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I am submitting herewith a dissertation written by Doris Marie Presley, entitled "Developmental Expression and Localization of Esterase, Malate Dehydrogenase, and Peroxidase Isoenzymes in Wheat." I recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Botany.

Otto J. Schwarz, Major Professor

We have read this dissertation
and recommend its acceptance:

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Graduate Studies and Research

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DEVELOPMENTAL EXPRESSION AND LOCALIZATION OF ESTERASE, MALATE DEHYDROGENASE, AND PEROXIDASE ISOENZYMES IN WHEAT

A Dissertation

Presented for the

Doctor of Philosophy

Degree

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ABSTRACT

The developmental expression for esterases, malate dehydrogenases, and peroxidases of Triticum vulgare Vill. var Lemhi seedlings was determined at 12 hour intervals during early development. Alteration of these patterns was examined by exposure to different photoperiods, to gamma radiation, and to the DNA synthesis inhibitor, 5-fluorodeoxyuridine (10^{-4} M FdUrd). Localization of the isoenzymes of each system at the organ and subcellular levels was attempted. Throughout these studies an attempt was made to carefully standardize all procedures in order to facilitate comparisons which are essential for the interpretation of possible physiological roles of these enzyme systems.

Ontogenetic zymograms of seedlings following all treatments show that the number of esterase and peroxidase bands increased during early development. Considerable variability of unknown cause was observed in MDH developmental patterns. The appearance of certain esterase bands extracted from normal seedlings were slightly delayed when grown in darkness. With respect to number and grouping of electrophoretic bands, the inhibition of an increase in the DNA content of wheat by gamma-irradiation (500,000 rads) or 10^{-4} M FdUrd did not greatly alter the enzyme complement of each enzyme system. However, a lag in the developmental expression of certain isoenzymes was observed. Total and specific activity results are presented for each enzyme and treatment during early development.

Separation of wheat organs revealed large amounts of esterase and peroxidase activity in the roots and MDH primarily in the leaf.

Zymograms illustrate differences in isoenzyme complements of each organ. Examination of the first leaf segments showed that the basal segment contained the highest peroxidase level while the MDH content increased with maturation and age of the leaf section. Esterases were most abundant in the second and third segments.

Preliminary subcellular preparations demonstrated larger amounts of esterase and peroxidase in the soluble fractions while most of the MDH activity was observed in mitochondrial and chloroplast fractions. Comparisons were made for each enzyme system following non-aqueous extraction of chloroplasts.

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I. INTRODUCTION

The widespread occurrence of isoenzymes in plant and animal tissues has been recognized. The isoenzyme content within a plant differs not only with the tissue type (59) but also in the timing of the appearance of the various isoenzymes during development (62). These variations may be qualitative and/or quantitative in nature (3). Characteristic zymograms of enzyme systems have implied specific roles for those enzymes and their isoenzymes during development (45). Since the expression of these isoenzymes seems to be related to differentiation of the cell, then a detailed analysis of their ontogenetic patterns may further the understanding of their possible physiological role in differentiation (60).

The ontogenetic zymograms of soluble proteins of wheat were first presented by Coulson and Slim (9). Reports of the enzymatic profiles in different plant parts and during stages of growth and development have followed (3, 42, 64, 67). However, none of these presented a detailed developmental pattern. Also the various growth conditions, irregular sampling times, diverse extraction methods, numerous staining techniques, and varied data measurements make these studies exceedingly difficult to compare.

Therefore in this study an attempt was made to standardize growth conditions, harvesting intervals (every 12 hours), extraction media, and enzyme extraction techniques. A timetable for the expression of certain wheat isoenzymes, i.e., multiple molecular forms of an enzyme (45), is presented in this study. Both electrophoretic patterns and activity

profiles for each of the enzyme systems were determined during a period from 12 to 168 hours after sowing. The possibility of altering these patterns was investigated by exposing the plants to different photoperiods, to high levels of gamma radiation, and to a DNA synthesis inhibitor. Studies involving the perturbation of the external environmental conditions (photoperiod) and of internal physiological and biochemical processes (gamma irradiation and DNA synthesis inhibitor) may give an indication of the extent to which the expression of the various isoenzyme complements is regulated internally or externally. Further knowledge of their possible physiological roles was explored through attempts to localize enzyme activities at the organ and subcellular levels.

The three enzyme systems selected for this study are esterase (non-specific esterase, E.C. 3.1), malate dehydrogenase (MDH) (L-malate: NAD oxidoreductase, E.C. 1.1.1.37), and peroxidase (Donor:H_2O_2 oxidoreductase, E.C. 1.11.1.7). Esterases were selected since very little work has been done on this enzyme system in plants, although changes in isoenzyme patterns have been observed during the course of development (2, 42). Also, the function of these esterases is not known. MDH was chosen for this study to serve as an internal reference which would remain constant throughout development (40, 58). Several important physiological functions within the cell have been attributed to MDH. These include activity in the Krebs cycle, the malate shuttle, and perhaps in CO_2 fixation in higher plants (81). Current interest deals with the subcellular localization of the different isoenzymes. Peroxidases were selected because their broad substrate specificity has implied a broad scope of functional roles (45). Their presence or absence

in certain tissues has been associated with specific developmental events such as the onset of meristematic activity (76) and cessation of division and senescence (52).

Wheat, Triticum vulgare, was the developmental system chosen for this study since its anatomical changes during development have been well-documented (16) and especially because certain of the developmental events can be altered through the use of gamma radiation (19, 20, 24, 25, 26) or an DNA synthesis inhibitor (27, 28).

II. MATERIALS AND METHODS

The Lemhi 66 variety of Triticum vulgare Vill. (T. aestivum L.) was obtained from the 1971 and 1973 grain crops of the Globe Feed and Seed Company, Twin Falls, Idaho. The germination observed in samples was approximately 90 percent.

Culture Conditions

All grains except those to be treated with 5-fluorodeoxyuridine (FdUrd) were washed with 0.5 percent NaOCl (10 percent v/v Chlorox) and 0.5 percent Tween 20 (31) for 15 minutes with agitation and then rinsed repeatedly with sterile distilled deionized water (referred to as water). This procedure was selected on the basis of its ability to control fungal contamination and its apparent lack of effect on the growth of seedlings. Twelve grains were sown under sterile conditions in autoclaved 9 cm Petri plates containing two layers of Whatman No. 1 filter paper discs moistened with 10 ml of water or the FdUrd solution. Each plate was not disturbed until the seedlings were harvested.

Dry grains which had been exposed to 500,000 rads of gamma radiation (2.28×10^3 rads/minute) from a ^{60}Co source (Gammacell 200, Atomic Energy of Canada, Ltd., Ottawa) were planted as above in water no later than 12 hours after exposure. Irradiated grains were supplied by the Plant Sciences Group, Biology Division of the Oak Ridge National Laboratories, Oak Ridge, Tennessee.

The grains germinated and seedlings grew for the 7-day test period in growth chambers maintained under one of the following

conditions: (a) constant dark at 20 ± 1°C, (b) constant illumination at 20 ± 1°C, or (c) 16 hours light at 21 ± 1°C and 8 hours darkness at 18.5 ± 1°C (referred to as standard conditions). The light intensity within these chambers measured 1.4×10^3 erg cm^{-2} $second^{-1}$.

Preparation of Extract

The embryonic axes with scutella were removed from the grains. Axes were held in cold deionized water in an ice bath until harvesting was complete and then they were rinsed with water to remove residual endosperm. These explants were ground with an ice-cold mortar and pestle with a small amount of sand in one of the following buffer solutions at pH 7.6: (1) 0.01 M tris [hydroxymethyl]aminomethane-hydrochloride (Tris-HCl); (2) 0.7 percent 2-mercaptoethanol (EtSH) and 10^{-3} M ethylenediamine tetracetic acid (EDTA) in 0.01 M Tris-HCl (3); (3) 2×10^{-3} M dithiothreitol (DTT) and 0.7 percent EtSH in 0.01 M Tris-HCl; (4) 2×10^{-3} M DTT in 0.01 M Tris-HCl (46); (5) 0.7 percent EtSH in 0.01 M Tris-HCl; (6) 0.7 percent EtSH and 10^{-3} M $MgCl_2$ in 0.01 M Tris-HCl; (7) 2×10^{-3} M DTT, 0.7 percent EtSH, and 10^{-3} M $MgCl_2$ in 0.01 M Tris-HCl. The number of plants ground in 2.5 ml of buffer was as follows: forty 12-168 hour gamma plantlets, forty 12-168 hour FdUrd-treated seedlings, forty 12-96 hour untreated water-grown (referred to as normal) seedlings and twenty 108-168 hour normal plants.

The crude extract was prepared by passing this homogenate through glass wool or four thicknesses of cheesecloth. Next it was centrifuged

at 27,000 x g for 30 minutes at 4°C in a refrigerated centrifuge (Sorvall RC 2-B, SS-34 rotor). A Pasteur pipet was used to draw off the 27,000 x g supernatant which was assayed spectrophotometrically and electrophoretically. During all extraction procedures the samples were held at 4°C. The 27,000 x g supernatants were examined throughout this study unless otherwise indicated.

Seedlings were harvested every 12 hours up to 168 hours after sowing in order to more completely identify the isoenzyme profiles. The samples taken at each of these times were prepared as described above.

Electrophoresis and Location of Enzyme Bands

An acrylamide step gradient gel (4, 6½, 8 percent) was prepared in an ORTEC vertical slab cell. The gels were polymerized with ammonium persulfate according to the ORTEC manual (51). Both citrate and sulfate gels were tested for their resolution of soluble proteins (non-specific stain) and enzyme bands utilizing the staining procedures described below. For the purpose of determining the developmental banding patterns, aliquots of the 27,000 x g supernatants were added to an equal volume of 50 percent sucrose solution in a gel buffer. Specific fractions from organelle separations were processed in the same manner. A total of 150 µl of this mixture containing 225 to 350 µg protein was placed in each sample well and then capped.

The polymerized gels were placed in 0.065 M Tris-borate buffers (pH 9.0) and run at 45 mA for 5 minutes, 87 mA for 10 minutes, and 115 mA for 60 minutes provided by the ORTEC 4100 pulsed constant power supply. Electrophoresis took place at 4°C. A 0.1 percent aqueous bromophenol blue solution added to the sample layer and upper tank

buffers served as the marker of the moving front. Following electrophoresis, the electrophoretic cells were held in an ice bath for 10 minutes to facilitate removal of the gels.

Immediately following staining described below, the gels were thoroughly rinsed and photographed with a Pentax camera using Ektachrome X film. The schematic drawings presented here were prepared using the R_f values measured immediately after the gels were photographed and are representative of two to twelve independent replicates. Bands were labeled for identification by using the first initial of the stain followed by those of the treatment, i.e., normal, gamma-irradiated, or FdUrd, light and dark (see Appendix); the relative mobility, i.e., slow-migrating defined here as an R_f up to 0.25, intermediate 0.26 to 0.70, and fast 0.71 to 1.00; and the numerical position within the migrating group starting toward the anode. The relative intensity of staining from faint, light, medium, to dark is indicated by increased density of the stipling on the diagrams.

Esterase stains. The staining technique of Markert and Hunter (44) as modified in the ORTEC manual (51) was used in this study. To prepare the gel for staining, it was first equilibrated in a solution of 4 ml of 1.0 M Tris-HCl buffer (pH 6.6) and 96 ml water at 37°C for 5 minutes with agitation. This equilibration solution was poured off the gel and a mixture of 0.1 g Fast Blue RR (Sigma Chemical Co.), 4 ml 1.0 M Tris-HCl buffer (pH 6.6), 96 ml water and 4 ml of 1 percent 1-naphthol acetate in acetone was added. Color development took from 15 to 30 minutes. The gels were rinsed with tap water rather than the ORTEC

stop solution of ethanol and acetic acid because the alcohol solution caused the bands to fade rapidly. The brown bands were immediately photographed.

An alternate staining method, modified after that of Schwarz (63), involved preincubation of the gel in a 0.01 M Tris-HCl buffer (pH 8.0) at 37°C followed by agitation in a solution of 4 ml 2.77 x 10^{-2} M indophenyl acetate in 96 ml 0.01 M Tris-HCl buffer (pH 8.0). Faint blue bands appeared after 15 to 20 minutes.

Peroxidase stains. The method of van Fleet (76) was employed for the visualization of peroxidase bands. The reaction mixture included 0.023 M Tris-HCl buffer (pH 7.5), 5 mM benzidine dihydrochloride, and 0.3 percent hydrogen peroxide. The gel was held in the dark at room temperature in the staining solution 10 to 25 minutes and was rinsed with tap water prior to photography.

Peroxidase banding was also examined using the guaiacol-hydrogen peroxide system of Ockerse (49). The stain solution consisted of 150 ml 0.1 M phosphate buffer (pH 5.8), 0.3 ml guaiacol, and 0.3 ml 30 percent hydrogen peroxide. The gels were incubated in the dark at room temperature for 30 minutes.

Malate dehydrogenase stain. Localization of bands with MDH activity took place according to the method of Endo (15). A stock solution of 0.02 M disodium malate, 0.1 M Tris-HCl buffer (1 M, pH 8.0), and 5 mM potassium cyanide was prepared. Immediately before use the following were added to the stock solution: 0.3 mg/ml nicotinamide adenine dinucleotide (NAD), 0.3 mg/ml p-nitroblue tetrazolium (NBT), and 20 µg/ml phenazine methosulfate (PMS). The gel was stained in the dark at room temperature for 1 to 3 hours.

Protein stains. According to the ORTEC instructions (51), 0.17 g amido black was added to one liter of 7.5 percent glacial acetic acid. The gels were stained at room temperature overnight and then destained by repeated washes in 10 percent glacial acetic acid.

An alternate stain, described by Caponetti, Harvey, and DeMaggio (7) was also used. The stain consisted of two parts: (1) a stock solution of 0.5 g Coomassie blue, 91 ml water, 91 ml methanol, and 8.2 ml acetic acid; and (2) the solvent which consisted of 91 ml water, 91 ml methanol, and 8.2 ml acetic acid. The staining mixture was prepared by adding one part of the stock solution to two parts of the solvent. The gels were stained overnight at room temperature and excess stain was removed by repeated washes in 7 percent acetic acid.

Enzymatic Assays

Supernatants and resuspended pellets were assayed in a Cary 14 dual beam spectrophotometer. One unit of enzyme activity is represented by a change of 0.01 O.D./minute.

The determination of the total enzymatic activity per seedling (T) was performed according to the following equation:

$$T = \frac{A \times D \times N \times V}{S}$$

where:

A = change in optical density per minute

D = dilution of initial extract

N = number of assay samples per milliliter

V = total volume of initial extract

S = number of seedlings harvested.

The specific activity of an enzyme is defined as the total activity per seedling divided by the number milligrams protein per seedling. Calculations of total activity per seedling and specific activity were based on averages of two independent replicates.

Esterase assay. The colorimetric determination was performed according to the method of Kramer and Gamson (35) as modified by Schwarz (63). The enzymatic activity of 0.1 ml of wheat extract in 4.45 ml of 0.05 M Tris-HCl buffer (pH 7.6) and 0.05 ml of 2.77 x 10^{-2} M indophenyl acetate was read at 625 nm. The enzyme blank was prepared as above except for the substitution of extraction buffer for the enzyme extract. The rate of hydrolysis was linear for over 15 minutes.

Peroxidase assay. Peroxidase activity was determined by the modified method of Sequi et al. (64). The reaction mixture included 2.75 ml of 0.05 M ammonium citrate-phosphate buffer (pH 5.4), 0.2 ml of 0.1 M hydrogen peroxide, 0.05 ml of 0.5 percent o-dianisidine, and 0.05 ml of the enzyme extract. In preparation of the reference cuvette, water was used instead of hydrogen peroxide. Absorbance was measured at 460 nm. The resulting plot was linear for 30 to 60 seconds.

Malate dehydrogenase assay. Malate dehydrogenase was assayed in the direction of malate oxidation according to the procedure reported by Grimwood and McDaniel (23). The reaction mixture contained the following: 3 ml of a solution of 25 mM sodium malate, 0.98 mM NBT, 13 µM NAD, 14 µM PMS, and 0.82 mM Tris-HCl buffer (pH 7.5); and 0.1 ml of the enzyme or extraction buffer for the reference cell. The initial linear activity was recorded at 650 nm for 1 to 2 minutes.

Protein Determination

An aliquot of each sample (the 27,000 x g supernatant or the indicated fraction) was added to an equal volume of 10 percent trichloroacetic acid (TCA) and held in an ice bath for 30 minutes. Following centrifugation at 2,000 x g for 5 minutes, the pellet was washed twice with 5 percent TCA and centrifuged as above. The final precipitate was resuspended in 0.1 N sodium hydroxide. Samples were assayed for soluble protein according to the method of Lowry et al. (41) using bovine serum albumin standards.

Analysis of DNA Content

Wheat embryonic axes treated with 10^{-3} to 10^{-7} M FdUrd were harvested every 24 hours for 7 days. The tissue was homogenized in 0.5 M NaCl and 0.1 M EDTA (pH 8.0) in a cold mortar and pestle. Nucleic acids were extracted according to the procedure of Smillie and Krotkov (68) as modified by Nitsan and Lang (47). Colorimetric determination was conducted according to the diphenylamine method of Hatcher and Goldstein (30). DNA standards were prepared following hydrolysis of 0.4 mg/ml salmon DNA in 5 x 10^{-3} M NaOH with an equal volume of 1 N perchloric acid at 90°C for 15 minutes.

Organ Separation

Seedlings were grown for 72 and 144 hours under standard conditions described earlier. These developmental times were selected since most of the early bands were observed by 72 hours, especially one peroxidase band which appeared for only a short duration, and because at

144 hours the late-appearing bands were present. Roots, leaves and coleoptiles were carefully separated and the 27,000 x g fractions were obtained. Each fraction was assayed directly and after electrophoretic separation in the usual manner.

First Leaf Segments

The shoots of 7-day-old seedlings grown under standard conditions were carefully excised and the leaves were removed from the coleoptile. Only the more mature first leaf was studied while the younger leaves enclosed in it were discarded. The basal 1 cm portion was removed and then succeeding 2 cm segments up to and including the tip were cut. Banding patterns and enzymatic activities were determined for each segment as described previously.

Organelle Enrichment

Crude homogenate. Four-day-old seedlings grown under standard conditions were gently ground for 2 to 3 minutes with an ice-cold mortar and pestle containing a very small amount of sand in 0.01 M Tris-HCl buffer (pH 7.6) and 0.5 M sucrose (58). This material was filtered through four layers of cheesecloth. The crude homogenate was then used directly in the preparation of the chloroplast or ribosomal fractions as indicated below.

Chloroplasts. The separation methods outlined by Bonner (4) were carried out. The crude homogenate was centrifuged at 100 x g for 5 minutes in a Sorvall RC 2-B centrifuge to remove nuclei and unbroken cells. The supernatant was recentrifuged at 2,000 x g for 10 minutes.

The resulting precipitate was washed with buffer and examined microscopically. Aliquots of the crude homogenate, 2,000 x g supernatant and pellet were brought to 80 percent acetone, centrifuged at 500 x g for 5 minutes and the amount of chlorophyll per sample was determined spectrophotometrically by the method of Arnon (2).

Non-aqueous extraction of chloroplasts were performed using modifications of the methods of Stocking (70) and Smillie (69). Two hundred, 4-day-old seedlings grown under standard conditions were lyophilized overnight. The plants were finely cut and placed in a pre-chilled mortar. The material was ground for 3 to 4 minutes in 20 ml of a solution of cold hexane and carbon tetrachloride with a density of 1.32. This homogenate was filtered through glass wool and then centrifuged at 10,000 x g for 5 minutes to remove large cellular debris. An equal volume of hexane was added to the supernatant and this mixture was centrifuged at 2,000 x g for 5 minutes. The resulting pellet was dried in a vacuum and then resuspended in 2 ml of 0.01 M Tris-HCl buffer (pH 7.6). Chlorophyll determinations, enzymatic assays and electrophoretic analysis were performed as described earlier.

Mitochondria. The supernatant resulting from the aqueous chloroplast separation was further centrifuged according to the method of Bonner (5) at 10,000 x g for 20 minutes to yield the mitochondrial pellet. The precipitate was washed and resuspended in 3 ml of extraction buffer for enzymatic analysis.

Mitochondria were also prepared by the modification of the method of Legris and Tsai (39). A medium containing 0.5 M sucrose, 0.165 M Tris-HCl buffer (pH 7.5), 10 mM KCl, 10 mM $MgCl_2$ 10 mM EDTA, and 5 mM

DTT was used for the extraction. Following centrifugation at 2,000 x g, the supernatant was centrifuged at 10,800 x g for 20 minutes. The pellet was resuspended in 8 ml of extraction buffer and 2 ml samples were layered over a step sucrose density gradient prepared as follows: 2.5 ml of 60 percent, 5.0 ml of 57 percent, 7.5 ml of 50 percent, 7.5 ml of 44 percent, and 3.5 ml of 33 percent sucrose (w/v in 10 mM EDTA, pH 7.5). Each tube was centrifuged at 41,000 x g for 4 hours in a Beckman model L3-50 ultracentrifuge using a SW 25.1 rotor. The bottom of each tube was punctured with a size 16 cannula and fractions were collected in one of two ways: (a) 10 drop fractions of the entire tube, or (b) 20 drop fractions of interfaces and intermediate regions. The sucrose content of each fraction was measured with a Bausch and Lomb hand refractometer. Each fraction was assayed for enzymatic activity as described above and active fractions were examined electrophoretically.

Ribosomes. After the removal of large particulates from the crude homogenate by centrifugation at 27,000 x g in a Sorvall RC 2-B centrifuge, the supernatant was centrifuged at 122,000 x g for 90 minutes in the Beckman model L3-50 ultracentrifuge using a type 50 rotor after the method of Lanzani and Galante (37). The light brown pellet containing ribosomes and endoplasmic reticulum membrane fragments (4) was resuspended in 2 ml of the extraction buffer. Sodium deoxycholate (1 mM) aided in the solubilization of this pellet (38). Aliquots of this 122,000 x g fraction were assayed kinetically and electrophoretically as described earlier.

III. RESULTS

Examination of Extraction Buffers

The selection of the buffer system is an important consideration in any enzymatic study. The isoenzyme patterns of the 27,000 x g supernatants extracted from 96 and 144 hour normal seedlings were examined in order to determine an appropriate buffer for the extraction of the three enzyme systems in wheat. The following buffers were employed in this study:

1. 0.01 M Tris-HCl, pH 7.6
2. 0.7 percent EtSH and 10^{-3} M EDTA in 0.01 M Tris-HCl, pH 7.6
3. 2 x 10^{-3} M DTT and 0.7 percent EtSH in 0.01 M Tris-HCl, pH 7.6
4. 2 x 10^{-3} M DTT in 0.01 M Tris-HCl, pH 7.6
5. 0.7 percent EtSH in 0.01 M Tris-HCl, pH 7.6
6. 0.7 percent EtSH and 10^{-3} M $MgCl_2$ in 0.01 M Tris-HCl, pH 7.6
7. 2 x 10^{-3} M DTT, 0.7 percent EtSH, and 10^{-3} M $MgCl_2$ in 0.01 M Tris-HCl, pH 7.6

Esterase. The results on esterase activity for each of the extractions of 4- and 6-day-old seedlings are shown in Figure 1. Band E_{BSi-3} was noticeably absent at 4 days in the Tris buffer and Tris plus DTT as was E_{BSf-1} in the EtSH and ADTA system. Similar differences were observed after 144 hours with respect to band E_{BSi-2}. In addition, the slow-moving band E_{BSs-1}, absent in 96 hour extracts, was present in all 144 hour systems except the Tris buffer. The relative intensities were greatest in the Tris, Tris plus EtSH and EDTA, and Tris plus DTT buffers.

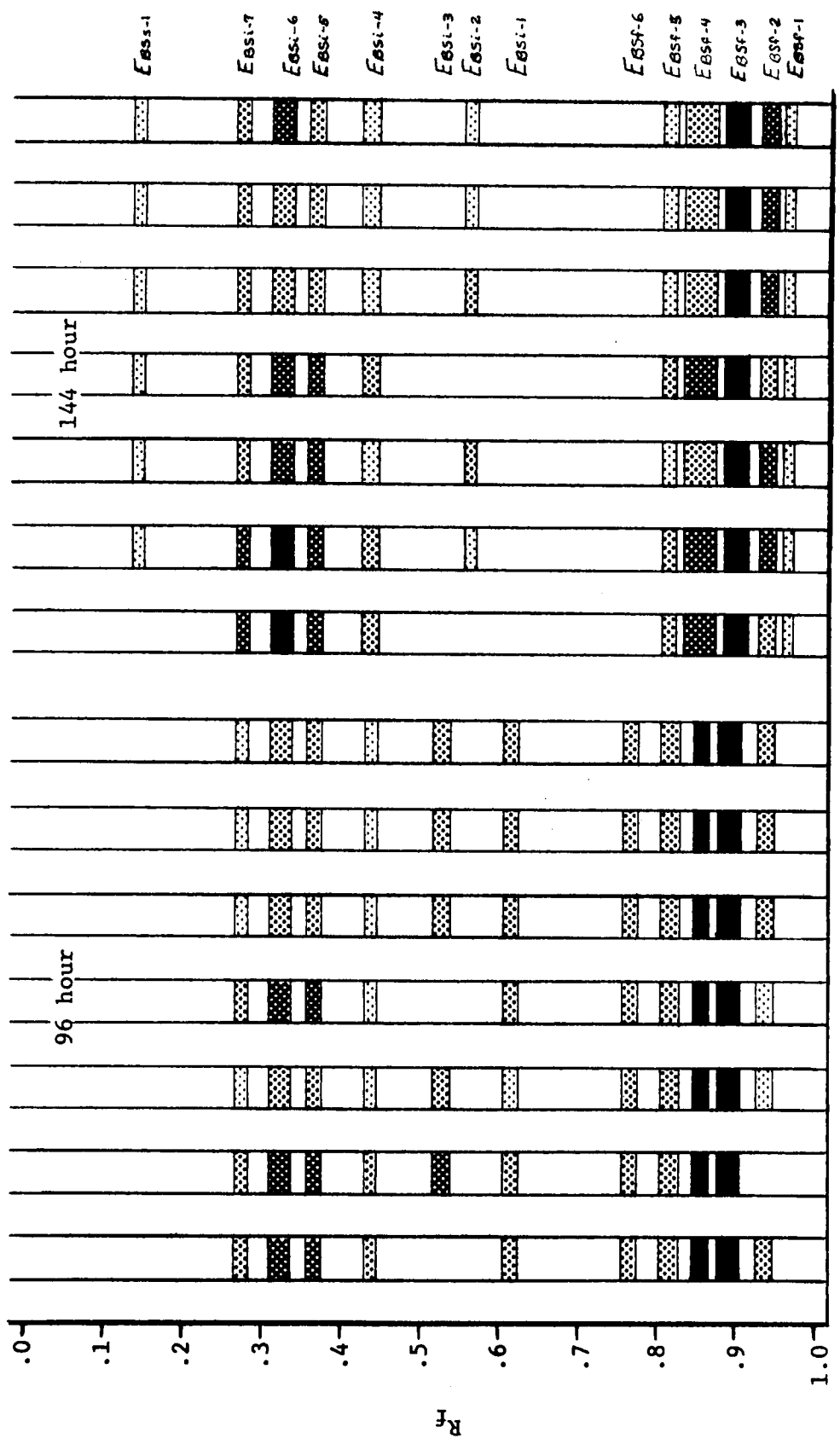

Figure 1. Electrophoretic patterns of esterases in 96 and 144 hour seedlings following extraction in a series of buffers. (1) Tris; (2) EtSH + EDTA; (3) DTT + EtSH; (4) DTT; (5) EtSH; (6) EtSH + $MgCl_2$; (7) DTT + EtSH + $MgCl_2$. Increased intensity of staining is reflected by increased density of stipling from faint (▒), light (▓), medium (▓), and dark (●).

Malate dehydrogenase. The bands with MDH activity are presented in Figure 2. The banding at 4 days after sowing showed considerable uniformity with the exception of one very faint band at R_f 0.65 in the Tris buffer only. In the older seedlings there was more variability. The very faint slow-migrating band at R_f 0.18 was observed in the Tris plus DTT, Tris plus EtSH, Tris plus EtSH and $MgCl_2$, and Tris plus DTT and $MgCl_2$ buffers. Also the fast-moving band between R_f 0.55 and 0.60 was absent in the DTT, and the DTT, EtSH, and $MgCl_2$ systems.

Peroxidase. The maximum number of bands with peroxidase activity was observed after both 4 and 6 days in samples extracted in Tris and Tris plus DTT (Figure 3). An additional band P_{BSi-3} appeared in both the DTT and the DTT and EtSH buffers only in the 4-day-old seedlings. There were fewer differences among the extracts of 6-day-old seedlings. Only the Tris and Tris plus DTT systems had bands P_{BSf-1} and P_{BSi-1}. One band, P_{BSs-2}, was not found in buffers with EtSH and $MgCl_2$ or EtSH, DTT, and $MgCl_2$.

The 0.01 M Tris-HCl buffer (pH 7.6) was adopted for the preparation of extracts for the determination of the ontogenetic zymograms.

Developmental Expression of Enzyme Bands in Normal Seedlings

To eliminate as much variability as possible in the developmental patterns, planting of grains was staggered so that the entire series was harvested and placed on a single gel for analysis.

No differences were observed between seedlings grown under continuous illumination and in a 16:8 hour light to dark cycle. Therefore, both of these treatments will be considered under the heading of light-grown patterns.

