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BIOCHEMICAL CHANGES DURING THE TIME-COURSE OF RHYTHMIC FLORAL INDUCTION IN *CHENOPODIUM RUBRUM*

by

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ABSTRACT

The previously established presence of an endogenous rhythm of floral induction induced in seedlings of Chenopodium rubrum by interrupting continuous light with a single dark period, prompted investigations of biochemical changes that occur during the time-course of this rhythmic behavior -- in the hope of further elucidating the basis for rhythmicity that is inherent in the seedlings, and the mechanism of floral induction.

Seeds of Chenopodium rubrum L. ecotype 60°47'N 137°32'W (selection 374) were germinated upon water-moistened filter paper within covered Petri dishes maintained under regular alternations of temperature and light intensity. Following the addition of Hoagland's nutrient solution at the end of the germination period, seedlings were transferred to a constant environment of temperature and light intensity. The seedlings were analysed either while under these conditions, or during darkness interrupting continuous light, or after a fixed light period following single dark periods of varied length, or during

continuous light following darkness; the specific circumstances depended on the experiment undertaken.

Qualitative thin-layer chromatographic and quantitative analytical investigations of changes in selected endogenous metabolites during darkness revealed irregular short-term fluctuations in the levels of total soluble carbohydrates and total free amino acids; however, no direct associations with the periodicity of floral induction were evident. Decreasing enzymatic activity during darkness in conjunction with lack of rhythmicity, suggested that the following enzymes were not directly allied with rhythmic processes in Chenopodium rubrum: peptidase, acid phosphatase, amylase, glucose-6-phosphate dehydrogenase, peroxidase, and glutamate-oxaloacetate transaminase. The observed activity patterns indicated that these enzymes were probably involved more with aspects of germination and seedling metabolism.

Oscillations in the activity of a flavin nucleotide-requiring nitrate reductase occurred after transfer of seedlings from the germination programme to constant temperature and light conditions. The presence of half-strength Hoagland's nutrient solution during the germination period increased the range of the oscillations but

did not affect the period. Analysis of nitrate and nitrite content within the seedlings indicated distinct correlations with enzyme activity. Nitrate reductase activity decreased if seedlings were placed in darkness, but the phasing of the oscillations was not altered by the transition from light to darkness; this was unlike rhythmic flower induction in which the same transition sets the phase of the rhythm. Furthermore, a relationship between the periodicities of variations in nitrate reductase activity and rhythmic floral induction was not apparent. Induction and oscillations of nitrate reductase activity were also observed during continuous light following darkness. This secondary rhythmicity was apparently superimposed upon the pattern of fluctuations previously established.

On the basis of the rhythmicity characterized, and also in light of observed effects of altered environmental conditions on the activity of nitrate reductase, hypotheses are presented to account for the endogenous oscillations of nitrate reductase activity in Chenopodium rubrum.

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TABLE OF CONTENTS

LIST OF PHOTOGRAPHIC PLATES

LIST OF TABLES

LIST OF FIGURES

CHAPTER I

INTRODUCTION

The impetus for this research study was provided by the delineation of rhythmic changes in the flowering response of Chenopodium rubrum L. ecotype 60°47'N 137°32'W (selection 374) (Cumming, Hendricks, and Borthwick, 1965). Rhythmicity could be characterized in seedlings at the cotyledonary stage by interrupting continuous light with single dark periods of varying length, and then assessing the percentage of plants flowering several days after the dark interruptions. It seemed apparent from the type of flowering data obtained, that some oscillatory process must have been occurring during darkness to account for the rhythmic induction. The following working hypothesis was formulated: "Flowering is controlled by a product of the enzymatic action of the far-red absorbing form of phytochrome (P_{fr}) on a single, but unknown substrate . . . The supply of substrate for P_{fr} action depends both upon a prior light period and on an eventual rhythmic change if the dark period is sufficiently long. The available substrate, if not utilized by P_{fr} action, is soon depleted by other reactions." The authors

concluded that the level of "substrate" was low during intervals of minimal flowering and high during maximal flowering periods. Subsequent data (Cumming, 1967a) disclosed that the rhythmic display of flowering could be intensified and partially sustained when glucose was supplied during the single dark period; if glucose was presented only in the phases of the dark period which elicited minimal flowering, there were inhibitory and non-stimulatory effects on the rhythmic flowering response. The experimental results obtained with glucose applications led to the postulation that under control conditions, the light period before darkness was functional in providing for the formation of sugar which directly or indirectly acted as substrate for P_{fr} action during the dark period. In an extended dark period the sugar reserves would be gradually depleted, thereby causing a reduction in flowering and a damping out of the rhythmic oscillations.

It was felt that it would be of value to attempt a survey of biochemical changes which might be occurring during the time-course of this rhythmic floral induction. Such an investigation could serve to elucidate both the rhythmicity inherent in Chenopodium rubrum seedlings and the mechanism of floral induction. Due to the dynamics of the Chenopodium rubrum system, the possibility existed

for the interaction of three distinct avenues of research, the biochemical basis of flower induction, seedling metabolism, and biochemical rhythmicity. It is with these topics in mind that the experimental investigations in this dissertation were carried out.

Garner and Allard (1920) were the first researchers to advocate the length of the day (photoperiod) as being of primary importance in the control of flowering in plants, though slightly prior to their report, nutrient status of plants had been proposed as the basis of flower induction (Klebs, 1918, cited in Cumming, 1967a; Kraus and Kraybill, 1918). As a result of experiments showing perception of the photoperiod in the leaves of vegetative plants, Chailakhyan (1937) first adhered to the existence of a flowering hormone which he termed "florigen." These pioneering studies referred to above, in association with the suggestion of Bünning (1936) that endogenous rhythmicity was involved in photoperiodic induction, opened far-ranging vistas in the study of floral induction. Albeit only one major facet of these investigations will be touched upon here, thorough discussion of all aspects of flowering may be found in the several excellent reviews available (Salisbury, 1963; Hillman, 1964; Lang, 1965; Zeevaart, 1962; Imamura, 1967; Searle, 1965; Evans, 1969,

1971; Bernier, 1970, 1971; Chailakhyan, 1968).

Referring back to the work of Cumming (1967a), it has been found that there was an optimal age for seedling response to the single inductive dark period. If the number of days from sowing to the start of darkness was too great, there was a lower percentage of flowering plants; likewise for seedlings which were too young. These observations indicated a sensitivity on the part of the seedlings to inductive conditions, a fact which might indicate the importance of definite shifting patterns of metabolism. Certainly seedling metabolism, as compared to that of a more mature plant, is in a relatively rapid state of flux, and the significance of this consideration should not be overlooked.

It becomes apparent on close scrutiny of the literature, that relatively few research studies have been concerned with metabolic or biochemical rhythmicity <u>per se</u> in higher plants, in contrast with the many more reports on aspects of the various rhythmic physiological display phenomena (Withrow, 1959; Cold Spring Harbor Symposium, 1960; Bünning, 1956, 1961, 1967, 1969; Hamner and Takimoto, 1964; Aschoff, 1965; Sollberger, 1965; Cumming and Wagner 1968; Sweeney, 1969a). An obvious contributing factor to the paucity of such studies has of course been the pro-

cedural considerations and labour involved in detailed experimental measurement of biochemical oscillations. It would appear though, that for an eventual thorough understanding of the mechanisms involved in the physiological display rhythms, more extensive biochemical investigation will have to be undertaken. As stated by Hess and Boiteux (1971), "oscillatory phenomena in biochemistry may differ from biological rhythms only in their degree of complexity and their frequency . . . mechanistic relationships between both organizational levels will certainly be elucidated in the future."

CHAPTER II

LITERATURE REVIEW

I. ENDOGENOUS METABOLIC CHANGES ASSOCIATED WITH FLORAL INDUCTION

A. Introduction

The promise of a simple biochemical explanation for floral induction has prompted a considerable number of investigations into metabolic changes occurring within the plant as a result of treatments which bring about the transition from a vegetative to a flowering state. These investigations have touched nearly all classes of compounds present in plants. Apart from the direct approach of measurement of biochemical changes transpiring as a result of photoperiodic or other treatments, there have also been extensive studies on the regulation of the inductive process by application to plants of metabolites, metabolic inhibitors, anti-metabolites, growth retardants, base analogues, organic and inorganic ions, and hormones. Although I feel that a complete discussion of findings attributable to this latter avenue of research is beyond

the scope of the present study, in some cases these two different approaches, if they may be designated as such, cannot be so easily separated.

B. Carbohydrate-Nitrogen Relationship

One of the earliest theories of floral induction was that involving the attainment of a suitable carbohydrate to nitrogen distribution within the plant before flowering could occur (Klebs, 1918, cited in Cumming, 1967a; Kraus and Kraybill, 1918) -- as stated by Kraus and Kraybill, "the relation between the amount of vegetative and reproductive growth made by a plant, is determined by the relative amounts of carbohydrate and nitrogenous material available within it." Using tomato, and varying the amounts of nitrates present in the nutrient medium, they concluded that reproductive activity would result from a high soluble carbohydrate to soluble nitrogen (C / N) ratio while a low C / N value would favor vegetative activity.

Somewhat later, although still implying that a relation between the carbohydrate and nitrogen status of a plant definitely existed with reference to its growth and physiological condition, Kraus (1925) perceived that an exact mathematical ratio could hardly represent such conditions; instead, he recognized the possibility that certain

compounds might be more functional than others in relation to specific aspects of plant behavior, even though he did find it remarkable that there was such a good correlation in some cases between the overall C / N ratio and floral induction. Sheard (1940), measuring chemical changes in the apices of chrysanthemum, cosmos, and tomato, did not find any significant change in C / N ratio correlated with the transition from the vegetative to the flowering condition; however, both cosmos and the two varieties of chrysanthemum tested, contained a high soluble sugar content in their growing points coinciding with flower bud appearance. As is the case unfortunately, in this type of study, the data did not readily indicate whether the change in sugar content was the cause or effect of the bud appearance.

Murneek (1937) pointed out the discrepancies inherent in the correlation of C / N ratios with photoperiodic induction, depending on which fractions of carbohydrate or nitrogenous compounds had been used in such calculations. Nevertheless, in general he found a lower C / N ratio in induced than in vegetative Biloxi soybean plants -- during the time of photoperiodic induction, the nitrogen content was only slightly higher, but the carbohydrate content was quite a bit lower than in the vegetative controls. This trend was reversed in the stages subsequent to the actual

inductive period, mainly due to a buildup of storage carbohydrates, especially starch, in the plants. He equated the importance of the carbohydrate-nitrogen relationship not with flower induction *per se* but more prominently with post-inductive flower and fruit development. This sentiment has been supported by Chailakhyan (1968) in pointing out that all plants, whatever their response type (short- or long-day plant), contain more carbohydrates under long-day conditions as compared to more nitrogenous compounds and proteins under short-day conditions. In his review, Chailakhyan (1968) concluded that, "in metabolism that precedes the formation of floral organs, the carbohydrate-nitrogen ratio does not condition the start of flowering at all, but it is of certain importance and can be considered a system related to the metabolic preparation for flowering."

C. Carbohydrate Status

Closely related to the investigations on C / N ratio have been explorations into the carbohydrate status of plants with respect to the "metabolic preparation" referred to above by Chailakhyan. Liverman and Bonner (1953) postulated that during high-intensity light prior to an inductive dark period, substrates were produced for processes which occurred during induction. They had observed, using *Xanthium*

pensylvanicum (cocklebur), that for an inductive dark period to be effective, it was necessary that it be preceded by a long enough period of high-intensity light, but this requirement could be replaced by sucrose or several other carbohydrates. Grainger (1964) reported investigations on 17 different plant species with respect to changes in "carbohydrate + ash" concentration in the vegetative shoot in relation to the transition from vegetative to flowering states, and from his data, suggested a mechanism for floral induction. He inferred that for a plant to flower, it was necessary that there be a sufficient number of leaf initials and a sufficiently high value of carbohydrate plus ash in the shoot. Of these two prerequisites, the carbohydrate content appeared to be most important, with an elevated level possibly having the role of causing an osmotic pressure increase in the cells of the growing plant; it was found that when both of these conditions were fulfilled, the plants always flowered.

Increased starch content has been found to be correlated with the initiation of flower primordia in the work of Harley, Masure, and Magness (1941) and Nakata and Watanabe (1966); however, in neither case could the authors demonstrate a causal relationship. The latter study, in which lychee was the experimental material, and a study by

Sadik and Ozbun (1968) utilizing cauliflower, both concluded for their respective cases that cold temperature treatment was a direct requirement for starch synthesis and floral initiation. Sadik and Ozbun found that flowering would occur in one-month old cauliflower plants subjected to two weeks of growth at 5°C -- during the first week, the starch and soluble sugar content showed a large increase as compared to the vegetative plants in which both exhibited a gradual decrease. Also, if carbohydrate synthesis was blocked by growing plants in darkness or in the light but minus carbon dioxide, neither the starch and soluble sugar content increased nor was there any flowering; higher temperatures which caused depletion of carbohydrates, given immediately following the cold treatment, similarly prevented flowering.

D. Hormonal Factors

Results such as those reported above would doubtlessly appear to attest to the importance of nutritional aspects in floral induction; however, in variance to this concept, when Murneek (1937) found that the initiation of flower buds in soybean had no relationship to the nutritional state of the plant, he concluded that whatever substance was required for floral induction might be present in very

small quantities. This inference of the existence of a hormone-like substance involved in floral induction, in conjunction with the classic paper of Chailakhyan (1937), foreshadowed the advent of the still continuing search for the elusive flowering hormone (floral stimulus) or "florigen."

The concept of the hormonal control of flowering should certainly take into account nutritional aspects, as the growth of the plant is a necessary precondition to later events, and in fact the production of hormonal factors may be directly dependent on the efficiency of metabolic pathways within the plant. Sadik and Ozbun (1968) in their study of flowering in cauliflower, proposed a mechanism consisting of a preliminary stage involving a buildup of carbohydrates, leading to the production of a "floral substance." Somewhat similarly, it has been proposed (Roberts and Struckmeyer, 1961; Roberts, 1961) that photoperiodic treatment leads to the synthesis of precursor metabolites upon which hormones could act.

Chailakhyan's theories of floral induction in long- and short-day plants (Chailakhyan, 1968) involve an interplay of nutritional and hormonal factors. However, relatively few studies have been carried out investigating such an interaction, but rather there has been a prevalence of

literature concerned with hormonal changes alone. It seems, therefore, appropriate to allow for a discussion of changes in hormone-like factors, both identified and unidentified, which have been characterized in the transition from the vegetative to the flowering state.

Several studies from the same laboratory reported the successful isolation of an extract both from tissues of flowering plants (Lincoln, Mayfield, and Cunningham, 1961; Lincoln et al., 1962; Mayfield et al., 1963) and surprisingly also from fungal cultures (Lincoln et al., 1966), which was capable of inducing flowering in Xanthium strumarium plants being maintained under non-inductive conditions. The active entity in these extracts was found to have properties similar to those of an organic acid (Lincoln, Cunningham, and Hamner, 1964), and hence the term "florigenic acid" was suggested.

Roberts and Struckmeyer (1961) obtained three lipid "hormones" from extracts of plants in various stages from induction to flowering, and they found that these "anthogens" could induce more than 70 species or varieties representing more than 20 families; however, good blossoming was not evident unless the treated plants had been grown for a short time in their typical inductive photoperiod (Roberts, 1961). More recently, the sterol fraction from

extracts of flowering chrysanthemum plants (Biswas, Paul, and Henderson, 1966), and also extracts of flowering cocklebur (Hodson and Hamner, 1970), have been found capable of inducing flowering in vegetative plants.

Moving on now to changes that are more strictly hormonal in nature, Harada and Nitsch (1959) succeeded in isolating substances showing activity in hormonal bioassay from Rudbeckia and Chrysanthemum species during the cold treatment necessary for induction of rapid bolting and flowering. Nitsch and Nitsch (1965) found a whole array of active substances characterized as both auxins and gibberellins in induced and vegetative Nicotiana species, but from their results it was difficult to say whether there was a direct link between variations in the levels of these growth factors and flower induction. More recently, Gifford and Nitsch (1970) have found changes in levels of growth substances showing auxin and gibberellin activity in bioassay, in both leaves and shoot tips of induced Plumbago indica plants.

The role of gibberellins in floral induction has received a great deal of attention, and it has been suggested that the presence of gibberellin is necessary for the production of the floral stimulus (Lang and Reinhard, 1961; Zeevaart and Lang, 1962; Zeevaart, 1969a). The notion

that gibberellin is in fact a universal "florigen" has been dismissed, in as much as it has been shown (Lang and Reinhard, 1961; Zeevaart and Lang, 1962) that added gibberellin can cause flowering in non-induced cold requiring and long-day plants, but not in non-induced short-day plants. Lang (1960) found higher gibberellin-like activity in young leaves of photoinduced Hyoscyamus niger plants than in those of the vegetative controls; similarly, in Bryophyllum daigremontianum (Zeevaart, 1969b, 1969c) an increase in gibberellin content was found to be correlated with the time at which floral induction occurred. Contrary to these results, Suge and Rappaport (1968) concluded from their experiments with radish that endogenous gibberellins were not functional in promoting flowering in the cultivar which they studied. A role of auxin, although not widely popularized, has also been implicated in the control of floral induction (Vlitos and Meudt, 1954; Kijosowa, 1960).

E. Nucleic Acid and Protein

The concept of the synthesis of a floral stimulus or hormone in response to inductive photoperiodic conditions would necessarily imply the involvement of an enzyme (or enzymes) responsible for this synthesis, and also possible associated changes in nucleic acid synthesis if actual

synthesis of the enzyme protein were required. On the other hand, the phenomenon of induction, signifying the impending transition from a vegetative to a flowering apex, might also trigger changes in nucleic acid and protein metabolism necessary for the morphological shift. There has been extensive investigation for the above reasons, on changes in nucleic acid and protein metabolism accompanying floral induction. Although the bulk of this work involves studies utilizing inhibitors and anti-metabolites, quite a few investigations concerned with endogenous changes certainly exist.

Histochemical and autoradiographic techniques have been used quite successfully to show that changes in ribonucleic acid (RNA) content occur in apices of induced plants. Gifford and Tepper (1962) demonstrated a rapid increase in the cytoplasmic RNA concentration of all cells of the shoot apex in Chenopodium album following photoperiodic induction. Pharbitis species showed an increase in RNA within one day after an inductive long night (Gifford, 1963; Healey and Jensen, 1965), and changes in Xanthium pensylvanicum apices were observable after two days (Gifford, 1963). Knox and Evans (1966) found localized increases in RNA of Lolium temulentum in areas between leaf primordia in shoot apices of induced plants; their

findings were consequently verified analytically (Rijven and Evans, 1967a) and by radioactive isotope incorporation studies (Rijven and Evans, 1967b; Evans and Rijven, 1967). High incorporation of uridine-5-^{3}H occurred in induced Pharbitis nil plants (Gressel, Zilberstein, and Arzee, 1970), and if flower induction was prevented by a light interruption of the inductive dark period, incorporation was lowered to a near control value. Labelled buds of photoperiodically induced Xanthium pensylvanicum plants synthesized more messenger RNA than those of vegetative controls (Cherry and van Huystee, 1965; Cherry, 1970).

In an investigation of the base composition of RNA species isolated from different fractions of Pharbitis nil cotyledons, Yoshida et al. (1967) discovered an altered nucleotide ratio only in messenger RNA as a result of an inductive night, and they proposed that this messenger RNA directed the synthesis of an enzyme protein responsible for synthesis of the floral stimulus.

Increases in protein concentration have been characterized in apices of induced plants (Healey and Jensen, 1965; Evans and Rijven, 1967), although in some cases showing a definite lag as compared to the time of attainment of higher levels of RNA (Gifford and Tepper, 1962; Gifford, 1963). Barber and Steward (1968) used polyacrylamide gel

electrophoresis to characterize the soluble protein patterns in Tulipa gesneriana at different times during the transition of the plant from a vegetative to a flowering state. As compared to the vegetative apex, which had a relatively low number and concentration of soluble components, apices just after inductive treatment contained a larger complement of proteins which were also present in higher concentration. These changes in soluble proteins preceded observable morphological differentiation, although several of the proteins characterized appeared later in the extracts of mature flower parts.

Nitsan (1962), in a non-specific study of the electrophoretic pattern of leaf extracts of vegetative and induced Xanthium pensylvanicum plants, found that photoperiodic treatment affected two of five identifiable protein compounds. Quantitative changes in the amino acid composition of leaf proteins of Kalanchoë blossfeldiana in response to inductive photoperiods have been described by Metzner (1955); also in this Kalanchoë species, Madan (1956) demonstrated altered levels of free amino acids in the root, shoot, and leaves in response to treatments causing floral initiation.

F. Other Biochemical Changes

Several miscellaneous biochemical changes have been

reported as occurring in response to photoperiodic conditions causing flower induction. Anthocyanin was detected only in leaves of Kalanchoë blossfeldiana plants grown under inductive short days (Neyland, Ng, and Thimann, 1963), and both flowering and anthocyanin formation, except for traces, could be prevented by exposure to long days, or short days with interrupted nights.

Bharti and Garg (1970) observed an increase in levels of ascorbic acid in lateral buds of soybean, correlated with exposure to inductive day length; high levels occurred prior to any morphological change signifying floral induction. In the post-inductive period during the onset of floral initiation, the ascorbic acid level dropped considerably, thus leading the authors to suggest that ascorbic acid increase was important in flowering in an actual causative capacity, and not merely as a coincident biochemical event.

Zucker, Nitsch, and Nitsch (1965) found that inductive treatment in both long- and short-day Nicotiana species led to an increase in the concentration of chlorogenic acid and its isomers in leaves, visible just prior to the onset of meristem differentiation; however, the levels of these decreased during the development of floral primordia. The authors felt that since chlorogenic acid

had been shown to be an inhibitor of indoleacetic acid (IAA) - oxidase, alterations in levels of chlorogenic acid, leading to respective changes in IAA content, might be important in flower formation. Also in relation to auxin metabolism, Aksenova (1963) and Chailakhyan and Boyarkin (1955) observed that peroxidase activity in leaves of both long- and short-day plants showed a decrease under long-day conditions and an increase under short-day conditions -- it was suggested (Aksenova, 1963) that if the peroxidase were active in auxin destruction, the results obtained might imply a relationship between photoperiodic induction and auxin metabolism. The effects of photoperiod on the activity of terminal oxidases led Chailakhyan and Aksenova (1959) to the conclusion that during floral induction of short- and long-day species of plants, different oxidase systems were activated.

In this section on metabolic changes associated with floral induction, an attempt has been made to point out the diversity and primarily descriptive nature of investigations pertaining to this avenue of research. It would appear as quite evident from the literature reported that changes in metabolism during floral induction which have been studied thus far, have not provided a lucid biochemical cause for the transition from a vegetative to a flowering state, but

have apparently merely reflected the metabolic differences between non-induced and induced plants.

II. ASPECTS OF SEEDLING METABOLISM

A. Protein and Carbohydrate Breakdown

Metabolism during seedling development is a very complex phenomenon, beginning with the emerging embryo's dependence on seed reserves broken down by various hydrolytic enzymes, and leading to a photosynthesizing and thereby self-sustaining plant. Cotyledons contain reserves which can be mobilized to nourish the developing seedling; breakdown products can be transferred to the seedling axis and used there, or utilized within the cotyledon itself.

Almost a century ago, Gorup-Besanez (1877, see Chibnall, 1939) was the first to postulate that during germination, reserve proteins within the seed were digested by enzymes with the resultant production of amino acids and amides. This postulate was borne out in extensive investigations by Schulze between 1878-1906 -- a description of this work and other early investigations has been outlined quite thoroughly by Chibnall (1939).

The majority of recent investigations on seedling metabolism have incorporated peas (Pisum sativum) as the test material. Danielsson (1951), examining the breakdown

of storage protein in peas, found that the level of total amino acids and other low molecular weight nitrogen products increased during the first ten days of germination, and a higher proteolytic enzyme activity during this period was suggested as the cause. In an analysis of different parts of pea seedlings germinated for one, three, or five days, Lawrence, Day, and Stephanson (1959) discovered a steady decrease of protein in the cotyledons paralleled by an increase in the other seedling parts, thereby labelling the cotyledons as the nitrogen source. Also they found that non-protein nitrogenous compounds were predominant in the root and shoot shafts in contrast to the meristematic tips which had a high protein content. This was an indication that soluble amino acids, released by the breakdown of cotyledonary protein, were being mobilized to other parts of the plant for utilization in new protein synthesis.

Quite similar patterns of degradation have been shown for both protein and starch in cotyledons of germinating peas (Juliano and Varner, 1969) -- a slower rate during the first five or six days of germination was followed by a much faster rate for the remaining ten days of the study; by day fifteen, starch was only at a level of 1% of its original content and protein only at 7%. Also in pea cotyledons, decreases in both starch and soluble sugars have

been characterized by Larson and Beevers (1965) and Bain and Mercer (1966). In the latter investigation, the total sugar level increased until it reached a maximum at about eight days of germination, followed by a relatively rapid decrease during the remainder of the experimental time-course.

Apart from the majority of investigations utilizing peas, several studies of other species have also revealed similar patterns of protein degradation during germination, such as in bean (Boulter and Barber, 1963), peanut (Cherry, 1963), and soybean (Treffry, Klein, and Abrahamsen, 1967). In pointing out that most of the work on breakdown of reserve materials in the seed or seedling involved species which contained their reserves in either the endosperm or non-photosynthetic cotyledons, Wiley and Ashton (1967) used Cucurbita maxima (squash), possessing cotyledons which do assume a photosynthetic role. However, they found that whether these seedlings were germinated in light or darkness, the patterns of protein breakdown were quite similar; also, the presence of the embryonic axis, in contrast to detached cotyledons alone, led to a higher level of protein hydrolysis.

As mentioned earlier, it had been known near the end of the nineteenth century that amino acids were released as

a consequence of protein breakdown during germination. This pioneering work also revealed that the amides, asparagine and glutamine, were often produced in large quantities in seedlings germinating in darkness. Prianischnikov (1910, 1922, in Chibnall, 1939) suggested that asparagine accumulation was a detoxification mechanism to utilize ammonia, which would otherwise accumulate due to increased storage protein breakdown with resultant stepped up deamination of the amino acids released; this same function has also been attributed to glutamine (Webster, 1959). The oxidative deamination of amino acids leads also to acid residues which can find their way into the organic acid pool (Beevers, Stiller, and Butt, 1966).

During dark germination of Phaseolus radiatus, Ganguli (1955) found that the various free amino acids, characterized chromatographically, appeared in higher concentration sooner than during germination in light. The non-protein amino acid, homoserine, is often very prominent during the initial few days of germination (Virtanen, Berg, and Kari, 1953; Lawrence and Grant, 1963; Larson and Beevers, 1965). Increase of soluble amino nitrogen in both the cotyledons and axis of pea seedlings, although occurring at a faster rate in the latter, coincided with a decrease of total nitrogen in the cotyledons (Beevers and

Guernsey, 1966; Beevers, 1968). This provided evidence of hydrolytic processes acting on cotyledonary protein. Characteristic amino acid profiles in root and shoot tips, and root and shoot shafts, along with different protein and non-protein amino acid compositions (Lawrence and Grant, 1963), have indicated a complex system of metabolism and interconversion of amino acids in germinating seedlings.

B. Enzymatic Activity

There have been many enzymatic investigations undertaken relatively recently in order to verify earlier theories that metabolic changes occurring during germination were caused by the activities of hydrolytic or transfer enzymes. Young and Varner (1959), studying enzyme synthesis in the cotyledons of pea seedlings, measured the activities of protease, amylase, and phosphatase between zero and eight days of germination. These authors found no significant change in protease activity during this period; however, amylase and phosphatase activities showed a tenfold and twenty-fold increase respectively; inhibitor experiments led to the conclusion that de novo synthesis from free amino acids was responsible for the development of activity. Following up this study, Young et al. (1960) and Varner, Balce, and Huang (1963) established that some

controlling factor or factors residing in the axis tissue of the seedlings, had to be present to allow for the development of normal enzyme activity in the cotyledons; more recently, this observation has been confirmed by Guardiola and Sutcliffe (1971), who suggested possible hormonal control.

At variance with the earlier conclusion of Young and Varner (1959) concerning the origin of enzymatic activity during germination, Presley and Fowden (1965) studying acid phosphatase and isocitratase production during germination of mung bean, pea, cucumber, and sunflower, inferred that these enzymes were derived from the activation of pre-formed proteins and not synthesized de novo from free amino acids. Also, Meyer, Mayer, and Hasel (1971) found that of nine different phosphatases isolated electrophoretically from germinating lettuce seedlings, only one had not been present in dry seed, and even the activity of this new adenosine triphosphatase was attributed to an activation phenomenon, not de novo synthesis. Data have been presented (Shain and Mayer, 1968) suggesting that the increase in the activity of a trypsin-like enzyme during germination of lettuce seedlings was due to the disappearance of an endogenous trypsin inhibitor caused by the degradative activity of other proteolytic enzymes.

Certainly, recent investigations of proteolytic enzyme activities have complemented the many studies of protein breakdown in germinating seedlings. Wiley and Ashton (1967) found that the activity pattern of the proteinase assayed in light- or dark-germinated squash seedlings, was quite similar to the pattern of protein breakdown in the cotyledons -- the enzyme activity increased to maximum at about two days, followed by a gradual decrease. Peptidase activity, measured using the synthetic peptide leucine p-nitroaniline (LPA) as substrate, has been found to be highest in non-germinated kidney beans with a subsequent decrease to a minimum after eight days of germination (Puztai and Duncan, 1971); a continuous decrease of protein nitrogen was also evident during the germination period. Similarly, Beevers (1968) demonstrated the activity of four proteolytic enzymes in cotyledonary extracts of germinating pea seedlings. These enzymes were subsequently characterized as two proteases and two peptidases (Beevers and Splittstoeser, 1968) as distinguished by substrate specificity and pH optima. In the cotyledons, the specific activity of both proteases increased similarly throughout the eleven day period studied, while the peptidases behaved exactly conversely. Interestingly, although the activities of these proteolytic enzymes were apparent during germina-

tion, they did not appear to bear a definitive relationship to the hydrolysis of reserve materials; only the timing of the maximum peptidase activity had been close to the period in which utilization of protein reserves was maximal. Furthermore, in the case of ribonuclease activity which had also been found, the relationship between enzyme activity and disappearance of ribonucleic acid reserves (Beevers and Guernsey, 1966) was opposite to that which might have been expected. Obviously the implications were that the enzymes involved in the release of amino acids and nucleotides during germination of pea seedlings differed from the ones which had been assayed. Guardiola and Sutcliffe (1971) have also concluded that protein hydrolysis in the cotyledons of germinating pea seeds was not directly related to the proteolytic activity measured. However, an increase in alpha-amylase activity previously described (Young and Varner, 1959), has been found to show a definitive correlation with the rate of disappearance of starch (Juliano and Varner, 1969).

Changes in levels of several enzymes during dark germination of Triticum durum seeds, as measured by Firenzuoli et al. (1968), revealed increases of activity for all of the enzymes tested -- these included: several glycolytic enzymes, glucose-6-phosphate dehydrogenase and

phosphogluconate dehydrogenase of the pentose phosphate shunt, isocitrate and malate dehydrogenases of Krebs' cycle, glutamate-oxaloacetate and glutamate-pyruvate transaminases, and acid phosphatase. The authors attributed the increase in activity of glutamate-oxaloacetate transaminase to a stimulated amino acid metabolism most likely correlated with degradation of protein and formation of asparagine. Mayer, Poljakoff-Mayber, and Krishmaro (1966) concluded that glucose-6-phosphate dehydrogenase may be involved in the initial stages of germination in lettuce because of its response to both a germination stimulator (thiourea) and inhibitor (coumarin); evidence has also been presented showing the importance of this enzyme in pea cotyledons during germination (Wray and Brown, 1966; Brown and Wray, 1968).

Measurement of the activities of sixteen non-photosynthetic enzymes in the young leaves of light- and dark-grown wheat seedlings, led Wolf (1968) to the conclusion that metabolism closely connected with photosynthesis was not the only way in which light- and dark-grown seedlings differed. Of the enzymes assayed, ten showed greater activity in light-grown seedlings while the remainder were more active in dark-grown seedlings. Certainly in seedlings germinated in the light, little or no amide formation

occurs (Mayer and Poljakoff-Mayber, 1963). If a nutrient supply is present, protein synthesis in light-grown seedlings will probably be primarily dependent on reduction of external nitrogen, and carbon skeletons formed by photosynthetic reactions, and not on amino nitrogen and amino acid residues released during protein breakdown. The presence of light and an external nitrate supply has proven to be necessary for the induction of nitrate reducing ability in several seedling types (Hageman and Flesher, 1960; Beevers et al., 1965; Tang and Wu, 1957).

III. BIOCHEMICAL OSCILLATIONS IN PLANTS

A. Definitions and Theoretical Considerations

1. Definitions

An inherent perplexity for persons unfamiliar with the literature of "rhythm research" has always been the jargon employed. Therefore, as a possible assistance, I am presenting a brief glossary of relevant terms which could occur in the context of the ensuing discussion. Definitions listed, unless otherwise indicated, have been obtained from the "circadian vocabulary" of Aschoff, Klotter, and Wever (1965).

Amplitude -- "difference between a maximum (or minimum) value and mean value in a sinusoidal oscillation. In a looser sense this term often is applied to oscillations of a general shape also."

Circadian Rhythm -- "oscillation with a period of about 24 hours." The term "circadian" (circa-about+dies-day) first used by Halberg et al. (1959).

Damped Oscillation -- oscillations which show a decrease in amplitude with time, tending toward a steady state.

Endogenous Rhythm -- ". . . biological processes which alter periodically although external conditions remain constant." (Bunning, 1956)

Entrainment -- "coupling of a self-sustained oscillation to a Zeitgeber (forcing oscillation) with the result that, either both oscillations have the same frequency (synchronization), or the frequencies are integral multiples (frequency demultiplication): possible only within

limited ranges of frequencies."

Free-running rhythms -- "self-sustained oscillations under constant conditions." Occurrence of a free-running period is "the only convincing evidence of an endogenous (circadian) periodicity" (Aschoff, 1960).

Frequency -- "reciprocal of period".

Frequency demultiplication (Pittendrigh and Bruce, 1957) -- "may result when a periodic energy input into an oscillator has a frequency which is roughly an integral multiple of the frequency of the oscillator. Frequency demultiplication occurs if the resulting oscillation is entrained to a whole number submultiple of the high frequency oscillation."

Pendulum-oscillator (Wever, 1960) -- wave form is smooth and oscillations occur evenly above and below an equilibrium position.

Period -- "time after which a definite phase of the oscillation reoccurs."

Phase -- "instantaneous state of an oscillation within a period."

Phase relation (Kalmus, 1966) -- "time relation between a specified phase in an external oscillator (for instance dawn) and a specified phase in a second synchronized oscillation." May also describe association between oscillators in a single system.

Phase shift -- "a single displacement of an oscillation along the time axis."

Range of oscillation -- "difference between maximum and minimum value."

Relaxation oscillator (Pittendrigh and Bruce, 1957) -- characterized by sudden changes occurring within a pattern of oscillations, i.e., periodically an unstable state is reached followed by a somewhat sudden shift to another state, e.g., the cycles of an automatic pipette rinser in which water flows in relatively slowly but empties quickly.

Synchronization (Kalmus, 1960) -- "state in which two or more oscillations have the same frequency due to mutual or unilateral influences."

Transients -- "temporary oscillatory states between two steady states."

2. Chemical and Biochemical Models

Just over sixty years ago, Lotka (1910) presented a series of strictly theoretical equations showing how damped oscillations of a transitory nature could exist in an autocatalytic chemical system in which a reactant was entering at a constant rate. Lotka (1920a) also demonstrated theoretically that undamped or sustained oscillations could exist in a chemical system, and he applied these derived relations loosely to a biological periodic system (Lotka, 1920b). Bray (1921) was the first to characterize an actual periodic chemical reaction by observing that in the non-enzymatic autocatalytic reaction between hydrogen peroxide and iodic acid, the formation of oxygen did not proceed smoothly but rather in a stepwise manner, as shown by continuous monitoring of the reaction. Also, readily visible, was the alternate appearance and disappearance of colouration in the reaction mixture due to respective formation and dissipation of iodine; period length varied between 3-13 minutes depending on the acidity of the reaction mixture. These surprising results were confirmed by Peard and Cullis (1951) who performed a detailed investigation of

the reaction in terms of factors affecting the periodicity. They concluded that the periodicity observed in the rates of evolution of oxygen and iodine liberation, was due to the establishment of a complex set of interrelationships in this autocatalytic system.

