Chlorophyll formation and the induction and development of photosynthesis during the greening of etiolated apical pea cuttings.

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CHLOROPHYLL FORMATION AND THE INDUCTION AND DEVELOPMENT OF PHOTOSYNTHESIS DURING THE GREENING OF ETIOLATED APICAL PEA CUTTINGS

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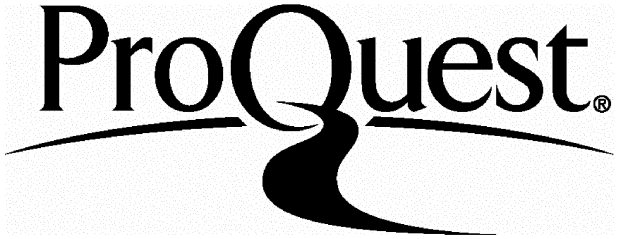

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A B S T R A C T

The development of chlorophyll, photosynthetic electron transport and carbon dioxide fixation was studied in greening pea cuttings treated with chloramphenicol, terramycin or light of low intensity. The cuttings were supported in agar solidified nutrient medium, in which the antibiotics were dissolved as required. All the treatments markedly inhibited the rate of total chlorophyll synthesis but the formation of chlorophyll a was more intensively inhibited by the antibiotics than chlorophyll b. The studies with varying light intensity indicated that normal pigment formation cannot proceed at intensities below 200 lux.

Chloramphenicol was shown to abolish electron transport in Light Systems I and II, maximal inhibition occuring at concentrations above 1 mg/ml. A survey of the partial reactions of photosynthetic electron transport in isolated chloroplasts, indicated that chloramphenicol inhibited Light System II, but stimulated ascorbate - DCIP donation of electrons to Light System I and cyclic photophosphorylation. Terramycin also inhibited Light System I but had no effect upon the ascorbate - DCIP oxidation, whilst cyclic photophosphorylation was stimulated. Generally, low intensity light inhibited all of these reactions.

Photosynthetic electron transport was first observed in chloroplasts isolated from untreated cuttings after 4 hours illumination, chloramphenicol and terramycin treated after 10 hours and low intensity light treatment after 12 hours. The chlorophyll content at all these times was 0.04 mg/g fresh weight. The onset of the ascorbate - DCIP oxidation in the antibiotic treated leaves was delayed by 4 hours when compared

with the Hill reaction. Photosynthetic carbon dioxide fixation in untreated cuttings occurred after 4 hours illumination and in the treated leaves after 12 hours. Carbon dioxide exchanges of the treated leaves demonstrated an unusual, but pronounced evolution of this gas in the light during the initial 10 hours of greening.

The normal development of the chloroplast is apparently controlled by the rate of chlorophyll synthesis and is dependent upon an adequate supply of substrates. A scheme is put forward describing the physiological relationships between the several biochemical pathways involved in chloroplast formation.

A B B R E V I A T I O N S

ADP	adenosine diphosphate
ATP	adenosine triphosphate
CMU	3-(3 - chlorophenyl)-1, 1-dimethyl urea
DCIP	dichlorophenol indophenol
DCMU	3-(3,4-dichlorophenyl)-1, 1-dimethyl urea
DNA	deoxyribonucleic acid
EDTA	ethylene diamine tetra acetic acid
GTP	guanosine triphosphate
NADP	nicotinamide-adenine dinucleotide phosphate
RNA	ribonucleic acid
TRIS	2-amino-2-hydroxymethyl propane-1, 3-diol

INTRODUCTION

The dark grown angiosperm seedling exhibits certain morphological characteristics which distinguish it from one grown in the light. In dicotyledons, the internodes and petioles are greatly extended and the leaves are rudimentary, whilst in monocotyledons it is the laminae which are elongated. However in both groups the most striking feature of etiolation is the lack of chlorophyll. The subsequent exposure of an etiolated plant to continuous white light initiates many biochemical processes which lead to the formation of chlorophyll and the eventual development of photosynthesis.

1. Changes in ultrastructure upon illumination

In the developing Elodea meristematic cell, the plastid is represented by an organelle consisting of a homogenous stroma surrounded by a double membrane (Muhlethaler and Frey-Wyssling, 1959). This membrane evaginates forming vesicles or tubes which later give rise to the prolamellar body. If however, the plant is not exposed to light the vesicles transform into long and evenly spaced tubes which associate to form a 'crystalline centre'. The further changes in structure which occur upon illumination have been described by many workers e.g. Hodge, McLean and Mercer (1956); Gunning and Jagoe (1966); Muhlethaler and Frey-Wyssling (1959); Virgin, Kahn and von Wettstein (1963); Kahn (1968), using electron microscopical techniques. In many of these studies the preparation of the plant material has involved fixation with potassium permanganate, which preserved much of the fine detail, but a recent report indicated that fixation with glutaraldehyde greatly enhanced the resolution of the fine structure (Gunning and Jagoe, 1966).

Virgin, Kahn and von Wettstein (1963) using permangante

fixatives recognised three major steps in the early development of the chloroplast. Immediately upon illumination the tubes of the 'crystalline centre' dissociated into a mass of small vesicles. This transformation was brought about by red or white light of low energy. Since far-red light had no effect it was suggested that the phytochrome system was not of importance. With continued illumination the vesicles quickly became dispersed throughout the stroma of the plastid; temperature variations between 3°C and 23°C have no effect upon the rate of dispersal. These two steps were essentially completed at the end of the lag phase of chlorophyll synthesis. The third step was that during which the individual vesicles aggregated forming the well known grana stacks and the rate of chlorophyll synthesis became exponential.

The use of glutaraldehyde as a fixative enabled Gunning and Jagoe (1966) to investigate the structure of the 'crystalline centre' and the light induced primary changes in much greater detail than was previously possible. After exposing etiolated oat leaves to white light the prolamellar body lost its regularity but retained its membranous structure (Gunning and Jagoe, 1966). The tubular connections of the prolamellar body then constricted forming two-dimensional, double-membraned sheets which gradually extended out into the stroma of the plastid in a roughly parallel fashion. The sheets of primary lamellae appeared to be perforated in many places and this construction probably gave rise to the ideas of vesicle dispersal reported by other workers (e.g. Eriksson, et al.,1961). These perforations have disappeared by the end of the lag period of chlorophyll synthesis. From an estimate of the total area

of membrane existing at this time, it was suggested that the lamellae are produced entirely at the expense of the prolamellar body.

The single double-membraned sheets begin to duplicate at about the same time as the rate of chlorophyll synthesis becomes logarithmic. After illumination for 4 hours many of the primary lamellae have become duplicated, forming grana stacks of two thylakoids. The number of thylakoids increased with continued illumination and by 10 hours there were 5 thylakoids present in each stack (Gunning and Jagoe, 1966).

2. Chlorophyll synthesis

The immediate precursor of chlorophyll in the etiolated plant is protochlorophyll (Frank, 1946) and this has been shown, in dark grown maize seedlings, to be completely converted to chlorophyll after 1 minute of illumination in white light (Koski, 1950). The rate of conversion was dependent upon temperature and the intensity of the incident light (Smith and Benitez, 1954). On the basis of a kinetic analysis these workers suggested that the conversion of protochlorophyll was not a strictly photo-chemical intramolecular process but also involved some intermolecular interactions. However it seems unlikely that two photoactivated holochromes are required to react together since the reaction rate is greatly affected by the light intensity.

After the initial rapid formation of chlorophyll *a* from protochlorophyll, the rate of chlorophyll synthesis does not immediately increase, a lag period often being observed (*e.g.* Koski, 1950; Virgin, 1955; Gassman and Bogorad, 1967 *a*). However it is not axiomatic that a lag period is always found in

the synthesis of chlorophyll. Sisler and Klein (1963) demonstrated that the lag phase did not occur in dark grown beans until they were 6 days old. Before reaching this age chlorophyll synthesis began immediately on illumination. The addition of δ-amino levulinic acid, a porphyrin precursor, to the 6-day old leaves abolished the lag phase and allowed chlorophyll synthesis to occur immediately upon illumination. However the chlorophyll species formed did not become properly integrated into the chloroplast but was rapidly destroyed by high intensity light. The lag phase could also be abolished by infiltrating the 6-day old leaves with a sucrose solution. It appears that the lag phase represents a period during which nutrients aremobilised, thereby supplying the necessary precursors for further chlorophyll synthesis (Wolff and Price, 1960). It has been suggested (Klein and Neuman, 1966) that the products of photosynthesis were also necessary for continued chlorophyll formation. Chlorophyll synthesis in greening bean leaves was prevented by CMU, a potent inhibitor of photosynthetic electron transport, but this inhibition could be overcome by the addition of sucrose. However in greening Euglena cells treated with DCMU (Schiff, Zeldin and Rubman, 1967), 70% of the total chlorophyll was formed, along with a morphologically recognisable chloroplast and an almost completely competent photosynthetic apparatus, in the absence of carbon dioxide fixation. It would seem that under these conditions the necessary precursors and energy for light induced chloroplast development were obtained from the reserves of the cell.

Apart from nutrient supply, other factors have been reported which abolish the lag phase of chlorophyll formation.

Short impulses of white light in rapid succession bring about the accumulation of chlorophyll a in etiolated barley plants, with no lag period (Augustinussen, 1964; Akoyunoglou, et al., 1966). The phytochrome system has also been implicated in the early stages of chlorophyll formation. Etiolated bean leaves, when irradiated by a short flash of red light, in the dark, synthesised chlorophyll immediately upon subsequent illumination by white light (Withrow, Wolff and Price, 1956). Exposure to far-red light after the initial flash partially reinstated the lag period. The phytochrome system may also be involved in chlorophyll b production (Augustinussen, 1964) although this may be due simply to the phytochrome affecting chlorophyll a synthesis, which may act as a precursor to chlorophyll b (Shlyk and Losev, 1963).

From a study of the fluorescence of chlorophyll, Butler (1965) reported that during the first 1.5 hours of illumination no change in light induced fluorescence could be detected, whilst the chlorophyll appeared as a single symmetrical absorption band at about 670 mμ. After illumination for 2 hours chlorophyll b was detected and the chlorophyll a differentiated into types which absorbed at 670 and 683 mμ. These changes became more pronounced upon further illumination. The type absorbing at 683 mμ appeared at about the same time as the thylakoids began to develop (Butler and Briggs, 1966). Upon illumination the chlorophyll type pigments became dispersed as they became phytylated, which occurred simultaneously with the loss of regularity of the prolamellar body. The pigments then reaggregated as their concentration increased.

The later stages of chlorophyll synthesis in greening plants

has been investigated using $^{14}CO_2$ (Vlasenok, Fradkin and Shlyk, 1965). After allowing green barley seedlings to metabolise $^{14}CO_2$ for 10 minutes, the specific activities in the porphyrin rings of protochlorophyllide, chlorophyllide a, chlorophyll a and chlorophyll b were measured at intervals over a 16 day period. A peak in the specific activity of protochlorophyllide occured after 12 hours, but this then declined rapidly. The highest activity for chlorophyllide a was reached a few hours after protochlorophyllide and also rapidly declined, whilst for chlorophyll a the peak in activity was seen after 24 hours. It was suggested that the later stages of chlorophyll synthesis proceeds via protochlorophyllide ⟶ chlorophyllide a ⟶ chlorophyll a. It has been reported (Shlyk, et al., 1963) that, in greening barley leaves, chlorophyll b is derived from chlorophyll a.

3. Nucleic acids and protein synthesis

De Deken-Grenson (1954) demonstrated that plastids from etiolated and green chicory leaves possessed fundamentally different structures when viewed with an electron microscope. However when exposed to light, the leucoplasts developed into normal chloroplasts and this transformation was accompanied by a net synthesis of chloroplastic proteins. It has been subsequently shown (Bamji and Jagendorf, 1966; Smillie, 1963) that mature chloroplasts are able to incorporate amino acids into proteins provided that ATP, GTP and magnesium ions are supplied. In the presence of light, the externally supplied ATP can be omitted, since the chloroplast light reactions provide the energy source for amino acid assimilation (Eisenstadt and Brawerman, 1962). The enzymes associated with protein synthesis

are sensitive to ribonuclease, puromycin and chloramphenicol (Smillie, 1963). Since amino acid incorporation was somewhat stimulated by ribonucleoside triphosphates and inhibited by puromycin (Eisenstadt and Brawerman, 1962) this may be evidence of DNA-dependent RNA-polymerase activity.

Chlorophyll synthesis in greening Euglena cells was inhibited by hydroxyurea (Buetow and Mego, 1967). This inhibitor also depressed the levels of DNA, RNA and protein, the level of inhibition of the synthesis of these components decreasing in the order listed. A suggestion that the synthesis of specific ribonucleic acid molecules occurred during the development of chloroplasts in Euglena (Brawerman and Chargaff, 1959) was confirmed and extended by Gnanaam and Kahn (1967 a; 1967 b; 1967 c). During the initial stages of development, the formation of a specific messenger RNA was noted, the synthesis of which was sensitive to streptomycin and 5-fluoro-uracil. This was in accordance with the hypothesis that the development of chloroplasts on exposure to light, constitutes an inducible system analogous to the initiation of specific enzymes by specific inducers and the extensive changes in cellular metabolism upon 'phage infection (Spiegelman, Hall and Storck, 1961). The formation of specific cytoplasmic ribosomes associated with the chloroplast during the early stages of development have not been demonstrated (Gnanaam and Kahn, 1967 c). However the chloroplast does contain ribosomes which differ in their sedimentation characteristics from those of the cytoplasm (Gnanaam and Kahn, 1967 c; Eisenstadt and Brawerman, 1962; Svetailo, Philippovich and Sissakian, 1967). Further experiments using ^{32}P confirmed that during chloroplast

development no new ribosomes formed, even though a new species of messenger RNA is synthesised (Gnanaam and Kahn, 1967 c). The site of amino acid incorporation by chloroplasts appears to be the lamellar membranes and thus it is possible that the proteins synthesised have a structural function (Gnanaam and Kahn, 1967 b).

Using antibiotics which are known to inhibit protein synthesis it has been shown that the formation of chlorophyll in the developing chloroplast is entirely dependent upon protein synthesis (Kirk, 1968; Margulies, 1962). Actidione prevents chlorophyll synthesis in the greening cells of Euglena (Kirk, 1968), whilst chloramphenicol achieves a high level of inhibition in greening bean leaves (Margulies, 1962). However carotenoid and anthocyanin synthesis are not so closely linked to protein synthesis since these pigments accumulate even when chloroplast development is prevented (Kirk, 1968).

4. Development of photosynthesis

(a) Utilisation of light energy

The current theories of the mechanism of photosynthetic absorption of light energy are discussed in Section 5. Proplastids derived from etiolated bean leaves possess no Hill reaction activity, with ferricyanide as electron acceptor (Anderson and Boardman, 1964). Unequivocal evidence of Hill reaction activity was only obtained after the plants had been illuminated for more than 6 hours. The rate of reduction then increased to the maximum value after 10 hours illumination. It is interesting to note that the highest rates of oxygen evolution in greening Euglena cells also occurred after about 10 hours exposure to light (Stern, Epstein and Schiff, 1964).

In plastids which had been illuminated for only 6 hours, the Hill reaction was insensitive to CMU in a concentration which would normally inhibit electron transport (Anderson and Boardman, 1964). Complete inhibition of the Hill reaction was achieved only after the plants had been illuminated for 12-14 hours. It was suggested that the ferricyanide reduction was not linked stoichiometrically to the oxygen evolving system in the immature chloroplast and that the electrons required for the reduction were not derived from water but from some other endogenous donor.

The natural hydrogen acceptor of the chloroplast light reactions is NADP (Vishniac and Ochoa, 1951; Davenport, 1963 a; Katoh and San Pietro, 1966; 1967). However reduction of NADP was not observed until the plants had been illuminated for 8 hours, and the rate increased to half that of mature chloroplasts only after 16 hours (Anderson and Boardman, 1964). NADP reduction had thus just commenced at the time when the rate of ferricyanide reduction was at its maximum. It was suggested that the rate of development of some component of the NADP reducing system restricted the flow of electrons to NADP and that this component was not involved in the reduction of ferricyanide.

Pigment development and electron transport in greening bean leaves has been studied using fluorescence measurements (Butler, 1965). Theoretical considerations suggest that light absorbed by Light System II pigments will bring about an increased fluorescence yield if electron transport occurs in this system. The converse is true of Light System I, and thus it is possible to detect when the two systems become operative

in the greening leaf. During the first 1.5 hours of illumination there were no changes in fluorescence yield, indicating that the Systems I and II were not functional. Between 1.5 to 2 hours the chlorophyll differentiated into the forms characteristic of Light Systems I and II and concomitant changes in fluorescence yield indicated that electron transport commenced as soon as the pigments had developed. It was subsequently suggested (Butler and Briggs, 1966) that the two Light Systems became functional as soon as the chlorophyll molecules begin to aggregate in the chloroplast lamellae.

The development of distinctive double membranes between the thylakoids appeared to be the key stage in the structural integration of the Light Systems (Rhodes and Yemm, 1966). From measurements of carbon dioxide exchanges in the dark in greening barley it was demonstrated that photosynthesis began after about 3 hours of illumination, and this was correlated with the formation of thylakoids in the chloroplasts. A minimum of structural development of the plastid appeared to be necessary before photophosphorylation was found in greening bean leaves (Gyldenholm and Whatley, 1968). Also in Chlorella thylakoid formation was required before phenozine methosulphate and flavin mononucleotide catalysed photophosphorylation, Hill reaction and carbon dioxide fixation were observed (Oh-hama, Shihira-Ishikawa and Hase, 1965). Indeed the early structural development of the chloroplast may occur without the photosynthetically supplied precursors, since illuminated Euglena chloroplasts differentiate normally in the presence of CMU (Schiff, Zeldin and Rubman, 1967). However it was suggested that exposure to light resulted in the activation of synthetic

systems external to the developing chloroplast and thereby providing the necessary precursors.

The appearance of phenazine methosulphate catalysed cyclic phosphorylation, ferricyanide and DCIP reduction activity in greening Linum seedlings was observed after 4 - 5 hours illumination (Dodge and Whittingham, 1966). The rate of the Hill reaction reached a peak after 12 hours but that of photophosphorylation became maximal only after 20 hours illumination. Changes in the plastoquinone content of the greening plants were estimated and it was concluded that this component of the electron transport system did not limit the development of photochemical activity. The amount of plastoquinone in the chloroplast had reached 61% of its final level after 4 hours illumination whereas the concentration of chlorophyll was only 15%. However in Chlorella the formation of plastoquinone closely paralleled that of chlorophyll (Oku, et al., 1968).

The content of ferredoxin-NADP-reductase, cytochrome 552 and cytochrome 561 in etiolated Euglena cells increased on exposure to light (Smillie, et al., 1967). Since this was prevented by chloramphenicol, it was suggested that the chloroplast ribosomes were responsible for the synthesis of these proteins. Similar inhibition was achieved by cycloheximide, but this was thought to be the indirect result of the antibiotic blocking the formation of certain structural components which were essential to the synthesis and integration of the electron transport enzymes. The activity of ferredoxin-NADP-reductase in dark grown bean leaves was also stimulated by light (Keister, Jagendorf and San Pietro, 1962). A brief illumination period (1.5 hour) brought about a 3-fold increase of activity during a

subsequent 48 hour dark period but a 50-fold increase was observed after 2 cycles of a 10 hour light - 14 hour dark regime. The increase in activity of this enzyme was apparently controlled by phytochrome and was correlated to the formation of the thylakoid membranes. Ferredoxin could not be detected in etiolated leaves, but after a brief illumination its content increased, in the dark, to equal that in plants which had been subjected to a 10 hour light - 14 hour dark regime (Keister, Jagendorf and San Pietro, 1962). The rates of increase in ferredoxin content and of ferredoxin-NADP-reductase with the illumination period were not however identical. The latter enzyme accumulated at the same rate as chlorophyll, whilst the synthesis of the former protein demonstrated no lag period but commenced immediately upon illumination (Melandri, Baccarini and Forti, 1969).

(b) The fixation of carbon dioxide

Irving (1910) suggested that etiolated leaves were not able to photosynthesise until they were fully green. The evolution of oxygen however, was demonstrated very soon after chlorophyll synthesis had reached its exponential phase (Inman, 1935), confirming the report of Briggs (1920). After illumination for 10 minutes etiolated barley leaves did not evolve oxygen, even though chlorophyll a was present (Smith, 1954). However, the evolution of oxygen was noted after illumination for 30 minutes and increased rapidly even though in many instances oxygen evolution occurred before the plant contained any chlorophyll b.

The first critical work on carbon assimilation in greening plants was that reported by Tolbert and Gailey (1957). Dark-grown wheat plants were exposed to light and the incorporation of $^{14}CO_2$ was determined. During the first 4-5 hours there

was very little fixation of $^{14}CO_2$, even though the rapid phase of chlorophyll synthesis had begun some 2 hours before. However, after 5 hours the rate of $^{14}CO_2$ assimilation increased until at 32 hours it compared favourably with that of the normal green plant. During the first 4 hours malic, aspartic and glutamic acids became labelled with ^{14}C (Tolbert and Gailey, 1957). However, at 4 hours some radioactivity had appeared in phosphoglyceric acid, which with alanine became heavily labelled after 5 hours. Also at this time the hexose phosphates and sucrose were becoming labelled at an increasing rate. Only trace amounts of ^{14}C were found in ribulose-1,5-diphosphate and sedoheptulose-7-phosphate at 5-6 hours, but by 25 hours the latter had accumulated significant amounts of ^{14}C, whilst the ribulose-1,5-diphosphate only contained traces. It was suggested that the availability of the ribulose-1,5-diphosphate could be a factor limiting carbon fixation during greening. In Euglena carbon assimilation was first detected after the dark-grown cells had been illuminated for 6 hours (Stern, Schiff and Epstein, 1964). After this lag period, seen also in pigment formation, lamellae formation and oxygen evolution, the rate of carbon fixation increased linearly with time.

The pathway whereby carbon is incorporated into carbohydrates and amino acids was elucidated by Calvin and his associates (e.g. Calvin, 1955). However the development of the enzymes involved in this pathway in the greening cell has been somewhat neglected. In greening Euglena cells the rate of chlorophyll formation was correlated with the appearance of NADP-linked glyceraldehyde-3-phosphate dehydrogenase (Brawerman and Konisberg, 1960). However the total protein content of the

cell did not change significantly. The activity of ribulose-1,5-diphosphate carboxylase increased by 5-6 fold in greening barley leaves after a period of 48 hours illumination (Hall, et al., 1959; Huffaker, et al., 1964). This was also found in greening bean leaves (Margulies, 1964). Although the dark grown plants possess some ribulose-1,5-diphosphate carboxylase (Margulies, 1964), its activity was markedly stimulated by light.

In greening maize, ribulose-1,5-diphosphate carboxylase activity increased after illumination for 3 minutes, reached a maximum after 3-6 hours and then began to decline (Chen, McMahon and Bogorad, 1967). The activity of ribose-5-phosphate isomerase did not change significantly until between 12-24 hours of illumination, whilst ribulose-5-phosphate kinase activity increased after a lag of about 6 hours. It was suggested that this course of events resulted from the sequential enzyme induction, including an initial light stimulated synthesis (e.g. ribulose-1,5-diphosphate carboxylase) and subsequent induction or derepression of the synthesis of other enzymes (e.g. ribose-5-phosphate isomerase).

The total protein content of the etiolated leaves of maize (Chen, McMahon and Bogorad, 1967), beans (Filner and Klein, 1968) and peas (Graham, Grieve and Smillie, 1968) increased on exposure to light; although in Euglena cells this did not occur (Brawerman and Konisberg, 1960). Red light induced the net synthesis of Fraction I protein and increased the activities of ribulose-1,5-diphosphate carboxylase, glyceraldehyde-3-phosphate dehydrogenase and fructose-1,6-diphosphatase in etiolated pea leaves (Graham, Grieve and Smillie, 1968). That these changes could be reversed by far-red light implicates

phytochrome, rather than protochlorophyllide as the primary photoregulator of enzyme synthesis (Graham, Grieve and Smillie, 1968). The total protein content of etiolated bean leaves increased in the dark following brief illumination (Filner and Klein, 1968). The increase in protein content and ribulose-1,5-diphosphate carboxylase activity, were prevented by cycloheximide but not by the DNA-synthesis inhibitor, 5-fluoro-deoxyuridine. Increased enzyme activity was also reversed by exposure to far-red light, and in common with cycloheximide, the inhibition could be effected at any time during 48 hours after the initial illumination. Since this period was quite extensive, it was concluded that the primary control of protein synthesis could not involve phytochrome.

The first product of carbon fixation by ribulose-1,5-diphosphate carboxylase, phosphoglyceric acid, is reduced to phosphoglyceraldehyde by NADP-linked glyceraldehyde-3-phosphate dehydrogenase. Etiolated pea seedlings do not possess this enzyme but upon illumination there was a rapid development of activity (Hageman and Arnon, 1955). This was similar to that reported for greening bean leaves (Margulies, 1964) and for Euglena (Brawerman and Konisberg, 1960). The activity of fructose-1,6-diphosphatase in Euglena was also stimulated by light (Smillie, 1963). Although this organism has two fructose-1,6-diphosphatase enzymes, cytoplasmic and chloroplastic bound, it was the latter which increased 4-fold when the cells were transferred to conditions which were suitable for chloroplast development.

