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Isoenzymatic Characterization of Cell Cultures of Bush Bean.

Isoenzymatic Characterization of Cell Cultures of Bush Bean, *Phaseolus vulgaris* L. cv. Contender

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ISOENZYMATIC CHARACTERIZATION OF CELL CULTURES OF BUSH BEAN (*Phaseolus vulgaris* cv. Contender).

by

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Ph.D. Biology

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Isoenzymatic characterization of cell cultures of bush bean (Phaseolus vulgaris cv. Contender).

ABSTRACT

Isoenzyme patterns of various enzymes were studied in callus and suspension cultures of root, hypocotyl and cotyledon of bush bean.

Isoenzyme patterns changed during the culture cycle and reflected the successive stages. Persistent differences in pattern were detected among the cultures and there was a correlation between cell expansion and peroxidase activity.

Effects of 2,4-D and kinetin on growth, cell form and the isoenzyme complements are reported.

The isoenzyme patterns of stock and newly established cultures were compared with those of the mature plant and seedling parts. Often the isoenzyme patterns of cultured cells were most similar to those of root; however, persistent isoenzymatic differences were detected among the cultures and some of these differences could be attributed to the tissue of origin.

The relevance of the results to the persistence and stability of characteristics in plant cell cultures and to differentiation is discussed.

D. Ph. Biologie

Paul G. Arnison

Etude des caractéristiques isoenzymatiques des cultures de cellules de Phaseolus vulgaris cv. Contender.

ABSTRAIT

Les bandes isoenzymatiques de certains enzymes ont été étudiées dans des cultures en callus ou en suspension de racine, d'hypocotyle et de cotylédon de Phaseolus vulgaris.

Les bandes isoenzymatiques se sont modifiées pendant le cycle de culture et reflétèrent ainsi les stades successifs de développement. De plus des différences persistentes dans les bandes ont été observées dans les cultures et une corrélation entre l'expansion cellulaire de l'activité de l'enzyme peroxydase a été démontrée.

Les effets de 2,4-D et de la kinétine sur la croissance, la morphologie cellulaire et les bandes isoenzymatiques sont décrits.

Les bandes isoenzymatiques de cultures établies et nouvelles ont été comparées avec celles de plantes mâtures et de plantules. Les bandes isoenzymatiques de cellules en culture étaient souvent semblables à celles de la racine, cependant des différences isoenzymatiques persistentes ont été découvertes parmis les cultures et certaines de ces différences sont attribuables au tissu d'origine.

L'importance de ces observations sur la persistence, la différentiation et la stabilité des caractéristiques de cultures de cellules chez les plantes est discutée.

Traduit par Marc-André Paré.

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ABBREVIATIONS

Tris	Trishydroxyaminomethane
EDTA	Ethylene diamine tetraacetic acid
NAD+	Nicotine adenine dinucleotide
NADP+	Nicotine adenine dinucleotide phosphate
NBT	Nitroblue tetrazolium
PMS	Phenazine methosulfate
DOPA	3,4-dihydroxy-phenylalanine
2,4-D	2,4-dichlorophenoxyacetic acid
IAA	Indole-3-acetic acid
NAA	Napthyleneacetic acid

AAP	Alanine amino peptidase
ADH	Alcohol (ethanol) dehydrogenase
Ald	Aldolase
AlP	Alkaline phosphatase
Amy	Amylase
AP	Acid phosphatase
Cat	Catalase
Est	Esterase
FDH	Folate dehydrogenase
GalDH	Galactose-6-phosphate dehydrogenase
GDH	Glutamate dehydrogenase
GOT	Glutamate-oxalacetate transaminase
G6PDH	Glucose-6-phosphate dehydrogenase
IAAOx	Indoleacetic acid oxidase
IndOx	Indophenol oxidase
LAP	Leucine amino peptidase
LDH	Lactate dehydrogenase
MDH	Malate dehydrogenase
PAL	Phenylalanine ammonia lyase
Per	Peroxidase
6PGDH	6-Phosphogluconate dehydrogenase
Phs	Phosphorylase
PPO	Polyphenol oxidase
RNase	Ribonuclease
SDH	Succinic dehydrogenase
TDA	Threonine deaminase
PP	Peroxidase (polyphenolic)

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I INTRODUCTION

Almost certainly our first scientific knowledge of a plant is based on observation of its growth and form. We recognize species by the distinctive characters of size, branching pattern, flower form, cross sections, size and shape of leaves, and so on. There exists, however, a wide gap between observation and description and the interpretation of the causal factors that lead to development. We know with certainty that the genetic information contained within the modest acorn will somehow lead to the development of a magestic oak. Of how this information is unlocked, channelled, monitored and regulated, we know in reality, very little.

In order to understand the control of development in a complicated organism useful facts and features can be studied in a simpler and more isolated system. To this end we turn to the study of single cells and small groups of cells participating in fundamental cellular activities and basic developmental processes.

Plant cell cultures can now be used for the study of metabolic processes, growth, differentiation, and development. Their potential value was stressed eloquently by Torrey (1971).

"Techniques of plant tissue culture offer some of the most promising approaches to an understanding of cytodifferentiation and its physiological and biochemical control. Analysis of the determination of morphological expression at the cell level by genetically and epigenetically controlled molecular events has hardly begun."

During the process of development differentiation into specialized tissues and organs may involve more or less irreversible cellular changes. The question of the transmission of these differentiated states in cell

lineages is fundamental to the understanding of developmental control mechanisms. It is not known to what extent cell cultures derived from different parts of the parent plant show persistent differences in vitro and if such differences do exist, to what extent they are related to the tissue of origin.

It is to this problem that the attention of the thesis is turned. The work reported herein is concerned with: the characterization of plant cells in culture and in particular the value of isoenzymes for such characterization, the degree to which differences between cell populations from different parts of the plant persist in culture, the degree to which these differences can be attributed to the point of origin of the culture. In addition, and as part of this study, the effects of the growth regulators 2;4-D and kinetin on the expression of morphological cell parameters and biochemical characters were studied.

Part of this work has been accepted for publication (Arnison and Boll, 1974, 1975a). The remainder has been submitted (Arnison and Boll 1975b, c), or prepared for publication (Arnison and Boll 1975d,e).

II REVIEW OF THE LITERATURE

A) Differences Between Cultures Derived From Different Parts of the Parent Plant

One of the most fundamental questions in development is whether differentiation into specialized tissues and organs may involve more or less irreversible changes which are then transmitted in cell lineages.

Plant tissue cultures in general, regardless of tissue or plant of origin, tend to be parenchymatous in nature and look remarkably alike. The variability of cell types and the ability to undergo morphogenesis tend to decrease with culture age (Steward and Mapes 1963, Stonier 1965, Torrey 1967, Syono 1969), although this is not true in every case (Khanna and Staba 1970). The changes that may occur during prolonged serial subculture are often attributed to changes in chromosome number and type (Fox 1963, Partenen 1963, 1965, 1972; Murashige and Nakano 1965, 1967; Torrey 1967; Shimada et al 1969, Van't Hof and McMillan 1969; Heinz et al 1969, Norstog et al 1969, Steward et al 1970; Shimada 1971). This assessment must be viewed with caution as regeneration has been demonstrated from old and very aneuploid tobacco cultures (Sacristan and Melchers 1969; Asuwa 1972).

The question of transmission of differentiated states through somatic cell lineages and the loss of the ability to regenerate differentiated states, is fundamental to the understanding of control mechanisms in development (Heslop-Harrison 1967, Halperin 1969). Related to this question is the still unresolved issue of whether cell cultures, derived from different parts of a parent plant or seedling, show persistent differences in properties in vitro. Considerable attention has been paid this matter by Heslop-Harrison (1967). Heslop-Harrison considers that there are three

possibilities, namely: a) there are no individual characters maintained in cell cultures and that all cultures regardless of point of origin in the parent plant will be identical, b) origin specific characters are initially present in cell cultures but these differences decline within a few passages and then all cultures will be identical, c) that origin specific characters are present in cell cultures and that these differences are maintained indefinitely in cell lineages.

Few detailed comparisons of plant cell cultures derived from different parts of the same plant have been published. Barker (1969) examined callus cultures derived from ovary wall, root, shoot, petiole and perimedullary region of basswood (Tilia americana L.) and various tissues of wheat (Triticum aestivum L.). Barker, using only criteria of appearance and the ability of calluses to meld together, concluded that the various cultures were not distinguishable.

In contrast, callus cultures derived from juvenile and adult forms of ivy (Hedera helix L.) showed persistent differences in growth, form, growth factor requirements and other characteristics (Stoutmeyer and Britt 1963, 1965, 1969). Similarly differences in the proliferative capacity of in vitro adult and juvenile clones of black locust (Robinia pseudoacacia) have been shown (Trippi 1963). The observations of Stoutmeyer and Britt were supported by those of Robbins and Hervey (1970). However, Street (personal communication) was unable to confirm these results. Caponeti et al (1971), from a study of black cherry (Prunus serotina) callus cultured from cambial zones of juvenile seedlings, mature trees and mature scions grafted on to juvenile trees, concluded that broad interclonal variation masked the differences in growth of tissue from juvenile and mature trees. Caponeti

et al used the term clone to refer to all calluses derived from the same tree.

Nutritional and morphological investigations of callus cultures derived from different parts of a cactus (Neomammillaria prolifera, Miller) showed varied callus formation from the different parts and no callus formation from the root (Minocha and Mehra 1974). Calluses that were established showed a high coconut milk and growth regulator requirement and showed no differentiation.

Callus cultures from root, stem and cotyledon of pinto bean (Phaseolus vulgaris L.) established by Nickell and Tulecke (1959) showed different responses to the growth hormone gibberellic acid. Root callus but not stem or cotyledon callus, was inhibited by 10 ppm gibberellic acid. Differences in activity of the enzymes isocitrate dehydrogenase, malate dehydrogenase and shikimate dehydrogenase were reported in suspension cultures derived from root, hypocotyl and cotyledon of mung bean (Phaseolus aureus)(Gamborg 1966). It was not stated either by Nickell and Tulecke or by Gamborg that the three cultures were established from the same seedling.

Previous work on bush bean (Phaseolus vulgaris cv. Contender) cell cultures has shown that although the cultures showed no marked differences in growth pattern or cell form there were marked differences in response to growth regulators, in tryptophan synthase activity, production of extracellular polysaccharide and color of cultures (Liau and Boll 1970, 1971, 1972; Liau 1971, Mante 1974, Sein 1974).

Recently Wu and Li (1970) reported comparisons of esterase isoenzymes from embryo, coleoptile, root, node, internode and scutellum and six-week-old

callus derived from those parts of rice plants (Oryza sativa, cv. Taichung No. 65). They concluded that all the cultures were identical because the patterns of the esterases were the same in all the cultures.

With this exception the criteria used to characterize tissue cultures have been: colour, texture, rate of growth, enzyme activities, and hormonal and nutritional requirements. This problem has recently been expounded by Dougall (1972).

"At the moment it is unlikely that adequate criteria for general use in the identification of plant cell lines could be proposed. This in part because the criteria have not been identified and in part because data on individual cell lines are not available. Data on cell lines which may lead to general criteria are desperately needed. Possibilities for such data include: comparative studies of isoenzymes in cell lines, comparative studies using immunological techniques particularly with purified antigens from cells, and studies of chromosome number and morphology."

B) Isoenzymes

1) Introduction to Isoenzymes

The study of isoenzymes seems to offer a highly useful index by which to judge cell and tissue individuality. There are, however, some very necessary considerations. This review will deal briefly with the nature, formation and significance of isoenzymes, whilst considering the major problems. Then, in some detail, isoenzyme studies that have been conducted with plants and plant tissue and cell cultures are reviewed.

2) Definition and Nomenclature

During the last few years because of increased awareness and technological advances there has been an explosive increase in the number of papers dealing with isoenzymes or isozymes. There exists considerable variation in the usage and the implied meaning of the terms isoenzyme and isozyme.

Initially Markert and Møller (1959) coined the term isozyme to describe the multiple forms of the enzyme lactate dehydrogenase (LDH). However, the alternative term isoenzyme was preferred and is officially recommended by the standing Committee on Enzymes of the International Union of Biochemistry (Wilkinson 1970).

Isoenzymes are thus generally described as different proteins with similar enzymatic activity occurring in a single taxonomic species. It is in this context that the term isoenzyme will be used throughout this thesis.

Many authors feel that this definition is too vague. However, there are no easy solutions. Some of the difficulties may be appreciated by the consideration of the following facts.

Multiple molecular forms of enzymes may be formed in many different

ways. Methods of formation and the occurrence of isoenzymes have been extensively reviewed (Shaw 1965, 1969a, b; Vessel 1968, Ogita 1968; Markert 1968; Shannon 1968, Latner and Skillen 1968; Scandalios 1969, 1974; Wilkinson 1970; Sing and Brewer 1971, Masters and Holmes 1972). The six most common types of isoenzymes are listed below:

a) Single molecules - protein molecules with completely different primary structure performing the same enzymatic activity.

b) Sub-unit enzymes - isoenzymes formed by the combination of sub-units often of more than one type forming dimers, trimers, tetramers etc. In most cases the monomers do not possess enzymatic activity and different sub-units function best under different physiological conditions.

c) Polymers - not as in b, but groups of isoenzymes of several different, often related, enzymatic functions in different states of polymerization resulting in the formation of multienzyme complexes.

d) Isoenzymes formed by ligand binding - enzyme molecules with small, possibly charged bound molecules or various amounts of attached carbohydrates.

e) Conformational isoenzymes - enzymes that possess interconvertible, thermodynamically stable, tertiary structures.

f) Combinational isoenzymes - specific combination of enzyme molecules with non-enzymatic proteins, i.e. carrier or membrane proteins.

Because of the many rather non-specific ways in which isoenzymes could be formed and the fact that an isoenzyme could belong in several of the above categories at the same time, it has been suggested (i.e. Shaw 1969) that the term isozyme or isoenzyme be restricted to multiple enzyme forms of the sub-unit type. Even if this were adopted the problems of nomenclature

would not be solved. For example malate dehydrogenase activity is known to exist in different cell compartments (soluble, mitochondrial and glyoxomal), each of the types differing in amino acid composition and physiological properties which may or may not allow interaction to form stable and active hybrids (Rocha and Ting 1970, Wilkinson 1970; O'Sullivan and Wedding 1972a; Höck 1973). Should we now consider MDH as one or three isoenzyme systems?

In consideration of these problems it is best for the present to stay with the simplest difinition until such time as more specific criteria can be clearly adopted.

Most of the problems that arise, however, are not due to lack of criteria by which to classify isoenzymes but rather the methods used to detect them. If the detection method is not very specific, perhaps because of the use of an artificial substrate, the possibility exists that because of overlapping specificities isoenzymes may be characterized in roles unrelated to in vivo function. An extreme example is that of peroxidase in that all that is required for peroxidase activity is a protein with a suitably situated haeme group (Galston et al 1968).

3) Biological Significance

Despite the many formidable problems associated with classification and the detection of isoenzymes they certainly exist and the advantages they afford are manifold. From consideration of the vast literature on isoenzymes it is obvious that they are of biological importance. The basis of this biological significance is the differing physiochemical nature of the individual isoenzymes which allows them to perform the same enzymatic reactions under diverse cellular conditions. Isoenzymes can be viewed as an expression

of the differentiation of cells and the control of isoenzyme expression during development may be fundamental to the developmental process. The mechanisms by which isoenzymes are formed and modulated are extremely complicated and sensitive. It is out of this sensitivity that the differential gene action of an organism is expressed.

4) Isoenzyme Changes During Development

Many electrophoretic studies in plants have been directed towards the detection of changes in isoenzyme complement with plant development. It has been found almost universally that there are changes in number and activity of isoenzymes with the growth and development of the plant.

a) Seedlings

Isoenzyme patterns have been studied extensively during the germination and the development of seedlings. Such studies included: Per, LAP, AP, Est, and GOT isoenzymes in the Saquaro cactus, Carnegiea gigantea, (Engelm)(Keswani and Upadhya 1969), Per, LAP, Cat, AP, and Est in onion, Allium cepa L., cv. Yellow Granex hybrid (Mäkinen 1968), Per MDH, ADH, LDH, FDH, Amy and RNase in bean, Phaseolus vulgaris cv. Balin de Alberga (Trippi and Guzman 1970) and Cat, MDH, and GDH in barley, Hordeum, (Mitra et al 1970). Peroxidase isoenzymes were found to change during the development of seedlings of Vanda (Alvarez and King 1969), wheat, Triticum aestivum cv. Marquis, (Bhatia and Nilson 1969), T. aestivum cv. Lee; (Macko et al 1967), peanut, Arachis hypogaea, (Thomas and Neucere 1974), and jack pine, Pinus banksiana Lamb., (Ramaiah et al 1971). Isoenzymes of glutamate dehydrogenase were studied during the development of safflower, Carthamus tinctorius cv. US-10, (Errel et al 1973) and isoenzymes of acid phosphatase in developing pea seedlings,

Pisum sativum cv. Alaska, (Johnson et al 1973).

b) Roots

Investigations of Per, LAAox, MDH, AP, and Est isoenzymes during the development of broad bean roots (Vicia faba cv. Chlumecky) showed some changes occurred with time but more pronounced differences were found between the different parts of the root (Sahulka 1969, 1970; Sahulka and Benes 1971, Hadacova 1972). Changes in the pattern of peroxidase isoenzymes occurred during the rooting of cuttings of black poplar Populus nigra, (Nanda, 1973) and mung bean, Phaseolus mungo, hypocotyls (Gurumurti and Nanda 1974). Fottrell (1968) studied the isoenzymatic changes of esterases, proteases and dehydrogenases during the growth of root nodules of eight different legumes. He found changes with growth and that the patterns of the isoenzymes were distinctive for each plant.

c) Leaves

Several detailed investigations of the isoenzymatic changes during leaf development have been conducted. Chen et al (1970) studied the isoenzyme patterns of 6PGDH, Phs, Ald, AP, Est, Per, G6PDH, MDH and amylase at various stages of development of single leaves of cockleburr, Xanthium pennsylvanicum. They found that many of the isoenzyme changes coincided with the cessation of cell division and the completion of leaf growth. Isoenzymes of peroxidase have been studied during the development of leaves of bean, Phaseolus vulgaris, cv. Pencil Pod Wax, (Racusen and Foote 1966), barley, Hordeum, variety Atlas 46, (Gupta and Stebbins 1969), maize, Zea mays, (Hamill and Brewbaker 1969), tobacco, Nicotiana tabacum L. cv. NC95, (De Jong 1972) and cotton, Gossypium hirsutum cv. Auburn 57, (Wise and Morrison 1971).

d) Fruit

Hobson (1974) studied the changes in isoenzyme pattern of 19 enzyme systems in developing and ripening fruit of tomato, Lycopersicon esculentum. They found that enzyme synthesis accompanies the climateric respiration rise at the expense of non-metabolic protein. Additionally, amylase isoenzymes were studied in developing barley seed Hordeum vulgare L. cv. Himalaya (Bilderback 1971).

5) Isoenzyme Patterns in Mature Plants

Concomitant with the many studies on changes of isoenzymes with development have been comparisons of the patterns produced, as a consequence of development, in the different mature parts of plants. These studies i.e. Jaspars and Veldstra (1965a); Siegel and Galston (1967); Barber and Steward (1968); Hall et al (1969); Sheen (1969), and Mitra et al (1970), have shown that generally organ specific isoenzyme patterns are the rule and that certain isoenzymes are found only in one specific tissue. Investigation, however, of MDH isoenzymes in cotton (Gossypium hirsutum L. cv. Delta Pine 16) led O'Sullivan and Wedding (1972a,b) to conclude that under appropriate conditions the same number of MDH isoenzymes were detected in all the leaves studied. These conclusions were reached from the discovery that the amount of total MDH activity applied to the gel column is critical for proper detection of isoenzyme bands. This is supported by the findings of Longo and Scandalios (1969) that the number of bands of MDH are the same in all organs of the young maize plant.

6) Isoenzymes and Taxonomy

Just as it is generally true that isoenzyme patterns change with development, and are different in the various tissues and organs of any

individual plant, it is generally true that the complements of isoenzymes are specific to individual species of plants and that these differences can be used to differentiate one species or variety from another. In this respect isoenzyme and protein electrophoresis has been used to aid taxonomy (Boulter et al 1966). Comparison of patterns between equivalent parts of unrelated and related plant species and many individuals of the same species has helped to establish relationships between plants, in the assessment of the variation within a genus or species and in the identification of hybrids.

Plants that are not closely related generally have isoenzyme patterns that are very distinctive (Fottrell 1968; Mäkinen and Macdonald 1968). This depends to a degree, however, on the enzyme studied, but is especially true of the isoenzymes of peroxidase and esterase (Schwartz et al 1964). Electrophoretic protein and isoenzyme patterns tend to be similar but not identical in more closely related plants (Sing and Brewer 1971). These studies have included: protein bands in wheat, Triticum species, (Johnson et al 1967), the genus Suaeda (Ungar and Boucaud 1974), and Yucca, (Smith and Smith 1970), GDH and FDH in the Fabaceae (Thurman et al 1967), Est and LAP in species of Phaseolus (West and Garber 1967), GDH, MDH, and Cat in species of Hordeum (Mitra et al 1970), MDH, Est and LAP in 32 isolates of Protosiphon (Thomas and Brown 1970), MDH, Est, LAP and GDH in three species of Chlamydomonas (Thomas and Delcarpio 1971), LAP, β-galactosidase and IndOx isoenzymes in natural populations of Baptisia (Scogin 1969), Est, Cat, LAP, AP, ADH, IndOx, Per in cultivars of peanut, Arachis hypogaea L. subsp. fastigiata cv. vulgaris and A. hypogaea L. subsp. hypogaea cv. hypogaea (Cherry and Ory 1973a,b), peroxidase in bush and vine forms of

squash, Curcurbita maxima and C. pepo, (Loy 1972), Per and MDH in species of Datura (Conklin and Smith 1971; Ganapathy and Scandalios 1973), Per Est, PPO, GalDH, AP, AlP in nine cultures of Polyporus (Shannon et al 1972), LAP in the genus Lupinus (Scogin 1973), Est and LAP in natural populations of Betula populifolia (Payne and Fairbrothers 1973), Per in populations of Gossypium (Cherry and Katterman 1971) and ADH in 1,553 varieties of safflower, Carthamus (Efron et al 1973).

Extensive studies on the variation of peroxidase and esterase isoenzymes were conducted among species, hybrids and amphiploids of Nicotiana (Smith et al 1970, Reddy and Garber 1971) and among 250 varieties of maize (Hamill and Brewbaker 1969). These studies showed that each species had a unique pattern, no isoenzyme band was common to all species, hybrids were most closely related in isoenzyme pattern to parents and additionally some hybrid enzyme forms were detected.

Isoenzymes would thus appear to offer a very convenient tool for taxonomical studies. Caution must be exercised, however, as Wennstrom and Garber (1965) were unable to detect differences in isoenzyme patterns of esterase and acid phosphatase in extracts of twelve species of the genus Collinsia and the work of Fieldes and Tyson (1972, 1973a,b,c) on genotrophs of flax, Linum usitatissimum, has shown that environmentally induced heritable changes in isoenzyme mobilities can occur. It is, therefore, possible that environmentally induced shifts in relative mobility may be a source of enzyme variation.

Nevertheless, isoenzyme patterns are very distinctive and have been used as markers in the identification of zygotic and nucellar seedlings of

citrus (Iglesias et al 1974) and as additional proof that somatic cell hybridization between two strains of tobacco (N. glauca x N. langsdorffi) had taken place (Carlson et al 1972). Malate dehydrogenase has been proposed as a hybridization marker in haploid and diploid species of Datura (Ganapathy and Scandalios 1973).

7) Isoenzymes: Genetic and Physiological Studies

Many of the studies of isoenzymes in plants have been directed towards an understanding of the genetic control of isoenzyme formation. This area has been reviewed by Scandalios (1969, 1974). The most extensive investigations have been concerned with the control of isoenzyme systems in maize, Zea mays, (Scandalios 1968; Schwartz 1960), the studies included: esterases (Schwartz 1965, 1967; Macdonald and Brewbaker 1974), catalase, (Scandalios 1968, 1969, 1974) ADH and MDH, Scandalios(1967, 1969, 1974), endopeptidase, (Melville and Scandalios 1972, Scandalios 1974) and transaminase (Macdonald and Brewbaker 1974).

Other genetic studies have included the control of esterase isoenzymes in cucumber, Curcurbita ecudorensis (Wall and Whitaker 1971), Solanum sp. (Desborough and Peloquin 1967) and oats, Avena Fatua (Clegg and Allard 1973), A. sativa and A. byzantina (Smith 1972). Genetic studies have been conducted on peroxidases in rice, Orza sativa (Endo 1971, Endo et al 1971), and O. perennis (Shani et al 1969), and flax, Linum usitatissimum (Tyson 1970, Tyson and Bloomberg 1971, Fieldes and Tyson 1972), esterase and amino peptidase in Phaseolus (West and Garber 1967, Wall 1968) and alcohol dehydrogenase in wheat, Triticum dicoccum (Hart 1969), sunflower, Helianthus annus (Torres 1974a,b) and narrow leafed lupine, Lupinus angustifolius L. (Marshall et al 1974).

Other studies of isoenzymes of whole plants have usually been directed toward finding changes in isoenzyme pattern associated with a specific physiological phenomenon. Studies related to injury, disease and disease resistance have included: bean leaves, Phaseolus vulgaris, afflicted with Halo Blight Disease (causal agent Pseudomonas phaseolicola) (Rudolph and Stahmann 1966) and infected with Agrobacterium tumefaciens (Curtis 1971), peroxidase isoenzymes of sweet potatoe, Ipomoea batatas L. cv. Norin No. 1, in relation to Black Rot (causal agent Ceratocystis timbriata) (Matsuno and Uritani 1972), Nicotiana glutinosa infected with tobacco mosaic virus and potato virus X (Chant and Bates 1970) and wheat stems infected with Stem Rust Disease (Seevers et al 1971). Sako and Stahmann (1972) studied 14 isoenzyme systems in barley leaves infected with Erysiphe graminis f. sp. Hordei (powdery mildew). Their results show an increase in the number of isoenzyme bands of 11 enzymes and a decrease in the number of 4 other enzymes after infection. These types of differences are often encountered between healthy and diseased tissues. It is not clear, however, that this additional synthesis of isoenzymes is not just a general reaction to injury (Seevers et al 1971).

Many other physiological, isoenzyme studies of whole plants have been in relation to hormonal regulation (Ridge and Osborne 1970; Ockerse and Mumford 1973, Gaspar et al 1973). Most of these studies have involved peroxidase isoenzymes and so will be examined in detail in the section on peroxidase. The remaining physiological studies have been: the effects of photoperiod on isoenzyme patterns (Warner and Upadhya 1968; Penel and Greppin 1972; De Jong 1973), the effect of nutritional régime on isoenzyme

pattern and mobility (Van Lear and Smith 1970; Fieldes and Tyson 1972, 1973a,b,c) and the usage of isoenzymes of MDH and GDH as markers of cytoplasmic and mitochondrial contamination of wall extracts of pea epicotyls (Ferrari and Arnison 1974).

8) Studies of Isoenzymes From Cultured Plant Cells

a) Hormonal Studies

A large number of isoenzyme studies conducted to date with cultured plant tissues have been related to the hormonal control of isoenzyme pattern expression (Kaur-Sawhney and Galston 1972). Many of these studies have used tobacco cell cultures or pith explants and have included the effects of cytokinins, gibberellic acid and IAA on IAA oxidase isoenzymes (Lee 1971a, b,c, 1972), IAA,2,4-D and kinetin on polyphenol oxidase isoenzymes (Stafford and Galston 1970; Vernon and Straus 1972), IAA and ethylene on isoenzymes of peroxidase (Galston et al 1968; Ritzert and Turin 1970, Birecka et al 1973; Birecka and Miller 1974), and GA on enzymes of starch metabolism (Thorpe and Meier 1973).

The in vivo and in vitro effects of gibberellic acid have been studied in barley, Hordeum vulgare (Jacobsen et al 1970; Montani and Kato 1972). Large increases in the activity and the synthesis of new isoenzymes of α-amylase were detected. Additionally the effects of the presence or absence of 2,4-D in the culture medium of carrot, Daucus carota L., have been studied (Lee and Dougall 1973). The greatest differences between the treatments was noted for isoenzymes of GDH, slight differences were detected in the patterns of esterase while the isoenzyme patterns of MDH, AP, aspartate amino transferase and γ-glutamyl transferase were essentially identical.

Studies on the hormonal control of isoenzyme patterns in tissue cultures have shown the responses to be quite diverse. The changes in patterns that do occur probably reflect the general changes in metabolism of the treated cells. It is not uncommon that growth regulators affect the activity and expression of indole acetic acid oxidase activity and peroxidase isoenzymes. This is of special interest because of the possible involvement of these enzymes in in vivo hormone metabolism.

b) Developmental and Physiological Studies

Related to the hormonal studies in plant tissue cultures are attempts which have been made to correlate the changes in isoenzyme patterns with developmental and physiological events occurring in tissue and cell cultures. Gradients in the activity and changes in the isoenzyme patterns of peroxidase, PPO and catalase were detected in explants of cultured tobacco pith, N. tabacum cv. Wisconsin 38 (Stafford and Galston 1970; Lavee and Galston 1968). The activity of peroxidase was greatest in the cultured explants from the more mature pith while the activity of catalase was exactly opposite.

Differences in the activity and isoenzyme patterns of peroxidase and catalase were detected in groups of peanut suspension cells of different sizes (Verma and Van Huystee 1970a,b). Additional activity and isoenzymes of peroxidase were present in the larger groups, while the opposite situation, that the greatest activity and number of isoenzymes were present in the groups of the smallest dimensions was the case for catalase.

Studies on the tumorous condition and reaction to wounding of tissue culture cells has shown that these conditions and reactions can be characterized by specific isoenzymes of Per, α-amylase, Est and AP (Jaspars and Veldstra

1965a,b; Foster and Weber 1969; Rousseaux et al 1971; Birecka et al 1973; Birecka and Miller 1974). It is also known that massive doses of irradiation of peanut (Arachis hypogaea) cells affect protein synthesis (Verma and Van Huystee 1971). The effects of ionizing radiation on plant cells have been reviewed by Verma (1973).

Different isoenzyme patterns of peroxidase were found to be characteristic of tobacco callus and tobacco callus induced to form buds and stems (Rücker and Radola 1971) and root characteristic enzyme levels of RNase Per, AAP, GOT were found in tissue cultures of Atropa belladonna cv. lutea that were forming roots (Simola 1972). All the tissue clumps that formed roots showed clear enzyme similarities irrespective of the culture medium used to initiate the differentiation.

Other physiological studies have included the effect of photoperiod and temperature on the isoenzyme patterns of Per, Est, AP and 6-PGDH in Nicotiana tabacum and Dianthus caryophyllus callus cultures (De Jong et al 1968; McGown et al 1970, De Jong and Olson 1972), the effect of ion concentration on peroxidase and lignification in apple callus and sunflower crown gall (Lipetz and Garro 1965; Lavee and Hoffman 1971), and the effect of enzymatic digestion of the cell wall of sugar cane suspension cells (Maretzki and Nickell 1973). These experiments have shown that certain isoenzymes only appear under some of the light and temperature régimes, that the levels of ions affect the peroxidases released by tissue culture cells and hence may influence lignification, and that the removal of the cell wall caused only quantitative differences in isoenzymes of esterase and GOT.

c) Enzyme levels in Plant Cell Cultures

Recently several investigators have reported changes in the levels of various enzymes during the culture cycle in batch culture. Simola and Sopanen (1970) reported the activities of 12 enzymes at four stages of the growth of Acer pseudoplatanus L. suspension cultures. Enzymatic activities during the phase of rapid cell division were different from those of the stationary phase. The activity of aldolase and LAP were highest in dividing cells while the activity of glucose-6-phosphatase, acid phosphatase, ribonuclease and peroxidase were highest in ageing stationary phase cells. The activity profiles of six dehydrogenase enzymes during the 12 day growth cycle of tobacco, Nicotiana tabacum L. cv. WR-132, suspension cultures were reported by De Jong et al (1967). The activity of ADH, SDH, GDH, and G-6PDH generally decreased over the culture cycle while MDH showed a sharp peak of activity on day four.

A study of invertase activity of cultured Acer pseudoplatanus L., cells showed that the activity of invertase rose during the period of active cell division reaching a peak which then declined during the stationary phase (Copping and Street 1972) These results are in contrast to the findings of Thorpe and Meier (1973) in that the invertase levels showed no correlation with the growth of tobacco callus. The fluctuations of invertase activity were similar to those of phenylalanine ammonia lyase activity reported earlier for cultured citrus fruit (Thorpe et al 1971).

Harland et al (1973) reported changes in some enzymes involved in DNA biosynthesis (DNA polymerase, thymidine kinase and thymidine monophosphate kinase) coincident with and dependant upon DNA replication in partially synchronized Jerusalem artichoke tissue cultures. Similarly King et al (1973)

measured the activity of thymidine kinase in synchronized Acer pseudoplatanus L. cells and found a peak of activity twelve hours after cytokinesis in cells with a mean generation time of 21.6 hours. King et al also reported results of Fowler (1971) on the activity of G6PDH and phosphofructokinase in batch cultures of Acer cells. Glucose-6-phosphate dehydrogenase was highest on day 4 of the 24 day cycle while phosphofructokinase showed no real peak of activity and was highest at the start and the end of the culture period. Studies on the levels of various nitrogen metabolizing enzymes have recently been reported in chemostat cultures of the same Acer pseudoplatanus L. cells (Young 1973). Culture steady states were perturbed by the addition of glutamate as an additional nitrogen source. The resultant changes in enzyme and amino acid levels were recorded. The transition caused an enhancement initially of alanine and with further time an elevation of glutamate-oxaloacetate and glutamatepyruvate transaminases and γ-glutamyl transferase activity and a decrease urease and nitrate reductase activity.

Davis (1971) reported the changes in activity of threonine deaminase and phenylalanine ammonia lyase activity during the growth cycle of rose suspension cells. The activity of TDA peaked at day 3 and that of PAL on day 6 of the 12 day culture period. The peak of PAL activity coincided with the period of maximal phenolics content.

Other studies on the levels of enzymes during the growth of tissue culture cells include the effects of the regulators α-naphthalene acetic acid (NAA) and α-naphthoxyacetic acid (NAO) in Atropa belladonna cells (Simola and Sopanen 1971) and the effect of 6-MP on the levels of G6PDH activity in cultured artichoke tissue (Aitchison and Yeoman 1973).

Marked differences in the levels of enzymes were also reported between shoot forming and non-shoot forming Acer (Simola 1972, 1973), and Nicotiana (Thorpe and Laishley 1973) cell cultures.

The general difficulty of interpretation of changes in enzyme activity associated with the growth cycle of batch cultures and the growth of differentiated structures is stressed by King and Street (1973).

9) Peroxidase and Polyphenol Oxidase Isoenzymes

A considerable portion of the work presented in this thesis deals with peroxidase isoenzymes and activity and hence this enzyme will be considered in some detail.

Peroxidase and polyphenol oxidase have been the subject of many investigations. The two enzymes are discussed together because of overlapping substrate specificities. Peroxidase is an enzyme which specifically utilizes hydrogen peroxide as an electron donor to oxidize a wide spectrum of phenolic and indole like compounds. Although most of the literature has been restricted to peroxidase activity PPO activity is often associated. Most isoenzymes that will oxidize monophenolic compounds will usually show some ability to oxidize polyphenolic compounds although enzymes have been detected that apparently possess only PPO activity (Wong et al 1971).

Peroxidases are haemoproteins and thus contain iron which was shown by Galston et al (1968) to be sufficient to give a positive peroxidase reaction. Polyphenol oxidase isoenzymes are copper containing enzymes (Van Lear and Smith 1970, Kertesz et al 1971). Peroxidase enzymes are known to be glycoproteins consisting of up to 18% by weight carbohydrate attached at various sites predominantly by lysine ε-amino groups. (Shannon et al 1966;

Wellinder and Smillie 1972; Darbyshire 1973).

Peroxidase activity was probably first described by Raciborski (1898a,b). Raciborski discovered an 'oxygenase' in the phloem of sugar cane stem sections which he called leptomin. Subsequent experimentation with other organs led him to postualte that leptomin fulfilled a similar role, as does haemoglobin of animals and haemocyanin of arthropods, in the carriage of oxygen, in this case from the leaves to the other organs.

Most of the earlier work on peroxidase as would be expected is histochemical in nature. Studies have been made on whole plant sections shoots, roots, stems and meristems by a variety of workers (Van Fleet 1947, 1959, Jensen 1955, Vanden Born 1963, De Jong 1967, Alvarez and King 1969, Sheen and Rebagay 1970, Hepler et al 1972; Ramaiah et al 1972). Tissues and structures that displayed the strongest activity were: root cap, epidermal layers, protophloem, and protoxylem in root; trichomes, epidermis, endodermis and phloem in stems; and sub-apical and pheripheral tissues in shoot tips.

In cellular terms peroxidase activity can be divided into wall associated and non-wall associated activities. Non-wall associated activity has been found in every cytoplasmic compartment examined (Lee 1974), i.e. in association with lysosomes and microbodies (Plesnicar et al 1967) and ribosomes and membranes (Penon et al 1970; Darimont and Baxter 1973). Wall associated activity was found both ionically and covalently bound to the cell wall matrix. The ionically bound activity is preferentially released by Na^{+} and Ca^{++} ions while the covalently bound enzymes may be released by pectinase (Lipetz 1965; Evans 1968; Ridge and Osborne 1970a,b, Osborne

et al 1972; Gordon and Alldridge 1971; Whitmore 1971; Birecka et al 1973).

Peroxidase activity has been studied cytochemically in tobacco tissue cultures by De Jong et al (1968). The strain of cells used by De Jong et al (Nicotiana tabacum cv. NC95) grew in filaments without well defined growth phases. Peroxidase activity was localized intensely at one end of a filament and scattered unevenly in the cell walls.

Activity detected biochemically, specific to polyphenol oxidase, appears to be predominantly associated with chloroplasts and mitochondria (Harel et al 1965; Mayer 1966; Anderson 1968; Tolbert 1973).

Much of the more recent work on peroxidase and PPO has been in relation to the wide occurrence of these enzymes and hence their usefulness as markers and tools. A large part of this work has been compiled and reviewed by Shannon (1968) and Scandalios (1974).

Changes in peroxidase isoenzyme patterns have shown a close correlation with many developmental phenomena. Peroxidase and PPO isoenzymes show shifts in activity and pattern with development (Racusen and Foote 1966; Alvarez and King 1969; Keswani and Upadhya 1969; Anstine et al 1970; Chen et al 1970; Evans 1970; Sahulka 1970; Ramaiah et al 1971; Khan et al 1972) which eventually result in the formation of organ specific isoenzyme patterns (Siegel and Galston 1967; Makinen 1968; Upadhya and Yee 1968; Hall et al 1969; Hamill and Brewbaker 1969; Sheen 1969; Wise and Morrison 1971). Peroxidase isoenzymes have been found to show changes in connection with: mutation (Gupta and Stebbins 1969), the size of clump in peanut cultures (Verma and Van Huystee 1970a,b) and x irradiation in Nicotiana (Chourey et al 1973), differentiation in tissue culture (Rücker and Radola 1971; Lee and Dougall

1973) with nutritional environment (Van Lear and Smith 1970, Fieldes and Tyson 1972, 1973a;b,c) and with light and temperature treatments (De Jong et al 1968; Olson et al 1969; McGown et al 1969, 1970; Penel and Greppin 1972; De Jong 1973).

Peroxidase is thus seen to be involved in or related to many developmental phenomena. Much of this evidence is purely circumstantial in nature as the actual in vivo role of peroxidase remains unclear.

One of the more attractive possibilities as to the function of peroxidase and PPO is the implication that it is involved in the metabolism of auxin (Galston et al 1953). This possibility has gained support because peroxidase isoenzymes can, when supplied with Mg^{++} and the appropriate cofactors, oxidize indole acetic acid and the findings that the appearance of certain peroxidase and polyphenol oxidase isoenzymes are under hormonal control. These isoenzymes have appeared in relation to IAA and 2,4-D (Galston et al 1968; Lavee and Galston 1968; Galston and Davies 1969; Lesham et al 1970; Ritzert and Turin 1970; Stafford and Galston 1970; Lee 1971a; Lesham and Galston 1971; Whitmore 1971, Birecka et al 1972; Meudt and Stecher 1972; Gaspar et al 1973), gibberellic acid (Birecka and Galston 1970; Lee 1971b; Rycher and Lewak 1971; Ockerse and Mumford 1973), ethylene (Gahagan et al 1968; Osborne et al 1972; Ridge and Osborne 1970a,b; Shannon et al 1971; Birecka and Miller 1974) and cytokinins (Lee 1971c, 1974; Gaspar et al 1973). In some cases the new isoenzyme forms that appeared have been shown by labelled amino acid incorporation (Gahagan et al 1968; Galston et al 1968; Kaur-Sawhney and Galston 1972), and by density labelling techniques (Anstine et al 1970, Quail and Varner 1971) to be formed de novo. The application of a hormone does not always elicit the formation of new

isoenzyme forms, in fact the formation of some isoenzymes may be repressed (Kaur-Sawhney and Galston 1972) or the total peroxidase activity decreased dramatically (Lee 1974).

The active site of peroxidase activity rests with the iron containing haeme prosthetic group while the IAA oxidase activity of the peroxidase which requires Mg^{++} and phenolic co-factors was thought to be a property of the apoenzyme (Galston et al 1968). The IAA oxidase activity of the apoenzyme has been disputed by Ku et al (1970) who found the apoenzyme to be devoid of peroxidative IAA destructive and ethylene forming ability.

There has been considerable dispute as to whether the levels of peroxidase and IAA oxidase are the controlling factors in the in vivo regulation of IAA levels (Stonier 1971; Raa 1971). Stonier has proposed that IAA levels are regulated by the levels of small molecular weight o-dehydroxyphenols which inhibit the oxidation of IAA by their action as anti-oxidants (Stonier 1971). A considerable amount of experimentation has been conducted on these auxin protector substances (Yoneda and Stonier 1966, 1967; Stonier and Yoneda 1967a,b, Stonier et al 1968a,b, Stonier 1969; Stonier et al 1970a,b, Stonier 1971; Novák and Galston 1971). Stonier suggested that anything that affects the levels of auxin protectors will cause a shift in the cell metabolism towards division or alternately towards differentiation. It has been shown that the levels of auxin protector substances are very high in meristematic, tumorous and wounded tissues. These studies have implicated peroxidase as involved in the maintenance of cellular redox potentials (Akasawa and Conn 1958, Stonier et al 1970a) by the oxidation of NADH or possibly SH groups (Pilet and Dubois 1968a,b).

In addition to peroxidase isoenzymes being stimulated by, and involved in, the destruction of auxin it has been postulated that peroxidase

itself could be responsible for the formation of another plant hormone, namely ethylene (Yang 1968; Kang et al 1971; Phillips 1972, Fowler and Morgan 1972). The generation of ethylene via methionine metabolism may explain the increases of peroxidase and PPO isoenzymes that accompany fruit ripening (Frenkel 1972; Haard 1973; Hobson 1974). Peroxidases have similarly been associated with plant responses to injury and disease resistance (Chant and Bates 1970; Curtis 1971; Rousseau et al 1971; Seevers et al 1971, Matsuno and Uritani 1972; Birecka et al 1973, 1974).

A more direct and specific role of peroxidase and PPO has been an involvement in the formation of lignin-like compounds (Lipetz 1965; Lipetz and Garro 1965; Brown 1966) and further involvement in the development of the cell wall (Ridge and Osborne 1970a,b; Osborne et al 1972). Peroxidase in vitro will polymerize simple phenolic compounds and elevated peroxidase levels are often associated with genetically dwarf plants (Siegel and Galston 1967, Evans 1968, Gordon and Aldridge 1971; Schertz et al 1971). Another rather novel and quite unrelated suggestion was made by De Jong (1966) that peroxidase is involved in a system for the transport of ions in roots.

Despite all of these many different but often related investigations of peroxidase and PPO activity their exact in vivo roles still remain very much a mystery.

III MATERIALS AND METHODS

A. Cell Cultures

1) Stock Cultures

The stock suspension and callus cultures of root, hypocotyl and cotyledon used in the present work were initiated in this laboratory in 1968 by Dr. D.F. Liau. Callus cultures were maintained on coconut milk containing M3 medium (Liau and Boll 1970; Liau 1971 Ph.D. thesis) on a 21 day culture cycle while suspension cultures were maintained in M4 medium (Liau 1971) on a 12 day culture cycle. The contents of M3 and M4 media are shown in Appendix I.

2) New Cultures

The general methods used for establishing cultures are described elsewhere (Liau and Boll 1970; Liau 1971). Root, hypocotyl and cotyledon explants of sterile five day old seedlings were set out on M3 or solidified synthetic medium (Maute 1974, contents given in Appendix II). The parts chosen for culture are shown diagramatically in Figure 1. The development and seed germination of bush bean (Phaseolus vulgaris cv. Taylors Horticultural) is discussed in detail by Walbot et al (1972). Calluses that formed from the explants were allowed to develop for 4 weeks and then transferred to solid or liquid medium (for the formation of suspension cultures) or used for experimental purposes directly.

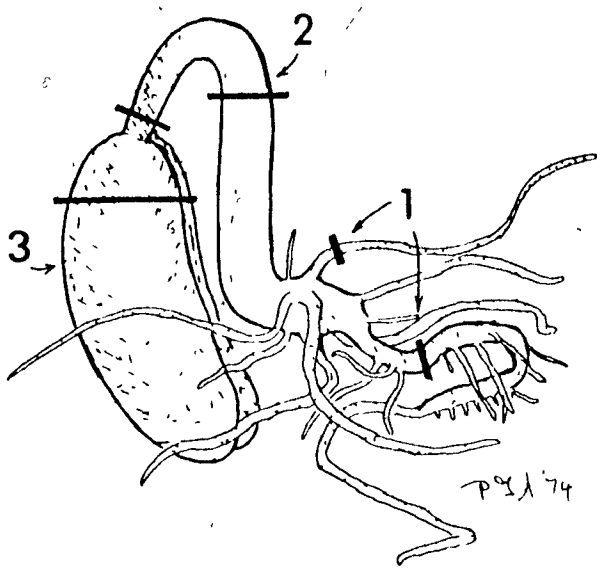


Fig. 1. Parts of the seedling chosen for culture: 1. - root, 2. - hypocotyl, and 3. - cotyledon.

Newly derived suspension cultures were analysed for isoenzyme patterns after one, two and three passages. Growth of the newly derived suspension cultures was considerably slower than the established stock cultures, therefore, the initial culture passages were of three or four weeks duration. Cells used at the end of this period were seived by the method described by Liau (1971) so as to exclude clumps of cells from the analysis.

B. Measurement of Growth

1) Fresh Weight

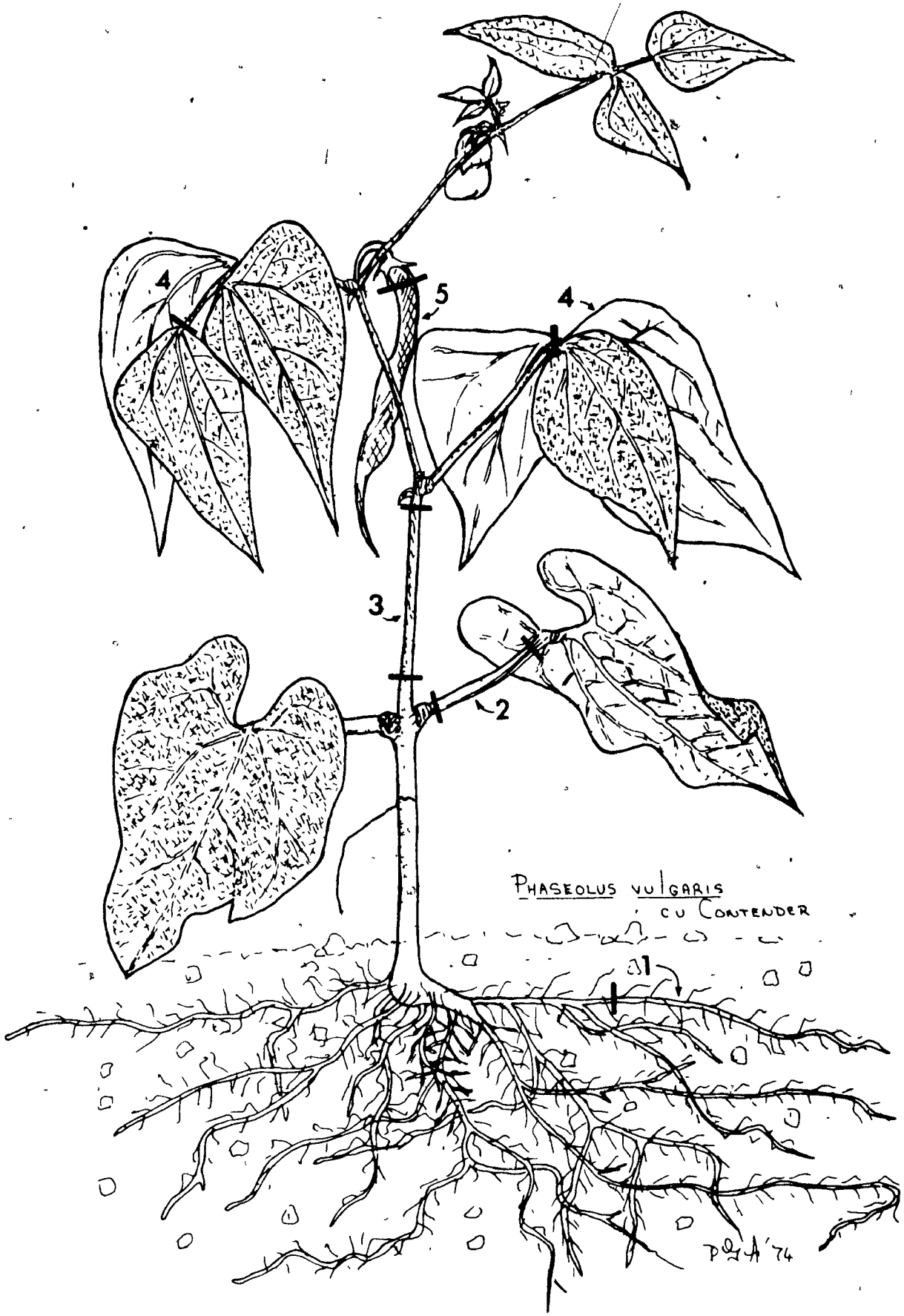
The fresh weight of cells was recorded simply by collection on filter paper in a Buchner funnel by suction. The cells were washed five times with dilute buffer or distilled water.

The weights of callus tissues used for analysis were measured directly

2) Cell Number

The number of cells per flask and per ml of culture were calculated from the chromic acid digestion of 1.0gm of suction dried cells, essentially

Fig. 2. Parts of the mature bean plant selected for isoenzyme pattern analysis. 1 - root, 2 - petiole, 3 - stem (second internode), 4 - leaf, 5 - immature pod.



PHASEOLUS VULGARIS
CV CONTENDER

PGA '74

as described by Liau (1971). After digestion for 12 hours the samples were diluted to 100 mls and agitated by expulsion through a syringe to separate the cells. The cells were then counted microscopically in a Sedgewick Rafter Plankton counting chamber with a capacity of one ml. The number of cells per culture was calculated from the average of fifty counts.

C. Electrophoretic Procedure

1) Preparation of Extracts

Sections of seedlings (Fig. 1) or the parts of whole, six week old plants grown under green house conditions (Fig. 2) were harvested and weighed. Samples of 5.0 gm of plant material were homogenized in 2.5 ml of chilled tris-HCl extraction buffer with a Virtis '45' high speed homogenizer and then subsequently in a motorized glass mortar and pestel. The extraction buffer, 0.59M tris-HCl pH 6.9, contained 5×10^{-3}M cysteine hydrochloride and 5×10^{-4}M EDTA. Homogenates were equilibrated with Polyclar AT* powder for 30 minutes at 4C. Samples (5.0 gm) of callus cells were homogenized directly in 2.5 ml extraction buffer with a glass mortar and pestel. Suspension cells from any one treatment were pooled, filtered from the medium in a Buchner funnel by suction and then washed with ten times dilute extraction buffer or distilled water to remove residual medium and medium macromolecules. Extracts were then prepared as with callus cultures.

All homogenates were centrifuged at 20,000g for 20 minutes in a Sorvall RC2-B refrigerated centrifuge. The resultant supernantant fluid was used for electrophoresis. Samples of various media retained after the filtration of cells were subjected to electrophoresis without further treatment.

Dialysis of the extracts was not included as part of the routine

* obtained from Geigy Corporation.

procedure because little clarification of isoenzyme patterns resulted from this process and there was considerable loss of some enzymatic activities.

Preservation of extracts for duplicate experiments was accomplished by freezing at -20C. Frozen extracts were analysed the following day in order to minimize any inactivation caused by freezing. On a few occasions callus and suspension cells were also preserved for short periods by freezing.

2) Disc Electrophoresis

Polyacrylamide electrophoresis was performed essentially as described by Davis (1964). Proteins were separated on 7.5% small pore gels at pH 8.9, at 4C in the dark. A large pore stacking gel was also used. Recipes for the acrylamide solutions and buffers are listed in Appendix III.

Aliquots of 0.2 ml of cell or tissue extract or 0.5 ml of medium were applied to each large pore gel and subjected to electrophoresis with a constant current of 2.0 milliamps per tube until the bromophenol tracking dye move into the gel. The current was then increased to 3.25 milliamps per tube. The protein extracts were diluted with forty percent sucrose solution to give a final concentration of either 100, 50, or 25 µg protein per ml. dependant upon the initial concentration and the enzymes studied. The electrophoresis was terminated when the tracking dye had moved to near the end of the tube (about 2-2 1/2 hours).

Gel tubes were removed from the electrophoresis tank and placed in a sink on a bed of crushed distilled water ice. Gels were cooled for 5 minutes and then removed from the tubes by a fine jet of water supplied by a hypodermic needle attached, by a hose, to a deionized water tap. The gels were then placed in the appropriate reaction mixtures.

3) Enzyme Visualization

a) Malate Dehydrogenase, (L-malate: NAD oxidoreductase, E.C. 1.1.1.37).

Malate dehydrogenase (MDH) activity was localized as dark blue staining bands against a light yellow background in reaction mixtures containing:

NAD^+	15 mg
NBT	25 mg
PMS	5 mg
0.2M tris-HCl, pH7.1	10 ml
0.05M Na-L-malate	25 ml
H_2O	15 ml
Incubation time;	20 minutes (in dark)
Protein concentration	25 or 50 µg/ml
Fix in 7% acetic acid.	

b) Glucose-6-Phosphate Dehydrogenase, (L-glucose-6-phosphate: $NADP^+$ oxidoreductase, E.C. 1.1.1.49). Glucose-6-phosphate dehydrogenase (G6PDH) activity was localized by method of Shaw and Prasad (1970).