It has been pointed out (Christiansen, 1961) that the possible periodic nature of other chemical reactions, both physical and biological, may be difficult to assess unless continuous and extremely accurate monitoring can be undertaken. Obviously, in the hydrogen peroxide-iodic acid reaction referred to above, if the oxygen output had not been monitored continuously but only at widely spaced times, the periodicity might never have been observed -- such a failure to observe periodicity has been termed the "stroboscopic effect" (Sollberger, 1965). Also, a phenomenon called "aliasing" (Sollberger, 1965; Mercer, 1960) can occur if the periods of observation or sampling are too long and not frequent enough as compared to the expected period of the oscillation under study. These relationships would hold true whether the rhythmicity being investigated were either strictly chemical or more pertinently, biological in nature (Chance, Pye, and Higgins, 1967).

The application of equations for undamped oscillations

in autocatalytic chemical reactions to biological systems (Lotka, 1920b), has been criticized by Spangler and Snell (1961) who used kinetical equations and also computer techniques to show that sustained periodic behavior was possible in biochemical (enzyme catalysed) reactions. Their model led them to conclude that chemical kinetic systems could underlie biological clocks. Similarly, Walter (1966) compiled computer simulations of several standard enzyme mechanisms and concluded that in certain cases oscillatory behavior could occur. It has been deduced theoretically (Morales and McKay, 1967; Walter, 1969) that if enzymes in a metabolic pathway, showing negative feedback inhibition, possess proper quantitative properties, there could be noticeable oscillations of levels of the intermediates. Goodwin (1966), in his lengthy, detailed analytical analysis of oscillating biochemical control circuits, has suggested that oscillations at a molecular level are a universal occurrence in all systems incorporating negative feedback; he perceived that rhythmic behavior by organisms is dependent on these internal biochemical oscillations (Goodwin, 1965, 1967).

More specifically, it has been concluded from an investigation of a peroxidase-like mechanism (Strickland and Ackerman, 1966) that, given certain values of kinetic con-

stants and substrate concentrations, kinetic equations could be derived that are characteristic of damped oscillations; however, the authors also suggested that such oscillations would be too small to be observable with present techniques. Significantly, in an experimental attempt to verify Lotka's (1910) original theoretical model for damped oscillations, Degn (1969) successfully observed damped oscillations in the reaction rate of the horseradish peroxidase catalysed oxidation of dihydroxyfumaric acid, indoleacetic acid, and reduced nicotinamide adenine dinucleotide. These oscillations, possessing a period of approximately two minutes, occurred only in a system open to O_2, a condition under which peroxidase catalysed oxidations are autocatalytic (Degn and Mayer, 1969).

Perhaps the most striking findings in recent years concerning the oscillatory behavior of enzymatic reactions have been characterized by Comorosan, Sandru, and Alexandrescu (1970). These authors found that when they irradiated an enzyme substrate (in powder form) with low-energy X-rays or ultraviolet light, and then used this substrate in an assay for activity of its respective enzyme, oscillations of the activity of the enzyme, dependent on the irradiation dosage given the substrate, were readily observable. The irradiated substrate-enzyme combinations used

were glucose-hexokinase, xanthine-xanthine oxidase, and glucose-6-phosphate-glucose-6-phosphate dehydrogenase; also, irradiation of the co-enzyme (NADP) for glucose-6-phosphate dehydrogenase activity led to similar oscillations. Moreover, it was shown both theoretically and experimentally that two different enzyme reactions which could utilize the same irradiated substrate, would demonstrate non-overlapping oscillations, i.e., the peaks and minima occurred with different energy levels of substrate irradiation for each enzyme. Also, in reversible enzyme reactions such as that involving malate dehydrogenase, if the substrates and co-factors in either direction were irradiated singly, similar non-overlapping oscillations resulted. The above two systems were termed "flip-flop" units and have been elaborated upon in a separate publication (Comorosan, 1970).

According to the authors, the doses of the X-rays or ultraviolet light given were too low to cause chemical transitions in the irradiated compounds. Because of the low energies required for an effect, they felt that biochemical energy-producing systems operating at the cellular level might have the same effect on enzyme substrates. Certainly one can envisage the relevance of this speculation in terms of cellular control mechanisms and biological

rhythmicity.

3. Biological Oscillating Systems

This section might more properly have been included in association with "definitions" (III.A.1.), as the terms themselves reflect inherent properties of biological rhythmicity. However, it was also felt that some acquaintance with rhythmic terminology might have proven useful prior to the preceding section.

The occurrence of biological feedback, especially in instances of hormonal regulation, has been a recognized phenomenon for many years. However, biological rhythms which must exhibit short-term feedback, capable of controlling a process so that it will occur at approximately the same time each day, have provided an interesting challenge to the researcher.

Most investigations of biological oscillatory phenomena with the exception of several biochemical systems, have been concerned with endogenous circadian rhythms. To those postulating a biochemical basis for such rhythmicity, possibly the most important findings in these research studies has been the relative "temperature-independence" of endogenous circadian rhythms (Sweeney and Hastings, 1960; Sollberger, 1965; Bünning, 1967). Endogenous rhythms

without environmental correlates tend to show an increase of frequency with a temperature increase (Berliner and Neurath, 1965a, 1965b). However, endogenous circadian rhythms that have the 24 hour lunar day as an external correlate, possess Q_{10}'s (Q_{10} = quotient of change in rate of metabolism for 10°C interval) in the range of 0.9-1.3 (Sollberger, 1962). The fact that the Q_{10} of most circadian rhythms is approximately "one" but not exactly unity, indicates that "temperature-independence" per se does not truly exist, but rather some form of compensatory mechanism is present (Pittendrigh, 1961). If temperature compensation did not exist in circadian rhythms, increases and decreases of temperature could cause the frequency of the oscillations to be altered outside of the range of control by natural light-dark cycles.

The importance of subtle external synchronizers in regulating endogenous rhythmicity, which could account for a degree of temperature insensitivity by means of strong coupling, has been stressed by Brown (1959, 1968, 1969), although often against strong criticism (Enright, 1965). He has suggested that organisms have the capacity to utilize input from these subtle geophysical variations in the external environment, and that endogenous biological rhythms are most likely actually co-operative systems

entailing both perception of such external input and utilization of certain innate endogenous timing mechanisms. In Brown's "auto-phasing" (Brown, 1962), an extrinsic rhythmic period could be used by the organism acting as a "variable frequency transformer," to set the phases of several internal rhythms possessing closely affiliated periods. Neville (1967) among others, has pointed out that phase shifting caused by light is adaptively significant in rhythmic systems since it shows the flexibility in biological clocks which allows organisms to synchronize with the alterations in day length during the year. The endogenous components would also contribute the plasticity necessary for adaptation of organisms to changing environments (Brown, 1966).

Such relatively temperature insensitive mechanisms as physico-molecular transformations and diffusion processes (Enright, 1971) have been suggested as playing a part in the timing mechanism of biological clocks. Ehret and Trucco (1967) incorporated a diffusion transport step as an important part of their model for timing of circadian rhythms in eucaryotic organisms. The basic tenet of this model was linear "sequential transcription" of messenger RNA from parent DNA; that is, a circadian cycle would be completed after transcription had proceeded from one end of the DNA molecule or "chronon," to the opposite terminus. Succes-

sive cycles would be initiated by diffusion-mediated feedback of specific initiator substances produced as a result of cellular metabolism which had been effected by the enzymes translated from the sequentially transcribed messenger RNA.

Temperature compensation in endogenous rhythms may be achieved by the interaction of several coupled temperature dependent reactions (Sollberger, 1962, 1965; Chance et al., 1967). In response to a temperature alteration, the rate of an inhibitory reaction could change at the same rate as the process exhibiting rhythmicity, Pavlidis (1969) proposed a mathematical model, incorporating equations for temperature compensation, which provided a mechanism for biochemical oscillations of short periods to couple together and result in overt circadian oscillations.

An oscillator in an organism might be entrained by a second endogenous oscillator if the coupling between them is sufficient and they have closely related frequencies (Hastings, 1959). Moreover, the stronger the coupling, the more disparate the frequencies could be. A physical model of coupled oscillators can be represented by two weights suspended from a length of string (Mercer, 1965). If one weight is set swinging it will set the other weight swinging and eventually cease moving itself, only to be conse-

quently set in motion again by the oscillations of the other weight. This process will continue with a smaller and smaller amplitude until motion can no longer be observed. However, if energy were applied, the oscillations would continue, and -- depending on the distribution pattern of the supplied energy -- the interaction of the oscillations could be more complex than in the model described above. Any number of coupled oscillators might be involved in biological time-keeping systems (Winfree, 1967a, 1967b; Pavlidis, 1971), resulting in an even more heightened complexity. Another complicating factor in cells might be shifts in energy distribution among the oscillators during development of the organism.

B. Unicellular Systems

1. Periodic Enzyme Synthesis in Bacteria

Bacteria have been included in this discussion of plant rhythmicity because the control of short-term periodic enzyme synthesis in bacteria, a lower organism, has been found to be mediated by feedback mechanisms -- one may infer that rhythmicity in higher organisms may be similarly controlled.

It was discovered that the activity of the enzymes invertase, histidase, and aspartate transcarbamylase,

measured in synchronous cultures of Bacillus subtilis, increased in a stepwise fashion (Masters, Kuempel, and Pardee, 1964); the time of the activity rise for each enzyme occurred at a different point in the cell cycle. This stepwise increase in enzymatic activity was attributed to periodic bursts of synthesis. However Donachie (1965), advocating continuous enzyme synthesis, ascribed the stepwise increases in activity to both periodic enzyme instability and end-product inhibition (Umbarger, 1961a, 1961b). Cultures of Escherischia coli containing sodium lactate exhibited periodic rapid tenfold increases of pyruvate concentration immediately followed by similarly rapid decreases (Sikyta and Slezak, 1965) -- each fluctuation occurred between 30-90 minutes apart. The authors could not provide a specific explanation for this observation but just proposed that a simple negative feedback mechanism might be operating. Kuempel, Masters, and Pardee (1965) have presented a model suggesting that these periodic phenomena in B. subtilis and E. coli may depend on changes in the degree of gene repression.

Given that enzyme synthesis itself is actually periodic in B. subtilis, Masters and Donachie (1966, 1967) have shown that the continuous presence of a repressor prevented the cyclic enzyme synthesis, and addition or removal of the

repressor could be used to set the time for enzyme synthesis to occur. On the other hand, when DNA synthesis was inhibited, the periodic enzyme synthesis could be maintained for at least two cell cycles, thereby indicating that periodic gene replication was not required. These results intimated that oscillations of repressor concentration might be the controlling factor in the periodic enzyme synthesis. Using this assumption, Goodwin (1966) proposed a model for periodic enzyme synthesis in bacteria, composed of control units containing messenger RNA, enzyme, and enzyme product (repressor) in a closed loop system incorporating feedback repression. It was suggested that as each gene locus on a DNA molecule was replicated during the cell cycle, at a specific point a small amount of messenger RNA would be synthesized and subsequently serve in modifying the enzyme of that locus to produce repressor (modified product). The repressor molecules thus formed could act in preventing further synthesis of the enzyme.

The association of periodicity with DNA replication inherent in Goodwin's model, would allow cycles of enzyme synthesis to be synchronized temporally, thereby maintaining the experimentally observed fixed phase relationships among the various enzymes. This model differs in principle from one incorporating only sequential enzyme synthesis as

the rhythmic factor (Ehret and Trucco, 1967), in that the formation of a repressor here is the actual controlling mechanism and modification of repressor levels could alter the equilibrium of the system.

2. Glycolytic Oscillations in Yeast

A very recent review on biochemical oscillatory phenomena (Hess and Boiteux, 1971) has stated that at present, glycolytic oscillations are the best understood biochemical oscillatory system. A major contributing factor allowing such a statement to be made has been the outstanding work done in the past decade by Britton Chance and his co-workers on glycolytic oscillations in yeast.

In 1964, Chance, Estabrook, and Ghosh established that in Saccharomyces carlsbergensis (baker's yeast) suspensions, previously observed rapid diphosphopyridine nucleotide (DPN=NAD) reduction and oxidation following either glucose addition or transfer from aerobic to anaerobic conditions, was actually the initial step in a series of damped sinusoidal oscillations. These damped oscillations of reduced diphosphopyridine nucleotide (DPNH=NADH) were also verified in a different laboratory (Hommes, 1964). Concurrently, it was observed that NADH oscillations similar to those obtained in intact cells, would occur also in

cell-free extracts of S. carlsbergensis (Chance, Hess, and Betz, 1964). However, these oscillations possessed a period of about seven minutes which was approximately ten times that observed in a yeast cell suspension at 25°C. This time disparity has been attributed to a dilution of the "glycolytic space" by cell sap released during extraction (Chance, Schoener, and Elsaesser, 1965). Unlike relatively stable endogenous circadian rhythms, the frequency of the oscillations of NADH level in both intact cells (Betz and Chance, 1965a) and cell-free extracts (Chance, Schoener, and Elsaesser, 1964) proved to be extremely temperature-sensitive; also, the amplitude and phase of the oscillations in the cell-free system could be altered by the addition of certain chemicals (Chance, Schoener, and Elsaesser, 1964).

Ghosh and Chance (1964) measured changes in glycolytic intermediates, in relation to the NADH oscillations in yeast cells induced by the aerobic-anaerobic transition caused by glucose addition. They found that oscillations of fructose diphosphate level were in phase with those of the reduced NAD, while fructose-6-phosphate and glucose-6-phosphate oscillations did not possess this similar phase relationship; from the data obtained, the phosphofructokinase enzyme reaction was identified as the control site for

the oscillations. With this information available, a general chemical mechanism for reactions showing oscillatory behavior was applied directly to a specific explanation of a mechanism for glycolytic oscillations in yeast (Higgins, 1964). The model, in simple terms, consisted of an initial substrate input step, a product-activated enzyme step (phosphofructokinase), and another enzyme step serving to remove the product; a more recent publication (Higgins, 1967) has elaborated on this mechanism. Sel'kov (1968) also described, and mathematically analysed, a kinetic model proposing a mechanism for glycolytic oscillations as found in yeast. This model incorporated both product activation and substrate inhibition of the phosphofructokinase reaction, whereas Higgins' (1964) model included only the former.

Detailed analysis of the phase relationships of glycolytic intermediate oscillations in yeast cells (Betz and Chance, 1965b) revealed that several of the intermediates were in phase with the NADH oscillations, and suggested that the mechanisms involved were more complex than had been previously proposed (Higgins, 1964). It was found that during in vivo glycolytic oscillations, the actual concentrations of metabolites involved in the oscillations were relatively small as compared to the flow of these metabolites

through the glycolytic pathway (Betz, 1966). Alternate incorporation of ^{32}P into fructose-1,6-diphosphate and then ATP (Betz, 1968), suggested that alternating feedback-controlled activation of the steps in glycolysis involving phosphofructokinase and glyceraldehydephosphate dehydrogenase, might be responsible for the observed oscillations. The results of ADP additions to oscillating extracts (Pye, 1969) suggested that ADP might be an integral part of the oscillation mechanism, serving as an activator of phosphofructokinase.

Addition of the disaccharide trehalose to a cell-free yeast extract has permitted the attainment of over 90 continuous oscillations of the level of NADH (Pye, 1966; Pye and Chance, 1966). Several applications of trehalose during an experiment could successfully maintain such sustained oscillations for periods of up to 20 hours (Hess, Brand, and Pye, 1966). It was suggested (Pye, 1966; Pye and Chance, 1966) that a slow continuous supply of glucose, derived from hydrolysis of trehalose by the yeast enzyme trehalase, was siphoned into the glycolytic system and allowed the persistent oscillations to occur. In actuality, free glucose fed into a cell-free extract at approximately the same rate that trehalose had been hydrolysed, did lead to the characteristic oscillations (Pye, 1969). The fact

that trehalose is also found *in vivo* within yeast cells indicates that a sustained type of oscillation may be possible in intact cells.

It has been postulated by Pye (1969) that biochemical rhythms such as these self-sustained glycolytic oscillations, although possessing relatively shorter frequencies as compared to those of most overt biological rhythms, might function internally in organisms as driving timers coupled to the physiological displays. The occurrence of beats in some of the trains of sustained oscillations in yeast (Pye and Chance, 1966) certainly suggests a possible timing mechanism. Mechanisms for temperature compensation and frequency multiplication would of necessity have to be incorporated in such a system.

3. Metabolic and Biochemical Oscillations in Algae

a. Gonyaulax

The marine dinoflagellate *Gonyaulax polyedra*, has proven extremely amenable for extensive investigation of its inherent rhythmicity. Endogenous circadian rhythms of bioluminescence and photosynthetic capacity in *Gonyaulax* possess similar properties to rhythms found in higher plants. Moreover, biochemical aspects of these rhythms which have been investigated, can provide further scope

for comparison with more advanced organisms.

The discovery that induced bioluminescent flashing, due to stimulation of sea-water cultures of the organisms by stirring or shaking, was most intense in the middle of dark periods in normal photoperiodic cycles (Sweeney and Hastings, 1957), led to the finding that this rhythmicity would continue under conditions of constant temperature and continuous darkness (Sweeney and Hastings, 1957) or dim light (Hastings, 1960). The presence of a related endogenous rhythm of persistent luminescent glow was also established (Hastings, 1960), with peaks of luminescence occurring 45 minutes later each day in continuous dim light; oscillations of this rhythm possessed a degree of sensitivity to certain inhibitors of macromolecular biosynthesis (Karakashian and Hastings, 1962, 1963).

Concomitant with the demonstration of endogenous circadian oscillations of induced luminescence, was the successful extraction and isolation of a soluble luminescent system (Hastings and Sweeney, 1957a). This system consisted of a substrate, luciferin, and an active enzyme, luciferase -- extractable activity corresponded to the rhythmic display of induced luminescence. Later experiments (Hastings and Bode, 1962; Bode and Hastings, 1963) revealed that the activity of the actual enzyme molecule,

and not the amount of enzyme or concentration of an inhibitor or activator molecule, was changing during the rhythmic display. Maximal luciferin concentration corresponded to the times of most intense luminescence in vivo (Hastings and Bode, 1962; Bode, De Sa, and Hastings, 1963). The major discrepancy in this system was that the bioluminescent rhythm featured 40-60 fold differences in intensity of flashing between minimal and maximal phases, whereas luciferase activity and luciferin content in the respective extracts did not exhibit such large divergences.

The discovery of luminescent crystal-like particles termed "scintillons" in Gonyaulax (De Sa, Hastings, and Vatter, 1963), suggested an alternate mechanism. These particles emitted a luminescent flash in response to a lowering of pH and appeared to react completely independent of the soluble system. Hastings and Keynan (1965) proposed that luciferin and luciferase were adsorbed to guanine crystals to form active scintillons, and it was intimated that the so-called "soluble system" was actually an artifact derived from the crystals during extraction! Yet more recently (Sweeney and Bouck, 1966; Sweeney, 1969b), it has been propounded that the soluble system was indeed responsible for the bulk of the light produced during display of the luminescent rhythm. The effect of the addition

of acetic acid (Sweeney, 1969b), which was found to minimize the difference between the luminescence of day and night phase cells, suggested that periodic *in vivo* sensitivity of the luminescent system to stimulation might be responsible for the rhythmicity. It was hypothesized that perhaps membrane permeability changes were involved in the response, with differences in permeability existing in the different phases of the rhythm.

An endogenous circadian rhythm of photosynthetic capacity has also been characterized in *Gonyaulax polyedra* cells (Hastings and Astrachan, 1959; Hastings, Astrachan, and Sweeney, 1960), occurring under similar light conditions as the bioluminescence rhythms although possessing a different phase relation. Neither changes in chlorophyll content nor periodicity in nucleic acid metabolism were found to be directly related to this rhythm (Hastings, 1960), although the generalization was put forth that if there were indeed some nucleic acid metabolism associated with the endogenous clock in this or any other organisms, the active proportion of the bulk nucleic acid content might be so small as to escape detection with the techniques employed. The photosynthetic process could be inhibited in *Gonyaulax* cells with minor effect on the rhythmicity (Hastings, 1960; Karakashian and Hastings, 1962), thereby indicating an

effect on the physiological display rather than on the endogenous clock itself.

In searching for a biochemical association with the photosynthetic capacity rhythm, Sweeney (1964) established that no periodicity existed in either the Hill reaction in intact cells or dichlorophenolindophenol reduction by cell homogenates; however, a close correlation was found with cycles of activity of the photosynthetic enzyme, ribulose diphosphate carboxylase (Sweeney, 1964, 1965, 1969b). The suggestion that oscillations in the activity of this enzyme could be responsible for the photosynthetic rhythm was implicit, although the basis for the differences of activity between "day" and "night" ribulose diphosphate carboxylase could not be ascertained (Sweeney, 1969b).

b. Other Algae

Several authors have characterized the presence of an endogenous circadian rhythm of photosynthesis (measured by oxygen evolution) in Acetabularia cells (Sweeney and Haxo, 1961; Schweiger, Wallraff, and Schweiger, 1964; Vanden Driessche, 1966; Terborgh and McLeod, 1967). This rhythm persisted under conditions of constant temperature and continuous light in both nucleated and enucleated cells (Sweeney and Haxo, 1961; Schweiger et al., 1964; Vanden

Driessche, 1966), for durations as long as 40 days (Schweiger et al., 1964). Rhythmicity in enucleated Acetabularia exhibited a high degree of insensitivity toward inhibitors of protein and nucleic acid synthesis (Vanden Driessche, 1966; Sweeney, Tuffli, and Rubin, 1967). An associated rhythm in chloroplast shape (Vanden Driessche, 1966), which was normally in phase with the photosynthetic rhythm in Acetabularia mediterranea, suggested the existence of a possible structure-function relationship.

Non-circadian oscillations in net photosynthetic oxygen evolution have been observed in cultures of Chlorella pyrenoidosa under strong illumination in the presence of either low CO_2 concentration or photosynthetic poisons (Bannister, 1965). The period of these oscillations was extremely short, between 4-60 seconds, and the amplitude was quite small.

In order to ascertain whether a correlation existed between enzyme activity and the maxima and minima of the photosynthetic oscillations in Acetabularia crenulata, Hellebust, Terborgh, and McLeod (1967) measured the activities of each of nine enzymes of the reductive pentose cycle at the midpoints of the "light" and "dark" phases of the rhythm -- no significant differences were found. An endogenous circadian rhythm of RNA synthesis in Acetabularia

mediterranea revealed by tritiated-uridine incorporation studies (Vanden Driessche and Bonotto, 1969), was apparently unrelated to the previously established photosynthetic oscillations, as shown by the inability of inhibited RNA synthesis to affect the photosynthetic periodicity (Vanden Driessche, Bonotto, and Brachet, 1970).

von Klitzing (1969) observed oscillations of several biochemical parameters with different frequencies in Acetabularia mediterranea cells. These included enzyme activities of lactate and malate dehydrogenase (frequency about 50 min^{-1}), ATP concentration (frequency 2-4 min^{-1}), and concentration of an unknown compound (frequency 150-360 min^{-1}); as previously shown for the endogenous circadian rhythms of photosynthesis (Sweeney and Haxo, 1961; Schweiger et al., 1964; Vanden Driessche, 1966), these oscillations were not affected by the removal of the nucleus. In an examination of enzyme activities in Hydrodictyon colonies grown under varying light and dark alterations, Richter and Pirson (1957) observed maxima and minima of activity of acid phosphatase, catalase, and starch phosphorylase corresponding to diurnal changes in the rates of photosynthesis and respiration; however, triose phosphate isomerase and glucose-6-phosphate dehydrogenase, enzymes more specifically involved in respira-

tory pathways, displayed no such periodicity. Feldman (1968) observed an endogenous circadian rhythm of amino acid incorporation in Euglena gracilis when cultures were transferred to continuous darkness, having previously been in a light-dark cycle in which a daily rhythm of incorporation was apparent. He perceived that this rhythmicity could have been related to oscillations either in the rate of protein synthesis or amino acid uptake.

C. Metabolic and Biochemical Rhythmicity in Higher Plants

Rhythmical aspects of respiratory and photosynthetic metabolism in higher plants has prompted several research studies. Endogenous rhythmicity in the rate of CO_2 evolution has been observed in leaves of Kalanchoë blossfeldiana (Schmitz, 1951, cited in Bünning, 1956), in excised leaves of Bryophyllum species (Wilkins, 1959), in callus cultures derived from Bryophyllum daigremontianum leaf tissue (Wilkins and Holowinsky, 1965), and in photoperiodically induced Lemna gibba plants (Miyata and Yamamoto, 1969). It was discovered that the endogenous circadian rhythm of CO_2 output in Bryophyllum fedtschenkoi leaves had as its origin a correlated rhythm in the rate of CO_2 dark fixation (Warren and Wilkins, 1961) -- the peaks of one rhythm corresponded to the troughs of the other. Oscillations in

net photosynthetic rate in attached leaves of cotton and pepper plants (Barrs and Klepper, 1968) coincided with cyclic variations in stomatal aperture (Barrs, 1968). Chia-Looi (1971, unpublished data) has recently accumulated evidence showing rhythmicity of net photosynthetic rate, chlorophyll content, and dark respiration in Chenopodium rubrum ecotypes 60°47'N and 50°10'N.

Bukatsch and Rudolph (1963) reported that under light-dark cycles (but constant temperature), rhythmic fluctuations in chlorophyll content occurred in Rumex alpinus leaves and in leaves and cotyledons of Perilla ocymoides; it had previously been established (Mitrakos, Bünning, and Eberhardt, 1957) that daily rhythmic variations in chlorophyll a and b levels in Perilla ocymoides would continue under conditions of continuous illumination and constant temperature. Alterations in a small fraction of the bulk chlorophyll in the plastids have been suggested as a possible origin for endogenous rhythmicity in the Hill-reaction activity of tomato chloroplasts (Hoffman and Miller, 1966). Wagner and Cumming (1970) described a rhythmic accumulation of chlorophyll and betacyanin in Chenopodium rubrum ecotype 50°10'N.

Quite a few years after Van de Sande-Bakhuysen's (1925) claim using wheat as his test material, that under constant

conditions of illumination and temperature, layering of newly synthesized starch did not occur in starch grains, several authors held the opposite to be true. Layering was observed to be present in starch grains of potato tubers grown under constant conditions of light and temperature (Roberts and Proctor, 1954; Mes and Menge, 1954; Hess, 1955, cited in Bünning, 1967). However, Buttrose (1960) found shells of starch deposition in barley starch granules, only in plants grown under normal day-night conditions. A detailed light- and electron-microscope study of both potato tuber and wheat endosperm starch granules disclosed that layering of starch in constant conditions occurred only in the former (Buttrose, 1962); it was postulated therefore, that in potato but not in wheat there was a functioning endogenous rhythm of starch production.

Extensive analytical investigations have revealed diurnal fluctuations of nitrogenous compounds in white spruce seedlings grown under natural conditions of light and darkness (Durzan, 1968a, 1968b). Under similar conditions, Becker (1953, cited in Bünning, 1956) observed diurnal fluctuations in auxin concentration in leaves and petals of Kalanchoë blossfeldiana, with maxima occurring in the light. Galston and Dalberg (1954) postulated the involvement of endogenous rhythmicity in adaptive (induc-

ible) synthesis of indoleacetic acid (IAA) oxidase, in the auxin-IAA oxidase system.

Diurnal variations in the activities of various enzymes have been observed in the phylloclades of cactus, Nopalea dejecta. It should be noted that this rhythmicity was neither circadian nor endogenous, as it did not occur in constant conditions but rather in natural day-night cycles. Sanwal and Krishnan (1960) observed such rhythmicity in the specific activities of aldolase and phosphatase, with aldolase having its highest activity during the evening and lowest during the day while the opposite was true for the phosphatase enzyme. It was suggested that the variations of activity were adaptive responses related to aspects of crassulacean acid metabolism in cactus. Glutamate-oxaloacetate transaminase (Mukerji, Sanwal, and Krishnan, 1961) showed the same activity pattern as the phosphatase enzyme mentioned above. The activity of mixed homogenates from periods of high and low activity indicated that neither activators nor inhibitors were involved, but rather that actual changes in the amount of active enzyme were being effected.

Khan and Sanwal (1965) found cyclic variations in the activities of the Krebs' cycle enzymes aconitate hydratase, succinate dehydrogenase, and fumarate hydratase in cactus

phylloclades, with maxima and minima of all three enzymes occurring coincidentally. A small portion of the variation in the activity of these enzymes was believed due to a periodic alteration of inhibitor levels. Peaks of activity of isocitrate dehydrogenase and glucose-6-phosphate dehydrogenase have been shown to occur during daylight hours (Khan and Sanwal, 1971). An analysis of the mitochondrial and supernatant fractions of extracts derived from cactus phylloclades (Khan et al., 1970), indicated the presence of diurnal rhythms in the activities of several enzymes in each fraction.

Similar diurnal cycles of enzyme activity have also been observed in species other than cactus. Srivastava and Krishnan (1962) found, in natural day-night conditions, a marked diurnal variation in both the activity of oxalic acid oxidase and oxalic acid content in extracts of leaves of Bougainvillea spectabilis, with peaks showing an inverse correlation. Diurnal variations in some enzymes of carbohydrate metabolism have been observed in extracts of tapioca leaves (Viswanathan, Srivastava, and Krishnan, 1962). Of the enzymes assayed, fructose-1,6-diphosphate aldolase, phosphorylase, and alkaline fructosediphosphatase demonstrated maxima during the day, while phosphoglucomutase exhibited a slightly higher activity at

night and glutamate-oxaloacetate transaminase activity remained constant throughout. Cyclic variations of the activity of acid phosphatases occurred in cowpea (Mishra and Mohanty, 1967) and rice leaves (Mishra and Panda, 1970), with maxima of activity evident during the daylight hours. Ehrenberg (1950, cited in Bünning, 1956) characterized rhythmic fluctuations of amylase and phosphatase activities in Phaseolus multiflorus, and also of phosphatase activity in leaves of Kalanchoë blossfeldiana (Ehrenberg, 1954, cited in Bünning, 1956). Nitrate reductase in corn leaves displayed a diurnal variation of activity (Hageman, Flesher, and Gitter, 1961), possessing maximal activity during the day and minimal activity at the end of the night.

Examples of enzymatic endogenous rhythmicity in higher plants have been few and far between. Venter (1956, cited in Bünning, 1956) demonstrated the occurrence of rhythmic activity of amylase in Spinacia oleracea in continuous light. More recently, Miyata and Yamamoto (1969) have described circadian damped oscillations in the activities of both NADP-linked and NAD-linked- glyceraldehyde-3-phosphate dehydrogenase (GPD), and acid phosphatase, in photoinduced Lemna gibba plants maintained under continuous light. In seedlings of Chenopodium rubrum ecotype 50°10'N,

Frosch (1971, unpublished data) has similarly characterized endogenous oscillations in the activities of both NADP-linked- and NAD-linked-GPD, having a period of fifteen hours; associated oscillations of adenylate kinase activity possessed a period of approximately thirty hours, with sub-peaks of 15 hour periodicity.

CHAPTER III

EXPERIMENTAL PROGRAMME

To ascertain the possible biochemical implications of rhythmic floral induction in Chenopodium rubrum ecotype 60°47'N, it seemed feasible initially to investigate levels of soluble metabolites extractable from seedlings grown under identical conditions to those eliciting the rhythmic flowering response (Cumming et al., 1965). Oscillations of specific small molecules, both carbohydrate and nitrogenous in nature, might have assumed a regulatory function through mechanisms such as repression of the synthesis of enzymes or other proteins (Vogel, 1961). Specifically, the ability of glucose to inhibit the synthesis of certain enzymes (Magasanik, 1961) could be more than coincidental with its observed effects on overt rhythmicity in Chenopodium rubrum (Cumming, 1967a) and other systems (Berliner and Neurath, 1966). Levels of soluble metabolites might also reflect cycles of breakdown of insoluble reserve materials such as starch or reserve protein (see Chapter II, part II). Along these lines, an analysis of starch content

in the seedlings could also yield interesting data, in light of the universality of starch in plants and the susceptibility of starch formation to induction by sugar (Gates and Simpson, 1968).

Results from the type of experiment referred to above, being inconclusive, suggested that the examination of more specific aspects of cellular metabolism such as enzymatic activities, might prove more rewarding. Taking into account previous data obtained, in conjunction with the consideration of reports in the literature and feasibility of methodological aspects, the levels of the activities of the following enzymes were analysed in an experimental situation: peptidase, acid phosphatase, amylase, glucose-6-phosphate dehydrogenase, peroxidase, glutamate-oxaloacetate transaminase, nitrate reductase. It could be seen that of these enzymes, only the activity of nitrate reductase, an inducible enzyme (Filner, Wray, and Varner, 1969; Kessler, 1964; Beevers and Hageman, 1969), showed significant indications of endogenous rhythmicity; hence, a more detailed investigation of this enzymatic activity was executed.

CHAPTER IV

MATERIALS AND METHODS

I. SOURCE OF EXPERIMENTAL MATERIAL

Seedlings used in most of the work described were grown from a seed sample of Chenopodium rubrum L. ecotype 60°47'N 137°32'W (selection 374) harvested in July 1966 (1000 seeds weighed approximately 0.1 g). The source plants had been soil-grown at the University of Western Ontario greenhouses under a continuous light regime in which natural daylength had been supplemented by incandescent illumination.

II. PREPARATION OF SEEDS FOR GERMINATION

Seeds were sieved and hand-rubbed so as to be free of all extraneous organic material. Cleaned seeds were then treated with a wetting agent (Cumming, 1967a), in order to promote water imbibition during germination. A 30 minute immersion in 1% "aerosol" (Fisher Scientific Company) was followed by several washes of boiled distilled water; seeds were allowed to dry completely before being sown.

III. GERMINATION MEDIUM

All operations were carried out in a manner so as to provide semisterile conditions for germination and subsequent growth (Cumming, 1967b). Small Petri dishes (6 x 1.5 cm) containing eight layers of 4.25 cm diameter Whatman No. 2 filter paper and an excess of distilled water, were placed in enamel trays covered by aluminum foil and autoclaved. Prior to the sowing of seeds, the excess liquid was poured off from each dish in sufficient quantity to allow for a suitable filter paper support for the seeds without flooding. Seeds could then be sown by hand into the dishes fairly uniformly, with the aid of a small seed envelope.

IV. GERMINATION AND GROWING CONDITIONS

The conditions employed were essentially those described previously by Cumming et al. (1965) and Cumming (1967a, 1967b). Germination occurred in growth cabinets under an alternation of high and low temperatures, namely 12 hr at 32.5°C and 12 hr at 10.0°C. This temperature alternation cycle was provided for either 72, 84, or 108 hr; the lengthening time was necessitated by a slight decrease in seed viability as experimentation proceeded over successive years. In all cases, the germination period

began and ended at the higher temperature. Light was provided continuously during the germination period in the form of cool-white fluorescent light with intensities averaging 600 ft-c at 32.5°C and 450 ft-c at 10°C. The light intensity was calibrated with a light meter (Weston Illumination Meter, Model 756, Weston Electrical Instrument Corporation, Newark 5, New Jersey) at the higher temperature, and the lower intensity at 10.0°C was a consequence of the cooling of the fluorescent tubes. Subsequent to the germination period, the temperature was always maintained at 20°C, whether in light or darkness.

Immediately following the end of the germination period, Hoagland's No. 1 nutrient solution (Hoagland and Arnon, 1938) was added to the Petri dishes either by using a large capacity plastic plunger syringe (Tomac Irrigating Syringe, 2 oz capacity, obtained from American Hospital Supply, Port Credit, Ontario) or a more practical autoclavable teflon squeeze-bottle (Cole-Farmer Instrument Company, Chicago, Illinois); iron was provided in the nutrient solution in the form of "Sequestrene" (sodium ferric ethylene-diamine tetraacetate, Giegy Chemical Company, Ardsley, New York). At that time the seedlings were given a 24 or 36 hr light treatment, the quality and intensity of which depended on the experiment being under-

taken.

If a dark period was to be given, it was inserted immediately following this light treatment -- Petri dishes were wrapped in aluminum foil or placed in black, light-proof cloth bags and maintained in a darkened growth cabinet. A complete range of dark period lengths could be obtained conveniently in an experiment by beginning the germination periods for groups of Petri dishes at 12 hr intervals. Subsequent to a dark period, if one was given, seedlings were either returned to the conditions that prevailed before darkness, or harvested immediately. The irradiation sources used were as follows:

Fluorescent white light -- Westinghouse cool white fluorescent lamps,

F40 T12/CW/SHO (for 3000 ft-c)

F20 T12 CW (for 600 ft-c)

Incandescent white light -- General Electric 300W incandescent lamps (for 1000 ft-c).

V. MEASUREMENT OF ENDOGENOUS METABOLITES

A. Harvesting Procedure

Petri dishes were removed from the darkened growth cabinet at their respective experimental times, and, while still wrapped in foil, carried in an enamel tray under

lightproof black cloth to a darkroom equipped with a green safelight (dim incandescent filament light filtered by two layers of No. 24 dark green "Cinemoid" plastic sheeting with a transmission maximum at approximately 520nm, purchased from Strand Electric and Engineering Co. Ltd., 755 Yonge St., Toronto, Ontario). The foil was stripped from a dish and the top layers of filter paper with attached seedlings were removed and immediately immersed in liquid nitrogen (-196°C) for 5-10 seconds; the frozen seedlings were then scraped off (minus roots) with a spatula into a pre-chilled mortar positioned on a block of dry ice (-78.5°C). This procedure was repeated for each dish in a sample until the required number had been utilized (25-30 dishes per sample). The collected seedlings were then ground in the mortar with a pre-chilled pestle following the addition of a small quantity of liquid nitrogen. The powder was placed in a small beaker and stored over dry ice until the end of the day, at which time all samples collected were freeze-dried overnight in a Thermovac freeze-drier. All dessicated samples were then stored in a dessicator containing phosphorus pentoxide and silica gel until extraction could be carried out.

