

# CAROTENOGENESIS IN THE CHLOROPHYTA

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## ABSTRACT

Carotenoids are essential in light reception and photo-oxidative protection in photosynthetic membranes. In some unicellular algae, secondary carotenoids are accumulated during cell senescence or when cells are exposed to nutritional and/or conditions unfavourable for growth. The carotenoids are usually  $\beta$ -carotene and /or its ketonic derivatives such as astaxanthin, canthaxanthin and echinenone. Such a phenomenon has been observed in the unicellular algae *Dunaliella salina* and *Haematococcus pluvialis*. In the present study a number of species and strains of algae belonging to the Chlorophyta were screened to determine which were able to produce the highest amounts of secondary carotenoids. The results identified that the strains of *H. pluvialis* were the most prolific producers of secondary carotenoids of the various species and strains tested.

Qualitative and quantitative analysis of the secondary carotenoid composition of *H. pluvialis* revealed that secondary carotenoids accounted for up to 5.4 % of the dry cell weight of the alga. Mono and diesters of astaxanthin were found to be the major carotenoids accumulated with smaller amounts of echinenone, canthaxanthin and adonirubin also being present. The accumulation of these secondary carotenoids was associated with morphological changes in the alga, including a thickening of the cell wall, a reduction in the growth rate, loss of mobility and a reduction in the photosynthetic rate of the algal cells.

Nutritional and environmental parameters affecting the synthesis of secondary carotenoids in *H. pluvialis* were investigated to determine the extent that each exerted on carotenogenesis. The results revealed that all the nutritional and environmental parameters investigated had some effect on inducing secondary carotenoid synthesis in the alga. These included light intensity, nitrate, phosphate, iron and salt concentration. The optimum levels of some of these parameters was investigated to determine which allowed for maximum rates of algal growth (i.e. light intensity, nitrogen concentration and temperature), and which resulted in maximum rates of secondary carotenoid synthesis (i.e. salt concentration and light intensity). The optimum levels were defined using response surface methodology.

This data was then used for the cultivation of the alga in a 30 l photobioreactor. A two stage fermentation process was designed for the pilot scale production of astaxanthin from *H. pluvialis*. During the first stage conditions within the reactor were optimised to achieve maximum rates of algal growth. When algal growth had ceased the conditions were changed to allow for maximum rates of astaxanthin production within the reactor. The results achieved within the reactor were comparable to those obtained when the alga was cultivated under small scale (i.e. 50 ml) laboratory conditions. The alga accumulated 40 mg of secondary carotenoid per unit litre of liquid culture, which accounted for 2.6% of the dry cell weight of the alga. However, in the 30 l reactor the length of time required to accumulate the secondary carotenoids was much longer under large scale culture conditions than under small scale laboratory conditions (probably due to limitations of light).

Further work involved studies on the biosynthetic pathway leading to astaxanthin formation in *H. pluvialis* using inhibitors of carotenoid synthesis. Some of the major carotenoid precursors involved in the biosynthesis of astaxanthin (i.e.  $\beta$ -carotene and lycopene) were identified and these results allowed possible pathways of astaxanthin synthesis to be postulated. However, the reactions of the latter stages of the pathway remain unresolved.

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# THE BIOCHEMISTRY OF CAROTENOIDS AND THEIR PRODUCTION FROM MICROALGAE

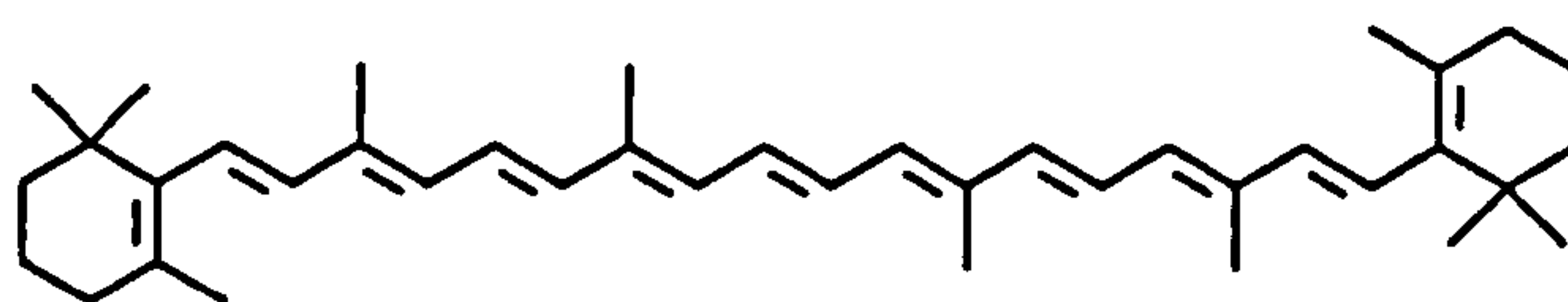
## 1.1 INTRODUCTION

Carotenoids are ubiquitous throughout nature and over 600 are known to exist in plants, animals, bacteria and fungi (Pfander, 1987). The carotenoids are yellow, orange or red and they are the major pigments of certain flowers, vegetables, berries, mushrooms, insects, feathers and egg yolk. In plants they are found in leaves, together with chlorophyll, and in animals they are dissolved in fats or combined with protein in the aqueous phase. Animals are unable to synthesise carotenoids and therefore accumulate them from extraneous sources in their diets. There are two main groups of carotenoids, the carotenes and the xanthophylls. The carotenes are hydrocarbons and are few in number. The xanthophylls are oxygenated derivatives of carotenes and make up the vast majority of the carotenoids.

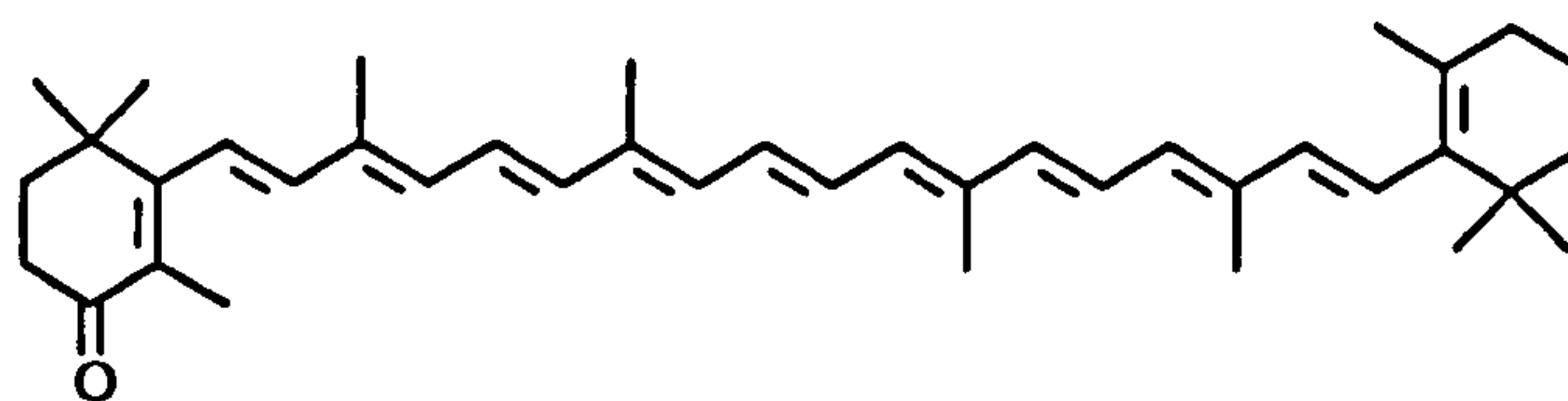
The microalgae are a diverse collection of chlorophyll *a* -containing organisms that include many divisions of the plant kingdom, including seaweeds and a number of single-celled and multicellular microscopic forms. Microalgae constitute the aquatic and marine phytoplankton and are common inhabitants of nearly all terrestrial and sub-aerial surfaces, including extreme environments in hot and cold deserts. Broad assemblages of microalgae are grouped into major categories, together with macroalgae, on the basis of pigmentation, cell wall composition, chemical constitution of food reserves, presence and type of flagella, and features unique to different groups (Metting and Pyne, 1986).

Microalgae accumulate a variety of unusual and characteristic carotenoids in their chloroplasts. In most members of the Chlorophyceae, so-called secondary carotenoids accumulate outside the chloroplast under certain adverse nutritional and environmental conditions. These should not be confused with those pigments found in the chloroplast which have specific functional roles in photoprotection and light-harvesting. The pigments concerned are usually  $\beta$ -carotene ( $\beta,\beta$ -carotene, I) and its ketonic derivatives echinenone

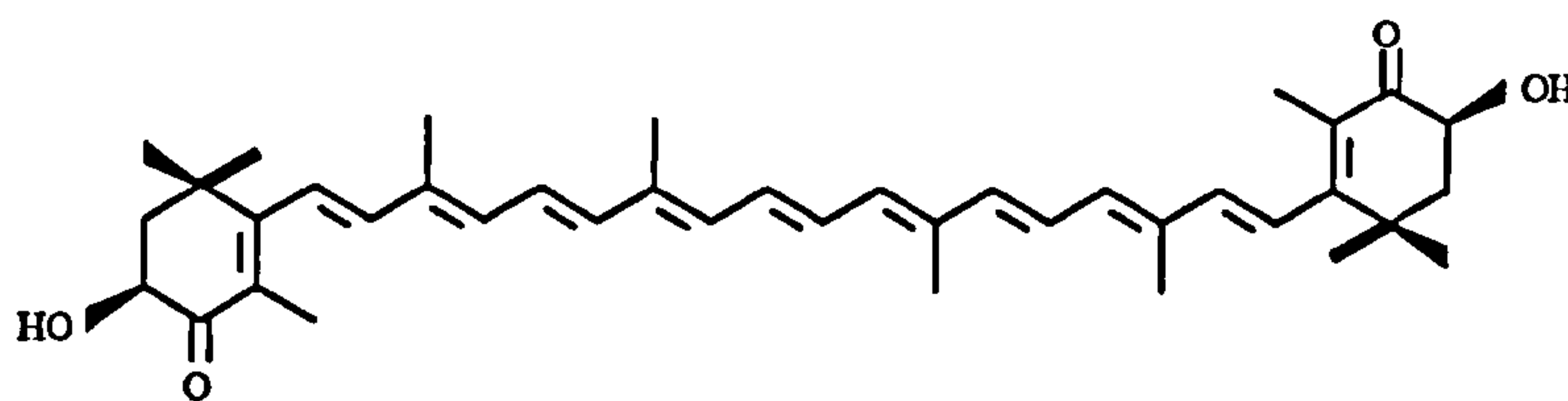
( $\beta,\beta$ -caroten-4-one, II), astaxanthin ((3*S*,3'*S*)-3,3'-dihydroxy- $\beta,\beta$ -carotene-4,4'-dione, III), canthaxanthin ( $\beta,\beta$ -carotene-4,4'-dione, IV) and adonirubin ((3*S*)-3-hydroxy- $\beta,\beta$ -carotene-4,4'-dione, V). These carotenoids are of potential commercial value if they can be produced in a highly concentrated form.



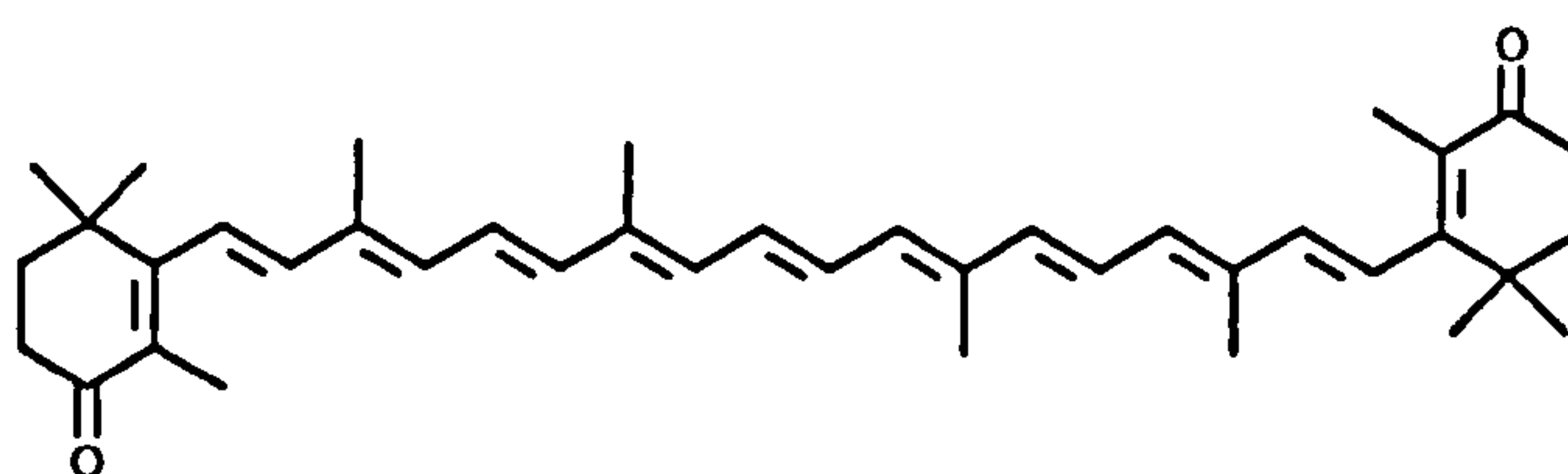
I  $\beta$ -Carotene



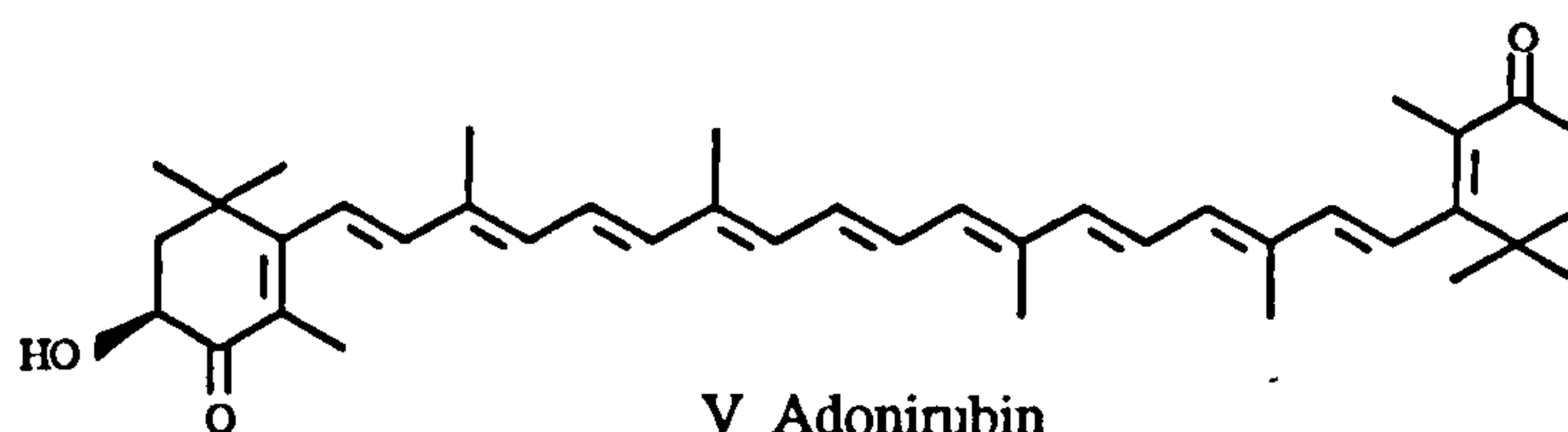
II Echineneone



III Astaxanthin



IV Canthaxanthin



V Adonirubin

Astaxanthin (III) is the principal carotenoid pigment of salmonids and imparts attractive pigmentation in the eggs, flesh and skin. World-wide production of farm-raised salmon has increased rapidly in the past decade, and more than 200,000T were produced in 1990. This figure is set to increase up to 460,000T by the year 2000 (Björndahl,



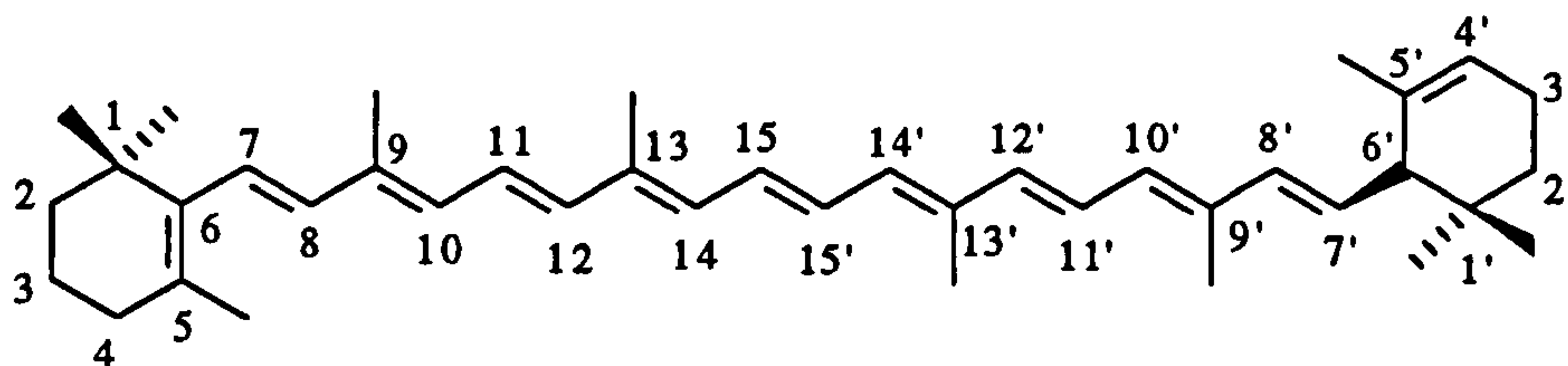
1990). The provision of pigments in feeds of farmed fish is very expensive. Currently the only available chemically synthesised astaxanthin (III) on a dry basis is sold for > \$4000/kg (marketed as Carophyll® Pink, Hoffman-LaRoche, Inc., Basel Switzerland, containing a minimum of 8% astaxanthin/kg). There is considerable interest within the aquaculture industry to develop the production of astaxanthin (III) from natural sources. Presently, the most promising recognised sources of astaxanthin (III) are crustacea (mainly the shrimp *Pandalus borealis* (Torrissen *et al.*, 1989) and the krill *Euphanasia pacifica*, *E. superba* (Lambertsen and Braekkan, 1971)), the microalga *Haematococcus* (Nakayama, 1962), and the yeast *Phaffia rhodozyma* (Andrewes *et al.*, 1976).

The work presented in this report is an investigation into the production of one specific carotenoid, astaxanthin (III), by microalgae. A number of species and strains of algae have been systematically screened to investigate which accumulates the greatest level of astaxanthin (III). The alga investigated most extensively was *Haematococcus pluvialis* (Flotow). A qualitative and quantitative examination of carotenoids in *H. pluvialis* was performed to determine the levels of astaxanthin (III) and associated secondary carotenoids within the alga. Morphological investigations of the alga revealed information concerning the alga's life cycle and its various morphological forms associated with the different stages of the cycle. Subsequent investigations were performed to determine the environmental factors responsible for initiating astaxanthin (III) synthesis in the alga, leading to the optimisation of the production of this particular carotenoid from *H. pluvialis*. The information gained from these studies was then applied to the cultivation of the alga in a 30 litre photobioreactor for the sole purpose of astaxanthin (III) production.

## 1.2 STRUCTURE AND NOMENCLATURE

Carotenoids are C<sub>40</sub> terpenoids that consist of eight isoprene units joined together so that the linking of the units is reversed at the centre of the molecule. The most prominent feature of the carotenoids is the polyene chain, which may extend from 3 to 15 conjugated double bonds. This chromophore is responsible for the characteristic absorption spectrum and colour of the molecule. Cyclization of the carbon skeleton, at one or both ends, also

occurs, while xanthophylls are formed from the hydrocarbon carotenes by the introduction of oxygen functions. In addition, skeletal modifications involving chain elongation to ( $C_{45}$  -  $C_{50}$ ) or degradation to apocarotenoids take place, and some carotenoids are biogenetically  $C_{30}$ . The basic system of numbering carotenoids is shown in the structure of  $\alpha$ -carotene ((6'*R*)- $\beta,\epsilon$ -carotene, VI). Individual carotenes are named by the specific end groups which they contain, while the xanthophylls are named according to the usual rules of organic chemical nomenclature.

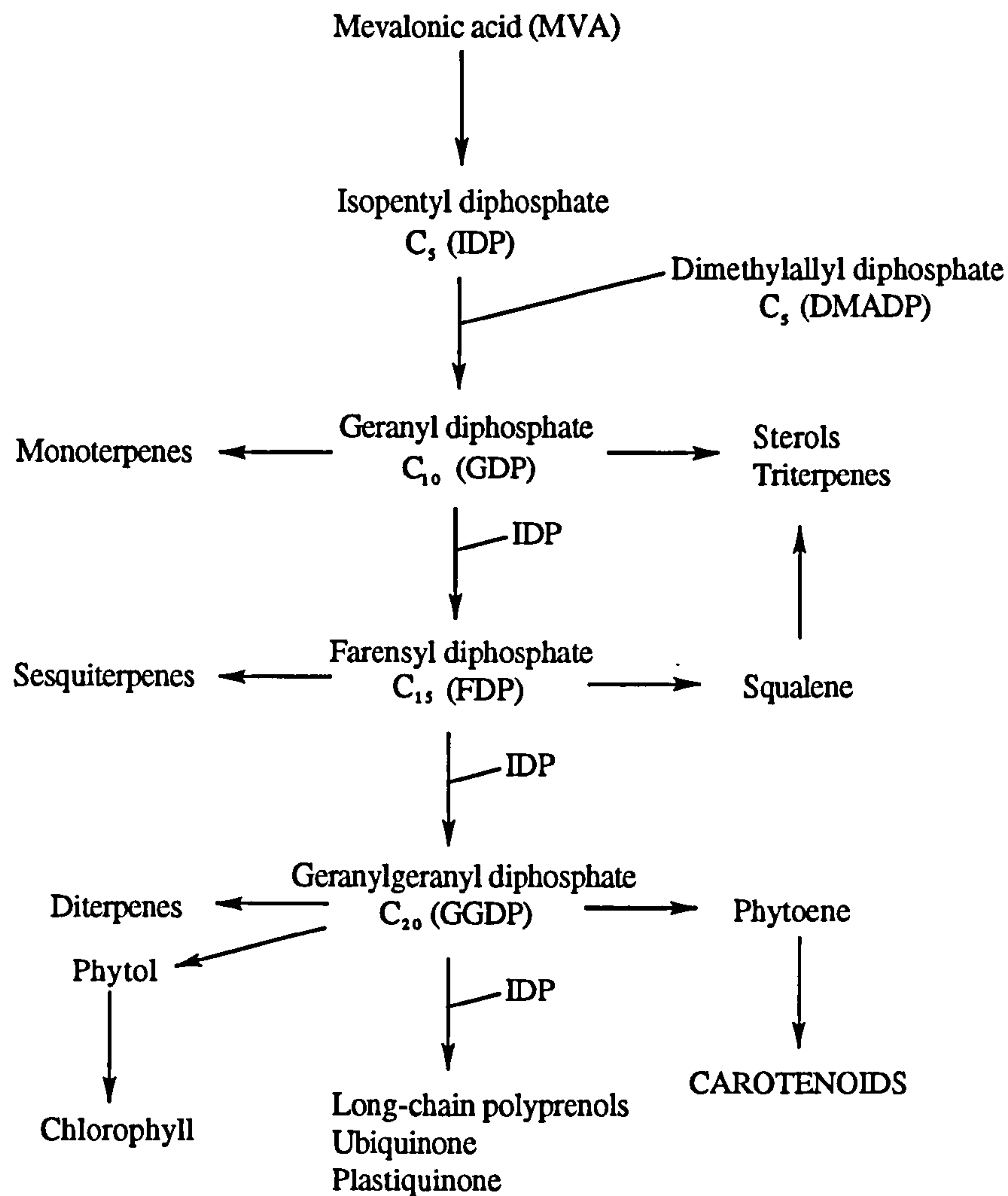


VI  $\alpha$ -Carotene

### 1.3 BIOSYNTHESIS OF CAROTENOIDS

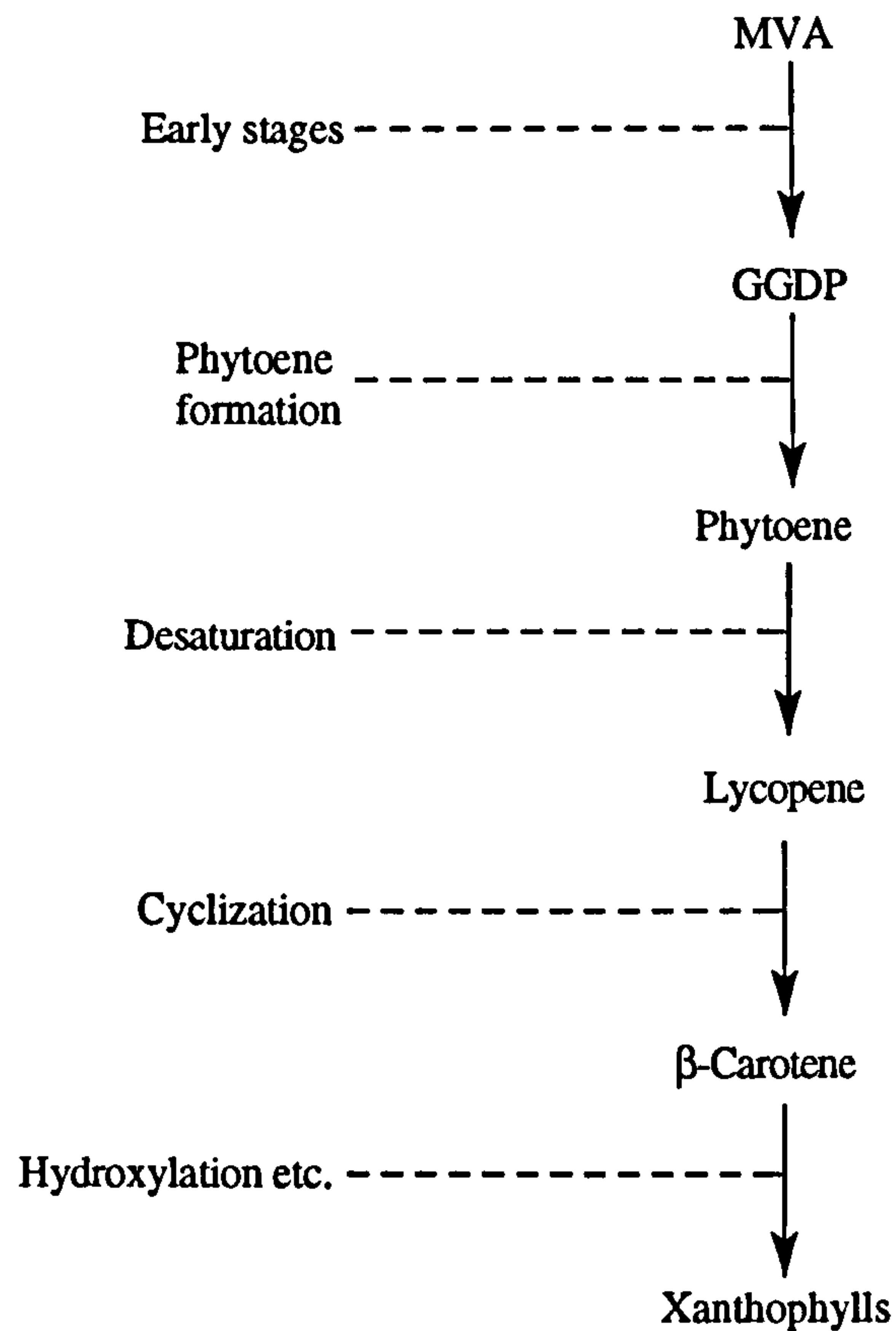
#### 1.3.1 Introduction

Since carotenoids are terpenoids they are related biosynthetically, and share a common early pathway, with other biologically important isoprenoids, as shown in figure 1.1 (Britton, 1976a).



**Fig. 1.1** Biosynthesis of isoprenoid compounds.

The early stages in which C<sub>5</sub> isoprenoid units are constructed and then used to build the required prenyl diphosphate intermediates, are common stages in the biosynthesis of all terpenoid compounds. The later stages after the formation of geranyl geranyl diphosphate are unique in the biosynthesis of carotenoids. The formation of phytoene (7,8,11,12,7',8',11',12'-octahydro- $\psi,\psi$ -carotene, VII and VIII) and its subsequent desaturation, cyclization and later modifications, are the main stages of carotenoid biosynthesis, as outlined in figure 1.2.

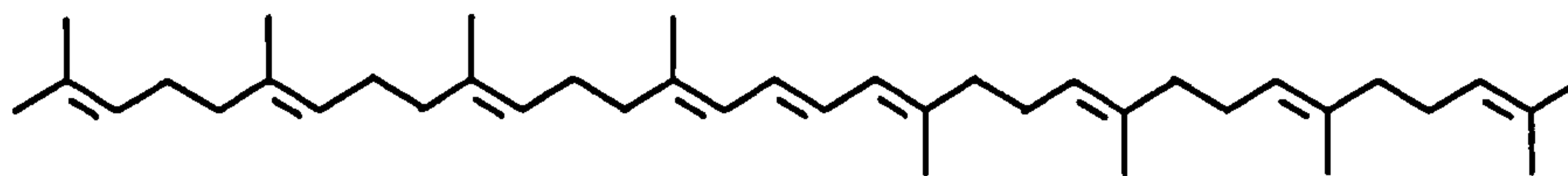


**Fig. 1.2** Summary of the main stages of carotenoid biosynthesis.

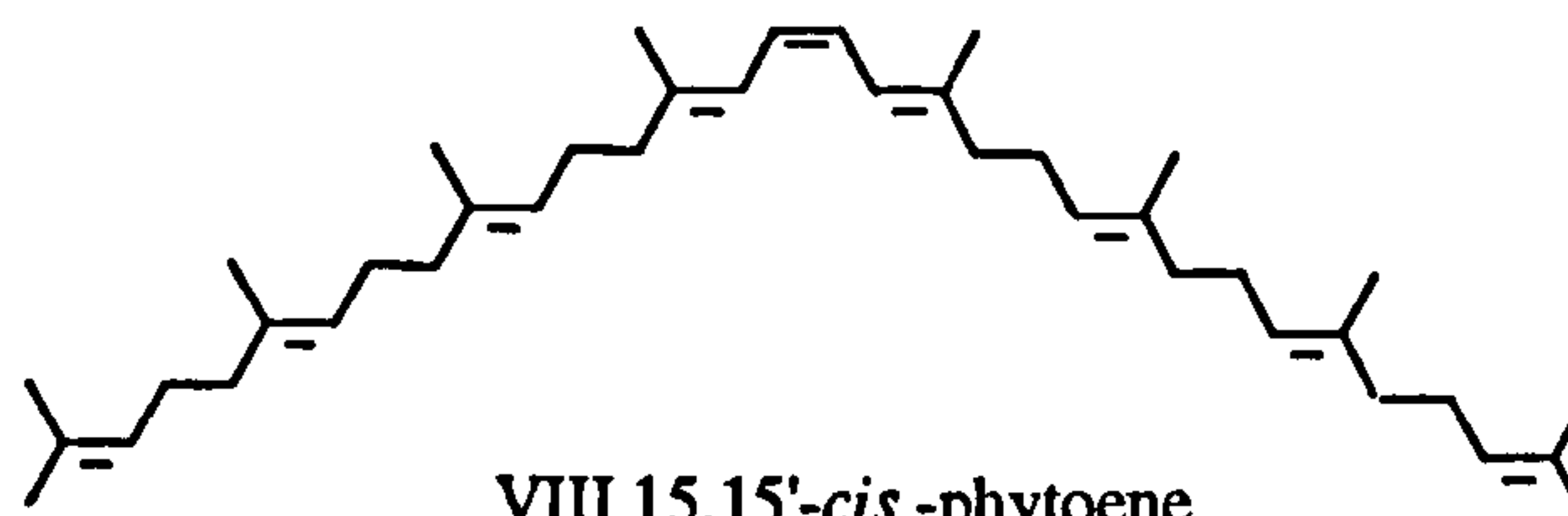
### 1.3.2 Formation of phytoene

The first specific precursor of all terpenoids is mevalonic acid (MVA), which is formed from acetyl-CoA via 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA). MVA is converted to isopentyl diphosphate (IDP), the universal isoprene unit, via a three step sequence involving the soluble enzymes MVA kinase, MVAP kinase, and diphosphomevalonate decarboxylase. Isomerization of IDP to dimethylallyl diphosphate (DMAPP), catalysed by IDP isomerase, is followed by a series of condensation reactions, resulting in the formation of geranyl diphosphate (GDP), farnesyl diphosphate (FDP), and geranyl geranyl diphosphate (GGDP). These condensation reactions are catalysed by prenyl transferases. One of six possible metabolic fates of GGDP is to undergo tail to tail dimerization to form the first C<sub>40</sub> carotene, phytoene (VII and VIII). This two-step conversion via prephytoene diphosphate (PPDP) is catalysed by two enzymes and form either 15-*cis* or all-*trans*-phytoene (VII and VIII) (Bramley, 1985).





VII all-*trans*-phytoene



VIII 15,15'-*cis*-phytoene

### 1.3.3 Desaturation

Phytoene (VII and VIII) undergoes a series of sequential desaturation reactions (Fig. 1.3) to give phytofluene (7,8,11,12,7',8'-hexahydro- $\psi,\psi$ -carotene, IX),  $\zeta$ -carotene (7,8,7',8'-tetrahydro- $\psi,\psi$ -carotene, X) or its 'unsymmetrical' isomer, neurosporene (7,8-dihydro- $\psi,\psi$ -carotene, XII), and finally lycopene ( $\psi,\psi$ -carotene, XIII). At each stage, two hydrogen atoms are removed by *trans*-elimination from adjacent positions to introduce a new double bond and extend the conjugated polyene chromophore by two double bonds (Davies and Taylor, 1976).

In organisms containing 15-*cis*-phytoene (VIII), the desaturation sequence must include a *cis* to *trans* isomerization step, since the more unsaturated carotenes are all-*trans* (except for tissues containing poly-*cis*-carotenes, e.g. tangerine tomatoes). In higher plants this step has been shown to be at the phytofluene (IX) stage, but it may occur at the phytoene (VII and VIII) level in *Flavobacterium* R1560 and *Phycomyces* (Britton, 1990).

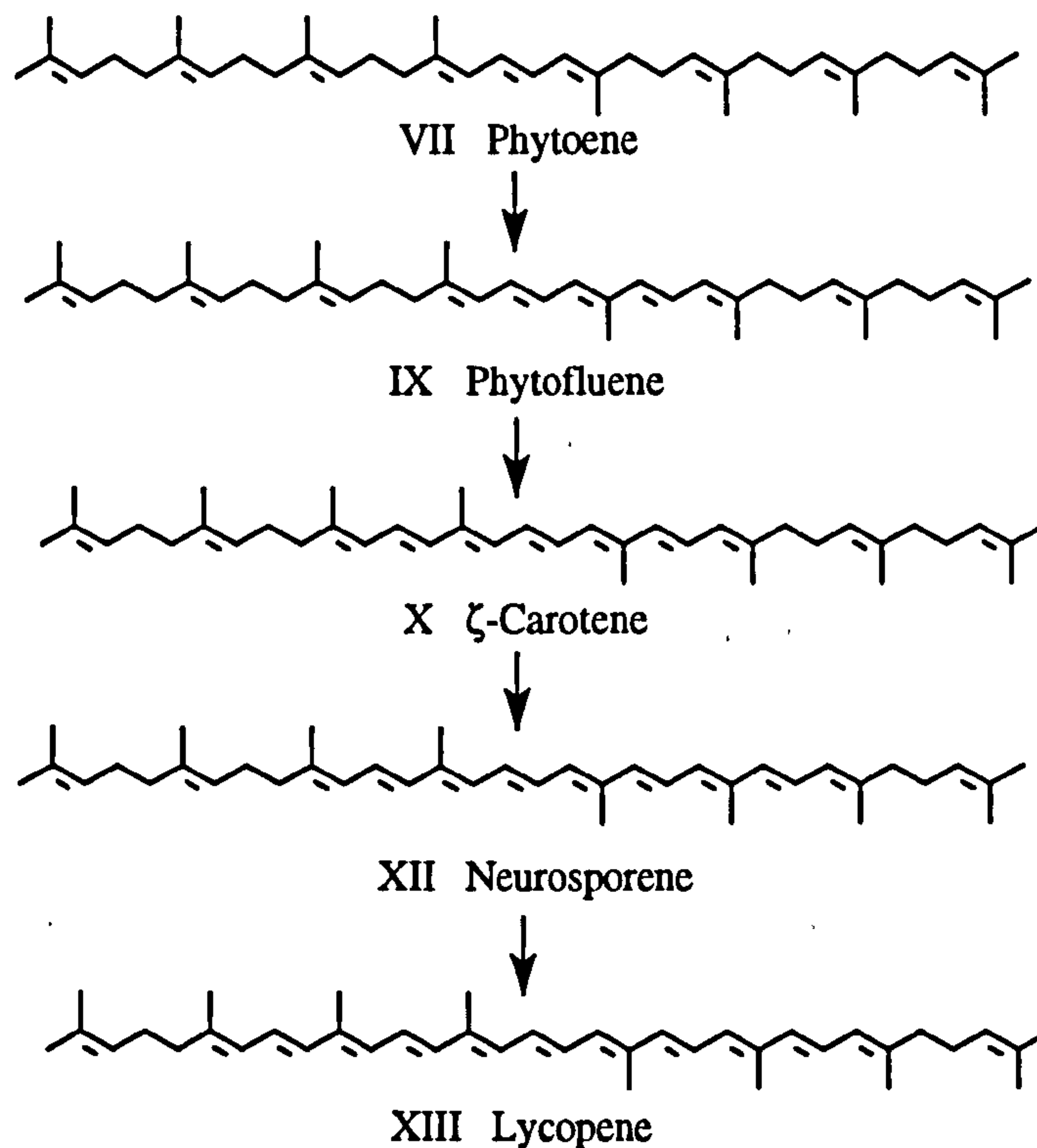
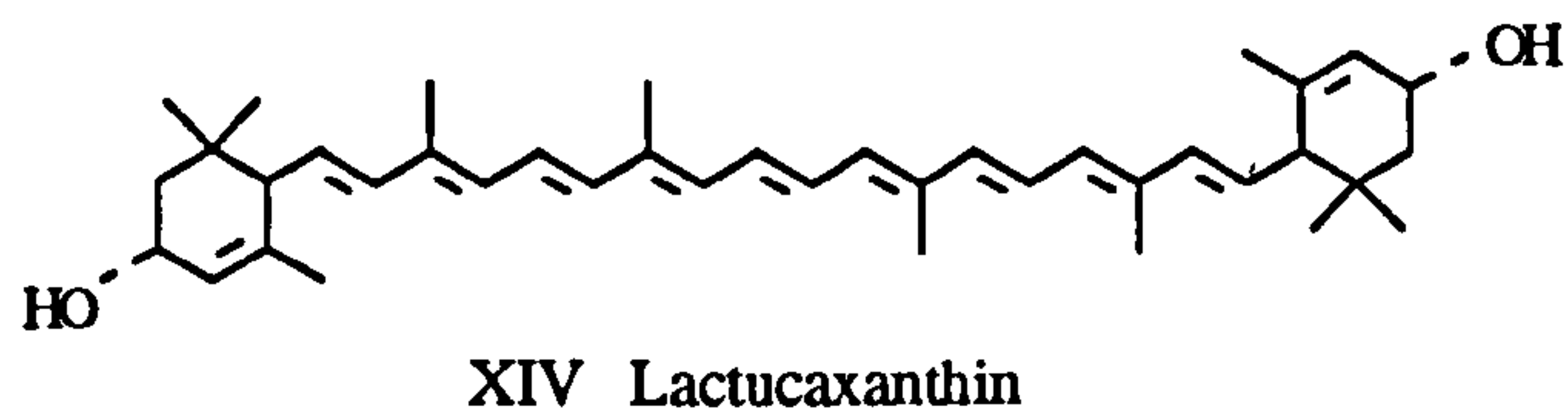


Fig 1.3 Sequence of desaturation reactions.

### 1.3.4 Cyclization

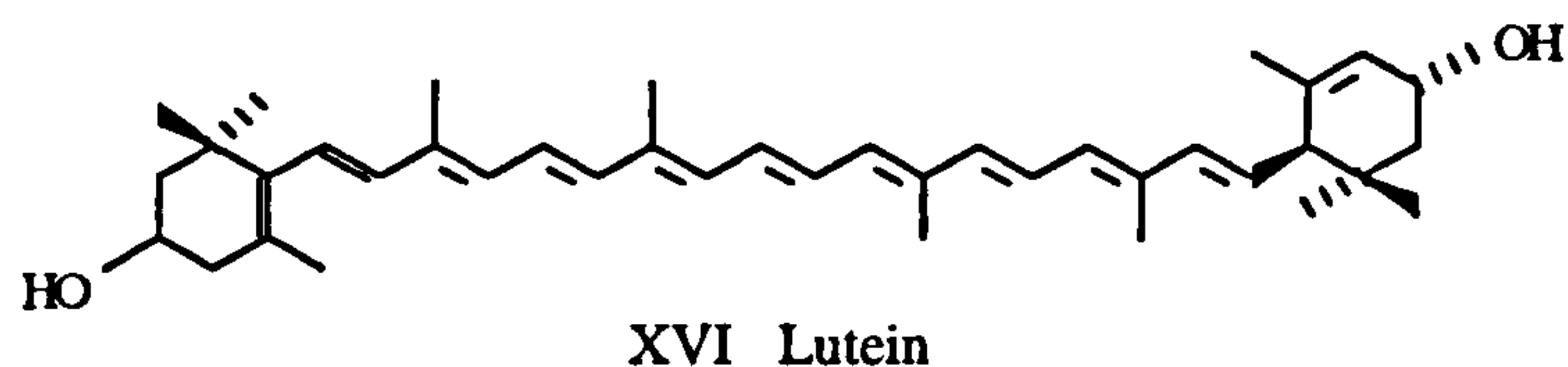
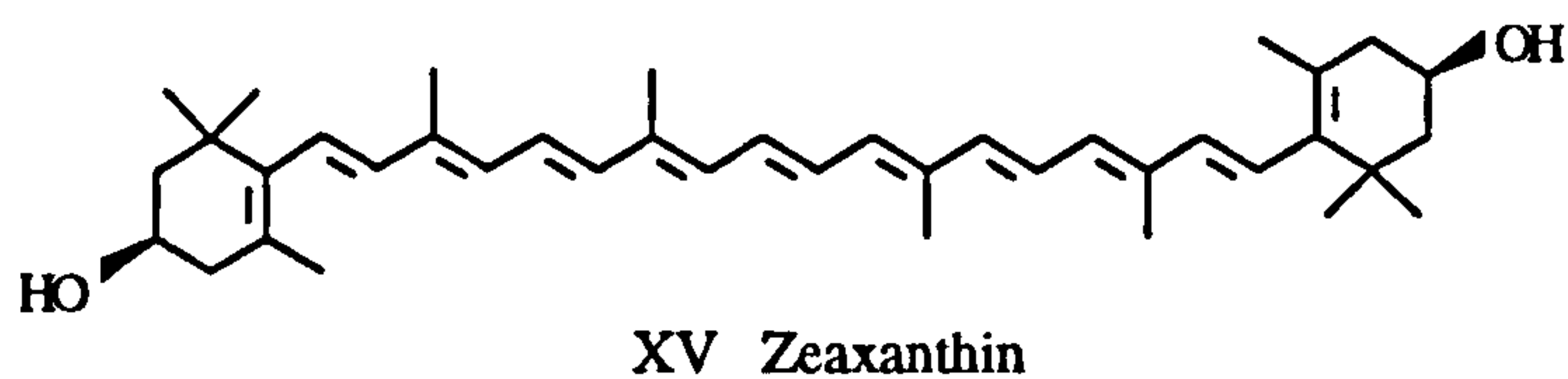
Once a carotenoid has reached the lycopene (XIII) level of desaturation, cyclization can take place to give a six-membered ring. Cyclization is initiated by proton attack at C-2 of the acyclic precursor, for either lycopene (XIII) or neurosporene (XII). Stabilization of the resulting transient C-5 'carbonium ion' intermediate is by the loss of a proton to form a  $\beta$ -ring,  $\epsilon$ -ring, or  $\gamma$ -ring depending on which proton is lost.

The removal of hydrogen in the cyclization reaction is stereospecific. It has been shown that in natural  $\epsilon$ -ring carotenoids such as lactucaxanthin (3*S*,6*S*,3'*S*,6*S*- $\epsilon,\epsilon$ -carotene-3,3'-diol, XIV), the configuration around C-6 is the *R* configuration. In the formation of the  $\beta$ -ring, the hydrogen atom lost is that which was originally the 2-pro-*S* hydrogen atom of MVA. The stereochemistry of the initial proton attack at C-2 is the same as in the formation of the  $\beta$ -ring, but the behaviour of the C-1 methyl groups has not yet been determined (Milborrow, 1982).



### 1.3.5 Hydroxylation and introduction of other O<sub>2</sub> functions

Xanthophylls commonly have hydroxyl substituents at C-3 and C-3'. It is generally accepted that the introduction of these groups occurs late in the biosynthetic pathway, i.e. after dehydrogenation has occurred. It has also been shown that the presence of oxygen is necessary for hydroxylation. In experiments using <sup>18</sup>O it was shown that the oxygen hydroxyl functions came from molecular oxygen and not water. Hydroxylation at C-3 of the β-ring, e.g. in zeaxanthin (3*R*,3'*R*-β,β-carotene-3,3'-diol, XV) or lutein ((3*R*,3'*R*,6'*R*)-β,ε-carotene-3,3'-diol, XVI), normally proceeds by the direct replacement of the (3-pro-*R*)-hydrogen atom of the hydrocarbon precursor (i.e. the one which was originally the (5-pro-*R*)-hydrogen of MVA) by OH. The reaction is assumed to be catalysed by a mixed-function oxidase enzyme, involving cytochrome-P<sub>450</sub> (Britton, 1976b).



The ketocarotenoid astaxanthin (III) occurs naturally in different enantiomeric forms. The form obtained in the algae is the (3*S*,3'*S*)-isomer. The assumption is made that in algae the C-3 hydroxy groups are introduced first, with normal stereochemistry, to give (3*R*,3'*R*)-zeaxanthin (XV), into which the C-4 keto groups are then introduced (Cooper *et al.*, 1975). However, later workers have suggested another route for the biosynthesis of secondary carotenoids in *H. pluvialis*, involving the conversion of β-

carotene (I) to astaxanthin (III) via the intermediates echinenone (II) and canthaxanthin (IV) (see below). Figure 1.4 outlines the proposed biosynthesis pathway of astaxanthin (III) from  $\beta$ -carotene (I) (Donkin, 1976; Grung, *et al.*, 1992).

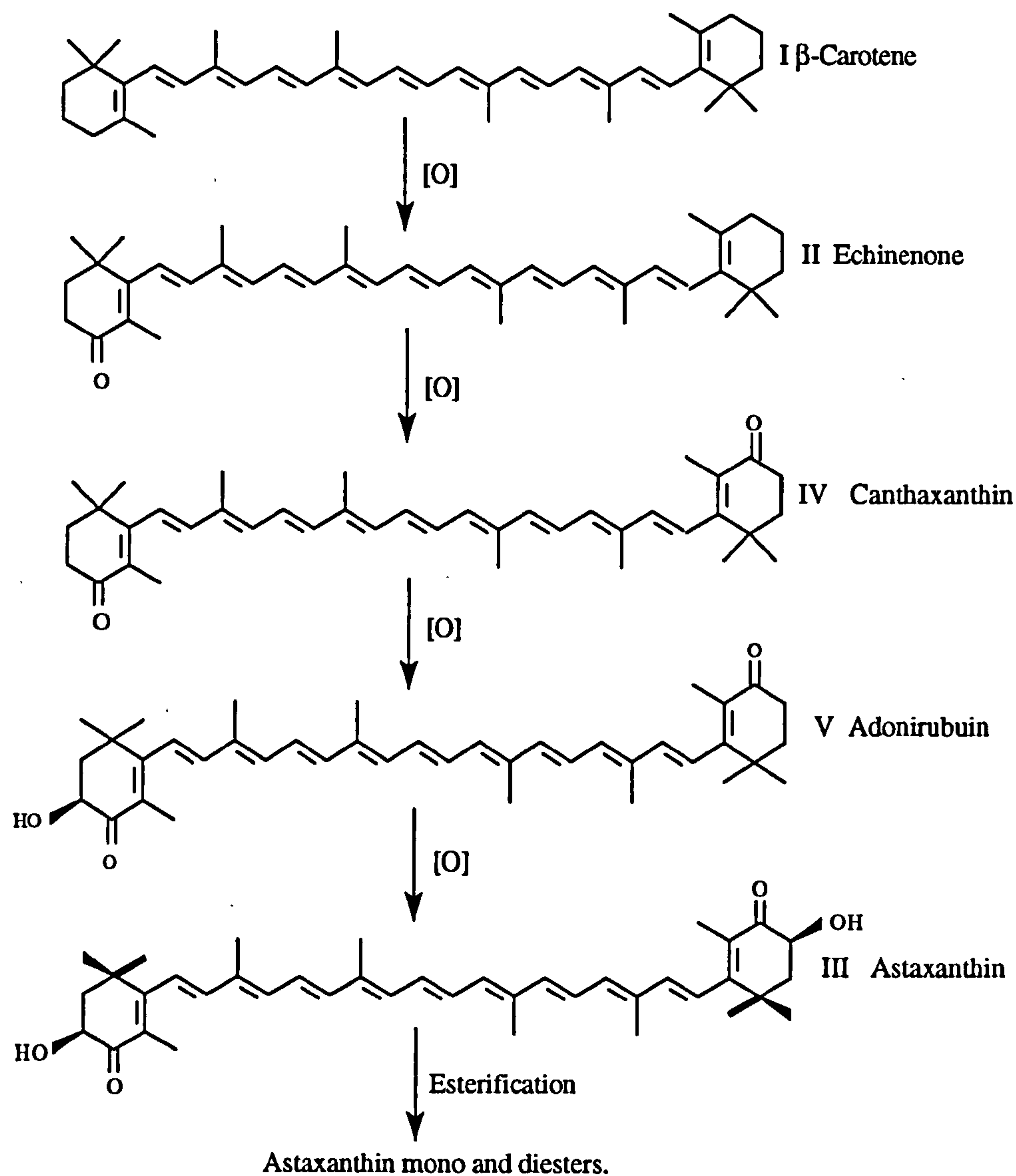


Fig. 1.4 Biosynthesis of secondary carotenoids in *H. pluvialis*.

### 1.3.6 Regulation of carotenoid biosynthesis in the Chlorophyta

The carotenoids found in the class Chlorophyta are usually those present in the chloroplasts of higher plants, namely  $\alpha$ -carotene (VI),  $\beta$ -carotene (I), lutein (XVI), violaxanthin (XVII), neoxanthin (XX), and zeaxanthin (XV). The latter is an occasional minor component of higher plant chloroplast carotenoids, but is widely distributed in the Chlorophyta. In addition to the normal complement of carotenoids, some species accumulate unusual xanthophylls. These exceptions will be discussed further in Chapter 3.



The complement of carotenoids an organism can synthesise is under genetic control, but the amount, and to some extent type, synthesised can be influenced by a number of external factors.

#### 1.3.6.1 Genetic control

The genetic control of carotenoid biosynthesis has been studied by the isolation and examination of carotenoid mutants. Isolated mutants of *Chlorella vulgaris* have been shown to form phytoene (VII and VIII), phytofluene (IX), and  $\zeta$ -carotene (X), or these compounds plus proneurosporene (XII) and prolycopene (XIII), when grown in the dark on glucose. One of these mutants also showed the disappearance of the more saturated carotenes, proneurosporene (XII) and prolycopene (XIII) on exposure to light, and the concomitant appearance of *trans*-lycopene (XIII) and  $\alpha$ - and  $\beta$ -carotene (VI and I) (Bramley and Mckenzie, 1988). A mutant strain of the green alga *Scenedesmus obliquus* accumulates  $\zeta$ -carotene (X) when grown in the dark, but is able to convert this into normal cyclic carotenoids when illuminated.

#### 1.3.6.2 Nutritional control

Under unfavourable nutritional conditions, especially nitrogen deficiency, some green algae may become yellow or red due to the formation of large amounts of  $\beta$ -carotene (I), echinenone (II), hydroxyechinenone (II), canthaxanthin (IV) and astaxanthin (III). These secondary carotenoids are located outside the chloroplast in globules or in the cell wall. In salinities above 5% NaCl (w/v) *Dunaliella salina* accumulates large amounts of  $\beta$ -carotene (I) in globules. In *Ankistrodesmus* spp., deficiency of  $\text{PO}_4^{3-}$ ,  $\text{SO}_4^{2-}$ ,  $\text{Ca}^{2+}$  or  $\text{Fe}^{3+}$  stimulates carotenogenesis. A low phosphate medium stimulates carotenogenesis threefold in a bleached strain of *Euglena* (Goodwin, 1980).

#### 1.3.6.3 Light

Carotenogenic organisms can be divided into three categories. In the first category are those organisms in which light plays little or no role in the regulation of carotenoid biosynthesis. In the second category are those organisms which can synthesise carotenoids in the absence of light, but synthesis is enhanced in the presence of light. Finally, there



are those organisms in which the synthesis of carotenoids is totally photo-induced. Algae generally belong the first category, however, photo-regulation of carotenoid biosynthesis has been documented in *Euglena* (Goodwin and Jamikorn, 1954a). Light intensity has been shown to be important for the accumulation of  $\beta$ -carotene (I) in *Dunaliella*; increasing the light intensity results in increasing amounts of  $\beta$ -carotene (I) per cell (Ben-Amotz and Avron, 1983). High light intensities also increase the level of accumulation of ketocarotenoids in *H. pluvialis*, however, light is not necessary for their synthesis (Kobayashi *et al.*, 1992). In a number of other cases light has been shown to influence carotenoid biosynthesis, but for the most part carotenoid biosynthesis in algae is independent of light (Rau, 1976).

#### 1.3.6.4 Chemical inhibitors

Certain chemicals have been identified which are able to interfere with the normal synthesis of carotenoids. While these are not a natural control mechanism, the use of these inhibitors has been of great value in studies on carotenogenesis. Some of the most effective inhibitors of normal carotenogenesis are diphenylamine (DPA), 2-hydroxybiphenyl, 9-fluorenone, nicotine, 2-(4-chlorophenylthio)-triethylamine HCl (CPTA) and norflurazon (Bramley and Mckenzie, 1988). The main target sites for these chemicals are the desaturation and cyclization reactions of carotenoid biosynthesis (see Fig. 1.2). There are no-known effective inhibitors of hydroxylation. This is probably due to the many other effects that such a chemical would produce in an algal cell.

#### 1.3.6.5 Chemical induction

There have been few reports of the induction of carotenoid synthesis by certain chemicals in microalgae. However, the addition of vinblastine to cultures of *H. pluvialis* has been reported to increase the accumulation of secondary carotenoids in the alga (Boussiba and Vonshak, 1991). While the chemical inhibits cell division, the ability of the alga to assimilate carbon is not affected.

## 1.4 FUNCTIONS OF CAROTENOIDS IN ALGAE

### 1.4.1 Location of carotenoids in the photosynthetic apparatus

The photosynthetic apparatus of algae is membrane bound, with the chlorophylls and carotenoids representing the major pigments of photosynthetic membranes. Chlorophylls and carotenoids are lipophilic and they can therefore be located either in hydrophobic domains of membrane proteins or in the lipid membrane matrix. Membrane models preferring either location have been put forward (Kreutz, 1964; Weier and Benson, 1966). More direct experimental evidence has shown that photosynthetic pigments are non-covalently bound to membrane proteins or membrane lipids.

For some higher plants, the protein bound nature of chlorophylls has been shown (Thornber, 1975). Carotenoids are removed more easily from their apoproteins than the chlorophylls when the membrane is exposed to detergent. The available data supports the view that photosynthetic carotenoids of higher plants are protein bound *in situ* (Siefermann-Harms, 1985). Only recently has 100% recovery of carotenoids during electrophoretic separation been possible for higher plant preparations (Bassi *et al.*, 1993; Ruban *et al.*, 1994). This is particularly difficult to achieve and requires the use of isoelectric focusing techniques (IEF) and mild detergents such as dodecyl maltoside. However, evidence that carotenoids of all photosynthetic apparatus are entirely protein bound still remains to be established. In the case of several algae, the fractionation of their photosynthetic apparatus requires rather harsh conditions. Under such conditions up to 60% of the carotenoids have been recovered in the 'free pigment' fraction, either because they have never been bound to proteins *in situ*, or, more probably, because they were freed during membrane fractionation.

Photosynthetic pigment proteins can be divided into two functional groups. First, reaction-centre proteins containing, in addition to antenna pigments, a primary electron donor pigment that is oxidized under illumination, and second, the light-harvesting proteins (LHC) containing antenna pigments only. These are photochemically inactive, but *in situ* they transfer absorbed light energy to the reaction-centre pigments. Although the reaction-centre proteins are normally contained within the membranes, the light-harvesting proteins are not always intrinsic membrane components.

## 1.4.2 Carotenoids in the photosynthetic apparatus of algae

The photosynthetic apparatus of algae consists of two photosystems that drive electron transport from water to NADPH through an electron carrier chain. This process is illustrated in figure 1.5. Photosystem I (PSI) and photosystem II (PSII) can be isolated in a photochemically active state. Preparations completely devoid of the major light-harvesting complexes have been obtained. In the case of green algae such photosystem preparations are still composed of several pigment proteins, namely, the reaction-centre proteins that bind both the primary electron-donor pigment, and a substantial amount of antennae pigments, and one or more light-harvesting proteins specifically associated with the reaction-centre-proteins.

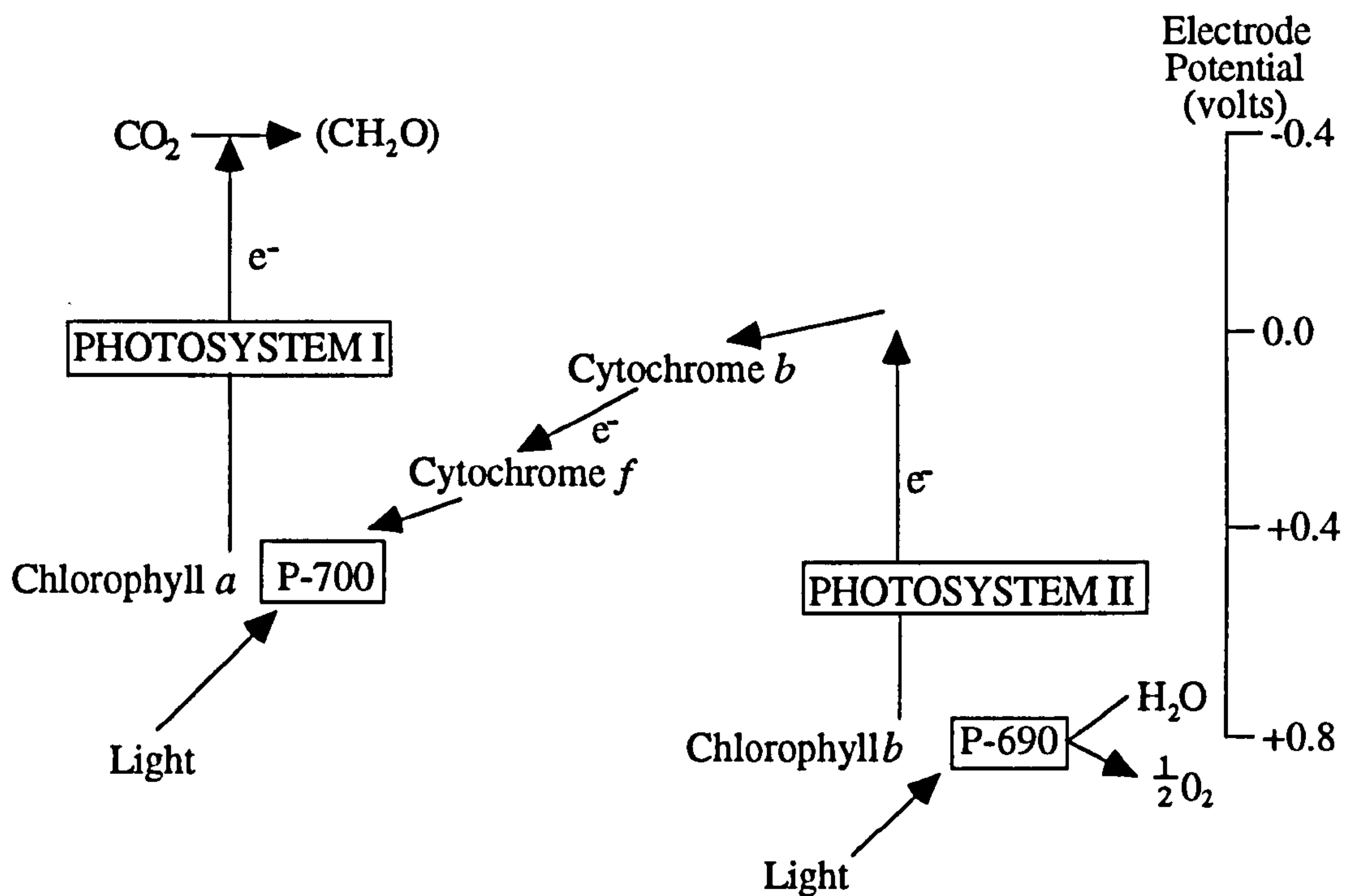


Fig. 1.5 Outline of the 'Z-scheme' indicating the two photosystems concerned with photosynthesis in aerobic organisms.

## 1.4.3 Reaction-centre proteins

### 1.4.3.1 Photosystem I

The reaction-centre protein of PSI contains the primary electron-donor pigment P-700 and numerous antenna pigments. It is composed of a variable number of polypeptides depending on the isolation procedure which has been used by the various workers. The



heaviest of these polypeptides (50 - 70 kDa) carries the pigments. In its photochemically active pigment-bearing state this polypeptide has been named subunit I. In green algae, two (Bengis and Nelson, 1977) or four (Vierling and Alberte, 1983) heavy polypeptides have been proposed to participate in the assembly of one functional subunit I, together with one P-700. Subunit I can be associated with 3 - 6 small polypeptides (8 - 20 kDa) that appear to be involved in electron transport around PSI (Lagoutte *et al.*, 1984). Reaction-centre I proteins have been isolated from red algae (Redlinger and Gantt, 1983), Cryptophyceae and Xanthophyceae (Wiedemann *et al.*, 1983), Phaeophyta (Duval *et al.*, 1983) and Chlorophyceae (Wiedemann *et al.*, 1983). As in the case for higher plants their pigment content usually consists of Chl *a* and  $\beta$ -carotene (I), often with  $\alpha$ -carotene (VI) (especially in the Rhodophyta) in addition to P-700.

#### 1.4.3.2 *Photosystem II*

PS II is located in grana membranes where it constitutes a supramolecular complex.

Reaction-centre II proteins consist of two pigment bearing subunits designated as CPa-1 and CPa-2 (Green and Camm, 1984). Each subunit contains a single polypeptide (40 - 50 kDa). Both subunits bind Chl *a* and  $\beta$ -carotene (I). Spectroscopic evidence indicates that the reaction-centre II pigment P-680 is located in CPa-1 (Nakatani *et al.*, 1984).

Furthermore, CPa-1 appears to contain phaeophytin, the primary electron acceptor of PSII. Therefore, CPa-1 can be considered to be the core complex of PSII. CPa-2, the lighter of the two reaction-centre II subunits, lacks photochemical activity and has been suggested to be an internal light-harvesting protein of PSII (Green, 1988).

The carotene and Chl *a* ratio for reaction II proteins from many species is very similar. When considering Chl *a*, reaction-centre II proteins contain more  $\beta$ -carotene (I) than reaction-centre I proteins.  $\beta$ -Carotene (I) and  $\alpha$ -carotene (VI) are present at similar levels in both the reaction-centre II core protein CPa-1, and the internal light-harvesting protein CPa-2. Minor pigment-proteins have been isolated from thylakoid extracts enriched in PSII that, in contrast to reaction centre proteins, contain significant amounts of Chl *b* (Chl *a* /*b*-ratio >3:1) (Camm and Green, 1980).

#### **1.4.4 Light-harvesting proteins**

All O<sub>2</sub> evolving photosynthetic organisms contain extended light-harvesting systems. The light-harvesting systems show enormous variations in the pigments present, their organisation and location within or adjacent to the membrane. The various types of light-harvesting pigment-protein complexes represent an important distinctive feature for the classification of algal groups. In spite of their structural heterogeneity they agree largely on the functional level, channelling harvested light energy predominantly in PSII (Fujimori and Livingston, 1957).

Properties of major light-harvesting pigment-proteins isolated from algae range from water-soluble phycobiliproteins of the dinoflagellates to hydrophobic membrane-intrinsic Chl *a/c* xanthophyll-proteins, or Chl *a/b* xanthophyll-proteins observed in the majority of algae. A general feature of all light-harvesting Chl-carotenoid-proteins is that, in contrast to the reaction-centre proteins, they bind xanthophylls rather than carotenes. Most (80%) of the total pigment composition is located in LHCII, as opposed to LHCI (Bassi *et al.*, 1993).

In contrast to higher plant LHC, in which the pigment composition is relatively conserved (usually 6 major xanthophylls), the LHC of algae contain a diversity of structures (more than 30 identified to date). This has enabled some workers to suggest that these compounds can be used as chemotaxonomic markers (see section 1.7.1). The Chlorophyceae, however, possess a similar pigment composition to that found in higher plants, although some species do contain additional and often unusual xanthophylls (see section 4.1).

#### **1.4.5 Light-harvesting function of carotenoids**

The light harvesting ability of carotenoids allows for utilization of blue-green light that is poorly or not absorbed by the Chls. This effectively extends the range of light available for photosynthesis and its importance has clearly been demonstrated in phototrophic bacteria. Its role in chl *a/b* containing plants and algae is less well developed due to SORET absorption of these chlorophylls (i.e. absorption of light in the blue region of the spectrum) at similar wavelengths to that of the carotenoids.



### 1.4.6 Energy transfer in photosynthetic structures

The reaction sequence beginning with carotenoid excitation and leading to some photosynthetic activity consists of several steps (Moore *et al.*, 1980):



The absorptive act, in which the carotenoid is transferred into its excited state,  ${}^1\text{Car.}^*$ :



Singlet-singlet excitation energy transfer from excited carotenoid to ground state Chl.



Singlet-singlet excitation energy transfer from  $\text{Chl}_1$  to  $\text{Chl}_n$ , and



transfer of the excitation energy into reaction centres, where the photochemical reactions are induced that finally drive photosynthetic activities like  $\text{O}_2$  evolution or  $\text{CO}_2$  fixation, or



de-excitation of excited Chl by fluorescence emission, a pathway especially favoured when reaction (1.4) is blocked or absent, as in isolated light-harvesting pigment-proteins. In algae, energy transfer from carotenoids to Chl *a* is generally observed, although efficiency may vary for different organisms and types of carotenoids.

### 1.4.7 Mechanism of singlet-singlet excitation energy transfer from carotenoids to chlorophyll

Singlet-singlet energy transfer from an excited donor  $^1\text{Don}^*$  to an acceptor in its ground state  $^1\text{Acc}$ .



may proceed via one of two basically different mechanisms:

(i) Coulombic or dipole-dipole resonance interaction between  $^1\text{Don}^*$  and  $^1\text{Acc}$ .

During energy transfer, resonance occurs between oscillations of the excited electron in  $^1\text{Don}^*$  and of a 'ground state' electron in  $^1\text{Acc}$ . The excited electron then relaxes its oscillatory motion, while the ground state electron is set into enhanced motion of an excited electron (Thrash *et al.*, 1979). Thus, the dipole-dipole resonance interaction is induced by an electromagnetic field originating in  $^1\text{Don}^*$  and does not require physical contact of the interacting partners (Förster, 1959).

(ii) Exchange resonance interaction between the excited electron of  $^1\text{Don}^*$  and a 'ground state' electron of  $^1\text{Acc}$ . During energy transfer, the interacting partners form a collision complex in which the electron clouds of donor and acceptor overlap. This situation allows the excited electron to move from  $^1\text{Don}$  to  $^1\text{Acc}$  while the ground state electron moves from  $^1\text{Acc}$  to  $^1\text{Don}$ . Thus exchange resonance interaction occurs via overlap of electron clouds and requires physical contact between interacting partners (Dexter, 1953).

### 1.4.8 Protective functions of carotenoids

For photosynthetic organisms, the presence of carotenoids in their photosynthetic apparatus is essential for protecting the cells against harmful effects of light and molecular oxygen ( $^3\text{O}_2$ ). Carotenoids can protect against light-mediated damage in two ways: (i) by preventing the formation of singlet oxygen by quenching triplet-state chlorophyll molecules, and (ii) by scavenging any singlet oxygen ( $^1\text{O}_2^*$ ) produced. Singlet oxygen is a powerful oxidizing agent which will result in the destruction of cell components

(membranes, proteins etc.), leading ultimately, to the death of the organism (Young, 1991).

The photophysical reactions preceding Chl destruction and the mechanism of protection by carotenoids are well established. In addition to the de-excitation pathways (1.4) and (1.5) of  $^1\text{Chl}^*$ , a third pathway is observed in photosynthetic membranes, i.e. the formation of  $^3\text{Chl}^*$  by intersystem crossing from the lowest excited electronic state with paired spins to that with unpaired spins (Mathis and Schenck, 1982):



This Chl species is difficult to detect in carotenoid containing membranes, since it is rapidly converted to ground state Chl, mainly via energy transfer pathway (1.8) (Fujimori and Livingston, 1957):



The triplet-state  $^3\text{Car}^*$  decays within a few microseconds via non-radiative intersystem crossing (Moore *et al.*, 1990):



This decay is strongly enhanced in the presence of  $^3\text{O}_2$ . In the absence of carotenoids,  $^3\text{Chl}^*$  transfers its energy to ground state  $^3\text{O}_2$ , thus generating excited singlet state oxygen  $^1\text{O}_2^*$ :



Pathway (1.10) is significantly less efficient for de-excitation of  $^3\text{Chl}^*$  than pathway (1.8). Since  $^3\text{Chl}^*$  has a sufficiently long lifetime to interact with other compounds, and since it readily forms reactive ions (Siefertmann-Harms, 1985), it has the potential to initiate membrane damage. More dangerous is the long lived  $^1\text{O}_2^*$  species, that readily reacts with

the unsaturated fatty acids of membrane lipids, with aromatic amino acids and purines (Krinsky, 1971). All of these reactions can ultimately lead to the death of the organism (Krinsky, 1978; Will and Scavel, 1990).

With the formation of  $^1\text{O}_2^*$ , carotenoids have a second chance to exhibit their protective role, by quenching  $^1\text{O}_2^*$  (Knox and Dodge, 1985):



While in photosynthetic membranes pathway (1.11) is of minor importance, it is central in protecting biological systems from accumulating  $^1\text{O}_2^*$  (Krinsky, 1979).

Carotenoids can act as protective agents only when the energy of  $^3\text{Car}^*$  is lower than that of  $^3\text{Chl}^*$  (pathways 1.8 and 1.10) or that of  $^1\text{O}_2^*$  (pathway 1.11) (Naqvi, 1980).

Carotenoids protect against a whole range of oxidative species ( $^1\text{O}_2$ ,  $\text{OH}^\cdot$ ,  $\text{O}_2^\cdot$ ,  $\text{H}_2\text{O}_2$ ). In the absence of carotenoids the oxygen radicals are able to initiate lipid peroxidation. Carotenoids, tocopherol, ascorbate, superoxide dismutase and catalase act in concert to deal with the range of oxidizing species. The peroxidation of lipids leads to the release of even more oxidative species which can initiate even further damage in the cell. Therefore, the need for this chain reaction to be controlled is of great importance for the effective functioning of the cell.



## 1.5 THE XANTHOPHYLL CYCLE

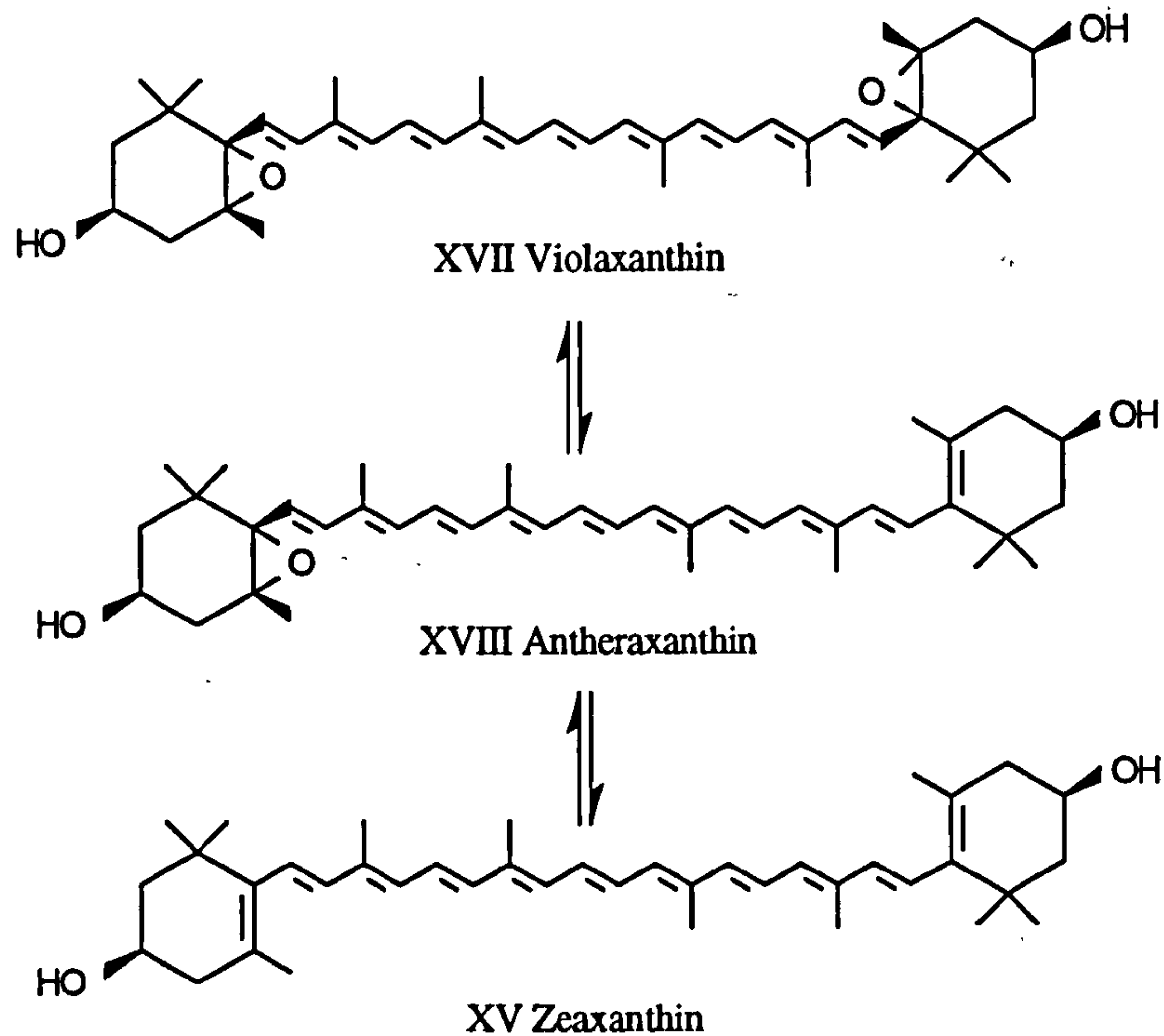
### 1.5.1 Introduction

The exposure of photosynthetic tissues to light in excess of that which can be utilized in photosynthesis results in photoinhibition. This causes a reduction in photosynthetic activity due primarily to a reduction in the photochemical efficiency of PSII. Several mechanisms exist to ameliorate photoinhibitory damage under such conditions. These include electron transport associated with non-photosynthetic processes such as photorespiration (Eickmeier *et al.*, 1993), the production of low efficiency PSII  $\beta$ -centres (Weis and Berry, 1987), cyclical electron transport around PSII (Foyer *et al.*, 1990), dissociation of light-harvesting complex (LHC) II and energy spillover to PSI (Anderson, 1986). The presence of antioxidant systems (Larson, 1988) and the radiationless dissipation of excess excitation energy in the chlorophyll pigment bed, is correlated with the formation of the xanthophyll pigment zeaxanthin (XV) (the xanthophyll cycle) (Demmig *et al.*, 1988; Young, 1991).

### 1.5.2 Zeaxanthin (XV) formation

In the xanthophyll cycle, zeaxanthin (XV) is formed through the de-epoxidation of violaxanthin ((3*S*,5*R*,6*S*,3*S*,5'*R*,6'*S*)-5,6,5',6'-diepoxy-5,6,5',6'-tetrahydro- $\beta,\beta$ -carotene-3,3'-diol, XVII) via antheraxanthin (5,6-epoxy-5,6-dihydro- $\beta,\beta$ -carotene-3,3'-diol, XVIII) in an enzymatic reaction catalyzed by a de-epoxidase (Hager, 1980; Yamamoto, 1979) (Fig. 1.6). There is also a second enzyme, an epoxidase, which reconverts zeaxanthin (XV) to antheraxanthin (XVIII) and violaxanthin (XVII). The de-epoxidase has a pH-optimum of 5.2 (Rees *et al.*, 1992), whereas the epoxidase exhibits maximum activity at pH 7.5 (Demmig-Adams, 1990). The de-epoxidase is thought to be located on the inner-side of the thylakoid membrane facing the acidic lumen, and the epoxidase on the outer-side facing the alkaline stroma as indicated in figure 1.7 (Hager, 1980).





**Fig. 1.6** The xanthophyll cycle involving the de-epoxidation of violaxanthin (XVII) to zeaxanthin (XV) via antheraxanthin (XVIII) and the epoxidation of zeaxanthin (XV) to violaxanthin (XVII) via antheraxanthin (XVIII).

### 1.5.3 Regulation of the cycle

In addition to pH, other factors involved in the regulation of zeaxanthin (XV) formation are the availability of (i) violaxanthin (XVII) for the de-epoxidase, which seems to depend on the redox reactions between PSI and PSII, and (ii) ascorbate which appears to act as an endogenous reductant for the de-epoxidation if it is reduced by PSI in the sequence NADPH-glutathione-ascorbate, although another electron carrier maybe involved after ascorbate (Yamamoto, 1979). Furthermore, the epoxidase requires O<sub>2</sub> and NADPH to act as co-substrates.

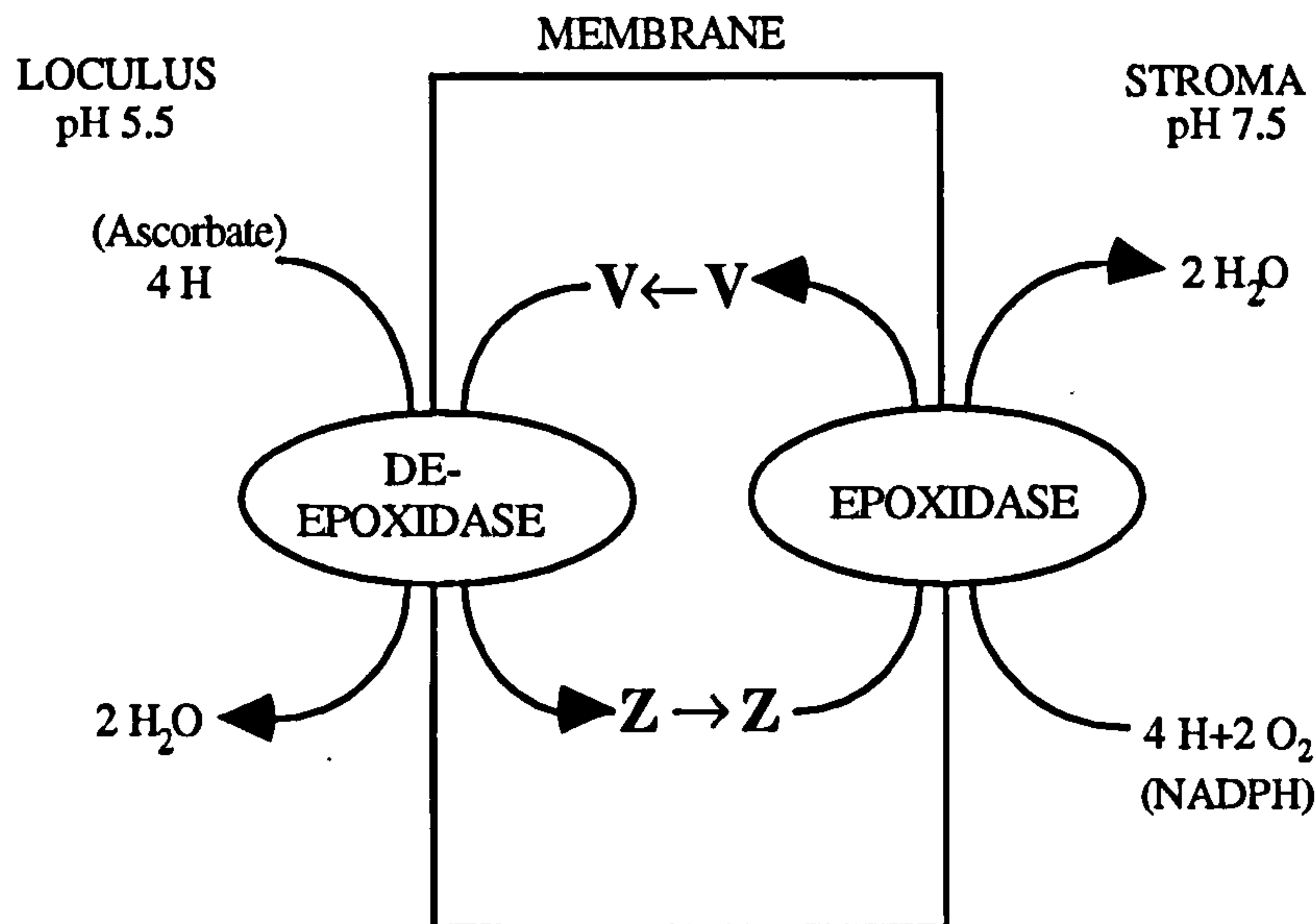


Fig. 1.7 Transmembrane model of the xanthophyll cycle (after Hager, 1980).

These regulating parameters are mostly measures of the balance between the rate of photon absorption and the rate of photochemistry or electron transport, i.e. they are indicators of whether or not light is in excess. Excessive light promotes the de-epoxidation of violaxanthin (XVII) to zeaxanthin (XV), evidently because the resulting build up of a pH gradient across the thylakoid membrane (Fig. 1.7) causes acidification of the lumen, thereby activating the de-epoxidase (Hager, 1980). The epoxidase activity is promoted under the pH and redox conditions that exist under limiting light.

#### 1.5.4 Location

Violaxanthin (XVII) bound to thylakoid membranes seems to be mainly located in LHCII and minor amounts are located in LHCI. Using IEF, the majority of LHCII can be split into the bulk light harvesting complex (LHCIIb), and the minor LH complexes (CP24, CP26, CP29). There is some disagreement as to the location of the xanthophyll cycle carotenoids within these complexes. Bassi *et al.* (1993) maintain that in maize, the minor complexes are solely responsible for the formation of zeaxanthin (XV) (i.e. LHCIIb, which contains 80% of the pigments, cannot de-epoxidase violaxanthin (XVII)). Contradictory results have been obtained by Ruban *et al.* (1994) who have shown that spinach LHCIIb contains 60% of the zeaxanthin (XV) formed during the xanthophyll

cycle. As similar procedures were used in both these reports, it is not clear which result is a more accurate reflection of the *in vivo* organisational state.

### **1.5.5 Evidence for zeaxanthin (XV)-mediated photoprotection**

Zeaxanthin (XV)-associated photoprotection has received considerable experimental investigation and support. At least five lines of evidence support the hypothesis that zeaxanthin (XV) is involved in protecting the photosynthetic membranes against the effects of high light: (i) both non-photochemical fluorescence quenching and the rate constant for radiationless heat dissipation vary in parallel with tissue zeaxanthin (XV) content (Gilmore and Yamamoto, 1991; 1993; Demmig and Björkman, 1987); (ii) the threshold for the onset of photoinhibitory damage coincides with the maximum zeaxanthin (XV) content generated upon exposure to high light (Demmig *et al.*, 1987); (iii) Dithiothreitol (DTT), which inhibits zeaxanthin (XV) formation, also enhances photoinhibitory damage (Bilger *et al.*, 1989; Demmig-Adams *et al.*, 1990); (iv) blue-green algae and lichens (containing blue-green algae) lack the xanthophyll cycle and more readily suffer photoinhibitory damage than green algae and lichens (containing green algae), which along with all higher plants possess the xanthophyll cycle (Demmig-Adams, 1990; Demmig-Adams and Adams, 1990); (v) high light preconditioning significantly increases the total size of the xanthophyll cycle pool, thereby increasing the concentration of zeaxanthin (XV) that can be generated at high light intensities (Thayer and Björkman, 1990; Demmig-Adams *et al.*, 1989). However, the precise mechanism(s) of zeaxanthin (XV) photoprotection at high light intensities are not known.

## **1.6 THE ROLE OF CAROTENOIDS IN PHOTORECEPTION**

The most likely candidates for photoreceptors in phototaxis and phototropism in the UV/blue region of the spectrum in higher plants are flavins and carotenoids. Flavins and carotenoids are considered as photoreceptors when the action spectrum of the corresponding response follows a characteristic pattern showing peaks or shoulders around 420, 450 and 480 nm. Photoreceptors in the UV/blue region with a main peak around 300

nm are also thought to exist. However, these reactions are not considered to involve carotenoids (Rau, 1988). The controversy over flavins or carotenoids is a long standing argument, although the majority of investigators favour a flavin at present (Gualtieri, 1993).

## **1.7 ALGAL CAROTENOIDS: CHEMOSYSTEMATICS AND PHYLOGENY**

### **1.7.1 Chemosystematics**

Carotenoids are generally located in chloroplasts and their use as a systematic and phylogenetic parameter is therefore restricted to photoautotrophic species (Bjørnland and Liaaen-Jensen, 1989). Table 1.1 shows the biosynthetic capability of algae to produce particular structural features of their carotenoids (Liaaen-Jensen, 1977). A number of features/trends are evident: monocyclic carotenoid glycosides are only found in the Cyanobacteria. Bicyclic xanthophylls are found in all algal classes and are of no chemosystematic value. 4-Keto-carotenoids are found in the Cyanobacteria, Euglenophyceae and Chlorophyceae when the algae are grown under stress conditions. Cyclization to  $\epsilon$ -rings is typical of certain algal classes. Acetylenic carotenoids are encountered in several classes. Epoxide carotenoids are synthesised by all except the three most primitive algal classes. Allenic carotenoids, 8-keto-carotenoids, and carotenol acetates are found as indicated. Butenolide formation and C-3 expulsion in carotenoid synthesis are unique properties of dinoflagellates. Oxidation of in-chain methyl groups and 19-ols is effected by some classes. 5,6-Glycol formation seems to be restricted to the Xanthophyceae and Euglenophyceae. Higher fatty acid esters of carotenoids are found in three classes, while the 2-hydroxy- $\beta$ -type is found in the Chlorophyceae (Arad *et al.*, 1993).



	Monocyclic glycosides	Bicyclic Xanthophylls	4-Keto	$\epsilon$ -rings	$C\equiv C$	O	$=C=$	8-Keto	-OAc	Butenolide C3-expelled	19-OH	5,6 Glycol	0-Acyl	2-OH
Cyanobacteria	+	+	+											
Rhodophyceae		+		+				.						
Cryptophyceae		+		+	+									
Raphidophyceae		+		+		+								
Chrysophyceae		+			+	+	+							
Phaeophyceae		+				+	+	+	+					
Haptophyceae		+		+	+	+	+	+	+					
Bacillariophyceae		+		+	+	+	+	+	+					
Dinophyceae		+			+	+	+	+	+	+				
Eustigmatophyceae		+				+	+	+	+		+			
Xanthophyceae		+			+	+	+	+			+	+	+	
Euglenophyceae		+	+		+	+	+	+				+	+	
Prasinophyceae/ Loxophyceae		+		+		+	+	+			+		+	
Chlorophyceae		+	+	+		+	+	+			+			+

**Table 1.1** Structural features of carotenoids particular to certain algal classes (from Liaaen-Jensen, 1977).

### 1.7.2 Phylogeny

The basic biosynthetic mechanism involved in the formation of phytoene (VII and VIII) seems to be the same in all organisms. The desaturation of phytoene (VII and VIII) to lycopene (XIII) is probably the major pathway leading to cyclic carotenoids. The subsequent metabolism of lycopene (XIII) in different phyla can be used in algal taxonomy and contributes to theories on algal evolution. These further changes in algae can be related to: (i)  $\beta$ -cyclase; (ii)  $\epsilon$ -cyclase; (iii) hydroxylase; (iv) 5,6-epoxidase; (v) 5,6-epoxidase



isomerase. This information can be used to devise the following evolutionary scheme for algae (Fig. 1.8) (Goodwin, 1971).

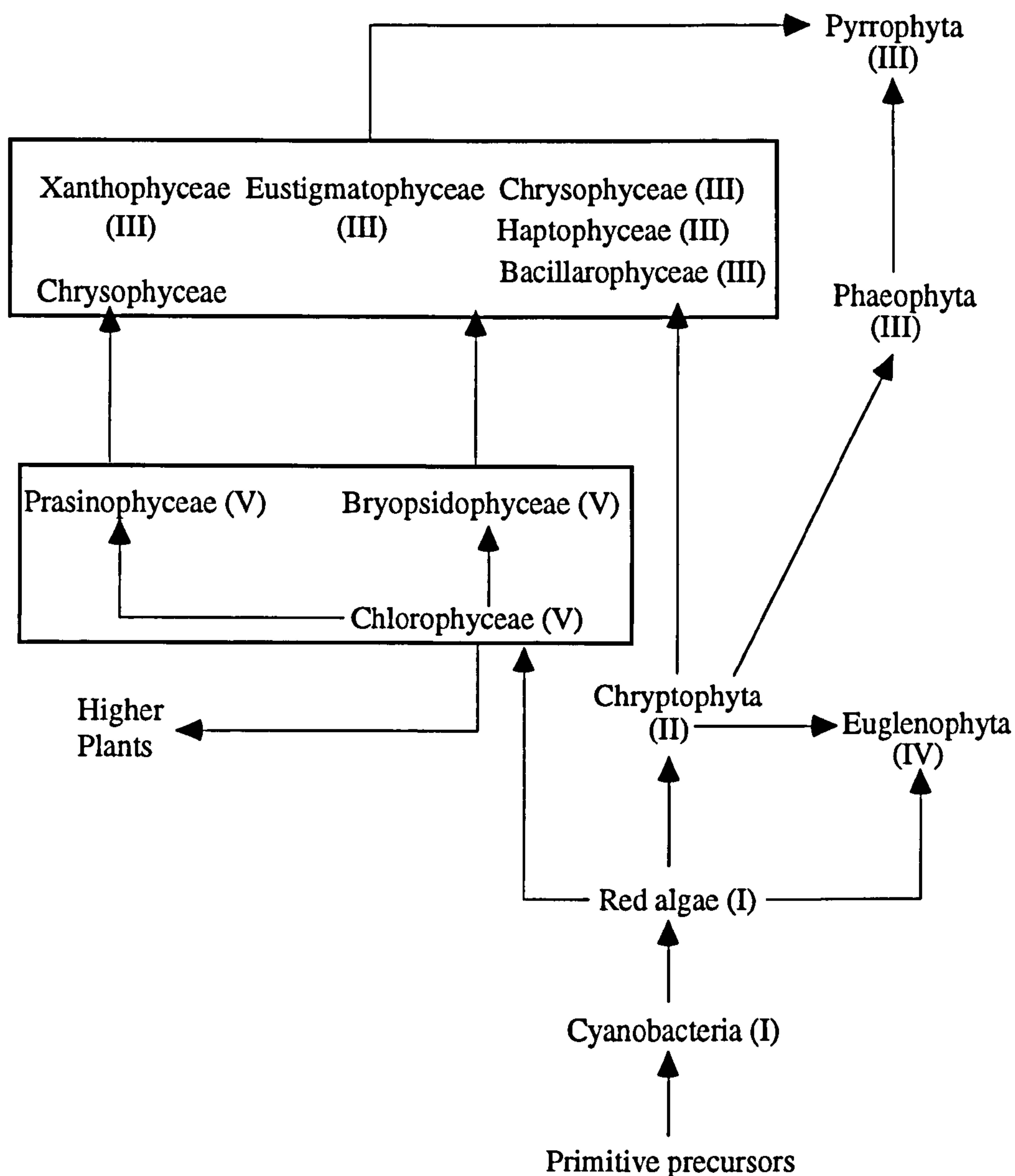
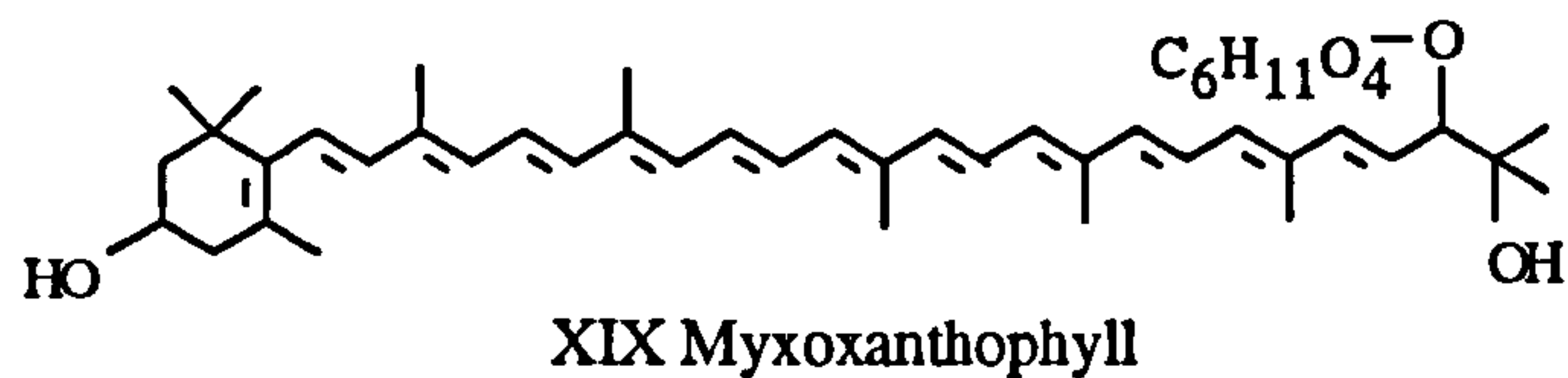


Fig. 1.8 A proposal for algal evolution based on carotenoid distribution from Goodwin, 1971. (Roman numerals indicate thylakoid type)

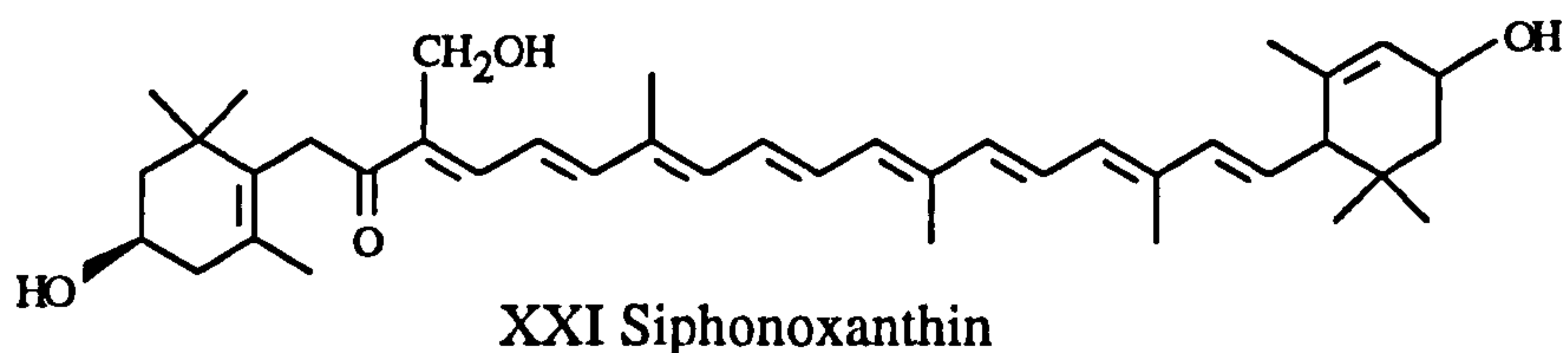
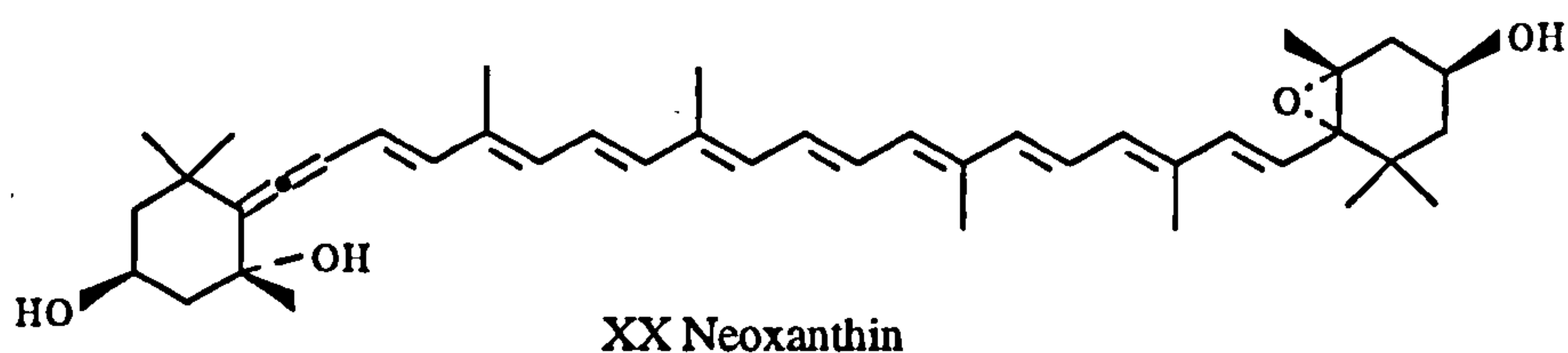
Assuming the primitive precursor can synthesise acyclic carotenoids, the first phylum to emerge would be the Cyanobacteria. Only  $\beta$ -cyclase is present because only  $\beta$ -carotene (I) and its derivatives echinenone (II) and canthaxanthin (IV) are present. The other major pigment is the monocyclic carotenoid glycoside myxoxanthophyll (2'-( $\beta$ -L-Rhamnopyranosyloxy)-3,4'-didehydro-1',2'-dihydro- $\beta,\psi$ -carotene-3,1'-diol, XIX). This type of carotenoid does not occur in other algae, but, as it contains the C-1,2 double bond

hydrated and the resulting hydroxyl group glycosylated, it is structurally very similar to many carotenoids of the photosynthetic bacteria. This correlation supports the evidence that the Cyanobacteria are prokaryotes and should not be classified within the algae.



The red algae could have evolved from the Cyanobacteria by developing an  $\epsilon$ -cyclase in some, but not all cases. At the same time they lost or repressed the ability to insert oxygen at C-4 and to form the enzyme concerned with glycosylating acyclic carotenoids. Alternatively, the Cyanobacteria may have evolved the latter enzymes after the red algae had branched off.

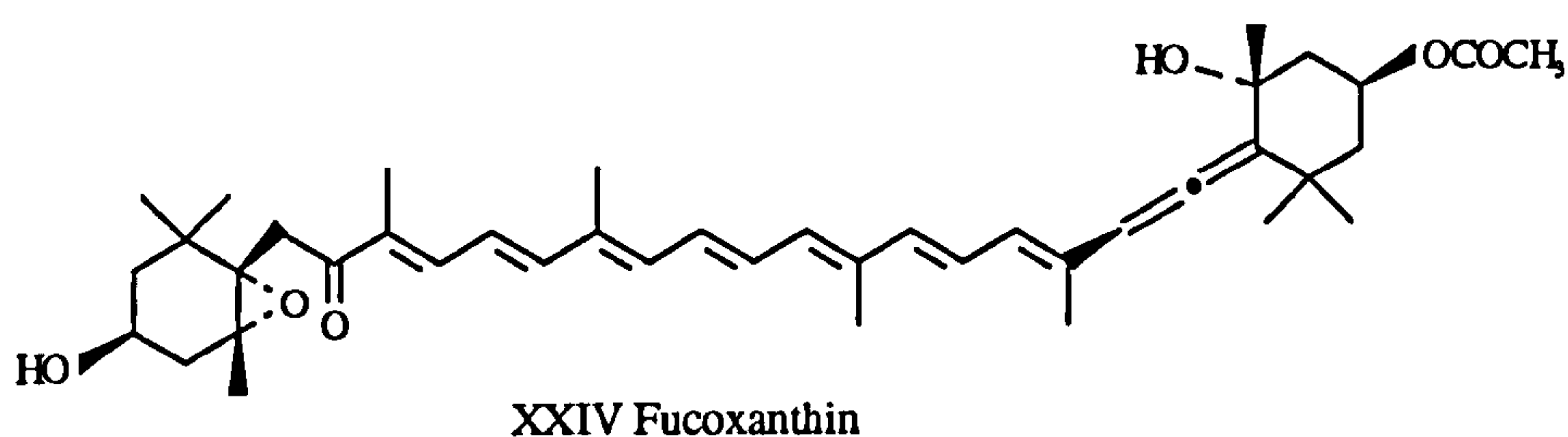
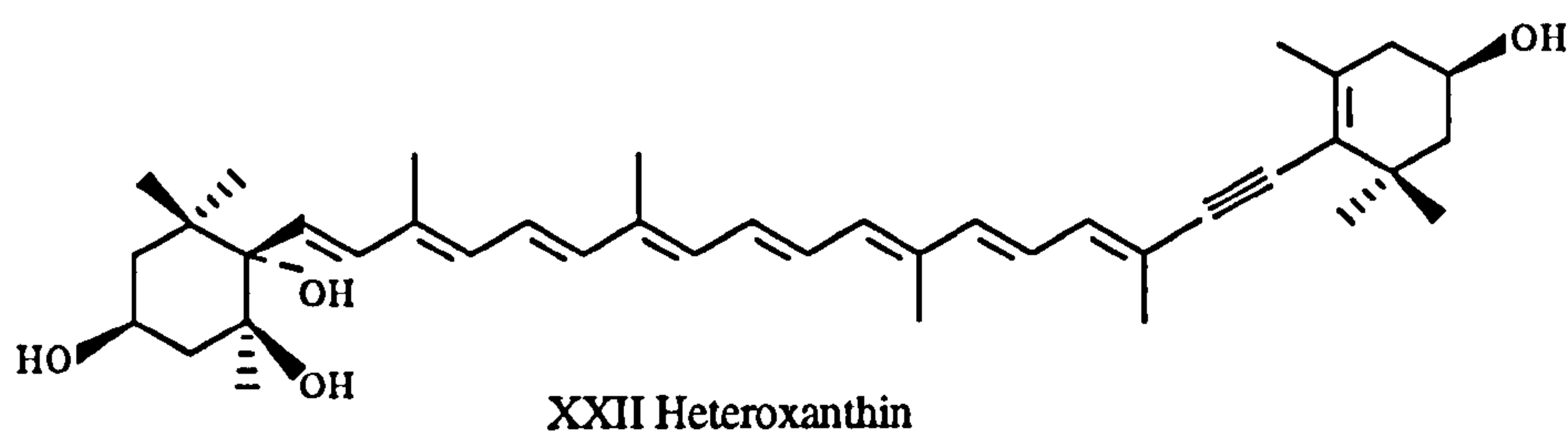
The main pigment development in the evolution of the Chlorophyta from the Rhodophyta is the development of two new enzymes, namely the 5,6-epoxidases and the 5,6-epoxidase isomerases. These would account for the consistent appearance of antheraxanthin (XVIII), violaxanthin (XVII) and neoxanthin ((3*S*,5*R*,6*R*,3'*S*,5'*R*,6'*S*)-5',6'-epoxy-6,7-didehydro-5,6,5',6'-tetrahydro- $\beta,\beta$ -carotene-3,5,3'-triol, XX). Within the classes Bryopsidophyceae and Prasinophyceae, siphonoxanthin (XXI), which is more oxygenated than normal Chlorophyte pigments is synthesised.

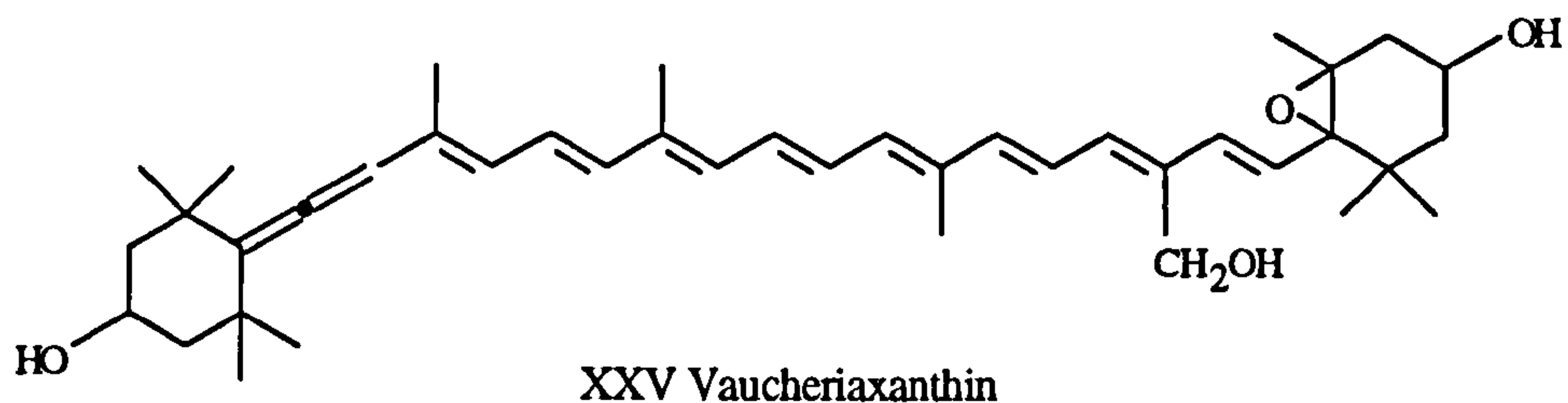


The next important evolutionary step is the appearance of the acetylenic bond in carotenoids, and those pigments which seem to be formed as the result of direct desaturation of pigments such as lutein (XVI) and zeaxanthin (XV). These are

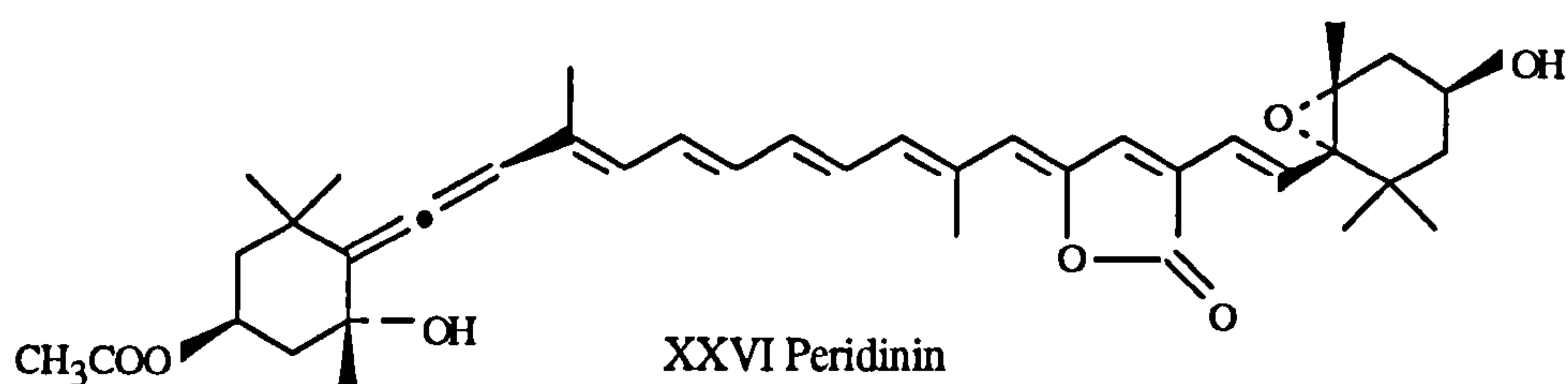
characteristic of the Cryptophyta, which can be considered as evolving directly from the red algae. The Euglenophyceae could have evolved from the Cryptophyta in a manner analogous to that by which the Chlorophyta developed from the Rhodophyta. The Euglenophyceae might also have developed from the Cyanobacteria directly, since they also cannot synthesise  $\epsilon$ -rings. They might have evolved from specific red algae which themselves never evolved the ability to synthesise  $\epsilon$ -rings.

All the classes except Chrysophyceae synthesise acetylenic carotenoids. The appearance of acetylenic carotenoids suggest that the Chrysophyceae evolved from the Cryptophyceae rather than from the Chlorophyta. However, the production of heteroxanthin ((3*S*,5*S*,6*S*,3'*R*)-7',8'-didehydro-5,6-dihydro- $\beta,\beta$ -carotene-3,5,6,3'-tetrol, XXII) in the Xanthophyceae, which involves in-chain methyl-oxidation characteristic of loroxanthin (XXIII), and fucoxanthin ((3*S*,5*R*,6*S*,3'*S*,5'*R*,6'*R*)-5,6-epoxy-3,3'-5'-trihydroxy-6',7'-didehydro-5,6,7,8,5',6'-hexahydro- $\beta,\beta$ -carotene-8-one 3'-acetate, XXIV) in the Chrysophyceae, Bacillariophyceae and Haptophyceae, involves the formation of a conjugated keto group characteristic of siphonoxanthin (XXI). Vaucherixanthin (5',6'-epoxy-6,7-didehydro-5,6,5',6'-tetrahydro- $\beta,\beta$ -carotene-3,5,3',19'-tetrol, XXV), which accompanies heteroxanthin (XXII) in the Xanthophyceae and Eustigmatophyceae, could have been formed by a loroxanthin-type (XXIII) oxidation of neoxanthin (XX) rather than from lutein (XVI). These observations indicate evolution from the Chlorophyta via the Briopsidophyceae. No  $\epsilon$ -rings are observed in any of the Chrysophyceae, which would suggest that they are not evolved from the Chlorophyta.





The major pigments in Phaeophyta and Pyrrophyta, fucoxanthin (XXIV) and peridinin (XXVI), also do not make  $\epsilon$ -rings. If peridinin (5',6'-epoxy-3,5,3'-trihydroxy-6,7-didehydro-5,6,5',6'-tetrahydro-10,11,20-trinor- $\beta,\beta$ -caroten-19',11'-olide 3-acetate, XXVI) is an oxidation product of fucoxanthin (XXIV), then the Pyrrophyta might have evolved from fucoxanthin-producing ancestors, i.e. the Chrysophyceae or Phaeophyta. The Chloromonadophyta appear to have very similar pigments to those in the Xanthophyceae. It is evident that the structural diversity and distribution of algal carotenoids fall into a nice pattern useful for chemosystematics, and in predicting possible theories for algal phylogeny.

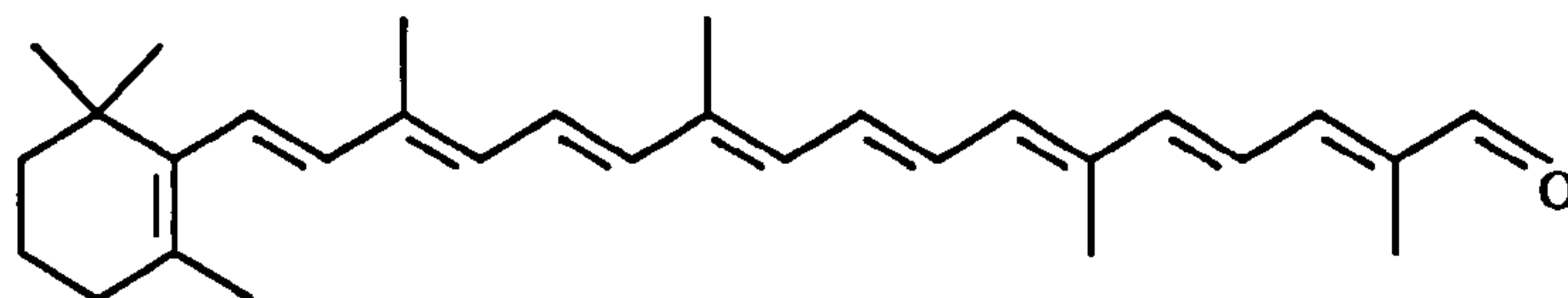


## 1.8 THE HISTORY OF MICROALGAE AS SOURCES OF CAROTENOIDS

### 1.8.1 Commercial uses of carotenoids

Colour is the common basic factor for the practical and industrial uses of carotenoids, and these may further be influenced by the metabolite functions, and particularly pro-vitamin A activity of some carotenoids. Commercial synthetic and natural carotenoids are mainly used for food *in vivo*, i.e. egg yolk and broiler pigmentation with  $\beta$ -apo-8'-carotenic acid ethylester (XXVIII) and pigmentation of farm raised salmon with astaxanthin (III).





XXVIII  $\beta$ -apo-8'-carotenic acid ethylester.

$\beta$ -Carotene (I) is present in all chlorophyll-containing plants and is the most widely distributed carotenoid. Carotenoids and in particular,  $\beta$ -carotene (I) appear to carry out functions in animals and humans which go far beyond acting as an optical signal and having pro-vitamin A activity (Mordi, 1993). For example  $\beta$ -carotene (I) enhances immunity to disease (Jyonouchi *et al.*, 1991) and acts as an intracellular antioxidant helping to prevent cancer (Zeigler, 1989). These findings point to important new uses of  $\beta$ -carotene (I), especially since it is non-toxic, even at very high doses.  $\beta$ -Carotene (I) is already used in livestock for enhancing fertility (Jackson, 1981) and is now an ingredient of many vitamin products for human use (Ben-Amotz, 1991).

Canthaxanthin (IV) is added to chicken feed which is deposited in the egg yolk, giving an orange-red colour to the yolk which is desirable to the consumer. Lutein (XVI) and zeaxanthin (XV) have also been identified as key natural pigments in poultry. Zeaxanthin (XV) acts synergistically with lutein (XVI), in that it intensifies the egg yolk colour obtained with the yellow base pigment. Carotenoids in the feed are deposited in the bodies of the poultry, being evident in the comb, beak and legs. Carotenoids enhance resistance to diseases and improve the shelf life and hatchability of the eggs (Paust, 1991). In the early 1960's, alga meals were extensively studied for pigmenting the skin and egg yolk of poultry (Nelis and de Leenheer, 1989). Although *Neosporangiococcum* spp, *Coccomyxa* spp, and several other algae were recognised as good sources of lutein (XVI), they are not presently being grown commercially. Lutein (XVI) is currently sold as a component of feeds such as cornmeal, alfalfa meal or powdered marigold petals (Marusich and Bauernfeind, 1981). All of these markets have potential for expansion by supplying the carotenoids more cheaply or by supplying 'new' carotenoids (Borowitzka, 1988).

A large number of studies have indicated that carotenoids act as anti-carcinogenic agents in animals treated with either ultraviolet light, ultraviolet light with chemicals, or with chemical carcinogens alone. Although pharmacological doses of carotenoids were



used in the early experiments, more recent evidence indicates that relatively small doses can be effective. These studies have been complemented by investigations in bacteria and mammalian tissues, either in cell culture or in organ culture, where it has been demonstrated that various carotenoid pigments can prevent mutagenesis, genotoxic effects, or malignant transformation. It would appear that these effects are intrinsic to the carotenoid molecule, and not necessarily due to the metabolic conversion to retinoids. Partially based on these observations, it has been suggested that carotenoids may function as chemopreventive agents for reducing the risk of cancer in humans. Numerous studies are underway to test this hypothesis (Mordi, 1993).

Carotenoids, notably  $\beta$ -carotene (I) and lycopene (XIII) as well as oxycarotenoids, e.g. zeaxanthin (XV) and lutein (XVI), exert antioxidant functions in lipid phases by free radical scavenging or  $^1\text{O}_2$  quenching. There are pronounced differences in tissue carotenoid patterns, extending also to the distribution between the all-*trans* and various *cis* isomers of the respective carotenoids. Antioxidant functions are associated with lowering DNA damage, malignant transformation, and other parameters of cell damage *in vitro*, as well as epidemiologically with lowered incidence of certain types of cancer and degenerative diseases, such as ischemic heart disease and cataract. They are also of importance in the process of reducing the effects of ageing. Reactive oxygen species occur in tissues and cells and can damage DNA, proteins, carbohydrates and lipids. These potentially deleterious reactions are controlled in part by antioxidants that eliminate pro-oxidants and scavenge free radicals. Their ability as antioxidants with radicals and  $^1\text{O}_2$  may explain some anti-cancer properties of the carotenoids independent of their pro-vitamin A activity, but other functions (e.g. membrane structure/organisation (A. Young and R. Bilton pers. comm.)) may play a role as well.

### 1.8.2 Microalgae

The microalgae are a large and diverse group of photosynthetic micro-organisms, spanning seven divisions and comprising several thousand species. It is therefore not surprising that they show great metabolic diversity, possibly only matched by the bacteria and fungi. Of this large number of species, only a few (< 50 species) have been studied in some detail with respect to their metabolism and chemical composition. Furthermore, details of

to their metabolism and chemical composition. Furthermore, details of physiology, biochemistry and potential for mass culture are known for only a few of these.

Since the 1940's extensive research has been carried out in many countries developing algal mass culture (Goldman, 1979; Soeder, 1980). Fundamental analyses of microalgal growth kinetics and photobioreactor design have been published (Pohl *et al.*, 1986; Tredici *et al.*, 1991). Despite this large governmental and private investment, which has run into many thousands of man years and hundreds of millions of dollars, there are few practical commercial enterprises and even fewer commercial products (Benneman *et al.*, 1987). The main metabolites of potential commercial interest which have been investigated are listed in figure 1.9.

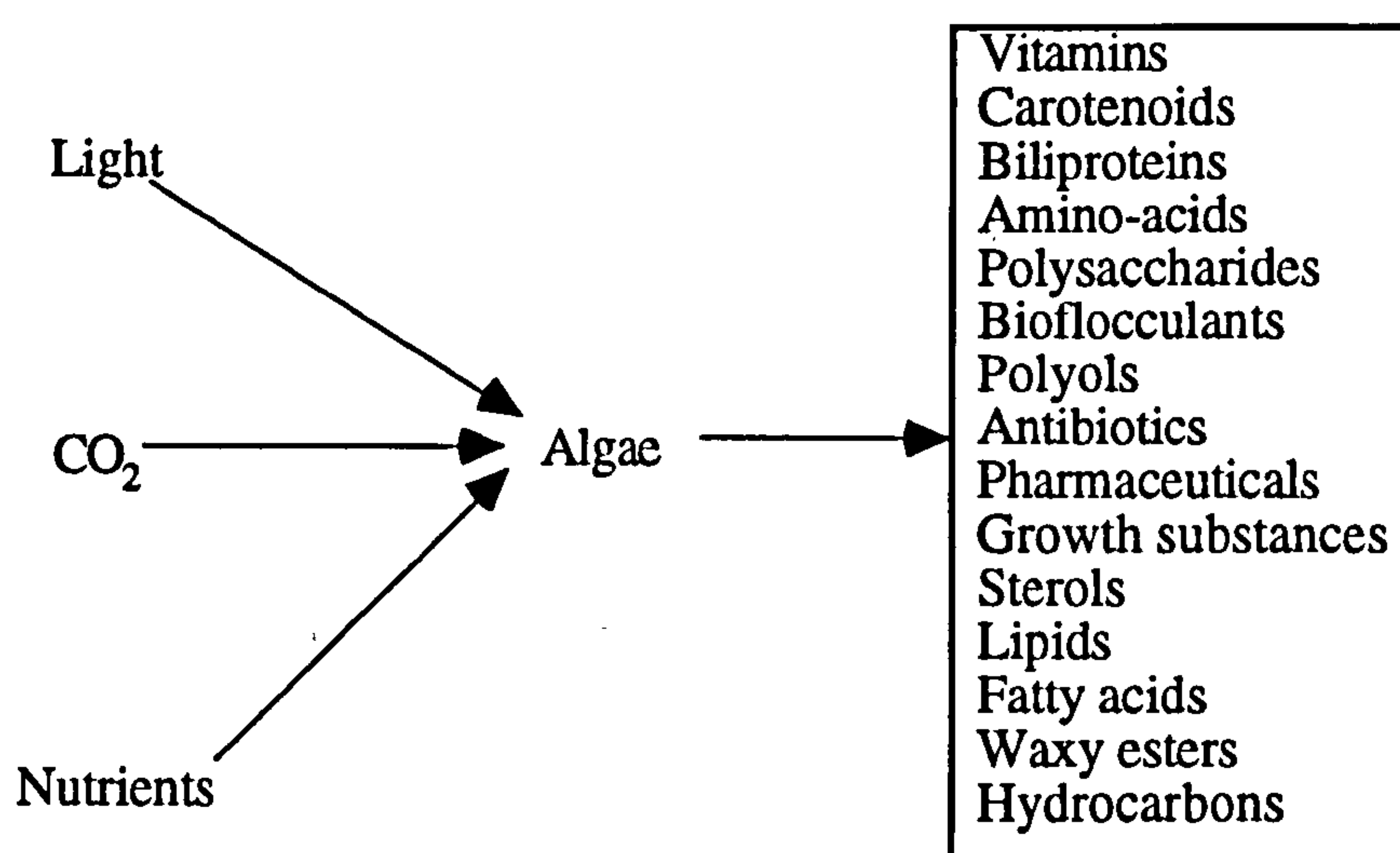


Fig. 1.9 Useful products with the potential for commercial production from microalgae.

### 1.8.3 Carotenoids in fish feeds

Carotenoids play a significant role in the commercial feeds developed for shellfish and fish. The bluish hues of many crustaceans result from carotenoproteins (Goodwin, 1984; Klausner, 1986), and the orange and red colouring of crustaceans and of many fish are due to xanthophylls. These animals do not synthesise carotenoids *de novo*, but metabolise those they ingest. The astaxanthin (III) content of salmon varies considerably depending on species, sex, nutrition, maturity and health of the fish (Torrissen *et al.*, 1989). Mature Atlantic salmon contain 3 to 8 ppm, while sock-eye salmon can have contents as high as 37 ppm. Astaxanthin (III) occurs mainly in the free form in salmonid flesh, and as the esterified form in the skin and ovaries (Torrissen *et al.*, 1989).

The uptake of astaxanthin (III) and other carotenoids from feed depends on the structure of the carotenoid, the proportion of *cis*-isomers, esterification and association of the carotenoid with fats or proteins. Carotenoids are generally absorbed with poor efficiency by animals and little is known of the uptake mechanism and of the means for enhancing uptake. Since astaxanthin (III) has two identical chiral centres, it exists as three configurational isomers in nature: (3*S*,3'*S*), (3*S*,3'*R*) and (3*R*,3'*R*). Each configurational isomer appears to be deposited equally well in salmonids (Storebakken *et al.*, 1984). During reductive metabolism of astaxanthin (III) to zeaxanthin (XV), epimerization from the 3*S* to 3*R* isomer was reported (Storebakken *et al.*, 1987). Rainbow trout appeared to preferentially hydrolyze the *R* configurational isomers of the palmitate esters, suggesting that the esterases have stereochemical preference for the *R* configuration. The (3*R*,3'*R*) astaxanthin (III) diester was deposited in the flesh of the rainbow trout two times more efficiently than the (3*R*,3'*S*) and four times more efficiently than the (3*S*,3'*S*) diesters (Katsuyama *et al.*, 1987).

The provision of pigments in feeds of farmed salmon is an expensive practice. Astaxanthin (III) is one of the most costly components of salmon feeds, accounting for 10 to 15% of total feed costs. On a dry weight basis, astaxanthin (III) is currently sold for > \$4000/kg, marketed as Carophyll® Pink (Hoffman-LaRoche, Inc, Switzerland), which contains a minimum of 8% (by weight) astaxanthin (III) per kilogram.

## **1.9 THE DEVELOPMENT OF A MICROALGAL CAROTENOID PRODUCT**

### **1.9.1 Introduction**

The first step in the development of a microalgal carotenoid product is the identification of a suitable algal species or strain with the potential to synthesise the desired carotenoid at a commercially viable level. Once identified, the algal species needs to fulfil certain criteria to ensure the species is suitable for mass cultivation. These include: (i) the ability to grow rapidly on inexpensive media (which may include effluents from industrial processes); (ii) non-complicated life history; (iii) short lag-phase on transfer to fresh media; (iv) cells



capable of withstanding the hydrodynamic forces and mechanical shear they will be subjected to in both a fermenter and as a result of downstream processing.

The potential use of the carotenoid product must also be considered, i.e. if the product is to be used as a feed supplement the alga must not be toxic or pathogenic (Day *et al.*, 1991).

### **1.9.2 Strain improvement**

Once a suitable species has been identified which produces the desired carotenoid, the need for this species to produce the carotenoid in a cost-effective manner is essential. Strain improvement is a factor which can be used to improve production efficiency. Many of the techniques mentioned in the following section have not yet been applied to algae on a commercial basis. However, the techniques mentioned are applicable to algae and should play a major role in the development of algal biotechnology.

#### **1.9.2.1 Screening**

Phenotypic variants may be isolated from heterogeneous cell suspension cultures using either screening or selection. Screening is defined as the analysis of a large number of cells to identify rare individuals with the desired trait, (in this case enhanced production of a carotenoid), and then to clone these cells and establish stable cell lines (Rhodes *et al.*, 1988). Selection procedures will be discussed later in the isolation of mutants. The most critical aspect of a screening operation is the analytical technique used, this is needed for the rapid and sensitive analysis of the carotenoid(s). Ideally, it should be non-intrusive allowing desirable cells to be identified and grown on. Analytical techniques used in cell screening to improve carotenoid production include visual screening and flow cytometry (Lister, 1988; An *et al.*, 1991). Having isolated a high producing line, it is not always possible to stabilise it. Given the heterogeneity of cultures, reversion will occur, albeit at varying rates. This necessitates the need for carotenoid re-isolation of the high yielding lines.

The only means so far developed for introducing exogenous DNA into green algae is by transformation (Rochaix and van Dillewijn, 1982). In *Chlamydomonas*, transformation was achieved by the use of protoplasts. Although transformants were obtained, the frequency was very low ( $10^{-6}$  -  $10^{-7}$ ). *Chlamydomonas reinhardtii* was transformed with a yeast plasmid vector carrying the complementing *arg<sup>4</sup>* gene. The yeast gene functioned poorly, and not all transformants were stable. However, shuttle vectors could be developed to enable manipulations to be done in more convenient hosts. Transformation frequencies at the level obtained are not a barrier to commercial applications, because sufficient DNA for successful transformation could be isolated from the alternative host.

Alternatives to transformation are the micro-injection of DNA into the nucleus (Graessman and Graessman, 1983), the encapsulation of DNA in viral envelopes and liposomes and their subsequent introduction into the cells by fusion (Vainstein *et al.*, 1983). Discoveries with yeast have opened the way for a general method for developing cloning vectors for practically any eukaryotic cell. The method involves the construction of a vector consisting of a bacterial plasmid, a eukaryotic biosynthetic gene, a eukaryotic chromosomal replicator sequence and a centromere sequence, the vector is called a minichromosome (Craig *et al.*, 1988). Despite their relative stability, artificial minichromosomes are still significantly less stable than natural chromosomes. However, this should not be a problem when a selective marker is present.

Other potential cloning vectors that have been developed for eukaryotes and higher plants include modified viruses, the transforming (T:) plasmid of *Agrobacterium* and the natural  $\mu$ m plasmid of yeast. Use of such vectors requires considerable fundamental research into the molecular biology of the parent replicon. The simplest method is simply to rely on integration into the host chromosome for replication of the cloned genes. A more sophisticated extension of the chromosomal integration method is the use of eukaryotic transposable genetic elements (Craig *et al.*, 1988).

Since the development of algal cloning vectors is at a very early stage, expression vectors have not been studied. However, significant work has been done with yeast, and many of these results are relevant. Vectors for the study of expression in yeast have been developed based on using the expression signal sequences to direct synthesis of  $\beta$ -



galactosidase. Analogous vectors would be very useful for the study and optimisation of expression in algae.

### 1.9.2.3 *Mutagenesis*

A wide range of mutagens have been used successfully with green algae. These include chemical mutagens such as various nitrosamines, alkylating agents, intercalating compounds and irradiation with ultra-violet light, laser light, X-rays or gamma rays (Kuchka and Jarvik, 1982; Nikolov *et al.*, 1981).

The frequency of a desired mutation depends on the nature of the mutation and the procedure used. Most mutations occur at a low frequency ( $10^{-6}$  -  $10^{-8}$ ). Obtaining the desired mutant generally depends on techniques to enrich or select for such mutants in a population of wild-type cells. Enrichment techniques (indirect selection) involves the killing of normal cells to increase the percentage of mutants in the population. With direct selection techniques (positive selection), mutant cells are selected for under conditions where the wild-type cells grow poorly or not at all.

The most laborious method of mutant isolation is direct analysis of colonies, the 'random screening' method. This is a reliable and cost-effective procedure that plays a central role in industrial strain improvement (Rowlands, 1984). Various methods have been developed to enhance efficiency, such as multi-level screening and the use of automation and miniaturization. These include flow cytometry to isolate high quality wild strains as well as mutant strains (Nonomura, 1988; Mohn, 1988). Flow cytometry and cell sorting has been used successfully to isolate hyper-producing astaxanthin (III) mutants of *Phaffia rhodozyma* (An *et al.*, 1991).

### 1.9.2.4 *Potential applications of strain improvement*

The techniques mentioned should play a major role in the development of algal biotechnology. These improvements should allow for faster growth rates and better utilization of substrates. A corollary of faster growth is improved competitive advantage over contaminating algae. A further advantage could be gained by genetically modifying the starting strain, so that it could grow well in conditions under which the original organism grows poorly or not at all.

The levels of metabolites in organisms are controlled by a variety of regulatory mechanisms operating at the levels of transcription, translation, processing and enzyme function. Appropriate mutations can eliminate or modify these controls, leading to enhanced or uncontrolled synthesis. For example, a slight change in enzyme structure can eliminate end-product inhibition, or a slight change in nucleotide sequence might eliminate repression. Genetic engineering can enhance product yields in a number of ways. Cloning a gene or genes on a multi-copy vector will increase expression simply by increasing the number of gene copies. If the gene is normally repressed, expression may be further increased if the number of genes exceeds the number of repressor molecules. Replacing the natural expression signals with stronger ones can lead to improved product yields.

Algae suitable for large scale cultivation can be made a more attractive proposition if a wider, or more profitable, range of products can be isolated from them. Mutagenesis can regenerate strains with improved or altered product compositions, for example by deregulation of a minor metabolic pathway, or by blocking a major pathway, thereby favouring side pathways or accumulating intermediates, or by affecting gene regulation, so that compounds produced at an undesirable stage in the life cycle are synthesised at a more convenient stage. Genetic engineering can potentially be used to add new peptide products, new enzymatic conversion steps, or even new biosynthetic pathways leading to the production of totally new products.

#### *1.9.2.5 Carotenoid production using algal carotenoid genes in biological systems.*

The carotenoid biosynthetic pathways in algae could be used as a genetic pool for the production of carotenoids in various biological systems. Recently, many genes coding for carotenoid biosynthetic enzymes have been isolated from bacteria, fungi, cyanobacteria, green algae, and higher plants (Table 1.2).

Organism	GGDP synthase	Phytoene synthase	Phytoene desaturase
<i>Rhodobacter</i>	crtE (Armstrong et al., 1989)	crtB (Armstrong et al., 1989)	crtI (Bartley and Scolnik, 1989)
<i>Erwinia herbicola</i>	crtE (Armstrong et al., 1990; Ausich et al., 1991)	crtB (Armstrong et al., 1990; Ausich et al., 1991)	crtI (Armstrong et al., 1990; Ausich et al., 1991)
<i>uredovora</i>	crtE (Misawa et al., 1990)	crtB (Misawa et al., 1990)	Misawa et al., 1990)
<i>Neurospora</i>	al-3 (Carattoli et al., 1991)	al-2 Schmidhauser et al., 1990)	al-1 (Schmidhauser et al., 1990)
<i>Synechocystis</i>			pds (Chamovitz et al., 1991)
<i>Synechococcus</i>		pys (Chamovitz et al., 1992)	pds (Pecker et al., 1992)
<i>Dunaliella</i>			pds (Pecker et al., 1992)
Tomato		pTOM5 (Ray et al., 1992) pys (Bartley et al., 1992)	
Soybean			pds (Bartley et al., 1991)
Pepper	GGDPS (Kuntz et al., 1992)	pys (Hugueney et al., 1992)	pds (Hugueney et al., 1992)
Maize		y1 (Buckner et al., 1990)	
	<b>Zeta-carotene desaturase</b>	<b>Lycopene cyclase</b>	<b>Beta-Carotene hydroxylase</b>
<i>Anabaena</i>	zds (Linden et al., 1993)		
<i>Erwinia herbicola</i> <i>uredovora</i>		crtY (Ausich et al., 1991) crtY (Misawa et al., 1990)	crtZ (Ausich et al., 1991) crtZ (Misawa et al., 1991)
	<b>Zeaxanthin glycosylase</b>		
<i>Erwinia herbicola</i> <i>uredovora</i>	crtX (Ausich et al., 1991) crtX (Misawa et al., 1990)		

Table 1.2 Genes isolated from different organisms coding for carotenoid biosynthetic enzymes.

Using recombinant DNA techniques, the wide variety of carotenoids produced in algae could be produced in alternative biological systems. The manipulation of the carotenoid biosynthetic pathway especially in plants which already accumulate large amounts of carotenoid (e.g. tomatoes), could lead to new and novel 'natural' carotenoids being produced on a commercial scale.

### 1.9.3 Optimisation for the autotrophic production of microalgae

Nutritional and physical parameters to which an alga is exposed significantly alters the carotenoid yield (Greasham and Inamine, 1986). Development for an economical production medium requires the selection of carbon, nitrogen, phosphorous, sulphur,



potassium and trace element sources. Equally important are the costs of the nutrients and their availability in bulk quantities. Other parameters important in medium optimisation include pH, CO<sub>2</sub>, temperature and light intensity.

These parameters can then be prioritised by adopting the Plackett-Burman experimental design (Plackett and Burman, 1946). The Plackett-Burman design allows for the investigation of up to X-1 variables in X experiments. The next stage in optimisation is to determine the optimum level of each key independent variable, as identified by the Plackett-Burman design, using response surface optimisation techniques (Bull *et al.*, 1992). This technique takes account of the linear interaction and quadratic effects of the key variables (i.e. nutrients, pH, temperature etc.). Once the level of each variable for optimal response has been determined, a confirmation experiment is performed, hopefully indicating that the optimised media permits better algal growth than the original media. The key elements of this technique will be discussed in Chapter 7.

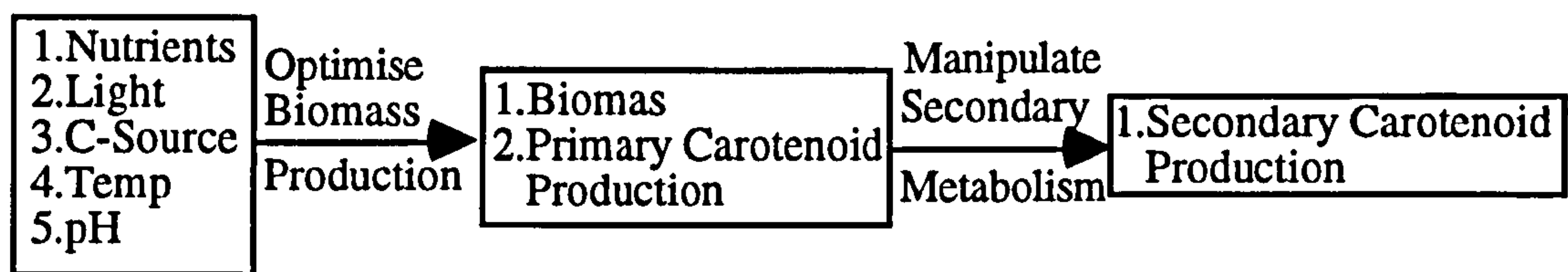


Fig. 1.10 Optimisation of secondary carotenoid synthesis in microalgae.

#### 1.9.4 System design for the autotrophic production of microalgae

Designs for microalgal mass culture systems reflect a need to balance the biological requirements of the algae with the physical characteristics of the engineered system. Both the productivity of algal systems and the cost of their construction and operation are determined to a great extent by the nature of the mixing systems which are employed. Culture mixing serves a variety of purposes, including prevention of cell settling, elimination of thermal stratification, distribution of nutrients and carbon dioxide, removal of photosynthetically produced oxygen and enhancement of light efficiency. There are two major trends in microalgal mass culture, cultivation in open (outdoor) systems and cultivation in closed systems, which can either be indoor or outdoor.



## 1.9.5 Open systems

### 1.9.5.1 Ponds

The simplest algal mass culture system is an open pond enriched with nutrients from either natural or artificial sources. Ponds of this type can either be constructed or make use of natural lakes and lagoons and can be anything up to 300 ha in size (Schlipalius, 1989).

The ponds can be operated at minimum cost but due to cell settling, low yields, unstable algal populations and the difficulty of distributing nutrients, unmixed ponds are not usually satisfactory. Consequently, mixing systems of various sorts have been added, increasing the capital and operating costs of these systems. Such mixing systems include the pumping of water and CO<sub>2</sub>-enriched air and the mechanical action of paddle wheels (Fig. 1.11) and rotary arms. However, pond systems can easily be contaminated by other micro-organisms and still show low productivity and unstable performance.

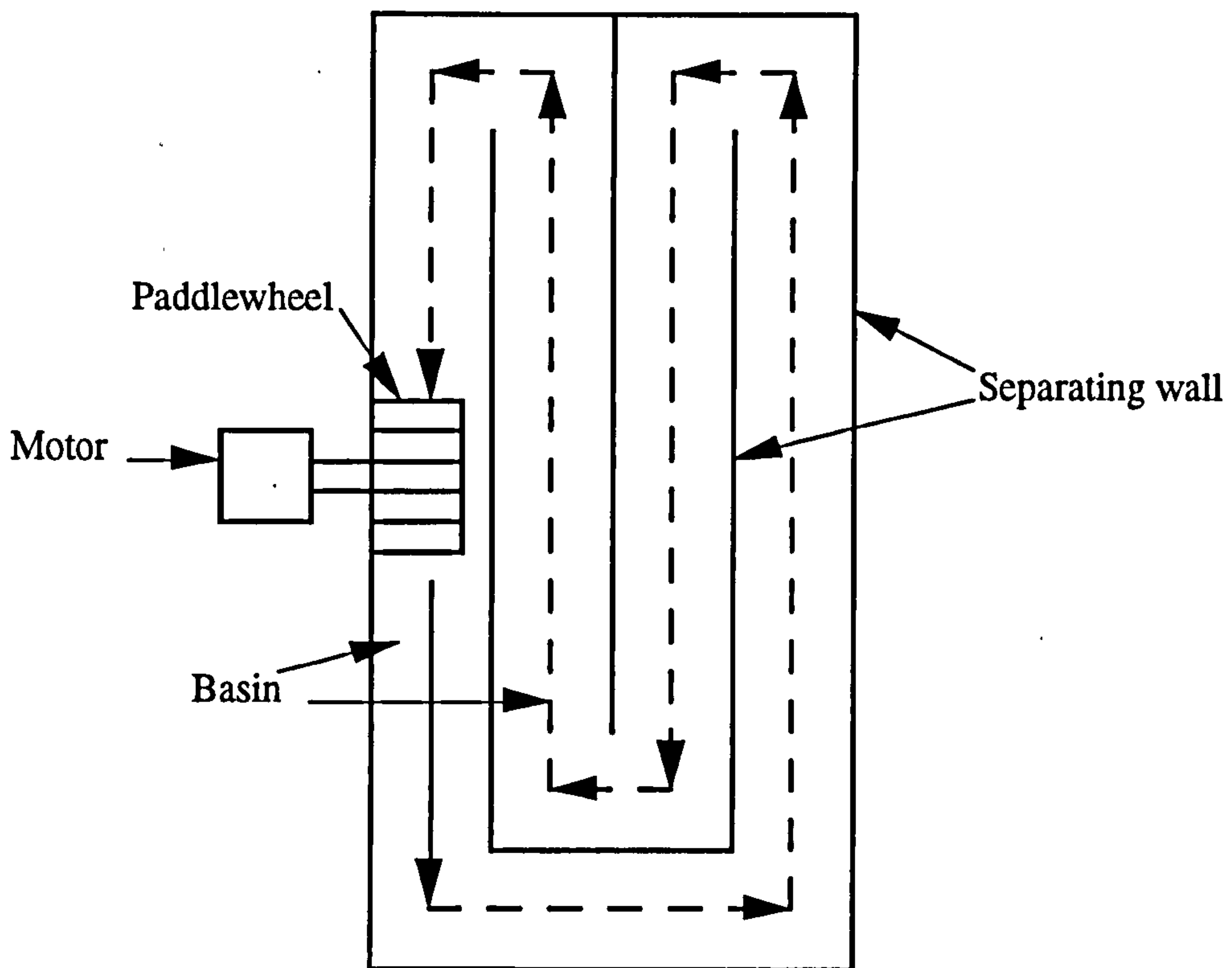


Fig. 1.11 Schematic drawing of pond in which the flow is generated by means of a paddle wheel from (Soong, 1980).

### 1.9.5.2 Deep channelled systems

In deep channelled systems the culture medium is circulated slowly through channels which in most cases form a closed, recirculating loop. The turbulence generated by the interaction of the flowing water with the bottom and sides of the containment is responsible

for culture mixing. The intensity of this turbulence varies with the rate of culture flow, while its impact varies with the depth of culture (Terry and Raymond, 1985). Channelled systems, while generally more productive than pond systems, are more costly to construct due to the need for channel dividers and for containment lining materials which are not eroded by the flowing culture. Pumps or other devices required to drive the circulation also represent a significant capital cost, and the energy required for their operation substantially increases operating costs.

### 1.9.5.3 *Shallow circulating systems*

These systems use the same basic design as deep channelled systems, but use shallow, circulating, heavily mixed systems. These systems employ intense mixing of either a turbulent or vortex nature. The shallow depths employed are required for effective utilization of mixing energy, but offer other advantages as well. For example, the dense cultures which are employed in shallow systems require less concentration at harvesting, reducing costs. However, the capital costs of these systems are high reflecting costs of the mixing devices, the culture lining materials and the more accurate grading required for the maintenance of uniform shallow depths over large areas.

## 1.9.6 **Closed systems**

Closed systems are complex in design and expensive. Temperature control of the culture vessel and the accumulation of waste products are often difficult to control. However, they allow the maintenance of axenic cultures in relatively controlled conditions, which is particularly important when producing pharmaceuticals and other fine chemicals (Pohl *et al.*, 1988). Numerous closed photobioreactors have been developed and this section will review the more successful of these bioreactors.

### 1.9.6.1 *Vertical glass or plastic columns*

The photobioreactor consists of a glass or plastic column which contains the algal suspension. Aeration and agitation are provided by the injection of air often enriched with CO<sub>2</sub>, through a sparge unit at the bottom of the column. Indoors the culture is illuminated by fluorescent lights placed around the column (Fig. 1.12). Outdoor columns suffer a

major setback because the reactor system is always at a large angle to the sun's rays, which results in a substantial amount of solar energy being reflected, and only a fraction being available for biomass growth (Lee, 1986).

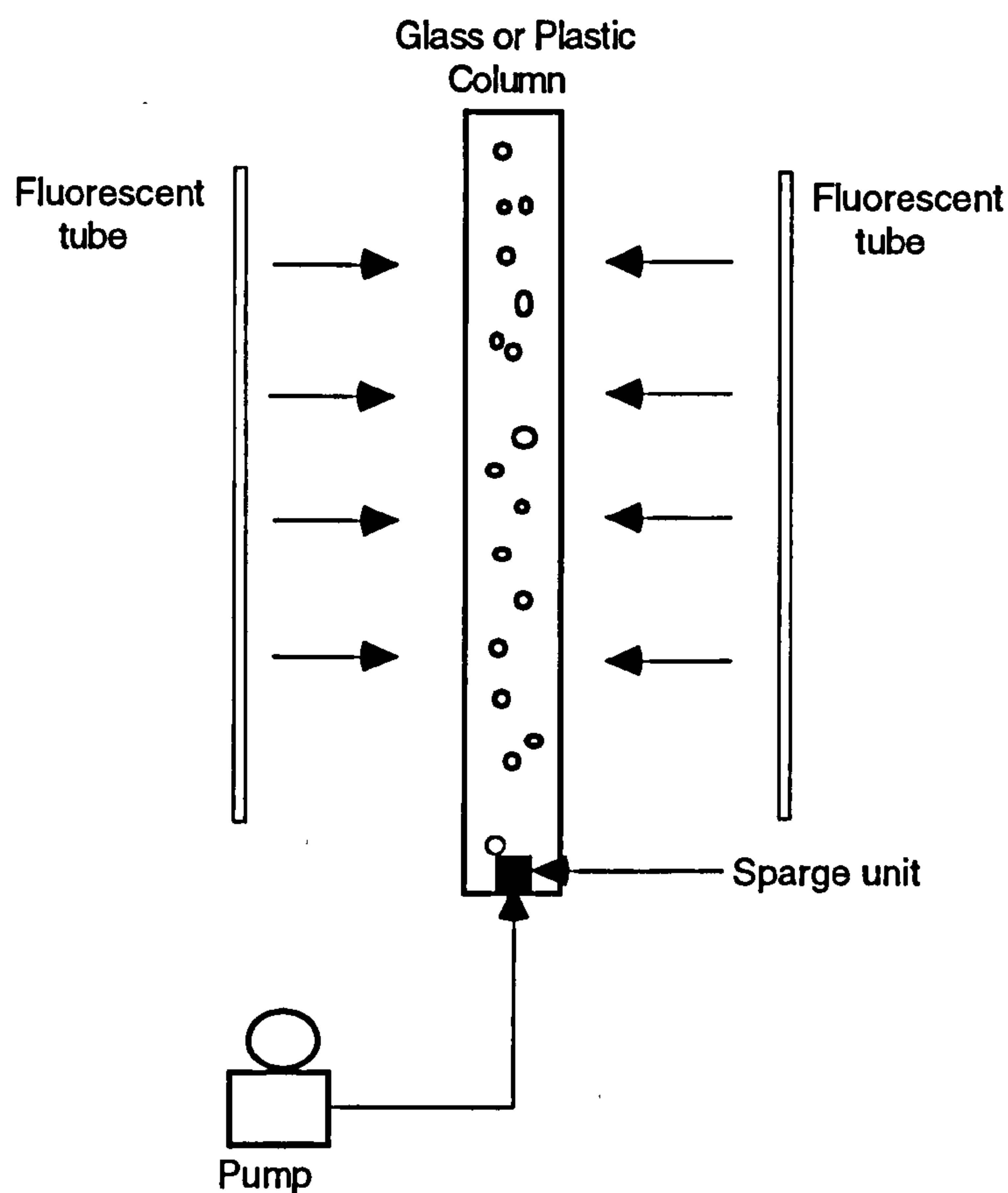
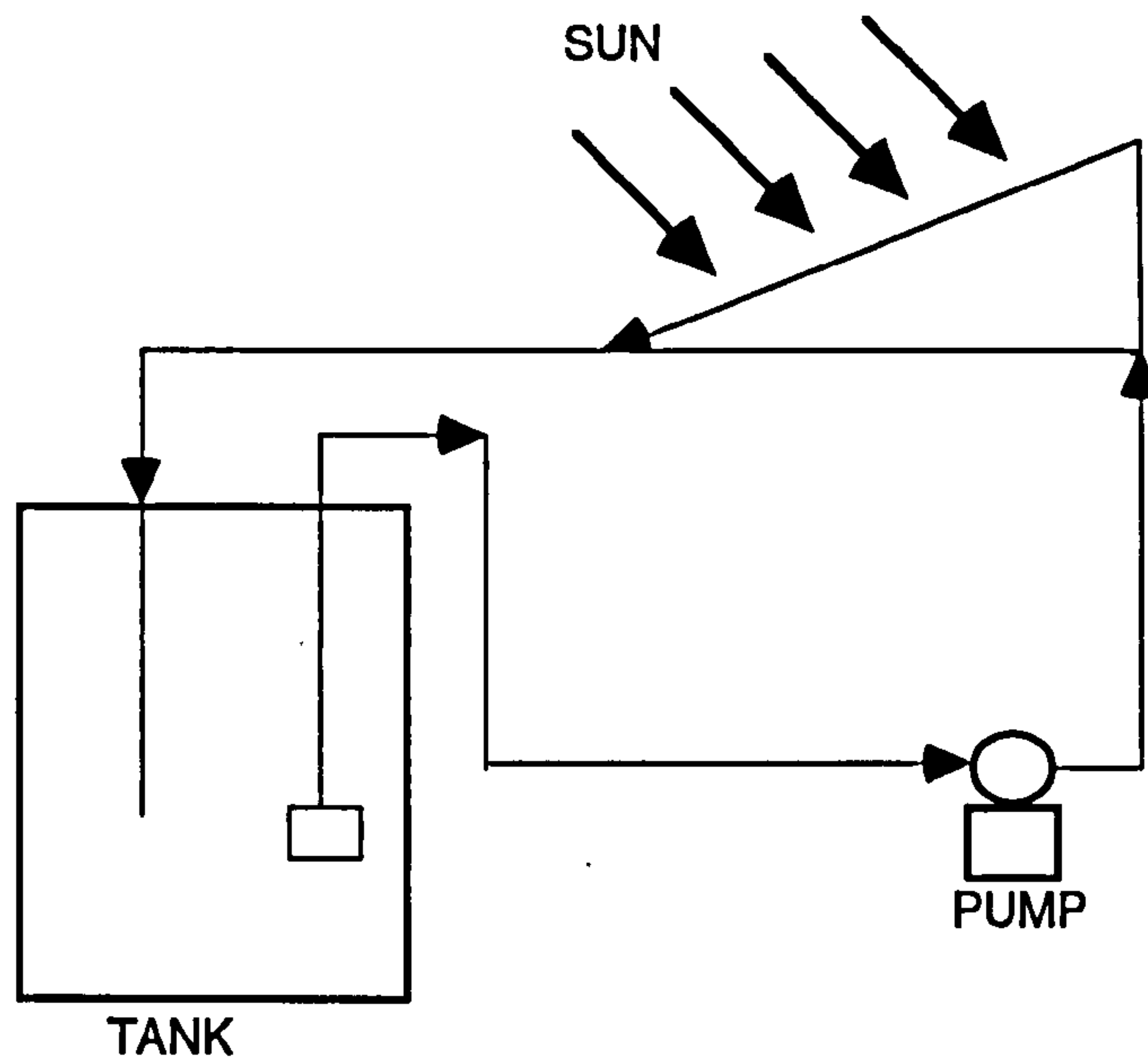


Fig. 1.12 Diagram of an air-lift type vertical column bioreactor.

#### 1.9.6.2 *Falling film photobioreactor*

The falling film photobioreactor consists of a tank which holds the algal suspension. Adjacent to the tank is a corrugated panel sloped at approximately  $20^\circ$ , with a relatively large surface area. A centrifugal pump conveys the algal suspension from the tank to the top of the panel. The suspension is then uniformly distributed over the top of the panel by a weir which produces a falling film effect. This allows for the absorption of solar or artificial irradiation by the algae. The algae are then returned to the tank for recirculation (Fig. 1.13) (Kläui, 1982).



**Fig. 1.13** Diagram of a falling film bioreactor in which is irradiated by the sun.

### 1.9.6.3 *Tubular reactors*

The reactor consists of glass or clear plastic tubing of a relatively narrow diameter (0.8 - 3 cm) which contains the algal suspension. The reactor can theoretically be of an infinite length (e.g. a 11,000 litre BIOCOIL<sup>®</sup> reactor has 3 miles of tubing (Biotechna pers. comm.)), especially if situated outdoors making use of solar irradiation. The cultures are circulated using a centrifugal or peristaltic pump or an air lift system, by which the cultures are injected with CO<sub>2</sub>-enriched air. The tubing may be emerged in water to control temperature. The tubular bioreactor is attractive for several reasons. Its construction allows: (i) effective sterilisation of the system; (ii) better control of gas transfer; (iii) effective illumination due to a large surface area to volume ratio; (iv) installation in any open space (Lee and Bazin, 1990).

### 1.9.6.4 *Fermenter-like bioreactors*

The algae are grown under axenic conditions in closed cylindrical tanks made of steel or polyethylene, with a capacity of 250 - 3000 l. The bioreactors are equipped with an internal illumination system. Four-water tight transparent glass tubes (each holding a fluorescent lighting tube) are inserted into the cylinder from the top of the bioreactor. Stirring and aeration are achieved by means of a T-shaped stirrer which rotates slowly (Pohl *et al.*, 1986). Sterile air is injected via the central tube of the stirrer into both blades



of the stirrer, from where the air penetrates into the culture medium through capillary holes (Fig. 1.14).

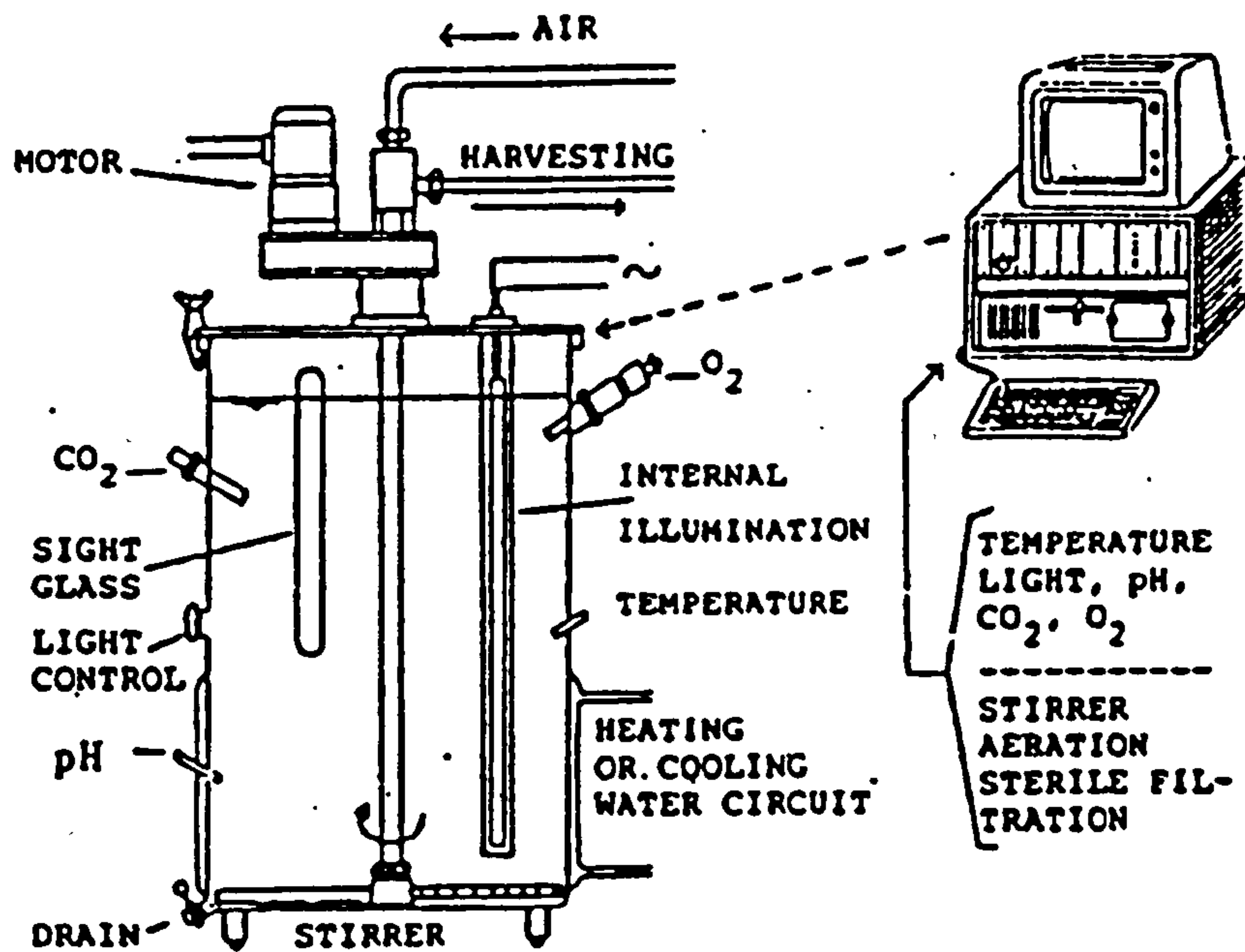


Fig. 1.14 Stainless steel bioreactor with internal illumination (from Pohl *et al.*, 1988).

### 1.9.7 Harvesting microalgal biomass and carotenoid recovery

The cost of harvesting is a major hurdle for microalgal production. Harvesting of microalgae is inherently expensive due to low cell densities which pose the problem of separating small, often motile particles from a large volume of medium. The final use of the algal carotenoid product will influence the harvesting method to be employed. If the algal biomass is to be extracted to produce a pure fine-chemical then toxic flocculants (e.g. iron-based) may be used for harvesting. However, if the algal biomass is to be used as a food or feed supplement, then other flocculation or harvesting methods must be developed. Techniques employed to date for the harvesting of microalgae are reviewed below.

#### 1.9.7.1 Centrifugation

Almost all types of microalgae can be separated reliably and without difficulty with the aid of centrifuges. Centrifugation machines developed include plate separators, nozzle centrifuges and decanters (Mohn, 1988). However, for most products, centrifugation is

prohibitively expensive due to the high volumes of algal culture which need to be harvested.

#### 1.9.7.2 *Chemical flocculation*

Particle size may be increased by adding flocculants, chemicals which cause the individual algal cells to agglomerate into larger particles which are easier to separate. Chemical flocculants may be broadly divided into two groups: (i) polyvalent metal ions such as  $Mg^{2+}$ ,  $Al^{3+}$ , and  $Fe^{3+}$  which form hydrates at suitable pH; (ii) polymer flocculants, which may themselves be anionic, cationic, and non-ionic. The type of flocculant used ultimately depends on the final use of the algal product, i.e. the addition of metal ions to the algal suspension is not acceptable when the biomass is to be used as a feed additive for fish and molluscs.

#### 1.9.7.3 *Floatation*

Flocs such as those including actively photosynthesising algae which are not sufficiently heavy to settle satisfactorily, may be more effectively separated by attaching them to gas bubbles, which will cause them to float to the surface of the medium.

#### 1.9.7.4 *Sedimentation*

Algal cells may be dense enough that, in the absence of any turbulence, the cells settle on the bottom of the pond or bioreactor. The supernatant is drawn off and the algal sediment removed. Flocculants may be used to agglomerate the cells, the agglomerates settling quicker than the single cells, which in any case may not be dense enough to settle.

#### 1.9.7.5 *Filtration*

Various filtering devices have been developed to separate algae. These include filter presses, vacuum band filters and sand filters. Pressure filtration employs low pressure in a closed flowing filter system in which the free medium passes through the filter concentrating the algae. Units consisting of hollow fibres, filter trays, porous tubing, etc. made of different materials provide continuous filtration for the algae.

### **1.9.8 Extraction**

The process of extracting carotenoids from algal biomass depends in part on the harvesting procedure employed and on the market requirements of the product. Of the extraction procedures available, the expense of supercritical fluids used extensively in the flavour additives industry and chromatography is currently far above commercial justification for carotenoids. Extraction with organic solvents may result in economically feasible concentrates. For example, solvent extraction might follow harvest with calcium hydroxide or other chromatographic materials. The alga and flocculant can be dried and packed directly into a column and carotenoids can then be selectively eluted. However, the use of certain solvents may not be acceptable to customers seeking a 'natural' product. Government and EU legislation also restricts the availability of certain solvents for use with food products. More acceptable alternative extraction methods use hot vegetable oil (Nonomura, 1987).

Extraction of the carotenoids is not always necessary. The harvested biomass may be dried (e.g. spray drying) rather than extracted. The dried algal meal is marketed as a carotenoid rich supplement for human health or animal feed.

### **1.10 *Dunaliella* - A CASE HISTORY**

The genus *Dunaliella* belongs to the class Chlorophyceae and the order Volvocales. It is a unicellular, biflagellate, naked green alga. *Dunaliella* is probably the most halo-tolerant eukaryotic organism known, showing a remarkable degree of adaptation to a variety of salt concentrations from as low as ~ 0.1 M to salt saturation, about ~ 5.0 M (Curtain *et al.*, 1987). *Dunaliella* osmoregulates by varying the intracellular concentration of glycerol in response to the extra cellular osmotic pressure (Ben-Amotz and Avron, 1980). When grown in media containing different salt concentrations, the intracellular glycerol concentration is directly proportional to the extra cellular salt concentration and is sufficient to account for most of the required osmotic pressure. This mechanism is aided due to the lack of a cell wall which permits the natural expansion and contraction of the cells. Under



high osmotic pressures the cells contract. This is followed by the adjustment of the glycerol levels within the cells and the cells return to their normal volume.

*D. salina* (*D. bardawil* is probably the same species) has the highest content of  $\beta$ -carotene (I) of any known alga (Schlipalius, 1989), with total carotenoid concentrations of up to 13% algal dry weight being reported (Nonomura, 1990). The extent of carotenoid accumulation depends on high salinity, high temperature and high light intensity (Ben-Amotz and Avron, 1983; Ben-Amotz, 1987; Borowitzka *et al.*, 1986, 1990; Lers *et al.*, 1990). The content is also enhanced under conditions of nutrient limitation, especially nitrogen (Borowitzka and Borowitzka, 1988a). The  $\beta$ -carotene (I) in *Dunaliella* accumulates within oily globules in the inter thylakoid spaces of the chloroplast (Ben-Amotz, 1991) and is composed mainly of two stereoisomers, 9-*cis* and all-*trans*. Both the amount of accumulated  $\beta$ -carotene (I) and the ratio of 9-*cis* : all-*trans*  $\beta$ -carotene (I), depends on the amount of light absorbed by the cell during one division cycle. The higher the light intensity and the lower the growth rate of the alga, the higher the cellular  $\beta$ -carotene (I) content and the ratio of 9-*cis* : all-*trans* (Nonomura, 1987).

