Heterotrophic growth of blue-green algae.

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HETEROTROPHIC GROWTH OF BLUE-GREEN ALGAE

By

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ABSTRACT

Twenty four strains of blue-green algae were screened for their ability to grow heterotrophically in complete darkness with sucrose. Eighteen of these strains proved capable of growth in the dark and all of these latter continued to grow on repeated subculture. Only six strains failed to grow in the dark and of these six, one was still viable after three months incubation in the dark. Among the organic substrates tested, sucrose (0.01M) was found to be the best substrate in allowing a considerable growth of the majority (ten out of eighteen) of the cultures in the dark.

Chlorogloea fritschii and four other strains were selected for obtaining their growth rates under different environmental conditions. C. fritschii was further used in a comparison of the dark growth rates of three heterotrophic cultures: material first subcultured from light to dark, material subcultured from dark to dark and material after three years of subculturing in the dark. The growth rates ($\hat{k}$) of all three heterotrophic cultures were found to be the same, thus suggesting that no physiological adaptation had taken place as a response to prolonged heterotrophic conditions.

The addition of sucrose to cultures of C. fritschii and four selected strains in the light (500 lux and up to 4000 lux with C. fritschii) resulted in an increase of the

growth rate (k̀).

Growth of five strains incapable of growth in dark was significantly stimulated by sucrose at 500 and 1000 lux. Only Anabaena variabilis did not respond significantly to sucrose.

Cultures grown in the dark were pigmented. Pigment analysis showed that the levels of phycocyanin and chlorophyll in dark-grown cells of selected strains are not appreciably lower than those in cells grown photoautotrophically at 500 lux.

Unlike the cultures of C. fritschii which consisted of aseriate colonies only in the dark, all other strains consisted of filaments.

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1. INTRODUCTION

Blue-green algae are widely distributed in nature. They frequently develop in lakes which are relatively highly polluted with organic matter, and may occur also in other habitats rich in organic matter such as estuarine muds. At least some strains seem to grow or remain viable under very low light intensities which would seem unfavourable for photoautotrophic growth. Besides free-living forms there are many blue-green algae that occur in very close association with other plants sometimes even growing inside these latter (HARDER,1917; WITTMAN et al., 1965). Therefore, the possible ability of blue-green algae to grow heterotrophically has long been of considerable interest to many investigators e.g. ALLEN (1952)

A number of investigators (ALLEN, 1952; KRATZ and MYERS, 1955a; VAN BAALEN, 1962; BAKER and BOLD, 1970; VAN BAALEN et al., 1971) and some reviewers (FOGG, 1947; HOLM-HANSEN, 1968; FOGG, 1969; WIESSNER, 1970) have concluded that most blue-green algae are obligate phototrophs and are unable to grow in the dark. This generalisation is, however, based on the early attempts to culture them in the dark and on observations in the literature. The phenomenon of obligate phototrophy seem well established for at least three pure cultures of blue-green algae, namely: Anacystis nidulans, Anabaena variabilis and Nostoc muscorum G. These organisms did not grow in the dark on any of the organic

substrates used by KRATZ and MYERS (1955a). Although the use of radioactive tracer technique has established that Anabaena variabilis, Anacystis nidulans, Coccochloris peniocystis, Gloeocapsa alpicola and Nostoc muscorum G can assimilate organic compounds in the light (HOARE et al., 1967; PEARCE and CARR, 1967; SMITH et al., 1967) these organic compounds have been found to make only a very small contribution to newly synthesised cellular carbon in Anacystis nidulans, Coccochloris peniocystis and Gloeocapsa alpicola (SMITH et al., 1967). The inability of the blue-green algae studied to assimilate organic compounds has been interpreted as due to failure of the organic substrates to penetrate the plasma membrane of the cell (KRATZ and MYERS, 1955b). HOARE et al. (1967) and SMITH et al. (1967) on the other hand have concluded that this was caused by specific biochemical deficiencies. Accordingly, HOLM-HANSEN (1968) has speculated that the absence of NADH oxidase in blue-green algae (SMITH et al., 1967) might account for the slow assimilation of organic compounds and failure to couple ATP synthesis with their oxidation. After reviewing the existing literature on the intermediary metabolism of blue-green algae SMITH (1973) concluded that impermeability and general toxicity are no longer adequate explanation for the inability of many blue-green algae to grow in the dark on organic media and that alternative theories must be sought. Since it was considered impossible at present, to determine with certainty the biochemical basis of the obligately phototrophic physiology

displayed by many species of blue-green algae, a comparison of the physiology and biochemistry of the obligately phototrophic and of heterotrophic species was suggested by SMITH (1973) as a useful line of attack on this problem.

Earlier investigators, working with impure cultures of blue-green algae did conclude that the addition to the medium of carbohydrates or other organic substances, stimulated growth, particularly in weak light or in complete darkness. BOUILHAC (1898) found that a unialgal culture of Nostoc punctiforme, contaminated with Azotobacter, could grow as well in weak light as in complete darkness, when supplied with sugar as a carbon source, and in the absence of combined nitrogen. HARDER (1917), however, conclusively proved the ability of axenic cultures of blue-green algae to grow heterotrophically in complete darkness at the expense of organic substances. His bacteria-free culture of N. punctiforme isolated from Gunnera, was capable of heterotrophic growth. The algal symbiont could grow autotrophically in light or in complete darkness if carbon compounds, particularly carbohydrates, were added to the medium. Among the organic substrates tested by HARDER in the dark, sucrose at 0.5% (0.014M) was found to be the best organic substrate to support growth in the dark, while dextrin, glucose, maltose and galactose came second. Two other strains of Nostoc, in pure cultures, one obtained from Cycas, and the other from Gunnera, showed

heterotrophic growth in complete darkness when fructose, glucose or mannose were added to the culture medium. Neither sucrose nor lactose supported growth under any condition (WINTER, 1935). ALLEN (1952), attempted to test the ability of 30 cultures of blue-green algae to grow in the dark on a glucose-yeast autolysate medium. Only six isolates; two strains of Oscillatoria sp., Lyngbya sp., Phormidium foveolarum, Plectonema notatum, and Nostoc muscorum A (ALLISON'S strain) showed very slow growth. In one culture (unidentified unicellular acid alga) the growth rate was found to be comparable to its growth rate in the light. ALLEN (1952) observed that the acid alga was morphologically a member of the Chlorophyceae although it had the pigments characteristic of a blue-green alga. The demonstration of the presence of a phycobilin is not of course satisfactory for recognising a blue-green alga, since not all phycobilin-containing organisms are blue-green algae (Rhodophyceae, Cryptophyceae and Chlorophyceae all have phycobilin-containing members) (O'HEOCHA, 1962). Cyanidium caldarium classified as a blue-green alga for many years, is now widely recognised as a member of the Rhodophyceae, although it has the pigments characteristic of a blue-green alga. Hence, the acid alga tested by ALLEN (1952) could well have been an eukaryotic organism. Although, the experimental evidence of ALLEN did not show a continuous growth in the dark, she was able to show that some growth did occur in the dark under the conditions used by her. Unfortunately, the limited range of factors which

she tested did not include those already shown by HARDER (1917) to permit the best heterotrophic growth of N. punctiforme. ALLEN used neither liquid culture medium, nor any carbohydrate other than glucose. ALLISON et al. (1937) proved that N. muscorum A, which was isolated from soil, could grow for months under strict heterotrophic conditions. Growth took place in complete darkness in liquid medium containing glucose or sucrose as carbon source.

Studies on nitrogen fixation by blue-green algae growing heterotrophically have been carried out successfully for a few species. ALLISON et al. (1937) found that growth and nitrogen fixation of Nostoc muscorum A continued slowly in the dark when either glucose or sucrose was present in the medium. In a similar study on Chlorogloea fritschii, FAY (1965) found sucrose, at 0.01M, to be the best organic substrate to support growth and nitrogen fixation in the dark, while maltose came second. No growth however, was observed on the other substrates tested, fructose, arabinose, glutamate or aspartate. Further, he found that carbon dioxide, while inhibitory to sucrose assimilation, was only slightly so to nitrogen fixation in the dark. WATANABE and YAMAMOTO (1967) have reported that Anabaenopsis circularis, can fix molecular nitrogen in the dark in the absence of combined nitrogen. Greatest growth and nitrogen fixation in the dark was found on glucose at a concentration of 0.5% (0.027M). Some growth also did occur on such substrates as fructose, sucrose and maltose, but

not on xylose, arabinose, glucose-1-phosphate or ethanol. In 1969, KOPTYEVA and TANTSYURENKO found that Calothrix braunii, Nostoc sp., N. punctiforme, and N. punctiforme f.n. can grow and fix atmospheric nitrogen in complete darkness in the presence of organic substances such as sucrose, glucose, and maltose in the medium. In contrast, investigations by KIYOHARA et al. (1960) on the heterotrophic growth of Tolypothrix tenuis showed no obvious increase in cell mass of this alga unless potassium nitrate was replaced by ammonium sulphate as a nitrogen source, in the presence of glucose (5.5×10^{-2}M). KIYOHARA et al. also suggested that ammonia can be used as a nitrogen source in a slightly acidic medium. HOARE et al. (1971) tested a range of organic compounds for the ability to support dark heterotrophic growth of the Nostoc-like isolate from the coralloid roots of Macrozamia lucida. The only compounds which supported heterotrophic growth in the dark were glucose, fructose and sucrose, while galactose, mannose, ribose, xylose, lactose, mannitol, glycerol, gluconic acid, acetate, pyruvate and citrate did not support growth in the dark. These authors obtained consistent growth of the isolate in the dark with glucose (0.05M) for five transfers. Consistent growth was also obtained on plates of glucose agar in anaerobic jars. Recently KENYON et al. (1972) screened 29 filamentous strains, both heterocystous and non-heterocystous, for their ability to grow heterotrophically in the dark on plates of

glucose (0.056M) agar, and their ability to grow in the light at the expense of glucose, in the presence of 10^{-5}M 3-(3,4-dichlorophenyl)-1, 1-dimethylurea (DCMU) (a concentration which completely inhibits growth in a mineral medium in the light). A total of 18 strains proved to be facultative photoheterotrophs,and 15 of them could also grow chemoheterotrophically, though more slowly, with glucose, in the dark.

In the course of their study on the photoheterotrophy and chemoheterotrophy of unicellular blue-green algae, STANIER et al. (1971) suggested that obligate photoautotrophy is the rule in this subgroup. None of the 40 axenic strains examined grew on agar plates of mineral medium supplemented with glucose, acetate, succinate, pyruvate or glutamate after incubation in the dark for one month, whereas good growth did occur on a parallel set of control plates incubated in the light. More recently, RIPPKA (1972) examined the same strains for their ability to grow photoheterotrophically in the light with glucose in the presence of 10^{-5}M DCMU, which inhibits photoautotrophic growth, and their ability to grow chemoheterotrophically in the dark with glucose (0.056M). Seven strains proved to be facultative photoheterotrophs. Six are members of the genus Aphanocapsa and one of the genus Chlorogloea. Three strains of Aphanocapsa and the only strain of the genus Chlorogloea could also grow chemoheterotrophically with glucose in the dark. RIPPKA (1972) found that consistent dark growth with glucose can

be obtained only if a liquid medium is used. The failure of STANIER et al. (1971) to detect the chemoheterotrophic growth of the four positive strains examined by RIPPKA (1972) is probably attributable to the fact that these workers used only solid media in screening for dark growth. In all cases the rates of dark heterotrophic growth were relatively slow. FAY (1965) found that the duration of the lag period of Chlorogloea fritschii grown under nitrogen fixing conditions with sucrose, was reduced to one week after subcultivation for one year (six subcultures) in the dark. Tolypothrix tenuis was found to grow heterotrophically in the dark at somewhat greater rates (KIYOHARA et al., 1960) The dark growth rate with glucose ($5.5X10^{-2}M$) and casamino acids ($3\ X10^{-2}M$) was three times less than the rate in mineral medium in the light. HOARE et al. (1971) observed that casamino acids stimulated dark heterotrophic growth of Nostoc-like isolate from the coralloid roots of the cycad Macrozamia lucida. The generation time with glucose (0.05M) alone was 48 hours, but this was reduced to 26 hours when casamino acids (0.1%) were also included. RIPPKA (1972) obtained the growth rates of one strain of the genus Aphanocapsa under a variety of environmental conditions. Glucose (0.056M) was found to increase the rate of photosynthetic growth, even at a light intensity (800-1000 lux) that supports a good rate of growth in a mineral medium. The dark growth rate with glucose was over three times less than the rate in mineral medium in the light; and about five times less than the rate with

glucose in the light.

During the course of their study of the chemoheterotrophy of marine planktonic algae CHENG and ANTIA (1970) observed that the inoculated control culture-flasks of several species, with no added organic compound (apart from EDTA, and traces of vitamins B_{12}, thiamin, biotin) showed resumption of normal growth on transfer to the light after 4 to 8 weeks incubation in darkness without growth. This observation prompted ANTIA and CHENG (1970) to undertake a more rigorous examination of the capacity of 31 species of marine unicellular algae to tolerate prolonged darkness in organic-free medium (apart from EDTA, and traces of vitamins B_{12}, thiamin, biotin). None of the species tested showed any growth in the dark, but six species, two of them blue-green algae (Agmenellum quadruplicatum, Anacystis marina) resumed normal growth in light after 24 weeks incubation in darkness. ANTIA and CHENG (1970) concluded that survival in the dark may have ecological relevance to the finding of planktonic algae in aphotic ocean depths. No attempt, however, was made to test the ability of various organic compounds to support growth in the dark. Another approach to the consideration of heterotrophic growth of blue-green algae was made by VAN BAALEN et al. (1971). They reported that while in dim light (100 lux) and in the presence of glucose as a carbon source, a unicellular species Agmenellum quadruplicatum and a filamentous species,

Lyngbya lagerheimii grew heterotrophically but not autotrophically. In complete darkness, no growth took place in the former, and only a marginal growth occurred in the case of the latter. The occurrence of marginal growth on transfer of some species to heterotrophic conditions does not however, constitute proof of an heterotrophic ability. This is emphasised by the observations of VAN BAALEN (1962) and of BAKER and BOLD (1970); in these studies of the heterotrophic potentialities of blue-green algae, the transfer of some strains to organic media resulted in marginal growth which did not occur on repeated subculture. A recent report (PAN, 1972) points out that an obligate phototroph Plectonema boryanum can grow on glucose (0.022M) in the dark, only if fresh sterile medium was continually replenished. Dark-grown cells were claimed to be devoid of chlorophyll; they proceeded to grow normally and synthesised chlorophyll on transfer to their normal mineral medium in the light. PAN (1972) suggested that, the reason for the lack of growth on glucose under stationary conditions may be due to the accumulation of toxic materials produced from the metabolism of glucose. No demonstration of an increase in cell mass was given. However, while the experimental results of PAN (1972) may be of interest in their own right, they do not constitute proof of an heterotrophic ability.

As mentioned earlier, the natural environment of many blue-green algae contains a relatively high concentration of dissolved organic matter. The Great Lakes

contain 22 to 99 mg/l of dissolved organic matter (ROBERTSON and POWERS, 1967); and glucose, acetate and glycollate have been detected in polluted waters at 0.02-0.05 mg/l (HOBBIE and WRIGHT, 1965). The study of the competition between planktonic bacteria and algae, hypothesised that the only significant uptake of dissolved organic matter present in natural waters is carried out by heterotrophic bacteria and that competition by algae for these compounds is insignificant (HOBBIE and WRIGHT, 1965), but recently MONHEIMER (1972) concluded that phytoplankton may conduct up to 50 and 25% of the dark and of the daily heterotrophic activity that occurs in the limnetic zone of a lake. It is not yet known to what extent blue-green algae utilise organic compounds present in their environment (FOGG, 1969), but recently VAN BAALEN et al. (1971) suggested that exogenous organic compounds may be of great importance to these organisms especially in situations where light intensity is low.

KANTZ and BOLD (1969) and BAKER and BOLD (1970) investigated semiquantitatively the effect of organic compounds on the growth of various blue-green algae in the light (1500-3000 lux, 12-12 hours light-dark cycle) and in the dark on agar plates of mineral medium supplemented with the equivalent amount of carbon of a 1% glucose solution. The compounds tested were glucose, fructose, D-and L- arabinose, ribose, xylose, pyruvate (sodium salt), and acetate (sodium salt). Growth of all of the isolates tested was enhanced by at least one of the organic compounds.

Ribose increased growth of most of the isolates, and glucose, fructose, D-and L-arabinose, and xylose came second. Pyruvate and acetate both supported growth of Microcoleus vaginatus var. fuscus and Schizothrix calcicola var. minuta. The latter grew quite well aerobically in acetate and anaerobically in pyruvate, where the former grew only slightly. Pyruvate supported slight growth of one member of the genus Schizothrix under both aerobic and anaerobic conditions and acetate supported slight growth of another member of the genus Schizothrix under aerobic conditions only. Carbohydrates (ribose, glucose, and fructose) were the only organic compounds to support growth in the dark. Only seven isolates could grow heterotrophically in the dark. Five are members of the genus Schizothrix and two, of the genus Oscillatoria. Available data on the response of several species of blue-green algae to various organic compounds reveal that, at high light intensities which were probably near to saturation, organic compounds did not increase the growth rate of these organisms (KRATZ and MYERS, 1955b; PEARCE and CARR, 1967; SMITH et al., 1967). The compounds tested were carbohydrates, mono- and di-carboxylic acids and amino acids. Carbohydrates were found to be the best organic compounds to stimulate the growth of blue-green algae but such effects have only been observed at low light intensities. The growth of Nostoc muscorum A (LAZAROFF and VISHNIAC, 1961) was stimulated by glucose at low light intensities (100-800 lux). Similar observations have been

reported by KIYOHARA (unpublished work by KIYOHARA cited by KIYOHARA *et al.*, 1962) with *Tolypothrix tenuis* and by HOARE *et al.* (1971) with a *Nostoc*-like organism isolated from *Macrozamia lucida*. Growth of these two organisms was stimulated by glucose at low light intensities which were barely sufficient to support autotrophic growth. Other organic compounds such as acetate, pyruvate and succinate were without effect under identical conditions as was glucose at high, near saturating light intensities. Another approach to the consideration of the effect of organic compounds on the growth of blue-green algae in the light was made recently by INGRAM *et al.* (1973a). They examined the growth of the same endophytic *Nostoc* used by HOARE *et al.* (1971), under a variety of culture conditions. At high light (3000 lux) and abundant CO_2 (1% CO_2 - enriched air), the addition of fructose, galactose, glucose, glycerol and acetate did not stimulate growth. However, at low light intensity (800 lux) and/or reduced CO_2 availability (air), the addition of reduced organic substrates produced a significant stimulation of growth. This stimulated rate of growth never exceeded that obtained under optimal autotrophic conditions (3000 lux and 1% CO_2). Neither acetate nor glycerol supported growth in the dark. In the light, in the absence of exogenously supplied CO_2 (N_2 - gassing), glycerol and acetate in addition to fructose, galactose and glucose were found to serve as sole sources of carbon. During photoheterotrophic growth, the pigmentation was identical to that of

autotrophic cultures. Autotrophic and photoheterotrophic cultures appear identical by light and electron microscopy. These authors suggested that respired CO_2 from substrate oxidation is assimilated by the photosynthetic machinery. Using the same approach INGRAM et al. (1973b) examined the photoheterotrophic growth of the blue-green alga Agmenellum quadruplicatum in the absence of CO_2 (N_2 - gassing) with glycerol as the sole source of carbon. The generation time on glycerol was 12 h, and on CO_2, 3 h. They found that glycerol carbon was converted into cellular carbon with a very high efficiency; most of the glycerol carbon was converted into new cellular material. Morphologically and ultrastructurally, photoheterotrophic cells were very similar to autotrophic cells. During photoheterotrophic growth, the pigmentation was very similar to that of autotrophic cultures.

Organic compounds may also have an indirect effect on the growth of blue-green algae. LANGE (1967, 1970) reported that, when atmospheric carbon dioxide was the growth limiting factor, the addition of sucrose to cultures of planktonic blue-green algae associated with bacteria, stimulated the algal growth. This stimulation appears to be due to the assimilation of the carbon source by bacteria and release of carbon dioxide. LANGE (1971) extended his studies of the effect on the algal growth of the symbiotic association of blue-green algae with bacteria, to include a wide range of aliphatic carbonaceous compounds and a representative selection of bacteria-associated cyanophyta

species. He found that the effect of specific carbonaceous compounds varied with algal species, and that the effects were only specific for the bacteria associated with the alga. An increased concentration of carbon dioxide in air was found to produce the same effect as that of the addition of organic compound. However, an increase of carbon dioxide concentration in the air was found to stop the growth-enhancing effect of added organic compound. More recently, PAN and UMBREIT (1972) reported that growth of a blue-green alga Plectonema boryanum was enhanced by the presence of heterotrophic bacteria; Pseudomonas fluorescens and a yeast; Saccharomyces cerevisiae, either directly mixed with the culture, or in parabiotic tubes separated by a membrane. They pointed out that the stimulating effect of such mixed cultures seemed to be highly specific.

The morphological differences between light-grown and dark-grown cultures of the ALLISON'S strain of Nostoc muscorum A were first noted by LAZAROFF and VISHNIAC (1961). They described the developmental cycle of this alga grown in culture using glucose as energy and carbon source. In complete darkness the alga was found to develop into sheathed undifferentiated cells (the aseriate stage), but a small amount of light or the addition of an aqueous extract of light-grown cells, allowed the completion of the developmental cycle. The inhibition of development in the dark was interpreted as being due to a lack of morphogenetic substances which are formed only in the light. In a similar study ROBINSON and MILLER (1970), have found

that white light, while regulating the development of motile trichomes in both Nostoc commune 584 and N. muscorum A at the aseriate stage, blocked this step in the former and promoted it in the case of the latter.

From a review of the work that had been carried out prior to the commencement of this thesis (January 1969), on the heterotrophic growth of blue-green algae, it seemed highly desirable to carry out further investigations. A diverse collection of blue-green algae was examined to determine their heterotrophic potentialities. The present studies report the reproducible growth of the majority of these cultures under strict heterotrophic conditions in the dark. Among the subsidiary aims of the present study was one to investigate the response (if any) of strains which would not grow heterotrophically in the dark, to sucrose at different light intensities.

2. MATERIALS AND METHODS

2.1 List of abbreviations in text

g	=	gramme
µg	=	microgramme
mg	=	milligramme
h	=	hour
min	=	minute
l	=	litre
ml	=	millilitre
log	=	logarithms
M	=	molar
mm	=	millimetre
µm	=	millimicron
nm	=	nanometre
OD	=	optical density
wt	=	weight
v	=	volume
EDTA	=	ethylenediaminetetra-acetic acid

2.2 Algal materials

The greater part of the present study is concerned with experiments on axenic cultures. Only four cultures were not axenic; they were unialgal and they were involved only in two experiments (Section 3.11 and 3.2). The Durham culture numbers are employed in the text to identify each strain. For instance, Plectonema boryanum, obtained from Indiana culture collection is referred to as Plectonema boryanum D181.

2.21 Axenic cultures

The cultures were obtained from various sources. In addition to those obtained from Cambridge and Indiana culture collections, one other was isolated from Saudi Arabia and obtained in an axenic state (Sections 2.23-2.25). Details of the cultures and their origins are given in Table I.

2.22 Unialgal cultures

Again the cultures were obtained from various sources and included one isolate made by the author from a sample from Aldabra Island (Section 2.23). Details of the cultures and their origins are given in Table II.

2.23 Isolation of the organisms

Anabaena sp. was isolated from the soil surface of a rice field in Al-Hassa, Saudi Arabia. The location of

Table I: Details of the axenic cultures and their origin

strain		culture collection	culture collection no.	Durham culture no.	source	isolator
Anabaena	cylindrica	Cambridge	1403 - 2a	166	pond, Surrey, England	Chu 1939
A.	inaequalis	"	1446 - 1a	164		Utrecht
A.	variabilis	"	1403 - 4b	165		Utrecht
Anabaena	sp.			145	paddy soil, Saudi Arabia	the author
Anabaenopsis	circularis	Cambridge	1402 - 1	160	Japan	Watanabe
Anacystis	nidulans	"	1405 - 1	33		
Calothrix	brevissima	"	1410 - 7	156	paddy soil, Palau Island, Japan	Watanabe
C.	membranacea	"	1410 - 1	179		Mitra
Chlorogloea	fritschii	Westfield College	50	50	soil, India	Mitra 1950
Lyngbya	sp.	Cambridge	1459 - 2	157		Manten, Utrecht
Nostoc	ellipsosporum	"	1453 - 16	173		Forest
N.	muscorum	"	1453 - 9	158	Scotland	Gibson
Nostoc	sp.	"	1403 - 5	159	soil, Scotland	Wassink, Utrecht
Oscillatoria	tenuis	"	1459 - 4	161	" "	Manten, Utrecht
Phormidium	luridum	Indiana	426	175		Boresch
Phormidium	sp.	"	485	176		Allen
Plectonema	boryanum	"	581	177		Dyer
P.	boryanum	"	594	181		Dyer
P.	calothricoides	"	598	178		Allen
Tolypothrix	tenuis	Cambridge	1482 - 3b	171	Japan	Watanabe

Table II: Details of the unialgal cultures and their origin

strain		culture collection	culture collection no.	Durham culture no.	source	isolator
Nostoc	commune			168	soil, Aldabra Island	the author
N.	punctiforme	Indiana	B 384	174	soil, Holland	Wassink, Utrecht
Nostoc	sp.	Cambridge	1453-4	167	soil, Holland	Wassink, Utrecht
Scytonema	sp.	"	B 1473-3	162	soil, Japan	George 1968

Al-Hassa is at a latitude of 25^{o},25̀, and at a longitude of 49^{o},40̀. The alga was collected on 5 August 1969, allowed to dry, and transported to Durham in a Polythene collecting bottle.

Nostoc commune was collected by Dr B.A. Whitton in January 1969 from soil in Aldabra Island, Indian Ocean. The location of Aldabra Island is at a latitude of 9^{o},10̀, and at a longitude of 46^{o},20̀.

2.24 Description of the organisms

Anabaena sp., filamentous, trichome straight, or entangled, vegetative cell barrel shaped or cylindric (3-4 µm wide and 4-5 µm long). Heterocyst ellipsoidal or spherical (4-5 µm wide and 6-8 µm long).

Nostoc commune, filamentous, trichome straight or entangled; vegetative cell, barrel shaped or spherical (3-4 µm wide and 4-5 µm long); heterocyst spherical and as wide as is long (5.5-6.5 µm).

2.25 Isolation of the organisms in axenic cultures

Isolation in axenic culture is essential to the study of heterotrophic growth. Different methods have been tried to obtain axenic cultures in the course of the present study, including repeated subculturing on agar plates, repeatedly washing cells in sterile medium (PRINGSHEIM, 1946), and treatment of the algae with dilute chlorine water (FOGG, 1942). The use of antibiotics seemed to offer a possible method for

freeing blue-green algae of bacterial contaminants. Therefore various antibiotics were employed, and a bacteria free culture of Anabaena sp. was eventually obtained by treatment of the alga with neomycin. Millipore filters (pore diameter 0.45 µm) were used for sterilising neomycin. Experiments were carried out in boiling tubes containing 5 ml of mineral medium plus a range of concentrations of neomycin (5-20 µg/ml). A small amount of inoculum was transferred into each tube and incubated in the dark at 35°C for 24 h. After the incubation period in the dark, 0.5 ml was transferred from each tube to neomycin-free medium in the light. After 10 days of incubation, the algal growth was obviously inhibited (the cells became colourless) in the high concentrations (14-20 µg/ml). Growth occurred in concentrations of 5-12 µg/ml (somewhat inhibited in 12 µg/ml concentration). 1 ml quantities of the cultures were tested for bacterial growth by incubation into bacterial tests (Section 2.26). The algal cells grown in the presence of 12 µg/ml neomycin showed no bacterial growth after incubation into the bacterial testing media. The alga has been maintained for several months by serial subculture in sterile medium. Regular sterility tests and direct microscopical examinations showed it to be still bacteria-free.

A similar series of experiments were carried out in attempts to obtain the Nostoc commune in a bacteria-free culture. Polymyxin B, streptomycin and neomycin were

effective at higher concentrations (16-20 µg/ml) to inhibit both the bacterial flora and the algal growth. At lower concentrations (5-14 µg/ml) the alga remained contaminated with bacteria. All attempts including irradiation with ultra-violet light at a distance of about 300 mm, and repeated subculturing on agar plates were without success.

2.26 Tests of purity of the cultures

Tests have been made with the following media:

(1) Beef peptone agar

(2) Malt extract agar

(3) Yeast extract agar

The compositions of the three media are shown in Table III. The media were sterilised by autoclaving for 15 min at 15 lb per sq inch. Incubation was in the dark at 35°C for 48 h. Direct microscopical examinations of the cultures were also made, and in fact absence of visible bacteria in old algal cultures is perhaps the single most reliable method of confirming that a culture is axenic.

2.3 Chemicals

Except for the items listed below, all chemicals were obtained from the British Drug Houses Ltd., London and were of Analar grade. The exceptions were:

Table III: The composition of beef peptone, malt extract, and yeast extract agar media.

Chemical	Beef peptone agar	Malt extract agar	Yeast extract agar
Bovril	3	-	-
Peptone	10	-	-
Malt-Difco	-	20	-
Yeast extract	-	-	30
Agar	18	18	18

concentrations are expressed in g/l

maltose	British Drug Houses Ltd., London
D(-) fructose, glucose-free	
D(+) galactose, glucose-free	
lysozyme (Muramidase) from egg white grade	Sigma Chemical Co. Ltd., U.S.A.
neomycin sulfate	
red papers	Griffin and George Ltd., London.

2.4 Culture methods

2.41 Cleaning of glass ware

Stock cultures were maintained and experiments were carried out in 100 ml conical Pyrex flasks. These were soaked overnight in a hot 2% (w/v) solution of 'Quadralene' laboratory detergent. After soaking they were scrubbed, and rinsed thoroughly in hot water. The flasks were finally rinsed three times in glass distilled water and dried in an oven at 100^{o}C.