B. Extraction Procedure

A 250 mg sample of dried plant material was refluxed

for one hour in 100 ml of 80% v/v ethanol; the heat source was an electric heating mantle and the suspension was stirred continuously using a magnetic stirrer. Then the resultant suspension was filtered through three layers of "Nitex" (Tobler, Ernst, and Traber Inc., Canadian Distributor B. and S. H. Thompson and Co. Ltd., Montreal 3, Quebec) monofilament nylon heavy duty screen cloth of pore size 149 microns, in a Büchner funnel without suction. In later experiments, a scintered glass filter Büchner funnel (medium porosity) was used in place of the nylon screening. This filtration removed most of the larger particulate matter; the residue remaining in the funnel was washed with an additional 100 ml of hot 80% ethanol. Then the 200 ml filtrate was concentrated to dryness by vacuum distillation in a rotary evaporator apparatus.

Then approximately 20 ml of distilled water was added to the dried residue and the resulting liquid was centrifuged at 15,000 revolutions per minute (27,000 x gravity) for 15 min in a Sorvall model SS-3 Automatic Superspeed Centrifuge. The supernatant was collected and the pellet was resuspended in approximately 10 ml of distilled water and recentrifuged. This was repeated once more. The three combined supernatant fractions were then concentrated to dryness as previously done.

To this residue was then added 10 ml of distilled water along with 500 mg of decolourizing carbon (Baker and Adamson Code 1551, Allied Chemical Co., Montreal, Quebec) as described by Bevenue (1949). This mixture was allowed to sit at room temperature for 30 minutes with occasional stirring. It was then filtered through a Millipore filter of pore size 0.8 microns. An alternative method for the clarification of plant extracts (Sanderson and Perera, 1966) would not remove the coloured components as thoroughly as this decolourizing carbon method. After washing the residue several times with small quantities of distilled water, the combined filtrate was concentrated to dryness. The precipitate which remained was taken up quantitatively in two ml of distilled water and stored frozen until quantitative or chromatographic analyses were undertaken.

C. Thin-Layer Chromatography

1. Preparation of Chromatoplates

Prior to use, the glass plates which measured 20 x 20 cm (Mondray Ltd., Montreal 26, Quebec) were washed with detergent and rinsed with tap water, distilled water, and methanol, in that order. The adsorbent used for all chromatographic procedures was microcrystalline cellulose (Vomhof and Tucker, 1965; Wolfrom, Patin, and de Lederkremer,

1965; Wolfrom, de Lederkremer, and Schwab, 1966) obtained as "Avicel" (Avicel Sales Division, American Viscose Division, FMC Corporation, Marcus Hook, Pennsylvania 19061).

The slurry was prepared by adding 3.4 parts distilled deionized water to one part cellulose powder (by weight) in a 500 ml filter flask. A smooth, two inch magnetic stirring bar was placed in the flask, the flask was sealed with a rubber stopper, and the side arm was connected to an aspirator. Using a magnetic stirring motor under the flask, stirring of the magnetic bar was begun and maintained at a moderate rate, while at the same time suction was started so that air bubbles in the cellulose slurry could be removed under vacuum. This concomitant suction and stirring was maintained for approximately 15 minutes. At the end of this time, the stirring was slowed and the suction was released as gradually as possible in order to avoid the formation of new air bubbles in the slurry. Then, as quickly as possible, the slurry was poured into the applicator, and a 0.4 mm thick layer was spread onto the glass plates. The applicator apparatus used was a "Unoplan Leveller - Model P with adjustable spreader" (Shandon Scientific Co., 515 Broad St., Sewickley, Pennsylvania 15143). In this apparatus, five chromatoplates could be prepared at one time -- this required 40 g microcrystalline

cellulose plus 136 ml of distilled deionized water. The final thickness of the dry cellulose layer was approximately 125 microns. Dried plates were stored in a cabinet under laboratory conditions. The above method, if followed carefully, ensured smooth, uniformly consistent layers on the glass plates.

2. Chromatography of Sugars

a. Development solvent

The solvent used was formic acid-tertiary butanol-methyl ethyl ketone-distilled deionized water (15:40:30:15, v/v) (Vomhof, Truitt, and Tucker, 1966). All reagents used were analytical grade.

b. Detection Reagent

Several reagents were tested (Hockenhull, 1953; Bryson and Mitchell, 1951; Lemieux and Bauer, 1954; Cifonelli and Smith, 1954; Mowery, 1957; Stahl and Kaltenbach, 1961); however, the reagent described by Mukherjee and Srivastava (1952) gave the best results in that it was extremely sensitive and reacted equally well with glucose, fructose, and sucrose standards on a chromatogram. This reagent was prepared as follows:

0.5 g of p-anisidine (Eastman Organic Chemicals, Rochester, New York) was dissolved in 2 ml of phosphoric

acid. This solution was diluted with 95% ethanol to 50 ml and then filtered to remove the p-anisidine phosphate precipitate. The filtrate (solution A) was retained and the p-anisidine phosphate was dissolved in the minimum amount of water, diluted with an equal volume of 95% ethanol, and to this solution was then added phosphoric acid to the extent of 2% (by volume) thus giving solution B. The two solutions (A and B) could then be mixed together or used separately. After spraying, detection of the sugars could be achieved by heating the chromatograms for several minutes at 95-100°C.

c. Chromatographic Procedure

The origin was marked 2.5 cm from the bottom of the plate. Samples and standards were applied at 1.5 cm intervals using a Hamilton microsyringe (700 Series, 50 microliter size, point style #3). Spots were dried between applications with a commercial hairdryer and a total of 40 microliters of the respective extract concentrate were applied at each locus.

Solvent was placed in the developing chamber ("Chromaflex" Developing Chamber, Kontes Glass Co., Vineland, New Jersey) to a depth of approximately 0.8 cm immediately before the plates were inserted (de Zeeuw, 1968). The plates

were developed twice one-dimensionally in an ascending manner at room temperature with an adequate drying time between developments. The solvent front was allowed to rise 10 cm from the origin each time; each development required approximately three and one-half hours.

After the final development and drying, the chromatograms were sprayed with the detection reagent, heated, and observed. Detection of spots was much improved under long-wave ultraviolet light.

3. Chromatography of Amino Acids

a. Development Solvent

The solvent system of Jones and Heathcote (1966) was used:

Solvent I -- 2-propanol-formic acid-water (40:2:10, v/v)

Solvent II-- tertiary-butanol-methyl ethyl ketone-ammonium hydroxide-water (50:30:10:10,v/v)

b. Detection Reagent

Ninhydrin-collidine chromogenic reagent (Jones and Heathcote, 1966):

Combine: 0.3 g ninhydrin

20.0 ml glacial acetic acid

5.0 ml collidine (2, 4, 6-trimethylpyridine)

Make up to 100 ml with 95% ethanol.

c. Chromatographic Procedure

i. One Dimensional

The origin was marked 2.5 cm from the bottom of the plate. The samples and standards were applied at 1 cm intervals using a Hamilton microsyringe; a total of 40 microliters of the respective extract concentrate was applied at each locus. By careful application, the spot size could be restricted to 4 mm in diameter.

The plates were placed in the chamber immediately after the addition of the solvent and ascending development was allowed to continue at room temperature until the solvent front had risen 12.5 cm from the origin. Solvent II was preferred for one-dimensional development.

After drying, the chromatogram was sprayed with the ninhydrin-collidine reagent and allowed to dry, after which time the amino acids could be detected.

ii. Two Dimensional

The procedure outlined by Jones and Heathcote (1966) was followed. The sample spot was applied at a point in the lower left corner of the plate 2.5 cm from the bottom edge and 2.5 cm from the side edge. Development with solvent I was always "along the grain" of the cellulose layer for consistency. Ascending development proceeded until the

solvent front had travelled 12.5 cm; this required approximately one and two-third hours. Upon drying, a line was etched in the cellulose layer just below a yellow band containing impurities which had formed as a result of the first development. This was necessary so that the solvent front would not be held back during development in the second dimension. A line was also etched 1 cm below the spot centre to insure uniformity of flow of the solvent. The plate was then turned 90° and developed in Solvent II in an ascending manner until the solvent front had travelled 12.5 cm from the origin; this required approximately one and one-quarter hours. After the final drying, the detection reagent was used to reveal the amino acid separation.

4. Chromatography of Sugar Phosphates

a. Development Solvent

The following solvents did not give good separations:

acetone-acetonitrile-1N hydrochloric acid (64:26:10, v/v) (Grassetti, Murray, and Wellings, 1965);

water-poor phase of: tertiary-amyl alcohol - water - formic acid (90:90:30, v/v) (Hanes and Isherwood, 1949);

tertiary-butanol- 50% formic acid- water (16:1:4, v/v) (Agarwal, Sanwal, and Krishnan, 1962);

tertiary-butanol- ammonium hydroxide- water (60:30:10, v/v) (Agarwal, Sanwal, and Krishnan, 1963);

ethylene glycol monomethyl ether (methyl cellosolve) - pyridine- glacial acetic acid- water (80:40:10:10, v/v) Runeckles and Krotkov, 1957);

methanol- aqueous ammonia (specific gravity 0.896)- water (7:1:2, v/v) (Davidson and Drew, 1966);

methanol- glacial acetic acid- water (8:1.5:0.5, v/v) (Davidson and Drew, 1966).

The one solvent which gave relatively good separations and which was subsequently used for the chromatography of the experimental extracts was one derived from Hanes and Isherwood (1949), composed of the water poor phase from: 60 ml tertiary-amyl alcohol- 30 ml water- 2 g p-toluene-sulphonic acid (Matheson, Coleman, and Bell, Norwood, Ohio).

b. Detection Reagent

Molybdate reagent (Hanes and Isherwood, 1949):

Combine: 1 ml 60% perchloric acid
2 ml 1N hydrochloric acid
5 ml 4% ammonium molybdate

Make up to 20 ml with distilled deionized water.

c. Chromatographic Procedure

The origin was marked 2.5 cm from the bottom of the

plate. The samples and standards were applied at 1 cm intervals using a Hamilton microsyringe; a total of 40 microliters of the respective extract concentrate were applied at each locus. After the solvent had been freshly prepared and added to the development chamber, the plates were positioned in the chamber and ascending development at room temperature was allowed to continue until the solvent front had risen 12.5 cm from the origin. After drying, the plate was run a second time in the same direction with fresh solvent; each development required approximately three and one-half hours.

Following the final drying, the chromatogram was sprayed with the molybdate reagent, allowed to dry, and then placed under a long-wave ultraviolet light source ("Black-Ray" Model X4, Ultra-Violet Products Incorporated, San Gabriel, California) for five minutes. This treatment allowed the spots formed by the reaction of the phosphorylated sugars with molybdate to become visible to the naked eye.

5. Chromatography of Organic Acids

a. Development Solvent

The solvent used was n-butanol- formic acid- water (25:3:7, v/v) (Marriage, 1969).

b. Detection Reagent

Bromphenol blue, 0.04% in 80% ethanol, made just basic with sodium hydroxide (Marriage, 1969).

c. Chromatographic Procedure

The origin was marked 2.5 cm from the bottom of the plate. The samples and standards were applied at 1 cm intervals using a Hamilton microsyringe; a total of 40 microliters of the respective extract concentrates were applied at each locus. Ascending development at room temperature was used and allowed to continue until the solvent front had risen 12.5 cm from the origin; only one development requiring approximately one and one-half hours was necessary. Upon drying, the chromatogram was sprayed with the bromphenol blue reagent.

D. Analytical Methods

1. Determination of Total Soluble Carbohydrates

a. Introduction

The phenol-sulphuric acid colorimetric method (Dubois et al., 1956; Hodge and Hofreiter, 1962) was used to determine the relative concentrations of the total soluble carbohydrates (mainly glucose and fructose) in the extract concentrates.

This method was used because it is simple, rapid,

accurate, sensitive, inexpensive and carbohydrate specific. The reactants are stable, easily available, and the method is virtually unaffected by the presence of amino acids and organic acids (Barnett and Tawab, 1957), and inorganic ions (Giudici and Fluharty, 1965). The colour produced is relatively stable for several hours, and there is an excellent linear relationship between absorbance and concentration (see Fig. 1a).

b. Reagents

sulphuric acid, 96%, reagent grade (Allied Chemical)

phenol, 5% -- made by diluting liquefied 90.5% phenol, reagent grade (Fisher).

i.e. 27.38 ml of 90.5%/500 ml solution.

c. Procedure

Between 50 and 100 microliters of the concentrated final extract was pipetted into the bottom of an 18 x 150 mm pyrex test tube using a Hamilton syringe (700 Series, 50 microliter size). The volume in this tube was then made up to 1.0 ml with distilled deionized water; blanks contained only 1.0 ml water. One ml of 5% phenol solution was added, and the solution in the tube was mixed by the action of a magnetic stirring motor on a 10 mm teflon coated magnetic stirring bar (Cole-Parmer Instrument Co., Chicago,

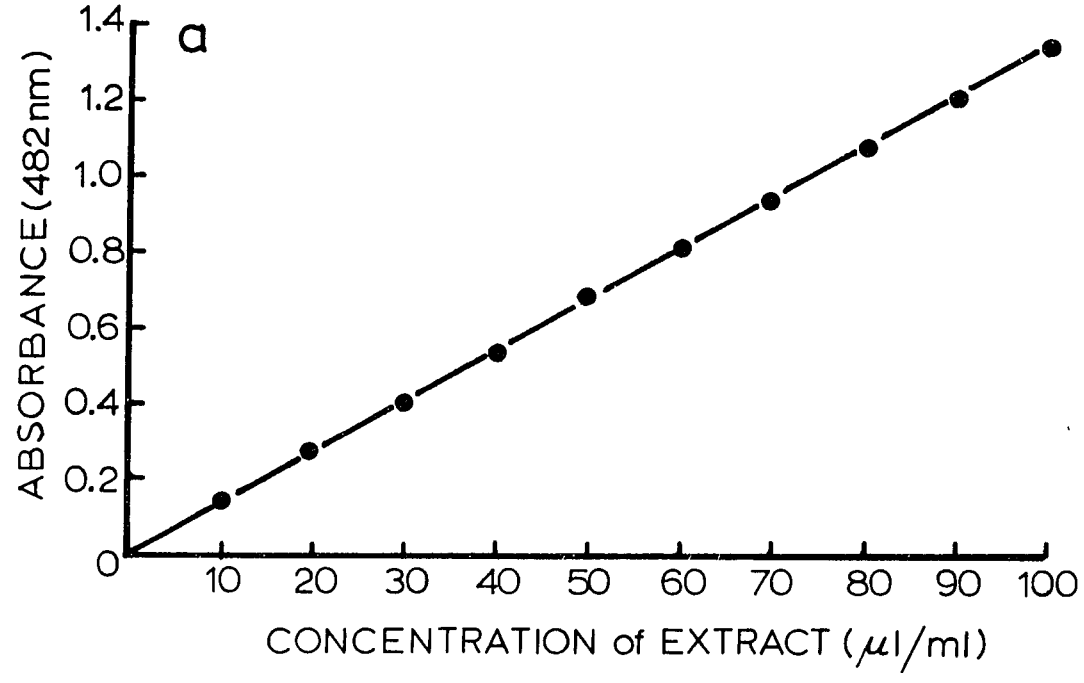
FIGURE 1

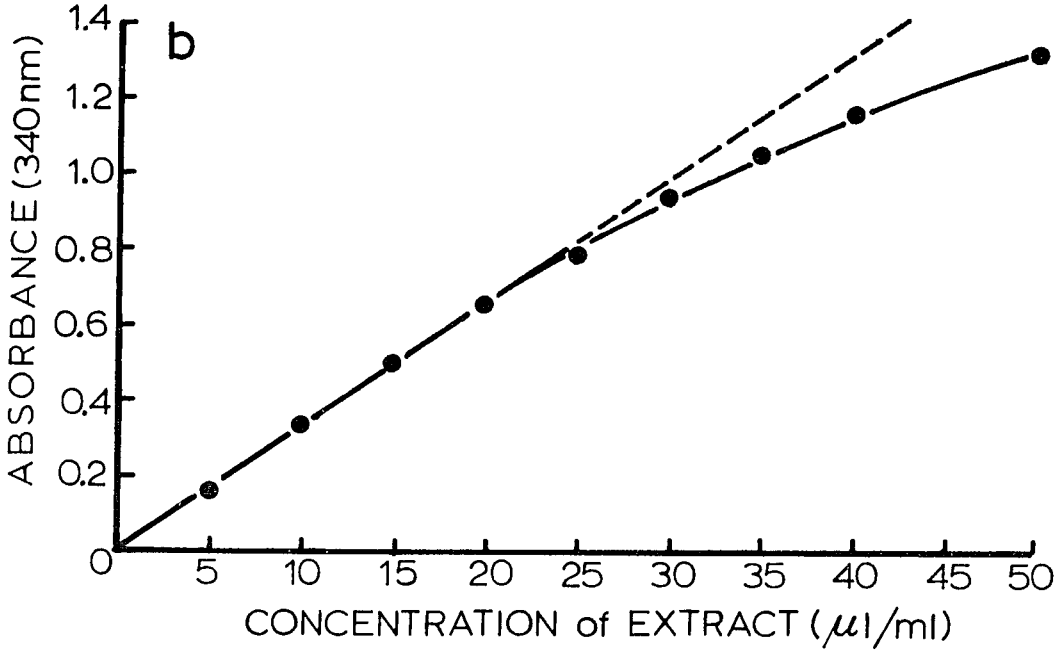
Concentrations of soluble metabolites vs. absorbance.

a. Relationship between relative concentration of total soluble carbohydrate in an extract and absorbance, for the phenol-sulphuric acid colorimetric method.

b. Relationship between relative concentration of total free amino acids in an extract and absorbance, for the TNBS colorimetric method.

In both cases, final volume made up to 1.0 ml with distilled deionized water.


a
ABSORBANCE (482nm)
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
10 20 30 40 50 60 70 80 90 100
CONCENTRATION of EXTRACT (μl/ml)


b
ABSORBANCE (340nm)
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
5 10 15 20 25 30 35 40 45 50
CONCENTRATION of EXTRACT (μl/ml)

Illinois) which had been placed at the bottom of the tube. Then, while the solution was being stirred continuously, five ml of concentrated sulphuric acid was added rapidly (within 15 seconds) from a 25 ml burette which had a cut-back tip in order to increase the flow rate. The tube was positioned so that the acid stream hit the liquid surface directly. The constant vortex action while the acid was being added assured good mixing and heat distribution -- this was essential for the reproducibility of the method (Giudici and Fluharty, 1965). The reaction product was stirred for exactly one additional minute and then the tube was set aside to cool at room temperature.

Presently, the absorbance was read in a Beckman Model DB Double Beam Spectrophotometer at 482 nm which had been found to be the absorption maximum for the carbohydrate mixture in the extracts.

2. Enzymatic Determination of Glucose and Fructose

a. Introduction

In order to determine whether the phenol-sulphuric acid method used in estimating total soluble carbohydrates was in fact showing changes in levels of glucose and fructose, since these two sugars had been shown to predominate in the chromatographic investigation, a specific enzymatic

method employing hexokinase and glucose-6-phosphate dehydrogenase was utilized to determine the glucose and fructose concentration in the extracts (Klotzsch and Bergmeyer, 1965).

Firstly, hexokinase was used to convert glucose and fructose to their respective hexose phosphates by the following reaction:

$$\text{hexose} + \text{ATP} \longrightarrow \text{hexose-6-phosphate} + \text{ADP}$$

Then glucose-6-phosphate concentration could be determined in the following manner, by the introduction of glucose-6-phosphate dehydrogenase:

$$\text{glucose-6-phosphate} + \text{NADP} \rightleftharpoons \text{6-phosphogluconate} + \text{NADPH} + H^+$$

The increase in absorbance due to the formation of NADPH was measured spectrophotometrically at 340 nm.

The concentration of fructose-6-phosphate could then be measured in this manner after its prior conversion to glucose-6-phosphate by the addition of phosphoglucose isomerase. This method also measured endogenous levels of glucose-6-phosphate and fructose-6-phosphate.

b. Reagents

Note: All reagents were made up in distilled deionized water unless otherwise noted.

0.05 M magnesium chloride ($MgCl_2.6H_2O$

0.017 M adenosine triphosphate ($ATP.Na_2H_2.3H_2O$, Sigma Chemical Co., St. Louis, Mo.)

0.012 M nicotinamide adenine dinucleotide phosphate (beta-$NADP-NaH_2$, Sigma Chemical Co., St. Louis, Mo.)

Hexokinase (Type C-300, from yeast, crystalline, ammonium sulphate suspension, Sigma Chemical Co., St. Louis, Mo.) Diluted with 3.2 M $(NH_4)_2SO_4$ to give a concentration of 1 mg protein / ml

Glucose-6-phosphate dehydrogenase (A grade, from yeast, in 3.3 M ammonium sulphate, Calbiochem, Los Angeles, California)

Diluted with 3.3 M $(NH_4)_2SO_4$ to give a concentration of 1 mg protein / ml

Phosphoglucose isomerase (Grade III, from yeast, crystalline, ammonium sulphate suspension, Sigma Chemical Co., St. Louis, Mo.)

Diluted with 2.4 M $(NH_4)_2SO_4$ to give a concentration of 1 mg protein / mg

0.05 M triethanolamine buffer, pH 7.6 (purchased as triethanolamine hydrochloride, Sigma Chemical Co., St. Louis, Mo.)

All solutions were stored in a cold room at 4^oC.

c. Procedure

Into sample and reference cuvettes, positioned in the cell compartment of a Beckman Model DB Double Beam Spectrophotometer, and being maintained at 25°C, were added the following:

2.62 ml triethanolamine buffer

0.10 ml $MgCl_2$ solution

0.10 ml ATP solution

0.10 ml NADP solution

0.01 ml hexokinase suspension

After allowing five minutes for temperature equilibration, 0.05 ml distilled deionized water was added to the reference cuvette and 0.05 ml plant extract to the sample cuvette; the contents were stirred and the absorbance (A_1) was adjusted to zero (i.e., $A_1 = 0$).

Then 0.01 ml glucose-6-phosphate dehydrogenase suspension was added and the absorbance was read after 10, 12, 14, and 16 minutes; the absorbance was extrapolated back to the time of the glucose-6-phosphate dehydrogenase addition (A_2) because of the possibility that the enzyme preparation might have contained traces of phosphoglucose isomerase activity. 0.01 ml phosphoglucose isomerase suspension was then added and after 20 minutes the absorbance was read (A_3).

From the measurements taken:

$A_3 - A_1 = \Delta A$ (glucose+fructose+glucose-6-phosphate+ fructose-6-phosphate)

$A_2 - A_1 = \Delta A$ (glucose+glucose-6-phosphate)

$A_3 - A_2 = \Delta A$ (fructose+fructose-6-phosphate)

Since the plant extracts contained endogenous glucose-6-phosphate and fructose-6-phosphate, the procedure was repeated but with the replacement of the hexokinase suspension by 3.2 M $(NH_4)_2SO_4$ solution.

Hence:

$A'_3 - A'_1 = \Delta A$ (glucose-6-phosphate+fructose-6-phosphate)

$A'_2 - A'_1 = \Delta A$ (glucose-6-phosphate)

$A'_3 - A'_2 = \Delta A$ (fructose-6-phosphate)

Therefore:

$\Delta A_{(glucose+glucose\text{-}6\text{-}phosphate)} - \Delta A_{(glucose\text{-}6\text{-}phosphate)} = \Delta A_{(glucose)}$

$\Delta A_{(fructose+fructose)} - \Delta A_{(fructose\text{-}6\text{-}phosphate)} = \Delta A_{(fructose)}$

The concentrations of the sugars or sugar phosphates in micrograms could be calculated according to Bergmeyer (1965).

3. Determination of Total Soluble Amino Acids

a. Introduction

The method of Satake et al.(1960) was used for the determination of the total soluble amino acid content, utilizing 2,4,6-trinitrobenzenesulphonic acid (TNBS) as the active reagent. This method has become increasingly popular in recent years (Habeeb, 1966; Nitecki, Stoltenberg, and Goodman, 1967; Mokrasch, 1967; Palmer and Peters, 1969) because in contrast to ninhydrin which is used most often for amino acid determination, TNBS is soluble in water, not affected by air, unreactive with ammonia, and effective under relatively mild conditions. TNBS reacts with the free amino groups of amino acids under alkaline conditions to produce an unstable complex (Palmer and Peters, 1969). Acidification will then convert this unstable complex to a stable trinitrophenyl derivative having an absorption maximum at 340 nm.

b. Reagents

4% $NaHCO_3$

0.10% TNBS (Eastman Organic Chemicals, Rochester, N.Y.)

1 N HCl

c. Procedure

It was found that for the complex mixture of amino

acids in the extracts, absorbance relative to concentration was linear only up to an absorbance of approximately 0.75 (see Fig. 1b). Therefore, a Hamilton Syringe (700 Series, 50 microliter size) was used to dispense an amount of each extract which would result in an absorbance value below this upper limit; an appropriate dilution factor was used to attain a level of equivalence between the different extract concentrates. 18 x 150 mm pyrex test tubes were used, with distilled deionized water being added to make the sample solutions up to 1.0 ml.

Then to each tube, 1.0 ml of $NaHCO_3$ solution was added, followed by 1.0 ml of TNBS solution.

The resultant solutions were mixed in a vortex shaker ("Fisher Mini-shaker," Fisher Scientific Co., Toronto, Ontario) and then the tubes were placed in darkness at a temperature of 40°C maintained by a water bath; an incubation time of two hours was used.

After the incubation, 1.0 ml of 1 N HCl was added and the resultant solution was mixed. The absorbance of the yellow product was read at 340 nm in a Beckman Model DB Double-Beam Spectrophotometer.

4. Determination of Starch Content in the Freeze-Dried Plant Material

a. Perchloric Acid Extraction

i. Introduction

Perchloric acid has been found to be an excellent solvent for the extraction of starch from plant tissues (Pucher, Leavenworth, and Vickery, 1948; McCready et al., 1950; Hassid and Neufeld, 1964). A relatively rapid analytical method which determined starch directly without previous acid hydrolysis (McCready et al., 1950) was utilized; however, in place of anthrone reagent, the phenol-sulphuric acid colorimetric procedure (Dubois et al., 1956) was used.

Since the original residue had previously been discarded after extraction of the soluble components from the bulk of the freeze-dried samples, only approximately 25-50 mg of dried plant material in each sample was available for the starch extraction. Fifteen milliliter conical bottom centrifuge tubes were used in the extraction as compared to the larger 50 milliliter size recommended in the original method, because of the reduced quantity of plant material available; the reagent volumes were reduced by an appropriate factor of five.

ii. Materials

80% ethanol -- 95% diluted with distilled deionized

water.

52% perchloric acid-- 72% (71.3%, British Drug Houses, Toronto, Ontario) diluted with distilled deionized water.

iii. Procedure

The freeze-dried sample was ground to a fine powder using mortar and pestle, and between 25-50 mg were weighed out and placed at the bottom of a 15 milliliter conical bottom centrifuge tube. A few drops of 80% ethanol were then added to wet the material and prevent clumping. One ml of distilled deionized water was added and the suspension was stirred thoroughly. The tube was then placed in a hot water bath at approximately 70°C, and 5 ml of hot 80% ethanol were added to the tube for the removal of soluble components. This mixture was stirred continually for 10 minutes, centrifuged at high speed in a table top centrifuge (International Clinical Model), and the supernatant discarded; this extraction was repeated three times.

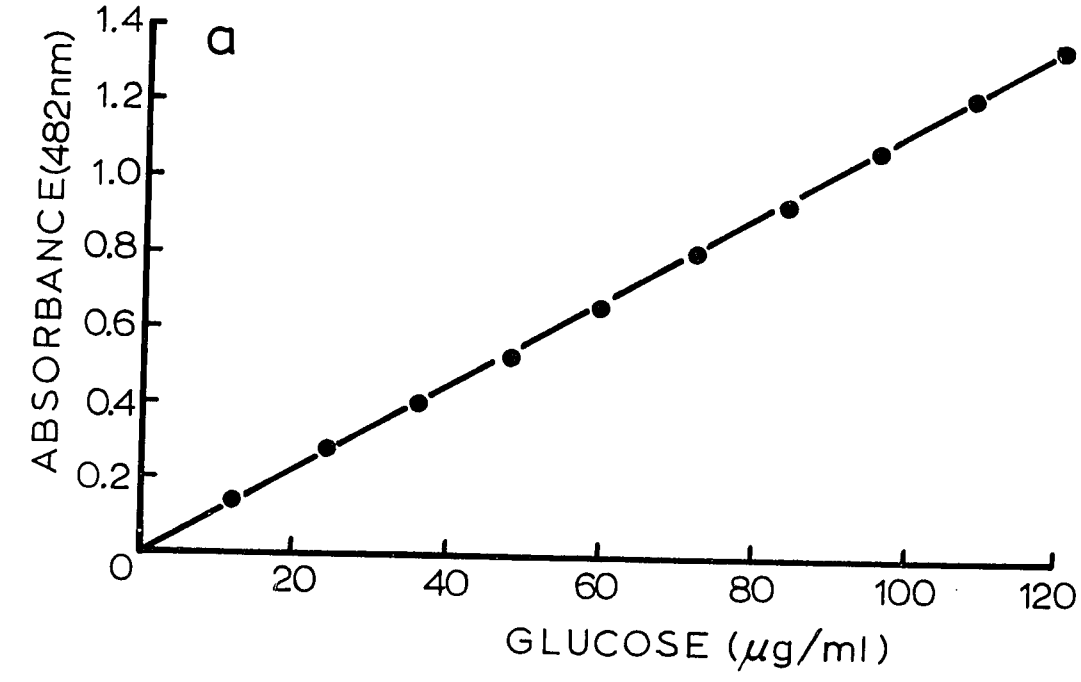
In order to gelatinize the starch, the residue was then heated for 15 minutes in a boiling water bath, after the addition of one ml of distilled deionized water. The suspension was cooled to room temperature, and 1.3 ml of

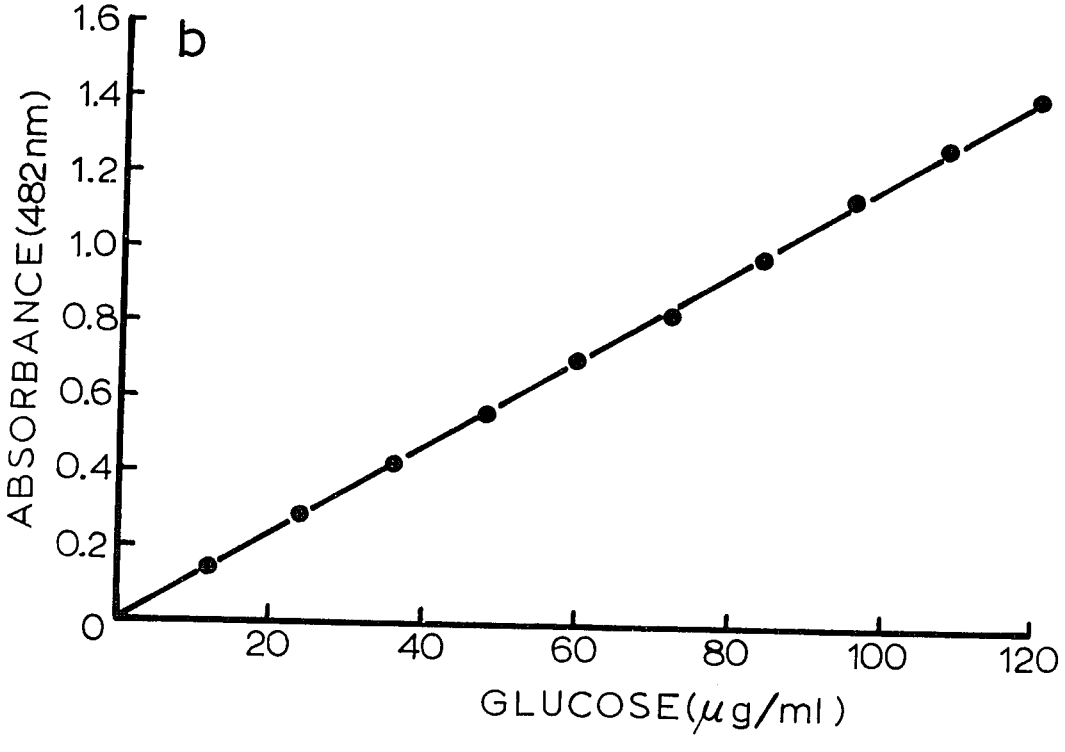
FIGURE 2

Standard curves for starch determination.

a. Perchloric acid extraction.

b. Alpha-amylase extraction.


a
ABSORBANCE(482nm)
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
20
40
60
80
100
120
GLUCOSE (μg/ml)


b
ABSORBANCE(482nm)
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
20
40
60
80
100
120
GLUCOSE(μg/ml)

52% perchloric acid was added while stirring with a glass rod and maintaining the tube at room temperature in a water bath. The stirring was continued for about 5 minutes and thereafter occasionally during fifteen additional minutes. Four ml of distilled deionized water were then added and the mixture was centrifuged. The aqueous starch solution was transferred to a 25 ml volumetric flask using a Pasteur pipette, and the extraction with perchloric acid was repeated without preliminary heating. The tube and glass rod were washed with water and the combined extracts and washes were diluted to the mark and filtered through glass wool.

Starch content was determined directly from an aliquot of this final solution using the phenol-sulphuric acid colorimetric method described previously (Dubois et al., 1956); the amount of glucose assayed was multiplied by a factor of 0.90 to convert to starch (Pucher et al., 1948).

b. Alpha-amylase Extraction

i. Introduction

During the analysis of starch content using perchloric acid extraction, it was discovered that an increased amount of perchloric acid in the extraction step would lead to elevated values for starch levels. It thus

appeared that some other components in the material, namely hemicellulose and cellulose, might have been undergoing degradation due to the acid present. It has been pointed out (Burris, Brown, and Blaser, 1967) that actively growing seedlings are prone to giving elevated starch levels when acids are used for extraction, because of the presence of these unstable structural materials. These authors described a truly starch-specific extraction technique utilizing the enzyme alpha-amylase. This method was used to determine the starch content in the samples from the short-term sampling experiment.

ii. Materials

Alpha-amylase, 250 micrograms / ml

Obtained by diluting concentrated enzyme (Type 1-A, Sigma Chemical Co., St. Louis, Mo.) with 0.5 saturated NaCl containing 0.003 M $CaCl_2.6H_2O$.

0.01 M phosphate buffer, pH 7.0

Made with 0.01 M KH_2PO_4 and 0.01 M Na_2HPO_4.

iii. Procedure

Extraction -- As the analysis of soluble components had not yet been accomplished for these samples, a modified extraction technique which would accommodate both starch content and soluble compounds was used.

The freeze-dried sample was ground to a fine powder using mortar and pestle; 200-500 mg were weighted out into a 250 milliliter round bottom flask. A few drops of hot 80% ethanol were added to wet the material. 100 ml of boiling 80% ethanol were then added and the suspension was stirred under reflux for 60 minutes. Then the suspension was washed quantitatively into four, 50 milliliter glass centrifuge tubes and centrifuged at top speed in a table top centrifuge (International Clinical Centrifuge, Model CL). The supernatant was collected and retained, and the residue was washed into one tube with 25 ml of hot 80% ethanol and extracted at 75°C for 10 minutes. This extraction was repeated twice more for ten minutes each time, with 25 ml 80% ethanol; then the residue was washed once with 25 ml of distilled deionized water at room temperature, centrifuged, and stored at -15°C for the starch determinations. The combined supernatants were concentrated for the analysis of soluble components.

Starch Determination -- 10 ml of 0.01 M phosphate buffer, pH 7.0, were added to the sample residue and the suspension was heated for 30 minutes in a boiling water bath. The tube was then allowed to cool to room temperature, 10 ml of alpha-amylase solution were added, and the tube was incubated at 25°C for 30 minutes with occasional

stirring. Unlike perchloric acid in the other procedure described, the amount of alpha-amylase solution added was not a factor in the level of starch determined.

After this incubation, the suspension was centrifuged, the supernatant decanted into a 100 ml volumetric flask, and the incubation step repeated on the residue. After this second incubation, the residue was washed twice with warm water and all washings were combined in the 100 ml flask; the volume was made up to 100 ml with water and aliquots of this solution were used for starch determinations using the phenol-sulphuric acid method.

