DEPARTMENT OF BIOCHEMISTRY
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XANTHOPHYLLS AS METABOLIC PRECURSORS

A THESIS SUBMITTED IN FULFILMENT FOR
THE DEGREE OF DOCTOR OF PHILOSOPHY
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DECLARATION

I hereby declare that this work has not already been accepted in substance for any degree and is not currently submitted in candidature for any degree.

CERTIFICATE

I hereby certify that the work embodied in this thesis is the result of my own investigation, except where reference has been made to published literature and where collaborators have been acknowledged.

Signed .. Candidate

Signed ... Director of Studies

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CHAPTER 4

CHICK EMBRYO EXPERIMENTS

ABBREVIATIONS

Dimethyl POPOP	1,4-di-2-(4-methyl-5-phenyloxazolyl) benzene
DBED salt of [2-^{14}C]MVA	N,N'-dibenzylethylenediamine-di-DL-[2-^{14}C] mevalonate
DMSO	dimethylsulphoxide
Ether	diethyl ether
EDTA-disodium salt	ethylenediaminetetra-acetic acid disodium salt
HMG CoA reductase	hydroxymethylglutaryl-CoA reductase
PPO	[1,4-di-(2-(5-phenyloxazolyl))benzene]

TABLE I.

Table of common and semi-systematic names of carotenoids.

Adonirubin	3-Hydroxy-β,β-carotene-4,4'-dione
Aleuriaxanthin	1',16'-Didehydro-1',2'-dihydro-β,ψ-caroten-2'-ol
Anhydrolutein	3',4'-Didehydro-β,β-caroten-3-ol
Antheraxanthin	5,6-Epoxy-5,6-didehydro-β,β-carotene-3,3'-diol
Astacene	β,β-Carotene-3,4,3',4'-tetrone
Astaxanthin	3,3'-Dihydroxy-β,β-carotene-4,4'-dione
Asteroidenone	3'-Hydroxy-β,β-caroten-4-one
Canthaxanthin	β,β-Carotene-4,4'-dione
α-Carotene	β,ε-Carotene
β-Carotene	β,β-Carotene
γ-Carotene	β,ψ-Carotene
ε-Carotene	ε,ε-Carotene
ζ-Carotene	7,8,7',8'-Tetrahydro-ψ,ψ-carotene
β-Carotene-triol	β,β-Carotene-3,4,4'-triol
ε-Carotene-diol	ε,ε-Carotene-3,3'-diol
β-Citraurin	3-Hydroxy-8'-apo-β-caroten-8'-al
Crustaxanthin	β,β-Carotene-3,4,3',4'-tetrol
α-Cryptoxanthin	β,ε-Caroten-3'-ol
β-Cryptoxanthin	β,β-Caroten-3-ol
Cynthiaxanthin	7,8,7',8'-Tetrahydro-β,β-carotene-3,3'-diol
Deepoxineoxanthin	6,7-Didehydro-5,6-dihydro-β,β-carotene-3,5,3'-triol
3'-O-Dehydrolutein	3-Hydroxy-β,ε-caroten-3'-one
Dehydroretinol	3,4-Didehydroretinol
α-Doradecin	3,3'-Dihydroxy-2,3-didehydro-β,ε-caroten-4-one
β-Doradecin	3,3'-Dihydroxy-2,3-didehydro-β,β-caroten-4-one
α-Doradexanthin	3,3'-Dihydroxy-β,ε-caroten-4-one
β-Doradexanthin	3,3'-Dihydroxy-β,β-caroten-4-one
Echinenone	β,β-Caroten-4-one

3'-Epilutein	(3R,3'S,6'R)-β,ε-Carotene-3,3'-diol
Fucoxanthin	5,6-Epoxy-3,5,3'-trihydroxy-6',7'-didehydro-5,6,7,8,5',6'-hexahydro-β,β-caroten-8-one-3'-acetate
Galloxanthin	10'-Apo-β-carotene-3,10'-diol
ε-Galloxanthin	10'-Apo-ε-carotene-3,10'-diol
3-Hydroxyechinenone	3-Hydroxy-β,β-caroten-4-one
3-Hydroxy-β-zeacarotene	7',8'-Dihydro-β,ψ-caroten-3-ol
Idoxanthin	3,3',4'-Trihydro-β,β-caroten-4-one
4-Ketocynthiaxanthin	3,3'-Dihydroxy-7,8,7',8'-tetradehydro-β,β-caroten-4-one
Isocryptoxanthin	β,β-Caroten-4-ol
Lutein	(3R,3'R,6'R)-β,ε-Carotene-3,3'-diol
Lycopene	ψ,ψ-Carotene
Neochrome	5',8'-Epoxy-6,7-didehydro-5,6,5',8'-tetrahydro-β,β-carotene-3,5,3'-triol
Neoxanthin	5',6'-Epoxy-6,7-didehydro-5,6,5',6'-tetrahydro-β,β-carotene-3,5,3'-triol
Neurosporaxanthin	4'-Apo-β-caroten-4'-oic acid
Neurosporene	7,8-Dihydro-ψ,ψ-carotene
Phillipsiaxanthin	1,1'-Dihydroxy-3,4,3',4'-tetradehydro-1,2,1',2'-tetrahydro-ψ,ψ-carotene-2,2'-dione
Phoenicopterone	β,ε-Caroten-4-one
Phoenicoxanthin	3-Hydroxy-β,β-carotene-4,4'-dione
Phytoene	7,8,11,12,7',8',11',12'-Octahydro-ψ,ψ-carotene
Phytofluene	7,8,11,12,7',8'-Hexahydro-ψ,ψ-carotene
Plectaniaxanthin	3',4'-Didehydro-1',2'-dihydro-β,ψ-carotene-1',2'-diol
Rubixanthin	β,ψ-Caroten-3-ol
Semi-astacene	3,3'-Dihydroxy-2,3-didehydro-β,β-carotene-4,4'-dione
Taraxanthin	5,6-Epoxy-5,6-dihydro-β,ε-carotene-3,3'-diol
Tilefishxanthin	3,2'-Dihydroxy-β,ε-caroten-4-one
Torularhodin	3',4'-Didehydro-β,ψ-caroten-16'-oic acid
Torulene	3',4'-Didehydro-β,ψ-carotene
Tunaxanthin	ε,ε-Carotene-3,3'-diol

Violaxanthin	5,6,5',6'-Diepoxy-5,6,5',6'-tetrahydro-β,β-carotene-3,3'-diol
Vitamin E	α-Tocopherol
β-Zeacarotene	7',8'-Dihydro-β,ψ-carotene
Zeaxanthin	β,β-Carotene-3,3'-diol

SUMMARY

Studies were undertaken to investigate the role of xanthophylls (particularly zeaxanthin and lutein) as precursors of other carotenoids and related compounds in some species of yeast, fish and birds.

In goldfish, β-carotene, lutein, zeaxanthin and canthaxanthin were shown by radiolabelling experiments to be precursors of retinol and 3,4-didehydroretinol. All these carotenoids, except lutein, were metabolised to astaxanthin in goldfish integument.

Turkey retinal oil droplet carotenoids were investigated and in some cases the absolute configurations defined e.g. (3R)galloxanthin, (3R,6'S) α-cryptoxanthin, astaxanthin (66% 3S,3'S-, 30% meso-, 4% 3R,3'R), zeaxanthin (72% 3R,3'R-, 28% meso-), epilutein (46% 3R,6'S,3'R-, 54% 3S,6'S,3'R-) and lutein (50% 3R,6'R,3'R-, 3R,6'S,3'S-). A new carotenoid structure is proposed for a retinal oil droplet carotenoid.

Chick retinal oil droplet carotenoids were qualitatively similar to turkey carotenoids. 3,4-Didehydroretinol, detected in chicken liver extracts, was not of dietary origin. The lutein and zeaxanthin of poultry feed were of plant origin i.e. (3R,6'R,3'R)lutein and (3R,3'R)zeaxanthin.

Zeaxanthin was shown by radiolabelling experiments in chick embryos, to be the precursor of astaxanthin, galloxanthin, ε-galloxanthin and α-cryptoxanthin. The conversion of zeaxanthin to ε,ε-carotene was not clearly defined. Zeaxanthin was deposited in chick feathers, intestine and livers during embryonic development, zeaxanthin and lutein being the major carotenoids present in these tissues.

Chick embryo experiments were carried out with [2-^{14}C]MVA as substrate to investigate whether or not de novo synthesis of carotenoids was possible in embryonic animal tissue. Low levels of radioactivity in the carotenoid zones subject these results to some doubt.

Microspectrophotometric studies on turkey oil droplets demonstrated that a mixture of carotenoids was present in each type of oil droplet. The same oil droplet types were present in turkey as in chicken and pigeon.

Previous work in the laboratory suggested that Phaffia rhodozyma metabolised zeaxanthin and canthaxanthin to astaxanthin. This had stereochemical implications. These metabolic transformations could not be demonstrated.

CHAPTER 1

INTRODUCTION

The carotenoids are yellow to red lipid-soluble isoprenoid (usually tetraterpenoid) pigments which are widely distributed in Nature. Although synthesised de novo only by plants, animals derive them via their food chains. As will be shown, the hydrocarbon carotenes are normally regarded as the biosynthetic precursors of the oxygenated xanthophylls. While this is certainly the case in the plant kingdom, this presentation considers the possibility that animals are capable of converting xanthophylls into carotenes (and, in the case of some fish, even into the vitamins A).

1.1 Distribution of Carotenoids.

These naturally occurring fat-soluble compounds are the most widely distributed pigments in the prokaryotic, plant and animal kingdoms. Even the lowest members of the plant kingdom, the algae and fungi, have carotenoid pigmentation as do the leaves, fruits and petals of higher plants. Likewise primitive animals such as the protozoan phylum contain carotenoids although the presence of carotenoids in animals is more conspicuous in some higher vertebrates. There are some exceptions, with various species of mammals and invertebrates not storing any carotenoids at all. Typically a given species will assimilate and store one carotenoid or type of carotenoid.

In animals, carotenoids may be detected in relatively high concentrations, although quantitatively this may vary from tissue to tissue. Carotenoids have been detected in many different tissues e.g. in the legs, body fat and eggs of domestic fowls, in the feathers of numerous wild birds and in the flesh and integument of marine invertebrates and fishes. They are to be found in minute oil globules of

the ovaries and eggs of many invertebrate and vertebrate species, in liver, fat depots and in the retinas and intra-ocular colour filters of eyes. They are also present in various secretions such as the waxy material from the ears of cattle and in mammalian milk.

The above serves to emphasise the almost universal occurrence of carotenoids and poses the question as to the function of carotenoids in Nature.

1.2 Functions.

Numerous functions have been ascribed to carotenoids. The functions of carotenoids themselves, as opposed to their metabolites, can be divided into two groups; photofunctions and non-photofunctions. Undoubtedly, one important effect of carotenoids is their apparent photoprotection, whereby carotenoid pigments can protect cells and tissues against the harmful effects of light. The generally accepted mode of action of this carotenoid-mediated photoprotection has been reviewed extensively (Krinsky, 1979). Many functions of carotenoids in photosynthesis have been proposed. Carotenoids can function as accessory pigments in photosynthesis, transferring radiant energy to chlorophyll. The efficiency of carotenoids functioning in this way varies and Dutton and Manning (1941) found that in marine algae fucoxanthin was as effective for photosynthesis as chlorophyll. It is also thought that carotenoids act as photoreceptors involved in the phototropism of green plants and in phototaxis of motile algae and some bacteria. These aspects of carotenoid function are reviewed by Krinsky (1971).

The functions of the second group (i.e. non-photofunctions) are less well understood and although many observations have been made relating to carotenoid pigments involved in various biological phenomena, there is little evidence of non-photofunctional roles for carotenoids. There is a possibility that carotenoid pigments stabilise their associated proteins

in carotenoprotein complexes. The presence of oil droplets containing carotenoids in the retinas of birds has caused much speculation about the role of these droplets in colour vision. The provitamin A activity of carotenoids in many species is generally accepted as is the involvement of carotenoids in reproduction although the precise mechanism of action is not defined. In humans carotenoids are thought to have some effects in cancer prevention (Mathews-Roth, 1985), and administration of β-carotene can relieve the symptoms of patients suffering from certain light-sensitive skin diseases (Mathews-Roth, 1982). As for a universal role throughout Nature the most plausible proposal is that carotenoids protect cells from photo-oxidative damage.

1.3 Absorption and transport of carotenoids in animals.

Relatively little is known about these aspects of carotenoid biochemistry. It has been established that intestinal absorption requires the presence of fats to allow the formation of micelles. Interaction of the micelles with the intestinal mucosal cell membrane results in the release of carotenoids into the intestinal epithelial cells. Hollander and Ruble (1978) showed that in unanaesthetised rats the absorption of carotenoids is passive, the diffusion rate being dependent upon the intestinal luminal concentration. The gradient can be maintained by rapid cleavage of β-carotene to retinal.

Carotenoids, once in the intestinal mucosa, are incorporated into chylomicrons. They enter the bloodstream via the lymph and appear in the lipoprotein fraction of the plasma. β-Carotene is found in the low density lipoprotein (LDL) fraction of human plasma (Krinsky et al., 1958; Mathews-Roth and Gulbrandsen, 1974; Bjornson et al., 1976) whereas xanthophylls are associated with the high density lipoprotein (HDL) fraction (Krinsky et al., 1958). In birds ketocarotenoids such as canthaxanthin and astaxanthin are present in the HDL fraction of plasma (Fox et al., 1965).

Carotenoid absorption can be increased in rats and birds by supplementing their diet with vitamin E (Grundboeck et al., 1977) but beyond a certain concentration the effect is lost (Arthur et al., 1979). As the rate of absorption of carotenoids is linearly related to the intestinal mucosal concentration, which in turn is dependent on the rate of formation of retinal, maintenance of a high cleavage rate of carotenoids is desirable. Protein, although ineffective for absorption of carotenoids is essential for rapid cleavage of carotenoids to retinal (Kamath and Arnich, 1973).

1.4 Carotenoid formation.

As animals cannot synthesise carotenoids de novo they immediately become dependent upon plants for the carotenoid carbon skeleton. This inability to produce carotenoids, although well documented, is quite remarkable in that the initial steps for carotenoid synthesis are common to other terpenoid biosynthetic pathways e.g. sterol pathway. Animals are capable of making geranylgeranyl pyrophosphate (GGPP) in a way depicted in Fig.1. The carotenes are products of the dimerisation of GGPP with a cyclopropylcarbinyl pyrophosphate (prephytoene pyrophosphate) as an intermediate (Altman et al., 1972). Phytoene is formed on the oxidative loss of the pyrophosphate from prephytoene pyrophosphate. Several mechanisms have been proposed for this (Epstein and Rilling, 1970; Altman et al., 1971; Edmond et al., 1971; Rilling et al., 1971; van Tamelen and Schwartz, 1971; Beytia et al., 1973). In higher plants the phytoene is of the 15-cis configuration (Jungalwala and Porter, 1965) but for certain non-photosynthetic organisms it is all-trans (Khatoon et al., 1972). It is assumed that the dimerisation of GGPP to phytoene is the stage which animals cannot manage. It is interesting to note that phytoene and other intermediates have been found in animals but it is assumed that these were derived directly from dietary plant material. Porter and Lincoln (1950) suggested that phytoene is sequentially desaturated to lycopene, with phytofluene, ζ-carotene and neurosporene

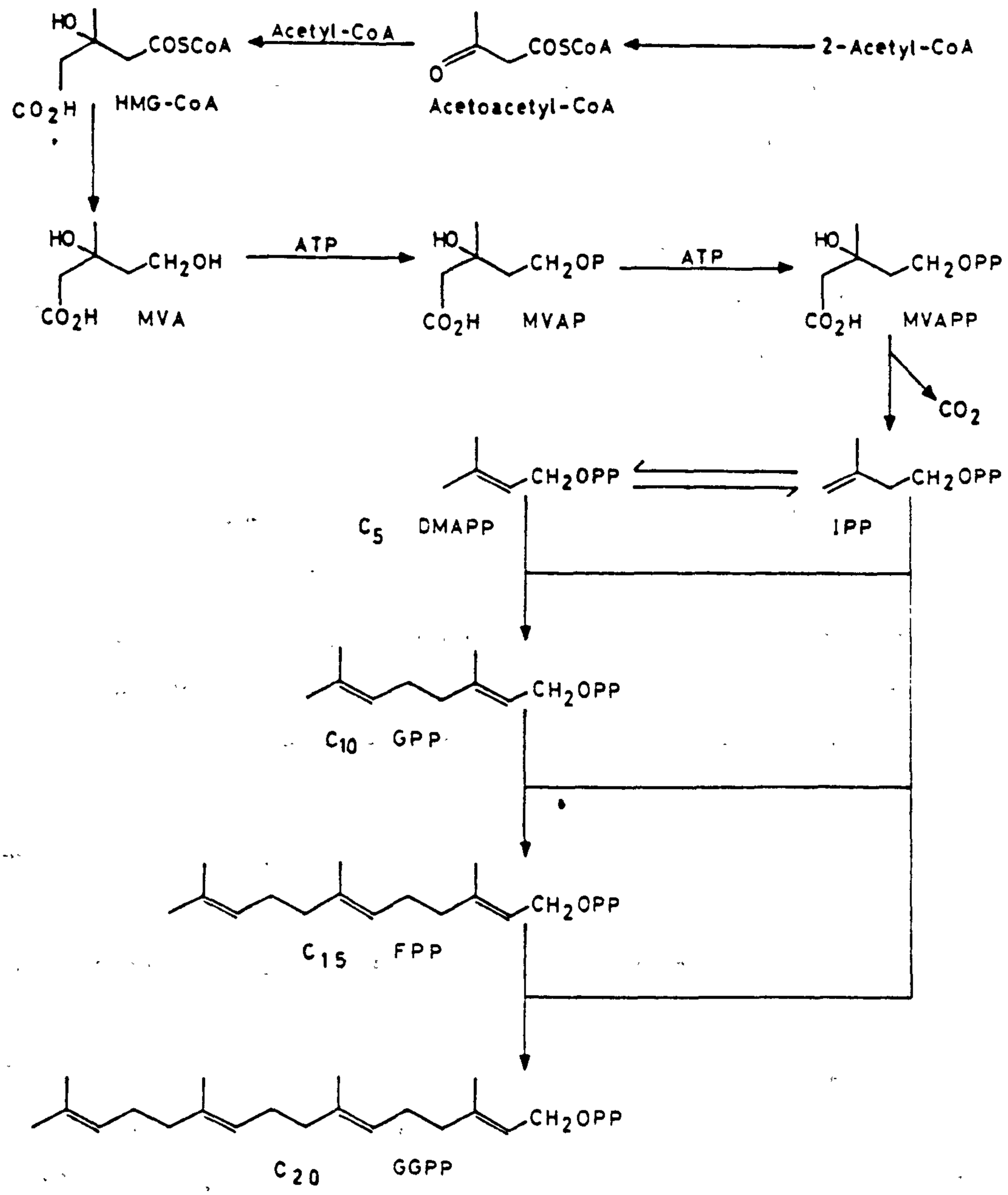


Fig. 1.

Pathway of formation of geranylgeranyl pyrophosphate (GGPP) from mevalonic acid (MVA).

IPP isopentenyl pyrophosphate

DMAPP dimethyl allyl pyrophospate

FPP farnesyl pyrophospate

GPP geranyl pyrophosphate

as intermediates. This occurs by the trans elimination of hydrogens. Cis-trans isomerisation must occur to account for the fact that in higher plants the phytoene is 15-cis but ζ-carotene, lycopene and neurosporene are of the all-trans configuration (Davies, 1970). Cyclisation may occur at a number of stages, notably at neurosporene to give β-zeacarotene and at lycopene to give γ-carotene and consequently β-carotene (Fig.2). Other modifications such as hydroxylations and modifications to the polyene chain are then possible.

The large number of carotenoids now documented in the literature is quite remarkable, illustrating the complexity of the structures based on a C_{40} skeleton. In 1976 the number of structures defined by Straub (1976) was over 400 but with the newer structures described in the proceedings of IUPAC International Symposia on carotenoids (Britton and Goodwin, 1982; Davies and Rau, 1985) the total number of carotenoids must have risen to well above 500.

1.5 De novo carotenoid synthesis in animal corpora lutea.

A noteworthy report conflicting with the accepted view that animals are incapable of forming carotenoids, has been published by Austern and Gawienowski (1969). These workers observed carotenoid biosynthesis in bovine corpora lutea, noting the incorporation of radioactivity from radiolabelled MVA into β-carotene. Similar experimentation with enzyme preparations from bovine corpora lutea in this laboratory revealed high incorporations of [2-^{14}C]MVA into unsaponifiable fractions from the incubation. After h.p.l.c. purification the β-carotene contained no radioactivity and no biosynthetic precursors were present (Davies and Akers, unpublished work). Although corpora lutea can reputedly synthesise retinol from β-carotene (Gawienowski et al., 1974) the evidence for total synthesis of carotenoids from their natural precursor MVA is not convincing.

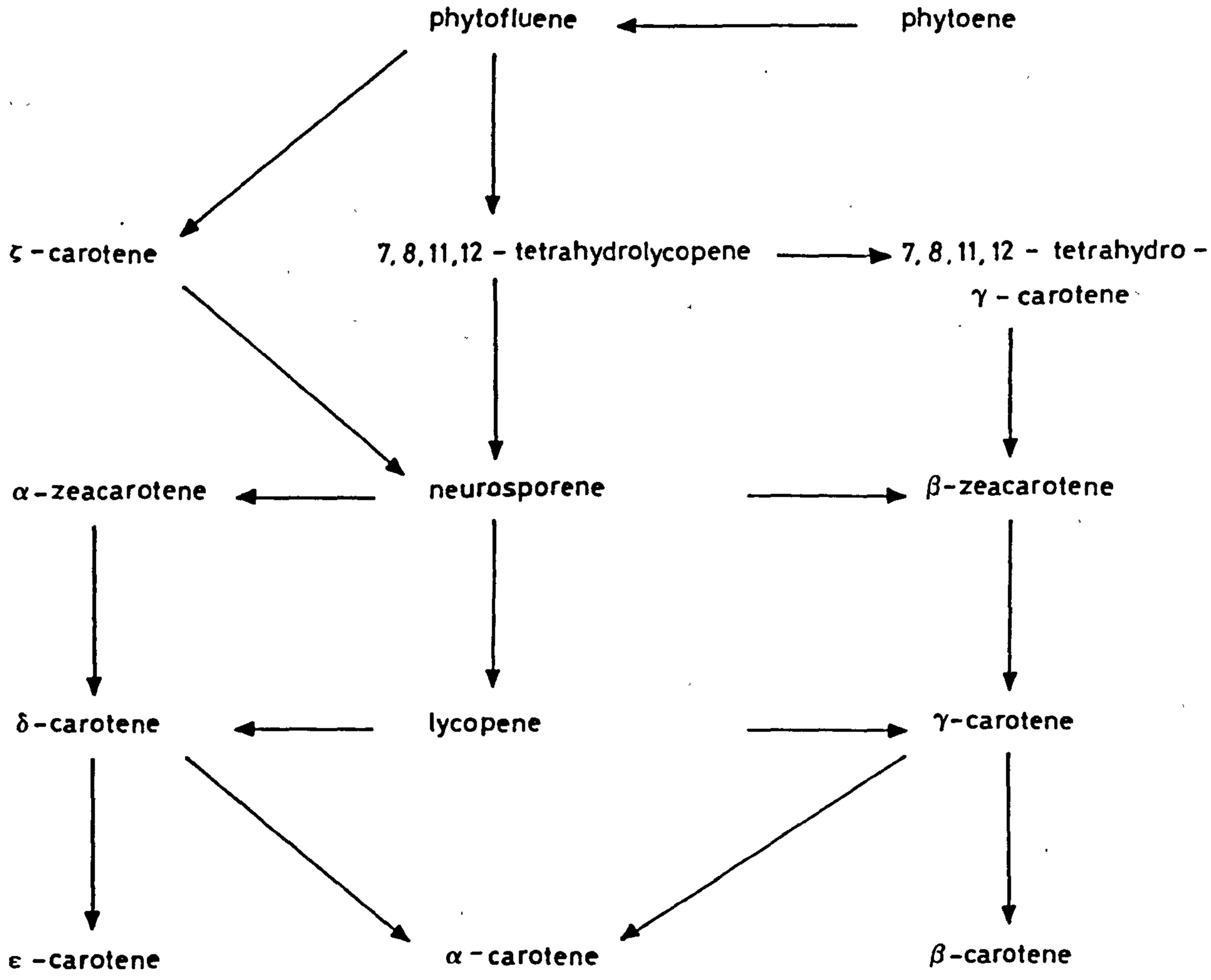


Fig. 2.

Carotene biosynthesis in higher plants and fungi, showing possible sites of cyclisation.

1.6 Animal metabolism of carotenoids.

Buchecker (1982), in a paper entitled "A chemist's view of animal carotenoids" drew attention to the limited knowledge and understanding of animal carotenoid biochemistry. It is readily agreed that the progress made in carotenoid biochemistry is inferior to advancements made by the organic chemist who, with the development of new techniques e.g. h.p.l.c., chiroptical methods and n.m.r., is now defining new carotenoid structures in great detail and in terms of absolute stereochemistry. The biologist is forever finding new species with carotenoid pigmentation requiring analysis but still the role of carotenoids in animals is ill-defined and many questions remain unanswered as to the metabolism of carotenoids. In the words of Buchecker "it would be desirable if the hypothetical mechanisms could be investigated by means of labelling experiments".

It is all too easy for the organic chemist to draw a putative arrow between two closely related structures; to prove that such an arrow represents a real metabolic transformation is more difficult. Part of the problem for the biochemist is that for reasonable studies of animal carotenoid metabolism the researcher is forced to undertake long term experiments which may or may not demonstrate any metabolism of carotenoids. At present the biochemist is proving metabolic transformations in animals using whole-animal feeding experiments with radioactively labelled carotenoids in the feed. Although this is satisfactory, more information and hence understanding may be generated from working with enzyme preparations. The only enzyme of animal carotenoid metabolism studied in any depth is the mammalian intestinal β-carotene-15,15'-dioxygenase. Considering the large number of carotenoids now documented, this is a poor reflection on the efforts of the biochemist in a difficult area of research.

Davies (1985) proposed that carotenoid metabolism in animals can take three forms each catalysed by different types of enzymes. These three reactions are:

a)the substitution of carotenoid end groups (see Fig. 3 for carotenoid end groups) by oxygen functions (-OH and -C=O)

b)the modification of end groups

c)cleavage of the polyene chain to give apocarotenoids and vitamin A

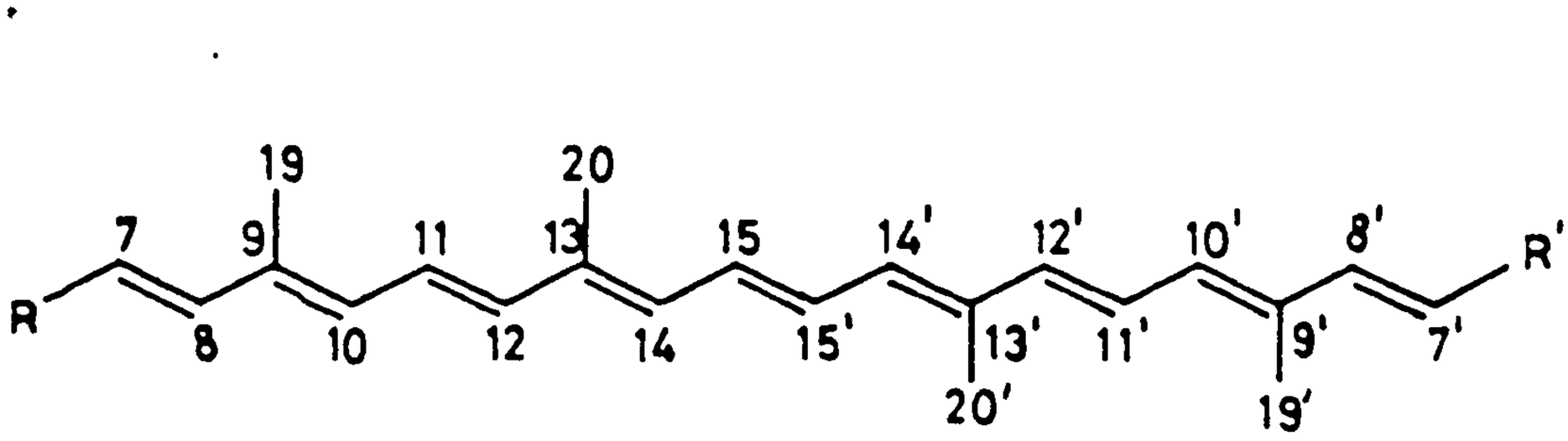
Each of these reaction types was investigated in a variety of animal and yeast species.

A. Goldfish

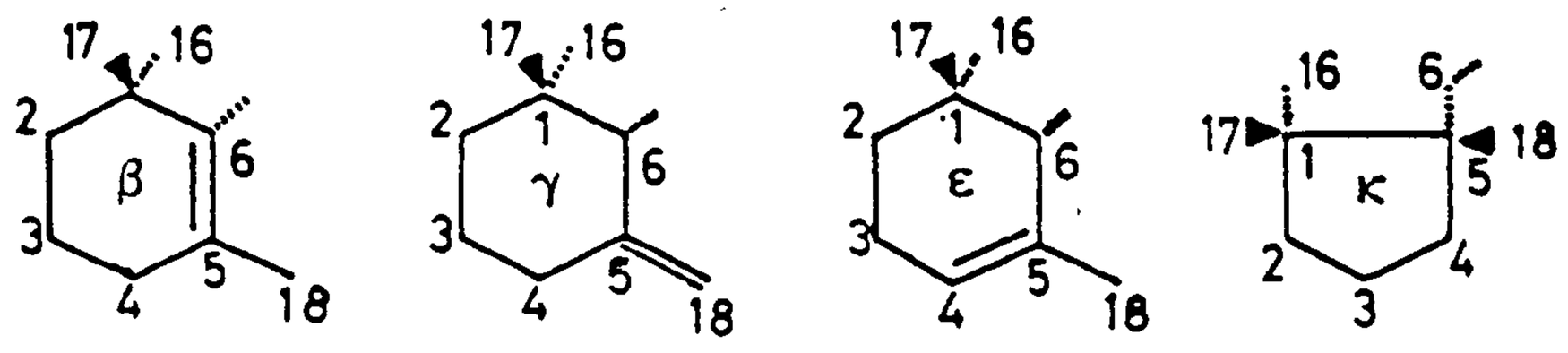
Freshwater fish produce 3,4-didehydroretinol (dehydroretinol, vitamin A_2) as well as retinol (vitamin A_1) and recent work suggests that lutein may be the precursor of this more highly unsaturated form of vitamin A. This hypothesis can be tested by whole organism feeding experiments. Goldfish are amongst those fishes forming dehydroretinol and using these fish as an experimental species would also prove whether or not lutein is converted into astaxanthin. As can be seen from Fig. 4 these two metabolic pathways represent all three reaction types put forward by Davies (1985).

B. Avian retinal oil droplets.

Establishment of the carotenoid content of avian retinal oil droplets has posed problems as to the metabolic origin of some of these carotenoids, in particular (6S,6'S)-ε,ε-carotene and galloxanthin both of which are unique to avian species. (3R,3'R)-Zeaxanthin can be envisaged



End Groups [R,R']



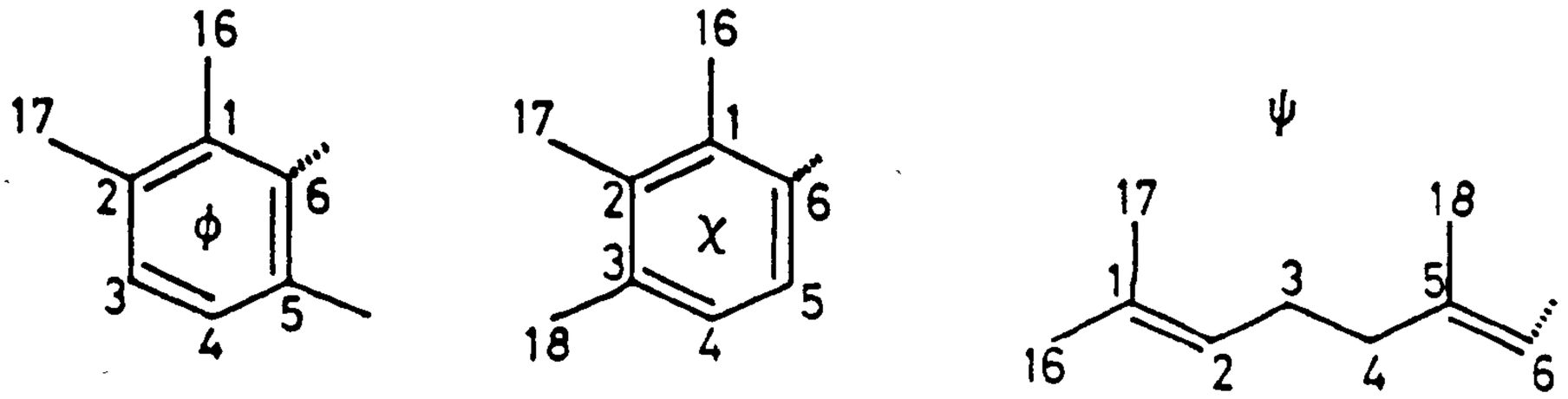


Fig. 3.

Carotenoid end groups.

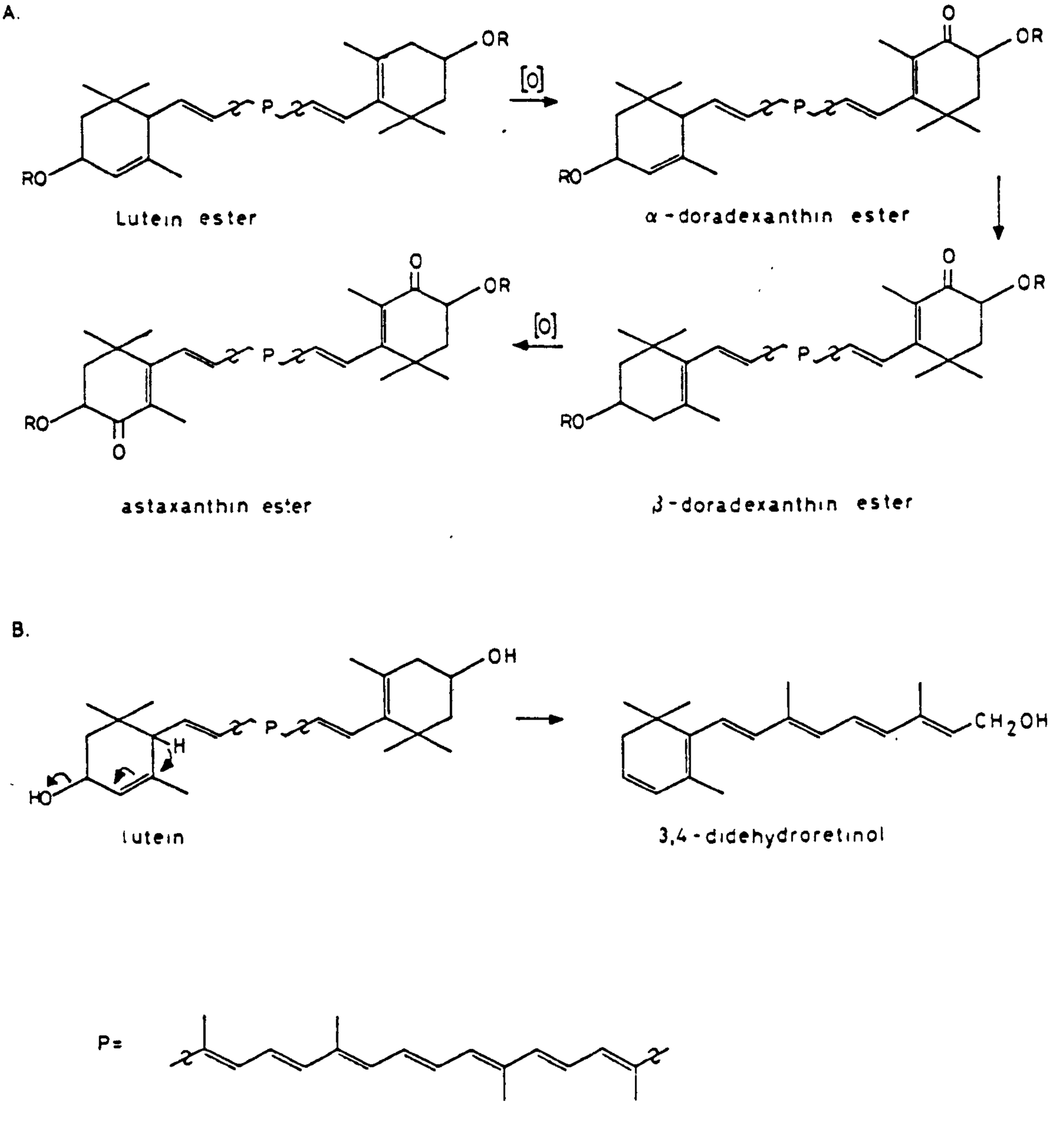


Fig. 4.

Metabolic pathways proposed for retinol, dehydroretinol and astaxanthin formation in goldfish.

as precursor as depicted in Fig. 5. A procedure has been developed to determine the role of zeaxanthin in the formation of astaxanthin, as well as galloxanthin and (6S,6'S)-ε,ε-carotene in chick embryos.

Again all three types of carotenoid reactions would be involved in these transformations.

C. Yeast

In animals many pathways have been postulated for the transformation of β-carotene to astaxanthin with all manner of intermediates isolated to substantiate these routes. Davies (1985) visualises that only three enzymes are required for any of these pathways provided that each enzyme has very broad specificity; a C-3 mono-oxygenase, a C-4 mono-oxygenase and a caroten-4-ol dehydrogenase. The existence of stereochemical isomers of astaxanthin demands that the enzyme responsible for 3-hydroxylations displays stereospecificity. Assuming that astaxanthin is formed from β-carotene by the action of the three aforementioned enzymes working in a random order and that the direction of attack of the 3-hydroxylation of the β-end group is influenced by whether or not there is a 4-keto group on the rings, this would be one way of forming a mixture of meso-astaxanthin and two enantiomeric astaxanthins.

Answers to this problem may be forthcoming using Phaffia rhodozyma, a red yeast which unusually forms astaxanthin as its main carotenoid. The astaxanthin of P. rhodozyma is (3R,3'R)astaxanthin rather than the more common 3S,3'S form.

Andrewes and Starr (1976) have ascribed this chirality difference to a different order of a) hydroxylation and b) ketonisation. The scheme of Fig. 6 illustrates this possibility. It is possible that the presence of a 4-keto substitution makes the 3-hydroxylase R-specific while hydroxylation of an unsubstituted ring is S-specific.

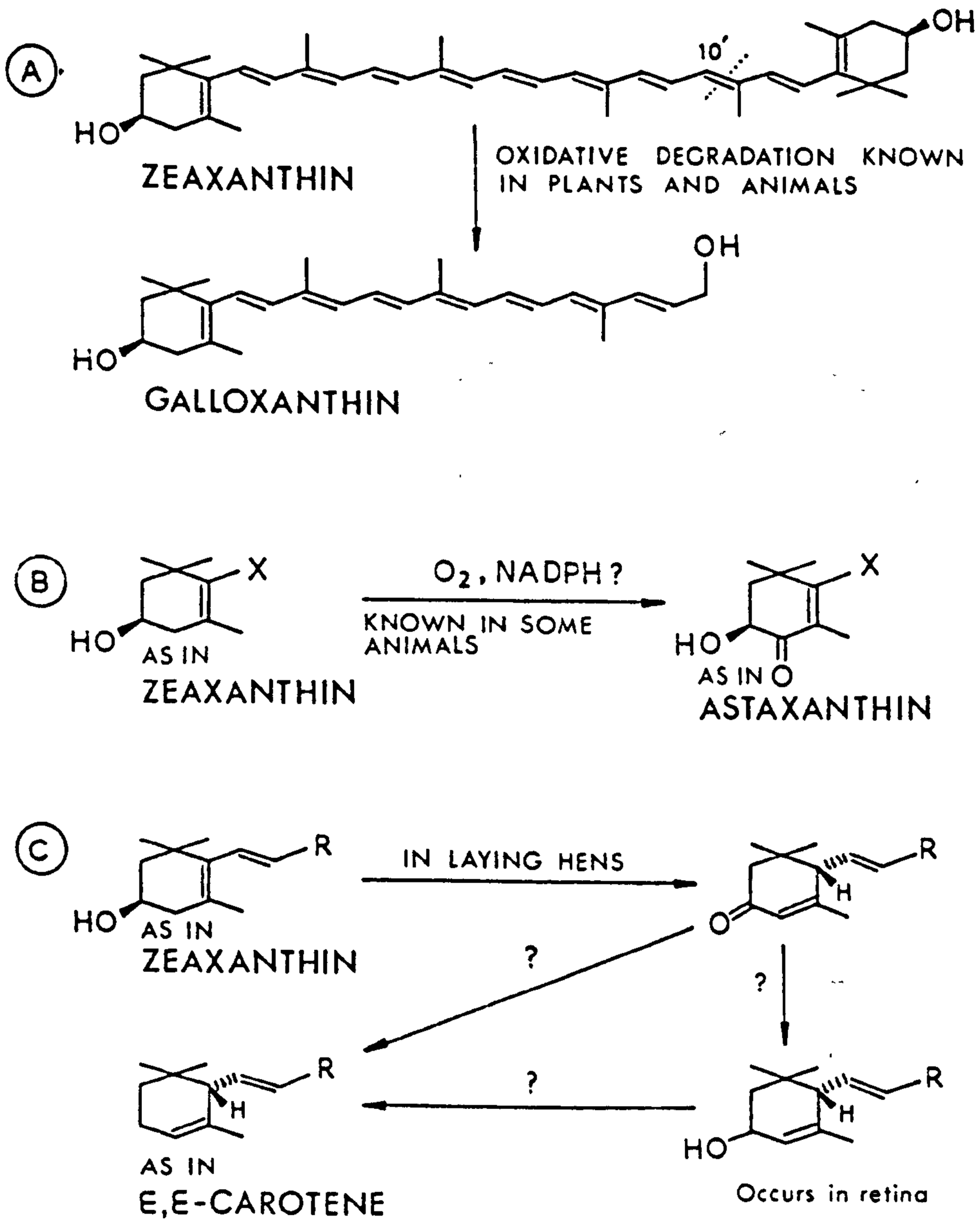


Fig. 5.

Proposed pathways of formation of (6S,6'S)-ε,ε-carotene, astaxanthin and galloxanthin from zeaxanthin in avian species.

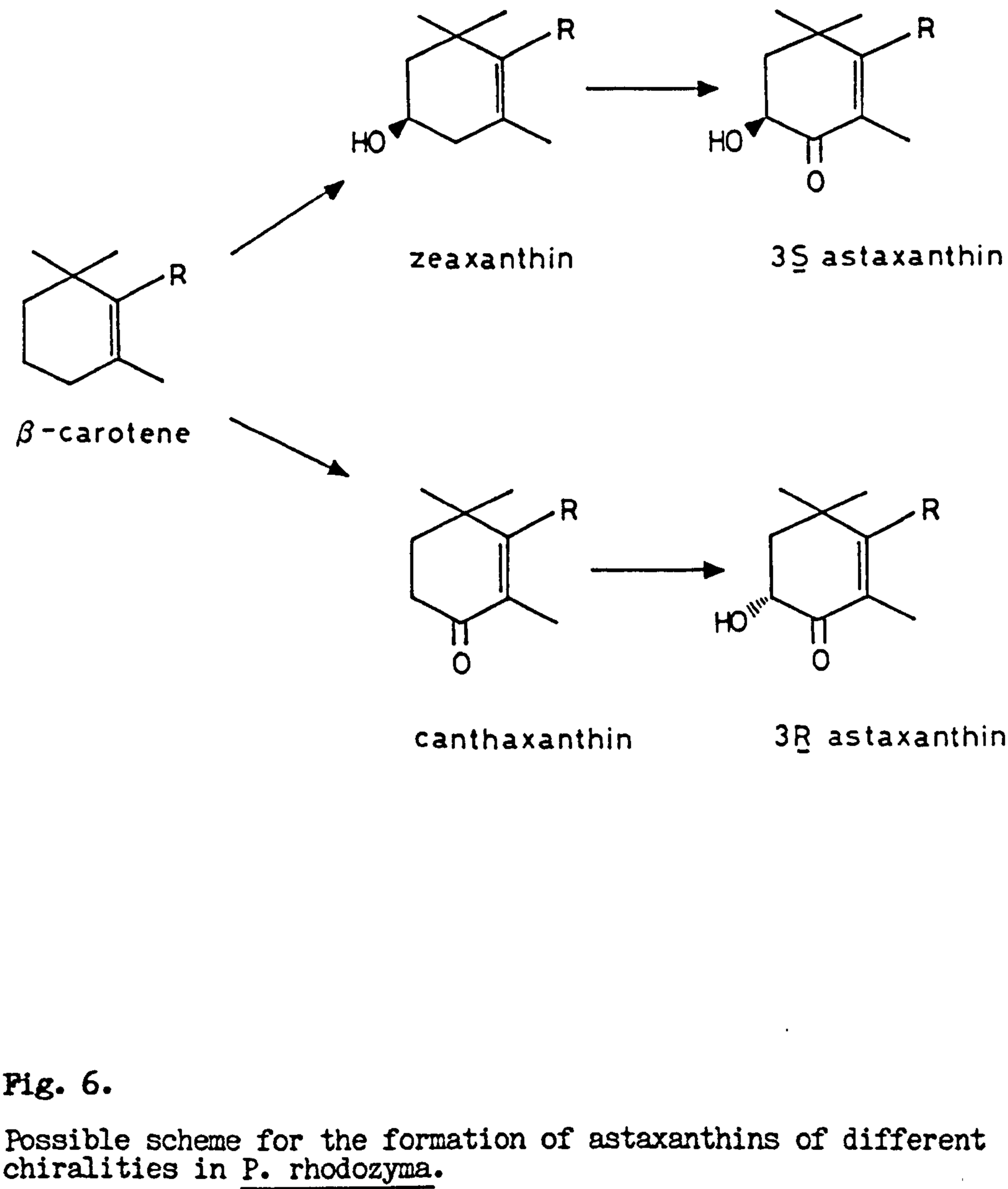


Fig. 6.

Possible scheme for the formation of astaxanthins of different chiralities in P. rhodozyma.

AIMS OF THE PROJECT

A series of comparative studies was undertaken to investigate the roles of xanthophylls (particularly zeaxanthin and lutein) as precursors of other carotenoids and related compounds in a variety of organisms including yeasts, fish and birds. A common experimental approach was to follow the metabolism of a ^{14}C-labelled xanthophyll in a whole organism or cell-free system.

CHAPTER 2

INTRODUCTION

2.1 General properties of carotenoids.

Carotenoids are notoriously difficult compounds with which to work because of their sensitivity to light, heat, oxygen, acids and, in some cases, alkali. This necessitates many precautions during their manipulation. Inadequate care during handling can lead to low recoveries, complete loss of especially labile carotenoids and the appearance of artifacts. In the case of the vitamins A the problems are even more severe and extreme care should be taken at all times.

General methods of manipulation of carotenoids are described in the following sections.

2.2 Preparation of radiolabelled carotenoids.

To follow the metabolism of a carotenoid in either an animal or microbial system requires the use of radiolabelled carotenoids.

Carotenoid feeding experiments in animals are not uncommon and the metabolism of any ^{14}C-labelled xanthophyll administered can be readily followed. Where labelled carotenoids are not available quantitative changes in metabolite levels must be used as an indication of metabolism; this is a far less reliable approach for metabolic studies.

Radiolabelled zeaxanthin and lutein were produced via biological systems. [^{14}C]Zeaxanthin was prepared via a broken cell suspension of Flavobacterium R1560 and [^{14}C]lutein was made using higher plants, either barley or maize.

The need for radiochemically pure ^{14}C-labelled carotenoids demanded that good h.p.l.c. separation systems were available for the purification of these xanthophylls.

The method of preparation and purification of ^{14}C-labelled xanthophylls are outlined in the following sections.

MATERIALS AND METHODS

2.3 Materials.

Solvents and general reagents were supplied by BDH Chemicals Ltd., Poole, England.

H.p.l.c. grade solvents were supplied by Rathburn Chemicals, Walkerburn, Scotland.

Goldfish were supplied by Mr. G. Evans, Machynlleth, Wales.

Compactin was a gift from Dr. R. Fears, Beecham Pharmaceuticals, Epsom, Surrey, England.

For alumina columns Alumina Woelm N, Akt 1., Woelm Pharma, Eschwege, Germany was used.

Maize, Brutus R2779 seeds were supplied by Nickerson RPB Ltd., Rothwell, Lincoln, England.

ATP and lysozyme were obtained from Sigma Chemical Company Ltd., Fancy Road, Poole, England.

15,15'[^{14}C]Canthaxanthin, [^{14}C] β-carotene, $^{15,15'}_{\Lambda}$[$^{3}H_2$]zeaxanthin, astacene, β-carotene, β-doradexanthin, lutein and 3,4-dehydroretinol were gifts from F. Hoffmann-La Roche and Co.Ltd., Basle, Switzerland.

2.4 General methods for manipulating carotenoids and vitamins A.

Carotenoids and vitamin A are unstable to light, heat and, in most cases, to acid and consequently exposure to any of these was avoided as far as possible.

Exposure to light, either ultraviolet or direct sunlight, causes cis-trans photoisomerisation and may lead to photodegradation of carotenoids. All containers were covered with black cloths or wrapped in aluminium foil. This applied especially during chromatographic procedures when columns were wrapped in aluminium foil and t.l.c. developing tanks covered with black cloths.

Heating of carotenoid solutions was avoided wherever practicable. The removal of solvent was carried out by rotary evaporation (Büchi Rotavapor-R, Büchi Laboratoriums Technik AG Flawil Switzerland) at reduced pressure (water pump). Low boiling point solvents were used wherever possible e.g. light petroleum (b.p. 40–60°C) and ether.

Carotenoid oxidation by oxygen or peroxides is a particular problem during chromatography, extraction and saponification. In the former case oxidation may be minimised by running t.l.c. in an atmosphere of nitrogen. During extraction and saponification oxidation may be reduced by the inclusion of an antioxidant such as pyrogallol.

Since exposure to acids can result in cis-trans isomerisations, oxidative degradation and the isomerisation of 5,6-epoxides to 5,8-epoxides, their use was avoided except for the extraction of acidic carotenoids after saponification.

Samples were stored in the dark, under an atmosphere of nitrogen in the deep freeze (-20°C). Containers used to store samples were allowed to reach laboratory temperatures before being opened.

High solvent purity was desirable to minimise the detrimental effect of solvent impurities on samples and to reduce complicating effects on analytical procedures such as h.p.l.c., m.s. and spectrophotometry. Light petroleum and ether were dried for 2 days over sodium wire. The dry ether was redistilled from reduced iron powder (removes peroxides). Dry light petroleum was redistilled. Ethanol and methanol were redistilled from KOH pellets. Acetone was redistilled before use. Pyridine was refluxed over KOH for 2h, redistilled in a moisture free system and stored over molecular sieve (type 4A, BDH Chemicals Ltd., Poole). Light petroleum (b.p. 100–120°C) was purified by the classical method involving treatment with concentrated sulphuric acid. After

shaking, the mixture was allowed to settle and the lower layer of acid was drawn off. The light petroleum was then shaken twice with water in order to remove most of the acid, once with 10% sodium carbonate and then finally dried over sodium sulphate. The light petroleum was column treated and fractionated (Vogel, 1959).

2.5 Extraction of total lipid.

Extraction procedures were the same for animal, plant and bacterial sources of carotenoids. The initial extraction was with either ethanol or acetone, acetone being avoided if aldol condensations with carotenals were likely (Davies, 1976). Homogenisation of the sample in its chosen solvent (Silverson Laboratory Homogeniser, Silverson Machines Ltd., Waterside, Chesham, Bucks.) was followed by centrifugation at 1000 g (Griffin Christ bench top centrifuge) and the residue was re-extracted 3 or 4 times with solvent, until extracts were colourless. The supernatant extracts from centrifugation were bulked in a separating funnel and ether added. Water was added until 2 phases appeared. The aqueous phase was removed and extracted a further 3 times with ether. The bulked ethereal layers were finally washed 3 times with water. After removal of the aqueous layer the ether could be removed by rotary evaporation.

Emulsions were dispersed by the addition of a few millilitres of alcohol or by adding a strong solution of sodium chloride.

2.6 Saponification.

In most cases, unwanted neutral lipids were removed by "cold" saponification. The total lipid was dissolved in ethanol and 60% (w/v) aqueous potassium hydroxide added, 1 ml for every 10 ml of ethanolic solution. The mixture was left for 12 - 16h in the dark at room temperature under nitrogen.

The cold alkaline solution was mixed with an equivalent volume of

ether in a separating funnel and water added until 2 phases separated, the carotenoids and vitamin A being in the ethereal layer. The aqueous layer was run off and extracted 3 times more with ether. The bulked ether extract was washed free of alkali by equivalent volumes of water until the washings were no longer alkaline to phenolphthalein.

The solution was then dried over anhydrous sodium sulphate and the solvent removed by rotary evaporation. Any residual water could be removed readily after treatment with ethanol and benzene (forms a stable azeotropic mixture). The unsaponifiable lipid was redissolved in a suitable solvent.

The aqueous phase contains any acidic carotenoids which may be present. To extract these carotenoids, the aqueous phase was acidified to pH 4 with glacial acetic acid and then extracted, washed and dried as above.

A report by Barua et al. (1973) stating that "cold saponification resulted in appreciable decomposition of dehydroretinol" was shown to be incorrect when no loss of dehydroretinol was observed after the saponification of a known amount of dehydroretinol. Studies on vitamins A by Collins et al. (1953) report no such ill effect of saponification on dehydroretinol.

Sterol removal was more difficult. In some cases it sufficed to dissolve the total unsaponifiable lipid in light petroleum (b.p. 40-60°C) and leave overnight at -20°C. The resultant sterol precipitate could be collected by filtration and washed with ice-cold light petroleum to remove residual carotenoids. Sterols could also be precipitated as their digitonides. The total unsaponifiable material (20 mg) was dissolved in 6 ml of 95% aq. ethanol in a thick walled centrifuge tube, antibumping granules added and the solution heated to boiling. Similarly digitonin (100 mg) was dissolved in 5 ml of 90% aq. ethanol in a boiling tube.

Antibumping granules were again added and the solution heated to boiling. The two boiling solutions were mixed and heated until a white precipitate formed. Complete precipitation was achieved by leaving the solution at -20° C overnight. The precipitate was collected by centrifugation (Griffin Christ bench top centrifuge, 1000 g) and washed with cold ether. This treatment was effective for removing most sterols, the notable exceptions being 4α-methyl and 4,4-dimethyl sterols.

2.7 Ultraviolet / visible spectrophotometry.

An SP8-150 (Pye Unicam) and a Beckman DU-7 spectrophotometer were used to determine all absorption spectra. The solvents used for the determination of spectra varied greatly but generally the absorption spectra of carotenes were determined in light petroleum and those of xanthophylls were determined in ethanol. In order to minimise instrumental error with the SP8-150, the appropriate bands from a holmium oxide filter were recorded onto each experimental absorption spectrum. In the case of the Beckman DU-7 this is unnecessary as the instrument automatically self calibrates.

2.7A Qualitative interpretation of spectra.

The profile of an absorption spectrum of a carotenoid is a function of its structure and purity. The number of conjugated double bonds in the molecule determines the absorption wavelength maxima, which may vary slightly with different solvents. The shape of an absorption spectrum can indicate various structural features. Acyclic carotenoids characteristically display greater persistence in the shape of their spectra than carotenoids with rings e.g. carotenoids with β-end groups. For many ketocarotenoids the typical 3 peaked spectra is lost altogether and only a symmetrical peak may be obtained. For preliminary identification purposes the main absorption maxima of carotenoids given

by Davies(1976) were used.

2.7B Estimation of carotenoids.

The carotenoids were dissolved in a known volume of solvent. The absorbance at the absorption maximum (λ_{max}) was determined and the amount of carotenoid then calculated as follows:

$$X = \frac{Ay}{A^{1\%}_{1cm} \times 100}$$

where X = g of carotenoid,

y = ml of solution,

$A^{1\%}_{1cm}$ = specific absorption coefficient.

Values of $A^{1\%}_{1cm}$ are quoted by Davies (1976).

2.8 Chromatographic procedures.

Three forms of chromatography were used routinely during the work. Preliminary separations were generally achieved by classical column chromatography, with final purifications or separations by h.p.l.c. or t.l.c.

Column chromatography was carried out by standard procedures (Davies, 1976). Columns were packed in light petroleum. In the case of alumina columns the alumina was deactivated with water (6% v/w water for Brockmann Grade III and 10% for Grade IV) before the addition of the packing solvent. MgO (MgO for chromatographic adsorption analysis, BDH Chemicals Ltd., Poole) and cellulose (Cellulose powder CF11 Whatman Ltd., Maidstone) were used directly. The columns were loaded with as small a volume of sample as possible.

Thin layers of Silica Gel G (Precoated Plates Merck Silica Gel 60F-254, Darmstadt, W.Germany.) were used for t.l.c. Samples were applied to the plates as narrow strips and the plates developed in chromatography tanks lined with chromatography paper (Whatman no.1, Whatman Biochemicals, Maidstone, Kent.) and covered with black cloths.

Two h.p.l.c systems were used for all work. For vitamin A work (requiring monitoring at 335 nm and hence a deuterium lamp) a Cecil instrument was used. The Cecil instrument CE212A variable wavelength u.v. monitor was used in conjunction with an Altex pump model 110A.

Detection of carotenoids was carried out at 440 nm. For carotenoid h.p.l.c. a Pye Unicam LC-UV detector and LC-XPD pump were used. A Bryans BS-271 chart recorder (Gould Bryans Instruments Ltd., Mitcham, Surrey.) was used with both systems.

Isocratic solvents were used for h.p.l.c. All solvents were filtered before use through a Millipore filter (Whatman glass microfibre filters GF/F, 4.7cm diam., Whatman Ltd., Maidstone) and degassed with helium in an attempt to stabilise the detector base line. Care was taken to equilibrate the columns in solvent before starting chromatography.

Absorption spectra of fractions collected were plotted using an SP8-150 (Pye Unicam) or a Beckman DU-7 spectrophotometer.

2.9 Mass spectrometry.

Mass spectrometry is a useful tool for establishing the molecular weight and basic composition of a compound. Also the fragmentation processes allow certain structural features to be elucidated. M.s. of carotenoids is difficult as they are degradable, necessitating low temperatures and short heating periods. Reproducibility may be poor, even with very pure samples.

The mass spectra were determined on a Kratos MS25 instrument by a direct insertion probe sampling technique. Probe temperatures were usually increased from 95°to 210°C and the ionising potential was 70eV. The data system was a Data General Nova 4 computer and samples were analysed using a data system DS55 programme.

2.10 Radioautography of t.l.c plates.

Under illumination from a Kodak Safelight (Filter No.2) the t.l.c. plates were put into contact with X-ray films (Kodak Direct Exposure film DEF-2) and left for an appropriate period of time. Light was excluded from the plates by wrapping in aluminium foil and black cloths.

For development of the films, Kodak DX-80 developer was used (diluted 1 part with 4 parts water). The X-ray film was submerged in the developer for 4 min (at 20°C) with periodic agitation. Before the fixing stage the film was rinsed with water. The film was fixed using Kodak FX-40 X-ray liquid fixer (diluted 1 part with 4 parts water) for 8 min (at 20°C). The film was rinsed with water for 30 min and left to dry.

2.11 Liquid Scintillation Counting.

The counter used for all the work was an Intertechnique SL30 liquid scintillation spectrometer (Lablogic, Sheffield).

The scintillation fluid used was either toluene-based with added PPO (5g/l toluene) and dimethyl POPOP (0.15g/l toluene) or Cocktail T 'Scintran' as supplied by BDH Chemicals Ltd, Poole.

For accurate estimation of radioactivity of a carotenoid sample an allowance must be made for colour quenching. Quenching of this sort does not occur with vitamin A.

Quench curves were determined by the ESCR method (external standard channels ratio). A plot was obtained from the determination of channels ratio values on a series of samples containing a known quantity of a known isotope and increasing amounts of a colour quencher i.e. carotenoid. For example, a series of vials was prepared, each vial containing a different amount of zeaxanthin, ranging from zero to 50 µg (vials 0-9). [^{3}H]Hexadecane (6810 d.p.m. per vial) and 10ml scintillation fluid were added and the vials counted for 2 x 10 min. The counting efficiency was calculated for each vial. Typical data are shown in Table 1. A graph was plotted of counting efficiency against ESCR enabling the linear regression to be calculated and hence co-efficients a to e, as depicted in Fig. 7. The instrument was programmed accordingly each time prior to counting samples. In later work the quench was calculated automatically by a d.p.m. program (copyright of Lablogic, Sheffield) in conjunction with an Epson HX-20 computer.