Reaction mixtures contained:

$NADP^+$	30 mg
NBT	20 mg
PMS	2 mg
0.5M tris-HCl, pH7.1	25 ml
Na_2 glucose-6-$PO_4 \cdot H_2O$	200 mg
H_2O	90 ml
Incubation time;	60 minutes (in light) (Chen et al 1970)
Protein concentration;	100 µg/ml
Fix in 7% acetic acid.	

c) Glutamate Dehydrogenase, (L-glutamate: NAD(P) oxidoreductase (deaminating), E.C. 1.4.1.3). Glutamate dehydrogenase (GDH) activity was localized as dark blue staining bands in reaction mixtures containing:

NAD^+	15 mg
(or $NADP^+$	15 mg)
PMS	5 mg
NBT	25 mg
0.2M Phosphate buffer pH8.0	25 ml
0.1M Na-glutamate	25 ml
Incubation time;	30 minutes (in dark)
Protein concentration;	50 µg/ml
Fix in 7% acetic acid.	

d) Peroxidase (polyphenolic)*

Peroxidase (polyphenolic) (PP) isoenzymes were localized by reaction mixtures containing hydrogen peroxide and polyphenols:

i) DOPA-H_2O_2, 8×10^{-3} M 3,4-dihydroxyphenylalanine plus 0.1 ml of 3% H_2O_2 per 10 ml. Gels were incubated for 15 and 30 minutes until dark brown bands appeared. Gels were fixed in 7% acetic acid.

ii) Catechol: H_2O_2, 5% 1,2 benzenediol in Sodium phosphocitrate buffer pH 6.5 + 0.1 ml 3% H_2O_2 per 10 ml reaction mixture. Incubation time; 15 or 30 minutes.

iii) Chlorogenic and caffeic acids: H_2O_2, Phosphate buffered, pH7.0, saturated solution of chlorogenic or caffeic acids + 0.1 ml of 3% H_2O_2 per 10 ml reaction mixture. Incubation time; 15 or 30 minutes.

*Peroxidase activity toward polyphenols is distinguished here as peroxidase (polyphenolic) without implying any specificity toward such polyphenols.

e) Catalase, (H_2O_2: H_2O_2 oxidoreductase, E.C. 1.11.1.6).

Catalase activity was located by the method of Mitra et al (1970). Soluble starch (0.25%) was incorporated into the small pore gel. After electrophoresis the gels were incubated for one minute in a solution containing 0.5% H_2O_2 and then washed with distilled water. Rinsed gels were dipped into a 1% potassium iodide solution acidified with acetic acid. Areas of catalase activity remain as light or white staining bands against a black background.

f) Peroxidase, (Donor: H_2O_2 oxidoreductase, E.C. 1.11.1.7).

Peroxidase (Per) isoenzymes were visualized by the following methods:

i) Guaiacol - H_2O_2. Gels were incubated in 5 x 10^{-3}M guaiacol (o-methoxyphenol) buffered with 0.2M Sodium acetate-HCl at pH5.0 with 0.1 ml of 3% H_2O_2 per 10 ml. Gels were incubated for ten or fifteen minutes and then fixed in 7% acetic acid which sometimes resulted in the appearance or intensification of some bands.

ii) Benzidine - H_2O_2. Gels were incubated in a reaction mixture made up as follows: One hundred ml of 7.0% acetic acid plus 16 gm of sodium acetate was saturated with EDTA. The solution was filtered and saturated with benzidine dehydrochloride (p-diamino-diphenyl·2HCl) Gels were incubated in the benzidine solution plus 0.1 ml H_2O_2 per 5 ml for 5 or 10 minutes. Peroxidase activity is localized as dark blue or yellow staining bands. Gels were fixed in 7% acetic acid.

iii) 3-amino-9-ethyl carbazole; H_2O_2. Gels were stained in a reaction mixture containing:

3 amino-9-ethyl carbazole (dissolved in Dimethyl formamide 1 mg/ml)	5 ml
0.05M Sodium acetate pH5.0	92.5 ml
0.1M $CaCl_2$	2 ml
3% H_2O_2	0.5 ml
Incubation time;	30 minutes

Fix in 50% glycerol (Shaw and Prasad 1970).

g) Indoleacetic Acid Oxidase: Indoleacetic acid oxidase activity was localized by the method of Endo (1968). Reaction mixtures contained:

1.0mM potassium -3 indoleacetate
0.5ml sodium 2,4,6 trichlorophenol
Fast Blue BB salt ..... 2 mg/ml
in 0.2M Sodium acetate buffer pH5.0

h) Glutamate - Oxaloacetate Transaminase, (L-aspartate: 2-oxoglutarate aminotransferase, E.C. 2.6.1.1). Glutamate - oxaloacetate transaminase activity was detected by the method of Schwartz et al (1963), cited by Shaw and Prasad (1970). The reaction mixtures contained:

L-aspartic acid	532 mg
α-ketoglutaric acid	73 mg
Pyridoxal phosphate	50 mg
Fast violet B salt	200 mg
0.1M phosphate buffer, pH7.0	100 ml
Incubation time,	30 minutes

Fix in 50% glycerol.

i) Esterase, (Carboxylic ester hydrolase, E.C. 3.1.1.1). Esterase (Est) isoenzymes were localized in reaction mixtures containing:

Fast Blue RR salt	25 mg
0.2M tris-HCl buffer pH7.0	10 ml
α - naphthyl acetate (1% in acetone)	0.1 ml
H_2O	40 ml

Incubation time; ..... 30 minutes
(changed after 15 minutes if solution became cloudy.)
Fix in 7% acetic acid.

Alternate substrates:

α-naphthyl proprionate, α-naphthyl butyrate
α-naphthyl laurate (lipase), Napthol AS acetate
and β-naphthyl acetate
Fast Garnet CBG salt was also used as a coupling dye (Lee and Dougall 1973).

Eserine and 5-Bromonaphthylacetate were used as inhibitors (Veerabhadrappa and Montgomery 1971a,b).

j) Acid Phosphatase, (orthophosphoric monoester phospho-hydrolase, E.C. 3.1.3.2). Acid phosphatase (AP) activity was localized as red-purple bands in reaction mixtures containing:

Na-α-naphthyl acid phosphate	100 mg
0.05M Na-acetate-HCl pH5.0	100 ml
Fast Black K salt	100 mg
or Fast Garnet CBG salt	100 mg

Incubation time; ..... 30 or 45 minutes
Fix in 7% acetic acid.
Method of Shaw and Prasad (1970).

k) Leucine Aminopeptidase, (L-leucyl-peptide hydrolase, E.C. 3.4.1.1)

Leucine aminopeptidase (LAP) isoenzymes were localized as blue bands against an orange background in reaction mixtures containing:

0.2M tris-maleate buffer pH6.0	50 ml
H_2O	50 ml
$L^=$ leucyl β-naphthylamide-HCl.....	20 mg
Fast Black K salt	50 mg
or Fast Garnet CBG salt	50 mg

Incubation time; ..... 30 or 45 minutes
Fix in 7% acetic acid.
Method of Shaw and Prasad (1970).

D. Protein Estimation

Protein estimation prior to electrophoresis was conducted by the convenient method of Waddel (1956). A sample of the protein extract, diluted 100 times, was scanned for absorbance in the uv range with a Unicam SP 800 scanning spectrophotometer. The difference in absorption between 215 and 225 nm when compared to a standard curve made using bovine serum albumin (BSA) gave a rapid estimate of the protein concentration. The values obtained were in good agreement with those obtained by the Lowry method. Protein estimates used in the calculation of specific activity of enzymes were conducted fluorometrically. Fluorometric assays using the fluoroscene reagent are very accurate and gave values similar to those of the more conventional Lowry method. No interference from secondary plant products was detected.

E. Recording of Electrophoretic Data

All gels were diagrammed immediately after electrophoresis. On some occasions gels were photographed using a Leica M2 camera equipped with a reflex housing and 50 mm lens. Gels were photographed in a petrie dish containing 7% acetic acid. Lighting was supplied by two 600 K flood lamps. Subsequently gel scans were made using a Zeiss LKB Spectrophotometer with a linear transporter. Dehydrogenase gels were scanned at 610 nm, Est, LAP and AP at 540 nm DOPA (PPO) at 325 nm and guaiacol (Per) at 295 nm. The mobilities of the individual isoenzymes were calculated directly from the diagrams and photographs.

The activities of the individual isoenzymes were calculated from the measurement of relative peak areas on the gel scans (Hart et al 1971). Activity is expressed as percent total enzyme activity and as Δ O.D. per

mg soluble protein, per gm fresh weight or per million cells. The accuracy of peak area calculations was checked using an ORTEC scanning and integrating spectrophotometer (EC&G Co., Oak Ridge, Tenn., U.S.A.).

Diagrams of the changes of intensity and changes in patterns of isoenzymes were calculated from the average of three and up to six separate experiments. Where data is presented for one specific culture cycle it was typical of repeated experimentation. The intensity of the isoenzymes activity is represented by an arbitrary eight-point scale of intensity. When composite diagrams were prepared the band intensity displayed was the average of all equivalent bands considered.

The nomenclature of isoenzyme bands was determined by the following general principles. Under anyone set of experimental conditions isoenzyme bands were designated with capital letters in order of decreasing migration towards the anode. In cases where isoenzyme bands appeared to be related or where large bands of activity were seen to divide into separable forms additional numerals were affixed for purposes of identification (e.g. A1, A2, A3). In the final section of the thesis isoenzyme patterns generated from numerous sets of experiments were compared. During the progress of the work certain equivalent isoenzymes were unavoidably designated with more than one symbol, therefore for clarity the isoenzyme bands where necessary were renamed. The same general principles were employed, however numerical additions were avoided as much as possible. Isoenzyme bands that migrated to the same point but showed differences in banding pattern or other properties were given small case letter subscripts for identification (e.g. K_r where r = root, and K_h where h = hypocotyl).

F. Ultraviolet and Colorimetric Enzyme Assays

Spectrophotometric assays of peroxidase, PP , MDH, GDH, LAP and AP were conducted. All readings were made with a Unicam SP-800 spectrophotometer temperature regulated to 25C.

1) Peroxidase

Peroxidase activity was calculated from the increase in absorbance at 470 nm of a 5×10^{-3} M guaiacol solution. Five drops (0.1 ml) of freshly prepared 3.0% H_2O_2 was added to 2.85 ml of the guaiacol solution buffered to pH 5.0 (Na-acetate/acetic acid) and quickly inverted several times. To this 0.05 ml of enzyme extract was added and quickly mixed. The change in absorption was recorded for 30 seconds. Each sample was tested five or six times and in the case when activity was very high dilutions of the extract were made and retested. Activity is expressed as Δ 0.D. units/minute per mg extract protein, per gm fresh weight, per million cells and per ml of medium.

2) Peroxidase (polyphenolic)

Peroxidase (polyphenolic) activity was measured from the increase in absorbance at 325 nm of a 5.0% catechol solution buffered to pH 5.0 with Na-acetate/acetic acid (0.2M). The procedure was the same as above.

3) Malate and Glutamate Dehydrogenase

Assays for dehydrogenases were carried out at 340 nm and monitored the rate of formation of NADH at this wavelength. The reaction mixture for malate dehydrogenase contained 0.9 ml of 100 mM tris-HCl buffer, pH 9.2, 1.0 ml of 50 mM sodium malate and 1.0 ml of NAD (1 mg/ml). The reaction was initiated by the addition of 0.1 ml enzyme extract. The reaction mixture

for GDH contained: 1.0 ml of 0.1 M phosphate buffer pH. 8.0, 0.2 ml of 0.1 M sodium glutamate, 0.5 ml NAD solution (2mg/ml), and 1.1 ml deionized water (Yamasaki and Suzuki 1969). The reaction is initiated by the addition of 0.2 ml enzyme extract. A unit of activity is defined as the increase in absorbance at 340 nm of 0.01 O.D. per minute.

4) Acid Phosphatase

Assays of acid phosphatase were conducted essentially as described in Sigma* Technical Bulletin No. 104. Reaction mixtures contained 0.5 ml sodium acetate buffer (0.05 M) pH 5.0, 0.5 ml substrate (Sigma 104 phosphatase substrate) 4 mg/ml, and 0.1 ml enzyme extract. After 15 minutes the reaction is stopped by the addition of 10.0 ml of 0.05 N NaOH. Each sample was tested five times. The intensity of the yellow color is read at 410 nm. Units of acid phosphatase activity are calculated from a calibration curve made with a p-nitrophenol standard solution (obtained from Sigma*). A typical curve is shown in Appendix 4.

5) Leucine Amino Peptidase

Assays of LAP were conducted as described in Sigma* Technical Bulletin No. 251. The amount of LAP activity is calculated from the colorimetric determination, at 530 nm, of β-Naphthylamide liberated from the substrate L-Leucyl β-Naphthylamide. Each sample was tested five times. A standard curve was prepared using Sigma* LAP calibration standard. A typical standard curve is shown in Appendix 5.

*Sigma Chemical Company, St. Louis, Missouri, U.S.A.

G. Enzyme Cytochemistry

Cells used for cytochemical enzyme location were filtered from the medium and weighed. Samples of 0.5 or 1.0 gm were put in small petrie dishes containing buffers and the appropriate reagents for staining. Some samples were washed to remove residual media-macromolecules. Cells not supplied with enzyme substrates were included as controls.

Peroxidase activity was localized using guaiacol and benzidine-H_2O_2 reaction mixtures similar to the methods of De Jong et al (1967) and Reiss (1973).

The PP activity was localized using DOPA-H_2O_2 reaction mixtures.

Glutamate dehydrogenase, MDH and G6PDH activities were localized using a buffered tetrazolium dye system containing: NBT, PMS and NAD as described by De Jong et al (1967).

Esterase activity was localized with an α-naphtholacetate/fast blue RR salt reaction mixture (Gomori 1952).

Acid phosphatase and LAP were localized at sites where enzymatically liberated α-naphthol and β-naphthyamide combined with the azo dye fast black K. The substrate used to localize AP was α-naphthol acid phosphate and that for LAP was L-leucyl-β-naphthylamide.

All cytochemicals were purchased from Sigma Chemical Co., St. Louis, Mo. except fast black K salt which was obtained from K & K Laboratories Inc., Plainview, N.Y.

IV RESULTS

A. Stock Callus Cultures: Changes in Isoenzymes During the Culture Cycle and Differences Between Root, Hypocotyl, and Cotyledon Cultures

1) Introduction

Isoenzyme patterns were exhaustively analysed over a six cycle period (passages 50-56) in order to fully appreciate, first, isoenzymatic changes during the culture cycle; second, persistent differences that occurred between the cultures; and third, the possibility of enzyme variability being of critical importance in interpretation.

Figures 3-6 show isoenzyme patterns from one culture cycle which was representative of recurring patterns of changes during all the culture cycles studied. It was found that a particular change might not be evident at exactly the same time in any one culture cycle, but nevertheless it always occurred within a period of a day or two.

Peroxidase, PP , Est, MDH, GDH and LAP enzymes were studied, in all three cultures, in three to six culture cycles. Catalase was studied once during cycle 51. Electrophoretic accuracy was checked both by running a duplicate sample of fresh extract and subsequently a preserved, frozen enzyme extract.

2) Peroxidase

The peroxidase patterns normally found with extracts from root, hypocotyl, and cotyledon tissues are summarized diagrammatically in Fig. 3. In general the isoperoxidase patterns of the three cultures were similar, the patterns of the isoenzymes were found to change during the culture cycle, and consistent differences were detected among the three cultures.

The peroxidase isoenzymes recorded here can be grouped into several categories according to their general properties during the culture cycle. Firstly, there are isoenzymes which are detectable at all stages and which show the maximum detectable activity as judged by band staining intensity. Examples are D1 and F in Fig. 3, which are present in all three cultures. Other enzymes, e.g. A1, B, C, D3, E (Root.), G, and H are variable and change in activity, sometimes disappearing completely during part of the cycle (e.g. A1, D3, E, G) or change in banding pattern (e.g. C1-C2).

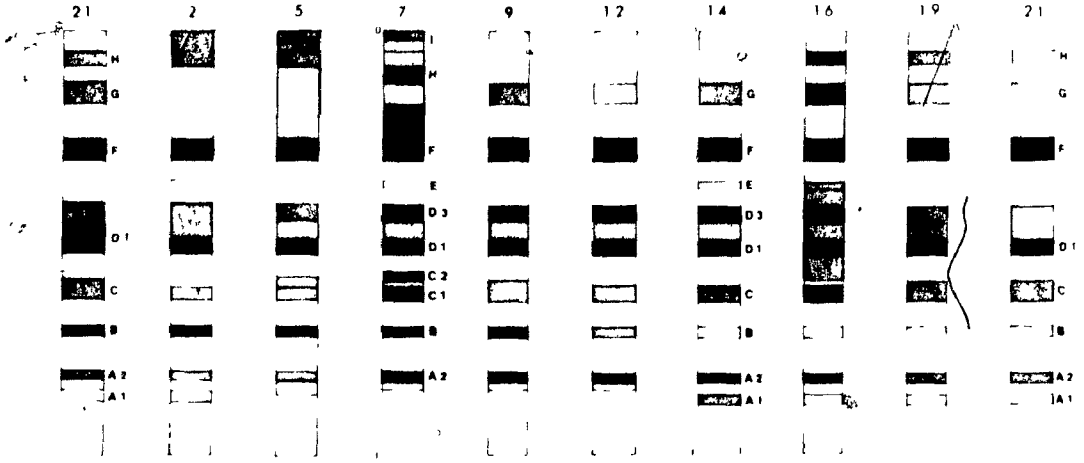
Certain isoenzymes were only present in one of the three cultures, Thus H, E, D3, and C2 occurred only in root, while A3 was detected only in hypocotyl. The maximum number of isoenzymatic differences, between the cultures, was found at day seven (see Fig. 7) during the phase of most active growth of the callus (Liau & Boll, 1970). In rare instances changes occurred which could not properly be grouped in any of the aforementioned categories. For example with isoenzyme B, in all three cultures in cycle 54, there was a decrease in activity to below that in the inoculum and, by day 21, the level of activity was not restored. This loss of activity was of a temporary nature because in subsequent passages the original level of activity was restored.

The isoperoxidases of these cultures display a certain degree of substrate specificity in that benzidine and guaiacol always gave a more intense reaction than did orthodianisidine and 3-amino-9 ethyl carbazole. Isoenzymes B, G, and H reacted more strongly with guaiacol as substrate, while isoenzymes A1, A2, A3, and D3 reacted more strongly with benzidine as substrate.

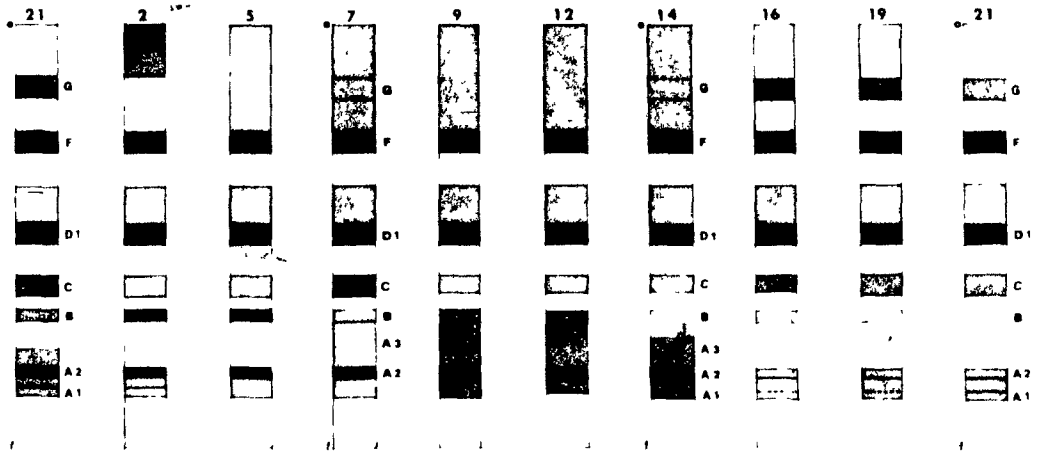
Fig. 3. Benzidine peroxidase isoenzymes. Diagrammatic comparison of isoenzymes from root, hypocotyl and cotyledon callus during cycle 54. The numbers at the top of the gels denote the days of the cycle (duration 21 days). The initial day 21 is from the preceding cycle (inoculum). Enzyme activity is represented by degree of band intensity.

Peroxidase Cycle 54

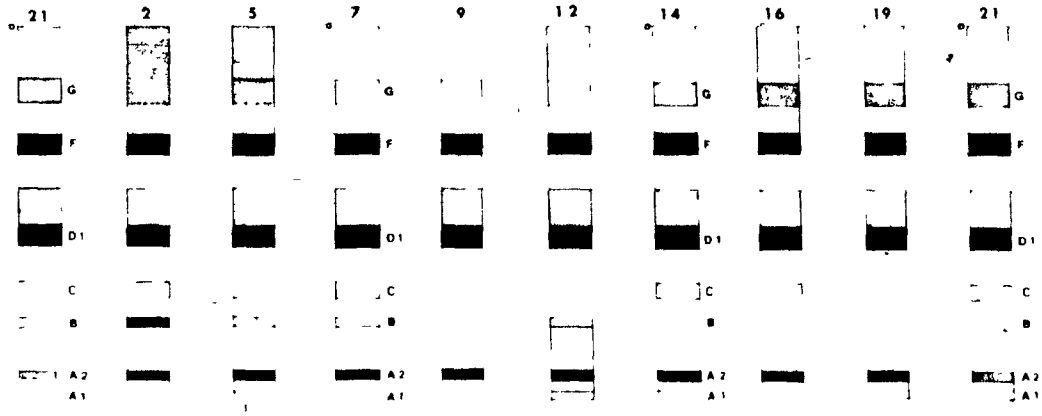
Root Callus



Hypocotyl Callus



Cotyledon Callus



Up to six of the isoenzymes, particularly A, C, and D3 or E showed considerable indophenol oxidase activity. Indophenol oxidase activity was localized as white or light staining bands present against the blue background of the dye, NBT, used in dehydrogenase staining systems. Control dehydrogenase gels, left in the light, slowly turn blue except for the regions of indophenol oxidase. Thus, on the basis of coincidence of Rf, indophenol oxidase activity appears to be a property of these peroxidases. The property is discussed by Brewer (1970).

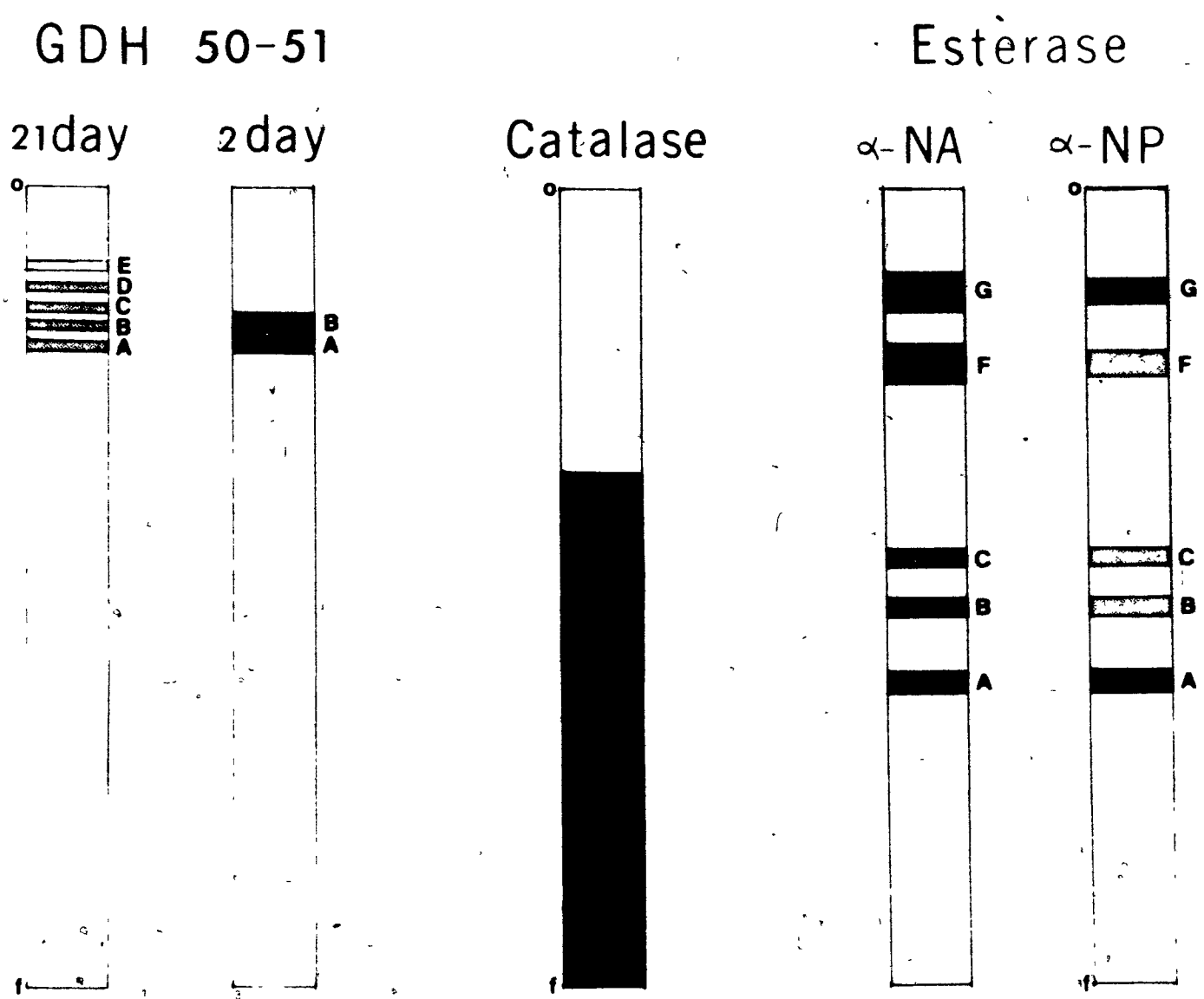
3) Peroxidase (polyphenolic)

Peroxidase (polyphenolic) activity was associated with at least eight of the most frequently occurring peroxidase isoenzymes. (Fig. 7). Only isoenzymes B, G, H, and I exhibited strong activity. The PP isoenzymes were very similar for all three cultures and, in contrast with the peroxidase activity, the activity of each PP isoenzyme varied little during the culture cycle. For this reason they are not illustrated. The main differences between the three cultures in regard to PP activity were the consistently high activity of isoenzyme B in the root and the higher levels of A2 activity in the hypocotyl (see Fig. 7).

4) Catalase

All cultures examined showed catalase activity as a rather large band at the top third of the gel (Fig. 4). No differences between the three cultures, or distinct changes during the growth cycle were evident. Because of this lack of promise as an enzyme marker, further analysis of catalase activity was not pursued.

Fig. 4. Diagrammatic representation of glutamate dehydrogenase, catalase and esterase isoenzymes. The GDH diagram shows the usual change in isoenzyme pattern upon re-inoculation. The catalase diagram is typical of all tissues studied. Enzymatic activity is shown by the white area. Esterase diagrams display a typical pattern from root cycle 51 (2 days) and show substrate specificity. NA = alphanapthylacetate. NP = alphanapthylproprionate.


GDH 50-51
21day
2day
Catalase
Esterase
α-NA
α-NP
E
D
C
B
A
B
A
G
F
C
B
A
G
F
C
B
A

5) Esterase

Of all of the enzymes studied, esterase isoenzymes were the most variable from growth cycle to growth cycle. Although changes in the number of isoenzymes and differences between cultures were observed, these were not reproducible with callus cultures. The study was also hampered by extremely low levels of esterase activity during a large part of the cycle. However, the esterases showed a considerable degree of substrate specificity and in this respect were similar to those described for Phaseolus by Veerabhadrappa and Montgomery (1971a). The most intense reaction was obtained with α-naptholacetate and α-napthol proprionate as compared with either β-napthol acetate or napthol AS acetate as substrates. In addition to differences in number of bands, the esterases also showed differing band intensities when the preferred α-napthol acetate and proprionate were used as substrates: (see Fig. 4).

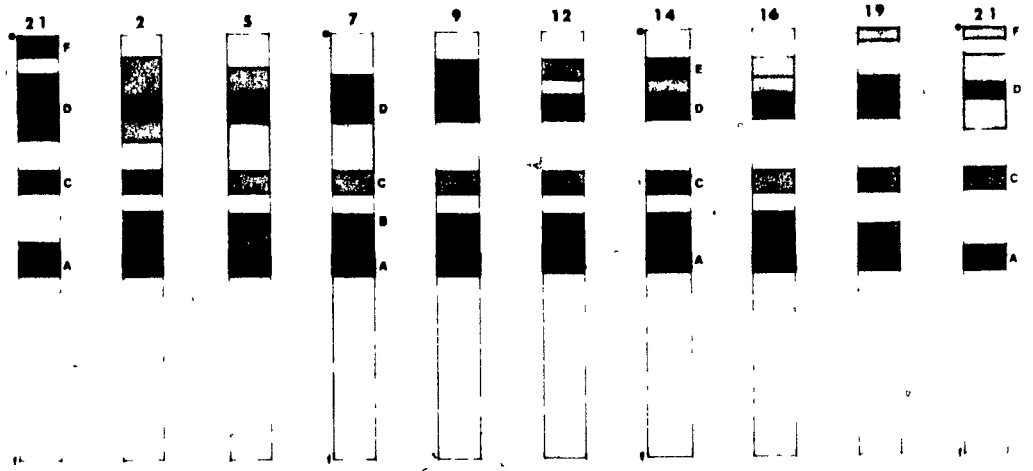
6) Malate Dehydrogenase

Observations on malate dehydrogenase activity are summarized diagrammatically in Fig. 5. The main difference among the three cultures was the predominance of the isoenzyme designated A in the root callus and the presence of isoenzyme E in the hypocotyl and cotyledon callus. Isoenzyme A was strong and present at all times in the root callus but only appeared briefly about day 12 in hypocotyl and cotyledon callus. Isoenzyme B was present in cotyledon and hypocotyl cells throughout the culture cycle and was at a peak at day 12, whereas in root cells isoenzyme B disappeared toward the end of the culture cycle and was at a peak about day 5.

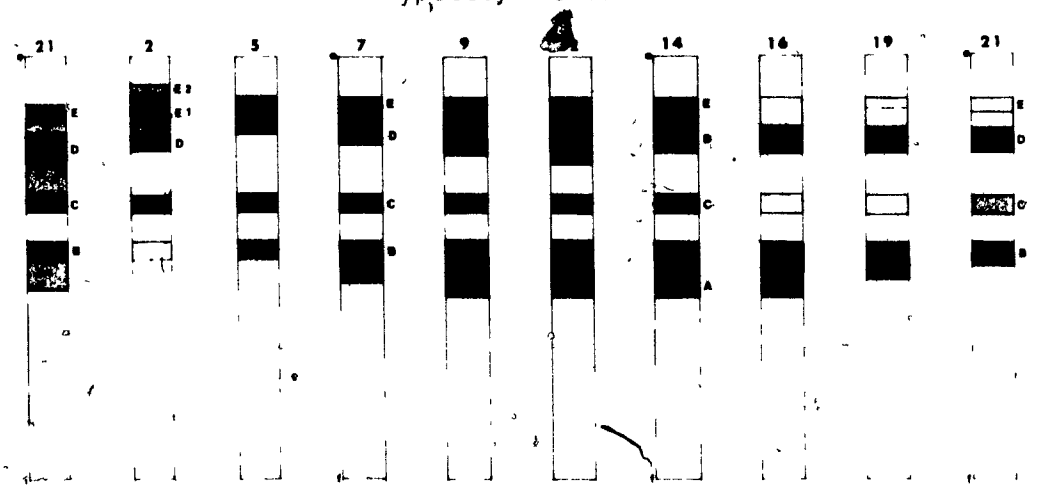
Fig. 5. Malate dehydrogenase isoenzymes. Diagrammatic comparison of enzyme activities during culture cycle 53. The darkness of the band represents relative activity.

Malate Dehydrogenase Cycle 53

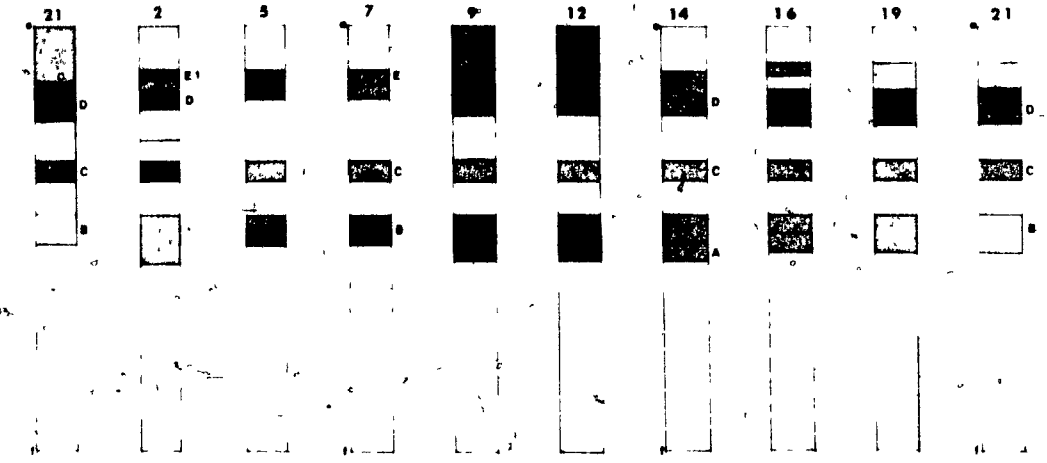
Root Callus



Hypocotyl Callus



Cotyledon Callus



7) Glutamate Dehydrogenase

A representative pattern for the glutamate dehydrogenase isoenzymes, which was the same for the three cultures, is shown in Fig. 4. GDH activity was resolvable into five distinct bands of closely migrating activity.

The isoenzymatic nature of GDH activity changed markedly during the culture cycle. Five bands predominated for mature 21 day old callus, but upon reinoculation onto fresh medium the pattern disappeared resulting in one broad band of activity. This band changed back to the five banded pattern by about day seven or nine.

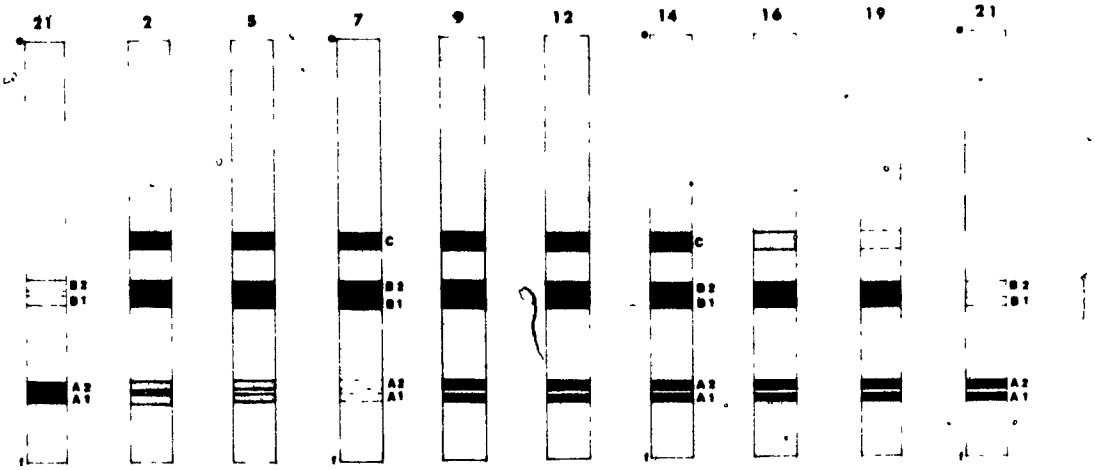
8) Leucine Amino Peptidase

Leucine amino peptidase activity (LAP) appeared as diagrammed in Fig. 6 (See also Fig. 7). The same isoenzymes of LAP were detected in each of the three cultures and each of the isoenzymes changed in activity during the culture cycle; however, each culture had a distinctive pattern of development during the cycle. Thus in root callus the isoenzyme designated C, in Fig. 6, rapidly increased in activity, following subculture, reaching a maximum at day 9 and then decreased. In contrast, isoenzyme C only appeared briefly in the hypocotyl cells at about day 12 and only, in any obvious amount, at the end of the culture cycle in cotyledon cells. The marked difference, between the cultures, in content of isoenzyme C is illustrated in Fig. 7. In root callus the activity changes in isoenzymes B1 and B2 paralleled those of isoenzyme C, while in cotyledon and hypocotyl callus the activity decreased after subculture and then increased later at day 9. Changes in the isoenzymes designated A1 and A2 were similar in all three cultures. The activity decreased to very low levels during the initial phases of the culture cycle and then increased during the later stages.

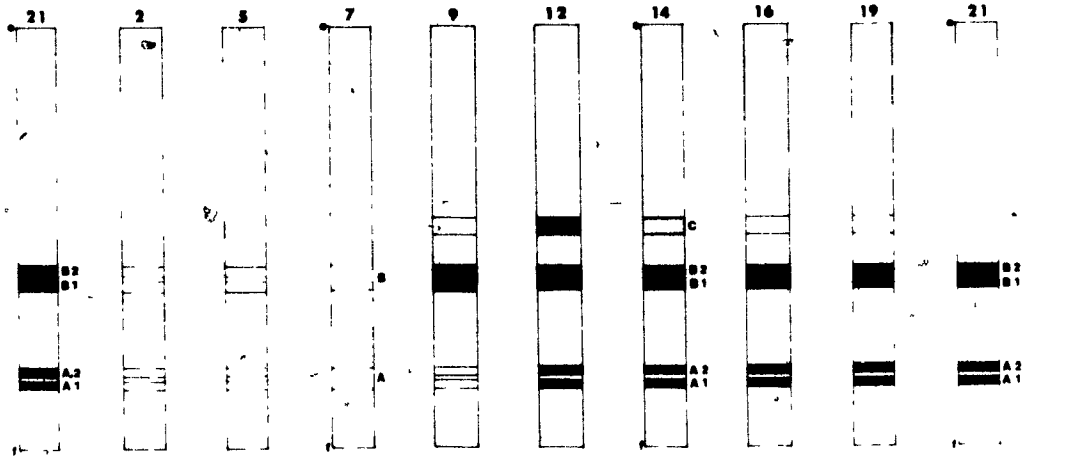
Fig. 6. Leucine amino peptidase isoenzymes. Diagrammatic representation of activities during cycle 53. Enzymatic activity is represented by band intensity.

Leucine Aminopeptidase Cycle 53

Root Callus



Hypocotyl Callus



Cotyledon Callus

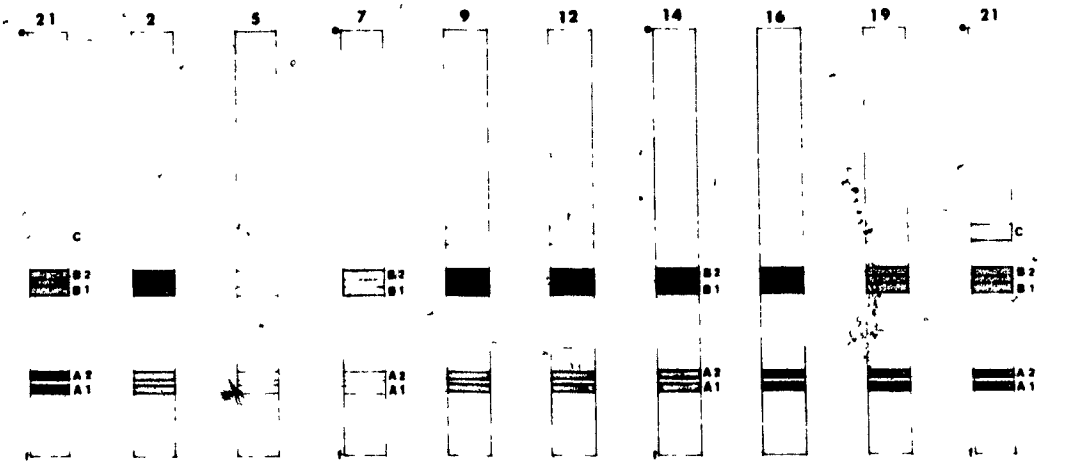
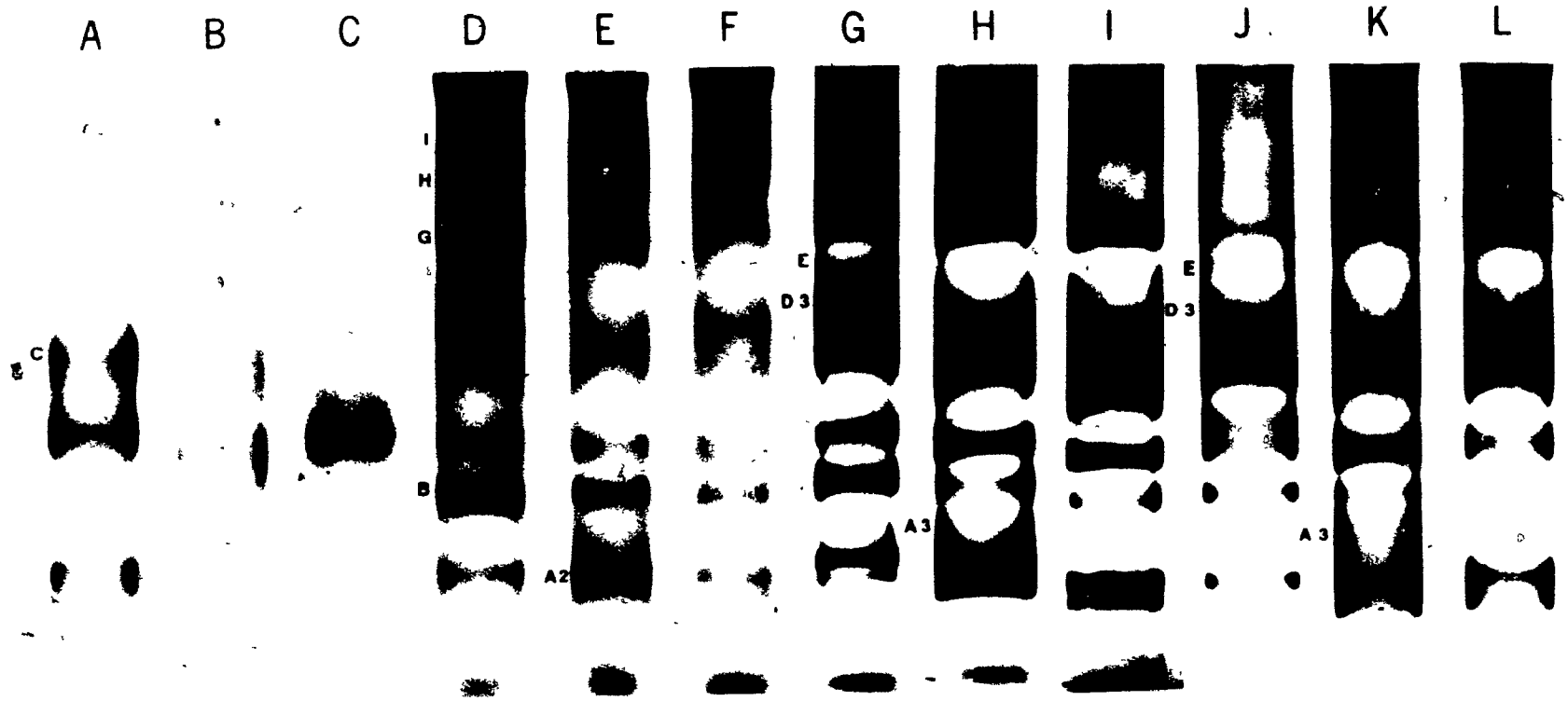


Fig. 7. Photographs of gels. A, B, C = LAP isoenzymes from root, hypocotyl and cotyledon, day 16, cycle 53. D, E, F = PP isoenzymes from root, hypocotyl and cotyledon cultures, day 14, cycle 53. G, H, I = Peroxidase isoenzymes from root, hypocotyl and cotyledon, day 5, cycle 53. Note the presence of D3 and E in root (G), and the activity designated A3 in hypocotyl (H). J, K, L = comparable peroxidase gels from cycle 54.


A
B
C
D
E
F
G
H
I
J
K
L
I
H
G
B
A2
E
D3
A3
E
D3
A3
C

B. Stock Suspension Cultures: Isoenzymatic Differences Between Root, Hypocotyl and Cotyledon Cultures

1) Introduction

Comparison of the isoenzyme patterns of eight enzymes studied intermittently over a three year period, showed persistent differences between the three cultures. Cell cultures were harvested on day twelve of passages: 65, 75, 90, 91, 112, 113, 119, 121, 124, 127, 141, and 143. The degree to which the isoenzyme patterns were different depended on the enzyme studied, but the differences have been maintained relatively unchanged for three years.

2) Peroxidase

Of all the enzymes studied the peroxidase isoenzymes showed the most striking differences. The patterns for the three cultures are shown in Figs. 8 and 9. The patterns for root and cotyledon cells are similar but that for hypocotyl cells is clearly different. The most evident differences are the absence of isoenzymes A1 and A2 in hypocotyl, the very high activity of these isoenzymes in cotyledon, the presence of isoenzyme F in cotyledon cells and the difference in intensity of other bands especially C1. The intensity of the various bands fluctuated somewhat but the differences persisted in successive cycles tested. Fig. 8 is for cells from passage 91. Fig. 9 is for cells from passage 121. It was noted that when the levels of total peroxidase activity per unit protein were determined spectrophotometrically by the method of Olson et al (1969) the level in hypocotyl cultures was always considerably below that of root or cotyledon cultures.

Peroxidase isoenzymes were detected in the media of all three cultures (see Fig. 8). As can be seen the isoenzyme patterns of peroxidase from the medium for the three cultures, were different. With the exception of the activities designated A1 and A2, the isoenzymes in the medium could not be clearly equated with those extracted from the cells. Therefore, to avoid possible confusion with isoenzymes of the media were given different symbols. The presence of A1 and A2 activity may be due to contamination of media with cytoplasm of ruptured cells.

3) Peroxidase (polyphenolic)

The PP activity appears to be a non-specific activity of some of the peroxidase isoenzymes. Thus isoenzymes A and B showed moderate activity. However, the isoenzymes showing the highest amount of DOPA oxidase activity, designated D and F (see Fig. 10) do not show peroxidase activity toward the monophenols tested. The three cultures differ in the relative amounts of the PP isoenzymes as well as in the absence of isoenzyme A in hypocotyl.

Fig. 8. Guaiacol peroxidase isoenzymes. A-Diagrammatic comparison of isoenzymes, day 12 passage 91. B-Diagrammatic comparison of isoenzymes from day 12 medium passage 91.

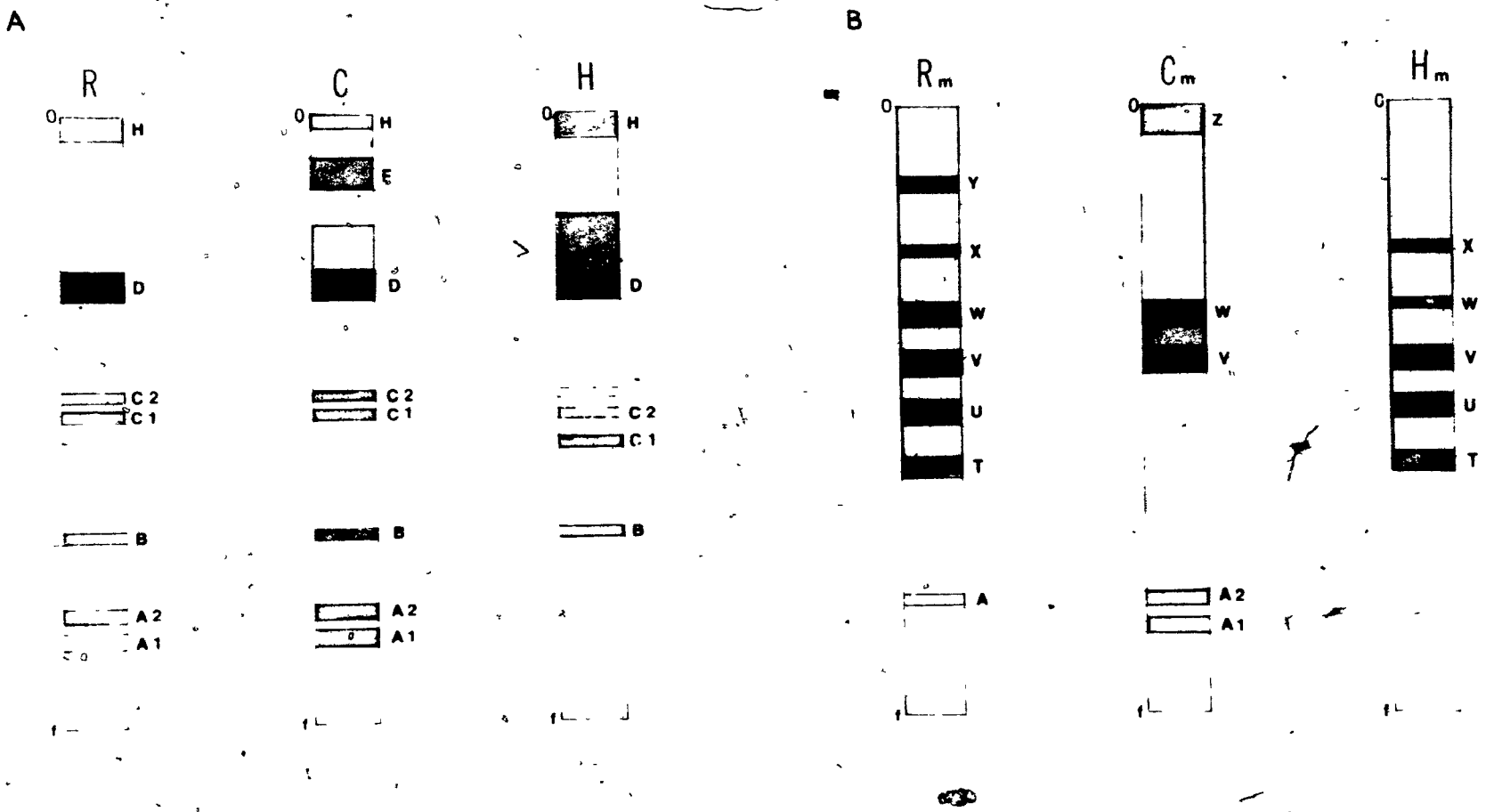

A
R
C
H
O
H
E
D
C 2
C 1
B
A 2
A 1
B
Rm
Cm
Hm
Z
Y
X
W
V
U
T
A

Fig. 9. Peroxidase isoenzymes. Tracings of gels, day 12 passage 121, scanned at 295 nm, guaiacol as substrate. R-root, H-hypocotyl, C-cotyledon, D-composite.

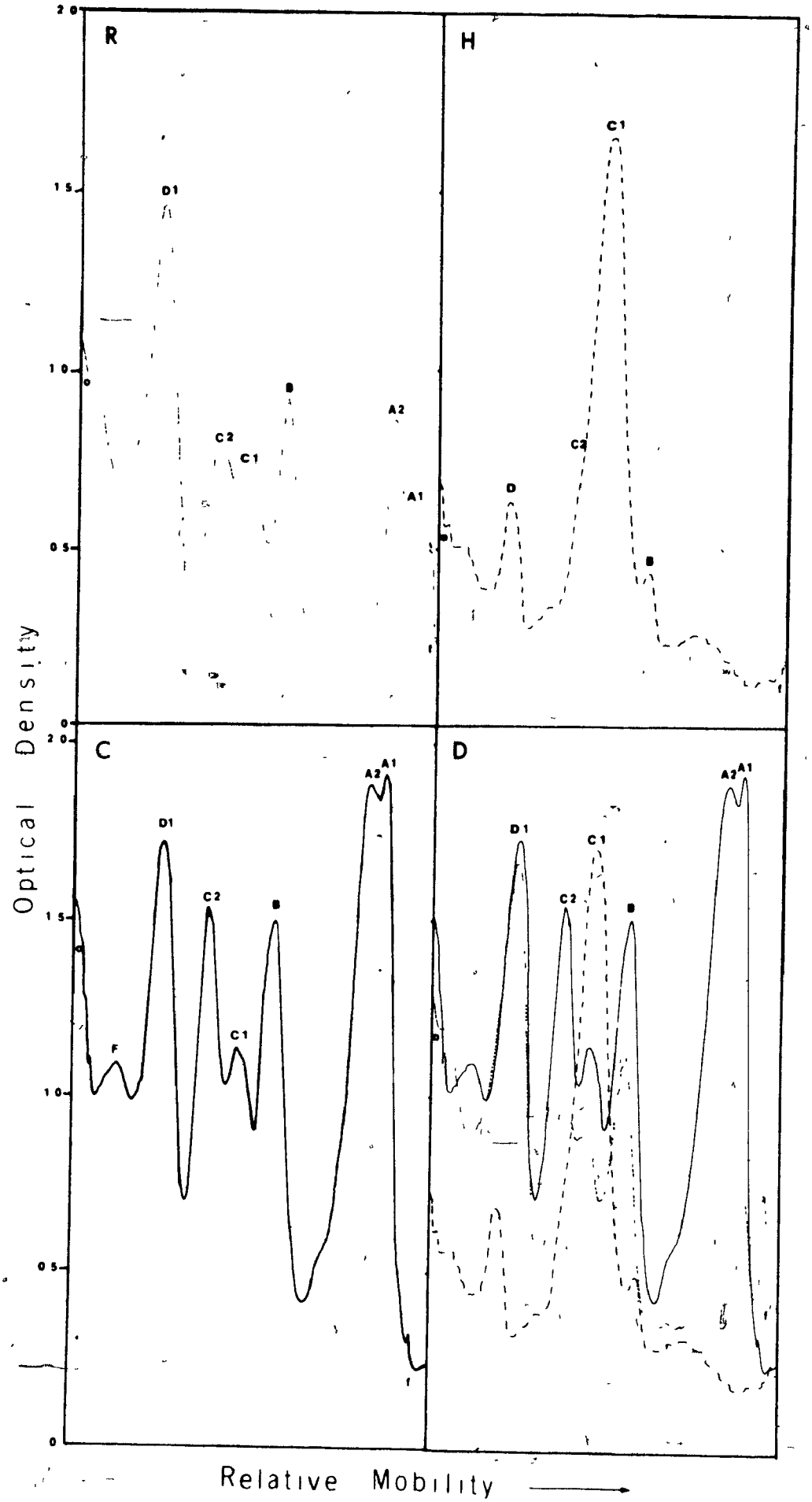
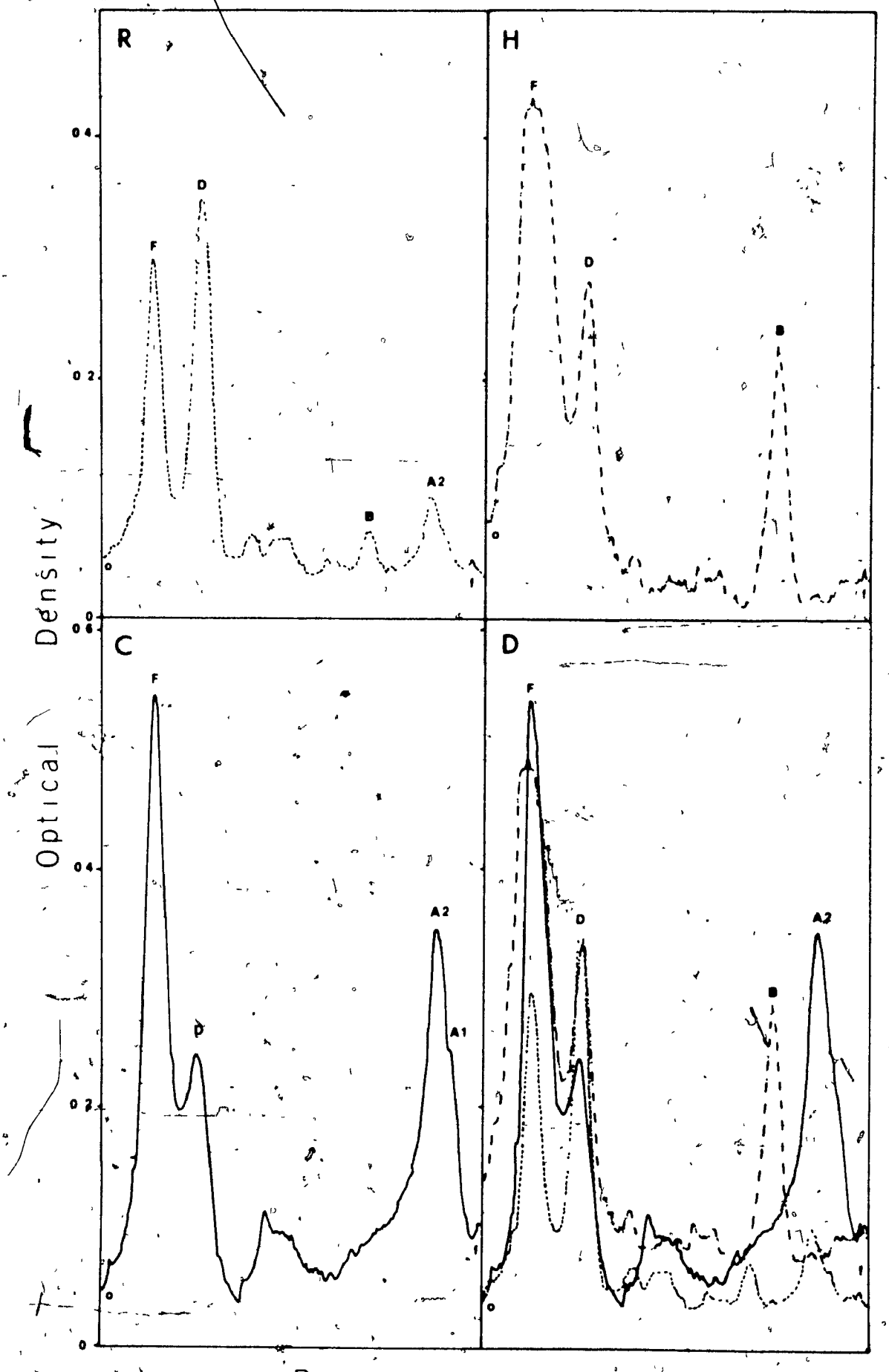

R
H
C
D
D1
C2
C1
B
A2
A1
F
Optical Density
Relative Mobility

Fig. 10. Peroxidase (polyphenolic) isoenzymes. Tracings of gels stained with DOPA, day 12 passage 124. R-root, H-hypocotyl, C-cotyledon, D-composite of three cultures. Scanned at 325 nm.