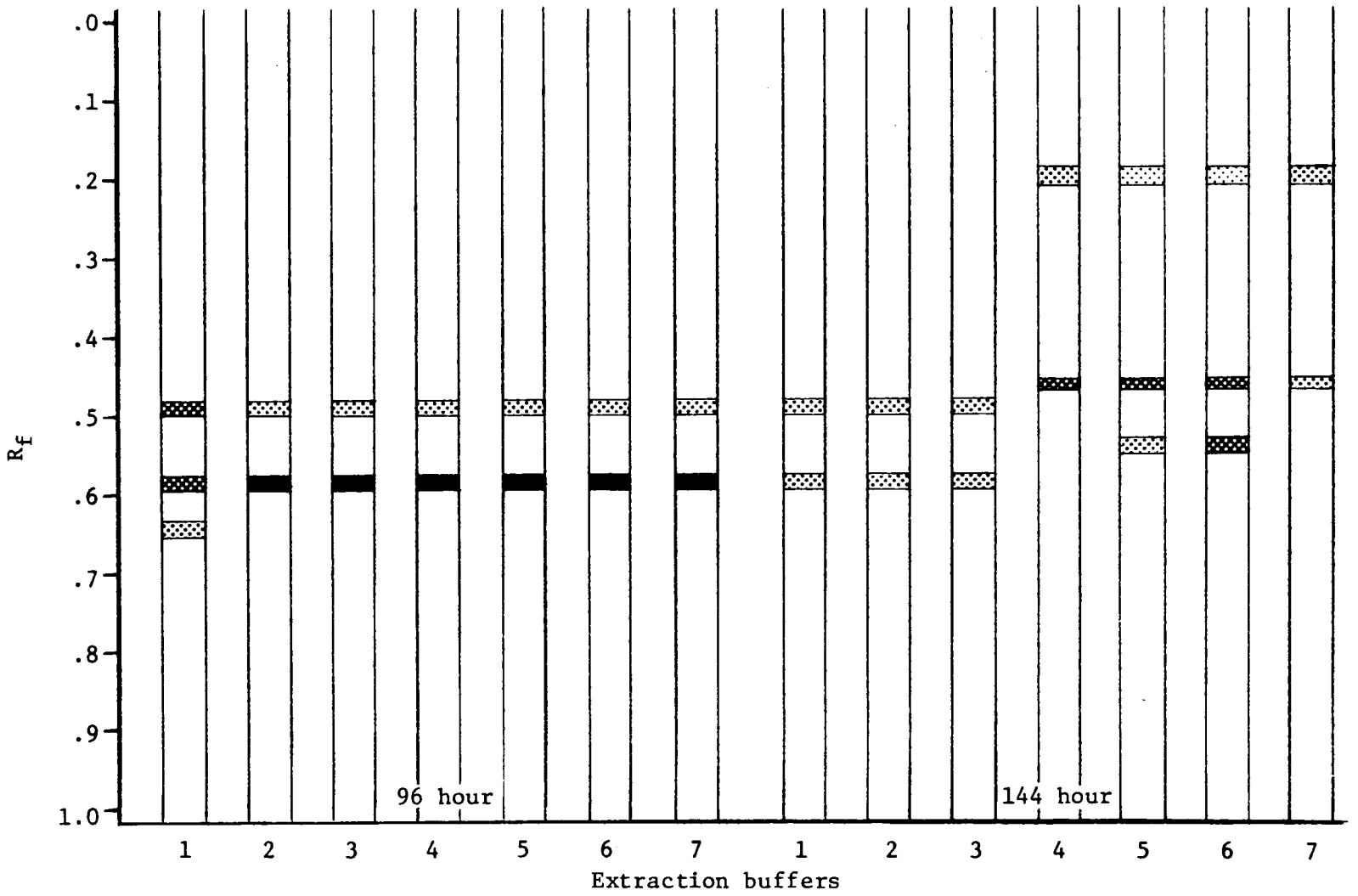

Figure 2. Electrophoretic patterns of MDH's in 96 and 144 hour seedlings following extraction in a series of buffers. (1) Tris; (2) EtSH + EDTA; (3) DTT + EtSH; (4) DTT; (5) EtSH; (6) EtSH + $MgCl_2$; (7) DTT + EtSH + $MgCl_2$.

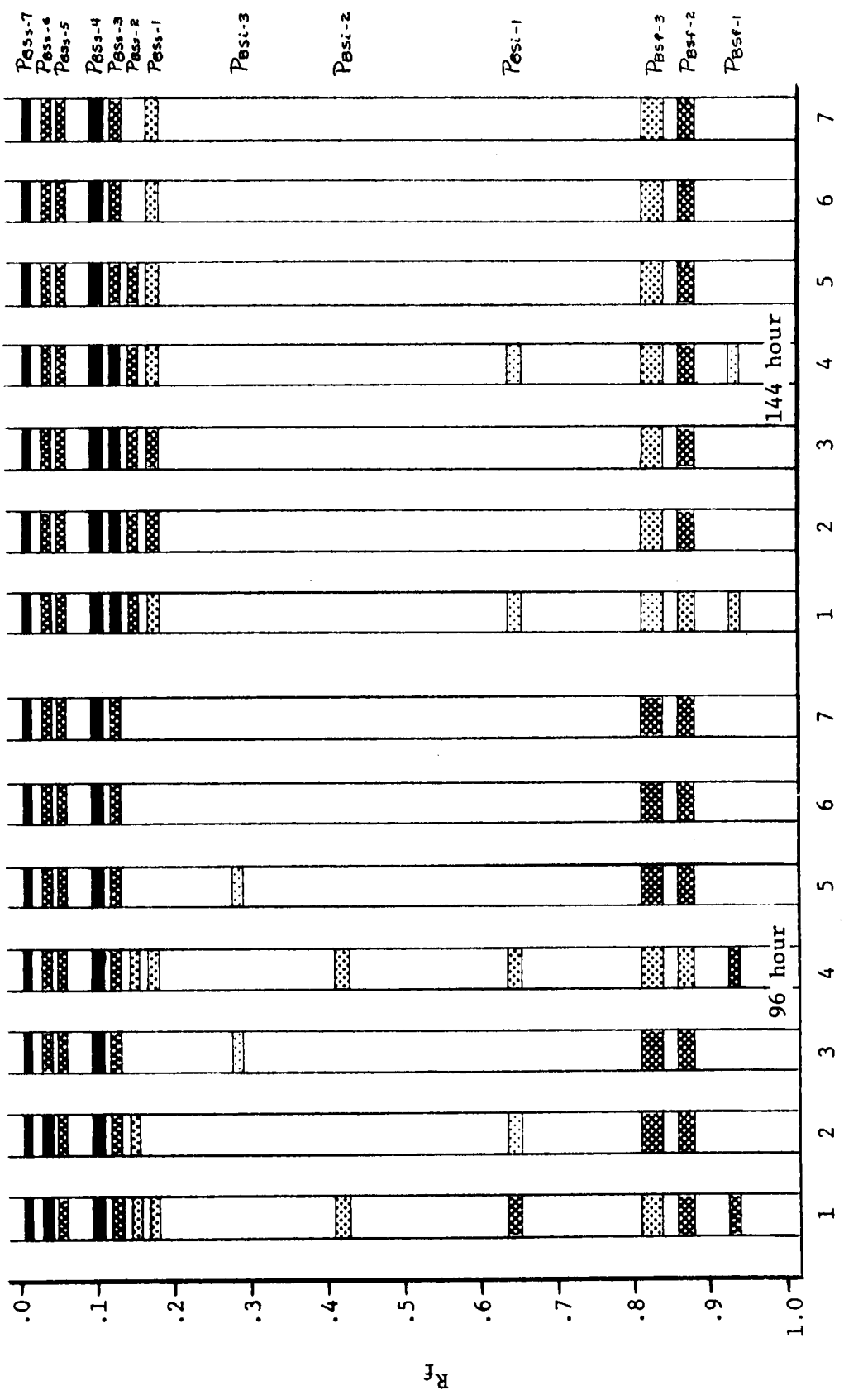

Figure 3. Electrophoretic patterns of peroxidases in 96 and 144 hour seedlings following extraction in a series of buffers. (1) Tris; (2) EtSH + EDTA; (3) DTT + EtSH; (4) DTT; (5) EtSH; (6) EtSH + $MgCl_2$; (7) DTT + EtSH + $MgCl_2$.

Protein. Resolution of protein bands using the amido black stain was better in sulfate buffered acrylamide gels than the citrate gels. However, due to the numerous protein species, it was impossible to determine distinct protein profiles. The Coomassie blue stain proved unsatisfactory in all attempts to demonstrate protein bands using the procedures defined earlier.

Citrate gels displayed better definition of bands for each of the three enzymatic systems and therefore were used in all procedures which are reported in the following material.

Esterase. Figure 4 illustrates the developmental banding pattern of esterases in normal seedlings grown under a light regime. A total of 13 bands--six fast, six intermediate, and one slow-migrating--was demonstrated. The three fastest moving bands appeared first during development. Slight variability was observed in the timing of the appearance of the E_{NLi-1} band during replicate runs from as early as 12 hours to as late as 36 hours after sowing. Most of the intermediate bands first appeared as a smear after 36 hours. One of the fast-moving bands, E_{NLf-4}, always appeared much later than other members of the group. Often this band was observed as early as 72 hours after sowing or not until 96 hours in other time course replicates.

The banding representative of esterase activities in dark-grown seedlings is shown in Figure 5. The three fastest migrating bands were the only bands to appear during the first 60 hours after sowing. The intermediate bands appeared in a light smear after 72 hours as did two other members of the fast group, E_{NDf-5} and E_{NDf-6}. It was noted that band E_{NDf-4} was seen at least 24 hours later than other members of its

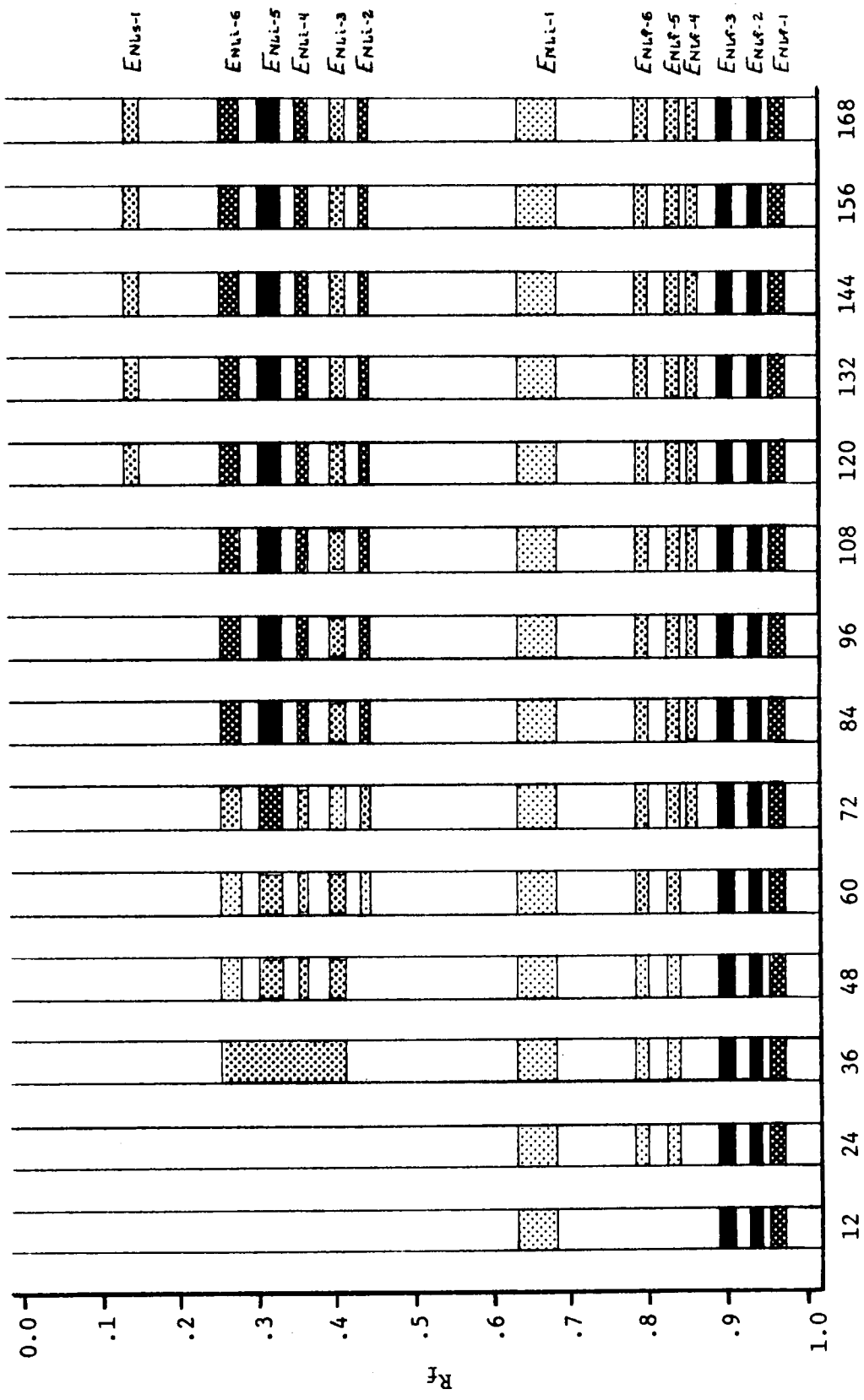

Figure 4. Developmental expression of esterase bands from normal light-grown seedlings.

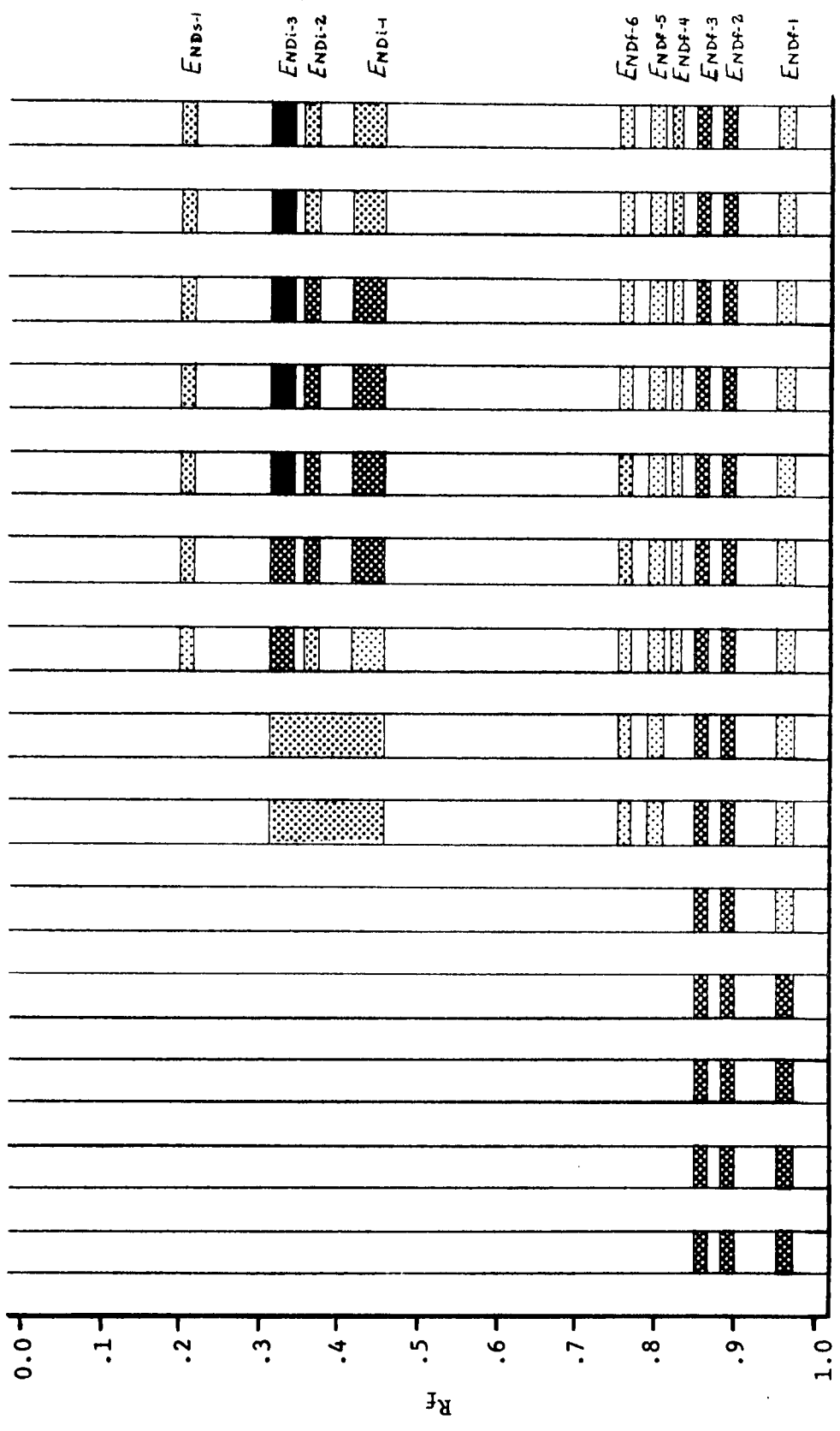

Figure 5. Developmental expression of esterase bands from normal dark-grown seedlings.

group. With the addition of a slow band E_{NDs-1} at 96 hours after sowing, the total complement of 10 bands was expressed.

Malate dehydrogenase. Throughout this study there was considerable difficulty in eliminating variability within zymograms exhibiting MDH activity. A representative developmental sequence for normal seedlings grown under standard conditions is shown in Figure 6. A total of five different, although overlapping, R_f values was observed. Also during development the dark-grown seedlings appeared to produce a total of five different bands with several containing similar R_f points (Figure 7). Arbitrary labels were not assigned to the individual bands because of their varying R_f values and the generally high level of non-reproducibility of the MDH zymograms.

Peroxidase. The pattern representative of peroxidase banding observed in normal seedlings grown in the light consisted of a total of 10 bands--three fast, two intermediate, and five slow-migrating (Figure 8). During the first 24 hours after sowing there were no bands or only two very faint slow-migrating bands. It was observed in some replicates that these bands, P_{NLs-3} and P_{NLs-5}, also may become intensely stained prior to the appearance of any other peroxidase bands. All bands appeared to maintain or increase their relative intensity except for P_{NLf-3} which showed a tendency to decrease by the seventh day. The band P_{NLi-2} occurred for a very short period during development, between 60 and 84 hours after sowing. The appearance of the first slow-migrating band, P_{NLs-1}, was observed as early as 96 hours; however, it was a constant feature after the sixth day.

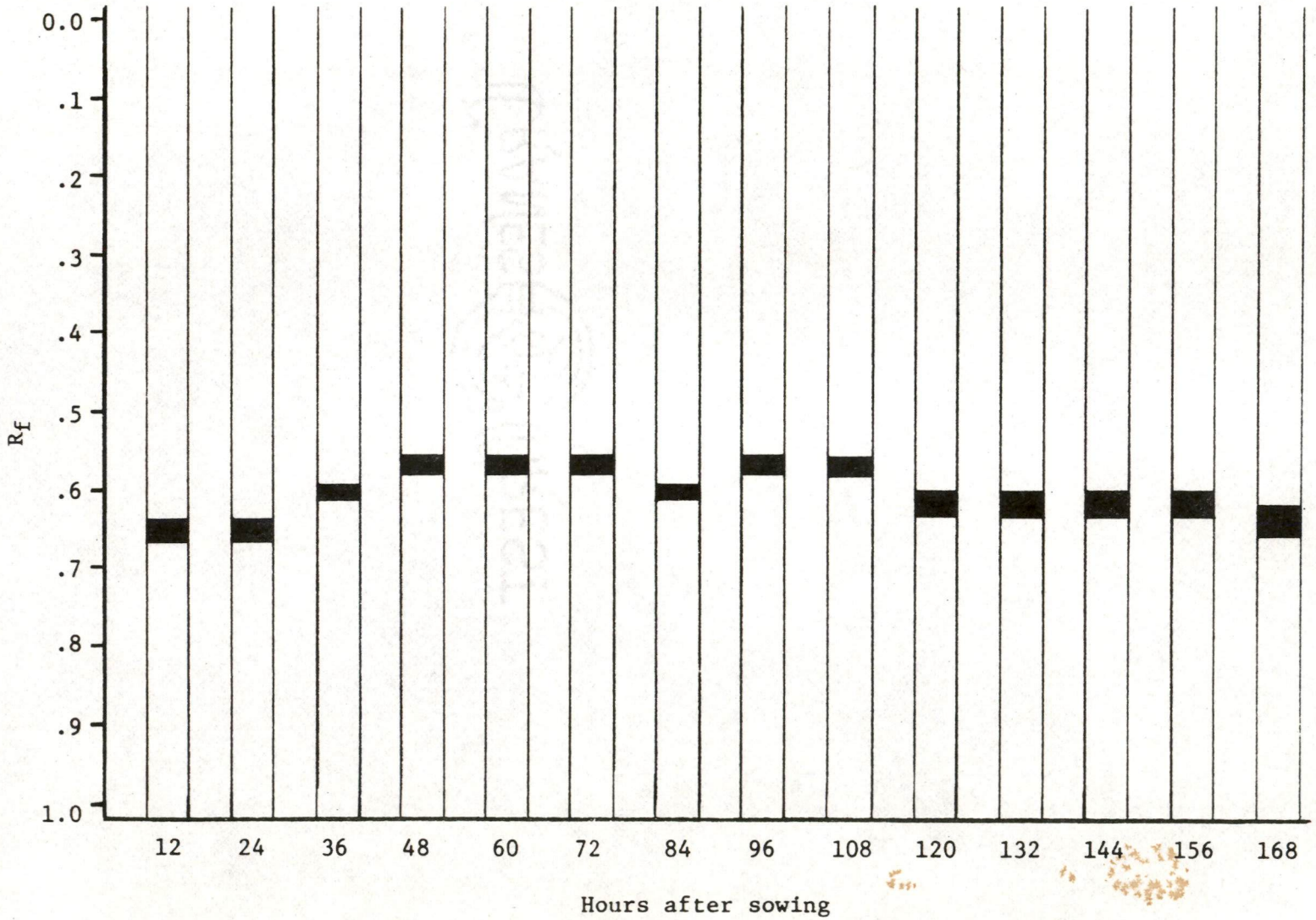

Figure 6. Developmental expression of MDH bands from normal light-grown seedlings.

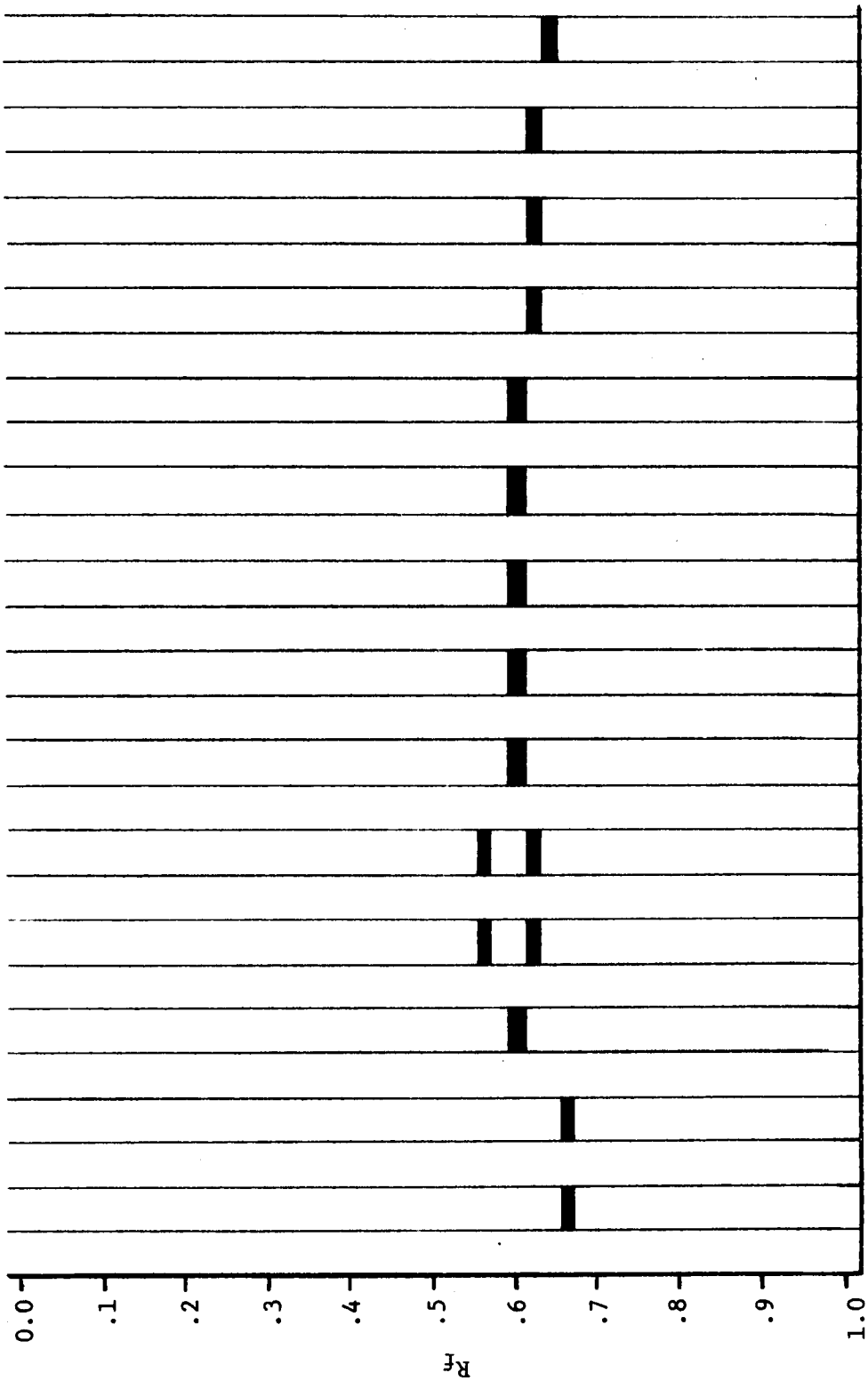

Figure 7. Developmental expression of MDH bands from normal dark-grown seedlings.

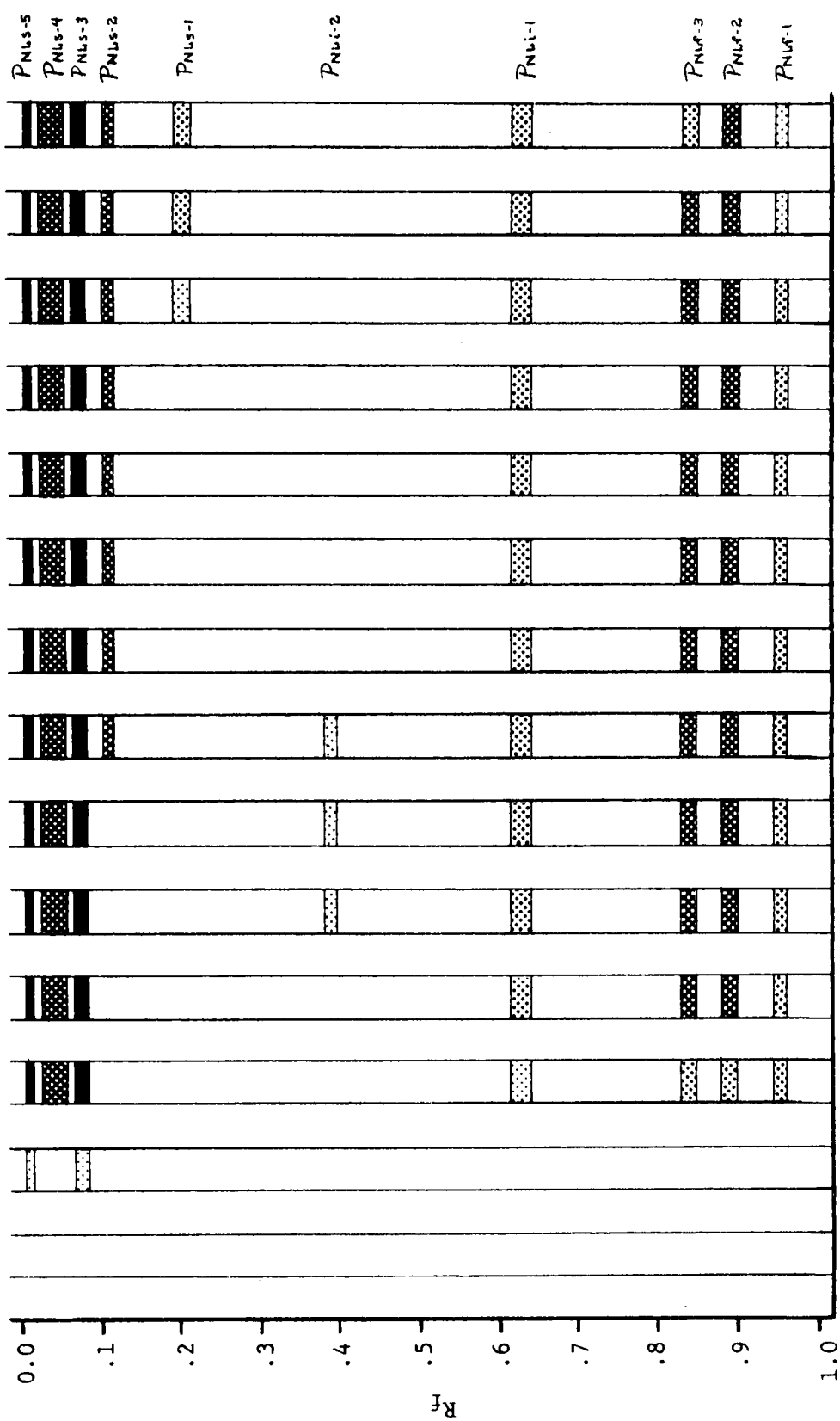

Figure 8. Developmental expression of peroxidase bands from normal light-grown seedlings.

The developmental peroxidase pattern of dark-grown seedlings is illustrated in Figure 9. The slow-moving bands appeared first and by 48 hours after sowing the total complement of nine bands was expressed. All of the slow-moving bands, including P_{NDs-1} and P_{NDs-2}, were observed by 48 hours.

Results obtained using guaiacol as a substrate, instead of benzidine dihydrochloride, in staining peroxidase bands from normal seedlings grown under standard conditions are presented in Figure 10. Two slow-moving bands, P_{Gs-3} and P_{Gs-5}, appeared 12 hours after sowing. After 36 hours the fast-migrating group was observed. The intermediate group, P_{Gi-1} and P_{Gi-2}, was present by 60 hours and remained throughout the rest of the 7-day test period. By 108 hours after sowing a total of nine bands--two fast, two intermediate, and five fast-migrating--was observed.

Early Growth and Development in the Absence of a Net Increase in DNA Content

The DNA content of seedlings treated with FdUrd (10^{-3} to 10^{-7} M) and grown under standard conditions is shown in Figure 11. Concentrations of 10^{-7} and 10^{-6} M FdUrd inhibited further increases in DNA content after 5 days of growth and development. Both 10^{-4} and 10^{-3} M FdUrd appeared effective in the inhibition of a net increase in the DNA content throughout the test period.

