm degradation allows a weakening

of a mechanical restraint but not if endosperm degradation allows the diffusion of large molecules out of the seed, lowering the osmotic pressure in the seed (extra embryonic fluid) and allowing water uptake by the radicle.

Cellular expansion and compression in nongerminating (thermodormant) seeds have been reported and may be due to the mechanical restraint of the endosperm (Foard and Haber, 1966). It has also been reported that sodium 2,4 dichloroisocyanuric acid (iC) inhibits the rupturing of the endosperm by an elongating embryonic axis (Pavlista and Haber, 1970) (Table 13). This compound, iC, is a chlorine-releasing compound (Lindner, 1961) of the s-triazine family which acts as selective preemergence herbicides (Pillai and Davis, 1973; Flater et al., 1974) and has been reported to increase activity of some enzymes in pea and sweet corn leaves (Wu et al., 1971). The endosperms of iC-treated seeds do not have signs of degradation (Figure 59). Furthermore, endoglucanase activity was not detected in low salt extracts of 9 hr imbibed seeds, although activity is unaffected in high salt extracts (Figure 58). The low salt extracted endoglucanase activities appear in time in seeds from the 1974 batch as in seeds from the 1971 batch; there is significantly less high salt extracted activity in the former seed batch but this activity does not change during the pregermination period (unreported data). These findings further indicate an involvement of low salt extracted endoglucanase activity and the noninvolvement of high salt extracted enzymatic activity during the processes

in the pregermination period.

The data presented here agreeing with the reports of Park and Chen (1974) and Jones (1974) demonstrates that the endosperm is degraded and endoglucanase may be responsible for this action. In addition, it is suggested that, due to the time of appearance of endoglucanase activity and of endosperm degradation (Figure 51), and the effects of light and temperature treatments (Table 5, 6, 12), an endoglucanase-mediated weakening of the endosperm at the micropylar region may be a prerequisite for normal germination. This suggestion is, therefore, supported by a) the effects of surgically induced changes in the endosperm (Ikuma and Thimann, 1963a), b) the observation of embryo expansion without protrusion induced by isocyanuric acid treatment, c) the occurrence of a peak in endoglucanase activity and d) the occurrence of a peak in endosperm degradation prior to germination.

Weakening of the endosperm may serve two purposes in lettuce seed germination. Endoglucanases, possibly in conjunction with other cell wall degrading enzymes, may remove the mechanical restraint of the endosperm, thereby facilitating radicle protrusion and inhibiting physical damage to the radicle's tip as it begins to expand. An additional role which endosperm weakening may have is to "signal" or "trigger" radicle elongation as may be speculated from the work on Striga lutea (Egley, 1972), species of Syringa (Juntilla, 1973a) and Stachys alpina (Pinfield et al., 1972). This is suggested by the induction of radicle protrusion by puncturing the endo-

sperm (Evenari and Neumann, 1952; Speer, 1974). Such a mechanism would require a "messenger" which would be produced and upon endosperm degradation would travel to the embryonic axis.

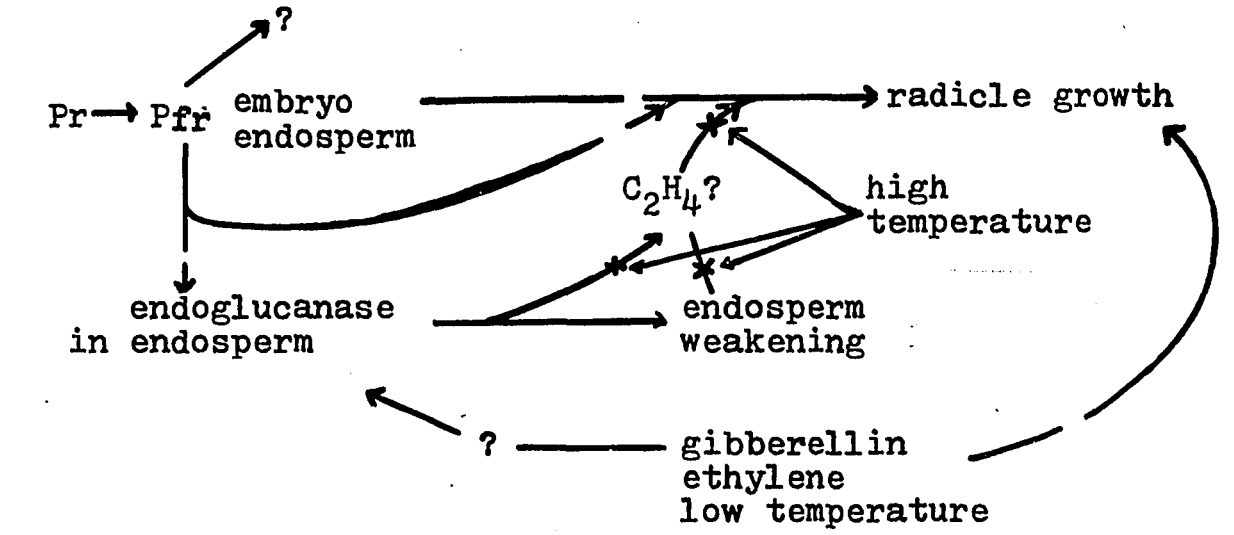
Ethylene may be such a "messenger" (Figure 60). Stewart and Freebairn (1969) reported that gibberellic acid stimulates ethylene synthesis and, in turn, lettuce seed germination. Using temperature-inhibited seeds which had been previously exposed to different light treatments and measuring ethylene released by ungerminating seeds separately from germinating ones, Abeles and Lonski (1969) suggested that germinating seeds produce ten-fold more ethylene that nongerminating seeds. Different light treatments did not seem to affect the results. They concluded that ethylene production is a result of germination. Burdett (1972c) did a time course study of ethylene production during the pregermination and germination periods of lettuce seeds. The reported ethylene levels increase 6 hr prior to germination at 20°C and the endogenous levels of ethylene appear to be sufficient to promote germination. Esashi and Leopold (1969) reported similar results with subterranean clover as the experimental plant material. At higher temperatures (30°C), ethylene production is inhibited and the level of ethylene measured does not appear until germination occurs. Burdett (1972b, c) hypothesized that endogenous ethylene is involved in the germination process and that the inhibition of germination by higher temperatures is due to an inhibition of ethylene production. This hypothesis

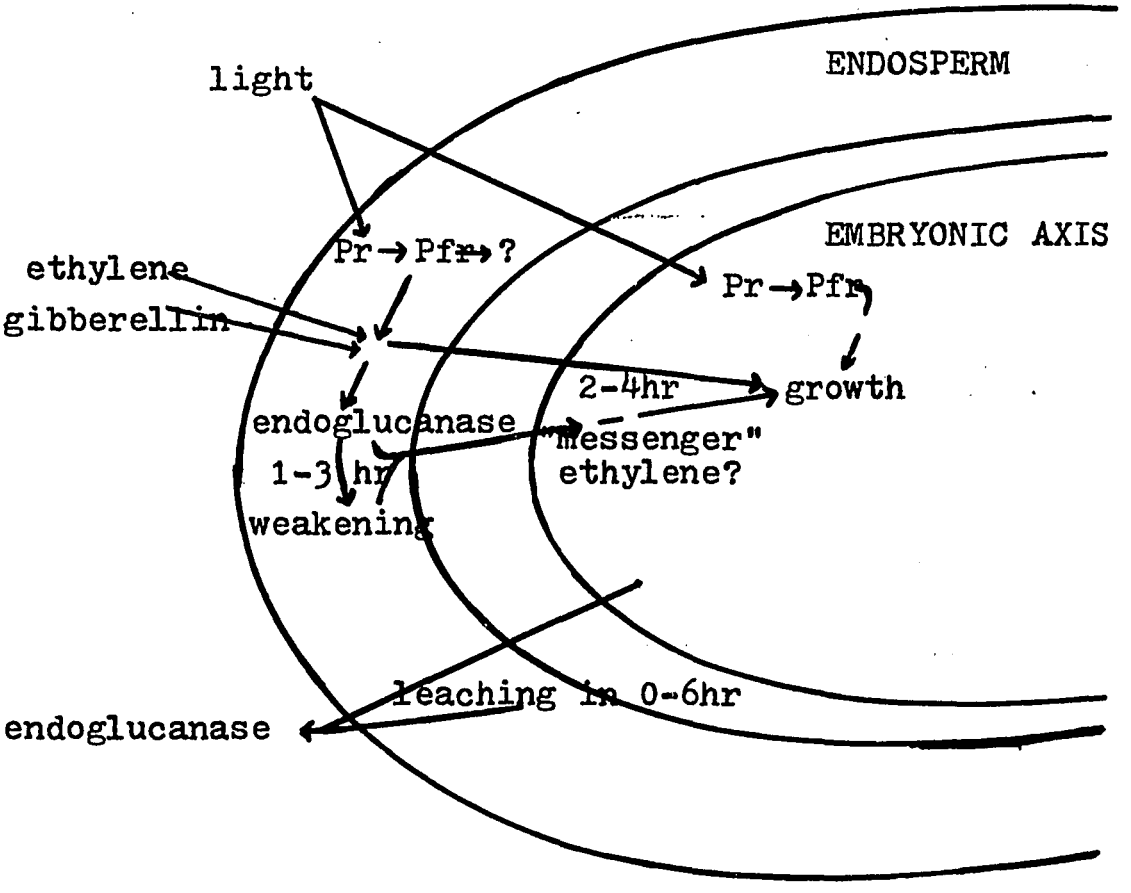
is further supported by studies on thermodormancy with the additional finding that endogenous carbon dioxide assists and is necessary for ethylene-stimulated germination (Negm et al., 1972; Negm et al., 1973; Keys et al., 1975).

Not only does exogenously applied ethylene induce germination, but also endogenous concentration of ethylene is sufficient for stimulation and arises within the seed during the latter part of the pregermination period (Burdett, 1972b, c). Furthermore, ethylene has been reported to be a "wounding" hormone, released as a result of cell damage, e.g., in fruit abscission (Cooper et al., 1969; Rasmussen, 1973). A model is proposed (Figure 61) which takes into account this "triggering" mechanism and the effects of light, temperature and some growth regulators. There is some progress in elucidating the steps, respiratory/energy, during hydration (Gesundheit and Poljakoff-Mayer, 1962; Evenari, 1965; Mayer, 1973; Mayer and Shain, 1974) and the steps, endoglucanase/endosperm weakening, near the end of the pregermination period (reported in this thesis). However, it is further noted that the steps between germination induction by temperature and light and endoglucanase activity are unknown (Figure 60b) and the role of ethylene is speculative.

Figure 61. Proposed model for lettuce seed germination involving endoglucanase activity and endosperm weakening.

Figure 61


?
Pr → Pfr
embryo
endosperm
radicle growth
C_2H_4?
high
temperature
endoglucanase
in endosperm
endosperm
weakening
?
gibberellin
ethylene
low temperature


light
ENDOSPERM
EMBRYONIC AXIS
Pr → Pfr → ?
Pr → Pfr
ethylene
gibberellin
growth
2-4hr
endoglucanase
"messenger"
ethylene?
1-3 hr
weakening
leaching in 0-6hr
endoglucanase

SUMMARY AND CONCLUSION

These studies indicate that endoglucanase activity and endosperm weakening precede embryo elongation and are correlated with seed germination. Endoglucanase activity peaks prior to the peak percentage of seeds with SEM-observable endosperm weakening and both these peaks occur prior to the peak in the rate of germination. Treatment of seeds with ribonucleic acid and protein synthesis inhibitors indicate that endoglucanases were synthesized de novo during the imbibition period. Extracts of endoglucanase activities were chromatographed in order to determine the presence of different enzymes. Five endoglucanases obtained from low and high salt extracts were characterized with respect to molecular weight, subunit composition and isoelectric point and these enzymes were related to bulk activity extracted from seeds imbibed for different times. Activity of these endoglucanases as well as bulk activity was localized in 0-1 and 9-10 hr imbibed seeds by extracting endoglucanases from surgically separated seed parts. Activities were predominantly in the radicle end and all activities (low salt extract) were in the endosperm of the radicle end (surrounding the embryonic axis) of 9-10 hr imbibed seeds. This suggests that endoglucanases

are synthesized de novo in the endosperm between 5½ and 9 hr from the start of imbibition. Both endoglucanase activities and SEM-observable weakening of the endosperm were inhibited by exposure to continuous darkness, far red light treatment and higher temperatures of 24 and 35°C. These affects were correlated with the inhibition of germination by these treatments. Furthermore, embryo elongation was separated from endosperm rupture by treatment of seeds with isocyanurate. Isocyanurate-treated seeds did not have either endoglucanase activity or endosperm weakening. Isocyanurate by inhibiting endoglucanase activity may inhibit the weakening of the endosperm and, since the embryonic axis elongates, this may result in buckling of the embryo within the endosperm sack.

