STUDIES ON THE HOST-PARASITE RELATIONSHIPS OF CUSCUTA CAMPESTRIS YUNCKER AND CUSCUTA REFLEXA ROXB.

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I. INTRODUCTION AND LITERATURE REVIEW.

From a study of the literature on the genus Cuscuta it was clear that many large gaps existed in the physiology of this parasitic genus. No experiments had been done to test its photosynthetic ability; and even though many writers have recorded the presence of chlorophyll in Cuscuta, modern text-books describe the plant as entirely heterotrophic.

Apart from casual references to the occurrence of chlorophyll in the parasite, no systematic quantitative analyses of the chlorophyll content of any member of the genus have been published. The carotenoid composition of the plant is even less fully investigated, with the single exception of MacKinney's analysis of the pigments of Cuscuta salina (1935). Despite the existence of a comprehensive anatomical account of the haustorial connections in Cuscuta, little attention has been given to the structure and occurrence of plastids in the genus. Again, enzymic studies on Cuscuta have not been investigated in any detail, at any rate by modern methods.

The following work was therefore undertaken in an attempt to fill in some of these gaps in our knowledge of the physiology of the genus, with the aid of such modern techniques as chromatography and the use of radioactive isotopes, and while any

conclusions which are drawn apply only to Cuscuta reflexa and Cuscuta campestris, they probably reflect to some extent the condition obtaining throughout this comparatively uniform genus.

Only two species of Cuscuta have been investigated in this work, C. campestris Yuncker and C. reflexa Roxb. C. campestris is grouped by Yuncker(1932) in the Section Cleistogrammica of the subgenus Grammica, and C. reflexa is classed as the only member of the Section Callianche of the subgenus Monogyna.

Cuscuta is world-wide in distribution, with the largest number of species occurring in North and South America. According to Yuncker, they extend in the Old World from about the 60th parallel north in Europe and Asia to the Cape region of South Africa, but appear to be absent from the Philippines. The wide and unnatural distribution of many species of Cuscuta is attributable to accidental sowings of the parasite's seeds along with seeds of economic crops.

The most recent comprehensive taxonomic study of the genus is by Yuncker (1932). In this monograph he describes a total of 158 species of Cuscuta, of which 25 were then new to science.

A great deal of detailed anatomical work, particularly concerning the haustorial region was done about the beginning of

the present Century, eg. Peirce (1893) and Thoday (1911). Since then the work has been in the main sporadic, but includes a number of investigations into the general physiology of the plant.

Early workers were much preoccupied with the nature of the intimate connections between host and parasite, the mechanism of haustorial penetration, whether mechanical or chemical, and the method whereby the phloem of the parasite is linked to that of the host. Von Mohl (1827) noticed that when a Cuscuta had wound about a polished silver rod, the position of the haustoria against this rod was indicated by spots of a viscid fluid, which he believed to be mucilaginous matter fastening the parasite to the host. Koch (1880) however considered it to be a solvent secreted for the purpose of softening if not dissolving the opposing tissues of the host, but he gives no experimental proof of this.

That a chemical solubilising agent is present in the haustoria of Cuscuta was conclusively demonstrated by Peirce (1894). He induced the parasite to form haustorial coils round a stick made of two parts of plaster of Paris to one of potato starch, and noticed marked corrosion of the starch grains in the region of the haustoria. Similarly by allowing a parasite to coil round a stick of elder pith he was able to show that the haustorial hyphae passed cleanly through the cells of the pith, without any evidence of distortion which would be caused by mechanical pressure.

In the same paper Peirce neatly shows that the haustoria of C. glomerata can also exert a considerable mechanical pressure. He wound a single sheet of tin-foil, 1/5mm in thickness, round a succulent Impatiens stem, and allowed the parasite to form haustorial coils round it. Eventually the foil was ruptured allowing the haustoria entry into the stem.

In a detailed paper on the structure of the haustoria of Phanerogamic parasites Peirce (1893) states that in C. americana the xylem elements of the haustorium apply themselves directly to the xylem elements of a fibro-vascular bundle in the host. The haustorial coils, once they have contacted the vessels, quickly differentiate into tracheids, the thickened and unthickened regions in their walls corresponding with the thick and thin places in the walls of the vessels to which they apply themselves. The phloem cells of the haustorium are united with the phloem cells of the same bundle by a direct union, and the companion cells of the haustorial sieve tubes unite directly with the companion cells of the sieve tubes of the foster plant. Of C. europaea he says that the conducting elements of both xylems and phloems unite directly with the corresponding elements in the host and furnish an unbroken connection between the conducting tissues of the parasite and its host.

According to Thoday (1911), the development of the sieve

plates and sieve fields in the phloem of Cuscuta reflexa is found to agree in all essentials with Vitis (Hill, 1908) and Laminaria (Sykes, 1908). The central hyphae of the haustorium push into the pith or fuse with the xylem of the host, the ones next surrounding these fuse with the sieve tubes, while the peripheral ones remain in the cortex. When a tip of a hypha is connected with a sieve tube of the host, this strand of cells is developed into a strand of short sieve tubes. Connecting threads and sieves are numerous in the subdivision walls, but are never formed in the fusion walls between the originally separated hyphae; that is they are only found in those cells which are genetically connected.

Preparatory to the formation of a junction with a host sieve tube, an invading hypha lays itself more or less alongside the sieve tube wall so that the two come into lateral contact. The mucilaginous wall of the parasite when it touches a sieve area is then absorbed and the naked protoplasm of the hypha applies itself to the sieve area of the host. It is nearly always with a sieve field, occasionally with a lateral plate, that a junction is made; the junction sieve plates and sieve fields in all cases exactly resemble those of the host under normal conditions. The protoplasm of the parasite is easily shrunk away from the sieve area and is probably never closely fused with the host protoplasm. The translocation of food substances from host to

parasite would appear to be of the nature of passive filtration, the contents of the sieve tubes forced by internal pressure, escaping through the lateral sieve fields into the parasite.

Thomson (1925) throws doubt on these observations of Peirce (1893) and Thoday (1911) that an intimate connection exists between the phloem elements of host and parasite. He states that the modified root hairs arising from the dermal cells of the haustorium of C. reflexa (in Cucurbita) never attach themselves to the phloem either on the outside or on the inside of the bicollateral bundle. He also mentions, contrary to the findings of Peirce and Thoday, that the strand of tracheids linking the xylem of the host with that of the haustorium is not accompanied by any phloem either in the host tissue or where it enters the haustorium; in fact that no definite connection exists between the host phloem and the haustorium. His view is that the nutrition of the parasite is derived from haustorial hyphae in the host cortex, and from organic substances transported via the xylem connections.

The origin of the haustorium, like that of lateral roots, is in the region of the pericycle (Peirce, 1893). The surface cells of the haustorium are sufficiently different to be called epidermis and in the young haustorium should be called dermatogen.

The cortex, consisting of several layers of cells at the base, is reduced to a single layer at the apex of the haustorium. It evidently originates from a periblem. The cells of the sucker are physiologically root hairs, but correspond in origin and position with the cap of ordinary roots; the haustorium is otherwise destitute of a cap.

There are differing reports in the literature on the anatomical effects produced by Cuscuta on the tissues of its hosts. Peirce (1894) states that the effect of the parasite on its hosts is not marked, though on the petiole of Solanum jasminoides, there was a general enlargement of the whole structure and decided enlargements in the zone where the haustoria were most abundant. It is interesting to note that this enlargement, though partly due to an increase in the number of layers of cortical parenchyma, was also partially due to an increase in the width of the band of xylem in which the proportion of fibres to ducts was greater than before.

The effects on its hosts brought about by C. reflexa are described by Thomson (1925). On Fuchsia, the change in the host is visible as a rupture of the outer tissues of the stem, caused by an increase in the host xylem and phloem in the vicinity of the haustorium, and in Geranium, the reaction of the host is the production of a warty protuberance at the point of infection, due

to an increased activity of the cork cambium with the resultant formation of an extensive phelloderm. In some stems, eg. Ficus carica, a distinct cambium originates in the cortex on either side of the haustorium. This cambial layer, formed between the cambial ring of the stele and the cork cambium, is distinct from any previously existing cambium, and results in the production of radial rows of parenchymatous cells, thereby adding to the width of the cortex. Though this increase in stem diameter may prevent the haustorium from reaching the vascular strands of the host, Thomson points out that the parasite must benefit from the flow of food material to the actively dividing zones.

Commenting on the ecological relationship of Cuscuta and its hosts, Peirce (1894) observes that unlike the fungi, which find easier entrance and more suitable conditions for growth and development when the host is ailing, Cuscuta flourishes best when the host is vigorous, and not liable to succumb to the drain on its food reserves. It is a distinct disadvantage to Cuscuta when its host dies before it itself has come to flower and set seed. It demands much food and strong mechanical support in order that its flowers may be advantageously displayed for insect pollination. Weak hosts cannot nourish well; hosts which easily succumb after being attacked do not afford the necessary mechanical support, and hosts which vegetate for only a short season cannot assist in

seed dissemination.

Peirce (1894) used the finding of Pfeffer (1885), that wet gelatine did not stimulate contact-sensitive tendrils, to determine the nature of the stimulus involved in the twining habit of Cuscuta. He placed a rod covered with wet gelatine in the path of an exploratory filament of Cuscuta, and noticed that the parasite made only long steep turns about the rod. Since Cuscuta, unstimulated, does not form close spirals, he concluded that the behaviour of the parasite provided positive evidence that a contact-irritation mechanism is normally involved. The same writer (1894) showed that haustoria commenced to develop when a branch of C. glomerata was grown between two leaflets of Phaseolus, the whole being confined between two sheets of glass so that no tight coils were possible. This proved that tight coils are not necessary for haustorial formation. He also demonstrated, however, that haustoria do not develop without physical contact, by placing branches of Cuscuta which were ready to form haustoria, in nutritive solutions and finding that under those conditions haustoria never developed.

An interesting series of experiments was performed by Harris (1924) on the osmotic relationships of Cuscuta salina and its host plants. Estimating the osmotic concentration of

the sap of C. salina, growing on the extremely halophytic Allenrolfea occidentalis, by means of freezing point depression, he found the osmotic pressure of Cuscuta sap to be 27 atmospheres, and that of its host to be 44 atmospheres. Estimations of chloride content revealed the sap of Allenrolfea to contain approximately four times as much chloride as Cuscuta (21.8 gm. chloride per litre of sap as opposed to 5.3 gm. for Cuscuta). These results are very different from conditions in the Loranthaceae found by Harris and Lawrence (1916) in which the osmotic pressure of the parasites Phoradendron, Phthirusa and Dendrophthora was nearly always higher than that of the host plants. They also differ from the results of Lilienstern (1932) who found that the ability of Cuscuta monogyna to attack a host successfully was conditional on its having a higher osmotic pressure than its host.

II. ANATOMY.

The two species described in this work, Cuscuta reflexa Roxb. and Cuscuta campestris Yuncker, which are not native to Britain, were obtained from the Botanic Gardens, Glasgow, by courtesy of the Curator, Mr.E.W.Curtis. Both parasites have long, waxy twining stems with scale leaves at irregular intervals (Figs. 1 and 2). The stem of C. reflexa is light-green, mottled with red, and individual filaments may reach a metre in length and up to 8mm in diameter, paricularly in the region of the haustorial coils (Fig. 3). Cuscuta campestris is orange-yellow in colour with stem filaments averaging about one millimetre in diameter (Fig. 4). On coming in contact with a host plant, both parasites make several tight coils around the host stem, and it is from these coils that the absorbing haustoria are developed. After the establishment of the tight haustorial coils the stem continues its growth as a comparatively straight exploratory filament which, on making contact with a new host or the original host at another locus will develop more haustorial coils.

As a preliminary to an investigation of some aspects of the physiology of the Cuscuta-host relationship, histological preparations of the haustorial connections were made. Of the several hosts employed in this work, Impatiens sultani proved

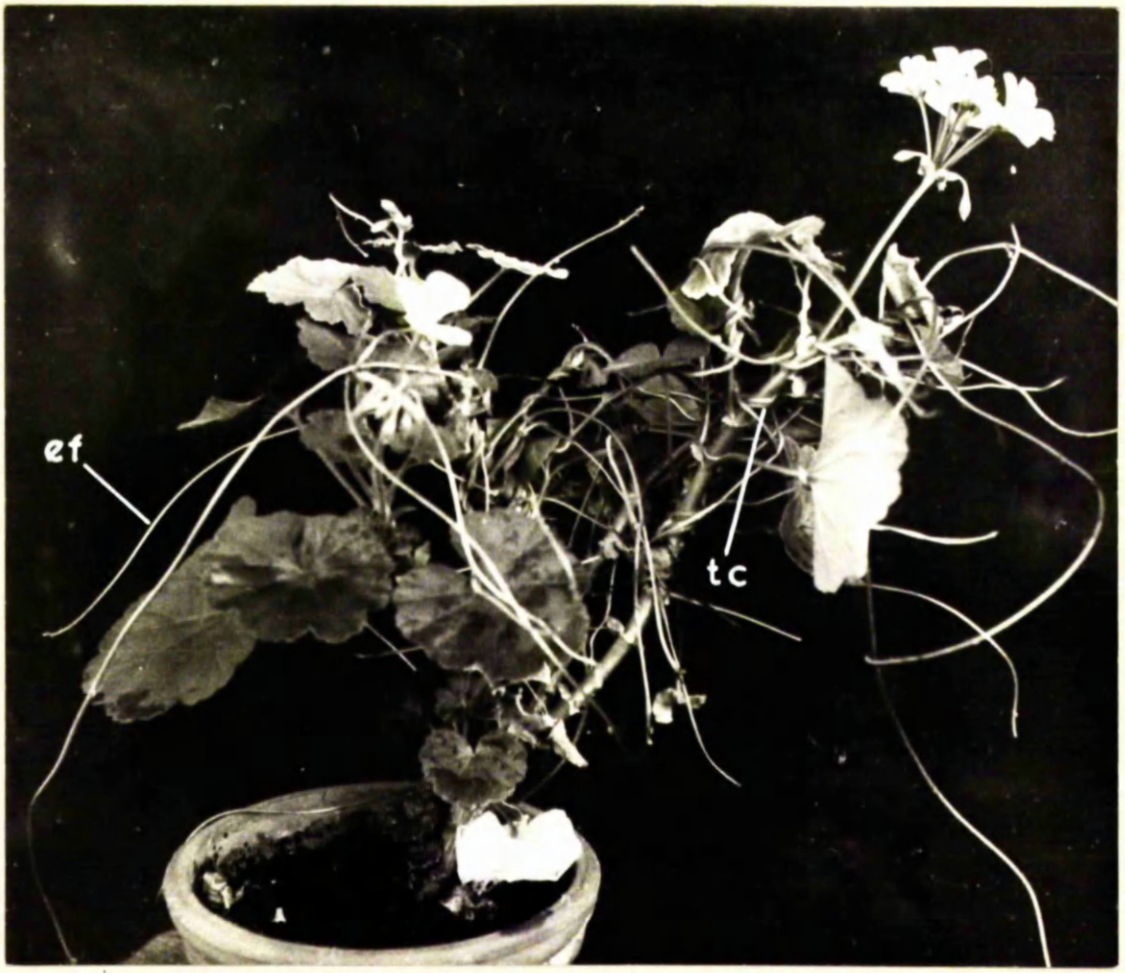


Fig. 1. Cuscuta reflexa on Pelargonium, showing tight coils(tc) and exploratory filaments(ef).

Fig. 2. Heavy parasitism of Cuscuta reflexa on Vitis.

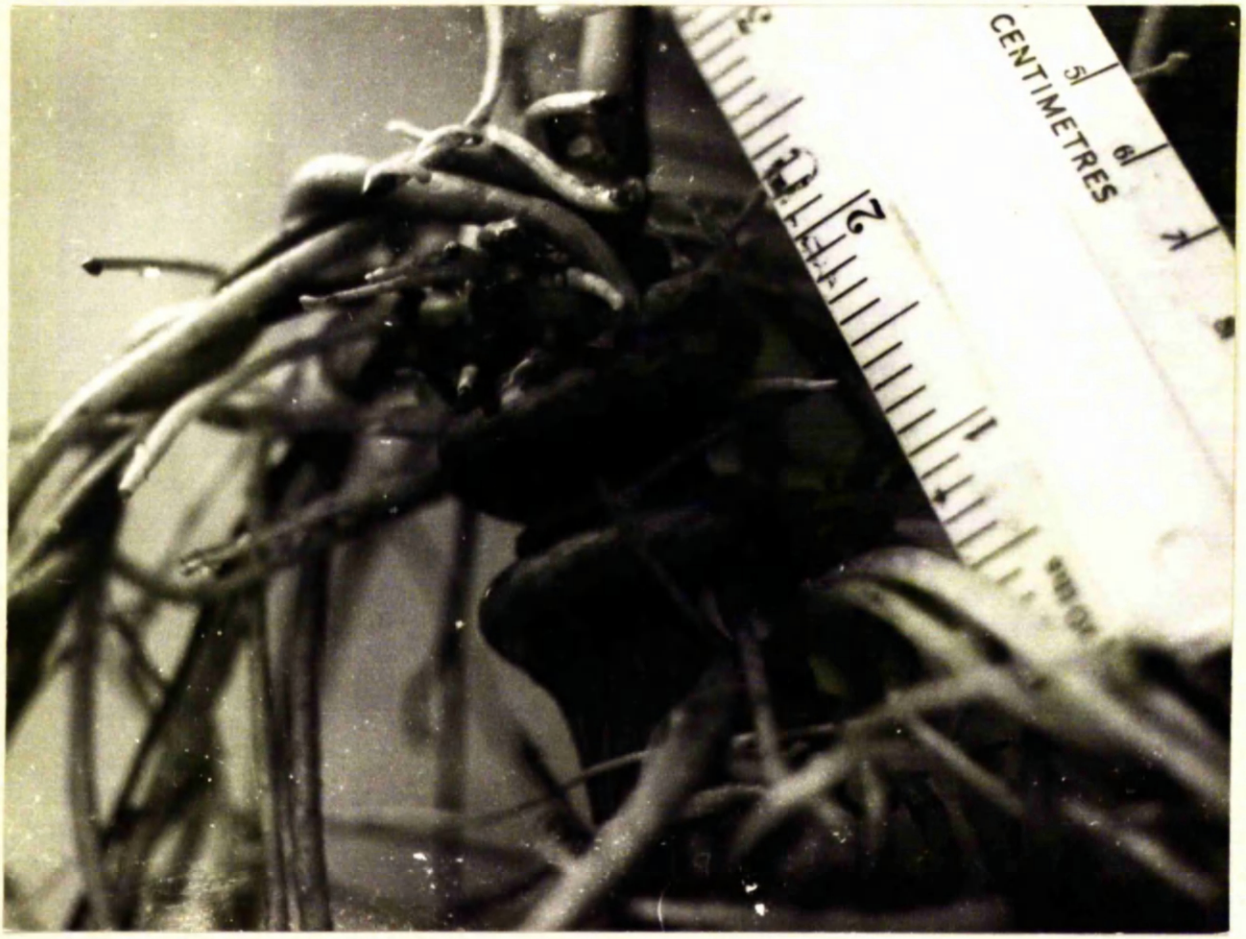


Fig. 3. Cuscuta reflexa on Vitis, showing massive haustorial coils.

Fig. 4. Flowering filaments of Cuscuta campestris on Bulbine. ($x\frac{1}{2}$).

most suitable. It is readily attacked by the parasite, and its comparatively succulent stem offers little resistance to invading haustoria.

a. Histological Techniques

Stems of C. campestris and C. reflexa growing on Impatiens stock were allowed to come in contact with vigorously growing plants of I. sultani. Within one to three days, tight haustorial coils of the parasites had formed on the host stems, and haustorial initiation could be observed. Approximately three weeks later, pieces of infected stem of 1cm. in length were harvested and fixed in Bouin's fluid. The fixed tissue was embedded in polyester wax (Steedman, 1957) and sectioned at 10, 15, and 20μ. The most satisfactory combinations of stain were safranin with light green or with haematoxylin.

1. Results (C. campestris)

Examination of the histological preparations showed the haustorium as a well defined structure of fairly constant form. It is elliptical in transverse section with its long axis vertical (Fig. 5). The haustorium measures approximately 0.6mm. in length, 1.2mm. in its long axis, and 0.4mm. along its short axis. With the above-mentioned stain combination it is usually easy to

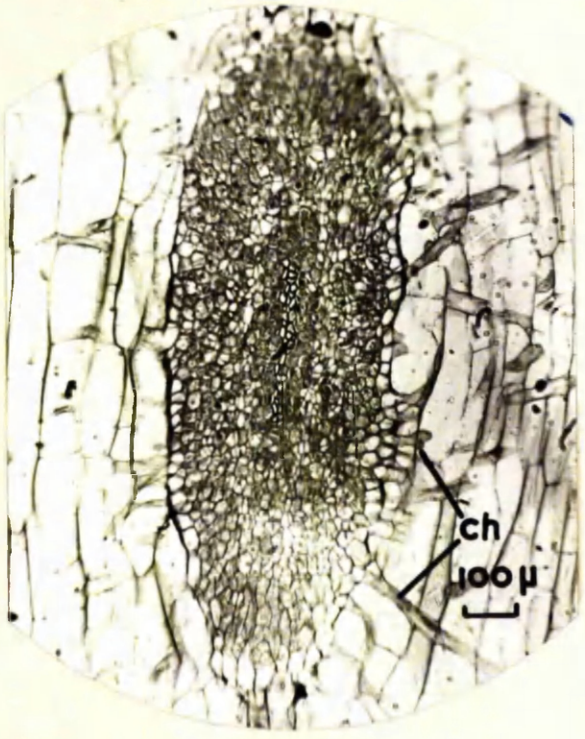


Fig. 5. Transverse section of haustorium of C. campestris on Impatiens. ch-cortical hyphae.

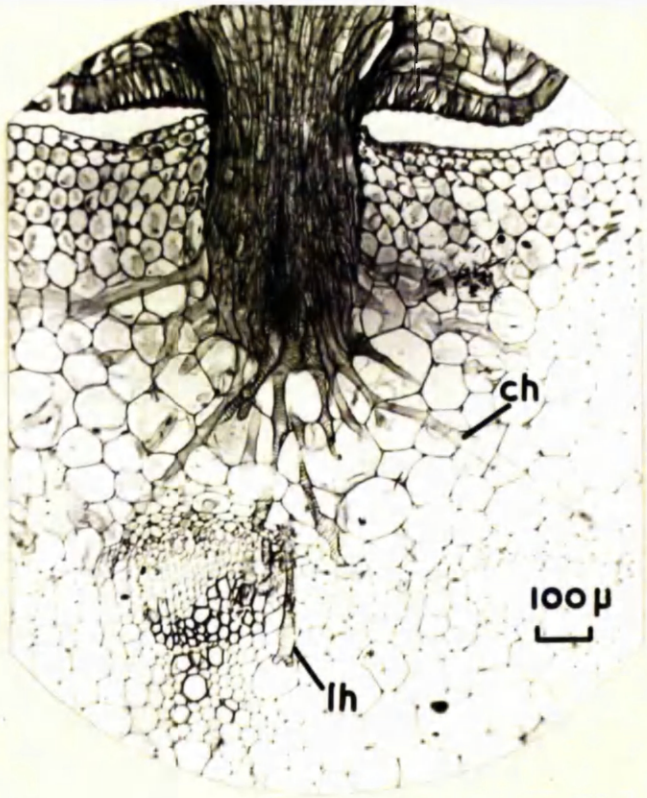


Fig. 6. Longitudinal section of haustorium of C. campestris on Impatiens. ch-cortical hyphae; lh-lignified hyphae.

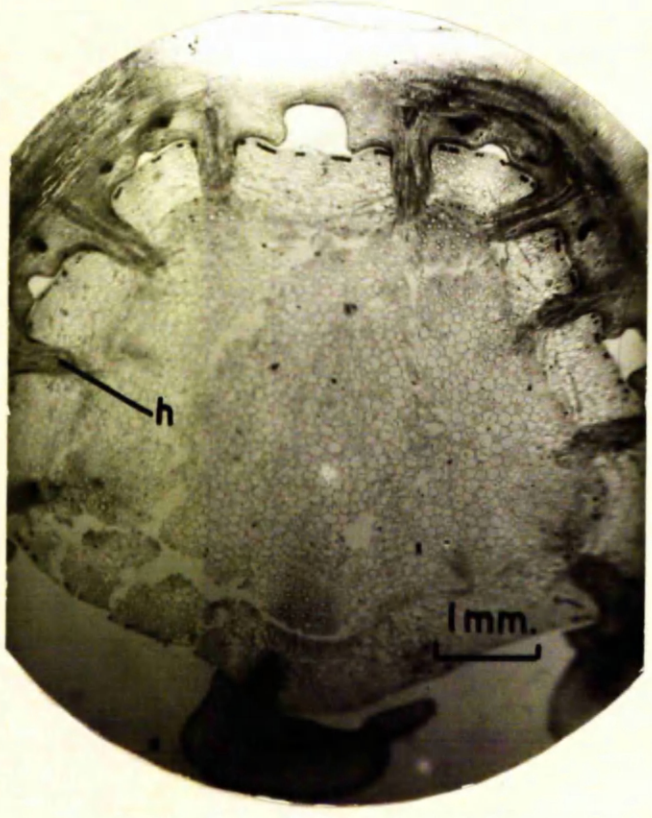


Fig. 7. L.S. haustorial coil of C. campestris on Impatiens. h-haustorium which has failed to contact a host vascular bundle.

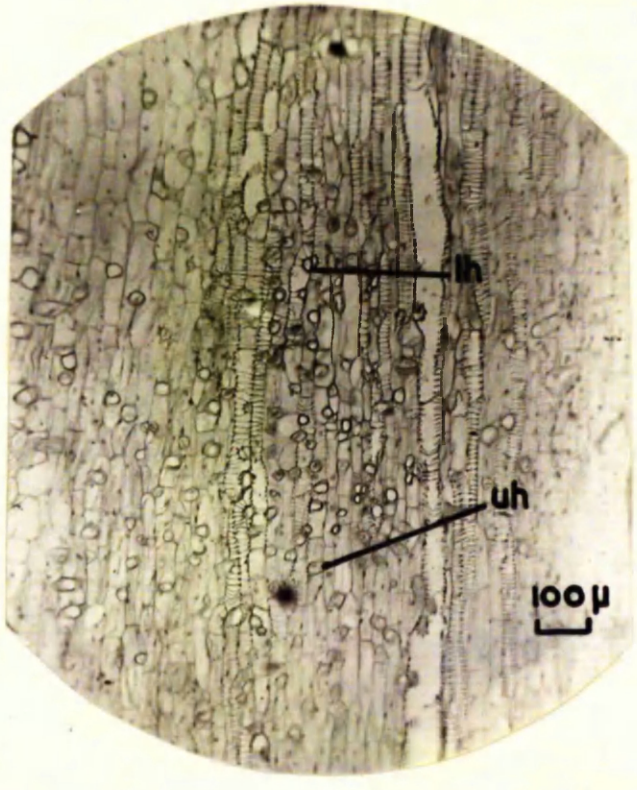


Fig. 8. Tangential longitudinal section of Impatiens infected by C. campestris. lh-lignified hyphae; uh-unlignified hyphae.

distinguish between host and invading tissue. This seems to be due to the dense protoplasm of the parasite adsorbing relatively more stain than surrounding host cells.

Arising from near the tip of the haustorium are long, uninucleate hyphae-like cells which invade the cortex of the host (Fig. 6). It is not clear to what extent these cortical hyphae contribute to the nutrition of the parasite. Hand sections of living parasitised material frequently show hyphae passing through host cells which contain a dense red pigment. A detailed study of a large number of such sections shows no evidence of any pigment leakage from these cells, nor of any pigment entering the hyphae. This indicates that any absorption by the cortical hyphae is to some extent selective, possibly comparable to the type of absorption associated with root hairs. This conclusion is supported by the work of Walzel (1952), who has shown that Cuscuta parasitising Nicotiana remains nicotine-free.

The root-like nature of the haustorium is clear from its appearance in transverse section in Fig. 5, which also shows that the cortical hyphae are extensions of the outermost layer of the haustorium, recalling the structure of root hairs. Lackey (1953) mentions that dodder haustoria are attracted to the vascular bundles in the sugar beet, and that the haustoria always penetrate

directly opposite to a conducting strand, and not into the greater space between the bundles. A study of the material here tends to confirm this view, but although the majority of haustoria enter the host stem at a point directly opposite a vascular bundle, this is not consistently the case; Fig. 7 shows a haustorium which has failed to establish contact with conducting tissue. In such cases penetration has never been observed much beyond the level of the vascular ring.

The method whereby the haustorium penetrates the host tissue has been described by Thomson (1925). He states that the hyphae advance through the host cells by a process of enzymic softening and eventual solution of the cell wall. Fig. 8 shows that the path followed by the majority of hyphae is closely associated with the radial walls of the host cells, though some hyphae enter the tangential walls without coming in contact with the other walls. Once contact with a host vessel has been established, lignification and differentiation of the invading hyphae commences (Figs. 9 and 10). Differentiation proceeds from the point of attachment backwards, with the formation of a number of reticulately thickened cells comparable in appearance to tracheids. Lignification of the haustorium does occur if contact with the host xylem is not made, but this is not common (Fig. 6).

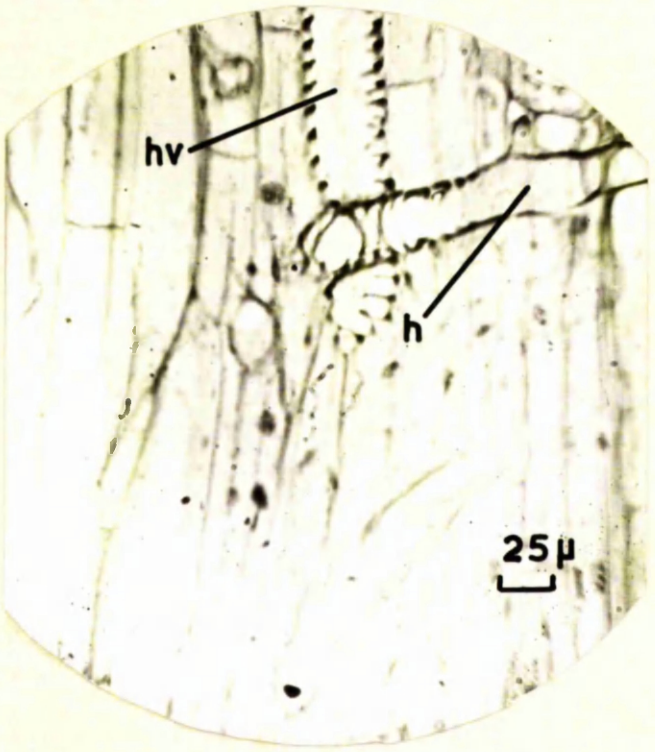


Fig. 9. C. campestris on Impatiens; initiation of lignification in hypha (h) which has contacted a host vessel(hv).

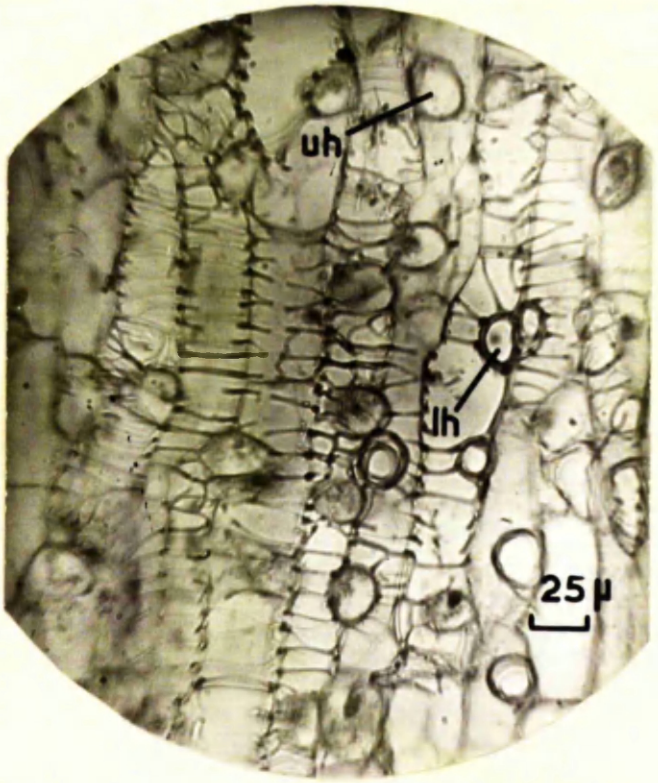


Fig. 10. Detail from Fig.8; lh-lignified hypha; uh-un--lignified hypha.

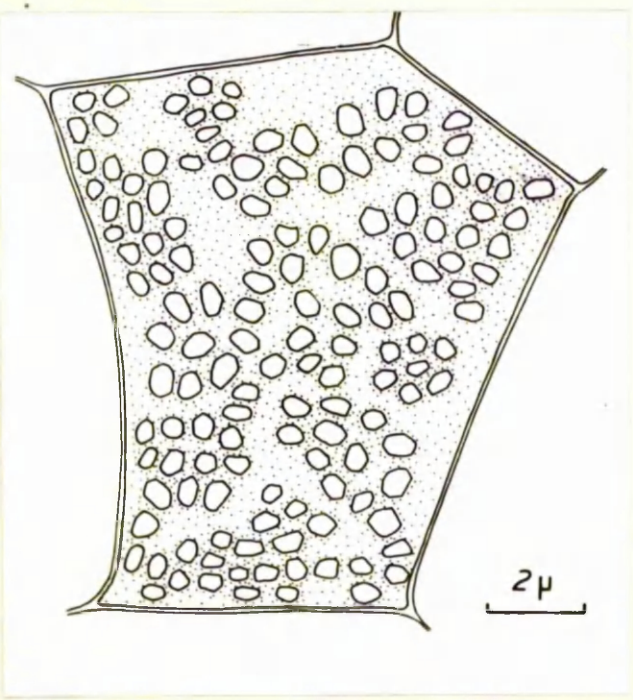


Fig. 11. Sieve plate from stem phloem of C. reflexa.

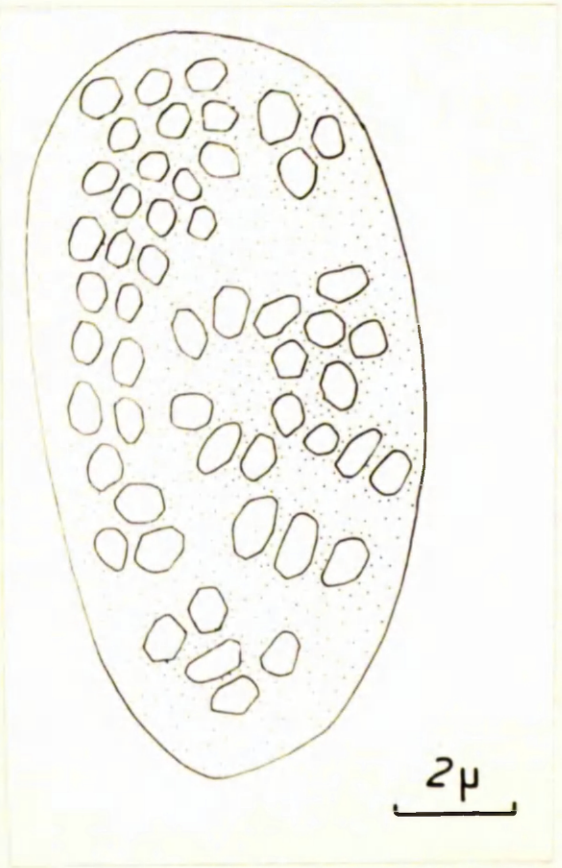


Fig. 12. "Sieve area" from lateral wall of cortical hypha of C. reflexa.

Besides the gross attraction of the entire haustorium for the host vascular system, a particular attraction is also exhibited by the individual haustorial hyphae. This is frequently well illustrated when the monocotyledon Bulbine is employed as a host. In Bulbine leaves, where the vascular bundles are relatively close to the epidermis, normal development of the haustorium takes its active absorbing region beyond the vascular zone of the host. In such cases lateral hyphae of the haustorium may be seen bending backwards through the host mesophyll to make intimate contact with a vascular strand (Fig. 13). The stimulus which is exerted on the hyphae by the vascular bundle appears to be effective at distances as great as 300μ.

2. Results (C. reflexa)

The structure and development of the haustorium of C. reflexa is fully described by Thomson (1925), who reported that no phloem is to be found in the haustorium. The present writer, however, finds that on the lateral walls of some cortical hyphae there are well defined sieve areas. The diameter of the pores and their spatial relationship to each other are similar to those in the normal sieve plate of the parasite stem phloem (Figs. 11 and 12). Thomson does not mention the presence of these structures, but it is possible that he may have seen them and regarded them as not

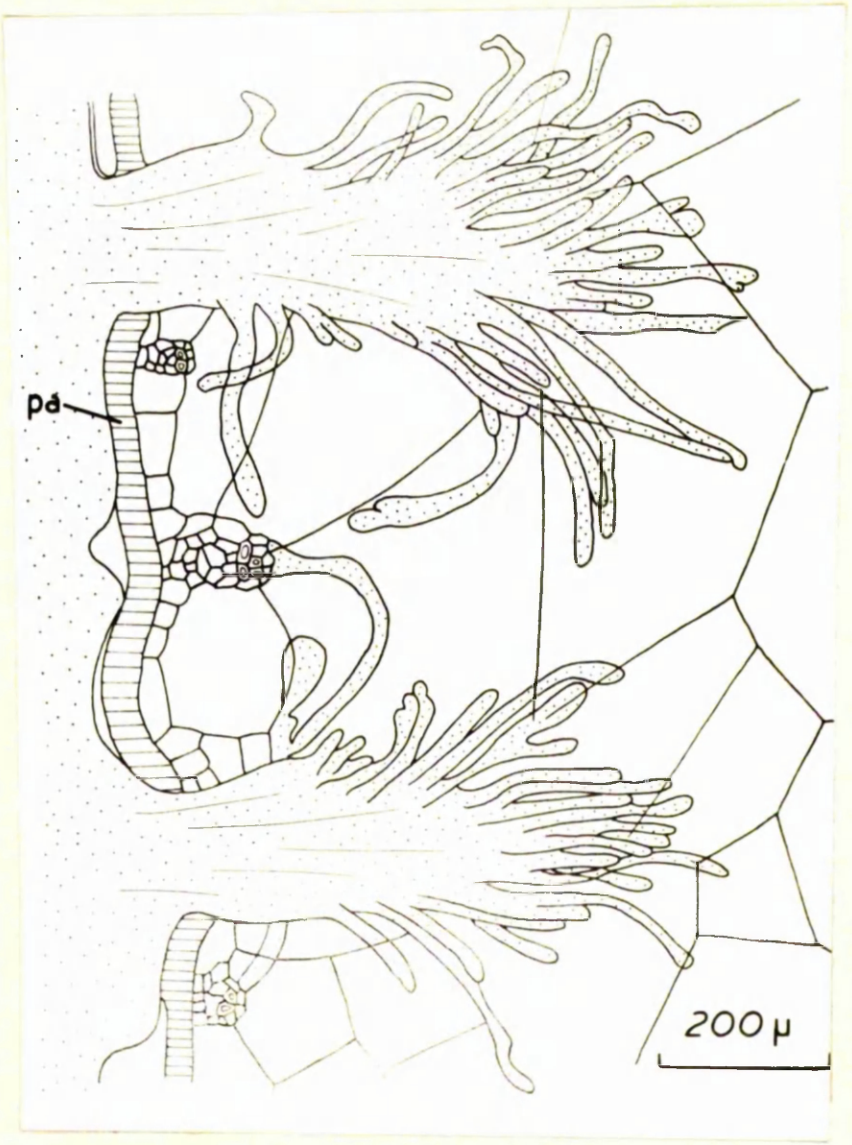


Fig. 13. Transverse section of Bulbine leaf, showing attraction of haustorial hyphae of C. campestris to host vascular bundle. pa-palisade mesophyll of host.

constituting true phloem.

b. Elucidation of haustorial connections by means of dye experiments.

The tapping of the host xylem sap by C. campestris was demonstrated by immersing the cut end of a parasitised Impatiens shoot in an aqueous solution of erythrosin. The progress of the dye was followed by cutting longitudinal and transverse sections of the haustorial region at various time intervals. It was found that often within ten minutes the dye had entered the parasite and formed an uninterrupted stream in the xylem of the host and parasite. Sections of the parasite stem taken less than about 1cm. from the haustorial connection generally showed the presence of dye in all vascular bundles, but at distances greater than this (up to 10cm.) all the dye was usually found in only one bundle. This would indicate that for any haustorial coil, one particular vascular bundle is mainly responsible for subsequent transport of xylem sap, even though a haustorial coil may contain as many as thirteen functional haustoria.

For any haustorial coil, dye movement occurred both upwards towards the stem apex of the parasite and downwards, irrespective of the same filament forming haustorial connections at higher or lower levels on the host stem. A long intact filament of C. campestris cut at its mid point underneath the

surface of the erythrosin showed that the dye was drawn into both cut ends and could be traced in all the vascular bundles up to 1cm. from the cut ends after one minute, and 6cm. after 18 hours. This shows that the xylem of the parasite is in a state of negative pressure.

Dye movement in C. reflexa follows a closely similar pattern to that exhibited by C. campestris, but the dye is carried from the host in all vascular bundles to a greater distance (about 2cm.) from the haustorial region and two vascular bundles are involved in transport beyond this distance.

III. DRY WEIGHT DETERMINATIONS

a. Variation in dry weight of parasites on different hosts.

Cuscuta spp. are on the whole highly non-specific, but even so, the literature on the subject contains references (Mirande; Lilienstern) to the degree of susceptibility of various hosts.

Since the control which the parasite establishes for a flow of nutrients must depend to some extent on the degree of hypertonicity which they attain, it seemed appropriate in the first place to ascertain the relative amounts of water in uninfected potential hosts, in infected hosts, and in the infecting Cuscuta. No record of any quantitative assessment of the dry:fresh weight ratio of Cuscuta on various hosts has been found in the literature, apart from references to the fact that it will or will not grow on certain plants.

Mirande (1901) states that the colour of C. japonica varies with its host; on Sambucus it was green, while on Forsythia it was a pronounced red. He suggests that the former colour represents unfavourable nutritional circumstances, and the latter favourable conditions.

In plasmolysis experiments on Cuscuta monogyna, Lilienstern

(1932) has shown that the ability of this parasite to attack a host successfully or not depends on osmotic pressure considerations. C. monogyna has a lower osmotic pressure than the cortex of Melilotus albus which is a resistant host, and a higher osmotic pressure than the cortex of Cicer sp. which is a susceptible host. The same author (1928) also found that C. monogyna parasitised plants which had a pH above 6.5, but more acid plants (pH 6.0-6.2) were not parasitised.

According to Mirande (1901) solanaceous hosts induce the formation of oil globules in the haustoria of Cuscuta. He suggests that this is a protective reaction against the alkaloids present in these hosts, since no trace of oil is found in parasites on innocuous hosts.

In view of the lack of published data on dry matter estimations, the ratio of dry matter to fresh weight was determined for both species of Cuscuta growing on a variety of hosts. The hosts chosen were Vicia faba, Nicotiana tabacum, Vitis vinifera, Bulbine sp., Pelargonium zonale, Pelargonium fragrans and Helianthus annuus.

1. Materials and methods.

Dry weight estimations were made during the month of July

when the parasites were vigorously growing. Freshly harvested samples (1 - 2gm.) of C. reflexa and C. campestris (collected between 10a.m. and 3p.m.) were cut into approximately one-inch lengths and weighed in 20-ml. glass dishes. These were then placed in an oven at 105°C. for 12 hours, allowed to cool in a desiccator and re-weighed. From these weighings the fresh and dry weights of each sample were obtained, and the dry:fresh weight ratios calculated. In the case of C. reflexa on Bulbine and C. campestris on Pelargonium fragrans and Vitis lack of material allowed the determination of only one sample; in all other cases from 8 to 12 separate determinations were made.

2. Results.

Figs. 14 and 15 summarise the results of these dry weight determinations on Cuscuta campestris and Cuscuta reflexa respectively. In the case of C. campestris, the dry:fresh weight ratio is highest when it is parasitising Nicotiana and becomes progressively lower on Pelargonium zonale, P. fragrans, Vicia, and Bulbine, reaching a minimum on Vitis. Cuscuta reflexa develops its maximum dry matter content on Helianthus, decreasing progressively on Vitis, Vicia, Nicotiana, Pelargonium spp. and Bulbine.