Growth measurements (Figure 12) were taken in order to define a FdUrd treatment which would produce seedlings comparable to the gamma-plantlet (Figure 13). Although the total length measurements of seedlings grown in 10^{-5} M FdUrd gave rise to seedlings similar to the

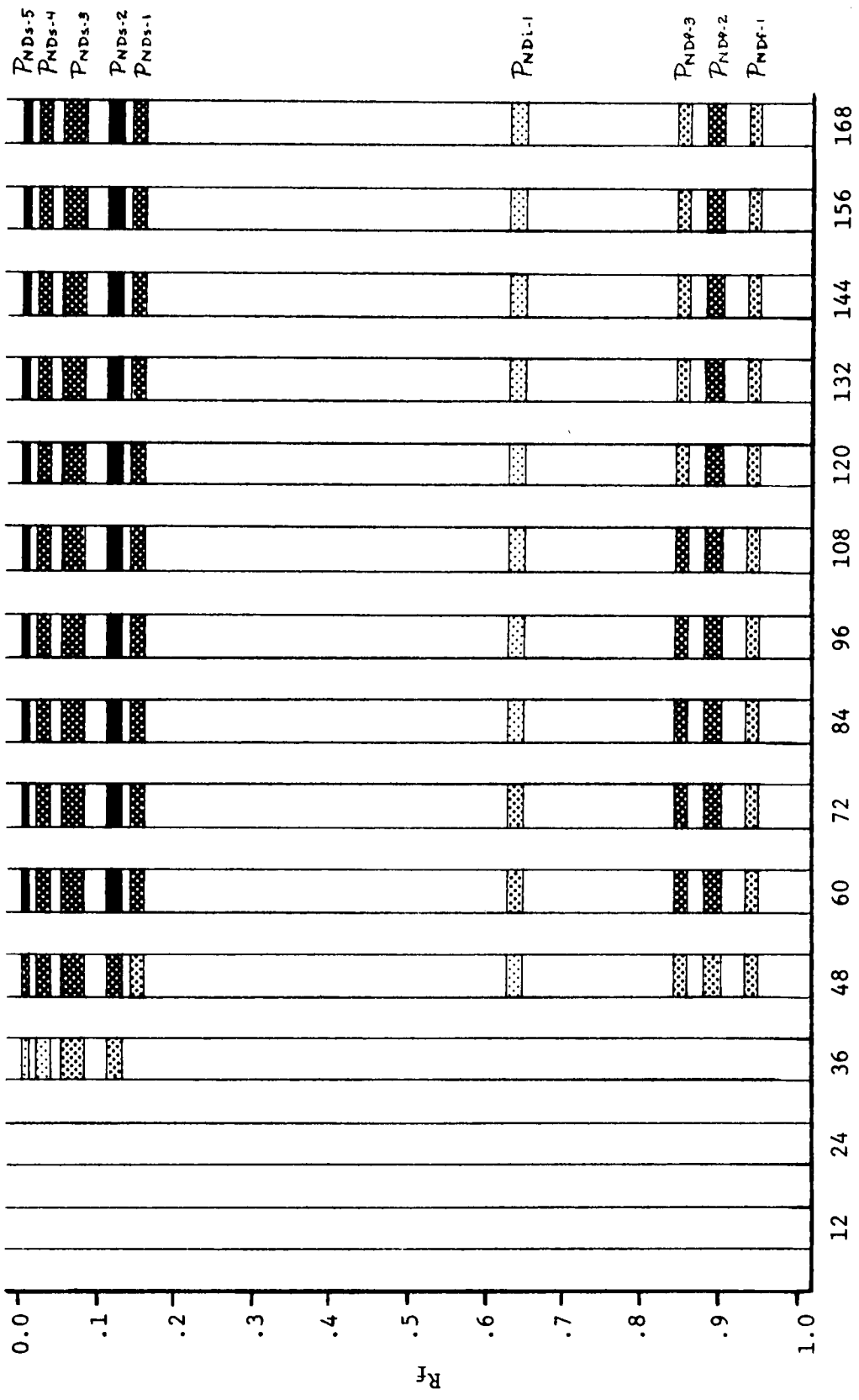

Figure 9. Developmental expression of peroxidase bands from normal dark-grown seedlings.

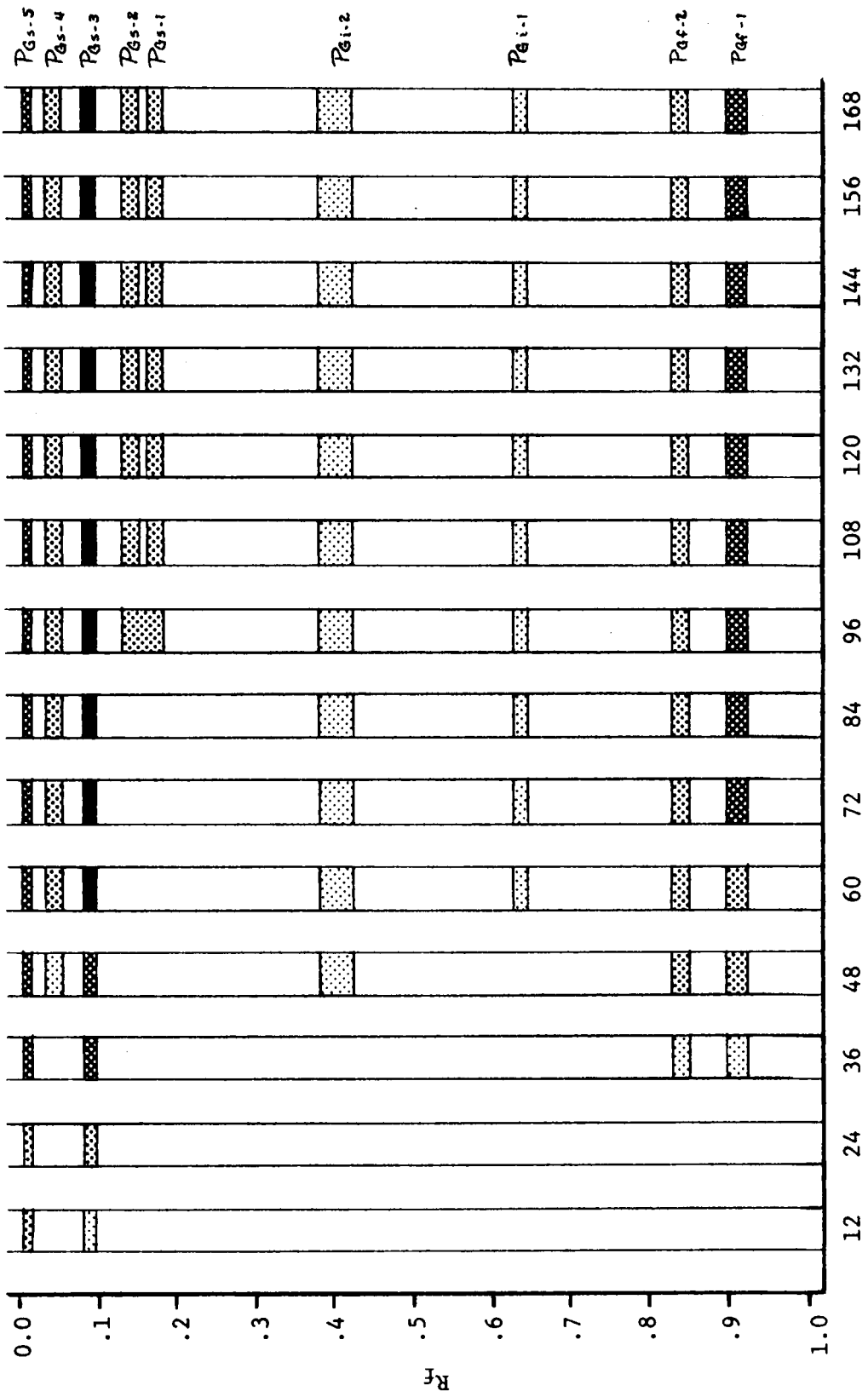

Figure 10. Developmental expression of peroxidase bands from normal light-grown seedlings using guaiacol as substrate instead of benzidine.

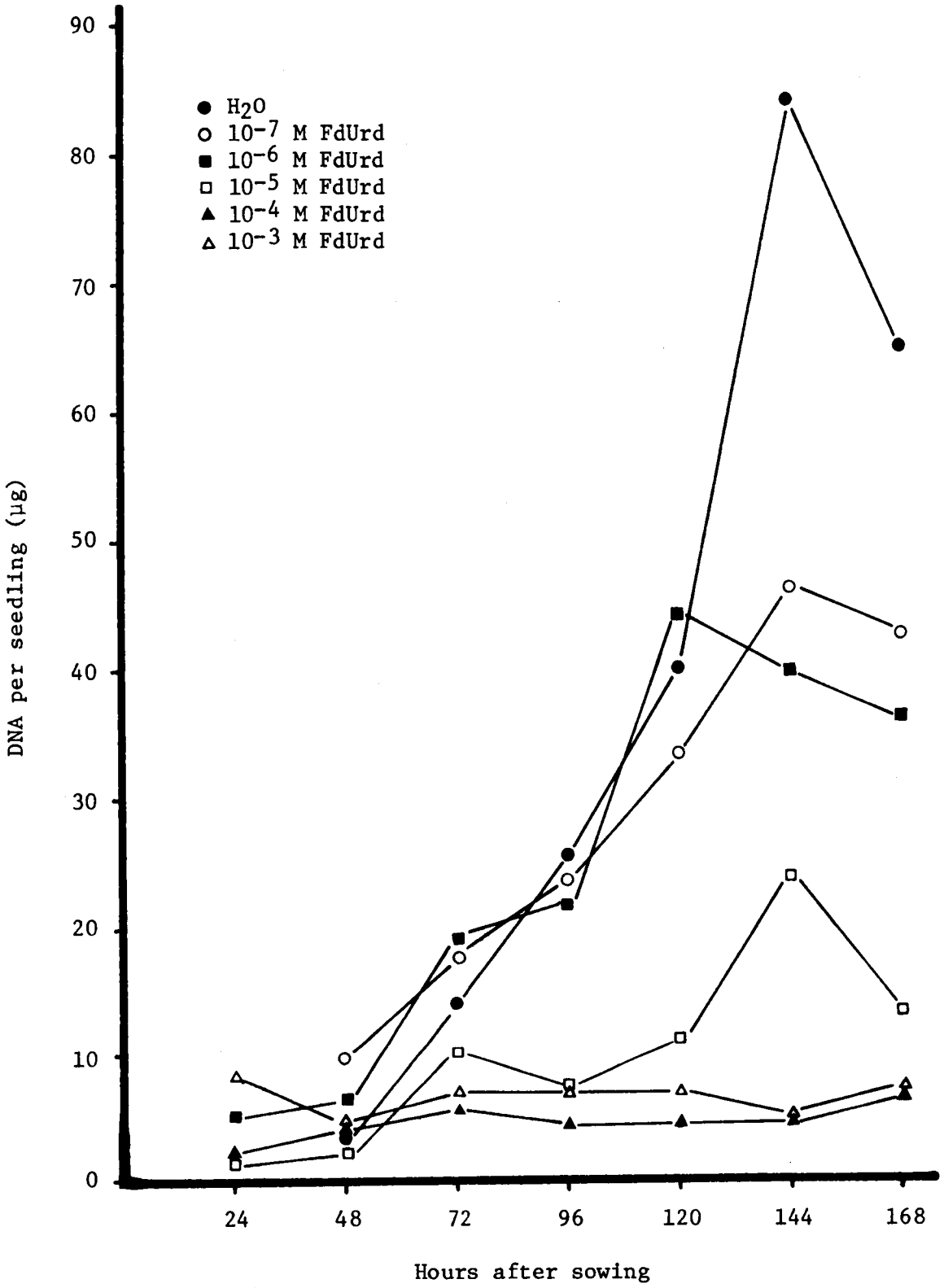

Figure 11. DNA content of FdUrd-treated seedlings grown under standard conditions.

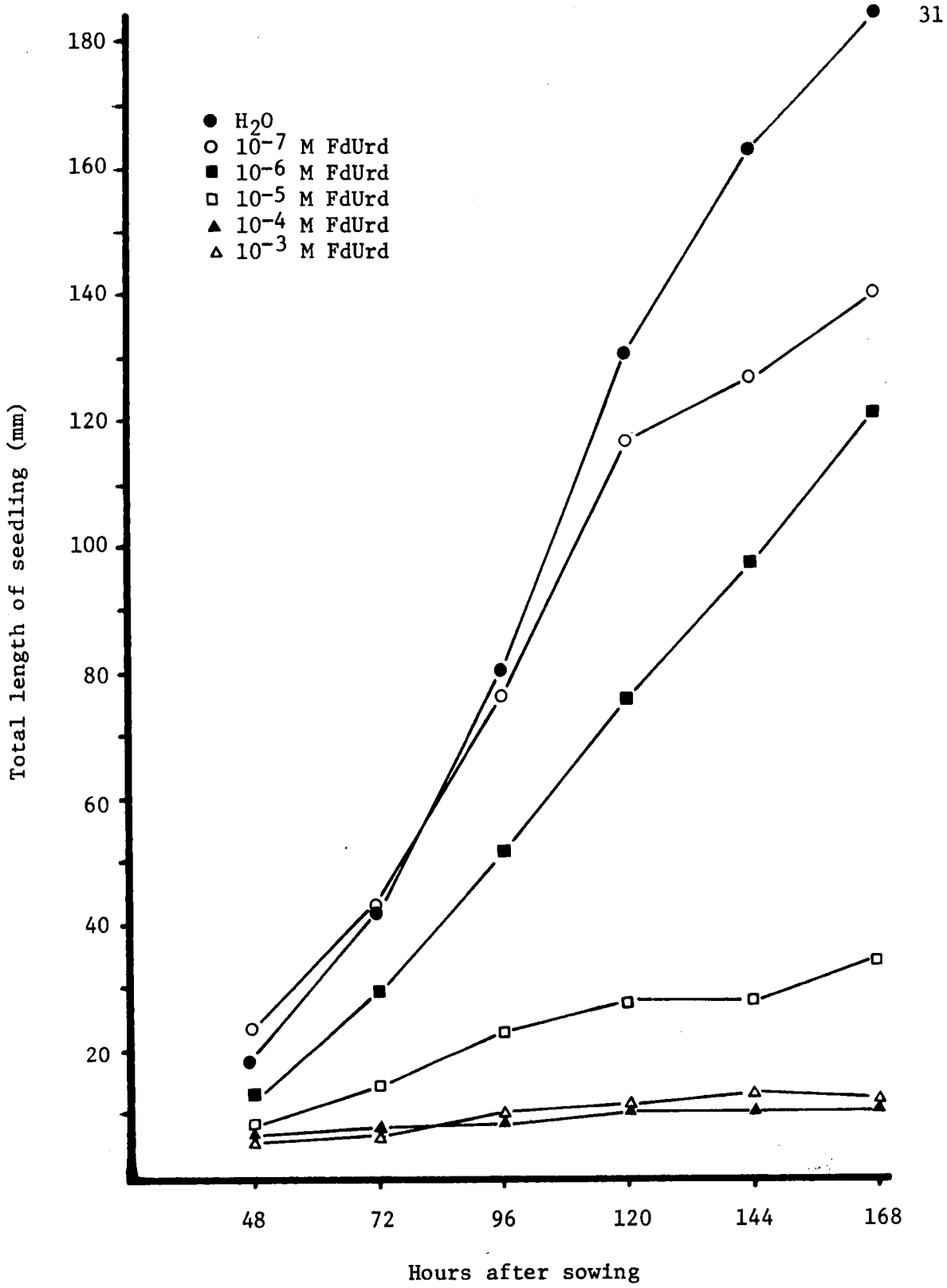

Figure 12. Total length of seedlings grown in concentrations of FdUrd.

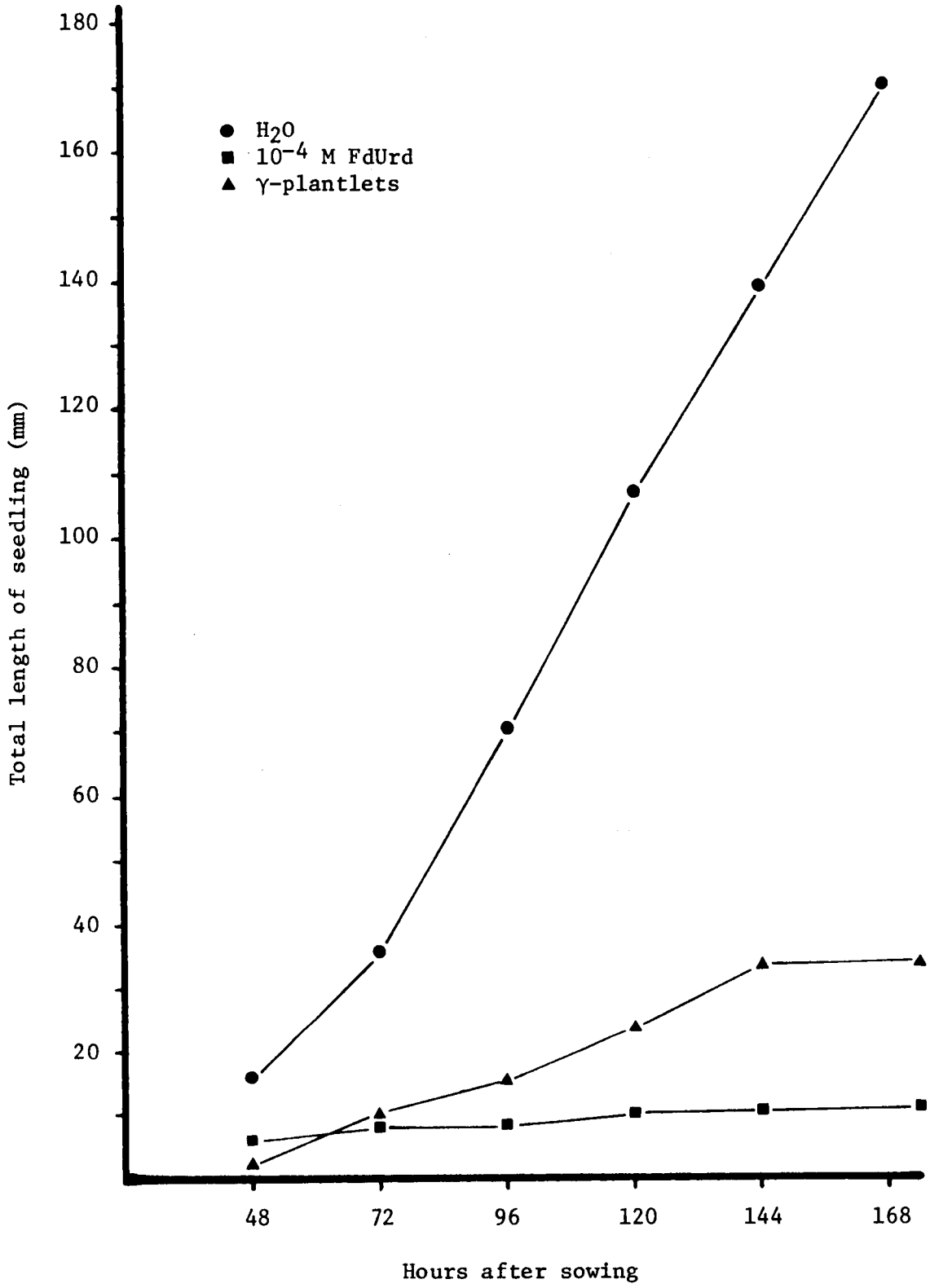

Figure 13. Comparison of growth responses of normal seedlings, FdUrd-treated seedlings and gamma-plantlets grown under standard conditions.

gamma-plantlets, selection of the lowest effective concentration of the inhibitor, 10^{-4} M FdUrd, was based on consideration of all data which included seedling length measurements, DNA content, and protein profiles (Figure 14).

Esterase. Gamma-plantlets grown under standard conditions demonstrated only the three fastest moving bands 12 hours after sowing (Figure 15). At 48 hours the fast group is complete with five bands. The fastest intermediate band, E_{GLi-1}, was observed between 72 and 120 hours after sowing. By the third day the total complement of 10 bands--five fast and five intermediate--had appeared.

Growth of the gamma-plantlet in the dark resulted in the production of a total of 11 esterase bands (Figure 16). The three fastest moving bands were seen at 12 hours after sowing. The remaining two bands of the fast group, E_{GDf-4} and E_{GDf-5}, appeared at 72 hours demonstrating a lag of 24 hours over the light-grown system. A lightly stained smear from R_f 0.29 to 0.38 appeared at 36 hours and then developed into the distinct intermediate bands, E_{GDi-4}, E_{GDi-5}, and E_{GDi-6}, by 48 hours after sowing. Next E_{GDi-3} was observed at 60 hours as a faint band which increased in intensity during development and then the last two bands, E_{GDi-1} and E_{GDi-2}, appeared after 96 hours. In this dark-grown system no band was observed to correspond with the short duration band E_{GLi-1}.

The esterase zymogram for FdUrd-treated seedlings grown in the light is presented in Figure 17. A total complement of 13 bands--seven fast and six intermediate-migrating--was observed. The three fastest moving bands were present at 12 hours and all of the fast-moving

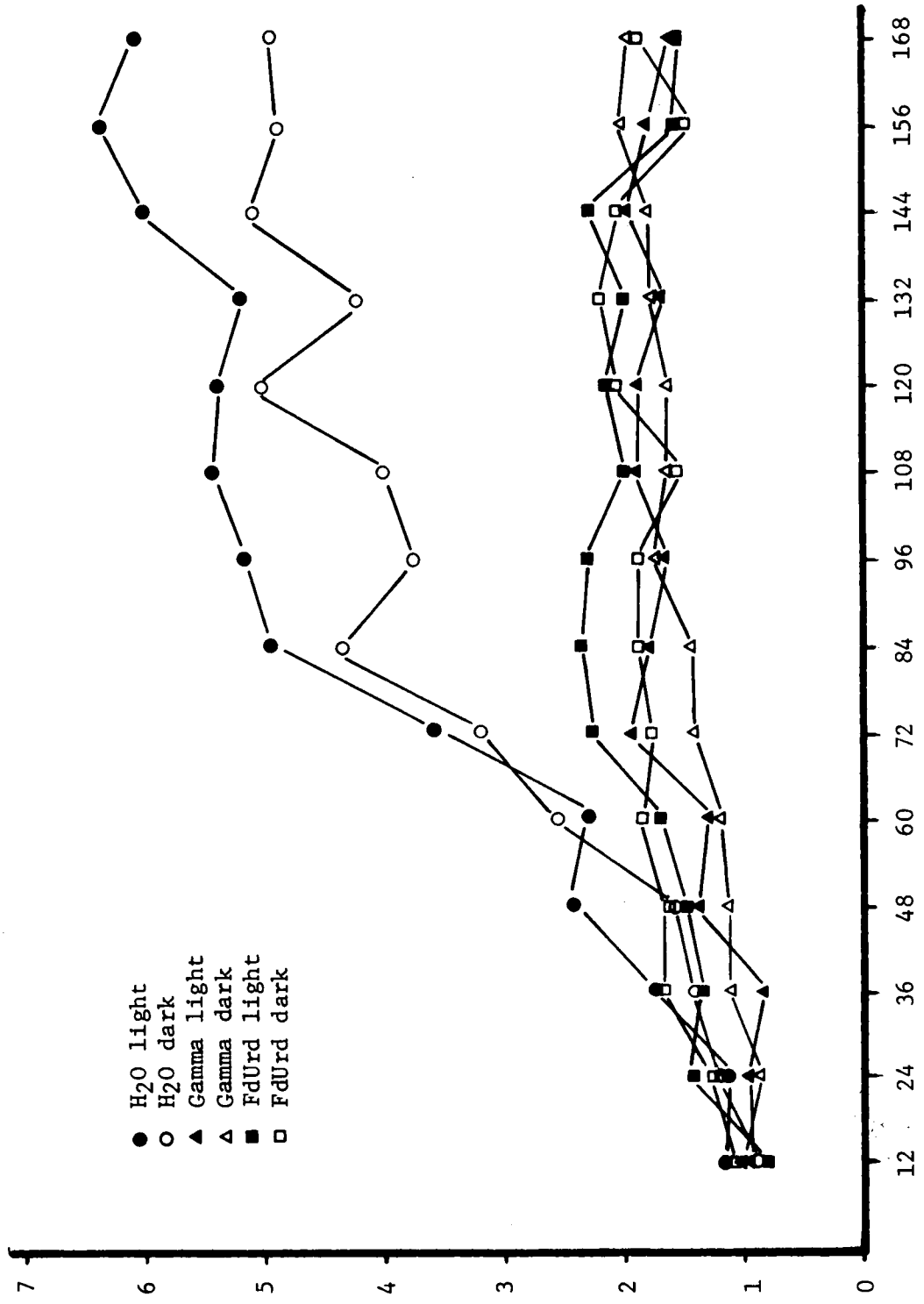

Figure 14. Protein content per seedling for each treatment.

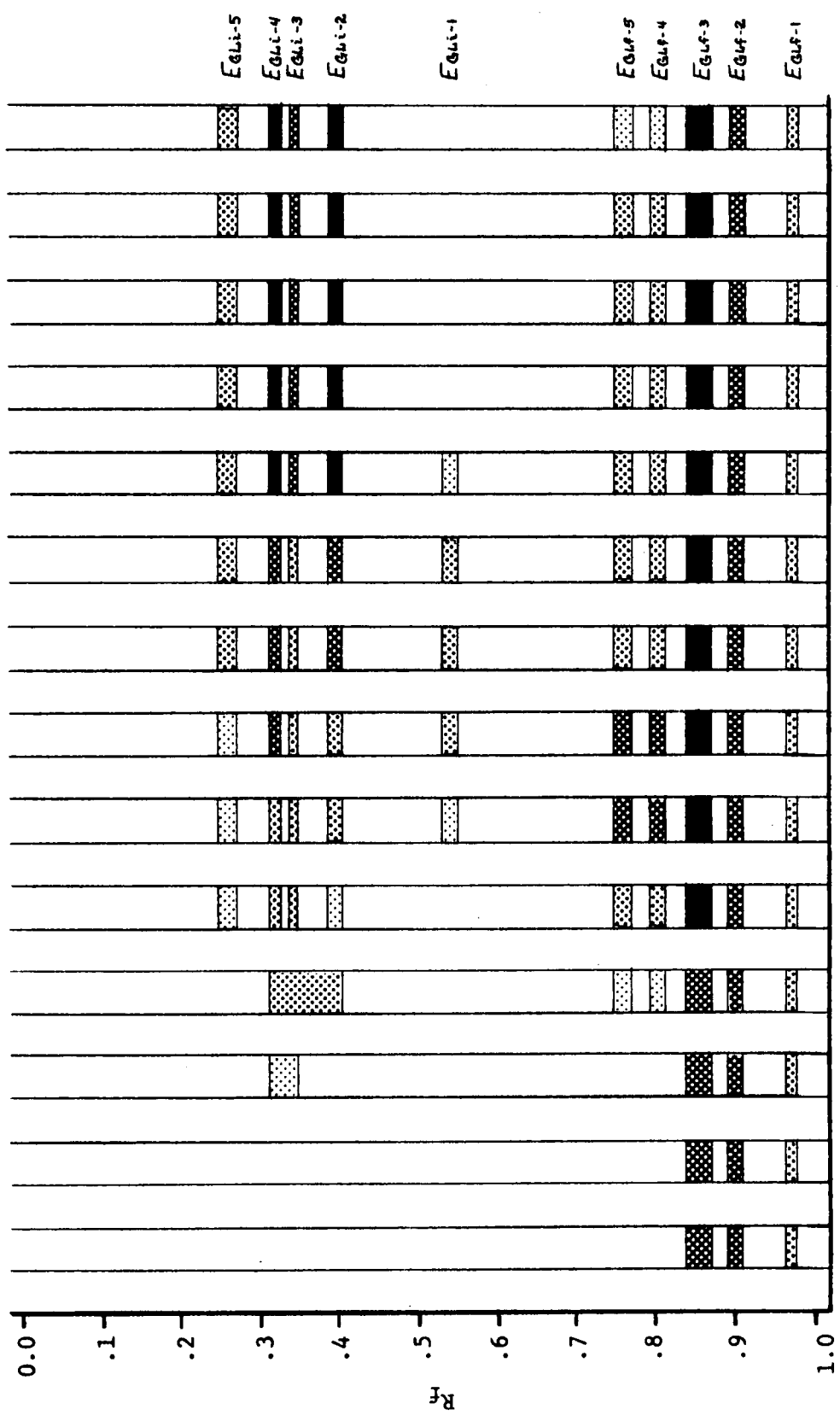

Figure 15. Developmental expression of esterase bands from light-grown gamma-plantlets.

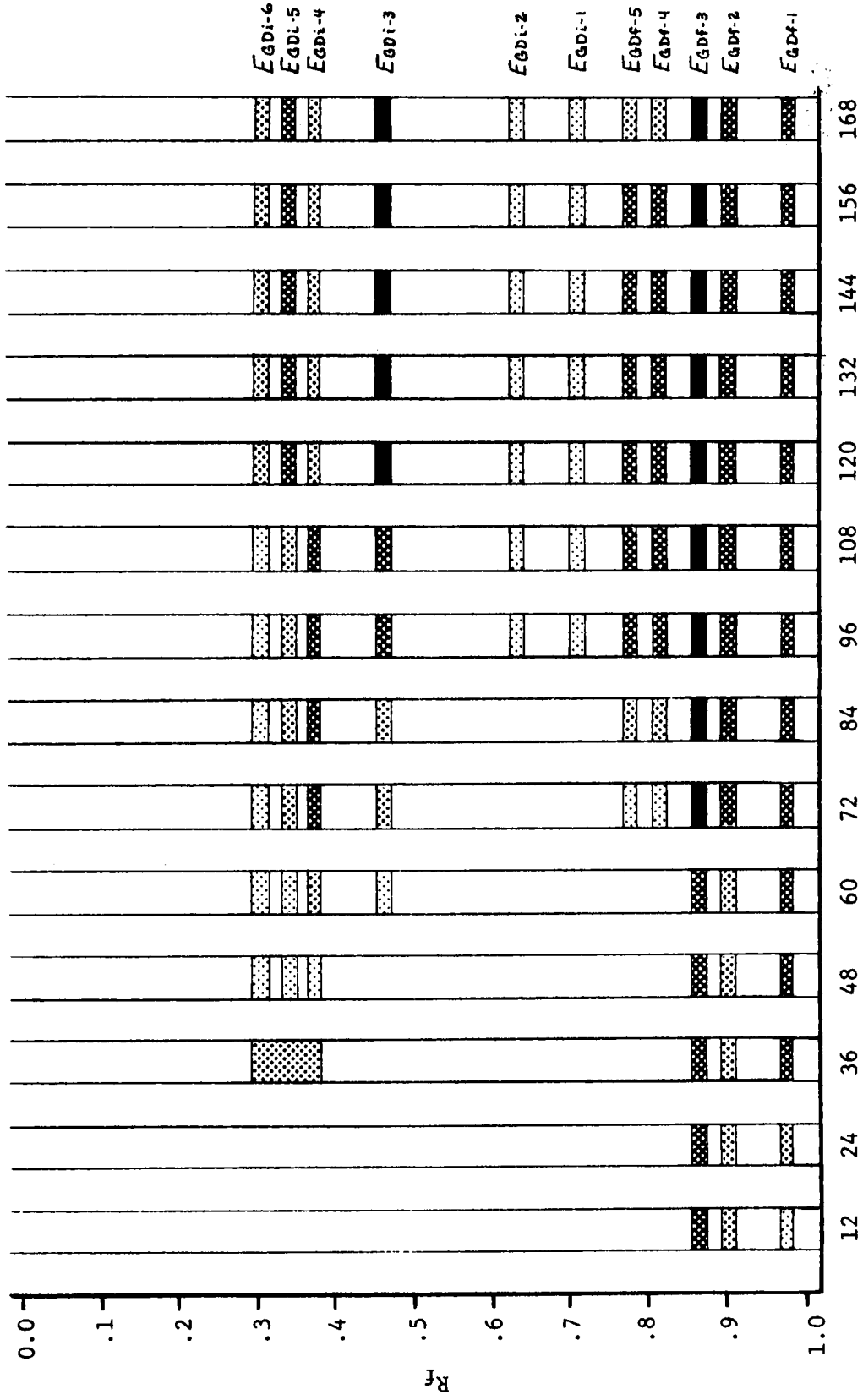

Figure 16. Developmental expression of esterase bands from dark-grown gamma-plantlets.