Pipettes were cleaned by soaking overnight in a mixture of one volume of saturated sodium nitrate and six volumes of concentrated sulphuric acid. They were rinsed thoroughly in tap water and were given a final rinse in glass distilled water.

2.42 Apparatus for growth of cultures

Growth was carried out in flasks incubated in tanks illuminated from below by a bank of fluorescent tubes (warm white). Light

intensities were adjusted by reducing the number of tubes and by wrapping layers of black fine-meshed cloth round the flasks. The apparatus was designed to make easy and rapid modifications of the incubation temperature and shaking rate.

An orbital incubator was modified to provide complete darkness conditions and was used for re-checking the growth of the cultures in the dark.

2.43 Media

The media were prepared with glass distilled water and chemicals of Analar grade. Two basic inorganic media were used, one containing a source of combined nitrogen, which is a modification of the 'C' medium of KRATZ and MYERS (1955a), and here termed 'AC' medium, and the other lacking combined nitrogen which is a modification of the medium of FOGG (1949), and here termed 'AD' medium. The compositions of the two media are shown in Table IV.

2.44 Sterilisation

Flasks were stoppered with non-absorbent cotton wool plugs (Best quality C 249, Robinson & Sons, Ltd., Chesterfield). Basal media, and media containing sucrose or sodium acetate, were sterilised by autoclaving at 15 lb per sq inch for 15 min, whilst those containing fructose galactose, glucose or maltose were sterilised by autoclaving at 10 lb per sq inch for 10 min. Pipettes were sterilised in a similar manner to the basal media.

Table IV: The composition of AC and AD media with their original description

Salt used	'C' medium of KRATZ and MYERS (1955a)	'AC' medium	medium of FOGG (1949)	microelements of ALLEN and ARNON (1955)	'AD' medium
*K_2HPO_4	1000	1000	200	-	250
$MgSO_4 7H_2O$	250	250	200	-	200
$CaCl_2$	-	-	100	-	-
$CaCl_2 2H_2O$	-	19.86	-	-	66.2
$FeCl_3$	-	-	0.4	-	-
$FeCl_3 6H_2O$	-	9.7	-	-	19.4
$Fe_2(SO_4)_3 6H_2O$	4.0	-	-	-	-
$Na_2EDTA\ 2H_2O$	-	12.7	-	-	25.4
NaCl	-	23.0	-	-	230
KNO_3	1000	1000	-	-	-
$Ca(NO_3)_2\ 4H_2O$	25.0	-	-	-	-
Na citrate $2H_2O$	165	-	-	-	-
H_3BO_3	2.86	2.86	0.1	0.5	0.5
$MnCl_2$	-	-	0.1	-	-
$MnCl_2 4H_2O$	1.81	1.81	-	-	-
$MnSO_4\ 4H_2O$	-	-	-	0.5	0.5
$ZnSO_4$	-	-	0.01	-	-
$ZnSO_4\ 7H_2O$	0.222	0.222	-	0.5	0.5
MoO_3 (85%)	0.0177	-	-	0.1	-
$Na_2MoO_4 2H_2O$	-	27.0	0.1	-	0.19
$CuSO_4$	-	-	0.01	-	-
$CuSO_4 5H_2O$	0.079	0.079	-	0.02	0.02
NH_4VO_3	-	-	-	0.01	0.01
$Na_2Vo_4 2H_2O$	-	-	-	0.01	0.01
$CoCl_2 6H_2O$	-	-	-	-	0.01
$NiSO_4 7H_2O$	-	-	-	0.01	0.01
$Cr_2(SO_4)_3 K_2SO_4 24H_2O$	-	-	-	0.01	0.01
$Co\ SO_4\ 7H_2O$	-	0.04	-	-	-
$Co(NO_3)_2\ 6H_2O$	-	-	-	0.01	-
$TiO(C_2O4)_x\ YH_2O$	-	-	-	0.01	-

concentrations are expressed in mg/l

* sterilised separately, cooled and added to the sterile medium.

Inoculation was carried out in a room partially sterilised by ultra-violet irradiation, with other precautions being taken to minimise contamination. Before inoculation the bench was cleaned with absolute ethanol and the room sprayed with absolute ethanol to remove suspended material from the atmosphere.

2.45 Inoculum material

In general, the inoculum material was composed of 1 ml of a culture between 23 to 40 days old, which had been grown in the light or in the dark under the standard growth conditions (Section 2.48).

2.46 Details for subculturing material

While subculturing material from dark to dark, special care was taken to avoid picking up any trace of light which might possibly have a photomorphogenetic effect on the algae. Before autoclaving, pipettes were wrapped in aluminium foil, and inoculation was carried out in the dark near the bunsen flame, in a sterilised room (Section 2.44). The modified flasks for growth in dark (Section 2.48) were immediately wrapped in aluminium foil and black polythene.

2.47 Measurement of physical features

The light intensities were measured with an EEL Light Master Photometer at the surface of the water, and oriented at right angles to the source of light. Light intensity is expressed in lux. Temperature is expressed on

the centigrade scale (^{o}C).

2.48 Standard growth conditions

The growth in light was carried out at 35^{o}C in flasks incubated in the tank at 2000 or 5000 lux and shaken 64 times per min through a horizontal movement of 30 mm. The growth in dark was carried out under the same conditions in flasks painted with black paint, and then wrapped in aluminium foil, and black polythene.

2.5 Growth measurements

During growth of the algae, estimations were made in various experiments, of the number of total cells, and dry weight of the algae.

The following standard procedure was used in harvesting. The culture was removed from the growth flask to a volumetric cylinder, and the volume was measured. It was then centrifuged at 5000 x g for 5 min. The algal material was washed with glass distilled water and re-centrifuged at 5000 x g for 5 min. This procedure was repeated twice. The material was then transferred to vitreosil crucibles (previously dried at 105^{o}C) and dried for 24 h at 105^{o}C.

Growth rates were expressed in terms of the relative growth constant, $\grave{k}$, in $\log_{10}$ day units (FOGG,1965).

$$\dot{k} = \frac{\log_{10} N - \log_{10} N_O}{t} ,$$

where t = Days after inoculation,

N = Yield after t days

N_O = The total amount of cell material in the culture at zero time.

The mean doubling time, G, can be derived from $\dot{k}$:

$$G = \frac{0.301}{\dot{k}}$$

For certain experiments (Section 6) 0.2 mm haemocytometer was used for counting the number of cells in filament/ml and the total number of cells/ml. Before counting of the cells, the culture was treated with an ultrasonic probe for 1 min to break the clumps. Three replicate counts were always made.

2.6 Pigment extraction

Pigment analyses were based on OD measurements, obtained using a Perkin-Elmer, 402, ultraviolet-visible spectrophotometer, using aqueous extracts and 90% methanol extracts. Percent chlorophyll was determined by multiplying the OD at 665 nm by 1390, the extinction coefficient of RICHARDS and THOMPSON (1952) as modified by TALLING and DRIVER (1963). Estimation of phycocyanin concentration was made on the aqueous extracts by use of the following equation

in which correction is made for chlorophyll absorption (MYERS and KRATZ, 1955):

$$\text{OD Phycocyanin} = 1.016\ OD_{618} - 0.203\ OD_{677}$$

Percent phycocyanin was then determined by dividing the corrected absorption of 618 nm by 0.073, the extinction coefficient of CRAIG and CARR (1968), and percent phycoerythrin was estimated by dividing the OD at 565 by 0.285, the molecular extinction coefficient used by HATTORI and FUJITA (1959). These values were then normalised to a mg dry weight alga basis.

In all extraction procedures, three 20 ml aliquots of freshly harvested or freeze-dried cell suspensions were centrifuged at 5000 x g for five min and washed three times with glass distilled water. After a third centrifugation of the washed cell suspensions, one aliquot was quantitatively transferred to preweighed vetreosil crucibles, dried for 24 h at 105°C and reweighed for dry weight determination. The second aliquot was used for chlorophyll extraction, and the third one was used for extraction of phycocyanin and phycoerythrin.

2.61 Chlorogloea fritschii

Quantitative extraction of the pigments of this alga proved quite difficult. The hot 90% methanol as well as cold methanol and grinding the alga with acid-washed fine sand with 90% acetone were not effective. Yellowish green pigments were generally extracted, but after filtration

through Whatman Glass Fibre Filter Paper (GF/C), the cells remained pigmented, even after repeated extraction. These results suggested that cell disruption was necessary for the quantitative extraction of pigments from this alga. Therefore, various methods were employed in an attempt to find effective methods for disrupting the cells of this alga. The various methods tried are described below.

Sonication

The washed algal material collected by centrifugation at 5000 x g was suspended in 90% cold methanol and disrupted using a type 1130A "Soniprobe" (Dawe Instruments Ltd., London) at maximum output for 30 min. Overheating was avoided by using an ice bath and by interrupting the sonication for one min after each completed two min. The supernatant was filtered through Whatman Glass Fibre Filter Paper (GF/C), and the residue was extracted by 90% hot methanol. This procedure was repeated several times, but the cells still remained pigmented.

Lysozyme

The enzyme lysozyme has been shown to be an effective method for digesting the cell walls of some blue-green algae (CRESPI et al., 1962; BERNS et al., 1966). It was, therefore, decided to use lysozyme in an attempt to facilitate leakage of pigments from the algal cells. TUCK (1968) has shown that treatment of freeze-dried cells of Chlorogloea fritschii

with 25 mg lysozyme per g of cells for 24 h, at room temperature, and repeating the procedure for another 24 h, to be the most effective method for extracting the cytochrome from cells of this alga. Therefore, washed algal material (freeze-dried) collected by centrifugation at 5000 x g was suspended in 0.03M phosphate buffer, pH 7, containing 25 mg lysozyme per g of cells (method described by TUCK, 1968). The suspension was incubated for 24 h at room temperature. After this incubation time, no phycocyanin was released from the cells. The suspension was centrifuged at 5000 x g and the residue was washed twice with distilled water. The residue was then resuspended in 90% cold methanol and the cells were sonicated, as described above, for 30 min. This was followed by filtration through Whatman Glass Fibre Paper (GF/C), and the residue was extracted by 90% hot methanol. The sonication of the lysozyme-treated cells increased the amount of chlorophyll, but the cells remained pigmented, even after repeated sonication. In this case, the incubation time was extended to 48 and 96 h, following the procedure described by TUCK (1968). Freeze-dried cells were incubated with 25 mg lysozyme per g of cells for 24 h, then the cells were centrifuged at 5000 x g,resuspended in fresh buffer containing 25 mg of fresh lysozyme, and were incubated for an additional 24 h period. Thus, the total incubation time was 48 h, and the total amount of lysozyme was 50 mg per g of cells. Again, this extended incubation time (48 h) did not facilitate leakage of phycocyanin from the cells. The

cells which were incubated with lysozyme for 48 h were found of course, to be contaminated with bacteria. Different concentrations of lysozyme were also tried without success. Neither extending the incubation time (96 h) nor increasing the sonication time of the lysozyme-treated cells increased the amount of chlorophyll; they did not facilitate extracting the cells to completion (cells still remained pigmented).

In vivo fluorescence

Since quantitative extraction of the pigments of Chlorogloea fritschii proved quite difficult and continuous in vivo chlorophyll a measurements by a sensitive fluorometer have proved to be a useful tool in broad ecological programs carried out at sea or lake (LORENZEN, 1966; NICHOLSON, 1970), it was decided to use such a method for measuring the chlorophyll a concentration of this alga. The instrument used was Aminco Fluoro-Microphotometer (Photomultiplier microphotometer, American Instrument Co. Inc., Silver Spring, Maryland, U.S.A.). Dilute suspensions were first sonicated for 5 min with an ultrasonic bath and the suspension was then placed in the fluorometer and the emission was noted. The concentration of chlorophyll a in the sample was calculated from the calibration curve. The amount of chlorophyll a indicated by the fluorometer was found far less than that obtained by 90% hot methanol.

2.62 Selected strains

Quantitative extraction of the pigment of the selected strains: Calothrix membranacea, Phormidium luridum, Phormidium sp., Plectonema boryanum D181, and P. calothricoides proved quite easy. One aliquot of fresh harvested cells was suspended in 90% hot methanol and filtered through Whatman Glass Fibre Filter Paper (GF/C). After a second and third extraction, the supernatants containing chlorophyll a were combined and made up to a known volume to obtain the absorption spectra. The blue cells remaining after extraction with 90% hot methanol showed a characteristic phycocyanin absorption with no trace of chlorophyll a absorption. Grinding the algae with acid-washed fine sand with glass distilled water for extracting phycocyanin and phycoerythrin proved quite a difficult technique and involved much physical effort, especially because more than one strain was involved. Therefore an enzymatic method was used to facilitate leakage of phycocyanin and phycoerythrin from the algal cells. Algal materials were suspended in 0.03M phosphate buffer, pH 7, containing 0.05% lysozyme and incubated overnight at room temperature. Clear blue supernatant was released from the cells, this was separated by centrifugation at 5000 x g for 5 min. The residue was sonicated (described above) for 5 min to ensure that all phycocyanin and phycoerythrin were extracted. The supernatants containing phycocyanin or phycocyanin and phycoerythrin were combined and made up to known volume to obtain the absorption spectra. Chlorophyll

was also extracted from lysozyme-treated cells, by 90% hot methanol. Extraction of chlorophyll from both freshly harvested cells and lysozyme-treated cells was equally successful.

2.7 Statistical analysis

The standard deviation was calculated for each set of data using the formula:

$$s = \sqrt{\frac{\Sigma (x-\bar{x})^2}{n-1}}$$

In Section 3.6, it will be noted that sucrose stimulates the growth of strains which failed to grow with sucrose in the dark, at low light intensities. Therefore significance test was carried out to test whether the stimulation of growth by sucrose is significant. A comparison of the effect of sucrose on the growth of Anabaena cylindrica at 500 lux will serve as an example of the calculations involved (PARADINE and RIVETT, 1968).

8 flasks were used; half of these were with sucrose and the other half were without it (control). The mean yield of cultures grown without sucrose (control) was 257.09 mg/l dry weight with a standard deviation = s_1 = 39.62, while the mean yield of cultures grown with sucrose was 374.14 mg/l dry weight with a standard deviation = s_2 = 50.89.

Treatment of the data to estimate the variance σ

afforded by the two samples:

$$\sigma^2 = \frac{n\ s^2 + n\ s^2}{n_1 + n_2 - 2} \qquad \sigma^2 = 2773.024$$

Therefore the two sets of data can be compared by a simple student t test, where t is found from the formula:

$$t = \frac{m_1 - m_2}{\sigma} \sqrt{\frac{n_1\ n_2}{n_1 + n_2}}$$

Therefore

$$t = \frac{117.05}{52.6595} \sqrt{\frac{4 \times 4}{4 + 4}} = 3.143$$

The value of P (probability) which gives a 95% certainty that there is a significant difference between the two sets of data is 2.447 (for 6 degrees of freedom : eight samples - two). In this case the probability that there is a significant difference in the amount of growth in cultures of Anabaena cylindrica grown with sucrose at 500 lux is between 95 and 98% certain (P = between 0.05 and 0.02 per cent level of probability).

RESULTS

3. Growth data

The results of systematic surveys on the heterotrophic growth of blue-green algae are meagre and often contradictory (Section 1). Therefore, the present study was commenced by screening the ability of 24 different strains of blue-green algae to grow heterotrophically in complete darkness on organic media. A subsidiary aim of the study was to determine whether sucrose can stimulate the growth of strains which failed to grow in the dark at different light intensities.

3.1 Cultural survey for heterotrophy

A qualitative survey of 24 strains was undertaken to screen their ability to grow in the dark. It was conducted by testing each strain for growth with sucrose in the dark and confirming it under critical conditions.

3.2 Standard test

The preliminary test was carried out for each strain to screen its ability for heterotrotrophic growth. The test was done in duplicate with 50 ml of basal inorganic medium. Strains capable of growth in the light, without a source of combined nitrogen, were subcultured to a similar medium in the dark. All strains were incubated in the dark together with 0.01M sucrose at a temperature known to be

favourable to their growth in light. Obvious growth was observed in most of the cultures after a month of incubation in the dark. The cultures were incubated for a further period of two months in the dark and the results are summarised in Table V.

The strains examined here are almost the same as those reported by KHOJA and WHITTON (1971). The exception was that Plectonema boryanum D181 which was included in the former was excluded and replaced by Anabaenopsis sp. in the latter.

As shown in Table V, Anabaena cylindrica, A. inaequalis, A. variabilis, Anabaena sp. and Tolypothrix tenuis, failed to grow in the dark on medium lacking a source of combined nitrogen. In these cases the test was repeated by subculturing from the material with an addition of combined nitrogen (KNO_3) to such a medium in the dark. Of these strains only Anabaena sp. showed obvious growth in the dark, while A. cylindrica, A. inaequalis, and A. variabilis failed to grow and the cells became colourless after three months of incubation in the dark. When Tolypothrix tenuis was subcultured, in the presence of KNO_3 as a nitrogen source, no increase in cell mass ever occurred in the dark. If KNO_3 was replaced with ammonium sulphate, an obvious growth occurred. The anomalous observation for this alga, that although it will grow in the light without combined nitrogen, it will not do so in the dark, is in agreement with the earlier observation of KIYOHARA et al. (1960). This problem is studied in further detail in

Table V: Effect of sucrose on the growth of the cultures incubated in the dark for three months.

strain		temperature °C	medium	growth in dark		remarks
				-N	+N	
Anabaena	cylindrica	25	AD	-	-	
A.	inaequalis	25	AD	-	-	
A.	variabilis	25	AD	-	-	
Anabaena	sp.	35	AD	-	+	
Anabaenopsis	circularis	35	AD	+		
Anacystis	nidulans	35	AC	-	-	
Calothrix	brevissima	25	AD	+		
C.	membranacea	35	AD	+		
Chlorogloea	fritschii	35	AD	+		
Lyngbya	sp.	35	AC	NT	-	viable
Nostoc	commune	35	AD	+		
N.	ellipsosporum	35	AD	+		
N.	muscorum	25	AD	+		
N.	punctiforme	25	AD	+		
Nostoc	sp. D139	35	AD	+		
Nostoc	sp. D167	25	AD	+		
Oscillatoria	tenuis	35	AC	NT	-	
Phormidium	luridum	35	AC	NT	+	
Phormidium	sp.	35	AC	NT	+	
Plectonema	boryanum D177	35	AC	NT	+	
P.	boryanum D181	35	AC	NT	+	
P.	calthricoides	35	AC	NT	+	
Scytonema	sp.	25	AD	+		
Tolypothrix	tenuis	35	AD	-	+	with NH_4 only

sucrose concentration = 0.01M; + = growth; - = no growth; NT = not tested

Section 3.43. One strain, Lyngbya sp., remained viable since growth occurred when the cultures were subsequently placed in the light (2000 lux) after three months of incubation in the dark, while Anacystis nidulans and Oscillatoria temuis failed to grow and the cells became colourless. In these cases the test was repeated by using ammonium sulphate in place of KNO_3 in AC medium, to preclude the possibility that nitrate is not a utilisable nitrogen source in the dark. The concentration of ammonium sulphate was the same as the concentration of KNO_3 in AC medium (1000 mg/l). The cultures were incubated for three months in the dark under the standard growth conditions (Section 2.48). After the incubation time Lyngbya sp. still remained viable, while the other two strains died.

3.12 Confirmation of growth under critical conditions

While carrying out the standard test as described above, it was suspected that trace light, which might possibly have a photomorphogenetic effect on the algae, could pass through flasks modified for dark growth (flasks painted with black paint, and then wrapped in aluminium foil and black polythene, Section 2.48). To verify this a test was carried out in a "dark room" using a very sensitive photographic film (Ilford PAN F 50 ASA). A piece of film was inserted in a container (a rectangular shaped tin) and the container closed with a firm lid, providing complete darkness; a second piece was inserted in the flask modified

for dark growth and both were subjected to the standard growth conditions (Section 2.48). After 15 min the two pieces of the film were developed and it was found that the piece of film in the flask had become slightly exposed, but not the one in the container thus necessitating further confirmation of growth in complete darkness.

Ten strains (Anabaena sp., Calothrix membranacea Chlorogloea fritschii, Nostoc ellipsosporum, Nostoc sp. D159, Phormidium luridum, Phormidium sp. Plectonema boryanum D177, P. boryanum D181, and P. calothricoides) were therefore chosen for rechecking the growth in dark under critical conditions (Section 2.42). All strains proved capable of growth under these conditions.

3.2 Effect of various organic substrates on growth in dark

Carbohydrates are the only organic compounds which have been shown to support dark heterotrophic growth of blue-green algae (Section 1). ALLISON et al. (1953), HOARE et al. (1967) and PEARCE and CARR (1967) on the other hand demonstrated that blue-green algae can assimilate acetate. Therefore, in the present study, both carbohydrates and acetate were tested for their ability to support growth of the strains in the dark in the presence and absence of combined nitrogen. The compounds were (0.01M) fructose, galactose, glucose, maltose, sucrose and acetate (sodium salt). The initial pH values varied from 7.3 to 7.5.

Strains which were not capable of growth on medium lacking a source of combined nitrogen (KNO_3) in the light, were tested in the dark on a medium containing it. The cultures were all tested for six weeks and the results are shown in Table VI.

Table VI shows the outcome of a qualitative screening of the organic compounds for supporting best growth. Maltose supported slight growth in six cultures in the absence of combined nitrogen. There was appreciable growth of two strains: Chlorogloea fritschii and Nostoc sp. D159 on maltose with nitrate but not without. In the presence of combined nitrogen, glucose supported growth in almost the same degree as fructose. Fructose and glucose allowed appreciable growth in six cultures, and allowed considerable growth in two others. In the absence of combined nitrogen however, fructose supported growth of seven strains while glucose supported growth of only five strains. Sucrose supported growth of all strains capable of heterotrophic growth in the dark. In the majority of cultures, best growth was on sucrose, in the presence and in the absence of combined nitrogen. Sucrose was outstanding among the substrates tested in allowing a considerable growth of the majority of the cultures. No growth, however, was found on either acetate with or without nitrate, or galactose with or without nitrate.

In the absence of combined nitrogen in the medium,

Table VI: Effect of various organic substrates on the growth of the cultures incubated in the dark for six weeks in the presence and absence of combined nitrogen in the mineral medium

strain		+ N						- N					
		fructose	galactose	glucose	maltose	sucrose	acetate	fructose	galactose	glucose	maltose	sucrose	acetate
Anabaena	cylindrica	-	-	-	-	-	-	-	-	-	-	-	-
A.	inaequalis	-	-	-	-	-	-	-	-	-	-	-	-
A.	variabilis	-	-	-	-	-	-	-	-	-	-	-	-
Anabaena	sp.	+	-	++	-	+++	-	-	-	-	-	-	-
Anabaenopsis	circularis	++	-	++	+	+++	-	+	-	+	-	++	-
Aphanocystis	nidulans	-	-	-	-	-	-	-	-	-	-	-	-
Calothrix	brevissima	+++	-	++	+	+++	-	++	-	+	±	++	-
C.	membranacea	+	-	+++	+	++	-	-	-	++	-	+	-
Chlorogloea	fritschii	-	-	+	++	+++	-	-	-	-	+	++	-
Lyngbya	sp.	-	-	-	-	-	-	NT	NT	NT	NT	NT	NT
*Nostoc	commune	-	-	-	-	-	-	-	-	++	++	+	-
N.	ellipsosporum	+++	-	-	-	++	-	++	-	-	-	+	-
N.	muscorum	++	-	+	-	+++	-	+	-	-	-	++	-
*N.	punctiforme	-	-	-	-	-	-	++	-	-	-	+	-
Nostoc	sp. D159	++	-	-	++	++	-	+	-	-	+	++	-
*Nostoc	sp. D167	-	-	-	-	-	-	-	-	-	+	++	-
Oscillatoria	tenuis	-	-	-	-	-	-	NT	NT	NT	NT	NT	NT
Phormidium	luridum	++	-	+	+	+++	-	NT	NT	NT	NT	NT	NT
Phormidium	sp.	++	-	++	+	+++	-	NT	NT	NT	NT	NT	NT
Plectonema	boryanum D177	++	-	+	++	+++	-	NT	NT	NT	NT	NT	NT
P.	boryanum D181	+	-	+++	-	++	-	NT	NT	NT	NT	NT	NT
P.	calothricoides	+++	-	++	-	++	-	NT	NT	NT	NT	NT	NT
*Scytonema	sp.	-	-	-	-	-	-	±	-	+	++	++	-
[x]Tolypothrix	tenuis	+	-	++	-	+++	-	-	-	-	-	-	-

substrate concentration = 0.01M; - = no growth; NT = not tested; + = light growth; ++ = moderate growth; +++ = heavy growth;

* - non-axenic cultures; [x] - with NH_4 only

fructose, glucose, maltose and sucrose supported appreciable growth of the unialgal (non-axenic) cultures: Nostoc commune, N. punctiforme, Nostoc sp. D167 and Scytonema sp..

When a source of combined nitrogen (KNO_3) was added to the carbohydrate cultures, bacterial action upon carbohydrate was fast and bacterial growth became abundant. The fast action of bacteria upon carbohydrate had caused the pH to drop to 6.5 and this hampered or inhibited the algal growth. In nitrogen free medium, however, bacterial growth was not abundant and bacterial action upon carbohydrate did not decrease the pH below 7.

3.3 Repeated subculture in dark for long periods

In order to ensure the capability of contimuous growth of strains in the dark,,those strains capable of heterotrophic growth were subcultured once every 4-6 weeks over a period of 12 months. This was extended to a further period of two years in the case of Chlorogloea fritschii. Contimuous growth was evident in all strains so subcultured.

Two strains of Calothrix (C. brevissima, C. membranacea) which were capable of heterotrophic growth in medium free of combined nitrogen in the first subculture, showed very poor growth or no growth at all in the second subculture in the same medium. This problem will be treated in detail in Section 3.41.

3.31 Growth rate of Chlorogloea fritschii

Growth rates were determined for three heterotrophic cultures: material first subcultured from light to dark, material subcultured from dark to dark, and after three years of subculturing in the dark. The aim of this was to detect any physiological adaptation to heterotrophic conditions, and also to study the effect of long-term dark incubation on the growth rate. The cultures were grown under the standard growth conditions (Section 2.48), using AC medium with 0.01M sucrose.

On first subculture from light to dark, inoculum containing 47.89 mg/l dry weight was taken from 28 day old culture grown under light intensity of 4000 lux, and at 35°C. Growth was noted at regular intervals, and the results are shown in Fig.1. The standard error of the mean was between ± 0.7-9.5%. Similarly growth was observed for material subcultured from dark to dark and after three years of subculturing in the dark. Inoculum containing 39.67 mg/l dry weight from 35 day old culture was taken for the former, and 89 mg/l dry weight from 42 day old culture was taken for the latter. The results are shown in Fig. 2. and Fig.3 respectively; the standard error of the mean was between ± 0.7-9.5% and ± 0.5-6.4% respectively.

With all three heterotrophic cultures, there was no detectable lag phase. There was no difference in the growth rate ($\hat{k}$) of these cultures, growth rate was 0.055 and the doubling time (G) was 131.4 h.

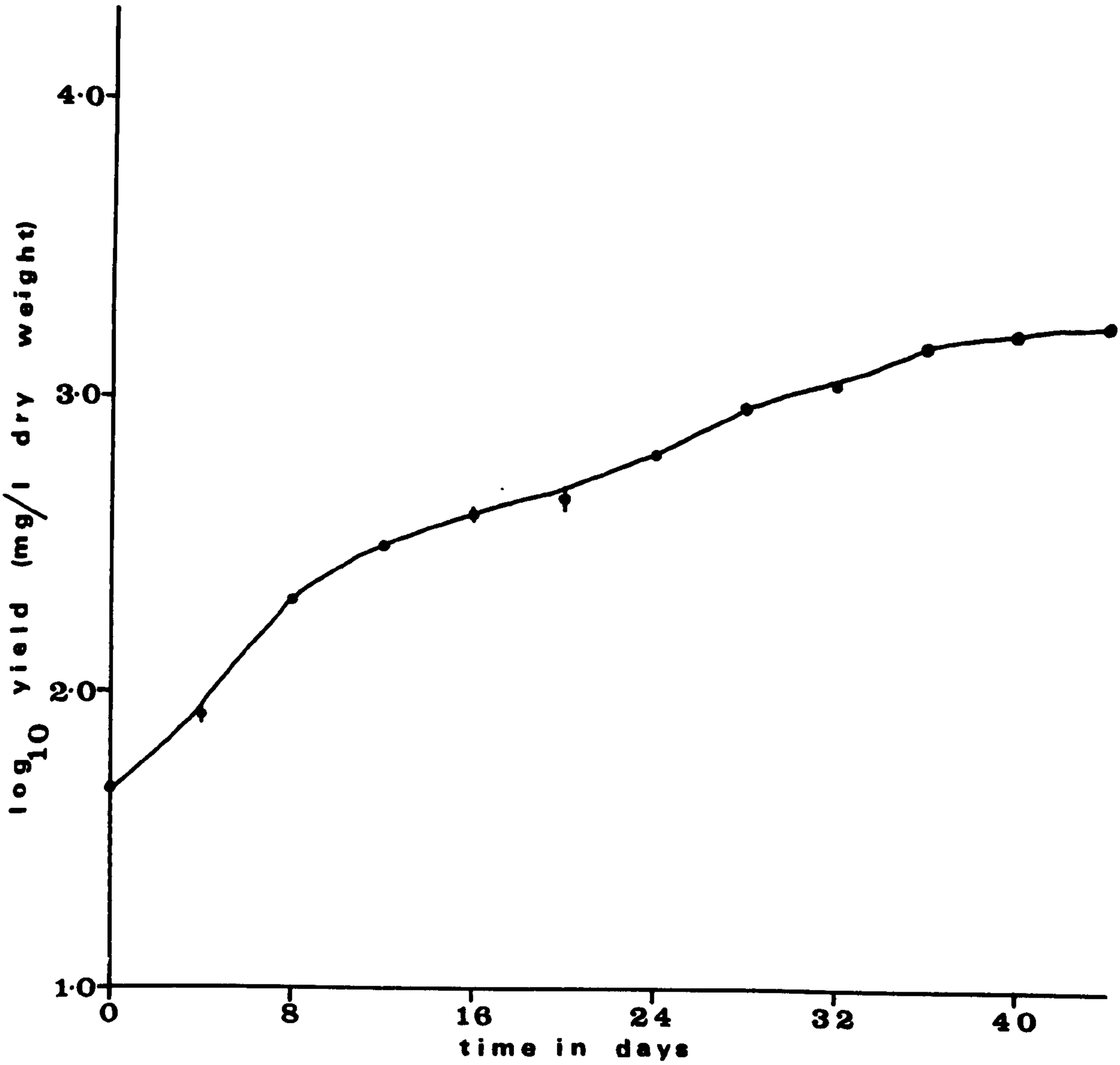

Fig. 1. Dark heterotrophic growth of Chlorogloea fritschii, (first subculture from light to dark) grown under the standard growth conditions (Section 2.48).