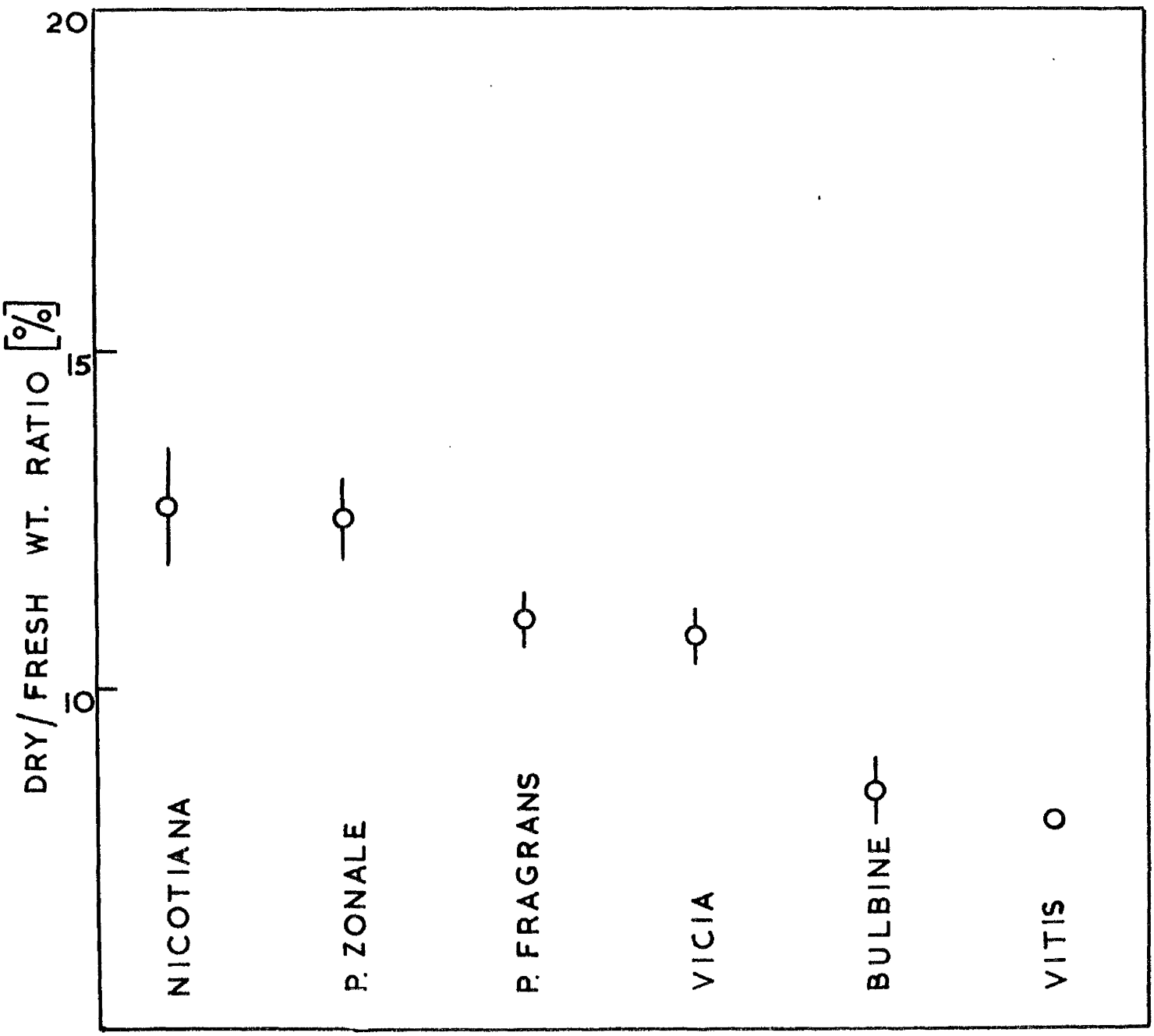


Fig. 14. Cuscuta campestris. Variation in dry/fresh weight ratio of parasite growing on different hosts. Circle denotes mean; standard deviation is represented by vertical line.

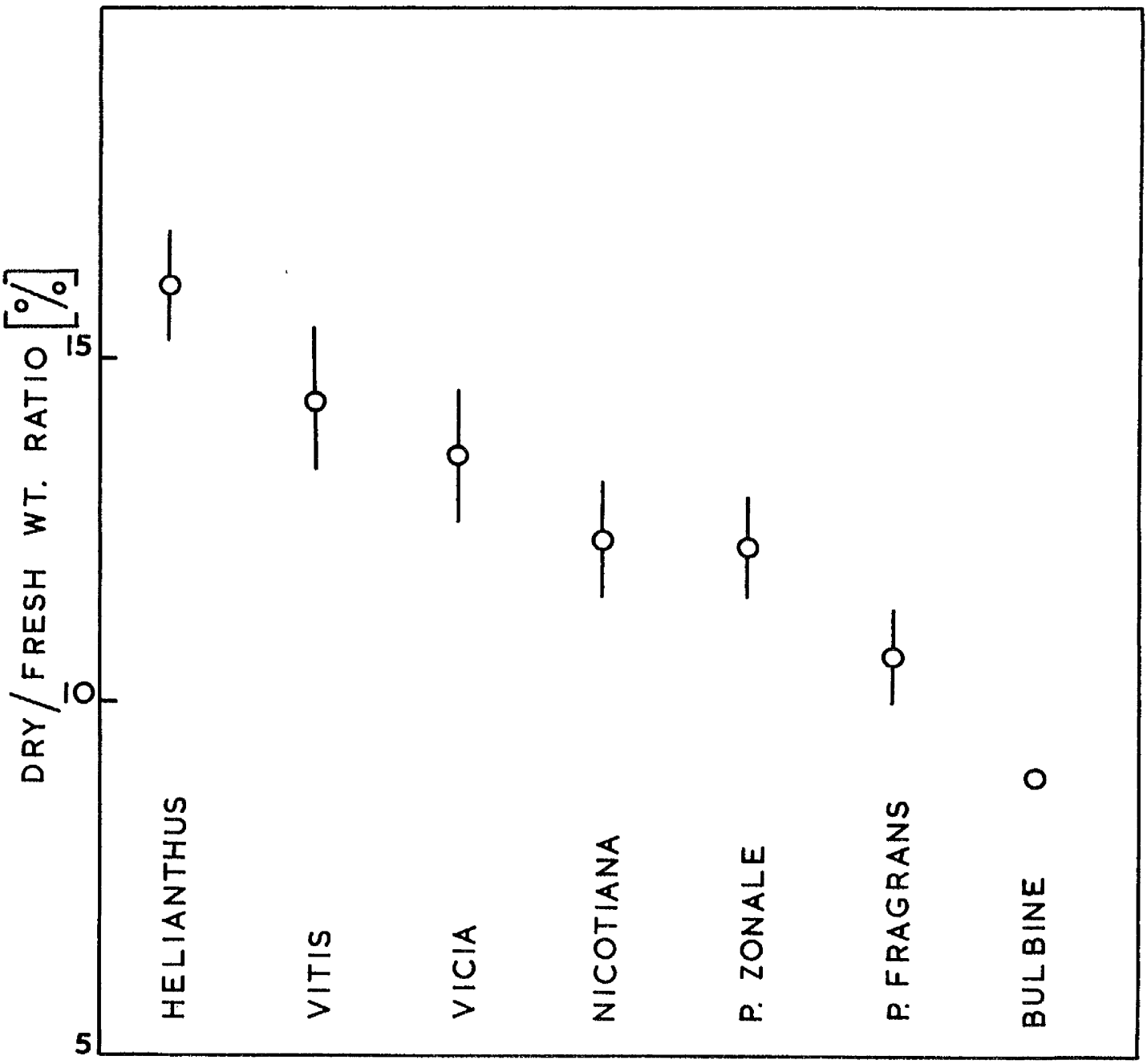


Fig. 15. Cuscuta reflexa. Variation in dry/fresh weight ratio of parasite growing on different hosts. Circle denotes mean; standard deviation is represented by vertical line.

It is clear from these results that no close correspondence exists in the reactions of the two Cuscuta species to the different hosts. Though both species have approximately similar dry:fresh weight ratios on Nicotiana and Pelargonium zonale, it is significant that on Vicia, the ratio is relatively higher in C. reflexa and lower in C. campestris. On Vitis, C. reflexa has a high dry matter content, whereas in C. campestris on Vitis the dry:fresh weight ratio is very low. It is on Helianthus that C. reflexa exhibits its maximum dry matter content.

There is a clear correlation between dry:fresh weight ratio in C. reflexa and the general appearance of the plant. On Helianthus and Vitis, with the highest ratios, the parasite was robust and vigorously growing with a stem diameter sometimes up to 5mm. On P. fragrans and Bulbine, with the lowest ratios, growth was slower, and the parasite stem seldom exceeded 2mm., while on Vicia, Nicotiana and P. zonale which show intermediate ratios, growth rate and stem diameter were also intermediate.

Rate of growth in C. campestris is not so obviously related to its host. It generally grows best on P. zonale, exhibiting on that host its most pronounced orange coloration. On Bulbine and Vicia, with relatively low dry:fresh weight ratios, the parasite is frequently yellow-green in colour, with a tendency

to a filiform growth habit. The very low value found for C. campestris on Vitis may be correlated with the fact that it is only with great difficulty that C. campestris can be induced to grow on the vine.

The growth pattern described above was maintained only on healthy host plants. As the hosts withered (largely as a result of the action of the parasite) the parasite stems gradually attenuated and dried up. Withering of C. reflexa stems nearly always proceeds distally (towards the stem apex), with the result that the apical portions remain alive after the death of the remainder of the stem. As the withered region approaches to within about 5cm. of the apex, a marked increase in the vigour of the apical region is apparent. There is a strong development of chlorophyll and an increase in stem diameter. Such apical portions are particularly effective in establishing haustorial connections with new hosts. Vigorously growing stem apices have been observed 21 days after cessation of host food supply. It is quite clear that distal portions of C. reflexa filaments draw on proximal regions for continuance of necessary food materials. There can be no doubt that this controlled withering has a high survival value for the parasite, particularly in view of the fact that only the apical region is effective in attacking new hosts.

b. Variation in dry weight of leaves of parasitised and unparasitised plants.

Prolonged parasitism by Cuscuta results in a deterioration in the vigour and general appearance of any particular host. So long as the host remains alive it affords a source of food for the parasite. Once Cuscuta has established a vigorous haustorial connection with a susceptible host (annual plants only) it will normally continue to grow until the supply of food materials is exhausted, i.e. the death of the host. When the parasite flowers, however, its vegetative growth is curtailed, and the host may continue its growth.

Estimations of dry matter content of leaves of parasitised and unparasitised plants were made to determine if any changes were attributable to the parasitism. The methods used in measuring dry:fresh weight ratios were similar to those described in section IIIa.(page 20). One entire leaf from each plant was used in the estimations, care being taken to ensure that corresponding leaves were as similar as possible in age and size. Only leaves which showed no external evidence of withering were selected from both healthy and parasitised plants. Each point in Fig. 16 represents the mean of three determinations; standard deviation is indicated by the vertical lines.

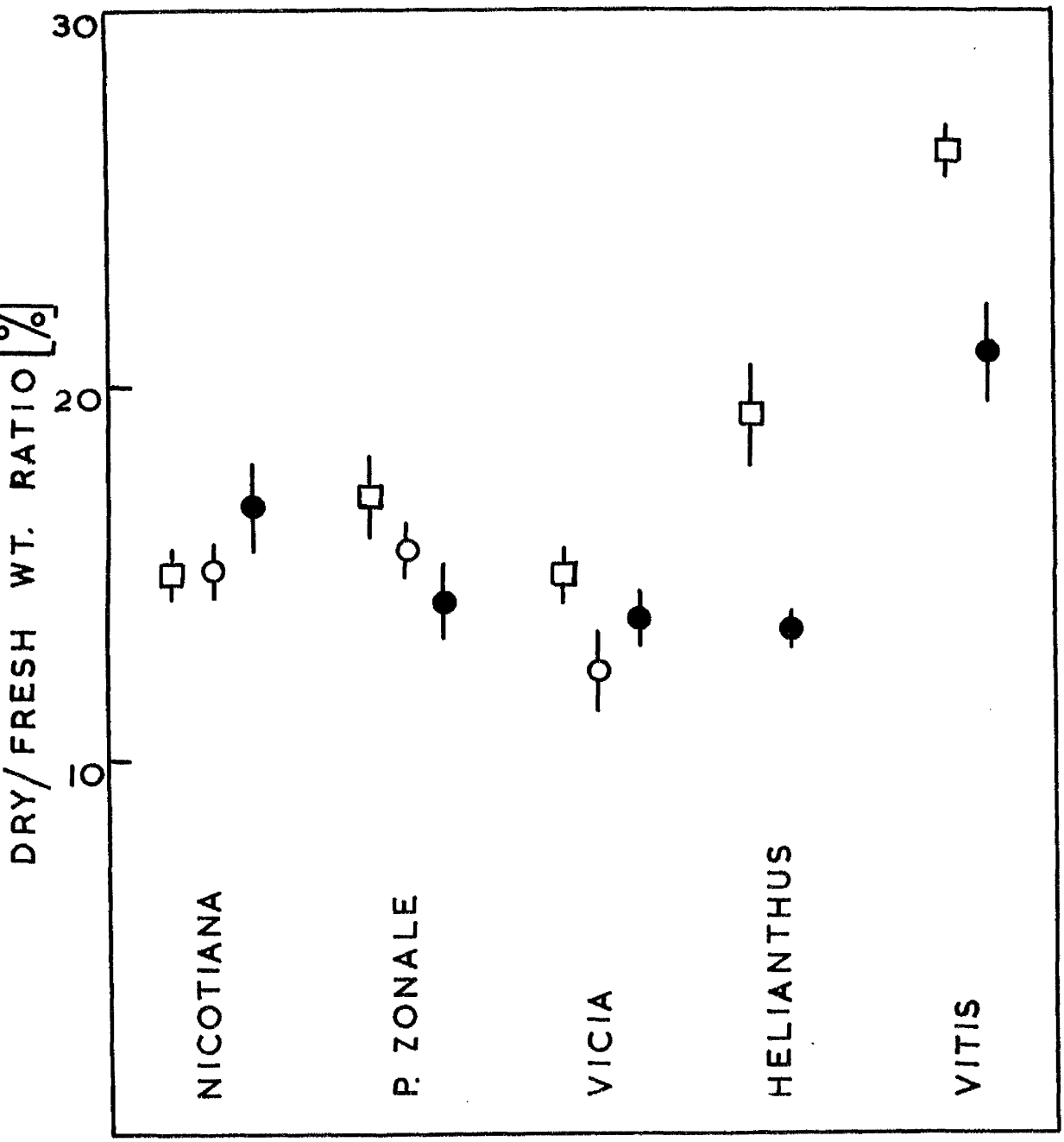


Fig. 16. Effect of parasitism by Cuscuta on dry/fresh weight ratio of various leaves. Squares represent unparasitised leaves; open circles represent leaves parasitised by C. campestris; closed circles represent leaves parasitised by C. reflexa.

Results.

With the exception of Nicotiana, the leaves of parasitised plants had a lower dry:fresh weight ratio than healthy leaves. This indicates that withdrawal of food materials from the host stem by the haustoria results in a drain of food reserves from the leaves. The slight increase in dry matter content found in parasitised Nicotiana leaves, while it might be a genuine response, is more likely to be the result of an inadequate number of assays, complicated by the comparatively succulent nature of Nicotiana leaves.

In the light of these observations it is probable that the death of the host plant as a result of parasitism is due to exhaustion of food reserves, rather than to toxicity or mechanical damage.

IV. STRUCTURE AND DISTRIBUTION OF CHROMOPLASTS IN CUSCUTA REFLEXA AND CUSCUTA CAMPESTRIS.

A survey of the literature reveals no reference to any detailed work on the plastids of the genus Cuscuta. One of the earliest records of green pigment in the Cuscutas is by Syme (1863), in which he refers to the green coloration of C. epilinum and C. europaea. MacKinney (1935) mentions that the reddish colouring matter in C. salina is aggregated in small plastid-like bodies. Mirande (1901) states that the green colouring in Cuscuta is primarily due to starch grains turning green, but that some green plastids are due to embryonic leucoplasts turning green.

A survey of the general literature indicates that the origin of plastids is a very complex problem, and that no unanimity as yet exists in this respect among cytologists. Some workers assume a common origin for plastids and mitochondria, while others believe that they are genetically unrelated. The following account is an attempt to trace the origin of the various plastid types found in the two species of Cuscuta. All observations are from sections mounted in water, the sections being hand cut from freshly harvested material

a. Cuscuta reflexa.

All the green colouring matter in this species is localised in discrete intracellular units, the chromoplasts, which vary greatly in size and structure. In the stem the pigmentation is densest in the cortex and around the vascular bundles, being almost absent in the pith (Fig. 17). All vegetative stem portions of C. reflexa, irrespective of host plant or nutritional circumstances possess a plastid distribution closely similar to that illustrated in Fig. 17. However, in the flowering branches, the plastid-distribution pattern of the flower stalks is strikingly different; almost all the pigmented bodies are found in cells surrounding the vascular strands, particularly the xylem elements (Fig. 18).

In the flower of C. reflexa green plastids are found in the ovules, ovary, nectaries, calyx and pedicel (Fig. 19). The chlorophyllous zones are markedly green, particularly the ovules and lower portions of the ovary. The plastids responsible for the green coloration, are, with the exception of the nectaries, similar to those found in vegetative portions of the plant; in the nectaries the plastids are yellow-green in colour and are responsible for the general colour of these organs. Green plastids of the same type as occur in the stem also occur

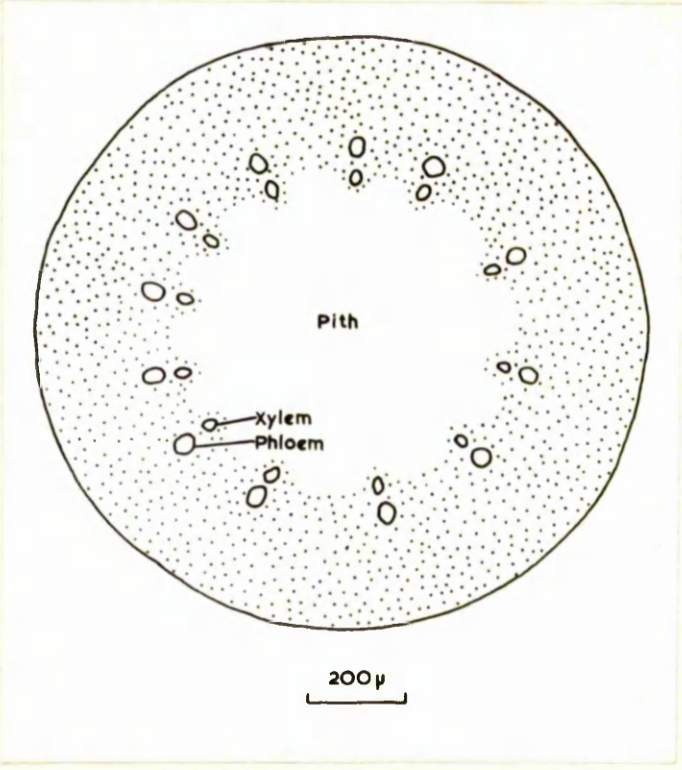


Fig. 17. Diagrammatic transverse section of C. reflexa stem, showing distribution of green pigmentation (stippled).

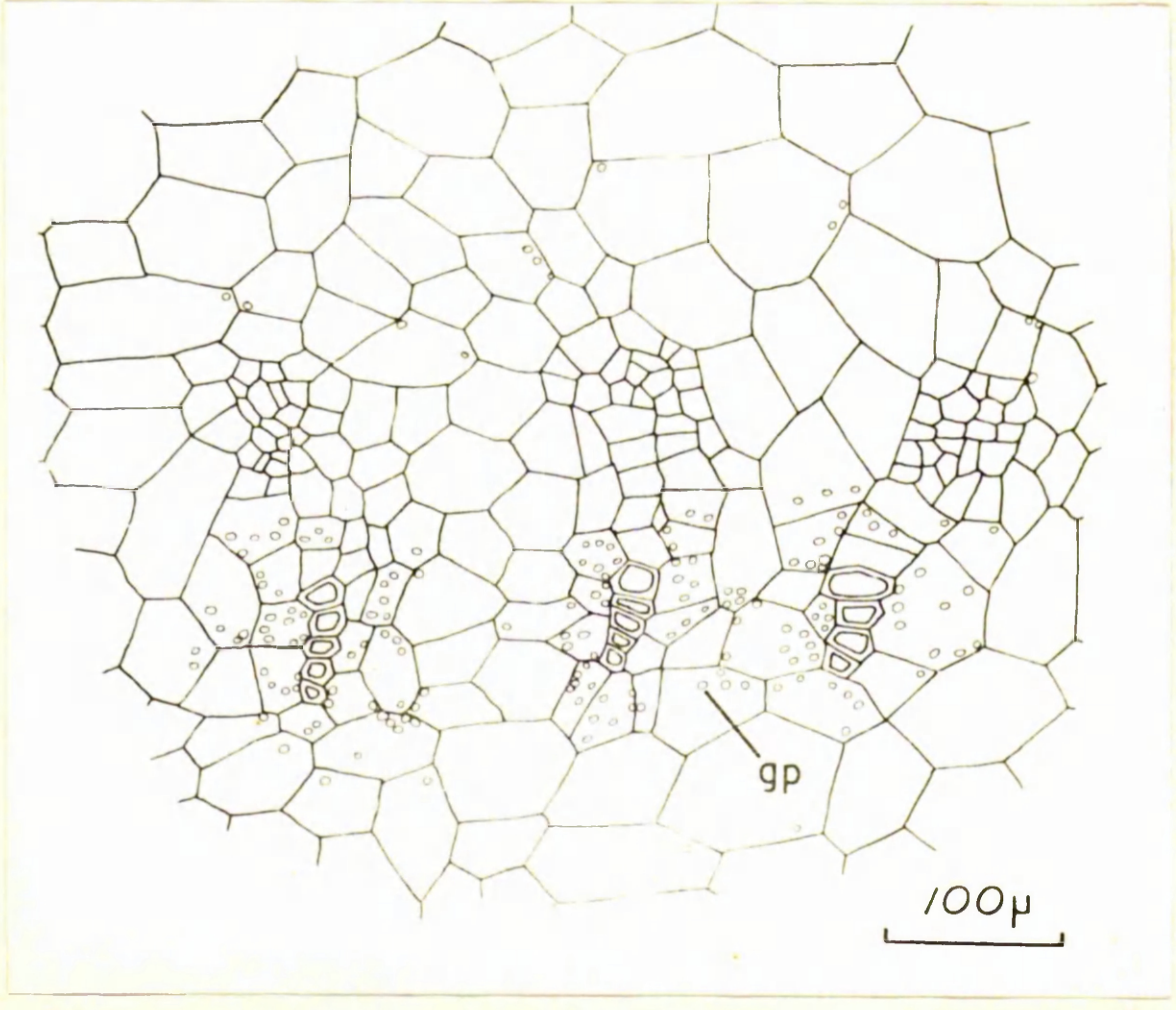


Fig. 18. Transverse section from pedicel of C. reflexa, showing distribution of green plastids(gp).

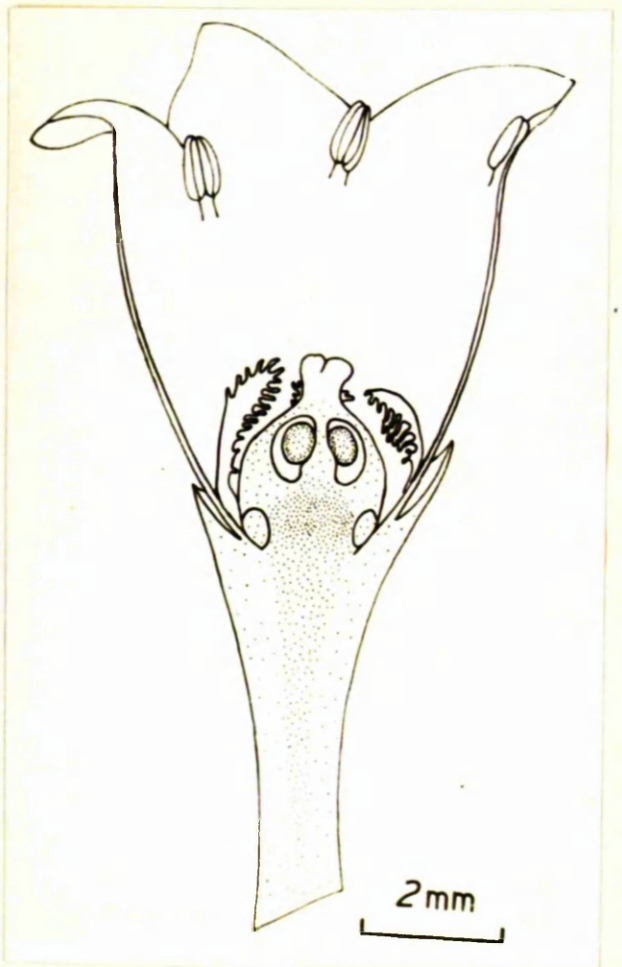


Fig. 19. Longitudinal section of flower of C. reflexa, showing chlorophyll distribution(stippled).

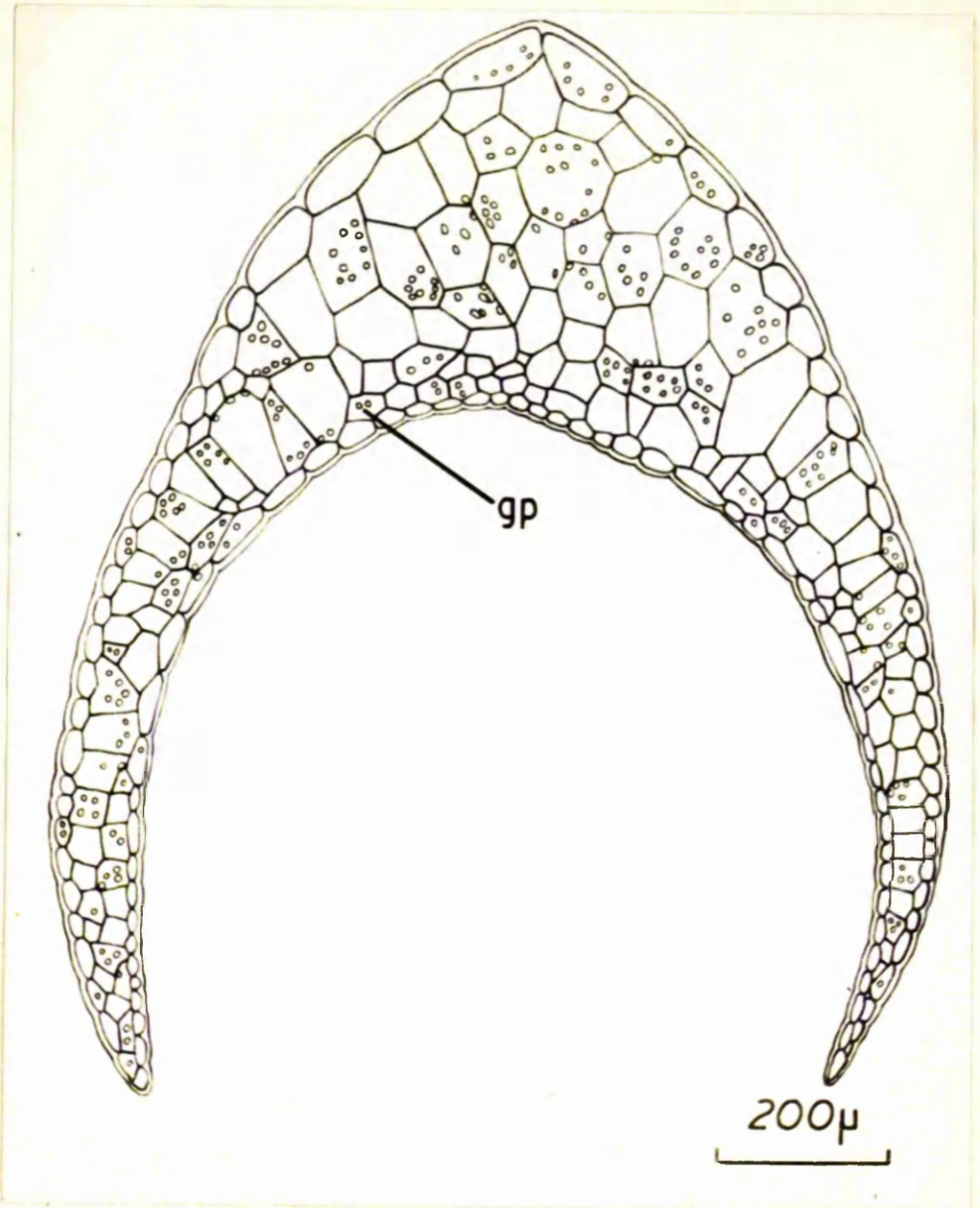


Fig. 20. Transverse section of scale leaf of C. reflexa, showing distribution of green plastids(gp).

in the scale leaves of C. reflexa, but the distribution of chlorophyllous cells is apparently a random one; there is no differentiation into palisade and spongy mesophyll (Fig. 20).

Inspection of a fresh section of C. reflexa stem reveals the presence of a variety of plastid types. No exact classification is possible on account of the gradation between these plastids. It is, nevertheless, convenient to group them into four broad divisions.

Type "a": Chloroplasts (4μ). In almost any section, plastids which are morphologically very similar to the chloroplasts of higher-plant leaves can be distinguished. The fact that their colour intensity is comparable to that found in leaf plastids suggests that the lower chlorophyll content of Cuscuta is due to the relative paucity of chloroplasts, rather than to a low chlorophyll density in the individual plastids.

Types "b" and "c" (5-7μ). Green plastids larger than type "a", and containing starch grains.

Type "b" plastids are those in which the volume of starch is less than 50% of the volume of the whole plastid (Fig. 21), and type "c" are those plastids in which starch occupies more than 50% of the plastid volume (Fig. 22).

Type "d" (5-12μ). Green amyloplasts with simple or compound starch grains (Fig. 23). In this section, the terms "green

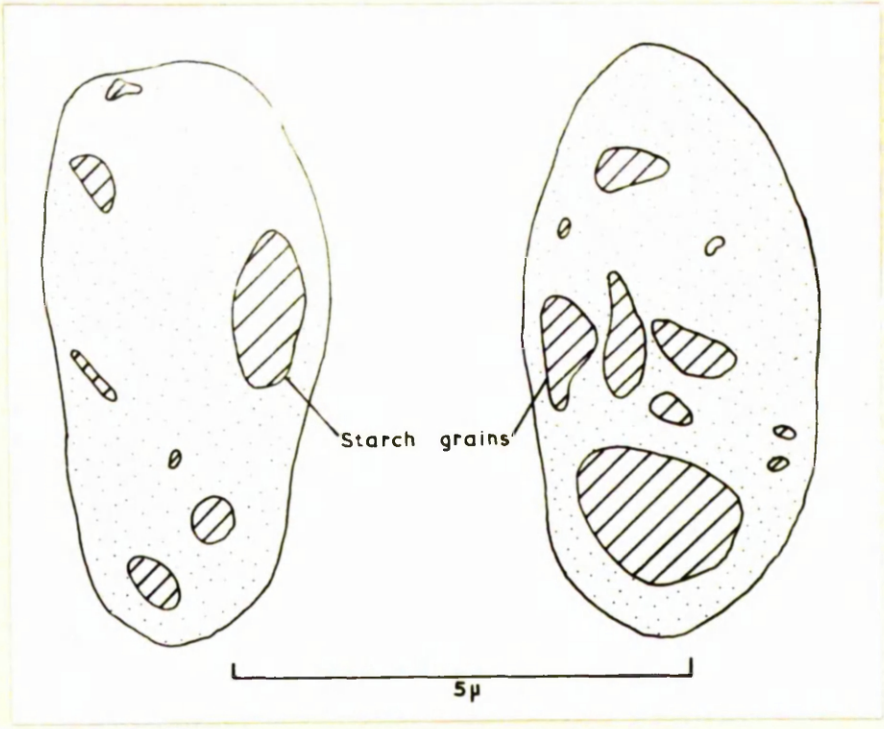


Fig. 21. Green plastids from stem of C. reflexa. Note that starch occupies less than half the plastid volume.

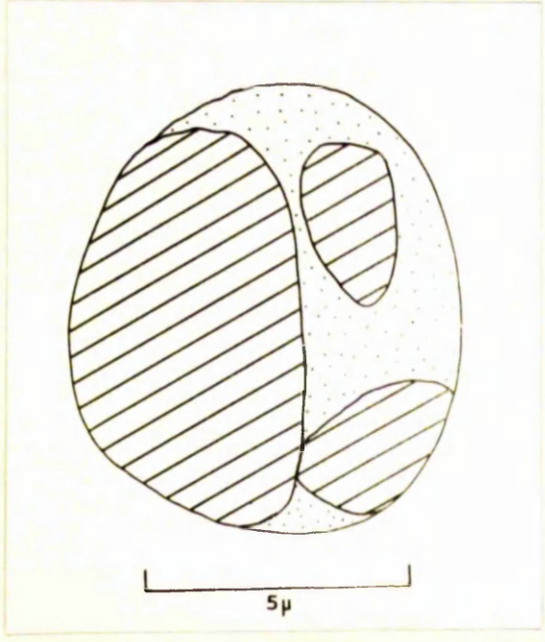


Fig. 22. Plastid in which starch occupies more than half the total volume of the plastid.(C. reflexa)

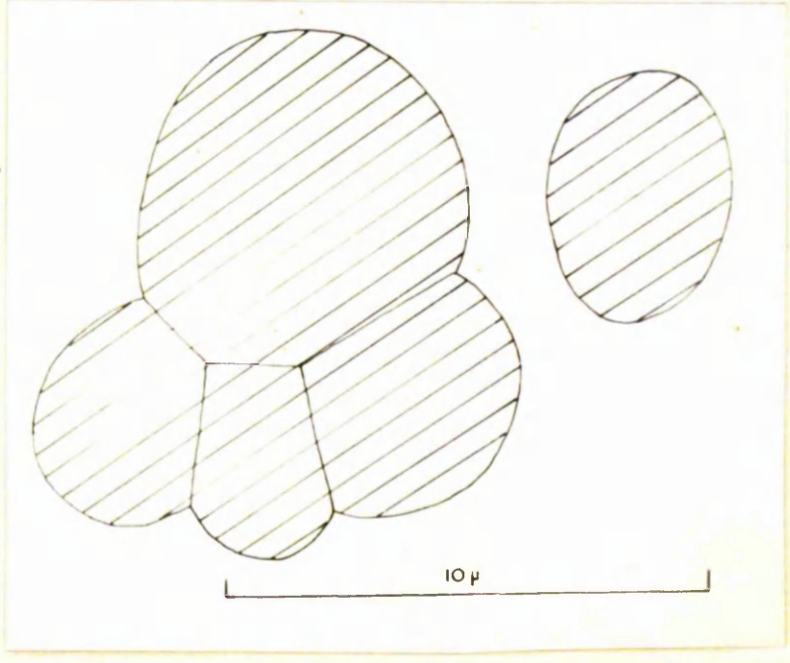


Fig. 23. Green amyloplast from stem of C. reflexa.

amyloplasts" or "green starch grains" refer to structures which have the microscopic appearance of green starch grains. It is assumed that the green coloration is due to a thin veneer of green plastid material enveloping the colourless starch grain.

The presence of this assortment of plastid types, whose relative abundance in any stem portion is very inconsistent, poses the general question of the origin of the various components. In plastid types "b" and "c" the starch is almost always present as several grains per plastid, embedded in a comparatively homogeneous green matrix. Where the volume of the granular material is small relative to the plastid volume (type "b"), the granules give only a weak reaction with iodine in potassium iodide solution. In both types "b" and "c" the starch grains are generally somewhat irregular in outline. Both plastid types "b" and "c" can easily be shown to be surrounded by a semi-permeable membrane. Plastids of this type which have escaped from damaged cells into the surrounding water, normally assume a spherical shape and increase markedly in volume.

Considering the variety of plastid-types found in the stem of C. reflexa, one may postulate that they constitute a transitional series from type "a" to type "d". If that is the case, the chloroplasts are capable of elaborating starch at

several foci, so that ultimately the chloroplast is almost entirely occluded with starch, associated with a residual chlorophyllous plasma which creates the visual image of a green starch grain. It is conceivable that the chlorophyll later disappears, leaving the colourless starch grains.

Though this developmental sequence may well occur in the plant, there is a certain amount of evidence that the green amyloplasts are derived from another source. Structurally no difference can be detected between starch grains which are colourless and those which are unquestionably green. This observation strengthens the case for a common origin of the two types, but the hypothesis that all the colourless grains have arisen from green plastids is unlikely on the grounds that it is not the rule in other organisms with large starch grains. In the higher plants, where storage organs are in any case usually underground or opaque to light, reserve starch grains occur in amyloplasts which are known to arise from colourless proplastids. The position in C. reflexa is probably therefore comparable to that found in the potato, where amyloplasts in the tuber turn green on exposure to light (Jungers and Doutreligne, 1943). Here, the amyloplast covering a portion of the starch grain was observed to turn green, and at the edges of the plastid a single layer of grana was observed. This particular observation provided

good evidence that chlorophyll is located in the grana of the chloroplast, since the individual grana were seen to be green, while the stroma appeared colourless against the white background of the starch grain.

The non-chloroplast origin of amyloplasts in C. reflexa is considerably strengthened by the fact that in C. campestris there are reserve starch grains which cannot have arisen from coloured plastids (see Section IVb). The argument used to demonstrate this conclusion in C. campestris is not readily applied to C. reflexa in view of the more general distribution of chromoplasts in vegetative stems of that species. In the pedicels of C. reflexa, however, starch grains must have arisen independently of chromoplasts, since the former are found in areas of the cortex normally almost devoid of chlorophyll.

In higher plants two types of starch grain may be distinguished; assimilation starch and reserve starch (Granick, 1953). The former is characteristic of actively photosynthesising chloroplasts, in which they may be formed in large numbers per plastid, but they remain small because they are continually being hydrolysed to soluble carbohydrates and transported elsewhere. Reserve starch, on the other hand, is usually in the form of large grains made up of concentric layers.

Type "b" plastids in C. reflexa are probably therefore derived from chloroplasts as a result of the plant's own photosynthetic activity; the small starch grains would therefore be assimilation starch. The presence of these small amyloid granules in chloroplasts is strong evidence in favour of the ability of Cuscuta to perform photosynthesis.

The origin of type "c" chromoplasts (Fig. 22) poses a more difficult problem. They may be derived from "b" types by an increase in size of the included starch grains, in which case they would represent a stage in the transitional series from "a" to "d", or they may have arisen from green amyloplasts as a result of downgrade metabolism of the starch in these bodies.

On the evidence presented in this section it is suggested that the chloroplasts of C. reflexa, by a photosynthetic process, elaborate starch in the form of small transient granules. It is also suggested that the chlorophyll-free starch grains of the stem arise, like the chloroplasts, from colourless proplastids, the raw material being derived partly from soluble carbohydrate absorbed from the host, and partly from the hydrolytic products of the assimilation starch produced in the parasite's own chloroplasts.

Green amyloplasts are also considered to be derived from

normal amyloplasts, as occurs in the potato tuber. Plastid types "c" and "d", which are intermediate between chloroplasts and green starch grains are thought to originate respectively from degeneration and elaboration of starch in the two extreme types. While further work is required for clarification of the inter-relationships of the various plastid types, the possibility is not excluded that the coloured components are labile and mutually interchangeable.

b. Cuscuta campestris.

In contradistinction to C. reflexa stems, where the pigment occurs largely in the outer stele and cortex, the pigmentation of C. campestris stems is almost exclusively confined to the inner stele (Figs. 24 and 25). As in C. reflexa, the pigment is located in discrete protoplasmic units, the chromoplasts.

All stem portions have an approximately similar distribution of pigment, although in juvenile regions of the stem plastids may be found in all tissues. In the floral pedicel, selective concentration of plastids around the vascular bundles, though evident in some sections, is not the characteristic feature it is in C. reflexa.

In the flowers of C. campestris pigment occurs in the

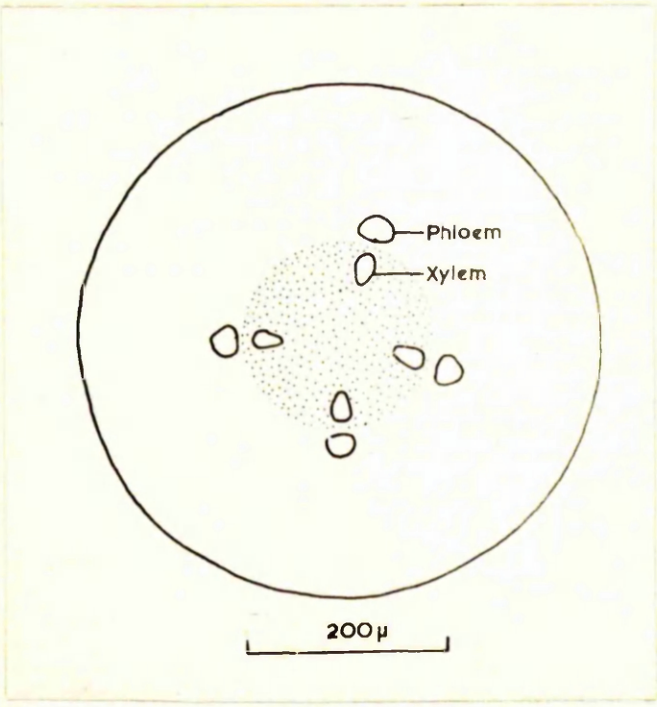


Fig. 24. Diagrammatic transverse section of stem of C. campestris, showing pigmented region(stippled).

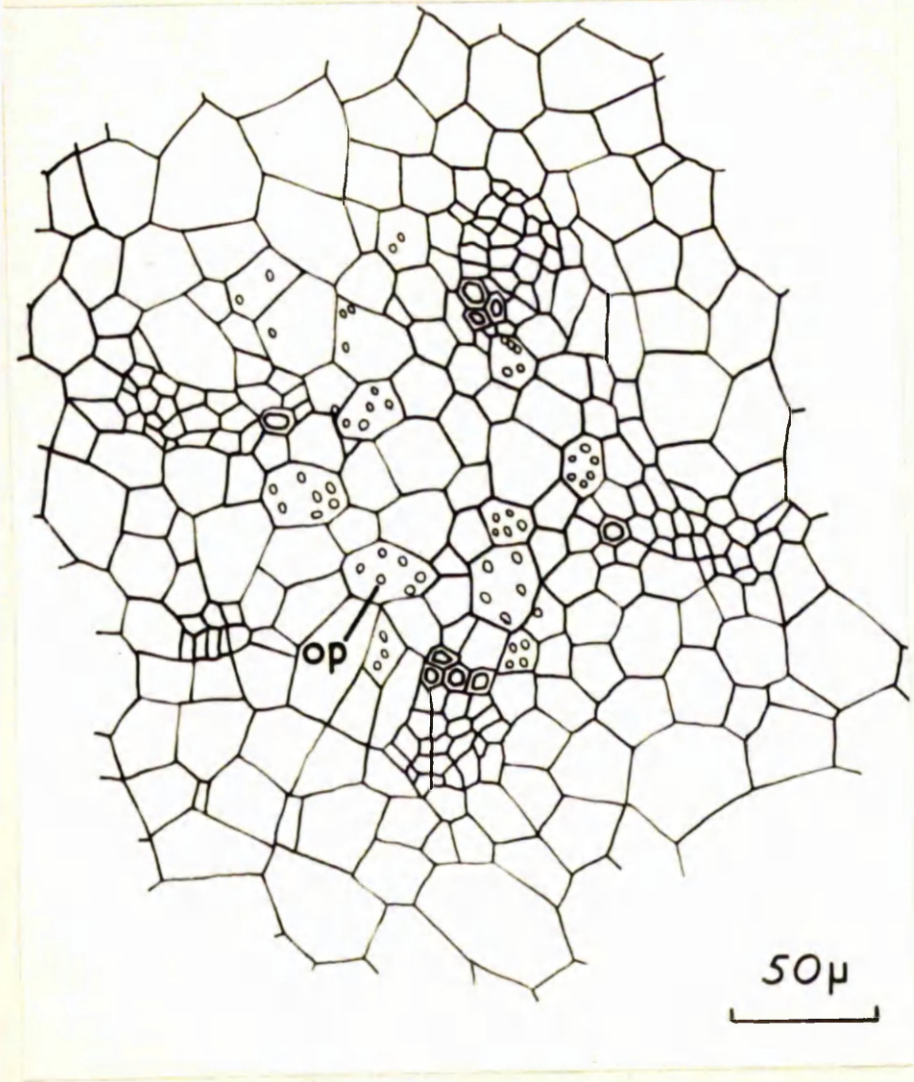


Fig. 25. Transverse section from stele of C. campestris stem, showing distribution of orange plastids(op).