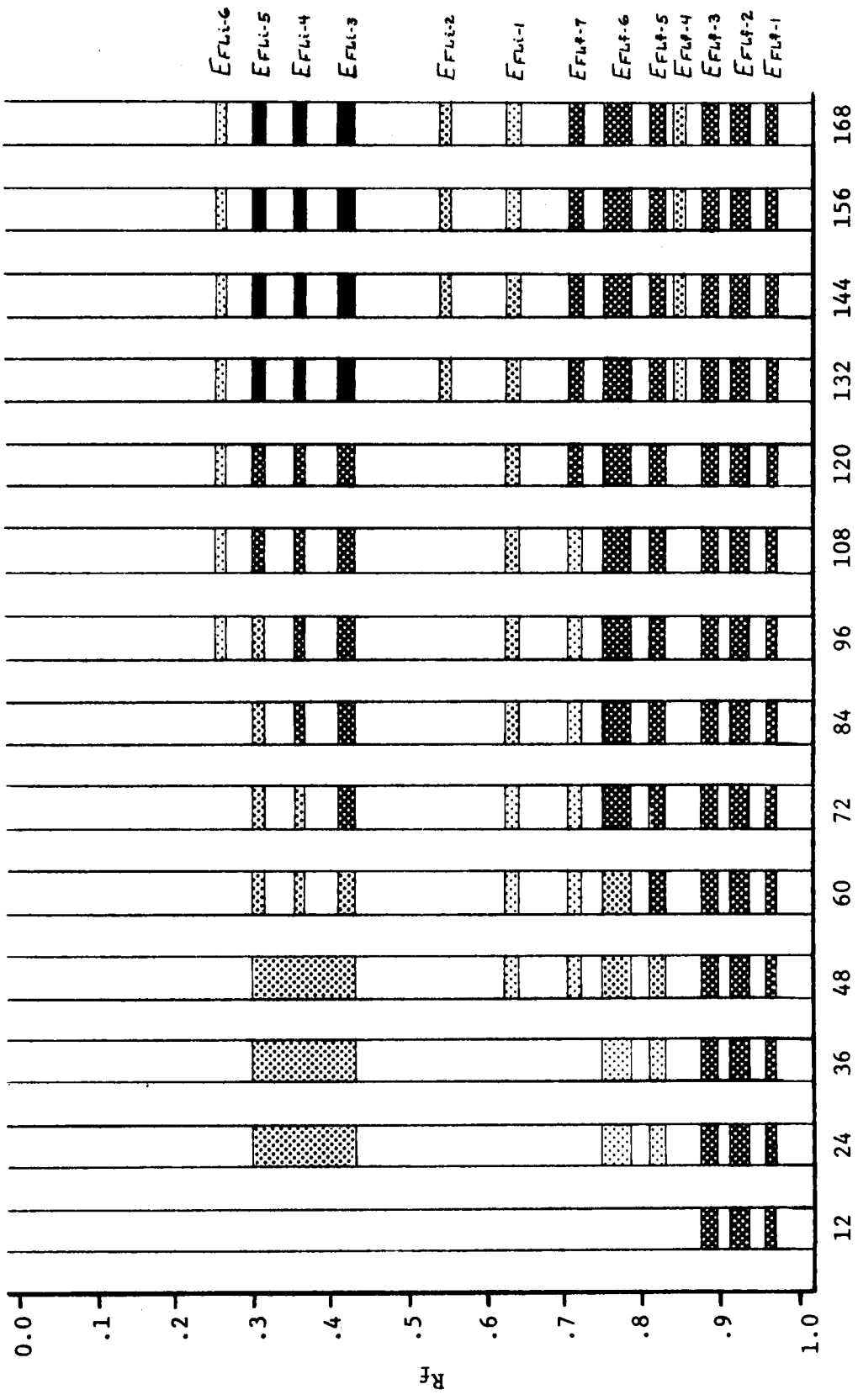

Figure 17. Developmental expression of esterase bands from light-grown FdUrd-treated seedlings.

group was observed by 48 hours except for E_{FLf-4} which did not appear until much later in development (at 132 hours). One of the intermediate bands E_{FLi-2} also did not appear until 132 hours after sowing.

The dark-grown FdUrd-treated seedlings displayed a total complement of 14 bands with esterase activity (Figure 18). The overall pattern is very similar to that presented under light conditions with the following differences: (a) the appearance of the slow-migrating band E_{FDs-1}; (b) two of the fast bands, E_{FDf-5} and E_{FDf-6}, appeared almost 2 days later than counterparts in the light-grown system; and (c) either the variance in the mobility of one band or the absence of bands comparable to E_{FLf-7} and E_{FDi-1} was noted under opposing growth conditions.

Malate dehydrogenase. Developmental MDH patterns of light- and dark-grown gamma-plantlets are presented in Figures 19 and 20, respectively. Again the variability in the R_f values and the overlapping of the bands with MDH activity was recorded in the FdUrd-treated seedlings grown in the light (Figure 21). The data shown in Figure 22 for dark-grown FdUrd-treated seedlings represented one of the few times that the bands migrated with the same relative mobility throughout the developmental sequence.

Peroxidase. A total of nine peroxidase bands--three fast, two intermediate, and four slow-migrating--was observed in the gamma-plantlets exposed to light during their development (Figure 23). Only P_{GLs-2} appeared at 36 hours while most of the bands did not appear until 48 hours after sowing. At 72 and 84 hours all of the bands were present since P_{GLi-2} occurred only over this very brief 12 hour period.

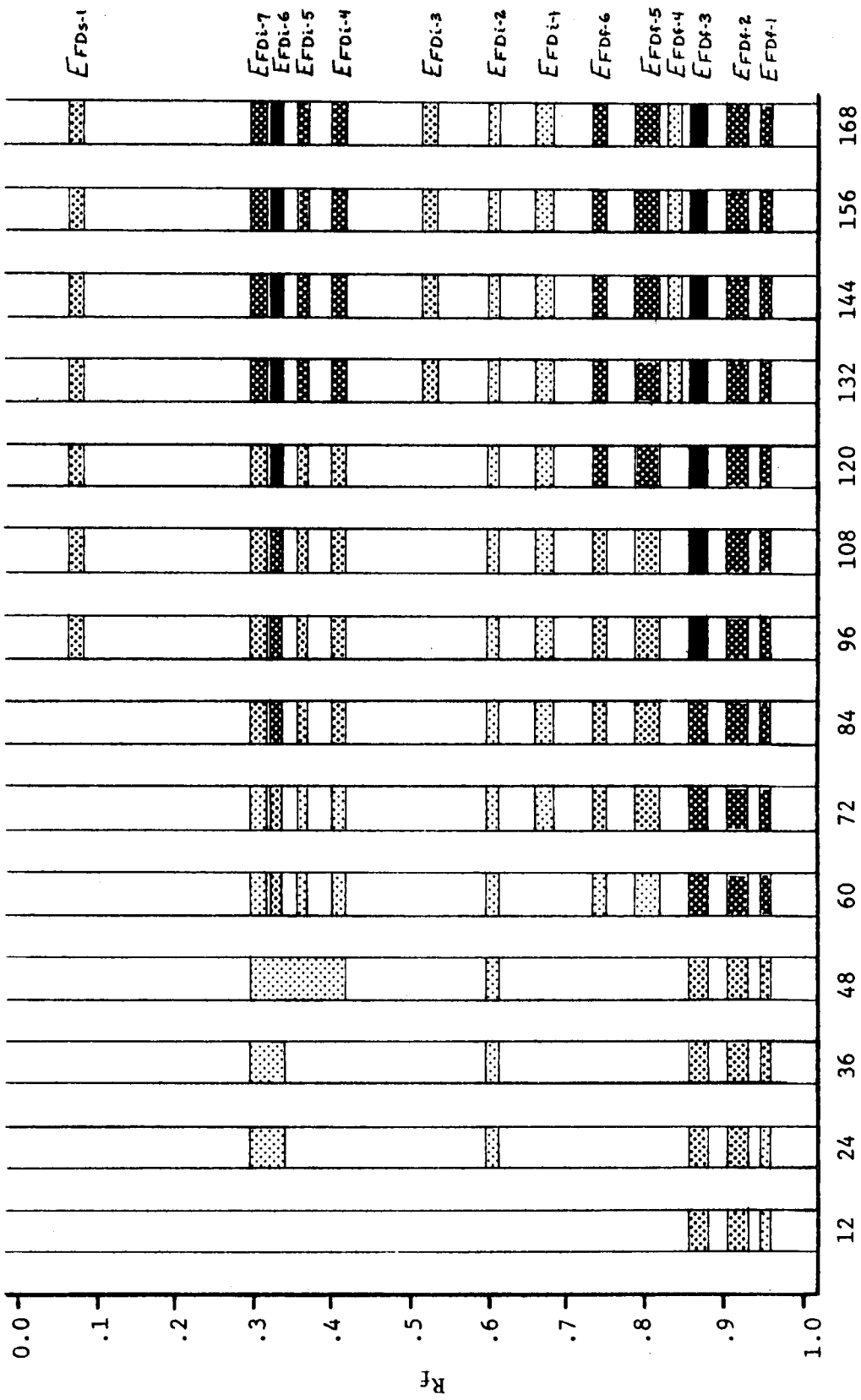

Figure 18. Developmental expression of esterase bands from dark-grown FdUrd-treated seedlings.

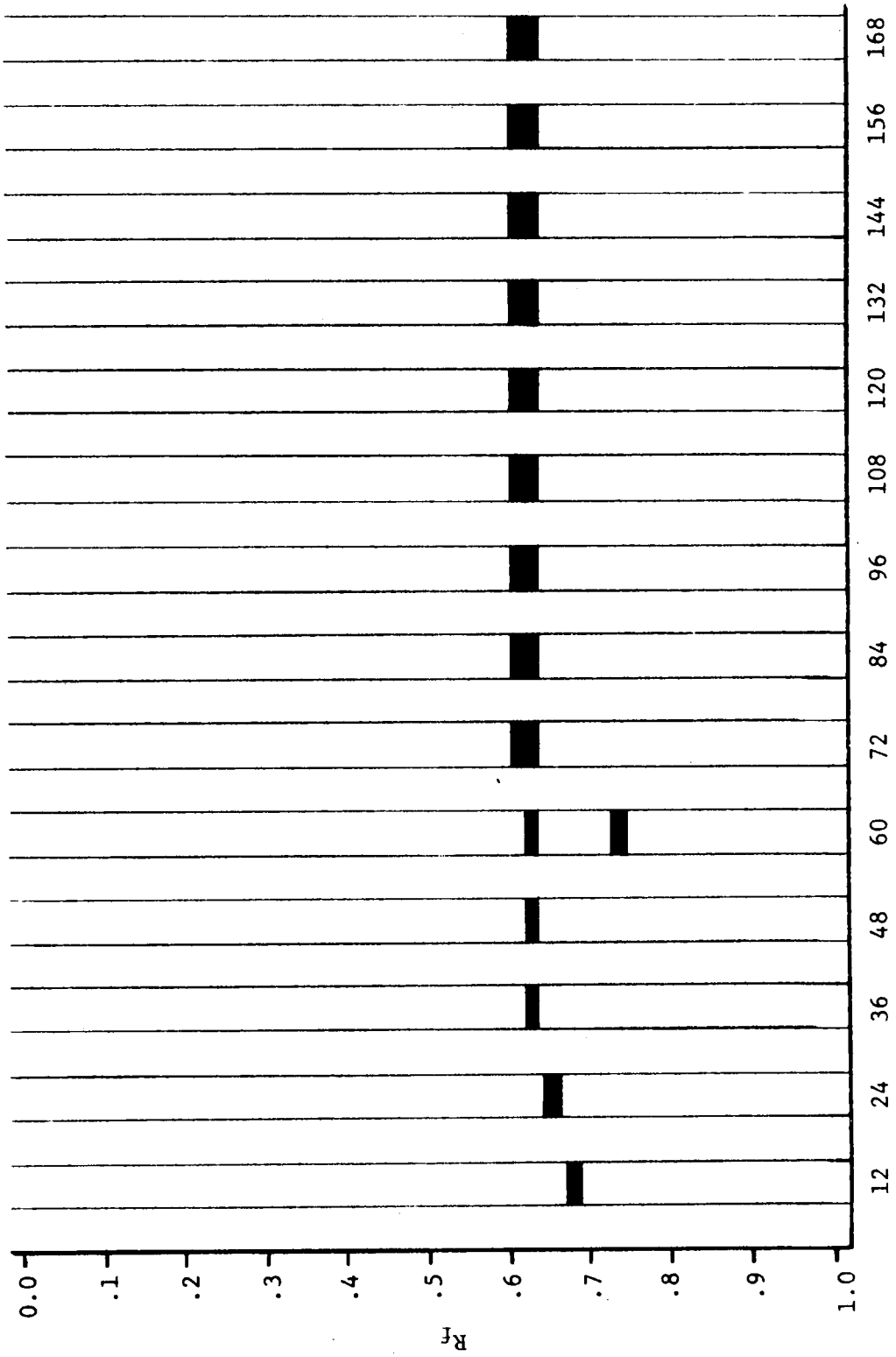

Figure 19. Developmental expression of MDH bands from light-grown gamma-plantlets.

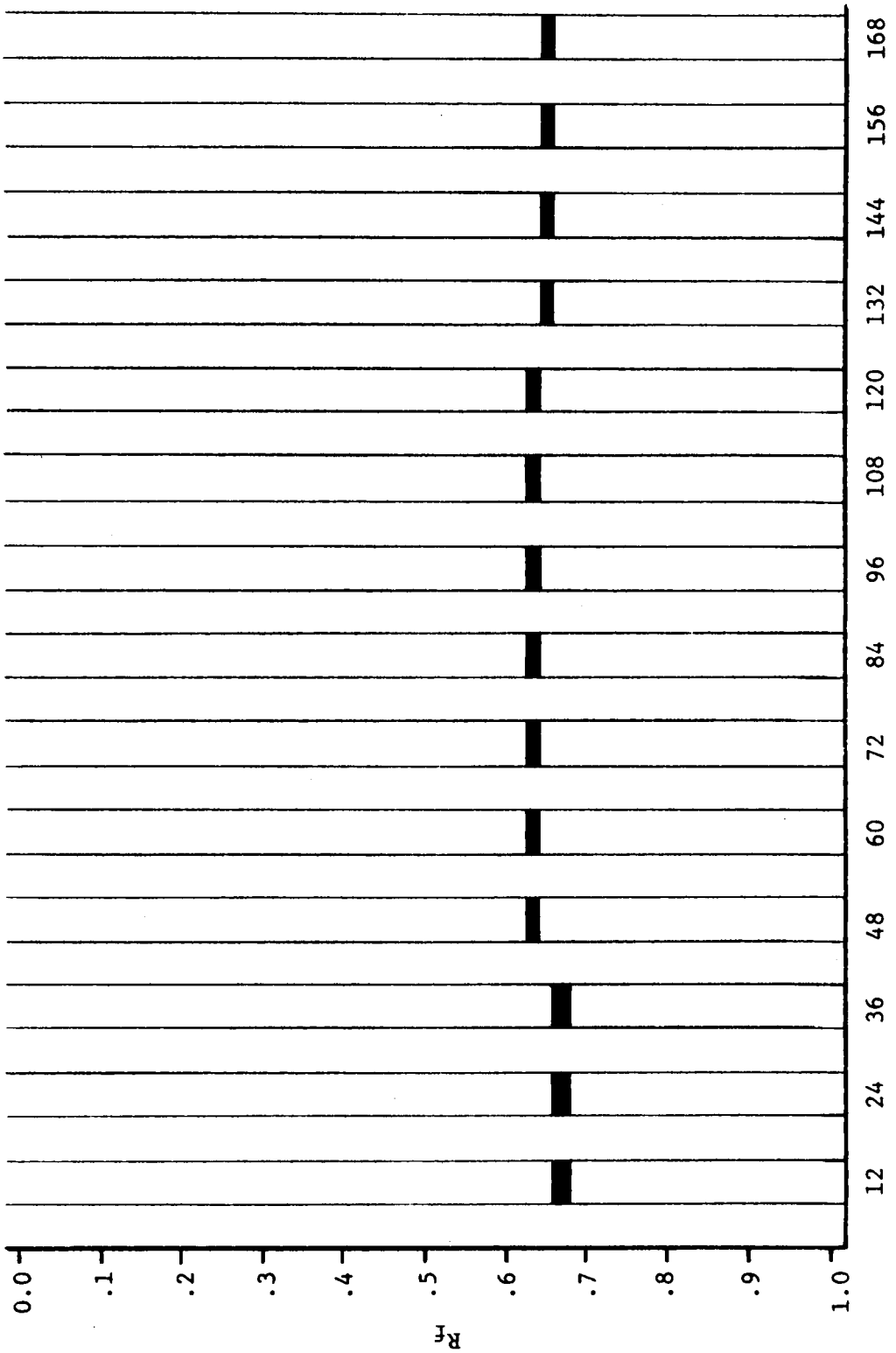

Figure 20. Developmental expression of MDH bands from dark-grown gamma-plantlets.

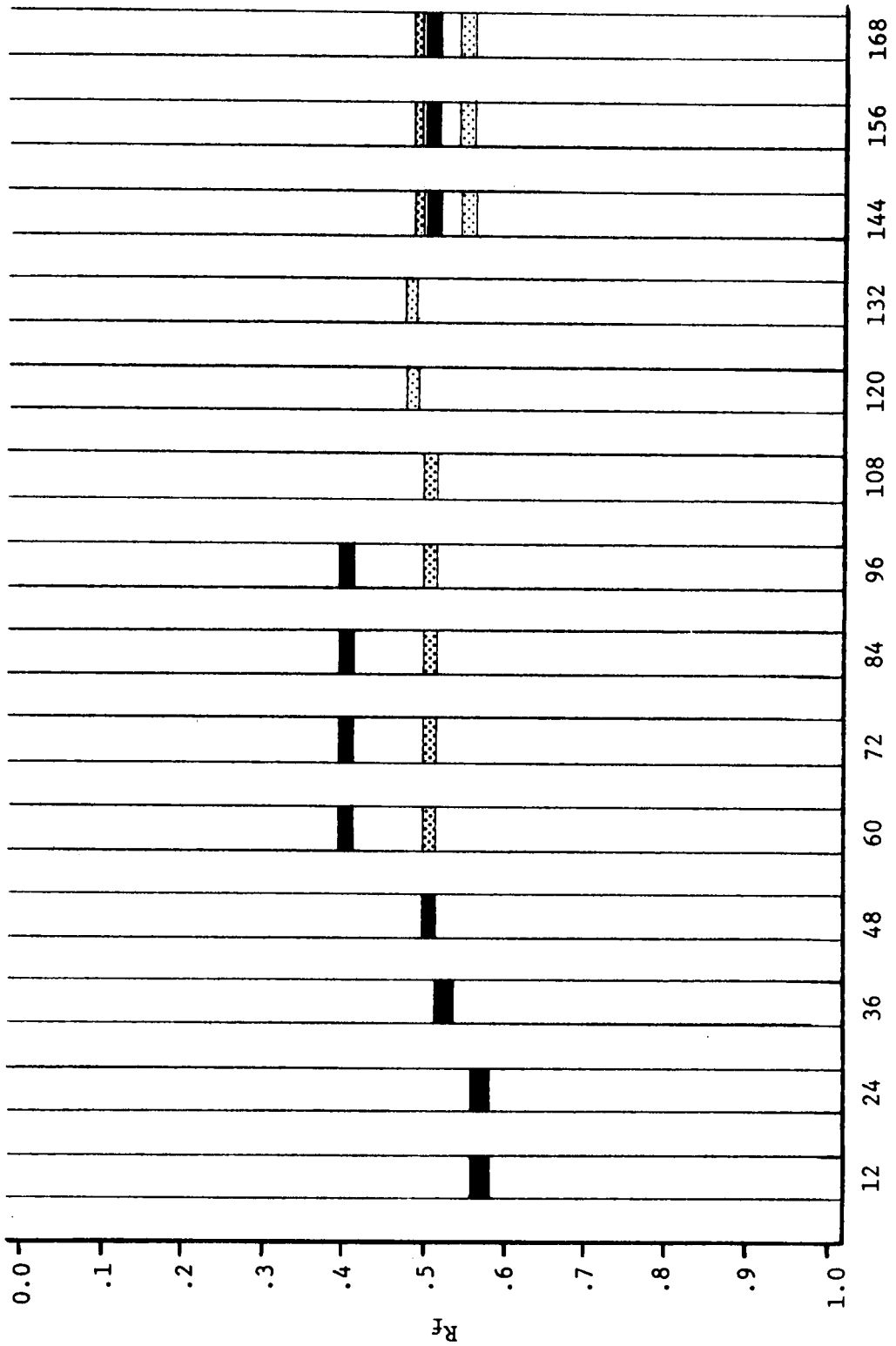

Figure 21. Developmental expression of MDH bands from light-grown FdUrd-treated seedlings.

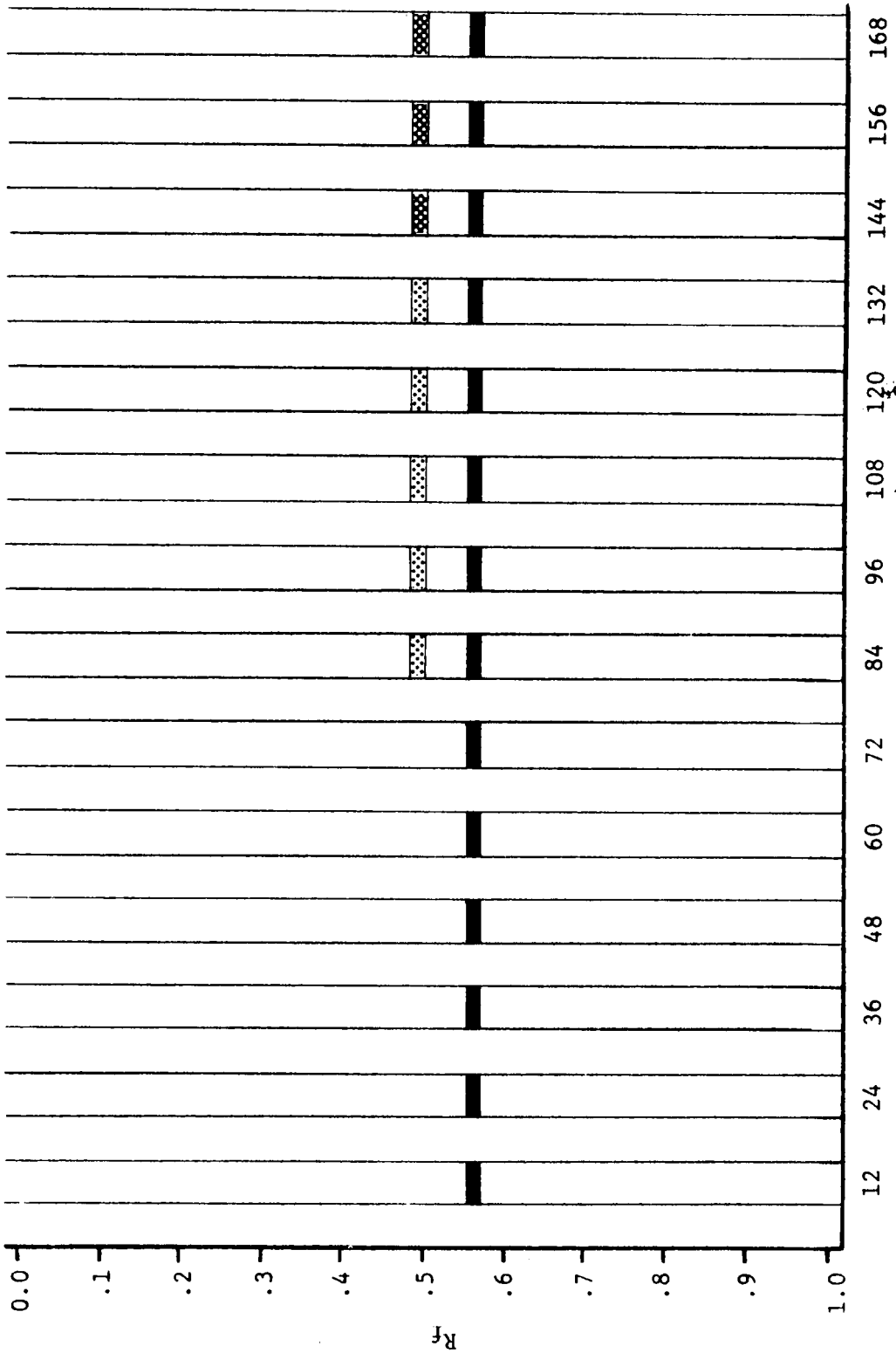

Figure 22. Developmental expression of MDH bands from dark-grown FdUrd-treated seedlings.

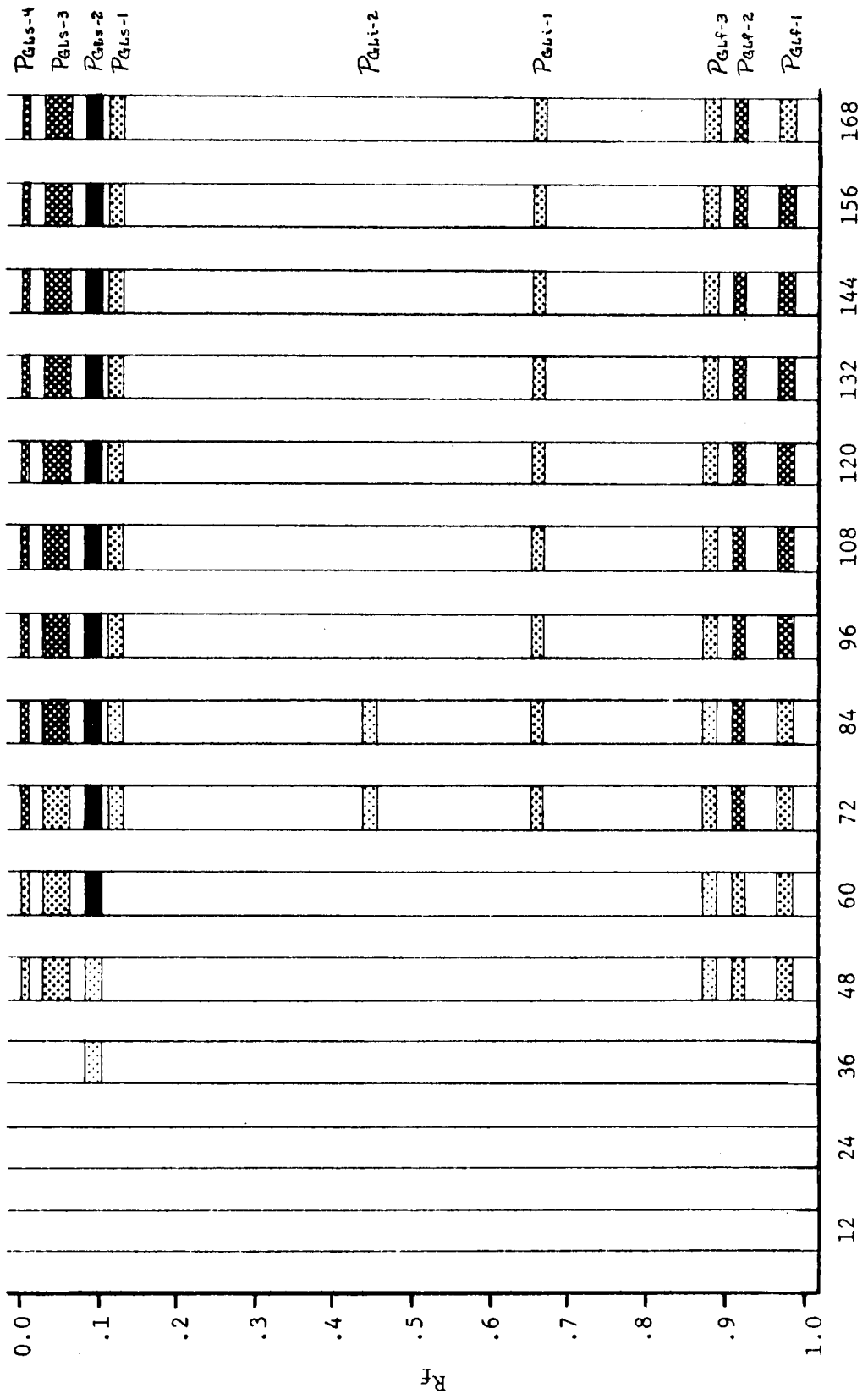

Figure 23. Developmental expression of peroxidase bands from light-grown gamma-plantlets.

Under dark conditions a total of 10 peroxidase bands was expressed (Figure 24). This number included one more slow band, P_{GDs-1}, than observed in the light-grown gamma-plantlet. All bands from the dark-grown system continued their appearance throughout the developmental sequence once expressed, except for P_{GDs-1} which occurred only from 72 to 96 hours after sowing.

Seeds treated with FdUrd whether grown under light or dark conditions, exhibited almost identical developmental patterns (Figures 25 and 26). In each, the second intermediate band, P_{FLi-2} or P_{FDi-2}, occurred only during the period of 48 to 132 hours after sowing. The appearance of the slow-moving bands was delayed until 36 hours in the dark while P_{FLs-4} occurred at 12 hours in the light and this band plus P_{FLs-2} and P_{FLs-3} also were present by 24 hours after sowing.

Enzymatic Activities During Development

Esterase. The total esterase activity per seedling increased under all treatments examined (Figure 27). All values showed approximately the same relative increase through the first 60 hours after sowing. The largest total activity was observed at 144 and 156 hours in the normal seedlings grown under standard conditions or in the dark. The values obtained for the gamma-plantlets and the FdUrd-treated seedlings were very similar showing an increase of approximately half that of the normal ones. The specific activity values graphed in Figure 28 show a very slight increase in esterases of the gamma-plantlets and the FdUrd-treated seedlings while the normal seedlings had final values similar to or less than initial ones.