The results summarized above suggest that germination is correlated with and preceded by endoglucanases, which, possibly in conjunction with other cell wall degrading enzymes, weaken the endosperm surrounding the embryonic axis. By this action, the mechanical restraint imposed by the endosperm against the embryo is lessened. This weakening of the endosperm layer, thereby, facilitates the ability of the elongating embryo to protrude through the endosperm at the radicle end, resulting in normal or typical germination.

APPENDIX A

PROCEDURES

Schedule I. Solutions for Extraction and Assay

1. Phosphate buffer, pH 5.9, 0.05 M

0.1 M K_2HPO_4	10 ml
0.1 M KH_2PO_4	90 ml
distilled H_2O	100 ml

2. Homogenizing Solution

   0.025 M sodium meta bi sulfite ($Na_2S_2O_5$) (phenolase inhibitor) added 1 hr prior to use to

   0.003 M (0.02%) sodium azide (NaN_3) (anti-microbial agent) dissolved in 0.05 M potassium phosphate buffer, pH 5.9.

3. Salt Fortified Homogenizing Solution

   1 M NaCl was added to homogenizing solution 1 hr prior to use.

4. Substrate Solution

   1.5% carboxymethylcellulose (CMC), 7 HS and 7H (Hercules Powder) dissolved with

   0.003 M (0.02%) NaN_3 in

   0.05 M potassium phosphate buffer, pH 5.9

   CMC was dissolved with a magnetic stirrer for 2 hr at room temperature

Final concentrations after mixing 1 part substrate solution and 1 part enzyme extract for assay are: 0.75% CMC, 0.003 M NaN_3, 0.05 M potassium phosphate buffer (pH 5.9) (salt fortified) 0.5 M NaCl.

Schedule II. Protein Determination (following Goa, 1953)

1. 3% sodium hydroxide (NaOH)
2. 11% trichloracetic acid (TCA)
3. Benedict's reagent:
   a) dissolve sodium citrate for a final concentration of 17.3% and sodium carbonate for a final concentration of 10% by heating without boiling; the solution is filtered warm.
   b) dissolve cupric sulfate ($\cdot 5H_2O$) for a final concentration of 1.73% by heating
   c) solutions a) and b) mixed in volumetric flask, cooled and filled to volume with distilled water; Benedict's solution was kept in a stoppered dark bottle.
4. Protein standard
   a) bovine serum albumin (BSA) was prepared in concentrations of 2.5, 2.0, 1.5 and 1.0 mg/ml
   b) one ml of each BSA solution was treated as described in 5 (following).
5. Procedure:
   a) one ml of protein solution (1 part extract: 1 part buffer) was mixed with 4 ml of Reagent 2.
   b) after letting a) stand for 10 min at room temperature

it was centrifuged at 2500 r/min for 15 min.

c) supernatant was drained and the pellet (TCA-percipitate) redissolved in 4 ml of Reagent 1.

d) 0.2 ml of Reagent 3 was added to c) and blank was prepared.

e) after 20 to 40 min at room temperature, optical density was measured at 330 nm with a Spectronic 20 (Bausch-Lomb).

f) protein content was calculated from standard curve obtained using bovine serum albumin (4).

Schedule III. Column Chromatography

1. Column dimensions - diameter = 2.5 cm, length = 45 cm
2. Bed height = 40.5 cm
3. Bed volume = 200 ml
4. Flow rate (Sephadex G-100) = 7.5 drops/min = 4 ml/cm^2 hr
5. Operating pressure (G-100) = 25-30 cm H_2O

   Operating pressure (G-200) = 10-15 cm H_2O

   Operating pressure was maintained using a Marriott flask made with a column reservoir (R25-Pharmacia)
6. Void volume was determined with blue bextran 2000 (1 mg/ml) and was about 50 to 52.5 ml.
7. Gel swelling:

   G-100; 16 gm were swelled in 300 ml phosphate buffer, pH 5.9 (Schedule I) plus 0.003 M (0.02%) NaN_3 for 5 hr in boiling water bath and 2-3 dy at room temperature

   G-200; 8 gm were swelled as above.

8. Column packing:

Column was leveled and after gel swelling, gel was poured into column with a long glass stirring rod. Sample applicator or flow adaptor as well as a Marriott flask were attached to column and entire assembly was placed in cold room at 8°C. Fractions were eluted with 0.05 M potassium phosphate buffer, pH 5.9, plus 0.003 M sodium azide.

Schedule IV. Molecular Weight Standards

1. Aldolase (158,000 Daltons) 100 mg (20-25% protein: 75-80% sucrose)

   Chymotrypsinogen A (25,000 D) 20 ml

   Sucrose 120 mg

   2 ml of eluant solution (Schedule III)

2. Ovalbumin (45,000 D) 20 mg

   Ribonuclease A (16,700 D) 20 mg

   Sucrose 200 mg

   2 ml of eluant solution

3. Blue Dextran 2000 ($2X10^6$ D) 2 mg

   2 ml of eluant solution

4. Bovine Serum Albumin (67,000 D) 20 mg

   Sucrose 200 mg

   2 ml of eluant solution

5. Partition Coefficients (Kav)

   $Kav = (Ve-Vo)/(Va-Vo)$

   Ve = elution tube for each protein

   Vo = void volume (tube) determined with blue Dextran 2000

   Va = bed volume (tubes)

Schedule V. Poly-Acrylamide Gel Electrophoresis

1. 1N HCl 24 ml
   Tris 18.1 gm
   Temed 0.12 ml dilute to 100 ml with distilled water
2. 1N HCl 48 ml
   Tris 5.98 gm
   Temed 0.46 ml dilute to 100 ml with distilled water
3. Acrylamide 28 gm
   Bis 0.735 gm dilute to 100 ml with distilled water
4. Acrylamide 20 gm
   Bis 5 gm dilute to 100 ml with distilled water
5. Riboflavin 4 gm in 100 ml distilled water
6. Sucrose 40 gm in 100 ml distilled water
7. Ammonium Persulfate 0.14 gm in 100 ml distilled water
8. All solutions were stored in dark bottles at 4°C.
   Solutions 6 and 7 were made on same day as used.
9. Separating Gel:
   1 part 1: 1 part 3: 2 parts 7
10. Rinsing solution:
    1 part 2: 1 part 5: 6 parts distilled water
11. Stacking Gel:
    1 part 2: 1 part 4: 1 part 5: 4 parts 6: 1 part distilled water
12. Gel tube:
    Separating gel = 5 cm topped with
    stacking gel = 1 cm
    rinsing solution used to wash tube after separating gel

hardens and prior to addition of stacking gel.

13. Tracking dyes

a) Bromcresol green 0.05% in distilled water
used at pHs below 7: 1 drop added atop each tube

b) Bromphenol blue 0.005% in distilled water
used at pHs above 8: 4 drops added to upper chamber of apparatus.

14. Citrate-Phosphate Buffer

I-0.1 M citric acid II-0.2 M dibasic sodium phosphate all solutions diluted to 100 ml with distilled water and titrated to proper pH when necessary

a) pH 4.0 : 30.7 ml I 19.3 ml II
b) pH 4.2 : 29.4 " 20.6 "
c) pH 4.3 : 28.6 " 21.4 "
d) pH 4.4 : 27.8 " 22.2 "
e) pH 4.5 : 27.25 " 22.75 "
f) pH 4.6 : 26.7 " 23.3 "
g) pH 4.8 : 25.2 " 24.8 "
h) pH 5.0 : 24.3 " 25.7 "
i) pH 5.1 : 23.8 " 26.2 "
j) pH 5.2 : 23.3 " 26.7 "

15. Phosphate Buffer

I-0.2 M monobasic sodium phosphate II-0.2 M dibasic sodium phosphate all solutions diluted to 400 ml and tritrated to proper pH

a) pH 6.0 : 87.7 ml I 12.3 ml II
b) pH 7.0 : 39.0 " 61.0 "

c) pH 8.0 : 5.3 ml I 94.7 ml II

16. Boric Acid-Borax Buffer

I - 0.2 M boric acid II - 0.05 M borax (0.2M sodium borate) all solutions diluted to 400 ml and titrated to proper pH

a) pH 9.0 : 50 ml I 59 ml II

Schedule VI. Scanning Electron Microscopy Solutions

1. Fixation

a) Cacodylate Buffer

I - 0.2 M sodium cacodylate II - 0.2 M HCl

pH 7.2 : 50 ml I 4.2 ml II

b) Gluteraldehyde Solution

6 ml of 50% gluteraldehyde solution was added to 44 ml of cacodylate buffer (a).

c) seeds were immersed in b) for 2 days at 4°C

d) after c), seeds were washed three times with cacodylate buffer (a) at 4°C.

e) after one to three days in (a), seeds were dissected and dehydrated.

2. Dehydration:

f) after dissection, seeds were placed in a wire basket

g) baskets (f) were placed in 10% ethanol for 10 min

h) after (g) baskets were placed for 5 min in each of: 20, 30, 40, 50, 60, 70, 80, 90 and 100% ethanol.

i) baskets were placed two additional times into 100% ethanol for 10 and 20 min respectively

j) baskets were placed in 10% Freon 113 for 10 min

k) after (j), baskets were placed for 5 min in each of: 20, 30, 40, 50, 60, 70, 80 and 90% Freon

l) baskets were placed three times into 100% Freon for 10 min, 20 min and overnight respectively

m) after above dehydration, baskets with seeds were placed into a critical point dryer (Technics), washed for 5 min with Freon 13 and critical point dried with Freon 13.

n) after critical point drying, seeds were placed in Petri dishes and let stand for one to three days at room temperature in air before prepared for viewing with SEM.

Schedule VII. Standard Error (Kruyt, 1954; Machlis and Torrey, 1956; Sokol and Rohlf, 1969)

1. arithmetic mean -- $\bar{x}$
2. individual deviation from the mean -- $/Xi-\bar{x}/$

   Xi = determination
3. square of 2 -- $/Xi-\bar{x}/^2$
4. sum of all 3s -- $\sum/Xi-\bar{x}/^2$
5. divide 4 by the number of determinations (N) less than one -- $\dfrac{\sum/Xi-\bar{x}/^2}{N-1}$
6. square root of 5 (standard deviation)

$$\sqrt{\frac{\sum/Xi-\bar{x}/^2}{N-1}} = SD$$

7. standard error: divide 6 (standard deviation) by square root of N -- $\frac{\sqrt{\frac{\Sigma/Xi-\bar{x}/^2}{N-1}}}{\sqrt{N}} = S.E.$

8. Standard error was calculated from three to ten determinations for measurement, and was represented by vertical bars superimposed on individual measurements.