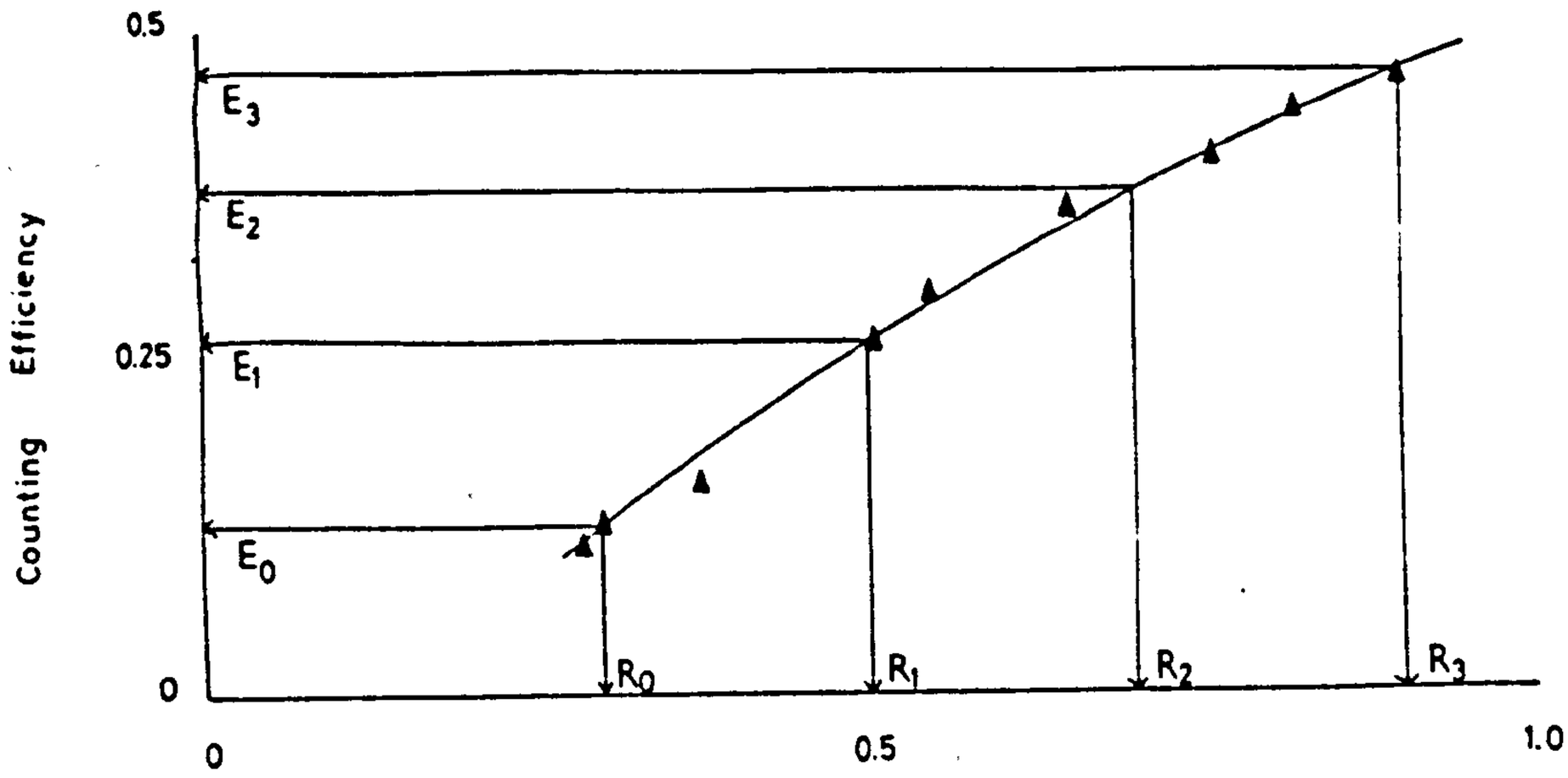
Bramley et al. (1974) found that one set of correction curves was applicable to all the carotenoids that they examined. They considered it improbable that any other carotenoid would be inconsistent with their data. Consequently, only one set of correction curves was determined (one curve for carbon-14 carotenoids and one for tritiated carotenoids).

For metabolic studies of the nature undertaken in this project, low level counting rates would be an inherent problem. A review of the assessment of the significance of low level count rates is given by Case et al. (1977). In all the work of the present study, the general rule was observed that for a net counting rate lower than 200 c.p.m. a 10 minute counting time is insufficient to give 95% accuracy.

TABLE 1.

Counting efficiencies and ESCR data for a series of vials each containing increasing amounts of carotenoid and a known amount of [^{3}H]hexadecane (6810 d.p.m.).

Sample	Carotenoid in µg	Average counts	Efficiency $\left[\frac{\text{Average counts}}{\text{Actual counts}}\right]$	Average ESCR
Blank	0	1.3	0	0.912
0	0	3155.8	0.4633	0.901
1	0.82	2967.5	0.4357	0.824
2	1.64	2733.2	0.4013	0.763
3	4.10	2490.4	0.3656	0.652
4	6.56	2072.4	0.3043	0.547
5	8.21	1801.5	0.2645	0.466
6	9.85	1415.5	0.2078	0.505
7	14.78	1242.9	0.1825	0.372
8	19.70	877.8	0.1289	0.3
9	25.45	759.9	0.1116	0.285



$E_0 = 0{\cdot}124$
$E_1 = 0{\cdot}272$
$E_2 = 0{\cdot}386$
$E_3 = 0{\cdot}466$

$R_3 = 0{\cdot}9$
$R_0 = 0{\cdot}3$

$$\Delta R = \frac{R_3 - R_0}{3} = 0{\cdot}2$$

$(\Delta R)^2 = 0{\cdot}04$

$(\Delta R)^3 = 0{\cdot}008$

$-E_0 = -0{\cdot}1243$	$+2E_0 = +0{\cdot}2486$	$-11E_0 = -1{\cdot}3673$
$+3E_1 = +0{\cdot}816$	$-5E_1 = -1{\cdot}36$	$+18E_1 = +4{\cdot}896$
$-3E_2 = -1{\cdot}158$	$+4E_2 = +1{\cdot}544$	$-9E_2 = -3\,474$
$+E_3 = +0{\cdot}466$	$-E_3 = -0{\cdot}466$	$+2E_3 = +0\,932$
$\Sigma A \ -0{\cdot}0003$	$\Sigma B \ -0{\cdot}0334$	$\Sigma C \ +0{\cdot}9867$

$$a = \frac{\Sigma A}{6(\Delta R)^2} = -0{\cdot}00625 \qquad b = \frac{\Sigma B}{2(\Delta R)^2} = -0{\cdot}4175 \qquad c = \frac{\Sigma C}{6(\Delta R)} = +0{\cdot}8225$$

$d = E_0 = 0{\cdot}1243$ $\qquad$ $e = R_0 = 0{\cdot}3$

Fig. 7.

Sample ESCR data and analysis of quench.

2.12 Preparation of [^{14}C]zeaxanthin.

Flavobacterium is a member of the Eubacteriales order. The bacteria are Gram negative rods and are characteristically orange or yellow. The colour of Flavobacterium is due to the presence of carotenoid pigments. The main carotenoid (95% of the total) of Flavobacterium strain R1560 is (3R,3'R)zeaxanthin but small amounts of 15-cis-phytoene, phytofluene, ζ-carotene, neurosporene, lycopene, β-zeacarotene, γ-carotene, β-carotene, β-cryptoxanthin, rubixanthin, 3-hydroxy-β-zeacarotene and several apocarotenals are present (Britton et al., 1977).

As Flavobacterium R1560 can efficiently incorporate MVA into its carotenoids, this bacterium is very suitable for the preparation of radioactive zeaxanthin.

A.Culture conditions.

Flavobacterium was cultured in the following medium:

	Grams per litre
Glucose	5
Yeast extract	10
Tryptone	10
NaCl	30
$MgSO_4.7H_2O$	5

The pH was adjusted to 7.2 with NaOH.

Stock cultures were maintained on slopes of liquid medium supplemented with agar (3% w/v). After 48 h incubation at 25°C slopes were stored at 5°C. Sub-culturing was necessary every 21 days for slopes stored at 5°C. Liquid cultures were incubated in an illuminated shaker (Gallenkamp) for 24 h at 25°C and 160 r.p.m. under illumination

(2000 lx).

B.Flavobacterium incubation

i. Culturing Flavobacterium R1560 prior to incubation.

Bacteria were grown in specially modified 250 ml conical flasks. Each flask had 3 indentations to act as baffles allowing greater aeration of the cultures. One flask, containing 50 ml of culture medium, was inoculated from an agar slope and incubated in an illuminated shaker (Gallenkamp) at 25°C and 160 r.p.m. for 24 h. From this liquid inoculum 1 ml of culture was taken to inoculate 8 flasks (50 ml per flask) which were then incubated as above.

Critical factors for the growth of these bacteria were high light intensities and thorough aeration. Cells cultured for longer than 24 h have lower enzyme activities (Britton et al., 1980).

The cells were harvested by centrifugation (Sorvall RC-2B or RC-5B Superspeed Refrigerated centrifuge, Du-Pont Instruments) at 10,000 g for 15 min at 0-4°C.

ii. Incubation conditions.

The incubation was carried out following the method of Britton et al. (1980). Cells harvested after 22 h growth were washed with 0.1 M Tris HCl buffer, pH 7, and resuspended in 30 ml of the buffer. The cell suspension was transferred to a 250 ml culture flask and the following were added in 4 ml of 0.1 M Tris HCl buffer:

2 µCi DBED [2-^{14}C]MVA (10 µCi/ml)	0.2 ml
Egg white lysozyme	40 mg
$MnSO_4.4H_2O$	4.4 mg
$MgSO_4.7H_2O$	25 mg
ATP	11.2 mg

Silicone antifoaming agent (BDH Chemicals Ltd., Poole) was added (1 drop) and the mixture incubated at 25°C on an orbital shaker at 160 r.p.m. (Gallenkamp Compenstat) for 18 h. Air was bubbled through the suspension to increase oxygen availability.

iii. Compactin incubation.

Compactin (see Fig. 8 for structure) is an inhibitor of HMG CoA reductase activity (Fears, 1981). The inclusion of compactin in the Flavobacterium incubation may, therefore, prevent the endogenous formation of MVA by the bacterium and so the utilisation of the [^{14}C] MVA may be increased. In the compactin incubation 0.1 ml of a 0.377 mM compactin solution was added to the 34 ml incubation mixture (this was equivalent to a 1 μM solution of compactin).

iv. Extraction.

The cells were harvested by centrifugation (10,000 g for 15 min at 0-4°C). The cells were extracted by standard procedures.

C. Large scale preparation of [^{14}C]zeaxanthin.

A liquid inoculum was prepared by inoculating 4 flasks, each containing 50 ml of culture medium, from an agar slope. After 24 h the total 200 ml culture was transferred to a 5 l conical flask containing 4 l of medium. Antifoam (1 ml of Silicone antifoaming agent, BDH) was added. Aeration was achieved by a magnetic stirrer and also by a continuous flow of air through the medium by means of a sparger. Fluorescent strips (6 Osram GEC fluorescent tubes, White 2ft, each of 20 W) were placed 20 cm from the conical flask. The ambient temperature was 25°C.

After 24 h the cells were harvested by centrifugation, washed with 0.1 M Tris HCl buffer and resuspended in 375 ml of the buffer. The incubation was carried out in a 1 l conical flask with stirrer and

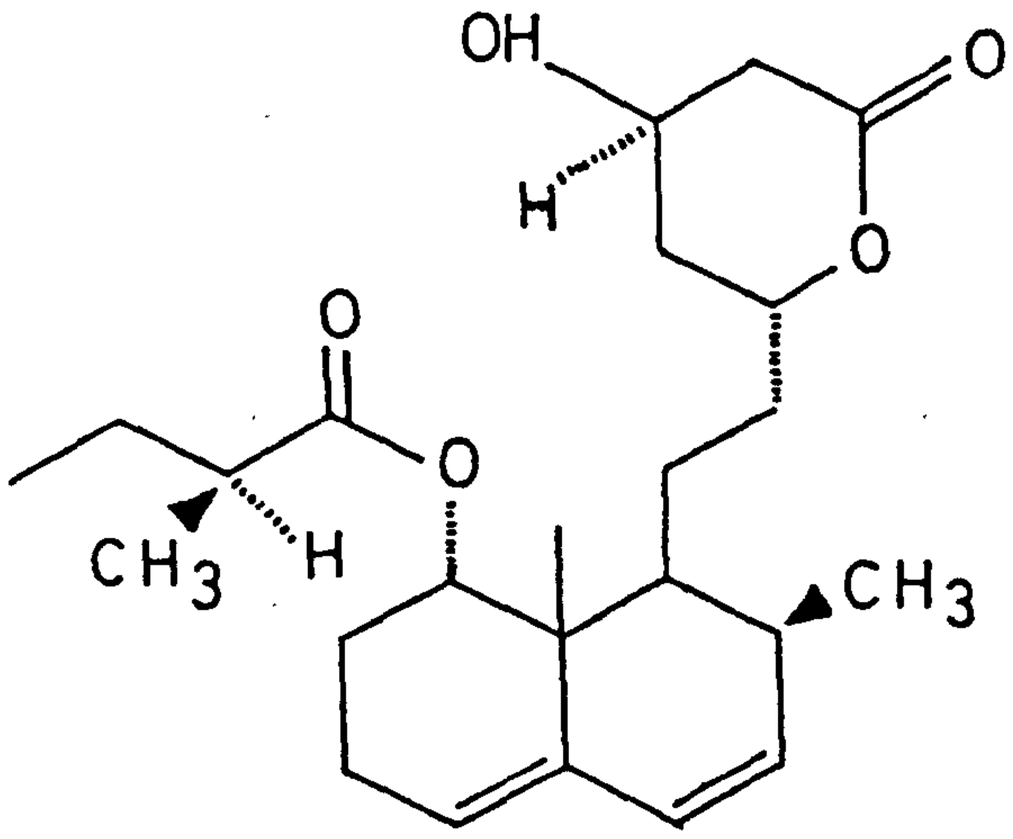


Fig. 8.

The structure of compactin.

sparger as before. The following incubation components were added in 50 ml of 0.1 M Tris HCl buffer:

25 µCi DBED[2-^{14}C]MVA (10 µCi/ml)	2.5 ml
Egg white lysozyme	500 mg
$MnSO_4.4H_2O$	55 mg
$MgSO_4.7H_2O$	313 mg
ATP	140 mg
Antifoam	1 ml

At the end of the 18 h incubation period the cells were harvested and extracted as before.

2.13 Treatment of Flavobacterium R1560 carotenoids

The lipid material was dissolved in light petroleum (b.p. 40 - 60°C) and left overnight in the deep freeze. Much of the zeaxanthin crystallised and could be isolated by filtration.

The remaining mother liquor, after removal of the zeaxanthin, was treated as described by Britton *et al.* (1977). Chromatography on a column of neutral alumina (Brockmann grade III) gave five fractions with 1, 20, 40, 60% (v/v) ether in light petroleum (b.p. 40 - 60 °C) and 2% (v/v) ethanol in ether as solvents. Fraction 5 (i.e. eluted with 2% ethanol in ether) was the zeaxanthin fraction. Also present in this fraction was a small amount of a *cis*-isomer of zeaxanthin and some antheraxanthin.

Many attempts were made to find an h.p.l.c. system that could resolve the zeaxanthin from its *cis*-isomer and antheraxanthin. Finally, a rapid, isocratic system was devised which adequately resolved these 3 components. The Pye Unicam system was used.

Column:	Nucleosil 50-5 (250 mm x 4.5 mm)
Chart speed:	30 cm/h
Detection wavelength:	440 nm
Solvent:	Hexane/dichloromethane/propan-2-ol (87:5:8, v/v)
Flow rate:	1 ml/min

2.14 Preparation of [^{14}C]lutein and [^{14}C] β-carotene

The method of Thomas and Huffaker (1981) was used elsewhere in the laboratory by B. Cooper (unpublished work) to incorporate $^{14}CO_2$ into maize (Zea mais) and barley (Hordeum vulgare) proteins; the residue from the procedure was recovered for lipid extraction.

A. Barley.

Barley seeds were grown in sterile soil in the dark at 25°C for 6 days. On day 7 approx. 150 plants were transferred to a sealed 6.4 l jar. $Ba^{14}CO_3$ (500 µCi; specific activity 296.2 µCi/mg) was suspended in a beaker inside the jar and $^{14}CO_2$ released by injection of 3 ml of 2 M lactic acid into the beaker through a Suba seal. The plants were exposed to light of effective photosynthetic light capacity 292 µEinsteins/m^2. Water filters were placed between the plants and the light source to maintain the temperature at less than 30°C. After 24 h, 5 ml of 2 M NaOH was injected into the beaker and absorbed by filter paper. The plants were left for another 24 h in the light.

The plants were harvested by cutting at the point of attachment of the first leaf to the stem. The tissue was homogenised in a Waring blender (3 x 1 min) with 150 - 200 ml 0.05 M Tris buffer containing 0.01 M EDTA-disodium salt and 0.01 M DL-dithiothreitol and sodium dithionite (5 g/l). The homogenate was strained through several layers of cheese cloth and the residue used for lipid extraction by standard

procedures.

B. Maize.

Essentially the same procedure was used for maize as for barley. Temperature and light conditions were comparable to those used for barley except that the maize was exposed to light throughout its growth period. In these experiments $^{14}CO_2$ was released from 100 μCi of $Na^{14}CO_3$ in the way described for barley.

2.15 Preliminary purification of lutein and β-carotene.

An extract of Griselinia littoralis leaves was used to devise a system to purify lutein and β-carotene. G. littoralis is an evergreen, broad leaved species of shrub close to the department, which is useful as a source of standard carotenoids from higher plant tissue. The carotenoids of G. littoralis, maize and barley are qualitatively similar.

The total unsaponifiable lipid dissolved in a small volume of light petroleum was chromatographed on a neutral alumina column activity Grade IV. The solvents were light petroleum (b.p. 40-60°C) with increasing concentrations of ether. The β-carotene and lutein fractions could be collected and further purified by h.p.l.c. (see section 2.16).

Maize and barley extracts were treated in the same way to purify [^{14}C]β-carotene and [^{14}C]lutein.

2.16 H.p.l.c. purification of lutein and β-carotene.

The h.p.l.c. system used for the purification of Flavobacterium zeaxanthin was used to separate G. littoralis carotenoids (see Section 2.13). The major requirement of this system was to separate lutein and zeaxanthin, a separation that is normally difficult to achieve without

chemical modification of the carotenoids (e.g. by acetylation). Lutein is the major carotenediol of maize and barley but traces of zeaxanthin are present. For the purpose of feeding experiments the lutein had to be totally free from zeaxanthin. Pure lutein could be obtained with this separation system. The [^{14}C]β-carotene and [^{14}C]lutein fractions from the barley or maize extractions were purified by this h.p.l.c. method.

RESULTS

2.17 Purification of [^{14}C]zeaxanthin from Flavobacterium R1560 extracts.

A. Preliminary column chromatography of Flavobacterium carotenoids.

The carotenoids of Flavobacterium have been characterised and identified by their chromatographic properties, light absorption spectra and m.s. elsewhere (Britton et. al., 1977). A summary of the separation procedure, and the components of the five fractions obtained with the solvents given in Section 2.13 are shown in Table 2.

Removal of crystalline zeaxanthin by filtration significantly reduced the amount of carotenoid which had to be separated by chromatography. During column chromatography the approximate proportion of zeaxanthin could be assessed. The anticipated % composition of zeaxanthin with respect to total carotenoid was 95%. In numerous cases it was evident that there was substantially more hydrocarbon present than expected, the effectiveness of aeration being the influencing factor. For efficient incorporation of [2-^{14}C]MVA into zeaxanthin, sufficient oxygen availability for the hydroxylation of β-carotene to zeaxanthin was crucial.

B. H.p.l.c. of the zeaxanthin fraction from Flavobacterium.

H.p.l.c. of fraction 5 gave the elution profile depicted in Fig. 9. The major peak (1) was identified as zeaxanthin by absorption spectra (λ_{max} in ethanol at (429) 451 and 478 nm) and m.s. data (m/z 568 (molecular ion M^+), 550 (M-18), 532 (M-18-18)) as shown in Fig. 10. Zeaxanthin retention time was 9 min. Peaks (2) and (3) were tentatively labelled as cis zeaxanthins. Peak (4) was representative of a more polar carotenoid than zeaxanthin and would be expected to be antheraxanthin (Britton et.al., 1977). A simple mono-epoxide test confirmed that peak

TABLE 2.

Results of classical column chromatography of Flavobacterium carotenoids reported by Britton et al., 1977.

Fraction	Solvent	Carotenoid component
1	1% E/P	phytoene, phytofluene ζ-carotene, neurosporene lycopene, β-zeacarotene γ-carotene
2	20% E/P	8'-apo-β-caroten-8'-al 10'-apo-β-caroten-10'-al 12'-apo-β-caroten-12'-al
3	40% E/P	rubixanthin, β-cryptoxanthin 3-hydroxy-β-zeacarotene
4	60% E/P	β-citraurin 3-hydroxy-10'-apo-β-caroten-10'-al
5	2% EtOH/E	zeaxanthin, cis-zeaxanthin antheraxanthin

E = diethyl ether
P = light petroleum
EtOH = ethanol

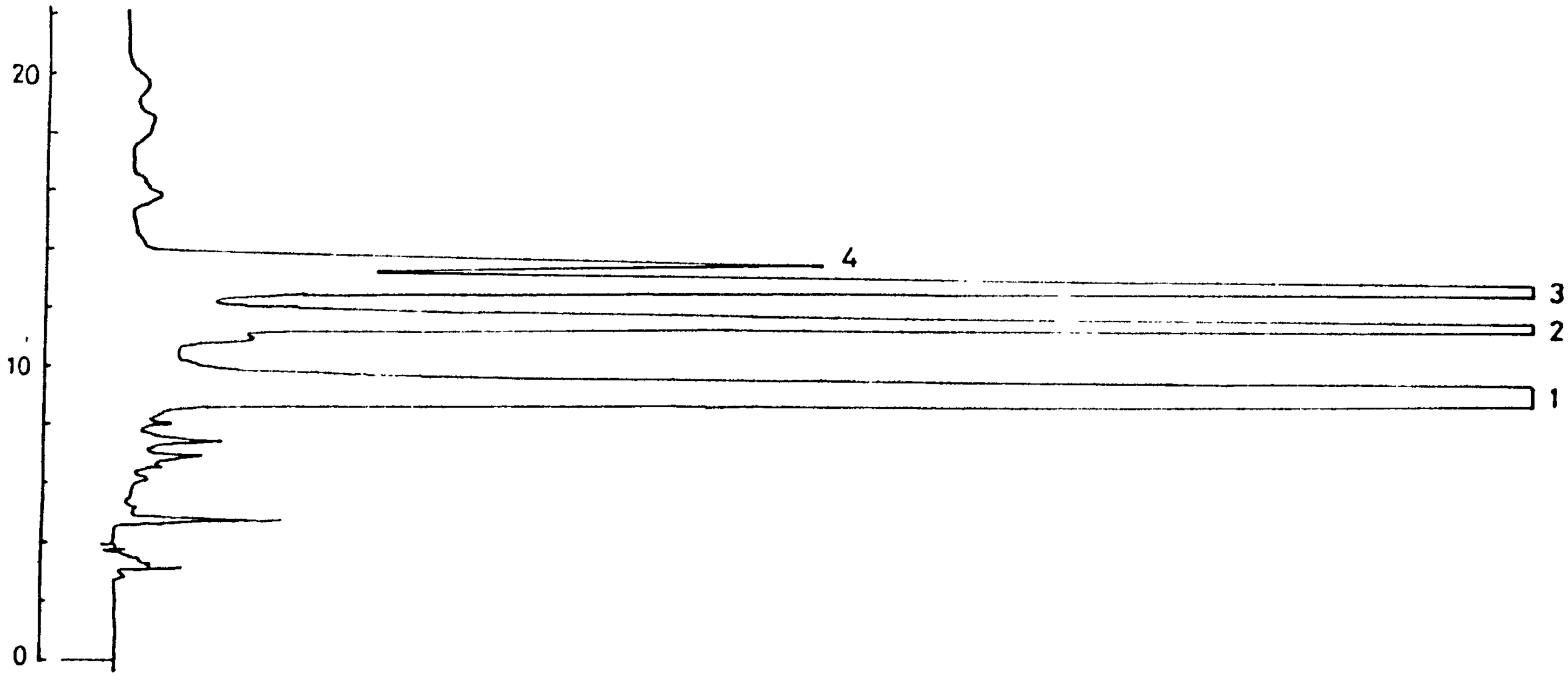


Fig. 9.

H.p.l.c. elution profile of Flavobacterium carotenoids on Nucleosil-50-5, solvent hexane/propan-2-ol/dichloromethane (87:8:5), flow rate 1ml/min.

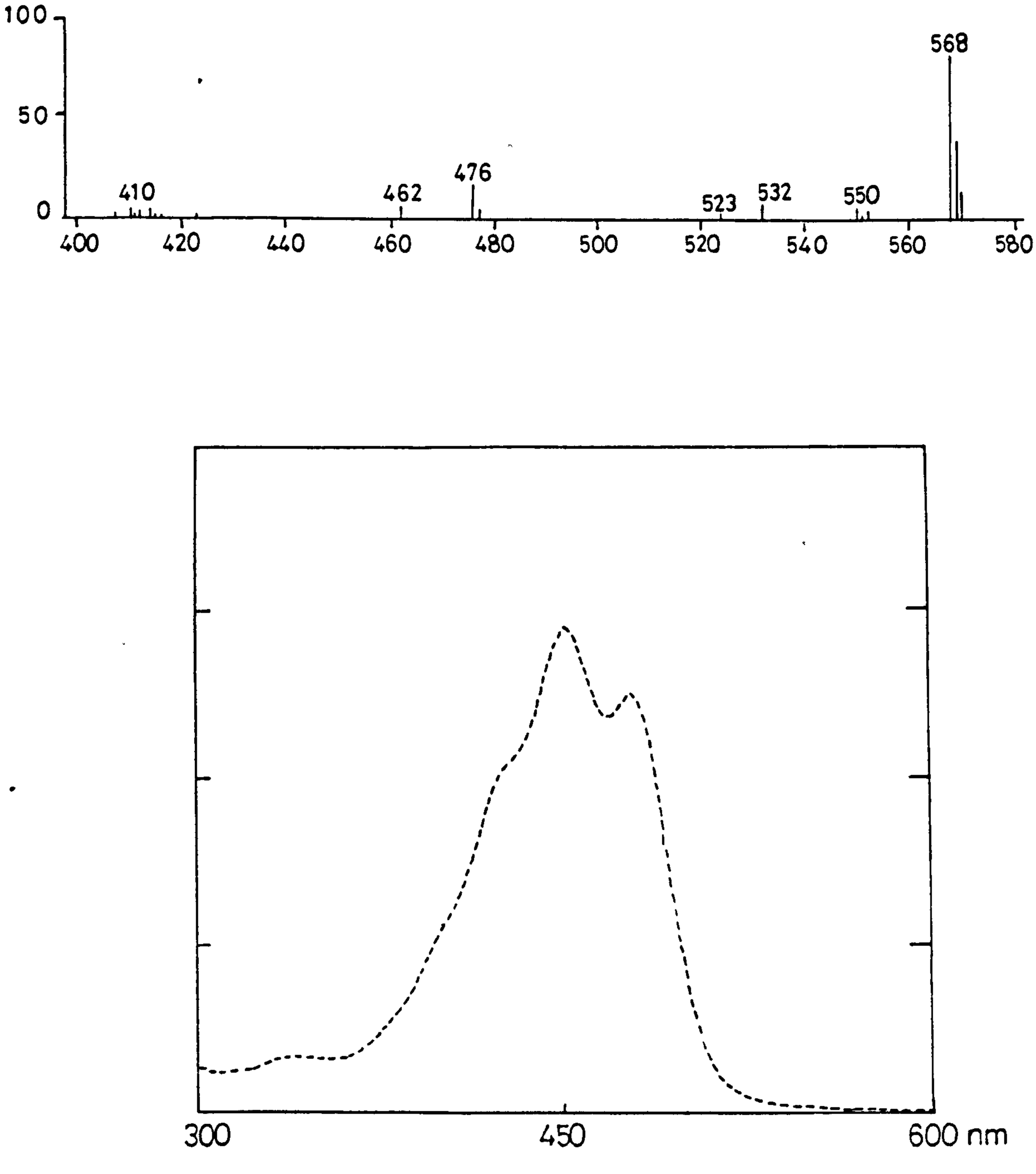


Fig.10.

Absorption spectrum and m.s. data for zeaxanthin.

(4) was indeed antheraxanthin. The addition of 1 drop of concentrated HCl to a cuvette containing an ethanolic solution of the carotenoid resulted in a shift of the wavelength maxima from (423) 445 474 nm to (405) 426 450 nm, a characteristic shift of 20 nm displayed by mono-epoxides.

Radiolabelled zeaxanthin for metabolic studies was purified by classical column chromatography followed by h.p.l.c.

2.18 Yield of [^{14}C]zeaxanthin from Flavobacterium incubations.

The final yields of zeaxanthin were estimated by spectrophotometric methods according to the procedure of Davies (1976). A specific absorption coefficient of 2340 was used for zeaxanthin dissolved in acetone.

Aliquots of zeaxanthin were removed for liquid scintillation counting for determination of the levels of incorporation of [^{14}C]MVA into the zeaxanthin fraction.

The following results are representative of the typical yields and incorporations attained.

A. Small-scale preparation.

On average the yield of pure zeaxanthin was 2.5 mg per incubation. The zeaxanthin contained approximately 0.2 µCi of ^{14}C corresponding to an incorporation of 10% of the [^{14}C] mevalonate.

The specific activity of the [^{14}C]zeaxanthin = 0.08 µCi/mg.

B. Compactin incubation.

The yield of zeaxanthin was 2.06 mg containing 0.418 µCi of ^{14}C.

The specific activity of the [^{14}C]zeaxanthin = 0.203 µCi/mg. It would appear that the inclusion of compactin has increased the % incorporation of [^{14}C]MVA into zeaxanthin as a result of inhibiting HMG CoA reductase.

C. Large scale preparation.

Numerous incubations were carried out from which varying amounts of zeaxanthin of different levels of incorporation were obtained. The average yield of zeaxanthin was 7.05 mg containing 3.07 µCi. The % incorporation was as high as 20% in some cases.

The specific activity of the [^{14}C]zeaxanthin = 0.44 µCi/mg.

The results of the small scale preparation are comparable to the results reported by Britton et al. (1980).

The large scale preparation produced higher specific activity [^{14}C]zeaxanthin than the small scale incubation, which is useful in metabolic studies where the absorption is likely to be poor.

2.19 Purification of [^{14}C] β-carotene and lutein.

A. Classical liquid chromatography of higher plant unsaponifiable lipid.

The use of classical l.c. reduced the need for time consuming and repetitive h.p.l.c. of small aliquots of total unsaponifiable lipid. Each h.p.l.c. run was of an hour's duration and with large amounts of material was an inappropriate method of purifying β-carotene and lutein.

A crude separation yielding 5 fractions was adequate as this produced a crude fraction of β-carotene and one of lutein. These fractions could then be purified by h.p.l.c. The chromatography was

essentially the same for Griselinia littoralis, maize and barley. Table 3 summarises the chromatography of higher plant unsaponifiable lipid.

B. HPLC of total unsaponifiable plant material.

The elution profile in Fig. 11 shows the separation achieved of total Griselinia littoralis carotenoids on the system desribed in Section 2.16.

A consideration of the structures of the carotenoids, chromatographic properties, light absorption spectra, chemical tests and m.s. has allowed the assignment of carotenoids to the peaks labelled 1-10 as depicted in Table 4.

Chemical tests consisted of acidification of ethanolic solutions of carotenoids, a test which allows identification of epoxides (Davies, 1976). Mono-epoxides exhibit a hypsochromic shift of approximately 20 nm in their absorption maxima and di-epoxides a shift of 40-45 nm.

C. H.p.l.c. of β-carotene from maize.

Fraction 1 from the classical l.c. of Section 2.15 of total maize was collected, and the solvent removed (rotary evaporator). The residue was redissolved in the h.p.l.c. running solvent. H.p.l.c. of this fraction gave the trace shown in Fig. 12. The appropriate fraction of pure β-carotene was collected.

D. H.p.l.c of lutein from barley or maize.

Fraction 3 of the classical l.c. was taken and the solvent removed (rotary evaporator) and the remaining solid redissolved in the h.p.l.c. solvent. H.p.l.c. of aliquots of this fraction resulted in traces as shown in Fig. 13. The lutein fraction (Peak 1) was collected each time.

TABLE 3.

Classical column chromatography fractions of higher plant unsaponifiable lipid e.g. Griselinia littoralis.

Fraction	Solvent	Absorption maxima in EtOH			Carotenoid component
1	P	(430)	449	475	β-carotene
2	50% E/P	(425)	444	470	β-cryptoxanthin
3	70% E/P	423	445	473	lutein
4	100% E	420	443	472	antheraxanthin/ violaxanthin
5	2% EtOH/E	413	436	465	neoxanthin

P = light petroleum
E = diethyl ether
EtOH = ethanol

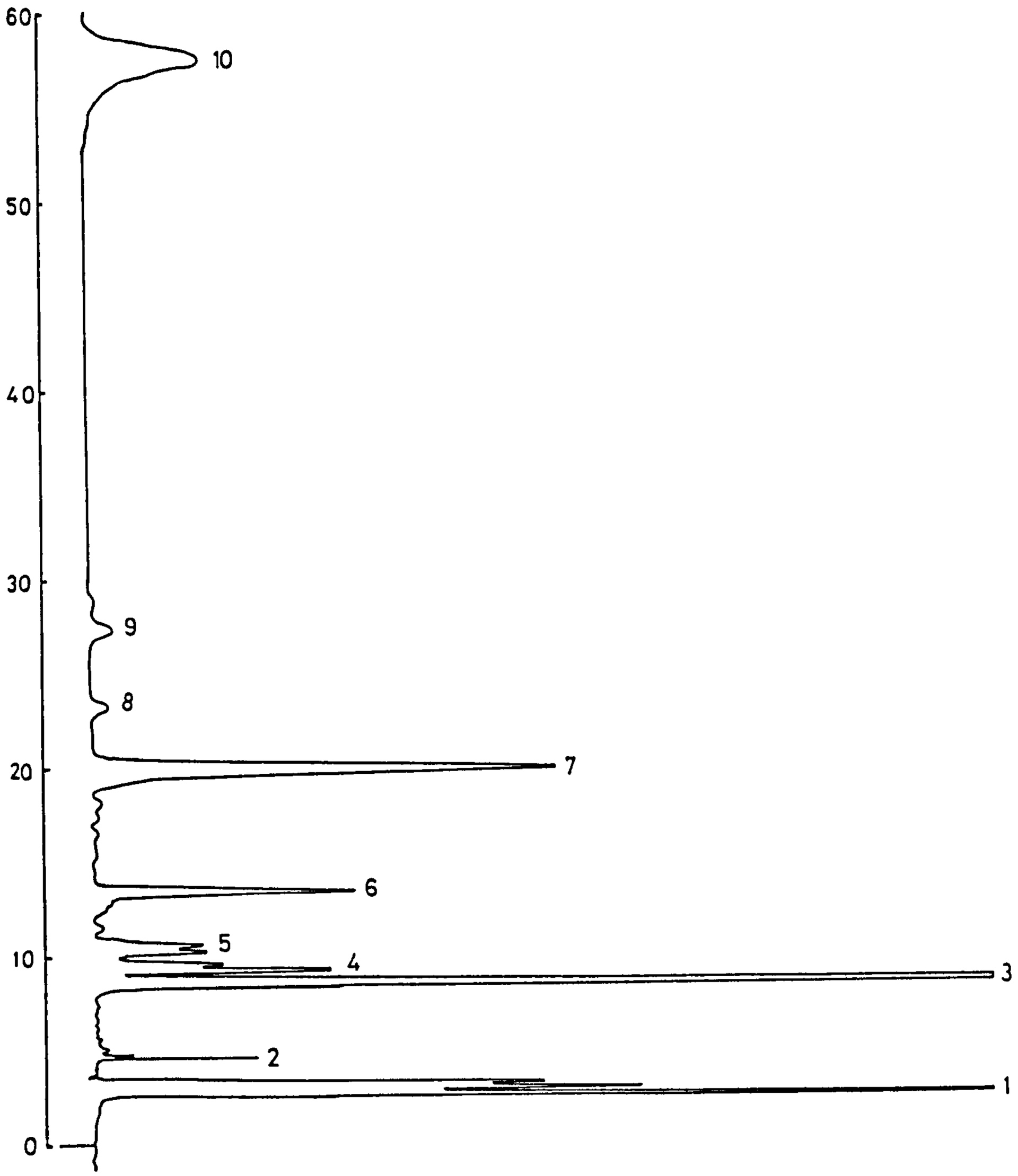


Fig.11.

H.p.l.c. elution profile of total unsaponifiable plant material on Nucleosil-50-5, solvent hexane/propan-2-ol/dichloromethane (87:8:5), flow rate 1ml/min (see Table 4 for peak identifications).

TABLE 4.

Assignment of carotenoids of peaks 1-10 from h.p.l.c. of Griselinia littoralis.

Peak	Absorption spectra max (in EtOH)			Carotenoid component	Retention time (min)
1	(427)	449	475	β-carotene	2.8
2	(428)	449	473	β-cryptoxanthin	3.6
3	(424)	445	473	lutein	8.8
4	(425)	447	473	zeaxanthin	9.0
5	(420)	440	468	cis-zeaxanthin	10.4
6	(423)	445	474	antheraxanthin	13.4
+HCl	405	426	450		
7	417	440	470	violaxanthin	19.8
+HCl	379	401	426		
8	414	435	465	cis-violaxanthin	23.2
+HCl	377	400	421		
9	411	435	463	cis-violaxanthin	27.2
+HCl	376	398	423		
10	413	436	465	neoxanthin	57.6
+HCl	398	421	448		

+HCl - chemical test for epoxides which results in a hypsochromic shift of some 20 nm for a mono-5,6-epoxide and of 35-40 nm for a 5,6,5',6'-diepoxide.

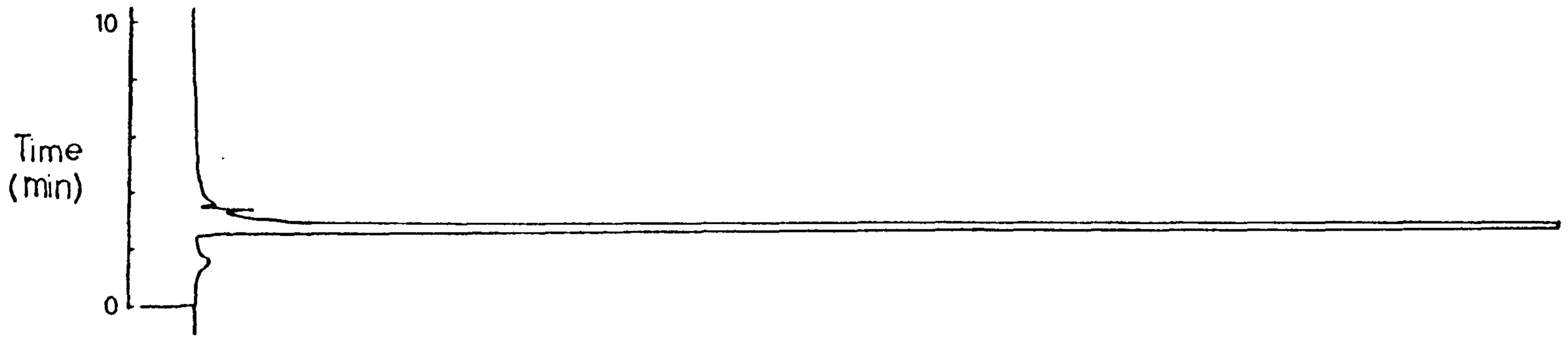


Fig.12.

H.p.l.c. elution profile of F1 from classical l.c. on Nucleosil-50-5, solvent hexane/propan-2-ol/dichloromethane (87:8:5), flow rate 1ml/min.

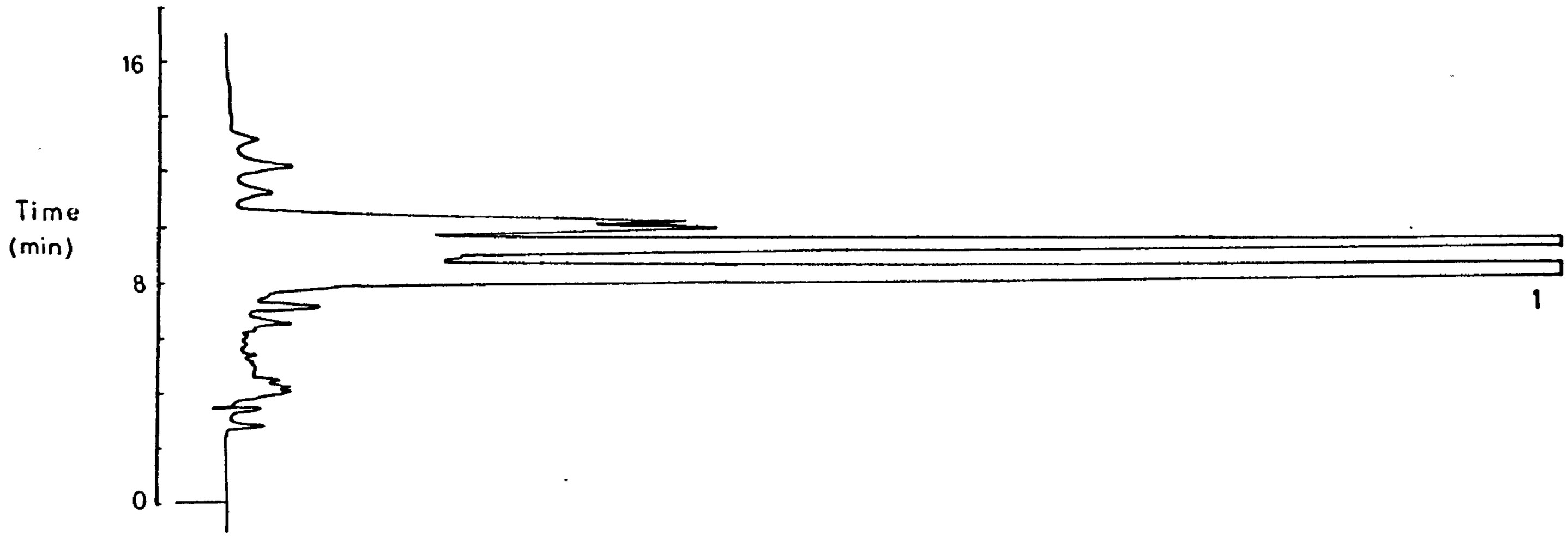


Fig.13.

H.p.l.c. elution profile of F3 from classical l.c. on Nucleosil-50-5, solvent hexane/propan-2-ol/dichloromethane (87:8:5), flow rate 1ml/min.

Each run was 10 min, a fraction of the time taken for a complete separation of all barley carotenoids (60 min), emphasising the importance of the inclusion of the classical l.c. stage.

2.20 Yield of ^{14}C-labelled carotenoids from maize.

Spectrophotometric estimation of the total β-carotene from maize in light petroleum using a specific absorption coefficient value of 2592 (Davies, 1976) gave a value of 18.9 mg. The total activity was determined by normal methods to be 0.318 μCi.

Specific activity of [^{14}C] β-carotene = 0.0168 μCi/mg.

The yield of [^{14}C]lutein was significantly lower than that of β-carotene and was of low specific activity. The lutein present amounted to 1.23 mg with total activity of 0.03 μCi.

Specific activity of [^{14}C]lutein = 0.0244 μCi/mg.

Low incorporations into maize may be accounted for by the presence of light in early growth stages. This will permit formation of carotenoids as normal, and hence when $^{14}CO_2$ is released there is less incorporation into total carotenoids.

2.21 Yield of [^{14}C]lutein from barley.

Lutein was estimated spectrophotometrically using a specific absorption coefficient of 2550 in ethanol (Davies, 1976). A known aliquot was withdrawn for liquid scintillation counting. The yield of lutein was 2.598 mg with a total activity of 5.9 μCi.

Specific activity of [^{14}C]lutein = 2.26 μCi/mg.

It should be noted that the results shown in Sections 2.20 and 2.21 are but examples.

DISCUSSION

The importance of radiochemically pure ^{14}C-labelled xanthophylls has already been stressed and it was to this end that a thorough investigation was carried out in order to produce good separations of Flavobacterium and higher plant carotenoids. The Nucleosil system devised is very successful and appropriate for use in many situations where a mixture of carotenes and dihydroxycarotenoids is anticipated. Of particular significance is the ready separation of lutein and zeaxanthin which would normally require derivatisation of the carotenoids followed by t.l.c. The m.s. data for zeaxanthin provide clear evidence for complete resolution of lutein and zeaxanthin. The absence of an ion corresponding to M-56 is proof that ε-end groups are absent.

The yields of ^{14}C-labelled xanthophylls varied considerably between experiments. In the case of zeaxanthin from Flavobacterium, optimal growth conditions were essential for good yields of zeaxanthin. Of paramount importance was the rate of aeration, which strongly influenced the level of hydroxylation and hence zeaxanthin formation. Compactin appeared to reduce the yield of carotenoids but enhanced the incorporation of [2-^{14}C]MVA into the carotenoids. A slightly increased specific activity of zeaxanthin was useful.

In the chick experiments, [$^{3}H_2$]zeaxanthin was also administered to the embryos. The advantage of this synthetically prepared sample over the [^{14}C]zeaxanthin of Flavobacterium is its high specific activity. The low specific activities of the biologically produced carotenoids is a problem. The anticipated low levels of absorption and incorporation intensifies the problems of detecting metabolites in a system where the starting material is of poor specific activity and the conversion will be minimal.

In all cases, including synthetically prepared [^{14}C]canthaxanthin

and β-carotene, the radiolabelled carotenoid administered to the fish, chick or yeast were radiochemically pure.

CHAPTER 3

INTRODUCTION

3.1 Goldfish.

The goldfish (Carassius auratus) is a member of the Cyprinidae or carp family, and therefore it is essentially a freshwater species. It is of Chinese origin and in China a marked distinction is made between the wild goldfish, a grey fish not much esteemed for ornamental waters and the domestic goldfish, the orange variety.

According to Hervey and Hems (1981), the first known literature reference to goldfish was made by Ke Li-Fang in recording the history of the monastery at Hu-chou for the period 618-906; "The three gates are each 100ft high and are called the Three Excellences. Moreover in the pool are Golden Chi which are seen once in several years". By 960 the goldfish had become a pet and its domestication is recorded by three writers. In the present context, the most interesting is the writing of the monk Tsan-ning (918-999) who noted "If goldfish eat the refuse of olives or soapy water then they die; if they have poplar bark they do not breed lice". In the T'ing Shih of Yo K'o (1173-1240) is written "At the present time there are fish breeders in Peking who can change the colour of fish to gold".

These historical references are significant not only because they describe the first of a long series of goldfish feeding experiments but also because they illustrate the natural curiosity and attraction to the colour of living things.

3.2 Carotenoids in Fish.

Reviews by Goodwin (1951), Fox (1974) and, more recently, by Goodwin

(1984) very adequately detail the distribution of carotenoids in fish. Goodwin (1984) defines the chirality of most of the carotenoids. Therefore this aspect of the carotenoids of fish is not covered here in depth but a few generalisations are made.

Generally fish tend to accumulate xanthophylls, the oxygenated derivatives of carotenes, rather than carotenes themselves. Originally it was thought that there were three main xanthophylls occurring in fish, both freshwater and marine, and these were lutein, taraxanthin and astaxanthin. Lutein and astaxanthin are common enough carotenoids but the nature of the third xanthophyll was uncertain although it had been detected in the large number of species examined. Spectroscopic and biochemical evidence suggested that the so-called taraxanthin was a α-carotene derivative, most probably lutein-5,6-epoxide. At this time fish were divided into two groups according to whether they accumulated lutein or taraxanthin. It appeared that astaxanthin could be present in fish of both classes but rarely occurred without either lutein or taraxanthin; notably it was uncommon to find both lutein and taraxanthin in the same species. In recent literature taraxanthin is conspicuous by its absence and consequently its position as a carotenoid of fish skin has to remain uncertain.

New groups of fish xanthophylls emerging in recent literature are the tunaxanthins and the tilefishxanthins. The tunaxanthins are known to exist as stereoisomers within the same species and as yet relatively little is known about the tilefishxanthins except that they are keto derivatives of hydroxylated α-carotenes.

It is interesting to note the high incidence of fish carotenoids that contain ε-end groups.

The common coexistence of stereoisomers within the same species of fish is in marked contrast to the situation in plant sources as typified

by astaxanthin which in plants is stereochemically pure. In both Atlantic and Pacific Salmon (Salmo salar and Oncorhynchus spp., respectively) three stereoisomers of astaxanthin have been found (Schiedt et al., 1981a). Similarly, 'lutein' from goldfish has been identified as its epimer, 3'-epilutein (Matsuno and Katsuyama, 1979a; Webber et al., 1973). The 'lutein' in fish may prove to be 3'-epilutein in many cases, as shown to be the case in Pacific Salmon and various trout species (Matsuno et al., 1980a,b,c), piranha (Matsuno and Katsuyama, 1979a) and Tilapia spp.(Matsuno and Katsuyama, 1979b).

3.3 Distribution of carotenoids within tissues.

The main store of fish carotenoids is the skin but additional supplies of carotenoids are found in the flesh, the ovaries and the liver. The carotenoids of the skin are always esterified and are localised in specialised cells. Fish pigments are restricted to cells known as chromatophores, of which there are two types. The carotenoids are localised in xanthophores or erythrophores (Goodrich et al., 1941) and non-carotenoid pigments are stored in the melanophores. The combination of the different sorts of cells produces a variety of effects. In the wild goldfish, the dark olive colour is made up of a mixture of xanthophores and melanophores together but on loss of the melanophores the goldfish displays the characteristic colour of domestic goldfish.

Also large amounts of carotenoids occur in the liver and ovaries of fish. In the liver xanthophylls are the major components, normally found in their unesterified form. The ovaries contain high levels of xanthophylls, particularly during sexual activity with small amounts of β-carotene also present in most species. Carotenoids have been detected in the eyes of some species, and astaxanthin specifically has been isolated from the intestine and kidneys of Salmo gairdneri and

S.irideus (Sivtseva, 1982).

Qualitative variations between species is small but the quantitative changes are significant. Moreover, qualitative and quantitative changes in the same species may be observed, for example, during growth. In Carassius auratus (goldfish) the developing fish changes from grey to black through orange and dark orange. Initially hydroxycarotenoids alone are found, red ketocarotenoids not appearing until the orange coloration is observed (Hata and Hata, 1971).

3.4 Functions of carotenoids in fish.

There are four primary functions of carotenoids in fish and each of these will be dealt with briefly as these topics have been covered in some detail by the three aforementioned reviews.

A. Photoresponse.

The pioneering microspectrophotometric studies by Steven (1948, 1949) demonstrated that in trout, lutein was concentrated within the xanthophores and astaxanthin in the erythrophores. Steven also calculated that a single xanthophore contained 11-28 pg lutein and each erythrophore contained between 200 and 340 pg of astaxanthin. The ability of some fish species either to concentrate or diffuse the pigments within their chromatophores allows a degree of change of skin colour. This is partly under the control of the nervous system but more frequently the intensity of colour is affected by the secretions of the ductless glands. The changes in colour are more dependent on the melanophores than the xanthophores or erythrophores as the microgranules of melanin can either disperse to give the fish a dark appearance or can be aggregated into one spot to give a pale appearance (Sumner, 1940). The advantage of this phenomenon to camouflage is obvious.

B. Reproduction.

Carotenoids have been intimated as functional in reproduction due to their active movement during development of the gonads and in the time leading up to spawning. The most obvious activity is seen in the colour changes of the skin during spawning, this secondary sexual response resulting from the transfer of liver carotenoids to the integument (Sumner and Fox, 1935).

Mobilisation of carotenoids from liver and skin to the developing ovaries and eggs also implies the involvement of carotenoids in reproduction (Sumner and Fox, 1935, Fundulus parvipinnis; Steven, 1948, 1949, Salmo trutta; Crozier, 1970, Kitahara and Saito, 1977, Loginova, 1969, Salmonidae). However, experimental evidence suggests that carotenoids are not needed for the normal development of embryos (Steven, 1949) as removal of 90% of carotenoids from the yolk sac of newly hatched brown trout did not lead to any problems in development. The carotenoids, in unesterified form, are transferred to the embryo, esterified and then deposited in the chromatophores of the integument. Therefore, it seems that large amounts of carotenoids in the yolk sac simply ensure enough pigment for natural markings of integument in the young animal, a protective mechanism.

Hartmann et al. (1947) proposed that the carotenoids may play a role at the fertilisation stage rather than in embryonic development but this work has not been substantiated.

More recently, the possible involvement of high carotenoid levels in improved viability in Salmonids has been put forward by Craik (1985).

C.Vitamin A precursors.

This particular aspect of fish carotenoids is dealt with directly in this presentation and consequently at this stage it will suffice to emphasise that β-carotene is generally accepted to be a precursor of vitamins A_1 and A_2 in freshwater and of vitamin A_1 in marine fish.

The limited availability of β-carotene in the natural diet of fish (mainly zooplankton) has to a certain extent substantiated proposals that xanthophylls may play an important role as vitamin A precursors in fish. These ideas will be discussed in detail later.

D. Vision.

Surprisingly, the presence of carotenoids in the retina of fish has provoked little interest. Nevertheless, it is possible that the carotenoids may reduce chromatic aberration and glare and hence increase visual acuity.

3.5 Carotenoid metabolism in fish.

As has already been mentioned there is a tendency for fish to accumulate xanthophylls and particularly common amongst fish is the general occurrence of astaxanthin. Schmidt-Nielsen and his co-workers investigated the carotenoids of several marine fish. They extracted 400 g of a red oil from the liver (1200 g) of a 70 kg Scythe fish Regalecus glesne (Schmidt-Nielsen et al., 1932a). The major component of this material was identified as astaxanthin. They later reported this same pigment to be present in the flesh of Salmo salar and in the red oil from some species of whales (Schmidt-Nielsen et al., 1932b). These early observations raise the question as to how such vast quantities of astaxanthin are formed, a problem which is central to animal metabolism of carotenoids in general.

In the review by Goodwin (1951) it is suggested that the dietary carotenoids are the source of fish pigmentation. It is questionable that the natural diet of the fish would be sufficient to provide the total complement of astaxanthin and other carotenoids.

One problem encountered is the variability of absorption of carotenoids from the gut e.g. the surf perch (Cymatogaster aggregatus) can absorb neutral xanthophylls but not astaxanthin, any astaxanthin and β-carotene being excreted quantitatively. The halibut (Hipoglossus) also excluded almost all dietary pigments, not appearing to store much astaxanthin at all although its diet is an astaxanthin-rich fish (cited in Goodwin, 1951).

It is therefore interesting that some marine species can modify dietary carotenoids. An example of this is the Killifish (Fundulus parvipinnis) which was shown to be able to modify dietary carotenoids by oxidation. However another species, the Garibaldi (Hypsypops rubicunda), given a diet containing only β-carotene failed to allow xanthophyll formation although some β-carotene accumulated in the skin.

More recent experiments have clearly demonstrated that astaxanthin can be formed from other carotenoids in some species. One such species is the goldfish where β-carotene can be metabolised to astaxanthin by the scheme shown in Fig. 14 (Rodriguez et al., 1973). Hata and Hata (1972) suggest that a more important route to astaxanthin is via zeaxanthin. Also lutein has been reported as a precursor of astaxanthin in goldfish; this will be discussed in the next section. Both prawns and lobsters use the same pathway as depicted in Fig. 14. In Fancy Red Carp (Cyprinus carpio) astaxanthin is formed from zeaxanthin with idoxanthin as a possible intermediate (Hata and Hata, 1975; Hata and Hata, 1976).

The occurrence of tunaxanthins in fish (mainly marine species but

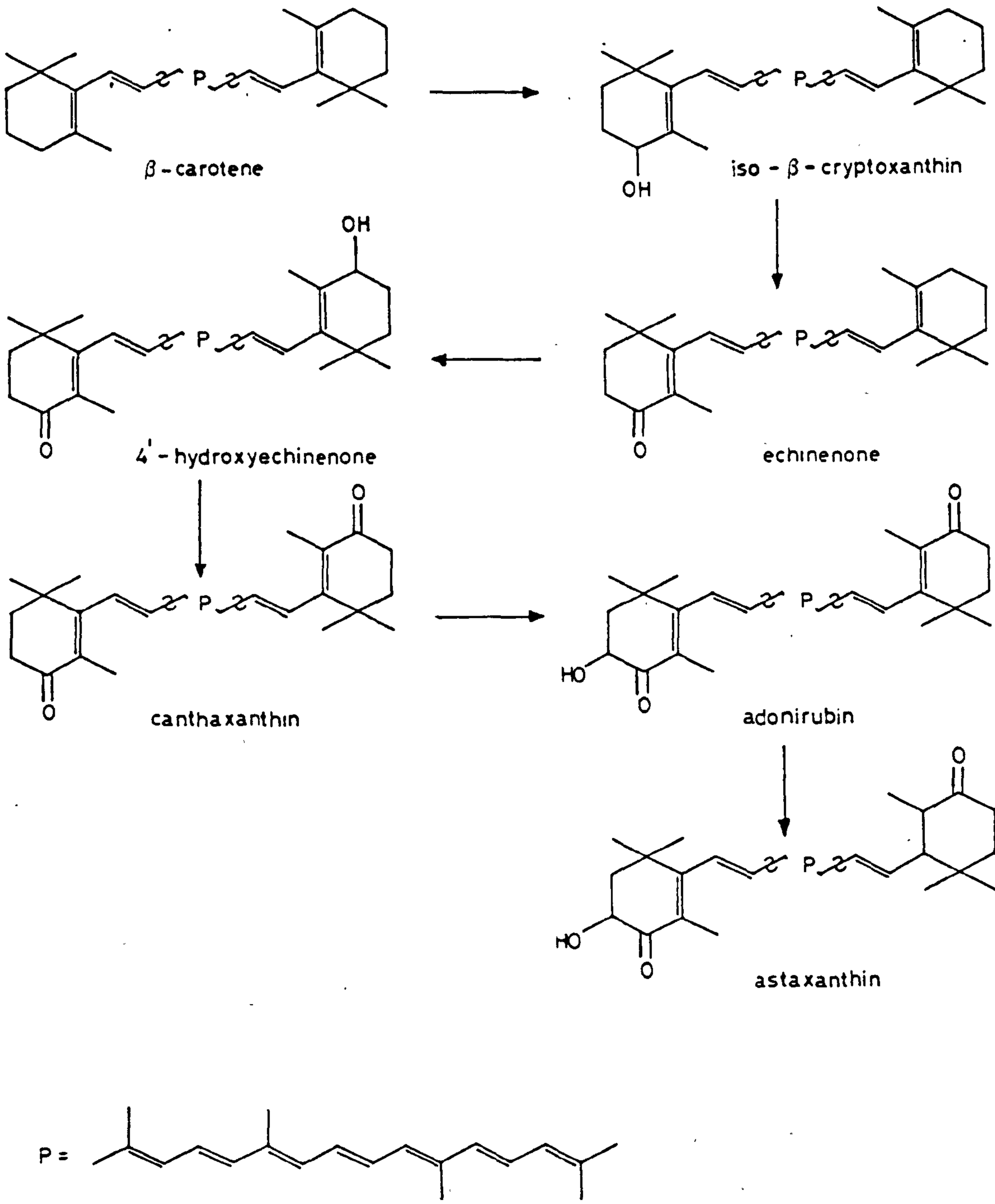


Fig.14.

Scheme for astaxanthin formation from β-carotene in goldfish.

also various goldfish and perch) poses an interesting biochemical problem in terms of both structure and stereochemistry. In some way a carotenoid with two ε-end groups with the appropriate chirality must be formed from dietary carotenoids. Several enantiomeric forms of the carotenoid have been isolated (tunaxanthins A, B, C) each of which has the opposite chirality at C-6 and C-6' to the chirality of C-6' in lutein and 3'-epilutein, which on first considerations would seem a possible precursor of the tunaxanthins. One tentative pathway has been suggested starting from β-carotene (Buchecker, 1982). Conversely, astaxanthin is a precursor of tunaxanthins in the yellowtail (Seriola quinqueradiata)(Fujita et al., 1983a; Miki et al., 1984; Miki et al., 1985) and the red Sea Bream (Chysophrys major)(Fujita et al., 1983b). The intermediates of the pathway are β-carotene-triol, zeaxanthin and 3'-epilutein. Noteworthy is the observation of Crozier (1969) that astaxanthin is formed from tunaxanthin (i.e. the reverse reaction) in the Californian Sheephead (Pimelometopon pulchrum).

Contrary to expectations little modification of dietary carotenoids is possible in Salmonidae species. Most of the work has been done on various species of trout where attempts at achieving pigmentation by supplementation of the diet with single pure carotenoids failed. Salmon, likewise, are unable to oxidise dietary carotenoids, but both salmon and trout can transfer ketocarotenoids from one part of the body to another. Peterson et al. (1966) confirmed that brook, brown and rainbow trout (Salvelinus fontinalis, Salmo trutta and S. irideus) derived their carotenoids unaltered from the diet. Therefore, unlike goldfish, salmon and trout cannot oxidise 3,3'-dihydroxy carotenoids.

In both marine and freshwater fish limited amounts of (3R,3'R)- and (3R,3'S)astaxanthin have been found and the origin of these carotenoids is a problem as the dietary source of astaxanthin was expected to be stereochemically pure.

Another fairly recent development in carotenoid biochemistry is that reductive pathways have been accepted as integral parts of animal metabolism. Fish are no exception to this, as exemplified by the formation of tunaxanthin from astaxanthin in marine species. A reductive pathway is also seen in rainbow trout, where the keto groups can be removed from astaxanthin to give zeaxanthin. Neither the removal of the hydroxyl groups of zeaxanthin was possible nor the elimination of the hydroxyl on C-3 of adonirubin (Schiedt et al., 1985). Numerous other examples can be found in the literature for this type of conversion.

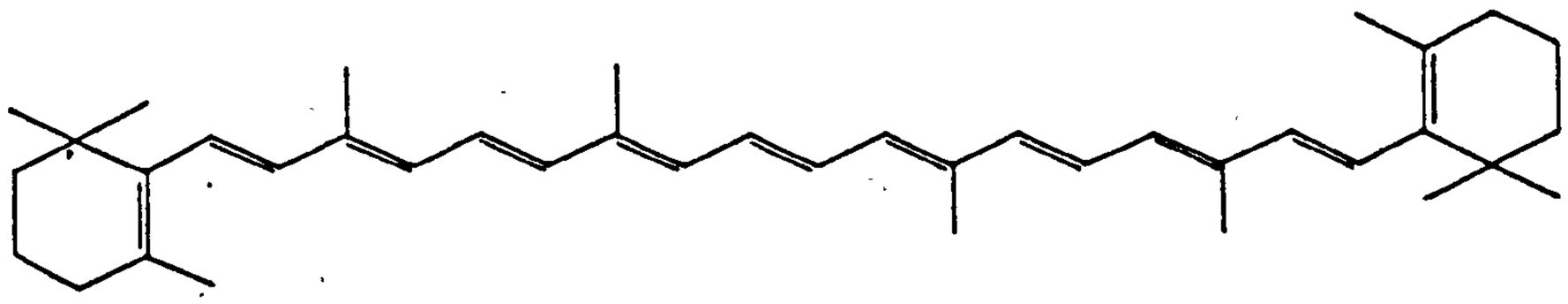
The ability of fish to perform reductive steps in carotenoid biochemistry widens the scope for the provitamin A activity of carotenoids. The early reports of xanthophylls acting as provitamin A carotenoids become more acceptable and their feasibility as precursors of vitamin A has been studied as described in the following Sections.