Commercial production of  $\beta$ -carotene (I) from *Dunaliella* requires: (i) optimisation of both cell production and product yield; (ii) development of a reliable growth system that can cope with the effects of weather and potential predators and competitors; (iii) a suitable low cost harvesting method. The upper limit for algal production appears to be in the range 30 - 40 g dry weight m<sup>-2</sup> day<sup>-1</sup>, with yields of 15 - 25 g dry weight m<sup>-2</sup> day<sup>-1</sup> being the level normally sustained for longer periods in most algal mass culture systems (Goldman, 1979).

Strain improvement of *D. salina* is extremely difficult and has been reported only occasionally on a commercial scale (Nonomura, 1990). Strain improvements, both in terms of growth characteristics and product yield, can be achieved using mutagenesis and selection programmes. Improved strain breeding using mating strains of *Chlamydomonas*, a closely related alga, is also possible (Craig *et al.*, 1988). In the future the use of cell fusion techniques or genetic engineering manipulations as developed for yeast cells could be applied to *D. salina* (Borowitzka and Borowitzka, 1989).

*D. salina* is highly suitable for mass cultivation; its ability to thrive in media with high salt concentrations enables it to be cultivated outdoors in relatively pure culture and



22.  
with few potential predators. Its high carotene content protects it from the intense solar irradiation in the areas where such cultivation is practicable (Ben-Amotz and Avron, 1990). Large natural populations of *D. salina* have recently been exploited in the Soviet Union (Geleskul, 1982) and Australia (Moulton *et al.*, 1987; Schlipalius, 1990). These natural systems have extensive biomass with up to 20 tons of  $\beta$ -carotene (I) in a lake system, but the concentration is low with 0.3 mg/l of  $\beta$ -carotene (I) or less. These systems also have high pumping energy requirements and harvesting costs.

In Israel and the USA, controlled high-density cultures have been developed. The system in Israel uses a two stage process where the alga grows rapidly in the green state and is then allowed to mature into the orange state (Avron and Ben-Amotz, 1980). In the USA, the green phase has been eliminated resulting in a one-step culture process with secondary metabolite production occurring during exponential growth (Nonomura, 1987). The largest systems have culture capacities of 10 million litres and contain over 50 mg of carotene per litre with cell densities of 1 g/l.

Cultures are initiated in the laboratory as axenic isolates. The alga is tested for hardiness to seasonally harsh environments in controlled laboratory systems. Different strains may be used for winter or summer weather, low or high salinity. Genetic manipulations and selections are made to improve the content and productivity of  $\beta$ -carotene (I). Isolates from sites around the world are used for creating mutants that are tailored for temperate to desert habitats. Selected strains are scaled up from laboratory clones originating from a single cell. A number of 200 - 500 litre cultures are used for initial field inoculation. The inoculum is taken up in steps from 200 litres to the final process bioreactors, each covering 4000 - 5000 m<sup>2</sup>. To grow from a 20 litre laboratory culture to a one million litre capacity field bioreactor requires approximately three months.

The design of the culture systems is basic to most photosynthetic algal field cultures. Shallowness for exposure to light, circulation for nutrient and algal distribution, and lines for processing. Process bioreactors are monitored carefully for  $\beta$ -carotene (I) productivity and algal growth. Features of these algal bioreactors include: (i) plastic lined containment vessels; (ii) control of contamination with NaCl; (iii) addition of inorganic nutrient salts; (iv) the use of free sunlight energy. Circulation is achieved by glass fibre paddle wheels which function as vane pumps for efficient movement of large volumes of

water with low energy input. Alternatively, the system may rely on wind to cause enough turbulence to mix the algal suspension.

Harvesting and extraction represent the major cost areas in the commercial production of  $\beta$ -carotene (I) from *Dunaliella*. *Dunaliella* is a single cell (with no protective cell wall) approximately 20 x 30  $\mu\text{m}$  in size, and is naturally buoyant in a high specific gravity, high-viscosity brine. Cell densities in large cultures tend to be about only 1 g/l and very large volumes therefore have to be processed. Efforts to centrifuge or filter the algae from the brine generally shear-damage the cells leading to  $\beta$ -carotene (I) loss by oxidation. The cells also distort and pass through filters with pore sizes less than 10  $\mu\text{m}$ . Corrosion of all metal equipment by the brine is also a major problem.

Patents issued to harvest *Dunaliella* include high pressure filtration using diatomaceous earth (Ruane, 1974a); exploitation of salinity-dependent buoyancy properties in stationary and moving gradients (Bloch *et al.*, 1982); exploitation of the phototactic and gyrotactic responses of the alga (Kessler, 1985); salinity-dependent hydrophobic adhesion properties of *Dunaliella* cells (Curtain and Snook, 1983); and flocculation (Sammy, 1987).

The process for extraction of  $\beta$ -carotene (I) from the biomass depends in part on the harvesting procedure used, and on the market requirements for the algal product. Extraction using conventional organic solvents is efficient (Ruane, 1974b), however, certain solvents cannot be used when the product is to be used for human or animal consumption. A more acceptable extraction method in these cases is the use of hot vegetable oil (Nonomura, 1987; Potts, 1987) or supercritical solvents (normally  $\text{CO}_2$  and organic modifiers). The flow chart depicted in figure 1.15 highlights the main stages of *Dunaliella* cultivation and downstream processing involved in producing highly concentrated  $\beta$ -carotene (I) products.

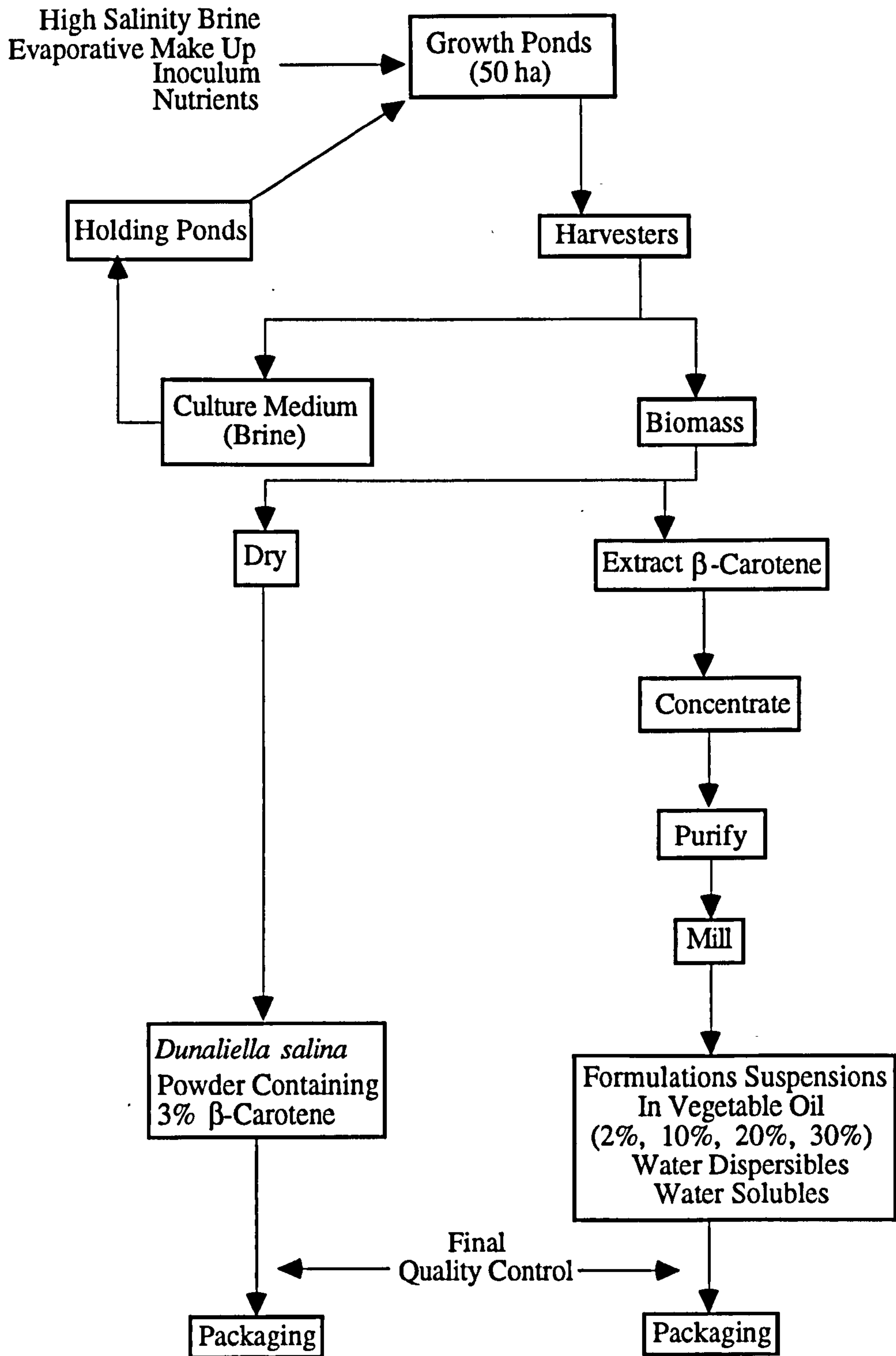


Fig. 1.15 Flow chart of the *Dunaliella salina*  $\beta$ -carotene production process used by Western Biotechnology Ltd (from Borowitzka and Borowitzka, 1989).

## GENERAL MATERIALS AND METHODS

### 2.1 METHODS FOR THE ISOLATION AND ANALYSIS OF CAROTENOIDS

#### 2.1.1 Isolation and purification of carotenoids

The procedures used for the extraction, purification and analysis of carotenoids are similar to those used for other classes of isoprenoid compounds. The conjugated polyene nature of the carotenoids makes it necessary for special and rigorous methods to be used when handling and purifying them (Britton, 1991). Carotenoids are sensitive to oxygen, heat, light, acids and, in some cases, alkali. Stringent precautions were observed throughout this study in order to avoid or minimise losses of material or induce unwanted structural changes which may be difficult to detect.

#### 2.1.2 Protection against oxidation

Carotenoids are highly susceptible to oxidation. Oxygen in combination with light and/or heat is particularly destructive. The presence of even trace amounts of oxygen in stored samples, of peroxides in solvents, or of any oxidising agents in crude samples containing carotenoids can rapidly lead to bleaching or to the formation of artifacts such as epoxides or apocarotenoids. Carotenoid samples were therefore always stored in an inert atmosphere ( $N_2$ ) in the complete absence of oxygen. Samples were sparged with  $O_2$ -free  $N_2$  for a few minutes prior to storage at  $-20^\circ C$ .

#### 2.1.3 Protection against light and heat

Exposure of carotenoids to light and/or heat was avoided whenever possible. However, direct isomerisation or photoisomerisation during normal manipulations usually occurs comparatively slowly and not to any great extent. Extracts which contain chlorophylls, or any potential sensitisers, are susceptible to photoisomerisation. This occurs via the carotenoid triplet state and can occur very rapidly so that appreciable amounts of carotenoid



Z-isomers can be produced. Even in the absence of a sensitiser, direct sunlight or UV-light may cause some geometrical isomerisation and must be avoided. Light was therefore excluded during all chromatographic procedures. Glass chromatography columns and developing tanks for TLC were covered from light sources.

Carotenoids may also undergo isomerisation and structural modification if heated, either as solids or in solution. Carotenoid-containing samples were not subjected to excessive heat. Solvents with low boiling points were used whenever possible, as these could subsequently be removed at low temperatures.

#### **2.1.4 Avoidance of acid or alkali**

Most carotenoids are susceptible to decomposition, dehydration or isomerisation if subjected to acid conditions. Carotenoid 5,6-epoxides undergo particularly facile isomerisation to the corresponding 5,8-epoxides if exposed to even traces of acid during extraction and purification. Plant tissues may be sufficiently acidic to bring about their isomerisation, although the use of neutralising agents such as NaHCO<sub>3</sub> during extraction can prevent this. Acidic adsorbents, especially silica gel, silicic acid and acid alumina, can cause isomerisation during chromatography. Acidic solvents such as chloroform were avoided.

Most carotenoids are stable to alkali and are not destroyed by mild saponification. However, those carotenoids containing the 3-hydroxy-4-oxo- $\beta$ -ring, as in astaxanthin (III), and the carotenoid esters are altered by treatment with even weak alkali. Normal saponification methods were avoided if it was suspected that any such compounds were present.

#### **2.1.5 Purity of solvents, adsorbents and reagents**

Pure solvents and reagents were used for all work with carotenoids. Solvents were dried and redistilled, and peroxides removed from diethyl ether by distillation from reduced iron powder. Solvents were stored in air-tight dark glass bottles.

When small samples (e.g. a few  $\mu\text{g}$ ) of a carotenoid are being prepared for analysis by MS (or NMR), impurities can be introduced during the purification procedure. Rigorous purification of solvents by double distillation and filtration through an activated

material such as alumina was required. TLC plates were pre-washed with a solvent at least as strong in polarity as that which was to be used for elution of carotenoids.

Small amounts of plasticisers, especially phthalates, are readily dissolved by organic solvents, and can cause a major contamination problem. All contact of samples, solvents etc. with plastic materials was avoided.

## **2.2 EXTRACTION**

### **2.2.1 Extraction of pigments from algae**

Undamaged fresh biological material was always used with the carotenoids being extracted as soon as possible in order to minimise oxidative or enzymatic degradation. Carotenoids were usually extracted with a water-miscible solvent such as acetone, ethanol or methanol. For unicellular algal material the algae was first separated from the suspension medium by centrifugation (3500 rpm for five minutes). The algal pellet was then transferred to a 10 ml bijoux glass bottle where the solvent and glass beads (2.5 - 3.5 mm dia.) were added. The glass bijoux bottles were then placed on a tissue disintegrator where mechanical disruption of the cells occurred. Following filtration the pigment extract was transferred to a snap-top glass vial, the sample was blown to dryness under a steady stream of N<sub>2</sub> and stored at -20°C.

For larger samples, the carotenoids were extracted with acetone. The mechanical disruption of the tissues was achieved using a pestle and mortar in the presence of clean sand. After filtration, the carotenoid-containing lipid extract was transferred to a separating funnel and an approximately equal volume of diethyl ether added. The solution was then thoroughly mixed and water or a saturated sodium chloride solution was added (to disperse emulsions), the amount added being approximately half that of the total volume. The funnel was then swirled vigorously to ensure efficient extraction and partition, and the two phases allowed to separate. The lower aqueous phase was run off and the upper ethereal layer, which contained the pigments was washed a further 2 - 3 times with water.

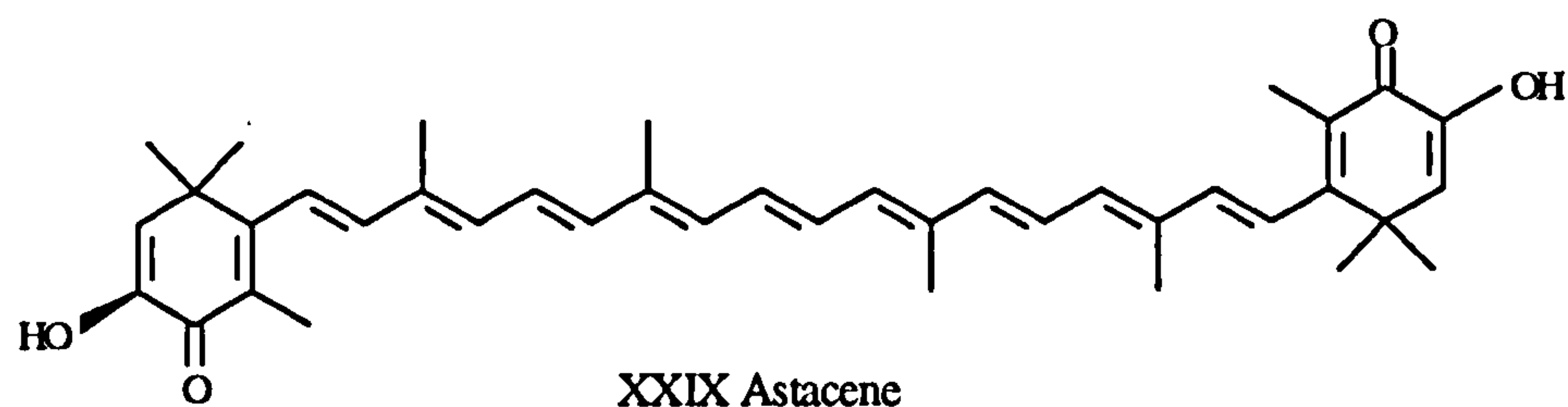
The ethereal pigment extracts were evaporated to dryness on a rotary evaporator at a temperature < 40°C. Once dried, the pigments were re-dissolved in a small volume of

diethyl ether and transferred to a snap-top vial, blown to dryness and stored as described above.

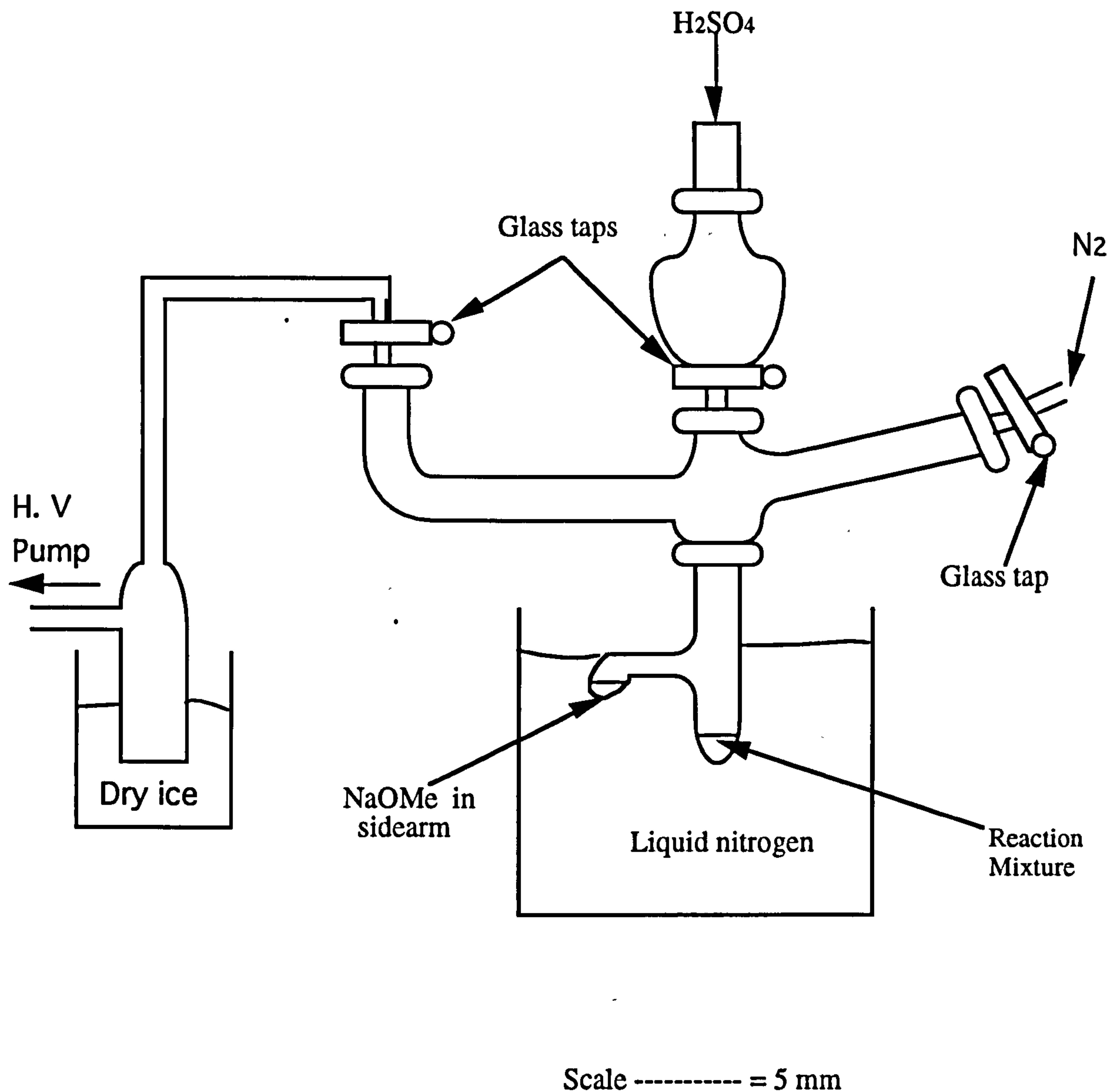
### 2.2.2 Saponification

The saponification of carotenoids removes large amounts of neutral lipid which can interfere with normal chromatographic procedures. Saponification also removes chlorophylls which may mask the presence of carotenoids, and allows for the hydrolysis of carotenoid acyl esters. Saponification is normally achieved by saponifying the carotenoid extract in 6% (w/v) ethanolic KOH at room temperature, in the dark, under N<sub>2</sub>. This mixture was normally left overnight to allow for the complete saponification of the carotenoids. An equal volume of diethyl ether was then added, followed by water, to which NaCl was sometimes added, until two layers formed. The lower aqueous phase was re-extracted with solvent until it was free of alkali. The extracts were then evaporated to dryness and stored in the usual manner.

The traditional method for the saponification of carotenoids with KOH is not satisfactory when attempting to saponify extracts containing astaxanthin (III) esters. The problem is that normal saponification leads to the formation of astacene (3,3'-dihydroxy-2,3,2',3'-tetrahydro- $\beta,\beta$ -carotene-4,4'-dione, XXIX), resulting in a loss of chirality. This can be prevented by carrying out the saponification in the absolute absence of oxygen. To achieve this, a special apparatus was developed (Fig. 2.1) which permitted anaerobic saponification (after Müller *et al.*, 1980).



The following procedure was applied: a modified glass tube, with a side-arm to allow for the addition of alkali in an inert atmosphere, was used (Fig. 2.1). The carotenoid extract



**Fig. 2.1.** Apparatus used for the anaerobic saponification of extracts containing astaxanthin (III) esters.

was dissolved in 1 ml of dichloromethane and transferred to the bottom of the glass tube which contained a small magnetic flea. The side-arm contained 1 ml sodium methylate and the whole system was flushed with nitrogen. The solutions were frozen in liquid nitrogen under vacuum and thawed carefully in order to degas the solvent. This step was repeated twice. The sodium methylate was pooled with the ester solution under high vacuum and stirred. Saponification took place within 10 minutes at room temperature. The reaction mixture was acidified with 1 ml 1M  $H_2SO_4$  and mixed well. The system was then opened and after dilution with water and ethanol, the mixture was extracted with hexane/ethanol



(1/1 v/v). The sample was then evaporated and stored in the usual manner (Müller *et al.*, 1980).

## **2.3 SEPARATION AND PURIFICATION**

### **2.3.1 Introduction**

Extracts of photosynthetic organisms or tissues normally contain a mixture of different carotenoids. For some purposes, particularly for all the routine quantitative determination of carotenoid compositions, all that is necessary is to separate the individual carotenoids from each other. The presence of colourless, non-carotenoid contaminants will not interfere with the subsequent spectrophotometric assay. In other kinds of work, however, especially when characterisation by MS, NMR or infrared spectroscopy is required or in work that involves isotopic labelling, all contaminants must be removed and the purification of carotenoids must be rigorous.

### **2.3.2 Non-chromatographic methods**

Non-chromatographic methods are mainly used in large-scale preparative work. They allow for the removal of major contaminants such as sterols and the partitioning of immiscible solvents to separate polar and non-polar carotenoids (Davies, 1976). However, the investigations presented in this report involved the analysis of small volumes of extracts, and therefore such techniques were not needed.

### **2.3.3 Chromatography**

Thin layer chromatography (TLC) was used extensively when separating and purifying extracts. High performance liquid chromatography (HPLC) was used extensively for qualitative and quantitative analysis of carotenoid-containing extracts.

### **2.3.4 Procedure**

#### **2.3.4.1 *Thin-layer chromatography***

TLC was used for the preparation of carotenoids for spectroscopic analysis and partial identification by comparison with authentic standards. Since the carotenoids are coloured (apart from the biosynthetic intermediates phytoene (VII and VIII) and phytofluene (IX)) they can be easily seen on TLC plates. Phytoene (VII and VIII), phytofluene (IX) and their derivatives are located by examining the chromatogram under UV light. Phytofluene (IX) fluoresces greenish-white, while phytoene (VII and VIII) is detected by its quenching of the fluorescence on silica gel GF<sub>254</sub> plates. Carotenoids on TLC are susceptible to oxidation, so the analysis was carried out as quickly as possible. All processes were carried out in dim light and the chromatogram left to run in the dark. Where necessary the chromatogram tank was flushed with N<sub>2</sub>.

#### 2.3.4.2 *TLC on silica gel*

Separation on silica gel occurs due to the polarity of the compounds, the most polar compounds being the most strongly adsorbed. Silica gel can be acidic enough to cause isomerisation of 5,6-epoxide groups and it is sometimes necessary to prepare the silica gel slurry with dilute KOH (~0.05 M) or pH 7.0 buffer in place of water. Astaxanthin (III) and related ketocarotenoids usually separated better with the inclusion of citric acid (~pH 4.0). Some acyclic carotenes may crystallise on the TLC plate causing streaking and poor resolution. This can be avoided by adding dichloromethane (<10%) in the developing solvent. Diethyl ether-hexane and acetone-hexane mixtures gave good resolution of carotenoids on TLC.

Once the carotenoids have separated, each carotenoid band was removed from the developing chromatogram. The bands were scraped from the plate and eluted with a solvent more polar than that used for development (usually 100% diethyl ether). The adsorbent was removed by filtration through a sintered glass funnel or by filtering through a small plug of non-absorbent cotton wool packed into a Pasteur pipette. Centrifugation was also used to remove the adsorbent. The eluting solvent was eluted through the adsorbent until the adsorbent was colourless. The solvent was then removed under a steady stream of N<sub>2</sub> and samples stored at -20°C.

#### 2.3.4.3 *Reversed phase TLC*

Separation using reversed phase TLC as with TLC on silica gel is again due to the polarity of the compounds, however, in this instance the most polar compounds are the least strongly adsorbed. Reversed phase TLC on commercial plates (Merck) also gave good separation of carotenoids. Once the chromatogram had developed the carotenoids were extracted as in TLC on silica gel. Hexane and ethyl acetate mixtures gave good resolution of carotenoids when using reversed phase TLC.

### **2.3.5 High performance liquid chromatography**

High performance liquid chromatography (HPLC) has become the widely used technique for the determination of the chlorophylls and carotenoids in plants and photosynthetic organisms. The invention of diode array detection systems has led to the development of fully computerised systems permitting simultaneous detection at several wavelengths, instantaneously and continuously memorising spectra during the evolution of a peak and offering convenient raw data handling after a chromatographic run (e.g. multi signal-, signal and spectra-, ratio of signals-, 3D plots, derivative spectra etc.) (Rüedii, 1985). Thus, optimal qualitative and quantitative information revealing the pigment composition of a sample is achieved with one single injection.

All solvents were thoroughly de-gassed in order to minimise baseline noise by reducing solvent out-gassing at the detector flow cell. When analysing extracts from complex matrices, a guard column of similar pore size and chemistry as the analytical column was incorporated between the injection valve and the column. Small bore tubing was used in all connections from the injection valve through to the detector in order to minimise dead volume and band broadening. Injection volumes were kept to a minimum (~30 µl) to prolong column life and reduce band broadening. The injection solvent was compatible with the HPLC mobile phase used.

### 2.3.5.1 *Algae*

Due to the particularly large range of structural features found in algal carotenoids, specialised chromatographic systems were used. The chromatography of pigments containing chlorophyll *c* was greatly improved by the addition of a modifier, triethylamine (0.5% v/v) to the mobile phase. The addition of this modifier proved particularly important when analysing extracts of brown algae.

### 2.3.5.2 *Reversed phase chromatography*

Reversed phase (RP) chromatography greatly reduces the risk of decomposition or structural modification of carotenoids during analysis. The vast majority of RP-chromatography methods use C<sub>18</sub> columns for the stationary phase, although not all columns are the same. Factors such as particle size and shape, pore diameter, surface coverage (carbon load), end capping and monomeric vs. polymeric synthesis influence the resultant separation (Craft, 1992). Acetonitrile/water and ethyl acetate were the most commonly used solvents in the mobile phase used in the present studies. Figure 2.2 shows the solvent gradient used when analysing samples containing ketocarotenoids using reversed phase HPLC. Figures 5.2 and 5.3 in Chapter 5 exhibit chromatograms obtained using reversed phase HPLC.

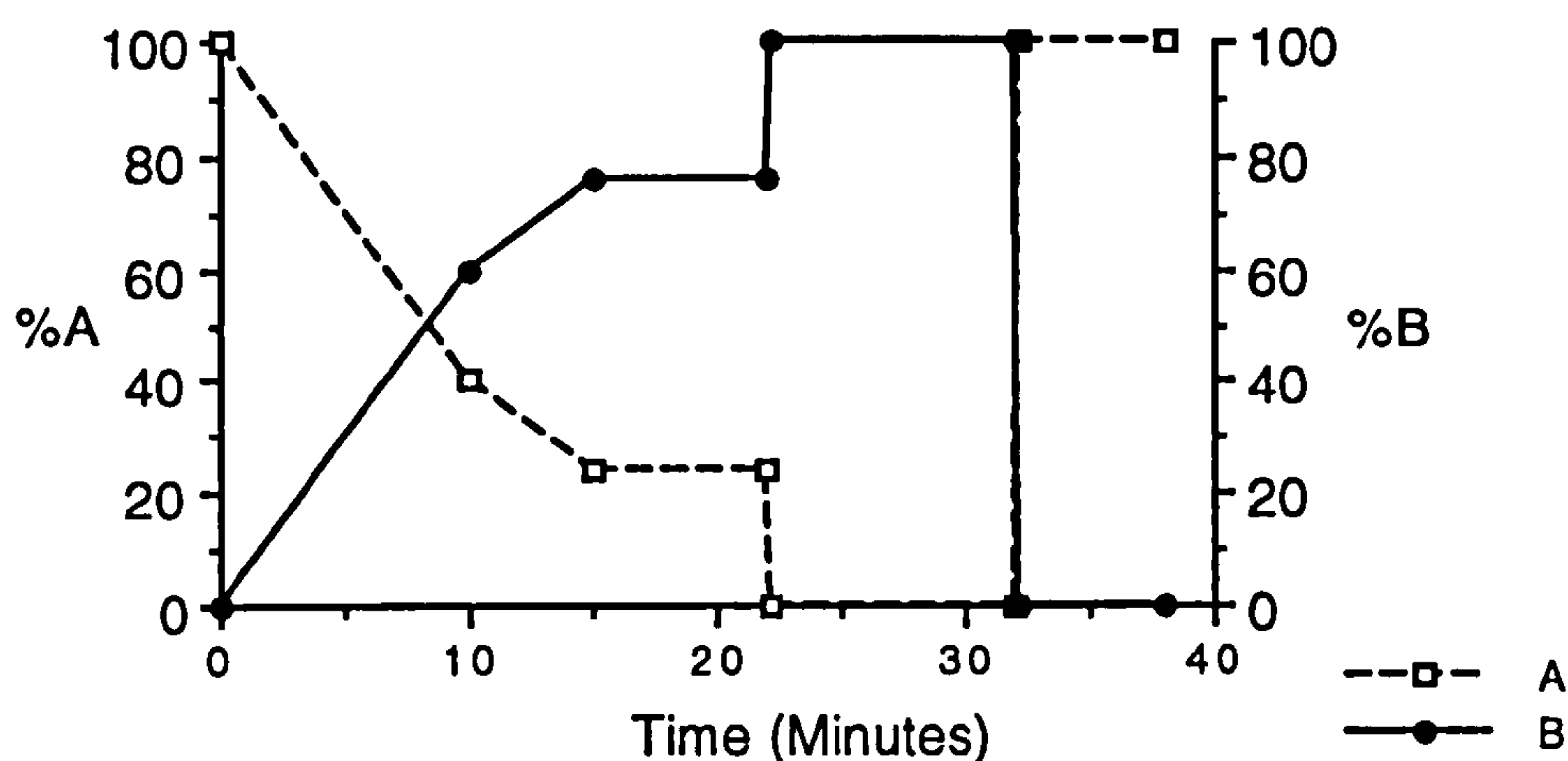


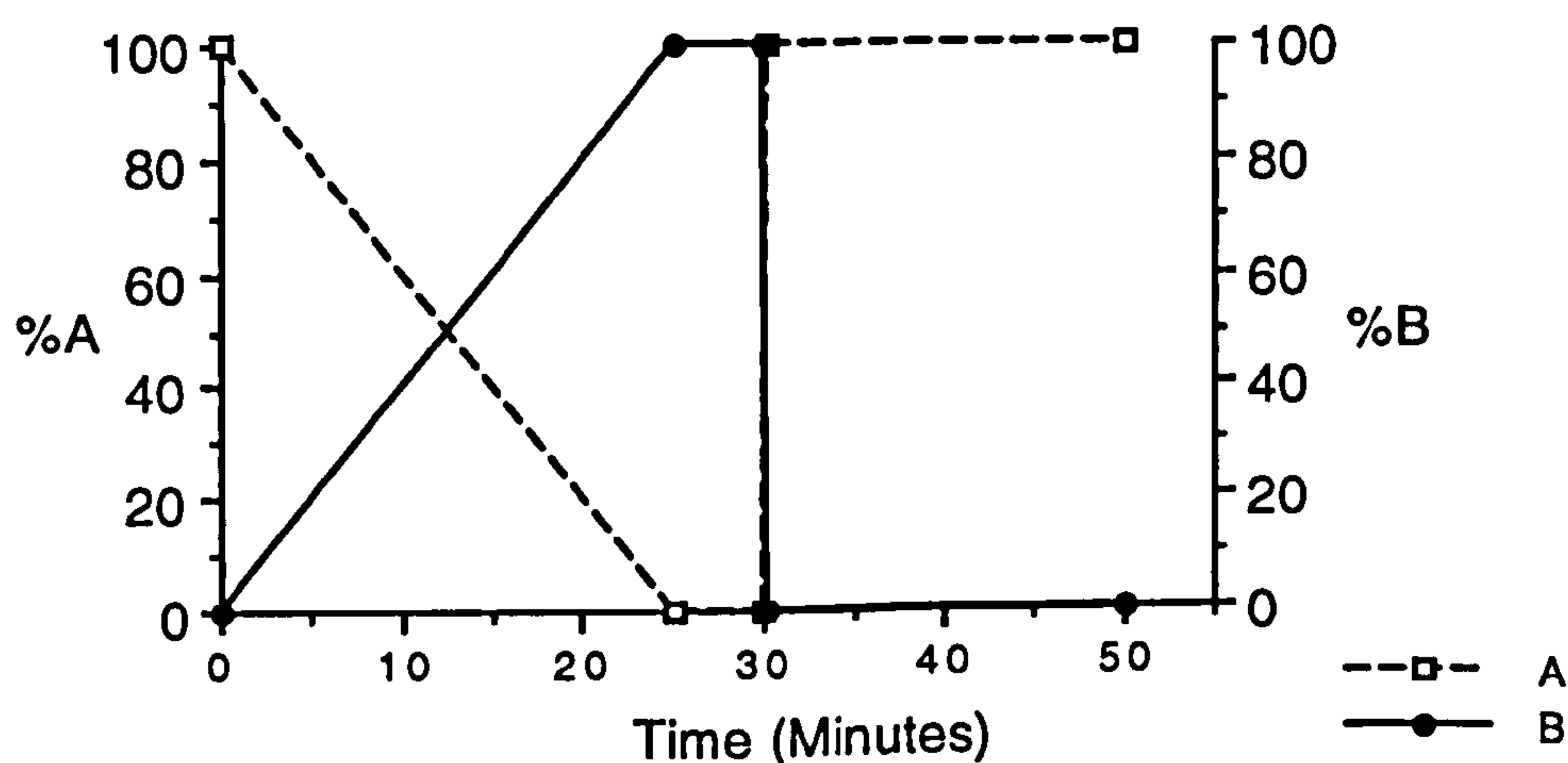
Fig. 2.2 Solvent gradient commonly used when using reversed phase HPLC.  
A = acetonitrile/water (9/1 v/v), B = ethyl acetate.



The recent interest in the functioning of the xanthophyll cycle in plants and algae has resulted in improved HPLC separation of lutein (XVI) and zeaxanthin (XV) (Thayer and Björkman, 1990; Juhler and Cox, 1990). In methods designed for the determination of all the major photosynthetic pigments (Brauman and Grimme, 1981), the two peaks overlap or are very closely related to each other and are therefore difficult to separate. Using reversed phase HPLC and the solvent gradient shown in figure 2.2, resolution and determination of carotenoids of the xanthophyll cycle is very reliable, even at very low levels.

### 2.3.5.3 Normal phase (adsorption) chromatography

Since efficient separation of carotenoids can be achieved by TLC, many silica columns are also extremely useful for HPLC separations. Most solvent systems employ hexane to which increasing amounts of a more polar solvent are added (in the present study acetone was used). The best separation was achieved by adding acetone in a stepwise manner as shown in figure 2.3. This eluted compounds of increasing polarity. Figure 2.3 shows the solvent gradient used to analyse extracts when using normal phase HPLC. Figure 5.4 shows a chromatogram obtained using normal phase HPLC.



**Fig. 2.3** Solvent gradient used when analysing extracts using normal phase HPLC.  
A = hexane, B = hexane/ethyl acetate (7/3 v/v).

An acid-coated column was also used to try and achieve improved resolution of ketocarotenoid esters. The acidic nature of the column prevented the ketocarotenoids

binding strongly to the silica stationary phase of the column. The mobile phase used an isocratic gradient of propan-2-ol/dichloromethane/hexane (0.8/10/88.2, v/v/v). Figure 5.5 shows a chromatogram obtained using this system.

## 2.4 UV/VISIBLE LIGHT SPECTROSCOPY

### 2.4.1 Chlorophylls

The absorption maxima of chlorophylls are found in the red and blue regions of the visible spectrum (Fig. 2.4). The position of  $\lambda_{\max}$  of chlorophyll *b* lies between those of chlorophyll *a* in all solvents. The shortest wavelength of  $\lambda_{\max}$  in the blue and red is found for both chlorophylls in water-free diethyl ether. The two major absorption maxima in the red and blue of both chlorophylls shift to longer wavelengths with increasing polarity and/or water content of the solvent.

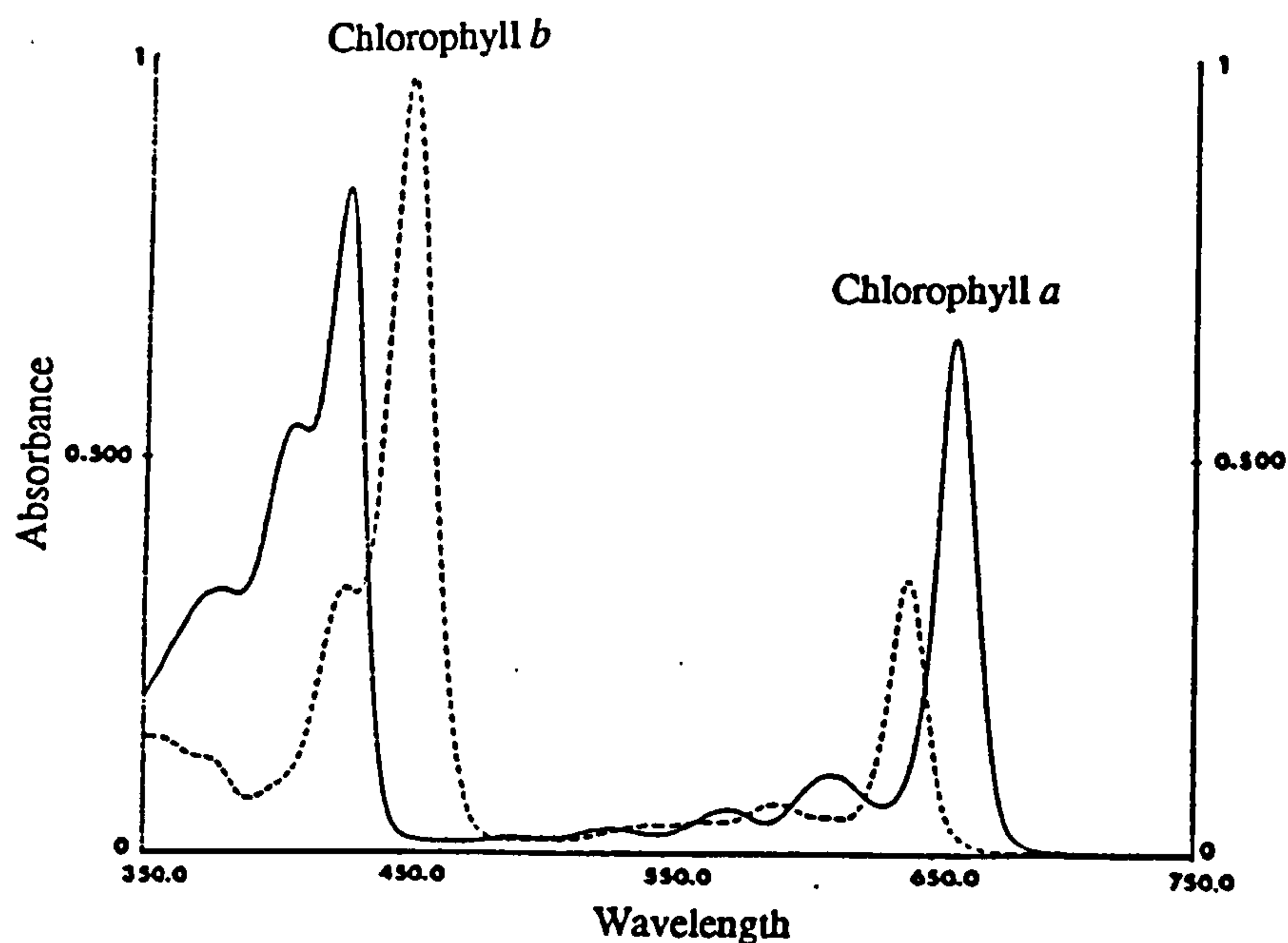


Fig. 2.4 Absorption spectrum of chlorophyll *a* and *b*.

### 2.4.2 Carotenoids

Due to their long conjugated double-bond systems, the carotenoids show absorption of ultra-violet (UV) and visible light. Both the position of absorption maxima ( $\lambda_{\max}$ ) and the

shape or fine structure of the spectrum are characteristic of the chromophore of the molecule.

### 2.4.3 Spectral fine structure

All chloroplast carotenoids exhibit a typical absorption spectrum which is characterised by three absorption maxima (e.g. violaxanthin (XVII) and neoxanthin (XX) figures 2.5 and 2.6 respectively) or two maxima with one shoulder (e.g. lutein (XVI) and  $\beta$ -carotene (I) Figs. 2.7 and 2.8 respectively) in the blue spectral region.

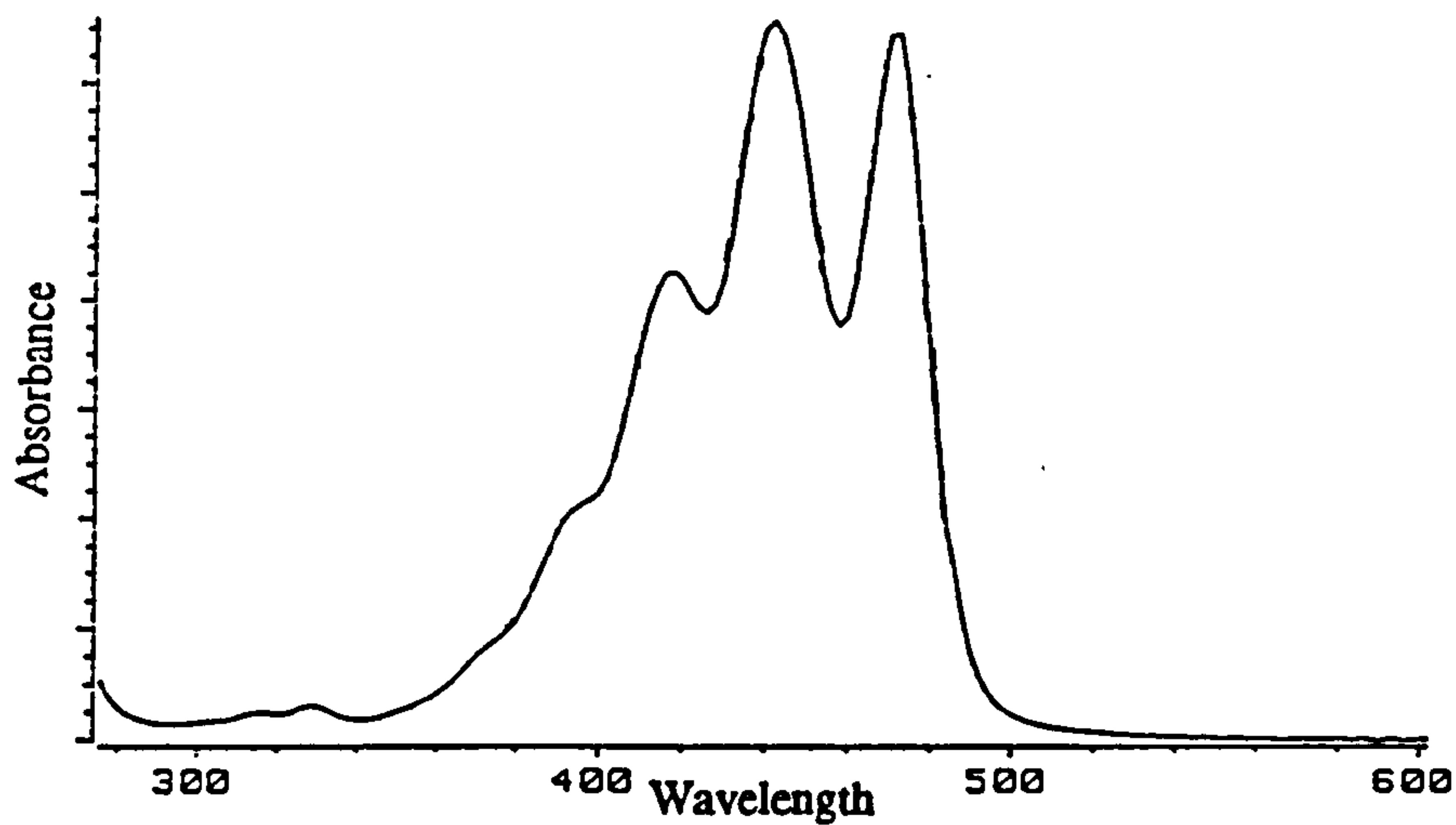


Fig. 2.5 Absorption spectrum of violaxanthin (XVII).

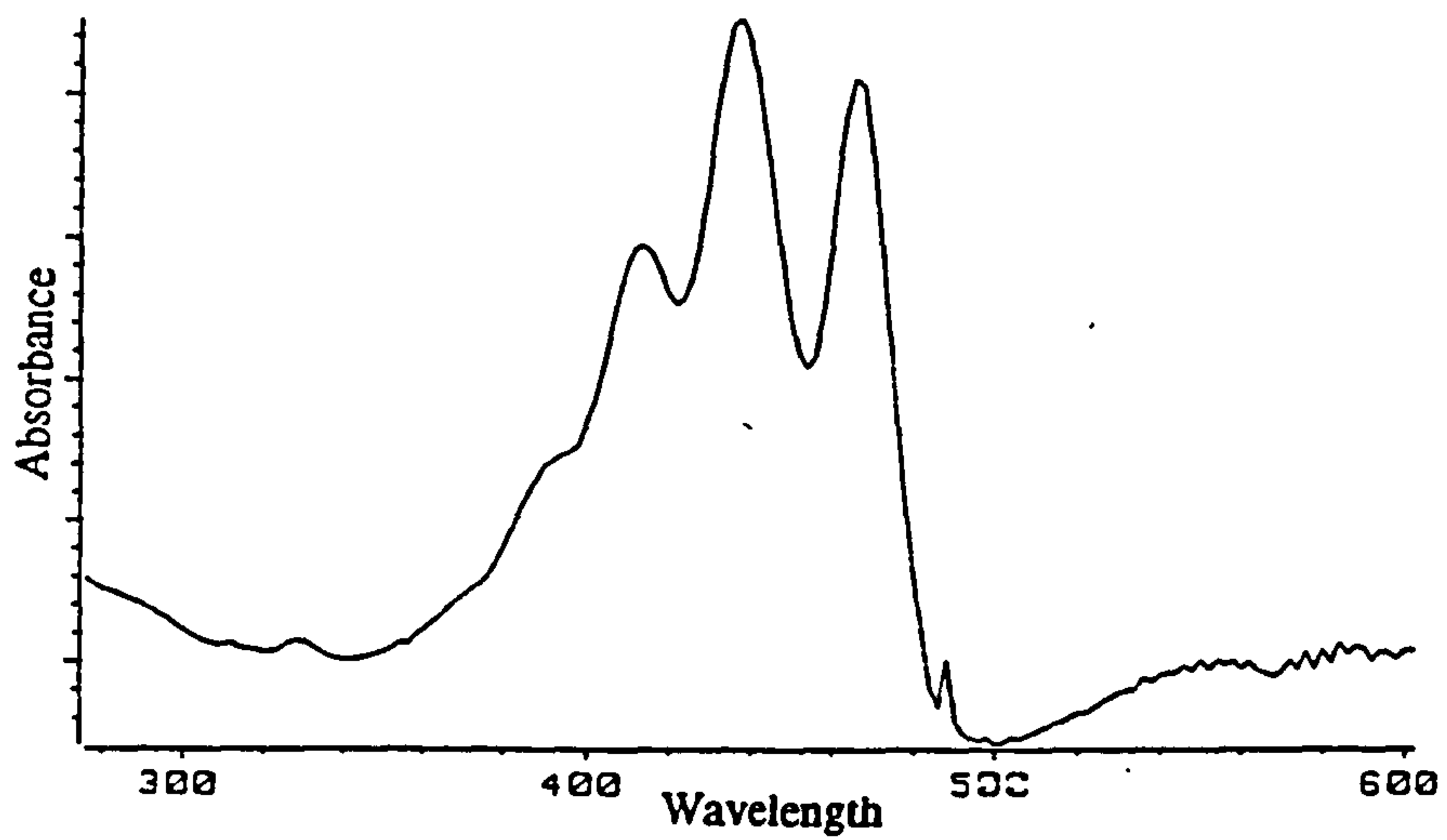


Fig. 2.6 Absorption spectrum of neoxanthin (XX).

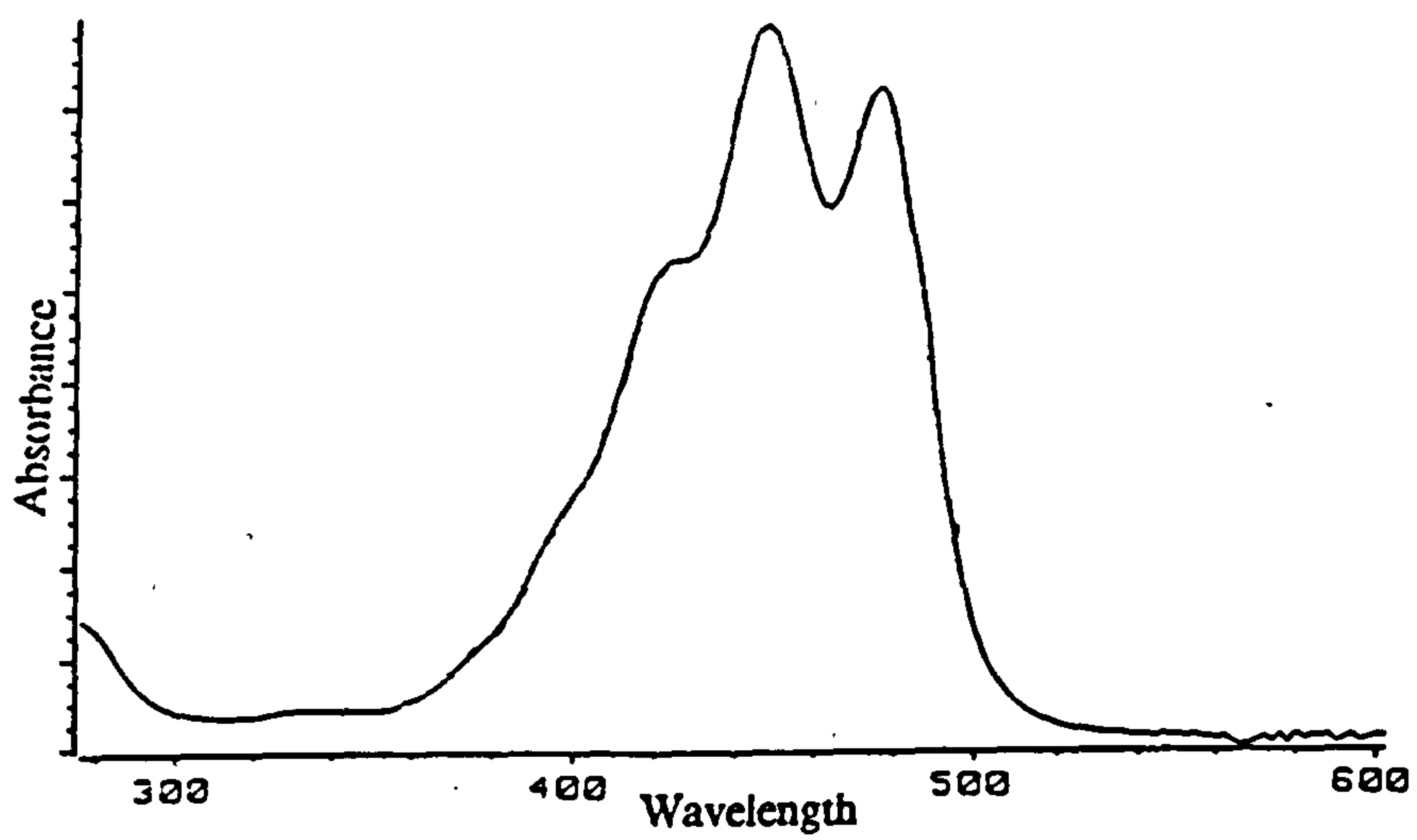


Fig. 2.7 Absorption spectrum of lutein (XVI).



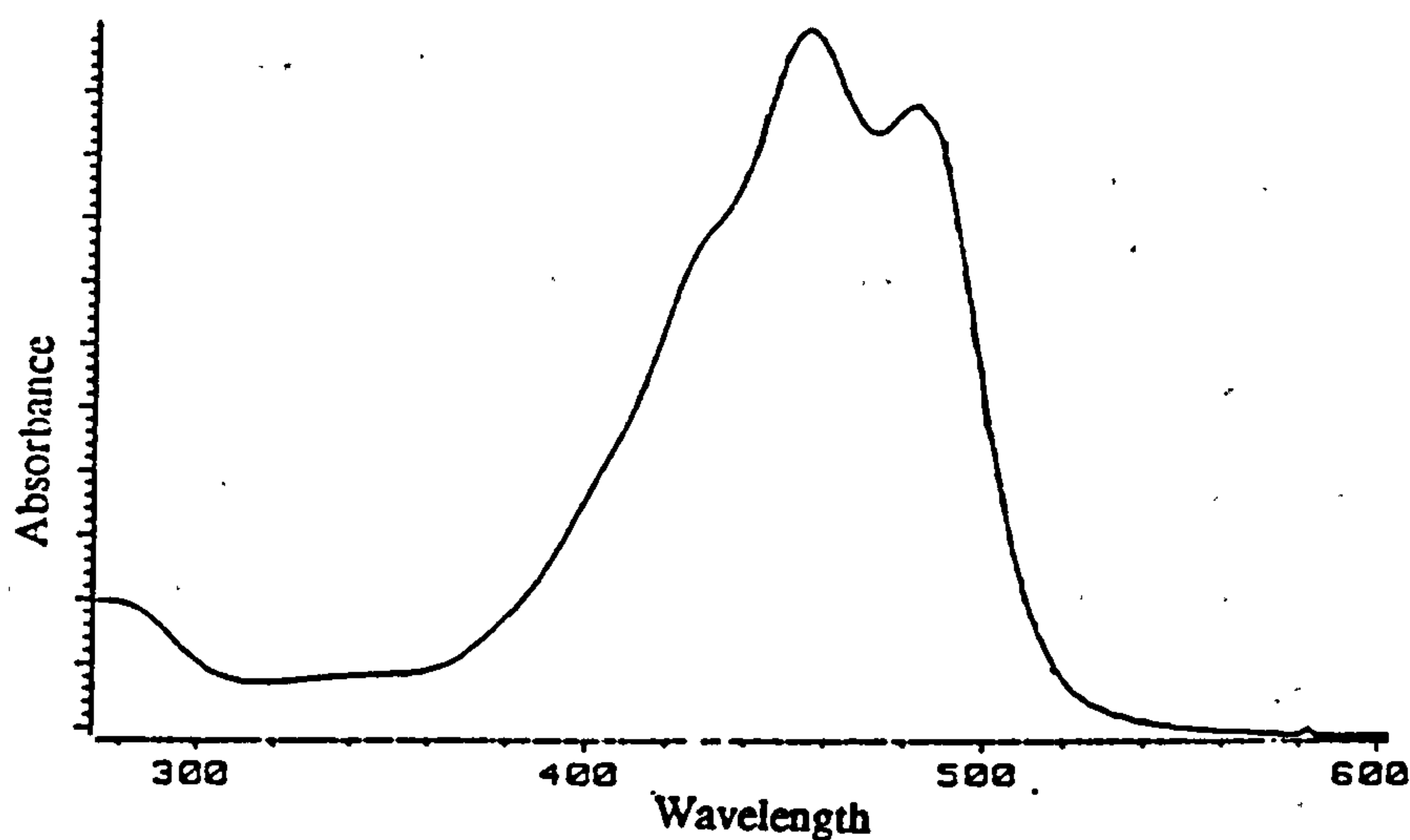


Fig. 2.8 Absorption spectrum of  $\beta$ -carotene (I).

The overall shape or degree of fine structure of the absorption spectrum of a carotenoid is also diagnostic and is generally dependent on the extent of planarity that the chromophore can achieve. The solvent used to determine the absorption spectra of carotenoids also has an influence on the degree of fine structure observed, as well as  $\lambda_{\text{max}}$ . (Goodwin, 1980; Davies, 1976).

#### 2.4.4 Position of the absorption maxima.

The absorption spectra of most carotenoids exhibit three maxima. Values of  $\lambda_{\text{max}}$  are markedly dependent on solvent. In any given solvent  $\lambda_{\text{max}}$  values increase as the length of the chromophore increases. Non-conjugated bonds do not contribute to the chromophore. Extension of the conjugated double bond system into a ring also does not extend the chromophore. However, because the ring double bond is not co-planar with the main polyene chain, the  $\lambda_{\text{max}}$  occurs at shorter wavelengths than those of the acyclic carotenoid with the same number of conjugated double bonds (e.g.  $\beta$ -carotene (I)  $\lambda_{\text{max}} = 455$  nm, lycopene (XIII)  $\lambda_{\text{max}} = 475$  nm,  $n = 11$  conjugated double bonds).

The wavelength position of the maxima of oxygen-free carotenoids such as  $\beta$ -carotene (I) are oriented to longer wavelengths than those of the oxygen bearing

xanthophylls. With increasing amounts of hydrophilic groups in the tetraterpenoid carbon skeleton of carotenoids the maxima (and shoulder) are shifted to shorter wavelengths.

#### 2.4.5 Geometrical isomers.

The absorption spectra of carotenoids that contain one or more *cis* double bonds in the chromophore show several characteristic differences from the spectrum of the *all-trans* compound. For the *cis*- isomers, the  $\lambda_{\text{max}}$  are generally 1 - 5 nm lower, the spectral fine structure is decreased and a new absorption peak (*cis*-peak) appears at a characteristic wavelength in the UV region,  $142 \pm 2$  nm below the longest wavelength peak in the main visible absorption region. These effects, especially the intensity of the '*cis*- peak', are greatest when the *cis*- double bond is located at or near the centre of the chromophore. This effect can be seen in figure 2.9 where the *cis* peak of a  $\beta$ -carotene (I) isomer is clearly distinguishable.

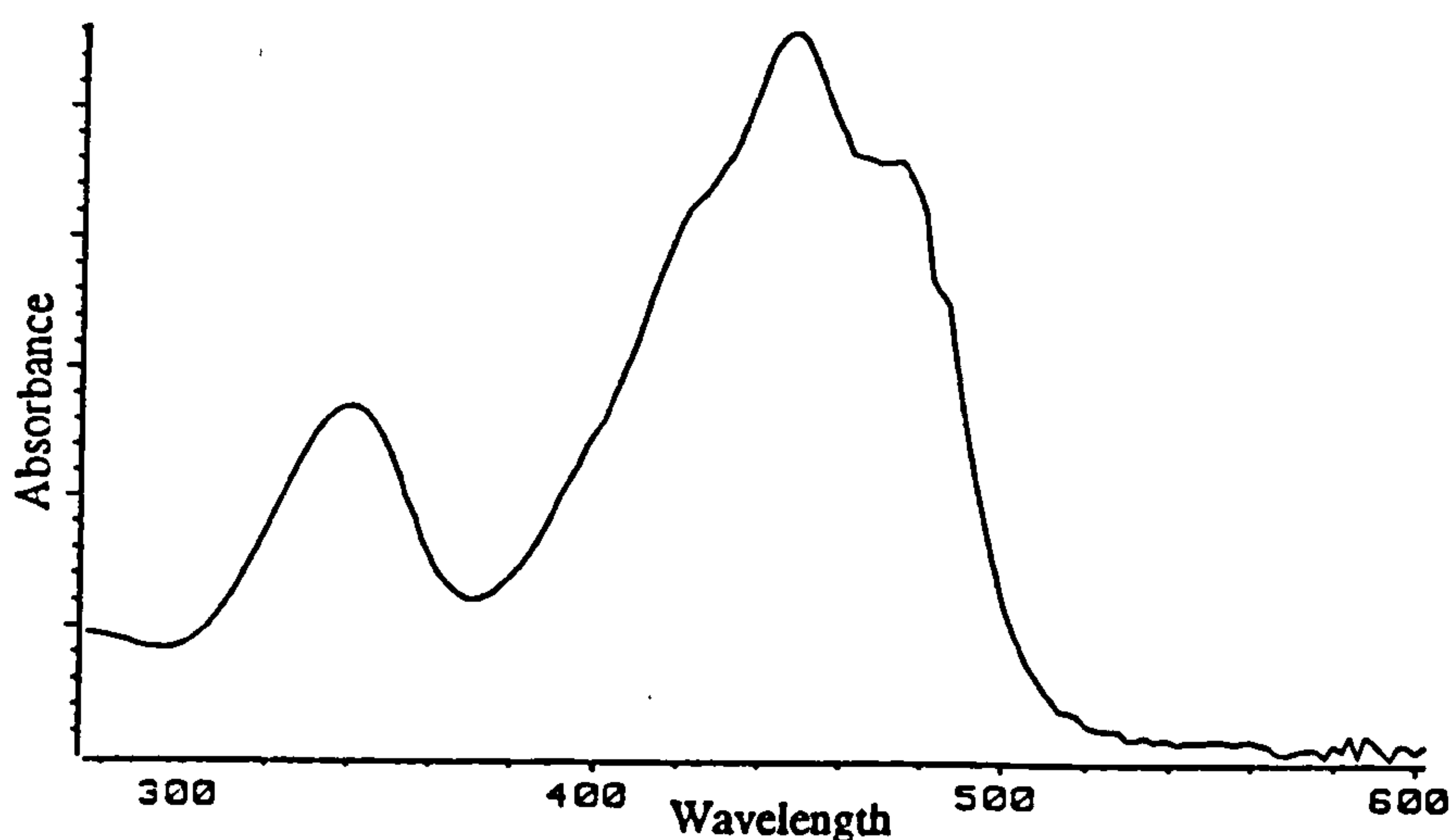


Fig. 2.9 Absorption spectra of a *cis*-isomer of  $\beta$ -carotene (I).

## 2.5 QUANTITATIVE DETERMINATION OF CHLOROPHYLL AND CAROTENOIDS

### 2.5.1 Spectroscopy

Various equations exist from different authors for different solvents for the determination of chlorophylls and carotenoids in pigment extracts. The problem with these equations is that the pigment values obtained in one solvent are not comparable with those of another. The discrepancies are particularly large in the values for the ratio of chlorophyll *a/b*.

More recently, work based on re-determined specific absorption coefficients in various solvents, has produced new equations for the simultaneous determination of total carotenoids, together with chlorophyll *a* and *b* in a pigment extract solution (Lichtenthaler, 1987). The sum of the carotenoids (xanthophylls +  $\beta$ -carotene (I);  $C_x + c$ ) was determined in pigment extracts together with chlorophylls by measuring the absorbance not only in the absorption maxima of chlorophyll *a* and *b*, but also at a wavelength where carotenoids show good absorption e.g. 470 nm (Figs. 2.5 - 2.9).

The absorbance of a pigment extract measured at 470 nm is due to light absorption by carotenoids; however, a smaller amount comes from chlorophyll *b*, whereas chlorophyll *a* contributes very little to the absorption at this wavelength. The concentration of total carotenoids ( $C_x + c$ ) is therefore determined by deduction of the relative absorption of chlorophyll *a* and *b* from the absorbency read at 470 nm, followed by division by the absorption coefficient of total carotenoids at 470 nm. Equations for the determination of  $C_x + c$  in different solvents are given in equations 2.1 - 2.4.

The advantage of these new equations are that the pigment values obtained in one solvent can be compared directly with those obtained in another solvent, including the ratio of chlorophyll *a* and *b*, and the total amount of carotenoids in the pigment extract. The equations are very reliable and the reproducibility of the results are high. The equations listed below were used in the present investigations;

Acetone, 100% (pure solvent): (Equ. 2.1)

$$C_a = 11.24A_{661.6} - 2.04A_{644.8}$$

$$C_b = 20.13A_{644.8} - 4.19A_{661.6}$$

$$C_a + C_b = 7.05A_{661.6} + 18.09A_{644.8}$$

$$C_x + c = \frac{1000_{470} - 1.90C_a - 63.14C_b}{214}$$

Acetone, 80% (v/v): (Equ. 2.2)

$$C_a = 12.25A_{663.2} - 2.79A_{646.8}$$

$$C_b = 21.50A_{646.8} - 5.10A_{663.2}$$

$$C_a + C_b = 7.15A_{663.2} + 18.71A_{646.8}$$

$$C_x + c = \frac{1000_{470} - 1.82C_a - 85.02C_b}{198}$$

Methanol, 100% (pure solvent): (Equ. 2.3)

$$C_a = 16.72A_{665.2} - 9.16A_{652.4}$$

$$C_b = 34.09A_{652.4} - 15.28A_{665.2}$$

$$C_a + C_b = 1.44A_{665.2} - 24.93A_{652.4}$$

$$C_x + c = \frac{1000_{470} - 1.63C_a - 104.96C_b}{221}$$

Methanol, 90% (v/v): (Equ. 2.4)

$$C_a = 16.82A_{665.2} - 9.28A_{652.4}$$

$$C_b = 36.92A_{652.4} - 16.54A_{665.2}$$

$$C_a + C_b = 0.28A_{665.2} + 27.64A_{652.4}$$

$$C_x + c = \frac{1000_{470} - 1.91C_a - 95.15C_b}{225}$$

The equations (Equ. 2.1 - 2.4) do not allow for the accurate determination of carotenoid levels in samples which contain astaxanthin (III). However, the equations do allow for the relative levels of carotenoids in samples containing astaxanthin (III) to be



ascertained. Quantitative determination of a specific carotenoid was carried out by simple spectrophotometric techniques. The absorption value was determined in the appropriate solvent (usually methanol, acetone or ethanol for carotenes and xanthophylls, respectively) at the  $\lambda_{\max}$  of the carotenoid. The specific absorption coefficient  $A_{1\text{cm}}^{1\%}$  (= specific extinction coefficient  $E_{1\text{cm}}^{1\%}$ ) was used to quantify the carotenoid under investigation. The  $A_{1\text{cm}}^{1\%}$  is the absorbance of a 1% (w/v) solution in a 1cm path cuvette at a defined wavelength. The accepted value of  $A_{1\text{cm}}^{1\%} = 2500$  was used when no specific values were available for an individual carotenoid or the extract contained a mixture of carotenoids. The amount of carotenoid present ( $x$  g) in  $y$  ml of solvent was determined as;

$$x = \frac{Ay}{(A_{1\text{cm}}^{1\%} \times 100)} \quad (\text{Equ. 2.5})$$

Tabulated values for  $A_{1\text{cm}}^{1\%}$  of carotenoids have been published (Britton, 1985; Davies, 1976).

### 2.5.2 HPLC

HPLC provides the most sensitive, accurate and reproducible method for quantitative analysis of carotenoids and chlorophylls, particularly when the instrumentation includes automatic integration facilities for measuring peak areas. The relative amounts of each component in the chromatogram were determined by calculating the peak area for each component at its  $\lambda_{\max}$  by using a multi-wavelength detector. This allowed the relative proportions of each chlorophyll/carotenoid component in a pigment extract to be determined. Having already calculated the total amount of chlorophyll and carotenoid present in the extract using equations 2.1 - 2.4, the absolute amount of each chlorophyll and carotenoid present in an extract could then be determined.

To estimate absolute amounts or concentrations using HPLC directly calibration is necessary using external standards. This can be achieved by injecting known amounts of pure carotenoid, determining peak areas, and creating a calibration graph, which allows the amount of each carotenoid to be estimated.

## 2.6 MASS SPECTROMETRY

### 2.6.1 Introduction

Mass spectrometry (MS) is an invaluable tool for structural elucidation in carotenoids. Most MS work on carotenoids has involved ionisation by electron impact (EI), although chemical ionisation has also been used (Lusby *et al.*, 1992). Carotenoids have very low volatility, and samples were inserted by means of a direct probe, heated to 200 - 220°C.

### 2.6.2 Molecular mass formula

Most carotenoids give good molecular ions, from which the molecular mass can be obtained. Unambiguous molecular formulae can be determined with a high resolution instrument.

### 2.6.3 Fragmentations

Fragmentation behaviour of carotenoids allow characterisation of the various terminal groups which is of importance for the structure elucidation of new carotenoids and to understand the mechanisms governing the elimination of parts of the polyene chain (Budzikiewicz, 1982). Among the types of ions produced are those resulting from in-chain losses of toluene, xylene and dimethyl-dihydronaphthalene moieties, and ions indicative of specific functional groups, such as aldehydes, alcohols and ethers. These fragment ions result from either a redistribution of excess kinetic energy deposited in the molecular ion during formation, or from the ionisation of thermally generated neutral species.

While for many carotenoids these mechanisms produce ions providing sufficient information for confirmation of structure, frequently the formation of ions with less internal energy is desirable. Reduction of the energy of electrons in the ionising beam from the usual 70 to 12 eV imparts high-energy processes, thereby decreasing the abundance of less massive fragments and, sometimes, accentuating otherwise obscured ions. There is, however, a concomitant attenuation of the absolute abundance of all ions. An alternative procedure for increasing the relative abundance of all ions at the upper end of the spectrum is to place the sample on a metallic surface, as opposed to glass or ceramic, and in close

proximity to the beam of ionising electrons, however, this was not needed in the present investigations.

## **2.7 PHYCOLOGICAL GROWTH MEASUREMENTS AND CULTURE METHODS**

### **2.7.1 Maintenance of algal cultures**

Algae can be kept under various conditions dependent upon the ecology of the algal species. The algae used in the current investigations were cultivated at room temperature (15 - 22°C), though growth may have been enhanced in some of the species at slightly higher temperatures. Temperature control was achieved by culturing the algae in a temperature controlled room at the appropriate temperature or, in a temperature controlled growth cabinet. The algae also needed to be illuminated, illumination was supplied by cool white fluorescent tubes. Illumination can be constant or follow a cyclic light/dark pattern, however, in the present studies the algae were illuminated continuously. The intensity of illumination also depends on the ecology of the algae, for the algae used in the present studies this was usually in the range 30 - 40  $\mu\text{mol m}^{-2} \text{s}^{-1}$  (PAR) to achieve good rates of growth. The algae (~50 ml of algal suspension) were kept in 250 ml Erlenmeyer flasks (cotton wool plugged), which were placed on a rotary shaker at 80 rpm to aid gaseous exchange. Approximately every 4 weeks (dependent on the species) the algal cultures were transferred to fresh media, by aseptically transferring ~5 ml of the 'old' culture to 40 - 50 ml of fresh media.

### **2.7.2 Media**

There are numerous recipes for the cultivation of microalgae under laboratory conditions. Most of them are modifications of previously published formulae and some are derived from analysis of the water in the native habitat and ecological considerations (Vonshak, 1985). The main considerations in developing a nutrient recipe for algal cultivation are: (i) the total salt concentration - largely dependent on the ecological origin of the alga; (ii) the composition and concentration of major ionic components such as potassium, magnesium, sodium, calcium, sulphate and phosphate; (iii) nitrogen sources - nitrate, ammonia and urea are widely used as the nitrogen sources, mainly dependent on the species performance and the pH optimum. Growth is highly dependent on the availability of nitrogen. Most microalgae contain 7 - 9% nitrogen per dry weight. Thus, for the production of 1.0 g of cells in 1.0 l of culture, a minimum of 500 - 600 mg/l  $\text{KNO}_3$  will be required; (iv) carbon sources - inorganic carbon is usually supplied as  $\text{CO}_2$  gas in a 1 - 5% mixture with air. Another means of supplying carbon is as bicarbonate. The preference is highly dependent on the pH optimum for growth; (v) pH - usually acidic pH values are used to prevent precipitation of calcium, magnesium and some of the trace elements; (vi) trace elements - usually supplied in a mixture at concentrations previously found to be effective (of the order micrograms per litre). However, the necessity of such components for growth has not always been demonstrated. For stability of the mixture of trace elements, chelating agents such as citrate and EDTA are used; (vii) vitamins - many algae require vitamins such as thiamine for growth.

The media used most extensively in the present investigations to cultivate the algae was Bold's Basal Medium (BBM) (Nichols and Bold, 1964) modified to pH 7.0. However, further experiments were carried out to try and modify the composition of the media to achieve improved growth rates (see later sections). The basic constituents of the media are detailed below;



Stock solutions:	<u>per 400 ml</u>
1. NaNO <sub>3</sub>	10.0 g
2. MgSO <sub>4</sub> .7H <sub>2</sub> O	3.0 g
3. NaCl	1.0 g
4. K <sub>2</sub> HPO <sub>4</sub>	3.0 g
5. KH <sub>2</sub> PO <sub>4</sub>	7.0 g
6. CaCl <sub>2</sub> .2H <sub>2</sub> O	1.0 g

	<u>per litre.</u>
7. Trace element solution:	
ZnSO <sub>4</sub> .7H <sub>2</sub> O	8.82 g
MnCl <sub>2</sub> .4H <sub>2</sub> O	1.44 g
MoO <sub>3</sub>	0.71 g
CuSO <sub>4</sub> .5H <sub>2</sub> O	1.57 g
CoNO <sub>3</sub> .6H <sub>2</sub> O	0.49 g
(Autoclave to dissolve)	

8. H <sub>3</sub> BO <sub>3</sub>	11.42 g
9. EDTA-KOH solution:	
EDTA	50.00 g
KOH	31.00 g
10. FeSO <sub>4</sub> .7H <sub>2</sub> O	4.98 g
+ H <sub>2</sub> SO <sub>4</sub> (conc)	1.00 ml

Final solution:

Stock solutions (1 - 6) 10 ml of each per litre of media.

Stock solutions (7 - 10) 1 ml of each per litre of media.

All media and equipment were sterilised by autoclaving at 121°C in pure saturated steam at 15 lb/in<sup>2</sup> above atmospheric pressure for 20 mins. Some equipment was kept in

sterile conditions after autoclaving (particularly pipettes), by storing the equipment at 300°C until needed.

All manipulations involving algae were carried out in a laminar air flow cabinet, preventing contamination of the algal cultures. The cabinet was swabbed with 70% ethanol and left for 30 minutes to reach the desired air flow rate before use.

### **2.7.3 Cultivation of large volumes of algal suspension**

In experiments which required a large volume of algal biomass special culture methods had to be adopted. The details of these culture procedures are outlined below.

#### **2.7.3.1 Apparatus**

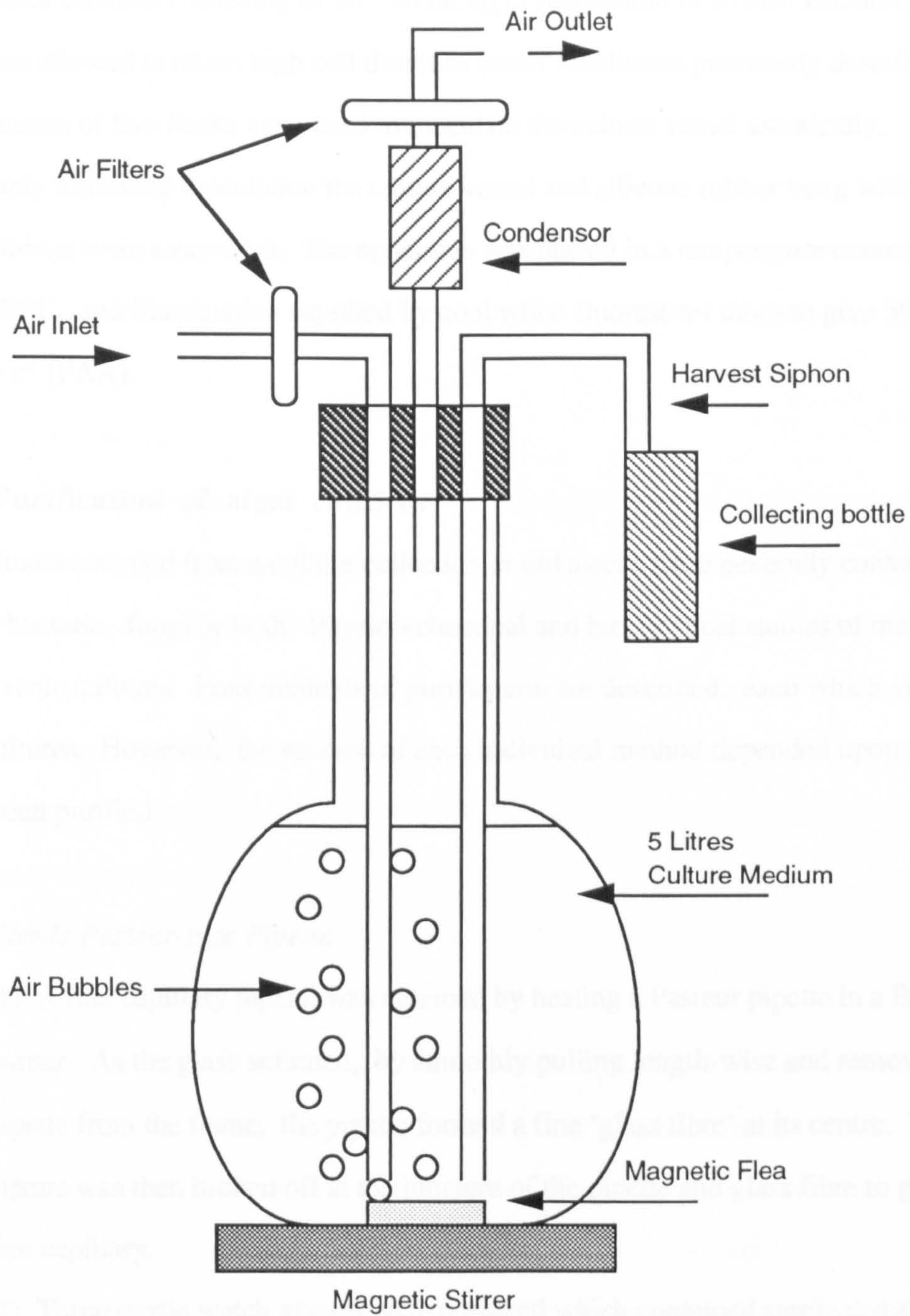
Figure 2.10 depicts the apparatus used to cultivate large volumes of algal material. The culture vessel consisted of a 5 l Pyrex glass flask, fitted with a silicone rubber bung. Three holes were bored through the bung to allow for glass tubing to be inserted through the bung for the following functions: (a) effluent gas; (b) influent gas; (c) harvest siphon.

(a) Effluent gas was allowed to escape through a short glass rod inserted in the bung. This was attached via silicone rubber tubing to a condenser to collect moisture. Gas then escaped through the condenser, through a membrane filter and into the atmosphere.

(b) Influent gas was delivered by an aerator, the air was filtered by a membrane filter to avoid contamination of the culture. A glass tube 10 mm dia reached to the bottom of the culture vessel and was connected to the aerator via silicone rubber tubing, enabling the air to be sparged into the algal suspension at a rate of 1.0 l/min.

(c) A silicone rubber tube reaching to the bottom of the culture vessel acted as a harvest siphon. The rubber tube was connected to a glass tube in the rubber bung which was connected to a 100 ml graded glass bottle. By blocking the effluent gas tube and opening the silicone rubber tube between the glass bottle and glass tube, the algal suspension was forced through the harvest siphon and into the graduated glass bottle allowing a known volume of algal suspension to be siphoned from the culture vessel.

(d) Agitation was supplied via a magnetic flea which rested on the bottom of the culture vessel.



**Figure 2.10** Apparatus used to cultivate large volumes of algae.

### 2.7.3.2 Operation

The culture vessel was filled with 5.0 l BBM modified to pH 7.0, a magnetic flea was placed inside the vessel and the mouth of the vessel plugged with cotton wool. The rubber



bung, effluent, influent and harvest tubes were assembled and placed in an autoclave bag which was then sealed. All parts of the assembly were autoclaved for 20 min at 15 lb/in<sup>2</sup>.

Stock cultures consisting of 50 - 60 ml algal suspension in 250 ml Erlenmeyer flasks were allowed to attain high cell densities under conditions previously described. The entire contents of five flasks were used to inoculate the culture vessel aseptically.

Immediately following inoculation the culture vessel and silicone rubber bung with its various fittings were assembled. The apparatus was placed in a temperature controlled room at 22°C, and illumination supplied by cool white fluorescent tubes to give 30 - 40  $\mu\text{mol m}^{-2} \text{s}^{-1}$  (PAR).

#### **2.7.4 Purification of algal cultures**

Algal cultures obtained from a culture collection or old stocks were generally contaminated by either bacteria, fungi or both. Physico-chemical and biochemical studies of microalgae require axenic cultures. Four methods of purification are described, each which yielded axenic cultures. However, the success of each individual method depended upon the algal species been purified.

##### **2.7.4.1 *Sterile Pasteur-type Pipette***

- (1) A fine capillary pipette was obtained by heating a Pasteur pipette in a Bunsen burner. As the glass softened, by smoothly pulling length-wise and removing the pipette from the flame, the pipette formed a fine 'glass fibre' at its centre. The pipette was then broken off at the juncture of the pipette and glass fibre to give a fine capillary.
- (2) Three sterile watch glasses were prepared which contained sterile distilled water (purification dishes).
- (3) The algal cells were located under a stereo-microscope.
- (4) The fine capillary pipette was heat sterilised in a Bunsen burner. An algal cell was then drawn up into the capillary by gently dipping the tip of the fine capillary into the algal suspension.



(5) The algal cell was then transferred to a purification dish containing sterile distilled water. The cell was 'washed' by transferring it with the capillary pipette to the sterile distilled water in the remaining two purification dishes.

(6) The algal cell was then transferred to a 100 ml conical flask containing 25 ml of sterile media.

(7) This process was repeated so that there were at least 20 purified cells in one conical flask. The conical flask was then placed under conditions suitable for growth.

#### **2.7.4.2 *Streak plating***

This method was particularly effective for purifying algal cells from fungal contamination.

(1) Petri dishes were prepared with growth media which contained no carbon source which was solidified with 1.0 - 1.5% w/v technical agar.

(2) 1 - 2 Drops of algal suspension were placed near the periphery of the agar. A wire loop, or a bent glass rod was heat sterilised by dipping the implement in 70% ethanol and flaming it in a Bunsen burner. Using either the loop or glass rod parallel streaks of the suspension were made on the agar.

(3) The plate was covered and inverted. The plate was incubated for seven days under suitable growth conditions.

(4) Using a stereo-microscope desired colonies were selected which were free from fungal hyphae. The desired cells were removed aseptically using a fine wire needle and transferred onto agar and incubated as in (3). This step was repeated a number of times until no fungal hyphae could be observed on the plates.

(5) Ensuring the algal cells were free from fungal contamination by observation with a stereo-microscope, the algal cells were transferred aseptically using a wire loop to 100 ml conical flasks containing 25 ml liquid medium.

(6) The conical flasks were placed under conditions suitable for growth.

#### **2.7.4.3 *Centrifugation***

Purification by washing was accomplished by repeated transfer of algal cells through a sterile liquid medium and centrifuging.

- (1) Algal cells were placed in sterile centrifuge tubes half filled with sterile distilled water.
- (2) The algal cells were centrifuged at 1000 rpm for 30 seconds and the supernatant decanted.
- (3) The algal cells were suspended in fresh media and re-centrifuged. This washing and centrifugation procedure was repeated 8 - 10 times.
- (4) Using a fine capillary pipette, algal cells were aseptically transferred to 100 ml conical flasks containing 25 ml of sterile media.
- (5) The conical flasks were then placed under conditions suitable for growth.

#### **2.7.4.4 Antibiotic treatment**

Antibiotics were used singly or in combination to establish axenic cultures (Hoshaw and Rosowski, 1973). The particular antibiotic or combination and concentration depended on the algal species been purified. Common antibiotics to establish axenic cultures were penicillin G, streptomycin, chloroamphenicol and cephataxine.

- (1) The antibiotic solution was prepared in sterile distilled water.
- (2) The antibiotic solution was filtered using a membrane filter.
- (3) 1 ml of algal suspension to be purified was placed in a 100 ml conical flask containing 25 ml culture medium.
- (4) Various amounts of the antibiotic solution were injected into the flask. Concentrations used gave penicillin levels ranging from approximately 20 - 500 mg/l and corresponding levels of the other antibiotics.
- (5) The conical flask was placed under conditions suitable for growth.
- (6) After 24 and 48, some of the algal cells were aseptically transferred to 100 ml conical flasks containing 25 ml sterile media
- (7) The flasks were placed under conditions suitable for growth.

#### **2.7.5 Sterility tests for axenic cultures**

To determine whether the four methods of purification previously described had established axenic cultures sterility tests were carried out. These involved aseptically transferring and streaking samples of algal suspensions (as previously described) onto agar. At least two

types of agar were used in the test, the growth medium was solidified with 1.0 - 1.5 (w/v) agar and Nutrient agar (used as directed on package). The plates were incubated under suitable conditions for growth for up to 2 weeks. Cultures supporting bacterial growth appeared cloudy, usually within 24 hours. Fungal contamination was usually be observed after 2 weeks using a stereo-microscope, with fungal hyphae protruding from the individual algal cells

## **2.8 GROWTH MEASUREMENTS**

### **2.8.1 Introduction**

Physiological and biochemical investigations of microscopic algae are generally conducted on cultures of algal cells as experimental objects. The obtained data reflects the activities of a multitude of cells. Quantitatively, they are usually referred to a unit of cell mass. Cell mass can be expressed as dry weight of cells or as packed cell volume of cells in a volume of given microbial culture. In a growing culture, dry weight and packed cell volume of cells per volume of cell suspension (of medium) increase with time, and this increase, termed growth, can be the specific subject of observation. Division rates may be determined from the time course increase in cell numbers. Division rate may be expressed in a number of different ways, and can be computed or derived graphically from cell count data. In turn, division rates may be used to calculate rates of increase of biomass, cell volume, or of other properties, if appropriate conversion factors are known.

For growth measurements a random sample of algal suspension is acquired. The sample is subject to the various analytical techniques designed to estimate reliably the growth/biomass of the algal cultures. Taking a sample, which is representative of a given culture is crucial for the reliable estimation of algal growth/biomass. Adequate stirring of the algal suspension and fast pipetting, prevented cell settling in the process of sampling, and were routine requirements for proper sampling.

### **2.8.2 Cell counts**

Cell counts were carried out using an Improved Neubauer haemocytometer, chamber volume  $2 \times 4^{-4}$  ml and a depth of 0.1 mm. A cover slip was used to cover the top of the haemocytometer which formed two chambers. Algal suspension was pipetted into the two chambers and the number of cells per chamber was counted using a light microscope. Cells touching the bottom and left hand grid lines in the chamber were counted, those cells touching the top and right hand grid lines in the chamber were not counted. The concentration of cells per unit volume of algal culture was then calculated. The algal culture sometimes needed to be concentrated or diluted so that the cell concentration counted was between  $10^5$  -  $10^7$  cells/ml (Guillard, 1973).

### **2.8.3 Dry weight**

Measurements of growth as increase in dry weight of cells involved taking samples of an algal suspension, drying samples to a constant weight and expressing the dry weight of cells per unit volume. Each measurement of dry weight of cells was done on at least three parallel samples.

A 5 ml sample of algal suspension (10 ml if cell densities were low) was taken and the cells separated from the medium by centrifugation (3500 rpm for 5 minutes). The pellet of cells was then re-suspended in distilled water and centrifuged again. This washing of the cells in distilled water removed salts (present in the nutrient medium) which can affect measurements of dry weight. The cells were then transferred with a pipette, in a small volume of distilled water, to pre-dried, pre-weighed foil weighing dishes. The weighing dishes were then incubated at  $100^{\circ}\text{C}$  for 24 hours after which, the dishes were then weighed and the dry weight of cells determined for each of the replicates. The average weight from the three replicates was used to calculate dry weight per unit volume of the culture.

### **2.8.4 Packed cell volume**

Determination of growth, as an increase in packed volume of cells involved centrifugation of the algal sample until the deposit of cells was compressed to a constant volume. To avoid changes in volume of individual cells, due to any possible osmotic effects,



measurements of packed cell volume were done in the same nutrient medium in which the cells were grown. Centrifugation to a constant packed volume was done in calibrated capillary tubes. The tube consisted of an enlarged upper portion, the receiver, and a lower calibrated portion, which is the capillary tube. The capacity of the calibrated portion was 0.05 ml. It was calibrated to 0.01 ml and, with an approximation, the volume of packed cells could be read to 0.0001 ml. A 5 or 10 ml sample of algal suspension (depending on cell density) was placed into the receiver and the tube was centrifuged at 1500 rpm for 10 minutes, or until the cells attained a constant volume. The packed cell volume of cells in cm<sup>3</sup> per litre was then determined. The packed cell volume of triplicate replicates from each sample was determined, and an average calculated to obtain a more accurate value (Sorokin, 1973).

## 2.9 STATISTICAL ANALYSIS

### 2.9.1 Standard errors

Standard errors were calculated to determine the variability of the means. Two methods were employed to determine the standard errors depending on how the standard errors were to be presented. When the standard errors were presented graphically equation 2.6 was used to determine the standard errors.

$$x_1 = \frac{\sigma n}{\sqrt{n}} \quad (\text{Equ. 2.6})$$

where

$n$  = number of observations

$x_1$  = standard error of the mean

$\sigma$  = standard deviation

When the standard error was presented numerically as a percentage, standard errors were determined using equation 2.7.

$$x_2 = \frac{x_1}{\bar{x}} \times 100 \quad (\text{Equ. 2.7})$$

where:

$x_1$  = standard error of the mean

$x_2$  = standard error (%)

$\bar{x}$  = the mean

### 2.9.2 Analysis of variance

One-way analysis of variance was carried out to determine any significant differences between the means of the treatments. The means of the treatments on a specific day (usually the final day) were subjected to an anova test to calculate the mean of the squares (*MS*). This value was then used to determine the least significant difference (*LSD*) of the means using equation 2.8.

$$LSD = t_{0.05[df_{within\ groups}]} \sqrt{\frac{2}{n} MS} \quad (\text{Equ. 2.8})$$

where:

*LSD* = least significant difference

*n* = number of observations

*t* = tabulated *t* value with 95% confidence limits

*df* = degrees of freedom within groups

*MS* = mean of the squares within groups

The equation produces a single value which once computed allows for the determination of significant differences between the means (Sokal and Rohlf, 1981). The value is represented as a single bar on a graph. Any points on the graph at the particular time on which the data was analysed, which are separated by a distance which is greater than the length of the bar are considered to be significantly different.

### 2.9.3 Spearman rank correlation

Spearman rank correlation's were carried out to determine if two sets of unbalanced data were correlated in some way. Equation 2.9 was used to determine the correlation coefficient  $r_s$ .

$$r_s = \frac{6 \sum^n (R_1 - R_2)}{n (n^2 - 1)} \quad (\text{Equ. 2.9})$$

where:

$n$  = number of observations

$R_1$  and  $R_2$  = paired variables

$r_s$  = Spearman rank coefficient

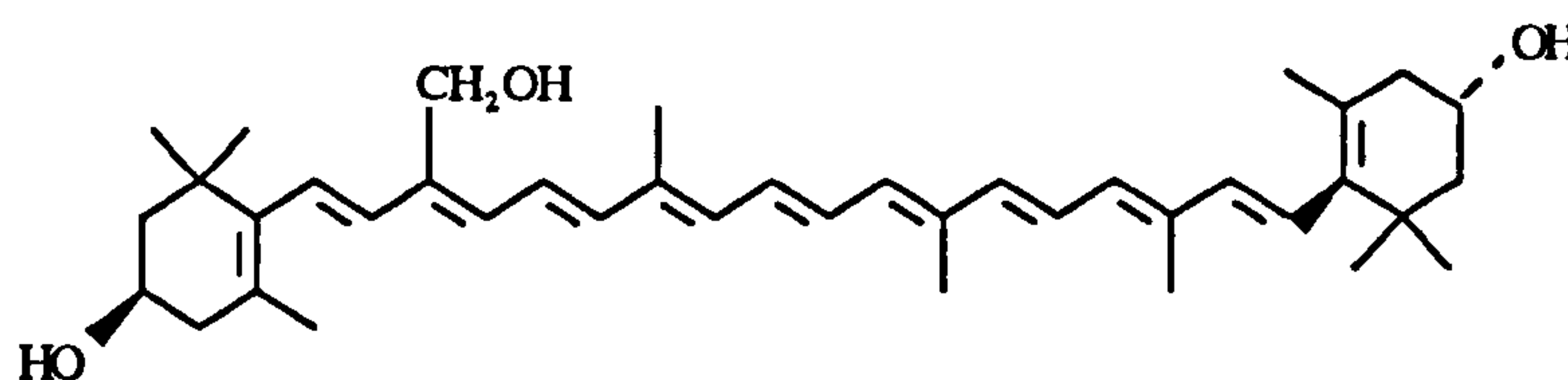
Computational analysis was carried out using Minitab. Release 9.1, Minitab Inc.

## SECONDARY CAROTENOID ACCUMULATION IN THE CHLOROPHYTA

### 3.1 INTRODUCTION

#### 3.1.1 Distribution

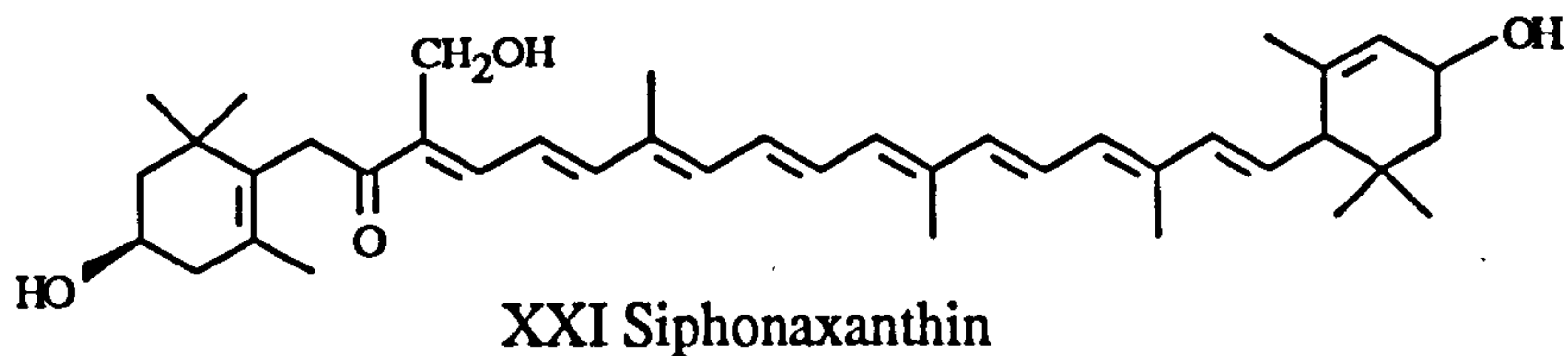
As previously described in section 1.3.6 the carotenoids found in the Chlorophyta are usually those present in the chloroplasts of higher plants, namely  $\alpha$ -carotene (VI),  $\beta$ -carotene (I), lutein (XVI), violaxanthin (XVII), neoxanthin (XX), and zeaxanthin (XV). However, as the pigment composition of more and more algae is determined by modern analytical techniques an increasing number of exceptions have been discovered. Small amounts of loroxanthin ( $3R,3'R,6'R$ )- $\beta,\epsilon$ -carotene-3,19,3'-triol, XXIII) a pigment not yet found in higher plants are found in four different orders of the classes Chlorophyceae and Bryopsidophyceae (Aitzmüller *et al.*, 1969). This pigment is characterised by the fact that an in-chain methyl at C-19 has been oxidized to hydroxymethyl.



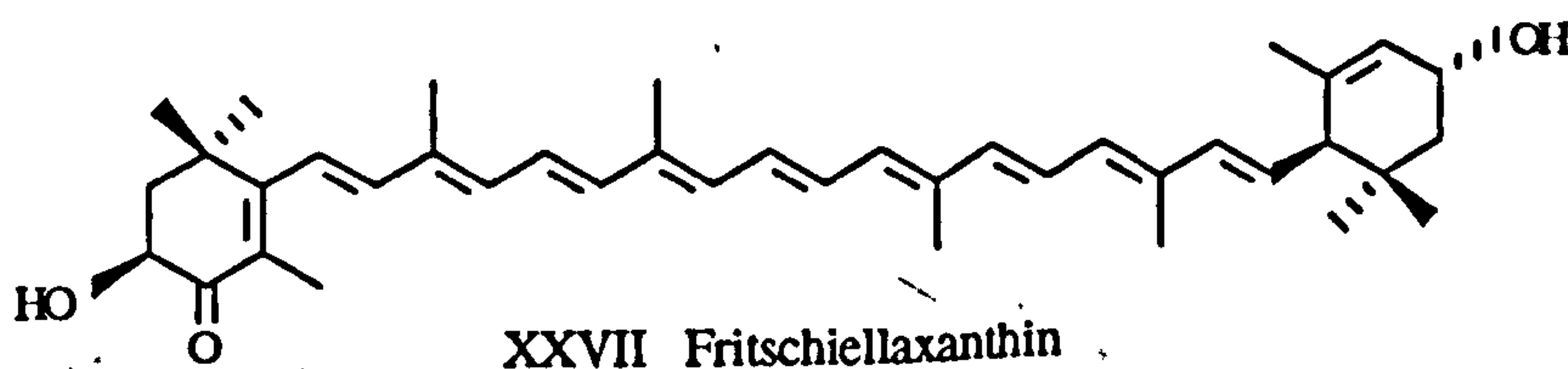
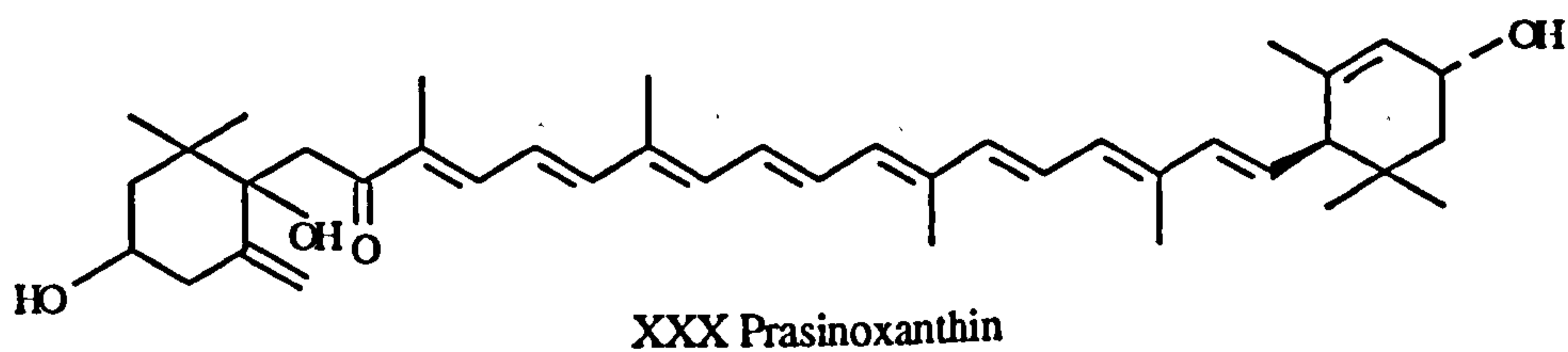
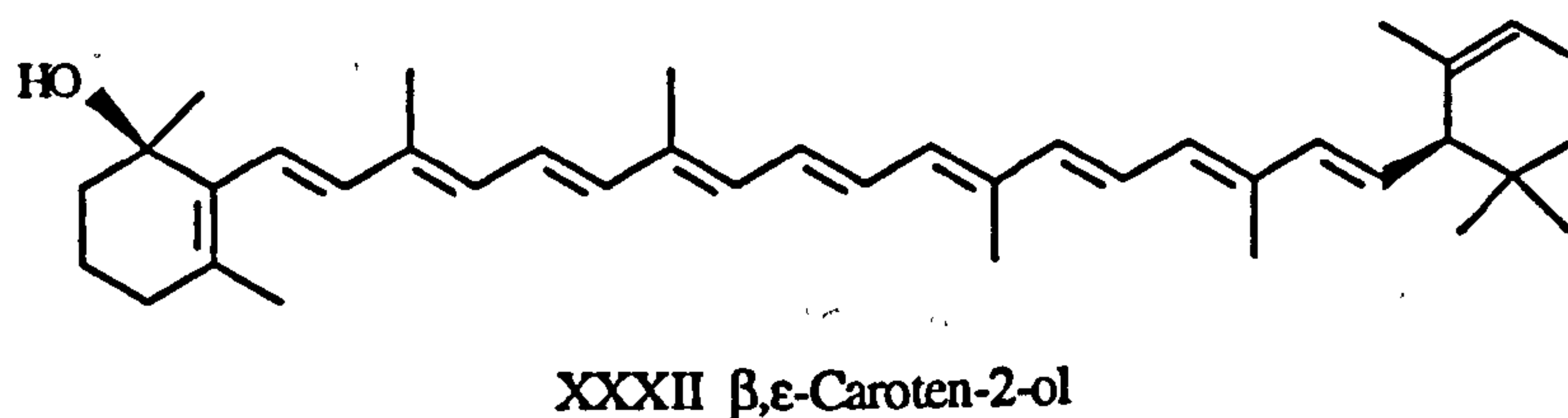
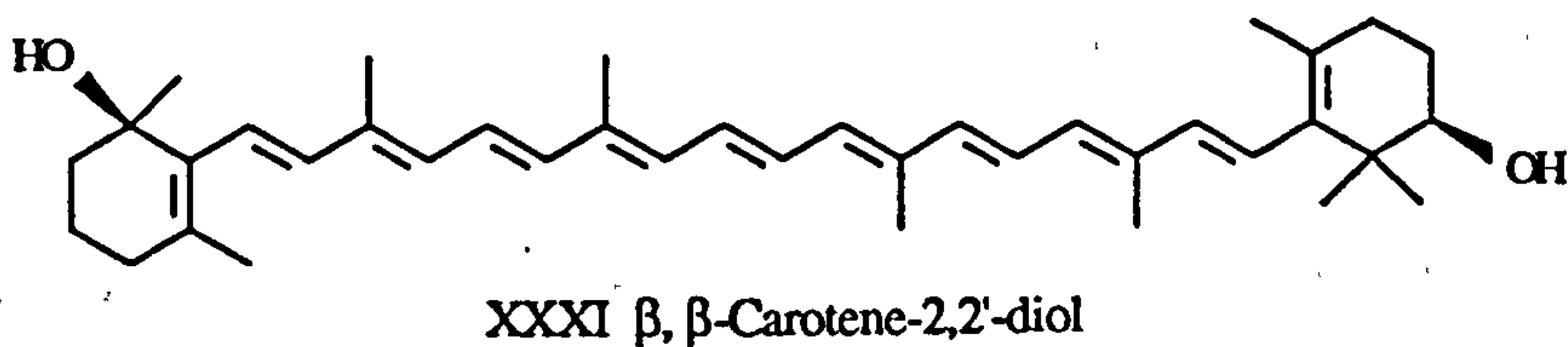
XXIII Loroxanthin

Siphonaxanthin (3,19,3'-trihydroxy-7,8-dihydro- $\beta,\epsilon$ -caroten-8-one, XXI) and siphonein, an esterified form of siphonaxanthin (XXI), has been observed in members of the Derbesidales, Codiales, Cauleropales, and to a lesser extent in Cladophorales and Siphonocladales (Bjørnland and Liaaen-Jensen, 1989). However, in some Dichtomosiphonales it is siphonein and not siphonaxanthin (XXI) which is found (Kleinig, 1969).





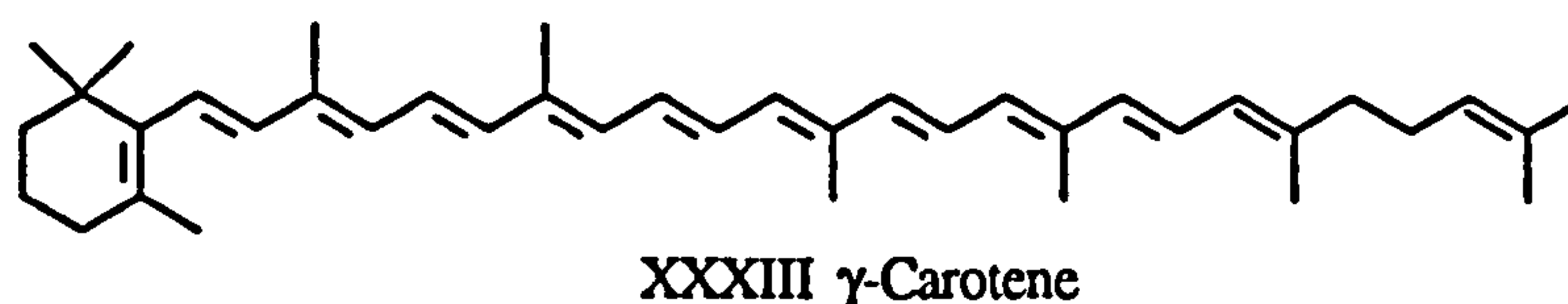
The green alga *Trentepohlia iolithus* contains  $\beta,\beta$ -carotene 2,2'-diol (XXXI) and  $\beta,\epsilon$ -caroten-2-ol (XXXII) (Nybraaten and Liaaen-Jensen, 1974). These were the first naturally occurring carotenoids to be discovered which are hydroxylated at position 2, such pigments have now been found in cyanobacteria but have opposite chirality at C-2 (*R*) from that in the *T. iolithus* pigments (2*S*). Some members of the Prasinophyceae contain siphonein (XXI) along with prasinoxanthin ((3'*R*,6'*R*)-3,6,3'-trihydroxy-7,8-dihydro- $\gamma$ , $\epsilon$ -caroten-8-one, XXX) (Liaaen-Jensen, 1985). Fritschiellaxanthin ((3*S*,3'*R*,6'*R*)-3,3'-dihydroxy- $\beta,\epsilon$ -caroten-4-one, XXVII) which is found in *Fritschiella tuberosa* is the 3'-epimer of  $\alpha$ -doradexanthin, a carotenoid which is found in many fish.



Under stress conditions, e.g. high light or nitrogen deficiency many algal species accumulate 'secondary carotenoids'. The function of these secondary carotenoids is not yet fully understood. However, some workers have suggested that they may be involved in photoprotection (Yong and Lee, 1991). Usually the secondary carotenoids which accumulate are ketocarotenoids. However, as already described in section 1.10 *Dunaliella salina* accumulates  $\beta$ -carotene (I) under conditions of high light, high temperature and high salinities (Ben-Amotz, 1987; Borowitzka *et al.*, 1990).

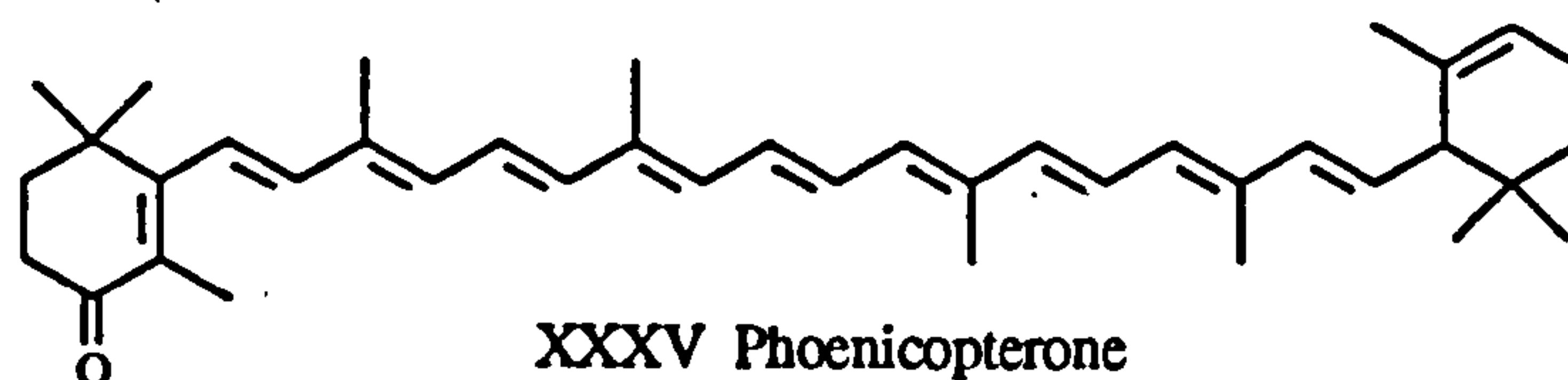
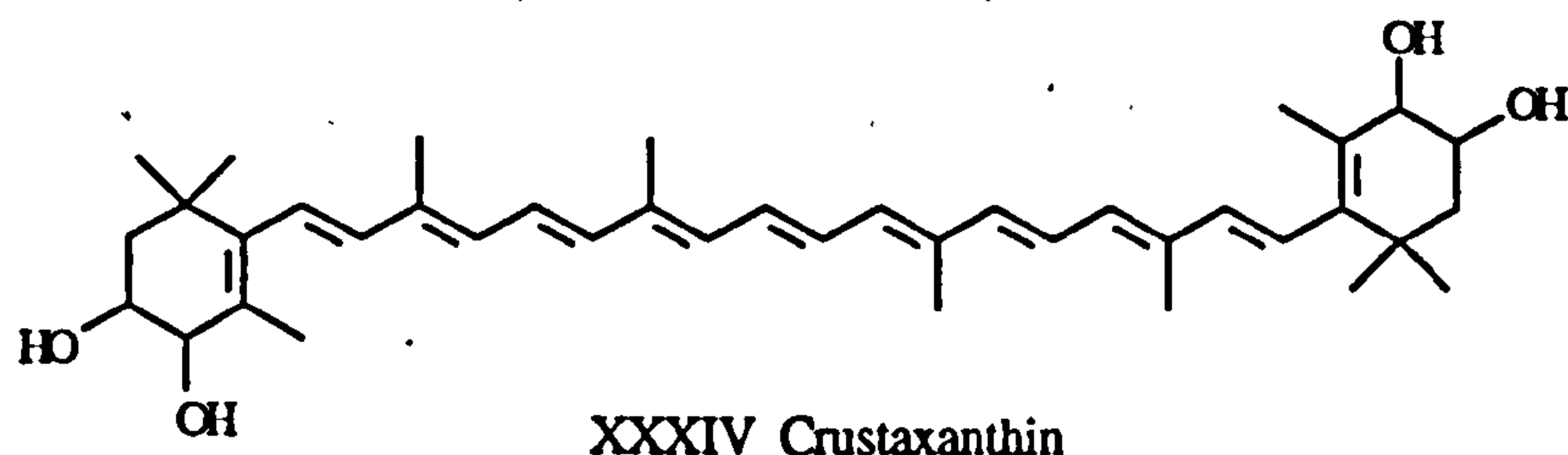
### 3.1.2 Localisation

As in higher plants the carotenoids of the Chlorophyta are usually located in the chloroplasts, although there can be differential distribution within the chloroplast. In the phototactic organism *Chlamydomonas reinhardtii* the eyespot is located in the chloroplast. The eyespots have been isolated by differential centrifugation and were found to contain  $\beta$ -carotene (I) (Ohad *et al.*, 1969). The eyespots of some algae such as *Euglena* contain keto-carotenoids (Heelis *et al.*, 1979). Carotenoids are found outside the chloroplast either in fruiting areas of certain colonial species or in the cytoplasm under certain favourable culture conditions. The gametes of the fruiting areas of *Ulva lobata* (Nybraaten and Liaaen-Jensen, 1974) and *Ulva lactuca* (Haxo and Clendenning, 1953) have a carotene content 4 - 6 times greater than in the non-fruiting areas, and this is mainly due to the specific synthesis of  $\gamma$ -carotene (XXXIII). This pigment and lycopene (XIII) accumulate in the antheridia of *Chara ceratophylla* and *Nitella syncarpa* (Karrer *et al.*, 1943).



The extra-plastidic pigments which are synthesised under adverse nutritional and/or environmental conditions are normally  $\beta$ -carotene (I) and its/or its ketonic derivatives, echinenone (II), canthaxanthin (IV), adonirubin (V), and astaxanthin (III), occasionally crustaxanthin (*trans*- $\beta$ , $\beta$ -carotene-3,4,3',4'-tetrol, XXXIV) and phoenicopterone ( $\beta$ , $\epsilon$ -

caroten-4-one, XXXV) are observed. The localisation of these carotenoids varies with species. They occur in intracytoplasmic deposits which have no limiting membrane in *Protosiphon botryoides* (Berkaloff, 1967), and in lipid vacuoles in *Ankistrodesmus braunii* (Lang, 1968).



In *H. pluvialis*, secondary carotenoids form in the ground substance of the cytoplasm and eventually fill the whole space among the lobes of the chloroplast, but are never observed within the chloroplast. During the accumulation of secondary carotenoids there may be a concomitant loss of plastid carotenoids (Czygan, 1970).

Various mutants of Chlorophyta show marked differences in carotenoid composition. A mutant of *Scenedesmus obliquus* C-6D lacks the ability to form  $\alpha$ - and  $\beta$ -carotene (VI and I) and xanthophylls in the dark. In the absence of light only precursors can be detected, i.e.  $\zeta$ -carotene (X), neurosporene (XII) and, lycopene (XIII). However, on transfer to light the levels of precursors decrease and carotenes and xanthophylls are synthesised (Humbeck, 1990).

### 3.1.3 Aims

The work in this chapter was designed to investigate the accumulation of secondary carotenoids in a range of species and strains of green algae. The first part of the investigation looked at 15 different species and strains to determine their secondary carotenoid composition. The algae were subjected to nutrient deprivation to enable high quantities of secondary carotenoids to be accumulated. The pigments were then extracted

and the carotenoid levels determined. This information was used to determine which species and strains were able to accumulate the highest concentration of secondary carotenoids. The carotenoid composition of the algae under favourable conditions (i.e. when green) was also determined so that the extent of secondary carotenoid synthesis could also be judged. The results of the screening experiment were used to identify the best algal species or strain, which could potentially be mass cultivated for the commercial production of secondary carotenoids.

The second part of the work in this chapter was designed to observe the accumulation of secondary carotenoids during nutrient deprivation. The ratio of secondary carotenoids, the decrease in chlorophyll levels, and the effect on plastid carotenoids was monitored. This work was designed to give an insight into any concomitant effects secondary carotenoid synthesis had on chlorophyll and plastidic pigments. In a second set of experiments this process was reversed by placing algal cells, which had accumulated high amounts of secondary carotenoids under nutrient deprivation into fresh media ('re-greening'). Again carotenoid and chlorophyll levels were monitored to observe the decrease in secondary carotenoids and the expected increase in chlorophyll and primary carotenoid levels.

## **3.2 MATERIALS AND METHODS**

### **3.2.1 Screening experiment**

Three 50 ml cultures of each algal species screened were cultivated in 250 ml Erlenmeyer flasks and maintained under normal growth conditions (see section 2.7.1). The cultures were subject to nitrogen deprivation to allow for the accumulation of secondary carotenoids within the cells. Each species/strain was cultivated for 16 weeks to allow for maximum secondary carotenoid accumulation. Green cells were obtained by sub-culturing some of the algal cultures into fresh media. The carotenoids were extracted from the algae in the normal manner and analysed using HPLC and UV/Vis spectrophotometry, to enable



quantitative and qualitative analysis of the pigments. Table 3.1 lists the algal species and strains used in the experiment and their source.

**Table 3.1** Algal species screened for secondary carotenoid production.

Species	Source
<i>Haematococcus pluvialis</i>	CCAP 34/6
<i>Haematococcus pluvialis</i>	CCAP 34/7
<i>Haematococcus pluvialis</i>	CCAP 34/8
<i>Haematococcus lacustris</i>	CCAP 34/11
<i>Haematococcus lacustris</i>	CCAP 34/1n
<i>Haematococcus lacustris</i>	CCAP 34/1k
<i>Haematococcus lacustris</i>	CCAP 34/1d
<i>Haematococcus lacustris</i>	CCAP 34/1f
<i>Oocystis minuta</i>	CCAP 257/5
<i>Oocystis minuta</i>	CCAP 257/6
<i>Gleomonas kupferri</i>	CCAP 33/1
<i>Muriellopsis sphaericum</i>	CCAP 256/1
<i>Monoraphidium contortum</i>	CCAP 245/1
<i>Bracteacoccus engadinensis</i>	CCAP 221/3
<i>Crucigenella rectangularis</i>	CCAP 218/2

### **3.2.2 Pigment levels during secondary carotenoid synthesis and re-greening of algal cells**

Three flasks of each algal species used in the experiment were cultured as in the screening experiment during nutrient deprivation to allow for the production of secondary carotenoids within the cells. When the cultures had accumulated high levels of secondary carotenoids they were transferred to fresh media to allow for the re-greening of the algal cells. Samples of algae were taken aseptically every seven days for pigment analysis. Samples were taken at shorter time periods during the re-greening experiment, since re-greening occurred in a relatively short time (10 - 14 days). The pigments were extracted in the normal manner and analysed using HPLC and UV/Vis spectrophotometry for quantitative and qualitative analysis of the pigments. The four species of algae used in the present experiment were selected on the basis of the relatively high levels of secondary carotenoids those species had accumulated in the previous experiment. Table 3.2 lists the algae used in this investigation and the source from where they were obtained.

**Table 3.2** Algal species used in secondary carotenoid synthesis and re-greening experiment.

Species	Source
<i>Haematococcus pluvialis</i>	CCAP 34/7
<i>Haematococcus lacustris</i>	CCAP 34/1n
<i>Oocystis minuta</i>	CCAP 257/6
<i>Crucigenella rectangularis</i>	CCAP 218/2

### 3.3 RESULTS

#### 3.3.1 Screening experiment

In the present investigation it was decided to compare and contrast the amount of the individual carotenoids accumulated as a percentage of the total pigment composition in the different algal species and strains screened. However, a variety of different parameters could have been used such as the carotenoid content of the cultures as a percentage of the dry cell weight of the cells, the absolute levels of carotenoids present in the cultures, or the amount of carotenoid per algal cell. The difficulty in choosing which parameter best represents the carotenoid content of the cultures arises because the amount of carotenoid in the cultures is dependant upon a number of factors which vary between different algal species and strains. These include the optimum growth conditions for each individual species and strain and the original algal inoculum used in the experiment. The value of each individual carotenoid as a percentage of the total pigment composition is a reliable indicator of the potential of each individual species and strain to accumulate a specific carotenoid/s. However, the best method of representing such data is unclear and many of the published results of similar experiments use a variety of parameters to express the data.

As it was not possible to identify the individual secondary carotenoids without the use of extensive analytical techniques including TLC and mass spectrometry, for rapid HPLC analysis the secondary carotenoids were grouped into ketocarotenoid mono- and diesters. Canthaxanthin (III), however, could be identified relatively easily using HPLC. The ketocarotenoid monoesters comprised of astaxanthin (III), astaxanthin (III) monoesters, echinenone (II), and adonirubin (V). The ketocarotenoid diesters comprised of astaxanthin (III) diesters only.

**Table 3.3** The pigment levels of the 15 species and strains of algae when the algae are grown under favourable conditions (S.E.  $\pm 4.7\%$ , n=3).

Species	Source	Neoxanthin		Violaxanthin		Lutein-5,6		Lutein		Canthaxanthin	
		$\mu\text{g/ml}$	%	$\mu\text{g/ml}$	%	$\mu\text{g/ml}$	%	$\mu\text{g/ml}$	%	$\mu\text{g/ml}$	%
<i>H. pluvialis</i>	34/6	0.43	2.5	0.52	3.0	0.13	0.7	1.76	10.1	0.00	0.0
<i>H. pluvialis</i>	34/7	0.25	2.3	0.28	2.5	0.10	0.1	1.04	9.4	0.00	0.0
<i>H. pluvialis</i>	34/8	0.38	2.7	0.43	3.1	0.28	2.0	1.53	11.0	0.00	0.0
<i>H. lacustris</i>	34/11	0.22	2.6	0.22	2.6	0.27	3.2	1.18	14.1	0.00	0.0
<i>H. lacustris</i>	34/1n	0.28	2.3	0.48	4.0	0.09	0.7	1.17	9.7	0.00	0.0
<i>H. lacustris</i>	34/1k	0.31	2.2	0.42	3.0	0.18	1.3	1.79	13.0	0.00	0.0
<i>H. lacustris</i>	34/1d	0.15	1.4	0.21	1.9	0.15	1.4	1.20	11.1	0.00	0.0
<i>H. lacustris</i>	34/1f	0.28	2.4	0.53	2.9	0.13	1.1	1.41	12.2	0.00	0.0
<i>O. minuta</i>	257/5	0.27	4.7	0.18	3.1	0.07	1.2	0.70	11.8	0.00	0.0
<i>O. minuta</i>	257/6	0.18	2.4	0.27	3.6	0.07	0.1	0.83	11.1	0.00	0.0
<i>G. kupfferi</i>	33/1	0.37	4.2	0.15	1.7	0.12	1.4	1.40	15.8	0.00	0.0
<i>M. sphaericum</i>	256/1	0.97	4.0	1.05	4.3	0.38	1.6	4.47	18.4	0.00	0.0
<i>M. contortum</i>	245/1	0.19	2.2	0.21	2.4	0.08	0.9	1.26	14.4	0.00	0.0
<i>B. engadenensis</i>	221/3	0.61	5.2	0.19	1.6	0.05	0.4	0.67	5.7	0.00	0.0
<i>C. rectangularis</i>	218/2	0.27	4.2	0.16	2.5	0.04	0.6	0.39	6.1	0.00	0.0

Species	Source	Chlorophyll a		Chlorophyll b		Mono-esters		Diesters		Beta-Carotene	
		$\mu\text{g/ml}$	%	$\mu\text{g/ml}$	%	$\mu\text{g/ml}$	%	$\mu\text{g/ml}$	%	$\mu\text{g/ml}$	%
<i>H. pluvialis</i>	34/6	9.20	52.8	4.90	28.1	0.00	0.0	0.00	0.0	0.22	2.9
<i>H. pluvialis</i>	34/7	5.60	50.8	3.50	31.7	0.00	0.0	0.00	0.0	0.26	2.4
<i>H. pluvialis</i>	34/8	6.68	48.0	3.96	28.5	0.00	0.0	0.00	0.0	0.65	4.7
<i>H. lacustris</i>	34/11	3.76	45.0	2.27	27.2	0.00	0.0	0.00	0.0	0.43	5.2
<i>H. lacustris</i>	34/1n	6.18	51.1	3.36	27.8	0.00	0.0	0.00	0.0	0.53	4.4
<i>H. lacustris</i>	34/1k	6.77	49.1	3.97	28.8	0.00	0.0	0.00	0.0	0.33	2.5
<i>H. lacustris</i>	34/1d	5.09	46.9	3.45	31.8	0.00	0.0	0.00	0.0	0.60	5.5
<i>H. lacustris</i>	34/1f	5.75	49.8	3.21	27.8	0.00	0.0	0.00	0.0	0.44	3.8
<i>O. minuta</i>	257/5	2.90	49.1	1.69	28.6	0.00	0.0	0.00	0.0	0.10	1.7
<i>O. minuta</i>	257/6	4.20	56.0	1.73	23.1	0.00	0.0	0.00	0.0	0.22	2.9
<i>G. kupfferi</i>	33/1	4.52	51.0	2.25	25.4	0.00	0.0	0.00	0.0	0.05	0.6
<i>M. sphaericum</i>	256/1	11.34	46.7	4.92	20.3	0.00	0.0	0.00	0.0	1.14	4.7
<i>M. contortum</i>	245/1	5.73	65.6	0.97	11.1	0.00	0.0	0.00	0.0	0.29	3.3
<i>B. engadenensis</i>	221/3	5.91	50.2	4.27	36.2	0.00	0.0	0.00	0.0	0.08	0.8
<i>C. rectangularis</i>	218/2	3.54	55.6	1.80	28.3	0.00	0.0	0.00	0.0	0.17	2.7



**Table 3.4** The pigment levels of the 15 algal species and strains when exposed to adverse nutritional conditions (S.E.  $\pm 5.2\%$ , n=3).

Species	Source	Neoxanthin		Violaxanthin		Lutein-5,6		Lutein		Canthaxanthin	
		$\mu\text{g/ml}$	%	$\mu\text{g/ml}$	%	$\mu\text{g/ml}$	%	$\mu\text{g/ml}$	%	$\mu\text{g/ml}$	%
<i>H. pluvialis</i>	34/6	0.00	0.0	0.00	0.0	0.00	0.0	0.15	0.1	2.13	1.7
<i>H. pluvialis</i>	34/7	0.00	0.0	0.00	0.0	0.00	0.0	0.14	0.1	1.38	0.9
<i>H. pluvialis</i>	34/8	0.00	0.0	0.00	0.0	0.18	0.3	0.04	0.1	1.03	1.5
<i>H. lacustris</i>	34/11	0.00	0.0	0.00	0.0	0.00	0.0	0.09	0.2	1.20	2.3
<i>H. lacustris</i>	34/1n	0.00	0.0	0.00	0.0	0.00	0.0	0.68	0.7	1.82	1.8
<i>H. lacustris</i>	34/1k	0.00	0.0	0.00	0.0	0.00	0.0	0.14	0.1	1.12	1.1
<i>H. lacustris</i>	34/1d	0.00	0.0	0.00	0.0	0.00	0.0	0.13	0.1	1.98	1.9
<i>H. lacustris</i>	34/1f	0.00	0.0	0.00	0.0	0.78	0.5	0.27	0.2	0.94	0.5
<i>O. minuta</i>	257/5	0.00	0.0	0.00	0.0	0.00	0.0	0.00	0.0	6.94	28.1
<i>O. minuta</i>	257/6	0.05	0.2	0.04	0.1	0.00	0.0	0.20	0.6	2.02	6.8
<i>G. kupfferi</i>	33/1	0.00	0.0	0.00	0.0	0.00	0.0	0.59	2.1	9.39	33.4
<i>M. sphaericum</i>	256/1	0.00	0.0	0.03	0.1	0.00	0.0	0.29	1.0	2.93	10.6
<i>M. contortum</i>	245/1	0.10	2.3	0.18	4.2	0.06	1.4	0.13	3.0	1.39	32.1
<i>B. engadenensis</i>	221/3	0.02	0.3	0.01	0.2	0.00	0.0	0.10	1.7	0.63	11.0
<i>C. rectangularis</i>	218/2	0.00	0.0	0.00	0.0	0.00	0.0	0.37	0.8	3.50	7.4

Species	Source	Chlorophyll a		Chlorophyll b		Mono-esters		Diesters		Beta-Carotene	
		$\mu\text{g/ml}$	%	$\mu\text{g/ml}$	%	$\mu\text{g/ml}$	%	$\mu\text{g/ml}$	%	$\mu\text{g/ml}$	%
<i>H. pluvialis</i>	34/6	2.44	2.0	3.77	3.0	83.06	66.9	30.81	24.8	1.85	1.5
<i>H. pluvialis</i>	34/7	8.10	5.1	5.77	3.6	93.11	58.6	47.13	29.8	3.06	1.9
<i>H. pluvialis</i>	34/8	2.14	3.1	1.89	2.7	45.34	64.8	17.10	24.2	2.26	3.2
<i>H. lacustris</i>	34/11	1.76	3.4	1.33	2.6	28.79	55.7	15.63	30.3	2.86	5.5
<i>H. lacustris</i>	34/1n	2.52	2.6	1.67	1.7	51.96	52.8	36.85	37.4	2.98	3.0
<i>H. lacustris</i>	34/1k	5.20	5.2	3.12	3.1	50.61	50.3	39.13	38.9	1.33	1.3
<i>H. lacustris</i>	34/1d	3.69	3.6	1.67	1.6	56.79	55.8	32.59	32.1	4.84	4.8
<i>H. lacustris</i>	34/1f	22.62	13.1	15.74	9.1	77.51	44.8	46.13	26.6	9.14	5.3
<i>O. minuta</i>	257/5	0.69	2.8	0.69	2.8	13.34	54.0	0.32	1.3	2.72	11.0
<i>O. minuta</i>	257/6	4.60	15.6	2.49	8.4	6.05	20.3	12.04	40.7	2.06	7.0
<i>G. kupfferi</i>	33/1	1.71	6.1	1.27	4.5	10.69	38.1	2.95	10.5	1.48	5.3
<i>M. sphaericum</i>	256/1	1.93	7.0	0.80	3.0	10.58	38.2	9.40	34.0	1.72	6.2
<i>M. contortum</i>	245/1	1.07	24.7	0.36	8.3	0.04	0.9	0.83	19.2	0.17	3.9
<i>B. engadenensis</i>	221/3	0.41	7.1	0.35	6.1	2.48	43.1	1.24	21.6	0.51	8.9
<i>C. rectangularis</i>	218/2	11.30	24.0	6.24	13.3	12.41	26.4	10.12	21.5	3.08	6.6

When the algae were grown under conditions favourable for growth (Table 3.3) the different species and strains contained similar pigment compositions at relatively similar levels, with only a few exceptions. The chlorophylls represented 70 - 83% of the pigments present, with chlorophyll *a* representing approximately two thirds of the chlorophyll present. Only *M. sphaericum* had slightly lower chlorophyll levels. The level of neoxanthin (XX) and violaxanthin (XVII) in each species and strain were very similar, except for the two *Oocystis* species, *G. kupfferi*, *C. rectangularis*, *M. sphaericum* and *B. engadinensis*. However, between species and strains the levels did fluctuate very



slightly, with *M. sphaericum* having relatively high levels (8.3%) and *H. lacustris* CCAP 34/1d having only 3.3% neoxanthin (XX) and violaxanthin (XVII) of its total pigment composition. The levels in the remaining species ranged from 4.8 - 7.7% of the total pigment composition.

The levels of lutein-5,6-epoxide (XVI) in the algae were generally very low with only *H. lacustris* CCAP 34/1 and *H. pluvialis* CCAP 34/8 having notable amounts present. The levels of lutein (XVI) in the *Haematococcus* and *Oocystis* species and strains were very similar, with levels ranging between 9.4 - 14.1%. *M. sphaericum*, *G. kupferri* and, *M. contortum* had slightly higher levels, while *C. rectangularis* and *B. engadinensis* had relatively low levels of lutein (XVI). The levels of  $\beta$ -carotene (I) fluctuated between the species and the strains, with levels ranging from 0.6 - 5.5%.

When the cells were grown under adverse nutritional conditions (Table 3.4), their pigment profiles changed considerably. Only *M. contortum* contained appreciable amounts of neoxanthin (XX), violaxanthin (XVII) and lutein-5,6-epoxide (XVI). The rest of the algal species and strains either did not contain any of these carotenoids or they were present at very low levels. The levels of lutein (XVI) were also very low. In all the *Haematococcus* and *Oocystis* species and strains and *C. rectangularis* the level was below 1.0%. The remainder of the algal species contained slightly higher levels with *M. contortum* containing the highest level of lutein (XVI) at 3.0%.

All the *Haematococcus* species and strains contained chlorophyll levels between 4.3 - 8.7%, except *H. lacustris* CCAP 34/1f which contained 22.2%. Again, approximately two thirds of the chlorophyll was present as chlorophyll *a*. The level of chlorophyll in the *Oocystis* strains varied greatly, with CCAP 257/6 having four times more chlorophyll than CCAP 257/5. *G. kupferri*, *M. sphaericum* and *B. engadinensis* all had similar levels of chlorophyll ranging between 10.0 - 13.2% of the total pigment composition, while *C. rectangularis* and *M. contortum* had much higher levels. The  $\beta$ -carotene (I) levels in the algal species and strains again varied considerably, with *H. lacustris* CCAP 34/1k having the lowest level and, *O. minuta* CCAP 257/5 having the highest level.

The levels of canthaxanthin (IV) in the *Haematococcus* species and strains were very similar. The *Oocystis* strains varied greatly with CCAP 257/5 having much higher levels than CCAP 257/6. *G. kupferri* and *M. contortum* had similar levels of

canthaxanthin (IV), as did *C. rectangularis*, *M. sphaericum* and, *B. engadinensis*. In all the species and strains screened no canthaxanthin mono- or diesters were detected.

The levels of monoesters and diesters of ketocarotenoids in the *H. pluvialis* strains were relatively constant. The level of monoesters of ketocarotenoids being particularly high in the *H. pluvialis* strains in relation to the other algal species. The levels of diesters of astaxanthin (III) were approximately half that of the monoesters of ketocarotenoids in the *H. pluvialis* strains. The levels of monoesters of ketocarotenoids in the *H. lacustris*, strains with the exception of CCAP 34/1f, were also relatively constant, with the levels of diesters being particularly high in relation to the other algal species. The ratio of monoesters and diesters were approximately 3:2 showing a high degree of esterification. *H. lacustris* CCAP 34/1f had lower levels of both monoesters and diesters.

There was a large variation in the levels of both monoesters and diesters of ketocarotenoid in the *O. minuta* strains. The strain CCAP 257/5 contained more than double the relative amount of ketocarotenoid than CCAP 257/6. However, CCAP 257/5 contained very low levels of diesters in contrast to CCAP 257/6 which contained relatively high levels of diesters of astaxanthin (III). The remaining species varied greatly in the levels of monoesters and diesters of ketocarotenoid accumulated. *M. sphaericum* accumulated the highest levels of monoesters and diesters of ketocarotenoids of the remaining species. *C. rectangularis* accumulated similar levels of monoesters and diesters. *M. sphaericum* and *G. kupferri* accumulated similar levels of monoesters of ketocarotenoid, but *M. sphaericum* accumulated more than three times more diesters of astaxanthin (III) than *G. kupferri*. *M. contortum* accumulated relatively low levels of diesters of astaxanthin (III), and only very small levels of monoesters of ketocarotenoid could be detected.

### 3.3.2 Pigment composition during secondary carotenoid synthesis and re-greening of algal cells

Table 3.5 Pigment composition of *H.pluvialis* 34/7 during secondary carotenoid synthesis (S.E.  $\pm 3.9\%$ , n=3).

Day	Neoxanthin		Violaxanthin		Lutein-5,6		Lutein		Canthaxanthin	
	$\mu\text{g/ml}$	%	$\mu\text{g/ml}$	%	$\mu\text{g/ml}$	%	$\mu\text{g/ml}$	%	$\mu\text{g/ml}$	%
0	0.06	1.1	0.06	1.1	0.02	0.4	0.47	8.7	0.00	0.0
7	0.31	1.8	0.26	1.5	0.16	0.9	1.80	10.4	0.02	0.1
14	0.22	1.3	0.18	1.0	0.00	0.0	1.61	9.3	0.31	1.8
21	0.21	0.6	0.17	0.5	0.07	0.2	0.99	3.0	0.09	0.3
28	0.22	0.7	0.22	0.7	0.10	0.3	1.37	4.4	0.15	0.5
35	0.20	0.7	0.18	0.6	0.06	0.2	1.02	3.6	0.16	0.6
42	0.21	0.6	0.20	0.5	0.08	0.2	1.15	3.1	0.31	0.8
49	0.18	0.5	0.16	0.4	0.05	0.1	0.94	2.4	0.39	1.0
56	0.19	0.4	0.08	0.2	0.21	0.4	1.14	2.4	0.58	1.2

Day	Chlorophyll a		Chlorophyll b		Monoesters		Diesters		Beta-Carotene	
	$\mu\text{g/ml}$	%	$\mu\text{g/ml}$	%	$\mu\text{g/ml}$	%	$\mu\text{g/ml}$	%	$\mu\text{g/ml}$	%
0	2.84	52.5	1.86	34.3	0.00	0.0	0.00	0.0	0.01	1.8
7	8.75	50.5	5.81	33.5	0.00	0.0	0.00	0.0	0.21	1.2
14	9.58	55.5	5.23	30.3	0.00	0.0	0.00	0.0	0.13	0.8
21	15.78	47.9	8.36	25.4	4.55	13.8	1.92	5.8	0.76	2.3
28	9.40	30.3	4.80	15.5	8.96	28.9	3.34	10.8	2.44	7.9
35	7.20	25.4	4.10	14.4	11.39	40.1	2.92	10.3	1.16	4.1
42	7.92	21.4	4.83	13.0	15.75	42.5	3.98	10.7	2.61	7.1
49	7.86	19.9	4.19	10.6	19.14	48.5	4.19	10.6	2.34	5.9
56	7.43	15.5	3.97	8.3	25.49	53.1	5.56	11.6	3.34	7.0

Fig. 3.1a

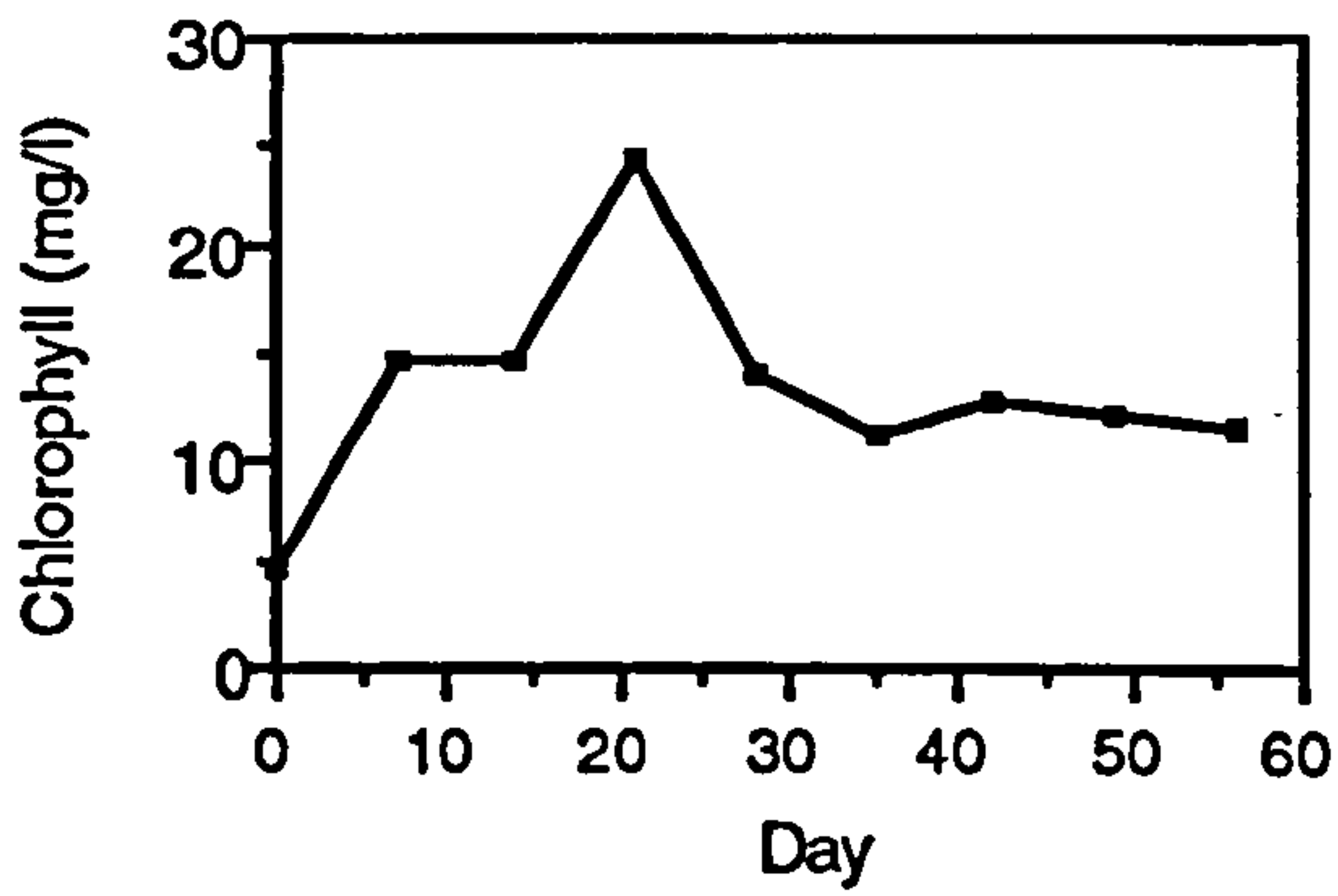


Fig. 3.1b

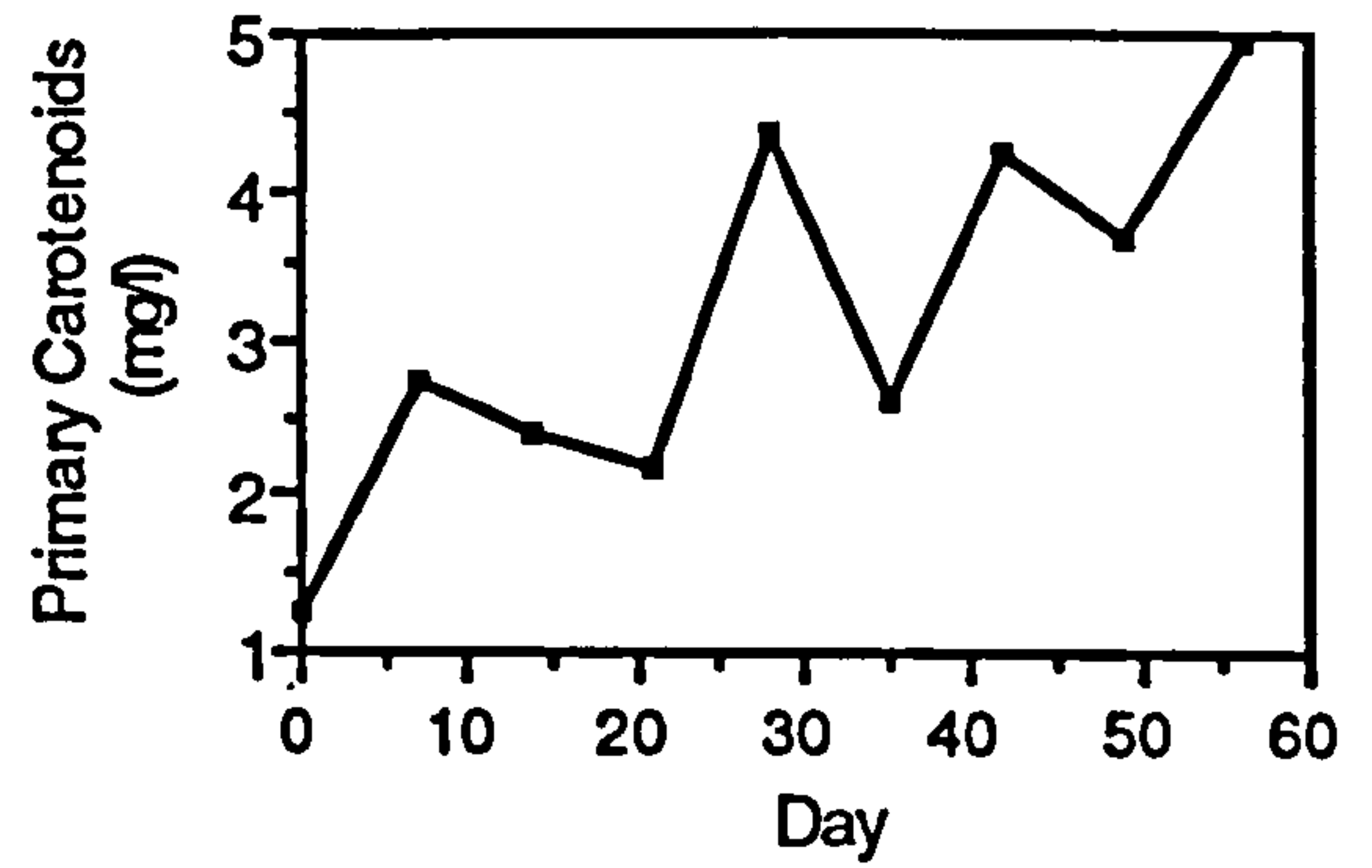


Fig. 3.1c

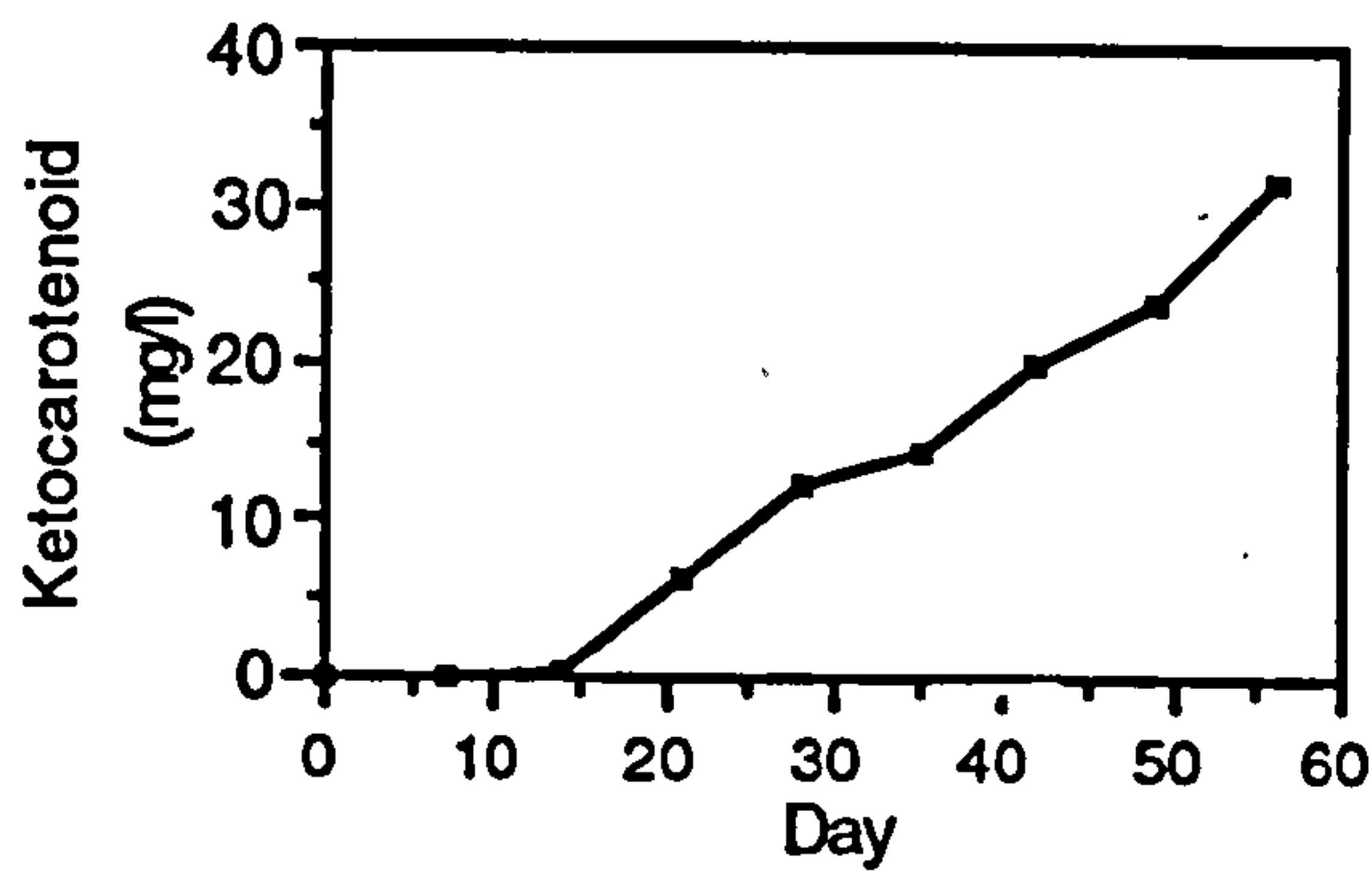


Fig. 3.1d

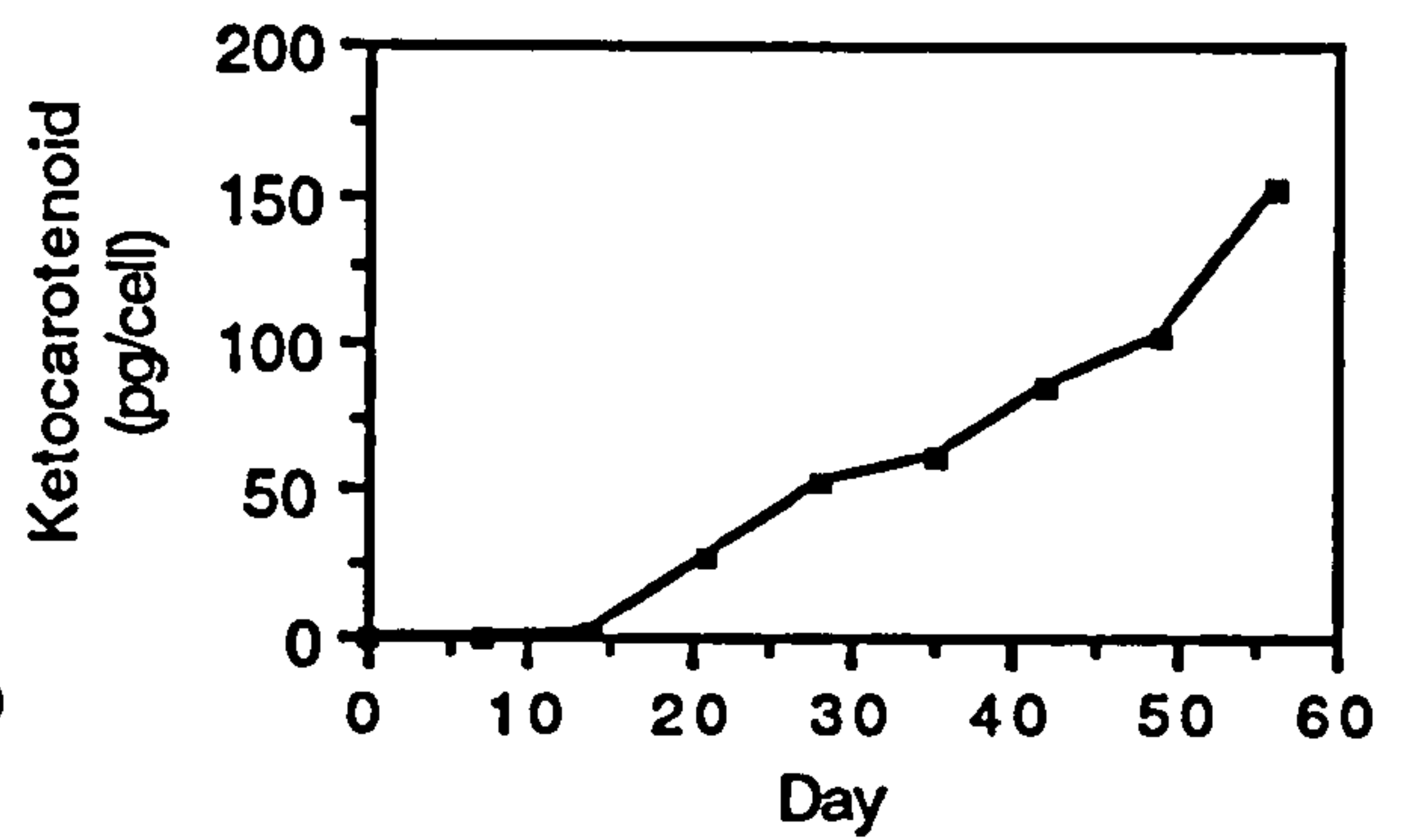
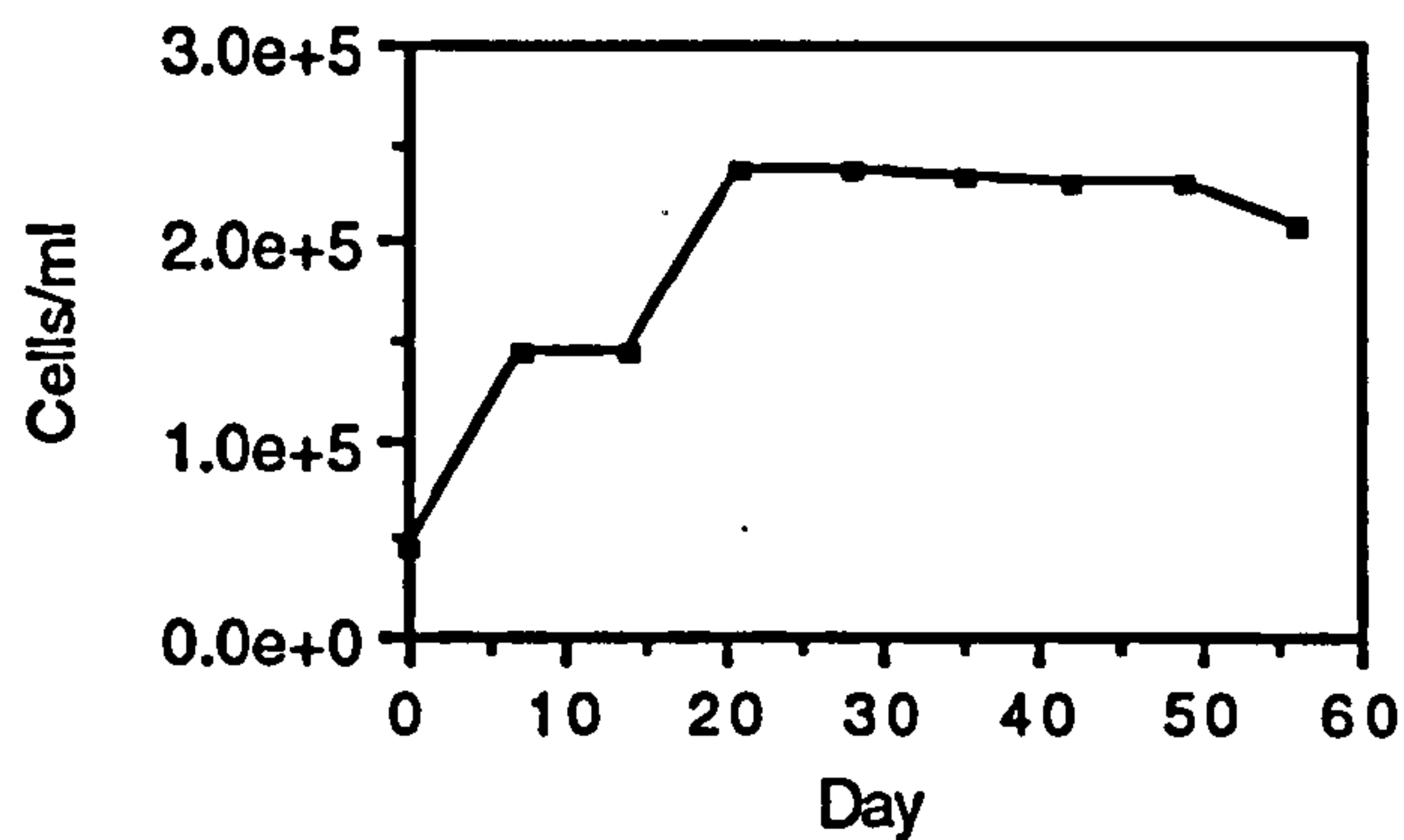


Fig. 3.1e



Figs. 3.1a-e Pigment levels of *H. pluvialis* 34/7 during secondary carotenoid synthesis (S.E.  $\pm 3.9\%$ , n=3).

The term 'secondary carotenoids' refers to carotenoids produced, usually in large quantities, under stress conditions such as nitrogen deficiency and high light intensity (Czygan, 1968b). These carotenoids are encountered outside the chromatophores. The term primary carotenoids (i.e. neoxanthin (XX), violaxanthin (XVII), lutein (XVI) and  $\beta$ -



carotene (I) is used to refer to the carotenoids produced during the exponential and linear growth phase. These carotenoids are located in the photosynthetic apparatus.

Table 3.5 and figures 3.1a-e show the accumulation of secondary carotenoids in *H. pluvialis*. This accumulation was induced in the algal cells by culturing the cells in nitrogen deficient BBM. The accumulation of secondary carotenoids started after 14 days and increased at a relatively constant rate throughout the experiment. The chlorophyll levels increased at a variable rate until day 21 after which the level of chlorophyll decreased rapidly. During the latter half of the experiment the chlorophyll level remained relatively constant. The number of cells increased at a variable rate until day 21, after which there was a steady decline in cell number. The level of primary carotenoids was extremely variable and fluctuated throughout the experiment. The ratio of monoesters to diesters of ketocarotenoid steadily increased during the experiment, the ratio on day 21 being 2.3:1 reaching 4.5:1 on day 56. The ratio of monoesters of ketocarotenoid to canthaxanthin (IV) remained relatively constant throughout the experiment at approximately 45:1, although canthaxanthin (IV) accumulation was apparent before that of the monoesters and diesters. The amounts of neoxanthin (XX) and violaxanthin (XVII) remained constant during secondary carotenoid synthesis, although their relative percentages decreased due to the accumulation of secondary carotenoids, as did the levels of lutein (XVI). The levels of  $\beta$ -carotene (I) increased steadily during the experiment although there was the odd fluctuation.