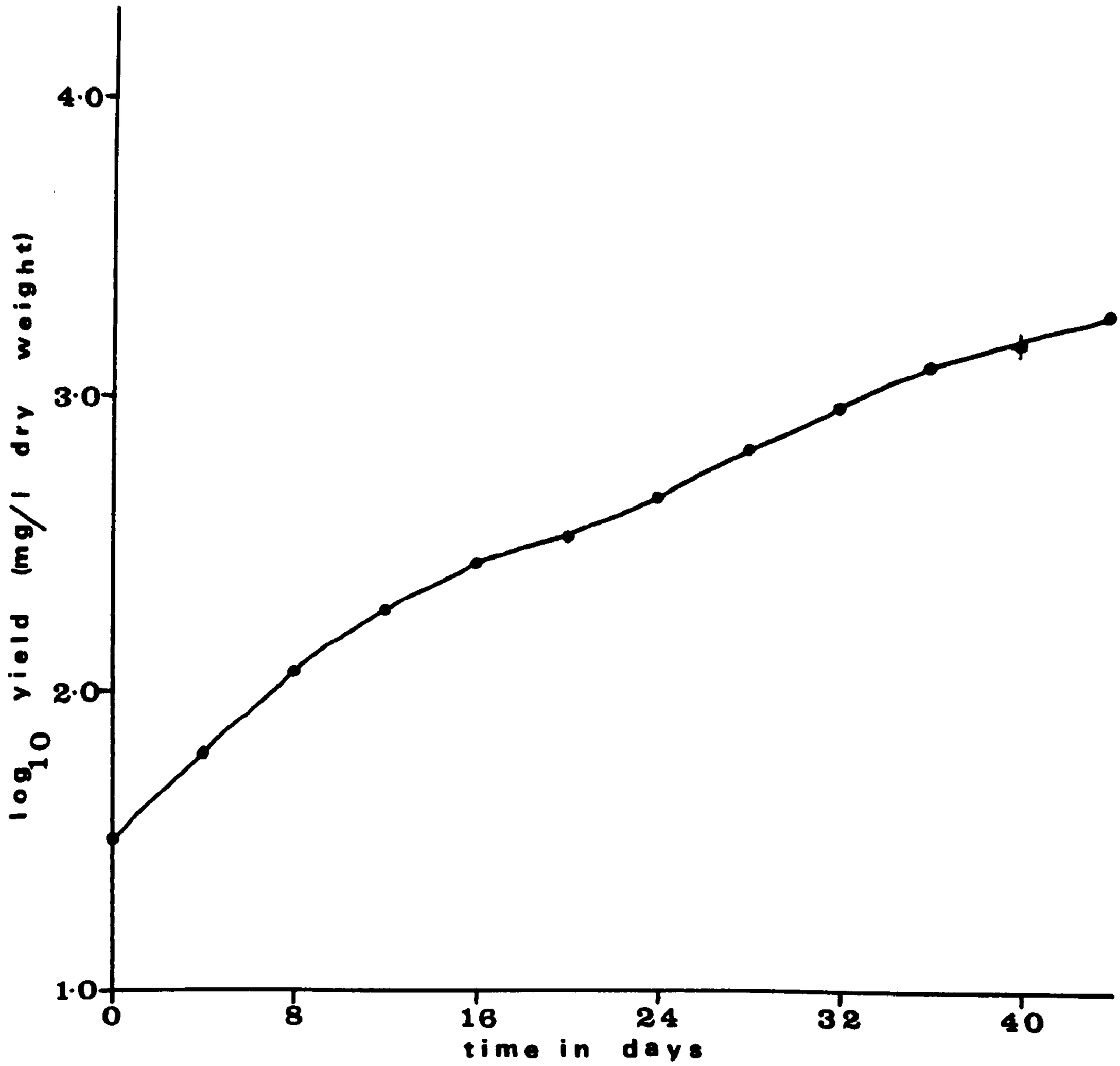

Fig. 2. Dark heterotrophic growth of Chlorogloea fritschii, (second subculture, from dark to dark) grown under the standard growth conditions (Section 2.48).

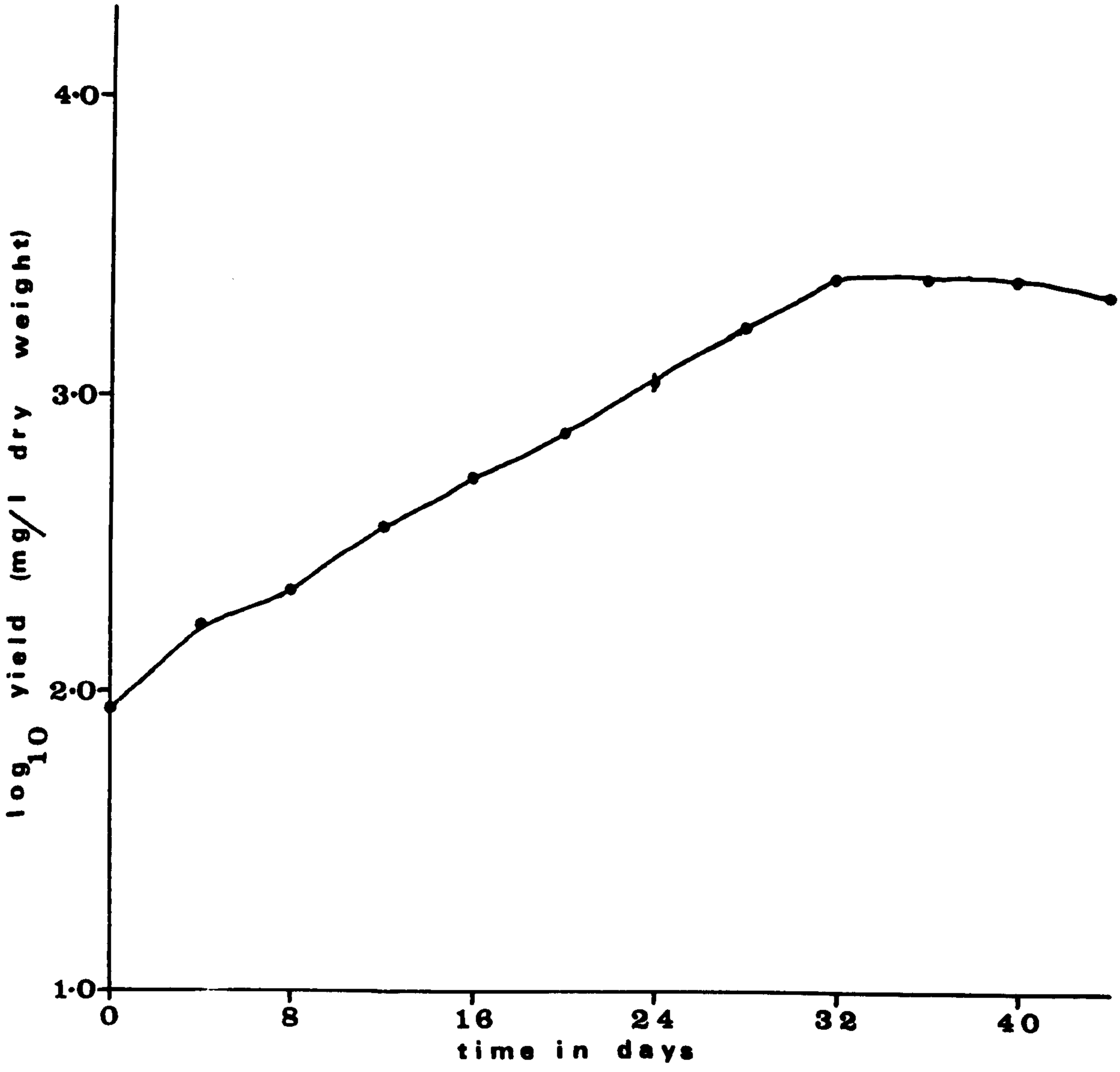

Fig. 3. Dark heterotrophic growth of Chlorogloea fritschii, (after 3 years in dark) grown under the standard growth conditions (Section 2.48).

3.32 Growth rate of the selected strains

Phormidium luridum, Phormidium sp., Plectonema boryanum D181, P. calothricoides which seemed to grow fairly fast with sucrose in the dark, were chosen for determining their growth rates. The strains were grown under the standard growth conditions (Section 2.48) in AC medium in the presence of 0.01M sucrose. Inocula were taken from 28-35 day old cultures, grown in the dark (first transfer from light to dark). Growth was followed at regular intervals, and the results are shown in Fig.4, 5, 6, and 7; the standard error of the mean was between ± 0.5 - 6.5, 0.2 - 4.7, 0.9 - 7.9 and 0.1 - 3.2% respectively, There was no detectable lag phase, and the growth rate declined after 12-26 days. Table VII shows the results of the growth rate ($\grave{k}$) together with the doubling time (G) in h. Growth rate of Phormidium luridum was the same as the growth rate of Phormidium sp..Plectonema boryanum D181 had a slow doubling time (92.62 h), while the doubling time of P. calothricoides was (55.15 h) the fastest among these selected strains.

3.4 General observations on growth in dark

In the first subculture from light to dark, when strains capable of growth on a nitrogen-free medium were subcultured to such a medium in the dark, an obvious increase in the cell mass did occur in all cultures after three weeks of incubation. In subsequent subculture from dark to

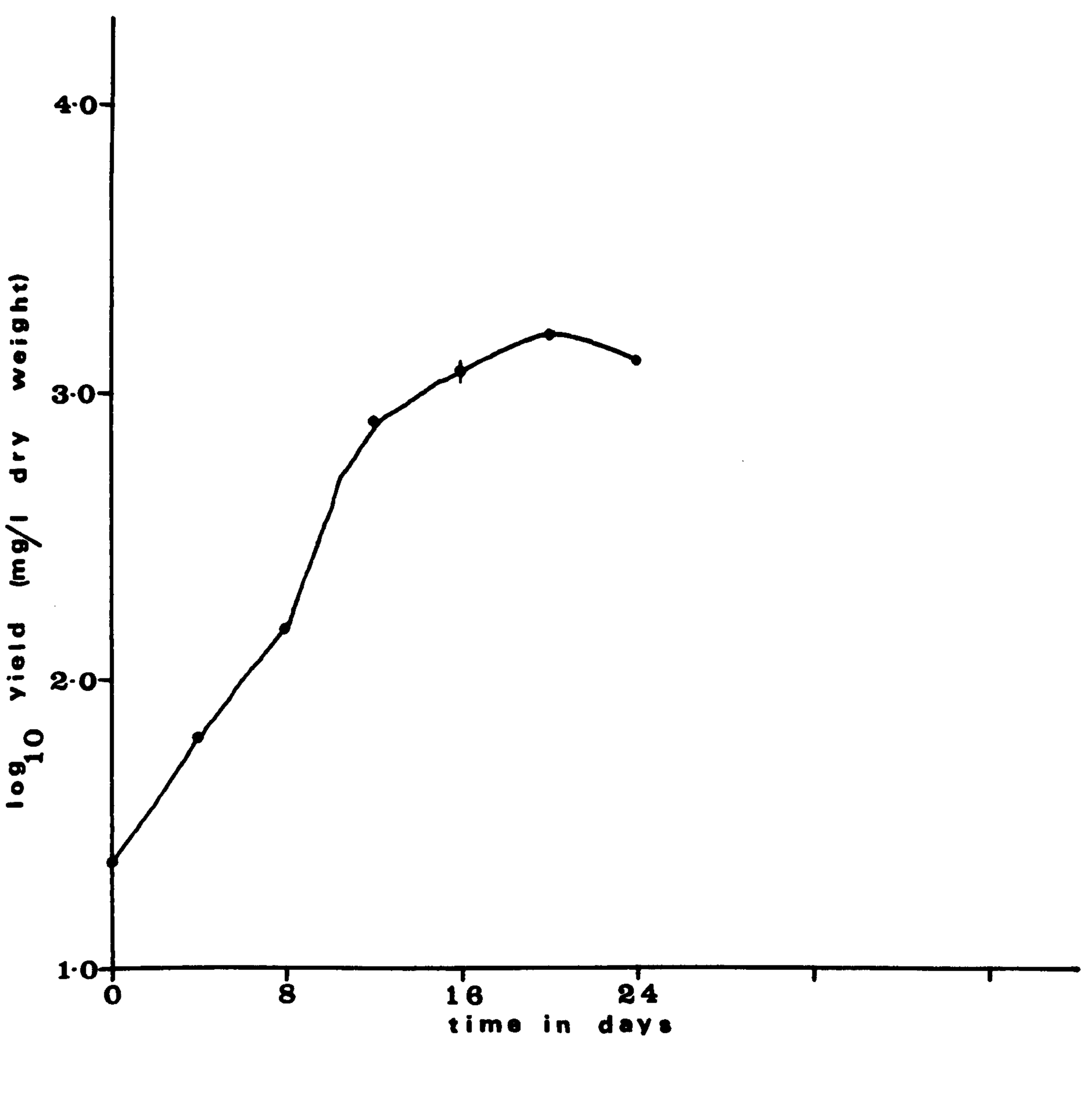

Fig. 4. Dark heterotrophic growth of Phormidium luridum, grown under the standard growth conditions (Section 2.48).

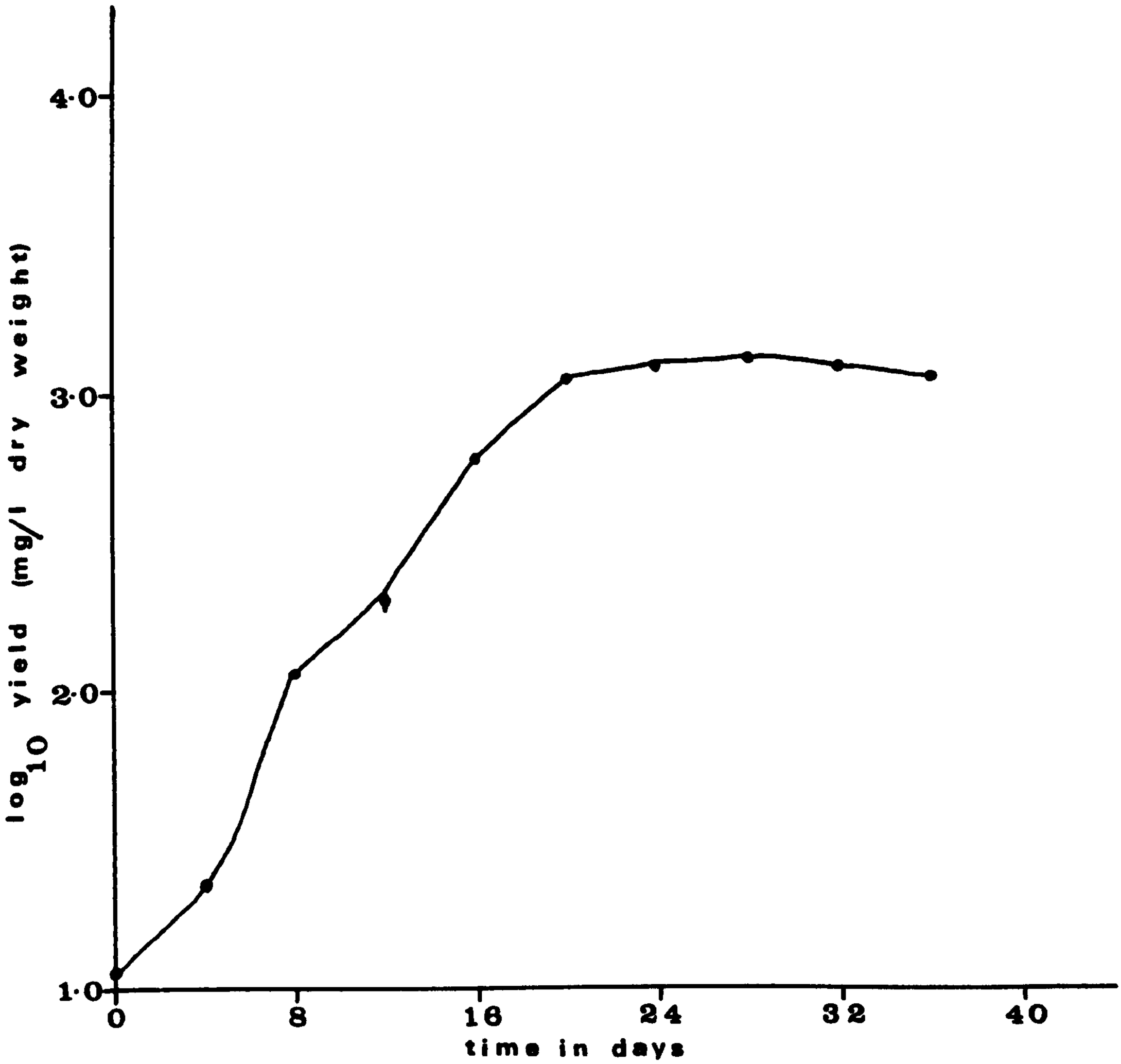

Fig. 5. Dark heterotrophic growth of Phormidium sp., grown under the standard growth conditions (Section 2.48).

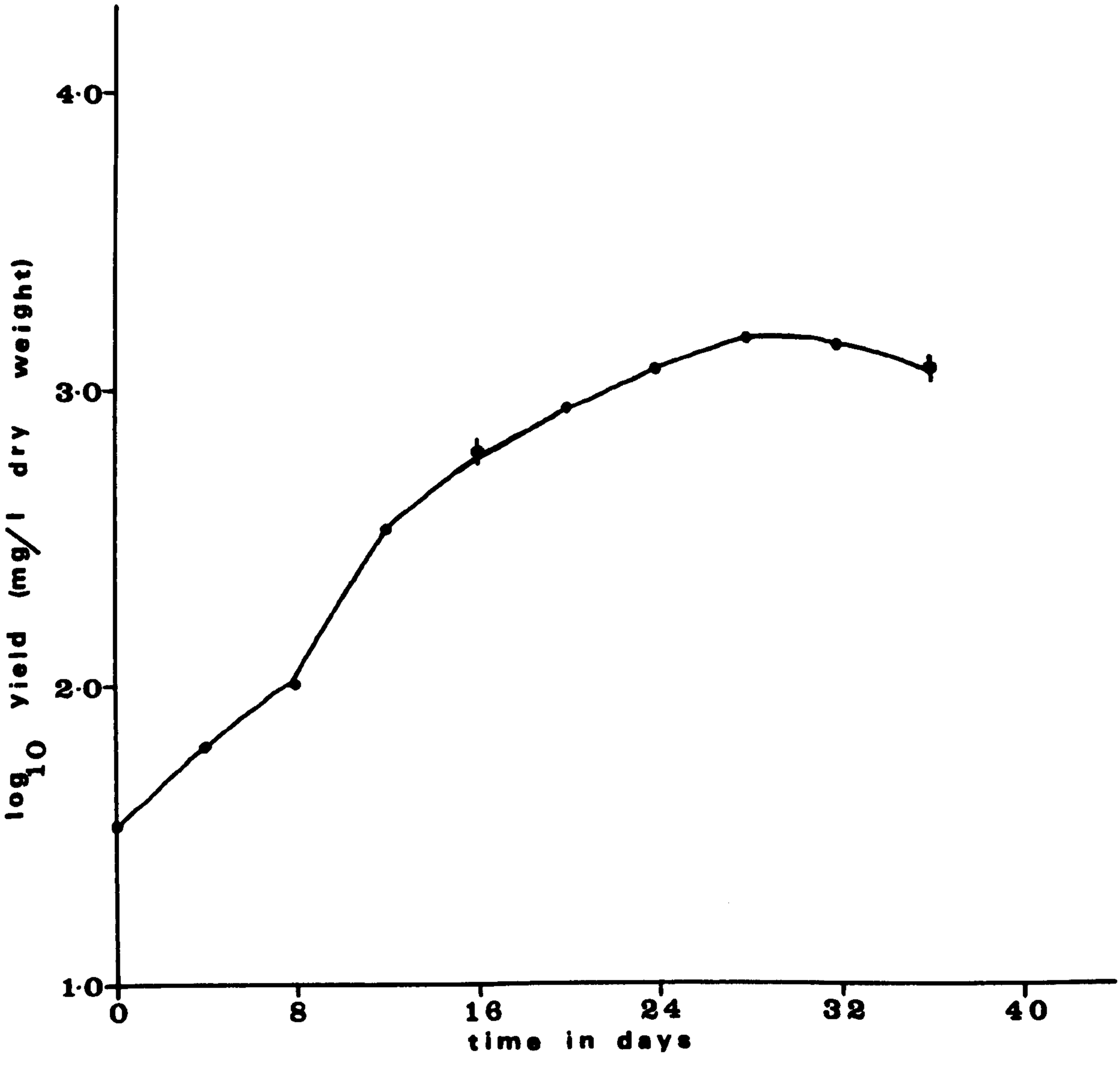

Fig. 6. Dark heterotrophic growth of Plectonema boryanum D181, grown under the standard growth conditions (Section 2.48).

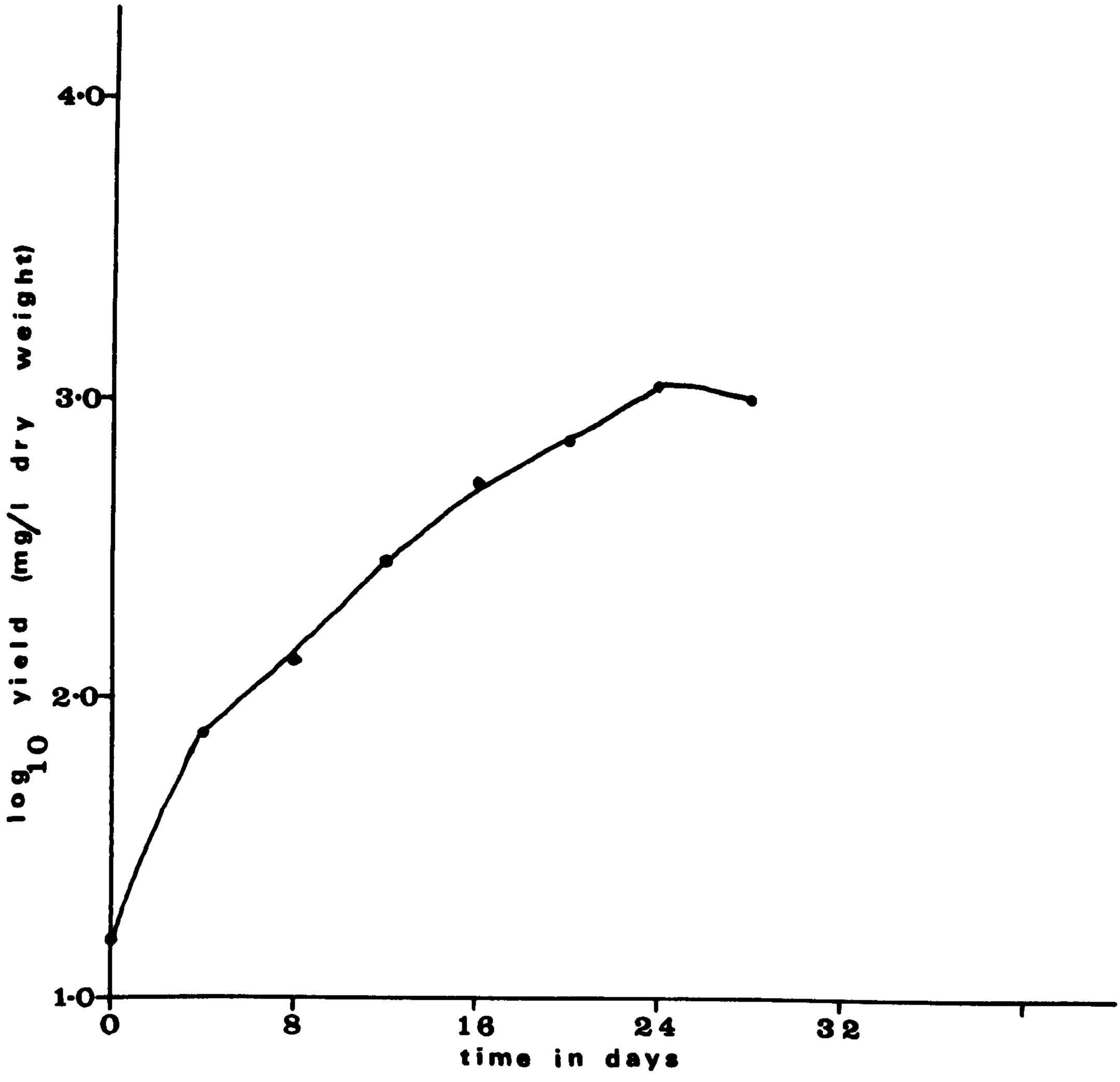

Fig. 7. Dark heterotrophic growth of Plectonema calothricoides, grown under the standard growth conditions (Section 2.48).

Table VII: Growth rate, $\dot{k}$, in $\log_{10}$, day units, and mean doubling time, G, in h, of selected strains grown in the dark under the standard growth conditions (Section 2.48)

strain			growth rate ($\dot{k}$)	doubling time (G)
Phormidium	luridum		0.112	64.5
Phormidium	sp.		0.112	64.5
Plectonema	boryanum	D181	0.078	92.62
P.	calothricoides		0.131	55.15

dark, however, unlike the remainder of the cultures, two strains of Calothrix (C. brevissima, C. membranacea) in a nitrogen-free medium showed extremely poor growth with hardly any increase in the cell mass.

3.41 Calothrix strains

When the two Calothrix strains (C. brevissima, C. membranacea) grown on a nitrogen-free medium showed no obvious increase in cell mass, in the second subculture in dark, they were subcultured into a medium containing a source of combined nitrogen (KNO_3). Obvious growth was seen and the strains were subjected to a repeated subculture in the dark for a long period (Section 3.3).

3.42 Strains viable but incapable of growth

The account of KHOJA and WHITTON (1971) reported that when Anabaenopsis sp. and Lyngbya sp. were subcultured they remained viable after three months of incubation in the dark in the presence of 0.01M sucrose. This raised a question on the mechanism of survival of these strains able to survive periods of darkness. Further experiments were therefore made on Lyngbya sp.. The first experiment was to test whether or not the alga can survive on a basal inorganic medium (AC) in the absence of a source of organic compound. The second was to show any resumption of normal growth on transfer to light after three months of incubation in the dark. Inoculum containing 3.45 mg dry

weight of 50 ml of medium in culture flask was made from 20 day old culture, grown under 2000 lux, and at 35°C. In the first experiment, 8 flasks, each containing 50 ml of AC medium, to 4 of which sucrose (0.01M) was added, were incubated in the dark at 35°C. After three months in the dark, it was found that, in the presence of 0.01M sucrose in the medium, the alga remained viable, and it was deep green in colour. The yield was 3.4 mg dry weight per flask. The difference between this yield and the yield of the initial inoculum is statistically not significant. P (Probability) amounting at 20 per cent level of probability. In the basal medium the alga died, and the cells became colourless. Good replication of these results were obtained in a further experiment.

The second experiment which was conducted to show any resumption of normal growth on transfer to light after three months of incubation in the dark, was done by using a set of flasks containing AC medium plus 0.01M sucrose. The cultures were incubated in the dark under the standard growth conditions (Section 2.48). After three months of incubation in the dark, the alga remained viable, and when transferred to light (2000 lux), showed resumption of normal growth.

3.43 Tolypothrix temuis

It was shown above (Section 3.11) that when Tolypothrix temuis was tested for growth in dark using the

standard test procedure (Section 3.11) there was no increase in cell mass unless ammonia was added to the medium. As a result, further experiments were considered desirable.