The Calvin cycle is completed by a series of transformations of the sugar phosphates which lead to the reformation of

ribulose-1,5-diphosphate. In Euglena the conversion of ribose-5-phosphate to ribulose-5-phosphate is catalysed by phosphoriboisomerase, which is found both in the cytoplasm and in the chloroplasts (Smillie, 1963). Etiolated barley seedlings also posses a high phosphoriboisomerase activity (Huffaker, et al., 1964), which is doubled after 24 hours of illumination. However, upon illumination the activity of phosphoribulokinase increased at a rapid rate after an initial lag period, the duration of which was inversely proportional to the light intensity (Huffaker, et al., 1966). Although the activity of the important enzymes of the Calvin cycle appears to be enhanced by light, the underlying mechanisms controlling such increases have yet to be elucidated.

5. The pathway of electron transport in mature chloroplasts.

Experiments by Emerson and Lewis (1942; 1943) demonstrated that the photosynthetic efficiency of Chlorella or Chroococcus cells was markedly reduced when they were illuminated by monochromatic light of wavelength 685 mμ. Investigation of the 'red-drop' of efficiency (Emerson, Chalmers and Cedarstrand, 1957) showed that the photosynthetic efficiency could be restored to above normal values if light of 420 mμ or 655 mμ wavelength was added to the red light. The light of longer wavelengths was absorbed predominately by chlorophyll a, but full photosynthetic efficiency was dependent upon the simultaneous absorption of light of a shorter wavelength by the accessory pigments.

The present hypothesis based on these findings was first put forward by Hill and Bendall (1960) and Whatley, Tagawa and Arnon (1963), and is shown in Fig. 1. (modified after Vernon and Avron, 1965). The two light reactions are thought to

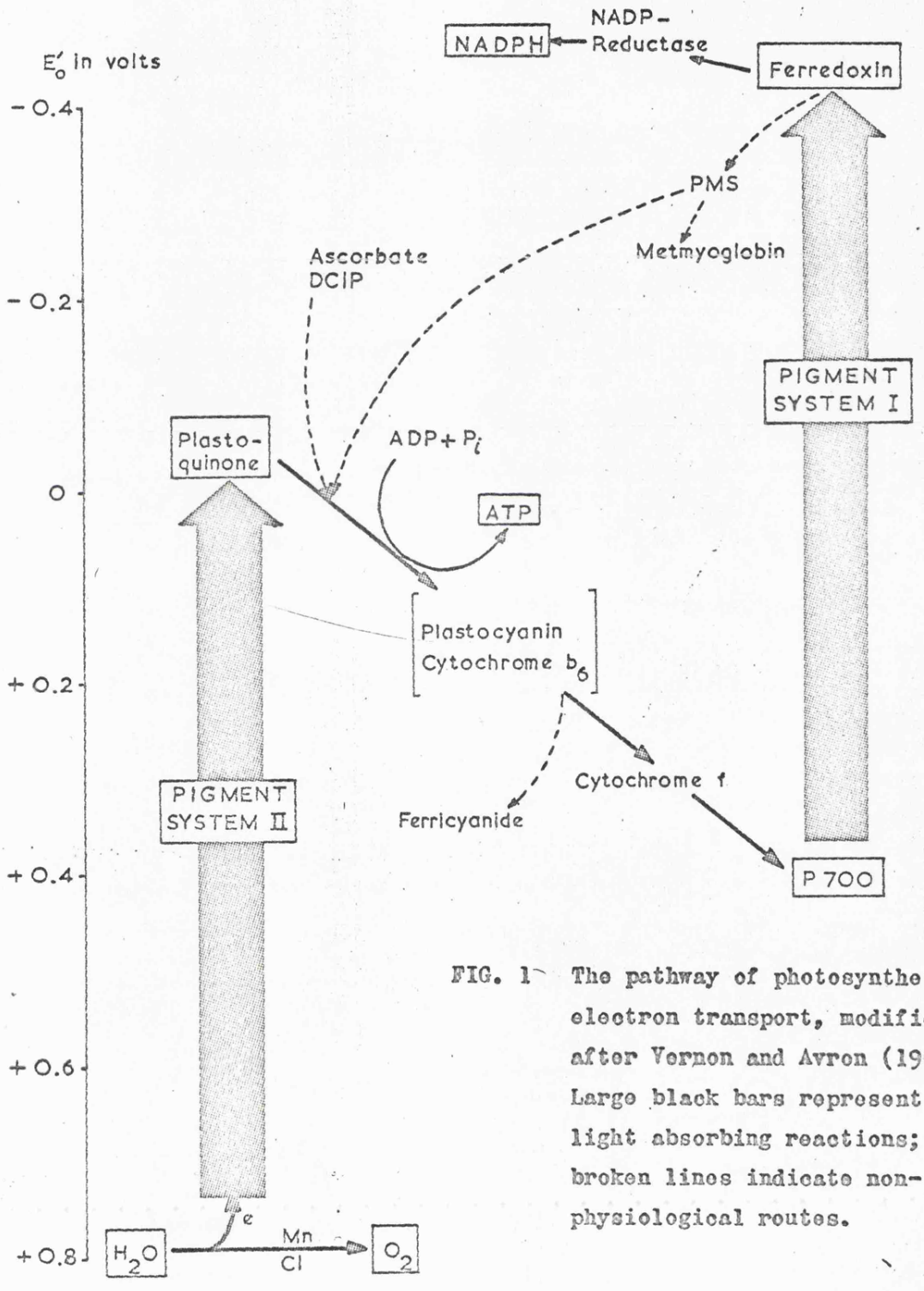


FIG. 1 The pathway of photosynthetic electron transport, modified after Vernon and Avron (1965). Large black bars represent the light absorbing reactions; broken lines indicate non-physiological routes.

collaborate directing the flow of electrons against the thermochemical energy gradient. The overall net change in redox potential for the interaction of the two systems is about 1.2 v, whilst for each of the systems the change is 0.8 v, suggesting that there is an overlap of the functional regions of the systems (San Pietro, 1967).

Light energy absorbed by the chlorophyll of Light System II is utilised to transfer electrons from water, the primary oxidant, to plastoquinone, the primary reductant of Light System II (Arnon and Horton, 1963; Friend and Redfearn, 1963), with the concomitant evolution of molecular oxygen (Hill, 1939). The second reaction, Light System I, transports electrons from the primary oxidant, P700 (Kok, 1961), to ferredoxin, the primary reductant of Light System I, and then *via* ferredox-NADP-reductase to reduce NADP (Vishniac and Ochoa, 1951; San Pietro and Lang, 1958; Shin and Arnon, 1965). P700 is then reduced by the electrons transferred spontaneously from plastoquinone *via* cytochrome *f* (Hill and Scarisbrick, 1951; Davenport and Hill, 1952), cytochrome b_6 (Olsen and Smillie, 1963) and the copper protein plastocyanin (Katoh, 1960; Katoh *et al.*, 1961), with the released energy being conserved as ATP. Oxygen evolution and phosphorylation may be both maintained by artificial electron acceptors *e.g.* ferricyanide and metmyoglobin. Ferricyanide is thought to be reduced by the Light System II - cytochrome complex (Gromet-Elhanan and Avron, 1963) and metmyoglobin by Light Systems I and II (Davenport, 1963 *a*). Light System I, in the absence of Light System II activity can reduce NADP and some bipyridyl salts, *e.g.* benzyl viologen, when the electrons are donated to the cytochrome chain by the ascorbate - DCIP couple (Vernon and Zaugg, 1960).

Phosphorylation in chloroplasts is associated with one of two pathways of electron flow. The 'non-cyclic' phosphorylation is coupled to NADP reduction, whilst the 'cyclic' phosphorylation is not (Arnon, 1967). Cyclic phosphorylation is mediated by Light System I and requires the presence of a cofactor e.g. phenazine methosulphate (Jagendorf and Avron, 1958), vitamin K_3 (Arnon, Whatley and Allen, 1955) or high concentrations of ferredoxin (Tagawa, Tsujimoto and Arnon, 1963). The change from non-cyclic to cyclic phosphorylation is thought to be controlled by the amount of available reduced NADP (Tagawa, Tsujimoto and Arnon, 1963).

The scheme shown in Fig.1 has been accepted for nearly 10 years and provides for the time being, the best explanation of the sequence of photochemical events in the chloroplast. However other hypotheses have been put forward, among them the suggestion that cyclic and non-cyclic electron transport are in parallel, with one light reaction step catalysing the transfer of electrons to ferredoxin from water (Kok, 1963). The results obtained by the differential uncoupling of phosphorylation by desaspidin (Gromet-Elhanan and Arnon, 1965) have led to the elaboration of this hypothesis (Arnon, 1967). However, the precise action of desaspidin has been the subject of some controversy (Gromet-Elhanan and Avron, 1966; Hind, 1966 a; Hind, 1966 b; Tsujimoto, McSwain and Arnon, 1966). Some support for the ideas of Arnon (1967) however has come from a recent study of the delayed emission of light from photosynthetically active chloroplasts (Arnold and Azzi, 1968).

As may be seen from the aforegoing review, the developing chloroplast is able to perform the reactions involved in

photosynthesis by the end of the lag period of chlorophyll synthesis, although the rates of these reactions are not maximal. By markedly reducing the rate of chloropyll synthesis some elucidation of the complex physiological relationships between chlorophyll synthesis and development of photosynthetic competence might be achieved. Examples of compounds known to retard or completely abolish chlorophyll synthesis are chloramphenicol (Margulies, 1962), actidione (Kirk and Allen, 1965), puromycin and actinomycin D (Gassman and Bogorad, 1967 b), terramycin (Netien and Lacharme, 1955), etamycin (Cercos, 1964), oleandomycin and lincomycin (Oh-hama, Shihira-Ishikawa and Hase, 1965) and light of low intensities (Huffaker, et al., 1966). The effects of some of these agents on chlorophyll synthesis and the development of photosynthetic electron transport and carbon assimilation in greening pea leaves have been investigated.

MATERIALS AND METHODS

The reagents used were of the highest purity available, solutions of which were prepared in distilled water obtained from an 'all glass' still (Quickfit, Ltd.) and were stored at 5°C. NADP, ADP and hexokinase were obtained from Sigma Chemicals Ltd., benzyl viologen and DCIP from British Drug Houses, Ltd., chloramphenicol from Parke-Davis, Ltd. and terramycin from Pfizer, Ltd. The following were gifts: etamycin (Bristol Laboratories, New York), oleandomycin (Pfizer, Ltd.), erythromycin (Abbot Laboratories, Ltd.), lincomycin (Upjohn, Ltd.) and CMU (Du Pont de Nemours, and Co.). Metmyoglobin was prepared by dissolving 0.5 g myoglobin from whale skeletal muscle (Seravac Laboratories, Ltd.) in the minimum volume of 0.3M TRIS-chloride buffer, pH 8.0. The solution was filtered through a celite pad and the volume adjusted to 15ml with additional buffer. The concentration of the metmyoglobin solution was equivalent to 2 μM/ml.

Cress seeds (Lepidium sativum) were sown on damp peat contained in waxed cartons. These were grown for 7 days at 20°C, either in light supplied by warm-white fluorescent tubes at an intensity of about 3000 lux, or in continuous darkness. Pea seeds (Pisum sativum, var. Meteor) were sown on damp expanded vermiculite contained in standard seed trays, and grown for 8 days at 20°C in continuous darkness.

Preliminary experiments on the inhibition of chlorophyll synthesis by various antibiotics utilised cress seedlings. These were sprayed with solutions of the inhibitors, under dim green light, and then allowed to dry before being exposed to light. Treatment of the etiolated pea plants was effected by supporting apical 3 cm cuttings in an aqueous solution of the

antibiotic [later experiments also included the medium of White (1943) Table 1] which had been solidified with 1% agar, for 18 hours before illumination. Fig. 2 shows details of the method and also a photograph of control and chloramphenicol treated cuttings after 48 hours illumination. In the experiments involving low light intensities, the glass dishes were coated on the outside with black paint, and lids covered with layers of neutral density filter (Kodak, Ltd.). The filters possessed a constant optical density value between 400 and 750 mμ. The ingress of light other than by way of the filter was prevented by masking the edges of the lids with black tape and paint.

All optical density measurements were performed on a Unicam SP 500 spectrophotometer.

1. Extraction and estimation of chlorophyll

The total chlorophyll content of the leaf and of suspensions of isolated chloroplasts were estimated using the method of Mackinney (1941) as modified by Arnon (1949). Leaf material of about 100 mg fresh weight was ground with a little sand in 80% (V/V) aqueous acetone. The homogenate was centrifuged at 1000 g for 5 minutes and the volume of the supernatant brought to 10 ml with additional acetone. An aliquot of the extract was used to determine the optical density at 645 mμ and 663 mμ. These values were converted by means of the Arnon-Mackinney equations into mg total chlorophyll per g fresh weight of leaf material.

The chlorophyll content of chloroplast suspensions was estimated as described above, excepting that the acetone extract was obtained by shaking 0.5 ml of the preparation in 5.0 ml of 80% aqueous acetone. The amount of chlorophyll was expressed

STOCK SOLN.	VOLUME OF STOCK SOLN./ 1 ℓ FINAL SOLUTION	N U T R I E N T (anhydrous)	STOCK SOLN. CONCN. g/ℓ .	FINAL SOLN. CONCN. mg/ℓ .
		$Ca(NO_3)_2$ †	2.0	200
		Na_2SO_4	2.0	200
1	100 ml	KNO_3	0.8	80
		KCℓ	0.65	65
		$Na\ H_2PO_4$	0.165	16.5
2	10 ml	$MgSO_4$	36.0	360
		$MnSO_4$	0.45	4.5
		$ZnSO_4$	0.15	1.5
3	10 ml	H_3BO_3	0.15	1.5
		KI	0.075	0.75
		$CuSO_4$	0.002	0.02
		Na_2MoO_4	0.021	0.21
4	10 ml	Fe^{+++} *	0.25	2.5
		Glycine	0.3	3.0
		Thiamine	0.01	0.1
5	10 ml	Pyridoxine	0.01	0.1
		Nicotinic Acid	0.05	0.5
		Sucrose	10.0	100

TABLE I The medium according to White (1943).

† adjust weight for water of crystallisation.

* iron chelated with EDTA.

The required volume of each stock solution was added to a large volume of glass distilled water and made up to 1ℓ . The solution was autoclaved at 15 lbs pressure for 15 minutes, the pH after sterilisation being 5.5 and osmotic pressure 1.8 atmospheres.

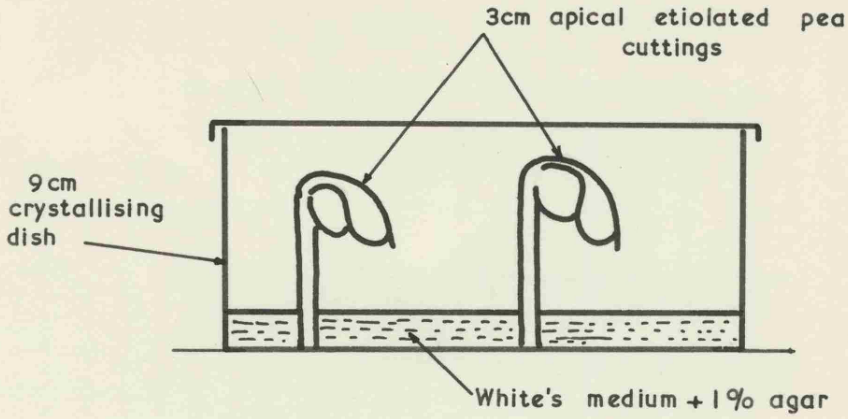


FIG. 2 Illustration of the method of treatment of pea cuttings. Above. Cross-sectional diagram of crystallising dish containing etiolated apical cuttings supported in agar solidified medium. Below. After 48 hours illumination, control on the left, chloramphenicol treated (1.5 mg/ml) on the right.

as mg total chlorophyll per ml chloroplast preparation.

2. Preparation of ferredoxin

Ferredoxin was isolated from parsley (Petroselinum sativum) leaves by a method similar to that described by Tagawa and Arnon (1962). The petioles were removed from washed parsley leaves and the laminae frozen. These were then ground to a powder in a chilled mincing machine and mixed with 0.05 M TRIS-chloride buffer, pH 8.0. The volume of buffer added was in the ratio of 800 ml to each kilogram of frozen leaves.

The mixture was allowed to thaw completely and then filtered through four layers of butter muslin. To the filtrate was added 50% (V/V) cold acetone (-20°C) with stirring, and left at 5°C for 15 minutes. After centrifugation at 1000 g for 15 minutes at 0°C, the acetone concentration of the supernatant was increased to 75% (V/V). The resulting precipitate was recovered by filtration through a pad of celite supported on filter paper, and then washed with a little cold acetone. The celite was powdered after the acetone had completely evaporated and was resuspended in a minimum volume of 0.05 M TRIS-chloride buffer, pH 7.3. The celite was removed by filtration to produce a clear brown liquid containing the ferredoxin.

The protein solution was dialysed overnight in cellophane tubing, against 0.005 M TRIS-chloride buffer, pH 7.3 at 5°C. The precipitate which formed during dialysis was removed by centrifugation at 6000 g for 20 minutes at 0°C, and the supernatant fractionated on Whatman DE 11 cellulose powder. A column (1 x 6 cm) was prepared from a slurry of the cellulose powder in distilled water. It was then washed several times with water and then equilibriated with 0.005 M TRIS-chloride

buffer, pH 7.3. The dialysed preparation was poured onto the column and first eluted with the equilibriating buffer until the flavone had been removed. Elution of the column with 0.2 M sodium chloride in 0.005 M TRIS-chloride buffer, pH 7.3, yielded the fraction containing flavoprotein and plastocyanin. The ferredoxin was recovered by elution with 0.8 M sodium chloride in 0.005 M TRIS-chloride buffer, pH 7.3, and stored at -20°C until required. For the purposes of this investigation it was not further purified.

3. Preparation of fragmented chloroplasts

The chloroplasts were isolated by a method similar to that described by Hill and Walker (1959). Leaves, taken from the pea cuttings and placed in a chilled mortar, were ground with a small quantity of washed sand in about 20 ml of 'grinding medium' at 0°C. This contained 0.1 M TRIS, 0.35 M sodium chloride and 0.03 M magnesium chloride at pH 7.7. The leaves were macerated until a thick homogenate was formed, which was filtered through 4 layers of butter muslin. The filtrate was centrifuged for 1 minute at 200 g at 5°C, precipitating the sand and larger debris. The supernatant was retained and centrifuged at 1500 g for 15 minutes to sediment the chloroplasts. After discarding the supernatant, the pellet was resuspended in 5 ml of 'breaking medium' at 0°C. This solution was a tenth dilution of the 'grinding medium' and was thus hypotonic to the chloroplasts, causing their fragmentation (Hill and Walker, 1959: Arnon, 1949). The fragmented chloroplasts were thoroughly resuspended in a Potter-Elvehjem homogeniser, and then filtered through glass wool into a tube immersed in an ice bath.

4. Estimation of photosynthetic electron transport

Unless otherwise stated, the reactions were carried out in silica spectrophotometer cuvettes of 1 cm light path and 3 ml capacity; the optical densities being recorded before and after illumination with saturating white light provided by the apparatus shown in Fig.3. All the reactions were performed with isolated, fragmented chloroplasts, the amount of which present in each reaction being equivalent to 10-20 µg total chlorophyll. Each reaction was done in duplicate for each of three chloroplast preparations from each treatment.

(a) Water as electron donor

i) Ferricyanide. Reduction of ferricyanide to ferrocyanide was determined by the decrease in optical density at 420 mµ (Jagendorf and Margulies, 1960). The reaction mixture consisted of 90 µ M TRIS-chloride buffer, pH 7.7, 10 µ M sodium chloride, 2 µ M potassium ferricyanide and water to give a total volume of 3.0 ml. The reaction blank was similar, but did not include the ferricyanide. The amount of ferricyanide reduced was calculated from a standard curve of µ M ferricyanide plotted against optical density at 420 m µ (Fig.4).

ii) Metmyoglobin. The reduction of metmyoglobin was estimated by an increase in extinction at 582 m µ (Davenport, 1960) in the presence of catalytic amounts of phenozine methosulphate (Davenport, 1963 b). The reaction mixture consisted of 90 µ M TRIS-chloride buffer, pH 7.7, 0.4 µ M metmyoglobin, 0.003 µ M phenozine methosulphate and water to 3.0 ml total volume. The reaction blank was similar, but did not include the phenozine methosulphate. Since a change of 0.1 optical density was equivalent to 0.0255 µ M of metmyoglobin at

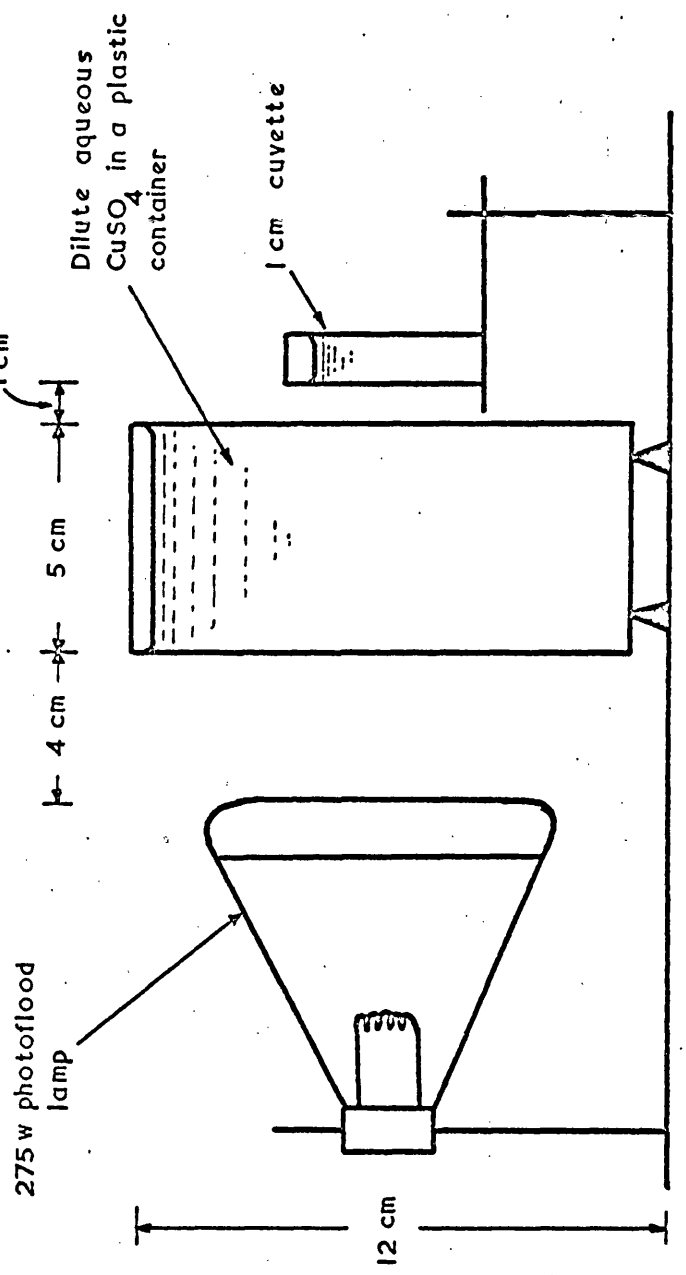


FIG. 3 Cross-sectional diagram of an illumination apparatus, for use in the estimation of photosynthetic electron transport in isolated chloroplasts contained within a spectrophotometer cuvette.