APPENDIX B

MODELS AND SCHEMES

ABBREVIATIONS:

A = abscisic acid

act = active form

C = cotyledon

D = darkness

EA = embryonic axis

EN = endosperm

fr = far red light exposure

G = gibberellin

inact = inactive form

K = kinetin

L = light

N_2 = nitrogen

O_2 = oxyten

P = phytochrome

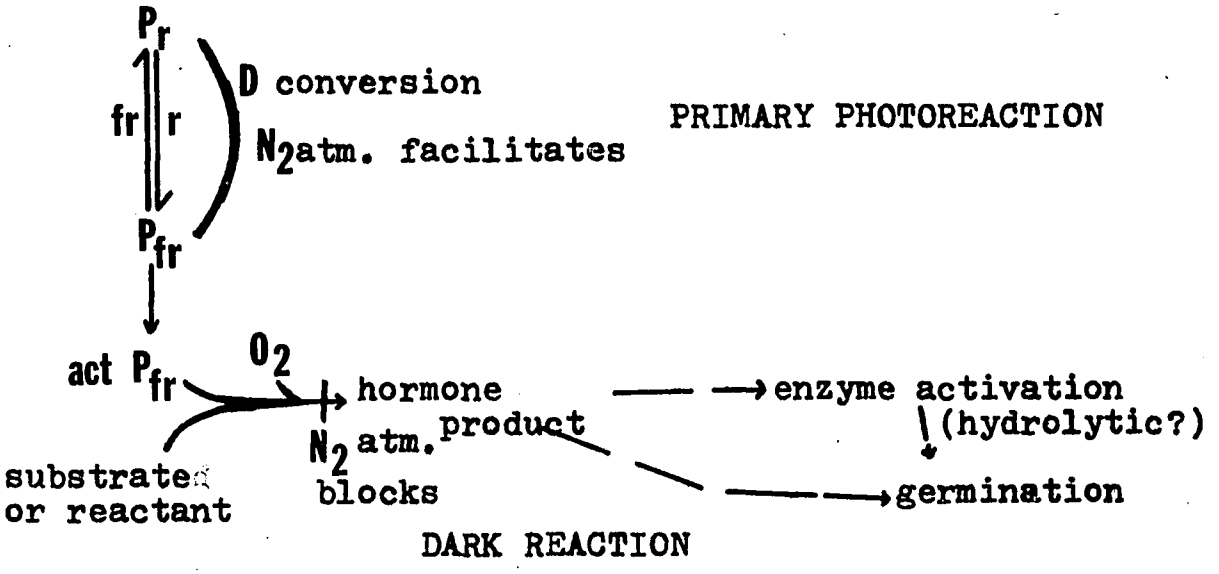
r = red light exposure

T = thiourea

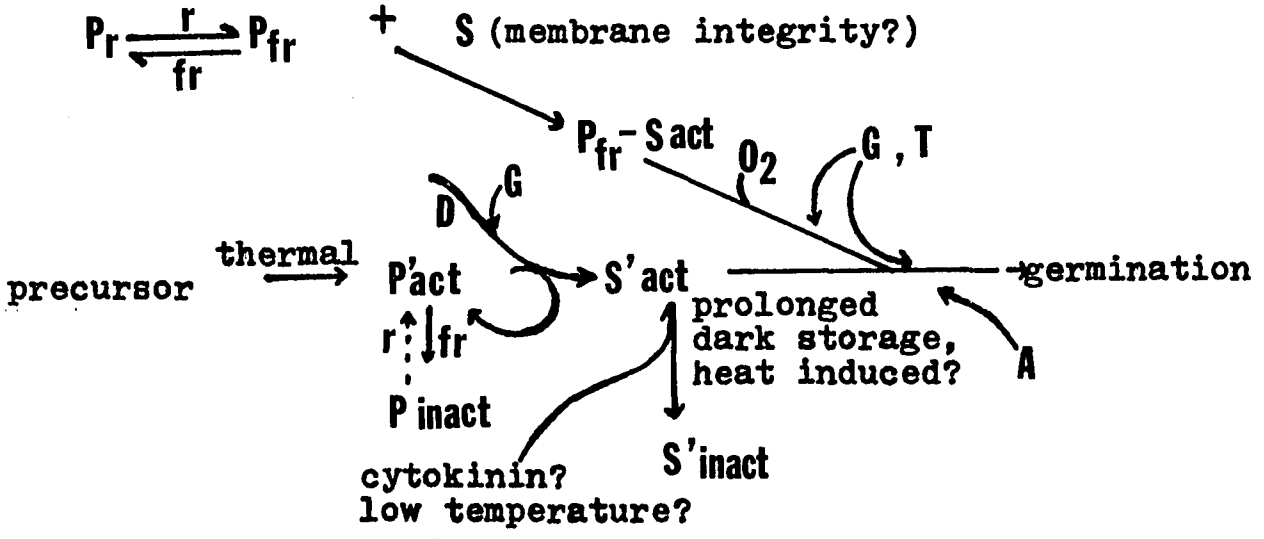
Z = zeatin

APPENDIX B

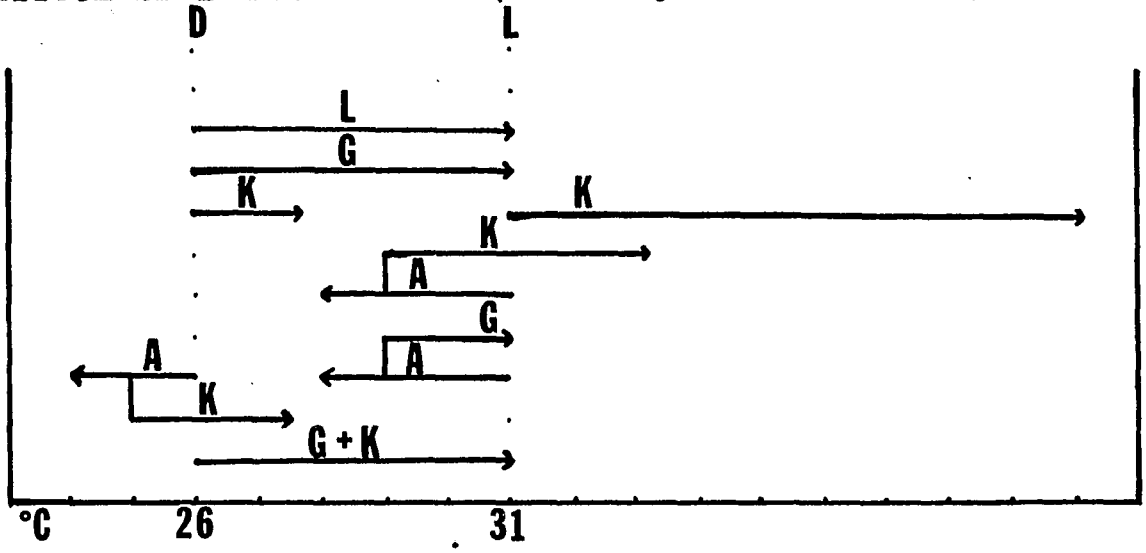
SCHEDULE I: PHYTOCHROME MODEL FOR LETTUCE SEED GERMINATION
(modified from Mancinelli and Borthwick, 1964 and
Ikuma and Thimann, 1964)



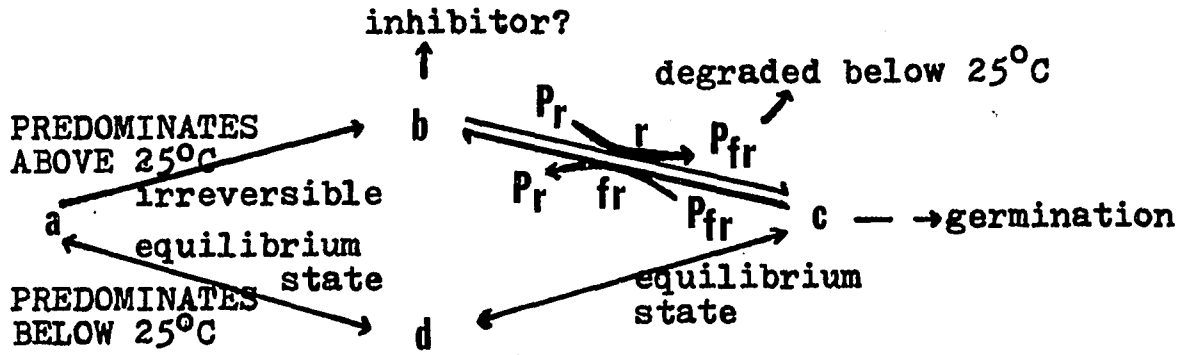
SCHEDULE II: TWO PIGMENT MODEL FOR LETTUCE SEED GERMINATION
(modified from Negbi et al., 1968 and Vidaver and Hsiao, 1974)



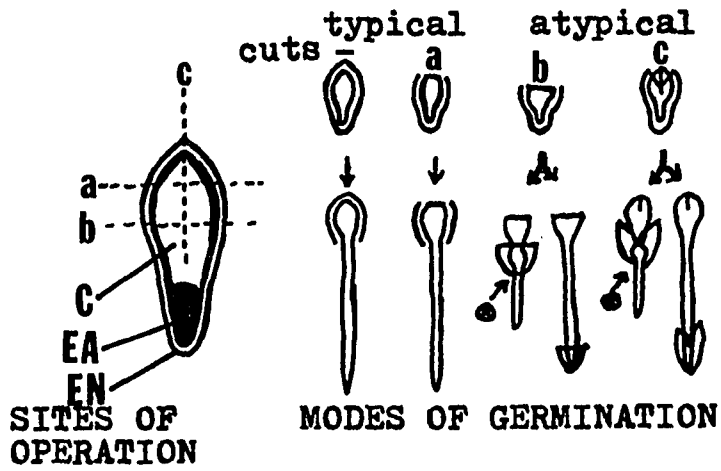
SCHEDULE III: HIGH TEMPERATURE CUT-OFF AND GROWTH REGULATORS AND LETTUCE SEED GERMINATION (from Reynolds and Thompson, 1973)



SCHEDULE IV: TEMPERATURE/LIGHT MODEL FOR LETTUCE SEED GERMINATION (modified from Berrie, 1966)



SCHEDULE V: SURGICAL CUTS AND LETTUCE SEED GERMINATION (from Ikuma and Thimann, 1963a)



⊛ note -- Radicle has also protruded through the endosperm.

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EXPLANATION OF FIGURES

Figures 40 through 50, 52 through 54 and 59 are scanning electron micrographs of the outer surface of the endosperm at the radicle end of lettuce seeds unless otherwise stated and are observations of seeds sown in distilled water at 20°C in continuous white light unless otherwise stated. Figures 56 and 57 are light micrographs.

AG---"atypically" germinated seed
B----break
BG---"buckling groove"
BS---"buckled" seed
C----crack
CO---cotyledon
D----degraded area
EN---endosperm
G----grain
HS---half seed
I----integument
L----large crack
N----net-like area
ND---nondegraded area
NG---"normally" germinated seed
NU---"normally" ungerminated seed
P----pit
PER--pericarp
PIN--"pinching"
R----radicle
W----cell wall (nondegraded)

Figure 40. Dry (0 hr imbibed) seeds showing nondegraded cell walls. a: X170, b: X560.

Figure 41. 3 hr imbibed seeds showing nondegraded cell walls. a: X180, b: X520.