3.431 Effect of various nitrogen sources in light

Two different nitrogen sources were used, potassium nitrate, and ammonium sulphate. The concentration of the nitrogen source was the same as the concentration of potassium nitrate in AC medium (1000 mg/1). Growth of T. tenuis with the two nitrogen sources was compared with its growth on nitrogen-free medium. The pH of the medium was about 7.3. Inoculum was made containing 19.1 mg/1 dry weight of 28 day old culture, grown under 4000 lux, and at 35°C. The cultures were incubated under the same conditions. Growth was observed at regular intervals, and the results are shown in Fig.8. As it can be seen from Fig.8, there was no lag phase. Exponential growth was fast, and lasted 4 days, with the two sources of nitrogen, while on nitrogen-free medium it lasted about 12 days. After 4 days, growth was slightly better with ammonia than with a nitrogen-free medium, but it was best with nitrate. The culture grown on ammonia showed loss in dry weight after day + 12. After 24 days the final yield on a nitrogen-free medium was 1064 mg/1 dry weight, while on medium containing nitrate it was 585 mg/1 dry weight. Exponential growth with ammonia was accompanied by a drop in pH from an initial pH

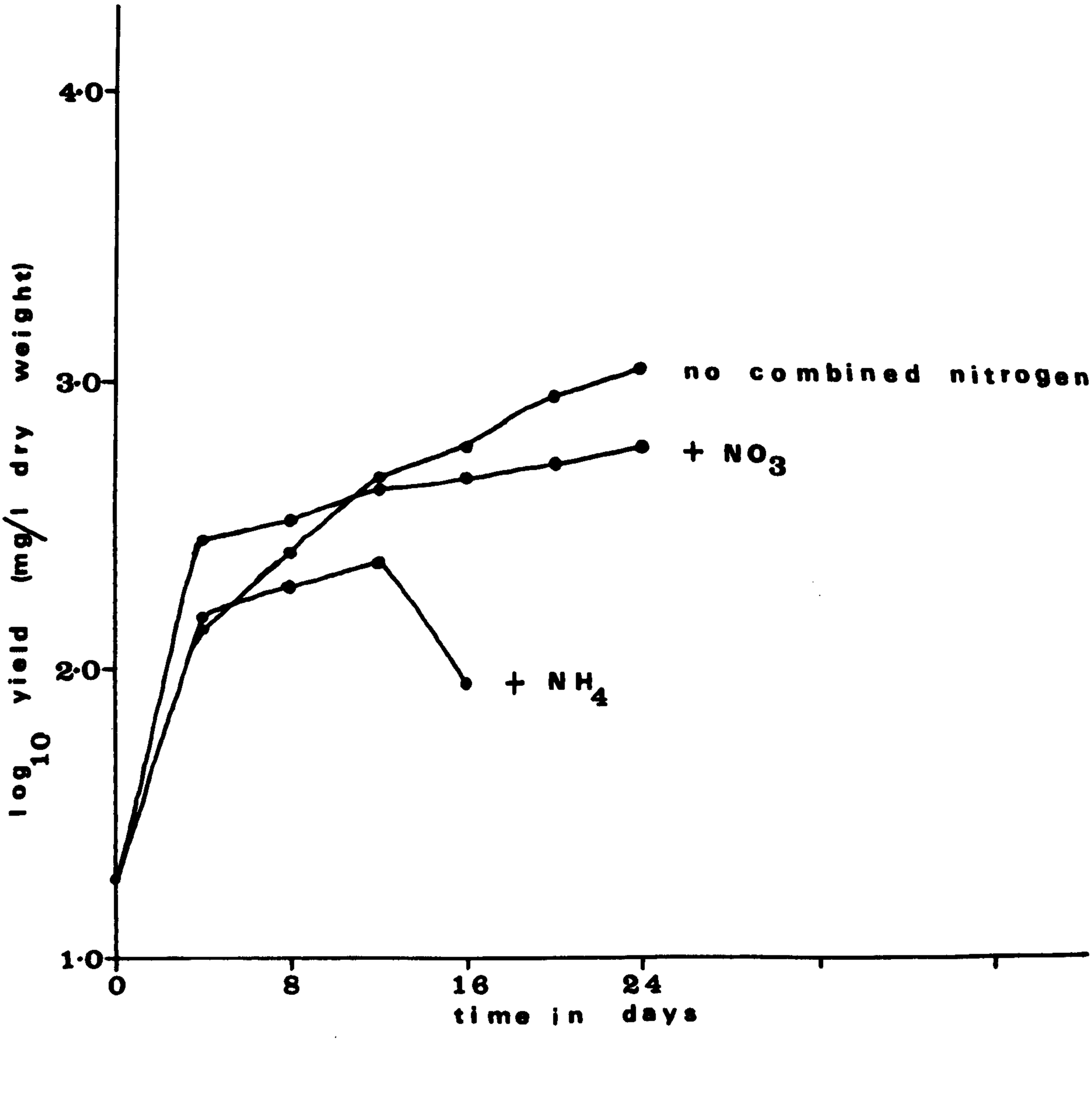

Fig. 8. Effect of various nitrogen sources on growth of Tolypothrix tenuis incubated at 35°C, 4000 lux and continuous shaking.

of 7.3 to a pH of 6.7 on day + 4. On day + 12 the pH of the medium dropped to pH 4.8 and the alga started to die.

3.432 Effect of sucrose at low light intensity

An experiment was performed to elucidate the effects of sucrose on the growth of Tolypothrix tenuis at low light intensity (500 lux). After maintaining Tolypothrix tenuis in liquid AD medium under 500 lux for 29 days, an inoculum was made containing 17.82 mg/l dry weight. Two sets of flasks, one containing 0.01M sucrose and the other without it, were incubated at 500 lux, 35°C and continuous shaking. Growth was observed at regular intervals, and the results are shown in Fig.9. The effect of sucrose at 500 lux on the growth of the alga resulted in a fast exponential growth. After 24 days the yield of organism, in cultures grown on sucrose, was about 6 times that of the control cultures.

3.5 Effect of sucrose in light

FAY (1965) found that the utilisation of sucrose by Chlorogloea fritschii resulted in a decrease of the generation time and a prolongation of the exponential phase of growth at a light intensity of 3000 lux. Thus, it was thought desirable to carry out further investigations on the effect of sucrose at different light intensities.

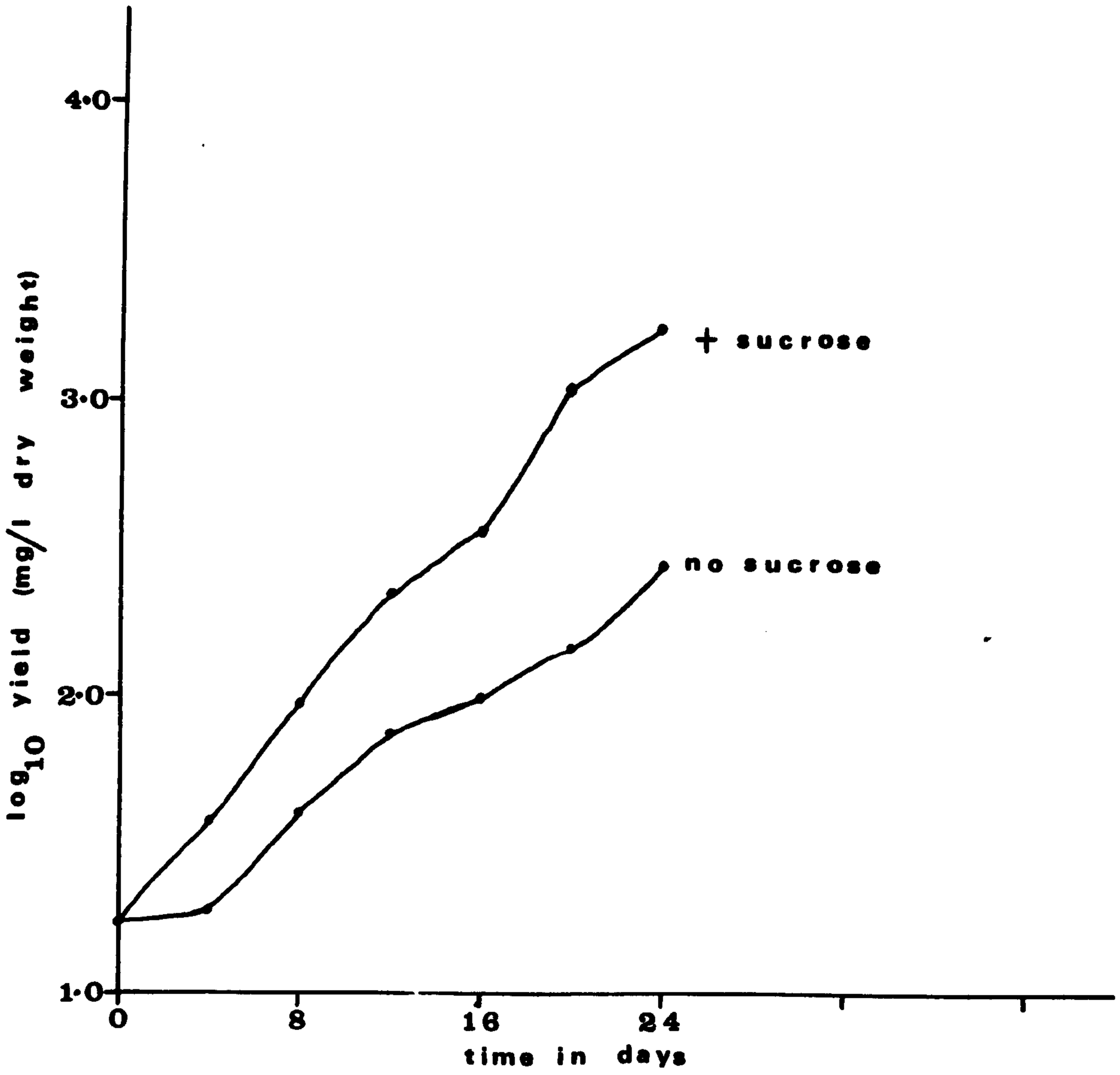

Fig. 9. Effect of 0.01M sucrose in the medium on growth of Tolypothrix tenuis incubated at 35°C, 500 lux and continuous shaking.

3.51 Growth rate studies on Chlorogloea fritschii

Growth was carried out in the presence and absence of 0.01M sucrose in AC medium, at 35°C, with continuous shaking. Inocula were taken from 30 day old culture, grown under similar light intensities to those used in the experimental series. These were approximately 500, 2000, and 4000 lux. Growth was noted at regular intervals and the results are shown in Fig. 10, 11, and 12; the standard error of the mean was between ± 0.1-7.5, 0.1-4.7, and 0.3-15% respectively.

With 500 lux, and basal medium, the doubling time (G) was about twice that of cultures with sucrose, Table VIII. As the light intensity was increased, sucrose was found to reduce the doubling time. The doubling time at 2000 lux with basal medium was 63.37 h, and at 4000 lux with the same medium, it was 57.33 h, whereas with sucrose the doubling time was reduced to 46.31 h and 41.76 h respectively. At the end of the exponential phase of growth, which lasted 8 days under 4000 lux, and 12 days under 500 and 2000 lux, the yield of organism in cultures grown on sucrose at 500 lux was 8 times that of control cultures. At 2000 lux the yield on sucrose was slightly more than three times that of the control, while it was just about three times that of control cultures at 4000 lux.

The results of the growth rate ($\dot{k}$) studies, using various light intensities and dark, are shown in Fig. 13. The effect of sucrose in light resulted in an increase of

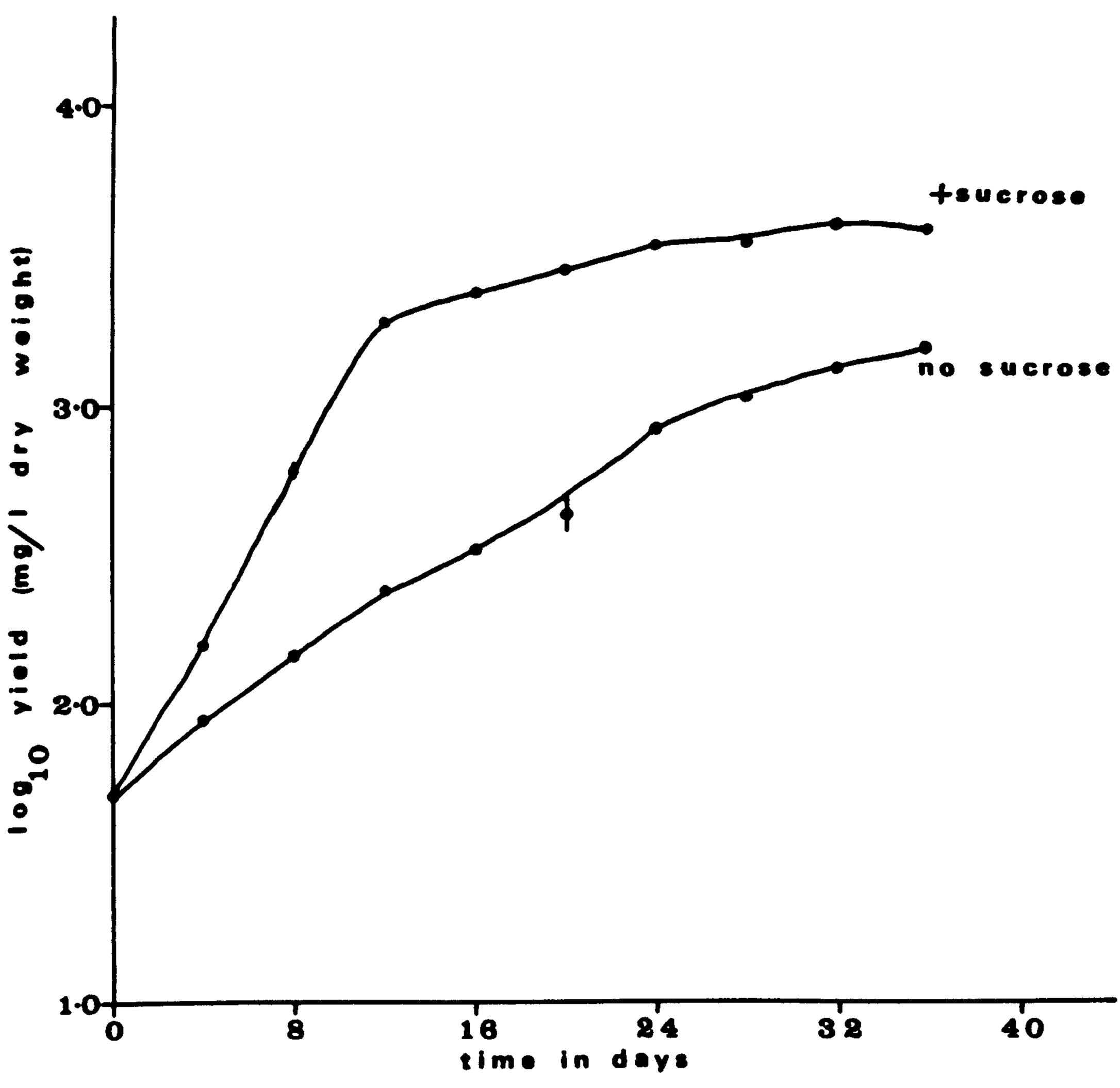

Fig. 10. Effect of 0.01M sucrose in the medium on growth of Chlorogloea fritschii incubated at 35°C, 500 lux and continuous shaking.

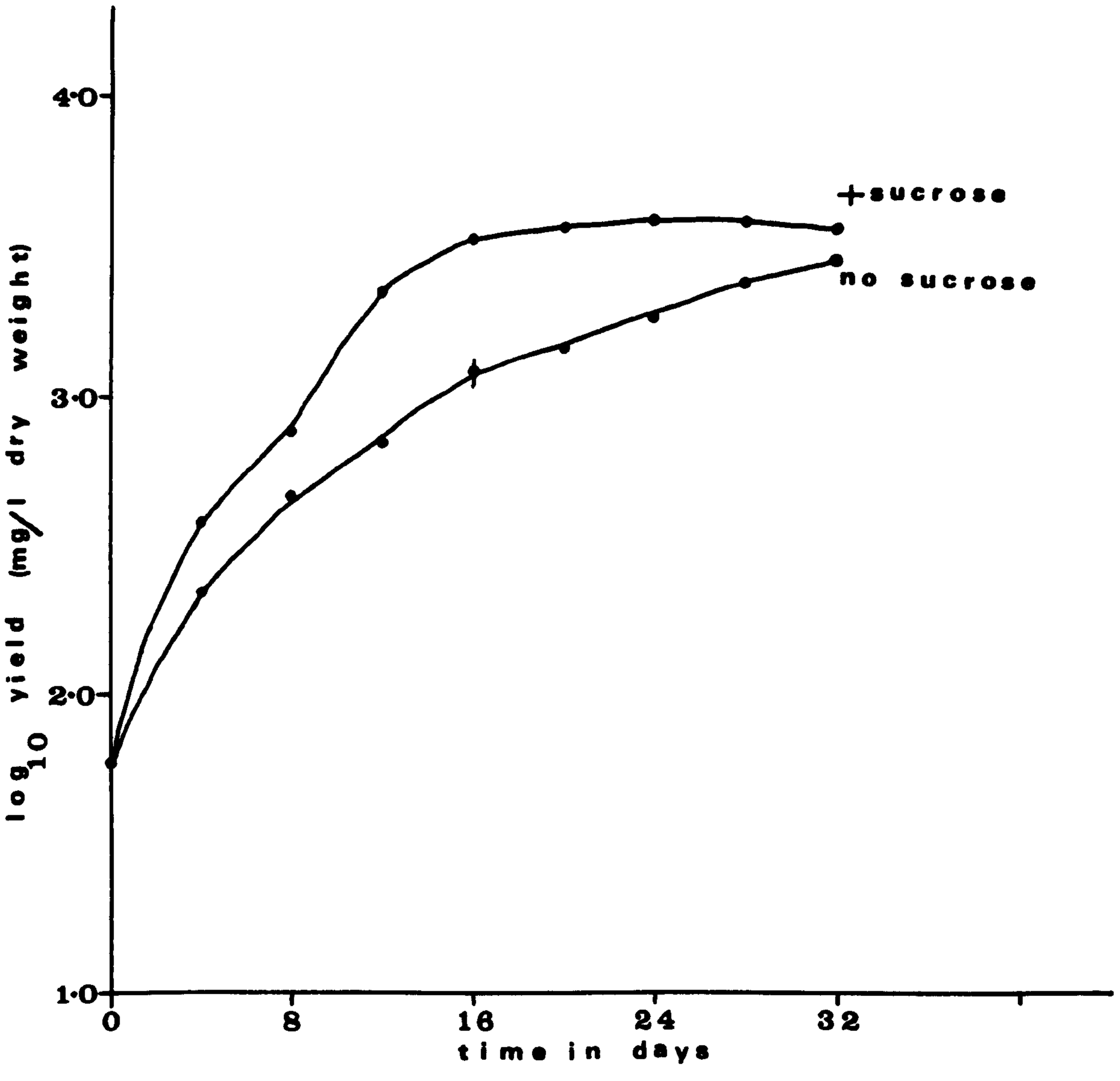

Fig. 11. Effect of 0.01M sucrose in the medium on growth of Chlorogloea fritschii incubated at 35°C, 2000 lux and continuous shaking.

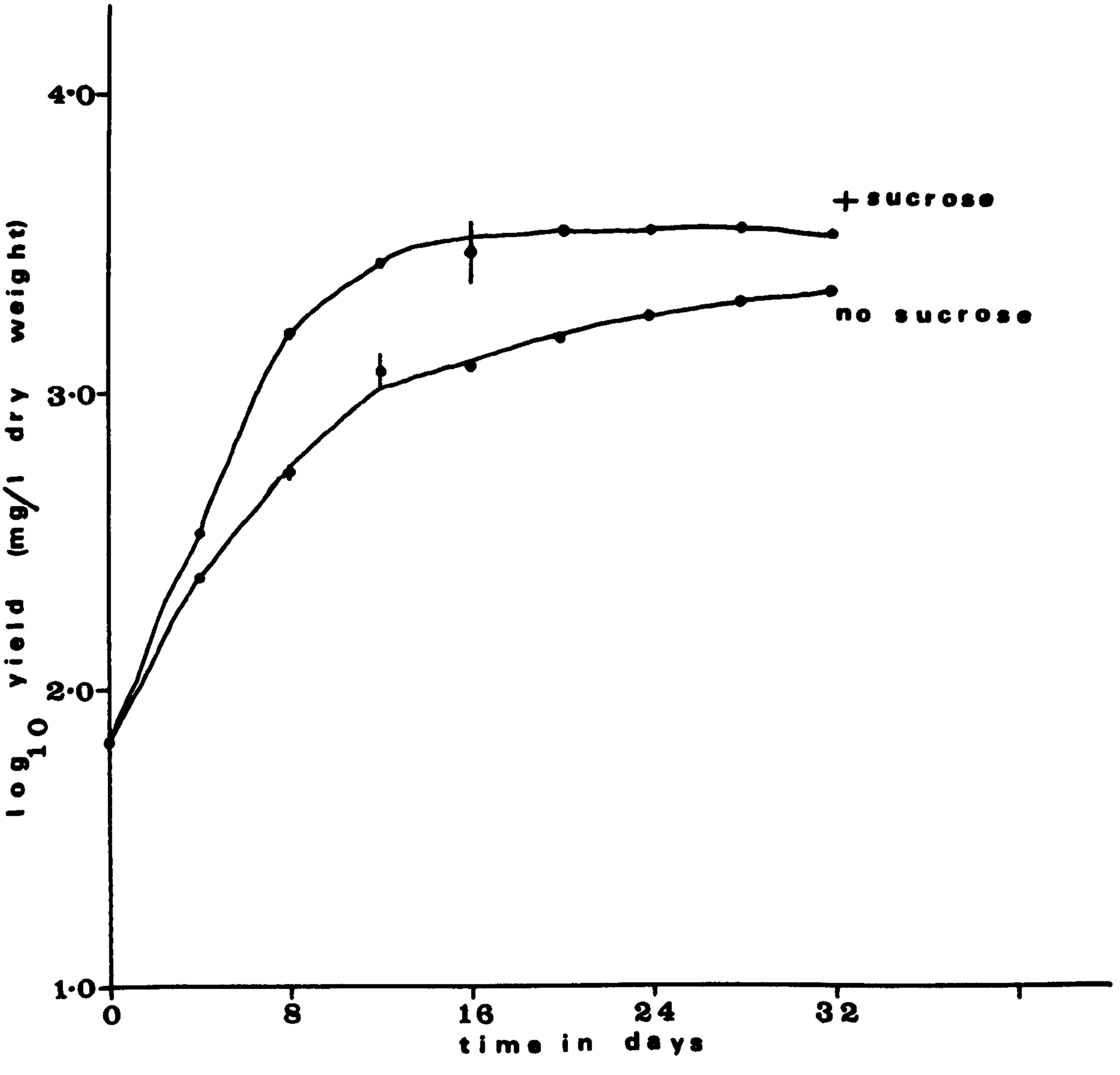

Fig. 12. Effect of 0.01M sucrose in the medium on growth of Chlorogloea fritschii incubated at 35°C, 4000 lux and continuous shaking.

Table VIII: Growth rate, $\dot{k}$, in $\log_{10}$ day units, and mean doubling time, G, in h, of Chlorogloea fritschii grown under different environmental conditions

light intensity (lux)	presence or absence of sucrose	growth rate ($\dot{k}$)	doubling time (G)
500	-	0.066	109.45
	+	0.137	52.73
2000	-	0.114	63.37
	+	0.156	46.31
4000	-	0.126	57.33
	+	0.173	41.76

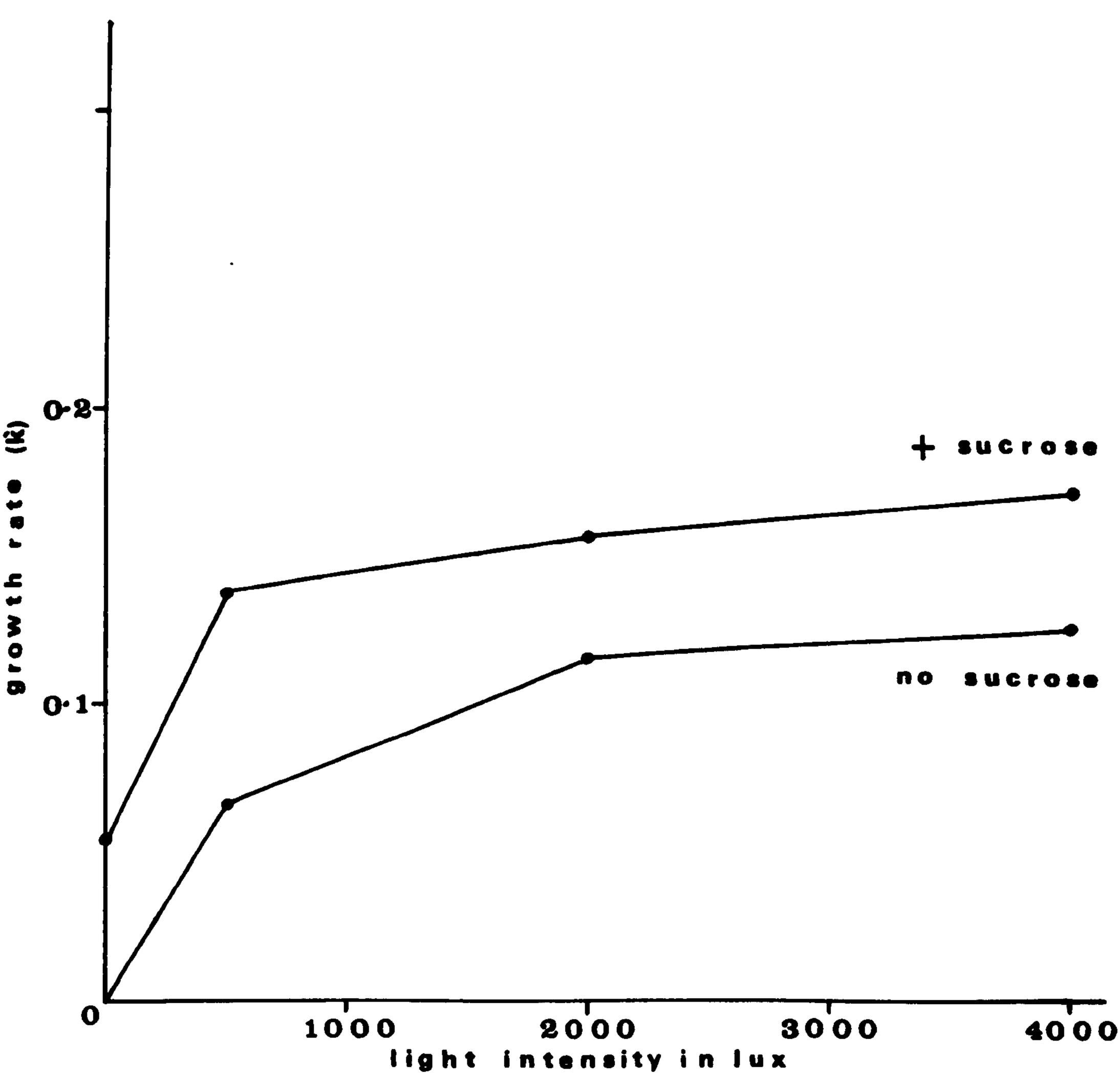

Fig. 13. Growth rates of Chlorogloea fritschii in the dark, and under different light intensities in the presence and absence of 0.01M sucrose in the medium.

the growth rate. As light intensity was increased, (up to 4000 lux covered in this experiment) sucrose was found to increase the growth rate. Sucrose increased the growth rate from 0.137 at 500 lux to 0.173 at 4000 lux.

3.52 Response of selected strains to sucrose at 500 lux

The strains studied were the same strains chosen for obtaining their growth rates in the dark (Section 3.32): Phormidium luridum, Phormidium sp. Plectonema boryanum D181, P. calothricoides. Growth was carried out at 35°C, in the presence and absence of 0.01M sucrose in AC medium. Before a run, the strains were grown for about 21 days under similar light intensity used in the experiments.

Response of Phormidium luridum to sucrose

There was no lag phase following inoculation into the medium to give a concentration of 27.39 mg/l dry weight. On sucrose, exponential growth was fast (doubling time 48.81 h), and lasted about 12 days (Fig. 14; the standard error of the mean was between ± 0.5-10.8%), while in the absence of sucrose the doubling time was, 95.1 h, about twice that of cultures grown on sucrose. After 16 days the yield of cultures with sucrose was about 5 times that of cultures without sucrose. Growth on sucrose was stationary on day + 32, and after day + 36 the alga showed gradual loss in dry weight.

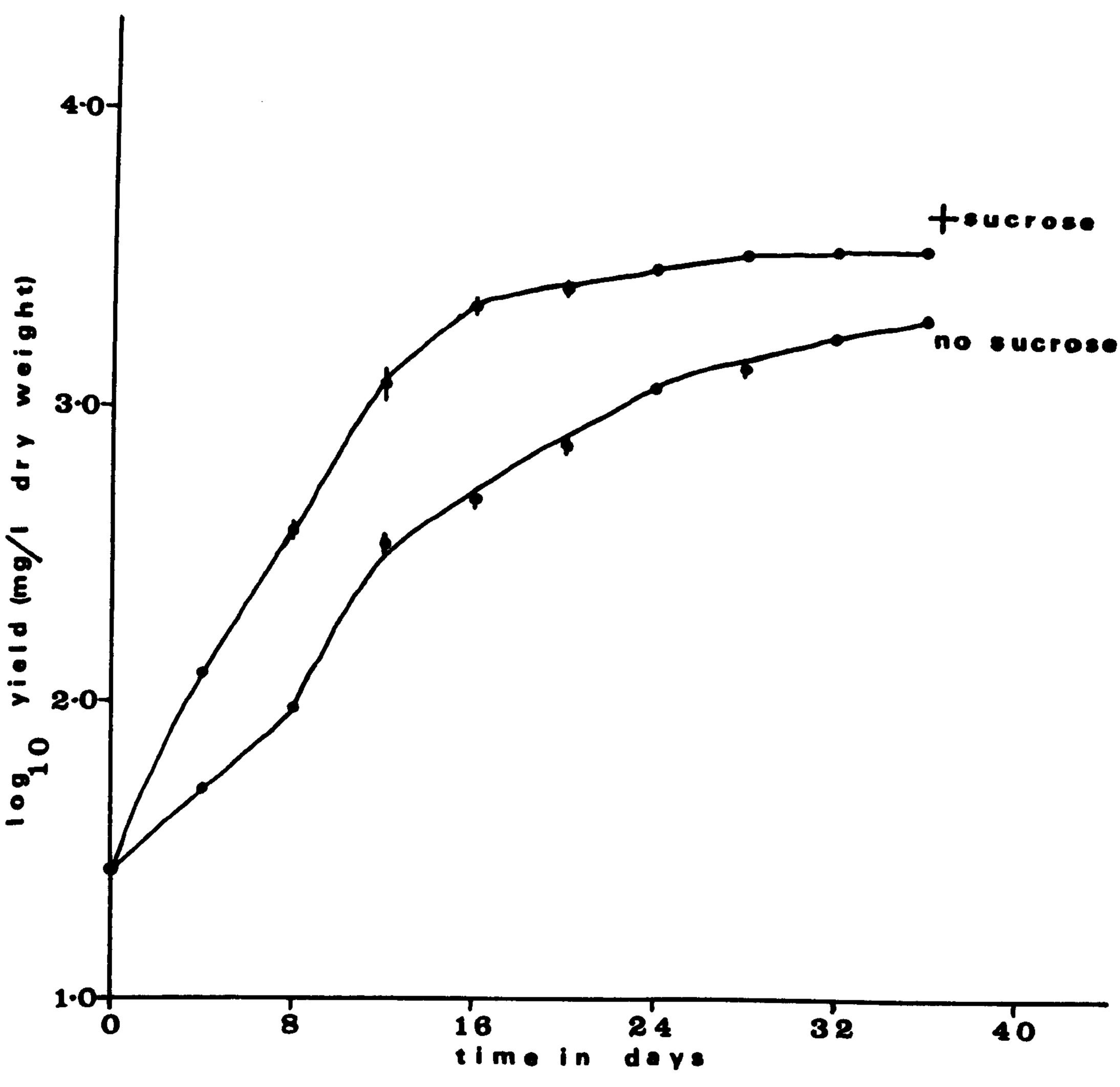

Fig. 14. Response of Phormidium luridum to 0.01M sucrose in the medium, grown at 35°C, 500 lux and continuous shaking.