**Table 3.6** Pigment composition of *H. pluvialis* 34/7 during re-greening (S.E.  $\pm 4.5\%$ , n=3).

Day	Neoxanthin		Violaxanthin		Lutein-5,6		Lutein		Canthaxanthin	
	$\mu\text{g/ml}$	%	$\mu\text{g/ml}$	%	$\mu\text{g/ml}$	%	$\mu\text{g/ml}$	%	$\mu\text{g/ml}$	%
0	0.01	0.1	0.04	0.6	0.05	0.7	0.21	3.0	0.35	5.0
2	0.06	0.9	0.08	1.2	0.02	0.3	0.37	5.3	0.38	5.5
3	0.10	1.1	0.20	2.2	0.04	0.4	0.45	4.9	0.29	3.2
6	0.19	1.8	0.30	2.8	0.06	0.6	0.80	7.4	0.14	1.3
10	0.21	1.8	0.34	2.9	0.04	0.3	1.07	9.2	0.12	1.0
13	0.19	1.9	0.29	3.0	0.07	0.7	0.98	10.0	0.02	0.2

Day	Chlorophyll a		Chlorophyll b		Monoesters		Diesters		Beta-Carotene	
	$\mu\text{g/ml}$	%	$\mu\text{g/ml}$	%	$\mu\text{g/ml}$	%	$\mu\text{g/ml}$	%	$\mu\text{g/ml}$	%
0	0.21	3.0	0.18	2.6	3.66	52.6	1.56	22.4	0.69	9.9
2	0.36	5.2	0.27	3.9	3.32	47.8	1.42	20.4	0.67	9.6
3	1.91	20.9	1.19	13.1	3.01	33.0	1.48	16.2	0.45	4.9
6	3.92	36.3	2.21	20.4	1.96	18.1	0.54	5.0	0.69	6.4
10	4.89	42.1	2.43	20.9	1.13	9.7	0.57	4.9	0.83	7.1
13	4.95	50.4	3.20	32.6	0.04	0.4	0.02	0.2	0.06	0.6

Fig. 3.2a

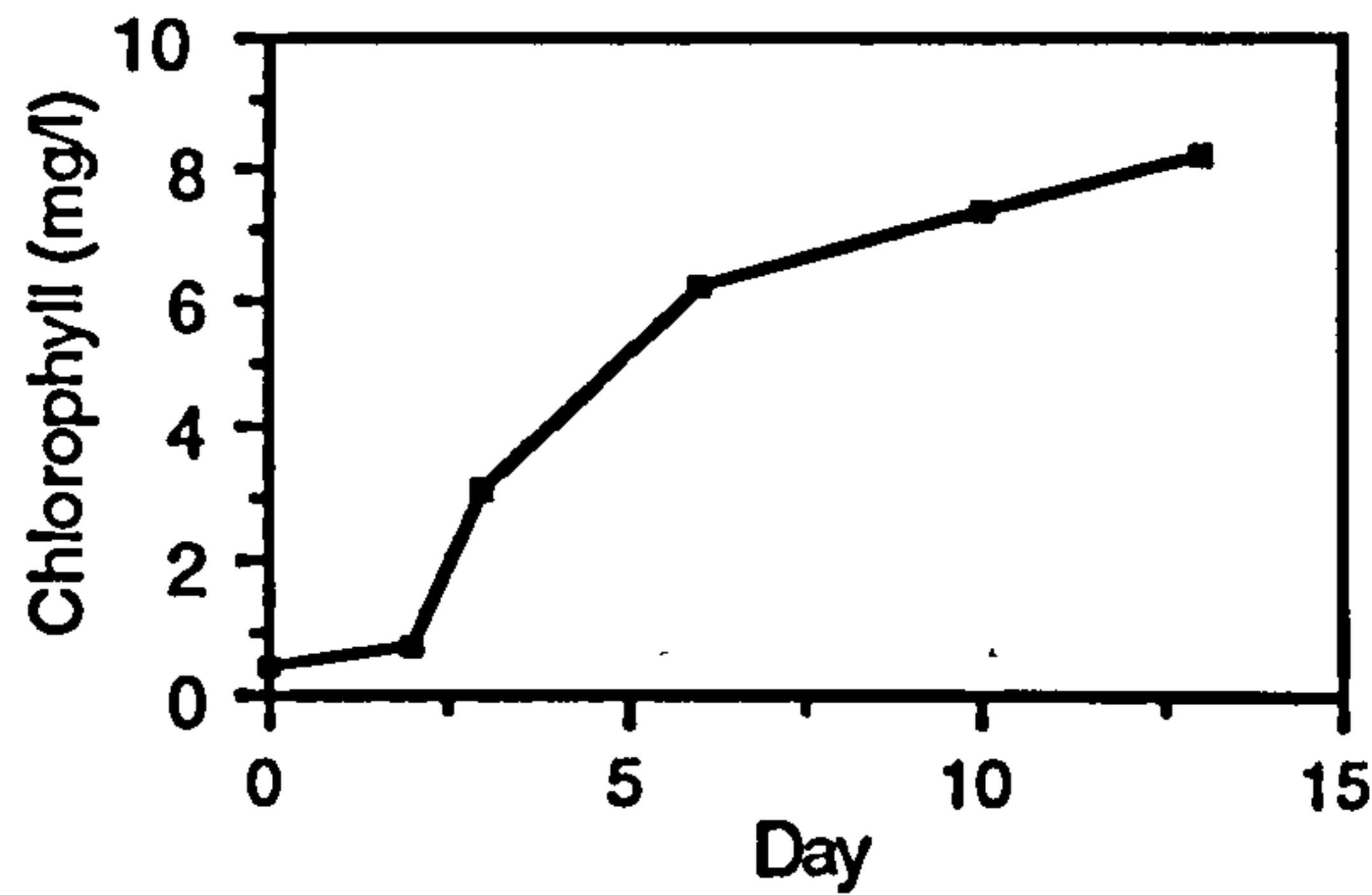


Fig. 3.2b

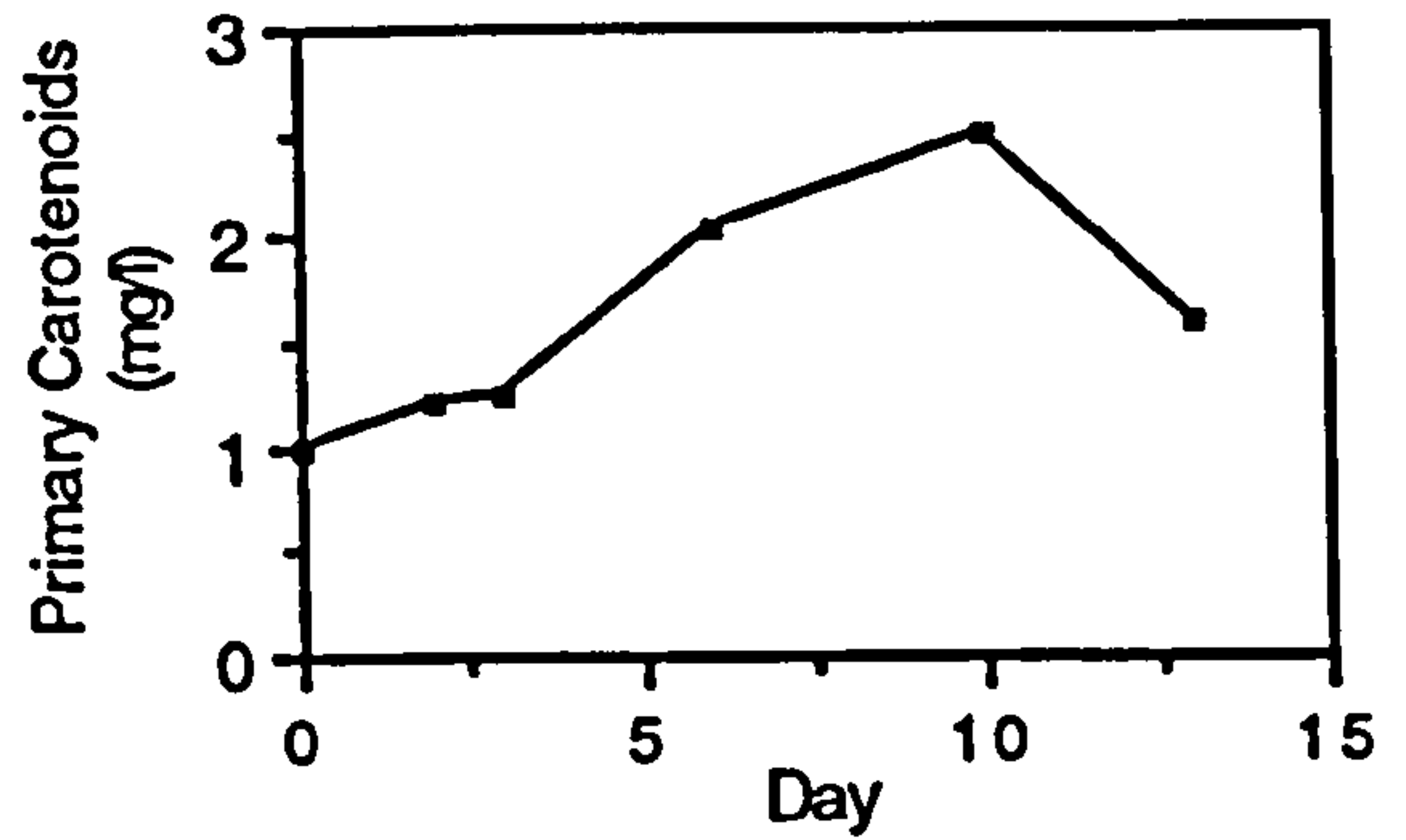


Fig. 3.2c

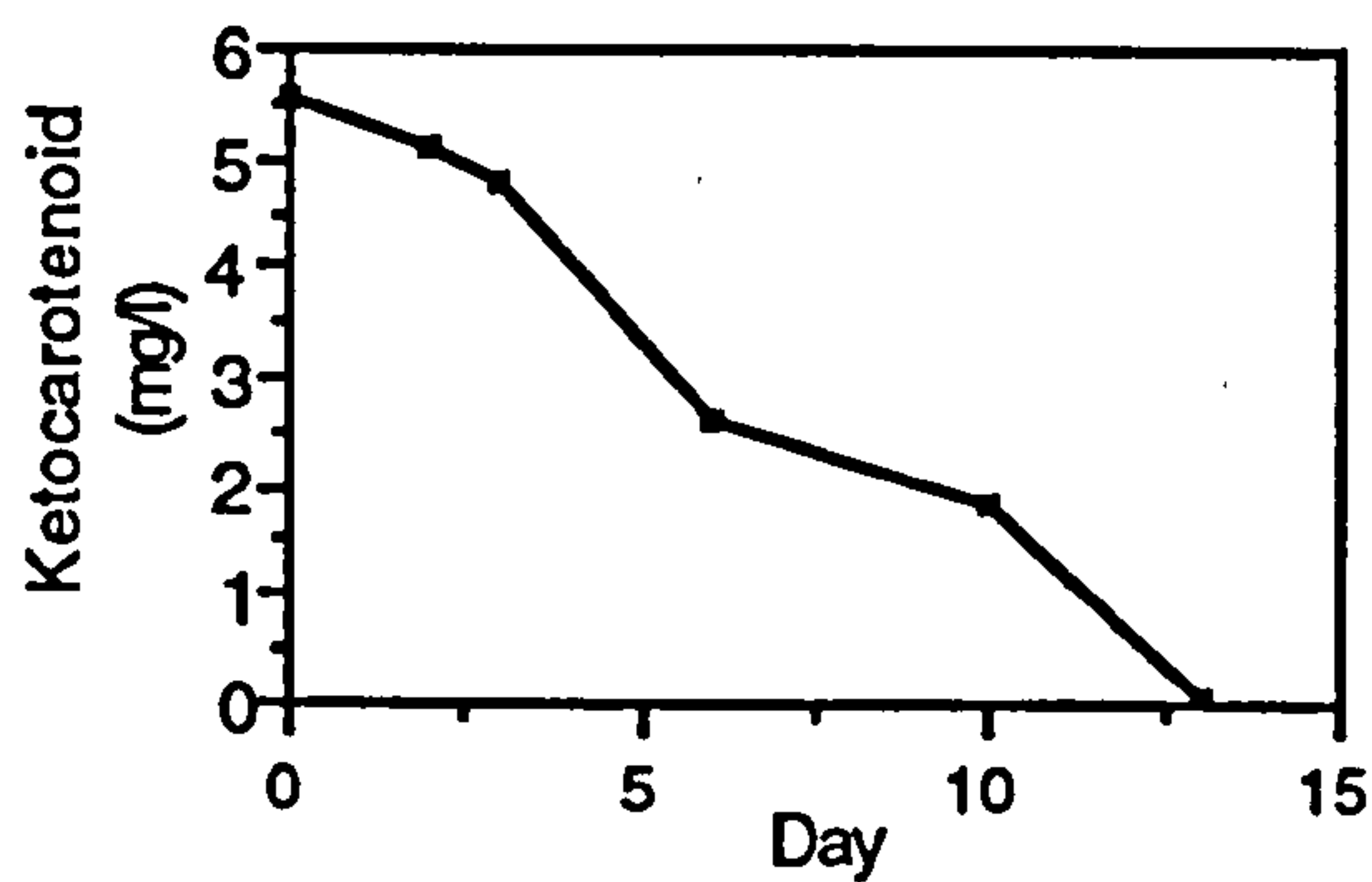


Fig. 3.2d

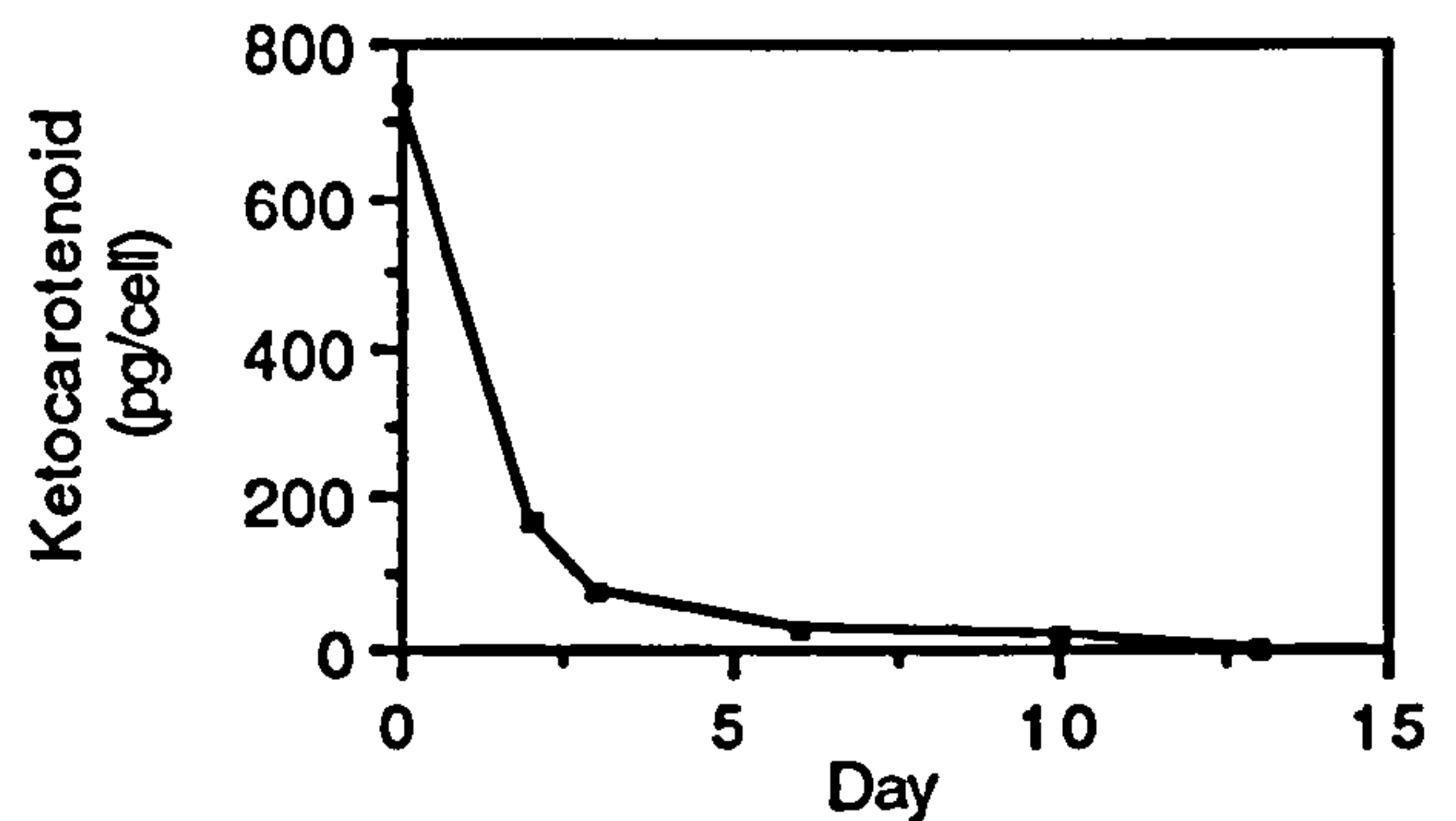


Fig. 3.2e

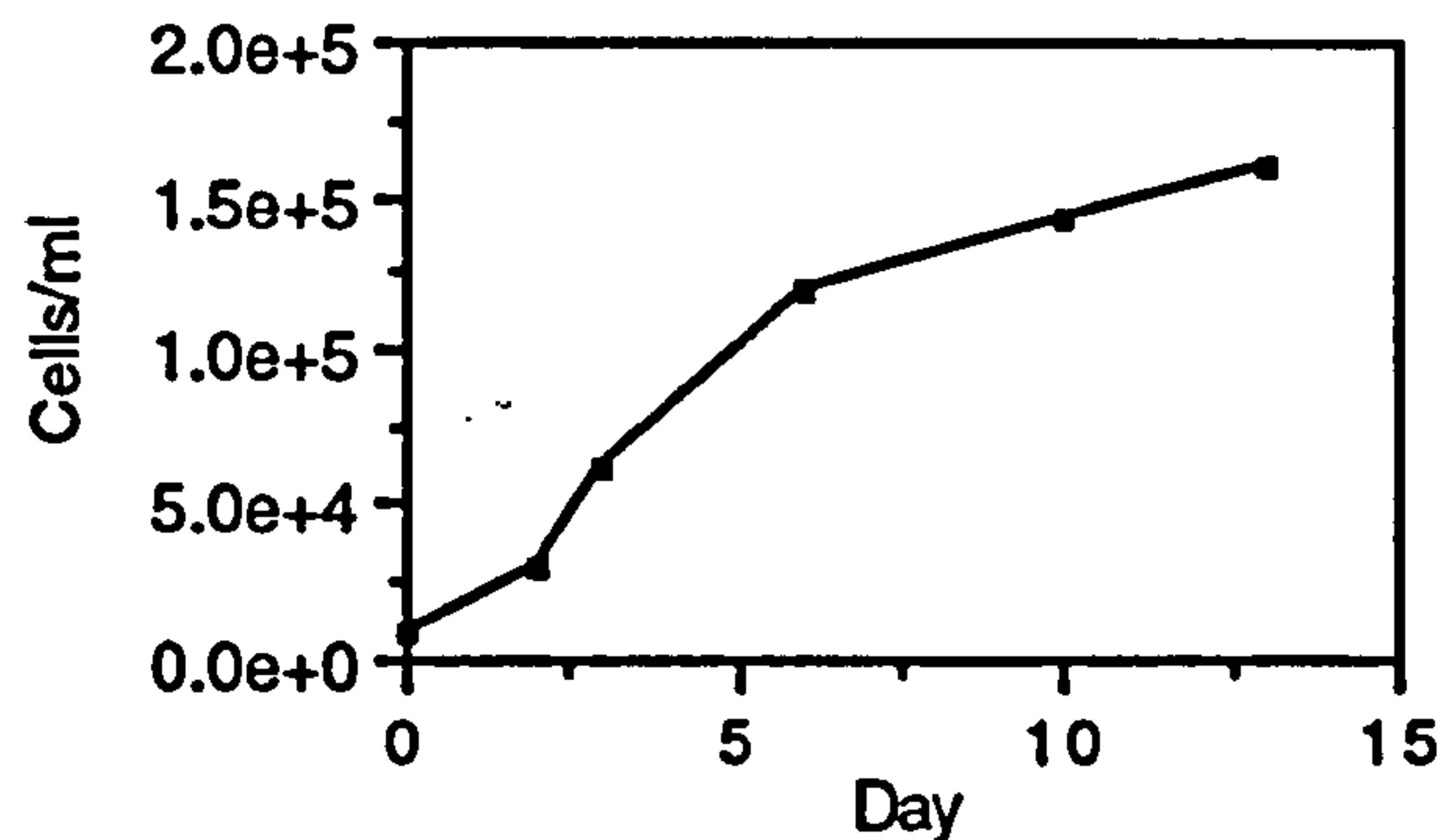
Figs. 3.2a-e Pigment levels of *H. pluvialis* 34/7 during re-greening (S.E.  $\pm 4.5\%$ ,  $n=3$ ).

Table 3.6 and figures 3.2a-e demonstrate the re-greening of *H. pluvialis*. The level of ketocarotenoid decreased steadily throughout the experiment, with the rate of decrease of ketocarotenoid per cell being very rapid at the start of the experiment. Chlorophyll levels and cell number steadily increased during the experiment although the rate decreased after day 6. The level of primary carotenoids steadily increased until day 10 after which the level declined. The ratio of monoesters to diesters of ketocarotenoid remained relatively constant

during the experiment at approximately 2.4:1. However, the ratio of monoesters of ketocarotenoid to canthaxanthin (IV) steadily decreased from 10:1 on day 0 to 2:1 on day 13. The levels of  $\beta$ -carotene (I) fluctuated slightly during the experiment, especially during the latter stages.

**Table 3.7** Pigment composition in *H. lacustris* 34/1n during secondary carotenoid synthesis (S.E.  $\pm 5.5\%$ , n=3).

Day	Neoxanthin		Violaxanthin		Lutein-5,6		Lutein		Canthaxanthin	
	$\mu\text{g/ml}$	%	$\mu\text{g/ml}$	%	$\mu\text{g/ml}$	%	$\mu\text{g/ml}$	%	$\mu\text{g/ml}$	%
0	0.03	1.8	0.04	2.3	0.01	0.6	0.15	8.8	0.00	0.0
7	0.12	2.5	0.13	2.7	0.03	0.6	0.59	12.1	0.00	0.0
14	0.16	1.3	0.14	1.2	0.12	1.0	1.16	9.6	0.00	0.0
21	0.31	1.5	0.15	0.7	0.21	1.0	2.39	11.2	0.00	0.0
28	0.42	2.2	0.32	1.7	0.19	1.0	2.24	11.8	0.00	0.0
35	0.46	2.5	0.45	2.5	0.16	0.9	2.03	11.2	0.03	0.2
42	0.32	1.6	0.27	1.4	0.13	0.7	2.22	11.2	0.12	0.6
49	0.38	1.5	0.32	1.3	0.14	0.6	2.64	10.6	0.24	1.0
56	0.34	1.3	0.32	1.2	0.14	0.5	2.41	8.9	0.45	1.7
63	0.36	1.1	0.28	0.9	0.26	0.8	2.52	7.8	0.63	2.0
70	0.26	0.8	0.18	0.6	0.21	0.7	1.84	5.9	0.75	2.4
77	0.25	0.7	0.20	0.5	0.23	0.6	1.97	5.0	0.87	2.3
84	0.22	0.5	0.19	0.4	0.14	0.3	1.78	4.1	1.05	2.4
91	0.14	0.3	0.14	0.3	0.06	0.1	1.21	2.8	1.28	3.0
98	0.14	0.3	0.16	0.3	0.06	0.1	1.45	2.6	1.23	2.2

Day	Chlorophyll a		Chlorophyll b		Monoesters		Diesters		Beta-Carotene	
	$\mu\text{g/ml}$	%	$\mu\text{g/ml}$	%	$\mu\text{g/ml}$	%	$\mu\text{g/ml}$	%	$\mu\text{g/ml}$	%
0	0.91	53.2	0.53	31.0	0.00	0.0	0.00	0.0	0.04	2.3
7	2.38	48.9	1.43	29.4	0.00	0.0	0.00	0.0	0.19	3.9
14	6.36	52.4	3.85	31.7	0.00	0.0	0.00	0.0	0.35	2.9
21	10.20	47.6	7.50	35.0	0.01	0.1	0.00	0.0	0.64	3.0
28	9.40	49.4	5.64	29.6	0.29	1.5	0.00	0.0	0.53	2.8
35	7.70	42.4	5.24	28.9	1.24	6.8	0.13	0.7	0.71	3.9
42	8.57	43.5	4.43	22.5	2.28	11.6	0.68	3.5	0.67	3.4
49	8.30	33.5	4.75	19.2	5.77	23.3	1.20	4.8	1.05	4.2
56	7.79	28.9	4.60	17.1	8.07	29.9	1.74	6.5	1.11	4.1
63	6.79	21.1	3.34	10.4	14.10	43.9	2.58	8.0	1.27	4.0
70	5.94	19.0	3.09	9.9	14.60	46.6	3.18	10.2	1.28	4.1
77	5.34	14.3	3.14	8.4	19.53	52.1	3.91	10.4	2.11	5.6
84	5.51	12.8	3.06	7.1	25.17	58.3	5.14	11.9	0.90	2.1
91	3.23	7.6	1.87	4.4	25.23	59.2	7.34	17.2	2.15	5.0
98	3.63	6.6	1.89	3.5	33.32	60.9	8.89	16.2	3.98	7.3



Fig. 3.3a

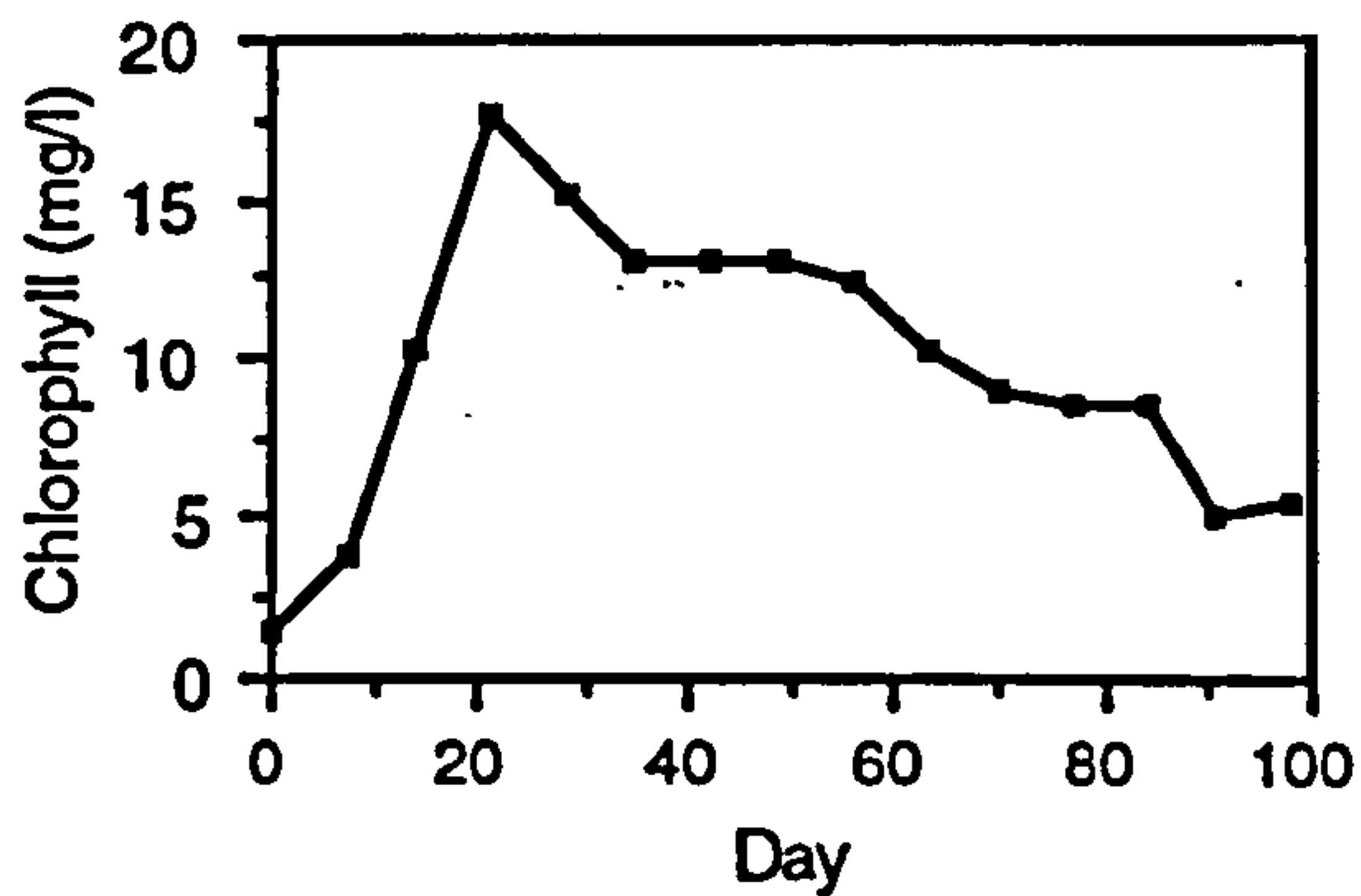


Fig. 3.3b

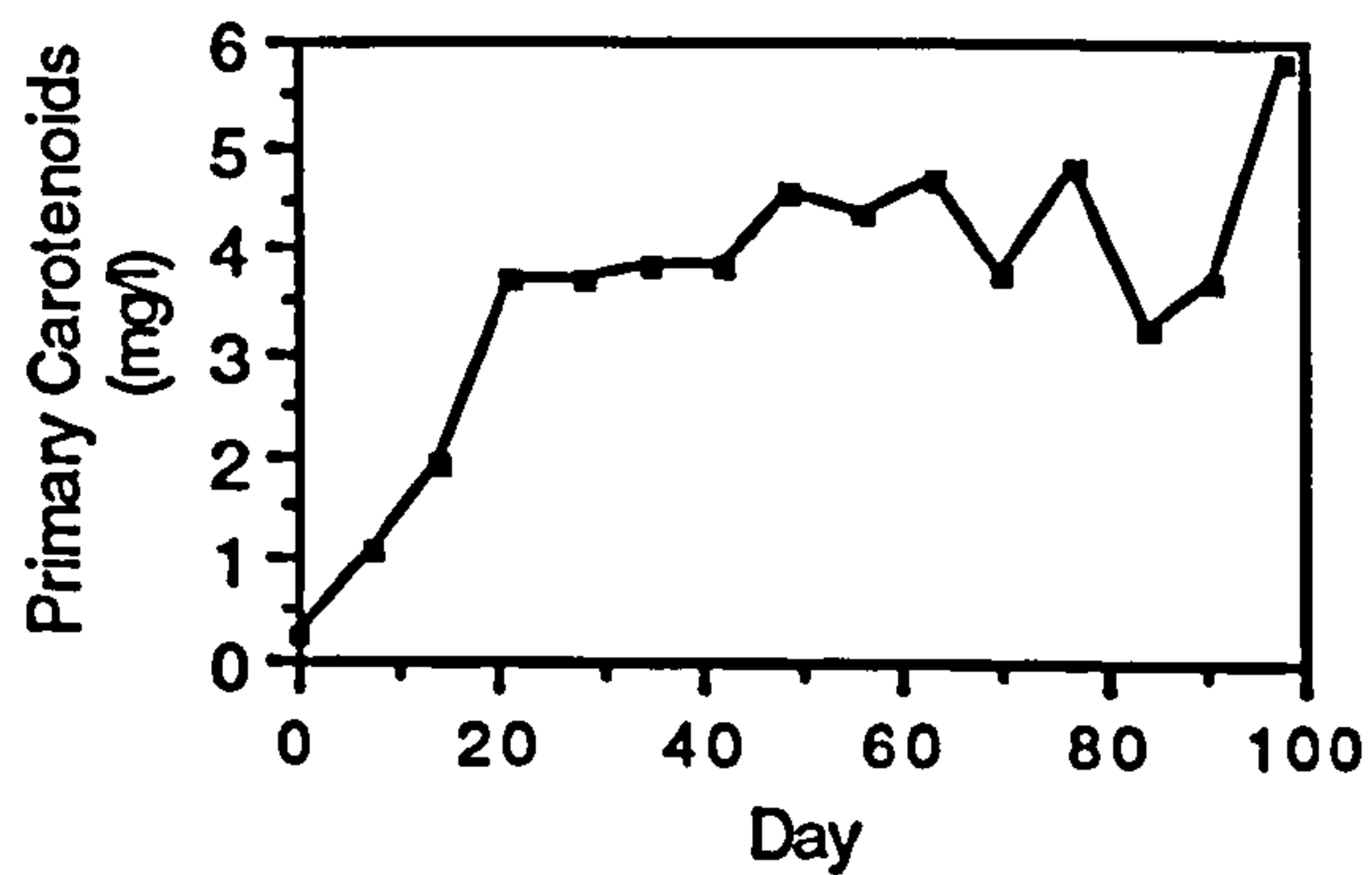


Fig. 3.3c

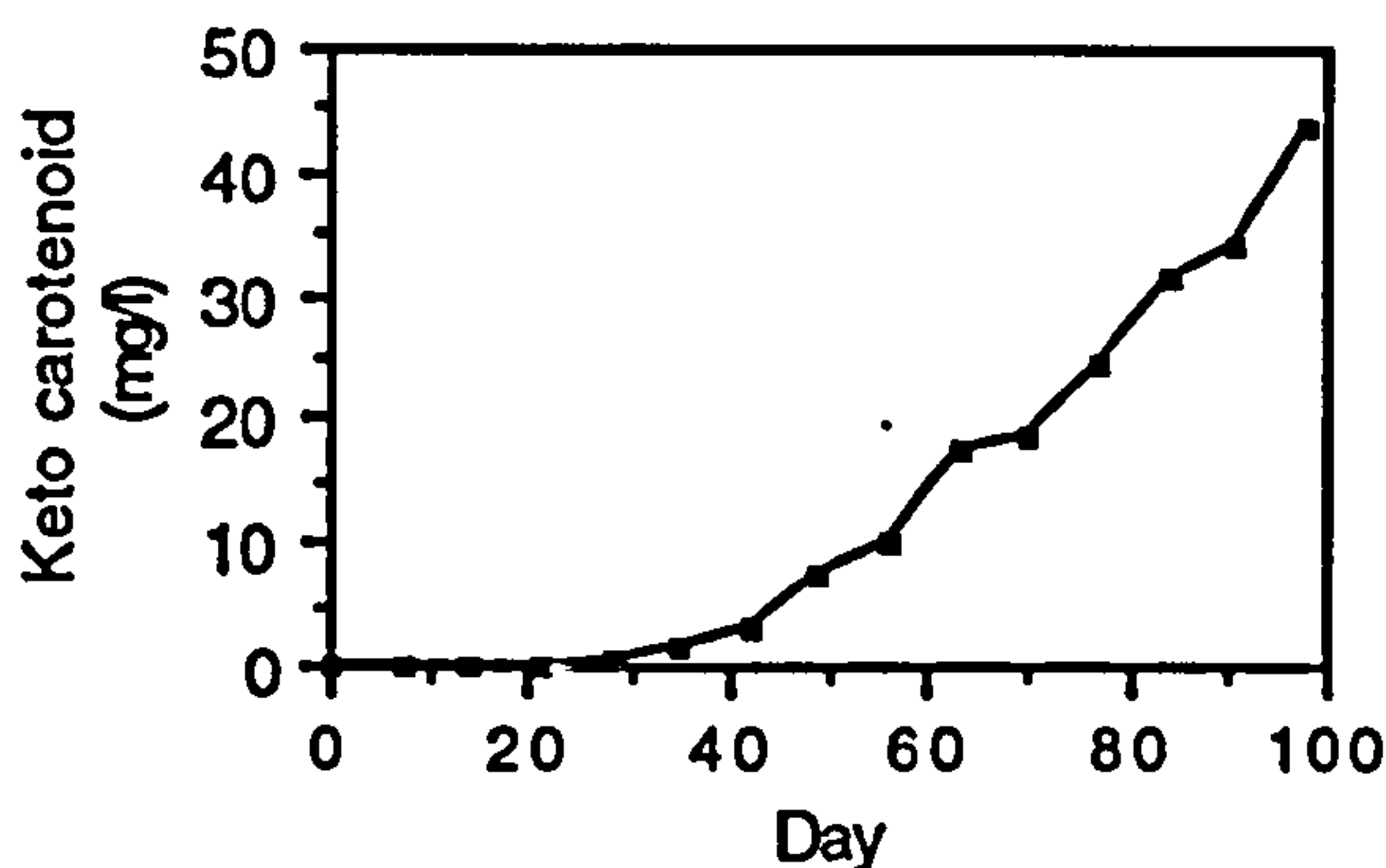


Fig. 3.3d

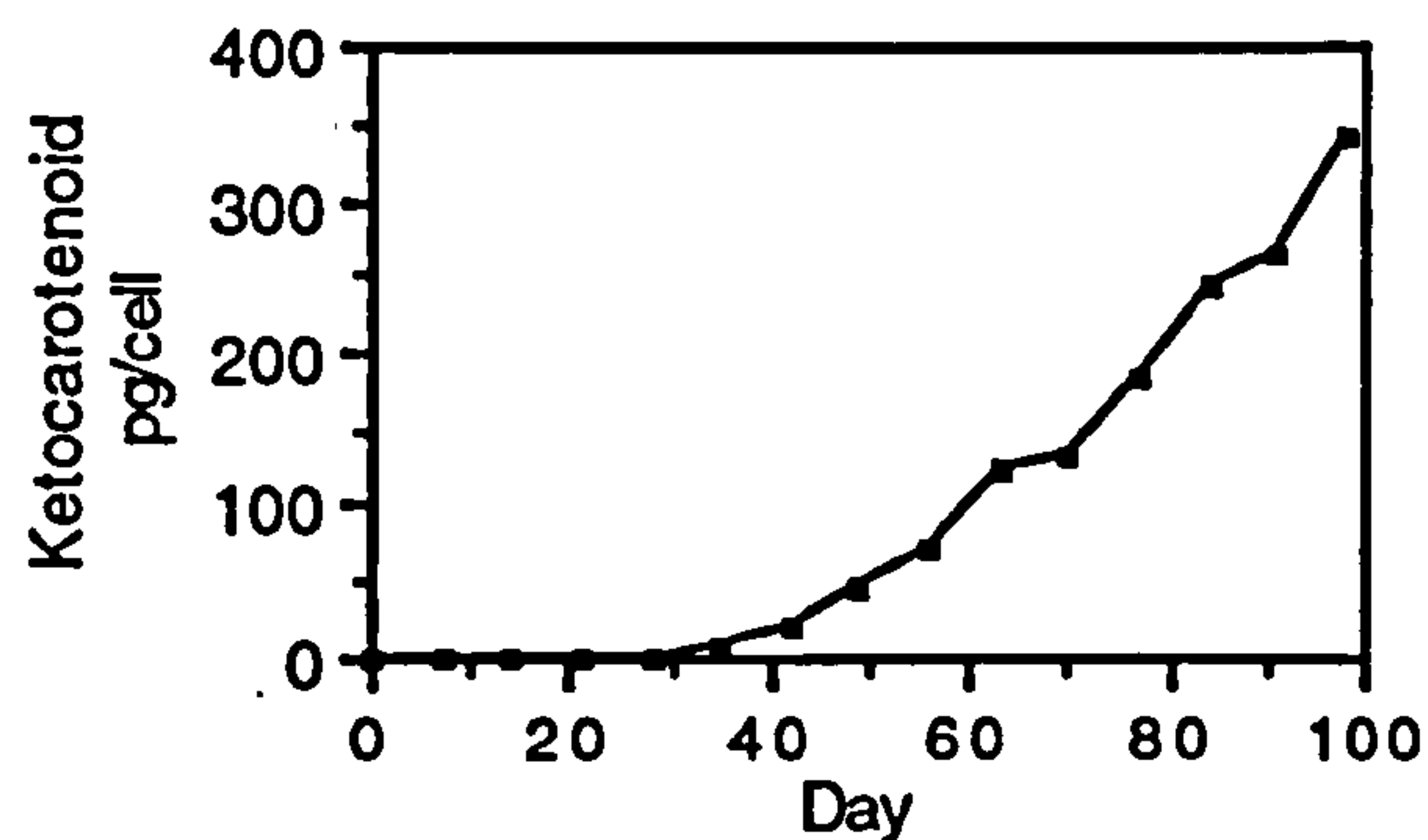
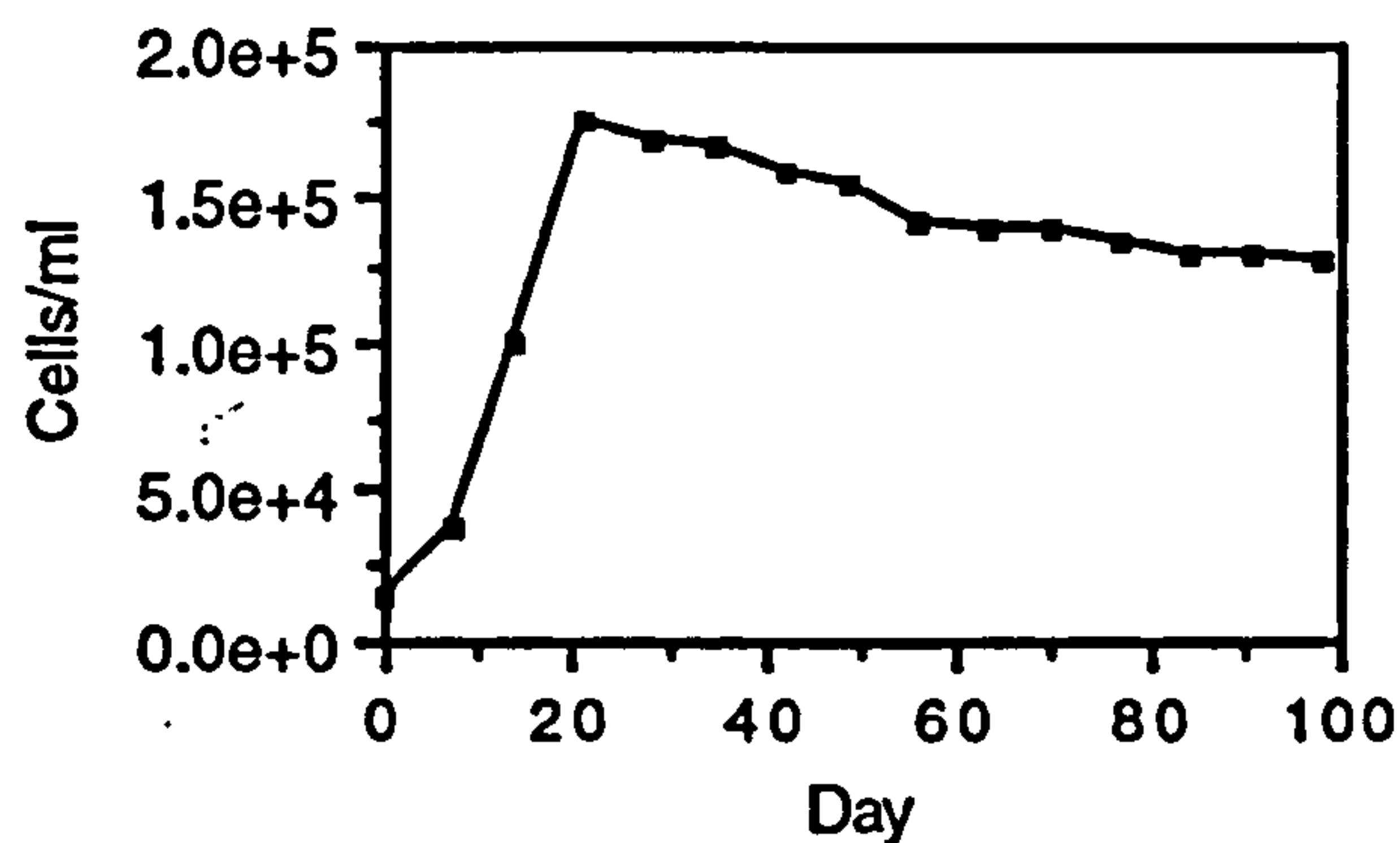


Fig. 3.3e



Figs 3.3a-e Pigment levels in *H. lacustris* 34/1n during secondary carotenoid synthesis (S.E.  $\pm 5.5\%$ , n=3).

Table 3.7 and figures 3.3a-e demonstrate the accumulation of secondary carotenoids in *H. lacustris*, when the cells were cultured in nitrogen deficient BBM. Chlorophyll and cell number reached a peak at day 21 and then steadily declined. Ketocarotenoid accumulation became noticeable on day 35 and increased at a constant rate throughout the experiment. The level of primary carotenoids increased rapidly until day 21

and then remained relatively constant until the latter stages of the experiment when the levels began to fluctuate. The ratio of monoesters to diesters of ketocarotenoid steadily increased until day 63 when the ratio was 5.5:1, after which the ratio began to decrease to a low of 3.7:1 on day 98. The ratio of monoesters of ketocarotenoid to canthaxanthin (IV) fluctuated constantly during the experiment however, after day 63 the ratio remained within the range 27-20:1. The levels of neoxanthin (XX), violaxanthin (XVII) and lutein (XVI) increased from the start and reached a maximum mid-way through the experiment. The levels then decreased at a constant rate during the second half of the experiment. The levels of  $\beta$ -carotene (I) increased during the experiment albeit at a very variable rate.

Table 3.8 Pigment composition in *H. lacustris* 34/1n during re-greening (S.E.  $\pm 3.8\%$ , n=3).

Day	Neoxanthin		Violaxanthin		Lutein-5,6		Lutein		Canthaxanthin	
	$\mu\text{g/ml}$	%	$\mu\text{g/ml}$	%	$\mu\text{g/ml}$	%	$\mu\text{g/ml}$	%	$\mu\text{g/ml}$	%
0	0.01	0.2	0.01	0.2	0.02	0.4	0.12	2.2	0.13	2.4
2	0.07	1.0	0.10	1.4	0.03	0.4	0.32	4.5	0.08	1.1
4	0.14	1.5	0.31	3.3	0.05	0.5	0.48	5.1	0.05	0.5
7	0.19	1.8	0.43	4.2	0.05	0.5	0.69	6.7	0.04	0.4
9	0.36	2.2	0.72	4.4	0.08	0.5	1.16	7.2	0.02	0.1
11	0.40	2.6	0.81	5.2	0.17	1.1	1.16	7.4	0.01	0.1
14	0.40	2.6	0.67	4.4	0.13	0.8	1.22	7.9	0.01	0.1

Day	Chlorophyll a		Chlorophyll b		Monoesters		Diesters		Beta-Carotene	
	$\mu\text{g/ml}$	%	$\mu\text{g/ml}$	%	$\mu\text{g/ml}$	%	$\mu\text{g/ml}$	%	$\mu\text{g/ml}$	%
0	0.45	8.4	0.30	5.6	3.69	68.6	0.48	8.9	0.17	3.2
2	1.63	23.0	0.66	9.3	3.38	47.6	0.56	7.9	0.27	3.8
4	3.29	35.2	1.63	17.5	2.61	27.9	0.44	4.7	0.34	3.6
7	4.01	38.7	2.19	21.1	2.00	19.3	0.37	3.6	0.40	3.9
9	7.17	44.2	3.69	22.8	1.90	11.7	0.36	2.5	0.70	4.3
11	6.40	40.8	4.52	28.8	1.20	7.7	0.20	1.3	0.80	5.1
14	6.64	43.1	4.56	29.6	0.86	5.6	0.08	0.5	0.82	5.3

Fig. 3.4a

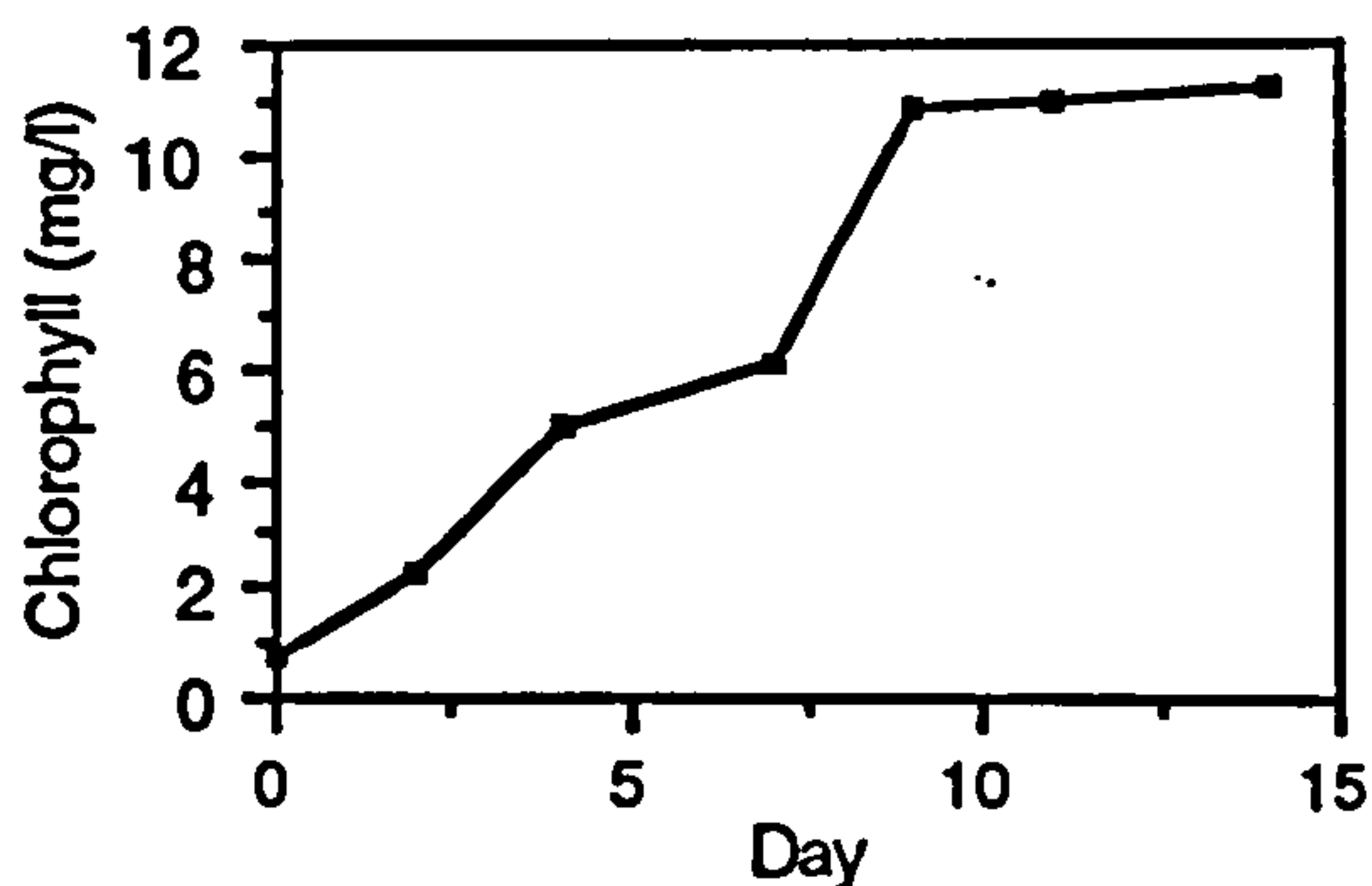


Fig. 3.4b

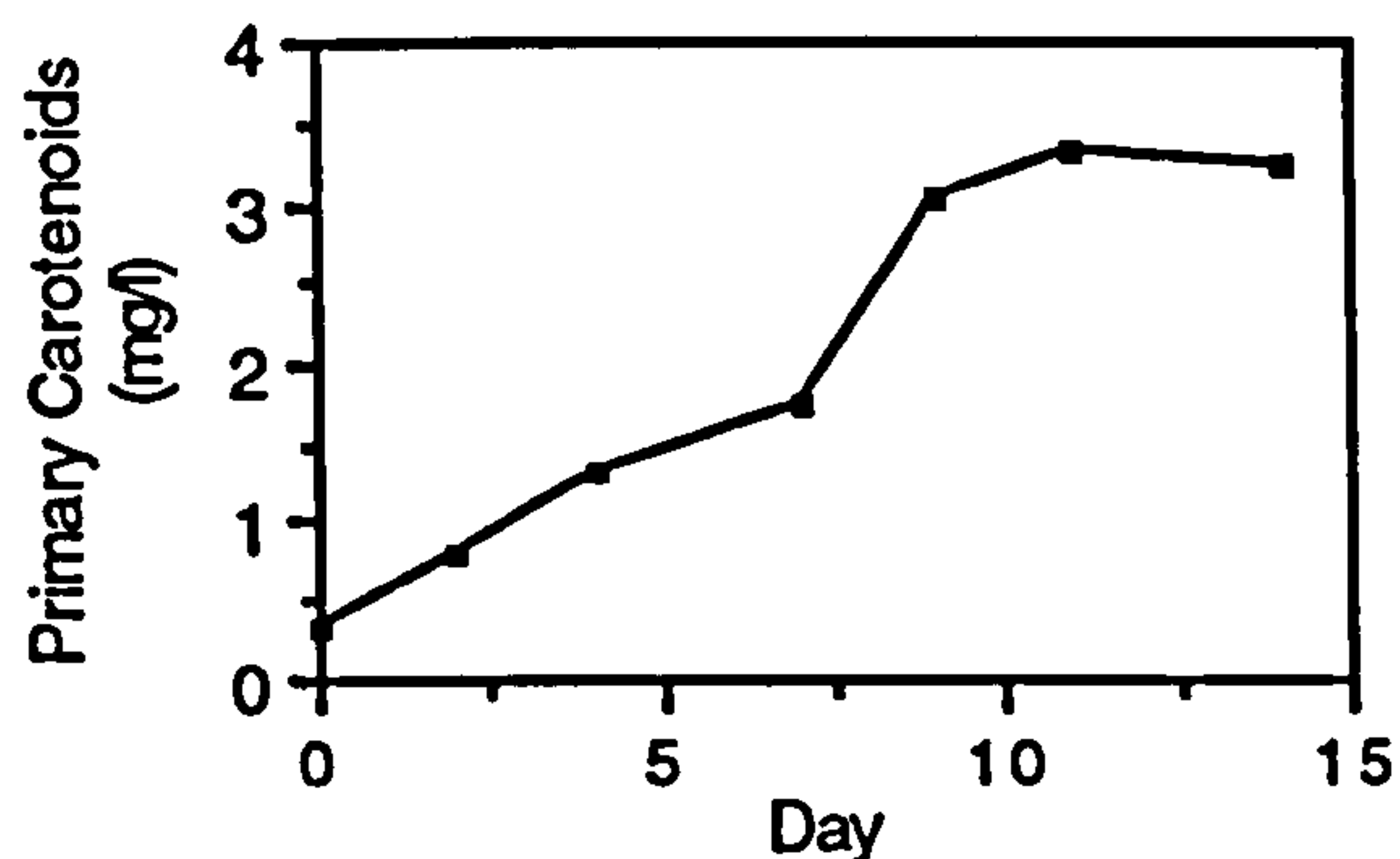


Fig. 3.4c

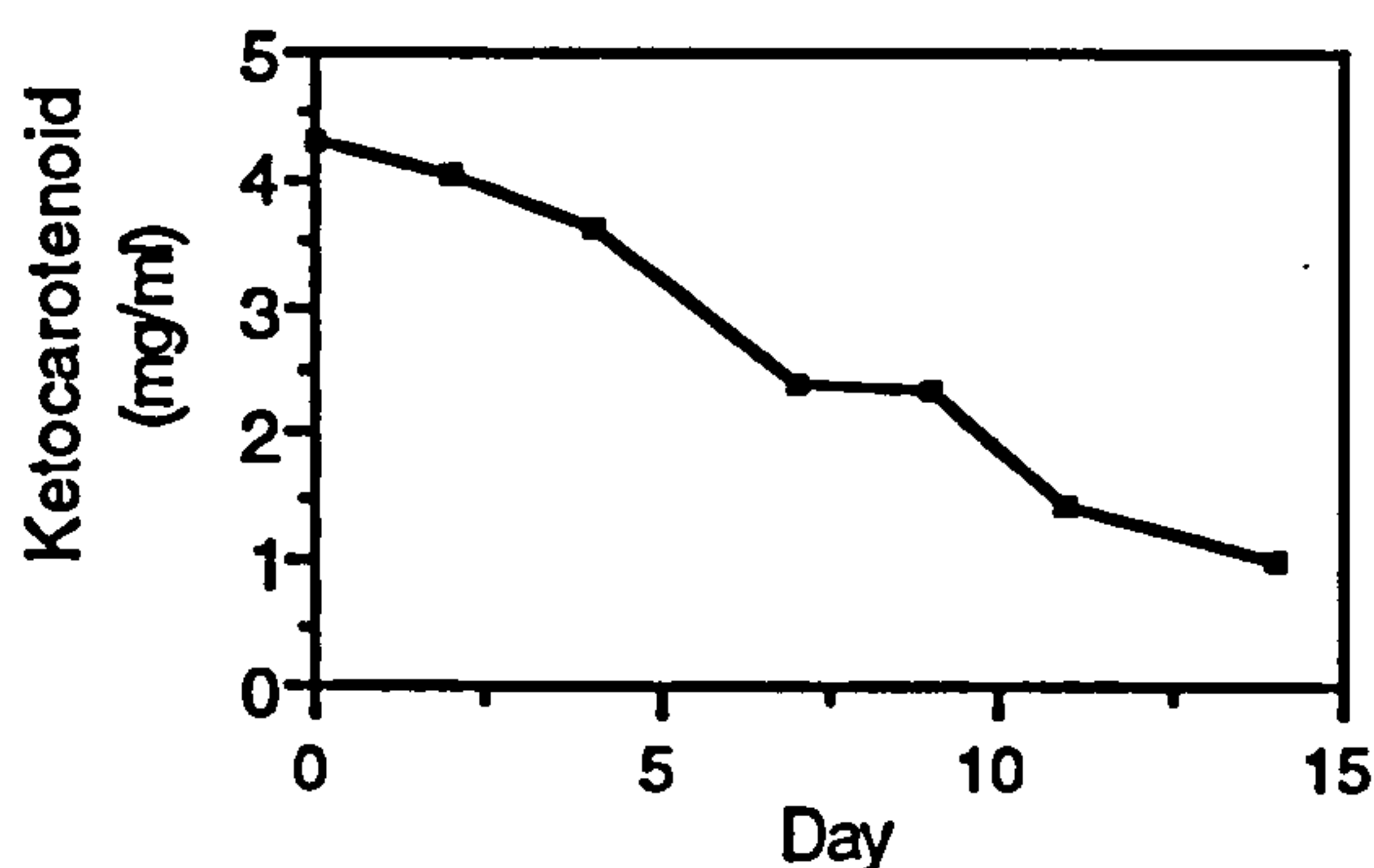


Fig. 3.4d

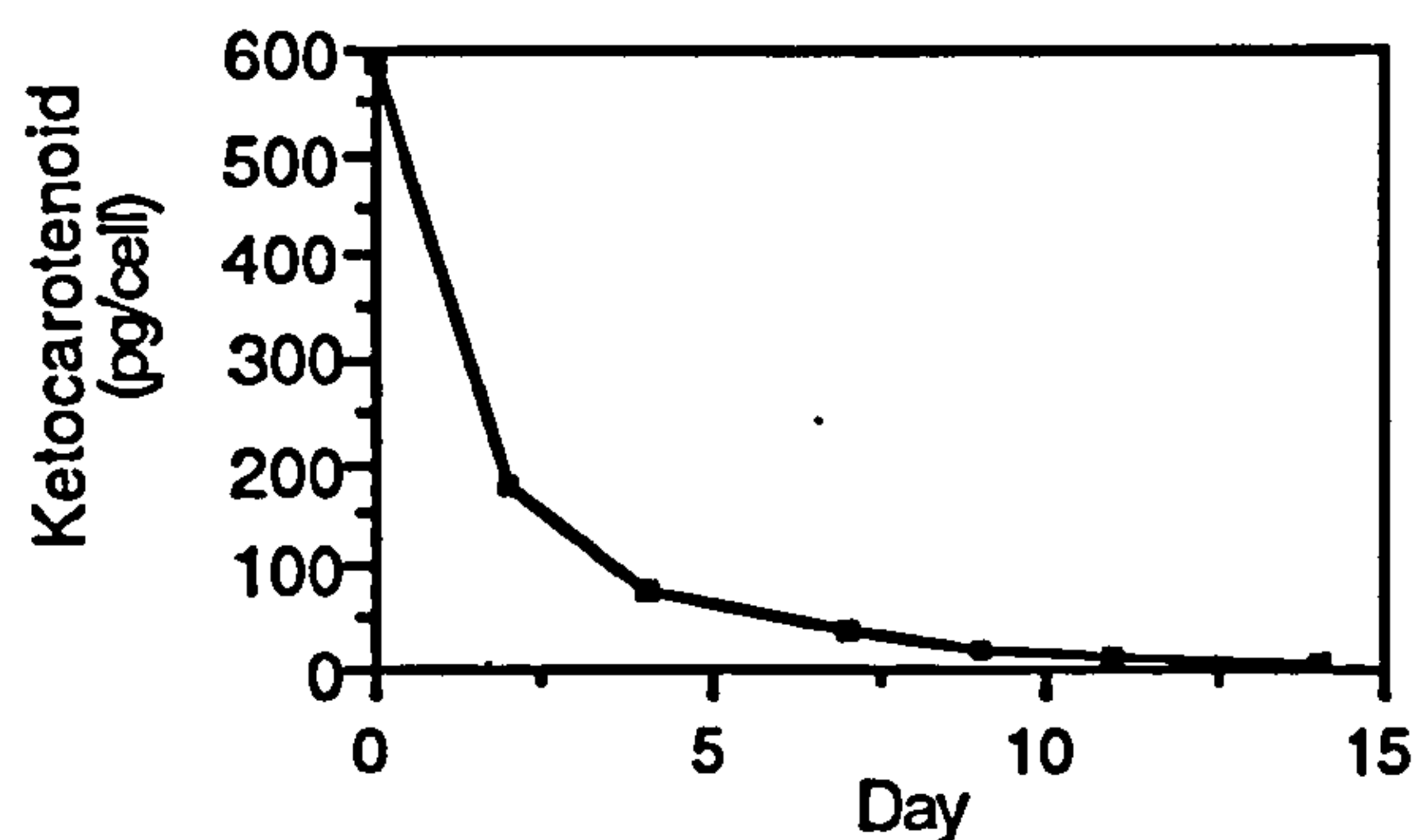
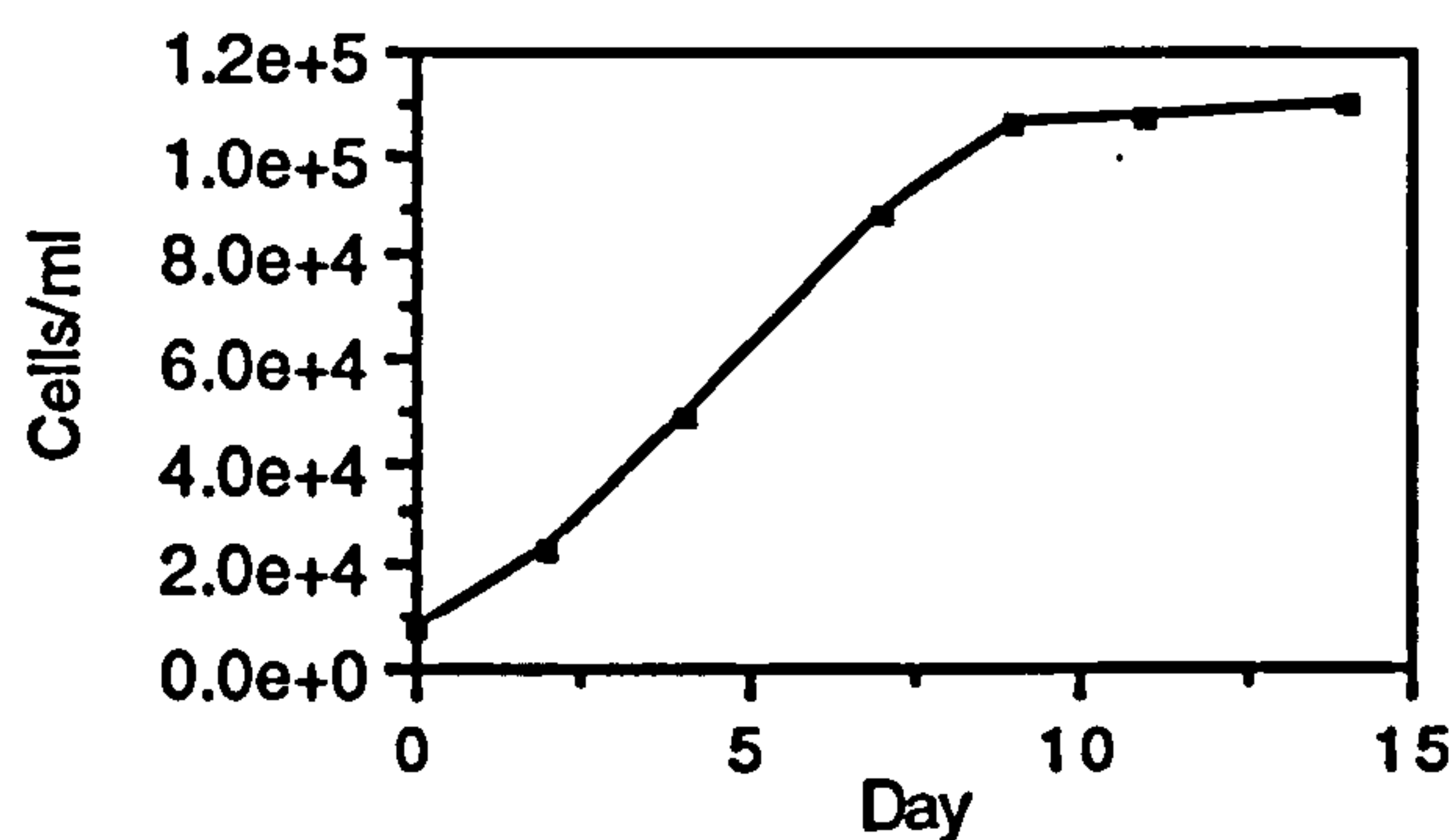


Fig. 3.4e



Figs 3.4a-e Pigment levels in *H. lacustris* 34/1n during re-greening (S.E.  $\pm 3.8\%$ , n=3).

Table 3.8 and figures 3.4a-e represents the re-greening of *H. lacustris* after encystment. The levels of chlorophyll and primary carotenoid steadily increased until day 9 after which the levels remained relatively constant. The number of cells increased rapidly upto day 9, after which the rate of increase receded. The levels of secondary carotenoids decreased throughout the experiment, especially the levels of ketocarotenoid per cell which decreased very rapidly between day 0 and day 4. The ratio of monoesters to diesters of

ketocarotenoid increased from ~8:1 on day 0 to 10:1 on day 14 during the experiment. The ratio of monoesters of ketocarotenoid to canthaxanthin (IV) also increased during the experiment from 28:1 on day 0 to 86:1 on day 14. The levels of  $\beta$ -carotene (I) showed a general increase during the course of the experiment.

**Table 3.9** Pigment composition of *O. minuta* 257/6 during secondary carotenoid synthesis (S.E.  $\pm 5.2\%$ , n=3).

Day	Neoxanthin		Violaxanthin		Lutein-5,6		Lutein		Canthaxanthin	
	$\mu\text{g/ml}$	%	$\mu\text{g/ml}$	%	$\mu\text{g/ml}$	%	$\mu\text{g/ml}$	%	$\mu\text{g/ml}$	%
0	0.03	1.6	0.04	2.2	0.03	1.6	0.14	7.7	0.03	1.6
7	0.20	2.1	0.21	2.3	0.07	0.8	0.71	7.6	0.02	0.2
14	0.42	3.4	0.41	3.3	0.10	0.8	1.34	10.8	0.04	0.3
21	0.33	2.2	0.19	1.3	0.13	0.9	1.62	10.9	0.03	0.2
28	0.55	2.8	0.70	3.5	0.12	0.6	2.07	10.4	0.07	0.4
35	0.42	2.1	0.35	1.8	0.15	0.8	1.90	9.6	0.04	0.2
42	0.83	3.1	0.58	2.2	0.25	0.9	3.87	14.5	0.54	2.0
49	0.65	2.1	0.55	1.8	0.17	0.6	3.00	9.8	0.75	2.4
56	0.69	1.9	0.66	1.8	0.22	0.6	3.08	8.4	1.19	3.3
63	0.71	1.6	0.57	1.2	0.54	1.2	3.33	7.3	2.08	4.6
70	0.56	1.1	0.44	0.9	0.72	1.4	2.65	5.3	2.79	5.6
77	0.39	0.9	0.46	1.0	0.68	1.5	1.75	3.9	3.18	7.1
84	0.28	0.6	0.47	1.1	0.10	0.2	1.82	4.2	3.99	9.1
91	0.19	0.4	0.20	0.4	0.10	0.2	1.50	3.2	3.71	7.9

Day	Chlorophyll a		Chlorophyll b		Monoesters		Diesters		Beta-Carotene	
	$\mu\text{g/ml}$	%	$\mu\text{g/ml}$	%	$\mu\text{g/ml}$	%	$\mu\text{g/ml}$	%	$\mu\text{g/ml}$	%
0	0.91	49.7	0.38	20.8	0.19	10.4	0.04	2.2	0.04	2.2
7	5.48	58.7	2.44	26.2	0.01	0.1	0.02	0.2	0.17	1.8
14	6.69	53.7	2.98	23.9	0.06	0.5	0.03	0.2	0.39	3.1
21	7.06	47.4	4.91	33.0	0.16	1.1	0.03	0.2	0.43	2.9
28	11.06	55.5	4.54	22.8	0.10	0.5	0.05	0.3	0.66	3.3
35	11.18	56.2	4.52	22.7	0.46	2.3	0.40	2.0	0.46	2.3
42	11.50	42.9	4.83	18.0	1.23	4.6	1.89	7.1	1.26	4.7
49	13.24	43.0	6.55	21.3	2.17	7.1	2.32	7.5	1.36	4.4
56	13.85	38.0	7.93	21.7	3.63	10.0	3.50	9.6	1.72	4.7
63	15.50	34.0	6.28	13.8	8.98	19.7	5.64	12.4	2.00	4.4
70	12.63	25.3	5.54	11.1	14.32	28.7	7.88	15.8	2.41	4.8
77	7.97	17.8	3.45	7.7	16.60	37.1	8.57	19.1	1.75	3.9
84	5.12	11.7	2.45	5.6	15.81	36.1	10.80	24.6	3.00	6.8
91	4.19	8.9	2.22	4.7	18.41	39.0	13.52	28.6	3.21	6.8



Fig. 3.5a

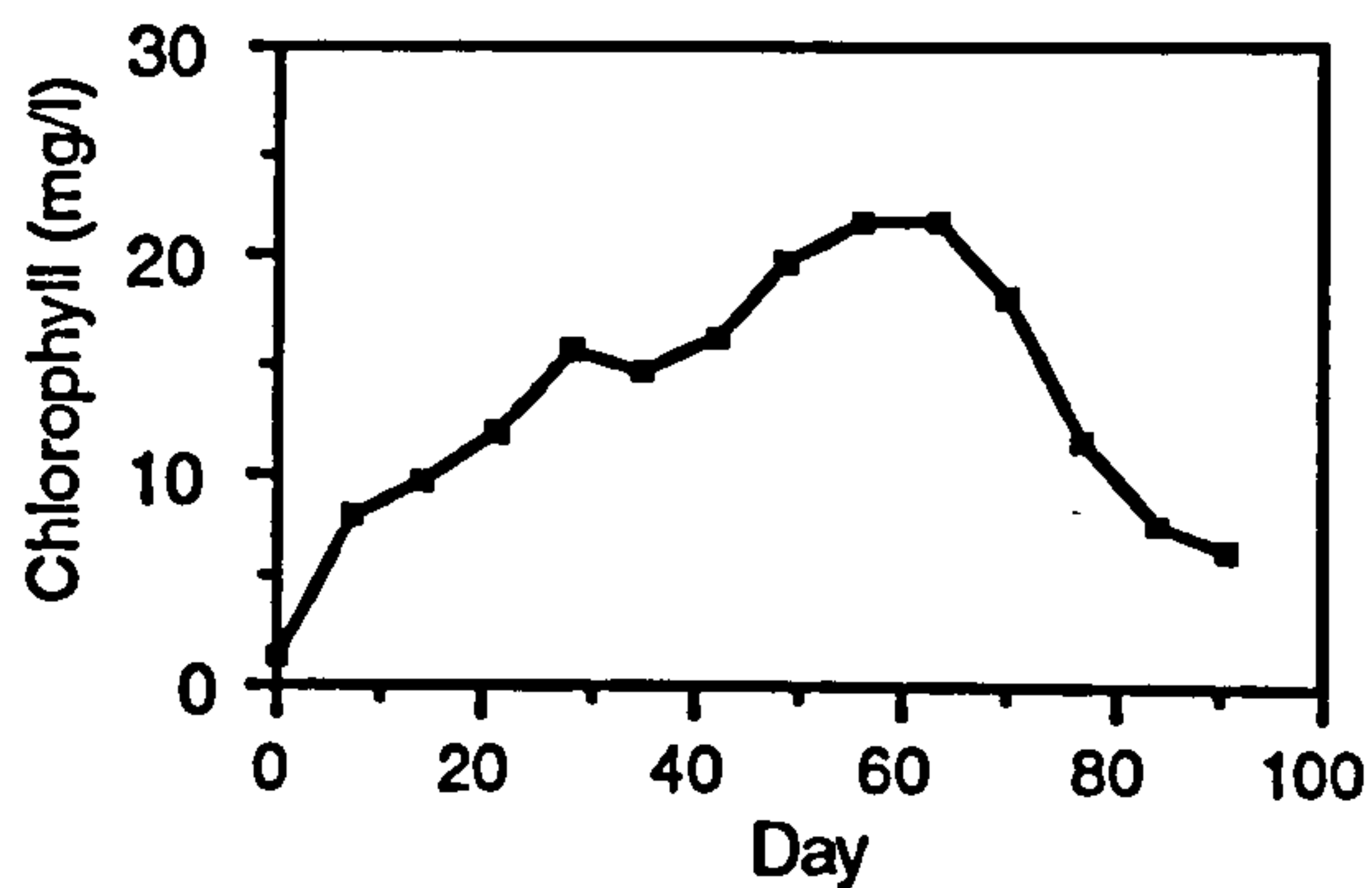


Fig. 3.5b

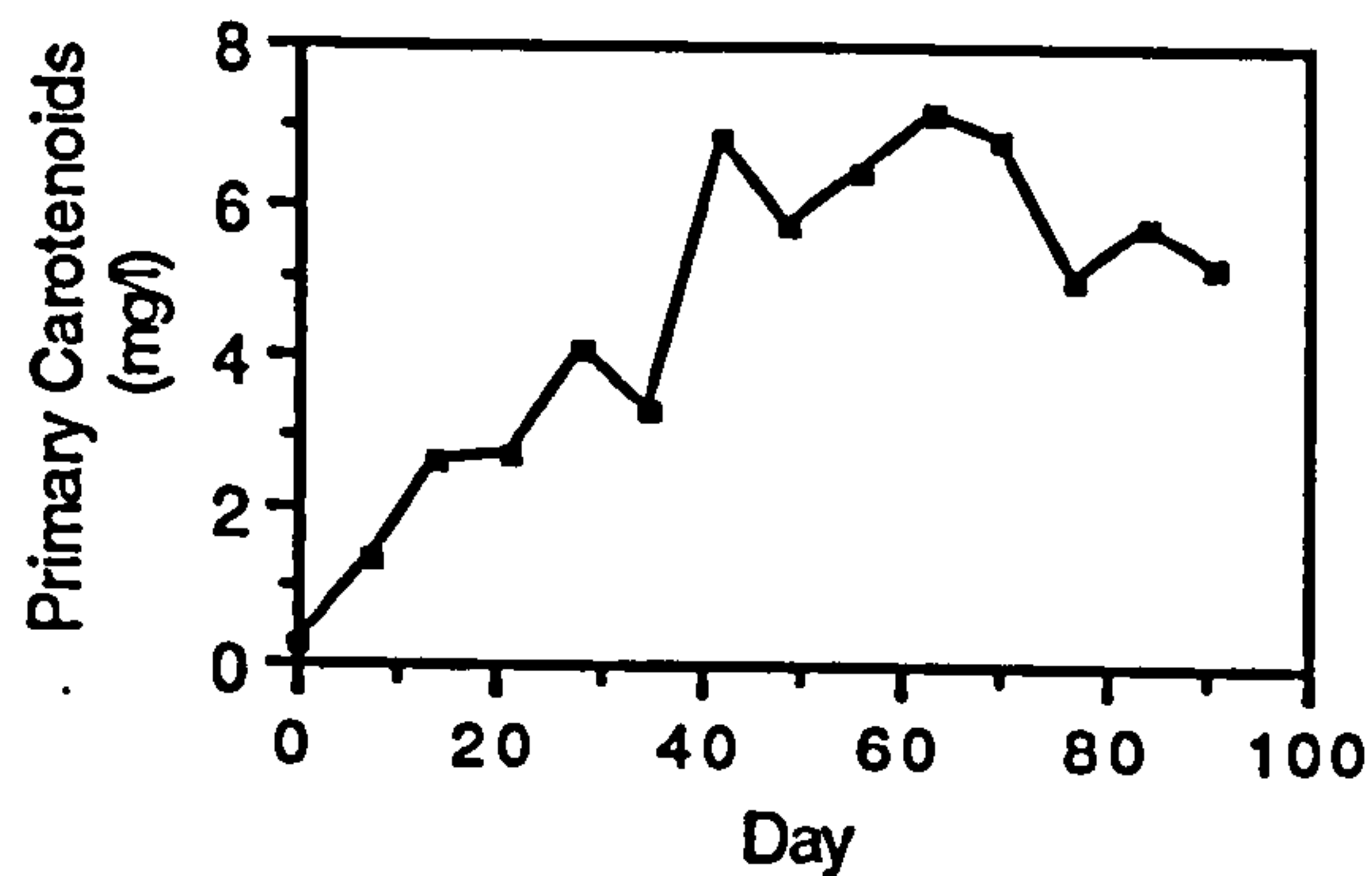


Fig 3.5c

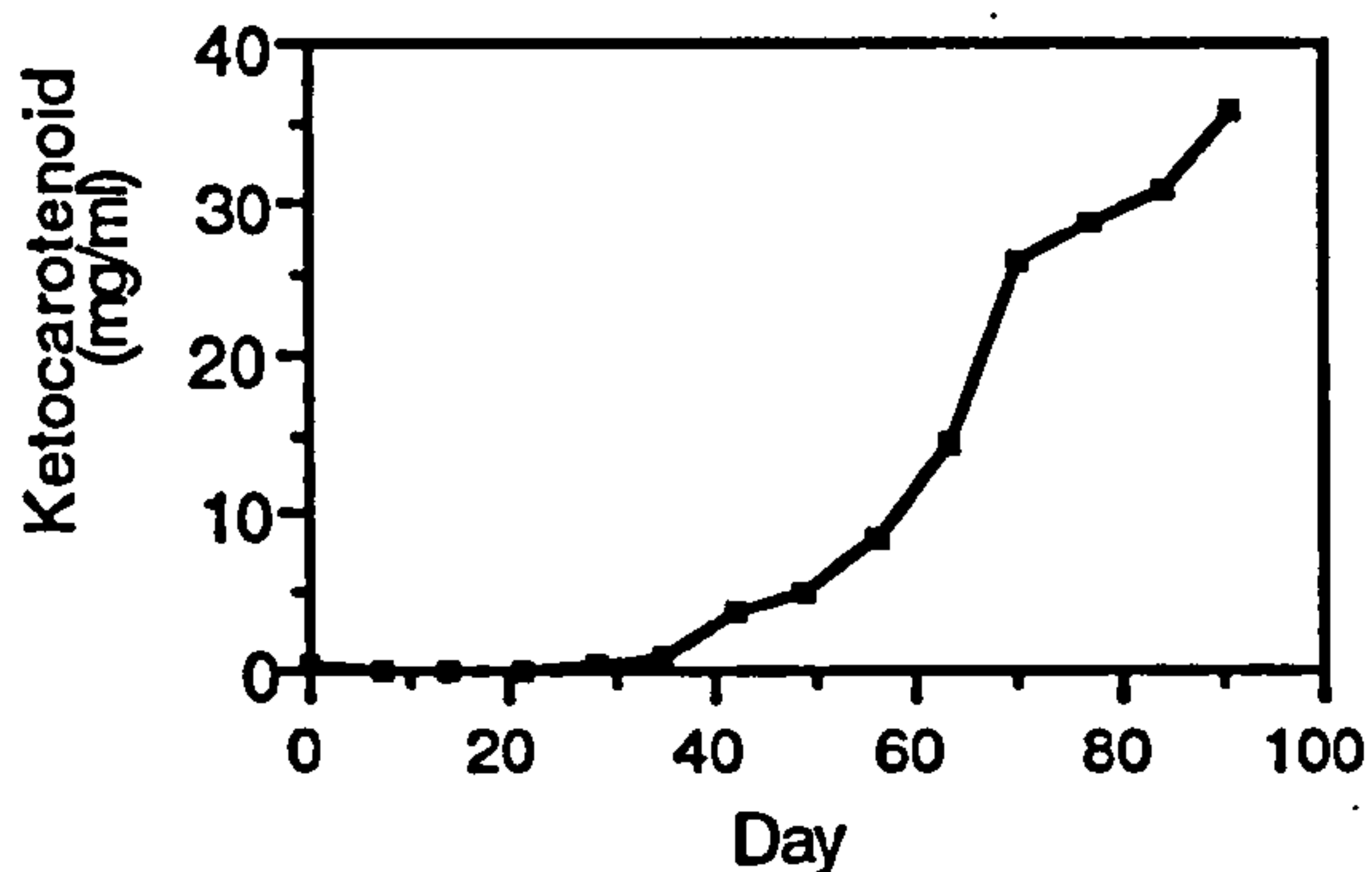


Fig 3.5d

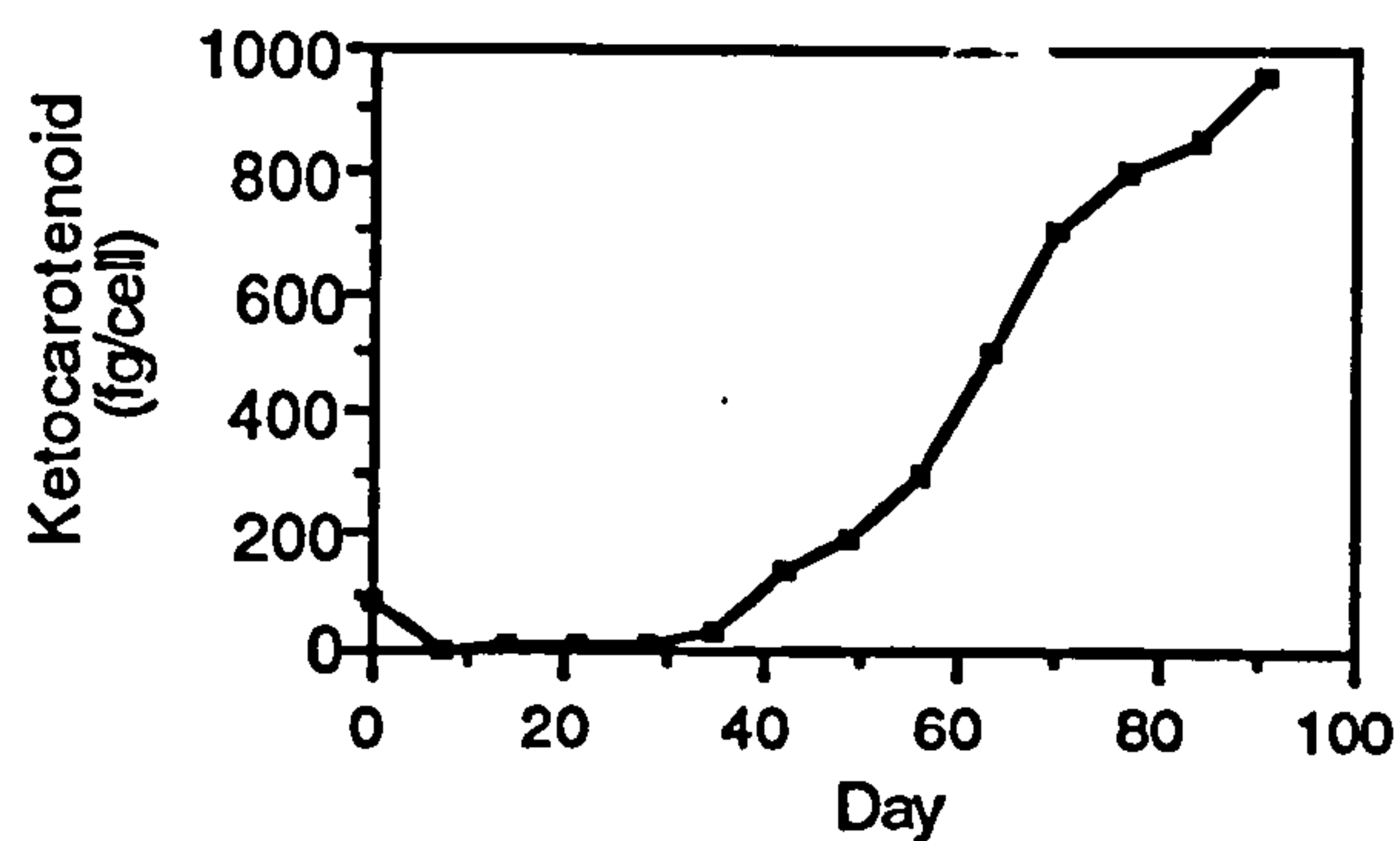
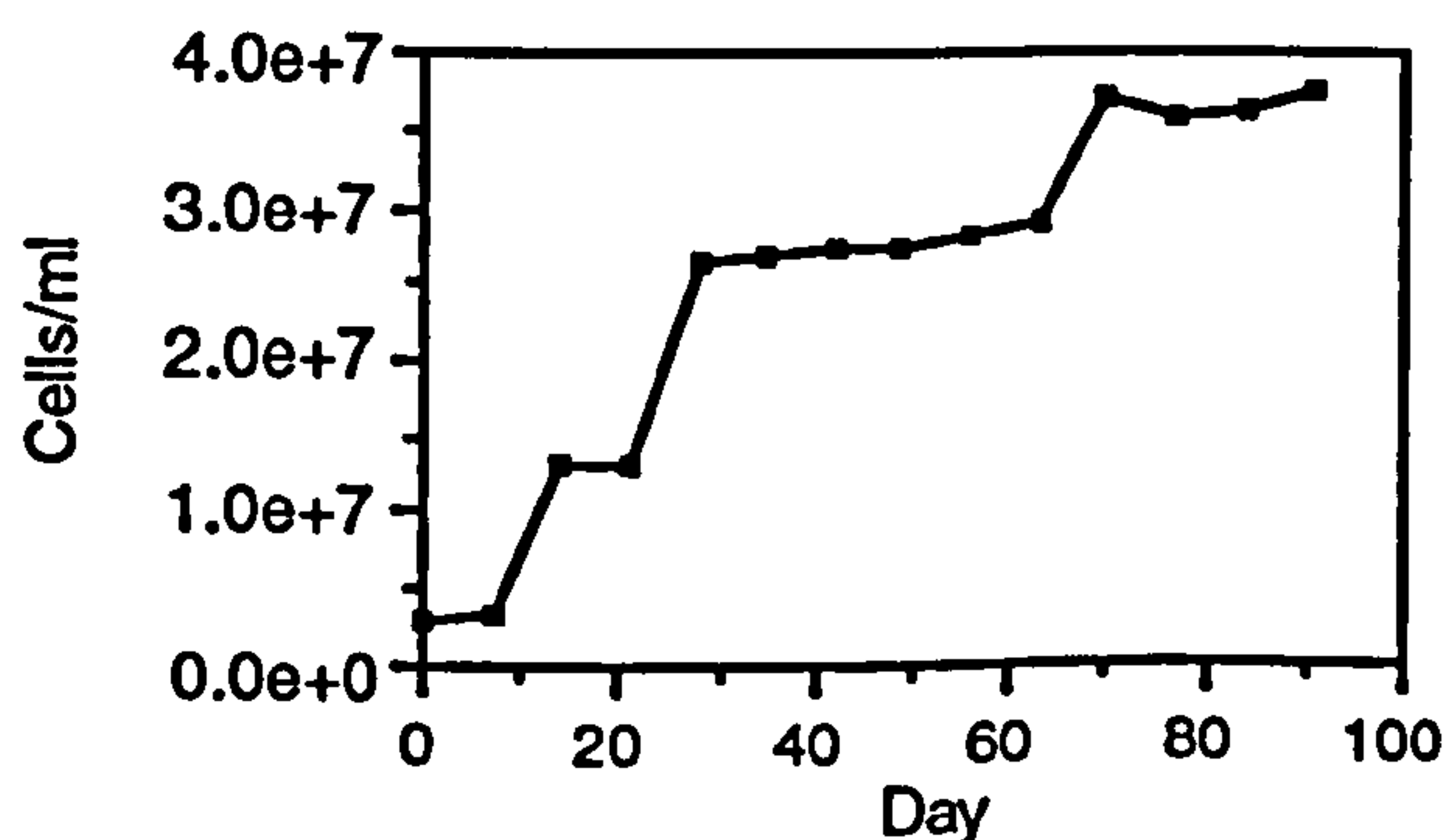


Fig. 3.5e



Figs 3.5a-e Pigment levels of *O. minuta* 257/6 during secondary carotenoid synthesis (S.E.  $\pm 5.2\%$ , n=3).

Table 3.9 and figures 3.5a-e show the accumulation of secondary carotenoids in *O. minuta* when the cells were cultured in nitrogen deficient BBM. The number of cells increased throughout the experiment, with sudden increases after days 7, 21 and 63. The accumulation of ketocarotenoids became noticeable after day 35. The rate of accumulation was relatively constant throughout the experiment. The chlorophyll levels increased at

varying rates until day 63 after which the level decreased rapidly. The level of primary carotenoids increased at a variable rate until day 70 after which the level began to decrease. The ratio of monoesters to diesters of ketocarotenoid fluctuated during the experiment, early in the experiment the ratio was 1:1 but increased to 2:1 mid-way through the experiment, then decreased to 1.4:1 in the final stages. The ratio of monoesters of ketocarotenoid to canthaxanthin (IV) increased from 1.4:1 on day 28 to 5.0:1 on day 91. However, canthaxanthin (IV) synthesis began very early in the experiment, with the carotenoid being present from the start of the experiment. The levels of neoxanthin (XX), violaxanthin (XVII) and lutein (XVI) all increased and reached maximum values mid-way during the experiment after which the levels decreased. The level of  $\beta$ -carotene (I) increased constantly throughout the experiment.

**Table 3.10** Pigment composition of *O. minuta* 257/6 during re-greening (S.E.  $\pm 3.5\%$ , n=3).

Day	Neoxanthin		Violaxanthin		Lutein-5,6		Lutein		Canthaxanthin	
	$\mu\text{g/ml}$	%	$\mu\text{g/ml}$	%	$\mu\text{g/ml}$	%	$\mu\text{g/ml}$	%	$\mu\text{g/ml}$	%
0	0.01	0.3	0.01	0.3	0.03	1.0	0.11	3.5	0.13	4.2
2	0.04	1.3	0.06	1.9	0.03	1.0	0.22	7.0	0.12	3.8
4	0.14	2.3	0.19	3.1	0.05	0.8	0.61	10.0	0.11	1.8
7	0.20	2.2	0.25	2.8	0.06	0.7	0.97	10.7	0.12	1.3
9	0.25	2.7	0.27	3.0	0.12	1.3	1.30	14.3	0.03	0.3

Day	Chlorophyll a		Chlorophyll b		Monoesters		Diesters		Beta-Carotene	
	$\mu\text{g/ml}$	%	$\mu\text{g/ml}$	%	$\mu\text{g/ml}$	%	$\mu\text{g/ml}$	%	$\mu\text{g/ml}$	%
0	0.38	12.1	0.20	6.4	1.23	39.3	0.86	27.5	0.17	5.4
2	0.77	24.4	0.29	9.2	0.86	27.2	0.60	19.0	0.17	5.4
4	2.56	41.9	0.99	16.2	0.53	8.7	0.65	10.6	0.28	4.6
7	4.21	46.5	1.68	18.5	0.66	7.3	0.58	6.4	0.33	3.6
9	4.26	46.8	1.61	17.7	0.48	5.3	0.37	4.1	0.42	4.6

Fig. 3.6a

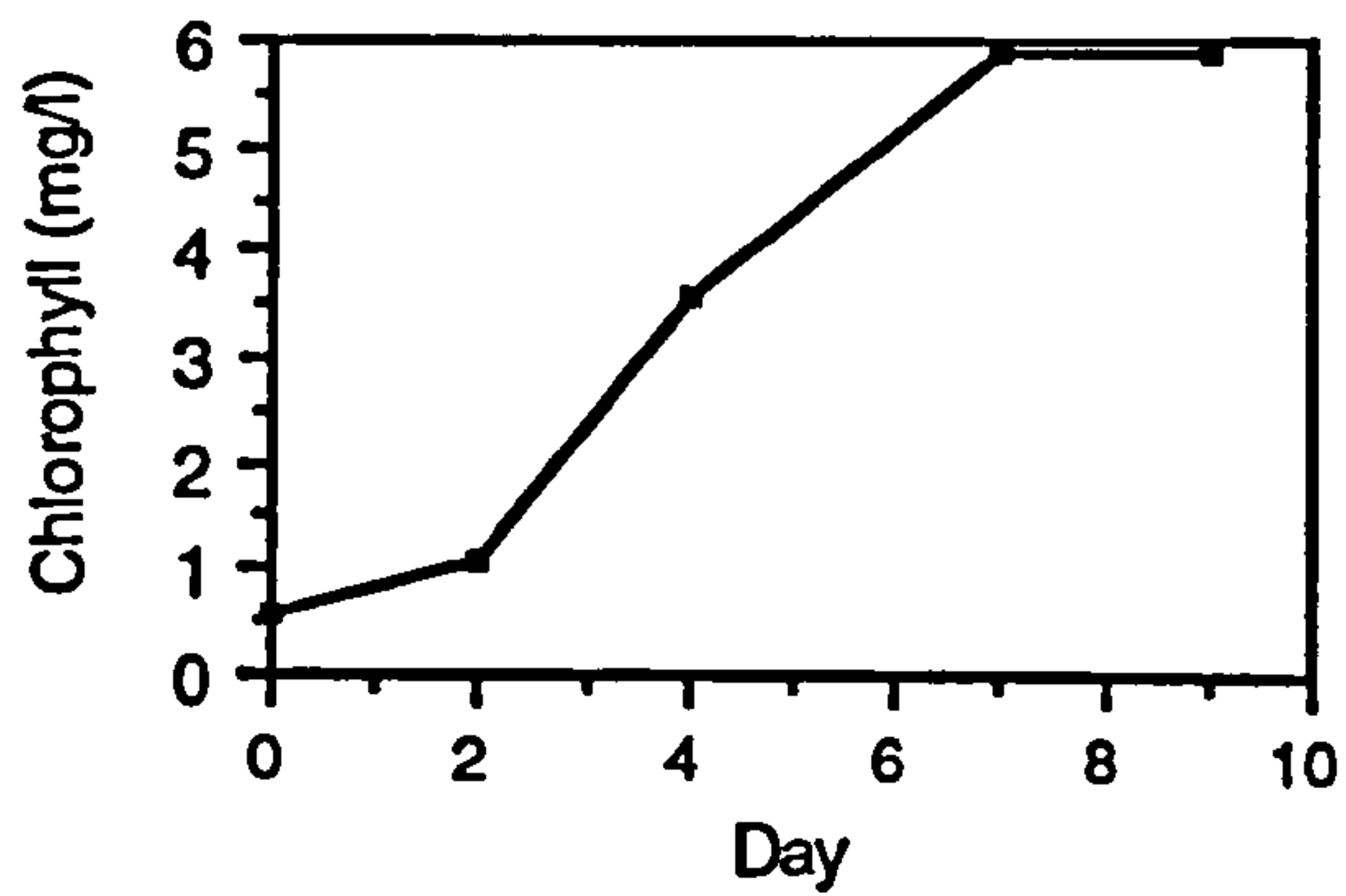


Fig. 3.6b

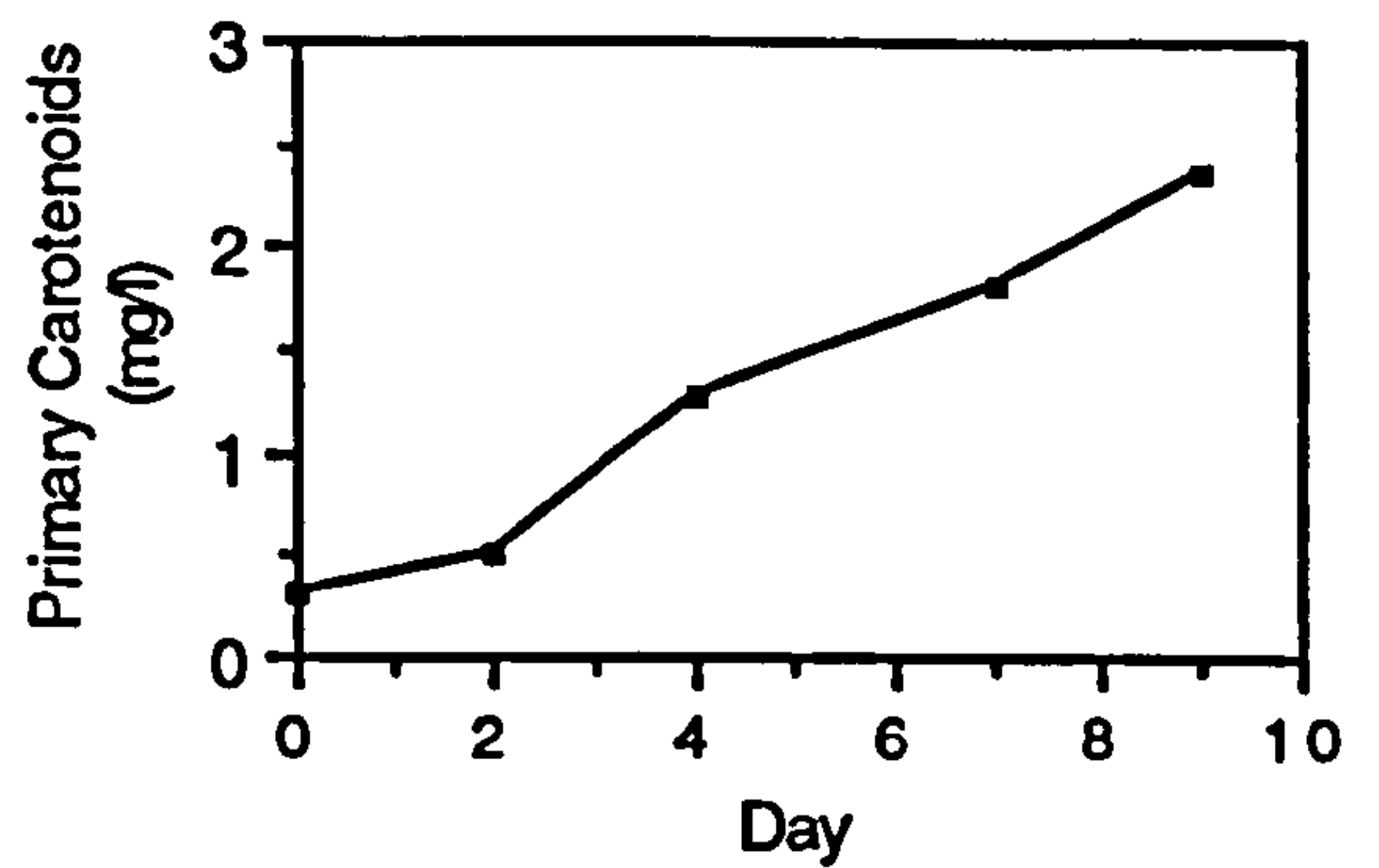


Fig. 3.6c

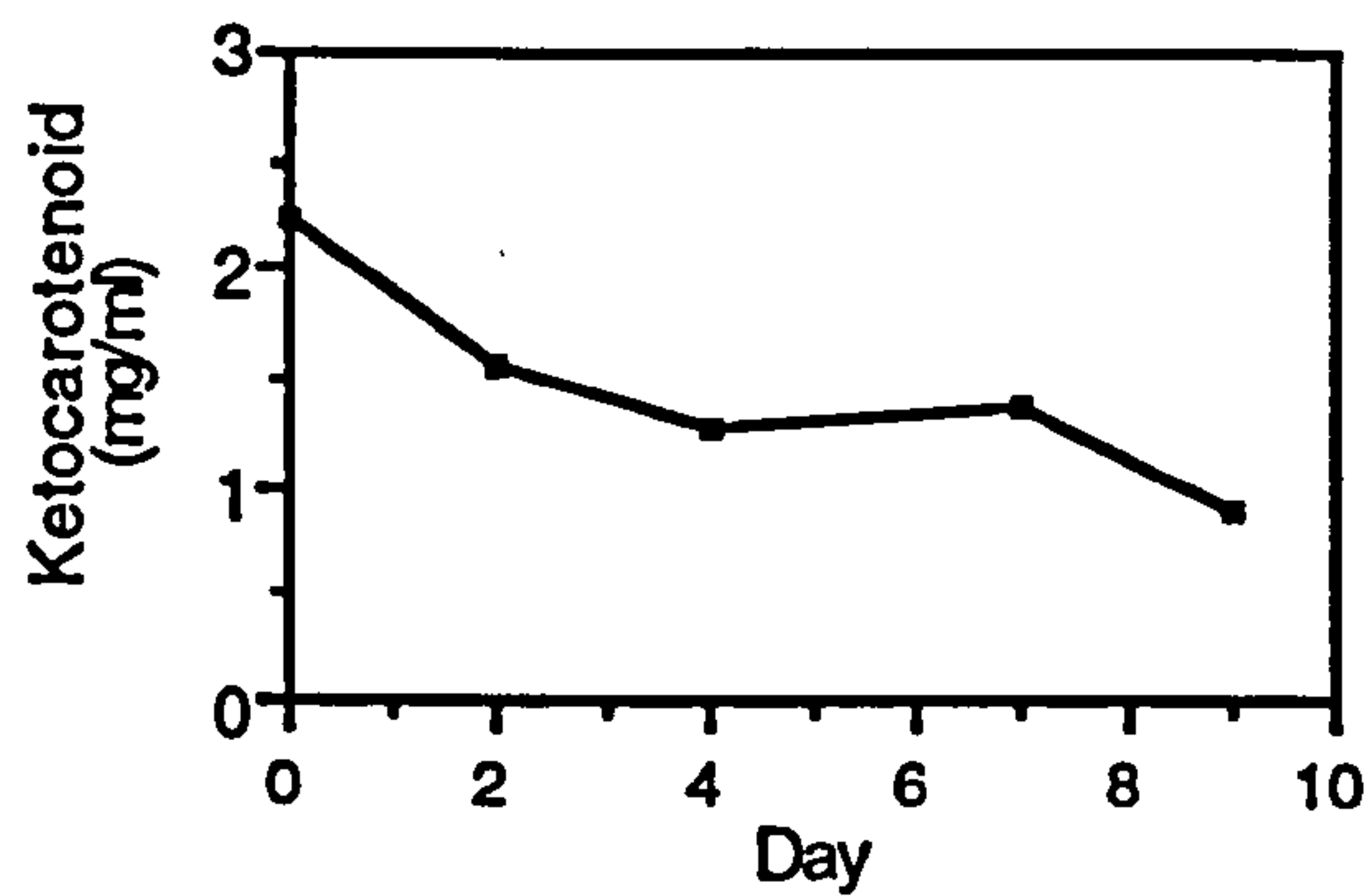


Fig. 3.6e

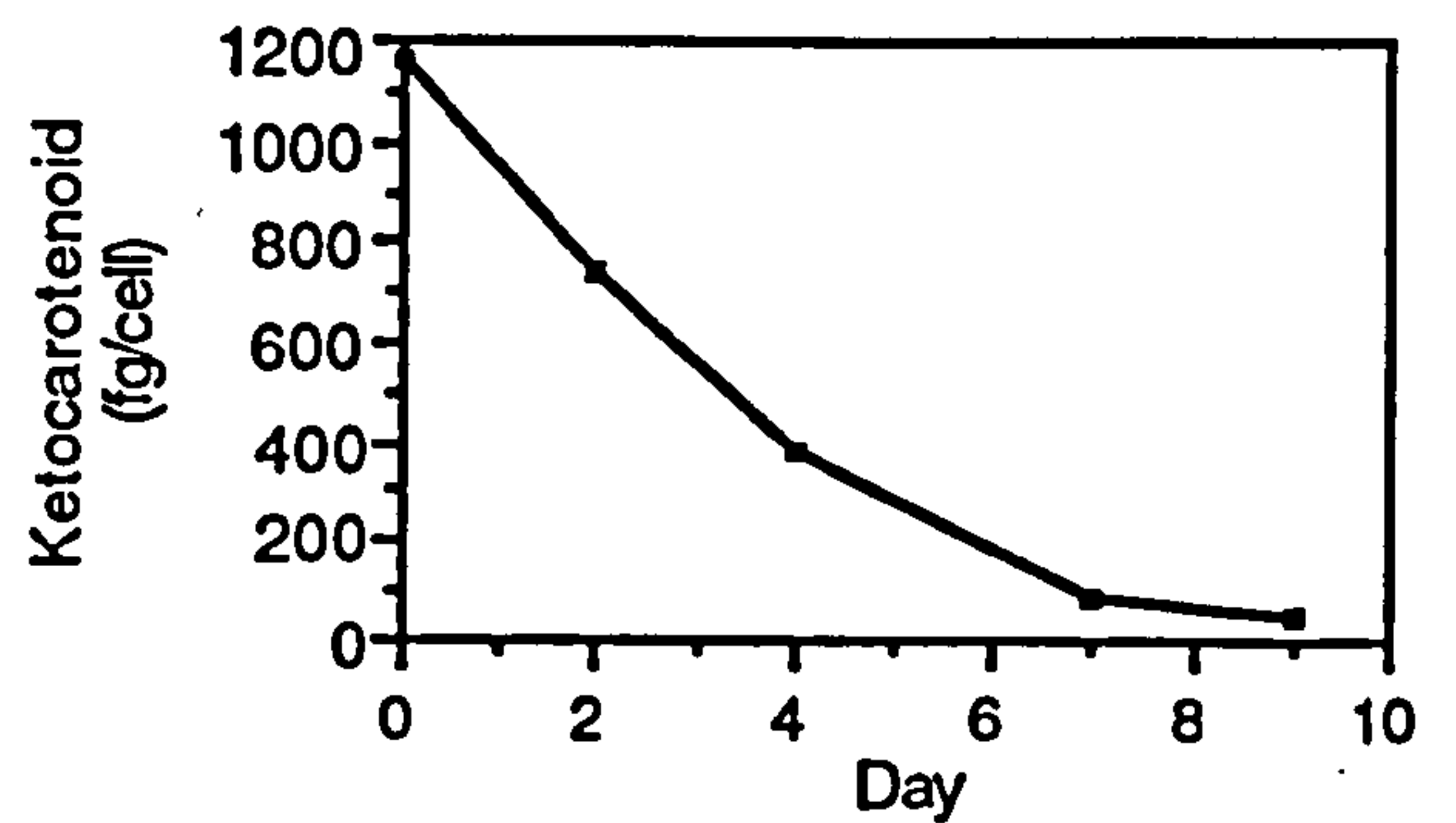
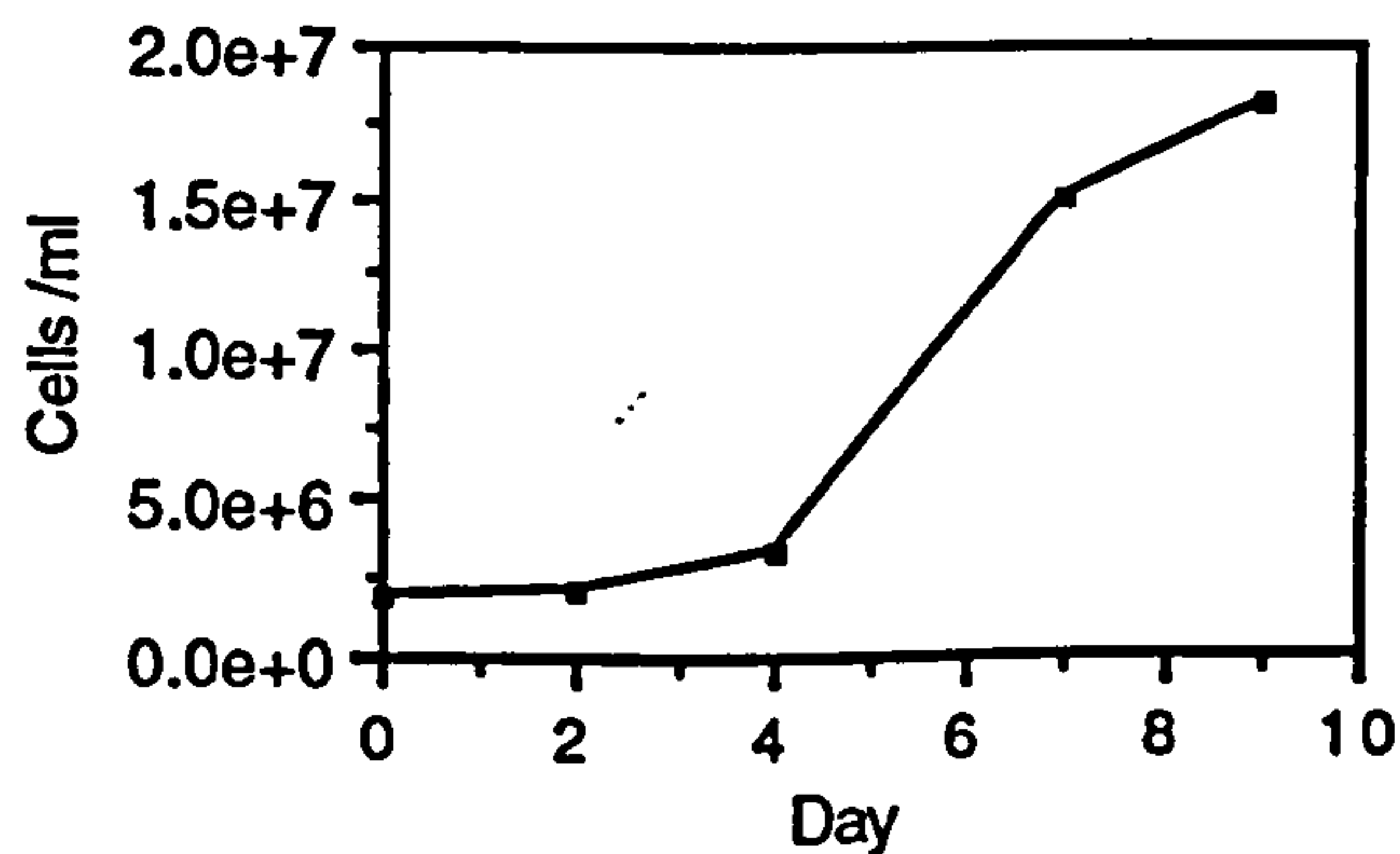


Fig. 3.6e



Figs. 3.6a-e Pigment levels of *O. minuta* 257/6 during re-greening (S.E.  $\pm 3.5\%$ , n=3).

Table 3.10 and figures 3.6a-e demonstrate the re-greening of *O. minuta* after encystment. The levels of chlorophyll and primary carotenoids increased throughout the experiment, with particularly high rates of chlorophyll synthesis between days 4 and 7. The number of cells also increased throughout the experiment, with the rate being particularly high between day 4 and 7 after an initial lag phase. The level of ketocarotenoid

per unit volume of culture declined at a relatively steady but slow rate during the experiment. The ratio of monoesters to diesters of ketocarotenoid remained relatively constant during the experiment at approximately 1.2:1. The ratio of monoesters of ketocarotenoid to canthaxanthin (IV) decreased from 9.5:1 on day 0 to 5.5:1 on day 7, however this ratio increased to 16:1 on day 9.

**Table 3.11** Pigment composition of *C. rectangularis* 218/2 during secondary carotenoid synthesis (S.E.  $\pm$ 4.6%, n=3).

Day	Neoxanthin		Violaxanthin		Lutein-5,6		Lutein		Canthaxanthin	
	$\mu\text{g/ml}$	%	$\mu\text{g/ml}$	%	$\mu\text{g/ml}$	%	$\mu\text{g/ml}$	%	$\mu\text{g/ml}$	%
0	1.10	7.4	0.94	6.3	0.18	1.2	2.66	17.9	0.12	0.8
7	0.99	6.0	0.74	4.5	0.21	1.3	2.77	16.8	0.19	1.2
14	0.78	3.3	0.76	3.3	0.07	0.3	3.08	13.7	0.36	1.5
21	0.60	2.4	0.31	1.2	0.26	1.1	2.98	12.0	0.63	2.5
28	0.48	1.9	0.44	1.8	0.23	0.9	2.67	10.8	0.70	2.8
35	0.48	1.8	0.44	1.7	0.19	0.7	2.94	11.2	0.92	3.5
42	0.29	1.2	0.23	0.9	0.15	0.6	2.38	9.8	1.05	4.3
49	0.13	0.8	0.14	0.8	0.17	1.0	1.36	8.1	1.03	6.1
56	0.12	0.8	0.14	1.0	0.08	0.5	1.18	8.0	1.22	8.3
63	0.07	0.5	0.09	0.7	0.01	0.1	0.80	6.1	1.30	10.0
70	0.05	0.4	0.08	0.7	0.05	0.4	0.49	4.3	1.43	12.5

Day	Chlorophyll a		Chlorophyll b		Monoesters		Diesters		Beta-Carotene	
	$\mu\text{g/ml}$	%	$\mu\text{g/ml}$	%	$\mu\text{g/ml}$	%	$\mu\text{g/ml}$	%	$\mu\text{g/ml}$	%
0	5.42	36.5	3.23	21.8	0.07	0.5	0.20	1.3	0.92	6.2
7	6.29	38.1	3.69	22.3	0.14	0.8	0.31	1.9	1.18	7.1
14	10.33	44.2	6.20	26.5	0.15	0.6	0.50	2.1	1.21	5.2
21	10.00	40.3	6.89	27.7	0.86	3.5	0.93	3.7	1.38	5.6
28	10.02	40.4	5.89	23.7	1.55	6.2	1.25	5.0	1.60	6.4
35	9.05	34.4	5.61	21.3	2.74	10.4	1.95	7.4	2.00	7.6
42	7.82	32.3	4.83	19.9	3.35	13.8	2.32	9.6	1.81	7.5
49	4.25	25.2	2.79	16.6	3.60	21.4	2.06	12.2	1.31	7.8
56	3.25	22.0	1.96	13.3	3.74	25.4	1.88	12.7	1.18	8.0
63	2.10	16.1	1.35	10.4	4.00	30.7	2.00	15.4	1.30	10.0
70	1.29	11.3	0.72	6.3	4.20	36.7	2.05	17.9	1.07	9.4



Fig. 3.7a

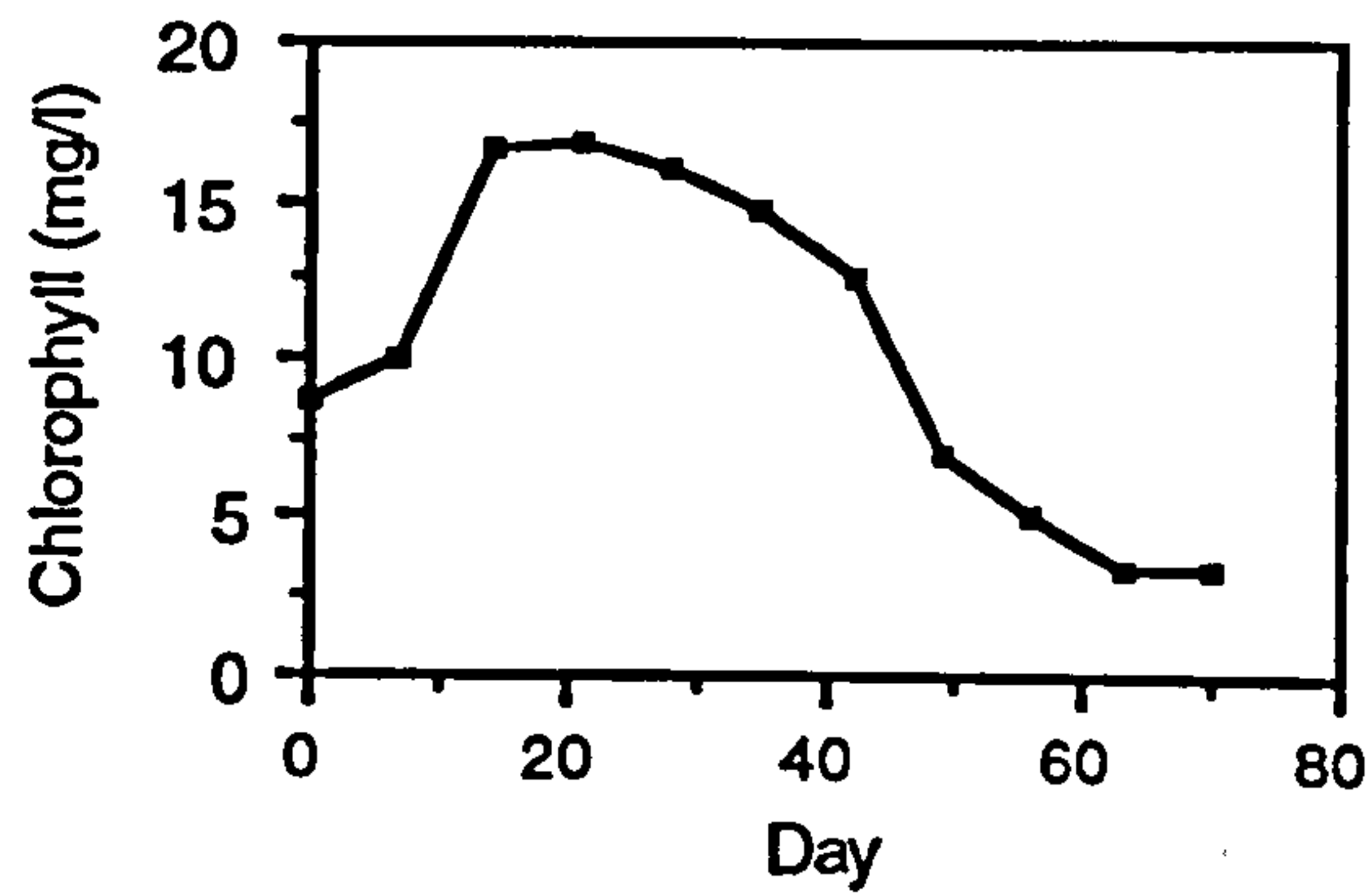


Fig. 3.7b

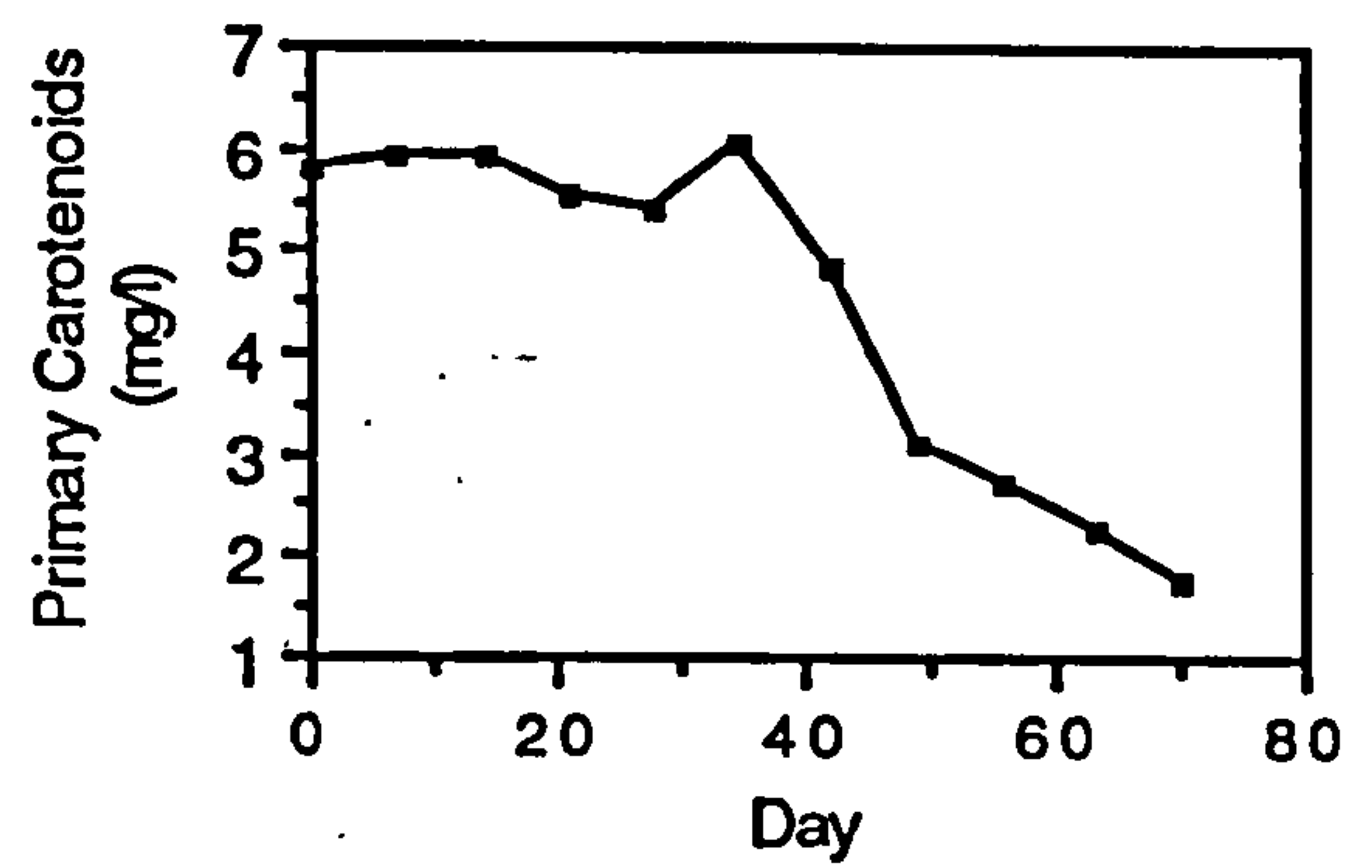


Fig. 3.7c

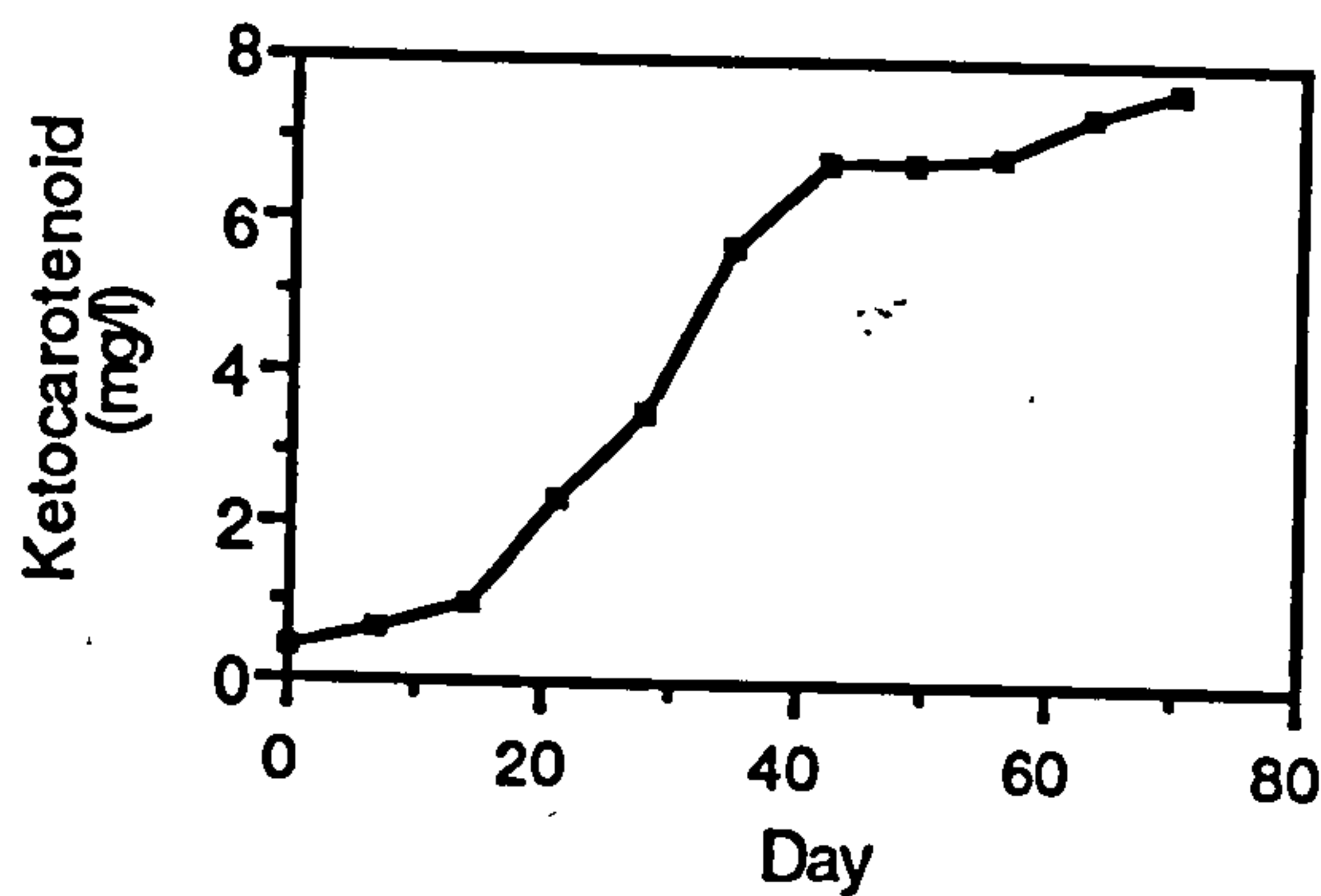


Fig. 3.7d

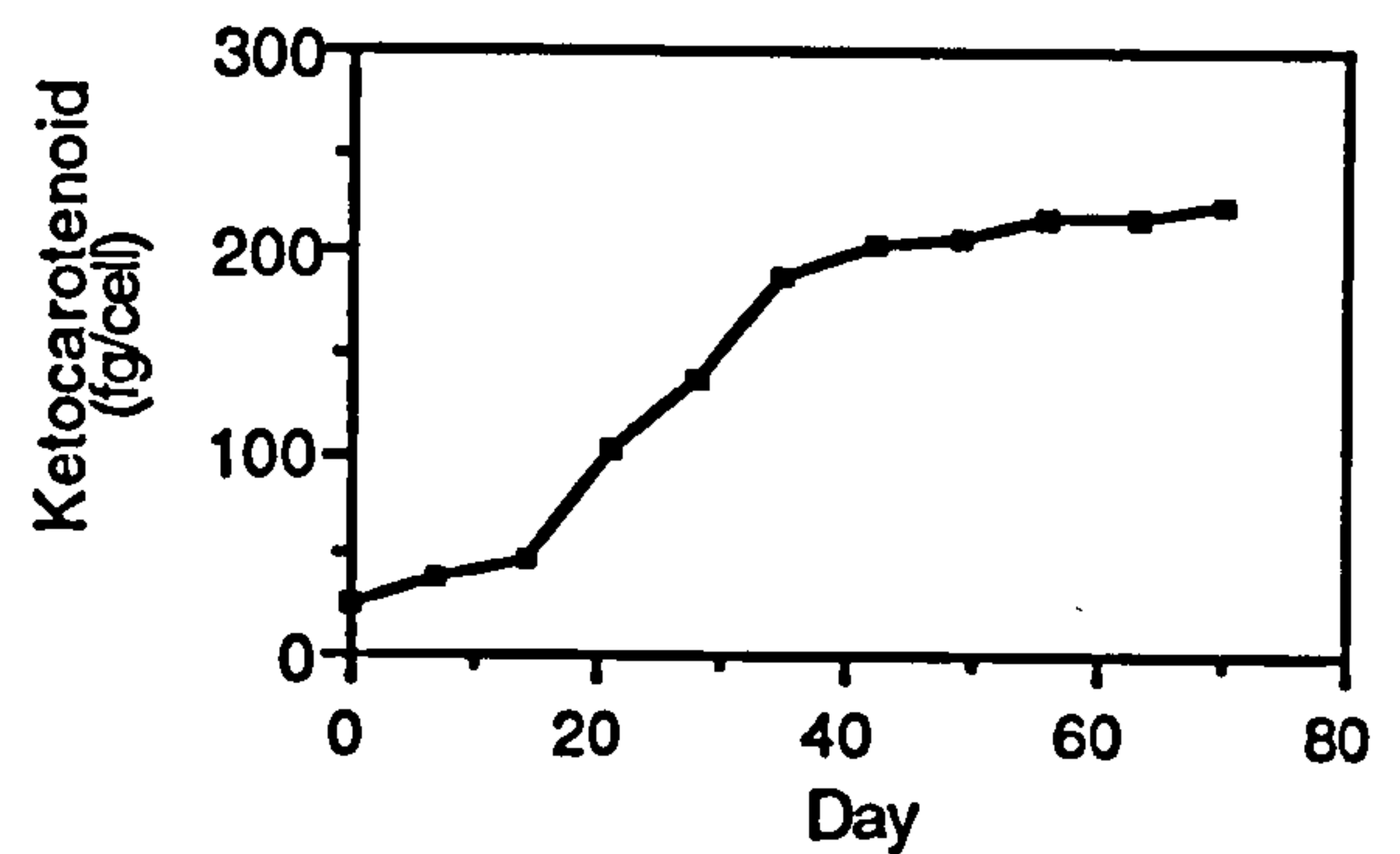
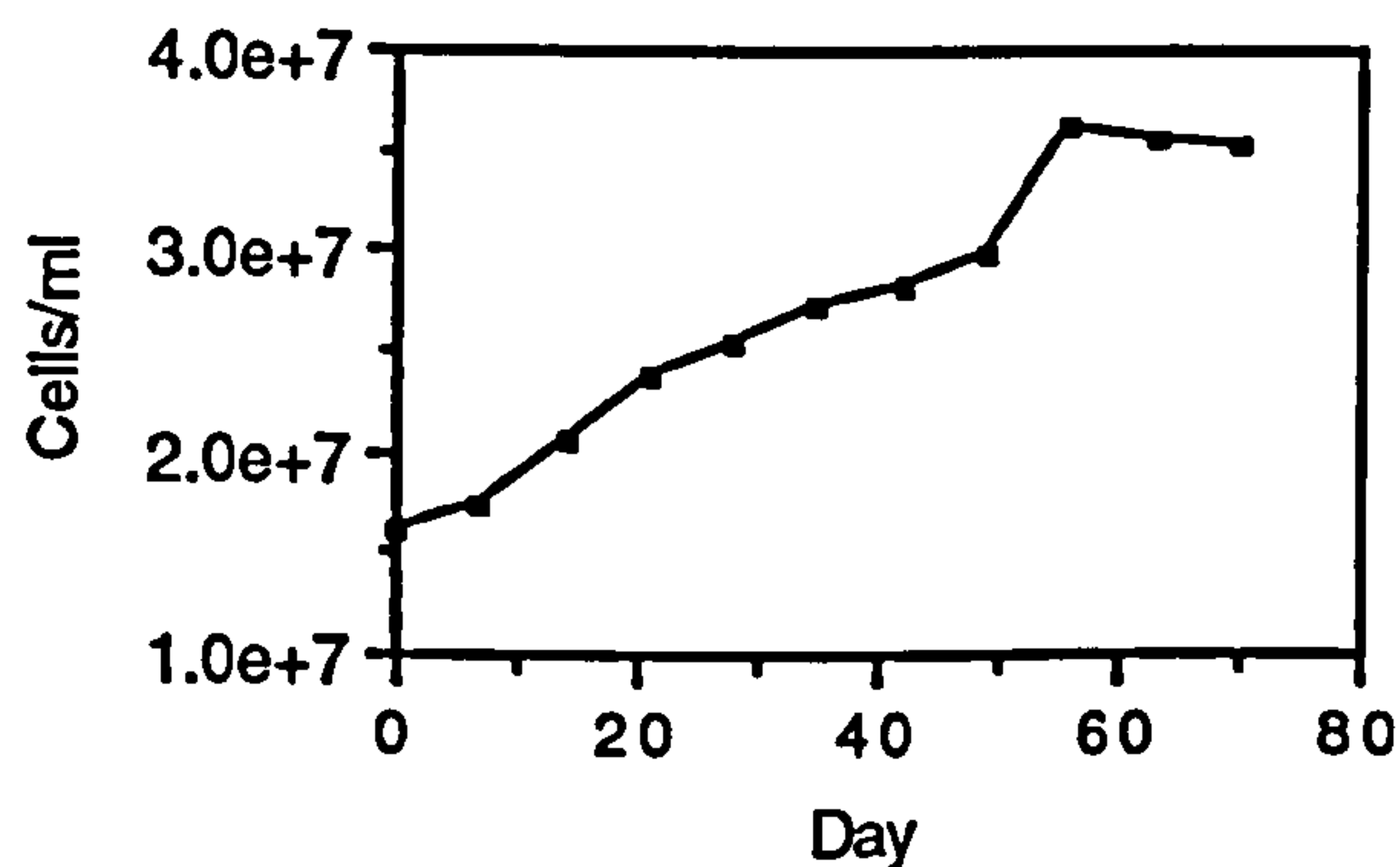


Fig. 3.7e



Figs. 3.7a-e Pigment levels of *C. rectangularis* 218/2 during secondary carotenoid synthesis (S.E.  $\pm 4.6\%$ ,  $n=3$ ).

Table 3.11 and figures 3.7a-e show the pigment levels of *C. rectangularis* during secondary carotenoid synthesis, when the cells were grown in nitrogen deficient BBM. The chlorophyll level increased until day 21 after which the level slowly decreased, this decrease became rapid after day 43. The level of primary carotenoids remained relatively constant up to day 30, when after a small increase the level decreased steadily for the

remainder of the experiment. The accumulation of ketocarotenoid became noticeable after day 14. The level increased rapidly between day 14 and 42 after which the rate of increase declined. The level of ketocarotenoid per cell followed a similar pattern as that of ketocarotenoid mg/l. The number of cells increased until day 56 after which the number of cells slightly declined. The ratio of monoesters to diesters of ketocarotenoid increased from 0.3:1 on day 0 to 2.0:1 on day 56, the ratio then remained at this level for the rest of the experiment. The ratio of monoesters of ketocarotenoid to canthaxanthin (IV) increased from 0.6:1 on day 0 to 3.0:1 on day 56, the ratio remained at this level for the rest of the experiment. The levels of neoxanthin (XX) and violaxanthin (XVII) decreased throughout the experiment. The level of lutein (XVI) steadily increased until day 14 after which the level slowly decreased for the remainder of the experiment. The level of  $\beta$ -carotene (I) was variable throughout the experiment but showed no significant fluctuations.

**Table 3.12** Pigment composition of *C. rectangularis* 218/2 during re-greening (S.E.  $\pm 4.5\%$ , n=3).

Day	Neoxanthin		Violaxanthin		Lutein-5,6		Lutein		Canthaxanthin	
	$\mu\text{g/ml}$	%	$\mu\text{g/ml}$	%	$\mu\text{g/ml}$	%	$\mu\text{g/ml}$	%	$\mu\text{g/ml}$	%
0	0.01	0.5	0.01	0.5	0.02	1.0	0.11	5.8	0.14	7.3
2	0.02	1.3	0.02	1.3	0.02	1.3	0.14	9.4	0.11	7.4
4	0.20	4.0	0.17	3.4	0.03	0.6	0.32	6.4	0.07	1.4
7	0.28	4.2	0.19	2.9	0.03	0.5	0.41	6.2	0.06	0.9
9	0.37	4.4	0.23	2.7	0.02	0.2	0.43	5.1	0.05	0.6

Day	Chlorophyll a		Chlorophyll b		Monoesters		Diesters		Beta-Carotene	
	$\mu\text{g/ml}$	%	$\mu\text{g/ml}$	%	$\mu\text{g/ml}$	%	$\mu\text{g/ml}$	%	$\mu\text{g/ml}$	%
0	0.39	20.4	0.19	9.9	0.65	34.0	0.29	15.2	0.10	5.2
2	0.41	27.5	0.17	11.4	0.38	25.5	0.12	8.1	0.10	6.7
4	2.48	49.5	1.27	25.3	0.17	3.4	0.07	1.4	0.23	4.6
7	3.30	49.9	1.92	29.1	0.10	1.5	0.04	0.6	0.28	4.2
9	4.51	53.8	2.38	28.4	0.04	0.5	0.04	0.5	0.32	3.8

Fig. 3.8a

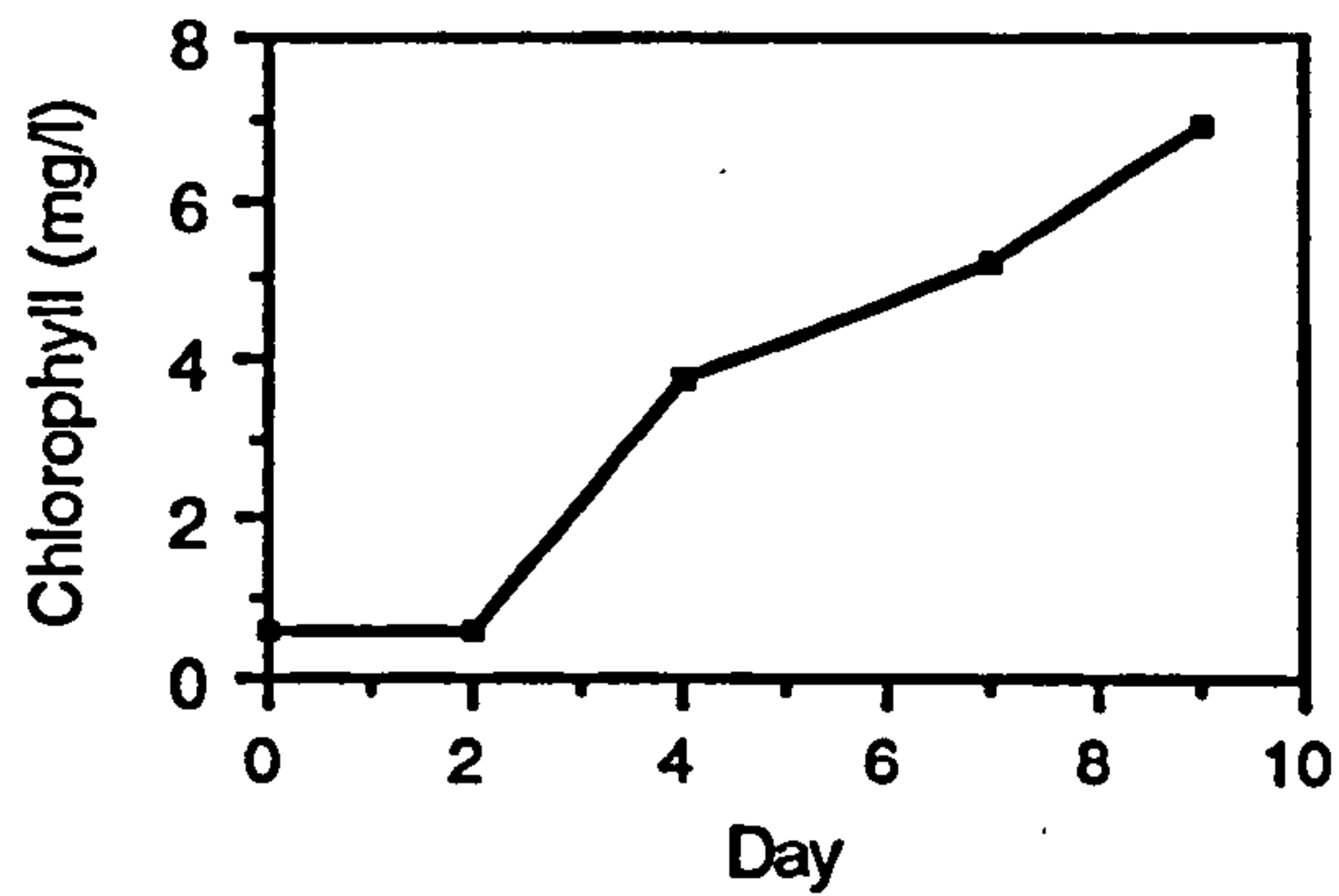


Fig. 3.8b

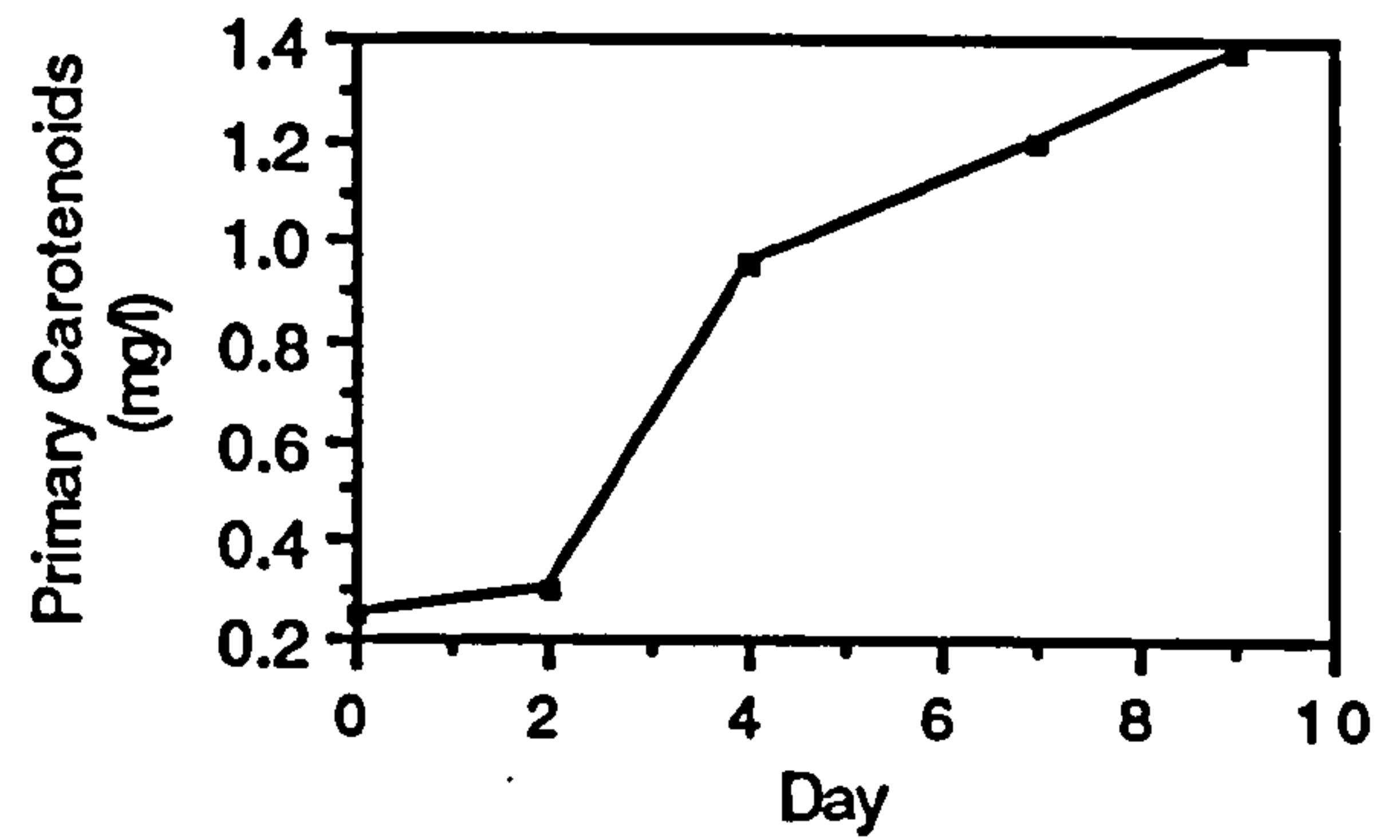


Fig. 3.8c

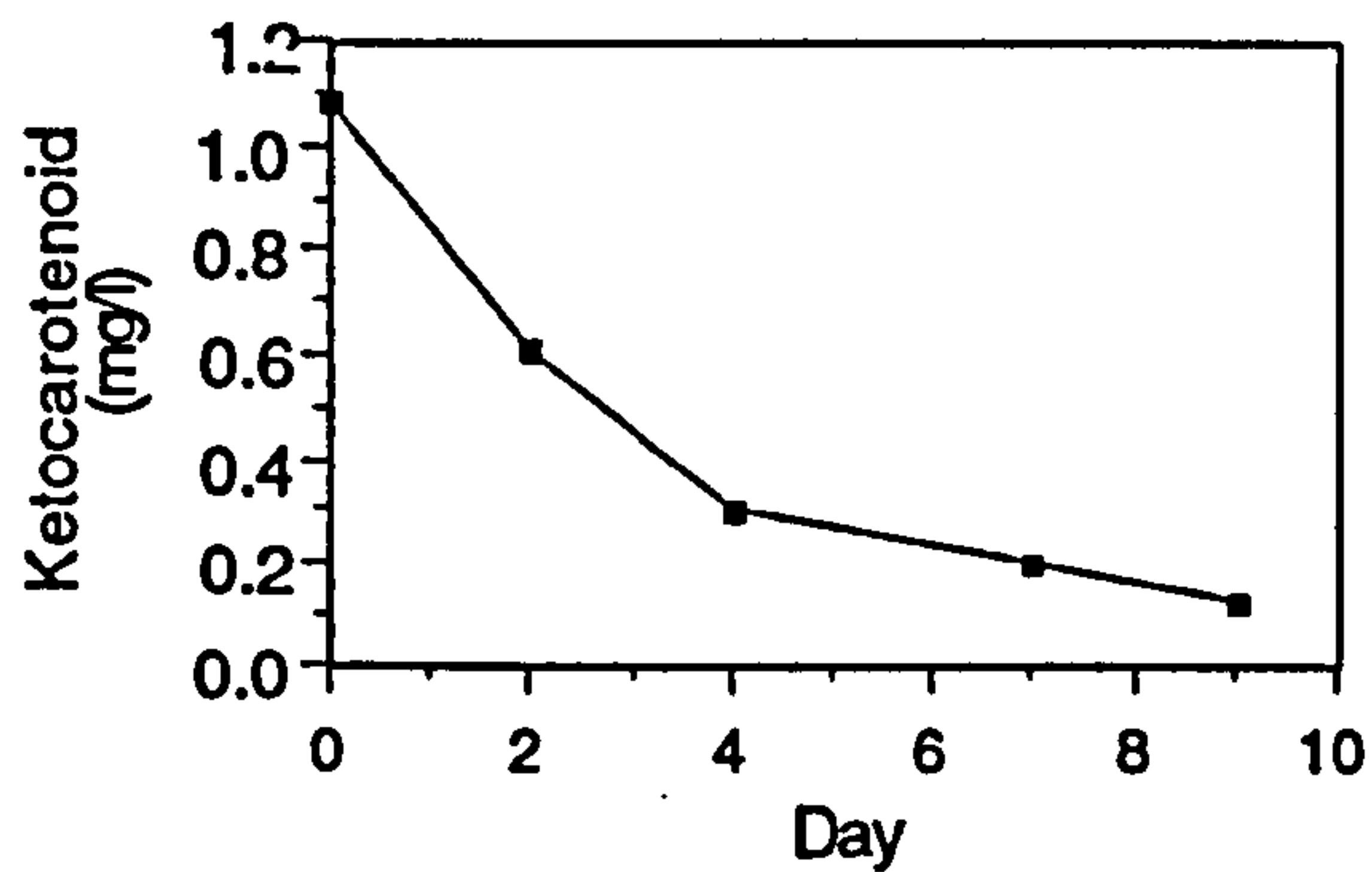


Fig. 3.8d

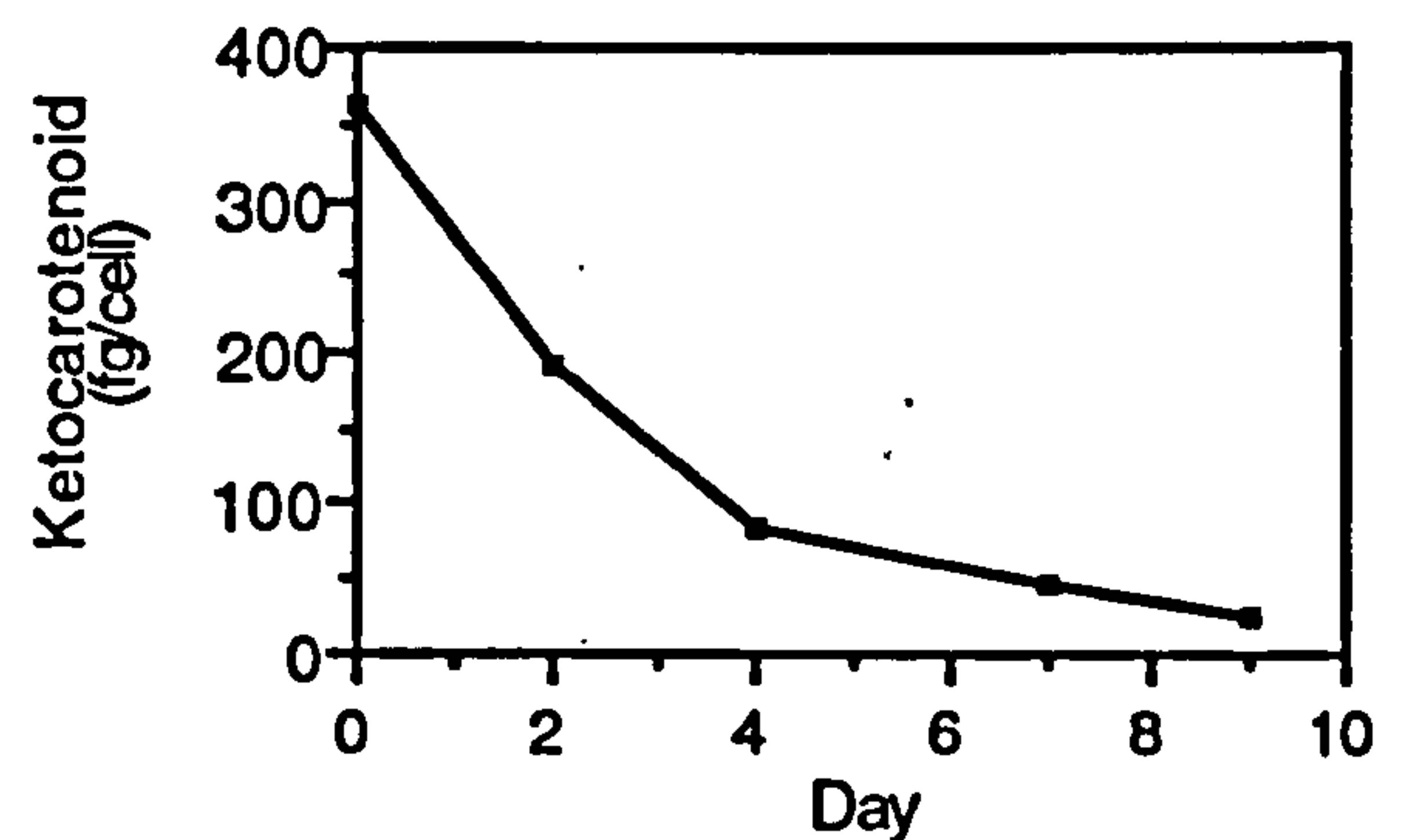
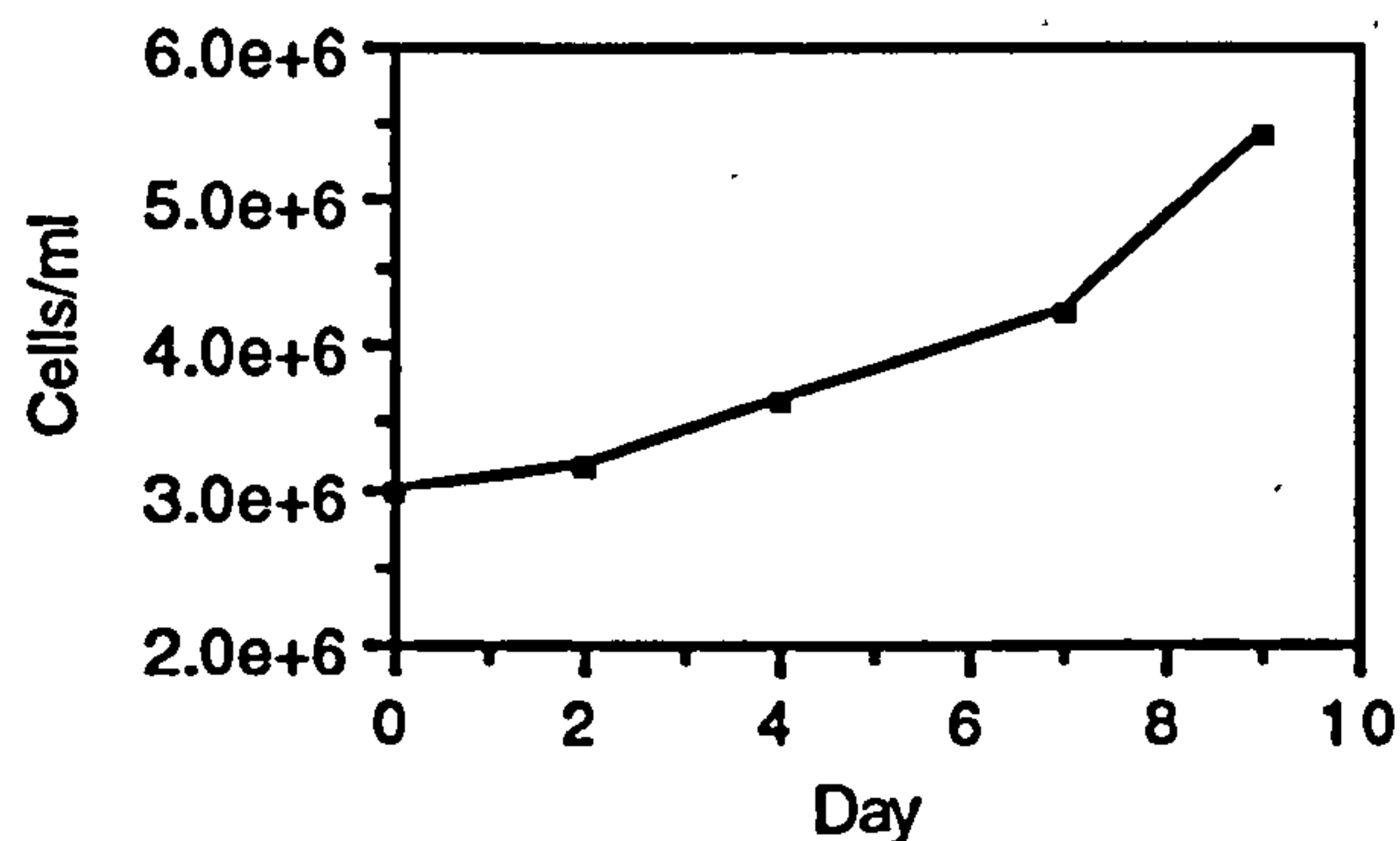


Fig. 3.8e



Figs. 3.8a-e Pigment levels of *C. rectangularis* 218/2 during re-greening (S.E.  $\pm 4.5\%$ ,  $n=3$ ).

Table 3.12 and figures 3.8a-e illustrate the changes in the level of pigments in *C. rectangularis* during re-greening. Chlorophyll and primary carotenoid levels increased very quickly after day 2 and continued to increase albeit at a slower rate throughout the experiment. The number of cells increased throughout the experiment with a rapid increase after day 7. The level of ketocarotenoid decreased during the experiment particularly

between days 0 and 4. The level of ketocarotenoid per cell followed a very similar pattern as that for ketocarotenoid mg/l. The ratio of monoesters to diesters of ketocarotenoid decreased from 2.2:1 on day 0 to 1:1 on day 9. The ratio of monoesters of ketocarotenoid to canthaxanthin (IV) decreased during the experiment, from 4.6:1 on day 0 to 0.8:1 on day 9. The percentage of  $\beta$ -carotene (I) fluctuated greatly and overall showed a slight decrease during the course of the experiment.

### 3.4 DISCUSSION

#### 3.4.1 Secondary carotenoid composition

The cells in the present study were cultured in a nitrogen-deficient medium. Deprivation of macro-elements causes a wide diversity of drastic changes in the structural and chemical organisation of algal cells (Piorreck *et al.*, 1984; Suen *et al.*, 1987). In the screening experiment one of the primary effects of nitrogen deficiency was to induce the synthesis of secondary carotenoids namely, astaxanthin (III) mono and diesters, canthaxanthin (IV), adonirubin (V) and echinenone (II) in the algal species tested. The primary carotenoids neoxanthin (XX) and violaxanthin (XVII) could not be detected in the encysted cells in the majority of these species. These two carotenoids were present in the green cells when the carotenoid composition and relative levels of carotenoids, were as expected for members of the Chlorophyceae under favourable growth conditions (Grung *et al.*, 1989).

When grown under favourable conditions the pigment composition and relative levels of carotenoids of the species and strains was relatively constant. When the cells were cultured in nitrogen-deficient medium the carotenoids present in the various species and strains again were very similar, but the relative levels of carotenoids varied considerably between particular species and in some cases between different strains as was the case with the two *O. minuta* strains.

The pigment composition of the secondary carotenoids in the algal species and strains screened agreed well with that of several orders of Chlorophyta grown in nitrogen deprivation. The secondary carotenoids astaxanthin (III), canthaxanthin (IV), and



adonirubin (V) are the main secondary carotenoids found in *Eremosphaera viridis*, Chlorococcales (Vechtel *et al.*, 1992a), in *Fritschiella tuberosa*, Ulotrichales (Weber, 1975), in *Neospongiococcum* sp., Chlorococcales (Deason *et al.*, 1977), in *Ankistrodesmus braunii*, Chlorococcales (Dersch, 1960), and in *Botryococcus braunii*, Chlorococcales (Grung *et al.*, 1989).

The secondary carotenoid composition in the *Haematococcus* species and strains on the whole was relatively constant. The fluctuations observed in the relative percentages of secondary carotenoids between the different species and strains of *Haematococcus*, could be due to particular species and strains requiring longer time periods to synthesise and accumulate secondary carotenoids. It would be fair to assume that when the aplanospores of the different species and strains were harvested, there would be some differences in their physiological and biochemical stage of encystment. Indeed the degree of esterification of astaxanthin (III) has been reported to be a function of the age of the cells (Grung *et al.*, 1992). It could therefore be assumed that the species and strains containing high levels of ketocarotenoid diesters are at a more advanced stage of encystment than those containing high levels of ketocarotenoid monoesters. It is conceivable that all the *Haematococcus* strains and possibly the two individual species would attain the same degree of esterification, and ultimately contain very similar secondary carotenoid pigment profiles given a longer period of time and the correct culture conditions. The *Haematococcus* species accumulated relatively low levels of canthaxanthin (IV) and  $\beta$ -carotene (I) relative to the other algal species screened.

The two strains of *O. minuta* accumulated very different relative levels of secondary carotenoids from each other, with *O. minuta* CCAP 257/6 showing a very high degree of esterification, although given a longer period of time the two strains may have reached similar levels of secondary carotenoid esterification. The remaining algal species accumulated very different levels of secondary carotenoids. *G. kupferri* and *M. contortum* accumulated relatively high levels of canthaxanthin (IV) relative to the other algal species. *M. contortum* showed a relatively high degree of esterification as did *O. minuta* 257/6, relative to the levels of mono ketocarotenoid esters the two species accumulated.

The species contained varying levels of  $\beta$ -carotene (I) with *O. minuta* CCAP 257/5 and 257/6 and *B. engadenensis* containing relatively high levels.

The accumulation of secondary carotenoids and the re-greening of red cells followed a relatively similar pattern in all the species investigated. The accumulation of appreciable amounts of secondary carotenoids within the cells required a relatively long period of time (8 - 14 weeks), in contrast the re-greening of the algal cells only required approximately 2 weeks. This is primarily due to the fact that the cells were not exposed to high light intensities which significantly increases the rate of secondary carotenoid synthesis in microalgae (see Chapter 6). During the re-greening of the algal cells the secondary carotenoids were rapidly metabolised and their levels quickly decreased, this decrease was concomitant with an increase in chlorophyll, primary carotenoid levels, and cell number.

The levels of ketocarotenoid diesters decreased rapidly in relation to the ketocarotenoid monoesters, this could have been because although the monoesters were constantly being metabolised, as the diesters were degraded they were metabolised initially into ketocarotenoid monoesters. Therefore, as ketocarotenoid monoesters were degraded they were replaced, in part, by the metabolised ketocarotenoid diesters. However, as the cells fully re-greened the levels of ketocarotenoid monoesters and diesters were relatively similar as the levels of secondary carotenoids became very low.

Once secondary ketocarotenoid synthesis in cells had commenced, the rate of accumulation in all the species occurred at a relatively constant rate. The levels of both monoesters and diesters of ketocarotenoid was initially similar, but as encystment continued the levels of monoesters increased in relation to the levels of the diesters. This would indicate that esterification of the diesters occurred at a slower rate than that of the free ketocarotenoid to monoesters. However, in the latter stages of the experiments the levels of diester ketocarotenoids increased relative to the levels of the monoesters, indicating an increase in the rate of esterification of the ketocarotenoid monoesters, or a reduction in the synthesis of ketocarotenoid monoesters.

The results indicate that the synthesis of secondary ketocarotenoids is initiated while cell number is still increasing, albeit at a reduced rate. This is in contradiction to previous

reports which state that an inhibition of cell division is a pre-requisite for secondary carotenoid synthesis (Droop, 1955; Boussiba and Vonshak, 1991), and that secondary carotenoid accumulation is induced whenever a disturbance in cell division is imposed. Although the number of cells is affected by the levels of macro-nutrients present in the growth medium, the synthesis of secondary carotenoids does not seem to be a function of cell division. Therefore, secondary carotenoid synthesis can occur in actively dividing cells; this is in agreement with Bubrick (1991). However, it would be fair to assume that if cells were exposed to an inhibitor of cell division which did not impair the ability of the cells to assimilate carbon, then secondary carotenoid synthesis would be induced.

The synthesis of secondary carotenoids can also commence while the levels of chlorophyll and primary carotenoids are also increasing. It has previously been suggested that under nitrogen deficient conditions the levels of chlorophyll and primary carotenoids decrease. This decrease is thought to be due to a decrease in the number of pigment-protein complexes (Vechtel *et al.*, 1992b). The pigment-protein complexes lose their protection by membrane lipids and after thylakoid destruction, the primary carotenoids within the pigment-protein complexes become freely accessible for degradation enzymes. Further hypotheses suggest that the phytol derived from degraded chlorophyll is converted to secondary carotenoids in the green alga (Czygan, 1968b). However, the results in the present study show that appreciable levels of chlorophyll can remain whilst the level of secondary carotenoids in the algal cells increases significantly.

### **3.4.2 Commercial production**

Considering the individual species and strains as potential producers of secondary carotenoids for commercial purposes on the basis of secondary carotenoid content, the species and strains of *Haematococcus* would seem to be potentially the most economically viable of the species and strains screened. All the *Haematococcus* species and strains produced over 92% ketocarotenoid of the total carotenoid content, higher than any of the other species. The strains of *H. pluvialis* and *H. lacustris* accumulated very similar levels of secondary carotenoids.



Table 3.13 presents the ketocarotenoid levels of the algae screened as percentages of the total carotenoid composition, and the amount of ketocarotenoid accumulated per litre of algal culture. These two criteria were used to determine which species and strains of microalgae would be most suitable for mass cultivation purposes for the production of secondary carotenoids. Each algal species and strain was rated 1 to 15 for each of the two criteria used in the evaluation. The species and strains assigned the lower numbers were those producing the higher amounts of ketocarotenoid per litre of algal culture and as a percentage of the total carotenoid present. The total of the two numbers assigned to each species and strain was then calculated. The alga with the lowest number was rated as the best potential candidate for the commercial production of secondary carotenoids, whilst the alga with the highest number was rated as the poorest potential candidate.

Table 3.13 Potential of algal species (studied) for the production of secondary carotenoids.

Species	Source	mg Ketocarotenoid per litre algal culture	% Ketocarotenoid of total carotenoid present	Rating of each alga as a possible candidate for the commercial production of secondary carotenoids
<i>H. pluvialis</i>	34/6	116	98.3	2
<i>H. pluvialis</i>	34/7	142	96.2	1
<i>H. pluvialis</i>	34/8	64	97.8	6
<i>H. lacustris</i>	34/11	46	93.9	8
<i>H. lacustris</i>	34/1n	91	96.1	6
<i>H. lacustris</i>	34/1k	91	98.4	3
<i>H. lacustris</i>	34/1d	91	94.8	4
<i>H. lacustris</i>	34/1f	125	92.4	4
<i>O. minuta</i>	257/5	21	88.3	13
<i>O. minuta</i>	257/6	20	89.5	12
<i>G. kupfferi</i>	33/1	23	91.8	10
<i>M. sphaericum</i>	256/1	23	91.8	9
<i>M. contortum</i>	245/1	2	77.9	15
<i>B. engadenensis</i>	221/3	4	87.2	14
<i>C. rectangularis</i>	218/2	26	88.3	11

Considering the data presented in table 3.13 both *H. pluvialis* 34/6 and 34/7 are rated as potentially the most economically viable algal strains in terms of commercial secondary carotenoid production. Due to the small differences in secondary carotenoid levels between the different strains of *H. pluvialis* and *H. lacustris*, this table should only be considered as a guide. The definitive technique of determining the best algal



species/strain when simply considering secondary carotenoid content alone, would have been to separately determine the optimum conditions required for growth and secondary carotenoid synthesis of each algal species/strain. Then, by cultivating each alga under its own optimum conditions required for maximum growth and secondary carotenoid synthesis under mass cultivation conditions, determine which species/strain produced the highest amounts of secondary carotenoids. Unfortunately, this was beyond the scope of the present study.

However, it should be recognised that other key factors are also important when determining the viability of a particular algal species for the commercial production of secondary carotenoids. In assessing the commercial potential of an alga for secondary carotenoid production a number of factors need to be addressed, these include: (i) the rate of growth of the alga; (ii) total level of ketocarotenoid synthesised; (iii) rate of synthesis of the ketocarotenoids; (iv) relative level of 'valuable' carotenoids (as a percentage of total carotenoid; (v) the need for the alga to have a relatively uncomplicated life history. All these factors need to be taken into account when considering an algal species as a possible commercial source of ketocarotenoids e.g. astaxanthin (III).

## MORPHOLOGICAL STUDY OF *HAEMATOCOCCUS PLUVIALIS* DURING SECONDARY CAROTENOID SYNTHESIS

### 4.1 INTRODUCTION

#### 4.1.1 Adaptive mechanisms in the Chlorophyta

The adaptation of green algae to extreme environmental conditions has been examined in many studies. Exposure to nutrient limitation, or suboptimal conditions of irradiance, temperature, and salinity results in an imbalance between the rate of photosynthesis and the substrates required for growth. The algae react to such imbalances in different ways by accumulating carbohydrates (Rhee, 1978), lipids (Shifrin and Chisholm, 1981), carotenoids (Ben-Amotz 1987) or by increasing excretion rates (Watanabe, 1980). Certain species have been shown to be capable of a morphological transformation, from vegetative cells to immobile aplanospores that are resistant to extreme environmental conditions, e.g. *Haematococcus* under nitrogen starvation (Droop, 1954) and *Chlamydomonas* under chilling conditions (Kawecka, 1981). Aplanospore formation has also been reported in the Dinophyceae (Tangen *et al.*, 1982) and Rhodophyceae (Hymes and Cole, 1983).