R
H
C
D
F
D
B
A2
A1
0.4
0.2
0
0.6
Optical Density
Relative Mobility

4) Esterase

Comparison of esterase activities from the three cultures was made somewhat difficult by the low levels of activity at day twelve. The most prominent difference between the three cultures was between root and cotyledon (see Fig. 11). The presence of a strong activity designated C2 in cotyledon is in contrast with the lower level in hypocotyl and the complete absence of activity in root. There are also clear differences among the minor enzyme peaks; the presence of A2 in cotyledon is particularly noticeable.

5) Malate Dehydrogenase

The differences between cultures in the complement of isoenzymes of malate dehydrogenase are shown in Fig. 12. Each of the three isoenzyme patterns shows two very prominent bands designated D and G. However, differences may be noted by the presence or absence of certain minor bands, B, E, H, and I which are indicated on the scans (Fig. 12) and which show clearly on the stained gels.

6) Glucose-6-phosphate Dehydrogenase

Glucose-6-phosphate dehydrogenase (G-6-PDH) activity was conspiciously low at all times in all three cultures. The presence of one lightly staining band was detected in cotyledon cells only. However, intact culture cells, supplied with buffered glucose-6-P, NBT and NADP (DeJong et al 1967), stained intensely in a reticulate manner. Thus it appears that the extraction or electrophoretic procedure had in some way eliminated the enzymatic activity.

Fig. 11. Esterase isoenzymes. Tracing of day 12 passage 124 gels scanned at 540 nm. R-root, H-hypocotyl, C-cotyledon, D-composite of three cultures.

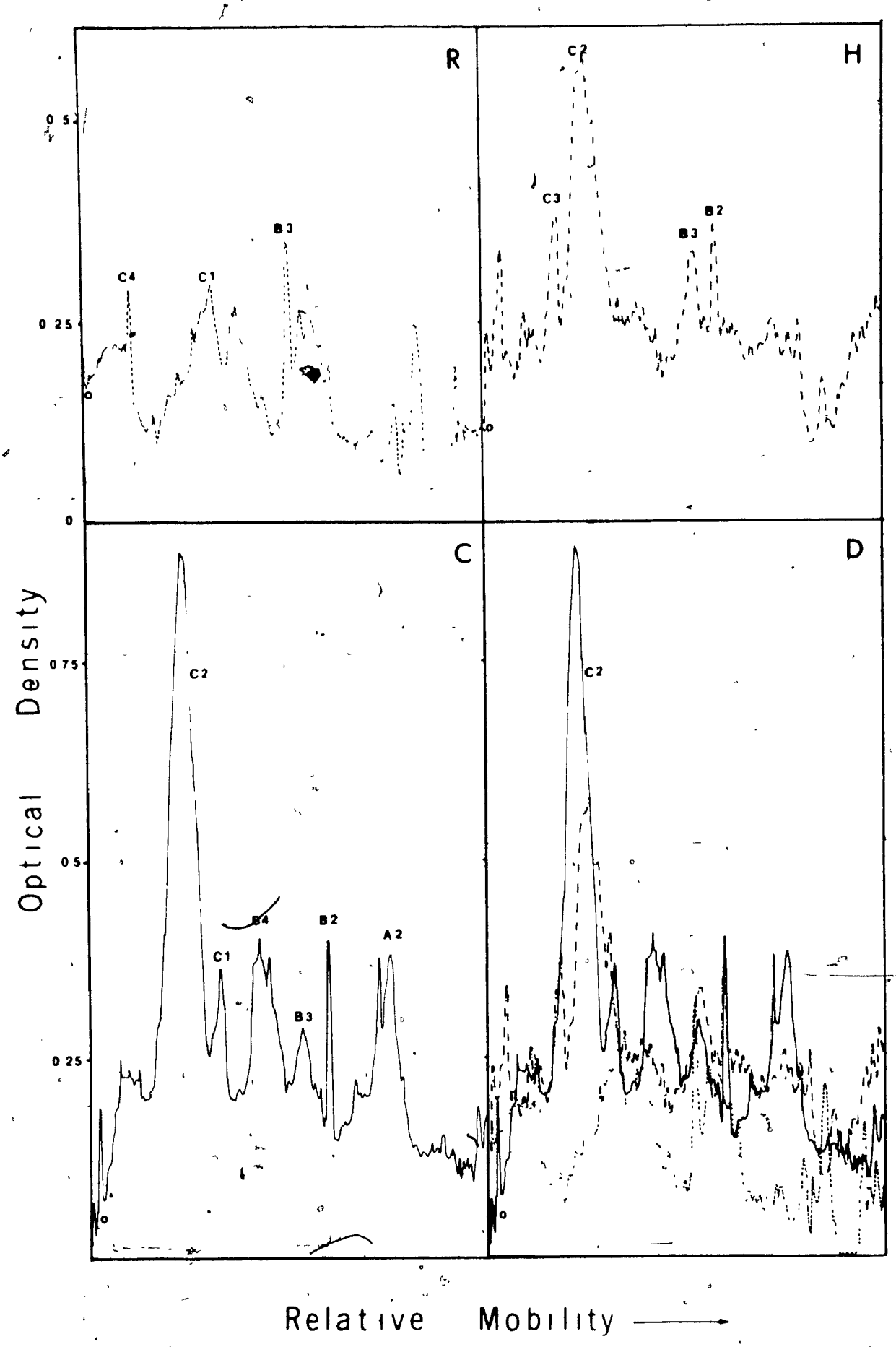
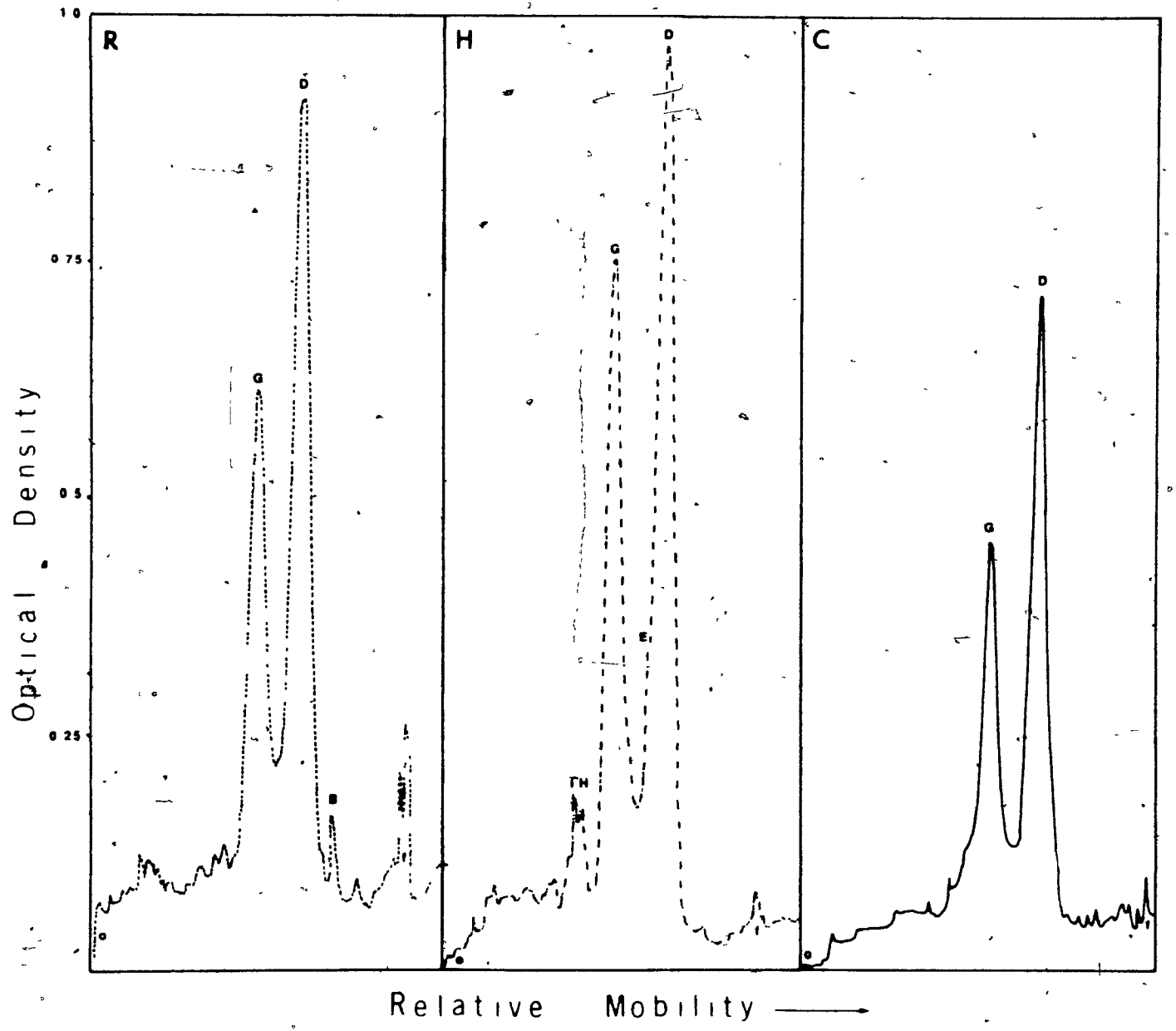

R
H
C
D
C4
C1
B3
C2
C3
B3
B2
C2
C1
B4
B2
A2
B3
C2
0 5
0 25
0
0 75
0 5
0 25
Optical Density
Relative Mobility

Fig. 12. Malate dehydrogenase isoenzymes. Tracing of day 12 passage 124, gels scanned at 610 nm. R-root, H-hypocotyl, C-cotyledon.


R
H
C
D
G
E
B
1.0
0.75
0.5
0.25
0
Optical Density
Relative Mobility

7) Leucine Amino Peptidase (LAP)

LAP activity, typical of root, hypocotyl, and cotyledon suspension cultures, is shown diagramatically in Fig. 13. The three cultures were very much alike. They differed only in the low levels of isoenzymes C1-C2 and B1-B2 in cotyledon cells.

8) Acid Phosphatase (AP)

The isoenzymes of acid phosphatase were found to be remarkably similar in all three cultures (see Fig. 14). The amount of AP activity was greater in cotyledon cultures. AP activity was detectable in the medium. However, activity was not detectable in the gels after medium samples were subjected to electrophoresis. Considerable AP activity was detected histochemically in the walls of the intact cells. This has also been reported by others (De Jong et al, 1967., and Johnson et al, 1973).

9) Glutamate Dehydrogenase (GDH)

Glutamate dehydrogenase activity was separable into five or six finely divided bands at day twelve. The pattern was essentially identical for all three cultures. The patterns for cycles 92, 126, and 127, from cotyledon cells, are shown in Fig. 15. The intensity of band staining and the height of densitometry peaks was low because of the low levels of GDH activity in stationary phase cells. With reinnoculation into fresh medium, as was the case with callus tissues, the total enzyme activity was stimulated and the five banded pattern changed to one or two. Subsequently up to seven bands may be evident. The bean cell cultures showed no extractable GDH activity using NADP as co-factor. After electrophoresis gels incubated with NADP showed no bands but when placed subsequently in a reaction mixture containing NAD the staining proceded as normal without inhibition.

Fig. 13. Leucine amino peptidase isoenzymes. Day 12 passage 91, diagrammatic representation of activity in R-root, C-cotyledon and H-hypocotyl cultures.

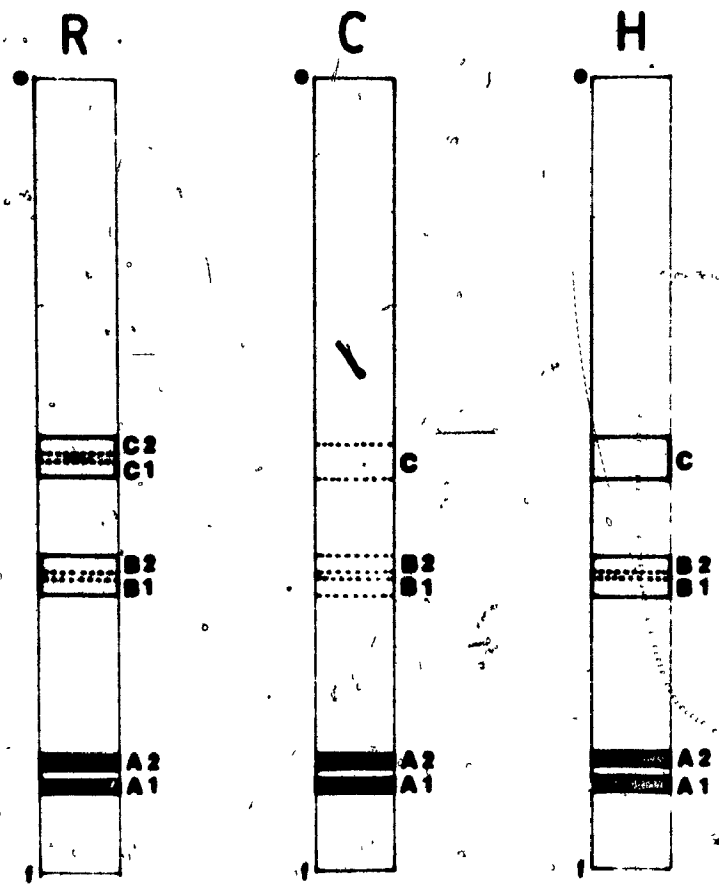

R
C
H
C2
C1
B2
B1
A2
A1
c
c
B2
B1
A2
A1
B2
B1
A2
A1

Fig. 14. Acid phosphatase isoenzymes. Tracing of gels, day 12 passage 124, scanned at 540 nm. R-root, H-hypocotyl, C-cotyledon, D-composite.

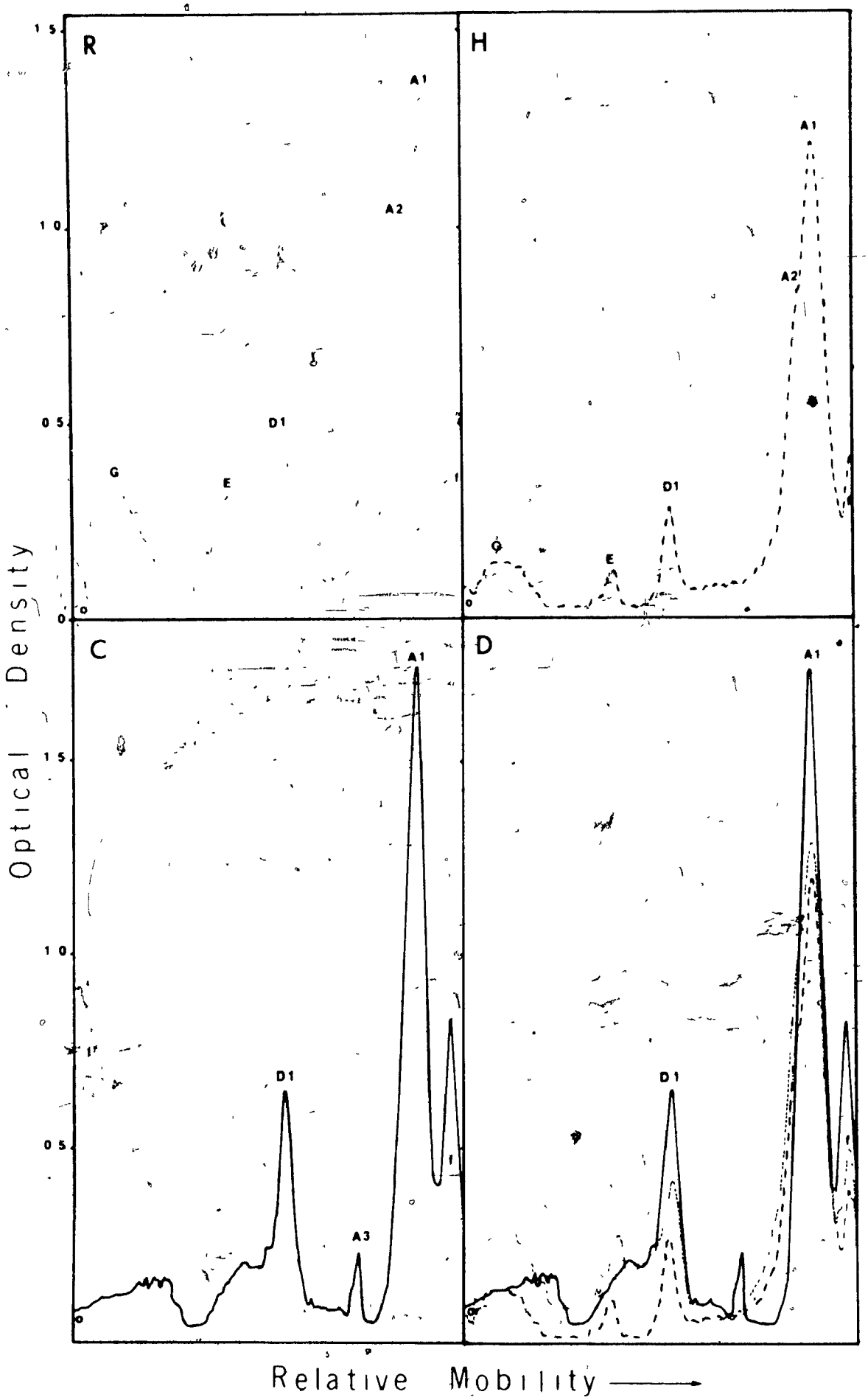
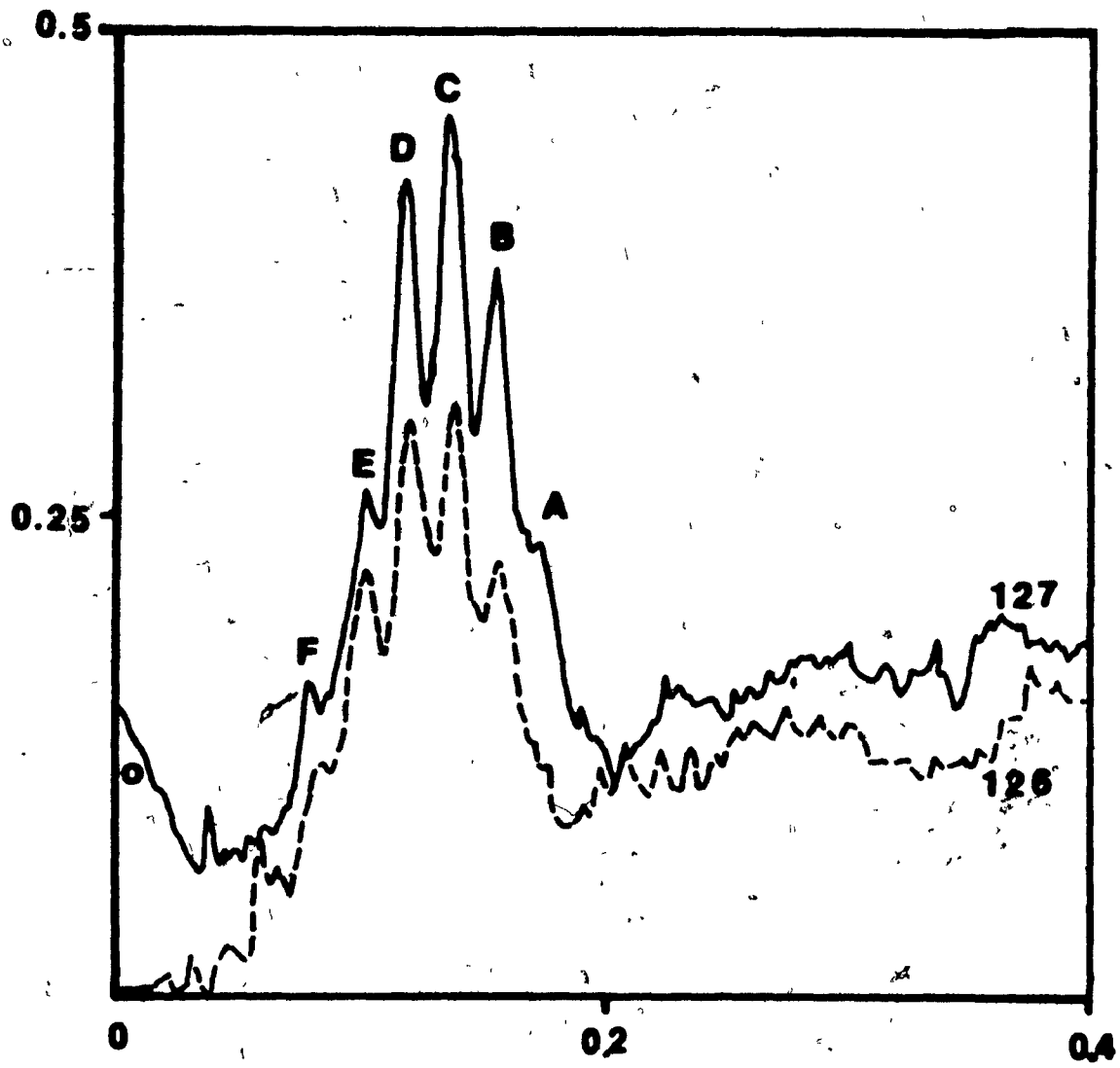

R
A1
A2
D1
G
E
H
A1
A2
D1
E
C
A1
D1
A3
D
A1
D1
1.5
1.0
0.5
0
Optical Density
Relative Mobility

Fig. 15. Glutamate dehydrogenase isoenzymes. Tracing of gels day 12 passage 126 and 127 from cotyledon scanned at 610 nm. Photograph of GDH activity, day 12 passage 92.


0.5
0.25
0
0.2
0.4
A
B
C
D
E
F
127
126

C. The Effect of 2,4-D and Kinetin on Growth and Isoenzyme Patterns of Cotyledon Suspension Cultures.

1) Introduction

Concomitant with studies designed to detect differences between cultures were studies designed to monitor changes in isoenzyme pattern that occurred during the culture cycle, as was done for callus cultures, and to detect possible effects of the growth regulators 2,4-D and kinetin on these isoenzyme patterns. Previous work by Liau and Boll (1970, 1971, 1972) and Liau (1971) showed that cotyledon cultures, unlike those of root or hypocotyl, maintained substantial growth rates in medium containing neither 2,4-D nor kinetin. These experiments showed the most striking comparative differences when both growth regulators were omitted. It was felt in consideration of these facts that cotyledon suspension cultures grown with and without growth regulators offered an excellent system for the study of growth regulator action.

Experiments on the effect of growth regulators were conducted for two consecutive passages for a total of 24 days. Cell cultures were harvested from passages: 90, 91, 92, 119, 120, 121, 125, 126, 127, 137, 138, 139, 141, 143, 146, 147, 148, 149, 154, 155, and 156.

Four ml of twelve day old cells were inoculated into 250 ml flasks containing 50 ml of media containing growth regulators, referred to as 'plus' cultures (L+) and into equivalent medium but without regulators designated 'minus' cultures (L-). Cells were transferred to fresh medium on day twelve of the experimental period. Because cultures maintained without 2,4-D and kinetin had less fresh weight and lower cell numbers after the first passage the plus cultures of passages 138

and 148 were inoculated with only 2.5 ml of cells in an attempt to compensate for any growth differences due to cell number and to cell density.

2) Growth Characteristics

The growth curve for cotyledon suspension cells, as measured by increase in fresh weight, is presented in Fig. 16. After inoculation into fresh medium the cells entered a short lag phase, followed by a phase of intense cell division, then a logarithmic growth and expansion phase and finally a stationary phase. The patterns of growth and cell division have previously been reported in detail by Liau and Boll (1971). The number of cells per culture and an index of cell size, namely cell number per gram fresh weight, are presented in Fig. 17 and 18 respectively. Comparison of the figures shows that during the lag phase there was no increase in cell number but there was an increase in cell size. The cells then divided rapidly. Most of the division was completed by day eight, after which time elongation and expansion accounted for the sharp rise in cell fresh weight. Cell size was at a minimum during the phase of cell division and at a maximum during the stationary phase. When plus cultures were inoculated with a lower cell density this resulted in a longer period of logarithmic growth, a slower increase in fresh weight and a smaller initial average cell size. By the end of the second culture cycle however the weight, number and size of the cells were comparable with values for day twelve in the first passage.

The growth kinetics were changed when cells were cultured in medium without growth regulators. Initially, minus cells divided sooner and more often producing comparatively larger cell numbers and

an increased total fresh weight by day four. However after day four without the regulators further cell division was restricted and the logarithmic increase in total fresh weight, until day twelve, was mostly due to cell enlargement. After twelve days the minus cultures contained about one third the number of cells and about one half the fresh weight of the plus cultures. Cells reinoculated into medium without growth regulators showed very little growth, as measured by increase in fresh weight, for the first six days (day 12-18). Initially cell division occurred predominantly during the first two days (day 12-14) and was mostly restricted to elongate cells. This resulted in the decrease in cell size, as shown by the cell size index (Fig. 18), and means that during the first six days of the second passage (day 12-18) the cells of the inoculum divided without any concurrent cell expansion. After day 18 there was again a sharp increase in the number of cells due this time to the rapid proliferation of previously small groups of tiny cells. This resulted in an increase in total fresh weight per culture which, despite the fact that the total number of cells approached that of the plus cultures, was only one fifth that of the plus cultures. Thus at this stage the minus cultures contained many very small cells growing in large clumps. An interesting and novel aspect of these observations is that the second passage without growth regulators, in effect, shows induced diauxic growth.

Fig. 16. Growth curve of cotyledon suspension cells grown with (plus) and without (minus) growth regulators 2,4-D and kinetin. Weight is expressed per culture flask. N.B. Cultures were inoculated into fresh medium on day twelve.

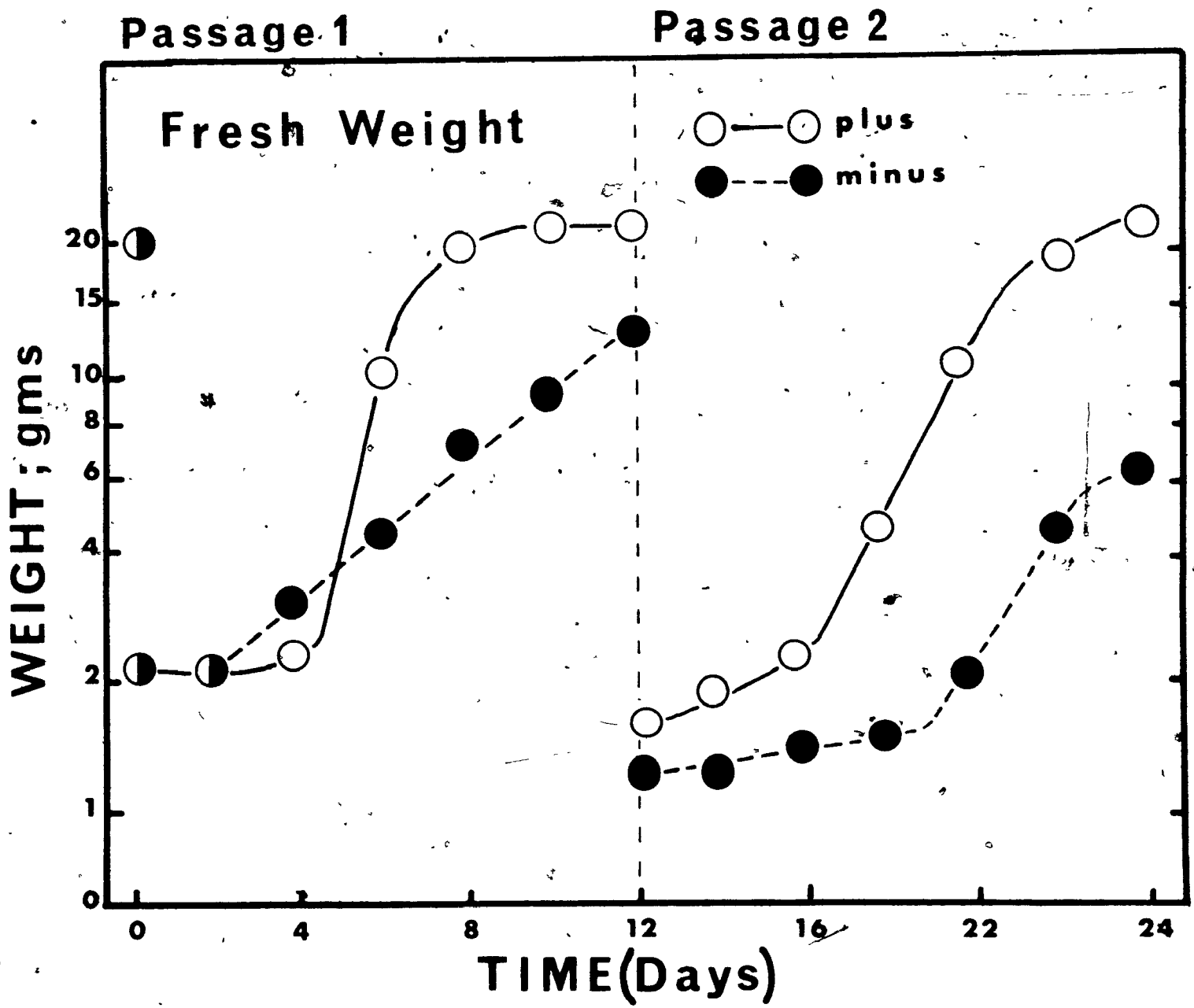

Passage 1
Passage 2
Fresh Weight
plus
minus
WEIGHT; gms
20
15
10
8
6
4
2
1
0
0
4
8
12
16
22
24
TIME(Days)

Fig. 17. The increase in cell number per flask of cultures grown with and without growth regulators. Cultures were inoculated into fresh medium on day twelve.

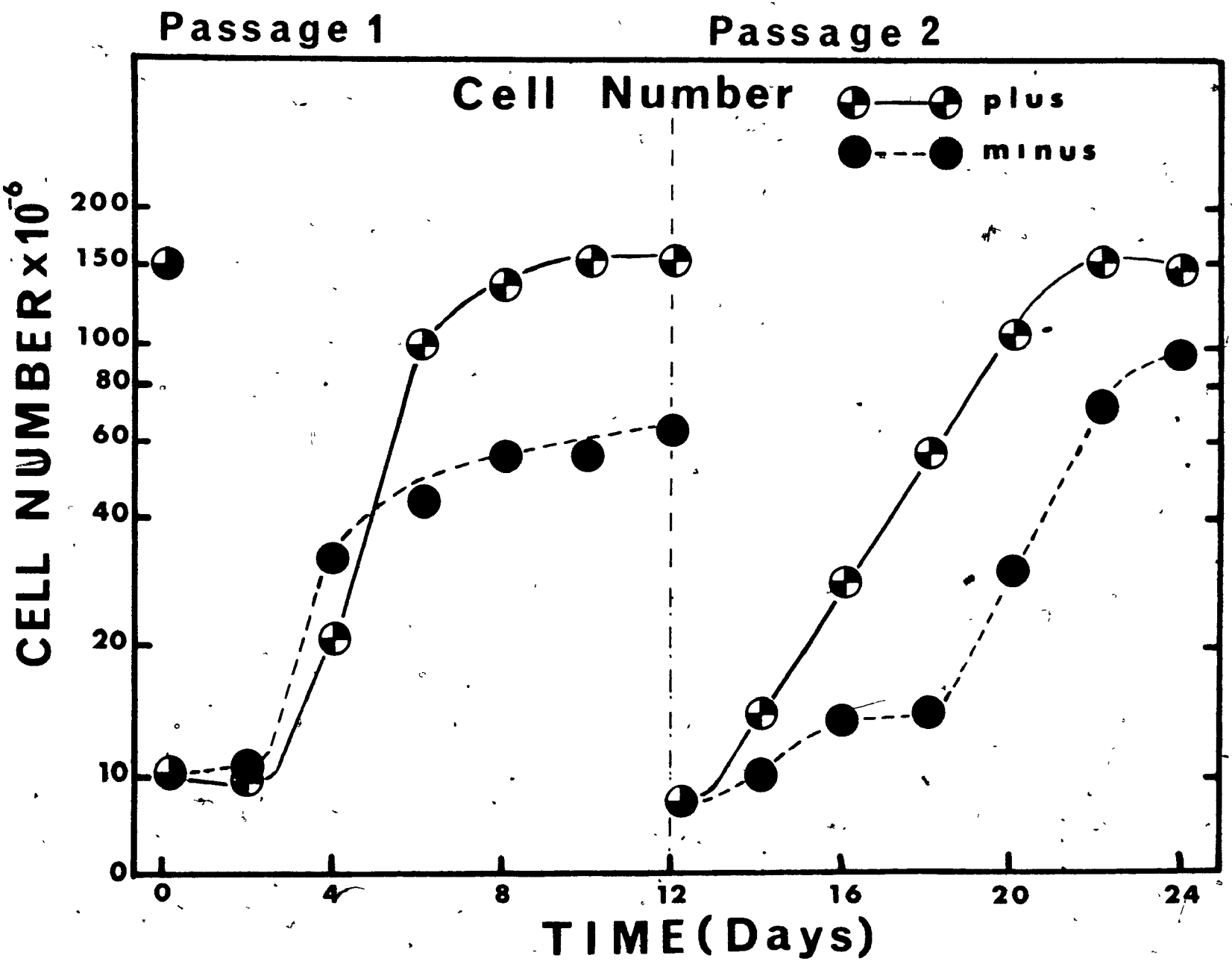
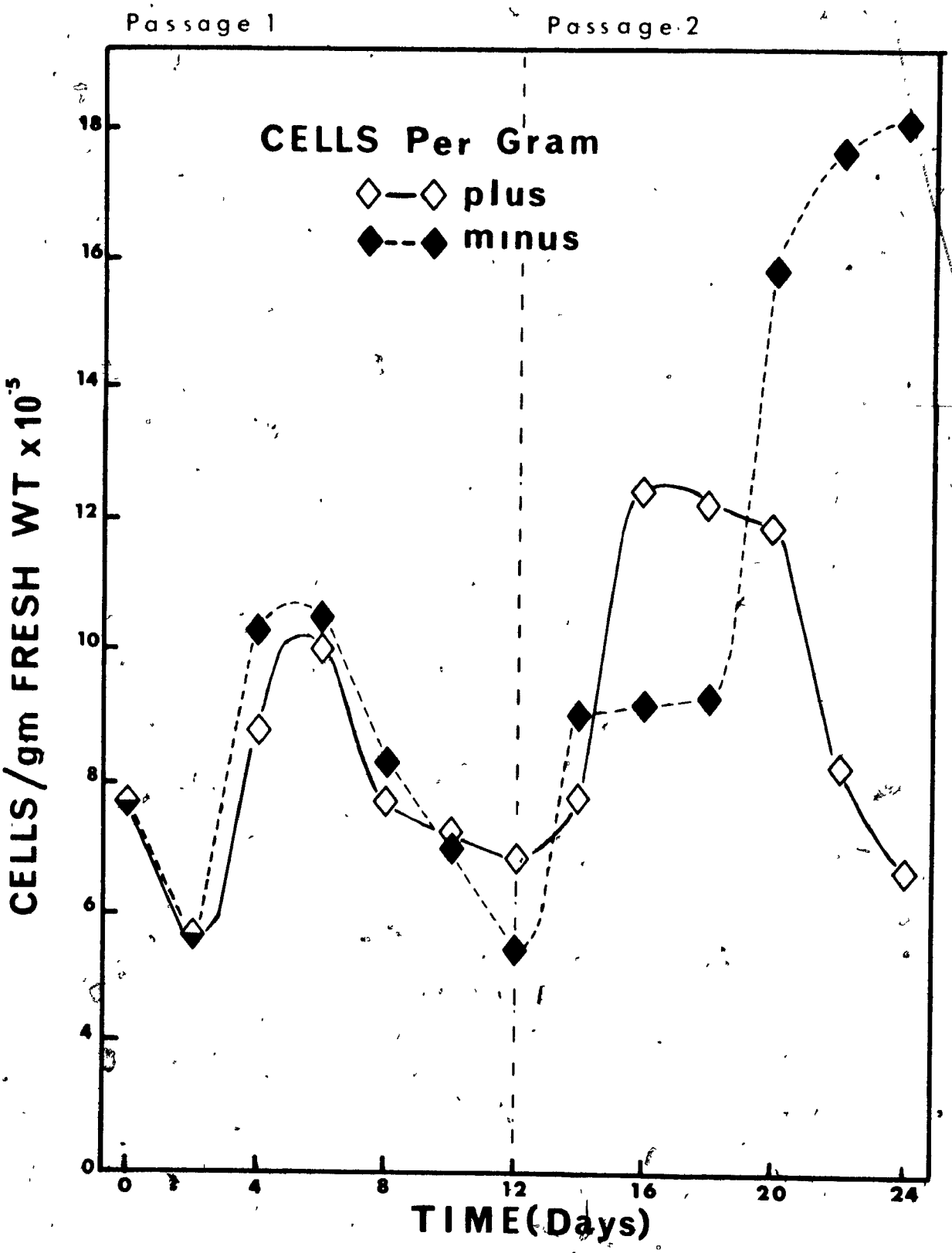

Passage 1
Passage 2
Cell Number
plus
minus
CELL NUMBER x10⁻⁶
200
150
100
80
60
40
20
10
0
0
4
8
12
16
20
24
TIME (Days)

Fig. 18. The number of cells per gram fresh weight for cultures grown with and without growth regulators. Cultures were inoculated into fresh medium on day twelve.


Passage 1
Passage 2
CELLS Per Gram
plus
minus
CELLS/gm FRESH WT x10⁻³
0
4
6
8
10
12
14
16
18
0
4
8
12
16
20
24
TIME(Days)

3) Morphological Characteristics

Twelve-day-old stock cultures used as inocula consisted of a mixed population of elongate, oval and round cells existing either singly or in small groups (Fig. 19-4). After the lag phase, clumps of actively dividing cells were formed (see Fig. 19-5). Some of the more elongate cells first divided by a series of internal cross walls and then proliferated in various ways (Fig. 19-6). These patterns of cell division are comparable to those described in detail by Liau and Boll (1971). By the end of twelve days in culture most of the cell clumps had dissociated into small groups of cells and single cells (Fig. 20-11). Growth characteristics during the second passage, with a lower inoculum density, were identical to those in the first passage (Figs. 21-17, 18, 22-26).

Cells cultured in the absence of growth regulators showed some marked morphological differences when compared with the plus cultures. By the sixth day, minus cultures were already markedly browner than the normally very white to pale yellow color of the plus cultures. Some of the cells had become very long and had a wavy or kinky appearance (Fig. 19-7,8,9,10). On close examination some cells appeared twisted with striations of wall thickening. It was common to find these cells spirally twined around each other (Fig. 19-10). Not all cells of the minus cultures became elongated and twisted. Thus, by day 12, the minus cultures consisted of a population similar in appearance to the plus cells (compare Fig. 20-11 and 20-14), but with a large number of long, coiled cells and groups of smaller dense cells.

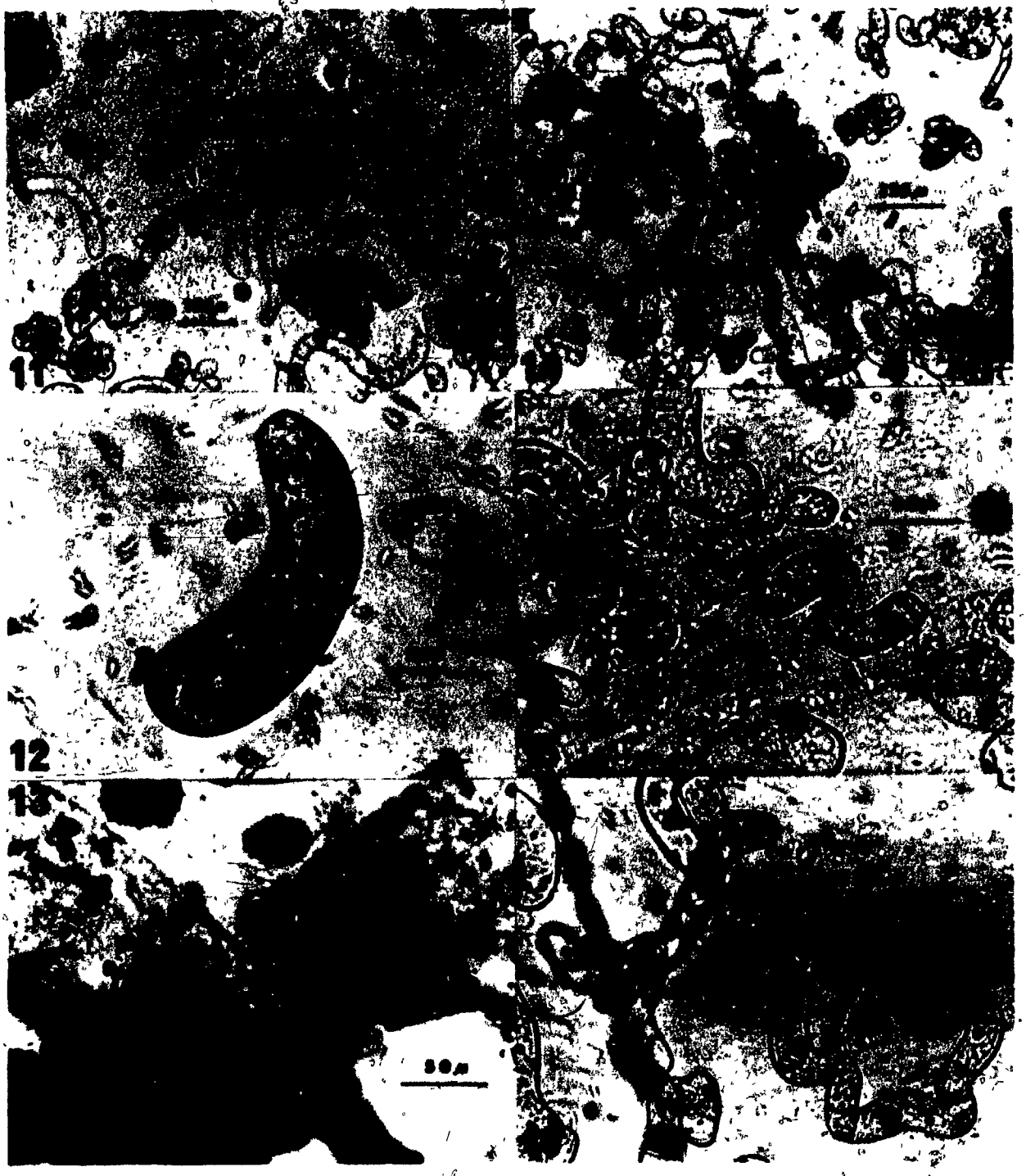
During the second passage without growth regulators the changes evident during the first passage became more exaggerated. By day 18 the minus cultures had become very dark brown and very little growth in fresh weight or cell number had occurred since day 12. The cultures consisted mostly of extremely long, thin, coiled cells and groups of very small cells with dense cytoplasm (Fig. 21-20,21,23, and Fig. 22-24,27). Some of the cells appeared to be plasmolysed or dead. The differences in size of cells, and in the nature of the plus and minus cultures after two passages, can be appreciated from a comparison of Figs. 22-26 and 22-29,30.

Cells maintained in minus culture for additional passages continued to grow but at a rate slower than that of the plus cultures. In order to maintain growth in minus cultures it was necessary to transfer ten ml of the culture. Cells so maintained in minus culture consisted of very dense groups of cells as in Fig. 22-29,30 and many small, round and oblong free cells. Many of the free cells and cell groups were very dense in appearance and the cultures remained dark brown. Transfer of cells cultured for one, two and three passages in minus medium to plus medium resulted in immediate cell proliferation and enlargement to produce populations of cells typical of plus cultures. Additionally the light yellow to whitish color of the cultures was regained with the first passage back in plus culture conditions. Cells cultured alternately in plus and then minus culture showed a complicated mixture of dense groups of small cells, some of which were very brown, elongated and twisted cells as well as a host of intermediate forms. The cultures were pale to medium brown in color.

Fig. 19. Representative cells from cultures grown with and without growth regulators.

Frame 4. Population of twelve day old cells from plus medium used as the inoculum. Frame 5 & 6. Cell groups from six-day-old plus culture showing a typical group of activity dividing cells and a long sub-divided cell proliferating at one end. Frames 7 to 10. Representative cells from six-day-old cultures grown without regulators. Note that the cells are long and wavy, some of which show wall thickenings and twisting. (arrow)

Fig. 20. Representative cells from cultures grown for twelve days with and without growth regulators. Frame 11. Cells from day 12 plus culture. Frame 12. Cell from day 12 plus culture stained for guaiacol peroxidase. Frame 13. Cells from day 12 plus culture stained for peroxidase showing 'bow tie' shaped crystals attached to the cell surfaces. Frame 14. Cells from day 12 minus culture, note elongate coiled cells (arrow) and groups of dividing cells. Frame 15 & 16. Very long highly coiled cells from day 12 minus culture..


11
12
13
50 nm

4) Peroxidase

a) Cytochemistry

The staining of plus and minus cultures for peroxidase activity at various times during the experimental period, showed that enzyme activity was predominantly cytoplasmic in young cells and very strongly associated with wall in older cells. Furthermore, cells cultured without growth regulators stained much more intensely. Cells that were used as the initial inoculum stained quite intensely for guaiacol peroxidase activity.

Young and actively dividing cells showed comparatively low activity that was localized through the cytoplasm (Fig. 21-18,19).

The peroxidase staining reaction gave a coarsely granular, crystalline deposit spread somewhat unevenly over the cell surface and a more even, and less crystalline, brown staining of the cytoplasm (Fig. 20-12). On some occasions larger 'bow tie' shaped crystals of oxidized guaiacol were found scattered over the cell surface (Fig. 20-13). Such 'bow tie' shaped crystals did not form when the spent medium itself was stained, although other larger and more amorphous crystals did form and were seen to float in the medium. 'Bow tie' crystals were easily washed off cells and then were observed in the medium. Cells that were washed with dilute buffers or distilled water before staining showed less intense cell wall staining and no 'bow tie' shaped crystals. Finally no crystal formation was observed either on the surface of cells that were washed with buffer and suspended in spent buffer or on cells that were washed, resuspended in spent medium and then this medium filtered off and replaced by fresh medium. Presumably the 'bow tie' shaped crystals, as

such, are a consequence of the location of the crystal growth rather than a crystal form produced by a special surface peroxidase. However, the observations do show that the peroxidase on the surface is not simply left there when the medium is filtered off but is loosely attached at numerous disperse sites on the surface. Crystal formation was not restricted to guaiacol oxidation products. Thus many long needle-like crystals of benzidine blue were formed when benzidine dihydrochloride was used as an alternative stain.

Cells that were grown without growth regulators showed the same distribution, and granular nature, of the peroxidase stain (Fig. 21-23 and 22-25). The long coiled cells of the day 18 minus cultures often stained so heavily that they appeared almost completely black (Fig. 22-24,27). Groups of small cells that were present in these cultures did not stain so intensely and, curiously, certain sections of some long coiled cells did not stain at all either in wall or cytoplasm. Such sections contained no obvious cytoplasmic contents (Fig. 22-27).

Surprisingly, negligible amounts of peroxidase activity, as judged by crystal formation, were present in the media of these cultures. Cells that were washed to remove peroxidase activity still stained very heavily (Fig. 22-25). Coiled cells that were present at the end of the experimental period stained extremely heavily but the more numerous clumps of small cells stained very little.

The Peroxidase (polyphenolic) activity showed a pattern of staining activity and distribution similar to that of peroxidase. A fairly even granular staining was evident in older and elongate cell walls. The major difference between peroxidase and PP activities, however,

was the preferential staining of the nucleus, and possibly cross cell walls, with DOPA oxidation products (Fig. 21-22 and 22-28).

Fig. 21. Representative cells from day 18 cultures grown with and without growth regulators. Frame 17. Sample of day 18 plus culture cells (equivalent to day six). Frame 18 & 19. Day 18 plus culture cells stained for guaiacol peroxidase showing light cytoplasmic staining. Frame 18, 20 & 21. Day 18 minus culture, representative cells showing elongated coils, small tightly packed groups and dense cytoplasm. Frame 22. Day 18 plus culture cells stained with DOPA to demonstrate PP activity. Activity is located through the cytoplasm, nucleus and possibly in the cross walls. Frame 23. Coiled day 18 minus culture cell stained for peroxidase activity.


18
19
22
23

Fig. 22. Representative cells from day 18 and day 24 cultures. Frame 24. Day 18 minus culture cells showing a very heavy peroxidase reaction. Frame 25. Enlarged portion of day 18 minus culture cell washed with buffer and stained for peroxidase activity. Note the granular nature of the stain deposit embedded in the cell wall. Frame 26. Sample population of day 24 plus culture cells. Frame 27 as in Frame 24 except sections of elongated cells did not stain. Frame 28. Enlargement of a portion of day 18 minus culture cell stained for PP activity. Note the granular nature of the reaction product and the staining of the nucleus. Frame 29-30. Representative clumps of small cells from day 24 minus cultures.


25

b) Peroxidase Activity

Peroxidase activity expressed per gram fresh weight, per million cells and per mg soluble protein is shown in Fig. 23, 24, 25 respectively. The activity is shown for cells grown with and without growth regulators over a two passage period of twenty-four days. The general conclusions are the same regardless of the base on which the enzyme activity is expressed.

After subculture, both plus and minus cultures showed an initial decrease in peroxidase activity which reached the lowest levels by day 4 and day 2 respectively. After this low point peroxidase activity increased to a maximum and then declined. Peroxidase activity increased most rapidly in the minus cultures which were previously found to have a shorter lag phase and a more rapid increase in fresh weight and cell number than the plus cultures. On a fresh weight basis, plus cultures showed highest activity on day 10 whereas minus cultures showed peaks of activity on day 6 and, during the second passage, on day 22. Peroxidase activity in plus cultures was considerably lower during the second passage (day 12-24) when the cultures were inoculated with lower numbers of cells. This lessening of activity was only temporary because activity was restored to levels comparable to those in the first passage if cells were kept for an additional four days without transfer. Cells cultured without 2,4-D and kinetin showed very high levels of peroxidase activity during the second passage.

Fig. 23. Changes in activity of peroxidase per fresh weight of cultures grown with and without growth regulators (plus and minus) during two successive culture cycles. Day twelve represents the point of reinoculation into fresh media. The inoculum of plus cells in the first passage was 4.0 ml and 2.5 ml in the second passage. Bar markings represent standard errors. Where no markings occur the standard error was smaller than the size of the dot.

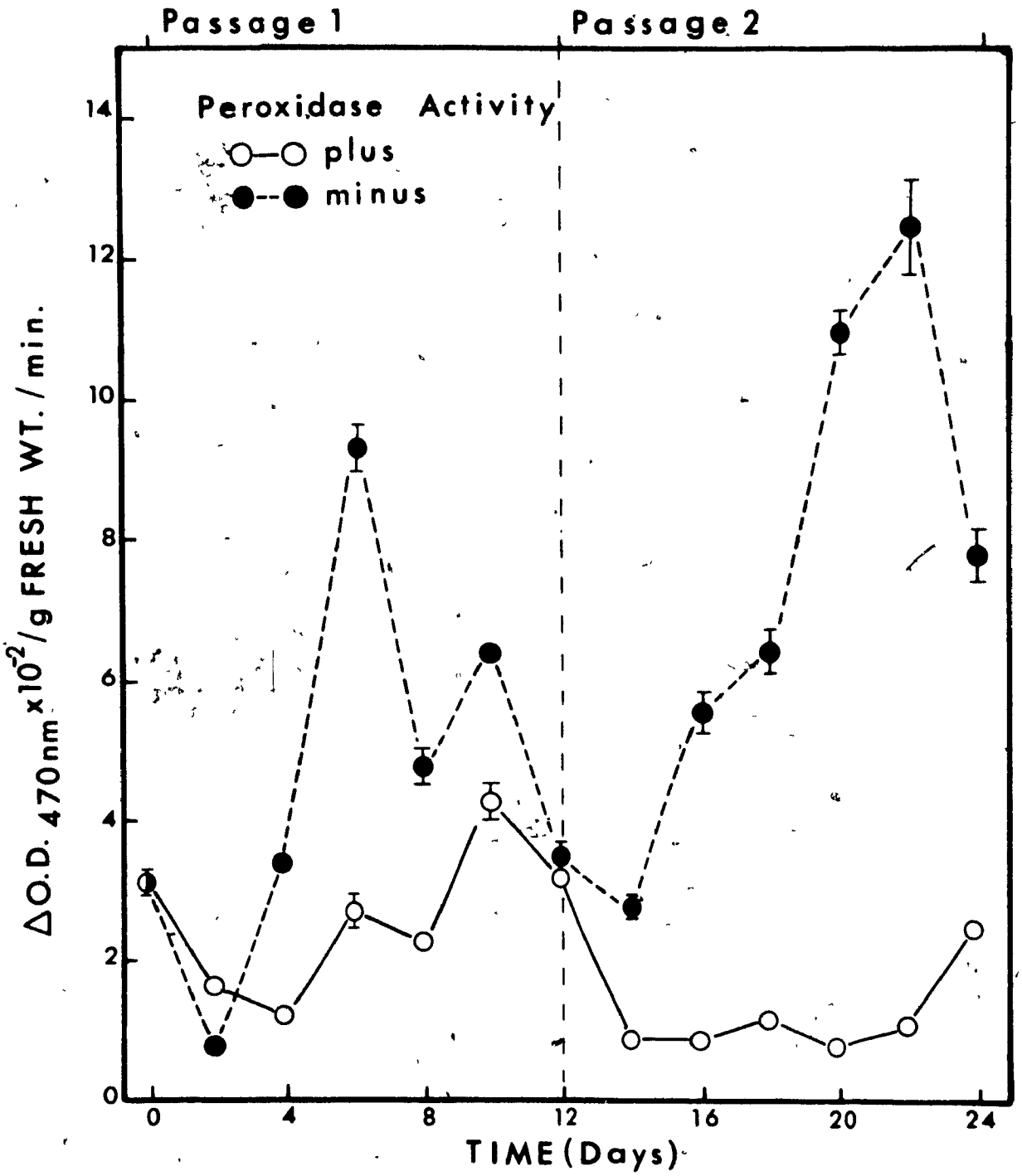

Passage 1
Passage 2
Peroxidase Activity
plus
minus
ΔO.D. 470nm x10⁻²/g FRESH WT./min.
TIME (Days)
0
2
4
6
8
10
12
14
0
4
8
12
16
20
24

Fig. 24. As for Fig. 23 but activity expressed per million cells. Inoculum sizes for plus cells were as for Fig. 23.