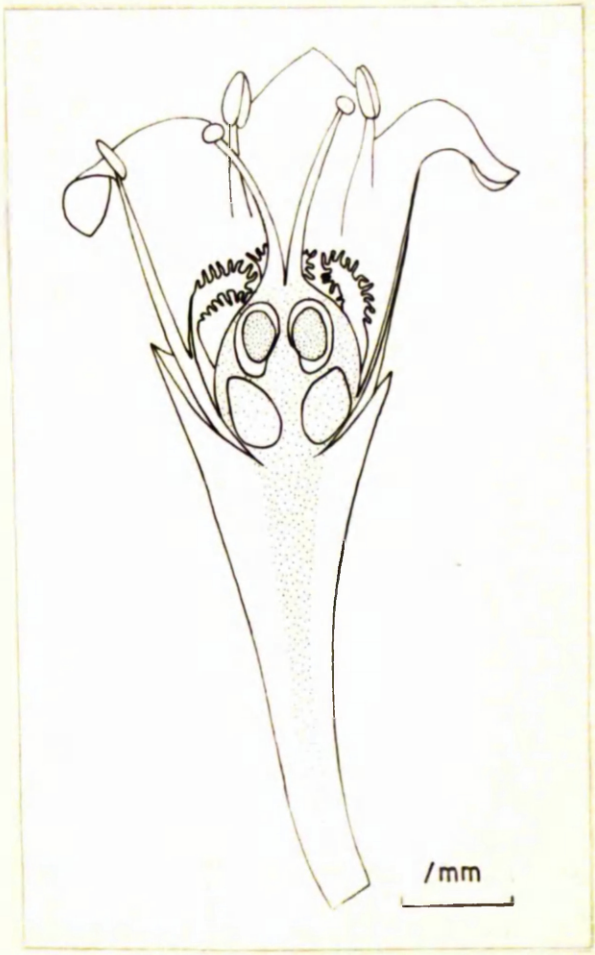


Fig. 26. Longitudinal section of C. campestris flower, showing chlorophyll distribution(stippled).

ovules, ovary, nectary and pedicel, together with smaller amounts in the anthers and the stigmas (Fig. 26). The highest concentrations are found in the ovules and ovary. Irrespective of the predominant colour of the stem, the pigmentation in the flowers is always green, or yellow in the nectaries. The cells of the stigmas contain small, green chloroplast-type structures (2.5μ); in the anthers, larger chloroplasts (5μ) are present. Nectary cells are yellow due to the presence of chloroplast-like structures (4μ) containing orange carotenoid granules.

The current investigation leads to the conclusion that the three colour-types of C. campestris stems, orange, yellow, and green, owe their coloration to a complement of coloured plastids whose relative proportions are characteristic of each type.

1. Orange stems.

All the pigmentation in this type of stem is due to red or orange plastids which vary considerably in size and structure; no green bodies can be detected in stems of this colour type. Type "a": Starch-free orange chromoplasts (3-6μ). These plastids show considerable internal variation and are markedly different both in pigmentation and structure. Each chromoplast, which has

a usually well-defined plastid membrane, consists basically of red carotenoid granules embedded in a plasma medium or stroma. The colour of the stroma of the plastid is most frequently pale yellow, though it may occasionally appear colourless. The carotenoid granules, which stain dark green with iodine in potassium iodide solution, never occupy more than 50% of the plastid volume. Such granules are broadly oval in shape, varying in length from 0.5-3.5μ, and their total number per plastid being limited by their combined volume. Sometimes vacuole-like oval sacs are visible in the plastid (Fig. 27).

Type "b": Chromoplasts containing usually small starch grains, but not completely occluded with starch. Plastids of this group vary from 4 to 7μ and are of comparatively infrequent occurrence in the stem. They consist of a usually well-defined membrane containing a yellow or colourless groundmass, in which are dispersed carotenoid granules and small irregular starch grains (Fig. 28).

Type "c": Colourless starch grains associated with chromoplast material. Considerable variation in the structure of these bodies exists, but in general, the starch grains, which are usually compound, occupy over 90% of the volume of the plastid. The chromoplast material consists of yellow or colourless basic plasma in which are embedded red carotenoid granules of varying

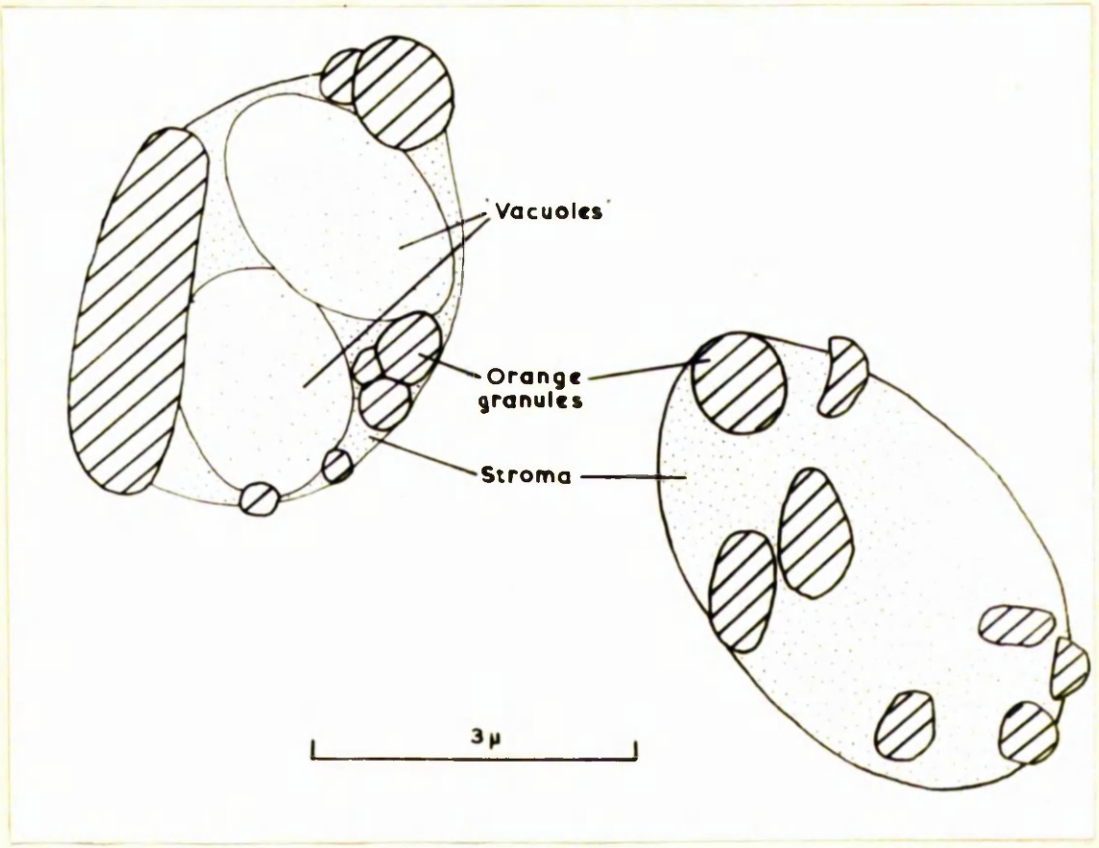


Fig. 27. Starch-free orange plastids from C. campestris stem.

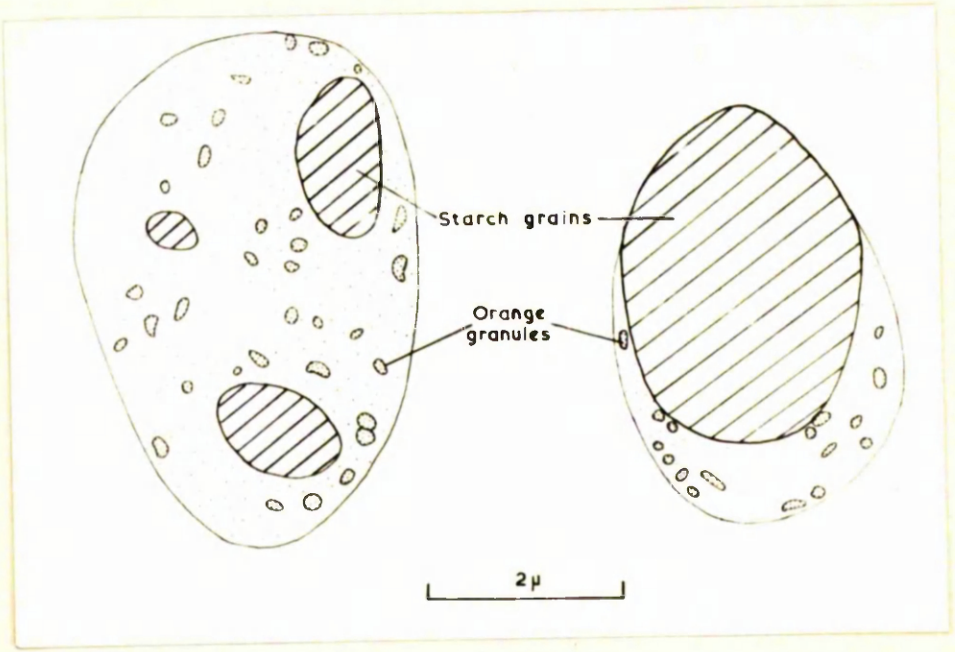


Fig. 28. Type "b" chromoplasts from orange stem of C. campestris.

size (Fig. 29). Occasionally the pigmentation of the amyloplasts is due to a homogeneous orange fluid without any evidence of granularity (Fig. 30). Although the pigmented material is normally located on the outside of the compound starch grains, examples have been observed in which the plastid material was embedded in the centre of a large compound starch grain. In any particular cell, all of the basic plastid types may be present (Fig. 31).

2. Yellow and green stems.

If an orange stem of C. campestris is allowed to grow under conditions of reduced illumination, eg. in the shade of its host, it may become yellow-green in the course of a few days. (Grown in total darkness, the stems become colourless). This colour change is due to the appearance of plastid types not found in orange stems. In addition to plastids of the type occurring in orange stems, at least four other types of plastid are present.

Type "A": Chloroplasts (3.6μ).

Type "B": Chloroplast-like bodies (3-6μ) containing variable amounts of carotenoid granules.

Type "C": Larger green plastids (6μ) with included granules of starch.

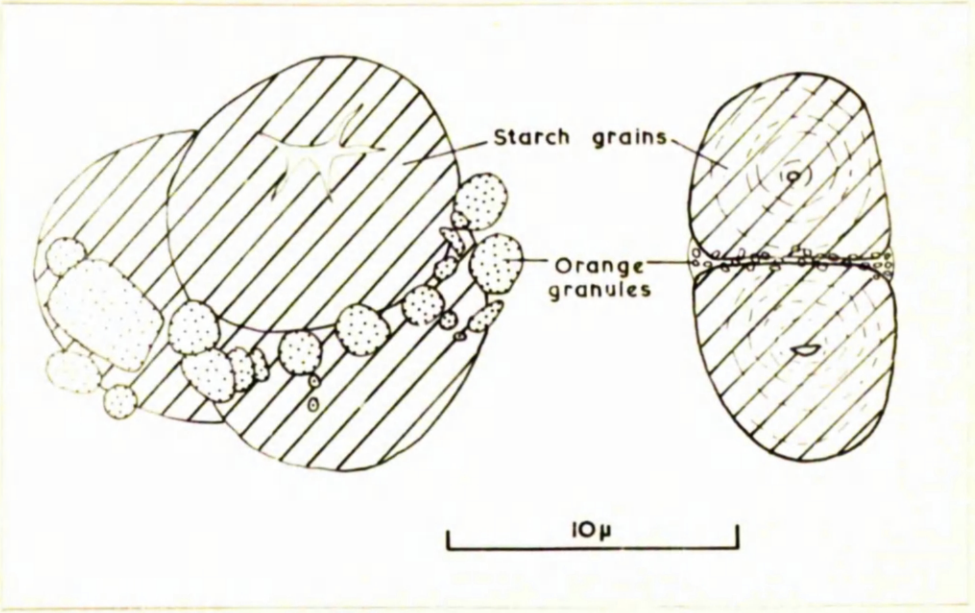


Fig. 29. Type"C" amyloplast from orange C. campestris stem. Note that the pigmented material is present as granules.

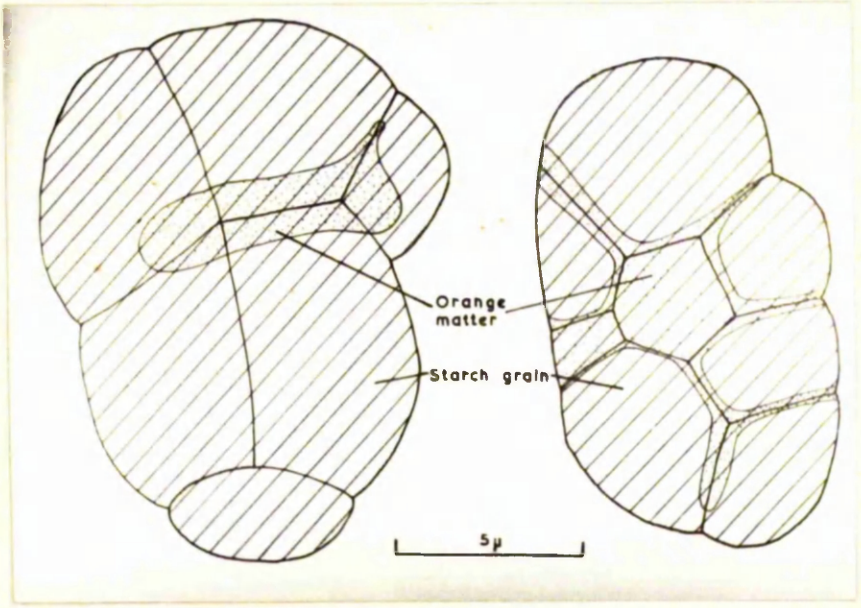


Fig. 30. Type"C" amyloplast from orange C. campestris stem. Note that pigmented matter is non-granular.

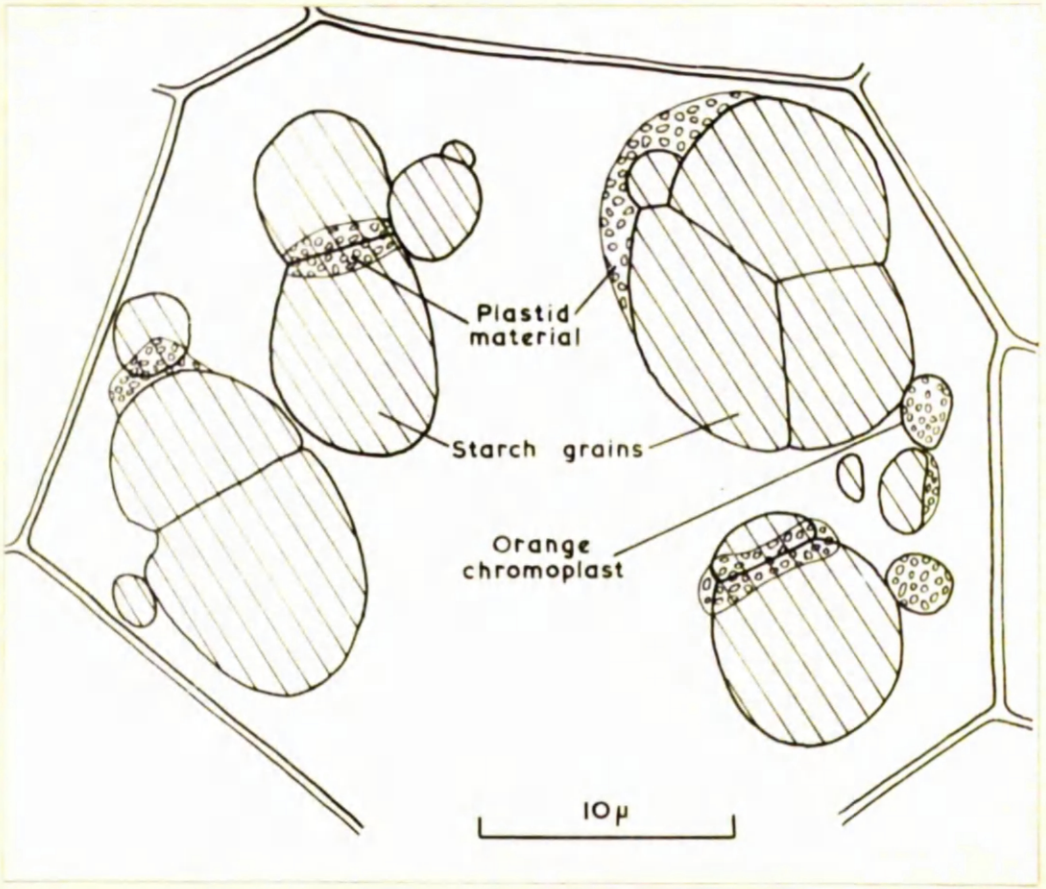


Fig. 31. Cell from stele of C. campestris stem, showing several types of chromoplast.

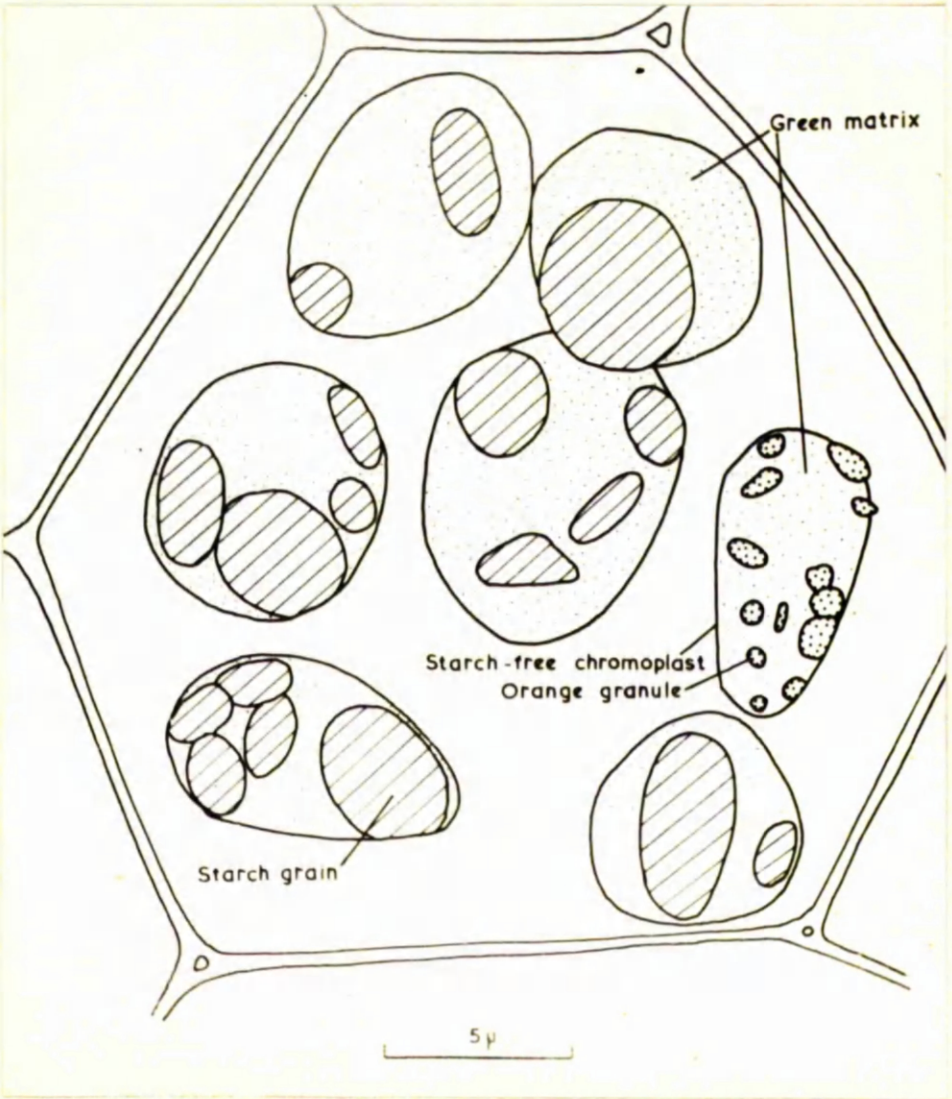


Fig. 32. Cell from region of protoxylem of yellow stem of C. campestris, showing presence of two plastid-types.

Type "D": Green amyloplasts (6-10μ).

It is quite clear that the colour change from orange to yellow or green in Cuscuta campestris stems is due to a reduction in the orange-plastid composition of the cells, associated with a concomitant increase in green plastids. Two possible mechanisms for this change in pigmentation may be suggested.

1. Annihilation or conversion into colourless plastids of the chromoplasts, accompanied by production of green plastids from pre-existing leucoplasts or proplastids.
2. Transformation of orange chromoplasts into green forms.

The first method requires the gradual disappearance of pigment from the plastids, with the formation of plastid bodies containing extremely variable amounts of colouring matter. Microscopic examination reveals no such transitional forms, certainly not in the large numbers which would be required for the modification of all the orange chromoplasts. The presence, in yellow and green stems of chloroplast-like bodies containing variable amounts of carotenoid granules is strong evidence in favour of the second hypothesis that orange chromoplasts are converted into green forms by gradual destruction of carotenoid.

In green stems, the pigmentation is almost exclusively

due to green plastids or amyloplasts, but even here, carotenoid granules exist in the plastids of cells bordering the protoxylem. A more intense orange coloration is similarly apparent in yellow stems in those cells of the medulla closest to the protoxylem (Fig. 32).

Because starch grains occur in tissues external to the central pigmented zone of C. campestris stems, they cannot have arisen from chromoplasts. The close morphological similarity of compound colourless starch grains and starch grains associated with carotenoid material in orange stems indicates a common origin, that is, they are both derived from colourless proplastids. In that case, association of the amyloplast with coloured material is a comparatively late development.

It frequently happens that starch-free chromoplasts are seen superimposed on starch grains giving the visual impression of an intimate association of starch grain and plastid. Indeed, it is almost certain that coloured amyloplasts (type "c") originate from a union of starch grains and one or more orange chromoplasts. Support is given to this view by the absence of any coloured starch grains containing only traces of carotenoid, which would be required by a de novo theory of development.

The fact that in orange stems a plastid sequence exists

from starch-free chromoplasts to coloured amyloplasts implies that some "c"-type plastids may have arisen from starch-free plastids by a progressive increase in the starch component; there is also no conclusive evidence that the reverse process may not occur. In this species, as in C. reflexa, the presence in the chromoplasts of a number of small starch granules must be taken as evidence of photosynthetic activity. It is conceivable that a mechanism comparable to that in the carrot root may exist in which chromoplasts develop from starch-containing leucoplasts; as the carotene increases in concentration, the starch disappears (Frey-Wyssling 1935). In the carrot root, however, where the plastids are non-photosynthetic, the older chromoplasts appear as large flat plates in the form of parallelograms with a typically crystalline appearance. No such structures are found in Cuscuta.

As chlorophyll has been shown to occur in all filaments of Cuscuta (Section V.A), and since all other cell components are colourless, chlorophyll must be present in at least some of the orange plastids, but masked by the carotenoids. According to Strain (1949), the yellow or orange colour of phaeoplasts (Phaeophyceae) appears to result from the physical condition of the pigments or from their geometrical arrangement in the plastids, rather than from a preponderance of the carotenoids. Extraction

of heated or fresh plants with alcohol yields green solutions in which the chlorophylls predominate. Similarly in the Thiorhodaceae and Athiorhodaceae the green colour of bacteriochlorophyll is masked by carotenoids (Rabinowitch 1945). The presence in a plant of orange-coloured plastids only is therefore not incompatible with the presence of chlorophyll in that organism.

In green stems of C. campestris, some, if not all green amyloplasts probably arise, as suggested for C. reflexa, from normal amyloplasts by the production of chlorophyll in the attenuated colourless plastid matter surrounding each granule. It is not thought that they have arisen from the fusion of a chloroplast and a starch grain. As in orange stem portions, the presence of a transitional series of plastids from green starch grains to chloroplasts suggests the possibility of mutual interconversion of the various plastid types.

Besides the several methods of plastid formation suggested in this section, there is good evidence that production of new plastids may occur by fission of existing adult plastids. Though the process was never observed directly, forms occur in which incipient division is almost certain. That chloroplasts can multiply by division was shown by Danegard (1947) who reported that the process can readily be seen in Elodea canadense; the

fission can occur even when starch grains are present in the chloroplast.

Electron photomicrographs reveal the chloroplast of the higher plant to consist of a (semipermeable) membrane about 100Å thick surrounding a colourless stroma or protein matrix in which about 50 dense grana are embedded. Heitz (1954) found a crystal-lattice arrangement of tiny granules in proplastids, but it is not known how these small granules are related to the lamellar structure of the grana of mature chloroplasts. Although the light microscope does not resolve the chloroplast contents of Cuscuta into distinct granules, it is possible that the carotenoid granules may represent the positions of the grana of the plastids. Electron microscopy of Cuscuta might therefore prove rewarding in research into the comparatively little known field of the localisation of chlorophyll, and particularly its association with carotenoid, within the grana of the chloroplast.

Cuscuta sp. might also be an ideal subject for an electron microscope study of plastid morphology and genesis. According to Sissakian (1958), there does not exist any unanimous concept either of the origin of plastids in general or of the possibility of interconversion of the various types. The theory of reversibility of the plastids is criticised by Frey-Wyssling et al. (1956) who

favour the concept of a monotropic-development path. They postulate the following scheme of monotropic-path formation.

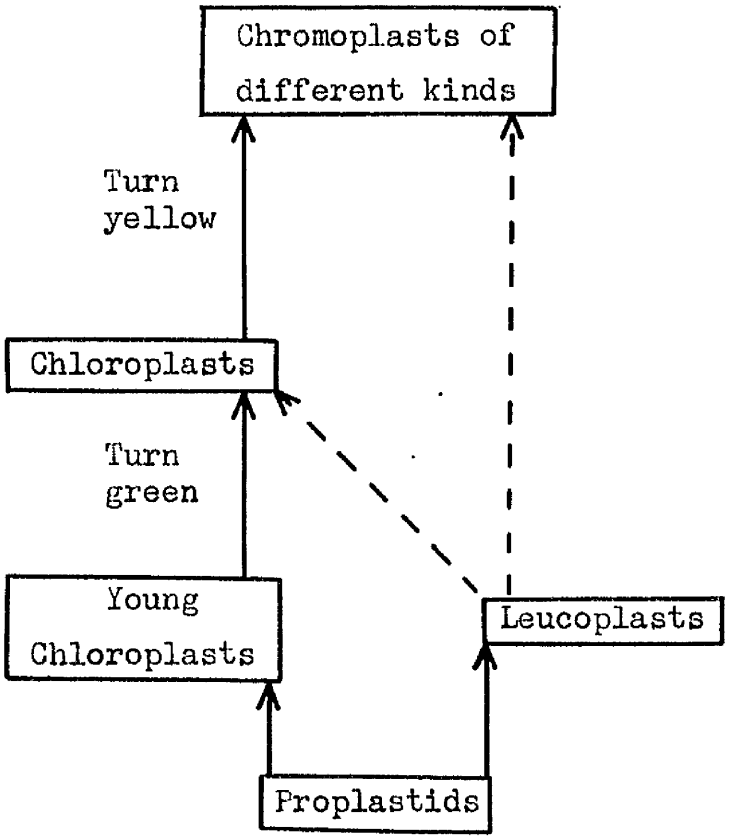


Fig. 33

According to this scheme, transformations against the direction of the arrows are not possible. Therefore the chromoplasts represent the final products of the plastid assembly line

and cannot be reconverted into any other type of plastid. This conflicts with the theory advanced in this work that the yellow or green modifications of C. campestris are derived from orange stems by the conversion of orange chromoplasts into green forms.

In Figs. 34 and 35 a suggested scheme of plastid genesis in C. reflexa and C. campestris is presented. All the pathways discussed in the text are included, but without any indication of the probability of their occurrence in the plant.

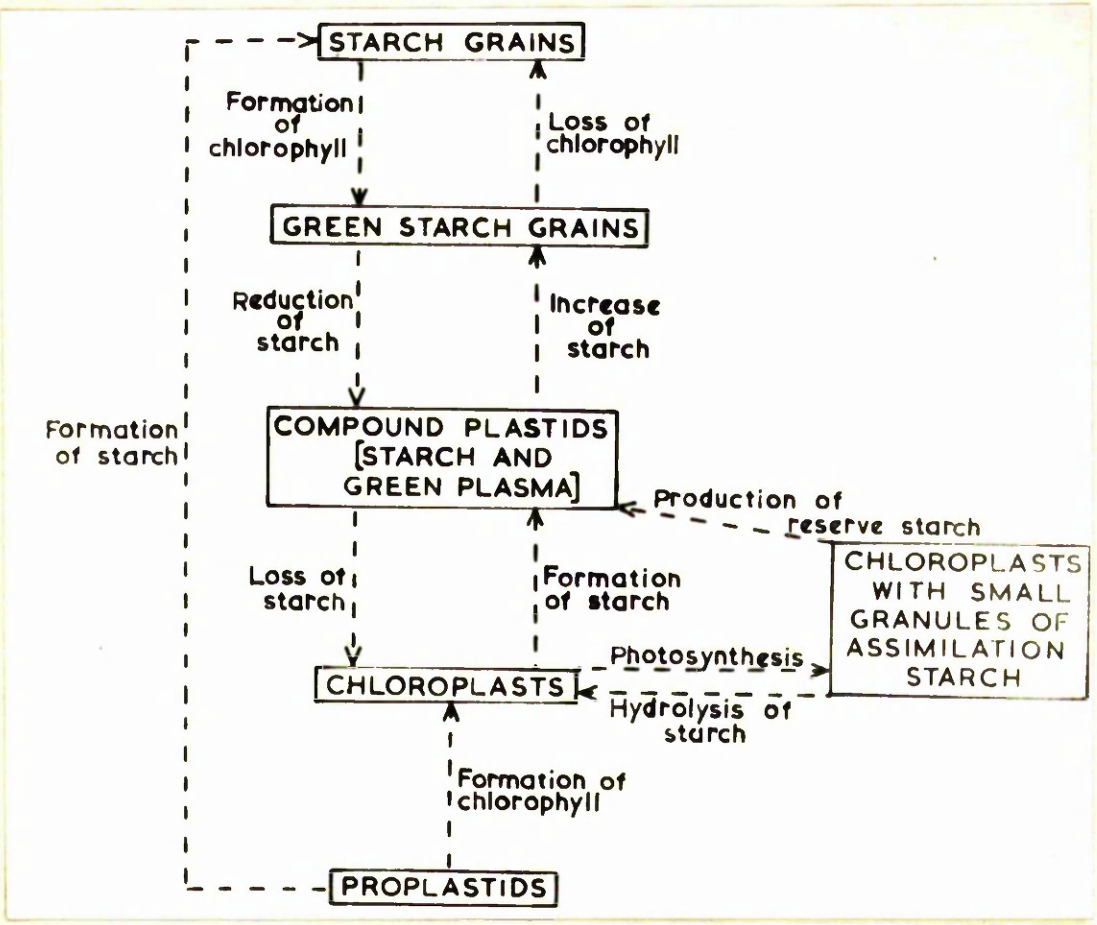


Fig. 34. Postulated scheme of plastid formation in Cuscuta reflexa.

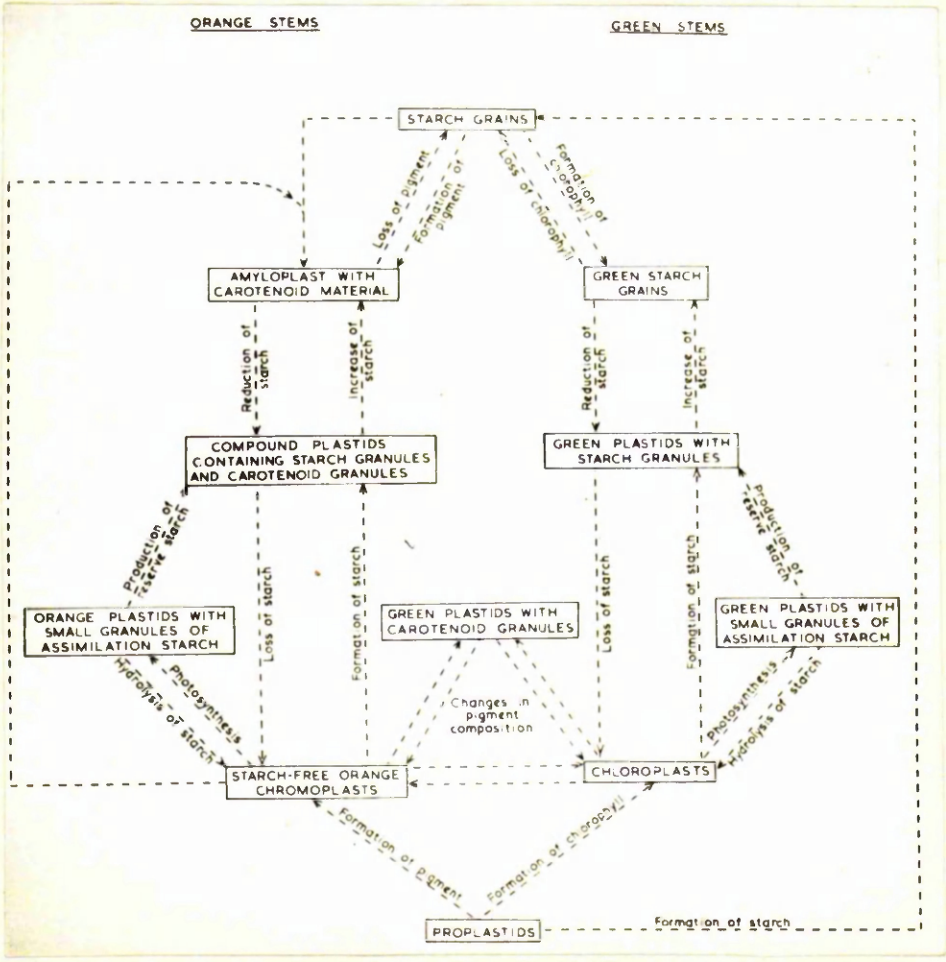


Fig. 35. Postulated scheme of plastid formation in Cuscuta campestris.

V. PIGMENT ANALYSES.

A. Isolation and Identification of Chlorophyll.

There are conflicting reports in the literature as to whether or not chlorophyll is present in Cuscuta. Willis (1955) describes the genus Cuscuta as a leafless and rootless total parasite which has no green tissue of its own, whereas Thomson (1925) notes the presence of chlorophyll in C. reflexa but states that it is generally regarded as totally insufficient for the carbohydrate requirements of the plant. MacKinney (1935) mentions that the shaded underside portions of Cuscuta salina are green, and he gives a brief description of the distribution of green pigment in the flowers and buds of the parasite. Describing the relationship between chlorophyll content and Vitamin C in Cuscuta gronovii, Walzel (1952) states that chlorophyll occurs in highest concentration in apical portions of the stem, being low in regions near the host.

Despite general observations in this vein, no worker appears to have subjected the occasional green coloration found among the Cuscutas to rigorous analytical tests which would confirm its identity. The following investigation was therefore undertaken in an attempt to confirm or disprove that the green

pigment is in fact chlorophyll, and further, if it is present, to determine whether chlorophyll "a" and chlorophyll "b" are in the same ratio as that found in autotrophic plants. It was considered important to ascertain these points as being of significance in assessing the degree of the plant's obligate parasitism. In this work, chlorophyll was isolated from Cuscuta extracts by paper and column chromatography and examined spectroscopically on a SP 600 spectrophotometer.

a. Isolation of chlorophylls by paper chromatography.

Paper chromatography has been extensively used to separate the fat-soluble pigments of plant tissues. Brown (1939), using a system consisting of a sheet of blotting paper between two plates of glass, succeeded in separating the two forms of chlorophyll ("a" and "b") from a carbon disulphide leaf extract. Extracts of the leaves of Tradescantia albiflora were analysed by one- and two-dimensional paper chromatography by Bauer (1952). His solvent system consisted of a mixture of benzene, petroleum ether, and acetone, including methanol for the second development in his two-dimensional chromatograms. By this technique he succeeded in separating chlorophyll "a" from chlorophyll "b".

Chlorophylls "a" and "b" have also been separated from

Soya-bean leaves with two-dimensional chromatography by Lind et al. (1953). They extracted the pigments in acetone and then transferred them to petroleum ether. The solvents used successively to develop the chromatograms in the first direction were:- acetone, petroleum ether, and 1% n-propanol in petroleum ether; in the second direction 25% chloroform in petroleum ether was used. By this method very clear separations of chlorophylls "a" and "b" were obtained.

More recently, Angapindu et al. (1958) tested the suitability of various papers and solvents in the separation of chlorophylls. One of the processes described, the reversed phase method, depends on the use of filter paper impregnated with water-repellent agents such as medicinal paraffin oil. For the separation in question, Whatman No. 4 filter paper was used, with methanol as irrigating solvent. A characteristic feature of this procedure is the final sequence of the plant pigments, which is almost exactly the reverse of the sequence obtained using unimpregnated paper. However, very distinct separation of chlorophylls "a" and "b" is obtained. With unimpregnated paper, Angapindu et al. used circular chromatograms, finding them superior to both ascending and descending methods. As irrigating solvents they used chlorobenzene, toluene or petroleum ether containing 0.5-1% n-propyl alcohol.

The methods used to test for chlorophyll in this work were based on the techniques of Angapindu et al. (1958). Attempts to separate the pigments on ascending or descending paper chromatograms were mainly unsuccessful. Very poor and inconsistent separations were obtained, with much tailing. However, good results were obtained by means of circular paper chromatography.

1. Preparation of pigment extracts.

Freshly harvested samples (1-10gm.) of Cuscuta from various hosts were thoroughly washed with cold water and dried by lightly pressing between layers of towelling. The filaments were then ground in a mortar under acetone with acid-washed silver sand, and the extract filtered under suction through a filter funnel containing a tight plug of cotton wool. The mortar and funnel were washed with acetone until the residue was colourless. The volume of acetone required for complete extraction varied with the amount of material used, but was usually about 100ml. The filtered extract was transferred to a separating funnel and an equal volume of petroleum ether added, together with sufficient water for the formation of two layers. This resulted in all the pigments being transferred to the epiphase. The lower acetone layer was run off and more water (20ml.) added, with careful shaking. The funnel was shaken gently to avoid the

formation of an emulsion which prolongs the separation into two distinct layers. Washing of the upper layer was repeated four or five times to remove most of the acetone from the petroleum ether. The extract was then dried for half an hour over anhydrous sodium sulphate, filtered, and transferred to a Buchner flask where it was evaporated to a small volume under reduced pressure. A bath of warm water was placed underneath the flask to speed up the rate of evaporation. It was this concentrated extract which was used to spot the chromatograms. Attempts at separation were made with reversed phase paper chromatography using paper dipped in paraffin, but as initial results were discouraging, the method was abandoned.

Spotting of the chromatograms was performed by two different methods. The simplest method was the application of a relatively large single spot (up to 50µl.) to the centre of the paper. On applying a few drops of petroleum ether to the centre of this spot, a clear zone surrounded by a circular band of pigment was formed. The second method entailed the application of usually four smaller spots (up to 25µl.) on the circumference of a 2.5cm. circle drawn at the centre of the paper. The latter method was preferred and was used throughout the following work.

2. Development of the chromatograms.

Following the application of pigment extract to Whatman No.1

(24cm.) circular filter paper, a small slit was cut in the centre of the paper, through which a filter paper wick (2mm. x 2cm.) was inserted. The irrigation system consisted of three petri dishes (5cm., 10cm., and 15cm.) arranged concentrically as illustrated in Fig. 36. The dishes were chosen so that their rims were co-planar. Into the small central dish was placed sufficient solvent to irrigate the whole filter paper (about 20ml.). Solvent was also poured into the two larger dishes to maintain a saturated atmosphere in the system.

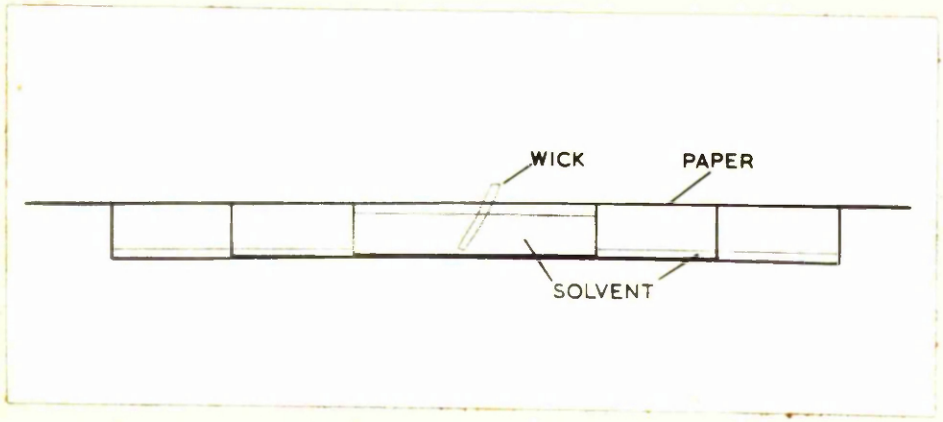


Fig. 36. Apparatus used for circular chromatography.

The spotted filter paper was then placed on the petri dishes so that the wick reached the bottom of the solvent in the central dish, and the whole system placed on a sheet of polythene and covered with a glass trough. The solvents employed to

develop the chromatograms were petroleum ether (boiling range 60/80) and toluene. After a few hours, when the solvent front had reached nearly to the edge, the filter paper was removed from the solvent and allowed to dry in air at room temperature.

3. Results.

The chromatogram described below was obtained when petroleum ether containing 1% iso-propyl alcohol was used as developing solvent (Fig. 37).

Carried along with the solvent front in extracts of either Cuscuta species was a deep orange band identified as β-carotene (Angapindu et al., 1958). Behind this, with an Rf value of approximately 0.9 was a faint orange zone, and inside this, with an Rf of 0.5, a composite zone corresponding to the monohydroxy xanthophylls. This zone was followed by two green zones, an outer lime-green zone and an inner moss-green zone corresponding with the chlorophyll "a" and chlorophyll "b" zones obtained by Angapindu et al.(1958) for sunflower leaves. Though the two chlorophyll bands were never separated by a pigment-free band there was frequently nevertheless a diminution in the pigment intensity at the interphase of the two green zones, indicating that separation was almost complete. In some chromatograms, a

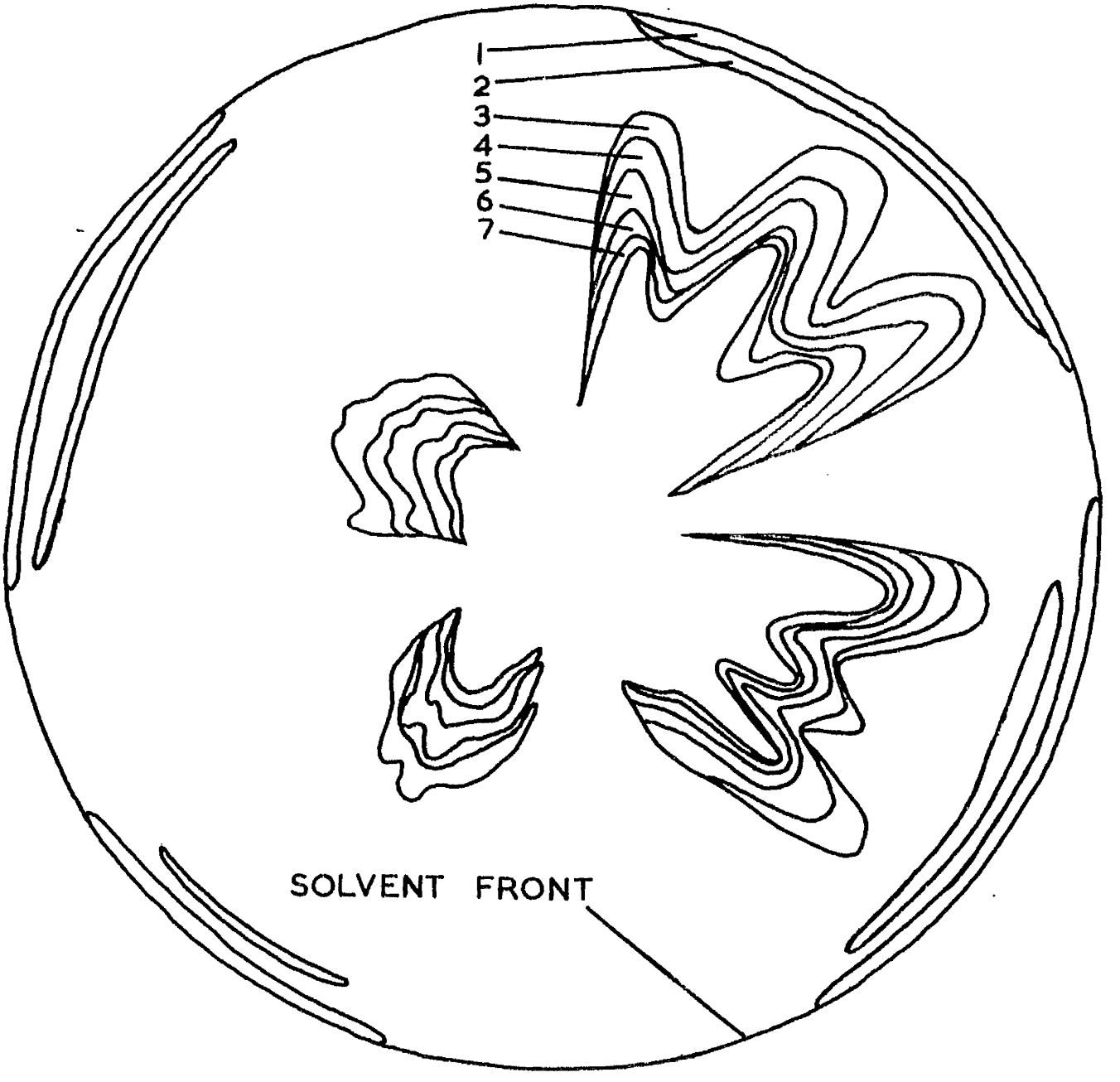


Fig. 37. Paper chromatogram of pigment extract of Cuscuta campestris, using petroleum ether containing 1% iso-propyl alcohol as developing solvent. 1=β-Carotene; 2=ɣ-Carotene?; 3 and 4 = mono-hydroxy xanthophylls; 5=Chlorophyll"b"; 6=Chlorophyll"a"; 7=di-hydroxy xanthophylls. This tracing shows the effect of high initial concentration(both right-hand spots) on Rf values.

faint yellow zone appeared between the chlorophyll "a" band and the origin. This was assumed to be dihydroxy xanthophylls (Angapindu et al., 1958).

