Phytochemistry, 1967, Vol. 6, pp. 465 to 471. Pergamon Press Ltd. Printed in England

SOLUBLE PROTEINS AND MULTIPLE ENZYME FORMS IN EARLY GROWTH OF WHEAT

V. MACKO, G. R. HONOLD and M. A. STAHMANN

Department of Biochemistry, University of Wisconsin, Madison, Wisconsin, U.S.A.

(*Received* 14 *September* 1966)

Abstract—Changes in the soluble proteins and enzymes of wheat (*Triticum aestivum L.*), var. Lee, in relation to germination and early plant development were studied. Extracts of dormant seed, germinated seed, etiolated coleoptile, and first green leaf were analyzed by polyacrylamide disc electrophoresis. Patterns of soluble proteins and the isoenzyme bands of the following enzymes were studied: glucose-6-phosphate, 6-phosphogluconate, malate, isocitrate, glutamate, and alcohol dehydrogenases; peroxidase; catalase; acid and alkaline phosphatases; esterase; and amylase. Photographs and densitometer tracings of gels showing changes during germination and growth are presented.

INTRODUCTION

THE changes in morphology and physiology during plant development are associated with changes in protein constitution of various tissues.[1–4] Recent techniques have revealed many previously unrecognized protein components in extracts of plants.[5]

Amido black is frequently used to stain soluble proteins after separation by zone electrophoresis. Differences have been shown between wheat varieties[6–7] and different stages of growth.[8] Phylogenetic affinities were investigated by Johnson and Hall.[9] The electrophoretic pattern of soluble proteins from the leaf of one variety was shown by Wrigley *et al.*[10]

Although multiple forms of enzymes and different electrophoretic mobilities have been extensively studied in various animal tissues,[11–18] little information is available concerning similar studies in plant tissue, especially about changes in isozymic composition after germination.

[1] F. C. STEWARD, R. F. LYNDON and J. T. BARBER, *Am. J. Botany* **52**, 155 (1965).
[2] F. C. STEWARD and D. J. DURZAN, In *Plant Physiology* (Edited by F. C. STEWARD), Vol. 4A, p. 379. Academic Press, New York (1965).
[3] R. L. CLEMENTS, *Phytochem.* **5**, 243 (1966).
[4] D. KOLLER, A. M. MAYER, A. POLJAKOFF-MAYBER and S. KLEIN, *Ann. Rev. Plant Physiol.* **13**, 437 (1962).
[5] M. A. STAHMANN, *Ann. Rev. Plant Physiol.* **14**, 137 (1963).
[6] C. B. COULSON and A. K. SIM, *Nature* **202**, 1305 (1964).
[7] C. B. COULSON and A. K. SIM, *J. Sci. Food Agr.* **16**, 458 (1965).
[8] C. B. COULSON and A. K. SIM, *J. Sci. Food Agr.* **16**, 499 (1965).
[9] B. L. JOHNSON and O. HALL, *Am. J. Botany* **52**, 506 (1965).
[10] C. W. WRIGLEY, H. L. WEBSTER and J. F. TURNER, *Nature* **209**, 1133 (1966).
[11] H. LAUFER, *Ann. N.Y. Acad. Sci.* **94**, 825 (1961).
[12] C. L. MARKERT and H. URSPRUNG, *Develop. Biol.* **5**, 363 (1962).
[13] D. T. Lindsay, *J. Exptl Zool.* **152**, 75 (1963).
[14] C. L. Markert, In *Second Int. Con. on Congenital Malformations*, p. 163. International Medical Congress, New York (1964).
[15] J. A. STEWARD and J. PAPACONSTANTINOU, *Biochim. Biophys. Acta* **121**, 69 (1966).
[16] B. FIELDHOUSE and C. J. MASTERS, *Biochim. Biophys. Acta* **118**, 538 (1966).
[17] M. EGUCHI and T. SUGIMOTO, *J. Insect Physiol.* **11**, 1145 (1965).
[18] C. R. SHAW, *Science* **149**, 936 (1965).

Recent work in this laboratory showed the isoenzyme patterns of dehydrogenases in wheat seed and wheat milling fractions.[19] It is the purpose of this paper to extend these studies to the electrophoretic patterns of protein and enzyme components during the major morphological changes in germination and early plant development of wheat (*Triticum aestivum L.*), var. Lee. Extracts containing proteins from dormant seed, germinated seed, coleoptile, and first leaf tissue were studied.