Figure 40

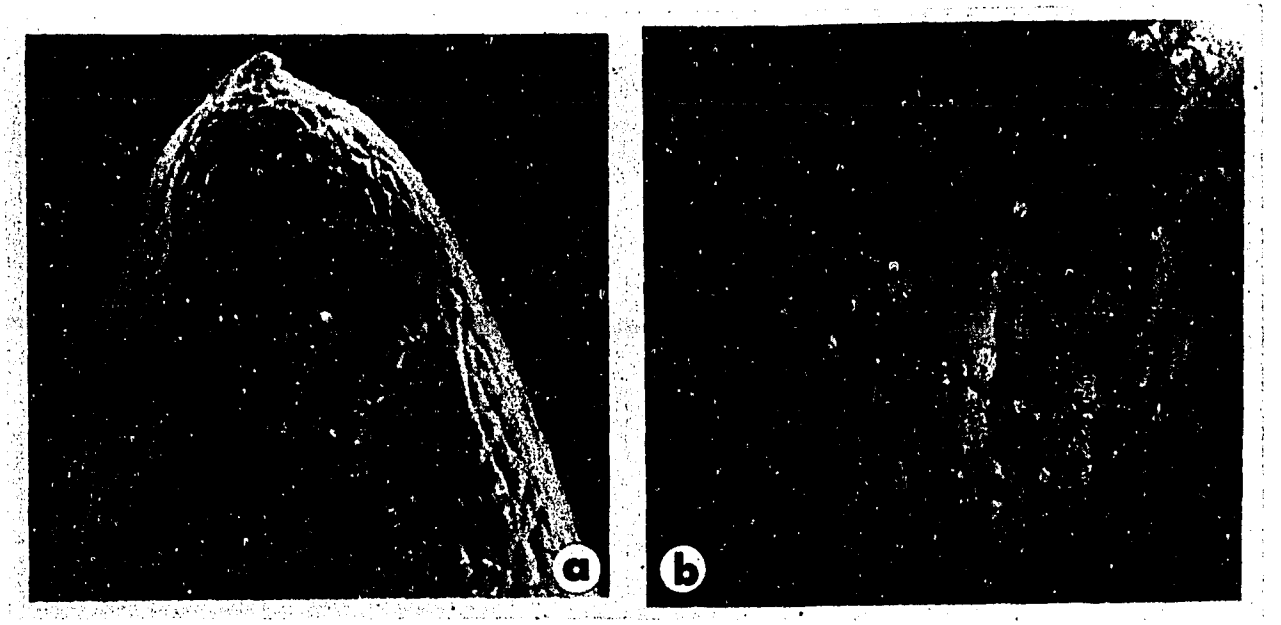


Figure 41

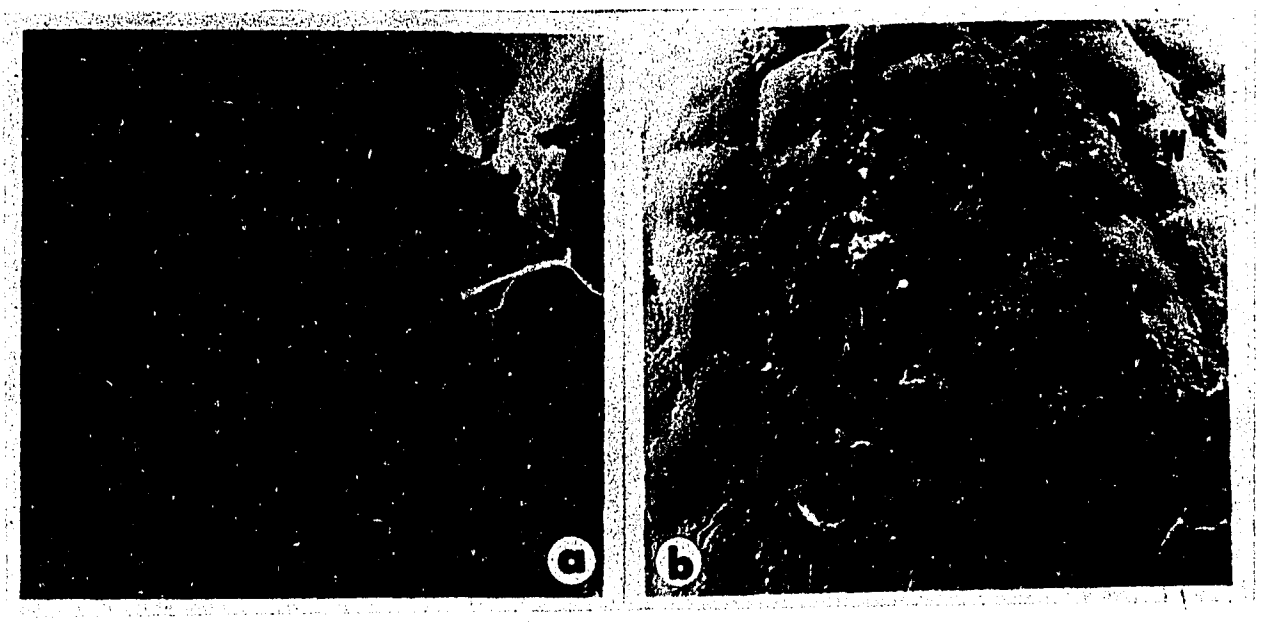


Figure 42. 6 hr imbibed seeds showing nondegraded cell walls. a: X120, b: X220, c: X600.

Figure 42

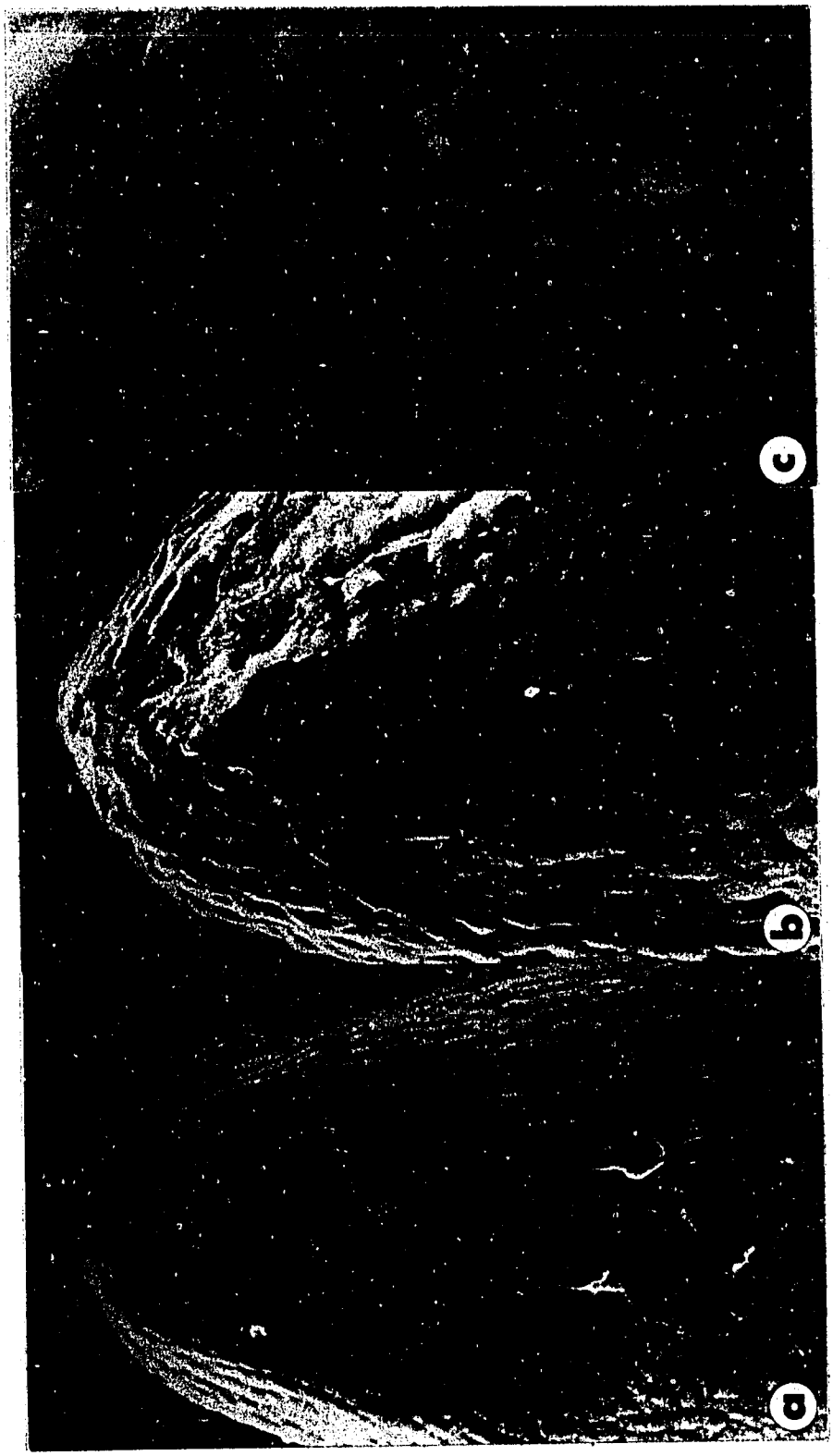


Figure 43. 8 hr imbibed seeds, note nondegraded (a, b, d) and degraded (a, c, d) areas. a: X180, b: X360, c: X340, d: X200.

Figure 43

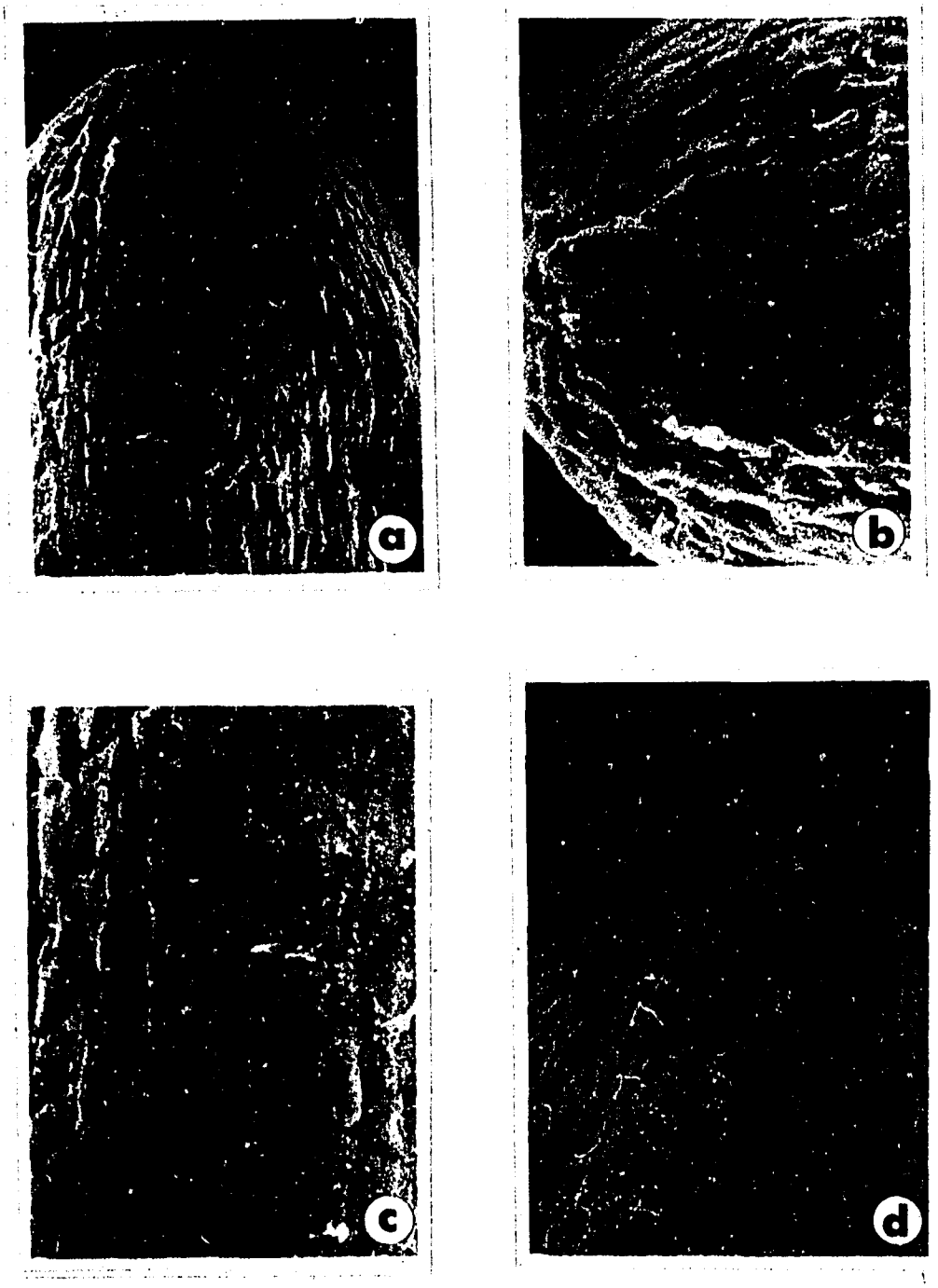


Figure 44. 9 hr imbibed seeds, note pits and cracks. a: X320, b: X530.