3.6 Carotenoids of goldfish.

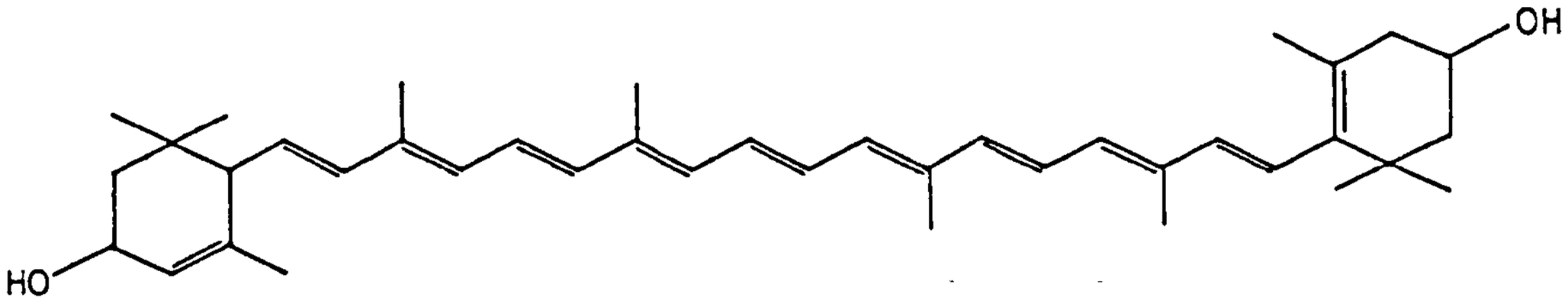
Astaxanthin is the predominant pigment of goldfish integument with relatively small quantities of β-carotene, lutein, α- and β-doradexanthin also present. All the carotenoids except β-carotene are esterified. In fact, the lutein of goldfish, as previously stated, has been identified as 3'-epilutein, an observation that is important with regards to the metabolism of "lutein" in this species (Matsuno and Katsuyama, 1979a and Webber et al., 1973). Figure 15 depicts the structures of the carotenoids of goldfish.

3.7 Carotenoid metabolism in goldfish.

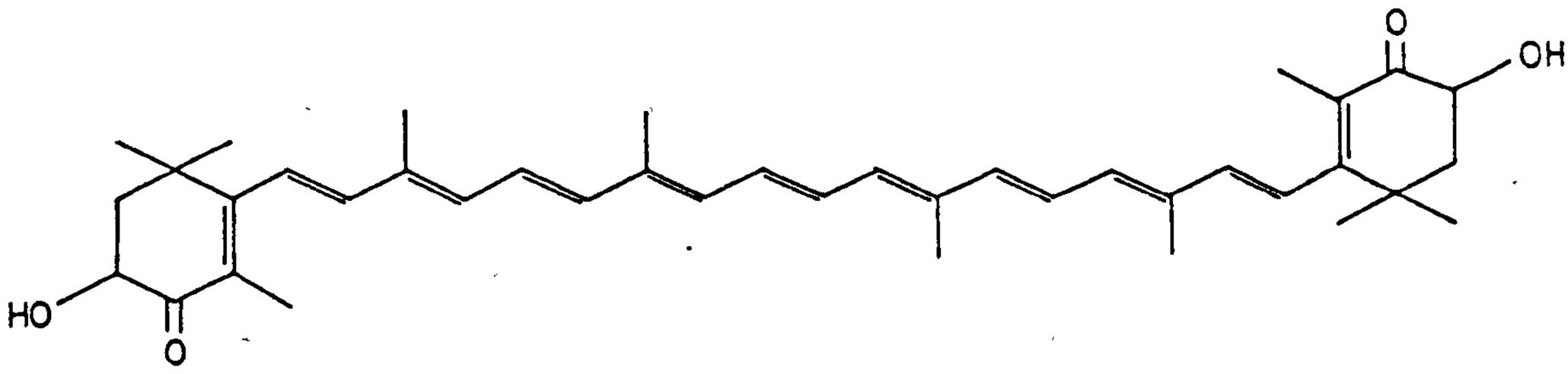
Over the years a great deal of research has been devoted to carotenoid metabolic studies in goldfish. One reason is the suitability of goldfish as an experimental species, but more important here is the



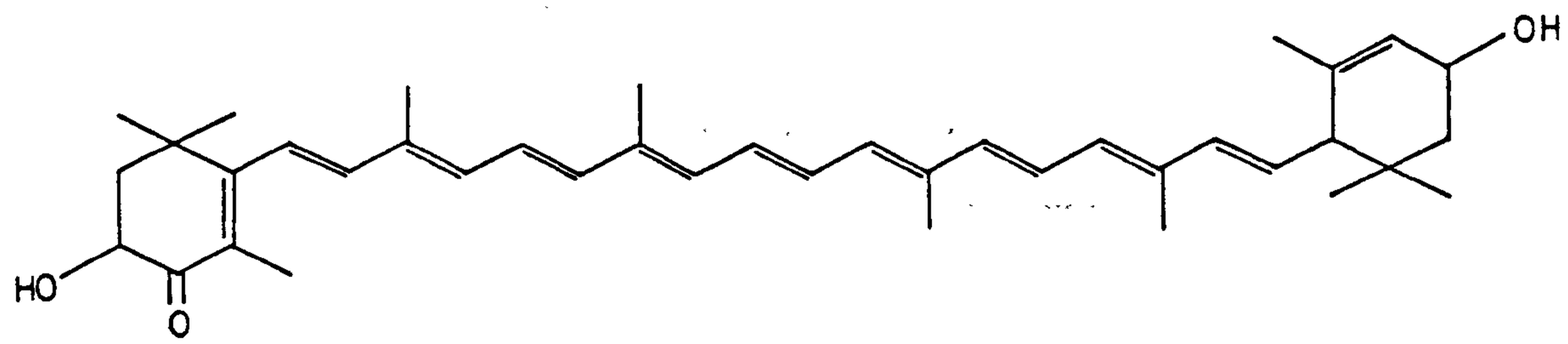
β-carotene



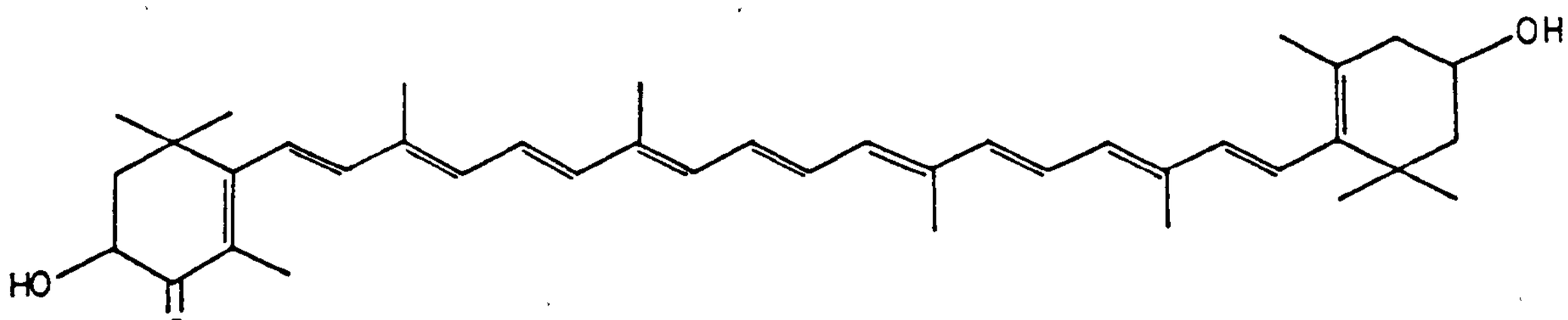
lutein



astaxanthin



α-doradexanthin



β-doradexanthin

Fig.15.

The structures of the carotenoids isolated from goldfish integument.

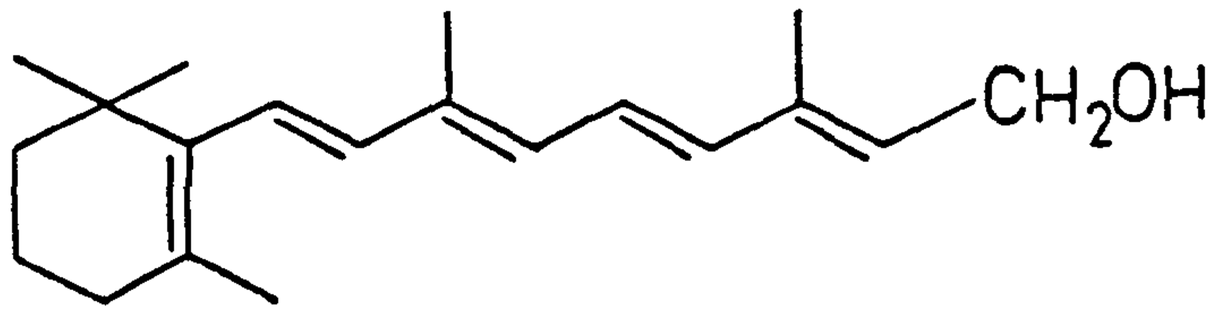
possibility of solving two problems with one series of experiments. Feeding experiments in goldfish may shed light on two matters, namely that of vitamin A (i.e. retinol and dehydroretinol) formation and that of astaxanthin formation. Each of these areas is dealt with separately in the following section.

A. The conversion of carotenoids into dehydroretinol.

Freshwater fish are unusual in that as well as forming retinol there are substantial quantities of dehydroretinol present (see Fig. 16 for structures). The existence of dehydroretinol was discovered in 1937 by Lederer and Rosanova (1937), whilst at Liverpool, Edisbury et al. (1937) made similar observations, finding the highest proportion of the new form in the eyes of goldfish. Dehydroretinol was eventually isolated in the form of its crystalline aldehyde by Salah and Morton (1948) and synthesised by Farrar et al. (1951).

There is little doubt that in freshwater fish dehydroretinol is the true vitamin A, as can be seen from the predominance of dehydroretinol over retinol (Balasundaram et al., 1956). Why there is a need for freshwater to form dehydroretinol remains uncertain. When given to rats, dehydroretinol displays only about half the biological activity of retinol (Weiser, 1964) but does maintain all functions of the vitamin (Howell et al., 1967).

Recently, the presence of dehydroretinol in human skin has been reported. Vahlquist (1980) showed the presence of dehydroretinol in the skin of psoriasis patients and later in normal skin (Vahlquist, 1982 and Vahlquist et al., 1982). The origin of the dehydroretinol is unknown but Vahlquist (1982) favours the opinion that it is formed in the skin from retinol. Also, the occurrence of dehydroretinol-based visual pigments in some amphibians, lampreys and swamp turtles is interesting (Bridges, 1972; Crescitelli, 1972; Knowles and Dartnell, 1977).



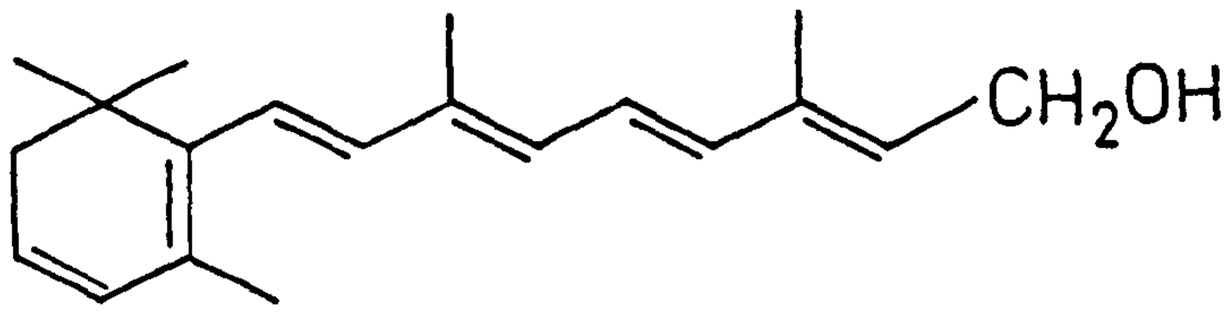


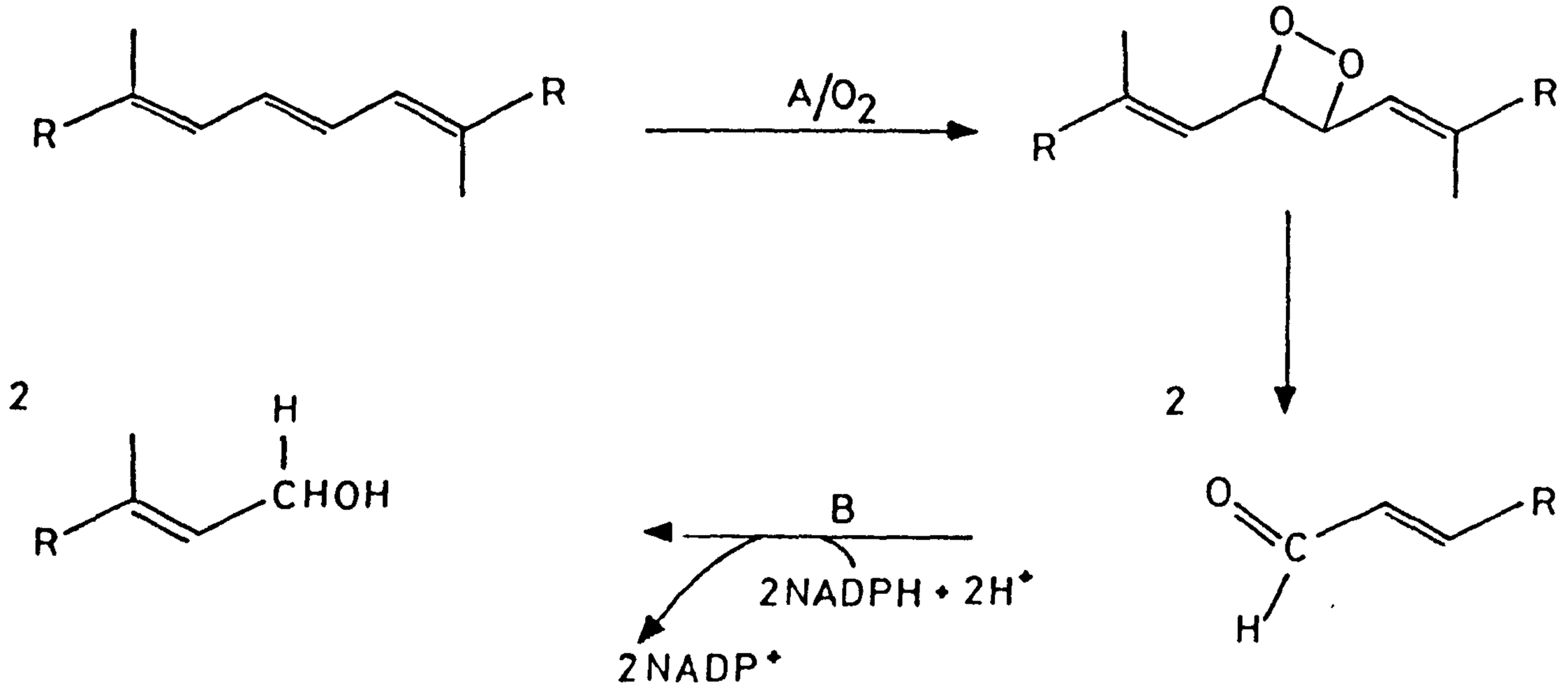
Fig.16.

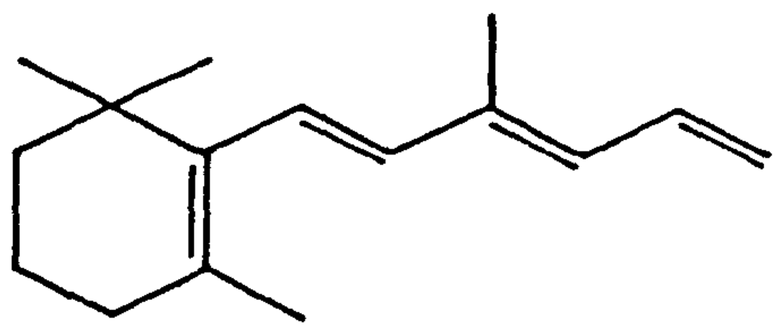
The structures of retinol and dehydroretinol.

The biosynthetic origin of dehydroretinol is not known, but there is some evidence from India that lutein may be the natural precursor (Barua et al. 1973). Zeaxanthin may also be postulated. Retinol is readily formed from β-carotene in many animals as depicted in Fig. 17 where the splitting of β-carotene to retinal is catalysed by β-carotene-15,15'-oxygenase. It is assumed that a transient peroxide is formed which is rapidly cleaved to 2 molecules of retinal and there then follows conversion of retinal to retinol by retinal reductase.

Thus dehydroretinol, which differs from retinol only by one additional double bond could be formed from lutein or zeaxanthin by a similar cleavage. In the case of lutein the allylic hydroxyl could be eliminated as water with consequent introduction of a double bond, followed by a rearrangement of the double bond to form anhydrolutein. This could then be cleaved to give dehydroretinol. Zeaxanthin could also be used, again with the loss of water (see Fig. 18). Barua and Das (1975) report the presence of anhydrolutein as well as lutein in the liver oil of the freshwater fish Sacchobranchus fossilis and furthermore, feeding of anhydrolutein to vitamin A depleted fish resulted in the accumulation of dehydroretinol. The Indian research groups, however, used unlabelled lutein (and anhydrolutein) for the experiments. Barua et al. (1977) have also isolated 3-dehydroretinol and 3-hydroxyretinol from fish; these are the expected products of the cleavage of anhydrolutein.

Another noteworthy pathway of retinal formation is by excentric cleavage of carotenoids to give apocarotenoids, which can then be whittled down to the C20 skeleton of retinal by successive stages of oxidation. This was first proposed by Glover and Redfearn (1954) and has been confirmed recently by Goswami (1985).



R = 

A = β-carotene-15,15'-oxygenase

B = retinal reductase

Fig.17.

Retinol formation from β-carotene as carried out in many animals.

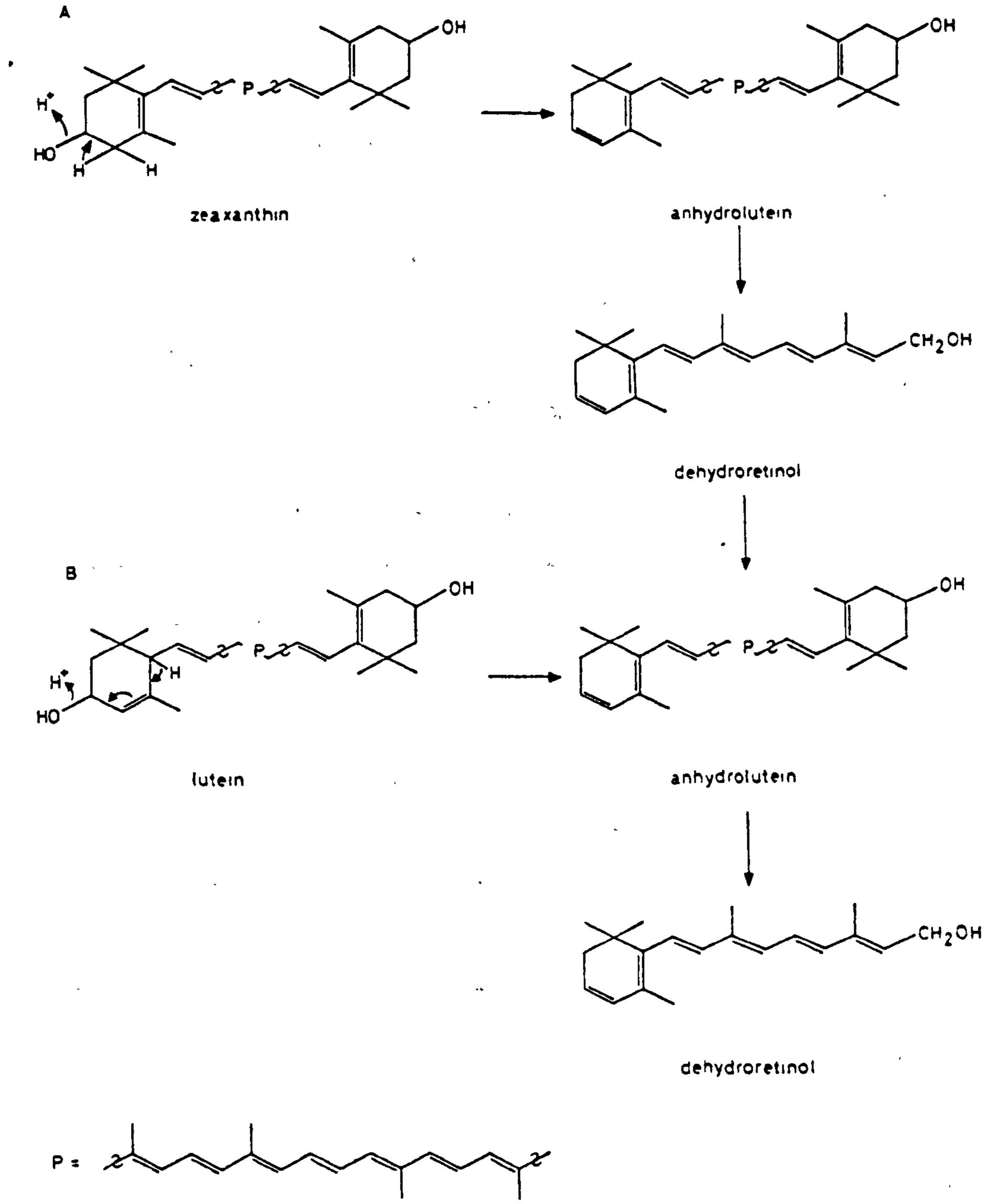


Fig.18.

Possible metabolic pathway for dehydroretinol formation from lutein and zeaxanthin in freshwater fish.

B. Lutein to astaxanthin conversion.

The carotenoids of goldfish have been the centre of much controversy for many years. The confusion arose when Hirao et al. (1963) suggested that the astaxanthin pigment of goldfish could be formed by the oxidation of lutein. Katayama et al. (1970a,b) investigated the carotenoids of goldfish and in the course of their work found a new ketocarotenoid which was shown to be 3-hydroxy-3',4'-diketo-α-carotene and was subsequently named α-doradecin. In nature α-doradecin most probably only exists as α-doradexanthin, these two compounds having the same relationship as astacene and astaxanthin. As a consequence of this finding Katayama and his colleagues suggested that astaxanthin was indeed formed by oxidation of lutein, as proposed originally by Hirao et al. (1963), with α- and β-doradexanthin as intermediates. A reaction scheme involving the oxidation of the β-ionone ring of lutein followed by the isomerisation of the α-ionone ring and its subsequent oxidation as depicted in Fig. 19, would satisfactorily account for the presence of astaxanthin and ketoderivatives in carotenoid extracts of goldfish.

Other oxidative pathways of carotenoid metabolism to astaxanthin were sought. Zeaxanthin, a dihydroxycarotenoid that is commonly a constituent of invertebrate diets could feasibly be a precursor of astaxanthin. Although zeaxanthin is present naturally in many organisms it is rarely a major constituent, and hence it is doubtful that this carotenoid is central to metabolism in fish. However, Hata and Hata (1970) demonstrated that goldfish could convert cynthiaxanthin (the diacetylenic analogue of zeaxanthin) to 4-ketocynthiaxanthin and then to the diacetylenic analogue of astaxanthin. These results suggest that goldfish may also be able to metabolise zeaxanthin to astaxanthin. The probable metabolic pathway is shown in Fig 20. Further work by Hata and Hata (1972, 1973) involving ^{14}C-labelled carotenoid feeding experiments illustrated the conversion of zeaxanthin to astaxanthin. By similar experimentation β-carotene was shown to be a poor precursor of

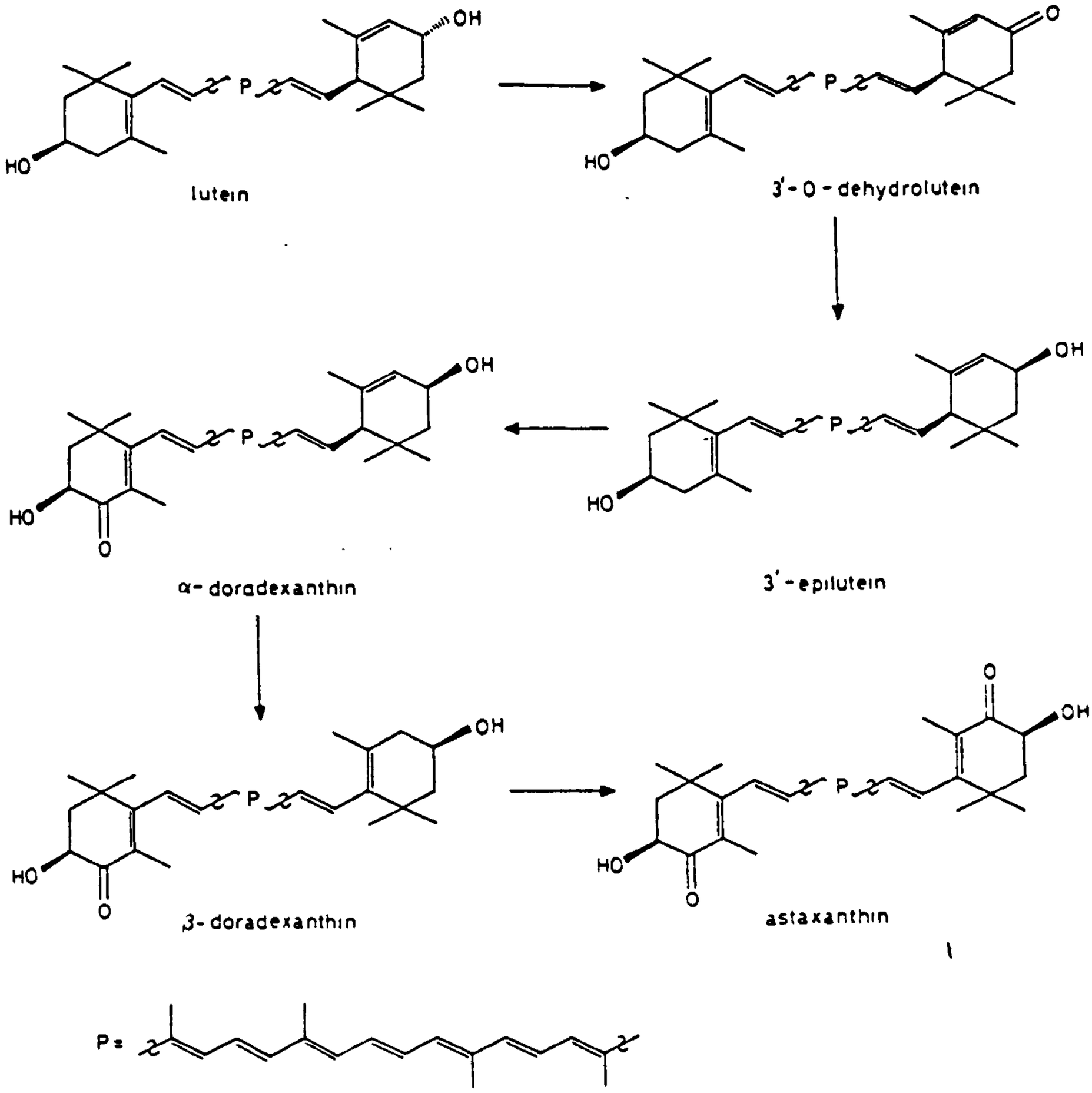


Fig.19.

Probable pathway for astaxanthin formation from lutein in goldfish.

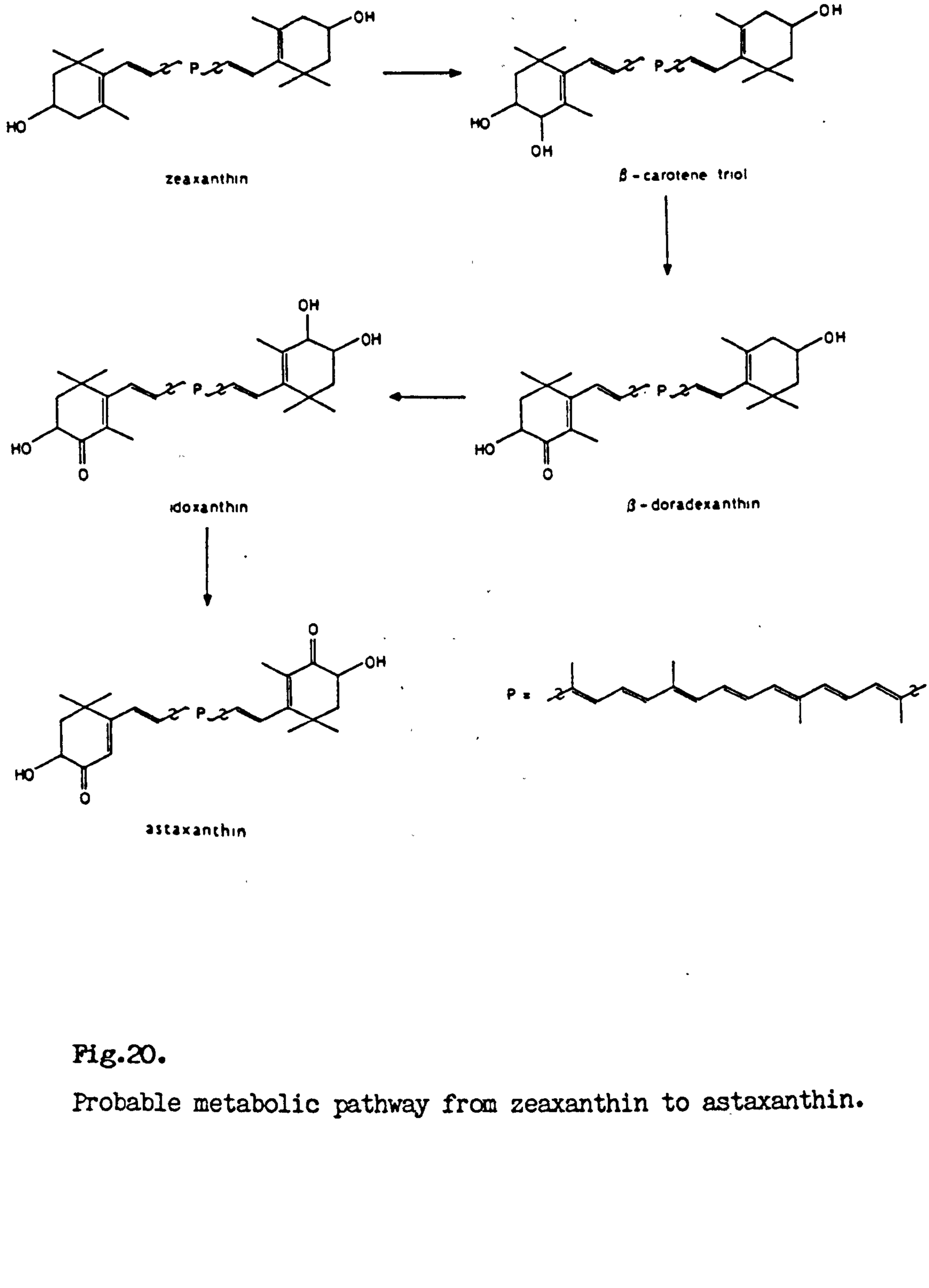


Fig.20.

Probable metabolic pathway from zeaxanthin to astaxanthin.

astaxanthin (see Fig. 14) and lutein was not converted beyond its 4-ketoderivative (i.e. α-doradexanthin).

Contrary to this are the views of Hsu et al. (1972). Hsu and co-workers were able to demonstrate the incorporation of radioactivity from [^{14}C]lutein into astaxanthin in goldfish, but their method of purification of the astaxanthin is questionable. Tanaka et al. (1976) used unlabelled zeaxanthin and lutein for feeding experiments and recorded an accumulation of astaxanthin after feeding in each case. It may be that the astaxanthin purity is the critical factor in this work and that Katayama's, Hsu's and Tanaka's groups are using similar methods that may be unsatisfactory. However, there is no doubt that the highly efficient absorption of 3,3'-dihydroxycarotenoids is important with respect to this work as was emphasised by Hata and Hata (1972).

In later experiments, Hata and Hata (1975, 1976) still did not see any evidence of the conversion of lutein to astaxanthin. Recently the chirality of the lutein from goldfish has been elucidated and this may have some bearing on the matter. Matsuno and Katsuyama (1979a) and Webber et al. (1973) report that the lutein is in fact 3'-epilutein which is of the correct chirality to give the α-doradexanthin of goldfish, which in turn has the same chirality at both C-3 atoms as astaxanthin. The epimerisation of lutein to 3'-epilutein could take place via 3'-O-dehydrolutein, and this putative intermediate has been isolated from goldfish by Buchecker et al. (1978).

Therefore the conversion of lutein to α-doradexanthin is generally accepted, and likewise the conversion of β-doradexanthin to astaxanthin is undisputed (Hata and Hata, 1975, 1976; Hsu et al., 1972). The only doubtful, but critical step is the conversion of α- to β-doradexanthin, the isomerisation reaction.

AIM OF THIS WORK

The aim of this work is two-fold. By feeding ^{14}C-labelled carotenoids to goldfish the role of xanthophylls as precursors of retinol and dehydroretinol will be investigated as will the role of lutein in astaxanthin formation. The ^{14}C-labelled carotenoids available were β-carotene, lutein, zeaxanthin and canthaxanthin.

MATERIALS AND METHODS

3.8 Maintenance of goldfish

The fish were kept in separate tanks of minimum capacity of 2 l water. All the fish were of similar physical dimensions. High oxygen levels were maintained by bubbling compressed air through each tank at a rate no greater than 2 ml/min as higher rates distress the fish. Tank water was changed regularly (every 2-3 days).

Fungal disease accounted for several fatalities but less severe infections could be cured using Liquetox (Interpet Ltd., Curtis Road, Dorking, England).

3.9 Goldfish diet.

The daily diet per fish was 0.03 g of Petcraft Freeze dried Magic Worms (distributed by Thomas's, Division of Mars Ltd., Halifax, England).

Each fish was trained over a period of a few weeks to consume all the food immediately. This was achieved by feeding the fish at the same time each day. Complete and rapid ingestion of the food would be important during the feeding experiment.

3.10 Extraction of goldfish food.

Goldfish food (5 g) was extracted by standard procedures and analysed for the presence of any carotenoids, retinol and dehydroretinol which may be present in the natural diet of the experimental animals. The h.p.l.c. systems described in Sections 3.15 and 2.13 were used to analyse the vitamin A fraction and carotenoid fraction respectively.

3.11 Administration of ^{14}C-labelled carotenoids to fish.

The ^{14}C-labelled carotenoid was dissolved in dichloromethane and pipetted onto cubes of food (0.5 g cube of Magic Worms). The solvent was removed under a stream of nitrogen.

A 0.5 g cube supplemented with a particular ^{14}C-carotenoid was fed to each fish over a period of 18 days i.e. approximately 0.03 g was given daily.

3.12 Dissection of goldfish.

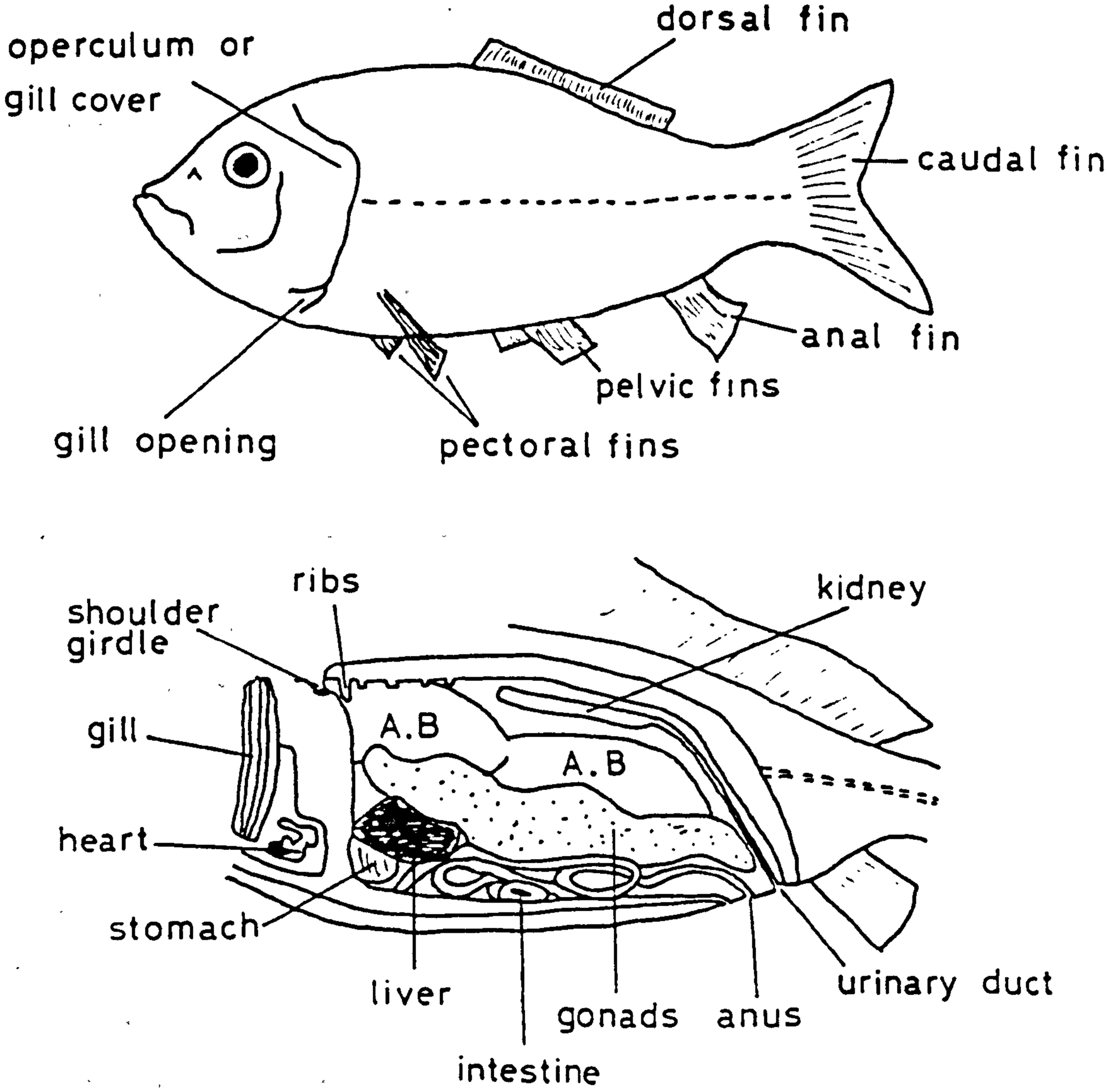
A. Eyes.

The eyes were removed from the head by cutting away the bony surroundings, the circum orbital bones, until the socket was fully exposed. Fine scissors were used to cut away muscles and gently excise the eye. Finally the optic nerve could be cut to completely free the eye.

B. Liver.

The liver is easily seen in the dissected fish as it is a large dark red tissue lying just behind the shoulder girdle. Due to its location care was taken not to disturb the tissue when the first incision was made. See Fig. 21 for arrangement of organs in body cavity.

The operculum was removed first to allow access to the gills and the shoulder girdle. The shoulder girdle was cut on the lower edge of the fish and the body cavity exposed by cutting along the lower side of the fish. The liver was then removed with scissors and forceps.



A.B - air bladder

Fig.21.

External features of the goldfish and a diagram of the arrangement of organs in the body cavity.

C. Intestine.

Once the body cavity has been exposed the intestine is readily seen and can be removed with scissors and forceps.

D. Integument.

All the integument was removed and extracted including the bony operculum and fins. Dissection and extraction of the flesh was avoided as this led to unmanageable samples due to sterol and oil contamination.

3.13 Treatment of goldfish tissues.

All tissues were extracted when fresh as freezing was suspected to cause losses, particularly of vitamins A.

A. Eyes.

The retina was exposed by making an equatorial cut around the eye. The retinal tissue was bleached (by exposure to bright light) and then extracted with ethanol twice followed by two ether extractions. At this early stage pyrogallol was added to minimise oxidative degradation. The total extract was "cold" saponified. The unsaponifiable lipid was stored at -20°C, under nitrogen in the dark.

B. Liver.

As in section 3.13A except that the bleaching stage was omitted.

C. Intestine.

As for liver.

D. Integument.

The integument, in acetone was homogenised in a Silverson Laboratory mixer emulsifier. The resultant suspension was centrifuged at 2000 g for 5 min in a Griffin Christ bench top centrifuge. The solid residue was extracted a further 3 times with ether. The bulked extract was washed several times with water and then dried.

Integument extracts were not saponified until a later stage.

3.14 Summary of procedures for the feeding experiments.

Three similar experiments were carried out at different times. In each case the special diet was fed to the fish every day over a period of 18 days. The fish were maintained for another 2 days on their normal diet after which time they were sacrificed by immersion in liquid nitrogen. The weight and length of each fish was measured before dissection as described in section 3.12.

Details of the specific activities of the ^{14}C-carotenoids or retinyl acetate fed are given in the results section. [^{14}C]β-Carotene, [^{14}C] canthaxanthin and retinyl-6,7-$^{14}C_2$ acetate were gifts from Roche but [^{14}C]zeaxanthin and [^{14}C]lutein were made via the biological systems previously described.

The various tissues were then treated as follows:

A. Eyes.

The ocular tissue was extracted as in Section 3.13A. Purification of retinol and dehydroretinol was as described in 3.15 and 3.16. In the first experiment the sample was spiked with "cold" authentic retinol and

dehydroretinol before h.p.l.c. The retinol and dehydroretinol fractions were collected, the h.p.l.c. solvent was removed and the pure vitamin A samples redissolved in scintillation fluid. The samples were counted for 10 min.

The second experiment was slightly different as specified in Section 3.16.

B. Livers.

The livers were extracted as outlined in Section 3.13B. The treatment was as for the eyes.

C. Intestine.

The intestines were extracted as described in Section 3.13C. The same treatment was applied as for the eyes.

D. Integument.

The tissue was extracted as desribed in Section 3.13D. The procedure of Section 3.17 was followed and radioautograms of the resultant t.l.c. plates were obtained as in Section 2.10.

E. Faeces.

The faeces were collected by filtration of the tank water through several layers of cheesecloth and then extracted by standard methods. The amount of radioactivity in the faeces was estimated by liquid scintillation counting of a known aliquot of the total extract.

3.15 Purification of retinol and 3,4-dehydroretinol (vitamins A_1 and A_2) by classical column chromatography.

A crude liver or ocular extract was chromatographed on a neutral alumina column (Brockmann Grade III) according to Heaton et al., (1957). The developing solvents were light petroleum (b.p. 40 - 60°C) with increasing concentrations of ether.

Both retinol and dehydroretinol could be visualised under short wavelength u.v. light (265 nm) in which they showed a yellow-green fluorescence.

The most widely reported method for the separation of retinol and dehydroretinol is by t.l.c. on Silica Gel G plates with a mixture of light petroleum (b.p. 30 - 45°C) and methyl heptenone (11:2, v/v) as solvent. Due to the very small quantities available in most cases this system was not considered suitable and an h.p.l.c. system was sought for this separation.

The preliminary column was suitable for all crude liver, intestinal and ocular extracts of both trout and goldfish. Trout liver extracts were used in the elementary stages to devise a method to purify retinol and dehydroretinol.

3.16 H.p.l.c. separation of retinol and dehydroretinol.

Based on the fact that the t.l.c. system described in Section 3.15 was on plates of silica the column used was Nucleosil 50-5. Various solvent systems were tried. Finally the solvent used was hexane/1,4-dioxan as used by Bridges et al., (1980). The Cecil instrument was used.

Column	Nucleosil 50-5 (200 mm x 4.5 mm)
Wavelength of detector	335 nm
Solvent	Hexane/dioxan (90:10, v/v)
Flow rate	1 ml/min
Chart speed	30 cm/h

Crude vitamin A fractions could be purified on this system and the retinol and dehydroretinol fractions collected separately.

In the first feeding experiment, the liver and ocular extracts were spiked, before chromatography, with cold authentic retinol and dehydroretinol to aid in their detection. Only the relevant fractions (i.e. retinol and dehydroretinol) were collected. The h.p.l.c. solvent was removed by evaporation on a hot-plate, under nitrogen. Scintillation fluid (10 ml) was added and the vials counted for 10 mins.

The second and third experiments were more sophisticated in that the amount of retinol and dehydroretinol was determined, enabling their specific activities to be calculated. Also a fraction collector was used (LKB BROMMA 2112 Redirac) to collect fractions every 30 s throughout the chromatography. Fractions were collected directly into vial inserts, the solvent removed and 2 ml of scintillation fluid added to each. The vials were counted. Quantification of retinol and dehydroretinol was achieved by integration. The integrator used was a Pye Unicam PU4810 computing integrator (Philips). Standard retinol, dehydroretinol and retinal were used to establish Response Factors (RF), which were then used to calculate the µg of compound present, based on the peak area.

3.17 Separation system for integument carotenoids of goldfish.

Separation of the integument carotenoids posed 2 problems. Firstly, all the carotenoids, except β-carotene, were esterified making chromatography very difficult as the ester group influenced the

chromatographic properties of the carotenoids. Secondly, astaxanthin ester was present and astaxanthin on saponification yields astacene which is difficult to chromatograph.

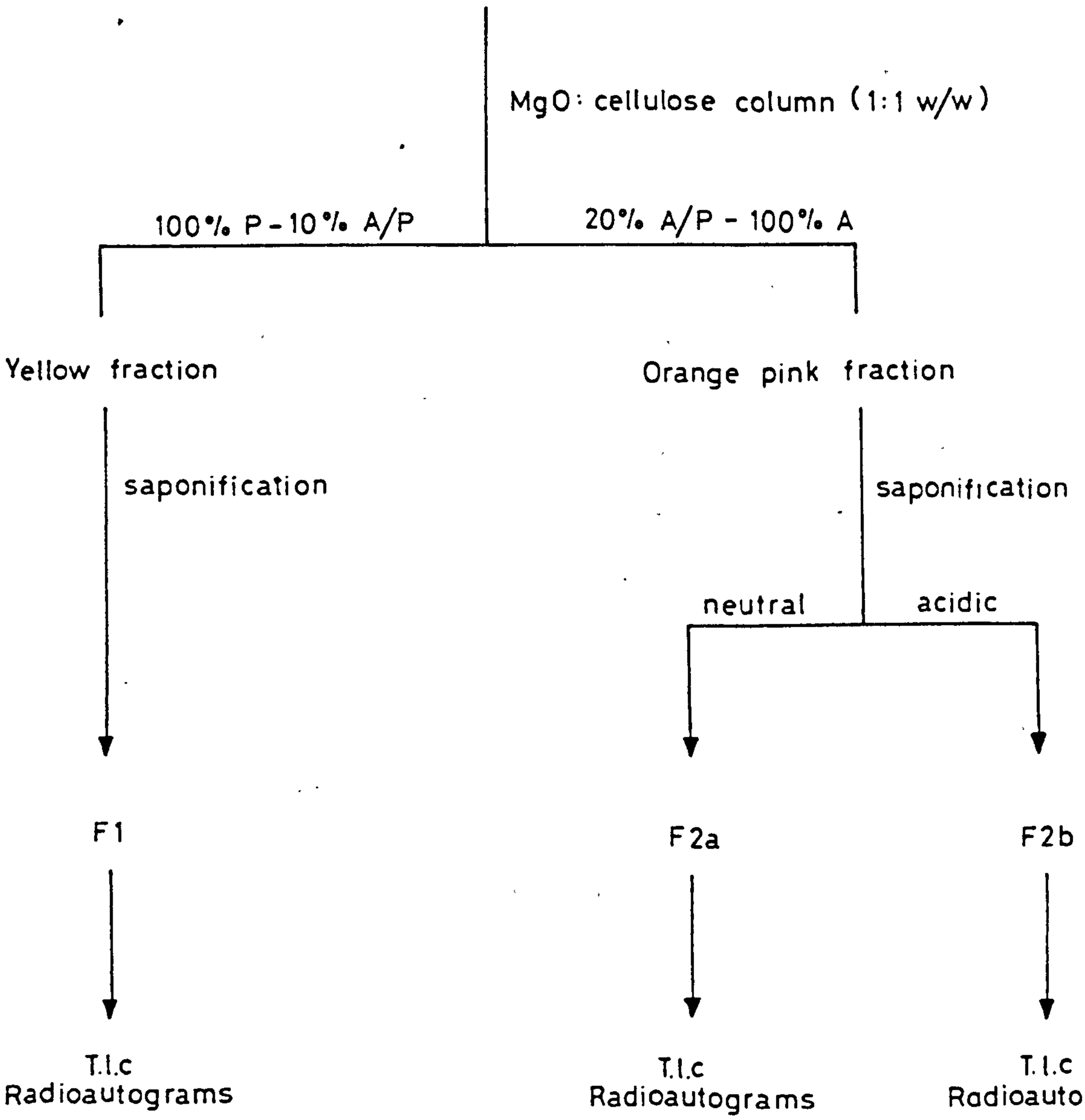
Initially a system for the separation of the esters was sought but little success was obtained with either h.p.l.c or t.l.c. A MgO:cellulose (1:1 w/w) classical column with light petroleum (b.p. 40 - 60°C) and acetone as solvent separated the carotenoid esters into 2 groups. This system was used as a preliminary separation to give a fairly non-polar fraction and a polar fraction. These 2 fractions were saponified separately. A suitable t.l.c. system for the separation of the free carotenoids was devised. Silica gel G t.l.c. (Precoated Merck 60F-254 plates) with 5% methanol in toluene as solvent was found to be successful.

A summary of the treatment of goldfish integument extracts is given in Fig. 22.

3.18 H.p.l.c. separation of acidic carotenoids.

In the first experiment the integument carotenoids were analysed by t.l.c. alone. H.p.l.c. analysis of the acidic carotenoids was carried out in the second experiment to clarify the labelling patterns observed by t.l.c. A column was prepared which achieved the separation of astacene, astaxanthin, β-doradecin and semi-astacene.

Nucleosil 50-5 (2.4 g) was suspended in 18 ml of methanol containing 1% ortho-phosphoric acid and ultrasonicated for 5 min. The suspension was packed into the column under pressure (48.3 MPa), the packing solvent being dichloromethane. The column was designated as Nucleosil/H_3PO_4.



A - acetone
P - light petroleum

Fig.22.

Separation system for integument carotenoids of goldfish.

Column	Nucleosil/H_3PO_4 (250 mm x 4.5 mm)
Wavelength of detector	460 nm
Solvent	Hexane- 10% CH_2Cl_2 (v/v), 0.75% Propan-2-ol (v/v)
Flow rate	1.8 ml/min
Chart speed	30 cm/h

In the second experiment, half of each of the integument samples was used for t.l.c. and radioautography. The remainder was analysed by h.p.l.c. The initial step was to isolate a polar and a non-polar fraction from each sample. This was achieved by t.l.c. (Silica gel 60G; 5% methanol in toluene). The regions corresponding to astacene, α- and β-doradecin were scraped off, eluted and applied to the h.p.l.c. system described above. The other regions of the chromatogram corresponding to β-carotene, lutein and zeaxanthin were scraped off, eluted and chromatographed on the h.p.l.c. system used to separate the carotenoids of Flavobacterium (Section 2.13).

In both cases 30 s or 1 min fractions were collected as appropriate, the solvent removed and scintillation fluid (10 ml) added to each vial.

RESULTS

3.19 Preliminary column chromatography of crude extracts from livers or eyes.

Chromatography as described by Heaton *et al.* (1957) gave six fractions, one of which was a crude fraction containing both vitamins A. The solvents used for the separation and the components of the resultant fractions are given in Table 5.

The vitamins A were eluted with 20% ether in light petroleum. Any β-carotene present in the extracts (e.g. after feeding experiments with [^{14}C]β-carotene) will have been eluted with a less polar solvent and [^{14}C]zeaxanthin, [^{14}C]canthaxanthin or [^{14}C]lutein require more polar solvents for elution. This procedure prevents contamination of vitamin A fractions with the ^{14}C-labelled carotenoids fed to the goldfish. If the vitamin A fraction was still coloured, however, a second classical column of neutral alumina (Brockmann grade IV) was prepared. The vitamin A was eluted with 2-20% ether in light petroleum (b.p. 40-60°C).

The vitamin A fraction (containing both retinol and dehydroretinol) could be readily detected under short wavelength u.v. light by its green fluorescence.

3.20 H.p.l.c. separation of retinol and dehydroretinol.

One of the earliest h.p.l.c. separations of retinol and dehydroretinol was described by Bridges *et al.* (1980). The column packing material for this separation was 10μm LiChrosorb and the solvent was 1,4-dioxan/hexane (1:9, v/v). This was the basis of the separation system outlined in Section 3.16, the significant difference being that the column packing material used in this work was Nucleosil 50-5. The retention times of retinol and dehydroretinol on LiChrosorb were approx.

TABLE 5.

Summary of classical column chromatography of liver or ocular extracts (Heaton et al., 1957).

Fraction	Solvent	Main component of fraction
1	100% P	hydrocarbons
2	2% E/P	aliphatic hydrocarbons - lycopene
3	4% E/P	ubiquinone
4	6–8% E/P	tocopherol
5	20% E/P	cholesterol and vitamins A
6	100% E	xanthophylls

P = light petroleum
E = diethyl ether

1h. Retention times were reduced considerably on Nucleosil as illustrated in Fig. 23.

From structural considerations it would be expected that retinol would migrate more quickly than dehydroretinol on a straightforward adsorption column such as Nucleosil. Collection of the two peaks followed by spectrophotometry gave the absorption spectra of Fig. 24. The reference solvent was ethanol. The expected absorption maximum of all-trans retinol in ethanol is at 324 nm corresponding closely to the absorption spectrum of fraction 1. All-trans dehydroretinol has an absorption maximum at 351 nm in ethanol with 2 new peaks appearing at 276 and 286 nm. This corresponds to fraction 2. M.s. data for fractions 1 and 2 substantiate that these peaks are retinol and dehydroretinol (Fig. 25). The spectrum for retinol displayed a molecular ion at m/z 286 as expected, with the following interpretative peaks also present (m/z 255, 119, 109, 95, 69, 43). Dehydroretinol displayed a molecular ion of 284 with m/z peaks at 266, 253, 149, 123, 43. In both cases there were high levels of lower m/z ions.

Goldfish and trout, liver, intestinal or ocular vitamin A fractions showed the same separation.

	Retention time (min)
Retinol	16.5
Dehydroretinol	18.5

3.21 Analysis of livers and eyes from untreated goldfish.

Ten goldfish were sacrificed and the eyes and livers were taken and extracted as normal. The retinol and dehydroretinol of the extracts were purified by the methods of Sections 3.15 and 3.16 and a quantitative estimation made by spectrophotometric means.

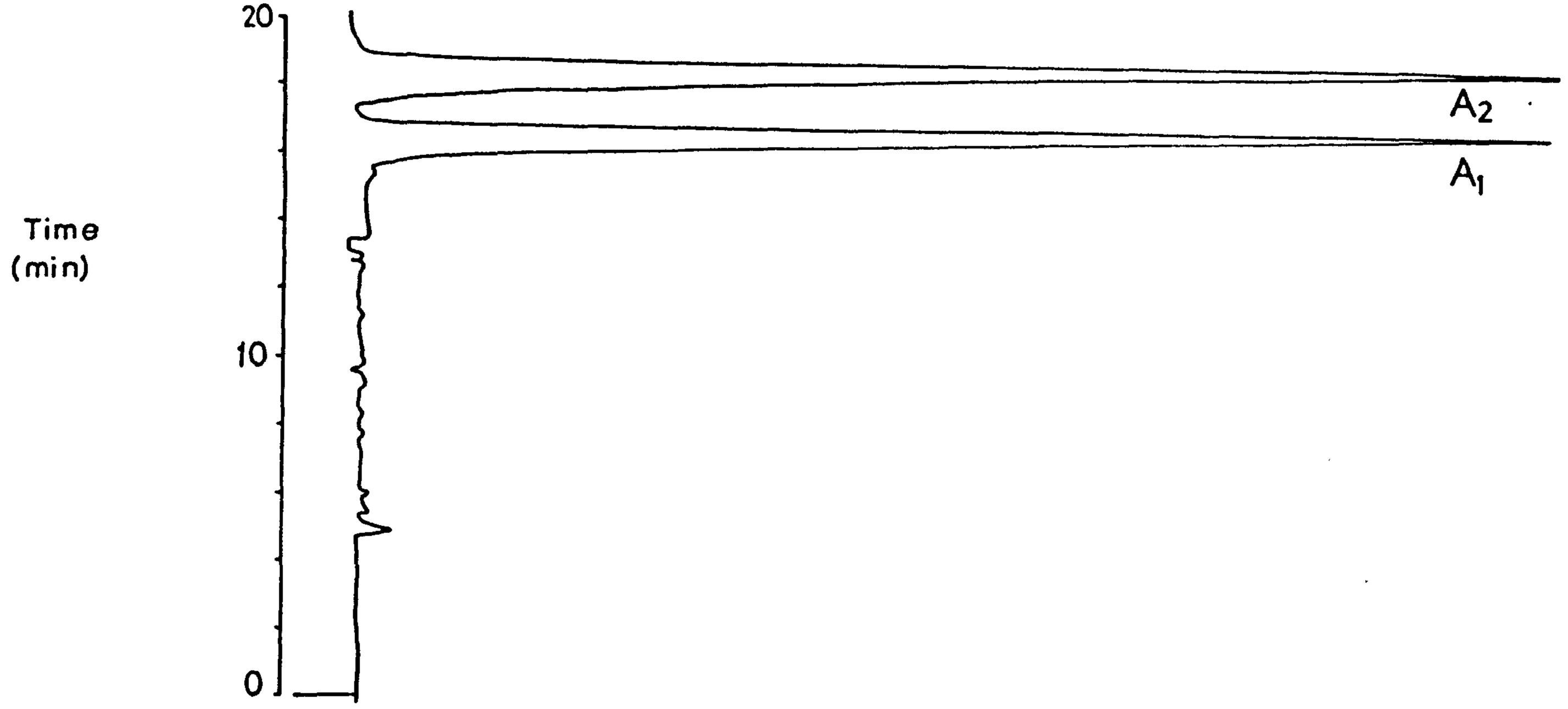


Fig. 23.

H.p.l.c. separation of retinol and dehydroretinol. Column: Nucleosil-50-5, 200 x 4.5 mm; solvent: hexane/1,4-dioxan, 9:1 (v/v) at 1 ml/min; detector: u.v. at 335 nm.