Response of *Phormidium* sp. to sucrose

An initial inoculum containing 15.25 mg/l dry weight was used in this experiment, and the results are shown in Fig. 15; the standard error was between ± 0.1-4.6%. The exponential growth (about 8 days) of cultures grown on sucrose was faster than that of control cultures. Sucrose reduced the doubling time from 53.51 h with the basal medium, to 27.78 h. Growth rate declined after day + 8, and with sucrose on day + 24, growth was stationary. On day + 28 cultures grown on sucrose showed gradual loss in dry weight. The yield of organism in cultures grown on sucrose after 12 days was about 4 times that of control cultures.

Response of *Plectonema boryanum* D181 to sucrose

Response of this strain to sucrose at 500 lux is shown in Fig.16; the standard error of the mean was between ± 0.1-3.8%. An initial inoculum containing 23 mg/l dry weight was followed by an exponential phase of growth for 8 days. The doubling time of control cultures was 66.28 h and this was reduced to 36.48 h in cultures grown on sucrose. The growth rate started to decline on day + 12, and the growth on sucrose was stationary on day + 24. After this day, cultures grown on sucrose showed loss in dry weight. After 12 days the yield of the alga in cultures grown on sucrose was 4 times that of control cultures.

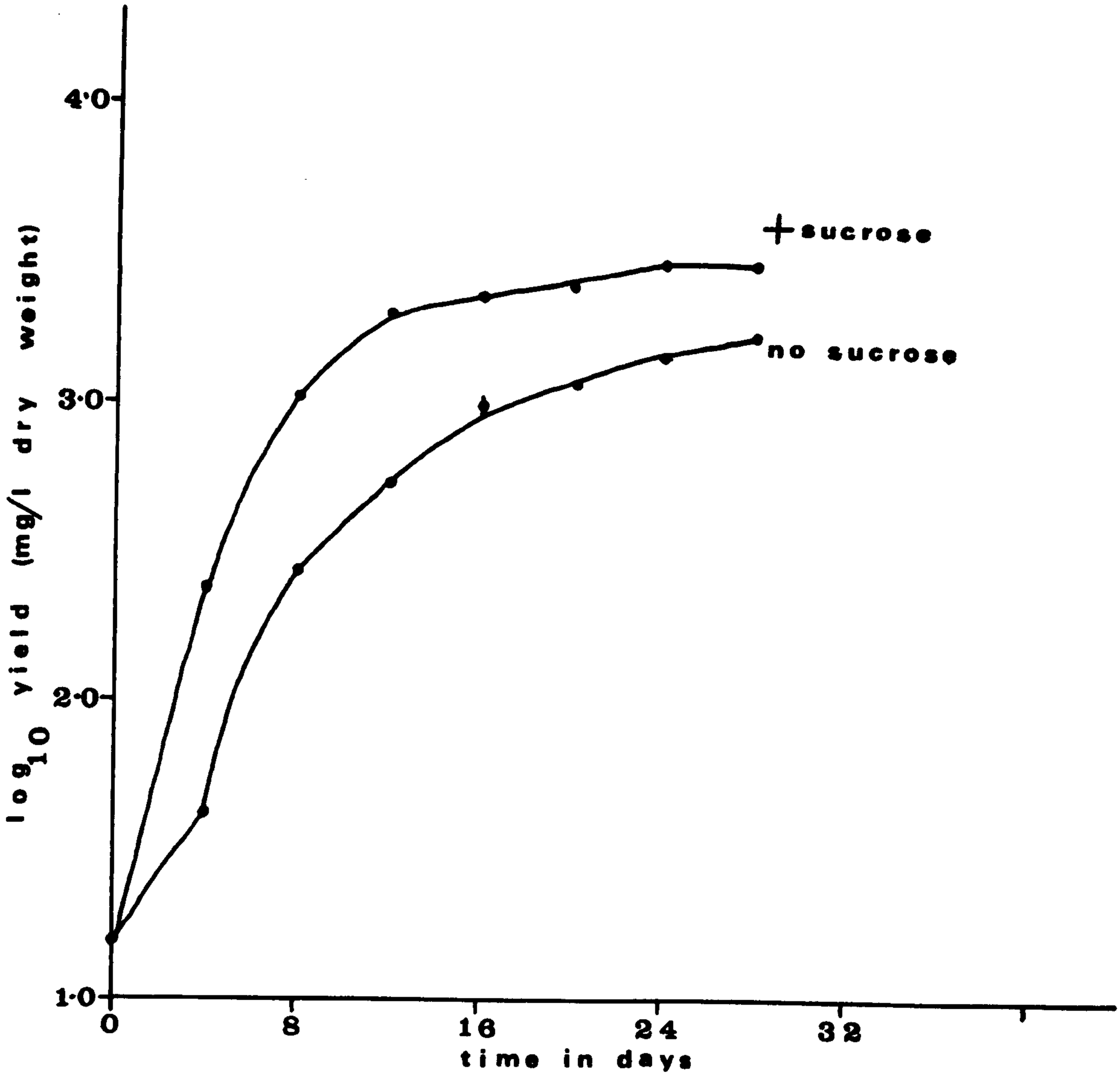

Fig. 15. Response of Phormidium sp. to 0.01M sucrose in the medium, grown at 35°C, 500 lux and continuous shaking.

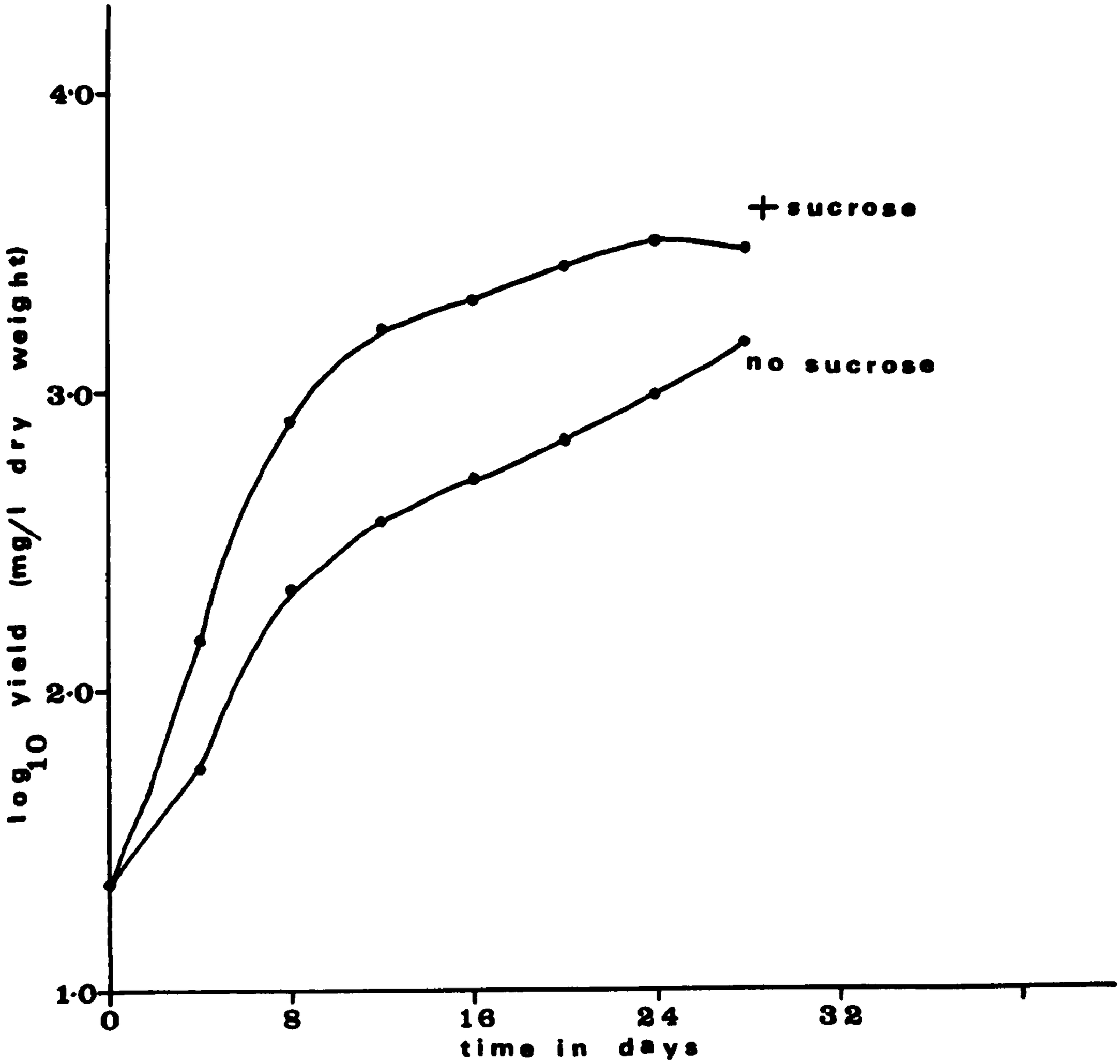

Fig. 16. Response of Plectonema boryanum D181 to 0.01M sucrose in the medium, grown at 35°C, 500 lux and continuous shaking.

Response of P. calothricoides to sucrose

Fig. 17 shows the effect of sucrose on the growth of this strain. The initial inoculum used, was 29.11 mg/l dry weight. As it can be seen from Fig. 17 (the standard error of the mean was between ± 0.1-8.6%) there was no lag phase, and the exponential growth lasted about 8 days. Growth rate declined after day + 8, and the growth of cultures grown on sucrose, was stationary on day + 24. On day + 28 cultures grown on sucrose showed loss in dry weight. The effect of sucrose on the growth of this alga resulted in a decrease of the doubling time from 44.1 h with basal medium to 30.48 h. The yield, after 8 days, in cultures grown on sucrose was 3 times that of control cultures.

3.53 Growth rate studies on selected strains

Growth was carried out in the presence and absence of 0.01M sucrose in AC medium, at 35°C, 500 lux, with continuous shaking. The growth rates ($\bar{k}$) of exponentially growing cultures of the strains selected (Phormidium luridum Phormidium sp., Plectonema boryanum D181, P. calothricoides) are shown in Table IX. The utilisation of sucrose at 500 lux by the strains selected resulted in an increase of the growth rate ($\bar{k}$) and a consequent decrease in the doubling time (G). Sucrose was found to increase the growth rate ($\bar{k}$) of Phormidium luridum, Phormidium sp. and Plectonema boryanum D181, to about twice that of control cultures. The

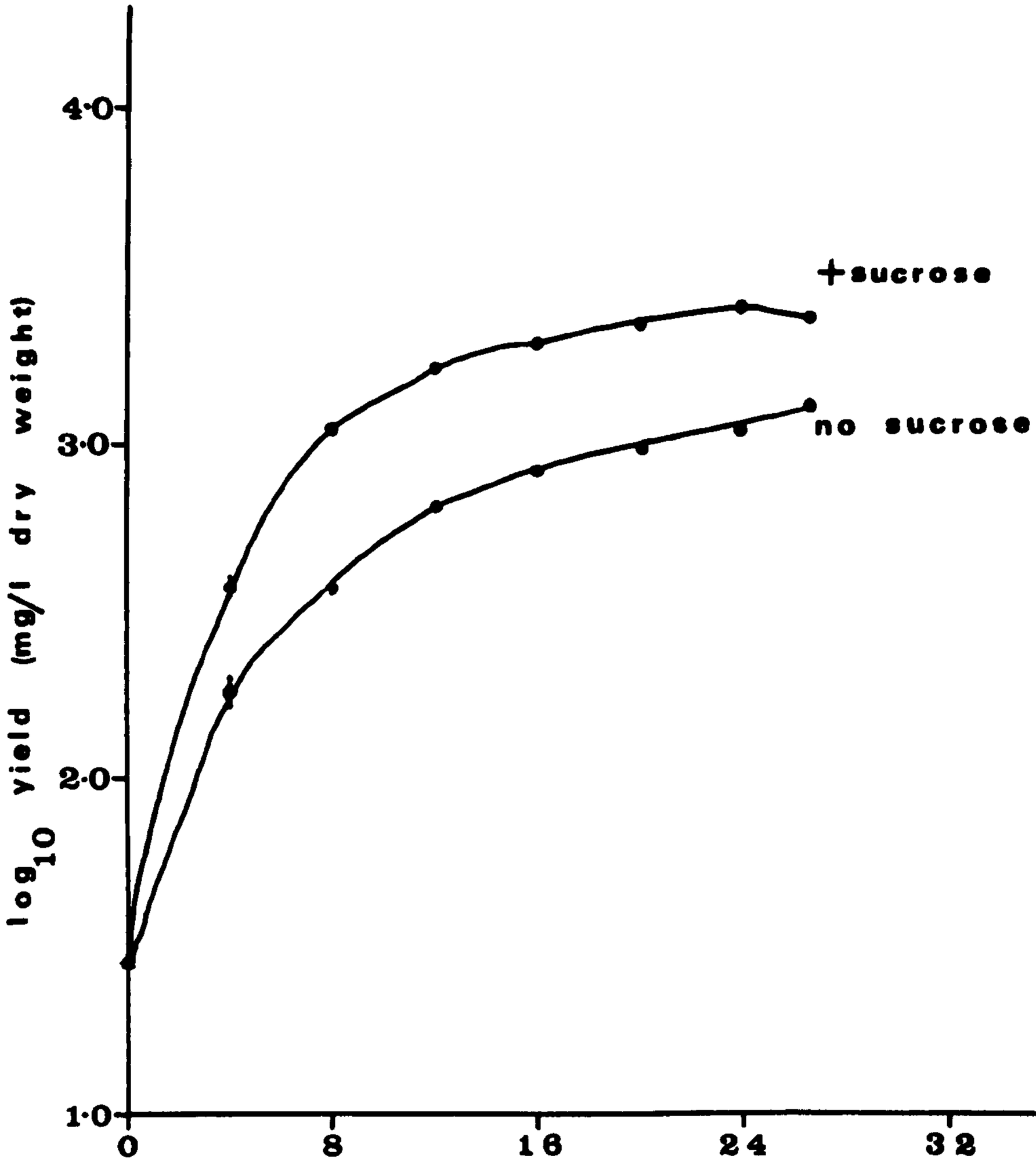

Fig. 17. Response of Plectonema calothricoides to 0.01M sucrose in the medium, grown at 35°C, 500 lux and continuous shaking.

Table IX: Growth rate, $\grave{k}$, in $\log_{10}$ day units, and mean doubling time, G, in h, of selected strains grown at 500 lux in presence and absence of 0.01M sucrose.

strain	presence or absence of sucrose	growth rate ($\grave{k}$)	doubling time (G)
Phormidium luridum	-	0.076	95.1
	+	0.148	48.81
Phormidium sp.	-	0.135	53.51
	+	0.261	27.78
Plectonema boryanum D181	-	0.109	66.28
	+	0.198	36.48
P. calothricoides	-	0.164	44.1
	+	0.237	30.48

growth rate (k̀) of Plectonema calothricoides in control cultures was 0.164. This was increased to 0.237 in cultures grown on sucrose.

3.6 Response of strains which failed to grow in the dark to sucrose at different light intensities

Studies on the effect of organic compounds on the growth of blue-green algae have revealed clearly, that, carbohydrates stimulate the growth of blue-green algae at low light intensities (Section 1). Therefore it was thought desirable to carry out further experiments with a view to determining whether sucrose can stimulate growth of the strains: Anabaena cylindrica, A. inaequalis, A. variabilis, Anacystis nidulans, Lyngbya sp., Oscillatoria temuis at different light intensities. The light intensities were approximately 20, 100, 500, and 1000 lux. Growth was carried out with two sets of flasks, one containing 0.01M sucrose, and the other without it. Anabaena cylindrica, A. inaequalis, A. variabilis were grown in AD medium at 25°C with contimuous shaking, while Anacystis nidulans, Lyngbya sp., and Oscillatoria temuis were grown in AC medium at 35°C, with contimuous shaking. All cultures were harvested after an incubation of 28 days.

Response of Anabaena cylindrica to sucrose

Initial inoculum consisting 10,06 mg/l dry weight was made from a culture grown for 15 days in AD medium, at

25°C, 2000 lux with continuous shaking. Fig. 18 shows the results of this experiment. The standard error of the mean was between±3.8-7.7%. Light intensities (20, 100 lux) which were insufficient for growth under photoautotrophic conditions did not produce a growth response in cultures with sucrose. Growth was slightly stimulated by sucrose at low light intensity of 500 lux. At this light intensity, the yield of organism, in cultures grown on sucrose was 374.14 mg/l dry weight, while on control cultures it was 257.09 mg/l dry weight. The difference between these two values is significant at the 5 per cent level of probability. Above 500 lux (1000 lux covered in the experiment) growth in cultures grown on sucrose decreased sharply.

Response of Anabaena inaequalis to sucrose

The results of this experiment are shown in Fig. 19; the standard error of the mean was between ± 0.94 - 9.5%. An initial inoculum containing 27.4 mg/l dry weight was made from a 21 day old culture, grown in AD medium, at 25°C, 2000 lux with continuous shaking. As it can be seen from Fig. 19, at low light intensities (20, 100 lux) which were not sufficient for growth under photoautotrophic conditions, there was no stimulation of growth by sucrose. At low light intensity of 500 lux, growth was slightly stimulated by sucrose, but at light intensity of 1000 lux, stimulation of growth by sucrose was better. At this light intensity the yield of the alga in cultures grown on sucrose was 949.46 mg/l dry weight, while on control cultures, it was 558.77 mg/l dry

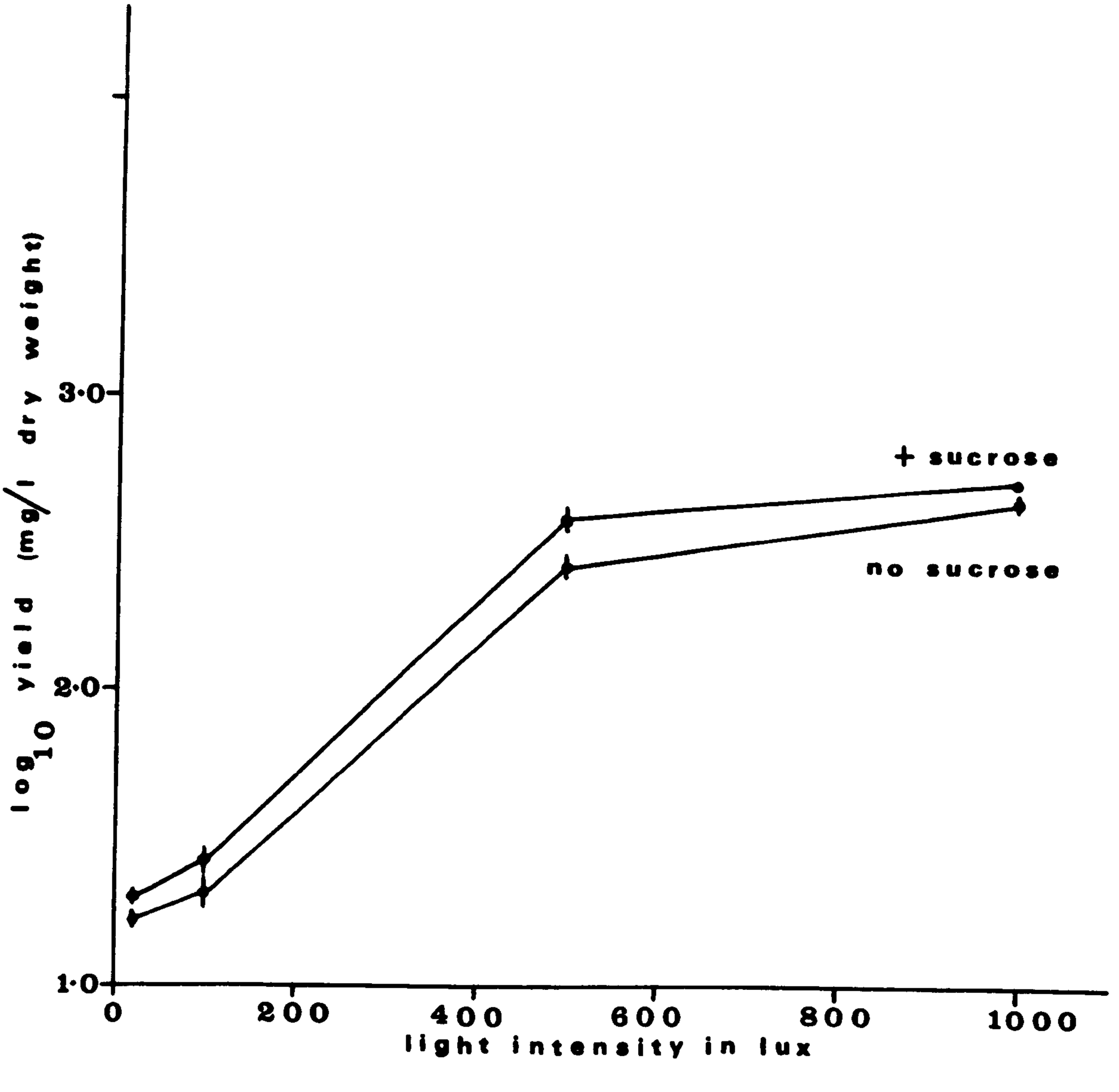

Fig. 18. Response of Anabaena cylindrica to 0.01M sucrose in the medium, grown at different light intensities, 25°C, and continuous shaking. Note the scale is different from that of Fig. 13.

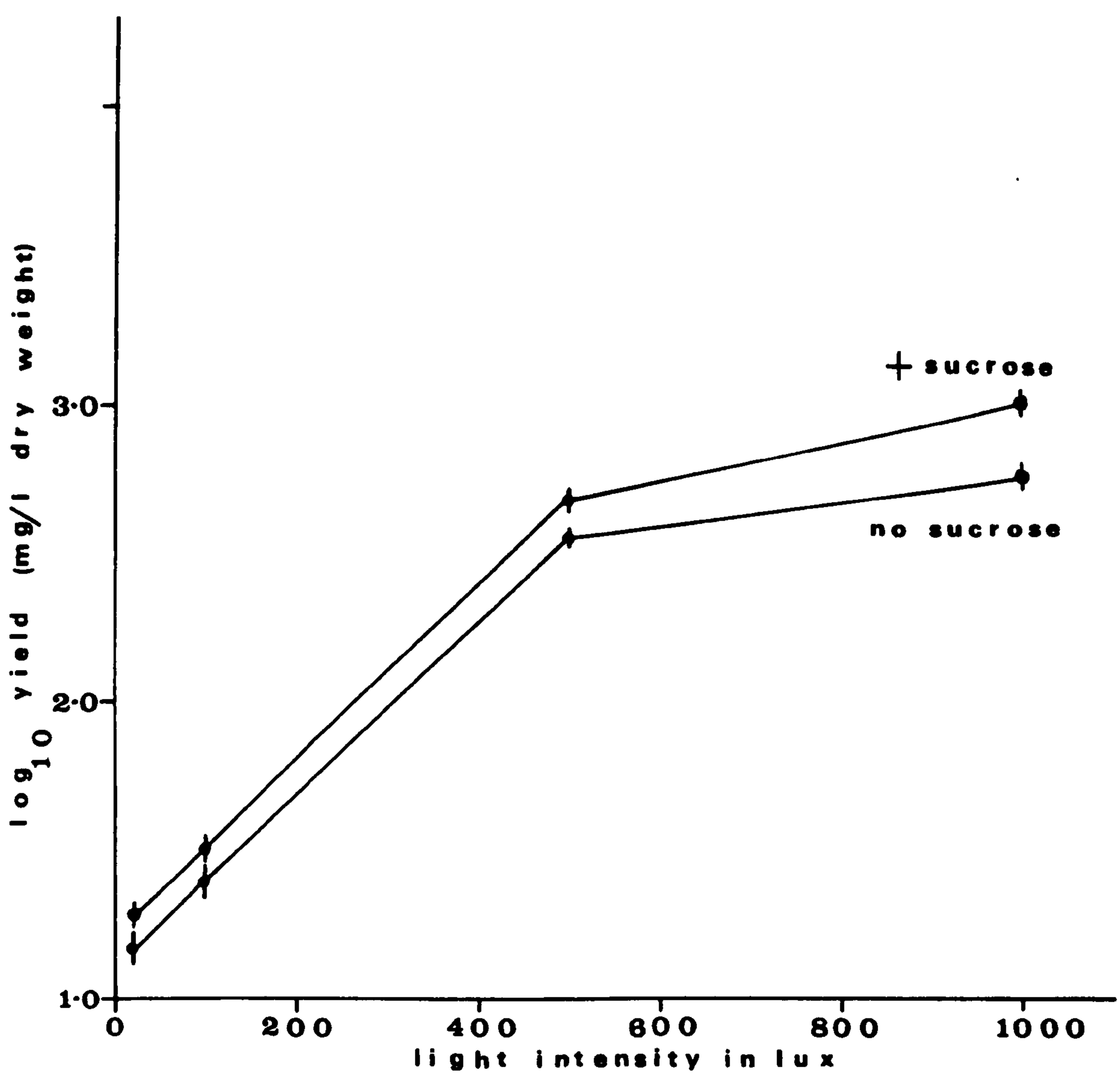

Fig. 19. Response of Anabaena inaequalis to 0.01M sucrose in the medium, grown at different light intensities, 25°C, and continuous shaking. Note the scale is different from that of Fig. 13.

weight. The difference between the two means is significant at the 1 per cent level of probability.

Response of Anabaena variabilis to sucrose

After maintaining this strain in AD medium at 25°C 2000 lux with continuous shaking, inoculum was made containing 46.95 mg/l dry weight. The results are shown in Fig. 20; the standard error of the mean was between ± 0.86-11.5%. There was no growth response to sucrose at low light intensity of 20, and 100 lux, which were insufficient to support photoautotrophic growth. Light intensity of 500 lux which was sufficient for growth under photoautotrophic conditions did not produce a growth response in cultures with sucrose. At light intensity of 1000 lux, growth was very slightly stimulated by sucrose, and the yield in cultures grown on sucrose was 905.3 mg/l dry weight, while on control cultures, it was 828.6 mg/l dry weight. The difference between these two values is not statistically significant. P (Probability) amounting at 30 per cent level of probability.

Response of Anacystis nidulans to sucrose

Inoculum containing 13.23 mg/l dry weight was made from an 8 day old culture, grown in AC medium at 35°C, 4000 lux with continuous shaking. The results of this experiment are shown in Fig. 21; the standard error of the mean was between ± 0.7-6.6%. At low light intensities (20, 100 lux)which

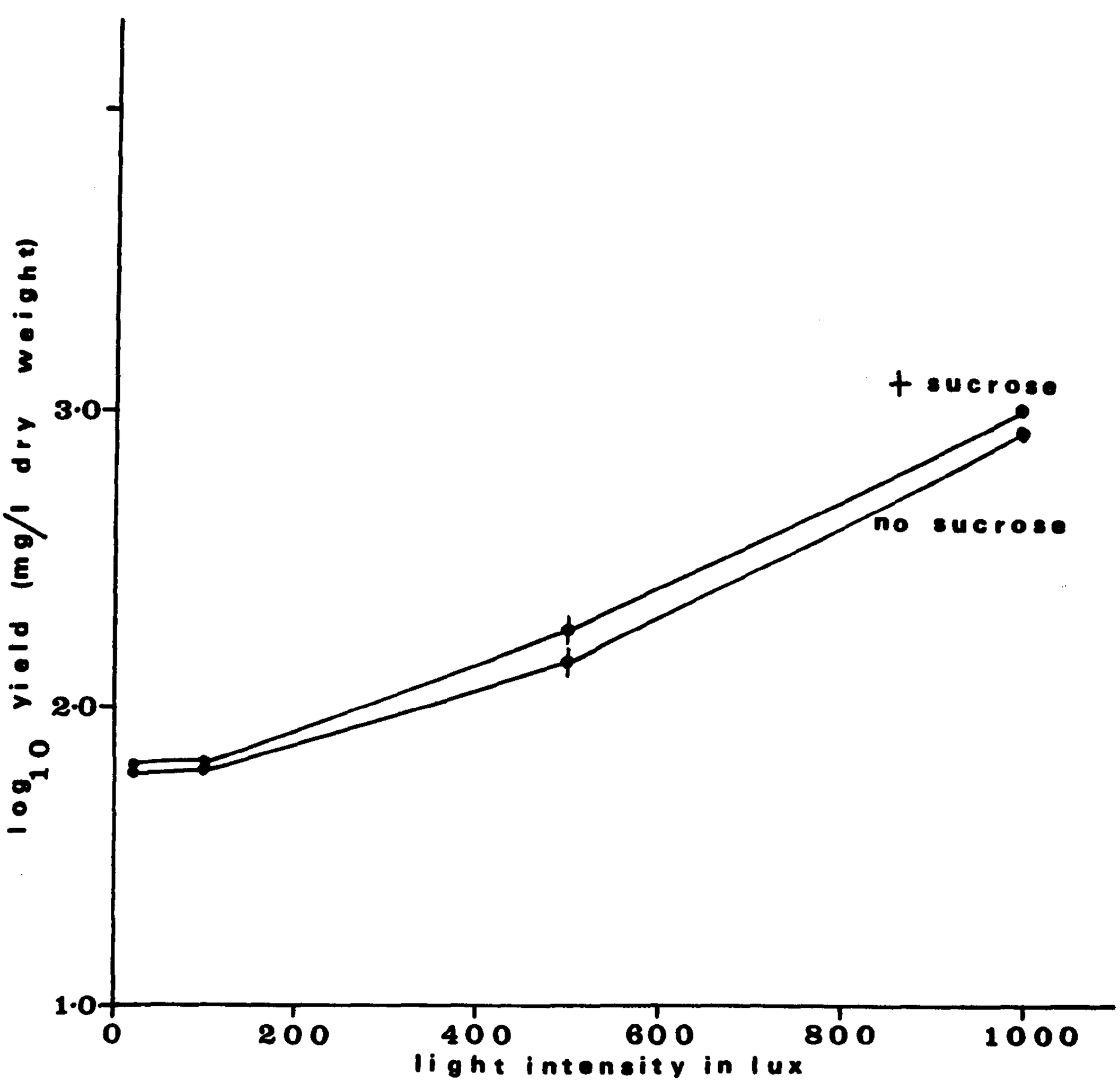

Fig. 20. Response of Anabaena variabilis to 0.01M sucrose in the medium, grown at different light intensities, 25°C, and continuous shaking. Note the scale is different from that of Fig. 13.