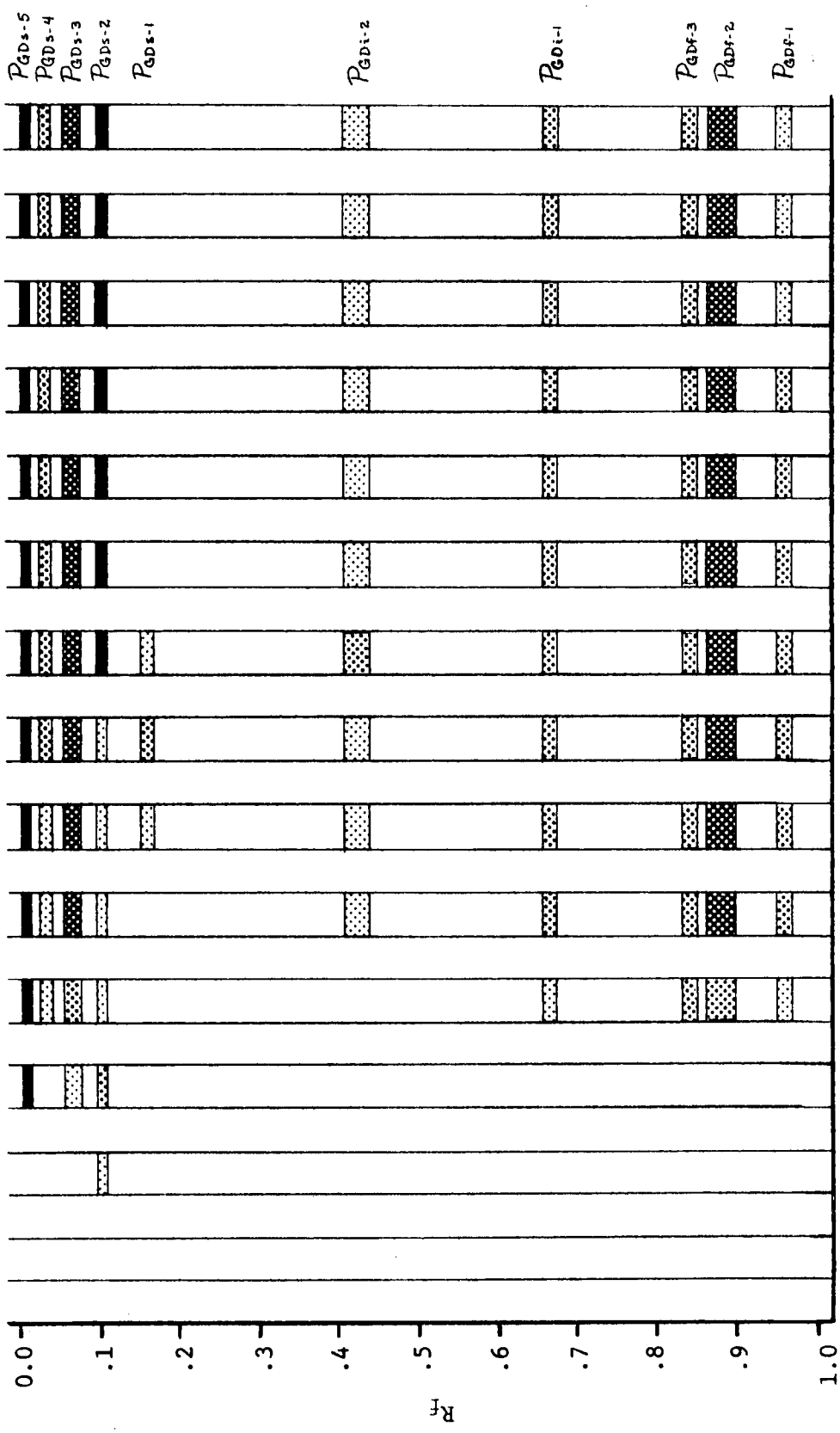

Figure 24. Developmental expression of peroxidase bands from dark-grown gamma-plantlets.

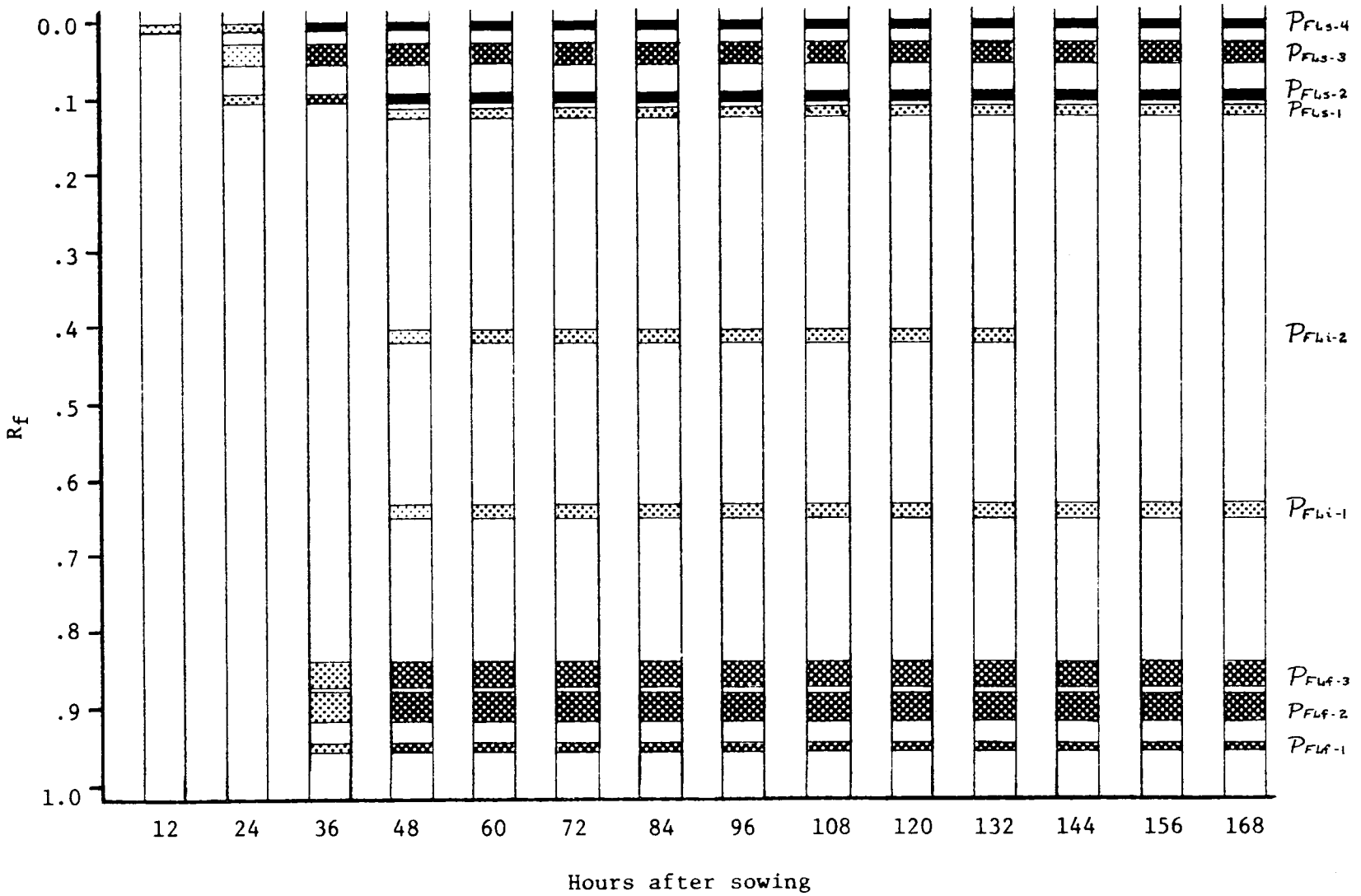

Figure 25. Developmental expression of peroxidase bands from light-grown FdUrd-treated seedlings.

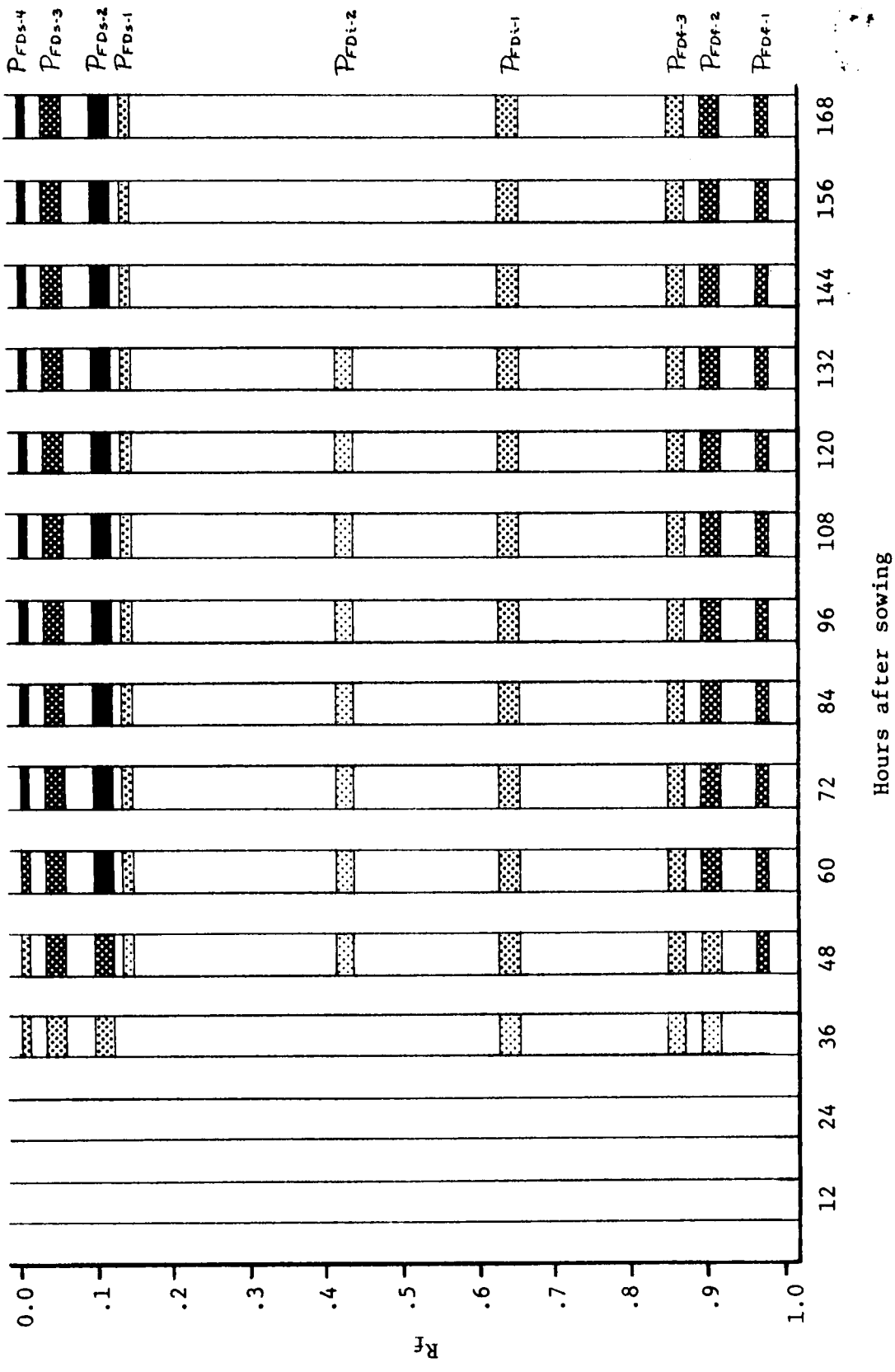

Figure 26. Developmental expression of peroxidase bands from dark-grown FdUrd-treated seedlings.

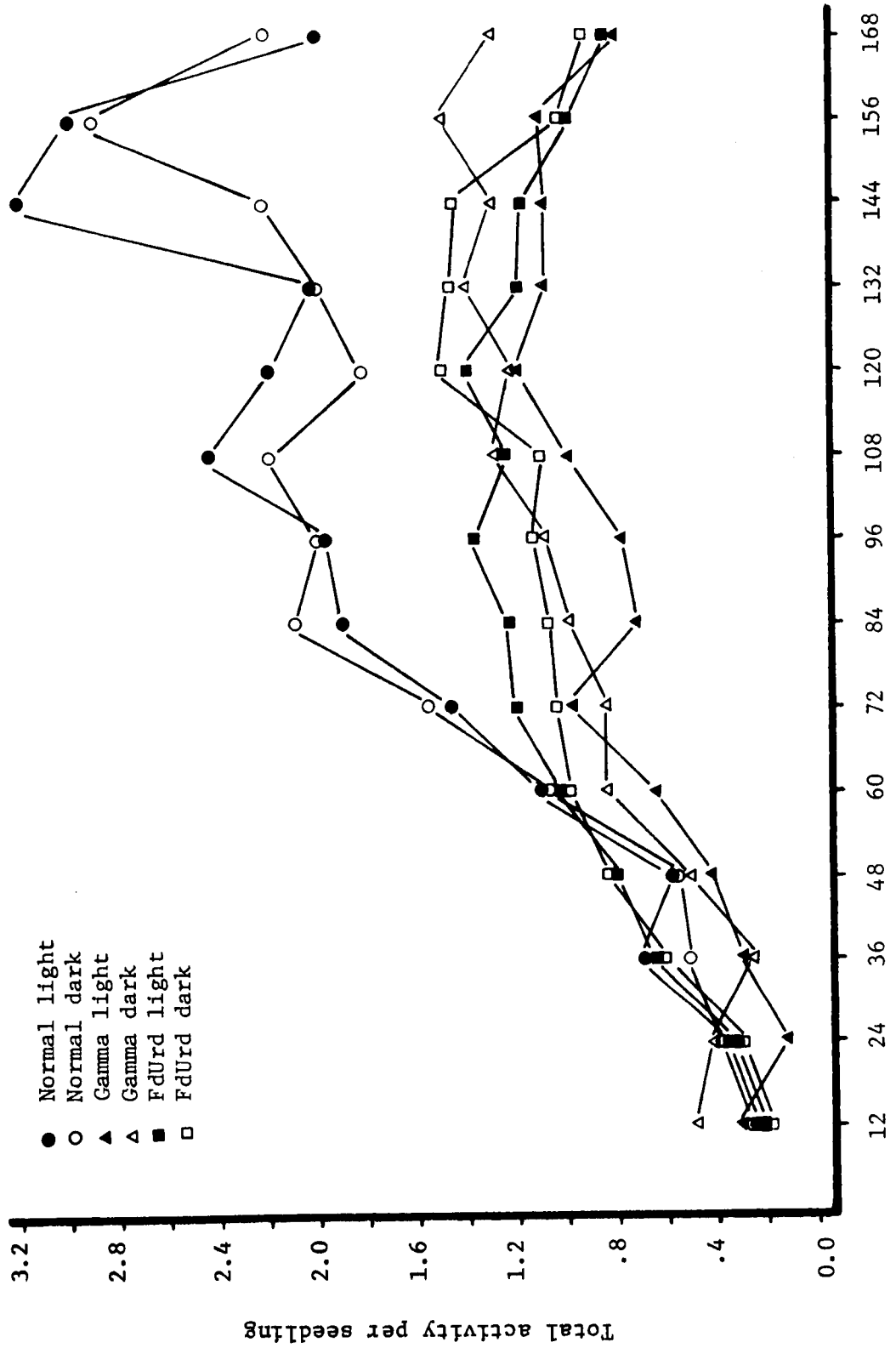

Figure 27. Total esterase activity per seedling for each treatment.

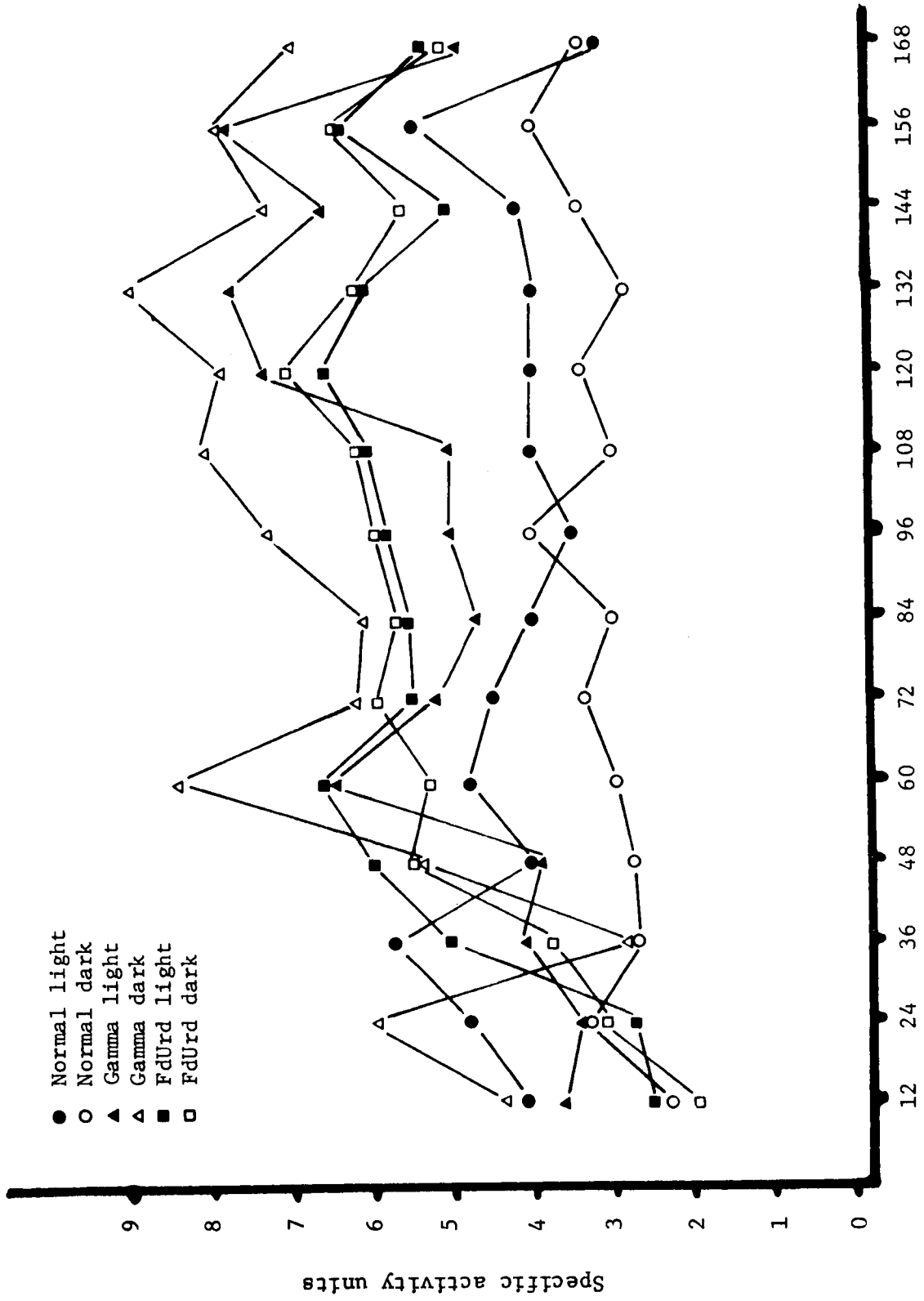

Figure 28. Specific activity of esterases for each treatment.

Malate dehydrogenase. During the developmental period under investigation essentially no change occurred in the total MDH activity in the gamma-plantlet or the FdUrd-seedling (Figure 29). There was a four- and six-fold increase in the total amount of MDH in the normal dark-grown and light-grown seedlings, respectively. The total activity values remained at approximately their initial levels during the first 60 hours after sowing. From the data presented in Figure 30, the specific activity of MDH decreased by the end of the 7-day period under most conditions examined or remained unchanged in the dark-grown FdUrd-treated seedlings.

Peroxidase. During the first 36 hours, very little peroxidase activity was detected in any of the seedlings examined (Figure 31). Then the total activity of the normal light- and normal dark-grown seedlings began to increase more rapidly by 48 hours than did the values for the other treatments. After 60 hours the peroxidase content in the FdUrd-treated seedlings appeared to change only slightly, while the activity in both the gamma-plantlets and the normal seedlings increased at a much greater rate. The normal seedlings grown under standard conditions attained the highest levels.

Figure 32 presents the specific activity of peroxidase observed in wheat seedlings. All values are small and closely clustered during the first 36 hours after sowing. The specific peroxidase activity in the gamma-plantlets increased very rapidly until 96 hours and then leveled off. Normal and FdUrd-treated seedlings had specific activities which displayed more moderate increases than the gamma-plantlets.

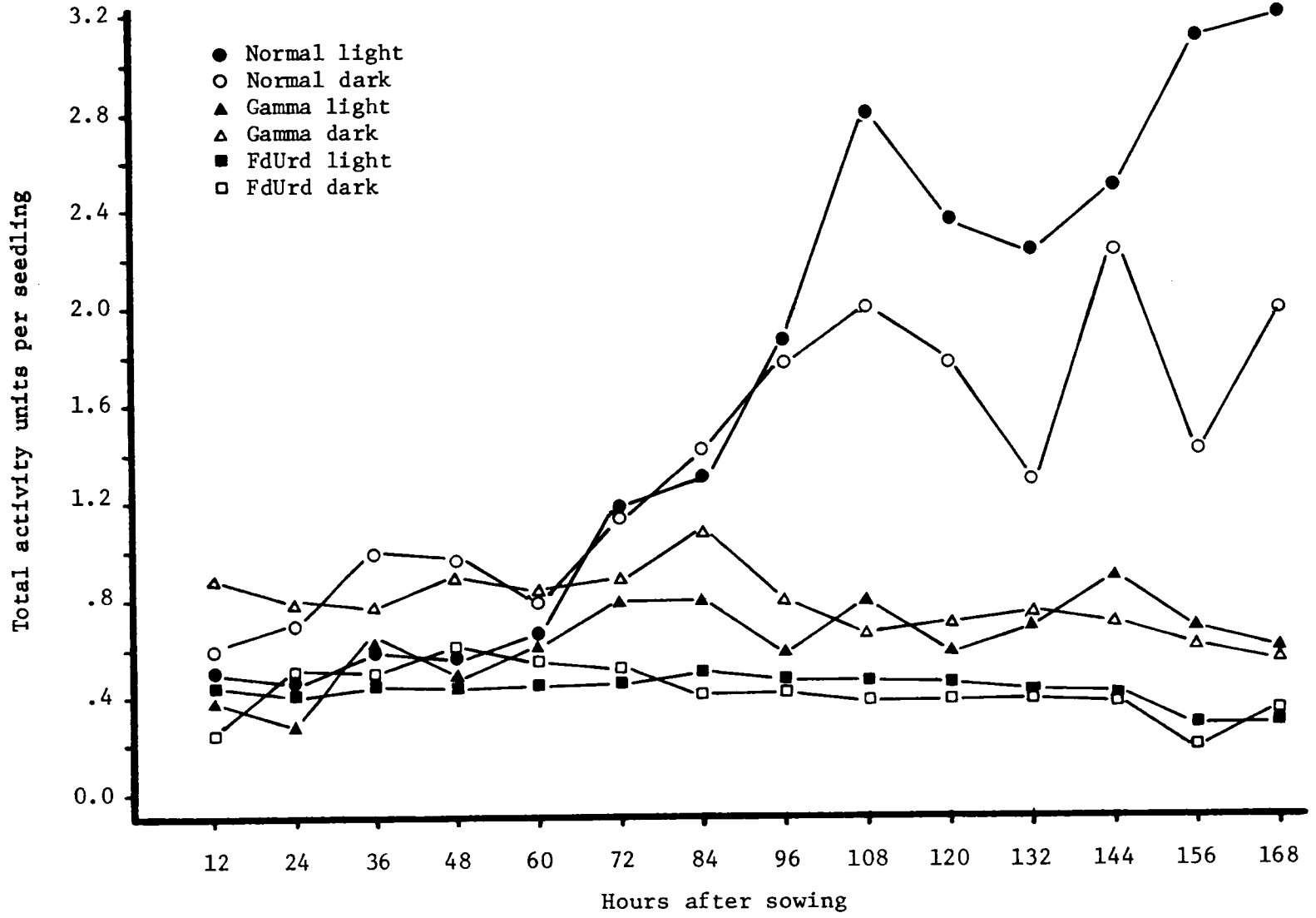

Figure 29. Total MDH activity per seedling for each treatment.

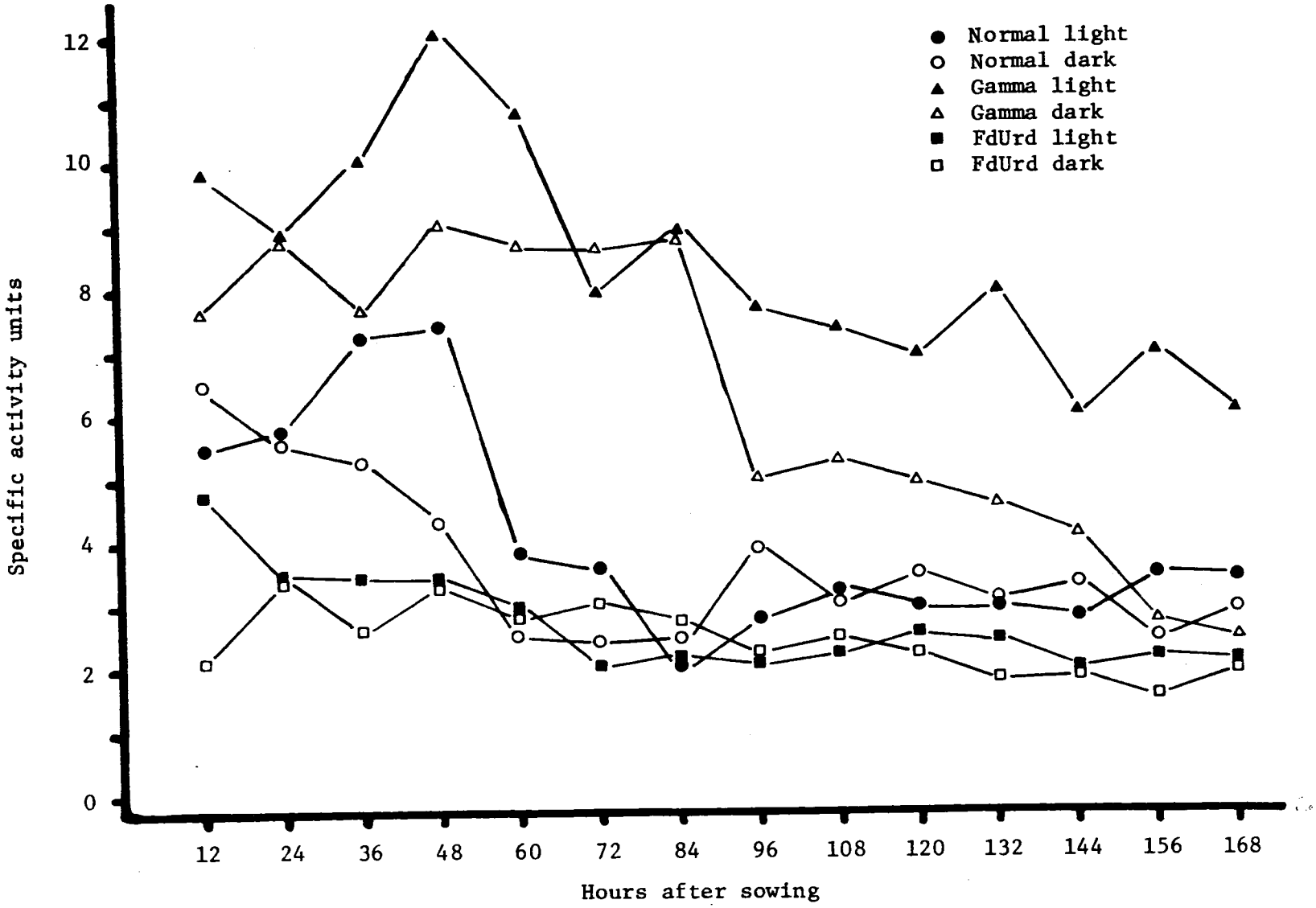

Figure 30. Specific MDH activity for each treatment.

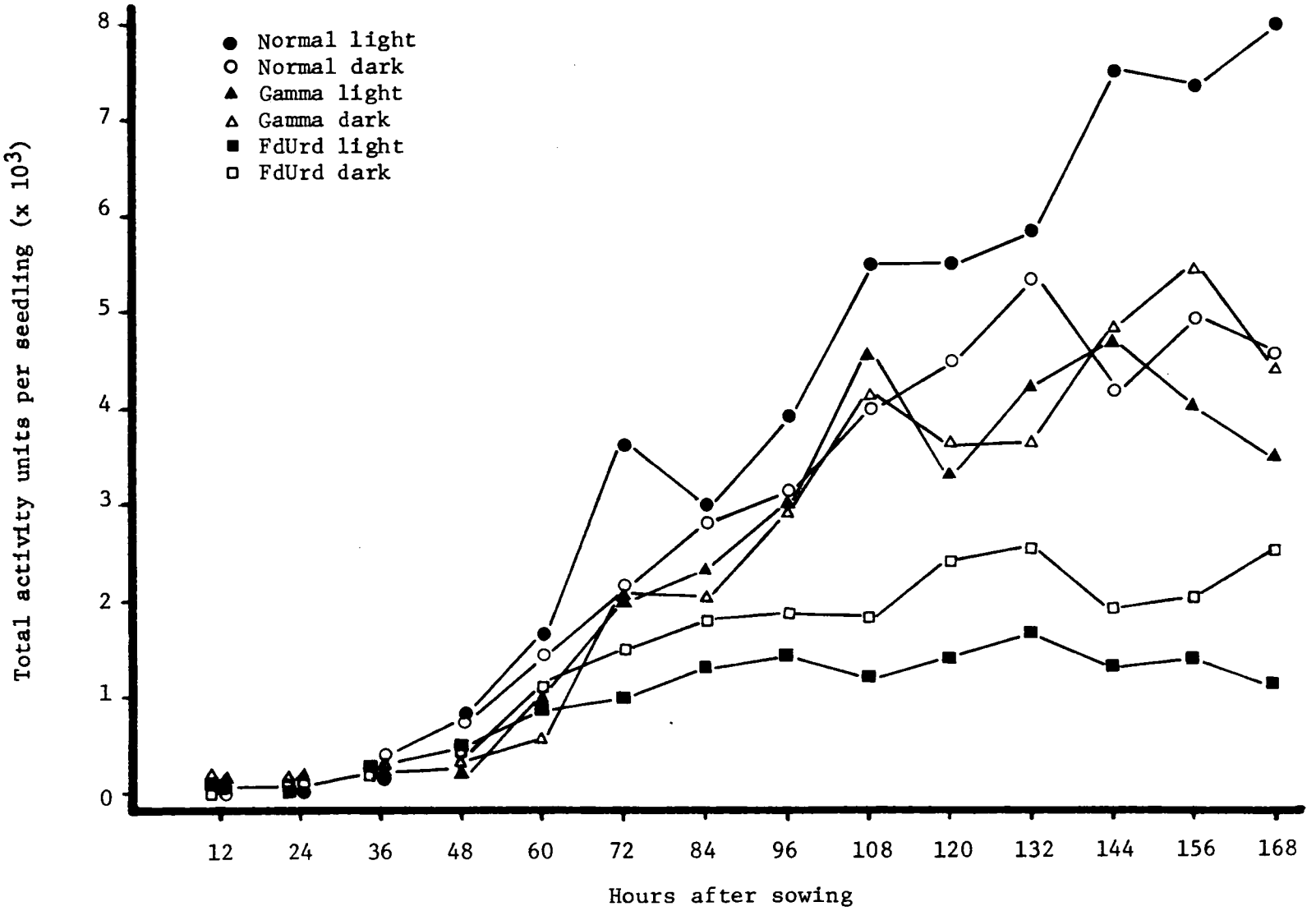

Figure 31. Total peroxidase activity per seedling for each treatment.