VI. ESTIMATION OF ENZYMATIC ACTIVITIES

A. Preparation of Plant Extracts

Petri dishes were removed from their respective treatments at pre-set times, and seedlings were cut off above the roots and placed on tissue for blotting. The plant material was then weighed on a small piece of aluminum foil and immediately placed into a pre-chilled glass-teflon pestle homogenizer ("Duall Homogenizer," 15 ml size, Kontes Glass Co., Vineland, N. J.). The foil was weighed separately to obtain the fresh weight of the material to be extracted; approximately 100 seedlings were used for each extract.

10 ml of pre-chilled 0.05 M tris-maleate buffer, pH 7.0 (purchased as "Trizma Maleate" crystals, Sigma Chemical Co., St. Louis, Mo.; made up in aqueous solution using 1 N NaOH for pH adjustment), were added to the plant tissue which was subsequently homogenized for four minutes by motor ("Coframo" stirrer Type RZR1-64, Wiarton, Ontario) in a cold room at 5°C.

The extract obtained was centrifuged at 20,000 X gravity (13,000 rpm in Sorvall RC-2B refrigated centrifuge) for ten minutes. The supernatant was then treated for 15 minutes with 0.5 g of "Polyclar AT" (General Aniline and Film Corporation, Dyestuffs and Chemicals Division, 435 Hudson St., New York, N.Y.). This step was undertaken in order to remove phenolic compounds in the extract which might cause some degree of inactivation of the enzymes present (Loomis and Battaile, 1966; Firenzuoli, Vanni, and Mastronuzzi, 1969). The "Polyclar AT" used did not require further purification -- as tests for impurities (Loomis and Battaile, 1966; Anderson and Sowers, 1968) had proven negative. During the 15 minute treatment period, the suspension was stirred several times to ensure complete adsorption of phenolics; then the suspension was placed in a centrifugal filter holder (Gelman Instrument Co., Ann Arbor, Michigan) fitted with a Gelman type GA-4, 0.8 micron

membrane filter, and centrifuged at 2,500 X gravity (3,000 rpm in Sorvall Model RC-3 refrigated centrifuge with swinging bucket head). The filtered extract thus obtained, and maintained between 0-4°C, was used for the determination of the activities of various enzymes.

B. Determination of Peptidase (Peptide Hydrolase) Activity

1. Principle

Peptidase activity was measured by a modification of the method of Erlanger, Kokowsky, and Cohen (1961). The increase in absorbance at 410 nm due to the release of free p-nitroaniline from the hydrolysis of the synthetic peptide leucine p-nitroanilide (LPA), was followed continuously, utilizing either a Beckman Model DB Double Beam Spectrophotometer equipped with recorder or a Beckman Kintrac VII A Kinetic Spectrophotometer.

2. Reagents

0.2 M citrate buffer, pH 4.0

Made up with citric acid and sodium citrate

0.2 M phosphate buffer, pH 7.3

Made up with KH_2PO_4 and Na_2HOP_4

5×10^{-4} LPA (Nutritional Biochemicals, Cleveland, Ohio)

Made up in each of the above buffers; due to a

low solubility, the LPA in buffer was maintained overnight before use on a hot plate between 35-40°C with continuous magnetic stirring.

3. Assay Procedure

The activity was measured at two pH values, pH 4.0 and pH 7.3.

Three ml of the buffered substrate solution was added to a one centimeter cuvette which was equilibrated in the temperature-controlled cell compartment of the spectrophotometer for 10 minutes at 25°C.

Then 0.2 ml enzyme extract was added to the sample cuvette with subsequent mixing. The reference cuvette contained three ml buffered substrate plus 0.2 ml extraction buffer.

The increase in absorbance at 410 nm was followed.

4. Effect of pH on Enzyme Activity

A pH range of 2.5-9.0 in increments of 0.5 pH units was tested using the following buffers:

pH 2.5-3.0 0.2 M glycine-HCl buffer

pH 3.5-5.5 0.2 M citrate buffer

pH 6.0-7.5 0.2 M phosphate buffer

pH 8.0-9.0 0.2 M tris buffer (purchased as "Trizma" from Sigma Chemical Co., St. Louis, Mo. and made

up in aqueous solution with 1 N HCl).

5×10^{-4} M LPA was made up in each of the above buffers.

5. Effect of Substrate Concentration on Enzyme Activity

A stock solution was made up of 5×10^{-4} M LPA in 0.2 M phosphate buffer, pH 7.3.

Substrate concentrations were varied by adding different volumes of stock solution to 0.2 M phosphate buffer, pH 7.3 to give a final buffered substrate volume in all cases of 3.0 ml.

C. Determination of Phosphatase Activity

1. Principle

Phosphatase activity was measured by the method of Linhardt and Walter (1965) utilizing p-nitrophenylphosphate as substrate. The enzyme liberated phosphate from this substrate, and the phosphate-free p-nitrophenol reacted with added NaOH to form a yellow anion which was measured at 400 nm. The addition of the strongly alkaline NaOH also served to inhibit any further phosphatase activity.

2. Reagents

0.05 M citrate buffer, pH 5.5

Made up with citric acid and sodium citrate

2.2×10^{-2} M p-nitrophenyl phosphate (Nutritional Biochemicals, Cleveland, Ohio)

Made up in citrate buffer (above)

0.1 N NaOH

3. Assay Procedure

1.0 ml of the buffered substrate was added to the blank and sample tubes. The tubes were equilibrated in a water bath at 25°C for approximately 10 minutes. Then 0.1 ml of the enzyme extract was added to each of the sample tubes while mixing. The tubes were allowed to incubate at 25°C for 10 minutes.

After the incubation period, 4 ml of 0.1 N NaOH were added to each tube to stop the reaction; 0.1 ml enzyme extract was added to the blank tube. The change in absorbance due to enzymatic activity was measured at 400 nm against the blank value.

4. Effect of pH on Enzyme Activity

A pH range of 4.0-9.0 in increments of 0.5 pH units was tested using the following buffers:

pH 4.0-5.4 0.05 M citrate buffer

pH 6.0-7.5 0.05 M tris-maleate buffer

pH 8.0-9.0 0.05 M tris buffer

2.2×10^{-2} M p-nitrophenyl phosphate was made up in each of the above buffers.

5. Effect of Substrate Concentration on Enzyme Activity

A stock solution was made up of 2.2×10^{-2} M p-nitrophenyl phosphate in 0.05 M citrate buffer, pH 5.5. Substrate concentrations were varied by adding different volumes of stock solution to 0.05 M citrate buffer, pH 5.5 to give a final buffered substrate volume of 1.0 ml.

D. Determination of Amylase Activity

1. Principle

Amylase activity was measured by a procedure based on the starch-iodine reaction as described by Filner and Varner (1967). Decreases in the intensity of the blue starch-iodine colour, dependent on the degree of amylolysis of the starch substrate, were measured spectrophotometrically at 620 nm.

2. Reagents

0.15% starch substrate

A 0.15% suspension of native (not solubilized) potato starch was boiled for one minute in a solution containing 0.04 M KH_2PO_4 and 0.2 mM $CaCl_2$. After cooling to room temperature, the

suspension was centrifuged at 3,000 X gravity for 10 minutes. The resultant supernatant was used as the substrate.

Iodine stock solution

6% KI plus 0.6% I_2 in distilled deionized water

Iodine reagent

The stock solution was diluted 100 fold with 0.05 N HCl.

3. Assay Procedure

One ml of the starch substrate solution was added to both sample and blank test tubes, followed by the addition of 0.7 ml of water. The tubes were allowed to equilibrate at 37°C for 10 minutes. Then 0.3 ml of the enzyme extract was added to the sample tubes. After mixing, the tubes were allowed to incubate at 37°C for 60 minutes.

The reaction was stopped by the addition of one ml of iodine reagent; 0.3 ml extract was then added to the blank. The resultant blue-coloured solution was diluted with 5 ml of water and the absorbance measured at 620 nm against water.

E. Determination of Glucose-6-phosphate Dehydrogenase Activity

1. Principle

Glucose-6-phosphate dehydrogenase activity was measured by the method of Löhr and Waller (1965). This enzyme catalyses the following reaction:

$$\text{Glucose-6-phosphate} + NADP^+ \rightleftharpoons \text{6-phosphogluconate} + NADPH + H^+$$

Activity was measured by following spectrophotometrically the increase in absorbance at 340 nm due to the formation of NADPH.

2. Reagents

0.05 M tris-maleate buffer, pH 7.5

0.2 M glucose-6-phosphate (disodium salt, Sigma Chemical Company, St. Louis, Mo.)
Made up in distilled deionized water

3×10^{-2} M nicotinamide adenine dinucleotide phosphate (NADP, sodium salt, Sigma Chemical Co.)
Made up in 1% $NaHCO_3$

3. Assay Procedure

The following was added into spectrophotometer cuvettes:

Blank	Sample
2.50 ml tris-maleate buffer	2.45 ml tris-maleate buffer
0.40 ml enzyme extract	0.40 ml enzyme extract
	0.05 ml NADP solution

The contents of the cuvettes were mixed and allowed to equilibrate for five minutes at 25°C in the spectrophotometer temperature-controlled cell compartment. Then to each cuvette was added 0.1 ml of the glucose-6-phosphate solution. The increase in absorbance at 340 nm was followed on a recorder.

4. Effect of pH on Enzyme Activity

A pH range of 4.0-9.0 in increments of 0.5 pH units was tested using the following buffers:

pH 4.0-5.5	0.05 M citrate buffer
pH 6.0-7.5	0.05 M tris-maleate buffer
pH 8.0-9.0	0.05 M tris buffer

0.1 ml of 0.2 M glucose-6-phosphate was used in this assay.

5. Effect of Substrate Concentration on Enzyme Activity

A stock solution was made up of 4×10^{-2} M glucose-6-phosphate in distilled deionized water. Substrate concentrations were varied by adding different volumes of stock solution to the incubation mixture giving a final volume in all cases of 3.0 ml.

F. Determination of Peroxidase Activity

1. Principle

Peroxidase catalyses the reaction

$$AH_2 + H_2O_2 \longrightarrow 2\ H_2O + A$$

where AH_2 is a hydrogen donor and A is its oxidized form. The method of Worthington and Teller (1969) was used, in which the rate of utilization of hydrogen peroxide (H_2O_2), with a dye, o-dianisidine, as hydrogen donor, was determined by measuring the rate of oxidation of o-dianisidine. This oxidation could be measured by following the colour development, due to production of oxidized o-dianisidine, in a spectrophotometer at 460 nm.

2. Reagents

0.05 M citrate buffer

Made up with sodium citrate and citric acid

Substrate stock solution, 0.3% H_2O_2 (purchased as 30% H_2O_2, Fisher Chemical Co., Toronto, Ontario)

Made up in distilled deionized water

Buffered substrate solution, 0.03% H_2O_2

Made up in 0.05 M citrate buffer, pH 5.0, fresh daily

0.5% o-dianisidine dihydrochloride (Sigma Chemical Co., St. Louis, Mo.)

Made up in methanol, fresh daily

5 N HCl (for rinsing cuvettes)

3. Assay Procedure

0.1 ml of the dye was added to 6.0 ml of the buffered H_2O_2 in a test tube. This solution was allowed to equilibrate at 25°C in a water bath for at least 10 minutes. Then 2.9 ml were transferred to the sample cuvette and the remainder into the control cuvette, in the spectrophotometer cell compartment. At zero time, 0.1 ml of the enzyme extract was added to the sample cuvette followed by rapid mixing. The rate of change in absorbance at 460 nm was recorded.

4. Effect of pH on Enzyme Activity

A pH range of 4.0-9.0 in increments of 0.5 pH units was tested using the following buffers:

pH 4.0-5.5 0.05 M citrate buffer

pH 6.0-7.5 0.05 M phosphate buffer, made up with KH_2PO_4 and Na_2HPO_4

pH 8.0-9.0 0.05 M tris buffer

0.03% H_2O_2 was made up in each of the above buffers.

5. Effect of Substrate Concentration on Enzyme Activity

A range of buffered H_2O_2 concentrations between

0.003%-0.3% was used in determining the saturating substrate concentration.

G. Determination of Glutamate-Oxaloacetate Transaminase Activity

1. Principle

The following reaction is catalysed by glutamate-oxaloacetate transaminase:

L-glutamate + oxaloacetate $\rightleftharpoons$ L-aspartate + alpha-oxoglutarate(alpha-ketoglutarate)

A method outlined by Bergmeyer and Bernt (1965) was used. This method measured the increase of oxaloacetate with time, as the reaction above proceeded reversibly from right to left. After a fixed incubation time, 2,4,-dinitrophenylhydrazine was added, thereby forming the 2,4-dinitrophenylhydrazone of oxaloacetate, which could be measured spectrophotometrically in alkaline solution at 546 nm.

2. Reagents

Buffered substrate solution, pH 8.0

Containing 0.1 M tris, 0.1 M L-aspartate, and 2×10^{-3} M alpha-ketoglutarate

The pH was adjusted to 8.0 with 1 N NaOH.

Ketone reagent, 10^{-3} M 2,4-dinitrophenylhydrazine
(British Drug Houses, Toronto, Ontario)
Dissolved in the minimum amount of 1N HCl and then made up to volume with distilled deionized water

0.4 N NaOH

3. Assay Procedure

One ml of the buffered substrate solution was added to both the sample and blank test tubes and these were allowed to equilibrate at 37°C for five minutes. Then 0.4 ml enzyme extract was added to the sample tubes and after mixing, the tubes were allowed to incubate at 37°C for 60 minutes.

The reaction was stopped by the addition of one ml of the ketone reagent followed by agitation; 0.4 ml enzyme extract was then added to the blank tube. After the tubes were allowed to stand for 20 minutes at room temperature, 10 ml of 0.4 N NaOH were added to each tube. The absorbance was read at 546 nm after five minutes. Units of activity were determined from the table given in Bergmeyer and Bernt (1965).

4. Effect of pH on Enzyme Activity

A pH range of 6.5-10.0 in increments of 0.5 pH units was tested using the following buffers:

pH 6.5-7.5 0.1 M phosphate buffer

pH 8.0-10.0 0.1 M tris buffer

In each case, a buffered substrate solution was prepared as described in the "Reagents" section. 1 N NaOH and 1 N HCl were used for final pH adjustment.

H. Determination of Nitrate Reductase Activity

1. Principle

Nitrate reductase catalyses the reaction:

$$NO_3^- + 2\ e \longrightarrow NO_2^- + H_2O$$

The activity of the enzyme can be measured by determining the amount of nitrite (NO_2^-) formed in an assay situation. A modification of the method of Ferrari and Varner (1969) in which flavin mononucleotide (FMN) served as the electron donor, was used to determine nitrate reductase activity.

2. Reagents

0.1 M phosphate buffer, pH 7.5

Made up with $NaH_2PO_4.H_2O$ and Na_2HPO_4

NOTE: In experiments involving nitrate reductase, phosphate buffer instead of tris-maleate buffer was used for extraction of the plant tissue, and the PVP step was omitted.

0.071 M KNO_3

Made up in 0.1 M phosphate buffer, pH 7.5

0.001 M FMN plus 0.001 M FAD (both obtained from Sigma Chemical Co., St. Louis, Mo.)

Made up in distilled deionized water

0.095 M $NaHCO_3$

Made up in distilled deionized water

1.6% $Na_2S_2O_3$ (sodium dithionite)

Made up in 0.095 M $NaHCO_3$

1% sulphanilamide (Eastman Organic Chemicals, Rochester, N. Y.)

Made up in 3 N HCl

0.02% N-1-naphthylethylenediamine dihydrochloride (Fisher Scientific Co., Toronto, Ontario)

Made up in distilled deionized water

3. Assay Procedure

To both the sample and blank tubes were added the following:

1.15 ml KNO_3 substrate in buffer

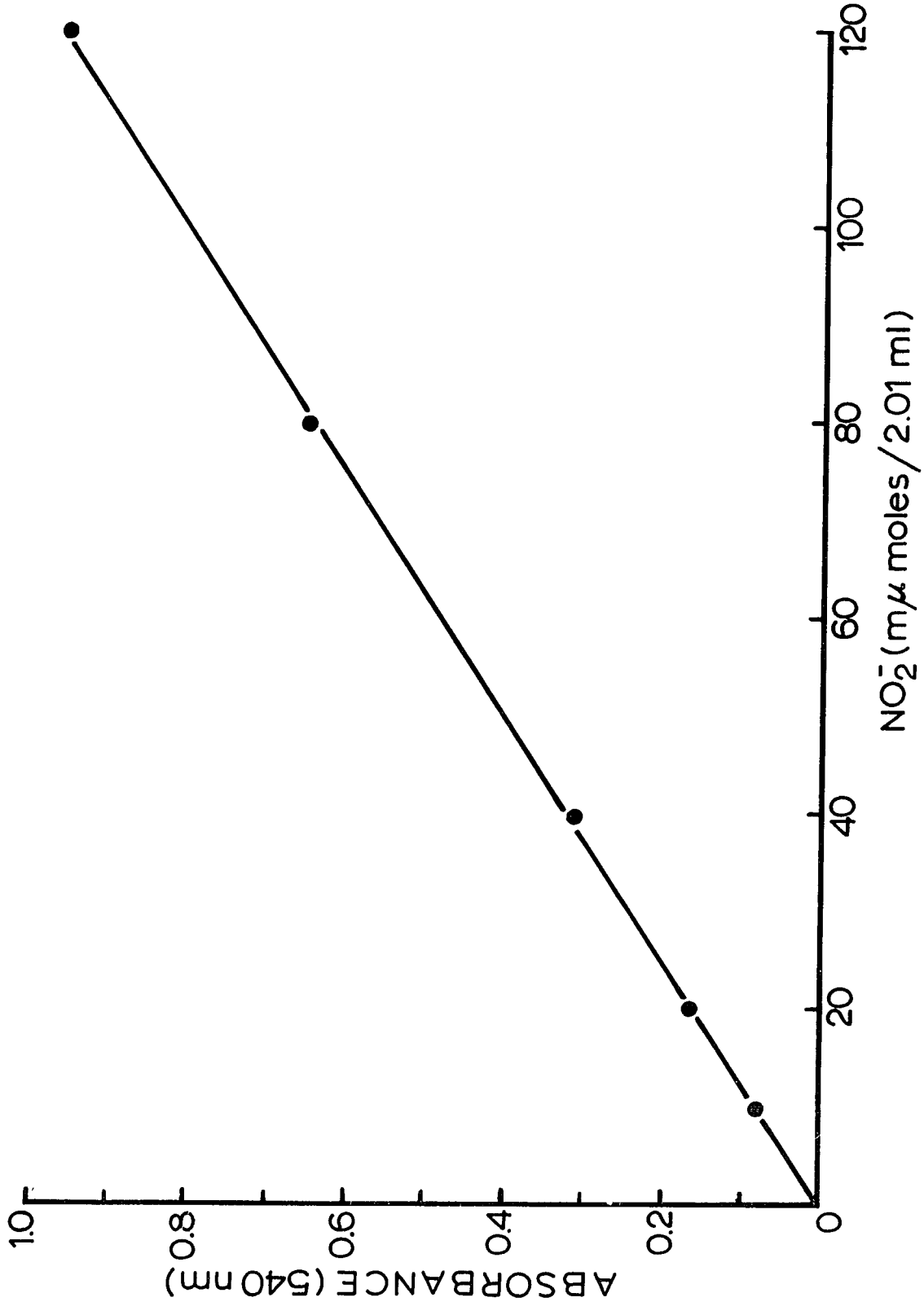
0.08 ml FMN-FAD solution

0.08 ml dithionite-bicarbonate solution

The tubes were allowed to equilibrate at 37°C for five minutes. Then 0.7 ml enzyme extract was added to the

FIGURE 3

Standard curve for nitrite concentration in nitrate reductase activity determinations.


ABSORBANCE (540 nm)
1.0
0.8
0.6
0.4
0.2
0
20
40
60
80
100
120
NO_2^- (mμ moles/2.01 ml)

sample tubes, and after gentle mixing, four drops of paraffin oil were layered onto the incubation mixture to prevent air-oxidation of the sodium dithionite. The tubes were incubated at 37°C for 30 minutes.

At the end of this time, the reaction was stopped by vigorously mixing the contents of the tubes using a vortex mixer, until a bright yellow colour appeared (as compared to the pale yellow-green previously present); 0.7 ml of enzyme extract was added to the blank tube immediately followed by rapid mixing. Then 1.2 ml of 1% sulphanilamide solution was added to all tubes followed by mixing. Finally 1.2 ml of 0.02% N-1-naphthylethylenediamine dihydrochloride solution was added also followed by mixing. After allowing 15 minutes for colour development, absorbance was measured at 540 nm.

4. Effect of pH on Enzyme Activity

A pH range of 6.0-9.0 in increments of 0.5 pH units was tested using the following buffers:

pH 6.0-7.5 0.1 M phosphate buffer

pH 8.0-9.0 0.1 M tris buffer

0.071 M KNO_3 was made up in each of the above buffers.

5. Effect of Substrate Concentration on Enzyme Activity

A range of KNO_3 concentrations between 0.008-0.08 M

was used in determining the saturating substrate concentration.

VII. DETERMINATION OF PROTEIN CONCENTRATION IN ENZYME EXTRACTS

A. Introduction

Protein concentration was measured by a relatively simple and extremely sensitive microassay, utilizing binding of protein to nitrocellulose membrane filters and quantitative staining of bound protein with amido black (Kuno and Kihara, 1967; Kihara and Kuno, 1968). The original procedure had been carried out using reduced pressure for the filtration steps; however, it was found that mild vacuum regulated by vacuum gauge and needle valve, proved to be more convenient and functioned quite successfully.

B. Materials and Reagents

Filtration apparatus (Pyrex Microanalysis Filter Holder, Millipore Ltd., Montreal, Quebec)

Nitrocellulose membrane filters (25 mm diameter, "Bac-T-Flex" Type B-6, Schleicher and Schuell Co., Keene, New Hampshire)

Vacuum gauge (Fisher Scientific Co., Toronto, Ontario)

Needle valve (Matheson of Canada Ltd., Whitby, Ontario)

"Fisher" Filtrator apparatus (Fisher Scientific Co.)

Unless otherwise noted, all reagents were made up in distilled deionized water:

0.2 M $MgCl_2$

0.1 M $MgCl_2$

0.1% Amido Black (Fisher Scientific Co.)

Made up in a solution of acetic acid-methanol-water in the proportions 1:5:4

1% acetic acid

0.1 N NaOH

Bovine Serum Albumin (BSA), 200 micrograms / ml

(Fraction V,B grade, Calbiochem, Los Angeles, California)

Dissolved in 0.05 M tris-maleate buffer, pH 7.0

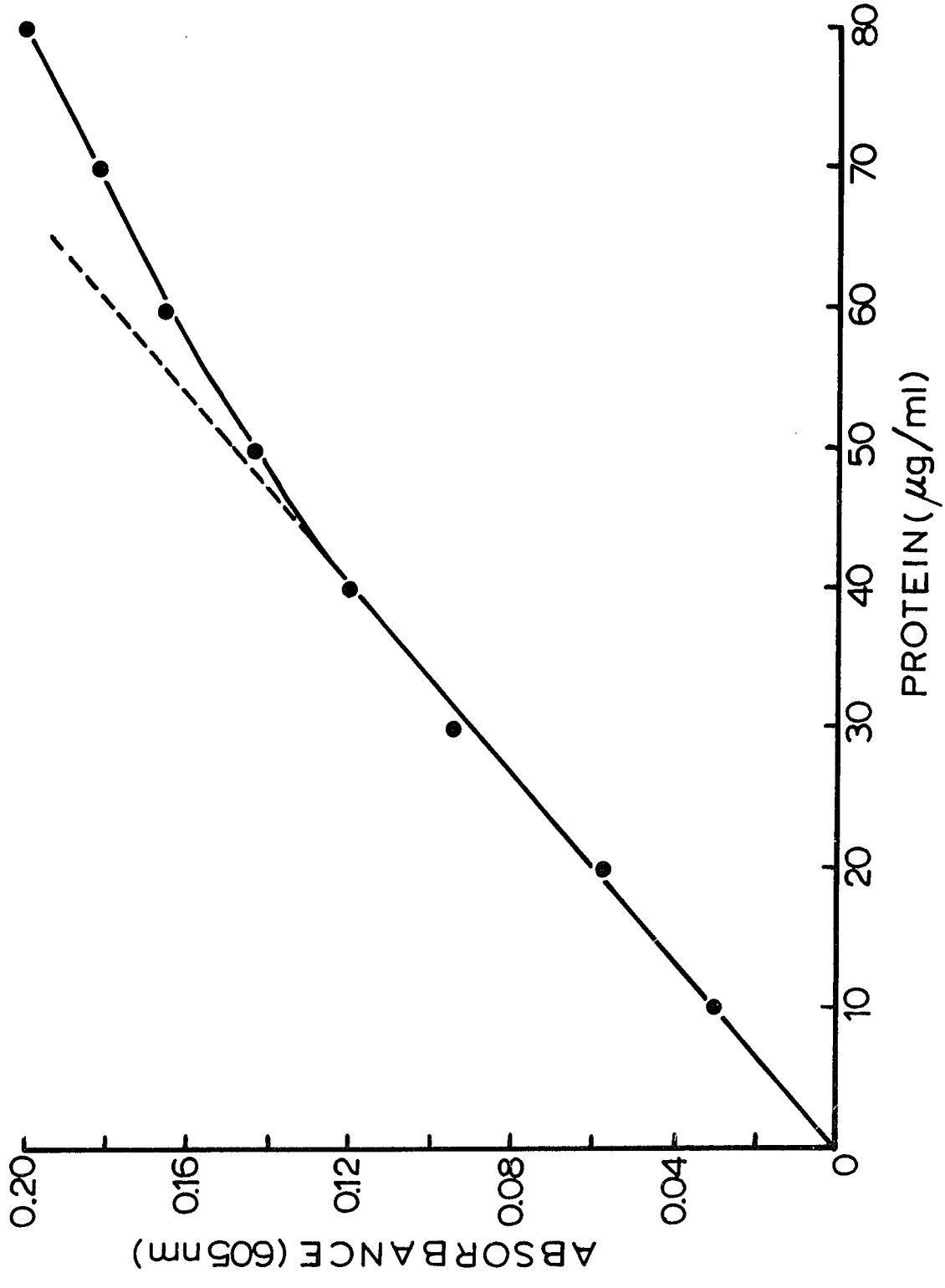
C. Procedure

Into a test tube in an ice bath were pipetted 1 ml enzyme extract, 1 ml 0.05 M tris-maleate buffer, pH 7.0, and 2 ml 0.2 M $MgCl_2$. With a membrane filter in place, the vacuum in the filtration apparatus was adjusted to 15 inches (a benchtop vacuum source was used). One ml of the above solution was slowly pipetted onto the surface of the filter in the holder. The filter was washed with 2.5 ml of 0.1 M $MgCl_2$. 3.0 ml of 0.1 amido black solution was

FIGURE 4

Standard curve for determination of protein concentration in enzyme extracts.

Protein as purified bovine serum albumin.


ABSORBANCE (605nm)
0.20
0.16
0.12
0.08
0.04
0
10
20
30
40
50
60
70
80
PROTEIN (μg/ml)

then passed through the filter; the stained filter was washed by passing through 3.0 ml of 1% acetic acid.

The dye adsorbed to the protein on the filter was eluted by passing through 3.5 ml of 0.1 N NaOH in four approximately equal aliquots, with the filter mounted on a "Fisher Filtrator" so that the eluate to be measured could be collected in a test tube supported inside. After this step, the filter holder was rinsed with distilled deionized water and vacuum dried before proceeding to the next replicate.

The absorbance of the released dye was measured at 605 nm, instead of 620 nm as used by Kuno and Kihara (1967) as the former wavelength was found to give the maximum absorbance. The observed values were compared to those on a standard curve for bovine serum albumin (Fig. 4), in order to have an approximation of the absolute protein concentration in the extracts.

VIII. DETERMINATION OF NITRATE AND NITRITE IN SEEDLINGS

A. Introduction

Nitrate and nitrite were determined by the method of Woolley, Hicks, and Hageman (1960), which is based on a reaction consisting of diazotization of sulphanilic acid by nitrite ion and subsequent coupling with 1-naphthylamine

to form a red dye; nitrate ion was determined as nitrite after first being reduced to nitrite by treatment with zinc. The dye formed could be measured spectrophotometrically at 520 nm.

B. Reagents

20% acetic acid

20% acetic acid containing 0.2 ppm copper as copper sulphate

Reagent powder (Nelson, Kurtz, and Bray, 1954)

1. For nitrate plus nitrite:

   100 g barium sulphate

   10 g manganese-sulphate monohydrate

   2 g finely powdered zinc

   75 g powdered citric acid

   4 g sulphanilic acid

   2 g 1-naphthylamine

2. For nitrite alone:

   Same as above but with omission of zinc and manganese sulphate

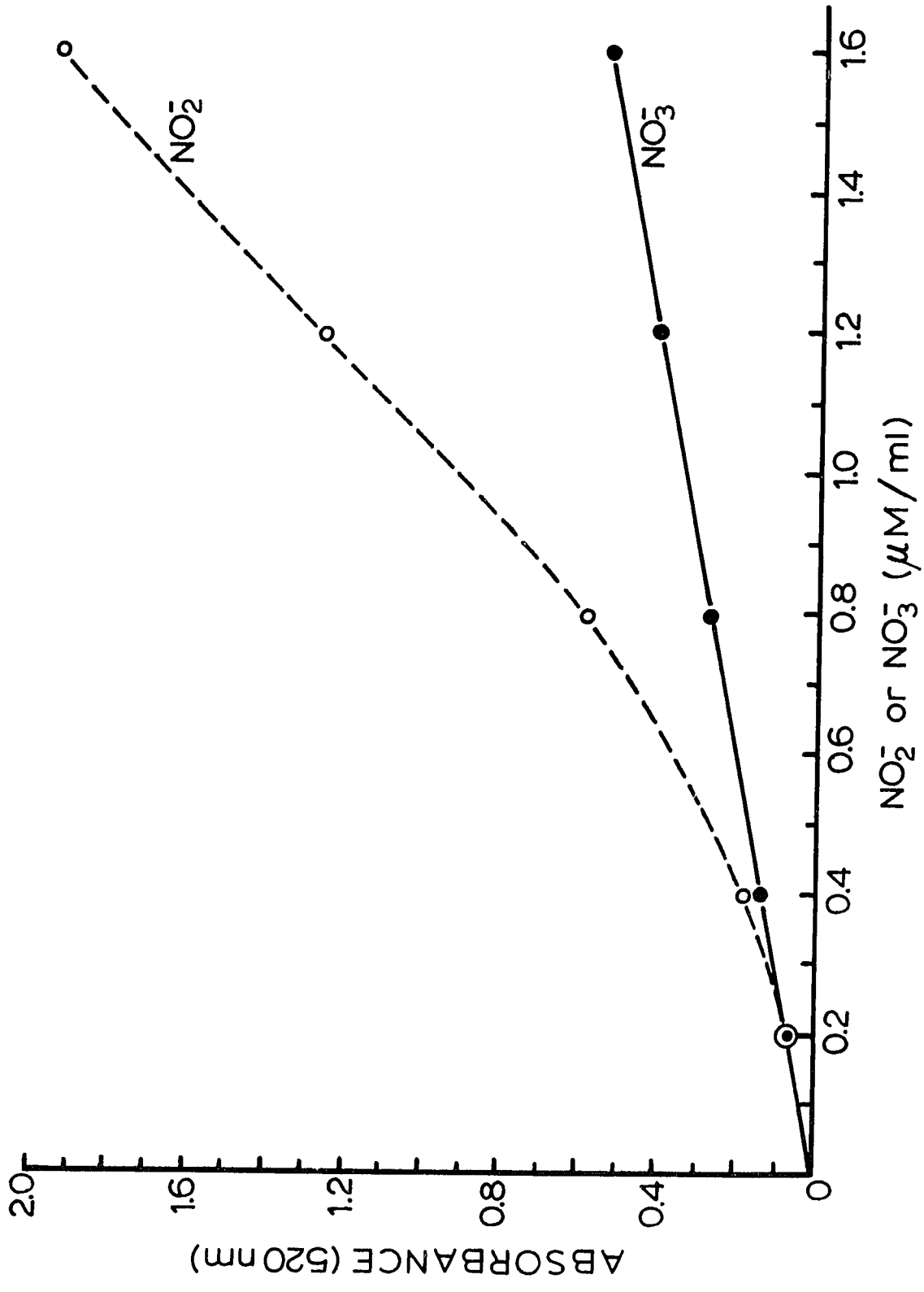
These powders were stored in blackened bottles, because 1-naphthylamine is adversely affected by light.

C. Procedure

The tissue extract was prepared as indicated in "VI.A."

FIGURE 5

Standard curves for the determination of nitrate and nitrate content in seedlings.


ABSORBANCE (520 nm)
2.0
1.6
1.2
0.8
0.4
0
0.2
0.4
0.6
0.8
1.0
1.2
1.4
1.6
NO_2^- or NO_3^- (μM/ml)
NO_2^-
NO_3^-

with the following modification: before being ground, the seedlings were washed in two changes of distilled deionized water (10 ml each) in a 15 milliliter medium porosity scintered glass funnel, using thirty seconds for each washing, and then blotted dry with tissue. This step was included in order to remove any nitrate ion which might have been present on the external surface of the seedling as a consequence of previous Hoagland's solution additions.

0.4 g of reagent powder "1" and 0.4 g of reagent powder "2", were weighed into separate 15 milliliter conical bottom centrifuge tubes. Into tubes containing reagent powder "1" were pipetted nine ml of 20% acetic acid containing $CuSO_4$; into tubes containing reagent powder "2," were pipetted nine ml of 20% acetic acid with no $CuSO_4$. Then, to designated blank tubes was added one ml of 0.05 M tris-maleate buffer, pH 7.0 (extraction buffer) and to the sample tubes, one ml of tissue extract. All tubes were covered with parafilm "M" (Marathon Products, Neenah, Wisconsin) and shaken vigorously for 15 seconds in succession and then shaken once more.

The tubes were centrifuged at top speed in a clinical centrifuge (International Clinical Model) and the supernatants were drawn out with Pasteur pipettes. The absorbance of the resultant coloured liquid was read at 520 nm against

the respective blanks, in a Beckman Kintrac VII A Kinetic Spectrophotometer.

CHAPTER V

EXPERIMENTAL RESULTS

I. LEVELS OF ENDOGENOUS METABOLITES

A. Thin-layer Chromatography

Characteristic chromatograms of soluble components contained within C. rubrum ($60^{o}47'N$) extracts from individual experiments are illustrated in Plate I (chromatograms of sugars proved too faint to reproduce photographically).

1. Sugars

Examination of thin-layer chromatograms of C. rubrum ($60^{o}47'N$) extracts revealed that the predominant sugars present were glucose and fructose (by comparison with standards), with a small proportion of sucrose also evident. Although the low absolute quantities present made detailed examination of the chromatograms difficult, there appeared to be identical alterations in levels of these sugars during the time-course of the experiments. For characterization of possible rhythmicity, a more

PLATE I

Characteristic thin-layer chromatograms of soluble components contained within C. rubrum ($60^{o}47'N$) extracts.

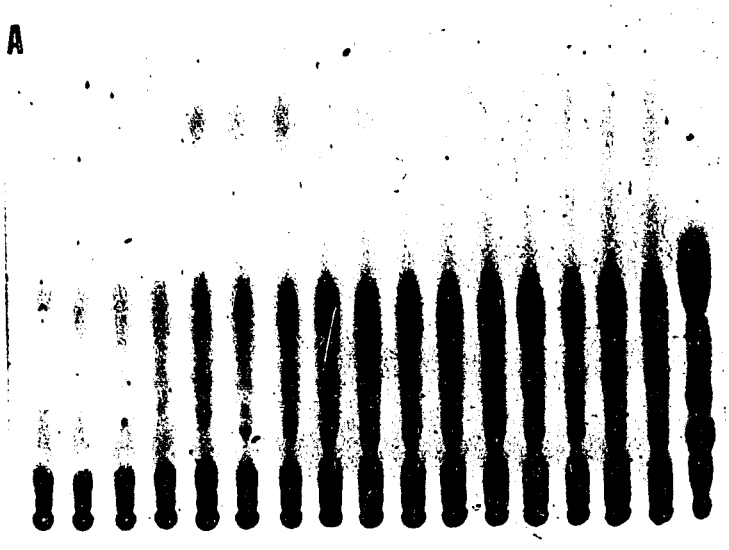
A. Amino acids.