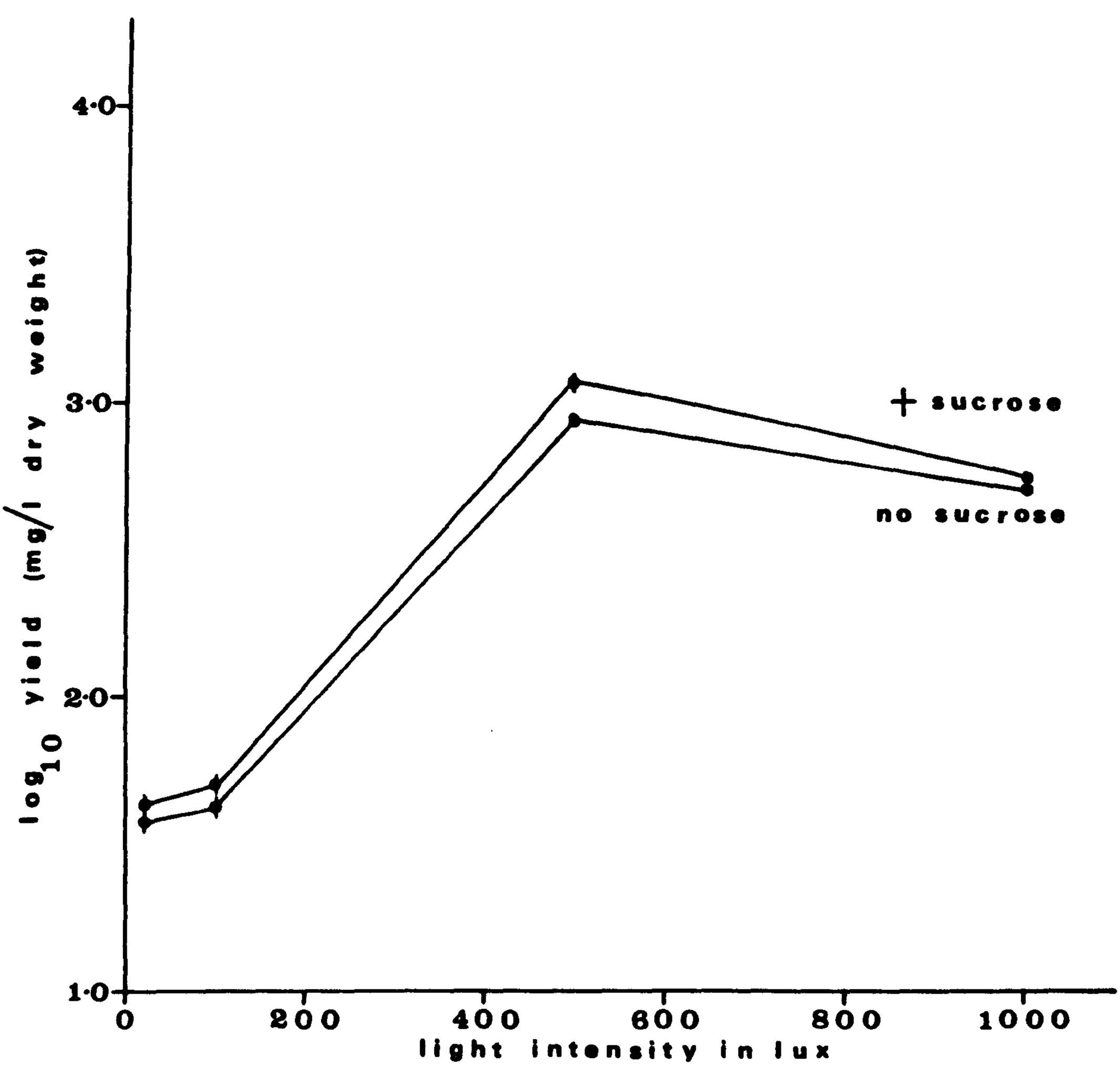

Fig. 21. Response of Anacystis nidulans to 0.01M sucrose in the medium, grown at different light intensities, 35°C, and continuous shaking. Note the scale is different from that of Fig. 13.

were insufficient for growth under photoautotrophic conditions, there was no growth response to sucrose. Sucrose stimulated the growth at low light intensity (500 lux), the yield in cultures grown on sucrose was 1134.55 mg/l dry weight, while on control cultures, it was 861 mg/l dry weight. The difference between the two means is significant at the 5 per cent level of probability. At a light intensity of 1000 lux, growth in cultures grown with or without sucrose decreased sharply.

Response of Lyngbya sp. to sucrose

The response of this strain to sucrose at different light intensities is shown in Fig. 22; the standard error of the mean was between±6.5-17.3%. An initial inoculum consisting of 18.48 mg/l dry weight, was made from a 14 day old culture, grown in AC medium at 35°C, 2000 lux with continuous shaking. Light intensities (20, 100 lux) which were insufficient for photoautotrophic growth did not produce a growth response in cultures with sucrose. Growth was stimulated by sucrose at low light intensity (500 lux). At this light intensity, the yield of organism in cultures, grown on sucrose was about twice that of control cultures. At light intensity of 1000 lux, growth in cultures with or without sucrose was decreased. Good replication of these results were obtained in a further experiment.

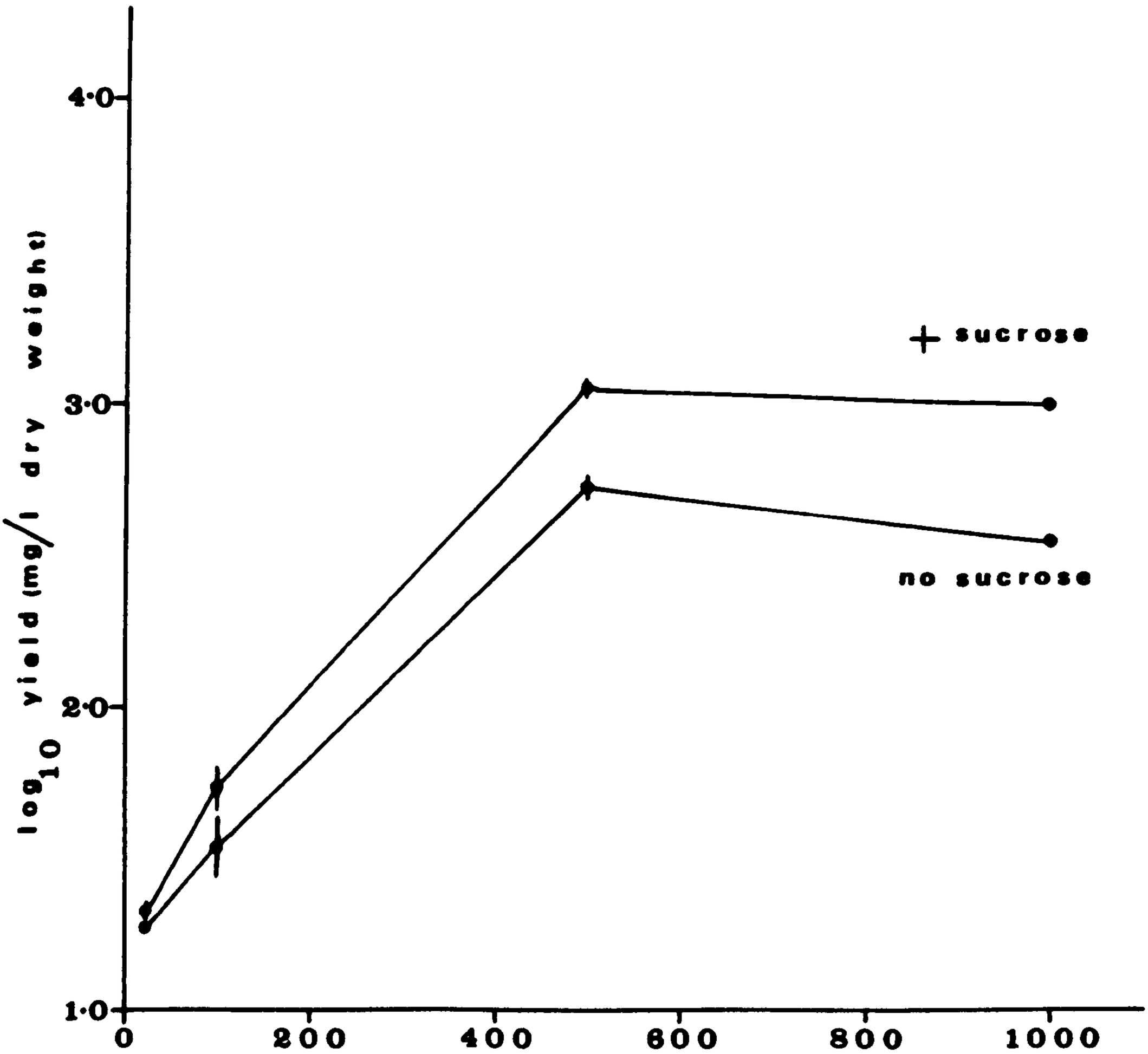

Fig. 22. Response of Lyngbya sp. to 0.01M sucrose in the medium, grown at different light intensities, 35°C, and continuous shaking. Note the scale is different from that of Fig. 13.

Response of Oscillatoria tenuis to sucrose

An initial inoculum containing 21.55 mg/l dry weight was made from a 15 day old culture, grown in AC medium at 35°C, 2000 lux with continuous shaking. The response of this alga to sucrose at different light intensities is shown in Fig. 23; the standard error of the mean was between ± 0.5-6.6 %. At low light intensities (20, 100 lux) which were insufficient for growth under photoautotrophic conditions there was no growth response. Best stimulation of growth was found at low light intensity (500 lux). At this low light intensity, the yield of organism in cultures, grown on sucrose was about 3 times that of control cultures. Growth in cultures grown with or without sucrose, decreased at light intensity of 1000 lux. Good replication of these results were obtained in a further experiment.

3.7 Growth rate studies in light

Growth rate under photoautotrophic conditions, was determined for Chlorogloea fritschii and for the same strains chosen for obtaining their growth rates under heterotrophic conditions (Section 3.32): Phormidium luridum, Phormidium sp., Plectonema boryanum D181, P. calothricoides.

3.71 Growth rate of Chlorogloea fritschii

Growth was carried out in AC medium, under optimum conditions (standard growth conditions, Section 2.48)

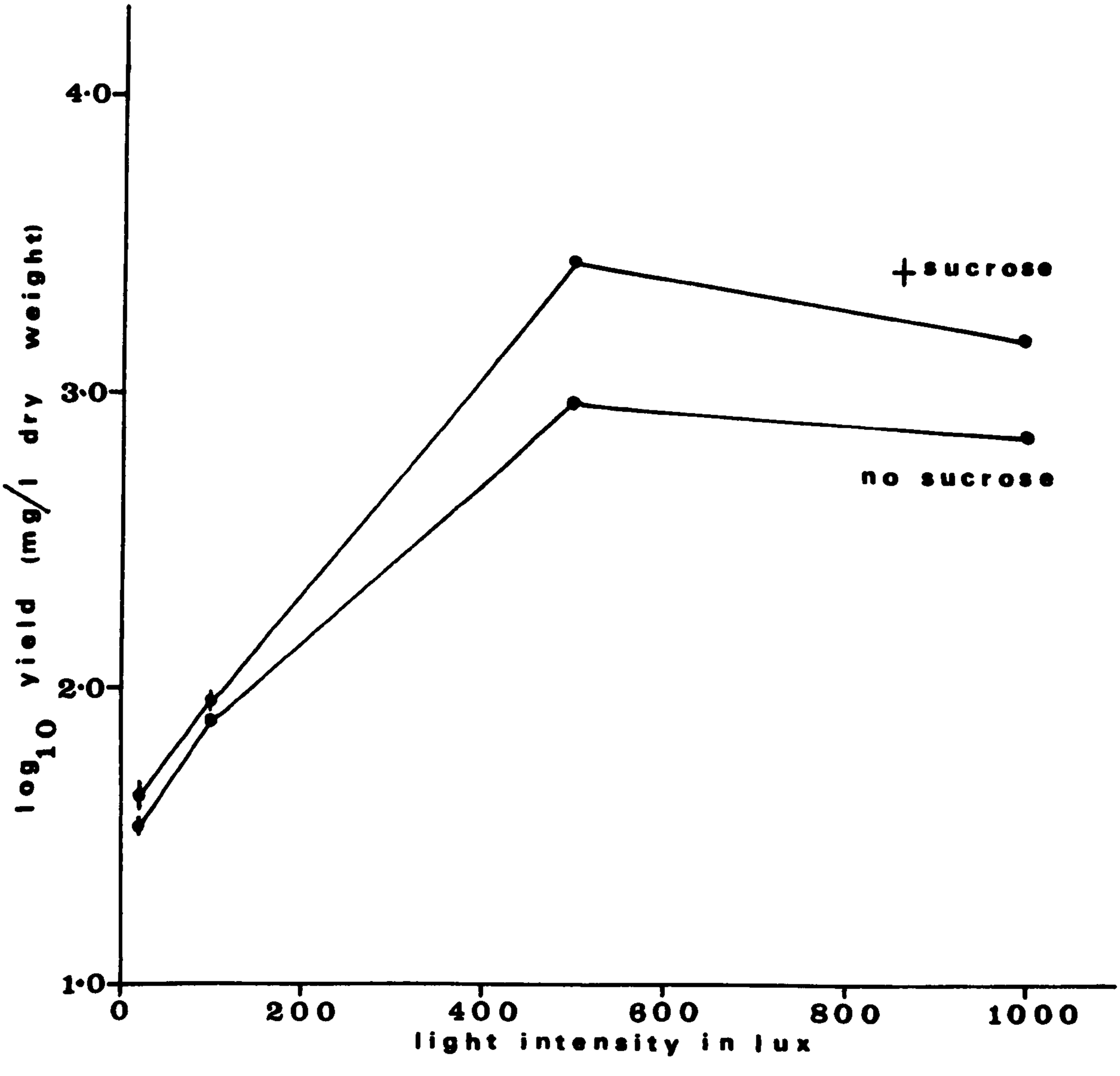

Fig. 23 Response of Oscillatoria temuis to 0.01M sucrose in the medium, grown at different light intensities, 35°C, and continuous shaking. Note the scale is different from that of Fig. 13.

obtained by FAY and FOGG (1962). An initial inoculum containing 60.21 mg/l dry weight was made from a 25 day old culture, grown in AC medium, under the standard growth conditions (Section 2.48). Growth was observed at regular intervals and the results are shown in Fig. 24; the standard error of the mean was between ± 0.2 - 6.7%. There was no lag phase, Exponential growth was slow (doubling time of 53.12 h, and growth rate ($\bar{k}$) of 0.136, Table X), and lasted about eight days. After day + 8, growth rate declined, and on day + 40 growth was stationary. The final yield obtained was 3207.8 mg/l dry weight.

3.72 Growth rate of selected strains

The strains studied here were the same strains chosen for growth rate studies (Section 3.32-3.53): Phormidium luridum, Phormidium sp., Plectonema boryanum D181 and P. calothricoides. These selected strains grow well in a variety of algal culture media but AC medium was the most satisfactory of those tested. From preliminary observations, it was clear that these strains grow best at 2000 lux, 35°C. However, the temperature levels used to determine the optimum temperature were fairly wide and it is probable that the optimum temperature is 35°C ± 3°C.

Growth was carried out in AC medium at 35°C, 2000 lux, with continuous shaking (Standard growth conditions, Section 2.48). Inocula were taken from a 12 day old culture, grown in AC medium under the standard growth

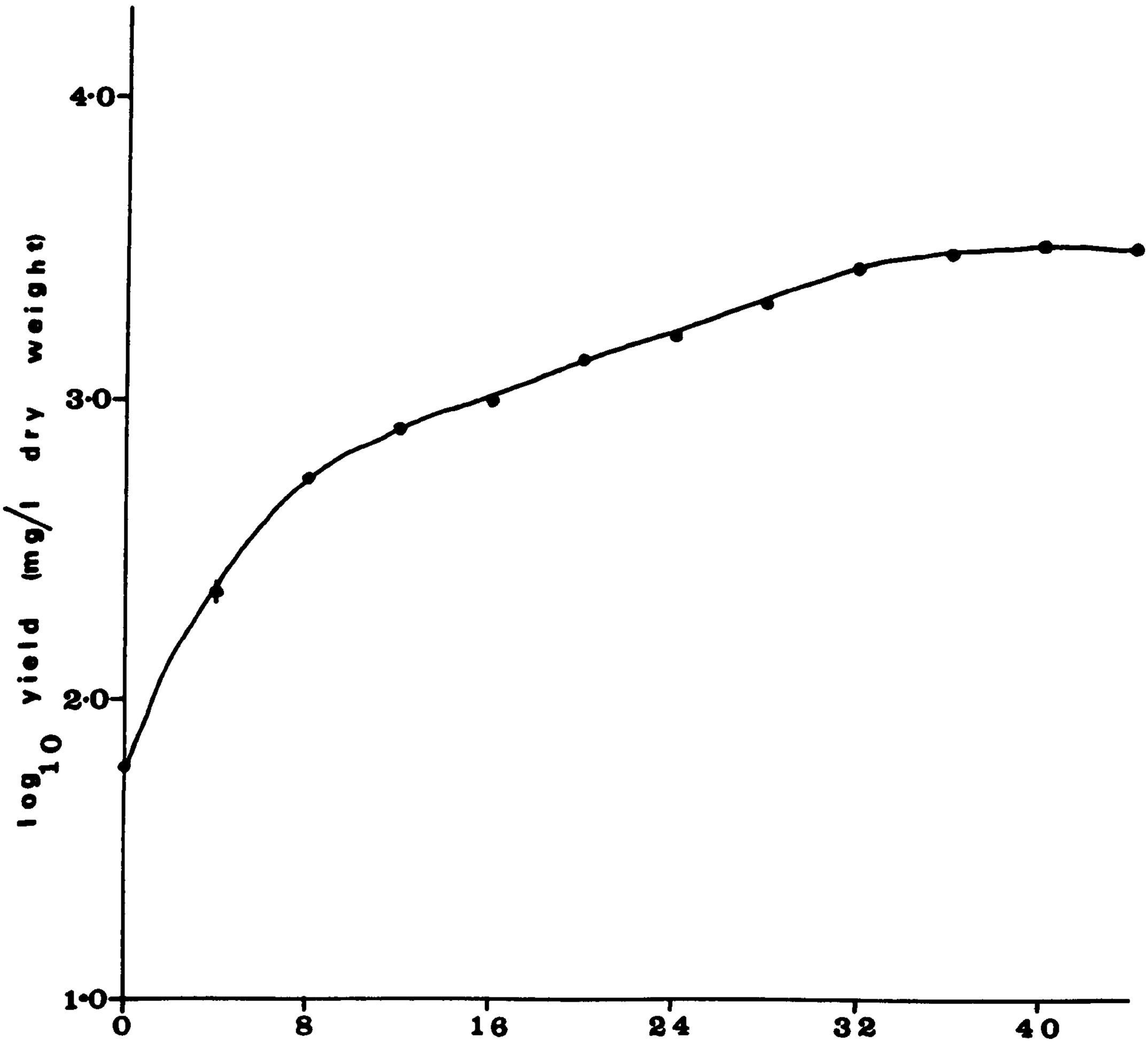

Fig. 24. Photoautotrophic growth of Chlorogloea fritschii, grown under the standard growth conditions (Section 2.48).

conditions (Section 2.48). Growth was followed at regular intervals, and the results are shown in Fig. 25, 26, 27, and 28; the standard error of the mean was between ± 2.8 - 6, 0.6 - 4.4, 1.3 - 3.8, and 1 - 7.6% respectively. There was no detectable lag phase. Exponential growth was fast, and lasted 32 - 48 h. Growth rate ($\bar{k}$) declined after 32 - 48 h. Table X show the results of the growth rate together with the doubling time in h. Growth rate of Phormidium luridum was 0.619 and had a doubling time (11.67 h) faster than Phormidium sp. (13.21 h). Plectonema boryanum D181 had a slow doubling time (14.8 h), while the doubling time of P. calothricoides was (10.99 h) the fastest among these selected strains.

4. Pigment content of light and dark-grown cultures

In order to test whether or not light plays any role in regulating the synthesis of the photosynthetic apparatus, pigmentation studies were made on light and dark-grown cultures of six strains: Calothrix membranacea, Chlorogloea fritschii, Phormidium luridum, Phormidium sp., Plectonema boryanum D181, and P. calothricoides. The heterotrophic cultures were incubated in complete darkness (Section 2.42) at 35°C in AC medium plus 0.01M sucrose. Growth in light was carried out in AC medium at 35°C, 500 lux, with contimuous shaking. Inocula for the heterotrophic cultures were made from 35 day old cultures (first subculture in dark), grown in AC medium plus 0.01M sucrose,

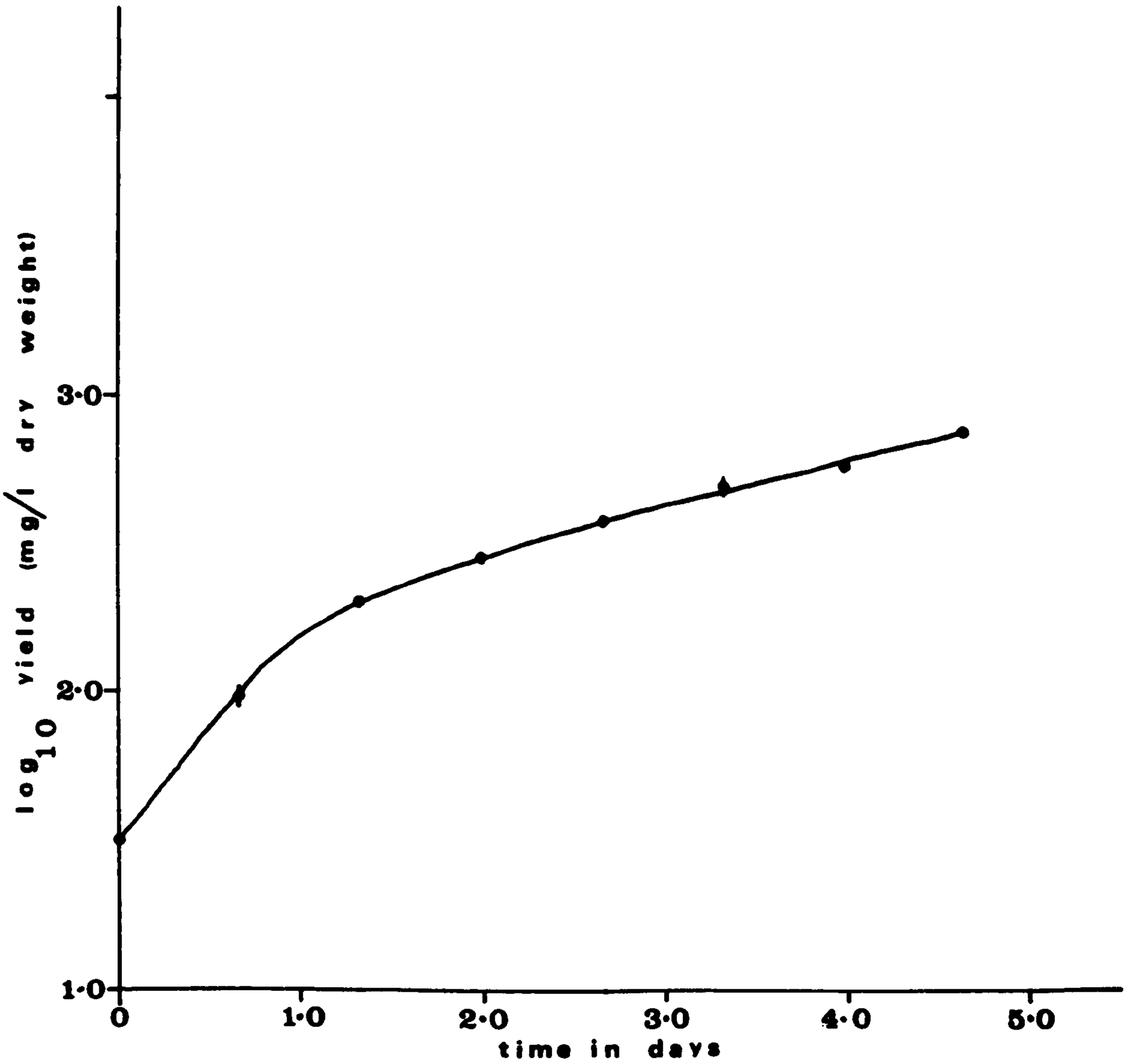

Fig. 25. Photoautotrophic growth of Phormidium luridum, grown under the standard growth conditions (Section 2.48)..Note the scale is different from that of Fig. 24.

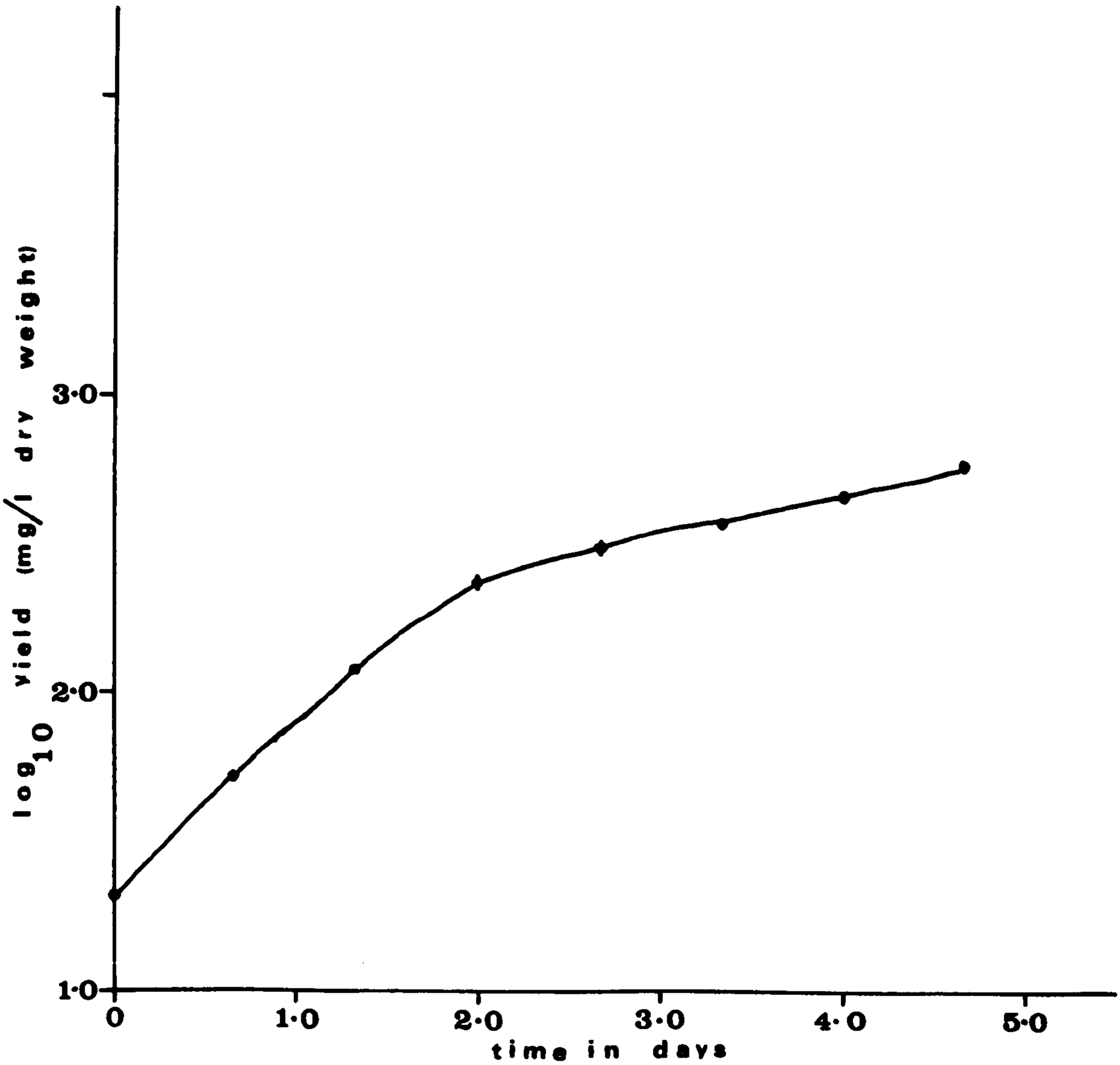

Fig. 26. Photoautotrophic growth of Phormidium sp., grown under the standard growth conditions (Section 2.48) Note the scale is different from that of Fig. 24.