RESULTS AND DISCUSSION

Soluble proteins and the multiple enzyme forms of dehydrogenases, oxidases, and hydrolases were detected.

Soluble Proteins

The electrophoretic patterns of soluble proteins stained by amido black are shown in Fig. 1 (A). Differences between dormant seed and germinated seed were observed in the fastest-migrating bands. The coleoptile and leaf extracts exhibited much different patterns than the dormant and germinated seed in addition to differing extensively from one another. A thick band of protein was always observed near the top of the gels containing leaf extracts Fig. 1 (A), No. 4. It is apparantly a nonspecific aggregate as shown by column chromatography in this laboratory[20] and may correspond to Fraction I protein. A similar aggregate has been found in a number of other plant tissue extracts.

Dehydrogenases

The most intense bands of activity for a given dehydrogenase on the gels had about the same R_f (distance of migration of a given band relative to the distance of migration of the bromphenol blue marker dye) in all samples; however, the number of bands varied. On zymograms of dehydrogenases, a dark band appeared at the marker dye front (see bottom of Fig. 1 (B,C,D)). This was reduction of nitro-blue tetrazolium by ascorbic acid in the extraction buffer.

Glucose-6-phosphate dehydrogenase (*EC* 1.1.1.49).[21] The isoenzyme patterns are shown in Fig. 1(B). The number of bands varied from four in extracts of the seed to six in first leaf extracts. The dormant seed and germinated seed fractions appeared to have nearly equal levels of activity as judged by band intensity. The coleoptile fraction contained the most intense bands, and the lowest apparent activity was observed in leaf extract. The wide bands that appear on the gel photographs are actually comprised of many bands close together, the R_f's of which are presented in Table 1.

6-Phosphogluconate dehydrogenase (*EC* 1.1.1.44). One heavy band of activity developed at R_f 0·37 in all samples. Upon prolonged incubation of gels in the substrate solution traces of additional light bands appeared at R_f 0·28 and 0·32.

The intensities of band stains on the gels indicate that there was an increase in glucose-6-phosphate and 6-phosphogluconate dehydrogenase activities in the germinated seed relative to the dormant seed and a still higher activity in the coleoptile as compared to lower activity in the first leaf. Both glucose-6-phosphate and 6-phosphogluconate dehydrogenases play a role in the metabolism of glucose via the pentose shunt. This pathway is not an important

[19] G. R. HONOLD, G. L. FARKAS and M. A. STAHMANN, *Cereal Chem.* **43**, 517 (1966).
[20] W. WOODBURY, Personal communication (1966).
[21] International Union of Biochemistry on the Nomenclature and Classification of Enzymes. In *Comprehensive Biochemistry* (Edited by M. FLORKIN and E. H. STOTZ), Vol. 13. Elsevier, Amsterdam (1965).

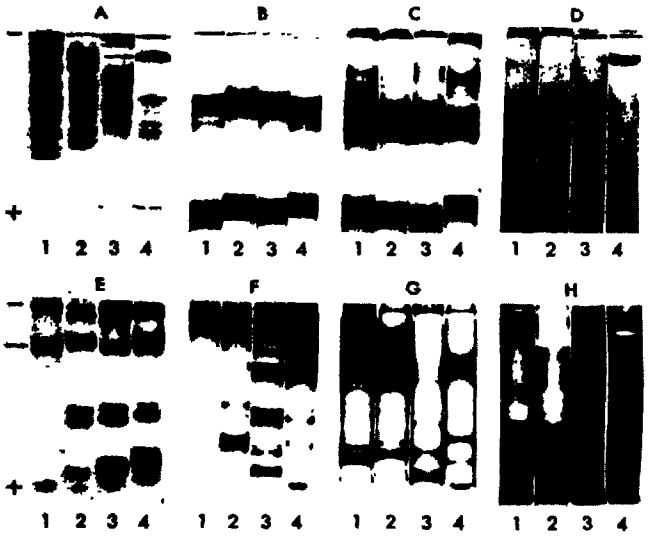

FIG. 1. ELECTROPHORETIC PATTERNS OF (A) SOLUBLE PROTEINS; (B) GLUCOSE-6-PHOSPHATE DEHYDROGENASE; (C) MALATE DEHYDROGENASE; (D) ALCOHOL DEHYDROGENASE; (E) PEROXIDASE, DONOR: CATECHOL; (F) PEROXIDASE, DONOR: BENZIDINE, (G) ESTERASE, AND (H) AMYLASE IN (1) DORMANT SEED; (2) GERMINATED SEED; (3) ETIOLATED COLEOPTILE; AND (4) FIRST LEAF OF WHEAT (*Triticum aestivum L.*), VAR. Lee.