B. Sugar phosphates.

C. Organic acids

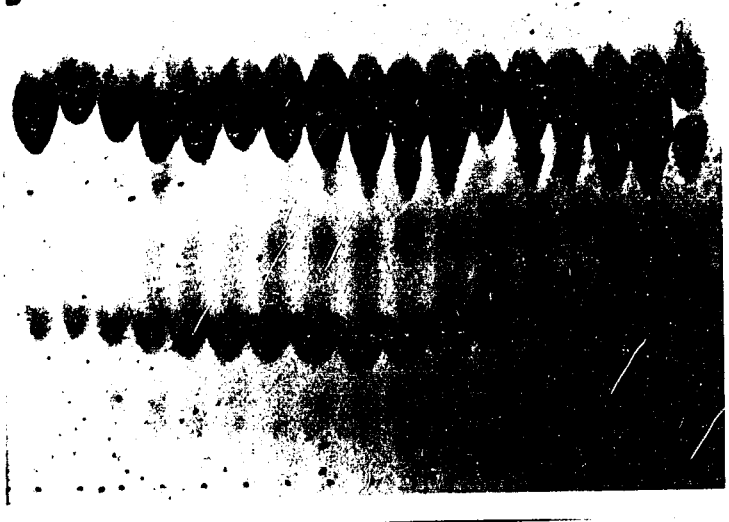
The time-course represented is 0-45 hours of continuous darkness at $20^{o}C$; measurements made at three-hour intervals. Previous treatment: standard germination programme for 72 hours, followed by 36 hours of high-intensity (3,000 ft-c) fluorescent illumination at $20^{o}C$ both preceded and terminated by addition of Hoagland's nutrient solution. In each chromatogram, the extreme-right band contains standard compounds.

A

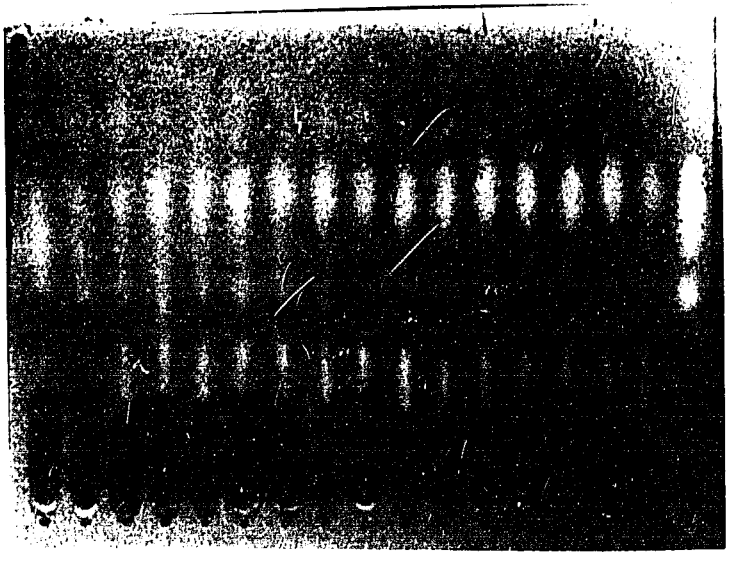


LEUCINE
ISOLEUCINE
METHIONINE
VALINE
TYROSINE
ALPHA-ALANINE
LYSINE
ASPARAGINE
GLUTAMINE

B



PHOSPHOENOLPYRUVIC ACID
INORGANIC PHOSPHATE
GLUCOSE-1-PHOSPHATE
FRUCTOSE-6-PHOSPHATE
FRUCTOSE-1,6-DIPHOSPHATE
GLUCOSE-6-PHOSPHATE

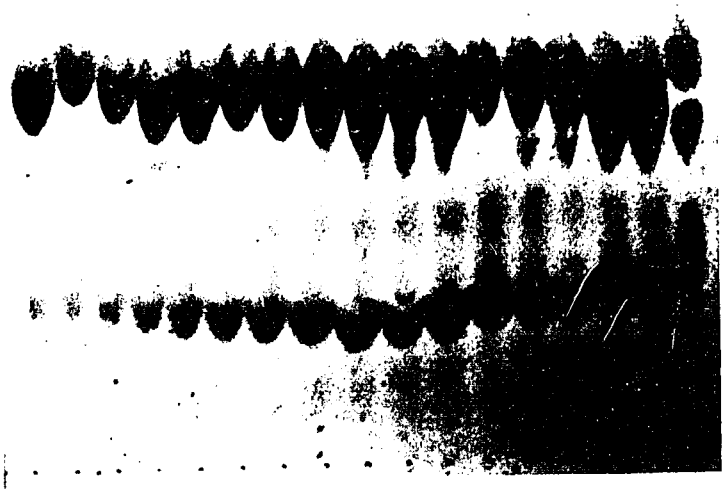


SUCCINIC ACID
MALIC ACID
CITRIC ACID
TARTARIC ACID

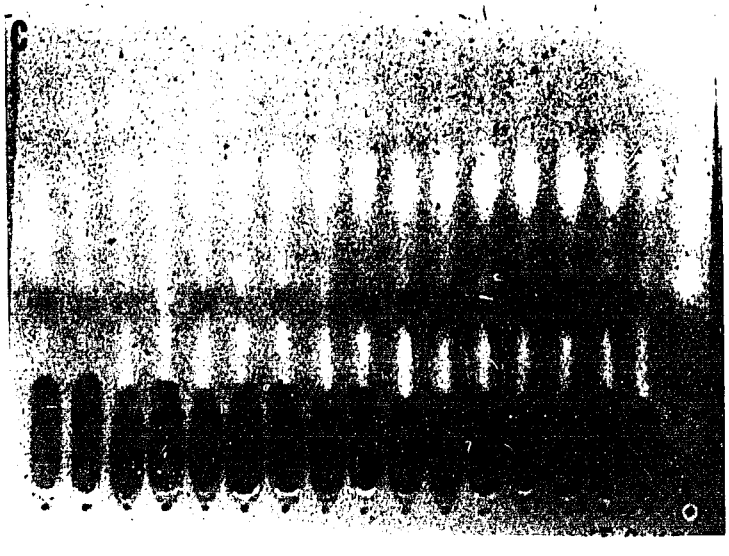
A


METHIONINE
VALINE
ALPHA-ALANINE
LYSINE
GLUTAMINE

B


PHOSPHOENOLPYRUVIC ACID
INORGANIC PHOSPHATE
FRUCTOSE-6-PHOSPHATE
FRUCTOSE-1,6-DIPHOSPHATE

C


MALIC ACID

analytical approach was obviously warranted.

2. Amino acids

Amino acids proved much easier to detect chromatographically than sugars had been. Examination of two-dimensional separations of selected extracts denoted the presence of at least 15 different amino acids. For purposes of assessing differences in relative concentration in an experimental situation, one-dimensional separation proved more feasible (see Plate I, A).

Changes in relative concentration were readily visible, and it appeared in general that the individual amino acids that were present followed identical patterns of fluctuation -- although some were of higher absolute concentration than others. Therefore, an analysis of total free amino acid content could provide a quantitative base of comparison and indicate the possible presence of rhythmicity.

3. Sugar Phosphates

Chromatographic separation of sugar phosphates revealed the presence in the extracts of glucose-1-phosphate and either or all of fructose-6-phosphate, fructose-1,6-diphosphate, and glucose-6-phosphate (Plate I, B). Only glucose-1-phosphate levels noticeably demonstrated some

fluctuation; however, the absolute concentration was quite low.

4. Organic Acids

From comparison with standards, the organic acids present in the extracts appeared to be succinic, citric, malic, and tartaric acids; of these, malic acid was the most readily visible and tended to show a gradual increase in concentration during the experimental time-course (Plate I, C). Both citric acid and tartaric acid seemed to occur in higher concentration initially, with a subsequent decline.

B. Analytical Investigations

As mentioned previously, chromatographic evidence indicated both that glucose and fructose were the predominant soluble sugars present in the seedling extracts, and that they appeared to undergo fluctuations in concentration during the time-course studied. It appeared then, that quantitative estimation of total soluble carbohydrates might easily and accurately reflect changes occurring in the content of these sugars, and indeed as shown by Figure 6, this proved to be the case. It can also be observed from this figure that levels of glucose-6-phosphate and fructose-6-phosphate, although relatively

FIGURE 6

Comparison of phenol-sulphuric acid colorimetric estimation of total soluble carbohydrates with enzymatic estimation of glucose and fructose, utilizing sample extracts prepared from seedlings of C. rubrum ($60^{o}47'N$) maintained in darkness at $20^{o}C$.

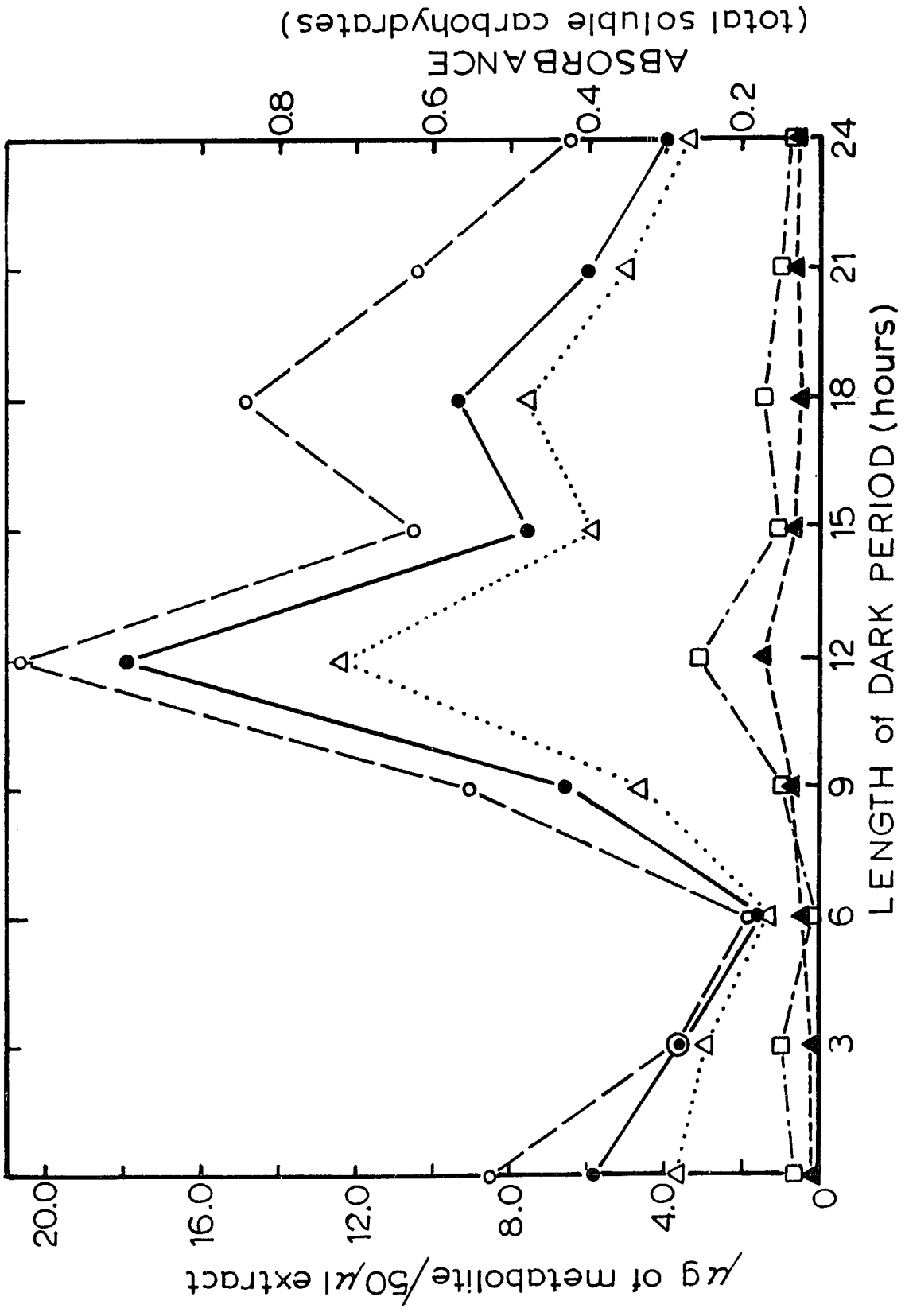
Total soluble carbohydrates: open triangle;

Glucose: closed circle;

Fructose: open circle;

Glucose-6-phosphate: closed triangle;

Fructose-6-phosphate: open square.


μg of metabolite/50μl extract
20.0
16.0
12.0
8.0
4.0
0
3
6
9
12
15
18
21
24
LENGTH of DARK PERIOD (hours)
ABSORBANCE
(total soluble carbohydrates)
0.8
0.6
0.4
0.2

low, tended to follow the pattern of change of the sugars.

Likewise, the determination of total free amino acid content, although possibly not reflecting the specific pattern of every individual amino acid present, appeared to give a quite accurate picture of changes occurring -- a fact observable from visual comparison of thin-layer chromatograms with analytical data from individual experiments. The relative simplicity of both the TNBS-amino acid and phenol-sulphuric acid-carbohydrate methods in allowing direct utilization of extract aliquots was a major consideration in choosing such procedures.

Figure 7 illustrates the changes in relative concentration of total soluble carbohydrates, total free amino acids, and starch in seedlings of C. rubrum ($60^{o}47'N$) maintained in darkness for up to 69 hours (for complete details see description of figure). Although marked short-term fluctuations in soluble carbohydrate and amino acid content are apparent, there is no obvious correlation with the rhythmic flowering curve of Cumming et al.(1965), which possessed distinct maxima at dark period lengths of approximately 15, 45, and 75 hours.

Relatively rapid initial decline in starch level for the first 27 hours of darkness was followed by a more gradual disappearance of this reserve carbohydrate; in

FIGURE 7

Relative concentrations of total soluble carbohydrates (closed circle), total free amino acids (open circle), and starch (closed triangle) in seedlings of C. rubrum (60°47'N) maintained in darkness at 20°C for periods ranging between 0-69 hours.
Measurements made at three-hour intervals. Previous treatment: standard germination programme for 72 hours, followed by 36 hours of high-intensity (3,000 ft-c) fluorescent illumination at 20°C both preceded and terminated by addition of Hoagland's nutrient solution.
Compilation of data from three experiments.

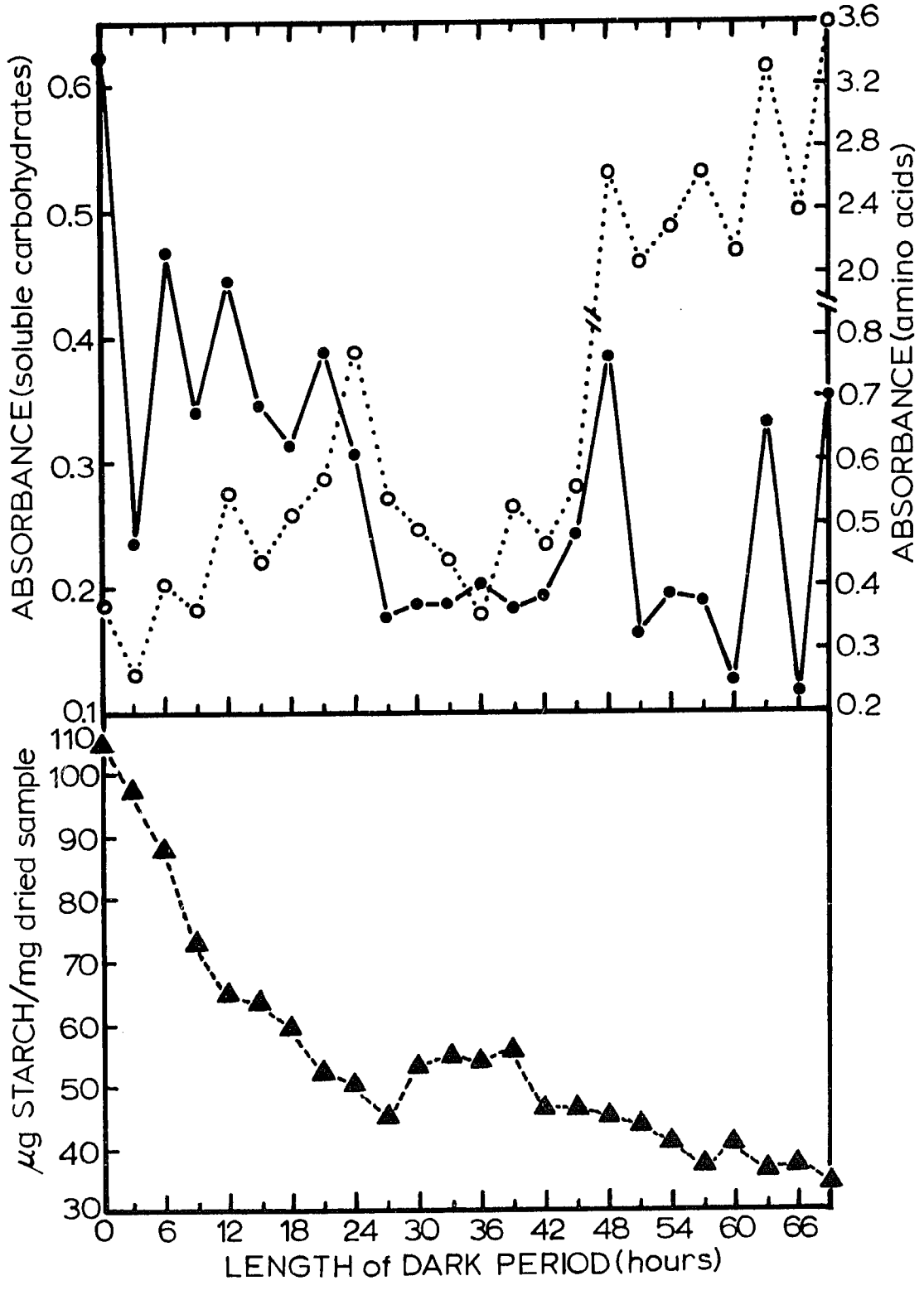
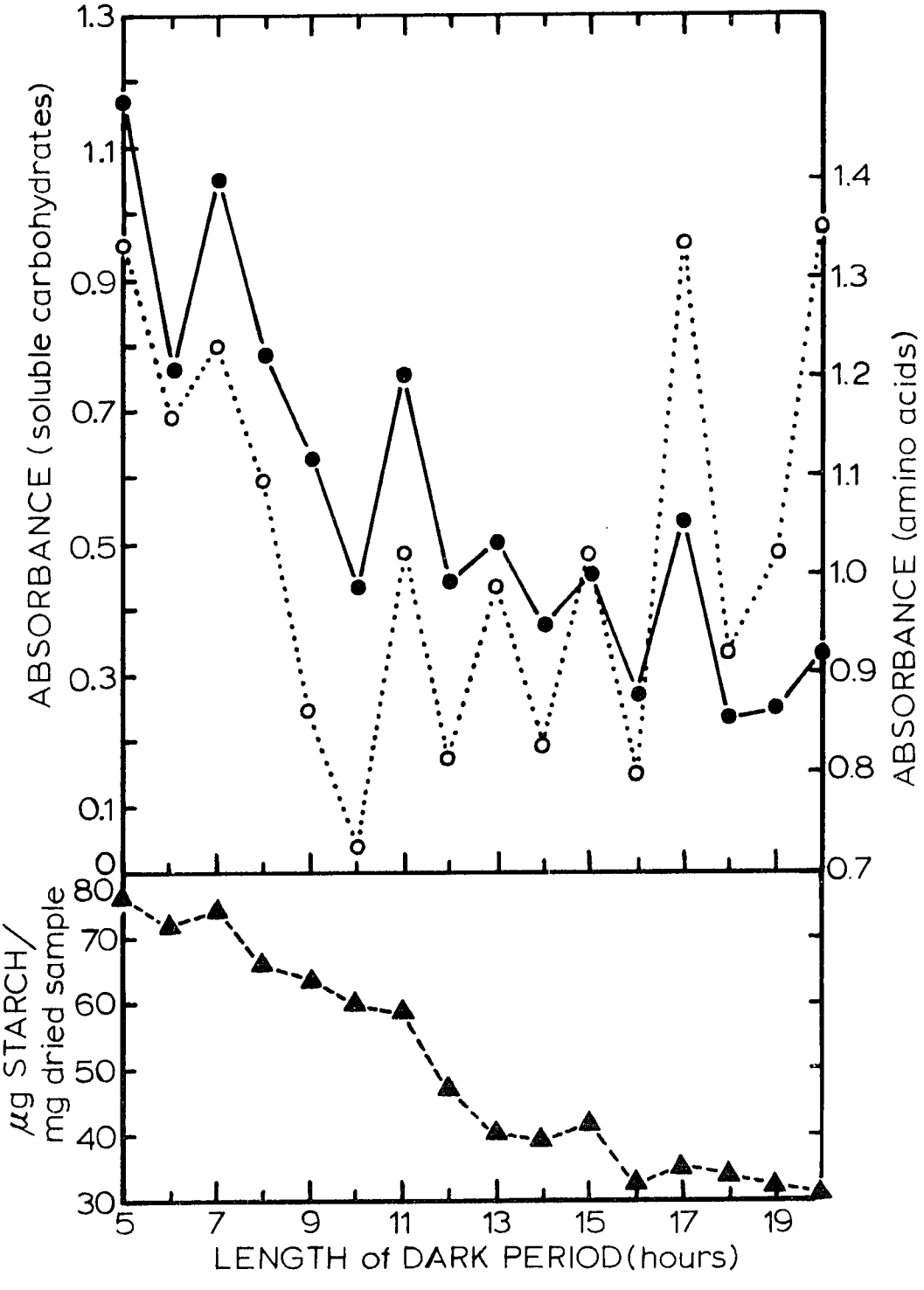

ABSORBANCE (soluble carbohydrates)
ABSORBANCE (amino acids)
0.6
0.5
0.4
0.3
0.2
0.1
3.6
3.2
2.8
2.4
2.0
0.8
0.7
0.6
0.5
0.4
0.3
0.2
μg STARCH/mg dried sample
110
100
90
80
70
60
50
40
30
0
6
12
18
24
30
36
42
48
54
60
66
LENGTH of DARK PERIOD (hours)

FIGURE 8

Relative concentrations of total soluble carbohydrates (closed circle), total free amino acids (open circle), and starch (closed triangle) in seedlings of C. rubrum (60°47'N) maintained in darkness at 20°C for periods ranging between 5-20 hours.
Measurements made at one-hour intervals. Previous treatment: standard germination programme for 84 hours, followed by 24 hours of high-intensity (3,000 ft-c) fluorescent illumination at 20°C both preceded and terminated by addition of Hoagland's nutrient solution.


ABSORBANCE (soluble carbohydrates)
ABSORBANCE (amino acids)
μg STARCH/ mg dried sample
LENGTH of DARK PERIOD (hours)
1.3
1.1
0.9
0.7
0.5
0.3
0.1
0
1.4
1.3
1.2
1.1
1.0
0.9
0.8
0.7
80
70
60
50
40
30
5
7
9
11
13
15
17
19

fact, between 27-42 hours there even appeared to be a small plateau-like increase in starch content. Timewise, this apparent dark-synthesis corresponded to a uniformly low level of soluble carbohydrate. The extremely elevated levels of total free amino acids in the lengthening dark period (note expanded scale) might signify an increasing degradation of protein reserves -- starch reserves had obviously been almost completely depleted by this time.

It was observed in individual experiments, that the short-term fluctuations in total soluble carbohydrate and total free amino acid content were not reproducible per se; that is, specific peaks and minima tended not to generally coincide. As this finding might have possibly been due to discrepancies resulting from the three-hour interval sampling programme, it was decided to test this hypothesis. Figure 8 shows the results of such a study. Indeed, it was discovered that even with this shorter sampling interval, pronounced short-term fluctuations were still readily apparent, thereby indicating relatively rapid metabolic transitions within the seedlings.

II. ENZYMATIC ACTIVITIES

A. Preliminary Information

Optimum conditions for the assay of the various en-

zymes have been delineated in Figures 9 to 14 inclusive.

The presence of two distinct pH optima for peptidase activity (Fig. 9a) suggested the existence of different molecular forms or isoenzymes (Markert and Moller, 1959) of this enzyme protein; the occurrence of such peptidase isoenzymes in plants has also been noted elsewhere (Shannon, 1968; Scandalios, 1969).

The phosphatase activity characterized, was observed to be that of an acid phosphatase enzyme possessing a pH optimum of 5.5, as distinct from that of the alkaline phosphatase type.

For assay of amylase activity, the procedure of Filner and Varner (1967) was followed without variation because it was observed that the reaction of iodine with starch was very sensitive to pH change other than between pH 3-4, digression leading to precipitation of the solubilized starch. However, in the incubation mixture itself, the pH was approximately 6 due to the weak buffering action of the 0.05 M, pH 7.0 extraction buffer, and the enzyme activity was readily demonstrable. Also, even though the starch substrate concentration used might have been suboptimal, a linear relationship between amylase activity and the colour of the starch-iodine reaction was

FIGURE 9

Optimum conditions for the assay of peptidase activity in crude extracts of C. rubrum (60°47'N) seedlings.

a. Effect of pH on enzyme activity (substrate concentration used: 5×10^{-4} M leucine p-nitroanilide).

b. Effect of substrate concentration on enzyme activity. Absorbance measured at 410 nm.

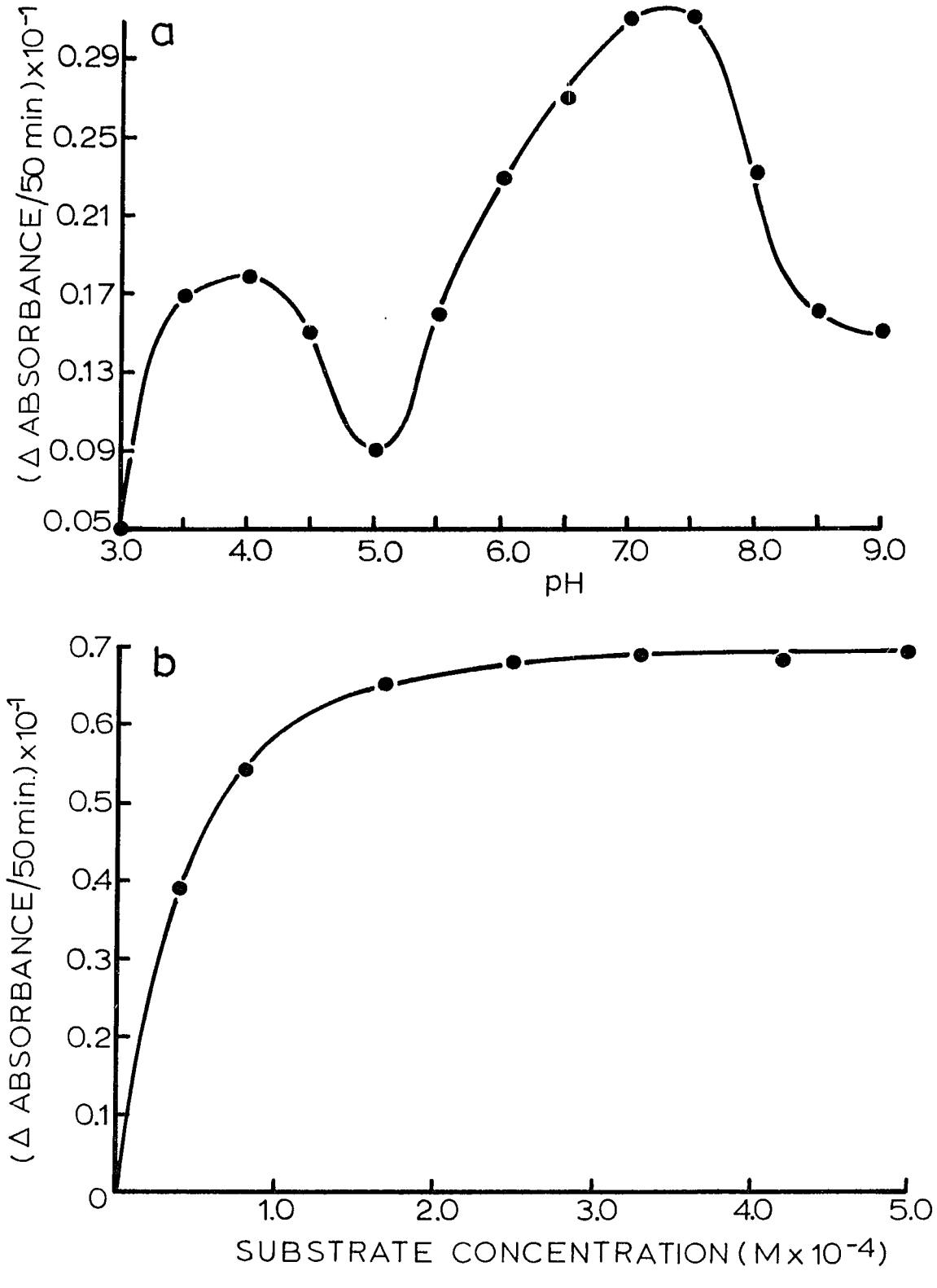
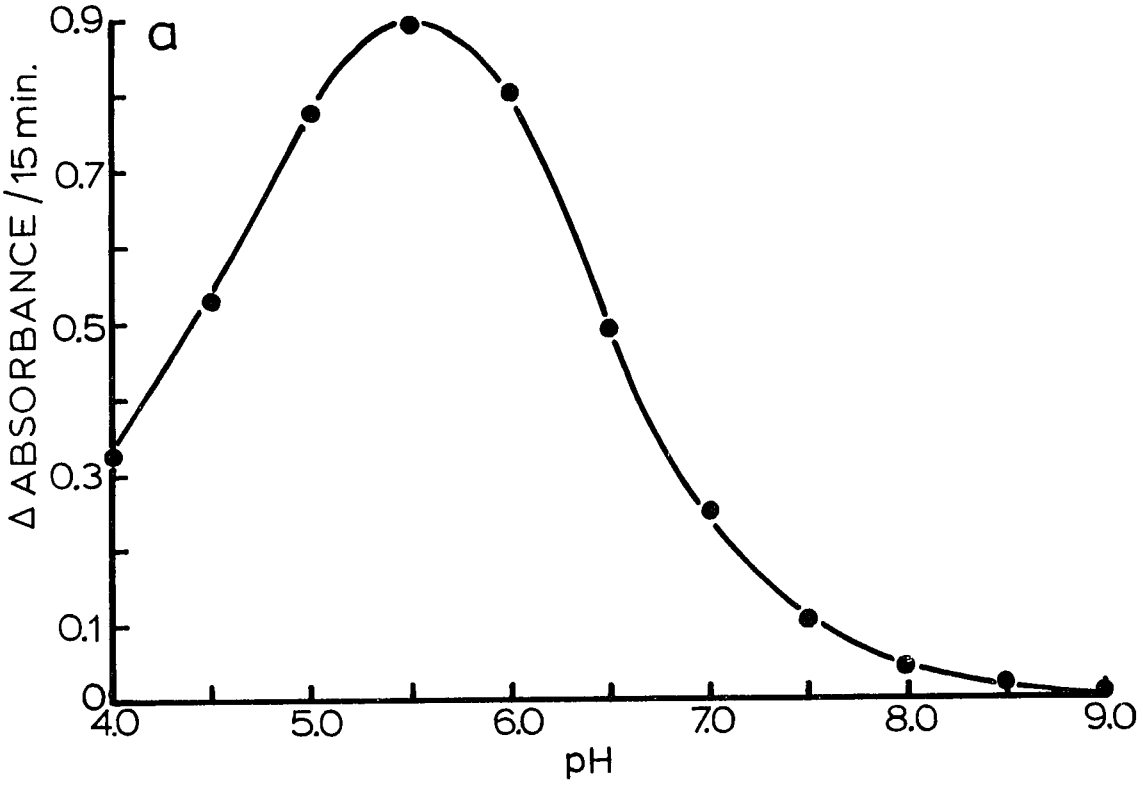

a
(Δ ABSORBANCE/50 min) x10⁻¹
0.29
0.25
0.21
0.17
0.13
0.09
0.05
3.0
4.0
5.0
6.0
7.0
8.0
9.0
pH
b
(Δ ABSORBANCE/50 min.) x10⁻¹
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
1.0
2.0
3.0
4.0
5.0
SUBSTRATE CONCENTRATION (M x 10⁻⁴)

FIGURE 10

Optimum conditions for the assay of phosphatase activity in crude extracts of C. rubrum ($60^{o}47'N$) seedlings.

a. Effect of pH on enzyme activity (substrate concentration used: 2.2×10^{-2} M p-nitrophenyl phosphate).

b. Effect of substrate concentration on enzyme activity. Absorbance measured at 400 nm.


a
0.9
0.7
0.5
0.3
0.1
0
Δ ABSORBANCE / 15 min.
4.0
5.0
6.0
7.0
8.0
9.0
pH

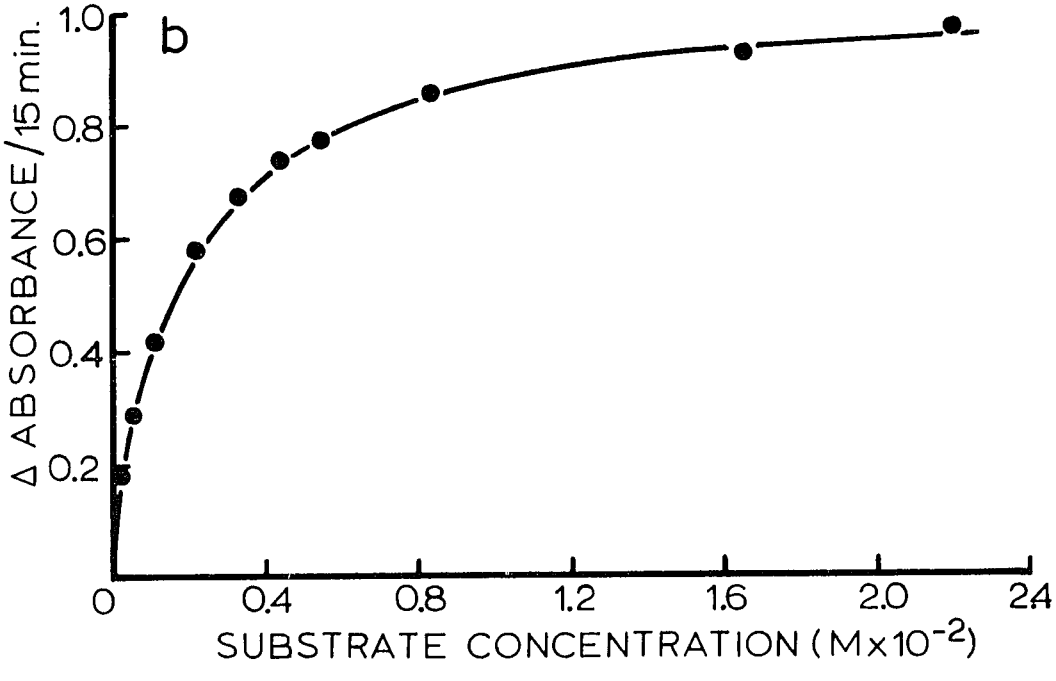
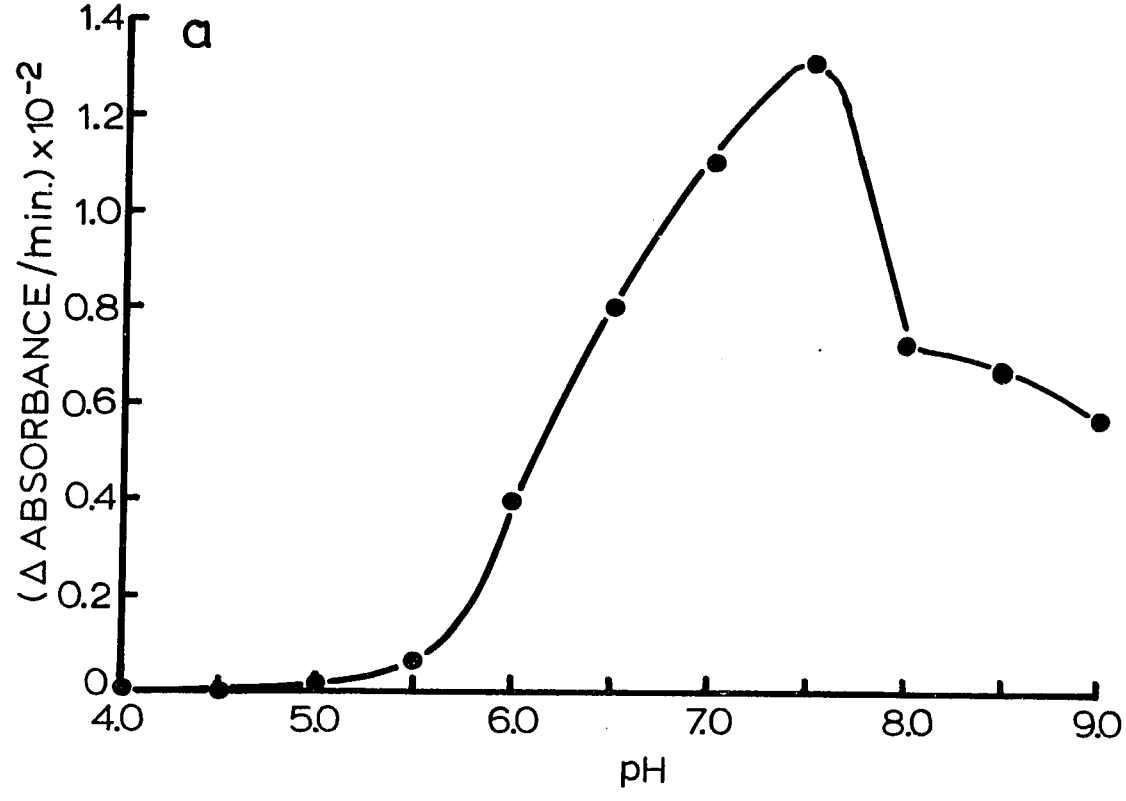

b
1.0
0.8
0.6
0.4
0.2
Δ ABSORBANCE / 15 min.
0
0.4
0.8
1.2
1.6
2.0
2.4
SUBSTRATE CONCENTRATION (M x 10⁻²)