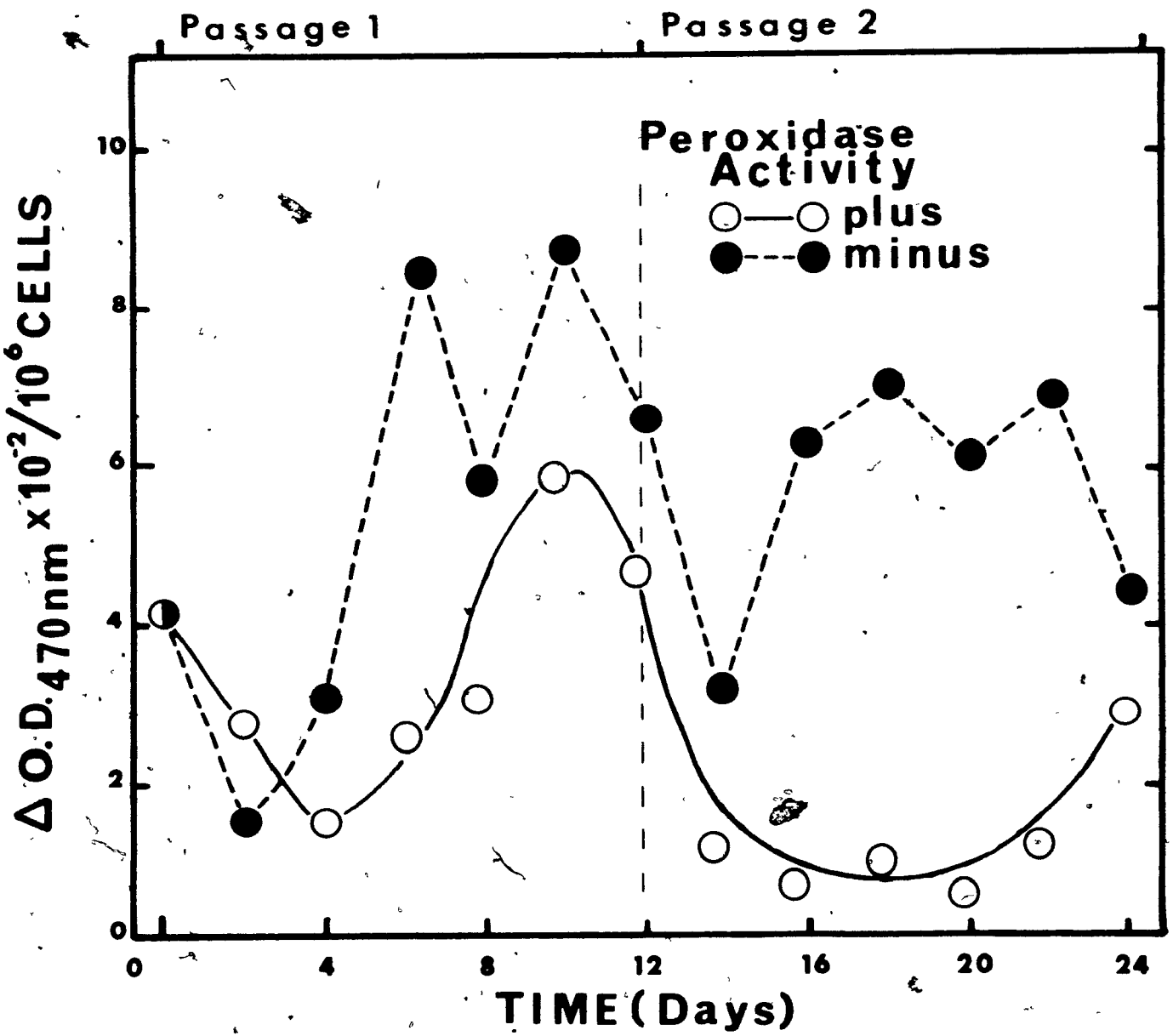
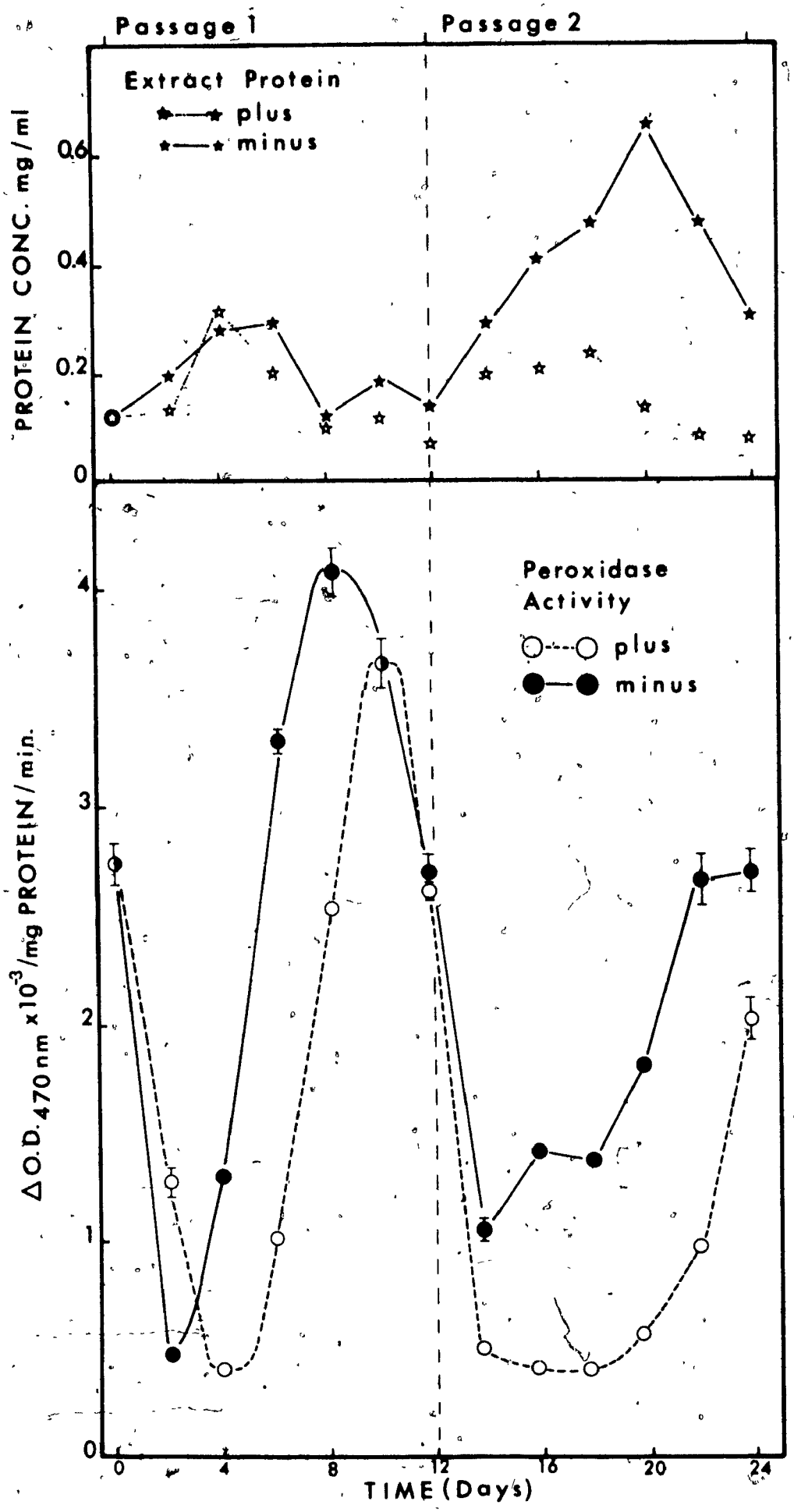

Passage 1
Passage 2
Peroxidase Activity
plus
minus
ΔO.D.470nm x10-2/10^6CELLS
0
2
4
6
8
10
0
4
8
12
16
20
24
TIME(Days)

Fig. 25. As for Fig. 23 and 24 but activity expressed per mg extract protein. The protein concentration in the extracts at different stages is also shown. Inoculum sizes for plus cells were as for Fig. 23.


Passage 1
Passage 2
Extract Protein
plus
minus
PROTEIN CONC. mg/ml
0
0.2
0.4
0.6
Peroxidase Activity
plus
minus
ΔO.D.470nm x10-3/mg PROTEIN/min.
0
1
2
3
4
0
4
8
12
16
20
24
TIME (Days)

c) Changes in Peroxidase Isoenzyme Patterns

The changes in isoenzymes of peroxidase are presented diagramatically in Fig. 26 and 27 for cells grown with and without growth regulators. Peroxidase isoenzymes changed both qualitatively and quantitatively during the culture cycle. The most noticeable qualitative changes were the appearance of isoenzymes designated E and D2 on day 2 and then the subsequent disappearance of isoenzymes D2 and C3 on day 4. Isoenzyme E no longer occurred after day 8 at which time isoenzyme C3 recurred. These changes in isoenzyme pattern, although not extensive, were found to be very consistent. Comparison of isoenzyme patterns of plus and minus cultures showed few qualitative differences although the patterns are very distinctive (see Fig. 27). Minus cultures showed a diffuse but noticeable band of activity designated A+ which appeared on day two and increased in intensity thereafter. Treatment of the extracts with reducing agents or subjecting extracts to dialysis or passage through Sephadex G-50, failed to remove this activity. It is also noteworthy that isoenzyme D2 persisted as a prominent peak until day 8 in minus cultures.

Most of the changes in peroxidase isoenzymes during the culture cycle, and differences between plus and minus cultures, were quantitative (see Fig. 26, 27). Changes in the activity of the individual isoenzymes D2, B and A2 are presented in Fig. 28, 29, 30 and 31. The most striking changes that occurred in plus cultures took place after inoculation into fresh medium. On day two there was a relative rise in the activity of isoenzymes E, F, G, C1, C2 and C3 even though the total peroxidase activity as measured spectrophotometrically was decreasing

rapidly. These isoenzymes subsequently decreased in activity and returned to the day twelve levels. Isoenzymes A2 and D1 which made up considerable portions of the peroxidase activity of the plus cultures reflect the total changes in peroxidase activity (see Table 1). This is exemplified in Fig. 28, 29 and 30 for isoenzyme A2. Figure 28 shows that the percent A2 activity changed relatively little during the culture cycle (day 0-12). However, the activity contributed by isoenzyme A2 when measured on either fresh weight (Fig. 29) or protein basis (Fig. 30), underwent considerable changes during the growth cycle. It can be seen by comparison of Fig. 23 and Fig. 29, as well as Fig. 24 and Fig. 30, that the changes in A2 activity paralleled those of the total activity. When calculated in a similar manner the activity of isoenzymes D2 and B followed a different pattern of change (Fig. 31). Isoenzyme D2, in plus cultures, was only prominent on day two and four during the time of lowest total activity (Fig. 23, 24 and 25) while isoenzyme B showed a different pattern in that percent activity was constant during the central portion of the growth cycle (Fig. 31). From these observations, and reference to Table 1, it can be seen that the isoenzymes that contributed larger portions of the total peroxidase activity generally changed as did the total activity but isoenzymes that overall, contributed less to the total activity changed more dramatically and at more specific times during the culture cycle.

Differences in quantitative changes between plus and minus cultures are also evident from Figs. 26-31 and Table 1 and 2. The most prominent difference in activity that resulted from transfer to minus medium was the immediate increase in activity of isoenzymes A1 and A2

Fig. 26. Diagrammatic representation of the patterns of activity of guaiacol peroxidase isoenzymes from cultures grown with and without growth regulators (plus and minus). Numbers at the top of the gels denote the day of sampling. Day 12 represents the point of reinoculation into fresh medium. The amount of activity is represented by band intensity calculated, as the average of at least three separate experiments.

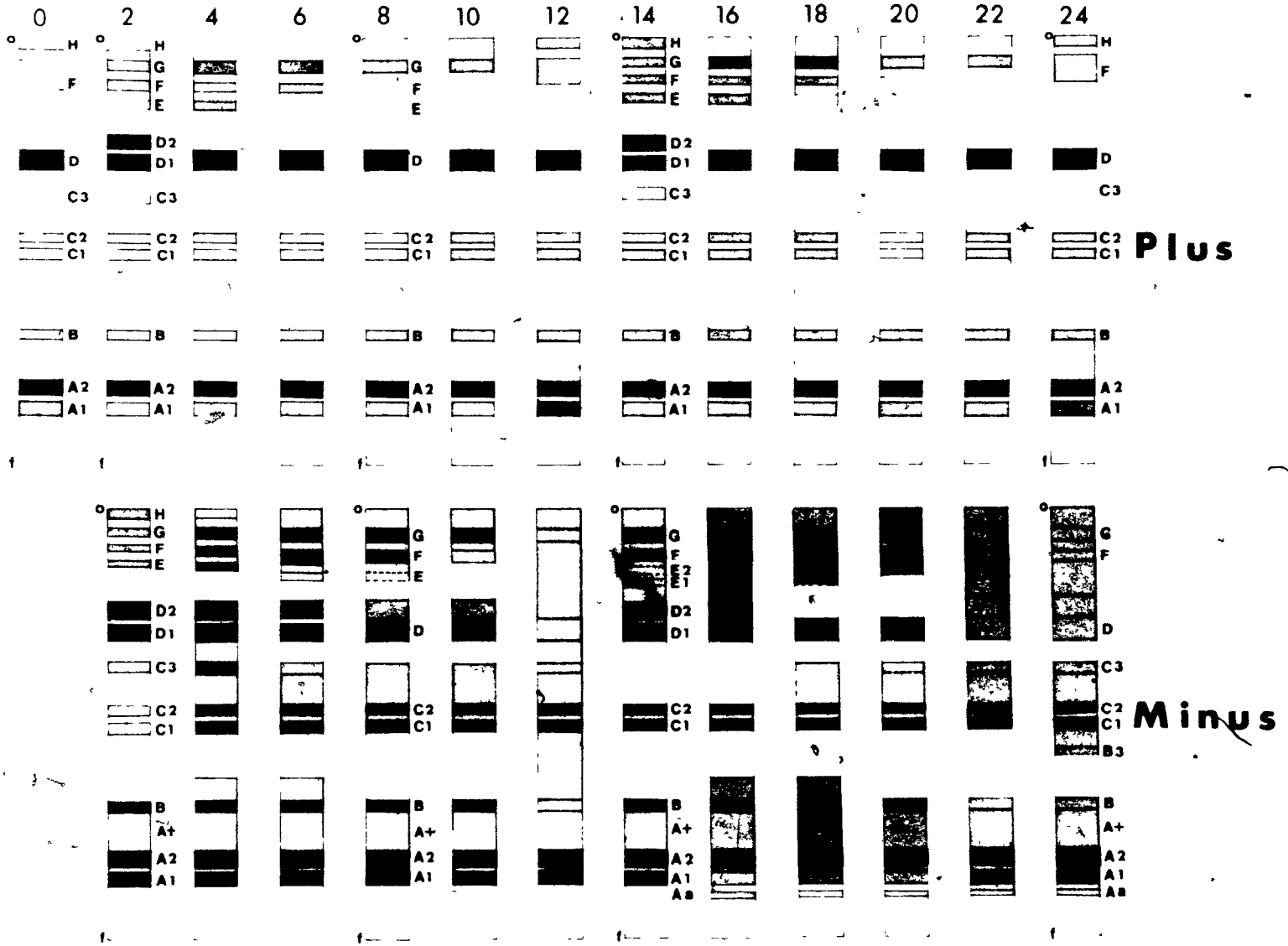

0
2
4
6
8
10
12
14
16
18
20
22
24
H
G
F
E
D2
D1
D
C3
C2
C1
B
A2
A1
A+
B3
Plus
Minus

Fig. 27. Tracings of peroxidase gel scans from cycle 126. Plus culture scans are represented with a solid line and minus culture scans with a dashed line.

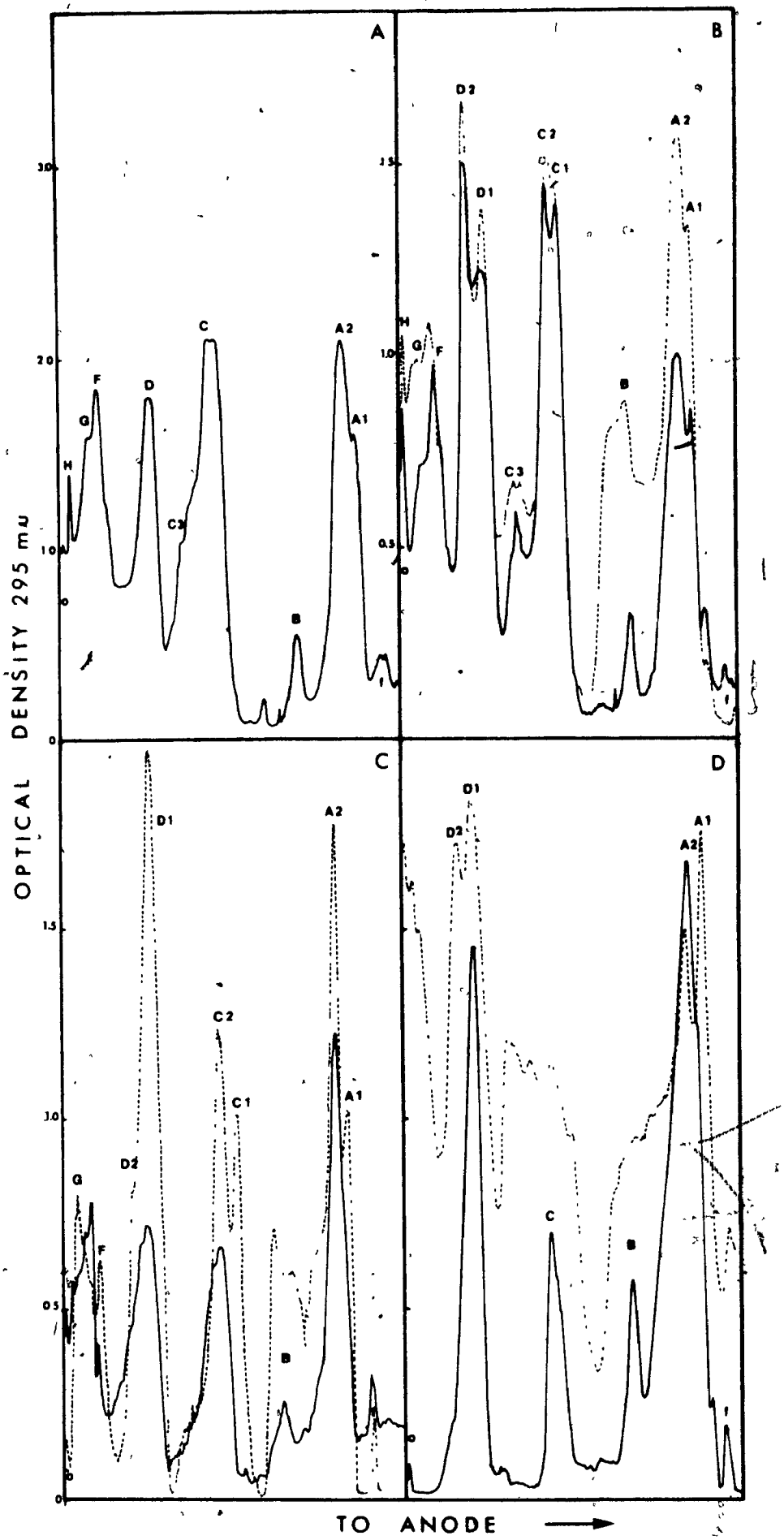
A.* Peroxidase isoenzymes of inoculum - 12 day passage 125.

B. Peroxidase isoenzymes 2 day passage 126.

C. Peroxidase isoenzymes 6 day passage 126.

D. Peroxidase isoenzymes 8 day passage 126.

* note A drawn 1/2 scale of B, C and D.


A
B
C
D
OPTICAL DENSITY 295 mu
TO ANODE
H
G
F
D
C3
C
B
A2
A1
D2
D1
C2
C1

and the activity designated A+ (Fig. 26 and Fig. 27-B). By day 6 there were considerably higher levels of D1, D2, C1 and C2 in the minus cultures. However, by day 12, at the end of the first culture passage, in contrast to the plus cultures, isoenzyme D1 had decreased considerably while isoenzymes C1-C2 had continued to increase (Fig. 26). During the second culture passage without growth regulators, high levels of D1 and D2 were again restored as in the plus cultures but as in the previous passage these activities were seen to decline to low levels by the completion of the cycle on day 24. The activities of isoenzymes C1 and C2 remained high in minus cultures through the second culture cycle while isoenzymes A1 and A2 continued to increase in activity. The most prominent difference between plus and minus cultures, by day 24, was the very low level of isoenzyme D2 in the minus cultures. Experiments in which extracts were treated with β-galactosidase indicated that peroxidase A2 and D2 are related in that β-galactosidase treatment resulted in a decrease of D2 and a concomitant increase in A2 activity.

The change in activity of the individual isoenzyme A2 is presented for minus cultures in Fig. 28-30. In contrast with the plus cultures the percent activity of A2 decreased during days 6-10 in the minus cultures. This was due in part to the persistence of isoenzyme D2 and the increase of isoenzyme C2 during this period. The actual levels of A2 as seen by density scans or measured per gram fresh weight were more or less equivalent however in plus and minus cultures (Fig. 29). The dramatic increase in D2 activity found in minus cultures (day 4-10) is shown in Fig. 31. The significant increase in activity of isoenzyme B in minus cultures as compared with plus cultures, during days 2 and 4

is probably due to the increase in absorbance on the gel scans due to the activity designated A+ (see Fig. 27). With increasing time in minus culture the scans obtained from the gels decreased in resolution due to smears of activity such as A+ (i.e. Fig. 27-D). This smearing made it difficult to calculate the activity actually contributed by the isoenzyme peaks. Therefore, it is possible that calculations of the activity contributed by the individual isoenzymes in the minus cultures may be somewhat imprecise. The percent activity of all the individual isoenzymes is presented in a tabular form in Table 2.

The substrate specificity of the peroxidase isoenzymes was tested by substitution of alternate enzyme substrates. No marked differences in isoenzyme pattern or staining activity were observed with benzidine as substrate, however, in comparison staining with chlorogenic acid and caffeic acid was much reduced. None of the isoenzymes showed any prominent activity towards either of these two substrates. Gels stained for IAA oxidase activity showed some low activity in the region of isoenzyme D2, C1 and C2 only. Isoenzymes stained for tyrosinase activity showed considerable activity in the region of A1-A2 and slight activity associated with isoenzymes B and D1. Tyrosinase activity was detected by the method of Jolley and Mason (1965).

Fig. 28. Changes in activity of peroxidase isoenzyme A2 expressed as percent of total peroxidase activity from cultures grown with and without growth regulators (plus and minus). Activity was calculated from areas on gel tracings.

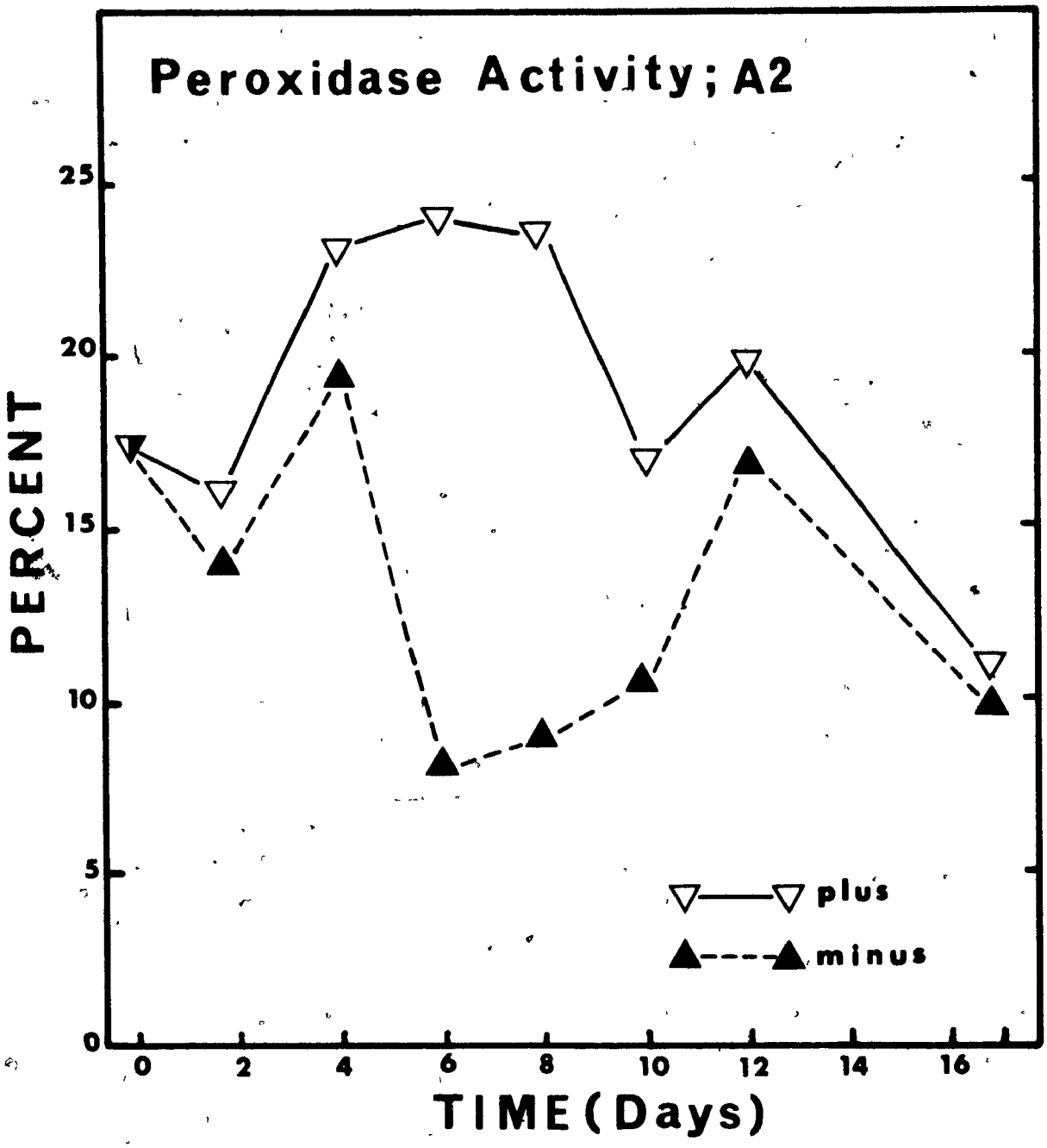

Peroxidase Activity; A2
PERCENT
25
20
15
10
5
0
0
2
4
6
8
10
12
14
16
TIME (Days)
plus
minus

Fig. 29. Changes in activity of peroxidase isoenzyme A2 expressed per gram fresh weight from cultures grown with and without growth regulators (plus and minus).

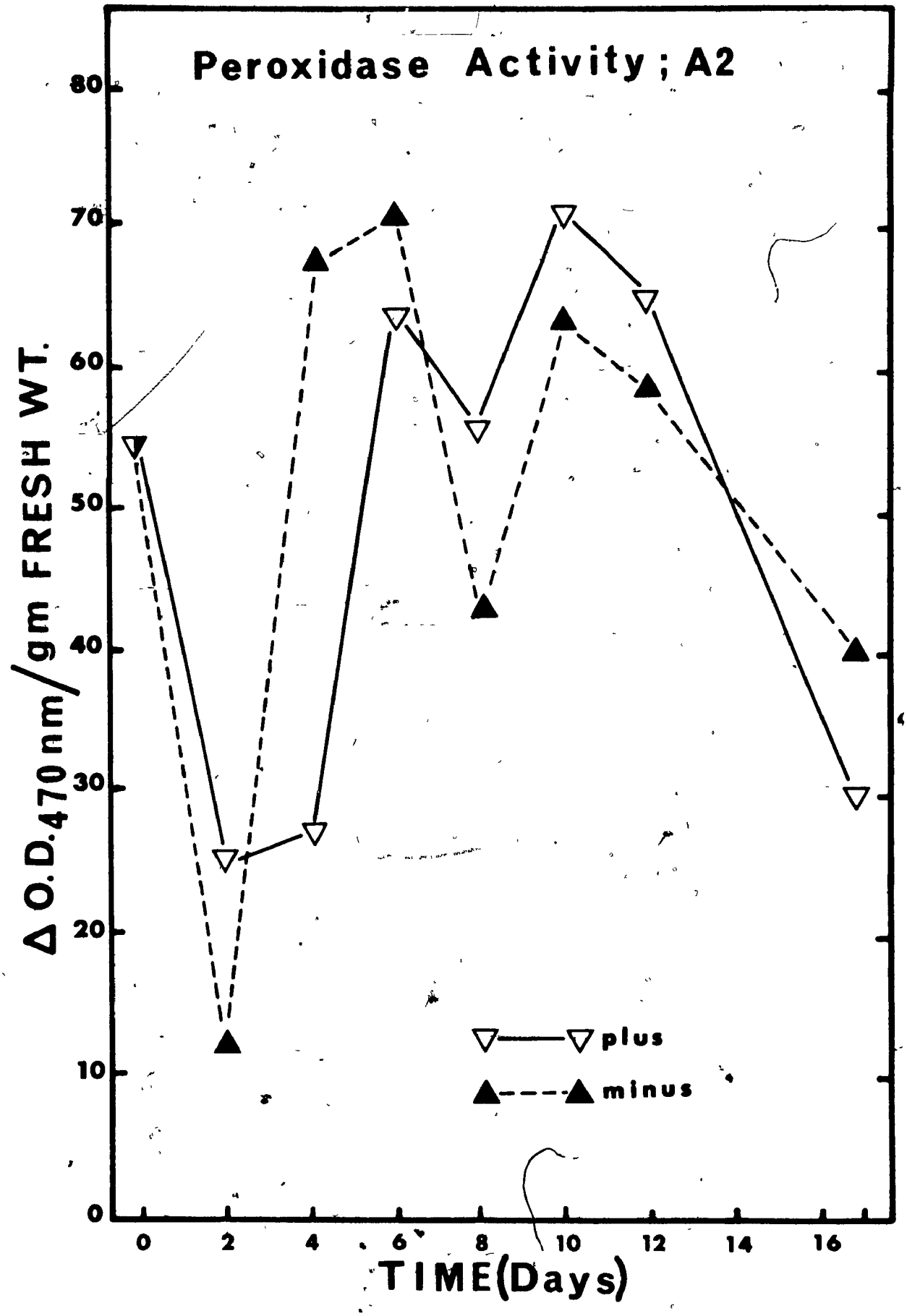

Peroxidase Activity ; A2
80
70
60
50
40
30
20
10
0
Δ O.D.470 nm/gm FRESH WT.
0
2
4
6
8
10
12
14
16
TIME(Days)
plus
minus

Fig. 30. Changes in activity of peroxidase isoenzyme A2 expressed per mg extract protein from cultures grown with and without growth regulators, (plus and minus).

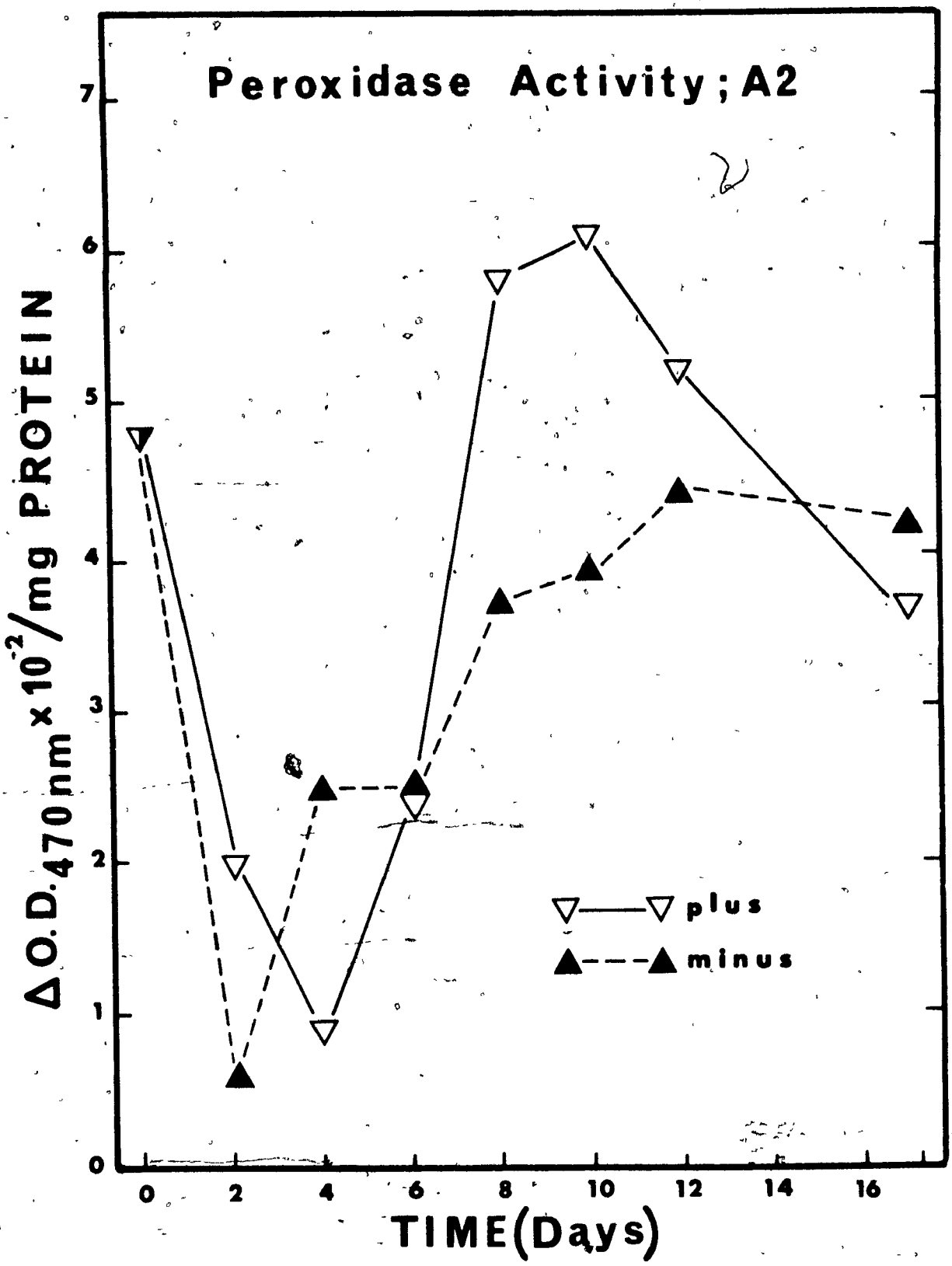

Peroxidase Activity; A2
ΔO.D.470nm x10-2/mg PROTEIN
TIME(Days)
plus
minus
0
1
2
3
4
5
6
7
0
2
4
6
8
10
12
14
16

Fig. 31. Changes in activity of peroxidase isoenzymes D2 and B expressed as the percent of the total peroxidase activity from cultures grown with and without growth regulators (plus and minus).

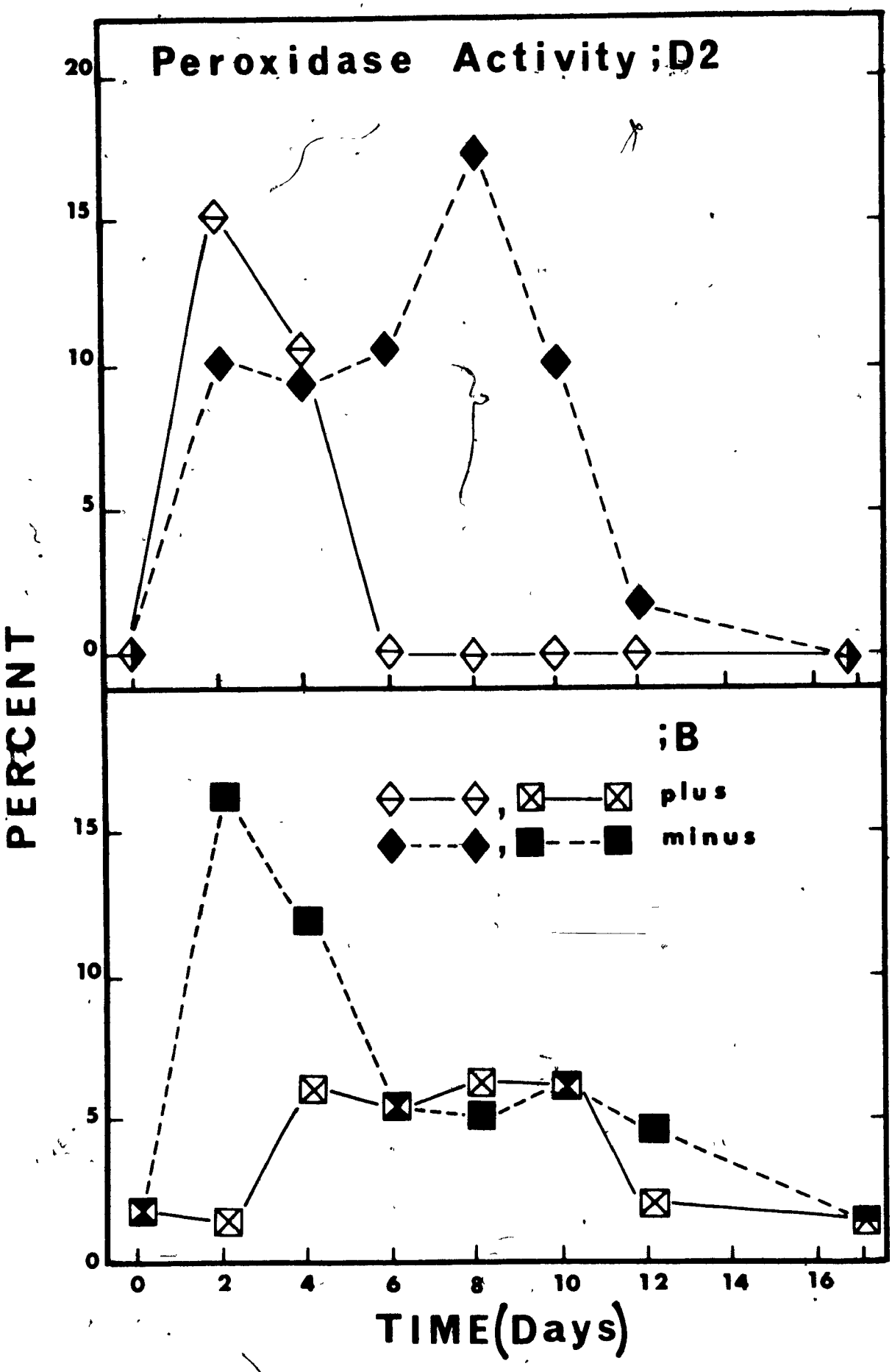

Peroxidase Activity ;D2
20
15
10
5
0
;B
plus
minus
PERCENT
15
10
5
0
0
2
4
6
8
10
12
14
16
TIME(Days)

Table 1

Percent of the total peroxidase activity contributed by the individual isoenzymes; Plus cultures passage 125-126.

Isoenzyme	Day 0	Day 2	Day 4	Day 6	Day 8	Day 10	Day 12
A1	6.5	4.0	3.5	5.5	3.0	5.0	9.0
A2	17.5	16.0	23.0	24.0	24.0	16.5	20.0
A+	2.0	-	-	-	-	5.5	4.0
B	2.0	1.5	6.5	6.0	6.5	6.5	2.0
C1	12.0	12.0	11.5	6.0	2.0	4.0	8.0
C2	12.0	14.5	10.0	9.0	8.5	12.0	13.0
C3	4.0	6.0	4.5	0.0	0.0	4.5	4.0
D1	16.0	9.0	16.0	18.0	28.0	24.0	23.0
D2	0.0	15.0	10.0	-	0.0	0.0	0.0
E	0.0	2.0	2.0	-	-	0.0	0.0
F	5.0	9.0	5.0	3.0	11.0	3.0	2.0
G	4.5	5.0	2.5	7.0	8.0	6.0	2.0
H	3.0	3.0	-	-	-	3.0	6.0
Other	15.5	3.0	5.5	21.5	9.0	10.0	7.0

Table 2

Percent of the total peroxidase activity contributed by the individual isoenzymes; Minus cultures passage 125-126.

Isoenzyme	Day 0	Day 2	Day 4	Day 6	Day 8	Day 10	Day 12
A1	6.5	5.0	3.5	4.0	5.0	4.0	7.0
A2	17.5	14.0	19.5	7.5	9.0	10.5	16.5
A+	2.0	4.0	5.5	15.0	6.5	6.5	8.0
B	2.0	16.0	11.0	5.5	5.0	6.5	5.0
C1	12.0	5.0	7.0	6.5	3.5	6.0	8.0
C2	12.0	11.5	12.0	10.0	9.5	10.5	8.0
C3	4.0	4.0	1.0	3.0	3.0	6.0	6.0
D1	16.0	7.0	22.0	9.0	18.0	24.0	15.0
D2	0.0	10.0	8.0	10.5	17.5	10.0	2.0
E	0.0	5.0	-	0.0	0.0	0.0	0.0
F	5.0	7.5	2.5	3.0	4.5	2.0	1.0
G	4.5	4.5	4.0	12.0	5.5	5.5	1.0
H	3.0	2.0	-	-	-	-	-
Other	15.5	4.5	3.0	14.0	13.0	8.5	20.5

d) Peroxidase (polyphenolic)

Peroxidase (polyphenolic) activity as measured per gram fresh weight and per mg protein extract are presented in Fig. 32 and 33. The PP activity curves were similar to those of peroxidase in that the levels of activity dropped markedly with subculture. During the first passage the increase in PP activity in plus cultures was gradual and in contrast to peroxidase only reached maximal levels by day 12 (Fig. 33). The increase of PP activities in minus cultures, however, paralleled that of peroxidase exactly (compare Fig. 23 and 32 and Fig. 24 and 33). The PP of plus cultures during the second passage inoculated with a lower number of cells, was, as was the case with peroxidase, lower in activity reaching the maximum on day 24. Minus cultures on the other hand, were found to have strikingly high PP activity. This activity was highest on day 18 when the cultures were found to contain many highly elongate and thickened cells.

Changes in PP activity with time are presented in Fig. 34 and 35. The PP isoenzymes with the possible exception of E and F also showed peroxidase activity. In contrast with peroxidase, changes during the culture cycle, and differences between plus and minus cultures did not involve differences in pattern although quantitative effects are evident. Most dramatic of the changes was the striking increase after subculture of isoenzyme B in the plus cultures and of isoenzymes E and F in the minus cultures (Fig. 34 and 35). The main difference between plus and minus cultures was the increase in the activity of isoenzyme A2 and the relative decrease of isoenzymes E and F in the minus cultures during the second passage (Fig. 34).

Fig. 32. Changes in activity of PP per gram fresh weight for cultures grown with and without growth regulators (plus and minus) during two successive culture cycles. Day 12 represents the point of reinoculation into fresh medium. Inocula as in Fig. 23.

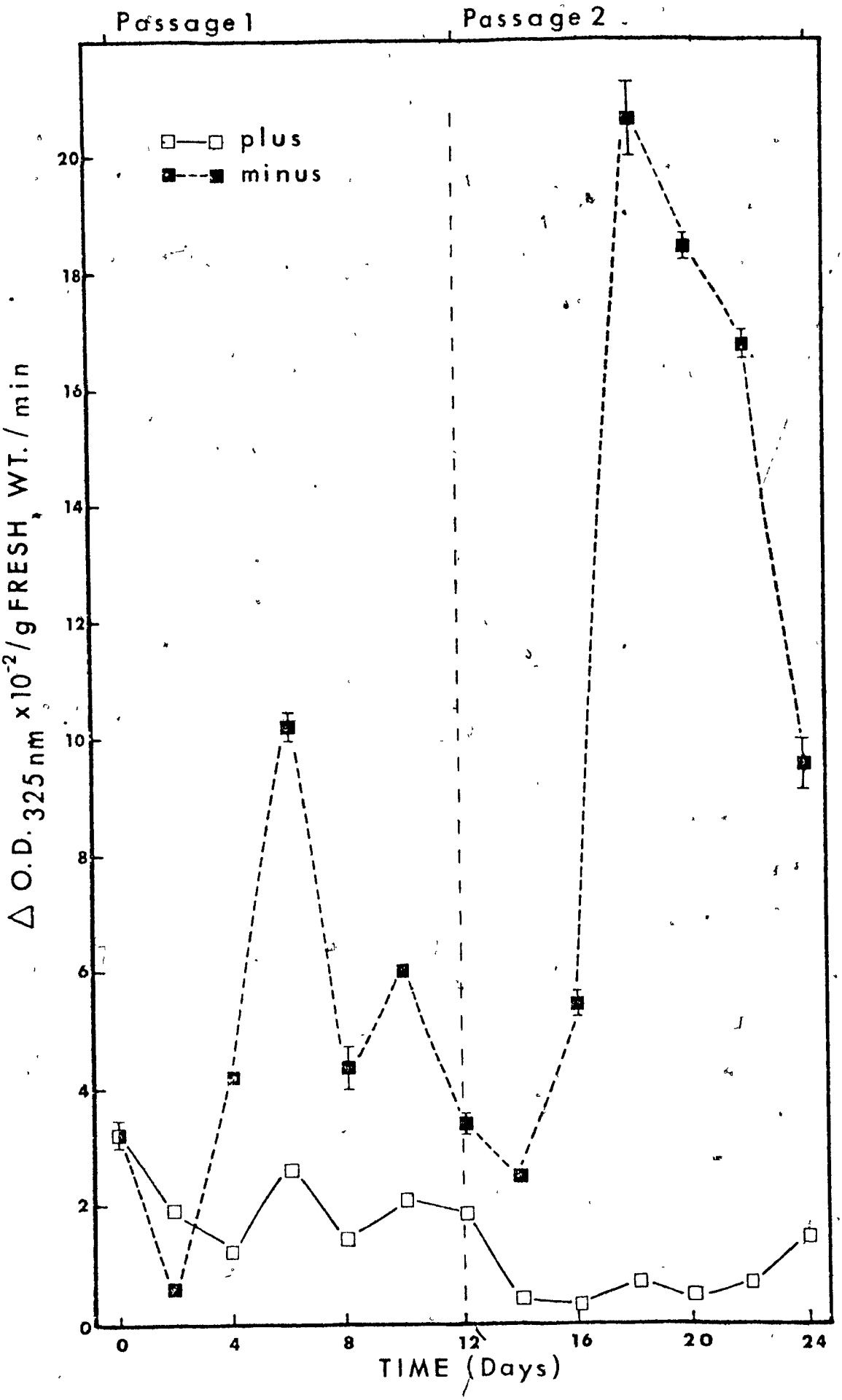

Passage 1
Passage 2
plus
minus
Δ O.D. 325 nm x10⁻²/g FRESH WT./min
TIME (Days)
0
2
4
6
8
10
12
14
16
18
20
0
4
8
12
16
20
24

Fig. 33. As for Fig. 32 but activity expressed per mg extract protein.

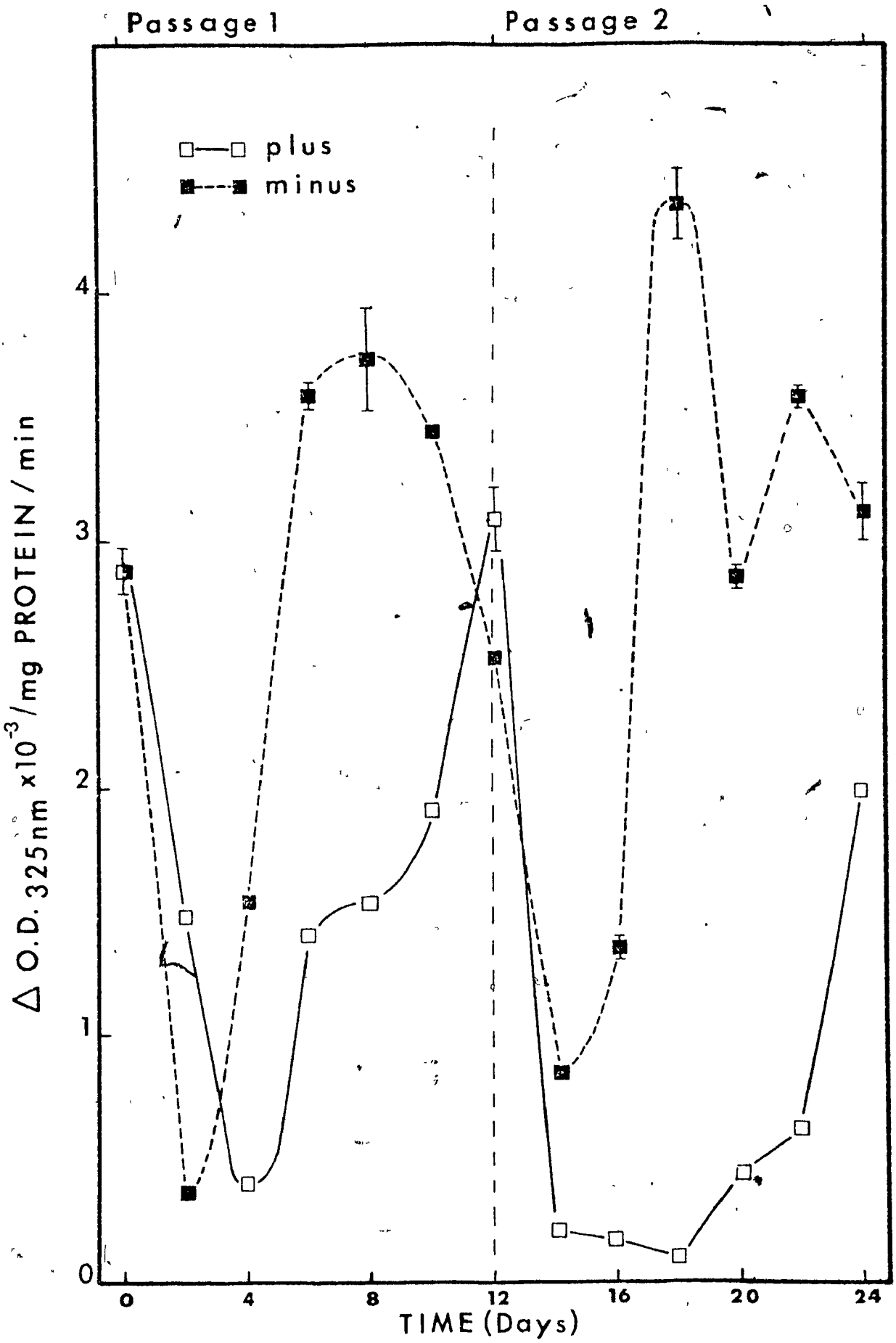

Passage 1
Passage 2
plus
minus
Δ O.D. 325nm x10^{-3}/mg PROTEIN / min
0
1
2
3
4
0
4
8
12
16
20
24
TIME (Days)

Fig. 34. Diagrammatic representation of the patterns of activity of PP isoenzymes from cultures grown with and without growth regulators (plus and minus). Numbers at the top of the gels denote the day of sampling. Day 12 represents the point of reinoculation into fresh medium. The amount of activity is represented by band intensity calculated as the average of at least three separate experiments.

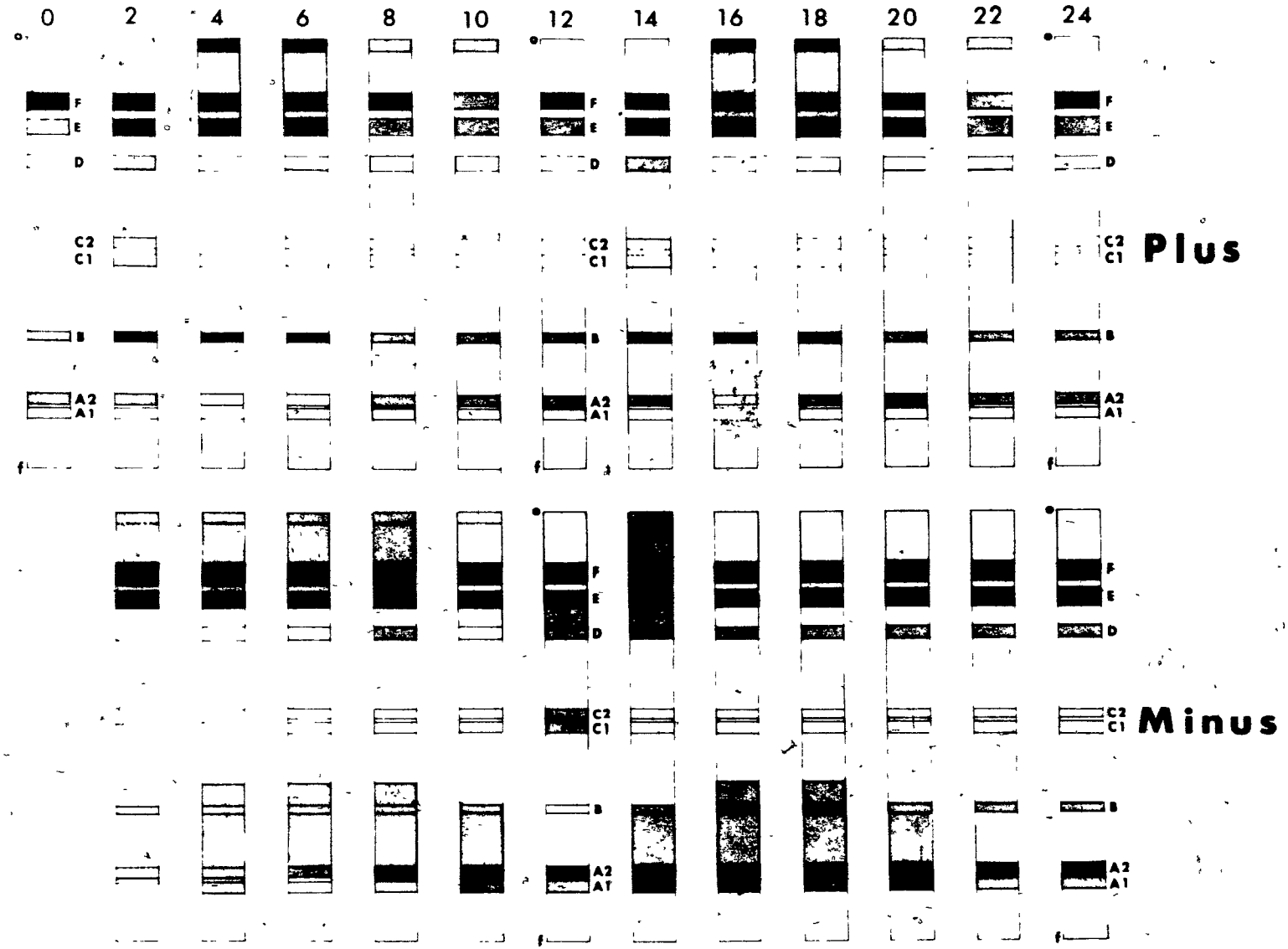

0
2
4
6
8
10
12
14
16
18
20
22
24
F
E
D
C2
C1
B
A2
A1
Plus
Minus

Fig. 35. Tracings of PP gel scans. Scans for plus cultures are drawn with a solid line and minus culture scans with a dashed line.