It was found that the Rf values of chlorophylls "a" and "b" varied with the amount of extract applied, high concentrations of pigment elevating the Rf values. Measurement of Rf values was further complicated by the irregular outline of the chlorophyll zones. The mean values were calculated (with toluene as solvent) from a large number of Rf measurements using the mid-points of the two green zones as reference points (Table 1).

Table 1. Rf values of chlorophyll "a" and chlorophyll "b".

	Chlorophyll "a"	Chlorophyll "b"
C. campestris	4.7	4.2
C. reflexa	5.1	4.5

Extracts for spectroscopic examination of the chlorophyll pigments were obtained by cutting out the appropriate zones from the paper and eluting with diethyl ether. The absorption curves for these extracts were in good agreement with the published values of Smith and Benitez (1955), but have not been included since column chromatography of the Cuscuta extracts described in

Section V.A.(b.) gives similar results.

When a mixture of Cuscuta extract and Acer pseudoplatanus leaf extract was co-chromatographed under conditions similar to those described above, the green zones of the Cuscuta coincided with those of the Acer, thus providing strong evidence that the green pigments of Cuscuta are chlorophyll "a" and chlorophyll "b". Chromatography of a mixture of extracts of the two species of Cuscuta similarly resulted in the formation of only two green zones.

b. Isolation of chlorophyll by column chromatography.

Although the methods of paper chromatography are suitable for the demonstration of the presence of chlorophyll in Cuscuta, they do not lend themselves easily to the isolation of sufficiently large quantities of pigment for quantitative and spectrophotometric measurements. It was decided, therefore, to use the techniques of column chromatography.

Most of the detailed attempts to separate the chlorophylls have entailed the use of columnar chromatography. It is important in this technique to use the correct adsorbent, since many adsorbents used in the general separation of plastid pigments cause decomposition of chlorophyll. This is particularly true of adsorbents such as aluminium oxide, calcium carbonate, sodium

sulphate (Winterstein and Stein, 1933), and magnesium citrate (Strain, 1942). The most satisfactory adsorbent appears to be powdered sucrose (Winterstein and Stein, 1933; Seybold and Egle, 1938; Strain, Manning and Hardin, 1943; and Koski and Smith, 1948). Petroleum ether, usually containing small amounts of polar liquids such as acetone, is the most commonly used solvent.

Chlorophylls "a" and "b" have been isolated in a chromatographically pure state by Stoll and Wiedmann (1952) by means of column chromatography. They used powdered sucrose in 10cm. x 50cm. columns, with a mixture of equal parts of benzene and petroleum ether as developing solvent. Once separation was complete, the green zones were mechanically removed from the column and eluted with diethyl ether.

Smith and Benitez (1955) have isolated chlorophylls "a" and "b" from fresh leaves. They disintegrated the leaves in a Waring blender with acetone and finally transferred the pigments to petroleum ether. All their operations were carried out under low light intensity in a cold-room at 4° to 6°C. The final extract was passed repeatedly through sucrose columns, until highly purified components were isolated and eluted with ether for spectroscopic determinations.

1. Materials.

The most satisfactory column size, in view of the quantities of pigment involved was found to be 1.4cm. x 14cm. Test-tubes proved to be ideal containers to adapt for this purpose. The closed end of the test-tube was heated and extended into a cone by pushing from the inside with a metal rod. The narrow apex thus produced was cut off and the rough edges rounded off in a bunsen flame.

A variety of adsorbents were tested, including aluminium oxide, light and heavy magnesium oxide with varying proportions of Hyflo Super-Cel or kieselguhr, zinc carbonate, and icing sugar. Of these, icing sugar was the most satisfactory

2. Preparation of columns.

Commercial icing sugar was dried overnight in a vacuum desiccator (Goodwin, 1958). A plug of cotton wool was inserted into the bottom of the column and small quantities of adsorbent added and lightly pressed down with a glass plunger. This process was repeated until the powder was within about 3cm. of the top of the tube. The lower narrow end of the tube was attached to a water pump via a Buchner flask and negative pressure applied. Pure petroleum ether was then poured in at the top of

the column until the whole column of sucrose was saturated. When the solvent level in the tube had reached within about 2mm. of the surface of the sugar, a small volume (about 1ml.) of the concentrated pigment extract (prepared as described on page 46) was introduced at the top of the column. When this had been absorbed by the powder, the upper portion of the column was washed with pure petroleum ether from a pipette and the washings allowed to sink into the sucrose. The washing was repeated until all the pigment had been adsorbed by the sugar column, whereupon fresh solvent was added until the tube was filled. Fresh solvent was continuously added throughout the separation.

Owing to the high concentration of masking carotenoid pigments, particularly with C. campestris, no green coloration could be observed during the early stages of separation. As elution proceeded, however, a definite green band appeared near the top of the column. When the lowest orange zone had passed through the column, 1-5% acetone was added to the developing fluid. This had the effect of removing most of the xanthophylls adsorbed below the green zone, and of separating the green zone into two distinct bands, an upper moss-green band and a lower band grass-green in colour. This result was obtained in both species of Cuscuta, irrespective of the host plant from which they were harvested.

To test if these two bands were chromatographically identical with chlorophylls "a" and "b" from green leaves, co-chromatograms were run with leaf extracts. Acer pseudoplatanus leaves were extracted with acetone in exactly the same manner as for Cuscuta extracts. Small aliquots of this extract were applied to columns prepared in the same manner as for Cuscuta. Using developing conditions as closely similar as possible to those employed for Cuscuta, the resulting chromatogram showed two green bands corresponding fairly well to those in both species of Cuscuta.

Chromatography of a mixture of extracts of Cuscuta stems and Acer leaves yielded only two green bands. A mixture of pigment extracts of C. campestris and C. reflexa, when passed through the column similarly yielded only two green bands. From these results it is concluded that the presence of chlorophyll in both species of Cuscuta is established, and that it is present as chlorophyll "a" and "b", thus supporting the results obtained from paper chromatography. A rough visual estimate of the ratio of chlorophyll "a" to chlorophyll "b", based on the width of the two zones on the column, puts its value at about 2:1.

3. Spectroscopic analysis of the chlorophylls.

Cuscuta extracts were prepared and chromatographed as

described on pages 53-55. When the two green zones were sufficiently separated and free from adjoining xanthophylls, the supply of solvent was discontinued and negative pressure applied to draw off most of the developing fluid from the column.

Removal of the green zones was a process attended with some degree of hazard. The most satisfactory method, when successful, was the extrusion of the intact column on to a sheet of paper by means of compressed air. This resulted in a banded column from which the relevant zones could easily be cut out. In this respect, sucrose proved most satisfactory as an adsorbing medium; the column retained its cylindrical form for a length of time sufficient for the removal of the pigmented zones. The alternative method, removing the bands mechanically by means of a spatula, suffered from the disadvantage that other pigments are liable to be removed along with the required zones. When either method was used, only the central, presumably purest portions of each zone, were selected for spectrophotometric measurements. The isolated zones were shaken up with 10ml. diethyl ether, filtered, and stored in sealed tubes in the dark at $4^{\circ}C$.

Spectroscopic analysis were performed on this extract using a Unicam SP 600 with a 1cm. light path. Extinction values were plotted at 10mμ intervals in the range 400mμ to 700mμ, and at

smaller intervals in the regions of maximum absorption to localise the exact turning points of the curves. The absorption curves for both green zones of C. reflexa and C. campestris are shown in Figs. 38-41. The two major absorption maxima for each extract, together with values obtained for chlorophyll "a" and chlorophyll "b" in grape leaves by Smith and Benitez (1955) are presented in Table 2.

Table 2. Absorption maxima of chlorophylls.

		Absorption Maxima
C. reflexa	Upper green zone	454 , 645
	Lower green zone	431 , 664
C. campestris	Upper green zone	455 , 647
	Lower green zone	433 , 665
Grape leaves (Smith and Benitez, 1955)	Chlorophyll "b"	455 , 644
	Chlorophyll "a"	430 , 662

In Table 2 it will be noted that there is a close correspondence between the absorption maxima obtained for the upper and lower green zones of both C. reflexa and C. campestris with the

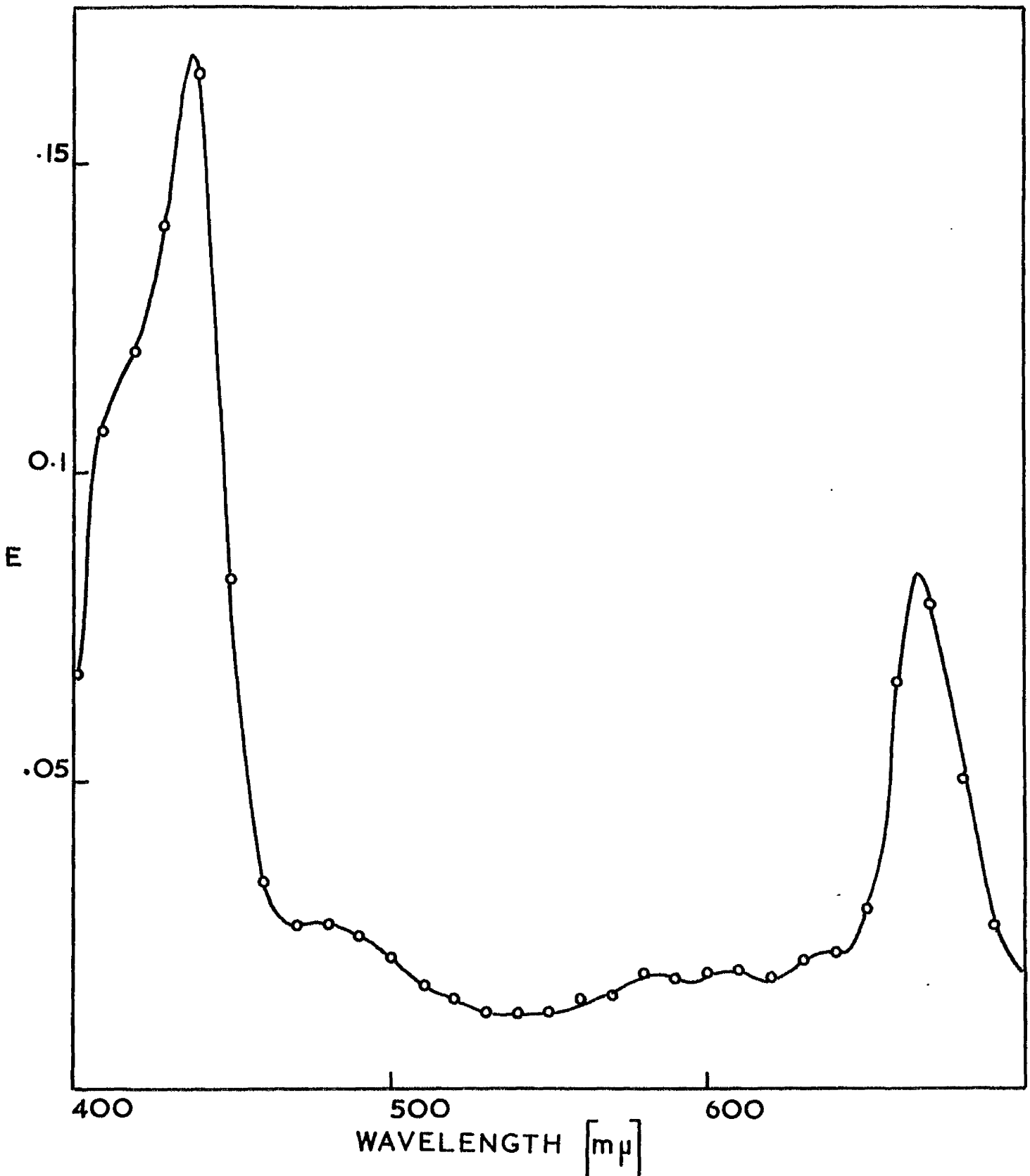


Fig. 38. Absorption Spectrum of Chlorophyll"a". (C. campestris)

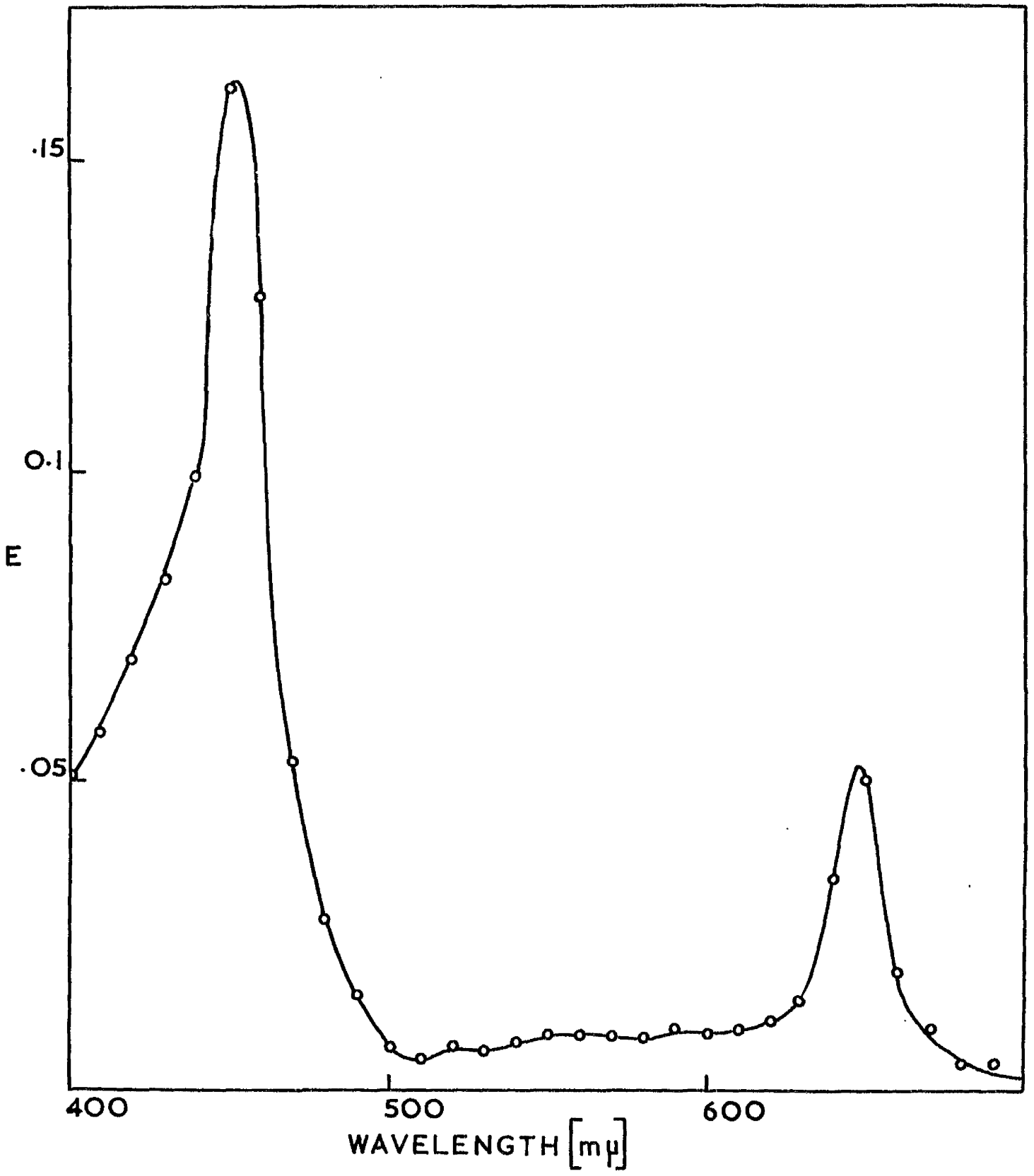


Fig. 39. Absorption Spectrum of Chlorophyll"b". (C. campestris)

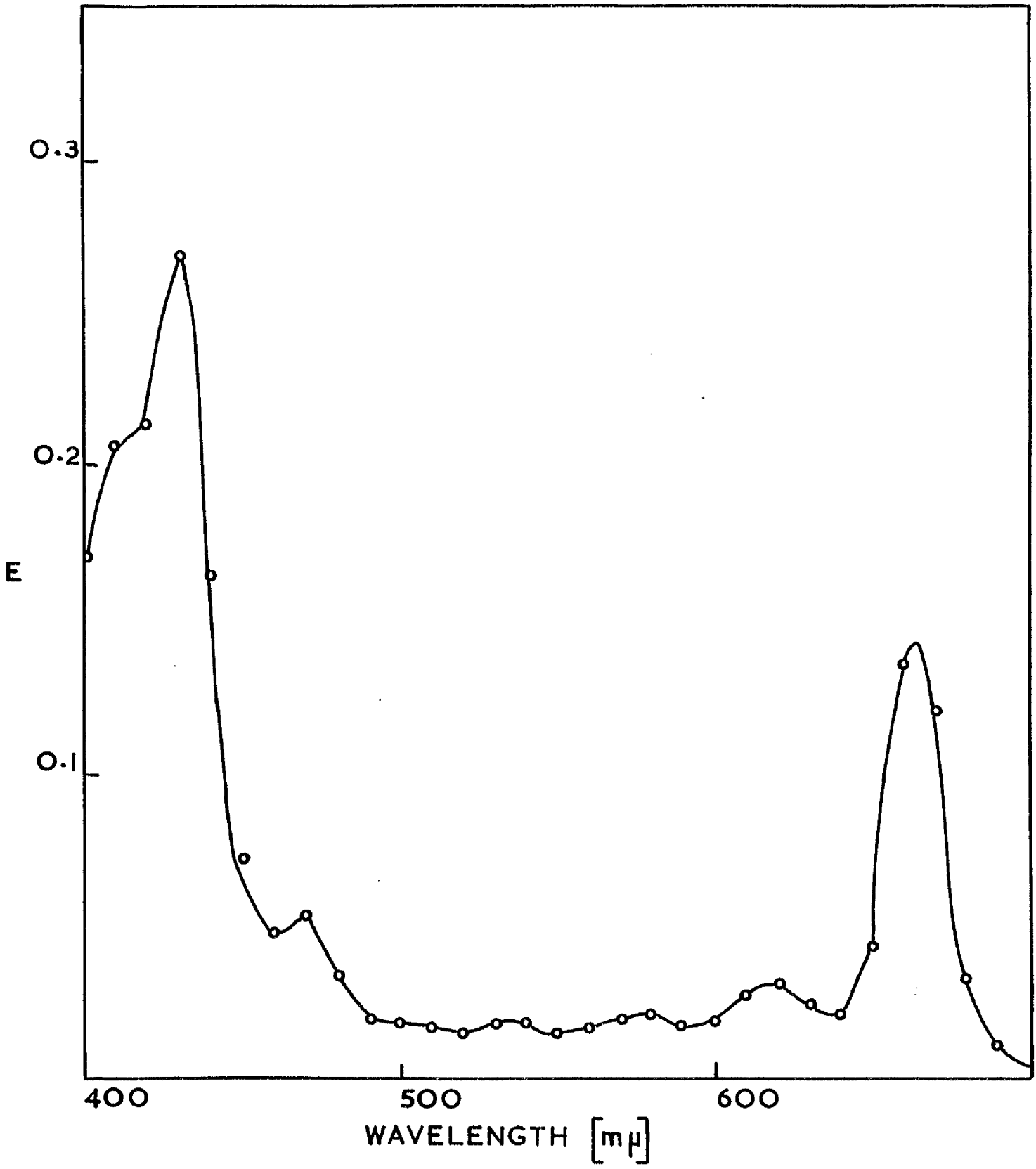


Fig.40. Absorption Spectrum of Chlorophyll"a". (C. reflexa)

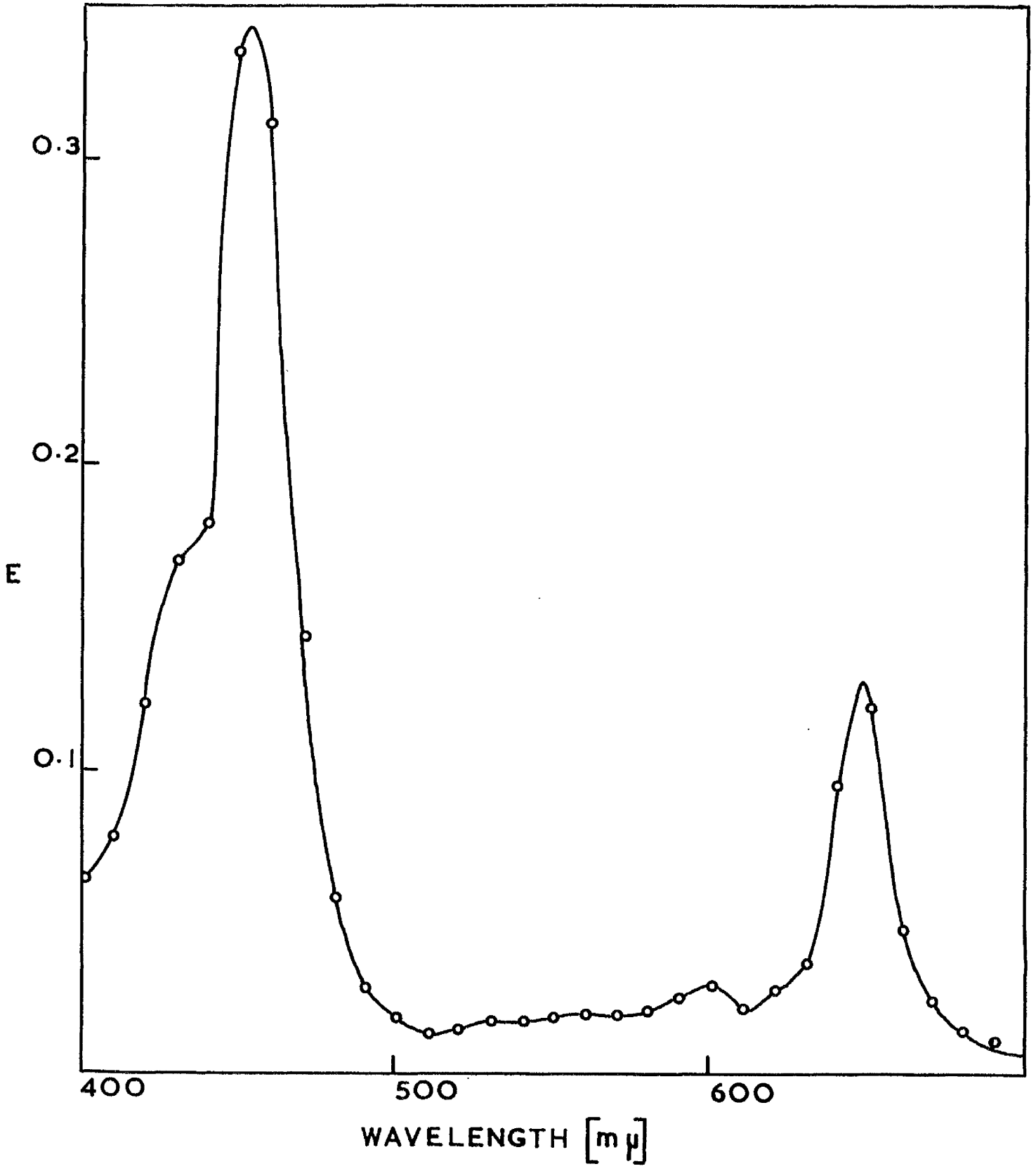


Fig. 41. Absorption Spectrum of Chlorophyll"b". (C. reflexa)

published values for chlorophyll "b" and chlorophyll "a" respectively (Smith and Benitez, 1955).

The conclusion drawn from these experiments involving both paper and column chromatography of Cuscuta extracts, combined with spectrophotometric analyses is that chlorophyll does occur in the two species tested, irrespective of the hosts on which they are grown. Furthermore, it is present in the form of chlorophyll "a" and chlorophyll "b", and if any other species of chlorophyll occur, they can be present only in insignificant amounts. This demonstration is of particular interest in view of the fact that Cuscuta has always been regarded as an obligate parasite.

c. Variation in chlorophyll content of parasites growing on different hosts.

In view of the apparently complete dependence of Cuscuta on its host, it was considered of importance at this stage to obtain quantitative measurements of its chlorophyll content. This value, when compared with chlorophyll levels in normal green plants could be used as a measure of the parasite's degree of autotrophism, on the assumption that its chlorophyll was able to function normally in carbon assimilation (See Section VI). It was

also considered of importance to test if growth on different hosts affected the amount of chlorophyll present in the parasite.

1. Methods.

Vigorously growing filaments of C. reflexa and C. campestris were harvested from their hosts, washed in cold water, dried and weighed. The quantities of material used varied between 1gm. and 5gm. The filaments were then ground under acetone as described in Section V.A.(a.), but additional care was taken in washing the residues to ensure that all the pigment had been extracted. To the filtered acetone extract an equal volume of diethyl ether was added, followed by sufficient water for the formation of two layers. The colourless hypophase was allowed to drain off, and the epiphase washed with successive 20ml. portions of water to remove the acetone. The ether layer containing the pigments was dried by transferring it to a tube containing anhydrous sodium sulphate for 60 min. The dry ethereal extract was filtered under negative pressure through a funnel containing a tight plug of cotton wool, and the sodium sulphate washed free of pigment by repeated washing with small volumes of pure diethyl ether. The extract was then made up with ether to a convenient volume, usually 50 or 100ml., depending on the colour intensity of the solution, and stored in the dark at 4^{o}C.

Estimations of chlorophylls "a" and "b" were made on these solutions by differential spectroscopy using the equations of Smith and Benitez (1955). These are:-

Chlorophyll "a" (gm./l.) = $0.0101\ D_{662} - 0.00101\ D_{644}$

Chlorophyll "b" (gm./l.) = $0.0164\ D_{644} - 0.00257\ D_{662}$

where D_{644} and D_{662} are the extinction values at 644 and 662mμ respectively, using a 1cm. light path. Four millilitre samples of the ether extracts described above were transferred to 1cm. cells and extinction measurements recorded at 644 and 662mμ.

2. Results.

Total chlorophyll was found by adding the values obtained for chlorophyll "a" and chlorophyll "b" (Tables 3 and 4). It is interesting to note that the ratio of chlorophyll "a" to chlorophyll "b" in C. reflexa and C. campestris is closely similar to that found in green leaves (Tables 5 and 6). From Fig. 42 it will be seen that in C. campestris the highest chlorophyll content occurs when it is parasitising Vicia, and its lowest on Nicotiana and Pelargonium, with an intermediate value on Bulbine. Cuscuta reflexa similarly exhibits its highest chlorophyll concentration on Vicia, but its lowest value is on Vitis, with intermediate values on Nicotiana, Pelargonium, and Helianthus (Fig. 43). These experiments show that of all the hosts on which they grow

Table 3 Chlorophyll content of C. campestris and Angiosperm leaves. Figures for leaves are calculated from data by Goodwin(1958)

Host	Total Chlorophyll	
	Fresh Weight	Dry Weight
Vicia	50.1	463.8
Bulbine	36.6	430.6
Pelargonium	25.2	200
Nicotiana	24.6	193.6
Acer pseudoplatanus		4000
Prunus nigra		8400
Quercus robur		6800

Table 4 C. reflexa: chlorophyll content, expressed as μ gm/gm fresh weight and dry weight.

Host	Total Chlorophyll	
	Fresh weight	Dry weight
Vicia	80.3	590.2
P. zonale	62.6	509
Nicotiana	52	419.4
Vitis	44	305.6
Helianthus	59.3	373

Table 5 C. campestris. Ratio of chlorophyll"a" to chlorophyll"b". Figures are µgm/gm fresh weight.

Host	Chlorophyll"a" (µgm/gm fresh weight)	Chlorophyll"b" (µgm/gm fresh weight)	Chlorophyll"a"/ Chlorophyll"b"
Vicia	39.1	11.1	3.5
Bulbine	27.2	9.8	2.8
Pelargonium	20.5	6.1	3.4
Nicotiana	18	6.2	2.9

Table 6 C. reflexa. Ratio of chlorophyll"a" to chlorophyll"b". Figures are µgm/gm fresh weight.

Host	Chlorophyll"a"	Chlorophyll"b"	Chlorophyll"a"/ Chlorophyll"b"
Vitis	29.8	10.8	2.8
Helianthus	35.8	18	2
Vicia	39.7	13.1	3
Pelargonium	38.2	18.3	2.1
Nicotiana	48.4	21.6	2.2

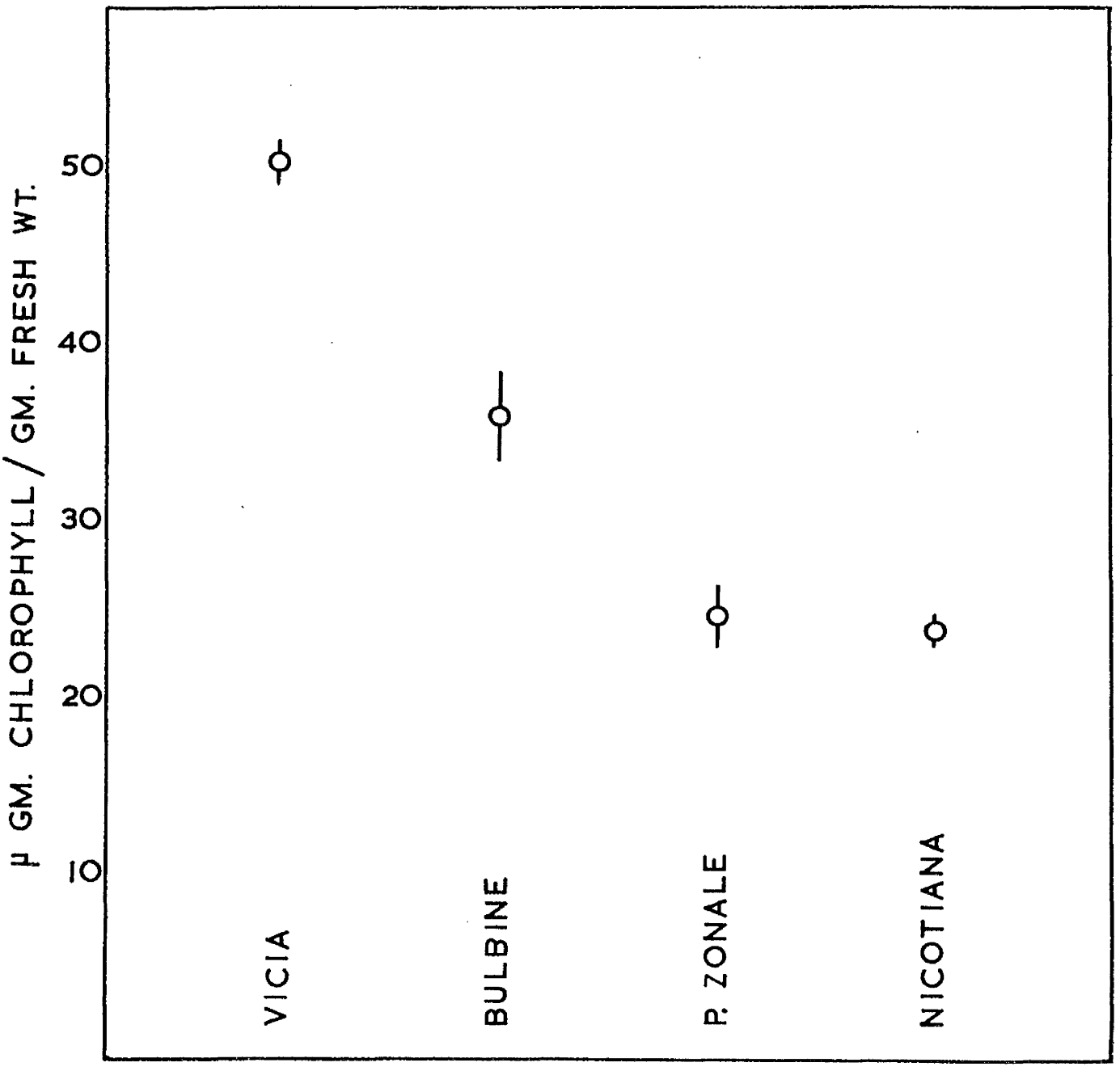


Fig. 42. Chlorophyll content of C. campestris, on Vicia, Bulbine, Pelargonium and Nicotiana hosts. Circle denotes mean; standard deviation is represented by vertical line.

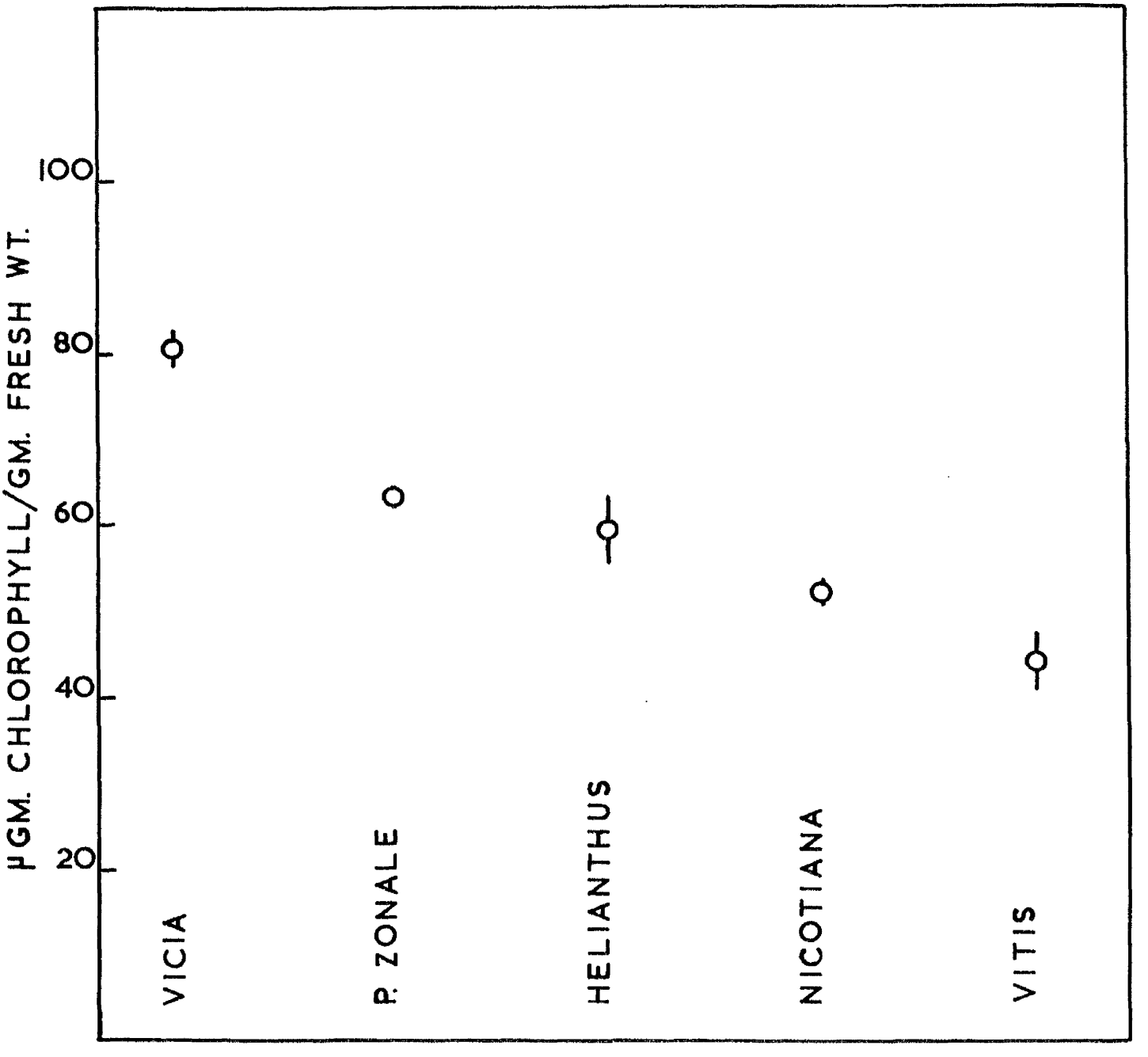


Fig. 43. Chlorophyll content of C. reflexa, on Vicia, Pelargonium, Helianthus, Nicotiana and Vitis hosts. Circle denotes mean; standard deviation is represented by vertical line.

successfully, both C. reflexa and C. campestris exhibit maximum chlorophyll content on Vicia. On Nicotiana and Pelargonium both parasites have a chlorophyll concentration which is always intermediate between the highest and lowest values obtained from plants on the various hosts. Estimations of differences in the green pigmentation which can frequently be made by visual examination, particularly in the case of C. campestris, are in accord with the more precise values found by spectroscopy.

As in the chromatographic estimations, no extracts have ever been found to be devoid of chlorophyll, even in the case of C. campestris on Pelargonium or Nicotiana, where in most filaments, no trace of green coloration is discernible to the naked eye. The highest chlorophyll content encountered was 190µgm./gm. fresh weight in an extract of stem tips from C. reflexa on a withering Vicia host. This figure is almost exactly 20% of the value (954µgm. per gm. fresh weight) obtained by the same analytical method for the chlorophyll content of a healthy Pelargonium leaf.

d. Estimation of the degree of autotrophism of Cuscuta, based on chlorophyll content.

On the basis of the results obtained in Section V.A.(c.), and assuming equal photosynthetic activity per µgm. of chlorophyll, portions of Cuscuta with such high chlorophyll content are auto-

trophic to a degree equal to approximately one fifth of the level found in Pelargonium leaves. Since most of the chlorophyll in Pelargonium is confined to the leaves, it follows that the ratio of chlorophyll content to the fresh or dry weight of the whole plant will be considerably less than for the leaves alone. In order to obtain an approximate measure of this ratio, a healthy Pelargonium zonale plant was subjected to the analysis summarised in Table 7.

Table 7. Fresh and dry weights of leaves, stem, and roots of Pelargonium zonale.

Fresh weight of leaves and petioles	11.79gm.
Fresh weight of stem	29.16gm.
Fresh weight of roots	6.24gm.
Fresh weight of entire plant	47.19gm.
Dry weight of leaves and petioles	1.15gm.
Dry weight of stem	4.20gm.
Dry weight of roots	1.93gm.
Dry weight of entire plant	7.28gm.

Assuming the value of 954μgm. as the chlorophyll content of the leaves and petioles per gram fresh weight (page 61), and neglecting the chlorophyll content of the stem, the chlorophyll content of the complete Pelargonium plant is $\frac{954 \times 11.79}{47.19} = 238.4\mu gm.$

per gram fresh weight. The value of 190μgm. per gram fresh weight found for a sample of *C. reflexa* (page 61) represents 79.7% of 238.4, so that under certain conditions *C. reflexa* develops a chlorophyll content, measured in μgm./gm. fresh weight of the whole plant, equal to nearly 80% of the level in *Pelargonium zonale*. If the dry:fresh weight ratio of *C. reflexa* is taken as 14% (Fig. 15), then the chlorophyll content of the parasite measured in terms of dry weight is $\frac{190 \times 100}{14}$ = 1357μgm./gm. dry weight, compared with $\frac{238.4 \times 47.19}{7.28}$ = 1545μgm./gm. dry weight for *Pelargonium*. Therefore on a dry weight basis, the chlorophyll content of *Cuscuta* is 87.8% of the level in *Pelargonium*.

It follows from the above discussion that under certain extreme circumstances, notably death of the host plant, *C. reflexa* can develop a chlorophyll content not much below that obtaining in the autotrophic plant *Pelargonium zonale*. It is therefore possible that in extreme conditions, *C. reflexa* can fix sufficient carbon for its requirements, its dependence on a host being in that case likely to be confined to the supply of water and mineral salts.

B. Carotenoid Estimations.

a. Total carotenoid.

The fact that the chlorophyll content of Cuscuta varies with its host plant (Section V.A.) suggests the possibility that the total carotenoid pigment complex might also be subject to variation by growing on different hosts. In order to test this hypothesis, estimations of total carotenoid pigment were made on C. reflexa and C. campestris from a range of host plants.

1. Methods.

The spectrophotometric method used for determining total carotenoid requires the preliminary removal of chlorophyll from the extract. This is achieved by saponification of the chlorophylls with potassium hydroxide and removal of the soaps thus formed by washing with warm water. The method of analysis used in this work is a modification of that of Smith and Benitez (1955).

Cuscuta samples were harvested, washed in cold water, and dried between sheets of towelling. Samples of one gram were ground under acetone with acid-washed silver sand, filtered, and the pigments transferred to diethyl ether as described in Section V.A. The volume of ether was made up to exactly 50ml. One 25ml. portion of this extract was dried over sodium sulphate and reserved

for β-carotene determination; the remainder was evaporated to dryness in a Buchner flask. To the solid pigment residue 10ml. of ethanol was added along with 2ml. of 60%(w/v) potassium hydroxide solution. The mixture was shaken to dissolve the pigment, and the solution then transferred to a 250ml. conical flask. The Buchner flask was washed out with a further 10ml. portion of ethanol which was added to the contents of the conical flask. The pigment extract was then boiled gently for five minutes to saponify the chlorophylls. To this hot solution was added 40ml. cold water and 50ml. diethyl ether. On being shaken, the carotenoid pigments were transferred to the ethereal layer, while the saponified chlorophylls remained in the aqueous phase. Extraction with 25ml. portions of ether was repeated until all the yellow pigment was removed from the hypophase. The ether extracts were combined and washed with 50ml. portions of warm water until the washings gave no alkaline reaction with phenolphthalein. This indicated that all the soaps had been removed from the ethereal layer. The ether extract was then dried for a minimum period of 60 min. over anhydrous sodium sulphate, and transferred to a Buchner flask for evaporation to dryness. The dried pigment was dissolved in 10ml. petroleum ether and stored in sealed test-tubes in the dark at $4^{\circ}C$.

Total carotenoid content was found by measuring the

extinction value at 446mμ on a SP 600 spectrophotometer using a 1cm. light path, assuming $E^{1\%}_{1cm}$ for the crude extract to be 2500 (Goodwin, 1955).

2. Results.

The values found are listed in Tables 8 and 9, and graphically represented in Figs. 44 and 45. Carotenoid levels in C. reflexa vary between 19.6 and 32.9μgm./gm. fresh weight (Fig. 45), and C. campestris between 34.1 and 50.5μgm./gm. fresh weight (Fig. 44). It is seen from Fig. 45 (C. reflexa) that the highest carotenoid content is found in the parasite growing on Pelargonium and Vicia, with intermediate values on Nicotiana and Helianthus, and a low value on Vitis. If these results are compared with the dry:fresh weight ratios of the parasite growing on different hosts, there is a rough inverse correspondence between carotenoid content and dry matter content. This can readily be seen in Fig. 46 where the carotenoid content is plotted with the reciprocal of the dry matter content.

Comparison of total carotenoid with chlorophyll content reveals that the lowest values of each are found when the parasite grows on Vitis. This may be the direct result of the considerable shading effect of the Vitis leaves, with consequent reduction in pigment production.