Figure 45. 10 hr imbibed seeds, note break (a), net-like area (b), pits and ripples along cell walls. a: X330, b: X500.

Figure 44

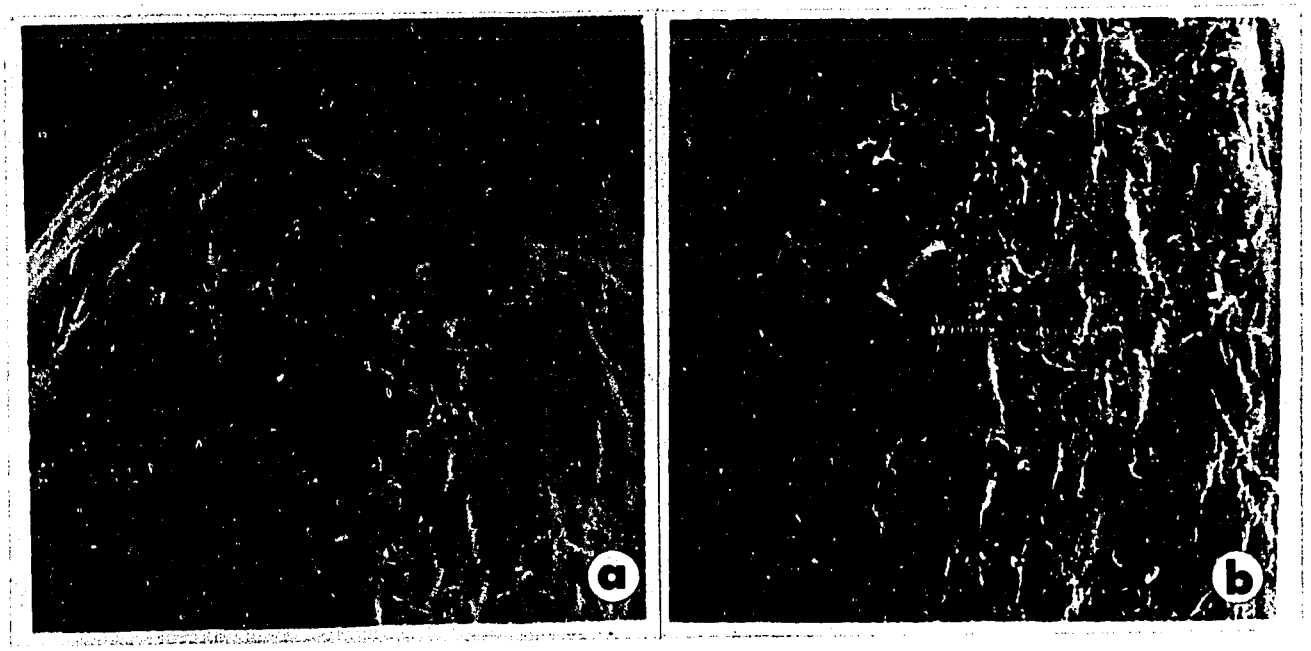


Figure 45

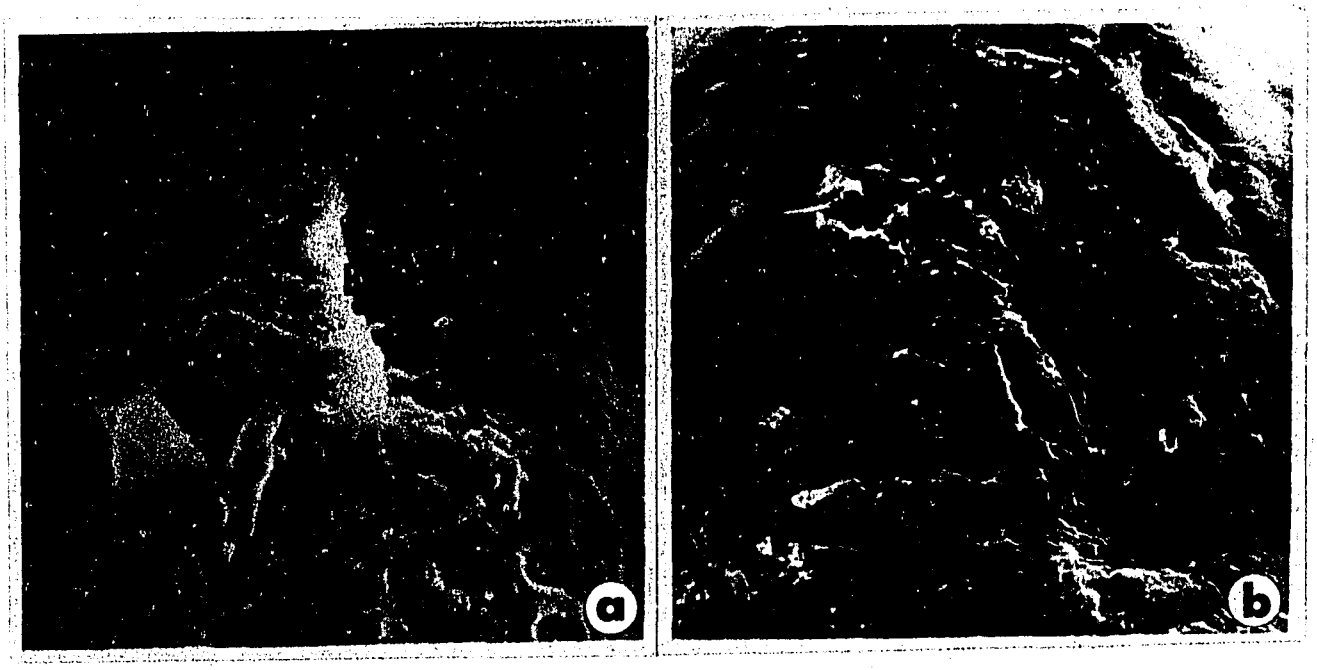


Figure 46. 12 hr imbibed seed, note collapsed area (a, b), large crack (a, c) and numerous pits and cracks (b, c, d). a: X200, b: X600, c: X530, d: X700.

Figure 46

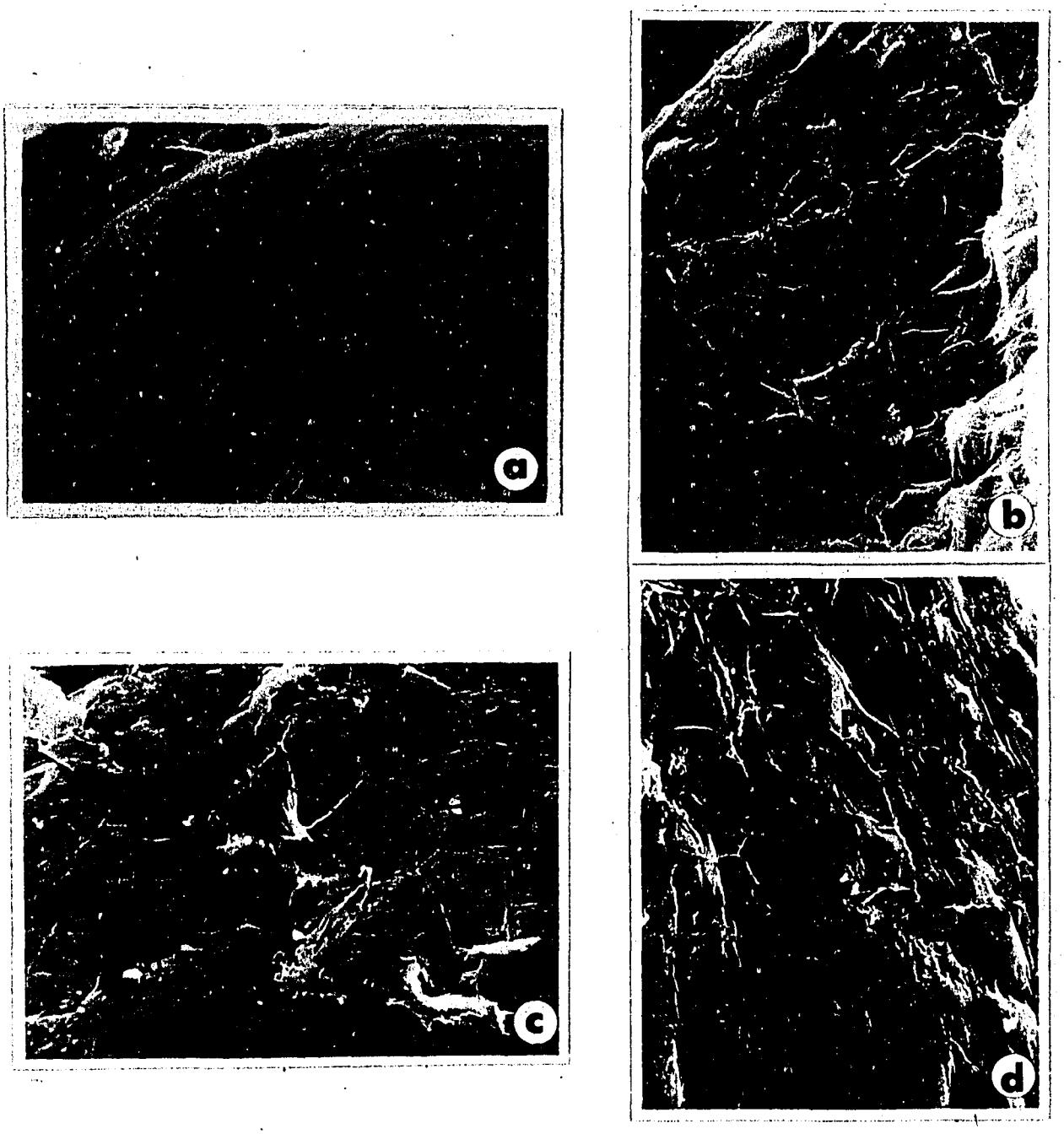
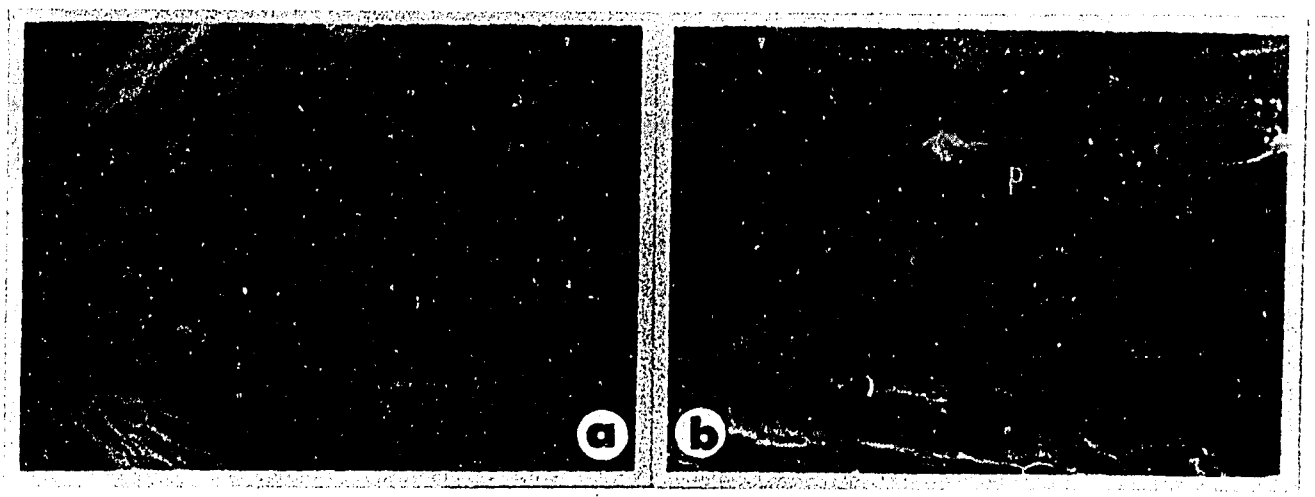


Figure 47. a, b, c: 12 hr imbibed seed, note net-like area (a) and numerous pits and cracks (a, b, c); d: 10 hr imbibed seed, note net-like area and pits. a: X270, b: X400, c: X700, d: X390.