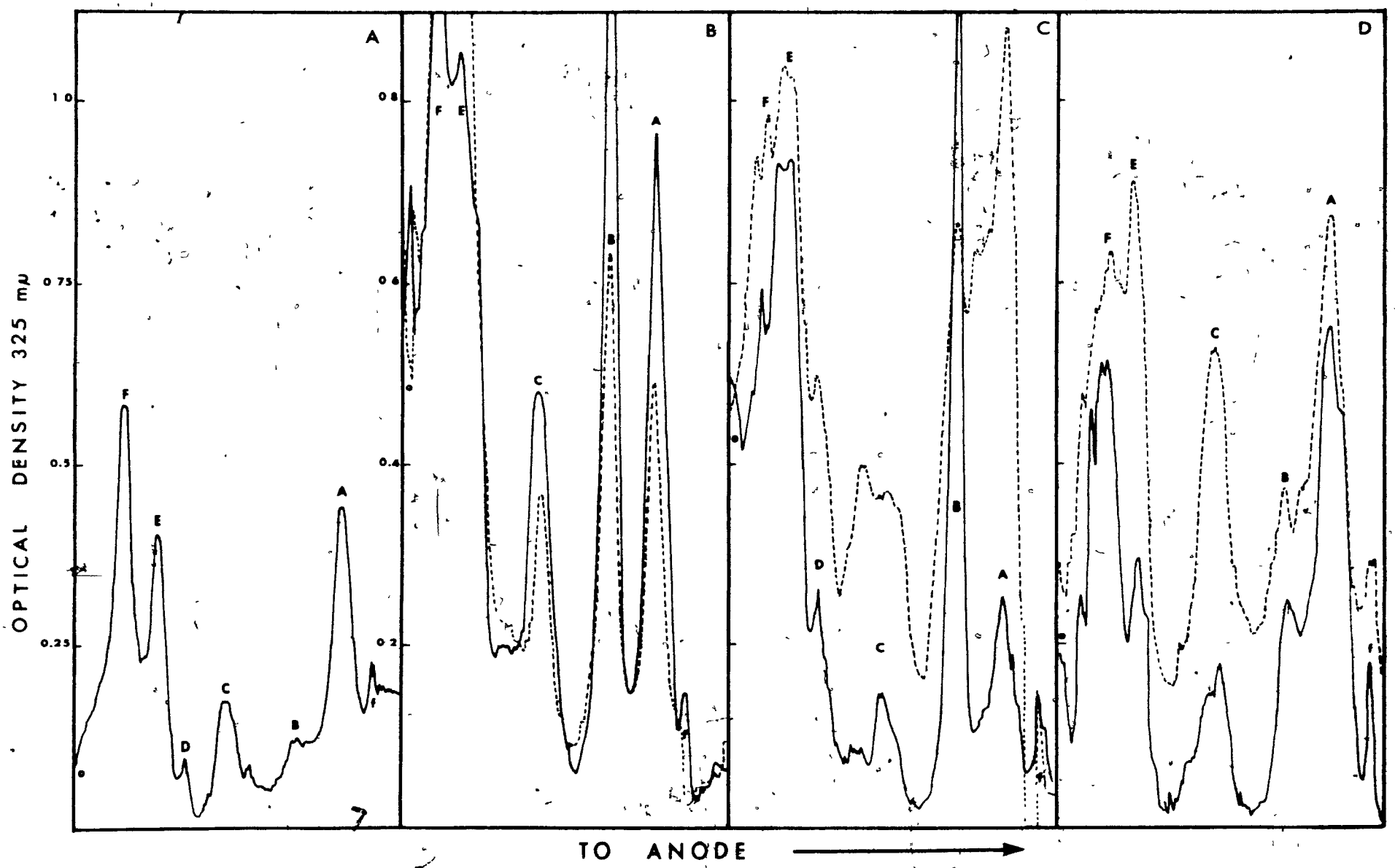
A.* PP isoenzymes 12 day 125 - inoculum.

B. PP isoenzymes 2 day 126.

C. PP isoenzymes 6 day 126.

D. PP isoenzymes 12 day 126.

* note section A drawn to 80% scale.


A
B
C
D
F
E
D
C
B
A
1.0
0.75
0.5
0.25
0.8
0.6
0.4
0.2
OPTICAL DENSITY 325 mμ
TO ANODE

e) Peroxidase Isoenzymes and Activity Detected In The Medium

Peroxidase and PP activity measured in the medium in which plus and minus cells were grown is presented in Fig. 36. Activity was low or nonexistent in the medium immediately after subculture but was seen to rise as soon as cell fresh weight increased. Cultures grown without 2,4-D and kinetin initially grow faster as shown by increase in fresh weight and showed higher levels of medium peroxidase and PP activity. However, by day 6 plus cultures had increased more in fresh weight and had secreted more peroxidase activity into the medium. Measurements of activity in passage two confirm that the occurrence and increase of peroxidase and PP in the medium was correlated with onset and increase in fresh weight due to cell expansion.

The isoenzyme patterns of peroxidase present in the medium and the changes that took place in these activities during the culture cycle, are presented in Fig. 37 and 38. The isoenzyme pattern of PP was identical to that of peroxidase and therefore is not illustrated. The electrophoretic mobilities of isoenzymes present in the medium were not the same as those of the cytoplasmic isoenzymes with the exception of A1-A2. As a consequence the medium isoenzymes were designated with different letters to avoid confusion. Figures 37 and 38 show that after subculture the first activity that appeared was isoenzyme V in both plus and minus cultures. Subsequently by day 6 W, Z1 and A2 were present in plus medium the activity of which, except for Z increased to maximal levels by day 12. Minus cultures differed from plus cultures in that initially there was a greater level of isoenzyme V, (Fig. 38-B) which however did not increase in activity after day 4. Minus cultures showed a much more rapid increase

in isoenzymes A2 and U (Fig. 38-C) and the presence of a previously undetected isoenzyme T (Fig. 37). By the end of the first culture passage the pattern of isoenzymes from minus medium was quite different from that for plus medium in that isoenzymes A2 and U were prominent and V and W were much reduced (Fig. 38-D). Initially during the second passage without growth regulators, day 12-24, very little activity was present in minus medium although the intracellular levels were extremely high at this time (Fig. 25, 35). With the onset of some growth, peroxidase activity at day 18 was found in the minus medium and, as in the first passage, showed a predominance of isoenzymes A2 and U(Fig. 37 and 38-E).

Fig. 36. Peroxidase and PP activity detected in the medium of cultures grown with and without growth regulators (plus and minus). Activity is expressed per ml of medium and a growth curve expressed as the increase in culture fresh weight is included for comparison. Day 12 represents the point of reinoculation into fresh medium.

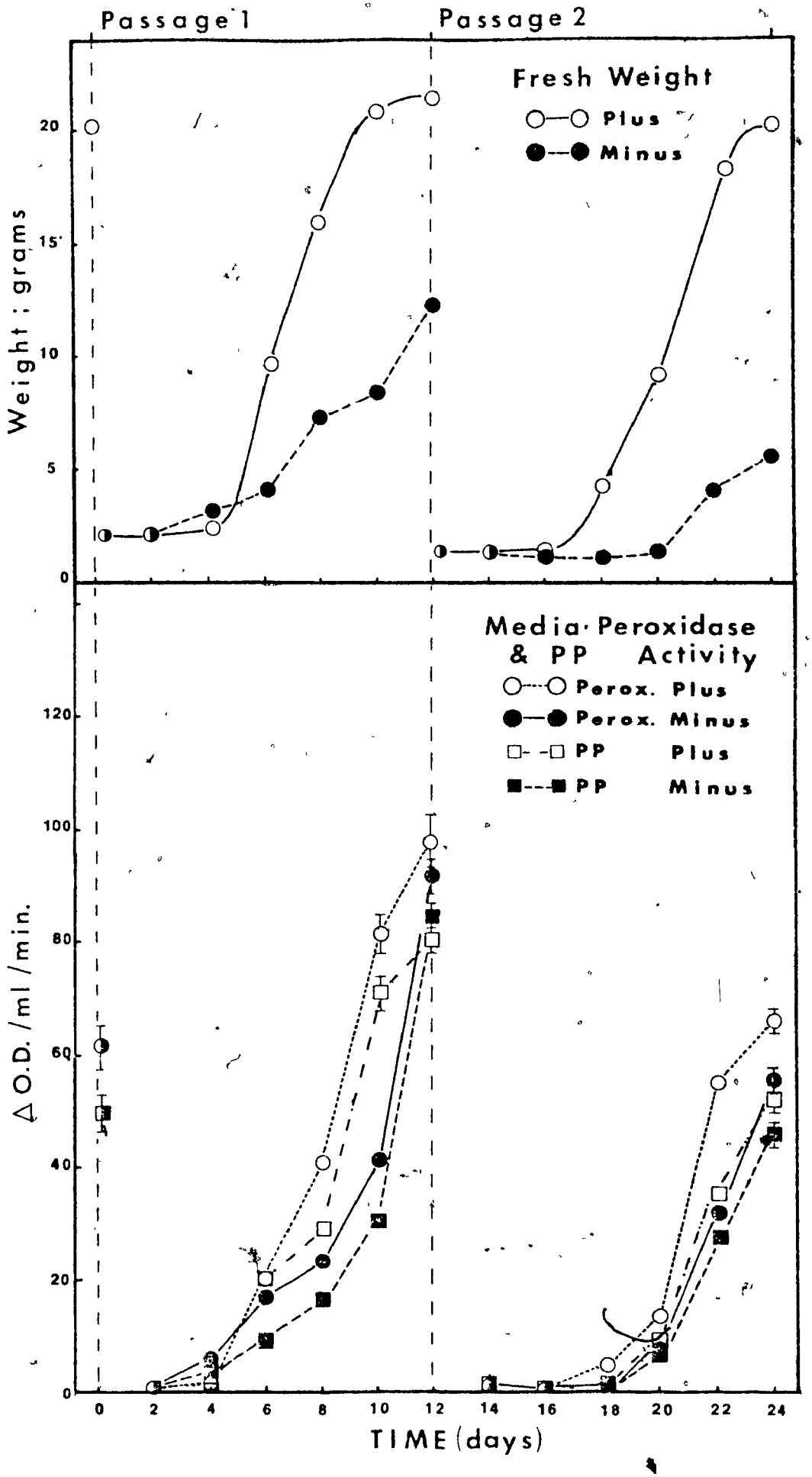

Passage 1
Passage 2
Fresh Weight
Plus
Minus
Weight ; grams
0
5
10
15
20
Media· Peroxidase & PP Activity
Perox. Plus
Perox. Minus
PP Plus
PP Minus
Δ O.D. /ml /min.
0
20
40
60
80
100
120
0
2
4
6
8
10
12
14
16
18
20
22
24
TIME (days)

Fig. 37. Diagrammatic representation of the patterns of activity of peroxidase isoenzymes detected in the medium from cultures grown with and without growth regulators (plus and minus). Numbers at the top of the gels denote the day of the experimental period. Day 12 represents the point of reinoculation into fresh medium. The amount of activity is represented by band intensity calculated as the average of at least three separate experiments.

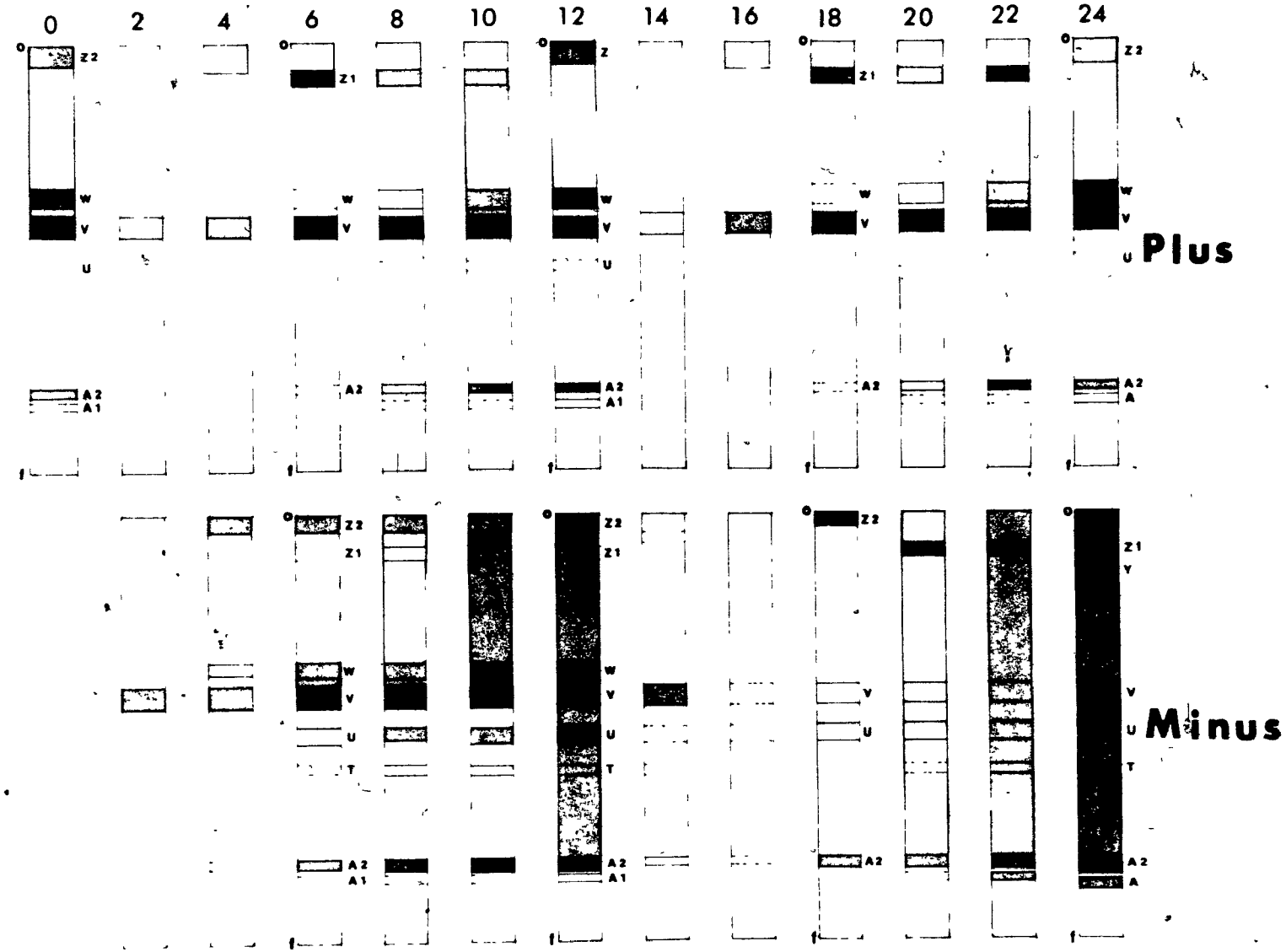

0
2
4
6
8
10
12
14
16
18
20
22
24
Plus
Minus

Fig. 38. Tracings of gel scans for media peroxidase. Scans for plus cultures are drawn with a solid line and minus culture scans with a dashed line.

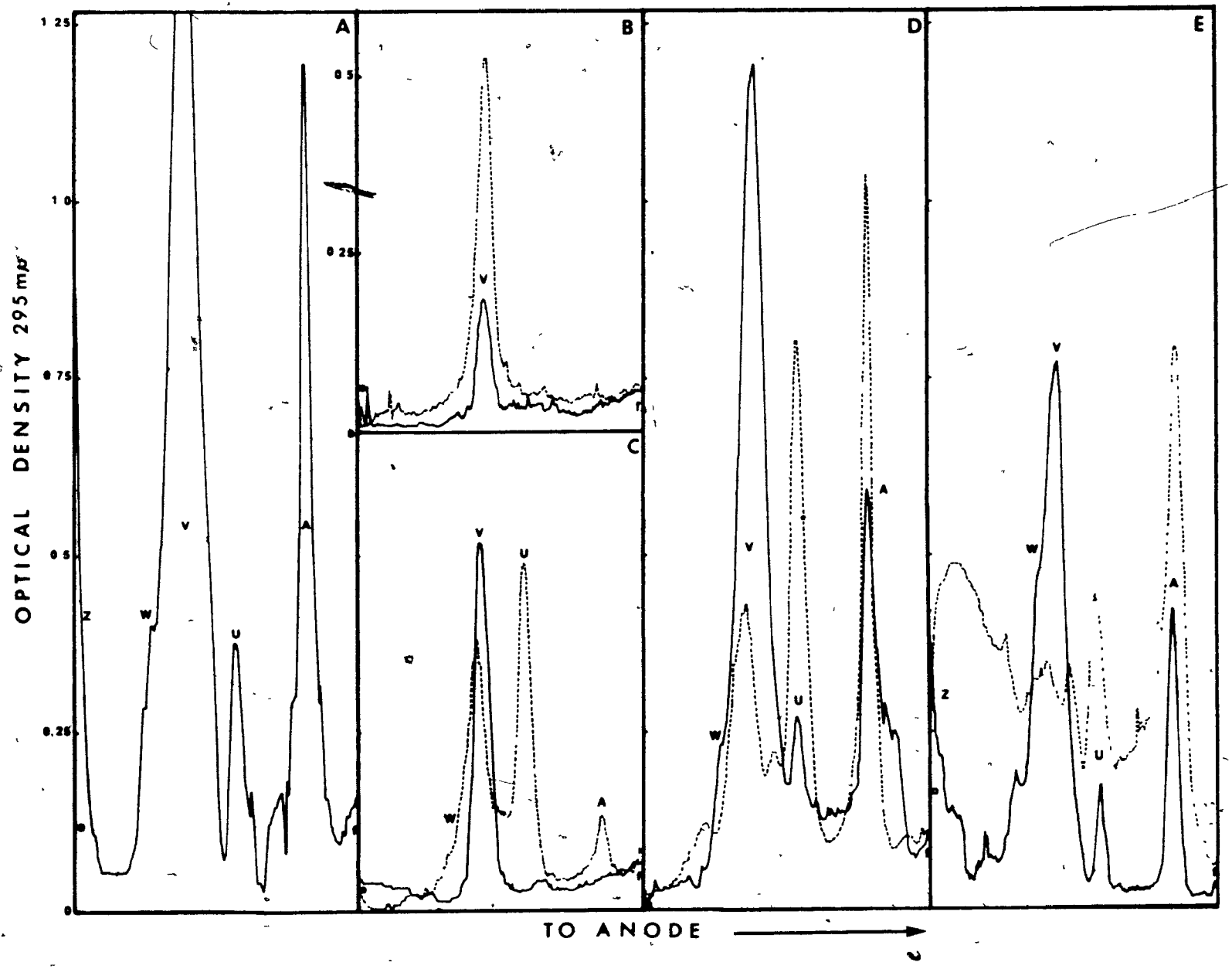
A. Peroxidase isoenzymes from 12 day 125 medium.

B. Peroxidase isoenzymes from 2 day 126 medium.

C. Peroxidase isoenzymes from 6 day 126 medium.

D. Peroxidase isoenzymes from 10 day 126 medium.

E. Peroxidase isoenzymes from 10 day 127 medium.


A
B
C
D
E
OPTICAL DENSITY 295 mμ
1.25
1.0
0.75
0.5
0.25
0
0.5
0.25
0
Z
W
V
U
A
TO ANODE

5) Acid Phosphatase

Acid phosphatase activity expressed per mg soluble protein and per gram fresh weight is shown in Fig. 39 and 40 respectively. The activity is shown for cells grown with and without growth regulators over a two passage period of twenty-four days. Acid phosphatase activity calculated per mg soluble protein showed a sharp drop with the inoculation of cells into fresh medium although the levels of soluble proteins increased at this time (see Fig. 25). AP activity then increased during the culture cycle to reach highest levels by day 12 and 24. AP activity expressed per fresh weight (Fig. 40) showed an initial decrease with inoculation into fresh medium but then rose sharply to reach a peak on day 6. The activity during the first passage followed essentially the same pattern in both the plus and minus cultures.

During the second passage plus cultures showed levels of activity equivalent with first passage values. Minus cultures showed considerably altered activity, however, the pattern of activity change during the culture cycle was similar to plus cultures but exaggerated on a fresh weight basis and much reduced on a per protein basis. This was due to the high levels of soluble protein in second passage minus cultures.

A comparatively small but significant amount of AP activity was detected in the medium in which cells had been grown (Fig. 40). The activity for plus cultures was seen generally to increase with duration of culture, although activity did not increase greatly after day 6. Minus culture medium contained equivalent amounts of AP activity until day 6

Fig. 39. Changes in activity of acid phosphatase per mg soluble protein for cultures grown with and without regulators (plus and minus) during two successive culture cycles (147-149). Day twelve represents the point of re-inoculation into fresh medium. The inoculum of plus cells in the first passage was 4.0 ml and 2.5 ml in the second passage. Bar markings represent standard errors. Where no markings occur the standard error was smaller than the size of the dot.

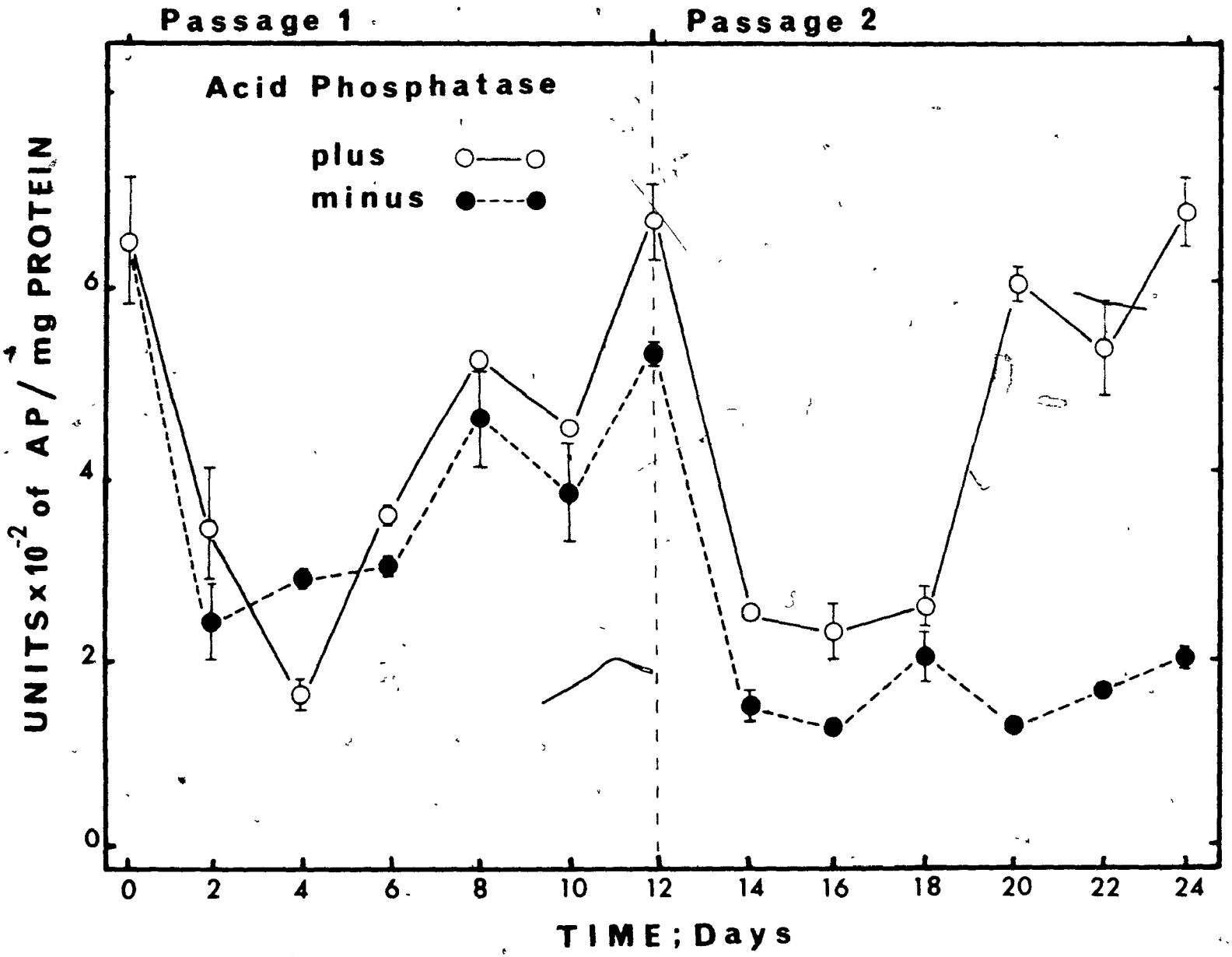
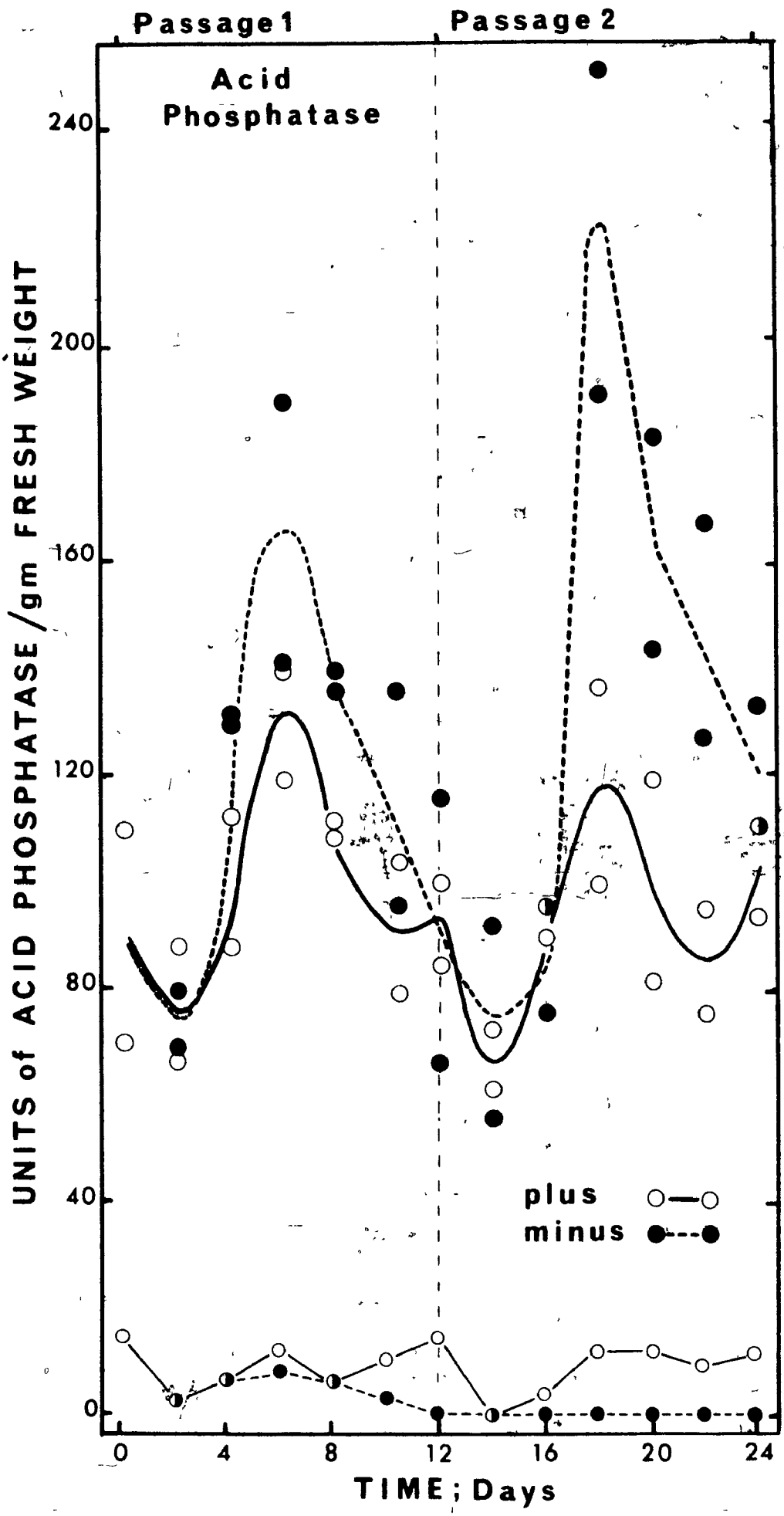

Passage 1
Passage 2
Acid Phosphatase
plus
minus
UNITS x 10^-2 of AP / mg PROTEIN
0
2
4
6
0
2
4
6
8
10
12
14
16
18
20
22
24
TIME; Days

Fig. 40. Changes in the activity of acid phosphatase per fresh weight of cultures grown with and without growth regulators (plus and minus) during two successive culture cycles (147-149). Acid phosphatase activity detected in the medium of plus and minus cultures expressed per ml medium is represented by the smaller circles. Day twelve represents the point of re-inoculation into fresh medium. The inoculum of plus cells in the first passage was 4.0 ml and 2.5 ml in the second passage.


Passage 1
Passage 2
Acid Phosphatase
240
200
160
120
80
40
0
UNITS of ACID PHOSPHATASE /gm FRESH WEIGHT
plus
minus
0
4
8
12
16
20
24
TIME; Days

at which time there was a considerable decrease. During the second passage without regulators the AP activity detected in the medium was negligible at all times even though the intracellular levels were very high at this time.

Concomitant with measurements of medium AP activity measurements of medium pH were recorded (Fig. 41). During the first passage the pH of both plus and minus cultures was seen to decrease quickly to reach lowest levels on day 4 and then to increase rapidly from days 6-10 to reach maximal levels on day 12. During the second passage without growth regulators this pattern of change was not maintained. The pH of the minus culture medium underwe t little change from the original medium pH during the second passage.

Acid phosphatase isoenzyme patterns and selected gel scans are shown diagrammatically in Fig. 42 and 43 respectively. The most prominent isoenzyme of the inoculum cells was isoenzyme A1 (see Fig. 43). This activity and all others are drastically reduced by day 2 after inoculation into fresh medium in both the plus and minus cultures (Fig. 42). By day 6 considerable activity of most isoenzymes was restored and by day 8 the pattern and activity of plus cultures was essentially identical to that of the inoculum. Minus cultures differed from plus cultures in the greatly increased activity of isoenzymes D1-D2 and G by day 12 (Fig. 43-B). No unique AP isoenzymes were detected in minus culture extracts. With reinoculation into fresh medium on day 12 AP isoenzymes all but disappeared in both plus and minus cultures, but in the case of the minus cultures the isoenzyme patterns were not restored by day 20.

A comparatively small amount of AP activity in the form of isoenzyme A1-A2 was detected at the end of the second passage on day 24 (Fig. 42). The low levels of staining on gels corresponded to low levels of AP per mg soluble protein.

Fig. 41. Change in the pH of the medium for cultures grown with and without growth regulators (plus and minus) during two consecutive culture cycles. Day twelve represents the point of inoculation into fresh medium. The pH of the medium was titrated to 5.8 before autoclaving. Each small dot represents the average of five replicate cultures.

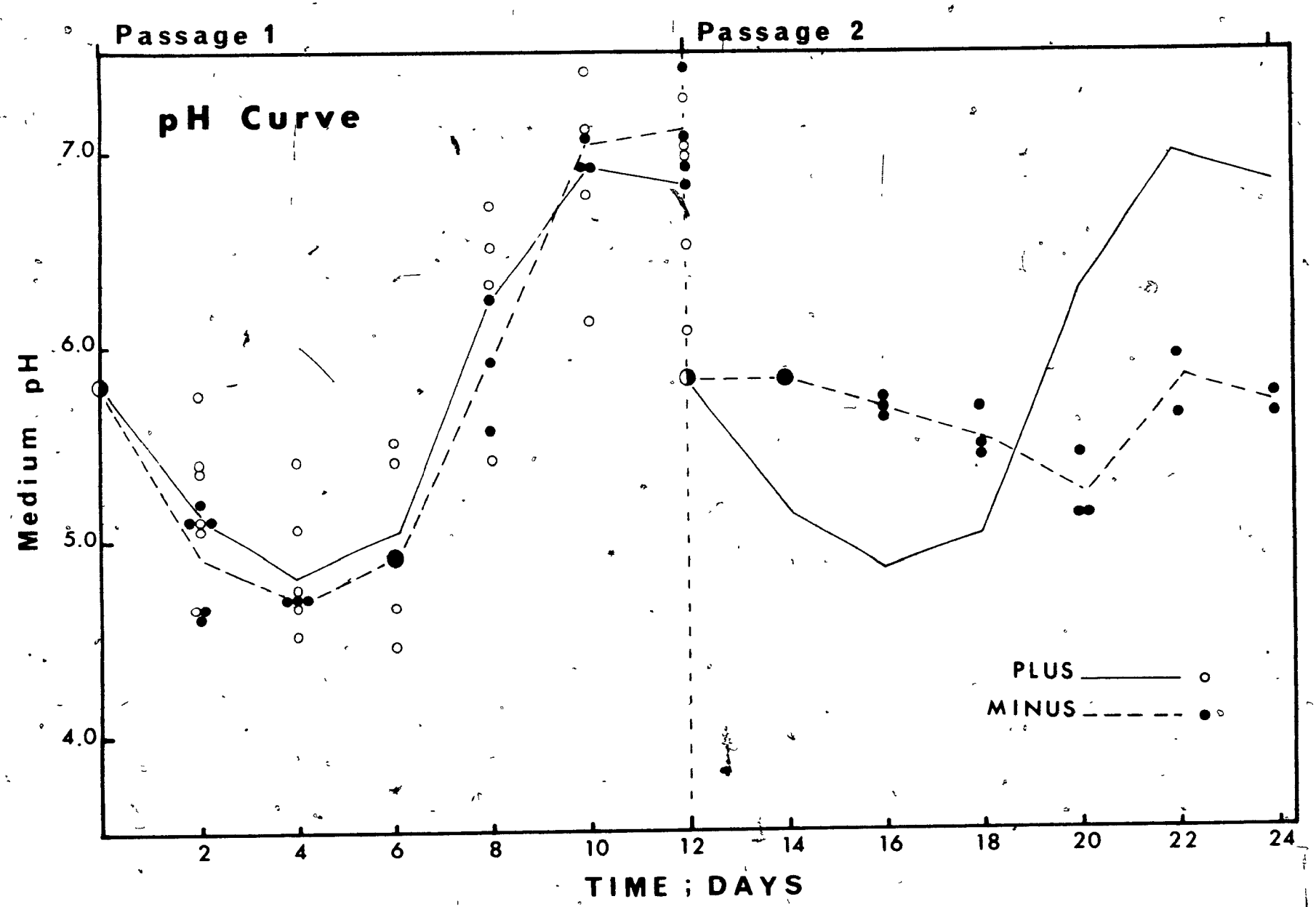

Passage 1
Passage 2
pH Curve
7.0
6.0
5.0
4.0
Medium pH
2
4
6
8
10
12
14
16
18
20
22
24
TIME ; DAYS
PLUS
MINUS

Fig. 42. Diagrammatic representation of the patterns of activity of AP isoenzymes from cultures grown with and without growth regulators (plus and minus) for two consecutive culture cycles. Numbers at the top of the gels denote the day of sampling. Day 12 represents the point of reinoculation into fresh medium. The amount of activity is represented by band intensity calculated as the average of at least three separate experiments.

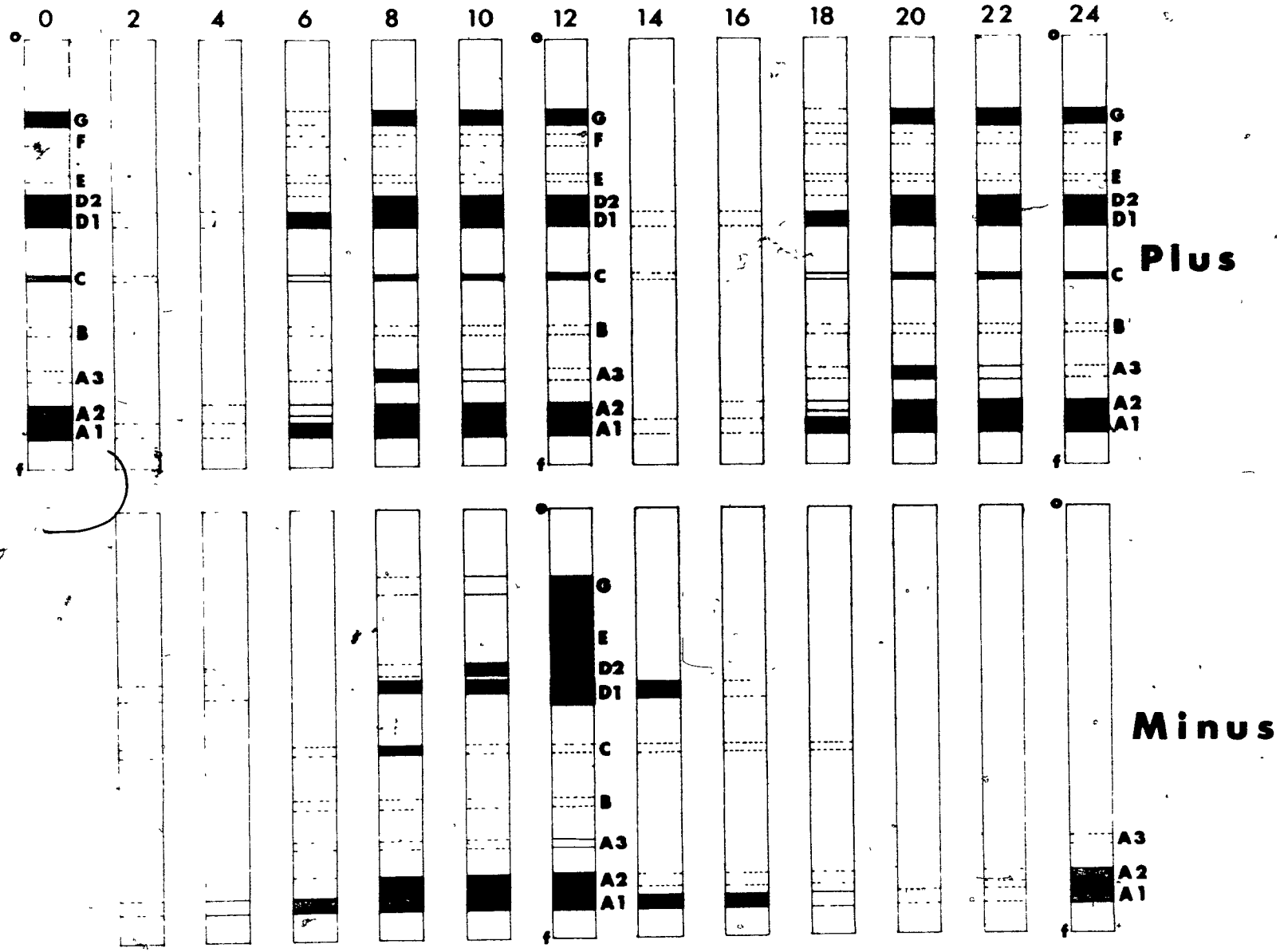
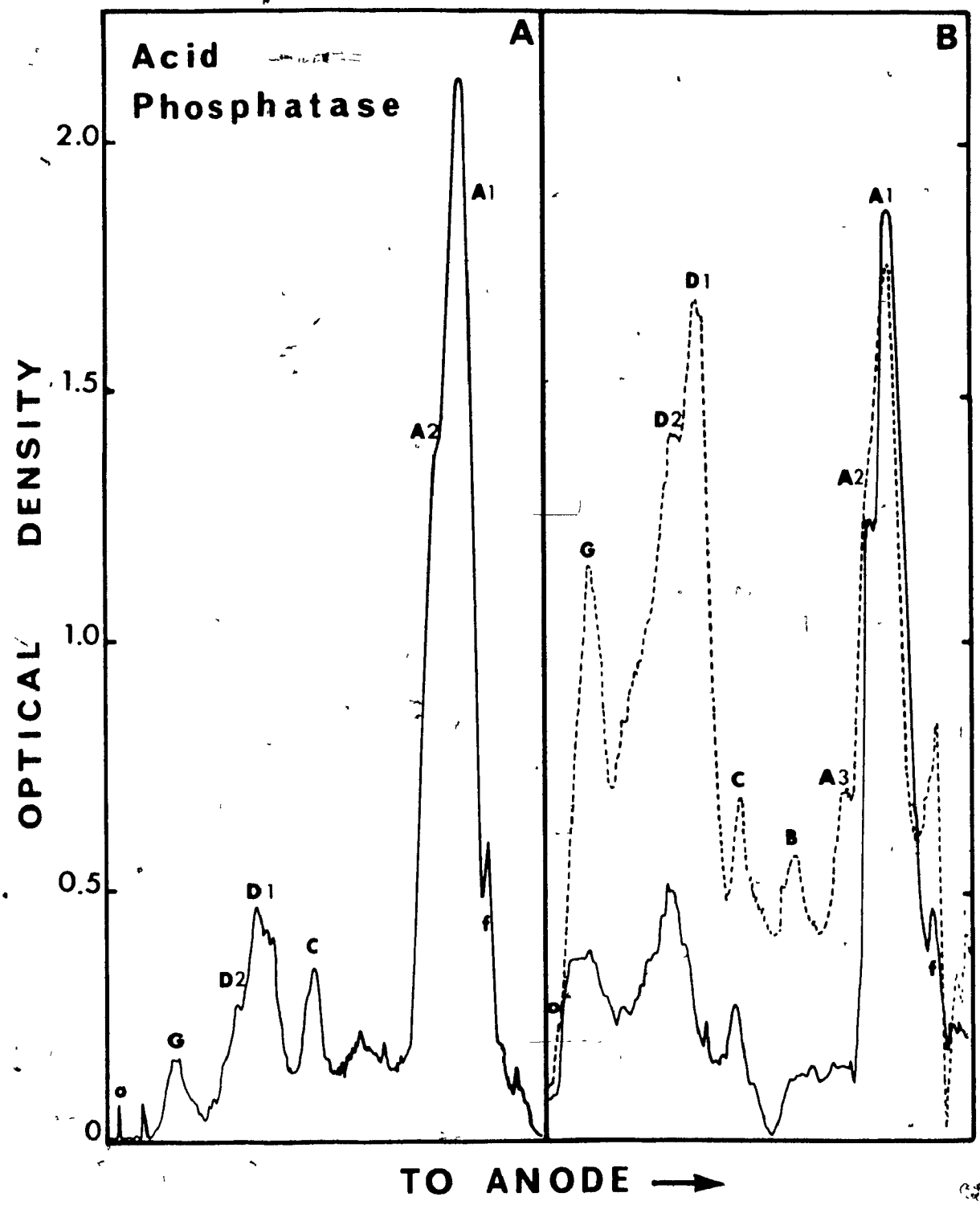

0
2
4
6
8
10
12
14
16
18
20
22
24
G
F
E
D2
D1
C
B
A3
A2
A1
Plus
Minus

Fig. 43. Tracings of AP gel scans. Scans for plus cultures are drawn with a solid line and minus culture scans with a dashed line.

A. inoculum 12 day cycle 125

B. 12 day cycle 126


Acid
Phosphatase
A
B
OPTICAL DENSITY
2.0
1.5
1.0
0.5
0
A1
A2
D1
D2
C
G
O
f
A3
B
TO ANODE

6) Leucine Aminopeptidase

The change in the activity of LAP expressed per mg soluble protein or per gram fresh weight is shown for cells grown with and without growth regulators in Fig. 44. The LAP activity of plus cells on a fresh weight basis increased to twice that of the inoculum by day 5 and then decreased to reach inoculum levels by day 8, whereas, LAP activity expressed per mg soluble protein showed only a slight peak on day 6-8.

The LAP activity of minus cultures expressed per mg soluble protein or per gm fresh weight decreased with inoculation into fresh medium and then increased sharply from day 4 to a maximum on day 8. LAP activity then decreased but by day 12 was maintained at higher levels than in plus cultures.

LAP isoenzyme patterns are shown diagrammatically in Fig. 45. The isoenzyme patterns did not change greatly during the culture cycle and thus are not illustrated extensively. The increased intensity of some bands and the occurrence of isoenzyme E not present in inoculum cells represented the only changes. The appearance of isoenzyme E may account for the increased LAP activity. The isoenzyme patterns of plus and minus cultures were essentially similar, however an elevated level of isoenzyme E was seen to persist in minus cultures.

During the second passage the activity and pattern of LAP isoenzymes was identical to that of the first passage. The minus cultures however, showed persistently much higher levels of LAP activity than plus cultures, but showed little fluctuation in activity. The isoenzyme pattern shown in Fig. 45-e was also maintained without significant

change from day 12 to 24. LAP activity was not detectable in the medium of plus or minus cultures at any time.

7) Glutamate-oxaloacetate Transaminase

Measurements of the total activity of GOT were not made but estimation of the activity from band intensity on stained gels indicated that activity in both plus and minus cultures was lowest in inoculum cells and highest in day 4-8 and 16-20 cells. The GOT isoenzyme patterns are shown in Fig. 45(f-i). The patterns of GOT isoenzymes changed little during the culture cycle, the greatest differences were evident between day 6 and day 12 cultures. Plus and minus culture isoenzyme patterns differed only slightly in a quantitative manner. The activity and isoenzyme patterns of both plus and minus cultures during the second passage were essentially the same as the first passage.

Fig. 44. Changes in activity of LAP per mg soluble protein and per gram fresh weight of cultures grown with and without growth regulators (plus and minus) during culture cycle 155.

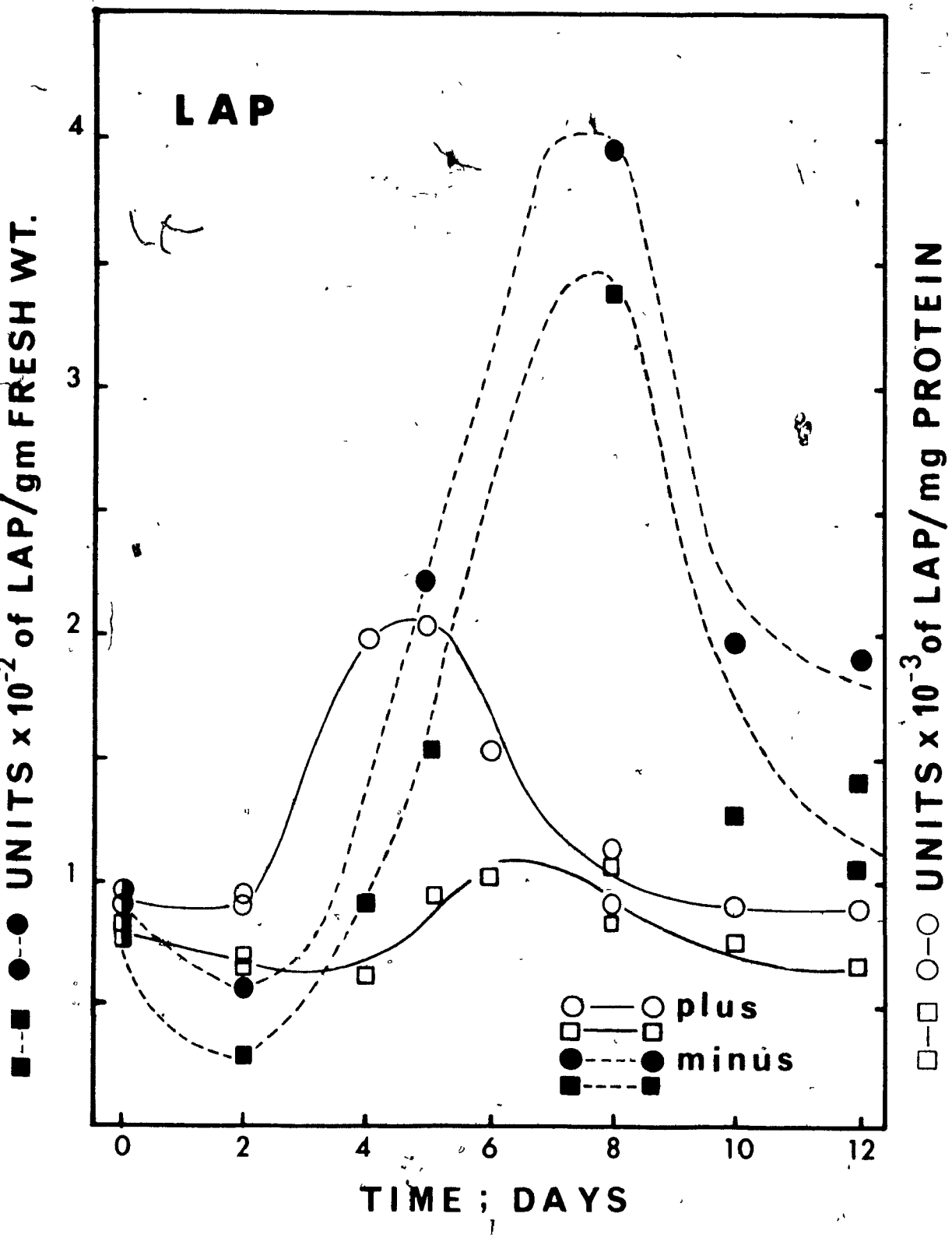
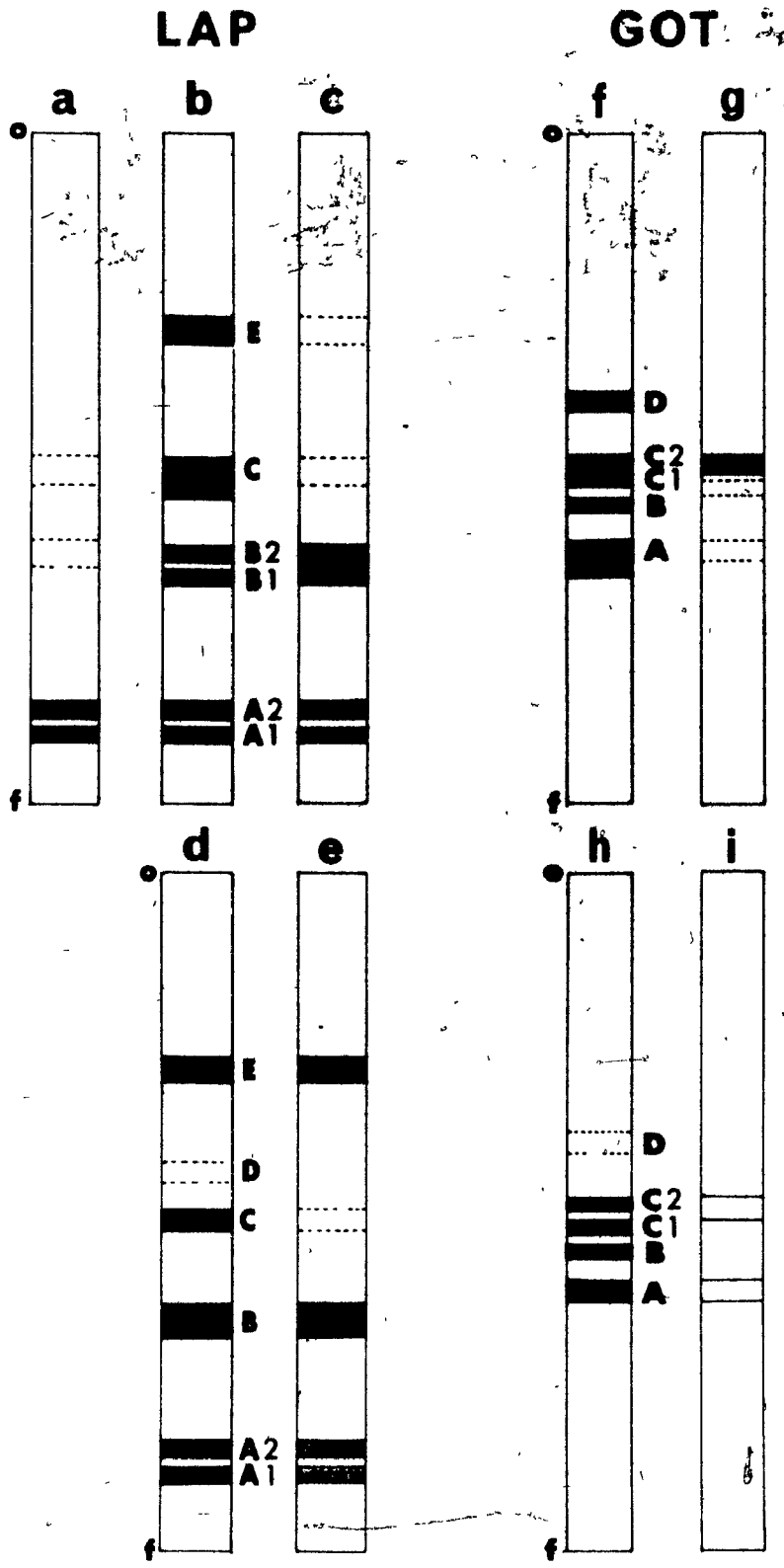

LAP
■-■ ●-● UNITS x 10^{-2} of LAP/gm FRESH WT.
□-□ ○-○ UNITS x 10^{-3} of LAP/mg PROTEIN
TIME ; DAYS
plus
minus
0
2
4
6
8
10
12
1
2
3
4

Fig. 45. Diagrammatic representation of the patterns of activity of LAP and GOT isoenzymes from cultures grown with and without growth regulators (plus and minus). The amount of activity is represented by band intensity.

a - LAP isoenzymes 12 day 147 plus culture (inoculum)
b - LAP isoenzymes 6 day 148 plus culture
c - LAP isoenzymes 10 day 148 plus culture
d - LAP isoenzymes 6 day 148 minus culture
e - LAP isoenzymes 10 day 148 minus culture

f - GOT isoenzymes 6 day 148 plus culture
g - GOT isoenzymes 12 day 148 plus culture
h - GOT isoenzymes 6 day 148 minus culture
i - GOT isoenzymes 12 day 148 minus culture


LAP
GOT
a
b
c
f
g
d
e
h
i
o
f
E
C
B2
B1
A2
A1
D
C2
C1
B
A

8) Esterase

Measurements of total esterase activity were not conducted because available techniques are somewhat sensitive and time consuming (see James and Smith 1974). Esterase activity as judged by staining intensity of gels was lowest in inoculum cells and highest in day 2-8 and 14-20 cultures.