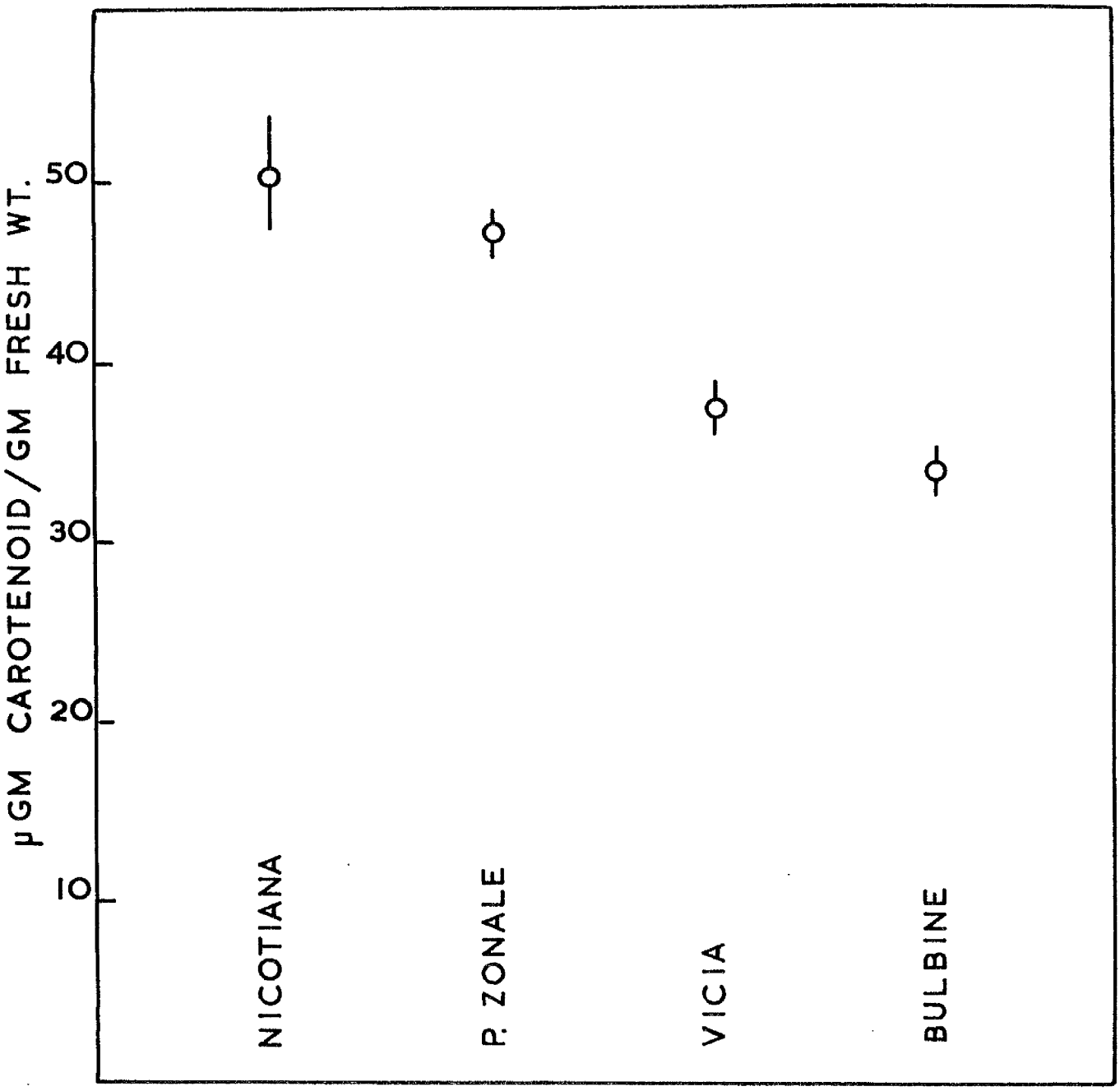


Fig.44. Carotenoid content of C. campestris, on Nicotiana, Pelargonium, Vicia and Bulbine hosts. Circle denotes mean; standard deviation is represented by vertical line.

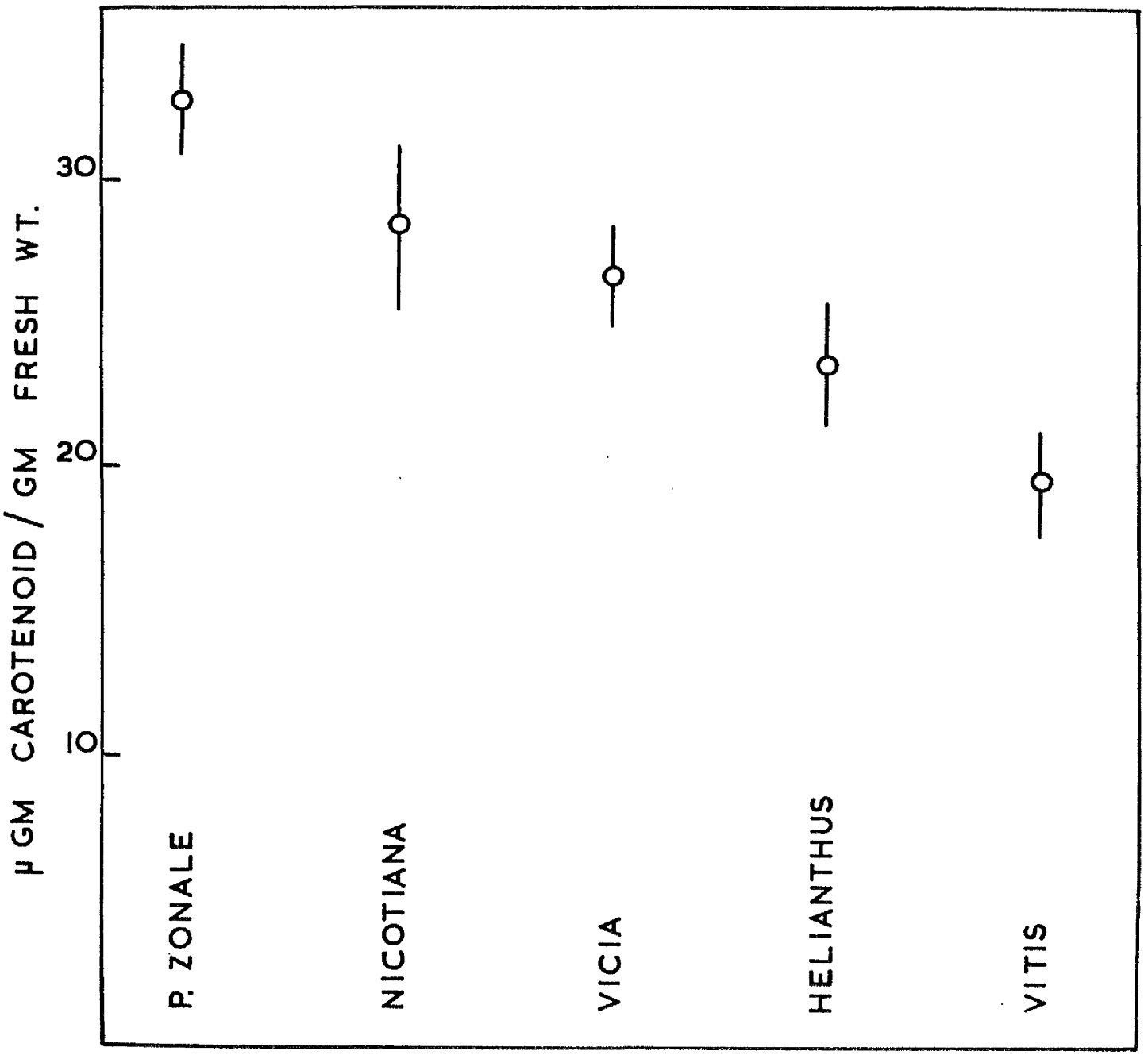


Fig. 45. Carotenoid content of C. reflexa, on Pelargonium, Nicotiana, Vicia, Helianthus and Vitis hosts. Circle denotes mean; standard deviation is represented by vertical line.

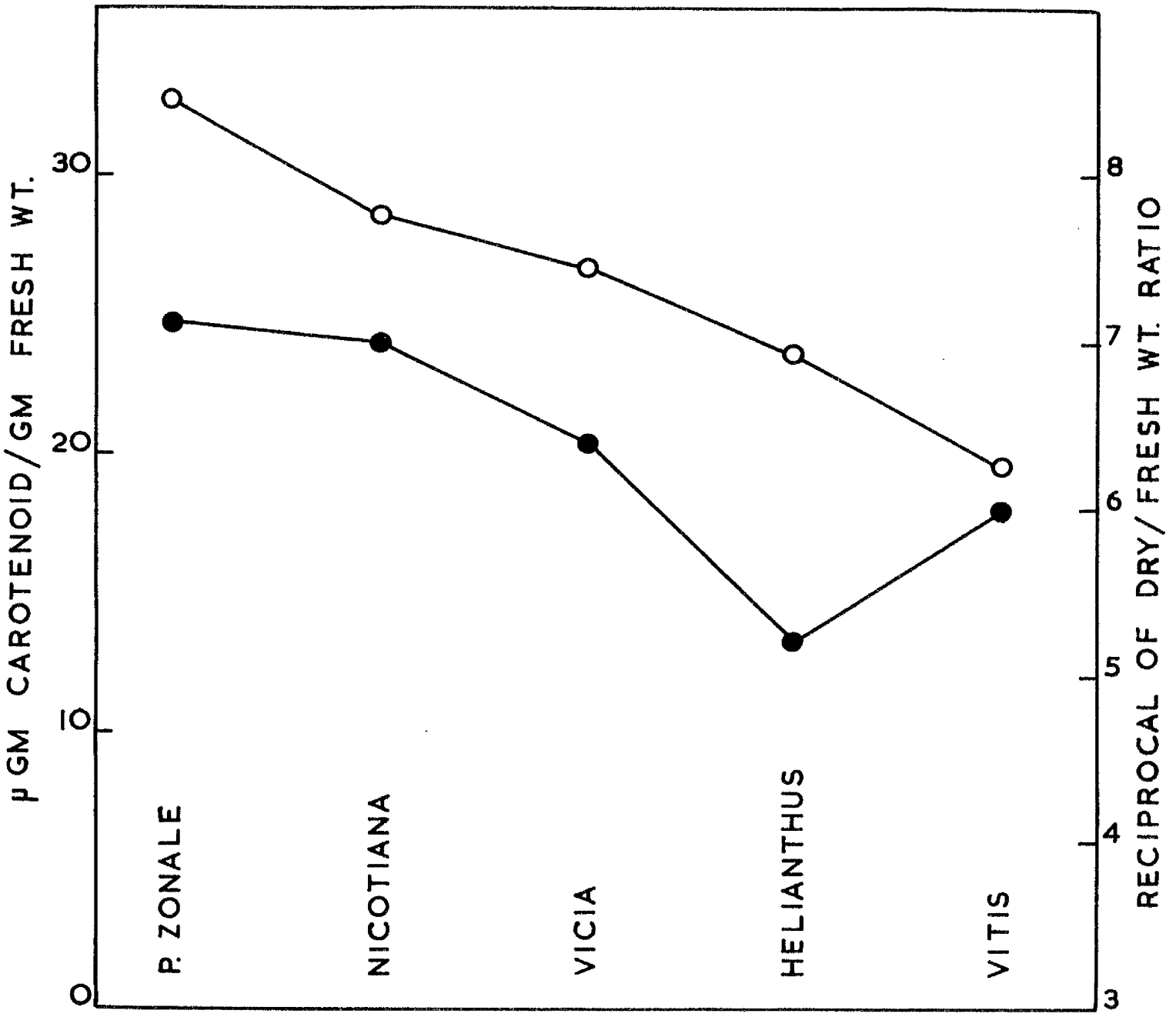


Fig.46. Relationship between Carotenoid content and dry weight content. (C. reflexa on various hosts.) Closed circles represent total carotenoid; open circles represent reciprocals of dry weight content.

Carotenoid levels in C. campestris are higher than in C. reflexa by from 41 to 78%. Relatively high values (50.5 and 47.5µgm./gm. fresh weight respectively) were obtained for plants growing on Nicotiana and Pelargonium, and lower values (34.1 and 37.7µgm./gm. fresh weight) for plants on Bulbine and Vicia. It is noteworthy that high total carotenoid values were obtained in plants with a high dry matter content, and lower values in plants with a low dry matter content. This result is consistent with gross visual estimations of carotenoid content based on the colour of the filaments, but is the reverse of the situation obtaining in C. reflexa, where an inverse relationship exists between total carotenoid and dry matter content (page 66).

When the carotenoid content of C. campestris is compared with chlorophyll content (Figs. 44 and 50), it can be seen that when the chlorophyll level is low (on Pelargonium and Nicotiana) carotenoid content is high, and on Vicia and Bulbine, when the parasite has a relatively high chlorophyll content, the carotenoid level is low. It is concluded, therefore, that in C. campestris, the total lipid-soluble pigment complex measured in µgm./gm. fresh weight remains at a substantially constant level; where a low level of either the chlorophyll component or the carotenoid fraction exists, it is compensated by a correspondingly higher level of the other component (Tables 8 and 9).

Table 8 C. campestris. Total Pigment analysis. All figures are µgm/gm fresh weight.

Host	Total Chlorophyll	Total Carotenoid	Total Pigment
Vicia	50.1	37.7	87.8
Bulbine	36.6	34.1	70.7
Pelargonium	25.2	47.5	72.7
Nicotiana	24.6	50.5	75.1

Table 9 C. reflexa. Total pigment analysis. All figures are µgm/gm fresh weight.

Host	Total Chlorophyll	Total Carotenoid	Total Pigment
Vitis	44	19.6	63.6
Helianthus	59.3	23.8	83.1
Vicia	80.3	26.8	107.1
Pelargonium	62.6	32.9	95.5
Nicotiana	52	28.3	80.3

With the exception of C. reflexa growing on Vitis, where extraneous factors may have been involved, the same relationship also appears to be operating in the case of this parasite.

b. β-Carotene.

β-Carotene is universally present in leaves, varying between 5 and 150 parts per million (Goodwin, 1955). MacKinney, (1935) has demonstrated the presence of α- and β-carotene in Cuscuta salina, together with γ-carotene; this is the only case of a report of γ-carotene in green tissues. The following investigation was undertaken to determine the presence or absence of β-carotene in C. reflexa and C. campestris, and its relation to the relative amounts of other carotenoids present.

1. Methods.

The ethereal extracts prepared during total carotenoid estimations (page 64) were evaporated to dryness in a Buchner flask, and dissolved in 1ml. of petroleum ether. This extract was chromatographed on a 1cm. x 5cm. sucrose column. The lowest zone, which passed straight through the column when pure petroleum ether was used as developer was collected in a 10ml. measuring cylinder and the volume made up to 5ml. with pure petroleum ether. The β-carotene concentration was calculated by measuring E max.

at 449mμ in a 1cm. cell, and using the equation $E^{1\%}_{1cm} = 2500$ (Goodwin, 1955).

2. Results.

The values obtained for C. reflexa varied between 2 and 9μgm. β-carotene per gram fresh weight, and for C. campestris between 6.5 and 10μgm. per gram fresh weight (Tables 10 and 11). Highest β-carotene values in both parasites occurred with Pelargonium and Nicotiana as hosts. Comparison of total carotenoid with β-carotene content from the same sample revealed a fairly close correspondence between the two constituents (Fig. 47). The ratio found for β-carotene to remaining carotenoid is within the range 0.1 to 0.33 (Goodwin, 1955) for β-carotene to xanthophylls in green leaves. The absorption spectrum of β-carotene from Cuscuta campestris and Cuscuta reflexa is plotted in Figs. 48 and 49.

The ratio of chlorophyll to carotenoid pigment in Cuscuta varies between 0.49 (C. campestris on Nicotiana) and 3.0 (C. reflexa on Vicia), Tables 12 and 13. The same ratio in the leaves of three deciduous trees was calculated from data published by Goodwin (1958):- Acer pseudoplatanus, 22; Prunus nigra, 70; and Quercus robur, 59 (Table 14). It is interesting to note that the carotenoid level in Cuscuta is comparable to that found in green leaves.

Table 10 C. reflexa. Ratio of xanthophyll/β-carotene. Figures are μgm/gm fresh weight.

Host	β-Carotene	Total Carotenoid minus β-Carotene	Xanthophyll/ β-Carotene
Pelargonium	9	28.5	3.2
Nicotiana	3.7	33.1	8.9
Vitis	3.3	11.3	3.4
Vicia	2.7	20.5	7.6
Helianthus	2	15.8	7.9

Table 11 C. campestris. Ratio of Xanthophyll/β-Carotene.

Host	β-Carotene	Total Carotenoid minus β-Carotene	Xanthophyll/ β-Carotene
Pelargonium	8.2	40.1	4.9
Nicotiana	10	47.4	4.7
Vicia	7.1	29.4	4.1
Bulbine	6.5	26.1	4

Table 12 C. campestris. Ratio of chlorophyll to carotenoid. All figures are expressed as µgm/gm dry weight.

Host	Total Carotenoid	Total Chlorophyll	Chlorophyll/ Carotenoid
Bulbine	400.7	430.6	1.1
Vicia	349.3	463.8	1.3
Pelargonium	377.2	200	0.53
Nicotiana	397.7	193.6	0.49

Table 13 C. reflexa. Ratio of chlorophyll to carotenoid. Figures are expressed as μgm/gm dry weight.

Host	Total Carotenoid	Total Chlorophyll	Chlorophyll/ Carotenoid
Vicia	197.2	590.2	3
Pelargonium	267.2	509	1.9
Nicotiana	228.2	419.4	1.8
Vitis	136.3	305.6	2.2
Helianthus	149.9	373.0	2.5

Table 14 Ratio of chlorophyll to carotenoid. Figures are μgm/gm dry weight, calculated from data by Goodwin(1958).

Species	Total Carotenoid	Total Chlorophyll	Chlorophyll/ Carotenoid
Acer pseudoplatanus	180	4000	22
Prunus nigra	120	8400	70
Quercus robur	115	6800	59

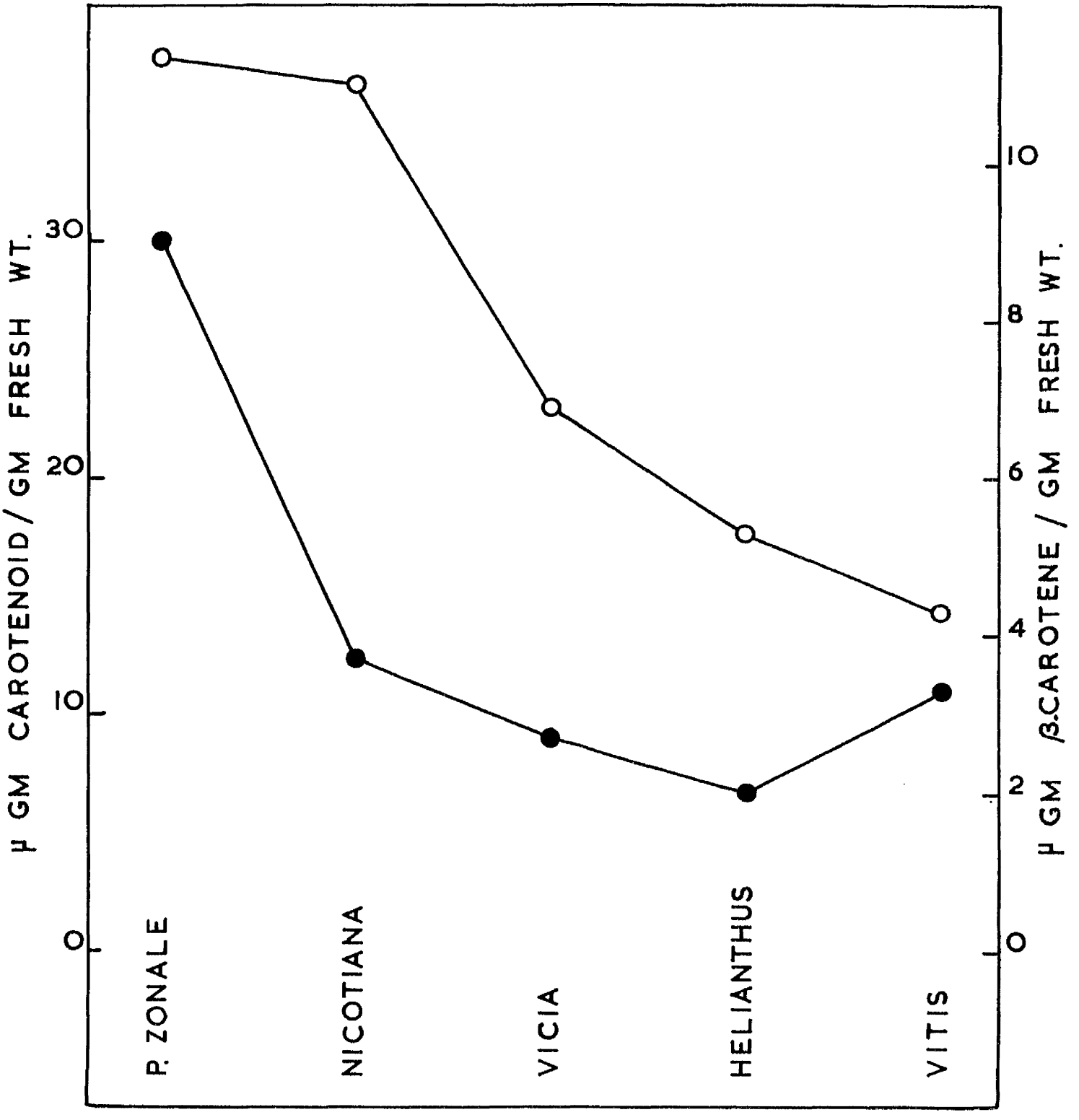


Fig. 47. Relationship between Carotenoid and β-Carotene content. (C. reflexa). Open circles are total Carotenoid; closed circles represent β-Carotene.

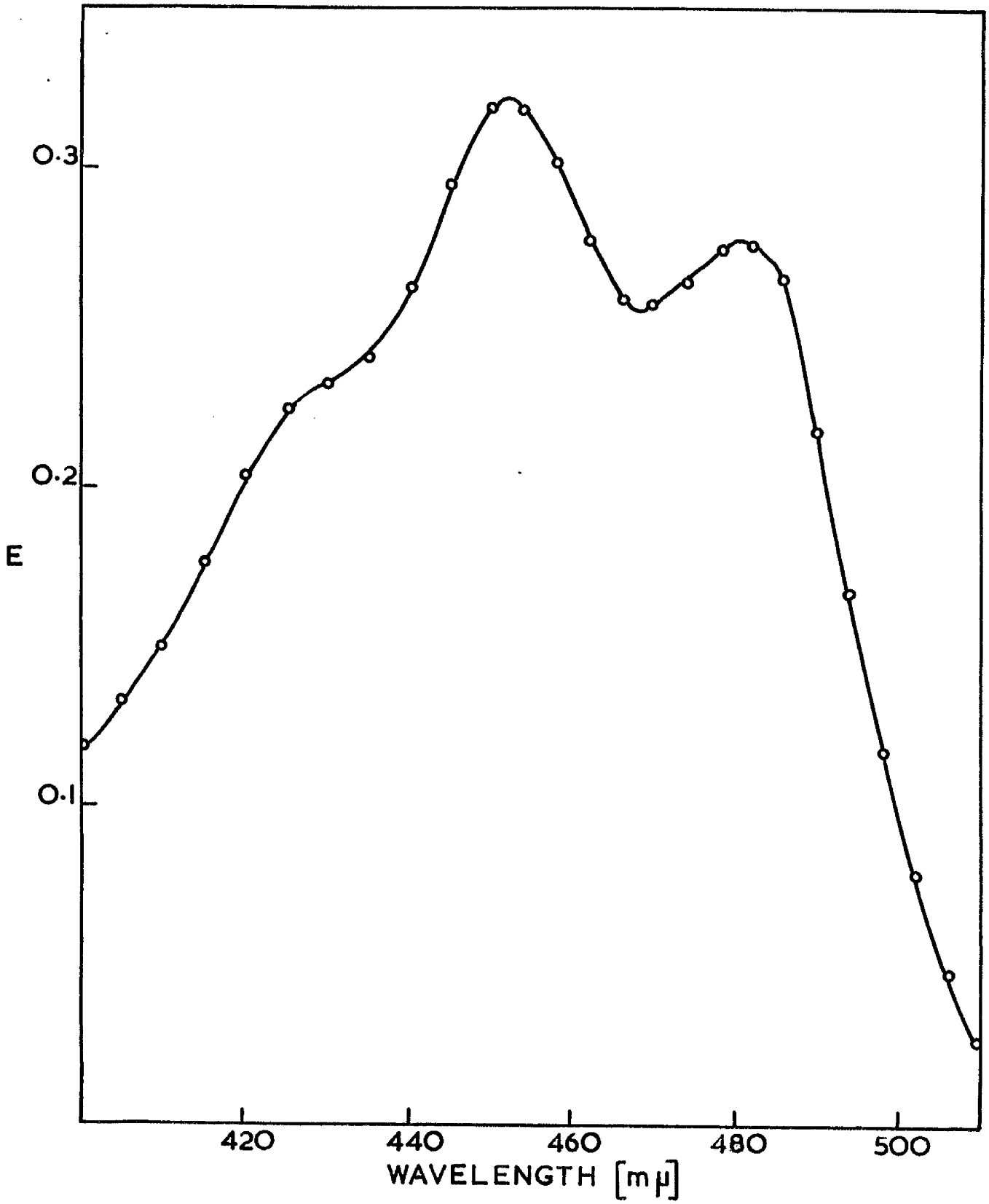


Fig. 48. Absorption Spectrum of β-Carotene. (C. campestris)

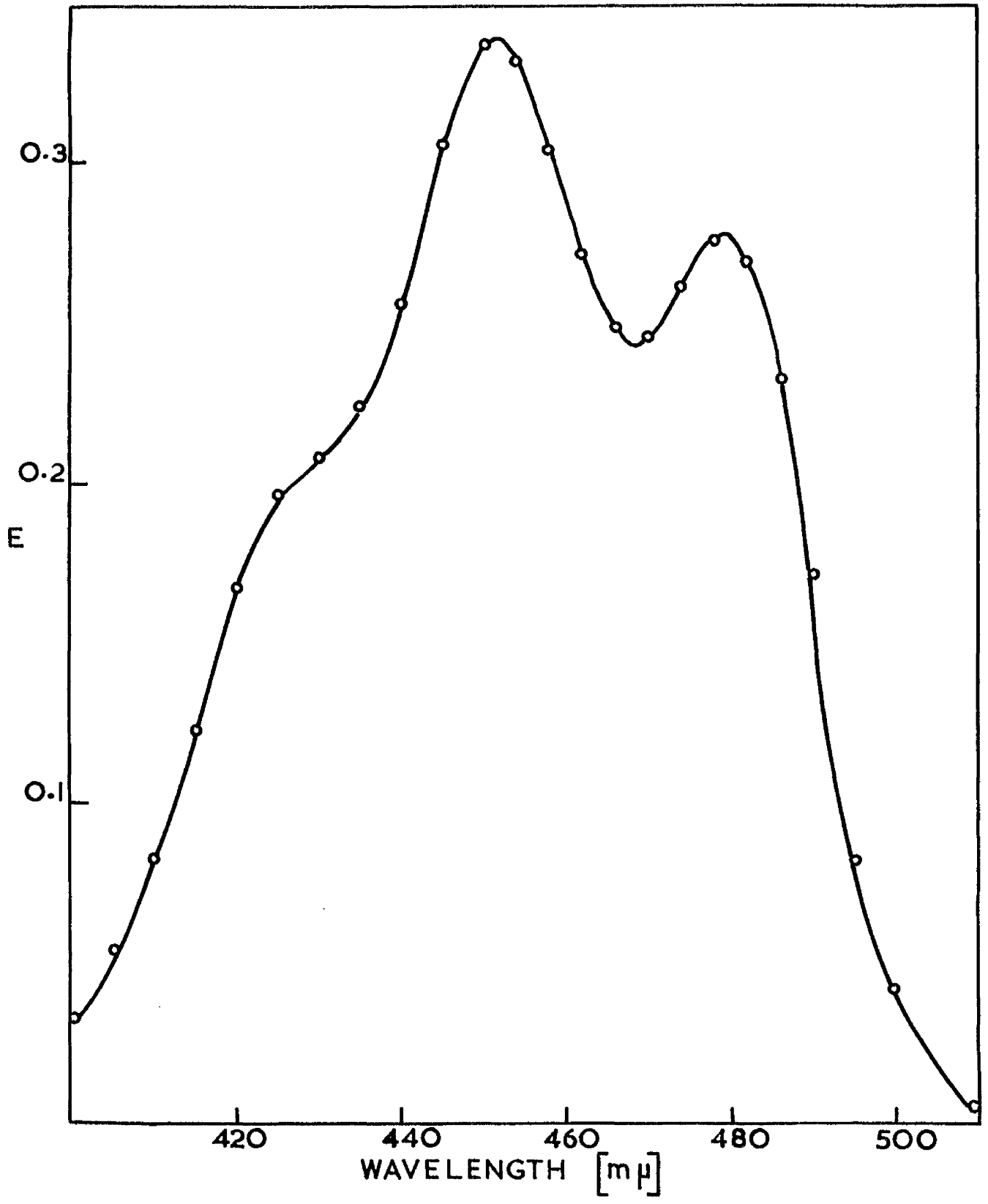


Fig. 49. Absorption Spectrum of β-Carotene. (C. reflexa)

A clue to the significance of this result may lie in the work of Griffiths et al.(1955). Working on the photosynthetic bacterium Rhodopseudomonas spheroides, they postulated a hitherto unsuspected function for carotenoids in photosynthesis, which could explain the universality of their association with the photosynthetic apparatus; namely protection of the cell from photodynamic destruction by chlorophyll. This is evidently not the sole function of carotenoids in photosynthetic organisms, but it is suggested that it may be the primary and most important one. Calvin,(1955), commenting on this hypothesis, suggests that the presence of visibly coloured carotenoid within the cell of R. spheroides when exposed simultaneously to oxygen and light, prevents the killing of the cell, and that this killing, or photo-oxidative destruction, is produced by light absorbed by the bacteriochlorophyll in the absence of coloured carotenoid. In this case, the use of carotenoids as ancillary photosynthetic pigments by some organisms may be a late evolutionary development.

c. Chromatography of the carotenoid pigments.

The only report in the literature on the resolution and identification of the carotenoid complex in the genus Cuscuta is an analysis by MacKinney (1935) of the pigments of the marsh dodder, Cuscuta salina. He succeeded in isolating α- and β-carotene

from this species, and obtained the quantitative value of 12.5µgm. α- + β-carotene per gram fresh weight, which is in accord with the figure obtained in this work for the β-carotene content of Cuscuta campestris (Table 11). His observation that in a crude petroleum ether extract the bulk of the colour was due to pigments more strongly adsorbed than either α- or β-carotene led him to investigate further the colouring matter in the parasite.

In his experiments, an extract of 16 Kilograms fresh weight of dodder was chromatographed on a magnesia column 35cm. x 6.5cm., using petroleum ether as developer; this washed the α- and β-carotenes through the column, leaving three reddish-coloured zones, surmounted by a greenish zone of chlorophyll or its derivatives. The red zones were partially separated by successive washings with benzene (2 litres) and dichloroethane (3 litres).

MacKinney obtained from the band immediately succeeding the β-carotene, 20mg. of a pigment identified as γ-carotene (absorption spectrum maxima in carbon disulphide 530, 496, 471mµ). From the next band, approximately 2mg. of lycopene were isolated, (absorption maxima in CS_2 544, 507, 472mµ). From the most strongly adsorbed band, 8mg. of rubixanthin were obtained (absorption maxima in CS_2 529, 495, 470mµ). When he used petroleum ether containing 15-20% dichloroethane as developing solvent he found

that neither the lycopene nor the δ-carotene bands appeared homogeneous.

Application of the methods of MacKinney to the separation of the pigments of C. reflexa and C. campestris, but using much smaller quantities of plant material (less than 15gm.) revealed at once that the pigment composition of these two species was much more complex than that found for C. salina.

Preliminary chromatograms on magnesium oxide columns with petroleum ether/acetone developer of saponified and unsaponified Cuscuta extracts showed the presence in these extracts of at least ten yellow or orange pigments. This observation was of considerable value in the selection of a suitable combination of adsorbents and solvent system which would separate the pigments sufficiently for isolation and characterisation; if considerably fewer than ten pigmented zones appeared on the column, it was assumed that some pigments were confluent, and the solvent-adsorbent system was abandoned for that reason.

A number of adsorbent and solvent systems were employed, including sucrose, zinc carbonate, aluminium oxide and magnesium oxide with hexane, benzene, and light petroleum containing varying amounts of polar solvents such as acetone. In view of the varying

degrees of success encountered in the use of the different solvents and adsorbents, and the large measure of inconsistency obtained in many of these analyses, it is proposed to describe only the results of one typical experiment, using MgO:Hyflo Super-Cel (1:1v/v) as adsorbent and benzene as developing solvent.

1. Methods.

Pigment extracts of C. reflexa and C. campestris were prepared from approximately 12gm. of fresh plant material as described in Section V.A.(a.). The final, dried petroleum ether extract was evaporated to dryness and taken up in a small volume of benzene (1-2ml.). In view of the low chlorophyll content of the two parasites, it was considered unnecessary to saponify the extracts; this measure consequently avoids the inadvertent loss, and possibly destruction of pigments which the process of saponification might entail.

Columns, 15cm. x 1.3cm. were closed at the bottom and half-filled with benzene. A small portion of adsorbent was added and the stopper at the base of the column opened. Gentle suction was applied to this end, while further portions of adsorbent and solvent were added, with intermittent stamping, until a compact column about 12cm. long was obtained. Portions of the concentrated extract were then introduced at the top of the column and the

supply of solvent maintained to ensure a continuous flow through the column. Each extract eventually separated into distinct coloured zones, the least strongly adsorbed of which were successively collected as they flowed from the column. The middle portions only of each zone were evaporated to dryness, dissolved in 5ml. of cyclohexane (Spectroscopic grade), and kept in sealed tubes in the dark at 4°C. for spectrophotometric analysis. The strongly adsorbed zones were isolated from the extruded column, eluted with ethanol, and extracts prepared for spectroscopy in the same manner as for the flowing zones.

2. Results.

In Fig. 50, composite drawings of the chromatograms of C. campestris and C. reflexa extracts are shown. The relative widths of the bands are accurately represented, but intervals between zones are compressed towards the base of the column. Tables 15 and 16 list the absorption spectrum maxima (in cyclohexane) for each zone, with, in some cases, a provisional identification of the pigment, based on its absorption spectrum and position on the chromatogram. Figs. 51-55 show the characteristic absorption curves of each zone.

A quantitative difference in the carotene and xanthophyll

Table 15 Characteristics of zones obtained by column chromatography of an extract of Cuscuta reflexa.

Number of zone on column, in order of decreasing adsorptive power.	Colour on column	Absorption Maxima(mμ, in Cyclohexane)	Provisional Identification
12	Green	—	Mixture of Chlorophylls"a"&"b"
11	Yellow	419,443,473	Violaxanthin
10	Pink	425,452,476	Zeaxanthin
9	Orange-yellow	424,446,477	Lutein
8	Orange-yellow	424,448,476	—
7	Yellow	422,446,477	—
6	Orange-yellow	426,451,480	Cryptoxanthin
5	Yellow	422,445,476	—
4	Yellow	—	—
3	Orange	—	—
2	Yellow	420,445,473	Leprotene
1	Yellow	430,455,484	β-Carotene

Table 16 Characteristics of zones obtained by column chromatography of an extract of Cuscuta campestris.

Number of zone on column, in order of decreasing adsorptive power.	Colour on column	Absorption Maxima(mμ, in Cyclohexane)	Provisional Identification
13	Green	—	Mixture of Chlorophylls"a"&"b"
12	Yellow	—	—
11	Orange	432,454,484	—
10	Orange-yellow	424,450,477	Zeaxanthin
9	Orange-yellow	426,450,479	Lutein
8	Pink	458	Mixture
7	Orange	427,451,479	Cryptoxanthin
6	Yellow	—	—
5	Pink	451,477,510	Rhodoxanthin
4	Orange-yellow	426,447,475	—
3	Pink	441,466,498	Neo-Lycopene A
2	Pink	438,462,493	-Carotene
1	Orange	414,438,468 and 452,480	Composite zone containing β-Carotene

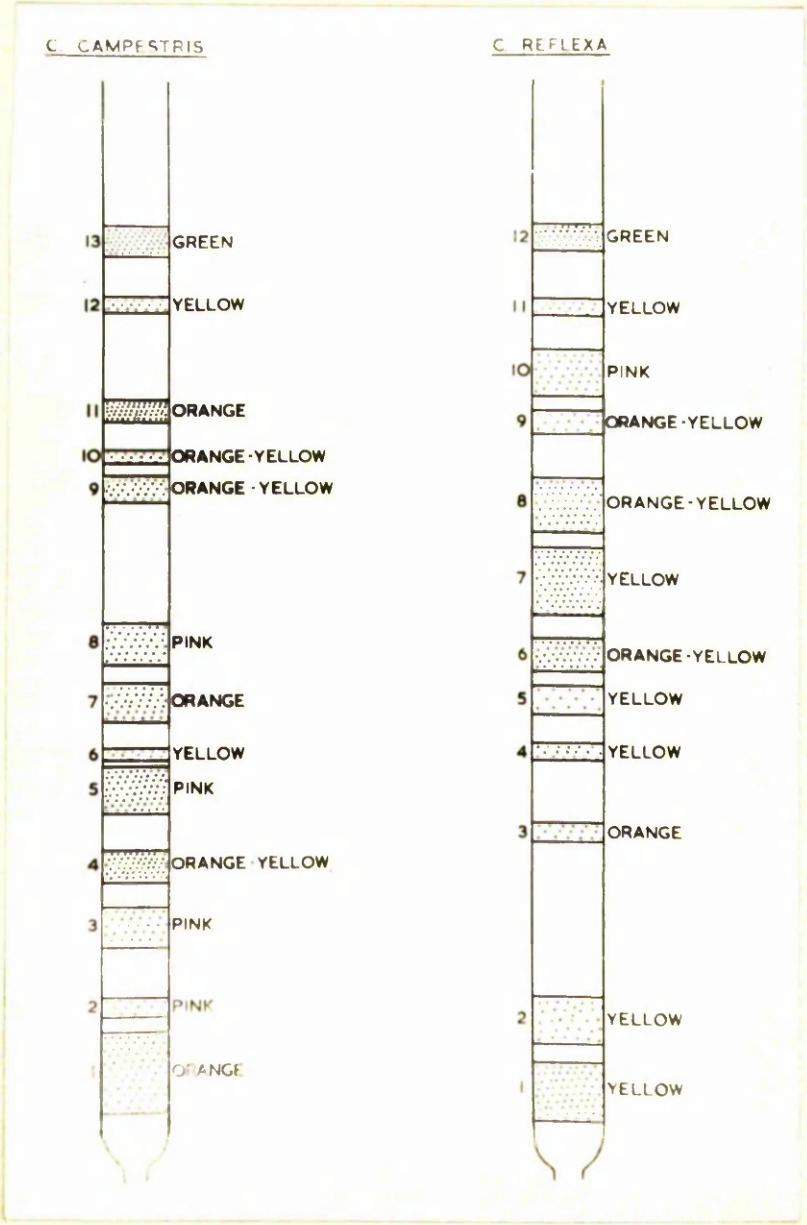


Fig. 50. Diagrammatic drawings of column chromatograms of pigment extracts of Cuscuta.

Key to Figs. 51-55.

Absorption spectra of zones obtained by column chromatography of *Cuscuta* extracts. Abscissae represent wavelength(mμ); ordinates represent optical density.

Fig. 51. a: Zone 1,(*C. reflexa*).
b: Zone 2,(*C. reflexa*).
c: Zone 5,(*C. reflexa*).
d: Zone 6,(*C. reflexa*).

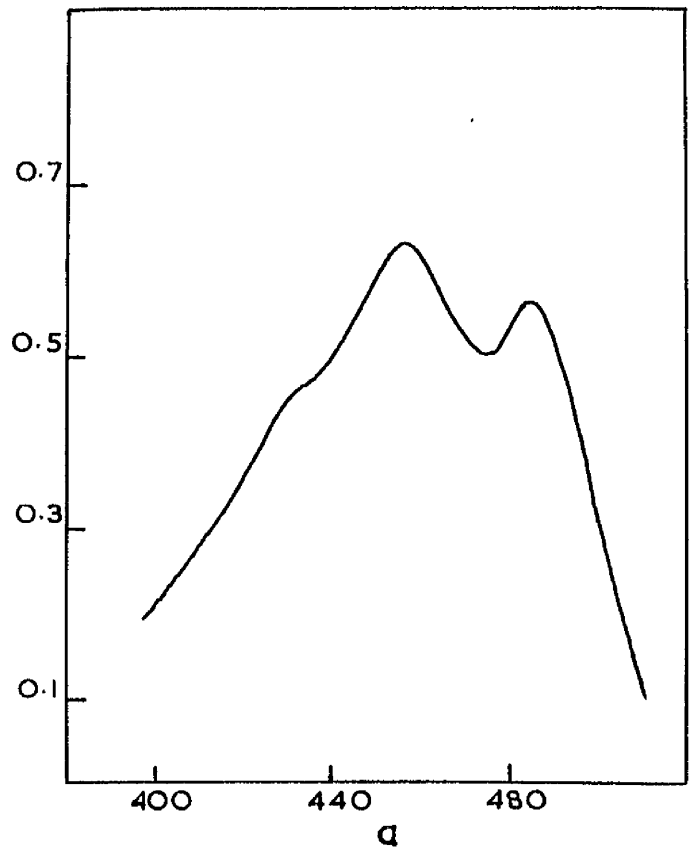
Fig. 52. a: Zone 7,(*C. reflexa*).
b: Zone 8,(*C. reflexa*).
c: Zone 9,(*C. reflexa*).
d: Zone 10,(*C. reflexa*).

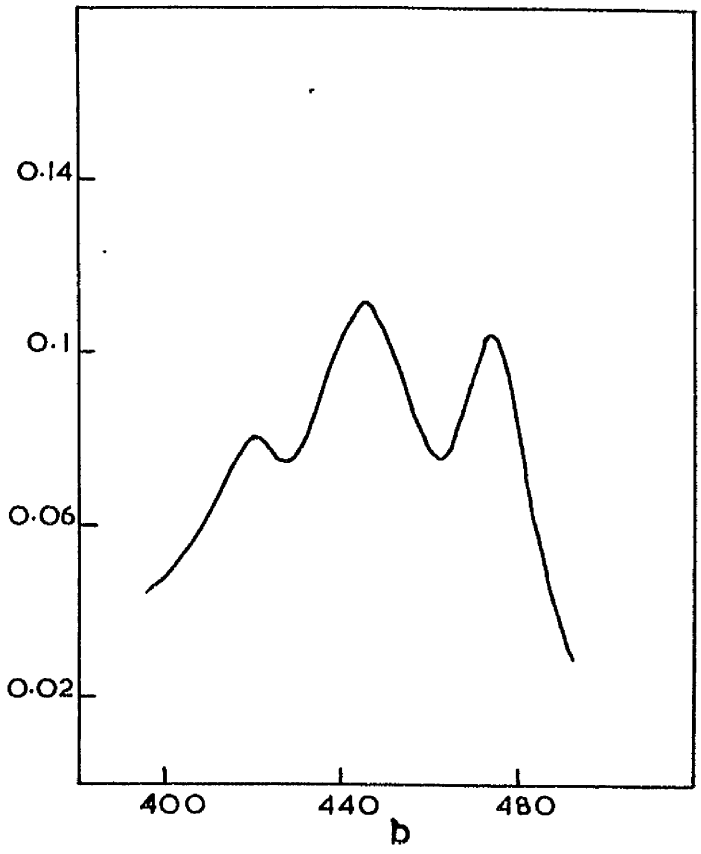
Fig. 53. a: Zone 11,(*C. reflexa*).
b: Lower portion of zone 1,(*C. campestris*).
c: Upper portion of zone 1,(*C. campestris*).
d: Zone 2,(*C. campestris*).

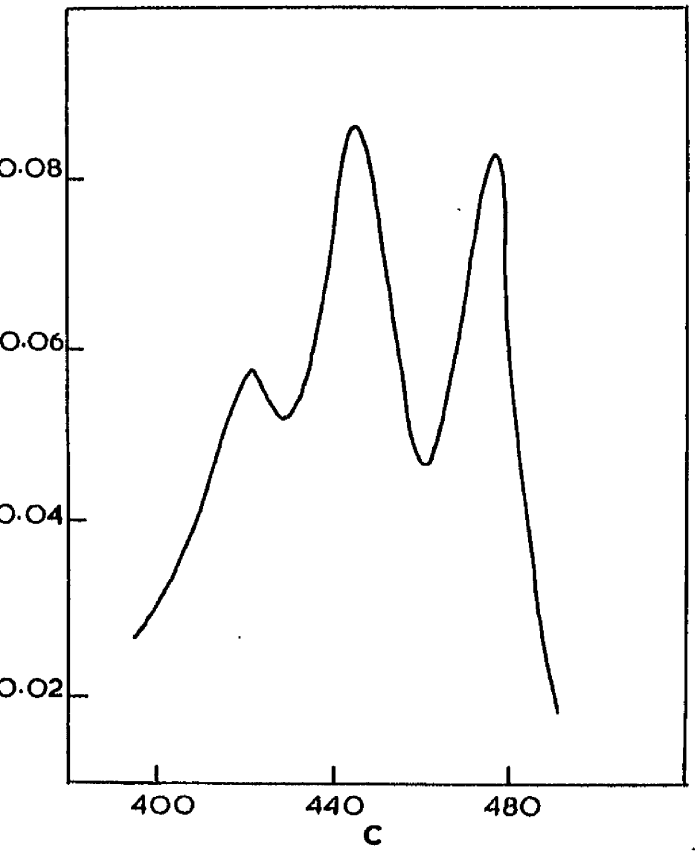
Fig. 54. a: Zone 3,(*C. campestris*).
b: Zone 4,(*C. campestris*).
c: Zone 5,(*C. campestris*).
d: Zone 7,(*C. campestris*).