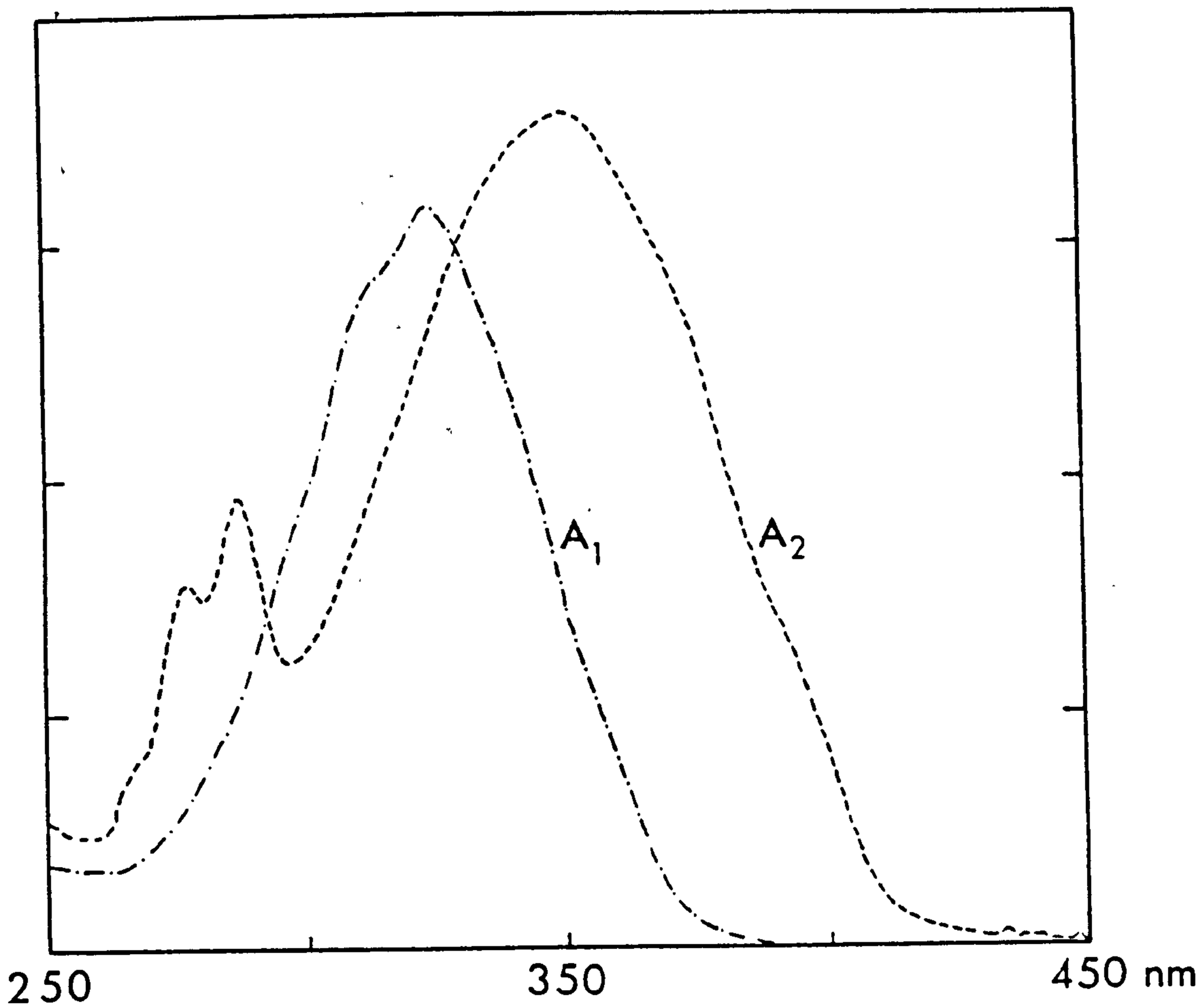
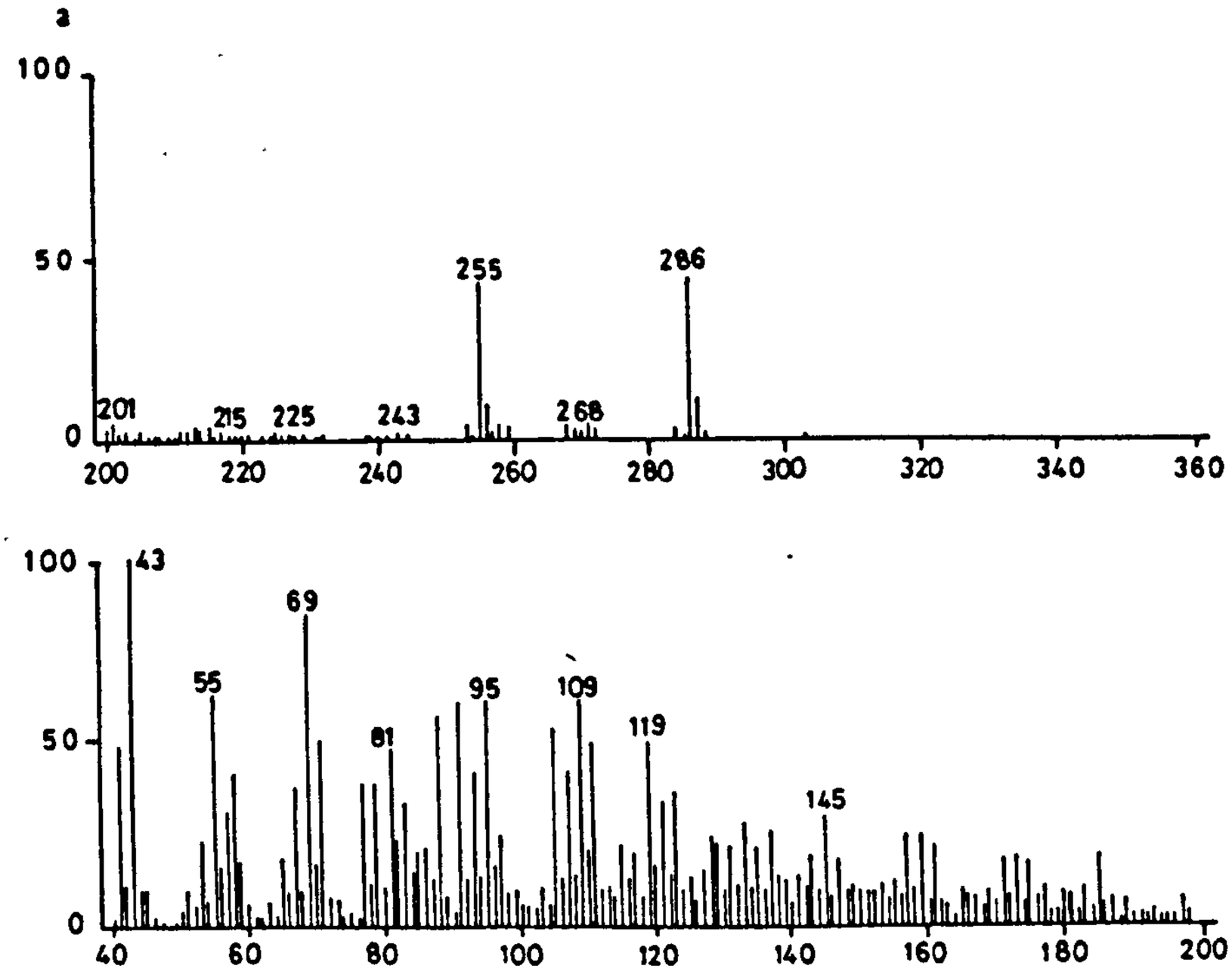


Fig. 24.

Absorption spectra of retinol (A_1) and dehydroretinol (A_2) recorded in ethanol.



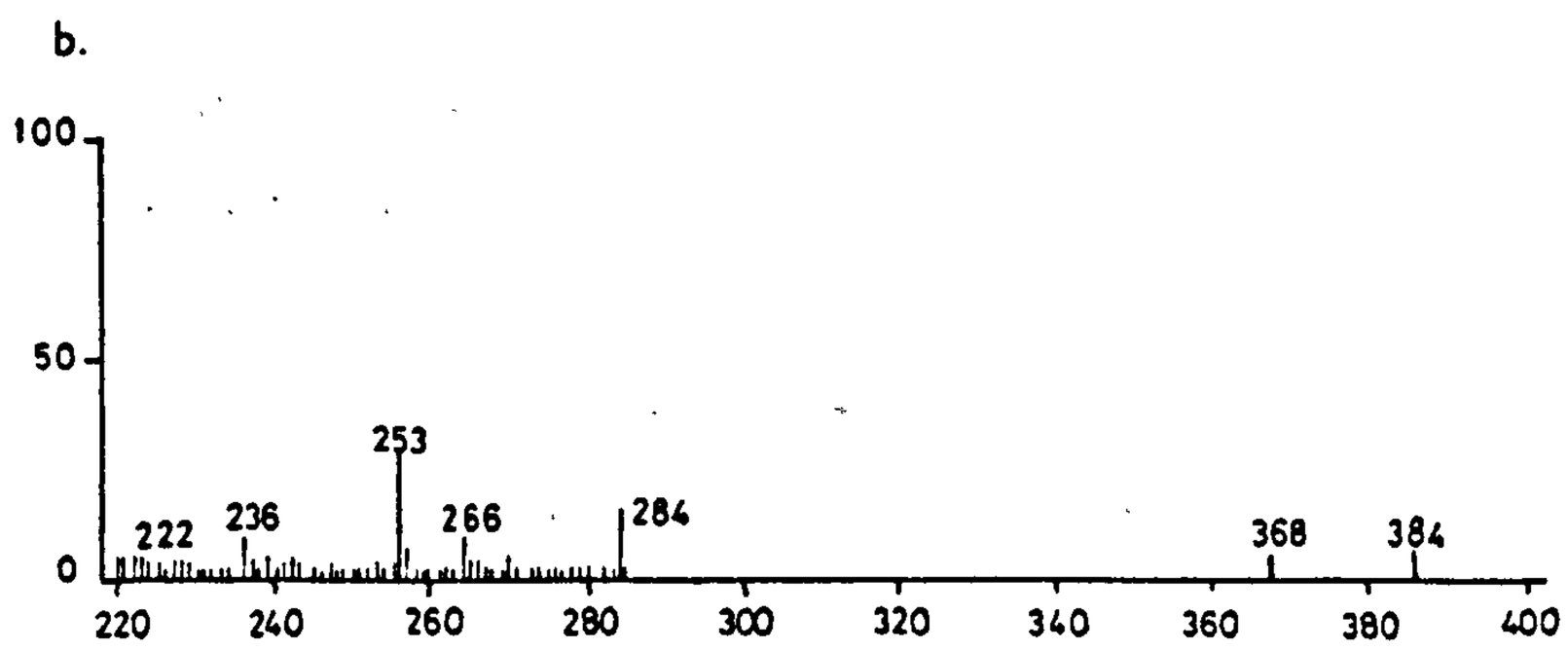


Fig. 25.

Mass spectra of retinol (a) and dehydroretinol (b).

$A^{1\%}_{1cm}$ for retinol (at 325 nm) and dehydroretinol (at 351 nm) in ethanol are 1850 and 1460 respectively (Planta et al., 1962).

A. Liver

Retinol per liver	=0.44 µg
Dehydroretinol per liver	=0.56 µg
Ratio of retinol:dehydroretinol	=1 : 1.27

B. Eyes

Retinol per eye	=1.295 µg
Dehydroretinol per eye	=6.39 µg
Ratio of retinol:dehydroretinol	=1 : 4.94

3.22 Classical column chromatography of carotenoid esters.

A preliminary analysis of goldfish carotenoid esters on a MgO:cellulose column gave the results shown in Table 6.

Some of the spectra obtained of the fractions were not as expected. The astaxanthin spectrum should have had an absorption maximum at 469 nm and F5, which was most probably β-doradexanthin should have displayed one symmetrical peak with wavelength maximum at 461.5 nm.

Although this separation was not very successful it was sufficient to separate the astaxanthin ester from all the other carotenoid esters. As the feeding experiment was primarily to investigate whether or not lutein is metabolised to astaxanthin, the purity of the astaxanthin was the major consideration.

The two groups of carotenoids i.e. in F1-F5 and F6 were saponified overnight.

TABLE 6.

Preliminary separation of carotenoid esters of goldfish by MgO:cellulose classical column chromatography.

Fraction	Solvent	Absorption maxima nm (in light petroleum)			Carotenoid component
1	5% A/P	420.5	436	466	β-carotene
2	10% A/P	420.5	442.5	471	lutein ester
3	10% A/P		447	469.5	α-doradexanthin ester
4	20% A/P		447	469.5	α-doradexanthin ester
5	30% A/P		451	471	α- and β-doradexanthin ester
6	A		470.5		astaxanthin ester

A = acetone
P = light petroleum

3.23 T.l.c. of saponified goldfish carotenoids.

Initially, standard carotenoids were used to establish R_f values for each carotenoid expected to be present in the extracts. Standard β-carotene, lutein, canthaxanthin, astacene, β-doradecin and zeaxanthin were all available and a sample of F3 (see Section 3.22) was purified and from comparison with m.s data from Katayama et al. (1970a) was identified as α-doradecin (see Fig. 26 for part of m.s. spectrum). These samples were applied to a thin layer plate and Fig. 27 is a representation of the typical separation achieved. Development of the chromatogram required 40 min.

Goldfish carotenoids when applied to the system were separated according to the above R_f values.

3.24 H.p.l.c. separation of goldfish integument carotenoids.

A typical separation of standard astacene (1), β-doradecin (2) and astaxanthin (3) is shown in Fig. 28. Retention times were 3.9, 4.3 and 20.2 min repectively.

3.25 Goldfish: physical data.

The data are summarised in Table 7. These data are included to illustrate the variation in the animals used even though an attempt had been made to use animals of uniform size.

3.26 Analysis of goldfish diet.

After saponification of the extract from goldfish feed there was little indication of any carotenoid or vitamin A in either the neutral or acidic fraction. Further analysis of the neutral fraction on an alumina column (Brockmann Grade IV) revealed the presence of small amounts of

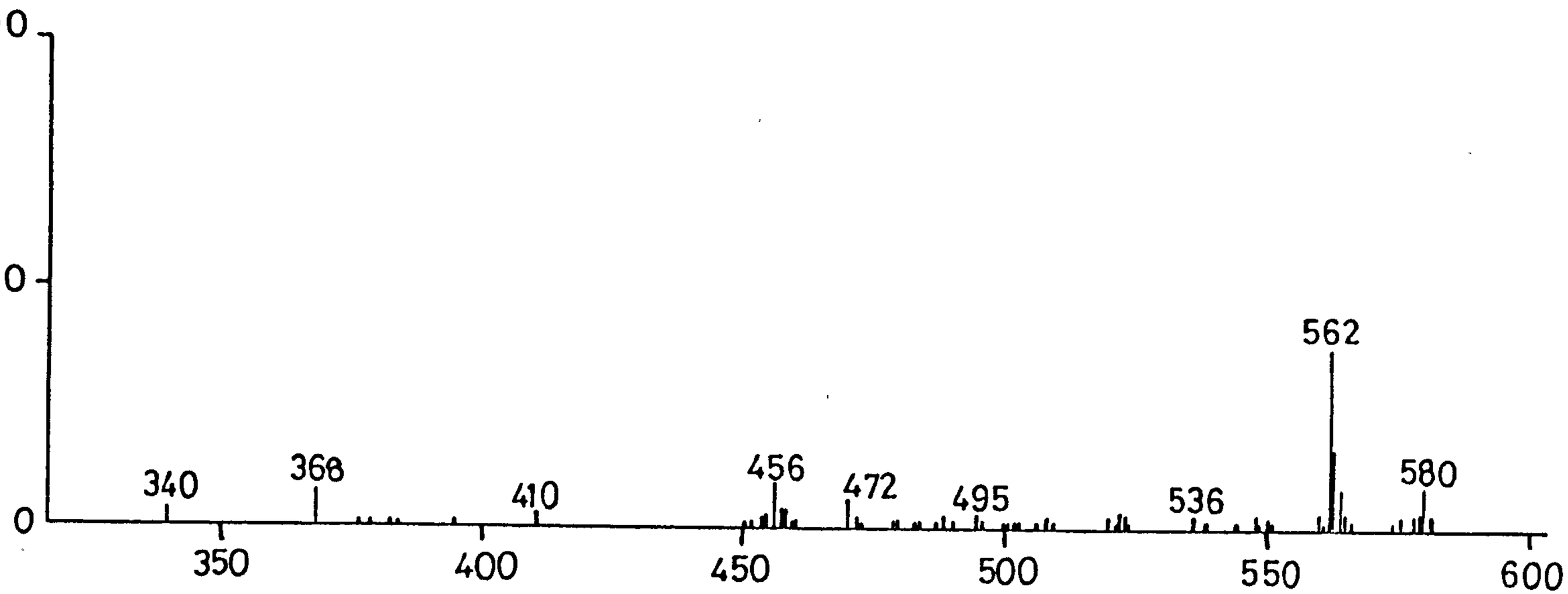
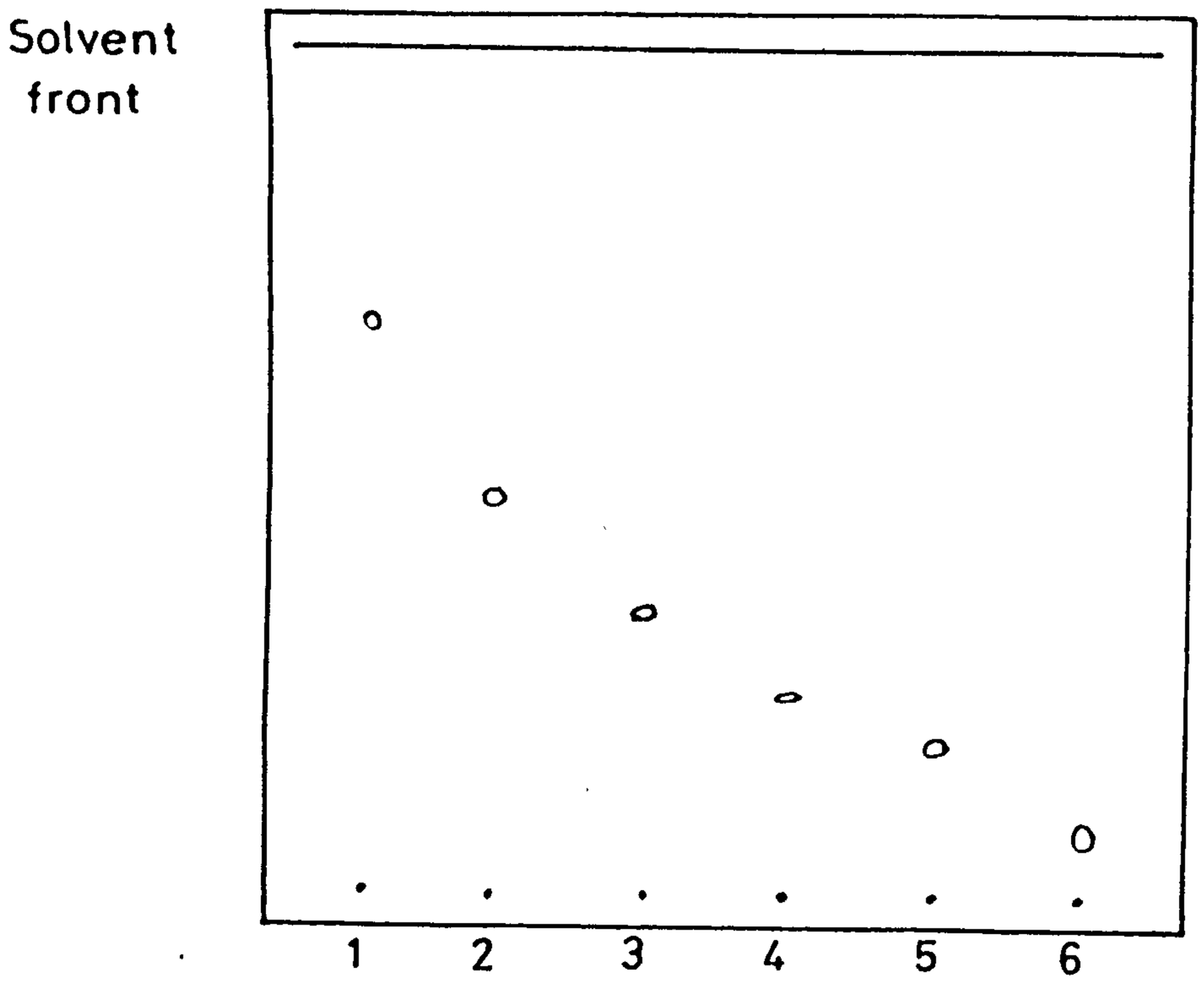


Fig. 26.

Mass spectrum of α-doradecin.



	CAROTENOID	R_f
1	β-carotene	0·7
2	canthaxanthin	0·46
3	astacene	0·34
4	α-doradecin	0·23
5	β-doradecin	0·2
6	lutein	0·1

Fig. 27.

Schematic representation of a typical t.l.c. separation of goldfish integument carotenoids on Silica Gel G, with 5% methanol in light petroleum as developing solvent.

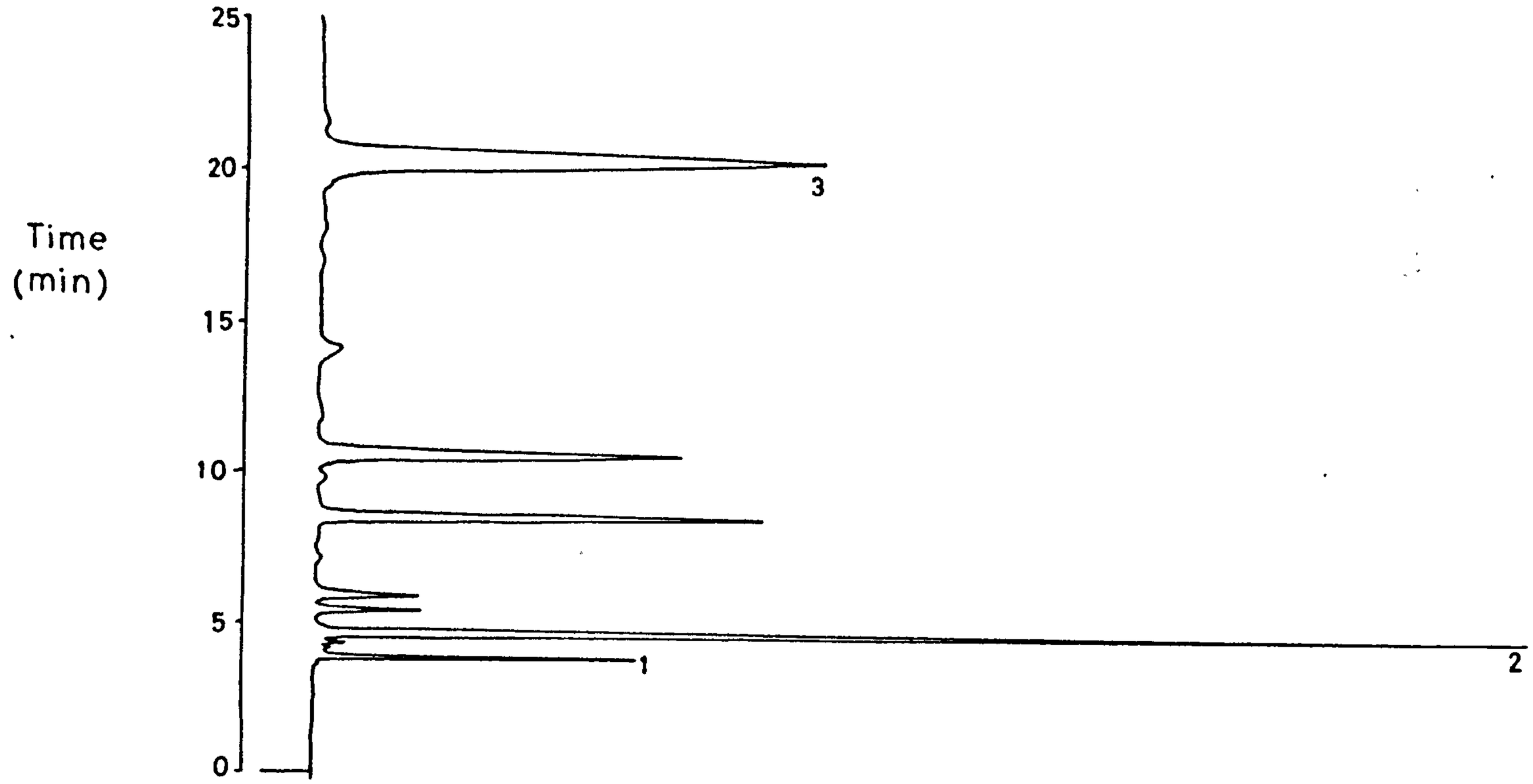


Fig. 28.

H.p.l.c. separation of astacene, β-doradecin and astaxanthin.
Column: Nucleosil/H_3PO_4, 250 x 4.5 mm; solvent: hexane–10% CH_2Cl_2, 0.75% propan-2-ol at 1.8 ml/min.

TABLE 7.

Physical data of goldfish used for experiments 1-3.

a. Experiment 1.

Fish	Weight (g)	Length (cm)
1	5.36	7.0
2	6.10	7.5
3	6.59	7.7
4	6.06	8.0
5	6.08	7.3
6	7.03	8.2
7	4.79	6.6
8	6.70	8.0
9	4.87	7.0
10	5.25	7.0
11	4.85	7.5
12	6.65	9.0
13	6.50	8.0

b. Experiment 2.

1	3.76	8.2
2	7.55	8.4
3	7.24	7.5
4	6.15	8.5
5	5.67	7.4
6	6.43	8.8
7	4.65	7.5
8	8.55	9.5
9	8.10	9.2

c. Experiment 3.

1	3.66	6.0
2	3.93	7.0
3	3.72	6.6
4	4.06	6.1
5	2.79	6.2
6	4.12	6.5
7	3.31	6.5
8	3.94	6.5
9	3.39	5.9
10	3.47	6.0

retinol. The total amount of retinol in 5 g of feed was determined by spectrophotometric means to be 5.63 µg. Carotenoids were not detected in the extracts at all. The low level of retinol (0.563 µg) in 0.5 g of feed (the diet per fish) would not be expected to interfere with the feeding experiment to any significant extent.

3.27 Radiolabelled carotenoids and retinyl acetate administered to goldfish.

Details of the carotenoids and retinyl acetaté administered to the goldfish in the three feeding experiments are summarised in Tables 8 a,b,c. The variation in specific activities of synthetic and biologically prepared carotenoids are obvious.

3.28 Results of feeding experiments.

A. Eye extract analysis.

The three tables (Tables 9a,b,c) summarise the results of incorporation from [^{14}C]β-carotene, [^{14}C]canthaxanthin, [^{14}C]lutein, [^{14}C]zeaxanthin and retinyl-6,7-$^{14}C_2$-acetate into retinol and dehydroretinol in goldfish eyes. Each of tables a,b,c correspond to experiments 1,2 and 3 respectively. The simplistic interpretation of Table 9a (Expt 1) whereby all except β-carotene are precursors directly of dehydroretinol cannot be substantiated by Expt 2 where the resultant specific activities shed more light on the metabolic process. From experiment 2 it was considered that each of the carotenoids may be precursors of retinol which can then be dehydrogenated to dehydroretinol. Experiment 3 aimed to prove that retinol could indeed be metabolised to dehydroretinol as has been demonstrated in other species (Schiedt et al., 1985). No pattern of incorporation of radioactivity into retinol or dehydroretinol could be detected in any of the fish.

TABLE 8.

Summary of radioactivity data of carotenoids and retinyl acetate fed to fish in experiments 1-3.

a. Experiment 1.

Fish	^{14}C carotenoid administered	Source	µCi per fish	mg	specific activity
1	canthaxanthin	Roche	4.08	0.136	30
2	canthaxanthin	Roche	1.46	0.048	30
3	β-carotene	maize	0.106	6	0.018
4	β-carotene	maize	0.106	6	0.018
5	β-carotene	maize	0.106	6	0.018
6	zeaxanthin	Flavobacterium	0.273	0.69	0.39
7	zeaxanthin	Flavobacterium	0.28	1.95	0.14
8	zeaxanthin	Flavobacterium	0.28	1.95	0.14
9	zeaxanthin	Flavobacterium	0.28	1.95	0.14
10	zeaxanthin	Flavobacterium	0.28	1.95	0.14
11	lutein	barley	0.5	0.22	2.27
12	lutein	barley	0.5	0.22	2.27
13	lutein	barley	0.5	0.22	2.27

b. Experiment 2.

1	β-carotene	Roche	4.5	0.115	39
2	β-carotene	Roche	4.5	0.115	39
3	β-carotene	Roche	4.5	0.115	39
4	lutein	barley	0.52	0.242	2.15
5	lutein	barley	0.52	0.242	2.15
6	lutein	barley	0.52	0.242	2.15
7	lutein	barley	0.52	0.242	2.15
8	lutein	barley	0.52	0.242	2.15

c. Experiment 3.

1	retinyl acetate	Roche	1.346	0.0142	94.65
2	retinyl acetate	Roche	1.346	0.0142	94.65
3	retinyl acetate	Roche	1.346	0.0142	94.65
4	retinyl acetate	Roche	1.346	0.0142	94.65
5	retinyl acetate	Roche	1.346	0.0142	94.65
6	retinyl acetate	Roche	1.346	0.0142	94.65
7	retinyl acetate	Roche	1.346	0.0142	94.65
8	retinyl acetate	Roche	1.346	0.0142	94.65
9	retinyl acetate	Roche	1.346	0.0142	94.65
10	retinyl acetate	Roche	1.346	0.0142	94.65

TABLE 9.

Percentage incorporations of dietary ^{14}C-carotenoids and retinyl acetate into retinol and dehydroretinol and specific activities of retinol and dehydroretinol in goldfish eyes after goldfish feeding experiments 1-3.

a. Experiment 1.

Fish	Carotenoid	Carotenoid sp. acty (µCi/mg)	Percentage incorporation A_1	Percentage incorporation A_2	Ratio of radioactivities $A_1 : A_2$
1	canthaxanthin	30	0.21	0.84	1:4
2	canthaxanthin	30	0.48	1.64	1:3.4
3	β-carotene	0.018	0.02	0.026	1:1.1
4	β-carotene	0.018	0.017	0.017	1:1
5	β-carotene	0.018	0.02	0.008	1:0.4
6	zeaxanthin	0.39	0.013	0.046	1:3.6
7	zeaxanthin	0.14	0.07	0.014	1:2
8	zeaxanthin	0.14	0.038	0.049	1:1.3
9	zeaxanthin	0.14	0.03	0.048	1:1.6
10	zeaxanthin	0.14	0.023	0.079	1:3.5
11	lutein	2.27	0.028	0.099	1:3.5
12	lutein	2.27	0.04	0.18	1:4.8
13	lutein	2.27	0.024	0.13	1:5.3

b. Experiment 2.

Fish	Carotenoid	Carotenoid sp. acty (µCi/mg)	Sp. acty of A_1 (µCi/mg)	Sp. acty of A_2 (µCi/mg)	Ratio of sp. acts. $A_1 : A_2$
1	β-carotene	39	7.06	7.13	1:1
2	β-carotene	39	3.43	3.95	1:1.16
3	β-carotene	39	5.71	6.67	1:1.7
4	lutein	2.15	0.06	0.04	1.5:1
5	lutein	2.15	0.053	0.08	1:1.5
6	lutein	2.15	0.29	0.124	2.3:1
7	lutein	2.15	0.164	0.075	2.2:1
8	lutein	2.15	0.197	0.07	2.8:1
9	lutein	2.15	0.06	0.05	1.2:1

c. Experiment 3.

No incorporation of radioactivity was detected in retinol and dehydroretinol fractions from any of the fish.

The histograms of Fig. 29 are typical data obtained from experiment 2 clearly showing that the incorporation of radioactivity from [^{14}C]canthaxanthin coincides exactly with the relevant peaks of retinol and dehydroretinol.

B. Liver extract analysis.

The results are shown in tabulated form for experiments 1-3 (Tables 10a,b,c, respectively). Similar observations apply to this section as for Section 3.28A.

C. Intestine extracts analysis.

Tables 11a and 11b summarise the data from intestinal extracts. In the first experiment intestinal tissue was not studied. The involvement of intestinal tissue in retinol and possibly dehydroretinol formation justified its analysis in experiments 2 and 3.

D. Integument.

Low levels (1-2%) of incorporation of radioactivity were observed for all fish, in both experiments 1 and 2. Table 12 shows the amount of carotenoid extracted from each fish in experiment 1. Table 13 gives the same information for experiment 2 but also shows final specific activities of the total carotenoid. For both experiments the shapes of the absorption spectra of each extract were not as expected. Katayama et al. (1970a) obtained spectra in light petroleum of total carotenoids from goldfish showing λ_{max} at 443 and 471-2 nm. In this work the absorption spectrum for any fish tended to reflect the spectrum of the carotenoid supplemented in its diet.

Samples in experiments 1 and 2 were analysed by t.l.c., with additional analysis of experiment 2 by h.p.l.c.

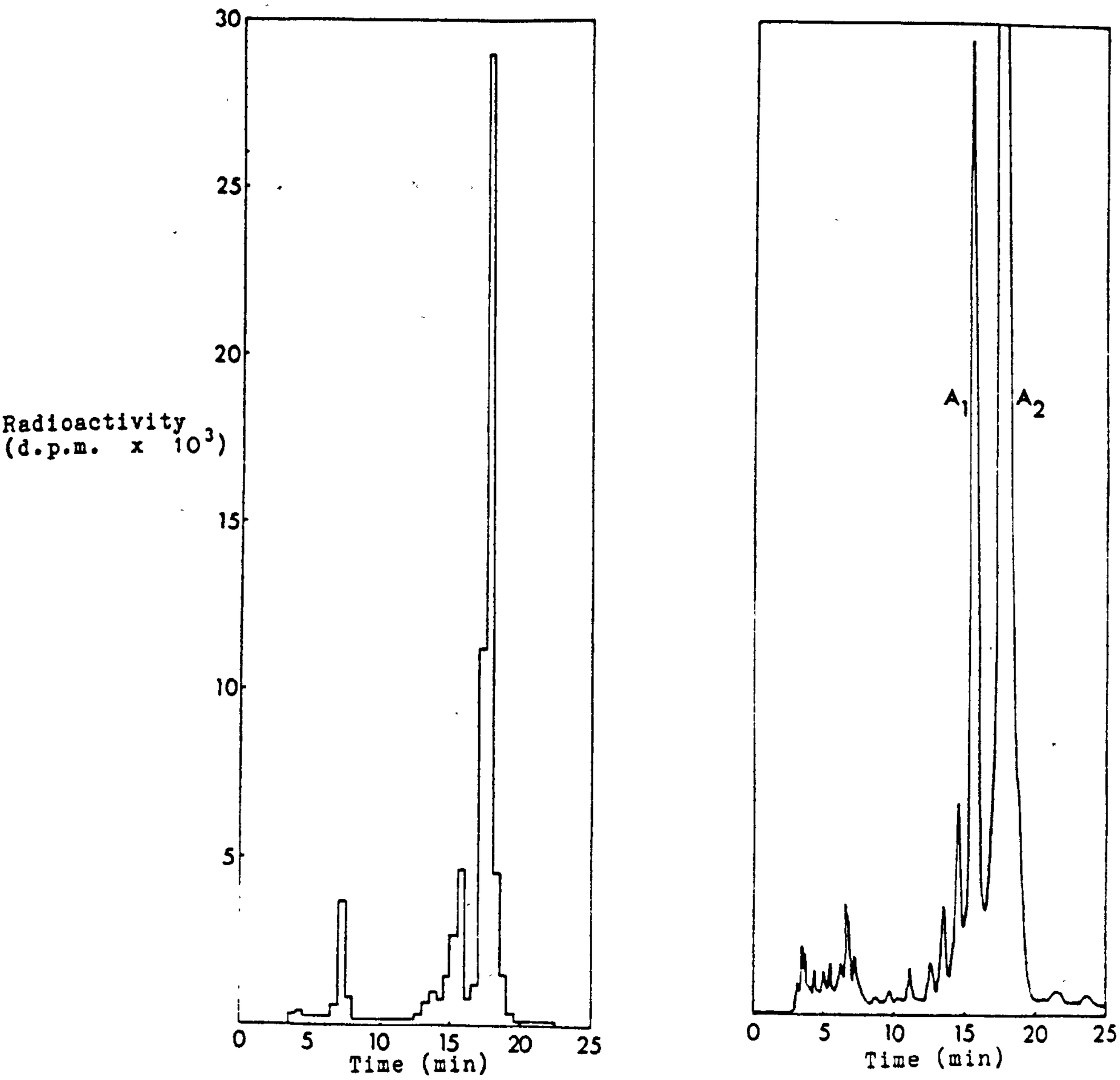


Fig. 29.

H.p.l.c. separation of retinol and dehydroretinol from eyes of goldfish treated with [^{14}C]canthaxanthin, with corresponding radioactivity profile. Column: Nucleosil-50-5, 200 x 4.5 mm; solvent: hexane/1,4-dioxan, 9:1 (v/v) at 1 ml/min; detector: u.v. at 335 nm.

TABLE 10.

Percentage incorporations of dietary ^{14}C-carotenoids and retinyl acetate into retinol and dehydroretinol and specific activities of retinol and dehydroretinol in goldfish liver after goldfish feeding experiments 1-3.

a. Experiment 1.

Fish	Carotenoid	Carotenoid sp. acty (µCi/mg)	Percentage incorporation A_1	Percentage incorporation A_2	Ratio of radioactivities $A_1 : A_2$
1	canthaxanthin	30	0.025	0.0046	1:0.2
2	canthaxanthin	30	0.33	0.39	1:1.2
3	β-carotene	0.018	0.016	0.002	1:0.13
4	β-carotene	0.018	0.019	0.0181	1:0.95
5	β-carotene	0.018	*	*	*
6	zeaxanthin	0.39	0.024	0.03	1:1.25
7	zeaxanthin	0.14	0.0057	0.006	1:1.05
8	zeaxanthin	0.14	0.024	0.031	1:1.29
9	zeaxanthin	0.14	0.019	0.029	1:1.5
10	zeaxanthin	0.14	0.0062	0.006	1:0.97
11	lutein	2.27	0.017	0.022	1:2.9
12	lutein	2.27	0.037	0.04	1:1.08
13	lutein	2.27	0.044	0.049	1:1.1

b. Experiment 2.

Fish	Carotenoid	Carotenoid sp. acty (µCi/mg)	Sp. acty of A_1 (µCi/mg)	Sp. acty of A_2 (µCi/mg)	Ratio of sp. acts. $A_1 : A_2$
1	β-carotene	39	15.15	5.54	2.7:1
2	β-carotene	39	11.28	3.3	3.4:1
3	β-carotene	39	*	*	*
4	lutein	2.15	0.93	0.15	6.2:1
5	lutein	2.15	*	*	*
6	lutein	2.15	*	*	*
7	lutein	2.15	1.15	0.41	2.8:1
8	lutein	2.15	1.36	0.57	2.4:1
9	lutein	2.15	0.7	0.15	4.7:1

c. Experiment 3.

No incorporation of radioactivity was detected in retinol and dehydroretinol fractions from any of the fish.

* - data not available

TABLE 11.

Percentage incorporations of dietary ^{14}C-carotenoids and retinyl acetate into retinol and dehydroretinol and specific activities of retinol and dehydroretinol in goldfish intestine after goldfish feeding experiments 1-3.

a. Experiment 2.

Fish	Carotenoid	Carotenoid sp. acty (μCi/mg)	Sp. acty of A_1 (μCi/mg)	Sp.acty of A_2 (μCi/mg)	Ratio of sp. acts. $A_1:A_2$
1	β-carotene	39	11.76	5.4	2.1:1
2	β-carotene	39	8.34	2.35	3.5:1
3	β-carotene	39	19.93	10.58	1.8:1
4	lutein	2.15	0.28	0.12	2.3:1
5	lutein	2.15	1.08	0.44	2.4:1
6	lutein	2.15	*	*	*
7	lutein	2.15	0.74	0.24	3.1:1
8	lutein	2.15	1.41	0.496	2.8:1
9	lutein	2.15	0.6	0.14	4.3:1

b. Experiment 3.

No incorporation of radioactivity was detected in retinol and dehydroretinol fractions from any of the fish.

* - data not available

TABLE 12.

Absorption maxima and quantitative data for integument extracts of fish from experiment 1.

Fish	Absorption maxima in ethanol (nm)					µg carotenoid
1	374	397	422	441	472	13.1
2		400	425	446	464	32.0
3				449	465	59.5
4			(425)	449	472	50.5
5			422	445	470	40.5
6				448	470	115.0
7			422	445	472	22.0
8				447	471	109.5
9				448	470	134.0
10				454	471	98.5
11				446	471	83.2
12				447	472	91.7
13				446	472	103.4

TABLE 13.

Table showing the amount of carotenoid extracted, and its specific activity, from the integuments of each fish in experiment 2.

Fish	Absorption maxima in ethanol nm		µg carotenoid	total d.p.m.	µCi	µCi/mg
1		474	69.2	83 750	0.038	0.549
2	454.5	474	36.8	94 000	0.0427	1.16
3	454.5	473	72.8	45 000	0.02	0.275
4		475	156.3	45 000	0.02	0.128
5	453	474.5	38.4	72 500	0.033	0.86
6	456.5	475.5	81.8	23 000	0.01	0.122
7		474	39.1	20 250	0.0092	0.235
8		475	134.9	33 750	0.015	0.111
9		474.5	101.0	35 125	0.016	0.158

i. T.l.c. analyses.

Difficulties were encountered with the t.l.c. in some cases, notably with the F1 samples, due to sterol interference. Even after numerous sterol precipitations and digitonin reactions considerable chromatographic problems remained. Unsatisfactory classical l.c. of carotenoid esters was revealed by the t.l.c. stage, in that polar carotenoids (e.g. astacene) were present in F1 as well as F2a and F2b fractions. Consequently, as this was unexpected, astacene standard was not run on the plates with F1 samples.

All 2a and 2b fractions were 'spiked' with 'cold' astacene, α- and β-doradecin and canthaxanthin as appropriate. This proved to be a vital precautionary step in the analysis.

As a direct consequence of sterol interference the t.l.c. was not as straightforward as had been anticipated but nonetheless yielded information about the metabolism of carotenoids by goldfish.

Typical radioautograms are shown in Figures 30-34. Relevant quantitative data for these radioautograms are shown in Tables 14-18.

[^{14}C]Canthaxanthin fed fish.

There is good evidence that canthaxanthin is metabolised to astaxanthin (astacene) as seen in Fig. 30 and Table 14. There is also significant incorporation into a very polar carotenoid near the origin, which may be an intermediate between canthaxanthin and astaxanthin. The most interesting aspect of the [^{14}C]canthaxanthin experiment is that t.l.c. of the non-polar carotenoid fraction (i.e. F1) revealed the presence of radiolabelled metabolites of non-polar nature (Fig. 31 and Table 15). There are 4 bands that show incorporation, one of which is

Fig. 30. Radioautogram of t.l.c. of integument carotenoids from goldfish fed [^{14}C]canthaxanthin.

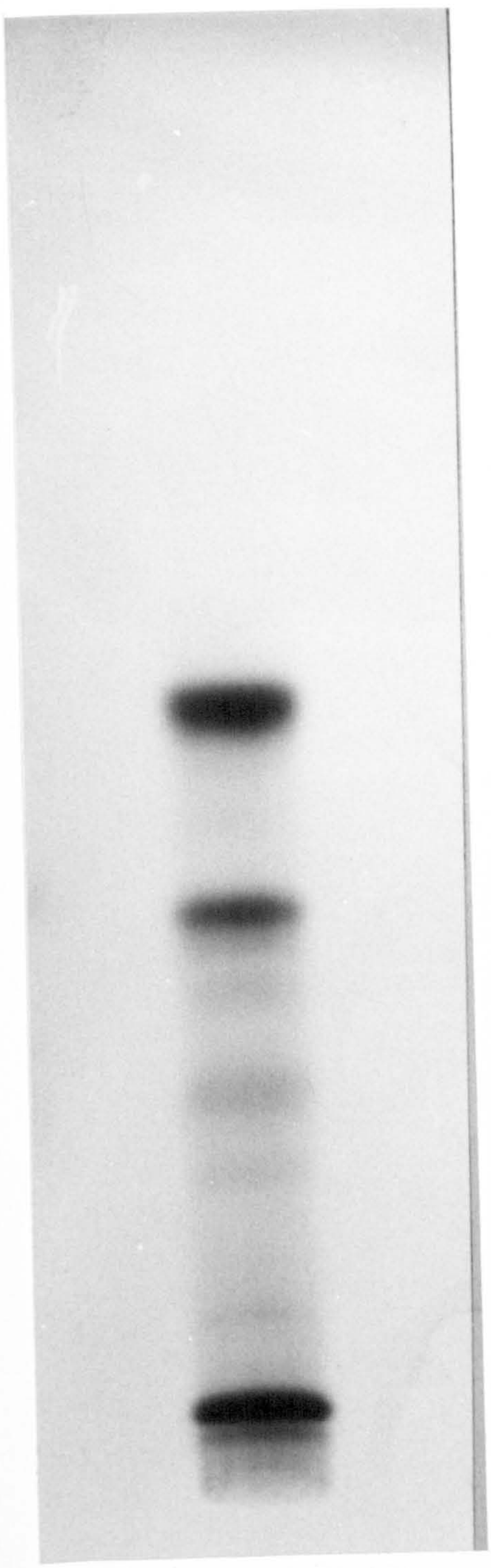


TABLE 14.

Radioactivity of zones, as labelled, from t.l.c. of integument carotenoids from goldfish fed [^{14}C]canthaxanthin.

		d.p.m. per zone
I	canthaxanthin	1613
II	astacene	995
III	unidentified	148
IV	unidentified	190
V	unidentified	115
VI	unidentified	1911

Fig. 31. Radioautogram of t.l.c. of integument carotenoids from a goldfish fed [^{14}C]canthaxanthin.

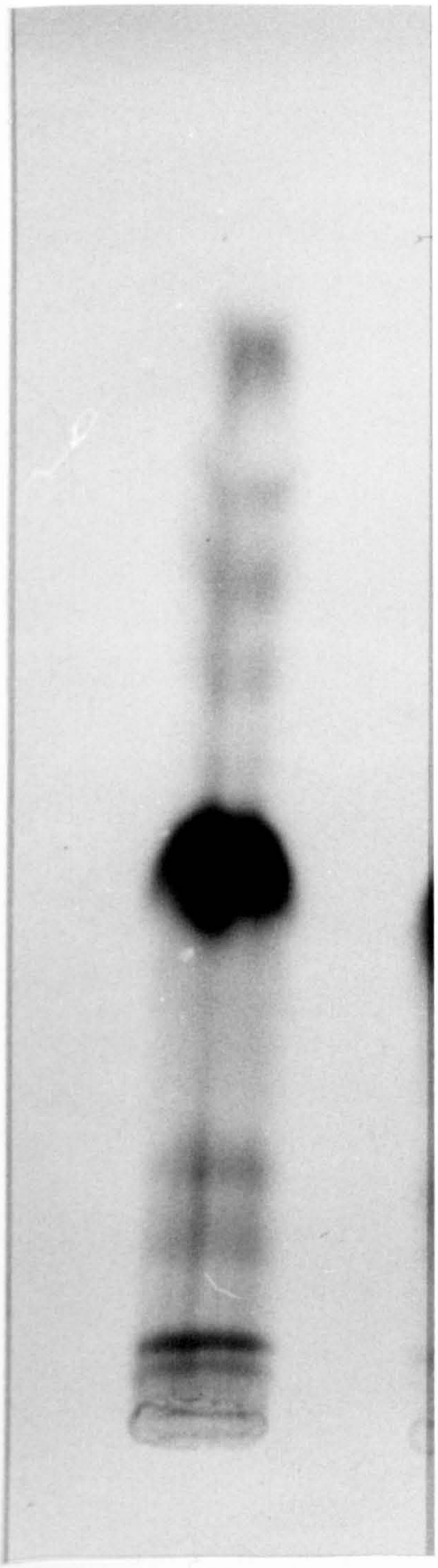


Table 15.

Radioactivity of zones, as labelled, from t.l.c. of integument carotenoids from a goldfish fed [^{14}C]canthaxanthin.

		d.p.m. per zone
I	β-carotene	288
II	unidentified	236
III	unidentified	208
IV	unidentified	192
V	unidentified	115
VI	canthaxanthin	7500
VII	astacene?	305
VIII	unidentified	172
IX	unidentified	318
X	unidentified	321
XI	unidentified	283
XII	unidentified	197

undoubtedly β-carotene. This is direct evidence for another 'back' reaction of carotenoid metabolism. Contrary to expectations there is also a carotenoid present in this fraction that has an R_f corresponding closely to astacene. As astacene was not expected to be present there was no astacene standard on the plate and so the identity of this heavily labelled band is not certain.

[^{14}C]Zeaxanthin fed fish.

A typical radioautogram of integument carotenoids from a goldfish fed [^{14}C]zeaxanthin is shown in Fig. 32. Incorporation into many carotenoid bands was readily demonstrated and the radioactivity from relevant carotenoid zones is summarised in Table 16. Incorporation occurs into a carotenoid which has an R_f value comparable to that of β-doradecin. There is also evidence for incorporation of radioactivity into both phoenicoxanthin and astacene. These are faint zones on the radioautogram, but as phoenicoxanthin is the expected intermediate between zeaxanthin and astaxanthin it seems likely that these zones are significant.

[^{14}C]β-carotene fed fish.

There are many zones showing incorporation (Fig. 33, Table 17), again suggesting that metabolism in goldfish is far more complex than initially anticipated. The large number of bands tends to indicate that the enzymes involved in carotenoid metabolism are of broad specificity, many substrates being used to give rise to a large number of intermediates. From previous work (Hata and Hata, 1972, 1973) echinenone, canthaxanthin and phoenicoxanthin would be anticipated as intermediates. A band corresponding to phoenicoxanthin shows incorporation as does a band with the same R_f as β-doradecin. There is substantial incorporation into a band corresponding to a fairly polar carotenoid which is presumably a late intermediate along the metabolic pathway. This carotenoid was not identified. There may be a very faint band corresponding to astacene but it is difficult to establish if this

Fig. 32. Radioautogram of t.l.c. of integument carotenoids from a goldfish fed [^{14}C]zeaxanthin.

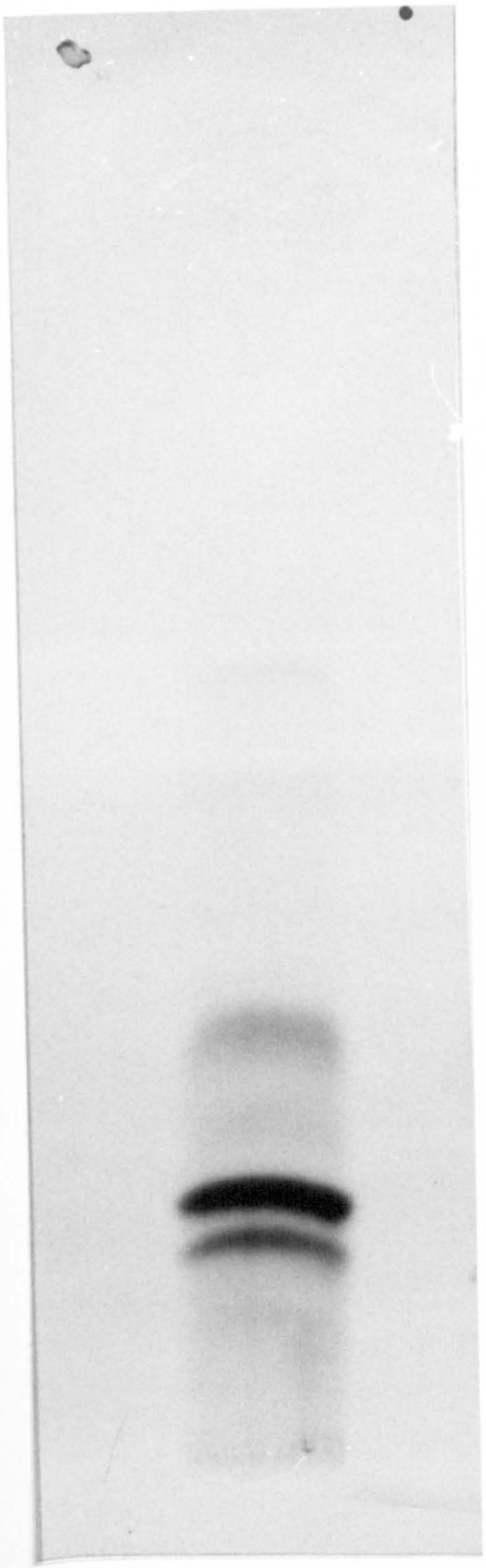


Table 16.

Radioactivity of zones, as labelled, from t.l.c. of integument carotenoids from a goldfish fed [^{14}C]zeaxanthin.

		d.p.m. per zone
I	phoenicoxanthin	30
II	astacene	61
III	β-doradecin	259
IV	unidentified	297
V	zeaxanthin	1581
VI	unidentified	558
VII	unidentified	209
VIII	unidentified	297

Fig. 33. Radioautogram of t.l.c. of integument carotenoids from a goldfish fed [^{14}C] β-carotene.

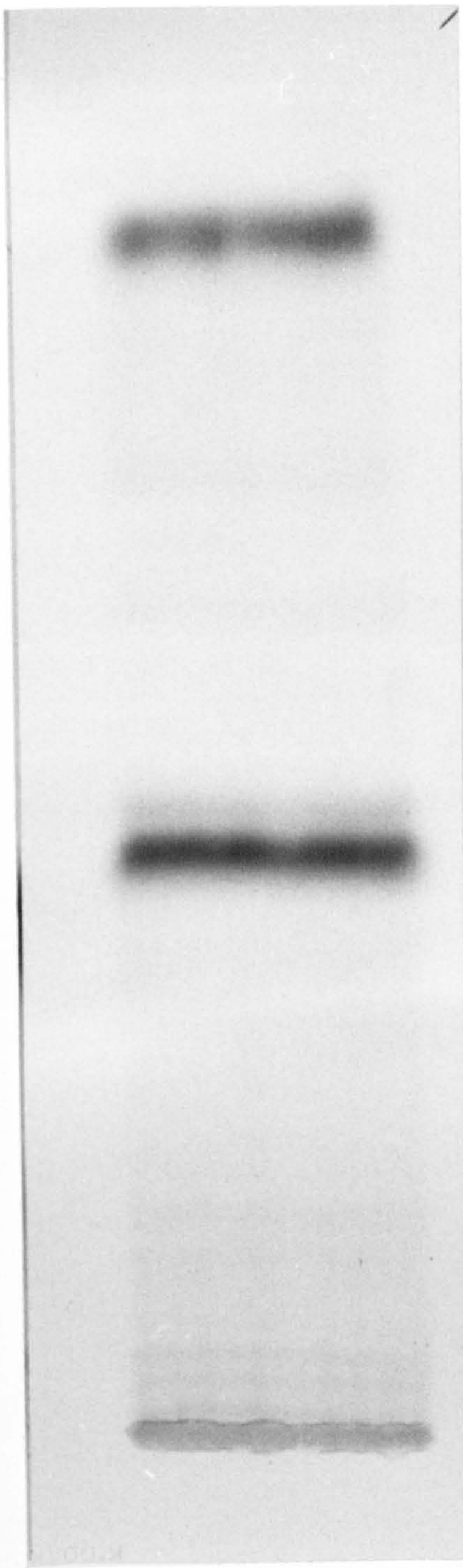


Table 17.

Radioactivity of zones, as labelled, from t.l.c. of integument carotenoids from a goldfish fed [^{14}C] β-carotene.

		d.p.m. per zone.
I	β-carotene	864
II	unidentified	175
III	unidentified	153
IV	phoenicoxanthin	125
V	astacene	144
VI	unidentified	1172
VII	β-doradecin	747
VIII	unidentified	119
IX	unidentified	341

is significant. The low levels of radioactivity in this band can be attributed to the multi-steps of the metabolic pathway. H.p.l.c. analysis of [^{14}C]β-carotene fed fish extracts yielded more information about the metabolism of β-carotene in goldfish.

[^{14}C]lutein fed fish.

The t.l.c. plates, and consequently the radioautograms, were difficult to analyse due to sterol contamination. However , there is certainly incorporation into the region corresponding to α- and β-doradecin (Fig. 34, Table 18). The zones were large diffuse areas which makes it difficult to assess whether or not incorporation occurs into both doradecins; an important distinction needs to be made between the two forms. Above this large area is a faint band which may result from incorporation of radioactivity into astacene. Again h.p.l.c. analysis was carried out, the results of which are shown in the following section.

ii. H.p.l.c. analyses.

H.p.l.c. served to emphasise the complexity of goldfish carotenoid metabolism. It also illustrated that t.l.c. was perhaps an inadequate method of separation of a complex mixture of carotenoids.

[^{14}C]lutein fed fish.

a) Neutral carotenoids.

A typical h.p.l.c. elution profile and corresponding histogram of radioactivity in 1 min fraction is shown in Fig. 35. As anticipated there is a relatively high proportion of dihydroxycarotenoids present. Generally levels of incorporation were low with evidence of incorporation into unidentified carotenoids of both more and less polar nature than the

Fig. 34. Radioautogram of t.l.c. of integument carotenoids from a goldfish fed [^{14}C]lutein.

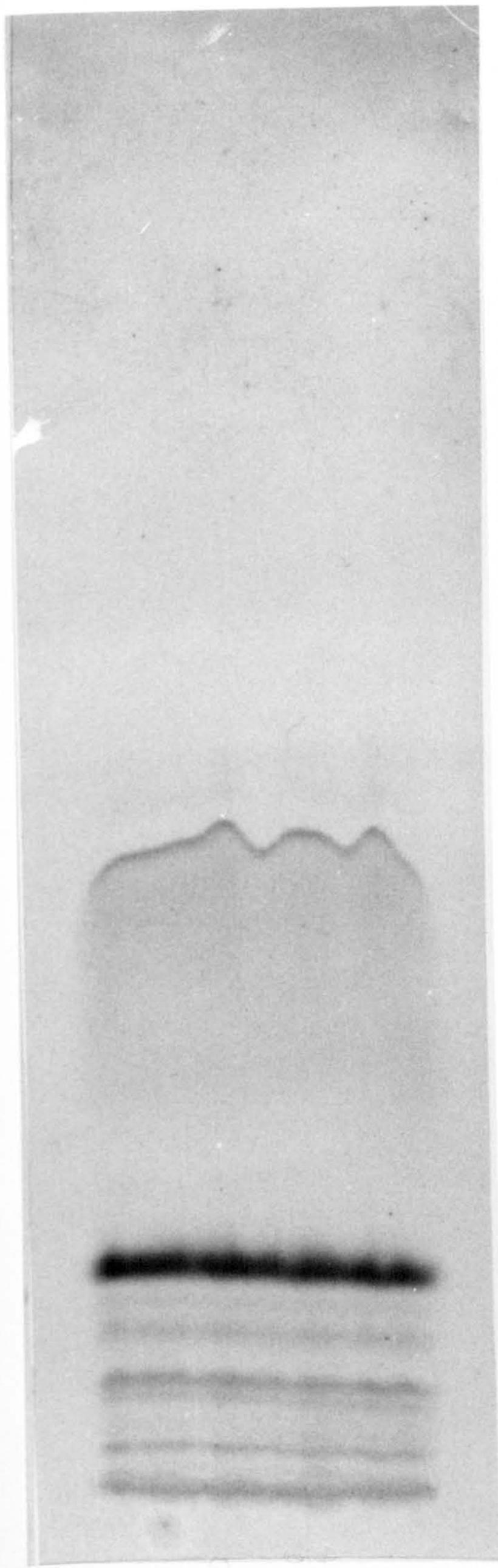


Table 18.

Radioactivity of zones, as labelled, from t.l.c. of integument carotenoids from a goldfish fed [^{14}C]lutein.

		d.p.m. per zone
I	unidentified	63
II	astacene	122
III	α- and β-doradecin	418
IV		675
V	lutein	850
VI	unidentified	706
VII	unidentified	339
VIII	unidentified	259
IX	unidentified	540

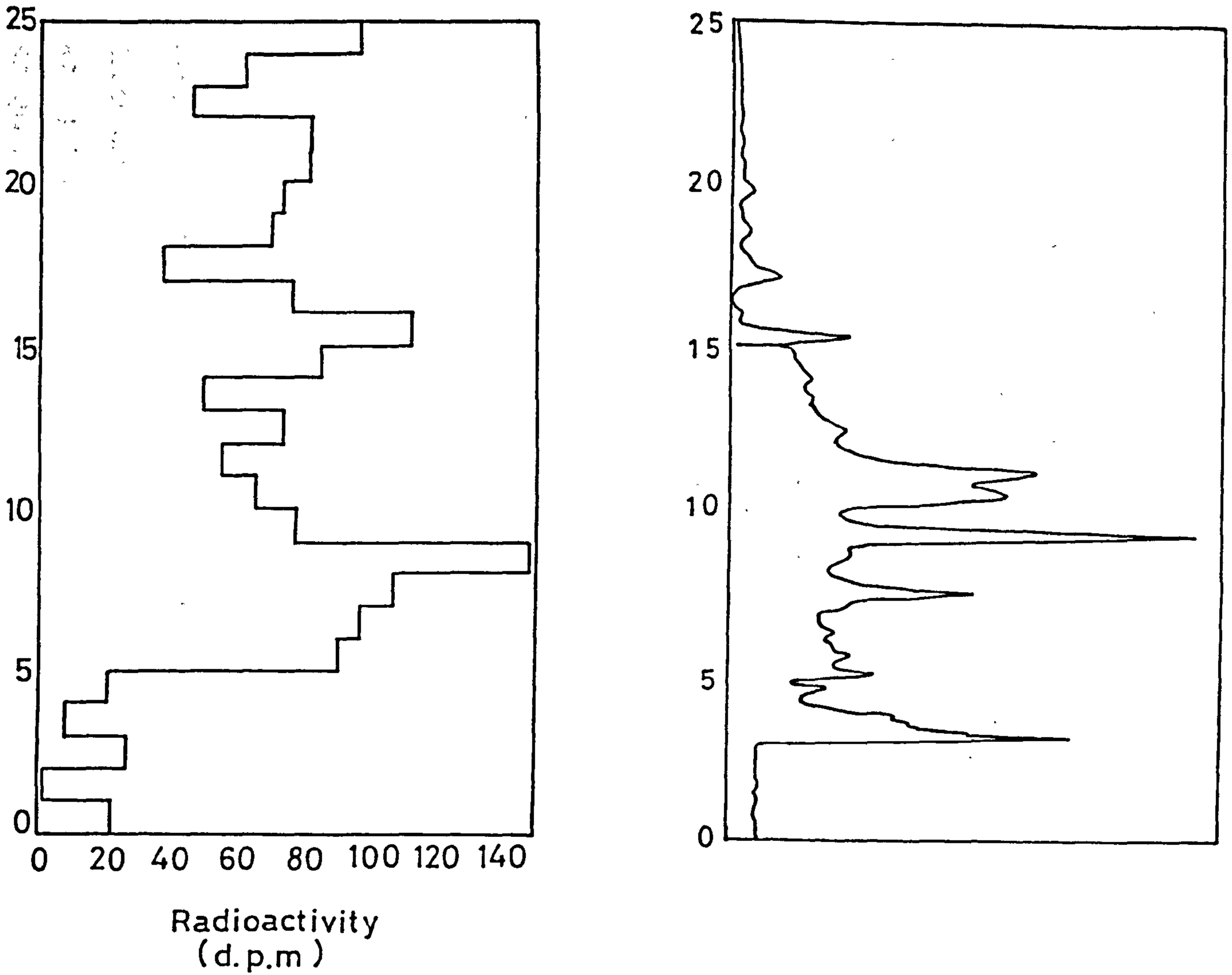


Fig. 35.

H.p.l.c. elution profile and corresponding radioactivity elution profile of neutral carotenoids from integument of goldfish fed [^{14}C]lutein. Column: Nucleosil-50-5, 250 x 4.5 mm; solvent: hexane/CH_2Cl_2/propan-2-ol (87:5:8 v/v) at 1 ml/min.

lutein itself.

b) Acidic carotenoids.

A typical h.p.l.c. elution profile is shown in Fig. 36. Also shown is a histogram of radioactivity incorporated into fractions 1-35. Fractions 1-10 correspond to 30 s fractions from time 0 to 5 min, the remaining fractions being 1 min fractions. Spectra of fractions 7 and 8 were comparable to that expected for astacene (λ_{max} in light petroleum at 473.5 nm). On average there was 1-2 µg of astacene present. Fraction 25 (retention time 20 min) was measured spectrophotometrically and λ_{max} were observed at 455 and 468 nm in light petroleum. As can be seen in the histogram low levels of radioactivity were present in fraction 7 suggesting that lutein is indeed metabolised to astacene in the goldfish.

[^{14}C]β-carotene fed fish.

a) Neutral carotenoids.

A typical h.p.l.c. elution profile with histogram of radioactivity in 1 min fractions is shown in Fig. 37. The second event marker indicates increased flow rate to 1.8 ml/min. As expected the major carotenoid present is non-polar β-carotene, which shows high levels of incorporation (3379 d.p.m.). A few other slightly more polar carotenoids are present, however, these do not display any significant incorporation except for fraction 11.

b) Acidic carotenoids.