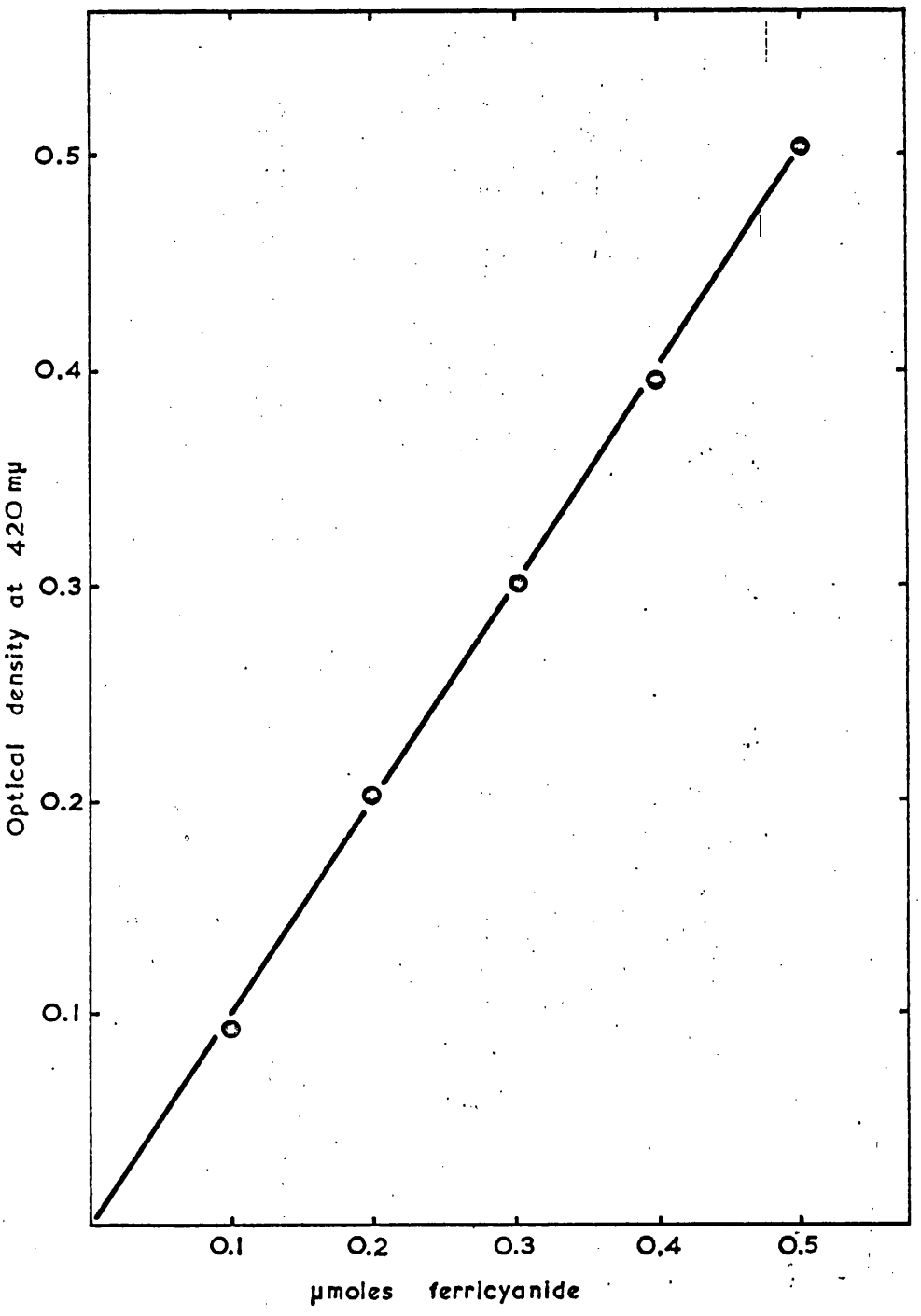


FIG. 4 Calibration curve for the spectrophotometric estimation of the ferricyanide radical.

pH 7.7 (Davenport, 1960), the amount of metmyoglobin reduced could be calculated.

iii) NADP. The photosynthetic reduction of NADP was estimated by an increase in optical density at 340 mμ in the presence of ferredoxin (San Pietro and Lang, 1958). Since a change of 0.1 optical density was equivalent to 0.048 μM NADP (Colowick, Kaplan and Ciotti, 1951), the amount of NADP reduced could be calculated. The reaction mixture consisted of 90 μM TRIS-chloride buffer, pH 7.7, 0.5 μM NADP, 0.1 ml of a ferredoxin preparation (approximately 1.8 mg protein) and water to 3.0 ml total volume. The reaction blank did not include the ferredoxin.

(b) Ascorbate - DCIP as electron donor

These estimations utilise Light System II only, since Light System I was inhibited by CMU (Wessels and van der Veen, 1956), and the electrons were donated by the ascorbate - DCIP couple (Vernon and Zaugg, 1960).

i) NADP. The reduction of NADP by ascorbate - DCIP was estimated as described above, but the reaction mixture also included 33 μM sodium ascorbate, pH 7.0, 10 μM DCIP and 0.1 μM CMU.

ii) Oxygen. Chloroplasts have been shown to reduce oxygen to hydrogen peroxide in the presence of a suitable mediator (Mehler, 1951 a; 1951 b), e.g. benzyl viologen. The oxygen uptake of a chloroplast suspension performing this reaction was measured manometrically in standard 15 ml single side-arm Warburg flasks. The reagents were kept at a constant temperature of 20°C in a water bath which was illuminated from below by fourteen 40 W tungsten lamps giving an intensity 11.8

kilolux at the flask level. The reaction mixture consisted of 90 µM TRIS-chloride buffer, pH 7.7, 33 µM sodium ascorbate, pH 7.0, 10 µM DCIP, 0.3 µM CMU, 0.01 µM benzyl viologen and water to 3.0 ml total volume. The flasks were immersed in the water bath and after thermal equilibriation, readings were taken at 5 minute intervals for 10 minutes in the dark. The flasks were then illuminated and readings taken at 10 minute intervals for 50 minutes. The changes in manometer readings with time were converted into µM oxygen reduced by the use of standard formulae (Umbreit, Burris and Stauffer, 1964).

(b) Cyclic photophosphorylation

Cyclic photophosphorylation with phenozine methosulphate as the cofactor (Jagendorf and Avron, 1958), was measured by the disappearance of inorganic phosphate caused by its esterification with ADP to form ATP. The ADP is regenerated by the utilisation of the newly formed ATP in the phosphorylation of glucose by hexokinase. The reaction mixture contained 90 µM TRIS-chloride buffer, pH 7.7, 10 µM sodium chloride, 20 µM magnesium chloride, 7.5 µM disodium hydrogen phosphate, 0.075 µM phenazine methosulphate, 7.5 µM ADP, 30 µM glucose, 1,400 units of hexokinase and water to 2.7 ml total volume. An unilluminated control was also prepared. After illumination for 5 minutes at 10°C the reaction was stopped by the addition of 0.3 ml of 11.5% trichloroacetic acid. The chloroplast reaction mixture was centrifuged and 0.3 ml of the supernatant added to 2.7 ml water, into which was pipetted 2.0 ml of a freshly prepared 'phosphate reagent' (see below) and left for 15 minutes. The optical density of the resulting blue colour was measured at 720 mµ and this was converted into µM phosphate by means of a calibration curve (Fig.5). The amount of phosphate esterified

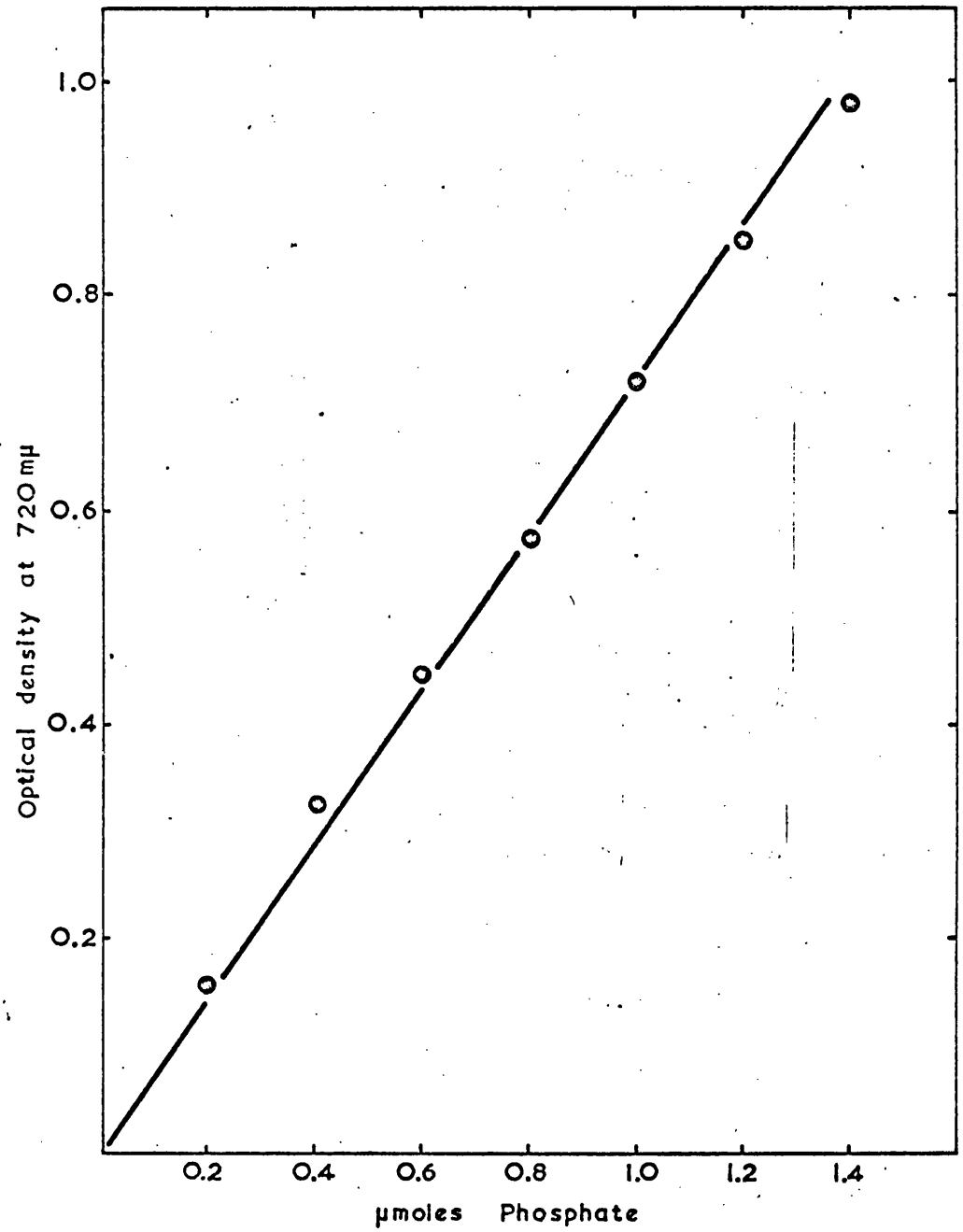


FIG. 5 Calibration curve for the spectrophotometric estimation of the phosphate radical.

as ATP was obtained by subtracting the phosphate concentration of the illuminated sample from that of the dark control. The 'phosphate reagent' consisted of a solution of hydrated ferrous sulphate (5% w/v) and ammonium molybdate (1% w/v) in 1N sulphuric acid, prepared as described by Taussky and Shorr (1953).

5. Measurement of carbon dioxide exchange

An infra-red gas analyser (Grubb Parsons, Ltd.) was used to measure changes in the carbon dioxide content of the air passing over plant material contained with a 5 x 1.5 x 100 mm perspex chamber. A cylinder of high purity compressed air provided the gas stream (see Fig.6), which, after passing over anhydrous calcium chloride (D_1) and through a combined pressure regulator and flow meter (F_1), was divided into two. One stream supplied the air for the reference tube of the instrument after passing through F_2, whilst the other supplied the sample tube. The sample stream, after passing through the flow meter F_4 entered the plant chamber where its carbon dioxide content was modified by 4-500 mg of leaf material. From the chamber, the air passed through a second drying tower, D_2, before entering the analyser. A facility was provided whereby the plant chamber could be bypassed via a flow meter F_3, thereby enabling the infra-red absorbtion in both the reference and sample tubes of the instrument to be equalised. The plant chamber was illuminated by a high intensity lamp, the evolved heat being absorbed by a screen of running water between the lamp and the plant chamber. The flow rate of the air stream in both reference and sample tubes was adjusted to 500 ml per minute. The results were expressed as μM carbon dioxide exchanged per g fresh weight of leaf material per hour.

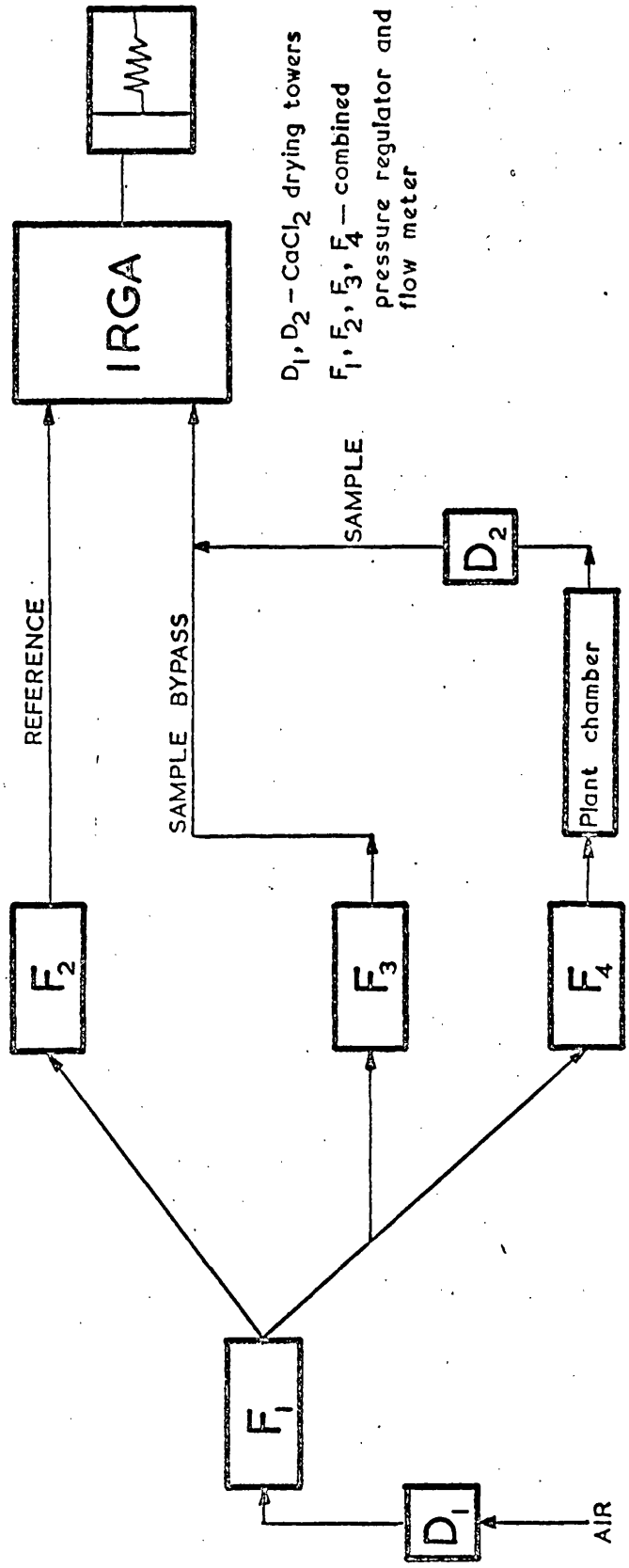


FIG.6 Diagram of the air stream supplying an infra-red gas analyser (IRGA), recording the carbon dioxide exchanges of plant material contained within the plant chamber.

RESULTS

1. Experiments on Chlorophyll Synthesis

(a) Control plants

It is well known that the rate of chlorophyll synthesis in greening leaves is markedly affected by the nutrient status of the plant (Kirk and Tilney-Bassett, 1967). Etiolated apical cuttings of peas were supplied with either 1% (w/v) sucrose, White's medium consisting of organic and inorganic nutrients, or water. The treated explants were kept in the dark for 18 hours before estimating the chlorophyll content at intervals during a subsequent 50 hours of illumination. The results shown in Fig.7 indicated that the rate of greening of the leaves was, to some extent, controlled by the availibility of nutrients. After 50 hours, the chlorophyll content of the cuttings supplied with sucrose increased by 20% and with White's medium by 60%, when compared with the water controls.

It is pointed out, however, that the formation of chlorophyll did not closely follow the accepted pattern (Virgin, 1955) in that the duration of the lag phase was reduced, with the rapid phase of synthesis commencing almost immediately. Since this was seen in cuttings with or without added nutrients it is thought that they contained sufficient endogenous nutrients to eliminate the lag period. However the nutrient supply to the explants would seem to be important in later chlorophyll synthesis.

(b) Inhibition by antibiotics

Several antibiotics are known to inhibit the formation of chlorophyll in the leaves of illuminated plants. Solutions of some available antibiotics, e.g. chloramphenicol, terramycin, etamycin, oleandomycin and lincomycin, were sprayed on to the leaves of light and dark grown cress seedlings. The leaves

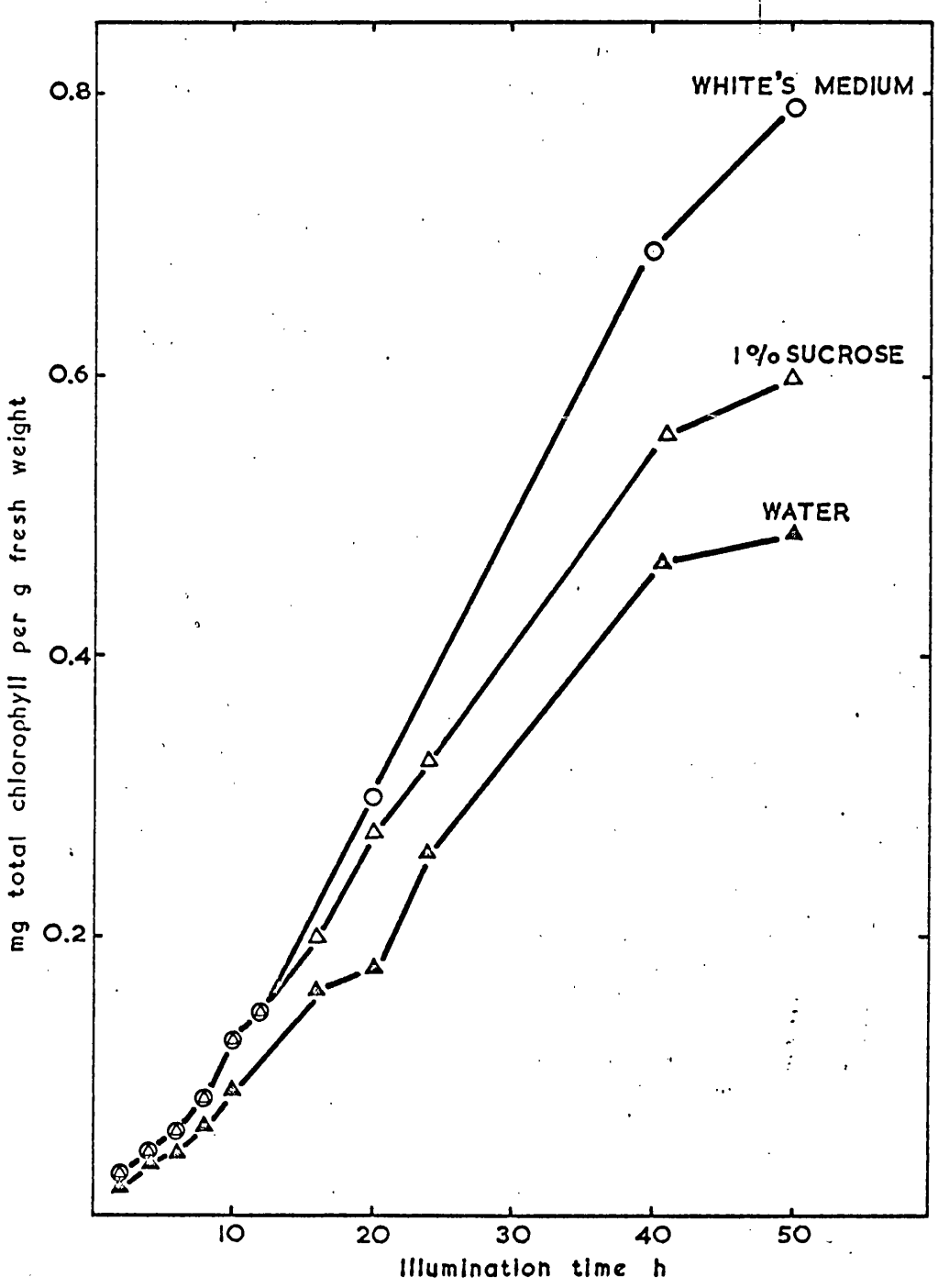


FIG. 7 The effect of sucrose and White's Medium on the rate of chlorophyll synthesis in untreated pea cuttings.

were allowed to dry in the dark, and were then exposed to continuous white light for 48 hours, after which the total chlorophyll content was estimated. The results summarised in Fig.8 demonstrate that the amount of chlorophyll which had been synthesised in all the treated dark grown plants was markedly less than the controls. None of the antibiotics had any effect upon the formation of chlorophyll in the light grown plants during the period of test. The variation in the chlorophyll content of the treated light grown plants was due to the use of two batches of seedlings in the experiment. The inhibition of chlorophyll formation in the higher plants by oleandomycin and lincomycin does not appear to have been reported previously. Subsequent work on the antibiotic inhibition of chlorophyll synthesis was restricted, however, to chloramphenicol and terramycin since the other compounds were either insoluble in water or required high concentrations to achieve their effect.

i) Chloramphenicol. Chloramphenicol is reported to inhibit the synthesis of chlorophyll primarily by preventing the formation of δ-aminolevulinic acid synthetase (Gassman and Bogorad, 1967 a, 1967 b), which is responsible for the production of δ-aminolevulinic acid, an early precursor of the porphyrins. However, it is not inconceivable that the antibiotic may also inhibit the production of other proteins important in the development of the chloroplast lamellae. The results shown in Fig.9 demonstrated that the chlorophyll content of the plants treated with varying concentrations of chloramphenicol are, even after 16 hours of illumination, essentially quite similar. However after 40 hours of exposure to light, the chlorophyll content had increased by an amount dependent upon the concentration

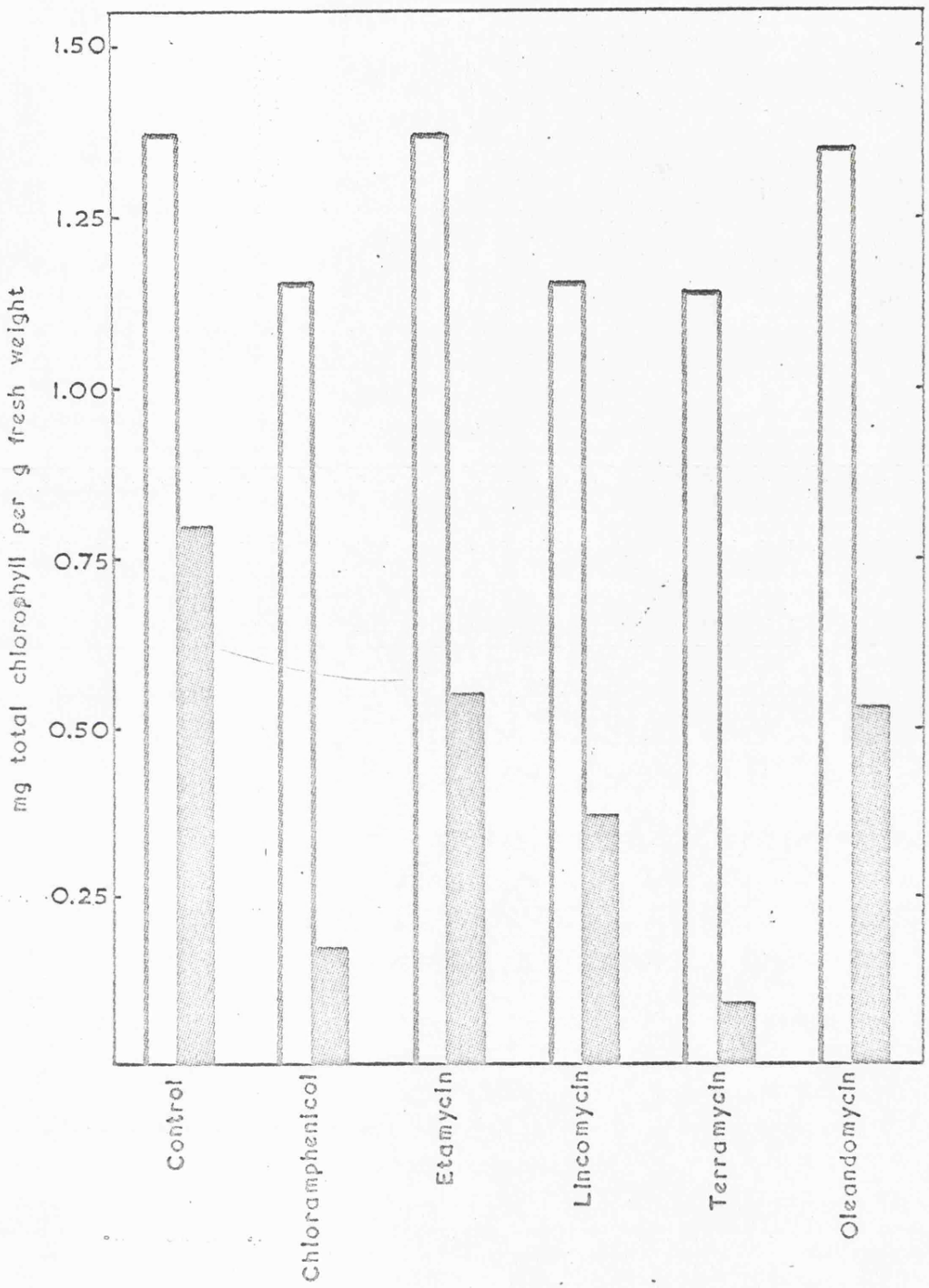


FIG. 8 The effect of various antibiotics upon the formation of chlorophyll in greening cress seedlings. Open bars - untreated; solid bars - treated. Chloramphenicol, etamycin, oleandomycin - 5 mg/ml in 50% ethanol; lincomycin - 50 mg/ml in water; terramycin - 5 mg/ml in water. Chlorophyll content estimated after 48 hours illumination.