Figure 47



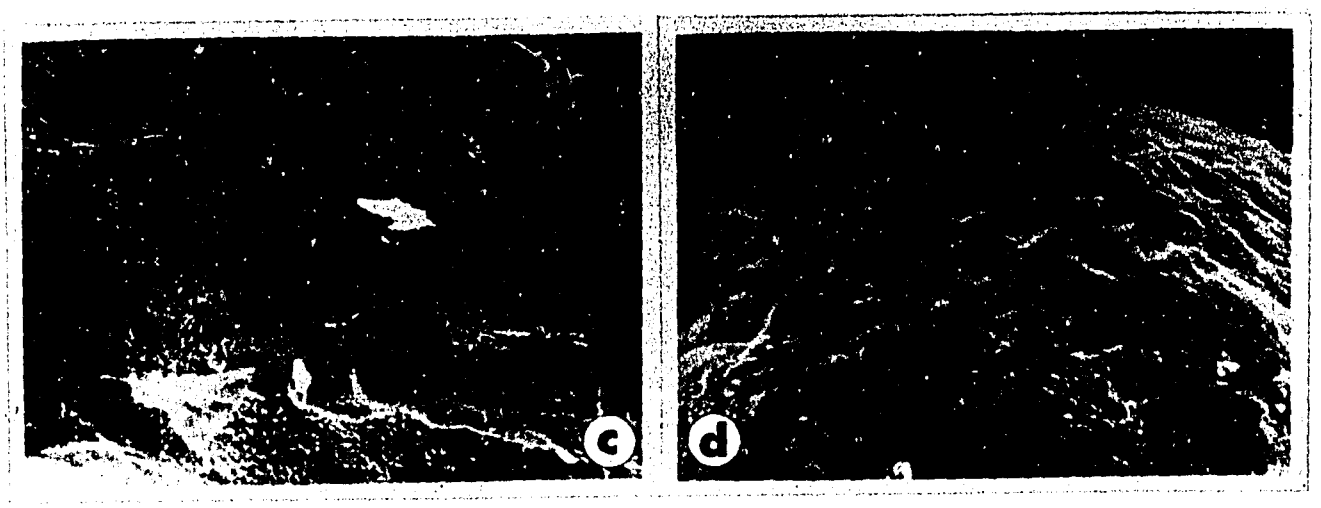


Figure 48. a: 12 hr imbibed seed, note break, b: 10 hr imbibed seed, c: 11 hr imbibed seed, d: 12 hr imbibed seed. Note open endosperm at radicle end. a: X170, b: X100, c: X100, d: X100.

Figure 48

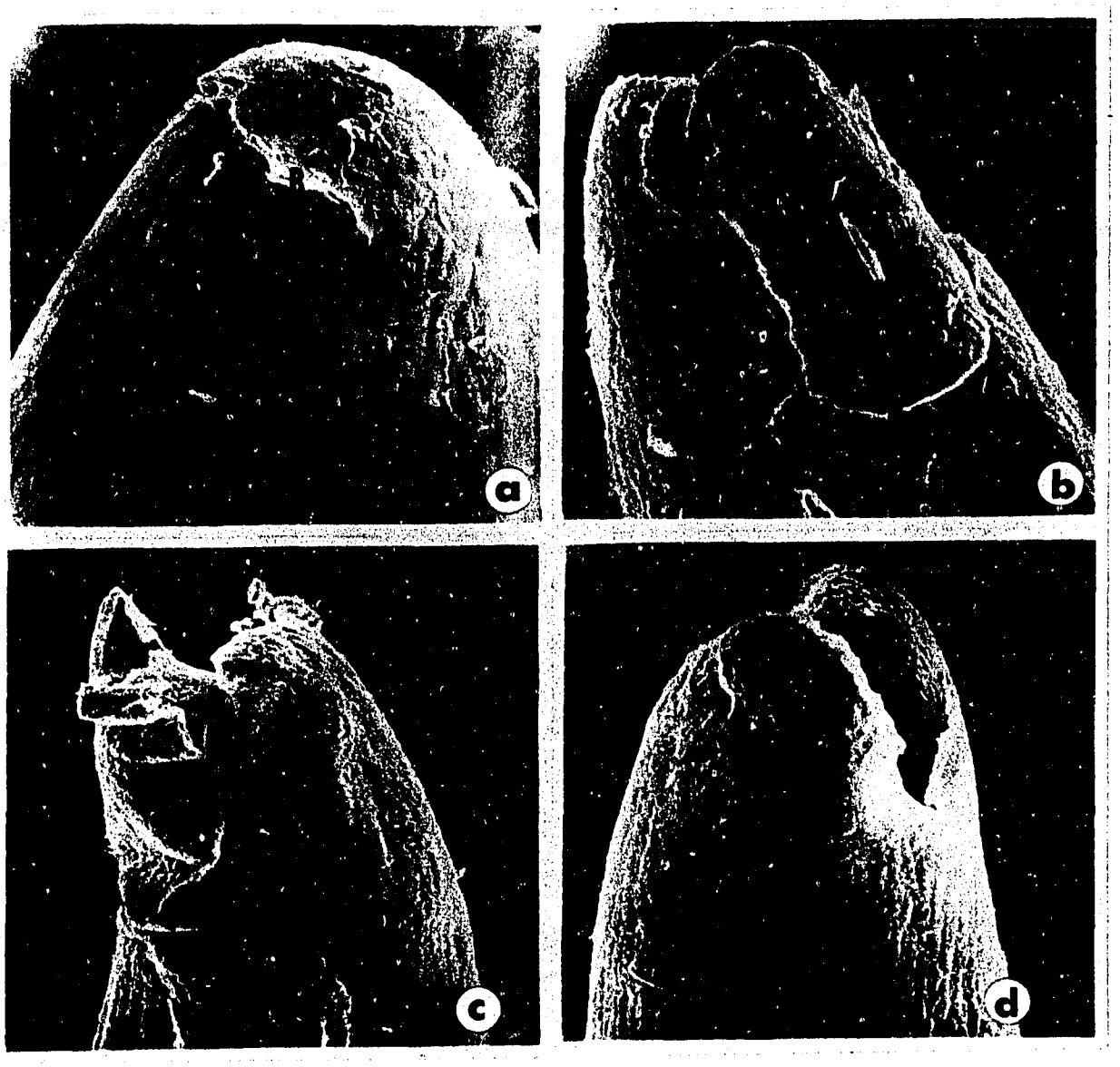


Figure 49. a: 12 hr imbibed seed, note large cracks and possible break or collapsed area, b: 12 hr imbibed seed, note break, c: 13 hr imbibed seed, note opening and net-like area. Note development of an opening in endosperm. d: mid section of an 11 hr imbibed seed (Figure 48c) which has an opening at tip. a: X160, b: X120, c: X160, d: X460.

Figure 49

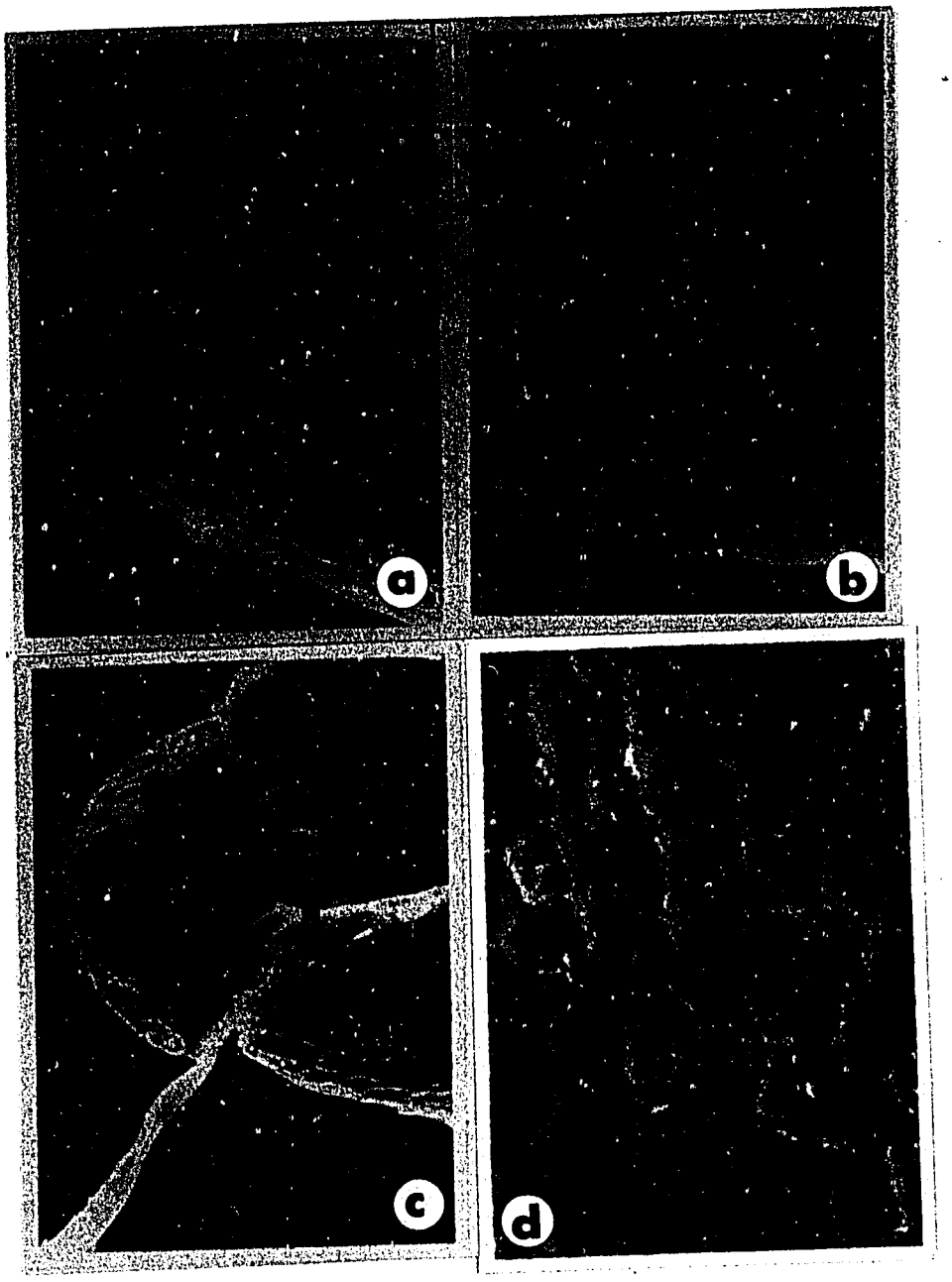


Figure 50: Scanning electron micrographs of inner side of endosperm flap of 12 hr imbibed sees with an opening; note breakup of cellular structure, grains in matrix and small section of cell wall. a: X1200, b: tilted 45°--approximately X1000, c: X1700, d: X3800 (closeup of c).