FIGURE 11

Optimum conditions for the assay of glucose-6-phosphate dehydrogenase activity in crude extracts of C. rubrum ($60^{o}47'N$) seedlings.

a. Effect of pH on enzyme activity (substrate concentration: 6.7×10^{-3} M glucose-6-phosphate).

b. Effect of substrate concentration on enzyme activity. Absorbance measured at 340 nm.


a
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
(Δ ABSORBANCE /min.) x10-2
4.0
5.0
6.0
7.0
8.0
9.0
pH

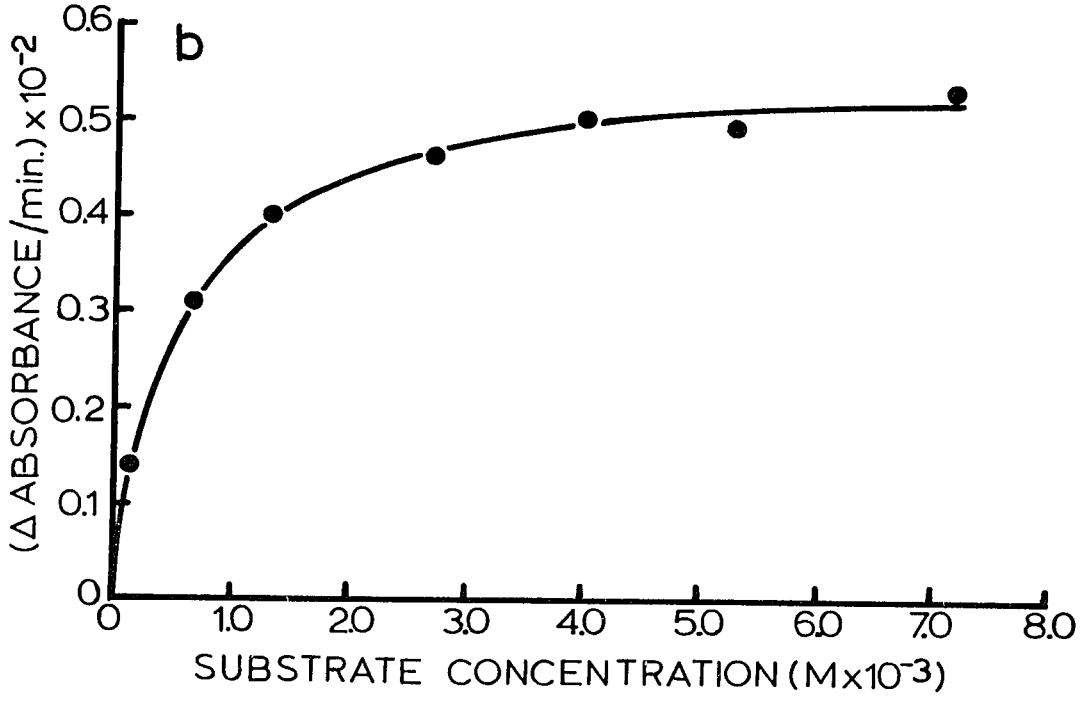
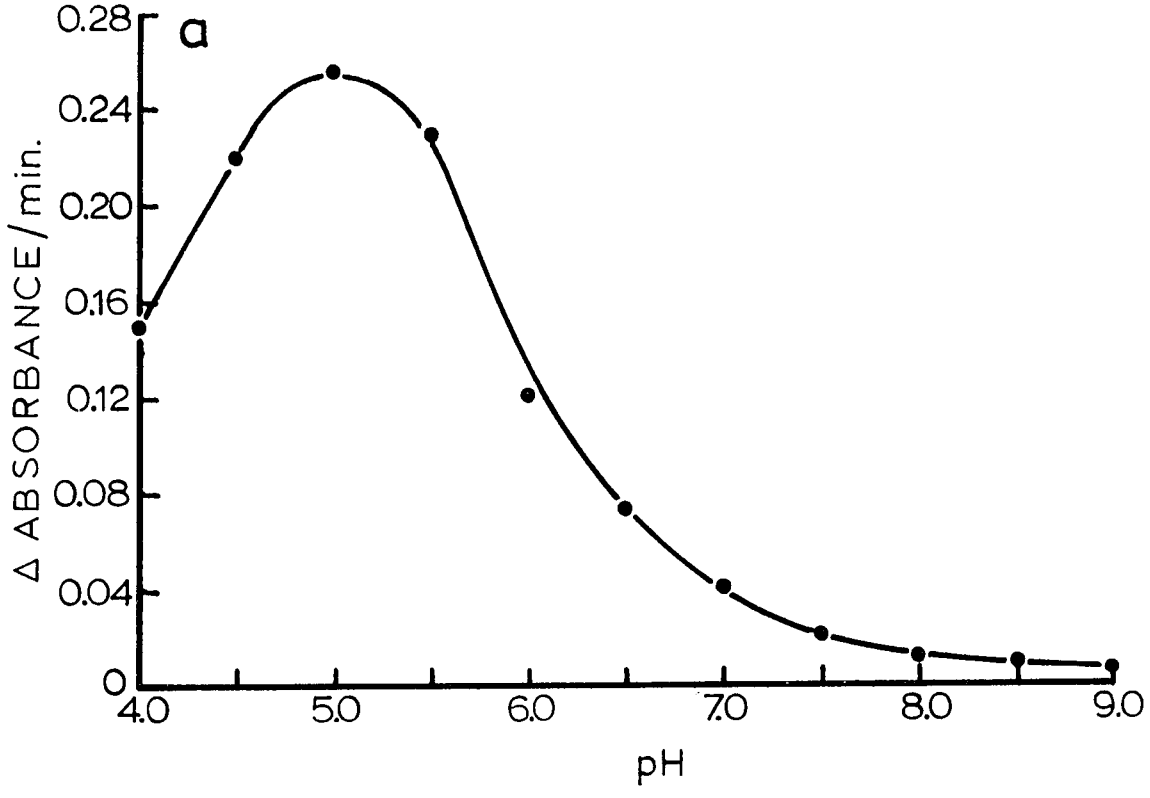

b
0.6
0.5
0.4
0.3
0.2
0.1
0
(Δ ABSORBANCE/min.) x10-2
0
1.0
2.0
3.0
4.0
5.0
6.0
7.0
8.0
SUBSTRATE CONCENTRATION (Mx10-3)

FIGURE 12

Optimum conditions for the assay of peroxidase activity in crude extracts of C. rubrum ($60^{o}47'N$) seedlings.

a. Effect of pH on enzyme activity (substrate concentration used: 0.03% v/v hydrogen peroxide).

b. Effect of substrate concentration on enzyme activity. Absorbance measured at 460 nm.


a
0.28
0.24
0.20
0.16
0.12
0.08
0.04
0
Δ ABSORBANCE/min.
4.0
5.0
6.0
7.0
8.0
9.0
pH

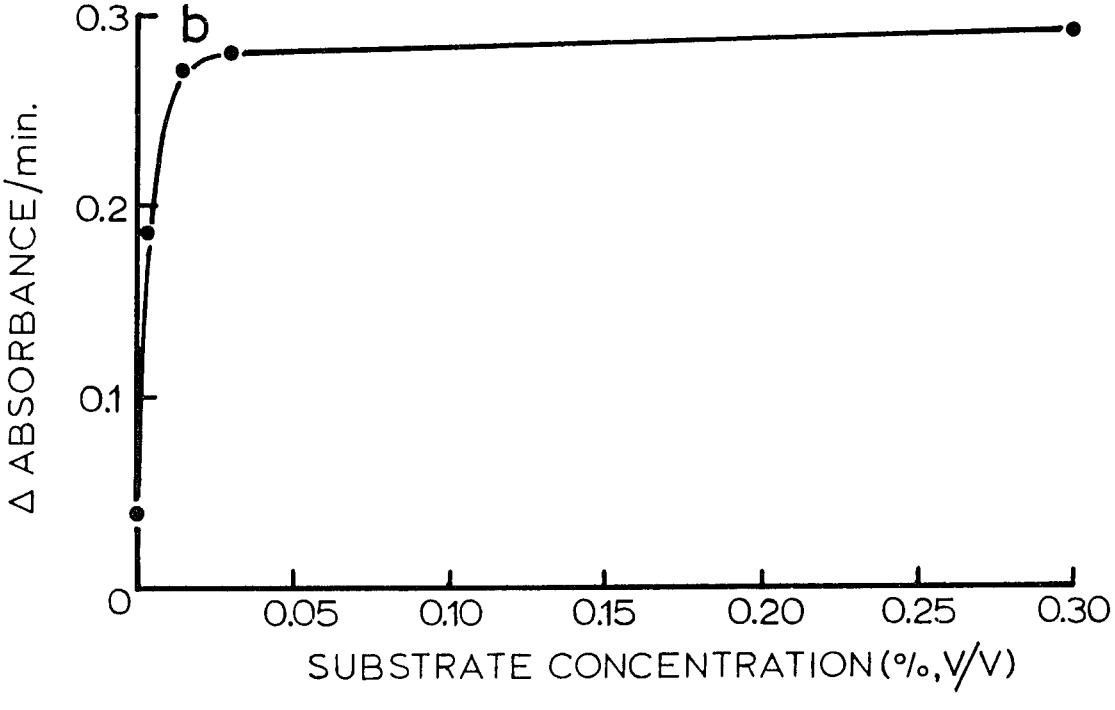

b
0.3
0.2
0.1
0
Δ ABSORBANCE/min.
0.05
0.10
0.15
0.20
0.25
0.30
SUBSTRATE CONCENTRATION (%, V/V)

FIGURE 13

Effect of pH on the activity of glutamate-oxaloacetate transaminase in crude extracts of C. rubrum ($60^{o}47'N$) seedlings (substrate concentration: 7×10^{-2} M L-aspartate plus 1.4×10^{-3} M alpha-ketoglutarate). Absorbance measured at 546 nm.

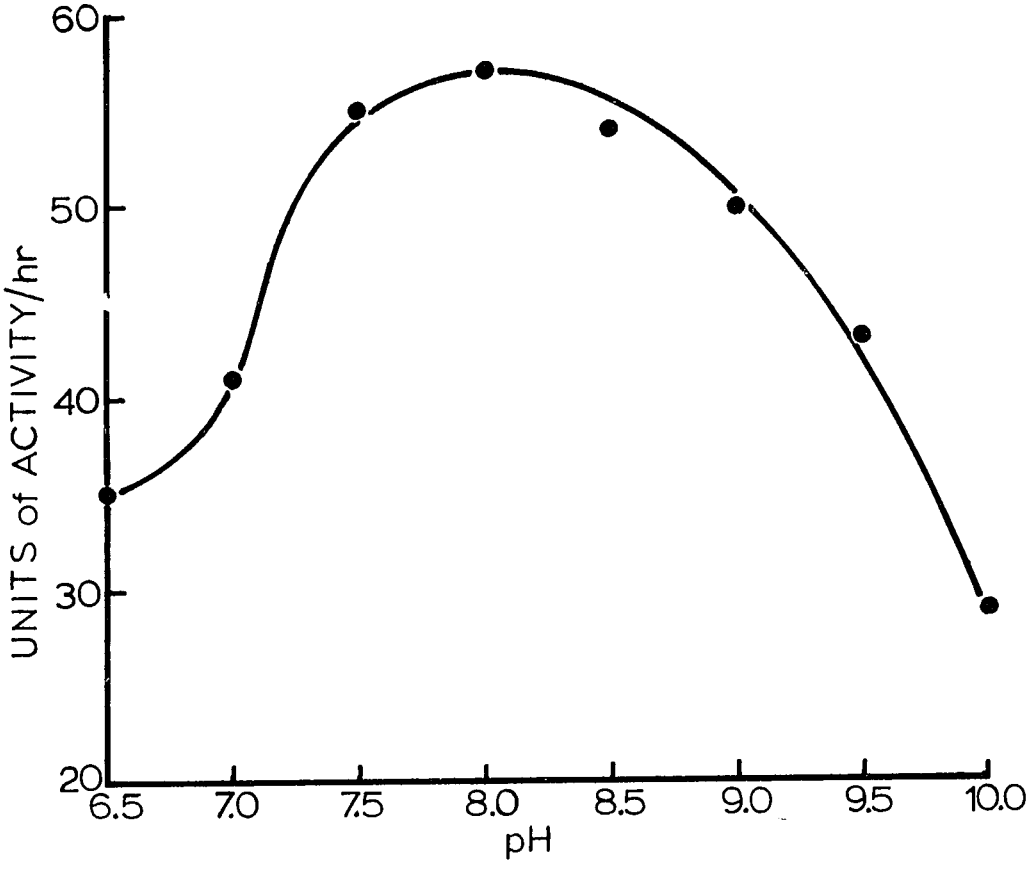
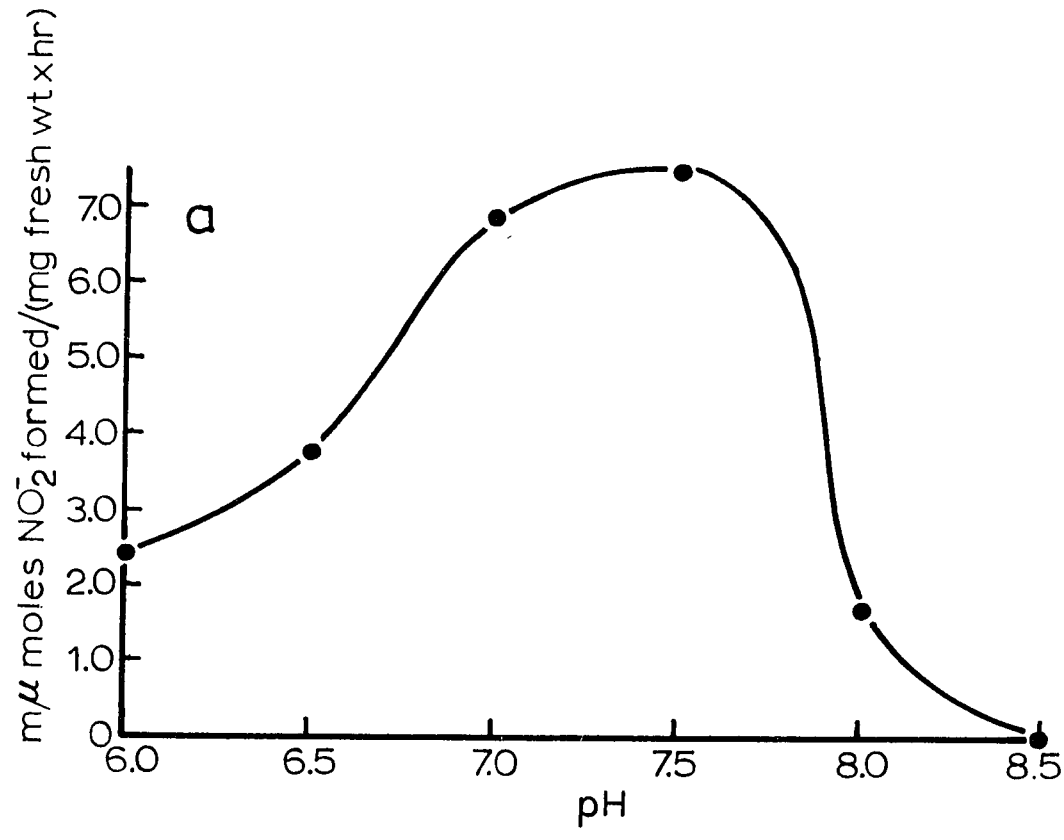

60
50
40
30
20
UNITS of ACTIVITY/hr
6.5
7.0
7.5
8.0
8.5
9.0
9.5
10.0
pH

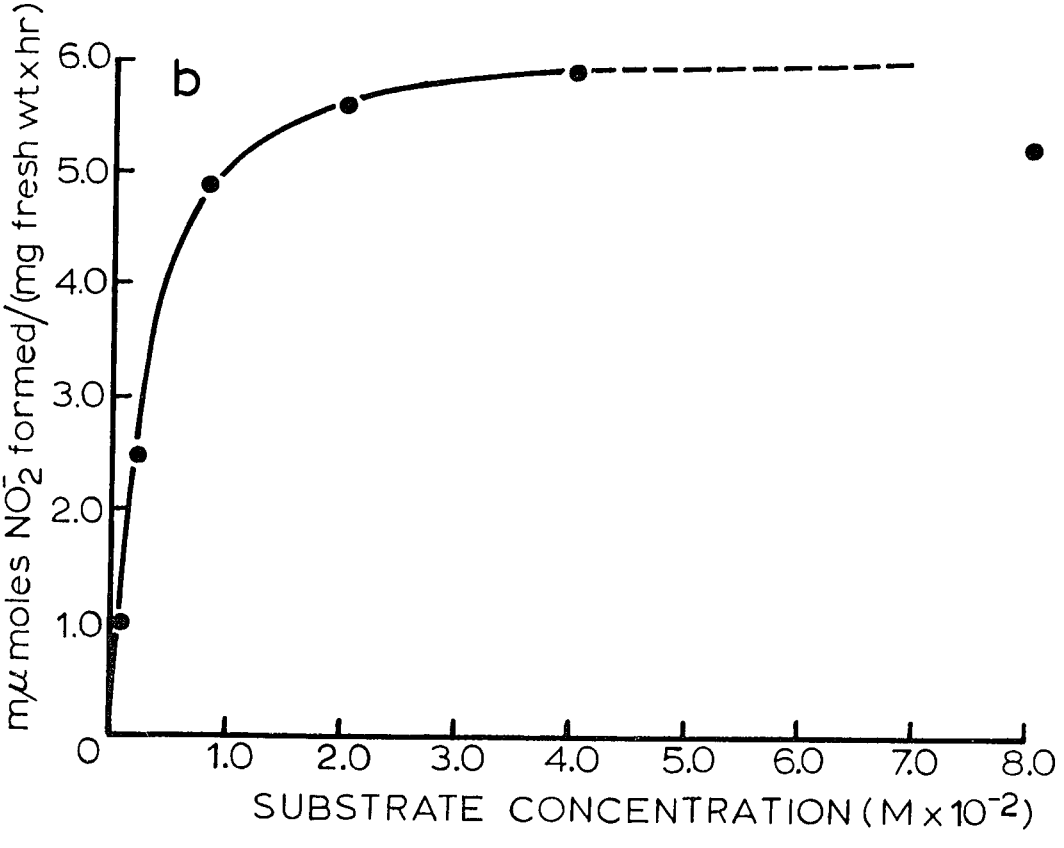
FIGURE 14

Optimum conditions for the assay of nitrate reductase activity in crude extracts of C. rubrum (60°47'N) seedlings.

a. Effect of pH on enzyme activity (substrate concentration used: 7.1×10^{-2} M potassium nitrate).

b. Effect of substrate concentration on enzyme activity. Absorbance measured at 540 nm.


a
mμ moles NO2- formed/(mg fresh wt x hr)
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0
6.0
6.5
7.0
7.5
8.0
8.5
pH


b
mμ moles NO2- formed/(mg fresh wt x hr)
6.0
5.0
4.0
3.0
2.0
1.0
0
1.0
2.0
3.0
4.0
5.0
6.0
7.0
8.0
SUBSTRATE CONCENTRATION (M x 10-2)

possible only within specific limits of absorbance. For my system, these limits occurred between 0.15-0.70 for an initial absorbance of 0.88.

As evident from the peroxidase substrate saturation curve (Fig. 12b), the hydrogen peroxide concentration reached approximate saturating level at about 0.03% since the value for 0.3% proved just a bit higher, and it would have been proper to use some intermediate level in the final assay. However, this peroxidase assay was based on a relatively rapid assessment of initial velocity, and it was observed that the reactions with the lower 0.003% and 0.015% hydrogen peroxide seemed to give the best initial linear reaction rate (for four and two minutes respectively), followed by 0.03% (approximately one minute). Therefore, since the rate of reaction at 0.03% hydrogen peroxide proved measurable especially under experimental conditions with three replicates, and since it was closer to the saturation level of the enzyme, it was decided that this concentration be used in further assays.

Optimum substrate concentrations utilized for assay of glutamate-oxaloacetate transaminase activity were those of Bergmeyer and Bernt (1965) and were not determined independently because of the relative difficulty of assessing the interdependence of L-aspartate and alpha-ketoglutarate.

TABLE 1

Electron donor requirement for the activity of nitrate reductase from crude extracts of seedlings of C. rubrum ($60^{o}47'N$).

Electron donor[1] (8×10^{-5}M)	Nitrate reductase activity (millimicromoles NO_2^- formed / mg fresh wt x hour)
FMN	3.53 ± 0.68
FAD	5.04 ± 0.37
FMN + FAD	5.80 ± 0.49
NADH	0.16 ± 0.15
NADPH	0.00

[1]In assay, flavin nucleotides reduced by sodium dithionite ($Na_2S_2O_4$).

The nitrate reductase activity in crude extracts of C. rubrum (60°47'N) did not appear to require the presence of a sulphydryl-group protector as has been the case for other plant systems (Sanderson and Cocking, 1964); in fact, slightly lower levels of activity were found when cysteine (Kannagara and Woolhouse, 1967) or insoluble polyvinylpyrrolidone were present in the extraction buffer. The saturating substrate concentration for this enzyme was found to be quite high (Fig. 14b), and the decrease in activity at the highest nitrate concentration utilized might have been an example of substrate inhibition; however, this was more probably due to a slight lowering of pH in the incubation mixture because of the acidity contribution of the increased potassium nitrate concentration. Table 1 shows the electron donor requirement for activity of nitrate reductase from C. rubrum extracts and indicates the preference for flavin nucleotides over nicotinamide nucleotides. Activity in the presence of FMN plus either NADH or NADPH was also found to be lower than with FMN alone.

B. Time-course Investigations

The activity patterns of the selected enzymes from extracts of C. rubrum seedlings maintained in darkness or subjected to 48 hours of light after different dark

period lengths, can be observed in Figures 15, 16, and 17. None of the enzymes analysed, apart from nitrate reductase, appeared to show significant fluctuations of activity which might be indicative of rhythmicity. For the former six enzymes there was an overall decline of activity with lengthening darkness, and treatment with 48 hours of light tended to stabilize the respective activities at an almost uniform level. As compared to the activities of these enzymes, the soluble protein content (Fig. 16d) showed more dramatic changes, indicating that possibly some other protein component(s) might have been oscillatory in nature.

In contrast to the other enzymes referred to above (Figs. 15, 16), only nitrate reductase activity (Fig. 17) showed greater variations in activity after a light period following darkness than during darkness itself; in fact, as can be observed, significant peaks and minima of activity definitely occurred. In continuous darkness, overall activity gradually declined; however, a decided correlation can be observed between the low-level variations in darkness and the ensuing oscillations of activity following 48 hours of light. Transfer of seedlings to light from darkness was obviously followed by a significant re-induction of activity. Moreover, under control

FIGURE 15

Time-course of the activities of peptidase(a), phosphatase(b), and amylase(c) in crude extracts of seedlings of C. rubrum ($60^{o}47'N$).
Seedlings maintained in darkness at $20^{o}C$ (closed circle), or subjected to 48 hours of high-intensity (3,000 ft-c) fluorescent illumination at $20^{o}C$ following the respective dark periods indicated (open circle); measurements made at three-hour intervals. Pre-dark treatment: standard germination programme for 108 hours, followed by 24 hours of high-intensity (3,000 ft-c) fluorescent illumination at $20^{o}C$ both preceded and terminated by addition of Hoagland's nutrient solution.

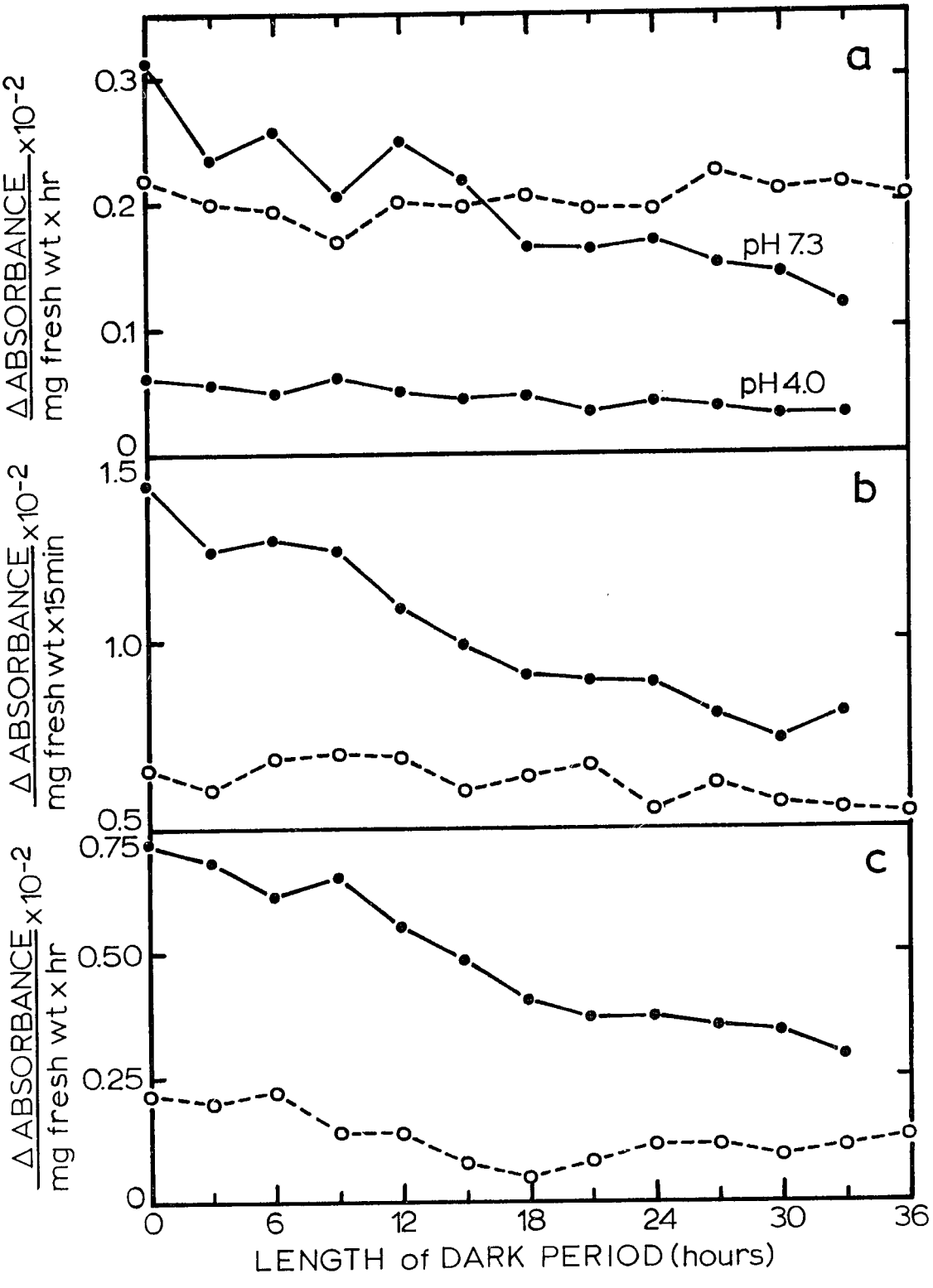

a
b
c
pH 7.3
pH 4.0
ΔABSORBANCE / mg fresh wt x hr x10-2
ΔABSORBANCE / mg fresh wt x 15min x10-2
ΔABSORBANCE / mg fresh wt x hr x10-2
0.3
0.2
0.1
0
1.5
1.0
0.5
0.75
0.50
0.25
0
6
12
18
24
30
36
LENGTH of DARK PERIOD (hours)

FIGURE 16

Time-course of the activities of glucose-6-phosphate dehydrogenase(a), peroxidase(b), and glutamate-oxaloacetate transaminase(c), and changes in soluble protein concentration(d), in crude extracts of seedlings of C. rubrum ($60^{o}47'N$).
Seedlings maintained in darkness at $20^{o}C$ (closed circle), or subjected to 48 hours of high-intensity (3,000 ft-c) fluorescent illumination at $20^{o}C$ following the respective dark periods indicated (open circle); measurements made at three-hour intervals. Pre-dark treatment: standard germination programme for 108 hours, followed by 24 hours of high-intensity (3,000 ft-c) fluorescent illumination at $20^{o}C$ both preceded and terminated by addition of Hoagland's nutrient solution.

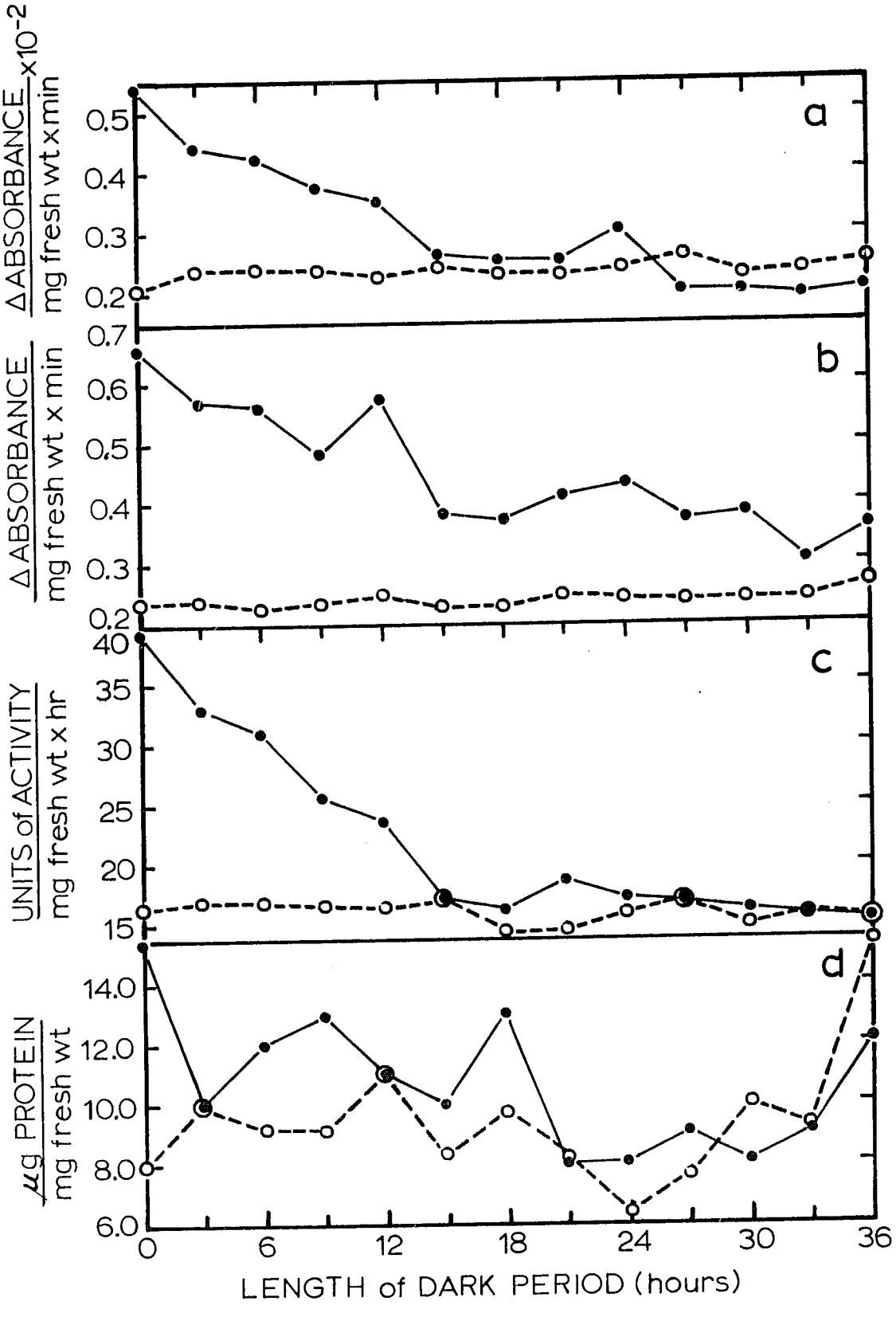
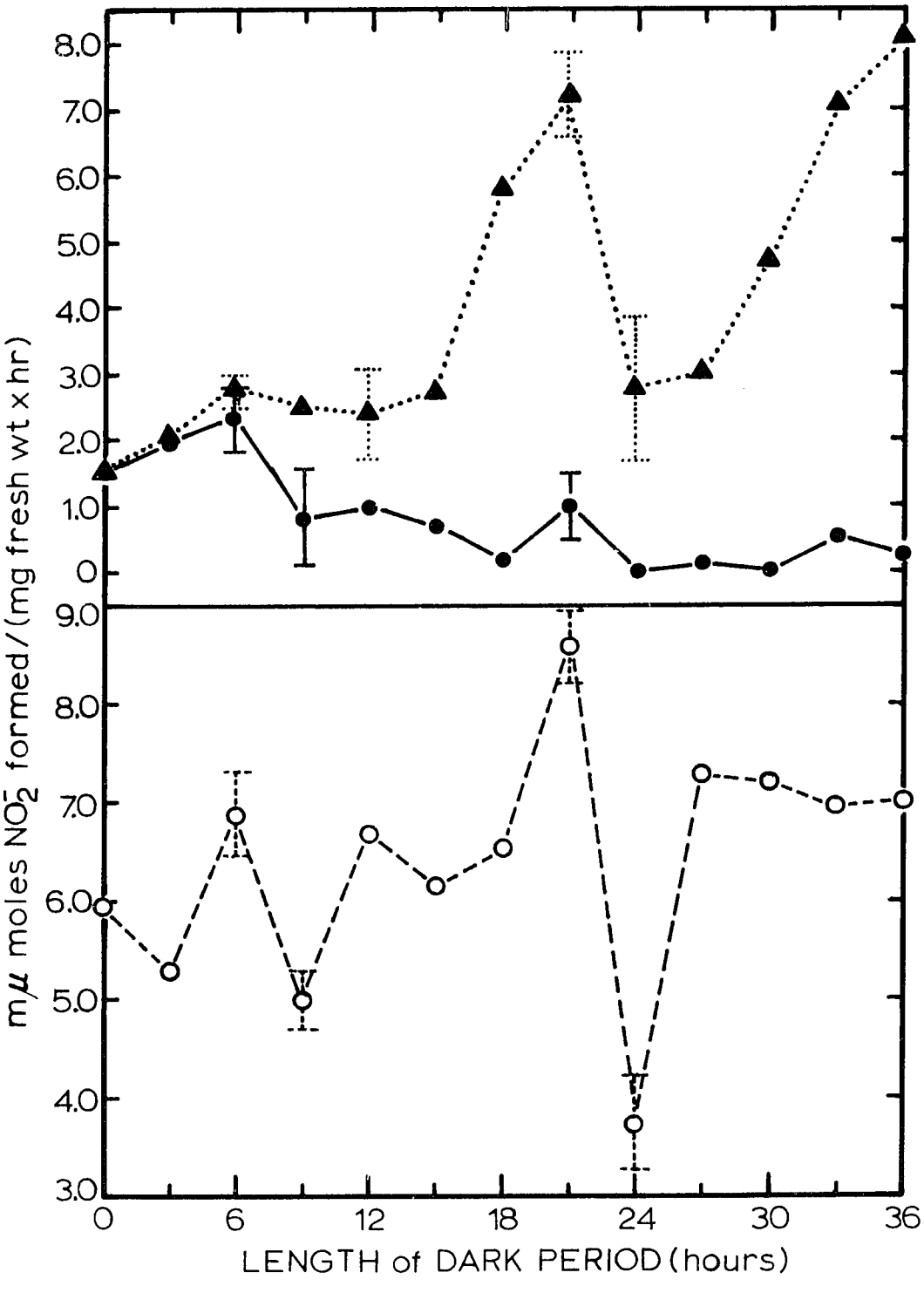

ΔABSORBANCE / mg fresh wt x min x10⁻²
0.5
0.4
0.3
0.2
a
ΔABSORBANCE / mg fresh wt x min
0.7
0.6
0.5
0.4
0.3
0.2
b
UNITS of ACTIVITY / mg fresh wt x hr
40
35
30
25
20
15
c
μg PROTEIN / mg fresh wt
14.0
12.0
10.0
8.0
6.0
d
0
6
12
18
24
30
36
LENGTH of DARK PERIOD (hours)

FIGURE 17

Time-course of the activity of nitrate reductase in crude extracts of seedlings of C. rubrum ($60^{o}47'N$). Seedlings maintained in darkness (closed circle) or in 600 ft-c fluorescent illumination (closed triangle) at $20^{o}C$, or subjected to 48 hours of 600 ft-c fluorescent illumination at $20^{o}C$ following the respective dark periods indicated (open circle); measurements made at three-hour intervals. Pre-dark treatment: standard germination programme for 108 hours, followed by 24 hours of 600 ft-c fluorescent illumination at $20^{o}C$ both preceded and terminated by addition of Hoagland's nutrient solution. Confidence limits expressed as $\pm$ standard deviation.