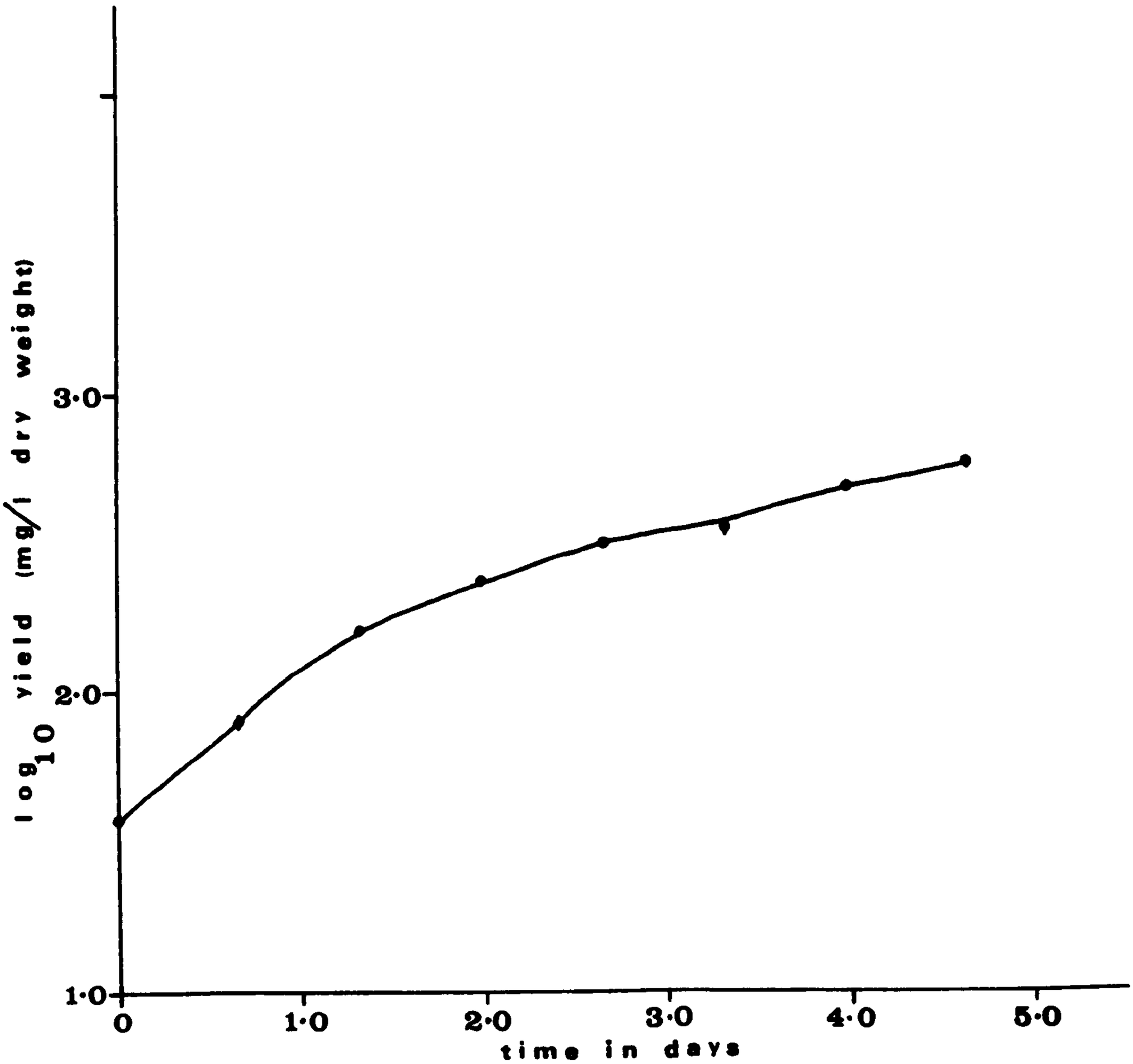

Fig. 27. Photoautotrophic growth of Plectonema boryanum D181, grown under the standard growth conditions (Section 2.48). Note the scale is different from that of Fig. 24.

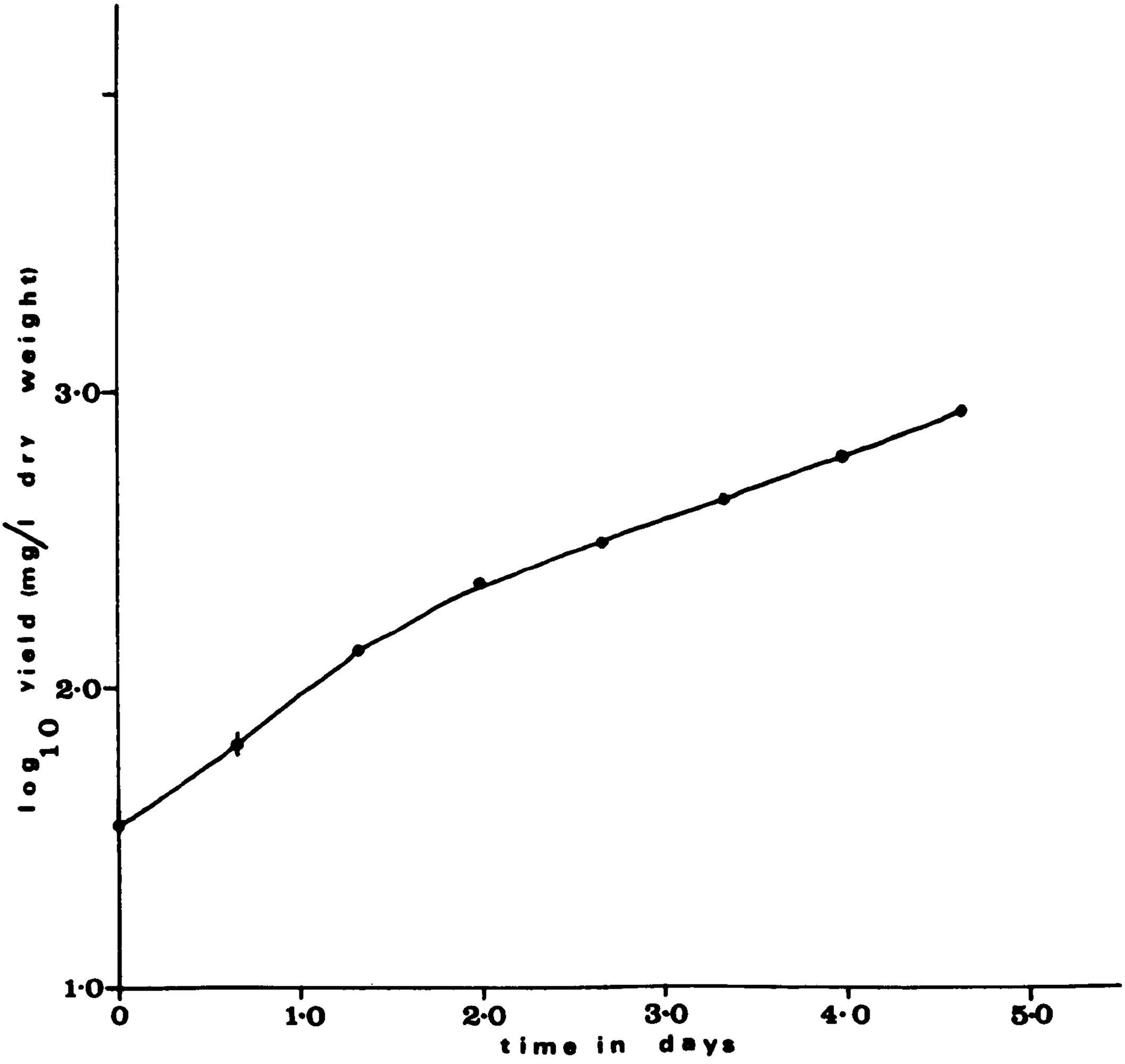

Fig. 28. Photoautotrophic growth of Plectonema calothricoides, grown under the standard growth conditions (Section 2.48). Note the scale is different from that of Fig. 24.

Table X: Growth rate, $\dot{k}$, in $\log_{10}$ day units, and mean doubling time, G, in h, of Chlorogloea fritschii, and selected strains, grown in the light under the standard growth conditions (Section 2.48)

strain		growth rate ($\dot{k}$)	doubling time (G)
Chlorogloea	fritschii	0.136	53.12
Phormidium	luridum	0.619	11.67
Phormidium	sp.	0.547	13.21
Plectonema	boryanum D181	0.488	14.8
P.	calothricoides	0.657	10.99

at 35°C in complete darkness (Section 2.42). Inocula for the photoautotrophic cultures were taken from 25 day old cultures, grown in AC medium at 35°C, 500 lux with continuous shaking. All cultures were harvested after an incubation of 21 days.

4.1 Chlorogloea fritschii

Quantitative extraction of the pigments of this alga presented a problem not normally associated with pigment extraction of other blue-greenalgae studied so far, and proved quite difficult. The hot methanol as well as cold methanol and grinding the alga with acid-washed fine sand with acetone were not effective. Yellowish-green pigments were generally extracted, but after filtration, the cells remained pigmented even after repeated extraction. These results suggested that cell disruption was necessary for the quantitative extraction of pigments from Chlorogloea fritschii. Therefore various methods were tried in an attempt to find effective methods for disrupting the cells of this alga. Sonication of fresh and freeze-dried cells did not facilitate extracting the cells to completion. Attempts to facilitate leakage of pigments from the algal cells by enzymatic digestion of the cell walls were also tried. Phycocyanin was not released from the cells even after an incubation of 96 h with lysozyme, whereas, with the selected strains (Section 4.2) phycocyanin was released from the cells after overnight

incubation with lysozyme. However, sonication of lysozyme-treated cells (24 h) increased the amount of chlorophyll, but the cells still remained pigmented, even after sonication for 40 min. The cell walls of different species of blue-green algae vary in sensitivity to lysozyme (CRESPI et al., 1962; BERNS et al., 1966). This is confirmed here by the lysozyme activity in digesting the cell walls of selected strains (Section 4.2) and by the insensitivity of C. fritschii to lysozyme.

Since contimuous in vivo chlorophyll a measurements by a sensitive fluorometer proved to be a useful tool in broad ecological programs carried out at sea or lake (LORENZEN, 1966; NICHOLSON, 1970), it was, thought that this technique might offer an alternative method for measuring the chlorophyll a concentration of this alga. Unfortunately the amount of chlorophyll obtained by the fluorometer was less than that obtained by 90% hot methanol. Experiments were repeated several times, particularly with Phormidium luridum which was selected for comparing the amount of its chlorophyll by the fluorometer, with the amount of its chlorophyll by 90% hot methanol. The amount of chlorophyll obtained by the fluorometer was far less than that by 90% hot methanol. For reasons that remain unclear, the fluorometer is not sensitive in measuring the in vivo chlorophyll a of cultures grown in the laboratory while it proved sensitive in measuring the in vivo chlorophyll a of phytoplankton in natural waters.

The assertion by PIKE (1970, 1971) that quantitative measurement of the pigments of Chlorogloea fritschii was successfully obtained by means of the freeze-drying and the french press must now be questioned in view of the results of the present studies. PIKE (1970) obtained 3.3 µg chlorophyll per mg dry weight for C. fritschii growing heterotrophically in the dark, and 7.7 µg chlorophyll per mg dry weight for the same strain growing at low light intensity with sucrose. In the present studies the highest amount of chlorophyll extracted from cells of C. fritschii was 7.1 µg per mg dry weight for cultures grown heterotrophically in complete darkness and 7.2 µg per mg dry weight for cultures grown photoautotrophically at 500 lux. Hence, it is extremely difficult to believe that the technique used by PIKE (1970) facilitated extracting the cells of C. fritschii to completion. Even, if one scarcely believes that PIKE (1970) extracted the cells to completion, it is very difficult to believe that C. fritschii has lower amount of pigments than other blue-green algae. However, the amount of pigments extracted from cells of C. fritschii indicates that the level of chlorophyll in dark-grown cells of this alga is not appreciably lower than that in cells which have been grown photoautotrophically at 500 lux. Furthermore, cells grown with sucrose in the dark for more than three years can initiate growth without a lag upon transfer to mineral medium in the light.

4.2 Selected strains

Of the strains studied here, four were the same strains chosen for obtaining their growth rates under various conditions (Sections, 3.32, 3.35, 3.72): Phormidium luridum, Phormidium sp., Plectonema boryanum D181, and P. calothricoides. In addition, Calothrix membranacea was chosen as a representative of those containing phycoerythrin.

Quantitative extraction of the chlorophyll of the selected strains was easily made by either extracting fresh cells by 90% hot methanol or treating the cells with lysozyme overnight and subsequently extracting the cells by 90% hot methanol. Both methods proved equally successful for extracting the chlorophyll from the selected strains.

Aqueous extracts obtained from cells subjected to repeated freezing and thawing, and subsequent grinding with acid-washed fine sand was not a convenient technique and involved much physical effort especially because more than one strain was involved. The enzyme lysozyme was effective in digesting the cell walls of the selected strains and proved a more satisfactory and effective procedure for the extraction of phycocyanin and phycoerythrin. After incubation the algal materials with lysozyme overnight, clear, blue supernatant was released from the cells.

The average values for the quantitative extraction of the pigments of light and dark-grown cultures of selected strains are presented in Table XI. The results

Table XI: Pigment content of selected strains grown in the light at 500 lux, and in complete darkness (Section 2.42)

strain	growth conditions	dry weight g/l	pigment concentrations in % dry wt. chlorophyll	phycocyanin	phycoerythrin	total	pigment ratios chlorophyll : phycocyanin : phycoerythrin
Calothrix membranacea	500 lux	0.43	2.11	11.74	4.51	18.36	1 : 5.6 : 2.1
	dark	0.515	1.69	13.4	-	15.09	1 : 7.9 : -
Phormidium luridum	500 lux	0.973	2.1	19.83	-	21.93	1 : 9.4 : -
	dark	1.79	1.64	19.07	-	20.71	1 : 11.6 : -
Phormidium sp.	500 lux	0.785	2.3	17.75	-	20.05	1 : 7.7 : -
	dark	1.23	1.59	17.26	-	18.85	1 : 10.9 : -
Plectonema boryanum D181	500 lux	0.678	2.25	22.09	-	24.34	1 : 9.8 : -
	dark	1.26	1.84	21.88	-	23.72	1 : 11.9 : -
P. calothricoides	500 lux	0.655	2.18	21.01	-	23.19	1 : 9.6 : -
	dark	1.035	1.73	19.91	-	21.64	1 : 11.5 : -

- = none found

show that the levels of phycocyanin and chlorophyll in dark-grown cells of selected strains are not appreciably lower than those in cells which have been grown photoautotrophically at 500 lux. Furthermore, growth in the dark does not appear to influence photosynthetic capacity: dark-grown cells initiate growth upon transfer to mineral medium in the light without lag.

Calothrix membranacea, the strain which was chosen as a representative of those containing phycoerythrin, grown photoautotrophically at 500 lux contained appreciable amount of phycoerythrin (Table XI). Phycoerythrin was never detected in cells of this alga grown heterotrophically with sucrose in the dark. Similar results were reported by KIYOHARA et al. (1960) for Tolypothrix tenuis. When this alga grew heterotrophically in the dark, phycoerythrin was never detected.

5. General observations on morphology of dark-grown cultures

It has been shown previously (FAY et al., 1964; PEAT and WHITTON, 1967) that Chlorogloea fritschii may show considerable morphological variation dependent on its previous growth conditions. Previous works (Section 1) have also shown that white light may regulate the developmental cycle of Nostoc muscorum A, and N. commune 584. Therefore it seemed highly desirable to examine the dark-grown cultures under the light microscope to see

whether there was marked variation in their morphology.

During sub-culturing material from dark to dark, special care was taken (Section 2.46) to avoid picking up any trace of light that would possibly have a photomorphogenetic effect on the algae. As shown earlier (Section 3.3) two strains of Calothrix (C. brevissima, C. membranacea) were incapable of continuous growth in dark (second sub-culture from dark to dark) on a nitrogen-free medium, but capable of doing so on medium containing a source of combined nitrogen (KNO_3), therefore, both cultures were examined under the light microscope. All cultures were grown for 3-6 weeks in complete darkness with continuous shaking.

In all cases, except that of Chlorogloea fritschii, there was no significant variation in the morphology of dark-grown cultures as compared with light-grown forms. The blue-green colour of light-grown cultures is, however, more intense than in the dark-grown form, and the filaments are brighter in colour.

Morphology of Anabaena sp.

Culture bluish-green; mostly short filaments, but some rather longer; trichome straight or entangled, vegetative cell barrel-shaped or cylindrical.

Morphology of Anabaenopsis circularis

Culture bluish-green; trichome straight or

entangled; fragmentations mainly at the heterocyst; heterocyst terminal and intercalary, and slightly coloured. A few akinete-like bodies seen.

Morphology of Calothrix brevissima

In dark-grown cultures, the filaments were not attenuated at all; sheath around the trichome clear. Vegetative cells much broader than long. Heterocyst both intercalary and terminal, as big as the vegetative cell or slightly bigger in places.

In light-grown cultures, the filaments were slightly attenuated; sheath around the trichome clear, trichomes short, slightly attenuated; heterocysts basal.

The morphology of cultures grown on medium containing a source of combined nitrogen (KNO_3), was similar to that grown on a nitrogen-free medium, except that heterocyst number was depressed.

Morphology of C. membranacea

In the dark, culture bluish-green in colour, filaments were not attenuated at all; trichomes straight or slightly irregularly curved; sheath around the trichome not visible. Vegetative cells blue-green or even deep blue-green in colour. Heterocyst both terminal and intercalary, the terminal heterocyst bigger than the vegetative cell with paler content.

In light-grown cultures, filaments long, mostly

curved; sheath around the trichome very thin, young short trichomes were slightly attenuated; heterocysts basal.

Culture grown on medium containing a source of combined nitrogen (KNO_3) had similar morphology to that grown on medium lacking it except that heterocyst number was depressed.

Morphology of Chlorogloea fritschii

In the dark, culture (second sub-culture in dark) was green. The heterotrophic material after three years sub-culturing in the dark, was also still green. Vegetative cells spherical or slightly polygonal; sheath around the colonies was prominent. No filament seen.

Light-grown cultures consisted of a filamentous stage, which later gradually disappeared and the shape of the cells changed from round to polygonal. The morphology of this strain will be studied in detail in Section 6.

Morphology of Nostoc commune

Culture bluish-green in colour; filamentous; trichome straight or entangled; vegetative cell barrel shaped or spherical; heterocyst spherical and bigger than the vegetative cell.

Morphology of N. ellipsosporum

Culture bluish-green; trichome long, healthy-looking, some short; a few degenerated vegetative cells;

heterocyst bigger than the vegetative cell, a few heterocyst-like cells, rather longer than others, but the same pale yellow-green in colour.

Morphology of N. muscorum

Culture bluish-green; trichome bright in colour, straight or entangled; heterocyst more or less rounded.

Morphology of N. punctiforme

Culture bluish-green; thallus forming; filamentous, filaments contorted and entangled; small irregular young colonies with a sheath and a heterocyst present. Cells more or less rounded, deeply constricted at their septa.

Morphology of Nostoc sp. D159

Culture bluish-green; trichome deep blue, straight or entangled; cells deeply constricted at their septa, fragmentations abundant; heterocyst terminal and intercalary of the same size as the vegetative cell or even bigger.

Morphology of Nostoc sp. D167

Culture light bluish in colour; trichomatous, mostly fragmented, cell structure similar to Nostoc sp. D159, but the colour bluish; heterocyst terminal and intercalary, terminal heterocyst bigger than the vegetative

cell with homogenous contents and vaculated at times; fragmentation mainly at the heterocyst.

Morphology of Phormidium luridum

Culture bluish-green; trichome straight, mostly short, but some rather long.

Morphology of Phormidium sp.

Culture bluish-green; trichome straight, mostly short, but some rather long; some unicells.

Morphology of Plectonema boryanum D181

Culture bluish-green; trichome straight, mostly short, but some rather long.

Morphology of P. boryanum D177

Culture green-blue in colour; trichome straight, some with visible sheath.

Morphology of P. calothricoides

Culture bluish-green; trichome straight, short, lots of unicells, yellowish in colour.

Morphology of Scytonema sp.

Culture bluish-green; mostly long trichome, but some shorter; sheath around the trichome visible; some of

the filaments were tapered.

Morphology of Tolypothrix temuis

Culture bluish-green; trichome pale blue in colour; sheath around the trichome visible; some of the filaments were tapered.

6. Detailed morphological investigations on Chlorogloea fritschii

The life cycle of Chlorogloea fritschii is more complex than those known for most blue-green algae. FAY et al. (1964), PEAT and WHITTON (1967) and FINDLEY et al. (1970), reported that C. fritschii showed marked morphological variation under different growth conditions. Previous works (FAY and FOGG, 1962; FINDLEY et al., 1970) have also shown that C. fritschii is capable of growth over a wide range of temperatures and light intensities. The development of motile trichomes from aseriate colonies in cultures of Nostoc muscoroum A, and N. commune 584 were found to be stimulated by red light (650 nm) (LAZAROFF and SCHIFF, 1962; ROBINSON and MILLER, 1970). Hence it was thought worthwhile to investigate further, the environmental conditions which would favour a high proportion of motile trichomes in cultures of Chlorogloea fritschii.

Growth was carried out in 100 ml flasks containing 50 ml AD medium plus 0.01M sucrose. Experiments were directed towards the understanding of the effect of red

light and temperature on the developmental stages of Chlorogloea fritschii. Light intensities used in the experimental series were approximately 500, 4000, 6000, 10000 lux and temperatures were between 25, 35, 40, 45°C. Red light was obtained by wrapping a layer of red paper round the flasks. The beam of light which passed through the red paper had a wave length of approximately 500 nm. Cultures were examined with a light microscope at 2 or 3 day intervals, and number of cells in filament/ml and total number of cells/ml was determined by using 0.2 mm haemocytometer.

At high light intensity (6000 lux) and at 35°C, the results corresponded most closely to those obtained by FAY et al. (1964) and PEAT and WHITTON (1967). Four distinct stages were observed during the life cycle. The various stages are illustrated in Fig. 29. After the transference to fresh medium, many small cells occurred from the mother cells (mature culture). Many of these daughter cells then gave rise to filaments usually 4-6 cells long (stage I). The cells at stage I were pale blue-green in colour. These cells grew in size and became more deep blue-green, dividing transversely so that the filamentous character was preserved (stage II). Later longitudinal division occurred and the filamentous character then gradually disappeared and the shape of the cells changed from round to polygonal (stage III). Finally the cells increased in size and the contents became yellowish green and markedly granular (stage IV).

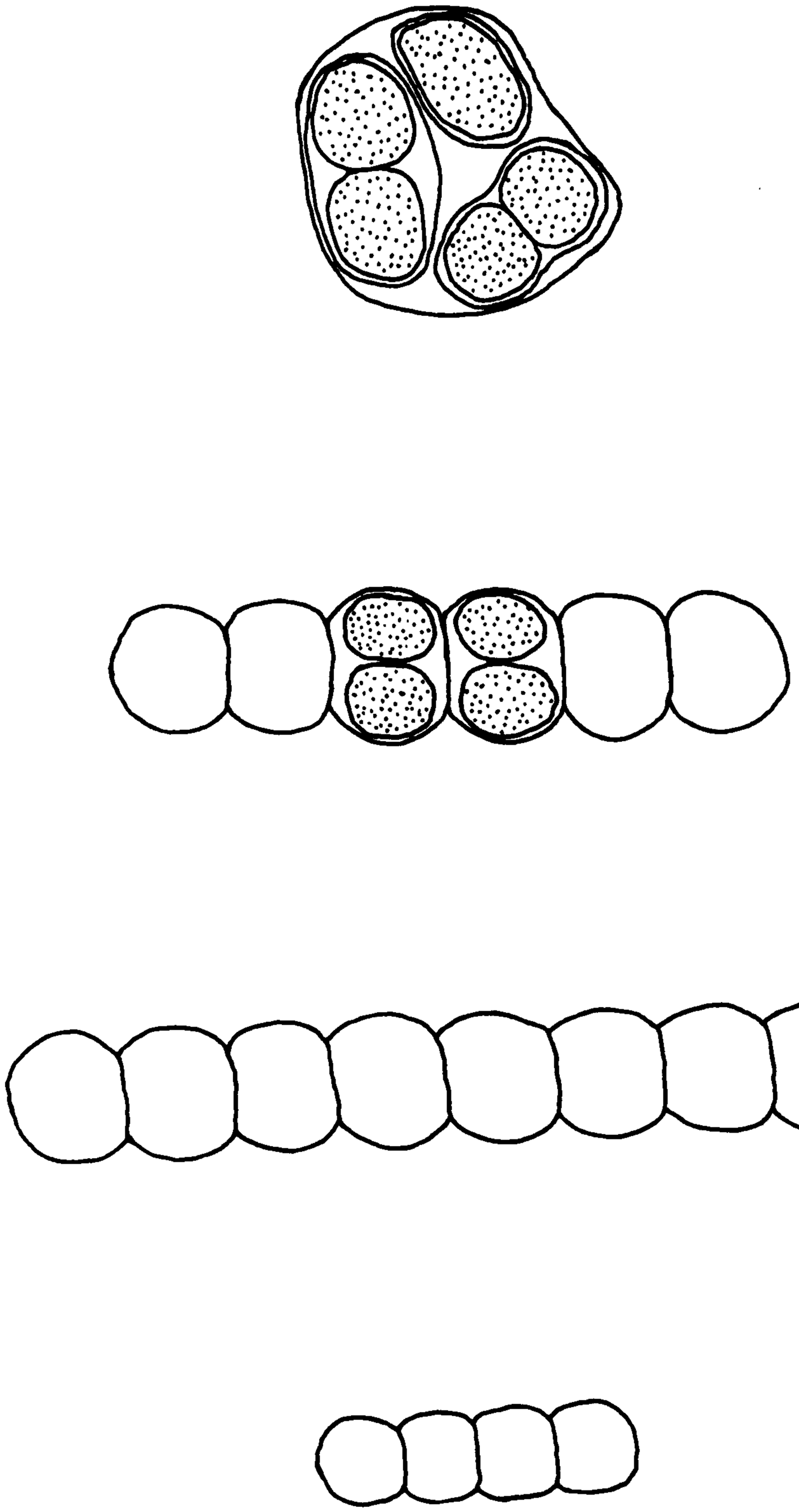

Fig. 29. A diagram of the four stages in 'life-cycle' of Chlorogloea fritschii.

At 35°C stage I cells were present in varying percentages in cultures grown under different light intensities. At low light intensity (500 lux) stage I cell type was seldom observed, whereas at high light intensity (6000 lux) stage I cells comprised 85% of the culture, the other 15% being stage IV aseriate. Transfer of a population rich in stage I cells to fresh medium and under the same conditions resulted in gradual change into stage IV rich population. At intermediate light intensity (4000 lux), stage IV cell was the prevalent cell type, stage I comprised only 20% of the culture. Higher light intensity (10000) did not favour a high proportion of stage I cells, it comprised only 51% of the culture.

Manipulation of light intensity from 500 to 10000 lux and temperature from 25 to 45°C did not result in an increase in the percentage of stage I cells in either aerated (5% CO_2 in air) or unaerated cultures.

Promotion of motility by media from motile cultures

It has been discovered that the addition of an extract of motile, light-grown Nostoc muscorum A to cultures subsequently grown in darkness allowed the formation of motile trichomes in the dark-grown cultures (Section 1). It was therefore decided to carry out similar investigations since stage IV cell type comprised 100% of the cells in the heterotrophic cultures of Chlorogloea fritschii. The extract was prepared from motile cultures (85% stage I) of red-light-grown Chlorogloea. The algal

material was ground with acid-washed sand. After grinding, the sand was separated by centrifugation and the supernatant sterilised by Millipore filters (Pore diameter 0.45 µm). 5-10 ml of supernatant were added into the heterotrophic cultures and then placed in darkness. Microscopical examination indicated that the presence of the extract did not enable the alga to pass through the various developmental stages previously observed only in the light.

The work of ROBINSON and MILLER (1970) showing that the supernatant from motile cultures of either Nostoc muscorum A or Nostoc commune 584 were reciprocally active in promoting motility in the other species, prompted me to test whether the supernatant from motile cultures of other blue-green algae (Anabaena inaequalis or Anabaenopsis circularis) could promote the motility in the heterotrophic cultures of Chlorogloea fritschii.

The extract was made from motile cultures of either light-grown Anabaena inaequalis or Anabaenopsis circularis. 5 to 10 ml of the supernatant were added aseptically into the heterotrophic cultures of Chlorogloea and then placed in darkness. Heterotrophic cultures were examined microscopically and showed no promotion of motility by the presence of either the extract of Anabaena inaequalis or Anabaenopsis circularis.