Aplanospores in the Chlorophyceae, in contrast to vegetative cells, are characterised by a lack of motility (Droop, 1954; Kawecka, 1981), ketocarotenoid accumulation in the protoplast (Lang, 1968), the presence of a cell wall resistant to extreme environmental conditions (Kawecka, 1981) and lower photosynthetic rates (Hagen *et al.*, 1992). The reduced ability of the algae to perform photosynthesis forces the aplanospore to save energy by cessation of growth and motility, and decreasing the rate of excretion. During the transition, the respiration rate increases due to significant morphological and biochemical changes (e.g. pigment content and composition). These reactions increase the ability of the aplanospores to survive under extreme environmental conditions (Zlotnik *et al.*, 1993). The ketocarotenoids generally accumulated in the

Chlorophyta under extreme environmental conditions are astaxanthin (III), canthaxanthin (IV), echinenone (II), adonirubin (V) and  $\beta$ -carotene (I). The hydroxy carotenoids typically occur as fatty acid esters.

The function of the ketocarotenoids present in lipid globules outside the chloroplast is unknown. In *Eremosphaera viridis*, astaxanthin (III) was detected in the light harvesting protein of PSI. The authors concluded that the astaxanthin (III) provided protection of the photosynthetic pigment protein complex from photosensitised damage (Vechtel *et al.*, 1992b), essentially in a manner similar to that of primary carotenoids (Siefermann-Harms, 1987). In aplanospores the main function of the secondary carotenoids is thought to be as a 'passive' photoprotectant (i.e. filter), minimising the amount of light available for absorption by the light-harvesting pigment-protein complexes of PSII (Yong and Lee, 1991). The esterification of the relatively polar, hydroxyl-containing astaxanthin (III) with fatty acids, represents a mechanism by which the chromophore can be localised within cytoplasmic globules to maximise the photoprotective efficiency of the astaxanthin (III) (Bidigare *et al.*, 1993).

However, protective activities of the extrachloroplastic secondary carotenoids are not explained by shading alone. Extrachloroplastic secondary carotenoids may represent a physico-chemical protective barrier, preventing photodynamic free radical-mediated damage. As secondary carotenoids initially surround the nucleus before filling the entire protoplast, this may suggest a specific protection of the genetic material. This 'shield' may stabilize membrane systems and act as a buffer for oxidative reactions as well as quenching radical reactions directly (Hagen *et al.*, 1993a).

#### 4.1.2 Aims

The morphological changes occurring in *H. pluvialis* cells during secondary carotenoid accumulation have been investigated. This involved the use of transmission electron microscopy (TEM), scanning electron microscopy (SEM), image analysis techniques and the use of phase contrast light microscopy. Changes in cell size and the ability of individual cells to synthesise and store astaxanthin (III) during secondary carotenoid formation were studied using electronic image analysis. Sites of astaxanthin (III) synthesis and changes in the ultrastructure of cells during the accumulation of this carotenoid were

monitored using TEM. Light microscopy was used to investigate the various stages of the life cycle of *H. pluvialis* as well as the morphology of the alga during the various stages.

## 4.2 MATERIALS AND METHODS

### 4.2.1 General morphology

*Haematococcus pluvialis* strain 34/7 was obtained from the Culture Collection of Algae and Protozoa, Windermere, U.K. The alga was cultured in nitrogen-deficient BBM medium to induce secondary carotenoid formation. Culture conditions were the same as those previously described in section 2.7.1.

Transmission electron microscopy was carried out using a Corinth 300 electron microscope. Samples were prepared by first separating the algal cells from the growth medium by centrifugation. The cells were then washed in distilled water to remove any impurities from the samples and fixed overnight at 4°C in 2.5% (w/v) glutaraldehyde solution. This treatment was found to buffer the system adequately and additional buffers were not necessary. Post-fixation using 4% (w/v) osmium tetroxide was carried out for 1 hr at 4°C, followed by dehydration through an ethanol series. The material was embedded in Spurr's resin (Spurr, 1969). Sections were post-stained with uranyl acetate and lead citrate before being examined.

Scanning electron microscopy was carried out on a Jeol JSM-840 electron microscope. Accelerating voltages of 1 - 15 kV were used. SEMs of the alga were taken while the alga was immobilised on small pieces of polyurethane foam. The foam was initially cut into ~1 cm cubes and boiled in distilled water for 15 minutes. The foam was boiled 5 times in total to leach out any toxins that may have been present in the foam. The foam was then autoclaved and aseptically transferred into 250 ml Erlenmeyer conical flasks containing 50 ml of green algal culture. The flasks were then placed on an orbital shaker under conditions previously described (see section 2.7.1). The foam was incubated with the algae until the algae had reached the aplanospore stage of development, during which time the algae had immobilised onto the surface of the foam. The small pieces of foam were then removed from the flasks and air dried at 22°C for 24 hrs in the dark. The foams



were then transferred to a Polaron E3000 critical point drying apparatus. The foams were further dried by raising the temperature in the apparatus to 35°C. The dried foams were then fixed to SEM aluminium stubs with double-sided sticky tape and coated with gold in a Polaron E5000 sputter coating unit.

Light microscopy was carried out using a Polyvar, Reichter-Jung light microscope. Computer-aided microscopic image analysis was carried out using the video signal of a coloured CCD camera (KYF30, JVC). This was mounted on top of a light microscope (Polyvar, Reichter-Jung) which was processed by a commercially available frame grabber board (DT2871, Data Translation) and analysed using image processing software (PC Image, Foster Findley Associates). The program included standard modules of image processing, pre-processing, optical isolation of the cells from the background by threshold operations, calculation of cell size and optical intensity. In addition the program allowed for optical isolation of the ketocarotenoid lipid vacuoles from the cell body.

In the following investigations involving image analysis the initial starter cultures contained cells which had reached the 'swimmer' or palmella stage of development, and contained ~20 mg of chlorophyll per litre of algal culture. The cells in these 'green' initial starter cultures contained no ketocarotenoid. Investigations involving aplanospores of *H. pluvialis* used cultures which contained ~25 mg astaxanthin (III) per litre of algal culture. The cells in these 'red' cultures contained relatively low levels of chlorophyll, ~5 mg of chlorophyll per litre of algal culture.

Standard errors and statistical analysis were calculated using the formulae described earlier (see section 2.9).

## 4.3 RESULTS

### 4.3.1 General morphology

Computer-aided image analysis allowed the monitoring of ketocarotenoid, chlorophyll and cell area during secondary carotenoid formation. The microscope image is projected onto the photocathode of a vidicon tube. The image produces a pattern of conductivity which varies according to the brightness of the different regions; when a fine beam of electrons is scanned in a regular raster fashion over the rear surface of the photocathode, charge neutralisation produces a potential difference in a collector which is proportional to the original input brightness pattern. If the value of this signal is sampled at regular time intervals, this produces a means of obtaining a numerical representation of the image. Each number represents an individual picture point or pixel. The image is digitised into  $512 \times 512$  pixels, the optical density or 'grey level' of each pixel is represented by a number from 0 (which represents black) to 255 (which represents white). The digitised image is then stored in the computer for subsequent processing and measurement (Wiess *et al.*, 1989).

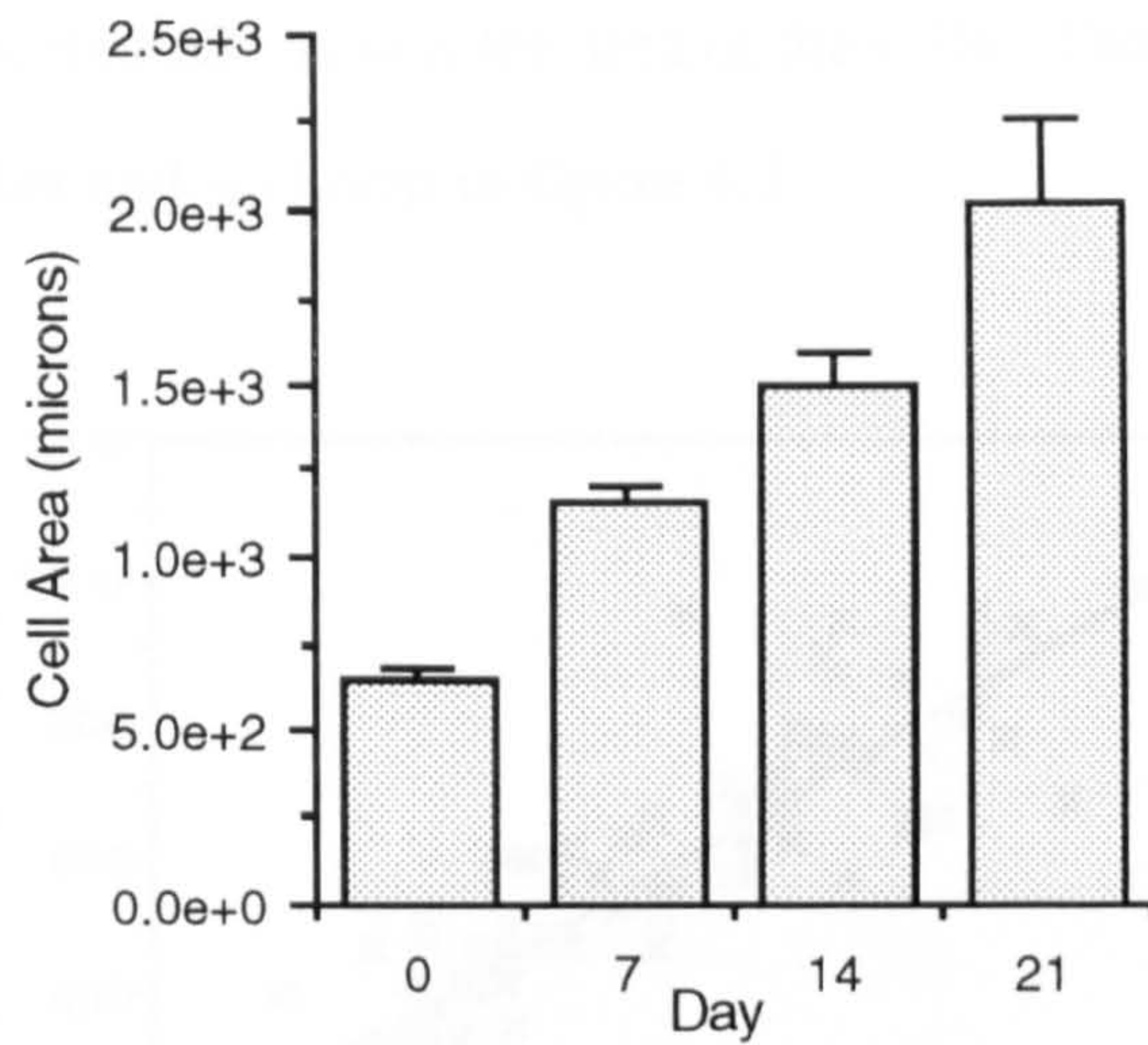
Before measurements can be made on a digitised image it is necessary to ensure that the computer only operates on those parts of the image which are significant. Once this is complete measurements are possible. Measurements may be either field-specific or object specific. Field specific data is derived from all the objects present in the field, whilst in the object specific mode each individually identifiable object gives rise to a set of measurements. The major measurements obtained from an image include total area, circumference, optical density and count. Calibration of the apparatus allows for measurement in absolute units i.e. mm,  $\mu\text{m}$  and nm etc. In the present study optical weight was defined as;

$$\text{Optical Density} = \log(255.5) - \log_{10}(g_i + 0.5)$$

where  $g_i$  = the grey level of the  $i^{\text{th}}$  pixel

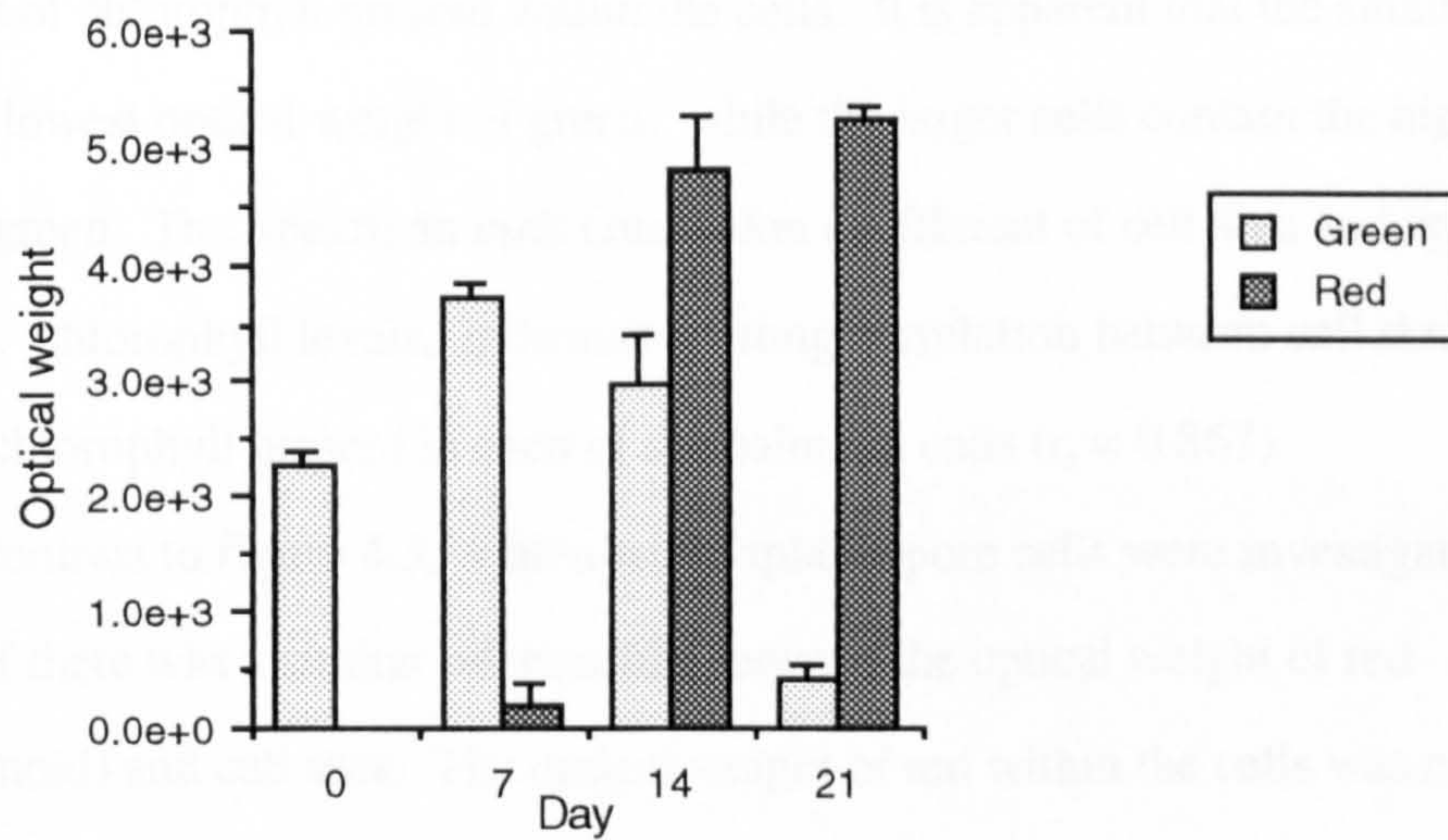
Figure 3.1 shows the increase in cell area during secondary carotenoid accumulation in *H. pluvialis* cells cultured in nitrogen deficient conditions.





**Fig. 4.1** Increase in cell area during secondary carotenoid formation (n=20).

During the experiment cell area increased by nearly 300%. The largest increases were observed between days 0 to 7 and days 14 to 21. This increase in cell area was concomitant with a decrease in the level of chlorophyll and an increase in the levels of ketocarotenoid in the cells as shown in figure 4.2.

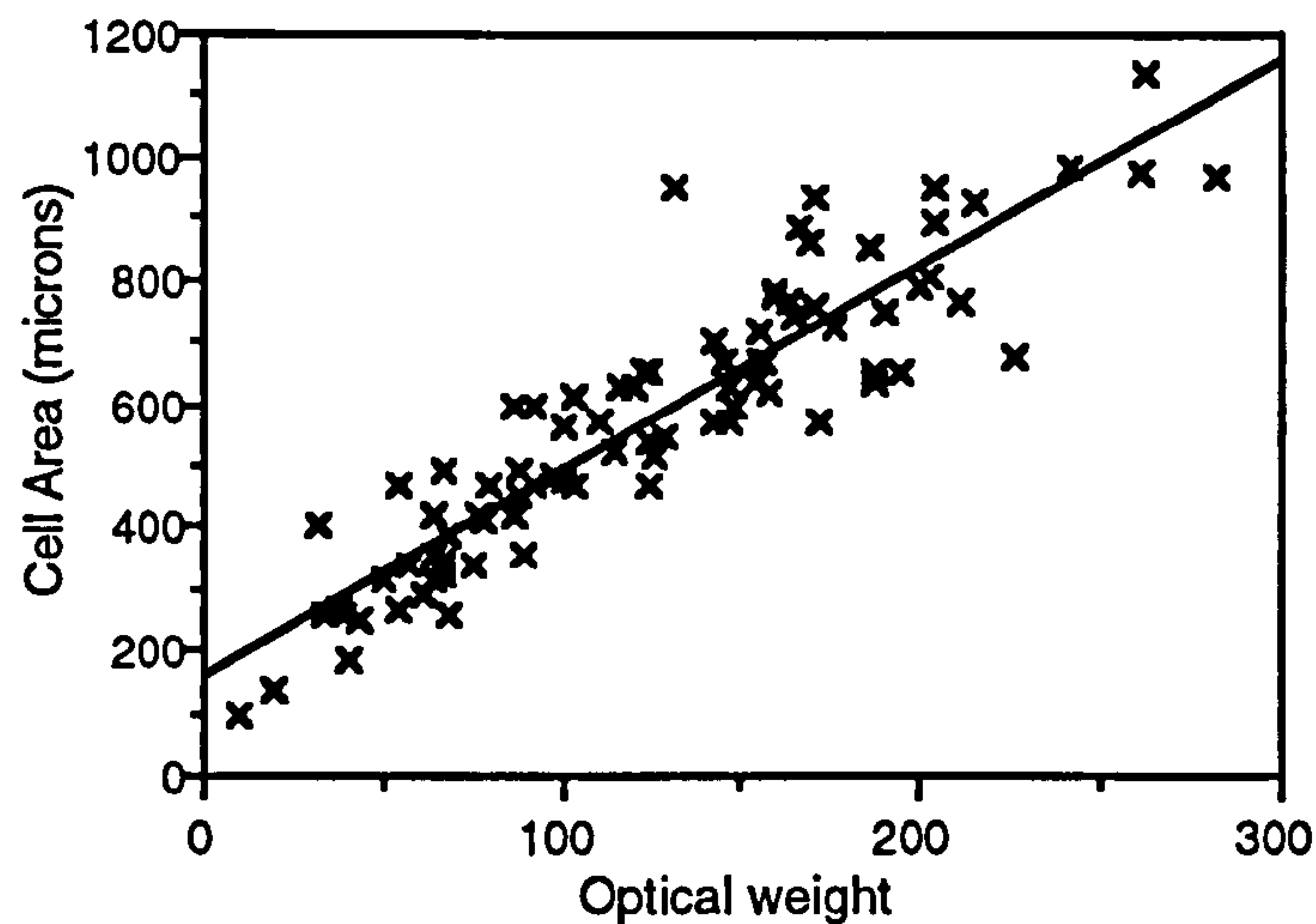


**Fig. 4.2** Changes in green (chlorophyll) and red (ketocarotenoid) during encystment (n=20).

The amount of green (chlorophyll) increased initially at the start of the experiment up to day 7, after which the level decreased particularly between days 14 and 21. Red (ketocarotenoid) accumulation became apparent on day 7. The levels increased extremely quickly between days 7 and 14, with a smaller increase between days 14 and 21.



The levels of green (chlorophyll) in palmella cells which contained no ketocarotenoid, were measured, as was the area of the cells. The two sets of data were plotted against each other and is shown in figure 4.3.

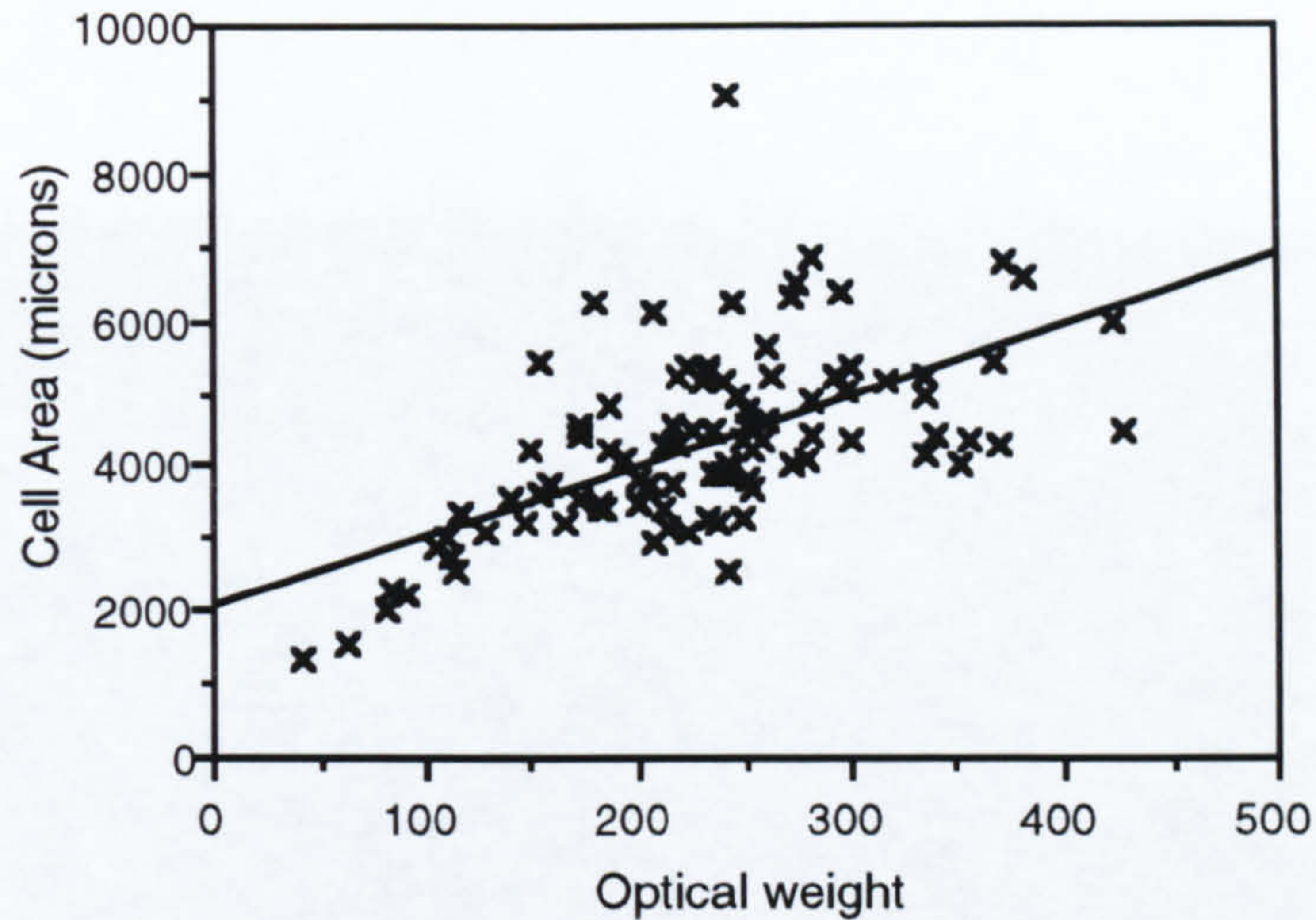


**Fig. 4.3** Optical weight of green (chlorophyll) present in palmella cells as a function of cell area ( $r_s = 100 = 0.867$ ,  $p < 0.05 >$ ).

Figure 4.3 indicates that as cell area increases so does the optical weight of green, i.e. amount of chlorophyll present within the cells. It is apparent that the smaller cells contain the lowest optical weight of green, while the larger cells contain the highest optical weights of green. The Spearman rank correlation coefficient of cell area and optical weight of green i.e. chlorophyll levels, indicates a strong correlation between cell size and the amount of chlorophyll present in each of the palmella cells ( $r_s = 0.867$ ).

In contrast to figure 4.3, a number of aplanospore cells were investigated to determine if there was a similar relationship between the optical weight of red (ketocarotenoid) and cell area. The optical weight of red within the cells was measured, as was the cell area. The two sets of data were plotted against each other as shown in figure 4.4. The image analysis equipment detected no chlorophyll within these cells.

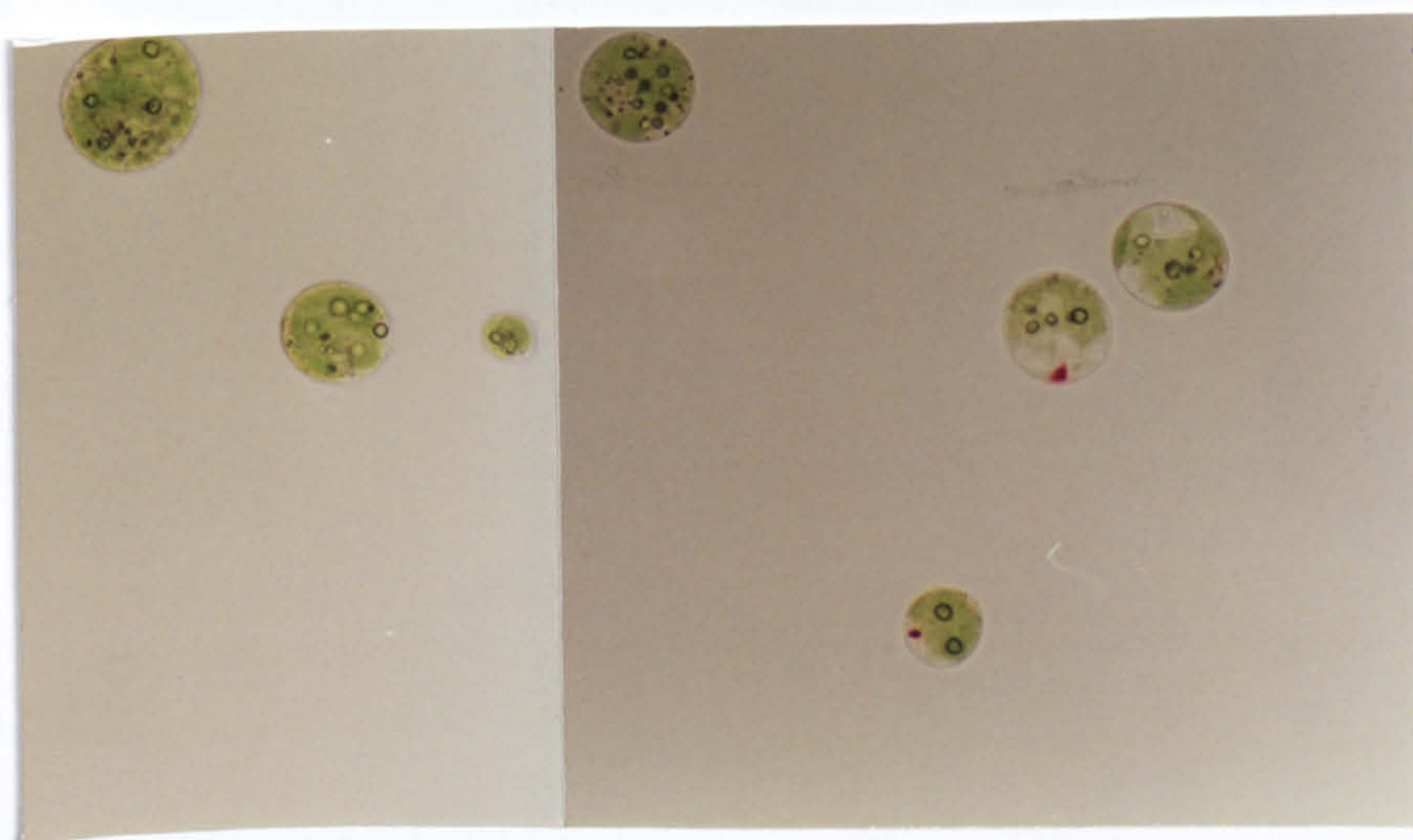




**Fig. 4.4** Optical weight (red) present in aplanospores as a function of cell area ( $r_s = 100 = 0.616$ ,  $p < 0.05$ ).

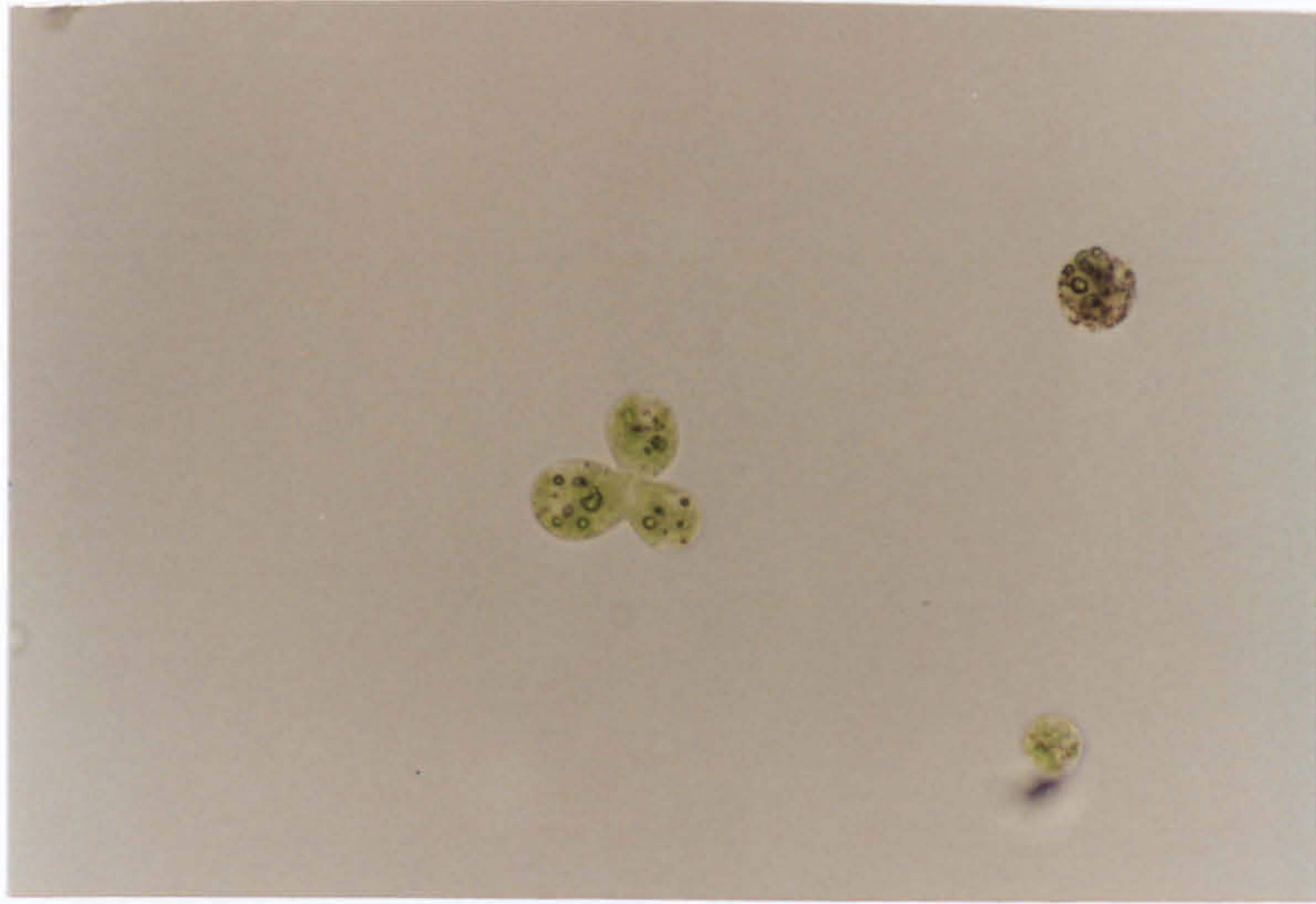
Figure 4.4 shows that the optical weight of red i.e. the amount of ketocarotenoid present in the cells can also be correlated with cell area. The Spearman rank correlation coefficient of cell area and intensity indicated that the parameters were correlated ( $r_s = 0.616$ ). However, this correlation is weaker than that observed between green (chlorophyll) and cell area in the palmella cells, which contained no astaxanthin (III) or other ketocarotenoids.

Plates 4.1 - 4.10 are light micrographs of *H. pluvialis* cells showing the different stages of the alga's life cycle.

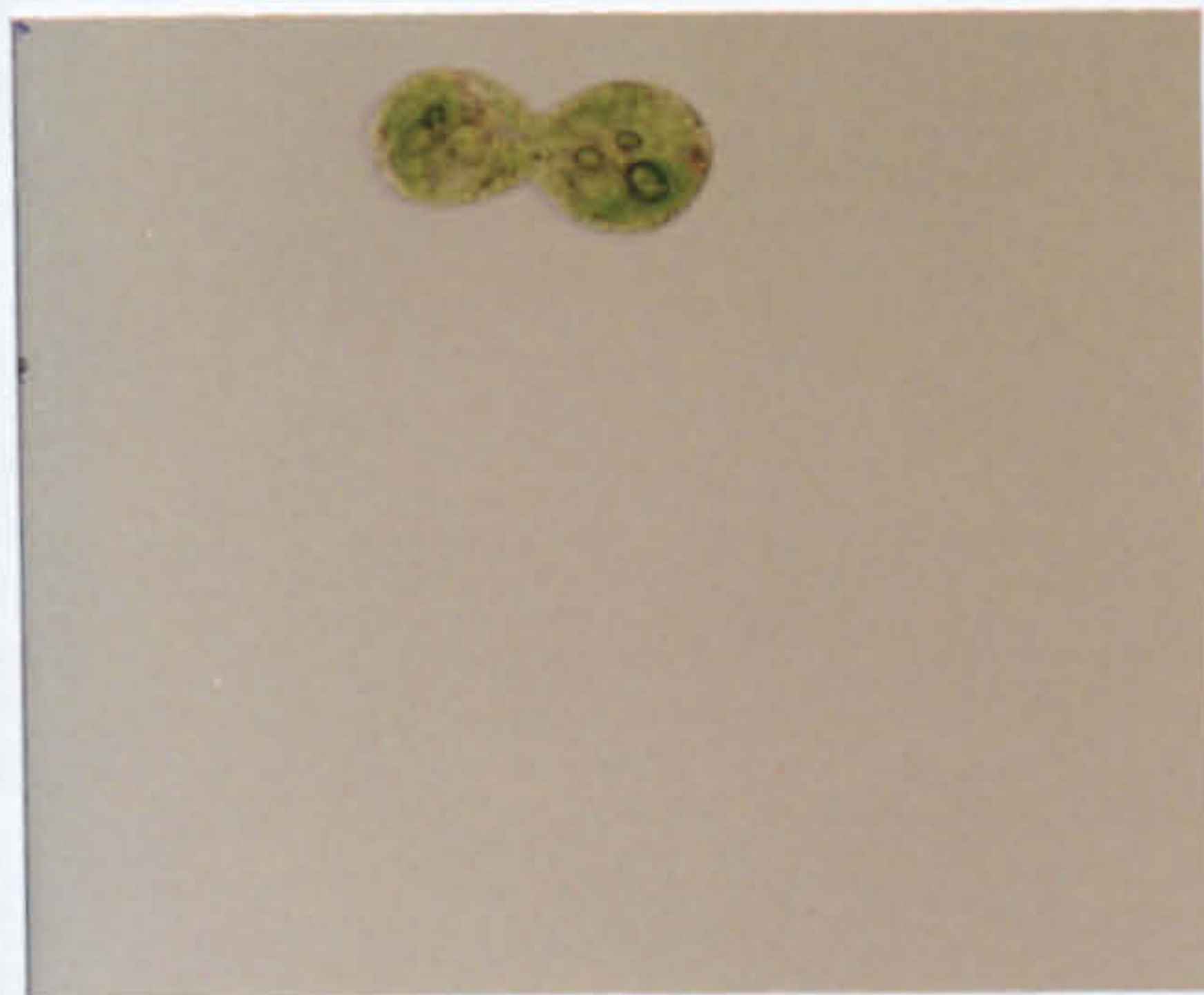


**Plate 4.1.** Green 'swimmer' cells of *H. pluvialis*. (mag. x 40)



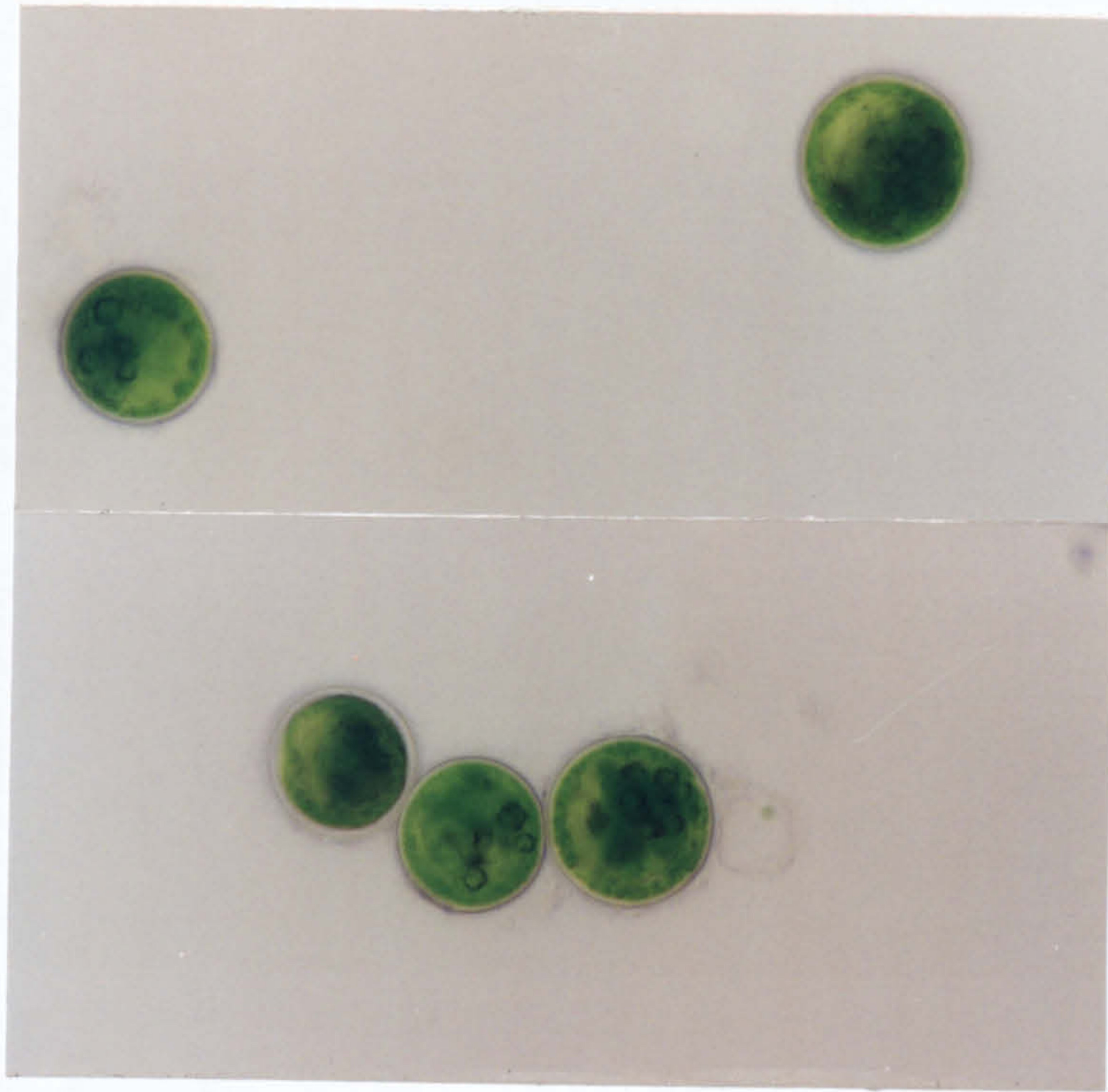


**Plate 4.2.** Green 'swimmer' cells of *H. pluvialis* dividing. (mag. x 40)

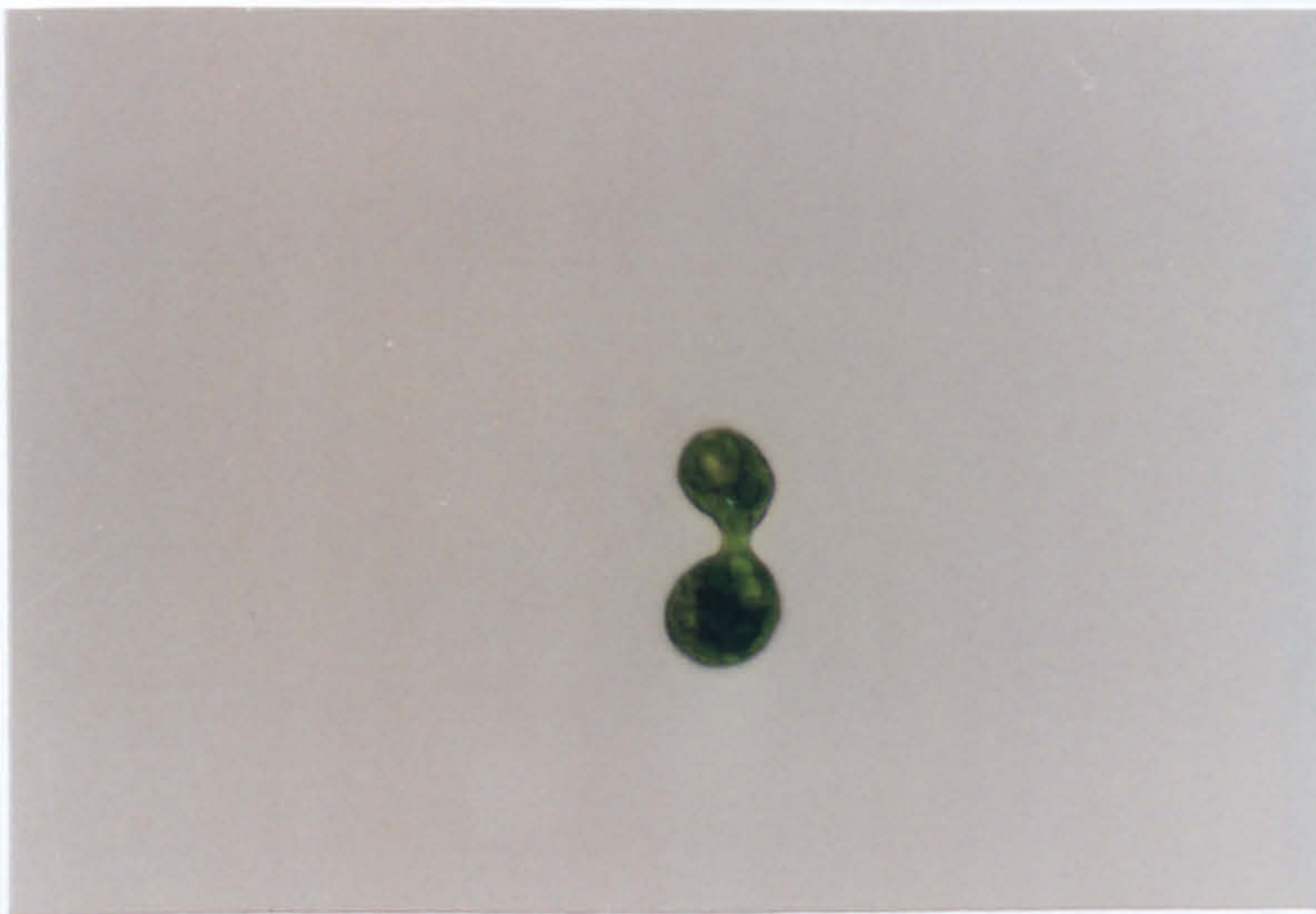


**Plate 4.3.** 'Swimmer' cells of *H. pluvialis* dividing which contain astaxanthin (III). (mag. x 40)



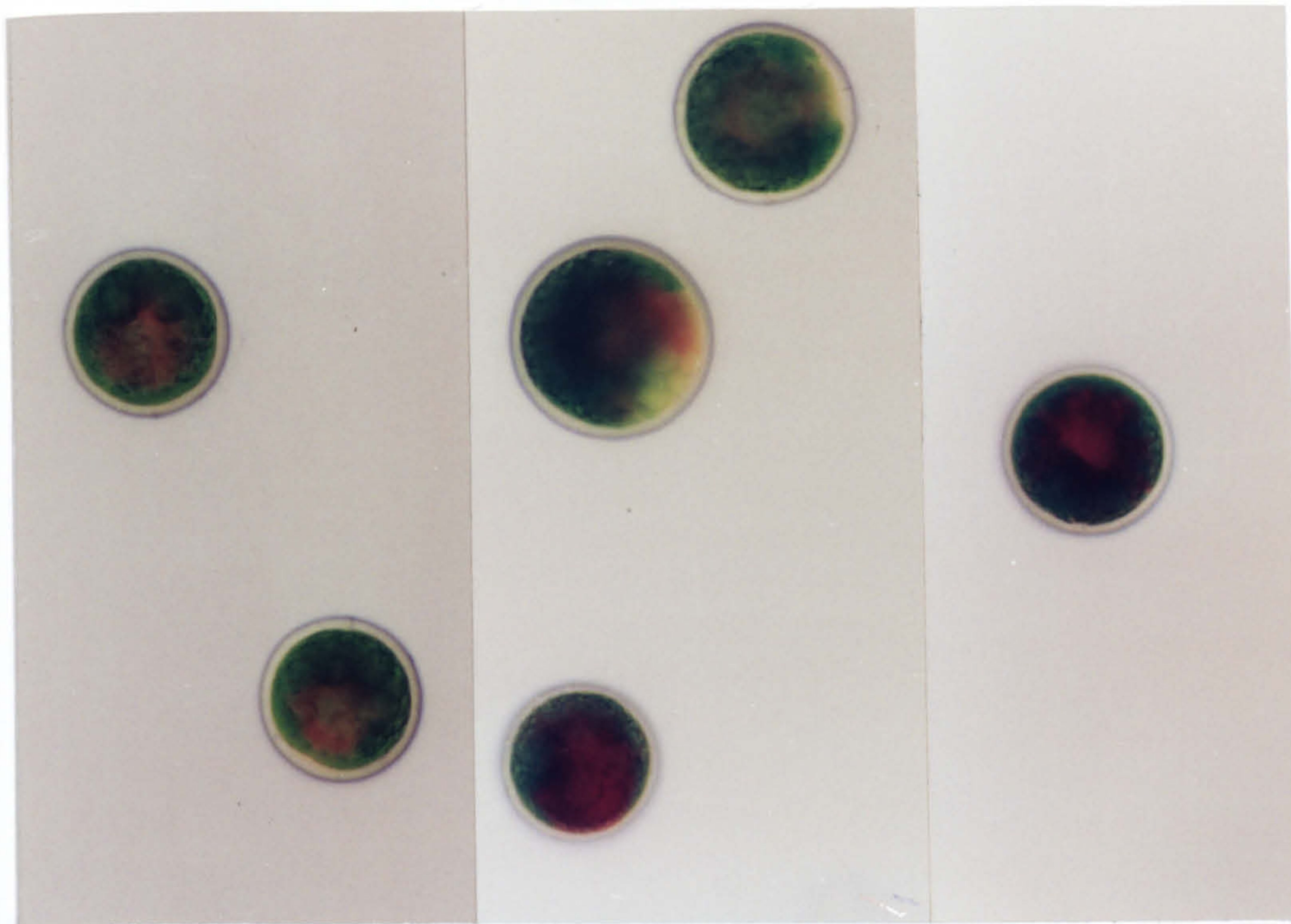


**Plate 4.4.** Green palmella cells of *H. pluvialis*. (mag. x 40)

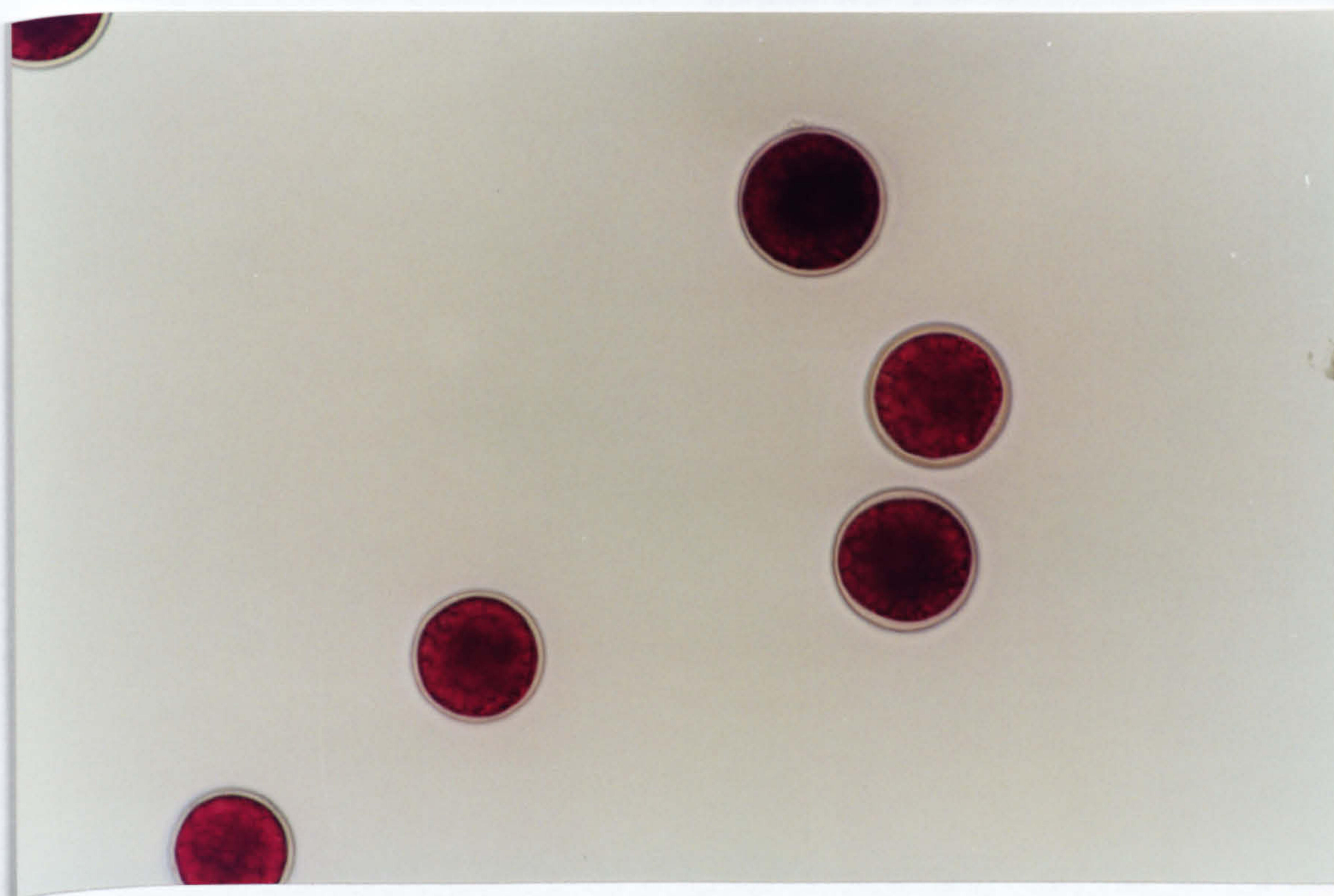


**Plate 4.5.** Green palmella cells of *H. pluvialis* dividing. (mag. x 40)



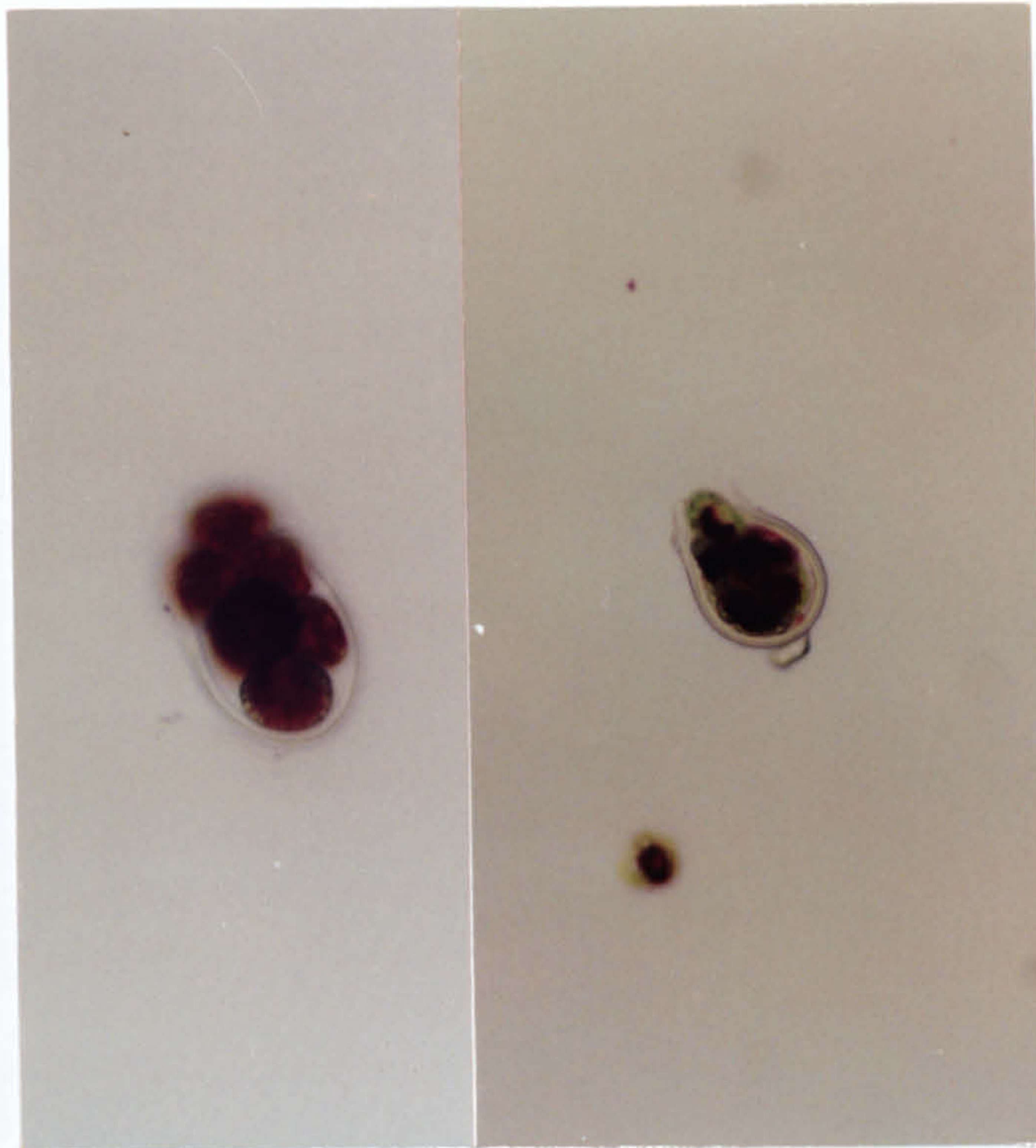


**Plate 4.6.** Palmella cells of *H. pluvialis* turning green to red. (mag. x 40)

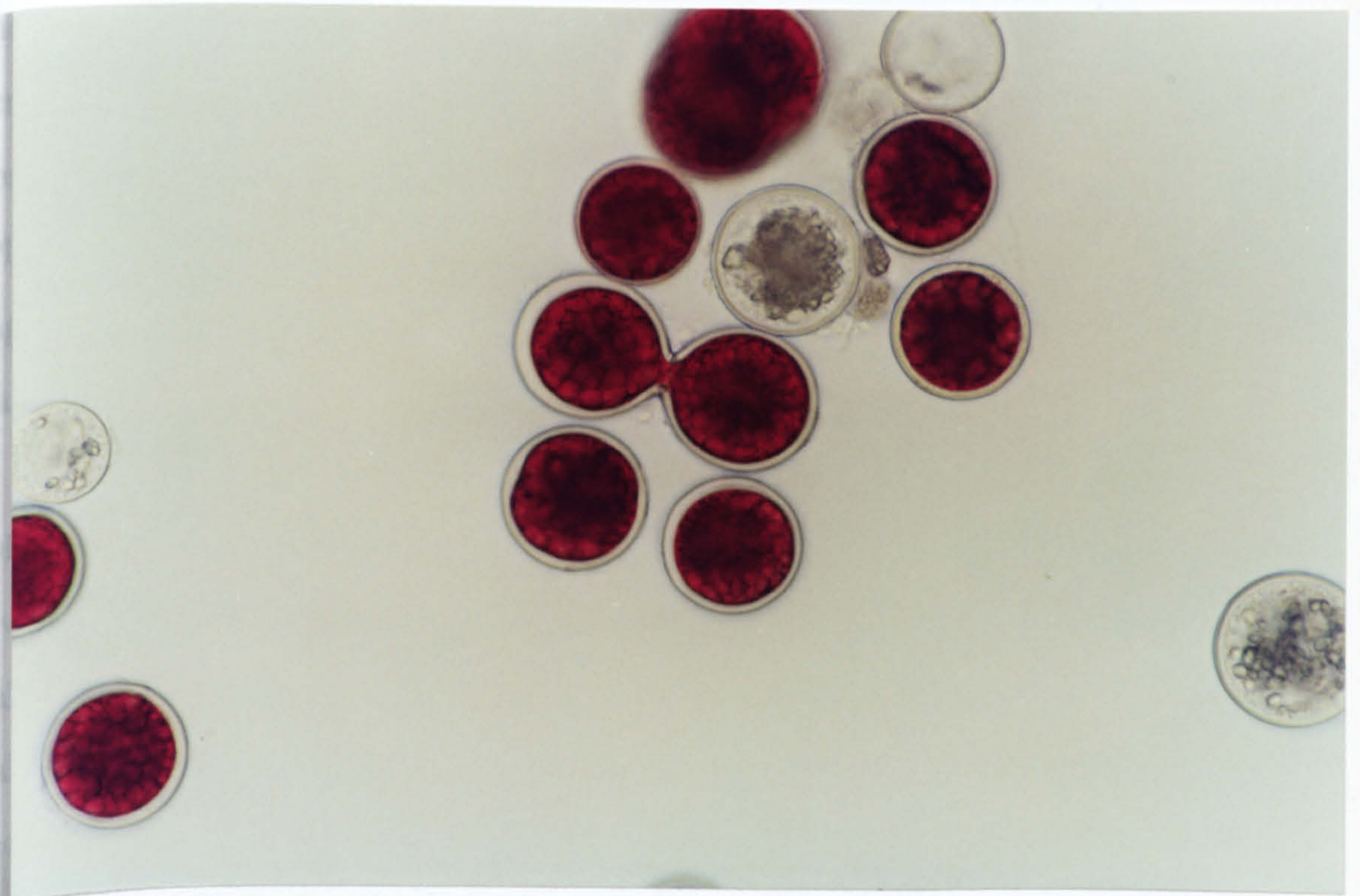


**Plate 4.7.** Red aplanospores of *H. pluvialis*. (mag. x 40)



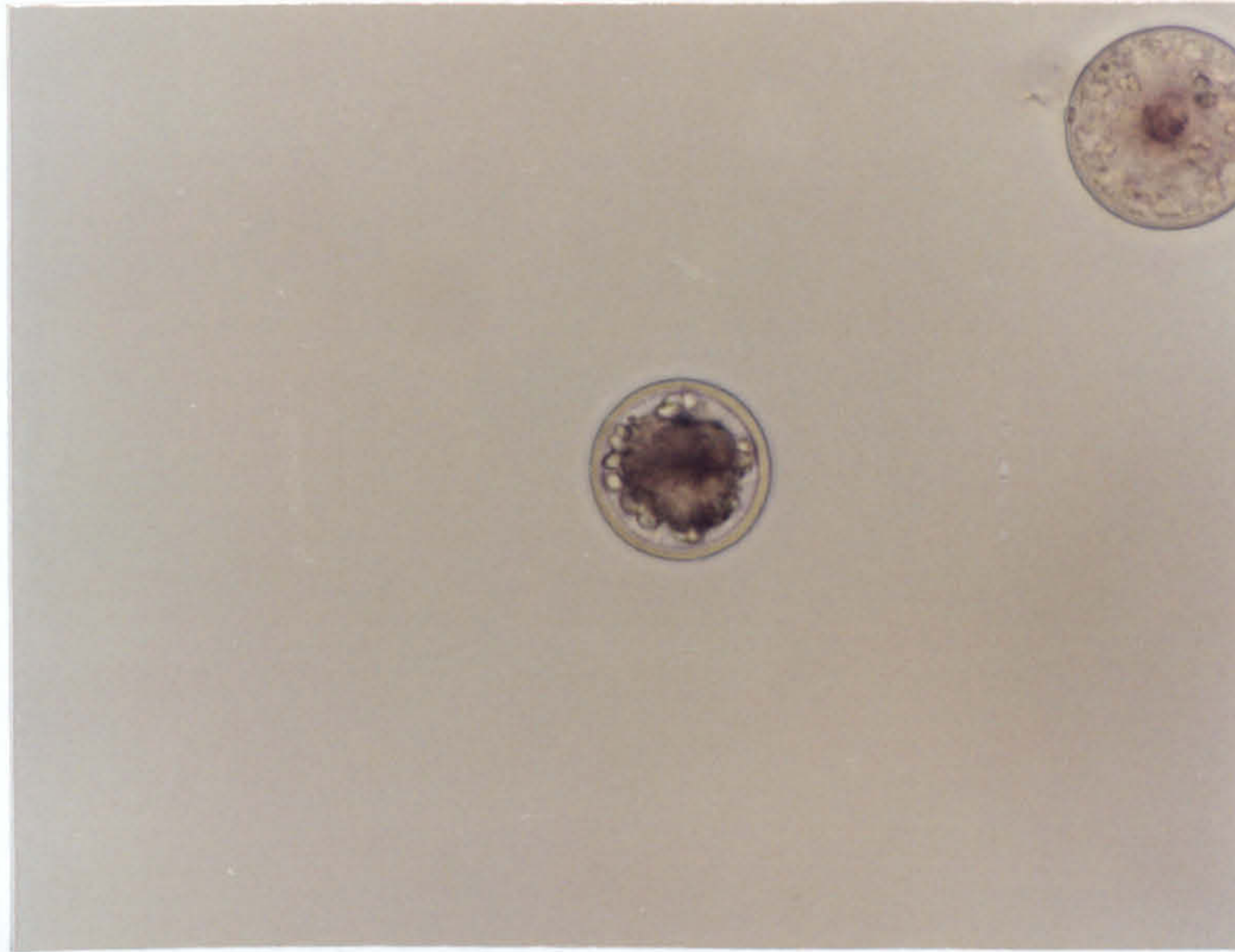


**Plate 4.8.** Rupturing aplanospore of *H. pluvialis* releasing 'swimmers'. (mag. x 40)



**Plate 4.9.** Red aplanospore of *H. pluvialis* dividing. (mag. x 40)





**Plate 4.10.** Bleached cells of *H. pluvialis*. (mag. x 40)

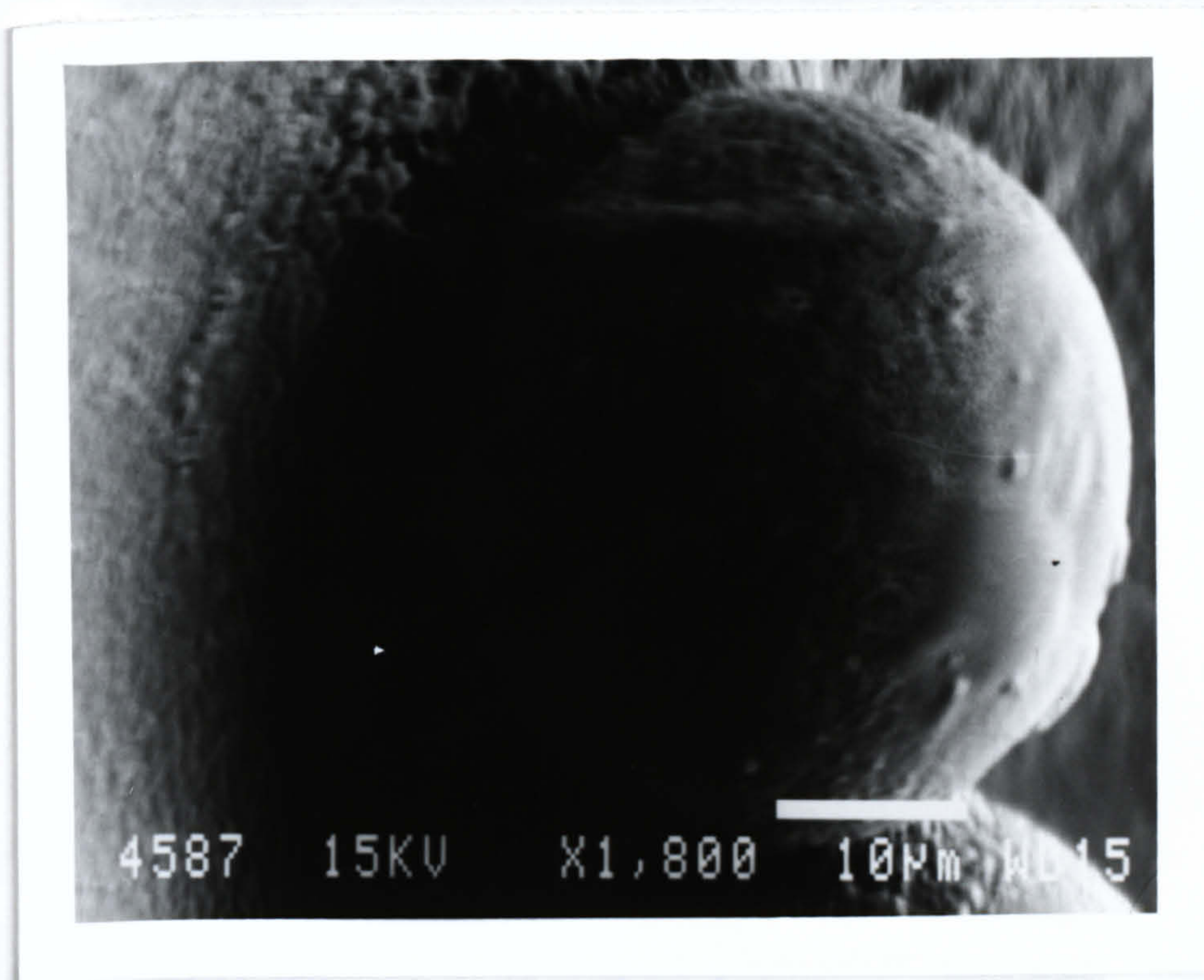
The micrographs of *H. pluvialis* illustrate the large morphological changes the cells undergo during encystment. The 'swimmer' cells (Plate 4.1) are pear shaped and have a very thin cell wall and two anterior flagella. Unfortunately, these flagella are not observable on the plate due to poor contrast. The 'swimmer' cells are capable of dividing (Plate 4.2) to produce more 'swimmer' cells or palmella cells. The swimmer cells are also capable of producing astaxanthin (III) (Plate 4.3). As the 'swimmer' cells age they transform into palmella cells.

The green palmella cells (Plate 4.4) contain no ketocarotenoid. The cells are spherical in shape and have lost their flagella. The cells are very green and do not possess a very thick cell wall as in the case of the aplanospores. In favourable growth conditions palmella cells divide to produce either more palmella cells or 'swimmers' (Plate 4.5). If the palmella cells are exposed to growth limiting conditions (i.e. nitrate or phosphate see section 6.3.1) they begin to synthesise ketocarotenoids. Initially the secondary carotenoids can be observed to accumulate in the centre of the cells around the nucleus. They then migrate to the periphery of the cells (Plate 4.6), this is concomitant with an apparent increase in the thickness of the cell wall. If the unfavourable growth conditions persist the palmella cells will develop into aplanospores.



The red aplanospores (Plate 4.7) are spherical in shape and contain large amounts of ketocarotenoid. The aplanospores possess a very thick cell wall. Under favourable growth conditions this wall will rupture, releasing 'swimmers' into the medium (Plate 4.8). The red aplanospores are also capable of dividing to produce more red aplanospores (Plate 4.9). In some cases during secondary carotenoid formation the cells lose all pigmentation and become bleached (Plate 4.10). This phenomena occurs particularly when the cells are exposed to extreme environmental conditions i.e. high light intensities or high salt concentrations.

Plates 4.11-4.14 show SEMs of *H. pluvialis* cells when immobilised on polyurethane foam.



**Plate 4.11.** Single *H. pluvialis* aplanospore immobilised on polyurethane foam.



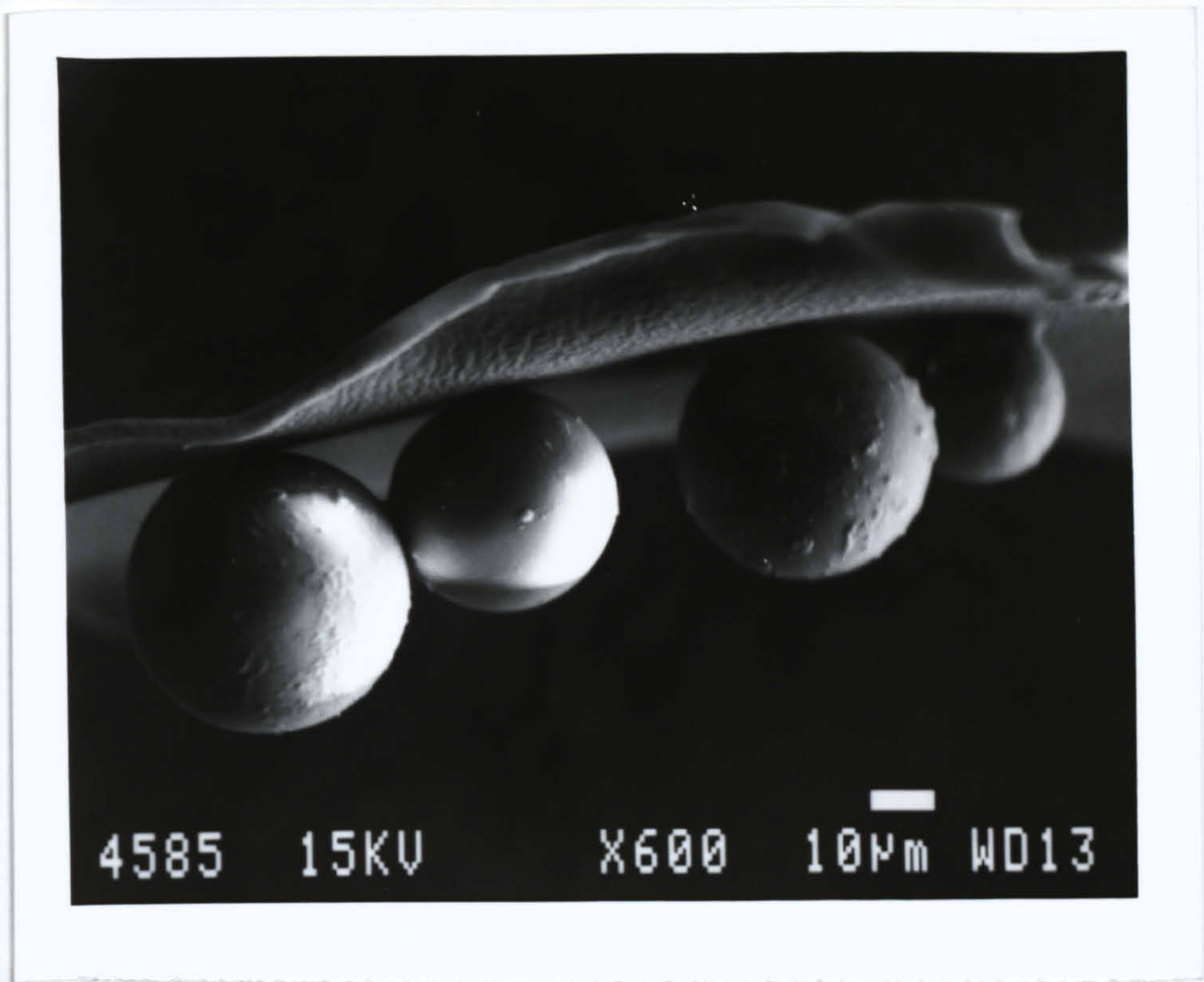


Plate 4.12 Four *H. pluvialis* aplanospores immobilised on polyurethane foam.

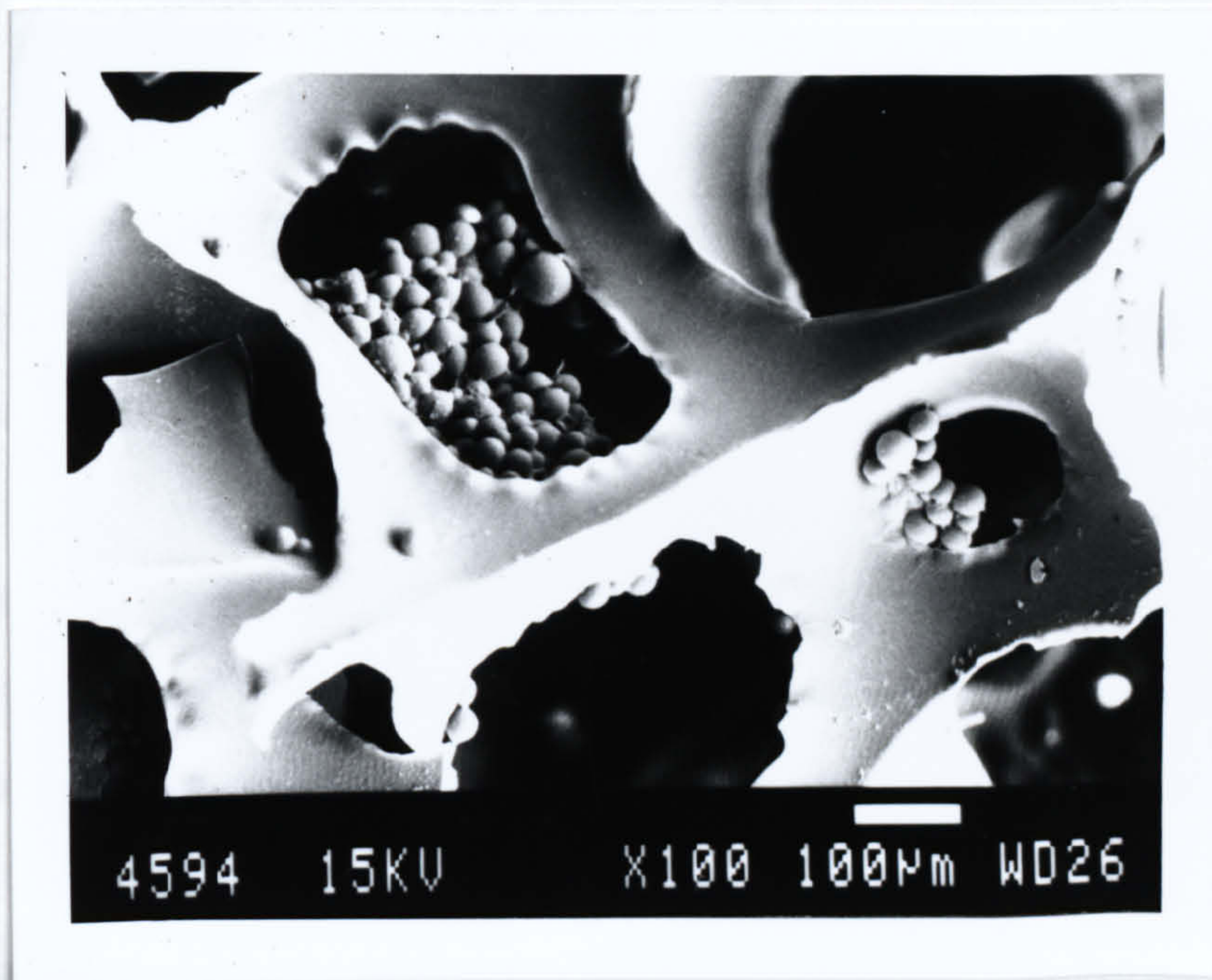
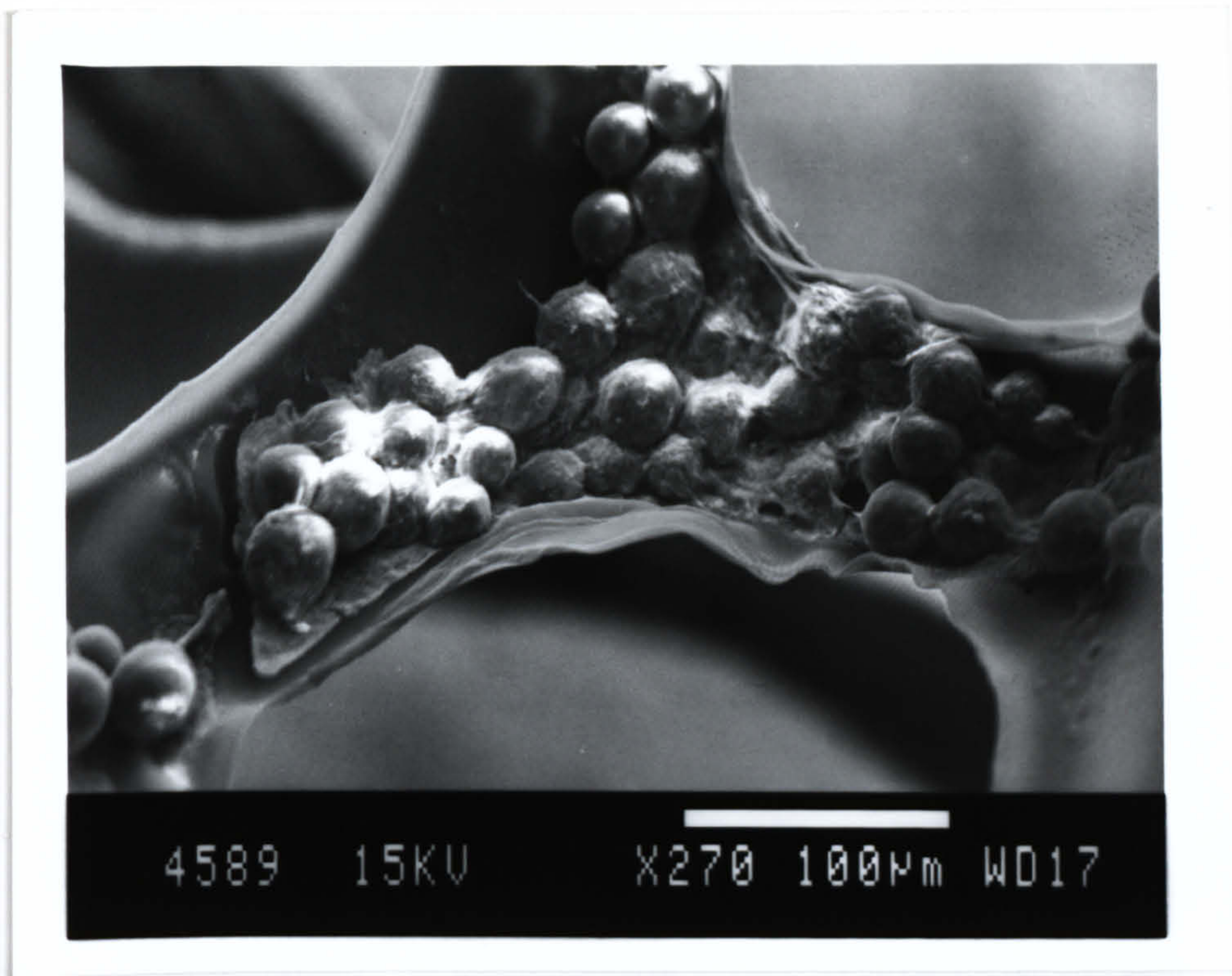


Plate 4.13 Group of *H. pluvialis* aplanospores immobilised on polyurethane foam.



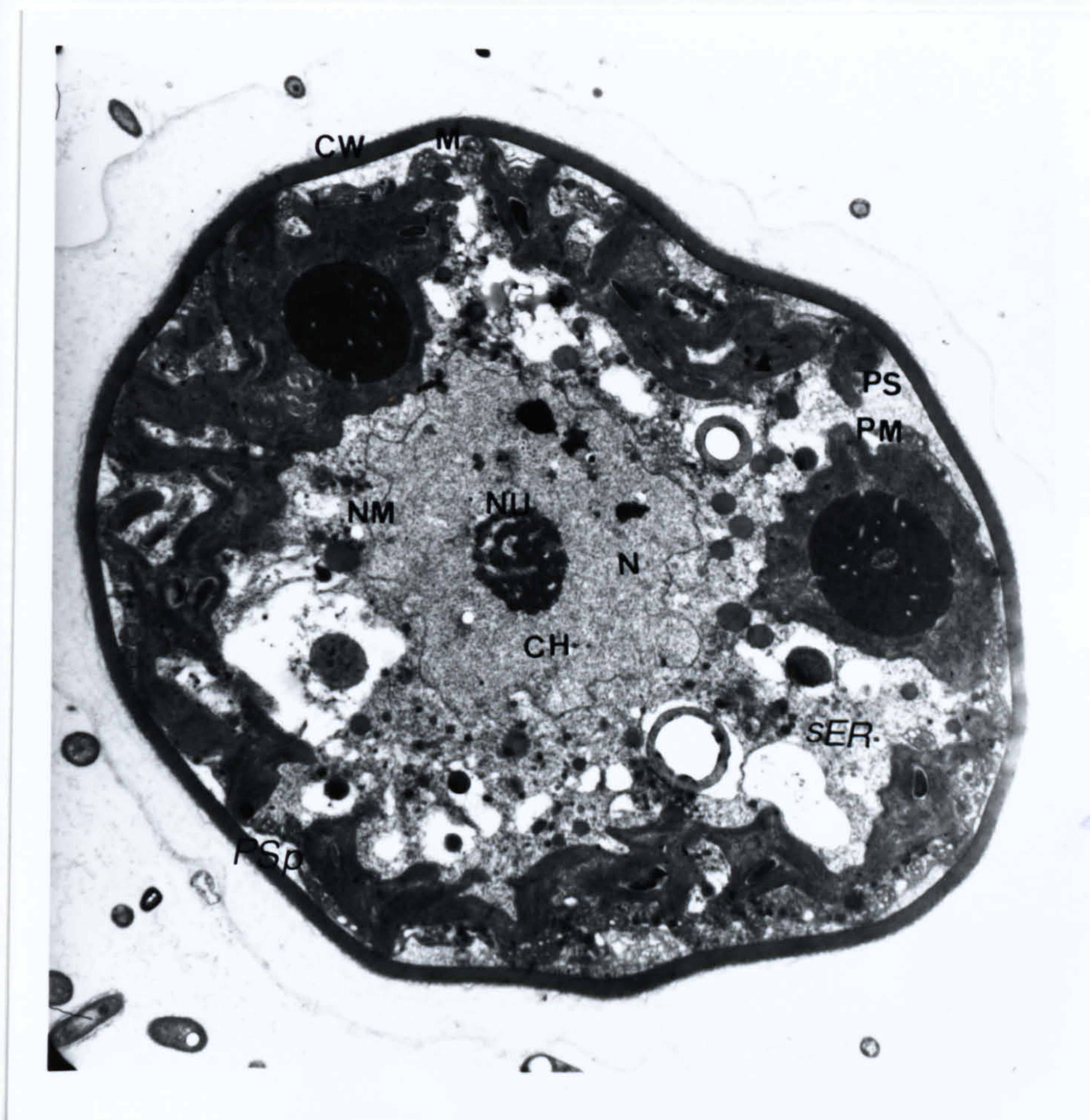


**Plate 4.14** Several groups of red *H. pluvialis* aplanospores immobilised on polyurethane foam.

The SEMs of the *H. pluvialis* aplanospores give an indication of their size. Plates 4.11 and 4.12 indicate that the aplanospores range from 35 - 50  $\mu\text{m}$  in diameter, these figures are in agreement with Elliot (1934) who stated that cells of *H. pluvialis* range in size from 8 - 50  $\mu\text{m}$ . The aplanospores can be seen to readily form agglomerates (Plates 4.13 and 4.14), binding to one another to form 'clumps' of aplanospores. This agglomeration would seem to involve some kind of secretion on the surface of the aplanospore which aids in adhesion to surfaces and neighbouring aplanospores. This secretion is especially apparent on the surfaces of the aplanospores in Plate 4.14. The exact nature of this secretion is unknown but it may have unforeseen consequences when the cells are grown under mass cultivation conditions.

Plates 4.15 - 4.20 show the ultrastructure of *H. pluvialis* cells during different phases of the life cycle of the alga.





**Plate 4.15** Green *H. pluvialis* cell. (mag. x 12,500)

Plate 4.15 of a whole green algal cell shows the cell to have a relatively large nucleus (N). In the centre of the nucleus is the nucleolus (NU) which is very dense. The nucleus also contains coagulated chromatin granules (CH) which are irregularly dispersed throughout the nucleolus. The nucleolus is separated from the cytoplasm by the nuclear membrane (NM), while the plasma membrane (PM) separates the cytoplasm from the cell wall (CW). Between the plasma membrane and cell wall is the perinuclear space (PSp), which contains protoplasmic strands (PS) linking the plasma membrane to the cell wall. Lighter coloured areas within the cell are areas of swollen endoplasmic reticulum (sER). Situated close to the plasma membrane are a number of elongated mitochondria (M).

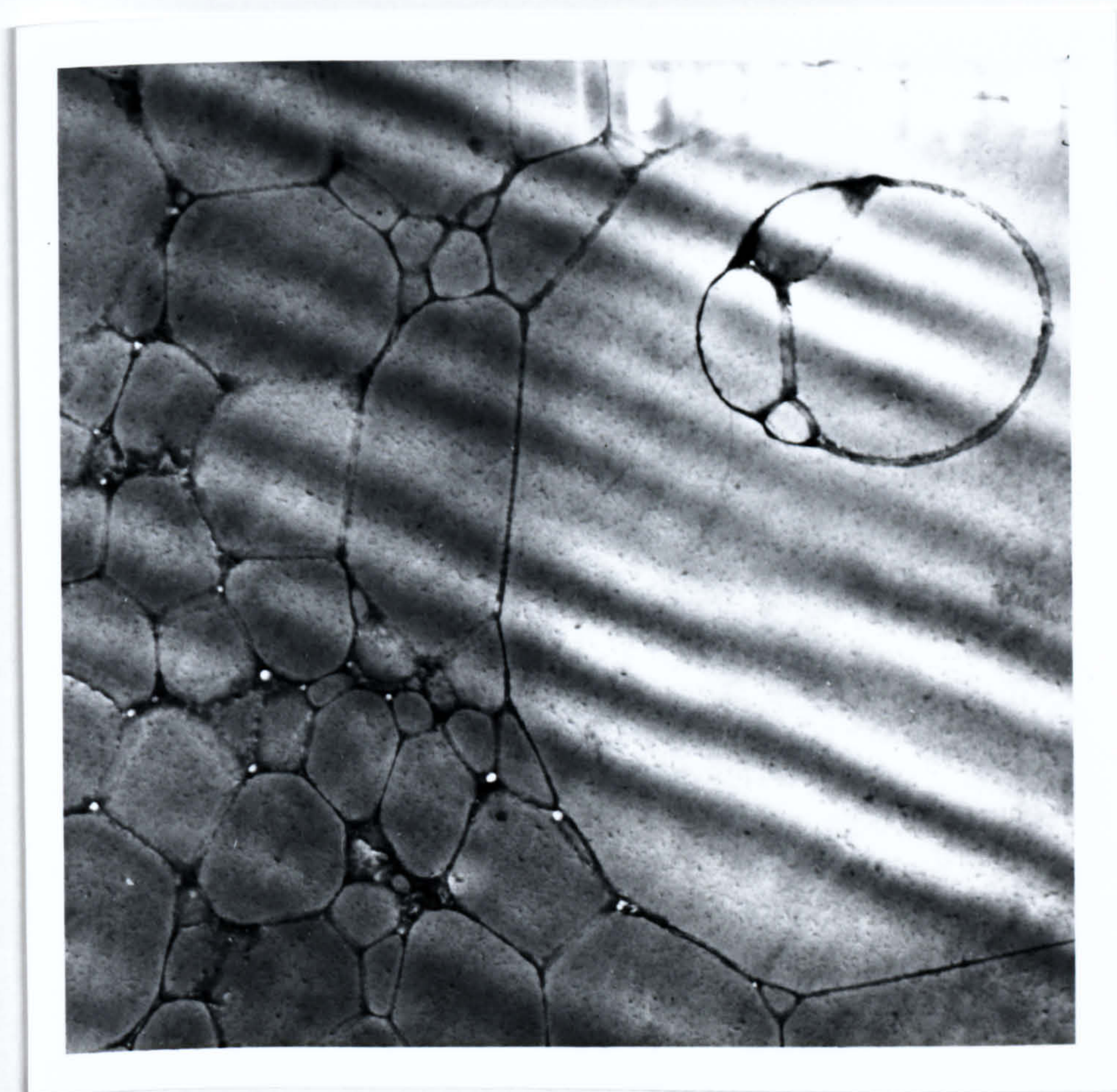




**Plate 4.16** Red aplanospore of *H. pluvialis*. (mag. x 4500)

In contrast plate 4.16 illustrates a red aplanospore which contains very large lipid globules which are observable throughout the whole cell. Very little internal structure can be discerned within the cell, apart from mitochondria (M) and vacuoles (V).

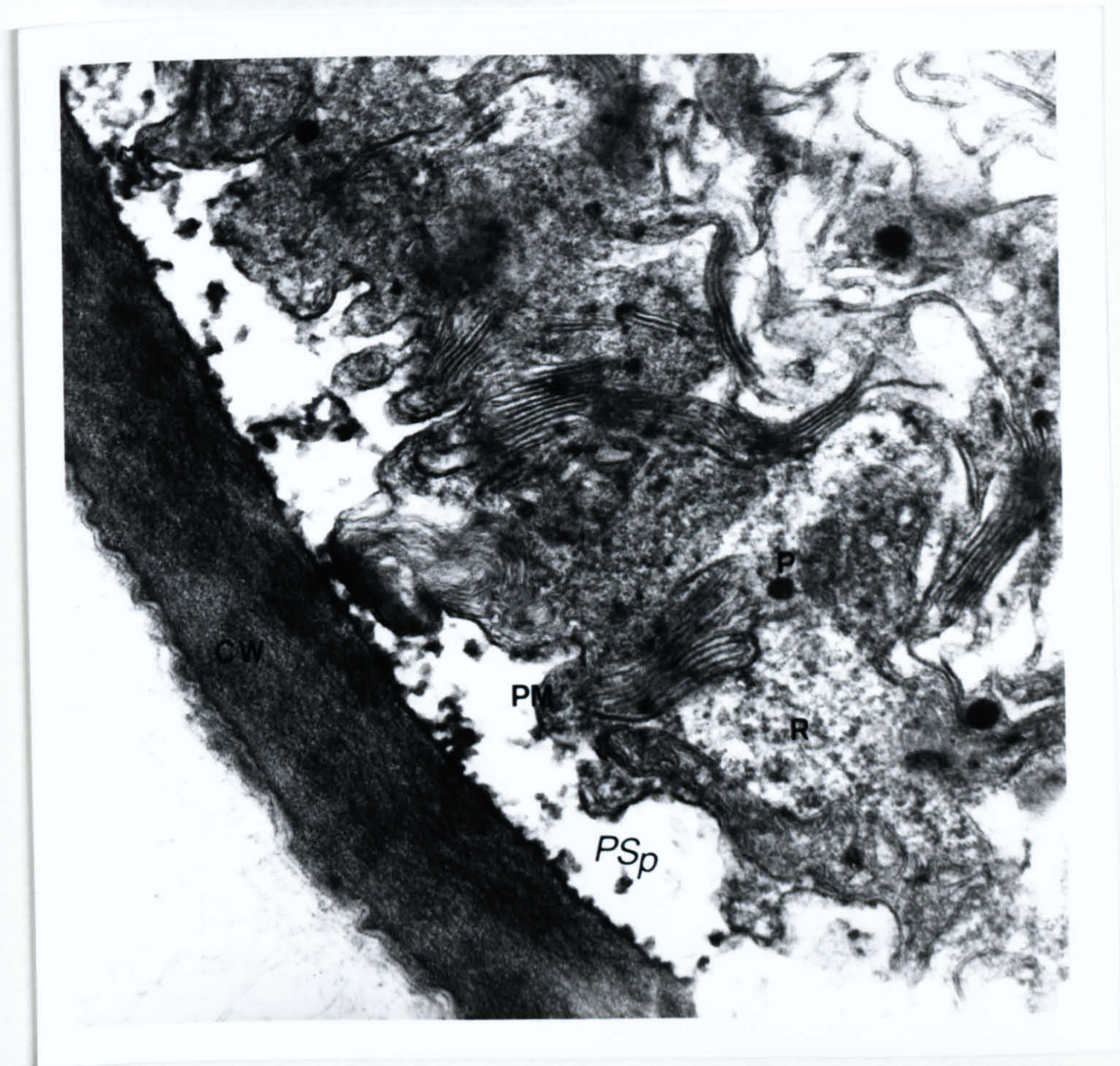




**Plate 4.17** Lipid containing globules in red *H. pluvialis* cells. (mag. x 20,000)

Plate 4.17 above shows the lipid globules which have been shown to contain the astaxanthin (III) and other related ketocarotenoids (Santos and Mesquita, 1984). The lipid globules vary in size and shape but are distinct structures from one another. It is not possible from the micrograph to determine if the lipid globules are membrane bound.

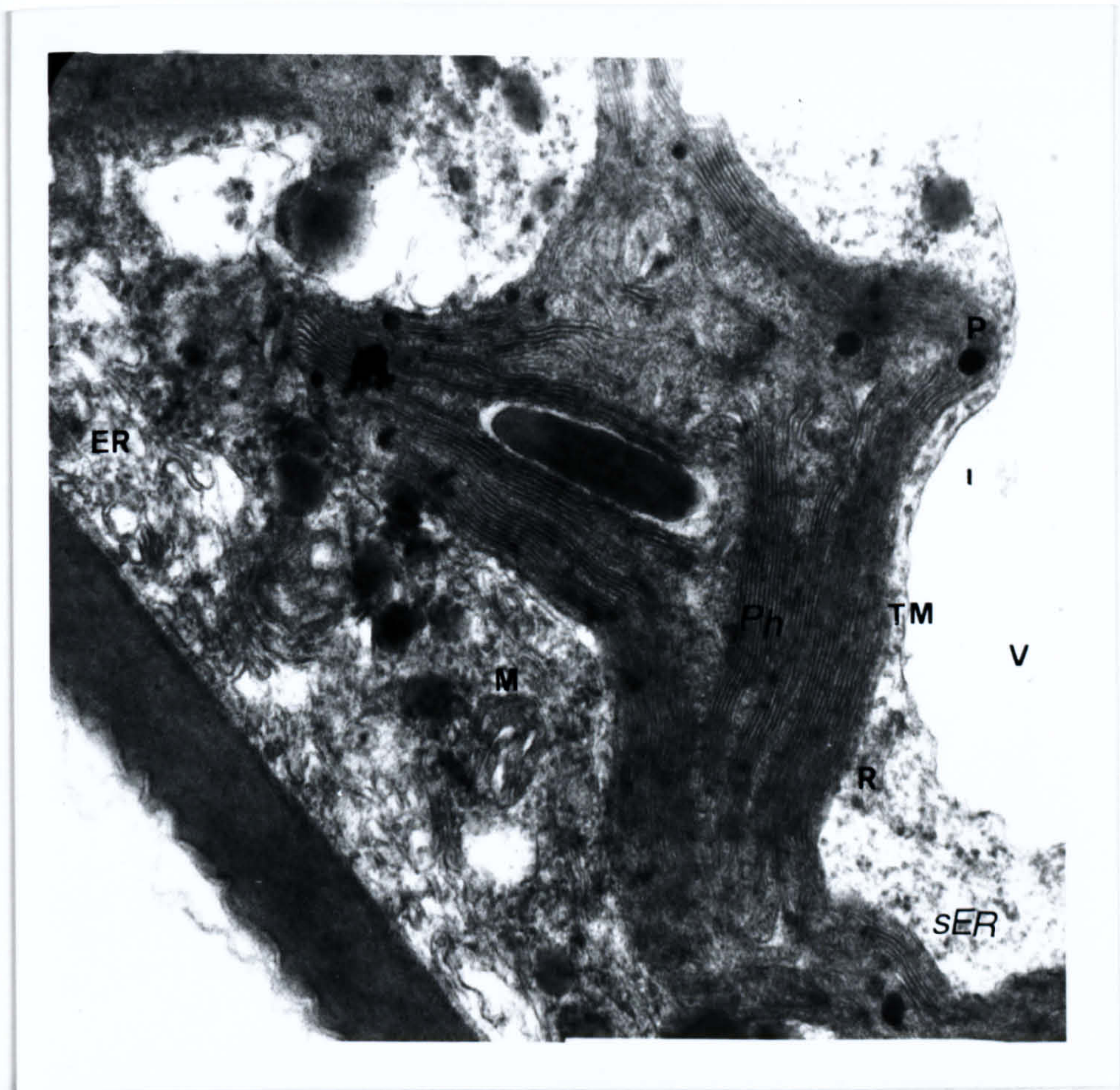




**Plate 4.18** Cell wall and membranes of *H. pluvialis* green cells. (mag. x 50,000)

The dense spherical structures shown in plate 4.18 are plastoglobuli (P) which are relatively abundant throughout the cell. The cell wall (CW) can be seen to be a fibrous structure. Ribosomes (R) contribute to the granular appearance of the ground substance of the cytoplasm, (polysomes may also be present but are not distinguishable in the plate). The plasma membrane (PM) and perinuclear space (PSp) are readily observed.





**Plate 4.19** Photosynthetic membranes of green *H. pluvialis* cells. (mag. x 50,000)

The photosynthetic membranes (Ph) are typical of normal green cells (Plate 4.19) with the membranes oriented parallel to each other. In the plate the membranes surround a central lipid globule. The tonoplast membrane (TM) is readily observed, which surrounds a vacuole (V) which is situated towards the bottom of the plate. Other cellular structures easily distinguishable in the plate are endoplasmic reticulum (ER), swollen endoplasmic reticulum (sER), plastoglobuli (P), mitochondria (M) and ribosomes (R).





**Plate 4.20** Stacked photosynthetic membranes of green *H. pluvialis* cells. (mag. x 75,000)

The photosynthetic membranes (Ph) in the plate form a 'stacked' structure (Plate 4.20). The membranes are in a region which contain large lipid bodies which are plastoglobuli (P).



## 4.4 DISCUSSION

### 4.4.1 Image analysis

The results of the image analysis indicate that as the cells accumulate secondary carotenoids they concomitantly increase in size. This is in agreement with previous studies (Borowitzka *et al.*, 1991; Elliot, 1934). These workers noted that the green 'swimmer' cells were approximately 8 - 20  $\mu\text{m}$  in diameter, while the red aplanospores could be anything up to 50  $\mu\text{m}$  in diameter. However, an explanation for this phenomenon has not yet been proposed.

The results also indicate the increase in the astaxanthin (III) content of the cells during encystment is not at the expense of chlorophyll, as has been previously suggested (Sprey, 1970). Previous observations led to the hypothesis of the involvement of chlorophyll breakdown products being involved in the synthesis of secondary carotenoids (Czygan, 1968b). The image analysis results presented in this investigation do not support this hypothesis. Figure 4.2 indicates that astaxanthin (III) synthesis began while the levels of chlorophyll in the culture were still increasing. The subsequent accumulation of astaxanthin (III) was also far greater than the concomitant metabolism of chlorophyll during encystment.

Observations of the apparent amount of chlorophyll present within the cells and the size of the cells indicate that as cell size increases, so does the amount of chlorophyll present within the cells, indicating a relationship between these two parameters. Increased chlorophyll levels may be a pre-requisite for cell growth, therefore the greater the amount of chlorophyll synthesised within a cell, the greater the potential the cell has for growth. Alternately, as the cell increases in size the ability of the cell to synthesise chlorophyll increases leading to high levels of chlorophyll. However, as was noted in the previous study an increase in cell size is also associated with secondary carotenoid formation within the cells. An increase in cell size during encystment would not appear to be related to chlorophyll levels and a different regulatory mechanism governing cell size seems to be in operation.

The ability to measure the amount of secondary carotenoids in cells *in vivo* using image analysis techniques has potential commercial implications. By coupling the image

analysis equipment to a cell sorter system, a population of cells could be screened very rapidly and those containing high levels of secondary carotenoids could be isolated and used to produce hyper-producing strains. This application could potentially be used to improve astaxanthin (III) production of a population in a fermenter situation. The cell lines isolated would not need to be stable for a long period of time, as new hyper-producing cell lines could be isolated at any time. Such cell lines have already been established for *Dunaliella* cultures (Nonamura and Coder, 1988).

#### 4.4.2 Life cycle

Observations of *H. pluvialis* cells using a light microscope allowed for the study of the life cycle of the alga. The life cycle of *H. pluvialis* is relatively simple where no sexual stage intervenes (Elliot, 1934). In the life cycle four types of cell are produced (see Fig. 4.5): macrozooids 'swimmers', microzooids 'swimmers', palmella cells and aplanospores. Macrozooids 'swimmers' range from 8  $\mu\text{m}$  - 20  $\mu\text{m}$  in diameter. During the juvenile stages the cells are pear-shaped with a pronounced papilla on the anterior end, while in older forms a spherical shape is attained. Palmella cells may divide to produce either flagellate organisms, macrozooids or juvenile palmella cells. The palmella cells produced grow into the 'adult' form and the cycle may be repeated. However, if flagellated macrozooids 'swimmers' are produced the cycle proceeds with the juvenile macrozooids 'swimmers' growing into the 'adult' macrozooids 'swimmers'. Periodically these macrozooids 'swimmers' lose their flagella and form palmella cells although this resemblance is only superficial, juvenile macrozooids 'swimmers' are formed from the palmella cells and the cycle repeats itself indefinitely. Both the microzooids and macrozooids are generally termed 'swimmers' because both possess flagella and are motile, also superficially both look very similar.

Aplanospores are formed under unfavourable, growth limiting, conditions either from macrozooids 'swimmers' or more usually from palmella cells. Aplanospores can also be formed by fission of other aplanospore cells. When conditions become favourable the aplanospores rupture releasing microzooids 'swimmers' and as many as 64 may be produced from one aplanospore. These microzooids 'swimmers' are soon converted into



juvenile palmella cells, these may develop into macrozoids or grow into 'adult' palmella cells. In either case the cycle proceeds from that point in the manner described above.

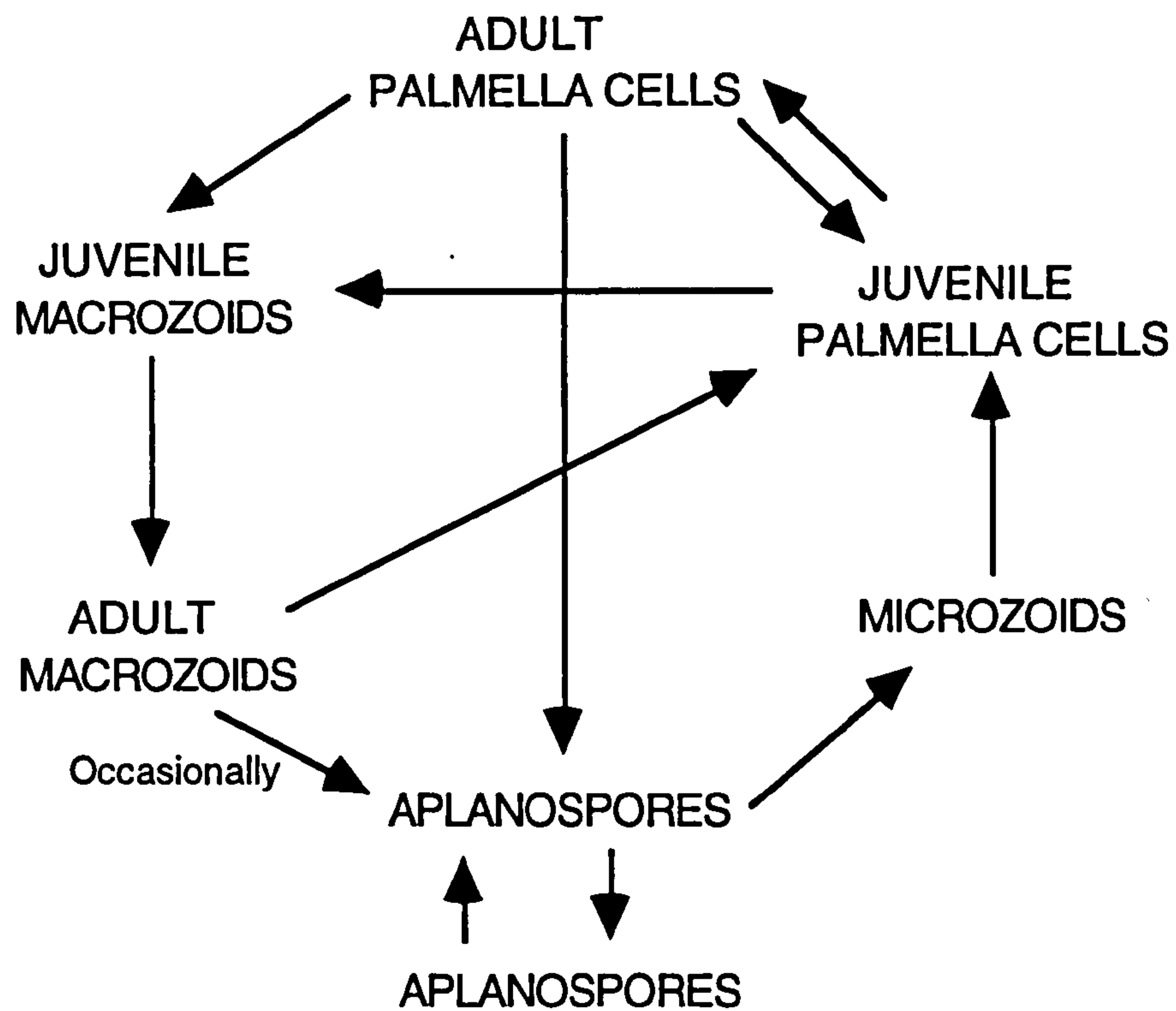


Fig. 4.5 Life cycle stages of *H. pluvialis*.

During fission the nucleus moves to the anterior end of the cell where a fusiform spindle is formed in the prophase or metaphase. The 20 - 30 chromosomes split and subsequently move to the poles in the anaphase. They then fuse into a chromatic mass which later breaks up into the granules of the daughter nuclei. The nucleolus disappears during fission but reappears about the time the nuclear membrane becomes visible. Two, four or eight cells may be formed in the palmella membrane before it ruptures, releasing the young macrozoids (Elliot, 1934).

For the mass cultivation of *H. pluvialis* it would be desirable to exert some control over the life cycle of the alga. This could be achieved by controlling the culture conditions of the alga very carefully, enabling the alga to only enter stages of its life cycle where it exhibits high rates of growth and secondary carotenoid synthesis i.e. those depicted in figure 4.6.

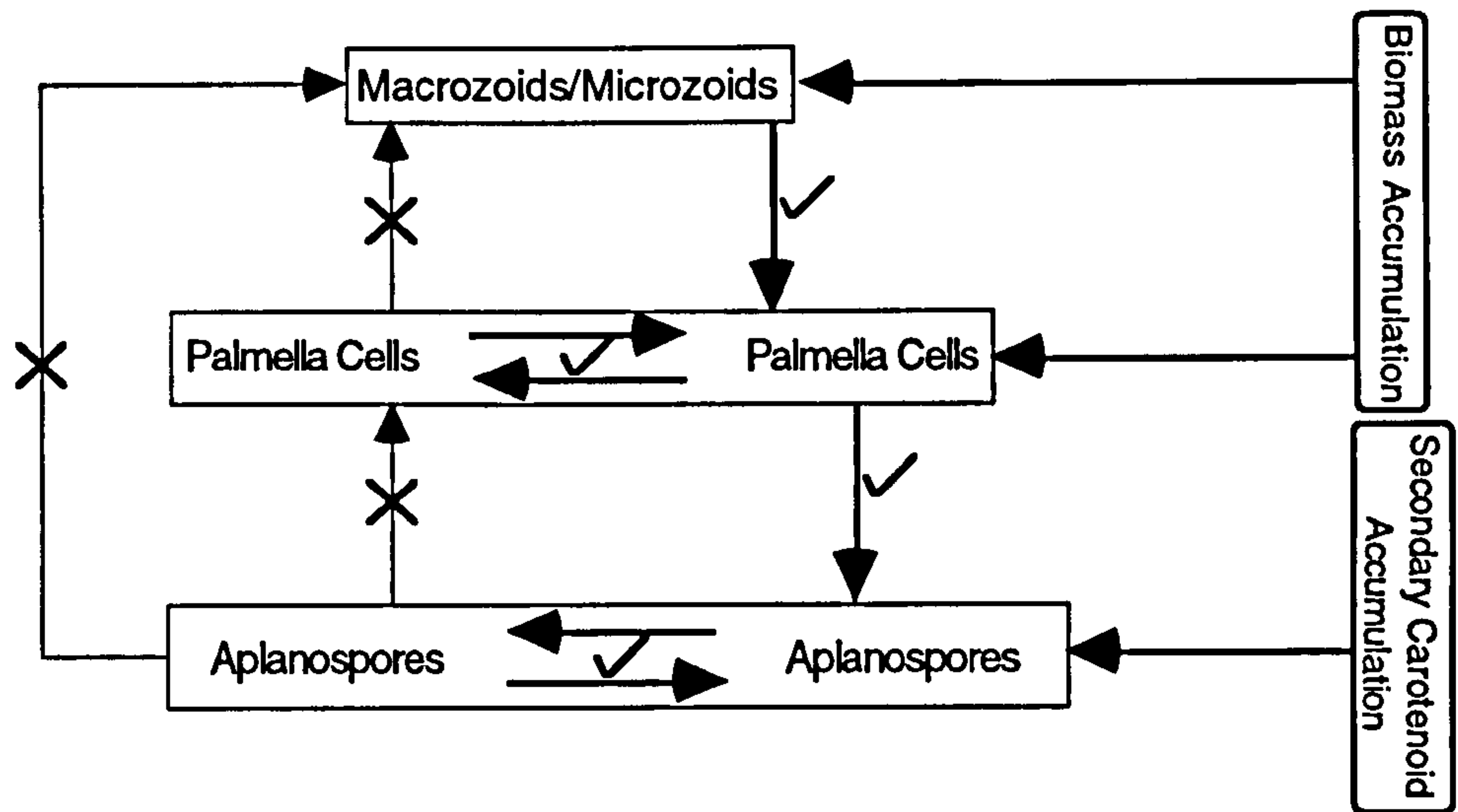


Fig. 4.6 Life stages of *H. pluvialis* desirable for mass cultivation.

The ultimate manipulation of the life cycle of the alga would eliminate any of the 'green' stages of the cycle. This would mean that the cells would be constantly red, while hopefully still showing relatively high rates of growth. This would significantly reduce the length of time required to cultivate the alga from the initial inoculation of a fermenter to harvest.

#### 4.4.3 General morphology

The transmission electron micrographs of the green palmella cells allowed for the observation of the main cellular constituents, including the nucleus, nucleolus, thylakoids, mitochondria, endoplasmic reticulum and the cell wall. The nucleus was spherical in shape, ranging from 2  $\mu\text{m}$  to 10  $\mu\text{m}$  in diameter, and was usually centrally located. A distinct nuclear membrane was also present. Characteristic cytoplasmic structures included the parietal chloroplast. The photosynthetic apparatus was differentiated into grana with stacks of 2 - 6 thylakoids and inter-grana, pyrenoids, extra-pyrenoidal starch grains and very small plastoglobuli in the stroma of the chloroplasts. Contractile vacuoles were also present which were localised near the cell surface and were surrounded by numerous vesicles, some of which may fuse with the membrane of the contractile vacuole (Lang, 1968).



The transmission electron micrographs of the cells failed to yield any useful information about the sites and location of astaxanthin (III) synthesis in the cells, probably because the cells were too advanced in their accumulation of astaxanthin (III) prior to fixation. This meant that the lipid bodies containing the astaxanthin (III) had coalesced and totally filled the protoplast. The nature of these bodies was characteristic of a high lipid structure because of their behaviour under sectioning and their appearance in subsequent TEM micrographs. The micrographs did show that the red cells contained very large highly pigmented lipid bodies which presumably contained the bulk of the ketocarotenoid. The size and shape of these lipid bodies varied considerably, and had the appearance of being compressed within the cells.

The red cells also contained large vacuoles which appeared to be empty. The sporopollenin-type cell wall surrounding the cells was very thick and dense. Although virtually no cell organelles could be observed in the cells it is assumed that the chloroplasts, thylakoids and the plastid envelope remain intact, and that the red cells are capable of photosynthesis albeit at a much reduced rate compared with green cells (data not shown).

Although not observed in the present study, during the formation of astaxanthin (III) the structure of the chloroplast is maintained (Lang, 1968), and astaxanthin (III) deposits, present as oil droplets within the chloroplast are never found. The granules of the stigma and rare plastoglobuli are the only osmophilic inclusions observed in the stroma of the chloroplasts. The number and dimensions of the plastoglobuli, as well as the ultrastructure of the chloroplast is not significantly modified during the process of secondary carotenoid accumulation (Santos and Mesquita, 1984). Even in very old red cultures where the chloroplast is pressed against the cell wall due to the great quantity of astaxanthin (III) present in the cell, the thylakoids and the plastid envelope are maintained intact (Santos and Mesquita, 1984).

The small globules of astaxanthin (III) form in the ground substance of the cytoplasm outside the endoplasmic reticulum cisternae. The globules increase in number and coalesce to form greater inclusions. These inclusions after surrounding the nucleus fill all the space among the lobes of the chloroplast. This is in contrast to *Dunaliella* where most of the stress-produced  $\beta$ -carotene (I) is located in oily globules inside the stroma fraction of the chloroplast (Ben-Amotz *et al.*, 1987). As the quantity of astaxanthin (III)

increases it progresses towards the periphery of the cell, resulting in a protoplast practically full of large astaxanthin (III) inclusions localised between the nucleus and the parietal chloroplast. Astaxanthin (III) esters are never found in the crystallised form within the protoplast, but exist as oil droplets.

Light microscopy was used to study *H. pluvialis* cells at the various stages during the life cycle of the alga. The microzooids 'swimmers' were cylindrical in shape, comparatively small at 20 µm or less, and swam much more actively than the macrozooids. Upon emerging, the microzooids swam actively for a few hours, after which they became ovoid and less active. Finally they became quiescent, losing their flagella and transformed into juvenile palmella cells.

Palmella cells were non-motile with a thick cell wall, under which a small periplasmic space with a rather sinuous plasmalemma was observed. The spherical protoplast retained the ultra-structural characteristics of the macrozooids, but there was no contractile vacuole. Astaxanthin (III) was either centrally located or evenly dispersed throughout the cell.

The aplanospores constituted the resting stage of the alga. These cells were able to accumulate massive amounts of ketocarotenoids, especially astaxanthin (III), both in free and esterified forms. Echinenone (II), canthaxanthin (IV) and adonirubin (V) were also formed but to a lesser extent (see Chapter 5). Astaxanthin (III) was first deposited in an area characterised by free ribosomes and an extensive network of ribosome coated endoplasmic reticulum segments (Lang, 1968). However, it should be noted that synthesis of astaxanthin (III) is not restricted to aplanospores; macrozooids and palmella cells readily synthesize astaxanthin (III) but at reduced levels in comparison to the aplanospores.

The 'reddening' of the cells is associated with a decrease in photosynthetic activity (data not shown; Lee and Ding, 1992), and a thickening of the cell wall. There is evidence to suggest that ketocarotenoids are involved in the formation of sporopollenin, the major cell wall polymer. Sporopollenin can comprise of up to 41% by weight of the cell wall material (Burczyk, 1987a). Sporopollenin is highly resistant to non-oxidative degradation and is thought to be formed by the oxidative polymerisation of carotenoids and/or their esters (Burczyk, 1987b).



Also observed in the cultures were colourless 'bleached' cells. The number of these 'bleached' cells increased considerably when the algae were exposed to severe adverse environmental conditions i.e. high light intensities and high salinities. These cells are thought to occur as a result of metabolic imbalances, combined with photo-oxidative events within the cells (Sandgren, 1983).

The scanning electron micrographs indicated that the red encysted cells were spherical in shape and 35 - 50  $\mu\text{m}$  in diameter. The covering of the cells in some kind of mucilage may explain why the cells 'clump' together in liquid culture, and why they readily adhere to the surfaces of glassware during experimental investigation. This heavy gelatinous matrix which surrounds the thick-walled aplanospores has been reported to contribute to the survival of the cell during periods of adverse environmental conditions (Sandgren, 1983).

## QUALITATIVE AND QUANTITATIVE ANALYSIS OF SECONDARY CAROTENOIDS IN *HAEMATOCOCCUS PLUVIALIS*

### 5.1 INTRODUCTION

#### 5.1.1 Carotenoid composition

The secondary carotenoid composition of *H. pluvialis* has been described by previous authors (Renstrøm *et al.*, 1981; Grung *et al.*, 1992), as has their fatty acid composition (Renstrøm and Liaaen-Jensen, 1981). The two groups of workers examining the carotenoid composition used TLC, HPLC, UV/VIS and mass spectrometry, Grung *et al.* (1992) also used  $^1\text{H}$  NMR. Renstrøm *et al.* (1981) esterified the astaxanthin (III) monoesters using (-)-camphanic acid and determined the optical purity of the astaxanthin (III) produced by saponification of the natural mono- and diesters of astaxanthin (III) in the absence of oxygen. Subsequent HPLC analysis of the diester of (-)-camphanic acid revealed that the alga synthesises pure (3*S*,3'*S*)-astaxanthin (III) esters.

Renstrøm *et al.* (1981) and Grung *et al.* (1992) examined the qualitative and quantitative characteristics of the carotenoid composition of different strains of *H. pluvialis* which were at different stages of encystment. Renstrøm *et al.* (1981) examined *Haematococcus pluvialis* Flotow em. Wille (clone NIVA CHL 9) during the early palmella stage, whilst Grung *et al.* (1992) examined *Haematococcus pluvialis* Flotow em. Wille (Strain MUR-145) during the aplanospore stage, by which time the alga had formed a very thick cell wall. The palmella cells were found to contain high levels of astaxanthin (III) monoesters (76%) and relatively low levels of astaxanthin (III) diesters (7%). The aplanospores contained similar levels of astaxanthin (III) mono- and diesters (46% and 34% respectively), indicating further esterification in the later stages of encystment. The esterifying acids in astaxanthin (III) monoesters were reported to be C<sub>16:0</sub>, C<sub>18:0</sub>, C<sub>20:0</sub> and C<sub>18:1</sub> (major) (Renstrøm and Liaaen-Jensen, 1981).



The epoxidic carotenoids violaxanthin (XVII) and neoxanthin (XX) associated with photosynthetic activity were absent from the aplanospores as were adonirubin (V) esters. No canthaxanthin (IV) or echinenone (II) were reported to be present in the palmella cells. Astaxanthin (III), lutein (XVI) and  $\beta$ -carotene (I) were present at similar levels in both the aplanospores and palmella cells. The results indicate that the stage of algal encystment is an important factor in determining the secondary carotenoid composition of the alga.

### 5.1.2 Aims

The work presented in this chapter was designed to identify and quantify the different secondary carotenoid components of *H. pluvialis*, strain 34/7 from the Culture Collection of Algae and Protozoa, Windermere, U.K. This alga was chosen as it had previously been identified as potentially the best algal species for the commercial production of secondary carotenoids (see section 3.4). The work involved extracting and isolating the different carotenoid components and analysing them using TLC, HPLC, UV/Vis and mass spectrometry. The cells used in this investigation were at a much later stage of encystment than those analysed by previous workers (Renstrøm *et al.*, 1981; Grung *et al.*, 1992).

## 5.2 MATERIALS AND METHODS

### 5.2.1 Carotenoid composition

*Haematococcus pluvialis* strain 34/7 was obtained from the Culture Collection of Algae and Protozoa, Windermere, U.K. Culture conditions were the same as those previously described in section 2.7.1. Extraction of carotenoids was the same as described in section 2.2.1, and anaerobic saponification was carried out as described in section 2.2.2.

Reversed phase HPLC was carried out using a Spherisorb ODS2, 5  $\mu$ m column (25.0 cm x 0.46 cm) with a 2 cm guard column. A solvent gradient of 0 - 60% A (0 - 10 min), 60 - 76% A (10 - 15 min), 76% A (15 - 22 min), 76 - 100% A (22.1 - 28.0 min), at a flow rate of 1 ml per minute (A = ethyl acetate, B = acetonitrile/water (9/1 v/v)) was used.

Normal phase HPLC was carried out using a 25.0 cm Techsphere 4.6  $\mu\text{m}$  silica column, with a 2 cm guard column. A solvent gradient of 0 - 100% A (0 - 25 min), 100% A (25 - 30 min), at a flow rate of 1 ml per minute (A = acetone/hexane (3/7 v/v), B = hexane) was used.

HPLC using an acid coated normal phase column was carried out on a 25.0 cm Nucleosil 4.6  $\mu\text{m}$  column. An isocratic mobile phase was used with propan-2-ol / dichloromethane / hexane (0.8:10:88.2, v/v/v), at a flow rate of 1.5 ml per minute.

Solvents were pumped using a CM4000 triphasic pump system. The samples were injected in 20  $\mu\text{l}$  aliquots via an on-line Rheodyne injector unit. A HP1040A diode-array detector was used to monitor spectra on line and to integrate chromatograms at up to 8 separate wavelengths.

TLC was carried out on Kieselgel 60 F<sub>254</sub> silica plates. R<sub>F</sub> values refer to solvent systems of acetone/hexane (3/7 v/v) and diethyl ether/hexane (1/1 v/v) respectively.

UV/Vis electronic absorption spectra were recorded in redistilled or HPLC grade acetone, methanol and in ethanol. The spectra were recorded using a Cecil CE 5501 computing double beam UV/Vis spectrophotometer.

Mass spectrometry was carried out using positive-ion EI on a VG 7070H double focusing magnetic sector mass spectrometer, operating at a low resolution (*ca* 1000). Data acquisition and processing was by a Finnigan INCOS 2300 data system. Full scan MS were recorded over the *m/z* range 40 - 700 at an accelerating voltage of 2 kV in a total cycle time of 3.5 sec. The probe temperature was raised gradually from ambient to >300°C in *ca* 5 min. The spectra were recorded at an ionisation potential of 70 eV. Authentic carotenoid standards were kindly provided by Hoffman-LaRoche, Inc., Basel Switzerland.



## 5.3 RESULTS

### 5.3.1 Carotenoid composition

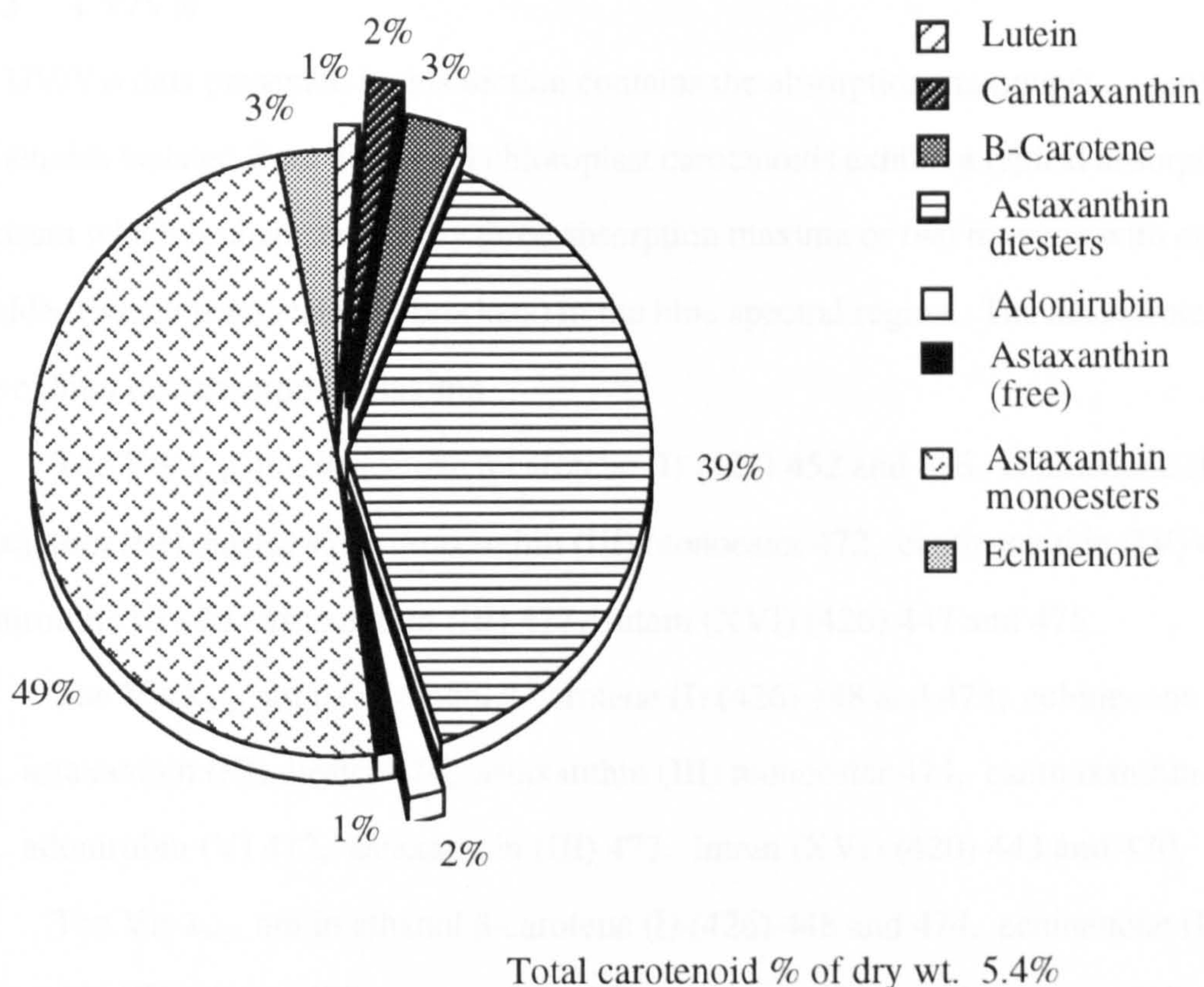


Fig. 5.1 Carotenoid composition of encysted *H. pluvialis* 34/7 cells.

The diagram above (Fig. 5.1) shows the carotenoid composition of heavily encysted *H. pluvialis* 34/7 cells as identified and quantified by mass spectrometry, TLC, UV/Vis absorption and, HPLC.

### 5.3.2 TLC

The individual carotenoids are treated in order of increasing adsorption by TLC (silica, acetone/hexane 3/7 v/v). Approximate  $R_F$ -values were  $\beta$ -carotene (I) 0.96, echinenone (II) 0.81, astaxanthin (III) diester 0.77 - 0.72, astaxanthin (III) monoester 0.62 - 0.58, canthaxanthin (IV) 0.51, adonirubin (V) 0.40, lutein (XVI) 0.29, astaxanthin (III) 0.28.

Re-chromatography was carried out on silica using diethyl ether/hexane (1/1 v/v), the individual carotenoids are again treated in order of increasing adsorption. Approximate  $R_F$ -values were  $\beta$ -carotene (I) 0.95, echinenone (II) 0.72, astaxanthin (III) diester 0.62 -



0.57, astaxanthin (III) monoester 0.45 - 0.41, canthaxanthin (IV) 0.34, adonirubin (V) 0.18, astaxanthin (III) 0.10, lutein (XVI) 0.07.

### 5.3.3 UV/Vis

The UV/Vis data presented in this section contains the absorption maxima ( $\lambda_{\max}$ , nm) of the carotenoids isolated from TLC. All chloroplast carotenoids exhibit a typical absorption spectrum which is characterised by three absorption maxima or two maxima with one shoulder (which is indicated in brackets) in the blue spectral region. The ketocarotenoids only exhibit one absorption maxima.

The Vis  $\lambda_{\max}$ , nm in acetone  $\beta$ -carotene (I) (428) 452 and 476, echinenone (II) 457, astaxanthin (III) diester 473, astaxanthin (III) monoester 472, canthaxanthin (IV) 470, adonirubin (V) 476, astaxanthin (III) 477, lutein (XVI) (426) 447 and 475.

The Vis  $\lambda_{\max}$ , nm in methanol  $\beta$ -carotene (I) (426) 448 and 473, echinenone (II) 456, astaxanthin (III) diester 474, astaxanthin (III) monoester 474, canthaxanthin (IV) 475, adonirubin (V) 472, astaxanthin (III) 477, lutein (XVI) (420) 443 and 470.

The Vis  $\lambda_{\max}$ , nm in ethanol  $\beta$ -carotene (I) (426) 448 and 474, echinenone (II) 461, astaxanthin (III) diester 478, astaxanthin (III) monoester 476, canthaxanthin (IV) 478, adonirubin (V) 474, astaxanthin (III) 478, lutein (XVI) (422) 444 and 472.

### 5.3.4 Mass spectrometry

$\beta$ -Carotene (I) MS  $m/z$  536 ( $[M]^+$ , 100%), 444 ( $[M-92]^+$ , 14%), 430 ( $[M-106]^+$ , 6%), 368 (100%). Inseparable from  $\beta$ -carotene standard by TLC (silica) and reversed phase HPLC ( $C_{18}$ ).

Echinenone (II) MS  $m/z$  550 ( $[M]^+$ , 100%), 548 ( $[M-2]^+$ , 6%), 458 ( $[M-92]^+$ , 17%), 444 ( $[M-106]^+$ , 4%), 368 (20%), 313 (46%), 275 ( $M^{2+}$ ), 133 (30%). Inseparable from echinenone (II) standard by TLC (silica) and reversed HPLC ( $C_{18}$ ).

Astaxanthin (III) diesters represented >10 peaks by reversed phase HPLC ( $C_{18}$ ). The bands were collected, purified and anaerobically saponified together. MS  $m/z$  596 ( $[M]^+$ , 100%), 594 ( $[M-2]^+$ , 200%), 580 ( $[M-16]^+$ , 400%), 578 ( $[M-18]^+$ , 700%), 504 ( $[M-92]^+$ , 90%), 490 ( $[M-106]^+$ , 60%), 313 (1000%). Inseparable from astaxanthin (III) standard by TLC (silica) and reversed phase HPLC ( $C_{18}$ ).



Adonirubin (V) MS  $m/z$  580 ([M]<sup>+</sup>, 100%), 578 ([M-2]<sup>+</sup>, 110%), 564 ([M-16]<sup>+</sup>, 25%), 488 ([M-92]<sup>+</sup>, 10%), 474 ([M-106]<sup>+</sup>, 10%), 313 (140%). Inseparable from adonirubin (V) standard by TLC (silica) and reversed phase HPLC (C<sub>18</sub>).

Astaxanthin (III) monoesters represented >10 peaks by reversed phase HPLC (C<sub>18</sub>). The bands were collected, purified and anaerobically saponified together. MS  $m/z$  596 ([M]<sup>+</sup>, 100%), 594 ([M-2]<sup>+</sup>, 60%), 580 ([M-16]<sup>+</sup>, 140%), 578 ([M-18]<sup>+</sup>, 200%), 504 ([M-92]<sup>+</sup>, 40%), 490 ([M-106]<sup>+</sup>, 25%), 313 (1400%), 109 (400%). Inseparable from astaxanthin (III) standard by TLC (silica) and reversed phase HPLC (C<sub>18</sub>).

Canthaxanthin (IV) MS  $m/z$  564 ([M]<sup>+</sup>, 100%), 562 ([M-2]<sup>+</sup>, 5%), 549 ([M-16]<sup>+</sup>, 20%), 472 ([M-92]<sup>+</sup>, 12%), 458 ([M-106]<sup>+</sup>, 5%), 282 (M<sup>2+</sup>), 133 (50%). Inseparable from canthaxanthin (IV) standard by TLC (silica) and reversed phase HPLC (C<sub>18</sub>).

Astacene (XXIX); astaxanthin (III) esters were aerobically saponified to produce astacene. MS  $m/z$  592 ([M]<sup>+</sup>, 100%), 577 ([M-15]<sup>+</sup>, 14%), 500 ([M-92]<sup>+</sup>, 11%), 486 ([M-106]<sup>+</sup>, 8%), 313 (30%), 203 (100%), 91 (32%). Inseparable from astacene (XXIX) standard by TLC (silica).

Lutein (XVI) MS  $m/z$  568 ([M]<sup>+</sup>, 100%), 566 ([M-2]<sup>+</sup>, 14%), 552 ([M-16]<sup>+</sup>, 22%), 550 ([M-18]<sup>+</sup>, 135%), 476 ([M-92]<sup>+</sup>, 15%), 462 ([M-106]<sup>+</sup>, 8%), 430 ([M-138]<sup>+</sup>, 5%), 91 (40%). Inseparable from lutein (XVI) standard by TLC (silica) and HPLC (C<sub>18</sub>).

### 5.3.5 HPLC

By reversed phase HPLC (C<sub>18</sub>) R<sub>T</sub>-values were lutein (XVI) 13.5 min, astaxanthin (III) 14.2, canthaxanthin (IV) 14.9 min, astaxanthin (III) monoesters 16.7 - 17.4 min, adonirubin (V) 16.9 min, β-carotene (I) 18.9 min, echinenone (II) 17.1 min, astaxanthin (III) diesters 19.3 - 20.4 min.

The following HPLC chromatograms of *H. pluvialis* cell extracts show the different results obtained when using the various HPLC techniques. Peak identification is as follows: (1) neoxanthin (XX); (2) violaxanthin (XVII); (3) lutein 5,6-epoxide (XVI); (4) lutein; (5) chlorophyll *b*; (6) chlorophyll *a*; (7) β-carotene (I); (7a) Z-isomers of β-

carotene (I); (8) ketocarotenoid monoesters; (9) astaxanthin (III) diesters; (10) canthaxanthin (IV).

The reversed phase chromatogram of the pigment extract (Fig. 5.2) from green cells shows the normal xanthophylls and chlorophylls associated with photosynthesis. The reversed phase chromatogram of the red encysted cells (Fig. 5.3) resolved the many peaks of the ketocarotenoids and their esters, as well as the xanthophylls and chlorophylls observed in the green extract. Most of the pigments separated well, or; only some of the ketocarotenoid monoesters separated poorly from one another.

The normal phase system (Fig. 5.4) resolved the ketocarotenoids and their esters quite well, but apart from  $\beta$ -carotene (I) the xanthophylls and chlorophylls were not identifiable from the chromatogram. Those pigments which were eluted from the column towards the end of the chromatographic run did not separate very well. This produced peaks of conglomerations of carotenoids which were not identifiable.

The chromatogram produced by the acid coated column (Fig. 5.5) showed no xanthophylls or chlorophylls because of the acidic nature of the column.  $\beta$ -carotene (I) was observed, but at lower concentrations than would be expected. The ketocarotenoids and their esters did not separate particularly well and converged into five main peaks.

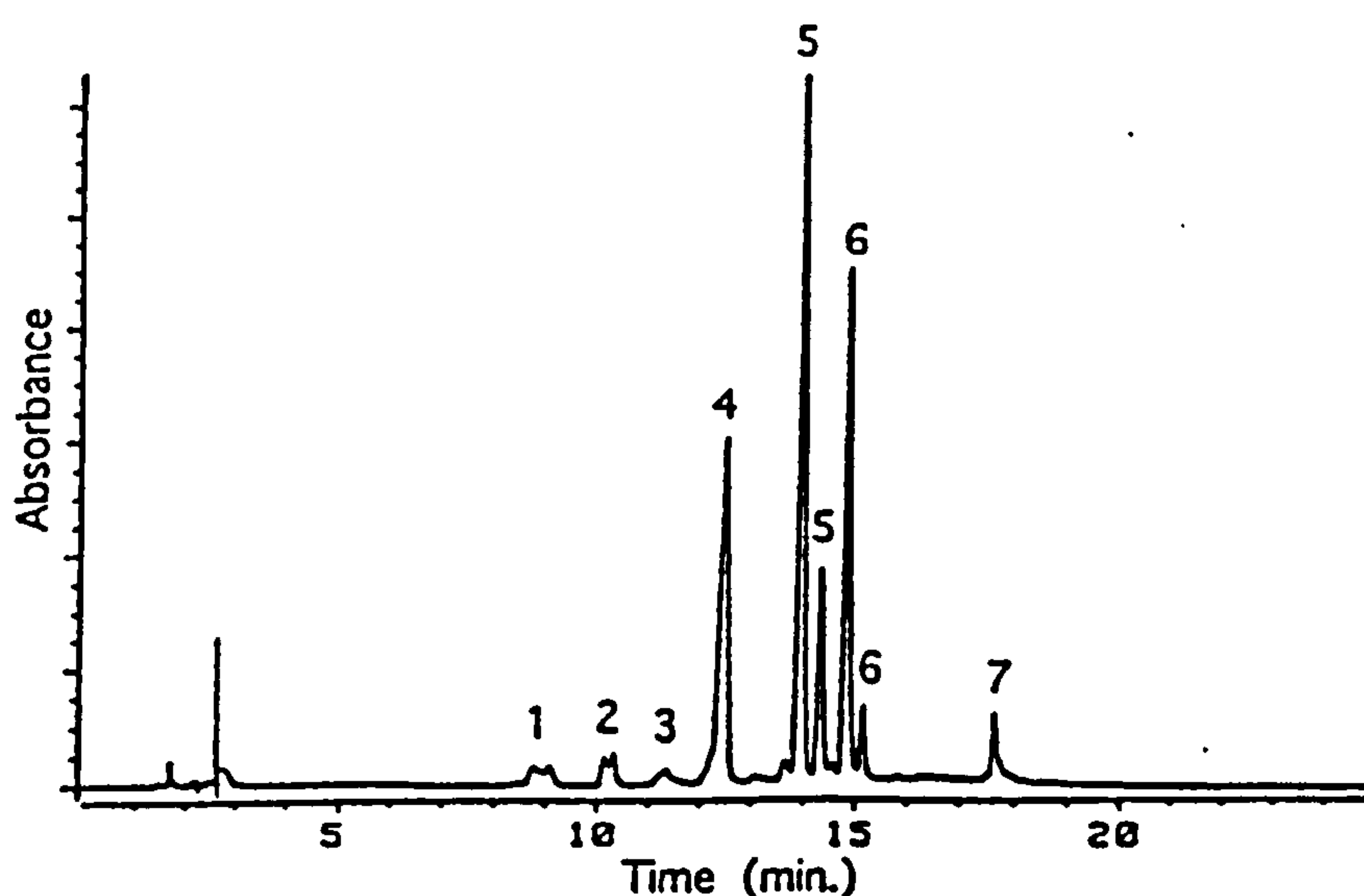


Fig. 5.2 Reversed phase HPLC chromatogram of green cells of *H. pluvialis*.



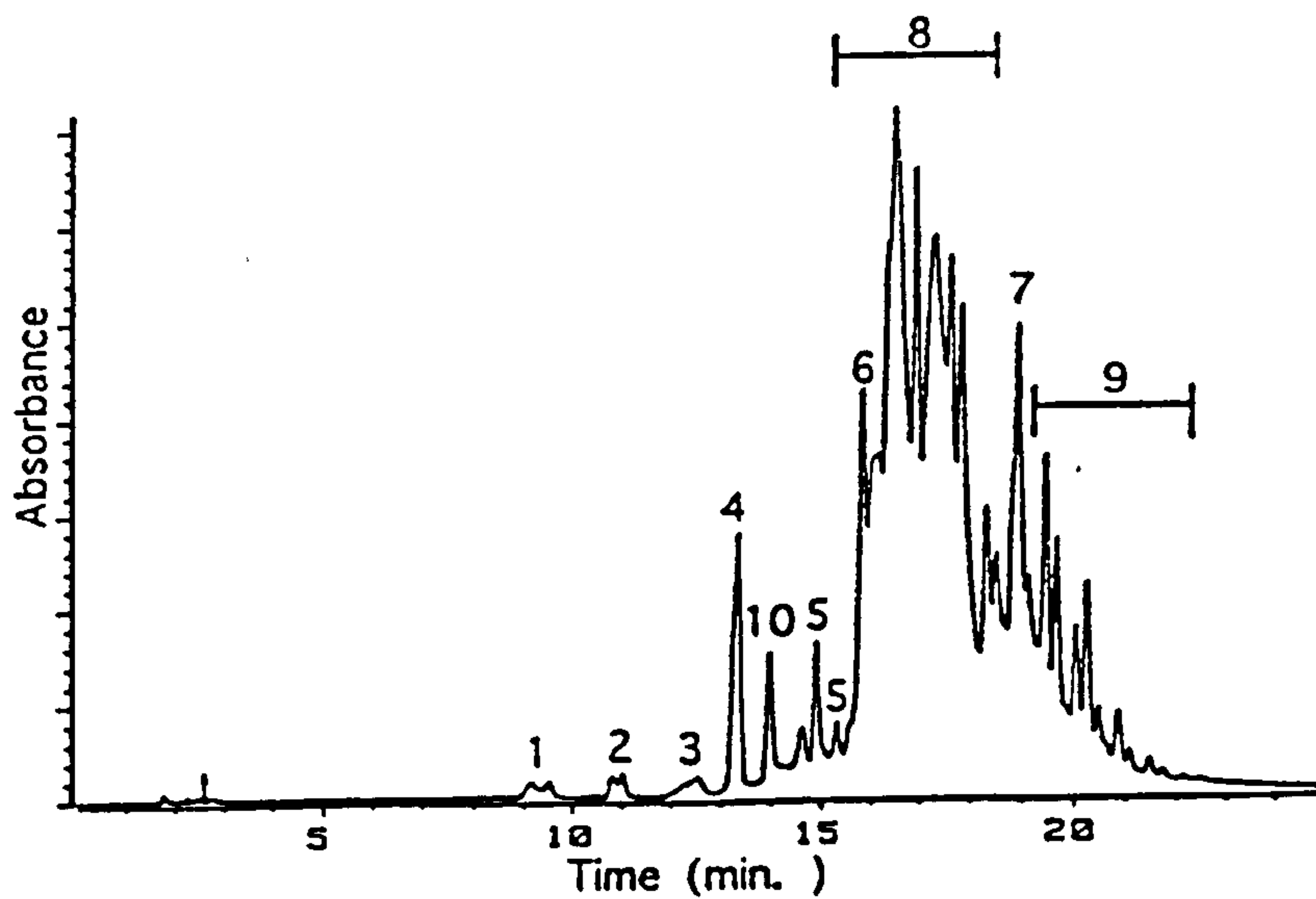


Fig. 5.3 Reversed phase HPLC chromatogram of red cells of *H. pluvialis*.

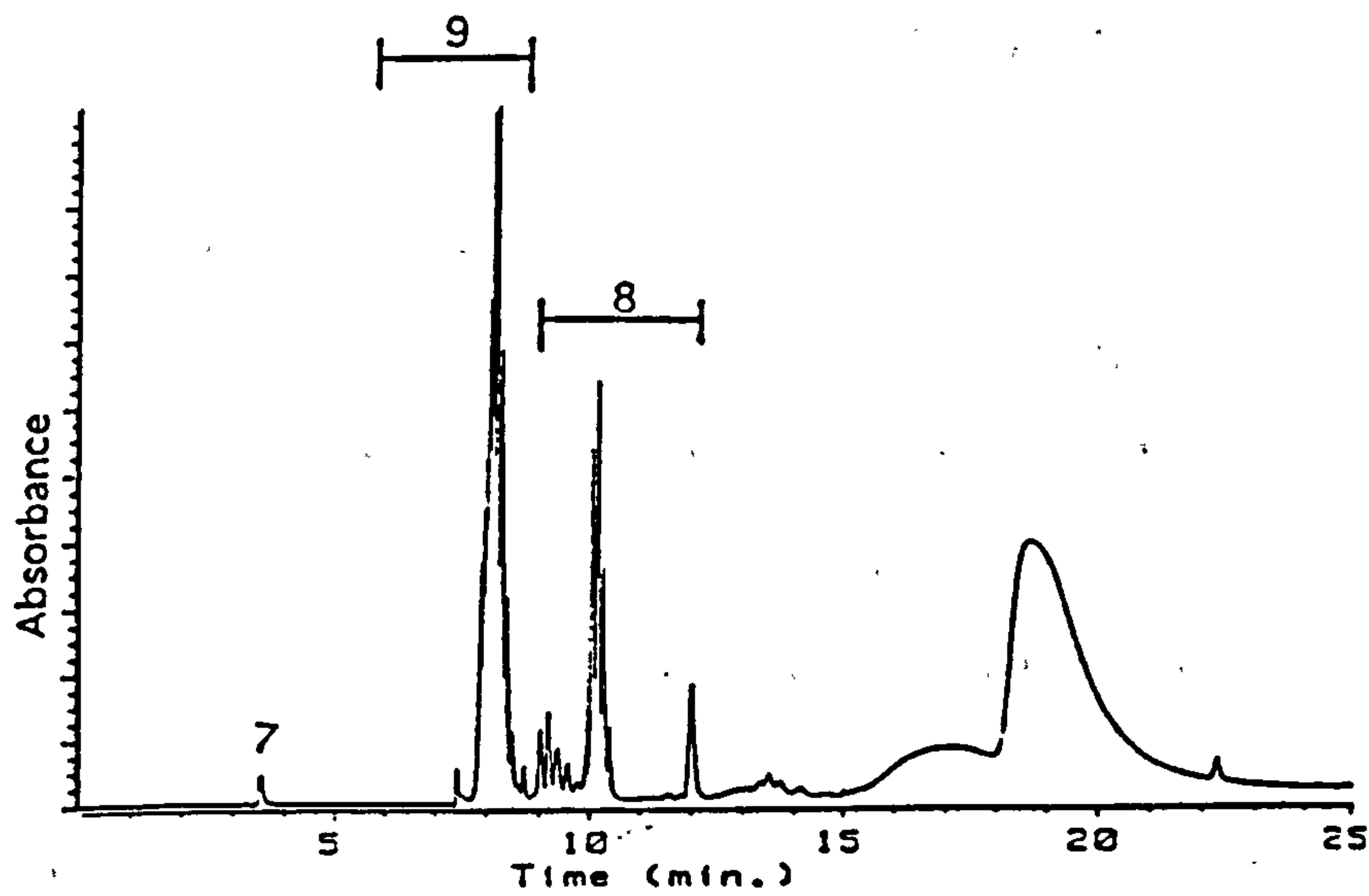


Fig. 5.4 Normal phase HPLC chromatogram of red cells of *H. pluvialis*.

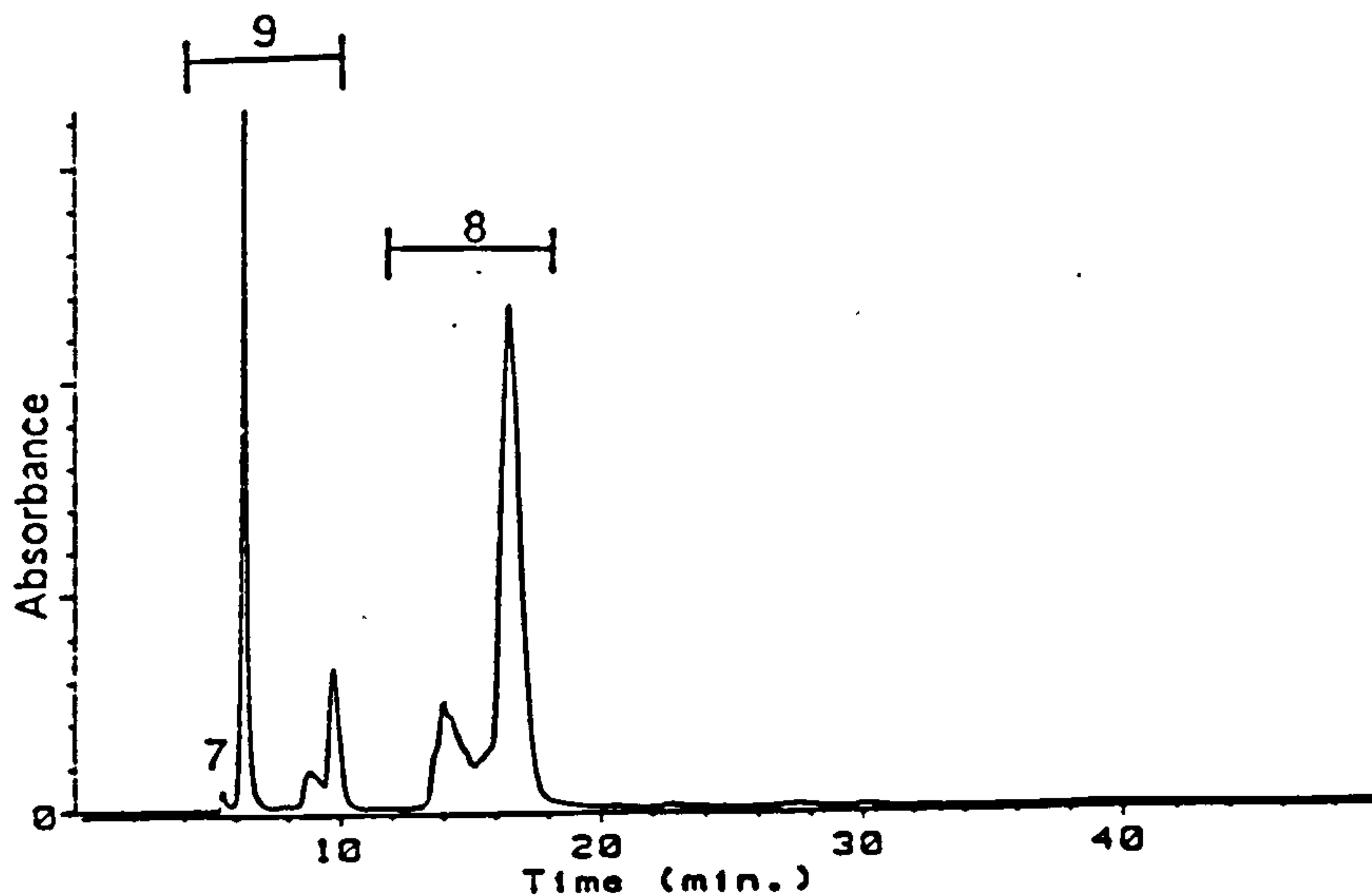


Fig. 5.5 HPLC chromatogram of *H. pluvialis* extract on an acid coated column.

#### 5.4 DISCUSSION

Highly encysted cells (aplanospores) of *H. pluvialis* 34/7 contained 5.4% carotenoid (dry wt. basis). The carotenoid composition was in agreement with that reported previously (Renstrøm *et al.*, 1981; Czygan, 1968a; Grung *et al.*, 1992). Table 5.1 shows that monoesters of astaxanthin (III) were the dominant pigments as in the previous study by Renstrøm *et al.* (1981), who analysed cells which were at a relatively early stage of encystment and showed relatively low levels of esterification. The levels of monoesters of astaxanthin (III) in the present study are similar to the levels of the astaxanthin (III) diesters. The cells used in this study were relatively old and highly encysted. Therefore, it can be concluded that esterification of astaxanthin (III) and astaxanthin (III) monoesters continues during secondary carotenoid accumulation. This esterification process may even continue after the cessation of secondary carotenoid synthesis. Previous studies (see section 3.3.2) confirmed that during the early stages of encystment the levels of monoesters and diesters are relatively similar i.e. 1:1. However, as encystment progresses the levels of monoesters relative to the levels of diesters increases up to 5:1.. During the latter stages



of encystment the levels of diesters increases relative to the levels of monoesters to a ratio approaching 1.5:1. This assumption is in agreement with Grung *et al.* (1992). They reported that highly encysted cells of *H. pluvialis* contain relatively similar levels of astaxanthin (III) mono and diesters.

**Table 5.1** Carotenoid composition of four strains of *Haematococcus pluvialis*.

Carotenoid	% of total carotenoid			
	Aplanospores CCAP 34/7	Aplanospores MUR 145*	Palmella stage NIVA CHL 9**	Palmella stage Strain A***
Beta-Carotene (I)	5	5	1	3
Echinenone (II)	3	4	-	-
Canthaxanthin (IV)	2	4	-	-
Adonirubin (V)	2	-	3	3
Astaxanthin (III) diester	39	34	7	16
Astaxanthin (III) monoester	49	46	76	71
Astaxanthin (III)	1	1	1	3
Lutein (XVI)	1	6	7	4
Violaxanthin (XVII)	-	-	2	1
Neoxanthin (XX)	-	-	1	1
Total carotenoid % of dry wt.	5.4	0.7	-	2.0

\* Grung *et al.* (1992).

\*\* Renstrom *et al.* (1981).

\*\*\* Grung *et al.* (1990).

The presence of adonirubin (V) is in agreement with Renstrøm *et al.* (1981), while the presence of echinenone (II) and canthaxanthin (IV) was previously reported by Czygan (1968a) and Sprey (1970). Violaxanthin (XVII) and neoxanthin (XX), carotenoids associated with photosynthetic activity, were not detected in the aplanospores in this study.  $\beta$ -Carotene (I) and lutein (XVI) were also present but at lower levels than those previously reported (Renstrøm *et al.*, 1981; Grung *et al.*, 1992). Overall the pigment composition was dominated by the presence of astaxanthin (III) mono and diesters. However, it should be noted that the age of the cells is a very important factor in determining which carotenoids are present, and the relative amounts of each carotenoid present within the cells.

The quantitative and qualitative analysis of astaxanthin (III)-containing extracts was particularly difficult due to the unstable nature of the ketocarotenoid. Astaxanthin (III) and its esters readily undergo rapid and irreversible oxidation to the 2,3-dihydro derivative astacene (XXIX). Astacene (XXIX) is a particularly unwanted artefact in chromatography as it binds to silica TLC plates and silica HPLC columns. This results in strong 'tailing' during TLC and HPLC operations, and good resolution of the free astaxanthin (III) and astaxanthin (III) mono and diesters is particularly difficult. This difficulty is accentuated when considering that the ketocarotenoids and their esters are chemically very similar, and therefore, very difficult to separate during chromatography even without the effects of 'tailing'.

The use of an acid coated column did not provide very good resolution of the ketocarotenoids, nor did the normal phase system. The best HPLC system for the analysis of extracts containing ketocarotenoids, xanthophylls and chlorophylls proved to be the reversed phase system. Good separation using TLC was achieved using both systems described. However, it should be noted that before the identification of these HPLC and TLC systems, many other HPLC and TLC systems were investigated but provided very poor resolution of the ketocarotenoids.



# A STUDY OF THE CONDITIONS RESPONSIBLE FOR THE ACCUMULATION OF ASTAXANTHIN IN *HAEMATOCOCCUS PLUVIALIS*

## 6.1 INTRODUCTION

### 6.1.1 Effects of nutrients

The occurrence of astaxanthin (III) in *Haematococcus pluvialis* has received increasing experimental investigation due to recent commercial interest in the pigment. Astaxanthin (III) can be used as a source of pigmentation for fish in aquaculture (Johnson and An, 1991) and also for its superior-antioxidant activity compared with  $\beta$ -carotene (I) and  $\alpha$ -tocopherol (Kurashige *et al.*, 1990; Palozza and Krinsky, 1992). However, relatively little research has been published on the practical aspects of astaxanthin (III) production from this organism. This is in part due to several disadvantageous characteristics of the alga when compared to other micro-organisms: (i) its relatively slow growth rate, (ii) its preference for low growth temperatures, (iii) a requirement of light for cultivation. Also much of the published data to date is contradictory, with workers from different laboratories reaching different conclusions as to the most suitable conditions for the growth of the alga and for astaxanthin (III) production from the alga. Therefore, no general consensus regarding the nutrient and culture condition requirements for *H. pluvialis*, with particular emphasis on those requirements which result in optimal production of astaxanthin (III), has yet emerged.

Many authors have reported that nitrogen-deficient conditions stimulate the induction of astaxanthin (III) synthesis and accumulation in *H. pluvialis* (Spencer, 1989; Goodwin and Jamikorn, 1954b; Borowitzka *et al.*, 1991). However, this hypothesis has been dismissed by some authors (Boussiba and Vonshak, 1991) with other workers stating that astaxanthin (III) accumulation can be independent of nitrogen concentration completely (Droop, 1954). Further work has shown astaxanthin (III) formation is

accompanied with the encystment of the cells induced under a high carbon/nitrogen ratio (Kakizono *et al.*, 1992).

Astaxanthin (III) accumulation has also been reported to be affected by the levels of phosphate present in the culture medium. Phosphate starvation has been reported to act as trigger for the accumulation of astaxanthin (III) (Boussiba and Vonshak, 1991).

However, other authors have suggested that high phosphate concentrations stimulate the production of astaxanthin (III) within the algal cells (Borowitzka *et al.*, 1991).

The exposure of the algal cells to increased salinity appears to lead to astaxanthin (III) formation in the alga (Spencer, 1989; Borowitzka *et al.*, 1991; Boussiba and Vonshak, 1991). All the available data agrees that exposure to increased salinities results in astaxanthin (III) formation. However, all three authors used different salt concentrations ranging from 50 - 170 mM NaCl. There appears to be no evidence of any experimental work to deduce the phytotoxic effects of the salt. There is also no data available indicating the optimum salt concentration for astaxanthin (III) production, or whether or not the form of salt used affects the level of astaxanthin (III) accumulated. Furthermore, there is no data available to indicate if it is more effective that the salt should be added all at once, or added in stages to improve astaxanthin (III) production in the alga.

The use of ferrous ion has also been investigated as a possible stimulant of astaxanthin (III) synthesis in *H. pluvialis*. Again conflicting results have been obtained as to the effect on astaxanthin (III) synthesis. The injection of ferrous Fe with acetate under high light intensities has been shown to stimulate astaxanthin (III) formation (Kobayashi *et al.*, 1991). The addition of EDTA-chelated  $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$  did not produce any significant differences in astaxanthin (III) formation when added at three different concentrations (Borowitzka *et al.*, 1991).

Most workers agree that high light intensities cause the accumulation of astaxanthin (III) in *H. pluvialis*. The production of astaxanthin (III) in the absence of light has also been reported, albeit at a much reduced rate (Droop, 1955). However, this is in direct contradiction to a previous paper which stated that astaxanthin (III) synthesis could only occur in the presence of light (Goodwin and Jamikom, 1954b). Astaxanthin (III) production is enhanced when the alga is grown under blue light as opposed to white or red light (Kobayashi *et al.*, 1992). The same authors also report that continuous illumination



rather than light and dark illumination cycles is more favourable for astaxanthin (III) formation. There is little information available on the effect of temperature on astaxanthin (III) formation in the alga, although some authors have reported that it is an important factor (Donkin, 1976; Borowitzka *et al.*, 1991).

It would appear that many nutritional and environmental parameters contribute to the accumulation of astaxanthin (III) in *H. pluvialis*. The degree to which each parameter contributes to the accumulation of astaxanthin (III) formation is unclear. This can in part be accounted for by poor experimental design in many of the published experiments. Most of the experiments from which the results have been published have measured the effect of one parameter in combination with that of a second and sometimes a third. Therefore, the results obtained do not always clearly indicate which parameter is having the greater effect, or whether or not the parameters are having any synergistic or antagonistic effect on each other. Therefore, no clear conclusion of the importance of each parameter can be deduced. The design of experiments in which combinations of parameters are tested, rather than testing each parameter independently could be why many of the published results are contradictory in their conclusions. However, almost all of the available data suggests that astaxanthin (III) accumulation is induced whenever a disturbance in cell division is imposed. Thus, a reduction in the rate of cell division would seem to be a prerequisite for the accumulation of astaxanthin (III).

### **6.1.2 Aims**

The work presented in this chapter was designed to evaluate the nutritional effects of nitrate, phosphate, iron and salt on the formation of astaxanthin (III) in *H. pluvialis*. The environmental effects of light and temperature were also investigated. Each parameter was tested independently. This enabled the contribution of each parameter on astaxanthin (III) formation by the alga to be determined. The effect of salt was determined, as was the concentration and form which produced the highest levels of astaxanthin (III) accumulation. A range of salts were tested, as was the way in which the salt was added to the cultures, to try and improve the production of astaxanthin (III) in *H. pluvialis* as a result of salt stress.

## 6.2 MATERIALS AND METHODS

### 6.2.1 Nitrate, phosphate and iron

A culture of *Haematococcus pluvialis* (Strain 43/7) was obtained from the culture collection of algae and protozoa, Windermere, U.K. The nitrate concentration of the media was varied by altering the level of  $\text{NaNO}_3$  in the culture medium. The phosphate concentration of the culture medium was varied by altering the levels of  $\text{K}_2\text{HPO}_4$  and  $\text{KH}_2\text{PO}_4$  in the media. The nitrate and phosphate concentrations investigated are shown in Table 6.1. The level of nitrate and phosphate in BBM is 3.0 mM and 1.5 mM respectively, and these levels were used as the controls in the experiment. Three 250 ml Erlenmeyer conical flasks containing 50 ml BBM modified to the appropriate nitrate or phosphate concentration were inoculated with 5 ml of *H. pluvialis* suspension, for each nitrate and phosphate concentration investigated. The concentrations of nitrate and phosphate present in the algal inoculum were regarded as negligible. The 250 ml Erlenmeyer conical flasks were kept on an orbital shaker at 80 rpm. The cultures were kept in a temperature controlled room at 22°C. Continuous light was supplied by cool white fluorescent tubes at an irradiance of 35  $\mu\text{mol photons m}^{-2} \text{sec}^{-1}$  (PAR).

**Table 6.1** Concentrations of nutrients used to determine effects on carotenogenesis.

Nutrient	Concentration	Concentration	Concentration	Concentration	Concentration
Nitrate	0.0 mM	0.75 mM	1.5 mM	3.0 mM	6.0 mM
Phosphate	0.75 mM	1.5 mM	3.0 mM	-	-
Fe	18.0 $\mu\text{M}$	36.0 $\mu\text{M}$	72.0 $\mu\text{M}$	-	-

Iron was added to the cultures as  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ . A known volume of the iron solution was filtered into 250 ml Erlenmeyer conical flasks containing 50 ml BBM using a 2  $\mu\text{m}$  Millipore filter, to avoid contaminating the cultures (autoclaving resulted in the iron precipitating out of solution). Three flasks of each iron concentration were prepared and inoculated with 5 ml of *H. pluvialis* suspension. The iron concentrations used in the experiment are given in Table 6.1. It should be noted that 1.0 mM is the normal iron concentration of BBM and was therefore used as the control. The flasks were incubated as described above. In all three experiments every seven days an aliquot was taken aseptically



from each flask, the pigments extracted and analysed for chlorophyll and carotenoid content as described previously in section 2.2.1.