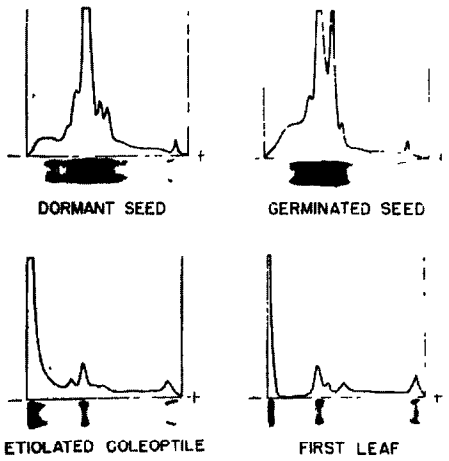

FIG. 2. ELECTROPHEROGRAMS AND DENSITOMETER TRACINGS OF ACID PHOSPHATASE IN EXTRACTS OF WHEAT (*Triticum aestivum L.*), VAR. Lee.

[*facing page* 466]

contributor of energy but it generates $NADPH_2$ and serves as a potential source of ribose-phosphate units that may be utilized in the synthesis of nucleic acids. It appears significant that the germinated seed and coleoptile contained the higher activity since there is extensive nucleic acid synthesis in these young, rapidly-growing tissues.[22] The relative importance of this pathway decreases during growth and development of seedlings, giving way to glycolysis and the citric acid cycle.[23]

TABLE 1. THE R_f'S AND RELATIVE INTENSITIES OF ISOENZYME BANDS OF GLUCOSE-6-PHOSPHATE DEHYDROGENASE IN FOUR STAGES OF DEVELOPMENT OF WHEAT

Dormant seed		Germinated seed		Etiolated coleoptile		First leaf	
R_f*	Intensity†	R_f	Intensity	R_f	Intensity	R_f	Intensity
						0·08	trace
				0·15	x		
0·31	trace						
0·37	xxx	0·37	xxx	0·37	xxxx	0·38	xx
0·43	xxx	0·42	xx	0·42	xx	0·43	x
		0·47	xx	0·47	xx	0·47	xx
0·50	x			0·51	x	0·52	x
		0·54	x			0·57	x

* $R_f = \frac{\text{distance that enzyme band migrates}}{\text{distance that marker dye migrates}}$

† Increasing intensity of bands is represented from x to xxxx.

Isocitrate dehydrogenase (*EC* 1.1.1.42). Development of bands for this enzyme required a much longer incubation time than other dehydrogenases. One light band appeared at R_f 0·40 in all samples. In the case of germinated seed, a second light band appeared at R_f 0·15.

Malate dehydrogenase (*EC* 1.1.1.37). Isoenzyme patterns are shown in Fig. 1 (C). Many bands of activity occurred and some of these were close together so that they appear in Fig. 1 (C) as a single band rather than individual moieties. More bands were observed for dormant and germinated seed than were observed for coleoptile. In the case of first leaf, just two broad dark bands were observed followed by a slower-migrating smear of activity. From the rate of band development, it was evident that the activity of malate dehydrogenase was much higher than any of the other dehydrogenases that were investigated. Malate dehydrogenase, an enzyme of universal distribution in wheat seed and milling fractions,[19] appeared equally active in all samples studied. In the dormant seed, malate dehydrogenase activity was more equally distributed among various isoenzyme forms than in other samples.

Glutamate dehydrogenase (*EC* 1.4.1.2). A long incubation time for band development on gels was required due to low activity. The number of bands observed ranged from one for dormant seed to three for germinated seed and coleoptile. A common band at R_f 0·25 was detected in all four samples with additional bands at R_f's 0·19 and 0·31 in germinated seed and coleoptile. Leaf tissue extract contained the R_f 0·31 band but not the R_f 0·19 band. The intensity of bands was highest in dormant seed and lowest in first leaf.

Alcohol dehydrogenase (*EC* 1.1.1.1). This enzyme appeared as one band of R_f 0·40 in dormant seed, germinated seed, and coleoptile as shown in Fig. 1 (D). No activity was

[22] J. E. VARNER, In *Plant Biochemistry* (Edited by J. BONNER and J. E. VARNER), p. 763. Academic Press, New York (1965).