Fig. 55. a: Zone 8,(*C. campestris*).
b: Zone 9,(*C. campestris*).
c: Zone 10,(*C. campestris*).
d: Zone 11,(*C. campestris*).

Zones refer to bands on chromatograms,(Fig. 50).


0.7
0.5
0.3
0.1
400
440
480
a


0.14
0.1
0.06
0.02
400
440
480
b


0.08
0.06
0.04
0.02
400
440
480
c

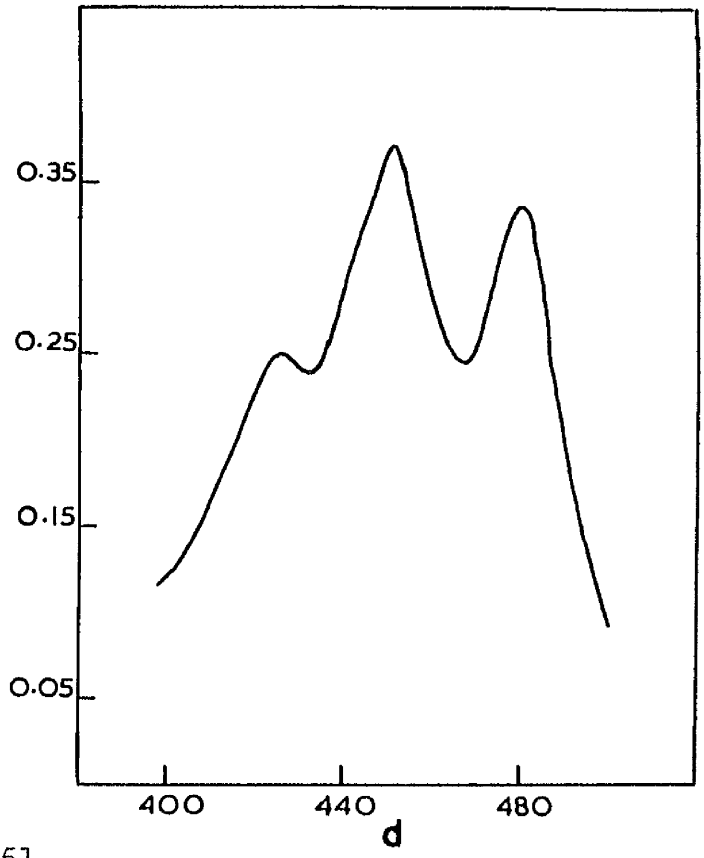
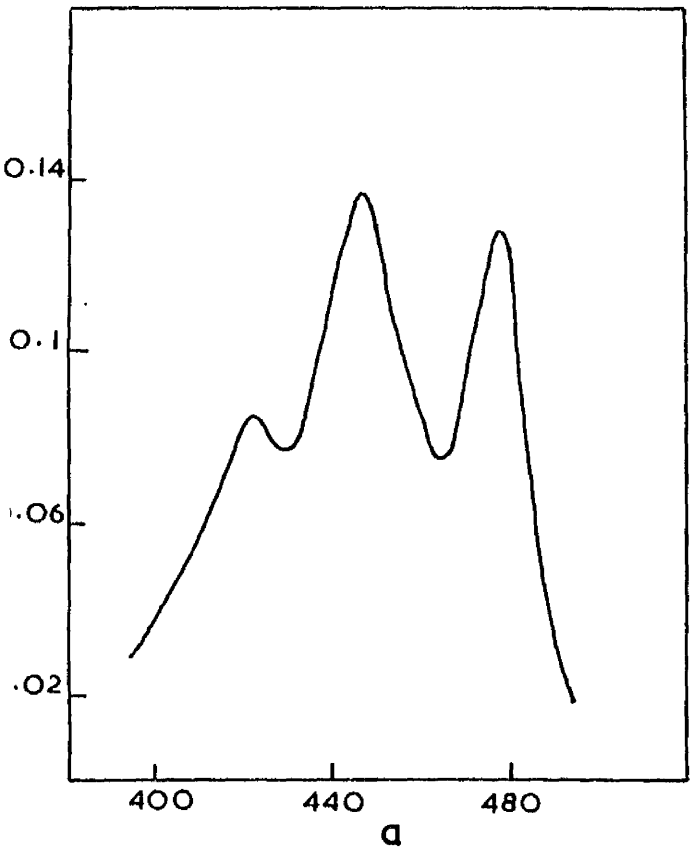

0.35
0.25
0.15
0.05
400
440
480
d

Fig. 51.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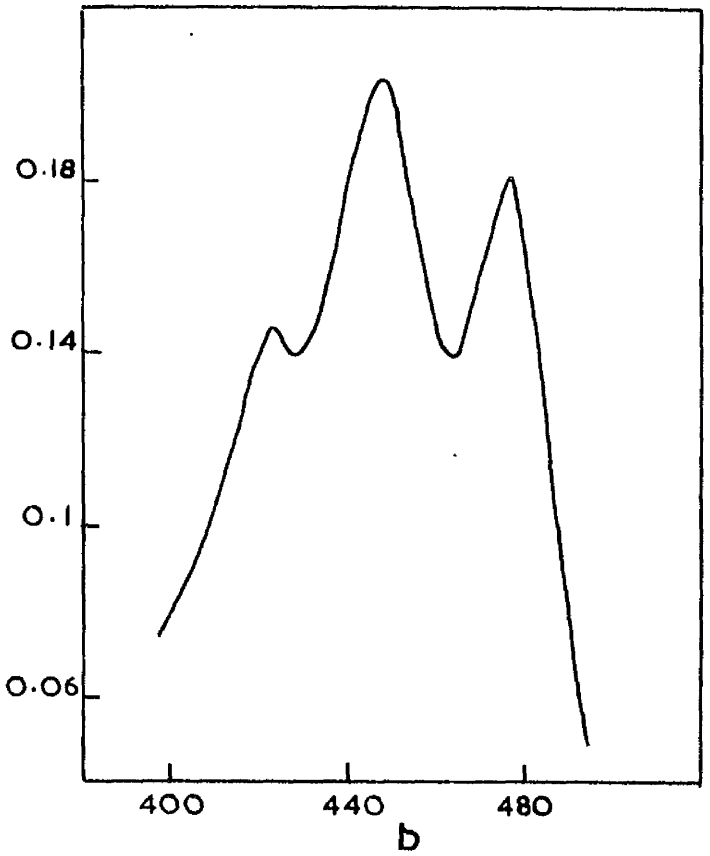
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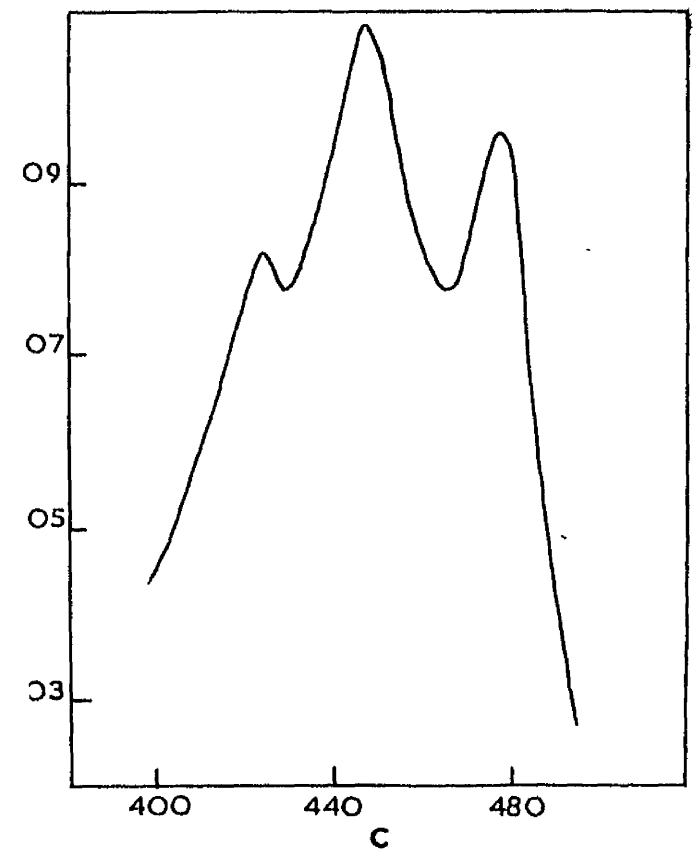
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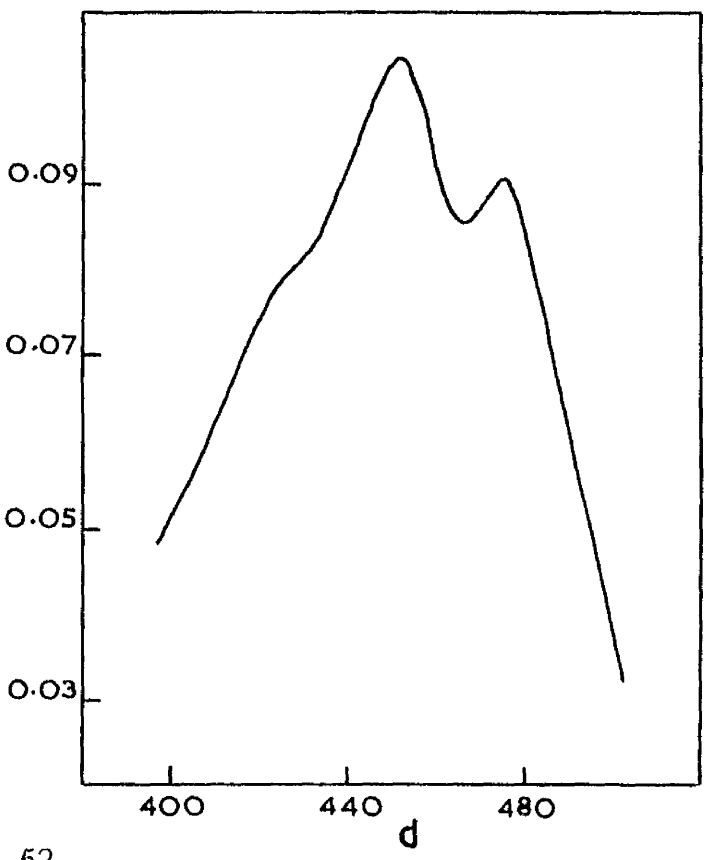
c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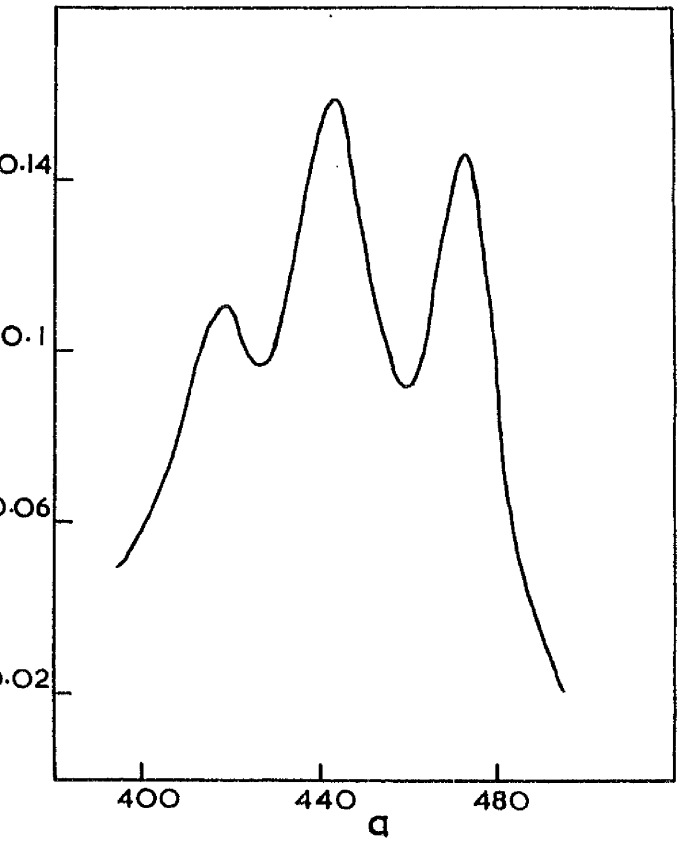
d

Fig. 52.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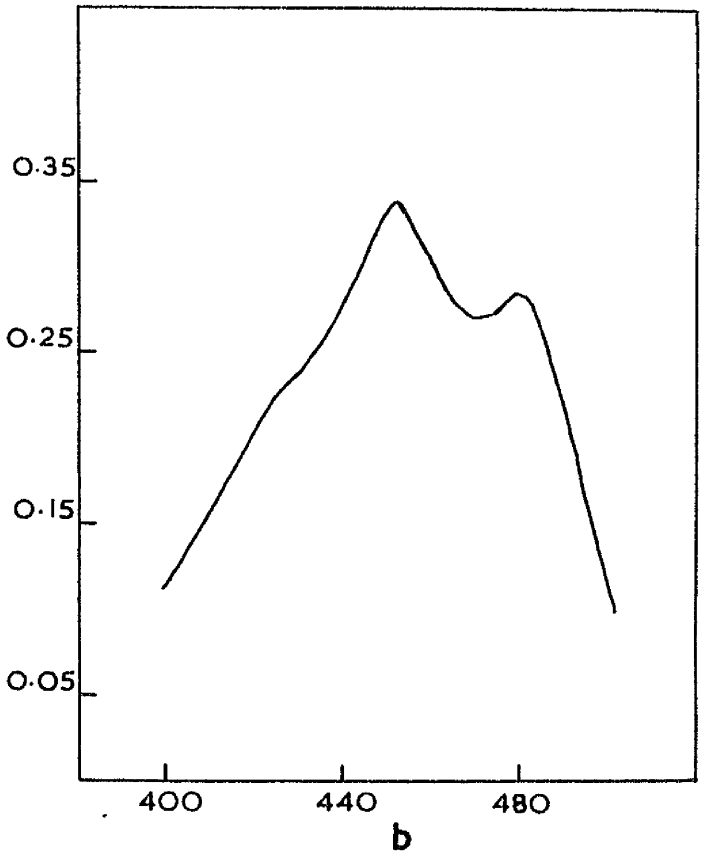
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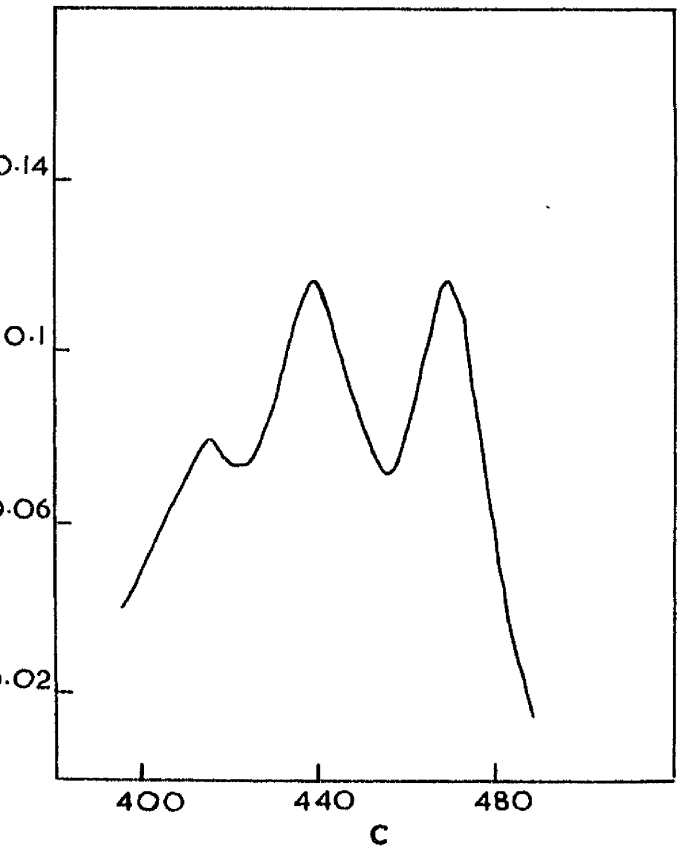
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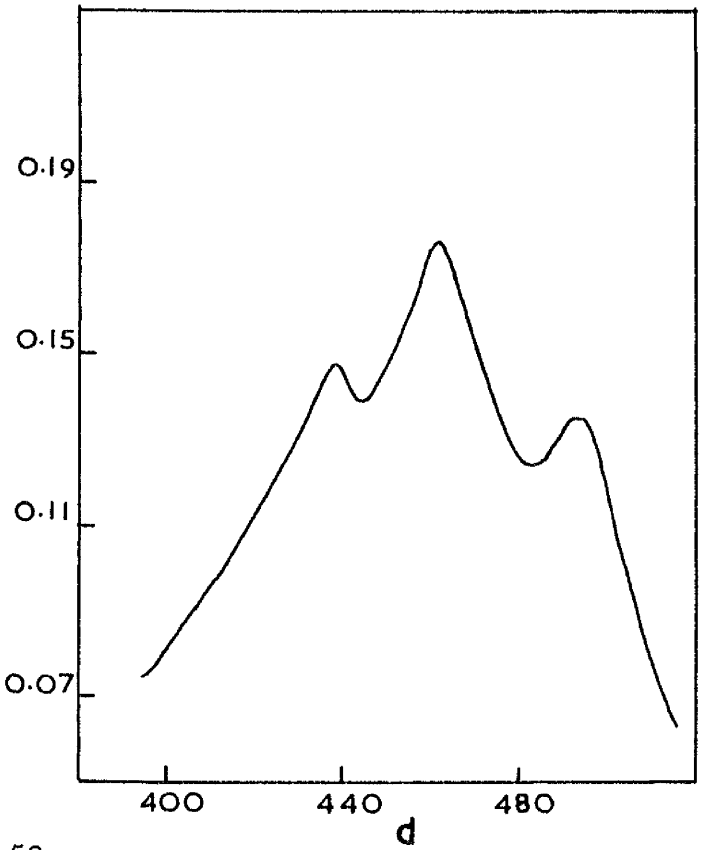
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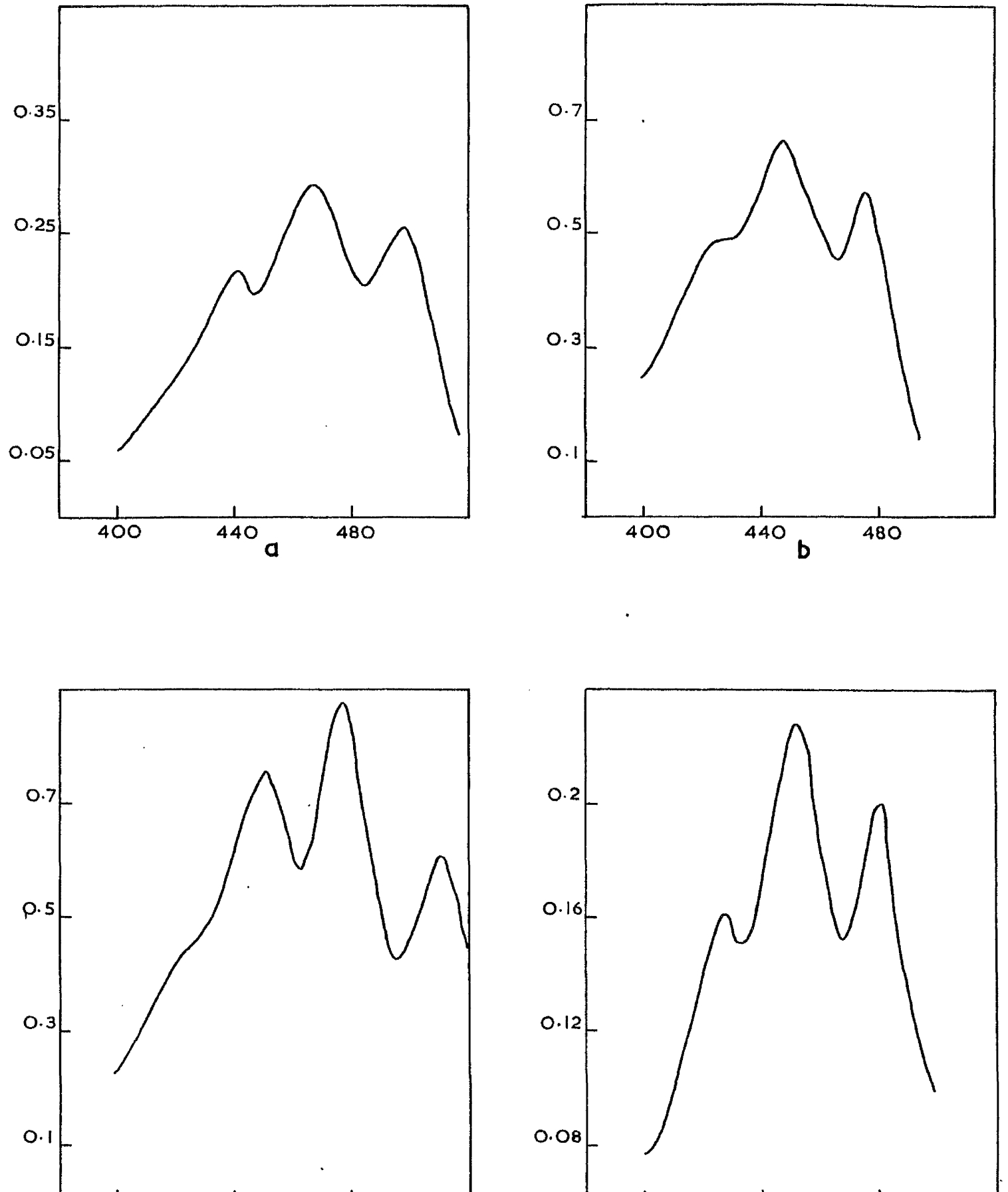
d

Fig. 53.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a
0.7
0.5
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b
0.7
0.5
0.3
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c
0.2
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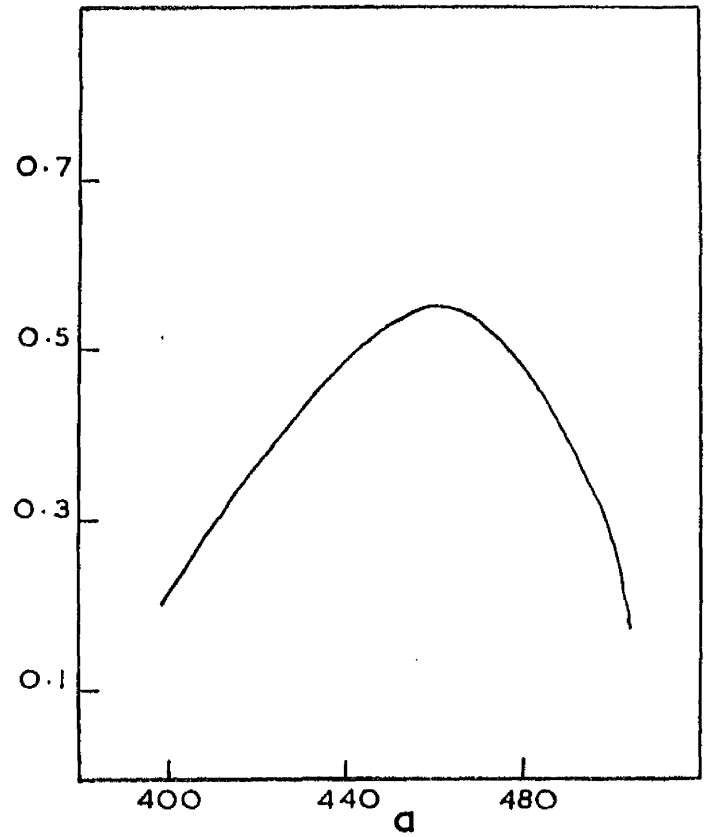
0.12
0.08
400
440
480
d

Fig. 54.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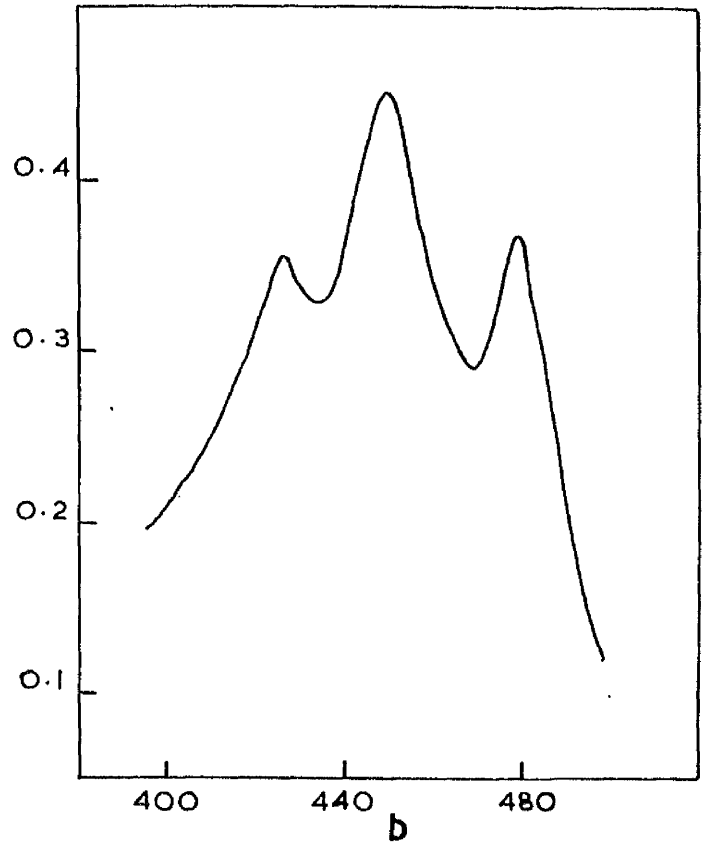
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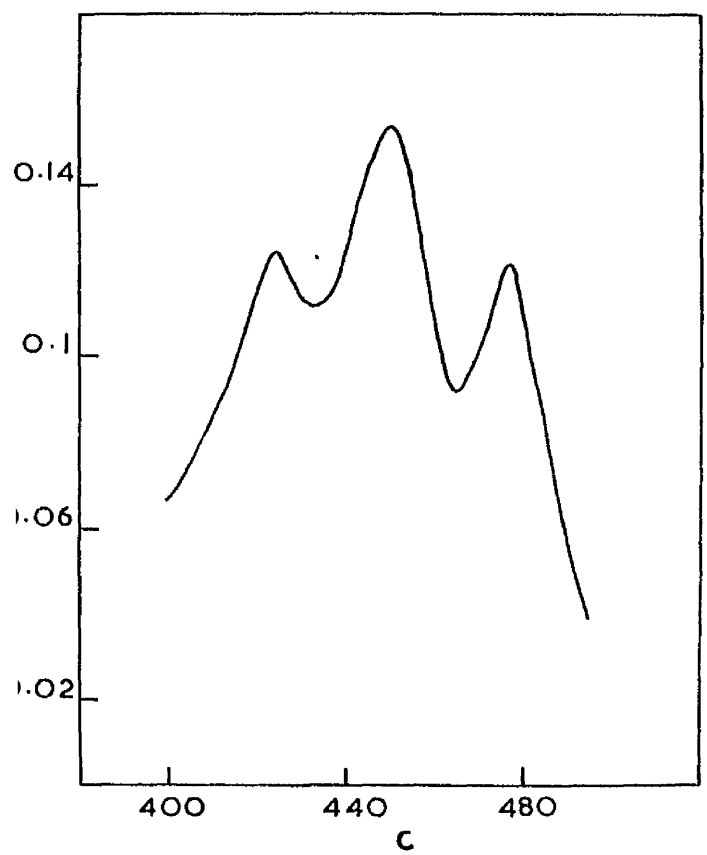
0·14
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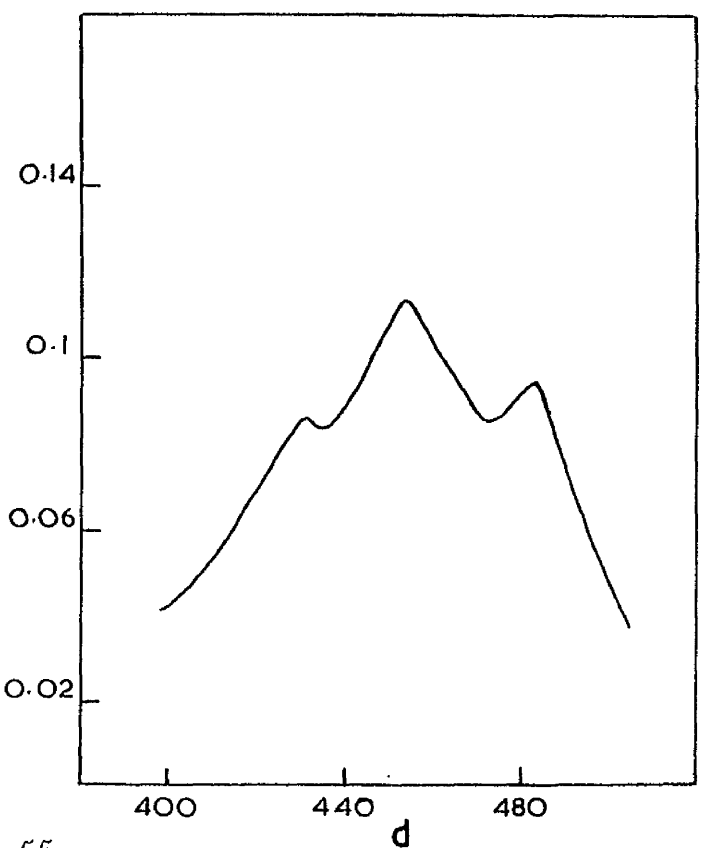
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d

Fig. 55.

content of these two species is always apparent from the chromatograms; C. campestris extracts always resolve into bands which are markedly denser than those of extracts of comparable weights of C. reflexa. It is apparent from Fig. 50 that qualitative differences also exist. The xanthophylls zeaxanthin, lutein and cryptoxanthin, together with β-carotene are common to both parasites. It is interesting that the two major xanthophylls of Cuscuta salina, rubixanthin and lycopene, do not seem to occur in either C. campestris or C. reflexa.

From the work described in this section it is shown that the carotenoid composition of C. reflexa is significantly different from that of C. campestris, and that both parasites have a higher number of carotenoid components than that described by MacKinney for Cuscuta salina. It is also apparent that the concentration in C. campestris and C. reflexa of such a large number of xanthophylls will require highly refined chromatographic techniques for complete elucidation.

C. Anthocyanin Pigments in Cuscuta reflexa.

No trace of anthocyanin-type pigments has been seen by the writer in C. campestris. C. reflexa, on the other hand, frequently shows epidermal cells entirely filled with a deep-red pigment. These cells usually occur in linear groups on the stem surface, giving the plant a mottled appearance.

Flavonoid pigments, particularly the anthocyanins have always been a troublesome source of disagreement among plant physiologists. The anthocyanins, which are usually dissolved in the cell sap are found chiefly in the flowers and fruits of higher plants, though, as in Cuscuta, they may occur in other plant organs also. Kosaka (1933) reported an increase in photosynthetic assimilation in red Oryza and Perilla plants due to the presence of anthocyanins or their chromogens. Sen (1940) noticed an increase in photosynthesis in red types of Eranthemum, although the chlorophyll content was lower than in green types. However, Rabinowitch (1945) reached the conclusion that all speculations and observations that relate anthocyanins and flavones to photosynthesis are unfounded so far. Stiles (1936) suggests that anthocyanins, as glycosides, can play a role in respiration of plants. A frequently expressed opinion is that the anthocyanins represent metabolic waste products in plants. Frey-Wyssling (1942)

regards the anthocyanidins as eliminated metabolic products which are glycosided and thus made water-soluble, allowing their excretion into the cell sap.

References to anthocyanins in the Convolvulaceae are rare in the literature. Yamaguchi (1924) isolated anthocyanin from the flowers of Ipomoea hederacea, and Phillip Smith (1931) showed that anthocyanins in the flowers of Ipomoea leerii exhibited a diurnal colour-range from magenta-pink to full blue, corresponding to a pH range of 6.0-7.8.

An analysis of the red pigment of C. reflexa was undertaken and a comparison made of its concentration in parasites growing on various hosts, with a view to ascertaining if any relationship existed between anthocyanin content and any of the other features, eg. chlorophyll content, already established for the species in this investigation.

a. Preparation of pigment extracts.

Samples (2-3gm.) of C. reflexa stems were extracted with acetone by the method used for carotenoid estimations (Section V.B.). It was assumed that acetone extraction did not remove the red pigments, and that the major portion remained in the solid residue (Goodwin, 1958). To extract the pigment, the residue was macerated in a mortar with ethanol containing 1% (v/v) hydrochloric acid, and

filtered. The filtered extract was then evaporated to near dryness under vacuum and taken up in a small volume of alcohol. Aliquots of the alcoholic extract were chromatographed by the circular method using butanol, acetic acid, water (4:1:5) as developing solvent. An extract of Prunus pissardii leaves, known to contain cyanidin-3'-glucoside (Goodwin, 1958) was prepared as for Cuscuta and co-chromatographed with the Cuscuta extracts. The resulting chromatogram resolved the red pigment of Cuscuta into two distinct zones, of Rf values 0.08 and 0.23, the latter corresponding exactly with cyanidin-3'-glucoside of the Prunus leaves (Fig. 56). The inner zone, which was present in approximately the same concentration as the outer was not identified. It is concluded from this experiment that the red pigment of the epidermal cells of C. reflexa consists of two chromatographically distinct units of approximately equal concentration, and that one of these components is cyanidin-3'-glucoside.

b. Variation in anthocyanin pigment in parasites growing on different hosts.

Quantitative estimations of anthocyanin concentration were made by measuring the extinction value of extracts at 538mμ, assuming the $E^{1\%}_{1cm}$ of the mixture of pigments to be 700 (Cyanidin-3'-glucoside, Geissman, 1955). Extracts of parasite stems (2.5gm.)

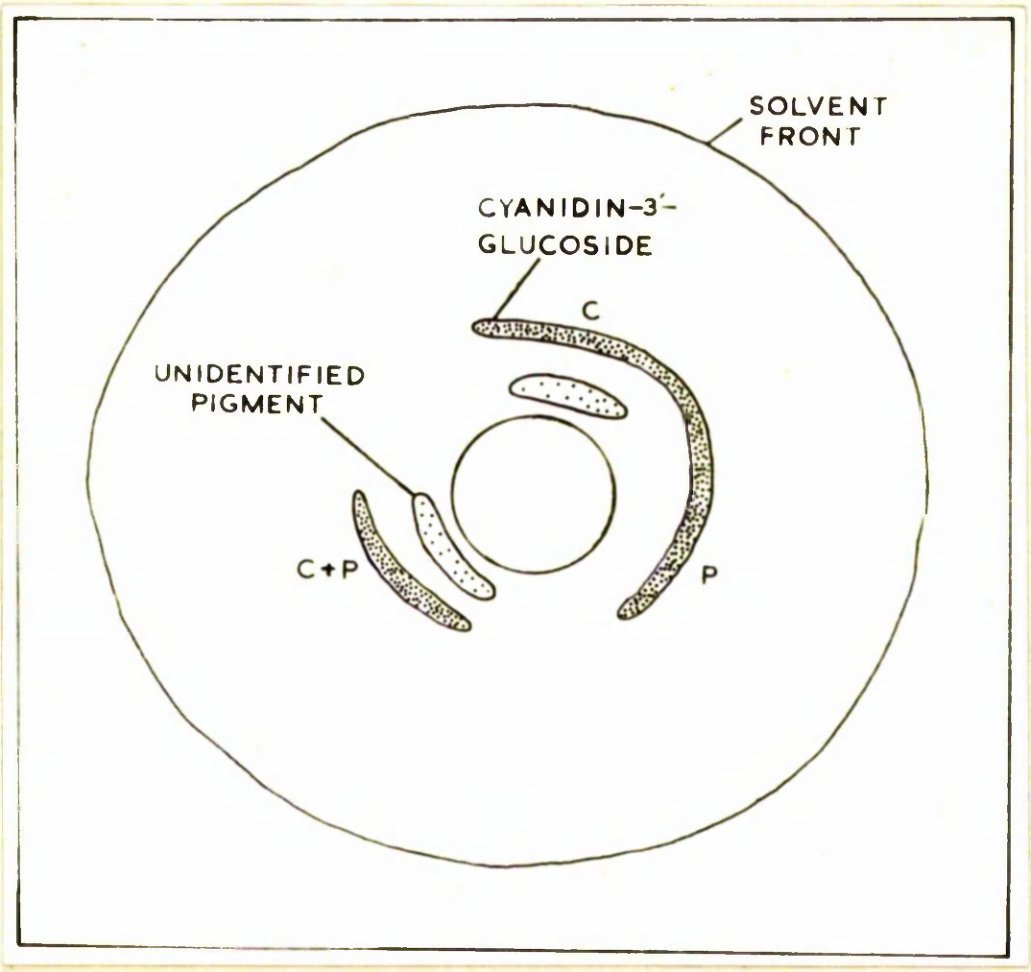

Fig. 56. Circular paper chromatogram of anthocyanin pigments of C. reflexa. C-Cuscuta extract; P-Prunus pissardii extract; C+P-mixture of both extracts.

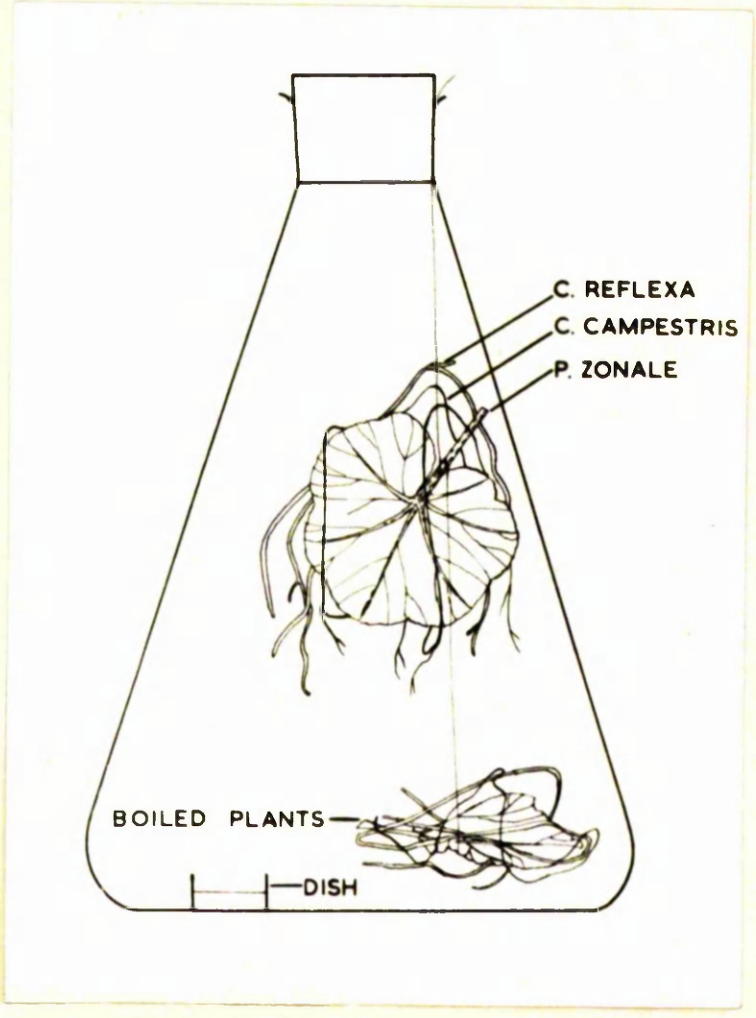

Fig. 57. Apparatus used to demonstrate CO_2-fixation in Cuscuta.

from Vicia, Pelargonium, Nicotiana, Vitis, and Helianthus hosts were prepared as in the previous experiment, filtered and made up to 60ml. with ethanol for spectrophotometric analysis. As a control, a similar extract of a stem which contained no visible red pigmentation was used as a blank solution. The results are presented in Table 17.

Table 17. Anthocyanin content of C. reflexa on various hosts.

Host Plant.	Anthocyanin content of C. reflexa (mg./gm. fresh weight).
Vitis	0.043
Helianthus	0.033
Vicia	0.032
Nicotiana	0.027
Pelargonium	0.013

The anthocyanin pigment was found in highest concentration (0.043mg./gm. fresh weight) in C. reflexa on Vitis, and in smallest amounts (0.013mg./gm. fresh weight) on Pelargonium. The maximum anthocyanin concentration recorded in this work (from deep-red filaments selected from several hosts) was 0.103mg./gm. fresh weight, which is approximately equivalent to 0.736mg./gm. dry

weight (assuming the dry:fresh weight ratio of C. reflexa to be 14%, Fig. 15). This should be compared with the value of 10.93mg. per gram dry weight found for Prunus pissardii leaves, a figure which compares closely with values found by Goodwin (1958) for the anthocyanin content of Prunus nigra leaves.

A significant fact which emerges from this analysis is that the anthocyanin content of C. reflexa on different hosts follows a closely similar pattern to the dry:fresh weight ratios (Fig. 15). This is consistent with the opinion of Blank (1953), that increased sugar supply stimulates the formation of anthocyanin in plants. It is also noteworthy that the anthocyanin-content pattern in C. reflexa follows exactly the same order on the different hosts as carotenoid content. Since carotenoid content has been shown to vary in an approximately inverse manner with chlorophyll content (Section V.B.), and considering the postulated promoting effect of anthocyanin on photosynthesis (Kosaka, 1933; Sen, 1940), variations in anthocyanin pigmentation may be due to the presence of a compensating mechanism which regulates the rate of photosynthesis in the parasite.

VI. PHOTOSYNTHESIS IN CUSCUTA.

In the literature, no reference has been found of conclusive evidence that the Cuscutas fix carbon dioxide,[1] though the presence of chlorophyll has been noted by many workers, notably Loo (1946), Mirande (1901), Lilienstern (1928a; 1928b), Thomson (1925), and Walzel (1952). Thomson suggests that C. reflexa may elaborate carbohydrate by means of the small amount of green colouring matter present in its cortex, but considers it improbable in view of the presence of an intact cuticle, uninterrupted by stomata, which would render unobtainable the necessary supply of carbon dioxide.

Loo (1946) succeeded in growing excised stem tips of C. campestris in vitro, through many transfers in the presence of light for a period of five months. The excised tips produced lateral buds and flowers, without the formation of leaves or roots. The chlorophyll produced in the stem tips seemed to play an important part in maintaining the growth of the culture.

Mirande (1901) points out that when Cuscuta grows in favourable conditions it is red, and in unfavourable conditions greenish. He mentions the occurrence of chlorophyll in the embryo of C. japonica. A more intense green coloration than occurs

1. The results of this section have been published by the writer in Experientia 17 542, (1961).

naturally was achieved by bending the ends of a C. japonica filament into a beaker of water; the greening process started after a few hours and reached a maximum in two days. This observation is supported by the work of Lilienstern (1928a) who showed that the chlorophyll content of C. monogyna increased when the parasite was grown in nutritive solutions; disappearance of starch was associated with an increase in chlorophyll content which reached a maximum in solutions of least nutritive value. The same author (1928b), however, states that chlorophyll development is never sufficient for even poor autotrophic growth.

Schmucker (1959), in a review article on heterotrophic plants, states that the genus Cuscuta contains chlorophyll, but that it is present at the most in small amounts only. It was also recorded that the chlorophyll content increases in the seedling if it fails to make contact with a suitable host, or if it has attached itself to a poorly suited host.

Syme (1863) describes the stems of C. epilinum and C. europaea as greenish-yellow, and the flowers of C. epilinum as greenish-white. Of C. trifolii he declares the stem to be reddish-yellow, but his accompanying illustration depicts the stems, and particularly the lower floral segments, as bright green. Most elementary botanical text-books describe the genus Cuscuta as devoid of chlorophyll and

entirely heterotrophic.

The demonstration of significant quantities of chlorophyll in C. campestris and C. reflexa (Section V.A.) suggested the possibility that photosynthesis may occur in these two species, despite the comparative absence of stomata or other anatomical discontinuities which would make gaseous exchange possible. The presence of stomata in the Cuscutas has been recorded by Schmucker (1953) who reports that they are present only in small numbers, comparatively several thousand times fewer than in autotrophs. The present writer has seen stomata on the stems of both C. reflexa and C. campestris and can confirm that their distribution, particularly in the latter species is exceedingly sparse.

a. Demonstration of carbon-fixation by Cuscuta sp.

The object of this experiment was to subject Cuscuta stems to an atmosphere containing $^{14}CO_2$ in the presence of light, and after a suitable period to analyse the tissues for radioactivity.