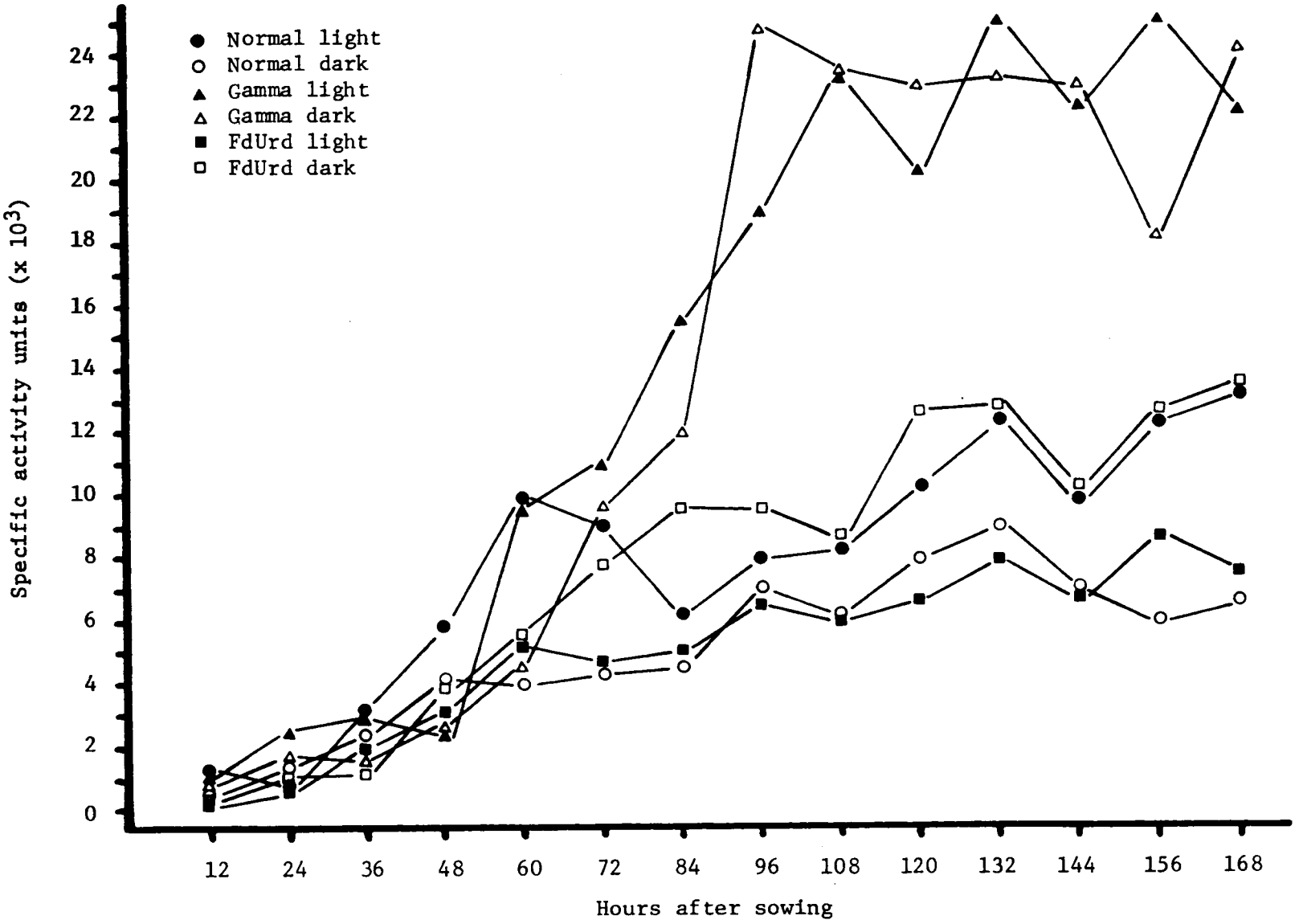

Figure 32. Specific activity of peroxidases for each treatment.

Enzymatic Profiles of the Root, Leaf, and Coleoptile

Esterase. At 72 hours after sowing a total of nine esterase bands was observed in the whole seedling (Figure 33). All of the intermediate bands were found in the leaf and the coleoptile, but E_{0i-1} was not observed in the root. In addition, the root lacked the fast bands E_{0f-3}, E_{0f-4}, and E_{0f-5}. At 72 hours after sowing only the leaf contained the fast-migrating band, E_{0f-3}, which later appeared in both the leaf and coleoptile. The differences after 144 hours were: (a) the absence of E_{0f-5} in all preparations, (b) the deletion of the fastest moving band, E_{0f-1}, in the coleoptile, and (c) the addition of the intermediate band, E_{0i-2}, in each 144 hour preparation except in the root.

Figure 34 shows that at 72 hours approximately two-thirds of the total esterase activity was located in the roots. The leaf and coleoptile each had only a very small amount of esterase. Also, at 144 hours the root possessed the largest proportion of the activity; however, the leaf contained only 12 percent less activity than the root while the coleoptile showed only 50 percent less activity found in the root. The organ with the highest esterase specific activity at both times samples was the root (Table 1). The data further indicates that the specific activity in the coleoptile was greater than that observed in the leaf.

Malate dehydrogenase. The basic banding pattern for the 72 hour seedlings showed no differences following the organ separation (Figure 35). Examination of the leaf 144 hours after sowing revealed two bands, the heavily-stained M_{0-2} which had been observed in all separations, and the faster-migrating M_{0-1}.

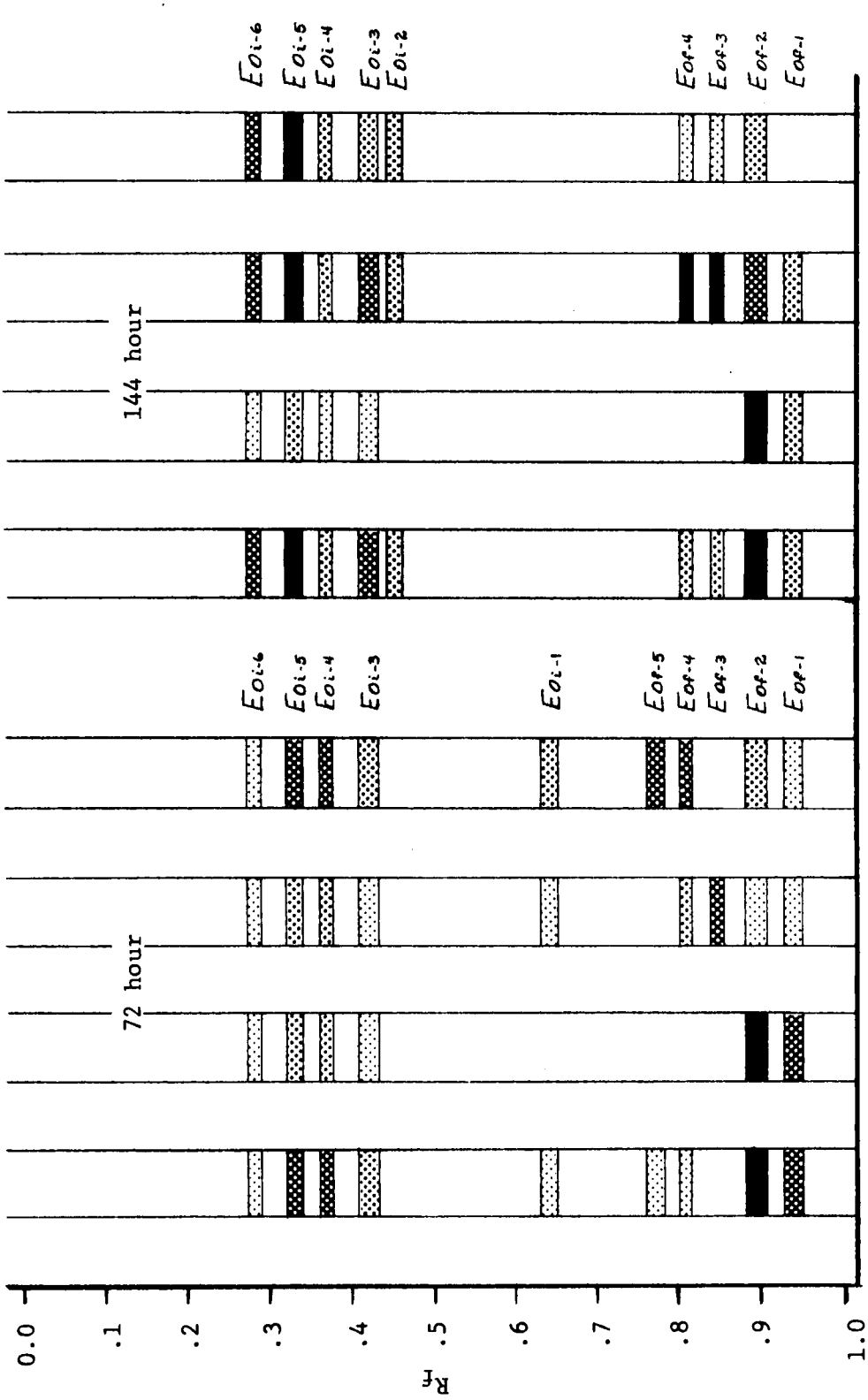

Figure 33. Esterase zymograms of whole seedlings (W), roots (R), leaves (L), and coleoptiles (C) sampled at 72 and 144 hours after sowing.

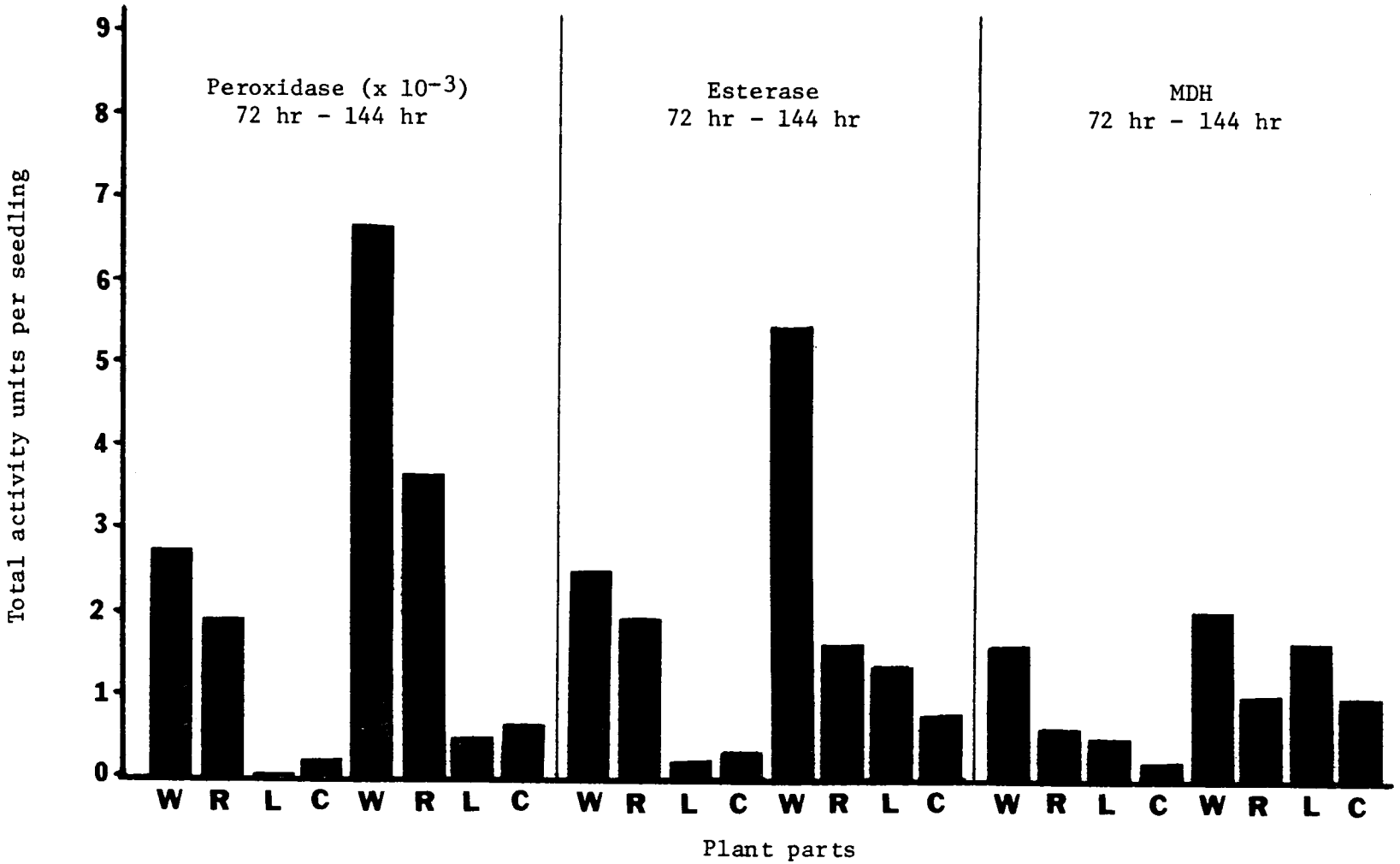

Figure 34. Total activities of organ separations sampled at 72 and 144 hours after sowing. Whole seedling (W), root (R), leaf (L), and coleoptile (C).

Table 1. Specific enzyme activities in wheat organs

Plant part	Age (hr)	Specific activity		
		Peroxidase (10^3)	Esterase	MDH
Whole	72	5.10	4.96	2.19
	144	4.58	3.91	1.73
Root	72	8.18	5.09	2.79
	144	13.90	5.54	3.27
Leaf	72	.30	2.68	4.04
	144	.84	2.51	9.54
Coleoptile	72	1.46	3.22	1.80
	144	4.60	4.08	2.17

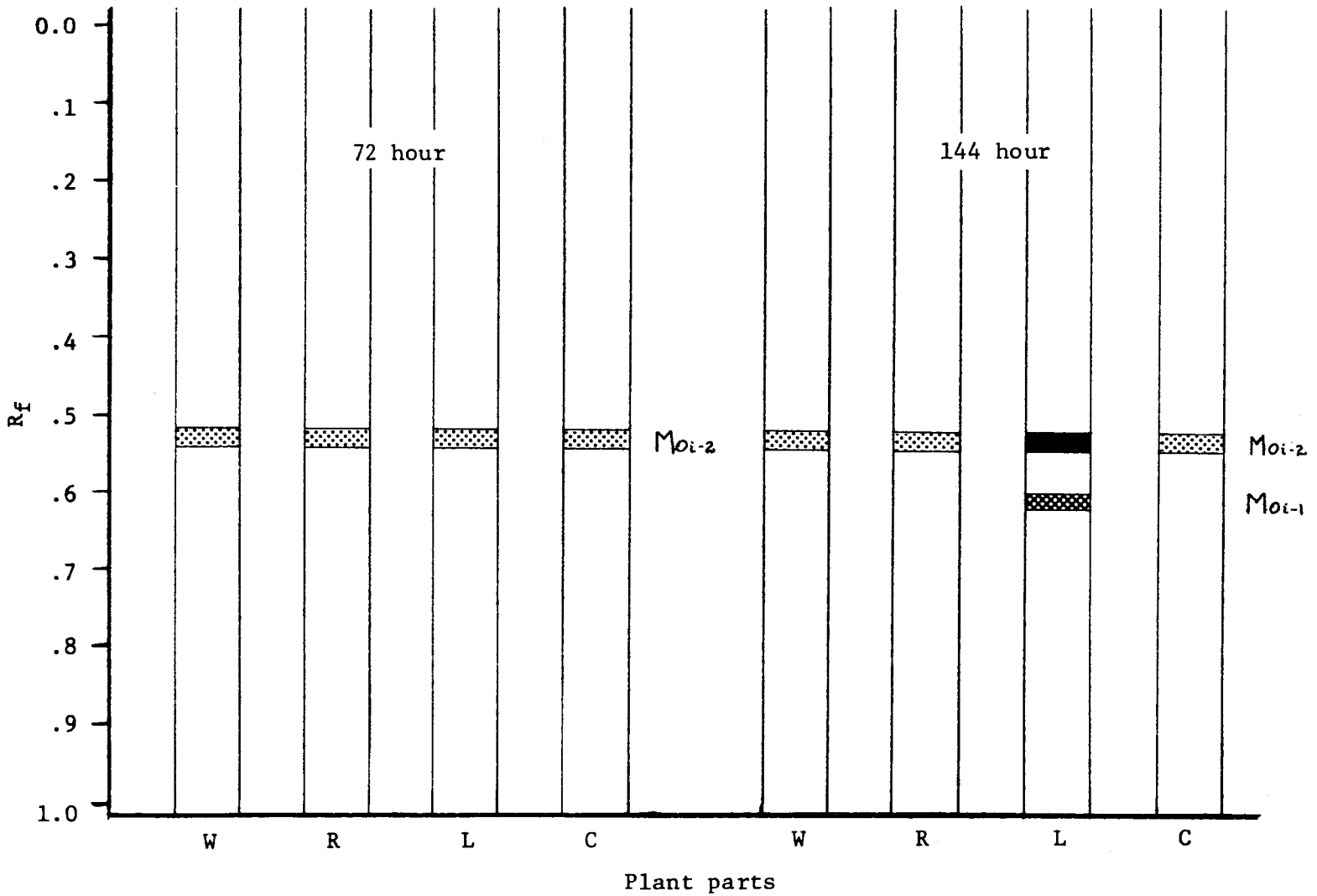

Figure 35. MDH zymograms of whole seedlings (W), roots (R), leaves (L), and coleoptiles (C) sampled at 72 and 144 hours after sowing.

Figure 34 shows that at 72 hours after sowing the root contained more of the MDH activity than the other organs. The data indicates that the leaf possessed about 10 percent less MDH than the root. At 144 hours most of the activity was observed in the leaf with 40 percent less in both the root and coleoptile. At both times sampled the specific MDH activity was highest in the leaf although the root did show considerable activity (Table 1).

Peroxidase. All the peroxidase bands recognized in the intact embryonic axis at both 72 and 144 hours after sowing were observed in the roots (Figure 36). In the younger leaf only three bands, P_{Os-4}, P_{Oi-1}, and P_{Of-2}, were observed. Later in its development two additional slow-migrating bands, P_{Os-2} and P_{Os-3}, appeared as did one fast bnad, P_{Of-1}. The band P_{Of-2} was observed only following extraction of leaf tissue. The 72 hour coleoptile displayed all of the slow-moving group, P_{Oi-1}, and only one fast band, P_{Of-3}; while at 144 hours all of the fast-moving group were present and P_{Os-1} was missing.

An average of about 65 percent of the total peroxidase activity per seedling (Figure 34) was located in the roots at both times sampled. In each case the coleoptile showed slightly more activity than the leaf; however, both of these values were small compared to the activity in the root. The specific activity of peroxidase (Table 1) was greatest in the roots and only small amounts were assayed in the leaf and coleoptile.

Examination of Segments of the First Leaf

Esterase. A total of nine bands with esterase activity was observed in the basal segment of the 7-day-old first leaf (Figure 37).

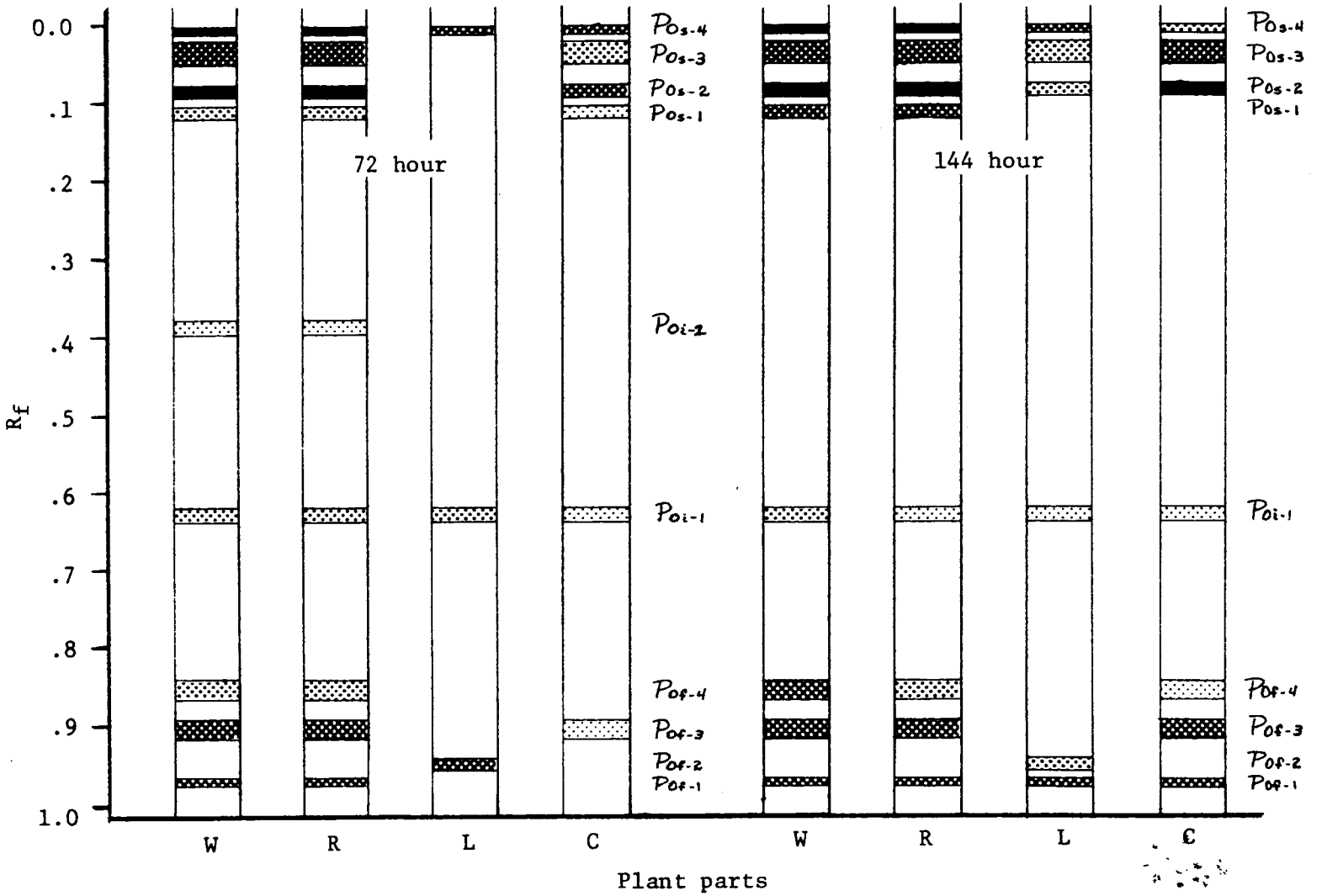

Figure 36. Peroxidase zymograms of whole seedlings (W), roots (R), leaves (L), and coleoptiles (C) sampled at 72 and 144 hours after sowing.

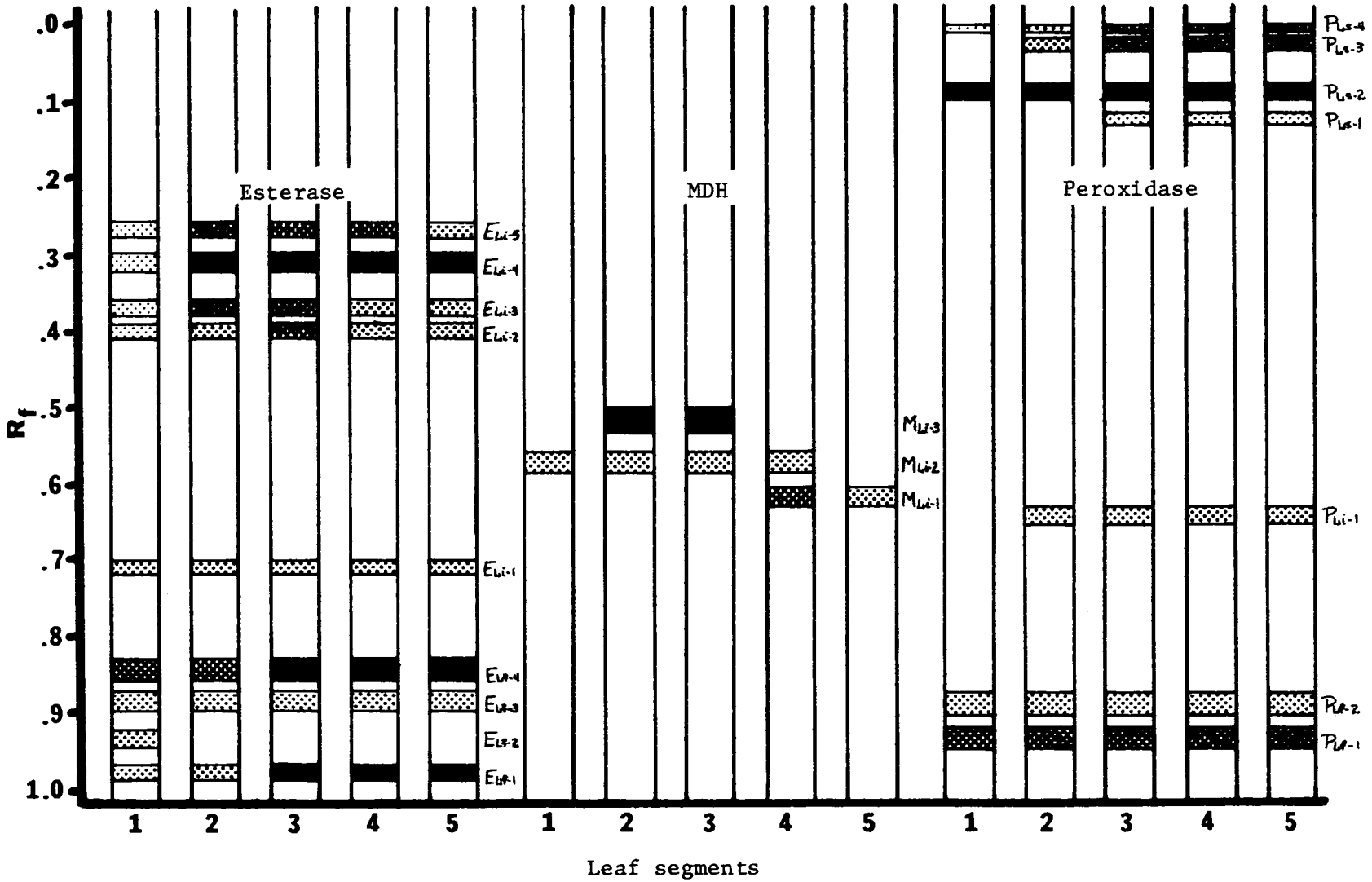

Figure 37. Electrophoretic patterns of segments from the 7-day-old first leaf (basal #1 through tip #5).

All of these bands except E_{Lf-2} appeared in the older segments through the tip of the leaf. The intensity of staining of certain bands decrease with distance from the base while the reverse is true for others. The total activity data in Figure 38 indicate that the esterase content of each of the first four segments is approximately the same. Table 2 shows that the esterase specific activity was highest in the second segment and gradually declined toward the tip.

Malate dehydrogenase. The bands stained for MDH activity are presented in Figure 37. A total of three different R_f values was observed with either one or two bands per segment. The total activity (Figure 38) was found to increase from the basal segment through the fourth segment. Specific activity values in Table 2 increased from the basal segment toward the tip of the leaf.

Peroxidase. The basal segment displayed four peroxidase bands, P_{Lf-1}, P_{Lf-2}, P_{Ls-2}, and P_{Ls-4} (Figure 37). In addition to these bands, P_{Ls-3} was observed in the second segment. The total complement of seven bands appeared in the third through the fifth segments. The total activity values (Figure 38) recorded for the first two segments were much greater than the succeeding segments. Table 2 shows that the specific peroxidase activity was highest in the basal segment and declined in subsequent segments excluding the tip (fifth segment) of the leaf which showed a slightly higher value than the forth segment.

Subcellular Localization of Enzymatic Activities

Esterase. All bands observed in the crude homogenate of the 4-day-old seedlings grown under standard conditions were found in

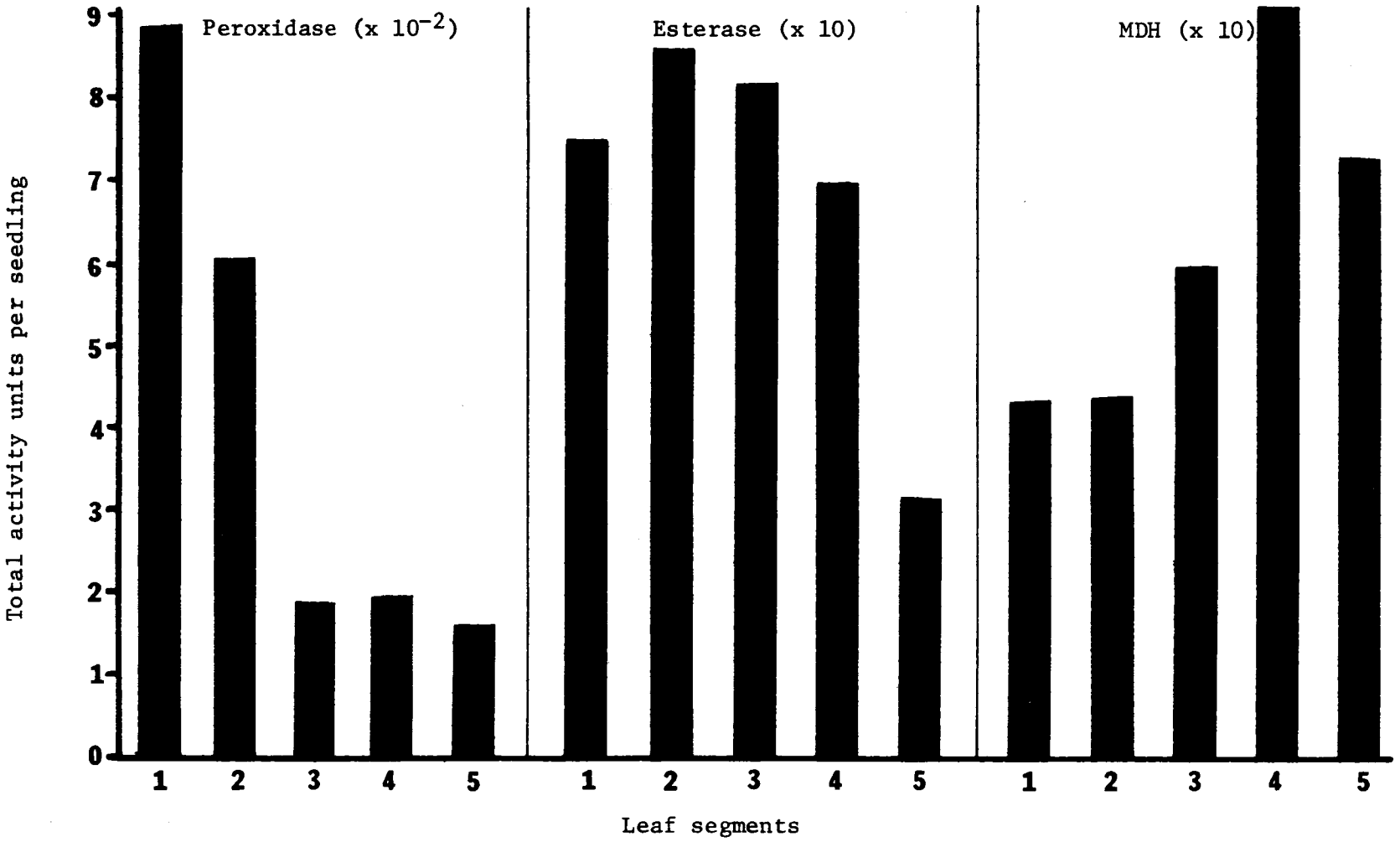

Figure 38. Total activities of segments from the 7-day-old first leaf (basal #1 through tip #5).