[23] M. CHAKRAVORTY and D. P. BURMA, *Biochem. J.* 73, 48 (1959).

detected in first leaf extract. The band which is shown on the first leaf zymogram in Fig. 1 (D) is the nonspecific protein aggregate discussed in relation to Fig. 1 (D), No. 4. The band intensity decreased from dormant seed to germinated seed to coleoptile. The results observed for alcohol dehydrogenase are of interest because of the trend from a very dark band in the dormant seed extract to a very light one in the coleoptile extract and no visual band in the extract of first leaf tissue. This is not surprising since the seed is more anaerobic than leaf tissue and anaerobic conditions seem to promote the formation of enzymes connected with fermentation, e.g. alcohol dehydrogenase.[24]

Peroxidase (*EC* 1.11.1.7). The isoenzyme pattern of peroxidase stained with catechol and hydrogen peroxide is shown in Fig. 1 (E). Only one dark band was observed in dormant seed extract. Four new isoenzymic bands were detected in germinated seed, coleoptile, and first leaf. The band marked by an arrow in Fig. 1 (E) seems to be a catalase. It did not stain with guaiacol as a hydrogen donor. Supporting evidence was provided by negative-staining of catalase activity on a starch-containing gel. When comparing peroxidase electrophoretic patterns, it is necessary to consider the hydrogen donor because the donor that is used will affect the pattern obtained. This was shown in our experiments with catechol, guaiacol, and benzidine as hydrogen donors for peroxidase. The electrophoretic patterns of peroxidase stained with benzidine and hydrogen peroxide are shown in Fig. 1 (F). The apparent activity was very low in dormant seed. A dramatic increase in peroxidase activity occurred upon germination. Qualitatively, the patterns from germinated seed, coleoptile, and first leaf are similar. Quantitatively, they differ considerably in intensity of individual bands relative to one another. It is interesting to note that the full complement of peroxidase isoenzymes was not observed in dormant seed. More peroxidase bands were observed in germinated seed, coleoptile, and first leaf. Ockerse *et al.*[25] suggested that peroxidase isoenzymes may be under hormonal control. A similar control mechanism may be involved in germination.

Hydrolases

Acid phosphatase (*EC* 3.1.3.2). Acid phosphatase isoenzyme patterns are shown in Fig. 2. Densitometer tracings are included to provide semiquantitative estimations of band intensities. Five bands were observed for dormant seed and coleoptile, whereas four were detected for germinated seed and first leaf. An interesting point is that most of the activity in the coleoptile and first leaf fractions did not migrate very far into the gel. This was not the case with the dormant seed and germinated seed. Nothing definite is known about the physiological role of nonspecific phosphomonoesterases. Although extensive changes were noted in acid phosphatase electrophoretic patterns during early plant development, the heterogeneity of this phosphatase activity may be attributable to the presence of several enzymes with broad and quite different substrate specificities.[26–33]

Acid phosphatase was assayed by the lead acetate method of Gomori as used in this

[24] R. H. HAGEMAN and D. FLESHER, *Arch. Biochem. Biophys.* **87**, 203 (1960).
[25] R. OCKERSE, B. F. SIEGEL and A. W. GALSTON, *Science* **151**, 452 (1966).
[26] J. BROUILLARD and L. QUELLET, *Can. J. Biochem.* **43**, 1899 (1965).
[27] D. W. A. ROBERTS, *J. Biol. Chem.* **219**, 711 (1956).
[28] D. W. A. ROBERTS, *J. Biol. Chem.* **222**, 259 (1956).
[29] D. W. A. ROBERTS, *J. Biol. Chem.* **226**, 751 (1957).
[30] D. W. A. ROBERTS, *J. Biol. Chem.* **230**, 213 (1958).
[31] D. W. A. ROBERTS, *Can. J. Biochem. Physiol.* **41**, 113 (1963).
[32] D. W. A. ROBERTS, *Can. J. Biochem. Physiol.* **41**, 1275 (1963).
[33] D. W. A. ROBERTS, *Can. J. Biochem. Physiol.* **41**, 1727 (1963).

laboratory[34] in addition to the diazonium salt method. The patterns of the two methods were qualitatively the same; however, the lead acetate assay was not as sensitive.

Alkaline phosphatase (*EC* 3.1.3.1). No activity was detected in any of the fractions.