### 6.2.2 Salt

Three 250 ml Erlenmeyer conical flasks containing 50 ml BBM modified to the appropriate NaCl or KCl concentration were inoculated with 5 ml of *H. pluvialis* suspension, for each salt concentration investigated. The culture conditions were the same as those described above. Table 6.2 shows the range of NaCl and KCl concentrations used in the experiment. Samples were taken and analysed as described in section 6.2.1. The first two salt experiments were designed to investigate the effect of increased salinity on astaxanthin (III) formation in *H. pluvialis*.

**Table 6.2** Concentrations of salts used to determine effects on carotenogenesis.

Salt	Concentration	Concentration	Concentration	Concentration	Concentration
NaCl	0 mM	86 mM	171 mM	342 mM	685 mM
KCl	0 mM	67 mM	134 mM	268 mM	537 mM

The second salt experiment was designed to establish an optimum NaCl concentration, i.e. that which produced the highest yields of astaxanthin (III) in *H. pluvialis*. The results of the first experiment were used to determine the range of NaCl concentrations used in the second experiment. *H. pluvialis* was initially grown in a 5 litre fermenter to produce sufficient algal biomass. Known volumes of algal suspension (50 ml) were then transferred into 250 ml Erlenmeyer conical flasks which contained a range of different NaCl concentrations. Table 6.3 shows the range of NaCl concentrations used in the experiment. Samples were taken and analysed as described in section 6.2.1 as are the culture conditions.

**Table 6.3** NaCl concentrations investigated to determine optimum concentration to induce carotenogenesis.

Salt	Concentration	Concentration	Concentration	Concentration
NaCl	0 mM	40 mM	70 mM	100 mM

The final experiment involving NaCl was to determine the most effective way of adding salt to the algal cultures in order to maximise astaxanthin (III) synthesis, without causing excessive reductions in cell number. Table 6.4 shows how the NaCl was added to the cultures. In all the treatments the final salt concentration added to the cultures was 40 mM NaCl. The experimental protocol was the same as that used in the previous salt experiment.

**Table 6.4** Methods of NaCl addition to the cultures to determine effects on secondary carotenoid synthesis.

	Treatment No.								
Salt Added	1	2	3	4	5	6	7	8	9
on Day	Concentration of Salt Added (mM)								
0	40	20	20	20	13.3	13.3	13.3	10	10
1	-	-	-	-	-	-	-	-	-
2	-	-	-	20	-	-	13.3	10	-
3	-	-	-	-	13.3	-	-	-	10
4	-	-	-	-	-	-	13.3	10	-
5	-	20	-	-	-	13.3	-	-	-
6	-	-	-	-	13.3	-	-	10	10
7	-	-	-	-	-	-	-	-	-
8	-	-	-	-	-	-	-	-	-
9	-	-	-	-	-	-	-	-	10
10	-	-	20	-	-	13.3	-	-	-

### 6.2.3 Light and temperature

Light and temperature were investigated as environmental factors which were expected to have an effect on astaxanthin (III) formation in *H. pluvialis*. The culture conditions were the same as those in 6.2.1. *H. pluvialis* was initially grown in a 5 litre fermenter to produce sufficient algal biomass. Known volumes of algal suspension (50 ml) were then transferred into 250 ml Erlenmeyer conical flasks. Algal cultures were exposed to various light and temperature intensities to monitor any effect such conditions produced in the alga. In the first experiment algal cultures were exposed to light intensities of 2, 37 and 89  $\mu\text{mol m}^{-2} \text{s}^{-1}$  (PAR) respectively. In the second experiment algal cultures were exposed to temperatures of 22, 32 and 42°C. The remaining experimental protocol was the same as used in the previous experiments.



#### **6.2.4 Statistical analysis**

The data was subjected to statistical analysis to determine the standard errors of the means. Significant differences between the treatments were calculated using the data obtained on the final day of the experiments. These were determined by calculating the least significant differences (LSD) of the means. The LSD is represented as a single bar on each of the graphs. For further information on the statistical analysis used in these experiments see section 2.9.

### **6.3 RESULTS**

#### **6.3.1 Nitrate**

Figures 6.1a-e show the response of cell number and pigment composition of *H. pluvialis* when cultivated in five different nitrate concentrations. The graphs indicate that growth was significantly improved in the cultures containing 3.0 mM and 6.0 mM nitrate. Reducing the nitrate content of the medium to 0.75 mM and 1.5 mM reduced the growth rate considerably. In the absence of nitrate algal growth was very poor, indeed what little growth that did occur in these cultures was probably due to some residual nitrate being present in the initial algal inoculum (this could be avoided by repeatedly washing the cells in future experiments). This growth ceased after 7 days and a slight decrease in cell number was observed after this point. The cells grown in the absence of nitrate had completely reddened and become encysted after 10 days. The cells continued to synthesise astaxanthin (III) throughout the experiment at a relatively rapid rate, accumulating significantly larger amounts of astaxanthin (III) per cell than the other treatments (> 500 pg/cell). In addition, the yield expressed per unit volume of culture exceeded that observed in some of the other treatments.

The cells in 0.75 mM and 1.5 mM nitrate showed very similar growth patterns; the cell number in both treatments continued growing throughout the experiment, but the growth rate was relatively low. These cells started accumulating astaxanthin (III) between days 14 and 21 and the cells had completely reddened by day 28, by which time the cells had accumulated > 200 pg of astaxanthin (III) per cell.

The cells grown in 3.0 mM and 6.0 mM nitrate showed very high growth rates up to day 21 when growth ceased. Astaxanthin (III) accumulation in the cultures was only observed after this point, but only relatively low amounts were present in the cells by the end of the experiment (< 100 pg/cell). The levels of astaxanthin (III) accumulated per cell were significantly lower than in the other three treatments. It is interesting to note that all the treatments described above accumulated relatively similar levels of astaxanthin (III) per unit volume of culture medium, except the cells grown in the highest level of nitrate (6.0 mM) which accumulated significantly lower levels than the other treatments.

Fig. 6.1a

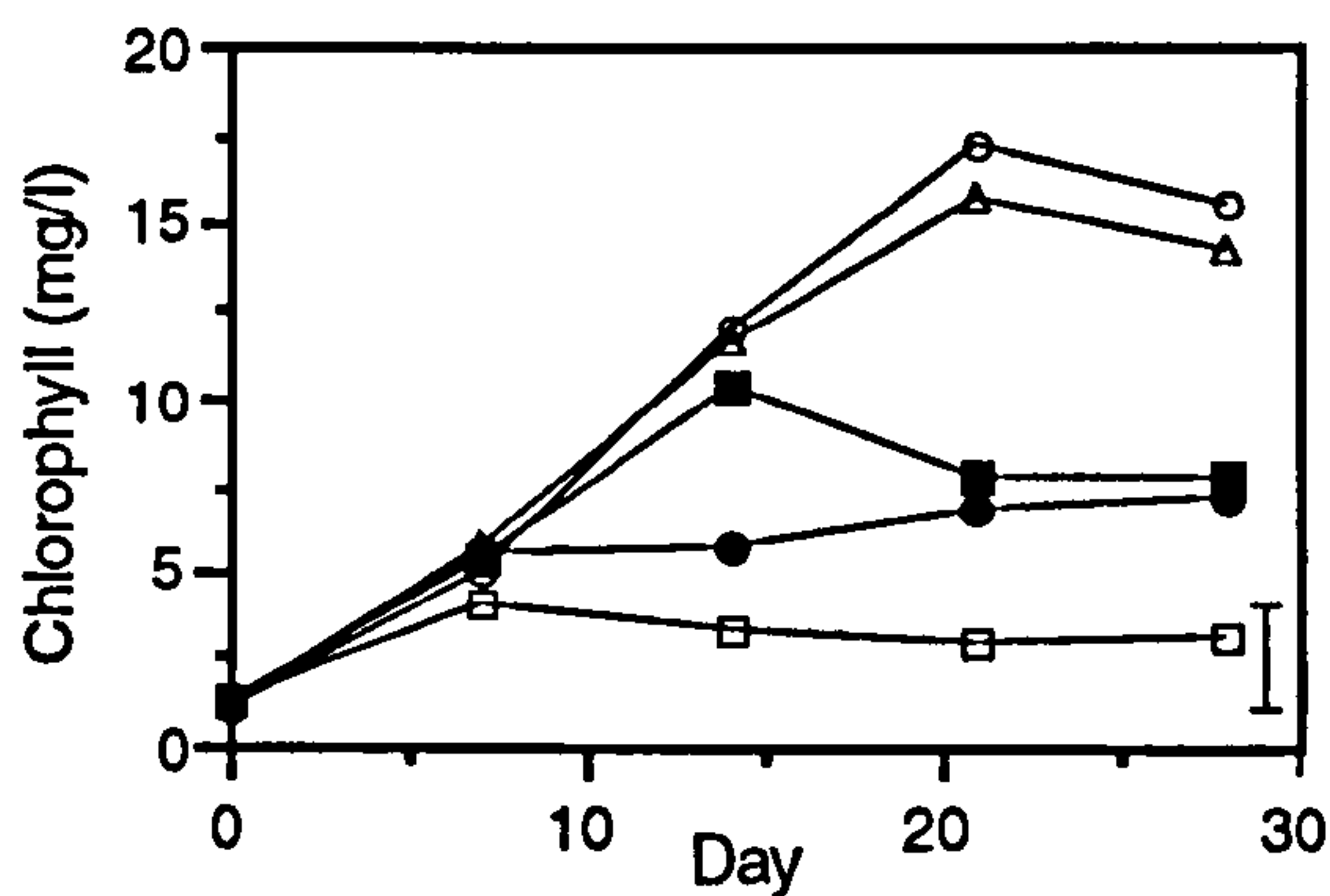


Fig. 6.1b

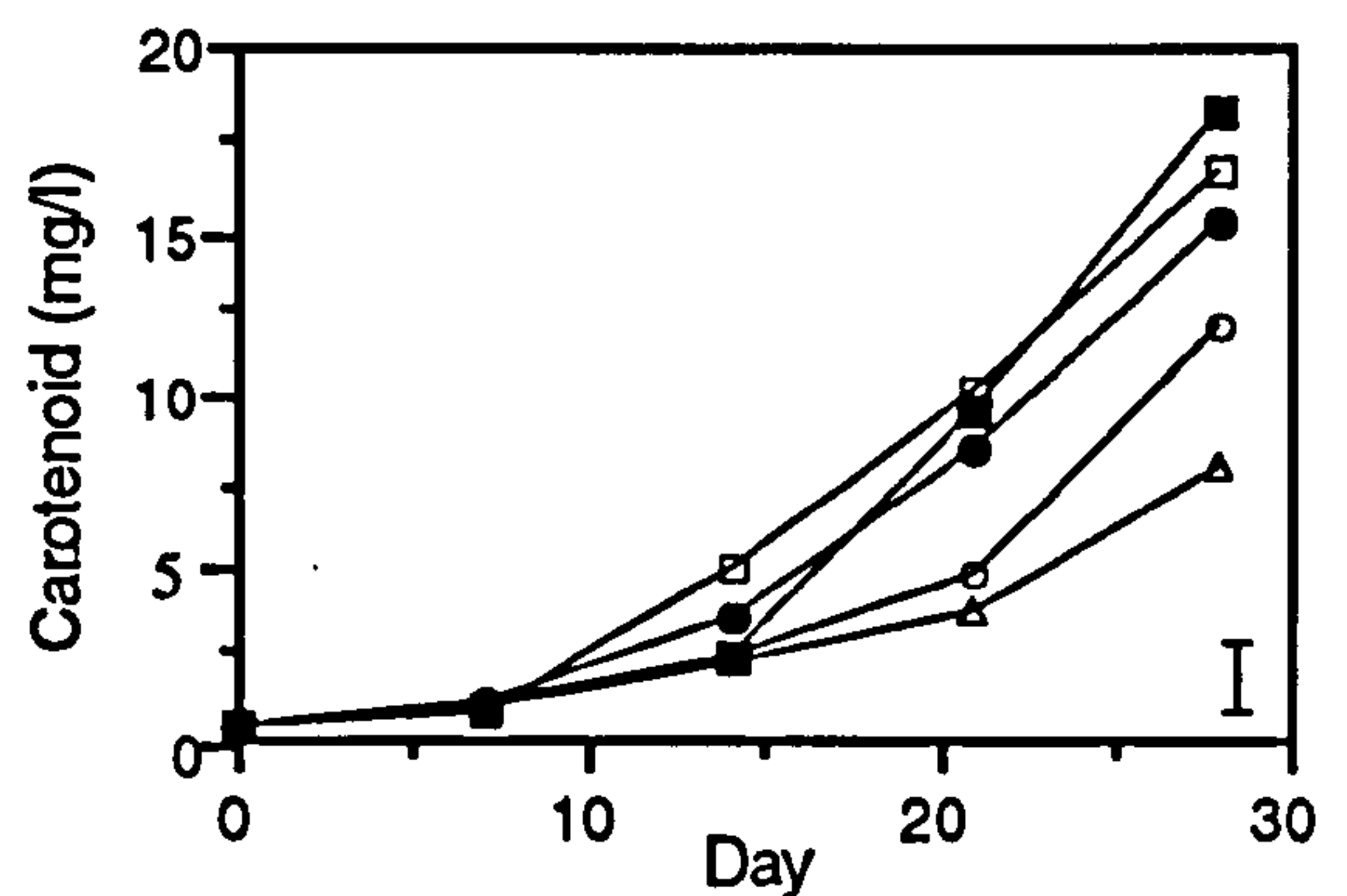


Fig. 6.1c

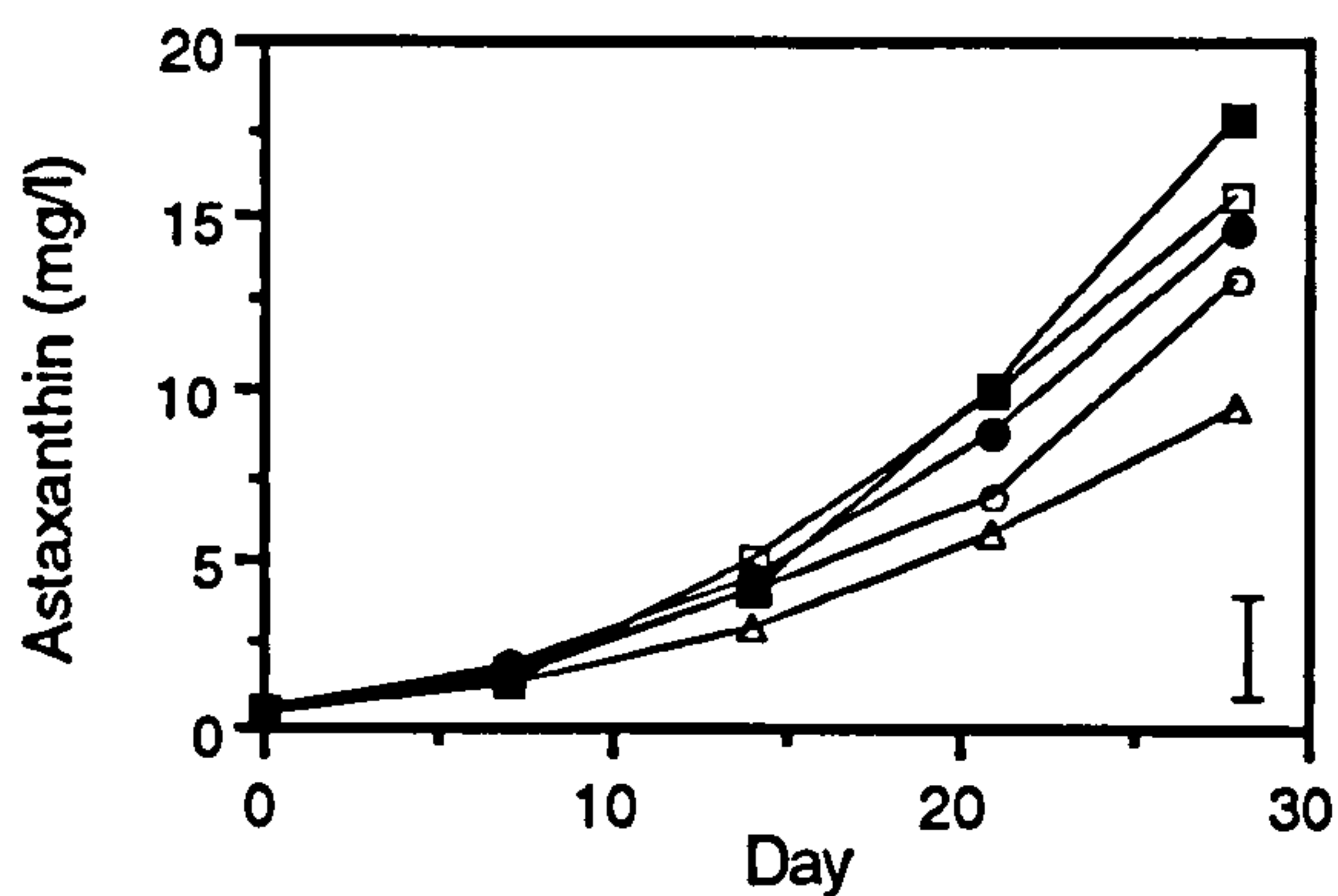


Fig. 6.1d

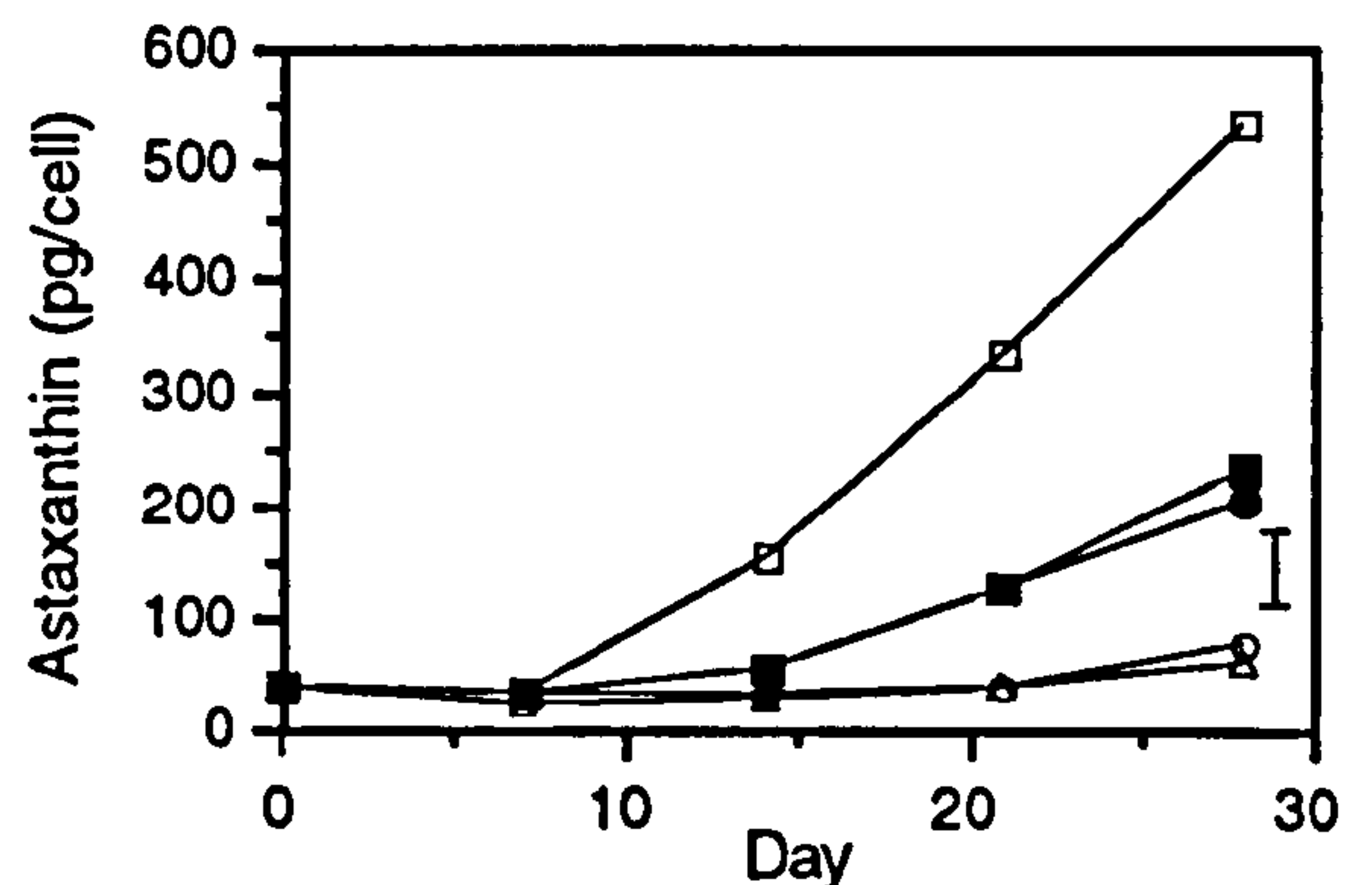
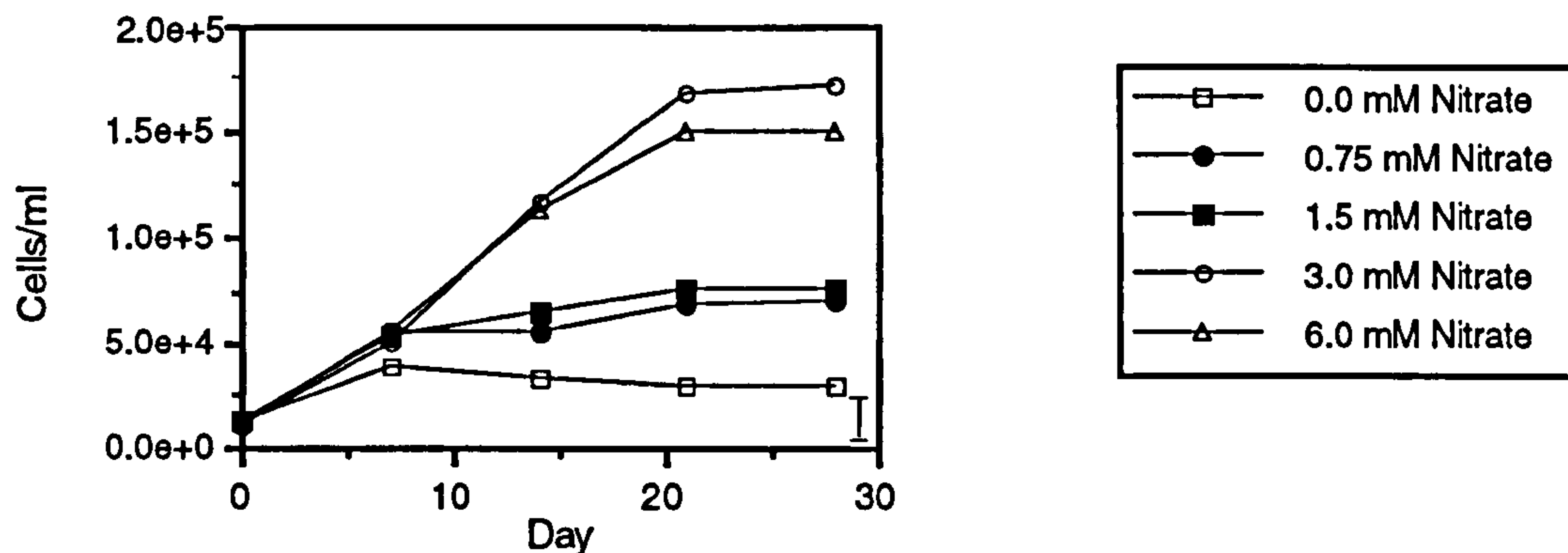




Fig. 6.1e



Figs. 6.1a-e Effect of nitrate concentration on growth and secondary carotenoid accumulation in *H. pluvialis* (S.E.  $\pm 6.2\%$ ; n=3).

### 6.3.2 Phosphate

Figures 6.2a-e show the response of cell number and pigment composition of *H. pluvialis* when cultivated in three different phosphate concentrations. The graphs indicate that algal growth was significantly better in the 1.5 and 3.0 mM phosphate cultures, with the cell number in both treatments still increasing at day 28 albeit at a reduced rate. However, these two treatments showed significantly lower levels of astaxanthin (III) accumulation than the 0.75 mM treatment, both in terms of astaxanthin (III) per unit volume of culture and astaxanthin (III) accumulated per cell.

In all the treatments astaxanthin (III) accumulation became noticeable after day 14, with relatively low amounts of astaxanthin (III) formed by day 28 in the 1.5 and 3.0 mM treatments. The cells grown in 0.75 mM phosphate showed a similar growth pattern as those grown in 1.5 and 3.0 mM phosphate cultures. These cells showed a relatively high growth rate up to day 14 after which the rate declined, with no growth between days 21 and 28. Astaxanthin (III) accumulation began after day 14 and was very rapid between days 21 and 28. The final levels of astaxanthin (III) accumulated were significantly higher relative to the other two treatments.

Fig. 6.2a

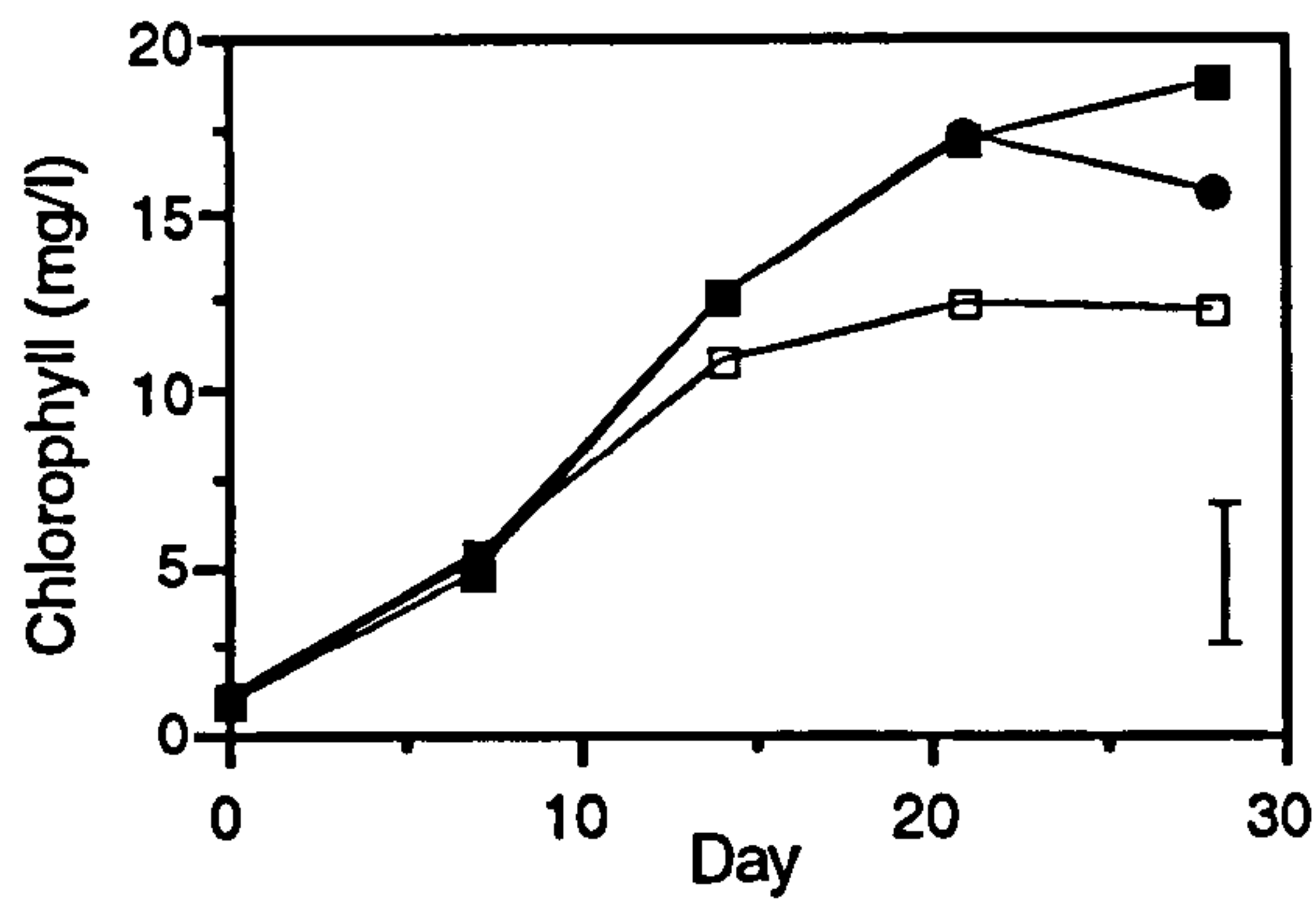


Fig. 6.2b

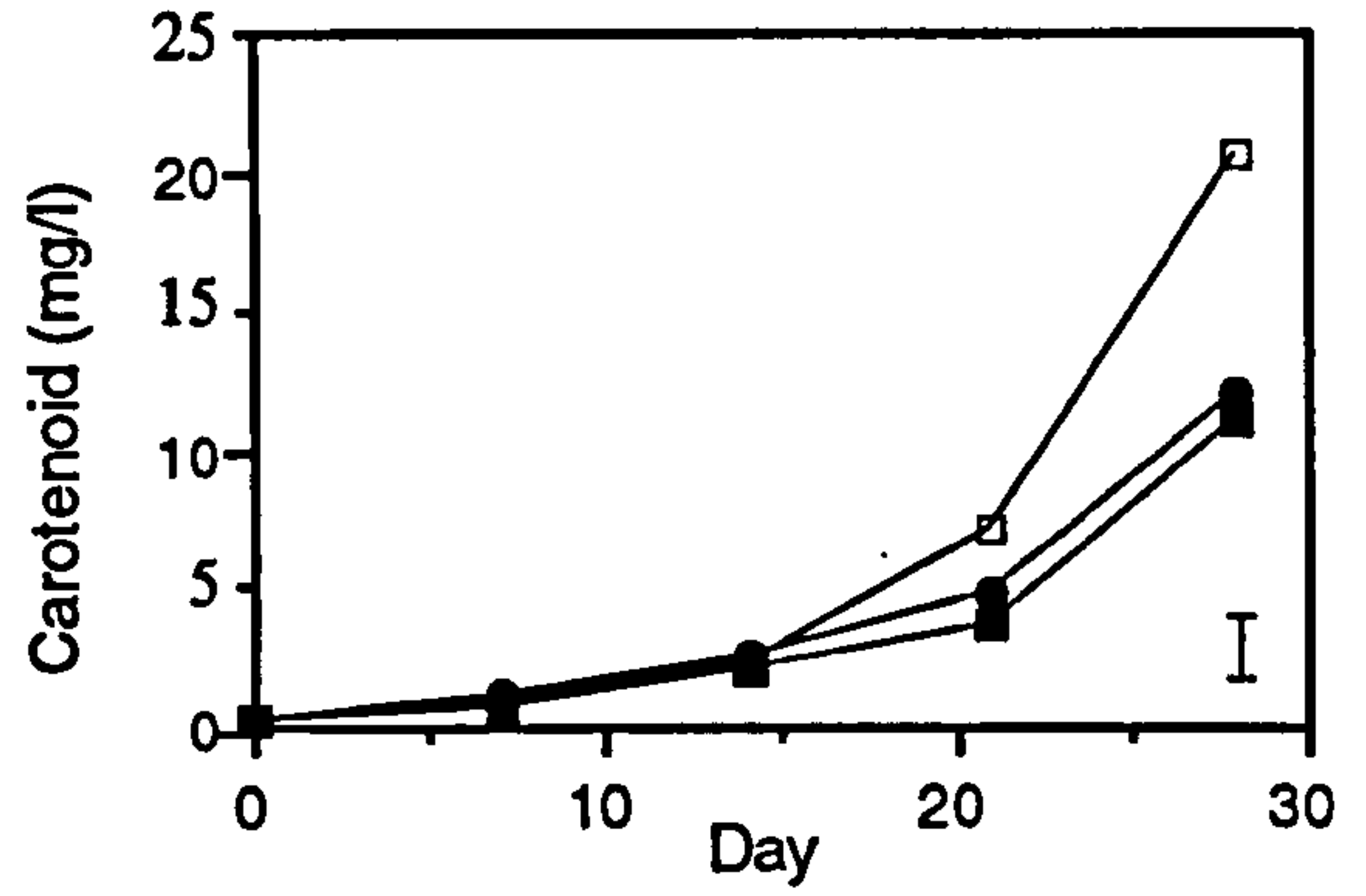


Fig. 6.2c

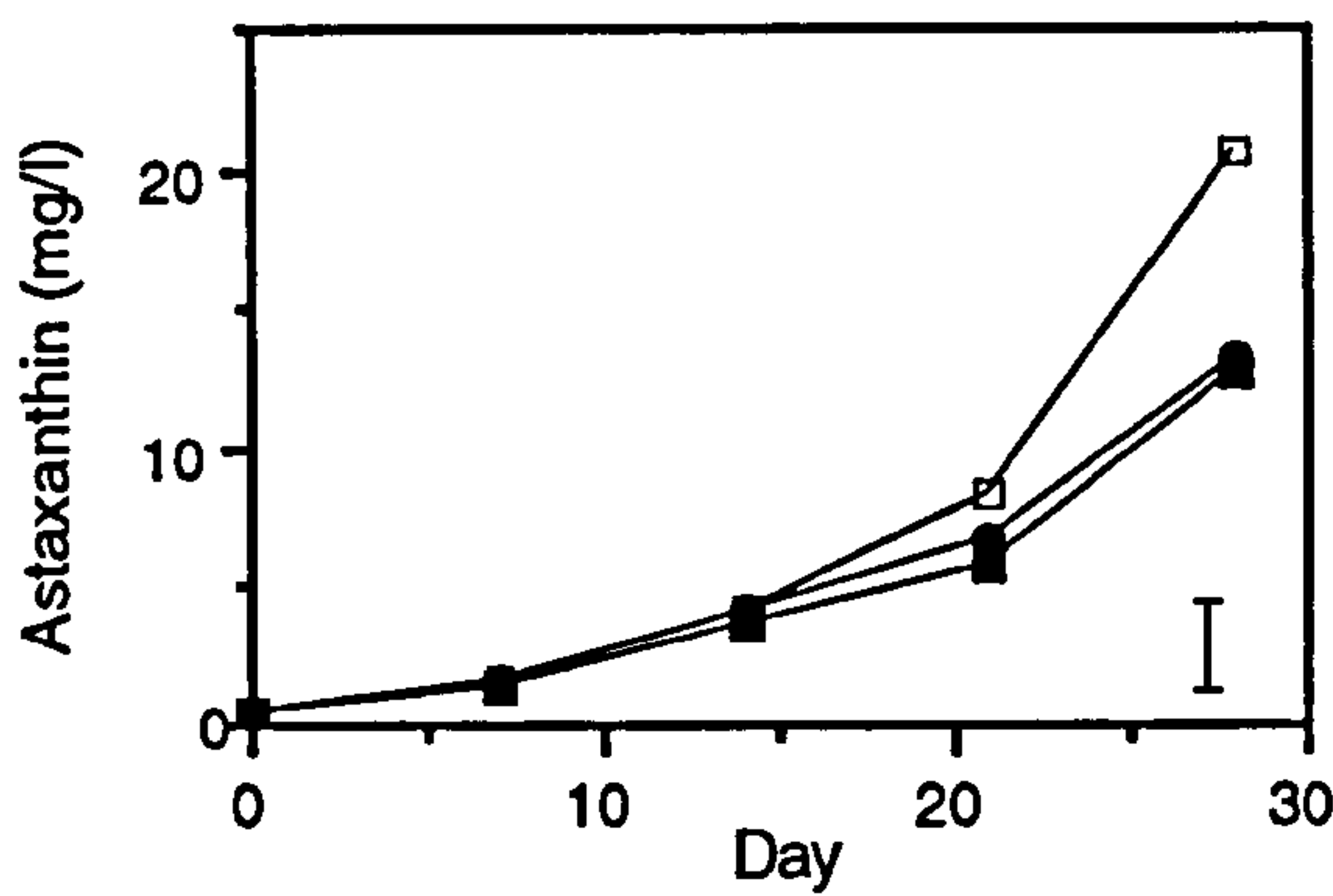


Fig. 6.2d

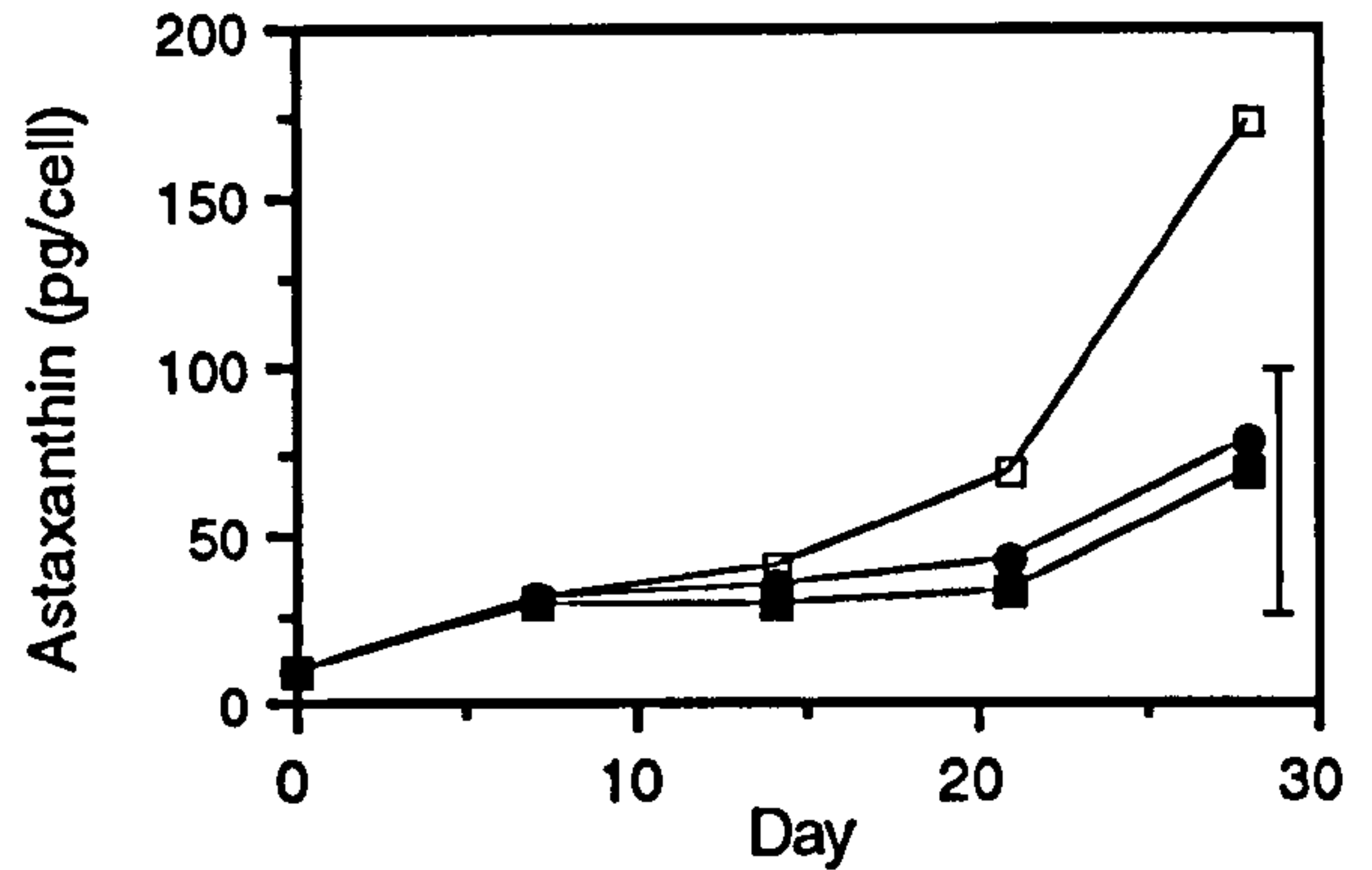
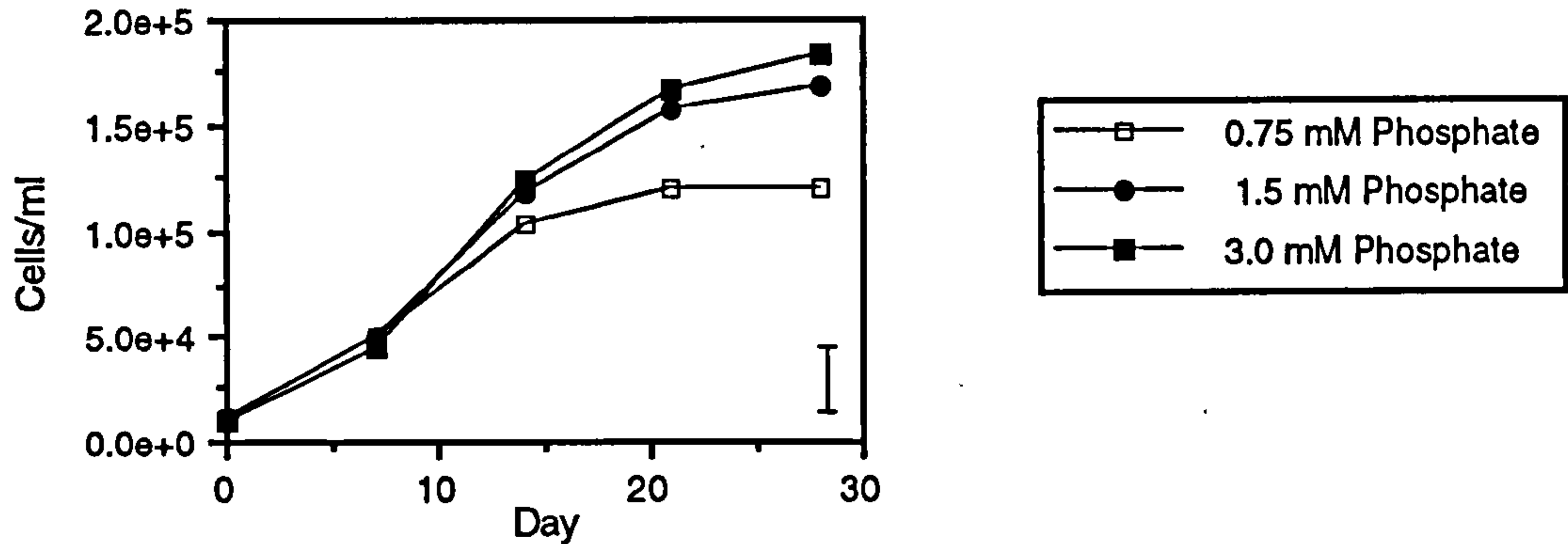


Fig. 6.2e



Figs. 6.2a-e Effect of phosphate concentration on growth and secondary carotenoid accumulation in *H. pluvialis* (S.E.  $\pm 5.8\%$ ;  $n=3$ ).

### 6.3.3 Iron

Figures 6.3a-e show the response of cell number and pigment composition of *H. pluvialis* when grown in three different concentrations of ferrous iron. The graphs indicate that cell growth was better in those cultures cultivated in the lowest iron concentrations. The



number of cells in the 72  $\mu\text{M}$  Fe cultures was significantly lower than in the 18  $\mu\text{M}$  Fe treatments. Astaxanthin (III) accumulation in all the treatments became noticeable after day 14. The rate of accumulation of secondary carotenoid per unit volume of culture was similar in all three treatments. Hence, there were no significant differences between the treatments in the amounts of astaxanthin (III) accumulated per unit volume of culture. However, the cells cultured in 72  $\mu\text{M}$  Fe contained significantly higher levels of astaxanthin (III) per cell than the cells cultivated in 18 and 36  $\mu\text{M}$  Fe.

Fig. 6.3a

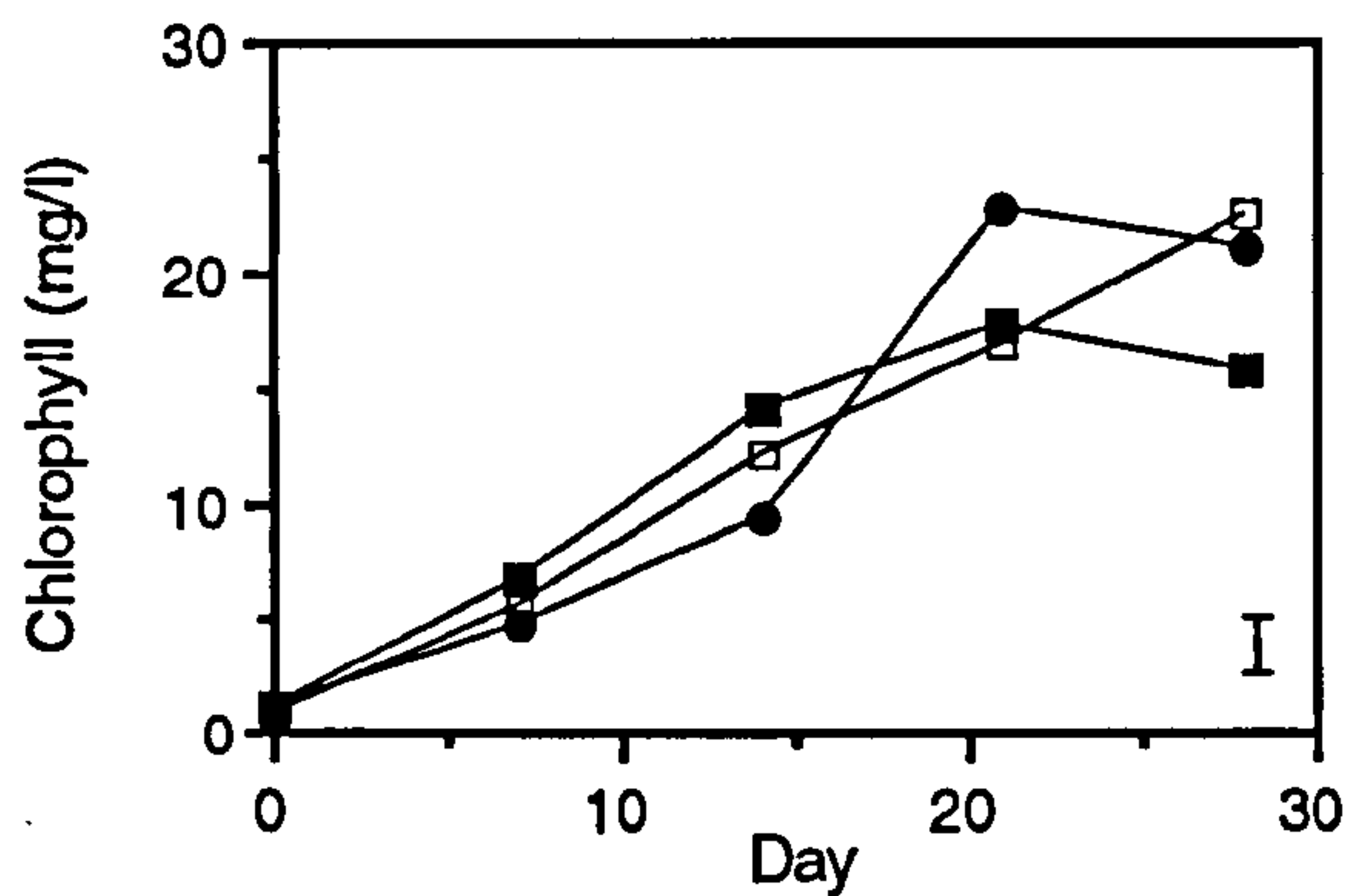


Fig. 6.3b

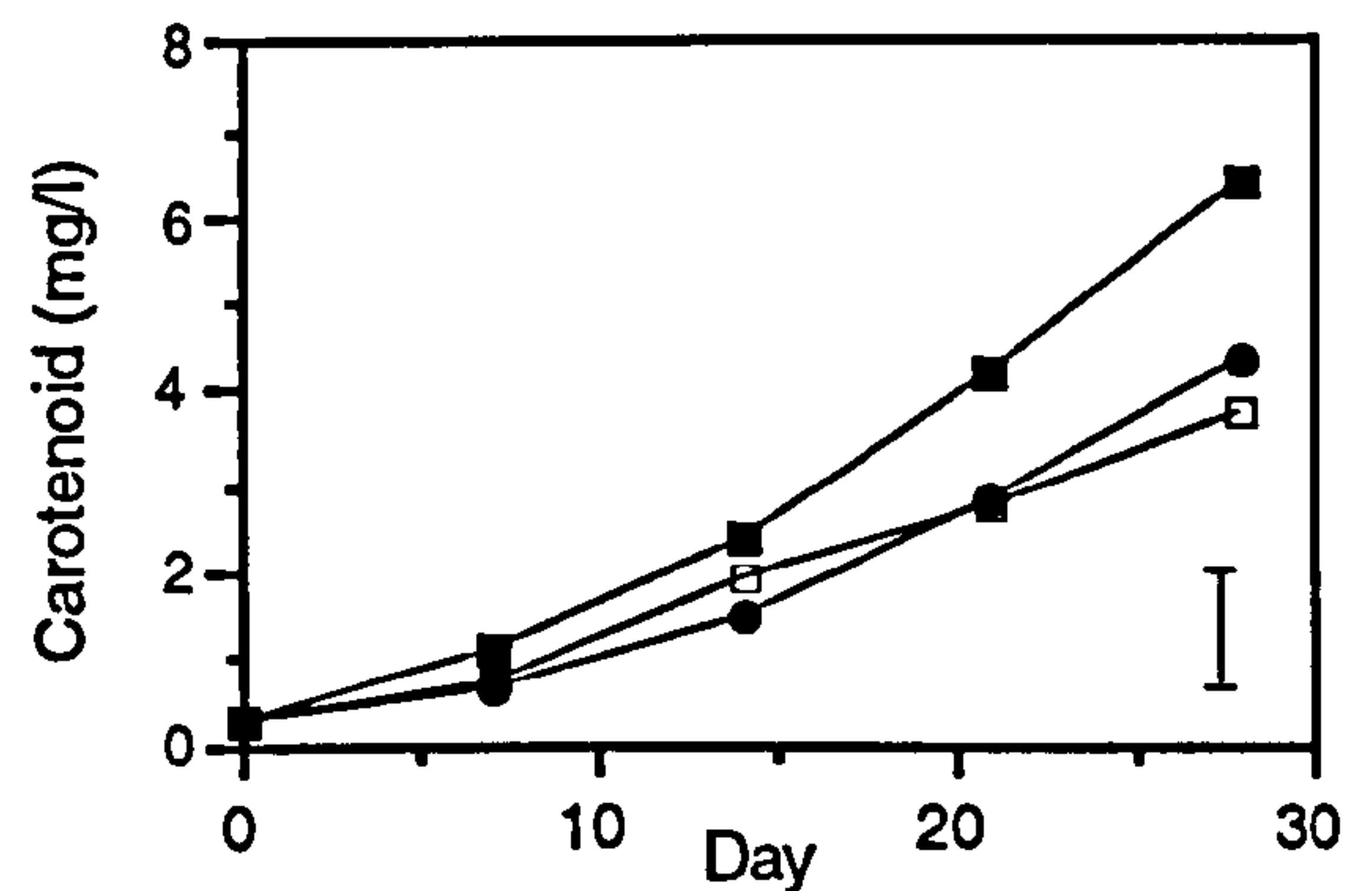


Fig. 6.3c

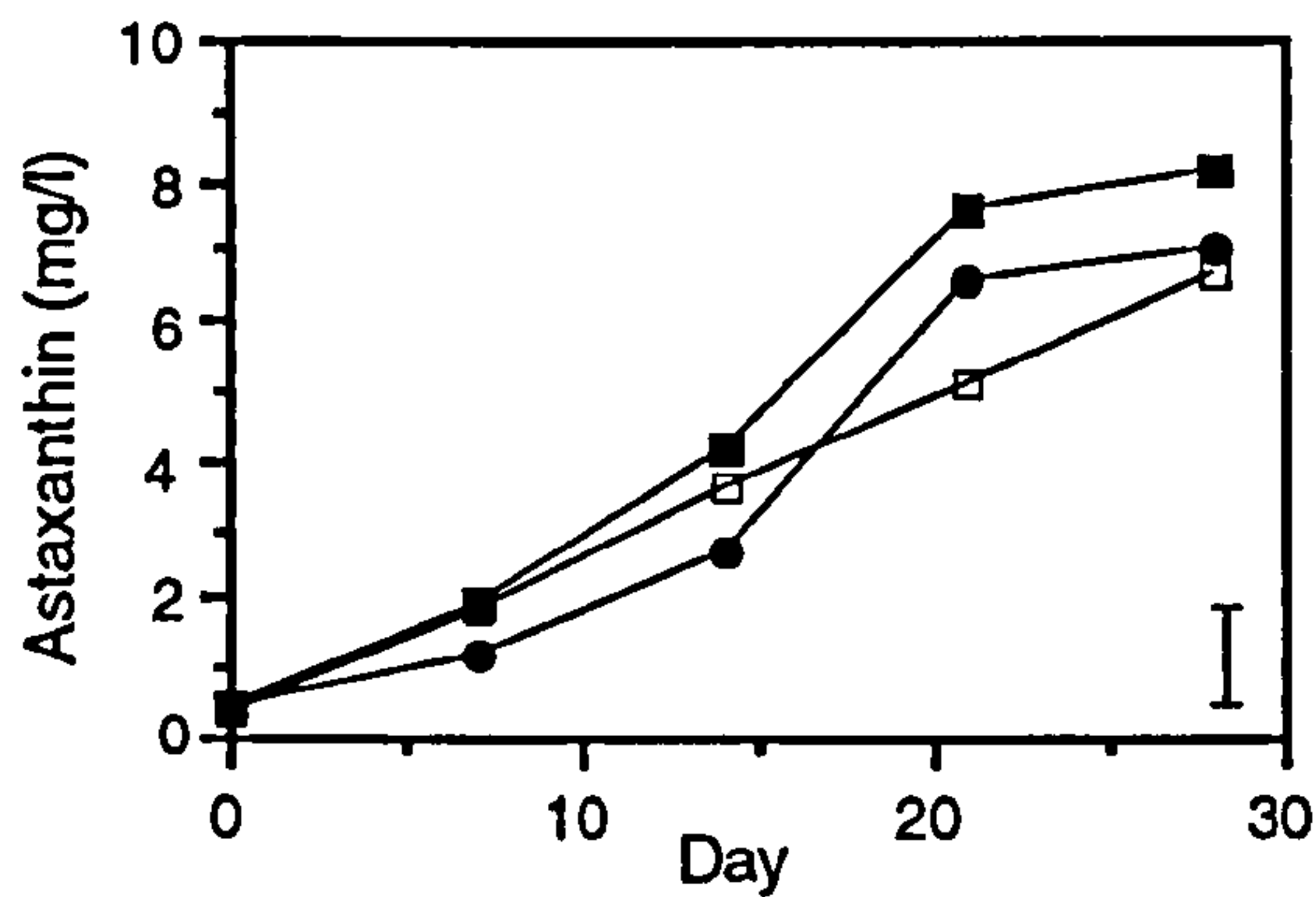


Fig. 6.3d

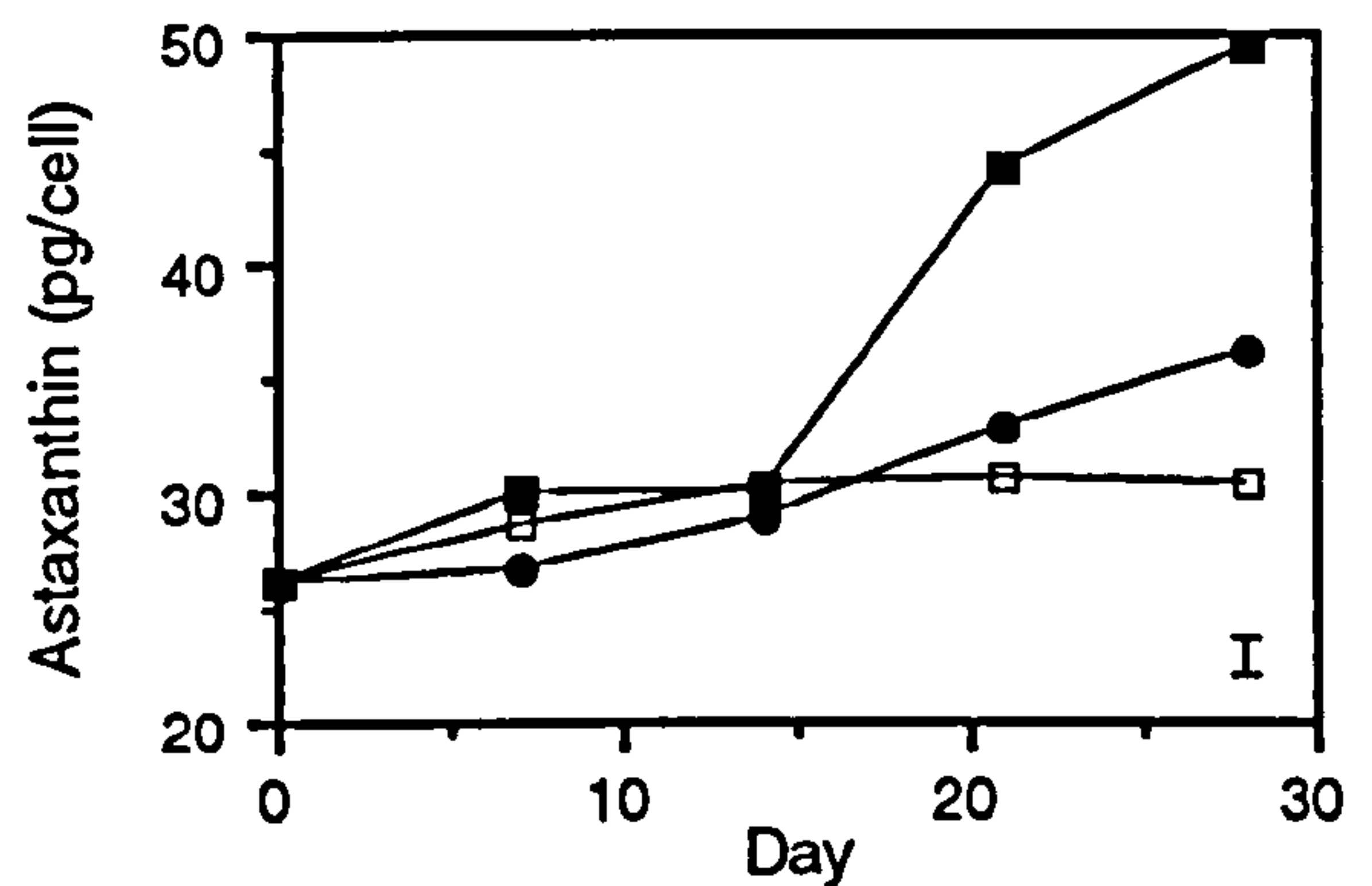
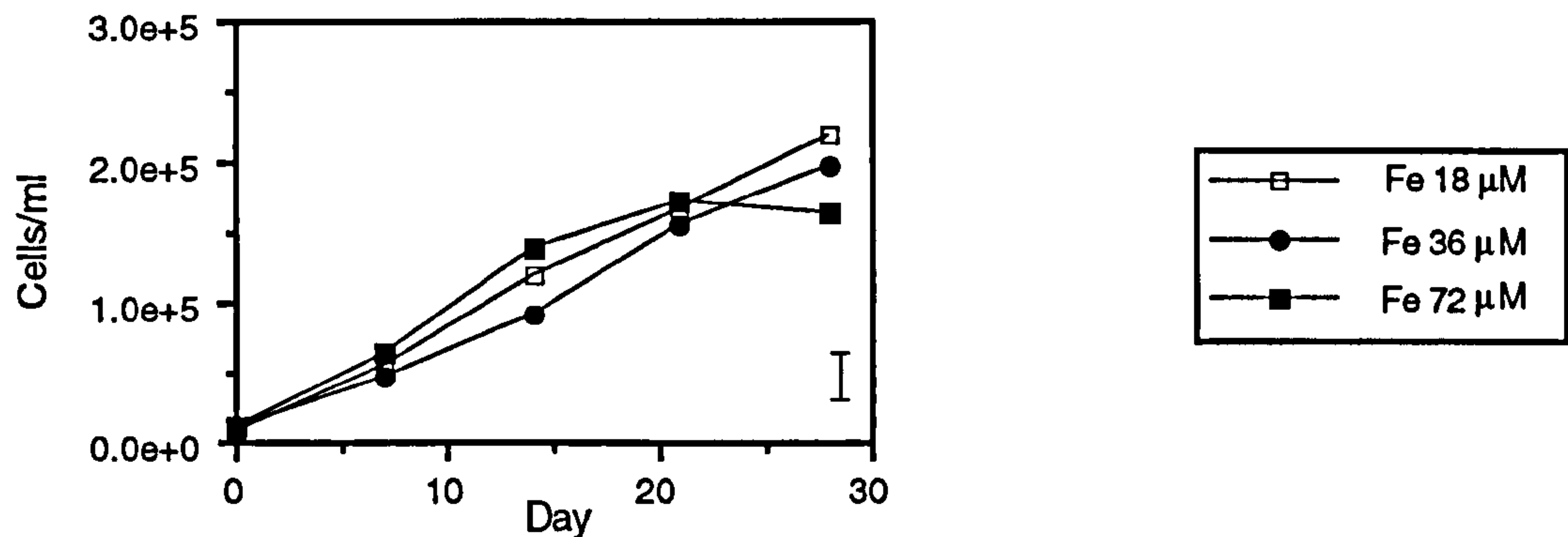


Fig. 6.3e



Figs. 6.3a-e Effect of Fe concentration on growth and secondary carotenoid accumulation in *H. pluvialis* (S.E.  $\pm 4.9\%$ ; n=3).

#### 6.3.4 NaCl

Figures 6.4a-e show the growth and pigment composition of *H. pluvialis* when cultivated in five different concentrations of NaCl. The graphs indicate that cultures exposed to NaCl were affected in different ways depending upon the NaCl concentration to which they were exposed. The addition of the salt had the effect of reducing growth rates, the effect being proportional to the amount of salt added to the cultures. This resulted in the number of cells on the final day of the experiment being significantly different between all the treatments. The addition of 342 mM NaCl proved to be quite toxic, while the addition of 685 mM NaCl was lethal after day 14 and no viable cells were observed in these cultures after this point. No cessation of growth was observed in the cells grown in 0.0 mM and 85 mM NaCl, however, the growth rate did recede after day 14 in both cultures. The addition of 171 mM NaCl inhibited cell growth after day 7 when a slight decrease in cell number was observed.

The levels of astaxanthin (III) per unit volume of culture on the final day were significantly different between all the treatments. Astaxanthin (III) accumulation became noticeable in the 86 and 171 mM NaCl cultures between days 7 to 14. The few remaining viable cells in the 342 mM NaCl cultures also began accumulating astaxanthin (III) at this time. The cells cultured in 86 mM NaCl accumulated the largest amounts of astaxanthin (III) with the control cultures accumulating the second highest levels. The cells cultured in 171 and 342 mM NaCl accumulated relatively low levels of astaxanthin (III). However, when this is compared to the levels of astaxanthin (III) per cell it can be observed that cells



cultured in 342 mM NaCl accumulated significantly higher levels of astaxanthin (III) per cell than cells in the other treatments. The levels of astaxanthin (III) per cell were very similar in the 86 and 171 mM cultures, whilst the control cultures (no NaCl added) accumulated relatively low amounts of astaxanthin (III) per cell.

Fig. 6.4a

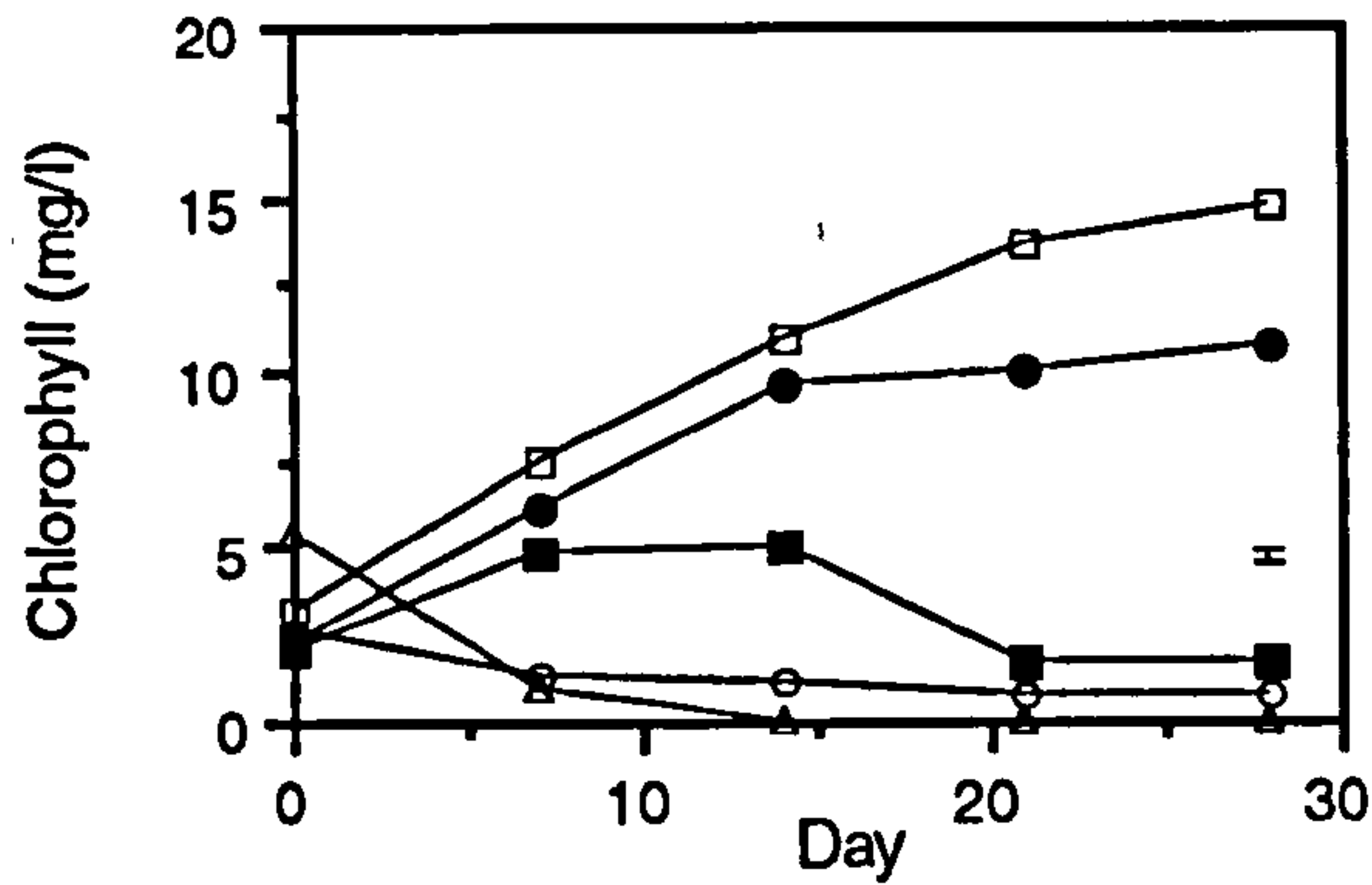


Fig. 6.4b

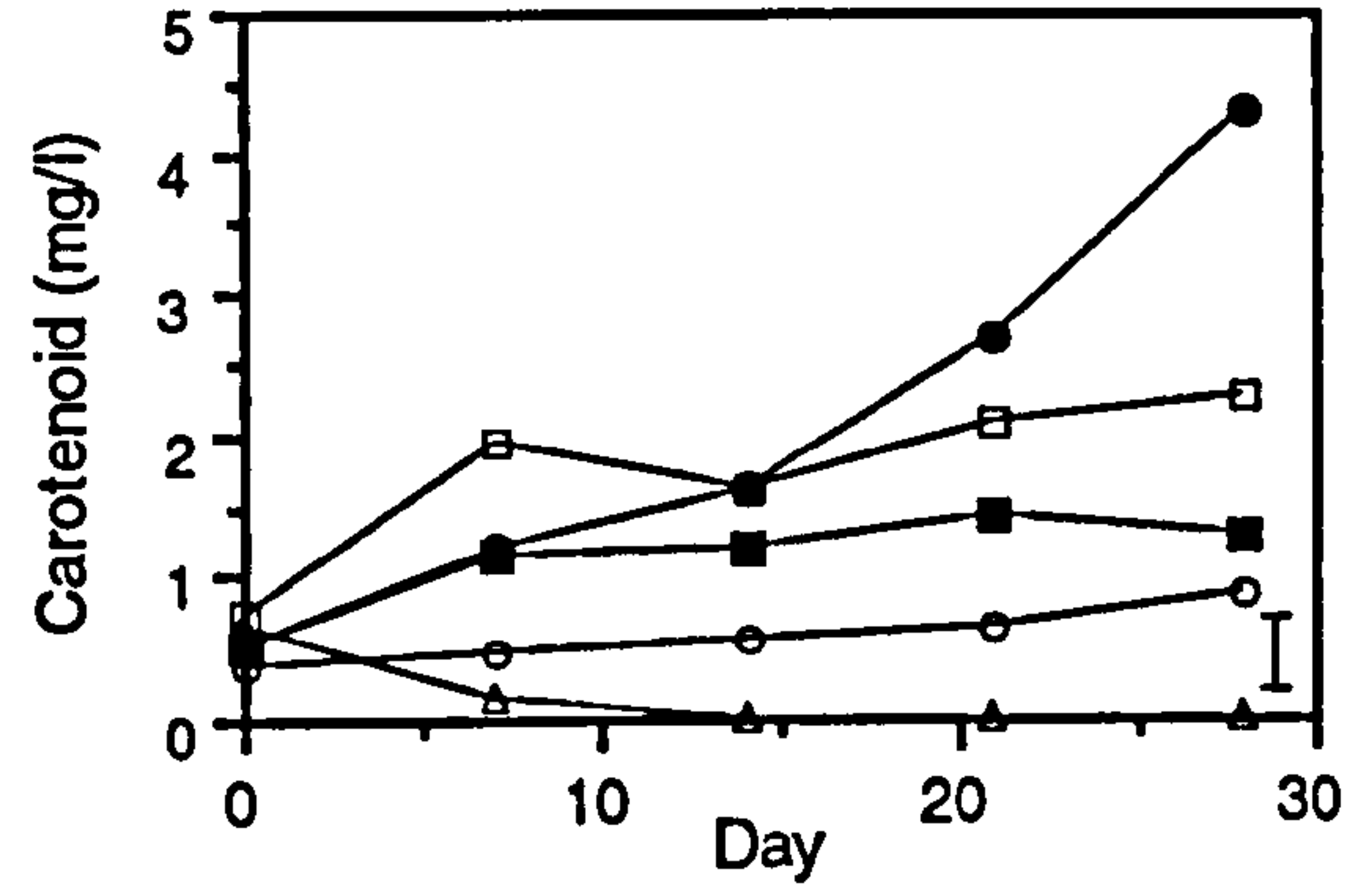


Fig. 6.4c

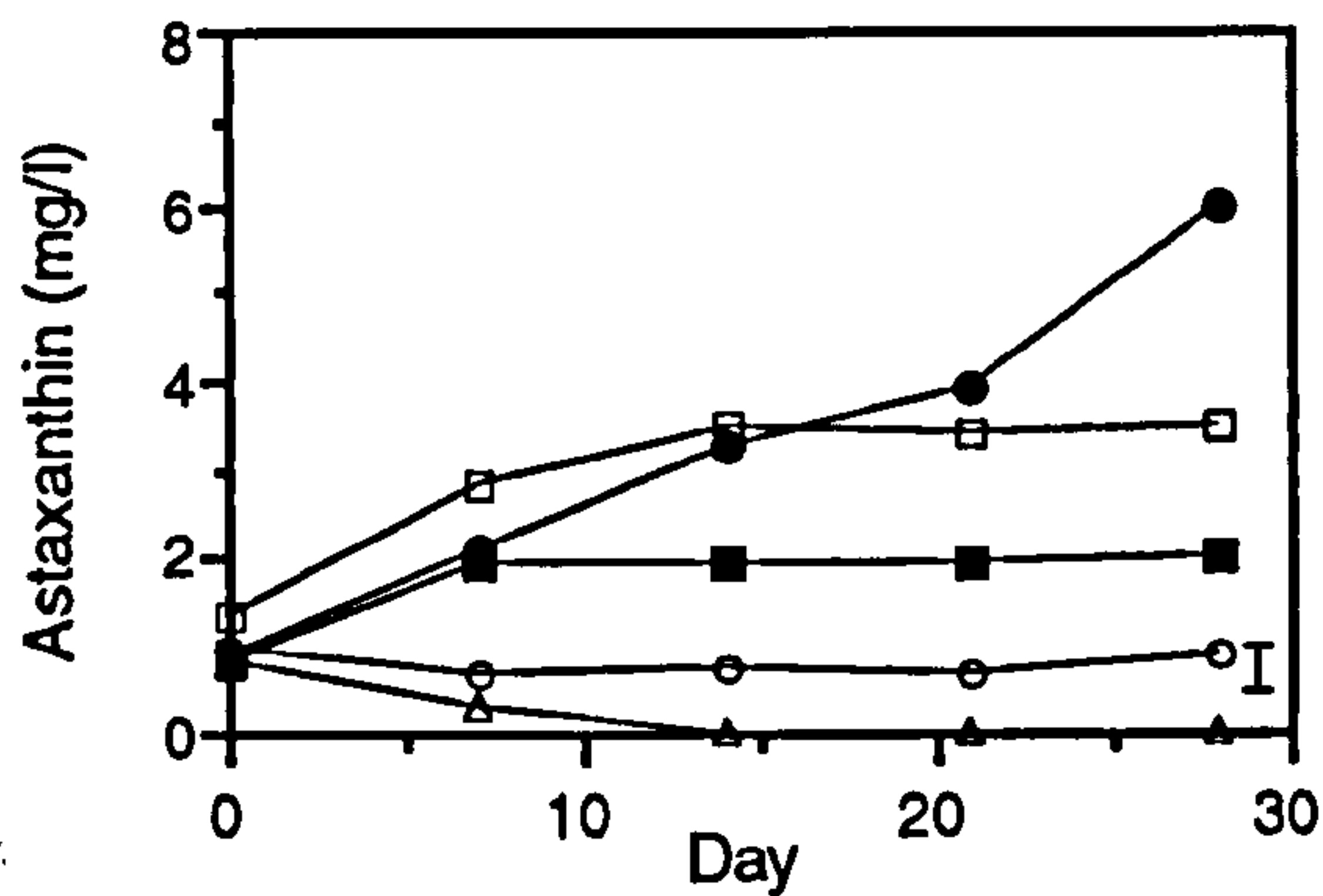


Fig. 6.4d

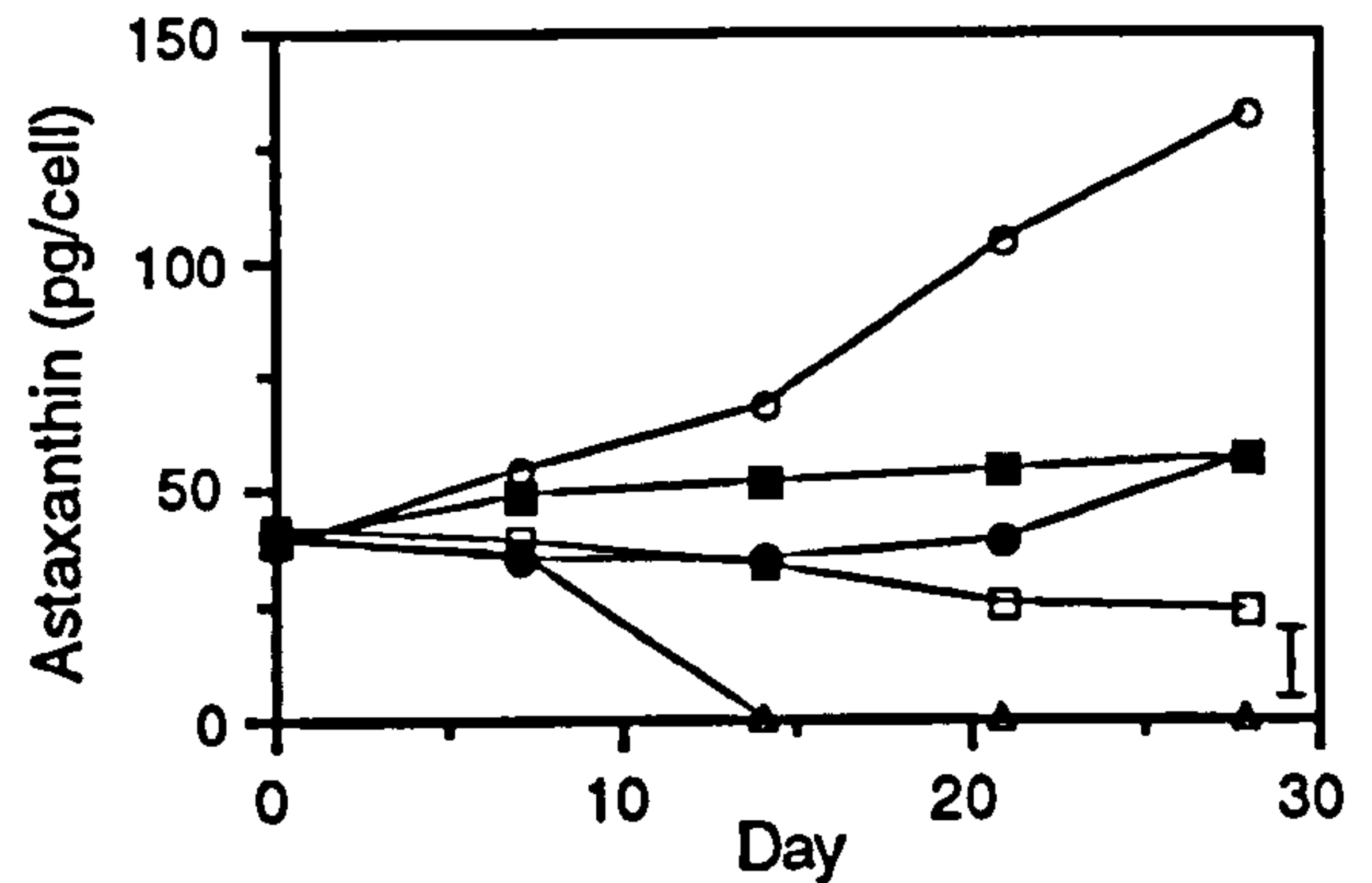
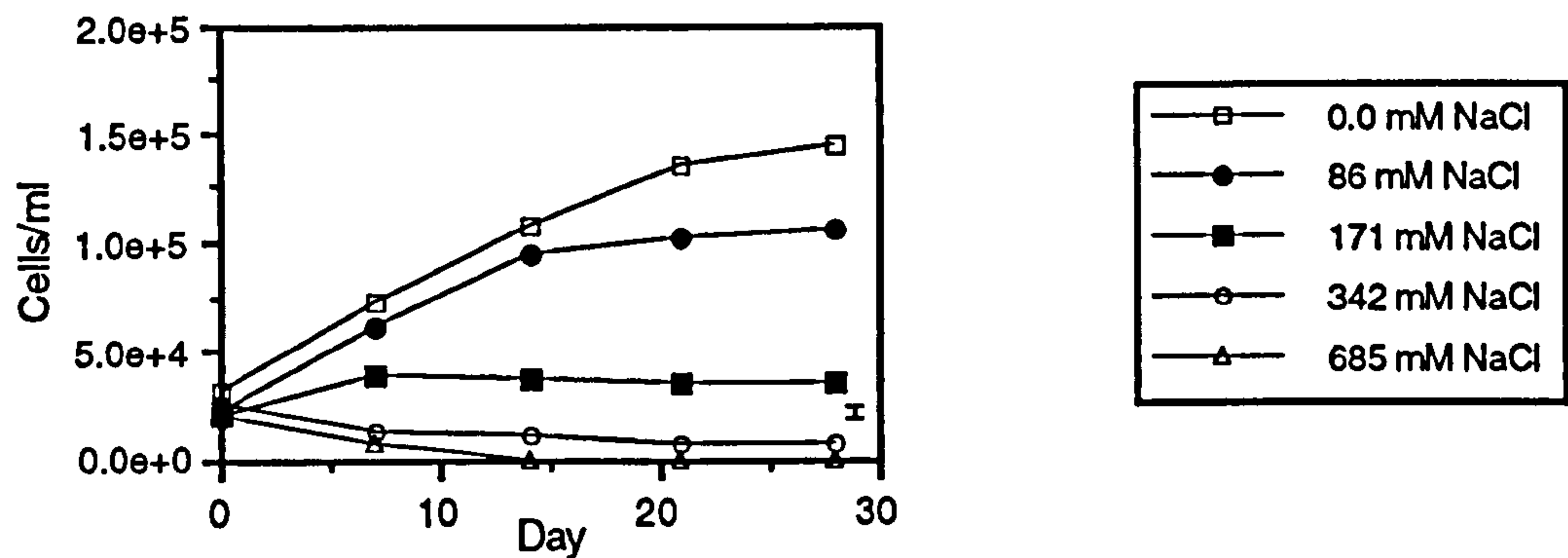


Fig. 6.4e



Figs. 6.4a-e Effect of NaCl concentration on growth and secondary carotenoid accumulation in *H. pluvialis* (S.E.  $\pm 5.7\%$ ; n=3).

### 6.3.5 KCl

Figures 6.5a-e show the response of cell number and pigment composition of *H. pluvialis* when grown in five different KCl concentrations. The graphs show that a similar trend was observed in these cultures as was observed in the cultures cultivated in NaCl. At high KCl concentrations the growth of *H. pluvialis* was inhibited; the extent of this inhibition was dependent on the concentration of KCl added to the cultures.

Similarly the amount of astaxanthin (III) accumulated per unit volume of culture appeared to be dependent on the amount of KCl added to the cultures. In the majority of the treatments astaxanthin (III) accumulation become noticeable between days 7 to 14. The cultures with the lowest amounts of KCl added accumulated significantly higher levels of astaxanthin (III) per unit volume of culture than those cultures subjected to the higher levels of KCl, with the control cultures (no KCl added) accumulating significantly higher levels of astaxanthin (III) than all the other treatments. At the highest concentrations of KCl (which had the poorest growth rates) the cultures accumulated the lowest amounts of astaxanthin (III) per unit volume of culture. When the data is expressed as the amount of astaxanthin (III) accumulated per cell, it can be seen that the levels were significantly lower in the 0.0 and 67 mM KCl cultures than the other treatments. Cells grown at 268 mM KCl accumulated significantly higher amounts of astaxanthin (III) per cell than any of the other treatments. The cells grown in 134 and 537 mM KCl accumulated relatively similar amounts of astaxanthin (III) per cell. Overall cell growth and astaxanthin (III) accumulation per unit volume of culture were significantly better in the control cultures indicating a strong phytotoxic effect of KCl on the alga.



Fig. 6.5a

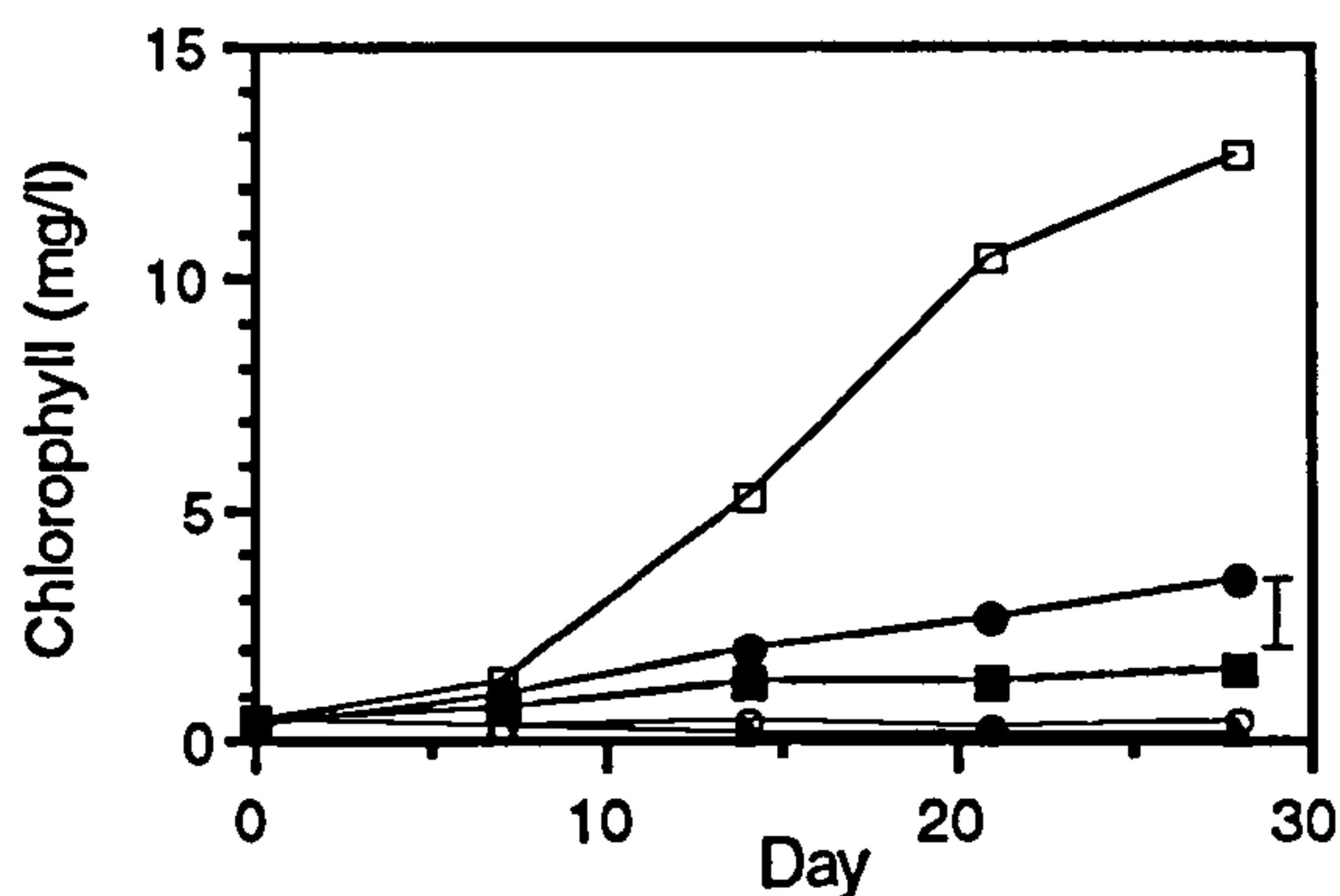


Fig. 6.5b

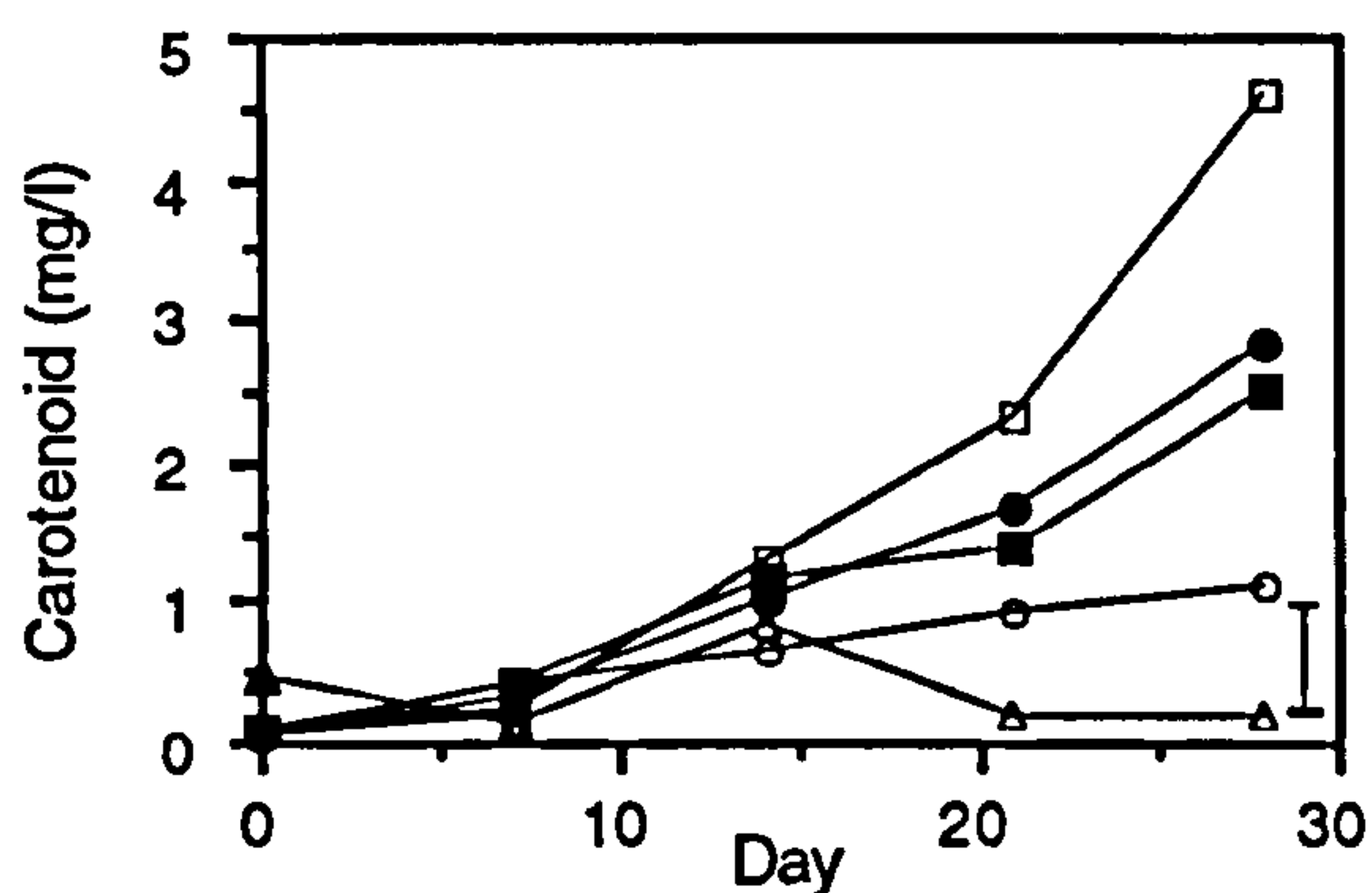


Fig. 6.5c

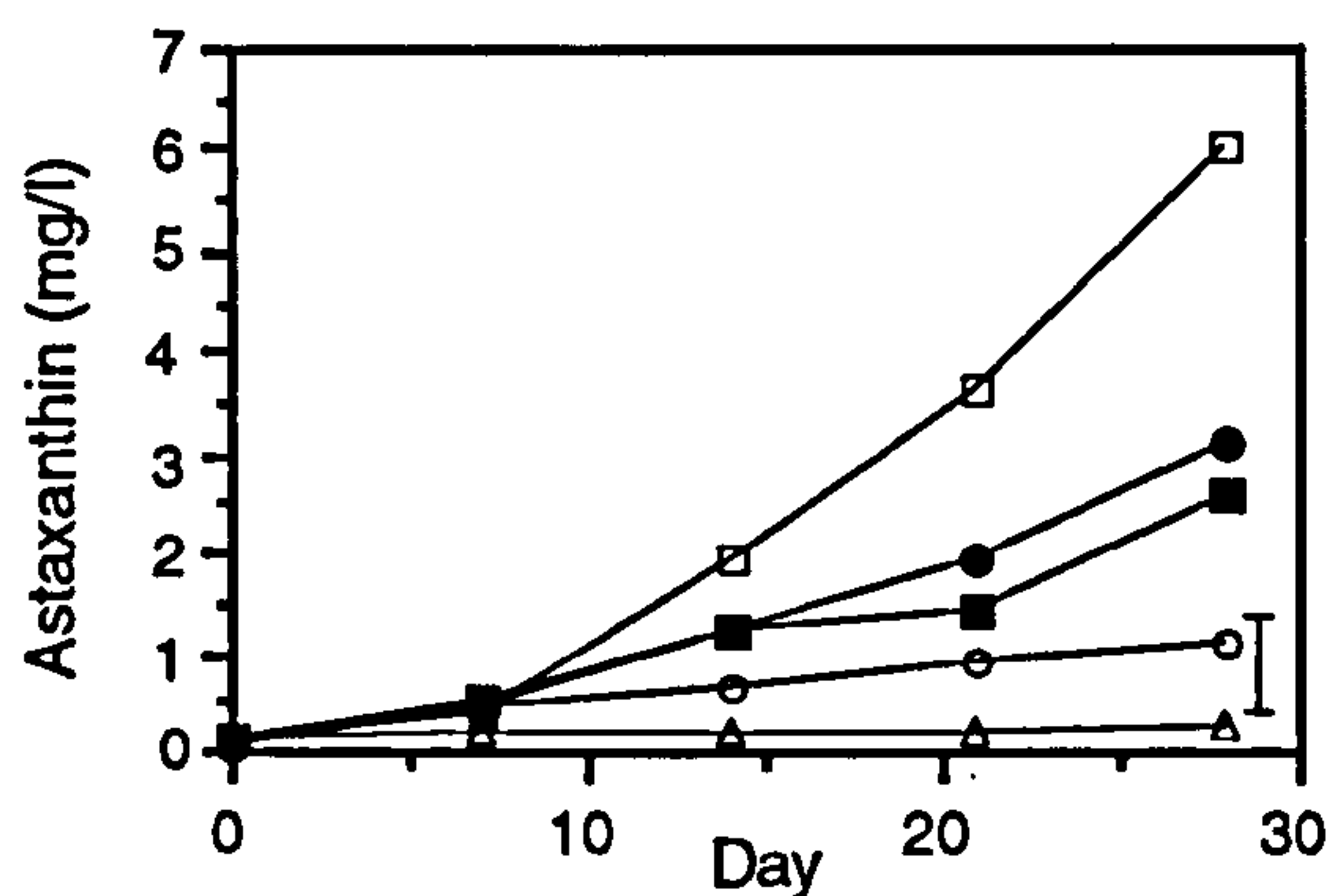


Fig. 6.5d

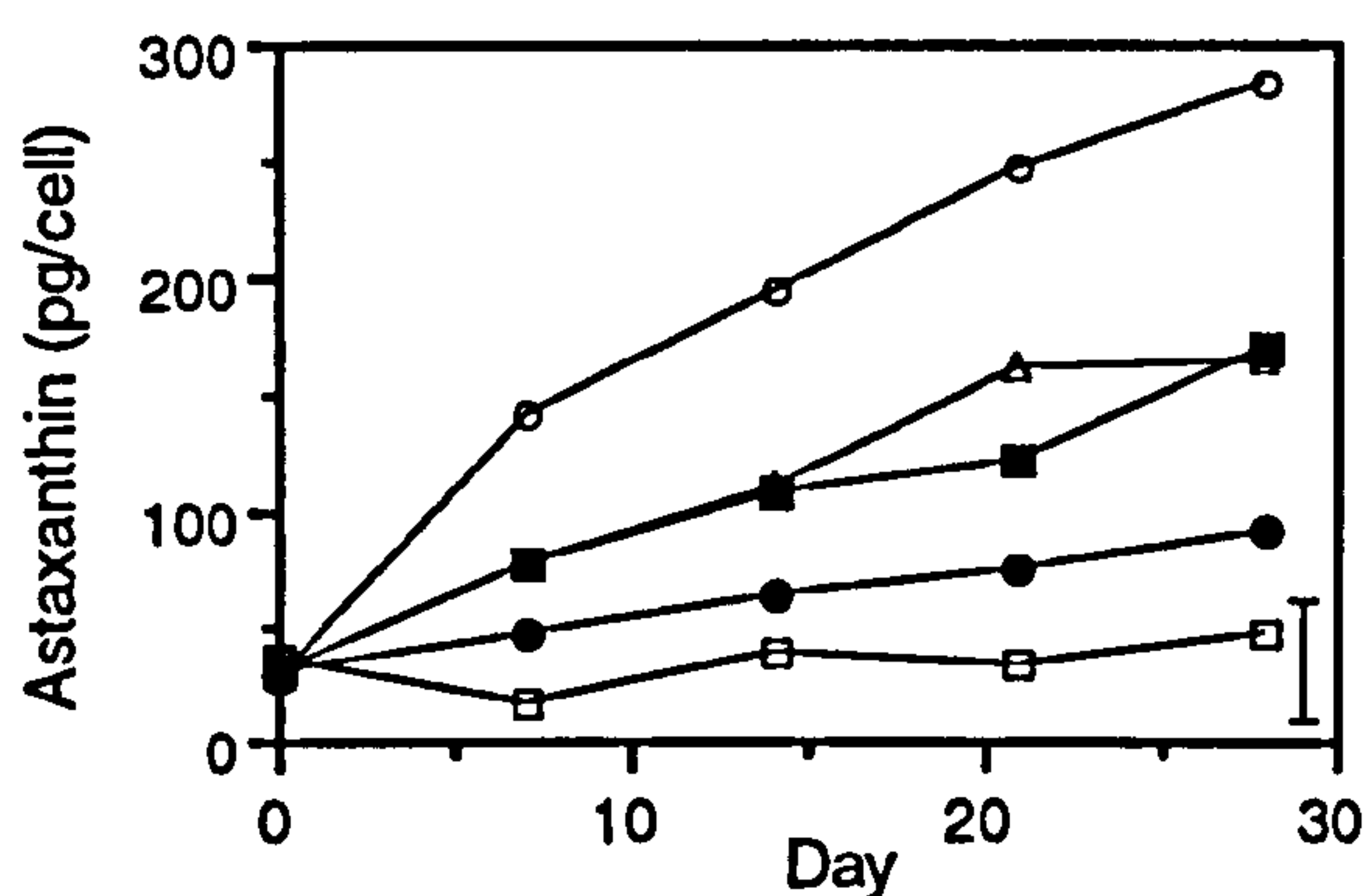
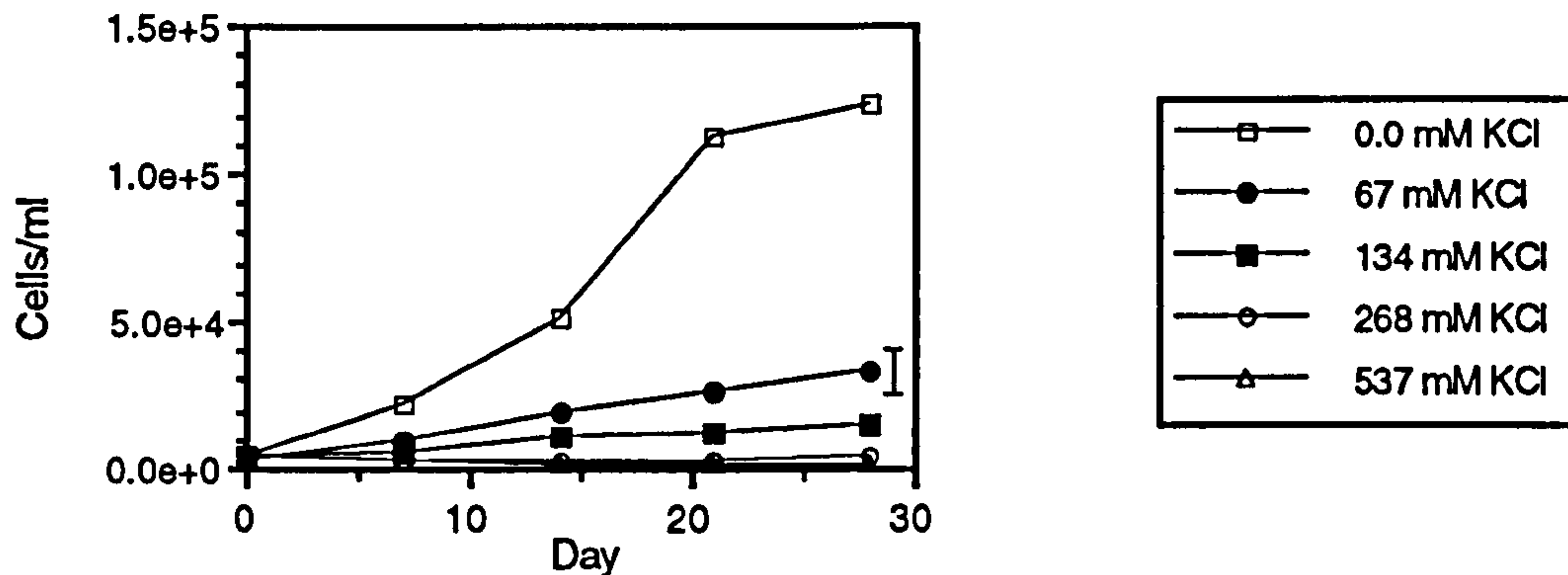


Fig. 6.5e



Figs. 6.5a-e Effect of KCl concentration on growth and secondary carotenoid accumulation in *H. pluvialis* (S.E.  $\pm 6.2\%$ ;  $n=3$ ).

### 6.3.6 Specific NaCl concentration

Figures 6.6a-e show the growth and pigment composition of *H. pluvialis* when grown in four different concentrations of NaCl to try and determine the optimum concentration for secondary carotenoid formation in the alga. In the first set of experiments using NaCl to induce secondary carotenoid synthesis in the alga, the range of salt concentrations

investigated was too great to identify the optimum concentration required to achieve maximum rates of secondary carotenogenesis (Figs. 6.4a-e). However, these results did indicate that the optimum concentration should be within the range 0 - 100 mM NaCl. NaCl was used in these experiments in preference to KCl because of the strong toxic effects KCl exerted on the alga in the previous experiment.

As with the previous experiments (Figs. 6.4a-e) the graphs indicate that cell growth was significantly better when the cells were grown in the absence of NaCl. These cultures continued to grow until day 14 after which growth ceased and there was a slight decline in cell number during the remainder of the experiment. In the cultures containing 40 and 70 mM NaCl no growth was observed during the experiment with cell number remaining relatively constant. The number of cells on the final day was significantly lower than in the control cultures. The number of cells in the highest NaCl concentration (100 mM NaCl) declined throughout the experiment, with the reduction being particularly rapid in the early stages of the experiment.

All the treatments showed a very similar pattern of astaxanthin (III) accumulation when expressed in terms of per unit volume of culture. The rate of accumulation in the cultures was initially slow but increased towards the latter stages of the experiment. The amounts of astaxanthin (III) accumulated per unit volume of culture were significantly higher in the 40 mM NaCl cultures than in any of the other treatments. The 0.0 and 70 mM NaCl cultures accumulated similar levels of astaxanthin(III), whilst the 100 mM NaCl cultures accumulated significantly lower levels of astaxanthin (III) than the other treatments. The amounts of astaxanthin (III) accumulated per cell was highest in the 100 mM NaCl cultures, with levels being significantly higher than in the other NaCl treatments. As before (Fig. 6.4e) in the absence of NaCl, cultures accumulated significantly lower levels of astaxanthin (III) per cell than the other treatments.



Fig. 6.6a

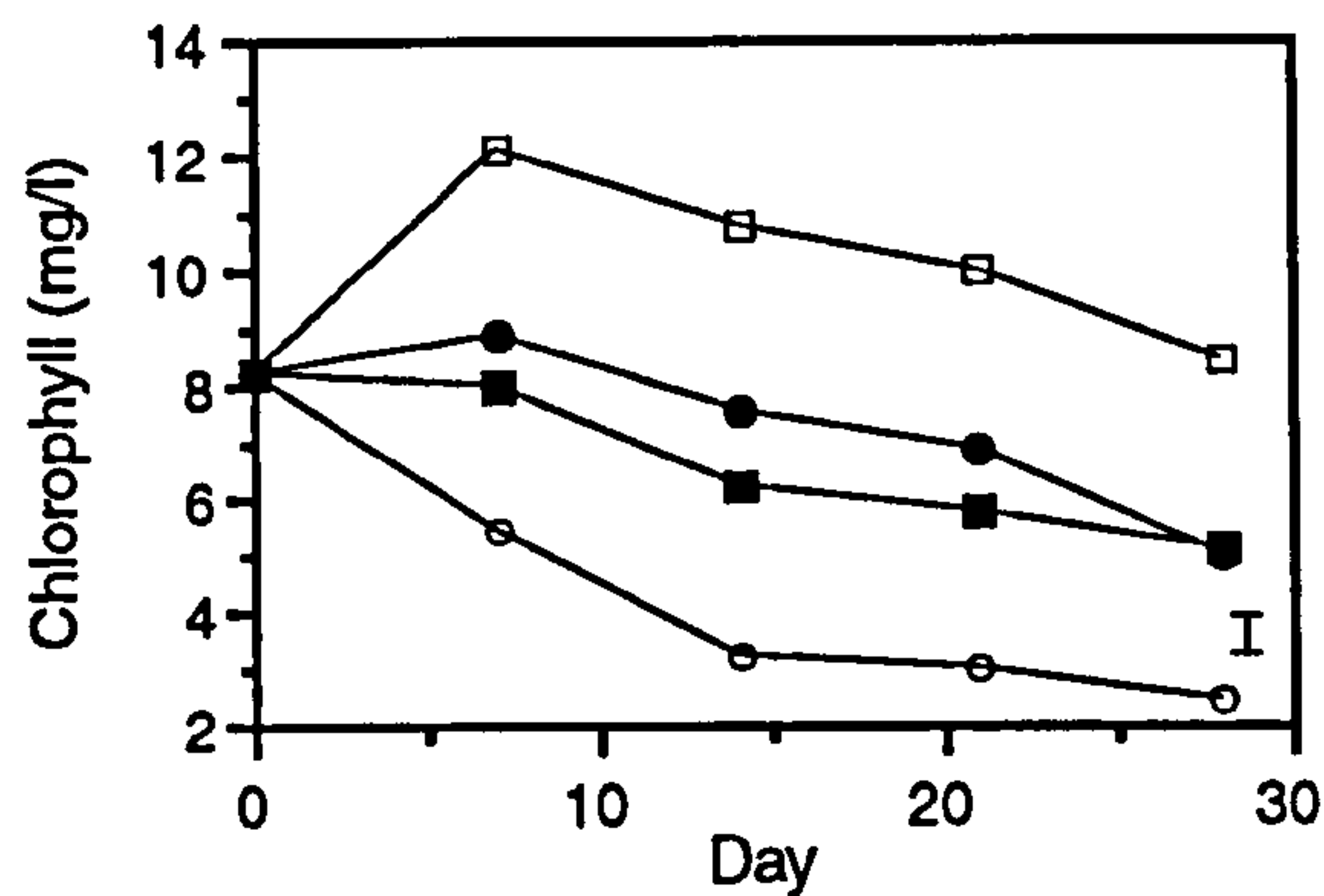


Fig. 6.6b

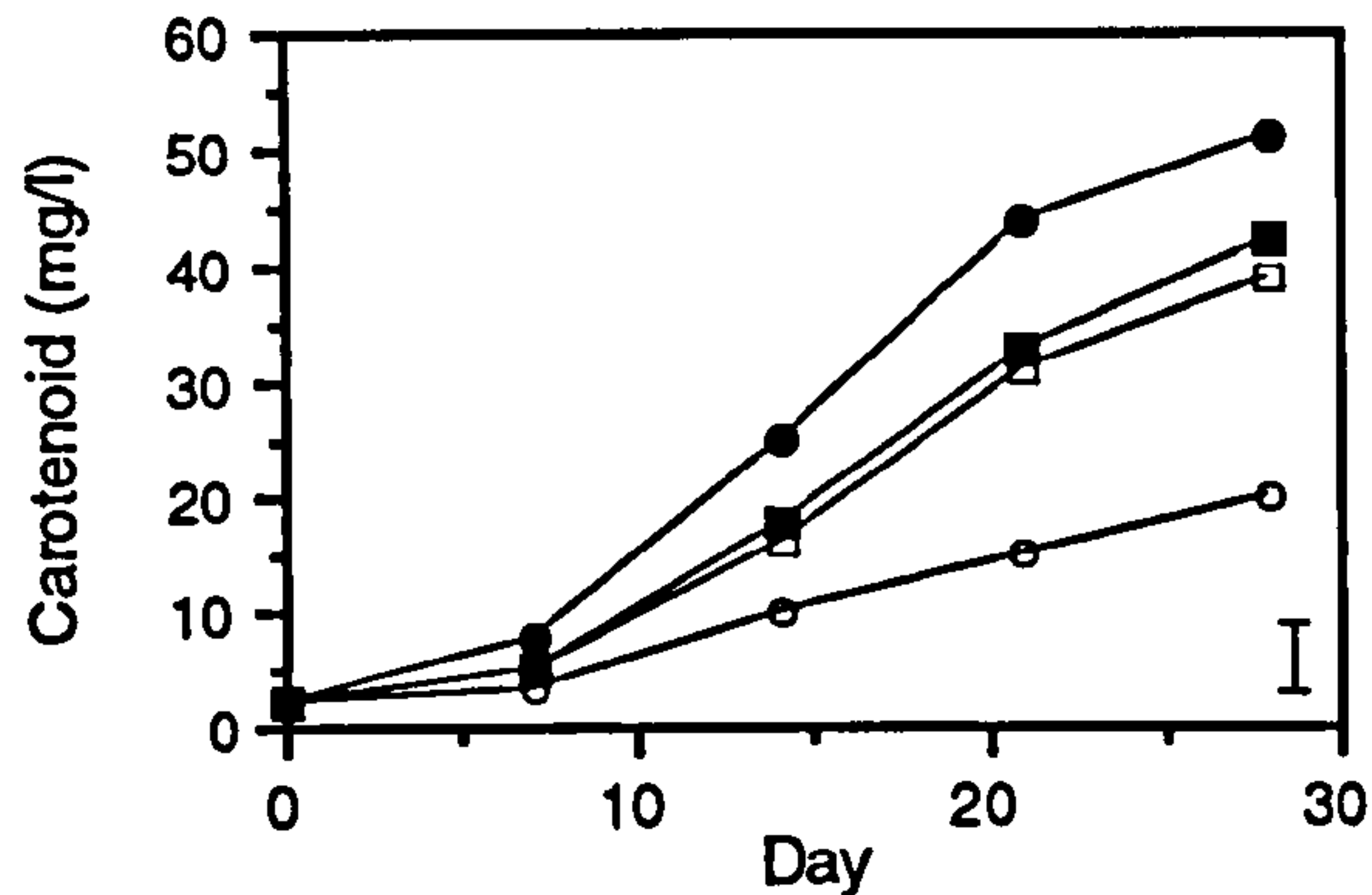


Fig. 6.6c

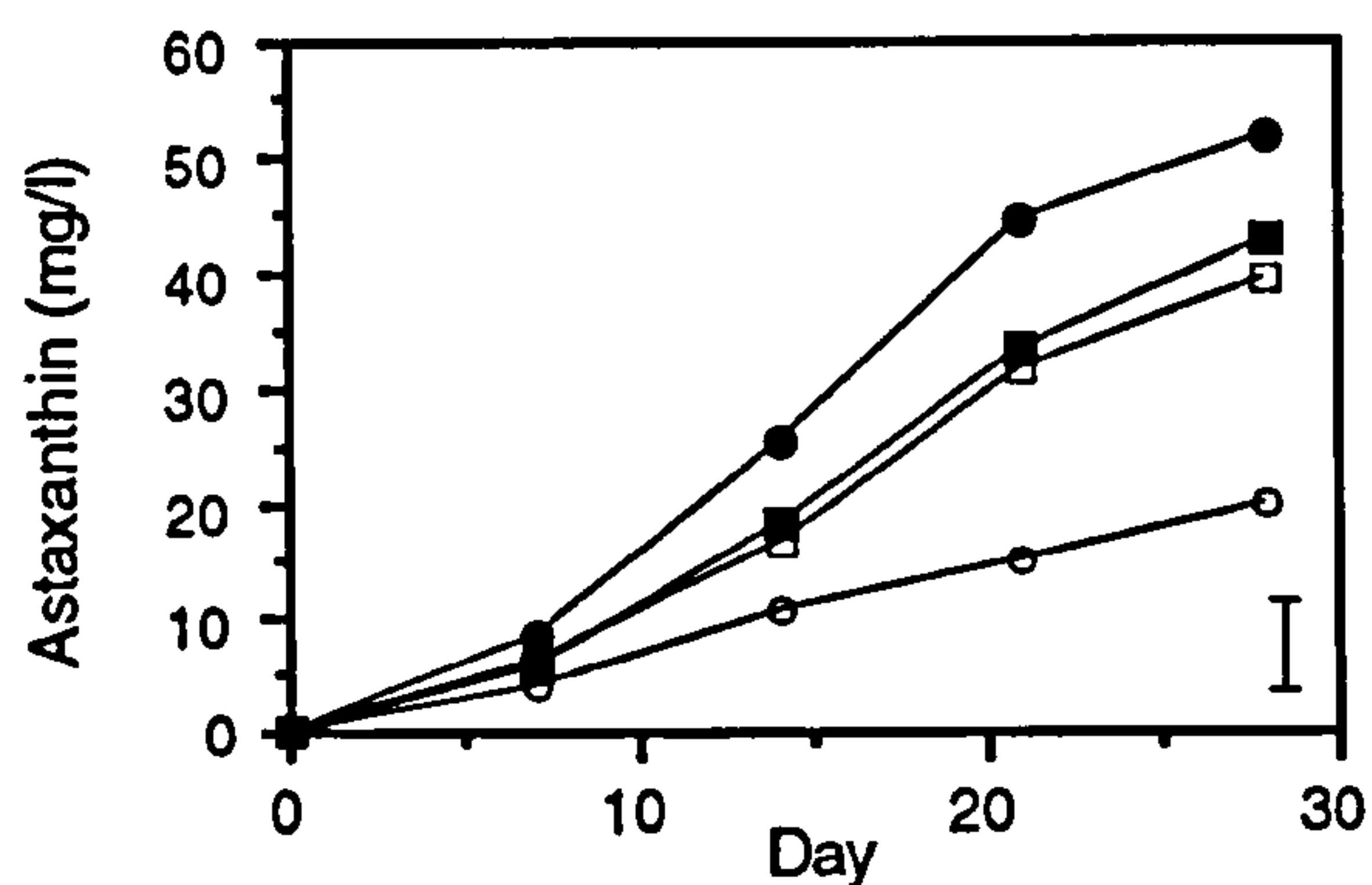


Fig. 6.6d

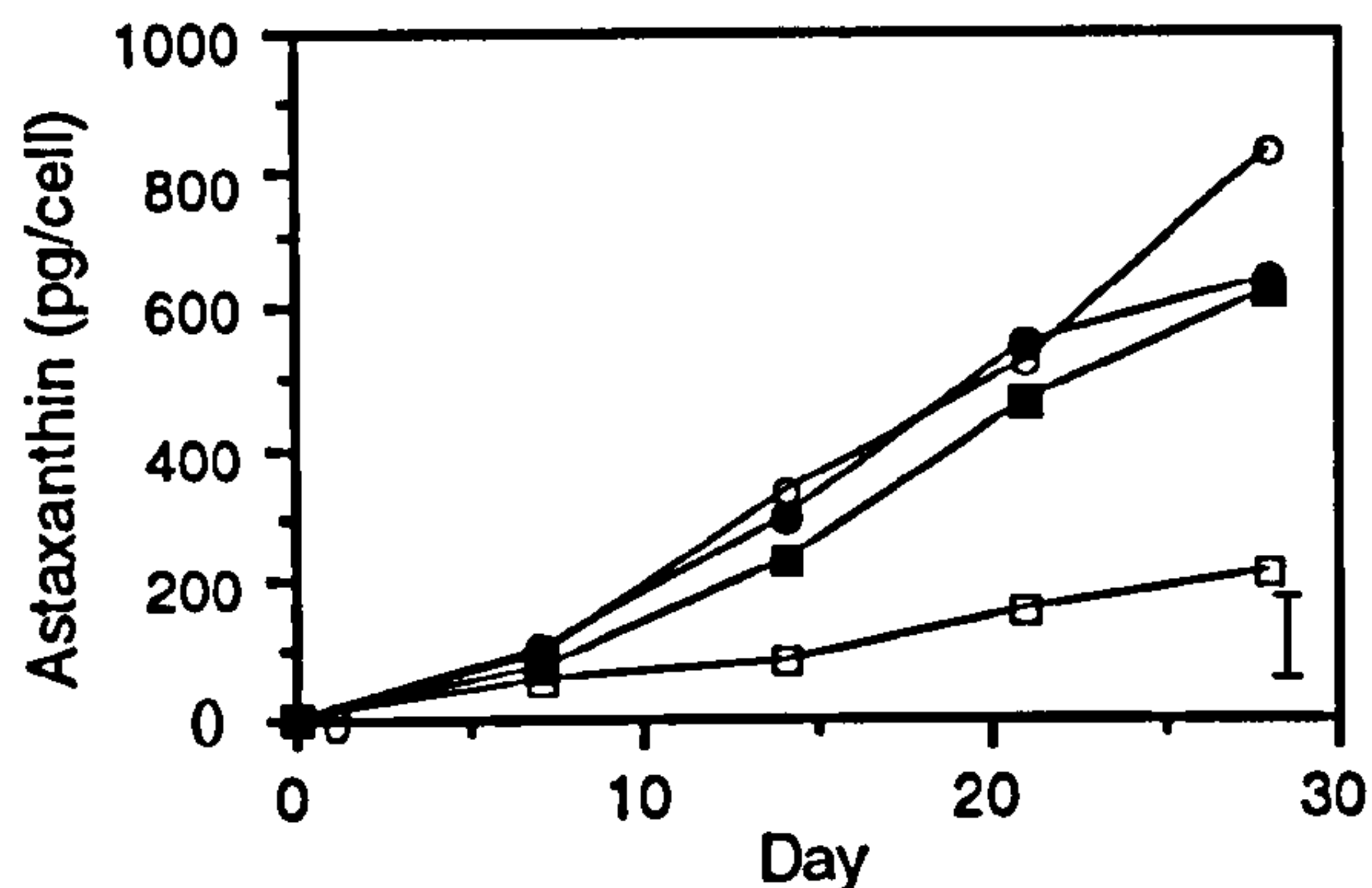
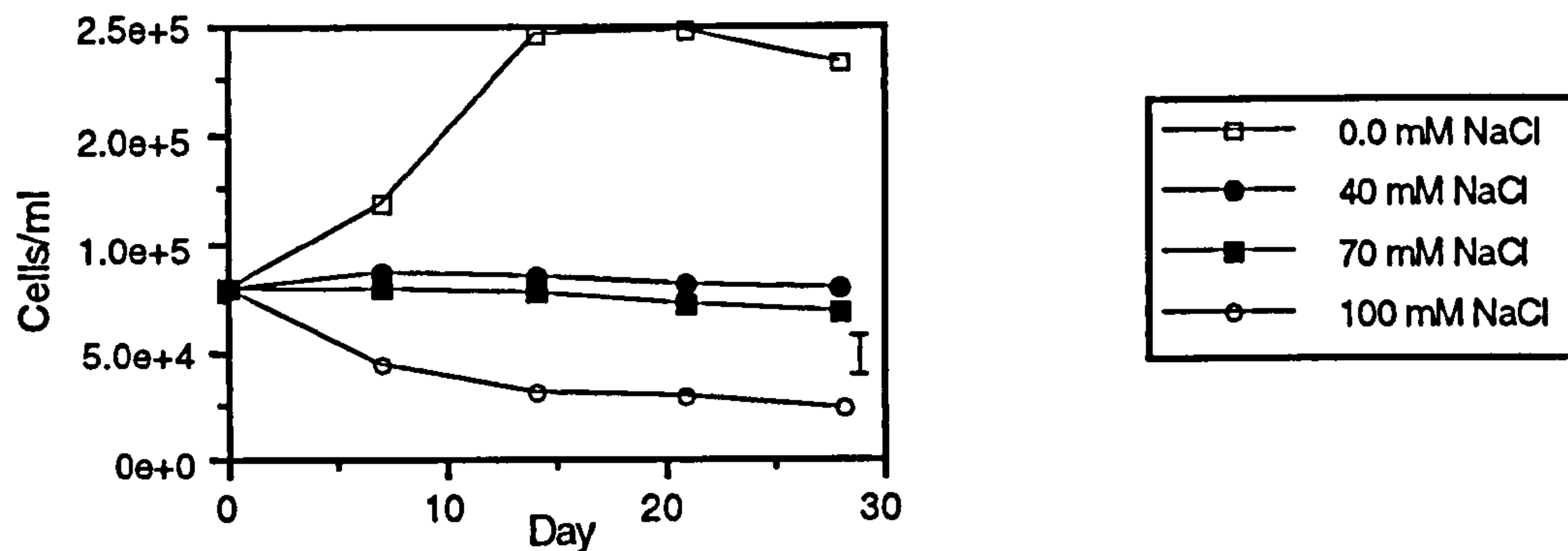


Fig. 6.6e



Figs. 6.6a-e Determination of optimum NaCl concentration for maximum secondary carotenoid synthesis in *H. pluvialis* (S.E.  $\pm 5.4\%$ ;  $n=3$ ).

### 6.3.7 NaCl stepwise addition

Figures 6.7a-e show the effects of adding NaCl to the algal cultures at different times to maximise secondary carotenoid accumulation. The graphs indicate that all the cultures followed relatively similar patterns of growth in the range of treatments investigated (for

treatment details see table 6.4). The cell number increased in the first seven days of the experiment and began to slowly decline during the middle stages of the experiment. There were very few significant differences in cell number between the treatments on the final day, however, the number of cells in treatment 3 on the final day was significantly higher than any of the other treatments. The number of cells in treatments 1 and 4 were significantly lower than a number of the other treatments.

As with previous salt experiments described earlier in this section, astaxanthin (III) accumulation in all the treatments became noticeable between days 7 and 14. All the treatments showed similar patterns of accumulation when expressed per unit volume of culture and per cell. The rate of astaxanthin (III) accumulation per unit volume of culture was relatively constant throughout the experiment, with the cultures in treatment no. 6 accumulating the highest levels of astaxanthin(III) per unit volume of culture. However, there were no significant differences in the amounts of astaxanthin (III) accumulated per unit volume between the majority of the treatments. The rate of astaxanthin (III) accumulation per cell was initially slow in all the treatments but increased steadily during the mid and latter stages of the experiment. Again there were no significant differences in the amounts of astaxanthin (III) accumulated per cell between the majority of the treatments, only treatment no. 3 accumulated significantly lower levels of astaxanthin (III) per cell than the majority of the other treatments.



Fig. 6.7a

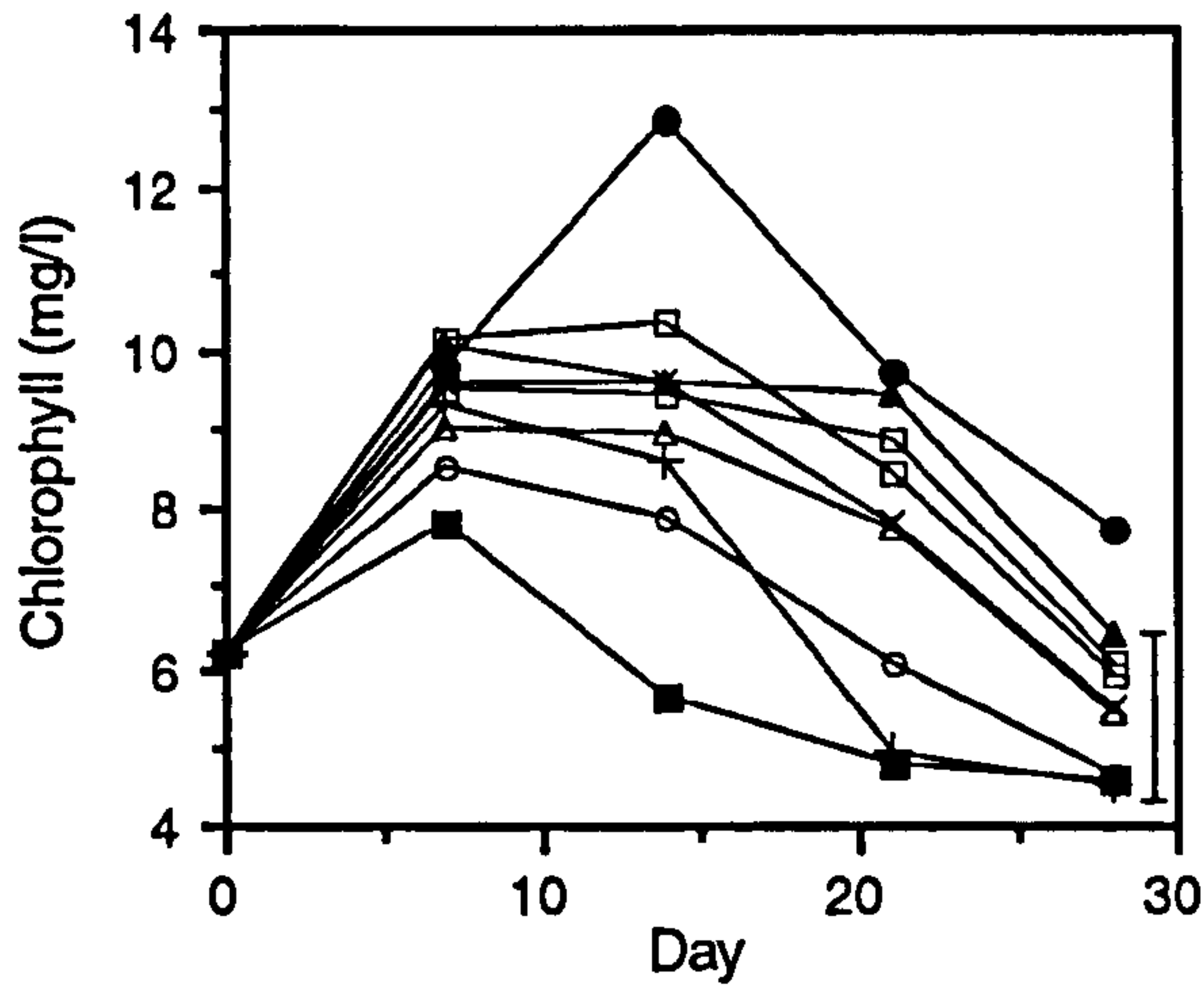


Fig. 6.7b

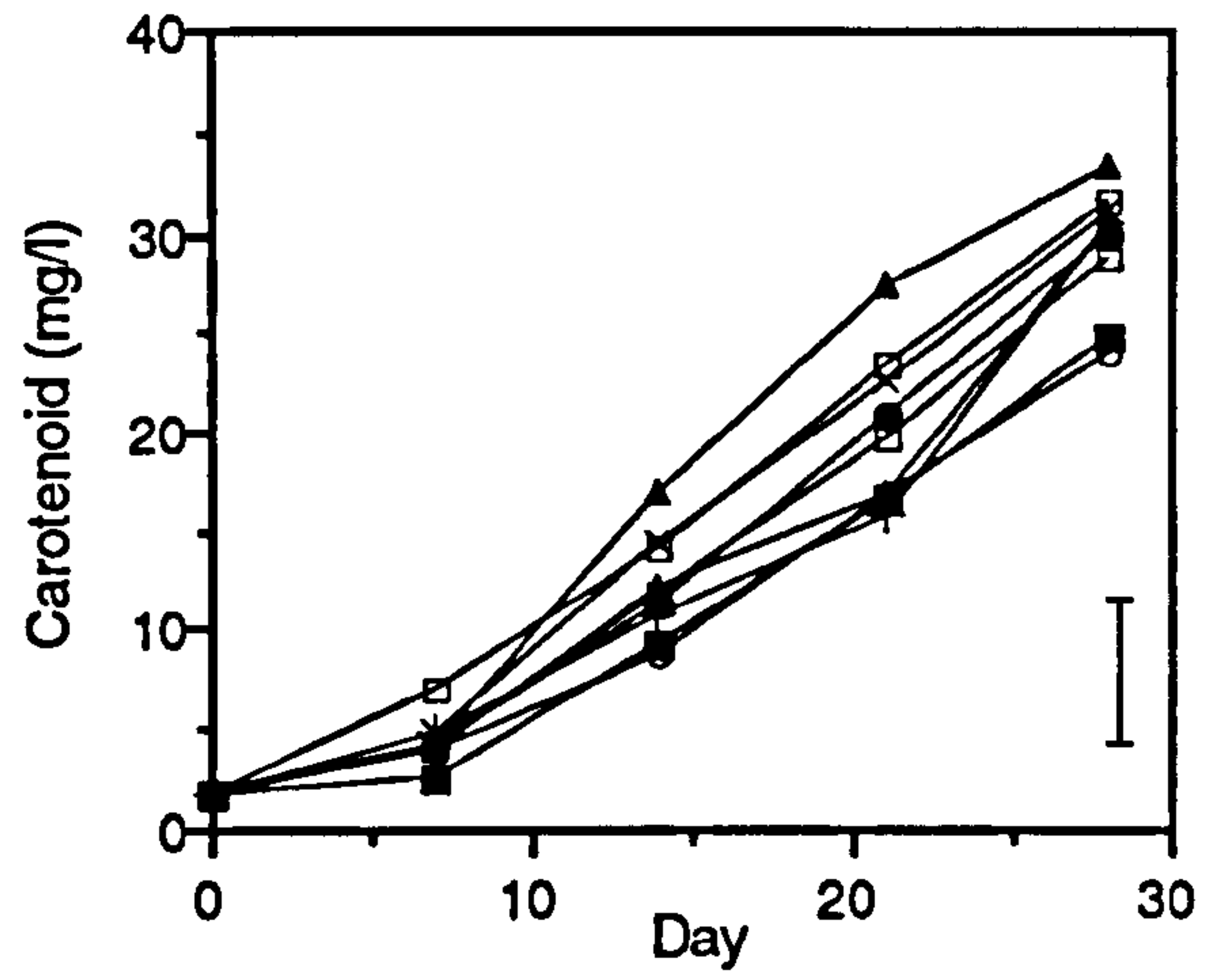


Fig. 6.7c

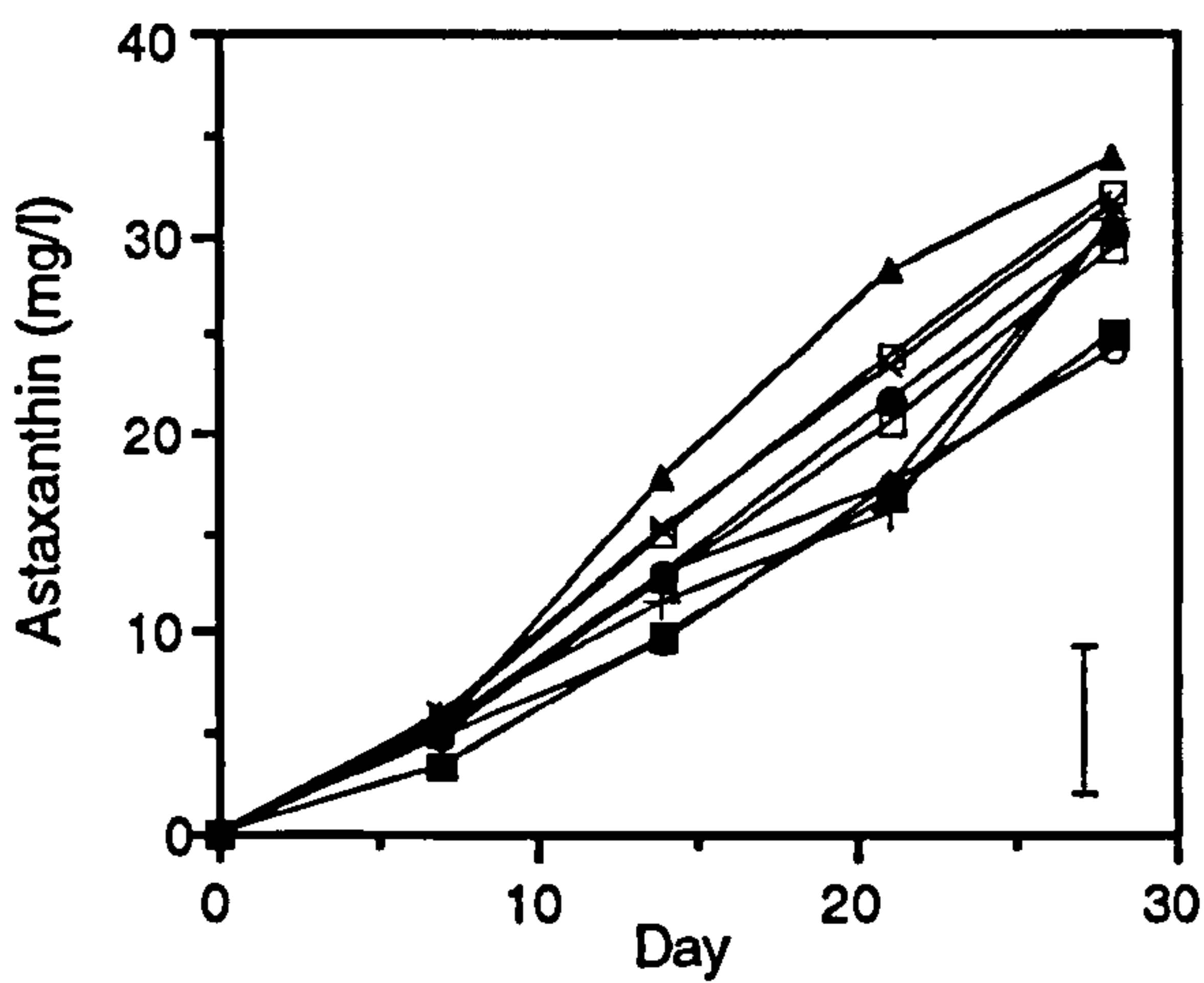


Fig. 6.7d

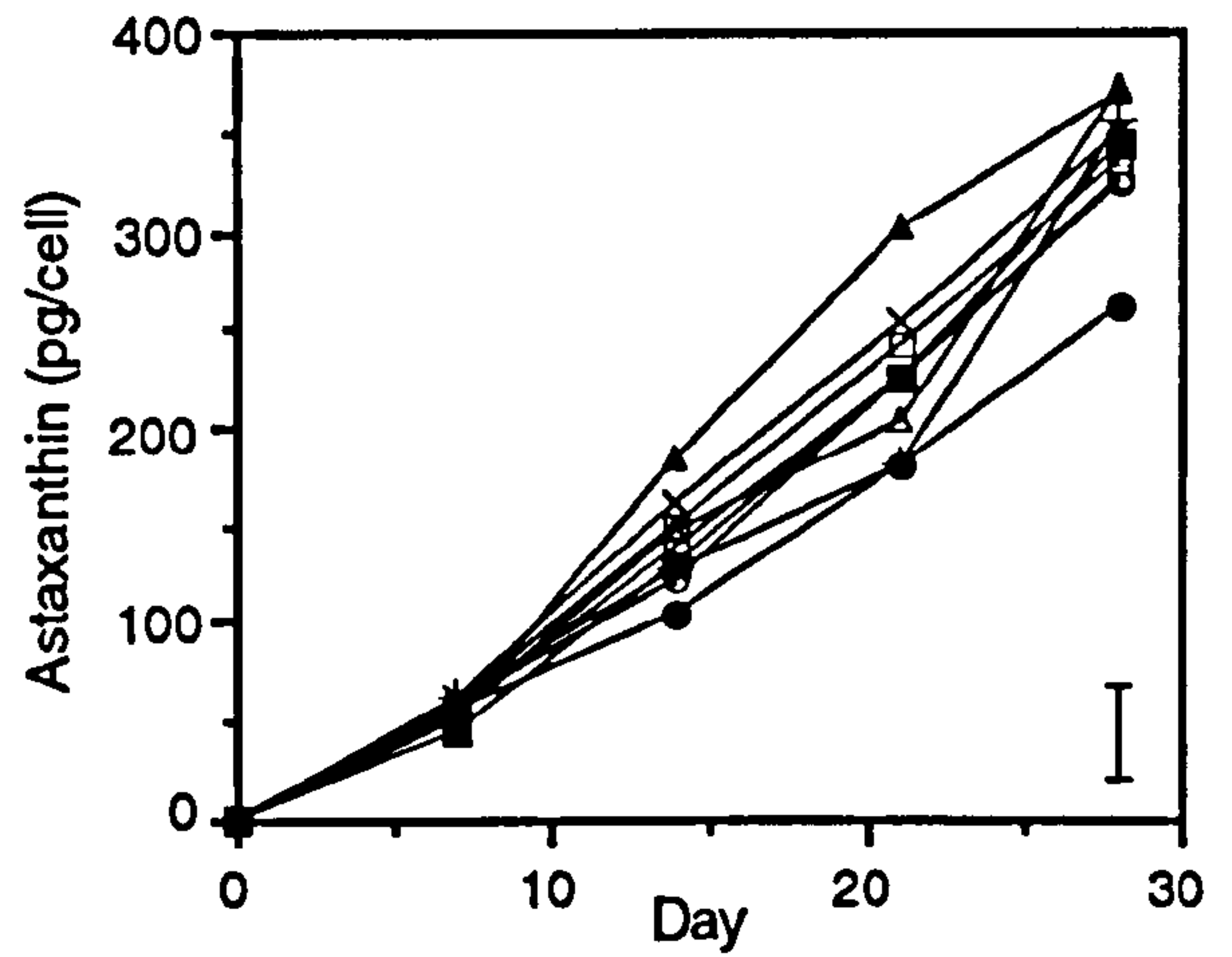


Fig. 6.7e

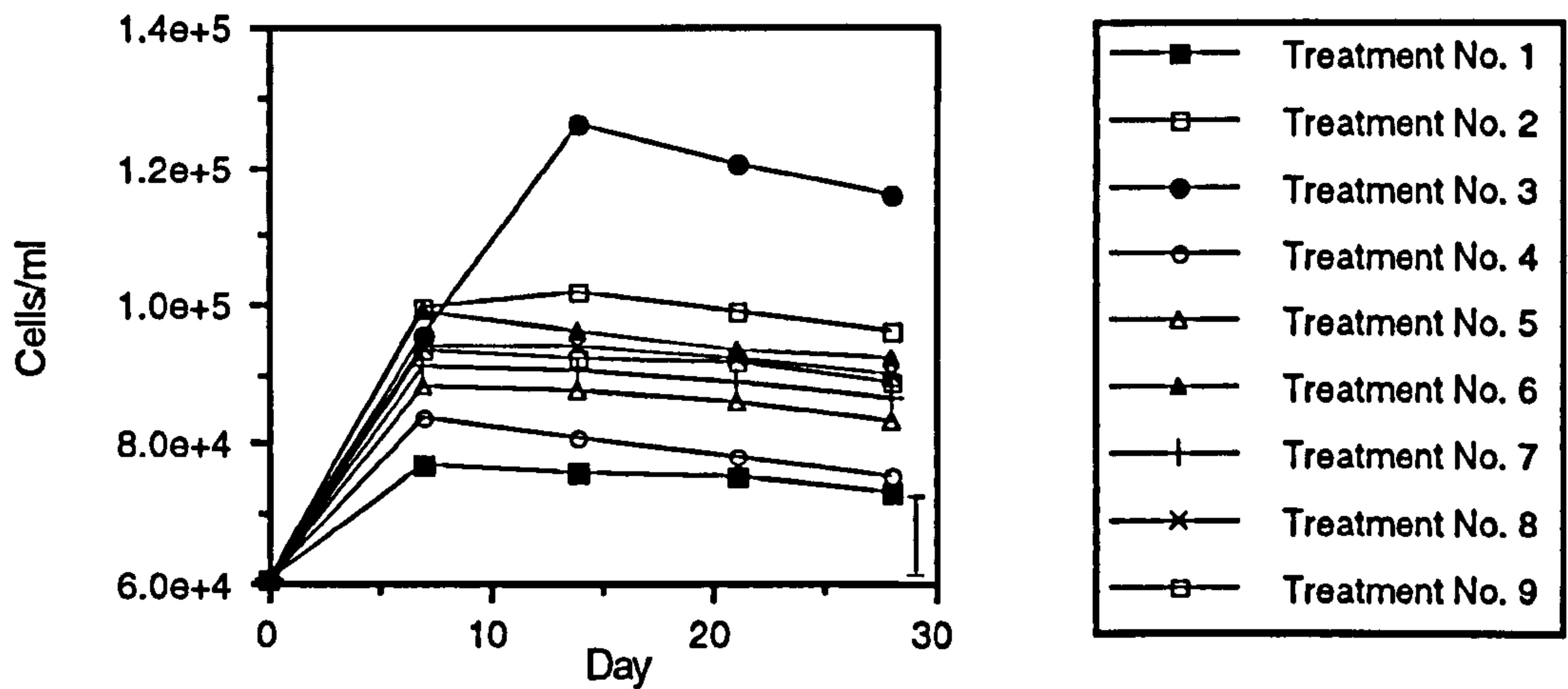


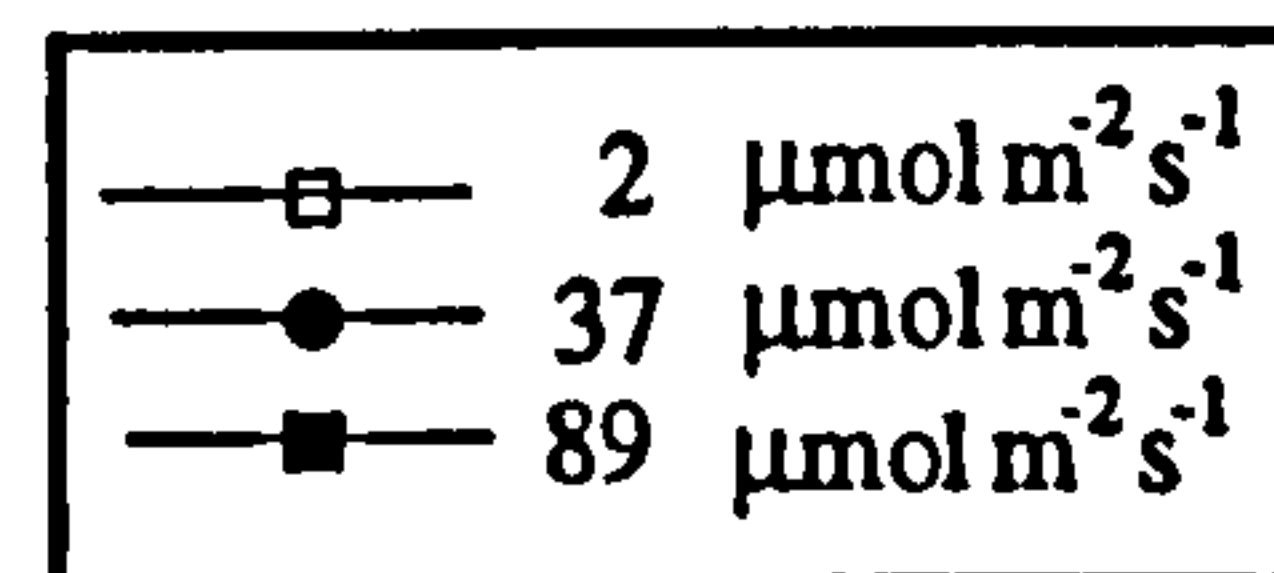
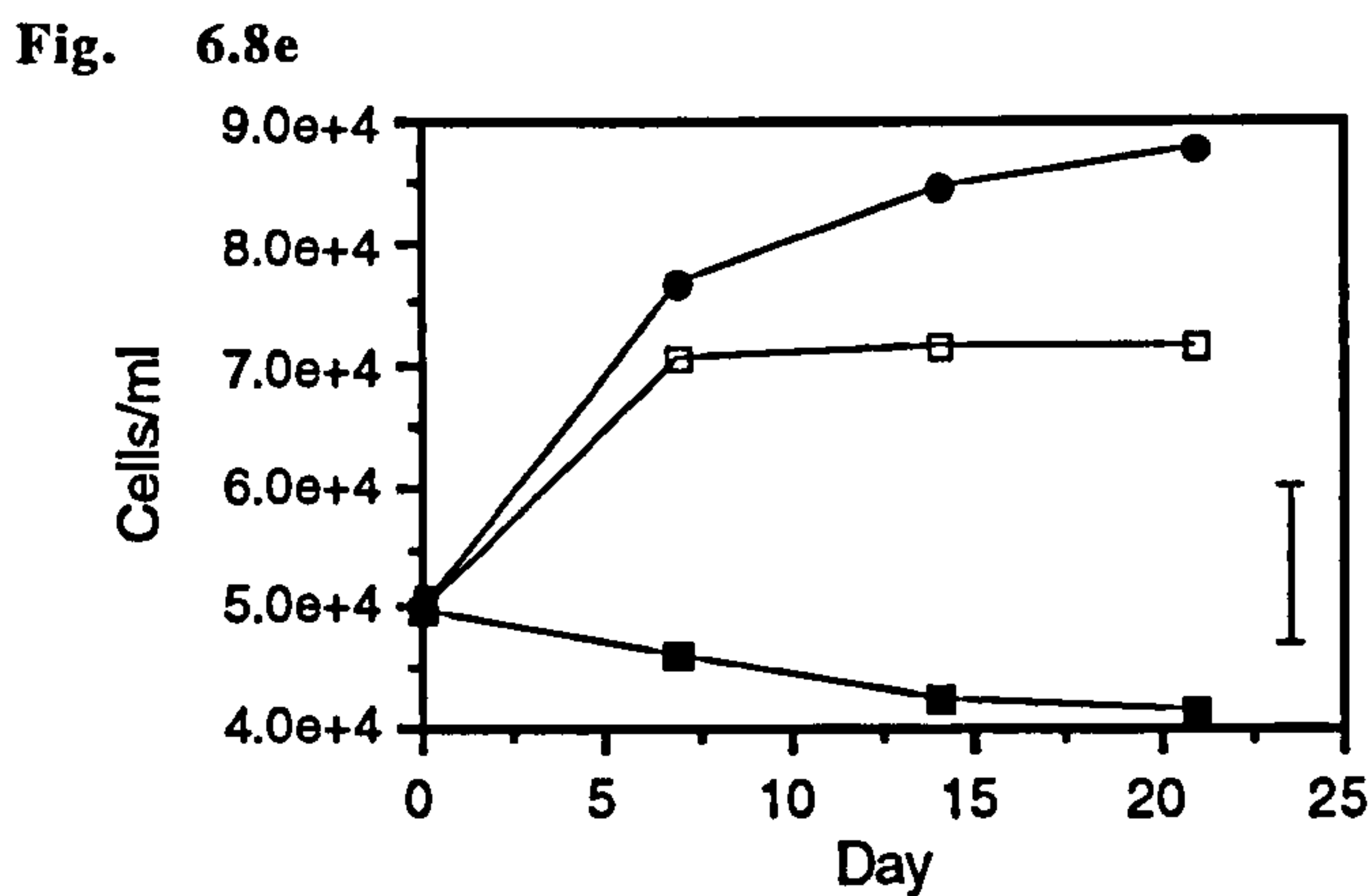
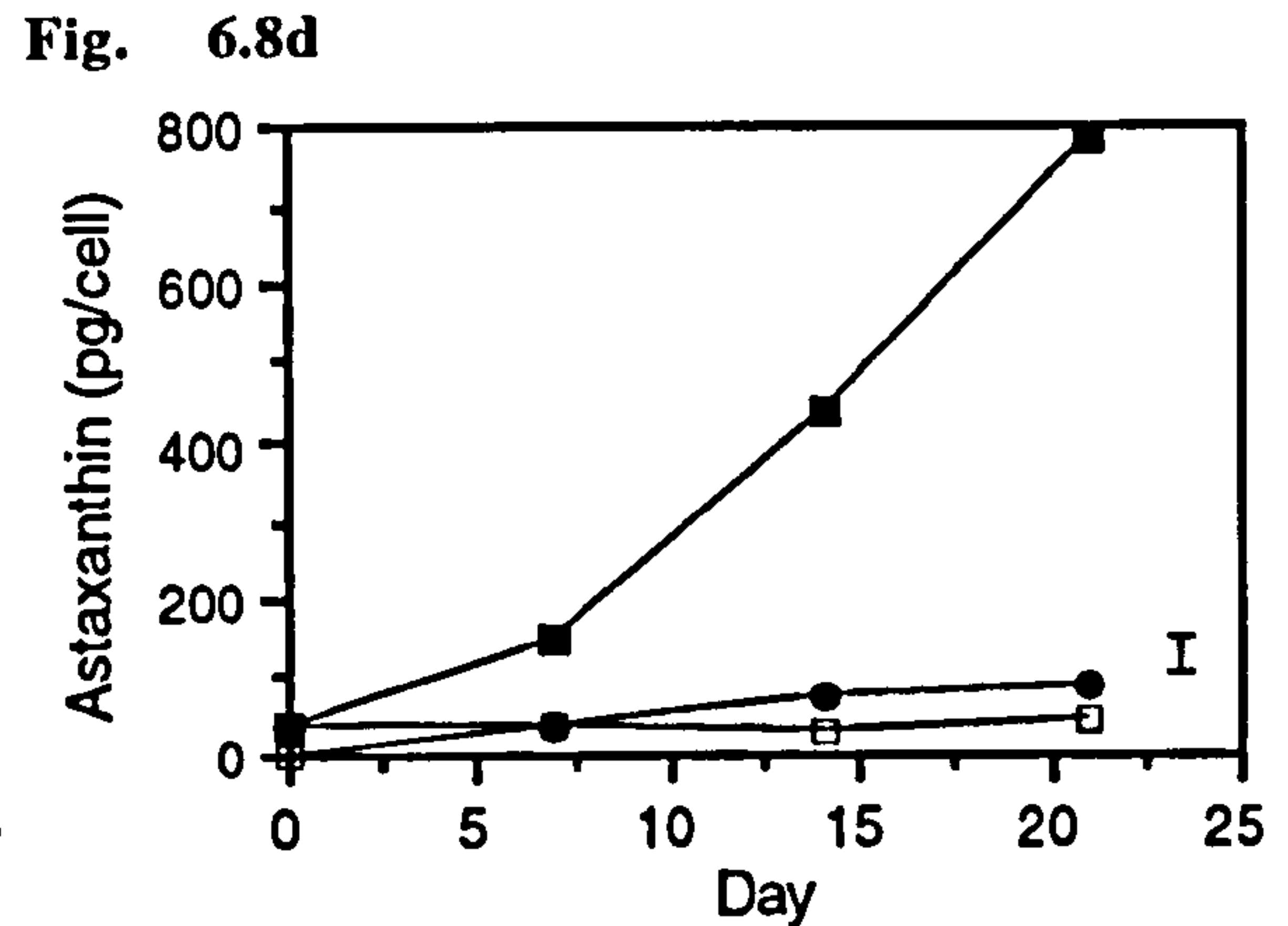
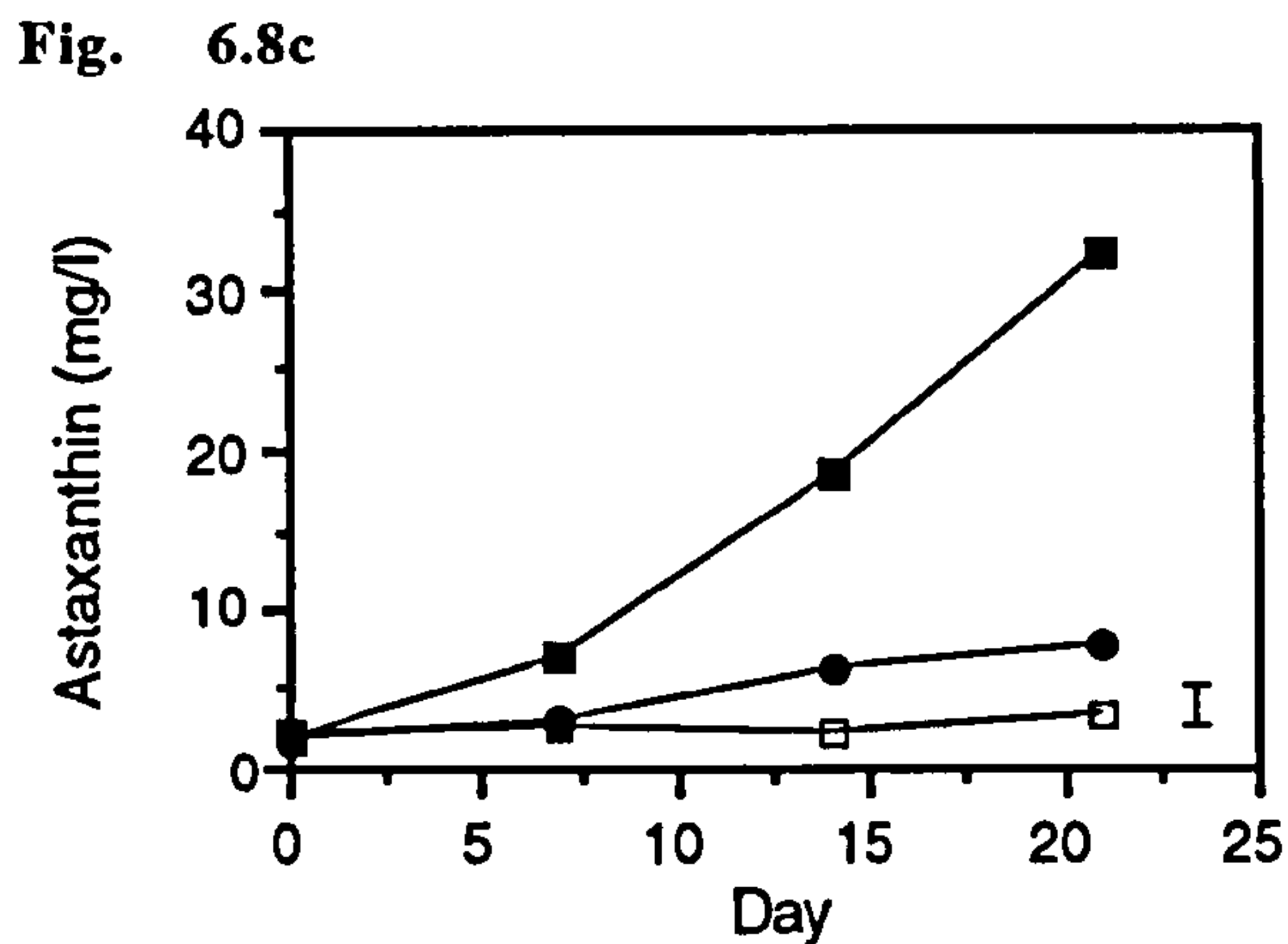
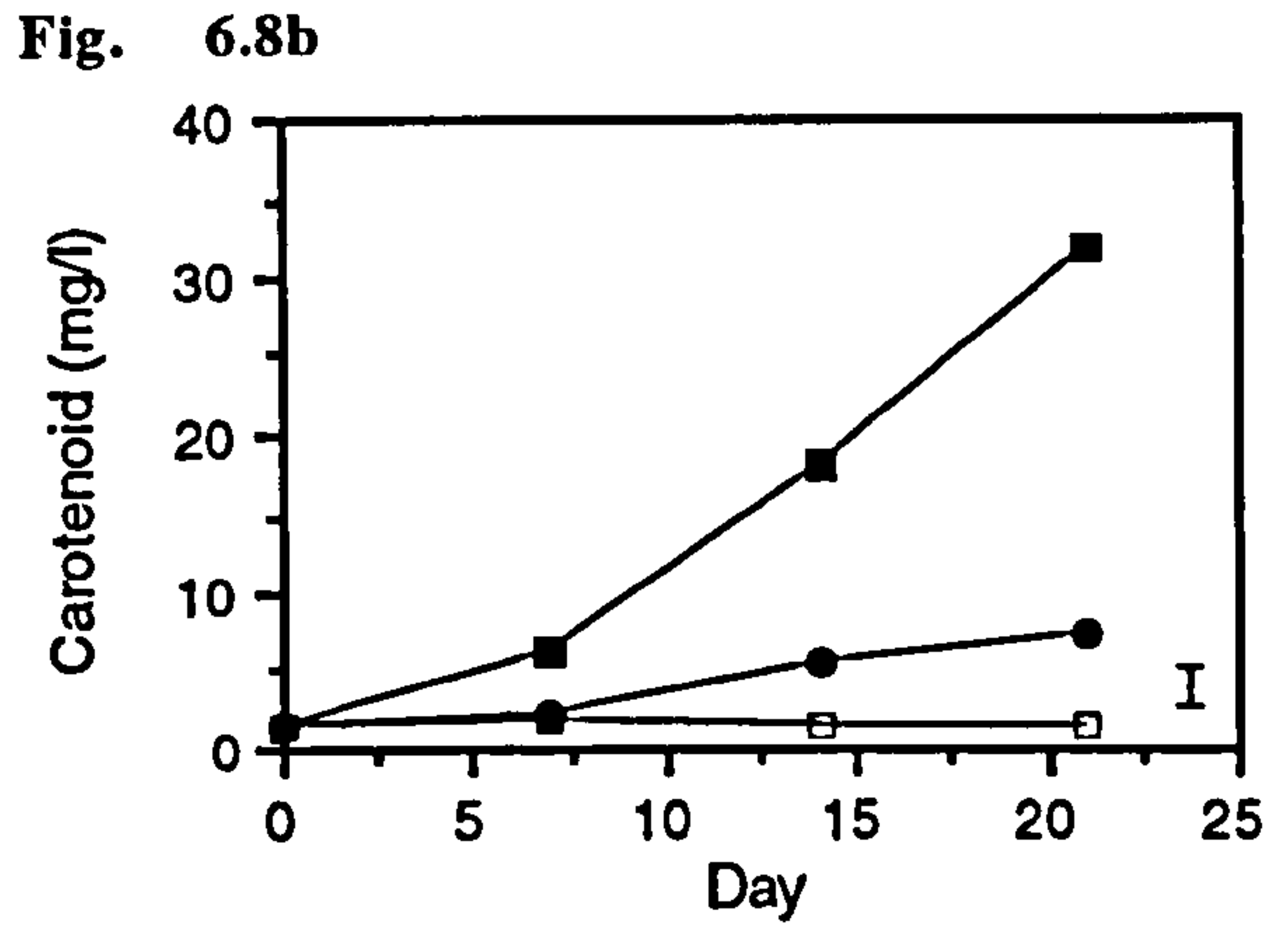
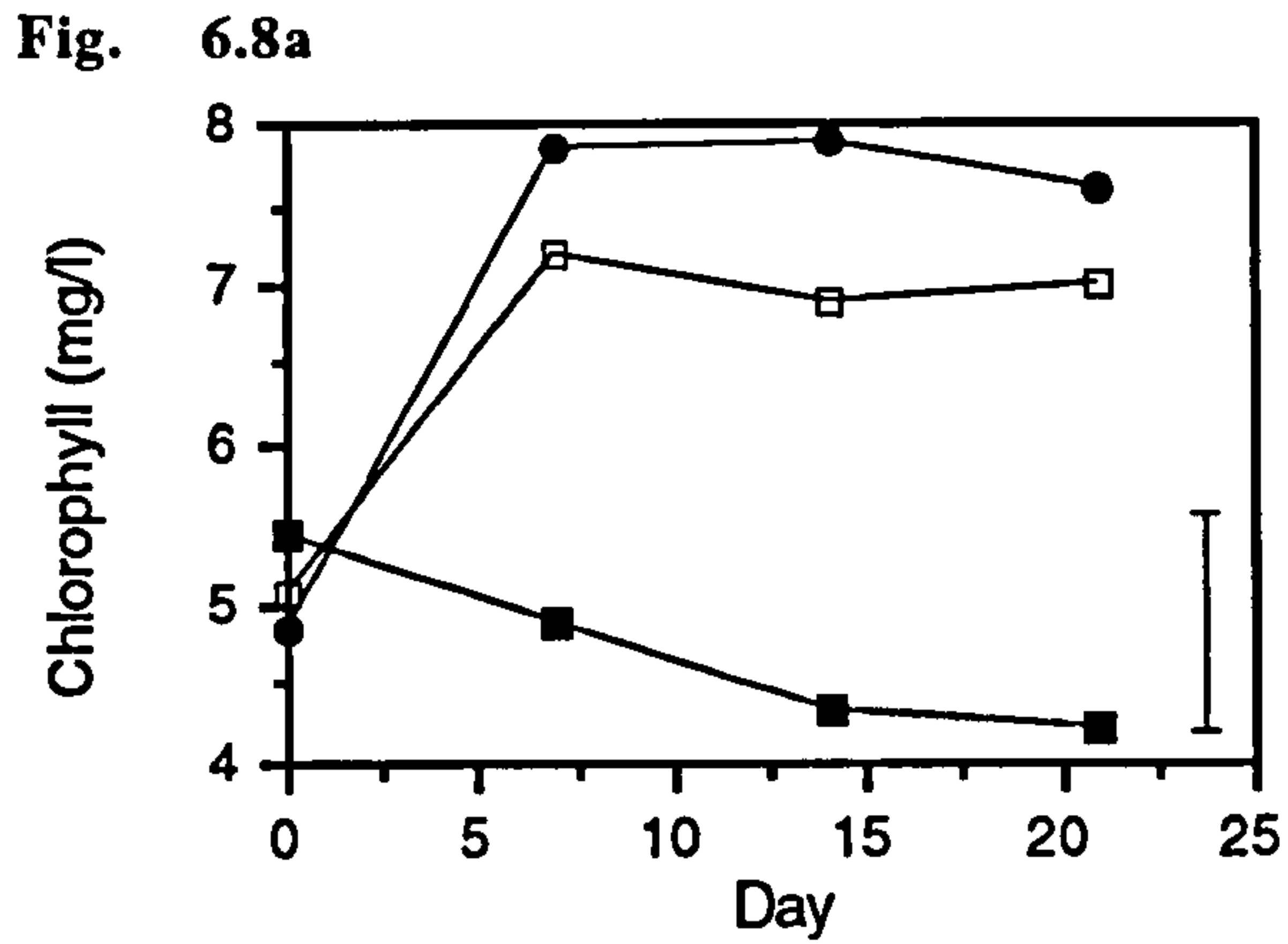
Fig. 6.7a-e The effect on astaxanthin (III) accumulation in *H. pluvialis* when NaCl is added to a final concentration of 40 mM at various time intervals (S.E.  $\pm 6.3\%$ ;  $n=3$ ). See Table 6.4 for details.

### 6.3.8 Light

Figures 6.8a-e show the effects of different light intensities on *H. pluvialis* both in terms of cell growth and pigment composition. The graphs indicate that when cultures are subjected to high light intensities it results in an inhibition of cell growth and relatively high rates of cell mortality. Exposure of cultures to low and intermediate light intensities (2 and 37  $\mu\text{mol m}^{-2} \text{s}^{-1}$  (PAR), respectively) resulted in an initial increase in cell number, especially in those cultures exposed to intermediate light levels. The rate of this increase declined after day 7, especially in the cultures exposed to low light intensities. The number of cells in cultures exposed to high light intensities declined steadily throughout the experiment. There were significant differences in cell number between all of the treatments on the final day of the experiment, the cultures exposed to 37  $\mu\text{mol m}^{-2} \text{s}^{-1}$  (PAR) showed the best rates of growth, while the cultures exposed to 89  $\mu\text{mol m}^{-2} \text{s}^{-1}$  (PAR) the poorest.