Table 2. Specific enzyme activities in first leaf segments

Segment	Specific activity		
	Peroxidase (10^2)	Esterase	MDH
1*	57.20	3.90	2.13
2	47.27	4.65	2.73
3	10.38	4.10	3.20
4	8.53	2.60	3.71
5	9.52	1.84	3.82

*The basal segment.

both the 27,000 and 122,000 x g supernatants (Figure 39). The 122,000 x g pellet displayed only very faint bands which included all of the intermediate bands observed in the crude homogenate and only one of the fast group, E_{SCf-3}. Neither the 2,000 x g nor the 10,000 x g pellet demonstrated any esterase banding.

The chloroplast fraction (2,000 x g pellet) prepared according to the aqueous extraction method showed a five-fold increase in the total chlorophyll content as compared to the crude homogenate (i.e., 45.10 mg to 9.55 mg, respectively). The mitochondrial fraction (10,000 x g pellet) and the 27,000 x g supernatant assayed only a trace of chlorophyll.

Esterase activity was detected in all aqueous fractions following differential centrifugation (Table 3). Most of the total activity found in the crude homogenate was lost in early preparative centrifugation steps during which large fragments of tissue were removed. The esterase specific activity values also reflected this sizeable loss in activity. The 2,000 x g and 10,000 x g pellets showed about the same level of specific activity as the soluble fraction (122,000 x g supernatant).

Following further fractionation of the mitochondrial pellet, esterase activity was found in the 33-44 percent sucrose interface which corresponds to the mitochondrial interface described by Legris and Tsai (39) (Figure 40).

Following non-aqueous extraction of chloroplasts from lyophilized 4-day-old seedlings with hexane and carbon tetrachloride (D = 1.32), the esterase zymogram in Figure 41 shows a total of six bands for the 2,000 x g pellet. The intermediate group observed in the non-aqueous

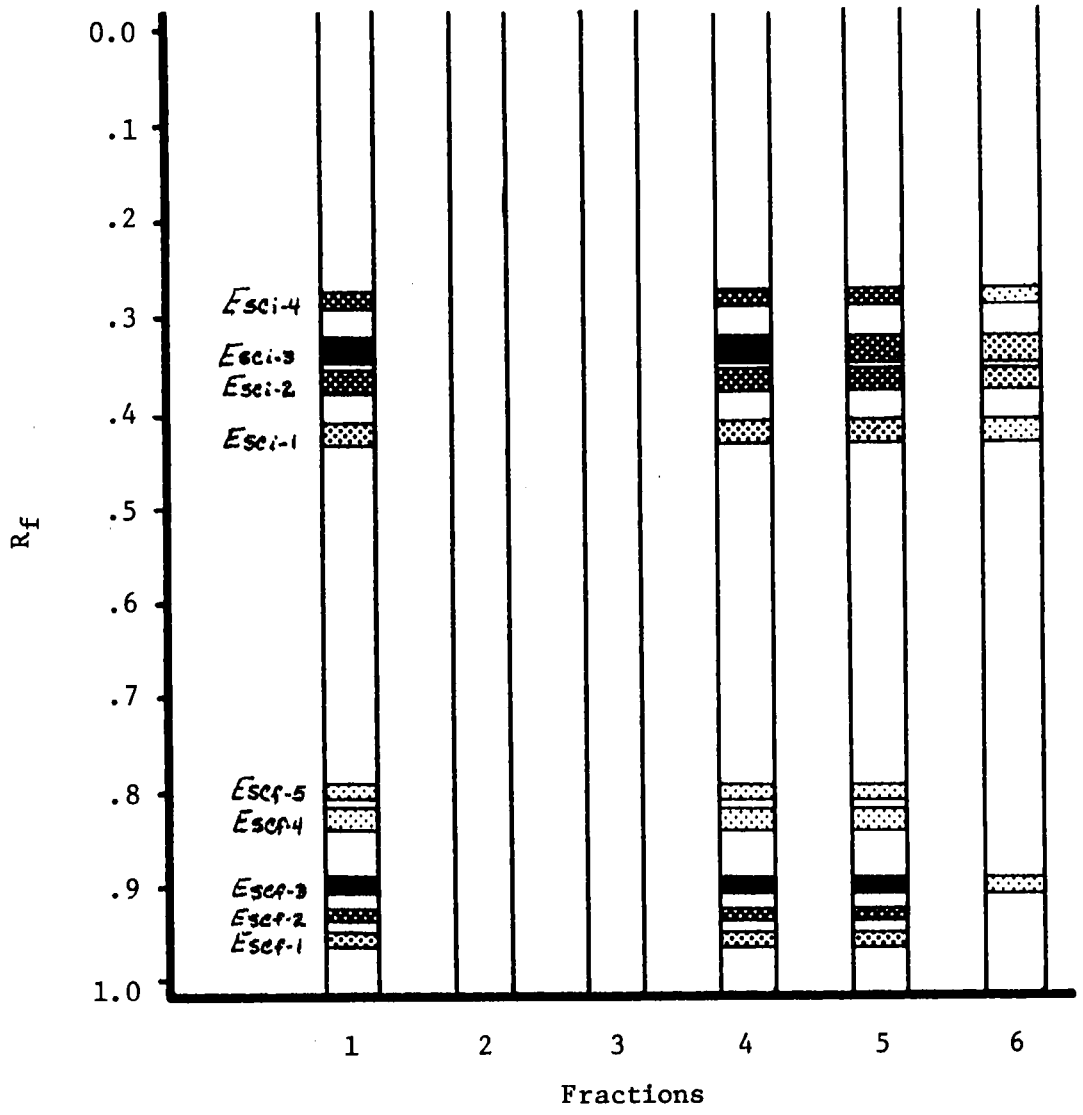

Figure 39. Electrophoretic patterns of esterase activity in the (1) crude homogenate, (2) 2,000 x g pellet, (3) 10,000 x g pellet, (4) 27,000 x g supernatant, (5) 122,000 x g supernatant, and (6) 122,000 x g pellet of 4-day-old seedlings.

Table 3. Activities of subcellular fractions from 4-day-old wheat seedlings

Fraction	Total activity			Specific activity		
	Peroxidase (x 10^2)	Esterase	MDH	Peroxidase (x 10^3)	Esterase	MDH
Aqueous extraction:						
Crude	65.15	15.18	11.98	13.28	27.18	23.05
2,000 x g P*	.56	2.16	.56	2.26	7.27	51.35
10,000 x g P	.59	.38	1.68	1.94	9.79	66.07
27,000 x g S*	41.75	5.92	3.28	12.67	10.44	10.24
122,000 x g S	38.26	2.20	1.32	11.10	9.58	5.50
122,000 x g P	2.45	1.32	.29	1.56	2.00	11.46
Non-aqueous extraction:						
Crude**	57.41	10.25	6.35	10.52	1.88	1.35
2,000 x g P	55.00	14.50	6.50	1.56	9.19	1.82

*S = supernatant; P = pellet.

**Crude homogenate of lyophilized seedlings.

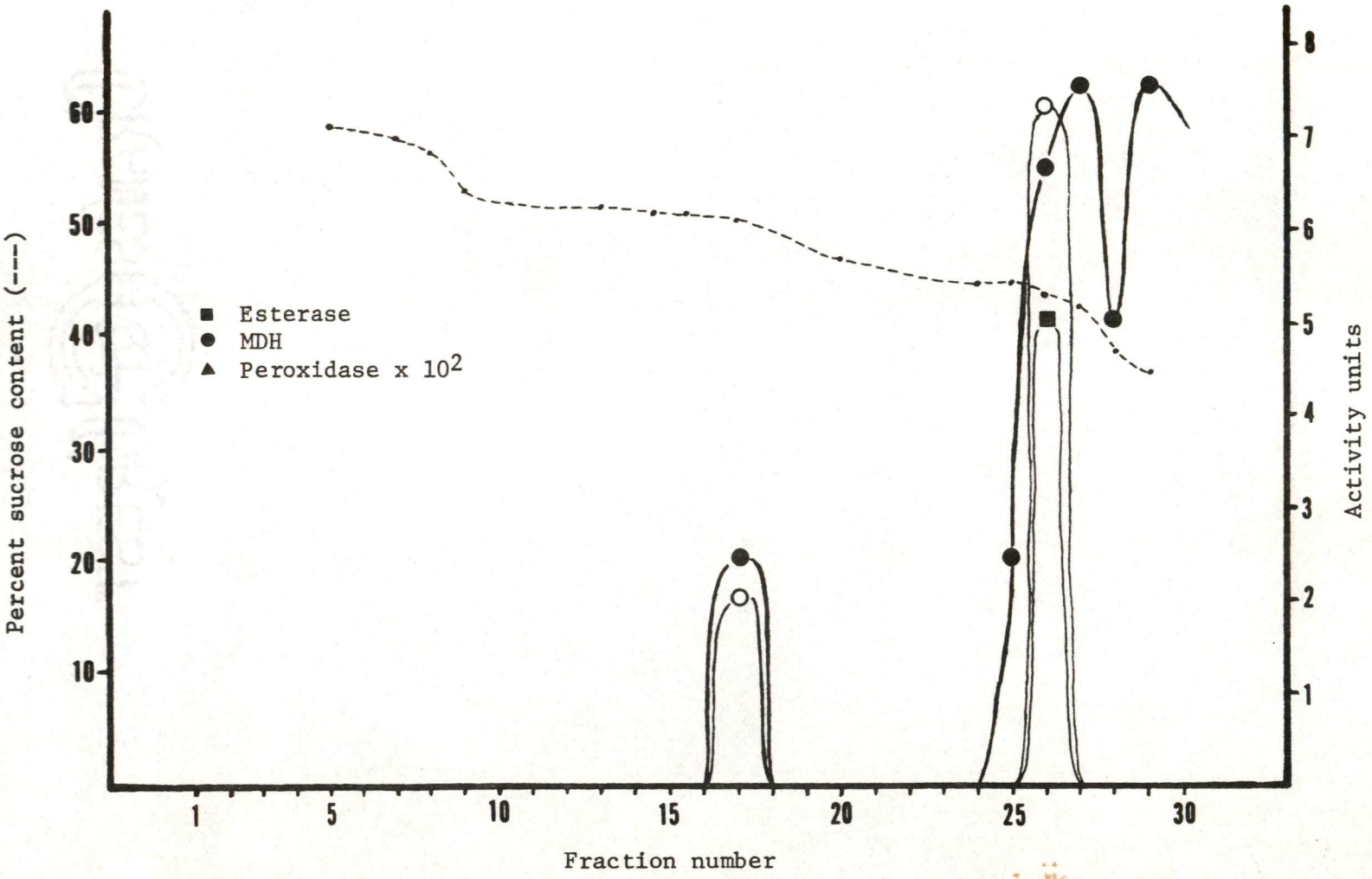

Figure 40. Enzymatic activities of fractions following separation of the 10,800 x g pellet by sucrose density gradient centrifugation.

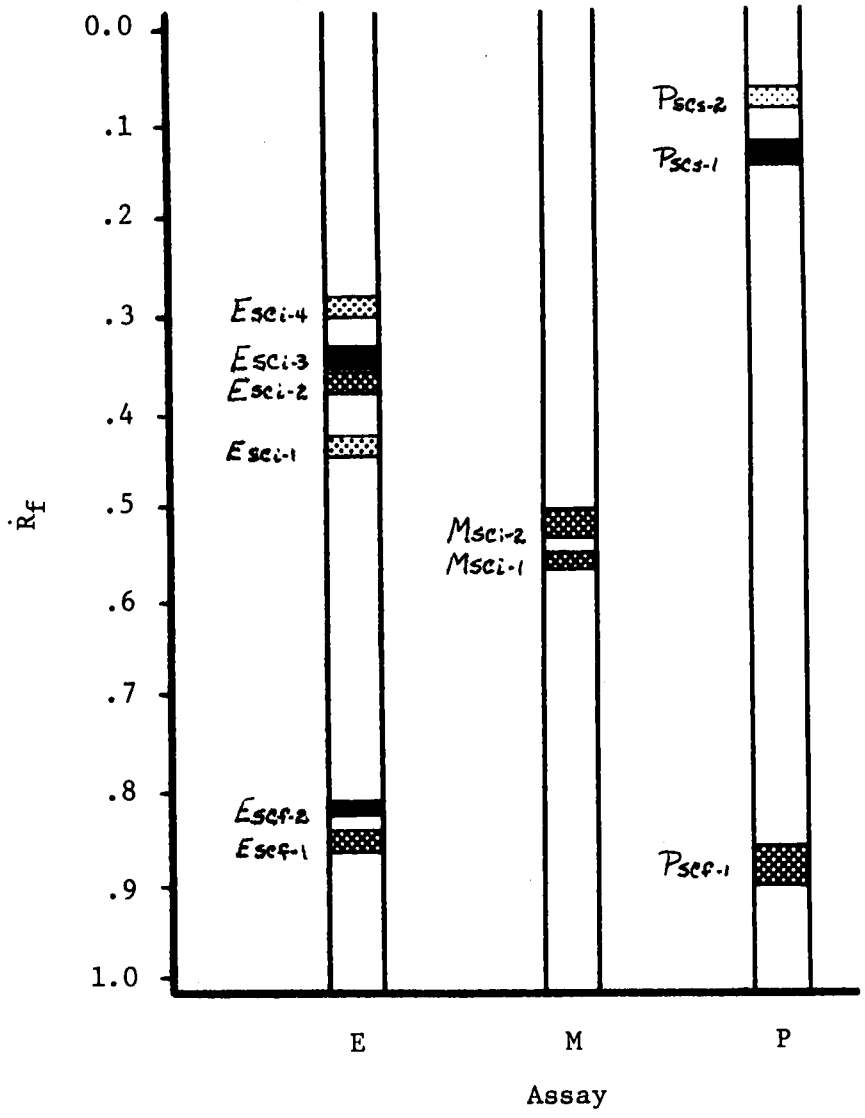

Figure 41. Electrophoretic patterns following non-aqueous extraction of the chloroplast fraction (2,000 x g pellet) for esterase (E), MDH (M), and peroxidase (P) of 4-day-old lyophilized seedlings.

chloroplast fraction was very similar to the aqueous crude homogenate (Figure 39, #1) and only two fast bands, R_f 0.81 and 0.85, which migrated a few millimeters farther than E_{SCf-4} and E_{SCf-5} of the aqueous crude homogenate were observed. The non-aqueous extraction procedure and subsequent centrifugation resulted in a three-fold enrichment in the chlorophyll content of the chloroplast fraction over the crude homogenate of lyophilized seedlings. According to the Arnon method (2) the crude homogenate demonstrated 9.11 mg chlorophyll while the 2,000 x g non-aqueous pellet had 29.30 mg chlorophyll.

The total esterase activity of lyophilized seedlings (Table 3) appeared to be slightly higher in the chloroplast fraction than in the crude homogenate. The specific activity was significantly higher in the 2,000 x g non-aqueous pellet than in the crude homogenate of lyophilized seedlings. A considerable difference between the specific activity values of crude homogenates from the extraction of fresh or lyophilized seedlings was observed.

Malate dehydrogenase. One band of MDH activity, R_f 0.575 to 0.600, was observed in the crude homogenate, the 2,000 x g pellet, and the 27,000 x g and 122,000 x g supernatants (Figure 42). No bands were evident following electrophoresis of the 122,000 x g pellet.

Table 3 presents the total and specific MDH activities observed in 4-day-old wheat seedlings grown under standard conditions. The specific MDH activity was highest in the 10,000 x g pellet, while the 2,000 x g pellet also has a large value.

Fractionation of the mitochondrial pellet was examined following sucrose density gradient centrifugation. Fractions containing MDH

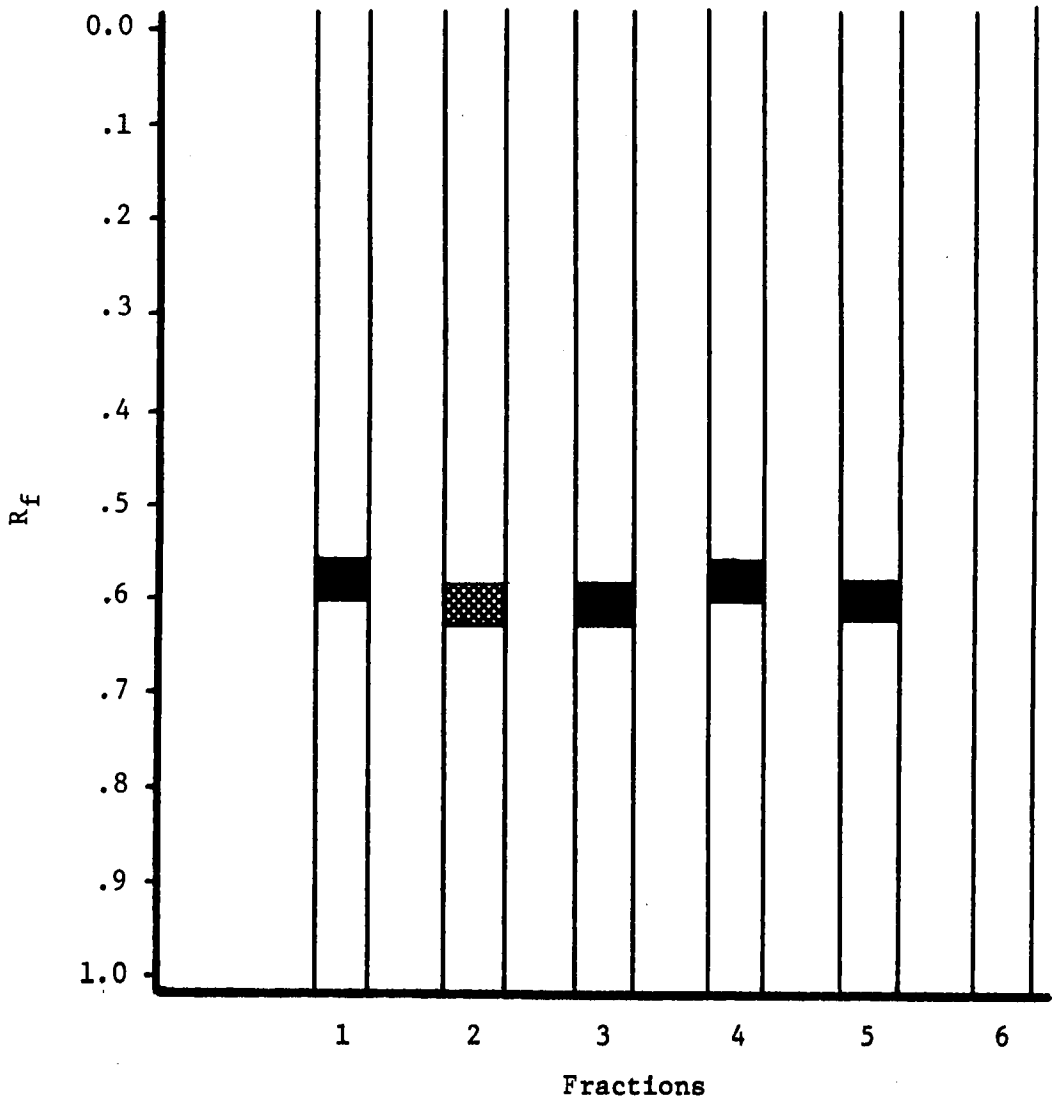

Figure 42. Electrophoretic patterns of MDH activity in the (1) crude homogenate, (2) 2,000 x g pellet, (3) 10,000 x g pellet, (4) 27,000 x g supernatant, (5) 122,000 x g supernatant, and (6) 122,000 x g pellet of 4-day-old seedlings.

activity were located at the 44-50 percent and 33-44 percent sucrose interfaces which correspond to the proplastid interface and the mitochondrial interface, respectively, as described by Legris and Tsai (39) (Figure 40). However, no MDH banding was observed in these fractions even though strong activity was detected in the mitochondrial interface and slight activity at the proplastid interface.

The non-aqueous method of extraction of chloroplasts resulted in the appearance of two bands at R_f 0.525 and 0.550 (Figure 41). Both the total and specific MDH activity values are approximately the same as those observed for the crude homogenate of lyophilized tissue (Table 3).

Peroxidase. All the bands with peroxidase activity observed in the crude homogenate were observed in the 27,000 x g supernatant and also in the 122,000 x g supernatant except for P_{SCs-4} (Figure 43). The ribosomal fraction (122,000 x g pellet) contained only two bands, P_{SCs-2} and P_{SCs-4}. The 2,000 x g pellet exhibited two bands with R_f values 0.080 and 0.140. These two bands plus those at R_f 0.875 and 0.930 were recorded for the 10,000 x g pellet which corresponds to the crude mitochondrial fraction (39).

Both the total activity and specific activity values following aqueous extraction were large in the 27,000 x g and 122,000 x g supernatants while those of the particulate fractions (2,000 x g, 10,000 x g, and 122,000 x g pellets) were much smaller (Table 3). Peroxidase activity was observed at the 33-44 and 44-50 percent sucrose interface during fractionation of the mitochondrial pellet by sucrose density centrifugation (Figure 40).

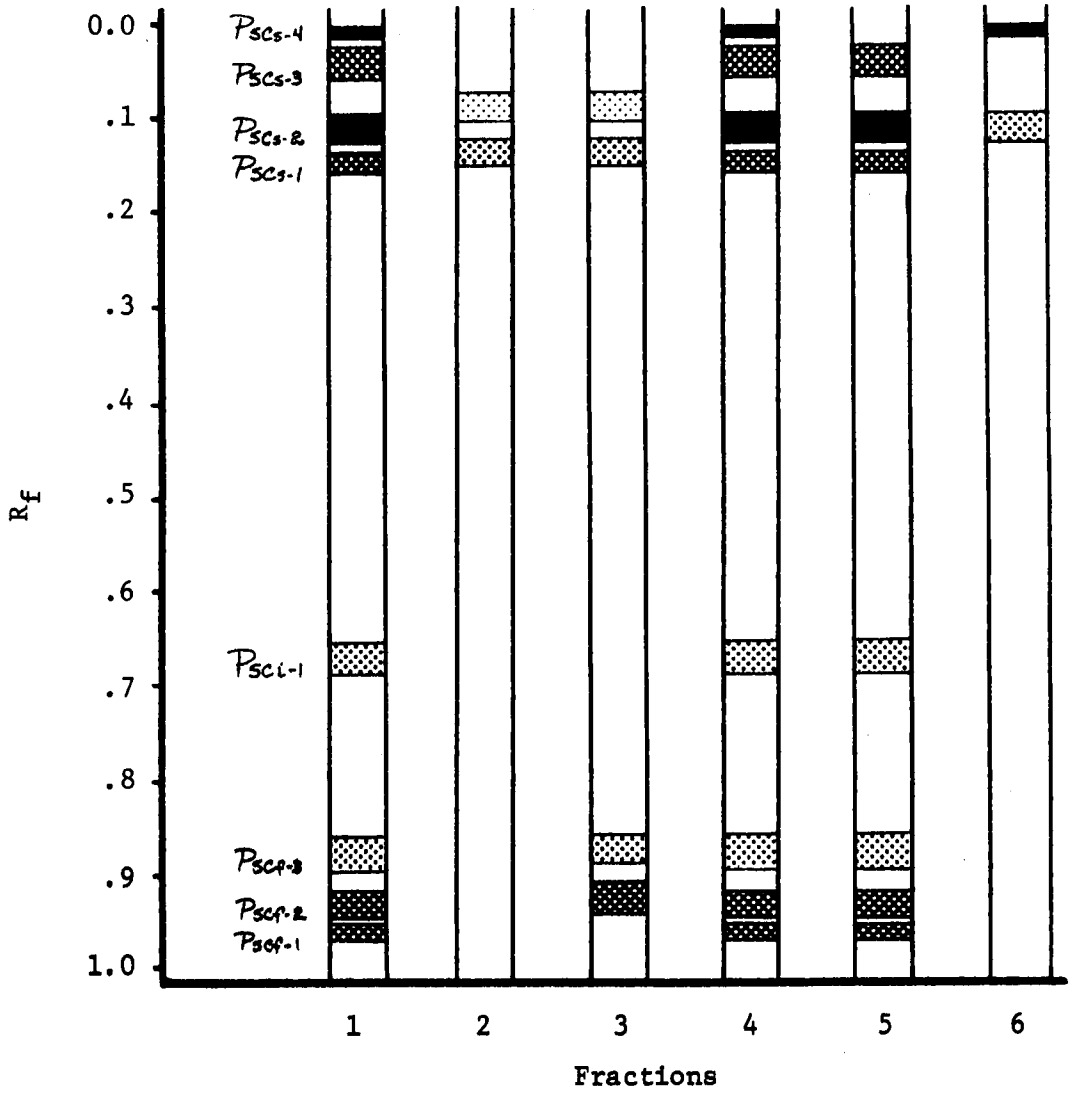

Figure 43. Electrophoretic patterns of peroxidase activity in the (1) crude homogenate, (2) 2,000 x g pellet, (3) 10,000 x g pellet, (4) 27,000 x g supernatant, (5) 122,000 x g supernatant, and (6) 122,000 x g pellet of 4-day-old seedlings.

The non-aqueous technique for the extraction of chloroplasts demonstrated three bands at R_f 0.075, 0.135, and 0.880 (Figure 41). The first two bands correspond to those observed in the 2,000 x g pellet obtained following aqueous extraction techniques (Figure 43, #2). The total peroxidase activity shown in Table 3 illustrated that the 2,000 x g pellet contained approximately the same level of total peroxidase activity as the crude homogenate of lyophilized tissue. There exists a marked difference between the peroxidase content of the chloroplast fractions following the different extraction techniques. Specific activity data show that only a small amount of the protein in the chloroplast fraction had peroxidase activity when compared to the crude homogenate of lyophilized tissue.

IV. DISCUSSION

Esterase

Extraction buffers. In the initial review of the literature, articles which discussed the basis for the selection of the buffer systems used in the extraction of plant isoenzymes for developmental studies were not found. Statements concerning the usefulness of certain inclusions such as EDTA and EtSH were noted (3). However no support was given for utilization of the chosen buffer system through comparative experimentation with other buffer systems.

The presence of the sulfhydryl-protecting reagent EtSH in the extraction buffer resulted in the demonstration of an additional band, E_{BSi-3}, in this study (Figure 1, p. 16). Analysis of extracts which contained buffered DTT showed banding comparable to the Tris buffer. The combination of DTT and EtSH demonstrated similar banding patterns to the EtSH buffer except that the intermediate group extracted in DTT and EtSH showed a slightly greater intensity at 144 hours after sowing. Studies by Oosterbaan and Jansz (50) on wheat germ esterases showed inhibition by certain other sulfhydryl reagents (eg., p-chloromercuric benzoic acid, etc.).

The apparent effect of EDTA in this study as inferred from a comparison of the banding in Figure 1 (p. 16) was to facilitate the extraction of E_{BSi-5} and E_{BSi-6} at both 96 and 144 hours after sowing. The loss of E_{BSf-2} at 96 hours cannot be explained. Previously Norgaard and Montgomery (48) noted that the chelating agent EDTA increased the activity of certain esterases and decreased variability among enzyme preparations.

The presence or absence of 10^{-3} M $MgCl_2$ in the buffer did not appear to effect the results as presented in Figure 1 (p. 16). Oosterbaan (50) also reported no apparent requirement for metal ions or other co-factors for esterase activity in peas. However, heavy metals were shown by Norgaard and Montgomery (48) to be inhibitory to pea esterases. In Vicia faba root tips, James and Smith (32), using the histochemical substrate naphthol AS-D acetate, found a requirement for 5 mM magnesium and noted that higher concentrations were inhibitory.

In this study, the least complex buffer system, 0.01 M Tris-HCl (pH 7.6) was used in an attempt to minimize secondary effects which may result from such inclusions in the extraction medium and to provide a basic extraction medium that could stand as a reference medium for further experimentation.

Normal developmental zymograms. Examination of the developmental zymograms observed in the present study showed that of the 13 bands displayed in the light (Figure 4, p. 21) only 10 appeared in the corresponding dark-grown seedlings (Figure 5, p. 22). The appearance of the intermediate group and two fast migrating bands, E_{NLf-5} and E_{NLf-6}, present in the light (Figure 4, p. 21) was delayed (repressed) for 48 hours in the absence of light during development. The repression of these esterase bands in seedlings grown in the dark is apparently due to some light related process.

Several studies have been conducted on the developmental banding patterns in wheat. As examined by van der Jooste and Moreland (34), the seeds of Triticum aestivum L. var Anderson following starch gel electrophoresis displayed a complement of five anodal bands (four fast and one

intermediate), a smear from the origin to the intermediate band, and one cathodic band near the origin. Macko et al. (42) reported a large number of fast-migrating bands with esterase activity in grains of the Lee variety using acrylamide gels. Bhatia and Nilson (3) examined the isoenzyme complement of the Marquis variety on acrylamide gels following 24, 48, 72, 120, and 168 hours of hydration. They demonstrated a total of 12 bands (six fast and six intermediate) in very early stages of development (24 to 48 hour embryo). These zymograms are considerably different from the ones presented in this material. The variation probably was due primarily to varietal differences in addition to variations in age of sampled seedlings, growth conditions, and extraction methods.

Patterns following inhibition of DNA synthesis. Haber and Schwarz (38) have shown that FdUrd concentrations from 10^{-3} to 10^{-1} M are both specific and effective in inhibiting DNA synthesis in Triticum vulgare Vill. var Lemhi. Therefore, they considered the wheat grown in 10^{-3} M FdUrd analogous to the gamma-plantlet, where each grew and developed in the absence of a net increase in its DNA content (27). Grown under standard conditions defined above, seedlings treated with 10^{-3} M FdUrd exhibited reduced growth (total root and shoot lengths) when compared to the gamma-plantlet (Figure 12, p. 31; Figure 13, p. 32). A FdUrd concentration of 10^{-4} M was effective in inhibiting a net increase in the DNA content (Figure 11, p. 30); however, these seedlings were smaller than the gamma-plantlets measured during the 7 day test period (Figure 13, p. 32). Even though the growth was not analogous to the gamma-plantlets, Figure 14 (p. 34) revealed similar protein content. The lower effective

concentration of FdUrd, 10^{-4} M, observed in this study may possibly be explained since the growth system used here differed from that of the earlier work. Haber and Schwarz (28) sowed grains in large Pyrex baking dishes in which the number of grains and the volume of FdUrd applied was not strictly controlled, and an unknown amount of water was added as needed over the developmental period probably diluting their effective inhibitor concentration.

The pronounced lag in the appearance of the intermediate bands under dark-grown conditions was not as apparent in either the gamma-plantlets or the FdUrd-treated seedlings as in the normal ones. However, a comparable lag in the darkness was observed in the fast group of the gamma-plantlets and the FdUrd-seedlings. Two fast bands in each of the treated groups, E_{GDf-4} and E_{GDf-5} (Figure 16, p. 36) or E_{FDf-5} and E_{FDf-6} (Figure 18, p. 39), were delayed in the dark for 24 to 36 hours when compared with E_{GLf-4} and E_{GLf-5} (Figure 15, p. 35) or E_{FLf-5} and E_{FLf-6} (Figure 17, p. 37). It should also be noted that the appearance of the bands E_{GLf-4} and E_{GLf-5} in the light-grown gamma-plantlets was delayed at least 24 hours beyond the development of comparable normal light-grown seedlings, E_{NLf-5} and E_{NLf-6}. While in the dark, each of the bands appear at 72 hours after sowing. Under all conditions tested, the fast-moving group consisted of six bands except for the gamma-plantlets, which lacked the late-appearing band E_{NLf-4} when exposed either to light or constant darkness.