Esterase isoenzyme patterns and representative gel scans are shown in Fig. 46 and 47. It is obvious that the esterase patterns are quite complicated. The isoenzymes have been labelled in three main groups based arbitrarily on mobility characteristics and termed A, B, and C.

Following inoculation into fresh medium the plus cultures showed a striking change in isoenzyme pattern. The predominant isoenzyme of inoculum cells, C2 almost vanished completely and the previously weak isoenzymes A3, A5 and B2 increased greatly. From day 2 to day 6, during the phase of cell division, the number of isoenzymes of the A and B group continued to increase (see Fig. 46, day 2-6, Fig. 47, D and E). As the cultures aged the prominence of isoenzymes of the A and B group diminished with the concomitant increase of isoenzyme C2 (Fig. 46).

Cultures that were transferred into minus medium very soon showed the increase of the A and B group isoenzymes but in contrast to plus cultures maintained considerable C2 activity. As was the case for the plus cultures additional A and B group isoenzymes appeared during the culture cycle. Examination of gel scans (Fig. 47) shows that there were frequent quantitative differences between plus and minus cultures among the isoenzymes of the A and B group but the major difference between

plus and minus cultures in the first passage was the maintenance of isoenzyme C2 in the minus cultures.

During the second passage minus cultures, as was the case for plus cultures, showed the increase and then decrease in number and activity of A and B group isoenzymes. The patterns of change were similar to the first passage except for the sudden decrease in minus cultures of isoenzyme C2 and its subsequent disappearance after day 20 (Fig. 46).

Fig. 46. Diagrammatic representation of the patterns of activity of esterase isoenzymes from cultures grown with and without growth regulators (plus and minus) for two consecutive culture cycles. Numbers at the top of the gels denote the day of sampling. Day twelve represents the point of reinoculation into fresh medium. The amount of activity is represented by band intensity calculated from the average of at least three separate experiments.

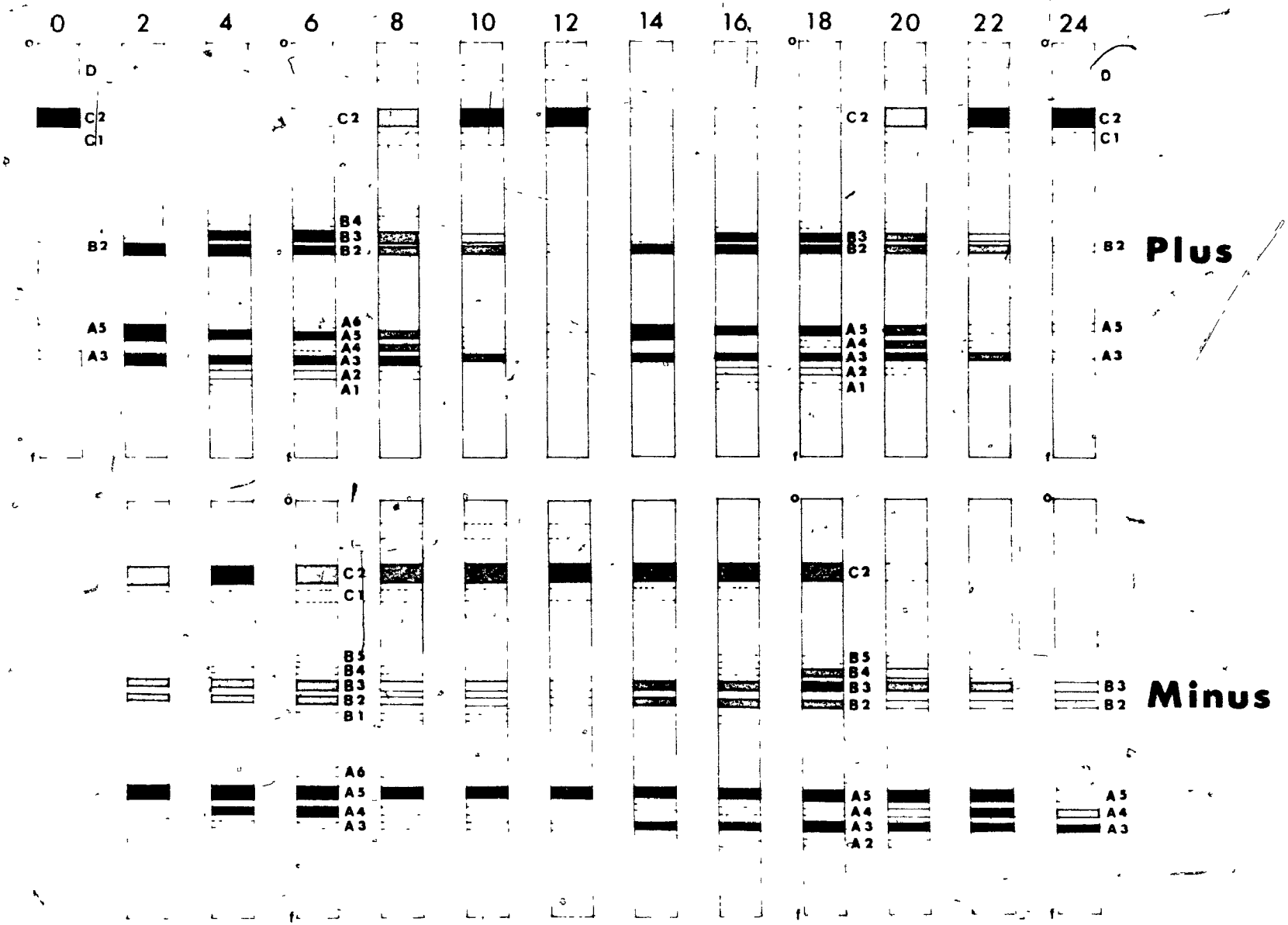
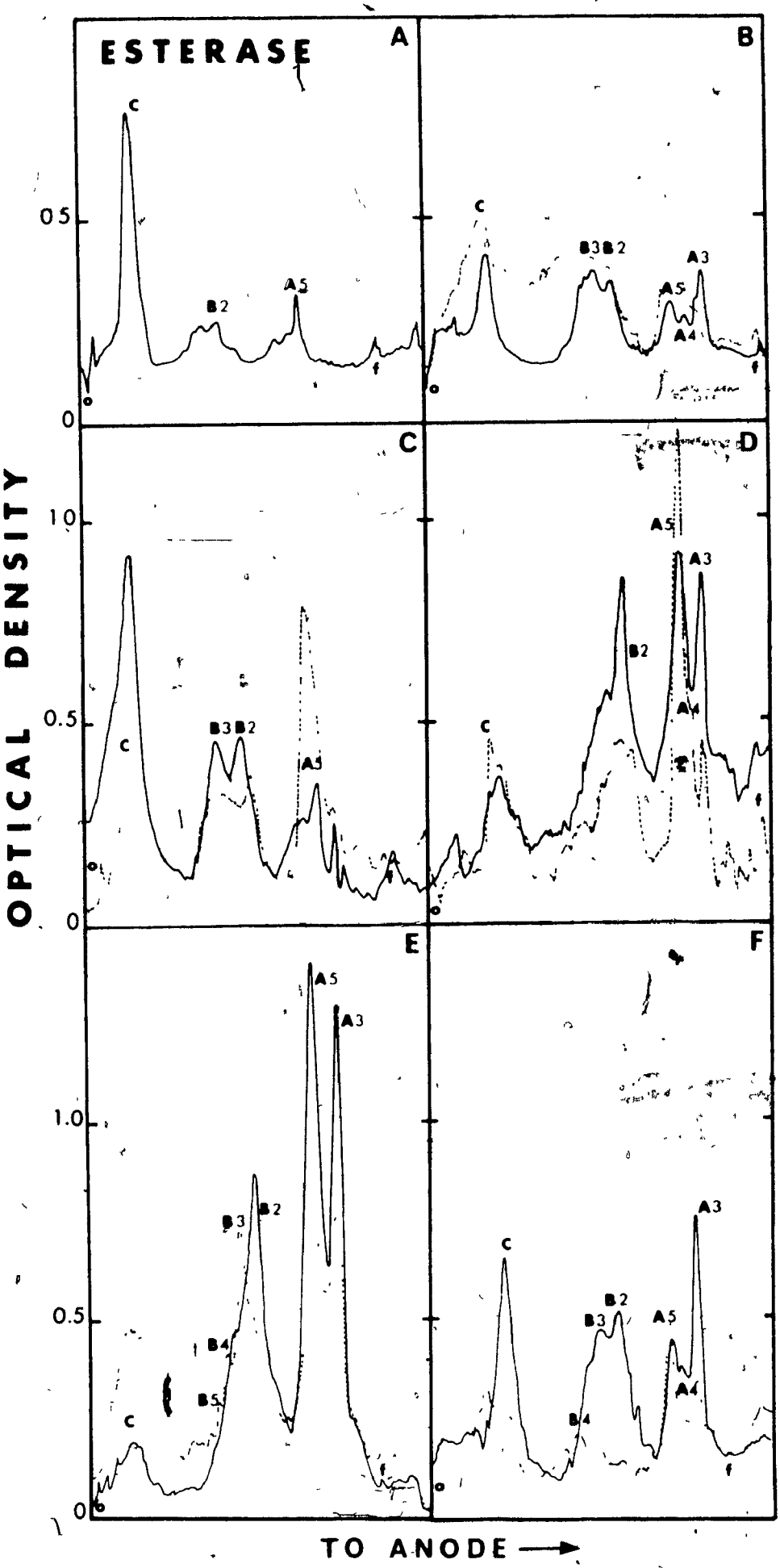

0
2
4
6
8
10
12
14
16
18
20
22
24
D
C2
C1
B4
B3
B2
A6
A5
A4
A3
A2
A1
Plus
B5
B1
Minus

Fig. 47. Tracings of esterase gel scans. Scans for plus cultures are drawn with a solid line and minus culture scans with a dashed line.

A - Est isoenzymes 12 day 125 - inoculum
B - Est isoenzymes 8 day 126
C - Est isoenzymes 10 day 126
D - Est isoenzymes 2 day 127 (14 day)
E - Est isoenzymes 6 day 127 (18 day)
F - Est isoenzymes 8 day 127 (20 day)


ESTERASE
A
B
C
D
E
F
C
B2
A5
B3 B2
A3
A4
B4
B5
OPTICAL DENSITY
0
0.5
1.0
TO ANODE

9) Malate Dehydrogenase

Spectrophotometric measurements of malate and glutamate dehydrogenase conducted by conventional methods (i.e., the increase in absorbance of NAD at 340 nm) were unsuccessful using the soluble protein extract prepared for electrophoresis. Evaluation of the intensity of staining on gels however, indicated that MDH activity was highest in cultures from days 2-6 and 14-18.

As was the case for esterase, MDH patterns showed striking changes with inoculation into fresh medium (Fig. 48 and 49). In the case of plus cultures isoenzyme G was seen to split into two peaks forming isoenzymes G and F, the relative mobility of isoenzyme D changed consistently in the direction of the anode and for purposes of identification is named isoenzyme B. Additionally on day 2 the appearance of isoenzymes A, H, I, and J were detected (see Fig. 48, 49). Isoenzyme A was most prominent on day 4 afterwhich time it decreased quickly and by day 8 the total MDH pattern was seen to change back that of the inoculum.

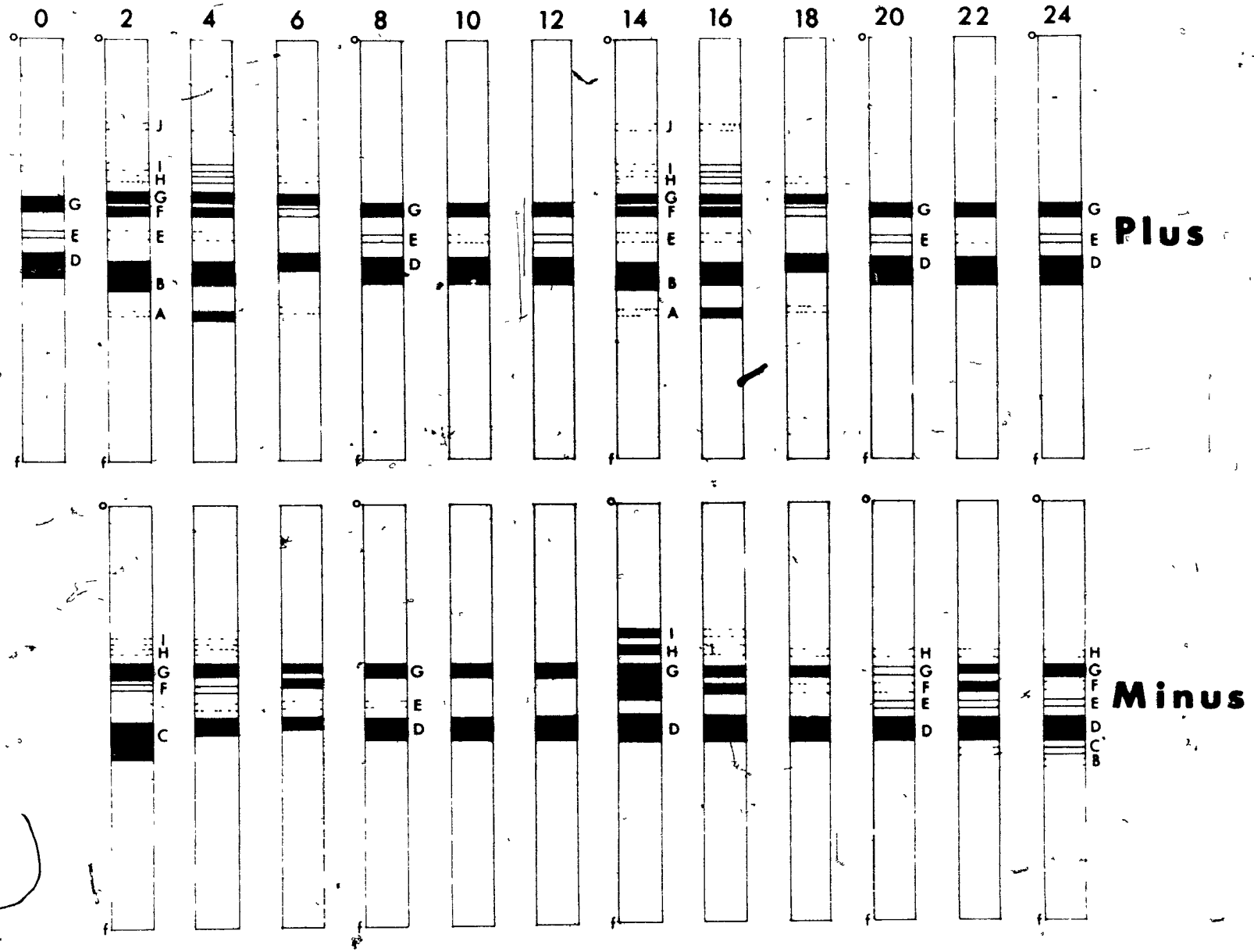
The pattern of change of MDH in minus cultures differed from plus cultures in many ways. Initially after subculture, minus cells showed much greater MDH activity than equivalent plus cultures but the pattern changed less strikingly. Isoenzyme G was not so strongly divided into F and G forms, the mobility of isoenzyme D changed less towards the anode and is so designated C for identification and isoenzyme A failed to occur at all. As was the case with plus cultures after day 6 the isoenzyme pattern became similar to that of the inoculum.

During the second passage in minus culture MDH activity was again found to be elevated after subculture but showed a less changed isoenzyme pattern than equivalent plus cultures. By day 20 the minus cultures showed numerous bands of minor activity some of which increased in strength until by day 24 the minus culture pattern, due to the presence of isoenzymes B, C, F and H, was considerably different than that of the plus cultures.

10) Glutamate Dehydrogenase

The changes in pattern of GDH isoenzymes are presented in tabular form in Tables 3 and 4. Representative gel scans are shown in Fig. 50. The GDH activity of inoculum cultures consisted of five or six closely migrating bands of low activity (Fig. 50-A). After inoculation into fresh medium both plus and minus cultures showed a dramatic shift in isoenzyme profile from the more slowly migrating D, E, F isoenzymes to the more quickly migrating A, B, C isoenzymes (consult Tables 3 and 4, Fig. 50). By day 6-8 isoenzymes D and E increased in activity to reach highest levels on, or near, day 8. As GDH activity diminished with culture age the activity of isoenzymes A and B assumed very low levels. Minus cultures differed from plus cultures in a somewhat slower transition from the slower to faster migrating isoenzyme forms and a slower increase in GDH activity which resulted in higher GDH levels in minus cultures at the end of one passage. During the second passage the GDH activity of minus cultures increased in activity and changed in pattern with reinoculation into fresh medium but after day 16 activity decreased to low levels. The isoenzyme pattern of minus cultures became very different than that of plus cultures after day 20 (see Table 4).

Fig. 48. Diagrammatic representation of the patterns of MDH isoenzymes from cultures grown with and without growth regulators (plus and minus) for two consecutive culture cycles. Numbers at the top of the gels denote the day of sampling. Day 12 represents the point of reinoculation into fresh medium. The amount of activity is represented by band intensity calculated as the average of at least three separate experiments.


0
2
4
6
8
10
12
14
16
18
20
22
24
J
I
H
G
F
E
B
A
D
C
Plus
Minus

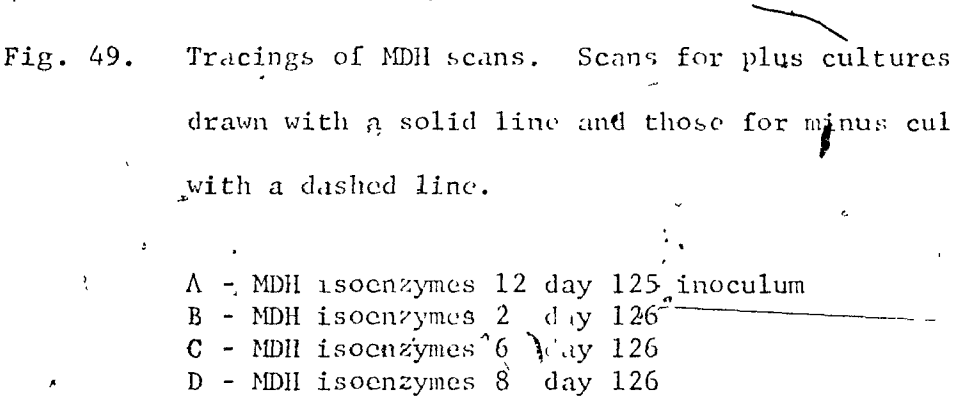


Fig. 49. Tracings of MDH scans. Scans for plus cultures are drawn with a solid line and those for minus cultures with a dashed line.

A - MDH isoenzymes 12 day 125 inoculum
B - MDH isoenzymes 2 day 126
C - MDH isoenzymes 6 day 126
D - MDH isoenzymes 8 day 126

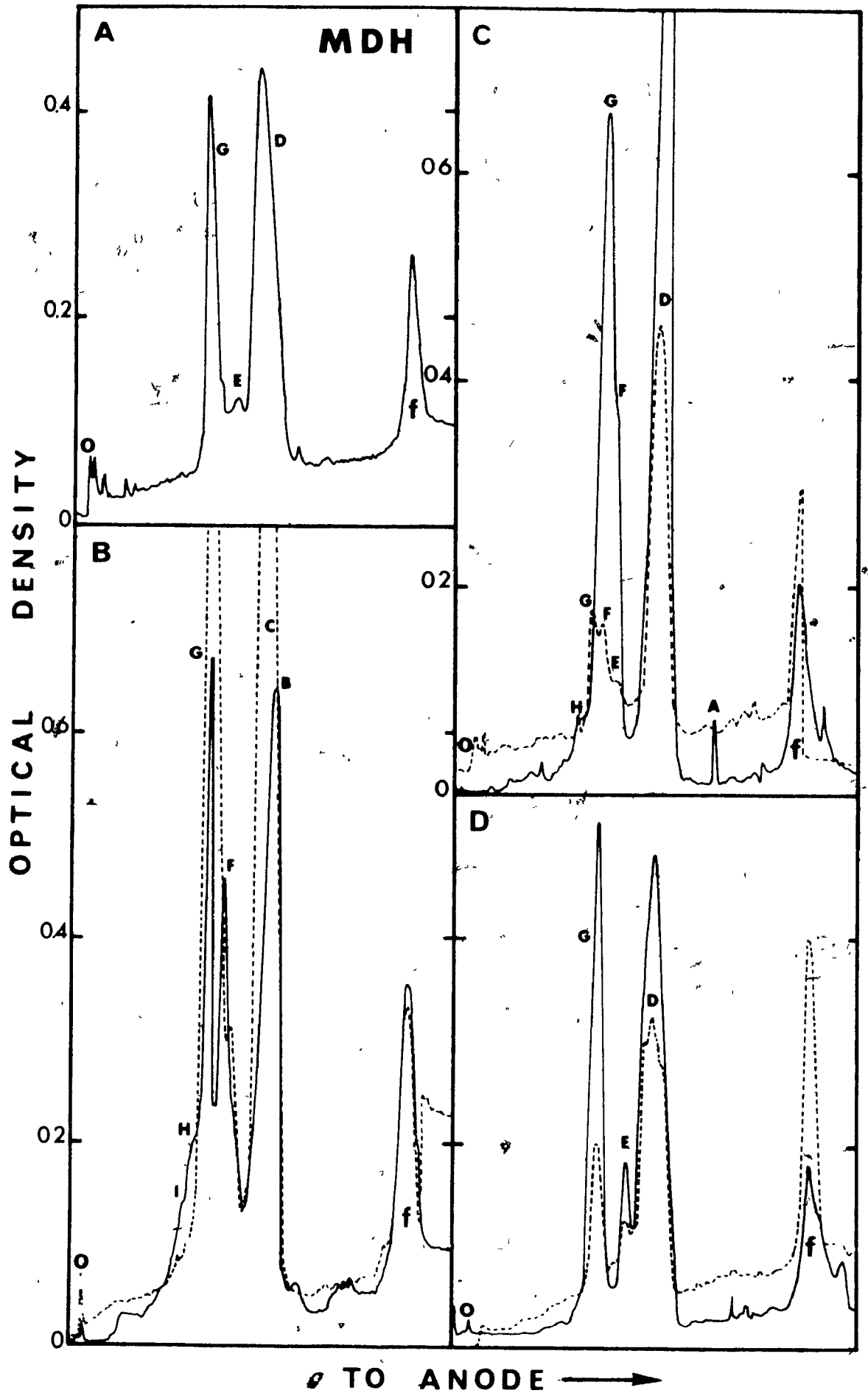

A
MDH
B
C
D
OPTICAL DENSITY
0.4
0.2
0
0.6
0.4
0.2
0
0.2
0
0.6
0.4
0.2
0
G
D
E
f
O
C
B
F
H
I
A
ø TO ANODE

Fig. 50. Tracings of GDH scans. Scans for plus cultures are drawn with a solid line and those for minus cultures with a dashed line.

A - GDH isoenzymes 12 day 125 (inoculum)
B - GDH isoenzymes 2 day 126
C - GDH isoenzymes 4 day 126
D - GDH isoenzymes 8 day 126
E - GDH isoenzymes 10 day 126
F - GDH isoenzymes 12 day 126

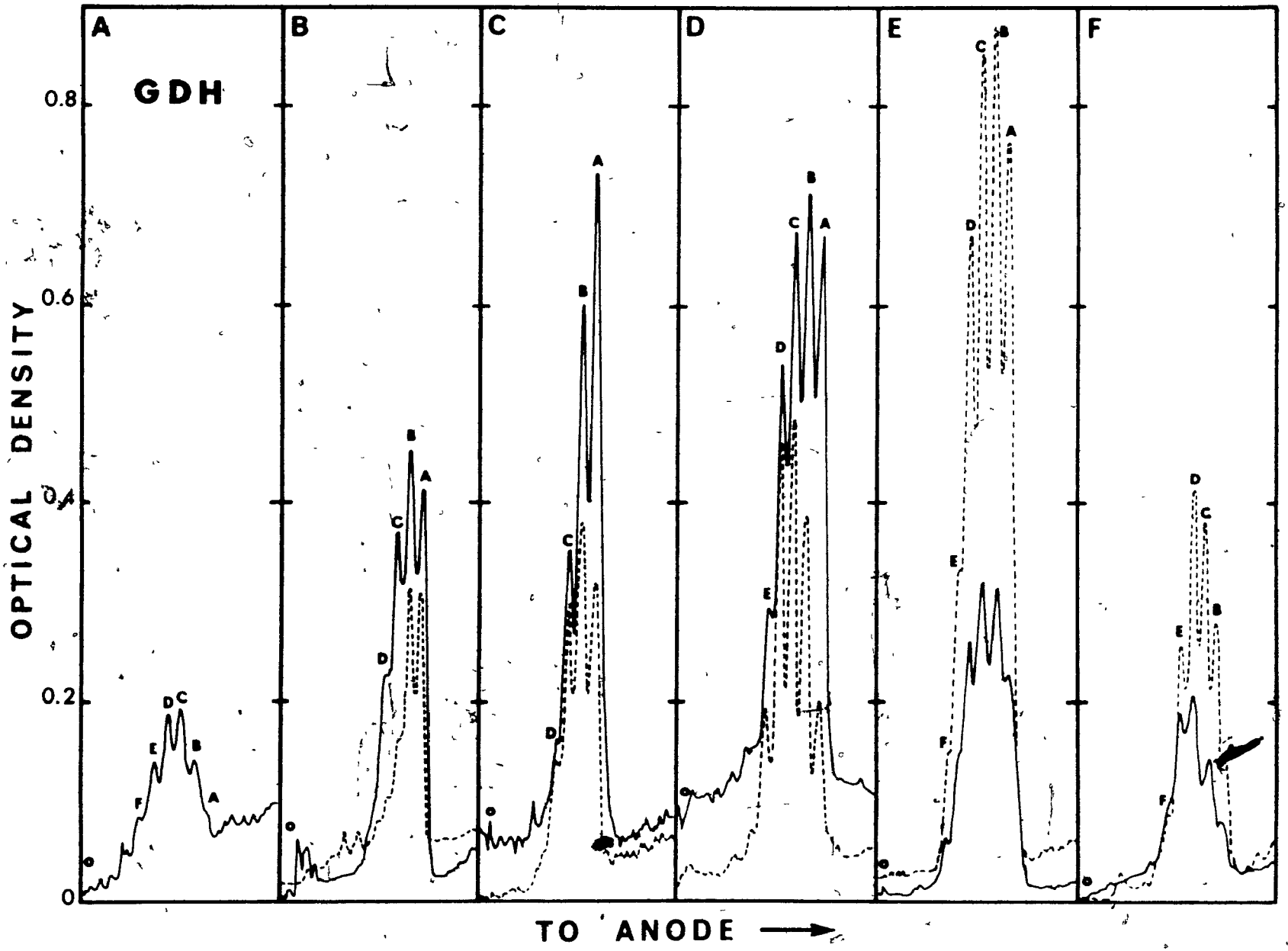

A
GDH
B
C
D
E
F
0.8
0.6
0.4
0.2
0
OPTICAL DENSITY
TO ANODE

Table 3

*Percent of the total GDH activity contributed by the individual isoenzymes; Plus cultures

Isoenzyme	Day 0	Day 2	Day 4	Day 6	Day 8	Day 10	Day 12
A	7	30	44	34	22	10	6
B	19	31	25	40	23	27	18
C	25	26	14	22	22	29	23
D	24	11	12	4	18	23	23
E	16	2	5	-	10	8	18
F	8	-	-	-	5	3	12
G	1	-	-	-	-	-	-

Isoenzyme	Day 12	Day 14	Day 16	Day 18	Day 20	Day 22	Day 24
A	6	40	43	27	25	18	8
B	18	35	24	31	31	26	19
C	23	20	14	23	26	28	28
D	23	5	13	16	14	21	25
E	18	-	5	3	4	6	14
F	12	-	1	-	-	1	6
G	-	-	-	-	-	-	-

* The percent activity was calculated from relative areas on gel scans.

Table 4

*Percent of the total GDH activity contributed by the individual isoenzymes; Minus cultures

Isoenzyme	Day 0	Day 2	Day 4	Day 6	Day 8	Day 10	Day 12
A	7	39	27	20	21	22	7
B	19	40	33	33	22	25	21
C	25	14	26	25	27	23	25
D	24	4	13	18	26	20	23
E	16	3	1	4	10	7	14
F	8	-	-	-	3	2	8
G	1	-	-	-	1	1	1

Isoenzyme	Day 12	Day 14	Day 16	Day 18	Day 20	Day 22	Day 24
A	7	27	49	16	14	23	32
B	21	33	35	19	11	25	24
C	25	26	12	26	11	22	17
D	23	12	4	23	19	20	15
E	14	2	-	15	23	6	12
F	8	-	-	1	17	4	-
G	1	-	-	-	5	-	-

* The percent activity was calculated from relative areas on gel scans.

D. The Growth of New Cultures of Root, Hypocotyl and Cotyledon, Comparison With Established Cultures, Seedlings and Mature Plant Parts.

1) Introduction

The results thus far have shown that persistent biochemical differences do exist in stock cultures derived by Dr. Liau (1971) from root, hypocotyl and cotyledon of a single seedling. This present section reports results from new cell cultures established from a number of different seedlings. The observations were made in order to test: 1 - whether differences between root, hypocotyl and cotyledon cultures is a general phenomenon, 2 - the degree of variation in the isoenzyme patterns of cultures derived from the same plant parts, and 3 - the possibility that the differences detected between cell cultures are related to the tissue of origin. In all a total of sixty-eight sets of root, hypocotyl and cotyledon cultures were started from five-day old seedlings on Liau medium. All attempts to start vigorous cultures directly on synthetic medium were unsuccessful.

Cultures which showed good and approximately equal growth from the three parts of the particular seedling were chosen for analysis. This was done for practical reasons and therefore to a certain extent, means that the cultures were not a random sample of the bean seeds.

Callus cultures were analysed at the end of two or three passages. Suspension cultures were started in both Liau and synthetic medium using first passage callus cells as inoculum. Growth in synthetic medium was generally successful only for cotyledon cultures.

2) New Callus Cultures

a) Morphology

Selected examples of representative cells of newly developing callus cultures, mechanically dispersed, are shown in Figs. 51 and 52. Cells in general were large and highly vacuolated with highly developed and intricate networks of cytoplasmic strands. As is commonly the case with such callus cultures many different shapes of cells were observed.

Initially, all callus cells that developed were white or yellowish in color and the callus was somewhat soft and friable in texture. With culture age and after transfer to fresh solid medium, the callus often became brown and more rubbery in texture. Selected cells from brown and white parts of the callus are shown in Fig. 51 (A-D) and 52 (A-F). There were no obvious differences in the variety, size and shape of cells. Great variation was evident within both white and brown callus. An obvious difference between white and brown callus was the large amounts of opaque material (possibly polysaccharide) present on the walls of brown callus cells.

The newly developing and actively growing callus cells were always white or yellowish in color. Newly derived callus cultures at first grew much more slowly than the established stock cultures. After the third passage, however, the rate of growth of callus cultures increased with the concomitant elimination of the brown cells. The wide variety of cell shapes and sizes present in all the new cultures made it impossible to distinguish one from another on a morphological basis.

In addition to the presence of large amounts of material external to the cell walls, and a variety of unusually shaped cells, newly derived cultures showed patterns of division and multinucleate cells previously now observed in the established stock callus and suspension cultures. Many of the new cultures showed an apparent chaining or budding form of cell division (Fig. 51-E). Multinucleate cells were observed in a variety of the newly established callus and suspension cultures (Fig. 52 C-G, Fig. 57-F). However, the proportion of multinucleate cells in any one culture was very low.

Fig. 51.

Selected cells from newly established callus cultures.

A - Cotyledon callus culture No. 2, second passage L+ medium, brown cells - note deposits of material external to the cells.

B - Hypocotyl callus culture No. 2, second passage L+ medium, brown cells.

C - Hypocotyl callus culture No. 2, third passage L+ medium, white cells - note no external wall deposits on typical highly vacuolated flask shaped cell.

D - Hypocotyl callus culture No. 2; third passage L+ medium, white cells - typical group of large elongate cells free of wall deposits.

E - Root callus culture No. 20, second passage white cells - chain-like or budding type of growth.

Fig. 52.

Selected cells from newly established cultures. All pictures taken with phase contrast.

A - Root callus culture No. 4, second passage L+ medium, highly vacuolated cell with cytoplasmic strands.

B - Cotyledon callus culture No. 4, second passage L+ medium, typical round cell.

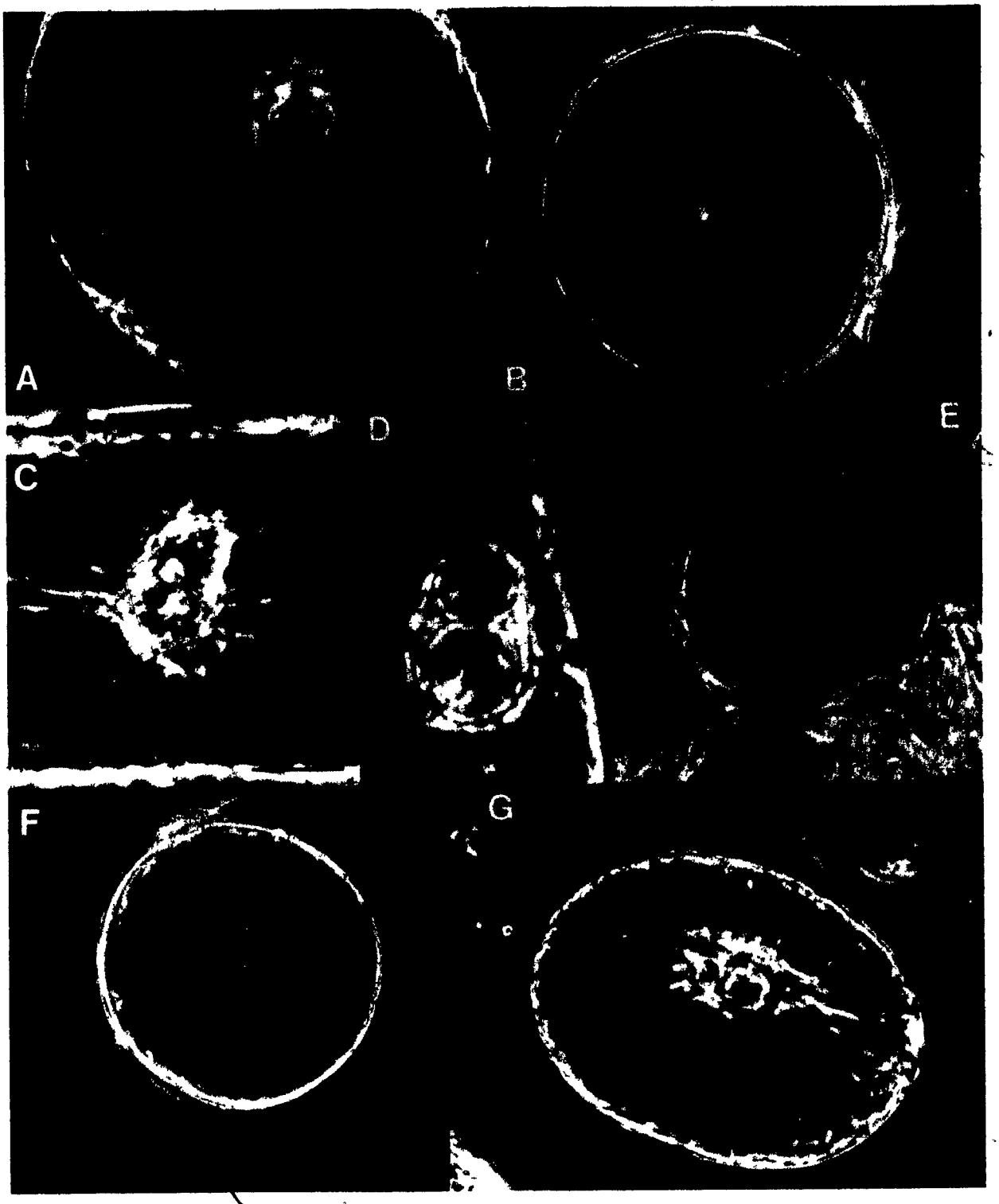
C - Hypocotyl callus culture No. 27, first passage synthetic medium, binucleate cell.

D - Cotyledon callus culture No. 27, first passage synthetic medium, binucleate cell.

E - Cotyledon callus culture No. 4, second passage L+ medium, binucleate cell from brown section of callus.

F - Root callus culture No. 4, second passage L+ medium, trinucleate cell.

G - Root suspension culture No. 17, first passage synthetic medium, multinucleate cell.


A
B
D
E
C
F
G

b) Peroxidase Isoenzymes and Activity

Because a difference in color of parts of the newly derived callus cultures may have meant a difference in certain enzymatic activities, especially peroxidase and PP , protions of white and brown callus were analysed separately.

Spectrophotometric measurements of peroxidase activity showed a variation of activity in units per mg soluble protein (1 unit = 1 Δ O.D. per min.). These ranged from 190-1490 for hypocotyl, 740-2090 for cotyledon and 880-2740 for root cultures. There was no significant difference in activity between white and brown cells.

The peroxidase isoenzyme patterns of new cultures are shown diagrammatically in Fig. 53. As can be seen from a comparison of a and b, c and d, e and f and k and l white and brown cells of any one tissue origin showed essentially identical isoenzyme patterns. The only difference between white and brown cells was the generally increased activity of isoenzymes M, L and K in the brown cells. The peroxidase isoenzyme patterns of the root, hypocotyl and cotyledon cultures are very similar. However, root cultures could be distinguished by the presence of isoenzyme F, C and the separation of isoenzyme I into two distinct bands I-1 and I-2. A comparison of the patterns of root cultures from different seedlings (Fig. 53 g-l) shows the considerable variation in the occurrence of F and C which limits the value of isoenzyme F as a useful marker of root cultures even though it was never detected in any other new culture type.

Fig. 53.

Peroxidase isoenzymes of newly derived callus cultures.

A. Benzidine peroxidase, composite diagrams of cultures No. 1-10.

a - root, w* b - root, b*

c - hypocotyl, w d - hypocotyl, b

e - cotyledon, w f - cotyledon, b

B. Guaiacol peroxidase

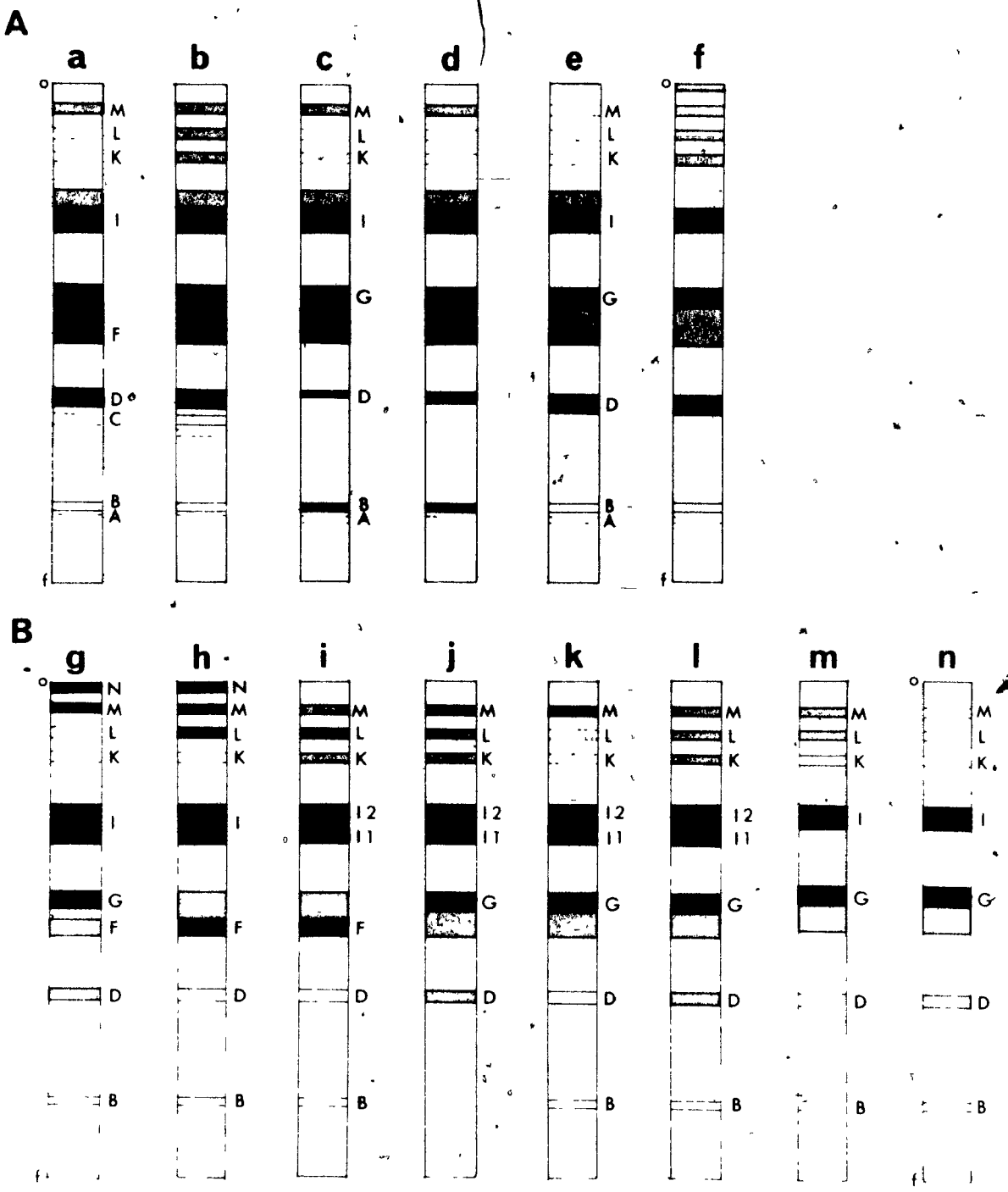
g to l - root culture No. 10-w*, 9-w, 8-w, 4-w, 2-w, 2-b*

m - hypocotyl, composite of cultures 1-10.

n - cotyledon, composite of cultures 1-10.

Peroxidase activity is represented by degree of band intensity.

*w - white cells, b - brown cells


A
a
b
c
d
e
f
M
L
K
I
G
F
D
C
B
A
B
g
h
i
j
k
l
m
n
N
M
L
K
I
12
11
G
F
D
B

c) Peroxidase (polyphenolic) Isoenzymes and Activity

The results of spectrophotometric measurements of PP activity in newly derived callus cultures gave the same general picture as for peroxidase. There was no significant difference in the amount of PP activity in extracts of white and brown callus; there was considerable variability in the amount of activity in the individual cultures and, as a whole, root cultures showed the most and hypocotyl cultures the least activity. PP activity ranged from 220-1,160 units (Δ O.D./min./mg soluble protein) in hypocotyl cultures, 470-1,130 units in cotyledon cultures and 620-1,780 units in root cultures.

The PP isoenzyme patterns representative of new cultures are shown diagrammatically in Fig. 54 (a-f). There was essentially no difference in pattern between root, hypocotyl and cotyledon cultures and no difference between white and brown cells. The presence of isoenzymes I, J, K and L in patterns a, b and f is considered to reflect peroxidase activity (see Fig. 53 a-f).

d) Malate Dehydrogenase

The MDH isoenzyme patterns of newly derived cultures are shown in Fig. 54-B. The patterns of root and hypocotyl were similar but those of cotyledon were quite distinctive due to the presence of isoenzymes D and E which were not present in root or hypocotyl. White cells also differed from brown cells in the increased activity of isoenzyme H in brown cells.

e) Glutamate Dehydrogenase

The GDH pattern of all the new cultures consisted of five or six closely migrating bands of low activity essentially identical to the pattern previously described for established callus cultures (i.e. Fig. 4).

Fig. 54.

A. PP isoenzymes, composite drawing of cultures No. 1-10.

All gels stained with DOPA.

a - root, w* b - root, b*

c - hypocotyl, w d - hypocotyl, b

e - cotyledon, w f - cotyledon, b

B. MDH isoenzymes, composite drawing of cultures No. 1-10.

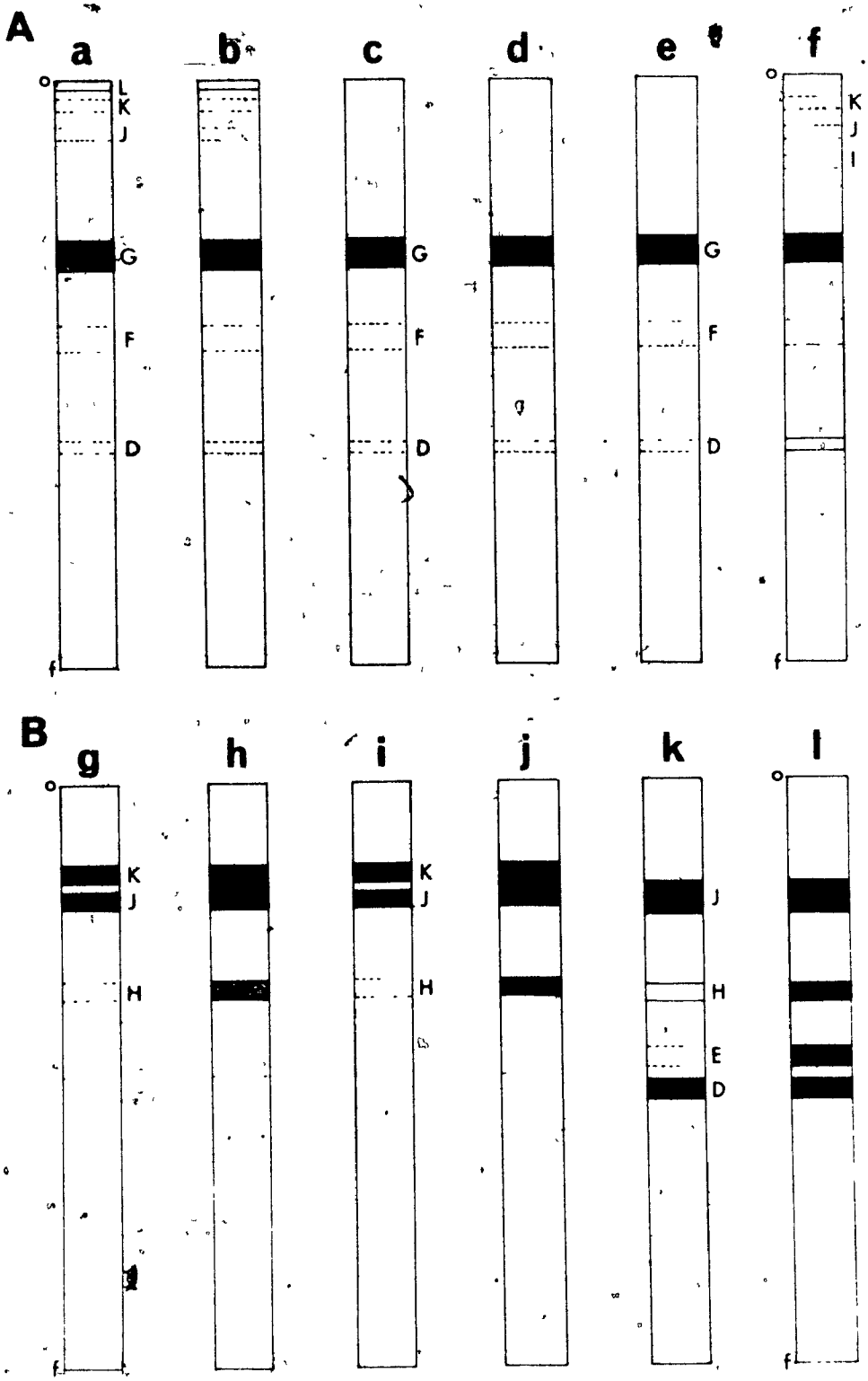
a - root, w* b - root, b*

c - hypocotyl, w d - hypocotyl, b

e - cotyledon, w f - cotyledon, b

The degree of enzymatic activity is denoted by band intensity.

*w - white cells, *b - brown cells


A
a
b
c
d
e
f
L
K
J
G
F
D
K
J
I
G
F
D
G
F
D
J
H
E
D
B
g
h
i
j
k
l
K
J
H
K
J
H
J
H
E
D

3) New Suspension Cultures

a) Morphology

As was the case with new callus, the new suspension cultures grew slowly in comparison with older stock suspension cultures. First passage suspension cultures contained many large, highly vacuolated cells. Many of these cells may have been present in the inoculum used to start the suspension culture. Examples of the cell types observed are shown in Fig. 55 to 57. After the first few passages in suspension culture the cell population became more homogeneous and increasingly smaller in cell size. This was reported and discussed by Liau (1971). The more irregular cell forms were no longer commonly observed and the cell populations tended to be made up of groups of actively dividing small cells (compare Fig. 55 A-E with F).

b) Cytochemistry

The morphological characteristics of first passage suspension cells, namely: large size and the ease of observation of the intricate internal construction, make these cells useful cytochemical material. Cells were stained for a variety of the enzyme activities used in the isoenzyme studies. Typical results are shown in Fig. 57.

Peroxidase activity was localized as a granular stain spread evenly throughout the cytoplasm and cell wall. Wall associated activity was much lower than that previously observed for stock cotyledon cultures and no crystals were observed on the surface of the cells. Staining for MDH, GDH and G6PDH activity generally produced a delicate mottled staining of the cytoplasm (Fig. 57 C-F). Magnification of the stained areas revealed the activity to be a highly stippled network (Fig. 57-E).

The cytoplasm adjacent to the nucleus often stained more intensely than other areas. Cells stained for esterase activity showed a granular deposit spread evenly throughout the cytoplasm. Additionally, some cells showed considerable esterase activity associated with the cell wall or located in material covering the outside surface of the cells.

Fig. 55.

Selected suspension culture cells.

A - Root suspension culture No. 59, first passage L+ medium - typical population of round cells.

B - Cotyledon suspension culture No. 59, first passage L+ medium - round cells with highly reticulate cytoplasmic network.

C - Root suspension culture No. 17, first passage synthetic medium - possibly a polyploid cell undergoing cell division.

D - Root suspension culture No. 18, first passage L+ medium - typical young free cell.

E - Hypocotyl suspension culture No. 14, first passage L+ medium - flask shaped cell.

F - Hypocotyl suspension culture, Stock culture 6 day passage 97 - small group of actively dividing cells for size comparison.

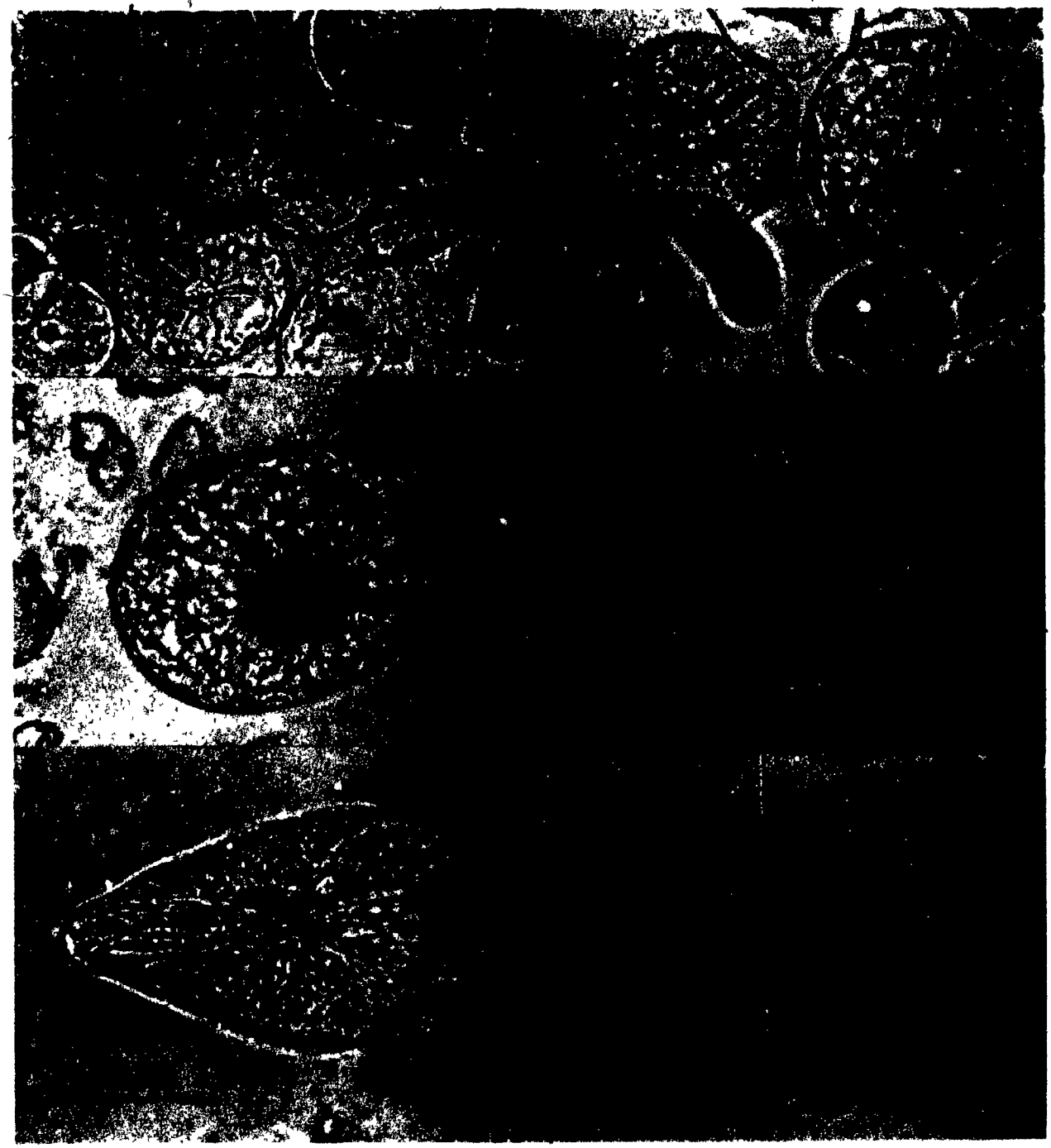


Fig. 56. Cotyledon suspension culture No. 14, first passage synthetic medium, large, highly vacuolated flask-shaped cell. Scale marking represents 100 microns.