Low levels of incorporation were observed again. However, the results tend to indicate incorporation into the astacene fraction and several others, especially fractions 15 and 19 (retention times 10 min

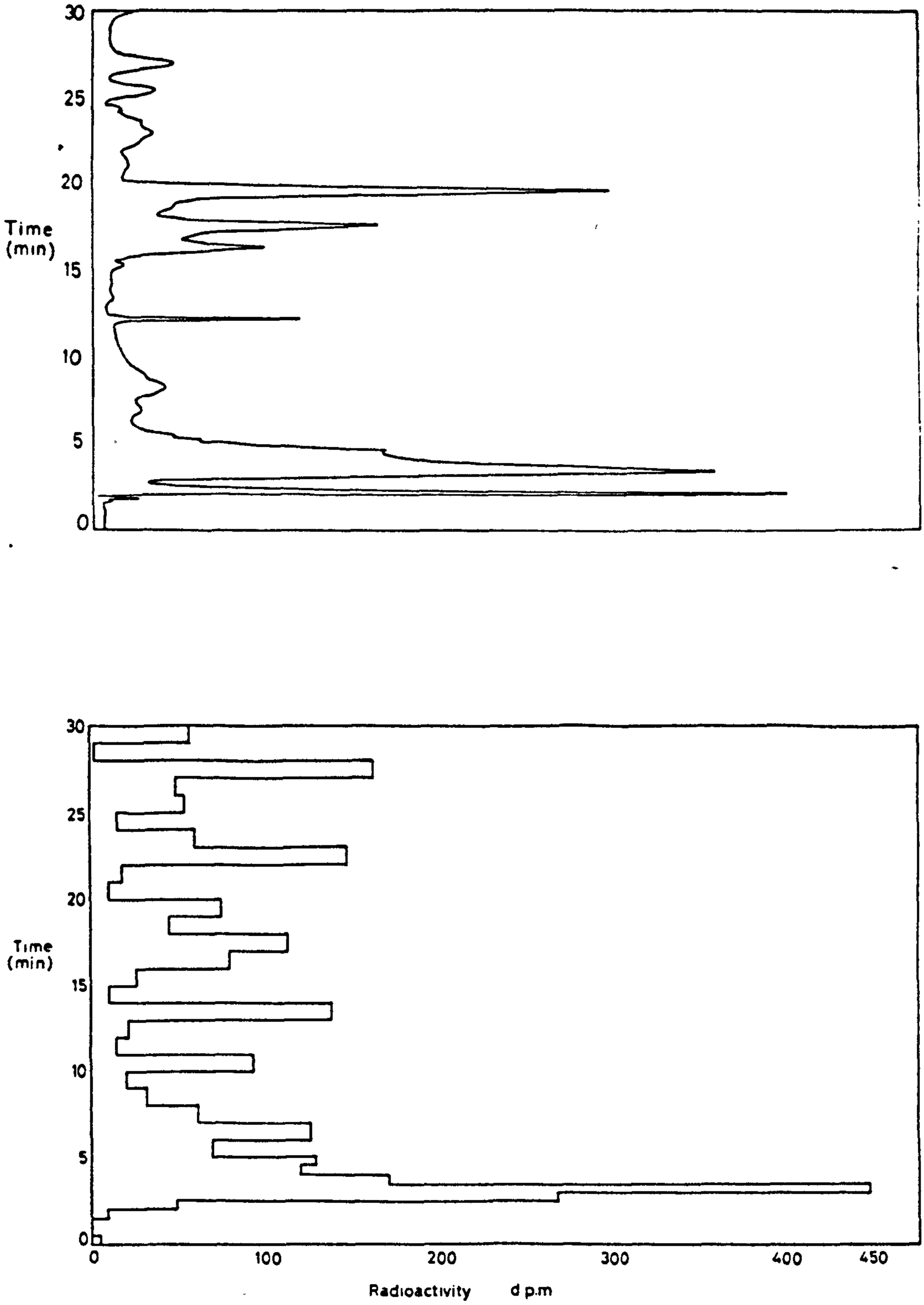


Fig. 36.

H.p.l.c. elution profile and corresponding radioactivity elution profile of acidic carotenoids from integument of goldfish fed [^{14}C]lutein. Column: Nucleosil/H_3PO_4, 250 x 4.5 mm; solvent: hexane–10% CH_2Cl_2, 0.75% propan-2-ol at 1.8 ml/min.

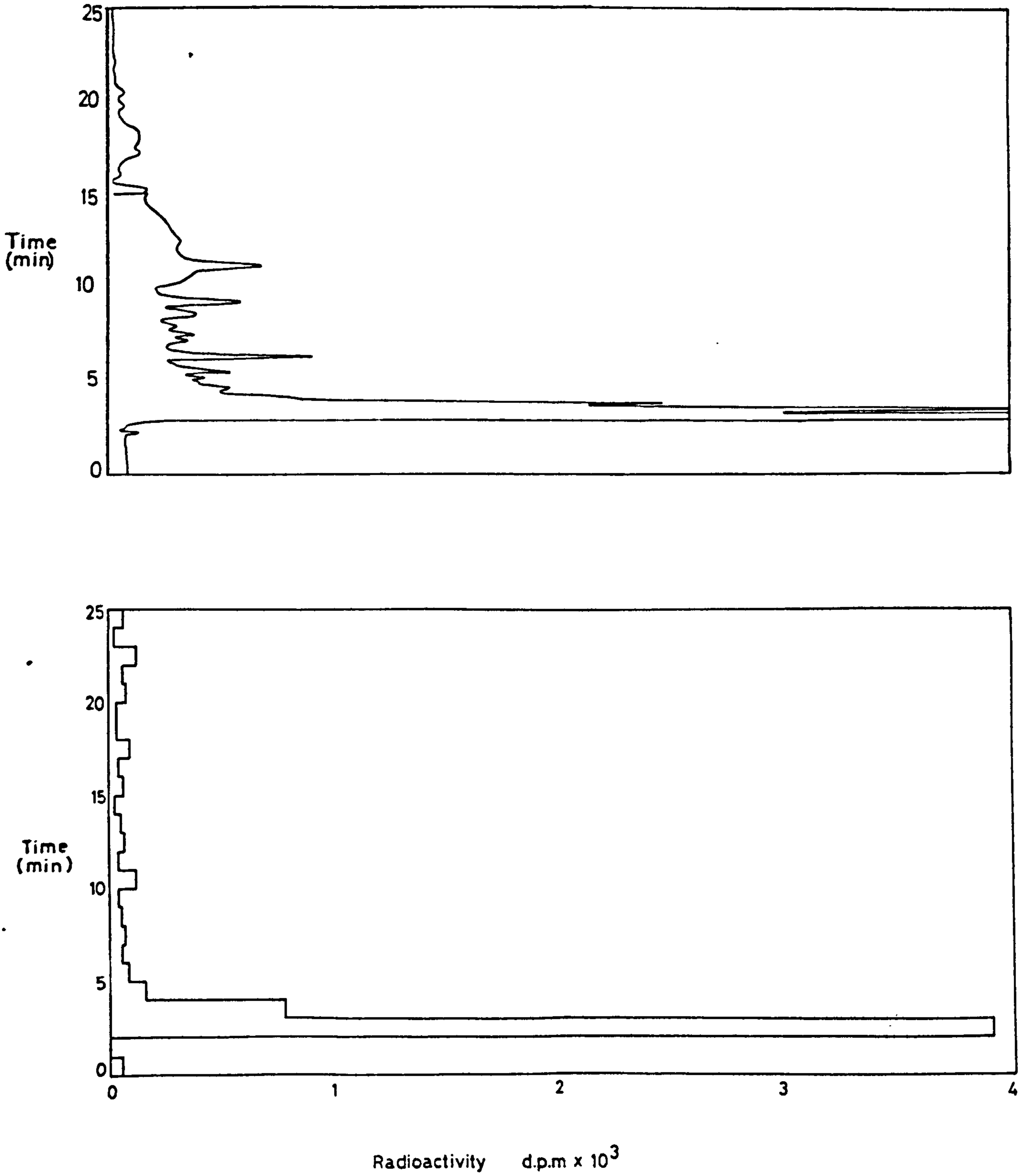


Fig. 37.

H.p.l.c. elution profile and corresponding radioactivity elution profile of neutral carotenoids from integument of goldfish fed [^{14}C]β-carotene. Column: Nucleosil-50-5, 250 x 4.5 mm; solvent: hexane/CH_2Cl_2/propan-2-ol (87:5:8 v/v) at 1 ml/min.

and 14 min respectively. Figure 38 shows a typical h.p.l.c. elution profile with histogram of radioactive incorporations.

E. Faeces.

Faeces were collected from the tank water of fish used in experiment 1. Table 19 summarises the percentage of the total carotenoid administered appearing in the faeces. The values vary greatly, from 1.2 to 28% but are consistently lower than expected. The absorption spectra of each faeces extract was characteristic of the [^{14}C]carotenoid. The value of this data is questionable and hence this particular aspect of the work was not pursued in subsequent experiments. This type of data may have been very revealing in experiment 3.

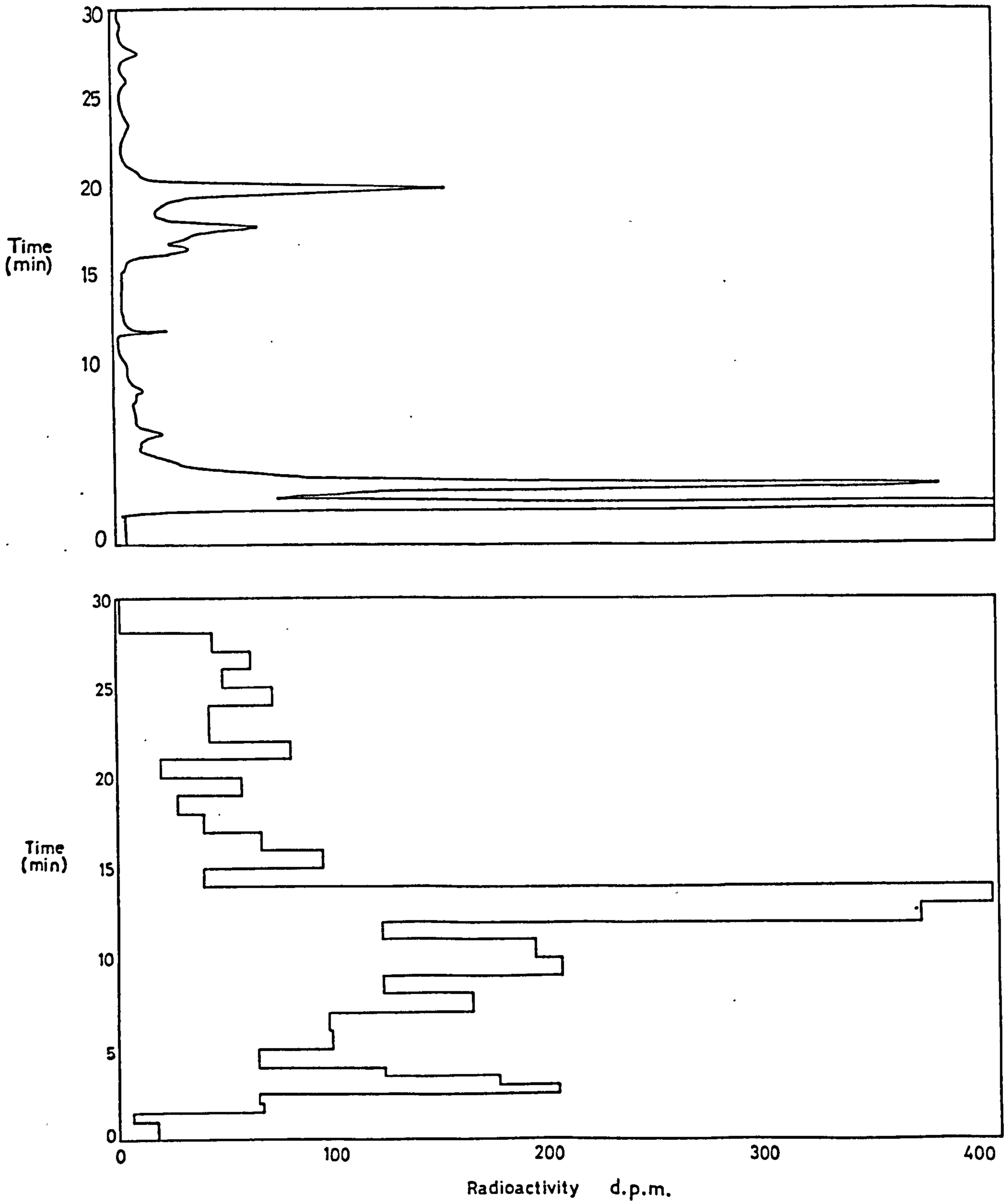


Fig. 38.

H.p.l.c. elution profile and corresponding radioactivity elution profile of acidic carotenoids from integument of goldfish fed [^{14}C]β-carotene. Column: Nucleosil/H_3PO_4, 250 mm x 4.5 mm; solvent: hexane-10% CH_2Cl_2, 0.75% propan-2-ol at 1.8 ml/min.

TABLE 19.

Estimation of [^{14}C]carotenoid in faeces from each fish in experiment 1.

Fish	Administered carotenoid	µCi administered	µCi recovered	Percentage of total in faeces
1	canthaxanthin	4.08	0.123	3.2
2	canthaxanthin	1.46	0.018	1.2
3	β-carotene	0.106	0.016	1.5
4	β-carotene	0.106	0.16	15
5	β-carotene	0.106	0.12	11.3
6	zeaxanthin	0.273	0.023	8.5
7	zeaxanthin	0.28	0.067	24
8	zeaxanthin	0.28	0.079	28
9	zeaxanthin	0.28	0.012	4.3
10	zeaxanthin	0.28	0.067	24
11	lutein	0.5	0.12	24
12	lutein	0.5	0.08	16
13	lutein	0.5	0.098	21

DISCUSSION

The results of this work will be discussed in light of the results and experimental approaches of past workers. In particular the retinol and dehydroretinol results will be compared with results recently obtained by the F. Hoffmann-La Roche and Co. Ltd., research group (Schiedt et al., 1985).

Although goldfish feeding experiments have been carried out by many different workers over the last 20 years, there can be no doubt that experimental protocol has varied greatly. The most obvious difference in approach is with respect to radiolabelled carotenoids. In some work quantitative changes in carotenoid levels have been taken as an indication of metabolism of a carotenoid supplemented in the diet. More recently, with the advent of a greater availability of radiolabelled carotenoids or systems capable of producing labelled carotenoids, experiments have tended to involve more satisfactory methods for the detection of carotenoid metabolites. Even though labelled carotenoids are more readily available, the low specific activities remain a problem. [^{14}C]Canthaxanthin supplied by Roche is of very high specific activity, but others produced by biological systems e.g. [^{14}C]zeaxanthin from Flavobacterium or [^{14}C]lutein from barley have relatively low specific activities. In animal studies of this nature low specific activities are a great disadvantage. The efficiency of absorption of carotenoids across the intestinal wall in animals may be as low as 1%. Optimisation of the efficiency of absorption of vitamins and provitamins is critical and many methods have been exploited to assist in absorption. These methods are discussed in detail by Bauernfeind et al. (1981). Factors affecting the absorption of carotenoids are the presence of emulsifying agents, fats, lecithin, protein and vitamin E. Vitamin E in moderate doses increases the efficiency of absorption of carotenoids by protecting them from oxidation in the intestinal tract. The advantages of oral administration as opposed to parenteral administration are also discussed.

The numerous goldfish feeding experiments recorded in the literature have varied with respect to these factors in addition to the methods of analysis of vitamins A and carotenoids.

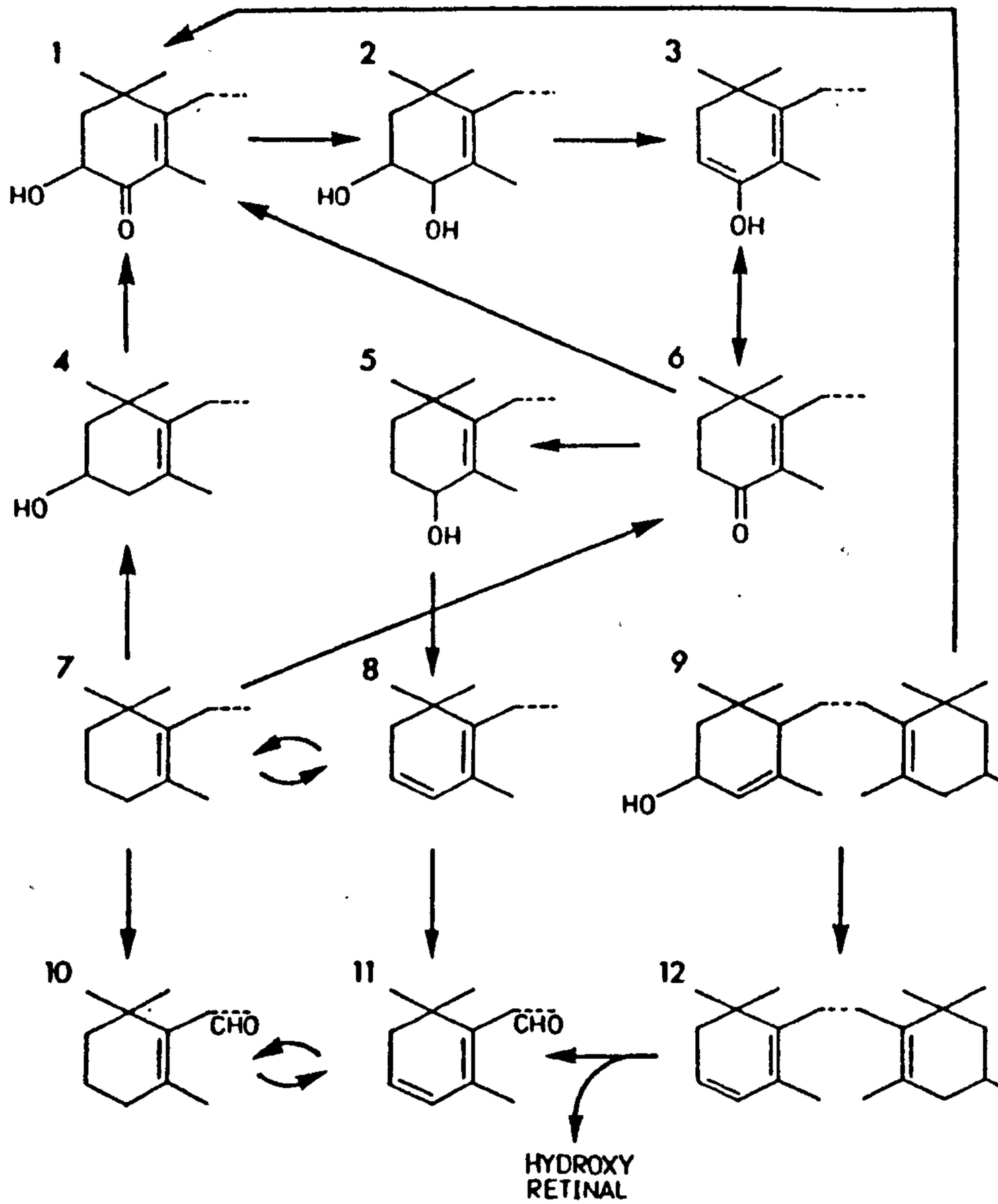
Retinol and dehydroretinol analysis.

Barua et al. (1973) and Barua and Das (1975) give a poor description of their purification methods. It would appear that the only purification stage is the chromatography of the total liver oil on deactivated alumina, a system whereby the separation of retinol and dehydroretinol would be incomplete and inadequate. Retinol and dehydroretinol were estimated spectrophotometrically using values of specific absorption coefficients of 1832 at 325 nm and 1455 at 350 nm respectively (Planta et al., 1962). No correction was made for irrelevant absorption, an important omission considering the purification. The methods of Gross and Budowski (1966) were somewhat more sophisticated. Chromatography of the total liver lipid on two successive deactivated columns of alumina resulted in a pure vitamin A fraction. T.l.c. of vitamin A was carried out on magnesia with light petroleum-acetone (17:3, v/v) as developing solvent. Pure retinol and dehydroretinol were estimated by the method of Carr and Price (1926). The amounts of retinol and dehydroretinol were calculated by comparison with pure reference samples following the principles of Plack and Kon (1961). The Carr-Price method is not very accurate, particularly when the amounts of vitamins A involved are small and when evidence for metabolism is based on quantitative data this type of inaccuracy may be quite significant. With labelling techniques the quantitative aspect is not as important and the most pressing requirement is the purity of retinol and dehydroretinol before liquid scintillation counting. The h.p.l.c. method used in this work satisfies this demand, and co-chromatography with standard retinol and dehydroretinol, spectral data and m.s. are very strong evidence for the purity of retinol and

dehydroretinol.

This presentation in conjunction with the work of Schiedt et al. (1985) together abolish the long held dogma that xanthophylls cannot be precursors of the vitamins A. The two independent studies of dehydroretinol allow the same conclusions to be drawn about dehydroretinol formation and confirm that in fish some 'back reactions' occur. 'Back reactions' here are those that are not the conventional oxidative reactions of carotenoids. If the carotenoid literature of the last 40 years is studied, a few isolated papers will be found reporting that xanthophylls can act as provitamins A in fish. The reactions which may be involved in retinol and dehydroretinol formation were summarised by Olson (1983) as depicted in Fig. 39. Morton (1940) suggested that, in fish, high levels of vitamins A might be maintained by astaxanthin in spite of its structure. Grangaud et al. (1956) reported the formation of retinol and dehydroretinol in depleted Gambusia holbrooki from astaxanthin. Gross and Budowski (1966) demonstrated the conversion of a number of xanthophylls to retinol and dehydroretinol in two species of freshwater fish. Barua et al. (1973) reported the conversion of lutein to dehydroretinol by Sacchobranchus fossilis with anhydrolutein as a possible intermediate (Barua and Das, 1975).

Excluding hypothetical pathways that involve the reduction of xanthophylls to β-carotene which is then cleaved to retinol (and thence to dehydroretinol), there are few reductive pathways in the literature. The elimination of a 4-oxo group from astaxanthin in rainbow trout has been confirmed by Schiedt et al. (1985). Feeding of non-radioactive canthaxanthin resulted in the appearance of echinenone and β-carotene, and feeding of adonirubin produced asteroidenone and cryptoxanthin. The 3-hydroxyl group from astaxanthin was not eliminated as shown in Fig. 40. In Atlantic salmon the feeding of (3R,3'R)astaxanthin resulted in increased amounts of (3S,3'S)zeaxanthin and meso-zeaxanthin, again a reductive process (Foss et al., 1984a,b). Kitahara (1983) reported a



KEY TO CAROTENOIDS:

1 Astaxanthin
2 Crustaxanthin
3 Canthaxanthin (enolic form)
4 Zeaxanthin
5 Isozeaxanthin
6 Canthaxanthin
7 β-Carotene
8 3,4,3',4'-Tetradehydro-β-carotene
9 Lutein
10 Retinal
11 3,4-Dehydroretinal
12 Anhydrolutein

Fig. 39.

Scheme of Olson (1983) to account for some of the carotenoid transformations reported to occur in fish. Only half molecules are represented.

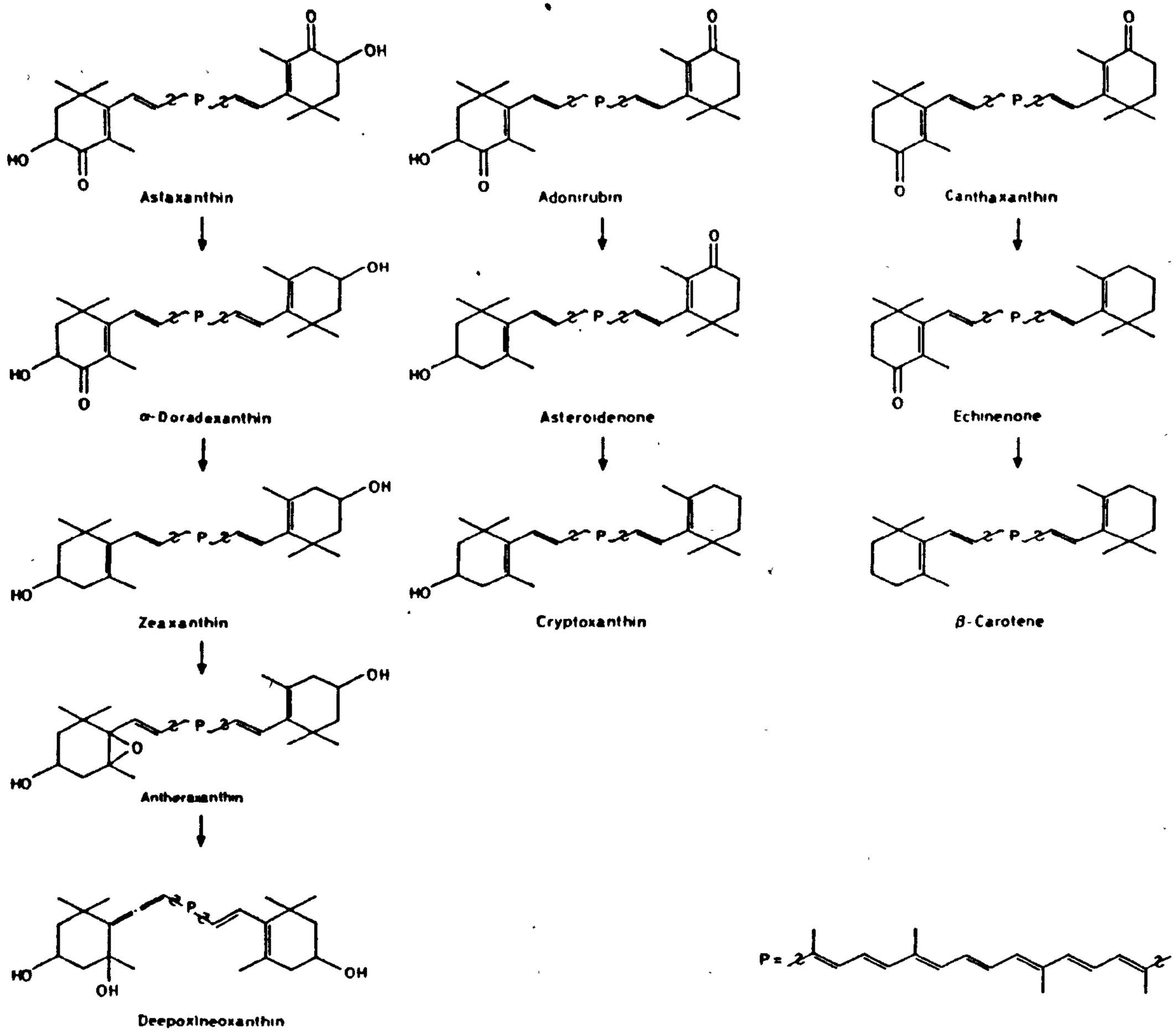


Fig. 40.

Metabolic pathways illustrating the elimination of the 4-oxo groups of astaxanthin, adonirubin (phoenicoxanthin) and canthaxanthin in rainbow trout.

reductive pathway in Oncorhynchus keta which involved astaxanthin reduction to idoxanthin and crustaxanthin. Fujita et al. (1983a,b) reported that astaxanthin diester from Antarctic krill meal was deposited in the integuments of red Sea Bream, and part of the astaxanthin was converted to tunaxanthin via β-carotene-triol, zeaxanthin and 3'-epilutein which necessitated reductive processes. Assuming that reductive reactions of carotenoids are possible, the initial results of Gross and Budowski (1966) and Schiedt et al. (1985) and this work become more acceptable.

The results of Schiedt et al. (1985) in principle are the same as the results in this report. They found a ratio of retinol to dehydroretinol of 1:4 in the intestines and livers of their experimental fish (i.e. rainbow trout), a comparable ratio to that found in goldfish eyes. The feeding experiment itself was over a longer period, the feeding time being 4-7 weeks, the pigment having been added to the basic feed as water dispersible beadlets. The water dispersible tritiated carotenoid preparations were administered in gelatine capsules 3x a week under slight anaethesia. As in this work, the fish were sacrificed 2 days after the last application. The details of the h.p.l.c. separation of retinol and dehydroretinol are not given. Schiedt et al. (1985) confirm that zeaxanthin and canthaxanthin are precursors of retinol and dehydroretinol and suggest that astaxanthin is also capable of forming the vitamins A. They found that the degree of incorporation of radioactivity into retinol and dehydroretinol was dependent on the size, age and vitamin A status of the fish. As retinol showed higher specific activities than dehydroretinol they concluded that retinol was the immediate precursor of dehydroretinol. This hypothesis was tested by the administration of retinyl-6,7-$^{14}C_2$ acetate in a feeding experiment. Incorporation did occur into the dehydroretinol which is in agreement with Hata et al. (1973), Lambersten and Braekken (1969) and Gross and Budowski (1966). This assumes that there is dehydrogenation of retinol to dehydroretinol. This could not be substantiated in this presentation

as there was no indication of absorption of the retinyl acetate administered. Other evidence put forward by Schiedt et al. (1985) against the postulation of Barua et al. (1973) and Barua and Das (1975) is that they could not detect any anhydrolutein or 3-hydroxyretinol (expected products of lutein cleavage) in the liver of trout. Schiedt et al. (1985) did not detect any β-carotene in the skin of their experimental animals and attribute this absence to the immediate conversion of β-carotene to retinol and dehydroretinol. Similarly anhydrolutein and 3-hydroxyretinol may be immediately transferred to the vitamins A.

Long before the structure of dehydroretinol was fully elucidated Morton and Creed (1939) observed that administration of β-carotene resulted in an increase of both retinol and dehydroretinol in some freshwater fish. Since the first observation of Morton et al. (1947), Naito and Wilt (1962) and Hata et al. (1973) reported that retinol could be converted to dehydroretinol in red hake (Urophycis chuss), sunfishes (Lapomis spp.) and raigyo (Channa argus) respectively. Contrary to this is the work of Morcos and Salah (1951), who failed to demonstrate this conversion in two species from the Nile, Clarius lazera and Tilapia nilotica. The inability of rats to perform the reverse reaction i.e. the hydrogenation of dehydroretinol to retinol, was demonstrated by Shantz et al. (1946). It was seen that the rat was able to use the dehydroretinol foreign to its own species when supplied in its diet, but was unable to convert this form to its own.

These references emphasise the conflicting views of research workers as to the mechanism of formation of dehydroretinol in fish. Schiedt et al. (1985) on the basis of their results and the observations of Morton et al. (1947) and other research groups, support the theory that xanthophylls are precursors of retinol directly, but are only indirect precursors of dehydroretinol i.e. they assume that freshwater fish interconvert retinol and dehydroretinol. From the retinyl acetate

feeding experiments there can be no doubt that retinol can be utilised for the formation of dehydroretinol when the fish is under stressful conditions of vitamin A depletion, but is this the natural precursor? It is feasible that lutein is the active precursor under normal conditions. Lutein was considered feasible as a precursor of dehydroretinol after it was shown to be present in the livers of freshwater fish by Balasundaram et al. (1958). Thus Barua et al. (1973) started feeding experiments and successfully showed that lutein was converted to dehydroretinol and retinol. Del Tito (1983) only showed conversion of lutein to dehydroretinol in goldfish, and Czeczuga and Czerpak (1976) witnessed conversion to dehydroretinol only in both the goldfish and the Far Eastern rudd (Leucaspias delineatus). Claims that anhydrolutein is a precursor were stimulated by work done on chicks and rats by Budowski and Gross (1965) and Savithry et al. (1972). There are no convincing reports of the occurrence of anhydrolutein in the plant kingdom.

The early reports by Grangaud and Moatti (1958a,b) that astaxanthin was a precursor of dehydroretinol have now been confirmed. As is often the case in animal carotenoid metabolic studies, the mechanism of transformation is not understood. A prime example is encountered in this work. It has been proven that xanthophylls are precursors of retinol and dehydroretinol but the mechanism is still undefined. For lutein and zeaxanthin, a pathway via either anhydrolutein or via β-carotene can be envisaged. In the case of canthaxanthin and astaxanthin it is more difficult to propose an alternative route to the β-carotene route. Nonetheless, since Schiedt et al. (1985) have pointed out that elimination of a 3-hydroxy group in rainbow trout is not possible, it seems contradictory that they should propose a scheme of astaxanthin to dehydroretinol via β-carotene. It would be more acceptable if the astaxanthin was converted to zeaxanthin which was then metabolised in a manner described by Barua et al. (1973).

Carotenoid analysis.

Many workers have used similar techniques for the separation of integument carotenoids. This is a direct result of the difficulties experienced during work on carotenoid esters, particularly astaxanthin ester. Very few reports are available on the chromatographic separation of carotenoid esters. Recent work on the xanthophyll esters in autumn senescing leaves of apple trees illustrates the problems encountered (Cardini, 1982). The fatty acids combined with the xanthophylls were analysed by g.l.c. and found to be C_7–C_{11} (1.5–1.8%), lauric acid (2–7%), myristic acid (9–15%), palmitic acid (36–44%), oleic (12–26%), stearic (8–10%), linoleic (5–6%), arachidic (0.7–2.5%) and linolenic-gadoleic acids(2.5–9.3%). The complex nature of the different esterifying fatty acids confuses the chromatography of the carotenoids themselves. Other papers relating to esterification of the xanthophylls are by Goodwin (1958), Eichenberger and Grob (1962a,b), Egger (1964) and Egger and Schwenker (1966).

Thus, the initial separation of goldfish carotenoids by most research groups was achieved by chromatography of the carotenoid esters on Microcel C with light petroleum and acetone as solvent. Papers by Ritacco et al. (1984a,b) report the observation of carotenoid reactions on Microcel C and propose a mechanism for these hydroxylation reactions. This reveals the unsuitability of Microcel C for this work and demands that a different adsorbent is used. The alternative approach is to saponify the carotenoids before chromatography, but this has its problems because astacene (the saponification product of astaxanthin) is difficult to chromatograph subsequently. Müller et al. (1980) have overcome this difficulty by constructing apparatus that permits anaerobic saponification, thus allowing the saponification of astaxanthin esters to give free astaxanthin. The whole procedure is carried out under high vacuum and argon atmosphere, the system only being opened after acidification with sulphuric acid. Ultimately, the reliability of the

results of the lutein feeding experiments is dependent on the final purity of the astacene or astaxanthin.

In the first experiment the final stage of purification of the astacene fraction was by t.l.c., and although this would normally be quite adequate the second experiment is more satisfactory as both t.l.c. and h.p.l.c. were used to achieve the same separation.

In both experiments the major problem was the presence of high levels of sterols. The chromatography was poor in some cases even though many stages of sterol removal had been carried out. It was evident from both t.l.c. and h.p.l.c. of the integument fractions that there is a very complex complement of carotenoids present. Although the major carotenoids have been identified, there are many other zones (t.l.c.) and peaks (h.p.l.c.) present, most of which are presumably unidentified carotenoid metabolites. In particular, h.p.l.c. analysis of the extracts emphasises the unexpectedly complex nature of the carotenoids of goldfish integuments.

As anticipated the levels of radioactivity present in the final extracts were low, thus making it difficult to assess the significance of any incorporation patterns. It was immediately apparent from the radioautograms that metabolism of the administered carotenoids was occurring. Many radioactive zones corresponded to carotenoid zones and hence it must be concluded that metabolism was occurring. The multitude of labelled bands (per ^{14}C-labelled carotenoid administered) supported the theory of Davies (1985), i.e. that there are relatively few enzymes associated with carotenoid synthesis/metabolism but that these are broad specificity enzymes. One important feature of carotenoid metabolism in goldfish demonstrated by this work was the relatively common occurrence of reductive metabolism. This was demonstrated both in the metabolism of carotenoids in integument and in reductive pathways in the formation of retinol and dehydroretinol from canthaxanthin, zeaxanthin and lutein.

Although the results from different fish within a dietary group were slightly variable quantitatively, the same incorporation patterns were observed. There is very strong evidence for the metabolism of canthaxanthin and zeaxanthin to astaxanthin in goldfish. Also, there is an indication that the β-carotene is metabolised to astaxanthin, although this is not as obvious due to the large number of steps in the metabolic pathway. These reactions are well documented in goldfish and other species, so their occurrence is unremarkable. The conversion of lutein to astaxanthin is not as well-defined. There is little doubt that bands (t.l.c.) and peaks (h.p.l.c.) of radioactivity correspond to the R_f and retention time respectively, of standard astacene.

A more sophisticated experiment can be envisaged which would give more information about the metabolism of lutein in goldfish. In goldfish, the lutein present is in fact 3'-epilutein; the epimerisation of lutein to 3'-epilutein in goldfish could take place via 3'-O-dehydrolutein, an intermediate that has been isolated from goldfish already (Buchecker et al., 1978). Feeding of [^{14}C]3'-epilutein to goldfish may give stronger evidence for this conversion, as the number of steps required for the transformation are fewer. Chemical oxidation of lutein using nickel peroxide produces 3'-dehydrolutein, resulting in a loss of chirality at C-3' (Liaaen-Jensen and Hertzberg, 1966). Chemical reduction by $LiAlH_4$ would give a 50:50 mixture of lutein and 3'-epilutein. Separation of these by h.p.l.c. (Rüttimann et al., 1983) would enable pure [^{14}C]3'-epilutein feeding experiments to be carried out.

From stereochemical considerations there is no reason why the above observation of the metabolism of lutein to astaxanthin cannot be a real effect. The occurrence of ε- to β-end group transformations in other species of fish illustrates the ability of fish to perform such reactions. Fujita et al. (1983a,b) demonstrated the reverse end group transformation in the integument of cultured yellowtail and Miki et al.

(1984, 1985) demonstrated this in the eggs of red sea bream. Matsuno et al. (1985) suggested reductive metabolic pathways of carotenoid metabolism in fish (astaxanthin to tunaxanthins) based on a stereochemical approach to the isolated carotenoids from eggs of four species of marine fish. It is interesting to note that Matsuno et al. (1981) did not detect conversion of lutein into astaxanthin in goldfish, although on stereochemical grounds it is quite feasible.

With respect to transportation of the dietary carotenoids in fish, a recent paper (Ando et al., 1986) gives evidence for a carotenoid-carrying lipoprotein (CCL) in the HDL and VHDL fractions of serum of chum salmon (Oncorhyncus keta). The CCL described was considered to transport the astaxanthin, which is the main carotenoid in muscle, into the skin and ovaries during spawning. It is possible that the CCL has the ability to transport other carotenoids as well. Ando and Hatano (1986) found that astaxanthin in egg yolk was bound to a protein arbitarily named E_1. Further characterisation of this protein by amino-acid composition, revealed its similarity to the egg protein lipovitellin.

Although slow progress is being made in the areas of metabolism and transportation of carotenoids in fish, the functions of the carotenoids, e.g. during spawning, is still unclear.

CHAPTER 4

INTRODUCTION

4.1 Carotenoids in birds.

Birds are considered to be xanthophyll accumulators, with carotenes only occurring in substantial quantities in the retina. Compared with fish there is relatively small variation in the types of xanthophylls occurring, with little evidence of many carotenoids present other than those well characterised in plant species e.g. zeaxanthin and lutein. The wide distribution of canthaxanthin, echinenone and astaxanthin as typified by the flamingos is noteworthy. Goodwin (1984) reviews the carotenoids present in many species of birds on the basis of their tissue distribution.

The following section outlines some of the avian literature and serves to demonstrate the limited knowledge we have of avian carotenoids and their metabolism.

4.2 Distribution of carotenoids within tissues.

The brightly coloured plumage of birds is quite often due to the presence of carotenoids. Although this is well established there still remain many unidentified carotenoids in avian species. Carotenoids which are well documented in the avian carotenoid literature are astaxanthin, canthaxanthin and phoenicoxanthin, all of which occur in the various species of flamingo (Fox and Hopkins, 1966; Fox et al., 1967). In some species, lutein may be the major carotenoid but as in fish this 'lutein' may prove to be 3'-epilutein (i.e. the opposite chirality to that of the plant form). Similarly, the stereochemistry of astaxanthin and other carotenoids is undetermined.

Generally speaking, yellow feathers are due to lutein or a similar type of pigment, and red feathers are pigmented with ketocarotenoids such as canthaxanthin and astaxanthin.

The yellow coloration of hen egg yolks is due to the presence of high levels of xanthophylls. The major xanthophylls (90% of which are esterified) are lutein and zeaxanthin although any other xanthophyll absorbed from the diet may also be deposited in the egg yolk. Again the stereochemistry of the carotenoids present is not well defined.

The skin of birds, such as the domestic hen, is invariably yellow due to the deposition of lutein. Much effort has been put into formulating carotenoid-rich diets for poultry with a long term plan to produce broilers of an acceptable colour. Likewise, the colour of egg yolks can be altered with diet and this is of great commercial importance (Marusich and Bauernfeind, 1981). In flamingos the skin coloration is due to the presence of astaxanthin, canthaxanthin and phoenicoxanthin as opposed to lutein. In some species e.g. Phoenicoparrus andinus (Andean flamingo) there is also a relatively high proportion of fucoxanthin present (Fox and Hopkins, 1965).

It is in the retina of avian eyes that more interesting carotenoids are encountered, in that carotenoids unique to avian species have been isolated and characterised from this tissue. Of particular interest in this work is the presence of galloxanthin and 6S,6'S-ε,ε-carotene. The significance of these 2 carotenoids is discussed later.

Carotenoids are also present in the blood plasma of some avian species e.g. the roseate spoonbill (Ajaia ajaja; Fox et al., 1965) where they are associated with high density lipoprotein (Trams, 1969). The crop milk of flamingos is bright red due to the presence of canthaxanthin (Fox, 1976).

4.3 Functions of carotenoids in birds.

The roles played by carotenoids in Nature are not easy to ascertain and carotenoid function in birds is no exception. The wide distribution of carotenoids within the tissues of birds suggests that there may be multiple functions for the carotenoids but in fact, only two functions of carotenoids have been put forward and remained undisputed.

As in other animals, appropriate carotenoids can be converted by birds into vitamin A, which is required for the normal operation of many metabolic processes.

The part played by carotenoids in achieving the natural plumage of birds is important in the sexual dimorphism of most species.

There does not seem to be an absolute requirement for carotenoids by birds. This was initially demonstrated by Palmer and Kempster (1919) who reared normal chicks on a xanthophyll-free diet. The offspring from these chicks hatched and developed normally. This work has been substantiated by Meyer et al., (1971) who reared Japanese quail (Coturnix coturnix japonica) on a carotenoid-free diet. A gradual reduction in blood and liver carotenoid levels was observed but again the offspring from these quail possessed normal growth and reproductive characteristics.

4.4 Carotenoid metabolism in birds.

The study of quail retinal carotenoids illustrated the inability of birds to form carotenoids de novo (Meyer et al., 1971) and thus there is no reason to doubt that birds, like other animals, derive all their carotenoids from dietary sources.

However, modification of dietary carotenoids by birds is possible,

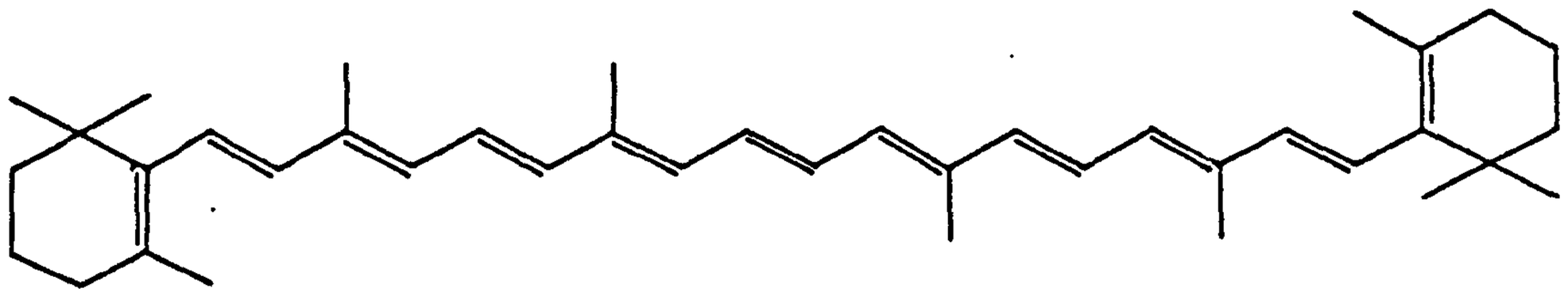
for example, the American flamingo (Phoenicopterus ruber) which displays, in its feathers and other tissues, rich red carotenoid pigmentation due to the accumulation of vast quantities of ketocarotenoids, can convert β-carotene into echinenone and canthaxanthin. This was demonstrated by the increase in blood levels of these pigments when β-carotene was fed to carotenoid depleted birds (Fox et al., 1969). Figure 41 shows the conversion of β-carotene to canthaxanthin via echinenone. This series of reactions is the same as that which is well proven in the Californian brine shrimp (Artemia salina; Davies et al., 1965; Hsu et al.,1970).

There is an impressive colour change of the feathers from white through yellow and orange to the pink of canthaxanthin under these experimental conditions. Whether the flamingo, in the wild, accumulates canthaxanthin derived directly from the diet (algae or crustacea) or whether it modifies other dietary carotenoids such as β-carotene is debatable but probably both processes occur simultaneously. The important consideration is that the flamingo does have the ability to oxidise β-carotene to canthaxanthin.

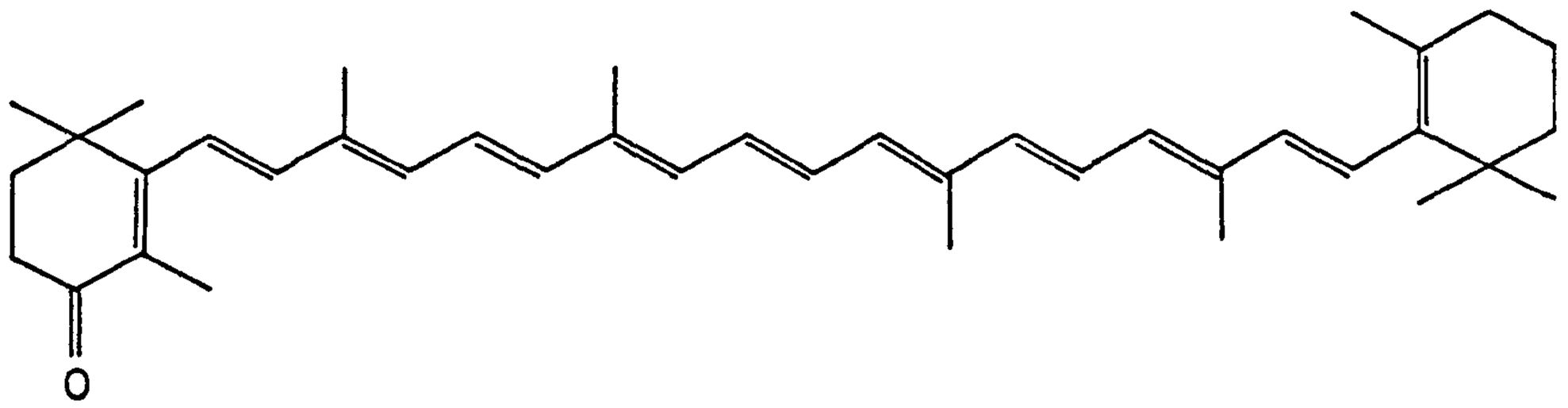
Other metabolic transformations have been seen in flamingos. Canthaxanthin was converted into phoenicoxanthin and astaxanthin and α-carotene was converted into phoenicopterone (Fox et al., 1970; Fox and McBeth, 1970).

Apart from these investigations of flamingo carotenoid metabolism relatively little work has been done on avian species.

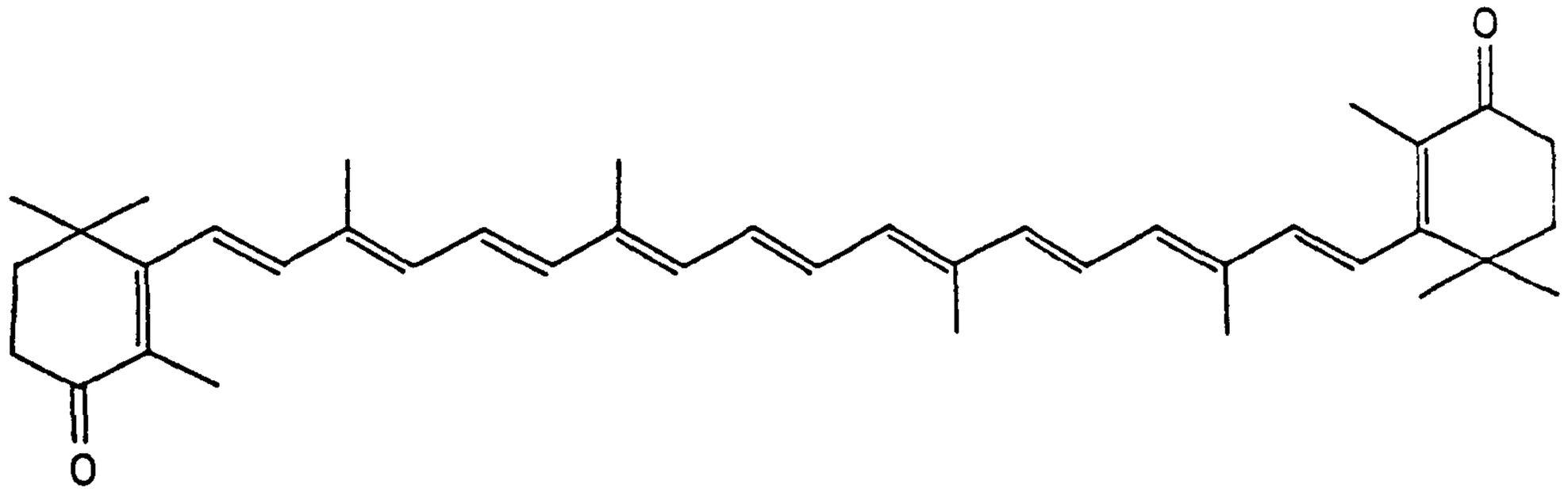
Fairly recently a very interesting metabolic conversion has been demonstrated in laying hens (Schiedt et al., 1981b). The implication of this observation are discussed in detail in Section 4.10.



β-carotene



echinenone



canthaxanthin

Fig. 41.

Pathway of metabolism of β-carotene to canthaxanthin in the American flamingo Phoenicopterus ruber.

4.5 Oil droplets.

The oil droplets in the sensory cells of retinas of birds and reptiles were first discovered by Hannover (1840). They usually only occur in cone cells (except for geckoes and teleosts) and the droplets have been shown to be deeply coloured with carotenoid resulting in red, orange, yellow or green globules. Smaller, colourless droplets occur in some retinas, sometimes in combination with coloured oil droplets or on other occasions without them.

The location of the oil droplets was shown by electron microscopy of chicken retinas (Morris and Shorey, 1967) to be at the choroidal end of the inner segment of the cone. Due to this location no light rays directed onto the cone can reach the visual pigment in the outer segment without first passing through the oil droplet (Fig. 42).

The cones can be identified by their different oil droplets or by the different electron density of the oil droplet as seen in electron microscopy.

4.6 Distribution of oil droplets.

The development of oil droplets as colour filters can be followed through evolution, the most primitive form of colour filter being the yellow lens as first seen in lampreys. The distribution of colours and proportions of carotenoids of oil droplets varies for different species within the following groups - chondrosteans, dipnoeans, amphibians, reptiles, birds, monotremes and marsupials, all of which may possess colourless droplets. Of these, amphibians, reptiles and birds can contain yellow droplets and reptiles and birds have additional red droplets. Starting at the lower end of the vertebrate scale, sturgeons have colourless droplets which led to the belief that the oil droplets

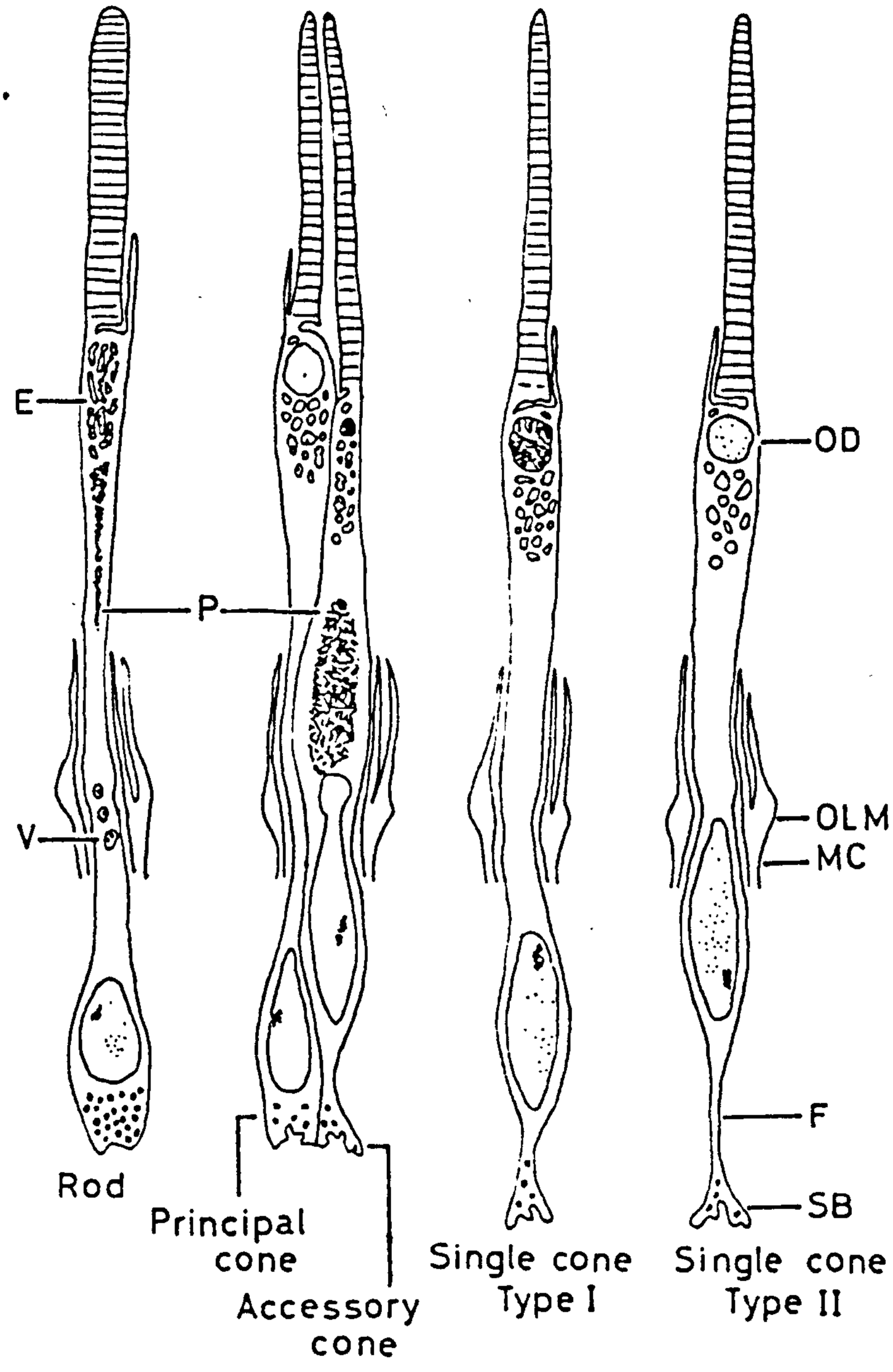


Fig. 42.

Schematic drawing of chicken visual cells based on an electron microscope study by Morris and Shorey (1967). Single rod: There are closely packed, elongated mitochondria in the ellipsoid (E). A parabloid (P) is present as well as prominent vesicles (V) at the level of the outer limiting membrane (OLM). Double cones: The chief member has a large yellow oil droplet, the accessory a small yellow-green oil droplet (OD). The glycogen-containing parabloid is seen in the accessory member. No Muller cells (MC) intervene between the two members of the double cone. Single cones: Two types have been identified, each containing different coloured oil droplets and both lacking a parabloid. (F)-fibre and (SB)-synaptic body.

developed as a colourless structure whose function was to project an intense beam of light onto the visual pigments of the outer segment. As sturgeons are nocturnal there would only be a need for colourless oil droplets. Colourless droplets have been shown to be present in three species of fish by Wald (1936a). These are the sea robin (Prionotus carolinus), black sea bass (Centropristes striatus) and scup (Stenotomus chrysops).

Unless oil droplets of the bull-frog (Rana catesbiana) are a reinvention (Wald, 1936b) the primitive amphibians were probably diurnal. This is likely because the oil droplets inherited from the ganoids would surely have had time to vanish as in snakes and placental mammals.

Nocturnal lizards have colourless oil droplets and although lizards gave rise to snakes there is an absence of oil droplets in nocturnal species of snakes. However, diurnal snakes have considerable lens pigmentation. This implies that the original snakes had a mode of life that did not require the oil droplets of its lizard ancestors but on subsequent differentiation diurnal snakes evolved which required and established the yellow lens.

Birds have a common ancestory with reptiles as can be seen from similar skeletal characteristics, one of which is the comparability of eye structure. The diurnal reptiles passed oil droplets onto birds. Diurnal birds, which have many more cones than rods are characterised by the presence of colourless, red, yellow or green droplets in their cones. Lonnberg (1930, 1934) found carotenoids in the eyes of 22 species of birds from different orders, e.g. Anseriformes (ducks, geese, swans), Galliformes (grouse, pheasants, domestic fowl), Gruiformes (cranes), Charadiiformes (gulls, auks), Strigiformes (owls) and Passeriformes (thrushes, warblers).

Early mammals possessed oil droplets as did the marsupials. Of the

marsupials only the diurnal forms e.g. kangaroo, have kept the oil droplet.

The above is consistent with the reasoning that once the oil droplet has been lost it cannot be re-established by a descendant group although it is possible for the yellow lens to be reproduced.

4.7 Properties of oil droplets.

Considerable confusion exists in the literature as to the types and relative numbers of cones present in the retina of any particular avian species. As the cones can be identified by the different colours of oil droplets it is important to establish the number of different oil droplets present in retinal tissue. It is readily possible to see 3 types of oil droplet (red, orange-yellow and yellow-green) under the light microscope. However, as early as 1881 and 1883, Waelchi measured the absorption spectra of chicken oil droplets and identified at least 4 types. In contrast to this Roaf (1929), Fujimoto et al., (1957) and Strother and Wolken (1960) only identified 3 types.

Morris and Shorey (1967) using electron microscopy counted 286 receptors of chick retina in which the cone types occurred in the following proportions; 45% double cones, and three types of single cones occurring in the ratio 5:2:1. Interestingly, they found that there was only a 'granular vesicle' in the accessory member of the double cone. This contrasts with the observation of Meyer and May (1973), who clearly showed the presence of an oil droplet in the accessory part of the double cone. They also found that red droplets were only present in single cones whilst double cones had yellow-green oil droplets.

With the advent of more sophisticated equipment for pursuing microspectrophotometric studies (MSP), more information is now available about the visual pigments and oil droplets of avian retinas. Work on

chicken retinas gives evidence for the presence of six types of oil droplets and three visual pigments (Bowmaker and Knowles, 1977) as opposed to two visual pigments as suggested by Wald (1937). Microspectrophotometric measurements indicate the presence of five types of cone - a double and four single cones. Three of the single cone types and both members of the double cone contain a pigment with λ_{max} 569 nm while the fourth type of single cone contains a pigment of λ_{max} 497 nm. The third visual pigment is found in the rod receptors (λ_{max} 506 nm).

As carotenoid concentrations in oil droplets are so high the direct measurement of carotenoid spectra is impossible in most cases. The flat-top and "cut-off" spectra commonly seen, although important for calculating spectral sensitivities of cone outer segments, do not yield useful information about the carotenoid composition of oil droplet types. Recently, methods have been developed to dilute particular oil droplets in such a way that a full absorption spectrum can be obtained (Liebman and Granda, 1975). A system of classification of avian oil droplets based on spectrophotometric and morphological properties has been proposed by Goldsmith et al., (1984).

Strother and Wolken (1960) originally suggested that the yellow droplets contained only lutein and zeaxanthin, the red droplets astaxanthin (prior to saponification) and the green droplets galloxanthin. The qualitative and quantitative composition of carotenoids in the oil droplets of chicken (Gallus domesticus) was determined by Stransky and Schultze (1977). As can be seen from Table 20 these were comparable to the carotenoids found in turkey retina (Meleagris; Pollard, 1980).

4.8 Functions of oil droplets.

Due to the position of oil droplets, between the incoming light and visual pigment, it has been assumed that the droplets play a significant

TABLE 20.

Comparison of the carotenoid components of chicken and turkey retinal oil droplets (percentages of total carotenoid).

	Turkey retina [1]	Chicken retina [2]
Astacene	17.0%	66.0%
14'-β-apo-caroten-14'-al *	13.7%	-
ε-Carotene	12.0%	-
ε-Carotene-diol	2.0%	-
β-Cryptoxanthin	5.6%	trace
Galloxanthin	27.0%	4.4%
Lutein / Zeaxanthin	8.0%	5.5%
Neochrome	-	trace
Phoenicoxanthin	-	15.6%
Unidentified	15.0%	8.0%

1 Pollard, 1980.
2 Stransky and Schulze, 1977.

* now known to be an artifact.

role in avian colour vision and, indeed, there has been much speculation about this aspect of carotenoid function. Krause (1863) first suggested that a visual pigment was broken down by all visible wavelengths thus initiating an impulse. The light had access to every cone but as there was only one pigment there could be no discrimination between wavelengths i.e. achromatic vision. Krause thus suggested that the role of the oil droplet of a given cone was to allow only certain wavelengths to affect the outer segments, hence discrimination was possible. Agreeing with this are Roaf (1933), Wald (1938), Donner (1960) and Hailman (1964). However, this is doubtful because teleosts have colour vision but no red droplets, Japanese quail when deprived of carotenoids are not effected (Meyer, 1971) and perhaps more significant is the revelation from MSP that there are several visual pigments as well as several types of oil droplet (Govardovski and Zueva, 1977; Bowmaker, 1977; Bowmaker and Knowles, 1977; Fager and Fager, 1981).

Many workers believed that the oil droplets served to concentrate the longer wavelengths into an intense beam directed into the outer segment (Honigmann, 1921; Erhard, 1924). Garten (1907) supported the theory that red droplets reduced chromatic aberration by removing the shorter wavelengths so resulting in a thinner image, the elimination of blur and consequently an increase in visual acuity. Henning (1920) also believed this, as did Walls and Judd (1933a,b) who also concluded that red droplets reduced glare in high intensities of light as well as taking over the functions of the yellow droplets. They considered that the yellow droplets under normal illumination would increase the visual acuity by reducing chromatic aberration, reducing glare and dazzle, and enhancing detail and contrast.

Strother (1963) suggested that oil droplets may modify colour vision by acting as "cut-off" filters to low wavelength light and increasing spectral sensitivity to red light. However, much evidence has been put forward against these theories and the function of oil droplets in

retinas is still unknown. For a review see Muntz (1972).