8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0
9.0
8.0
7.0
6.0
5.0
4.0
3.0
0
6
12
18
24
30
36
mμ moles NO2- formed/(mg fresh wt x hr)
LENGTH of DARK PERIOD (hours)

light conditions (replacing darkness) the main peaks of activity also showed a similar correspondence, indicating that transition to darkness was not necessary for these oscillations of activity to be established.

As was the case for the soluble components, there was not a readily apparent correlation of the variations in nitrate reductase activity with the flowering rhythm (Cumming et al., 1965), the first peak of which would occur at approximately a 15 hour dark period length.

C. Oscillations of Nitrate Reductase Activity

The fluctuations in the activity of nitrate reductase, as presented in Figure 17, suggested the presence of persistent endogenous rhythmic activity of this enzyme in C. rubrum. Since the "light control" appeared to possess a pre-set oscillation of activity, examination of nitrate reductase activity beginning immediately following the end of the germination programme (i.e. 24 hours before "0" in Figure 17) and continuing for a relatively long duration in the light, could provide more specific information on this speculation. Figure 18 shows the results of such experiments. It can be observed that in fact a free-running oscillation of nitrate reductase activity did exist under constant conditions following germination, with peaks of activity at 18, 30, and 45 hours. An

FIGURE 18

Time-course of the activity of nitrate reductase in crude extracts of seedlings of C. rubrum (60°47'N) maintained under continuous fluorescent (600 ft-c) illumination at 20°C beginning immediately after germination.

Germination either in normal water-medium conditions (closed circle) or with one-half strength Hoagland's nutrient solution provided (open circle); measurements made at three-hour intervals. Previous treatment: standard germination programme for 108 hours terminated by the addition of full strength Hoagland's nutrient solution.

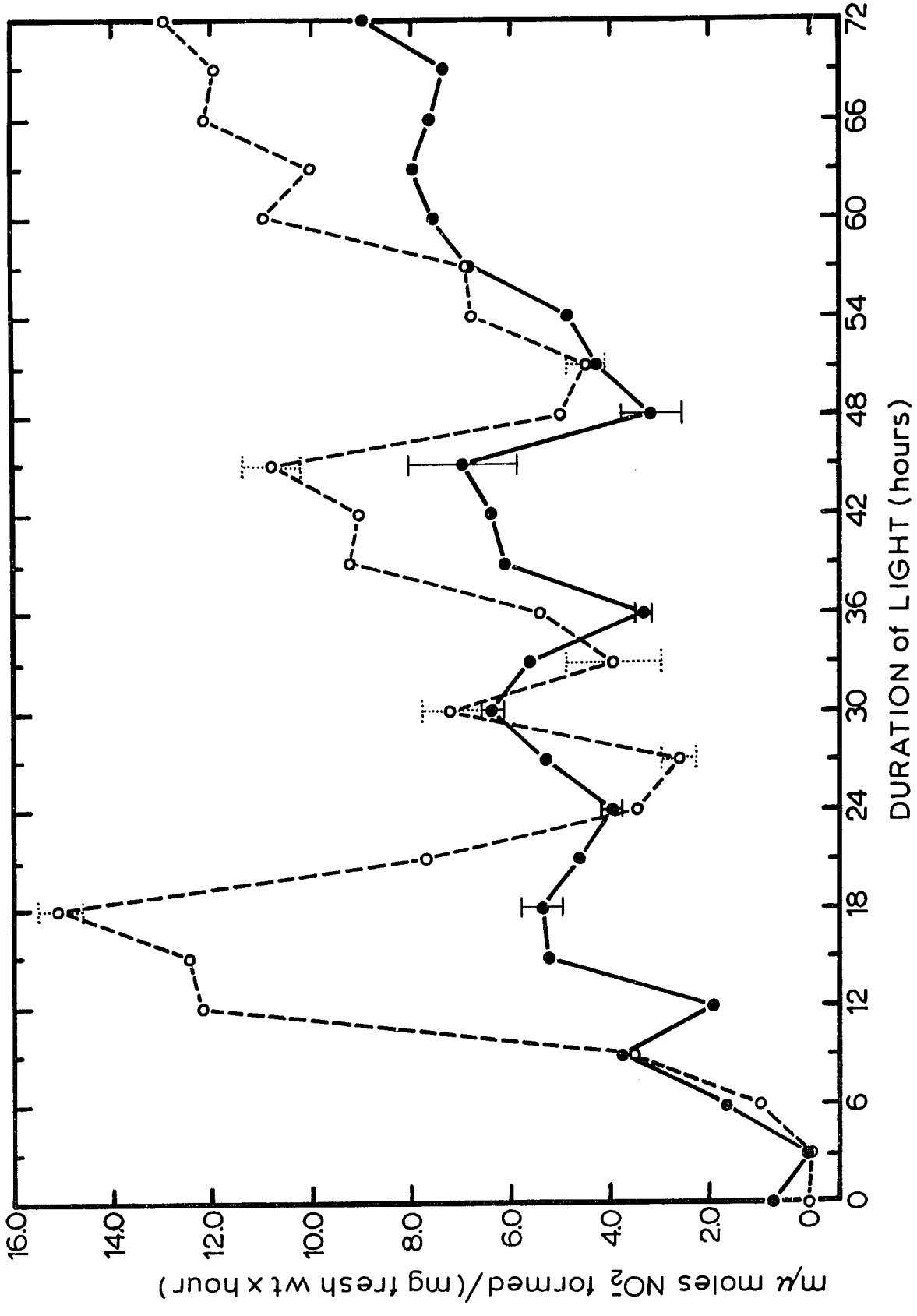

mμ moles NO2 formed/(mg fresh wt x hour)
16.0
14.0
12.0
10.0
8.0
6.0
4.0
2.0
0
0
6
12
18
24
30
36
42
48
54
60
66
72
DURATION of LIGHT (hours)

FIGURE 19

Changes in the fresh weight of seedlings of C. rubrum ($60^{o}47'N$) maintained under continuous fluorescent (600 ft-c) illumination at $20^{o}C$.

Measurements made at three-hour intervals. Previous treatment: standard germination programme for 108 hours, terminated by the addition of Hoagland's nutrient solution.

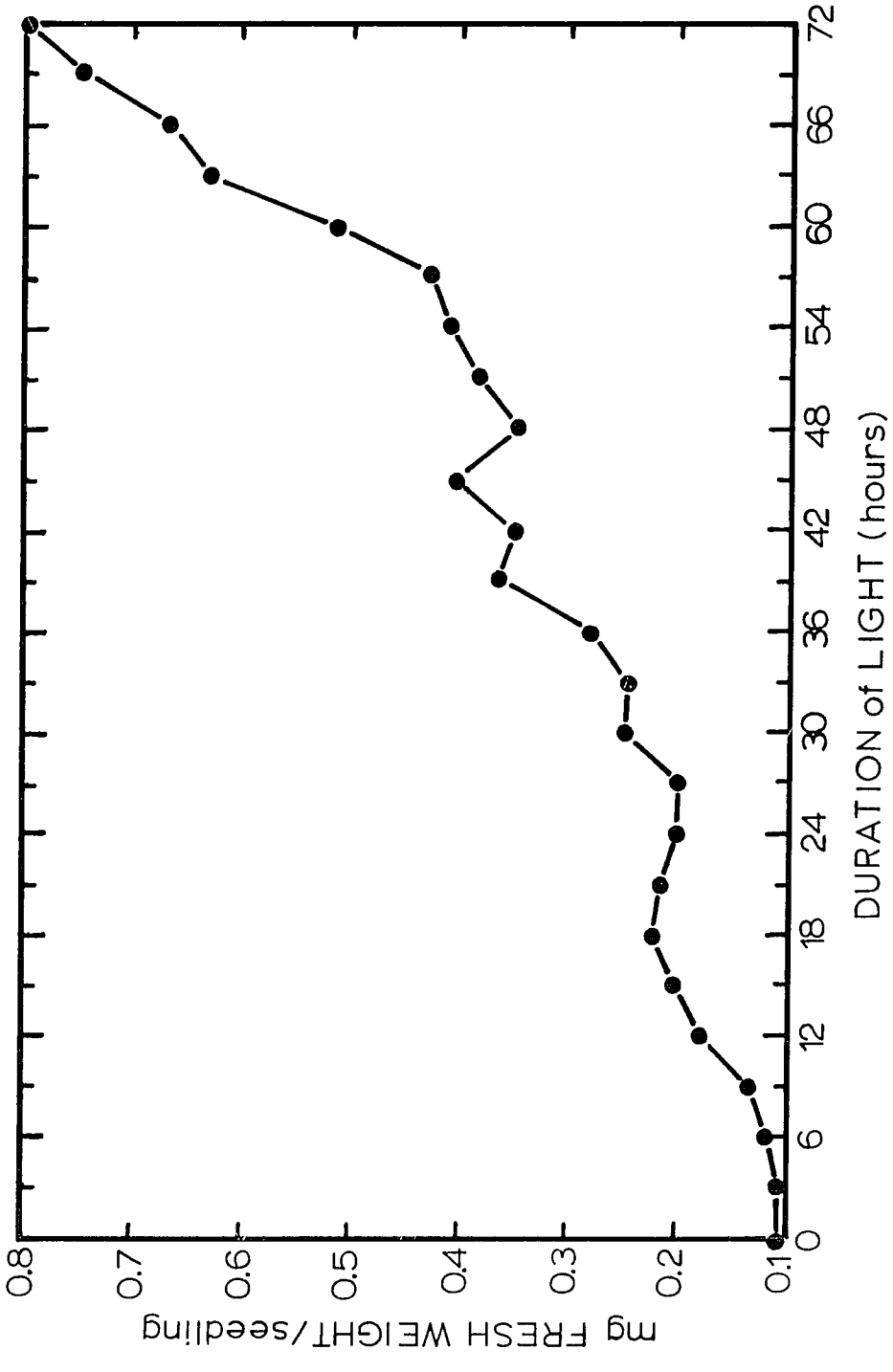
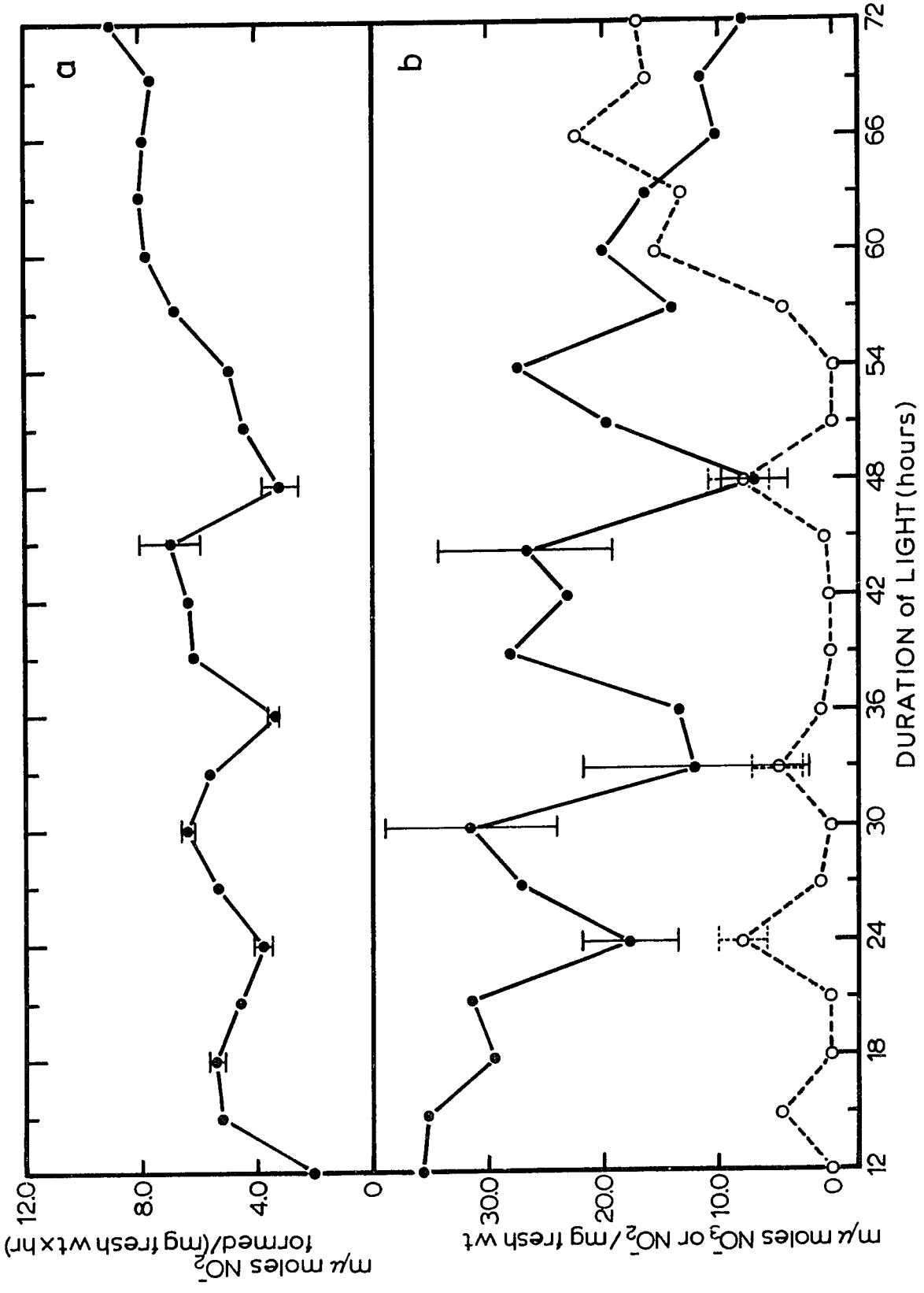

mg FRESH WEIGHT/seedling
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
6
12
18
24
30
36
42
48
54
60
66
72
DURATION of LIGHT (hours)

FIGURE 20

Comparison of the time-course of nitrate reductase activity(a) in crude extracts of seedlings of C. rubrum ($60^{o}47'N$) with changes in endogenous content(b) of nitrite (closed circle) and nitrate (open circle). Seedlings maintained under continuous fluorescent (600 ft-c) illumination at $20^{o}C$; measurements made at three-hour intervals. Previous treatment: standard germination programme for 108 hours, terminated by the addition of Hoagland's nutrient solution.


a
b
12.0
8.0
4.0
0
30.0
20.0
10.0
0
12
18
24
30
36
42
48
54
60
66
72
DURATION of LIGHT (hours)
mμ moles NO2− formed/(mg fresh wt x hr)
mμ moles NO3− or NO2− / mg fresh wt

enhancement of these oscillations was observed when one-half strength Hoagland's nutrient solution was provided instead of distilled water during the germination period. In both cases, enzyme activity was initially at a very low level, and apparently was subsequently induced following the addition of full strength nutrient solution and transfer of the seedlings to constant light and temperature conditions.

Timing of the oscillations in both instances was practically identical, and this indicated that the application of nutrient solution at the end of the germination period was probably not the cause per se of the oscillations, but merely allowed through the presence of nitrate, the induction of nitrate reductase activity according to a pattern predetermined during germination.

The occurrence of the three major peaks and minima was followed by a fairly rapid, relatively linear increase of enzyme activity; this increase was paralleled by a shift in the seedlings to a more rapid increase in fresh weight (Fig. 19).

Analysis of nitrate and nitrite content within the seedlings (Fig. 20) during the time-course of the oscillations of enzyme activity in continuous light, did not provide extremely precise data, because the nitrate

and nitrite concentrations were at the lower limit of sensitivity of the procedure employed. Nevertheless, the results indicated that in general there was a positive correlation between the enzyme activity and nitrite content and a negative correlation between the enzyme activity and nitrate content. Also, towards the end of the period under investigation, nitrate content increased while nitrite content decreased to a level below that of nitrate.

Referring back to the data on oscillations of nitrate reductase activity in light, 48 hours after dark periods of varied length (Fig. 17), it has been shown that the variations of activity during darkness were both stabilized and potentiated by the succeeding light period. Figure 21 shows the time-course of development of nitrate reductase activity during (and beyond) such a light period following either 12 or 24 hours of darkness. Re-induction of enzyme activity can be seen, and both sets of seedlings exhibited quite similar oscillations with a periodicity of approximately 18 hours. Because the absolute age of the 12 and 24 hour lots differed by 12 hours, one might have expected the respective maxima and minima to be 12 hours out of phase if these oscillations of activity were simply a continuation of the oscillations already shown to be present in the seedlings following the ger-

FIGURE 21

Time-course of the activity of nitrate reductase in crude extracts of seedlings of C. rubrum ($60^{o}47'N$) maintained under continuous fluorescent (600 ft-c) illumination at $20^{o}C$ begun immediately following a dark period of either 12 hours (closed circle) or 24 hours (open circle) duration.

Measurements made at six-hour intervals. Previous treatment: standard germination programme for 108 hours terminated by the addition of Hoagland's nutrient solution, followed by 24 hours of fluorescent (600 ft-c) illumination previous to the respective dark periods.

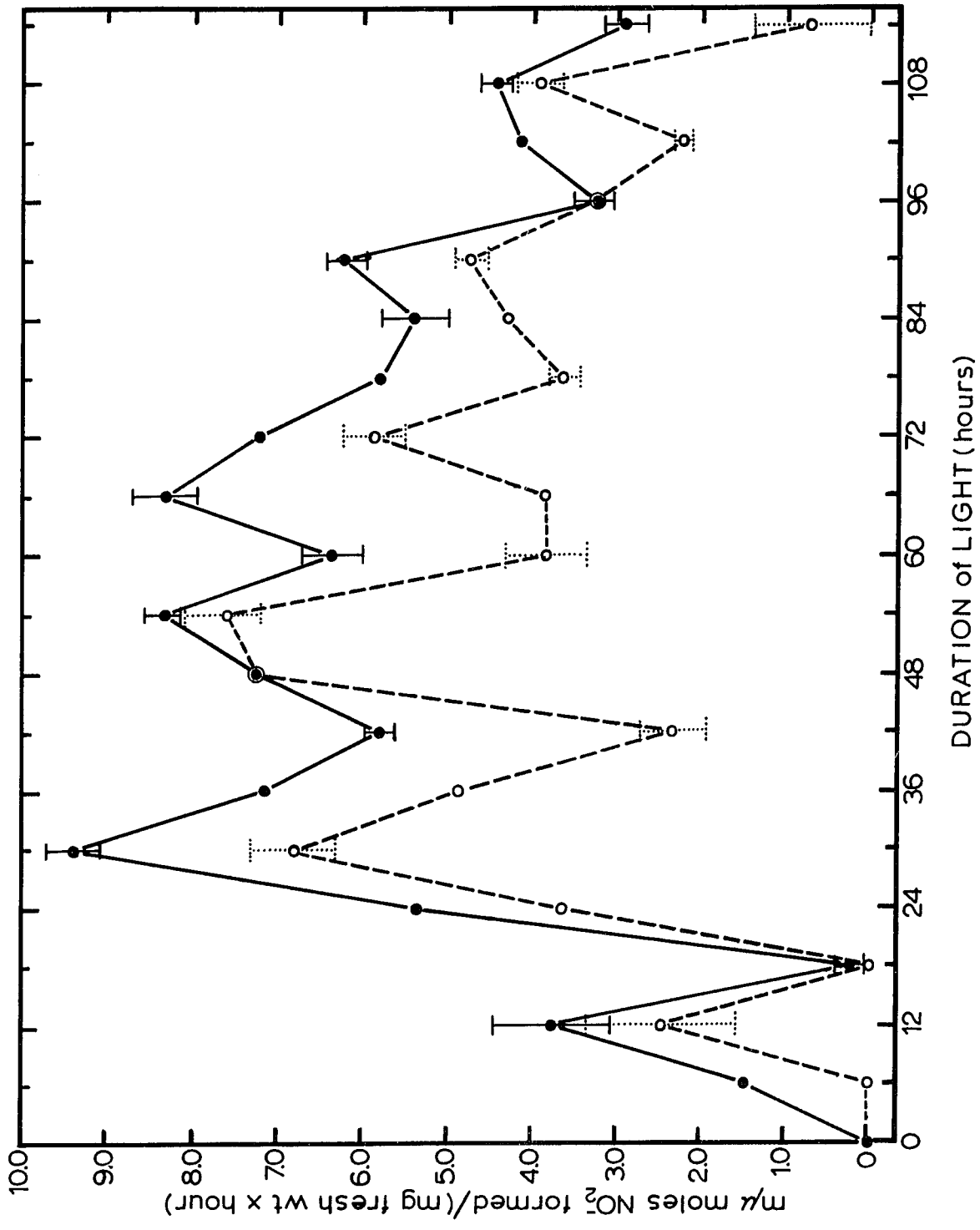
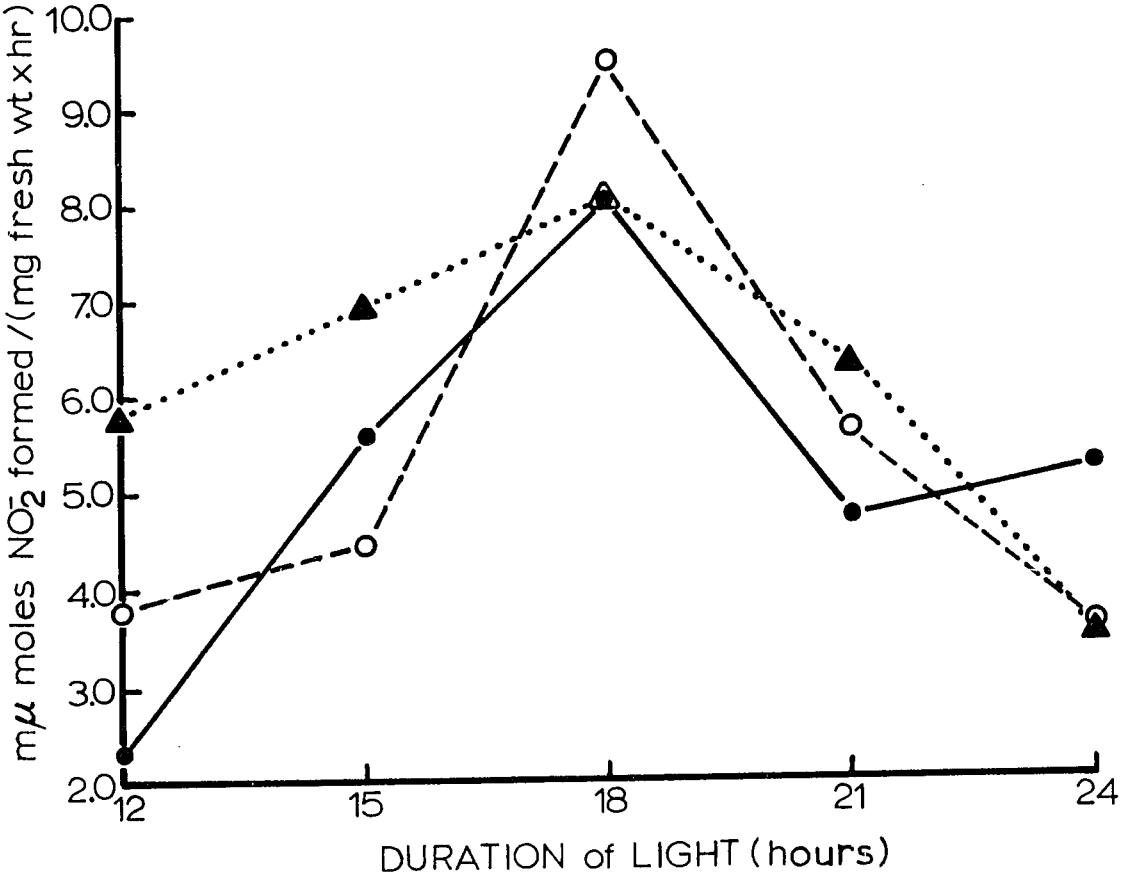

mμ moles NO_2^- formed/(mg fresh wt x hour)
10.0
9.0
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0
0
12
24
36
48
60
72
84
96
108
DURATION of LIGHT (hours)

FIGURE 22

Effect on nitrate reductase activity of alternations in the concentration of NO_3- in the Hoagland's nutrient solution added to seedlings of C. rubrum (60°47'N) immediately following the end of the germination programme. Concentrations represented are normal (closed circle), twice normal (open circle), and four times normal (closed triangle). Seedlings maintained under continuous fluorescent (600 ft-c) illumination at 20°C; measurements made at three-hour intervals. Previous treatment: standard germination programme for 108 hours.


mμ moles NO_2^- formed /(mg fresh wt x hr)
10.0
9.0
8.0
7.0
6.0
5.0
4.0
3.0
2.0
12
15
18
21
24
DURATION of LIGHT (hours)

mination period. However, the two sets of oscillations paralleled one another very closely, and this indicated that a new rhythm may have been initiated by the transition from darkness to light. The level of activity was in general lower for the "24 hour dark period" seedlings, most likely because of the effect of the increased dark period in reducing the level of nitrate reductase activity to that observed in Figure 17. Mixed extracts from points of high and low activity gave the expected average values, thereby ruling out the possibility that simple changes in level of stimulatory or inhibitory substances caused the oscillations of activity. The overall decline in enzyme activity observed in both cases, with continuous light longer than 72 hours, was probably due to a gradual decrease in nitrate content in the medium surrounding the seedlings in their Petri dishes, because no fresh Hoagland's solution had been introduced during this time and nitrate reductase is a relatively labile enzyme (Afridi and Hewitt, 1964).

Figure 22 shows that increased nitrate concentration in the Hoagland's nutrient solution provided at the end of the germination period, did not significantly alter the level of induction of nitrate reductase activity. Together with earlier data showing the effect of the

FIGURE 23

Effects of light quality and intensity on nitrate reductase activity in seedlings of C. rubrum (60°47'N). Following standard germination programme terminated by the addition of Hoagland's nutrient solution, seedlings maintained at 20°C under either continuous fluorescent illumination at 600 ft-c (closed circle), or 3,000 ft-c (open circle), or continuous incandescent illumination at 1,000 ft-c (closed triangle); measurements made at three-hour intervals.

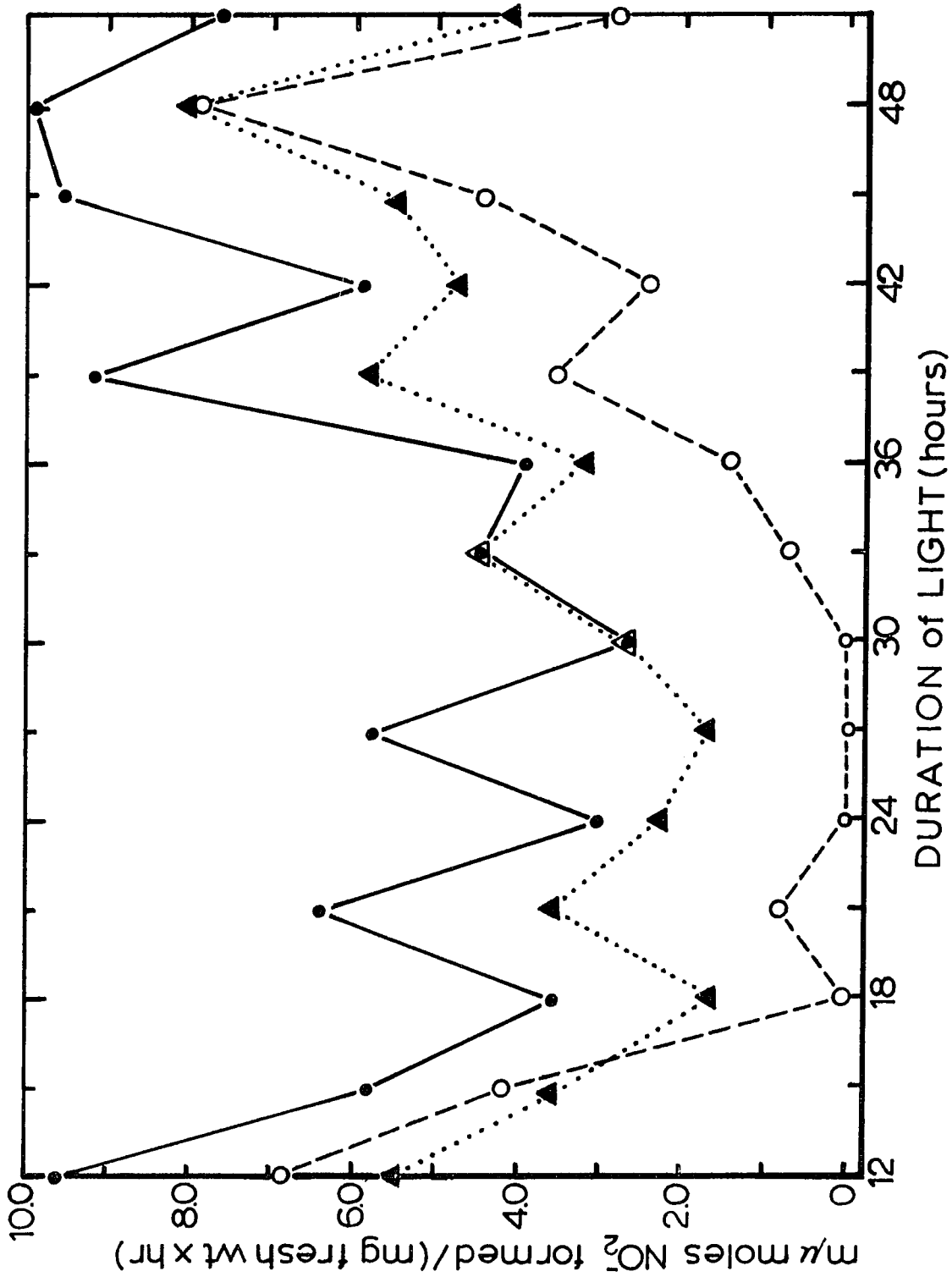
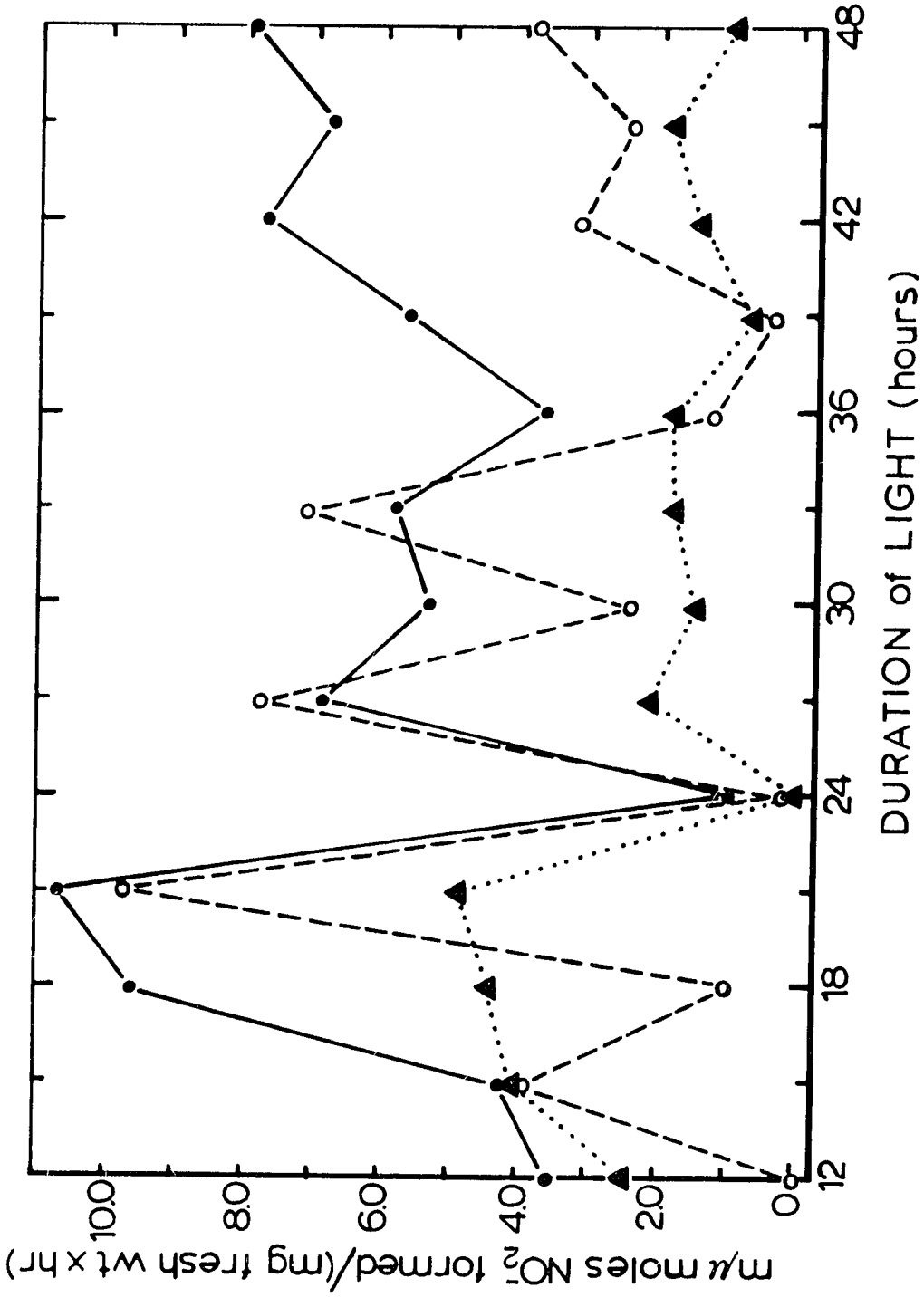

mμ moles NO_2^- formed/(mg fresh wt x hr)
10.0
8.0
6.0
4.0
2.0
0
12
18
24
30
36
42
48
DURATION of LIGHT (hours)

FIGURE 24

Effects of temperature on nitrate reductase activity in seedlings of C. rubrum (60°47'N).
Following standard germination programme terminated by the addition of Hoagland's nutrient solution, seedlings maintained under continuous fluorescent (600 ft-c) illumination at either 10.0°C (open circle), 20.0°C (closed circle), or 32.5°C (closed triangle); measurements made at three-hour intervals.


mμ moles NO_2^- formed/(mg fresh wt × hr)
0.0
2.0
4.0
6.0
8.0
10.0
12
18
24
30
36
42
48
DURATION of LIGHT (hours)

presence of mineral nutrients during germination (Fig. 18), this suggests that overall growth of the seedling was probably more important than nitrate supply (within limits), for the induction of nitrate reductase activity under these conditions.

It can be noted that the previously described pattern of oscillations of nitrate reductase activity was not as characteristic in the results displayed in Figures 23 and 24 as might be expected. This fact was believed to be indirectly due to as yet unexplained difficulties in obtaining optimal germination and growth of C. rubrum seedlings when these experiments were undertaken. Such difficulties were also experienced by co-workers in the laboratory, although flowering periodicity was not as severely affected. Nevertheless, the experimental results expressed in these two figures do provide several insights into the development of nitrate reductase activity in C. rubrum seedlings.

Figure 23 shows that one could obtain higher levels of nitrate reductase activity if fluorescent illumination of 600 foot-candles rather than either that of 3,000 foot-candles or incandescent illumination of 1,000 foot-candles was provided after the germination programme. It appeared that high-intensity fluorescent illumination might even

have been inhibitory at certain stages. Of course, as a complete range of light intensities was not tested, the actual optimal intensity for development of activity could not be ascertained. In general, the pattern of fluctuation of enzyme activity appeared to be quite similar under all three treatments.

Apart from an optimal light intensity, there also appeared to be a definite optimal temperature for development of nitrate reductase activity in continuous light following germination (Fig. 24). Oscillations of activity were much more pronounced at 10°C than at either 20°C or 32.5°C, although at this lowest temperature, the activity level eventually decreased. Surprisingly, 32.5°C appeared to be inhibitory to fluxes in enzyme activity and average activity was lower than that at either of the other two temperatures. Thus, of the three treatments, 20°C proved optimal in that only at this temperature did activity appear to be developing relatively normally. Decided effects on the phasing of the enzyme periodicity (i.e. phase advance or delay) by the high and low temperatures were not readily apparent, although some alterations could be observed.

CHAPTER VI

DISCUSSION

I. NON-RHYTHMIC COMPONENTS

A. Endogenous Metabolites

The preceding investigations showed no pronounced periodicity in levels of endogenous metabolites, although irregular short-term fluctuations in total soluble carbohydrate and amino acid concentrations were evident (Figs. 7, 8). If endogenous changes in these components were indeed correlated with rhythmic floral induction (Cumming et al., 1965; Cumming, 1967a, 1969, 1971), then one might postulate the presence of a system incorporating "frequency demultiplication," in which a given number of short-term biochemical fluctuations could correspond to a single oscillation at a higher level possessing a longer rhythmic period; interrelated rhythms, each possessing a distinct frequency, could occur at various cellular and organismal levels.