The zymogram of light-grown FdUrd-treated seedlings displayed a complement of 13 bands. Although this is the same number of bands observed in the normal light-grown seedlings (Figure 4, p. 21), no complementary bands for the following were observed in the two systems: neither

E_{FLf-7} nor E_{NLs-1}. The gamma system closely resembled the FdUrd-seedlings except for E_{FLf-4}, E_{FLf-7}, and E_{FDs-1}. The isoenzyme complements of the light-grown gamma-plantlets and the dark-grown normal seedlings are very similar except that E_{GLi-1} and E_{DNf-4} do not resemble bands in corresponding zymograms (Figure 5, p. 22).

Endo (15) studied the post irradiation effects of gamma-rays (50,000 rads) on enzyme systems in maize. After 4 to 5 days of dark incubation the gamma-irradiated seedlings showed an appreciable decrease in the number of cathodal bands as compared to the unirradiated shoots. Slight variations in the intensity of staining were also observed. He noted that the damage intensified with time after treatment and that there was no effective repair mechanism. No records were kept on the condition of DNA synthesis following the irradiation of the dry maize seed. In the present study, a few differences were observed in the wheat system irradiated with a large dose (500,000 rads) of gamma radiation. These include the deletion of the comparable bands (E_{NLf-4}, E_{NLi-3}, and E_{NLs-1}) in the light-grown gamma-plantlets. Otherwise the grouping, developmental appearance (except for previously noted lag in two fast bands), and relative intensity of bands are similar for both normal and gamma-irradiated systems.

High level irradiation of dry wheat grains prevents mitotic divisions and cytokinesis without killing the embryo (26). Haber and Foard (25) observed no net increase in the DNA content in the gamma-plantlet. Further, they observed that RNA synthesis occurred in the nucleus of the gamma-plantlet and that some of this RNA migrated into the cytoplasm. Therefore, this large dose (500,000 rads) of gamma radiation prevented cell division but did not inhibit all nuclear

functions. The relative physiological normalcy observed in the gamma-plantlet may result primarily because it is a non-dividing system. According to Schwartz and Bay (61),

> the genetic complement of a cell in which a large number of chromosome breaks have been induced will remain intact as long as the cell does not divide. Distribution of its genes on many fragments rather than on whole chromosomes would not be expected to impair their functions.

Close examination of all the normal, gamma-irradiated and FdUrd-treated seedling zymograms reveal that certain bands, especially members of the intermediate group, were variable in their R_f values. This may be due to incomplete extraction of the isoenzymes from membrane fragments or to the presence of isoenzymes differing in subunit composition. This might also explain the variability observed in different buffer extractions. Desborough and Peloquin (14) have proposed that esterase isoenzymes in the potato tuber are tetramers composed of one, two, or three types of monomers. Also, in studies of rat organs, Choudhury (8) has suggested a subunit structure of esterases and has inferred from overlapping substrate specificities that many of these may have common subunits. However, much more biochemical and genetic data are needed before such a statement can be offered for the wheat system.

Enzyme activity. The esterase content of the 27,000 x g fraction was observed to increase under all growth conditions for the first 60 hours after sowing (Figure 27, p. 49). A similar observation was made in Figure 14 (p. 34) on the soluble protein content per seedling. This may indicate that during the first 60 hours after sowing the developmental events occurring in the gamma-plantlet and the FdUrd-treated seedlings were keeping pace with the normal seedlings. However, after that point

(60 hours) the total esterase content of the normal seedlings increased more rapidly, as the result of extensive cell division and development, than those in which DNA synthesis had been inhibited. For each treatment the total esterase content is not significantly different in seedlings grown with light or in continuous darkness.

Organ separation. The coleoptile (Figure 33, p. 57) demonstrated the same banding pattern as in the whole embryonic axis for each sampling time except that the 144 hour coleoptile did not show the fastest band, E_{Of-1}. The 72 hour leaf extracts displayed a fast band, E_{Of-3}, which did not appear in whole seedlings of that age. This band may correspond to E_{NLf-4} (Figure 4, p. 21) which routinely occurred later in development of intact seedlings. Bhatia and Nilson (3) recorded 15 bands (five fast, nine intermediate, and one slow) in 3 to 7-day-old wheat roots. Of these 15 bands only 12 were observed in the 24 to 48 hour embryo, 3 to 7-day-old coleoptiles, and 3 to 25-day-old leaves. Further, they reported that after emergence of the leaf, its isoenzymes remained unchanged through the developmental stages examined.

Unlike the results reported by Bhatia and Nilson (3) on the Marquis variety of wheat, the esterase banding pattern of the leaf of the Lemhi variety changes over time. These differences include variations in the staining intensity as well as the total band complement. Although Bhatia and Nilson (3) observed more bands in the root than in the young embryo, coleoptiles, or leaves, the present study shows fewer bands in the 72 and 144 hour root than in the leaf, coleoptile, or whole embryonic axis.

The roots contained the highest levels of esterase activity, both total and specific. The total activity in the root increased less from 72 to 144 hours after sowing than did the other organs over this time period (Figure 34, p. 58). The root and coleoptile showed increases in esterase specific activity from the third to sixth day after sowing (Table 1, p. 59).

First leaf segments. Examination of serial segments of the first leaf of wheat provides a system for the study of development and aging. A basal meristem provides the new cells for the growth and expansion of the leaf (16). Therefore, the cells near its tip are the oldest developmentally and chronologically.

Esterase zymograms in Figure 33 (p. 57) and Figure 37 (p. 63) show that the esterase complement of the basal segment of the leaf is very similar to the entire 72 hour leaf. The basal one centimeter has the full complement of esterase isoenzymes. All but one band, E_{Lf-2}, were found in subsequent segments. The basal segment is essentially non-photosynthetic, and unlike other non-photosynthetic tissues it did not demonstrate a reduction in band number but instead the entire leaf complement was expressed.

Both the total (Figure 38, p. 65) and specific activity values (Table 2, p. 66) were highest in the second segment and then decreased toward the tip of the leaf. Since no conclusive information is available concerning the biochemical and physiological function of esterases it is not possible to speculate on the importance of these esterases in the elongation, differentiation, and development of leaf cells.

Subcellular localization. Following aqueous extraction of freshly harvested material, the crude homogenate, the 27,000 x g supernatant, the 122,000 x g supernatant, and the pellet demonstrated bands, while the 2,000 x g and 10,000 x g pellets did not show esterase banding (Figure 39, p. 68). However, data in Table 3 (p. 69) show that activity was detected in each fraction. The absence of bands in the chloroplast and mitochondrial fractions is not understood since the low level of activity in the ribosomal fraction produced banding. Specific activity values for the mitochondrial, 27,000 x g supernatant, and soluble (122,000 x g supernatant) fractions are approximately the same while that of the ribosomal fraction is considerably smaller. The total activity decreased sharply following centrifugation of the crude homogenate. Approximately one-third of the total activity in the crude homogenate remained in the 27,000 x g supernatant which was the sample routinely assayed electrophoretically to determine the developmental isoenzyme patterns. This initially high total esterase activity in the crude homogenate may be due to the presence of small cell fragments which were able to pass through the glass wool but large enough for easy removal by low-speed centrifugation. Therefore, part of the activity in the 2,000 x g pellet may also be due to contamination by cell wall fragments.

The non-aqueous extraction of lyophilized tissue designed to provide an enriched chloroplast preparation demonstrated two fast and four intermediate bands in the chloroplast fraction (Figure 39, p. 68). The total activity data show that large amounts of esterase were retained in this chloroplast fraction. Specific activity values demonstrate a considerable increase in the level of esterase activity among chloroplast proteins when compared to the crude homogenate. Since

lyophilization of cell membranes reduces leakage from the organelles (69), the higher total activity values in the 2,000 x g pellet of lyophilized seedlings may indicate the presence of esterases in the chloroplast which had been leached out during aqueous extractions. The specific activity data further supports this suggestion. It would appear that lyophilization of seedlings is a valuable preparative step in the study of esterases in wheat. However, Fink and Hay (17) have found that esterases of wheat germ were extensively inactivated by lyophilization.

Since esterase activity was detected in all fractions, a specific functional label for them is difficult to derive from these data. Other reports have concentrated on localization of plant esterases. Fluck and Jaffe (18) observed that the acetylcholinesterase activity in Phaseolus aureus was highest in the roots and aerial buds. Further, they noted that 95 percent of this activity in the roots was localized in the cell wall. Working with Vicia faba, Gahan and McLean (21) used histochemical techniques to demonstrate esterases in small vesicles passing through the plasmalemma, in vesicles associated with the formation of the primary wall, and in cell wall fibers laid parallel to the middle lamella. This information is consistent with the marked decrease in esterase activity following filtration and centrifugation of the crude wheat extract.

Malate Dehydrogenase

Extraction buffers. Only slight differences in the MDH banding patterns were observed in the 96 hour normal wheat seedlings grown under standard conditions and extracted in several buffer solutions including

EtSH (Figure 2, p. 18). These data support the findings of Ahmadi and Ting (1) on spinach leaf MDH which indicated that EtSH did not significantly effect activity. However, working with wheat germ, Kuramitsu (36) reported that MDH activity was inhibited when sulfhydryl groups were involved in the extractions.

The variability expressed in 144 hour seedling extracts is unexplainable since similar extractions of 96 hour seedlings displayed relative uniformity. It was unfortunate that the gels had only 12 sample wells which necessitated the use of a second gel for analysis. The highly irregular zymograms were electrophoresed on a separate gel during a second run. Particular precautions were taken to keep these samples at 4°C so that heat denaturation would be avoided.

Normal developmental zymograms. The results from developmental banding patterns of MDH isoenzymes were extremely variable. In the series of buffer extractions (Figure 2, p. 18) four different bands were observed depending on the buffer employed. Developmental patterns displayed one to three bands, typically one (Figure 6, p. 24; Figure 7, p. 25). Before a suitable technique for the surface sterilization of the wheat seed was developed, zymograms prepared from seedlings apparently free from contamination or from slightly contaminated culture dishes often showed as many as six MDH bands.

Two possible reasons for the variability in banding were explored. First, the possibility of NBT oxidase activity was rejected because no bands appeared when the malate substrate was omitted from the staining solution. The second possibility concerned the instability of MDH conformers which has been reported to decrease on the addition of NAD

to the medium (78). For this purpose, 10^{-3} M NAD was introduced into the enzyme extract before electrophoresis. There was no observable difference between zymograms with the sample containing NAD and the Tris buffered wheat extract prepared as described earlier.

DeJong and Olson (13) employed deoxycholate to solubilize the mitochondrial MDH. They found that a 2 to 5 percent concentration of deoxycholate was needed to insure the mobility of their particulate fraction of tobacco away from the origin during electrophoresis. Deoxycholate was not used in the present investigation since the bands appeared to migrate well into the gel during electrophoresis. However, the slight variability in the R_f values of a band may be due partially to the incomplete dissociation of the enzymes from membrane fragments of varying sizes.

Earlier studies on wheat have reported from two (6) to ten bands (66) of MDH activity. Generally the normal seedlings grown under standard conditions demonstrated one band during the time course of development (Figure 6, p. 24). The normal dark-grown seedlings typically had one band except for the 48 and 60 hour seedlings which showed two bands (Figure 7, p. 25). No explanation is attempted for this variability.

Patterns following inhibition of DNA synthesis. The MDH banding in the gamma-plantlets was very similar to the normal seedlings. The only difference is that a second band was observed in the 60 hour light-grown gamma-plantlets (Figure 19, p. 40) and not in the dark-grown ones (Figure 20, p. 41). The light-grown FdUrd-treated seedlings displayed the greatest number of bands observed during development (Figure 21, p. 42) with two bands in the 60 and 96 hour seedlings and three bands in

the 144 to 168 hour seedlings. The dark-grown FdUrd seedlings (Figure 22, p. 43) had two bands only after 86 hours of hydration.

Endo (15) found that the intensities and migration rates in maize shoots, grown from gamma-irradiated (50,000 rads) seeds, were very similar to controls over a 4 to 9 day developmental period. The irradiation of dry wheat grains with a high dosage (500,000 rads) of gamma radiation in this study also did not seem to have altered significantly the MDH pattern during development. Again, as described in the esterase section above, the higher dose of radiation prevented mitosis and cytokinesis in the wheat so that physiological events were relatively unimpaired during early development.

Enzyme activity. In the normal wheat seedlings (Figure 29, p. 52) the total MDH activity increased very slightly during the first 60 hours after sowing and then showed a marked increase later in development corresponding primarily to the extensive growth of the leaves. The MDH content of those seedlings in which the net increase in DNA content was inhibited remained essentially unchanged. The information obtained from organ separation studies (Figure 34, p. 58; Table 1, p. 59) show that the large increase in the MDH activity of normal seedlings was primarily due to the growth and expansion of the leaf. Similar enlargement was not observed in the gamma-plantlets or in the FdUrd-treated seedlings. The specific activity values (Figure 30, p. 53) decrease with time after sowing in the gamma-plantlets, declining as the leaves became progressively chlorotic. A slight decrease in specific activity in the normals and light-grown FdUrd-treated seedlings was observed while the initial and terminal values for dark-grown FdUrd-treated seedlings were the same.

Organ separation. The MDH patterns in wheat (Lee variety) have been examined by Macko et al. (42). Using acrylamide gels, they observed more bands in the dormant and germinating grains than in the coleoptile. The leaf had two broad bands and a slow-migrating smear. Starch gel electrophoretic studies by Weimberg (77) revealed approximately six different R_f values for MDH isoenzymes in wheat. Because of the large number of bands he concluded that the isoenzyme patterns were not the same in various tissues of the wheat plant. These experiments also illustrated banding with slight variability in the mobilities of certain bands although it never was mentioned.

In the present study the zymograms of the 27,000 x g supernatants following organ separation of 72 hour seedlings showed a single band, R_f 0.525, in all organs (Figure 35, p. 60). The leaf of the 144 hour seedlings displayed a second band with MDH activity, R_f 0.600. As noted earlier, once surface sterilization procedures were initiated only a few bands were observed with MDH activity. The workers cited above did not employ similar measures to check contamination of their cultures.

At 72 hours after sowing the root possessed slightly more total MDH activity units than the leaf (Figure 34, p. 58). As the leaf developed from the small structure enclosed completely inside the coleoptile at 72 hours to the organ expanding beyond the coleoptile at 144 hours, it became the organ containing the largest proportion of the total activity. The leaf was shown in Table 1 (p. 59) to have the highest specific activity at both sampling times. The increased number of MDH bands and activity values in the older leaf may be the quantitative indication for the initiation of a key physiological process such as photorespiration. The presence of peroxisomes and photorespiration in wheat leaves

have been reported (33, 74). To test the latter possibility, comparisons of zymograms and kinetic data in etiolated leaves are needed.

First leaf segments. The basal one centimeter and last two centimeter segments of the first leaf showed one band while the other segments had two bands (Figure 37, p. 63). A total of three different R_f values were observed for these bands. The total MDH content of each segment increased from the basal segments toward the tip (Figure 38, p. 65). The lower MDH activity observed in the fifth segment may be explained since this fraction did not always contain a full two centimeter segment of tissue. This idea is supported by a parallel increase in specific activity from the basal to tip segments (Table 2, p. 66).

Subcellular localization. Several investigators have reported the subcellular location of MDH activity in higher plants. Suggested sites of MDH activity include: (1) the soluble or cytoplasmic fraction (10, 39, 40, 46, 58, 71, 72, 80, 82, 83); (2) mitochondrial fraction (10, 39, 40, 46, 58, 71, 72, 80, 82, 83, 84); (3) peroxisomal fraction (39, 40, 46, 58, 80, 84); and (4) chloroplast fraction (39, 46, 80).

Each of the subcellular fractions prepared in this study using an aqueous extraction medium displayed one band of MDH activity as illustrated in Figure 42 (p. 73) except for the ribosomal fraction (122,000 x g pellet) which showed no activity. Two bands were observed in the non-aqueous preparation of the chloroplast fraction (2,000 x g pellet) (Figure 41, p. 71). Mobilities of the two non-aqueous bands were slightly less than those extracted using aqueous methods; however, they are within the range of R_f values observed in developmental zymograms presented in Figure 6 (p. 24).

Following aqueous extraction of fresh seedlings approximately one-tenth of the total MDH activity assayed in the crude homogenate was observed in the mitochondrial fraction (10,000 x g pellet) (Table 3, p. 69). The aqueous extract of chloroplast demonstrated very little total MDH activity. However, specific activity data show high levels of activity in both the mitochondrial and chloroplast fractions. The largest MDH content was recorded in soluble fractions.

Non-aqueous extraction of the chloroplast fraction yields a markedly higher total MDH activity value which probably reflects the compartmentalization of chloroplast enzymes as a result of an intact chloroplast envelope (69) and also contamination by peroxisomes associated with this membrane system (64).

Fractionation of the crude mitochondrial preparation according to the method of Legris and Tsai (39) demonstrated a large amount of MDH at the mitochondrial interface (33-44 percent sucrose) and a very small amount at the proplastid interface (44-50 percent sucrose) (Figure 40, p. 70). However, no bands with MDH activity were observed following electrophoresis of these active fractions. This anomaly remains unexplained. Legris and Tsai also observed the highest total activity in the mitochondrial fraction and a very small amount in the proplastid fraction. In addition, however, they observed an intermediate, although quite low, level of activity in the glyoxysomes.

Peroxidase

Extraction buffers. Extraction in EDTA and EtSH reduced the number of bands expressing peroxidase activity as compared to extraction in Tris buffer (Figure 3, p. 19). Three bands, P_{BSi-1}, P_{BSi-2}, and

P_{BSf-1}, were lacking in the EDTA and EtSH system at 96 hours while only the latter two bands were absent after 144 hours. DeJong (13) also reported a loss in peroxidase activity in onion roots resulting from EDTA extraction. However, pea shoots showed an increase in the number of peroxidase bands according to Macnicol (43).

Normal developmental zymograms. Developmental patterns for peroxidase of wheat have been presented by Macko et al. (42), Bhatia and Nilson (3), Sequi et al. (64), and Singh and Singh (67). Macko and his colleagues reported very low levels of activity in the dormant seed. In their study they showed a dramatic increase in banding occurring with germination. However, Bhatia and Nilson observed an addition of only one band after the seed had been hydrated 24 to 48 hours. With the exception of one very fast-moving band, these bands in the wetted seed represented the total complement of peroxidases. The work by Sequi and co-workers showed the number of anodal peroxidases increasing gradually through development, while the opposite was found for the basic group of isoenzymes. The wheat seed was observed by Singh and Singh to have only one fast-moving band while by the fifth day after sowing the seedling had a total complement of eight bands (three fast, two intermediate, and three slow).

In the present study a rapid appearance of peroxidase bands also was observed in the normal wheat seedlings grown under standard conditions following 24 to 48 hours of hydration (Figure 8, p. 26). However, no bands were stained in the initial samples taken at 3 to 12 hours after sowing. Thus, the activity observed by others (3, 42, 67) probably resided in portions of the grain other than the embryo and scutellum.

Germination, defined as the emergence of the radicle (16), can be detected macroscopically in this study between 24 and 36 hours after sowing. Therefore, the marked increase in peroxidase activity associated with germination observed by Macko et al. (42) and Bhatia and Nilson (3) was also noted in the Lemhi variety. Although contrary to those works, no peroxidase banding was detected in the embryo until 24 hours after sowing.

The zymograms for normal seedlings grown under standard conditions demonstrated a total complement of 10 bands (Figure 8, p. 26) while the dark-grown seedlings showed only nine bands (Figure 9, p. 28). Under conditions of darkness the appearance of the early slow-moving bands was retarded for a period of at least 12 hours. The patterns reported by Singh and Singh (67) show three fast, two intermediate, and three slow-migrating bands and these resemble the banding groups presented here except for the number of slow bands.

In addition to benzidine, guaiacol was examined as a substrate for peroxidase reactions. Except for the variation in the timing of the appearance of certain bands, the banding pattern using guaiacol was very similar to the normal patterns with the benzidine-hydrogen peroxide system. The one major difference was the absence of the fastest moving band using guaiacol. According to the discussion presented by Macko et al. (42), the band might be best classified as a catalase. Positive identification of this band by the potassium iodide negative staining method presented by Shaw and Prasad (65) was unsuccessfully attempted using starch in the acrylamide gel.

Patterns following inhibition of DNA synthesis. There were only a few differences among the banding patterns of seedlings in which a net increase in DNA content was inhibited. All gamma-irradiated (Figure 23, p. 44; Figure 24, p. 46) and FdUrd-treated seedlings (Figure 25, p. 47; Figure 26, p. 48) demonstrated three fast, two intermediate, and four slow-migrating bands, except for the dark-grown gamma-plantlets which had five slow-moving bands similar to the normal dark-grown system (Figure 9, p. 28). A lag of 12 hours in the appearance of the fast group and 36 hours for P_{GLi-1} was observed in the gamma-plantlets grown under a light regime. No comparable lag in the intermediate bands was observed in dark-grown gamma-plantlets.

Contrasting results were observed by Endo (15) in maize following gamma-irradiation of seeds. Large increases in staining intensities of bands and a new heavily stained slow band appeared in the irradiated maize samples. Again, as stated in the esterase section above, the absence of mitotic divisions and cytokinesis in the gamma-plantlet makes possible continued nuclear function.

Enzyme activity. Initially very little peroxidase activity was observed by spectrophotometric measurements (Figure 31, p. 54). After 36 hours of incubation the level of activity began to increase rapidly. The total activity assayed in the FdUrd-treated seedlings increased at a slower rate than other treatments. The maximum value for FdUrd-seedlings was approximately one-third of the total activity for the normal light-grown seedlings at 108 hours after sowing and remained at the level throughout the 7 day developmental period. The normal light-grown seedlings displayed the maximum activity values and these increased

throughout the study. Parish (53) and Wolf (79), however, observed that the total peroxidase activity was greater in the dark-grown wheat seedling and showed decreasing activity in the light-grown leaf over time.

Figure 31 (p. 54) shows that the total peroxidase content of the normal dark-grown seedlings and all gamma-irradiated seedlings was very similar and was slightly less than that of the normal light-grown seedlings. The specific activity data presented in Figure 32 (p. 55) show that the rate of increase was much greater in the gamma-plantlets than in the FdUrd-treated or normal seedlings. This accelerated rate of increased activity demonstrates that for peroxidases the gamma-plantlet does not approach normalcy. According to the studies of Parish (52), these data may indicate an accelerated rate of senescence within the gamma-irradiated seedlings.

Organ separation. The localization of peroxidase activity within the plant body has been of considerable interest to researchers. Early work done by van Fleet (76) showed that the peroxidase activity decreased with development in all plant tissues except the phloem. Also peroxidase activity appeared just prior to cell division and declined in senescing tissues. Additional support of this concept was provided by the work of Vanden Born (75) who observed abundant peroxidase activity in regions of high mitotic activity. These concepts that a high level peroxidase activity is indicative of meristematic activity (75, 76) or senescence (52, 53) seem to be completely opposite physiologically. Analysis of isoenzyme activity and additional localization of the isoenzyme complement is needed before the role or roles of the peroxidase system can be determined.

The total peroxidase activity in this study was found to be highest in the roots (Figure 34, p. 58). Both the leaf and coleoptile possessed relatively small amounts of peroxidase. The roots also displayed a very high specific activity (Table 1, p. 59). Similarly, DeJong (12) reported the greatest concentration of peroxidase in the roots of onions.

Zymograms for each organ are illustrated in Figure 36 (p. 62). The roots which show a large amount of peroxidase activity also display the total complement of bands observed in the whole seedling. The leaf demonstrated fewer bands of peroxidase activity. These results support the work of Bhatia and Nilson (3) who found the total complement of peroxidase bands in the roots while the coleoptile and 24 to 48 hour embryo showed a similar yet reduced number of bands. Singh and Singh (67) also observed the maximum number of bands in the 7-day-old roots. An earlier report of Macko et al. (42) indicated no qualitative differences in the patterns of the coleoptile, first leaf, and germinated seed; however, quantitative changes with respect to the intensity of bands following organ separation were observed. The present study shows both quantitative and qualitative differences with respect to the organ and development of that organ (Figure 36, p. 62).

First leaf segments. The basal one centimeter segment of the wheat leaf contains the intercalary meristem (16). The banding pattern for the basal segment and succeeding two centimeter segments up to and including the tip illustrates an increasing number of peroxidase bands (Figure 37, p. 63). However, both Figure 38 (p. 65) and Table 2 (p. 66) show that the total and specific activities were highest in the basal

segment and declined in older segments. These results seem to be in opposition to those of Parish (52) who observed an increase in the total peroxidase activity with maturity in wheat coleoptiles.

The present study tends to support the findings of van Fleet (76) who observed high peroxidase activity in meristematic cells. It would be interesting to examine the development of the first leaf in the gamma-plantlets and FdUrd-treated seedlings where a net increase in the DNA content is inhibited. However, the study was not attempted because these leaves were too small to work with even after several days of growth. One might speculate that the basal segment in these non-dividing systems would contain approximately the same amount of peroxidase activity as other segments. However, a high peroxidase content in the basal segment could be observed because the basal cells actually may be preparing to divide although the process is not completed. No physiological markers have been identified which delineate a cell preparing to divide.

Subcellular localization. Considerable interest has been generated in the localization of peroxidase at the subcellular level. Certain of these cytochemical, electrophoretic, and kinetic studies have indicated the presence of peroxidases in the (1) cell wall (12, 22, 43, 57); (2) chromosomes (57); (3) cytoplasm (12, 22, 38, 54, 57); (4) endodermis (29); (5) endoplasmic reticulum (22, 54, 56); (6) epidermis (29); (7) Golgi vesicles (22); (8) lysosomes (56); (9) microbodies (56); (10) mitochondria (11, 12, 13, 54); (11) nucleoli (57); (12) plasmalemma (22); and (13) ribosomes (11, 22, 37, 55).

Using the subcellular enrichment techniques described in this study, peroxidase activity was detected in all fractions. However, only a very small amount was found in the 2,000 x g pellet (chloroplast fraction) and the 10,000 x g pellet (mitochondrial fraction) under aqueous extraction (Table 3, p. 69). Examination of specific activity values in crude homogenates of fresh and lyophilized tissue and chloroplast fractions reveals comparable peroxidase activity resulting from the two extraction methods. However, the variance between total peroxidase contents of the chloroplast fractions is very large. The high total peroxidase activity observed in the chloroplast fraction derived by non-aqueous extraction possibly results from contamination by peroxisomes which have been suggested to contain peroxidase activity by Plesnicar et al. (56). Under aqueous methods, these microbodies are easily ruptured and contribute to the activity assayed as the soluble fraction.

Following aqueous and non-aqueous extraction of 4-day-old wheat seedlings, peroxidase banding was observed in all fractions. Quantitative and qualitative differences were observed among the various preparations. The apparent ubiquitous occurrence of members of the peroxidase system may imply varied functional roles although the nature of these has not been defined.

V. SUMMARY

1. The ontogenetic zymograms for esterase, malate dehydrogenase, and peroxidase of normal light- and dark-grown wheat seedlings were determined at 12 hour intervals from 12 to 168 hours after sowing.
2. A slight retardation in the appearance of certain esterase bands was observed in the normal dark-grown seedlings. Only a few other light-dark effects were detected in any of the other treatments.
3. During the first 24 hours after sowing, no strongly stained bands with peroxidase activity were detected in most of the treatments. The enzymatic activity assayed over this period of development was very low. Later development resulted in an accelerated increase in the concentration of peroxidase per seedling.
4. Considerable variability was observed in the MDH zymograms for each treatment. The cause of this variability remains unknown.
5. Inhibition of an increase in the DNA content of wheat seedlings by high level gamma radiation (500,000 rads) or 10^{-4} M FdUrd did not drastically alter the isoenzyme complements of the enzyme systems with respect to the number and grouping of electrophoretic bands, although a lag in the developmental expression of certain isoenzymes was observed.
6. The total activity of peroxidase in the light-grown gamma-plantlet demonstrated a faster rate of increase than did all of the other treatments. Therefore the gamma-plantlet may not so nearly approach normalcy. This change in rate of appearance may be due to an acceleration of the senescence process brought about by radiation exposure.

7. The changes observed in the total activity and specific activity for each enzyme studied are uniquely different. This indicates that these enzyme systems are clearly independently controlled during development of wheat seedlings.
8. The results from organ separation show large amounts of esterase and peroxidase activity in the roots while MDH was localized primarily in the leaf. The zymograms illustrate differences in the enzyme complements in each organ.
9. The examination of the first leaf segment revealed that the area of highest total and specific activity for peroxidases was located in the basal segment. Since this basal segment in grasses contains the intercallary meristem, these data tend to support the work of van Fleet (76) who histochemically showed an increase in peroxidase levels in cells just prior to cell division.
10. At the subcellular level the larger amounts of esterase and peroxidase activities were observed in the soluble fraction (122,000 x g supernatant) while MDH activity occurred in the mitochondrial (10,000 x g pellet) and the chloroplast (2,000 x g pellet) fractions.

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APPENDIX

Table 4. Table of electrophoretic experiments

Experiment	Independent replicates	Banding nomenclature
Buffer series	2	BS
Normal light-grown seedling development	12	NL
Normal dark-grown seedling development	3	ND
Light-grown gamma-plantlet development	5	GL
Dark-grown gamma-plantlet development	2	GD
Light-grown FdUrd-seedling development	7	FL
Dark-grown FdUrd-seedling development	3	FD
Organ separation	4	O
First leaf segments	4	L
Aqueous organelle enrichment	8	SC
Non-aqueous organelle enrichment	3	SC

VITA

Doris Marie Presley was born in Bristol, Virginia on November 19, 1946. After the age of three days she was taken home to Bristol, Tennessee and has remained a resident of Tennessee. Early education was obtained through the city public school system with graduation from Bristol Tennessee High School in 1964.

In the fall she entered East Tennessee State University where she followed a secondary education curriculum. She graduated cum laude with a Bachelor of Science degree in Biology and Mathematics in June, 1968. During these four years she was very active in the Phi Mu sorority serving as treasurer, pledge trainer, and chaplain; in student government with election to the House of Representatives and Judicial Review Board; and in special interest groups such as serving as president and vice-president of Senior Orchesis and as a member of Kappa Delta Pi, national honorary educational fraternity. In 1968 Miss Presley was listed in Who's Who Among Students in American Universities and Colleges.

She entered the Graduate School at East Tennessee State University in September, 1968 and received a Master of Science degree in Biology August, 1970. During this time she served as a graduate teaching assistant in the Department of Biology. Her area of special interest was plant morphology and embryology and her thesis was entitled "Embryology of Clintonia". During the academic year 1970-71 she was employed as a mathematics instructor at Cleveland City High School, Cleveland, Tennessee. She taught advanced algebra and trigonometry.

In September, 1971, Miss Presley was awarded a three-year NDEA fellowship to pursue a doctoral degree in the Botany Department of the University of Tennessee. In her fourth year of study in plant physiology she accepted a graduate teaching assistantship. Following election by fellow graduate students, she served as student representative on the Graduate Council of the University of Tennessee (1973-74). She received the Doctor of Philosophy degree in Botany in August, 1976, while employed in the Biology Department of Virginia Intermont College.