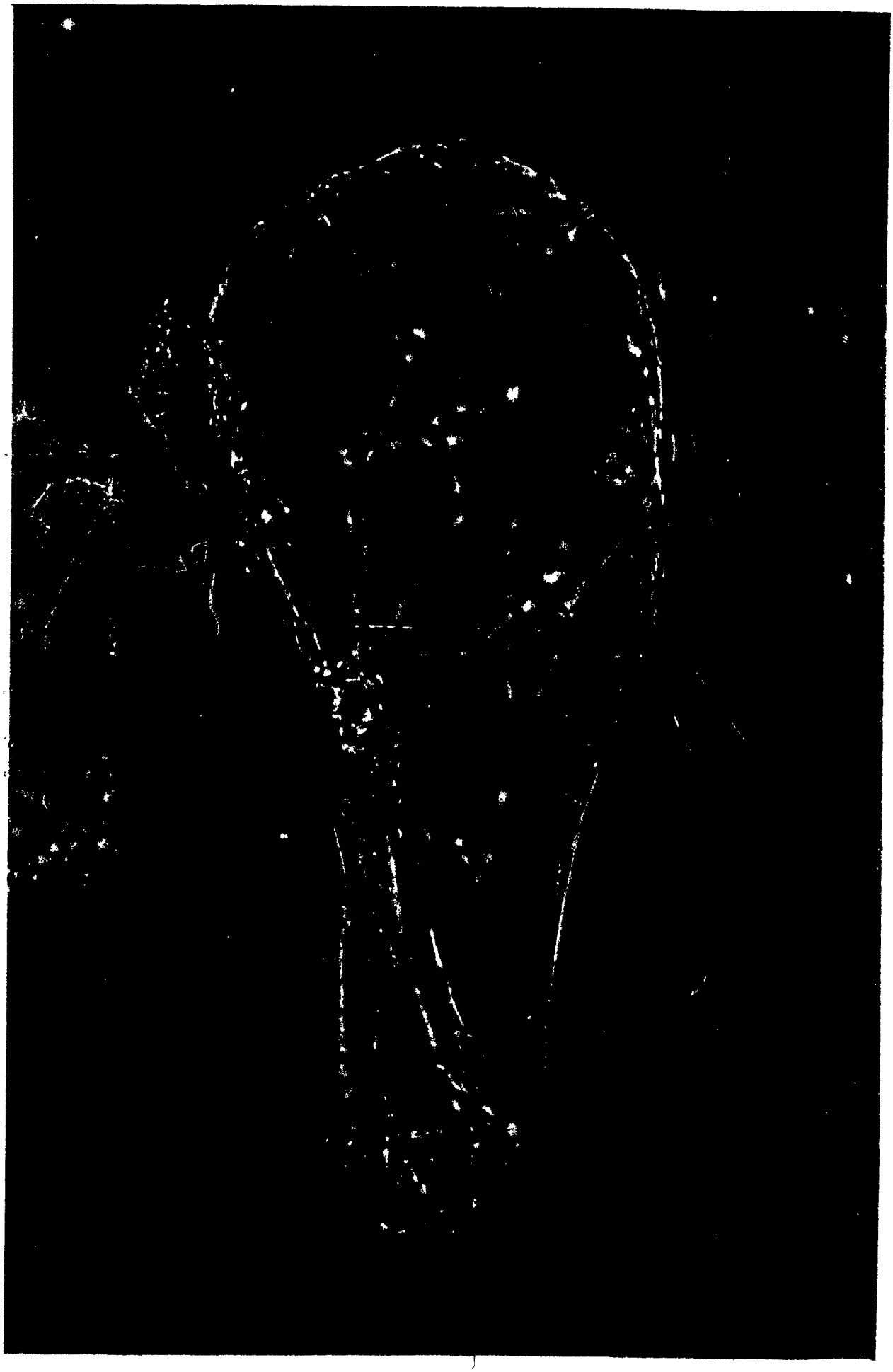


Fig. 57.

Cytochemistry of selected first passage No. 59; root, hypocotyl and cotyledon suspension culture cells grown in L+ medium.

A - Root cell stained for benzidine peroxidase - note reticulate staining of cytoplasm and cell wall.

B - Root cell stained for guaiacol peroxidase - staining identical to A.

C - Root cell stained for GDH activity - general reticulate staining of the cytoplasm.

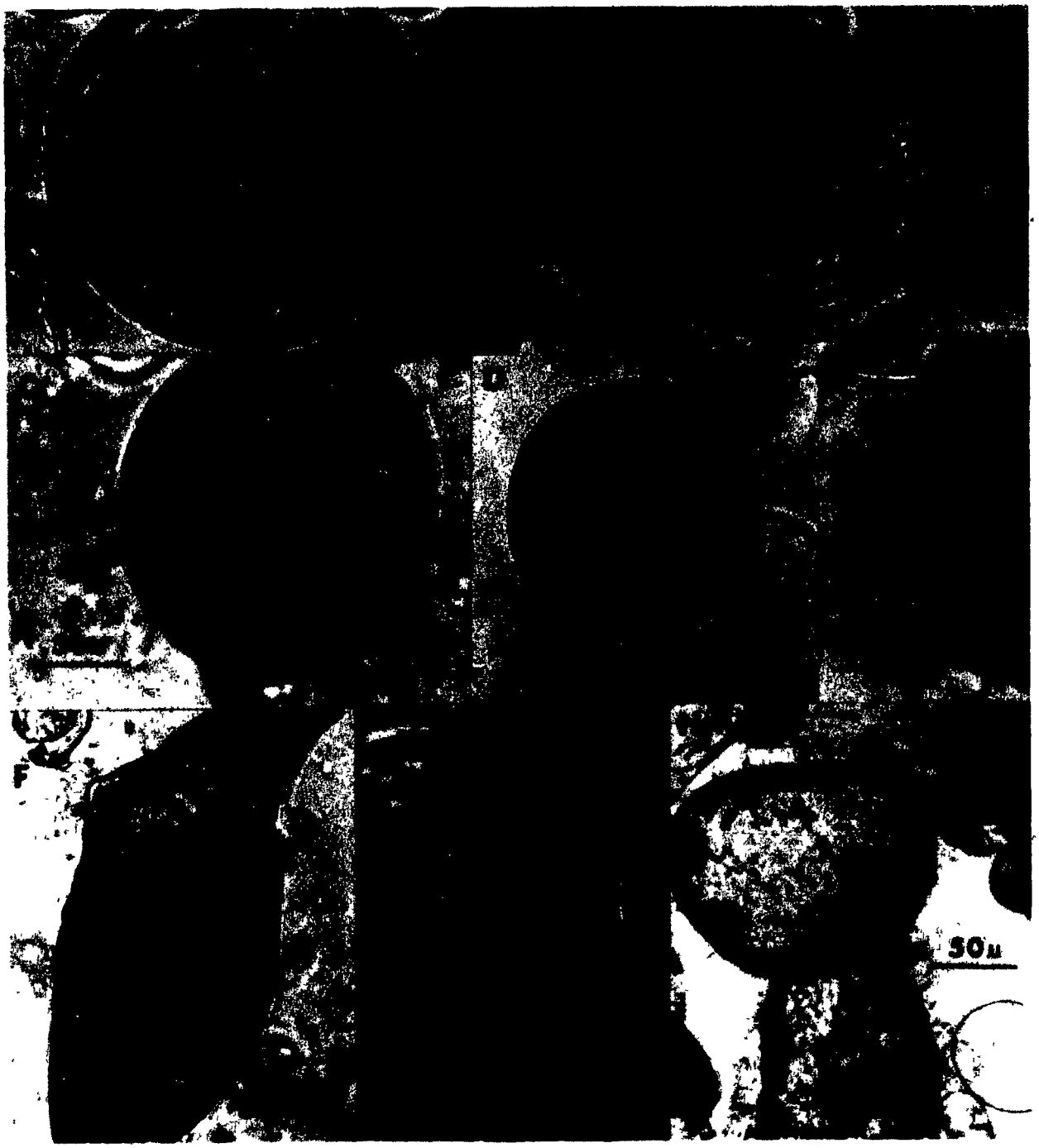
D - Root cell stained for G6PDH activity - general staining of cytoplasm around the nucleus.

E - Cotyledon cell stained for MDH activity - enlargement of cytoplasmic staining.

F - Root cell stained for GDH activity - staining cytoplasmic in nature but especially heavy between the nuclei.

G - Hypocotyl cell stained for Est activity - granular stain spread evenly throughout the cytoplasm.

H - Cotyledon cells stained for Est - staining of cytoplasm with some cells showing considerable staining on the cell wall surface.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c) Peroxidase Isoenzymes

The guaiacol isoenzyme patterns of selected root, hypocotyl and cotyledon cultures are shown in Fig. 58. As can be seen there is a considerable amount of variation among the cultures derived from the same part of different parent seedlings. Nevertheless there are consistent differences between the three cultures. The most prominent difference is the consistent presence of isoenzyme A and B and G and F in cotyledon cultures, the presence of strong isoenzyme M, L, K activity in root and cotyledon cultures and the presence of isoenzyme H in hypocotyl cultures.

As can be seen from a comparison of Fig. 58 p and q, the isoenzyme patterns of peroxidase were essentially the same in cells grown in L+ and synthetic medium. First passage callus cells inoculated into synthetic medium usually showed little growth. When sufficient growth occurred for isoenzyme analysis to be carried out, the patterns of isoenzymes of MDH and PP , as well as peroxidase, were found to be essentially identical in cells from the two media.

Analysis of the spent medium in which new suspension cultures were grown showed that cell cultures secreted or lost peroxidase activity into the medium. This is shown diagrammatically in Fig. 58 r-t. The pattern of medium peroxidase from cotyledon, root and hypocotyl cultures are distinctive.

Fig. 58.

Guaiacol peroxidase isoenzymes of newly derived suspension cultures.

a - e Root cultures* No. 64, 67, 68, 59 and 63.

f - j Hypocotyl cultures* No. 64, 67, 68, 59 and 63.

k - o Cotyledon cultures* No. 64, 67, 68, 59 and 63.

p - Cotyledon culture No. 38 L+ medium.

q - Cotyledon culture No. 38 synthetic medium.

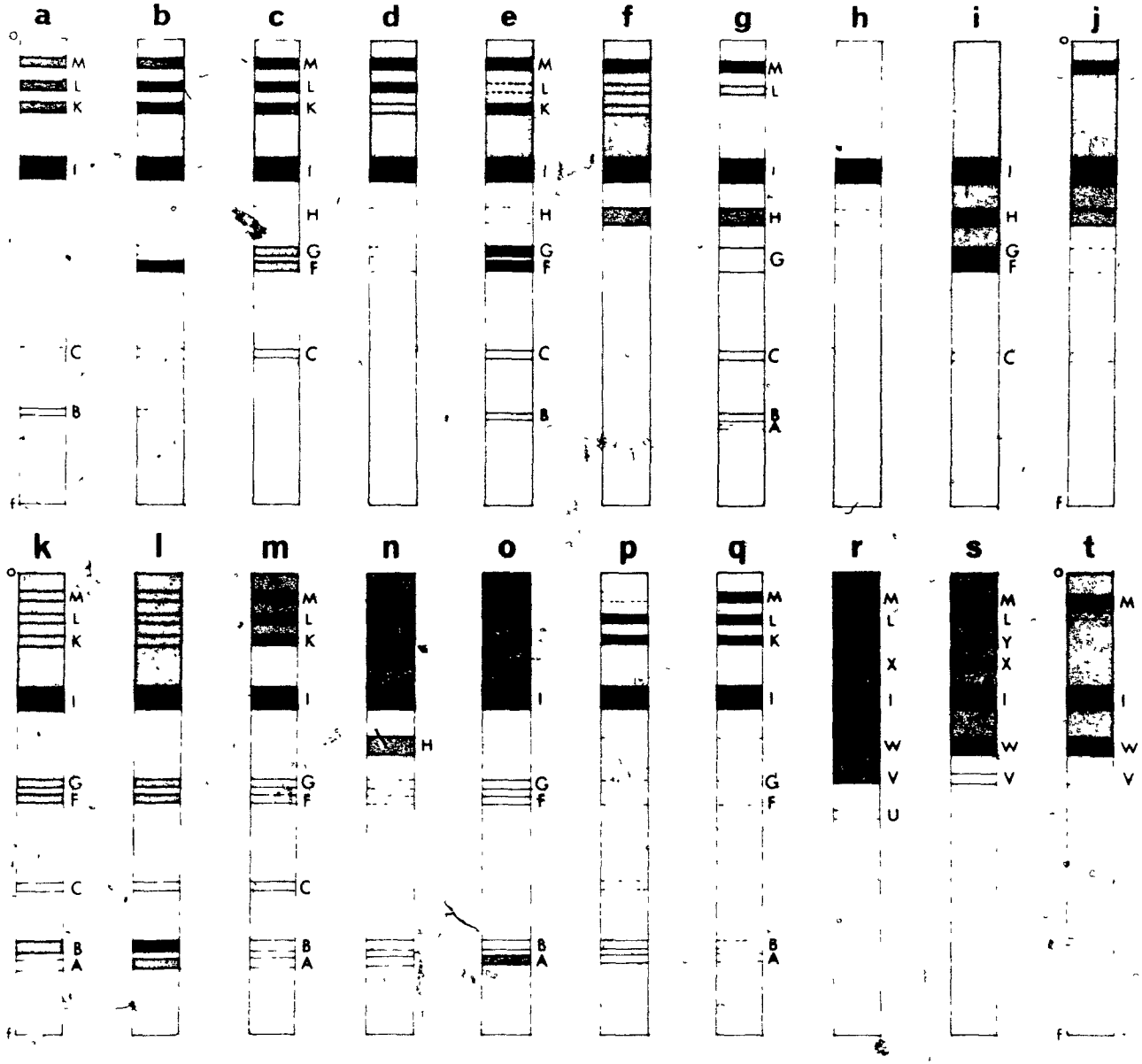
r - Root, medium peroxidase, composite diagram.

s - Hypocotyl, medium peroxidase, composite diagram.

t - Cotyledon, medium peroxidase, composite diagram.

The degree of enzyme activity is represented by band intensity.

*grown in L+ medium.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d) Peroxidase (polyphenolic) Isoenzymes

The isoenzymes of PP for newly derived suspension cultures are shown diagrammatically in Fig. 59 a to i. The patterns of hypocotyl cultures can be distinguished from those of root and cotyledon by the presence of isoenzyme J and the absence of isoenzyme H. Isoenzyme G, present in all of the cultures, is equivalent to peroxidase isoenzyme I (Fig. 58).

e) Malate Dehydrogenase and Other Isoenzymes

Many of the isoenzyme patterns of MDH, Est, AP, LAP and GOT were very weak in the first passage suspension cultures and in this respect not very dependable as enzyme markers. The patterns of MDH and esterase did show reproducible culture specific isoenzyme patterns when the level of activity was reasonably strong. Typical patterns of MDH are shown in Fig. 59 j-l Root cell cultures characteristically showed the presence of isoenzyme B not present in hypocotyl or cotyledon.

The esterase activity of most 28 day new cultures was usually so low as to make comparisons between cultures difficult. It was evident, however, that isoenzyme R was only present in cotyledon cell cultures (see Fig. 64).

The patterns of AP, LAP and GOT isoenzymes showed little variation in the newly established cultures. All newly established suspension cultures exhibited a relatively simple pattern shown in Fig. 64 and 65.

Fig. 59.

PP and MDH isoenzymes of newly derived suspension cultures grown in L+ medium.

a - c PP isoenzymes from root cultures No. 59, 64 and 67.

d - f PP isoenzymes from hypocotyl cultures No. 59, 64 and 67.

g - i PP isoenzymes from cotyledon cultures No. 59, 64 and 67.

j - l MDH isoenzymes from root, hypocotyl and cotyledon cultures No. 64.

The degree of enzyme activity is represented by band intensity.


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j
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l
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F1
E

4) Comparison of Isoenzyme Patterns From Cultured Cells With Those of Seedling and Mature Plant Parts

a) Introduction

A difficult aspect of a comparison of isoenzyme patterns from many different sources is the problem of nomenclature. Isoenzyme bands reported here have been designated in accord with the general principles outlined in the section on materials and methods namely that isoenzymes are labelled with capital letters in order of decreasing migration towards the front. Isoenzymes that occurred in close proximity and may possibly be related are given subscripts of the same letter, e.g. A1, A2, A3, etc. During the course of this study some isoenzymes detected in various different experimental conditions, although identical in electrophoretic mobility, have been given different letter designations.

In order to make easier the comparisons between the many isoenzyme patterns reported in this section, composite figures were first drawn for each enzyme based on all analyses for that enzyme including analyses reported in previous sections. The isoenzymes were then relabelled when required according to electrophoretic mobility. Furthermore, numerical subscripts were only used in cases where a large band of activity, e.g. D of Fig. 60-c was also found as subbands (D1,D2).

Additionally, when the electrophoretic mobilities of certain isoenzymes were essentially identical but the banding pattern characteristics of the isoenzymes were not the same, small case letter subscripts were affixed for purposes of identification. For example,

the banding of guaiacol peroxidase isoenzymes G and F are different but very characteristic in callus and suspension cultures. It is not known whether these bands represent identical enzymatic activity and thus are labelled with subscript c for callus cultures and subscript s for suspension cultures (see Fig. 60 e and f). In the same manner, it is not always certain, when comparing certain isoenzymes of stock cultures with newly derived cultures that the isoenzymatic activities are identical. In this case established culture bands are given subscript o (old) and newly established culture bands are given subscript n (new). Any isoenzyme band that differed slightly in mobility or other characteristics from the average band was given a subscript to identify the point of origin of the band. The subscripts used were: r - root, h - hypocotyl, x - cotyledon, m - medium, q - stem, p - petiole, l - leaf and y - pod. Certain of the isoenzymes of the medium could not be clearly equated with any of the cytoplasmic isoenzymes; therefore, their designation with completely different symbols as used previously was retained.

b) Guaiacol Peroxidase

The isoenzyme patterns of guaiacol peroxidase for whole plant and seedling parts and new and established cultures are presented diagrammatically in Fig. 60. The patterns of peroxidase are very complicated and up to 18 isoenzymes were observed.

The peroxidase activity of one day imbibed seeds was very low. Only one isoenzyme (K) with very low activity was detected in both the embryo and cotyledons (Fig. 60 a and u). It is noteworthy that catalase activity (not depicted) was extremely high in cotyledons at this time. By day 5 peroxidase activity had increased very markedly

Fig. 60.

Comparison of Guaiacol Peroxidase Isoenzymes

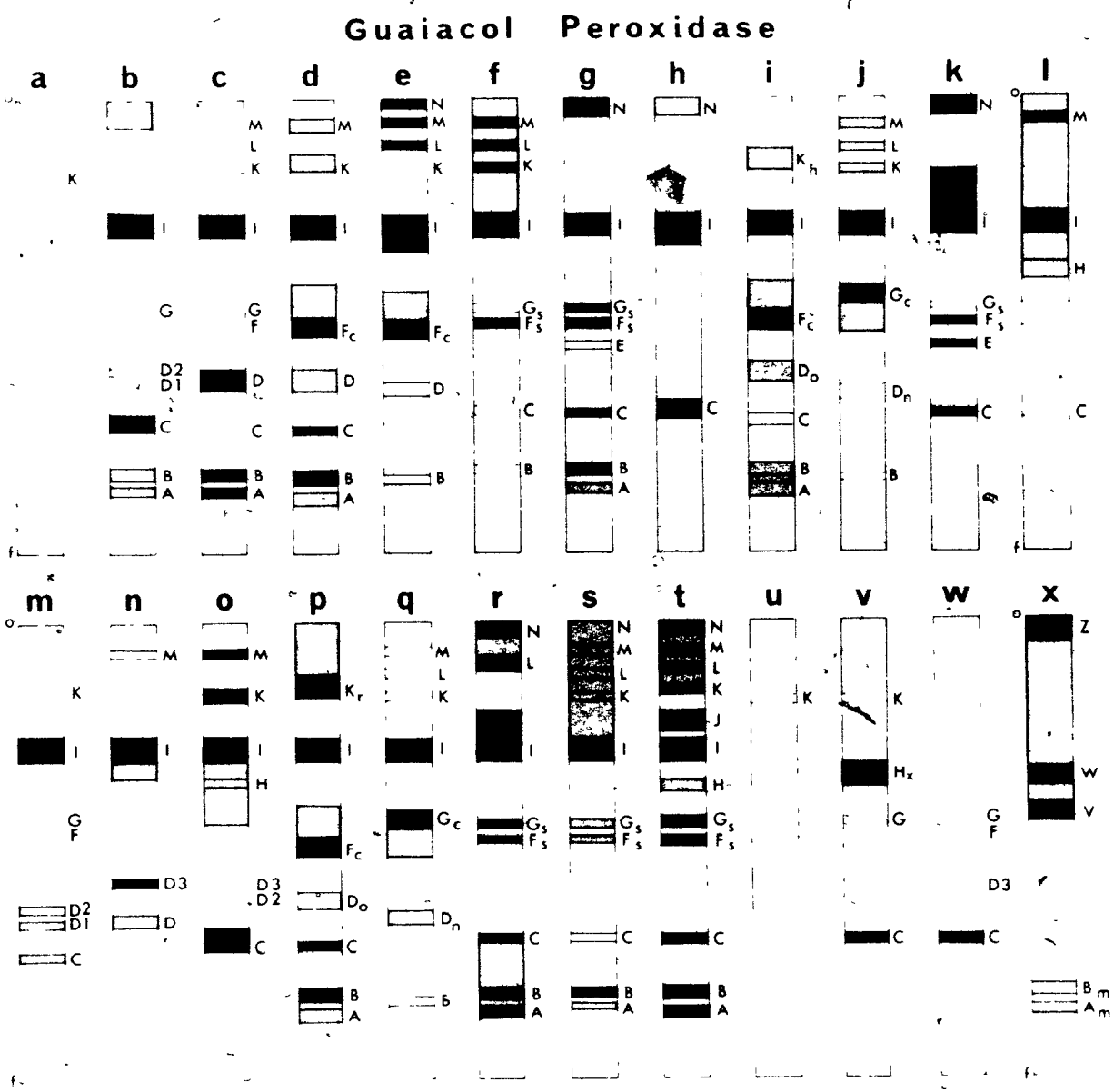
a - 1 day embryo (24 hr. imbibed seed).
b - 5 day root.
c - mature root (6 weeks).
d - root callus, stock culture, 21 day passage 53.
e - root callus, new culture No. 9, (28 days).
f - root suspension, new culture No. 67, (28 days).
g - root suspension, stock culture, 12 day passage 121.
h - 5 day hypocotyl.
i - hypocotyl callus, stock culture, 21 day passage 53.
j - hypocotyl callus, new culture No. 9, (28 days).
k - hypocotyl suspension, stock culture, 12 day passage 121.
l - hypocotyl suspension, new culture No. 63, (28 days).
m - mature stem, second internode, (6 weeks).
n - petiole (fully expanded leaf).
o - leaf (fully expanded).
p - cotyledon callus, stock culture, 21 day passage 53.
q - cotyledon callus, new culture No. 9, (28 days).
r - cotyledon suspension, stock culture, 12 day passage 121.
s - cotyledon suspension, new culture No. 67, (28 days).
t - cotyledon suspension, stock culture, 2 day passage 146.
u - 1 day cotyledon (24 hr. imbibed seed).
v - 5 day cotyledon.
w - immature bean pod (without seeds).
x - cotyledon medium peroxidase 12 day passage 121.

Peroxidase activity is represented by the degree of band intensity.

Subscripts: c - callus, s - suspension, h - hypocotyl

o - stock culture, n - new culture

r - root, x - cotyledon, m - medium


Guaiacol Peroxidase
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and the root, hypocotyl and cotyledon showed distinctive isoenzyme patterns (Fig. 60 b, h and v).

The seedling root displayed the strongest peroxidase activity and the greatest number of isoenzyme bands. Isoenzymes A, B, D1 and D2 were not present in either hypocotyl or cotyledon. The hypocotyl and cotyledon patterns were comparatively simple with bands of low intensity. Isoenzyme H_x seems to occur only in 5 day cotyledons although it may be equivalent to isoenzyme w detected in spent culture medium (Fig. 60 v and x). Comparison of 5 day root with mature root (Fig. 60 b and c) shows that the predominant changes with time were quantitative and the characteristic isoenzymes of root, A and B, were intensified.

Comparison of the various parts of the mature plant (Fig. 60 c, m, n, o and w) shows, not surprisingly, that each plant part gives a distinctive peroxidase isoenzyme pattern. Although the patterns are unique, parts of the plant that are related share many isoenzymes in common (e.g. Fig. 60 m, n and o).

Comparison of the isoenzyme patterns of different cultured cells with each other and various parts of the mature plant show some interesting points. The patterns of callus cultures are similar to those of suspension cultures but differ in the banding patterns of isoenzyme F and G (given subscripts c and s for purposes of identification) and the presence of isoenzyme D in callus cultures (compare Fig. 60 p, q, r, s). In the case of root cultures (Fig. 60 d - g) older stock cultures, both callus and suspension, differed from newly established cultures. Older cultures showed much more pronounced

activity of isoenzymes A and B and much less pronounced activity of isoenzymes M, L and K. The isoenzyme patterns of the root cultures show close similarities with the patterns from mature and seedling roots (compare d - g to b and c).

Isoperoxidase patterns in hypocotyl cultures are depicted in Fig. 60 i - l. As was the case with root cultures callus and suspension culture patterns show similarities but again, differed in G and F banding patterns. Hypocotyl suspension cultures differ from those of root and cotyledon in the characteristic lack of isoenzymes A and B. The patterns from hypocotyl stock callus (i) are very similar to those of root (d) and cotyledon callus (p). In this respect they are much more like the patterns of mature root (c) than 5 day hypocotyl (h) or mature stem (m). The newly derived callus and suspension cultures (j and l) lacked isoenzymes A and B and thus are more like 5 day hypocotyl.

Patterns of isoperoxidase from cotyledon cultures are shown in Fig. 60 p - t. The isoperoxidase patterns of cotyledon callus and suspension cultures are the most uniform of the three groups. Callus and suspension cultures again differed in the banding pattern of isoenzymes G and F but contained considerable activity of isoenzymes A and B. In this respect, and because of the presence of isoenzymes K, L, M and N, cotyledon isoperoxidase isoenzyme patterns are most like those of the root. The peroxidase patterns of passage one and passage 121 cotyledon suspension were remarkably alike (Fig. 60 r and s) whereas those of root suspensions (Fig. 60 f and g) were not, although with time they showed a pattern similar to that of cotyledon cultures.

Peroxidase isoenzymes were found to change somewhat during the culture cycle; isoenzyme J (see Fig. 60-t) was present on day 2-4 of the culture cycle. Isoenzyme J was not detected at any other time or in any other part of the plant.

The results for peroxidase agreed with many other reports of plant isoperoxidases in that patterns of seedling and mature plant parts are distinctive and that the patterns of activity change with development. The isoperoxidase patterns of callus and suspension cells from any one plant part showed differences, differences were detected among calluses derived from root, hypocotyl and cotyledon and the isoenzyme patterns of all cultured cells most closely resembled those of root.

c) Benzidine Peroxidase

The isoenzyme patterns of benzidine peroxidase for whole plant and seedling parts and cultures are presented diagrammatically in Fig. 61. In general the isoenzyme patterns of benzidine peroxidase are very similar to those of guaiacol peroxidase but there were many quantitative differences in isoenzymes and a few minor qualitative differences. Isoenzyme Ia was only observed when benzidine was used as a stain.

The benzidine peroxidase isoenzyme patterns of the callus and suspension cultures were identical to those obtained with guaiacol and hence showed the same results. Callus culture patterns were similar (Fig. 61 d, g, n) whereas suspension culture patterns were different (Fig. 61 e, h, o). It is important to remember that differences detected between root, hypocotyl and cotyledon callus cultures were most evident on day 7-10 of the 28 day culture period and not on day 28 (see Fig. 3). The isoenzyme profiles of all three calluses and cotyledon suspension most closely resemble the patterns of mature root. Finally the isoenzyme patterns of stem, petiole, leaf and pod showed more intense bands and a more complicated isoenzyme pattern when benzidine was used as a stain (compare Fig. 60 m, n, o, w and Fig. 61 i, j, k and p).

Fig. 61.

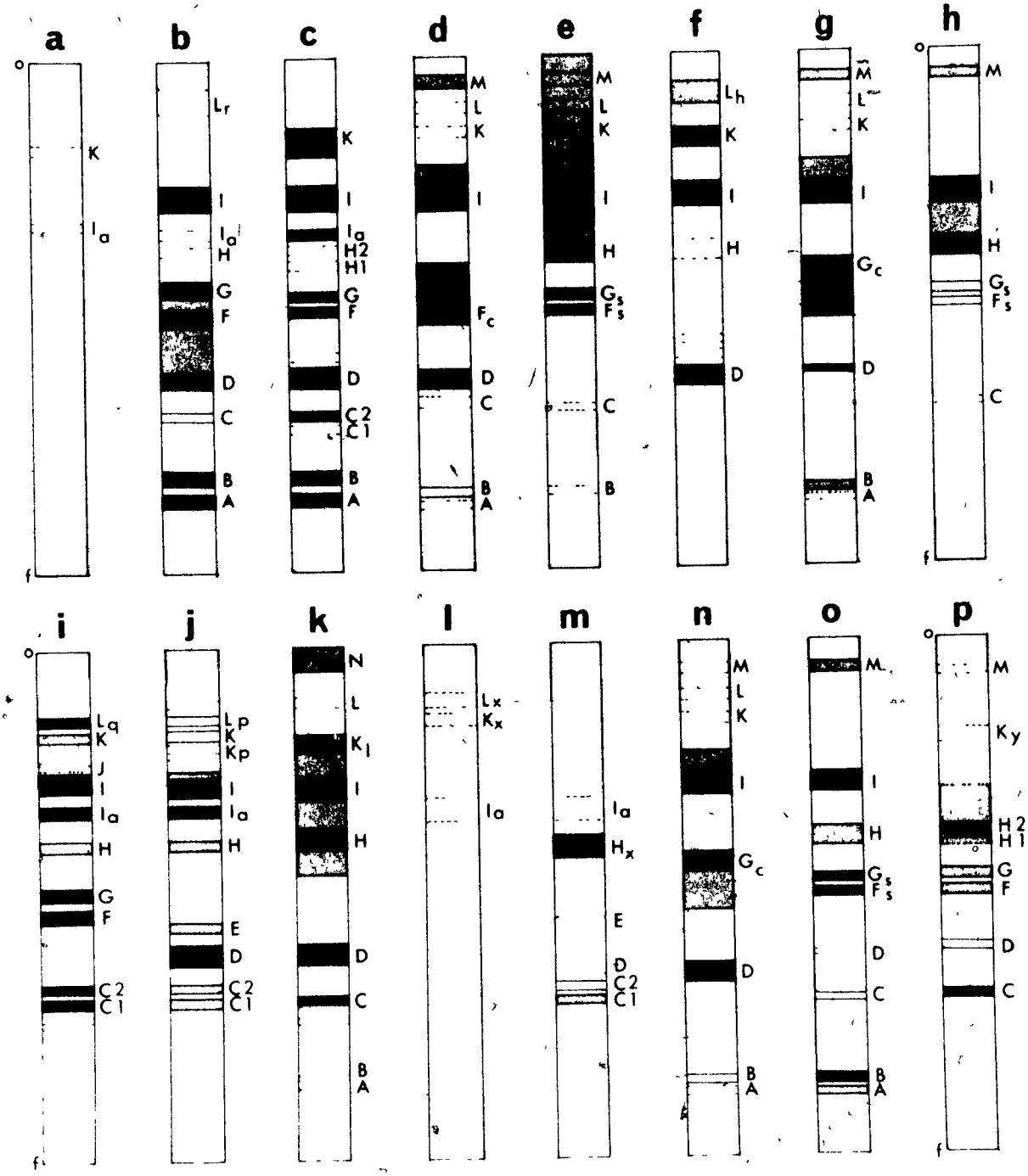
Comparison of Benzidine Peroxidase Isoenzymes

a - 1 day embryo (24 hr. imbibed seed).
b - 5 day root.
c - mature root (6 weeks).
d - root callus, new culture, composite of No. 1-10, (28 days).
e - root suspension, new culture, composite of 59, 63, 64, 67, and 68, (28 days).
f - 5 day hypocotyl.
g - hypocotyl callus, new culture, composite of No. 1-10, (28 days).
h - hypocotyl suspension, new culture, composite of 59, 63, 64, 67 and 68, (28 days).
i - mature stem, second internode (6 weeks).
j - petiole (fully expanded leaf).
k - leaf (fully expanded).
l - 1 day cotyledon.
m - 5 day cotyledon.
n - cotyledon callus, new culture, composite of No. 1-10, (28 days).
o - cotyledon suspension, new culture, composite of 59, 63, 64, 67 and 68, (28 days).
p - immature bean pod (without seeds).

Peroxidase activity is represented by the degree of band intensity.

Subscripts: a - i.e. Ia, subband of I detected with benzidine only, r - root, s - suspension, c - callus, h - hypocotyl, q - stem, p - petiole, l - leaf, x - cotyledon, y - pod.

Benzidine Peroxidase



d) Peroxidase (polyphenolic)

The PP isoenzyme patterns of cultures and of the parts of seedlings and mature plants are shown diagrammatically in Fig. 62. The patterns are considerably less complicated than those for peroxidase. A total of eleven isoenzymes was observed.

The PP activity of cotyledon of one day imbibed seeds and of two day embryo, was very strong but only one band, isoenzyme I in the embryo and isoenzyme G in the cotyledon, was detected in each case (Fig. 62 a and t). This activity decreased with time especially in cotyledon (Fig. 62 t and u). The PP patterns of 5 day root and hypocotyl (Fig. 62 b, h) are similar but that of 5 day cotyledon is different (Fig. 62-u).

The isoenzyme patterns of established callus were quite similar. They differed mainly in the activity of isoenzyme C and showed the most complex PP isoenzyme profiles detected (Fig. 62 d, i, p). The isoenzyme patterns of stock suspension cultures were readily distinguished on the basis of isoenzyme A and B activity (Fig. 62 f, k, r).

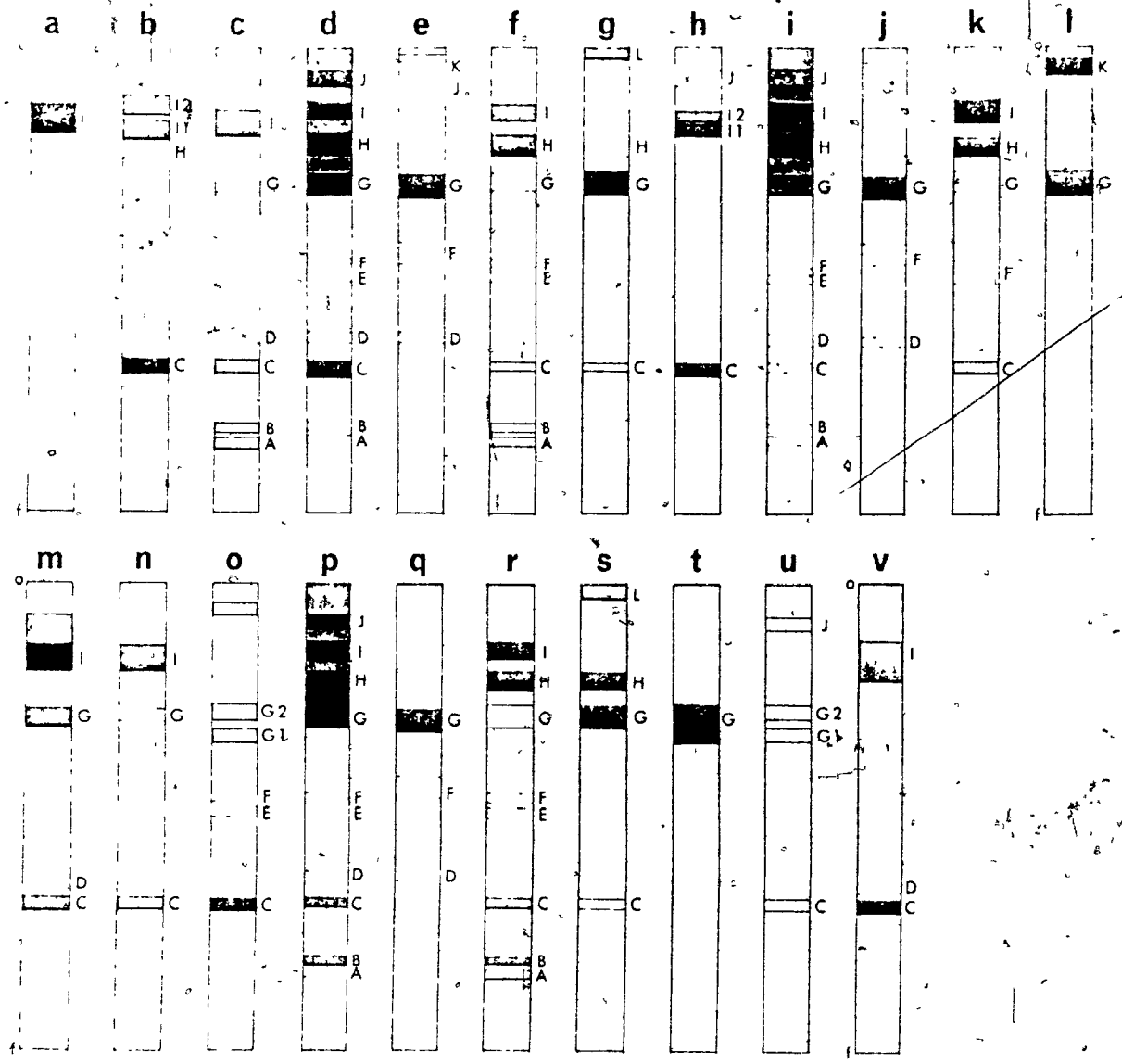
The most striking differences in PP isoenzyme profiles are seen between newly established and stock suspension and callus cultures (e.g. root callus, d and e). The isoenzyme patterns of newly derived cultures are much less complicated. Especially noticeable is the absence of isoenzymes C and I (see Fig. 62 e, g, j, l, q, s). In general the isoenzyme patterns of established cultures are unique whereas the patterns of new cultures are not distinctive and are much like those of many parts of the parent plant.

Fig. 62.

Comparison of PP Isoenzymes

a - 2 day embryo.
b - 5 day root.
c - mature root (6 weeks).
d - root callus, stock culture, 21 day passage 53.
e - root callus, new culture, composite of No. 2, 4, 8, 9, and 10, (28 days).
f - root suspension, stock culture, 12 day passage 121.
g - root suspension, new culture, composite of No. 59, 63, 64, 67 and 68, (28 days).
h - 5 day hypocotyl.
i - hypocotyl callus, stock callus, 21 day passage 53.
j - hypocotyl callus, new culture, composite of No. 2, 4, 8, 9 and 10, (28 days).
k - hypocotyl suspension, stock culture, 12 day passage 121.
l - hypocotyl suspension, new culture, composite of No. 59, 63, 64, 67 and 68, (28 days).
m - mature stem, second internode (6 weeks).
n - petiole (fully expanded leaf).
o - leaf (fully expanded).
p - cotyledon callus, stock culture, 21 day passage 121.
q - cotyledon callus, new culture, composite of No. 2, 4, 8, 9 and 10, (28 days).
r - cotyledon suspension, stock culture, 12 day passage 121.
s - cotyledon suspension, new culture, composite of No. 59, 63, 64, 67 and 68, (28 days).
t - 1 day cotyledon (24 hr. imbibed seed).
u - 5 day cotyledon.
v - immature pod (without seeds).

Activity is represented by the degree of band intensity.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e) Malate Dehydrogenase

The isoenzyme patterns of MDH for cultures and seedling and whole plant parts are shown diagrammatically in Fig. 63.

The MDH isoenzyme patterns of 1 day embryo and cotyledon were very strong and quite distinctive (Fig. 63 a and u). Isoenzyme B was only found in embryo and in root or root cell cultures. The patterns of 5 day old root and mature root are distinct from those of 5 day hypocotyl, 5 day cotyledon, stem, petiole, leaf and pod which are very similar (Fig. 63 compare b, c with h, m, n, o and x).

The patterns of callus cultures are distinct from each other and quite unlike any other MDH patterns detected (Fig. 63 d, i, p). The slow migrating isoenzymes J and K were only found in callus cultures. In stock callus cultures isoenzyme D was detected only in callus cultures of root (Fig. 62-d). However, isoenzyme D was detected in newly established cotyledon callus cultures (Fig. 62-q).

It is quite remarkable that the patterns of MDH in cultures are so diverse. The patterns for suspension cultures are less unique than those for callus. The patterns of stock suspension cultures resemble one another and in this respect are all similar to the pattern for 5 day hypocotyl or cotyledon (compare Fig. 63 f, k and r with h and w). The patterns of newly derived suspension cultures differed from each other and differed from the patterns of established suspension cultures in the case of root and cotyledon. The newly established root suspension pattern resembled that of mature root or embryo (Fig. 63 g, a, c). The newly established hypocotyl pattern resembled 5 day hypocotyl and stem (Fig. 63 l, h, m). The newly

established cotyledon suspension gave a pattern similar to the isoenzyme patterns of stock suspension cultures during the division stage of the growth cycle (Fig. 63 u, s and t).

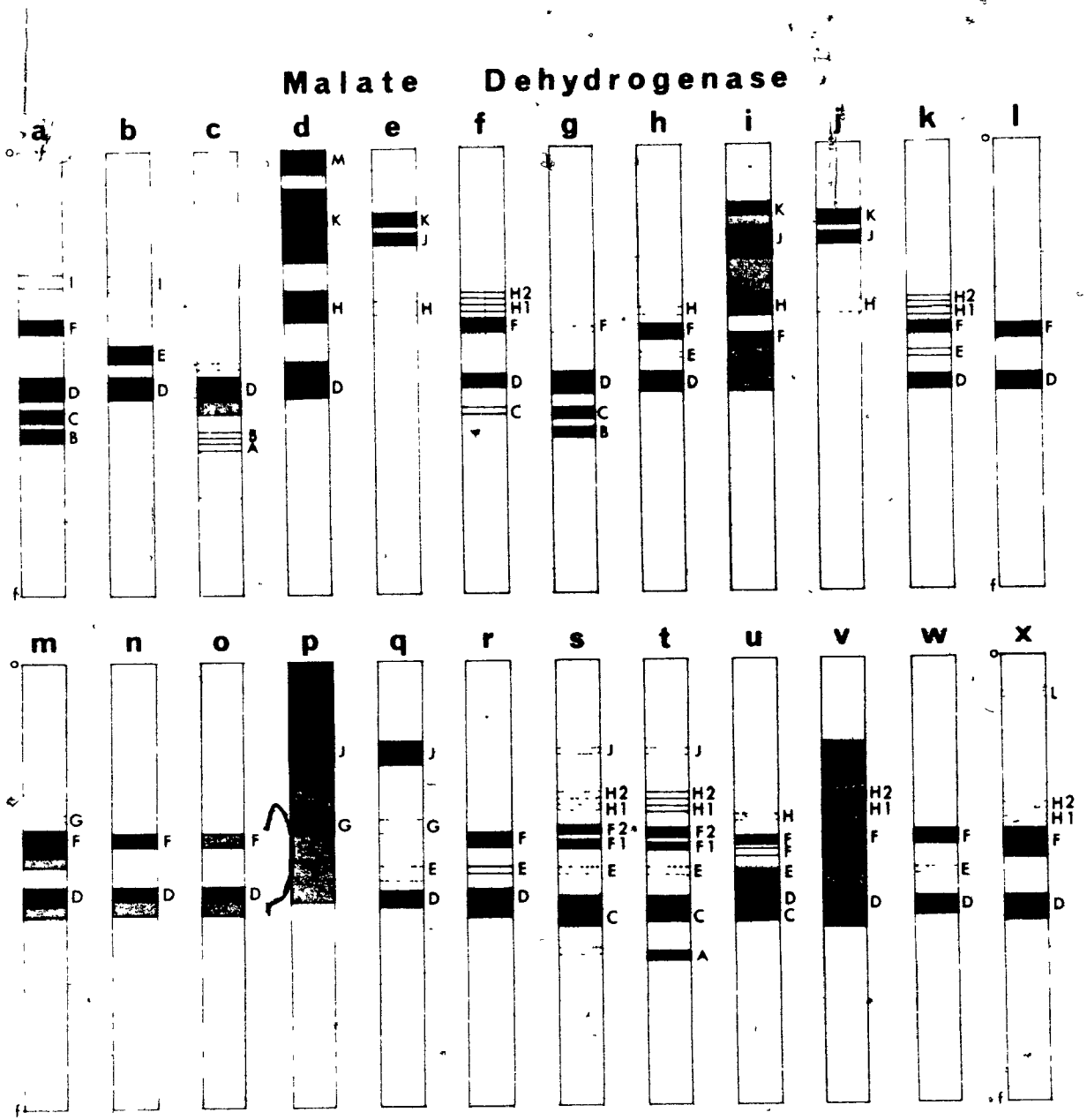
It appears from a consideration of these results that initially the suspension cultures showed a pattern similar to that of the tissue of origin but with increasing duration of sub-culture the patterns of new root and cotyledon suspension changed until all three cultures were similar in pattern to that of 5 day hypocotyl (or cotyledon).

Fig. 63.

Comparison of Malate Dehydrogenase Isoenzymes

a - 2 day embryo.
b - 5 day root.
c - mature root (6 weeks).
d - root callus, stock culture, 21 day passage 52.
e - root callus, new culture, composite of No. 2,4,8,9, 10, (28 days).
f - root suspension, stock culture, 12 day passage 141.
g - root suspension, new culture, composite of No. 59, 63, 64, 67, 68, (28 days).
h - 5 day hypocotyl.
i - hypocotyl callus, stock culture, 21 day passage 52.
j - hypocotyl callus, new culture, composite of No. 2, 4, 8, 9, and 10, (28 days).
k - hypocotyl suspension, stock culture, 21 day passage 52.
l - hypocotyl suspension, new culture, composite of No. 59, 63, 64, 67 and 68, (28 days).
m - mature stem, second internode (6 weeks).
n - petiole (fully expanded leaf).
o - leaf (fully expanded).
p - cotyledon callus, stock culture, 21 day passage 52.
q - cotyledon callus, new culture, composite of No. 2, 4, 8, 9 and 10, (28 days).
r - cotyledon suspension, stock culture, 12 day passage 141.
s - cotyledon suspension, stock culture, 2 day passage 147.
t - cotyledon suspension, stock culture, 4 day passage 147.
u - cotyledon suspension, new culture, composite of No. 59, 63, 64, 67 and 68, (28 days).
v - 1 day cotyledon (24 hr. imbibed seed).
w - 5 day cotyledon
x - immature pod (without seeds).

Malate dehydrogenase activity is represented by the degree of band intensity.


Malate Dehydrogenase
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f) Esterase

The esterase isoenzyme patterns of cultures and parts of mature plants and seedlings are shown diagrammatically in Fig. 64-A. Up to nineteen isoenzymes were detected.

As was the case with other enzyme systems discussed so far, the different seedling and mature plant parts gave different isoenzyme profiles (compare Fig. 64: a - embryo, g - 5 day cotyledon, b - 5 day root, e - 5 day hypocotyl, k - leaf, l - pod, m - stem and n - petiole).

As can be seen from a comparison of c, f and h the patterns of stock suspension cultures are distinctive in that root cultures never contained isoenzyme R. Similarly, newly derived suspension cultures (compare d and j) show no detectable isoenzyme R in root culture. Isoenzyme R is found in 5 day cotyledons (g) and in the immature pod (l). In this respect the presence of isoenzyme R may have potential value as a marker enzyme for cotyledon suspension provided it is recognized that isoenzyme R was very low in activity during the division phase of cotyledon suspension growth cycle (compare Fig. 63 h and i, See Fig. 46).

g) Acid Phosphatase

The acid phosphatase isoenzyme patterns of cultures and parts of the mature plant and seedling are shown in Fig. 64-B. A total of nineteen isoenzymes was detected.

The strongest activity and the most distinctive pattern were found in 5 day old roots (o). Isoenzymes D, E, H and I were not

detected in any other plant part or cultured cells. Comparison of the pattern from mature root (p) with that of 5 day roots (o) shows some very striking changes with development. Isoenzymes C, D, E, H, I, M and Q disappeared and isoenzymes S and R were formed. The latter were found in considerable amounts only in mature roots.

The isoenzyme patterns of 5 day hypocotyl (q) and cotyledon (v) are very different from that for 5 day root. The pattern of 5 day cotyledon (u) is very simple in that only one major band (isoenzyme A) was detected.

The AP isoenzyme patterns of mature leaf, stem and petiole are complex but nevertheless very similar (compare r, s, t). As previously described (Section IV, B-8), the AP isoenzyme patterns of root, hypocotyl and cotyledon suspension cultures were found to be essentially identical. A typical pattern is shown in Fig. 64-v. Comparison of this pattern with those for different parts of the plant or seedling shows that the 12 day culture pattern is most like that of stem (r) or petiole (s). It must be remembered that the AP pattern is known to change considerably during the growth cycle and to show very low levels at some points (see Fig. 42). All of the newly derived cultures showed low levels of AP and the simple pattern as shown in Fig. 64-w which is representative.

Fig. 64.

Comparison of Esterase and Acid Phosphatase Isoenzymes

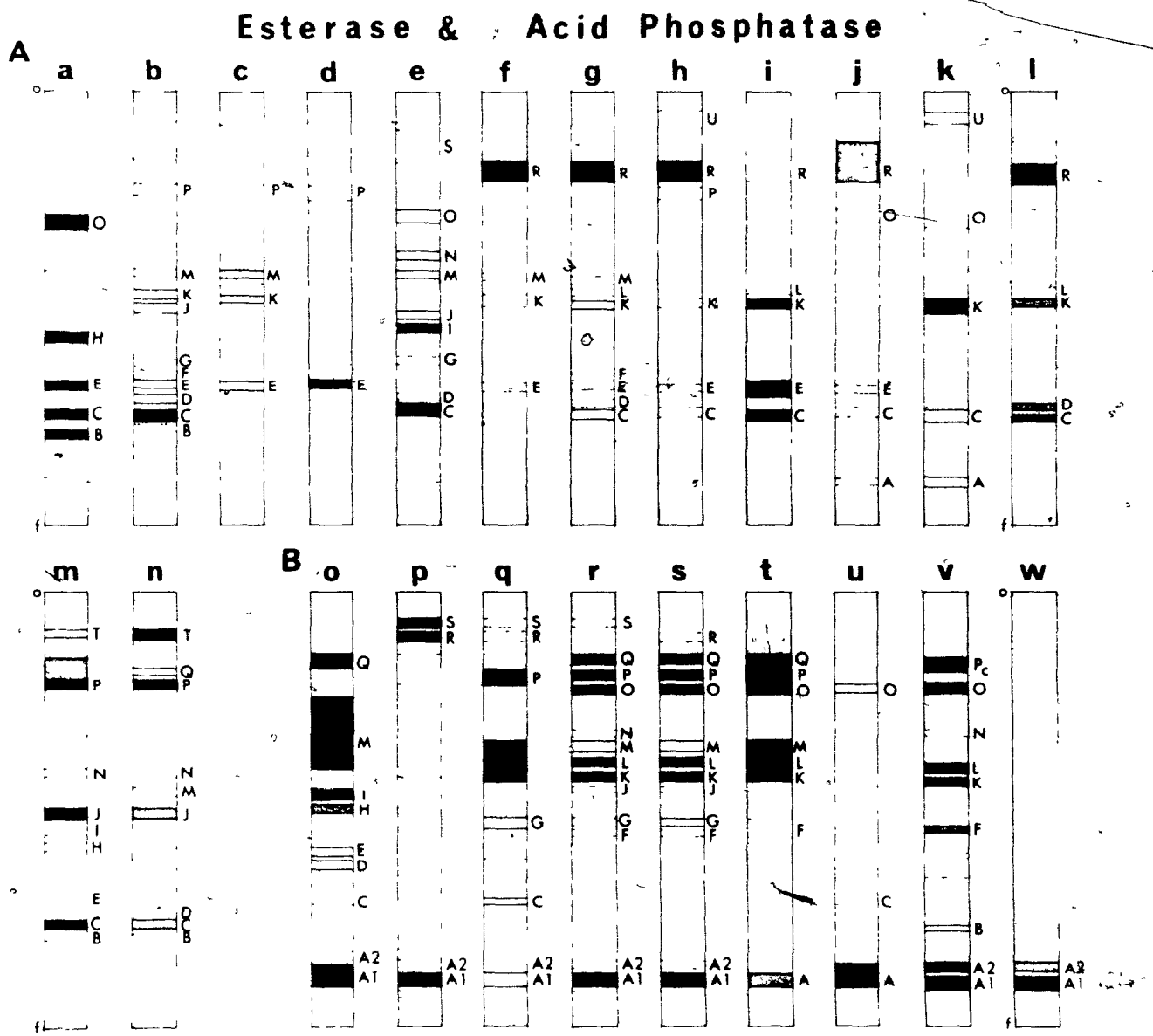
A. Esterase

a - 1 day embryo (24 hr. imbibed seed).
b - 5 day root.
c - root suspension, stock culture, 12 day passage 121.
d - root suspension, new culture No. 67, (28 days).
e - 5 day hypocotyl.
f - hypocotyl suspension, stock culture, 12 day passage 121.
g - 5 day cotyledon.
h - cotyledon suspension, stock culture, 12 day passage 145.
i - cotyledon suspension, stock culture, 2 day passage 146.
j - cotyledon suspension, new culture No. 67, (28 days).
k - leaf (fully expanded).
l - immature pod (without seeds).
m - mature stem, second internode (6 weeks).
n - petiole (fully expanded leaf).

B. Acid Phosphatase

o - 5 day root.
p - mature root (6 weeks).
q - 5 day hypocotyl.
r - mature stem, second internode (6 weeks).
s - petiole (fully expanded leaf).
t - leaf (fully expanded).
u - 5 day cotyledon.
v - cotyledon suspension, stock culture 12 day passage 126.
w - new suspension, typical pattern.

Acid phosphatase activity is represented by the degree of band intensity.


Esterase & Acid Phosphatase
A
a
b
c
d
e
f
g
h
i
j
k
l
B
m
n
o
p
q
r
s
t
u
v
w

h) Glutamate-Oxaloacetate Transaminase

The isoenzyme patterns of GOT from cultures and parts of seedlings and mature plants are presented in Fig. 65-A. Compared with the many other enzyme activities studied GOT patterns were relatively simple and remarkably uniform throughout all the tissues and cultures studied.

Isoenzymes C and A occurred in all samples analysed, isoenzyme D appeared in root hypocotyl and stem and isoenzymes E and F in intact cotyledon only. The isoenzyme patterns detected in cell cultures, both old and new, were essentially identical and equivalent to enzyme patterns of the parts of the mature plant. Isoenzyme D was not present in the 12 day cultures illustrated (h) but was detected in 6 day cultures (Fig. 45).

i) Leucine Amino Peptidase

The LAP isoenzyme patterns of cultures and mature plant parts are presented diagrammatically in Fig. 65-B. As was the case for GOT the isoenzyme patterns of LAP were relatively simple with all tissues and cultures examined.

Isoenzymes A1 and A2 were present in all but 5 day cotyledon (t) and 5 day hypocotyl (n) and isoenzyme D was present in all but mature root (k), petiole (r) and new suspension cultures (x). The most complex isoenzyme pattern was observed in 6 day suspension cultures (v). A total of six isoenzymes were detected, one of which (G) was only observed in 6 day suspension cultures.

The patterns from the established cultured cells are similar but all resemble the pattern from 5 day root rather than from hypocotyl or cotyledon. This is because of the absence of A1 and A2 in 5 day hypocotyl and cotyledon. Thus only the cell cultures from root gave a pattern similar to that of the tissue of origin (compare u, v, w, j, n, and t).