4.9 Development of oil droplets.

Again there is great confusion in the literature concerning this aspect of oil droplet biochemistry. Walls and Judd (1933a,b) showed that all droplets regardless of their final condition are at first colourless and that coloured kinds are derived independently from these and not from each other, the first colour to appear being red. Cooper and Meyer (1968) considered that the yellow droplets developed first from the colourless, followed by the green-yellow and finally red. All the research groups agree that development takes place during embryonic development, although there is speculation about the precise time of appearance of the droplets as summarised in Table 21.

4.10 Carotenoid metabolism in oil droplets.

The carotenoids present in turkey and chicken retinas are summarised in Table 20. Of these the presence of the majority is unremarkable, in that zeaxanthin, lutein and β-cryptoxanthin are commonly found in what may be considered to be a normal diet for these domestic birds. These carotenoids are also encountered in other avian tissues. More interesting is the presence of galloxanthin, ε,ε-carotene and astaxanthin in retinal tissue. Galloxanthin is interesting as it appears to be a carotenoid unique to avian retinas. The astaxanthin may be interesting depending on its chirality which, at the onset of this study, was not defined. The ε,ε-carotene, of all the avian retinal carotenoids, is of the most significance. It is unusual that the sole carotene of avian retina should be ε,ε-carotene as it is a rare carotenoid found only in a few algal species and the fruit of 'delta' mutant tomatoes. The two asymmetric carbons of ε,ε-carotene allow for different chiral forms of the carotene. Pollard (1980) showed, by circular dichroism, that

TABLE 21.

Day of appearance in embryo of the various types of droplet (literature sources as indicated).

	Type of oil droplet			
	Colourless	Red	Yellow	Green
Coulombre (1955)	13	15		
Walls and Judd (1933a,b)	14	15	17	
Schultze (1873)	17-18	18	19	19
Wald and Zussman (1937,1938)	15	19	>19	>19
Davies et al. (1984)	18	19	19	19

ε,ε-carotene of turkey was of the 6S,6'S configuration. This is of the opposite chirality to that of the plant form e.g. from Ulva lactuca. This eliminated the possibility that the turkeys were being fed a somewhat unusual diet and were merely absorbing the carotene from ingested material.

The presence of (6S,6'S)-ε,ε-carotene, in particular, in turkey retina raised questions as to the origin of this carotenoid. Galloxanthin and astaxanthin posed similar problems.

There are two possible ways in which these unusual carotenoids may arise. Firstly, de novo synthesis of the carotenoids may be taking place but it is assumed that this is improbable. Secondly, and more likely to be the case, is that considerable modification of ingested carotenoids can occur. As oil droplets form during embryonic development, carotenoids from the yolk must be precursors of ε,ε-carotene, astaxanthin and galloxanthin. Turkey yolk contains zeaxanthin, β-cryptoxanthin, ɣ-carotene, β-zeacarotene, β-carotene and phytofluene (Pollard, 1980).

It is reasonably straightforward to postulate oxidative pathways for the production of galloxanthin and astaxanthin. In fact, (3R,3'R)zeaxanthin would be a satisfactory precursor of these metabolic conversions (Fig. 5a,b).

On first reflection it is more difficult to envisage a suitable precursor of ε,ε-carotene from carotenoids which, at best, have only one ε-ring. Recently, it has been shown that a β- to ε-ring conversion is possible in laying hens (Schiedt et al., 1981b). (3R,3'R)Zeaxanthin can be converted via (3R,6'S)-3-hydroxy-β,ε-carotene-3'-one into (6S,6'S)-ε,ε-carotene-3,3'-dione. Lutein, although originally thought to be a feasible precursor of ε-carotene, when administered to laying hens was metabolised to (6S,6'R)-ε,ε-carotene-3,3'-dione via (3R,6'R)-3-hydroxy-β,ε-carotene-3'-one. A reductive pathway must be envisaged to

accommodate the conversion of zeaxanthin to ε,ε-carotene (see Fig. 5).

Although the majority of the analytical work has been carried out on turkey retina, the technical problems associated with a metabolic study using turkeys were far too great. As the oil droplets develop before hatching, the experimental work has to involve embryonic studies. The well characterised embryonic development of chicks combined with the relatively easy access to fertile chicken eggs makes for a far more suitable system with which to work. It was assumed that the carotenoids of the chicken were comparable to those of the turkey. Particularly important was the detection of ε-carotene in chick retinal extracts (Davies, 1983; unpublished work). Another assumption was that the embryonic carotenoids would be the same as the adult complement of carotenoids.

A method has been developed (Davies et al., 1984) to introduce radioactive zeaxanthin into chick embryos. The incorporation of radioactivity into retinal tissue coincided precisely with the formation of oil droplets in embryo. Using this system it was hoped that the metabolism of these carotenoids could be followed.

4.11 Embryology of chicks.

Immediately after its fertilisation the ovum enters upon a series of mitotic divisions - segmentation or cleavage. In the egg of a bird undergoing cleavage, the disc of active protoplasm at the animal pole (blastodisc) is a white circular area about 3 mm in diameter. It is in the central region of the blastodisc that cleavage furrows first appear. When segmentation has progressed to the stage in which the succession of cleavages is irregular, the term blastoderm is applied to the entire group of blastomeres formed by cleavage of the blastodisc.

Segmentation is ended with the onset of aggregation and

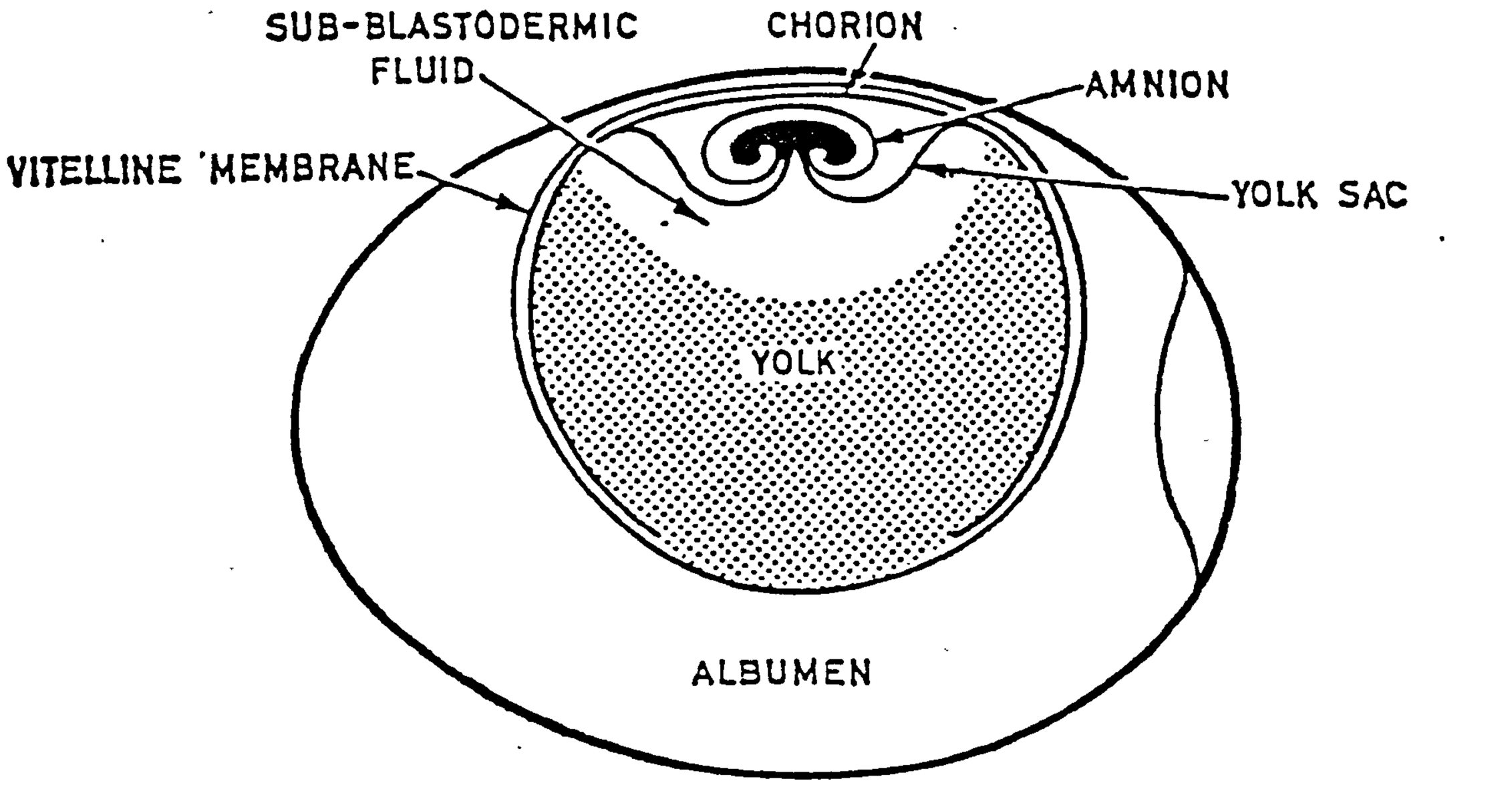
differentiation of cells. A rearrangement of cells occurs to form the blastula. The space between the blastoderm and the yolk is the blastocoele and when this is formed the embryo is said to be at the blastula stage.

Gastrulation then follows which results in the formation of two of the three germ layers. The single cell layer of the blastula doubles up on itself to give rise to the ectoderm (outer layer) and the endoderm (inner layer). The next stage is the formation of the mesoderm. The endoderm and ectoderm merge in a mass of rapidly dividing cells. Cells extend to both sides of the cell mass, between the ectoderm and endoderm to form the mesoderm. Development of the different tissues takes place from these three germ layers. The ectoderm gives rise to epithelial cells (skin, glands, feathers), the nervous system and sensory organs. The endoderm gives rise to the epithelial lining of the gut and respiratory organs and their associated glands. The mesoderm becomes differentiated to form fibrous and rigid connective tissues, muscle, the epithelial lining of the body cavities, the blood, organs of the circulatory system, lymphatic organs and the urino-genital system of the adult.

The distribution of egg contents is given in Fig. 43. By the fifteenth day of incubation the embryo has fully developed, from which time forth it grows and will hatch approximately on the 21st day of incubation.

4.12 Embryology of eyes.

Eyes appear very early as outgrowths of the brain called optic vesicles. They grow out laterally until they touch the head ectoderm and so stimulate lens formation. The vesicles remain attached to the forebrain by the optic stalks. The optic cup is formed by the optic vesicles becoming indented and so the cup-shaped structure with double



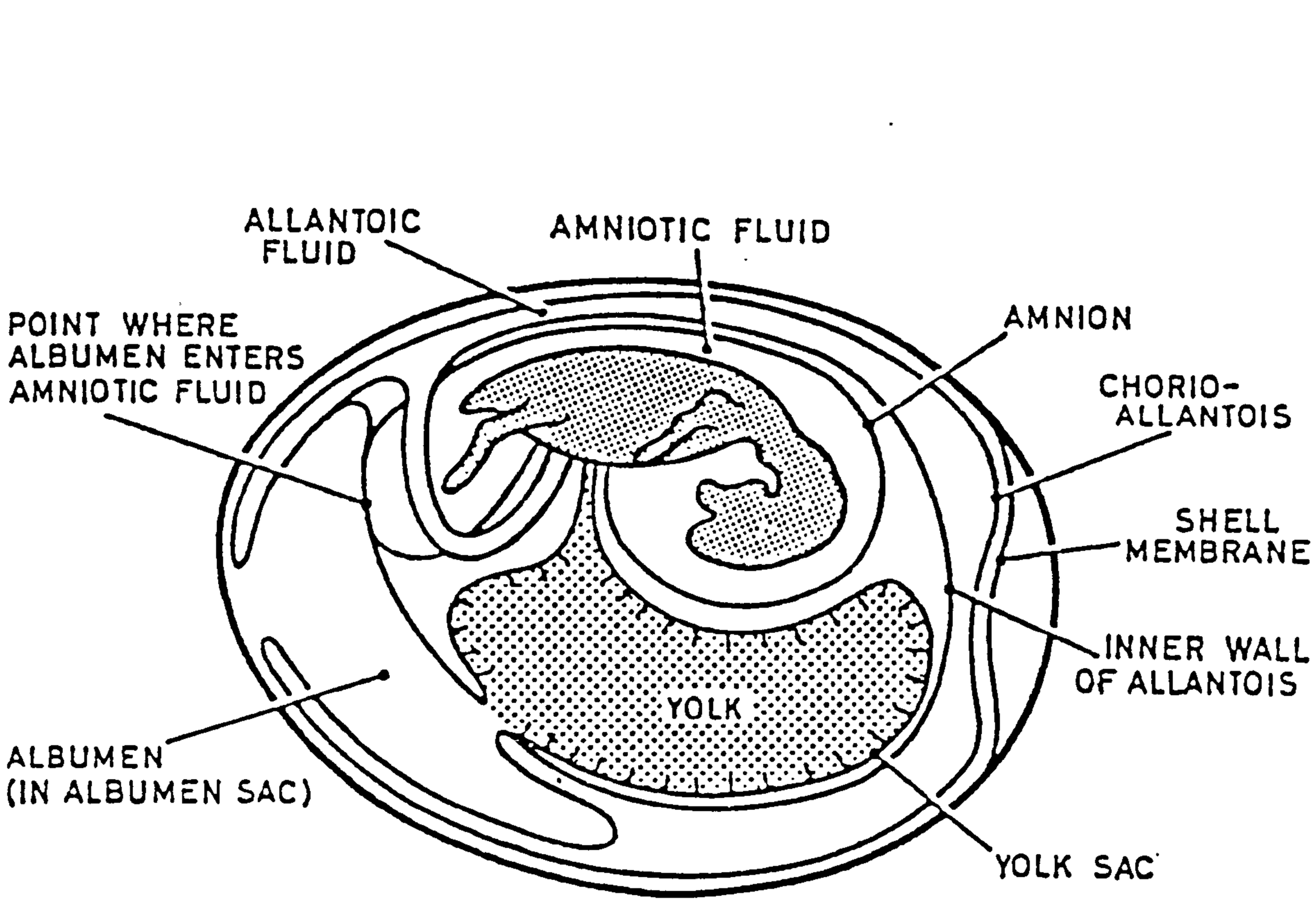


Fig. 43.

Distribution of egg contents before incubation and on approximately the 15th day of incubation.

walls forms. This process is not complete and a small hole is left, the choroid fissure. This allows blood vessels and mesenchyme to enter the cup, hence it is an outlet for the optic nerve. The lens fits into the cup itself and the overlying ectoderm, with some mesenchyme, becomes converted into the transparent cornea.

Of most interest in the present context are the layers within the optic cup. There are two layers within the cup; a thick inner layer, which is the nervous portion of the retina, and a thin outer layer which is the pigmented part of the retina. The rods and cones develop in the nervous layer. The cells nearest the cavity of the optic cup differentiate into neurones which send fibres along the inner surface of the cup to the optic stalk. The fibres eventually reach the brain and the cells of the optic stalk become converted into supporting tissue. Therefore light initially falls on the nerve fibres and then passes through to reach the light sensitive rods and cones. The process is complete within 4 days of incubation. Details of chick embryology can be obtained from New (1966).

AIM OF THIS WORK

A thorough analytical study of the turkey retinal carotenoids will be undertaken with special emphasis on the assignment of the astaxanthin chirality. Also, the metabolism of (3R,3'R)zeaxanthin by chick embryos will be investigated with an aim to prove that zeaxanthin is the natural precursor of astaxanthin, ε,ε-carotene and galloxanthin.

MATERIALS AND METHODS

4.13 Materials.

Turkey heads were supplied by Mrs B. Roberts, Crugiau Farm, Rhydyfelin, Aberystwyth. Chicken heads were supplied by Mrs. M. Dalton, Gelly Garneddau, Llangybie, Lampeter.

The fertile eggs were supplied by Churchstoke Hatchery, Montgomery, Wales.

4.14 Isolation and extraction of retinal tissue from turkey and chick eyes.

The availability of large numbers of turkey heads locally made this tissue more suitable than chicken for the initial isolation and identification work. The qualitative similarities between the two species already observed justified the time spent on this research.

Fresh turkey or chicken heads were obtained in batches of 100. The eyes were removed from the head by cutting away the eyelid tissue until the socket was exposed. At this stage the eyeball could be displaced slightly to allow access to behind the eye with fine scissors. Muscular tissue could be cut away and finally the optic nerve was severed. The excised eye could be kept on ice for short periods before dissection of the retina from the eye. Retinal tissue was isolated by making an equatorial cut with scissors. On removal of the lens, humour and pecten the retina was readily visible and could be scraped off using a spatula. Retinas were placed in ethanol for the first extraction. Acetone was avoided throughout extraction procedures to avoid the formation of the aldol condensation artifact, 14'-β-apo-caroten-14'-al (Shufflebotham, unpublished work).

The more delicate nature of the embryo eye demanded a more cautious

approach to dissection. Extraction was as for adult retinas.

4.15 Chicken liver extraction.

Frozen livers (purchased from Market Fresh Superstores) were extracted and saponified by normal procedures. Liver extracts were difficult to handle due to the presence of substantial amounts of sterols. Chromatographic resolution was poor and inconsistent. Consequently sterol precipitation was an important step and as this did not completely remove all sterols, the production of sterol digitonide precipitates was necessary (Section 2.6)

4.16 Isolation and extraction of embryonic tissues.

The method followed for embryonic retina has been described elsewhere (Section 4.14). Other embryonic tissues studied were the liver and intestine. These were isolated in a similar manner. A vertical cut along the length of the body of the embryo was made and the connective tissue removed. The liver was fully exposed and could be removed with forceps and scissors. Likewise the intestine was removed. Feathers were readily obtained by plucking with blunt forceps. Care was taken to wash the body before taking the feathers to ensure that no yolk carotenoids were removed and extracted.

Extraction, saponification and sterol removal was carried out by standard methods for each tissue.

4.17 Treatments of retinal, hepatic, intestinal and feather extracts.

A. Turkey and chick retinal extracts.

Each extract generated two fractions, a neutral and an acidic fraction. These were dealt with separately.

i. Neutral carotenoids.

Many systems of t.l.c. and h.p.l.c. were devised for the separation of the component carotenoids of turkey retina. The best separation was achieved by straight forward adsorption h.p.l.c. This system proved to be suitable for chick extracts as well.

Column:	LiChrosorb Si-60-5 (4.5 mm x 250 mm)
Solvent:	Hexane/Propan-2-ol/CH_2Cl_2 (92:5:3, v/v)
Flow rate:	1.5 ml/min
Chart speed:	30 cm/h
Detector wavelength:	440 nm and/or 380 nm

Detection at 440 nm enabled the carotenoids to be studied. At 380 nm retinol and any shortened chromophore carotenoids could be detected.

Appropriate fractions were collected during the chromatography and spectra of the carotenoids corresponding to each detector peak were then obtained. Further investigative steps undertaken for each carotenoid are described in the results section.

ii. Acidic carotenoids.

The major acidic carotenoid was anticipated to be astaxanthin. Its saponification product astacene was chromatographed on the Nucleosil/H_3PO_4 system described previously (Section 3.18).

An extract of turkey retina was submitted to Roche, principally for chiral analysis of astaxanthin but a thorough investigation of the sample was undertaken. An outline of the methodology is given in Appendix 1.

B. Chicken liver analysis.

H.p.l.c. of a saponified, sterol-free liver extract was carried out on the system described for Flavobacterium analysis (Section 2.13) i.e. a Nucleosil column and hexane/propan-2-ol/CH_2Cl_2 87:8:5 (v/v). Monitoring was carried out at both 440 nm and 340 nm.

From the h.p.l.c. elution profile it was possible to divide the total nonsaponifiable lipid into 3 crude fractions; a carotene fraction, a vitamin A fraction and a xanthophyll fraction. These three crude fractions could be generated most easily by classical l.c. on Grade III Alumina (neutral) with light petroleum and ether as developing solvents. Each fraction was dealt with separately.

i. Carotene fraction.

A previously established system was routinely used for carotene analysis, of liver, retinal and intestinal carotene fractions alike. Reverse phase h.p.l.c. was more successful for the separation of ε-carotene and β-carotene for which a clean separation was crucial.

Column:	ODS Hypersil 5μ (4.5 mm x 250 mm)
Solvent:	Acetonitrile/ Propan-2-ol (95:5, v/v)
Flow rate:	2 ml/min
Chart speed:	30 cm/h
Wavelength detection:	440 nm

ii. Vitamin A fraction.

The crude vitamin A fraction from chicken liver was studied further on the system devised for goldfish vitamins A_1 and A_2 (see Section 3.16).

iii. Xanthophyll fraction.

Again the Flavobacterium Nucleosil system was used for this analysis (Section 2.13).

For each crude fraction (i.e. i to iii) the initial identification procedure was to collect fractions corresponding to the major peaks, record absorption spectra and compare with synthetic standards where possible. In this way the key components were identified.

C. Chick intestine analysis.

Although standard material was unavailable it was anticipated that a similar separation system as used for liver would be acceptable for intestinal samples. Pollard (1980) analysed turkey intestine and found zeaxanthin to be the predominant carotenoid with small amounts of β-cryptoxanthin, α- and β-carotene and phytofluene also present.

Consequently embryonic intestinal fractions were treated in the same manner as the liver extracts. It transpired that a crude vitamin A fraction was absent at the classical column chromatography stage and indeed no vitamin A was detected in intestinal fractions. The carotene and xanthophyll fractions were dealt with as for liver (Section 4.17B).

D. Chick feather analysis.

Little was known about the carotenoid composition of this tissue. Due to the nature of the tissue it was anticipated that extraction would be difficult and sterol contamination problematic. No material was available except the test material of which there was very little.

Embryonic material was chromatographed on the LiChrosorb system described in section 4.17 A because this is a well characterised

separation system.

4.18 Diet extraction.

All feeds were supplied by Wynnstay Farmers Ltd., Llansantffraid, Powys. "Starter crumbs" (221 g), "Minipellets" (320 g), "Grower no. 1" (183 g), "Grower no. 2" (523 g) and "Finisher" (444 g) were extracted and saponified by standard procedures. The resultant total carotenoid from each feed was dissolved in light petroleum and the absorption spectrum recorded quantitatively. Classical l.c. (Alumina Brockmann Grade IV) generated a crude carotene, retinal, monohydroxy- and dihydroxycarotenoid fraction. Further analysis was undertaken of the retinal and dihydroxycarotenoid fractions.

4.19 Metabolic studies.

It has been postulated that zeaxanthin is central to the metabolism of carotenoids in chicken in particular, in the formation of ε-carotene, galloxanthin and astaxanthin (Davies, 1985). Incorporation of radioactivity into ε-carotene, galloxanthin and astaxanthin fractions following labelling experiments carried out on chick embryos would be conclusive evidence for these metabolic pathways.

Consequently, radiolabelled zeaxanthin was prepared in a form suitable for injection into fertile eggs. [^{14}C]Zeaxanthin was produced via a biological system (Flavobacterium; Section 2.12) and its chirality was defined as (3R,3'R). The synthetic [$^{3}H_2$]zeaxanthin supplied by F. Hoffmann-La Roche and Co. Ltd. was a 50:50 mixture of (3S,3'S)- and (3R,3'R)zeaxanthin.

The procedure for injection of the radiolabelled zeaxanthin into fertile eggs is described in the following section. Embryos had to be maintained through to hatching to accommodate the late development of

retinal oil droplets. The first appearance of oil droplets is normally on the 19/20th day of development of the chick embryo (Davies et al., 1984).

Batches of eggs were injected at weekly or fortnightly intervals, depending on availability of fertile eggs. Each batch was injected with either [2-^{14}C]MVA, [$^{3}H_2$]- or [^{14}C]zeaxanthin. The newly hatched chicks from each batch were sacrificed as soon as possible and dissected. The tissues studied were retina, liver, intestine and feathers. The extraction and subsequent saponification of these tissues was by standard procedures. As very small quantities of carotenoids were anticipated from each tissue, per chick, all extracts of a particular tissue were bulked. Therefore four extracts (retinal, hepatic, intestinal and feathers) were generated per batch. As there were three treatments (i.e. MVA, [$^{3}H_2$]- and [^{14}C]zeaxanthin) the final number of extracts was twelve. The 12 extracts were stored in the deep freeze until the last batch of eggs was hatched. A record was kept of the number of eggs injected with a particular, known amount of either [^{14}C]- or [$^{3}H_2$]zeaxanthin or [2-^{14}C]MVA so that percentage absorptions could be calculated.

Nineteen dozen eggs were injected with radiolabelled substrate over a period of 6 months.

The extracts were dealt with in the appropriate manner as described in Section 4.17.

4.20 Injection of fertile eggs.

The zeaxanthin had to be prepared in a form suitable for uptake by the embryo in a way which was not harmful to the embryo or which would not cause infection. Several problems had to be overcome.

1. All aspects of the work had to be done under sterile conditions.

Zeaxanthin is not autoclavable.

2. In animal development work it is normally possible to follow the metabolism of a certain compound by giving the compound to the embryo in its natural form. A window would be cut in the egg shell and the test compound droppered onto the egg membrane from where it could diffuse through the albumen to the embryo. The insolubility of carotenoids in water is an obvious problem. This problem was not encountered with the MVA experiments.

3. Any solvent used to dissolve the carotenoid may be harmful to the embryo.

4. The embryo has to survive through to hatching.

An ethanolic solution of zeaxanthin dissolved in sterile L-α-phoshatidylcholine was the most suitable form for injection into fertile eggs. L-α-Phoshatidylcholine is a major constituent of egg yolk and is consequently of no potential harm to the embryo. Chick embryos are known to be able to tolerate up to 2% ethanol (Appleton and Kemp, 1974) and this stipulation was followed. A 5% L-α-phosphatidylcholine solution was prepared by dissolving 0.5 g of phosphatidylcholine in 10 ml of distilled water. Difficulties encountered with dissolving the phosphatidylcholine could be overcome with the use of a shaker (Griffin and George Microid flask shaker). The solution was then autoclaved at 103.5 kPa for 20 min.

Into this 100 µl of an ethanolic solution of labelled zeaxanthin was added aseptically. As the specific activity of the labelled zeaxanthin was known the amount of label added could be estimated spectrophotometrically. Assuming that 0.5 ml of this final solution was administered to each egg, the total ethanol given would be 1%.

The procedure for injection into eggs was the same for either form

of zeaxanthin with minor differences for MVA experiments.

The fertile eggs were incubated in a Western PW incubator for 60 h prior to injection. During this time the eggs were positioned such that their pointed ends were downwards. Five minutes before injection the eggs were turned on their side so that the embryo would move to the top. At this stage of development a blood ring and possibly a blood network should be clearly visible and hence the viability of the embryo easily determined.

The top of the egg and the pointed end were wiped with ethanol and a small hole made in the pointed end using the tips of scissors. Using a 2 ml syringe and sterile needle (50mm 11/10 Gillette 19G x 2), 2 ml of albumen were withdrawn slowly out of the egg causing an air space to form directly above the embryo. A small square of sticky tape was placed over this hole. Another small hole was made in the top of the egg with the sterile scissors and a drop of Hanks Basic Salt Solution (Adams, 1980) containing penicillin and streptomycin was deposited over this hole. The solution was drawn into the egg by capillarity. A piece of adhesive (Scotch) tape (5 cm x 2 cm) was used to cover the hole and reinforce the shell so that an oval window (1 cm x 1.5 cm) could be cut out of the shell.

The window if cut correctly was positioned directly over the developing embryo, the blood ring almost entirely covering the top of the yolk. Using a sterile 1 ml syringe and needle (25 mm 5/10 Gillette 25G x 15/16), 0.5 ml of the labelled zeaxanthin solution was injected into the sub-blastodermic fluid just outside the blood ring and directly below the embryo. The window was resealed with adhesive (Scotch) tape. Care was taken to avoid injecting air bubbles into the egg yolk and to avoid rupturing the yolk.

The risk of infection was reduced by performing the above in a

sterilising cabinet (Centrionic Europe Ltd.).

The eggs were then placed in an incubator at 37.5°C. The required high levels of humidity were maintained by leaving a vessel full of water in the incubator. The chicks were allowed to hatch and were then immediately sacrificed (strangulation).

For the MVA experiments the window was cut in the same way but the MVA solution (water soluble) could be pipetted onto the albumen from where it could diffuse across to the embryo.

4.21 Analysis of chick tissues from embryos developed in the presence of radiolabelled zeaxanthin.

Tissues dissected from embryos which had developed in the presence of radiolabelled zeaxanthin were extracted and saponified by normal procedures. An estimation of total carotenoid and incorporation of radioactivity into each tissue was carried out by spectrophotometry and liquid scintillation counting, respectively. Each of the tissues was treated in the manner previously outlined in Section 4.17.

4.22 Analysis of chick tissues from embryos developed in the presence of [2-^{14}C]MVA.

This work presented an opportunity to explore whether or not any embryonic de novo synthesis of carotenoids could occur in chicks. This possibility was stimulated by the report of Austern and Gawienowski (1969), who allegedly observed the incorporation of radioactivity from [2-^{14}C]MVA into β-carotene in an incubation system using bovine corpora lutea.

The major problem with the extracts from MVA-treated chick embryos

was to ensure total removal of sterols from the extracts. The heavy incorporation of MVA into the sterol fraction could easily confuse and disguise the real effect of MVA in any carotenoid biosynthesis in chick embryos.

A. Preparation of samples for chromatography.

All the tissues were treated in a similar way. Each extract was treated with digitonin twice and several sterol precipitations were carried out. Classical column chromatography on alumina (Brockmann Grade II; 3% water, v/w) with light petroleum (b.p. 40–60 °C) as solvent eliminated the squalene fraction and removed the less polar sterols.

B. T.l.c. of samples from the MVA experiment.

Two-dimensional t.l.c. was performed to resolve the carotenoids and sterols in a way comparable with that used by Austern and Gawienowski (1969). Silica Gel G plates (precoated plates) were used with 100% light petroleum as developing solvent. Radioautograms of the resultant t.l.c. plates were prepared as described in Section 2.10 and allowed to develop for 10 weeks. The radioactive bands were scraped from the plates and counted by standard procedures.

4.23 Microspectrophotometric studies of turkey retinas.

A particularly thorough review of microspectrophotometry (MSP) of photoreceptors (Liebman, 1972) gives details of theory, design and results of MSP analysis.

A. Preparation of tissue for MSP.

The eye was opened by a longitudinal incision. The retina and attached tisue was immersed in a saline solution (normally mammalian

Ringer solution; Altman and Dittmer, 1964). A small piece of retina was withdrawn and chopped into small pieces with a razor blade. Suitable pieces were then transferred to a microscope slide, covered with a cover slip and then wax sealed.

For droplets which produce "cut-off s", i.e. droplets with very high carotenoid concentrations, it is possible to dilute individual droplets prior to MSP. The eye was opened as before and the retina placed in saline. A small amount of retinal tissue was transferred to a watch glass and distilled water added. This causes some oil droplets to be released to the surface of the water. A drop of water was transferred onto an agar plate where the individual oil droplets were visible under a light microscope (Zeiss). A micromanipulator (Singer Micromanipulator, Singer Instruments Ltd., Reading, England) was used in conjunction with a glass rod with a 10 µm loop to collect individual droplets from the agar. Droplets were transferred to a minute globule of mineral oil on a microscope cover slip. For relocation of the globule under the microspectrophotometer the cover slip was marked. Droplets of the same colour were transferred to mineral oil until the globule was visibly coloured. Cover slips cannot be placed on the prepared slides as this may disrupt the globules.

B. MSP techniques.

All the work needs to be carried out in red light so Kodak Safelight filters (No. 2) were installed. The basic units of the MSP equipment consisted of a light source, monochromator with associated optics, aperture plate and chopper to form and pulse the microbeams, a pair of microscopes (inverted and upright), photomultiplier and electronic circuits and a sophisticated computer system. The light source for visible spectra measured in this work was a tungsten ribbon filament lamp (100 W). The lamp was operated from a constant current regulated d.c. supply (Kepco ABC d.c. regulator) to ensure stable average quantum flux

output. Narrow wavelength bands were obtained with a grating monochromator (Bausch and Lomb, Sigma Instruments.) The wavelength advance is linear with shaft rotation. The exit slit of the monochromator is focused onto the ocular of an inverted microscope consisting of the Zeiss 32X Ultrafluor objective and quartz ocular. These optics reduce and project an image of the aperture plate, which is placed at the field lens of the monochromator, onto the object plane of the microscope. The aperture plate obscures all but 2 tiny holes which when reduced in size by the demagnifying inverted microscope creates an image of approx. 2 x 2 μm. The reference and sample beams are allowed to transmit light for about 6 msec, sample and reference pulses being separated by about 1.5 msec of darkness. A rotating sector disc achieves this time separation. The detector is a photomultiplier tube (EMI S-20 cathode). The instrument was operated in conjunction with a Minc computer system (digital Minc 11 and RXO2) which advances the wavelength drum on the monochromator, interrogates the photomultiplier tube via an A/D converter, stores and substracts baselines and calculates and displays the absorption spectrum. Spectra were printed out on a Digital Decwriter IV graphic printer.

Before running MSP of samples a background had to be measured. This was done by focusing two beams of light on air initially. The correct focusing of light beams was very important. Test spectra were recorded and stored on files which can take up to 11 spectra per file.

Multiple spectra were recorded for each type of droplet and an average can be calculated by the computer. Sometimes the oil droplets were attached to tissue and the reference beam was focused on an area associated with tissue. This causes slight problems in that there are artificially high levels of light scatter. This can be overcome by measuring a 'tissue' background.

Spectra were routinely measured by scanning from 750 nm to 380nm.

Spectra were measured of oil droplets from turkey eyes. Spectra of droplets from frozen tissue (-20°and -80°C) were compared with spectra measured of oil droplets from fresh tissue.

RESULTS

The results of this chapter can be split into three groups. These are analytical data, metabolic studies and microspectrophotometric studies (MSP).

4.24 Analytical data.

A thorough analysis of turkey retinal carotenoids was carried out. This species was investigated, as opposed to the chicken, for two reasons. Firstly, a local turkey farm ensured a reliable and convenient supply of turkey heads. Secondly, preliminary studies (Pollard, 1980) had already been carried out on this species and hence, were a good basis for subsequent work. As chicken and turkey were known to have qualitatively similar carotenoids, it was assumed that a direct comparison could be made between the two species. A brief investigation of adult chicken retinal carotenoids was undertaken to substantiate this assumption.

Analyses of chick liver and turkey diet were also carried out.

Other tissues of interest in the metabolic studies were intestine and feathers, neither of which became available for analysis before the onset of the metabolic work. The embryonic extracts had to be dealt with in a manner deemed suitable. Pollard (1980) analysed turkey intestine and as it was anticipated that the two species would compare well, an overall picture of the intestinal carotenoids of chicks could be assumed.

The most extensive analytical study was carried out on turkey retinal carotenoids. Data from the F. Hoffmann-La Roche and Co. Ltd. analysis is included in the preceding sections to substantiate the findings of this work and to further define carotenoid structures e.g. by p.m.r. and c.d. analysis. The methodology employed by the Roche group is

described in Appendix 1.

4.25 Turkey retinal carotenoids.

A. Neutral carotenoids.

The h.p.l.c. elution profile of the total neutral carotenoids of turkey retina on the LiChrosorb system (see Section 4.17A) is given in Fig. 44. As can be seen there are five major peaks, with seven other minor peaks also present. Following multiple injections and subsequent collection of peak fractions, spectra were obtained of the twelve peaks as indicated. Table 22 shows the absorption maxima for each fraction and also gives a tentative indication of the carotenoid component of some of the peaks.

Further analysis of the fractions resulted in identification of the carotenoid content of most of the peaks. Some of the carotenoid structures present were defined in terms of absolute stereochemistry.

i. Fraction 1.

Due to its non-polar nature (indicated by a short retention time) this peak was assumed to be a carotene. Pollard (1980) studied the carotene of turkey retina in some detail and established the presence of ε,ε-carotene. In this work the persistent nature of the absorption spectrum (λ_{max} in light petroleum at 414 437 and 467 nm) was consistent with this structure (Fig. 45).

H.p.l.c. analysis of the fraction also gave evidence for the presence of ε,ε-carotene. The system used was ODS Hypersil 5μ column with solvent and flow rate as described in section 4.17B. The retention time of the turkey carotene fraction was identical to that of synthetic ε,ε-carotene (21.2 min).

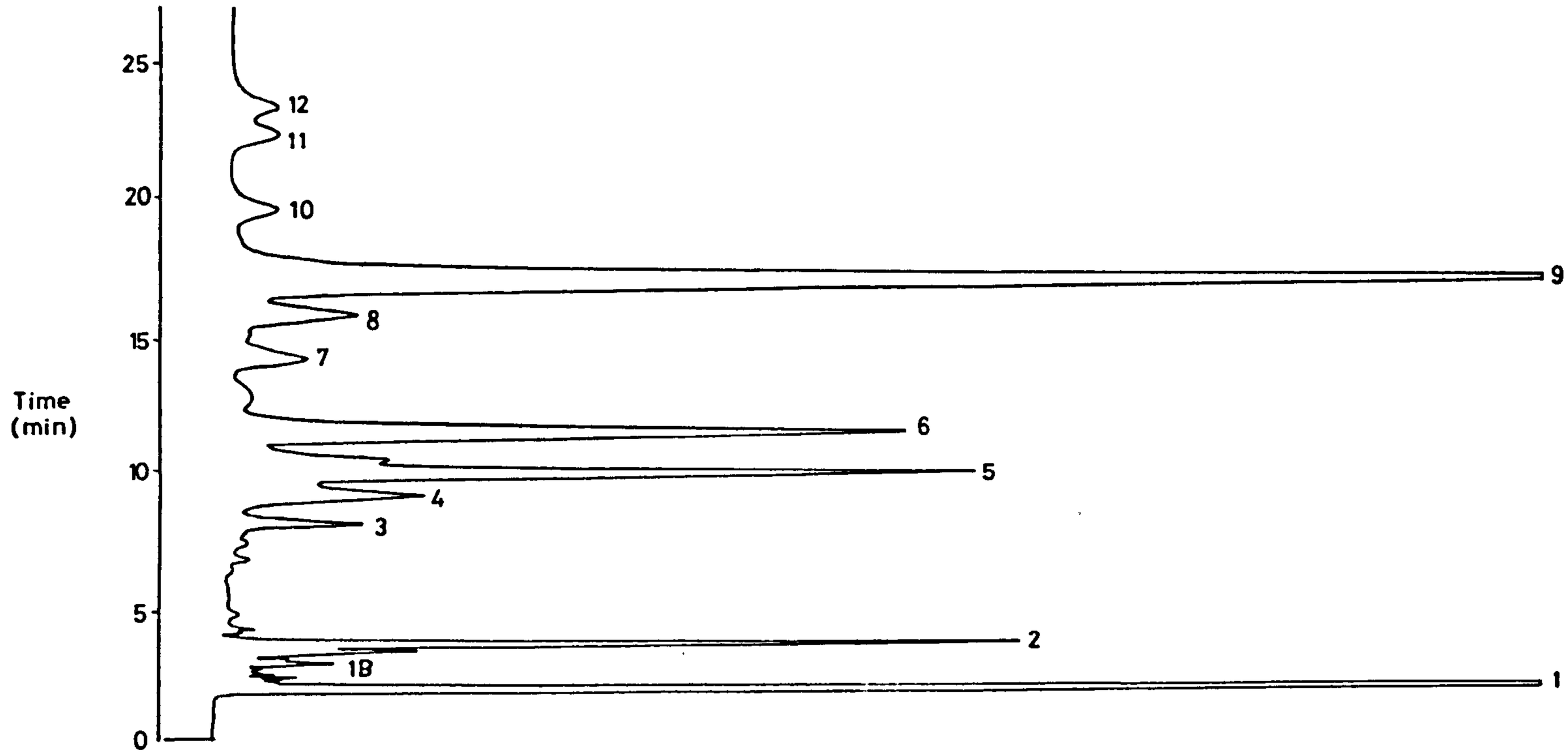


Fig. 44.

H.p.l.c. elution profile of total neutral carotenoids of turkey retina oil droplets on LiChrosorb Si-60, solvent hexane /propan-2-ol/ CH_2Cl_2 (92:5:3, v/v) at 1.5 ml/min (see Table 22 for peak identifications).

TABLE 22.

Absorption maxima for fractions 1-12 from h.p.l.c. of turkey retina neutral carotenoids on LiChrosorb Si-60, with hexane/propan-2-ol/ CH_2Cl_2 as solvent at 1.5 ml/min.

Fraction	Absorption maxima in ethanol (nm)					Carotenoid
1			415	438.5	468.5	ε,ε-carotene
2			421.5	444.5	473	cryptoxanthin
3				438.5		?
4			418.5	440.5	468.5	ε,ε-carotene diol
5			424.5	444.5	474	lutein
6			(428)	451.5	478.5	zeaxanthin
7			374	395	419	?
8	353.5	379	396.5	416.5	444.5	?
9			(380)	401	423	galloxanthin
10	290.5			398	418	?
11	293		379.5	397.5	417.5	?
12	295			397.5	418	?

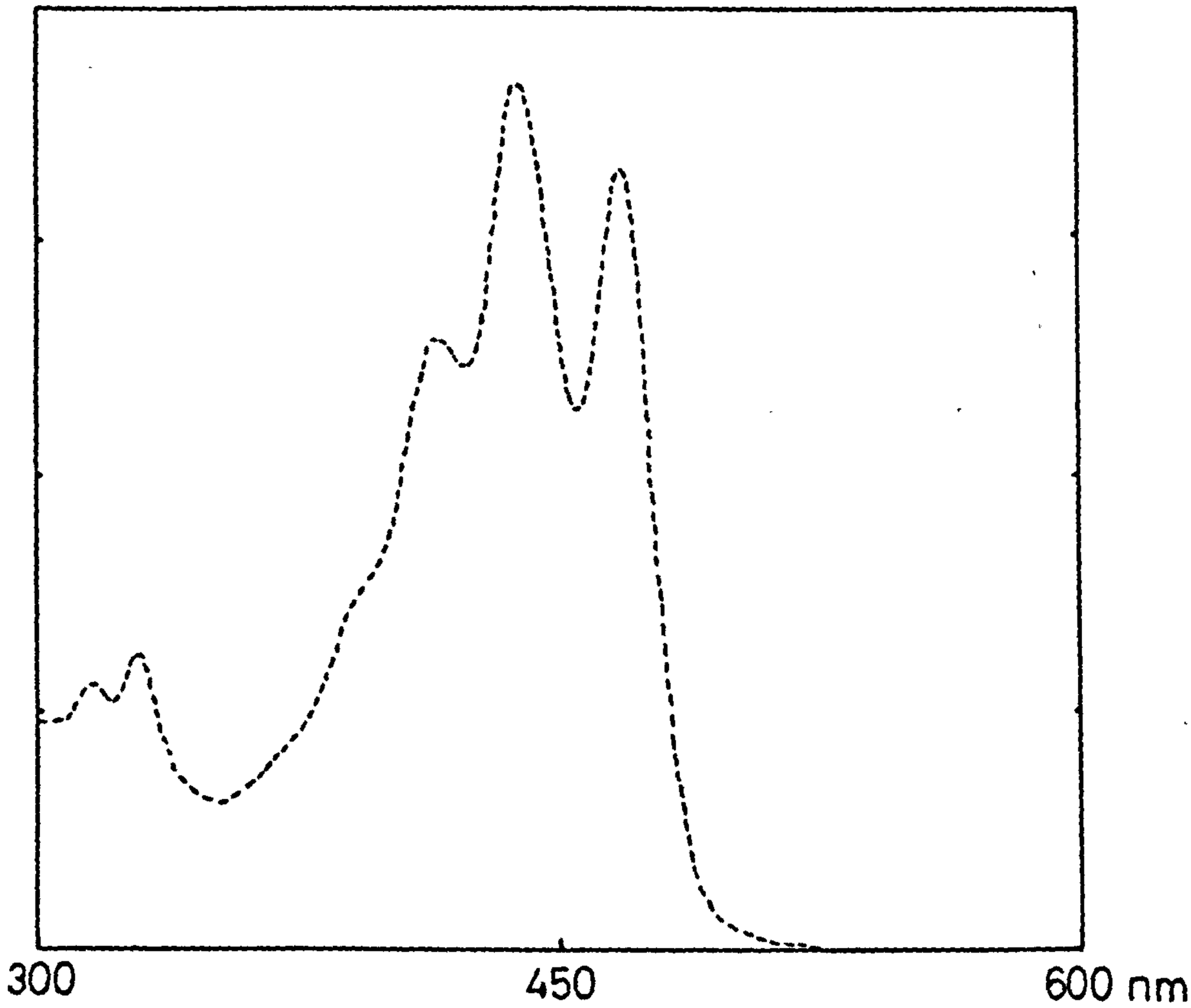


Fig. 45.

Absorption spectrum of ε,ε-carotene recorded in light petroleum.

Further confirmation of structure was given by n.m.r. data.

For analysis of all n.m.r. data, reference was made to general reviews of n.m.r. of carotenoids (Vetter et al., 1971; Moss and Weedon, 1976; Englert, 1982, 1985). These give chemical shifts (mean values; in p.p.m.) and coupling constants (in Hz) expected for certain structural features e.g. partial spectra for different end groups and the chain of conjugated double bonds.

ε,ε-Carotene ^{1}H-n.m.r. δ (p.p.m.)[$CDCl_3$]: 6.0-6.7 [m, vinyl-H]; 5.525 [2H, dxd, J_1 9.1 J_2 9.45, H-C(7) and H-C(7')]; 5.407[2H, s, H-C(4) and H-C(4')]; 2.182 [2H, d, J 8.9, H-C(6) and H-C(6')]; 2.015 [4H, s, H_2C(3) and H_2C(3')]; 1.96 [6H, s, H_3C(20) and H_3C(20')]; 1.91 [6H, s, H_3C(19) and H_3C(19')]; 1.583 [6H, s, H_3C(18) and H_3C(18')]; 0.82 and 0.903 [12H, 2s, H_3C(16), H_3C(17), H_3C(16') and H_3C(17')]. This is consistent with the structure of ε,ε-carotene. The characteristic chemical shifts compare well with those quoted by Vetter et al. (1971).

M.s. data also confirmed the ε,ε-carotene structure: m/z 536 (molecular ion M^+), and fragment ions at m/z 480 (M-56), 444 (M-92), 388 (M-92-56), 368. The fragmentation ion at M-56 is characteristic of an ε-ring.

Pollard (1980) showed that the chirality of ε,ε-carotene from turkey retina was 6S,6'S. This was verified during the course of this analysis.

A c.d. spectrum of ε,ε-carotene (concentration 0.078 mg/ml) in EPA (diethyl ether / isopentane / ethanol 5:5:2) was recorded over a range of 197 to 410 nm in a 0.2 cm cell pathlength. C.d. (EPA): 204 (-20.06), 220.3 (-7.63), 223.9 (-7.89), 237.6 (-5.01), 263.7 (-15.25), 292.6 (-0.91), 326 (-2.72), 350.4 (-1.18), 410 (-9.12). The wavelength is shown first, with the appropriate molar circular dichroism absorption coefficient ($\Delta\varepsilon$) value given in brackets.

The c.d. spectrum is shown in Fig. 46a. The spectrum is identical to the spectrum of synthetic (6S,6'S)-ε,ε-carotene.

ii. Fraction 1b.

Considerable difficulties were encountered whilst analysing this fraction.

An initial spectrum of the crude fraction gave general absorption between 280 and 320 nm. When this fraction was rechromatographed through the same system with the detector monitoring at 380 nm, two peaks were observed (1A and 1B). Spectra of 1A (λ_{max} in hexane at 364 nm) and 1B (λ_{max} in hexane at 276 282 313 328 366 nm) suggested the presence of retinal and retinol in turkey extracts.

Dual wavelength monitoring (380 and 440 nm) using a Hewlett Packard 1040A high speed spectrophotometric detector plus integrator and plotter, clearly demonstrated that there was no overlap of fractions 1A and 1B with either fraction 1 or 2 (Fig. 47). This may be an important consideration in the metabolic studies.

Further h.p.l.c. analysis of fractions 1A and 1B was unsuccessful. However, the Roche research group were able to identify retinol and retinal homologues from turkey retinal extracts.

The retinol fraction was acetylated and, by h.p.l.c. analysis, was identified as 66.9% all trans-retinol and 33% cis-retinol (11-cis-retinol, 10.4; 13-cis-retinol, 7.4; 9,13-cis-retinol, 2.7; 9-cis-retinol, 12.5).

Pollard (1980) identified 14'-apo-β-caroten-14'-al in extracts of turkey retinas. Shufflebotham (1982; unpublished work) showed that this apocarotenoid could be formed by an aldol condensation of retinal and

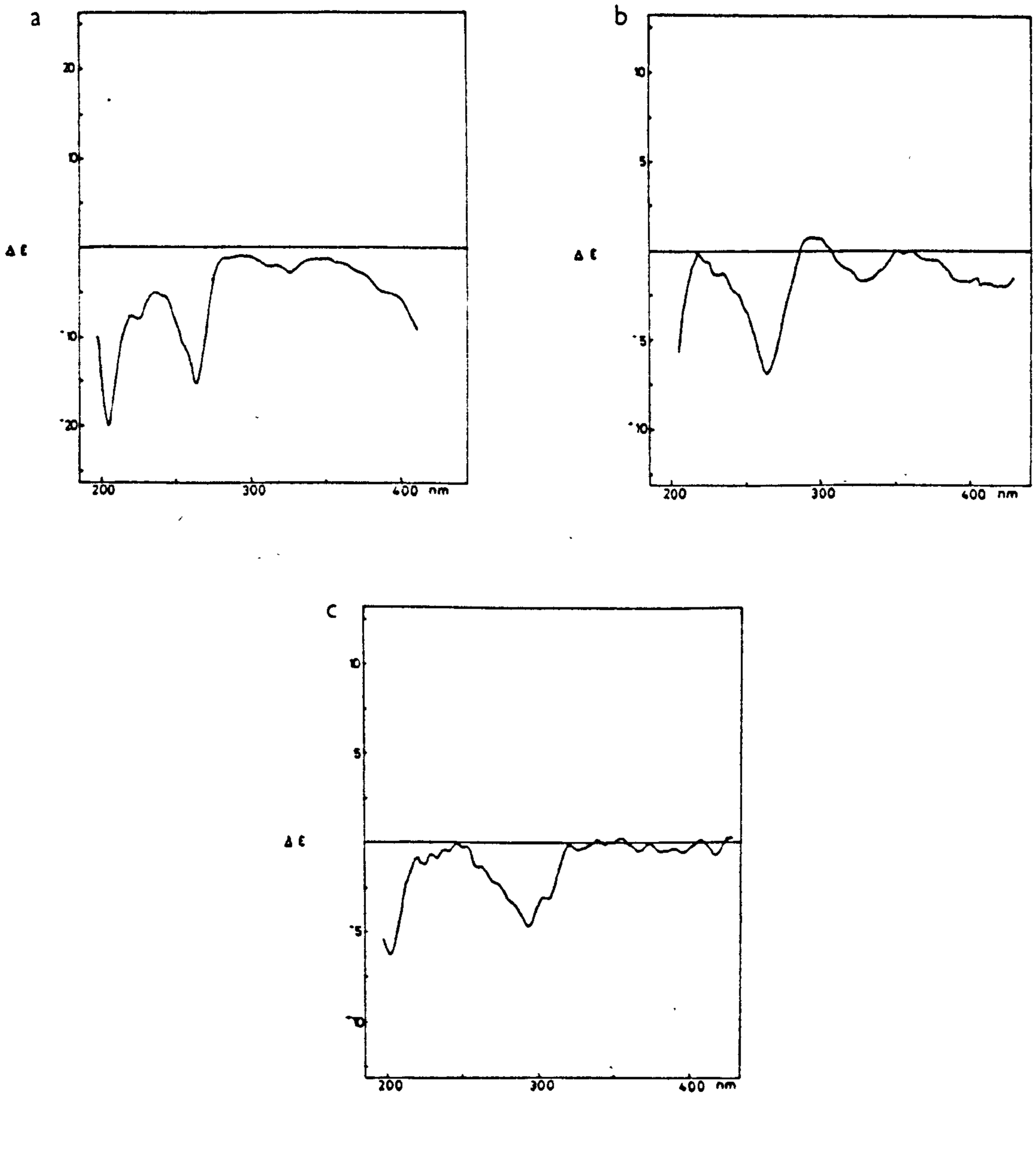


Fig. 46.

Circular dichroism spectra of ε,ε-carotene (a), α-cryptoxanthin (b) and galloxanthin (c).

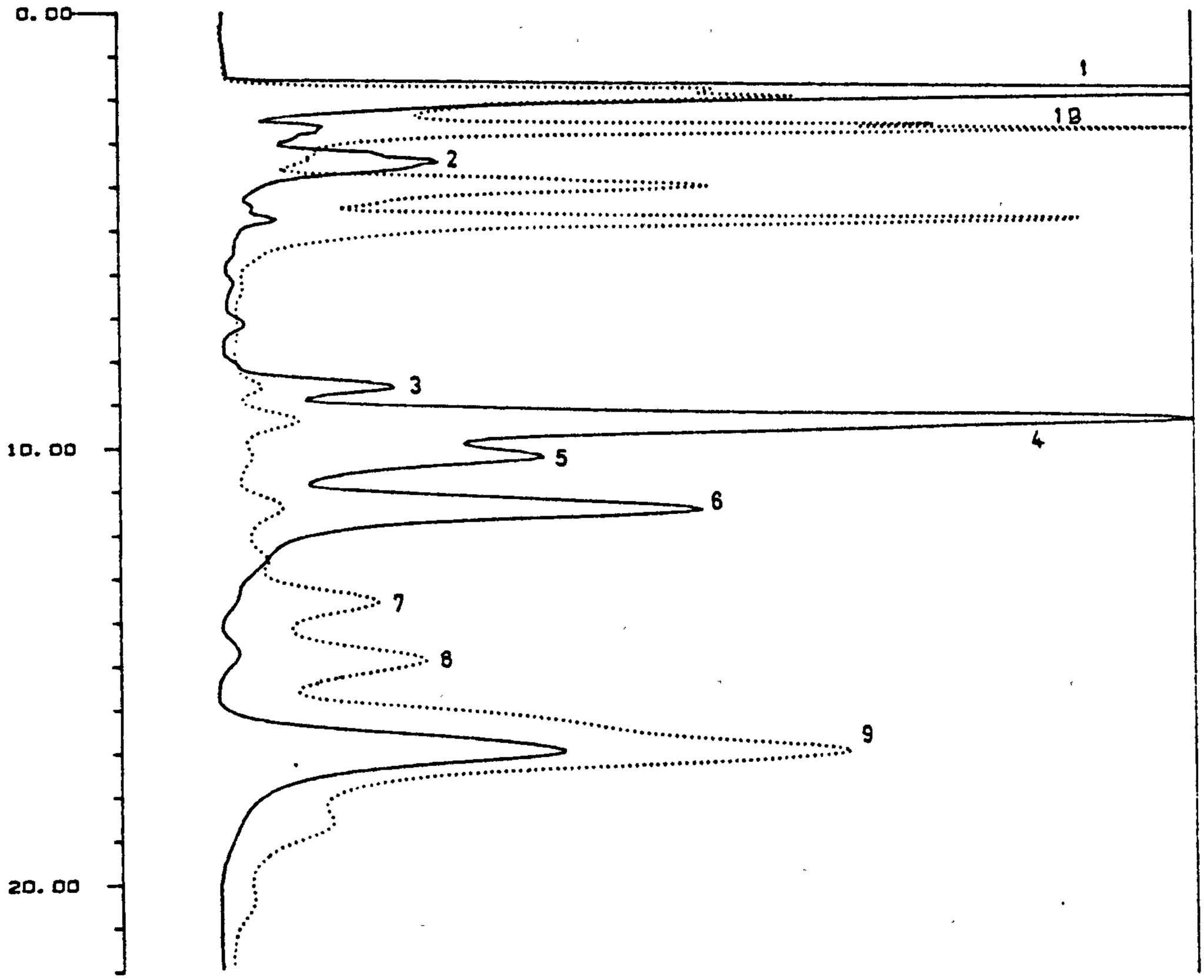


Fig. 47.

H.p.l.c. elution profile of total neutral carotenoids of turkey retina oil droplets on LiChrosorb Si-60, solvent hexane/propan-2-ol/ CH_2Cl_2 (92:5:3, v/v) at 1.5 ml/min with dual wavelength monitoring (..... 380 nm and _____ 440 nm).

acetone, and proposed that this compound was an artifact formed during extraction of the retinas. In the present study, acetone was avoided at all times. The Roche research group produced data (uv/vis, m.s. and n.m.r.) which indicate the presence of a compound $C_{23}H_{32}O$ (Fig. 48a). Uv/vis: λ_{max} 378 nm. M.s.: m/z 324 (M^+), 309 ($M-CH_3$), 281 ($M-CH_3CO$), 251, 239, 149, 105, 43. ^{1}H-N.m.r.: δ (p.p.m)[$CDCl_3$]; 6.0-7.0 [m, vinyl-H]; 2.297 [3H, s, $H_3OC(15)$]; 2.083 [3H, s, $H_3C(20)$]; 2.02 [2H, s, $H_2C(4)$]; 1.995 [3H, s, $H_3C(19)$]; 1.716 [3H, s, $H_2C(18)$]; 1.611 [2H, m, $H_2C(3)$]; 1.472 [2H, m, $H_2C(2)$]; 1.032 [6H, s, $H_3C(16)$ and $H_3C(17)$].

From h.p.l.c. analysis a vitamin A_2 aldehyde was tentatively identified in turkey retina extracts. This identification was based on retention times alone, the test material having an identical retention time to that of dehydroretinal.

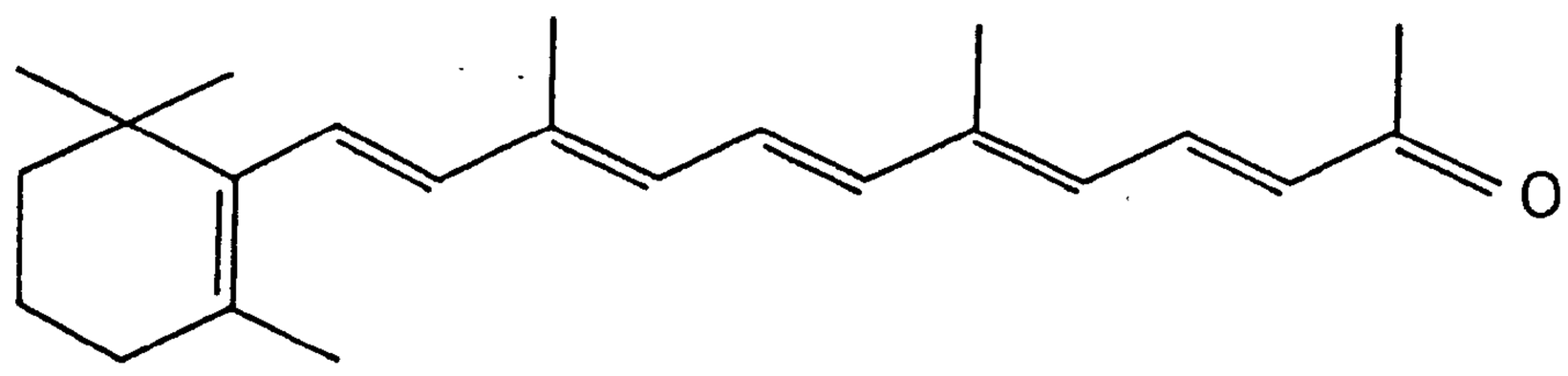
A third retinal based structure was also detected. Uv/vis: λ_{max} 385 nm. M.s.: m/z 350 (M^+), 335 ($M-CH_3$), 307 ($M-CH_3CO$), 267, 251, 237. This is consistent with the structure shown in Fig. 48b. An aldol condensation reaction between acetone and 14'-apo-β-caroten-14'-al would give rise to this structure. Further confirmation of this structure was provided by the n.m.r. spectrum. ^{1}H-N.m.r. δ (p.p.m.)[$CDCl_3$]; 6.0-7.0 [m, vinyl-H]; 2.277 [3H, s, $H_3OC(13')$]; 2.016 [2H, s, $H_2C(4)$]; 1.984 [6H, s, $H_3C(19)$ and $H_3C(20)$]; 1.714 [3H, s, $H_3C(18)$]; 1.616 [2H, m, $H_2C(3)$]; 1.467 [2H, m, $H_2C(2)$]; 1.029 [6H, s, $H_3C(16)$ and $H_3C(17)$]. There was considerable likeness between this spectrum and that for $C_{23}H_{32}O$.

The relative proportions of the retinal based compounds were $C_{23}H_{32}O$ 37.7%, dehydroretinal 7.0% and $C_{25}H_{34}O$ 55.3%.

iii. Fraction 2.

The initial absorption spectrum of this fraction was indicative of an α- or ε-chromophore (λ_{max} in ethanol at 421.5 444.5 473). On the

a



b

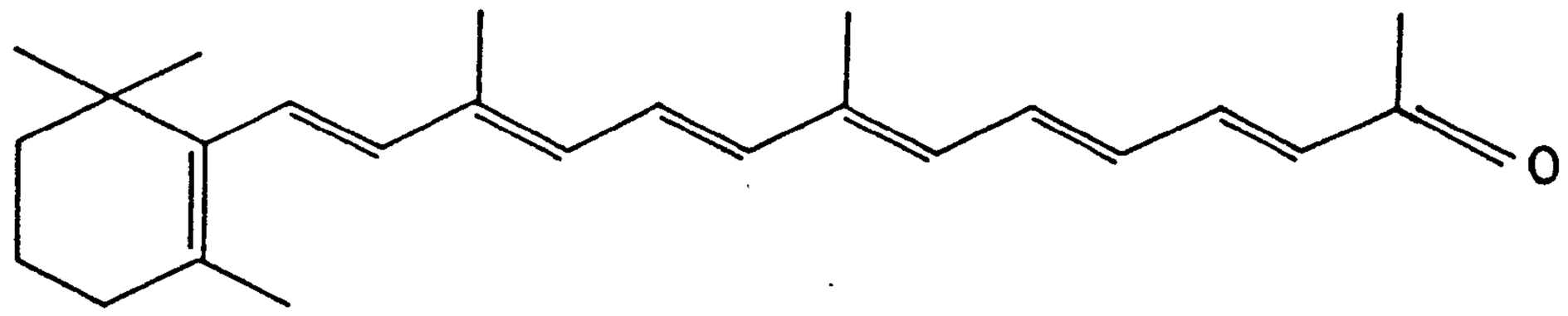


Fig. 48.