Esterase (*EC* 3.1.1.2). A characteristic of esterase is the high number of isoenzyme bands that appear in the fast-migrating section of the gel as shown in Fig. 1 (G). The level of esterase activity appeared to be much higher in the dormant seed and germinated seed than in the coleoptile and first leaf. Similar observations were made by Schwartz[35] in studies on maize. Three prominent bands appeared with high R_f's in addition to broad smears of activity closer to the origin. Coleoptile had four fast-migrating bands plus one a little slower but the broad smear of activity was lacking. The extract of first leaf contained four bands which migrated quite fast and three discernible bands in the area on the gel where the smear of activity appeared for dormant seed and germinated seed.

More information is available on the electrophoretic properties of esterases than of other enzymes in plants.[34, 36, 37] Plants contain a multiplicity of esterases which vary in different species, in different strains of the same species, and even in different parts of the same plant.[37] The same situation exists in animals.[38, 39]

The qualitative and quantitative changes in the esterases observed in these experiments may be related to breakdown of storage materials such as proteins in seeds during germination. It has been suggested[37] that carboxylic ester hydrolase activity may be due to proteinases or peptidases that are rather nonspecific.

Amylase (*EC* 3.2.1.1 *and EC* 3.2.1.2). The activation of amylase during germination is well-documented.[40, 41] Figure 1 (H) demonstrates the amylase activation in germinated seed. Amylase activity was low in dormant seed; however, much activity (starch degradation) was noted in the gels containing extracts from the germinated seed. No amylase was noted in coleoptile but low activity was detected in the first leaf extract.

GENERAL DISCUSSION

The analysis of electrophoretic variants of enzymes has application in genetics, biochemistry, and pathology of plants and animals.[18, 42–46] Although the cells of a plant have the same genotype, the phenotypic expression is manifested in different ways through the synthesis of proteins specific for each stage of development. The differential activation of genes in developing cells is an essential aspect of cell differentiation.[14] The combination of subunits of some enzymes is apparently controlled by different genes and gives rise to hybrids with different electrophoretic properties.[14,43,45] Whether the isozyme patterns of all enzymes could be the consequence of random association of subunits remains to be determined. The

[34] K. RUDOLPH and M. A. STAHMANN, *Plant Physiol.* **41**, 389 (1966).
[35] D. SCHWARTZ, *Proc. Natl Acad. Sci.* **48**, 750 (1962).
[36] J. VAN DER JOOSTE and D. E. MORELAND, *Phytochem.* **2**, 263 (1963).
[37] H. M. SCHWARTZ, S. I. BIEDRON, M. M. VON HOLDT and S. REHM, *Phytochem.* **3**, 189 (1964).
[38] J. PAUL and P. FOTTERELL, *Biochem. J.* **78**, 418 (1961).
[39] J. M. ALLEN and R. L. HUNTER, *J. Histochem. Cytochem.* **8**, 50 (1960).
[40] J. E. VARNER and G. R. CHANDRA, *Proc. Natl Acad. Sci.* **52**, 100 (1964).
[41] L. G. PALEG, *Ann. Rev. Plant Physiol.* **16**, 291 (1965).
[42] J. H. WILKINSON, *Isoenzymes*, p. 158. E. & F. N. Spon, London (1965).
[43] D. SCHWARTZ, *Proc. Natl Acad. Sci.* **46**, 1210 (1960).
[44] D. SCHWARTZ and T. ENDO, *Genetics* **53**, 709 (1966).
[45] J. G. SCANDALIOS, *Proc. Natl Acad. Sci.* **53**, 1035 (1965).
[46] M. A. STAHMANN, Proc. U.S.–Japan seminar on *The Dynamic Role of Molecular Constituents in Plant-Parasite Interactions* (In press).

mechanisms by which genes are turned on or off are still unknown; however, they are the subject of increasing research. It has been suggested that hormones may act in depression of genes.[47]

Heterogeneity in the molecular composition of an enzyme would not necessarily imply greater biological importance. However, examination of our zymograms from different tissue reveals that each tissue has its own specific pattern of isozymes with some of the enzymes which suggests metabolic significance that has not, as yet, been elucidated.

MATERIALS AND METHODS

A hard red spring wheat (*Triticum aestivum L.*), var. Lee, was analyzed. Extracts were made from dormant seed, etiolated coleoptile (including the leaf), germinated seed, and the first leaf of the seedlings.

Dormant seed. The seed was ground in a small Wiley mill with medium mesh screen for 20 min and the ground seed served as a starting material for extraction.

Germinated seed and coleoptile. Seeds were germinated in the dark on moist filter paper in a saturated atmosphere of water in enclosed glass trays at 19°. When the coleoptiles were about 15 mm long, they were separated from the seed. The roots were cut from the germinated seed and discarded.