1. Materials.

Radioactive carbon (^{14}C) was obtained in the form of sodium bicarbonate from the Radiochemical Centre, Amersham, England. The activity of the original solution (1.5m Curies per ml.) was reduced

to 20 μCuries per ml. by dilution with distilled water. Saturated citric acid solution was used to release the CO_2 from the bicarbonate; it was chosen on account of its non-toxicity and low volatility. Flasks of 250ml. capacity were used to contain the parasites in the $^{14}CO_2$ atmosphere. The reacting solutions were contained in 5-ml. specimen bottles placed in the bottom of each flask. Samples for radioactivity measurements were placed on 2.5-cm. planchettes, and analysed on a Geiger-Muller end-window counter.

The efficiency of the counter for measuring particle emissions from the type of samples used was determined as follows. Stem portions of C. reflexa and C. campestris were harvested, washed and surface dried. One gram of each was separately placed in 25ml. of 80% ethanol, macerated in a mortar and the extracts filtered. Each extract was evaporated to dryness and then taken up in 1ml. distilled water. 0.5ml. of this extract was placed on a planchette together with 0.1ml. of saturated sodium bicarbonate solution containing 0.01 μCuries ^{14}C sodium bicarbonate. The addition of saturated $NaHCO_3$ solution maintained an excess of bicarbonate ions in the final mixture and therefore prevented decomposition of the radioactive bicarbonate by organic acids or other agents present in the plant extract. Evaporation to dryness was effected by means of a current of warm air. The planchette was then placed in a

Geiger-counter and readings taken for periods of five minutes. Taking the activity of 0.01 μCuries to be 3.7×10^2 disintegrations per second, the observed disintegration rate in extracts of both plants corresponded to almost exactly 1% of this. From these results it was assumed, for the particular methods and materials employed in the following experiment, that the efficiency of the counter was 1%.

2. Methods.

Into each of seven 250-ml. flasks was placed a 5-ml. specimen bottle, and 0.2ml. of saturated sodium bicarbonate solution was added to each bottle by means of a graduated pipette. (Assuming the value of 9% at 15°C. as the solubility of $NaHCO_3$, 0.2ml. of saturated bicarbonate solution would liberate 5ml. CO_2, thus giving a 2% atmosphere of carbon dioxide within the flask). In each bottle was also placed 4 μCuries (0.2ml.) of ^{14}C sodium bicarbonate solution.

Stems of C. campestris and C. reflexa were then harvested from a variety of hosts and each species cut into seven 1-gm. portions. Seven small leaves of Pelargonium zonale (0.3-0.35gm.) were also weighed, to be included in the experimental flasks as standards. Into each flask was placed a 1gm. portion of each parasite, together with a Pelargonium leaf. The plant material

was suspended above the base of the flask by means of a thin cotton thread which was tied round the neck of the flask (Fig. 57). This precaution was necessary to prevent any of the plants coming into direct contact with any spillage of the radioactive solution. The remaining 1gm. portions of plant material were placed in boiling water for 5 minutes to kill the cells, and similarly suspended in a flask. The flasks were then placed out of doors in bright sunlight.

To release the CO_2, 0.1ml. saturated citric acid solution was rapidly added to the contents of each specimen bottle, and the flasks stoppered immediately. Each flask was rotated successively through 90° at intervals of five minutes to ensure uniform illumination and prevent undesirable shading effects. The flasks containing the living plants were left in sunlight for periods of 15 min., 30 min., 1 hr., 2 hrs., 3 hrs., and 4 hrs. respectively; the boiled material was exposed to sunlight for 4 hours.

At the end of the experimental periods, the Cuscuta and leaf samples were removed from the flasks, washed in a large volume of cold water to remove any surface radioactive contamination caused by effervescence in the specimen bottle, and macerated in a mortar with warm 80% ethanol. The extracts were then filtered and the mortar and filter paper washed with 80% ethanol until the final volume of the original filtrate and washings was 25ml. A few drops

(0.1ml.) of sodium bicarbonate solution, followed by a similar volume of saturated citric acid solution were added to the filtrate to remove any remaining traces of radioactive bicarbonate contamination. Each extract was placed in a porcelain basin for evaporation to dryness in an oven at 100°C., and the dry residue taken up in 1ml. distilled water. 0.5ml. of this extract was evaporated to dryness on a planchette and analysed for radioactivity on a Geiger-counter (Table 18).

3. Results.

The results show that in all the extracts of living material, significant amounts of radioactive carbon had been incorporated. The degree of carbon-fixation was approximately similar in C. reflexa and C. campestris, increasing progressively with time of exposure to the isotope. In the Pelargonium leaves, carbon fixation, calculated on a fresh weight basis, was approximately 10 times higher than in Cuscuta. The low levels of radioactivity in the boiled controls indicate that assimilation was necessarily a vital process.

Assuming the average disintegration rate of the two Cuscuta samples for exposure of one hour to light as 330 counts per 5 min., and using the value 3.7×10^4 disintegrations per second as the activity of 1 μCurie, then after correction for the efficiency of

Table 18. CO_2-fixation in Cuscuta and Pelargonium (in sunlight). Results given as counts per 5 min. Background: 63 counts per 5 min. Efficiency of counter: 1% approximately.

Exposure time to $^{14}CO_2$	C. reflexa	C. campestris	Pelargonium leaf
15 min.	124	160	340
30 min.	200	263	508
1 h.	280	379	528
2 h.	567	468	1009
3 h.	811	604	2044
4 h.	1065	938	4218
Control(boiled material).	68	71	71

Table 19. CO_2-fixation in Cuscuta and Pelargonium (in darkness). Results given as counts per 5 min. Background: 60 counts per 5 min. Efficiency of counter: 1% approximately.

Exposure time to $^{14}CO_2$	C. reflexa	C. campestris	Pelargonium leaf
4 h.(living material)	121	113	207
4 h.(boiled material)	65	71	63

the counter, the rate of CO_2-fixation under the conditions of the experiment was 7.3 µgm. CO_2 per gm. per hour. If it is assumed that all the carbon had been incorporated in sucrose, this corresponds to the formation of 4.73 µgm. carbohydrate per gram of tissue per hour. The figure for Pelargonium leaves is approximately ten times this value.

From the results of the foregoing experiment it was not possible to say that CO_2-fixation was a light-activated reaction. To test this possibility, two flasks similar to the above, one containing living, and the other boiled material were kept in the dark for a period of four hours after the liberation of the radioactive CO_2. The plant matter was removed from the flask in subdued light, washed in water, and separately transferred to dishes containing 25ml. warm 80% ethanol. They were left in alcohol in the dark for one hour to ensure complete death of the cells. The material was then extracted and analysed in the same manner as before (Table 19).

The relatively high counts obtained for the living material, while they may represent dark-fixation, may also be partly accounted for by the short exposures to the subdued light necessarily incurred during the course of the experiment. However, in no case is the value obtained higher than the lowest value found for the material

exposed to light. These results demonstrate conclusively that both C. reflexa and C. campestris are capable of fixing carbon dioxide by a process which requires the presence of light.

b. Identification of radioactive compounds in extracts.

In order to demonstrate that the carbon-fixation observed in the two Cuscuta species was a photosynthetic process similar to that found in normal chlorophyllous tissues, it was necessary to demonstrate that the early radioactive products of the reaction were carbohydrates. The procedure adopted was the standard method of paper chromatography combined with autoradiographic techniques.

1. Materials.

For chromatography of the extracts, Whatman No.1 24cm. circular filter paper was used. The chromatographic apparatus was similar to that employed for pigment chromatography (Section V.A.); n-butanol, pyridine, water (3:2:1.5) was used as developing solvent (Jeanes et al., 1951). Analar sucrose, maltose, glucose, and fructose were used as reference sugars. To reveal the sugar zones, the paper was sprayed with a mixture of 0.2% naphthoresorcinol in absolute ethanol and syrupy phosphoric acid (9:1 v/v) Bryson and Mitchell, 1951. Industrex type "D" X-ray film was used to detect the radio-

activity on the paper.

2. Methods.

A preliminary qualitative analysis of the sugar composition of normal Cuscuta stems was considered desirable for two reasons, as providing a basis on which a mixture of standard sugars could be prepared for co-chromatography with the experimental extracts, and also of being of sufficient interest in itself to justify investigation, particularly since no reference was found in the literature to carbohydrate analyses of any member of the genus, apart from general microchemical tests (Mirande, 1901).

Samples (5-10gm.) of C. reflexa and C. campestris were surface-washed with cold water and macerated in a mortar with 100ml. of 80% ethanol. The extracts were filtered, washed with further portions of 80% ethanol, and transferred to porcelain basins for evaporation to a small volume in a current of warm air. This extract was then filtered and aliquots spotted in small fractions on the paper, with drying between each application. Spots containing a mixture of sucrose, maltose, glucose, and fructose were co-chromatographed along with the Cuscuta extracts. Once the solvent had reached the edge, the paper was air-dried, sprayed with naphthoresorcinol, and heated at 100°C. until coloured zones appeared. As a check to identification of the sugar zones, duplicate

chromatograms were sprayed with aniline phthalate reagent.

3. Results.

The sugar pattern was very similar in the two species of Cuscuta. Fig. 58, C. campestris, shows that sucrose, glucose, and fructose with Rf values of 4.4, 5.0, and 5.4, produced the most pominent coloured zones. Their relative proportions in all extracts tested were substantially constant. Inside the sucrose zone, with an Rf value of 3.7, maltose could generally be detected. A faint zone, with an Rf value of 6.0 beyond the fructose band, was identified by means of co-chromatography as the pentose sugar xylose. Two reddish-blue zones near the origin, with Rf values of 0.18 and 0.08, were always detectable but were not identified; they were presumably oligosaccharides. It is clear from these results that the sugar composition of C. campestris and C. reflexa is qualitatively similar, consisting of maltose, sucrose, glucose, fructose, and xylose, along with at least two unidentified oligosaccharides.

The pattern is not dissimilar to some recent findings on plant materials made through paper chromatography and compiled by Bell (1955). Williams and Bevenue (1951) found the following free sugars in an 80% ethanol extract of wheat flour:- arabinose, fructose, glucose, sucrose, maltose, and raffinose. This compares fairly

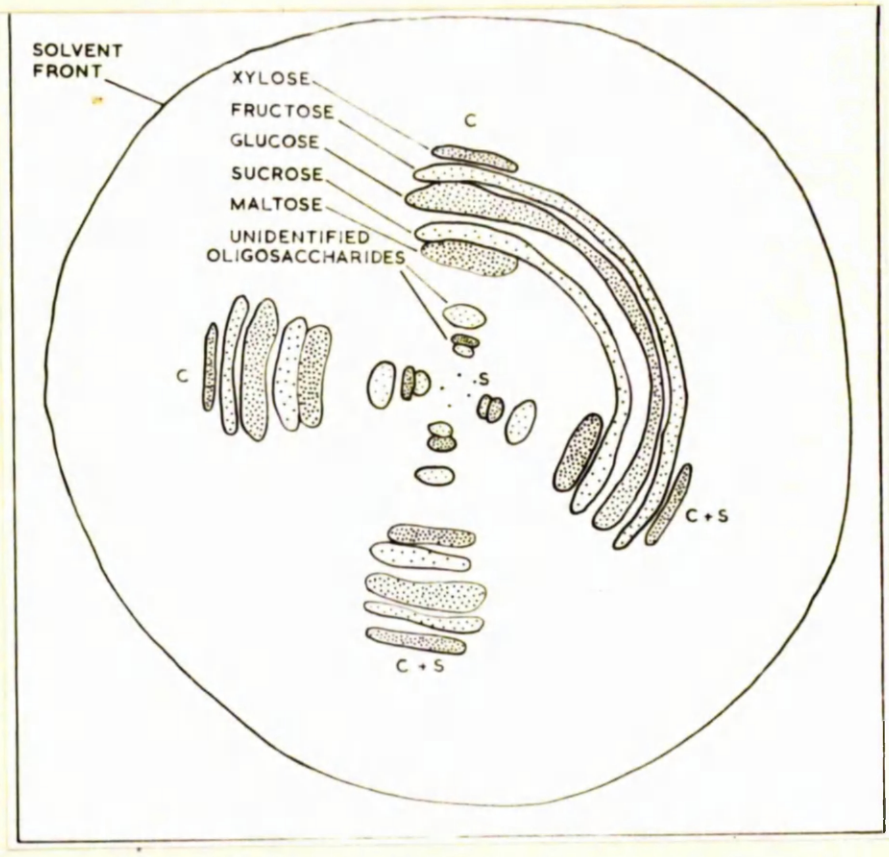

Fig. 58. Circular paper chromatogram of 80% ethanol extract of C. campestris, using as solvent system: butanol:pyridine:water(3:2:1.5). S-standard sugars; C-Cuscuta extract; S+C-mixture of both extracts.

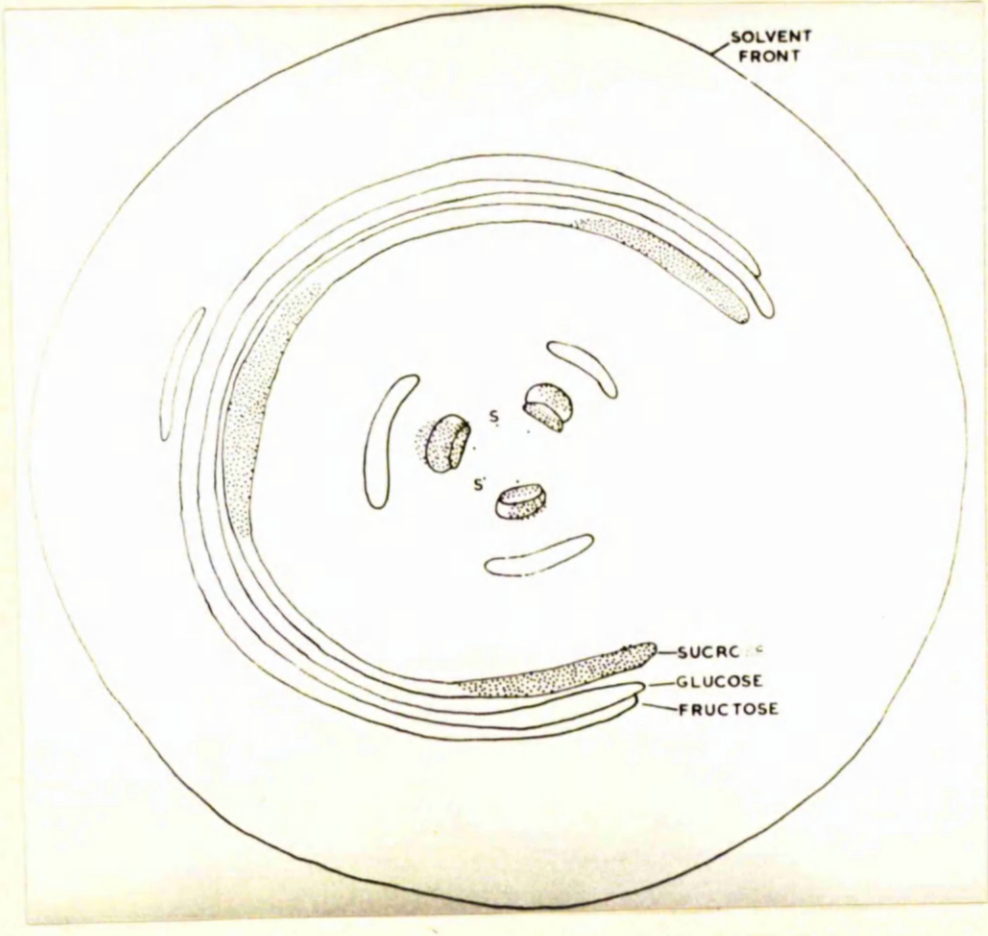

Fig. 59. Chromatogram of radioactive extract of C. reflexa, with areas of radioactivity (stippled) superimposed.

closely with a more recent analysis of young wheat leaves (Jain and Pelletier, 1958) which revealed fructose, sedoheptulose, glucose, sucrose, maltose, melibiose, and raffinose, together with some unidentified sugars. In an analysis of representatives of 27 families of Spermatophyta, Bidwell et al. (1952) have shown that sucrose was the predominant sugar in most land plants. Analyses of a number of species of the Saxifragaceae (Nordal and Oiseth, 1952) and of the Crassulaceae (Nordal and Klevstrand, 1951) revealed sedoheptulose, glucose, fructose, and sucrose in both families.

c. Analysis of radioactive extracts.

Cuscuta stems were exposed to radioactive CO_2 in the presence of sunlight as described in Section VI.(a). The flasks, which contained 8 μCuries ^{14}C were exposed to sunlight for periods of from 15 min. to four hours. Extracts were prepared and chromatographed as described in Section VI.(b). A standard mixture of glucose, fructose, sucrose, and maltose (each constituent present as a 2% solution) was co-chromatographed on the same paper. After development, the filter paper was allowed to dry overnight in air at room temperature. The filter paper was then placed on a sheet of X-ray film, wrapped in black paper, and kept weighted down for periods of up to 8 weeks at $4^{\circ}C$. A needle was passed through the package at several points so that the original superposition of the

paper could be obtained when required. At the end of the exposure period the film was developed and the paper sprayed to reveal the sugar zones.

Results.

Examination of the X-ray plates showed that in every extract, virtually all of the radioactivity was confined to one zone. Comparison with the sprayed paper revealed that this zone corresponded exactly with the sucrose zone on the chromatogram (Fig. 59). Two other minor areas of radioactivity occurred near the origin (Rf values of 0.05 and 0.1), but did not coincide with any spots revealed by the sprays.

These results have shown that both C. reflexa and C. campestris are able to fix carbon dioxide by a process which occurs only in the presence of light; furthermore, after periods of exposure to $^{14}CO_2$ ranging from 15 min. to four hours, nearly all the ^{14}C had been incorporated in sucrose. It is concluded from this investigation that in the two Cuscutas tested, carbon-fixation is a photosynthetic process, and that on a fresh weight basis the level of photosynthesis in C. reflexa and C. campestris is similar, and equal to about one tenth the level in Pelargonium leaves.

d. Effect of splitting Cuscuta stems on the rate of photosynthesis.

Thomson (1925) dismisses the photosynthetic ability of C. reflexa as insignificant in view of the absence of stomata and the resultant difficulty in the maintenance of a supply of carbon dioxide to the pigmented region of the parasite stem. This conclusion appears reasonable especially if one considers the diameter of the stem (up to 8mm.) and its possession of a relatively uninterrupted cuticle. In C. campestris, where the pigmented region is entirely within the narrow stele, the problem of CO_2 supply is presumably more acute.

It accordingly seemed desirable to test if photosynthetic activity was accelerated by allowing the carbon dioxide to come into more intimate contact with the plastid-containing tissues. To effect this, the stems were cut longitudinally by means of a scalpel blade into two similar halves.

1. Methods.

Into three 500-ml. flasks were placed 1gm. portions of C. campestris and C. reflexa stems, together with 1gm. portions which had been cut longitudinally immediately before the experiment. To minimise damage as a result of sectioning, robust stem portions, taken from Vicia, Pelargonium, and Nicotiana hosts, were chosen

for this experiment. An atmosphere of 2% CO_2 containing 8 μCuries was created within the flasks which were immediately stoppered and left in continuous bright sunshine for 30 min. To ensure uniform illumination the flasks were rotated every five min. The plant material, at the end of the exposure period, was extracted and analysed for radioactivity as described in Section VI.(a). Due to technical difficulties, the radioactivity measurements in this and the succeeding section were made on a different instrument from that used in Section VI.(a).

2. Results.

It can be seen from Table 20 that the activity of extracts of sectioned plants is significantly higher than that of intact filaments. This difference corresponds to an increase in photosynthetic activity of from 27.6% (C. campestris on Vicia) to 70.1% (C. reflexa on Vicia) in the treated plants.

The fact that the rate of photosynthesis in both parasites from Vicia hosts is higher than in the parasites from Pelargonium or Nicotiana is probably due to the higher chlorophyll content normally associated with Cuscuta growing on that host (Section V.A.). From these observations it follows that the effect of splitting Cuscuta stems longitudinally is to increase, over a limited period,

Table 20 Photosynthesis in C. campestris and C. reflexa, measured as counts per minute per sample. Readings are corrected for background.

	Host	Intact Parasite	Sectioned Parasite	% Increase
C. CAMPESTRIS	Vicia	355	453	27.6
	Pelargonium	279	408	46.2
	Nicotiana	306	448	46.4
C. REFLEXA	Vicia	395	672	70.1
	Pelargonium	291	480	64.9
	Nicotiana	311	515	65.6

the photosynthetic activity of the parasite by approximately 50%. It is concluded from this that the rate of photosynthesis in Cuscuta is limited by the non-availability of carbon dioxide supply. One would expect, if the CO_2 could be introduced to the pigmented regions of the stem by some means other than by splitting the stem with its concomitant damage to the tissues, thet the increase in photosynthetic activity would be greater than that found in this experiment.

A feature of this experiment is that splitting of the stem has a relatively weaker promoting effect on photosynthesis in C. campestris than in C. reflexa. If it is recalled that the plastids in C. reflexa are in the cortex and therefore near the epidermis (Fig. 24), whereas in C. campestris they are embedded in the narrow stele (Fig. 17), this result is unexpected. Certainly the filaments of C. campestris are narrower than in the other species. It is also unlikely that the selective distribution of plastids in the vicinity of the vascular strands of C. campestris is without significance.

Despite the relatively high efficiency of photosynthesis per milligram of chlorophyll in Cuscuta (Section VI.e.), the only mechanism that can be suggested at this stage whereby carbon dioxide can reach the chromoplasts is by diffusion across the intervening

stem tissues. There are almost no air spaces in Cuscuta stems.

e. Estimation of the efficiency of the photosynthetic process in Cuscuta.

In the course of experiments on photosynthetic activity and chlorophyll content, it became clear that the photosynthetic ability of Cuscuta relative to its chlorophyll content compared favourably with that of its host plants. In the following experiment, designed to obtain a numerical value for this relationship, the rate of photosynthesis and chlorophyll content were measured in closely similar samples

1. Materials.

Healthy leaves of Pelargonium, Nicotiana, Vicia, and Helianthus were cut longitudinally through the midrib into two similar halves. Stem filaments of C. campestris and C. reflexa on Nicotiana were selected and also split longitudinally. Each sectioned portion was weighed

2. Methods.

One set of sectioned material was analysed for chlorophyll content by differential spectroscopy as described in Section V.A.(b.). The other half-portions were placed in a 500-ml. flask and allowed

to photosynthesise in the presence of 8 μCuries $^{14}CO_2$ for 45 min. (The atmosphere in the flask contained approximately 2% CO_2). At the end of the experimental period, the tissues were extracted and analysed for radioactivity as described in Section VI.a.

3. Results.

The rate of photosynthesis per milligram chlorophyll in the two species of Cuscuta was found to be comparable to that in the leaves examined (Table 21). The efficiency in C. campestris was 30.5% above that in C. reflexa, and more than twice as high as in Pelargonium leaves. The highest efficiency (in Nicotiana leaves) is only 26% above that of C. campestris.

Willstatter and Stoll (1918) showed that chlorophyll content of normal green leaves may have little relation to the rate of photosynthesis. In their experiments with green- and yellow-leaved varieties of Ulmus, where the yellow varieties contained only 6.4% as much chlorophyll as the green on a fresh weight basis, they found that the rate of assimilation per unit weight of chlorophyll was nearly twelve times higher in the yellow leaves. It follows from this observation, that in green leaves the chlorophyll content is in such an excess that other factors (CO_2 pressure and light intensity) are normally limiting.

If it is assumed, on a basis of these results, that 11/12th.

Table 21 Comparison of photosynthetic rate in Cuscuta and host plants.

Plant Analysed	Chlorophyll Content (μ gm/gm fresh weight)	Rate of Photosynthesis (c.p.m./gm fresh weight)	Rate of Photosynthesis per mg Chlorophyll
Pelargonium	954	7091	7433
Nicotiania	845	18,532	2193
Vicia	2120	39,239	1866
Helianthus	2046	36,313	1774
C. reflexa on Nicotiana	56	675	1205
C. campestris on Nicotiana	24	416	1734

of the chlorophyll in autotrophs is superfluous to its photosynthetic requirements, then a new comparison of the useful chlorophyll content in green leaves and the chlorophyll content of Cuscuta can be made.

This comparison reveals C. reflexa to be furnished with adequate chlorophyll for autotrophic development, with C. campestris somewhat less well equipped. Comparison of the normal chlorophyll content of Cuscuta with 1/12th. of the chlorophyll content of an entire Pelargonium plant reveals C. campestris and C. reflexa (on Vicia) as containing respectively 250% and 400% of the useful chlorophyll content of Pelargonium. The relatively high photosynthetic efficiency found in the stems of C. reflexa and C. campestris is therefore likely to be the direct result of low chlorophyll density; the limiting factor in the plants' potential independence may be their undoubted anatomical unsuitability as photosynthetic organs.

VII. ENZYME ACTIVITY IN CUSCUTA.

Nitrogen is of basic importance in metabolism as a constituent of amino acids, the essential building blocks of proteins. Cuscuta presumably derives all of its nitrogen from the host plant, but it is of considerable importance to determine if this is achieved by withdrawal of nitrogen as organic or inorganic compounds, since this knowledge would shed some light on the degree of dependence shown by the parasite towards its host.

For this reason several enzymes which form nitrogenous compounds (amino acids) were investigated to establish the extent of the synthetic capabilities of the parasite. Glutamic dehydrogenase and several transaminase enzymes were selected for detailed study, since they are involved in the incorporation of inorganic nitrogen into a bound organic form by way of reductive amination of α-keto acid followed by amino-group transfer to give a wide range of amino acids.

Lilienstern (1928a) showed that the peroxidase and diastase activity of Cuscuta monogyna was higher in portions of the parasite embedded in the host than in free stems. The same writer also found that when Cuscuta was grown in nutrient solutions, the peroxidase activity of the parasite was greatest in solutions of least nutritive value. Apart from this work, very few references

exist in the literature to the host-parasite-enzyme complex in Cuscuta. It is therefore clear that the whole field of enzyme composition and relationships in the genus is almost completely uninvestigated, and some contribution to the subject is therefore highly desirable, if only as a minor contribution to the large amount of data rapidly becoming available in the complementary field of fungal host-parasite relationships. Enzymic studies in Cuscuta have the advantage that it is a comparatively simple matter to detach the parasite completely from the host; there is no extensive ramification of parasitic tissue into the host, which is such a characteristic feature of fungal hyphae.

A. Transaminase.

The following work describes the examination of the parasite for transaminase activity, its variation with host plant, and the effect of parasitism by Cuscuta on the transaminase activity of its hosts.

Transamination, in its usual form, is the transference of an amino group from an amino acid to an α-keto acid, without the formation of ammonia. The reaction was first observed by Herbst in vitro in 1936, and in 1937, Braunstein and Kritsmann demonstrated enzymic transamination in pigeon breast muscle. Since then, enzymic

transamination reactions have been shown to occur in all groups of plants; by Wilson et al., (1954) in angiosperms; by Feldman and Gunsalus, (1950) in Escherichia coli; by Millbank, (1953) in Chlorella; and by Fincham, (1951) in Neurospora.

In a system where some amino acids essential to protein synthesis are lacking, the presence of transaminase enzymes which can promote the synthesis of amino acids from corresponding α-keto acids is obviously of critical importance. Transaminase reactions also form a vital link between amino acids and the analogous α-keto acid intermediates of the citric acid cycle. The demonstration of transaminase in Cuscuta would indicate that a large number of metabolic processes are probably undertaken by the parasite itself, and it is therefore of considerable interest to determine if such an enzyme system exists in Cuscuta.

a. Range of amino acid-α-keto glutarate transamination in Cuscuta.

A total of 16 amino acids were tested for their ability to transaminate with α-keto glutaric acid in crude extracts of Cuscuta campestris and Cuscuta reflexa stems. Portions of parasite stems (5gm.) were washed with cold water, surface dried, and ground with 10-15ml. of chilled phosphate buffer of pH 8.0. The extract was then filtered through a double layer of muslin, and dialysed against

distilled water at 4°C. for 18 hours. The dialysed extract was then made up to 20ml. with phosphate buffer.

For the qualitative assay of transaminase activity of the Cuscuta extracts, reaction mixtures were prepared as follows:- 0.5ml. enzyme extract, 0.1ml. of 0.1M amino acid, 0.1ml. of 0.1M α-keto glutaric acid, 1.2ml. of 0.2M phosphate buffer (pH 8), together with 0.1ml. of 0.03M pyridoxal phosphate which is considered by Meister (1957) to be involved in all transaminase reactions. The reacting mixtures were incubated in test-tubes at 37°C. for two hours, and the reaction stopped by plunging the tubes in boiling water for three minutes.

Aliquots (10 μlitres) from each tube were then chromatographed on Whatman No. 1 filter paper ascending chromatograms with 80% aqueous phenol as developing solvent, and the paper allowed to dry overnight at room temperature. The papers were then sprayed with 0.25% ninhydrin reagent, heated at 60°C. for 30 minutes, and allowed to stand at room temperature for a further period of three hours to achieve optimum intensity of the ninhydrin colour complex. If, in addition to the original amino acid spot, a second spot (of glutamic acid) was present (identified by its Rf value and co-chromatography) transamination was considered to have occurred. Transamination occurred in C. reflexa with aspartic acid, alanine,

leucine, iso-leucine, valine, and asparagine; and in C. campestris with aspartic acid, alanine, valine, asparagine, and methionine. With the exception of aspartic acid, the glutamic acid spots formed with all the other acids were faint and in some cases uncertain (Table 22).

Since the glutamic-aspartic transaminase system was the most active of the amino acids tested, it was selected for a more detailed study of its occurrence in C. reflexa and C. campestris.

Preliminary experiments were performed on the crude dialysed extracts to determine the optimum pH of the reaction:- aspartic acid + α-keto glutaric acid ⇌ glutamic acid + oxaloacetic acid. Only the forward reaction in the above equation was studied, although the back reaction was shown to occur in both parasites, but at a considerably slower rate than the forward reaction. By varying the pH of the buffer used in an aspartic acid reaction mixture similar to that described on page 103, and incubating at 37°C. for 10 minutes, it was found that the optimum pH, indicated by maximum glutamic acid formation, occurred in both parasites when buffer of pH 8.0 was used (Figs. 60 and 61). pH values 6-8 were obtained by mixing KH_2PO_4 and K_2HPO_4 solutions in the requisite proportions; buffers of higher pH (up to pH 9.0) were obtained using borax:boric acid mixtures.

Table 22. Transamination to α-ketoglutaric acid by crude extracts of Cuscuta reflexa and Cuscuta campestris.

Amino group donors	C. reflexa	C. campestris
Aspartic acid	++	++
Alanine	++	++
Leucine	+	-
Iso-leucine	+	-
Lysine	-	-
Valine	+	++
Asparagine	++	+
Arginine	-	-
γ-amino butyric acid	-	-
Tryptophane	-	-
Methionine	-	+
Tyrosine	-	-
Phenylalanine	-	-
Proline	-	-
Glycine	-	-
Serine	-	-

++ indicates that the glutamic acid spot is easily detectable.
+ indicates that the glutamic acid spot is very faint.
- indicates that the glutamic acid spot is absent.

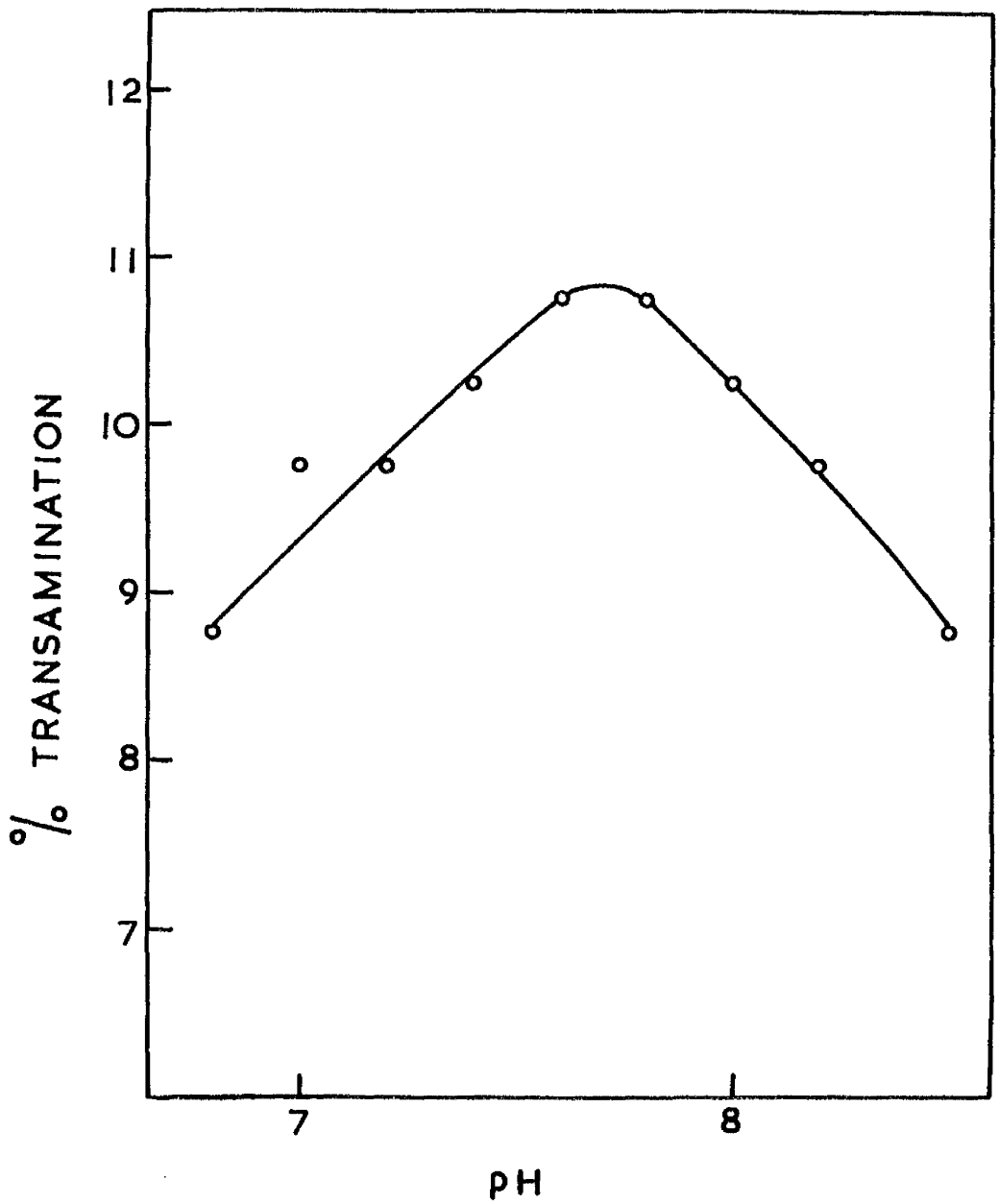

Fig. 60. The effect of pH on glutamic-aspartic transaminase in C. campestris.

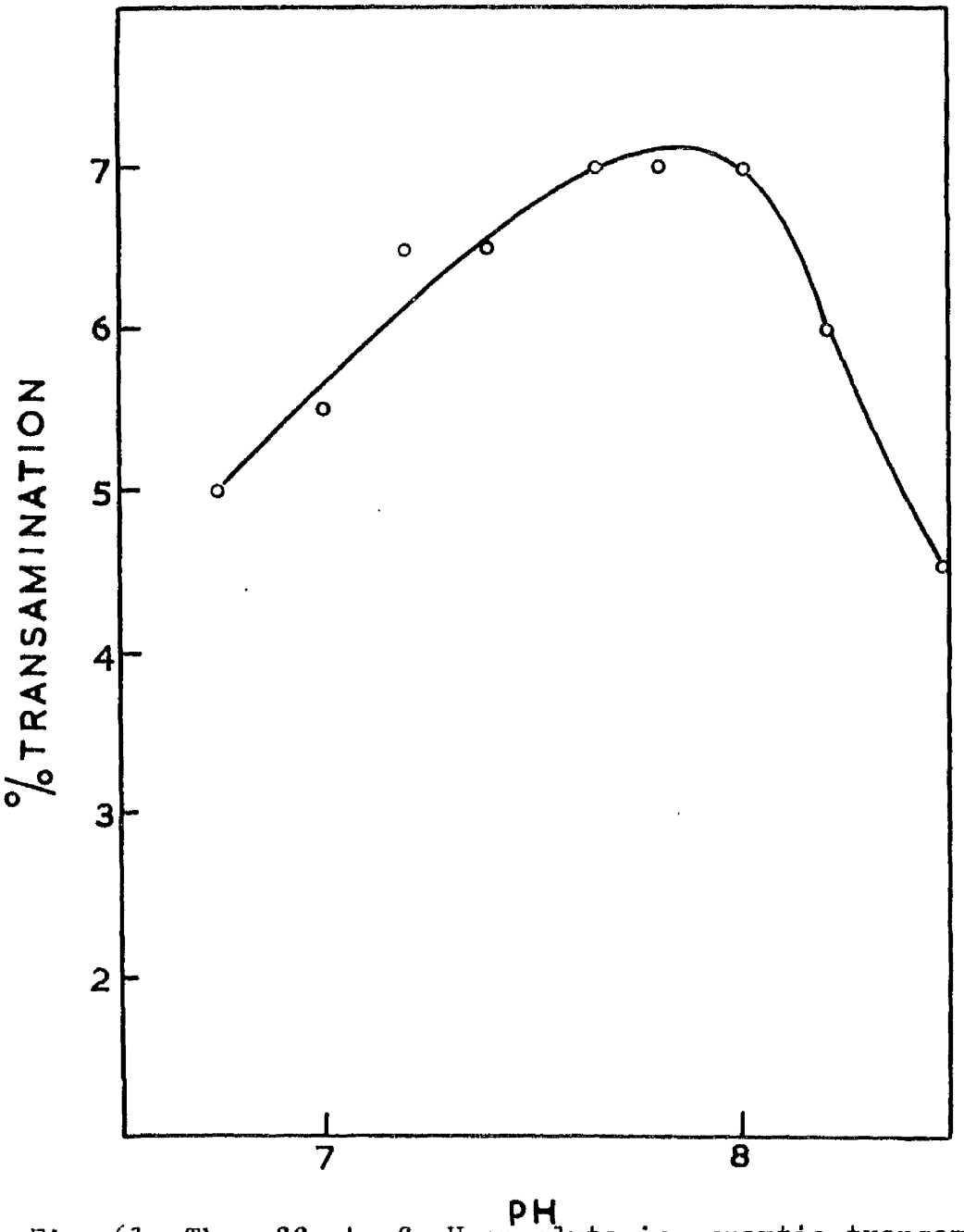

Fig. 61. The effect of pH on glutamic-aspartic transaminase in C. reflexa.

b. Measurement of transaminase activity of parasites from different host plants.

The following experiment describes an attempt to establish if any correlation existed between the transaminase activity of the parasite and that of its host plant.

Leaves of Vicia, Nicotiana, Vitis, Pelargonium, and Helianthus were harvested, surface washed, and dried. Portions of 5gm. were then extracted with buffer of pH 8.0, filtered and dialysed for 18 hours as described on page 102. Extracts of C. reflexa and C. campestris stems from individual host plants were similarly prepared. Standard reaction mixtures (page 103) were then incubated at 37°C. Samples were removed at intervals of 5, 10, and 15 minutes, deproteinised by immersing in boiling water for three minutes, and then aliquots (10 µlitres) spotted on chromatograms. Control tubes, lacking α-keto acid were incubated along with the test solutions.

Quantitative estimations of transaminase activity, measured as the amount of glutamic acid formed, were made using the method of Smith (1959). The glutamic acid spots revealed by spraying the chromatograms with ninhydrin are cut from the paper, eluted in buffered 50% ethanol for 18 hours at 4°C. and the intensity of

the eluted pigment measured spectrophotometrically at 570mμ, and compared with a standard curve for glutamic acid (Fig. 62). Appropriate corrections were made for the blank determinations.

In this experiment it was found that the leaves of Nicotiana, Vicia and Helianthus showed positive transamination, varying from 0.32 mMoles glutamic acid formed per gram dry weight of extract per 10 minutes incubation (in Nicotiana) to 0.19 mMoles in Helianthus. No trace of glutamic acid formation could be detected in extracts of Vitis or Pelargonium leaves (Fig. 63). All Cuscuta extracts, on the other hand, exhibited positive transamination, with C. campestris approximately 30% more active than C. reflexa. In C. reflexa extracts, the highest transaminase activity (0.28 mMoles glutamic acid formed per gram dry weight of extract after 10 minutes incubation) was found in parasites from Vicia hosts, with progressively lower values on Nicotiana, Pelargonium, Vitis, and Helianthus (Fig. 64). The values for C. campestris vary from 0.36 mMoles glutamic acid formed per gram dry weight of extract after 10 minutes incubation (on Vicia) to 0.16 mMoles glutamic acid formed (on Pelargonium), Fig. 65. No values were found for Vitis or Helianthus hosts, since C. campestris does not grow well on these plants.

It is clear from these results that the pattern of transaminase activity (on a host-plant basis) is similar in the two

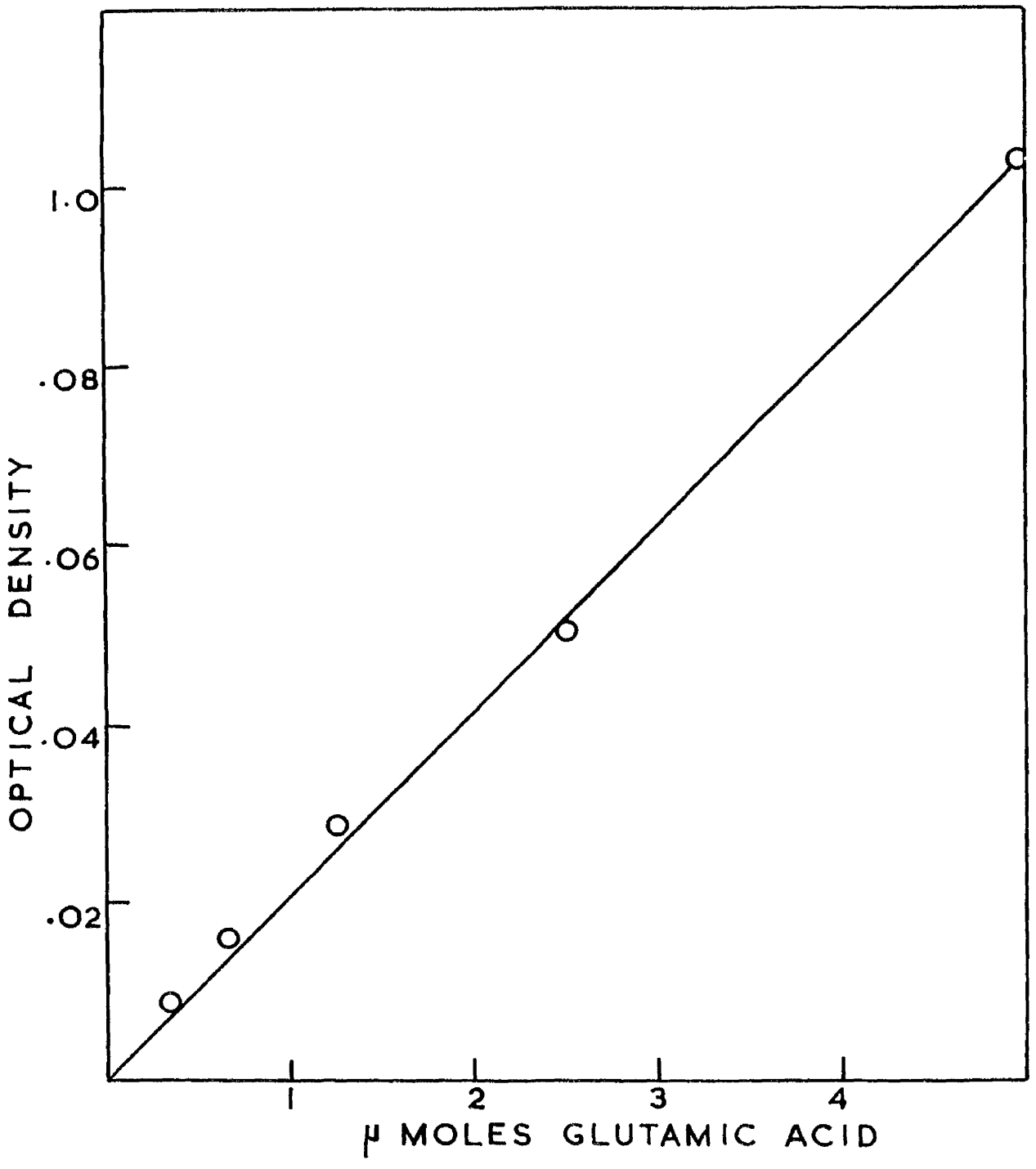

Fig. 62. Standard curve for glutamic acid.