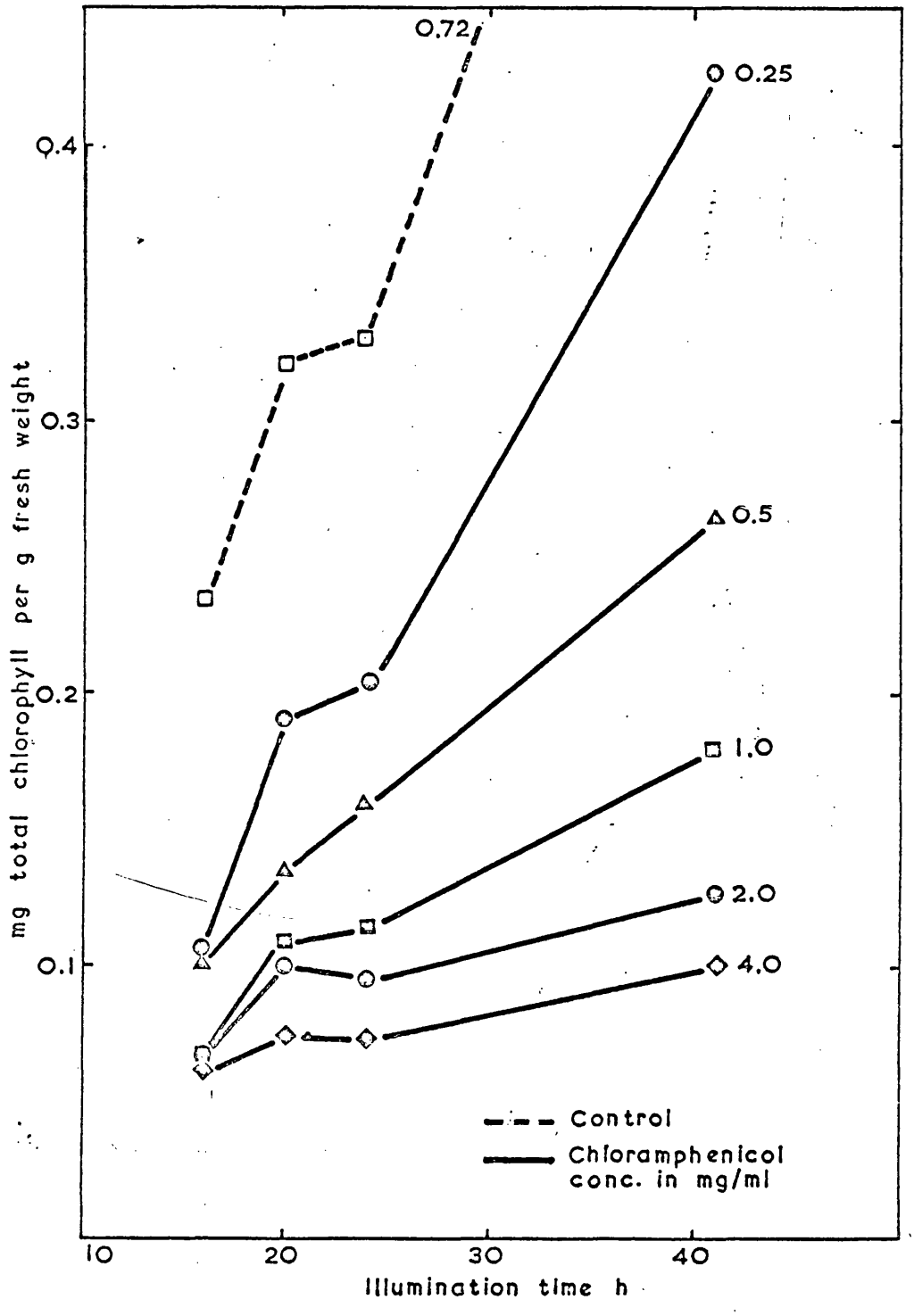


FIG. 9 The rate of chlorophyll formation in greening pea cuttings treated with various concentrations of chloramphenicol.

of the inhibitor. It is obvious that as the chloroplast developed the pool size of the δ-aminolevulinic acid synthetase increased in spite of the presence of low concentrations of antibiotic. The inhibition of chlorophyll formation was intensified with increasing amounts of antibiotic, until at 4 mg/ml the inhibition was 85%. This was the greatest inhibition observed, which suggested that chloramphenicol had no effect upon the conversion of protochlorophyll to chlorophyll (Gassman and Bogorad, 1967 a).

Margulies (1962) reported that the ratios of chlorophyll a to b in chloramphenicol treated and control greening bean leaves were approximately equal. However the data presented in Fig.10 showed that the synthesis of chlorophyll a in greening peas was inhibited to a greater extent by chloramphenicol than was that of chlorophyll b. The content of chlorophyll b remained constant over a 4-fold increase in the concentration of inhibitor, excepting for a small decrease at 0.25 mg/ml. The ratio of chlorophyll a to b in the treated plants was lower than in the controls, over the whole range of inhibitor concentrations. The ratios after 40 hours illumination were greater than those at 20 hours (Fig.11). It is difficult to explain the insensitivity of chlorophyll b synthesis to chloramphenicol unless one accepts that either the synthetic pathway to chlorophyll a and b are not identical, or, at least, the immediate precursor of chlorophyll b is not chlorophyll a.

ii) Terramycin. Although the effect of terramycin on the metabolism of higher plants has received little attention, its mode of action is better understood. It appears that the antibiotic has an affinity for magnesium and manganese ions

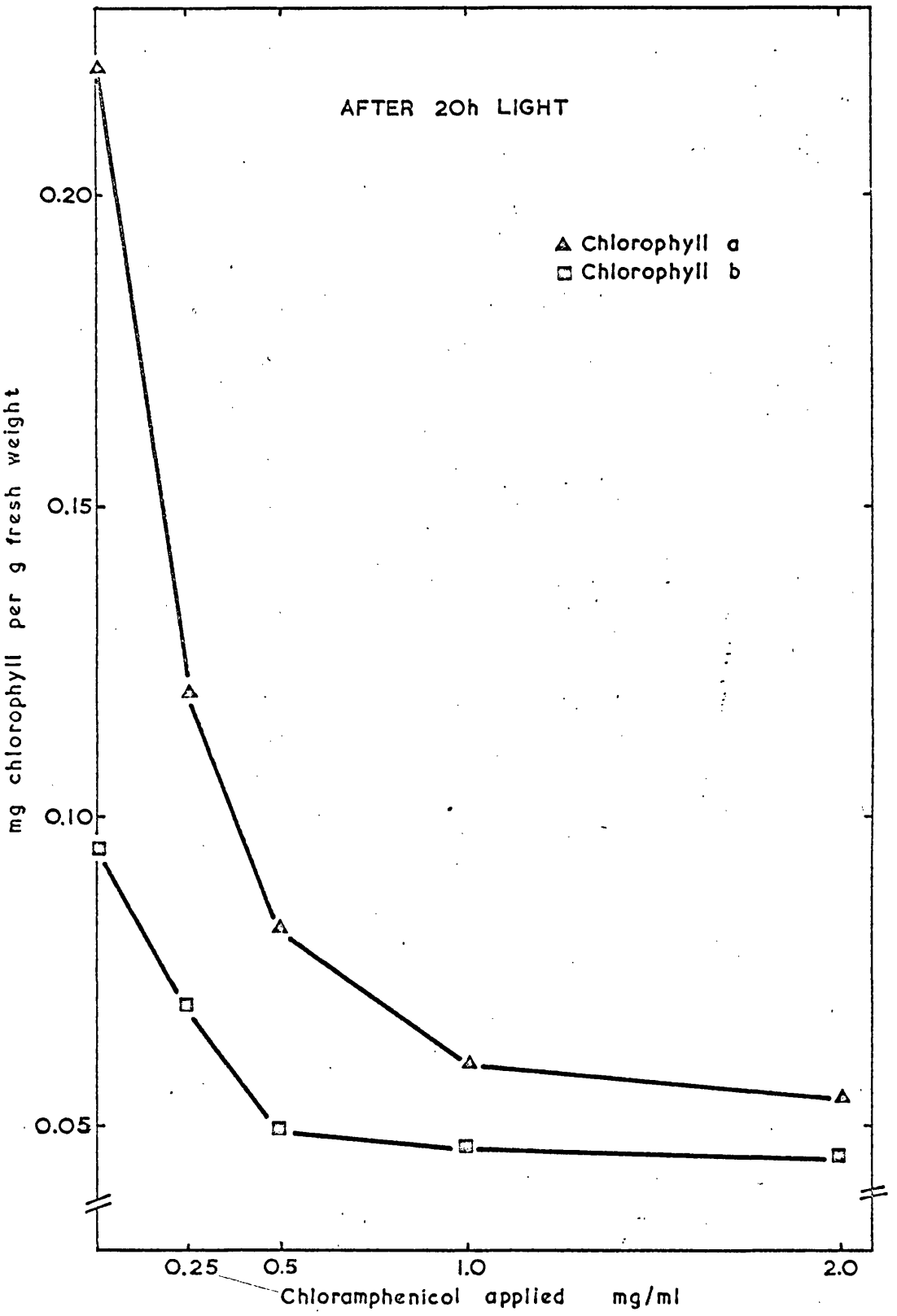


FIG. 10 The content of chlorophyll a and chlorophyll b in greening pea cuttings treated with various concentrations of chloramphenicol.

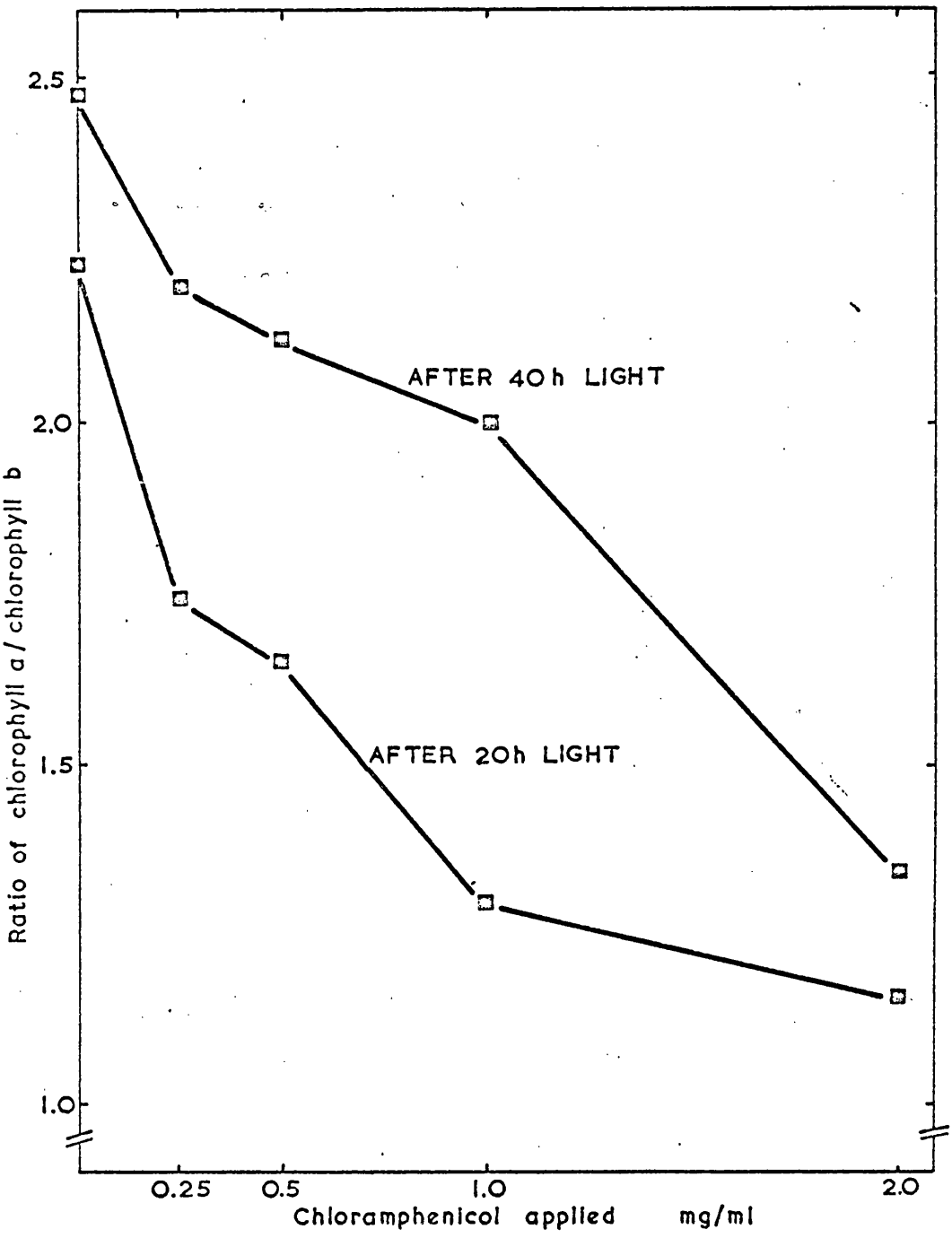


FIG. 11 The effect of various concentrations of chloramphenicol on the ratio of chlorophyll a to b in greening pea cuttings, after 20 and 40 hours illumination.

(Gale and Folkes, 1953, cited by Franklin, 1966) in addition to inhibiting the formation of protein from messenger RNA (Brody, Hurwitz and Bain, 1954, cited by Franklin, 1966). Hence the prevention of chlorophyll synthesis in treated plants may be the result of the terramycin induced deficiency of magnesium ions in the leaf, as well as the inhibition of the synthesis of the enzymic and/or structural proteins responsible for pigment production.

The results shown in Fig.12 demonstrate that increased concentrations of terramycin bring about an increased inhibition of chlorophyll synthesis. Netien and Lacharme (1955), reported that radish seeds, when dusted with terramycin powder germinated into seedlings deficient in chlorophyll. In the treated plants there was no chlorophyll a even though traces of chlorophyll b were present. In the present study however, chlorophyll a was detected at all antibiotic concentrations, although its synthesis was more inhibited than that of chlorophyll b (Fig.13). At the higher concentrations the chlorophyll a to b ratio was greater after 40 hours of illumination than at 20 hours (Fig.14); the later chlorophyll synthesis being relatively unaffected by the antibiotic.

Since the results of the terramycin induced inhibition of chlorophyll formation were similar to those obtained using chloramphenicol, it would seem that their modes of action were similar, although terramycin achieved a greater inhibition of pigment synthesis per mg of antibiotic applied, than did chloramphenicol. The greater efficiency of terramycin may either be a consequence of its ion chelating properties, or due to a larger amount of the compound being actually present at the site of protein synthesis.

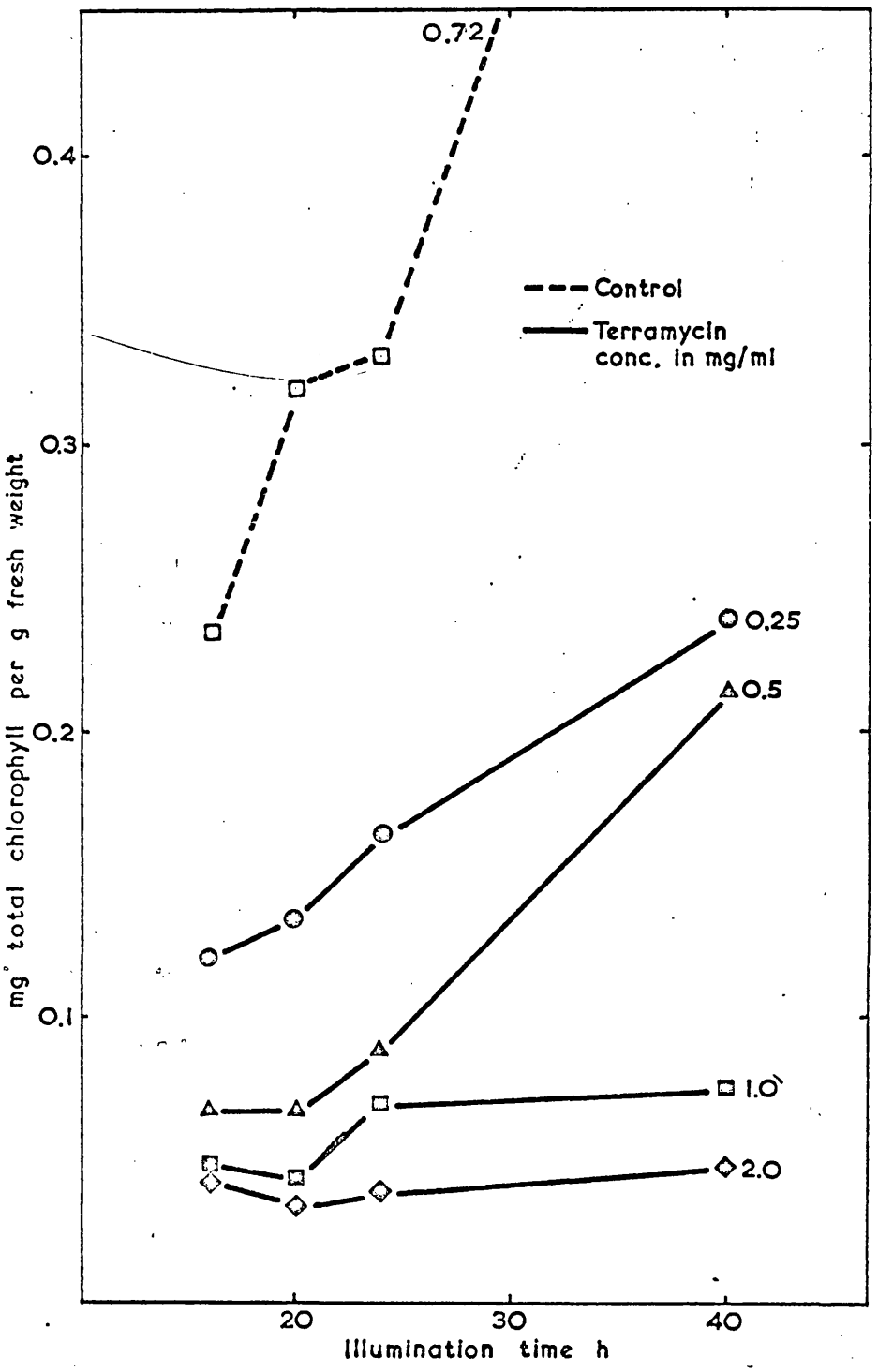


FIG. 12 The rate of chlorophyll formation in greening pea cuttings treated with various concentrations of terramycin

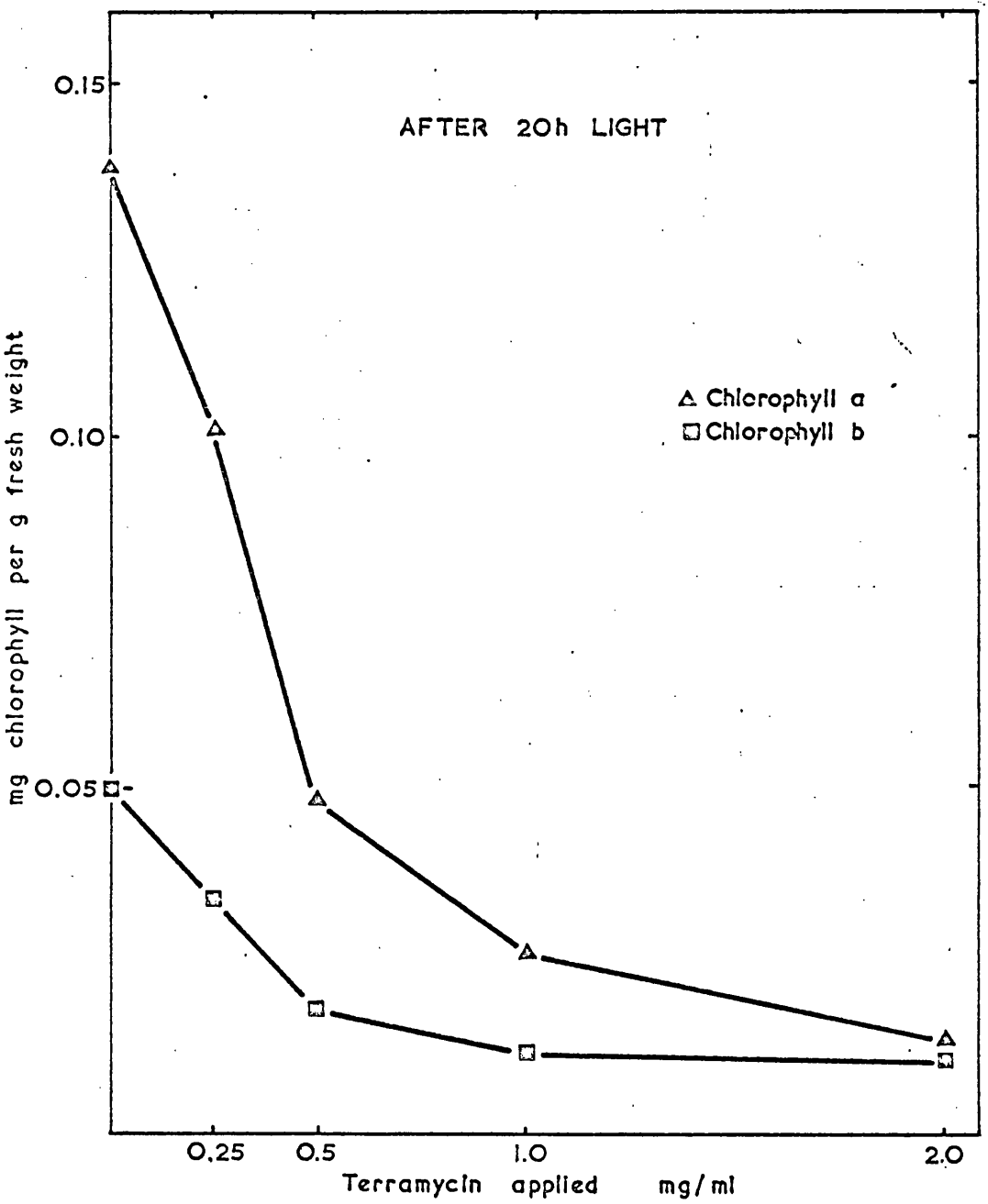


FIG. 13 The content of chlorophyll a and chlorophyll b in greening pea cuttings treated with various concentrations of terramycin.

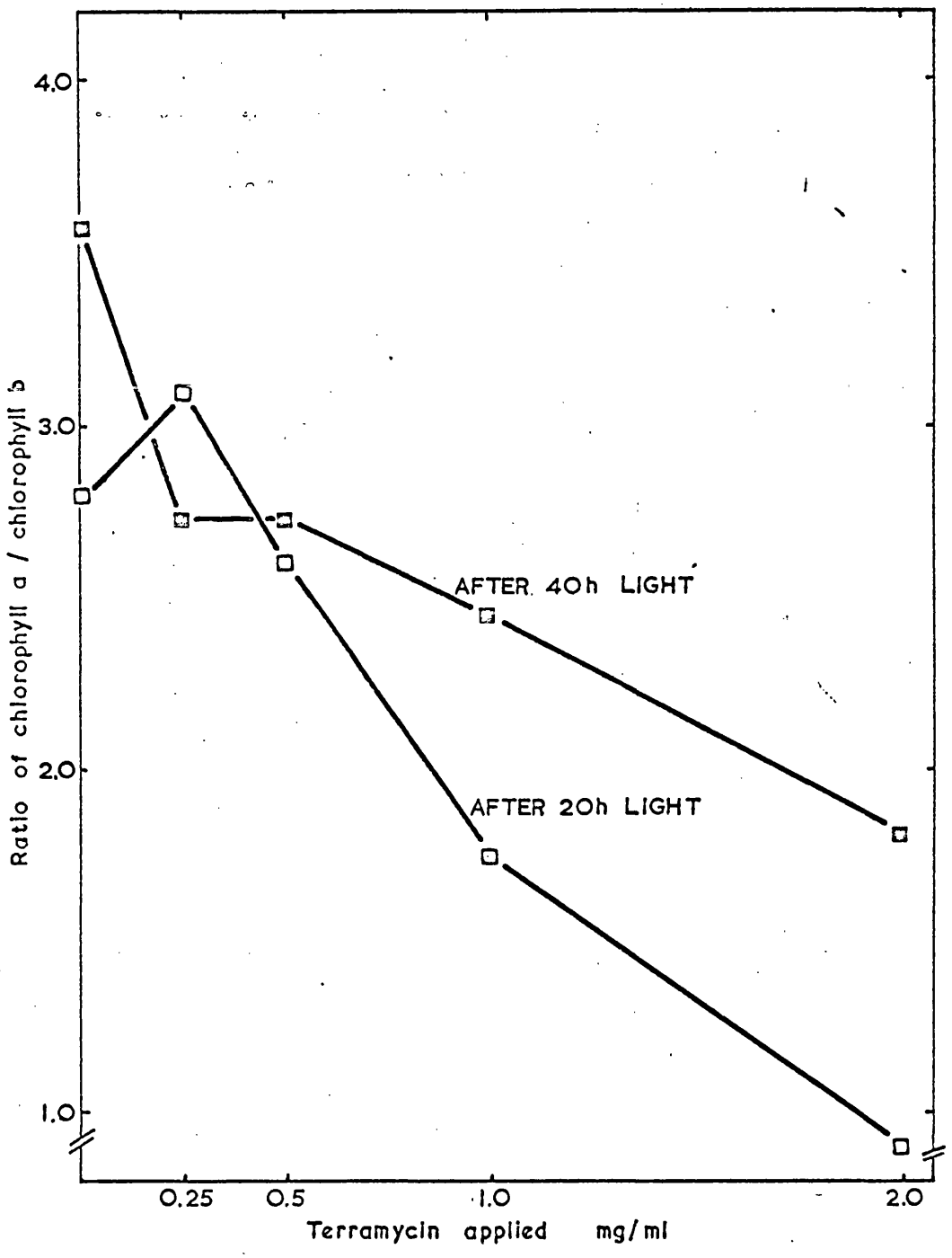


FIG. 14 The effect of various concentrations of terramycin on the ratio of chlorophyll a to b in greening pea cuttings after 20 and 40 hours illumination.