In cultures grown at the highest light intensity the levels of astaxanthin (III) per unit volume of culture increased relatively rapidly during the experiment, especially after day 7. The astaxanthin (III) levels in the cultures grown at 37  $\mu\text{mol m}^{-2} \text{s}^{-1}$  (PAR) showed a steady but relatively slow rate of increase, whilst the cultures grown at the lowest light intensity showed very little increase in astaxanthin (III) concentration during the experiment. On the final day of the experiment there were significant differences between all the treatments in the amounts of astaxanthin (III) accumulated per unit volume of culture. The pattern of astaxanthin (III) accumulation per cell in the three treatments followed a similar pattern as that observed when the data was expressed per unit volume of culture. On the final day the cells exposed to 89  $\mu\text{mol m}^{-2} \text{s}^{-1}$  (PAR) had accumulated significantly more astaxanthin (III) per cell than the other two treatments, between which there were no significant differences.





**Figs. 6.8a-e** Effect of light intensity on secondary carotenoid synthesis in *H. pluvialis* (S.E.  $\pm 5.1\%$ ;  $n=3$ ).

### 6.3.9 Temperature

Figures 6.9a-e show the pigment composition and cell number of cultures cultivated at three different temperatures, namely 22°C, 32°C and 42°C. The graphs indicate that when *H. pluvialis* cells are exposed to relatively high temperatures it results in an inhibition of

cell growth and high rates of cell mortality. This was especially apparent in the cultures exposed to 42°C where the number of cells declined rapidly, especially during the early part of the experiment. The number of cells in the cultures exposed to 32°C declined throughout the experiment, but the rate of decline increased during the later stages of the experiment. The cultures exposed to 22°C showed a moderate increase in cell number up to day 21, after which the number of cells receded slightly. There were significant differences between all the treatments in the number of cells present in the cultures on the final day of the experiment. The cultures grown at 22°C contained the most cells, while the cultures grown at 42°C contained the least number of cells.

The accumulation of astaxanthin (III) expressed in terms of per unit volume of culture and per cell in the cultures grown at 22°C was initially slow but increased relatively quickly throughout the mid and latter stages of the experiment. On the final day the astaxanthin (III) levels in these cultures in terms of per unit volume of culture were significantly higher than in the other two treatments. The cultures exposed to 32°C showed no observable increase in astaxanthin (III) levels during the experiment both in terms of per unit volume of culture or per cell. The levels of astaxanthin (III) per unit volume in the cultures exposed to 42°C had decreased considerably by the final day of the experiment, these cultures contained significantly lower levels of astaxanthin (III) per unit volume than the other two treatments. However, the number of cells in these cultures was very low, so those cells which did survive contained relatively high levels of astaxanthin (III) (> 150 pg/cell), which was significantly higher than the levels in the cells cultivated at 32°C.



Fig. 6.9a

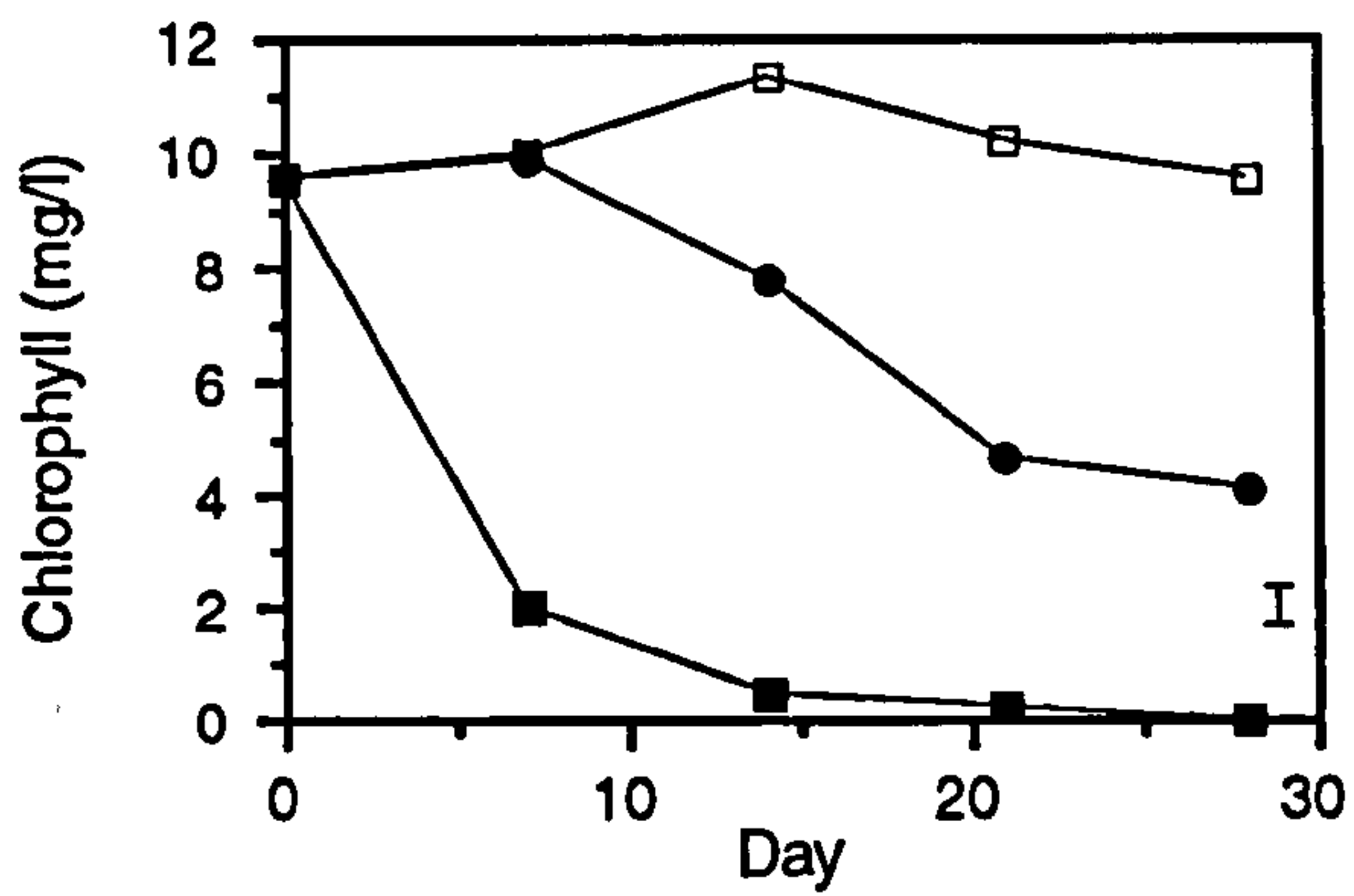


Fig. 6.9b

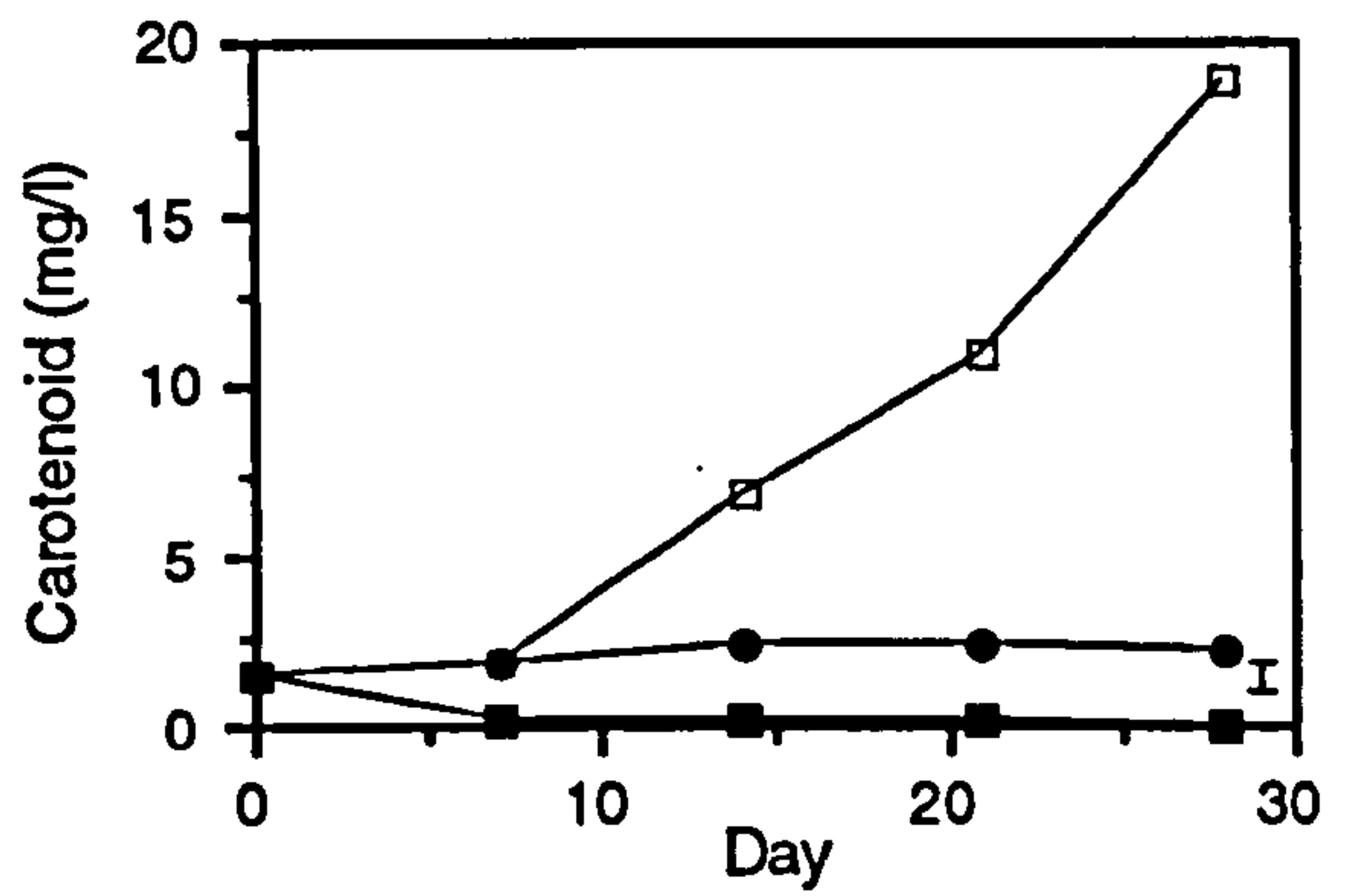


Fig. 6.9c

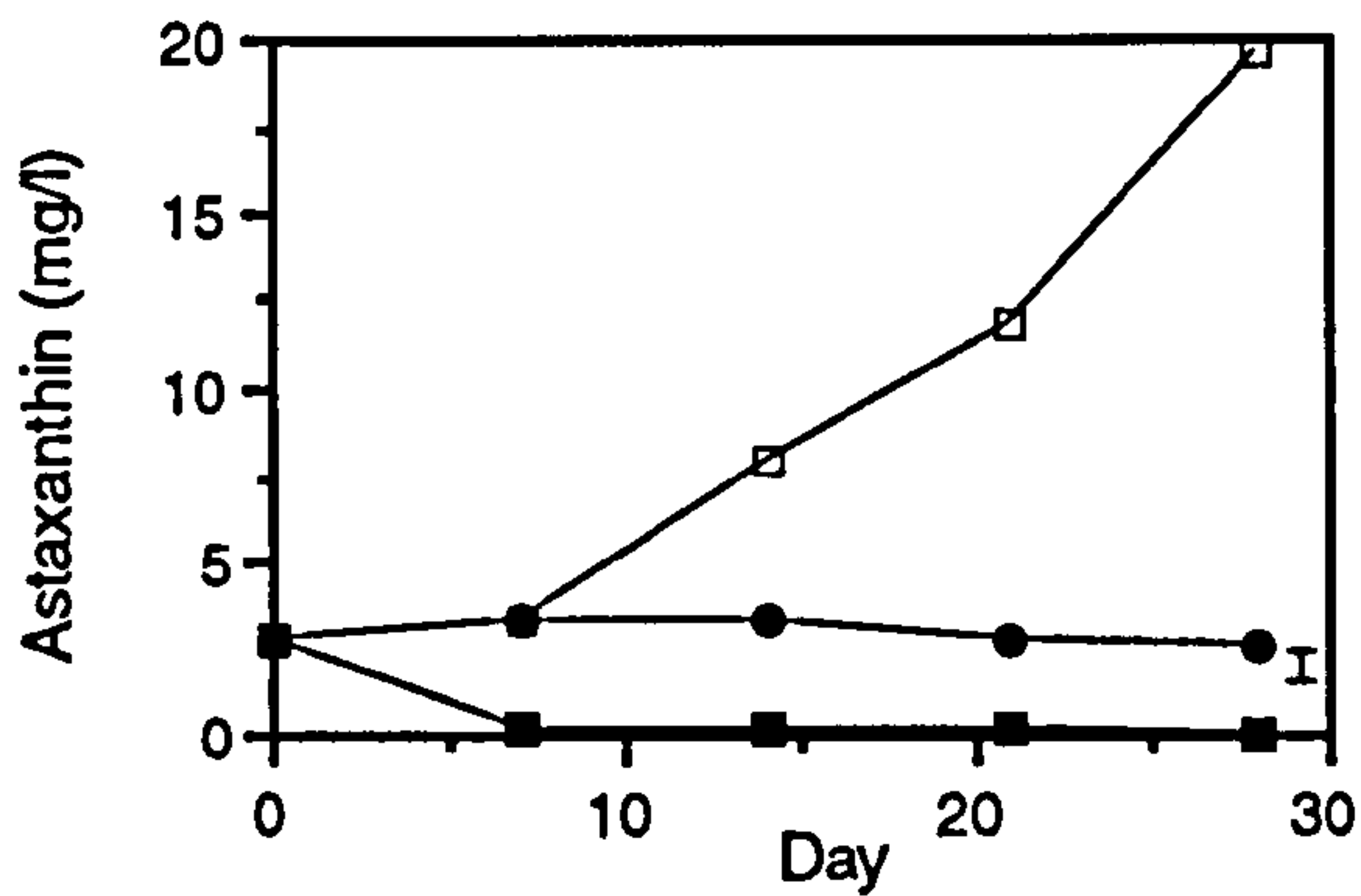


Fig. 6.9d

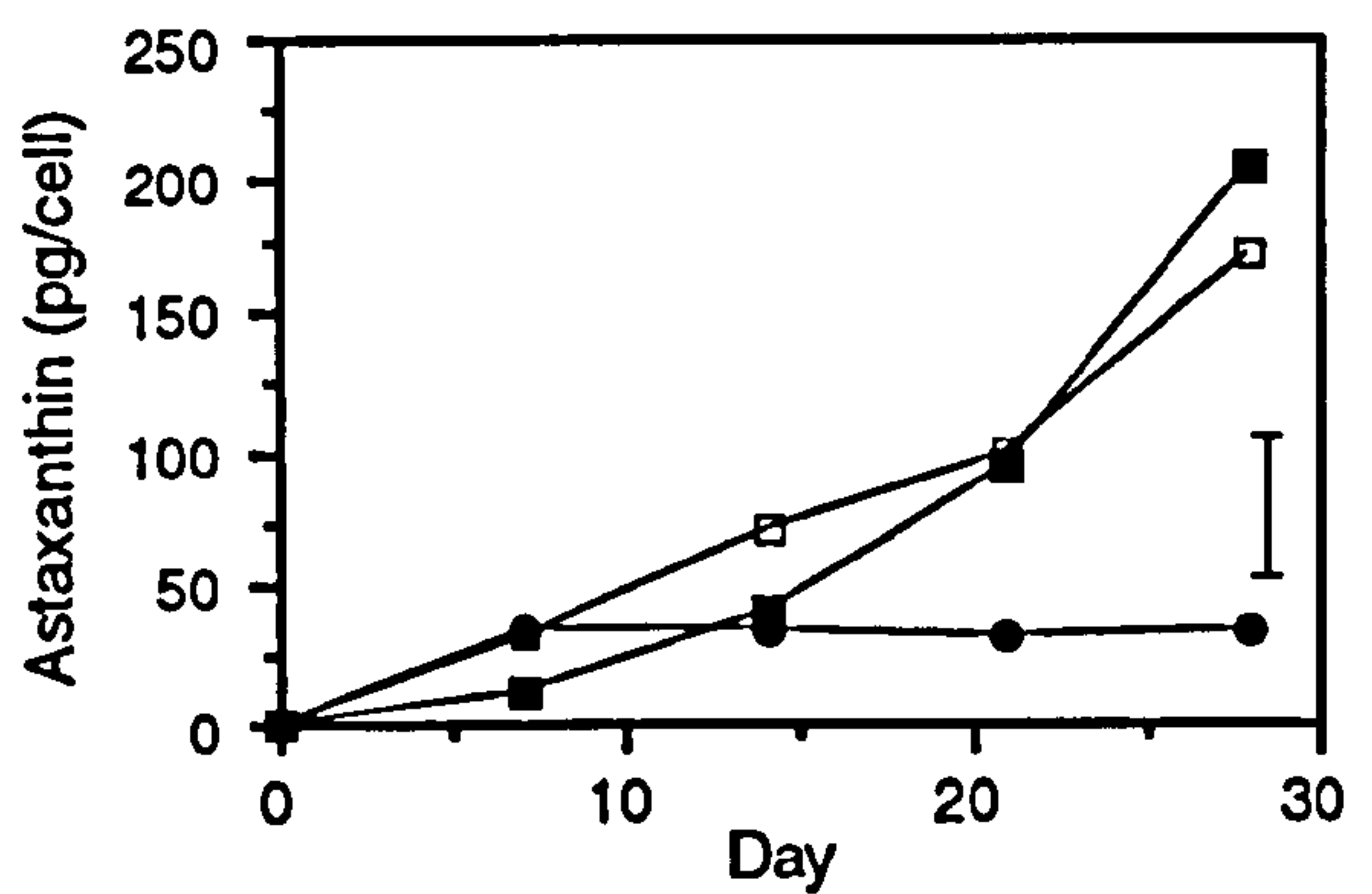
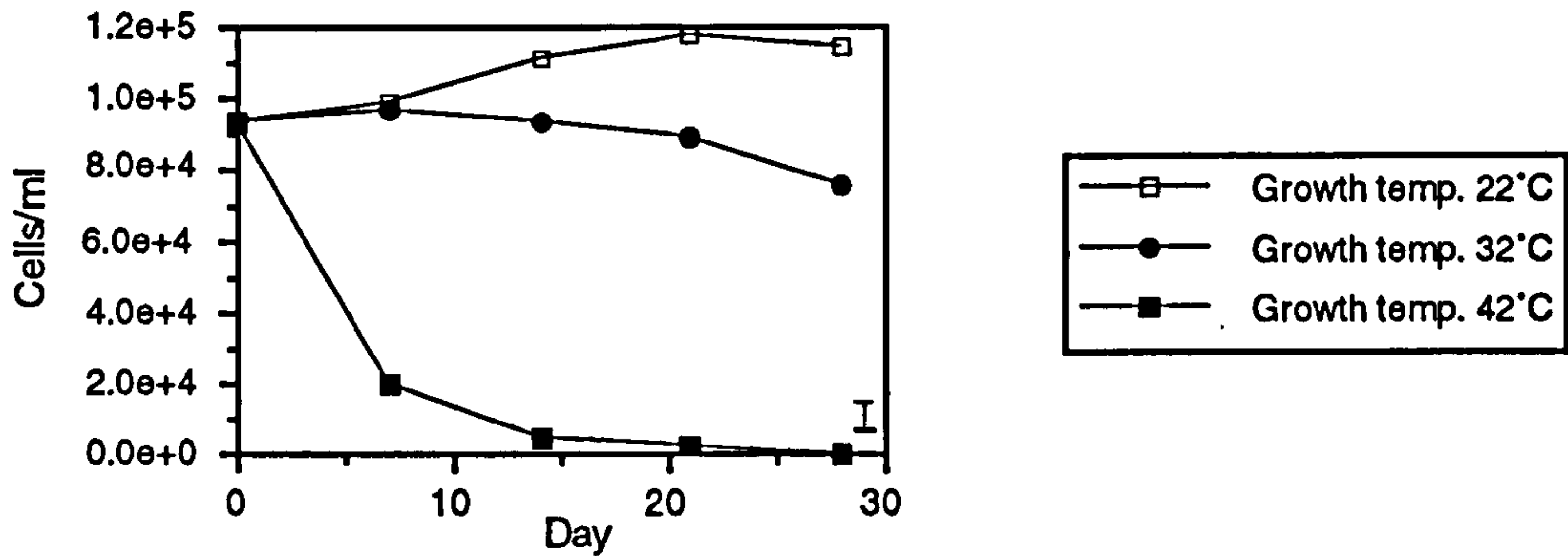


Fig. 6.9e



Figs. 6.9a-e Effect of temperature on cell growth and secondary carotenoid synthesis in *H. pluvialis* (S.E.  $\pm 4.7\%$ ; n=3).

## 6.4 DISCUSSION

The results clearly indicate that many nutritional and environmental parameters are potential inducers of secondary carotenoid synthesis in cells of *H. pluvialis*. By varying the concentration of nitrogen in the medium, the alga can be manipulated with respect to both growth and secondary carotenoid production. At the cellular level, the lower the nitrogen concentration the higher the concentrations of secondary carotenoids accumulated in the cells (Fig. 6.1d). However, in production terms it is important to consider the levels of secondary carotenoids per unit volume of the culture medium. The highest levels of secondary carotenoids per unit volume of culture are observed in those cultures containing intermediate levels of nitrogen i.e. 1.5 mM of nitrate. This is because initial cell growth in these cultures is greater than that observed in cultures containing lower nitrogen concentrations. However, the cells soon exhaust the nitrogen present in the original medium and are then exposed to nitrogen-deficient conditions. Thus, a relatively high number of cells begin to synthesise secondary carotenoids, which results in relatively high levels of secondary carotenoids per unit volume of culture medium. The results presented here are in agreement with Spencer (1989) and Goodwin and Jamikom (1954b) in that nitrogen deficient conditions result in secondary carotenoid synthesis in *H. pluvialis*. These findings contradict the results obtained by Boussiba *et al.* (1992) who concluded that nitrogen is an important requirement for astaxanthin (III) synthesis in the alga, and that nitrogen deficient conditions do not induce astaxanthin (III) formation in the alga.

The effect of phosphate-limiting conditions on *H. pluvialis* cells would seem to be similar to the effects observed in nitrogen limited cultures. Low phosphate levels (0.75 mM) in the culture medium induced secondary carotenoid formation, especially when expressed per cell (Fig. 6.2d). However, it should be noted that when dealing with phosphate in liquid media it is inherently difficult to determine the actual concentration of phosphate present. This is due to the phosphate present readily adhering to and leaching out of any glassware used in the experiments. Unless the phosphate concentration of the growth medium can be accurately determined, only broad assumptions as to the effects of phosphate on the algal cells can be made. From the results presented it would be reasonable to assume that the level of phosphate in the medium does have an effect on the



initiation of secondary carotenoid synthesis in *H. pluvialis* cells. This assumption is in agreement with the results obtained by Boussiba and Vonshak (1991) but not with those obtained by Borowitzka *et al.* (1991).

The concentration of ferrous iron present in the culture medium on growth and secondary carotenoid accumulation only had an effect at relatively high concentrations (Figs. 6.3c-e). Cells grown in the two lower iron concentrations (18 and 36  $\mu\text{M}$  Fe respectively), showed very few differences both in terms of cell growth and pigment composition. Cells grown at the highest iron concentration (72  $\mu\text{M}$  Fe) showed relatively high levels of secondary carotenoid formation at the cellular level, and a slight decrease in cell growth. The cause of this elevation in secondary carotenoid formation due to increased iron concentrations remains to be elucidated, although the phenomena has been observed by other workers, e.g. Kobayashi *et al.* (1991).

The ferrous form of Fe in particular is known to give rise to free radical formation (especially hydroxyl radicals  $\cdot\text{OH}$ ) *via* Fenton chemistry. Radical initiation of cellular processes is thought to be an integral part of many biological systems and it has been suggested that free radicals may play a role in the accumulation of  $\beta$ -carotene (I) in *Dunaliella* (Ben-Amotz and Avron, 1983). It is likely that  $\text{Fe}^{2+}$  stimulated radical production does have an important role in inducing astaxanthin (III) synthesis in *H. pluvialis*. This could be further investigated through the use of other radical generators or other forms of iron (e.g. ferric) which will not readily give rise to  $\cdot\text{OH}$  production. Light will also play an important part in this process (Kappus, 1991).

An increase in the salinity of the culture medium can result in the initiation of secondary carotenoid synthesis in the alga. However, if the salinity of the culture medium is raised too much high rates of cell mortality are observed (Figs. 6.4e and 6.5e), and secondary carotenoid formation either does not occur or occurs at a reduced rate (Figs. 6.4c and 6.5c). Therefore, the concentration of salt added to the algal cultures needs to be carefully monitored to avoid killing too many cells whilst keeping the rate of secondary carotenoid synthesis high. The problem of increasing the salinity of the cultures above that which the alga can tolerate was observed in both the initial NaCl and KCl experiments (Figs. 6.4a-e and 6.5a-e). In both experiments the addition of relatively large amounts of

salt (> 500 mM) to the culture medium resulted in high rates of cell mortality and low rates of secondary carotenoid synthesis.

Relatively moderate salinities of approximately 300 mM NaCl resulted in high levels of secondary carotenoids being accumulated per cell e.g. approximately 150 pg astaxanthin (III)/cell, while the overall levels of secondary carotenoids present per unit volume of culture were relatively low at approximately 1.0 mg/l. This trend was also observed in the *H. pluvialis* cultures exposed to KCl. This is due to the high rates of cell mortality in these cultures when the cells are exposed to relatively high saline conditions, as outlined above. However, the cells which are viable in these conditions are able to accumulate very high levels of secondary carotenoids per cell (over 250 pg per cell in the cultures exposed to 268 mM KCl (Fig. 6.5d)). It may be that the increased capacity some cells possess for producing high levels of secondary carotenoids is one of the reasons why particular cells are able to survive under such severe conditions.

The addition of KCl to the algal cultures even at relatively low concentrations i.e. 67 mM (Fig. 6.7e) resulted in poor rates of cell growth and relatively low rates of secondary carotenoid synthesis per unit volume of culture. It would appear that the addition of potassium to the cultures, even at low concentrations, produces a phytotoxic effect. The strong phytotoxic effects of the chloride ions from KCl can be discarded, as such an effect is not observed when NaCl is added to the cultures and it is likely that the K<sup>+</sup> are toxic. It has been suggested that algal cells do not possess an efficient extrusion mechanism for K<sup>+</sup> as they do for Na<sup>+</sup> and Cl<sup>-</sup> (Pick *et al*, 1986). Whatever the origin of the phytotoxic effect, the use of KCl to induce secondary carotenogenesis in *H. pluvialis* is not a viable option and it is recommended that NaCl should be used as a salt source whenever possible.

The high rates of cell mortality caused by the addition of NaCl at high concentrations resulted in the need to determine the optimum NaCl concentration for maximum secondary carotenoid synthesis per unit volume of culture. The optimum level needs to be a balance between inducing a response within the alga and keeping cell mortality rates to a minimum. Over the range of NaCl concentrations investigated (Figs. 6.6a-e), the optimum NaCl concentration required to obtain the maximum yield of secondary carotenoid production per unit volume of culture was 40 mM. This NaCl



concentration also resulted in the highest levels of secondary carotenoid accumulation per cell.

In section 7.3.3 the NaCl concentration identified as the optimum to maximise astaxanthin (III) production in *H. pluvialis* using response surface methodology is 30 mM, which is very similar to the value obtained in the present study. The optimum NaCl concentration obtained in both these studies is similar to the concentration used by Spencer (1989) who used 50 mM NaCl to induce secondary carotenogenesis in *H. pluvialis* on a commercial basis. However, it should be noted that although many workers use NaCl to induce secondary carotenogenesis in *H. pluvialis*, none of these groups have presented any data, or inferred, that they have tested the alga in a range of NaCl concentrations to achieve maximum secondary carotenoid production.

The addition of a given amount of NaCl to the cultures in stages had only a small effect on the levels of secondary carotenoids accumulated, both in terms of per unit volume of culture and per cell. By adding the salt in stages a small increase in the level of secondary carotenoids accumulated was observed when compared with adding the salt all at once. However, these differences were not significant in the majority of the treatments. Therefore, it would be more practical to add the salt to the required concentration all at once and not in stages as this would save time, money and reduce the risk of contaminating the cultures while the salt is being added.

The light intensity that the cultures were exposed to had a very large effect on the levels of secondary carotenoids accumulated in the cultures. High light intensities produced a very large increase in the accumulation of secondary carotenoids, both at the cellular level and per unit volume of culture. Even though high light intensities resulted in high rates of cell mortality, the cells which did survive produced massive amounts of secondary carotenoids. At lower light intensities the amount of secondary carotenoids accumulated was in comparison very low but the survival rates of the alga were greatly increased. Further studies described in section 7.3.3 indicate that the optimum light intensity for the induction of astaxanthin (III) synthesis in the alga is in the range 1550 - 1650  $\mu\text{mol m}^{-2} \text{s}^{-1}$  (PAR), which is considerably higher than any of the light intensities investigated in the present study. However, these results illustrate along with those of other groups that light is one of the most important factors for astaxanthin (III) production

in *H. pluvialis* (Donkin, 1976; Boussiba and Vonshak, 1991; Borowitzka *et al.*, 1991; Kobayashi *et al.*, 1992).

The highest light intensity used in the present study are well below those which would be encountered in an outdoor production system. Early work with *Dunaliella* concentrated on optimising growth and carotenogenesis of the cultures under laboratory conditions. However, once the cultures were transferred outdoors into open ponds it was apparent that the high intensity of natural sunlight the cultures were exposed to caused high rates of cell mortality within the populations (Post *et al.*, 1983). The *Dunaliella* populations were commonly exposed to irradiances in excess of  $2000 \mu\text{E m}^{-2} \text{s}^{-1}$ . These high levels of irradiation killed a large proportion of the green cells of *Dunaliella* in the population. However, the cells which had already accumulated appreciable amounts of  $\beta$ -carotene (I) were able to survive these high irradiances (Borowitzka *et al.*, 1984).

Although the early work with *Dunaliella* identified the importance of light for inducing  $\beta$ -carotene (I) synthesis (Ben-Amotz and Avron, 1983), the need to control the level of irradiance at times when the cells had little or no protection against high light intensities also became apparent. These earlier results and the results obtained in the present study indicate that by controlling the irradiance level to which *H. pluvialis* is exposed to during the different phases of the alga's life cycle, is an important factor in maximising secondary carotenoid production in the alga. If the irradiance levels to which the 'swimmer' and palmella cells are exposed is too large, then high rates of cell death will be observed. A gradual increase in the light intensity to which the cells are exposed, enables the cells to accumulate low levels of secondary carotenoids. As a result, the cells would show improved viability rates when exposed to the higher light intensities. This would enable maximum rates of secondary carotenoid accumulation to be obtained, whilst minimising the number of cells lost due to photo-bleaching effects.

The exposure of the cells to various temperatures failed to yield much useful information. The two highest temperatures ( $32^\circ\text{C}$  and  $42^\circ\text{C}$ ) proved to be too high for the alga; resulting in poor cell growth and high rates of cell mortality. Culturing the alga at  $42^\circ\text{C}$  proved to be lethal for the alga, which is important when considering that temperatures greater than  $42^\circ\text{C}$  may be encountered in areas of the world where the alga is likely to be cultivated outdoors e.g. Australia, Israel and Portugal. The alga did survive at



32°C, but was unable to synthesise secondary carotenoids. The cells grown at the lower temperature showed the typical pattern of secondary carotenoid accumulation. Considering *H. pluvialis* is often termed as a 'snow alga' due to its ability to survive in polar environments, the inability of the alga to tolerate high temperatures is not unexpected. The results obtained in sections 7.3.2 and 7.3.3 indicate that the optimum temperature for growth and secondary carotenoid production in the alga is approximately 14 - 15°C, which more closely reflects the polar habitat of the alga.

The experiment may have yielded more useful information if the cultures had been exposed to a heat shock for a short period of time i.e. hours instead of days. From personal observations (data not shown), it would seem that such a shock can be used to induce secondary carotenogenesis and may be more representative of the conditions found in areas of the world where the alga may be grown (see above).

All the various parameters studied did have an effect on the induction of secondary carotenogenesis in the alga. It can be concluded that any factor which interferes with certain cellular processes within the alga (and hence limits growth) can act as a trigger for the process of secondary carotenoid production. It would appear that microalgae synthesise and store secondary carotenoids with environmental and nutritional factors regulating this function. This is interpreted in most of the literature as being due to the inhibition of mitosis observed under the given conditions (Suen *et al.*, 1987). The cell cycle is thought to be interrupted at a position primarily responsible for the synthesis of lipids. As mitosis is impeded, lipid content increases which leads to the formation of cytosolic lipid bodies.

Under nitrogen stress, lipid accumulation produces substantially fewer polar bodies and significantly more neutral bodies (Piorreck *et al.*, 1984). No one has yet elucidated the mechanism by which stress conditions promote the production of lipids in microalgae. However, a role for abscisic acid and other higher plant hormones such as ethylene has been suggested (Maillard, 1993). Moreover, it has not yet been determined whether the enhanced lipid production is the result of catabolism of pre-formed cellular carbohydrates and/or proteins or the result of *de novo* synthesis by regulation (or deregulation) of the photosynthetic pathway.

The results presented in the current investigation indicate that the various environmental and nutritional factors invoke a differential response within the alga. The degree of this response is dependent upon the individual factor (stress) to which the alga is exposed. Different factors induce secondary carotenogenesis at different rates. This attribute of the alga to perceive various stresses and to respond to them differentially could be manipulated when culturing the alga for the commercial production of secondary carotenoids. By exposing the alga to specific conditions at particular stages of the alga's life cycle the levels of secondary carotenoids accumulated could be greatly improved, and the time required to achieve this significantly reduced. Table 6.5 lists the various factors responsible for inducing secondary carotenogenesis determined in the present study in order of effectiveness.

**Table 6.5** Factors responsible for inducing secondary carotenogenesis in *H. pluvialis*

Rating	Parameter	Parameter needs to be		Comments
		in excess	: limited	
[1]	Light	+		Costly capital investment; heat generated difficult to control.
[2]	Salt	+		Increases running costs; problems of corrosion with equipment.
[3]	Nitrogen		+	Difficult to control.
[4]	Phosphate		+	Difficult to control.
[5]	Fe	+		Inclusion makes alga unsuitable as a feed additive.
[6]	Temperature	?	?	Difficult to control on an industrial scale.

The addition of certain chemicals to a fermenter (e.g. salt) or increasing the level of light or temperature, is much easier to control than when a factor needs to be present at a limiting concentration i.e. nitrogen and phosphate. This is especially the case when considering that relatively high levels of nitrogen and phosphate need to be present in the growth medium initially to produce enough algal biomass to synthesise high levels of secondary carotenoids. To be able to significantly reduce the levels of nitrogen and phosphate in a fermenter chemically would be very difficult and probably prove to be very costly. However, by optimising the algal growth rate the levels of nitrogen and phosphate would be rapidly reduced naturally, whilst allowing high rates of algal biomass to be accumulated. Once the cultures had accumulated sufficient biomass, to induce secondary carotenogenesis the cultures would then be exposed to increased salinities and irradiances. This two phase fermentation system is investigated in proceeding chapters.



The inability of the alga to tolerate high temperatures and the resultant reduction in secondary carotenoid production poses problems if high light intensities are to be used to induce secondary carotenoid synthesis. This inability also raises doubts as to whether or not the alga could be cultivated outdoors in countries with 'hot' climates, to make use of natural sun light to stimulate algal growth and to promote secondary carotenoid induction. These problems could be overcome by the production of mutant strains of the alga which are able to tolerate high temperatures. Another method could be to identify individual cells by some kind of selection method which can also tolerate increased temperatures. The use of such techniques could also be used to produce cell lines which can tolerate increased salinities, this would reduce the threat of contamination and predation within the reactor system. It would then be possible to cultivate the alga outdoors in open ponds in countries which receive high amounts of sunlight during the year. The cultivation methods and downstream processing could be similar to those which have proved successful in the cultivation of *Dunaliella* (see section 1.10). The adoption of such methods could result in the production of a feed additive product derived from *H. pluvialis* becoming widely available on the market.

## **OPTIMISATION OF ASTAXANTHIN PRODUCTION IN *HAEMATOCOCCUS PLUVIALIS* USING RESPONSE SURFACE METHODOLOGY**

### **7.1 INTRODUCTION**

#### **7.1.1 Nutritional improvement of fermentation processes**

Major improvements in the productivity of many microbial fermentation processes are generally ascribed to the development of superior strains via genetic manipulation. As yet, such techniques have been limited to only a few cases in the development of large scale algal fermentation processes. There are however other parameters such as the nutritional and physical environment to which an alga is exposed, which are known to significantly alter algal growth and product yield. Media commonly used to screen for industrially important microbial metabolites frequently do not become part of the process definition. Undesirable characteristics of these media include economically unattractive nutrients, support of sub-optimal productivity and support of the synthesis of closely related product components. Therefore, efforts to improve the medium should commence early in the development of a successful fermentation process and continue throughout in support of 'improved' culture introduction and fermentation scale-up activities.

Optimum values of temperature and pH are also important factors in the optimisation of the fermentation process. However, consideration should also be given to interactions between the parameters and to the effects of increased scale on so called optimum conditions (Winkler, 1987). The process design needs to concentrate on adequate formulation of the process objectives and the identity of key parameters.

Development of an economical production medium requires the selection of carbon, nitrogen, phosphorus, sulphur, potassium, and trace element sources as well as an energy source. These should support not only good algal growth but also maximise product yield, reduce the synthesis of compounds closely related to the product and enhance product recovery.



### 7.1.2 Statistical-mathematical methods of optimisation

The use of models, therefore, may be advantageous in producing a simplified description of the process under investigation, and in assisting understanding and design. In the context of optimisation the design process comprises the elements shown in figure 7.1. The problem itself will be relatively easy to define, whether it is biomass production or the production of secondary metabolites. A statement of the objectives of the problem needs to be in quantitative terms in order to facilitate mathematical treatment. Solutions to the problem are evaluated against a set of criteria and constraints. The former enables one to identify the superior solution in terms of a specified criterion, while the latter defines a condition or conditions that must be satisfied before a solution is acceptable. For example, if a process has to achieve a certain productivity, candidate solutions that do not meet these specifications are discarded. Optimisation is the means to which the best solution is defined. Finally, it is necessary to determine those results needed to evaluate solutions and those data needed to apply the model respectively, in order to compare the results within the objective.

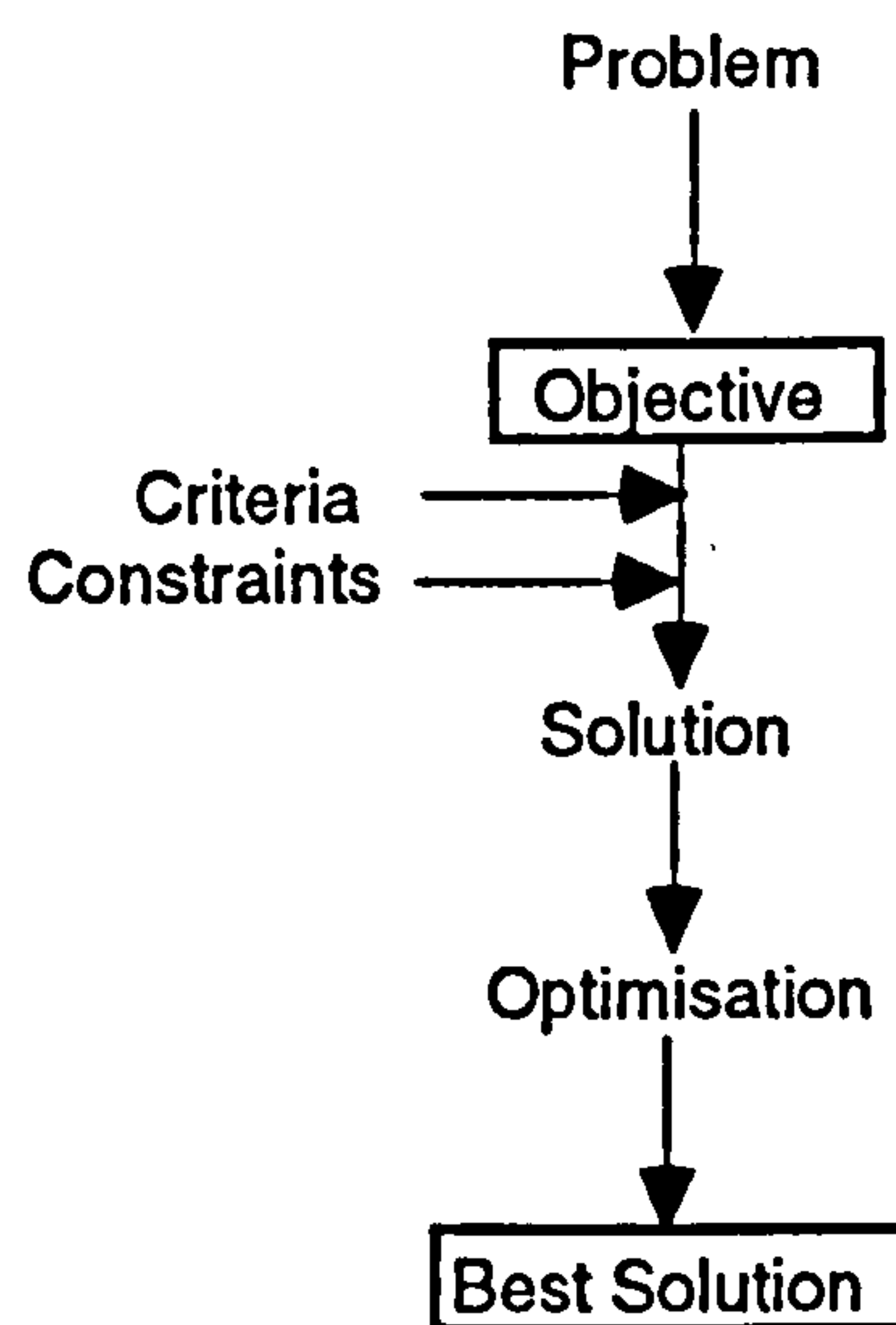


Fig. 7.1 Optimisation logic.

In summary, the route to optimisation can be viewed as the application of a hierarchy of models which, if successful, allows one to proceed from the 'mess' of reality to the realisation of the best solution. The multi-parameter analysis shown in figure 7.2 is

some form of simulation model, the inputs and outputs of which enable their relationships to be established through a regression model. Some familiarity with the process under consideration is necessary in order to initiate the screening of the parameters.

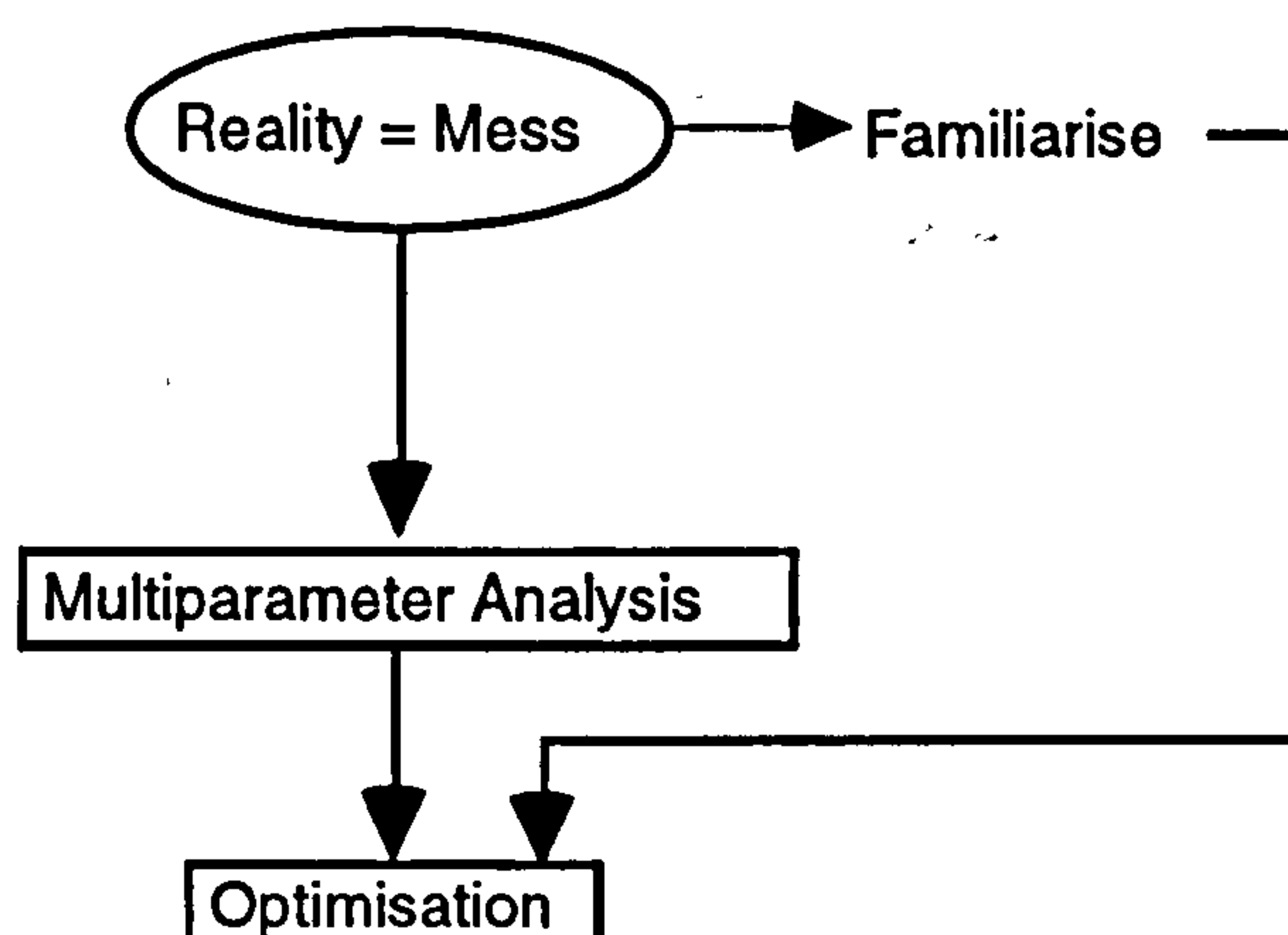


Fig. 7.2 Optimisation: a hierarchy of models.

Once a suitable strain for a microbial product has been selected (*H. pluvialis* 34/7 for astaxanthin (III) production), a medium for optimal productivity is devised and the critical mechanism determined following a series of experiments or observations (Fig. 7.3). The process may then be categorised, for example, as growth linked or growth dissociated (Gaden, 1959), as subject to repression by a specific nutrient assimilation rate or as a secondary metabolite (Bushell, 1989). Having determined the mechanism, it may be possible to enhance production by deregulation as a consequence of genetic modification. After a further observation stage to determine the critical mechanism of the new strain, the iteration is complete (Fig. 7.3) and a suitable bioreactor configuration may be implemented and scaled up.



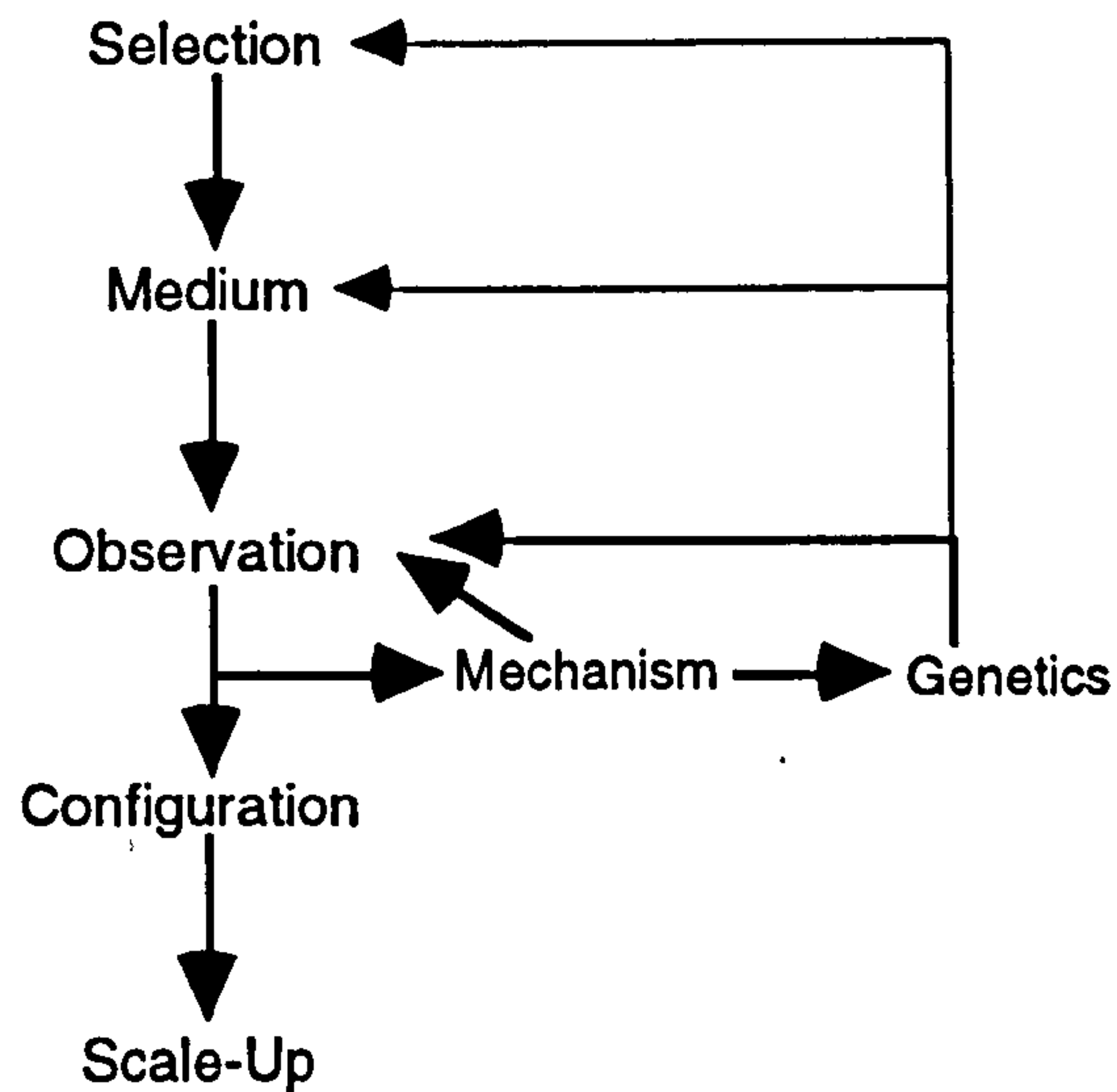


Fig. 7.3 Process Development: logical.

### 7.1.3 Plackett-Burman design

It is necessary to carry out preliminary formulation work using an empirical approach. The likelihood of success of such an approach can be enhanced considerably by the use of factorial and other numerical experimental designs (Greasham and Inamine, 1986). The first step in the use of statistical designs is to identify the most important nutritional and physical parameters independently, and determine the effect of these on growth and/or product yield. This can lead to an inordinately high number of trials. Evaluating four nutrients at four concentrations, for example would require 256 separate trials.

The problem of being confronted with many variables which may be of possible importance, and the pressure to select and optimise the most important variables as efficiently as possible, led to the widespread adoption of the procedure developed by Plackett and Burman (1946). These authors suggested a series of designs for up to 100 experiments using an experimental rationale known as balanced incomplete blocks. This allows for the evaluation of  $X-1$  variables by  $X$  experiments.

### 7.1.4 Response surface design

The next step in the optimisation procedure is the determination of the optimum level of each of the independent variables (previously identified). Response surface methodology (RSM) is one such technique that can be applied. RSM is a heuristic approach and does not guarantee the achievement of the unique optimum. Optimisation needs to take account of

non-linear responses, and for this purpose a full quadratic model is required. For three independent variables  $x_1$ ,  $x_2$  and,  $x_3$  the model will be:

$$y = b_0 + b_1x_1 + b_2x_2 + b_3x_3 + b_{12}x_1x_2 + b_{13}x_1x_3 + b_{23}x_2x_3 + b_{11}x_1^2 + b_{22}x_2^2 + b_{33}x_3^2$$

where  $y$  is the dependent variable (predicted output):  $b_1$ ,  $b_{12}$  and,  $b_{11}$  are the linear, two-way interaction, and quadratic coefficient respectively: and  $b_0$  is the regression coefficient at the centre point.

Each variable is tested at a minimum of three values, the upper and lower values being spaced equally from a centre point value. Once again the experimental design is a fraction only of the full factorial, but here the Plackett-Burman design cannot be used because it does not evaluate non-linear responses where they occur. Instead, designs such as the Box-Wilson (Box and Wilson, 1951) which accommodates two-way and quadratic effects are used. Trials are made in random sequence and the coefficients calculated. The output from these computations is depicted as contours of responses plotted against two independent variables. Several contour plots are required to accommodate three or more variables. While it is possible and certainly hoped that these plots (the response surfaces) will reveal the optimum condition, or suggest the region where the optimum is to be found, it is also possible that  $x^*$  does not indicate the unique optimum but rather a saddle point or ridge. Finally, confirmation experiments are carried out once the value of each variable for optimum response has been estimated.

### 7.1.5 Aims

The work in this section reports on the use of RSM to optimise the production of astaxanthin (III) from *H. pluvialis*. This initially involved using RSM to optimise the production of biomass from *H. pluvialis*, with the identification of three key independent variables responsible for biomass production. The key independent variables investigated were selected on the basis of personal observations i.e. familiarisation (Fig. 7.2), and



previous reports (Borowitzka *et al.*, 1991; Pringsheim, 1966). The first stage of this work involved the identification of an optimum nitrogen source for the growth of the alga.

RSM techniques were then applied to astaxanthin (III) production in the alga. Again, the three key independent variables were selected on the basis of personal observations and previous reports (Kobayashi *et al.*, 1992; Spencer, 1989). The aim of the two RSM experiments was to optimise the nutritional and physical parameters to support maximum biomass production in *H. pluvialis* in the shortest time period and then, to manipulate these parameters further so that maximum rates of astaxanthin (III) production in the alga could be obtained.

## **7.2 MATERIALS AND METHODS**

### **7.2.1 Optimum nitrogen source for the growth of *H. pluvialis***

*H. pluvialis* 34/7 CCAP was cultured in 250 ml Erlenmeyer flasks under normal cultivation conditions. The media the alga was cultivated in was modified BBM (as described in section 2.7.1) but with a range of nitrogen sources incorporated into the media. The nitrogen sources investigated were sodium nitrate, sodium nitrite, ammonium chloride, urea and potassium nitrate. The concentration of nitrogen in the cultures containing the various nitrogen sources was 3.0 mM. The dry cell weight of the cultures was determined after 3 weeks cultivation.

### **7.2.2 Optimisation of biomass production**

Two sets of biomass optimisation experiments were carried out to determine the optimum conditions. In the first biomass optimisation experiment green 'swimmer' cells of *H. pluvialis* 34/7 CCAP were cultured in 250 ml Erlenmeyer flasks in modified BBM under normal cultivation conditions (see above). The three key independent variables identified for *H. pluvialis* growth were light intensity, temperature and nitrogen concentration. Algal cultures were grown at three different levels of each parameter and in all combinations. The nitrogen concentrations employed were 0.15, 1.47 and 2.79 mM of urea, the light intensities used were 10, 40 and 70  $\mu\text{mol m}^{-2} \text{s}^{-1}$  (PAR), and the

temperatures used were 15, 22 and 29°C. Urea was used as the nitrogen source in these experiments, since it had previously been identified as the best nitrogen source for algal growth (see above). The dry cell weight of the cultures was determined after 3 weeks cultivation.

In the second set of biomass optimisation experiments the culture conditions were the same as in the first biomass optimisation experiments. However, the nitrogen concentrations employed were 0.37, 1.47 and 2.57 mM of urea, the light intensities were 10, 50 and 90  $\mu\text{mol m}^{-2} \text{s}^{-1}$  (PAR), and the temperatures used were 11, 15 and 19°C. The dry cell weight of the cultures was determined after 3 weeks cultivation.

### **7.2.3 Optimisation of astaxanthin (III) production**

Two sets of astaxanthin (III) optimisation experiments were carried out to determine the optimum conditions for astaxanthin (III) production. In the first astaxanthin (III) optimisation experiment, green palmella cells of *H. pluvialis* 3477 CCAP were cultured in 250 ml Erlenmeyer flasks in modified BBM under normal cultivation conditions (see above). The three key independent variables identified for astaxanthin (III) production were: (i) salt concentration; (ii) temperature; and (iii) light intensity. The algal cultures were exposed to three different levels of each parameter and in all combinations. The salt concentrations used were 100, 170 and 240 mM NaCl, the light intensities were 1, 45 and 90  $\mu\text{mol m}^{-2} \text{s}^{-1}$  (PAR) and, the temperatures used were 22, 32 and 42°C. The astaxanthin (III) content of the cultures (mg/l) was determined after 3 weeks cultivation.

In the second set of astaxanthin (III) optimisation experiments the culture conditions were the same as those in the first astaxanthin (III) optimisation experiment. However, because of the high light intensities employed in the second set of experiments it was not possible to simultaneously investigate the optimum temperature for astaxanthin (III) production. Therefore, only two key independent variables were investigated in this set of experiments. The salt concentrations were 10, 50 and 90 mM NaCl and the light intensities used were 30, 630 and, 1230  $\mu\text{mol m}^{-2} \text{s}^{-1}$  (PAR). The cultures were cultivated at 24°C and harvested after five days when their astaxanthin (III) content (mg/l) was determined.



#### 7.2.4 Statistical design and analysis

Statistical design and analysis was achieved using three-level factorial experiments which constituted the basis of the statistical designs used. These are experiments in which each of the key independent factors (three in this case) is varied at three levels and in all combinations. Computation was carried out using a Ness-286 computer using the University of Nebraska Response Surface Methodology program for regression analysis. The program used for response surface plotting was Systat 5.1.

### 7.3 RESULTS

#### 7.3.1 Optimum nitrogen source for *H. pluvialis* growth

The dry cell weights of the algal cultures cultivated in the range of nitrogen sources for 3 weeks were plotted (Fig. 7.4). For the details of statistical analysis see section 2.9.2.

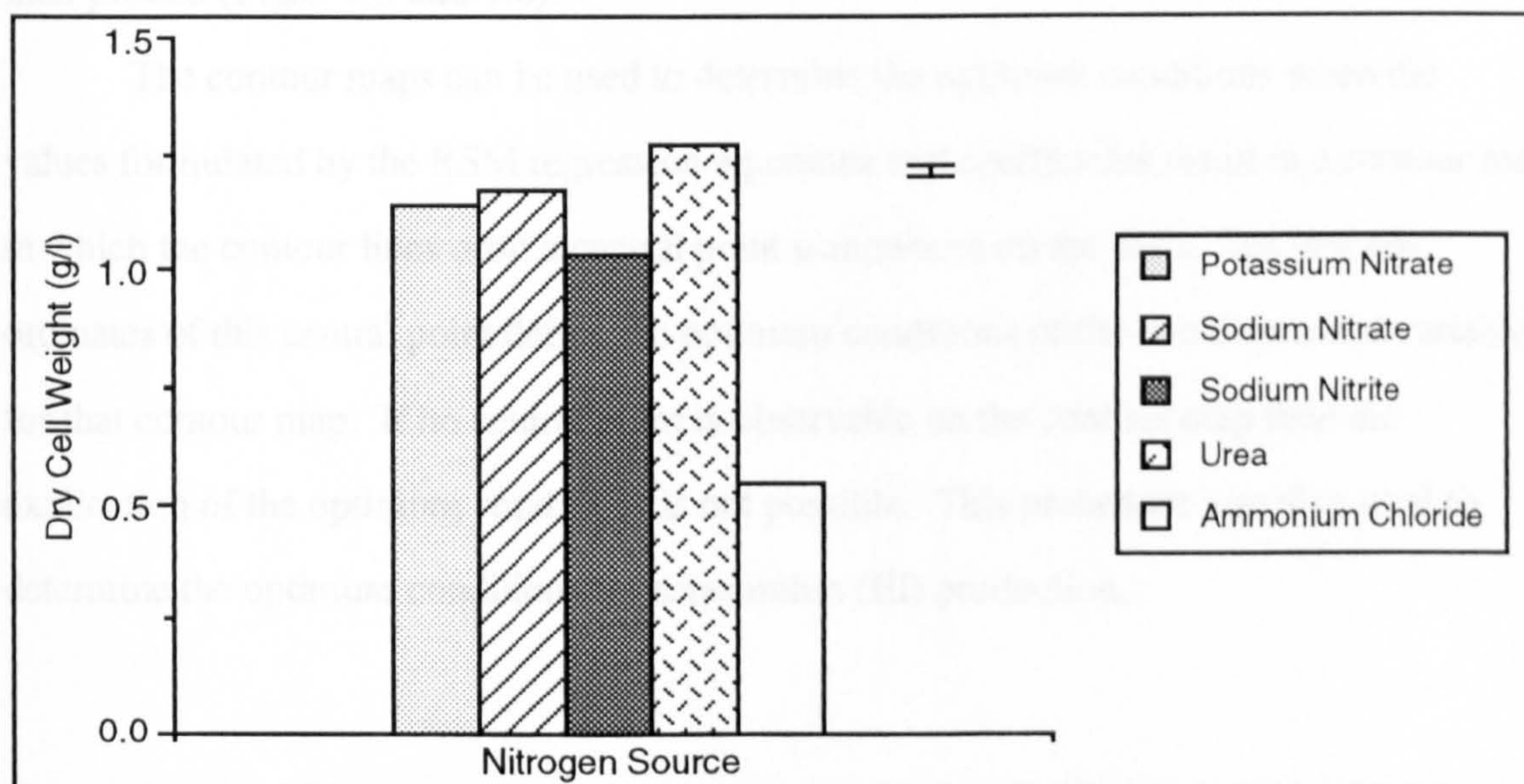


Fig. 7.4 Optimum nitrogen source for the growth of *H. pluvialis* (S.E.  $\pm 3.9\%$ ,  $n=3$ ).

Figure 7.4 indicates that urea is the best nitrogen source for supporting maximum algal growth. The growth of *H. pluvialis* in urea was significantly better than the growth of the alga in any of the other nitrogen sources. The next best nitrogen source for algal growth was sodium nitrate with the extent of algal growth being significantly better than that achieved in potassium nitrate. Sodium nitrite was the fourth best nitrogen source for



algal growth with growth in ammonium chloride significantly lower in comparison to the other nitrogen sources. The alga grew very poorly in ammonium chloride, probably due to the large pH fluctuations observed in these cultures during cultivation (data not shown).

### **7.3.2 Optimisation of biomass production**

The dry cell weights of the algal cultures cultivated under the range of physical and nutritional conditions after three weeks is shown in table 7.1. These values were used to calculate the RSM regression equations and coefficients. The regression equations calculated by the RSM program allow for the prediction of algal biomass at any concentration/intensity of two of the three key independent variables tested. All the RSM equations follow the same format with the constants and coefficients being the numeric variables in each specific equation. Therefore, by altering the values of the two key independent variables in the equation, the value of the predicted algal biomass varies accordingly. Using the values obtained from the regression equations contour maps were then plotted (Figs. 7.5 and 7.6).

The contour maps can be used to determine the optimum conditions when the values formulated by the RSM regression equations and coefficients result in a contour map in which the contour lines orbit a central point somewhere on the map. The two coordinates of this central point define the optimum conditions of the two dependent variables for that contour map. If no central point is observable on the contour map then the explication of the optimum conditions is not possible. This procedure was also used to determine the optimum conditions for astaxanthin (III) production.



**Table 7.1** Summary of the data collected in the first series of *H. pluvialis* biomass experiments to determine the optimum conditions for algal growth.

Temperature (C)	Light Intensity ( $\mu\text{mol}/\text{m}/\text{m}/\text{s}$ )	Urea Concentration (mM)	D.C.W (g/l)	$\pm$ S.E. (n=3) (%)
15	10	0.15	0.33	2.0
15	10	1.47	0.52	0.8
15	10	2.79	0.44	0.5
15	40	0.15	0.63	4.3
15	40	1.47	0.77	0.6
15	40	2.79	0.61	3.5
15	70	0.15	0.54	1.6
15	70	1.47	0.73	3.6
15	70	2.79	0.57	1.8
22	10	0.15	0.39	4.4
22	10	1.47	0.44	5.2
22	10	2.79	0.40	2.5
22	40	0.15	0.51	2.8
22	40	1.47	0.66	2.3
22	40	2.79	0.47	2.5
22	70	0.15	0.66	3.0
22	70	1.47	0.59	4.8
22	70	2.79	0.58	3.5
29	10	0.15	0.53	5.1
29	10	1.47	0.46	1.0
29	10	2.79	0.44	4.9
29	40	0.15	0.50	5.3
29	40	1.47	0.47	3.2
29	40	2.79	0.35	5.4
29	70	0.15	0.44	4.9
29	70	1.47	0.24	1.1
29	70	2.79	0.48	5.8

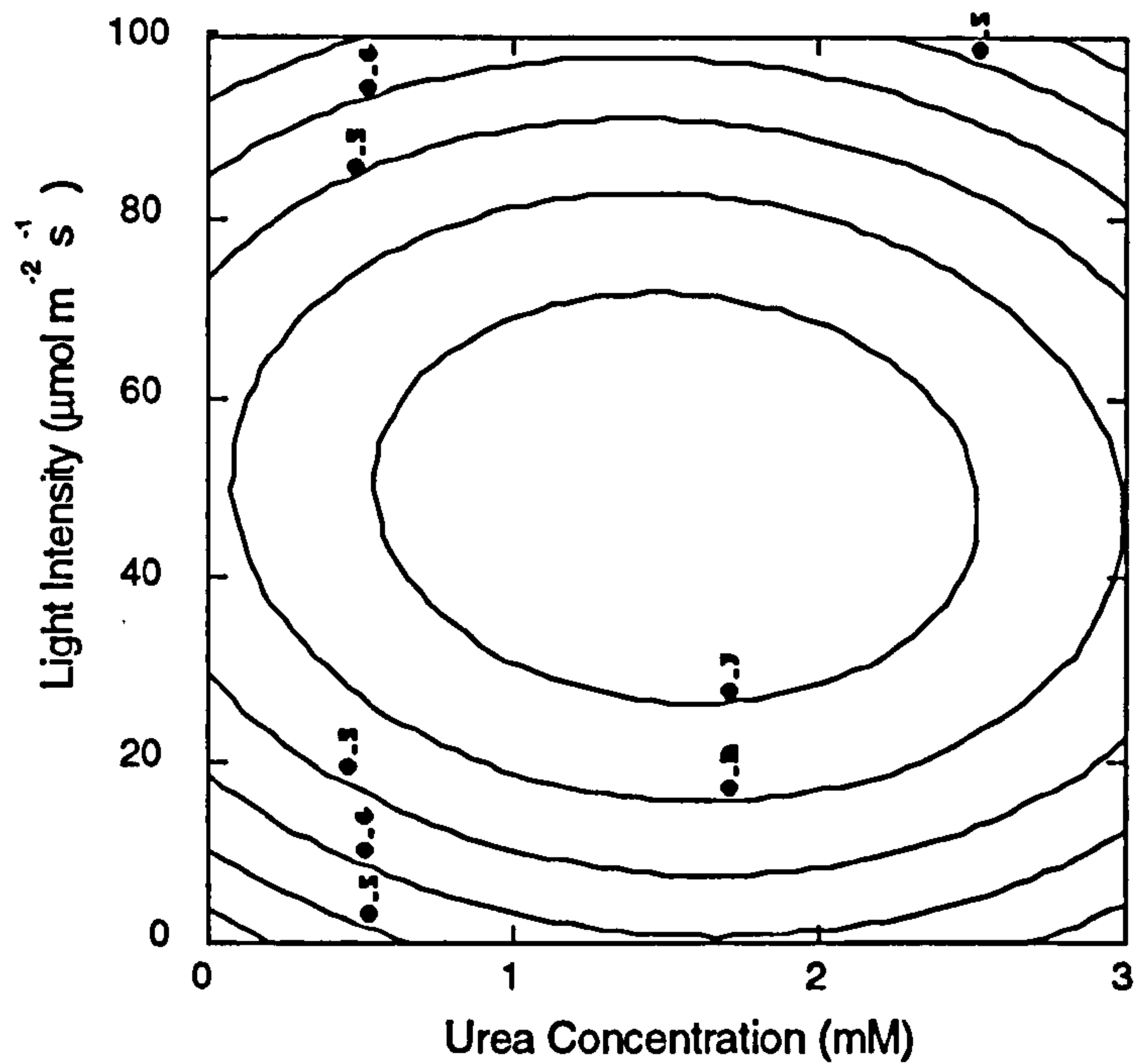


Fig. 7.5 Contour map to determine the optimum urea concentration and light intensity for algal growth.

Regression equation;

$$\begin{aligned} \text{Predicted biomass} = & 0.144164 + 0.2940778 * \text{urea concentration} + \\ & 1.698319\text{E-}02 * \text{light intensity} + -5.050581\text{E-}04 * \text{urea} \\ & \text{concentration} * \text{light intensity} + -8.800137\text{E-}02 * \text{urea} \\ & \text{concentration}^2 + -1.64815\text{E-}04 * \text{light intensity}^2 \end{aligned}$$

Regression coefficients;

$$\begin{aligned} \text{Constant} &= 0.144164 \\ \text{Urea Concentration} &= 0.2940778 \\ \text{Light Intensity} &= 1.698319\text{E-}02 \\ \text{Coefficient of determination (R}^2\text{)} &= 0.9781811 \\ \text{Coefficient of multiple correlation} &= 0.9890304 \\ \text{Standard error of estimate (SE)} &= 3.288476\text{E-}02 \end{aligned}$$



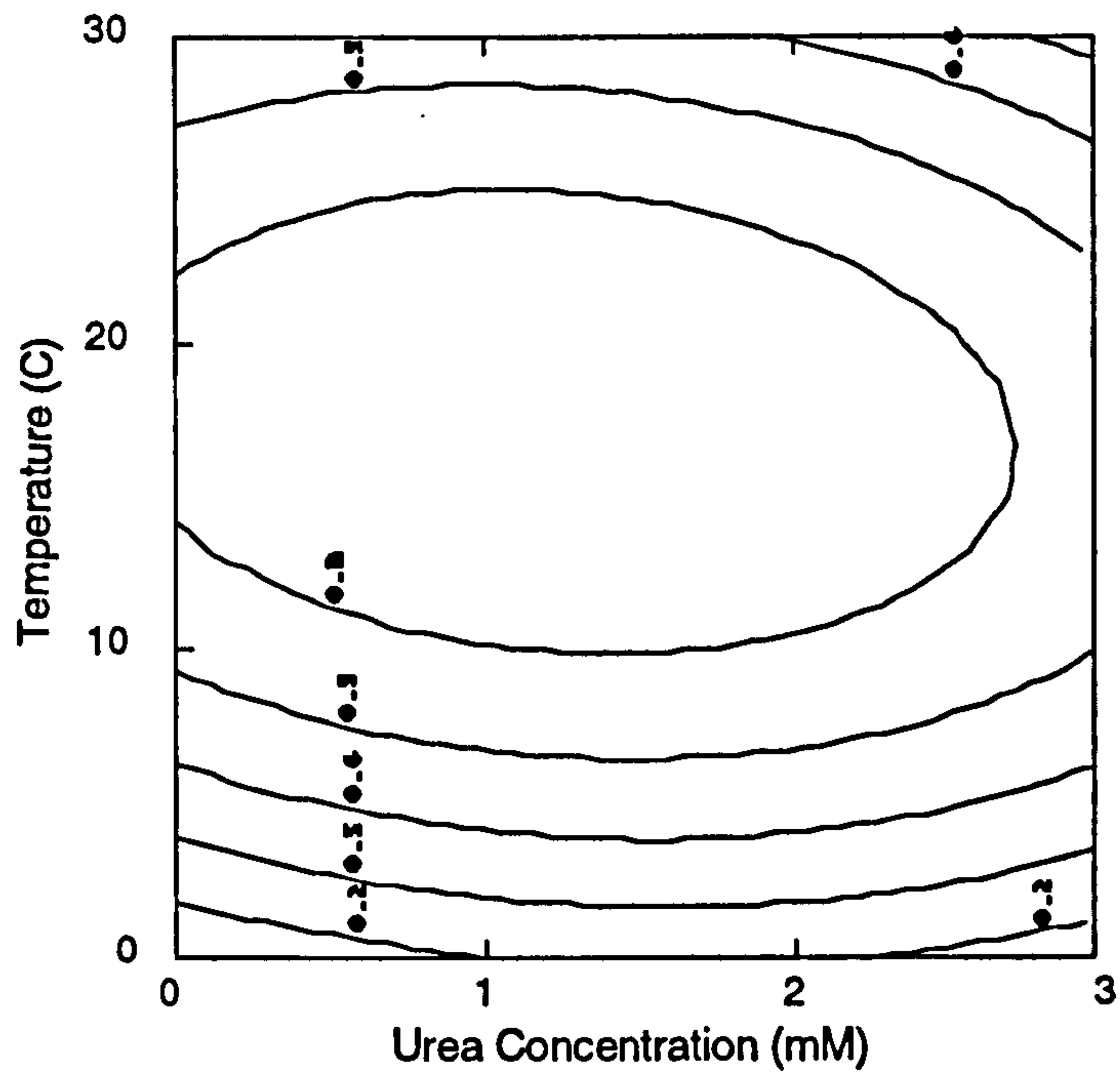


Fig. 7.6 Contour map to determine the optimum urea concentration and temperature for algal growth.

Regression equation;

$$\begin{aligned} \text{Predicted biomass} = & 0.1000167 + 0.1400093 * \text{urea concentration} + \\ & 5.810618\text{E-}02 * \text{temperature} + -1.955085\text{E-}03 * \text{urea concentration} \\ & * \text{temperature} + -4.290296\text{E-}02 * \text{urea concentration}^2 + \\ & -1.601872\text{E-}03 * \text{temperature}^2 \end{aligned}$$

Regression coefficients;

$$\text{Constant} = 0.1000167$$

$$\text{Urea Concentration} = 0.1400093$$

$$\text{Temperature} = 5.810618\text{E-}02$$

$$\text{Coefficient of determination (R}^2\text{)} = 3.342268\text{E-}03$$

$$\text{Coefficient of multiple correlation} = 5.781236\text{E-}02$$

$$\text{Standard error of estimate (SE)} = 2.851449$$

The contour maps (Fig. 7.5 and 7.6) indicate that the urea concentration corresponding to maximum algal biomass production was approximately 1.5 mM. The light intensity corresponding to the maximum level of algal biomass production was approximately 50

$\mu\text{mol m}^{-2}\text{s}^{-1}$  (PAR), and the optimum temperature required for the maximum production of algal biomass was approximately 17°C.

Since in these initial optimisation experiments the optimum temperature for the maximum production of algal biomass was not clearly defined a second series of experiments was carried out similar to the first set. This second set of experiments was again designed to try and determine the optimum conditions for algal biomass production. The results of the first experiments were used as a guide to determine the levels of the physical and nutritional parameters to be tested in the second set of experiments. Table 7.2 shows the dry cell weights of the algal cultures cultivated under the range of physical and nutritional conditions after three weeks. The values obtained were used to calculate the RSM regression equations and coefficients. Using the values formulated from the regression equations and coefficients contour maps were then plotted (Figs. 7.7 and 7.8).



**Table 7.2** Summary of the data collected in the second series of *H. pluvialis* biomass experiments to determine the optimum conditions for algal growth.

Temperature (C)	Light Intensity ( $\mu\text{mol}/\text{m}/\text{m}/\text{s}$ )	Urea Concentration (mM)	D.C.W (g/l)	$\pm$ S.E. (n=3) (%)
11	10	0.37	0.14	3.4
11	10	1.47	0.28	5.8
11	10	2.57	0.27	1.7
11	50	0.37	0.55	1.8
11	50	1.47	0.95	1.4
11	50	2.57	0.88	0.6
11	90	0.37	0.54	1.0
11	90	1.47	0.90	0.5
11	90	2.57	0.82	1.1
15	10	0.37	0.24	2.2
15	10	1.47	0.71	0.4
15	10	2.57	0.65	1.5
15	50	0.37	0.75	2.4
15	50	1.47	1.09	0.9
15	50	2.57	1.01	0.7
15	90	0.37	0.61	1.2
15	90	1.47	0.96	0.5
15	90	2.57	0.83	0.6
19	10	0.37	0.23	5.9
19	10	1.47	0.35	2.7
19	10	2.57	0.31	0.9
19	50	0.37	0.63	2.2
19	50	1.47	0.94	0.5
19	50	2.57	0.82	0.6
19	90	0.37	0.73	0.1
19	90	1.47	0.87	1.4
19	90	2.57	0.77	1.1

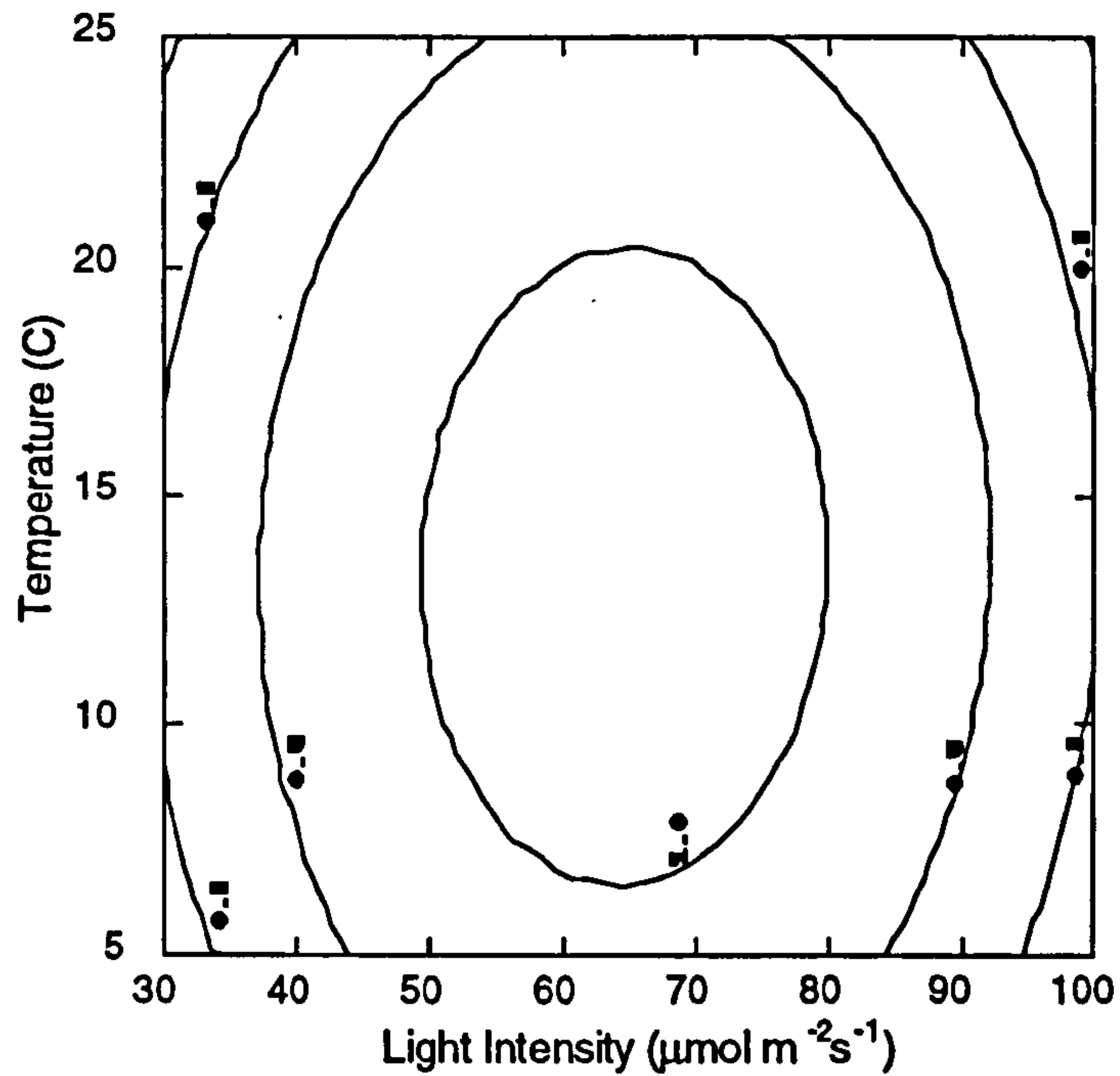


Fig. 7.7 Contour map to determine the optimum light intensity and temperature for algal growth.

Regression equation;

$$\begin{aligned} \text{Predicted biomass} = & 9.143954\text{E-}02 + 2.460869\text{E-}02 * \text{light} \\ & \text{intensity} + 2.365008\text{E-}02 * \text{temperature} + 2.226146\text{E-}05 * \text{light} \\ & \text{intensity} * \text{temperature} + -1.927704\text{E-}04 * \text{light intensity}^2 + \\ & -9.306559\text{E-}04 * \text{temperature}^2 \end{aligned}$$

Regression coefficients;

$$\text{Constant} = 9.143954\text{E-}02$$

$$\text{Light intensity} = 2.460869\text{E-}02$$

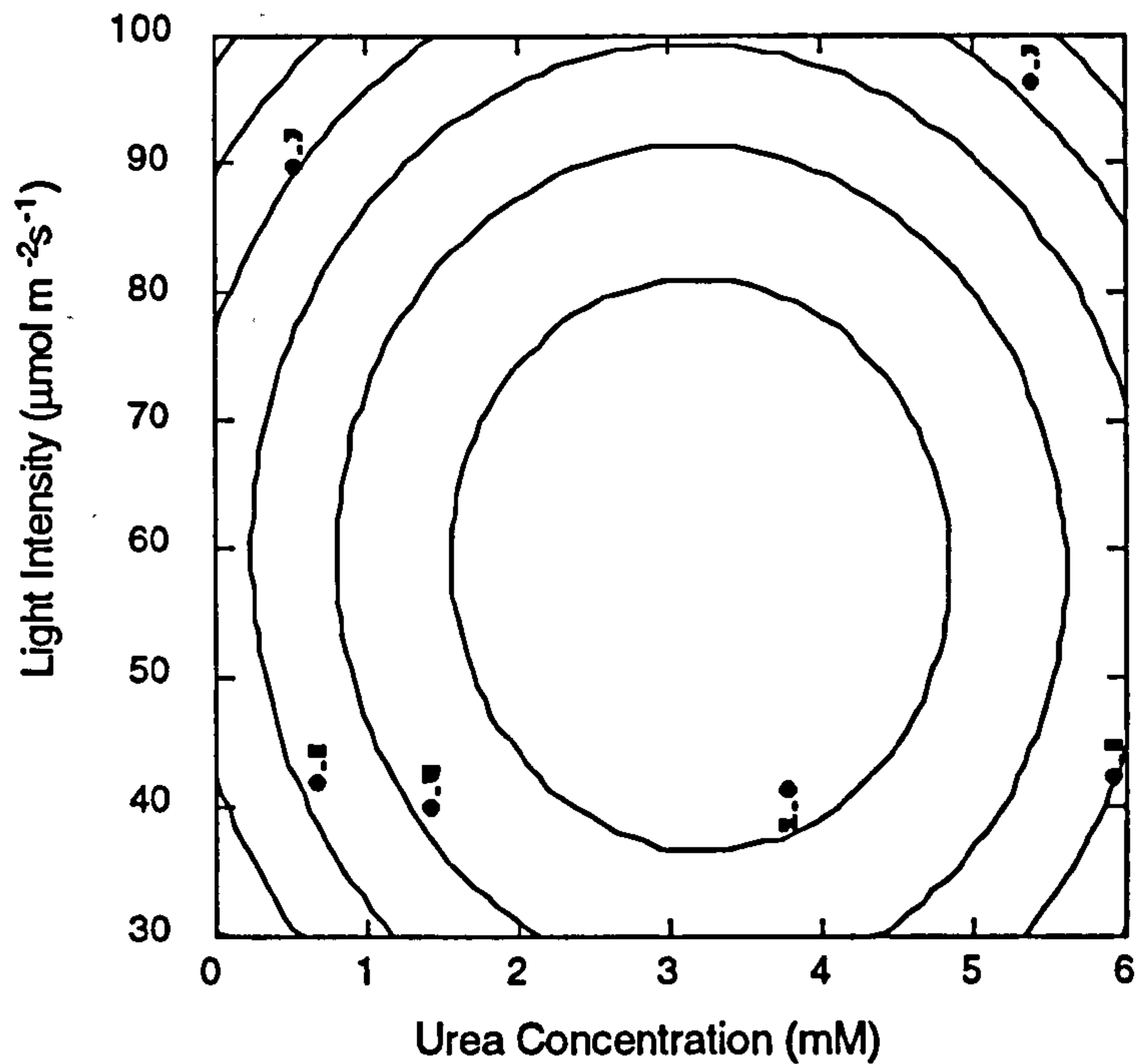
$$\text{Temperature} = 2.365008\text{E-}02$$

$$\text{Coefficient of determination (R}^2\text{)} = 0.0363675$$

$$\text{Coefficient of multiple correlation} = 0.1907026$$

$$\text{Standard error of estimate (SE)} = 1.965685$$





**Fig. 7.8** Contour map to determine the optimum urea concentration and light intensity for algal growth.

Regression equation;

$$\begin{aligned} \text{Predicted biomass} = & 0.1194797 + 0.2160638 * \text{urea concentration} + \\ & 2.122043\text{E-}02 * \text{light intensity} + -1.04673\text{E-}04 * \text{urea} \\ & \text{concentration} * \text{light intensity} + -3.287115\text{E-}02 * \text{urea concentration}^2 \\ & + -1.772189\text{E-}04 * \text{light intensity}^2 \end{aligned}$$

Regression coefficients;

$$\text{Constant} = 0.1194797$$

$$\text{Urea concentration} = 0.2160638$$

$$\text{Light intensity} = 2.122043\text{E-}02$$

$$\text{Coefficient of determination (R}^2\text{)} = 5.464244\text{E-}02$$

$$\text{Coefficient of multiple correlation} = .2337572$$

$$\text{Standard error of estimate (SE)} = 1.395523$$

The contour maps of the second set of *H. pluvialis* biomass optimisation experiments (Figs. 7.7 and 7.8) indicate that the light intensity corresponding to the maximum rate of algal biomass production was approximately 60  $\mu\text{mol m}^{-2} \text{s}^{-1}$  (PAR). The optimum

temperature to achieve maximum algal growth was 14°C, whilst the optimum urea concentration for algal growth was approximately 3.0 mM.

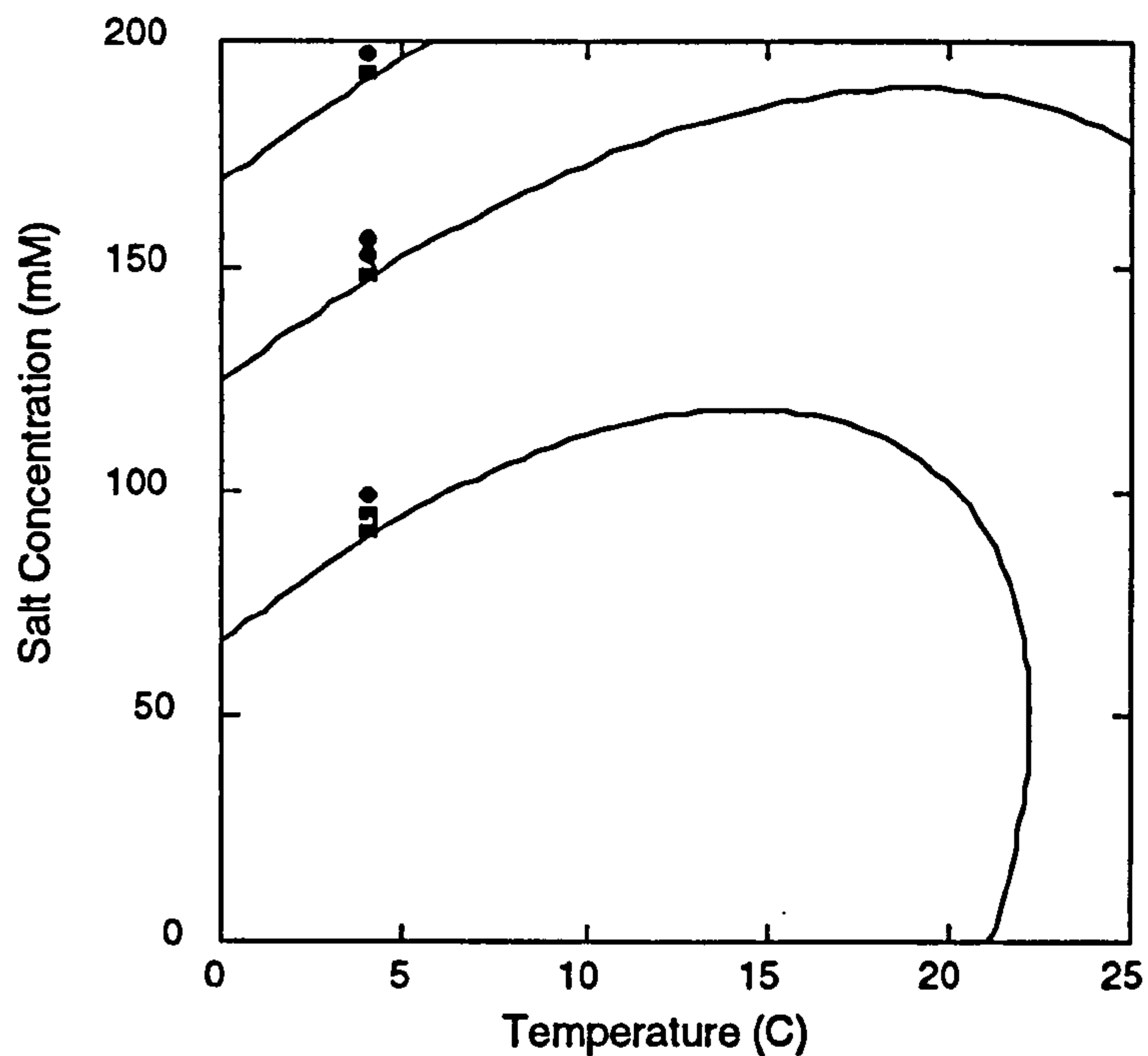
### 7.3.3 Optimisation of astaxanthin (III) production

The astaxanthin (III) content of algal cultures cultivated under a range of physical and nutritional conditions for 3 weeks is shown in table 7.3. The values shown in the table were used to calculate the RSM regression equations and coefficients. The values obtained from the RSM analysis were then used to plot the contour maps figures 7.9 and 7.10.

**Table 7.3** Summary of data assembled to construct the first set of contour graphs to determine the optimum conditions for astaxanthin (III) production.

Temperature (C)	Light Intensity ( $\mu\text{mol}/\text{m}^2/\text{s}$ )	Salt Concentration (mM)	Astaxanthin (mg/l)	$\pm$ S.E. (n=3) (%)
22	10	100	17.6	4.6
22	10	170	13.5	2.4
22	10	240	27.1	1.1
22	50	100	10.1	2.4
22	50	170	25.0	2.3
22	50	240	41.9	0.3
22	90	100	29.1	1.9
22	90	170	42.8	1.3
22	90	240	41.3	2.4
32	10	100	4.1	1.3
32	10	170	5.9	2.2
32	10	240	14.4	0.6
32	50	100	6.9	3.5
32	50	170	12.8	2.7
32	50	240	20.7	4.6
32	90	100	5.4	2.8
32	90	170	16.0	2.5
32	90	240	23.9	3.7
42	10	100	0.3	4.1
42	10	170	0.3	0.6
42	10	240	0.4	3.7
42	50	100	0.5	2.8
42	50	170	0.6	1.1
42	50	240	0.3	4.8
42	90	100	0.6	3.3
42	90	170	0.6	0.9
42	90	240	0.4	1.7





**Fig. 7.9** Contour map to determine the optimum temperature and salt concentration for astaxanthin (III) production from *H. pluvialis*.

Regression equation;

$$\begin{aligned} \text{Predicted astaxanthin (III) content} = & 184.6898 + 2.839204 * \\ & \text{temperature} + -0.3448339 * \text{salt concentration} + 2.761079\text{E-}02 * \\ & \text{temperature} * \text{salt concentration} + -0.2118014 * \text{temperature}^2 + \\ & -2.68544\text{E-}03 * \text{salt concentration}^2 \end{aligned}$$

Regression coefficients;

$$\text{Constant} = 184.6898$$

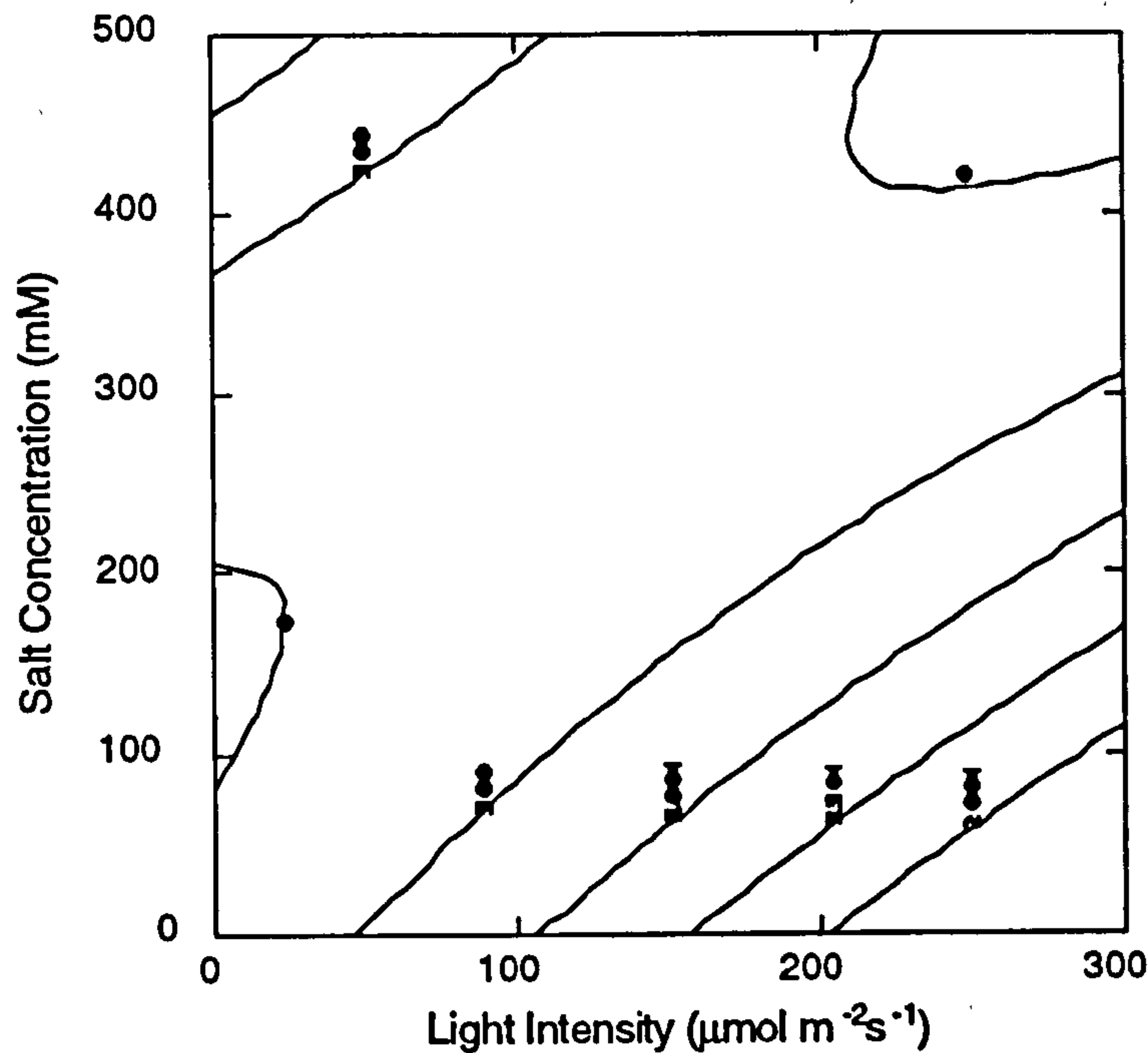
$$\text{Temperature} = 2.839204$$

$$\text{Salt concentration} = -0.3448339$$

$$\text{Coefficient of determination (R}^2\text{)} = 0.1889917$$

$$\text{Coefficient of multiple correlation} = 0.4347317$$

$$\text{Standard error of estimate (SE)} = 277.8283$$



**Fig. 7.10** Contour map to determine the optimum light intensity and salt concentration for astaxanthin (III) production from *H. pluvialis*.

Regression equation;

$$\begin{aligned} \text{Predicted astaxanthin (III) content} = & 176.7243 + 6.361646 * \text{light} \\ & \text{intensity} + -3.077811 * \text{salt concentration} + -3.054856\text{E-}02 * \text{light} \\ & \text{intensity} * \text{salt concentration} + 1.319896\text{E-}02 * \text{light intensity}^2 + \\ & 1.073807\text{E-}2 * \text{salt concentration}^2 \end{aligned}$$

Regression coefficients;

$$\text{Constant} = 176.7243$$

$$\text{Light intensity} = 6.361646$$

$$\text{Salt concentration} = -3.077811$$

$$\text{Coefficient of determination (R}^2\text{)} = 0.9494989$$

$$\text{Coefficient of multiple correlation} = 0.9744224$$

$$\text{Standard error of estimate (SE)} = 31.86875$$

The contour maps in figures 7.9 and 7.10 do not indicate the salt concentration corresponding to the maximum rate of astaxanthin (III) production. The optimum temperature needed to achieve maximum astaxanthin (III) production is also not apparent



from the contour plots. The optimum light intensity for astaxanthin (III) production would seem to be much greater than the light intensities investigated in the present experiments and cannot therefore be estimated. Since the first astaxanthin (III) optimisation experiments failed to define the optimum levels of the parameters investigated a second set of astaxanthin (III) optimisation experiments was carried out. Due to practical problems of temperature control at the high light intensities used in the experiment, only two key independent variables were tested, namely salt concentration and light intensity.

The astaxanthin (III) content of algal cultures cultivated under the various salt concentrations and light intensities after five days is shown in table 7.4. The values shown in the table were used to calculate the RSM regression equations and coefficients. The values obtained from the RSM analysis were then used to plot the contour map figure 7.

11.

**Table 7.4** Summary of data assembled to construct the second set of contour graphs to determine the optimum conditions for astaxanthin (III) synthesis.

Light Intensity ( $\mu\text{mol/m}^2/\text{s}$ )	Salt Concentration (mM)	Astaxanthin (mg/l)	$\pm$ S.E. (n=3) (%)
30	10	25.8	3.8
30	50	28.5	0.4
30	90	28.3	0.1
630	10	37.6	0.2
630	50	39.8	0.7
630	90	28.5	0.8
1230	10	41.9	0.1
1230	50	43.6	0.7
1230	90	35.1	0.2

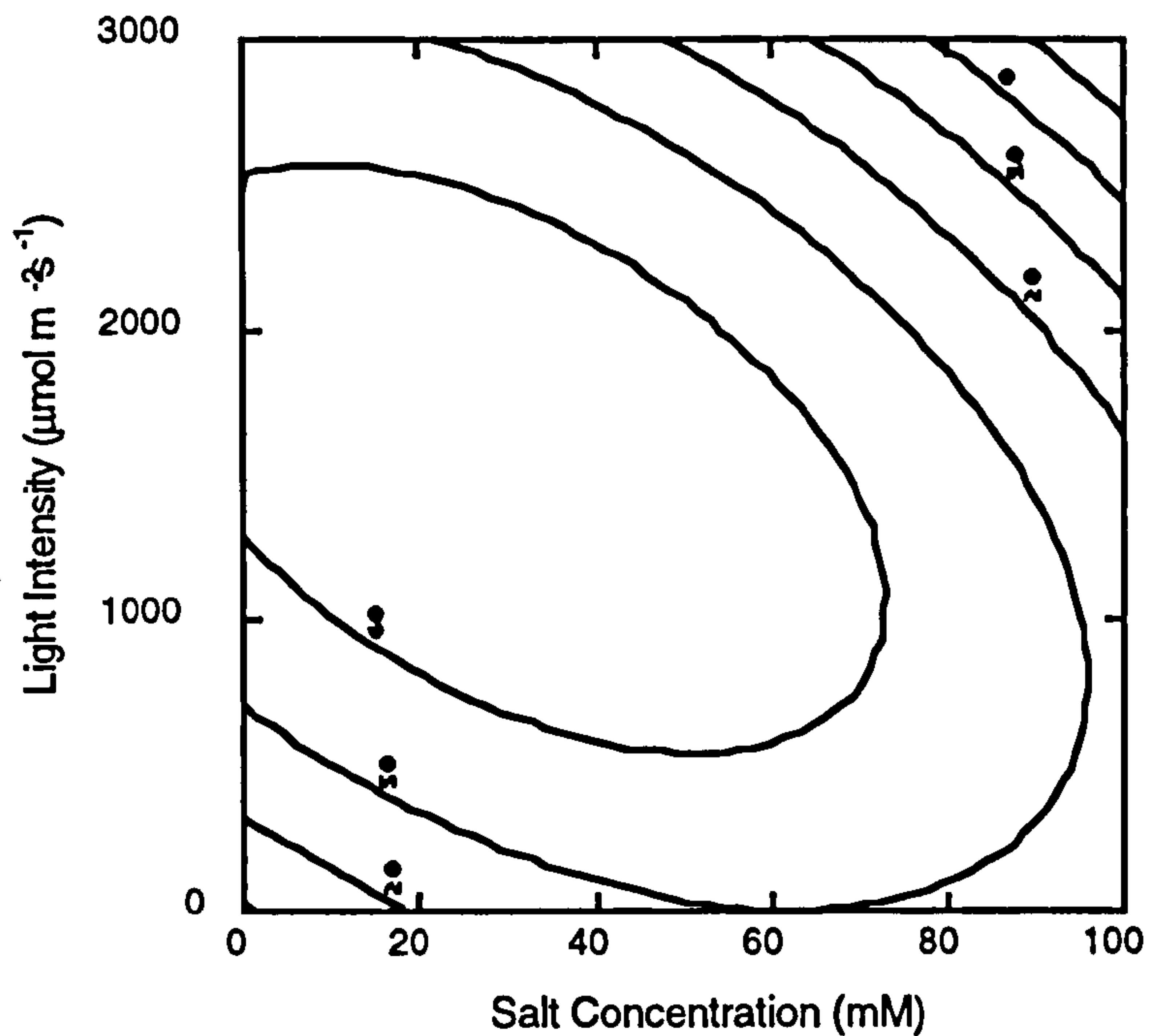


Fig. 7.11 Contour map to determine the optimum light intensity and salt concentration for astaxanthin (III) production from *H. pluvialis*.

Regression equation;

$$\begin{aligned} \text{Predicted astaxanthin (III) content} = & 9.259102 + 0.6659855 * \text{salt} \\ & \text{concentration} + 3.630979\text{E-}02 * \text{light intensity} + -2.214323\text{E-}04 * \\ & \text{salt concentration} * \text{light intensity} + -5.347514\text{E-}03 * \text{salt} \\ & \text{concentration}^2 + -9.530493\text{E-}06 * \text{light intensity}^2 \end{aligned}$$

Regression coefficients;

$$\text{Constant} = 9.259102$$

$$\text{Salt concentration} = 0.6659855$$

$$\text{Light intensity} = 3.630979\text{E-}02$$

$$\text{Coefficient of determination (R}^2\text{)} = 1.147608$$

$$\text{Coefficient of multiple correlation} = 1.071265$$

$$\text{Standard error of estimate (SE)} = 4.340081$$

The contour map (figure 7.11) indicates that the optimum light intensity for algal growth is in the range 1550 - 1650  $\mu\text{mol m}^{-2} \text{s}^{-1}$ , whilst the optimum salt concentration for algal biomass production lies between 25 - 35 mM NaCl. The nutrient status of the cultures was



not studied in the current astaxanthin (III) production optimisation experiments, although nutrient deficient conditions are known to be an important factor for inducing secondary carotenogenesis in the alga. This was due to the difficulty of controlling the nutrient status of the media (i.e. chemically) without significantly altering the chemical environment to which the cells are exposed.

## 7.4 DISCUSSION

There have been few studies of the nutrient and culture condition requirements for *H. pluvialis*, with particular emphasis on those requirements which result in the optimal production of astaxanthin (III). The few previous investigations into the growth requirements for *H. pluvialis* have shown up complex relationships between nutrients, growth, cell yield, cell type and astaxanthin (III) formation.

In the present studies the best nitrogen source for biomass production was identified as urea. This finding is in agreement with a previous report (Borowitzka *et al.*, 1991), who tested four possible nitrogen sources (potassium nitrate, ammonium chloride, ammonium nitrate and, urea) to determine which source resulted in the highest levels of growth in *H. pluvialis*. Their results agreed that urea was the best nitrogen source for *H. pluvialis* cultivation with the nitrate sources also providing a favourable nitrogen source for algal growth. The use of an ammonium salt as a possible nitrogen source in the present study resulted in particularly low levels of algal growth. Previous investigations with *H. pluvialis* have also shown that nitrate nitrogen sources are preferred to ammonium nitrogen sources (Proctor, 1957), although Stross (1963) noted that exponentially growing cells at acid pH preferred ammonium nitrogen sources. *H. pluvialis* has been reported to differ from most other microalgae in preferring nitrate nitrogen sources to ammonium nitrogen sources, at least under laboratory culture conditions (Syrett, 1962). Cultivation of the alga in fast growing, high cell density cultures in ammonium nitrogen sources may lead to high cell mortality rates because of the rapid acidification of the medium due to ammonium uptake and metabolism (Borowitzka and Borowitzka, 1988b).

The key independent variables tested in the biomass and astaxanthin (III) production optimisation experiments, all proved to be important factors in determining the overall levels of cell growth/product yield. Unfortunately, the initial optimisation experiments failed to clearly identify the optimum levels of all the key independent variables tested. A second set of optimisation experiments were therefore carried out. However, the data obtained in the initial optimisation experiments was used in the second set of optimisation experiments, since the initial results indicated the range of parameter values where the optimum could be found. Just as important the initial optimisation experiments also indicated the range of parameter values where the optimum could not be identified. This trial and error approach is a significant component in the optimisation procedure when using RSM.

The biomass optimisation experiments identified the optimum levels of all the key independent variables tested. Table 7.5 shows the optimum levels identified to achieve maximum biomass production in *H. pluvialis*. The temperature required for optimum biomass production was 14 - 15°C. This is in agreement with the findings of a previous study (Borowitzka and Borowitzka, 1988b) who identified 15°C as the optimum growth temperature for *H. pluvialis*. The optimum urea concentration for algal growth was 1.5 - 3.0 mM, while the optimum light intensity for algal growth was 50 - 60  $\mu\text{mol m}^{-2} \text{s}^{-1}$  (PAR).

Key independent variable	Optimum level of the key independent variable
Temperature	14 - 15 °C
Light intensity	50 - 60 $\mu\text{mol m}^{-2} \text{s}^{-1}$ (PAR)
Urea concentration	1.5 - 3.0 mM

**Table 7.5** Optimum levels of the three key independent variables for biomass production in *H. pluvialis*.

The astaxanthin (III) production optimisation experiments identified the optimum levels of two of the three key independent variables investigated. Table 7.6 shows the optimum levels identified to achieve maximum astaxanthin (III) production in *H. pluvialis*. The optimum NaCl concentration required for astaxanthin (III) production was 25 - 35



mM. The optimum light intensity identified for astaxanthin (III) production was 1550 - 1650  $\mu\text{mol m}^{-2} \text{s}^{-1}$  (PAR). This value is much greater than any reported value in the literature for the induction of astaxanthin (III) formation in *H. pluvialis*.

Unfortunately, the optimum temperature for astaxanthin (III) production could not be identified due to practical considerations. The light sources used in the experimental design radiated large amounts of heat and this meant that it was impossible to cultivate the cultures below 24°C. It was considered that even had 24°C been used as a minimum point to investigate the optimum temperature, this value would have been greater than the optimal temperature required for astaxanthin (III) production. On a commercial scale because of the temperature problems associated with using high light sources, the best light source for astaxanthin (III) production would appear to be natural sunlight.

Key independent variable	Optimum level of the key independent variable
Temperature	~14 °C
Light intensity	1550 - 1650 $\mu\text{mol m}^{-2} \text{s}^{-1}$ (PAR)
NaCl concentration	25 - 35 mM

**Table 7.6** Optimum levels of the three key independent variables for astaxanthin (III) production in *H. pluvialis*.

Overall the use of RSM as a technique to optimise biomass production and ultimately astaxanthin (III) production in *H. pluvialis* proved to be very effective. The optimisation experiments were designed around a two stage production process using *H. pluvialis* to produce astaxanthin (III). The first stage involved the bulking up of algal biomass, and the second involved induction of astaxanthin (III) synthesis in the alga. These experiments involved carrying out a relatively low number of trials whereas if an empirical approach had been employed to optimise the key independent variables in the optimisation experiments, it would have been necessary to vary each nutrient and physical parameter independently and determine the effect of this on algal growth/product yield. This would have resulted in the need to carry out an inordinately large number of experiments. RSM avoids the need to carry out this number of trials, but is nonetheless a potent technique in optimising algal fermentation processes.

RSM allows the visual representation of response surfaces as a direct means of locating maximum points, and with the availability of suitable computer programs, the mathematical manipulations are reduced to routine procedures. This leaves the way open for RSM to be applied to any algal fermentation process to optimise key parameters and achieve maximum algal growth/product yield. The next stage would be to continue the optimisation process during the scale-up of the fermentation process. This can be achieved using the simplex optimisation method. The simplex method (Spendley *et al.*, 1962) for two parameter optimisation would involve three initial trials which may be represented by the vertices of a triangle in a plot with concentrations of the parameters as axes. Having determined the algal growth/product yield, the trial with the lowest growth/yield is identified and a further triangle, a reflection of the first, is constructed opposite the lowest growth/yield trial. The process is continued until the optimum point is located at which point the 'simplex' circles around it. This enables a fully optimised large scale algal fermentation process to be developed.



## THE PRODUCTION OF ASTAXANTHIN FROM *HAEMATOCOCCUS PLUVIALIS* IN A 30 LITRE PHOTOBIOREACTOR

### 8.1 INTRODUCTION

#### 8.1.1 A historical review of microalgal mass culture technology

Research into microalgae originated in the search during the fifties and sixties for a cheap and abundant protein source for humans, cattle and poultry feed (Fisher and Burlew, 1953; Milner, 1953; Lipstein and Hurwitz, 1980). During the seventies the interest in microalgae centred around the treatment of wastewater (Shelef *et al.*, 1980), either combined with classical activated sludge processes or as a separate tertiary treatment. Since the world energy crisis in 1975, microalgae have been proposed as an alternative source of fuel and as sources of bulk and fine chemicals either directly (Gudin and Thepenier, 1986), or with an intermediate fermentation step (Goldman, 1979; Wagener, 1981). This latter strategy is supported by the fact that microalgae utilise solar energy more efficiently than any other plants (Richmond, 1983).

As a consequence of this frequent change of objective, various process designs have been published in the literature, some of them highly specific, while others are adaptable for a variety of purposes. However, a discrepancy clearly exists between the recognised commercial potential of many microalgal species and their actual industrial development. The major reason for this is the prohibitively high costs of production which reflects current ignorance of the basic physiological aspects of mass production of many microalgal processes (Richmond, 1988).

#### 8.1.2 Current methods for the production of astaxanthin from *Haematococcus*

Commercial cultivation of astaxanthin (III) from *Haematococcus* has so far been limited to the growth of the alga in ponds, containing 30,000 to one million litres of algal culture (Bubrick, 1991). The production ponds have been located both indoors and outdoors, with the former having the advantage of being under a certain degree of environmental

control. This is an important consideration when growing *Haematococcus* since it is a freshwater alga and is susceptible to competition and contamination by other organisms. However, indoor locations are more expensive for production. The outdoor ponds are shallow and are lined with white plastic. Owing to the potential for contamination, cultivation is usually carried out by a batch process. After the pond is cleaned it is filled with fresh water that has usually been treated by chlorination, UV irradiation or ozone to reduce the number of potential competitors. Nutrients are then added for algal growth. For autotrophic growth, it is necessary to add a nitrogen source, such as ammonia or nitrate and a source of phosphorous. Usually, ammonium bicarbonate, potassium phosphate, ferric chloride and EDTA are added to the pond.

*Haematococcus* may also be cultured heterotrophically. Heterotrophic growth requires an organic carbon source, a nitrogen source, phosphorous and vitamins. Acetate is usually the preferred carbon source. Urea is the preferred nitrogen source, and thiamine is required as a growth factor (Pringsheim, 1966).

In autotrophic culture the pond is seeded with a set-up culture, usually ranging from 0.5 to 5% of the production volume. After inoculation, the pond is slowly mixed with a paddlewheel at one end of the pond. The pond is gassed with CO<sub>2</sub> to provide carbon for autotrophic growth and to control the pH at 7.3. Best growth is obtained under relatively low light intensities. Growth is complete in about 5 days and the cell density usually reaches 3 - 6 × 10<sup>5</sup> cells per ml. Once this stage is reached, encystment is encouraged by nutrient deprivation and by increasing the salinity of the medium. Cells are allowed to encyst for an additional 5 days. The non-motile, thick-walled, red-pigmented encysted cells are then harvested from the pond. Mixing is stopped and the cells are allowed to settle. The sedimented cells are collected and further concentrated by centrifugation. The paste is often heated to 70°C to concentrate the cells and to inactivate any contaminating organisms. Further washing and purification of the cells may also be carried out. The cells are then subjected to downstream processing procedures to produce a commodity suitable for marketing (Spencer, 1989).

This open pond system for the production of astaxanthin (III) from *Haematococcus* appears promising. However, there are a number of commonly encountered problems during the scale-up process. These include the contamination of ponds with protozoal



predators. Contamination with fast growing, unicellular green and/or blue-green algae can also be a rare cause of pond failure. Protozoal predators however, can be the limiting step in pond productivity. When fully established, predators may eliminate 90% of the *Haematococcus* biomass within 72 hours (Spencer, 1989). The control of these predators accounts for a significant proportion of the resources within *Haematococcus* research programmes.

### 8.1.3 Aims

Due to the problems of open pond cultivation of *Haematococcus*, this study has concentrated on developing a large scale production process for *Haematococcus* using closed systems. For this purpose a 30 litre glass column, air lift, photobioreactor was designed and constructed to act as a pilot scale production reactor. This was seen as part of the scale-up process from laboratory to full scale commercial production for the mass culture of *Haematococcus* for astaxanthin (III) production.

The commercial production of astaxanthin (III) from *Haematococcus* requires;

- (i) optimisation of cell production and product yield;
- (ii) development of a reliable production system;
- (iii) a suitable low cost harvesting method.

The optimisation of cell production and product yield was carried out in Chapters 6 and 7 and the results of these investigations were incorporated into the present study. A suitable harvesting system soon became apparent whilst carrying out earlier experiments involving *Haematococcus*. The red aplanospores of *H. pluvialis* are very dense and were observed to readily settle on the base of culture vessels once agitation of the algal culture had ceased. Therefore, it was concluded that a simple cell sedimentation technique would be an effective and cheap harvesting method.

The primary objective of the current investigation was to develop a reliable production system for *Haematococcus*, that could also cope with and/or eliminate the effects of potential predators and competitors. A two stage production system was adopted for the production of astaxanthin (III) from *Haematococcus* designed to allow for biomass accumulation followed by an astaxanthin (III) accumulation stage. The two stages involved

manipulating the culture conditions in the photobioreactor to achieve maximum algal growth followed by maximum rates of astaxanthin (III) production.

## 8.2 MATERIALS AND METHODS

*H. pluvialis* 34/7 CCAP was cultured in 250 ml Erlenmeyer conical flasks under axenic conditions, each flask contained 50 ml of algal culture (for culture conditions see section 2.7.1). These cultures were incubated until they had reached cell densities of  $2 \times 10^5 - 3 \times 10^5$  per ml. Five of these flasks were then used to aseptically inoculate a 5 litre glass aspirator (for culture conditions see section 2.7.3). When the algal suspension in the aspirator had reached  $2 - 3 \times 10^5$  cells per ml, the 5 litre culture was used to inoculate the 30 l photobioreactor (Fig. 8.1).

In the closed photobioreactor the alga was grown under semi-axenic conditions. Prior to inoculation of the algae in the photobioreactor, the reactor was sterilised by means of a 0.2% sodium hypochlorite solution. The photobioreactor was cylindrical in shape and made of glass (Fig. 8.1). The photobioreactor was equipped with an external illumination system (Fig. 8.1), which supplied  $50 \mu\text{mol m}^{-2} \text{s}^{-1}$  (PAR) to the outside of the culture vessel. The photobioreactor was surrounded by a plastic insulating sheet to try and maintain a relatively constant temperature within the environment surrounding the reactor.

The alga was grown in BBM modified to pH 7.0, with 1.5 mM urea acting as the nitrogen source. Stirring and aeration were achieved by means of a sparger unit located in the base of the photobioreactor (Fig. 8.1). Sterile air was passed via the air inlet tube (Fig. 8.1) into the sparger, from which the air penetrated into the culture medium through capillary holes in the sparger. The turbulence caused by air passing up through the culture vessel was enough to agitate the cells sufficiently to keep the cells in constant suspension in the culture medium. The aeration was also sufficient to supply the cells with a continuous source of  $\text{CO}_2$ . The air was pumped through the sparger at a rate of 1.5 - 3.0 litres per minute, depending upon the age of the culture.

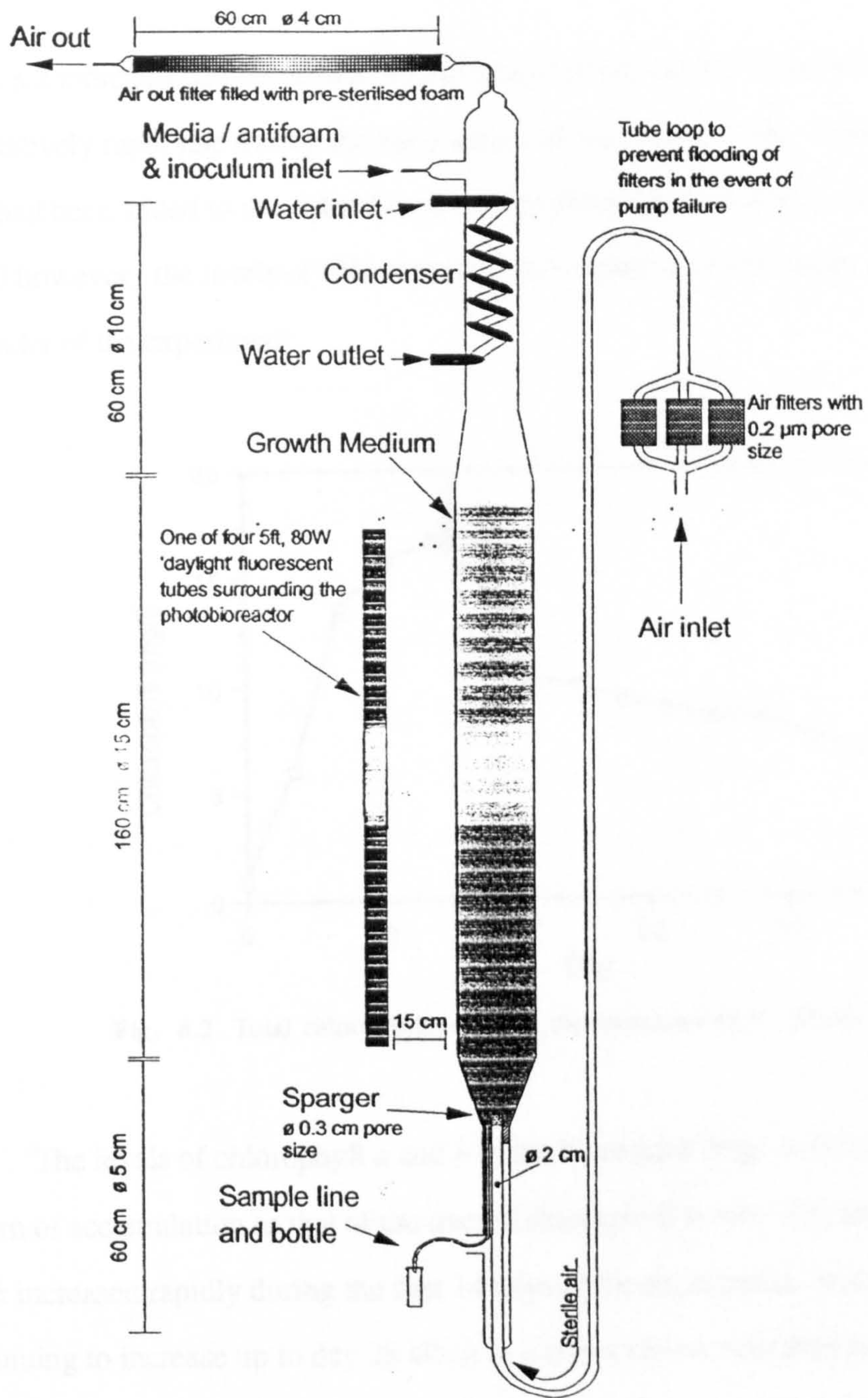
Antifoam (Basildon Chemicals Ltd.) was added to the culture at a rate of 0.2 ml per litre of algal culture when required. When the alga had reached the stationary phase of



growth NaCl was added to the culture medium. NaCl was added on days 30, 35 and 40 to make a final NaCl concentration of 40 mM in the reactor (indicated by 3 arrows in figures 8.2 - 8.13). The NaCl was added in three stages over a period of ten days since the results of experiments carried out in section 6.3.7 had indicated that this method would induce the culture to produce slightly more astaxanthin (III) than if the salt was added all at once. The design of the photobioreactor also meant that the NaCl could be added to the culture aseptically, thereby eliminating the risk of contaminating the culture whilst adding the NaCl.

When the astaxanthin (III) levels of the cells in the reactor had reached the stationary phase of accumulation, aeration of the culture vessel was stopped. The alga was allowed to settle on the base of the reactor and then harvested as a thick algal suspension by allowing the concentrated alga to flow out of the reactor via the sample line (Fig. 8.1). Once the concentrated algal suspension had been removed from the reactor, the algal-free medium remaining was allowed to flow out of the reactor via the sample line and was discarded. The reactor was then washed and sterilised ready for the next batch culture of *H. pluvialis*. The thick algal suspension was frozen and kept for subsequent analysis.

Samples of the algal suspension were taken every 7 days via the sample line (Fig. 8.1). Initially 200 ml of the culture was removed from the reactor and discarded, since it contained algal cells which had been lodged in the sample line and had not been exposed to the culture conditions in the reactor. A second sample containing 200 ml of algal culture was taken and used for pigment and growth analysis. For practical details of these analyses see sections 2.2.1, 2.3.5, 2.5.1, 2.8.2, 2.8.3 and 2.8.4).



**Fig. 8.1** Diagram of the 30 l photobioreactor used for the production of astaxanthin (III) from *H. pluvialis*.



### 8.3 RESULTS

Figure 8.2 indicates that the levels of chlorophyll of the culture in the bioreactor increased at a relatively rapid rate during the early stages of the reactor cycle. However, once the NaCl had been added to the culture medium the chlorophyll levels decreased sharply. After day 40 however, the levels of chlorophyll in the culture decreased only slightly during the remainder of the experiment.

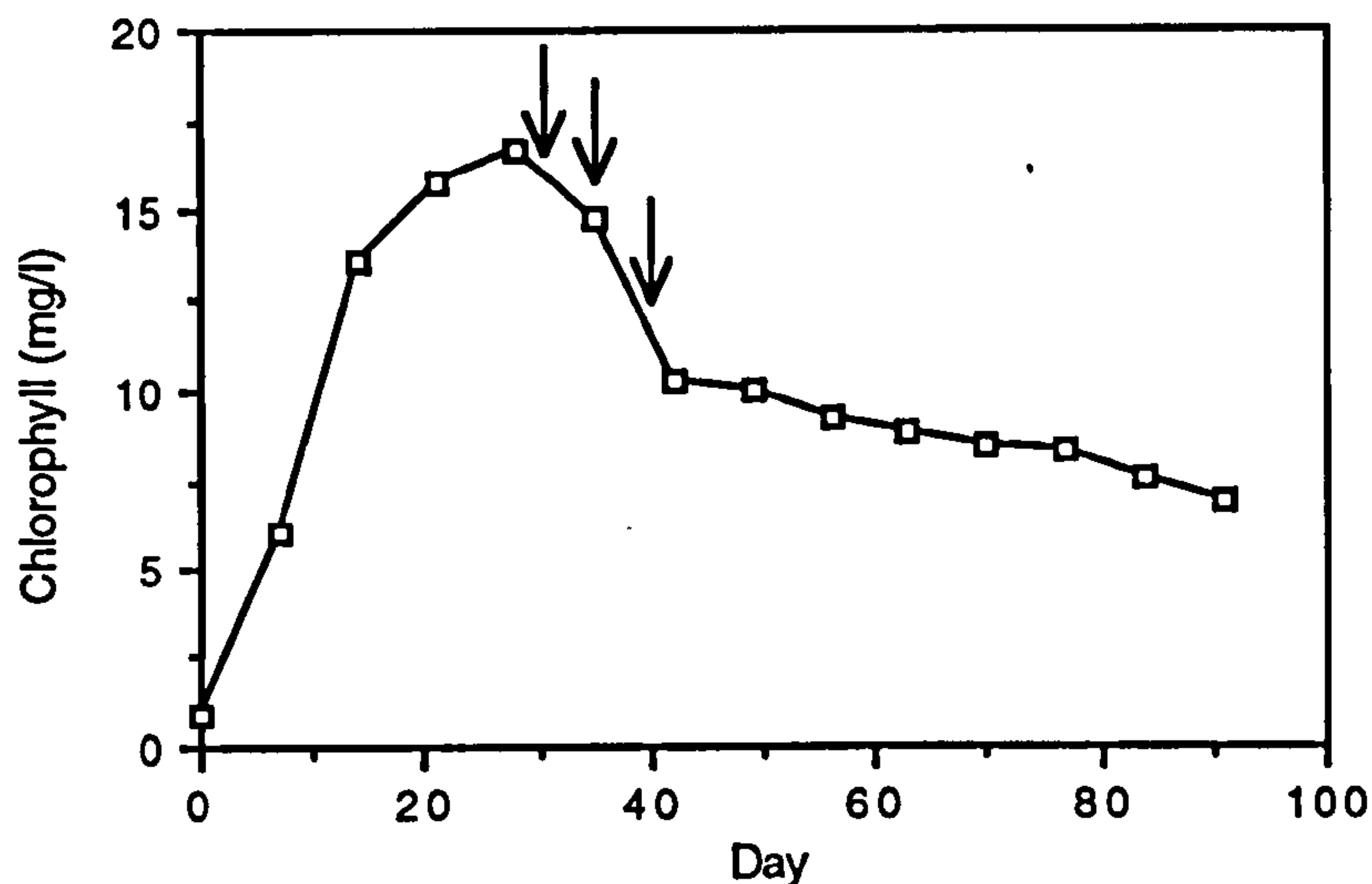


Fig. 8.2 Total chlorophyll levels in the bioreactor (S.E.  $\pm 3.6\%$ ,  $n=3$ ).

The levels of chlorophyll *a* and *b* in the bioreactor (Fig. 8.3) followed a similar pattern of accumulation as that of the overall chlorophyll levels. The levels of chlorophyll *a* and *b* increased rapidly during the first 14 days of the experiment, with the levels continuing to increase up to day 28 albeit at a rather slower rate than previously. However, with the addition of NaCl to the culture medium the levels of chlorophyll *a* and *b* decreased rapidly particularly between days 28 and 42. After day 42 the levels of chlorophyll *a* and *b* declined at a relatively low rate for the remainder of the experiment. Throughout the experiment the ratio of chlorophyll *a*:*b* remained relatively constant at 3:2.

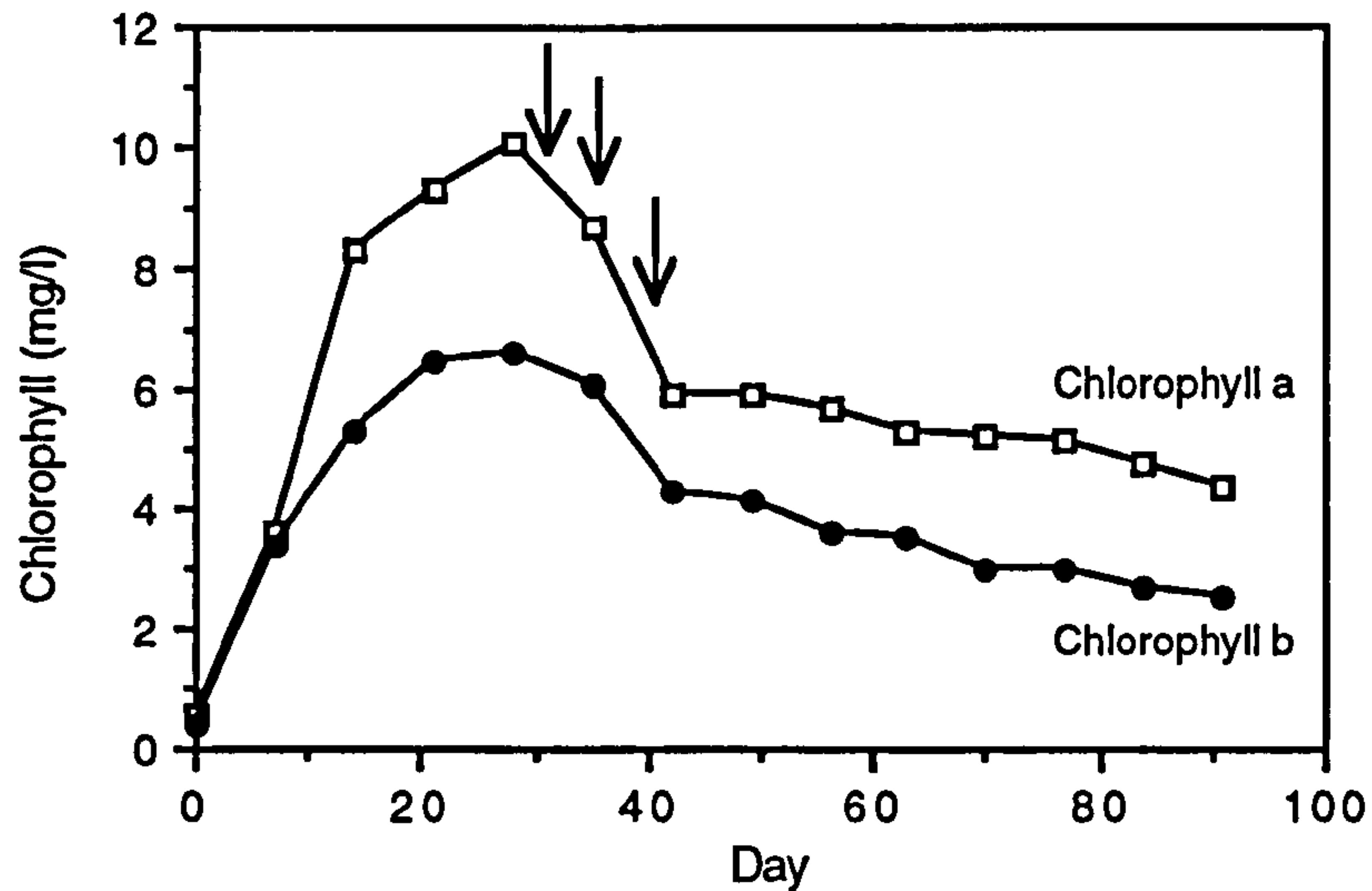


Fig. 8.3 Chlorophyll *a* and *b* levels in the bioreactor (S.E.  $\pm 2.8\%$ ,  $n=3$ ).

Figure 8.4 indicates that the total levels of carotenoid of the algal culture in the bioreactor increased throughout the course of the reactor run. The rate of increase was initially relatively slow, but after the NaCl had been added to the culture medium the rate increased considerably. However, during the latter stages of the experiment the rate of increase of the carotenoid levels in the culture receded.

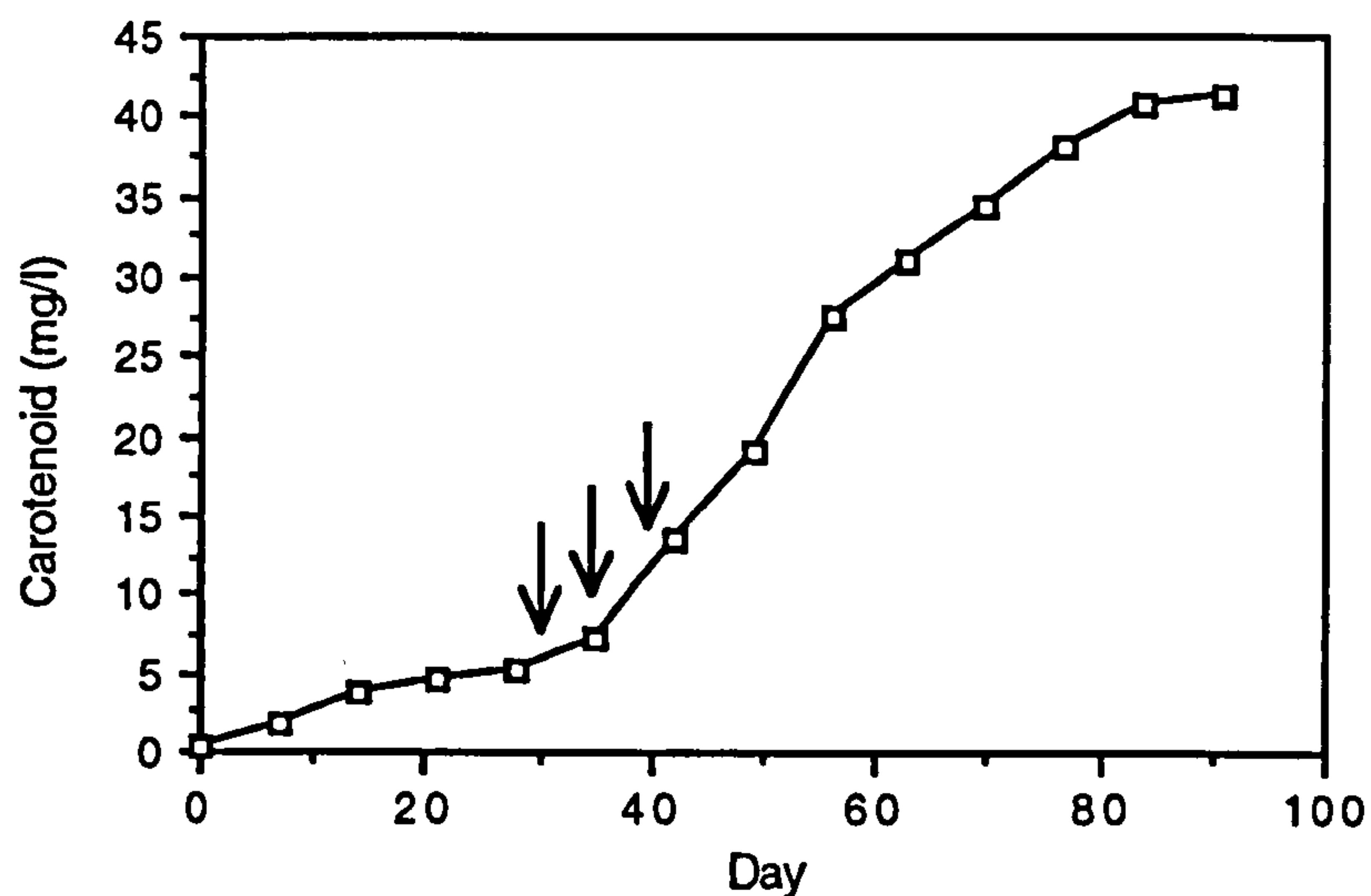


Fig. 8.4 Carotenoid concentration of the cells in the bioreactor (S.E.  $\pm 4.7$ ,  $n=3$ ).

The levels of primary carotenoids in the bioreactor (Fig. 8.5) varied considerably between the individual carotenoids present. The levels of lutein (XVI) increased considerably in the early stages up to day 28. Once NaCl was added to the culture the



levels of lutein (XVI) declined rapidly. This rate of decline receded towards the end of the experiment. The levels of neoxanthin (XX) and the xanthophyll cycle pigments (violaxanthin (XVII), antheraxanthin (XVIII), and zeaxanthin (XV) (VAZ)) were very similar throughout the experiment. The levels increased slowly up to day 28 after which the levels declined. For the second half of the experiment the levels of neoxanthin (XX) and VAZ remained relatively constant. The levels of  $\beta$ -carotene (I) increased at varying rates up to day 70, after which the level of  $\beta$ -carotene (I) dropped.

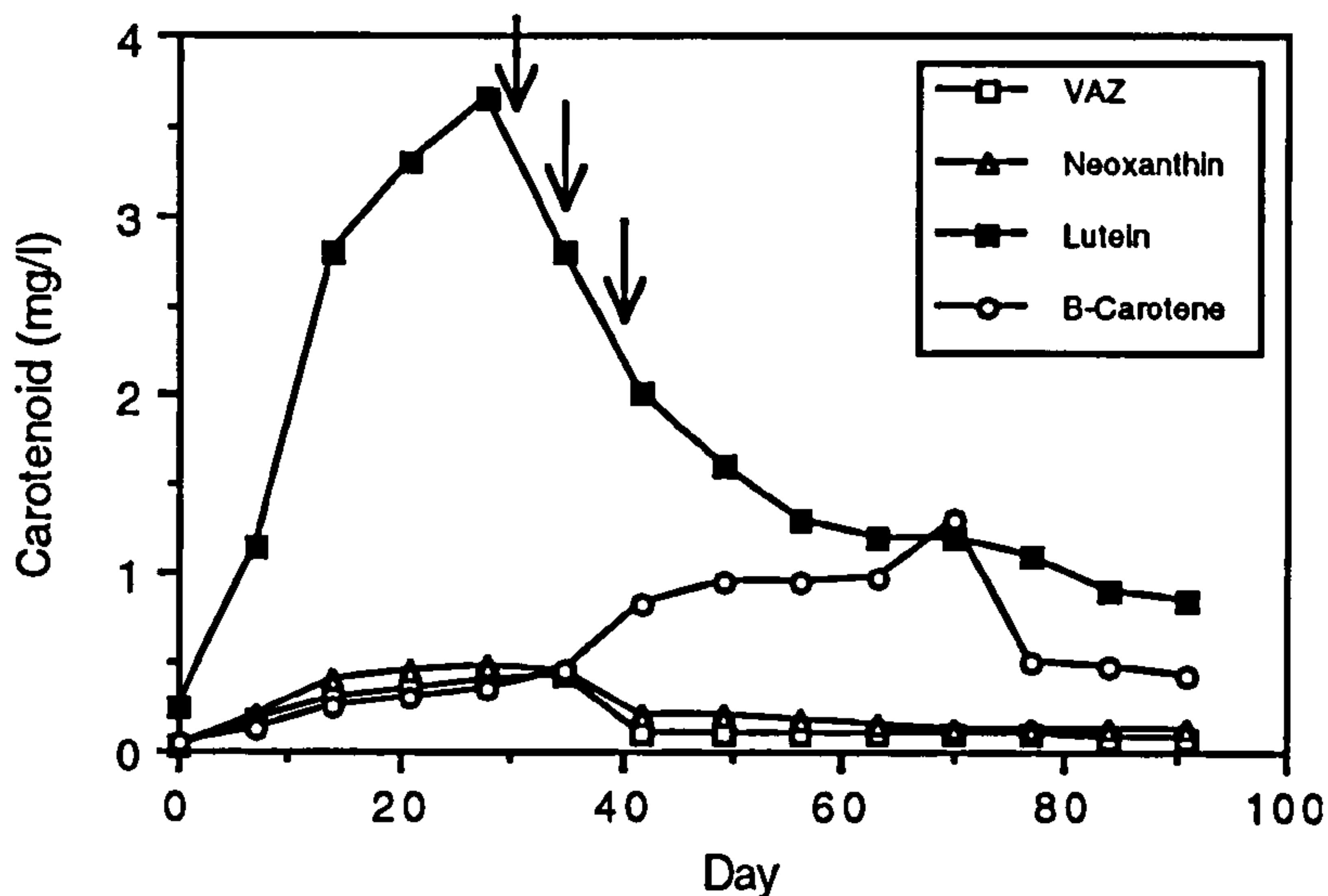


Fig. 8.5 Primary carotenoid levels of the cells in the bioreactor (VAZ=sum of violaxanthin (XVII), antheraxanthin (XVIII) and zeaxanthin (XV)) (S.E.  $\pm 4.6$ ,  $n=3$ ).

The levels of secondary carotenoids in the bioreactor (Fig. 8.6) did not show appreciable increases until after day 28 with the addition of NaCl. Before NaCl was added to the culture medium the levels of secondary carotenoids in the culture was very low. However, once NaCl had been added to the culture medium there was an exponential increase in the levels of secondary carotenoids present within the algal culture. Towards the end of the experiment the accumulation of secondary carotenoids began to reach a stationary phase.

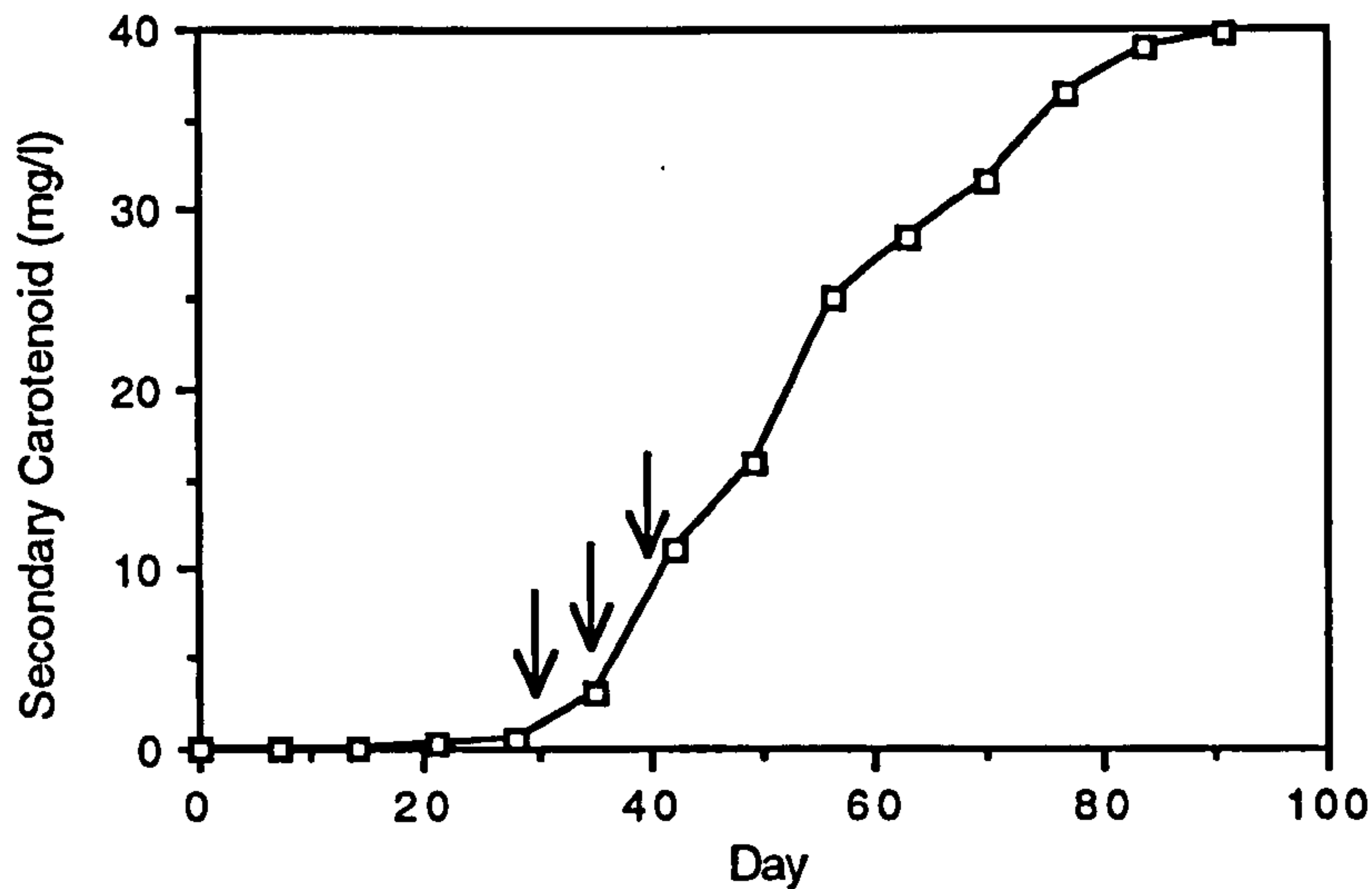


Fig. 8.6 Secondary carotenoid concentration of the cells in the bioreactor (S.E.  $\pm 1.7$ ,  $n=3$ ).

The composition of secondary carotenoids in the bioreactor (Fig. 8.7) indicated that the levels of canthaxanthin (IV) remained relatively low throughout the experiment and there was no appreciable accumulation of canthaxanthin (IV) within the cells. The levels of astaxanthin (III) monoesters increased at a relatively constant rate throughout the experiment after the addition of NaCl. The accumulation of astaxanthin (III) diesters after the addition of NaCl was much more rapid than that observed for the astaxanthin (III) monoesters. The levels of astaxanthin (III) diesters increased at a relatively rapid rate until the latter stages of the experiment, when their levels reached a stationary phase of accumulation.

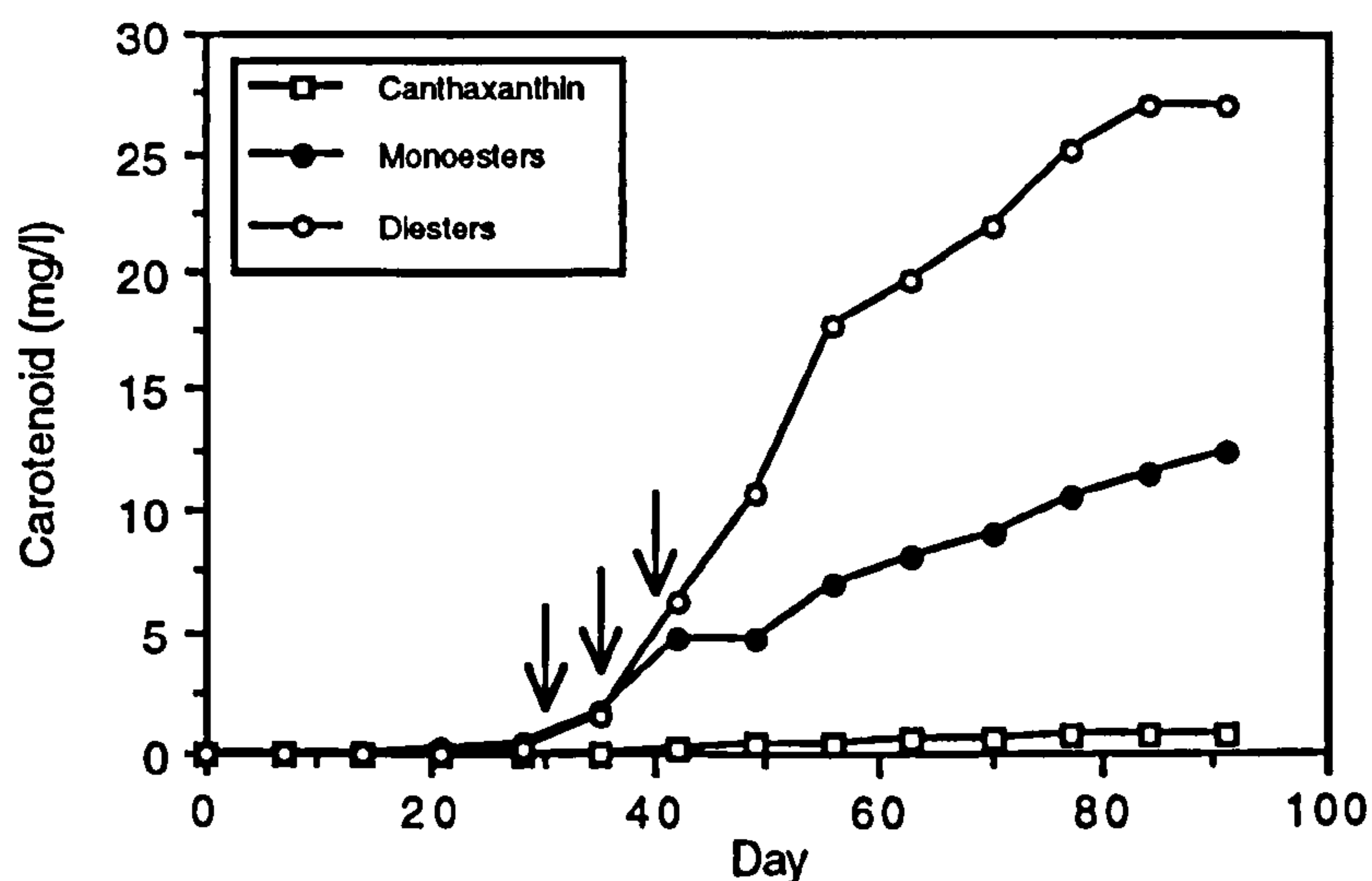


Fig. 8.7 Composition of secondary carotenoids of the cells in the bioreactor (S.E.  $\pm 3.4$ ,  $n=3$ ).



The number of cells in the algal culture in the bioreactor (Fig. 8.8) increased rapidly during the early stages of the experiment and then reached a stationary phase, which lasted for approximately 14 days until NaCl was added to the culture medium. The addition of NaCl immediately resulted in a sudden decrease in the number of cells in the algal culture in the bioreactor. However, following this initial response to the addition of NaCl cell number receded at only a relatively slow rate.

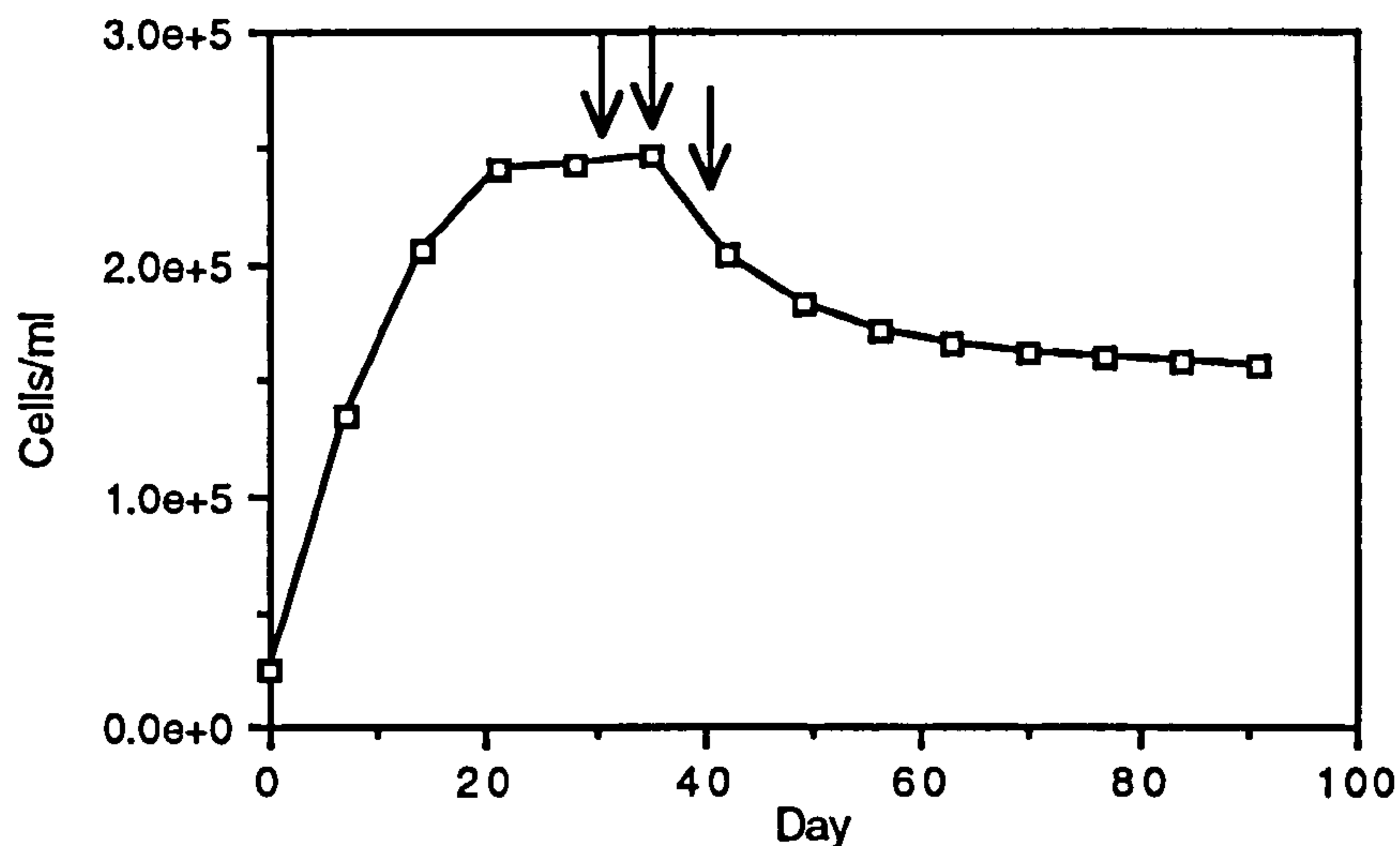


Fig. 8.8 Number of cells in the bioreactor (S.E.  $\pm 4.9\%$ ,  $n=3$ ).

The packed cell volume of the algal culture in the bioreactor (Fig. 8.9) increased rapidly during the first 14 days of the experiment. The level then increased only slightly until day 28 when again the level rose quite markedly until day 35. After this point the packed cell volume of the cells increased slightly up to day 70, after which the level declined at a relatively slow rate for the remainder of the experiment. The two major increases in packed cell volume of the culture corresponded with the initial exponential phase of cell growth of the green 'swimmer' and palmella cells, and the formation of the aplanospores after the addition of NaCl, respectively.

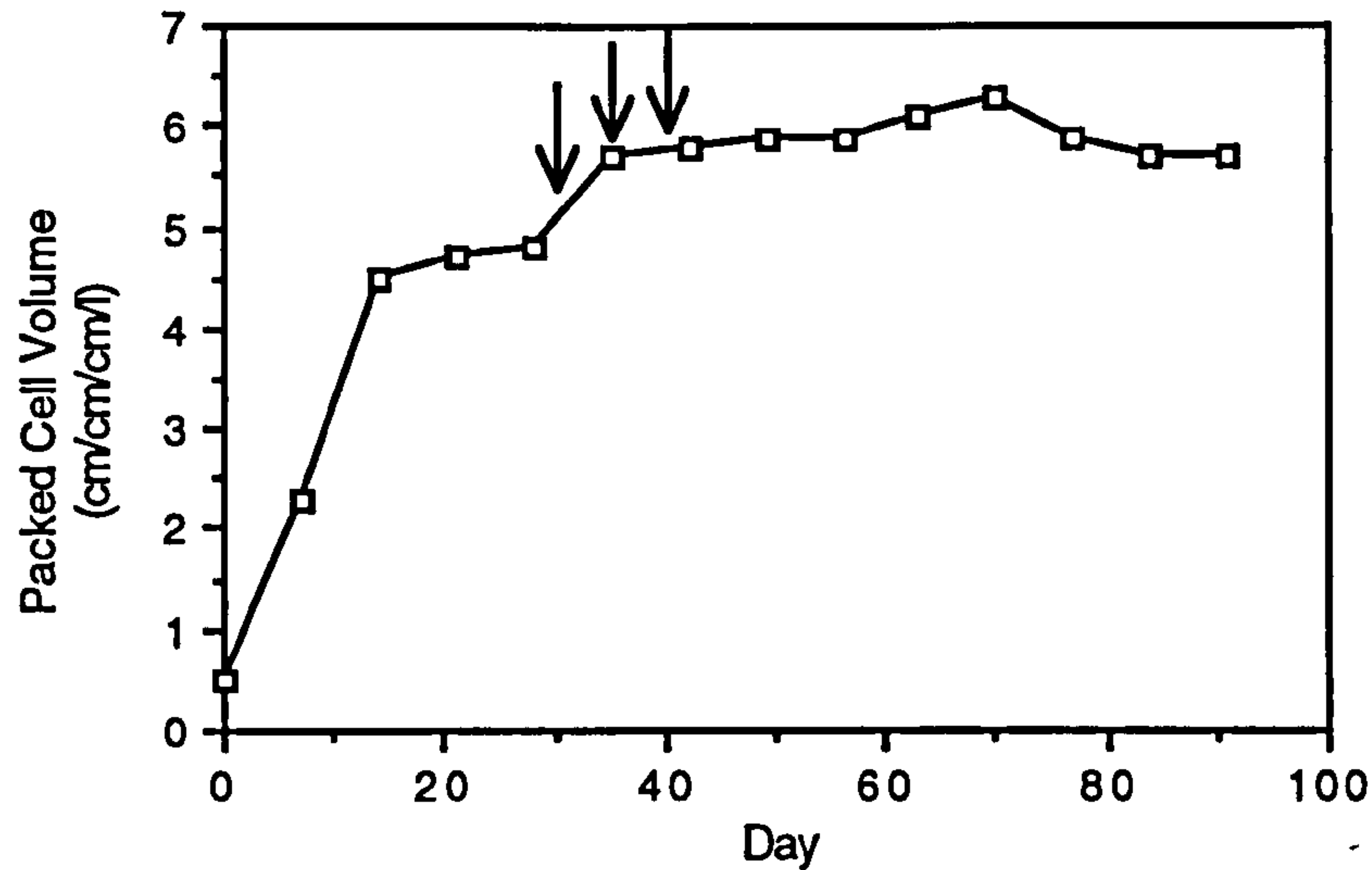


Fig. 8.9 Packed cell volume of the culture in the bioreactor (S.E.  $\pm 4.8\%$ ,  $n=3$ ).

Figure 8.10 indicates that the dry cell weight of the culture in the reactor increased throughout the reactor run. The rate of increase was relatively constant during the first half of the experiment, with the dry cell weight of the culture increasing at a relatively fast rate. After the mid-way point of the experiment the rate of increase declined slightly. During the final 14 days of the experiment very little increase in the dry cell weight of the culture was observed.

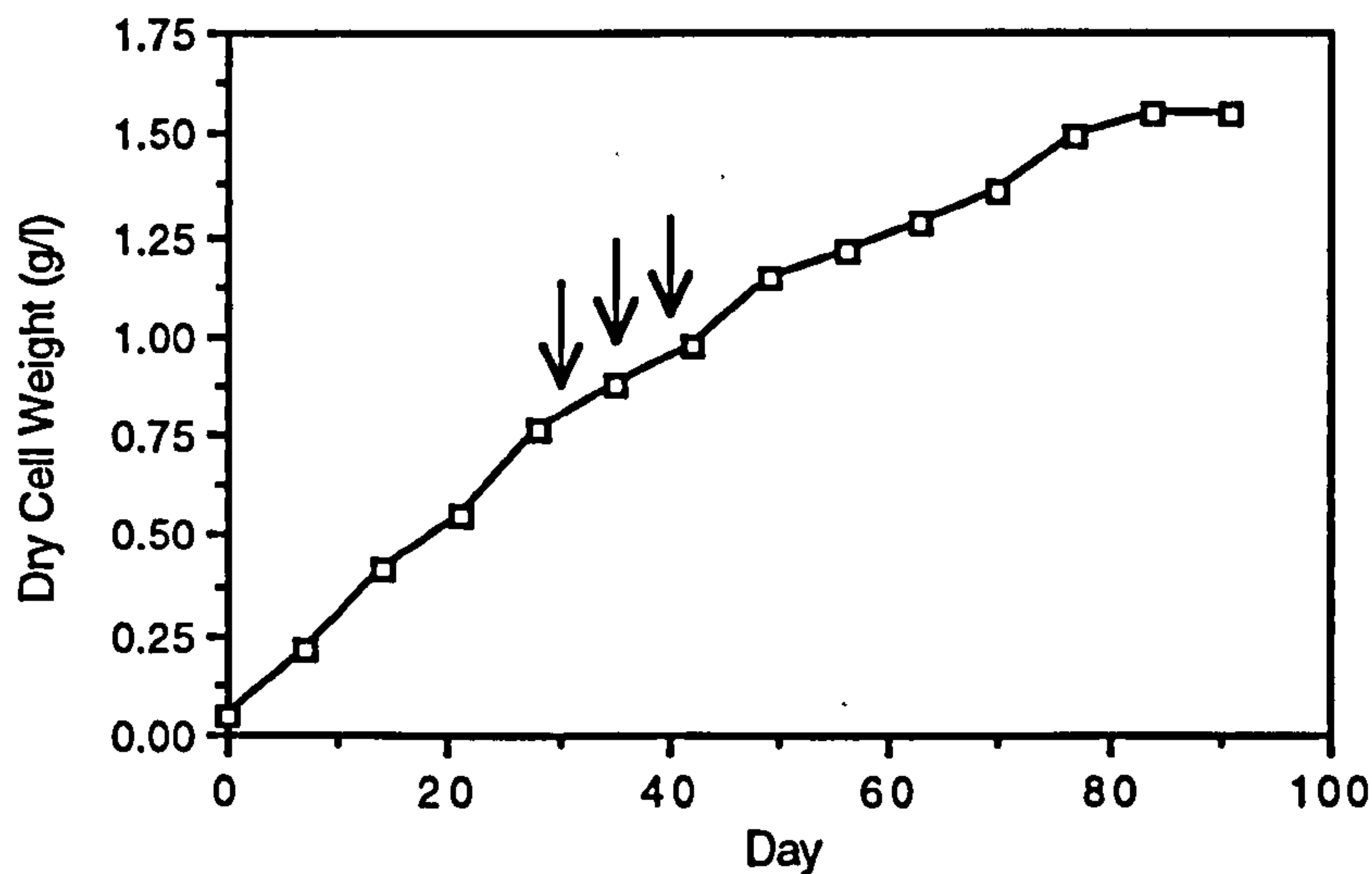


Fig. 8.10 Dry cell weight of the algal suspension in the bioreactor (S.E.  $\pm 3.3\%$ ,  $n=3$ ).

The secondary carotenoid content of the *H. pluvialis* cells in the bioreactor (Fig. 8.11) shows that there was no observable accumulation of secondary carotenoids in the



cells until NaCl had been added to the culture medium. Once the NaCl had been added to the culture medium the levels of secondary carotenoids in the cells rose at an exponential rate until day 81. Although the levels continued to increase after day 81, the rate of increase receded considerably during the final phase of the experiment.