The isoenzyme pattern of newly derived cultures in all cases simply consisted of isoenzymes A1 and A2 and thereforethey too most closely resemble the pattern of root.

Fig. 65.

Comparison of Glutamate-oxaloacetate Transaminase and Leucine Aminopeptidase Isoenzymes

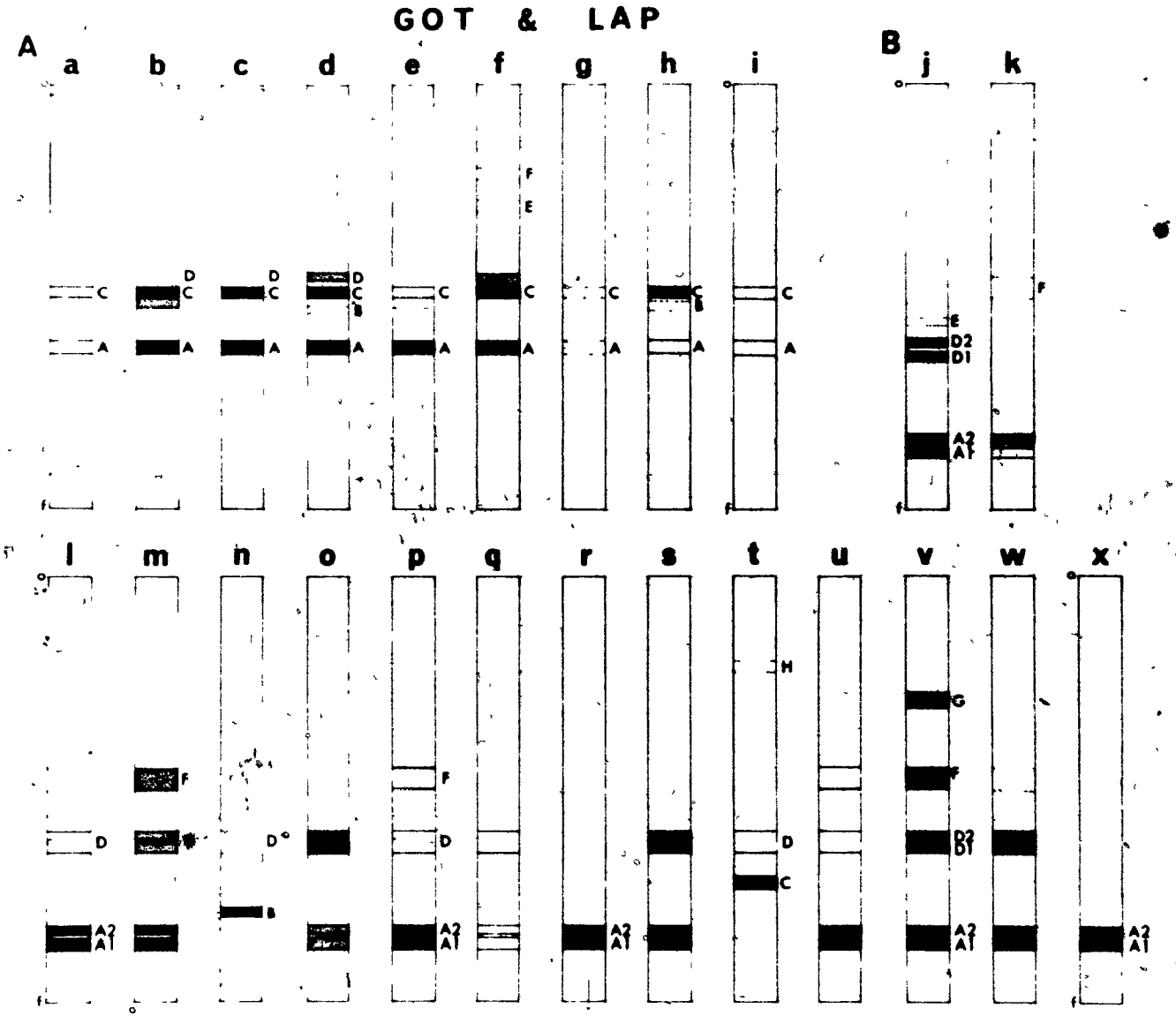
A. GOT

a - 5 day root.
b - mature root (6 weeks).
c - 5 day hypocotyl.
d - mature stem, second internode (6 weeks).
e - petiole (fully expanded leaf).
f - 5 day cotyledon.
g - leaf (fully expanded).
h - cotyledon suspension, stock culture, 12 day passage 148.
i - new suspension culture, typical pattern.

B. LAP

j - 5 day root.
k - mature root (6 weeks).
l - root callus, stock culture, 21 day passage 53.
m - root suspension, stock culture, 12 day passage 91.
n - 5 day hypocotyl.
o - hypocotyl callus, stock culture, 21 day passage 53.
p - hypocotyl suspension, stock culture, 12 day passage 91.
q - mature stem (6 weeks).
r - petiole (fully expanded leaf).
s - leaf (fully expanded).
t - 5 day cotyledon.
u - cotyledon callus, stock culture, 21 day passage 53.
v - cotyledon suspension, stock culture, 6 day passage 147.
w - cotyledon suspension, stock culture, 12 day passage 147.
x - new suspension culture, typical pattern.

The degree of enzyme activity is represented by band intensity.


GOT & LAP
A
a
b
c
d
e
f
g
h
i
B
j
k
C
D
A
F
E
D2
D1
A2
A1
l
m
n
o
p
q
r
s
t
u
v
w
x
H
G

V DISCUSSION

A) Introduction

In order to discuss the fundamental question of whether cell cultures, derived from different parts of the parent plant or seedling, show persistent differences in properties *in vitro*, and how such differences, once detected, can be related to the plant as a whole demands that many different aspects of the problem be considered simultaneously. In order to make this possible the discussion will be presented in the chronological order in which the experimentation was conducted. During the course of the work a great deal of care was exercised in order to establish what are hopefully the correct bases for comparison. Only when all the avenues of isoenzyme variation have been explored can proper comparisons be made. Many of the experiments conducted not only shed light on broader problems of growth and differentiation but additionally provided information as to the nature of the enzymes themselves.

B) Stock Callus Cultures

In order to establish isoenzyme patterns as bases for comparison it was necessary to detect the patterns during the various stages of the callus culture cycle so that any variation due to the growth of the cultures could be accounted for. After this was done comparisons between the cultures could be made on a strong footing.

1) Changes During The Culture Cycle

All enzymes studied in callus cultures, with the possible exception of catalase, underwent changes during the growth cycle. Enzyme levels are known to change during tissue culture cycles (De Jong 1967;

Olson et al 1969; Simola and Sopanen 1970, 1971; Copping and Street 1972, Thorpe and Meier 1973, Harland et al 1973), but relatively little attention has been paid to isoenzyme changes.

The present study shows differences in number and development, during the standard culture cycle, for isoenzymes of Per, LAP, and GDH. The isoenzyme patterns were seen to change the most as callus entered a phase of vigorous growth (day 6-14) after subculture. The PP isoenzymes underwent only slight changes during the culture cycle while the patterns of esterase were often too weak and too variable to be classified. GDH isoenzymes changes after subculture from a five to a one banded pattern and then in time returned to the five banded pattern in all three cultures.

2) Differences Among Stock Callus Cultures

In addition to the occurrence of changes in particular isoenzymes during the culture cycle, differences between the three cultures were established for complements of Per, MDH, and LAP. Isoenzyme patterns of PP were essentially identical in all three cultures but clear differences in the levels of certain isoenzymes were detected. The isoenzyme patterns of GDH and Cat were the same in all three cultures and in the case of Est no culture specific patterns were detected simply as a consequence of the low activity of esterase.

C) Stock Suspension Cultures

1) Comparison with Callus Cultures and Comparison of 12 day Root, Hypocotyl and Cotyledon Cultures

Comparison of the isoenzyme patterns of suspension cultures with their counterpart callus cultures shows that patterns of certain enzymes, i.e. GDH and LAP, were very similar in both types of cultures.

The isoenzymes of MDH, in contrast, were completely different in callus and suspension cultures. On the other hand certain of the faster migrating peroxidase isoenzymes are readily identifiable as present in both suspension and callus cultures, but certain of the more slowly migrating isoenzymes are quite distinct.

As was the case for callus many (up to 7) GDH isoenzymes were detected. Seven bands of GDH activity have been shown with carrot suspension cultures (Lee and Dougall, 1973) and various plant tissues (Lea and Thurman, 1972; Pahlich, 1972). The lack of extractable GDH activity, from bean cell cultures using NADP as cofactor is in contrast to results with carrot suspension cultures reported by Lee and Dougall (1973). An unusual phenomenon with the GDH from bean cell cultures was that the gels incubated with NADP after electrophoresis showed no bands but subsequent incubation in a reaction mixture containing NAD gave normal staining. It is difficult to give an explanation for this, however, relatively little is known about higher plant GDH. Glutamate dehydrogenase has been reviewed recently by Goldin and Frieden (1971).

Peroxidase as well as other enzymes, (α-amylase, I.A.A. oxidase, acid phosphatase, pectin methyl esterase, ribonuclease, ascorbate oxidase, and β-galactosidase) have been detected in medium in which plant cell cultures have been grown (Straus and Campbell 1963; Jaspars and Veldstra 1965; Olson et al 1969; Yamaoka et al 1969, Olson 1971, Misawa & Martin, 1972; Srivastava and Van Huystee 1973). Of the many enzymes studied peroxidase was the only one which showed a large amount of activity in the medium of suspension cultures. One of the most interesting aspects

of the peroxidase isoenzyme patterns studied here is that the intra and extracellular isoenzymes have different mobilities and that the isoenzyme patterns for media of cotyledon suspension differ from the corresponding patterns for root and hypocotyl cultures.

The Peroxidase (polyphenolic) activity detected in the bean medium was identical in pattern to that of peroxidase. Polyphenol oxidase activity was reported to be compartmentalized in Phaseolus mitochondria by Anderson (1968), while peroxidase activity has been found in many plant cell walls (Ridge and Osborne 1970; Gordon and Alldridge 1971). Therefore, it seems likely that the PP activity in the medium may be attributed to the peroxidases present here.

It is evident from the results with suspension cultures that considerable differences exist, between the cultures, in content and activity of isoenzyme bands of different enzymes. The most pronounced differences were detected in the case of peroxidase, polyphenol oxidase and esterase. It is important to note that the isoenzyme patterns remained relatively unchanged in the case of suspension cultures for a period of almost three years.

2) Cotyledon Suspension Cultures And The Effect of Omission of 2,4-D and Kinetin.

a) Growth, Morphology and Cytochemistry of Peroxidase Activities

Most plant cell cultures studied to date, at least to some degree, require auxin and possibly cytokinin for growth (Street, 1966a, b,

Yeoman and Aitchison 1972). Auxin requirements of tissue cultures can usually be met by the synthetic regulator 2,4-D. Tumorous and so called habituated cultures grow without an exogenous supply of growth regulators but these cultures are known to synthesize their own (Butcher 1972). Cotyledon suspension cultures used in the present studies grew without added 2,4-D and kinetin and hence presumably received from the basal medium, or synthesized, sufficient auxin and some form of cytokinin (Liau and Boll, 1971, Liau, 1971).

Inoculation of cotyledon cells, grown with 2,4-D and kinetin, into medium without regulators resulted in many marked changes. Cells in minus cultures divided faster, but stopped dividing sooner, resulting in a smaller number and fresh weight of cells than in plus cultures. A similar result was reported by Street et al (1968) for suspension cultures of Acer pseudoplatanus L. cells cultured without 2,4-D.

Concomitant with the very early increased rate of growth in minus cultures, that is without 2,4-D, was marked browning of these cells. A similar change in color of Nicotiana cells cultured without 2,4-D was reported by Vernon and Straus (1972). Cells cultured here without growth regulators showed many changes in form, most noticeable of which were the elongated, twisted cells with thickened cell walls. These cells could be regarded as being more highly differentiated than the cells in the plus medium. Very similar cells have been reported recently in cotyledon cultures of almond, Prunus amygdalus (Mehra and Mehra 1974) grown on Murashige and Skoog (1962) medium with N.A.A. as the growth regulator source.

It has been shown that, generally, the presence of 2,4-D as the auxin source leads to a state of repressed differentiation which in some cases can be overcome by the removal of 2,4-D or its replacement by some other regulator such as I.A.A. (Street 1966a & b; Steward et al 1970; Steward and Israel 1972; Vasil and Vasil 1972; Steward and Bleichert 1972; Yeoman and Aitchson 1972; Lee and Dougall 1973; Havranek and Novák 1973). The tissue cultures of almond (Mehra and Mehra 1974) that contained the elongate coiled cells also produced tracheids and under appropriate conditions could be made to regenerate plantlets. Many tissue cultures, predominantly callus, that do not regenerate whole plants still maintain the capacity to form differentiated cells such as vessel elements (Wetmore and Reir 1963; Jeffs and Northcote 1967; Fosket and Torrey 1969; Mizuno et al 1971; Torrey 1971; Wilber and Riopel 1971; Minocha and Halperin 1974). The coiled thickened and twisted cell walls of the cells that are found as a consequence of removal of 2,4-D may represent a rudimentary differentiation. This possibility is further supported by the fact that secondarily differentiating tissues are known to have high peroxidase activity associated with wall (Van Fleet 1947; Jensen 1955; De Jong 1967; Ramaiah et al 1971) such as was the case in the coiled and elongate cells formed in minus cultures.

Cells in minus medium cultured for a second passage without regulators, showed exaggerated and more disorganized forms of the coiled cells present in the first passage but by day 18 a considerable number of very small but actively dividing cells were present. These cells were presumably those adapted to growth in the absence of the growth regulators whereas the apparent differentiation of cells was caused by the switch

from one medium to another. The view that the small cells produced after two passages in minus culture represent adaptation rather than selection is supported by the fact that when an inoculum, consisting mainly of such cells, was put back into plus medium the cells in the resulting culture were the same in form and color as those in the original inoculum transferred to minus medium.

Studies of the cytochemical localization of peroxidase and PP activity showed no obvious differences between cells in plus and minus medium. It was found that activity was low in young cells and was mostly cytoplasmic in nature. Activity was much higher in older cells. The increased activity was most apparent in the wall. These findings are in general agreement with the findings of De Jong (1967) and Makinen (1968) in that onion root tips showed little or no activity in young dividing cells and increased wall associated activity with older cells. The findings are also in agreement in the observations on gradients of peroxidase activity, increasing with age of tissue, in excised tobacco pith (Galston et al 1968). Lipetz (1965) reported that peroxidase is associated with lignifying but not lignified plant cell walls. It can be suggested from the findings with bean cell cultures here that, in view of the increased activity with age and the correlation with the phase of cell expansion, peroxidase may be involved in cell wall expansion, and in the formation of differentiated structures.

The Peroxidase (polyphenolic) activity, detected cytochemically, was distributed in the cell walls in a manner identical to that of peroxidase and probably represents activity due to the same enzymes. The only noticeable difference in the localization of PP, stain as compared with

peroxidase was the staining of the nucleus or, as may be the case, the cytoplasm adjacent to the nucleus.

Results from the cell culture studies in general showed that there were only one or two PP isoenzymes that did not show strong peroxidase activity.

b) Peroxidase Activities and Changes in Isoenzyme Patterns

Experimentation on the levels and isoenzyme changes of peroxidase and PP during the culture cycle showed striking correlations with culture growth. The data will be discussed in three sections: the first two in relation to what was observed and in the third section the possible relationship of this data to fundamental problems of hormone action and growth is discussed.

i) Changes In Total Activity During the Culture Cycle

Total activity of both peroxidase and PP activity was found to be lowest during the division phase and highest during the elongation phase when measured either per unit fresh weight, per million cells or per mg extract protein. This was true in both plus and minus cultures. The increase in peroxidase activity correlated with cell expansion, together with the observed increase in activity in the cell wall during cell growth, supports the general view that peroxidase may be involved in cell wall expansion and development. A study conducted by Verma and Van Huystee (1970) with peanut suspension cultures revealed an increase in

peroxidase activity and isoenzyme complement with increasing cell clump size and heterogeneity. Peroxidase activity is known to increase with the age of plant tissues and the degree of differentiation (see Literature Review).

Furthermore, peroxidase activity was detected in the medium in considerable amounts only when the cells were actively growing. Peroxidase activity has been detected in the medium in which various plant cells have been grown (Straus and Campbell 1963, De Jong et al 1967; Olson et al 1969; Yamaoka et al 1969; Olson 1971; Misawa and Martin 1972; Srivastava and Van Huystee 1973). As was the case with the tobacco cultures of De Jong et al (1967), the electrophoretic properties of peroxidase activity detected in the medium here were not the same as those of the cytoplasmic isoenzymes and the amount of activity increased in the medium in proportion to the increase in culture fresh weight.

The studies conducted without 2,4-D and kinetin also support the view that peroxidase is involved in cell growth. In the first passage in such medium the characteristic growth produced highly elongated cells accompanied by large increases in peroxidase activity both in the cells and in the medium. During the second passage, without growth regulators, there was very little growth from days 12-18. The cells contained very high levels of peroxidase at this time but produced almost no activity in the medium. Activity then began to increase with the increase in fresh weight which began on day 18 when the proliferation of small nondifferentiated cells began. Thus the presence of peroxidase in the medium was correlated with cell expansion and was not a consequence of levels of internal or wall associated peroxidase.

ii) Changes in Isoenzyme Patterns and Activity During The Culture Cycle

As was the case for callus cultures the isoenzyme patterns of both peroxidase and PP in suspension cultures changed during the culture cycle. Certain isoenzymes of peroxidase were only present or prominent during specific phases of the culture cycle. For example, regardless of treatment, isoenzyme D2 was only present when cells were entering the division phase. Isoenzyme D2 was most prominent during the time of lowest cell peroxidase activity and hence indicates a specialized role perhaps unrelated to isoenzymes which may be associated with the wall and involved in cell expansion. This is also the case with isoenzyme B which also shows strong PP activity which is prominent during the middle of the culture cycle.

The effects of omission of growth regulators were mostly quantitative in nature as found with carrot tissue culture (Lee and Dougall 1973). However, there were some qualitative changes in the isoenzyme pattern. Most striking of these was the smearing, with resulting loss of resolution, of isoenzyme bands in the minus cultures. Evans (1968) reported a smearing of peroxidase activity which had been recovered from the cell wall of pea. Peroxidase isoenzymes are known to be glycoproteins (Shannon et al 1966; Welinder and Smillie 1972; Darbyshire 1973). It is therefore possible that culture in minus medium may result in a peroxidase glycoprotein which is more heterogeneous as a consequence of a more limited addition of polysaccharide. In support of this view is the fact that with increasing time in minus culture there was an increase in both smearing and in the predominance of the smaller molecular weight isoenzymes in both the cytoplasm

and the medium. Preliminary experiments with β-galactosidase treated media indicate the enzyme affected the peroxidase banding pattern.

PP isoenzymes present in the medium displayed isoenzyme patterns identical with those of peroxidase and therefore probably reflect activity of the same enzymes.

iii) Speculation on the Role of Peroxidase in Cell Growth

The purpose of the present section is to relate some of the findings from bean cell cultures, together with the data of other workers, to the question of hormone action and cell growth. The ideas that have been generated extend considerably beyond the scope of the present study; therefor, they will not be stated in detail. However, the main aspects are brought out in the hope that they may stimulate interest in the possibilities for further research.

The mechanism by which cell walls elongate or grow, and how this process is stimulated by auxin, and possibly other hormones, remains a mystery. Inspection of the properties of peroxidase may shed new light on this problem.

As was true with the bean cell cultures described here it has been shown, with elongating Avena coleoptiles, that increases in activity of various peroxidase isoenzymes are correlated with cell elongation (Chappet and Dubouchet 1972; Gordon and Henderson 1973). The bean cell cultures showed a release of large quantities of polysaccharide correlated with growth (Mante 1974). Recently Labavitch and Ray (1974) have shown a relationship between auxin promoted liberation of xyloglucan polymers from cell walls and elongation in pea. It is known that large amounts of peroxidase activity are associated with cell walls and that peroxidase is

related in some way to the hydroxyproline-rich protein network found in plant cell walls (Ridge and Osborne 1971). If we assume the cell wall matrix to be somewhat like the model proposed by Keegstra et al (1973) it is possible that peroxidase may be related to the release of xyloglucans from elongating cell walls by the peroxidative breakage of the serine-hydroxyl connection between the cell wall protein and the polysaccharide network.

This idea becomes more plausible in consideration of some additional properties of peroxidase. For some time now many workers have considered that the in vivo role of peroxidase is the oxidation and hence inactivation of auxin as a hormone. The mode and site of action of auxin is thought of separately and remains obscure. Peroxidase is thus envisioned to control growth by controlling the levels of IAA. Much of the confusion in relation to the function of peroxidase stems from the properties of the enzyme itself. It must be remembered that peroxidase is a redox enzyme, and the methods used to detect peroxidase activity are very non-specific. The specificity of the different isoenzymes of peroxidase are actually quite varied (Chmielnicka et al 1971), and it is therefor likely that peroxidase is widely involved in many cellular activities. Certainly, one of these is in relation to cellular redox levels. It is known that cell redox levels may be important in the control of cell activities, and furthermore, it has been reported that the reduced state favors cell division and a more oxidized state favors differentiation (Van Fleet 1954; Szent-Gyorgyi 1968, Stonier 1971). It has also been shown that peroxidase, with Mg^{++} and IAA or monophenolic cofactors, can oxidize reduced pyridine nucleotides (Akazawa and Conn 1958). It is suggested

that IAA and similarly shaped small molecular weight monophenols function as electron transport cofactors in a system that channels the reducing power of cellular NADH or some comparable agent, into the reduction of bonds which will allow cell wall expansion. In this way the mode of action of auxin is seen as directly related to cellular activity. When the store of cellular reducing power is depleted or channelled in some other direction the indoleacetic acid will all become oxidized. It is readily apparent that small molecular weight monophenolic 'auxin protectors' (Stonier 1971) could function as redox buffers.

With this idea in mind that IAA may, in effect, have some direct action as a cofactor in an enzyme system leading to a loosening of cell wall structure, rather than as an agent affecting production of an enzyme protein, it is tempting to speculate on the mode of action of other plant hormones. It is known that plant growth substances possess metal chelating properties and that some chelating agents can mimic the action of plant growth regulators (Heath and Clark 1956 Cohen et al 1958; Maheshwari and Seth 1966, Chopra and Rashid 1969, Oota and Tsuozuki 1971; Kochlar et al 1971) although it is evident that chelation cannot explain all the known actions of regulators. However, kinetin, a compound that specifically causes cell division chelates iron, (Oota and Tsuozuki 1971), and in this respect could, under some circumstances, affect peroxidase activity. It was found by Lee (1974) that the addition of high kinetin levels to the medium of tobacco callus resulted in a 90% drop in peroxidase activity. It is therefor suggested that one of the actions of kinetin may be directed towards the control of cellular redox levels by the modulation of peroxidase activity. If kinetin did work in this manner its presence would encourage a generally reduced state, exactly that which is known to occur during cell division (Szent-Gyorgyi 1968).

The point of the digression has now been made. It is conceivable that the mode of action of auxin in growth is associated with cellular redox levels through interaction with peroxidase and that peroxidase (probably different isoenzymes) itself located in the cell walls results in the breakage of specific bonds by a redox mechanism. It is conceded, of course, that in a highly structured and interconnected cell wall matrix other cell wall metabolizing enzymes could play additional important roles.

c) Activity and Isoenzyme Patterns of Other Enzymes

Other studies on the levels of enzymatic activity during cell culture cycles have shown, as might be expected, that the activities of enzymes during the phase of cell division were very different from those of stationary phase cells (De Jong et al 1968; Simola and Sopanen 1970; Fowler 1971; Copping and Street 1972; Thorpe and Meier 1973; Aitchison and Yeoman 1973; Harland et al 1973).

In the present study changes in LAP, MDH, GDH and GOT showed generally increased levels in young dividing and expanding cells as compared to stationary phase cells. This is in agreement with results of De Jong (1968) and Simola and Sopanen (1970) for tobacco and Acer suspension cultures respectively.

Acid phosphatase showed a different pattern of change in that activity was highest in stationary phase cells and lowest during cell division. This too, is in agreement with the findings of others (Simola and Sopanen 1970; Suzuki and Sato 1973). Acid phosphatase activity has been localized in cell walls (De Jong et al 1968; Johnson et al 1973) and has been found in the medium in which plant cells have been grown (Straus and Campbell 1963; Yamaoka et al 1969, Ueki and Sato 1971). AP activity is increased in cell walls and the medium as a consequence of low inorganic phosphate and has been suggested as part of a transport system (McLean and Gahan 1970; Ueki and Sato 1971, Suzuki and Sato 1973).

Acid Phosphatase in the present study increased in the medium with time and reached maximum levels on and after day 6. Medium

from cultures grown two passages without growth regulators showed no detectable AP activity. Thus as was the case for peroxidase there is a correlation between medium AP activity and cell growth and may indicate the stoppage or sharp slowdown in energy dependent transport processes in these cells.

As was the case with callus cultures, all the isoenzymes studied in suspension cultures showed changes in pattern during the culture cycle. These changes in pattern can be separated, generally, into three types depending on when, during the culture cycle, the changes were most striking. The types are as follows:

i) The pattern and activity of AP isoenzymes were strongest and most complex during the stationary phase. The activity and isoenzyme pattern almost completely disappeared during cell division and reappeared during the cell expansion phase.

ii) The isoenzyme patterns of MDH, GDH and Est changed very strikingly when the cells were inoculated into fresh medium. These changed patterns were maintained during the period of cell division but then returned to the original stationary phase pattern during the log growth and expansion phase. The most dramatic shift in pattern was shown by the esterases.

iii) The third type of isoenzyme pattern change was exhibited in the case of LAP and GOT. The activity and isoenzyme patterns changed less quickly and became most pronounced during the middle portion of the culture cycle and then decreased again during the stationary phase.

Thus in total the isoenzyme pattern changes closely reflect changing growth phases during the culture cycle.

The changes that occurred in MDH and GDH banding pattern may be due to changes in subunit conformations. Conformers of both MDH and GDH are believed to exist (Kitto et al 1966; Roberts 1969; Fahlich 1972; Zeigler 1974). The evidence is most substantial in the case of GDH. The six subunits of bovine liver GDH have been sequenced and found to be identical in amino acid content (Moon and Smith 1973). Similarly the amino acid sequence of chicken liver GDH subunits were found to be identical (Moon et al 1973). The two immunologically distinct Neurospora GDH subunits were found to be products of the same gene as a single mutation removed both forms of the enzyme (Roberts 1969). The change in pattern and activity of the individual GDH isoenzymes of both callus and suspension cultures is consistent with the idea that there is a conformational change from an inactive, or a less active, to an active form of subunit. Isoenzyme A is envisioned as containing 6 active subunits, isoenzyme B, five, etc. The change in conformation occurred when the cells entered the division phase or a period of increased metabolic activity.

The effects of omission of growth regulators on the expression of isoenzyme pattern were, as in the case of peroxidase and PP , predominantly quantitative in nature. With the exception of MDH isoenzyme A, and perhaps a minor band of both LAP and Est, all the isoenzymes were detected in cultures grown both with and without growth regulators. Without exception, however, there were quantitative differences in isoenzyme bands and many differences in the time of detection during the culture cycle. These

differences were greatest during the second passage without growth regulators. It is apparent that the isoenzyme pattern and isoenzyme pattern changes can most clearly be related to the phase of the culture cycle regardless of whether the cultures were grown with or without growth regulators. The only known comparable study was conducted with wild carrot suspensions (Lee and Dougall 1973) and gave essentially analogous results. The carrot cells were grown in medium with and without 2,4-D. The cultures without 2,4-D differentiated and many embryoid-like structures were formed. It was found, however, that there were no differences in the patterns of MDH, AP, GOT, γ-glutamyl transferase. Quantitative differences in peroxidase and a minor qualitative difference in esterase were detected. The largest differences were detected in the pattern of GDH isoenzymes, but these differences could be due to the different conformational forms associated with different levels or type of activity.

It would appear that there is a good basis for the conclusion that the isoenzyme profile displayed by the cells is very diagnostic of the cellular activities. In the case of the bean cells the pattern of isoenzymes can be used to identify the different phases of the culture cycle. Cells that are undergoing a stage of rapid cell division display phase typical isoenzymes regardless of the factors that resulted in the cell division.

D) Growth of New Cultures and Comparison of Isoenzyme Patterns With Those of Seedling and Mature Plant Parts

1) Introduction

In the present section of the discussion the isoenzyme patterns of newly established cultures are compared and evaluated. In order to establish relationships, if they exist, with the plant as a whole, the culture patterns are then compared with those of both seedling and mature plant parts. The key issues in this section are: can culture specific isoenzyme patterns be identified and is there any evidence that differences between cultures are related to the tissue of origin?

2) Isoenzyme Patterns of Newly Established Callus Cultures

The results from callus cultures showed that generally the isoenzyme patterns were quite similar in all cultures examined, however, some potential marker enzymes in the case of peroxidase isoenzyme C2 and F (Fig. 60) and MDH isoenzymes D and E (Fig. 63) were detected. The variation in enzyme patterns made it difficult to establish clear marker isoenzymes. The patterns of some enzymes, e.g. PP and GDH, were essentially identical in all cultures studied and hence provided no origin specific isoenzyme markers. However, the observations were made on four-week-old callus and it must be noted that most of the differences detected between stock callus cultures were much more apparent during the stage of active growth of callus (Fig. 3-7).

3) Isoenzyme Patterns of New Suspension Cultures

Culture specific isoenzyme patterns were detected in suspension cultures for peroxidase, PP , MDH, and Est. The patterns of LAP, GOT and AP

were essentially identical in all cultures examined. Some variation in the isoenzyme patterns of peroxidase accounted for some overlap of potential marker isoenzymes. For example, strong isoenzyme A and B activity was detected in all cotyledon cultures examined, weak activity for these bands sometimes occurred in root cultures and in one occasion in a hypocotyl culture. Nevertheless, the isoenzyme patterns were clearly culture specific.

The isoenzyme patterns of MDH were also very different in the three cultures. The interpretation of these differences is somewhat complicated by the fact that MDH patterns changed considerably during the culture cycle (Fig. 48, 49). The esterase, medium peroxidase and PP patterns of new suspension cultures were quite similar but certain consistent differences were detected.

Thus, undoubtedly the differences detected between the cultures were not artifacts or chance variations and thus differentiation into specialized tissues may involve changes, irreversible under the conditions of these experiments, that were transmitted in cell lineages. There are, however, some other considerations because differences in enzymes between cultures have been attributed to other factors. It is possible that some differences between cultures may be a consequence of selection during the initiation and early subculture of cells and hence could represent a type of frozen accident. It is well known that the growth of cell clones and even further subclones has resulted in the isolation of stable culture lines differing in many respects. Differences in clones have been detected in growth, color, friability, adaptability to different media, ploidy and nitrogen requirements (Muir et al 1958; Arya et al 1962; Cooper et al 1964;

Sievert and Hildebrandt 1965; Davey et al 1971; Lutz 1971; Wright and Northcote 1973; Harvey et al 1973). It must be noted, however, that single isolated cells of tobacco when induced to regenerate plantlets produced large numbers of apparently identical plants (Vasil and Hildebrandt 1967). It seems unlikely that the processes that account for the differences reported between cloned cultures could account for the differences observed here between the bean cultures in as short a period as one or two passages.

Observations on the morphology and growth properties of cultures studied here, at least with suspension cultures, showed a simplification of form and a decrease in the size of the cells with time (Liau 1971). In this respect it could be argued that the differences between the cultures whether short-term or long-term cultures, were due to selection and were possibly related to changes in chromosome number and type which are known to occur in many plant cell cultures (Partanen 1963, 1965; Murashige and Nakano 1967, Torrey 1967; Shimada et al 1969; Heinz et al 1969; Steward et al 1970; Shimada 1971). Such increases and duplications of chromosomes might therefore account for variations in enzyme levels between cultures of the same or different origins. Experimentation with the trisomic lines of Datura stramonium has shown that a simple dosage relationship is often found between the number of structural gene copies and the enzymatic activity expressed by the cultured cells (Carlson 1972). Thus although changes in chromosome complement might affect the level of activity they would not necessarily affect the isoenzyme pattern. Furthermore, it is clear from the work reported here that isoenzyme patterns of the stock cultures remained stable for a period of more than three years regardless

of any changes on ploidy which might have occurred. Quantitative estimation of changes in ploidy in these cultures is difficult, for various reasons, but some changes have been recorded (Sein 1974).

The results with bean cells therefor suggest that specific and characteristic differences are detectable in the isoenzyme patterns of root, hypocotyl, and cotyledon cell cultures.

4) Comparison of Isoenzyme Patterns of Cultures and Seedling and Mature Plant Parts

Comparison of the isoenzyme patterns of the seedling parts from day one through five, and of the various mature plant parts, confirmed many established characteristics of isoenzyme patterns (see Literature Review). Thus, during development, there were changes in the activity and isoenzyme patterns of all enzymes studied and the patterns of isoenzymes were generally specific for the various parts of the seedling and mature plant.

Comparison of the isoenzyme patterns of suspension cultures with those of callus cultures revealed that characteristic differences in isoenzyme pattern exist between suspension and callus cultures in the case of Per, PP and MDH. These differences are most striking in the case of MDH. Thus, callus cultures all showed wide MDH bands of slow mobility which were not detected in any other cultures or part of the mature plant. Apparent oligomers of high molecular weight MDH have been detected in bean, (Phaseolus vulgaris cv. Pencil Pod Black Wax), (Habig and Racusen 1968, 1974), and cotton (O'Sullivan and Wedding 1972a, b).

The isoenzyme patterns of newly derived cultures were in many cases different from those of established stock cultures. Again this

was most apparent in the case of MDH patterns. The MDH patterns were strikingly different in all three new cultures but with time the cotyledon and root patterns changed to be more like but not identical to, the pattern of hypocotyl suspension which remained relatively unchanged.

The peroxidase isoenzyme pattern of newly derived cotyledon suspension cultures, after the first passage, was essentially identical to that of six-year-old stock suspension cultures. The pattern of root cultures, however, was different in newly derived and established suspension cultures. With time the pattern of root cultures became more like that of cotyledon cultures.

The isoenzyme patterns of PP , LAP and AP were very similar in the newly established cultures and in comparison with established cultures were relatively simple. With duration of subculture the PP patterns became more complex and the isoenzyme patterns of the three cultures appeared to diverge.

Comparison of the isoenzyme patterns of cell cultures with parts of the seedling and the mature plant shows a variety of different correlations. In the case of peroxidase it is quite surprising to find that the characteristic isoenzymes A and B, which can most easily be used to identify the newly derived cotyledon suspension cultures, were only found in root (Fig. 59b, c). The isoenzyme patterns of 5 day hypocotyl or cotyledon were very simple and bore no resemblance to any cell culture pattern. The patterns of stock root and hypocotyl callus, and somewhat less so those of newly derived root cultures, also showed close similarities to the pattern in roots. The hypocotyl suspensions, however, lacked the

characteristic isoenzymes A and B of root. In this and other respects the newly established hypocotyl cultures show some similarities to the pattern of 5 day old hypocotyl but overall, the patterns of all cultured cells most closely resemble the pattern of intact roots.

The patterns of PP of stock cultures were similar to those of peroxidase and showed no close correlation with the PP patterns of seedlings or mature plants. In contrast, the patterns of new cultures because of their relative simplicity showed patterns not unlike many parts of the seedling and mature plant.

The patterns of SDH isoenzymes in newly derived suspension cultures were strikingly like those of the tissue of origin. This is most clear in the case of root cultures due to the presence of isoenzymes B, C and D (Fig. 62). It is equally striking that the patterns of root and cotyledon with time became more like that of hypocotyl cultures. The pattern of the hypocotyl suspension cultures changed little and maintained the resemblance to 5 day hypocotyl which was like the pattern from various parts of the mature plant.

Comparison of the patterns of esterase are difficult due to the large number and variability of the isoenzyme bands, however, one marker isoenzyme was identified. Isoenzyme R, although sometimes low in activity during the division phase of the cotyledon culture cycle, was not present in either root or hypocotyl cultures. In the intact plant isoenzyme R was detected only in 5 day cotyledon and perhaps in mature leaf (Fig. 63).

The LAP isoenzyme patterns of 5 day hypocotyl and cotyledon were distinctive due to the absence of isoenzymes A1 - A2 which were present in all other tissues and all of the cultures examined (Fig. 65). Because of this and the presence of other isoenzyme correlations the patterns of cultures were most like those of the root. In contrast the isoenzyme patterns of AP, which were identical in all stock cultures showed the closest resemblance to the patterns from mature leaf or petiole (Fig. 64).

The isoenzyme patterns of GDH and GOT were found to be similar in all tissues and cultures examined and therefore were of no obvious value as marker enzymes in this study.

A particularly interesting observation from the comparisons of isoenzyme patterns is that the isoenzyme patterns of root, hypocotyl and cotyledon, whether identical or not, all tended to resemble the pattern of one part of the mature plant. The pattern of the cultured cells, in most cases, for example peroxidase, resembled those of root. The culture patterns of MDH and AP, however, more closely resemble the patterns of hypocotyl, stem or petiole. It is possible that culture conditions (e.g. oxygen tension) and the lack of chloroplasts and thus the lack of chloroplast associated activities and other factors result in the predominance of patterns that most closely resemble root. The implication is, however, that the culture conditions cause the development of isoenzyme patterns, which in the intact plant, represent at least part of the process of differentiation of one particular part. Presumably, therefore, the cultures could provide the opportunity to analyse the control of that part of the process of differentiation.

In summary the following general principles are recognized as a result of comparisons of the isoenzymes:

1) Isoenzyme patterns of cell cultures are, in general, characteristic and persistent differences between cell lines are maintained in culture.

2) In some cases, (Per, MDH, Est), the differences detected in cell cultures were related to the tissue of origin; the stability of these differences varied depending upon the enzyme studied.

3) The isoenzyme patterns of cultured cells often tended to be very similar, particularly GDH, LAP, GOT, and regardless of tissue of origin most isoenzyme patterns were more like the pattern of one part of the plant, this was most often root.

The issue of whether differentiation into specialized tissues and organs may involve irreversible changes and differences that are transmitted in cell lineages, that these differences may be related to the tissue of origin and the way that plant cells might provide evidence is discussed most explicitly by Heslop-Harrison (1967).

Heslop-Harrison states that "there is abundant evidence of the transmission of differentiated states or tendencies through tissues cultured in vitro where the explant has been relatively massive." The extent of the value of the work cited in support of this statement is somewhat doubtful although there is other supportive evidence (see Literature Review). The results reported here with bean cell cultures which were derived from relatively large explants because of the use of electrophoretic techniques and the resultant detection of isoenzyme markers, represent the

best positive evidence to date. Heslop-Harrison considered that proof of the transmission of differentiated states in cell cultures would be obtained if "clones derived from single cell explants of different tissues of the one parent indefinitely betray signs of their origin, yet cells from which can be shown in other conditions to be totipotent."

The establishment of cell cultures from single cells would ensure that the differentiative behavior in individual cells was not governed by cell-cell interactions or metabolites emanating from other parts of the founding explant. In this respect it is clear that the logical extension of the present work with bean cultures is to continue with studies of single cell clones preferably derived directly from parent plant tissues. Preliminary experimentation with established stock cultures has shown that the establishment of single cell clones is possible.

To eliminate the possibility that clonally transmitted differentiated states were caused by mutational changes that had occurred, and were not the result of intercellular controls that were not switched to a ground condition, Heslop-Harrison maintained that the cells in question must be shown to be totipotent. The demonstration of totipotency must be considered an important factor, however, it does not necessarily preclude the fact that genetic change has occurred. For example aneuploid plants have been regenerated from tobacco tissue cultures, the number of chromosomes did not affect the plants ability to show totipotency although they may be deficient in other ways (Sacristan and Melchers 1969).

In a similar light the fact that certain plant cultures do not show totipotency does not necessarily mean that this is a result of genetic change. Halperin (1973) suggests "that the failure of many tissues to form organs in culture is a manifestation of incomplete dedifferentiation, in the sense that the cells retain epigenetic machinery characteristic of their differentiated function in the tissue of origin."

The bean cell cultures used in the present experiments have, as yet, shown little tendency toward plantlet regeneration. There are favorable indications of asymmetric and polar patterns of growth of suspension cultures (Liau 1971) which suggests that the generation of plantlets may be possible under the appropriate conditions. It has been reported (Wu and Li 1970) that the esterase patterns of rice callus cultures derived from different parts of the parent plant were identical. It is known that rice cultures may readily regenerate plantlets of many ploidy levels (Nish[illegible] and Mitsuoka 1969). This is scant evidence as yet, but the possibility exists that there may be an inverse relationship between ability to express totipotency and the maintenance of differences in culture. It is therefore clear that the bean cell culture work must be continued to include the establishment of single cell clones and to explore the possibilities of plantlet regeneration from these cultures.

It is evident from this discussion that the data available at present are too limited to formulate generalities as to the persistence of replicative differentiated states in plant cell cultures. The results from bean cell cultures show that persistent differences, which may represent such states, can be detected between cell cultures and that some of these differences bear a relationship to the tissue of origin. The results also show the obvious value of isoenzyme pattern analysis in studies of this type.

VI CLAIMS OF ORIGINAL CONTRIBUTION TO KNOWLEDGE

To the best of my knowledge the work reported in this thesis is the most comprehensive study of isoenzymatic characterization of plant cell cultures conducted to date. The following points are, in the author's opinion, the most important items:

1) The detection of persistent isoenzymatic differences in stock callus and suspension cultures derived from different parts of a single bean seedling is reported.

2) The comparison of isoenzyme patterns of newly derived cultures with those of seedling and mature plant parts showed that isoenzymatic differences related to the tissue of origin could be detected. The stability of these differences varied with the enzyme studied; nevertheless, evidence for the persistence of a replicative differentiated state in plant cell cultures was obtained.

3) The isoenzyme patterns of cultured cells from root, hypocotyl and cotyledon, whether different or not, tended to resemble most closely one part of the parent plant, in most cases this was root.

4) Changes in isoenzyme patterns during the culture cycle were detected and the patterns of change correlated with the different phases of growth of the culture cycle.

5) Omission of the growth regulators from the medium of cotyledon suspension cultures affected the growth, cell form, the levels of enzyme activities, the timing of isoenzyme changes and the relative amounts of the different isoenzymes, but apart from the smearing of peroxidase activity, it did not affect the isoenzyme complements.

6) A correlation was observed between culture growth and the peroxidase levels in both cells and medium. This suggests a possible role

of peroxidase in cell expansion. It is suggested that this could involve a redox mechanism with auxin.

7) The value of isoenzyme patterns for the characterization of plant tissue cultures is demonstrated by the work.

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APPENDIX 1.

The composition of M3 and M4* medium. (referred to as Liau medium L^+ or L^-).

A. Inorganic salts

Major Elements		Minor Elements	
Compound	mg/l	Compound	mg/l
NH_4NO_3	1650	$MnSO_4 \cdot 4H_2O$	22.3
KNO_3	1900	$ZnSO_4 \cdot 4H_2O$	8.6
$CaCl_2 \cdot 2H_2O$	332	H_3BO_3	6.2
$MnSO_4 \cdot 7H_2O$	370	KI	0.83
KH_2PO_4	170	$Na_2MoO_4 \cdot 2H_2O$	0.25
		$CuSO_4 \cdot 5H_2O$	0.025
		$CoCl_2 \cdot 6H_2O$	0.025

B. Organic Constituents

Substance	mg/l	Substance	mg/l
Coconut milk	150 mls	Riboflavin	0.1
2,4-D**	2	Thiamine HCl	1
Kinetin**	0.64	Nicotinic acid	0.4
Sequestrene Na Fe-EDTA	50	Pyridoxal HCl	1
Meso-inositol	200	Choline chloride	1
Glycine	6	Calcium pantothenate	1
L-Glutamic acid	50	Nicotinamide	1
L-Aspartic acid	50	Biotin	1
Agar*	8400	L-Glutamine	100
Sucrose	20000	Urea	50
		L-Asparagine	25
		Adenine SO_4	5
		Vitamin B_{12}	0.0015
		Folic acid	1

*M4. The liquid media M4 is the same as M3 solid except that the inorganic salts are used at 1/4 strength and the agar is omitted.

** The growth regulators are omitted from L^+ to form L^- medium.

The pH of the medium was adjusted 5.6 with 0.2N NaOH, and medium was distributed in flasks and then autoclaved.

APPENDIX 2.

Synthetic Medium

The composition of Kaa medium. (Modified Gamborg BV by Mante.)

A. Inorganic Salts

Major Elements		Minor Elements	
Compound	mg/l	Compound	mg/l
KNO_3	2500	$MnSO_4 \cdot 4H_2O$	10
$(NH_4)_2SO_4$	400	$ZnSO_4 \cdot 7H_2O$	2
$MgSO_4 \cdot 7H_2O$	370	H_3BO_3	3
$CaCl_2 \cdot 2H_2O$	150	KI	0.75
$NaH_2PO_4 \cdot H_2O$	150	$Na_2MoSO_4 \cdot 2H_2O$	0.25
		$CoCl_2 \cdot 6H_2O$	0.025
Na Fe EDTA	40		

B. Organic Constituents

Compound	mg/l	Compound	mg/l
2,4-D	2	m-Inositol	100
Kinetin	0.64	Glycine	1.2
Adenine SO_4	1.0	L-Glutamic acid	10
Riboflavin	0.1	L-Aspartic acid	10
Thiamine	1.0	L-Glutamine	20
Nicotinic acid	1.0	Urea	20
Pyridoxyl HCl	1.0	Asparagine	5
		Sucrose	30,000

APPENDIX 3.

Stock Solutions for Electrophoresis*

Solution A.

1N HCl	24	ml
tris	18.1	gm
TEMED	0.12	ml
water	to 100	ml

(pH 8.9)

Solution B.

1N HCl	48	ml**
tris	5.98	gm
TEMED	0.46	ml
water	to 100	ml

(pH 6.7)**

Solution C.

acrylamide	28.0	gm
bis acrylamide ..	0.735	gm
water	to 100	ml

Solution D.

acrylamide	20.0	gm
bis acrylamide	5.0	gm
water	to 100	ml

Solution E.

riboflavin ... 4mg/100 ml

Solution F.

sucrose ..... 40% w/v

Solution G.

catalyst: ammonium persulfate ..... 0.14 gm/100 ml

Solution H.

tank buffer .... (pH 8.3)

tris	6.0	gm
glycine	28.8	gm
water	to 1000	ml

(diluted x 5 for usage)

Solution I.

Extraction buffer

0.059M tris HCl pH 6.9

containing 0.005M cysteine hydrochloride

0.0005M EDTA

Small Pore gel: 1 pt A, 1 pt C, 2 pts G

Large Pore (spacer) gel: 1 pt B, 1 pt D, 1 pt E, 2.5 pts F

Rinse solution: 1 pt B, 1 pt E, 6 pts H_2O.

* Essentially as described by Davis (1956).

** pH adjusted with 1N HCl.

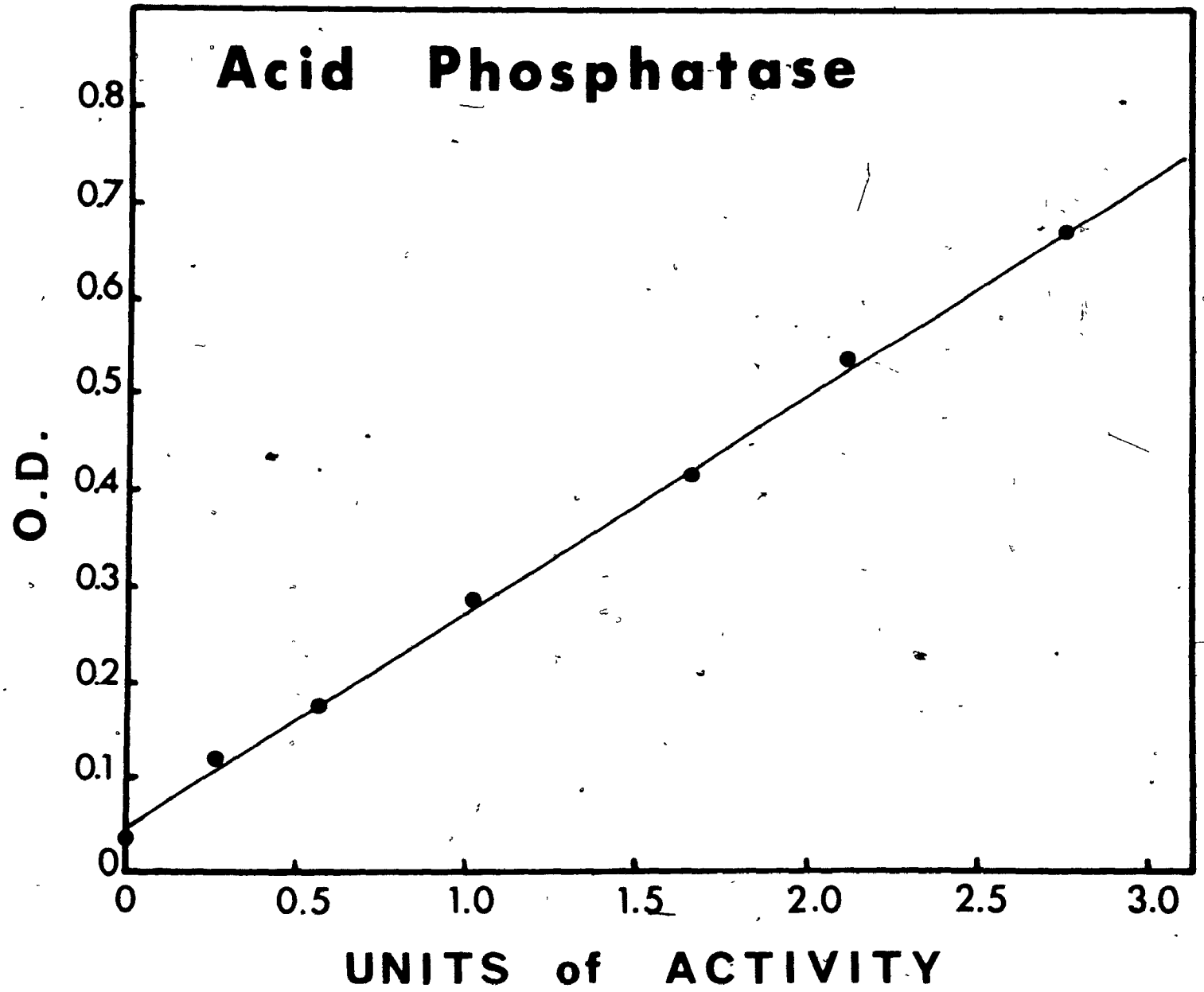
Acrylamide used for electrophoresis was obtained from BDH Chemical Company. Recrystalized acrylamides were purchased from Eastman Kodak Ltd. and TEMED (N,N,N',N' - tetramethylethylenediamine) from Canalco (CANAL Industrial Corp., Rockville, Md). All other chemicals were obtained locally.

APPENDIX 4.

Standard curve used for the determination of acid phosphatase activity was described in Sigma Technical Bulletin No. 104. One Sigma Unit of phosphatase will liberate one micromole of p-Nitrophenol per hour. (1μM = 0.1391 mg). All assay conditions were temperature regulated to 25C.

APPENDIX 5.

Standard curve used for the determination of leucine aminopeptidase activity as described by Sigma Technical Bulletin No. 251. One Aminopeptidase Unit will release one micromole of β-Naphthylamide from L-Leucyl-β-Naphthylamide per hour at pH 7.1. All assay conditions were temperature regulated to 25C.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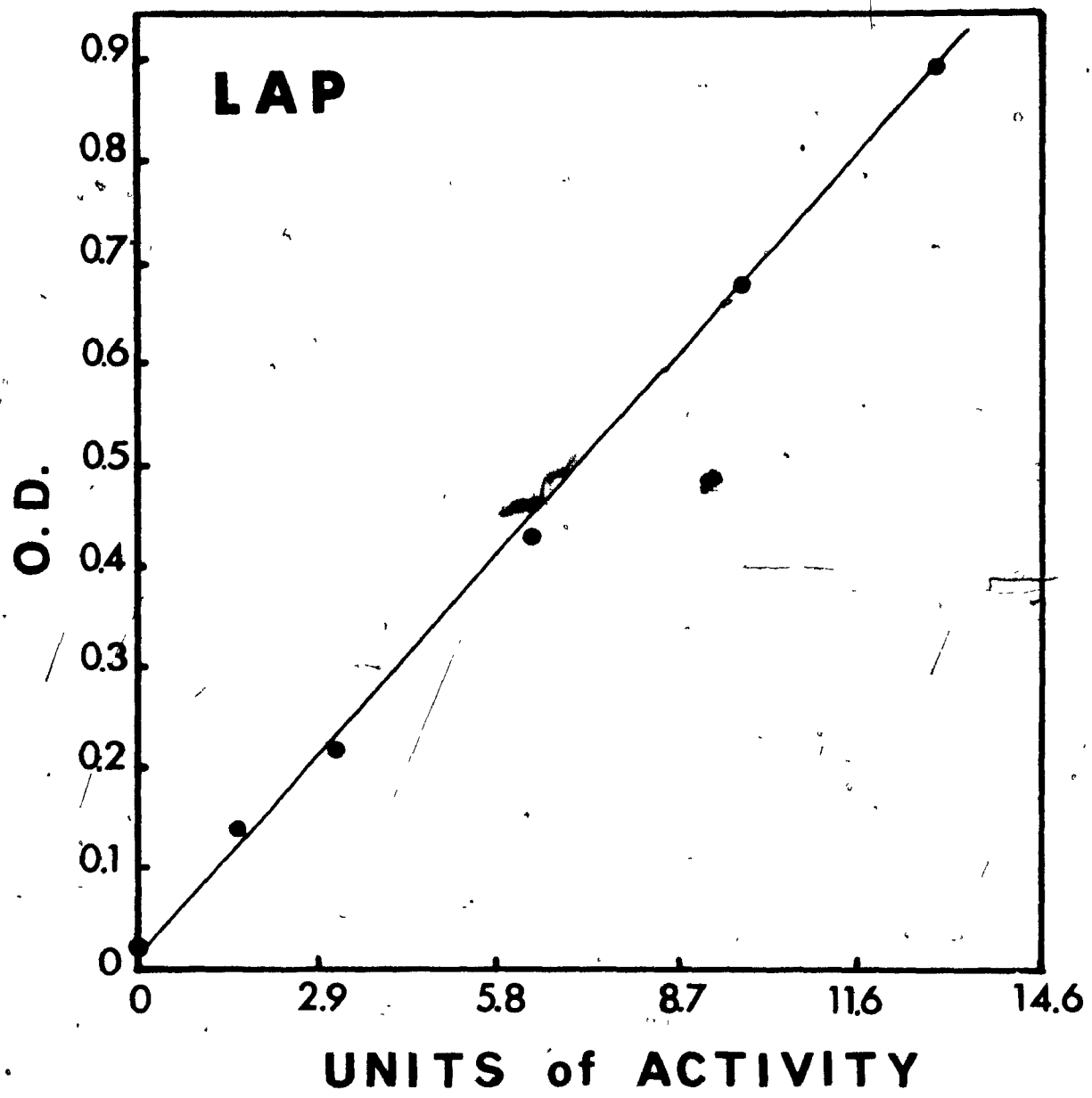
UNITS of ACTIVITY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