(c) Inhibition by light of low intensity

The synthesis of chlorophyll in illuminated, etiolated leaves is limited by the intensity of the incident light. Dark grown apical pea cuttings were illuminated by white light which was reduced in intensity by means of neutral density filters. The chlorophyll content, estimated at intervals during the 40 hour illumination period increased with increasing light intensity. At intensities below 160 lux, the maximal rate of chlorophyll formation occurred after about 36 hours of illumination, but at intensities greater than this the rate was submaximal even after 40 hours (Fig.15). Huffaker, et al. (1966) report that in barley, no significant increases in the synthesis of chlorophyll occurred after 36 or 30 hours of irradiation by light of 1076 or 32 lux respectively. The data suggest that pigment synthesis cannot occur normally below a threshold intensity of about 200 lux. This concept was further supported by the results shown in Fig.16. After 20 hours of illumination, a light intensity above 160 lux resulted in a continued increase in the chlorophyll a content, but only a negligible change in that of chlorophyll b. The ratio of chlorophyll a to b at 20 hours continued to increase with increasing light intensity due to the further synthesis of chlorophyll a (Fig.17), but at 40 hours the ratio was unaltered at intensities greater than 160 lux. Thus it seemed that radiant energy over and above that required to initiate chlorophyll formation was necessary to bring about a normal chlorophyll content and chloroplast structure.

2. Experiments with isolated chloroplasts

The data shown in Fig.6 demonstrate that the rate of formation of chlorophyll may be enhanced by exogenously supplied

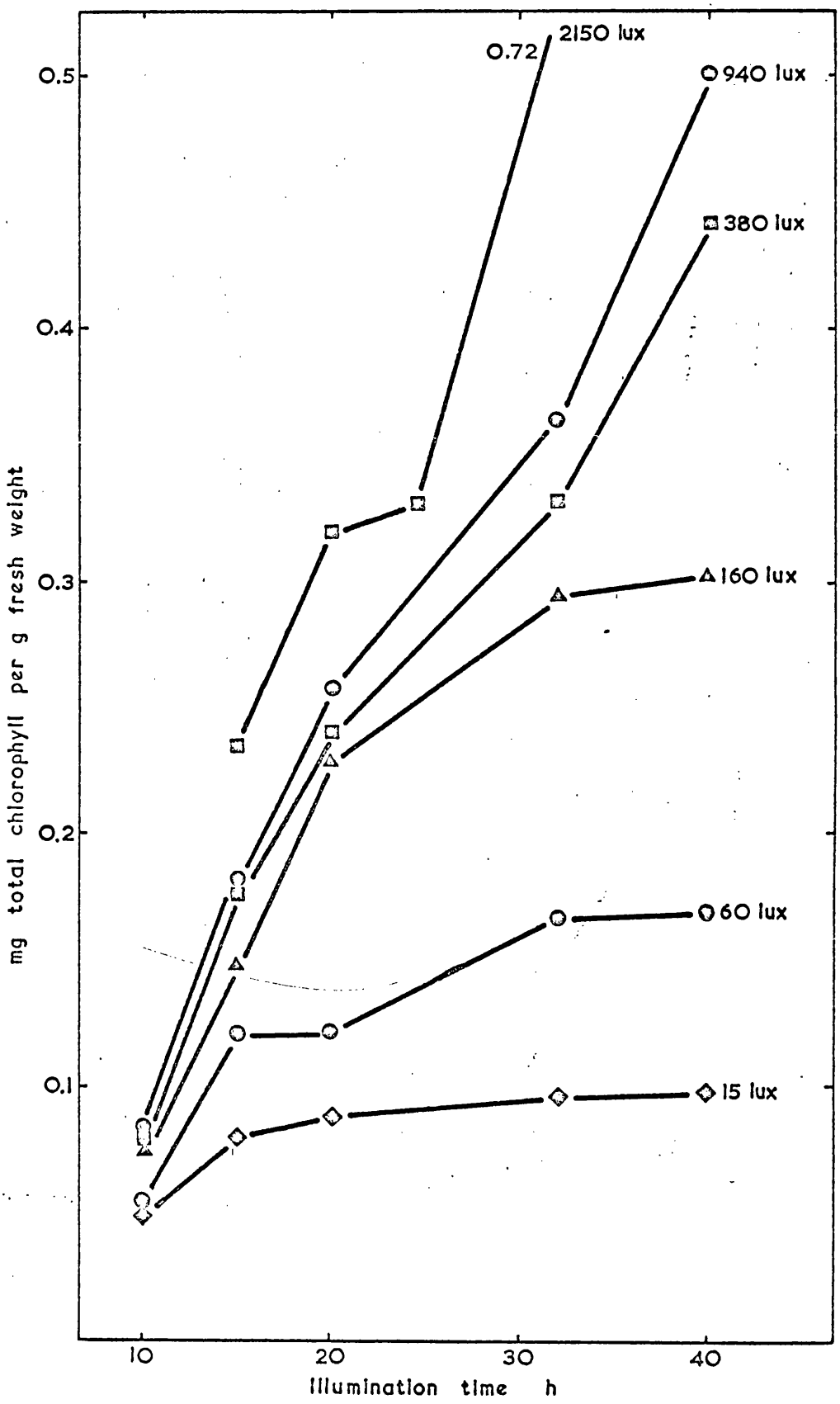


FIG. 15 The rate of chlorophyll formation in greening pea cuttings exposed to various intensities of light.

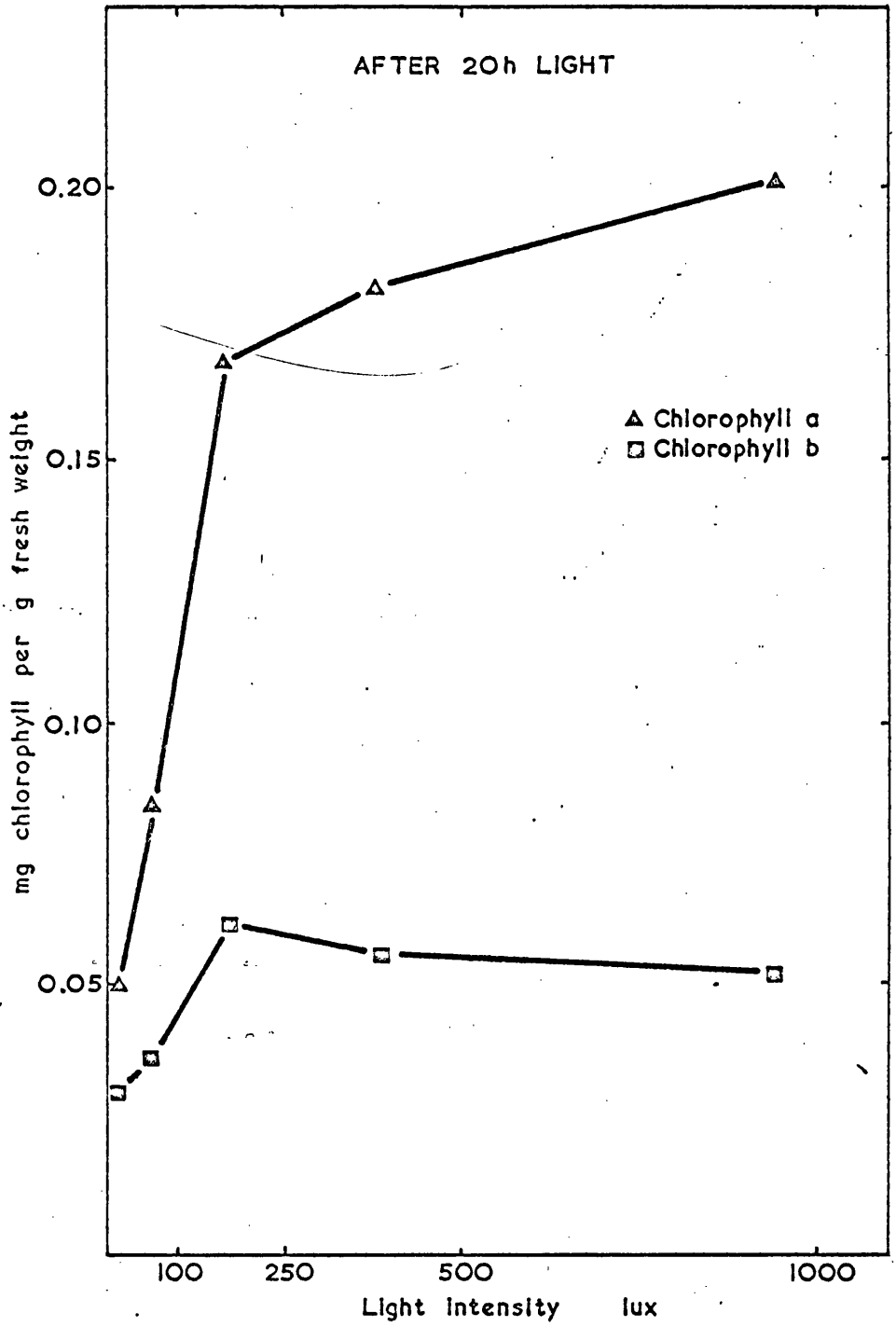


FIG. 16 The content of chlorophyll a and chlorophyll b in greening pea cuttings exposed to various intensities of light.

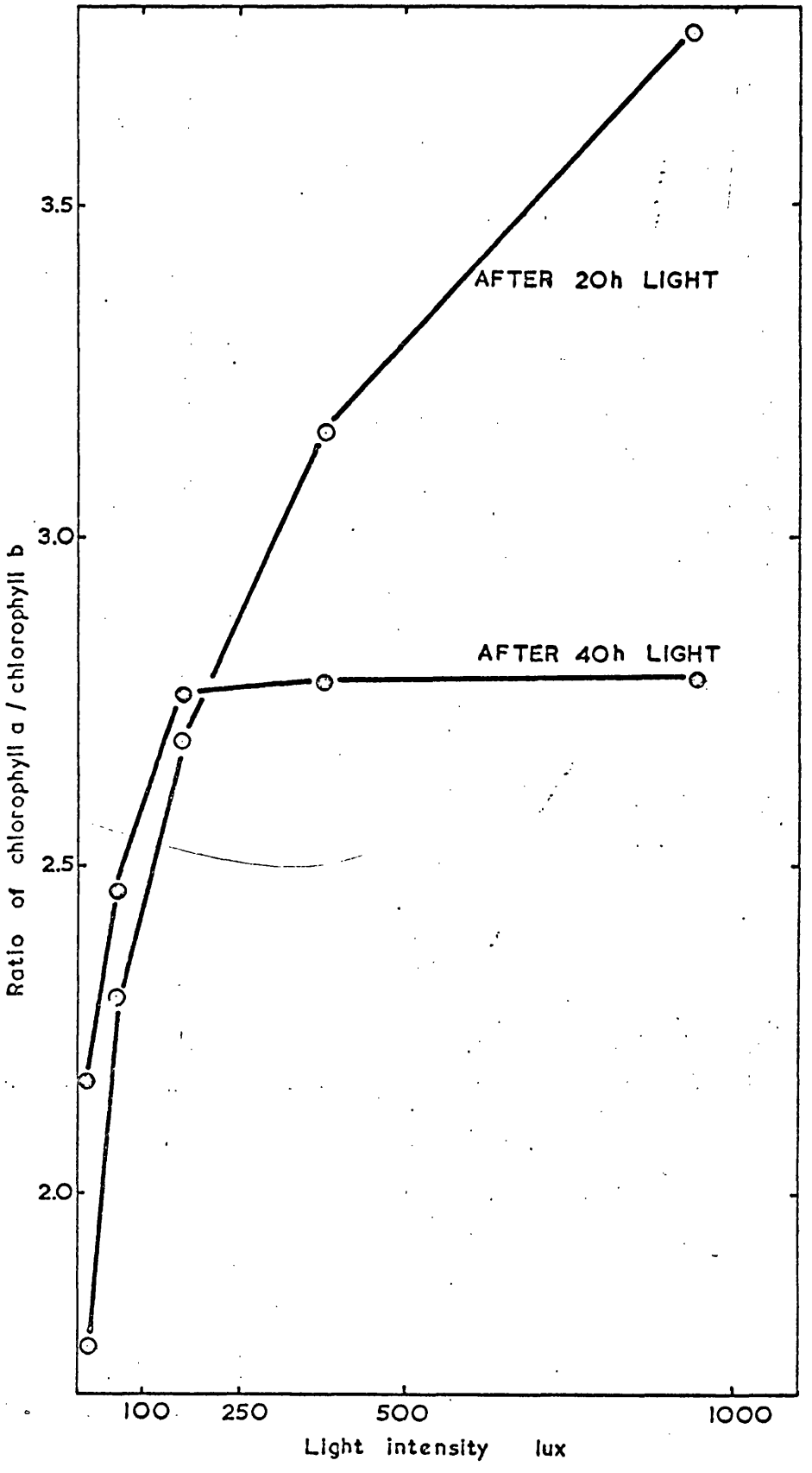


FIG. 17 The effect of various intensities of light on the ratio of chlorophyll a to b in greening pea cuttings after 20 and 40 hours illumination.

nutrients. These results, along with those of the antibiotic and low light intensity treatments, point to the importance of photosynthetically derived assimilates in the continued synthesis of chlorophyll and the normal development of the chloroplast. The techniques used in the isolation of functional chloroplasts from green leaf tissue are now well developed. The currently accepted rationale for the mechanism of photosynthetic electron transport has been described earlier, and with this theoretical background, the photochemical reactions of chloroplasts derived from antibiotic and low intensity light treated leaves were studied.

(a) Control

The change in the rate of photosynthetic electron transport in untreated pea cuttings are reported in Section 3.

(b) Chloramphenicol

Margulies (1962, 1964) reported that both carbon dioxide fixation and electron transport were abolished in chloroplasts isolated from greening bean leaves which had been treated with high concentrations of chloramphenicol prior to illumination. The effect of this inhibitor on chloroplasts electron transport was investigated using etiolated pea cuttings which had been supplied with 4 mg/ml of chloramphenicol which, after a period of 18 hours in the dark, were illuminated for 48 hours. The electron transport activity of chloroplasts isolated from the leaves at the end of this time was assayed using ferricyanide, metmyoglobin and oxygen as electron acceptors. Two controls were also included, one of which was illuminated for 48 hours and the other for 8 hours, the latter having a chlorophyll content similar to the chloramphenicol treated plants.

TABLE 2

	Control 48 h light	Control 8 h light	CM 4 mg/ml
Total chlorophyll, mg/g fresh wt.	0.688	0.085	0.080
Electron acceptors, μM reduced per mg fresh wt per h			
Ferricyanide	37.7	7.0	0
Metmyoglobin	158.2	6.8	0
Oxygen (electrons from ascorbate - DCIP)	13.2	10.9	1.0

The results shown in Table 2, demonstrate that chloramphenicol, at high concentration, effectively abolished the flow of electrons in Light System I (oxygen), Light System II (ferricyanide) and in the combined systems (metmyoglobin). In comparison, chloroplasts isolated from untreated plants which had been illuminated for only 8 hours possessed appreciable electron transport activity.

Since these results essentially confirm the data given by Margulies (1962), the rate of photosynthetic electron transport in chloroplasts isolated from cuttings treated with varying amounts of chloramphenicol were also estimated, after 48 hours illumination. The photochemical activity was estimated by the reduction of ferricyanide and NADP (both with water as the electron donor) and oxygen and NADP (both with ascorbate - DCIP as electron donor). As may be seen in Fig.18 an increasing concentration of antibiotic initiated a steady decline in electron transport activity of both Light Systems, with the maximal inhibition at concentrations above 1.0 mg/ml.

Etiolated cuttings treated with sufficient chloramphenicol to permit the formation of approximately 0.2 mg total chlorophyll

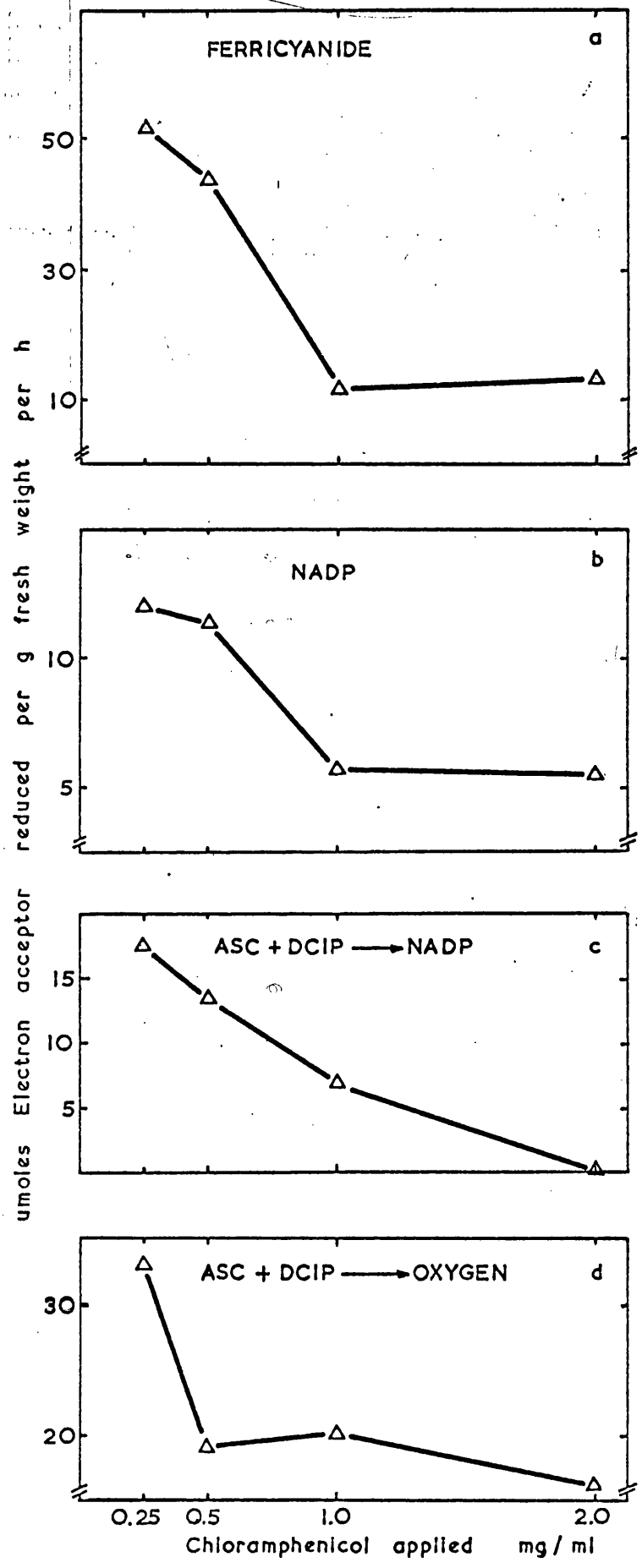


FIG. 18 The rates of some partial reactions of photosynthetic electron transport in chloroplasts treated with various concentrations of chloramphenicol after 48 hours illumination.

per g fresh weight of leaves after 48 hours of illumination, provided chloroplasts which demonstrated low but reproducible rates of electron transport. The terramycin and low intensity light treatments were also adjusted to allow a similar level of chlorophyll after the same period of illumination. The conditions for this level of chlorophyll were 1.5 mg/ml chloramphenicol, 0.7 mg/ml terramycin and light of 60 lux intensity. The chlorophyll content of the untreated plants was also standardised at the above level by limiting the illumination period to 18 hours.

(c) Comparison of the effects of all the treatments

The photochemical activity of chloroplasts derived from chloramphenicol, terramycin or low intensity light treated plants all containing similar amounts of chlorophyll was compared with chloroplasts isolated from untreated plants which had been illuminated for 18 or 48 hours. Electron transport was assayed by the reduction of ferricyanide and metmyoglobin (water as the electron donor), NADP and oxygen (ascorbate - DCIP as electron donor) and also cyclic photophosphorylation with phenozine methosulphate as the cofactor. Each treatment was performed in triplicate and each measurement of electron transport was duplicated. The average results are shown in Fig.19, with the ratios of chlorophyll a to b given in Fig.19E for completeness.

i) Chloramphenicol. When compared with the 18 hour controls (C_2), the antibiotic appeared to inhibit the development of Light System II since the reduction of ferricyanide and metmyoglobin were inhibited by 41% (Fig.19A) and 72% (Fig.19B) respectively. However the rates of the reactions requiring a functional Light System I and an artificial or cyclic supply of

electrons were stimulated. The rate of cyclic photophosphorylation was enhanced by 42% (Fig.19F) and the donation of electrons from ascorbate - DCIP to NADP by 86% (Fig.19C) and to oxygen by 41% (Fig.19D). A similar pattern of inhibition and stimulation was seen when the results were compared with the 48 hour controls (C_1), excepting that the rate of cyclic photophosphorylation was then less than the control.

It would seem that the light absorbing centres of the two light reactions are relatively unaffected by chloramphenicol, since ferricyanide and NADP are both reduced at reasonable rates. The outstanding feature however was the marked stimulation of the rate of electron donation of ascorbate - DCIP. These results suggest that this antibiotic has induced some structural aberration of the chloroplast lamellae, particularly in the intermediate electron transport system. The efficiency of electron donation of ascorbate was thus increased, whilst the non-cyclic flow to metmyoglobin and ferricyanide was impeded, as was photophosphorylation.

ii) Terramycin. Treatment with terramycin, when compared with the 18 hour controls (C_2), inhibited the non-cyclic flow of electrons from water to ferricyanide by 15% (Fig.19A) and to metmyoglobin by 55% (Fig.19B). No significant stimulation of the rate of electron donation by ascorbate - DCIP to NADP (Fig.19C) or to oxygen (Fig.19D) was observed, although the rate of cyclic photophosphylation was enhanced by 83% (Fig.19F). However, when compared with the 48 hour controls (C_1) the inhibition of metmyoglobin reduction was increased to 91% (Fig.19B), and the inhibition of ferricyanide was relieved to 23% (Fig.19A). The donation of electrons by ascorbate -

DCIP to NADP was inhibited by 33% (Fig.19C) whilst no appreciable difference was observed in the donation to oxygen. Cyclic photophosphorylation was inhibited, but the inhibition was relieved to 15% (Fig.19F).

iii) Light of low intensity. Etiolated cuttings exposed to light of low intensity yielded isolated chloroplasts in which the reduction of ferricyanide was inhibited by 48% (Fig.19A) and of metmyoglobin by 41% (Fig.19B) in comparison with the 18 hour controls (C). In this respect the results of this treatment were similar to the effects of the antibiotics, but whilst both antibiotics markedly stimulated the rate of cyclic photophosphorylation over the 18 hour control (C) rate, low light only enhanced the rate by 8% (Fig.19F). The rate of donation of electrons from ascorbate - DCIP to NADP was stimulated by 14% (Fig.19C) but to oxygen was inhibited by 38% (Fig.19D).

However, when the results are compared with the 48 hour controls (C), the ascorbate - DCIP electron donation to NADP (Fig.19C) and oxygen (Fig.19D) was inhibited by 20% and 33% respectively, whilst the rate of cyclic photophosphorylation was inhibited by 58% (Fig.19F). The non-cyclic flow of electrons from water to ferricyanide (Fig.19A) and metmyoglobin (Fig.19B) was also inhibited by 50% and 88% respectively.

From this comparison of the effects of the treatments on the partial reactions of photosynthetic electron transport it was concluded that the inhibition of chlorophyll synthesis had far reaching effects upon the proper integration of the developing chloroplast. Although the 18 hour control cuttings contained a similar amount of chlorophyll to the treated cuttings, they had not received a similar amount of radiant energy.