Figure 50

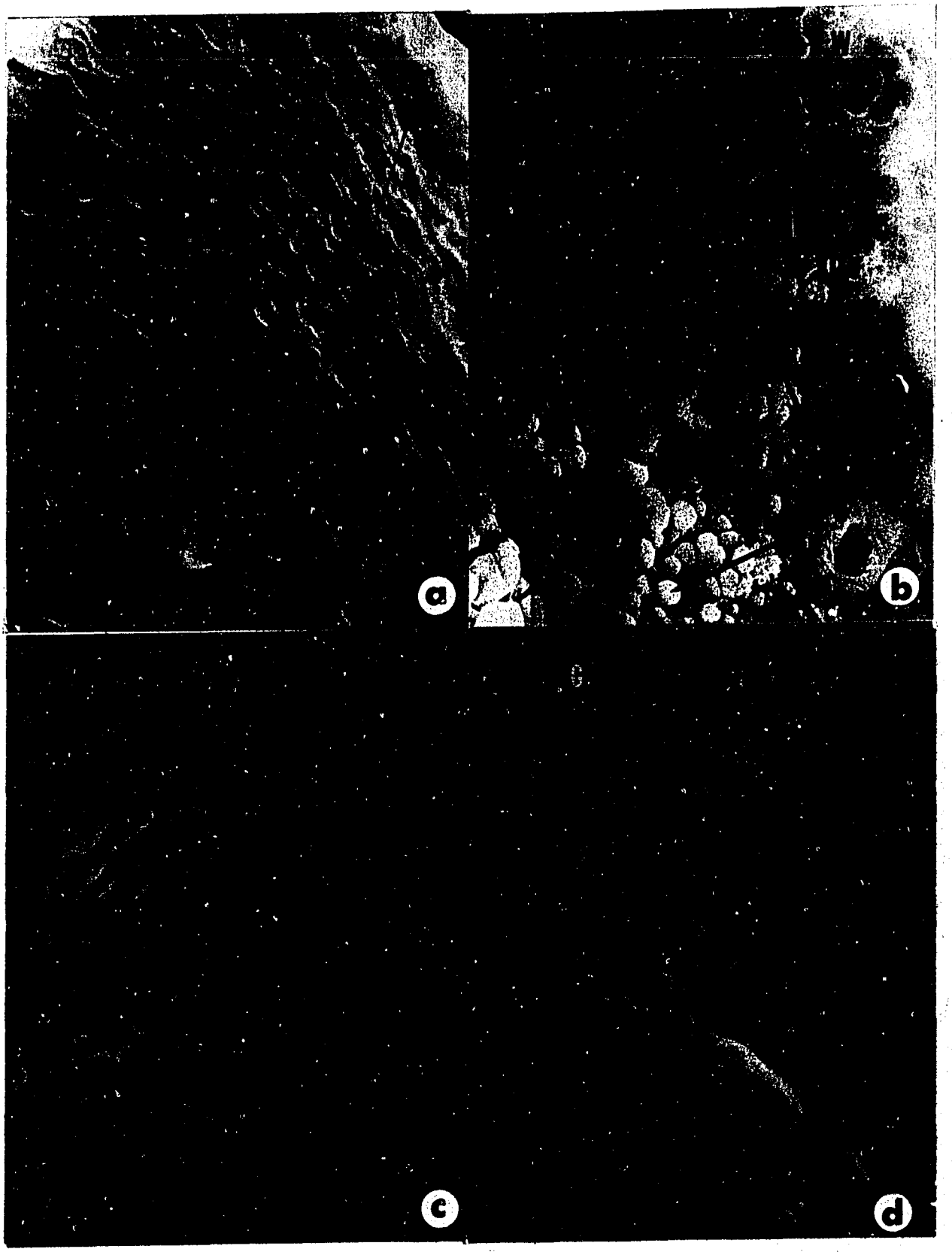


Figure 52. 12 hr imbibed seeds sown in darkness and exposed to far red light between the 3rd and 4th hr of imbibition. a: X340, b: X780.

Figure 52

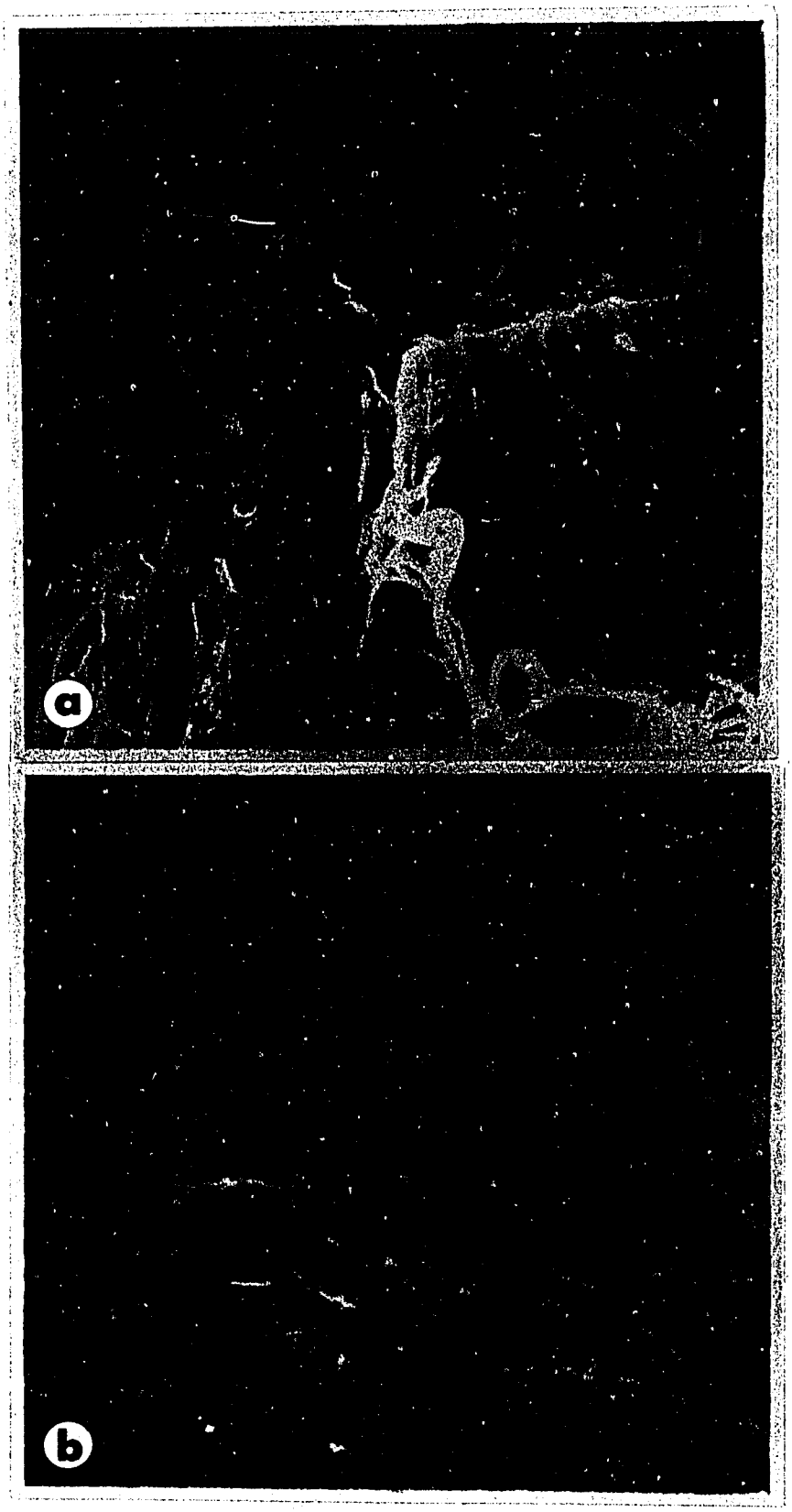


Figure 53. Effects of temperature (24°C) on surface appearance of endosperms of 12 hr imbibed seeds sown in continuous white light (a, b) or in continuous darkness (c). a: X210, b: X660, c: X200.

Figure 53

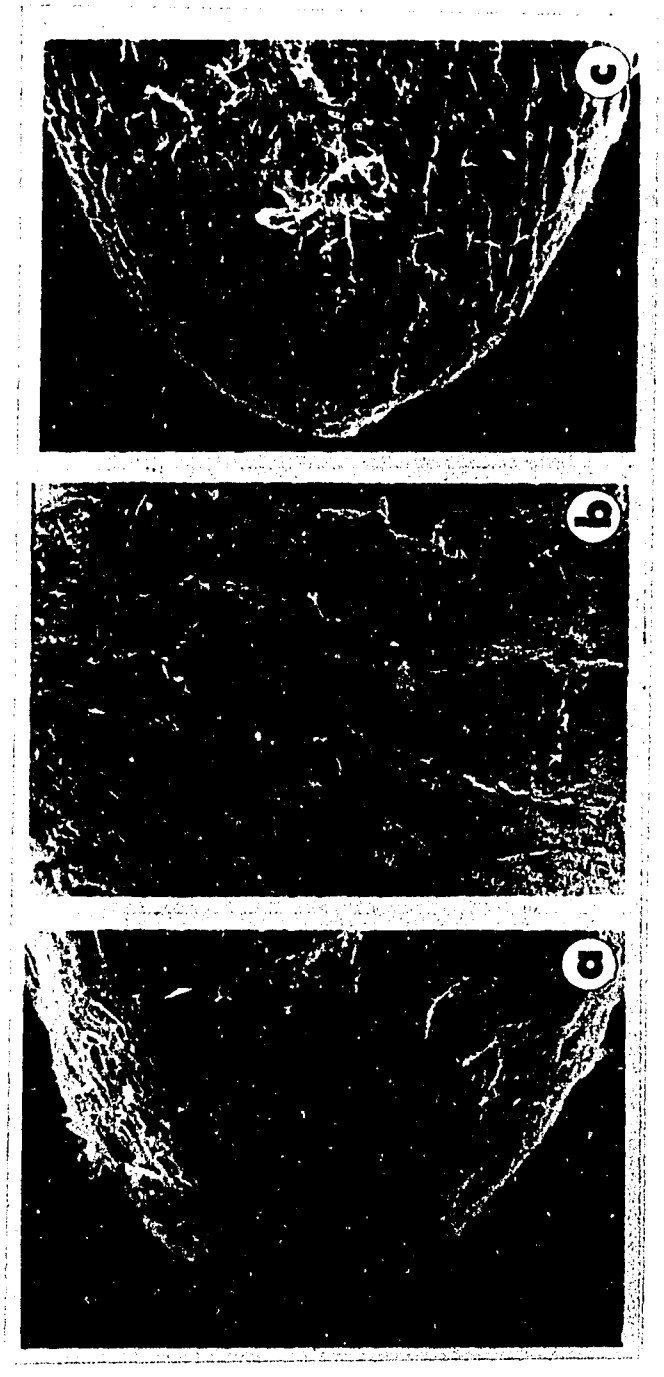


Figure 54. Effects of temperature (35°C) on surface appearance of endosperms of 12 hr imbibed seeds sown in continuous white light (a, b, c) or in continuous darkness (d). a: X280, b: X350, c: X410, d: X220.