7. DISCUSSION

7.1 Heterotrophic growth of blue-green algae

Growth in the dark has been previously reported for Tolypothrix tenuis by KIYOHARA et al. (1960), for Chlorogloea fritschii by FAY (1965) and for Anabaenopsis circularis by WATANABE and YAMAMOTO (1967). The present studies have confirmed these reports and have found that not less than 18 filamentous strains of blue-green algae, both heterocystous and non heterocystous, that have been examined are facultative heterotrophs, as shown by their ability to grow with sucrose in the dark. Under these conditions these strains must be capable of using sucrose as a carbon and as an energy source. Only six axenic strains (Anabaena cylindrica, A. inaequalis, A. variabilis, Anacystis nidulans, Lyngbya sp., Oscillatoria tenuis) consistently failed to grow with sucrose in the dark. Of these six, one strain (Lyngbya sp.) was still viable in the presence of sucrose since growth occurred when the cultures were subsequently placed in the light after three months of incubation in the dark. These strains can not presumably use sucrose as a source of energy, but can presumably use it as a source of carbon (Section 3.6). Hence, it appears that these strains are not impermeable to organic compounds. These strains will be classified as obligate phototrophs; this places the emphasis more on the energy source rather than the carbon source for although these strains can utilise sucrose in the light (Section 3.6),

all of them appear unable to dispense with light for significant growth. Even Agmenellum quadruplicatum and Lyngbya lagerheimii which can utilise organic compounds in amounts which are sufficient to account for new cell synthesis show an absolute requirement for light for growth on organic media (VAN BAALEN et al., 1971).

A general impermeability of the cell to organic compounds, once offered as an explanation of obligate autotrophy, has been disproved in many cases of filamentous and unicellular blue-green algae (HOARE et al., 1967; PEARCE and CARR, 1967; SMITH et al., 1967; BAKER and BOLD, 1970; HOARE et al., 1971; KENYON et al., 1972; RIPPKA, 1972). SMITH et al. (1967) were unable to detect either α-ketoglutarate dehydrogenase or NADH oxidase in cell-free extracts of three unicellular blue-green algae. They proposed accordingly, that obligate autotrophy has a specific metabolic basis, i.e. inability to couple the breakdown of organic substrates with the generation of ATP. It has now become evident (PELROY et al., 1972) that facultative chemoheterotrophy among blue-green algae is based on a mode of energy-yielding respiratory metabolism which does not require the operation of the tricarboxylic acid cycle, and for which carbohydrates are the only utilisable primary substrates, namely: the pentose phosphate pathway. In the particular cases of Anabaena cylindrica, A. inaequalis, A. variabilis, Anacystis nidulans, Lyngbya sp., Oscillatoria tenuis, ability to use sucrose as source of carbon in the light (Section 3.6) constitutes prima facie

evidence for the absence of a permeability barrier, therefore some other factor (s) presumably prevents their growth with sucrose in the dark. Similar results were reported by RIPPKA (1972) for some Aphanocapsa strains; although these strains can grow photoheterotrophically with glucose in the presence of DCMU, they will not grow with glucose in the dark. As mentioned earlier, at the present time, these algae which can utilise organic compounds in the light, but apparently unable to dispense with light for significant growth, are best classified as obligate phototrophs.

There is no very obvious correlation between ability of the alga to grow in the dark and taxonomic position. However, it is worth noting that if it is assumed that the names given to the strains are an indication of their morphology when isolated, those unable to grow in the dark might have been expected to be relatively free of mucilage while nearly all those capable of dark growth would have had an obvious sheath or abundant mucilage layer.

Before discussing heterotrophic growth in more detail, it is imperative to decide what constitutes unequivocal proof of growth in the dark on organic media. Firstly, every possible precaution must be taken to ensure complete exclusion of light from cultures under test. The importance of such a step is emphasised by the recent study of VAN BAALEN et al. (1971); in this study of the heterotrophic growth of blue-green algae, organic media on

which growth in the dark was negligible, supported appreciable growth of two species at low light intensity (100 lux). Secondly, if complete exclusion of light is ensured, the demonstration of an increase in cell mass on repeated subculture in organic media would constitute unequivocal proof of dark heterotrophic growth. The observations of VAN BAALEN (1962) and of BAKER and BOLD (1970) emphasise the importance of this second condition; they showed that the transfer of some strains to organic media resulted in marginal growth which did not occur on repeated subculture.

From the above consideration of the experimental definition of dark heterotrophic growth, most of the reports in the literature of dark heterotrophic growth of blue-green algae are unaccompanied by sufficient evidence. A recent report has described the growth of Plectonema boryanum in the dark on glucose using a dialysis-flowthrough culture technique (PAN, 1972). Unlike other strains capable of dark heterotrophic growth, the dark-grown cells obtained by this procedure were devoid of chlorophyll; however, they were claimed to grow normally and synthesised chlorophyll on transfer to their normal mineral medium in the light. So far, no other author has found a similar result with a blue-green alga. Up to now, the work of FAY (1965) on Chlorogloea fritschii and of HOARE et al. (1971) on a Nostoc-like isolate from the coralloid roots of Macrozamia lucida, provide the most convincing evidence for

heterotrophy amongst blue-green algae. FAY (1965) observed an increase in cell mass over a period of several weeks in the dark in media containing either maltose or sucrose. HOARE et al. (1971) obtained consistent growth of the cycad symbiont in the dark with glucose for five transfers.

In the present studies growth in dark has been confirmed under critical condtions (Section 2.42) in which complete exclusion of light from cultures under test was ensured. Consistent growth of all strains capable of heterotrophic growth in the dark has been obtained on sucrose for one year (8-12 transfers) and Chlorogloea fritschii has been grown on sucrose for more than three years. Subculturing material from dark to dark has been carried out in the dark (Section 2.46) to avoid picking up any trace of light which might possibly have a photomorphogenetic effect on the algae. The importance of such a step was emphasised by the observations of LAZAROFF and VISHNIAC (1961), who reported that Nostoc muscorum A (ALLISON's strain) grew as a mass of large sub-globose cells (the aseriate stage) in complete darkness. Either glucose or sucrose served as carbon and energy source; but small amount of light or the addition of an aqueous extract of light-grown cells, was required for differentiation of filamentous stages. The inhibition of development in the dark was interpreted as being due to a lack of morphogenetic substances which are formed only in the light.

In a similar study ROBINSON and MILLER (1970), confirmed the existence of the nostoc**ac**ean developmental cycle in both N. commune 584 and N. muscorum A.

The anomalous observation for Tolypothrix tenuis, that although it will grow in the light without combined nitrogen, it will not do so in the dark, is a confirmation of the earlier observation of KIYOHARA et al. (1960). It has also been observed that although the two Calothrix strains (C. brevissima, C. membranacea) will grow on a nitrogen-free medium in the light and in the first subculture in the dark, they will not do so in the subsequent subculture from dark to dark. From preliminary observations, it was clear that C. brevissima and C. membranacea grow best at 25°C and 35°C respectively. However, the results of a more detailed investigation on the effect of a wide range (23.5°C - 52°C) of temperatures on the growth of C. membranacea showed that the maximum temperature is 42.5°C and the optimum is 36°C. Preliminary experiments with C. membranacea grown for five weeks in the light at 25°C and at 32°C and transferred to the dark for the period of the incubation indicated that the rate of acetylene reduction in the dark was considerably greater at 25°C than at 32°C over a 2 h incubation period (C. SINCLAIR, personal communication). Nitrogen fixation may be specifically inhibited by extremes of temperature. Anabaena cylindrica for instance had an optimum temperature between 30°C and 35°C for photosynthesis, but nitrogen fixation was reduced to 80% with an increase in temperature

from 30°C to 35°C (FOGG and THAN-TAN, 1960). PATTNAIK (1966) found that maximum growth of Westiellopsis prolifica occurred at 40°C. The percentage nitrogen content of the cells decreased above 35°C. Similarly, maximum growth of Chlorogloea fritschii occurred at 45°C but maximum nitrogen fixation occurred at 40°C (FAY and FOGG, 1962). Hence, it is probable that the temperatures used for growth of Calothrix brevissima, C. membranacea and Tolypothrix tenuis were not optimum for nitrogen fixation and may have had a greater inhibitory effect.

ALLEN (1952), BAKER and BOLD (1970) and STANIER et al. (1971) attempted to test the ability of large numbers of separate isolates of blue-green algae to grow heterotrophically in the dark on plates of glucose agar. These attempts have given results which imply that heterotrophy is a rare phenomenon amongst blue-green algae. The results of a more recent survey by RIPPKA (1972) indicate that reproduceable dark growth with glucose can be obtained only if a liquid medium is used. Thus, it appears possible that more isolates examined by ALLEN (1952), BAKER and BOLD (1970) and STANIER et al. (1971) might have been induced to grow heterotrophically if proper conditions (e.g. liquid media) were used. The occurrence of dark growth on organic media may be largely dependant on the organic substrate provided and the conditions under which the cultures are incubated. This factor together with the fact that the strains incapable of dark growth include some of those which

have been selected for their fast growth rates and capacity to produce high yields in laboratory cultures, may have contributed to the false conclusion that blue-green algae are obligate photoautotrophs.

Carbohydrates are the only organic compounds which have been shown to support heterotrophic growth of blue-green algae in the dark. Only the four sugars, fructose, glucose, maltose and sucrose supported growth of the strains examined in the dark. The utilisation of fructose, glucose and maltose is either limited or dependant on the presence of combined nitrogen in the medium. Sucrose supported growth of all strains capable of heterotrophic growth in the dark. The finding (Table VI) that sucrose is outstanding among the substrates tested in allowing a considerable growth of the majority of the cultures in the dark, contrasts with the reports concerning other blue-green algae (HOARE et al., 1971 ; KIYOHARA et al., 1960; WATANABE and YAMAMOTO, 1967) for which glucose was the best substrate for growth in the dark. Sucrose was most effective in supporting dark growth of Chlorogloea fritschii (FAY, 1965) and Nostoc punctiforme (HARDER, 1917).

The results of the effect of various organic substrates (Section 3.2) on the growth of the unialgal (non-axenic) cultures (Nostoc commune, N. punctiforme, Nostoc sp. D167,. Scytonema sp.) indicate that fructose, glucose, maltose and sucrose supported appreciable growth of these strains in the absence of combined nitrogen in the medium. In the presence of combined nitrogen (KNO_3) in the

medium fast action of the bacteria upon carbohydrates result in an abundant bacterial growth which lowers the pH below 7 and this hampers or inhibits the algal growth. Similar observation was reported by LANGE (1970) for cultures of Cyanophyta containing bacteria; when sucrose was added during the dark period (16-18 h cycle of light and dark) and the bacterial action upon the carbohydrate had caused the pH to drop below 7, the algal growth was hampered during the first few days or inhibited. Since carbon dioxide was not the growth limiting factor in the case of the unialgal cultures, the stimulation of algal growth by bacteria and their increased production and release of carbon dioxide is invalid. It appears possible that the unialgal (non-axenic) cultures (Nostoc commune, N. punctiforme, Nostoc sp. D167, Scytonema sp.) could compete with bacteria for carbohydrate in the dark. The uptake of carbohydrates by these strains seems to be significant since appreciable algal growth did occur in the dark in the presence of bacteria. This significant uptake of organic compounds by algae in natural waters has been pointed out recently by MONHEIMER (1972). Strains (Anabaena cylindrica, A. inaequalis, A. variabilis, Anacystis nidulans, Lyngbya sp. Oscillatoria temuis) which failed to grow with sucrose in the dark under the standard procedure (Section 3.11) also failed to grow in the dark with any of the organic substrates tested. At the present time, the inability of acetate and galactose to support growth in the dark can best be explained by the inability of such organic compounds

to be readily convertible to glucose-6-phosphate, as only exogenous substrates readily convertible to glucose-6-phosphate can support growth in the dark (STANIER, 1973).

The rates for dark heterotrophic growth of blue-green algae are relatively slow, as compared with rates in the light. FAY (1965) found that the duration of the lag period of Chlorogloea fritschii grown under nitrogen fixing conditions with sucrose, was reduced to one week. A generation time of 48 h was reported by HOARE et al. (1971) for a Nostoc-like isolate from the coralloid roots of Macrozamia lucida, grown heterotrophically in the dark with glucose and of 24 h for the same strain grown with glucose in the light (320 lux). RIPPKA (1972) obtained the growth rates of one strain of the genus Aphanocapsa under a variety of environmental conditions. The dark growth rate with glucose was about five times less than the rate with glucose, in the light (800-1000 lux). In the present studies, the results of dark growth rate studies (Section 3.31) of the three heterotrophic cultures (material first subcultured from light to dark, material subcultured from dark to dark and material after 3 years of subculturing in the dark) of Chlorogloea fritschii, grown under non nitrogen fixing conditions indicate that there is no physiological adaptation to heterotrophic conditions and the growth rates (k) of all three heterotrophic cultures were the same. The dark growth rate with sucrose was over two times less than the rate with sucrose in the light (500 lux). The dark

growth rate of Phormidium sp. and of Plectonema boryanum D181 was over two times less than the rate with sucrose in the light (500 lux), while the dark growth rate of Phormidium luridum and Plectonema calothricoides with sucrose was under two times less than the rate with sucrose in the light (500 lux).

7.2 Effect of organic compounds on the phototrophic growth of blue-green algae

FAY (1965) showed that sucrose decreased the generation time and prolonged the exponential phase of Chlorogloea fritschii at a light intensity of 3000 lux. The present studies have confirmed the report of FAY (1965) and found that the utilisation of sucrose by Chlorogloea fritschii and selected strains (Phormidium luridum, Phormidium sp., Plectonema boryanum D181, P. calothricoides) in the light result in an increase of the growth rate ($\dot{k}$) and a consequent decrease in the generation time. With Chlorogloea fritschii sucrose has been found to increase the rate of photosynthetic growth even at a light intensity (4000 lux) that support a good rate of growth in mineral medium (Table VIII). However, growth was more stimulated by sucrose at low light intensity (500 lux). At this light intensity the growth rate ($\dot{k}$) with mineral medium was two times less than the rate with sucrose. At the end of the exponential phase of growth, the yield of organism in cultures grown on sucrose at 500 lux was 8 times that of control cultures. At 2000 lux the yield on sucrose was

slightly more than 3 times that of the control, while it was just about 3 times that of control cultures at 4000 lux. The growth of Phormidium luridum, Phormidium sp., Plectonema boryanum D181, and Plectonema calothricoides, was stimulated by sucrose at light intensity of 500 lux (Table IX). At this light intensity the growth rate ($\hat{k}$) of Phormidium luridum, Phormidium sp. and Plectonema boryanum D181, with mineral medium alone was about 2 times less than the rate with sucrose, while the growth rate ($\hat{k}$) of Plectonema calothricoides with mineral medium was under 2 times less than the rate with sucrose. At the end of the exponential phase of growth, the yield of culture of Phormidium sp., Plectonema boryanum D181 with sucrose was about 4 times that of cultures without sucrose; while it was about 5 times for Phormidium luridum and 3 times for Plectonema calothricoides.

On the basis of the data available at the present time, it is clear that at high light intensities which were probably near to saturation, organic compounds did not increase the growth rate of blue-green algae (KRATZ and MYERS, 1955b; PEARCE and CARR, 1967; SMITH et al., 1967). Among the organic compounds tested, carbohydrates were found to be the best organic compounds to stimulate the growth of blue-green algae but such effects have only been observed at low light intensities. LAZAROFF and VISHNIAC (1961) found that growth of Nostoc muscorum A was stimulated by glucose at low light intensities (100-800 lux). Similar observations have been reported by KIYOHARA

(unpublished work by KIYOHARA cited by KIYOHARA et al., 1962) with Tolypothrix tenuis, by HOARE et al. (1971) with a Nostoc-like organism isolated from Macrozamia lucida and by VAN BAALEN et al. (1971) with Lyngbya lagerheimii and Agmenellum quadruplicatum. Growth of these organisms was stimulated by glucose at low light intensities below that required for photoautotrophic growth.

Although carbohydrates clearly stimulate the growth of some species of blue-green algae in the light, sufficient data are not available at the moment to permit generalisation regarding the effect of organic compounds on the growth rate of all blue-green algae. It is, nevertheless, reasonable for one to speculate that some blue-green algae in dimly lit situations are able to derive significant benefit from organic compounds present in the medium.

7.3 Response of strains incapable of growth in dark to sucrose in light

The results of the effect of sucrose on the phototrophic growth of strains incapable of growth in dark (Anabaena cylindrica, A. inaequalis, A. variabilis, Anacystis nidulans, Lyngbya sp. Oscillatoria tenuis) show that these strains respond to sucrose (Section 3.6). The light intensities effective in stimulating growth varied from species to species. Low light intensities (20, 100 lux) which were insufficient for growth under photoautotrophic conditions did not produce a growth response in cultures with sucrose. Apparently, light

stimulated the use of sucrose for growth. At low light intensities (500, 1000 lux) sucrose produced significant increase in growth of Anabaena cylindrica, A. inaequalis, Anacystis nidulans, Lyngbya sp. and Oscillatoria temuis. Only one strain (Anabaena variabilis) did not respond significantly to sucrose, although some increase in the growth did occur. These observations show a high degree of variability which may be found in the response of different species or of varieties of the same species. The response of these strains to sucrose in the light is striking in view of their inability to grow on organic media in the dark. These strains can presumably use sucrose as a source of carbon, but not as a source of energy. Recent observations (VAN BAALEN et al., 1971) have clearly shown that growth of Agmenellum quadruplicatum and Lyngbya lagerheimii was stimulated by glucose at low light intensity (100 lux) which was barely sufficient to support autotrophic growth. In light of the present studies and the results of LAZAROFF and VISHNIAC (1961), HOARE and MOORE (1965), HOARE et al. (1967), PEARCE and CARR (1967), SMITH et al. (1967), PEARCE and CARR (1969), BAKER and BOLD (1970), HOARE et al. (1971), VAN BAALEN et al. (1971), KENYON et al. (1972) and RIPPKA (1972) who demonstrated that many blue-green algae assimilate a wide range of organic compounds in the light, it appears that any tendancy to regard all blue-green algae as obligate photoautotrophs would be unjustified as well as premature.

Consequently, blue-green algae as a whole are not impermeable to organic compounds and therefore not restricted to carbon dioxide as carbon source. Strains: Anabaena cylindrica, A. inaequalis, A. variablis, Anacystis nidulans, Lyngbya sp. and Oscillatoria tenuis which will not grow on organic media in the dark, but will do so in the light are best classified as obligate phototrophs, but not obligate photoautotrophs.

7.4 Pigments and morphology of dark and light-grown cultures

The various methods tried in attempts to facilitate pigments extraction from cells of Chlorogloea fritschii to completion were unsuccessful. The enzyme lysozyme was very effective in digesting the cell walls of selected strains (Section 4.2) but was inactive in digesting the cell walls of C. fritschii. CRESPI et al. (1962) and BERNS et al. (1966) found that the cell walls of different species of blue-green algae vary in sensitivity to lysozyme. It appears that the cell walls of C. fritschii are not sensitive to lysozyme.

PIKE (1970, 1971) asserted that quantitative measurement of the pigments of C. fritschii could successfully be obtained by means of the freeze-drying and the french press. PIKE (1970) claimed that C. fritschii contains 3.3 µg chlorophyll per mg dry weight when it is grown heterotrophically in the dark and contains 7.7 µg

chlorophyll per mg dry weight when it is grown at low light intensity with sucrose. In the present studies the highest amount of chlorophyll obtained was 7.1 µg chlorophyll per mg dry weight for cells grown heterotrophically in the dark and 7.2 µg chlorophyll per mg dry weight for cells grown photoautotrophically at 500 lux. Hence, it is very difficult to believe that the technique used by PIKE (1970) succeeded in extracting the cells to completion. Even, if one presumes that PIKE (1970) extracted the cells to completion, it is very difficult to believe that C. fritschii has lower amount of pigments than other blue-green algae. However, the results of the qualitative extraction of the pigments of C. fritschii and the results of the pigment analysis (Table XI, Section 4.2) of Calothrix membranacea, Phormidium luridum, Phormidium sp., Plectonema boryanum D181 and P. calothricoides show that the levels of phycocyanin and chlorophyll in dark heterotrophic cells are not appreciably lower than those in cells which have been grown photoautotrophically at 500 lux. Furthermore, cells grown with sucrose in the dark can initiate growth without a lag upon transfer to a mineral medium in the light. Growth in the dark, therefore, does not appear to influence photosynthetic capacity. Similar observations were reported by HOARE et al. (1971) for a Nostoc-like isolate from the coralloid roots of Macrozamia lucida, and by RIPPKA (1972) for Aphanocapsa. The chlorophyll and phycocyanin contents of dark-grown cells

of the two strains were not appreciably lower than those in cells grown in the light. Calothrix membranacea grown photoautotrophically at 500 lux contained an appreciable amount of phycoerythrin. Phycoerythrin was never detected in cells of this alga grown heterotrophically with sucrose in the dark (Table XI). Similar results were reported by KIYOHARA et al. (1960) for Tolypothrix tenuis. Phycoerythrin was not found in cells of this alga grown heterotrophically in the dark, while an appreciable amount was present in cells grown in light.

The observation of a sequence of morphological stages in light grown cultures of Chlorogloea fritschii confirmed the previous observations of FAY et al. (1964) PEAT and WHITTON (1967), and FINDLEY et al. (1970). When cultured under light the organism completes its developmental cycle, but when cultured in darkness motile trichomes are not formed and cultures which consist of aseriate colonies result. LAZAROFF and SCHIFF (1962) and ROBINSON and MILLER (1970) demonstrated for Nostoc muscorum A and for N. commune 584 that red light was effective in promoting motility. Cultures of Chlorogloea fritschii which were subjected to continuous illumination (6000 lux) with red light comprised the highest percentage of motile trichomes (85%). Neither manipulation of light intensity from 500 to 10,000 lux nor temperature from 25°C to 45°C result in an increase in the percentage of motile trichomes in either aerated (5% CO_2 in air) or

unaerated cultures. LAZAROFF and VISHNIAC (1961) have shown that additions of extracts of motile, light-grown Nostoc muscorum A to cultures subsequently grown in darkness allowed the formation of motile trichomes in the dark grown cultures. In the case of Chlorogloea fritschii the addition of such extracts or extracts from motile cultures of other blue-green algae (Anabaena inaequalis or Anabaenopsis circularis) did not enable the alga to pass through the various developmental stages previously observed only in the light.

In all other strains examined in the present studies, the morphology of dark grown cells remain almost the same as those of cells grown in the light. The blue-green colour of light grown cultures is, however, more intense than in dark-grown form and the filaments are brighter in colour. The slight attenuation of trichomes in light-grown cultures of Calothrix brevissima and the slight attenuation in the young short trichomes of C. membranacea was not seen in those grown in the dark.

SUMMARY

Studies were carried out on the ability of 24 strains of blue-green algae to grow heterotrophically in the dark on 0.01M sucrose (Section 3.11). 18 of these strains were capable of growth in the dark. Only six strains failed to grow in the dark and of these six, one was still viable after three months of incubation in the dark. Growth in dark was confirmed (Section 3.12) under critical conditions in which complete exclusion of light from cultures under test was ensured. Reproduceable dark growth of all strains capable of growth in dark was obtained on sucrose for one year and Chlorogloea fritschii was grown on sucrose for more than three years (Section 3.3). The results of the effect of carbohydrates and acetate (Section 3.2) on growth of the strains in the dark revealed that only the four sugars, fructose, glucose, maltose and sucrose supported growth. Sucrose supported growth of all cultures capable of growth in dark and in the majority (ten out of eighteen) of these cultures best growth was found on sucrose.

C. fritschii and four other strains were chosen for detailed studies in which their growth rates were obtained under different environmental conditions (Section 3.31, 3.32, 3.5, 3.7). C. fritschii was investigated further in which its growth rate was determined for three heterotrophic materials; material first subcultured from light to dark, material second subcultured from dark to dark and material

after three years subculture in the dark. It was found that the growth rates ($\hat{k}$) of all three heterotrophic cultures were the same, thus suggesting that no physiological adaptation had taken place as a response to prolonged heterotrophic conditions.

The addition of sucrose in the light resulted in an increase of the growth rate ($\hat{k}$) of exponentially growing cultures of C. fritschii, and of selected strains.With C. fritschii sucrose was found to increase the growth rate even at a light intensity (4000 lux) that support a good rate of growth in mineral medium (Section 3.51). However, stimulation of growth by sucrose was greater at 500 lux. At this light intensity growth rate with sucrose was two times more than that of control cultures. The growth rate of selected strains with mineral medium was under two times less (at least) than the rate with sucrose at 500 lux.

The results of the effect of sucrose on the phototrophic growth of the six strains incapable of growth in dark revealed that these strains respond to sucrose at 500 and 1000 lux (Section 3.6). At these light intensities sucrose produced significant increase in growth of five strains. Only one strain (Anabaena variabilis) did not respond significantly to sucrose, although some increase in the growth did occur.

The various methods tried in attempts to facilitate pigment extraction from cells of Chlorogloea fritschii to completion were unsuccessful and the results

obtained by these attempts have been discussed in detail and compared with the results obtained by PIKE (1970) (Section 4.1). The enzyme lysozyme was very effective in digesting the cell walls of selected strains (Section 4.2) but was inactive in digesting the cell walls of C. fritschii which suggest that the cell walls of C. fritschii are not sensitive to lysozyme. However, the results of the qualitative extraction of the pigments of C. fritschii and the results of the pigment analysis (Section 4.2) of selected strains showed that the levels of phycocyanin and chlorophyll in dark heterotrophic cells were not appreciably lower than those in cells which have been grown photoautotrophically at 500 lux. Thus, it appeared that growth in the dark does not influence photosynthetic capacity.

General observations on the morphology of light and dark-grown cultures (Section 5) showed that unlike the cultures of C. fritschii which consisted of aseriate colonies only in the dark, all other strains consisted of filaments. However, the trichomes of Calothrix brevissima and the young trichomes of C. membranacea which were slightly attenuated in light-grown cultures were not so, in those grown in the dark.

The effect of red light and temperature on the developmental stages of Chlorogloea fritschii showed that, at 35°C and continuous illumination (6000 lux) with red light, motile trichomes comprised the highest percentage

(85%) of the culture (Section 6). Neither manipulation of light intensity from 500 to 10,000 lux nor temperature from 25°C to 45°C resulted in an increase in the percentage of motile trichomes in either aerated (5% CO_2 in air) or unaerated cultures. The addition of extracts of motile (85%) light-grown C. fritschii or extracts from motile cultures of other blue-green algae to cultures of C. fritschii subsequently grown in darkness did not enable the alga to pass through the various stages previously observed only in the light.

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Heterotrophic Growth of Blue-Green Algae

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Some recent reviews have concluded that most blue-green algae are obligate phototrophs and unable to grow in the dark (e.g. Holm-Hansen, 1968; see also Van Baalen *et al.*, 1971). This generalization is, however, based mostly on scattered observations in the literature rather than any systematic study. The present communication reports the results of a survey of the ability of 24 strains of filamentous blue-green algae (including the only occasionally filamentous *Anacystis nidulans*) to grow in the dark.

The algae were all tested at a temperature and with a basal inorganic medium known to be favourable to relatively rapid growth in the light. They were incubated in the dark together with 0.01 M sucrose, using liquid culture with gentle shaking. Strains capable of growth in the light without a source of combined nitrogen were subcultured to such a medium in the dark. In the one instance (*Tolypothrix tenuis*) where growth then failed, the test was repeated by subculturing from material with a source of combined nitrogen to a similar medium in the dark. All the cultures were tested for at least three months, and subcultured at least once.

The results summarized in Table 1 indicate that the majority of these blue-green algae are capable of growth in the dark. Only 7 out of 24 strains failed to grow well and of these 7, 2 were still viable after 3 months when grown in a medium with combined nitrogen. In all instances where growth did occur in the dark, the growth rate was, however, relatively slow. With *Chlorogloea fritschii*, for instance, the fastest doubling time found for dark growth was about 5 days, as opposed to about 1 day for growth with sucrose at 500 lux. The anomalous observation for *Tolypothrix tenuis*, that although it will grow in the light without combined nitrogen, it will not do so in the dark, is a confirmation of the earlier observation of Kiyohara *et al.* (1960).

Table 1. *Growth of blue-green algae in dark*

Organism	Source	Presence or absence of combined N	Growth in dark	Comments
Anabaena cylindrica	C.1403-2a	—	—	
Anabaena inaequalis	C.1446-1a	—	—	
Anabaena variabilis	I.377	+	—	
Anabaena sp.	D.145	+	+	
Anabaenopsis circularis	C.1402-1	—	+	
Anabaenopsis sp.	W.51	—	—	viable after 3 months in dark
Anacystis nidulans	C.1405-1	+	—	
Calothrix brevissima	C.1410-7	—	+	
Calothrix membranacea	C.1410-1	—	+	
Chlorogloea fritschii	W.50	—	+	
Lyngbya sp.	C.1459-2	+	—	viable after 3 months in dark
Nostoc commune	D.168	—	+	
Nostoc ellipsosporum	C.1453-16	—	+	
Nostoc muscorum	C.1453-9	—	+	
Nostoc punctiforme	I.384	—	+	
Nostoc sp.	C.1403-5	—	+	
Nostoc sp.	C.1453-4	—	+	
Oscillatoria tenuis	C.1459-4	+	—	
Phormidium luridum	I.426	+	+	
Phormidium sp.	I.485	+	+	
Plectonema boryanum	I. 581	+	+	
Plectonema calothricoides	I.598	+	+	
Scytonema sp.	C.1473-3	—	+	
Tolypothrix tenuis	C.1482-3b	+	+	growth in dark with NH_4^+ only, but also NO_3^- and N_2 in light

C. = Cambridge collection number; D. = Durham isolate; I. = Indiana collection number; W. = Westfield College, London, number. D.145 was isolated from soil in Saudi Arabia; D.168 from soil on Aldabra Island, Indian Ocean.

There is no very obvious correlation between ability of the alga to grow in the dark and taxonomic position. However, it may be pointed out that if it is assumed that the names given to the strains are an indication of their morphology when isolated, those unable to grow in the dark might have been expected to be relatively free of mucilage

while nearly all those capable of dark growth would have had an obvious sheath or abundant mucilage layer. It is also worth noting that the strains incapable of dark growth include some of those most widely used as research organisms, and this fact may have helped to give rise to the false impression that most blue-green algae are obligate phototrophs.

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