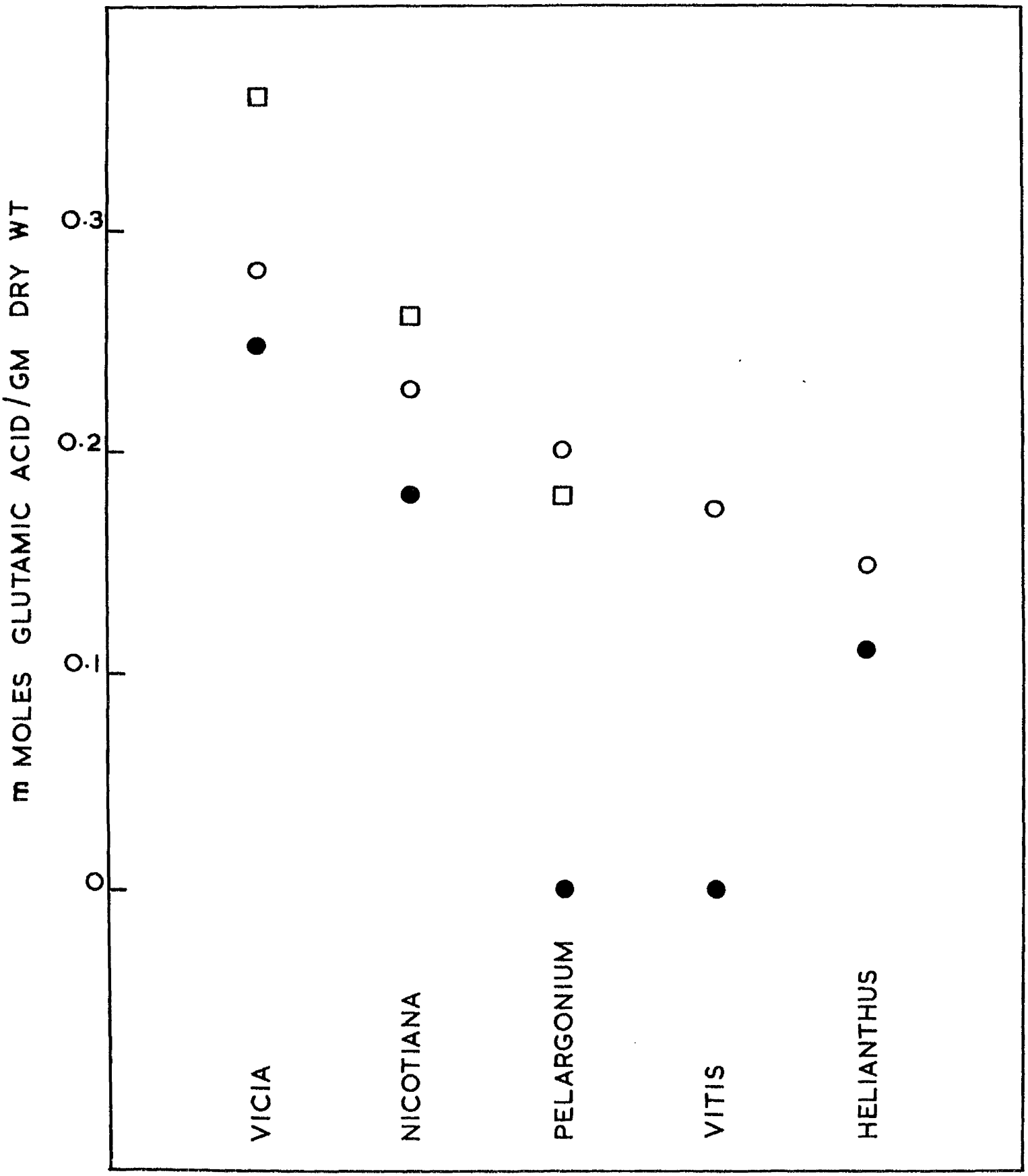

Fig. 63. Comparison of transaminase activity of leaves and parasites. ●=leaves after 5 mins. incubation; ○= C. reflexa after 10 mins. incubation; □ = C. campestris after 10 mins. incubation.

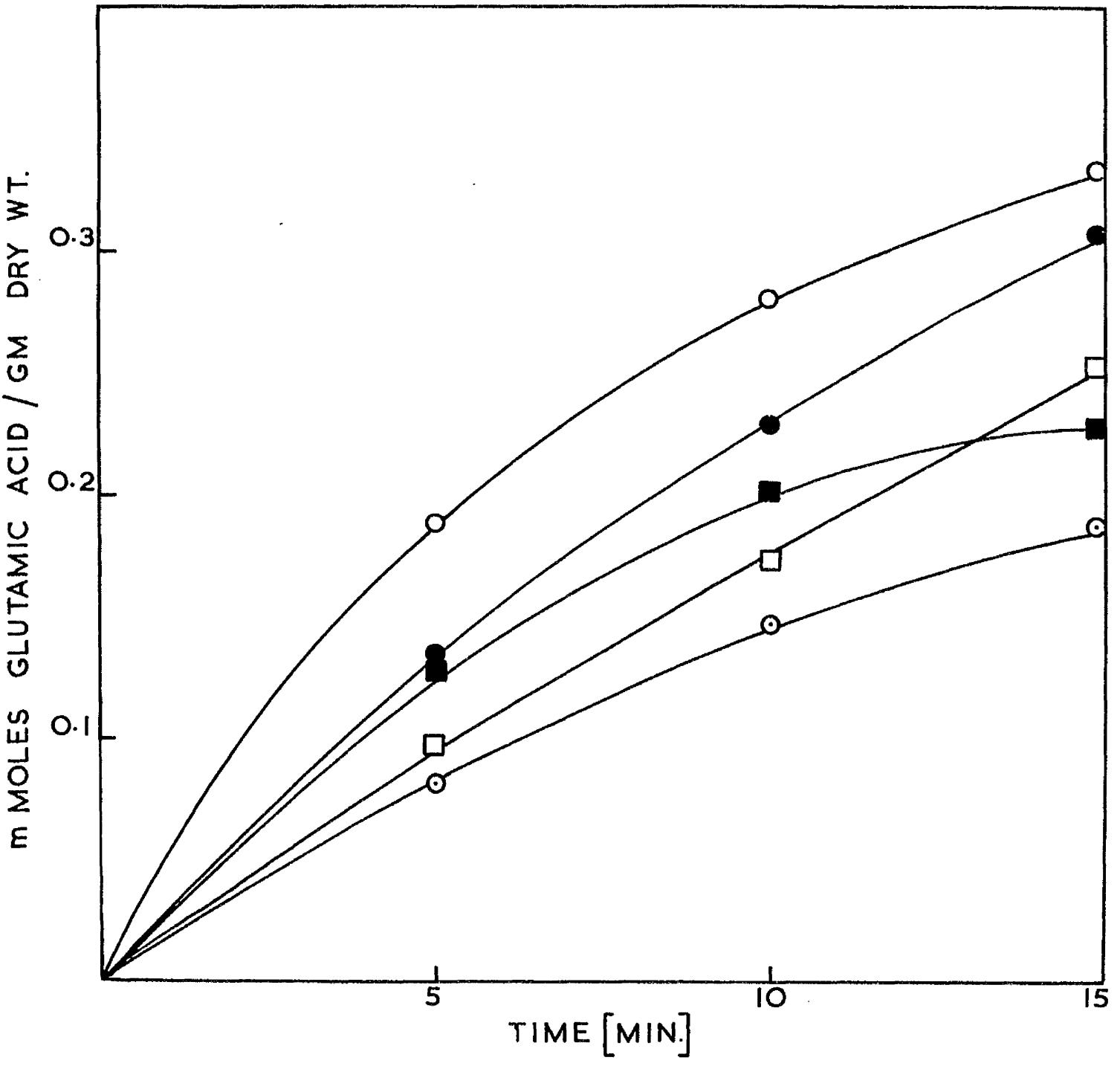

Fig. 64. Transaminase activity in C. reflexa on various hosts. Open circles- on Vicia; closed circles- on Nicotiana; open squares- on Vitis; closed squares- on Pelargonium; dotted open circles- on Helianthus.

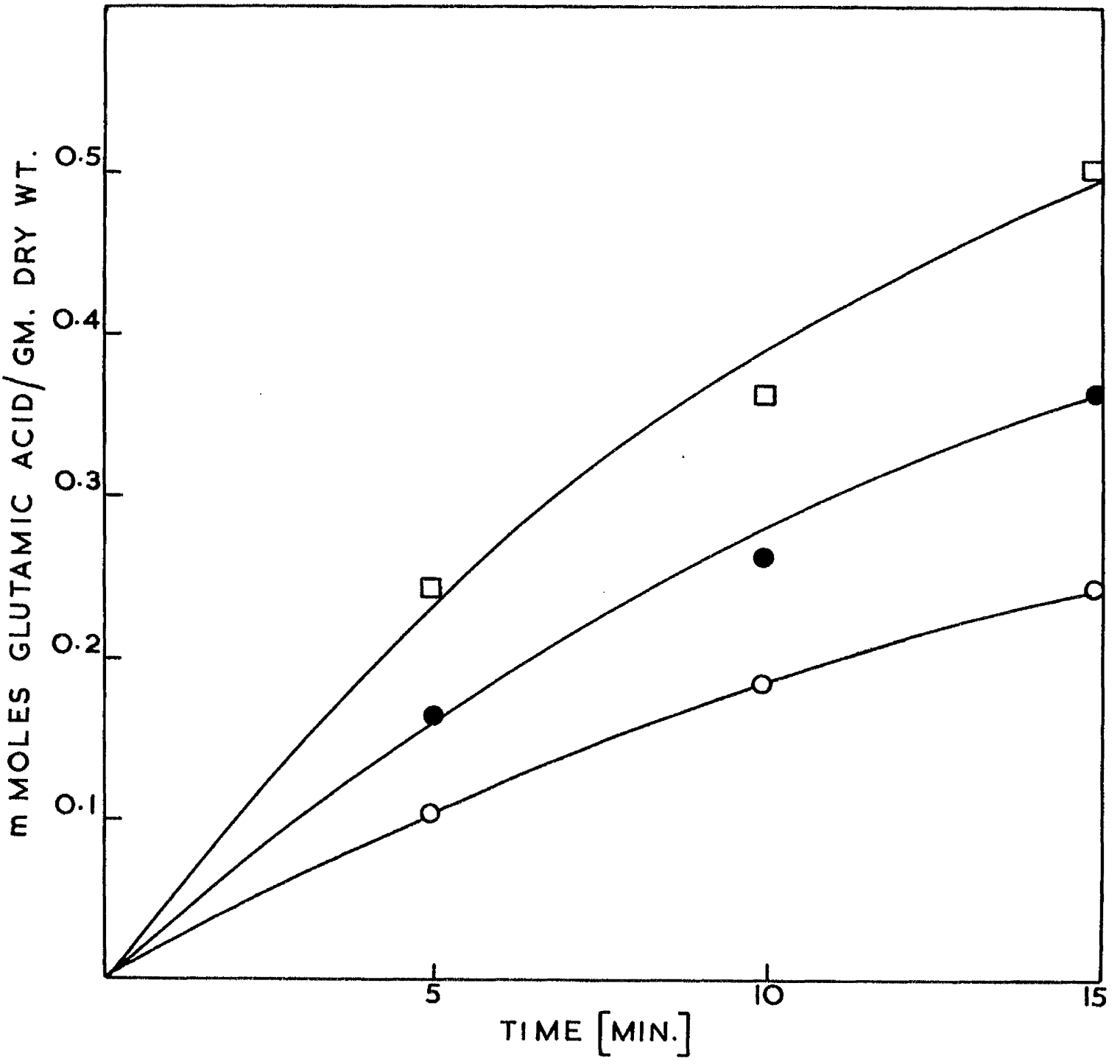

Fig. 65. Variation in transaminase activity of C. campestris on various hosts. Open circles-- parasites on Pelargonium; closed circles- parasites on Nicotiana; squares- parasites on Vicia

parasites. A correspondence also exists between the transaminase activity of the parasite and its host. Of the leaves examined, Vicia showed the highest transaminase activity, and it is on this host that both C. reflexa and C. campestris exhibited their maximum enzyme activity. The transaminase activity of Cuscuta on Nicotiana and Helianthus similarly reflects the transaminase activity of their host plants.

It is interesting to note that although no transaminase activity could be demonstrated in Pelargonium and Vitis leaves (at any rate in the pH range 7-8), the enzyme was present in parasites growing on these hosts. While an investigation of Pelargonium and Vitis leaf extracts over a wide pH range might reveal the presence of transaminase activity, this demonstration must be taken as evidence that the seat of transaminase formation is within the parasite itself, and not in the host.

c. Effect of parasitism by Cuscuta on the transaminase activity of host-plant leaves.

Leaf samples were harvested from healthy unparasitised plants, from plants parasitised by C. reflexa, and from plants parasitised by C. campestris. Care was taken to ensure that leaf samples from parasitised plants showed no major necrosis, and that

all samples from each host species were reasonably uniform. The leaves were extracted, incubated at 37°C. with aspartic acid and analysed for transaminase activity by the methods described in Section VII.A.(b).

Results.

As in the previous experiment, no transaminase activity was detected in Vitis or Pelargonium leaves; parasitised leaves of these two plants similarly showed no activity. The transaminase activity of Vicia leaves appeared to be little affected by the parasitism of either species of Cuscuta (Fig. 66). In Nicotiana and Helianthus, however, parasitism by Cuscuta has a distinct promoting effect on transaminase activity (Figs. 67 and 68). This finding should be compared with the results of Smith (1959) who found that the transaminase activity of sunflower cotyledons parasitised by Puccinia helianthi was higher than in healthy plants.

d. Intra-cellular localisation of transaminase in Cuscuta.

The primary purpose of this investigation was to determine if the locus of the glutamic-aspartic transaminase was on the particulate (mitochondrial) or soluble (cytoplasmic) fraction of the cell. According to Rowsell (1951), the transaminase enzymes in animal tissues are primarily located in the mitochondria. The

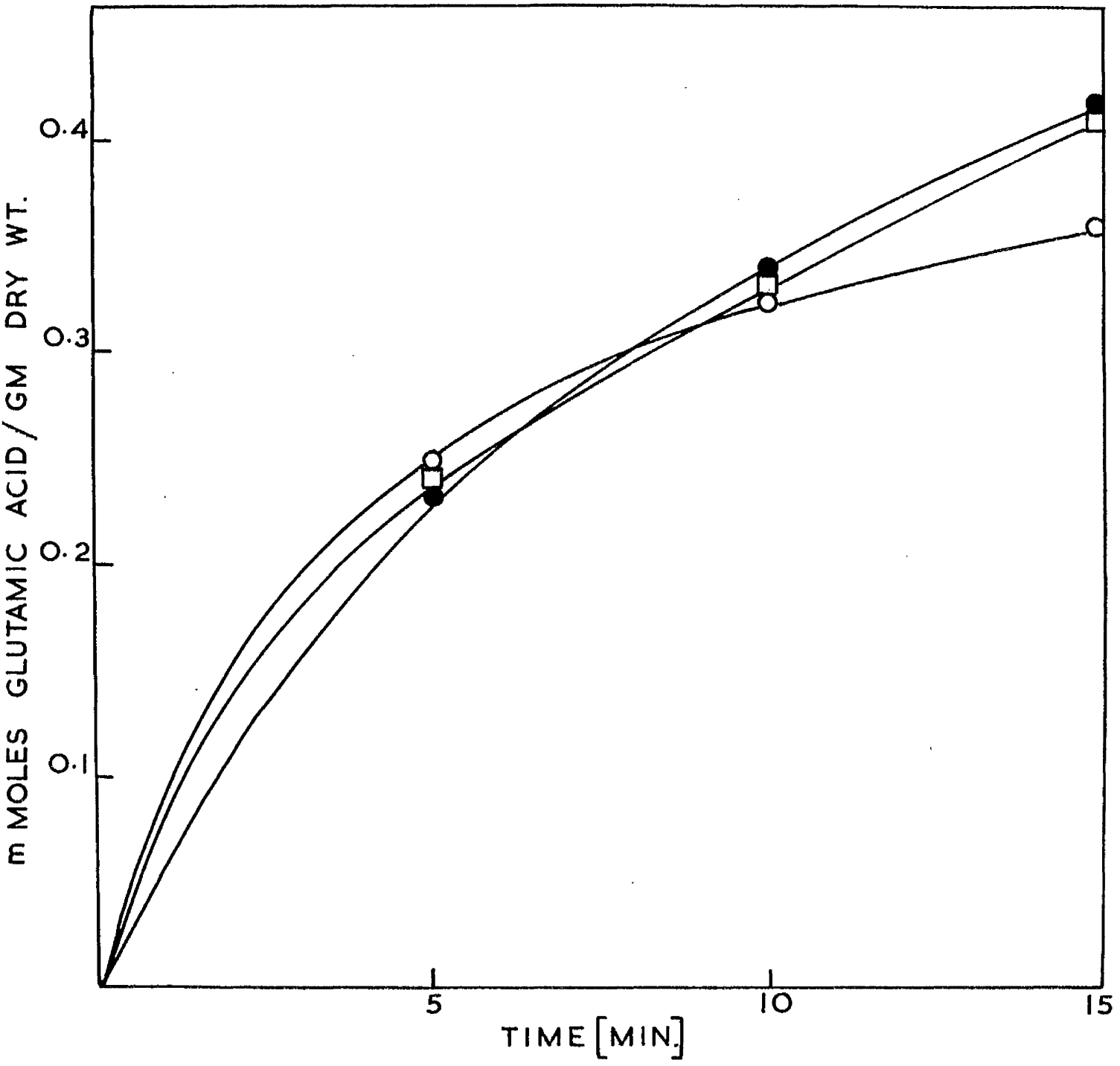

Fig. 66. Effect of parasitism by Cuscuta on transaminase activity of Vicia leaves. Open circles represent unparasitised plants; closed circles represent Vicia plants parasitised by C. campestris; squares represent plants parasitised by C. reflexa.

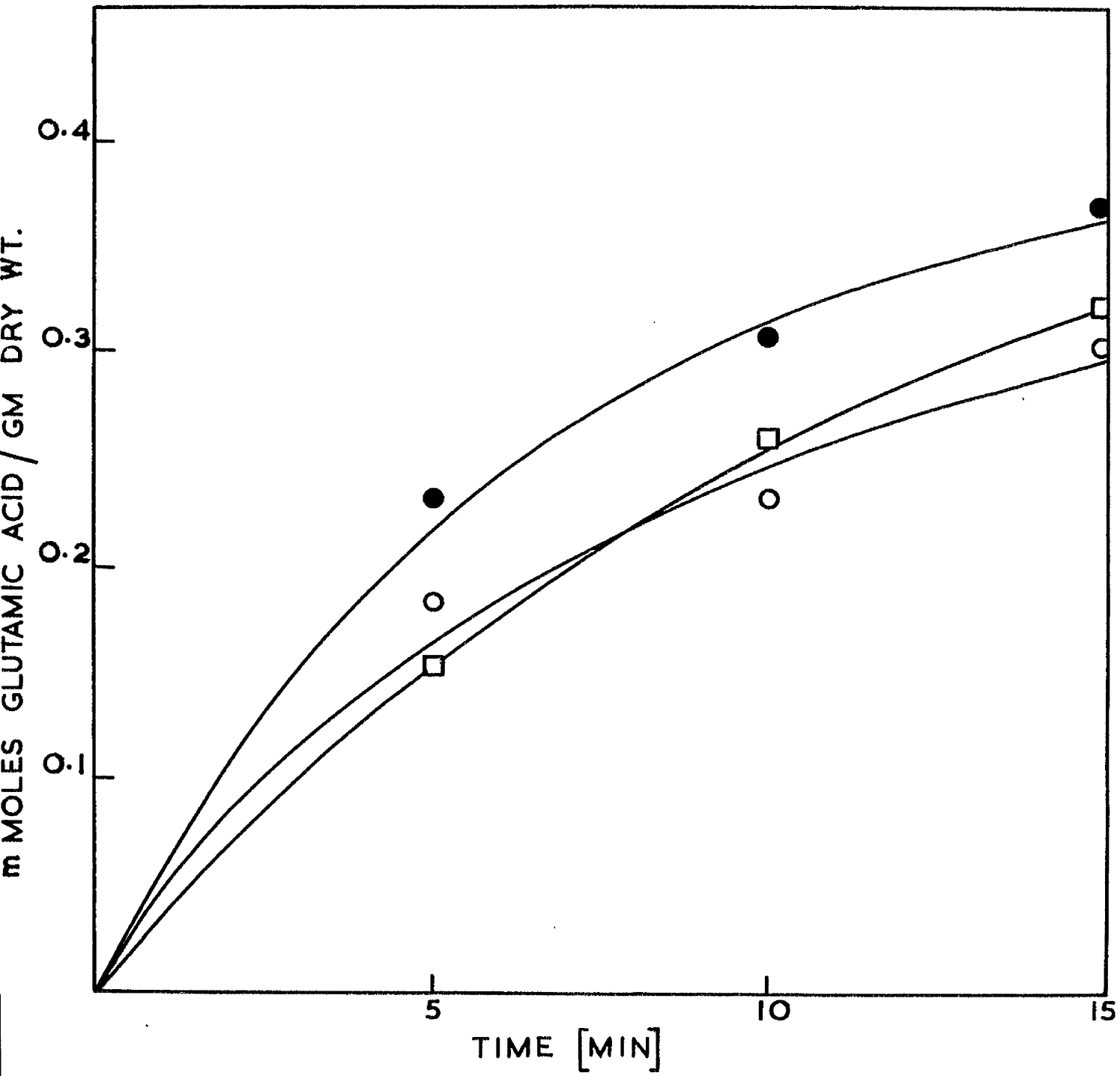

Fig. 67. Effect of parasitism by Cuscuta on transaminase activity of Nicotiana leaves. Open circles represent unparasitised leaves; closed circles represent Nicotiana plants parasitised by C. campestris; squares represent plants parasitised by C. reflexa.

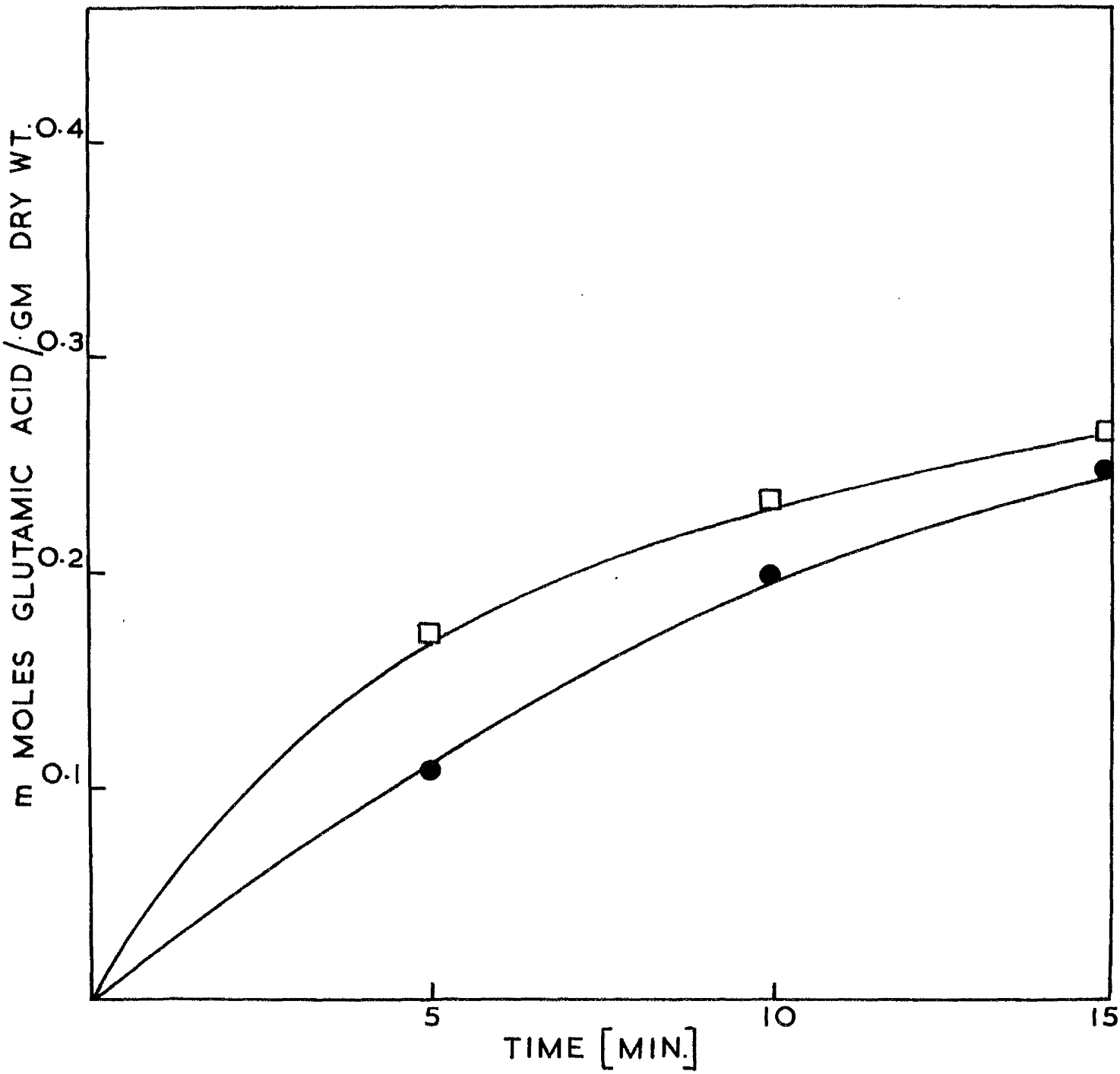

Fig. 68. Effect of parasitism by C. reflexa on transaminase activity of Helianthus leaves. Closed circles represent unparasitised leaves; squares represent parasitised leaves.

localisation of the enzyme in plant cells appears to depend on the particular species, eg. Wilson et al. (1954) have produced evidence that the transaminase enzyme of lupin is present both in the cytoplasm and the mitochondria, while Bone and Fowden (1960) have shown that the highly active glutamate-aspartate transaminase of mung-bean seedlings is predominantly mitochondrial. The cytoplasmic fraction is regarded as the site of transaminase activity in Neurospora by Fincham (1951) and of Fusarium by Sanwal (1958). Smith (1962) has shown that the glutamate-aspartate transaminase of sunflower hypocotyls is largely associated with the mitochondria; he suggests that cytoplasmic activity may be due to leaching of the enzyme from the mitochondria during preparative procedures.

1. Methods.

Crude enzyme extracts of Cuscuta (from 10gm. of fresh material) were prepared as described in Section VII.A.(a), with the difference that 0.4M sucrose-phosphate buffer was used as extracting medium (Smith, 1962). This assured a medium of sufficiently high tonicity to prevent rupture of the mitochondrial membrane. The crude extract (total volume 30ml.) was centrifuged in a M.S.E. "High Speed 17" centrifuge for 5 min. at 1000xg to remove coarse cell debris, and then at 20,000xg for 30 min. to sediment the mitochondria. The mitochondrial pellet was drained free of supernatant

and taken up in 10ml. sucrose buffer of pH 8.0 containing 0.005M ethylene diamine tetra acetic acid to prevent aggregation of the mitochondria. This extract was than divided into two 5-ml. portions, and to one of tham was added approximately 0.1% of a non-ionic detergent (Nonidet P40). The ability of non-ionic detergents to free particulate-bound enzymes in plant tissue has been demonstrated by Bone (1959), and by Smith (1962).

The transaminase activity of the treated and untreated mitochondrial extracts was then assayed by the method described on page 105, using 0.5ml. of mitochondrial suspension and 0.4M sucrose buffer. Besides the mitochondrial extracts, the final supernatant was dialysed against sucrose buffer for 18 hours and tested for transaminase activity along with the mitochondrial preparations.

2. Results.

In both C. reflexa and C. campestris, the addition of detergent promoted the ability of the mitochondrial extract to transaminate aspartic acid. The promoting effect was higher in C. reflexa (approximately doubling the activity) than in C. campestris (Figs. 69 and 70). However, the actual level of mitochondrial transamination in C. campestris was between two and three times higher than in C. reflexa. Examination of the transaminase activity of the supernatant fraction in both parasites showed that it

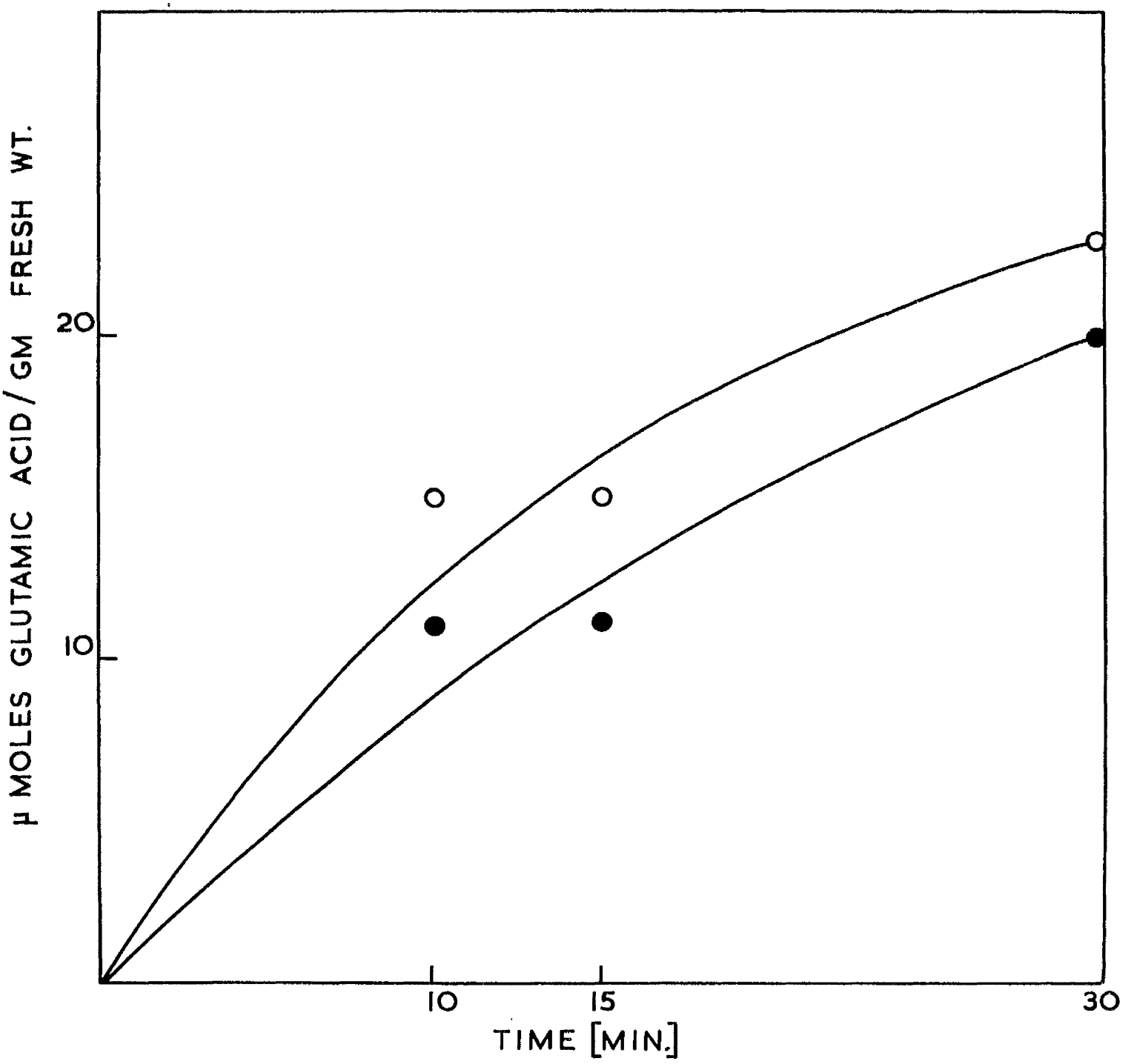

Fig. 69. Effect of addition of detergent on transaminase activity of mitochondrial fraction of C. campestris extract. Closed circle represents untreated mitochondria; open circle represents opened mitochondria.

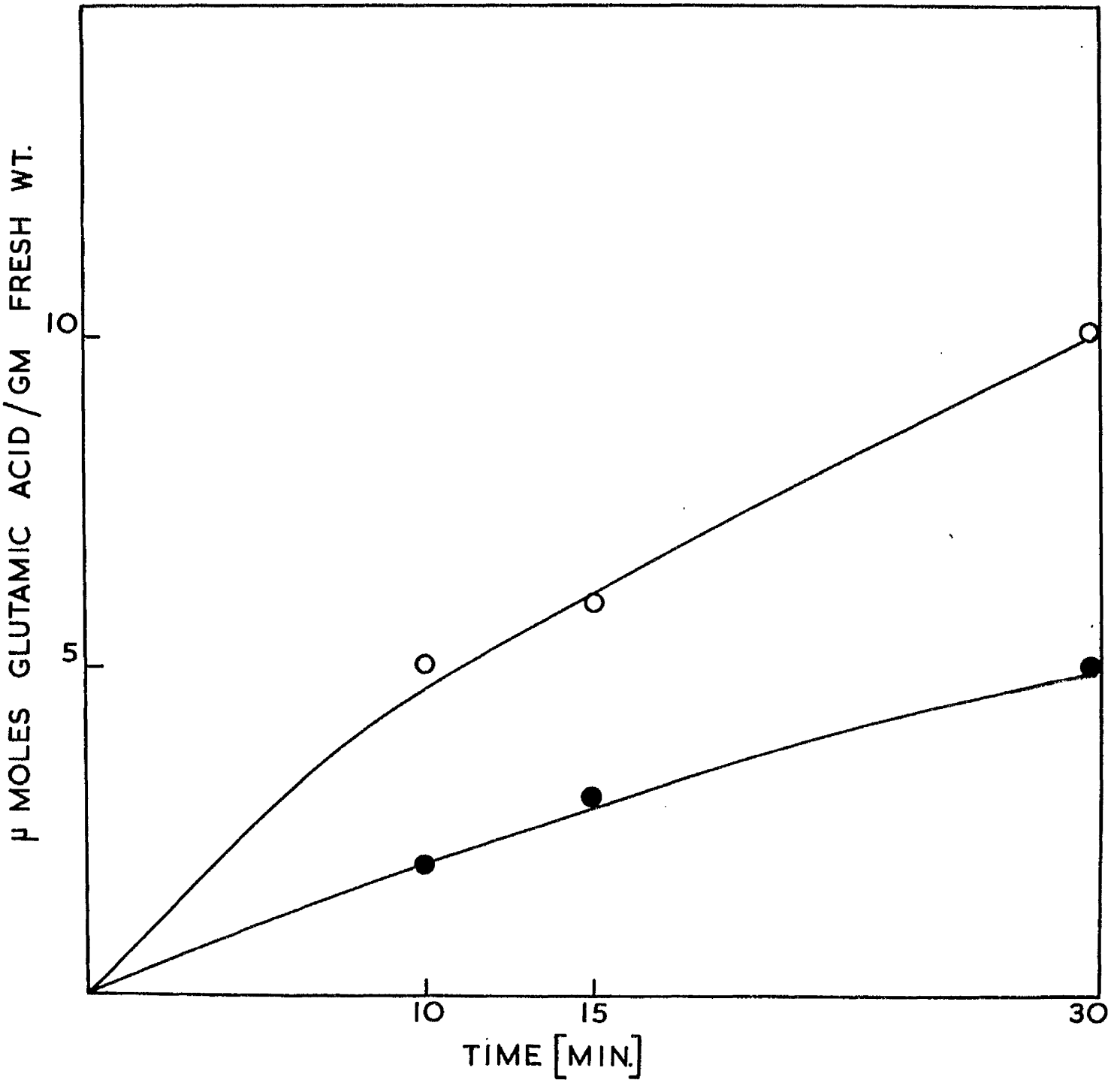

Fig. 70. Effect of addition of detergent on transaminase activity of mitochondrial fraction of C. reflexa extract. Closed circle represents untreated mitochondria; open circle represents opened mitochondria.

contained a considerable residual activity, corresponding to 66% of the total in C. campestris, and 90% of the total in C. reflexa.

From these results it is shown conclusively that at least part of the transaminase activity of the crude enzyme extract in both C. campestris and C. reflexa is localised in a particulate fraction of the cell, presumably the mitochondria. This result is similar to that found in lupins by Wilson et al. (1954), but as Smith (1962) suggests, the possibility is not excluded that the cytoplasmic activity derives from enzyme dislodged from the mitochondria during extraction.

B. Glutamic Dehydrogenase.

Glutamic dehydrogenase catalyses the reaction:- glutamate + pyridine nucleotide = α-ketoglutarate + NH_4^+ + reduced pyridine nucleotide + H^+. The first demonstration of this enzyme in plants (pea seedlings) was by Damodaran and Nair (1938). It has since been shown to be present in a wide variety of plant tissues, eg. in maize leaves (Bulen, 1956) and in Neurospora crassa (Fincham, 1951). Sanwal and Lata (1961) have demonstrated the presence of two different dehydrogenases in Neurospora, one DPN-specific and the other TPN-specific.

The fact that a transaminase enzyme exists in Cuscuta which utilises or produces glutamic acid, suggests the possibility that other enzymes may be present which also regulate the glutamic acid level in the parasite. The following investigation was undertaken to establish the presence or absence of glutamic dehydrogenase in Cuscuta, and to ascertain if it is DPN- or TPN-specific. Because of insufficient suitable material of Cuscuta campestris, only Cuscuta reflexa was analysed for enzyme activity.

a. Methods.

Approximately 5gm. of C. reflexa was extracted with phosphate buffer as described on page 102. This undialysed extract

was then centrifuged at 10,000g for 15 min. to remove coarse cell debris. The following method of analysis is a modification of the method of Sanwal and Lata (1961).

Into 1-cm. cuvettes were placed 300 µlitres of 0.1M α-keto glutaric acid, 50 µlitres of enzyme extract, 2.3ml. of 0.1M phosphate buffer (pH, 8.0), and 100 µlitres of DPNH or TPNH. To initiate the reaction, 200 µlitres of 1.5M ammonium sulphate were added, and the cuvette inverted to mix the contents of the cell. The cuvette was immediately placed in a SP 500 spectrophotometer, and absorption values measured at 340mµ against a mixture similar to the above, but lacking ammonium sulphate. Readings were taken at various intervals over a total period of 8 minutes.

Table 23. Activity of glutamic dehydrogenase in C. reflexa. Figures are absorption values at 340mµ.

Time.	Control (TPNH, no $(NH_4)_2SO_4$).	Test sample (TPNH).	Control (DPNH, no $(NH_4)_2SO_4$).	Test sample (DPNH).
1 min.	1.398	1.015	1.310	1.326
2 min.	1.397	1.005	1.296	1.316
4 min.	1.395	0.998	1.296	1.310
6 min.	1.395	0.994	1.290	1.310
8 min.	1.395	0.989	1.290	1.309

b. Results.

From Table 23 it can be seen that a progressive decrease of TPNH and DPNH takes place with time in the test mixtures, thus indicating the presence of glutamic dehydrogenase in the Cuscuta extracts. It can also be seen from the table that the enzymic activity with TPNH is significantly higher (relative to the controls) than with DPNH. From these results it is concluded that two dehydrogenase enzymes are present in Cuscuta reflexa, one TPN-specific, and the other DPN-specific. It would appear, therefore, that in Cuscuta reflexa the level of glutamic acid is controlled by at least two separate enzyme systems, transaminase and glutamic dehydrogenase. The presence of both TPN- and DPN-specific dehydrogenase in Cuscuta recalls the situation obtaining in Neurospora crassa (Sanwal and Lata, 1961).

VIII. DISCUSSION.

This thesis has dealt with an anatomical study of the haustorial connections of *C. campestris* and *C. reflexa*, and with an investigation of some aspects of the physiology of these two species. The anatomical studies have shown that the haustoria of *C. campestris*, which have not been described hitherto, conform substantially to the existing descriptions of the haustoria of other members of the genus. The writer tends to favour the view of Thomson (1925) regarding *C. reflexa*, that no definite connection is made between the phloem of host and parasite, which is contrary to the findings of Peirce (1893) and Thoday (1911). Despite an intensive examination of numerous sections of the haustorial regions, using callus-specific stains, no evidence of any sieve plates or areas was found, either in the cortex between the haustorial apex and the host vascular bundle, or in the haustorium proper. (Normal phloem is easily demonstrated in the parasite stem). Certainly, hyphae have been observed which were in intimate contact with host sieve tubes, but there was no evidence that these contacts were more frequent than would be expected by random penetration of parasitic hyphae through the peripheral tissues of the host. It must be remembered that the relatively small haustoria of *C. campestris* make detailed observation difficult, and the fact

that the writer was not able to demonstrate undoubted phloem in the haustorial regions cannot be taken as evidence of its non-existence.

The findings of Harris (1924) that the osmotic pressure of the sap of Cuscuta salina was lower than that of its host, and the work of Lilienstern (1932) who found that parasitism was successful only if the osmotic pressure of the parasite exceeded that of its hosts, strongly indicates that the same osmotic mechanism cannot be operating in these two cases, and suggests that some other mechanism must be involved in the nutrition of this group of plants. The writer's experiments with erythrosin dye showed conclusively that the xylem of host and parasite are continuous with each other. The rapid flow of xylem sap through the haustorial connections from host to parasite suggests the possibility, in conformity with the views expressed by Thomson (1925), that this may be the main channel of food supply to the parasite; comparatively large volumes of even an extremely dilute xylem sap might be expected to furnish adequate organic and inorganic nutrients for the parasite's requirements.

Detailed examination of the chromoplast composition of C. campestris and C. reflexa has revealed a complicated assortment of plastid types, associated with fluctuations in the pigment

complement of the parasite. Commenting on the pigmentation of Cuscuta, Peirce (1894) states that when occasion demands, eg. lack of food, Cuscuta can develop a very considerable quantity of chlorophyll, together with the appearance of the plastids which contain it, justifies the belief that it is a highly functional element in the plant, and is most interesting confirmation of the hypothesis that this genus of plants has become parasitic within comparatively recent times and after having attained a fairly complex development such as the other non-parasitic members of the Convolvulaceae possess. Although Peirce noticed that the orange colour of C. glomerata was due to small orange chromoplastids, he was unable to distinguish chlorophyll granules.

The close association of chromoplasts with the xylem elements in the pedicel of C. reflexa and in the stem of C. campestris poses the interesting question of the significance of the juxtaposition. Since a great deal of the plant's nourishment is derived from the host and channelled through the vascular system of the parasite, a satisfactory mechanism must exist for the transport of food materials from the conducting system to peripheral tissues of the stem. It is conceivable that this mechanism is unidirectional, being more efficient centrifugally, so that upgrade metabolites manufactured in the outer cortex could be adequately translocated.

Any substances produced as a result of photosynthesis in the pedicel of C. reflexa are almost certainly used in the nutrition of the developing flowers and ovules. This will undoubtedly be the case for the photosynthetic products of the highly chlorophyllous flowers themselves. An interesting speculation in this connection is that for full development, the flowers may require substances which can be supplied only by the photosynthetic apparatus of the parasite. A re-examination of other postulated chlorophyll-free angiospermous parasites might yield useful results.

Quantitative estimations of the chlorophyll content of C. campestris and C. reflexa show it to be approximately the same in the two species (somewhat higher in C. reflexa), and equal to about 5% of the level obtaining in green leaves, though the chlorophyll levels have been shown to vary with ecological conditions such as illumination and the nature of the host plant. The demonstration of the presence of chlorophylls "a" and "b" in the same general proportions as obtaining in Angiosperms indicates that the reduction in chlorophyll content which is postulated in the evolution of parasitism in Cuscuta involved the simultaneous and equal loss of both components.

This work has shown that the level of carotenoid is approximately the same in C. campestris and C. reflexa, and of the

same order as the carotenoid content of leaves. Since no microscopical evidence has been found that carotenoid exists on separate plastids from those bearing chlorophyll, it is probable that it occurs in close association with chlorophyll in the grana of the plastids, which are therefore homologous to the chloroplasts of green leaves, rather than to the chlorophyll-free chromoplasts of carotenoid-containing organs such as the carrot.

Though carotenoids may occur in plant organs which are devoid of chlorophyll, it is, according to Strain (1949) a rule without known exceptions that carotenoids accompany chlorophylls in the photosynthetic apparatus of all phototrophs from bacteria to the higher plants. In view of the low chlorophyll density in Cuscuta, it is likely that the high carotenoid content is much in excess of that necessary for photosynthesis, even assuming a low efficiency of energy transfer from carotenoid to chlorophyll. It may be that the carotenoid content of Cuscuta, corresponding closely as it does to that of autotrophs, represents the condition in an ancestral stage of the plant's evolutionary history. In that case, if the ratio of carotenoid to chlorophyll in the ancestral types was similar to that obtaining in present-day green plants, it would have had a chlorophyll content of the same order as modern autotrophs. This observation lends physiological support to the

view that Cuscuta represents a degeneration from a free-living to a parasitic habit.

The demonstration of photosynthesis in Cuscuta at a level approximately one tenth of that in Pelargonium leaves, together with the fact that the chlorophyll content of the parasite, may, under the appropriate physical and ecological conditions closely approach that of autotrophic plants, must be taken as evidence that the conception of Cuscuta as an obligate parasite is not applicable, at any rate to the two species investigated in this work; instead they should be regarded as highly facultative as far as carbohydrate nutrition is concerned, and as such, their physiological affinities are more towards the semi-parasites of the Loranthaceae.

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