Figure 54

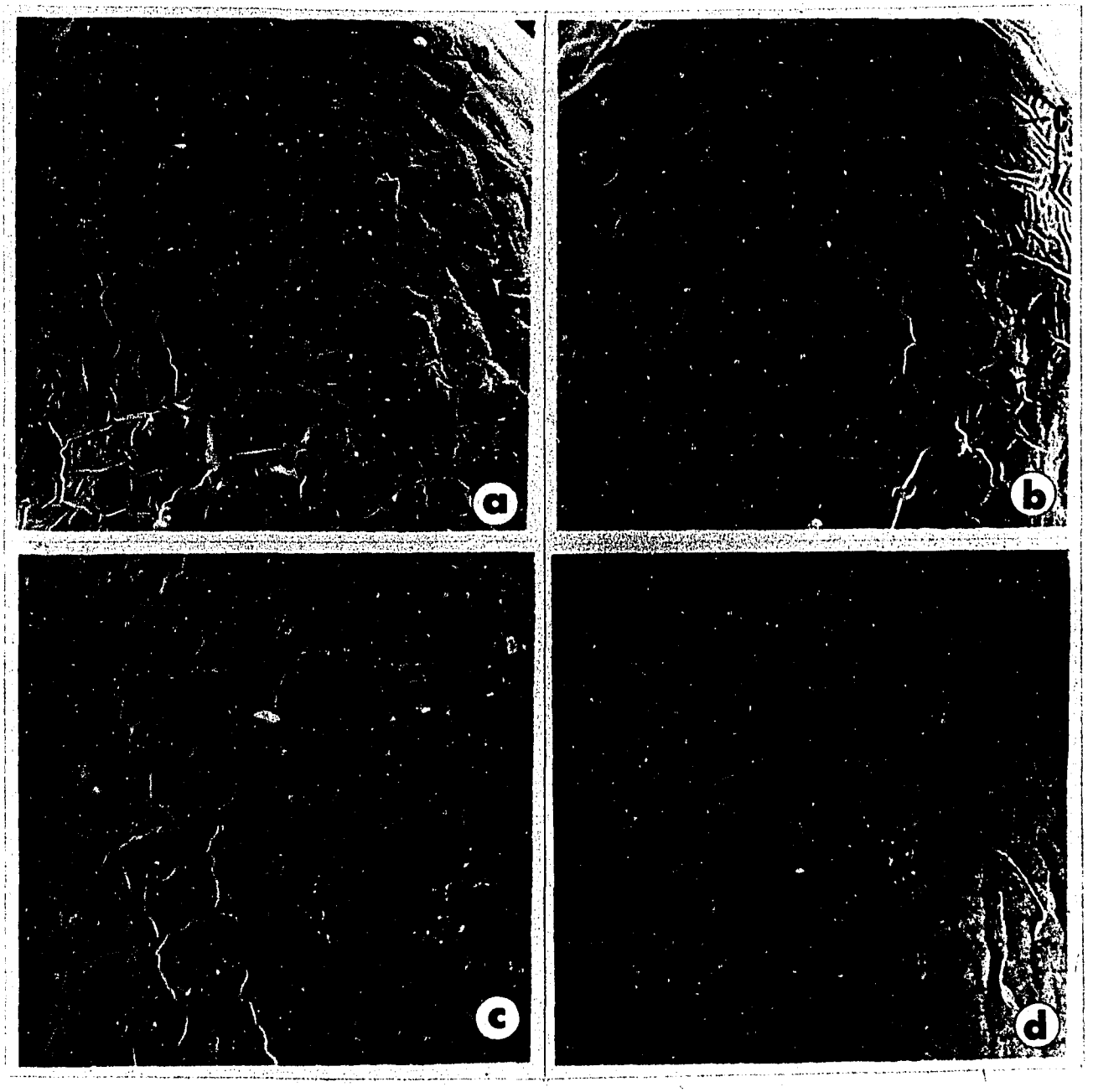


Figure 56. Light micrographs. a: Normal germinated and ungerminated lettuce seed. b: Normal germinated and ungerminated seeds, "buckled" seed sown in 5 mg/ml iC for 4 dy (pericarp removed for micrograph) and half seed sown in 5 mg/ml iC. c: Seeds sown in 5 mg/ml iC--green seedling with groove indicating the probability of having been "buckled," half seed and two "buckled" seeds. a: X15, b: X13, c: X13.

Figure 56

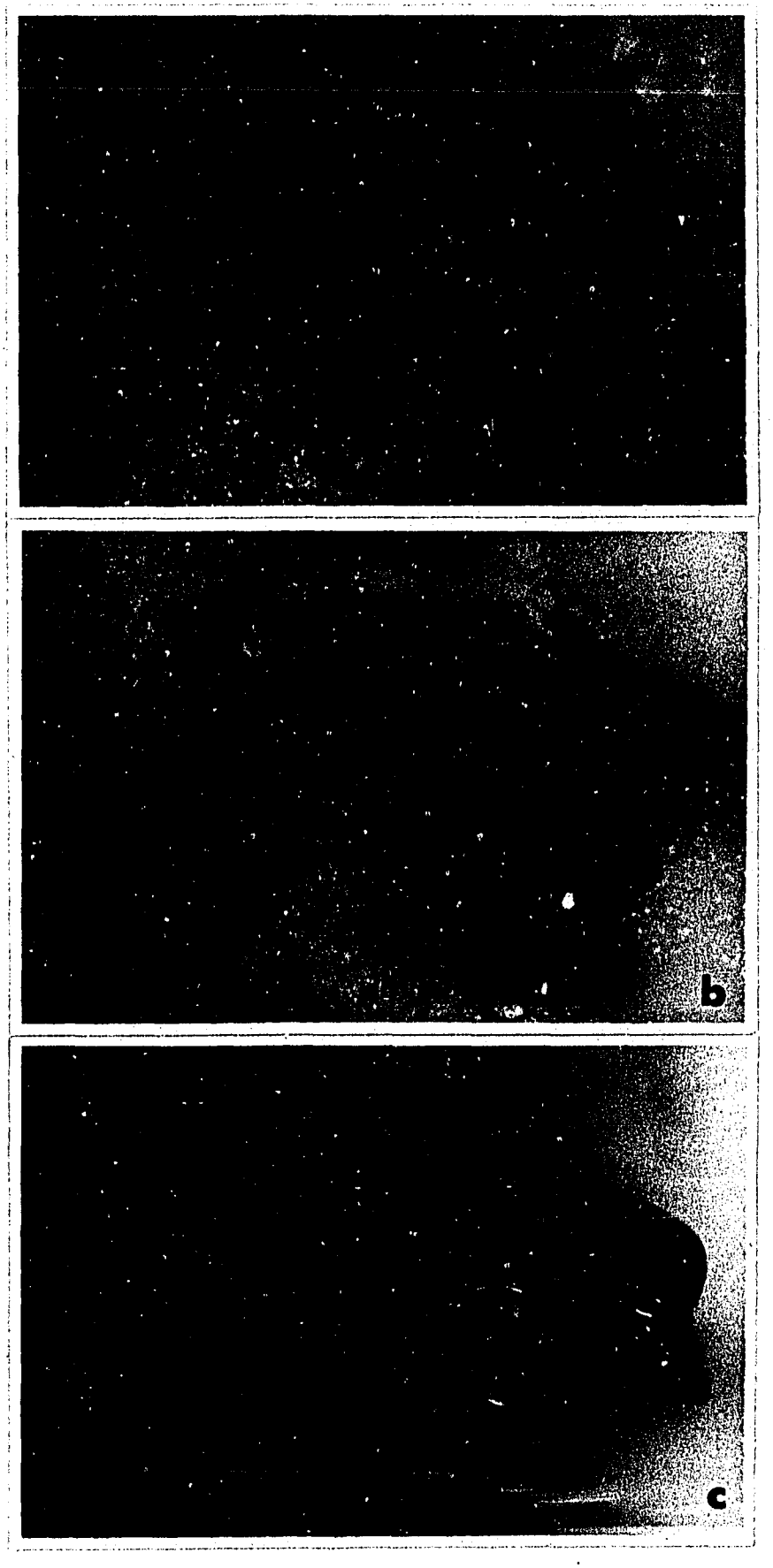


Figure 57. Seeds sown in 5 mg/ml iC for 4 dy show "buckled" seeds (a, b, c, d) and seeds atypically germinating through mid section (d, e) and through cotyledonary end (f). a: X11, b: X15, c: X23, d: 9.0, e: X15, f: X15.

Figure 57

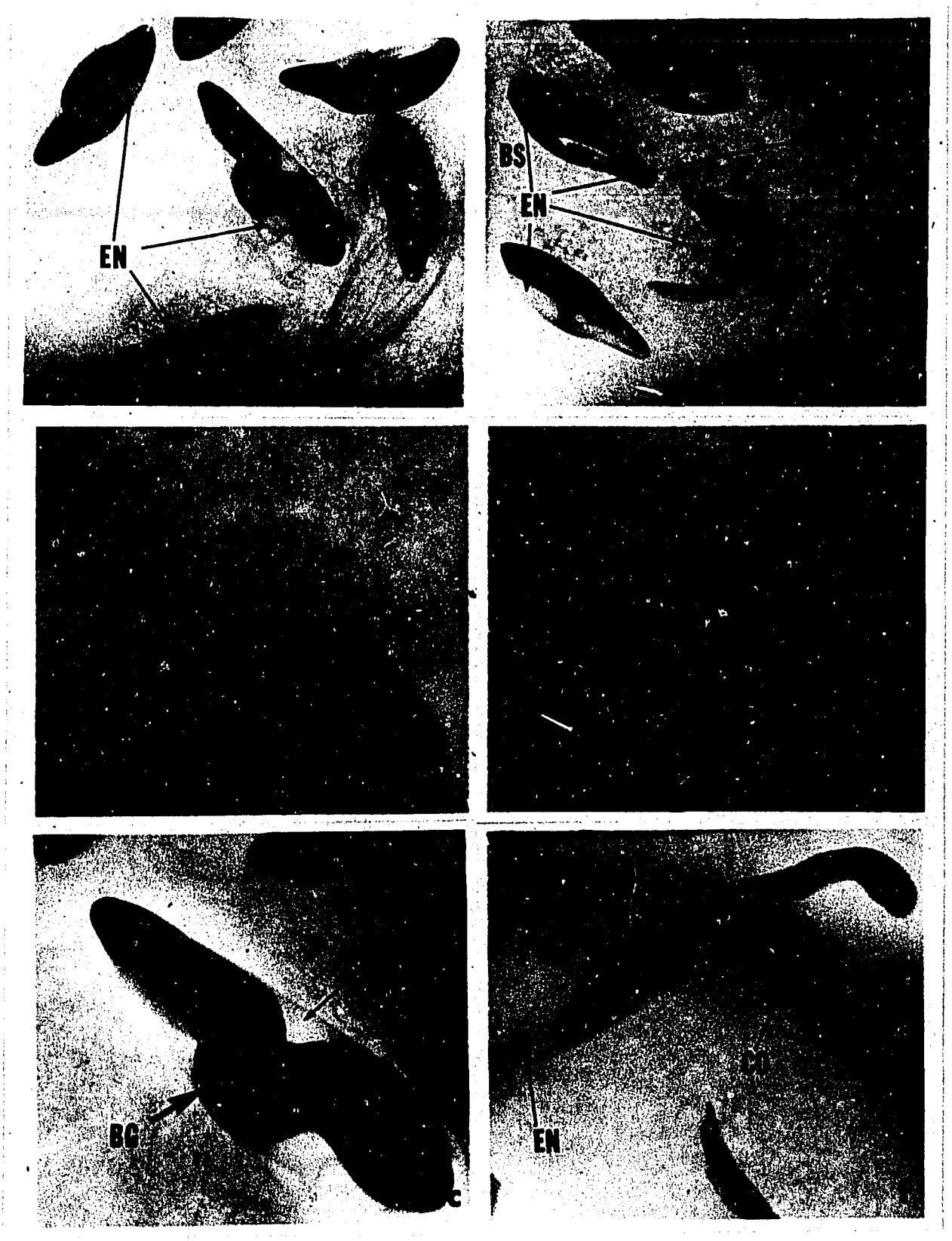


Figure 59. Scanning electron micrographs of seeds sown in 5 mg/ml iC for 4 dy at 20°C and in 16 hr photoperiod. a, b: "Buckled" seeds, note lack of degradation. c: germinated seed, note the "pinching" of radicle as it emerges. a: X250, b: X170, c: X120.

Figure 59