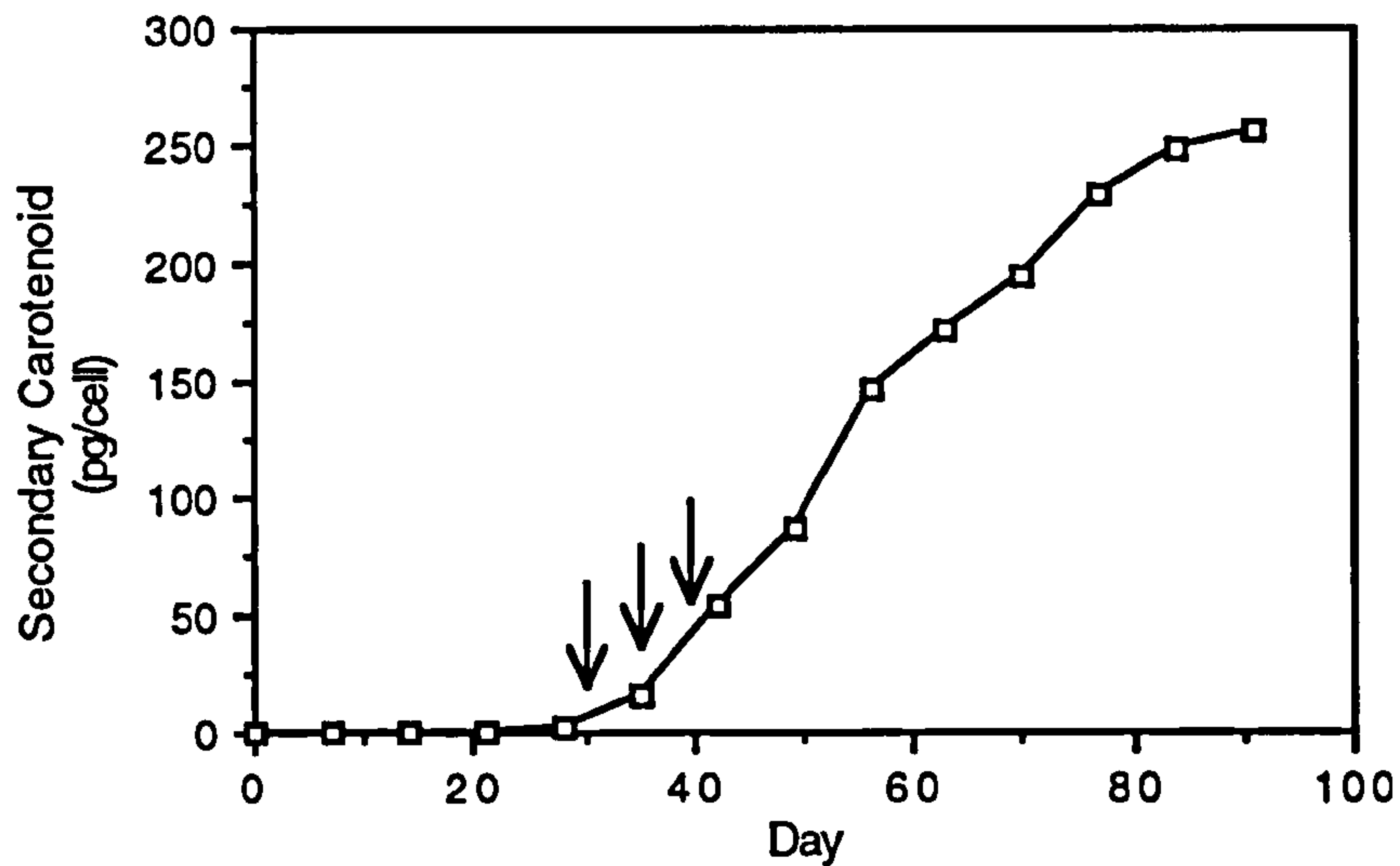


Fig. 8.11 Secondary carotenoid content of the *H. pluvialis* cells in the bioreactor (S.E.  $\pm 3.1$ ,  $n=3$ ).

Figure 8.12 indicates the secondary carotenoid content of the culture as a percentage of the dry cell weight of the culture. The percentage was initially very low and only increased slightly during the early stages of the experiment. However, after day 28 and the addition of NaCl the level began to rise exponentially after the initial lag phase. This high rate began to decline after day 56, but the level continued to rise albeit at a reduced rate until the end of the experiment.

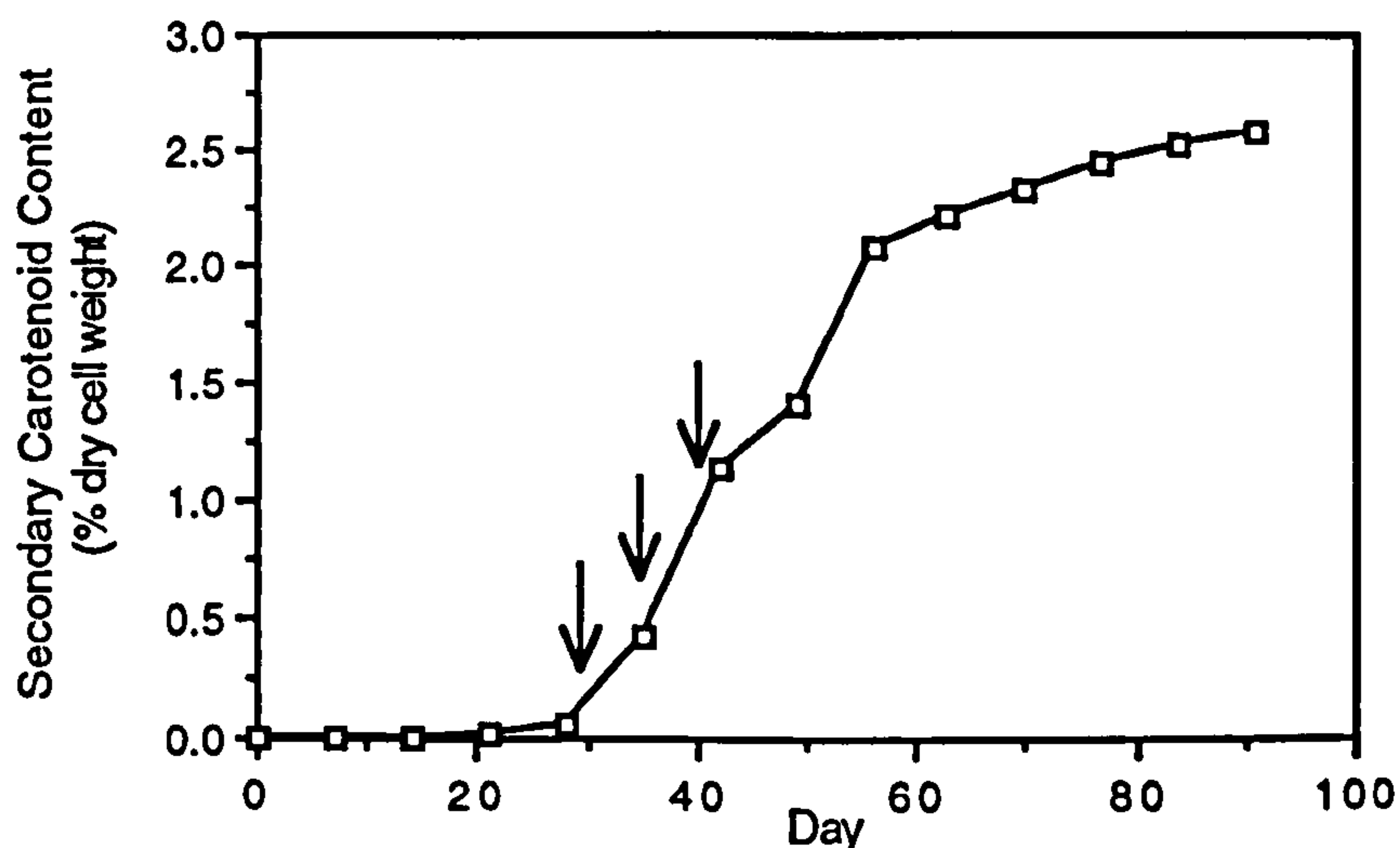


Fig. 8.12 Secondary carotenoid content of the culture as a % of the dry cell weight of the cells in the bioreactor (S.E.  $\pm 3.3\%$ ,  $n=3$ ).

The pH of the culture medium in the bioreactor (Fig. 8.13) increased relatively rapidly during the early stages of the experiment to a maximum of 8.2. However, the pH quickly declined with the first addition of NaCl to the culture media on day 30. The pH of the culture media continued to decrease after the addition of NaCl until day 70, when a slight increase in the culture pH was observed during the final stages of the experiment.

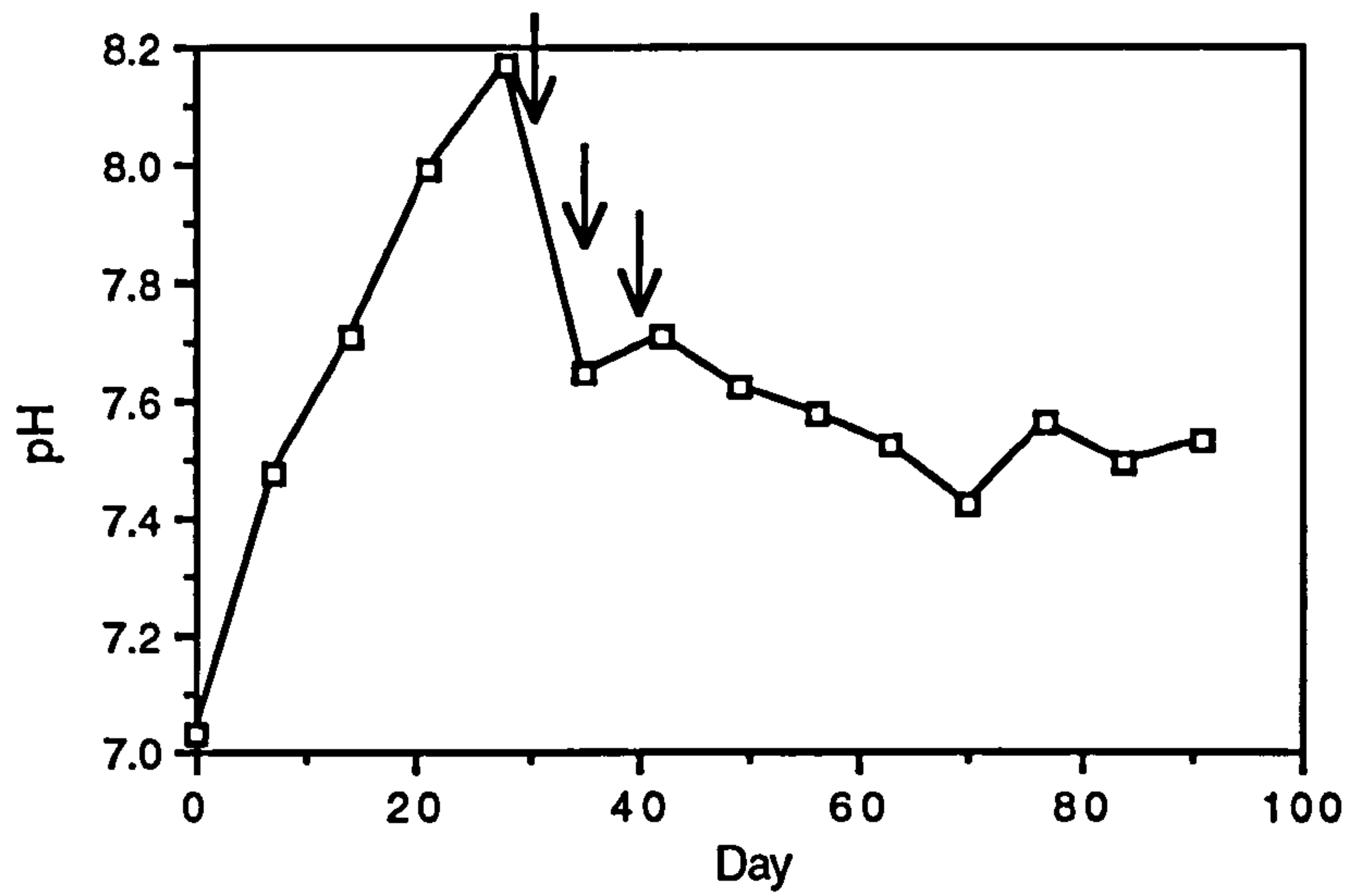
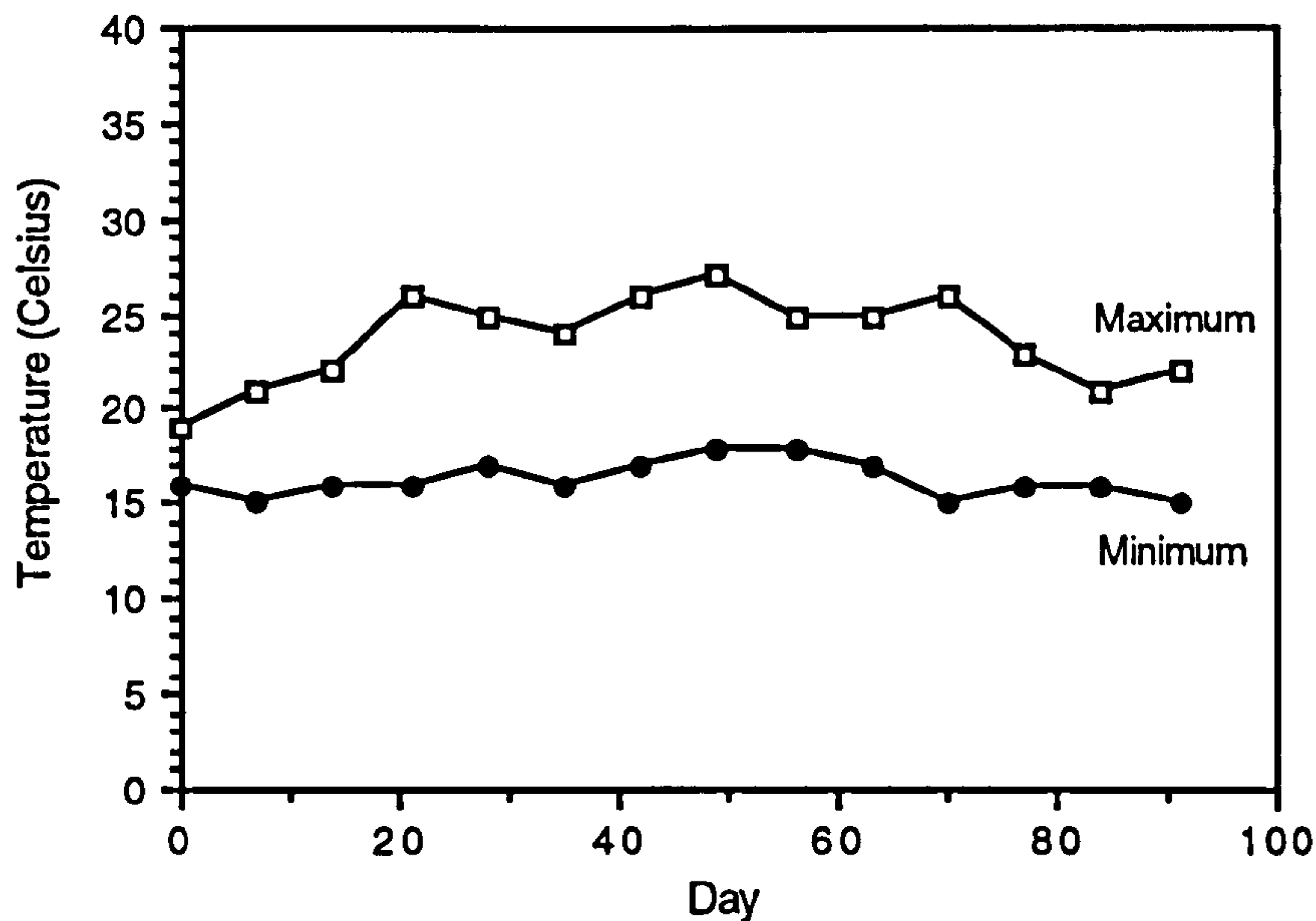


Fig. 8.13 pH of the culture media in the bioreactor (S.E.  $\pm 2.3\%$ ,  $n=3$ ).

The minimum temperature of the environment surrounding the bioreactor (Fig. 8.14) only fluctuated by  $4^{\circ}\text{C}$  throughout the experiment, declining to a minimum of  $14^{\circ}\text{C}$ . The maximum temperature of the surrounding environment of the bioreactor fluctuated by only  $6^{\circ}\text{C}$ , with the maximum temperature not exceeding  $27^{\circ}\text{C}$ . Overall, there were no major temperature fluctuations in the environment surrounding the bioreactor, and the mean temperature was approximately  $19 - 20^{\circ}\text{C}$  during the reactor run.





**Fig. 8.14** Maximum and minimum temperatures of the surrounding environment of the bioreactor.

#### 8.4 DISCUSSION

The cell number and the dry cell weight of the cells in the bioreactor compared favourably with the yields obtained when the cells were grown in shake flask culture (see sections 3.3.2 and 7.3.1). This is also true of the levels of secondary carotenoids accumulated within the culture as a whole (see section 3.3.2). The pattern of secondary carotenoid accumulation was again similar to that observed when the alga is cultivated in shake flask culture (see section 3.3.2). Esterification of the free form and monoesters of astaxanthin (III) was again observed to increase with the age of the aplanospores. The addition of NaCl to the culture had a significant effect on the growth characteristics of the culture and the rate of synthesis of secondary carotenoids. The addition of NaCl had the effect of causing an increase in the cell mortality rates within the culture, which corresponded to a marked increase in the packed cell volume of the cells in the culture. This would indicate that the cells which did survive showed a significant increase in their volume. The pH of the culture medium significantly decreased with the addition of NaCl to the culture medium.

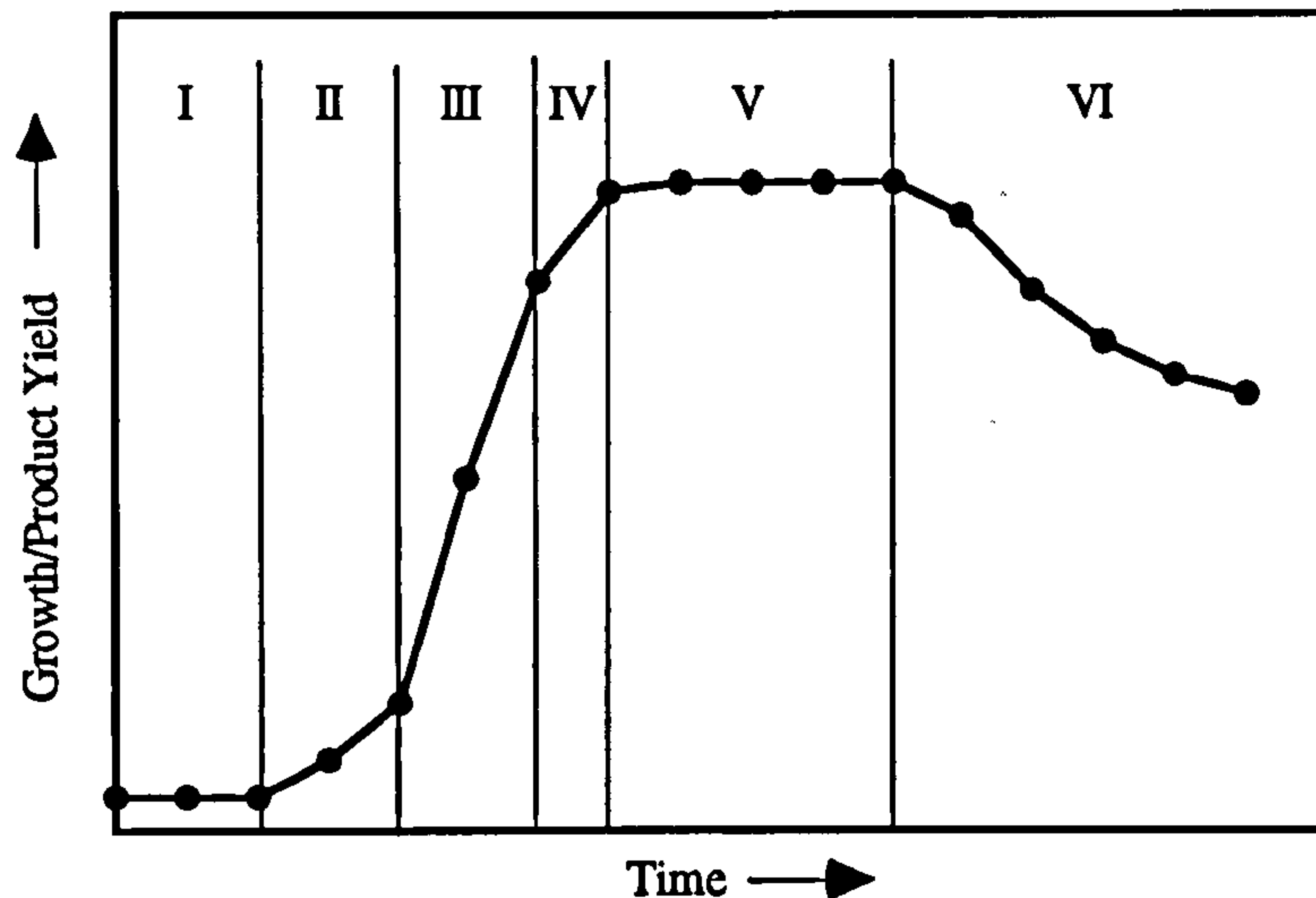
Overall, the cells in the bioreactor behaved in a very similar fashion as observed when the cells are grown in 50 ml shake flask cultures. The one major difference was the

actual rate of cell growth/secondary carotenoid synthesis. Although the final yields of cell biomass/secondary carotenoid accumulation in the bioreactor were very similar to those obtained in shake flask conditions, the actual time taken to achieve these final yields was slightly longer (see section 3.3.2 and 6.6).

Unfortunately, the temperature of the environment surrounding the bioreactor could not be strictly controlled due to practical considerations. However, the temperature did not exceed 27°C or fall below 14°C. This meant that the temperatures the culture was exposed to were not lethal. The temperatures observed during the experiment were within the range to allow for optimum rates of cell growth and astaxanthin (III) production (see section 7.4). This was also true for the pH of the culture medium. During the bioreactor run there were no means of controlling the pH of the culture medium. However, for the majority of the experiment the pH of the medium was within 0.6 pH units of the optimal pH for cell growth (pH 7.0).

The pattern of cell growth and secondary carotenoid accumulation was as expected for an algal batch production system. In a closed, batch culture system the growth rate of the algae and the product yield tends toward zero. This is due to either the accumulation of a product which cannot be tolerated, or because of the lack of a particular nutrient or light. The commercial cultivation of algae in batch cultures means, in practical terms, that the growth of the culture is permitted to proceed until the termination of the exponential growth phase (end of phase III in Fig. 8.15). The biomass is then normally removed and a new batch culture is initiated, or the biomass is harvested only to the concentration found at the onset of accelerating growth (phase II in Fig. 8.15). In cultures which are correctly maintained, decelerating growth (phase IV in Fig 8.12) occurs because the increase in population density causes such intense mutual shading, that net growth declines rapidly due to the lack of sufficient irradiance for the individual cells in culture (Richmond, 1983).





**Fig. 8.15** Classical batch growth curve with six phases: I lag; II accelerating growth; III exponential growth; IV decelerating growth; V stationary; VI decline (after Richmond, 1983).

The fermentation process used in the present study was slightly different to the normal commercial cultivation of algae in batch culture. This was because the algal biomass was not considered as the final product for this particular system. The fact that a secondary metabolite was the product meant that harvesting did not take place with the termination of the exponential growth phase. An inhibition in cell growth is considered a prerequisite in this fermentation process for the production of the secondary metabolite (astaxanthin (III)). Therefore, harvesting of the reactor did not occur with the onset of the stationary growth phase. However, in the present system the concepts of commercial algal cultivation in batch culture still seem to hold true. The classical batch growth curve (Fig. 8.15) can be applied to (at least in part) most of the algal growth/secondary carotenoid production graphs (Figs. 8.2 - 8.12). It would be expected that given a longer period of time most of the algal growth/ secondary carotenoid graphs (Figs. 8.2 - 8.12) would have exhibited the six phases observed in the classical batch growth curve (Fig. 8.15).

Since the graphs (Figs. 8.2 - 8.12) contain the features of the classical batch growth curve (Fig. 8.15) it can be concluded that one or more components of the system is a limiting factor in cell growth/secondary carotenoid production during the fermentation process. This is an expected feature of the algal growth curve in the present study. The algae soon exhaust the nutrients in the medium which results in a reduction and finally cessation of the growth of the algae. During this period the density of the culture also

greatly increases restricting the amount of light each cell receives. The bioreactor constructed for the present study had a low surface area to volume ratio. This is an important implication when considering the importance of light for algal growth and secondary carotenoid production. Rather than considering the total solar energy impinging on the culture surface, the most important factor is the quantity of energy available at the cellular level. These are concepts of 'light per cell' and 'light regime' expressed by Richmond (1986), which describe the duration of each exposure of the average cell in the photic zone, below the compensation point, or in darkness. Soeder (1980) was among the first to elaborate the idea of 'areal density', thus taking into account the mutual shading of cells.

To overcome the phenomena of light limitation and to achieve the best light regime, high levels of mixing are required to reach a turbulent flow of the culture (Gudin and Chaumont, 1991). In microalgal mass culture, productivity is affected by the mixing system. When light is considered as a limiting factor of growth, the effective self shading of a cell has a significant effect on the growth rate/secondary carotenoid production. This means that an efficient turbulent flow (high liquid velocity) must be maintained within the culture, so each cell is able to receive enough light in order to improve the photosynthetic efficiency of the culture. The mixing system employed in the present study had to take into account the phase of the life cycle the alga was currently at (see section 4.3.1). The culture of green vegetative cells in the exponential phase of growth required lower liquid velocity because of their fragility and the relatively low cell concentration. The aplanospores are more robust than the green vegetative cells due to their thick cell wall and their culture in the stationary phase required a higher liquid velocity to prevent cell sinking, and to ensure the best light regime for the cells for the process of secondary carotenogenesis. This was even more important since the light intensity during the stationary growth phase and the inducement of secondary carotenogenesis could not be increased because of practical difficulties.

Contamination of the bioreactor by microorganisms i.e. protozoal predators, bacteria and fungi was not a major problem in the present study. Although, initially the conditions in the bioreactor were axenic, because of the location of the bioreactor and the length of time required for the fermentation process some minor contamination of the

bioreactor did occur. Small numbers of protozoal predators were observed but their numbers were relatively low and did not cause a significant loss of algal biomass. Some bacteria and fungi were also present in the bioreactor but were not able to grow abundantly due to the culture conditions in the bioreactor. It would be fair to assume that by optimising the conditions for cell growth in the bioreactor the *Haematococcus* cells are able to grow relatively rapidly. This coupled with the ambient culture conditions in the bioreactor restricted the growth of contaminants and therefore did not pose a major problem in the production system.

The investigation allowed a preliminary evaluation of the potential of the bioreactor for the mass cultivation of *H. pluvialis* for secondary carotenoid production. The main advantages and disadvantages of the fermentation process are summarised in table 8.1. The investigation demonstrated the technical feasibility of the mass cultivation of *H. pluvialis* for secondary carotenoid production in the bioreactor. Problems and limitations of the system have been highlighted, thereby identifying improvements that can be made to the system to improve cell growth/secondary carotenoid production in the future.

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*Advantages:*

- [1] Effective mixing and oxygen removal achieved by air bubbling, which minimises the shear force to which the cells are exposed;
  - [2] Good yields of algal biomass and secondary carotenoids
  - [3] Contamination by other algae, bacteria and fungi not a major problem;
  - [4] Once installed-low maintenance and running requirements;
  - [5] Potentially adaptable for the growth of any autotrophic microorganism.
- 
- 

*Disadvantages:*

- [1] Low surface to volume ratio, leading to poor light distribution within the culture;
  - [2] Difficult and costly to construct;
  - [3] No control over some important culture parameters (pH, temperature etc.), although such control systems could be added at a later date;
  - [4] Time for each batch culture from inoculation to harvest relatively long.
- 
- 

**Table 8.1** Main advantages and disadvantages of the photobioreactor constructed for the pilot scale production of secondary carotenoids from *H. pluvialis*.



Commercially the results of the investigation pose the question of when is the best time to harvest such a fermentation process. In the current investigation the bioreactor was operated for fundamental research purposes only. However, when operating such a bioreactor for commercial purposes the shorter the time required for the fermentation process, the more cost effective such an operation will be. Certain authors have suggested that it is commercially viable to harvest *Haematococcus* once the secondary carotenoid content of the culture has reached 1.0 - 1.5% of the dry cell weight of the culture (Spencer, 1989; Bubrick, 1991). In the present study this would have meant harvesting the bioreactor between days 49 and 56. However, the amount of secondary carotenoid in the bioreactor per unit volume of culture on day 49, was only approximately one third of the final amount of secondary carotenoid accumulated in the bioreactor on a per unit volume basis. This discrepancy between the levels of secondary carotenoid per unit volume of culture and as a percentage of the dry weight of the algae requires further investigation. Such an investigation would hopefully determine the most commercially viable time to harvest the bioreactor. The results would need to offset the running costs of the bioreactor against the increased yields of secondary carotenoids observed when the fermentation process is allowed to operate over an extended period of time. In the present investigation a suitable time to harvest the reactor would have been day 56. At this point the percentage of secondary carotenoids of the dry weight of the algae was approximately 2.0%, and the levels of secondary carotenoid per unit volume of culture was approximately 25.0 mg/l. A 30 l bioreactor such as this would therefore yield 750 mg of astaxanthin (III).

Using the final yields obtained in the bioreactor and the price of astaxanthin (III) from *Haematococcus* on the world market at \$US ~4000/kg, the value of the culture grown in the bioreactor on the final day would have been approximately \$US 4.80. If the bioreactor had been harvested on day 56 the final value of the culture in the bioreactor would have been \$US 3.00. The cost effectiveness of determining the best point to harvest such a fermentation process is vital in improving the commercial viability of this kind of operation. The operating costs and capital investments have of course to be considered. However, if this kind of fermentation process was scaled up, the relative costs of the overheads should decrease. If the final yields achieved in the bioreactor could be repeated

on a commercial scale i.e. a 100,000 l photobioreactor, the final value of the algal product at the end of each operation would be \$US 16,000. An assessment of the commercial viability of such an operation, however, is beyond the scope of the present study.

# INHIBITION OF SECONDARY CAROTENOID SYNTHESIS IN *HAEMATOCOCCUS PLUVIALIS* USING INHIBITORS

## 9.1 INTRODUCTION

### 9.1.1 Inhibition of carotenoid biosynthesis

The carotenoid biosynthetic pathway involves a complex sequence of reactions (outlined in section 1.3) and this pathway is a well known target for herbicides. Many compounds have now been identified which inhibit the formation of coloured carotenoids (Wakabayashi and Yukiharu, 1992), or the build up of chlorophylls (Böger, 1989). Both types of inhibitor result in the bleaching of photosynthetic tissues. Inhibition of carotenoid formation by herbicides results in the accumulation of colourless carotenoid precursors which cannot protect against photooxidation (Krinsky, 1971).

### 9.1.2 Herbicides inhibiting carotenoid synthesis

Different inhibitors affect the enzymatic reactions leading from phytoene (VII and VIII) through to the xanthophylls. Many compounds are known which inhibit phytoene desaturase resulting in the accumulation of phytoene (VII and VIII), which is generally not found in healthy, green tissues (Britton *et al.*, 1989). Two of the best known examples are the phenylpyridazines-norflurazon (SAN 9879; 4-chloro-5-methylamino-2-(3-trifluoromethylphenyl)-pyridazin-3(2H)one) (Fig. 9.1; Sandmann and Böger, 1983), and metflurazon (SAN 6707) (Bartels and McCullough, 1972). The phenylpyridazinones act by direct interference by norflurazon and metflurazon with phytoene desaturase, resulting in decreased activity, and not by inhibiting the synthesis of the phytoene desaturase enzyme (Mayer *et al.*, 1989).

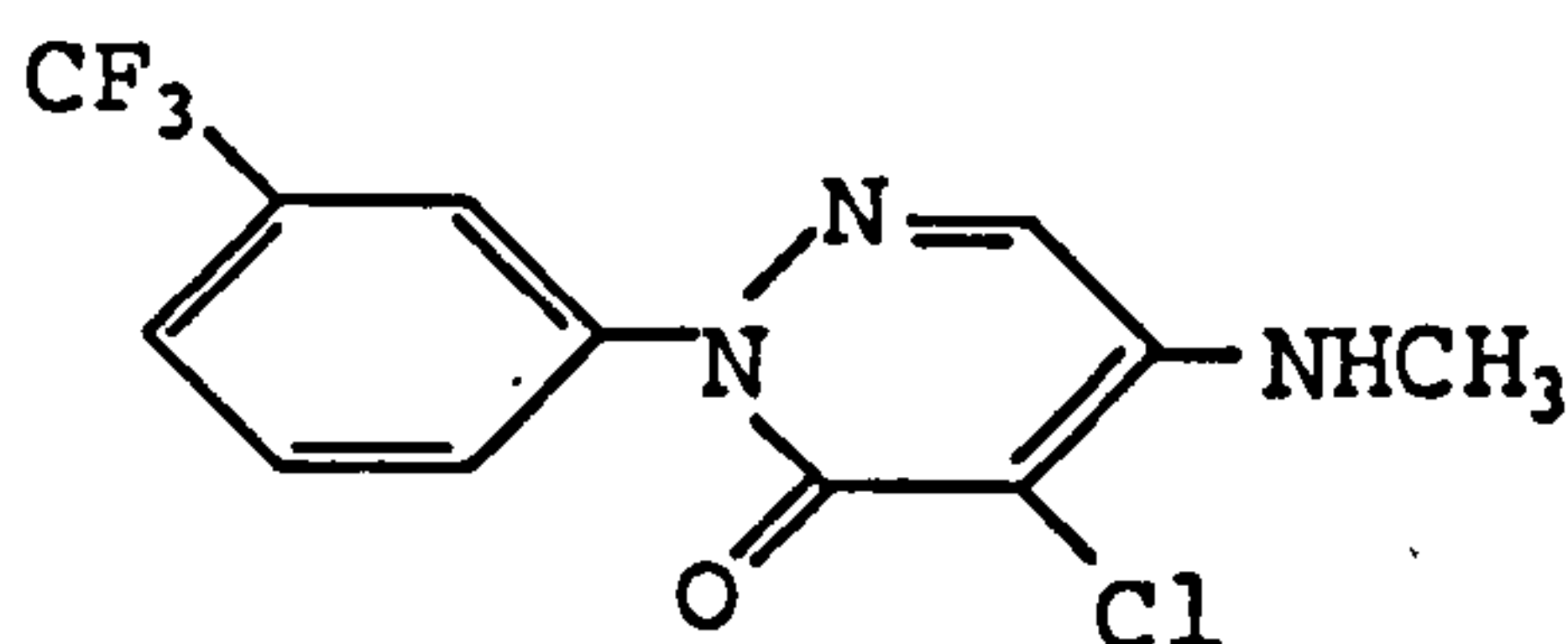


Fig. 9.1 Structure of the inhibitor norflurazon.



One of the first compounds known to inhibit the desaturation of phytoene (VII and VIII) were bis-phenyl derivatives such as diphenylamine (DPA) (Fig. 9.2; Goodwin and Land, 1956).

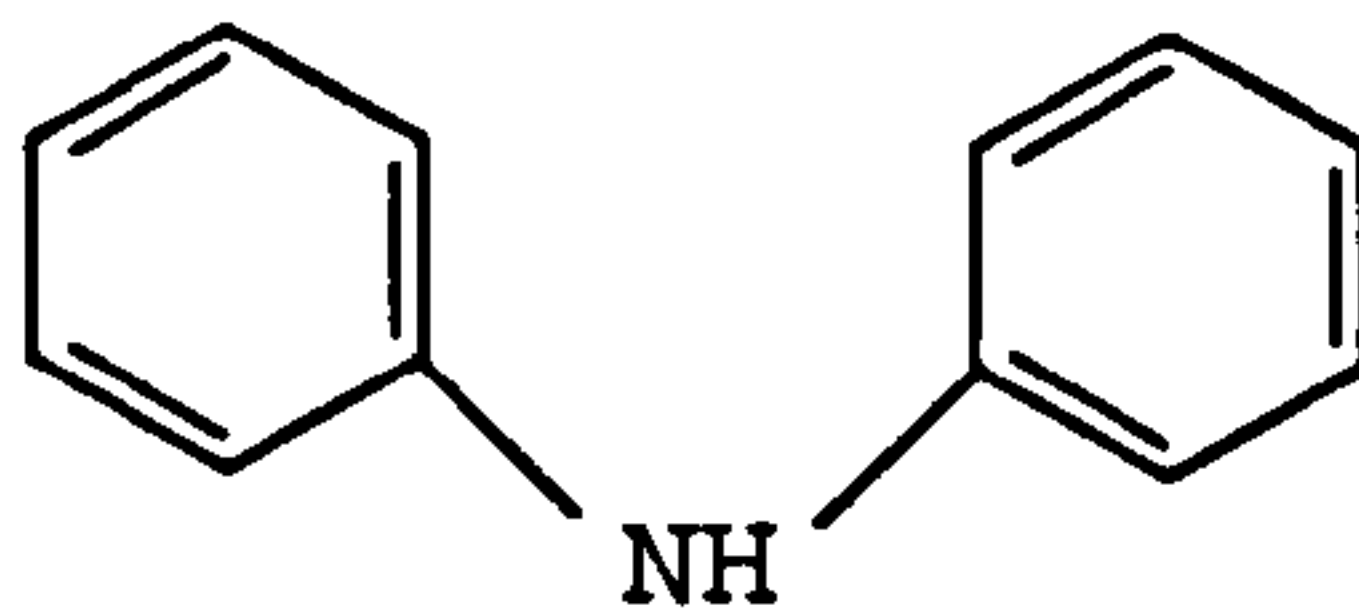


Fig. 9.2 Structure of the inhibitor diphenylamine.

Other phytoene desaturase inhibitors include fluridone (1-methyl-3-phenyl-5-(3-trifluoromethylphenyl)-4(1H)-pyridone) (Bartels and Watson, 1978) and diflufenican (MB 38544; *N*-(2,4-difluorophenyl)-2-(3-trifluoromethylphenoxy) nicotinamide) (Fig. 9.3; Sandmann *et al.*, 1985). There are many other less effective phytoene desaturase inhibitors, for a review see Böger and Sandmann (1993).

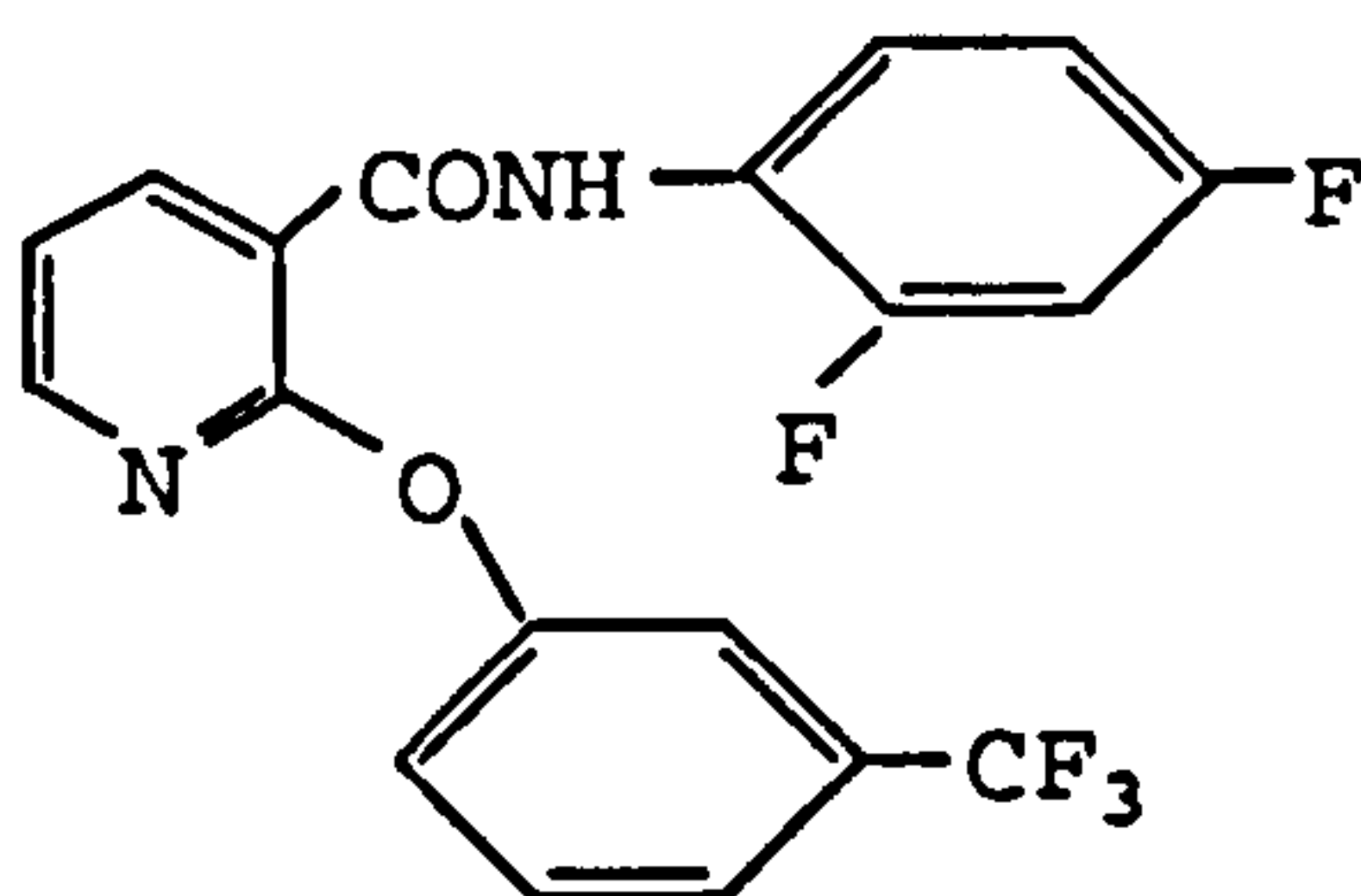


Fig. 9.3 Structure of the inhibitor diflufenican.

Relatively few compounds are known which inhibit the desaturation of  $\zeta$ -carotene (X), which results in the accumulation of  $\zeta$ -carotene (X) in photosynthetic tissues. Amitrole (3-amino-5-triazole) (Fig. 9.4) and dichlormate have been reported to cause the accumulation of  $\zeta$ -carotene (X) in whole plants (Burns *et al.*, 1971).

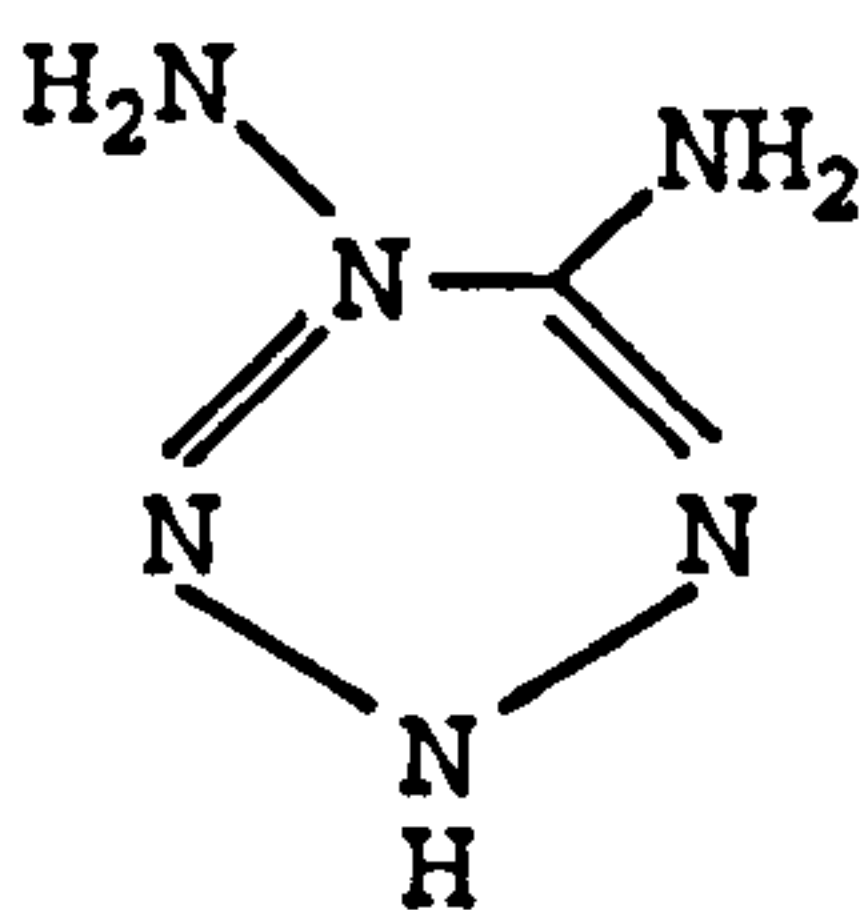


Fig. 9.4 Structure of the inhibitor amitrole.

There are relatively few known specific inhibitors of lycopene cyclase, the only known compounds are the triethylamines e.g. CPTA (2-(4-chlorophenylthio)-triethylamine HCl) (Fig. 9.5) and nicotine (Britton *et al.*, 1977), both of which result in the accumulation of lycopene (XIII).

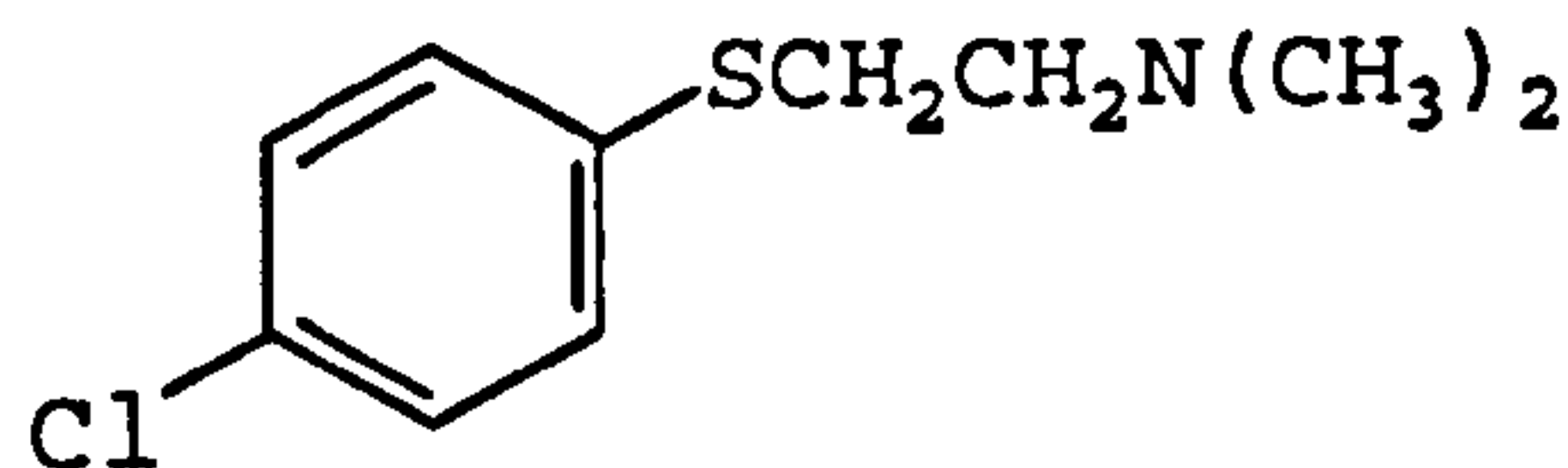
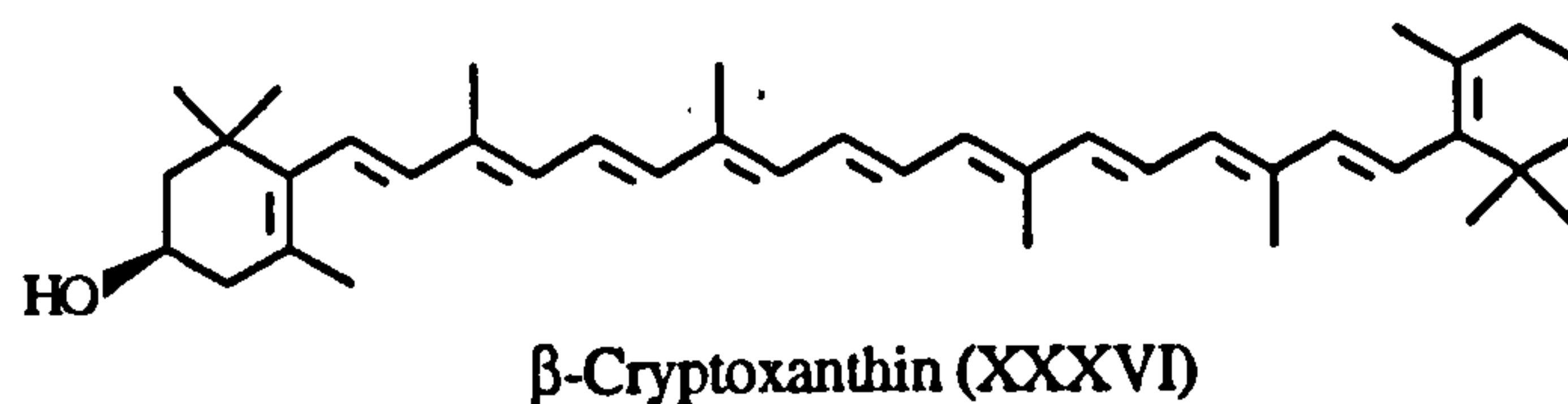


Fig. 9.5 Structure of the inhibitor CPTA.

No inhibitors targeted against the mixed function oxygenase reactions of xanthophyll formation are available yet (Sandmann and Böger, 1989). However, the presence of the bioregulators tetcyclacis (LAB 102883; 5-(4-chlorophenyl)-3,4,5,9,10-pentazotetracyclo-5,4,1,0<sup>2,6</sup>,0<sup>8,11</sup>-dodeca-3,9-diene) and LAB 117682 (3-(1,2,4-triazolyl-1)-1-(4-chlorophenyl)-4,4-dimethylpentane-1-one) inhibits the *in vitro* hydroxylation of  $\beta$ -carotene (I) to  $\beta$ -cryptoxanthin ((3*R*)- $\beta$ , $\beta$ -caroten-3-ol (XXXVI)) (Sandmann and Bramley, 1985).



Other compounds have also been reported to inhibit xanthophyll formation these include nicotine (McDermott *et al.*, 1973) and 1-aminobenzotriazole (Fig. 9.6) (A. Young, pers. comm.).

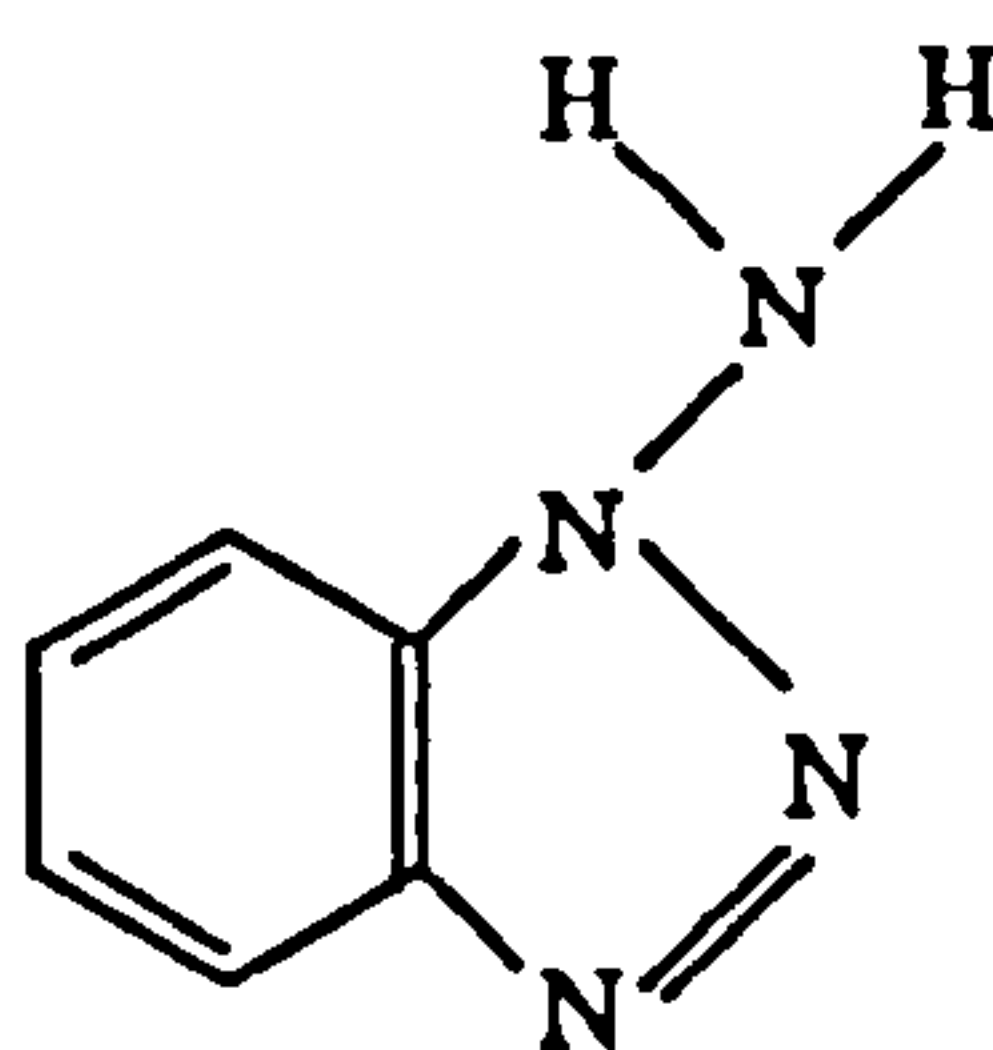


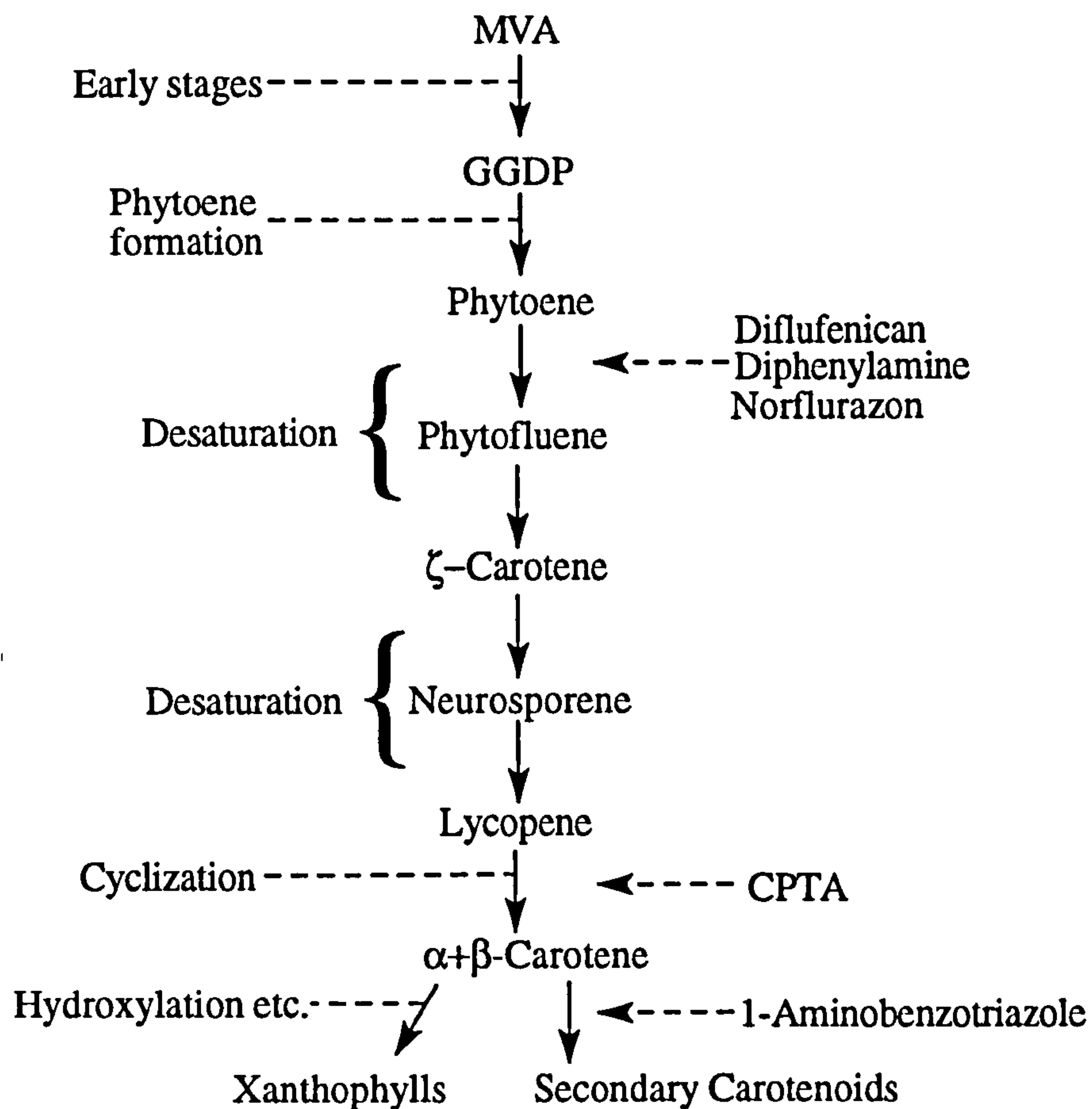
Fig. 9.6 Structure of the inhibitor 1-aminobenzotriazole.

The main effects of inhibitors which interfere with carotenoid biosynthesis is an accumulation of carotenoid precursors which are prevented from forming the normal cyclic carotenoids present in higher plants and algae. The absence of these cyclic carotenoids prevents the normal assembly of the photosynthetic apparatus, resulting in poor photosynthetic performance. The absence of the cyclic carotenoids leads to photooxidation which ultimately results in cell death (Kunert and Dodge, 1989).

### **9.1.3 Aims**

There is no available information on the activity of herbicidal compounds on the accumulation of secondary carotenoids in *H. pluvialis*. Such information would be useful in indicating whether or not the carotenoids associated with photosynthesis act as precursors for secondary carotenoids; or that the biosynthesis of secondary carotenoids and those involved in photosynthesis occur along separate but parallel pathways. The results may also provide some information about the biosynthetic pathway leading to ketocarotenoid formation in *H. pluvialis* and reveal the identity of some of the precursors involved in the pathway. In the present study a number of inhibitors were investigated to determine their effect on secondary carotenoid accumulation in *H. pluvialis*. The inhibitors used and their 'predicted' mode of action are shown in figure 9.7, for more information on the inhibitors used see Bramley (1993).





**Fig. 9.7** Predicted sites of carotenoid inhibition caused by inhibitors used in the present investigation.

In a second series of experiments *H. pluvialis* cells were incubated with an inhibitor to allow the accumulation of a specific precursor. This was then removed and a second inhibitor added to the cells to inhibit any further carotenoid synthesis. The pigment content of the cells was then monitored to determine if the precursor accumulated could be converted into the accumulating secondary carotenoids. The results would determine whether there is a functional role for the specific precursor accumulated in the synthesis of secondary carotenoids.

## 9.2 MATERIALS AND METHODS

The alga was initially grown in a 5 litre fermenter to produce sufficient algal biomass (see section 2.7.3). Once the algal suspension had reached a cell concentration of approximately  $1.5 \times 10^5$  cells / ml and the green palmella stage of development, 50 ml of algal suspension was transferred aseptically into 250 ml Erlenmeyer conical flasks. A stock solution of each inhibitor was made up in 10 ml vials. The inhibitors needed to be dissolved in a small volume of ethanol before sterile distilled water could be added to make up the stock solutions (stored at 4°C). The final ethanol concentration did not exceed 0.2% of the final volume. Aliquots of inhibitor were added to the algal cultures via a 0.2 µm Millipore filter to avoid contamination of the cultures. The flasks were then placed on an orbital shaker (80 rpm) and incubated at 15°C.

For each inhibitor, cultures were exposed to inducing and non-inducing conditions for the biosynthesis of secondary carotenoids. This was controlled by incubating the cultures at specific light intensities. The light intensities used were  $10 \mu\text{mol m}^{-2} \text{s}^{-1}$  (PAR) for non-inducing conditions and  $75 \mu\text{mol m}^{-2} \text{s}^{-1}$  (PAR) to induce secondary carotenoid biosynthesis. Each inhibitor treatment was replicated three times. The inhibitors used and the concentrations at which they were added to the algal cultures are given in Table 9.1. The particular concentration of each inhibitor used in the study was identified by initial screening experiments and by previous reports concerning carotenoid inhibitors (Böger and Sandmann, 1993). Aliquots of the alga were taken aseptically at given time intervals and pigments were extracted as described in section 2.2.1 and analysed using reversed-phase HPLC as described in section 2.3.5.2.

**Table 9.1** Inhibitors and concentrations used in the first series of experiments.

Inhibitor	Concentration (µM)
Norflurazon	100
Diphenylamine	100
Diflufenican	100
CPTA	10
1-Aminobenzotriazole	1000

In a second series of experiments combinations of two different inhibitors were added to the cultures. Initially CPTA or DPA was added to the cultures which had reached the green palmella stage of development. The cultures were placed under inducing conditions as in the previous experiments and allowed to accumulate either lycopene (XIII) or  $\beta$ -carotene (I), respectively. After 14 days the CPTA or DPA treated cells were centrifuged (3000 rpm) and the supernatant containing the inhibitor removed from the algal pellet. The cells were then resuspended in sterile distilled water and centrifuged again to wash the cells. This procedure was repeated a number of times to ensure all the inhibitor had been removed from the cells. The cells were then resuspended in 50 ml of BBM in 250 ml Erlenmeyer conical flasks. Norflurazon was added to the cultures which were again placed under secondary carotenoid inducing conditions at 15°C. Table 9.2 shows the concentrations of inhibitor used in the experiments.

**Table 9.2** Inhibitors and concentrations used in the second series of experiments.

1st Inhibitor	Concentration ( $\mu$ M)	2nd Inhibitor	Concentration ( $\mu$ M)
CPTA	10	Norflurazon	100
Diphenylamine	100	Norflurazon	100

Definition of the terms used in the keys of the results section: Primary carotenoids; neoxanthin (XX), violaxanthin (XVII), antheraxanthin (XVIII), zeaxanthin (XV), lutein (XVI) and  $\beta$ -carotene (I). Xanthophylls; neoxanthin (XX), violaxanthin (XVII), antheraxanthin (XVIII), zeaxanthin (XV) and lutein (XVI). Secondary carotenoids; astaxanthin (III), astaxanthin (III) mono and diesters, echinenone (II), adonirubin (V) and canthaxanthin (IV).



## 9.3 RESULTS

### 9.3.1 Norflurazon

Figures 9.8a-d show the effect on carotenoid composition when cells of *H. pluvialis* were exposed to norflurazon during secondary carotenoid synthesis. Note that in figures 9.8 - 9.21 to improve clarity, the standard errors for the individual experiments are given in the figure legends.

Fig. 9.8a No norflurazon: non-inducing conditions.

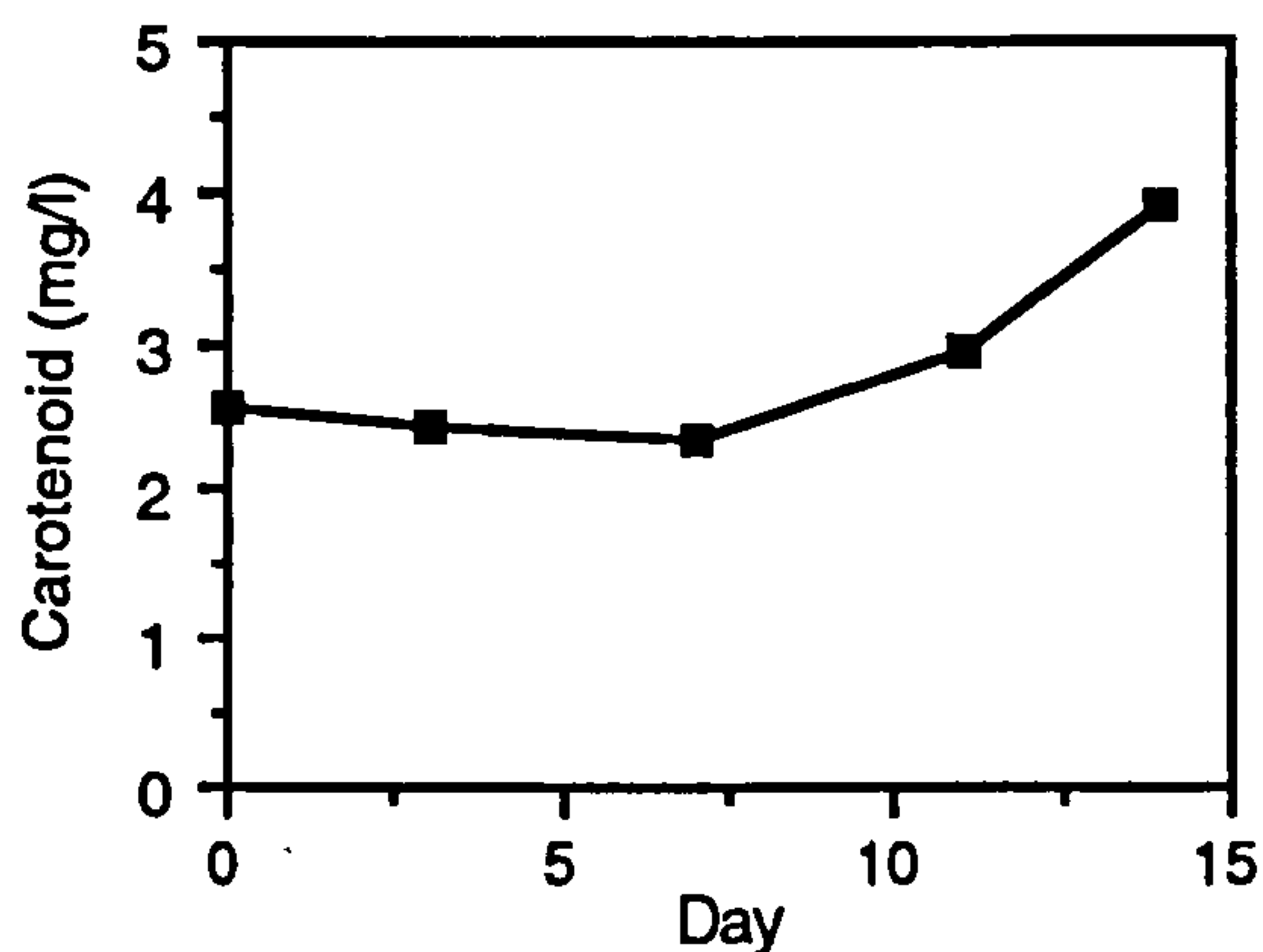


Fig. 9.8b 100  $\mu$ M norflurazon: non-inducing conditions.

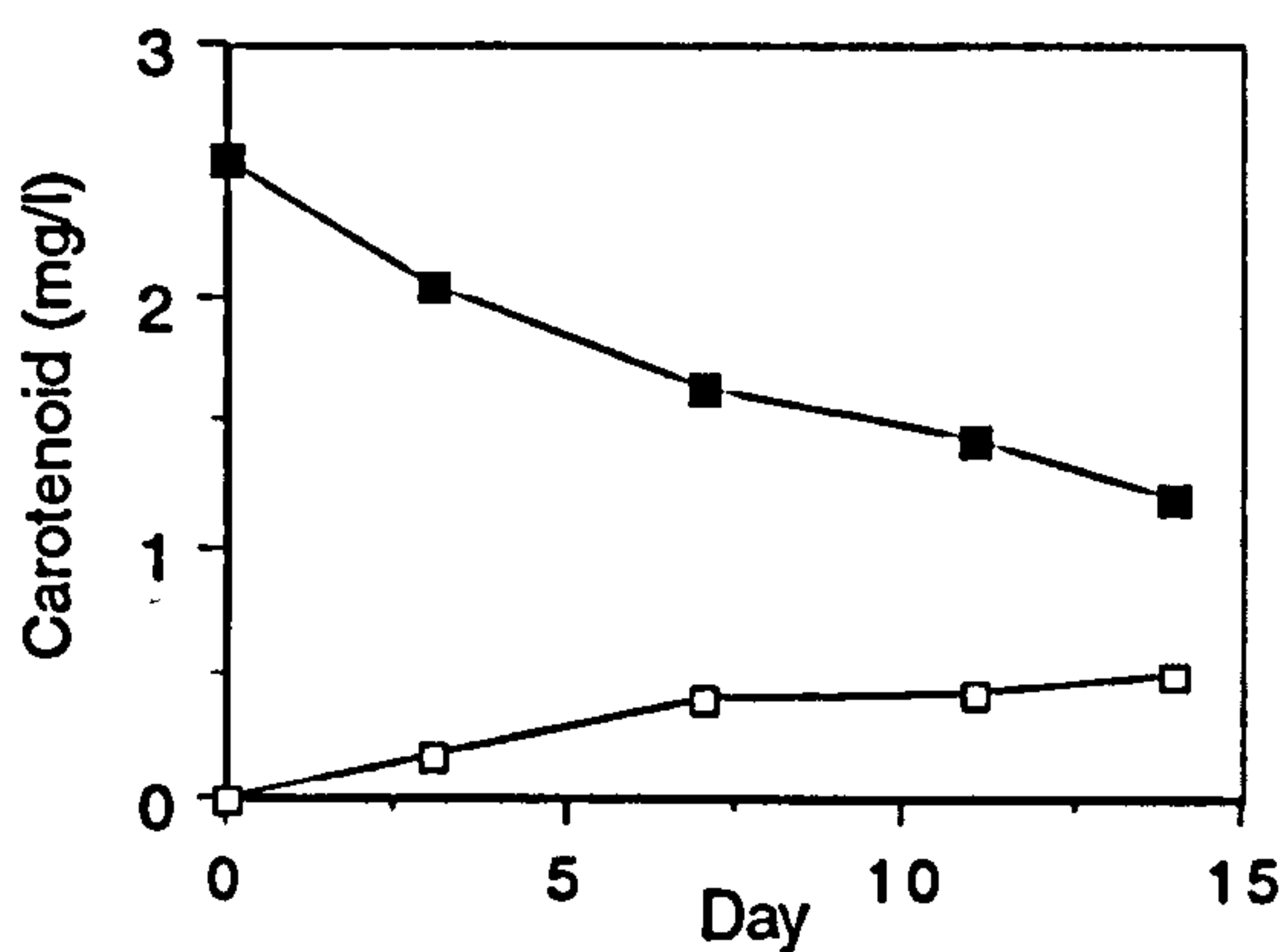


Fig. 9.8c No norflurazon: secondary carotenoid inducing conditions.

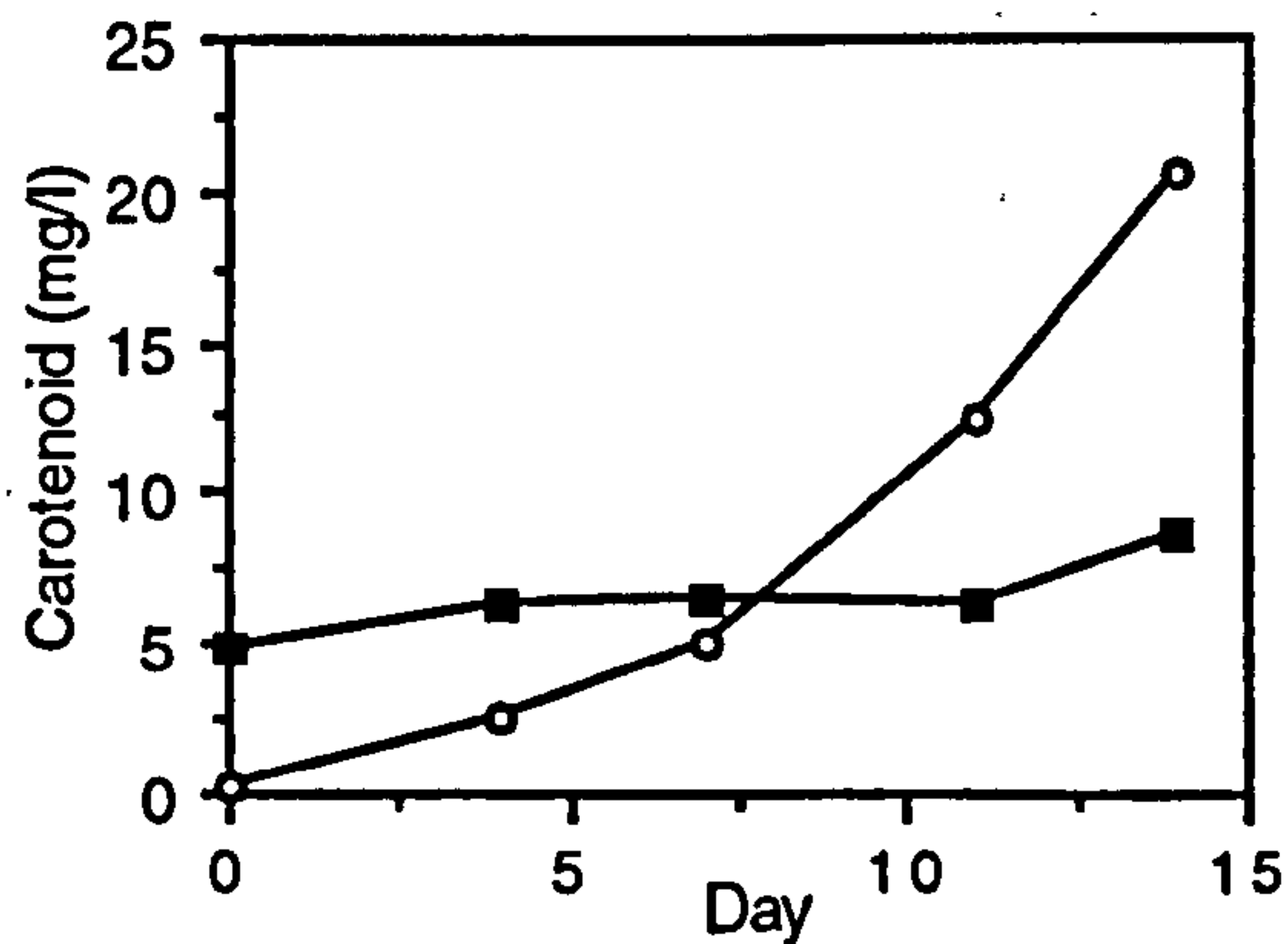
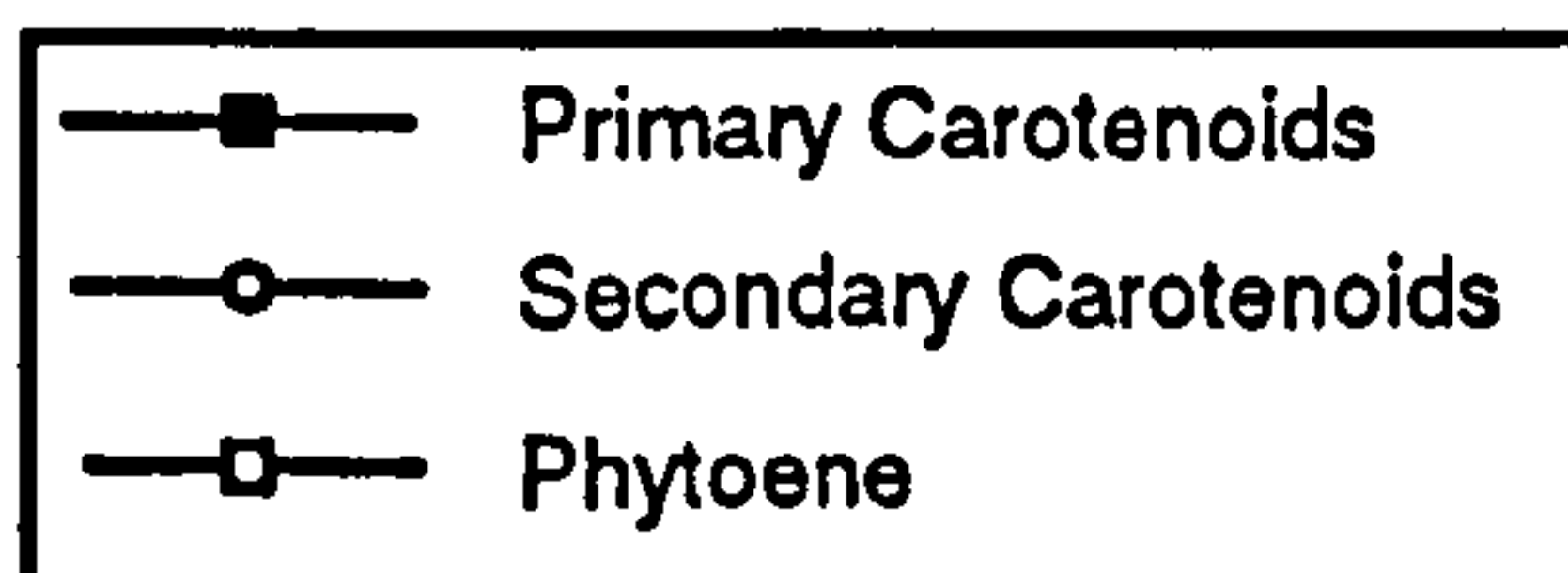
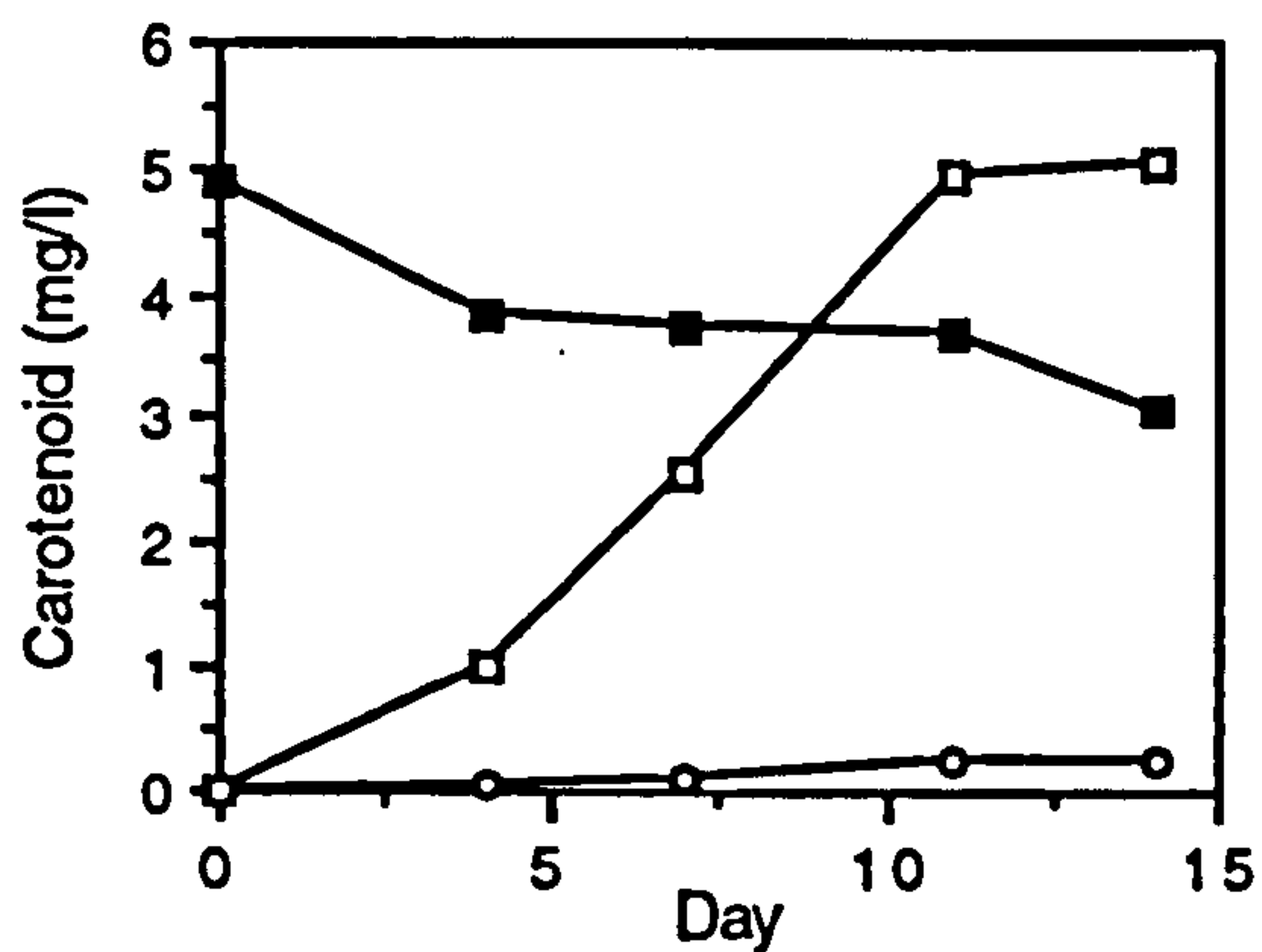


Fig. 9.8d 100  $\mu$ M norflurazon: secondary carotenoid inducing conditions.



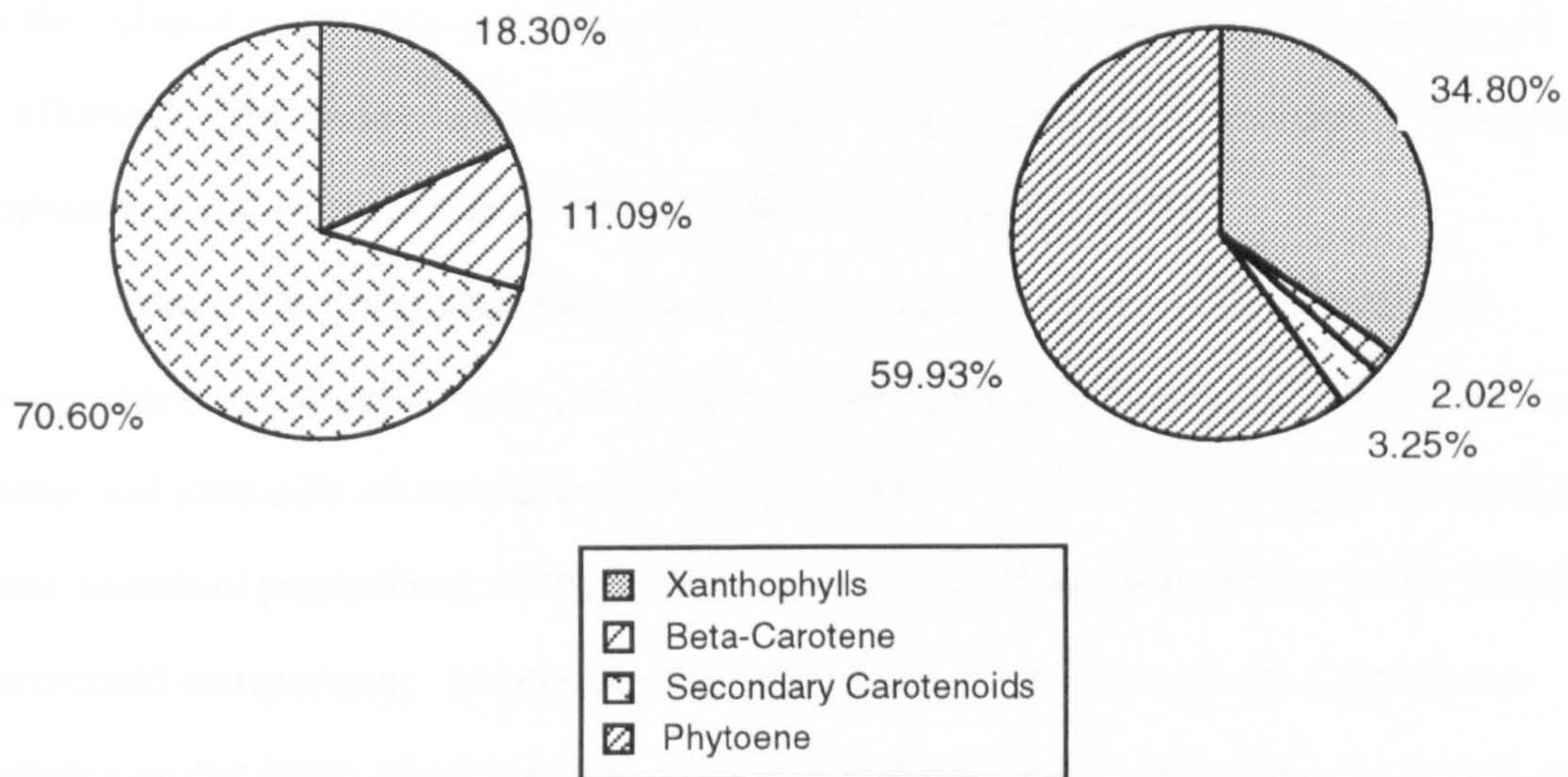
Figs. 9.8a-d The effect of norflurazon on secondary carotenoid composition in *H. pluvialis* (S.E.  $\pm$ 3.6%, n=3).



Figures 9.9a and 9.9b show the carotenoid composition of cells of *H. pluvialis* on the final day of the experiment when exposed to norflurazon in conditions leading to the synthesis of secondary carotenoids.

**Fig. 9.9a** No norflurazon: secondary carotenoid inducing conditions.

**Fig. 9.9b** 100  $\mu$ M norflurazon: secondary carotenoid inducing conditions.



**Fig. 9.9a-b** The final carotenoid composition of cells of *H. pluvialis* cells when incubated under secondary carotenoid inducing conditions with and without norflurazon (S.E.  $\pm 4.1\%$ , n=3).

Figure 9.8a indicates that the cultures exposed to non-inducing secondary conditions in the absence of norflurazon showed a slight increase in the levels of primary carotenoids during the experiment. As expected, no secondary carotenoids, phytoene (VII and VIII) or phytofluene (IX) were detected in the cultures. The cultures exposed to non-inducing conditions in the presence of norflurazon (Fig. 9.8b) did accumulate small amounts of phytoene (VII and VIII), while the levels of primary carotenoids declined slightly during the experiment. Again no secondary carotenoids or phytofluene (IX) were detected in these cultures.

Cultures exposed to secondary carotenoid inducing conditions in the absence of norflurazon (Fig. 9.8c) accumulated relatively large amounts of secondary carotenoids (up to double the amount of primary carotenoids). The levels of primary carotenoids only showed a slight increase during the experiment, but no phytoene (VII and VIII) or phytofluene (IX) were detected in these cultures. Figure 9.8d shows the effect on *H. pluvialis* cultures when they were exposed to secondary carotenoid inducing conditions in



the presence of norflurazon. The cultures accumulated relatively high levels of phytoene (VII and VIII) compared to the cultures exposed to non-inducing secondary carotenoid conditions in the presence of norflurazon (Fig. 9.8b). These cultures contained nearly ten times more phytoene (VII and VIII) than the cultures depicted in figure 9.8b. The occurrence of secondary carotenoids was observed, albeit at relatively low levels compared to the cultures exposed to secondary carotenoid inducing conditions in the absence of norflurazon (Fig. 9.8c). The levels of primary carotenoids declined slightly during the experiment and no phytofluene (IX) was detected in these cultures.

Figure 9.9a indicates that on the final day of the experiment the carotenoid composition of cultures exposed to inducing conditions in the absence of norflurazon was composed primarily of secondary carotenoids (70.60%). The xanthophylls were the next most abundant pigments at 18.30%, while  $\beta$ -carotene (I) accounted for 11.09% of the final carotenoid composition. In contrast over half of the carotenoid content found in the cultures grown in the presence of norflurazon under inducing conditions comprised of phytoene (VII and VIII) (59.93%). The xanthophylls made up over one third of the final composition (34.80%) with secondary carotenoids accounting for only 3.25%. The level of  $\beta$ -carotene (I) was also very low at 2.02% of the final carotenoid composition compared to 11.09% in the control cultures (Fig. 9.9a).

### 9.3.2 Diflufenican

Figures 9.10a-d show the effect on carotenoid composition when cells of *H. pluvialis* were exposed to diflufenican during the synthesis of secondary carotenoids.

Fig. 9.10a No diflufenican: non-inducing conditions.

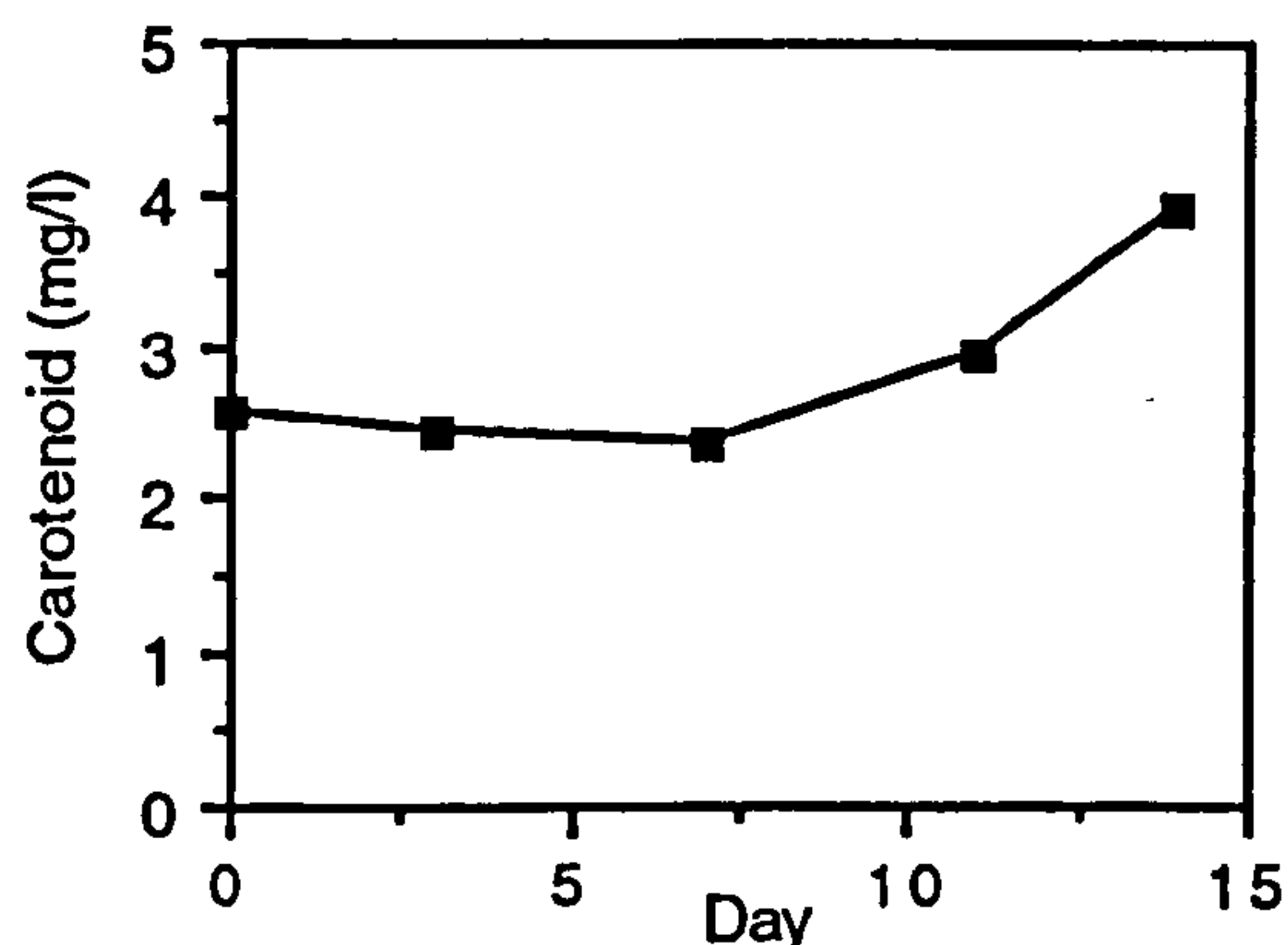
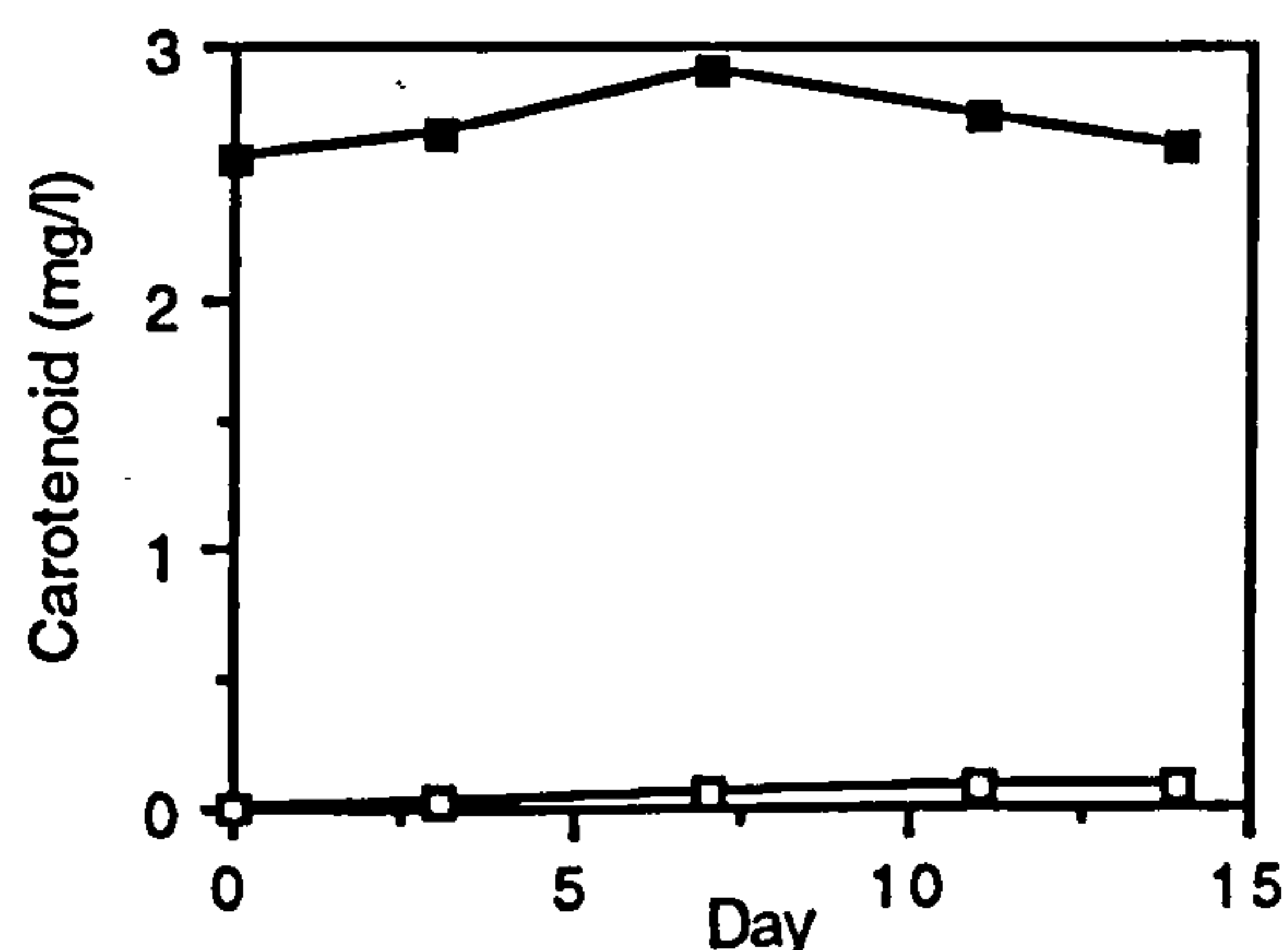
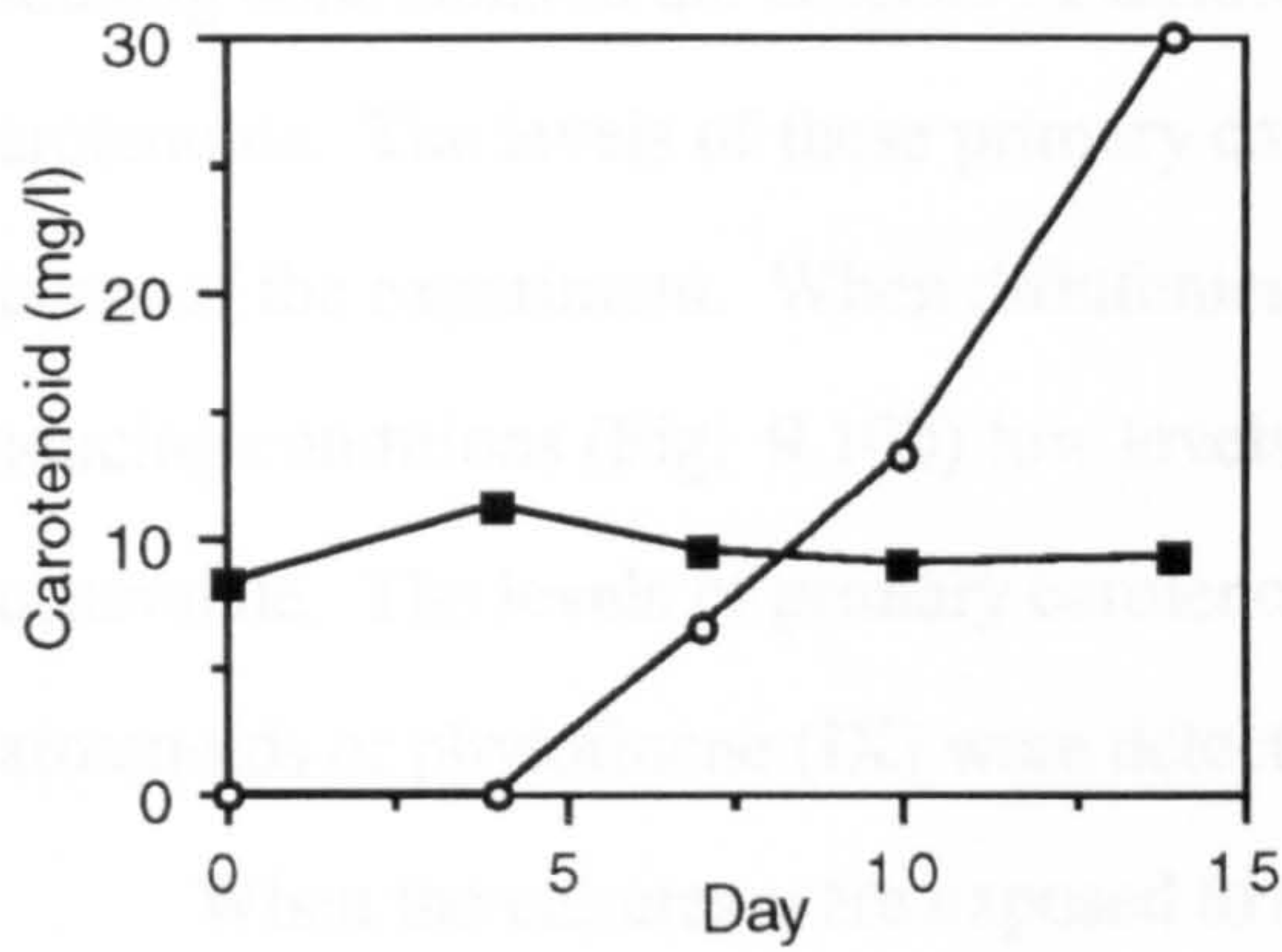


Fig. 9.10b 100  $\mu$ M diflufenican: non-inducing conditions.

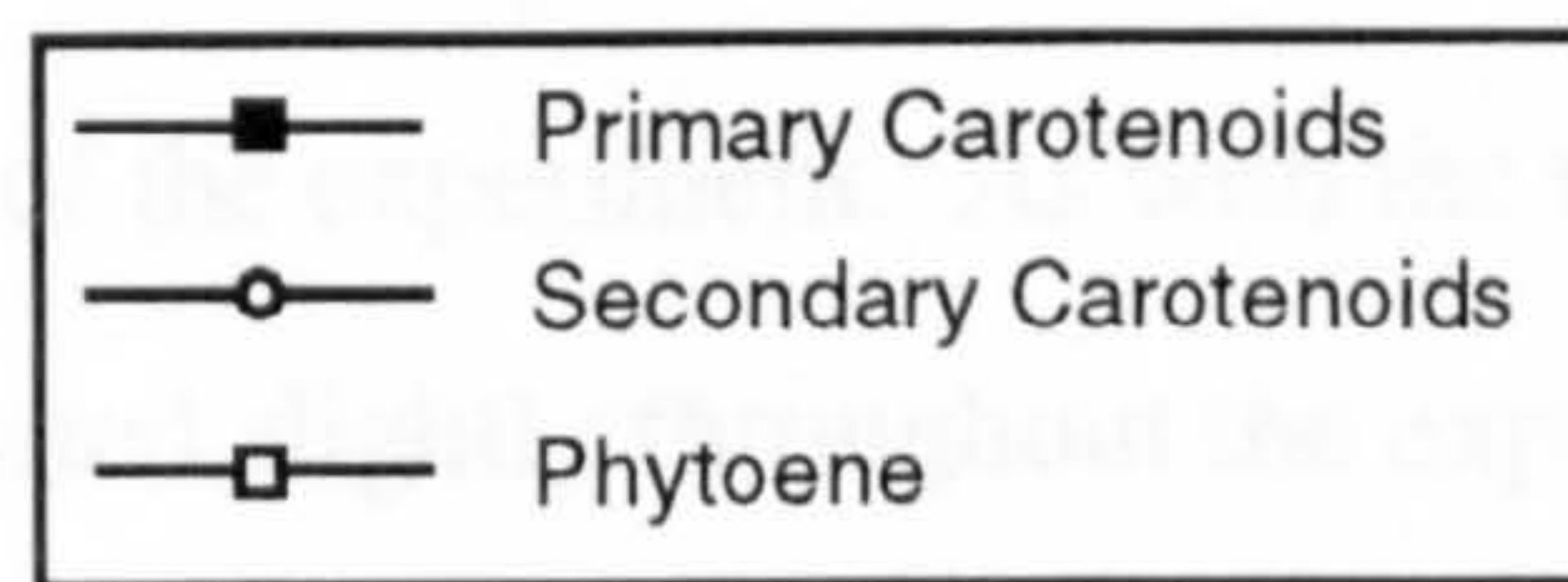
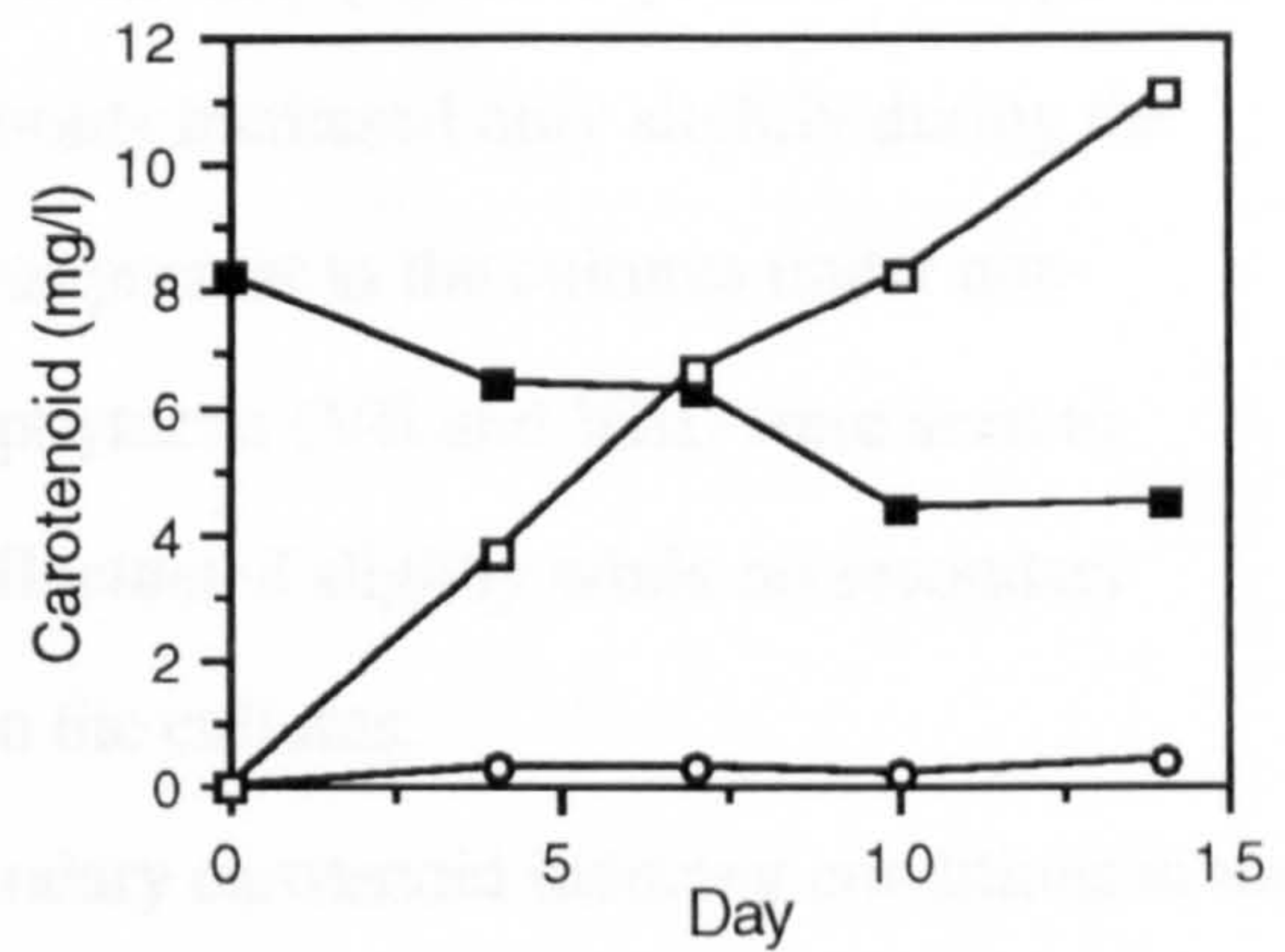




**Fig. 9.10c** No diflufenican: secondary carotenoid inducing conditions.



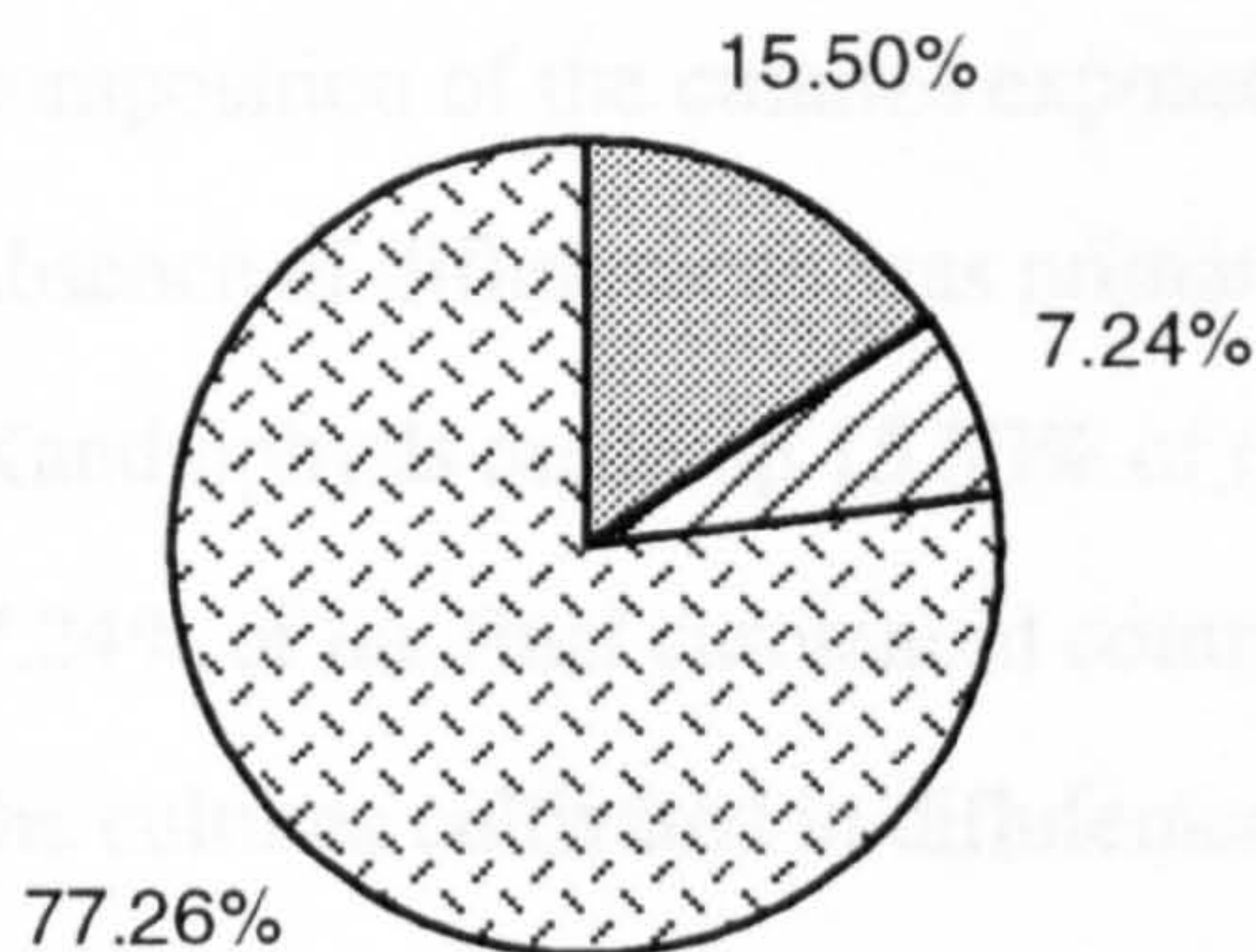
**Fig. 9.10d** 100  $\mu$ M diflufenican: secondary carotenoid inducing conditions.



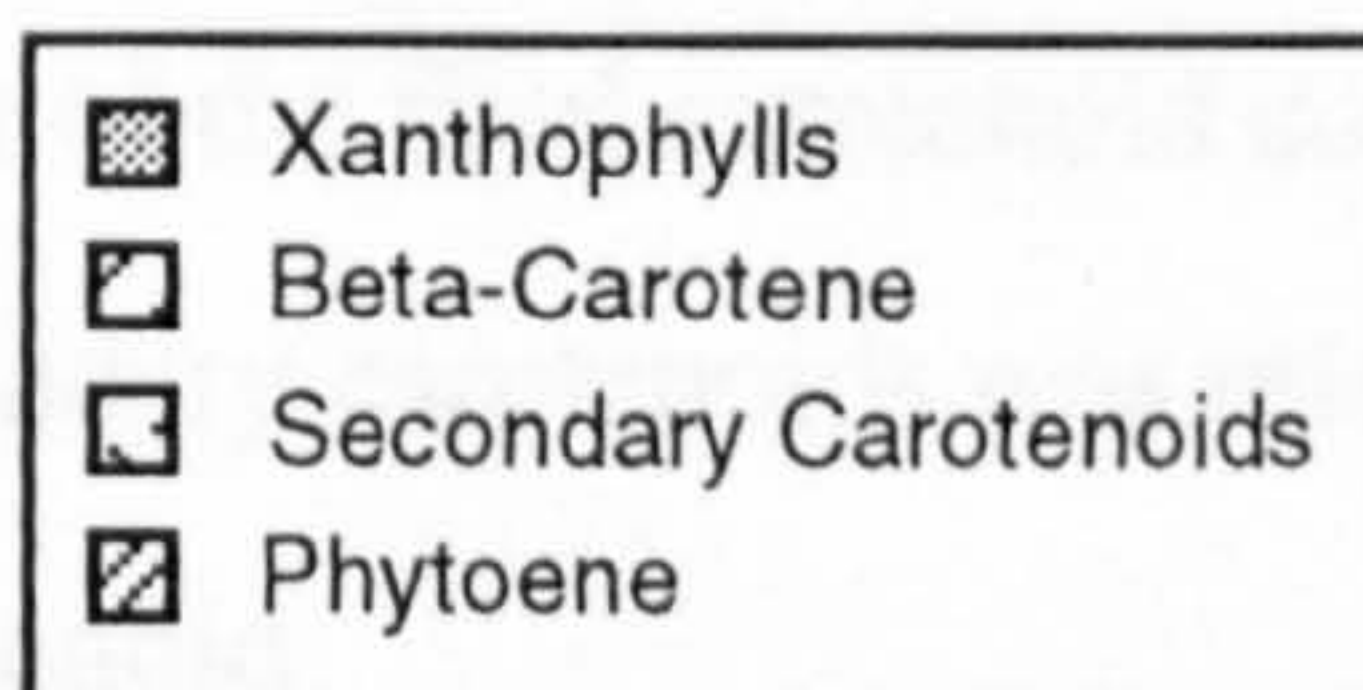
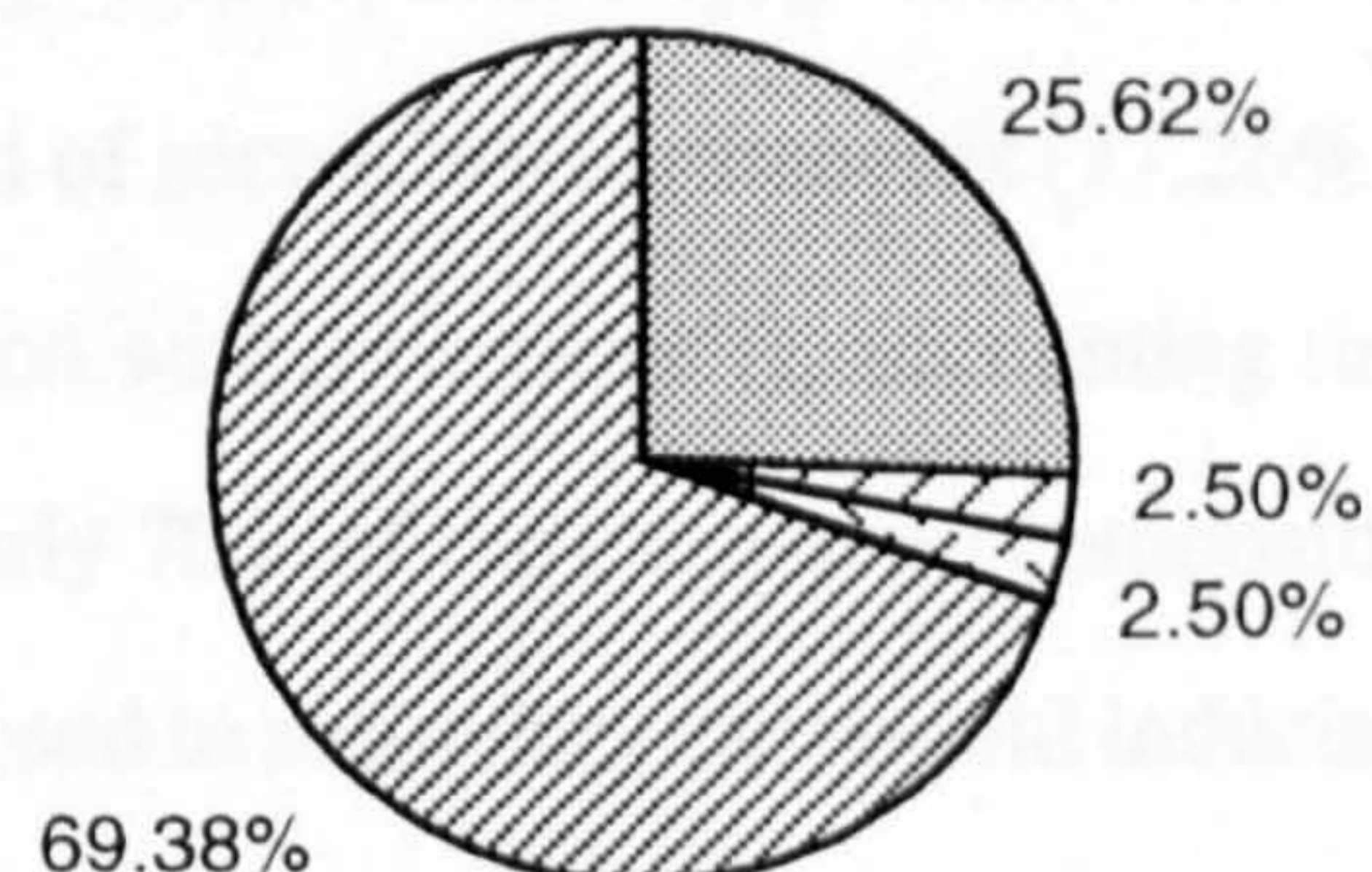
**Fig. 9.10a-d** The effect of diflufenican on carotenoid composition in *H. pluvialis* (S.E.  $\pm 4.6\%$ ,  $n=3$ ).

Figures 9.11a and 9.11b show the carotenoid composition of cells of *H. pluvialis* on the final day of the experiment when exposed to diflufenican during secondary carotenoid synthesis.

**Fig. 9.11a** No diflufenican: secondary carotenoid inducing conditions.



**Fig. 9.11b** 100  $\mu$ M diflufenican: secondary carotenoid inducing conditions.



**Fig. 9.11a-b** The final carotenoid composition of cells of *H. pluvialis* when incubated under secondary carotenoid inducing conditions with and without diflufenican (S.E.  $\pm 3.0\%$ ,  $n=3$ ).



Figure 9.10a indicates that when *H. pluvialis* cultures were cultivated under non-inducing conditions in the absence of diflufenican the only pigments present were primary carotenoids. The levels of these primary carotenoids increased only slightly during the course of the experiment. When diflufenican was present in the cultures under non-inducing conditions (Fig. 9.10b) low levels of phytoene (VII and VIII) were seen to accumulate. The levels of primary carotenoids fluctuated slightly while no secondary carotenoids or phytofluene (IX) were detected in the cultures.

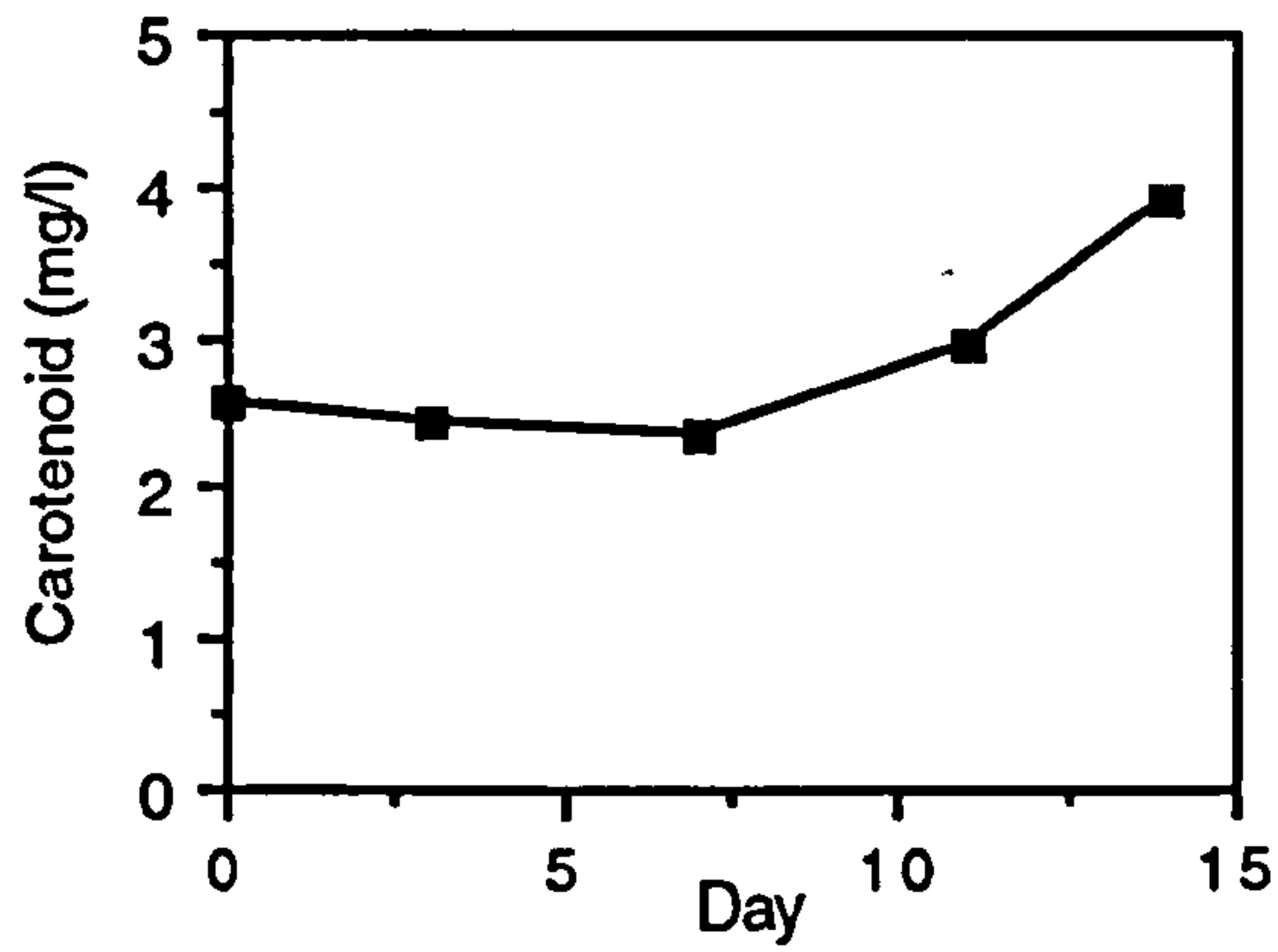
When the cultures were exposed to secondary carotenoid inducing conditions in the absence of diflufenican (Fig. 9.10c) high levels of secondary carotenoids were observed, especially in the latter stages of the experiment. As with the norflurazon control the levels of primary carotenoids fluctuated slightly throughout the experiment, and phytoene (VII and VIII) and phytofluene (IX) were not detected in these cultures. Figure 9.3d indicates that when the cultures were cultivated under secondary carotenoid inducing conditions in the presence of diflufenican relatively large levels of phytoene (VII and VIII) accumulated within the cultures. The levels of primary carotenoids gradually decreased during the experiment while only relatively low levels of secondary carotenoids were observed in the cultures. During the course of the experiment phytofluene (IX) was not detected in the cultures.

Figure 9.11a shows that on the final day of the experiment the carotenoid composition of the cultures exposed to secondary carotenoid inducing conditions in the absence of diflufenican was primarily composed of secondary carotenoids (77.26%). Xanthophylls made up 15.50% of the composition with  $\beta$ -carotene (I) accounting for only 7.24% of the final carotenoid composition. Nearly 70% of the carotenoid composition in the cultures cultivated in diflufenican when exposed to secondary carotenoid inducing conditions (Fig. 9.11b) comprised of phytoene (VII and VIII). The xanthophylls accounted for just over a quarter of the final carotenoid composition (25.62%), while the levels of  $\beta$ -carotene (I) and secondary carotenoids was relatively low, with each one only comprising 2.50% of total carotenoid.

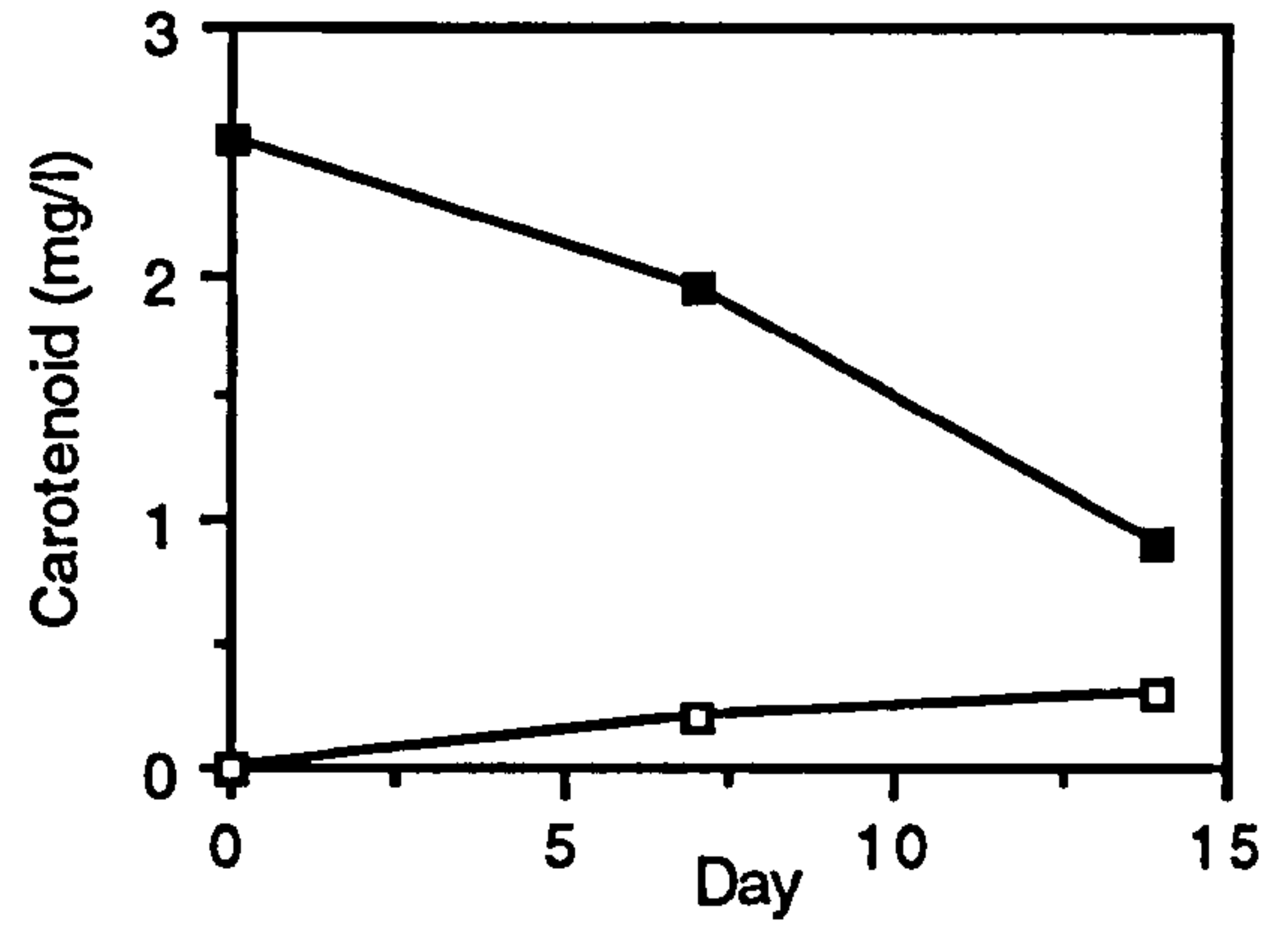
### 9.3.3 CPTA

Figures 9.12a-d show the effect on carotenoid composition when cells of *H. pluvialis* were exposed to CPTA.

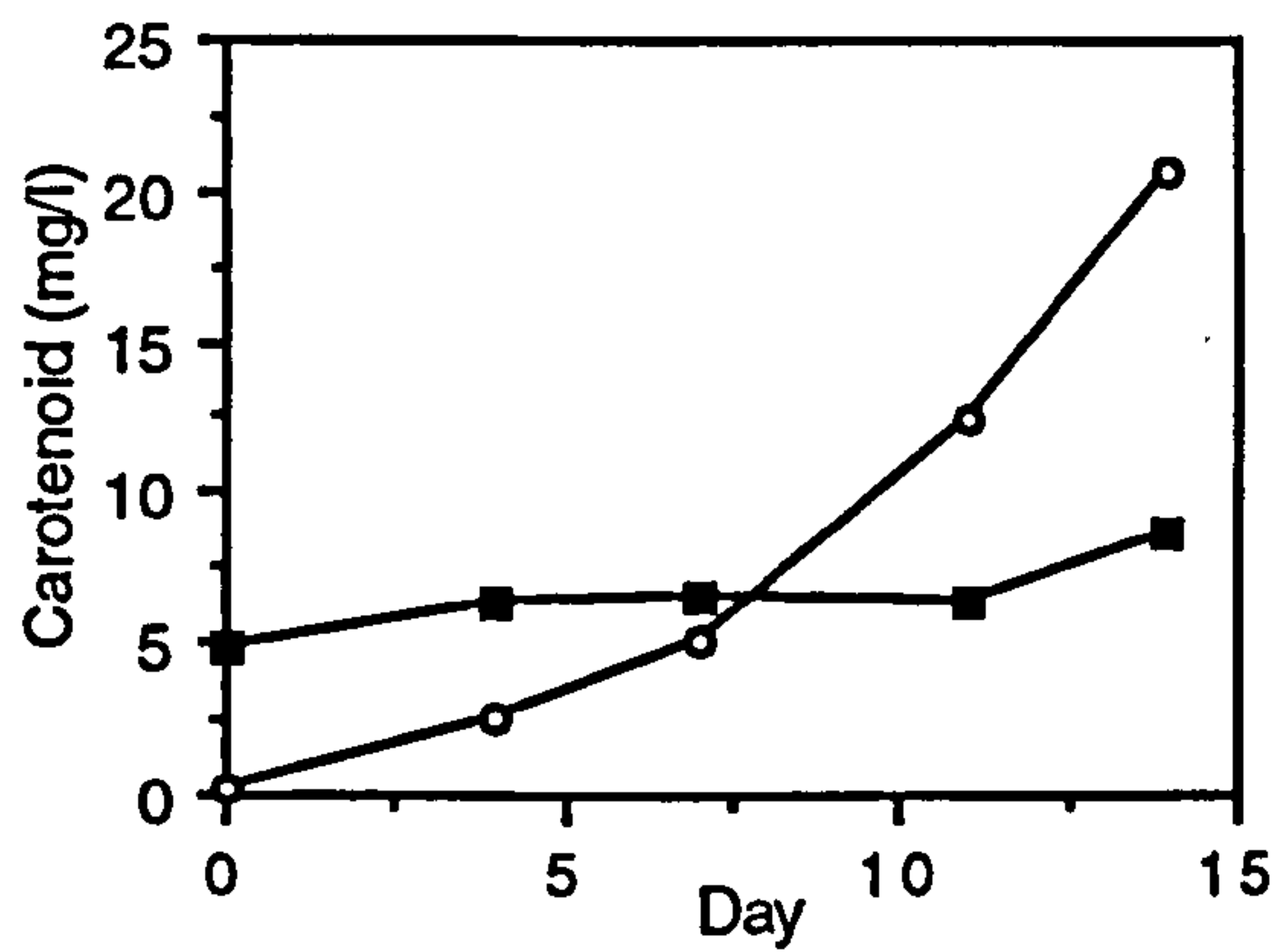
**Fig. 9.12a** No CPTA: non-inducing conditions.



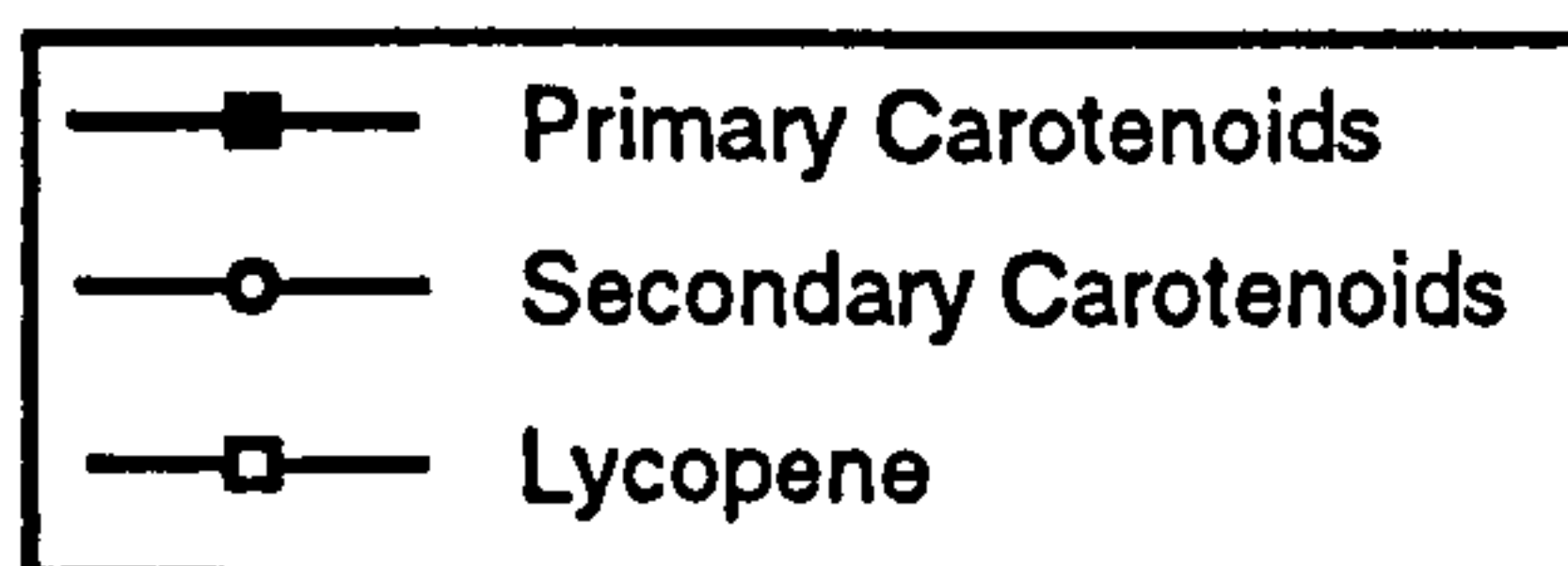
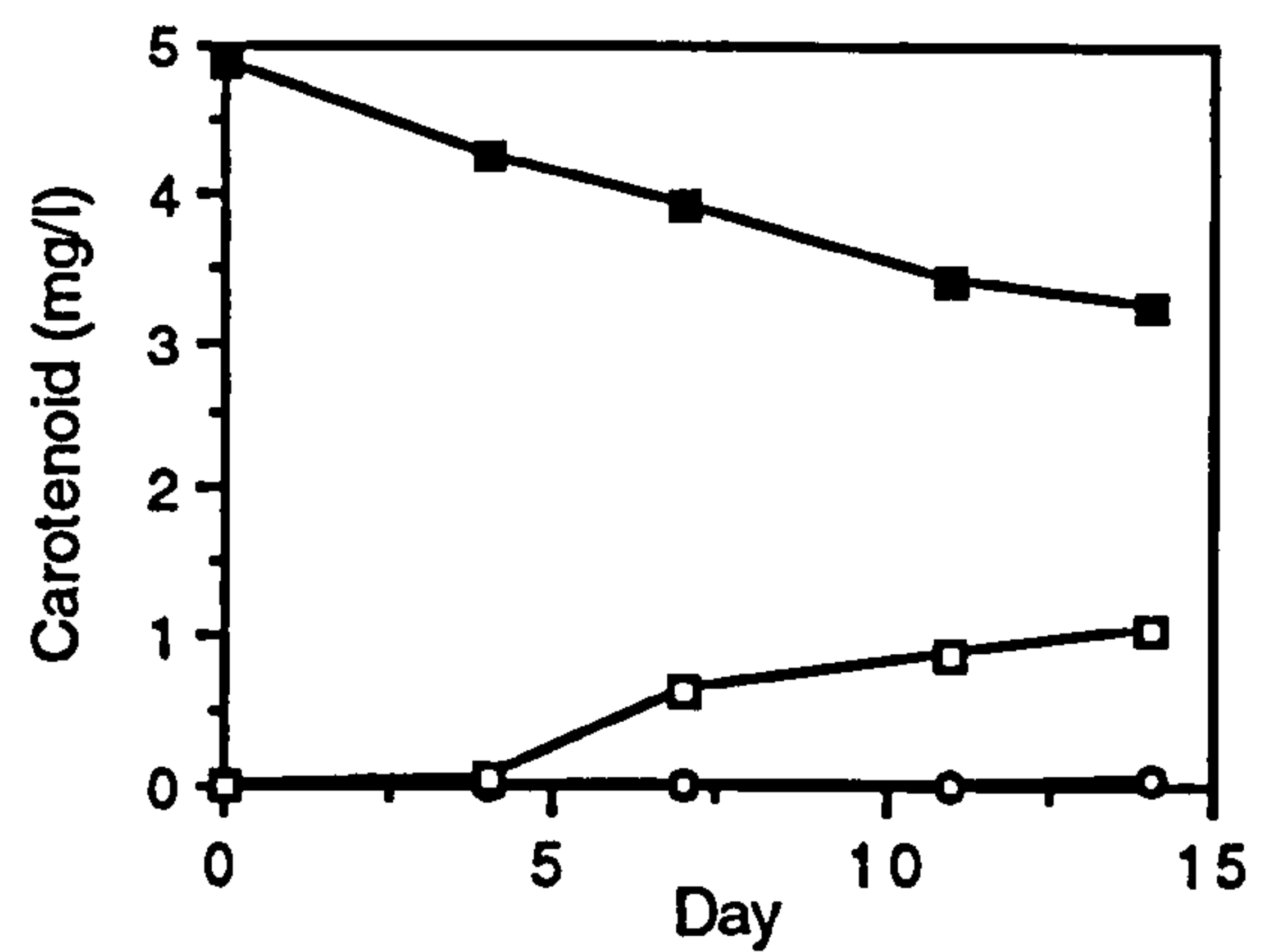
**Fig. 9.12b** 10  $\mu$ M CPTA: non-inducing conditions.



**Fig. 9.12c** No CPTA: secondary carotenoid inducing conditions.



**Fig. 9.12d** 10  $\mu$ M CPTA: secondary carotenoid inducing conditions.

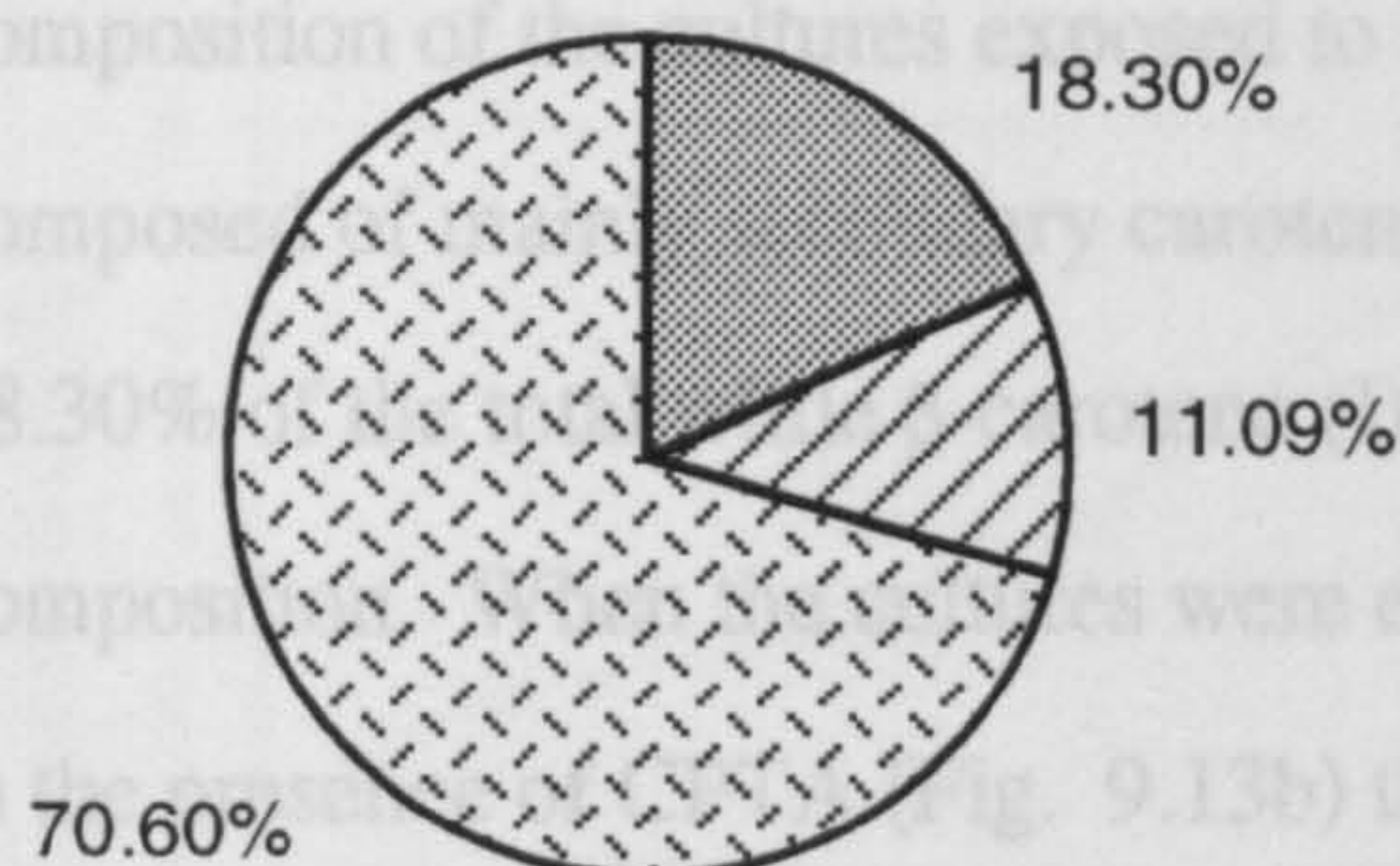


**Fig. 9.12a-d** The effect of CPTA on carotenoid composition in *H. pluvialis* (S.E.  $\pm 5.1\%$ ,  $n=3$ ).

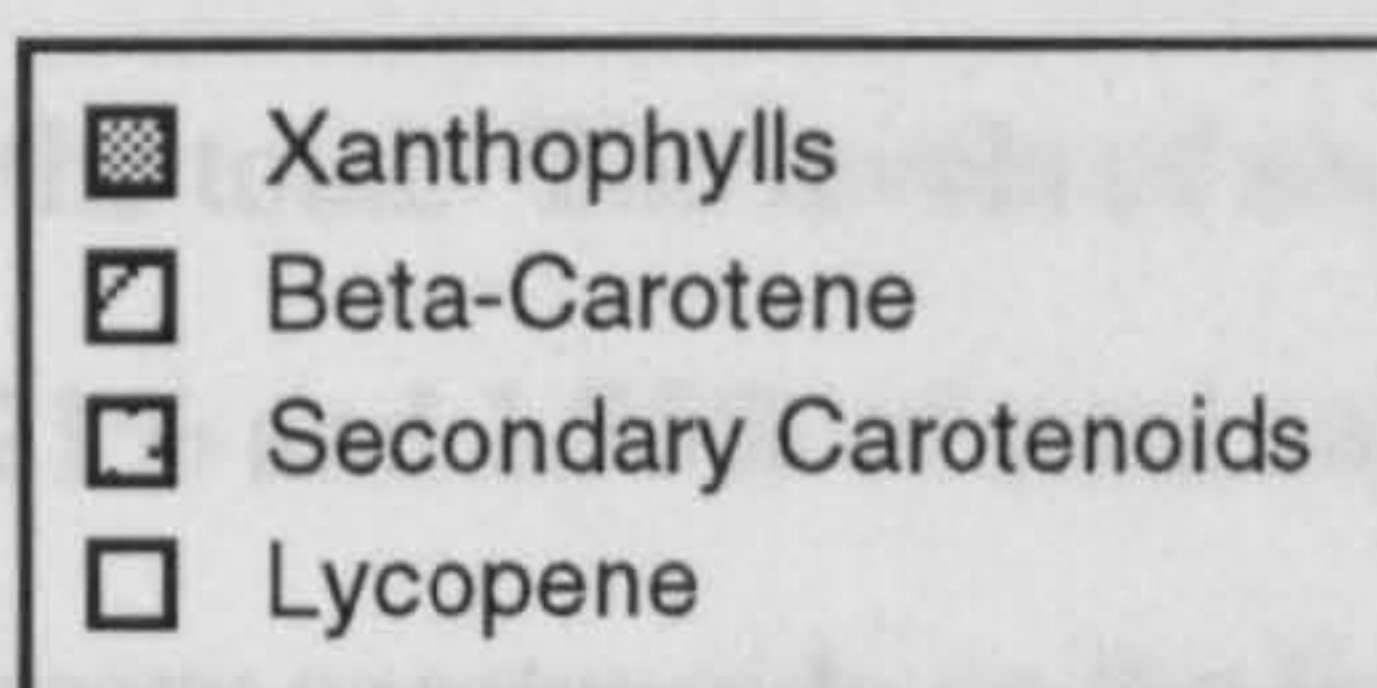
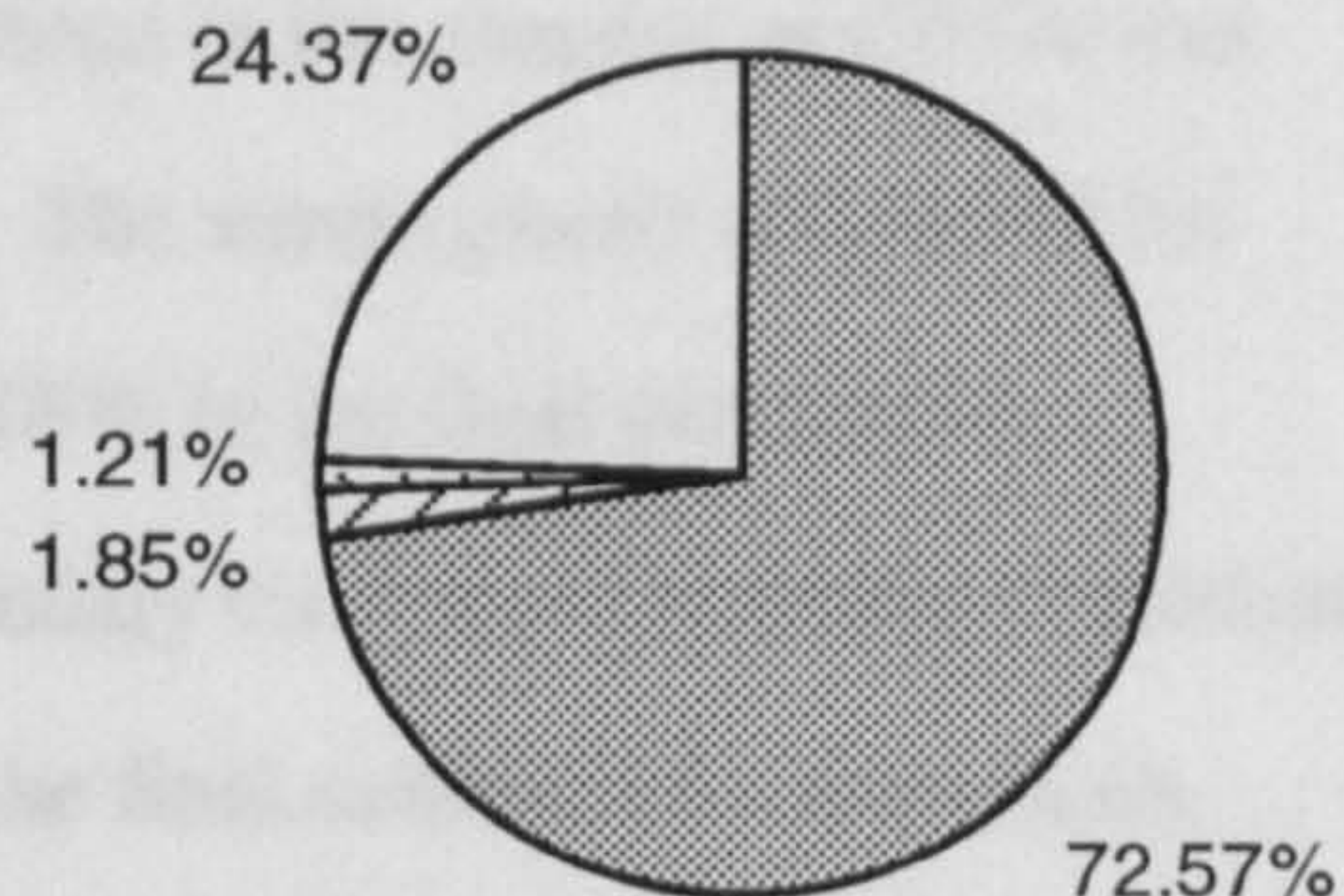


Figures 9.13a and 9.13b show the carotenoid composition of cells of *H. pluvialis* on the final day of the experiment when exposed to CPTA under secondary carotenoid inducing conditions.

**Fig. 9.13a** No CPTA: secondary carotenoid inducing conditions.



**Fig. 9.13b** 10  $\mu$ M CPTA: secondary carotenoid inducing conditions.



**Fig. 9.13a-b** The final carotenoid composition of cells of *H. pluvialis* when incubated under secondary carotenoid inducing conditions with and without CPTA (S.E.  $\pm 4.6\%$ ,  $n=3$ ).

When the cultures were cultivated in the absence of CPTA under non-inducing secondary carotenoid conditions (Fig. 9.12a) no phytoene (VII and VIII), phytofluene (IX), lycopene (XIII) or secondary carotenoids were detected. Only primary carotenoids were present with the levels increasing slightly during the experiment. Figure 9.12b shows that when the cultures were exposed to non-inducing conditions in the presence of CPTA, the levels of primary carotenoids declined at a relatively constant rate throughout the experiment. The cultures also accumulated moderate amounts of lycopene (XIII) although no phytoene (VII and VIII), phytofluene (IX) or secondary carotenoids were observed in the cultures.

The cultures exposed to secondary carotenoid inducing conditions (Fig. 9.12c) in the absence of CPTA accumulated relatively high levels of secondary carotenoids. The levels of primary carotenoids fluctuated during the experiment, while no lycopene (XIII) accumulated in the cultures. Figure 9.12d indicates that when the cultures were exposed to inducing conditions in the presence of CPTA moderate amounts of lycopene (XIII) did



accumulate. Very low levels of secondary carotenoids accumulated in the cultures, while the levels of primary carotenoids fluctuated throughout the experiment. The presence of phytoene (VII and VIII) or phytofluene (IX) was not detected in these cultures during the course of the experiment.

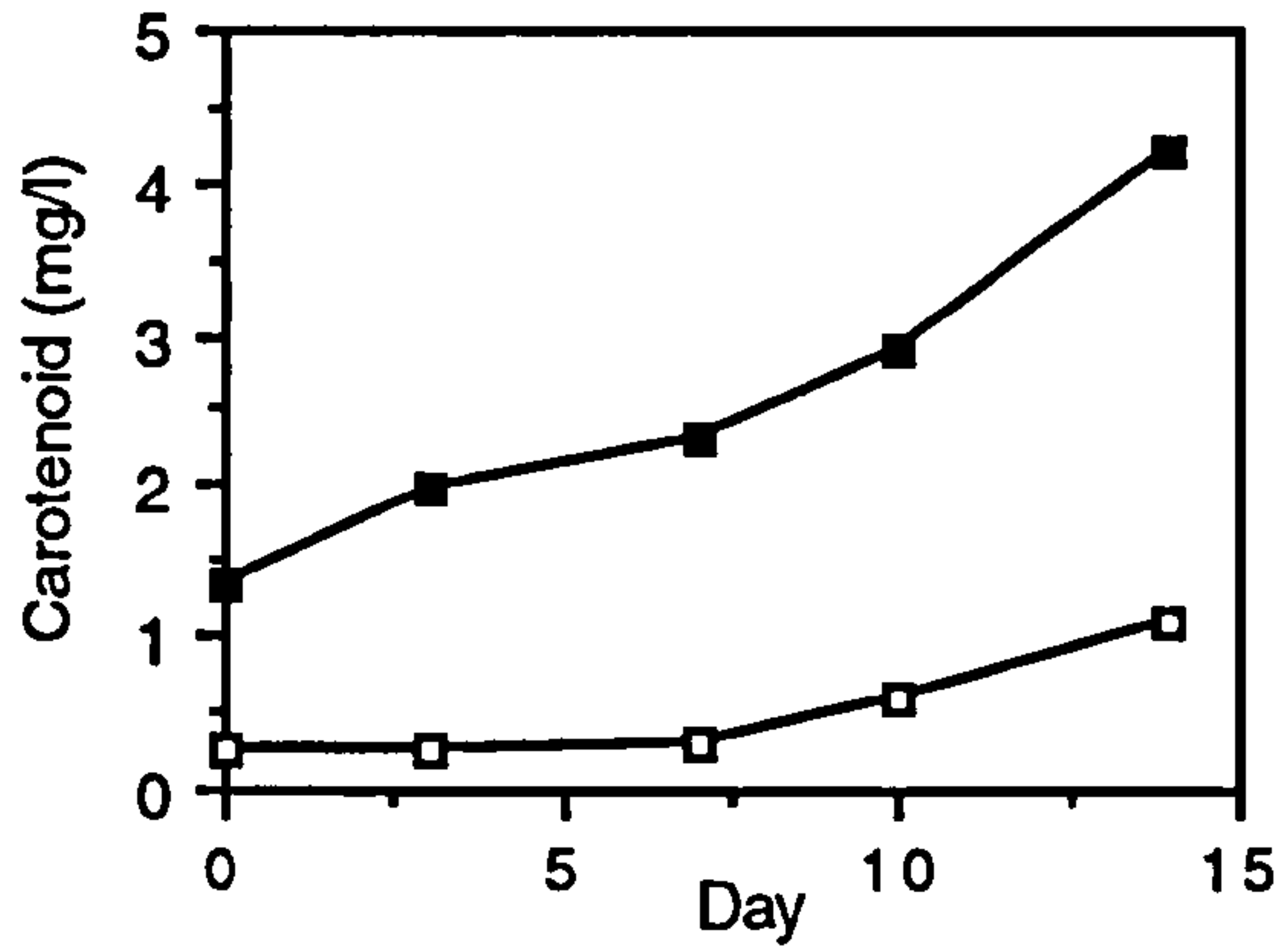
Figure 9.13a shows that on the final day of the experiment the carotenoid composition of the cultures exposed to inducing conditions in the absence of CPTA was composed of mainly secondary carotenoids (70.60%). The xanthophylls accounted for 18.30% of the total while  $\beta$ -carotene (I) comprised 11.09% of the final carotenoid composition. When the cultures were exposed to secondary carotenoid inducing conditions in the presence of CPTA (Fig. 9.13b) the majority of the final carotenoid composition comprised of the xanthophylls (72.57%). Lycopene (XIII) was the next major pigment which accounted for 24.37% of the total. The levels of secondary carotenoids and  $\beta$ -carotene (I) were very low at 1.21% and 1.85% of total carotenoid, respectively.

The relative levels of primary carotenoids on the final day between the two treatments indicate that CPTA exerts a differential effect on the  $\epsilon$ - and  $\beta$ - cyclases, respectively. In the control treatments the relative proportion of primary carotenoids were neoxanthin (XX) 6.5%, violaxanthin (XVII) 3.4%, antheraxanthin (XVIII) 2.7%, lutein (XVI) 46.6%, zeaxanthin (XV) 3.1% and  $\beta$ -carotene (I) 37.8%. However, in the CPTA treated cells the relative proportions of primary carotenoids was neoxanthin (XX) 12.6%, violaxanthin (XVII) 8.6%, antheraxanthin (XVIII) 0.8%, lutein (XVI) 70.3%, zeaxanthin (XV) 5.0% and  $\beta$ -carotene (I) 2.6%. The high proportion of lutein (XVI) in the CPTA treatments indicates that the  $\epsilon$ - cyclase is not as strongly inhibited as the  $\beta$ -cyclase in these treatments.

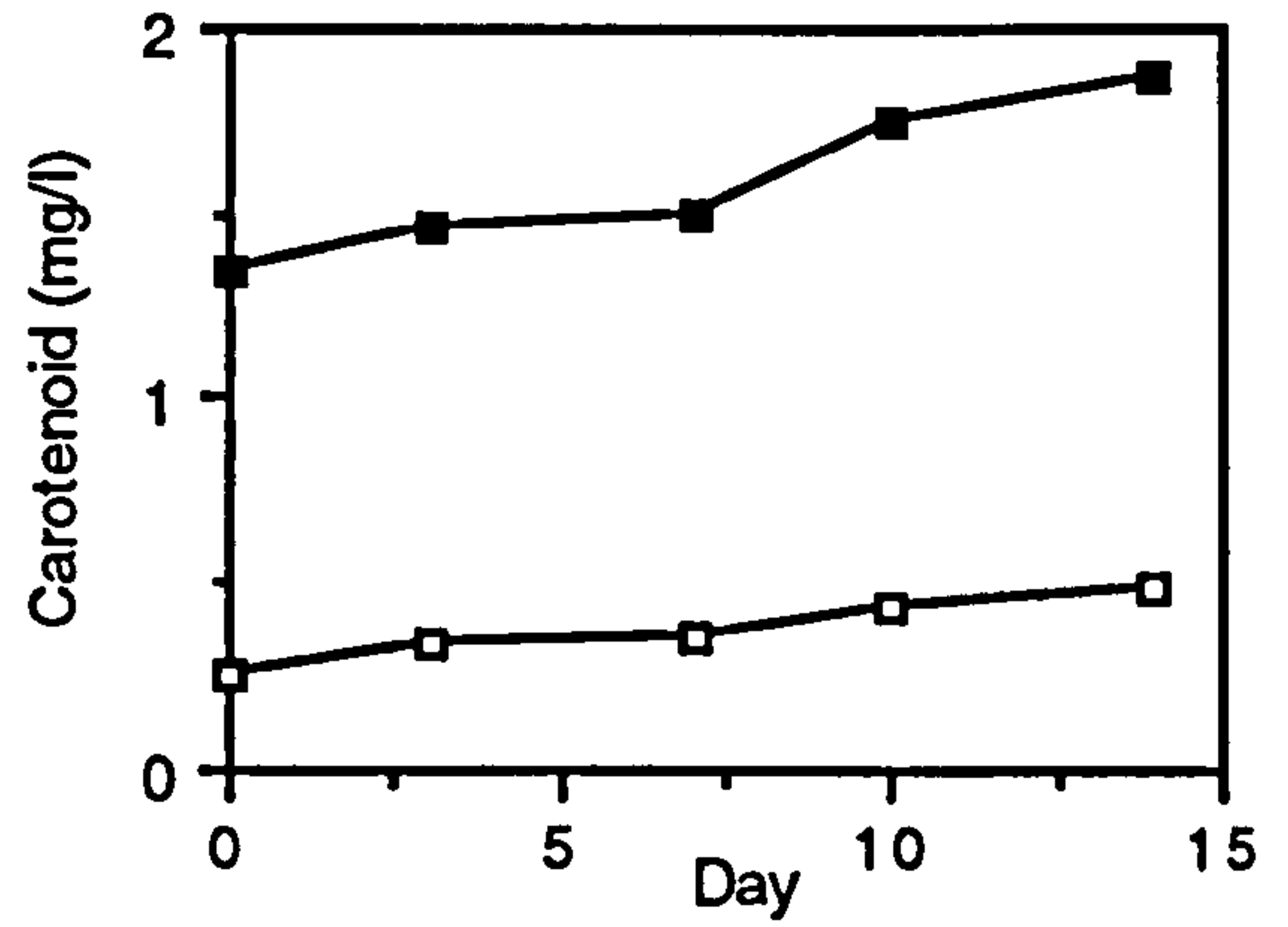
### 9.3.4 Diphenylamine

Figures 9.14a-d show the effect on carotenoid composition when cells of *H. pluvialis* were exposed to 100  $\mu$ M DPA during the synthesis of secondary carotenoids.

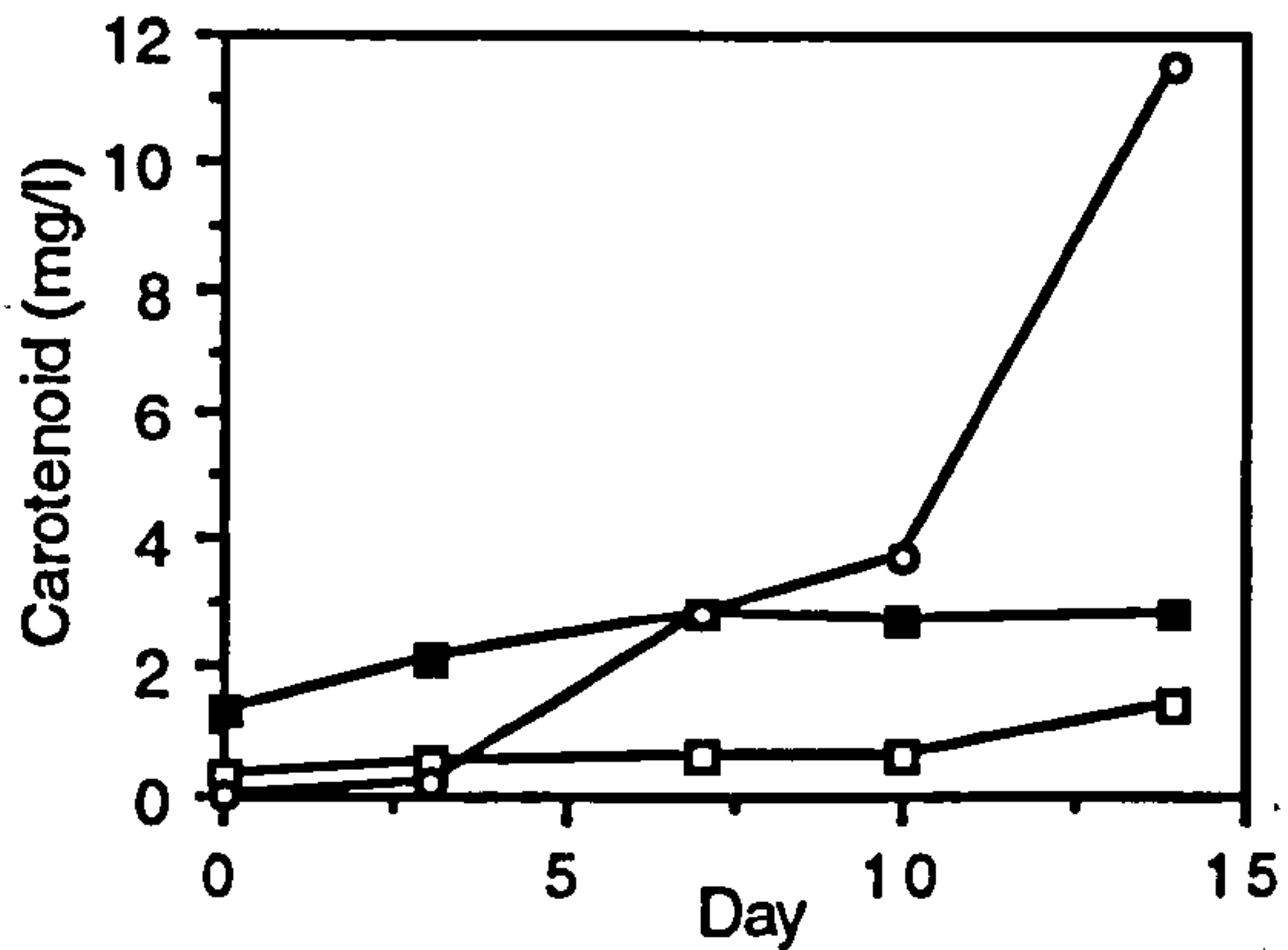
**Fig. 9.14a** No DPA: non-inducing conditions.