Possible structures of two retinal based compounds, a) $C_{23}H_{32}O$ and b) $C_{25}H_{34}O$, isolated from turkey retina oil droplet extracts.

basis of retention time this fraction was anticipated to be a monohydroxy carotenoid. Allegedly, β-cryptoxanthin is present in turkey retinas (Pollard, 1980); however, the absorption spectrum tends to indicate α-cryptoxanthin. The crude monohydroxy carotenoid was further purified on an ODS Hypersil 5μ column (200 mm x 4 mm) with acetonitrile/propanol (96:4, v/v) as solvent with flow rate of 2 ml/min. The absorption spectrum of the pure carotenoid is shown in Fig. 49. The presence of α-cryptoxanthin as opposed to β-cryptoxanthin was confirmed by the Roche research group.

The configurational isomers of cryptoxanthin can be determined after derivatisation to the carbamates and h.p.l.c. separation (Rüttimann et al., 1983). H.p.l.c. of cryptoxanthin carbamates was used to verify the monohydroxy carotenoid structure. Although an α-cryptoxanthin carbamate standard was not available, the β-cryptoxanthin carbamates were used for analogy studies. The turkey monohydroxy carotenoid was eluted at a time between 3S- and 3R-β-cryptoxanthin:

	retention time (min)
3S-β-cryptoxanthin	15.94
α-cryptoxanthin	17.41
3R-β-cryptoxanthin	17.83

The mass spectrum of the purified monohydroxy carotenoid was consistent with the α-cryptoxanthin structure. M.s.: m/z (M^+), and fragment ions at m/z 460 (M-92), 404 (M-92-56), 159, 119.

N.m.r. analysis of the fraction verifies the α-chromophore. ^{1}-H n.m.r.: δ (p.p.m.)[$CDCl_3$]; 6.0-6.7 [m, vinyl-H]; 5.53 [1H, dxd, J_1 9.4 J_2 9.6, H-C(7')]; 5.4 [1H, s, H-C(4')]; 2.183 [1H, m, H-C(6')]; 1.964 [9H, s, H_3C(19), H_3C(20) and H_3C(20')]; 1.91 [3H, s, H_3C(19')]; 1.74 [3H, s, H_3C(18)]; 1.584 [3H, s, H_3C(18')]; 1.074 [6H, s, H_3C(16) and H_3C(17)]; 0.822 and 0.903 [6H, 2s, H_3C(16') and H_3C(17')]. This compares very well

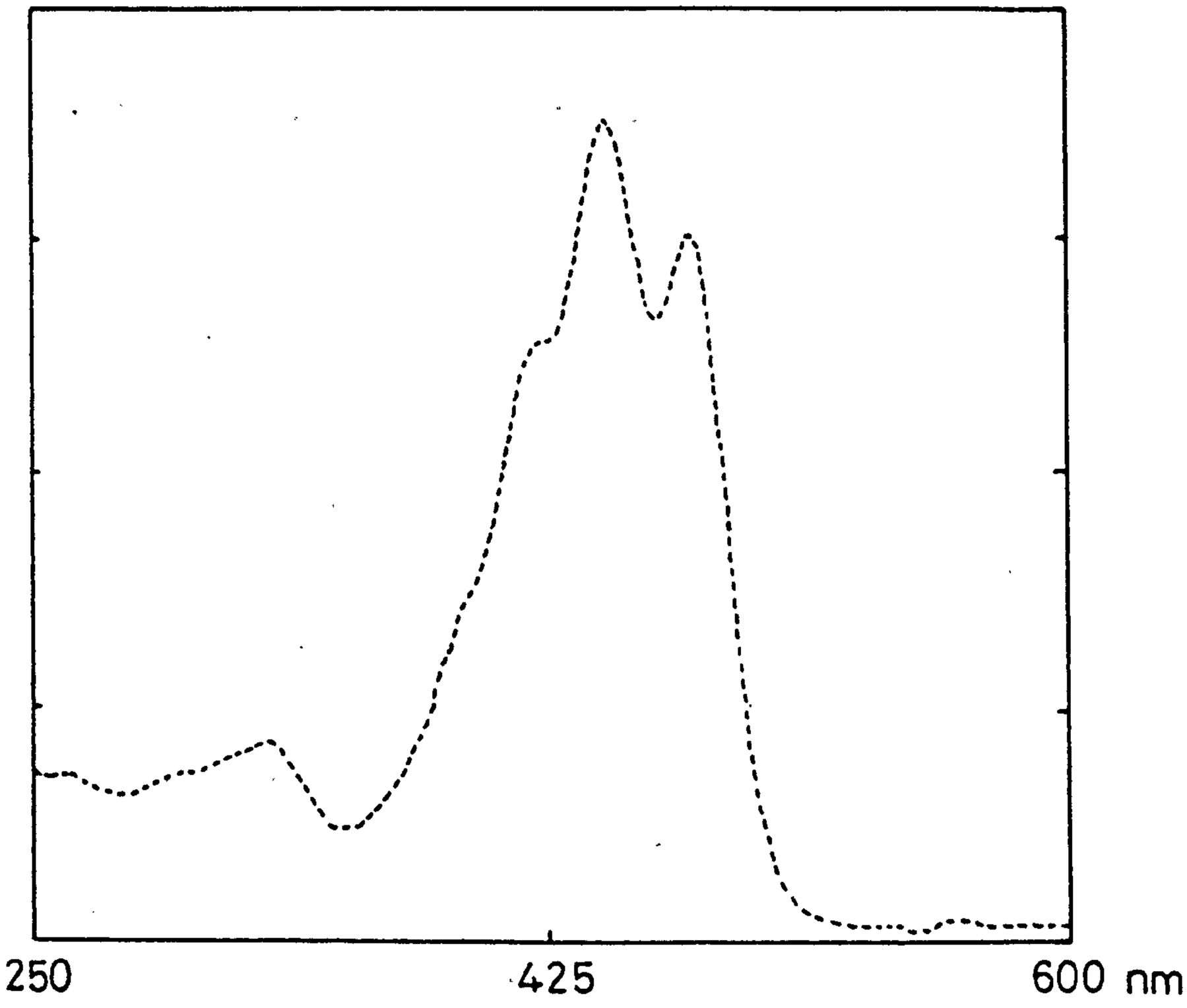


Fig. 49.

Absorption spectrum of purified fraction 2 (α-cryptoxanthin) recorded in ethanol.

with n.m.r. data for α-cryptoxanthin from Buchecker and Eugster (1979).

The absolute configuration of the α-cryptoxanthin was defined as 3R,6'S by c.d. analysis. The c.d. spectrum (see Fig. 46b) of α-cryptoxanthin (concentration 0.021 mg/ml) in EPA was recorded over the range 205-430 nm in a 0.5 cm cell pathlength. C.d. (EPA); 217.8 (-0.18), 264.3 (-6.9), 295.2 (0.76), 327 (-1.68), 351.9 (0.04), 394.7 (-1.68).

iv. Fraction 3.

The first absorption spectrum (see Table 22) indicated the presence of a ketocarotenoid but on further purification of this fraction by h.p.l.c. on the same LiChrosorb system the shape of the absorption spectrum became far more persistent (λ_{max} in ethanol at 419 440 and 467). The retention time of the fraction (7.7 min) required that the carotenoid was more polar than a monohydroxy carotenoid but less polar than ε,ε-carotene-diol. Schiedt et al. (1981b) detected 3'-O-didehydrolutein in chicken egg yolk. Although the spectrum of fraction 3 was not identical to that from the work of Schiedt et al. (1981b), this peak was tentatively identified as 3'-O-didehydrolutein. The Roche research group later confirmed the presence of dehydrolutein in turkey retinal extracts.

v. Fraction 4.

From its position on the chromatogram it was anticipated that this fraction was a dihydroxy carotenoid. The less polar nature (shorter retention time) than lutein implied that this carotenoid must have two ε-rings. The absorption spectrum compares well to that of ε,ε-carotene-diol isolated in turkey retinal extracts in previous studies (Pollard, 1980).

On rechromatography of fraction 4 on the same LiChrosorb system two peaks were observed (4.1 and 4.2). These fractions were collected separately and absorption spectra obtained. Fraction 4.1 had an absorption spectrum corresponding to ε,ε-carotene-diol and on the basis of absorption spectrum and retention time, fraction 4.2 was tentatively identified as lutein.

A methylation reaction consisting of the addition of HCl to a methanolic solution of carotenoid is a specific test for allylic hydroxyl groups. ε,ε-Carotene-diol would react to form a monomethoxy- and dimethoxy carotenoid.

Hydrochloric acid (2 drops of 0.2M HCl) was added to 10 ml of methanolic carotenoid and the reaction allowed to proceed for 3 h at room temperature. At the end of this time, the carotenoid was re-extracted into ether and the products of the reaction separated by h.p.l.c. on the LiChrosorb system (see Fig. 50a). Peak 4 corresponds to unreacted material but as previously stated only 2 other peaks were anticipated. Spectra were obtained of fractions 1-3 and from these 1 and 2 were identified as ε,ε-carotene-diol derivatives. Contamination of the starting material (fraction 4.1) with lutein would result in one product of methylation of lutein. A sample of standard lutein was methylated, as above, and the products separated by h.p.l.c. on the LiChrosorb system (Fig. 50b). Comparison of retention times clearly demonstrates that peak 3 of Fig. 50a is the lutein methoxy product.

M.s. data, although not indicative of a structure with ε-end groups, confirms the dihydroxycarotenoid structure. M.s.: m/z 568 (M^+), and fragment ions m/z 550 (M-18), 532 (M-18-18), 422, 408, 309.

Rønneberg et al. (1978), Bingham et al. (1979) and Siefermann-Harms et al. (1981) confirm the structure of ε,ε-carotene-diol on the basis of mass spectral data, giving only M-18 and M-18-18 as diagnostic peaks.

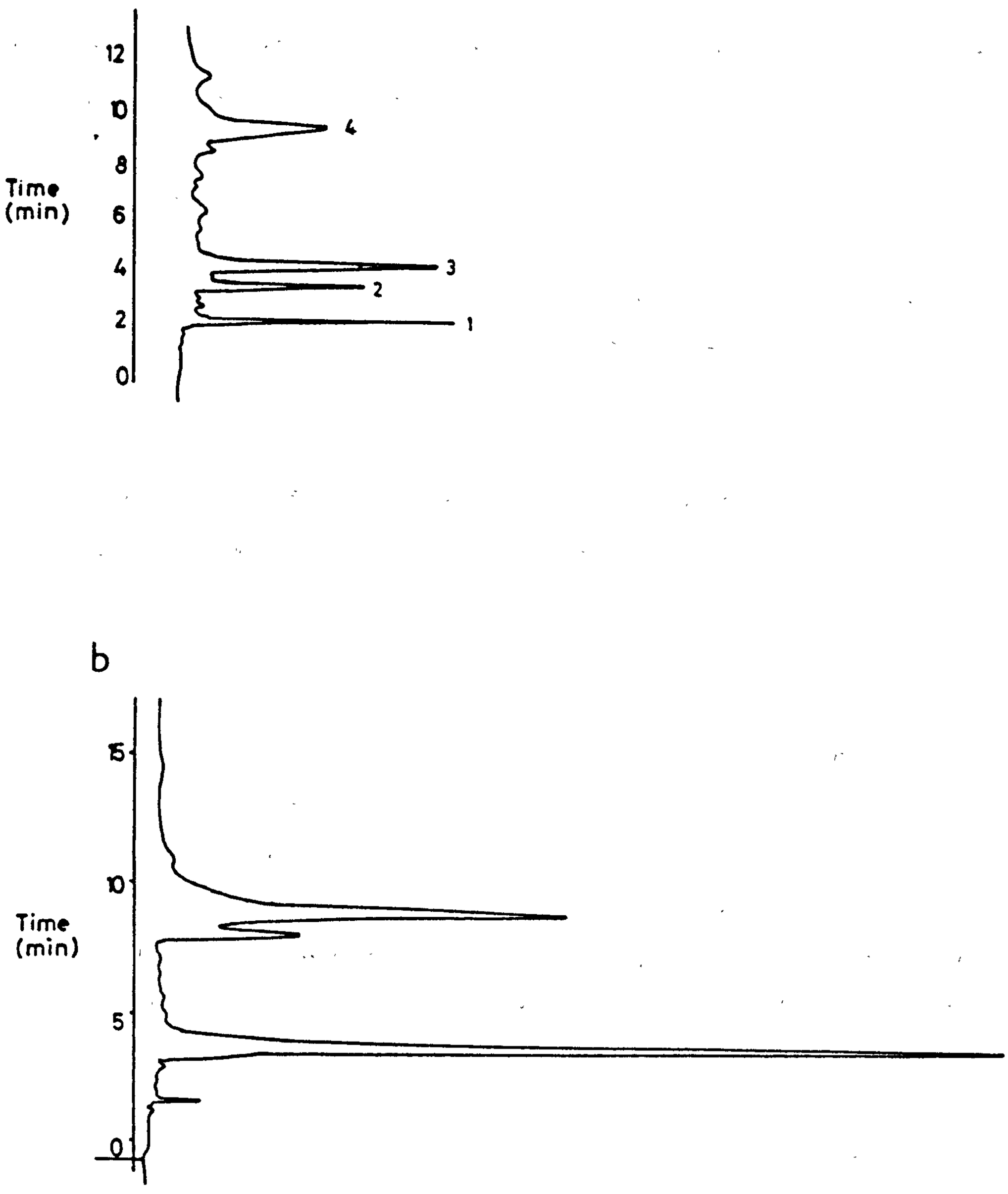


Fig. 50.

H.p.l.c. elution profile of a) fraction 4.1 methylation products and b)lutein methylation products, on LiChrosorb Si-60, solvent hexane/propan-2-ol/CH_2Cl_2 (92:5:3, v/v) at 1.5 ml/min.

The allylic character of the two hydroxyl groups was proved by the methylation reaction in each case.

vi. Fraction 5.

The initial absorption spectrum of this peak indicated the presence of lutein. More detailed analysis by Roche afforded the chirality of the lutein and 3'-epilutein present.

Both the lutein and epilutein fractions were derivatised to form their respective dicarbamates. In this form configurational isomers of the compounds could be resolved (Rüttimann et al., 1983).

On h.p.l.c. analysis of the lutein dicarbamate, two peaks were obtained which were identified, by comparisons with standards, as 3R,3'R,6'R- and 3R,3'S,6'S or 3S,3'R,6'R lutein. After c.d. analysis the second peak was assigned the 3R,3'S,6'S chirality. The c.d. spectrum was not good but on the basis of the negative cotton effect displayed by the compound the lutein was assigned as 3R,3'S,6'S. The c.d. spectrum was recorded over a range 204-420 nm with a 1 cm cell pathlength. The solvent used was EPA with a final carotenoid concentration of 0.027 mg/ml. C.d. (EPA): 204 (-20.42), 248.9 (-7.55). 267.6 (-8.57), 366.1 (-1.43), 379.8 (-1.83), 420 (1.06). The relative proportions of 3R,3'R,6'R - and 3R,3'S,6'S lutein was approximately 1:1.

By the same experimentation, the epilutein was found to be 3R,3'R,6'S-epilutein (46%) and 3R,3'S,6'R or 3S,3'R,6'S-epilutein (54%). C.d. spectroscopy distinguished between these latter two forms. The c.d. spectrum was recorded over the range 197-430 nm with a 0.2 cm cell pathlength. A carotenoid concentration of 0.051 mg/ml was used. C.d. (EPA): 197 (-10.14), 238.6 (0.41), 273.3 (-7.76), 338.8 (-0.31), 367.4 (-0.75). This was consistent with 3S,3'R,6'S-epilutein.

vii. Fraction 6.

This fraction was shown to be zeaxanthin by its absorption spectrum and its cochromatography with standard zeaxanthin.

H.p.l.c. of the zeaxanthin dicarbamates (Rüttimann et al., 1983) from turkey retina revealed that the zeaxanthin was not enantiomerically pure, as previously reported. The zeaxanthin was found to be (3R,3'R)zeaxanthin (72%) and (3R,3'S)zeaxanthin (28%).

viii. Fraction 7.

The absorption spectrum of F7 is of interest because Pollard (1980) failed to detect any carotenoid displaying similar properties. However, the presence of a carotenoid with a comparable absorption spectrum was noted in chicken retinal extracts by Stransky and Schultze (1977).

Considering the absorption spectrum (Fig. 51) and chromatographic properties of Fraction 7 a possible explanation of its structure is that it is the ε,ε-carotene-diol equivalent of zeaxanthin cleavage to galloxanthin i.e. an ε-galloxanthin.. This would account for its being slightly less polar than galloxanthin and for its very persistent absorption spectrum at appropriate wavelengths (i.e. approximately corresponding to the presence of 7 conjugated double bonds).

The proposed structure of this hitherto-undefined carotenoid is depicted in Fig. 52.

M.s. data are essential for verification of a structure. From previous experience with galloxanthin it has been found that m.s. analysis of the acetylated carotenoid is more successful. Acetylation would also confirm the dihydroxy- nature of the carotenoid.

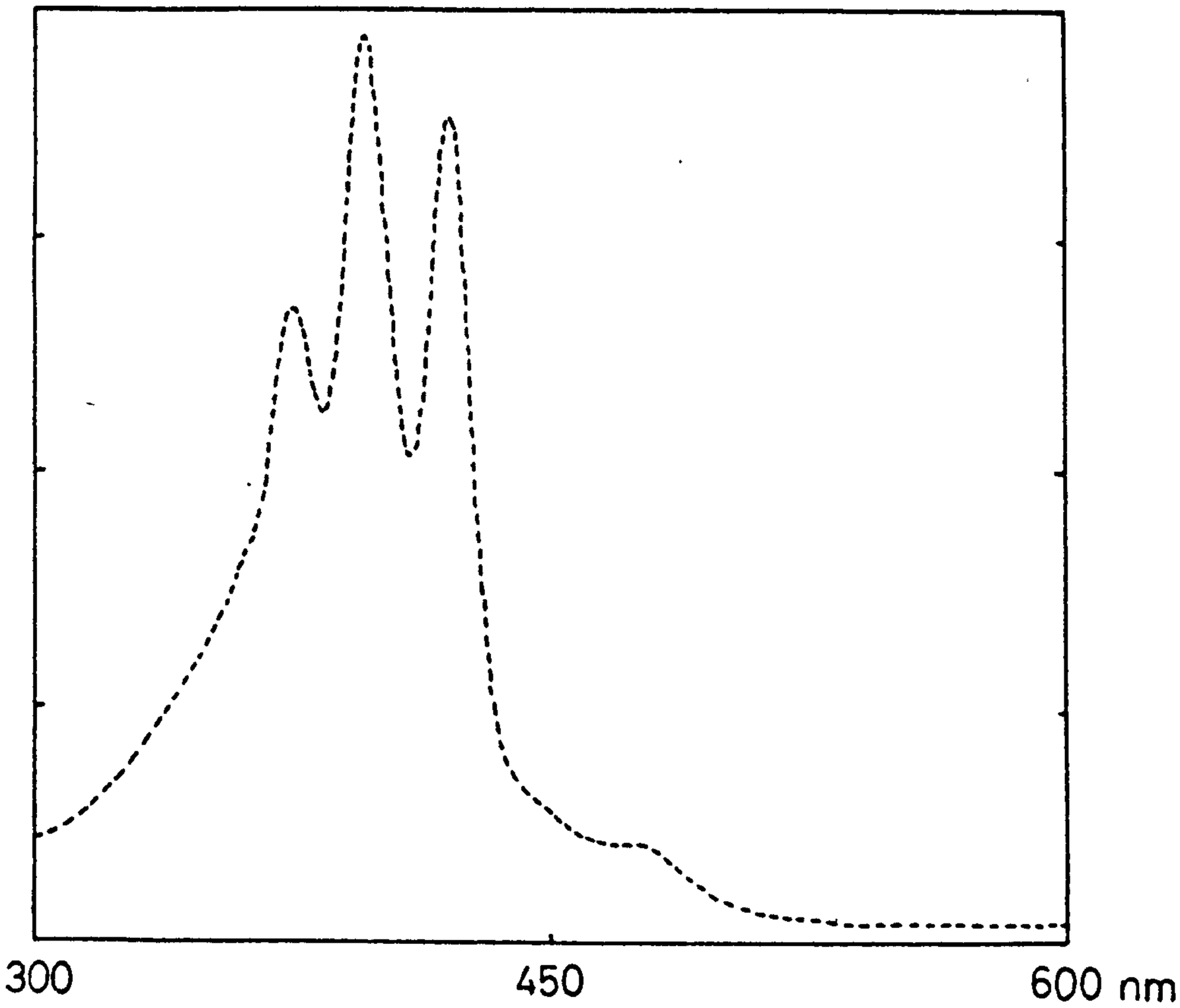


Fig. 51.

Absorption spectrum of fraction 7 recorded in ethanol.

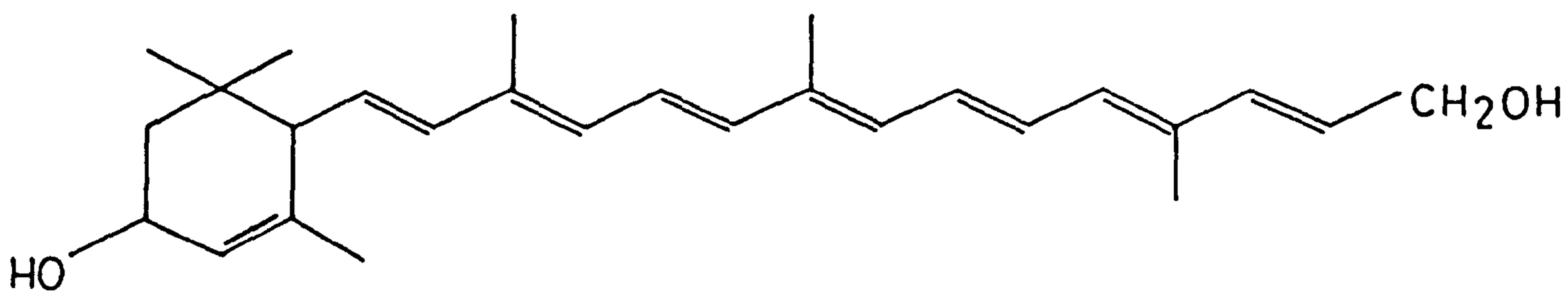


Fig. 52.

The proposed structure of the ε-galloxanthin of fraction 7.

Acetylation was carried out by a method modified from sterol acetylation procedures routinely used in this laboratory. Acetylation was achieved by the addition of acetic anhydride and pyridine (1:2, v/v) to the 'dry' carotenoid. The mixture was left at 40-45 °C for 30 min after which time the reaction was terminated by the addition of ice-cold water. The carotenoids were extracted into ether.

On the LiChrosorb h.p.l.c. system used throughout this work 2 peaks were observed for the acetylation products; a monoacetate- and diacetate-carotenoid. The diacetate was submitted for m.s. analysis but insufficient material was available to produce a spectrum.

The Roche research group confirm the presence of a carotenoid with appropriate characteristics (absorption spectrum and chromatographic properties) for the ε-galloxanthin structure. Further evidence is still required for the absolute confirmation of the structure (m.s., n.m.r. and c.d.)

ix. Fraction 8.

This is clearly a mixture of two carotenoids. It is feasible that one component may be 'ε-galloxanthin'. Further analysis failed positively to identify the components of the fraction.

x. Fraction 9.

The absorption spectrum of fraction 9, collected directly from h.p.l.c. of total neutral carotenoids clearly indicates that this fraction is galloxanthin (λ_{max} in ethanol at (380) 401 and 423 nm).

M.s. data are essential for the conclusive identification of a carotenoid structure. The polar nature of galloxanthin results in poor

m.s. data. Consequently, m.s. of the diacetate of galloxanthin was obtained.

The galloxanthin was acetylated by the addition of 1 ml acetic anhydride and 2 ml dry pyridine and the reaction allowed to proceed for 30 min at 40 - 45°C. Galloxanthin diacetate was separated from unreacted galloxanthin and its monoacetate by h.p.l.c. on the LiChrosorb system (retention times of 2.2, 15.6 and 4.6 min, respectively). M.s. data are consistent with the galloxanthin diacetate structure. M.s.: m/z 478 (M^+), 418 (M-60), 368 (M-110), 358 (M-60-60).

N.m.r. data are also consistent with the structure of galloxanthin diacetate. ^{1}-H n.m.r. δ (p.p.m.)[$CDCl_3$]; 6.0-6.7 [m, vinyl-H]; 5.772 [1H, dxt, J_1 15.6 J_2 6.2, H-C(11')]; 5.057 [1H, dxdxdxd, H-C(3)]; 4.653 [2H, d, J 6.4, H-C(10')]; 2.446 [1H, dxd, J_1 3.6 J_2 ?, H_{eq}-C(4)]; 2.049 [3H, s, H_3CCOOC(3)]; 1.967 [6H, s, H_3C(19) and H_3C(20)]; 1.902 [3H, s, H_3C(20')]; 1.72 [3H, s, H_3C(18)]; 1.0747 and 1.106 [6H, 2s, H_3C(16) and H_3C(17)].

A c.d. spectrum was recorded of a solution (concentration 0.12 mg/ml) of galloxanthin diacetate in EPA. The curve was recorded from 195 to 430 nm in a 0.1 cm cell pathlength. C.d. (EPA); 203 (-5.75), 248.2 (-0.18), 293.5 (-4.62), 321.3 (-0.12), 340 (0.14), 367.4 (-0.48), 409.1 (0.15). The c.d. spectrum in EPA solution is shown in Fig. 46c. This spectrum is consistent with the 3R configuration of galloxanthin.

xi. Fractions 10 -12.

These fractions were tentatively identified as cis-isomers of galloxanthin and ε-galloxanthin from their absorption spectra.

B. Acidic carotenoids.

The major component of the acidic fraction from turkey retinal extracts was astacene (saponification product of astaxanthin). From metabolic considerations, the chirality of the astaxanthin was crucial.

The configurational isomers of astaxanthin can be determined after derivatisation to the (-)-dicamphanates and h.p.l.c. separation (Vecchi and Müller, 1979). After anaerobic saponification (to yield free astaxanthin from the astaxanthin esters) and derivatisation, the astaxanthin of turkey retinal carotenoids was established as (3S,3'S)-(66%), meso- (30%) and (3R,3'R)astaxanthin (4%).

These proportions correlate well with the corresponding enantiomeric zeaxanthins (note that the 3S,3'S hydroxyls have the same orientation as the 3R,3'R hydroxyls of astaxanthin).

SUMMARY

Table 23 summarises the composition of turkey retinal carotenoids. The quantitative data are provided by the Roche research group.

4.26 Chicken retinal carotenoids.

A. Neutral carotenoids.

Essentially, the h.p.l.c. elution profile is the same for chick retinal and turkey retinal carotenoids (Fig. 53 shows that of the chicken retinal carotenoids). One noticeable difference can be seen in peak 5 where the chick extract trace shows a more prominent peak, rather than a shoulder, as well as the main lutein peak. The qualitative differences are obvious, especially, the low levels of galloxanthin.

TABLE 23.

Composition of turkey retinal carotenoids.

Carotenoid	Percentage of total carotenoid
Astaxanthin	23.2
ε,ε-Carotene	12.0
α-Cryptoxanthin	2.4
3'-Dehydrolutein	0.01
Epilutein	8.0
Galloxanthin	29.5
ε-Galloxanthin	1.3
Lutein	1.0
Retinal derivatives	6.0
Zeaxanthin	8.9
Unidentified	8.0

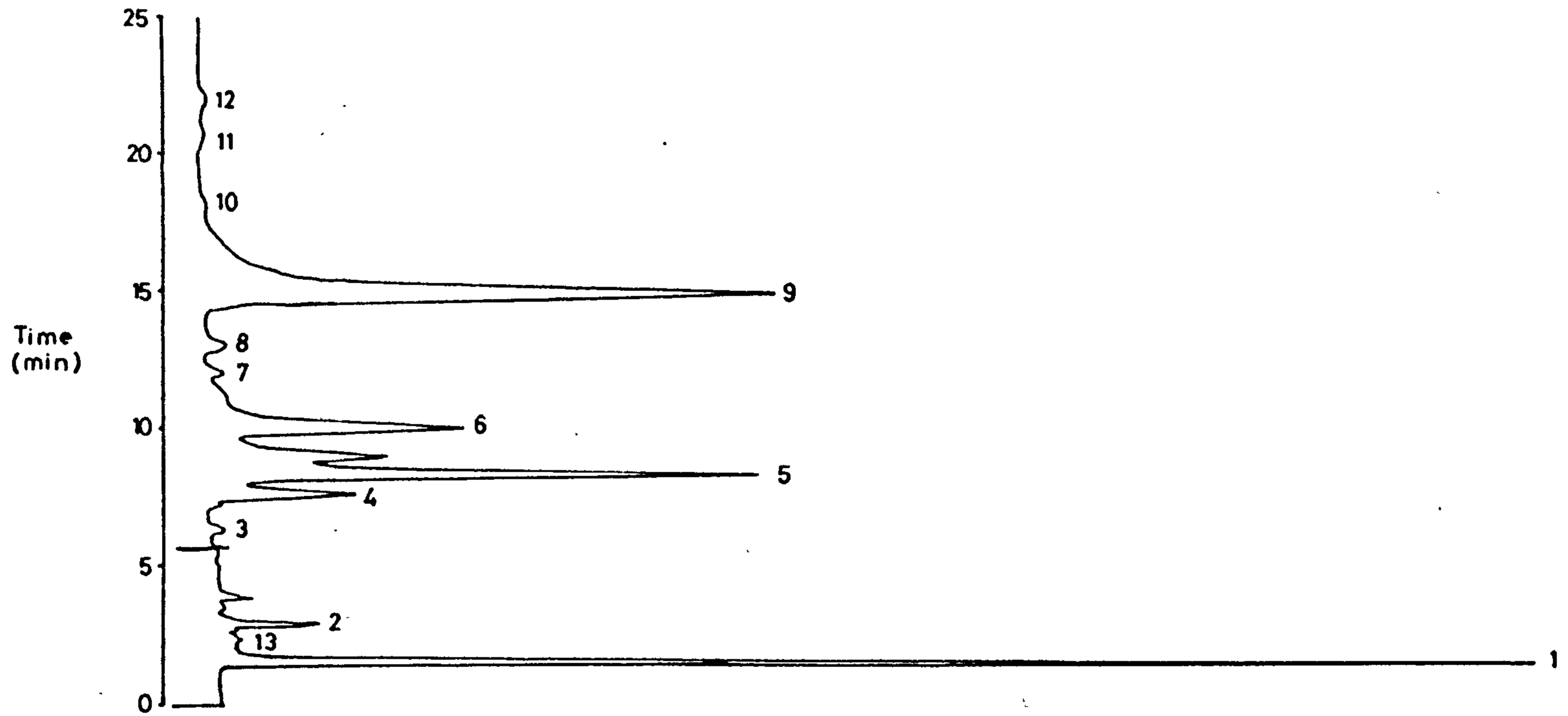


Fig. 53.

H.p.l.c. elution profile of total neutral carotenoids of chicken retinal oil droplets on LiChrosorb Si-60, solvent hexane/propan-2-ol/ CH_2Cl_2 (92:5:3, v/v) at 1.5 ml/min.

There was no evidence to suggest the presence of a carotenoid of similar structure to galloxanthin, but two carbons shorter. The presence of this carotenoid structure was inferred by microspectrophotometric examination of chicken oil droplets (Goldsmith et al., 1984). This C_{25} carotenoid 'fringillixanthin' was not investigated in any way and thus it is feasible that the Goldsmith et al. (1984) fringillixanthin may be ε-galloxanthin.

As Stransky and Schultze (1977) did not detect any carotene fraction in chicken retina at all, further work was carried out on the h.p.l.c. fraction assumed to be a carotene (peak 1). Previous work (Davies, 1983; unpublished work) suggested that there was indeed ε,ε-carotene in chick retina as shown by chromatographic properties, absorption spectra and m.s. data.

From a metabolic standpoint, the nature of the cryptoxanthin present in chick retinal carotenoids is important. α-Cryptoxanthin can be postulated as intermediate between zeaxanthin and ε,ε-carotene. Bearing in mind the high degree of comparability between chick and turkey retinal carotenoids thus far, and the positive identification of α-cryptoxanthin in turkey retinas, the nature of the chick cryptoxanthin (fraction 2) was investigated.

i. Chicken carotene fraction (fraction 1).

The chick retinal carotene fraction was collected from successive injections of total neutral carotenoids on the LiChrosorb h.p.l.c. system. An absorption spectrum of the fraction gave a fairly persistent profile.

The carotene fraction was chromatographed on ODS Hypersil as described in Section 4.17B. Three peaks were observed as shown in the

h.p.l.c. elution profile of Fig. 54. The relevant fractions were collected and the absorption spectrum measured of each; fraction 1 (λ_{max} in light petroleum at (425) 445 471 nm; retention time, 16.0 min), fraction 2. (λ_{max} in light petroleum at 414 438 468 nm; retention time, 21.4 min) and fraction 3 (λ_{max} in light petroleum at (425) 445 470 nm; retention time, 25.8 min). The anticipated order of elution of the carotenes from this system (Pollard, 1980) was ε-, α- and finally β-carotene. Standard β-carotene when chromatographed on this system had retention time of 25.8 min.

From the above information it can be concluded that ε,ε-carotene is not the sole carotene of chick retinal extracts. There is little doubt that there is some β-carotene and another unidentified carotene present.

The presence of β-carotene amongst the carotenoids of chick retinal extracts means that radioactivity detected, if any, in the carotene fraction of extracts from embryos developed in the presence of radiolabelled zeaxanthin is not necessarily due to the metabolism of zeaxanthin to ε,ε-carotene. To eliminate the possibility that any radioactivity was due to the metabolism of zeaxanthin to β-carotene, the carotene fraction in the metabolic experiments was collected each time and further resolved on the ODS Hypersil system. Any radioactivity in the carotene fraction could then be assigned to the appropriate carotene.

ii. Chick cryptoxanthin fraction (fraction 2).

Stransky and Schultze (1977) describe the cryptoxanthin of chick retina as "identical with β-cryptoxanthin". An absorption spectrum of a h.p.l.c.-pure sample of the cryptoxanthin fraction was persistent and more typical of α-cryptoxanthin than β-cryptoxanthin(λ_{max} in ether at 423 446 and 474).

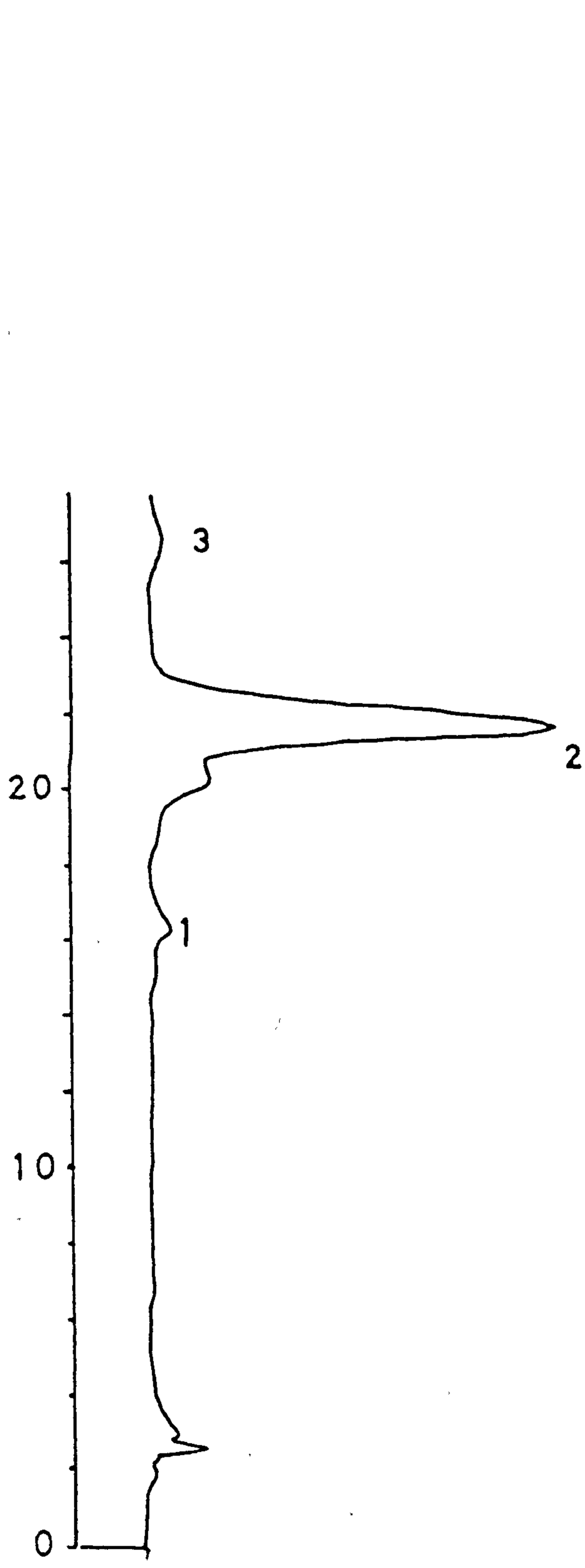


Fig. 54.

H.p.l.c. elution profile of the carotene fraction from chicken retinal oil droplet extracts on ODS Hypersil 5μ, solvent acetonitrile/propan-2-ol (95:5, v/v) at 2 ml/min.

B. Acidic carotenoids.

As with turkey retina, astacene was detected in the extracts. The proportion of astacene in chick retinal extracts is substantially higher than for turkey extracts. Although several attempts were made to detect phoenicoxanthin in chicken retinal carotenoid extracts, on no occasion was there any evidence for the presence of this carotenoid.

4.27 Chicken liver analysis.

Digitonin treated liver extracts afforded relatively sterol-free non-saponifiable material.

An absorption spectrum of total chick liver carotenoids was indicative of the presence of mainly α-chromophore carotenoids (λ_{max} in light petroleum at 422 443 and 473 nm). Pollard (1980) isolated substantial quantities of lutein from turkey livers.

H.p.l.c. of the extract on the Flavobacterium system (Section 2.13) was carried out, with monitoring at 380 and 440 nm. With detection at 440 nm, 2 major peaks were observed with retention times of 2.7 and 6.1 min. Of these two peaks the latter was by far the major carotenoid present. At 380 nm a single large peak was observed with retention time of approximately 4 min. At this stage it was assumed that this fraction was vitamin A although no vitamin A was detected in turkey liver extracts (Pollard, 1980).

From the h.p.l.c. trace it was possible to divide the total non-saponifiable lipid into three fractions; carotene, vitamin A and xanthophyll fractions. Each fraction was analysed separately.

i. Chicken liver carotene fraction.

On the ODS Hypersil system described in Section 4.17B the carotene of chicken liver was identified as β-carotene alone.

ii. Chicken liver vitamin A.

The crude vitamin A fraction from chicken liver extract was studied further on the h.p.l.c. system devised for goldfish extracts (see Section 3.16). Three fractions were collected from the h.p.l.c. as indicated in Fig. 55. From its absorption spectrum fraction 2 was clearly retinol. In fraction 3 the absorption maxima at 276 and 286 nm were indicative of vitamin A_2, although the third maximum (340.5 nm) was too low. On rechromatography of fraction 3 on the system, two clear peaks were seen which corresponded to the retention times of vitamin A_1 and A_2. An absorption spectrum of the second fraction verified the presence of vitamin A_2 in chick liver extracts (λ_{max} in ethanol at 274 286 and 351 nm). The origin of this dehydroretinol is unknown. Analysis of a standard chicken feed may reveal the presence of retinol and dehydroretinol.

iii. Chick xanthophyll fraction.

The xanthophyll of chick liver had a retention time of 6.1 min on the Flavobacterium system. An absorption spectrum of this fraction implied the presence of lutein (λ_{max} in acetone at 422 445 and 472 nm). The fraction was then chromatographed on the LiChrosorb system. Co-chromatography with lutein confirmed that the xanthophyll fraction was composed of lutein. The presence of lutein was not unexpected as this was seen to be the major carotenoid of turkey liver (Pollard, 1980).

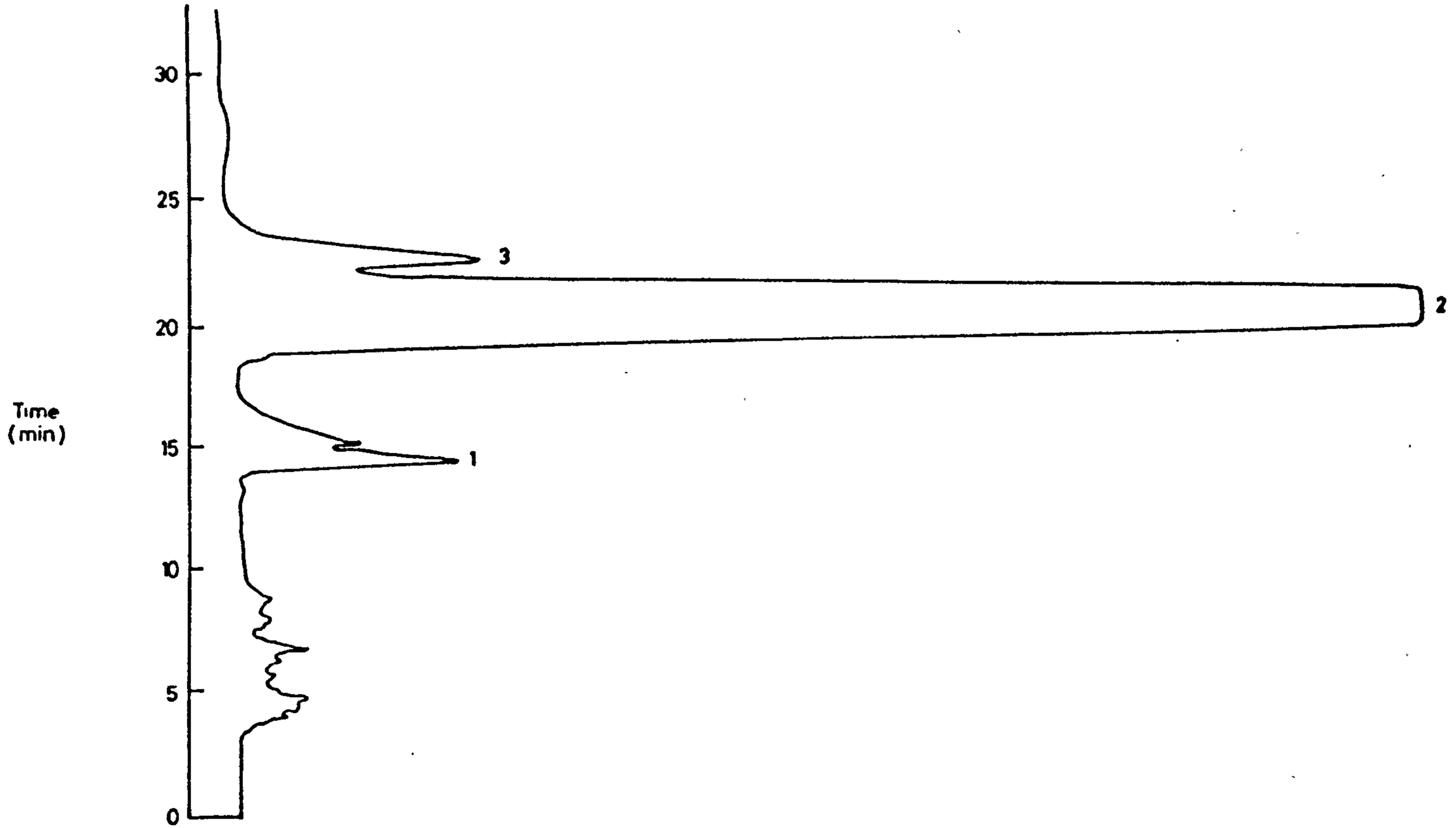


Fig. 55.

H.p.l.c. elution profile of the crude vitamin A fraction of chicken liver extracts on Nucleosil 50-5, solvent hexane/1,4-dioxan (9:1, v/v) at 1 ml/min.

4.28 Turkey feed carotenoids.

A thorough quantitative analysis of turkey feeds was carried out by Pollard (1980). Consequently, the purpose of this analysis was to look at some of the carotenoids present in more detail. Pollard (1980) observed that there were no acid carotenoids in the feed. Absorption spectra of the total carotenoid from the different feeds were very similar with α-carotene type persistence. The findings of this work were consistent with the above. The major components in all feeds were lutein and zeaxanthin (63 - 76% total carotenoid; Pollard, 1980) with other unidentified xanthophylls also present. The carotenes (α-carotene and β-carotene; Pollard, 1980) accounted for only 4 - 15% of the total carotenoid. No ε-carotene was present in any feeds.

Classical l.c. of the feed extracts on neutral Alumina (Brockmann Grade III) generated several fractions. The carotenes were eluted with 100% light petroleum, the vitamin A fraction eluted with 20 - 50% ether in light petroleum and the dihydroxycarotenoids with 2% ethanol in ether. Only the vitamin A fractions and the dihydroxycarotenoids were analysed further.

A. Vitamin A fraction.

Retinol was present in all feeds but in relatively low amounts. Dehydroretinol was not present in any of the extracts at all. Its absence was clear from h.p.l.c. of the crude vitamin A fractions on the Nucleosil system devised for the separation of retinol and dehydroretinol from goldfish (Section 3.16).

B. Dihydroxycarotenoids.

The dihydroxycarotenoid fractions were rechromatographed on Alumina (Brockmann Grade III) to eliminate contamination of the zeaxanthin and

lutein with cryptoxanthin and any more polar carotenoids. The lutein/zeaxanthin fractions from each feed were sent to Roche for analysis. Details are given in the Appendix.

By h.p.l.c. the ratio of lutein to zeaxanthin was shown to be approximately 9:1 in all feeds. Epi-lutein was shown to be present in varying amounts (0 - 2.7% total dihydroxycarotenoid fraction) as well as other unidentified carotenoids (2.6 - 9.3% total dihydroxycarotenoid fraction). Cis-isomers accounted for 13 - 20.3% of the fractions.

The dihydroxycarotenoid fractions from the five feeds were bulked prior to classical l.c. Chromatography of the fractions on MgO:celite (2:1, w/w) allowed complete separation of the zeaxanthin and lutein. Lutein was eluted with 1% methanol in ether and zeaxanthin with 2% methanol in ether. The dicarbamates of the lutein fraction and zeaxanthin fraction were prepared and from comparison of the h.p.l.c. elution profiles with standard racemic mixtures, the chirality of the zeaxanthin and lutein present in the feeds could be assigned.

The lutein (95.65% (3R,3'R,6'R)-trans-lutein and 3.64% cis-lutein) and zeaxanthin (99.88% (3R,3'R)zeaxanthin) were stereochemically pure.

4.29 Metabolic studies.

The high risk of infection of embryos following treatment resulted in few chicks surviving to hatching and hence, relatively little material was generated for analysis. Over the 6 months, during which time 19 dozen eggs were injected with radiolabelled substrate, a total of 53 chicks hatched. A few others survived to the $19^{th}/20^{th}$ day of incubation and were considered to be of some use as the oil droplets would be in the early stages of formation. Of the embryos injected with [^{14}C]zeaxanthin 28 survived, of those with [$^{3}H_2$]zeaxanthin 19 survived and 11 [2-^{14}C]MVA

treated embryos survived.

The chicks were killed and the retina, liver, intestine and feathers dissected and extracted as described in Section 4.16.

The extracts from embryos developed in the presence of [2-^{14}C]MVA were treated differently to the extracts from radiolabelled zeaxanthin treated embryos. The results will be presented in two parts; firstly, the zeaxanthin experiments and secondly, the MVA experiment.

4.30 Analysis of chick tissues from embryos developed in the presence of radiolabelled zeaxanthin.

Tables 24 and 25 summarise the results of quantitative spectrophotometry ($A^{1cm}_{1\%}$ 2500) and liquid scintillation counting of a known aliquot from each tissue extract for both [^{14}C]- and [$^{3}H_2$] zeaxanthin treated embryos.

The low levels of radioactivity and small amounts of material present are obvious and as expected. The percentage absorption of carotenoids is lower than anticipated (approximately 1% had been anticipated from previous work, Davies, 1983; unpublished work).

In the following sections the data from the [$^{3}H_2$]zeaxanthin experiments will be shown predominantly. The higher specific activity of the [$^{3}H_2$]zeaxanthin resulted in higher levels of radioactivity and hence metabolism of zeaxanthin was more clearly seen. In all cases, the [^{14}C]zeaxanthin results were consistent with the [$^{3}H_2$]zeaxanthin experiments.

Throughout the work low specific activities were being dealt with and so, where possible, 'spiking' of samples with appropriate standards

TABLE 24.

Summary of quantitative estimations of amounts of carotenoid present and incorporation into each tissue of embryos developed in the presence of [^{14}C]zeaxanthin. The data are for tissue extracts of 28 embryos which reached an acceptable stage of development and correspond to a total administration of 2.522 µCi of [^{14}C]zeaxanthin.

Tissue	Total volume (ml)	Volume counted (ml)	d.p.m.	Total d.p.m.	µCi	Percentage absorption
Feathers	10	0.5	100	2 000	0.0009	0.036
Intestine	25	0.5	341	17 050	0.0077	0.3
Liver	25	0.5	75	3 750	0.0017	0.07
Retina	25	0.5	86	4 300	0.00194	0.08

Tissue	Absorbance at λ_{max}	Wavelength maxima (in acetone nm)			Total µg	Specific activity µCi/mg
Feathers	0.22	415	422	470	8.8	0.1
Intestine	0.2	(424)	446	472	20	0.39
Liver	0.69	(426)	449	472	69	0.025
Retina	0.39	436	440	470	39	0.05

TABLE 25.

Summary of quantitative estimations of amounts of carotenoid present and incorporation into each tissue of embryos developed in the presence of [$^2H_2$]zeaxanthin. The data are for tissue extracts of 19 embryos which reached an acceptable stage of development and correspond to a total administration of 60.42 µCi of [$^2H_2$]zeaxanthin.

Tissue	Total volume (ml)	Volume counted (ml)	d.p.m.	Total d.p.m.	µCi	Percentage absorption
Feathers	10	0.1	267	26 700	0.012	0.02
Intestine	25	0.1	597	149 250	0.067	0.12
Liver	25	0.1	1997	499 250	0.225	0.376
Retina	25	0.1	1232	308 000	0.139	0.23

Tissue	Absorbance at λ_{max}	Wavelength maxima (in acetone nm)				Total µg	Specific activity µCi/mg
Feathers	0.11		(424)	446	470	4.4	2.77
Intestine	0.15		(424)	446	470	15	8.0
Liver	1.175		(424)	445	467	117.5	1.93
Retina	0.24	399	423	448	470	24	5.83

was avoided. In this way the total samples could be injected onto h.p.l.c. systems without loss of resolution due to the overloading of columns. Another advantage of not spiking the extracts was that a direct comparison could be made between adult and embryonic carotenoids in various tissues.

4.31 Analysis of retinal extracts from chick embryos treated with [^{14}C]zeaxanthin or [$^{3}H_2$]zeaxanthin.

As has been seen from the h.p.l.c. elution profile of Fig. 53, the LiChrosorb system gives clean separation of all the neutral carotenoids of importance from a metabolic consideration i.e. ε-carotene, α-cryptoxanthin, lutein , zeaxanthin, ε-galloxanthin and galloxanthin. Consequently, the analysis of the neutral retinal carotenoids was carried out by h.p.l.c.

The astacene fractions were dealt with separately as was the case for the carotene fraction, once separated from the other neutral carotenoids.

A. Neutral carotenoids.

i. [$^{3}H_2$]zeaxanthin treated embryos.

The initial chromatography of these carotenoids on the LiChrosorb system was poor although the general profile was consistent with that for adult retinal extracts. Quantitative differences were apparent, notably there was much more galloxanthin and ε-galloxanthin in the embryonic sample. After 25 minutes, the time required to elute all the neutral carotenoids, the chart speed was reduced to 10 cm/h and the system left running. Unexpectedly, a large peak emerged at retention time 45 min. An absorption spectrum revealed the ketocarotenoid nature of this unidentified carotenoid (λ_{max} in acetone at 469 nm). The very polar

nature of the carotenoid would accommodate a structure such as phoeniconone, which, undetected in chick retinal extracts in this present study, was recorded in chicken retinas by Stransky and Schultze (1977). Alternatively, this carotenoid may be astacene. The solvent was removed by rotary evaporation, the residue redissolved in a small volume of solvent and transferred to a scintillation vial. The solvent was removed under a stream of nitrogen, scintillation fluid added (10 ml) and the vial counted. The total radioactivity of this fraction was 18,401 d.p.m. (8.36×10^{-3} µCi).

The total neutral carotenoid fractions from the first h.p.l.c. analysis were collected, bulked and 'spiked' with 'cold' adult chicken retinal sample and rechromatographed on the LiChrosorb system. The h.p.l.c. elution profile of Fig. 56 shows the chromatography and relevant fractions collected. Fractions were collected directly into scintillation vials every 30 s along the chromatogram, except for peaks, which were collected as separate fractions regardless of the time element. Several fractions, where chromatography was poor, were further purified by rechromatography on the same system e.g. zeaxanthin (fraction 16), ε,ε-carotene-diol (fraction 13), α-cryptoxanthin (fraction 5) and ε-galloxanthin (fraction 20; an absorption spectrum of the final fraction demonstrated its purity).

The fractions of importance in terms of zeaxanthin metabolism are labelled at the appropriate place along the chromatogram of Fig. 56.

Solvent was removed (under a stream of nitrogen) from all the vials except number three (the carotene fraction) and scintillation fluid (10 ml) added. The vials were counted for a statistically acceptable length of time. The histogram of Fig. 57 represents the radioactivity per vial along the chromatogram. It can readily be seen that vials containing significant amounts of radioactivity coincide with the zeaxanthin, α-cryptoxanthin, ε-galloxanthin and galloxanthin peaks. This

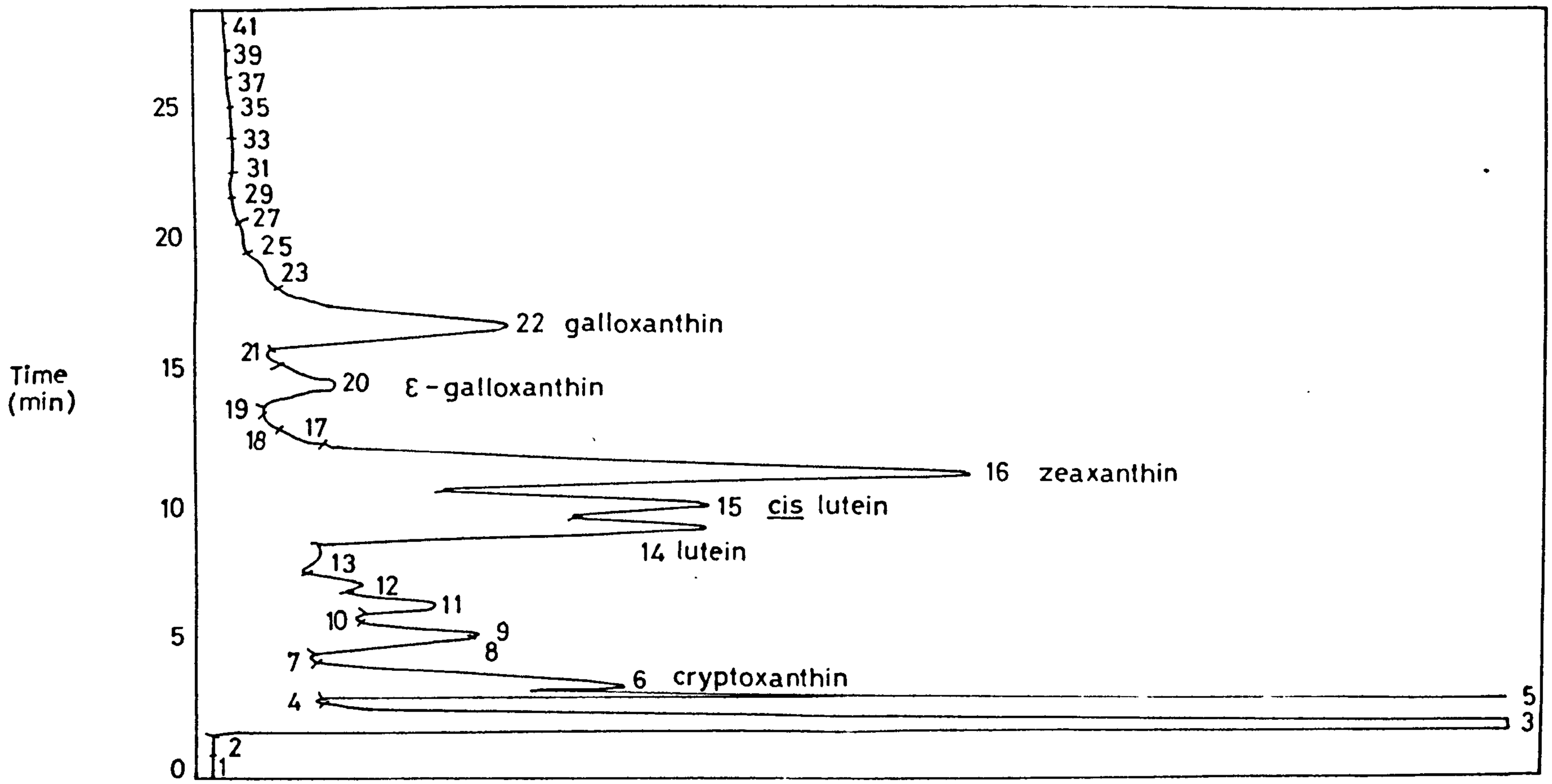


Fig. 56.

H.p.l.c. elution profile of retinal neutral carotenoids from chick embryos developed in the presence of [$^2H_2$]zeaxanthin and 'spiked' with 'cold' adult chick retinal sample. Column: LiChrosorb (250 mm x 4.5 mm); solvent: hexane/propan-2-ol/CH_2Cl_2 (92:5:3, v/v) at 1.5 ml/min. Fractions were collected as shown on the chromatogram and the fractions of importance in terms of zeaxanthin metabolism are labelled.

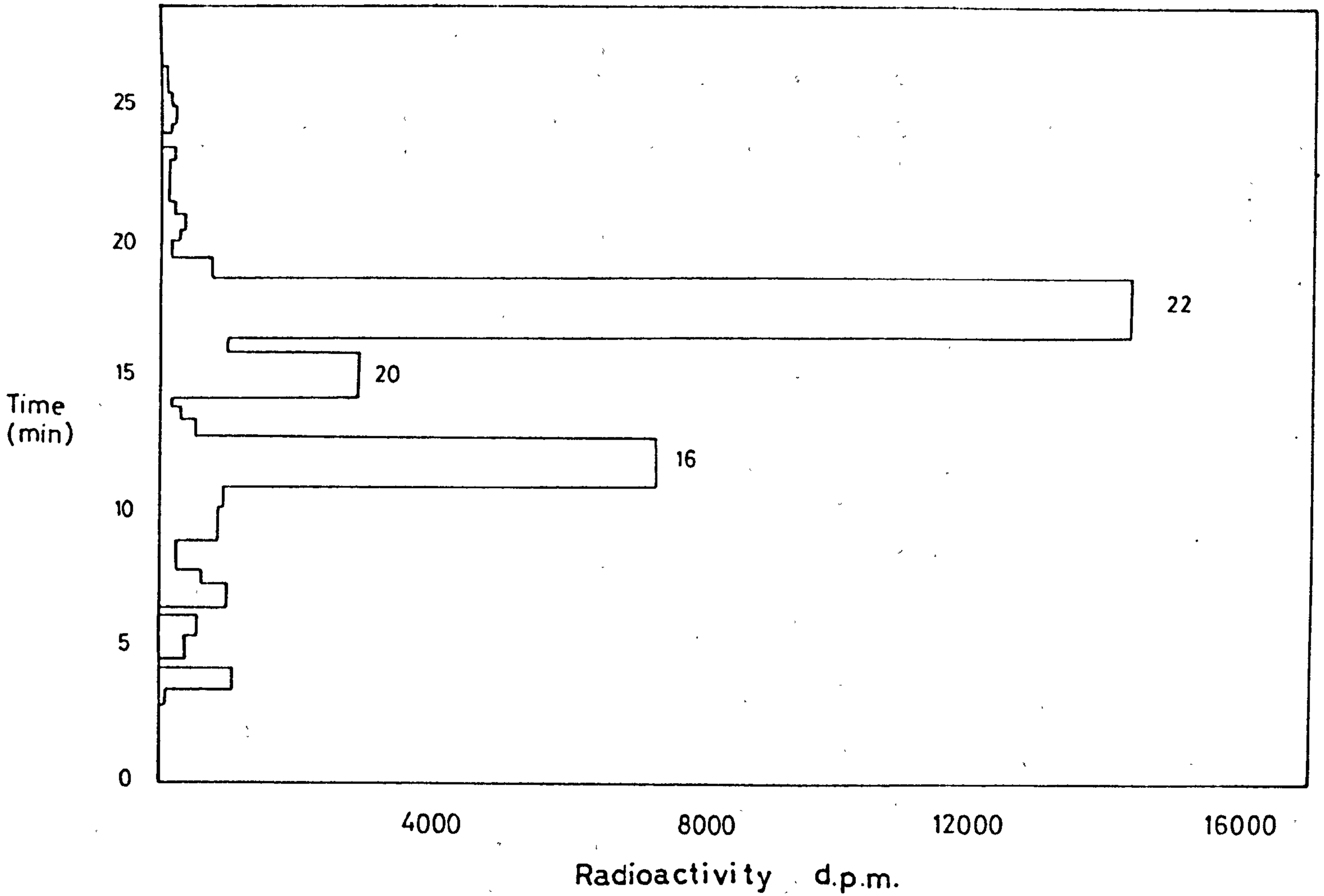


Fig. 57.

Radioactivity elution profile of chick embryo neutral carotenoids on the LiChrosorb system. Fractions of importance in terms of zeaxanthin metabolism are labelled.

conclusively demonstrates the metabolism of zeaxanthin to α-cryptoxanthin, ε-galloxanthin and galloxanthin in chick retinas. There is evidence for several intermediates of zeaxanthin metabolism at the early stages of the chromatogram. Presumably, these are monohydroxy carotenoids and hence additional examples of reductive metabolism in animals.