First leaf. The seeds were planted in vermiculite in growth chambers under controlled conditions (day: 16 hr, 22°; night: 8 hr, 17°; light intensity: 250 lux at plant height) and irrigated with Hoagland's solution. The first leaf was harvested 10 days after planting.

Extraction was done by grinding in a mortar using 0·1 M pH 8·0 Tris-HCl (tris[hydroxymethyl]aminomethane-hydrochloride) buffer containing 0·5 M sucrose, 0·006 M ascorbic acid, and 0·006 M cysteine-hydrochloride for extraction according to Staples and Stahmann.[48] Samples were centrifuged 1 hr at 100,000*g*. Extraction of samples, centrifugation, and electrophoresis were conducted at 4°. Samples containing 250 μg of protein were applied to each gel for electrophoresis. The protein content of extracts was determined by the biuret method.[49] Disc electrophoresis was performed according to Ornstein[50] and Davis.[51] Soluble proteins were stained with amido black (C.I. 20470) in 7% acetic acid and excess dye was removed by electrophoretic destaining. Staining for enzymatic activities on gels was done by histochemical methods[42, 52] as modified in this laboratory.[19, 34, 48]

Dehydrogenases were assayed by coupling the enzymatic activity to reduction of nitro-blue tetrazolium (NBT). The incubation solution contained the appropriate substrate, $MgCl_2$, NAD or NADP as required, electron-transfer agent (phenazine methosulfate), NaCN, Tris-HCl pH 7·5 buffer, and NBT.[19]

Peroxidase was assayed using the hydrogen donors catechol, guaiacol, and benzidine. For catechol or guaiacol, the gels were immersed in 0·02 M donor for 30 min, washed, and then incubated in 0·1% H_2O_2 for band development.[48] For benzidine, an aqueous saturated solution of benzidine was used which contained 0·1% H_2O_2.

Catalase was assayed in gels containing 0·3% hydrolyzed starch. The gels were first immersed in 0·1% H_2O_2 for 1 min and then incubated in 1·5% KI for negative staining of

[47] J. BONNER, *J. Cell. Comp. Physiol.* **66**, 77 (1965).
[48] R. C. STAPLES and M. A. STAHMANN, *Phytopathology* **54**, 760 (1964).
[49] A. G. GORNALL, C. J. BARDAWILL and M. M. DAVID, *J. Biol. Chem.* **177**, 751 (1949).
[50] L. ORNSTEIN, *Ann. N. Y. Acad. Sci.* **121**, 321 (1964).
[51] B. J. DAVIS, *Ann. N. Y. Acad. Sci.* **121**, 404 (1964).
[52] M. S. BURSTONE, *Enzyme histochemistry*, p. 621. Academic Press, New York (1962).

bands, i.e. the whole gel stained a deep blue due to the starch-iodine reaction except where catalase destroyed H_2O_2, thus preventing the oxidation of iodide to iodine.

For amylase, 0·3% hydrolyzed starch was incorporated into the gel before electrophoresis. The gels were incubated in a solution of 0·004% iodine and 1·5% KI in 0·2 M acetate buffer at pH 4·8. Bands were observed by negative staining. The amylase assay was used as a control for catalase, since starch destruction by amylase in the gels results in a negative stain that could be interpreted as catalase.

Acid phosphatase was assayed by the diazonium dye method using α-naphthyl phosphate and Diazo Blue B in pH 5·1 sodium acetate buffer.[34] Acid phosphatase staining was also conducted using sodium glycerophosphate and lead nitrate in pH 5·1 sodium acetate buffer.[34]

Alkaline phosphatase was assayed by the latter method using 0·1 M Tris-HCl buffer at pH 9·5.

Esterase activity was detected on gels with Fast Blue RR (diazotized) and α-naphthyl acetate in Tris-HCl buffer at pH 7·4.[34]

Densitometer tracings of gels were obtained by using a Joyce Chromoscan densitometer. A green filter with a maximum transmittance at 532 mμ was used for scanning enzyme patterns and a red filter with a maximum transmittance at 660 mμ was used for scanning soluble protein patterns.

Acknowledgement—This study was supported by Agricultural Research Service, U.S. Department of Agriculture Grant No. 12-14-100-7654 (74) administered by the Western Utilization Research and Development Division, Albany, California. Published with the approval of the Director of the Wisconsin Agricultural Experiment Station.