The ability of glucose to prolong and stimulate

flowering rhythmicity (Cumming, 1967a) could be mediated through induction of enzymes for conversion of the sugars to phosphate esters (Marre, Cornaggia, and Bianchetti, 1968) which can exist in the cell as stable control points for metabolic pathways (Banks and Vernon, 1970); glucose-mediated repression of enzyme synthesis (Magasanik, 1961) could serve as the counterbalancing force in a rhythmic system. Sulebele and Rege (1968) found that low and high concentrations of glucose (also fructose and mannose) would respectively both stimulate and inhibit induced enzyme synthesis in yeast. Thus, changes in level of a single endogenous component, itself possibly regulated through a feedback system, might conceivably initiate a succession of periodicities analogous to a train of gears, which would ultimately result in an overt physiological display phenomenon such as flowering.

The experimentally observed gradual decline of starch content (Fig. 7), characteristic of seedling metabolism (Juliano and Varner, 1969; Larson and Beevers, 1965; Bain and Mercer, 1966), was not suggestive of the complex fluctuation pattern of the sugars. Starch might serve as a continuous source of glucose, feeding it into the system at a more or less constant rate, with subsequent metabolic transitions and interconversions occurring

independently of starch breakdown. The hydrolysis of trehalose, a storage disaccharide in yeast, has been found to mediate sustained glycolytic oscillations of that organism in this manner (Pye, 1969). Although starch content of plants may not disappear entirely in an extended dark period (Komuro, Yano, and Hattori, 1966), the residual content might exist in a non-utilizeable form; additional starch could be synthesized through the addition of excess energy sources such as glucose (Wolf, 1967; Gates and Simpson, 1968), thus prolonging a rhythmic display (Cumming, 1967a).

However, in the absence of an additional energy source, one may observe that in seedlings maintained in an extended dark period, following depletion of carbohydrate (or fat) reserves, there occurs a breakdown of proteins to their constituent amino acids which can consequently function as respiratory substrate by contribution of their carbon skeletons (Yemm, 1965). This fact could readily explain the dramatic increases in total free amino acid content which occurred in C. rubrum (60°47'N) seedlings during darkness, coinciding with the retarded rate of starch disappearance (Fig. 7). The chromatographically observed similarity between the fluctuation patterns and overall increases of individual amino acids, would be an

obvious consequence of increased protein hydrolysis during darkness. In a study of the diurnal variation of amino acids in tobacco leaves, Noguchi and Tamaki (1962) found that of the twenty-one amino acids characterized, all (excluding aspartic acid) behaved similarly in showing maximum concentrations during the day and minima at night, and in not exhibiting any obvious interrelationships.

In the short-term fluctuations that were apparent in C. rubrum seedlings (Fig. 8) both in the case of the amino acids and carbohydrates, it would be necessary to invoke extremely complex metabolic patterns in order to account for such a rapid alternation of either synthetic and degradative states or interconversions. As the methods of analysis employed were sensitive to all soluble components in their respective categories, and assuming that the data obtained truly reflected what was occurring in the seedling, one would have to envisage rapid transitions between soluble and insoluble forms. Certainly, the uniformly low level of total soluble carbohydrate between 27-42 hours corresponding to a higher level of starch content (Fig. 7), suggested interconversion in darkness between soluble and insoluble states.

The data presented in Figure 7 indicates that initially in darkness, there was a relatively high soluble

carbohydrate to soluble nitrogen (C / N) ratio, but with the eventual large increase in free amino acid content, this ratio was lowered considerably. It appears doubtful that this situation played any direct importance in rhythmic floral induction in C. rubrum; however, as pointed out by Chailakhyan (1968), such alterations in metabolism could effect a necessary precondition which would allow flowering to occur. Observations on the effects of exogenously supplied free amino acids on flower induction in Lemna gibba (Nakashima, 1964, 1965) have suggested that in vivo concentrations of amino acids might be an important factor in photoperiodic floral induction.

B. Enzymatic Activity

Recently, periodicity of adenylate kinase activity in C. rubrum (50°10'N) seedlings (Frosch, 1971, unpublished data), has been found to be coincident with the rhythm controlling flowering in this ecotype. In the present study, decreasing activity during darkness, in conjunction with lack of periodicity in light following darkness, suggested that the enzymes which were analysed initially (Figs. 15, 16) could not be directly correlated with rhythmic floral induction in C. rubrum (60°47'N). The activity patterns obtained for these enzymes indicated instead that they were probably more innately involved

with aspects of germination and seedling metabolism.

The activity in seedlings of peptidase enzymes, that contribute to protein hydrolysis in association with proteases (proteinases), has generally been found to decrease gradually following the initial hours of germination (Beevers and Splittstoeser, 1968; Puztai and Duncan, 1971; Guardiola and Sutcliffe, 1971). One might have expected the pattern of peptidase activity in C. rubrum to have shown a closer correlation with the fluctuations in total free amino acid content observed previously (Fig. 7); however, it should be realized that in plants a large variety of peptidases might exist with different specificities (Holley, 1965; Dixon and Webb, 1964) contributing in unison to protein degradation.

Although increases in the activities of acid phosphatase (Young and Varner, 1959; Presley and Fowden, 1965; Firenzuoli et al., 1968), amylase (Young and Varner, 1959; Juliano and Varner, 1969), and glucose-6-phosphate dehydrogenase (Firenzuoli et al., 1968) have been observed during germination in some plants, this was obviously not the case for C. rubrum seedlings during the period of investigation in darkness. However, due to the limitations of this study, it was not known whether increased enzymatic activity might have preceded the observed declines -- in

peas, rising glucose-6-phosphate dehydrogenase activity during the first three or four days of germination was followed by a diminishing activity of this enzyme (Wray and Brown, 1966; Brown and Wray, 1968). The ability of added glucose to prolong the flowering rhythm (Cumming, 1967a), might have been due to stimulation of respiratory processes mediated by the pentose phosphate pathway (Ap Rees and Beevers, 1960); here the rate-limiting step is catalysed by glucose-6-phosphate dehydrogenase (De Flora, 1968). Inorganic phosphate in the Hoagland's nutrient solution introduced at the end of the germination programme, may have caused the repression of acid phosphatase synthesis (Price, 1962), thereby contributing to the decreasing acid phosphatase activity observed. Although a declining level of amylase activity occurred at the time of rapid starch breakdown (Fig. 7), one would suppose that the amount of enzyme present possessed sufficient catalytic power for this degradation to proceed.

Rhythmicity in the activity of peroxidase, an enzyme believed to be involved in indoleacetic acid destruction (Galston, Bonner, and Baker, 1953; Ray, 1958), could suggest a hormonal link with the flowering rhythm. However, the activity of this enzyme showed no significant oscillations.

The fluctuations in amino acid content observed previously might have been due to reversible "intermolecular transfer of the alpha-amino group from an amino acid to an alpha-keto acid, without the intermediate participation of ammonia" (Meister, 1955), catalysed by transaminase enzymes. Although examination of glutamate-oxaloacetate transaminase activity in darkness revealed no oscillations of activity, but rather a rapid decline in the first fifteen hours, this transamination is only one of many characterized in plants (Leonard and Burris, 1947; Stumpf, 1951; Wilson, King, and Burris, 1954) and general involvement of these enzymes cannot be ruled out.

The observed decline of the enzyme activities during darkness could have been due to the action of proteolytic enzymes in reducing such enzyme proteins to sources of amino nitrogen and respiratory substrate. Nonetheless, general information on the breakdown of enzyme proteins by proteolytic enzymes is scanty (De Flora, 1968). The observed changes in soluble protein level during darkness (Fig. 16d) suggest that protein synthesis did occur through the utilization of free amino acids released from degraded pre-existing proteins; however, protease activity in extracts of C. rubrum could not be detected. The lessened activity of the selected enzymes in light, in

contrast with the increased protein concentration, implies that the enzymes characterized were of minor importance in the subsequent growth and development of the seedlings.

II. RHYTHMICITY IN NITRATE REDUCTASE ACTIVITY

A. Characteristics of the Enzyme Activity

Nitrate reductase, the enzyme which catalyses the first step in the reduction of nitrate to ammonia (Evans and Nason, 1953), has been widely characterized in both higher and lower plants (Kessler, 1964; Beevers and Hageman, 1969). For activity, the enzyme requires the presence of bound molybdenum (Spencer and Wood, 1954; Nicholas and Nason, 1954; Candela, Fisher, and Hewitt, 1957; Randall, 1969; Vega et al., 1971), and a suitable electron donor -- specifically, either reduced nicotinamide adenine nucleotide (NADH or NADPH) or reduced flavin nucleotide ($FMNH_2$ or $FADH_2$), depending primarily on the species of plant. The nitrate reductases that require reduced nicotinamide adenine nucleotide as co-factor preferentially utilize NADH (Spencer, 1959; Beevers, Flesher, and Hageman, 1964; Schrader et al., 1968; Ferguson, 1969). The reduced flavin nucleotides tend to vary in effectiveness when they are required (Evans and Nason, 1953; Spencer, 1959; Ramirez et al., 1964; Paneque et al., 1965; Losada et al., 1965; Oji and Izawa, 1969). Nitrate reduc-

tase activity in extracts of C. rubrum seedlings was optimal when a mixture of $FMNH_2$ and $FADH_2$ was provided as electron donor (Table 1): the almost complete lack of activity with NADH suggested that flavin nucleotide might be the in vivo electron donor. Increases of both FAD concentration and the activity of the enzyme responsible for its synthesis, have been found in germinating Phaseolus radiatus seedlings (Giri et al., 1960).

The increased activity of nitrate reductase following the addition of Hoagland's nutrient solution containing nitrate to C. rubrum seedlings, strongly suggested a substrate-inducible property of this enzyme in light. This has been observed in many plants, including algal species (Hattori, 1962; Morris and Syrett, 1963; Ohmori and Hattori, 1970), rice (Tang and Wu, 1957; Shen, 1969), cauliflower and mustard (Hewitt and Afridi, 1959; Afridi and Hewitt, 1962, 1964), pea (Wallace and Pate, 1965), radish (Beevers et al., 1965; Ingle, Joy, and Hageman, 1966), tobacco culture (Filner, 1966), barley (Ritenour and Huffaker, 1964; Ferrari and Varner, 1969; Wray and Filner, 1970; Smith and Thompson, 1971), apple (Klepper and Hageman, 1969), corn (Beevers et al., 1965; Travis, Huffaker, and Key, 1970; Travis and Key, 1971), darnel Bowerman and Goodman, 1971), and duckweed species (Joy,

1969; Ferguson, 1969; Swader and Stocking, 1971). That this substrate-inducible activity is due to de novo protein synthesis, rather than an activation phenomenon, has been inferred both from the inhibitory effects on induction of nitrate reductase activity by inhibitors of protein and RNA synthesis (Beevers et al., 1965; Ingle et al., 1966; Ferrari and Varner, 1969; Shen, 1969; Smith and Thompson, 1971), and from radioactive isotope incorporation studies (Zielke and Filner, 1971). Nevertheless, Ingle (1968) found that the formation of actual nitrate reductase protein was not significantly increased in nitrate-induced radish cotyledons, even though induction of nitrate reductase activity could be prevented by inhibitors of protein and RNA synthesis. It was suggested that the synthesis of an effector protein might be necessary for the enzyme activity and that its synthesis was being affected by the inhibitors.

In contrast to lower plants such as Chlorella (Morris and Syrett, 1963; Losada et al., 1970), nitrate reductase induction in higher plants cannot as a rule be repressed by the presence of ammonia, the end-product of the nitrate reduction pathway (Afridi and Hewitt, 1964; Schrader and Hageman, 1967; Ingle et al., 1966; Minotti, Williams, and Jackson, 1969). An example of partial inhibition has how-

ever, been reported (Smith and Thompson, 1969). Occasionally, the addition of ammonium ions has even tended to slightly enhance the induction of activity (Schrader and Hageman, 1967; Shen, 1969); this may be attributed to increased amino acid synthesis leading to protein synthesis.

The overall decline in activity of nitrate reductase that occurred when C. rubrum seedlings were transferred from light conditions to darkness (Fig. 17), is a commonly observed feature of this enzyme in plants (Candela et al., 1957; Hageman and Flesher, 1960; Ritenour and Huffaker, 1964; Beevers et al., 1965; Kannagara and Woolhouse, 1967; Shibata, Kobayashi, and Takahashi, 1969). The first rise in activity closely paralleled the pattern of the control (light) treatment during the first six hours. The subsequent decline suggests that certain inducers (or possibly co-factors) favoring nitrate reductase activity may have been formed in the preceding light period and that they were eventually depleted in darkness. It has been found in barley leaves that the normal decline of nitrate reductase activity in darkness could be suppressed by inhibitors of protein synthesis (Travis, Jordan, and Huffaker, 1969), thereby possibly preventing the formation of an inactivating protein. The presence of inhibitory proteins,

specific for certain enzymes, has certainly been indicated in other systems (Bechet and Wiame, 1965; Pressey, 1966; Kenney, 1967).

Another characteristic of nitrate reductase in plants is that light is generally required for induction of activity in the presence of nitrate (Candela et al., 1957; Ritenour and Huffaker, 1964; Beevers et al., 1965; Kannagara and Woolhouse, 1967; Shibata et al., 1969, Chen and Ries, 1969; Travis, Jordan, and Huffaker, 1970). This feature was apparent from the increase in level of activity following transfer of C. rubrum seedlings from darkness to continuous light (Figs. 17, 21). The light requirement could be mediated through the photosynthetic generation of electron donor (Beevers and Hageman, 1969, and references within; Klepper, Flesher, and Hageman, 1971), through nitrate uptake (Beevers et al., 1965), or through the synthesis of the enzyme itself (Hageman and Flesher, 1960). Kannagara and Woolhouse (1967) showed that if light and nitrate were provided to Perilla frutescens leaves, but in a CO_2 free atmosphere, no activity of nitrate reductase was induced. Also, if induced leaves were placed in a CO_2 free atmosphere, activity dropped as in darkness. They therefore suggested that nitrate reductase was being continuously degraded both in light and

darkness, but the difference in activity between light and darkness could be attributed to the presence or absence of photosynthetic processes. Nitrate reductase has been localized both within and outside of chloroplasts in green leaves (Ritenour et al., 1967; Grant, Atkins, and Canvin, 1970). Lips and Roth-Bejerano (1969) have proposed that light indirectly induces nitrate reductase activity by effecting a change in hormonal balance within the plant -- they observed that specific proportions of gibberellic acid and kinetin would allow dark induction of activity in leaves of nitrate-treated tobacco.

Nitrate reductase is not the only plant enzyme to be induced by light. Others include glycolic acid oxidase (Tolbert and Cohan, 1953), $NADP^+$-dependent glyceraldehyde-3-phosphate dehydrogenase (Ziegler and Ziegler, 1965), cinnamic acid 4-hydroxylase (Amrhein and Zenk, 1968), and phenylalanine ammonia-lyase (Zucker, 1965, 1969; Durst and Mohr, 1966; Nitsch and Nitsch, 1966; Engelsma, 1967a, 1967b; Scherf and Zenk, 1967a, 1967b; Creasy, 1968). Investigations on the induction of phenylalanine ammonia-lyase activity have revealed similarities to that of nitrate reductase: the characteristic decline in activity on transfer to darkness; the ability of protein synthesis inhibitors to prevent this reduction of activity (Zucker,

1969). Incorporation of labelled amino acids has indicated that in darkness, synthesis of the enzyme was still occurring (Zucker, 1970), thereby suggesting that formation of a protein inactivator was responsible for the diminishing activity -- since inhibition of photosynthesis prevented light-induction of phenylalanine ammonia-lyase activity, it was felt that the light requirement was in effect a photosynthetic requirement (Zucker, 1969).

B. Oscillations of Activity

Unlike the circadian rhythm of flower induction in C. rubrum ($60^{o}47'N$) which was initiated by the transition from continuous light to darkness (Cumming et al., 1965), endogenous oscillations of nitrate reductase activity were already established in the light period following the germination programme (Figs. 17, 18) and did not appear to be reset by the transition to darkness. The results of Wagner and Frosch (1971) suggested analogous characteristics for an endogenous circadian rhythm of betacyanin accumulation in a different C. rubrum ecotype; they also observed that there was a rhythm in darkness correlated with the oscillations found in continuous light 48 hours after dark periods of varied length. Although oscillations of nitrate reductase activity in C. rubrum ($60^{o}47'N$) seedlings were not well pronounced in darkness

due to enzyme inactivation, the rhythmic potential was obviously still present (Fig. 17). Hoshizaki, Brest, and Hamner (1969) in a study of circadian leaf movement rhythms in Xanthium pensylvanicum, also observed a rapid damping out of rhythmicity in continuous darkness.

In C. rubrum, both in darkness and after 48 hours of continuous light following varying dark periods, peaks of nitrate reductase activity occurred with dark period lengths of 6 and 21 hours, unlike rhythmic flowering induction which displayed its first peak when seedlings were given a dark period of approximately 15 hours duration (Cumming et al., 1965). If a phase relationship between these two periodicities exists, it is not an obvious one. In other investigations of C. rubrum rhythmicities (Wagner and Cumming, 1970; Wagner and Frosch, 1971; Frosch, 1971, unpublished data; Chia-Looi, 1971, unpublished data) no direct causal relationship with flowering periodicity is readily apparent. Nevertheless, the study of specific biochemical periodicities may be the only truly definitive technique in determining metabolic transitions underlying such overt rhythmicity (Cumming, 1971).

Continuous light can inhibit endogenous rhythmicity (Sweeney, 1960). The replacement of oscillations of

nitrate reductase activity by continuously rising enzyme activity (Fig. 18) appeared to be more a consequence of a shift in the seedlings to a new developmental stage -- a contention supported by the shift to a more rapid increase in fresh weight (Fig. 19) and the dramatic increase in nitrate content accompanied by a lessened nitrite concentration (Fig. 21). Nitrate reductase is an enzyme intimately concerned with post-seedling growth in plants, and these oscillations of activity in continuous light may have been a consequence of metabolic interactions indicating the transition between the end of primarily reserve-dependent seedling germination and the initiation of more autotrophic development. Lack of rhythmicity in the other enzymes analysed (Figs. 15, 16), which are more associated with early germination processes, also support this hypothesis. The oscillations of nitrate reductase activity that occurred after germination, may have been entrained or simply initiated by the alternating temperature and light (temperature-dependent) conditions during the germination programme -- a possibility which will be discussed in more detail later.

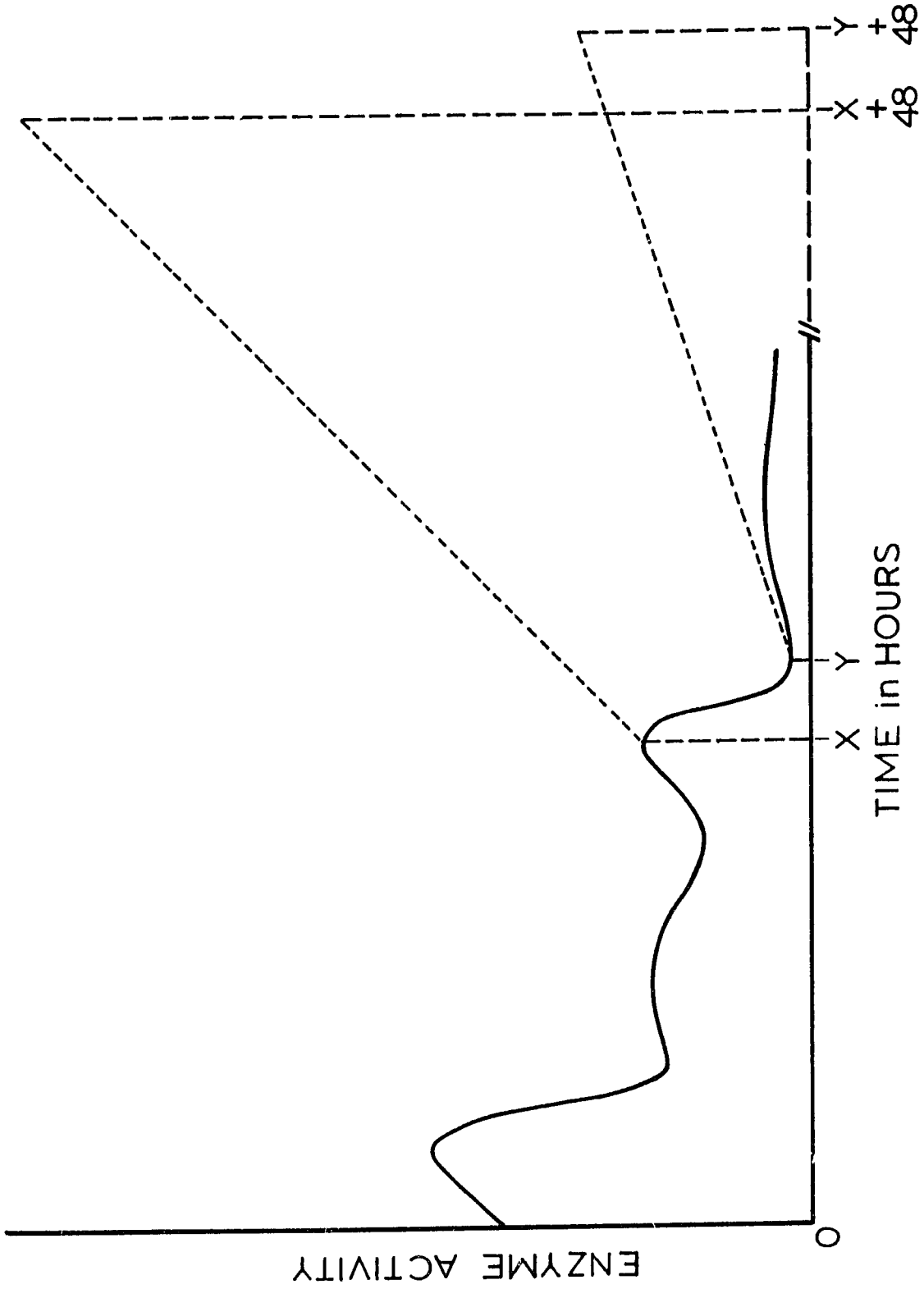
If induction of nitrate reductase activity is dependent on nitrate uptake (Beevers et al., 1965), rhythmicity in the latter could lead to oscillations of enzyme activity

by periodic activation of the enzyme. The data expressed in Figure 20 accords with this premise. Nevertheless, since nitrate and nitrite content and not actual nitrate uptake were measured, the fluctuating levels of these ions could just as easily be a consequence of the oscillating synthesis of enzyme protein. In agreement with this latter possibility, Hageman et al., (1961) observed a negative correlation between nitrate reductase activity and nitrate content, and found a positive correlation between nitrate reductase activity and protein content. However, their protein analysis was non-specific; moreover, periodic ion uptake under constant conditions has been observed both in C. rubrum (Andreae, 1968, unpublished data) and other plants (Wallace, Soufli, and Hemaidan, 1966). Thus, without further detailed investigation, it cannot be ascertained whether either of these mechanisms or a combination of both was responsible; nonetheless, in a singularly rhythmic plant such as C. rubrum, several overlapping periodicities have already been shown to occur (Cumming, 1971).

The oscillations of nitrate reductase activity that occur in light 48 hours after the end of various length dark periods (Fig. 17), indicate that the low-level oscillations during darkness were subsequently stabilized

FIGURE 25

Diagrammatic representation of a possible mechanism for the expression of enzymatic activity oscillations in light following exposure of seedlings to dark periods ("X" and "Y") of varying length.


ENZYME ACTIVITY
0
X
Y
TIME in HOURS
X + 48
Y + 48

and potentiated by the succeeding light period. The simplest way to explain this phenomenon would be to postulate different rates of increase of nitrate reductase activity in the light, depending on the time of removal of the seedlings from darkness. The rate of increase would be greatest for a peak of activity and least for a minimum point, yielding after 48 hours in the light a series of values reflecting the oscillations occurring in darkness, as shown diagrammatically in Figure 25. This hypothesis was tested by measuring the enzyme activity during the light period beginning immediately after the transfer of seedlings from darkness (Fig. 21). Obviously, the increase in enzyme activity following darkness was not linear but oscillatory in nature. Therefore, the inference is that a new rhythm was initiated by the transfer of seedlings from darkness to light. Yet this new rhythm was superimposed on the original oscillations present in darkness, since the relationships established by the original dark (or light control) oscillations still held after a light period of 48 hours. Hoshizaki and Hamner (1969) have shown the existence of both light-on and light-off rhythms in the flowering response of Pharbitis nil. Although periodicity of flower induction in C. rubrum is normally considered a light-off response

(Cumming et al., 1965; King, 1971; King and Cumming, 1972), these "light-on" oscillations of nitrate reductase activity further attest to the complexity of rhythmicity in this species.

It could be expected that a complex rhythmic system might be subject to some degree to factors causing internal disorganization within an organism. This unfortunately appeared to be the case in the experiments on the effects of light and temperature (Figs. 23, 24), in which unexplained decreased vigour of the germinating seedlings led to a somewhat altered rhythmicity. If coupling of closely related oscillating systems in an organism normally resulted in derivative oscillations of intermediate frequency by a process of "mutual entrainment" (Pittendrigh and Bruce, 1957), then alterations of metabolism at only one of many levels might interfere with normal periodicity. For instance, the oscillatory activity of an enzyme could require all of the following steps (Goodwin, 1963), possessing varying degrees of sensitivity to disturbance: diffusion-mediated derepression of messenger RNA (mRNA) synthesis, mRNA synthesis, diffusion-mediated movement of mRNA out of nucleus into cytoplasm, enzyme synthesis, diffusion-mediated repression of mRNA synthesis.

The partially inhibitory effect of high-intensity

fluorescent light on nitrate reductase activity (Fig. 23) suggested either inhibition of nitrate reductase synthesis or inactivation by the formation of some endogenous inhibitor -- certainly the pigmentation of the seedlings (reddish cast) was different under this light condition as compared to the others. In contrast to this finding, Harper and Paulsen (1968) observed no significant effect of high light intensity on nitrate reductase activity in wheat leaves.

The results from temperature alteration indicated that seedlings maintained at 10°C showed surprisingly strong fluctuations of activity, while those at 32.5°C demonstrated less pronounced changes and possessed an overall lower level of activity than at either 20°C or 10°C (Fig. 24). The literature reveals that there is often decreased nitrate reductase activity in light after exposure to increased temperature (Afridi and Hewitt, 1965; Younis et al., 1965; Mattas and Pauli, 1965); also, in darkness the normally observed decline of activity can be hastened by increasing the temperature (Kannagara and Woolhouse, 1969). Conversely, low temperature can significantly impair the loss of nitrate reductase activity in darkness (Ritenour, 1964; Ritenour and Huffaker, 1964; Travis et al., 1969). These effects of high and low

temperature could be mediated through the respective promotion or suppression of the synthesis of an inactivating protein.

Although endogenous rhythms tend to exhibit temperature independence which may be due to compensation in extremes of temperature (Pittendrigh, 1961), there is usually an optimal temperature at or near the middle of the range (Bünning, 1959). Certainly this appeared to be true overall in the case of the oscillations of nitrate reductase activity in C. rubrum. Bünning and Tazawa (1957, cited in Bünning, 1967) found that when Phaseolus multiflorus plants were transferred from 21°C to 10°C, the first period of the endogenous leaf movement rhythm was longer than normal but each succeeding period was shorter than the first; in fact, an overcompensation actually occurred since the period length for several cycles went below that of the average length at 20°C. Ball and Dyke (1957) found a similar overcompensation in the endogenous growth rate rhythm of Avena sativa coleoptiles. Positive effects of high temperature have ranged from a slightly lengthened period in the endogenous luminescence rhythm of Gonyaulax polyedra (Hastings and Sweeney, 1957b) to inhibition of rhythmic CO_2 output in Bryophyllum fedtschenkoi (Wilkins, 1962). Since the C. rubrum seedlings

had been transferred directly from the alternating-temperature germination regime to the various experimental temperatures, and the oscillations of nitrate reductase activity were not as clear-cut as had been previously observed, definitive conclusions on the degree of temperature compensation cannot be made from this experiment, although it was observed that the fluctuation patterns at all three temperatures were in general quite similar.

Given the occurrence of free-running oscillations of nitrate reductase activity in constant conditions, one may well ask what could be the causes and significance of this endogenous periodicity. One possible direct explanation for such endogenous oscillations might be alternating states of synthesis and degradation of the nitrate reductase enzyme protein itself (Marcus, 1971; Schimke, 1969). More indirectly, perhaps a fluctuating equilibrium between rates of synthesis of effector and inactivating proteins, whose presence has been postulated in relation to the activity of this enzyme in higher plants (Ingle, 1968; Travis et al., 1969), could act on a relatively constant level of nitrate reductase protein in causing fluctuations between functional and non-functional forms. Such mechanisms could be mediated through a controlling rhythmicity of either DNA-directed mRNA synthesis or mRNA-directed

protein synthesis, self-regulated by changes in repressor level.

The activity of several enzymes has been found to be dependent on minor changes in structure which can have considerable effects on their catalytic ability (De Flora, 1968); such structural changes leading to a modification of activity may be due not only to the action of another protein on the enzyme molecule, but also to the effect of small molecules becoming bound to the enzyme protein and causing an "allosteric transition" (Monod, Changeux, and Jacob, 1963; Monod, Wyman, and Changeux, 1965). Allosteric enzyme molecules such as haemoglobin (Perutz, 1967) possess several active sites, and an enzyme-substrate attachment at one site has the ability to cause a greater affinity for substrate and higher potential for activity at the other sites; however, in some cases the activating compound need not always be the substrate -- co-factors or even molecules not involved in the specific reaction could participate. Also, a metabolite could act as an inactivating substance; that is, attachment to a site on an enzyme molecule could cause a resultant lower affinity for substrate and consequent lower potential for activity. If nitrate uptake in C. rubrum were rhythmic, one could postulate that oscillations of nitrate reductase activity

might be caused by alternate activation of the enzyme protein by nitrate taken up and inactivation by nitrite formed in the enzymatic reaction.

On the other hand, since flavin nucleotide was found to be necessary for the expression of nitrate reductase activity in C. rubrum (Table 1), a mechanism involving regulation only by this co-factor could also be postulated. Recent investigations (Kaufman, Vratsanos, and Erlanger, 1968; Bieth et al., 1969) have shown how the in vitro activity of an enzyme, itself insensitive to light, could be photoregulated through reversible configurational changes in a low-molecular weight, light-sensitive (photochromic) molecule. The characteristic light-induction and dark-decline of nitrate reductase activity suggests that such molecules may be functional in vivo in plant tissue, and one such candidate could well be a flavin nucleotide since it has been expressed that riboflavin-containing compounds might be implicated in certain light-sensitive responses of plant organs (Galston, 1967). Therefore, if there were endogenous successive alternations of configurational states of flavin nucleotide in C. rubrum seedlings under constant conditions, and the nitrate reductase protein could behave in both a positive and negative allosteric manner toward these respective

alternate forms, then one could expect to observe upon provision of nitrate, oscillations of enzyme activity reflecting the periodic changes in active and inactive flavin nucleotide. Sustained oscillations of flavin compounds have indeed been recently observed (Degn and Harrison, 1971), but these occurred in bacteria and possessed a very short period of approximately eight minutes.

Whatever the direct cause of the endogenous oscillations of nitrate reductase activity under constant conditions in C. rubrum may be, there still must be an underlying basis for the rhythmicity per se. Repeated temperature alternations between 10°C and 32.5°C during germination in continuous light may have served as a stimulus to initiate (although not necessarily entrain) rhythmicity; also the slight alternation of light intensity (temperature-dependent) may have contributed. Several investigators (Hillman, 1956; Schwemmle, 1960; Went, 1962) have pointed out the importance of temperature alternations in conjunction with light-dark cycles in sustaining optimal plant growth and development. While Hoffman and Miller (1966) found that temperature alternations between 18°C and 29°C would not initiate a rhythm in the Hill-reaction activity of tomato chloroplasts, Oltmanns (1960, cited in Bünning, 1967) observed that temperature cycles

with the difference between the two alternating temperatures being as small as 1°C, could effectively entrain rhythmic petal movement in Kalanchoë blossfeldiana. The results shown in Figure 24 certainly indicate the capacity of nitrate reductase activity in C. rubrum to be influenced by different temperatures.

One specific manner in which alternating temperatures might effect rhythmicity has been suggested in the work of Jones (1969, 1970a, 1970b, 1971). He found that in leaves of both Chenopodium rubrum (Jones, 1969, 1970a) and Phaseolus vulgaris (Jones, 1970a), a higher concentration of intracellular ATP was present when the plants were at 4°C as compared to 20°C; this temperature-dependent modification of ATP content was also shown to be reversible. Jones (1969, 1970b, 1971) hypothesized that oscillations of ATP level, caused by a shifting equilibrium between synthesis and degradation, might be a central feature of biological clocks. Apropos to nitrate reductase, the in vitro inhibition by ADP but not by ATP of activity of this enzyme from tomato leaves (Nelson and Ilan, 1969), suggested that alterations of ATP levels may play a part in the regulation of nitrate reductase activity.

What might be the significance of endogenous oscillations of nitrate reductase activity? Only one other en-

dogenous rhythm under constant conditions in nitrate reducing ability has been reported (Kessler and Czygan, 1963), and this was a seasonal rhythm found in the green alga Ankistrodesmus braunii; however, several investigators have observed diurnal variations in nitrate reductase activity under normal day-night conditions (Hageman et al., 1961; Shibata et al., 1969; Roth-Bejerano and Lips, 1970; Bowerman and Goodman, 1971). Considering the previously discussed characteristic behavior of this enzyme in light and darkness, one might expect such rhythmic variations to occur in a much broader range of plants than have as yet been researched. It has been proposed that in multicellular organisms, at least one of the internal oscillators would be coupled to the natural light-dark cycle, thus helping to establish temporal organization in the organism by coupling to other internal oscillators (Pittendrigh and Minis, 1964). The light- and substrate-inducible nature of nitrate reductase activity in plants has suggested that the nitrate reductase step could be the logical control point for the input of reduced nitrogen and consequent amino acid and protein synthesis. Also, the nitrate assimilation system has been implicated in the evolutionary history of photosynthesis and respiration (Olson, 1970) -- it was postulated that, nitrate respiration preceded O_2 res-

piration, and photosynthesis based on nitrite (as electron donor) preceded that requiring water. Alternation of day with night throughout the evolutionary history of plants may have produced a genetically controlled endogenous rhythm of nitrate reduction, which under normal conditions would be entrained by the natural day-night cycles, but under constant experimental conditions could show a free-running oscillation.

CHAPTER VII

SUMMARY AND CONCLUSIONS

One may undertake biochemical investigations of organisms displaying overt physiological rhythmicity, in the hope of more clearly defining the rhythmic systems and elucidating the mechanisms involved. In the present study, there was no evidence of coincidence or apparent correlation between changes in any of the endogenous components investigated and the rhythm of floral induction in Chenopodium rubrum (60°47'N) seedlings. Certainly, these findings do not as yet rule out the possibility of there indeed being a biochemical basis for flowering rhythmicity in this plant species.

Specifically, irregular short term fluctuations in levels of soluble carbohydrates and free amino acids occurred during darkness that is inductive to flowering; the major soluble carbohydrates present were glucose and fructose, and approximately fifteen different amino acids were represented. One could envisage a system of "fre-

quency multiplication" as being of importance in respect to the relationship of such changes to a rhythmicity possessing a long period of oscillation.

Activity of the following enzymes did not display rhythmicity either during darkness or after a fixed light period following single dark periods of varied length: peptidase, acid phosphatase, amylase, glucose-6-phosphate dehydrogenase, peroxidase, and glutamate-oxaloacetate transaminase. Instead, declining activities during darkness and low levels of activity in light after darkness, indicated that these enzymes had been more involved with metabolism during earlier germination stages.

Oscillations in the substrate- and light-induced activity of a flavin-nucleotide requiring nitrate reductase, were evident during darkness inductive to flowering and under fluorescent illumination provided both before and after a dark period. Transition of the seedlings from light to darkness did not alter the phasing of the oscillations established in the preceding light period; however, during continuous light after a dark period, there was evidence of a secondary rhythmicity. Changes in levels of nitrate and nitrite within the seedlings were consistent with the periodicity of nitrate reductase activity

during the light period following germination.

The occurrence in C. rubrum (60°47'N) seedlings of nitrate reductase activity oscillations in contrast to the more linear levels of the other enzymes characterized in this study, indicates that in an organism capable of displaying overt physiological rhythmicity, only certain biochemical processes may exhibit rhythmicity while others do not. In other words, all reactions involved in the various cellular metabolic pathways need not be linked to the "clockwork" within the organism. The difficulty arises in attempting to assess the value of reactions that do oscillate, to the organism as a whole.

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