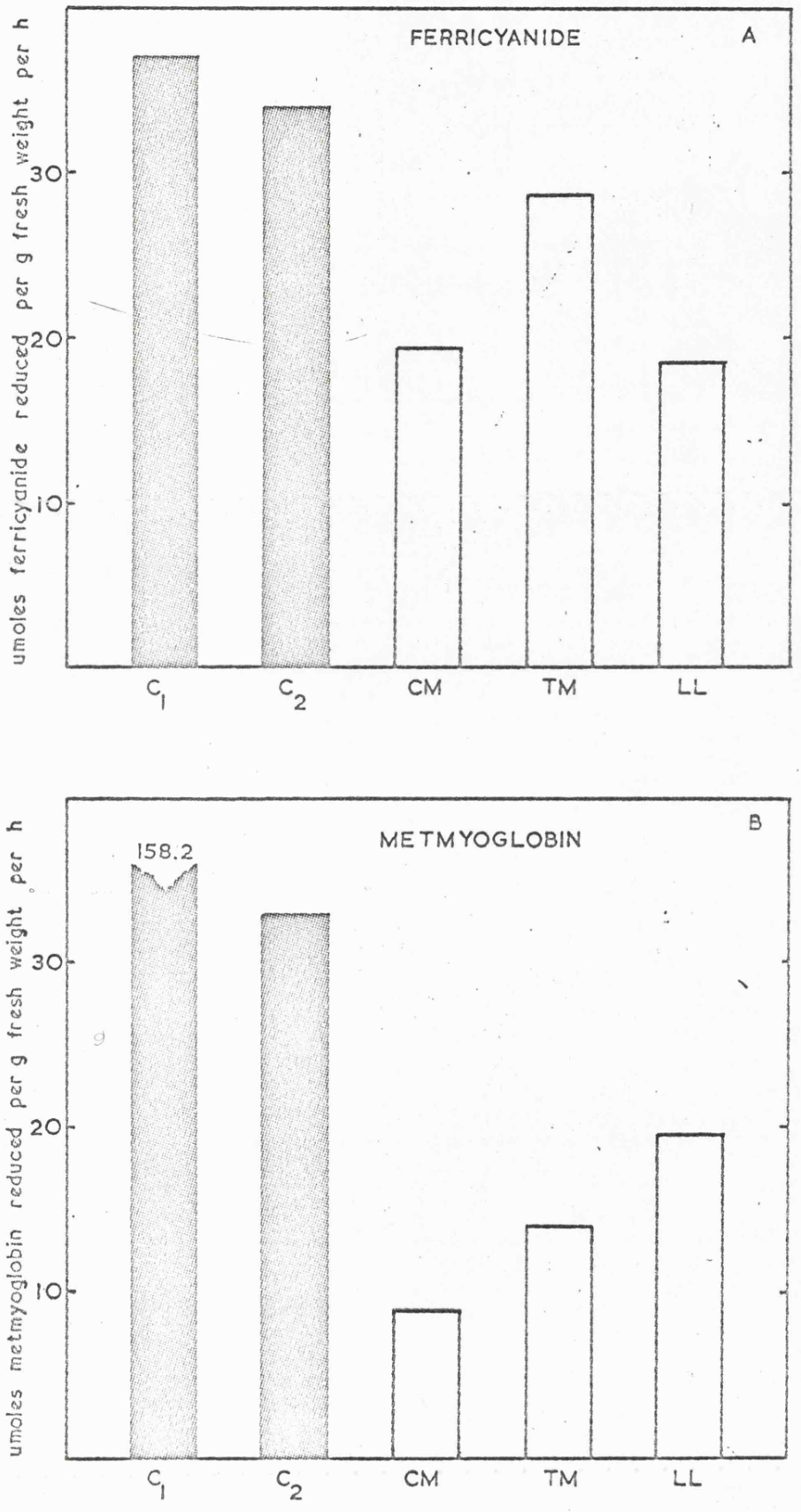
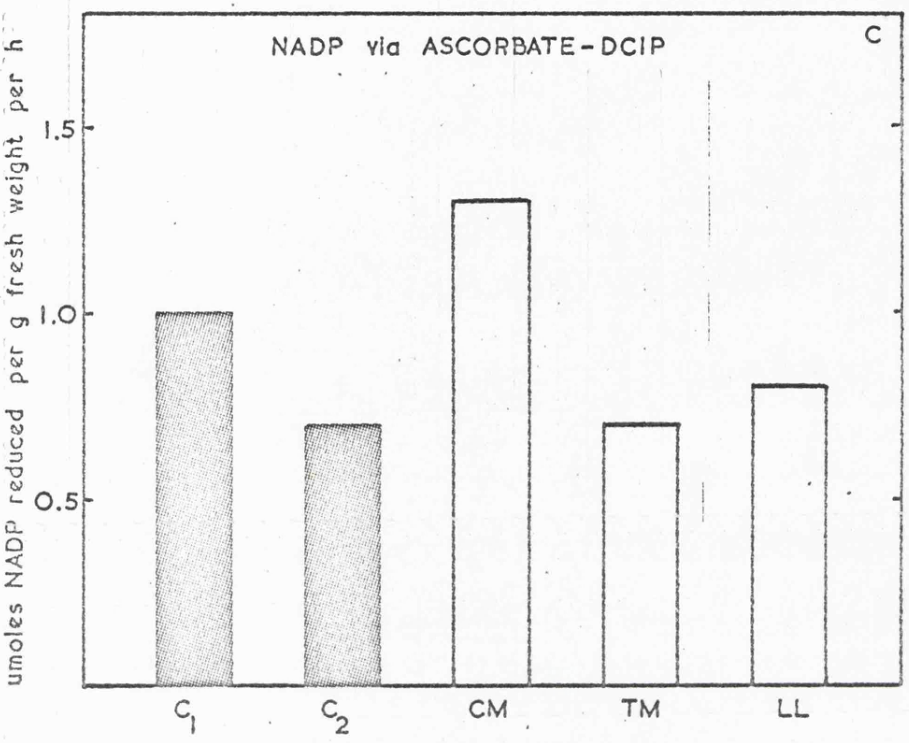


Fig. 19 A, B The effect of chloramphenicol (CM, 1.5 mg/ml), terramycin (TM, 0.7 mg/ml) and low intensity light (LL, 60 lux) on the rates of some partial reactions of photosynthetic electron transport in isolated chloroplasts, after 48 hours illumination. The controls were illuminated for 48 hours (C_1) and 18 hours (C_2).


NADP via ASCORBATE-DCIP
C
umoles NADP reduced per g fresh weight per h
1.5
1.0
0.5
C1
C2
CM
TM
LL

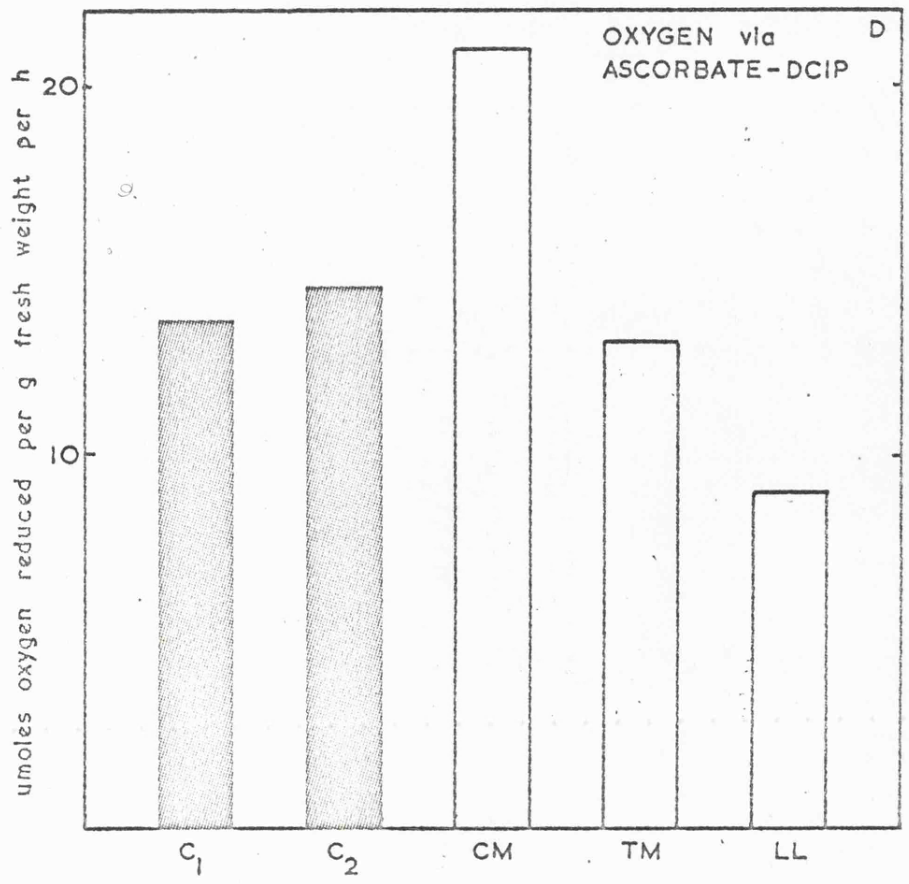
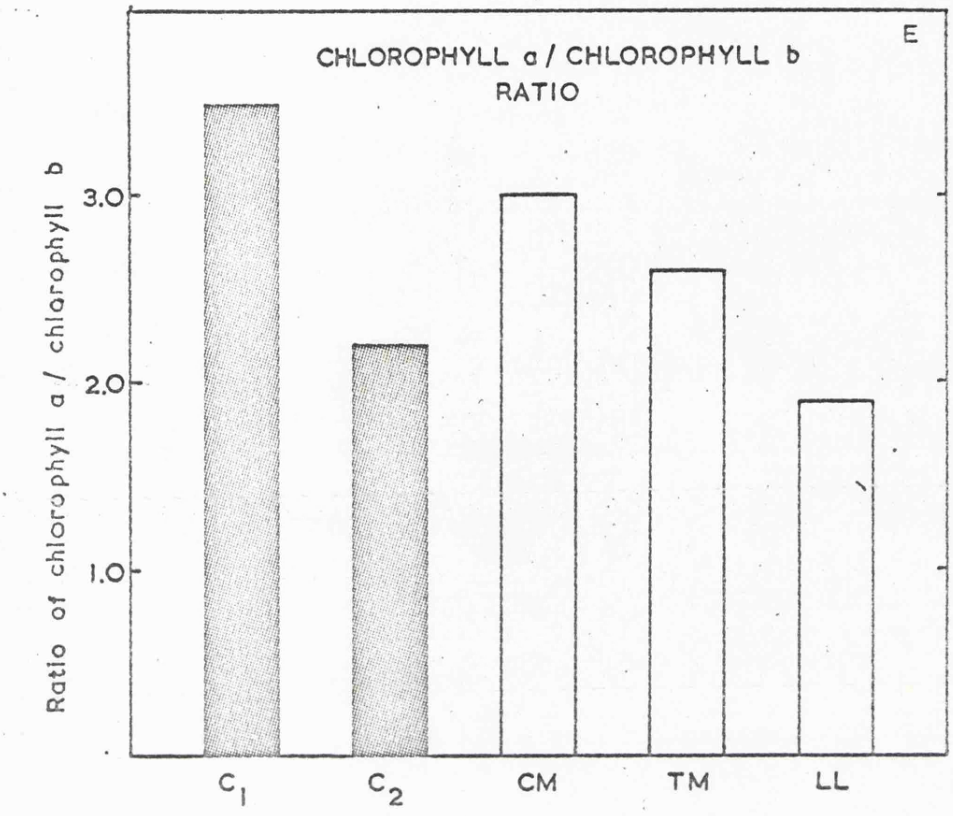

OXYGEN via
ASCORBATE-DCIP
D
umoles oxygen reduced per g fresh weight per h
20
10
C1
C2
CM
TM
LL

FIG. 19 C, D


E
CHLOROPHYLL a / CHLOROPHYLL b
RATIO
Ratio of chlorophyll a / chlorophyll b
3.0
2.0
1.0
C1
C2
CM
TM
LL

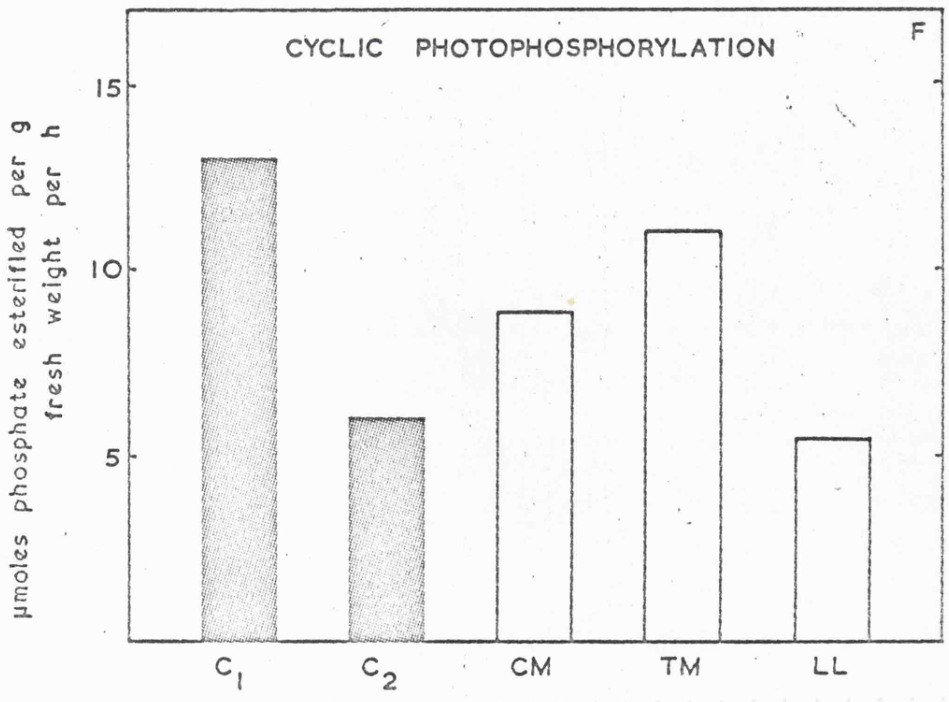

F
CYCLIC PHOTOPHOSPHORYLATION
µmoles phosphate esterified per g
fresh weight per h
15
10
5
C1
C2
CM
TM
LL

FIG. 19 E, F

It appears therefore that some components of the chloroplast may require either high light intensity, or a longer period of time before complete development is achieved. It was thought necessary to investigate the onset and later development of some of the partial reactions of electron transport in the chloroplasts of greening cuttings.

3. The onset and later development of electron transport

(a) Controls

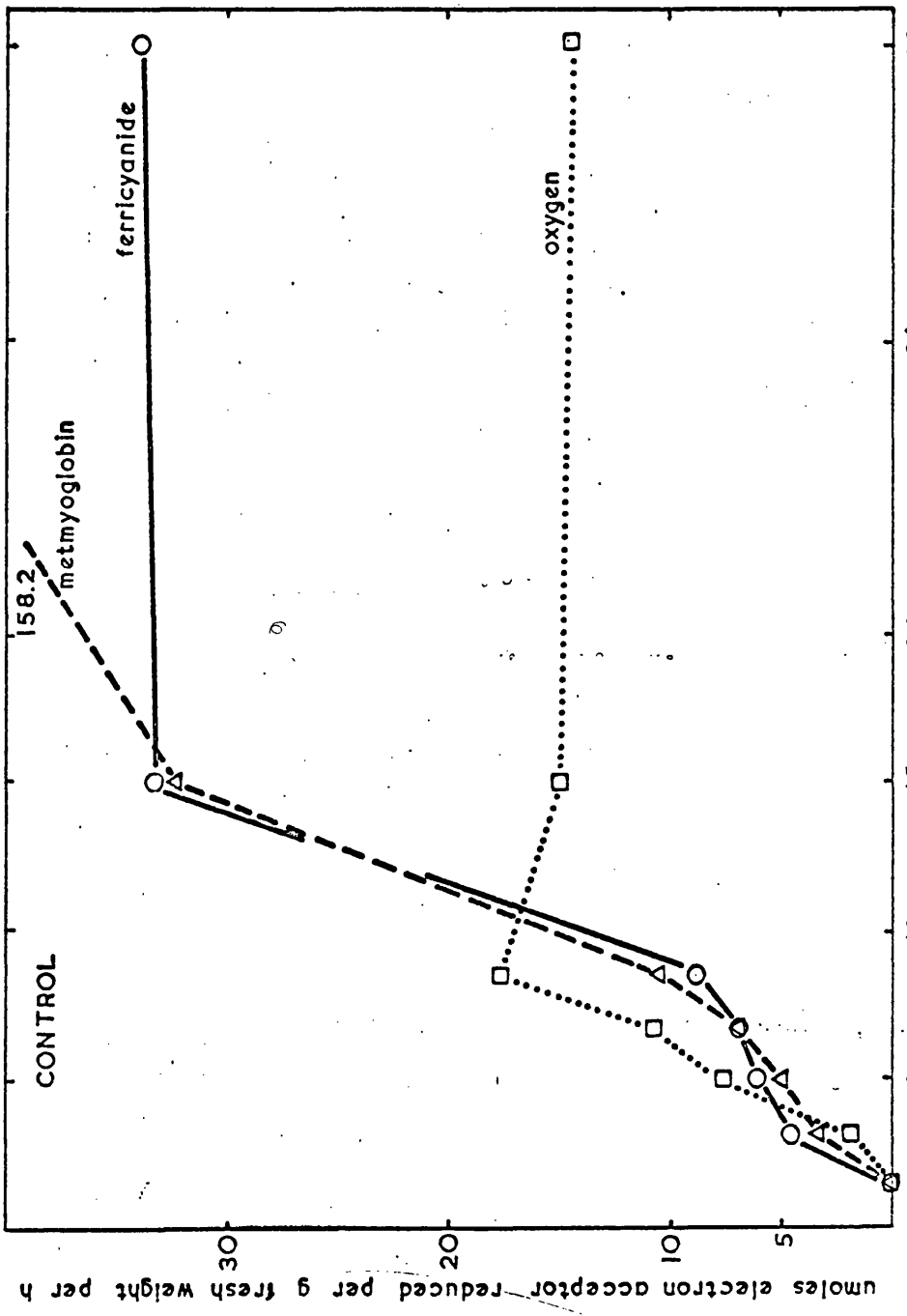
Cuttings were illuminated by white light for a period up to 48 hours. The total leaf chlorophyll was determined along with the reduction of ferricyanide, metmyoglobin and oxygen (ascorbate - DCIP as electron donor), by chloroplasts isolated at intervals during the period of illumination. Evidence of the activity of Light Systems I and II in reducing these electron acceptors was found after 4 hours illumination, at which time the chlorophyll content was 0.04 mg/g fresh weight (Fig.20). The rates of reduction of these electron acceptors continued to increase until a maximum was reached for oxygen at 10 hours, and ferricyanide at 18 hours, but metmyoglobin had not reached a maximum even after 48 hours illumination.

It appears from these results that Light System I and II become fully functional, independently after about 18 hours of illumination, since no further increase in rate was observed after 48 hours, even though the chlorophyll continued to increase. However it appears that complete co-operation between the two Light Systems required an extensive period of illumination since the rate of reduction of metmyoglobin had not achieved a constant value even after 48 hours.

For comparative purposes, Fig.21 is included, wherein

FIG. 20

The onset of photosynthetic electron transport in chloroplasts isolated from untreated, greening pea cuttings.



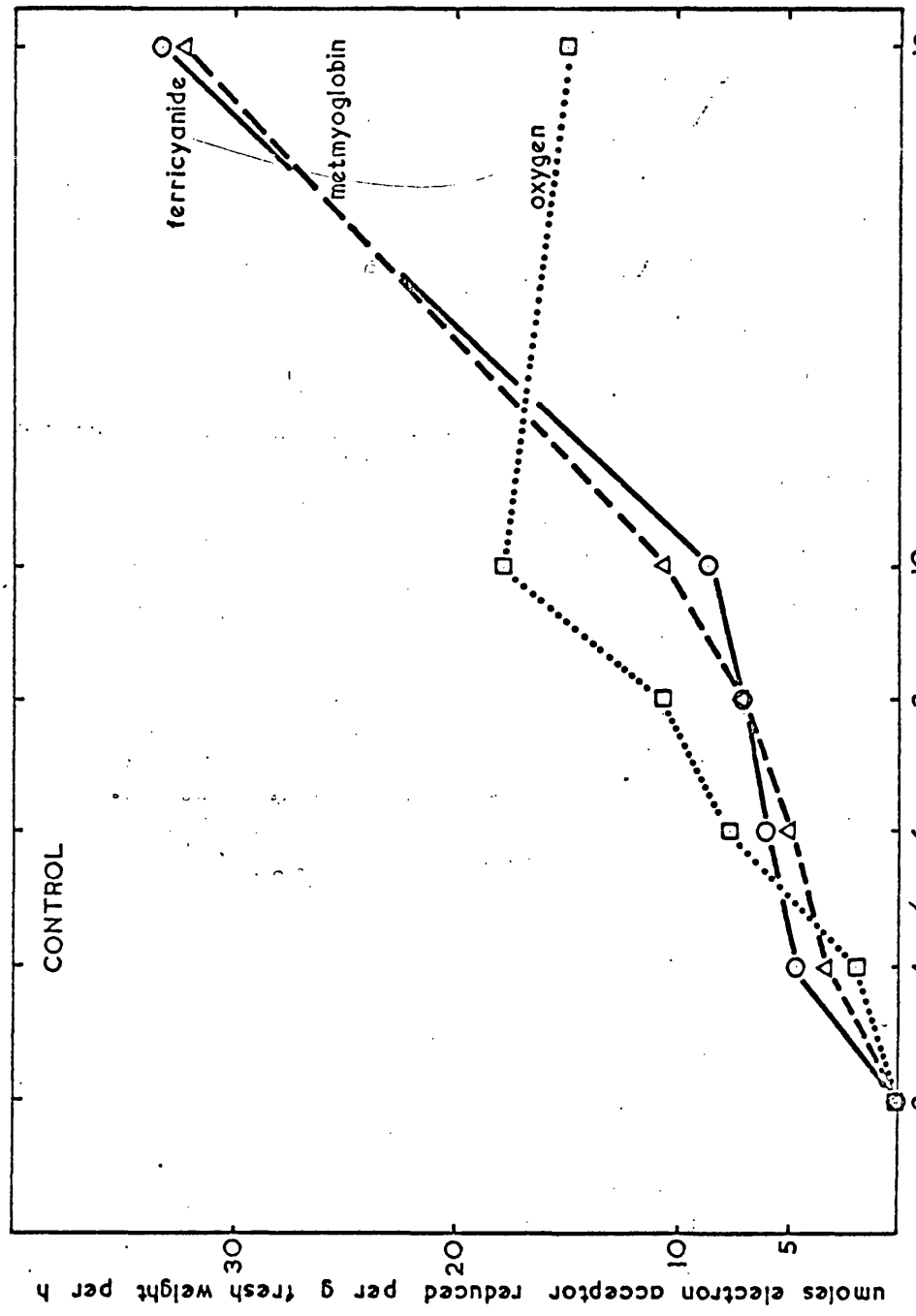


FIG. 21

The onset of photosynthetic electron transport in chloroplasts isolated from untreated, greening pea cuttings. The time scale has been adjusted to put 18 hours equivalent to 48 hours in linear measurement.

the time scale has been adjusted to place the untreated 18 hours equivalent in linear measurement to the 48 hour treated plants. Justifiable comparisons of the rate of chloroplast activity of treated and untreated plants can be made since the chlorophyll contents at these times are identical.

(b) Comparison of the effects of the treatments

The chloroplast electron transport activity and leaf chlorophyll content were estimated at intervals, as described for the controls, in cuttings supplied with 1.5 mg/ml chloramphenicol, 0.7 mg/ml terramycin or illuminated with light of 60 lux intensity. Each treatment was set up in triplicate and the electron transport determinations were duplicated for each treatment.

Electron transport (assayed by ferricyanide and metmyoglobin) in chloroplasts isolated from the leaves of both chloramphenicol (Fig.22) and terramycin (Fig.23) treated cuttings was detectable after 10 hours illumination. However in both these treatments evidence of the reduction of oxygen with electrons donated by ascorbate - DCIP was not obtained until after 16 hours had elapsed. The total chlorophyll content at 10 hours was 0.042 mg/g fresh weight for the chloramphenicol treatment and 0.050 mg/g fresh weight for the terramycin treatment, whilst at 16 hours the values were 0.085 and 0.110 mg/g fresh weight respectively.