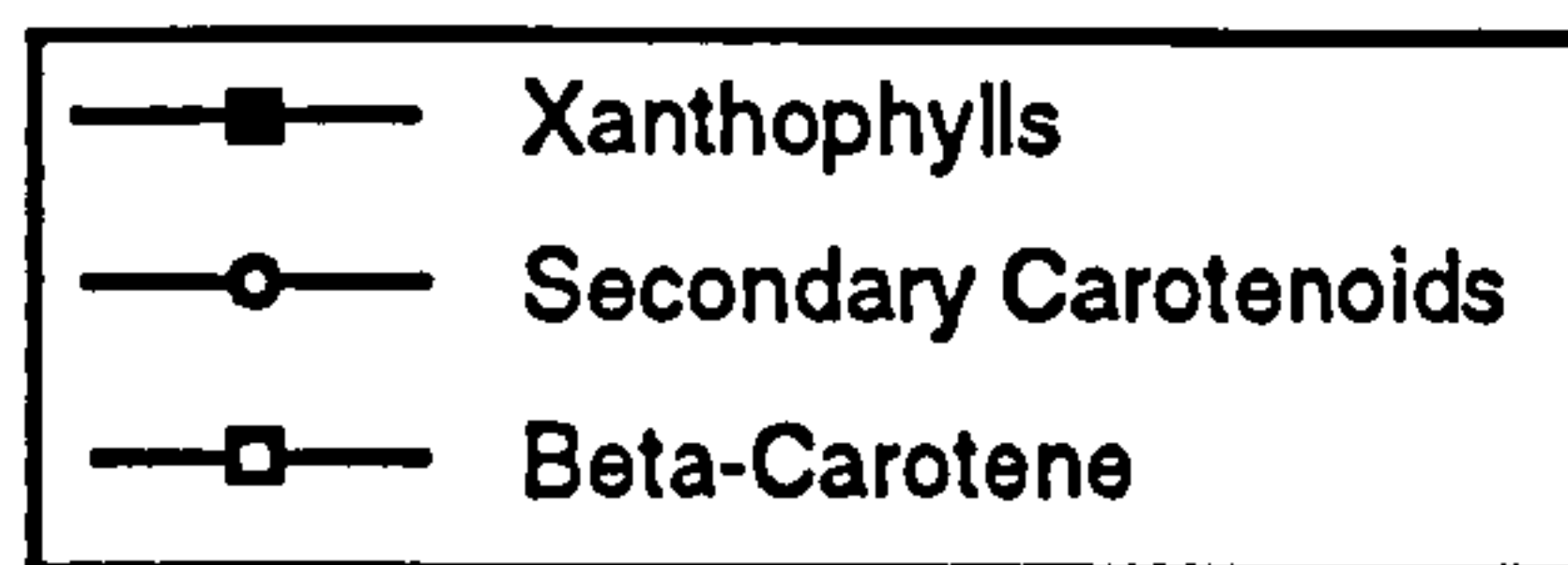
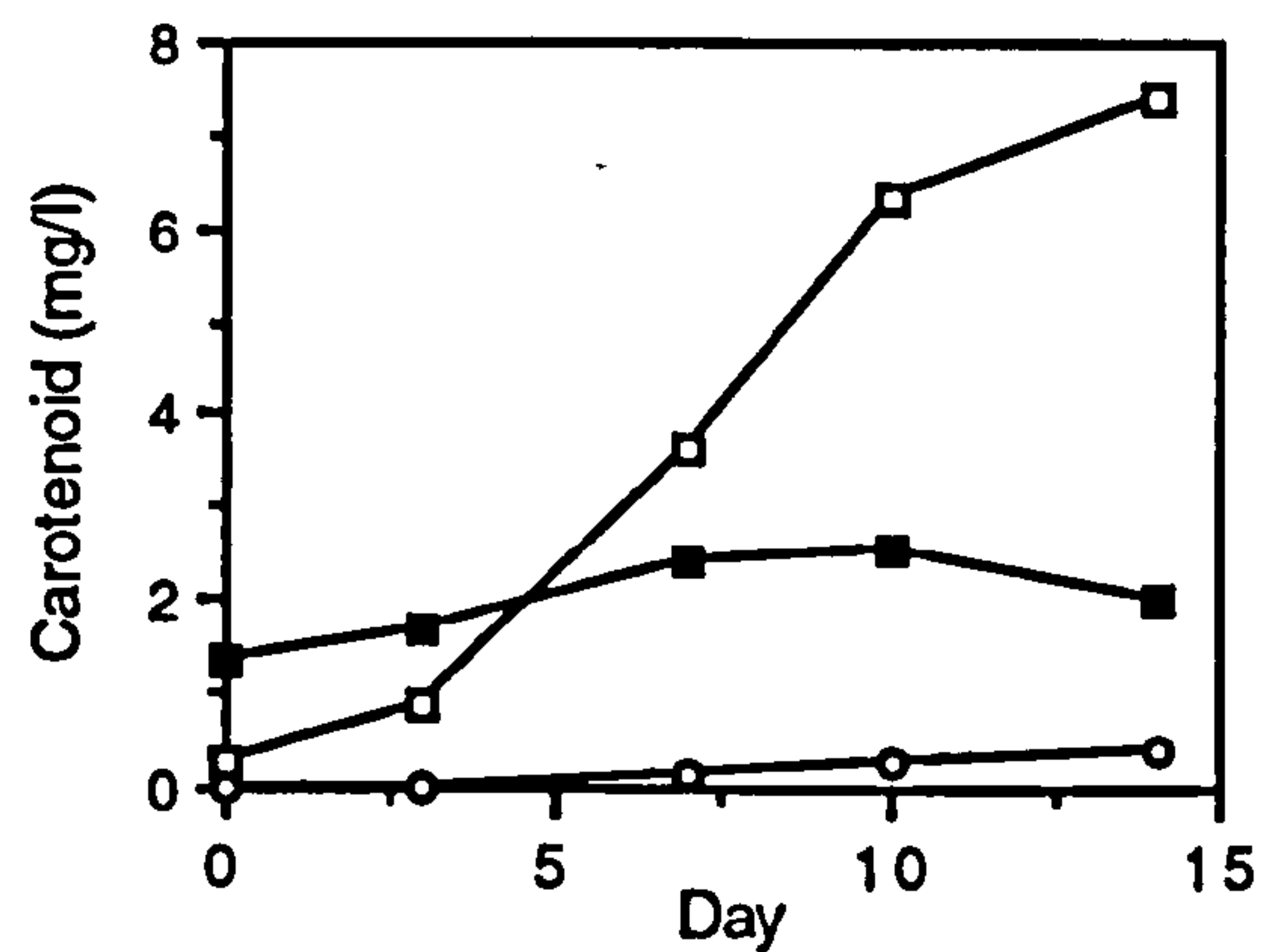
**Fig. 9.14b** 100  $\mu$ M DPA: non-inducing conditions.



**Fig. 9.14c** No DPA: secondary carotenoid inducing conditions.



**Fig. 9.14d** 100  $\mu$ M DPA: secondary carotenoid inducing conditions.

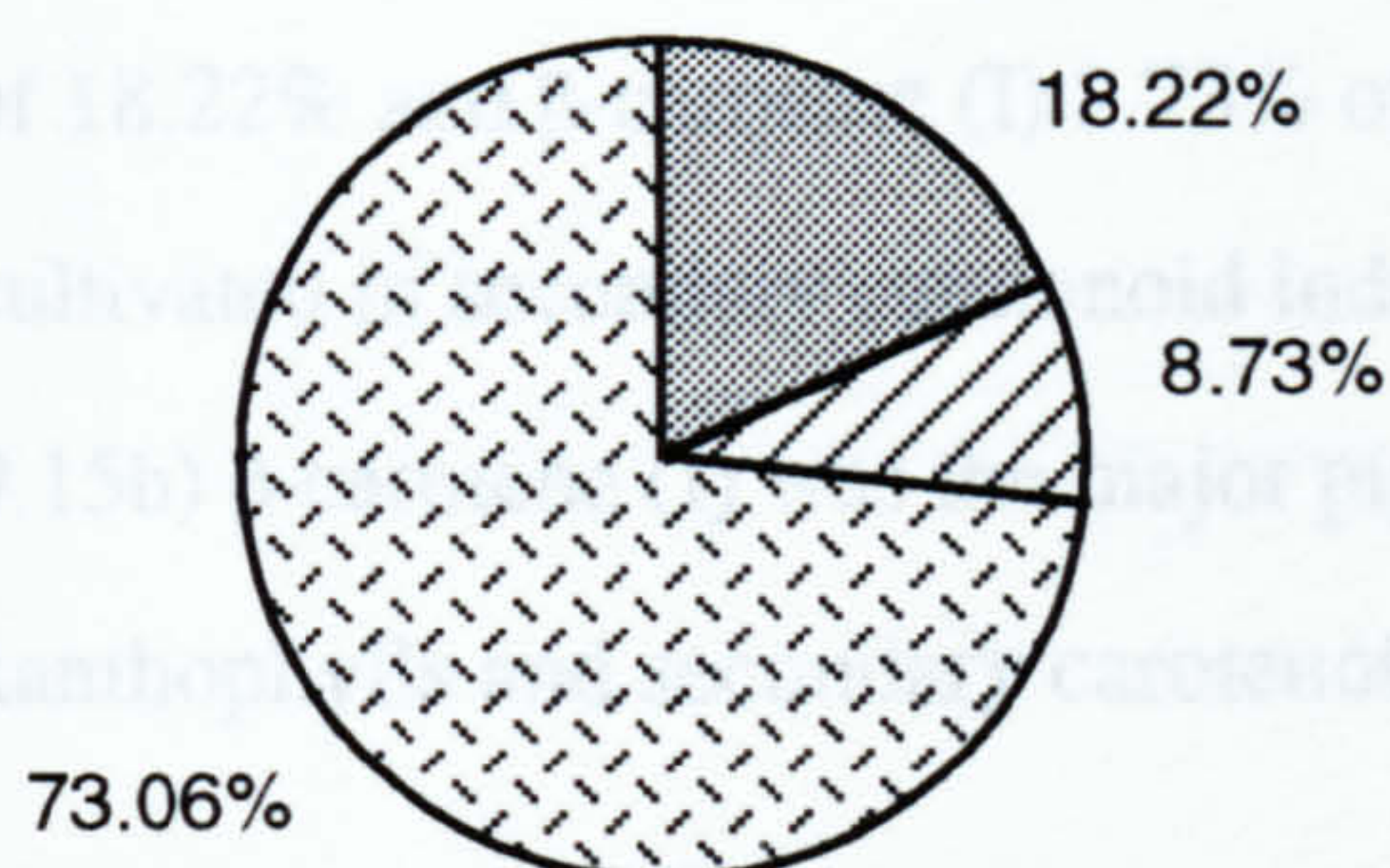


**Fig. 9.14a-d** The effect of DPA on carotenoid composition in *H. pluvialis* (S.E.  $\pm$ 3.5%, n=3).

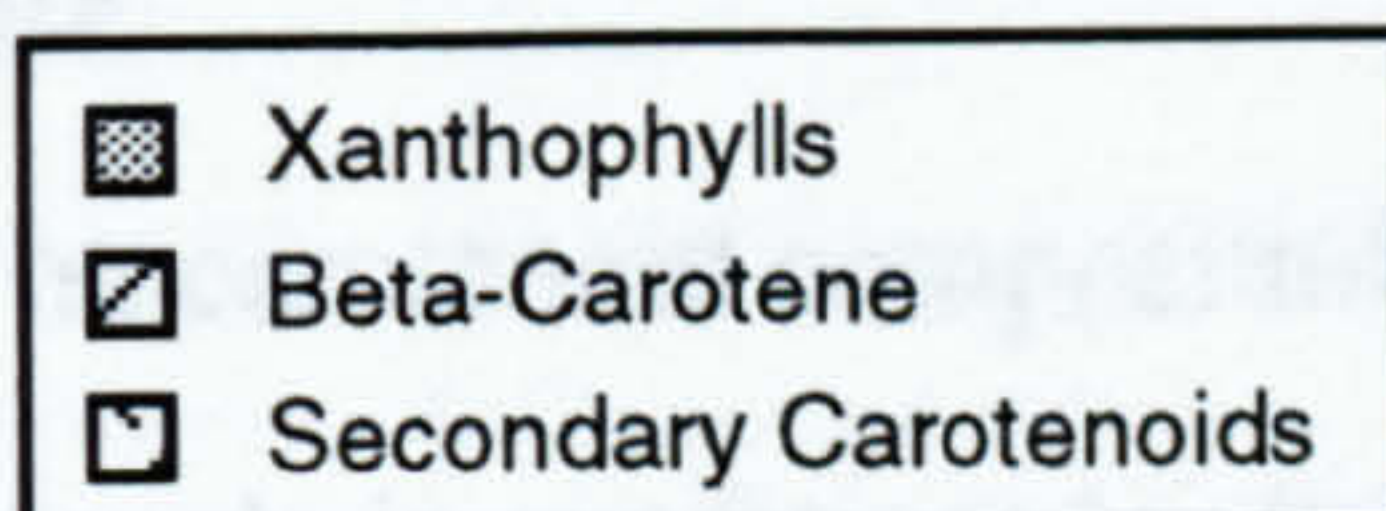
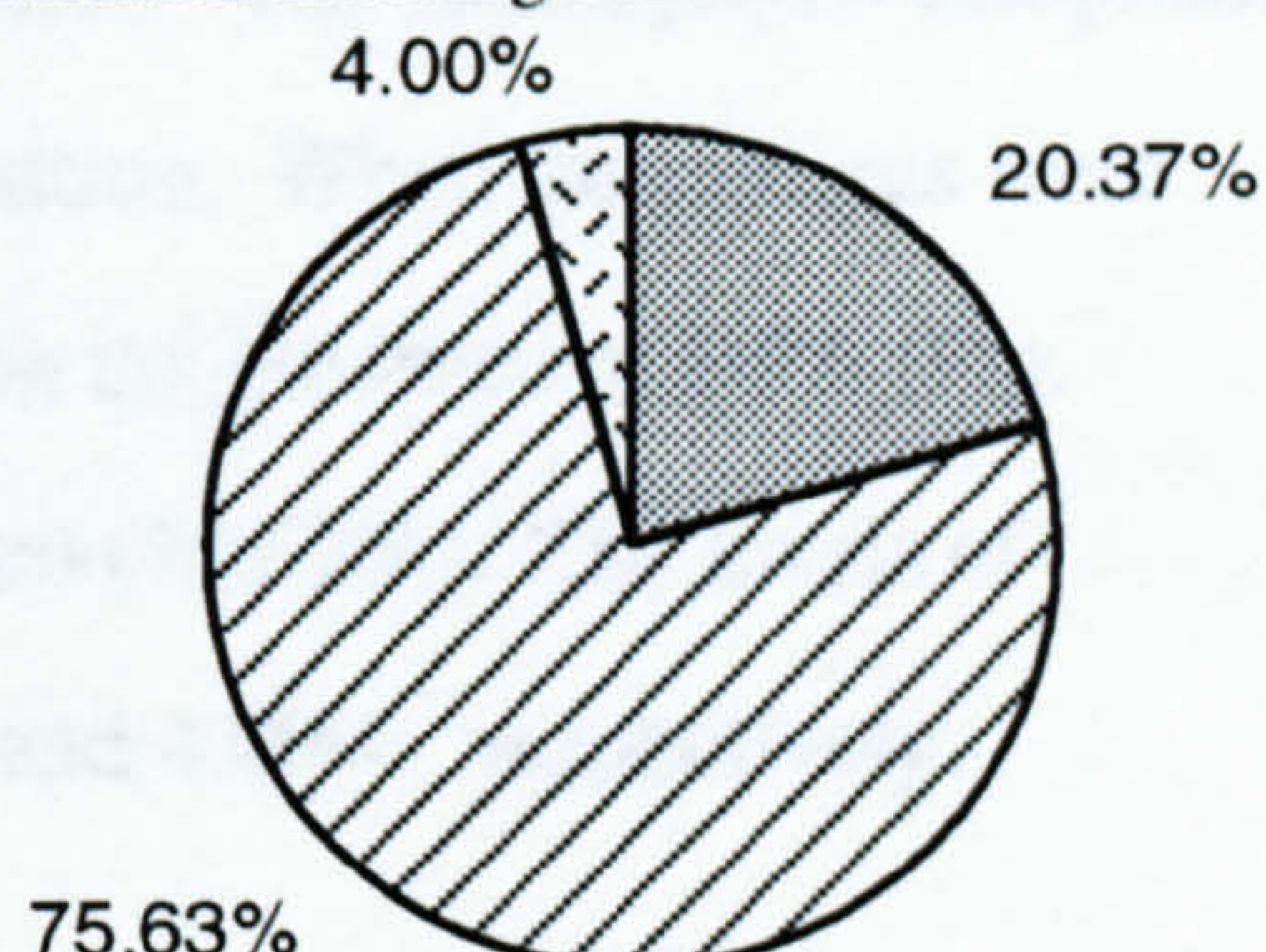


Figures 9.15a and 9.15b show the effect on carotenoid composition on the final day on the final day of the experiment when cells of *H. pluvialis* were exposed to 100  $\mu$ M DPA in conditions leading to secondary carotenoid synthesis.

**Fig. 9.15a** No DPA: secondary carotenoid inducing conditions.



**Fig. 9.15b** 100  $\mu$ M DPA: secondary carotenoid inducing conditions.



**Fig. 9.15a-b** The final carotenoid composition of cells of *H. pluvialis* when incubated under secondary carotenoid inducing conditions with and without DPA (S.E.  $\pm 2.9\%$ ,  $n=3$ ).

Figure 9.14a shows that the cultures exposed to non-inducing conditions in the absence of DPA showed a relatively large increase in the levels of xanthophylls during the experiment. The levels of  $\beta$ -carotene (I) also increased throughout the experiment albeit at a lower rate than that of the xanthophylls. No secondary carotenoids or precursors were detected in these cultures. When the cultures were exposed to non-inducing conditions in the presence of DPA (Fig. 9.14b) the cultures showed relatively low increases in the levels of xanthophylls and  $\beta$ -carotene (I) during the experiment. Again no secondary carotenoids or precursors were detected in these cultures.

Figure 9.14c indicates that cultures exposed to secondary carotenoid inducing conditions in the absence of DPA showed a relatively large accumulation of secondary carotenoids. The levels of xanthophylls increased slightly during the experiment as did the levels of  $\beta$ -carotene (I). When the cultures were exposed to secondary carotenoid inducing conditions in the presence of DPA (Fig. 9.14d) they accumulated relatively large amounts of  $\beta$ -carotene (I). The levels of xanthophylls initially increased but then decreased in the latter part of the experiment. The levels of secondary carotenoids began to increase slowly



during the latter stages of the experiment. During the course of the experiment no phytoene (VII and VIII) or phytofluene (IX) were detected in the cultures.

Figure 9.15a shows that the final carotenoid composition of cultures exposed to secondary carotenoid inducing conditions in the absence of DPA results in secondary carotenoids accounting for 73.06% of the total composition. The xanthophylls comprised of 18.22% and  $\beta$ -carotene (I) 8.73% of the total composition. When the cultures were cultivated in secondary carotenoid inducing conditions in the presence of DPA (Fig. 9.15b)  $\beta$ -carotene (I) was the major pigment accumulated (75.63%). The levels of xanthophylls and secondary carotenoids were 20.37% and 4.00%, respectively.

### 9.3.5 1-Aminobenzotriazole

Figures 9.16a-d show the effect on carotenoid composition when cells of *H. pluvialis* were exposed to 1-aminobenzotriazole in conditions leading to secondary carotenoid synthesis.

Fig. 9.16a No 1-aminobenzotriazole: non-inducing conditions.

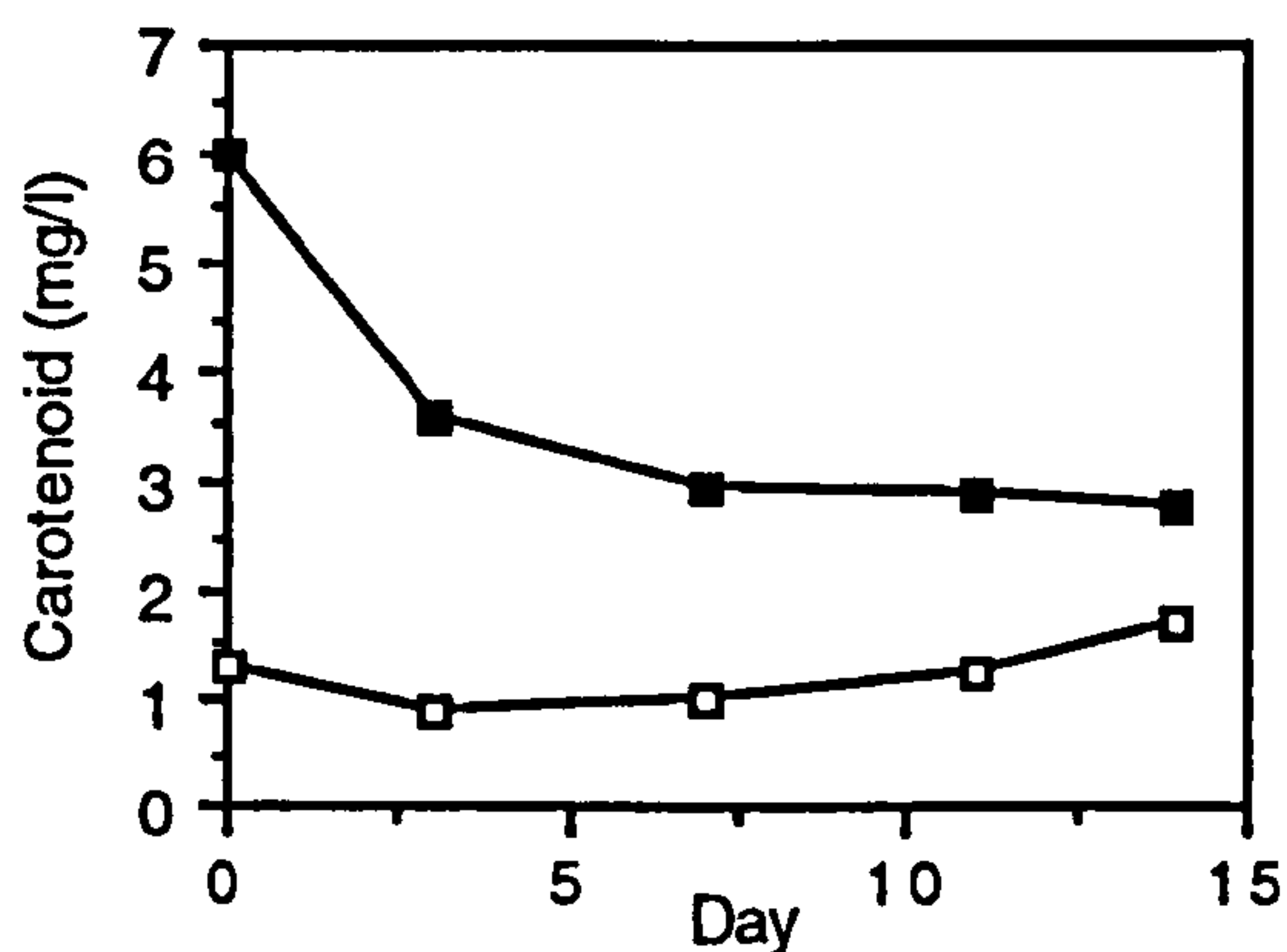
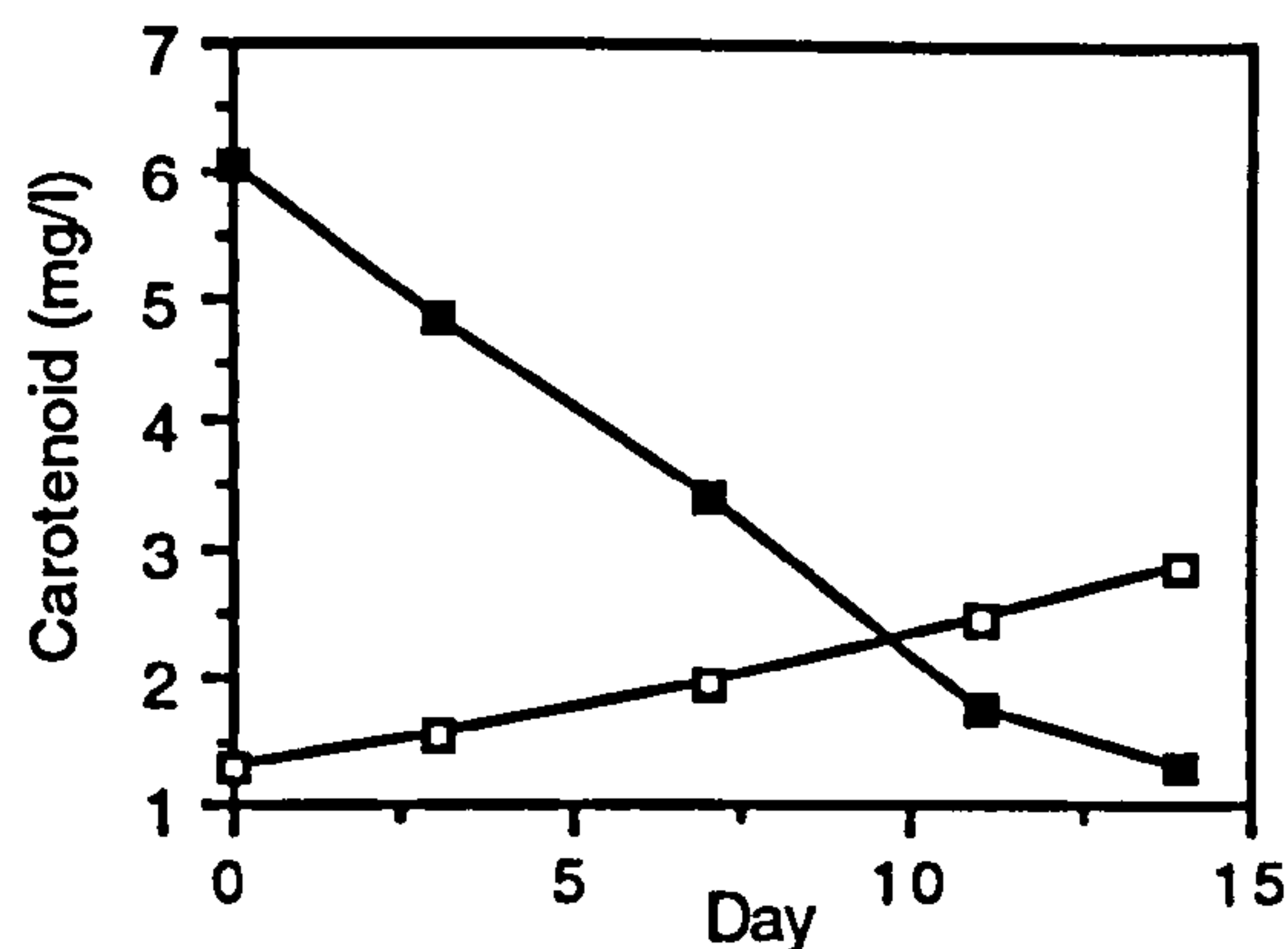
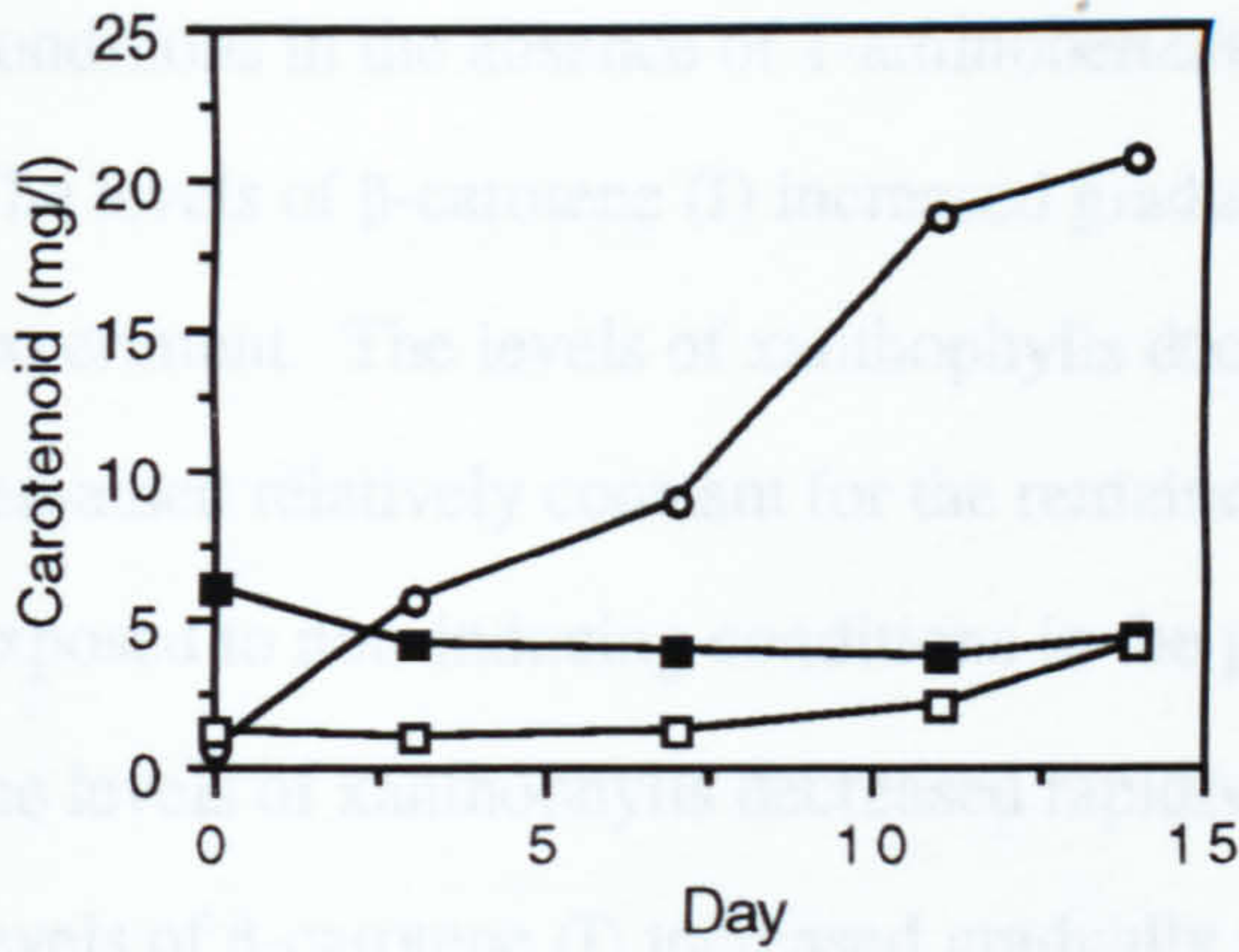


Fig. 9.16b 1000  $\mu$ M 1-aminobenzotriazole: non-inducing conditions.

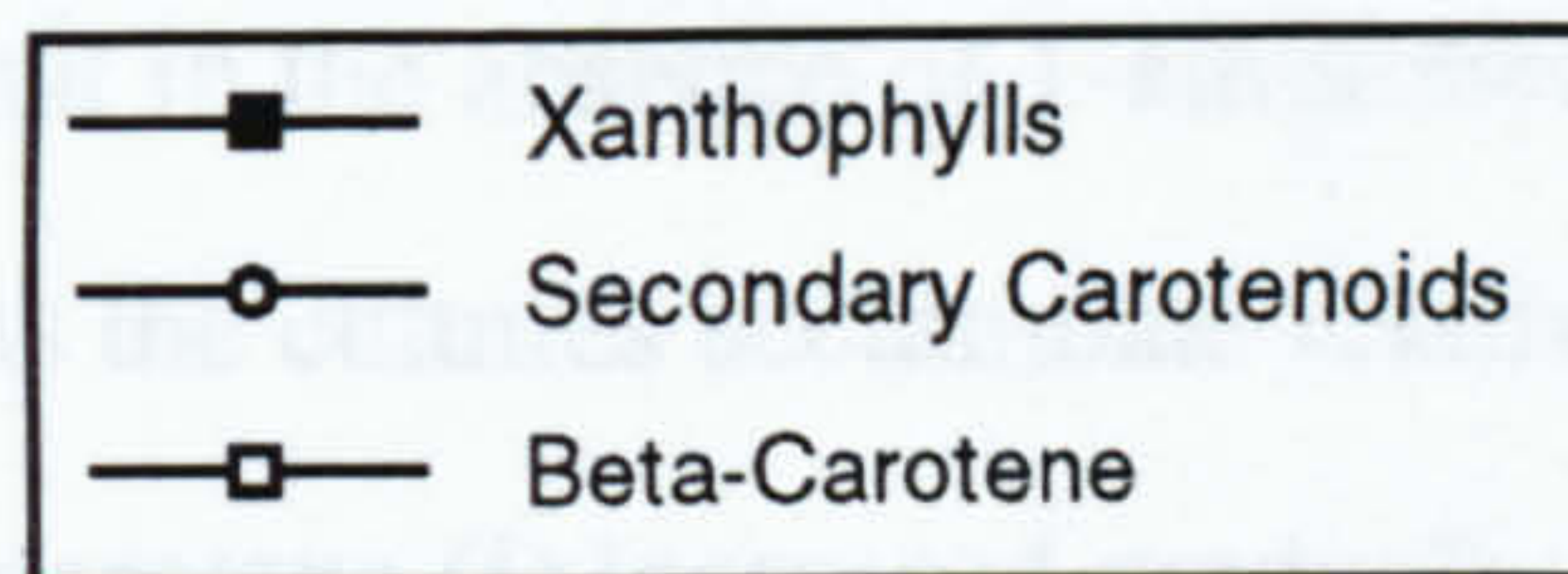
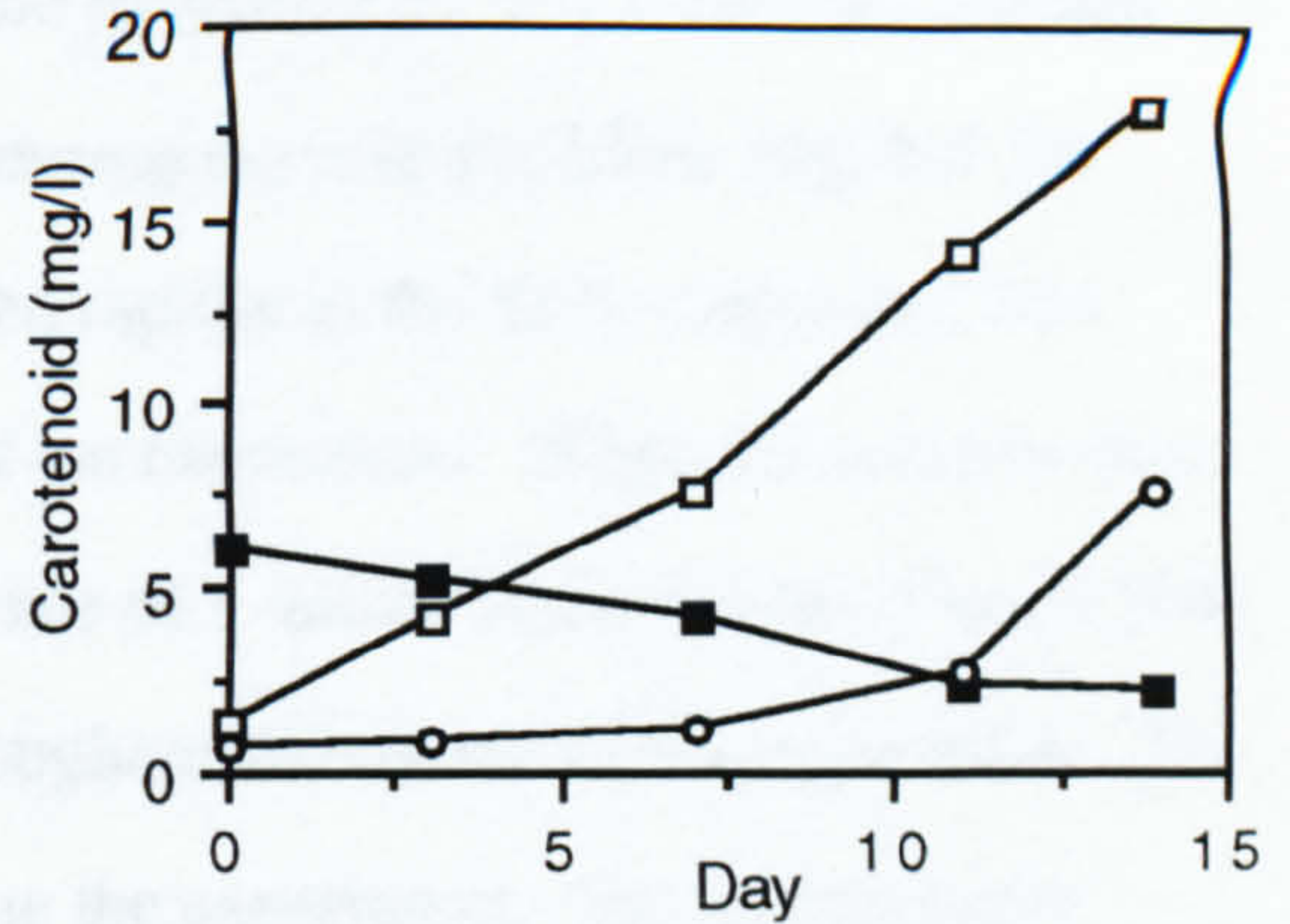




**Fig. 9.16c** No 1-aminobenzotriazole: secondary carotenoid inducing conditions.



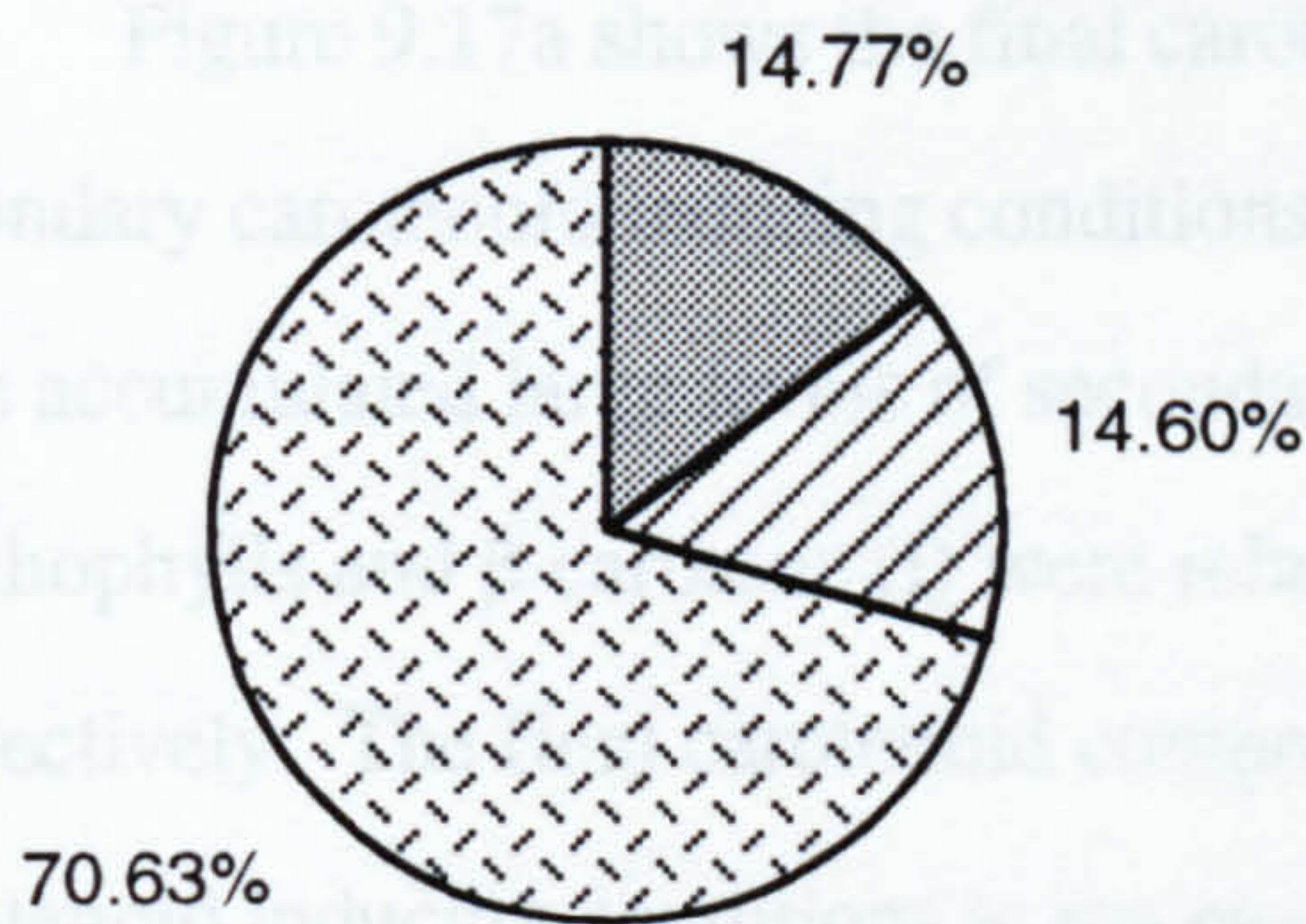
**Fig. 9.16d** 1000  $\mu$ M 1-aminobenzotriazole: secondary carotenoid inducing conditions.



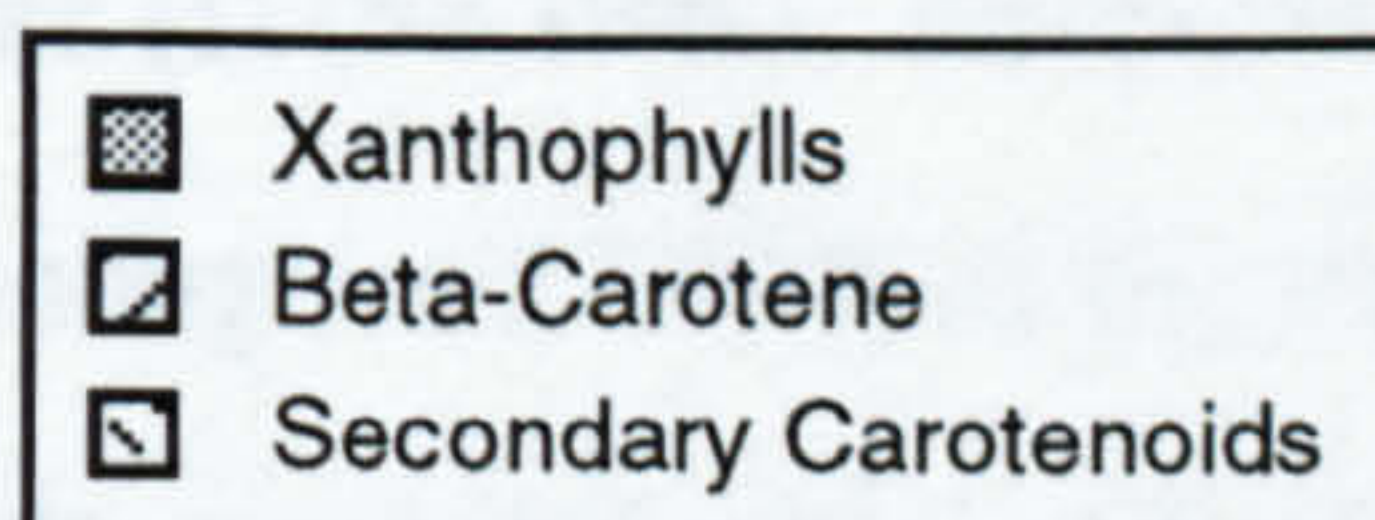
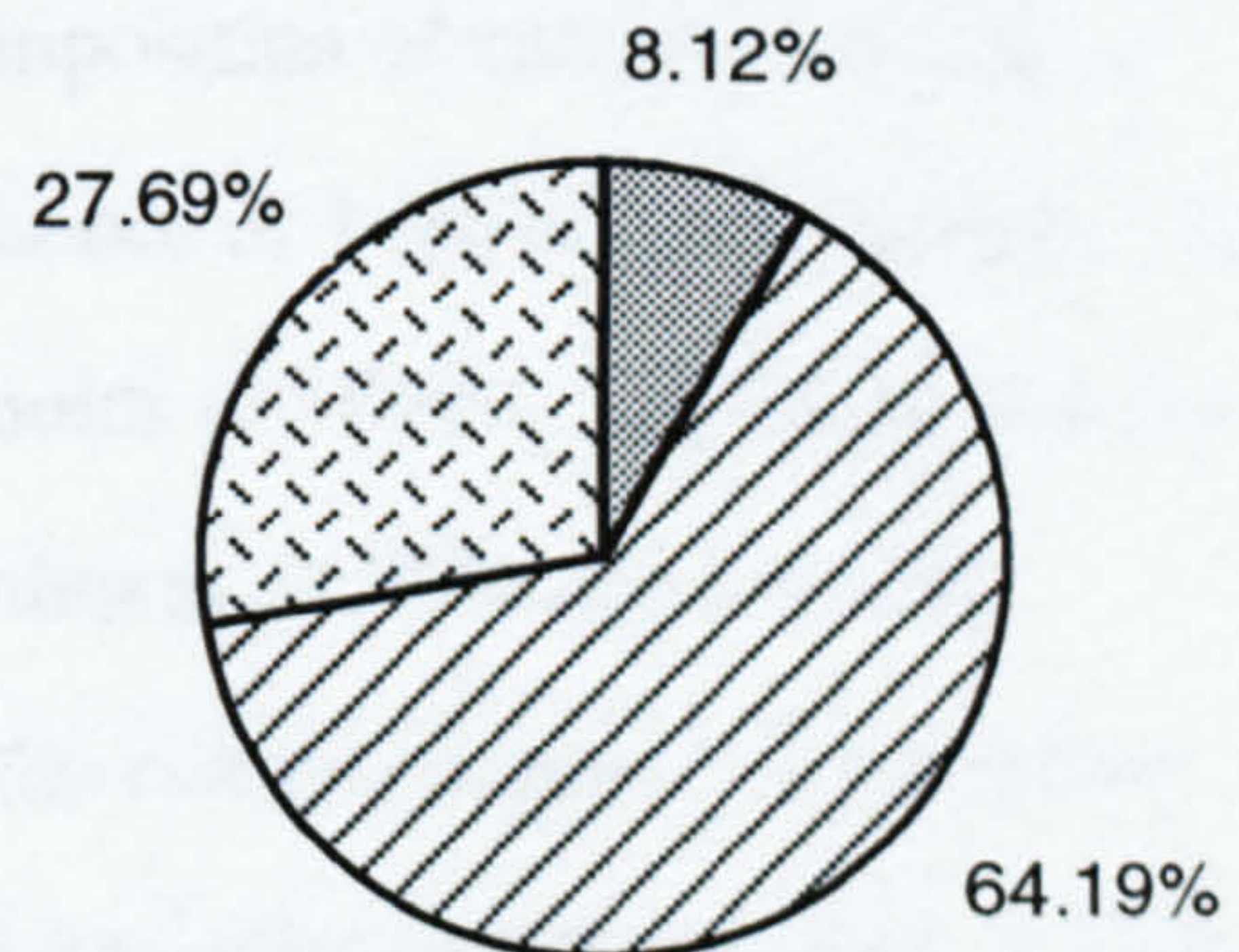
**Fig. 9.16a-d** The effect of 1-aminobenzotriazole on carotenoid composition in *H. pluvialis* (S.E.  $\pm 2.0\%$ ,  $n=3$ ).

Figures 9.17a and 9.17b show the effect on carotenoid composition on the final day of the experiment when cells of *H. pluvialis* were exposed to 100  $\mu$ M 1-aminobenzotriazole in conditions leading to secondary carotenoid synthesis.

**Fig. 9.17a** No 1-aminobenzotriazole: secondary carotenoid inducing conditions.



**Fig. 9.17b** 1000  $\mu$ M 1-aminobenzotriazole: secondary carotenoid inducing conditions.



**Fig. 9.17a-b** The final carotenoid composition of cells of *H. pluvialis* when incubated under secondary carotenoid inducing conditions with and without 1-aminobenzotriazole (S.E.  $\pm 1.5\%$ ,  $n=3$ ).



Figure 9.16a indicates that cultures of *H. pluvialis* cultivated under non-inducing conditions in the absence of 1-aminobenzotriazole produced no secondary carotenoids. The levels of  $\beta$ -carotene (I) increased gradually during the mid and latter stages of the experiment. The levels of xanthophylls decreased rapidly in the early stages and then remained relatively constant for the remainder of the experiment. When the cultures were exposed to non-inducing conditions in the presence of 1-aminobenzotriazole (Fig. 9.16b) the levels of xanthophylls decreased rapidly throughout the course of the experiment. The levels of  $\beta$ -carotene (I) increased gradually during the experiment, and no secondary carotenoids or precursors were detected in the cultures.

Figure 9.16c shows that in the absence of 1-aminobenzotriazole under secondary carotenoid inducing conditions the cultures accumulate relatively large levels of secondary carotenoids. The levels of  $\beta$ -carotene (I) increased gradually, while the levels of xanthophylls decreased slightly during the experiment. When the cultures were cultivated under secondary carotenoid inducing conditions in the presence of 1-aminobenzotriazole (Fig. 9.16d) the cultures accumulated relatively large levels of  $\beta$ -carotene (I). The levels of secondary carotenoids increased slightly up to day 11 after which the level rose quite rapidly. The levels of primary carotenoids decreased at a constant rate throughout the experiment. During the course of the experiment phytoene (VII and VIII) and phytofluene (IX) were not detected.

Figure 9.17a shows the final carotenoid composition of cultures exposed to secondary carotenoid inducing conditions in the absence of 1-aminobenzotriazole. These cells accumulated large levels of secondary carotenoids (70.63%), whilst the levels of xanthophylls and  $\beta$ -carotene (I) were relatively similar at 14.77% and 14.60%, respectively. The final carotenoid composition of the cultures exposed to secondary carotenoid inducing conditions in the presence of 1-aminobenzotriazole (Fig. 9.17d) comprised primarily of  $\beta$ -carotene (I) (64.19%). Secondary carotenoids were the next major carotenoids present accounting for 27.69% of the total, indicating only partial inhibition of the biosynthetic pathway, while the xanthophylls accounted for only 8.12% of the total.



### 9.3.6 CPTA and norflurazon

Fig. 9.18a Removal of 10  $\mu\text{M}$  CPTA (day 0) under secondary carotenoid inducing conditions.

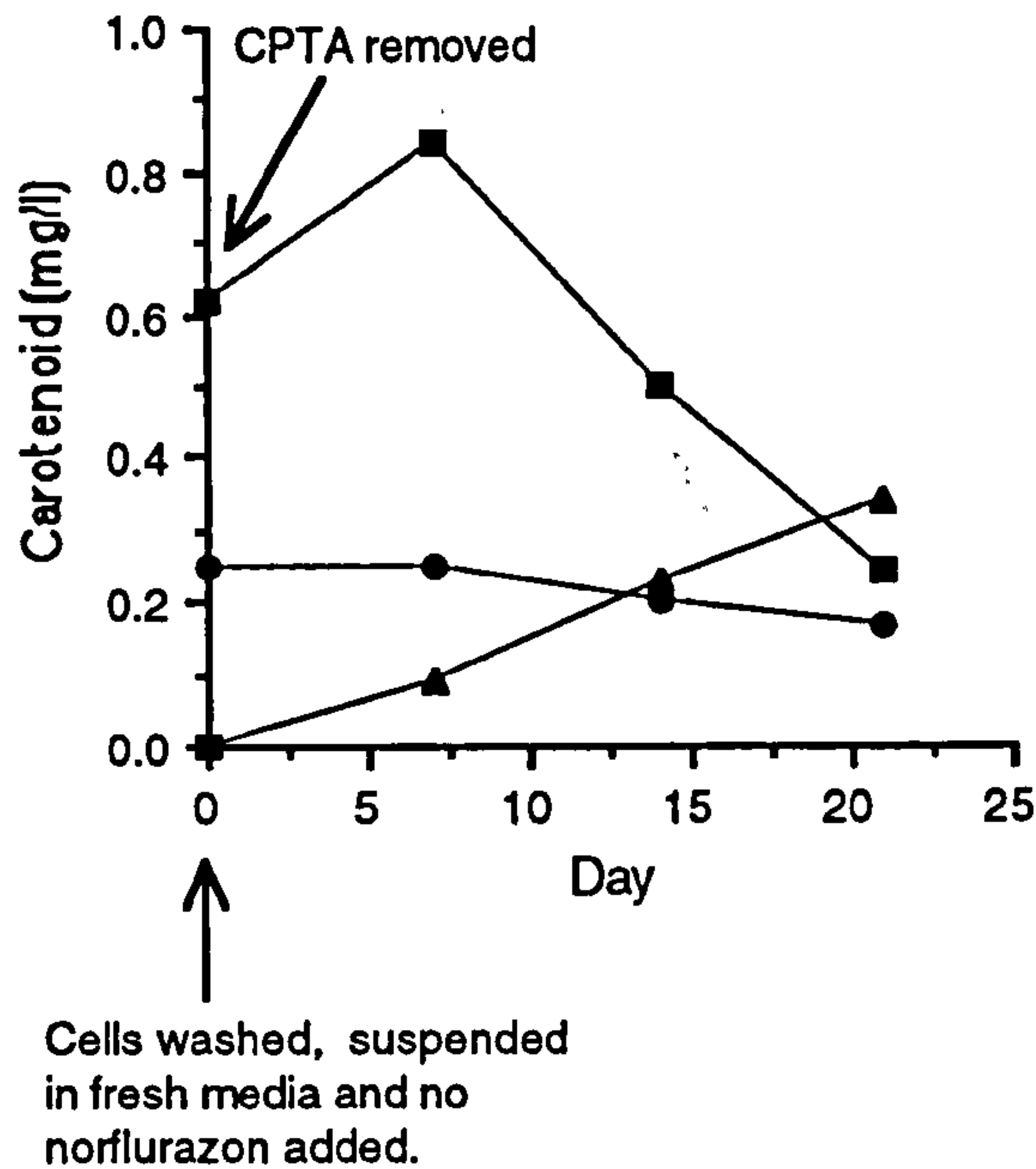


Fig. 9.18b Removal of 10  $\mu\text{M}$  CPTA followed by the addition of 100  $\mu\text{M}$  norflurazon (day 0) under secondary carotenoid inducing conditions.

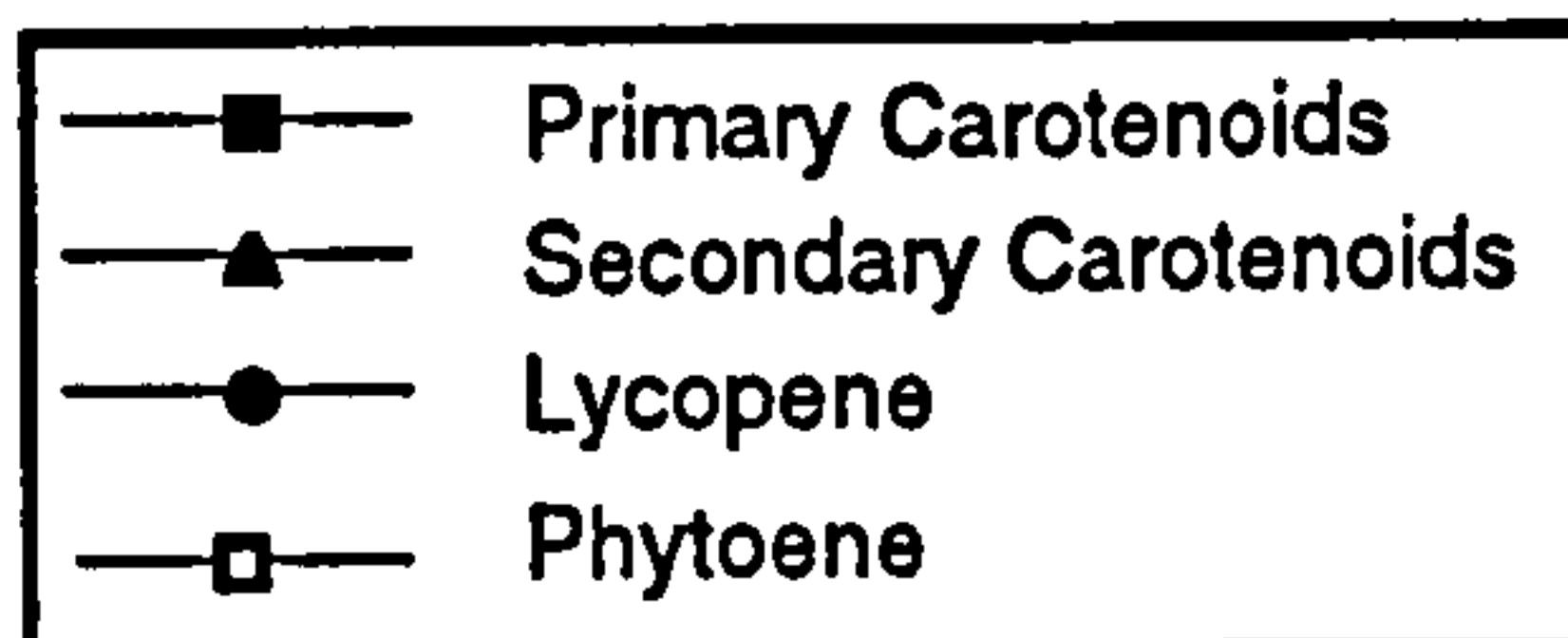
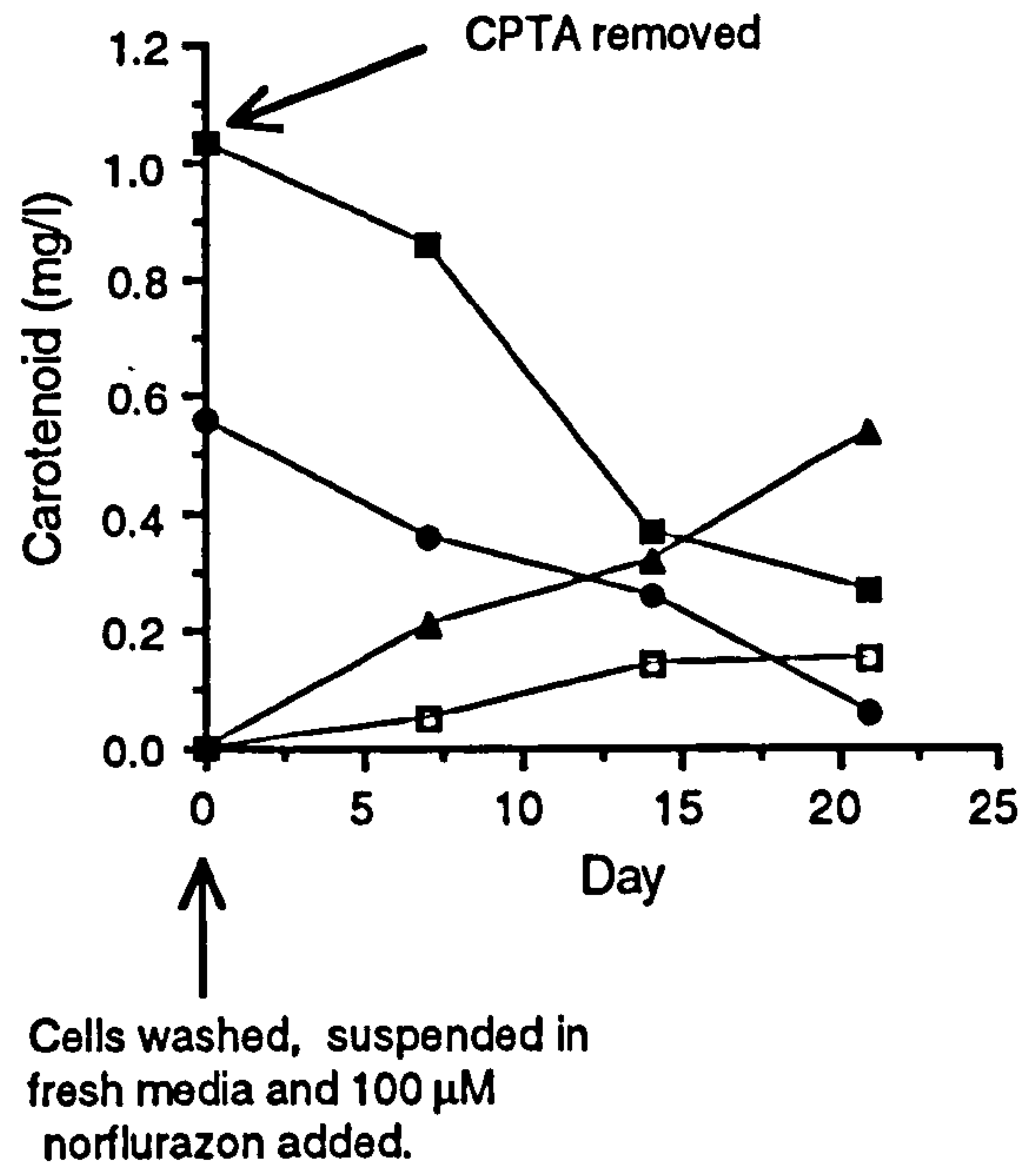


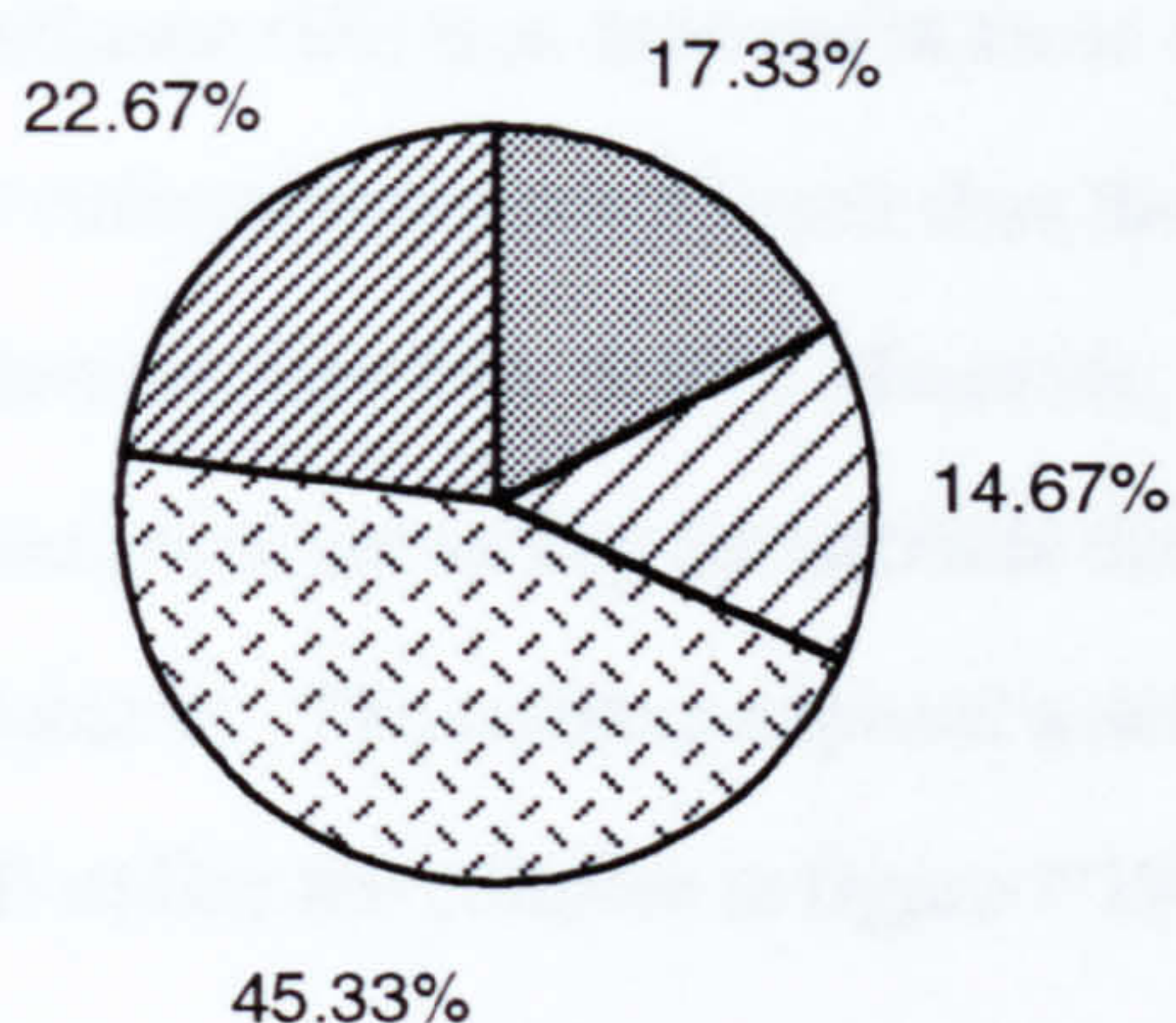
Fig. 9.18a-b The effect of removing CPTA followed by the addition of norflurazon on carotenoid composition during the synthesis of secondary carotenoids in *H. pluvialis* (S.E.  $\pm 5.1\%$ ,  $n=3$ ).

Figure 9.18a shows the pigment profile of cultures allowed to accumulate lycopene (XIII) due to treatment with CPTA, and then exposed to secondary carotenoid inducing conditions in the absence of norflurazon. The levels of primary carotenoids decreased rapidly during the mid and latter stages of the experiment. The levels of lycopene (XIII) in the cultures declined slightly, while the levels of secondary carotenoids increased steadily throughout the experiment. Figure 9.18b shows the pigment profile of cultures which had initially been treated in the same manner, but then exposed to secondary carotenoid inducing conditions in the presence of norflurazon. Again the levels of primary carotenoids decreased relatively rapidly during the experiment. The levels of lycopene (XIII) decreased at a constant rate during the experiment, the rate of which was similar to the rate of increase of the secondary carotenoid levels in the cultures. These cultures also accumulated

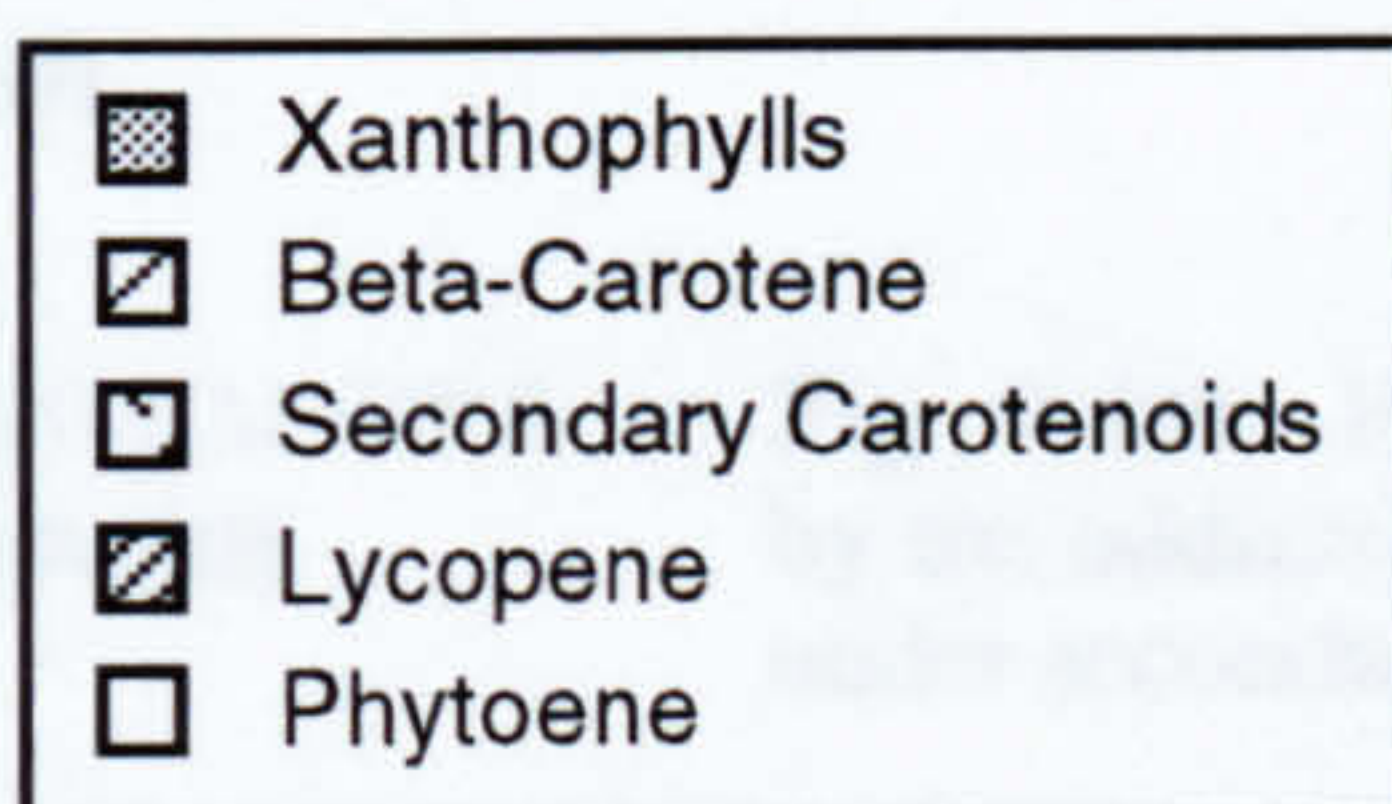
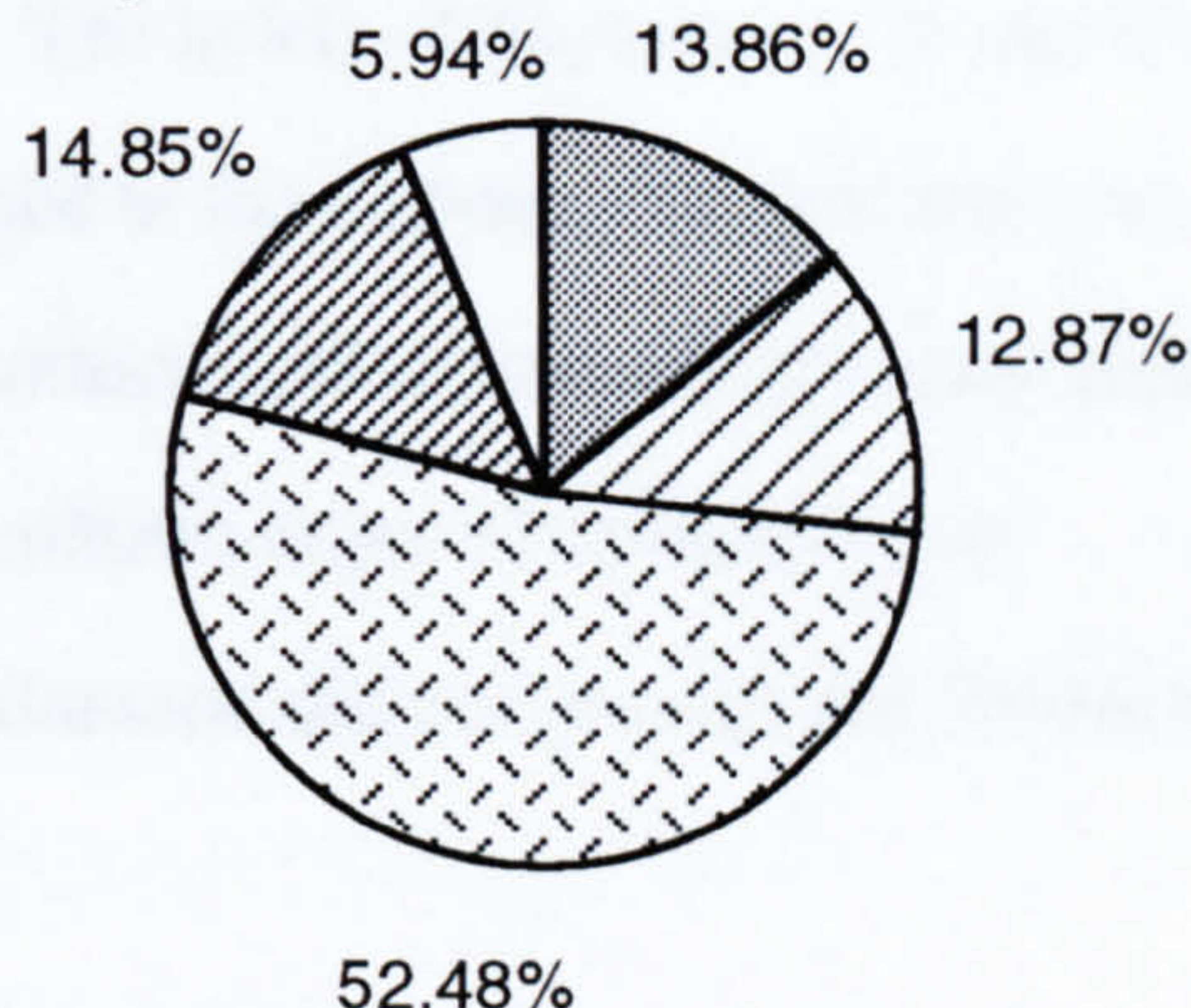


phytoene (VII and VIII), due to the dimer action of norflurazon on *de-novo* carotenogenesis. However, no phytofluene (IX) was detected in these cultures during the course of the experiment.

**Fig. 9.19a** Removal of 10  $\mu$ M CPTA (day 0) under secondary carotenoid inducing conditions.



**Fig. 9.19b** Removal of 10  $\mu$ M CPTA followed by the addition of 100  $\mu$ M norflurazon (day 0) under secondary carotenoid inducing conditions.



**Fig 9.19a-b** The final carotenoid composition of cells of *H. pluvialis* treated with CPTA followed by the removal of the inhibitor and the addition of norflurazon under secondary carotenoid inducing conditions (S.E.  $\pm 2.5\%$ ,  $n=3$ ).

Figure 9.19a shows the carotenoid composition on the final day of the experiment of cultures which had accumulated lycopene (XIII), and then been exposed to secondary carotenoid inducing conditions in the absence of norflurazon. The major pigments present were the secondary carotenoids which accounted for 45.33% of the final carotenoid composition. Lycopene (XIII) accounted for 22.67% of the total, whilst the levels of xanthophylls and  $\beta$ -carotene (I) were lower at 17.33% and 14.67%, respectively. These cultures contained a significantly higher proportion of secondary carotenoids and  $\beta$ -carotene (I) than those cultures exposed to CPTA only (see Fig. 9.13b). However, the proportion of xanthophylls was considerably lower in these cultures than those exposed exclusively to CPTA.

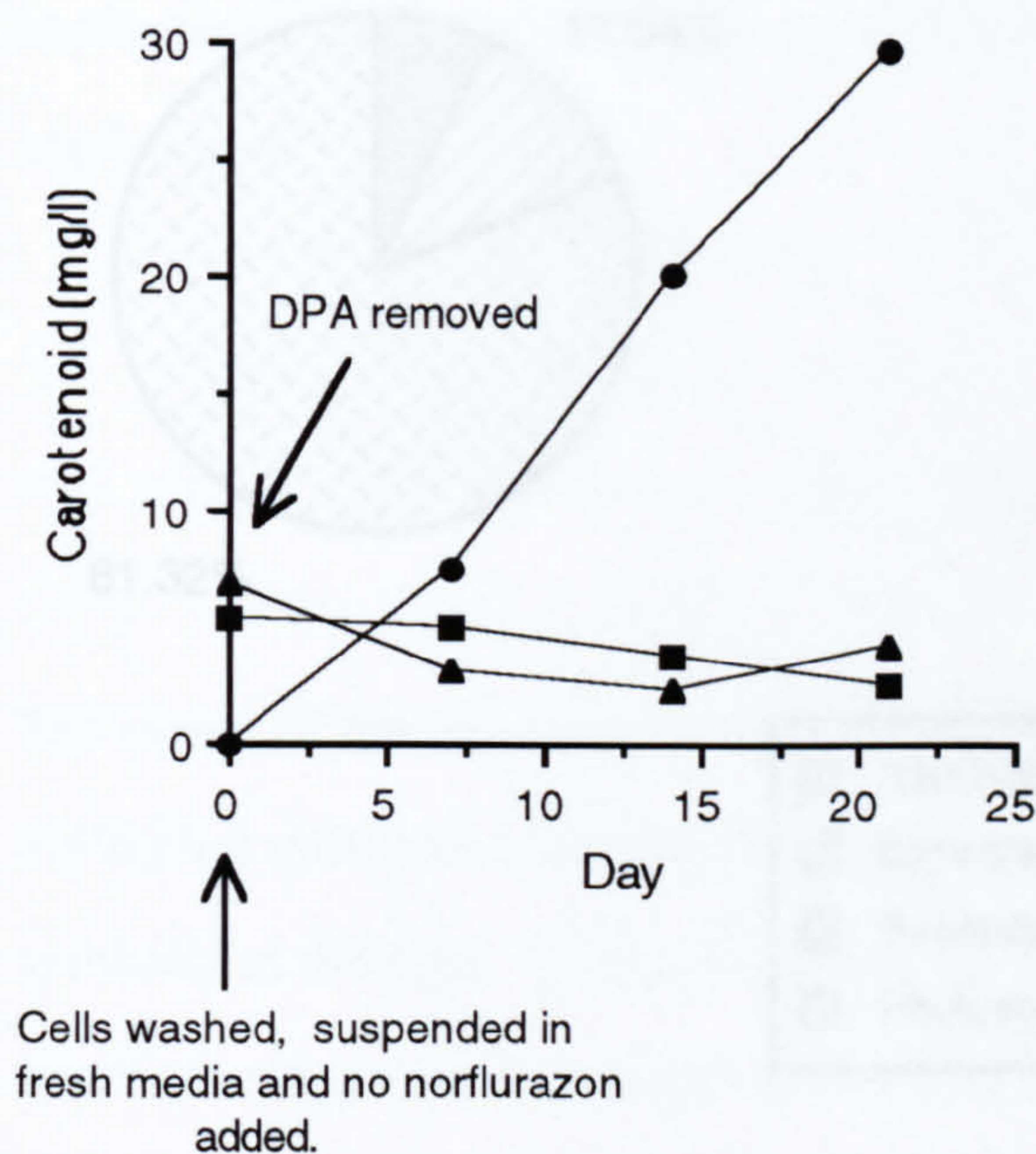
Figure 9.19b shows the final carotenoid composition of the cultures which had been allowed to accumulate lycopene(XIII), and then been exposed to secondary



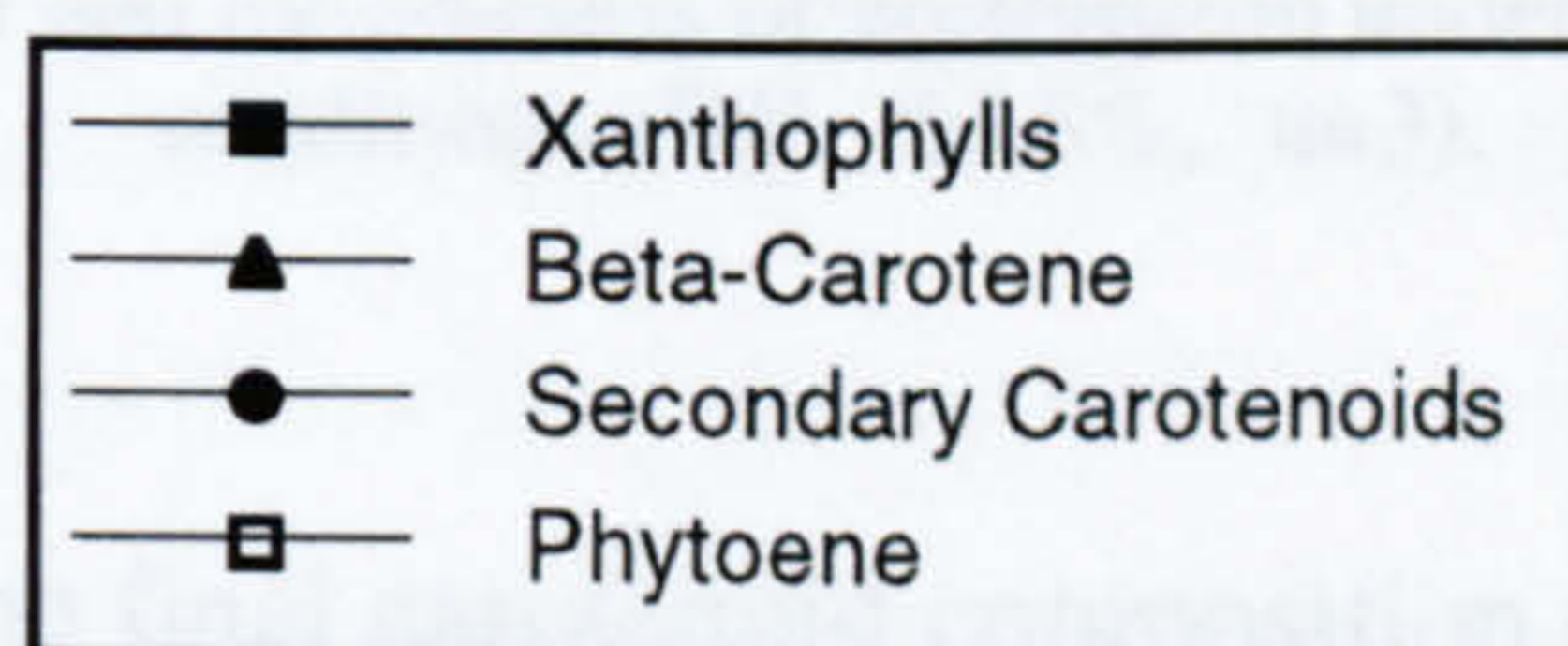
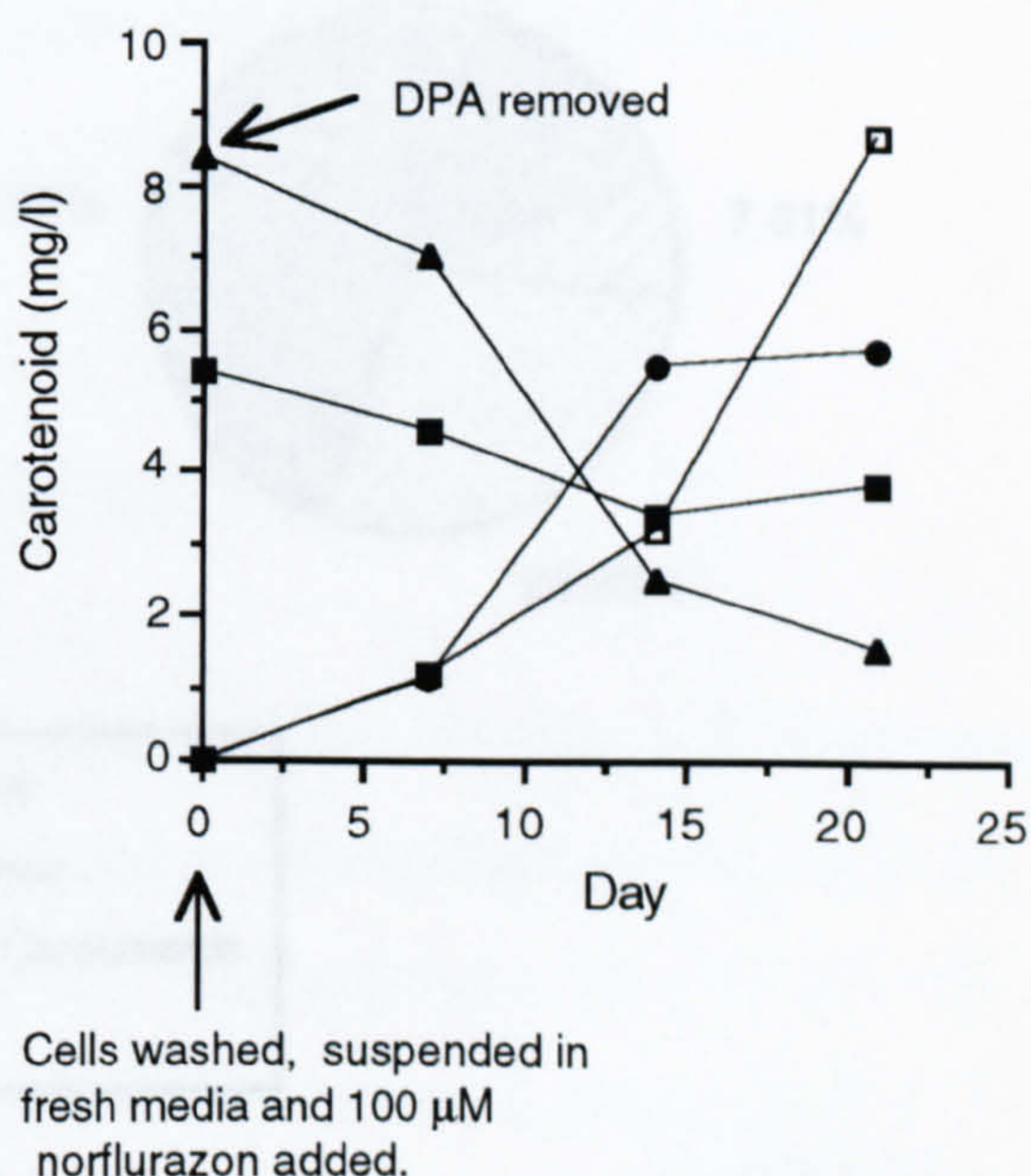
carotenoid inducing conditions in the presence of norflurazon. Again the major pigments present in the cultures were the secondary carotenoids, which accounted for 52.48% of the total. The levels of xanthophylls,  $\beta$ -carotene (I) and lycopene (XIII) were all relatively similar at 13.86%, 12.87% and 14.85%, respectively. The cells at the end of the experiment contained a relatively small amount of phytoene (VII and VIII) (5.94%), but no phytofluene (IX) was detected in these cultures. The levels of phytoene (VII and VIII) in these cultures were much lower than those detected in the cultures exposed solely to norflurazon (see Fig. 9.9b). However, these cultures contained a significantly higher proportion of secondary carotenoids than those cultures exposed exclusively to norflurazon. The cultures exposed solely to norflurazon did not contain any lycopene (XIII) unlike the cultures in figure 9.19b.

### 9.3.7 DPA and norflurazon

**Fig. 9.20a** Effect of removal of 100  $\mu$ M DPA (day 0) during secondary carotenoid inducing conditions.



**Fig 9.20b** Removal of 100  $\mu$ M DPA followed by the addition of 100  $\mu$ M norflurazon (day 0) under secondary carotenoid inducing conditions.



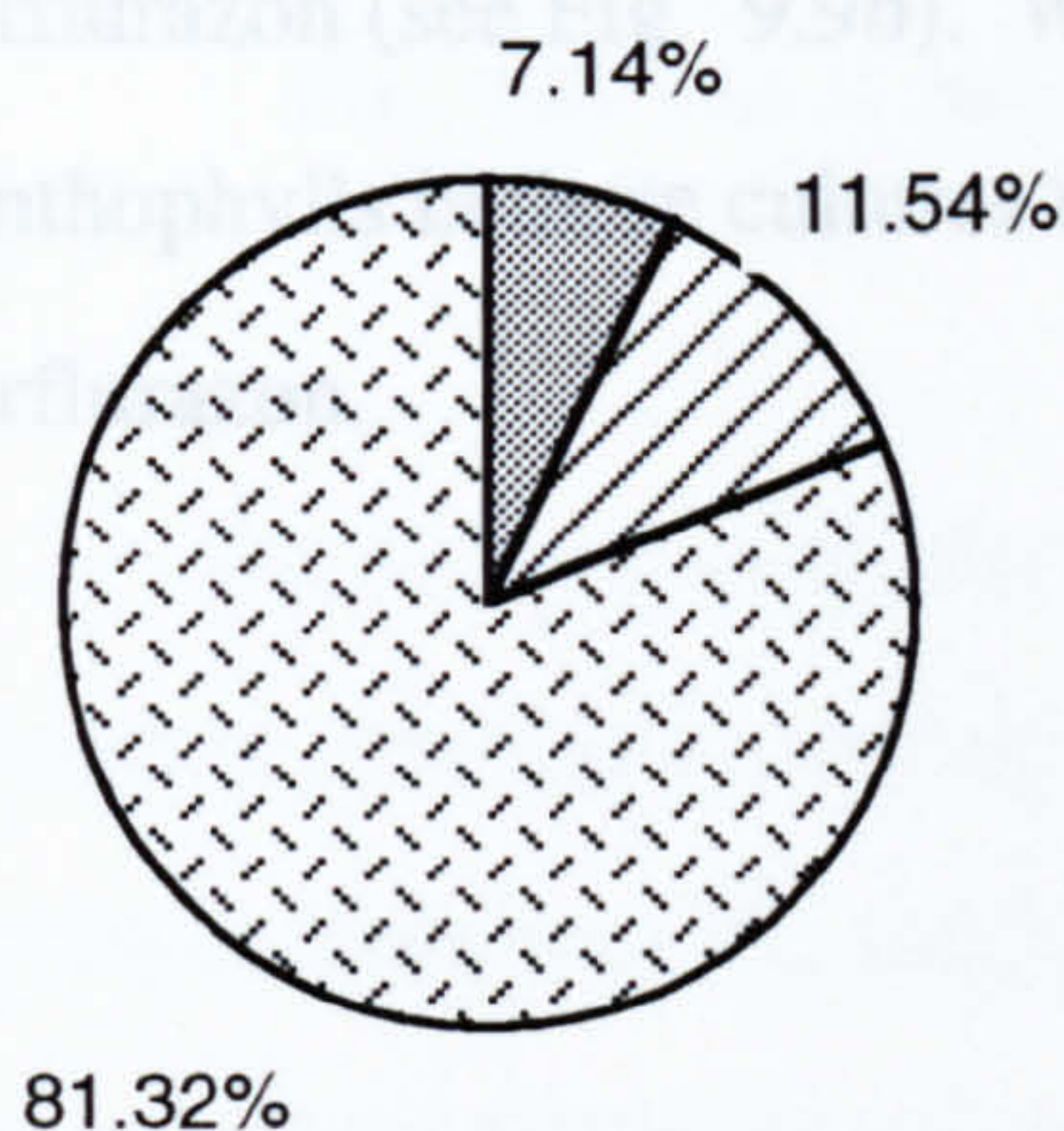
**Fig. 9.20a-b** The effect of removing DPA followed by the addition of norflurazon on carotenoid composition during secondary carotenoid synthesis in *H. pluvialis* (S.E.  $\pm$ 5.0%, n=3).



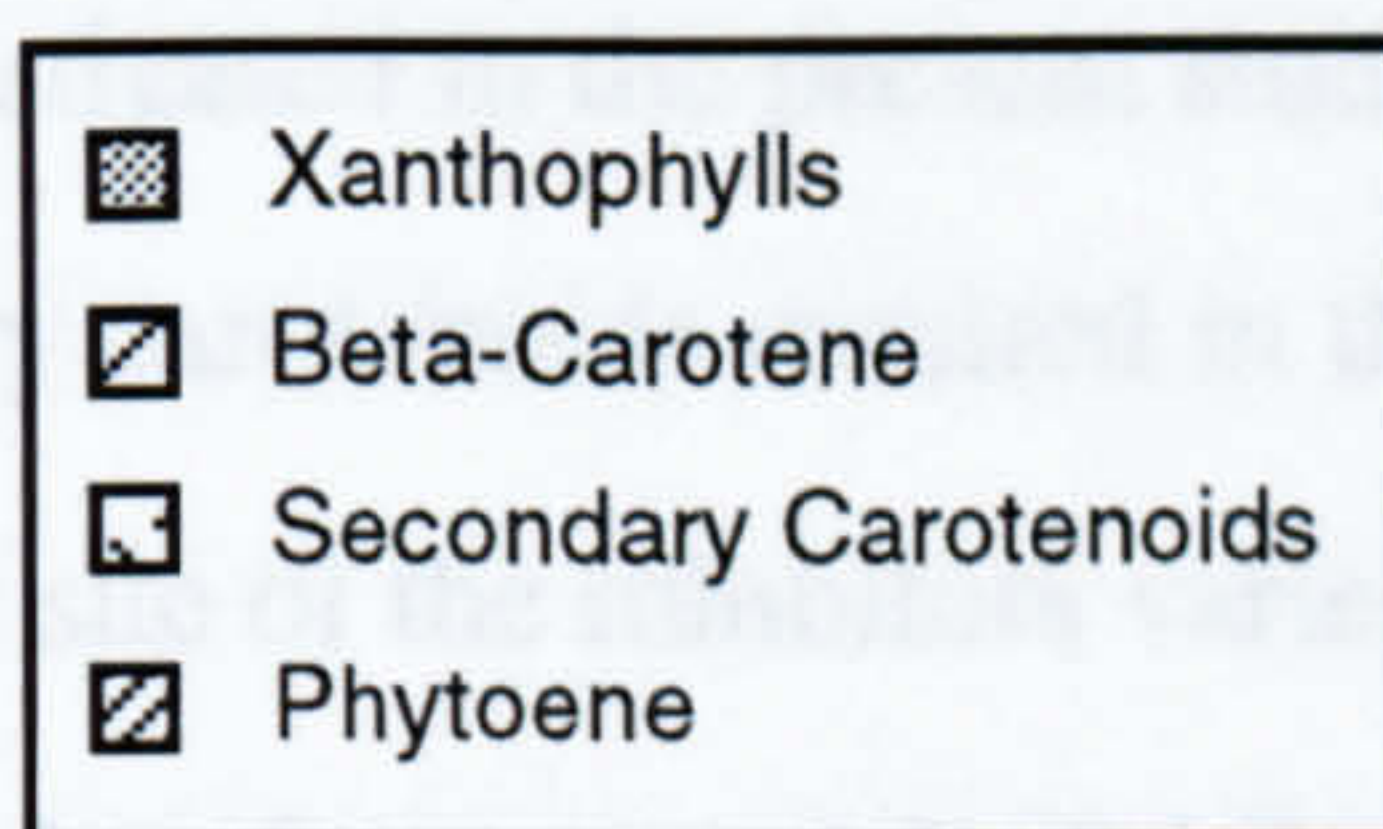
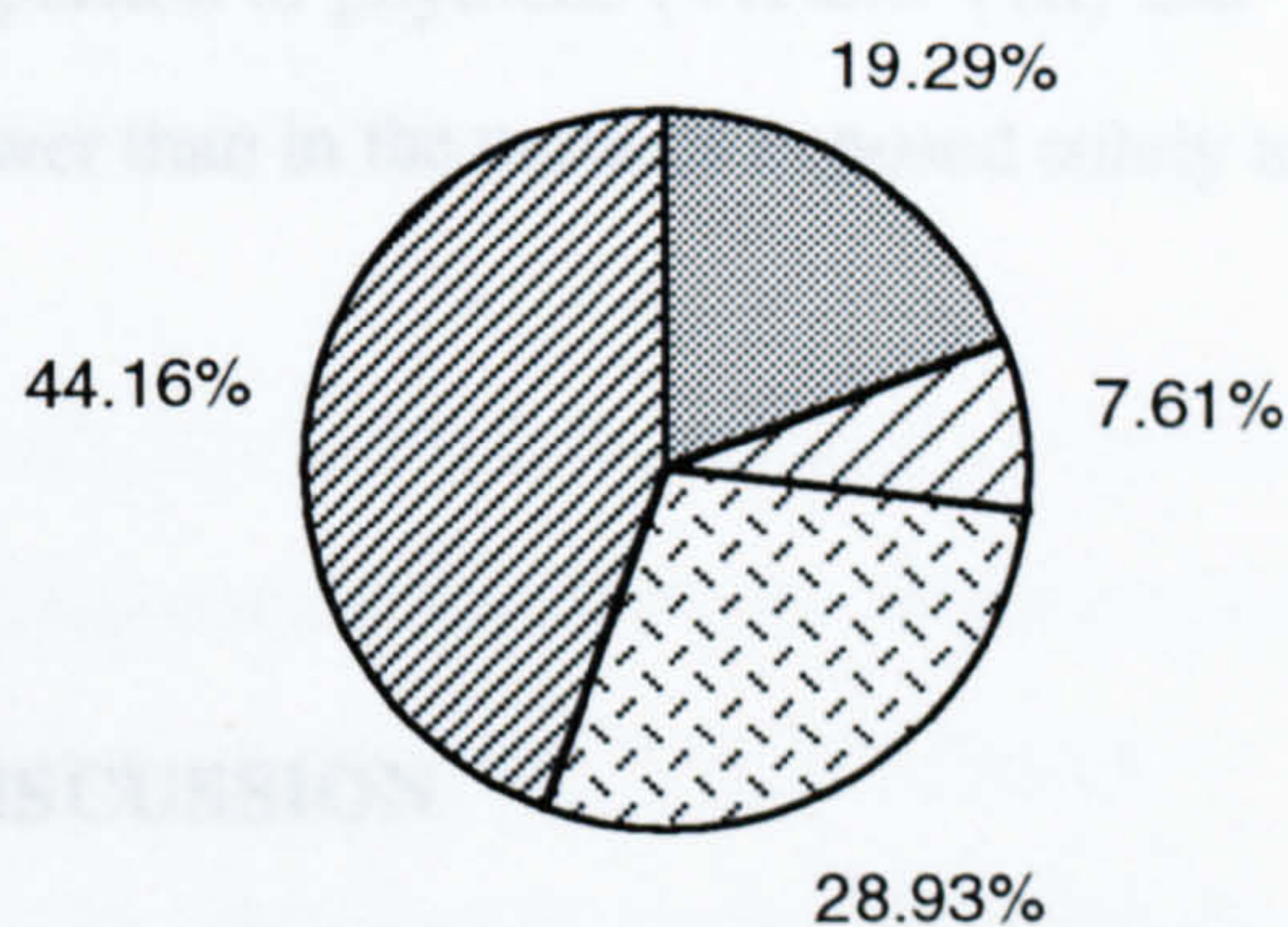
Figure 9.20a shows the pigment profile of *H. pluvialis* cultures which had been allowed to accumulate  $\beta$ -carotene (I) due to exposure to DPA. On removing the DPA the cultures were exposed to secondary carotenoid inducing conditions in the absence of norflurazon, which lead to the cultures accumulating massive amounts of secondary carotenoids. The levels of xanthophylls decreased slightly while the levels of  $\beta$ -carotene (I) fluctuated throughout the experiment.

When the cultures which had been allowed to accumulate  $\beta$ -carotene (I) were transferred out of the DPA and exposed to secondary carotenoid inducing conditions in the presence of norflurazon (Fig. 9.20b), relatively large amounts of phytoene (VII and VIII) accumulated in the cultures. The levels of  $\beta$ -carotene (I) decreased while the levels of secondary carotenoids increased during the experiment, concomitantly the levels of xanthophylls decreased at a relatively rapid rate throughout the experiment.

**Fig. 9.21a** Effect of removal of 100  $\mu$ M DPA (day 0) during secondary carotenoid inducing conditions.



**Fig 9.21b** Removal of 100  $\mu$ M DPA followed by the addition of 100  $\mu$ M norflurazon (day 0) under secondary carotenoid inducing conditions.



**Fig 9.21a-b** The final carotenoid composition of cells of *H. pluvialis* cells treated with DPA followed by the removal of the inhibitor and the addition of norflurazon under secondary carotenoid inducing conditions (S.E.  $\pm 4.8\%$ , n=3).

Figure 9.21a shows the final carotenoid composition of the cultures allowed to accumulate  $\beta$ -carotene (I) and then exposed to secondary carotenoid inducing conditions in the absence of norflurazon was primarily composed of secondary carotenoids (81.32%).

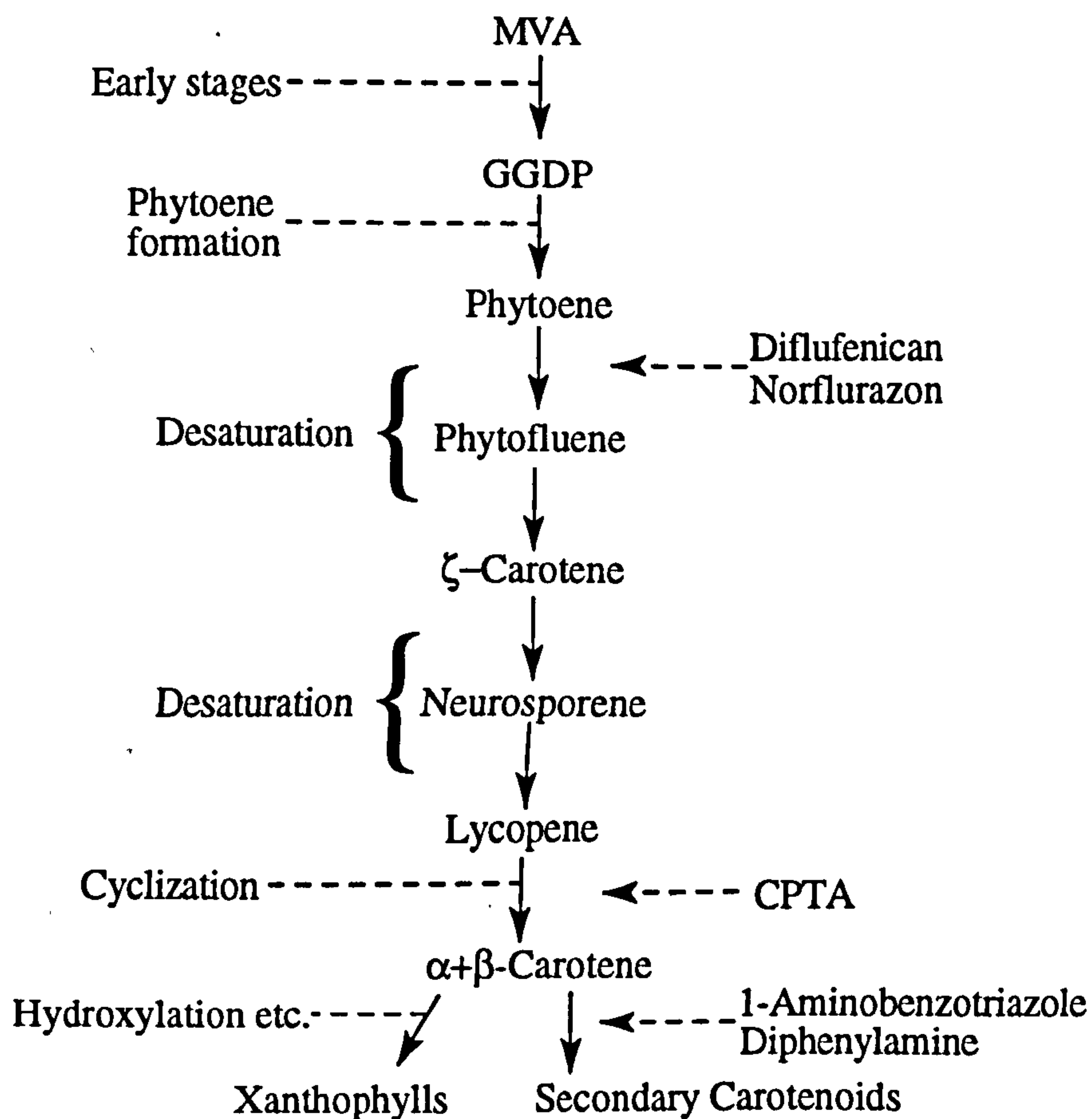


$\beta$ -Carotene (I) accounted for 11.54% of the total while the xanthophylls were responsible for only 7.14% of the final carotenoid composition. These cultures contained a significantly lower proportion of  $\beta$ -carotene (I) than those cultures exposed exclusively to DPA (see Fig. 9.15b). However, the proportion of secondary carotenoids in these cultures was considerably higher than in the cultures exposed solely to norflurazon, whilst the proportion of xanthophylls was nearly 3 times greater in the cultures exposed exclusively to norflurazon, than in those cultures exposed to both DPA and norflurazon.

The final carotenoid composition of the cultures allowed to accumulate  $\beta$ -carotene (I) and then exposed to secondary carotenoid inducing conditions in the presence of norflurazon (Fig. 9.21b), contained phytoene (VII and VIII) (44.16%) as the major carotenoid. The secondary carotenoids were the next major pigments present accounting for 28.93% of the total. The xanthophylls and  $\beta$ -carotene (I) were responsible for 19.29% and 7.6% of the total respectively. The proportion of secondary carotenoids and  $\beta$ -carotene (I) was considerably higher in these cultures than in those cultures exposed exclusively to norflurazon (see Fig. 9.9b). While the proportion of phytoene (VII and VIII) and xanthophylls in these cultures was much lower than in the cultures exposed solely to norflurazon.

#### 9.4 DISCUSSION

The action of the inhibitors investigated in the present study of their effects on *H. pluvialis* during the synthesis of secondary carotenoids resulted in the inhibition of astaxanthin (III) synthesis in the alga. The target site of the inhibitors varied depending upon the inhibitor under investigation. The target sites for most of the inhibitors were as predicted from previous studies in other organisms (see figure 9.7) except for DPA which did not inhibit phytoene desaturation, but rather the oxygenase reactions of  $\beta$ -carotene (I). The target site of each inhibitor examined in the present investigation is schematically represented in figure 9.22.



**Fig. 9.22** Site of carotenoid inhibition in cells of *H. pluvialis* under secondary carotenoid inducing conditions.

The addition of norflurazon and diflufenican to the cultures when exposed to secondary carotenoid inducing conditions resulted in the accumulation of the carotenoid precursor phytoene (VII and VIII). The presence of phytoene (VII and VIII) in photosynthetic tissues is a clear indication of the inhibition of carotenoid synthesis. Under normal conditions phytoene (VII and VIII) is rapidly desaturated, and subsequent HPLC analysis of the photosynthetic tissue fails to exhibit the presence of any phytoene (VII and VIII) (Britton *et al.*, 1989). Both norflurazon and diflufenican are thought to act directly on the phytoene desaturase protein (Bramley, 1993) thereby reducing the activity of the protein, and do not affect the synthesis of the protein.

Phytoene (VII and VIII) was also detected in the cultures which were incubated with norflurazon and diflufenican but not subjected to secondary carotenoid inducing conditions. This finding indicates that the synthesis of primary carotenoids was also inhibited resulting in the build up of relatively small amounts of phytoene (VII and VIII). The low amounts of phytoene (VII and VIII) accumulated in the cells exposed to non-



inducing conditions could be due to low rates of carotenoid turnover in these cells.

However, under inducing conditions the cells exhibited high rates of carotenoid turnover which resulted in the cells accumulating high levels of phytoene (VII and VIII).

Small amounts of secondary carotenoids were present in the cultures cultivated under secondary carotenoid inducing conditions in the presence of norflurazon and diflufenican. This was probably due to 'leakage' i.e. incomplete inhibition by the inhibitor. This so called 'leakage' is a result of increased protein synthesis due to the break down of the feedback inhibition mechanisms in the carotenoid biosynthetic pathway, since carotenoid synthesis has been inhibited. This coupled with the fact that large amounts of precursor i.e. phytoene (VII and VIII) are available for desaturation in the cells results in the inhibitor (i.e. norflurazon or diflufenican) becoming saturated. This allows some of the accumulated precursor, in this case phytoene (VII and VIII), to undergo desaturation and advance along the carotenoid biosynthetic pathway, resulting in the formation of secondary carotenoids. However, as can be observed (figures 9.8c - d and 9.10c - d) the levels of secondary carotenoids synthesised in this manner are small, when compared with the levels produced in the cultures exposed to inducing conditions in the absence of norflurazon or diflufenican.

The combined levels of phytoene (VII and VIII) and secondary carotenoids present in the cultures exposed to secondary carotenoid inducing conditions in the presence of norflurazon or diflufenican, were much lower than the levels of secondary carotenoids accumulated in the cultures in which neither of these inhibitors was present. It might have been expected that the total amount of carotenoid (including precursor) produced in both cultures (i.e. with and without inhibitor) would be similar. This may be due to the inherent instability of acyclic carotenoids such as phytoene (VII and VIII) within the cells of *H. pluvialis* especially when exposed to light. The secondary carotenoids are present in lipid globules within the cell cytoplasm which provides a stable environment for the ketocarotenoids (Lang, 1968). However, no transmission electron micrographs are available for comparison of the inhibitor treated cells to determine the presence or absence of such globules, or the sites of accumulation of phytoene (VII and VIII). It would be fair to assume that if the phytoene (VII and VIII) was stabilised somehow within the cells, the combined levels of phytoene (VII and VIII) and secondary carotenoids in the inhibitor

treated cells, would be similar to the levels of secondary carotenoids accumulated in cells exposed to secondary carotenoid inducing conditions in the absence of the inhibitors.

When CPTA was added to the cultures of *H. pluvialis*, secondary carotenoid synthesis was again inhibited. As with the previous experiments, some 'leakage' of the inhibitor occurred resulting in the formation of small quantities of secondary carotenoids. The inhibition resulted in relatively modest amounts of lycopene (XIII) accumulating within the cells. Lycopene (XIII) was also observed in the cultures maintained under non-inducing conditions in the presence of this inhibitor, indicating that the synthesis of primary carotenoids had also been inhibited. The precise mechanism/s by which CPTA inhibits the cyclization reactions is unknown. However, the mode of action of CPTA is thought to be very complex and may have multiple effects in plants and algae (Hsu *et al.*, 1972; Bucholtz *et al.*, 1977).

The exposure of cells of *H. pluvialis* to DPA and 1-aminobenzotriazole resulted in the accumulation of  $\beta$ -carotene (I) and the inhibition of secondary carotenoid synthesis. Again low levels of secondary carotenoids were observed in the cultures exposed to secondary carotenoid inducing conditions in the presence of DPA and 1-aminobenzotriazole due to some 'leakage'. In the cultures treated with 1-aminobenzotriazole the levels of  $\beta$ -carotene (I) increased under non-inducing secondary carotenoid conditions, whilst the levels of xanthophylls decreased in these cultures indicating that their synthesis had also been inhibited. The levels of  $\beta$ -carotene (I) in the DPA cultures exposed to non-inducing conditions increased substantially. However, the levels of xanthophylls also increased slightly in these cultures. The levels of xanthophylls in the cultures exposed to secondary carotenoid inducing conditions in the presence of DPA also increased. These results fail to clearly indicate whether the synthesis of xanthophylls is inhibited by DPA.

DPA was shown to inhibit  $\beta$ -carotene (I) synthesis in response to stress factors (i.e. high light intensity and high salt concentration) in the chloroplasts of the green alga *Dunaliella bardawil* (Vorst *et al.*, 1994). However, in the present study the results showed that  $\beta$ -carotene (I) synthesised in the cytoplasm of *H. pluvialis* cells during exposure to stress factors was not inhibited by DPA. The results indicate that the  $\beta$ -carotene (I) accumulated under stress conditions is probably synthesised at different sites



within the two algal species, or that differential transport mechanisms of the synthesised pigment exist between the two species.

The inhibition of the reactions of secondary carotenoid formation in *Haematococcus* using DPA has been reported previously (S. Boussiba pers. comm.). The use of DPA as an inhibitor of phytoene (VII and VIII) desaturation in higher plants and photosynthetic organisms is widely known (Davies, 1970; Sandmann *et al.*, 1984). The unique effect of DPA in *H. pluvialis* by inhibiting the mixed function oxidase reactions was also imitated by the action of the compound 1-aminobenzotriazole, a compound well known for inhibiting mixed function oxidase reactions in animals. The precise mechanism/s by which these compounds inhibit secondary carotenoid synthesis in algae are unknown.

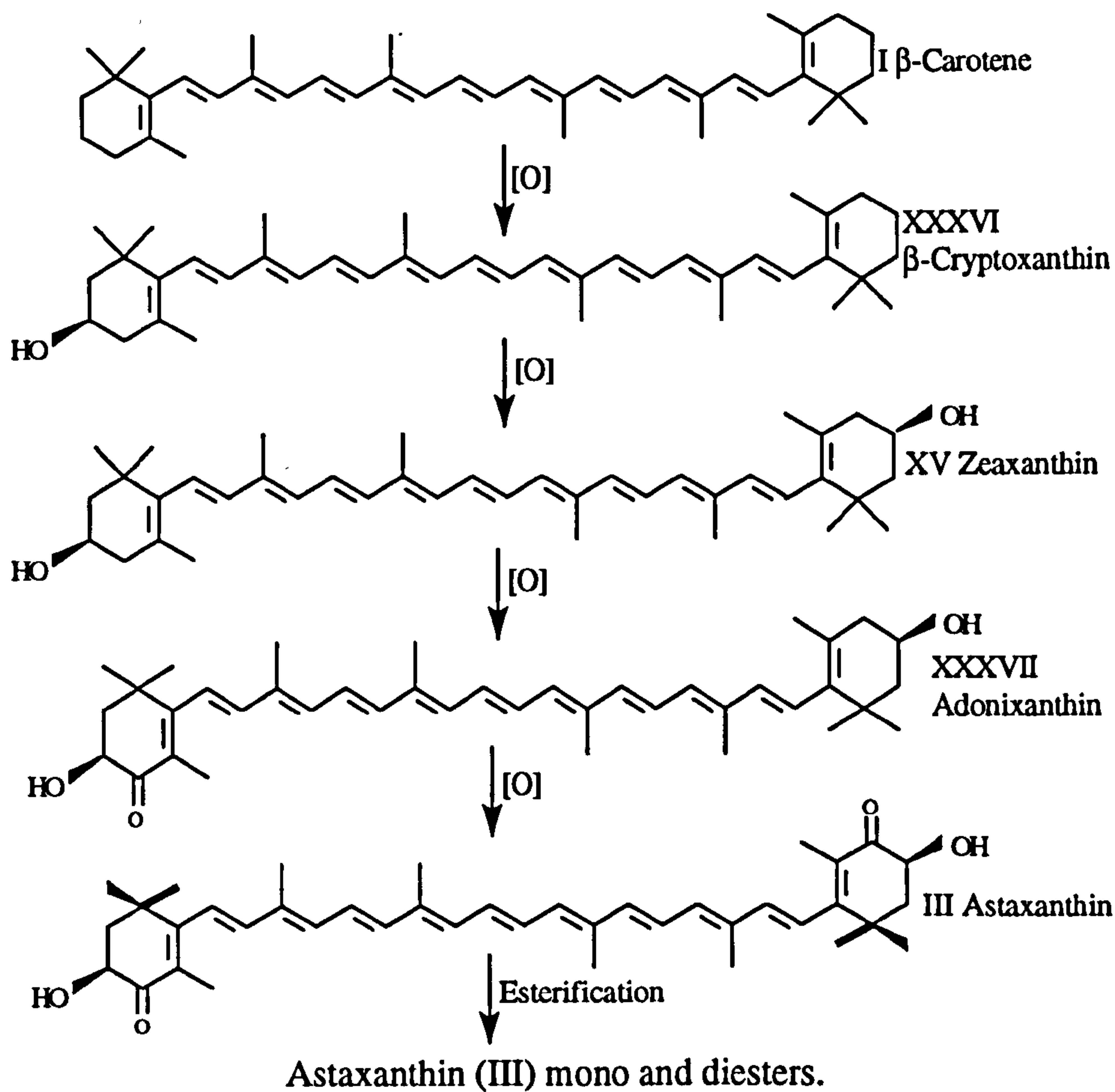
Previous reports of the use of inhibitors to inhibit secondary carotenoid synthesis in *Haematococcus* have not presented any data indicating the presence of carotenoid precursors (Hagen *et al.*, 1993b; Tjahono *et al.*, 1994). Hagen *et al.* (1993b) used norflurazon and tetcyclacis to inhibit secondary carotenoid production in *H. lacustris*, and reported that the former caused the stimulation of the deepoxidase enzymes of the xanthophyll cycle. This resulted in the cells containing relatively high levels of zeaxanthin (XV) and low levels of violaxanthin (XVII). These workers also reported that tetcyclacis caused the inhibition of cell division and photosynthesis, although no reference was made as to the accumulation of any precursors by either inhibitor. Therefore, the site of inhibition in the carotenoid biosynthetic pathway caused by the inhibitors in this investigation is not known. However, it would be expected that, as in this study, norflurazon would inhibit at the point of phytoene (VII and VIII) desaturation.

Tjahono *et al.* (1994) obtained a series of mutants resistant to inhibitory compounds (norflurazon, fluridone and nicotine) by cultivating colonies of *H. pluvialis* on medium plates containing the inhibitors. By visually detecting colonies on the plates still able to produce astaxanthin (III), they were able to breed hyper-producing astaxanthin (III) strains of the alga. Hybrid strains generated by protoplast fusion between these resistant mutants were found to possess a two-fold increase in the level of ploidy, and a three-fold increase in the level of carotenoid formation than the parental and wild type strains.

The fact that CPTA and DPA resulted in the accumulation of lycopene (XIII) and  $\beta$ -carotene (I), respectively, and the inhibition of secondary carotenoid synthesis, indicated that lycopene (XIII) and  $\beta$ -carotene (I) act as precursors in secondary carotenoid biosynthesis. This hypothesis was tested by inhibiting cultures of *H. pluvialis* with either CPTA or DPA, to allow the cells to accumulate either lycopene (XIII) or  $\beta$ -carotene (I). The CPTA or DPA was then removed from the cells, which were resuspended in fresh media and cultivated under secondary carotenoid inducing conditions in the presence of norflurazon to inhibit any further *de novo* carotenoid synthesis. If the cultures were to produce appreciable amounts of secondary carotenoids as opposed to those formed through 'leakage', the carotenoids accumulated subsequent to exposure to norflurazon (in this case lycopene (XIII) or  $\beta$ -carotene (I)) would need to act as precursors. This hypothesis would seem to be correct, as cultures allowed to accumulate lycopene (XIII) were able to convert this into secondary carotenoids, as did the cultures previously allowed to accumulate  $\beta$ -carotene (I). Indeed in both experiments the rate of decrease of either lycopene (XIII) or  $\beta$ -carotene (I) showed some relation to the rate of increase of the levels of secondary carotenoids in the cultures. The results of these experiments confirm that both lycopene (XIII) and  $\beta$ -carotene (I) act as precursors in secondary carotenoid biosynthesis.

The fact that  $\beta$ -carotene (I) acts as a precursor in the synthesis of secondary carotenoids suggests that the next step of secondary carotenoid biosynthesis after  $\beta$ -carotene (I) is either (i) the introduction of a hydroxy group at C3 of the  $\beta$ -ring to give  $\beta$ -cryptoxanthin (XXXVI), or (ii) the addition of a keto group at C4 of the  $\beta$ -ring to produce echinenone (II). These two alternative pathways available to  $\beta$ -carotene (I) suggest two possible pathways for astaxanthin (III) synthesis. Details of the pathways are schematically represented in figures 9.23 and 9.24, respectively. The pathway represented in figure 9.23 involves the stereospecific introduction of the 3,3'-hydroxy groups first to produce (3*R*,3'*R*) zeaxanthin (XV), into which the C4 keto groups are then introduced. This route was proposed by Cooper *et al.* (1975) based upon studies involving the synthetic synthesis of astaxanthin (III).



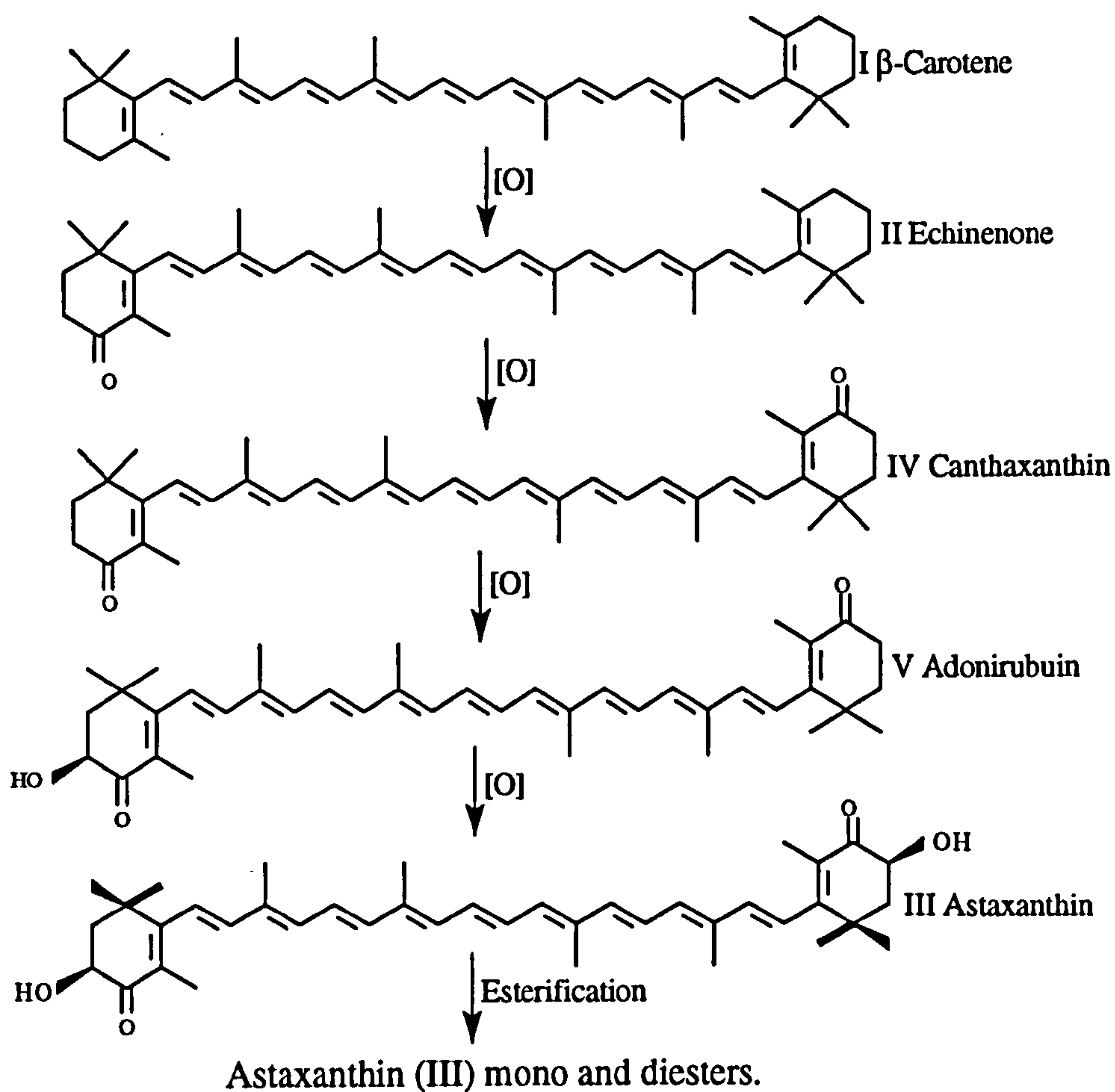


**Fig. 9.23** One possible biosynthetic pathway for the synthesis of astaxanthin (III) in *H. pluvialis* involving the production of zeaxanthin (XV) as a probable intermediate.

The second pathway (Fig. 9.24) involves the introduction of the 4,4'-keto groups first to produce canthaxanthin (IV) followed by the stereospecific introduction of the 3,3'-hydroxy groups. This second pathway has been proposed by Donkin (1976) and Grung *et al.* (1992). Both groups of workers proposed this pathway on the basis of the structural configuration of the secondary carotenoids (i.e. echinenone (II), canthaxanthin (IV) and adonirubin (V)) encountered in *H. pluvialis*, which are thought to act as precursors in astaxanthin (III) biosynthesis.

In the present studies the same secondary carotenoids have been observed as those encountered by Grung *et al.* (1992) and Donkin (1976) (see chapter 5). However, it would be imprudent to suggest that either of the pathways is responsible for astaxanthin (III) synthesis simply on the basis of the presence or absence of certain possible intermediates. It could be that neither pathway is responsible for astaxanthin (III) synthesis in the alga, or maybe both pathways exist in parallel with each other. At the present time

there is still insufficient data available to expound unequivocally the precise mechanism/s of astaxanthin (III) synthesis in *H. pluvialis*.



**Fig. 9.24** One possible biosynthetic pathway for the synthesis of astaxanthin (III) in *H. pluvialis* involving the production of canthaxanthin (IV) as a probable intermediate.

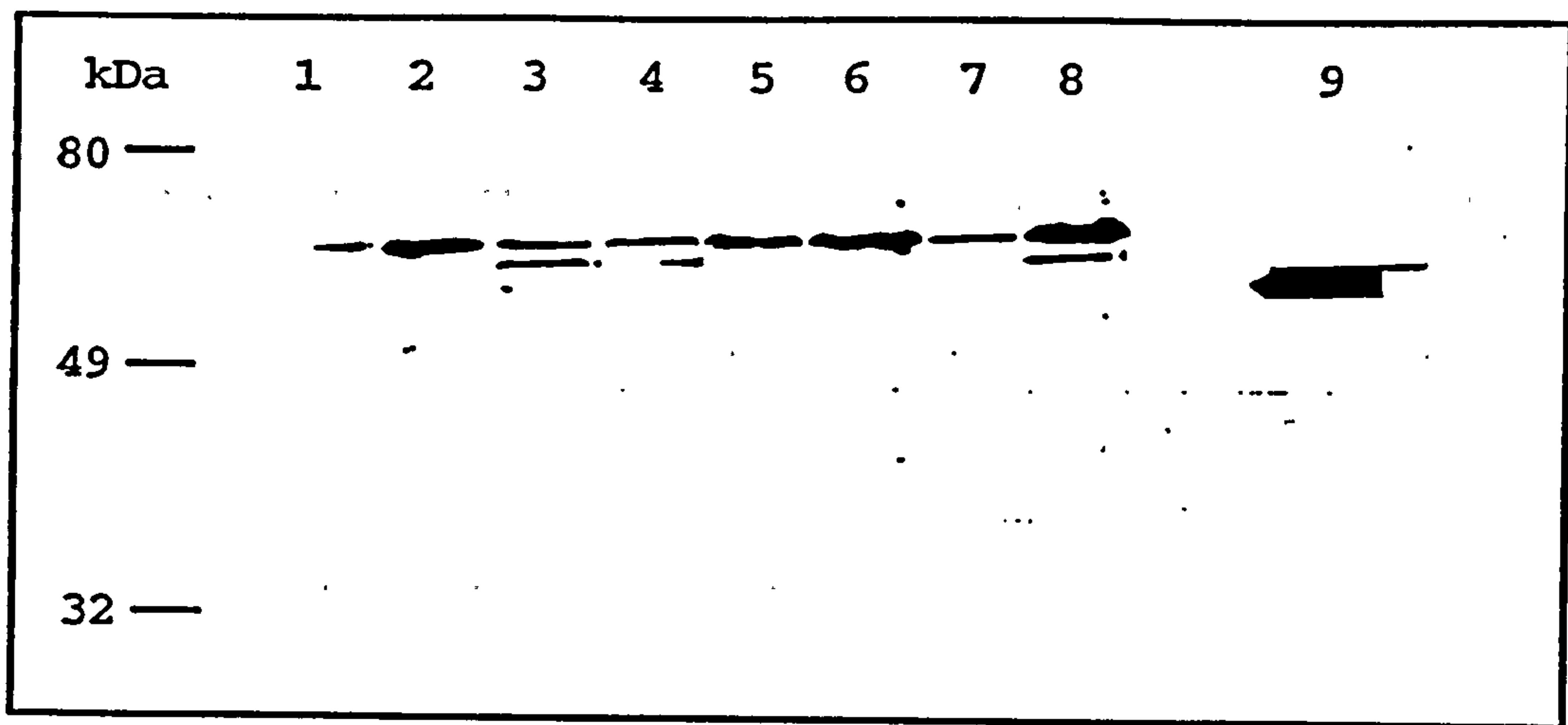
One method which could be used to determine if one or both of the aforementioned secondary carotenoid biosynthetic pathways exist would be to discover any inhibitors which cause the accumulation of either zeaxanthin (XV) or canthaxanthin (IV). In both pathways it would be fair to assume that two proteins are responsible for the conversion of  $\beta$ -carotene (I) to astaxanthin (III), due to the stereospecificity of the reactions. By inhibiting the second of these proteins either zeaxanthin (XV) or canthaxanthin (IV) would be accumulated. Then by transferring the cells to fresh media in the presence of norflurazon, (inhibiting any further carotenoid synthesis), the fate, if any, of the accumulated zeaxanthin (XV) or canthaxanthin (IV) could be ascertained. The results of such an experiment would indicate the existence of one of the pathways as a route for astaxanthin



(III) biosynthesis. Although, the existence of one of the pathways (Figs. 9.23 and 9.24) would thus be confirmed, unless two different inhibitors were discovered which caused the accumulation of zeaxanthin (XV) and canthaxanthin (IV), the results would not rule out the participation of both pathways in astaxanthin (III) biosynthesis, it would merely confirm the existence of one of the pathways. Alternatively, molecular biological techniques could be used to identify the genes responsible for astaxanthin (III) synthesis thereby elucidating the astaxanthin (III) biosynthetic pathway in *H. pluvialis*.

One further question raised by using inhibitors to inhibit secondary carotenogenesis in *H. pluvialis* is - are the proteins responsible for the synthesis of primary carotenoids (i.e. phytoene synthase, phytoene desaturase and lycopene cyclase) the same proteins required for secondary carotenoid synthesis? Although the inhibitors used in the present investigation inhibit these early reactions of carotenoid biosynthesis thereby inhibiting secondary carotenoid formation, it is not clear whether or not the inhibitors are acting upon more than one protein. It may well be that primary carotenoids and secondary carotenoids are encoded by divergent genes, encoding for different proteins which have the same function. This would signify that primary carotenoids and secondary carotenoids are synthesised along parallel but divergent biosynthetic pathways, which involve the same precursors.

Early studies carried in collaboration with the laboratory of Prof. J. Hirschberg at the Hebrew University of Jerusalem, Israel support this hypothesis. Immunological detection of phytoene desaturase in *H. pluvialis* extracts indicated the presence of two phytoene desaturase proteins in red aplanospores of *H. pluvialis*, while in green palmella cells only one phytoene desaturase protein was encountered (Fig. 9.25). The Western blot also shows an induction experiment where green palmella cells were exposed to secondary carotenoid inducing conditions. The results indicate that as secondary carotenoid synthesis is initiated two phytoene desaturase proteins are observed to be present, as opposed to only one when the cells are exposed to favourable conditions for growth. Although only a preliminary investigation the results do indicate the presence of two phytoene desaturase proteins in red aplanospores of *H. pluvialis*. Such a system of divergent genes synthesising the same carotenoids, using two parallel carotenoid biosynthetic pathways within the same organism has recently been reported for tomatoes (Fraser *et al.*, 1994).



**Fig. 9.25** Western blot of membrane and 'whole cell' fractions of *H. pluvialis* with monoclonal anti-phytoene desaturase. Each lane contained 25  $\mu$ g of protein. Molecular mass markers are shown on the left. (Lanes 1, 2, 3 and 4) whole cell fractions of *H. pluvialis* after 0, 3, 6 and 9 days, respectively of secondary carotenoid induction. (Lanes 5 and 6) whole cell and membrane fractions respectively, of green palmella cells. (Lanes 7 and 8) whole cell and membrane fractions respectively, of red aplanospores. (Lane 9) whole cell fraction of *Dunaliella*.



# THE COMMERCIAL PRODUCTION OF ASTAXANTHIN FROM *HAEMATOCOCCUS PLUVIALIS*: ITS POTENTIAL AS A FEED ADDITIVE FOR FARMED SALMONIDS

## 10.1 ASTAXANTHIN FROM *HAEMATOCOCCUS*

### 10.1.1 Introduction

Astaxanthin (III) is widely distributed throughout nature and is the principal pigment in crustaceans, salmonids, a range of bird species (including the flamingo and the scarlet ibis) and many other organisms (Steven, 1948; Goodwin, 1986; Brush, 1990). The carotenoid imparts distinctive orange-red coloration to the eggs, flesh and skin of salmonids. The red coloration of the salmonid flesh contributes to consumer appeal and therefore affects the price of the final product. Since animals cannot synthesise carotenoids, the pigments must be supplemented to feeds, at considerable cost to the producer.

### 10.1.2 Sources of carotenoids

A variety of carotenoids, synthesised both synthetically and naturally, are available or are being developed for use in aquaculture. These include synthetically produced  $\beta$ -carotene (I), canthaxanthin (IV) and natural materials such as krill, *Spirulina*, crustacean-meals, marigold, *Capsicum* and other xanthophyll-containing vegetable meals. Commercially available sources of astaxanthin (III) include the astaxanthin (III)-rich yeast *Phaffia rhodozyma* (Johnson and An, 1991) and the synthetically produced astaxanthin (III) marketed as Carophyll Pink<sup>®</sup>, Hoffman-LaRoche, Inc., Basel, Switzerland.

### 10.1.3 The potential production of astaxanthin using recombinant DNA techniques

The key limitation to the use of biological systems for carotenoid production is the low productivity of these systems compared to chemical synthesis. One way to increase the

productivity of carotenoid production in biological systems is the use of recombinant DNA technology. However, before recombinant DNA technology can be applied to improve carotenoid productivity in biological systems, the genes encoding the carotenoid biosynthetic enzymes need to be isolated and characterised.

Only recently have there been major advances in the field of carotenoid molecular biology. The majority of these advances have occurred over the past four years. Many genes coding for enzymes catalysing specific steps in the biosynthesis of carotenoids have been isolated from bacteria, cyanobacteria, green algae and higher plants (Ausich, 1994). Currently, this research has concentrated primarily on the genes and enzymes which catalyse the early reactions of carotenoid synthesis. This means that still relatively little is known about those genes and enzymes which mediate the biosynthesis of xanthophylls from carotene precursors. These later hydroxylation reactions in carotenoid biosynthesis involve mixed function oxidase reactions. *In vitro* biosynthesis of  $\beta$ -cryptoxanthin (XXXVI) from  $\beta$ -carotene (I) in *Aphanocapsa* membranes suggests the involvement of a mono-oxygenase reaction (Sandmann and Bramley, 1985). A single gene product (CrtZ) was suspected of being responsible for the conversion of  $\beta$ -carotene (I) to zeaxanthin (XV) in *Erwinia uredovora* (Misawa *et al.*, 1990), but the oxygenase has yet to be isolated and characterised.

There is still obviously a great deal of work to be done before astaxanthin (III) can be produced via a genetically altered host organism. To this end *Haematococcus* could have a crucial role to play. The isolation and characterisation of the genes responsible for the mixed function oxidase conversion of carotenes to astaxanthin (III) in *Haematococcus* would enable the oxidase genes to be available for manipulation. The genes responsible could then be transferred into a more convenient host organism for carotenoid production purposes i.e. higher plants. Astaxanthin (III) is also found in prokaryotic systems (e.g. marine bacteria) which are easier systems to manipulate for molecular biology purposes than eukaryotic systems. The possibility should not be dismissed of transferring the genes directly into the salmonids, thereby creating a self pigmented fish.



#### **10.1.4 *Haematococcus* as a source of astaxanthin**

The current investigations have revealed that certain green algae in the subphylum *Chlorophyceae* are capable of synthesising astaxanthin (III) when exposed to unfavourable environmental conditions. The green alga *H. pluvialis* accumulated the highest concentrations of astaxanthin (III) of the eight algal species analysed. *H. pluvialis* was able to accumulate 5.4% astaxanthin (III) on a dry weight basis and 40 mg astaxanthin (III) per litre of algal culture. The astaxanthin (III) accumulated by *H. pluvialis* is the pure (3*S*,3'*S*) isomer (Renstrøm *et al.*, 1981). The majority of the astaxanthin (III) accumulated was present in an esterified form (88%) which are not thought to affect the deposition and metabolism of astaxanthin (III) in salmonids (Torrissen *et al.*, 1989). The red aplanospores of *H. pluvialis* which contain the high concentrations of astaxanthin (III) occur in a highly encysted form, which is surrounded by a thick cell wall composed of the biopolymer sporopollenin (Burczyk, 1987b). These biopolymers are highly resistant to oxidative degradation and animal digestion, thus impeding the absorption of the pigments by salmonids.

#### **10.1.5 Cellular location of astaxanthin in *H. pluvialis***

The cellular location of astaxanthin (III) within the algal cell is of considerable importance for the development of mutants and hyper-producing strains that are capable of producing large quantities of carotenoids. Since carotenoids are accumulated as intracellular products the maximum concentrations will depend on the precise location/s of the carotenoid, i.e. are these pigments confined to specific organelles or are distributed throughout the cell? TEM images of *H. pluvialis* indicated that lipid globules thought to contain carotenoids accumulated during encystment were evenly distributed throughout the cell cytoplasm. The potential of *H. pluvialis* as a source of astaxanthin (III) is enhanced since the accumulation of astaxanthin (III) is not restricted to a specific organelle. Since astaxanthin (III) is distributed throughout the cell cytoplasm, the limiting anatomical feature of astaxanthin (III) accumulation in *H. pluvialis* would appear to be the actual size of the algal cell. The results of the image analysis experiments (see section 3.3.1) also support this theory by establishing a correlation between cell size and astaxanthin (III) content of *H. pluvialis* cells.

### **10.1.6 Environmental regulation of astaxanthin biosynthesis**

The induction and rate of astaxanthin synthesis in *H. pluvialis* is affected by many environmental factors including light, temperature, the availability of specific nutrients, and other factors. Many of these effects are non-specific and the production of astaxanthin (III) is brought about due to alterations of overall cellular metabolism. The influence of environmental and nutritional conditions on secondary carotenogenesis has been reviewed extensively in Chapter 6.

### **10.1.7 Strain improvement - genetic approaches**

Various approaches exist for the genetic improvement of potentially commercially viable strains of *H. pluvialis*. These include mutagenesis, recombination of mutants (e.g. by protoplast fusion), and gene cloning and amplification. Each approach has potential benefits and limitations. However, microalgae are prolific producers of secondary carotenoids. *H. pluvialis* is capable of producing 26,000 µg secondary carotenoid per gram dry cell weight (Fig. 8.12) compared with only 500 µg secondary carotenoid per gram dry cell weight in *Phaffia rhodozyma* (Johnson and An, 1991). This high level of carotenoid production indicates that strain development for the industrial production of astaxanthin (III) in *H. pluvialis*, is not as crucial as in some other potential microbial sources of astaxanthin (III). However, genetic improvements in *H. pluvialis* could be utilised to increase the alga's tolerance to increased salinities and temperatures. These improvements would improve the performance of the alga under mass cultivation conditions. If the alga could be grown in a high salinity medium this would eradicate the problems of contamination by predators and other green and blue/green algae. Increasing the optimum growth temperature of the alga would improve the biomass production rate under mass cultivation conditions. Currently, the alga achieves maximum rates of growth at ~15°C, which is significantly lower than the ambient temperature in the areas of the world in which microalgae are currently produced e.g. Australia, Hawaii etc. where the ambient temperatures are ~25 - 35°C.



### **10.1.8 Screening - image analysis and cell sorting**

Screening of pigmented algal colonies/cultures by visual examination has limited utility and is not capable of detecting hyper-producing cells. The current investigations have successfully applied image analysis to isolate hyper-producing cells of *H. pluvialis*. Unfortunately, this technique has not yet provided a hyper-producing 'active' culture. This is because the particular image analysis technique used in the present study could not select individual cells quickly or aseptically. The application of image analysis needs to be coupled with a cell sorting device. This would enable single cells to be analysed and sorted at a rate of 4000 to 10,000 cells per second. Cell sorting devices currently available which could be used for this purpose include the Coulter counter and fluorescence activated cell sorters.

### **10.1.9 The biosynthetic pathway of astaxanthin in *H. pluvialis***

The biosynthetic pathway/s from phytoene (VII and VIII) to astaxanthin (III) have been indirectly inferred by chemical identification of suspected carotenoid precursors in astaxanthin (III)-producing organisms. The pathways proposed are based on the logical sequence of carotenoid biosynthetic reactions desaturation → cyclization → oxygenation. The enzymes catalysing the mixed function oxidase reactions have not yet been isolated in astaxanthin (III) producing organisms, and the actual sequence of the mixed function oxidase reactions and precursors still remain unknown. Possible pathways and precursors are discussed in section 9.4.

### **10.1.10 Chemical inhibition of secondary carotenogenesis**

Studies with inhibitors of the isoprenoid pathway have been very useful in revealing some information about the biosynthesis of astaxanthin (III) in *H. pluvialis*. The principal inhibitors used to inhibit specific steps of the secondary carotenoid pathway were:

- (i) Phytoene desaturase (phenylpyridazones e.g. norflurazon and diflufenican);
- (ii) lycopene cyclase (substituted triethyl amines e.g. CPTA);
- (iii)  $\beta$ -carotene hydroxylase/ketolase (diphenylamine and 1-aminobenzotriazole).

Both norflurazon and diflufenican inhibited secondary carotenogenesis which resulted in the accumulation of phytoene (VII and VIII). CPTA inhibited lycopene cyclase

causing the accumulation of lycopene (XIII).  $\beta$ -carotene hydroxylase/ketolase was inhibited by diphenylamine and 1-aminobenzotriazole causing the accumulation of  $\beta$ -carotene (I).

#### **10.1.11 Laboratory to pilot scale production of astaxanthin from *H. pluviialis***

From the initial screening of a number of species and strains of the phylum *Chlorophyceae*, to the pilot scale production of astaxanthin (III) from *H. pluviialis* required a large amount of man hours and expenditure. Figure 10.1 indicates the various steps involved and the progression of the work from laboratory scale cultivation to the pilot scale fermentation process. *H. pluviialis* had proved to be the most prolific producer of astaxanthin (III) in all the *Chlorophyceae* species screened. Initial investigations into the morphology and life history of the alga suggested that the alga would be a suitable species for mass cultivation. Qualitative and quantitative analyses of the alga indicated that in terms of carotenoid composition and content the alga had the potential to provide a reliable and relatively abundant source of astaxanthin (III). Ideally during the scale-up process from laboratory to pilot scale production further improvements in the astaxanthin (III) content and growth requirements of the alga could have been achieved (see sections 10.1.7 and 10.1.8). Unfortunately, due to the lack of facilities this was not possible. However, during the sub-culturing of the alga visual examinations of the cultures allowed for the selection of cultures which contained high levels of astaxanthin (III).

Research into the basic physiological aspects of astaxanthin (III) synthesis in the alga proved to be very important. The results indicated that the culture conditions responsible for the growth of the alga during the 'swimmer' and palmella stages of the life cycle were very different from those responsible for inducing astaxanthin (III) synthesis in the aplanospores of the alga. These results implied that during large scale cultivation of the alga for astaxanthin (III) production, two distinct fermentation phases would be required. This would lead to the development of a two stage fermentation process. During the first stage, the alga would be subjected to culture conditions which would allow for maximum rates of growth and biomass accumulation. The second stage would expose the alga to the culture conditions required for maximum rates of astaxanthin (III) production. The



optimum conditions required for the two fermentation stages were defined using response surface methodology.

These results were used in the scale-up process (except for light intensity due to practical considerations), firstly the alga was transferred from 50 ml cultures in 250 ml conical flasks to a 5 litre glass aspirator, and then to a 30 litre glass column, air lift, photobioreactor. During the scale-up process the biomass and astaxanthin (III) yields were comparable to those achieved in the 250 ml conical flasks, only the length of time required to achieve the final yields increased slightly at each scale-up point. During the pilot scale production of the alga contamination of the culture proved not to be a major problem. Harvesting also proved to be a relatively simple process *via* sedimentation of the aplanospores.

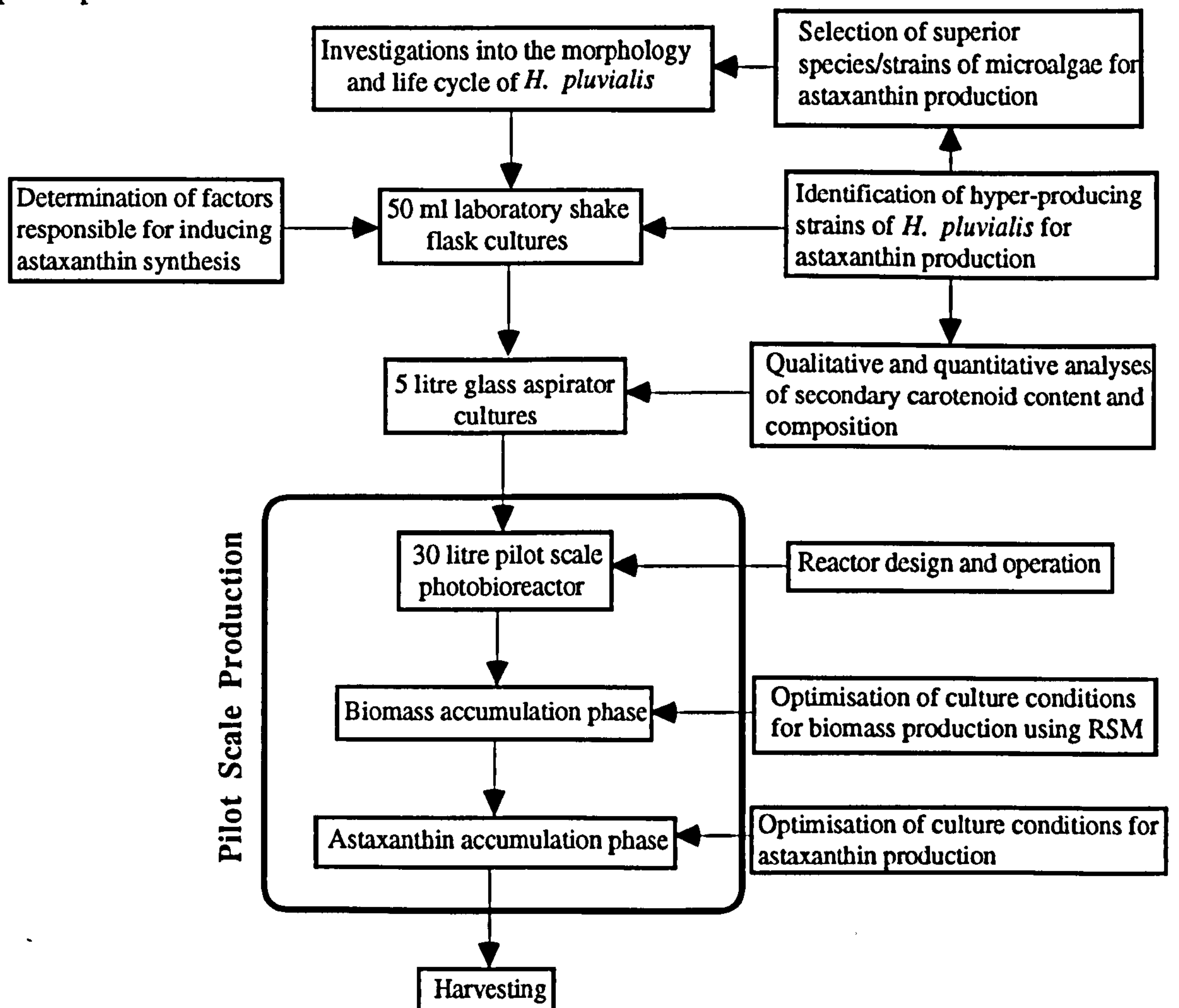


Fig. 10.1 Step-wise progression of work from laboratory to pilot scale production of astaxanthin (III) from *H. pluvialis*.

### 10.1.12 Pilot scale to full scale commercial production

The full scale commercial production of astaxanthin (III) from *H. pluvialis* still requires a considerable amount of research and developmental work. One of the major problems to be overcome is the design and operation of a large scale closed photobioreactor. The design of the glass column, air lift, photobioreactor used in the present study has limitations in the volume of algae which can be cultured within in it. Currently, a number of studies are being carried out on the development of large scale tubular reactors for the cultivation of *H. pluvialis*. These tubular reactors have a far superior surface area to volume ratio than column type photobioreactors, this ensures the efficient distribution of light throughout the algal suspension. This a particularly desirable feature considering the importance of light for astaxanthin (III) synthesis in the alga. The high surface area to volume ratio of tubular reactors also ameliorates the problem of high cell densities in the photobioreactor, which result in poor light distribution throughout the algal suspension. Tubular reactors can also be constructed which allow for relatively large volumes of algae to be cultured within them i.e. 10,000 litres. Tubular reactors have been constructed for the commercial production of  $\beta$ -carotene (I) from *Dunaliella* e.g. in Spain. One problem of tubular reactors is that cleaning the tubing which can be up to 3 miles in length can be very difficult. The tubing material also needs to be able to remain in good working condition over a long period of time e.g. 5 years, especially considering the severe light intensities to which the tubing will be exposed.

Further problems of the scale-up process to a full scale commercial production system include the identification of cheap sources of media components which can be utilised efficiently by the algae to achieve good rates of growth. Another problem of large scale cultivation of the alga is the control of important culture parameters within the photobioreactor i.e. CO<sub>2</sub>, O<sub>2</sub> and pH. The technology of biomonitoring and control of such parameters is available and could be readily applied to the cultivation of *H. pluvialis*. The acquisition of land and building costs of a photobioreactor represent the major investment required to make the transition from pilot scale to full scale commercial production of astaxanthin (III) from *H. pluvialis*. Such capital costs are very high, however, once in operation the running costs of a photobioreactor utilising solar radiation are relatively low. The problems of large scale commercial cultivation of *H. pluvialis*



mentioned should not pose a serious threat to the development of *H. pluvialis* as a commercial source of astaxanthin(III). Figure 10.2 highlights the main aspects of the commercial production of astaxanthin from (III) *H. pluvialis*:

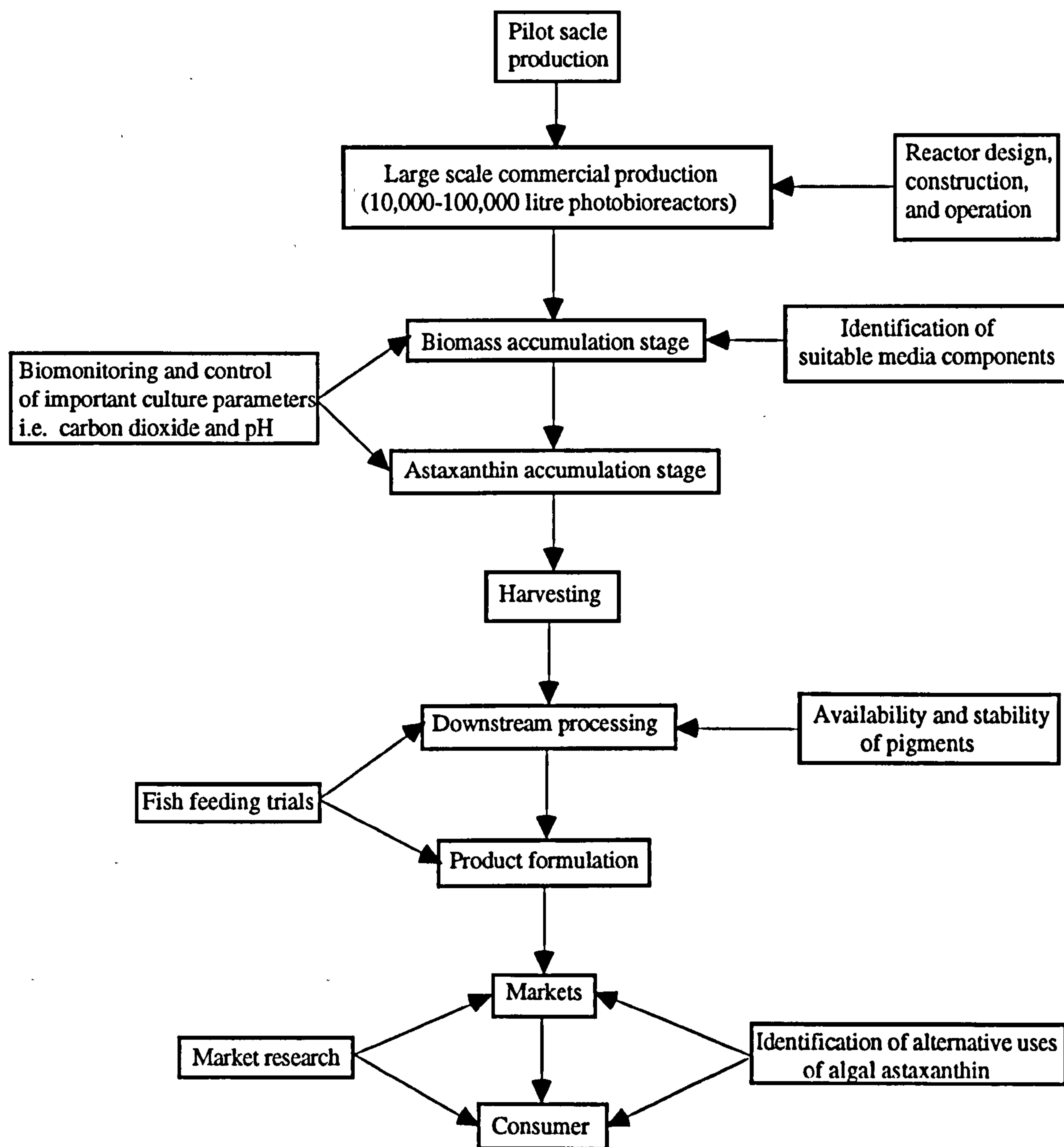


Fig. 10.2 Possible route for the commercial production of astaxanthin (III) from *H. pluvialis*.

### 10.1.13 Downstream processing and product formulation

Post-harvest treatment of the algae requires the liberation of the pigments from the dried, cleaned cells of *Haematococcus* which are then prepared for product formulation. There

are two problems when preparing the cells as a fish feed supplement. The first problem is that the cell wall of the alga is resistant to animal digestion (see section 10.1.4). The second problem is that free astaxanthin (III) and its esters are very susceptible to oxidation, forming the biologically inert pigment astacene (XXIX). The process of product formulation needs to overcome these two problems and has been described by Spencer (1989). Dry cells are mixed with liquid nitrogen and a suitable antioxidant powder (i.e. butylated hydroxytoluene or ethoxyquin) and cryogenically ground at -170°C. The dry antioxidant is therefore dissolved directly into the liquid phase, preventing the oxidation of astaxanthin (III) to astacene (XXIX), while the cell walls are fractured releasing the pigment and making it biologically available. Recommended usage of the algal product is from 25 to 100 ppm pure astaxanthin (III) in the appropriate feed (depending on fish species and age). The final product will include free, mono- and diesters of astaxanthin (III) which make up the bulk of the carotenoid composition. Lesser amounts of other carotenoids in the supplement will include  $\beta$ -carotene (I), lutein (XVI), echinenone (II), canthaxanthin (IV) and adonirubin (V), along with chlorophylls *a* and *b*.

## **10.2 SALMONID PRODUCTION**

### **10.2.1 Introduction**

The world-wide harvest of salmon in 1991 was approximately 720,000 MT., of which 25 - 30% were produced in a variety of aquaculture facilities and enclosure designs (Meyers, 1994). Many salmonid species are responsible for this world-wide production, including rainbow trout, Atlantic salmon, chinook salmon, coho, pink and chum salmon. The production of Atlantic salmon has seen the largest rises, especially in Norway and Chile. Since 1979, production in Norway has expanded from approximately 4,000 MT. to over 150,000 MT.

### **10.2.2 Carotenoids in salmonids**

The pinkish hue of salmonid flesh is due to carotenoids derived from their diets. The predominant carotenoid in wild salmonids is astaxanthin (III), which is derived from



feeding on carotenoid-rich crustaceans. The range of carotenoid levels in salmonids varies, reflecting differences in the prey, fish size, stage of maturity, and specific pigment metabolism. The levels of carotenoids range from 26 - 39 mg/kg for sockeye salmon to 8 - 9 mg/kg for chinook salmon and > 3 mg/kg in rainbow trout (Torrissen *et al.*, 1989). In farmed salmonids, astaxanthin (III) and canthaxanthin (IV) are supplemented to feed to impart the desired flesh coloration. Rainbow trout fed diets containing synthetic astaxanthin (III) achieved flesh concentrations of approximately 6.5 - 9 mg/kg (Storebakken and No, 1992). In larger fish, flesh levels of 20 -25 mg/kg may occur. Feeds used to pigment rainbow trout or Pacific salmon generally contain 50 -100 mg/kg feed. Flesh pigment levels of 4 - 5 mg/kg are considered to be the minimum to impart acceptable flesh coloration (Torrissen, 1989).

### **10.2.3 Biological activities of carotenoids in aquatic species**

The biological role of astaxanthin (III) in fish is not confined to that of a pigmentation agent. Carotenoids in general enhance both the specific and non-specific immune system (Bendich, 1989). Proposed functions include protection against UV light, serving as provitamin A, enhancing tolerance to elevated ammonia levels and low oxygen levels, stimulation of growth, maturation rate, fecundity reaction as a fertilisation hormone and improvements of egg quality (Meyers, 1994).

The function of astaxanthin (III) as a powerful antioxidant in marine animals has been demonstrated, providing a defence mechanism against oxygen free radicals (Miki, 1991). Astaxanthin (III) is a strong inhibitor of lipid peroxidation and has been shown to play an active role in the protection of biological membranes from oxidative injury by inhibition of mitochondrial lipid peroxidation (Kurashige *et al.*, 1990). It has also been shown that astaxanthin (III) can function as a potent antioxidant both *in vivo* and *in vitro*.  $\beta$ -Carotene (I) and astaxanthin (III) were shown to exert immunomodulating effects on mouse lymphocytes, independent of provitamin A activity (Jyanouchi *et al.*, 1991). Astaxanthin (III) and canthaxanthin (IV) which possess oxo groups at the C4 and C4' positions in the  $\beta$ -ring were proved to be more effective antioxidants than  $\beta$ -carotene (I) in free radical stabilisation (Terao, 1989).

#### **10.2.4 Factors affecting carotenoid function in fish**

Pigment source, form and concentration, diet composition, especially fat content, fish size, physiological state and stage of sexual maturation, and genetic background all affect the carotenoid function in fish (Torrissen *et al.*, 1989). Research has focused on carotenoid digestibility, intestinal absorption, metabolism, and excretion, all of which affect dietary carotenoid utilisation and retention (Foss *et al.*, 1987; Torrissen, 1989). Astaxanthin (III) absorption also depends on the concentration provided in the diet and whether it is provided in its free form or as a mono or diester. The effects of abiotic factors, i.e. temperature and salinity, have also been examined in both mature and immature salmonids and were found not to significantly alter carotenoid digestion and retention in the fish (No and Storebakken, 1991, 1992).

#### **10.2.5 Salmonid feeding trials with diets supplemented with astaxanthin**

Research into dietary carotenoids of salmonids have indicated that dietary lipids directly affect carotenoid deposition levels. Free astaxanthin (III) is more efficiently utilised than canthaxanthin (III), with astaxanthin (III) also giving the flesh a more reddish hue at comparable flesh concentrations. Absorption occurs in the intestine with conversion to vitamin A occurring mainly in the intestinal wall. Carotenoids are transported in the blood by lipo-proteins with the liver being the major organ for carotenoid metabolism (Meyers, 1994).

The capacity for carotenoid deposition in the flesh of rainbow trout varies from start of feeding to sexual maturation (Bjerkeng *et al.*, 1992). Flesh concentrations of carotenoids can vary from 6 mg/kg to 20 - 25 mg/kg in large trout. When dietary levels of astaxanthin (III) exceed 59 mg/kg, there is a low corresponding increase in flesh pigmentation. In both Atlantic salmon and rainbow trout flesh pigmentation follows a saturation curve (Bjerkeng *et al.*, 1992). During active growth, high concentrations of carotenoids are deposited in the skin. During sexual maturation, carotenoids are redistributed from the flesh to skin, eggs, and gonads (Torrissen *et al.*, 1989; Choubert and Blanc, 1989).

In epimerization experiments involving different optical isomers of astaxanthin (III) ((3*S*,3'*S*) (3*R*,3'*S*) (3*R*,3'*R*)) in Atlantic salmon, there was no significant preferential



utilisation of the three optical isomers tested. Epimerization did not occur in the flesh at the chiral centres C-3 and C-3' in astaxanthin (III). The results confirmed that the ratio of astaxanthin (III) isomers in wild salmon reflects the dietary carotenoid deposition rather than a metabolic C-3,3' epimerization. Investigations involving rainbow trout discovered that dietary astaxanthin (III) diesters were primarily absorbed and accumulated in the integument. The diesters kept their configurations and were partially metabolised to the three stereo-isomers of zeaxanthin (XV) (Katsuyama *et al.*, 1987).

Diets with the three optical isomers of astaxanthin (III) and canthaxanthin (IV) were evaluated in rainbow trout from start of feeding to sexual maturation (Bjerkeng *et al.*, 1992). Astaxanthin (III) stereoisomers deposited in the flesh retained their optical configuration. No significant difference was observed between optical isomer composition of astaxanthin (III) in the flesh and that in the feed. Skin of fish fed astaxanthin (III) mainly contained astaxanthin (III) esters while skin of fish fed canthaxanthin (IV) contained canthaxanthin (IV) and its reductive metabolites. Zeaxanthin (XV) in the skin was a mixture of all three optical isomers. The liver and gonads of mature fish contained astaxanthin (III) with an isomeric ratio close to that of the dietary astaxanthin (III).

Recent trials involving astaxanthin (III) derived from *Haematococcus* have established that the alga can be used as an effective pigmenter of salmonids (Sommer *et al.*, 1991; Choubert and Heinrich, 1993). Choubert and Heinrich (1993) obtained the best results of the two groups of workers previously mentioned. Using trout fed with diets supplemented with *Haematococcus* to achieve 100 mg carotenoid / kg of feed, the study confirmed that the feed caused significant carotenoid deposition in trout muscle. The final muscle concentration was 6.2 mg / kg which is considered as acceptable (Foss *et al.*, 1984). The amount of carotenoids in the trout flesh was higher than that reported by Sommer *et al.* (1991).

However, the effect of the synthetic carotenoids i.e. astaxanthin (III) on trout pigmentation also investigated by Choubert and Heinrich (1993) was higher than that of carotenoids derived from *Haematococcus*. This could be due to the low availability of the carotenoids from the alga because of the thick cell wall, which is highly resistant to digestion. Another possibility is that free astaxanthin (III) found in the synthetic feed supplements may be more efficiently utilised by the fish than the esterified astaxanthin (III)

forms found in *Haematococcus* (Schiedt *et al.*, 1985; Foss *et al.*, 1987), indicating that the cleavage of astaxanthin (III) esters may be a limiting step in pigmentation.

#### **10.2.6 The future**

By the year 2000 it is estimated that farmed salmon production will have reached 460,000 MT. (Johnson and An, 1991). A reduction in wild stocks through over fishing and by environmental problems will also serve to increase the demand for farmed salmonids. This large industry will need to supply a high quality product to the consumer. This will stimulate the search for alternative 'natural' sources of astaxanthin (III) and result in a large array of production systems. Potentially, *Haematococcus* could be a major contributor to this market providing a high quality and reliable source of astaxanthin (III). Further work in salmonid diet development, especially in the understanding of lipid metabolism and carotenoid transport, will play a major role in improving astaxanthin (III) deposition and retention rates in salmonids.



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