However chloroplasts which had been isolated from the cuttings exposed to light of low intensity, furnished evidence that all the electron acceptors were being reduced after 12 hours illumination (Fig.24), there being no delay observed for the ascorbate - DCIP to oxygen system. The total chlorophyll

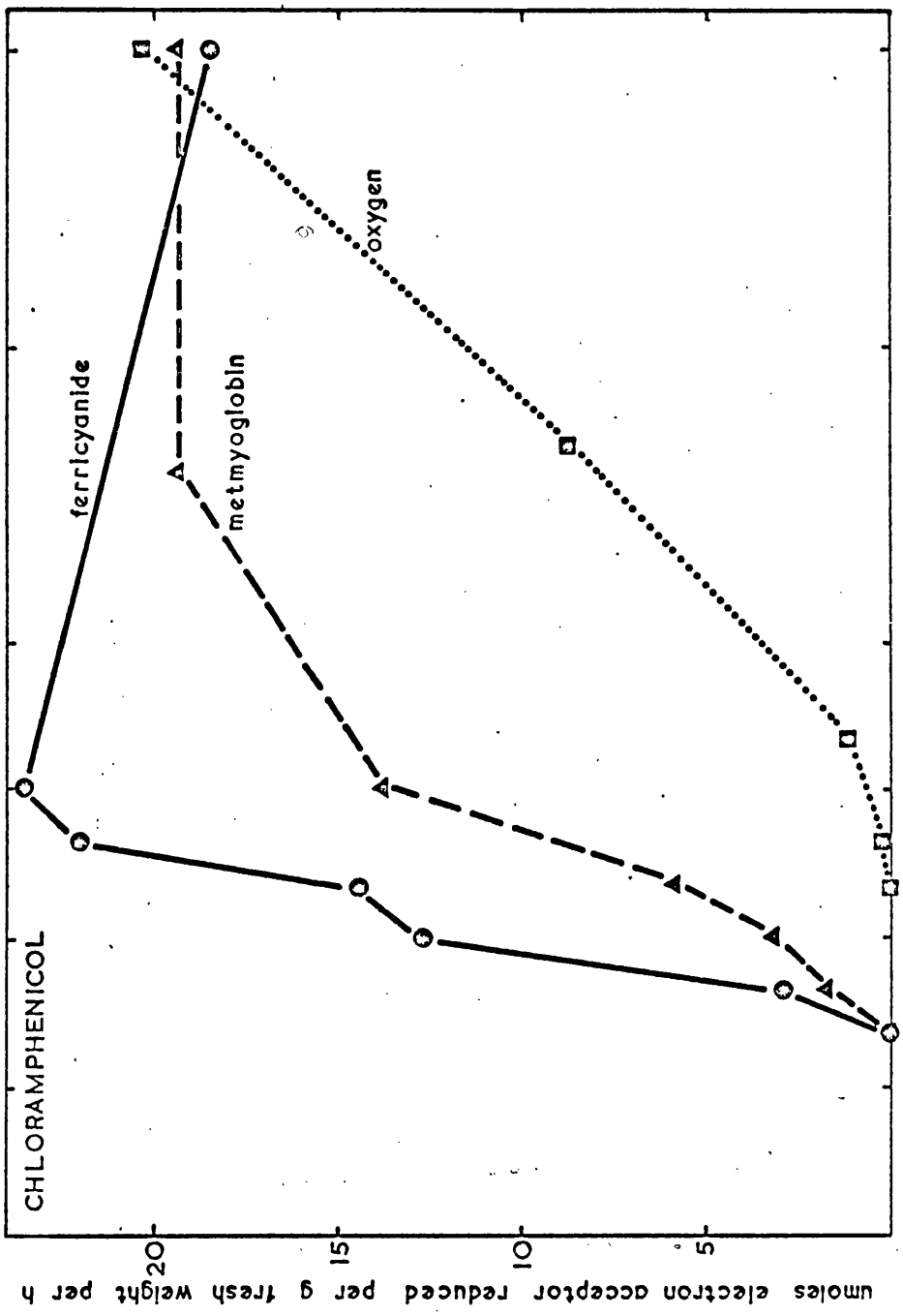


FIG. 22

The onset of photosynthetic electron transport in chloroplasts isolated from chloramphenicol treated (1.5 µg/ml) greening pea cuttings.

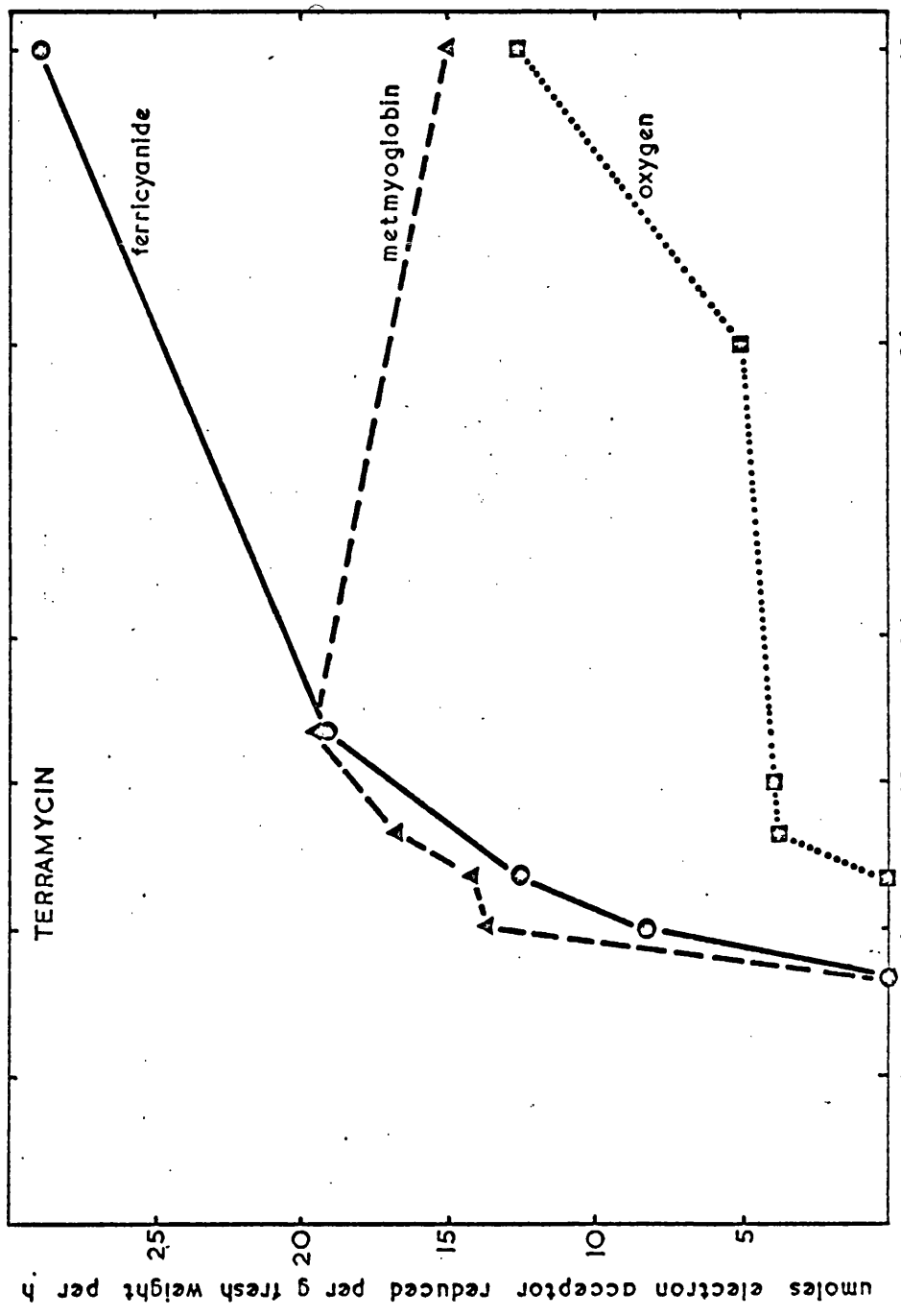


FIG. 23

The onset of photosynthetic electron transport in chloroplasts isolated from terramycin treated (0.7 µg/ml) greening pea cuttings.

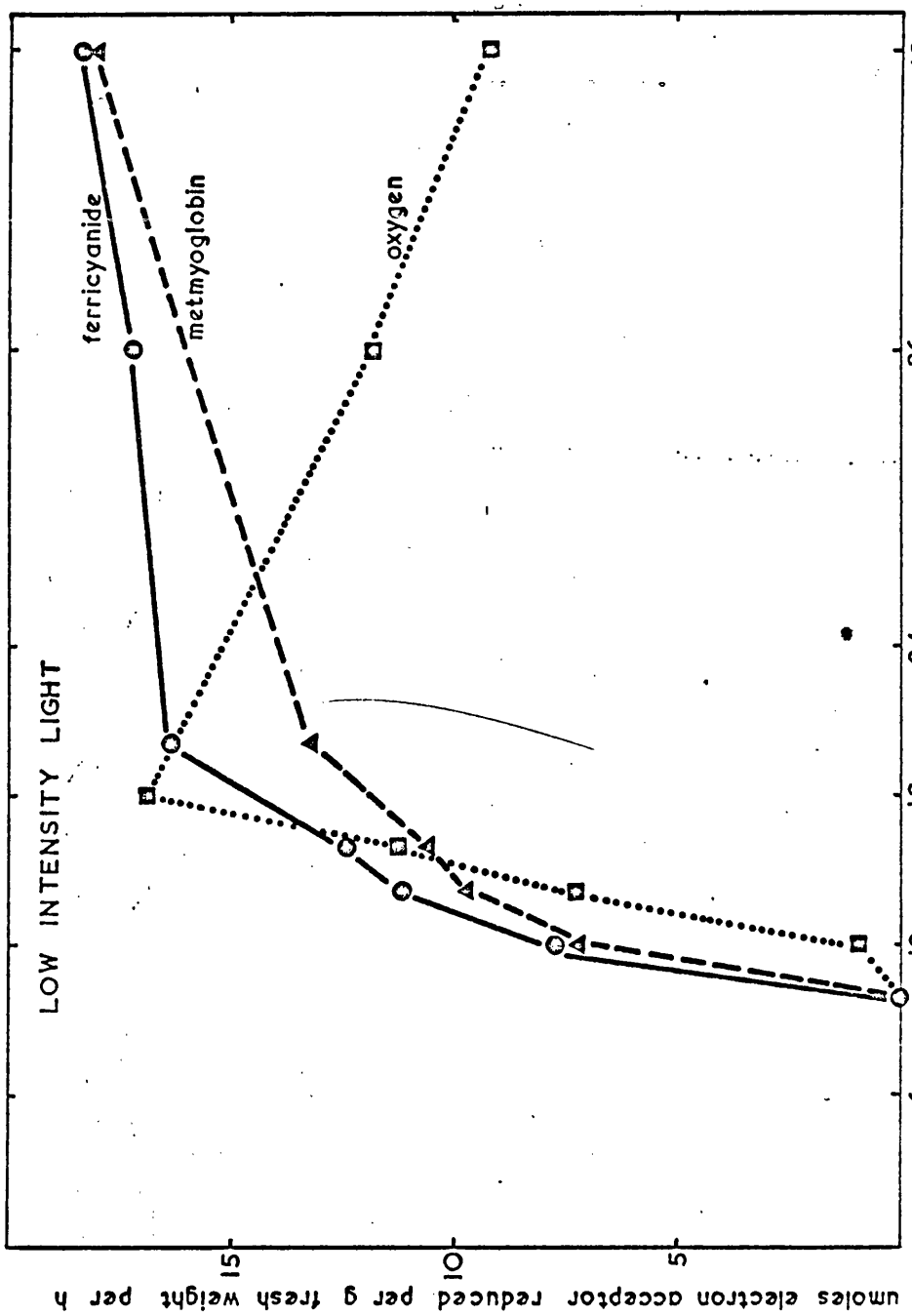


FIG. 24

The onset of photosynthetic electron transport in chloroplasts isolated from greening pea cuttings exposed to low intensity (60 lux) illumination.

content at 12 hours was 0.040 mg/g fresh weight.

The rates of reduction of the electron acceptors continued to increase with the further development of the chloroplast. In the chloramphenicol treatment, the maximum rate of reduction of ferricyanide was seen at 18 hours; for metmyoglobin at 30 hours, whilst oxygen reduction was still submaximal at 48 hours (Fig.22). The results of the terramycin treatment differed from these in that although the rate of reduction of metmyoglobin was at a maximum at 20 hours, the rates of reduction of the other electron acceptors were still increasing at 48 hours. Chloroplasts isolated from cuttings exposed to light of low intensity, however, showed the maximal rate of reduction of oxygen at 18 hours and ferricyanide at 20 hours, whilst metmyoglobin reduction was still increasing at 48 hours illumination.

In general the start of electron transport activity in the treated chloroplasts was not detectable until about 8 hours after it was so in the controls. It would appear that the initiation of electron flow is largely determined by the actual amount of chlorophyll present, since the pigment content of both treated and untreated leaves at the time of commencement of electron transport were remarkably similar. This is supported by the observation that the increase in the rate of reduction of the electron acceptors is greater in treated than untreated chloroplasts (compare Fig.21 with Figs.22,23 and 24). In the case of the antibiotic treatments this would suggest the synthesis of essential components of the chloroplast is not only insensitive to these compounds but is completed before sufficient chlorophyll has been incorporated to catalyse the light mediated flow of electrons. However since the low

intensity light treatment also gave results similar to that of the antibiotic treatments, it might be concluded that the development of the chloroplast structural and enzymic proteins is either in advance of or in strict parallel with chlorophyll formation thus achieving a functional chloroplast immediately electron flow commences.

It is interesting to note that the antibiotic treatments delayed the onset of the ascorbate - DCIP mediated oxygen reduction by 6 hours longer than that of either ferricyanide or metmyoglobin, whilst the low intensity light treatment had no differential effect. Hence it would appear that the synthesis of the protein(s) of that part of the intermediate electron transport chain responsible for the acceptance of electrons donated by the ascorbate - DCIP couple is retarded by antibiotic treatments. The integration of this part of the chain may not become normal, even after an extensive illumination period and thus give rise to the observations reported in Section 2.

4. Development of photosynthetic carbon dioxide fixation

(a) Controls

The data shown in Fig.25 indicated that the light induced assimilation of carbon dioxide was first observed in untreated greening pea cuttings after 4 hours illumination, the compensation point was reached after 17 hours and thereafter the apparent photosynthetic carbon dioxide fixation exceeded the respiratory evolution. The dark respiration rate of the greening leaves increased slowly to reach a maximum after 8 hours of illumination, and then remained fairly constant over the remainder of the illumination period. This time of commencement of apparent photosynthesis of the whole leaf

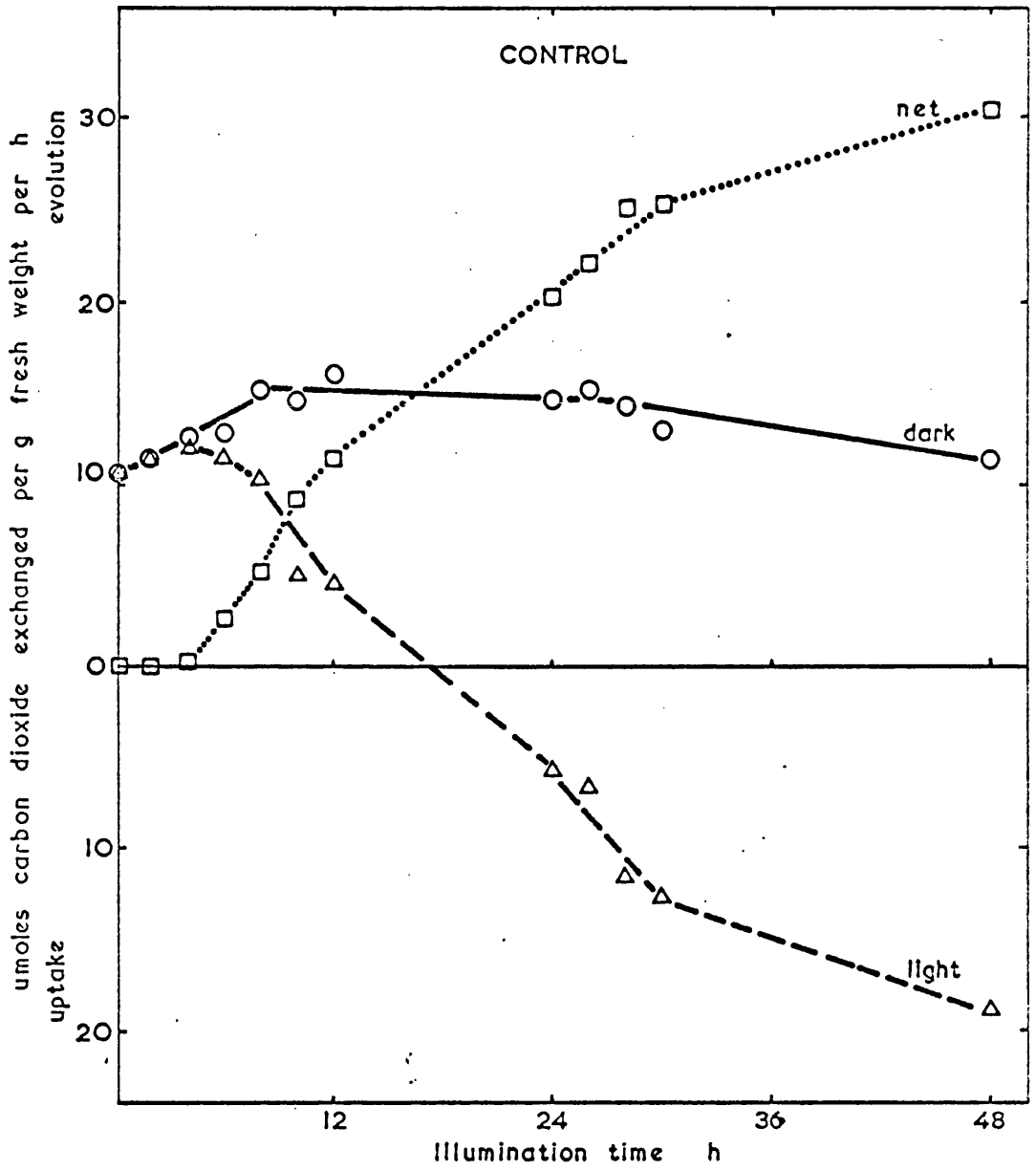


FIG. 25 **The changes in respiratory and photosynthetic carbon dioxide exchange in greening <u>untreated</u> pea cuttings. The curve labelled 'net' represents the 'dark' rate minus the 'light' rate.**

compares favourably with the start of electron transport in untreated isolated chloroplasts.

(b) Comparison of the effects of the treatments

An outstanding feature of all the carbon dioxide exchanges of the treated leaves is the marked increase in the evolution of carbon dioxide in the light, during the first 8 - 10 hours of greening (Figs.26,27,28). During this period photosynthesis was known to be non-existent since photosynthetic electron transport was absent. Hence the light induced evolution of carbon dioxide cannot be related to the 'light respiration' described by Krotkov (1963) which requires a functional photosynthetic system. A report by Schiff (1963) demonstrated a light enhanced rate of oxygen uptake during the early stages of chlorophyll formation in Euglena cells. It was suggested that this phenomenon was caused by the light induced activation of synthetic pathways responsible for the formation of chloroplast constituents. It is thought that the increase in the rate of carbon dioxide evolution in the light, observed in the antibiotic and low intensity light treatments was also a manifestation of this phenomenon.

Some difficulty was experienced in the interpretation of results of the measurement of the light and dark carbon dioxide exchanges in the treated cuttings. The light stimulated carbon dioxide evolution seen for the initial 8 - 10 hours illumination very rapidly disappeared as the photosynthetic fixation of this gas commenced. The biochemistry responsible for this phenomenon could not be assumed to cease immediately photosynthesis started, but the physiological requirement for this effect obviously became less as soon as photosynthetic products were available.

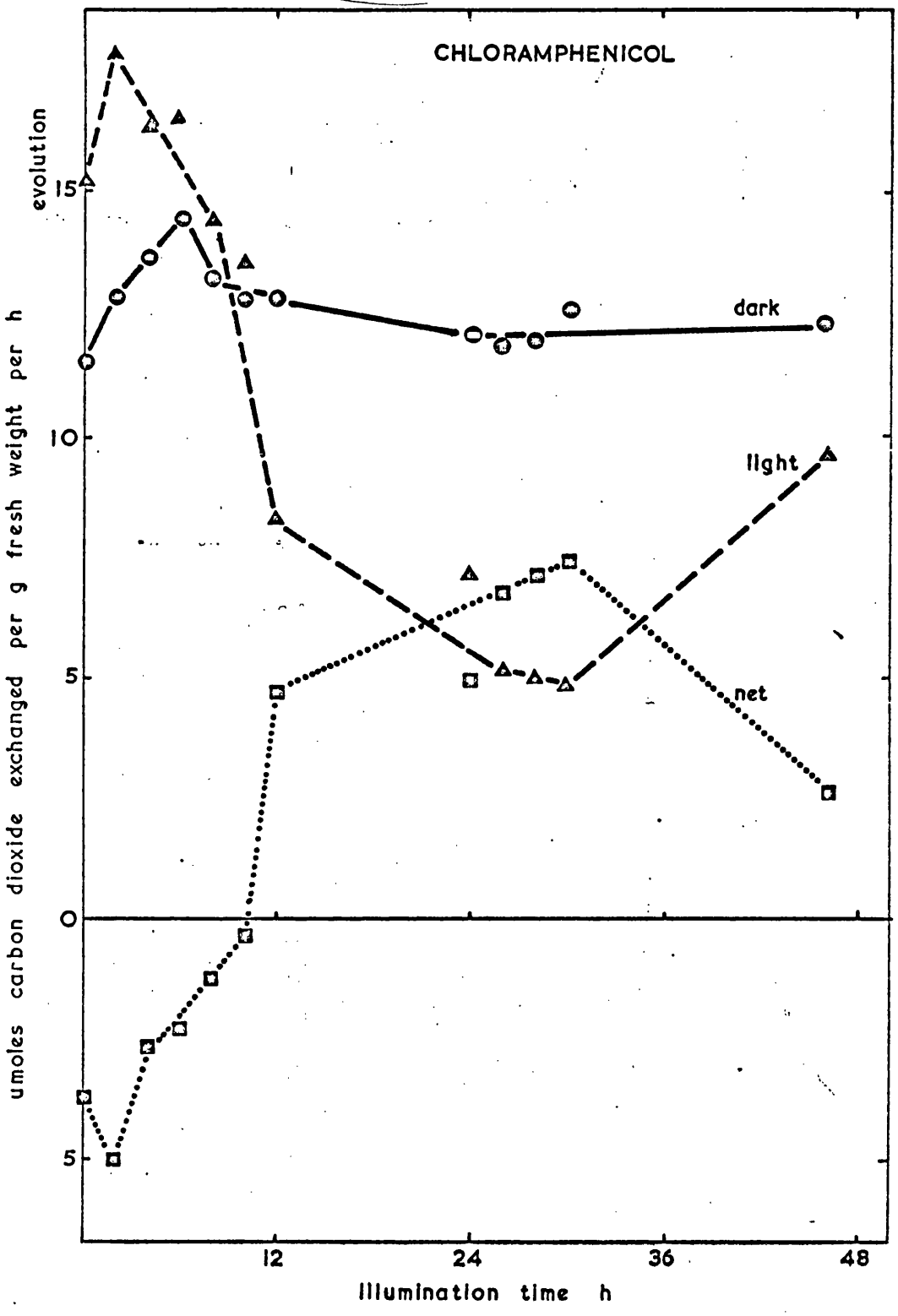


FIG. 26 The changes in respiratory and photosynthetic carbon dioxide exchange in greening chloramphenicol treated (1.5 mg/ml) pea cuttings. The curve labelled 'net' represents the 'dark' rate minus the 'light' rate.

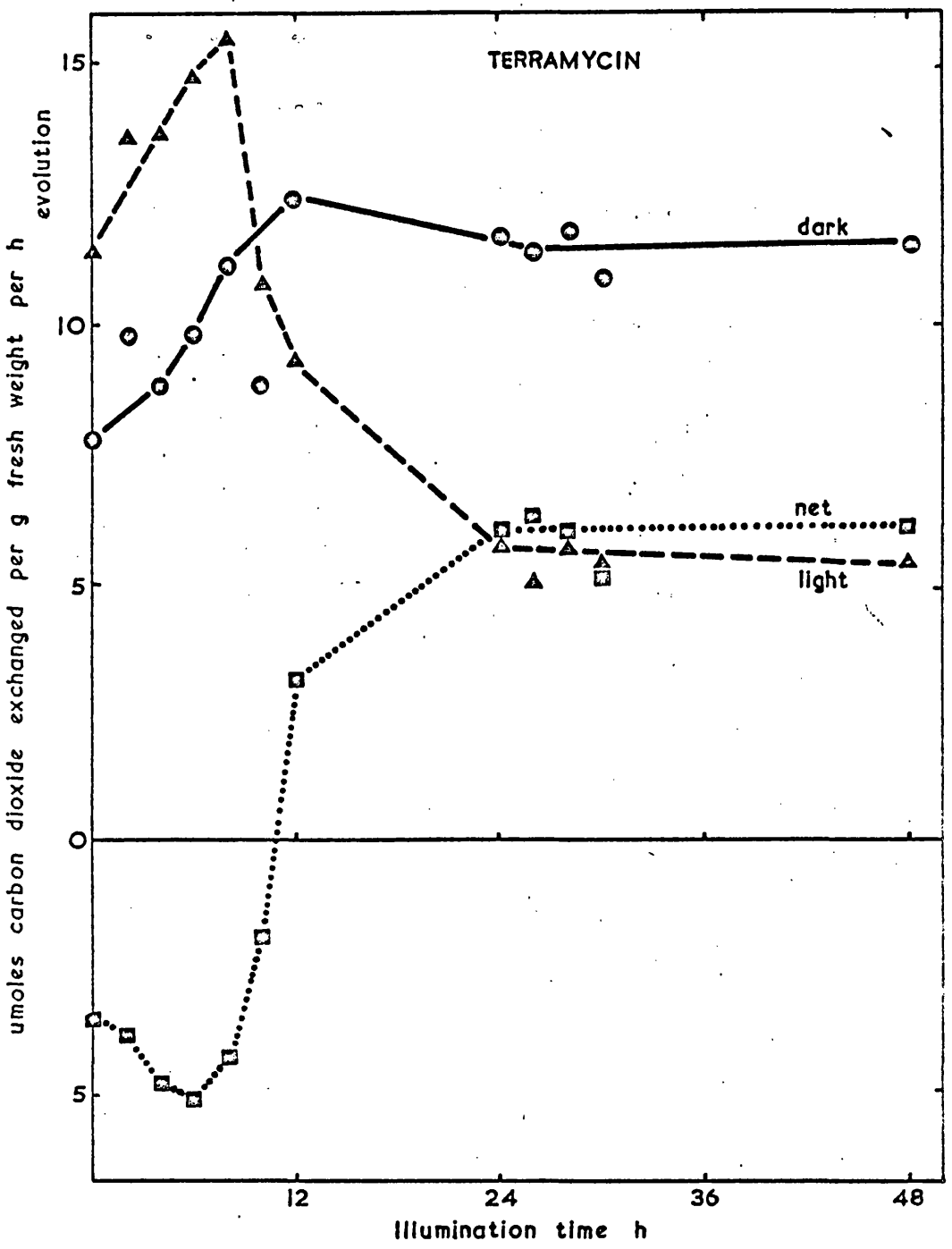


FIG. 27 The changes in respiratory and photosynthetic carbon dioxide exchange in greening terramycin treated (0.7 mg/ml) pea cuttings. The curve labelled 'net' represents the 'dark' rate minus the 'light' rate.

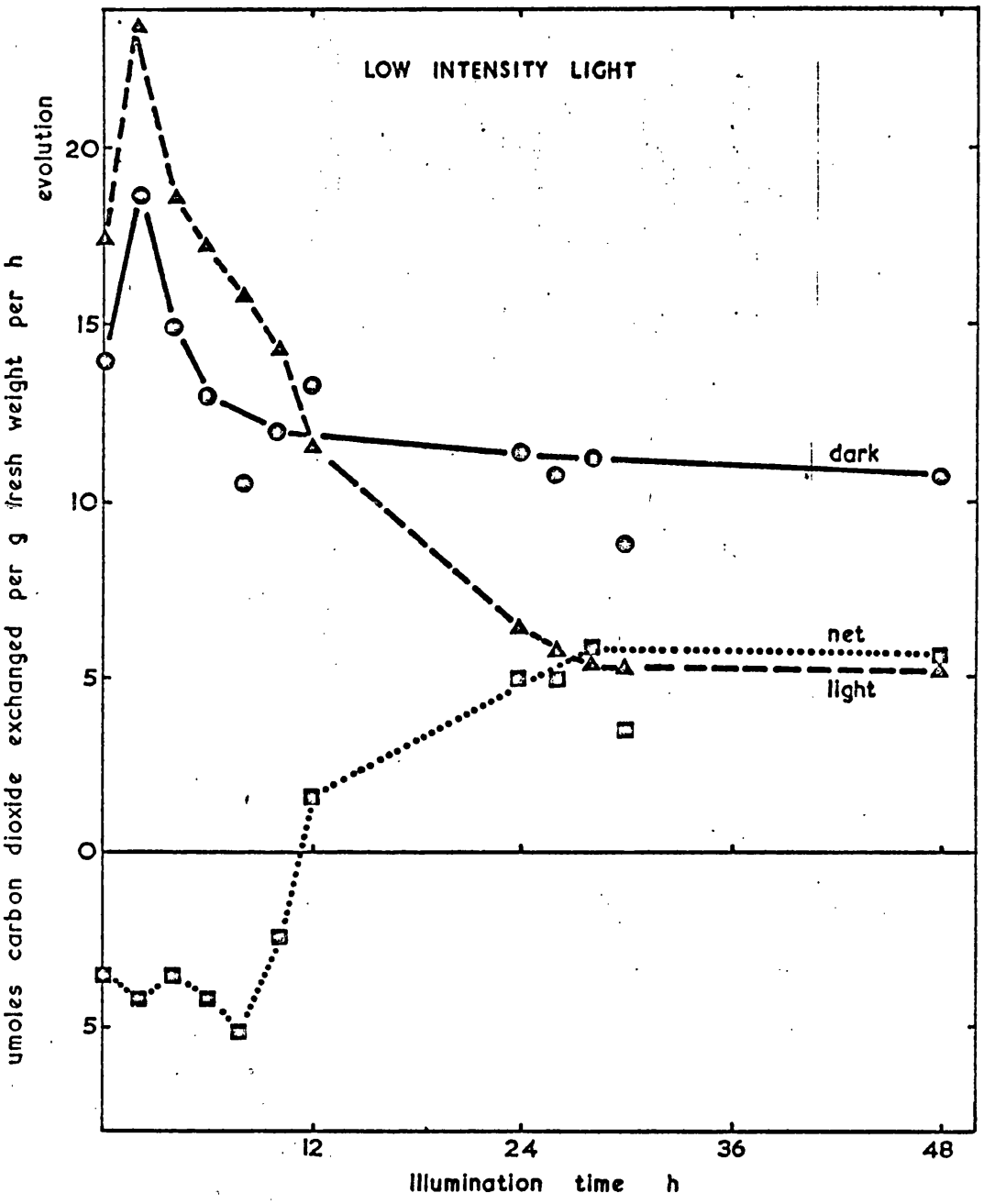


FIG. 28 The changes in respiratory and photosynthetic carbon dioxide exchange in greening pea cuttings exposed to light of low intensity (60 lux). The curve labelled 'net' represents the 'dark' rate minus the 'light' rate.

Although for the purposes of this investigation, photosynthesis was taken to have commenced once the illuminated rate of carbon dioxide exchange was less than the dark rate, the attainment of this criterion may well have occurred sometime after the actual beginning of photosynthesis. This objection however does not invalidate the comparison of these results with those of the onset of electron transport, since the techniques of chloroplast isolation and assay of electron transport are probably insufficiently sensitive to the very low activities found at the onset.

The beginning of photosynthetic carbon dioxide assimilation occurred after about 10 - 12 hours of illumination in all of the treatments (Figs.26,27,28). Since there was little or no discrepancy between this time and that of the onset of the chloroplast electron transport it appears likely that the chloroplast possesses all of the enzymes associated with the Calvin cycle at this stage of development.

The rate of apparent photosynthesis continued to increase with continued illumination to reach a maximum at about 30 hours and this level of activity was maintained in all but the chloramphenicol treatment. The restricted rate of carbon dioxide assimilation in terramycin and low intensity light treatments probably reflected the limited electron transport activity observed in chloroplasts isolated from the cuttings exposed to these agents.

However the effect of chloramphenicol on the fixation of carbon dioxide appeared to be different from the other treatments (Fig.26). Margulies (1964) has reported that chloramphenicol inhibited the formation of ribulose-1,5-diphosphate carboxylase

in greening bean leaves. The eventual decline in the rate of carbon dioxide assimilation after 30 hours illumination may be attributed to the antibiotic preventing the further synthesis of at least this one enzyme of the Calvin cycle.

It is noted that none of the treatments have any marked effects upon the rate of dark carbon dioxide evolution.

DISCUSSION

It is apparent that photosynthetic competence in the greening chloroplast is attained only after quite extensive changes in fine structure have occurred, the completion of which is dependent upon prolonged illumination providing photosynthetic assimilates, ATP and reduced NADP (Klein and Neuman, 1966; Rhodes and Yemm, 1966). However the structure of a chloroplast which had been treated with the protein synthesis inhibitor chloramphenicol, or exposed to light of low intensity is very different from an untreated chloroplast. Margulies (1966) has reported that in chloramphenicol treated bean leaves the chloroplasts have no readily identifiable fret-work structure, the thylakoids being represented by vesicles, which are seen singly or in chains, the membranes of which are not markedly different from those of untreated chloroplasts. Also in the chloroplasts of bean leaves which had been exposed to light of low intensity, ring-like structures of vesicles, together with rows of lamellae or tubuli, were observed (Eliam and Klein, 1962). The aberrations of fine structure induced by these agents would be expected to lead to a malfunction of the photosynthetic electron transport system. This is indeed so with chloramphenicol (Margulies, 1964). There appear to be no reports of fine structural changes in higher plant chloroplasts resulting from terramycin treatments.

The application of chloramphenicol to illuminated dark grown leaves (Margulies, 1962) and algae (Pogo and Pogo, 1965) inhibited protein synthesis leading to the almost complete prevention of chlorophyll synthesis. This study has confirmed that chloramphenicol prevented chlorophyll formation in greening pea cuttings, the chlorophyll content of which appeared to be inversely proportional to the amount of inhibitor applied.

Chlorophyll synthesis is dependant upon a continual supply of δ - aminolevulinic acid synthetase (Gassman and Bogorad, 1967 a; Margulies, 1967). They suggest that it is the synthesis of this enzyme, controlled by a short-lived messenger RNA, which is inhibited by protein synthesis inhibitors, e.g. chloramphenicol and actidione. It would seem therefore that at the lower concentrations of antibiotic applied the formation of δ - amino - levulinic acid synthetase from the messenger RNA is not completely inhibited, thus allowing some synthesis of chlorophyll.

It might be surmised that the terramycin induced inhibition of chlorophyll synthesis may be the result of a similar interference with protein formation from RNA, since in bacteria this antibiotic prevents protein synthesis from RNA (Gale and Folkes, 1953, cited by Franklin, 1966). In addition, the extent of the inhibition of chlorophyll may be heightened by the affinity of terramycin for magnesium and manganese ions (Brody, Hurwitz and Bain, 1954, cited by Franklin, 1966).

In leaves not treated with antibiotics, the rate of chlorophyll synthesis was limited by the intensity of the incident light. At intensities below 160 lux, up to 36 hours of illumination were required before the rate of chlorophyll synthesis became maximal, but at greater intensities the rate increased with time. Greening bean seedlings exposed to light of 20 lux intensity attained a maximal chlorophyll content after only 5 hours illumination and this level remained constant for 40 hours (Eliam and Klein, 1962). However in greening barley, 30 hours exposure to light of 32 lux intensity was required before the maximal rate was attained (Huffaker, et al., 1966).

In the present study after illumination for 20 hours the ratio of chlorophyll a to b increased with the intensity of the incident

light, but after 40 hours, no change in the ratio was observed at intensities greater than 160 lux. The formation of chloroplasts in Euglena was initiated by light of low intensity (below 70 lux) and was completed by exposure to light of high intensity (300-1000 lux) (Stern, Epstein and Schiff, 1964). The reaction responsible for the initiation of development may be the photoreduction of protochlorophyll, a reaction which is known to require small amounts of energy (Eriksson, et al., 1961). Proplastid expansion, formation of lamellar discs, a limited synthesis of chlorophyll and a restricted photosynthetic capacity are induced by the low light intensity. High light intensity would then be responsible for the completion of chloroplast development and the attainment of maximal photosynthetic competence (Stern, Epstein and Schiff, 1964). Thus in greening pea seedlings, it would seem that both these light steps are essentially complete by 40 hours of illumination at above 160 lux intensity. However after only 20 hours illumination, the development induced by high light intensity is still continuing and hence may be stimulated by an increase in the incident light intensity. These findings suggest that the requirement for high light intensities is a manifestation of the need for an adequate supply of photosynthetic assimilates during the later stages of chloroplast development (Klein and Neuman, 1966). The importance of photosynthesis is also indicated by the increase in the ratio of chlorophyll a to b with increasing duration of illumination, at all but the highest concentrations of chloramphenicol or terramycin.

It is interesting to note that the synthesis of chlorophyll a is more sensitive to chloramphenicol and terramycin than is chlorophyll b. There is some disagreement on the final stages

of chlorophyll b synthesis, but extensive studies (Shlyk, et al., 1963; Shlyk and Losev, 1963; Shlyk, Losev and Shevchuk, 1967) appear to substantiate the view that chlorophyll a is the immediate precursor of chlorophyll b. The greening of etiolated barley leaves in flashing light has also indicated that at least the major part of the biosynthetic route is common to both chlorophylls a and b (Augustinussen, 1964). It is difficult to reconcile the observed antibiotic sensitivity of chlorophyll a synthesis with these reports. It may well be that the pathway leading to chlorophyll b is not identical with that of chlorophyll a. More probably however, due to the antibiotic - induced malformations in lamellar structure, the integration of all the chlorophyll a produced may be limited, and the excess pigment destroyed by photo-oxidation.

In the study of the photosynthetic electron transport system of the chloroplasts of treated leaves, the choice of comparable untreated material is important. The results from treated leaves can be compared with control cuttings which had been illuminated for 18 hours, these having a chlorophyll content similar to that of treated leaves. This comparison assumes that chlorophyll content is a reliable indicator of the degree of chloroplast development and that the formation of the other components of the chloroplast do not require longer periods of illumination to attain functional levels. This latter objection may be overcome by using untreated leaves which had been illuminated for 48 hours, i.e. for the same duration as the treated leaves, but of course the chlorophyll content is much greater. The evidence presented here appears to support the contention that chlorophyll content is indeed a reliable indicator of at least the extent of

development of photosynthesis.

The development of chloroplast electron transport activity in greening leaves has been studied previously (Smith, French and Koski, 1952; Anderson and Boardman, 1964; Smillie, Evans and Lyman, 1963), but as far as is known, no investigation has been made of the onset of electron transport in chloroplasts in which the rate of chlorophyll synthesis has been artifically retarded.

The state of the electron transport system assessed in chloroplasts from treated and untreated leaves, after 48 hours illumination, provided the data presented in Fig.19. It is noted that although each of the treatments partially inhibited the formation of chlorophyll to the same extent, their effects upon chloroplast electron transport are rather different. Accepting the reservations about the choice of control material, it is seen that all the treatments have markedly interfered with the development of Light System II, whether assayed by ferricyanide or metmyoglobin. It is well known that this part of the photosynthetic electron transport system is very sensitive to disruptive agents, e.g. sonication (Katoh and San Pietro, 1966), detergents (Vernon, Shaw and Ke, 1966; Anderson and Boardman, 1966) and heat or age of prepartion (Vernon and Kamen, 1954; Vernon and Zaugg, 1960; Okayama, 1967). Thus it is reasonable to propose that the treatments used here hinder the transport of electrons via Light System II by the induction of structural defects in the chloroplast lamellae. The site of these aberrations appears to be localised in the intermediate electron transport chain between Light Systems I and II, since chloramphenicol treatment induces a stimulation in the rate of

electron donation by ascorbate - DCIP, via Light System I, to either NADP or oxygen, whilst the other treatments inhibit this reaction. It is speculated that this stimulation of the rate of this reaction is caused by the abnormal 'exposure' of the protein responsible for accepting electrons from ascorbate - DCIP thus improving the efficiency of donation. This concept is supported by the fact that treatment of chloroplasts with ultra-sound or heat can result in conformational changes which increase the accessibility of a site to which ascorbate can donate electrons directly, without the need of DCIP (Davenport and Dodge, 1969). Chloroplasts treated with digitonin (Anderson and Boardman, 1966) also demonstrate an enhanced rate of Light System I mediated ascorbate - DCIP photo-oxidation.

The inhibition of this reaction by terramycin or light of low intensity may be the result of the prevention of the synthesis of the protein which accepts electrons from ascorbate - DCIP, whilst the low rates of cyclic photophosphorylation also support the contention that the treatments induce structural deformities in the chloroplast lamellae. It appears therefore that all the treatments have effected the integration of the many components of the intermediate electron transport chain resulting in chloroplasts in which the two Light Systems are not properly co-ordinated. It may well be that the physical separation of Light System I from Light System II by detergent treatments (Anderson and Boardman, 1966; Hind, 1968 a; Wessels, 1968) occurs at a position in the intermediate electron transport chain which is identical with the site of action of antibiotic or low intensity light treatments, and is associated with plastocyanin (Davenport and Dodge, 1969).

Investigations into the onset and later development of electron transport activity in untreated chloroplasts have confirmed that this is absent in etioplasts, but commences 3 - 4 hours after the beginning of illumination and attains the maximal rate after 10 - 18 hours. However the reduction of metmyoglobin continues to increase with prolonged illumination, providing a further indication that the full co-operation of the two Light Systems requires an extensive period of development.

The treatment of the etiolated cuttings with agents which extend the duration of the chlorophyll synthesis lag period also causes a delay in the onset of photosynthetic electron transport. Generally the photoreduction of the electron acceptors did not occur until some 12 - 14 hours after the beginning of the illumination period. The significant observation is that in both treated and untreated leaves, the chlorophyll content at the onset of electron transport is identical, at 0.04 mg/g fresh weight. This further substantiates the view that a correlation exists between the increasing structural complexity of the greening chloroplasts and the development of photosynthetic activity (Rhodes and Yemm, 1966; Gyldenholm and Whatley, 1968).

The photo-oxidation of ascorbate - DCIP by Light System I in chloramphenicol and terramycin treated leaves, however, required a 4 hour longer illumination period to develop than did either ferricyanide or metmyoglobin reduction. The low intensity light treatment also delayed the onset of this reaction by 1 - 2 hours. Gyldenholm and Whatley (1968) have observed that the onset of phenazine methosulphate mediated cyclic photophosphorylation in greening bean leaves occurs before that of the ascorbate - DCIP mediated NADP reduction. However in this study,

as all the electron acceptors are artificial, i.e. do not require ferredoxin, the delay in the onset of photoxidation of ascorbate - DCIP in comparison with that of ferricyanide or metmyoglobin, is probably due to a slowed development and integration of the protein(s) which accept the electrons from the donor. If this is the case, then during the initial period of electron transport, the pathway utilised by electrons reducing ferricyanide or metmyoglobin must bypass the as yet undeveloped site of ascorbate - DCIP donation. In a study of the light induced absorbance changes of cytochrome b_6 in spinach (Hind and Olson, 1966; Hind, 1968 b) it has been proposed that cytochrome f is a component of the electron transport chain containing a phosphorylation site and that cytochrome b_6 lies on a non-phosphorylating bypass. This scheme also accounts for the independance of basal and coupled electron flow systems revealed by the use of phlorizin and other inhibitors (Izawa, et al., 1966). Unfortunately due to practical difficulties it was not possible to study the effects of various phosphorylation uncouplers on treated chloroplasts and therefore the site of antibiotic and low intensity light inhibition of ascorbate - DCIP photo-oxidation cannot be associated with either of the two routes proposed by Hind (1968 b).

The onset of carbon dioxide assimilation by the greening leaves of treated and untreated cuttings was found to be coincident with that of photosynthetic electron transport. Although chloramphenicol had no effect upon cell respiration (Havinga, et al., 1953), a report by Margulies (1964) indicated that it inhibited the formation of ribulose-1, 5-diphosphate carboxylase and hence may be expected to delay the onset of carbon dioxide fixation in comparison with electron transport. This did not occur, although

the eventual inhibition of the synthesis of this enzyme is indicated by the decreasing rate of carbon dioxide assimilation after 30 hours illumination. In the terramycin and low intensity light treatments, steady rates of carbon dioxide exchange in the light were observed after 30 hours. Thus it appeared that none of the treatments markedly affect the initial formation and (with the exception of chloramphenicol) continuing synthesis of the enzymes of the carbon fixation cycle, the rate of carbon assimilation being apparently limited by the photosynthetic supply of ATP and reduced NADP. It is perhaps unlikely that the treatments used would have prevented the dark synthesis of the enzymes responsible for the assimilation of carbon dioxide, since most of the enzymes would be present in the etioplast before treatment (Bradbeer, 1969). Previous studies of the development of carbon dioxide fixation have indicated that it follows closely that of chlorophyll synthesis (Irving, 1910; Tolbert and Gailey, 1957; Stern, Schiff and Epstein, 1964; Oh-hama, Shihira-Ishikawa and Hase, 1965; Rhodes and Yemm, 1966) and hence is dependant upon a functional electron transport system. Also the presence of at least one completed lamella per plastid is required in Euglena cells, before carbon dioxide fixation occurs (Ben-Shaul, Schiff and Epstein, 1964).

An outstanding feature of the effect of the treatments on exchanges of carbon dioxide is the marked increase in the evolution of this gas, in the light, during the first 8 - 10 hours of illumination; this phenomenon was not detected in the untreated leaves. It has been reported that in dark grown wheat leaves, increased oxygen consumption occurs immediately upon exposure to light (Gabrielsen, Madsen and Vejlby, 1961).

It has been suggested (Schiff, 1963) that the photostimulated increase in oxygen uptake observed during the initial stages of greening Euglena cells, may result from the light induced activation of the oxidative pathways responsible for the formation of chloroplast constituents. This proposal may also explain the unusual carbon dioxide exchanges observed here. It is not considered that this phenomenon is related to 'light respiration' (Krotkoy, 1963; Bunt, 1969), since this effect is dependent upon a functional photosynthetic system, which is absent at the time at which the phenomenon was observed.

A scheme summarising the findings of many workers and the results of this investigation (Fig.29) provides a picture, albeit incomplete, of the physiological relationships between the many biochemical pathways involved in chloroplast development. This study has shown that the development of the photosynthetic electron transport system is supported by the supply of intermediates and energy, derived initially from respiration and later from photosynthesis. Thus the fully grown chloroplast results from a highly complex sequence of inter-related reactions, initiated by light and controlled partly by the photomorphogenic receptors, e.g. phytochrome (Graham, Grieve and Smillie, 1968; Withrow, Wolff and Price, 1956; Mitrakos, 1961) and protochlorophyll (Smith and Benitez, 1954). The stimulation of such receptors leads to the production of a chloroplast specific messenger RNA (Gnanaam and Kahn, 1967 a) synthesising the structural and enzymic proteins of the chloroplast (Gnanaam and Kahn, 1967 b). It is obvious that in the etiolated condition, the development of the proplastid is entirely dependant upon precursors and energy supplied by the cell. Even after the light initiated conversion

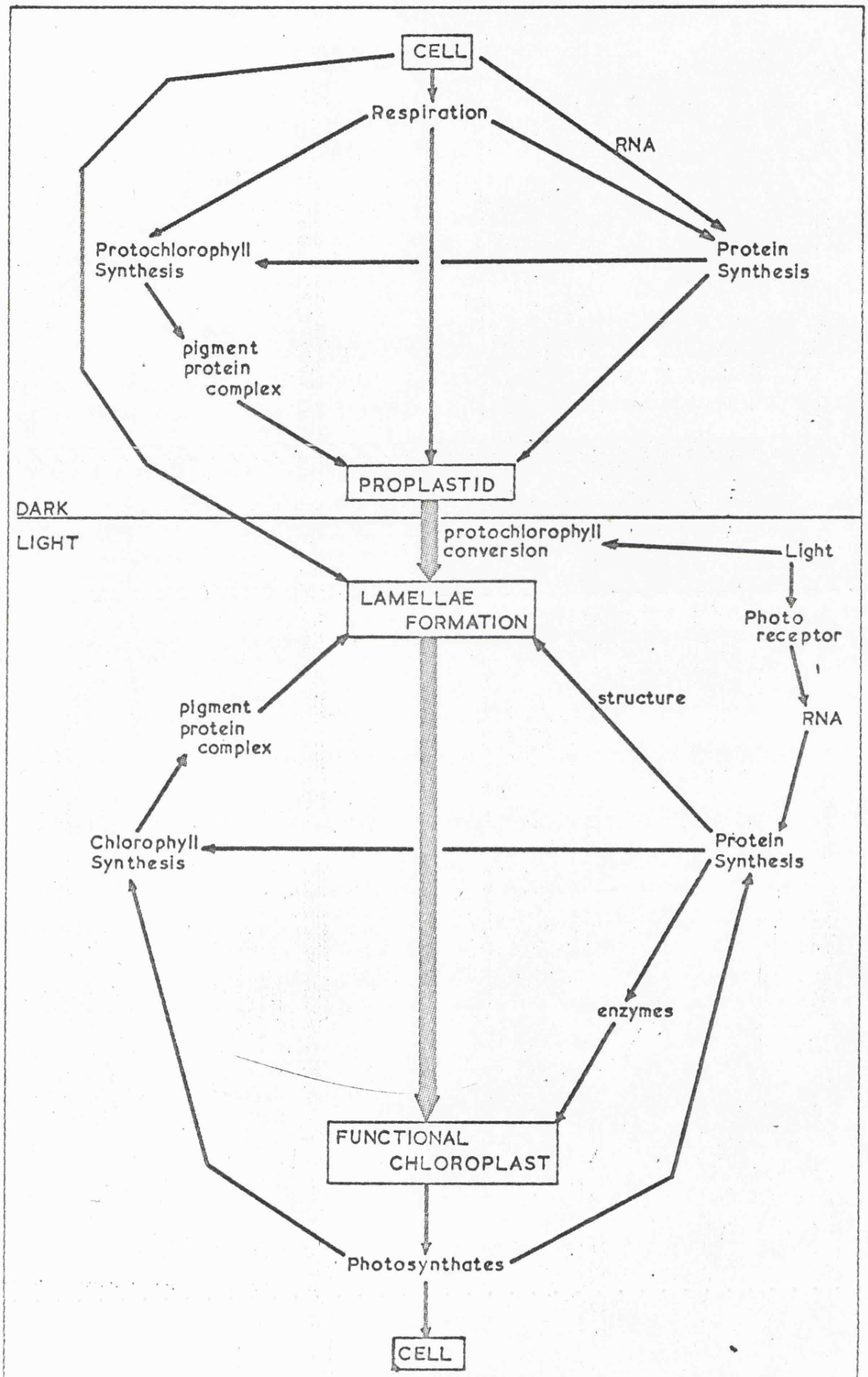


FIG. 29 A schematic representation of the physiological relationships of several biosynthetic pathways involved in the development of the chloroplast.

of the proplastid into a chloroplast, a large proportion of the necessary energy and intermediates are still provided by the cell during the early stages of development (Klein and Neuman, 1966; Wolff and Price, 1960). The experimentally induced deficiency of photosynthetic assimilates in greening Euglena cells treated with DCMU also demonstrated that, by drawing on the reserves of the cell, chloroplast development can proceed normally (Schiff, Zeldin and Rubman, 1967). Only after the onset of photosynthetic electron transport and carbon dioxide assimilation does the developing chloroplast become increasingly self-sufficient.

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