Analysis of ε,ε-carotene fraction (fraction 3).

Chromatography of the carotene fraction was carried out on the ODS Hypersil 5u system as described in Section 4.17B. The fraction was not 'spiked' with standard carotenes. The chromatography was very poor as shown in Fig. 58. The major peaks (fractions 32-34) were assumed to be the unidentified carotene known to be present in chick retina and consequently an absorption spectrum of F32-34 was not recorded. Subsequent chromatography of standard ε,ε-carotene on this column immediately after the analysis of the fractions from [$^{3}H_2$]- and [^{14}C]zeaxanthin treated embryonic extracts was problematic. The retention time of the standard ε,ε-carotene was reduced from 21.4 min to 17.6 and the peak was distorted. Thus it is quite possible that F32-34 are ε,ε-carotene.

Again fractions were collected every 30 s except for peaks which were collected as one fraction even if the time for elution exceeded 30 s. The histogram of Fig. 58 shows radioactivity per fraction, and is drawn such that each bar of the histogram (i.e. each fraction) corresponds to the time period on the chromatogram from which it was collected.

Low levels of radioactivity make it very difficult to draw any conclusions about the metabolic conversion of zeaxanthin to ε,ε-carotene. Although radioactivity is detected in the appropriate fractions, the significance of this is uncertain. The presence of other, more

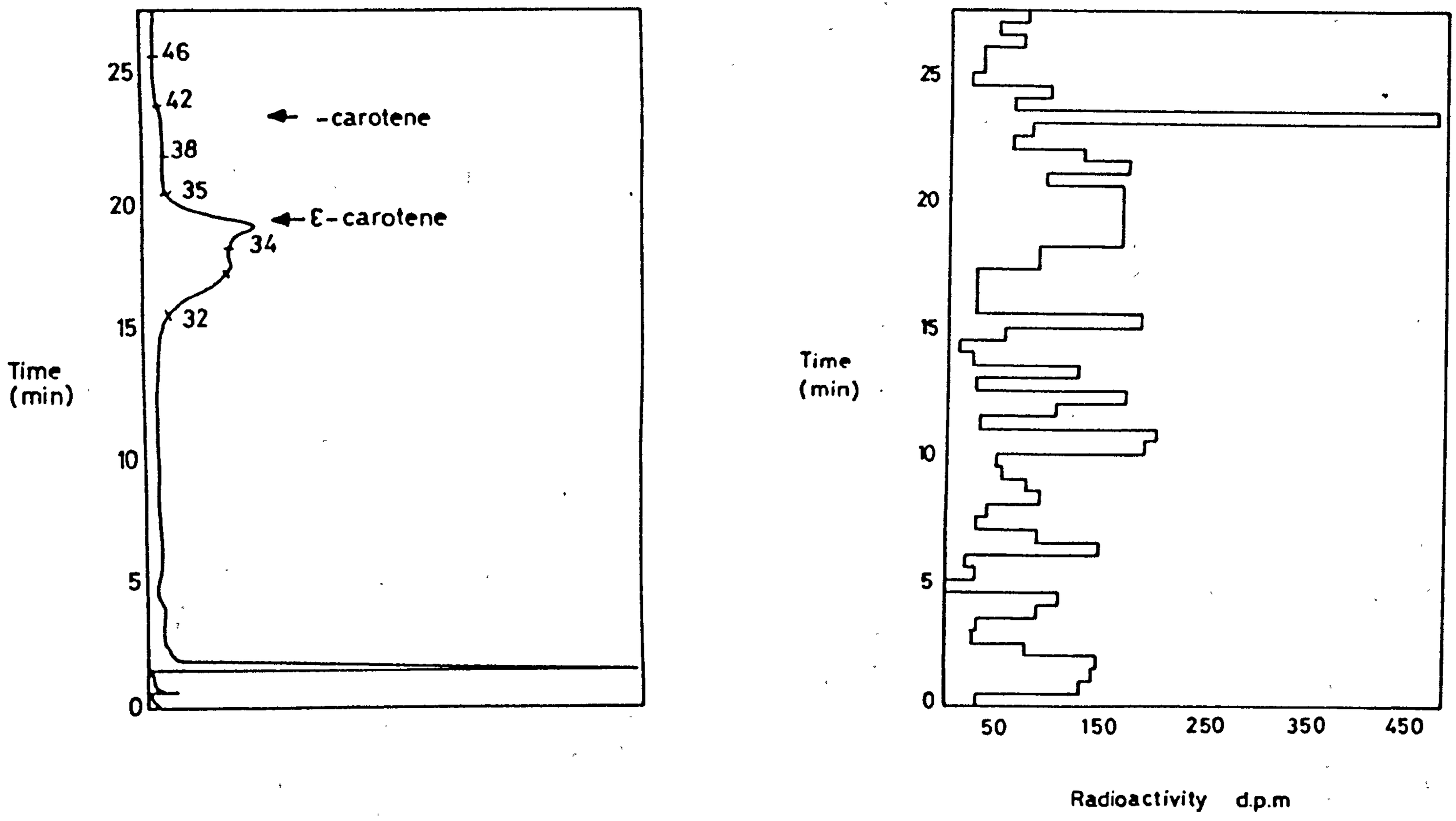


Fig. 58.

H.p.l.c. elution profile and corresponding radioactivity elution profile of the carotene fraction from retinal oil droplet carotenoids of chick embryos developed in the presence of [$^2H_2$]zeaxanthin. Column: ODS Hypersil 5μ (250 mm x 4.5 mm); solvent: acetonitrile/ propan-2-ol (95:5, v/v) at 2 ml/min.

radioactive, components in the carotene fraction distracts from the possible conversion of zeaxanthin to ε,ε-carotene. Considering the presence of these more highly radioactive metabolites in the carotene fraction it was fortuitous that the fraction was treated in this way.

ii. [^{14}C]zeaxanthin treated embryonic retinas.

The results from this experiment were directly analogous to those shown in the preceeding section. The h.p.l.c. elution profile was consistent with that for adult neutral carotenoids. As for the [$^{3}H_2$]zeaxanthin experiment, several fractions (α-cryptoxanthin, lutein, ε,ε-carotene-diol and ε-galloxanthin fractions) were further purified by rechromatography on the LiChrosorb system. Low levels of radioactivity were detected in vials corresponding to α-cryptoxanthin, ε-galloxanthin and galloxanthin thus substantiating the findings of the [$^{3}H_2$]zeaxanthin experiment.

A polar fraction from the LiChrosorb system was again identified. The radioactivity detected in this fraction was 224 d.p.m.

Analysis of the ε,ε-carotene fraction (fraction 3).

Analysis of the carotene fraction was difficult. The chromatography was not particularly successful but an ε,ε-carotene peak could be identified, although it had reduced retention time (20.4 min). Again low levels of radioactivity (maximum d.p.m. detected in any fraction being 108 d.p.m.; the fraction corresponding to ε,ε-carotene was 46 d.p.m.) make it extremely difficult to interpret the results. Therefore, the role of zeaxanthin in the formation of (6S,6'S)-ε,ε-carotene is still unclear.

B. Acidic carotenoids.

i. [$^{3}H_2$]zeaxanthin treated embryos.

The acidic fraction was chromatographed on the Nucleosil/H_3PO_4 system described in Section 3.18. Standard astacene had a retention time of 6.3 min. The major peak (Fig. 59) chromatographed at retention time 8.3 min. An absorption spectrum of this fraction (F17) was not helpful in the identification of this carotenoid (λ_{max} in ether at 476.5 nm). An aliquot of F17 (1/20th) was withdrawn, the solvent removed by evaporation on a hot-plate (40 °C) and under a stream of nitrogen, and scintillation fluid (10 ml) added. The radioactivity for the total fraction was 43,428 d.p.m. (0.0197 µCi). This carotenoid was clearly a metabolite of zeaxanthin. The fraction was resaponified and rechromatographed on the Nucleosil/H_3PO_4 system. The retention time of the fraction on this occasion was 6.2 min which correlates well with standard astacene. The original retention time was consistent with semi-astacene. This demonstrates the conversion of zeaxanthin to astacene, and thus by implication astaxanthin.

ii. [^{14}C]zeaxanthin treated embryos.

The same difficulties were encountered with this fraction as for the corresponding fraction from the [$^{3}H_2$]zeaxanthin study. After resaponification, the fraction was eluted from the column at a time corresponding to astacene. The radioactivity detected in the fraction was 473 d.p.m.

4.32 Analysis of liver extracts from chick embryos developed in the presence of [^{14}C]zeaxanthin or [$^{3}H_2$]zeaxanthin.

Liver tissue is always a difficult tissue to work with as there are vast quantities of sterols present. Sterol contamination is a problem

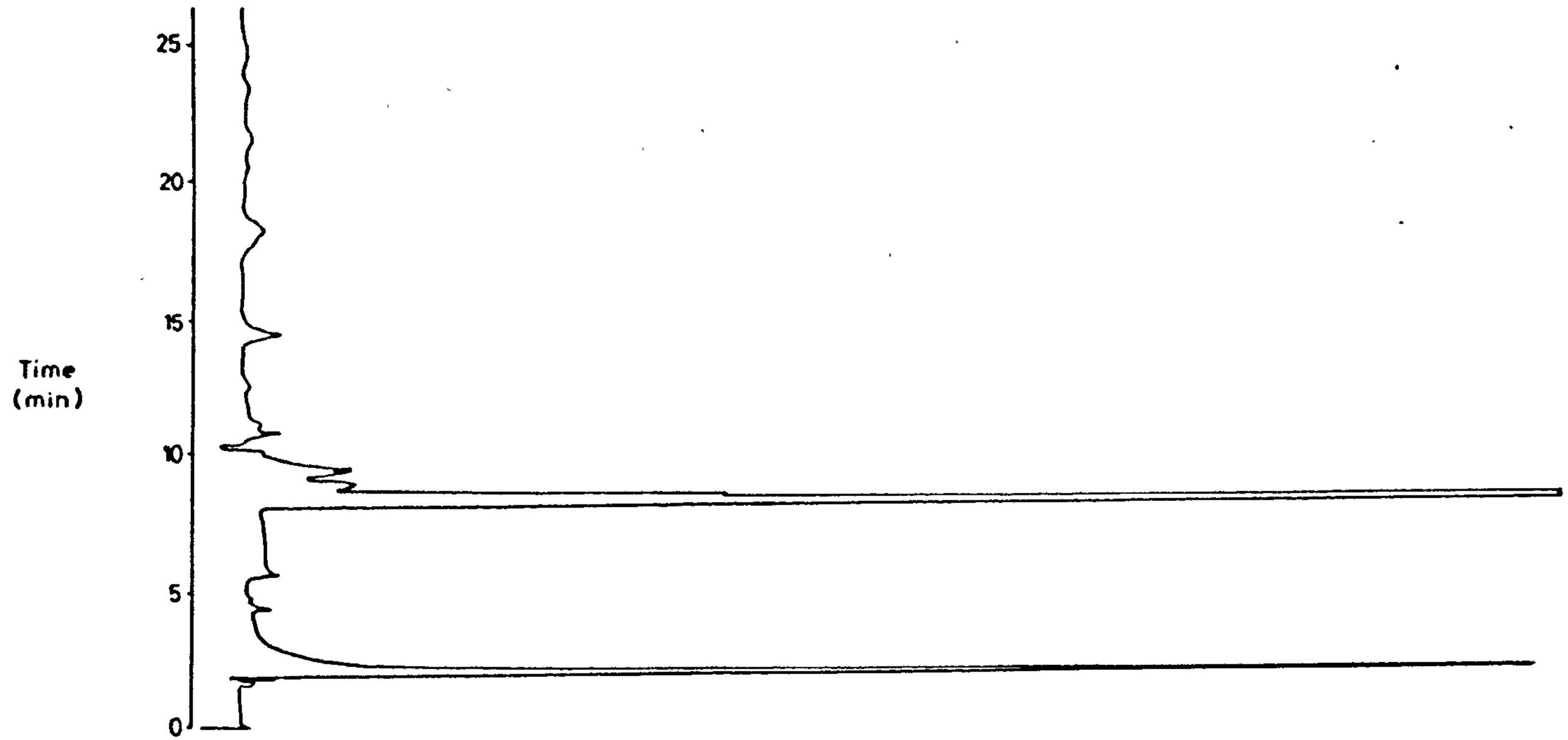


Fig. 59.

H.p.l.c. elution profile of acidic retinal carotenoids from chick embryos developed in the presence of [$^3H_2$]zeaxanthin. Column: Nucleosil/H_3PO_4 (250 mm x 4.5 mm); solvent: hexane-10%CH_2Cl_2, 0.75% propan-2-ol at 1.8 ml/min.

during chromatography especially. Successive treatments of the liver extracts with digitonin were undertaken in an attempt to alleviate this potential problem.

A study of turkey liver carotenoids by Pollard (1980) gives an indication of the carotenoids that may be present in chick liver extracts. These are lutein, 11-apo-β-caroten-11-al, ε-carotene, β-carotene and phytofluene. From a study of chicken livers in this presentation it was anticipated that vitamin A_1 and A_2 may also be present.

It was evident that deposition of relatively large amounts of carotenoids was occurring in the livers of the embryos. This can be seen in Plates 1 and 2, where the liver tissue is clearly yellow. Plate 2 also shows the relatively large sized eyes, in relation to the total body size, of the newly hatched chick.

To give a clearer guideline about the liver extract composition, the equivalent extract from MVA-treated chick embryos was chromatographed on the LiChrosorb system previously described. It was assumed that there would be little or no difference between these embryonic samples. From the h.p.l.c. elution profile (Fig. 60) it can be concluded that there are trace amounts of carotene (peak 1), vitamin A (peak 2), α-cryptoxanthin (peak 3) and larger amounts of lutein (peak 4) and zeaxanthin (peak 5) present.

Several different stages of analysis of liver extracts was required. The vitamin A fraction needed to be resolved into vitamin A_1 and A_2 before analysis, and the carotene fraction needed to be resolved into its appropriate carotene constituents. As relatively large amounts of material were present, classical l.c. was carried out in an attempt to isolate carotene, monohydroxycarotenoid, vitamin A and dihydroxycarotenoid fractions. Chromatography on Alumina (neutral, Grade

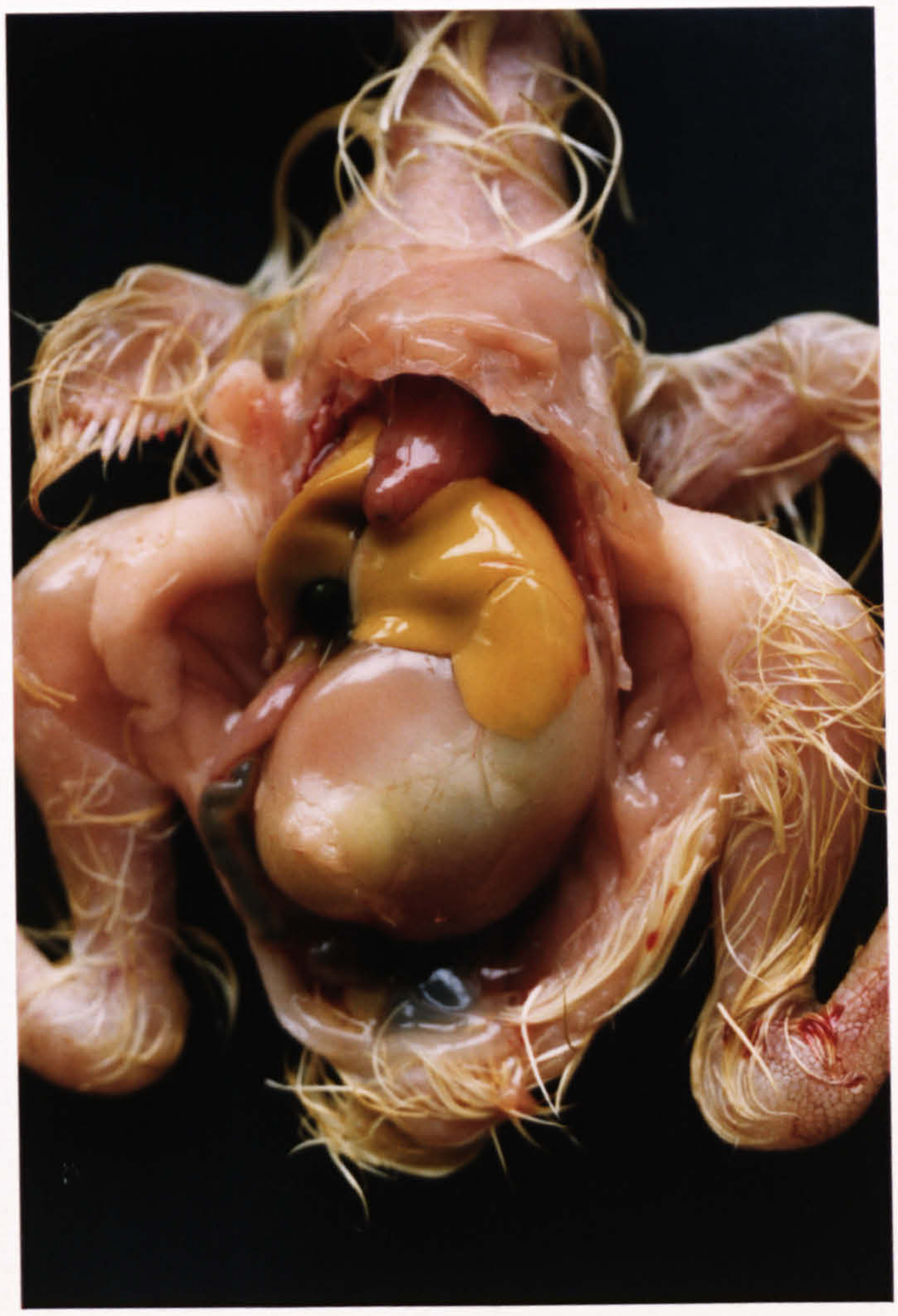


Plate 1.

Dissection of chick illustrating the deposition of carotenoid into the liver.

Plate 2.

Dissection of chick illustrating the large size of the eyes in relation to body size.

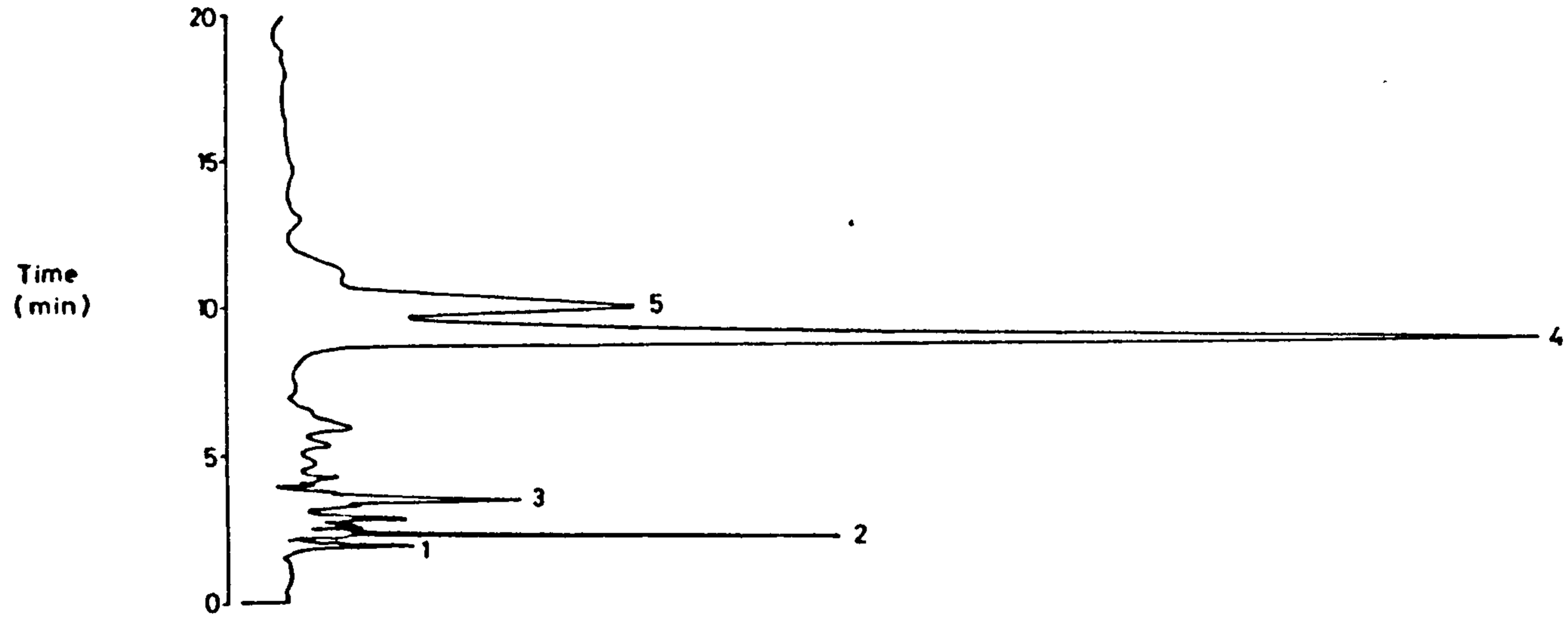


Fig. 60.

H.p.l.c. elution profile of total unsaponifiable lipid from livers of chick embryos developed in the presence of [2-^{14}C]MVA. Column: LiChrosorb Si-60 (250 mm x 4.5 mm); solvent: hexane/propan-2-ol/ CH_2Cl_2 (92:5:3, v/v) at 1.5 ml/min.

III) gave 4 clearcut fractions for liver extracts from both [$^{3}H_2$]zeaxanthin and [^{14}C]zeaxanthin treated embryos (see Table 26a and b).

H.p.l.c. analysis of the fractions was carried out in 3 parts. Firstly, for each isotope, fractions 1 and 2 were bulked and the sample analysed on the ODS Hypersil system (carotene system). Secondly, fraction 3 from each isotope was analysed for vitamin A and thirdly, the dihydroxycarotenoid fraction was analysed on the LiChrosorb system.

A. Carotene analysis (fractions 1 and 2).

i. [$^{3}H_2$]zeaxanthin treated embryos.

Chromatography of the combined fractions 1 and 2 on the ODS Hypersil system was not as anticipated. There was no indication of the presence of any carotenes, with a single major peak occurring at a retention time corresponding to a considerably more polar carotenoid such as cryptoxanthin (Fig. 61). The retention time of standard cryptoxanthin was subsequently found to be 7 min. Several attempts were made to get m.s. data for this fraction (λ_{max} in ethanol at (418) 443 (460) nm; λ_{max} in ether at (420) 437 459 nm) but all were unsuccessful due to the interference from vast quantities of ethyl stearate (m.s.: m/z 312 (M^+)).

Again, fractions were collected every 30 s, except peaks which were collected as one fraction regardless of the time element. In Fig. 61, fractions 1-12 correspond to time 0-6 min, fraction 13 corresponds to unidentified carotenoid and fractions 14-53 correspond from time 7 min to the end of the chromatography. The solvent was removed from each fraction by evaporation on a hot-plate (40 °C), under a stream of nitrogen. Scintillation fluid (10 ml) was added and the vials counted. There was no indication of incorporation of radioactivity into any of the fractions. The maximum radioactivity detected in any vial was 216 d.p.m.

TABLES 26a and 26b.

Fractions from classical column chromatography (Alumina Grade III) of livers from chick embryos developed in the presence of [$^{3}H_2$]zeaxanthin or [^{14}C]zeaxanthin.

a. [$^{3}H_2$]zeaxanthin.

Fraction	Solvent	Absorption maxima (in light petroleum) nm			Possible carotenoid composition
1	100% P	422.5	442.5	468.5	carotene
2	20% E/P	421	441.5	467.5	carotene/ cryptoxanthin
3	50% E/P		327.5		vitamin A
4	2% EtOH/E	423	445	473	lutein

b. [^{14}C]zeaxanthin.

Fraction	Solvent	Absorption maxima (in ether nm)			Possible carotenoid composition
1	100% P		441	462	carotene
2	20% E/P	420	441	462	carotene/ cryptoxanthin
3	50% E/P		322		vitamin A
4	2% EtOH/P	424	445	473	lutein

P = light petroleum

E = ether

EtOH = ethanol

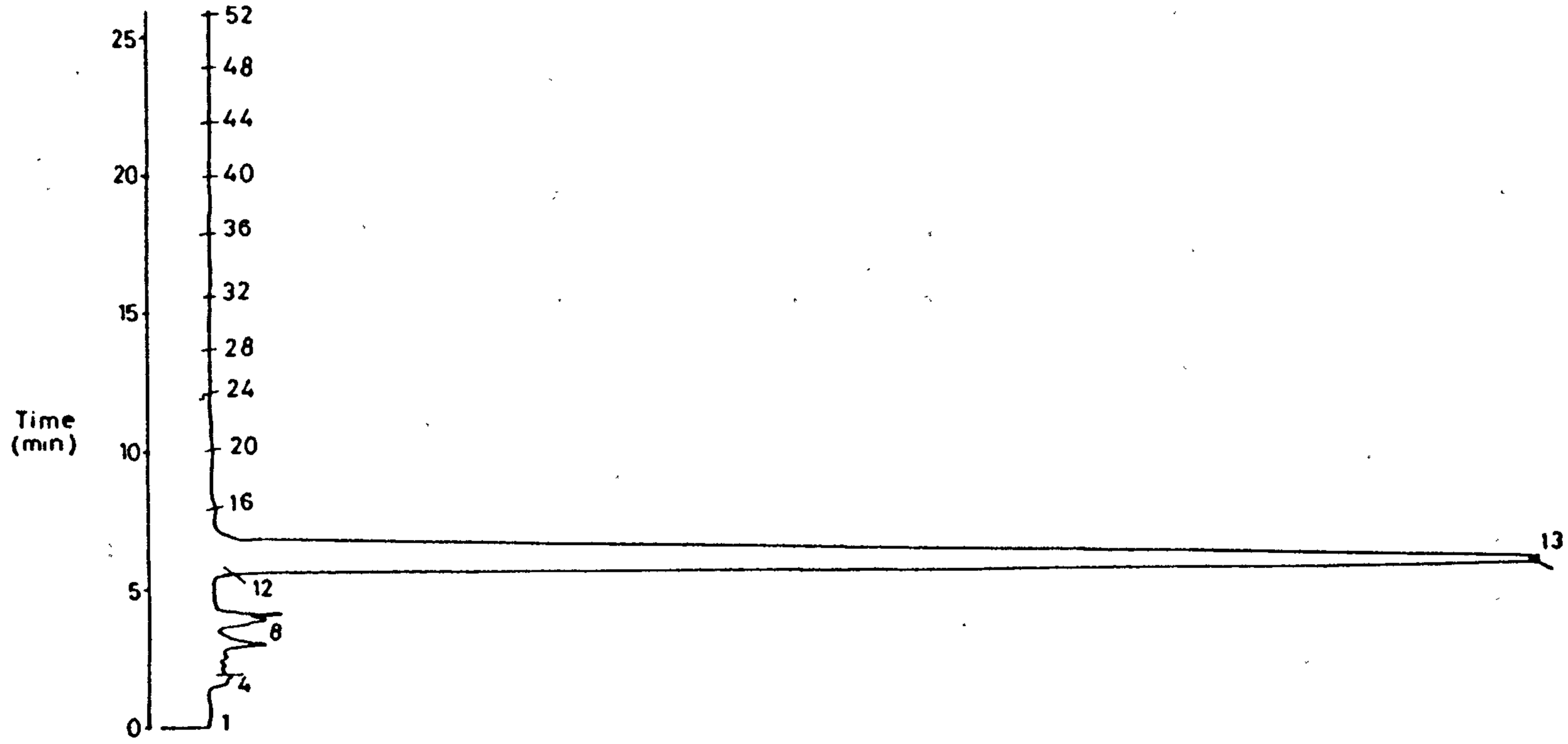


Fig. 61.

H.p.l.c. elution profile of the carotene fraction from livers of chick embryos developed in the presence of [$^2H_2$]zeaxanthin. Column: ODS Hypersil 5µ (250 mm x 4.5 mm); solvent: acetonitrile/propan-2-ol (95:5, v/v) at 2 ml/min. Fractions were collected as shown.

and this constituted 4.7 % of the total radioactivity of the fraction.

ii. [^{14}C]zeaxanthin treated embryos.

The chromatography of this sample was identical to that seen for the equivalent [$^{3}H_2$]zeaxanthin sample. Attempts at identification of the only significant peak (retention time, 6.2 min) were again unsuccessful. A random distribution of radioactivity into fractions was observed.

B. Vitamin A fraction.

i. [$^{3}H_2$]zeaxanthin treated embryos.

A small portion of the total crude vitamin A fraction was chromatographed on the Nucleosil h.p.l.c. system devised for the goldfish experiments (Section 3.16). Even at this early stage there was an indication of the presence of dehydroretinol in embryonic chick tissue (Fig. 62, peak 4). The predominance of retinol (peak 3) over dehydroretinol, and the large amounts of material made the analysis of the fraction more complex.

A Pye Unicam PU4810 computing integrator was used for the initial analysis. The integration parameters were set up as for the goldfish work (Section 3.16). Analysis of a small undefined aliquot of sample determined the ratio of A_1:A_2 of 14:1.

The fractions were collected as indicated on the h.p.l.c. elution profile of Fig. 62. Multiple injections of the sample through the system was necessary because of the large amounts of material. Fractions 1-4 were then dealt with separately.

Fraction 1.

Chromatography of this sample was carried out on the Nucleosil

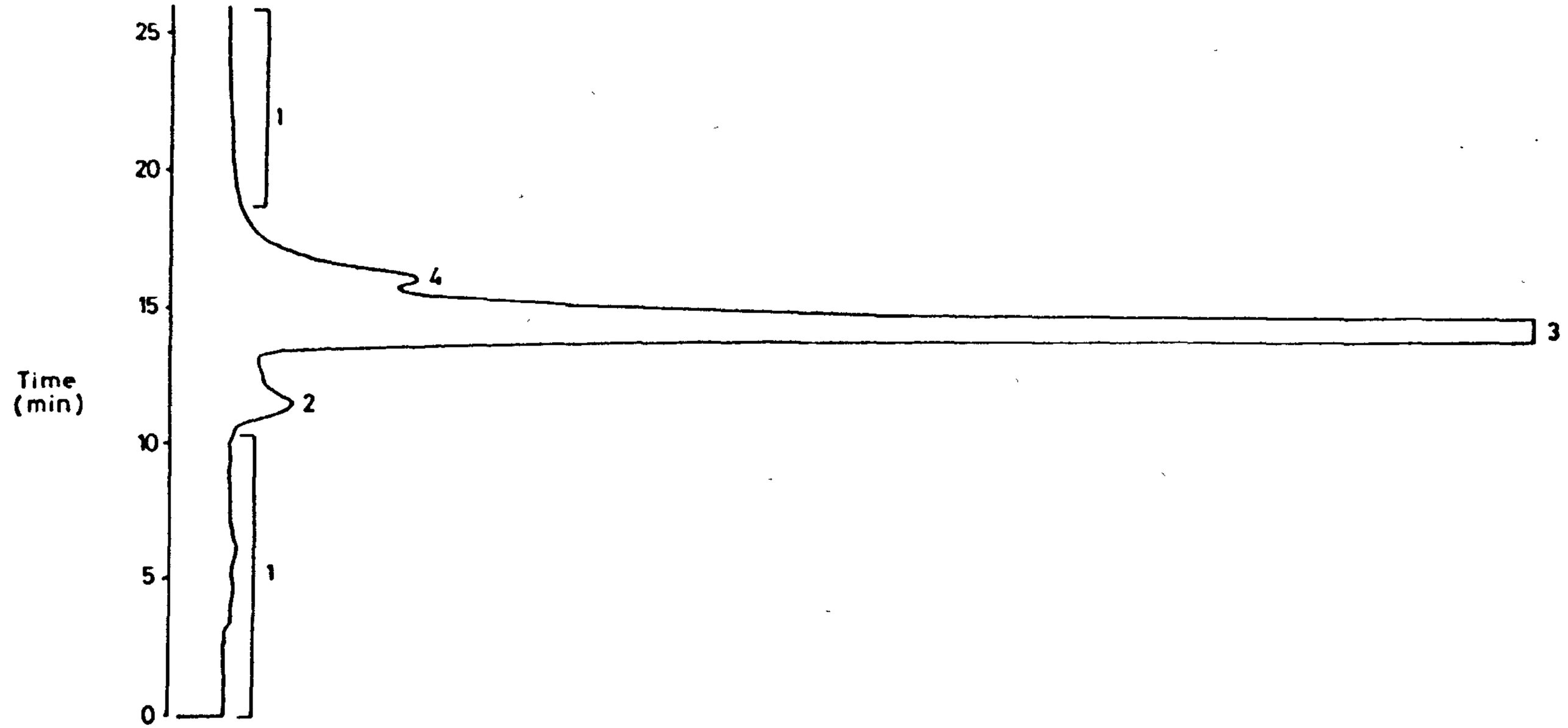


Fig. 62.

H.p.l.c. elution profile of the vitamin A fraction from livers of chick embryos developed in the presence of [$^3H_2$]zeaxanthin. Column: Nucleosil 50-5 (200 mm x 4.5 mm); solvent: hexane/1,4-dioxan (9:1, v/v) at 1 ml/min.

system for vitamin A, as above. Fractions were collected every 30 s from time 0-10.5 min and 18-25 min. Each fraction was collected directly into vial inserts, the solvent removed by evaporation on a hot-plate (40°C), under a stream of nitrogen and finally, scintillation fluid (2 ml) added. The fractions were numbered 1-21 and 27-40.

Fraction 2.

Two fractions were collected from chromatography of this fraction on the Nucleosil system. Fraction 22 corresponded to the peak and fraction 23 to the trough between peak 2 and the following peak. An absorption spectrum of F22 did not allow its identification but confirmed that it was some form of vitamin A (λ_{max} in light petroleum at 326 nm).

Fraction 3.

Two fractions were collected from rechromatography of this fraction (F24 and F25). Fraction 24 was clearly pure retinol and F25 corresponded to the trough between the peaks of retinol and dehydroretinol.

Fraction 4.

This fraction was resolved into two components. Three fractions were collected. These were F24 (retinol), F25 (the trough) and F26 (dehydroretinol).

The amounts of retinol and dehydroretinol present in the total liver extract, as determined by spectrophotometric methods, were 21.9 µg and 1.5 µg, respectively. This is consistent with the ratio already established.

Solvent was removed from fractions F22-F27 by evaporation on a hot-plate (40°C) under a stream of nitrogen, scintillation fluid added (2 ml) and the vials counted for a statistically acceptable length of time.

The levels of incorporation were very low once again. The maximum

radioactivity in any vial (F6) was very low (117 d.p.m.) and constituted 7% of the total radioactivity in the whole fraction. There was a random distribution of radioactivity and hence it cannot be concluded from this study that zeaxanthin is the precursor of retinol and dehydroretinol in chicks.

ii. [^{14}C]zeaxanthin treated embryos.

The analysis was carried out in a precisely comparable manner. The ratio of retinol to dehydroretinol was 12.3:1. More retinol and dehydroretinol were present in this extract (112.4 µg and 9.5 µg, respectively). There was no indication of metabolism of zeaxanthin to either form of vitamin A.

C. Dihydroxycarotenoid fraction (fraction 4).

i. [$^{3}H_2$]zeaxanthin treated embryos.

After treatment with digitonin the sample was chromatographed on the LiChrosorb system (Section 4.17B). Figure 63 depicts the h.p.l.c. elution profile of embryonic liver dihydroxycarotenoids. The fractions from h.p.l.c. were collected in 30 s fractions or as whole peaks. Fraction 20 was identified as lutein and fraction 21 as zeaxanthin. Other unidentified carotenoids were present, the most prominent having a retention time of 4.3 min (F9). An absorption spectrum of F9 (λ_{max} in light petroleum at 453.5 476 nm) did not allow a tentative identification of the carotenoid. Spectrophotometric estimation of zeaxanthin and lutein showed the presence of 0.944 µg of zeaxanthin and 2.34 µg of lutein.

Each fraction was prepared for liquid scintillation counting in the normal way for carotenoids (i.e. in glass vials with scintillation fluid (10 ml) added). The histogram of Fig. 63 depicts the results of counting

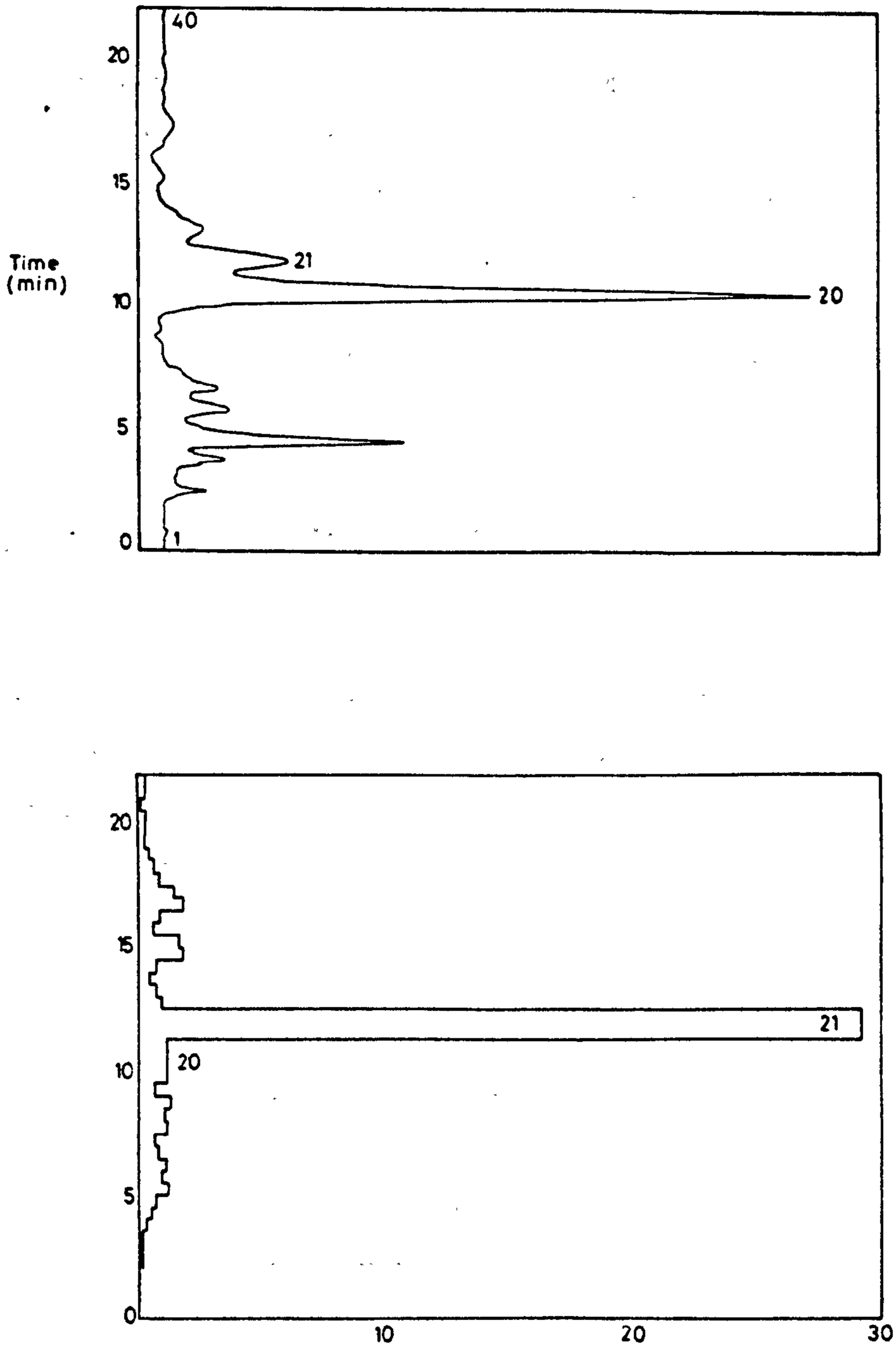


Fig. 63.

H.p.l.c. elution profile and corresponding radioactivity elution profile of the dihydroxycarotenoid fraction from livers of chick embryos developed in the presence of [$^3H_2$]zeaxanthin. Column: LiChrosorb Si-60 (250 mm x 4.5 mm); solvent: hexane/propan-2-ol/ CH_2Cl_2 (92:5:3. v/v) at 1.5 ml/min.

the vials for a suitable time period. As can be seen over 50% of the radioactivity was detected in the zeaxanthin fraction. There may also be incorporation into two other carotenoids of a more polar nature than zeaxanthin.

The final specific activity of the zeaxanthin was calculated as 13.94 μCi/mg.

ii. [^{14}C]zeaxanthin treated embryos.

Lutein and zeaxanthin were the major components of this fraction. Spectrophotometric estimation of zeaxanthin and lutein showed the presence of 18.62 μg and 4.36 μg of carotenoids, respectively. Again, the majority of the radioactivity was detected in the zeaxanthin fraction (over 36%). The final specific activity of the [^{14}C]zeaxanthin was calculated to be 0.084 μCi/mg.

4.33 Analysis of intestinal extracts from chick embryos developed in the presence of [^{14}C]zeaxanthin or [$^{3}H_2$]zeaxanthin.

Pollard (1980) identified zeaxanthin, β-cryptoxanthin, ζ-carotene, β-carotene, α-carotene and phytofluene in turkey intestine extracts. For comparison purposes the intestine extract of embryos developed in the presence of MVA was chromatographed on the LiChrosorb system. From the h.p.l.c. elution profile (Fig. 64) there is evidence for a dihydroxy carotenoid and carotene fraction. Thus the intestinal fractions were treated in a way similar to the liver extracts. Classical l.c. on Alumina (Brockmann Grade III) was carried out as for liver. Three clearcut fractions were obtained for both the [^{14}C]- and [$^{3}H_2$]zeaxanthin experiments. The absorption maxima of each fraction are shown in Tables 27 a and b.

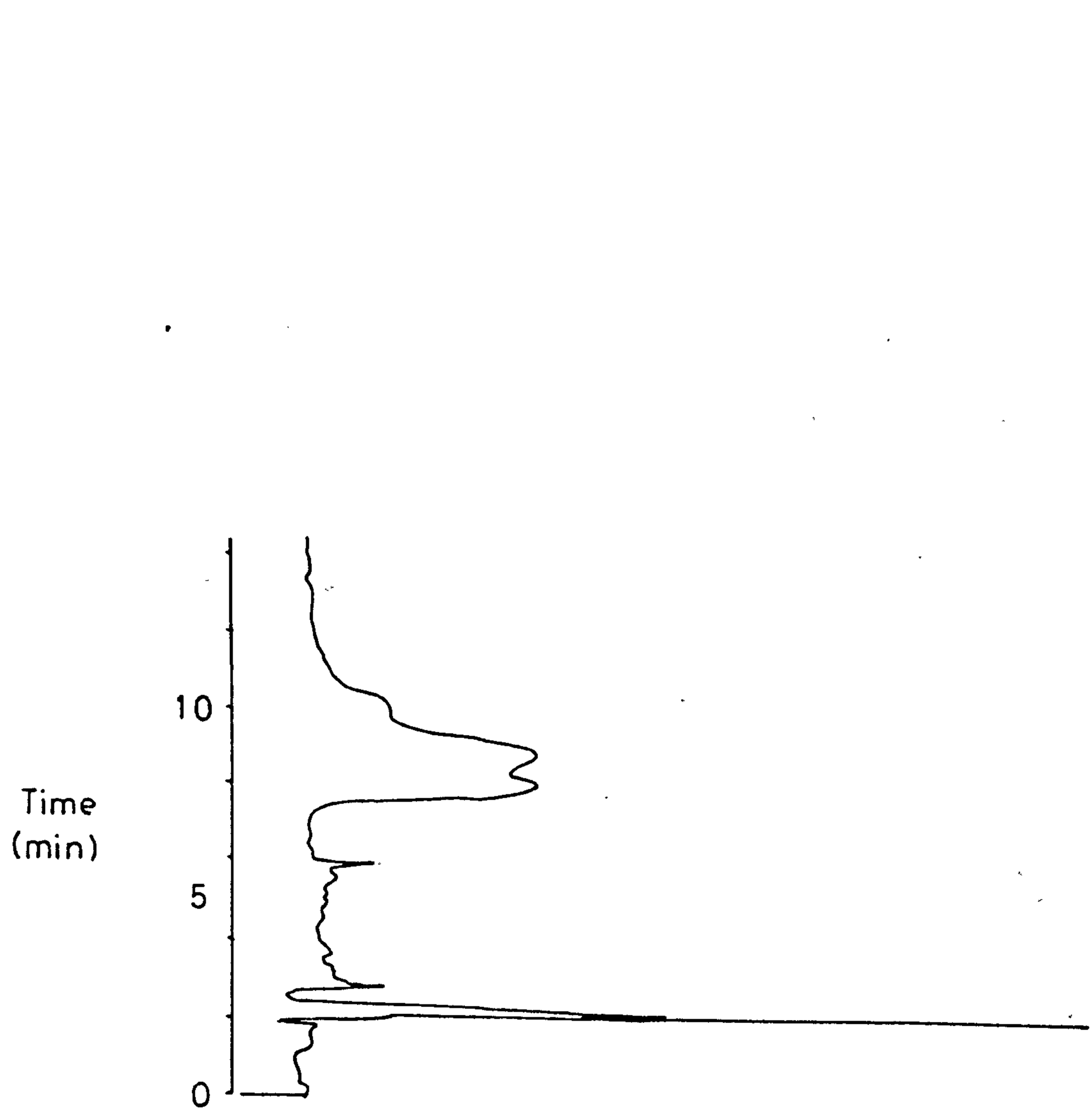


Fig. 64.

H.p.l.c. elution profile of total unsaponifiable lipid from intestines of chick embryos developed in the presence of [2-^{14}C]MVA. Column: LiChrosorb Si-60 (250 mm x 4.5 mm); solvent: hexane/propan-2-ol/CH_2Cl_2 (92:5:3, v/v) at 1.5 ml/min.

TABLES 27a and 27b.

Fractions from classical column chromatography (Alumina Grade III) of intestines from chick embryos developed in the presence of [$^{3}H_2$]zeaxanthin or [^{14}C]zeaxanthin.

a. [$^{3}H_2$]zeaxanthin.

Fraction	Solvent	Absorption maxima (in light petroleum) nm			Possible carotenoid composition
1	100% P - 20% E/P	422	444	463.5	carotene/ cryptoxanthin
2	50% E/P	not detected			-
3	2% EtOH/E	424	447	474	zeaxanthin/ lutein

b. [^{14}C]zeaxanthin.

Fraction	Solvent	Absorption maxima (in light petroleum) nm			Possible carotenoid composition
1	100% P - 20% E/P	432	442	467.5	carotene
2	50% E/P	430	445	467.5	cryptoxanthin
3	2% EtOH/E	424	445	473.5	zeaxanthin/ lutein

P = light petroleum

E = ether

EtOH = ethanol

A. Carotene fraction (fractions 1 and 2).

i. [$^{3}H_2$]zeaxanthin treated embryos.

It was hoped that this non-polar fraction (resulting from the combination of fractions 1 and 2) would give more information about the possible metabolism of zeaxanthin to ε,ε-carotene. Unfortunately, the very small amounts of carotenoid present made the analysis of this fraction more difficult.

The sample was chromatographed on the system used for carotene analysis (ODS Hypersil, Section 4.17B). The observed peaks (see Fig. 65) had shorter retention times than anticipated for carotenes. The retention times (1.3 min, F3; 3.7 min, F8; 5.5 min, F11 and 12; 7.2 min, F15; 7.7 min, F16) of the observed peaks did not coincide with any retention times of standard carotenoids which may be present e.g. ε-carotene, 17.3 min; zeaxanthin, 3 min; β-carotene, 22 min; β- and α-cryptoxanthin, 7.1. and 8.0 min respectively). Fraction 15 may be α-cryptoxanthin.

Fractions were collected directly into scintillation vials every 30 s along the chromatogram. The solvent was removed from each vial by evaporation from a hot-plate and under a stream of nitrogen. Scintillation fluid (10 ml) was added to each fraction and the vials counted for an appropriate time period.

Generally, levels of incorporation were low, thus any metabolism was more difficult to demonstrate. However, there may be significant incorporation into fraction 8, an unidentified metabolite.

ii. [^{14}C]zeaxanthin treated embryos.

This sample chromatographed in a similar way to the [$^{3}H_2$]zeaxanthin

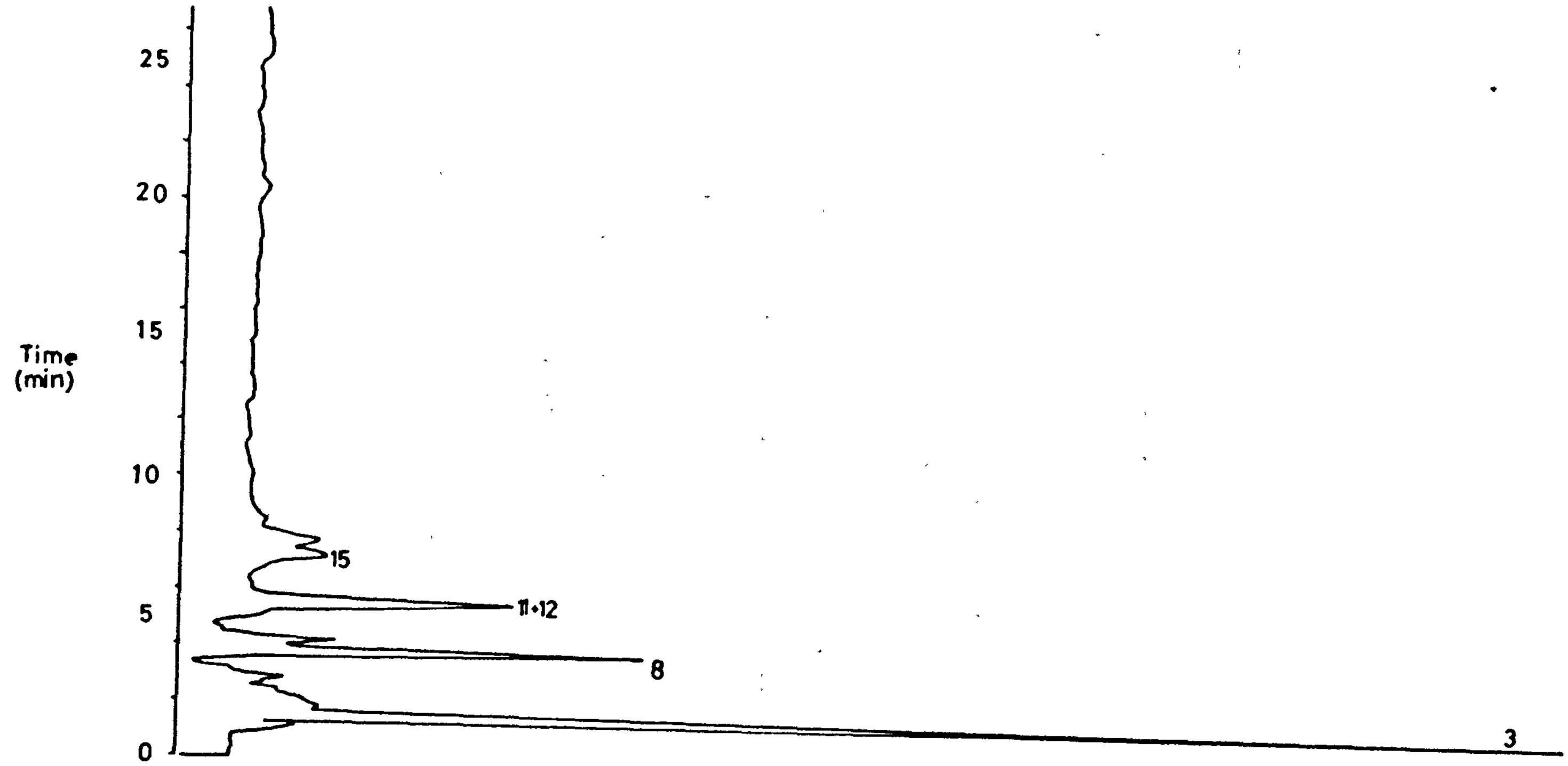


Fig. 65.

H.p.l.c. elution profile of the carotene fraction from intestines of chick embryos developed in the presence of [$^3H_2$]zeaxanthin. Column: ODS Hypersil 5µ (250 mm x 4.5 mm); solvent: acetonitrile/propan-2-ol (95:5, v/v) at 2 ml/min.

sample. Again, the metabolites were not identified. The levels of radioactivity were extremely low and no significance was attached to the random distribution of radioactivity in the fractions.

B. Dihydroxycarotenoid fraction (fraction 3).

i. [$^{3}H_2$]zeaxanthin treated embryos.

This fraction was anticipated to be predominantly zeaxanthin (Pollard, 1980).

The fraction was chromatographed on the LiChrosorb system. The h.p.l.c. elution profile of embryonic intestinal dihydroxycarotenoids is shown in Fig. 66. As can be seen, there is relatively little present in the fraction except dihydroxycarotenoids. Contrary to expectations, there is a considerable proportion of lutein present in the fraction. The fractions were collected every 30 s except for the peaks corresponding to lutein (F21) and zeaxanthin (F22). By spectrophotometric analysis it was determined that there was 2 µg of lutein and 1.832 µg of zeaxanthin present.

The fractions were collected directly into scintillation vials, the solvent removed by evaporation on a hot-plate and under a stream of nitrogen and scintillation fluid (10 ml) added. Each vial was counted and the histogram of Fig. 66 is a representation of the results. As can be seen, over 40% of the total radioactivity was located in the vial (F22) corresponding to zeaxanthin. The final specific activity of the zeaxanthin was calculated as 4.8 µCi/mg.

ii. [^{14}C]zeaxanthin treated embryos.

The fraction was almost entirely composed of zeaxanthin (2.94 µg) and lutein (3.62 µg). Only the fraction corresponding to zeaxanthin

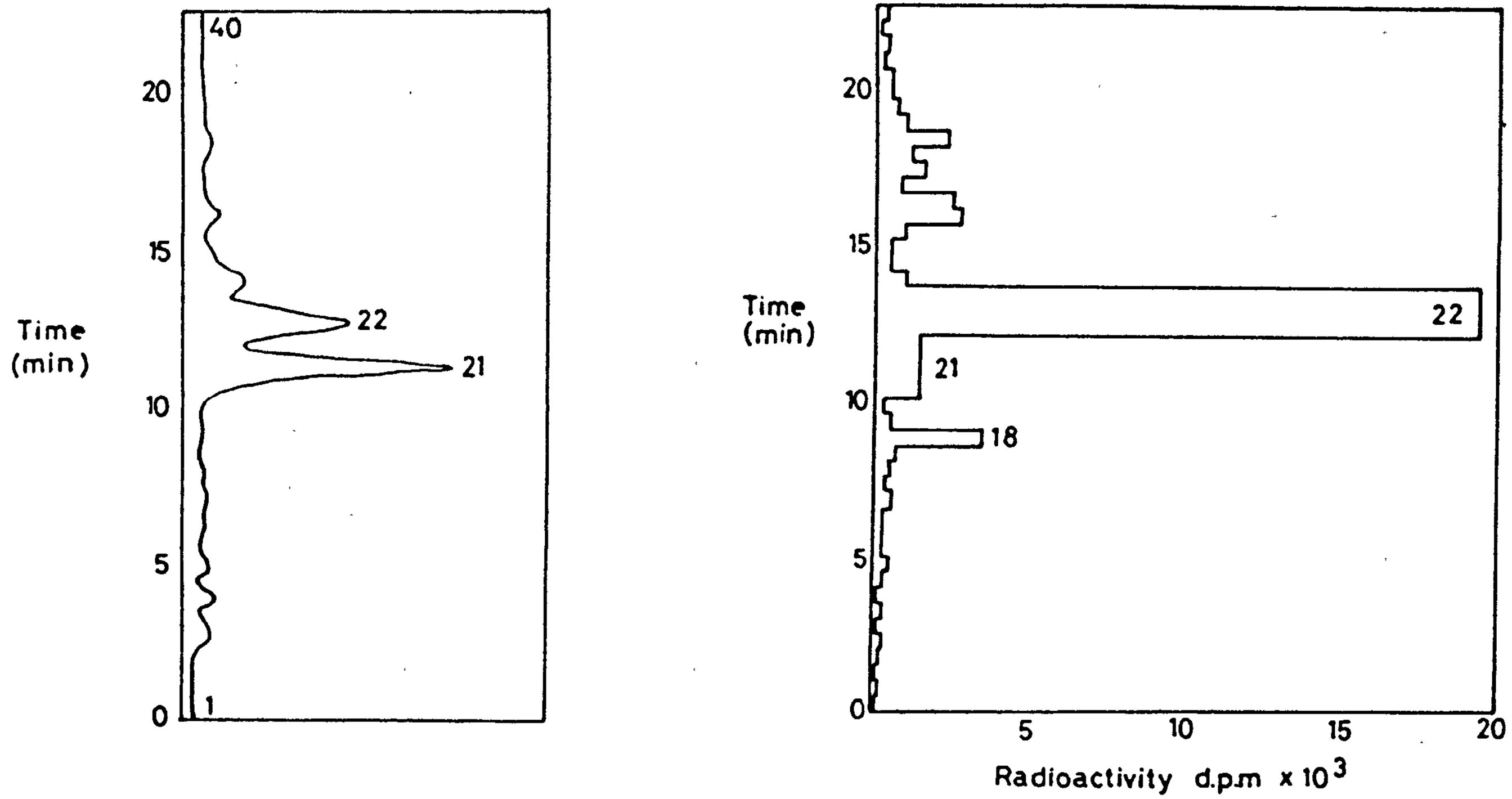


Fig. 66.

H.p.l.c. elution profile and corresponding radioactivity elution profile of the dihydroxycarotenoid fraction from intestines of chick embryos developed in the presence of [$^3H_2$]zeaxanthin. Column: LiChrosorb Si-60 (250 mm x 4.5 mm); solvent: hexane/propan-2-ol/CH_2Cl_2 (92:5:3, v/v) at 1.5 ml/min.

displayed significant levels of radioactivity (478 d.p.m). This accounted for 21% of the total radioactivity in the sample.

4.34 Analysis of feather extracts from chick embryos developed in the presence of [^{14}C]zeaxanthin or [$^{3}H_2$]zeaxanthin.

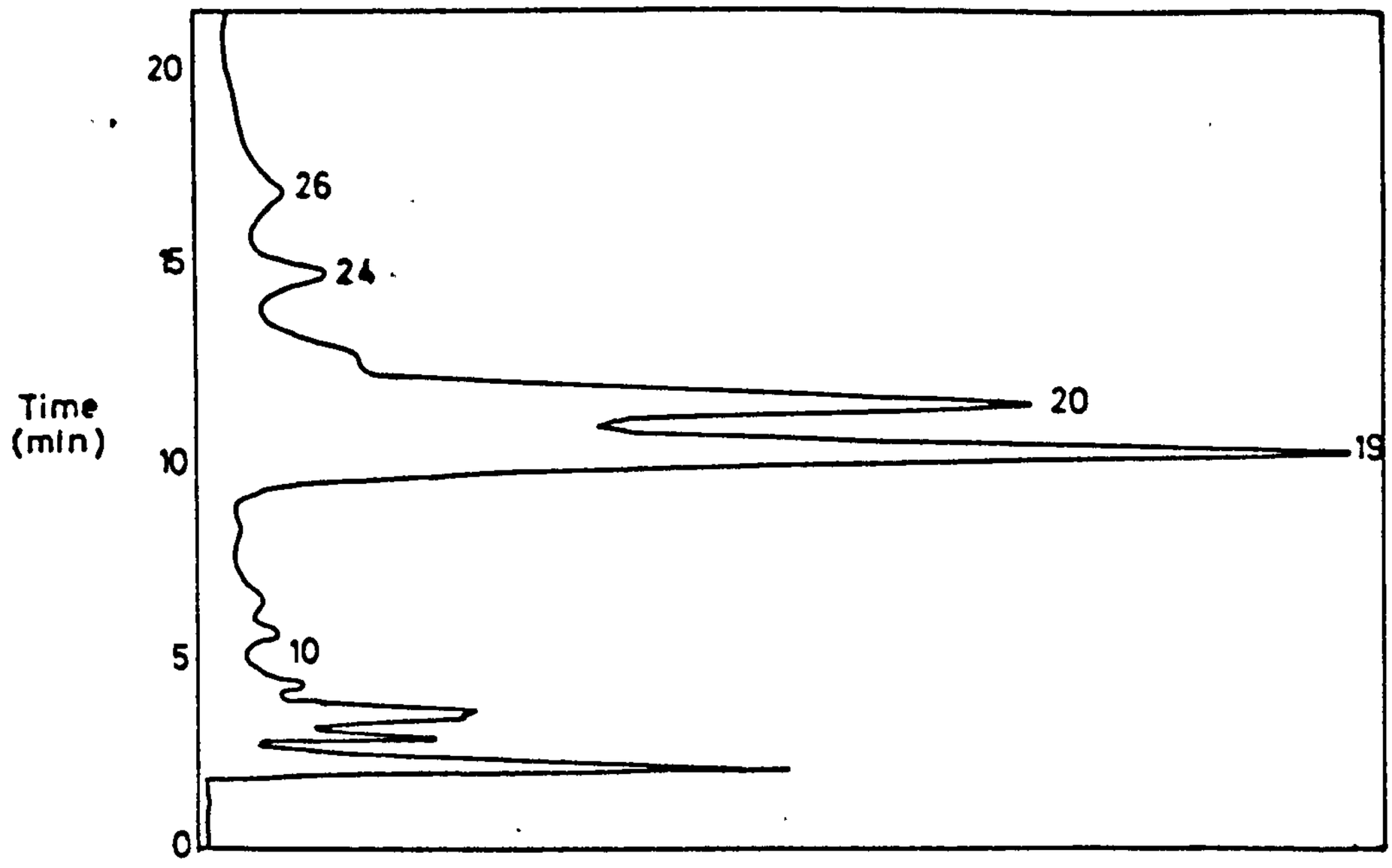
Limited information is available about the carotenoids of chicken feathers. The feathers themselves were difficult to extract due to their fibrous nature, and the resultant extract awkward to handle because of the interference from sterols. The final amount of material available was extremely low (8.8 µg, [^{14}C]zeaxanthin experiment; 4.4 µg, [$^{3}H_2$]zeaxanthin experiment) and so the subsequent analysis was not comprehensive.

i. [$^{3}H_2$]zeaxanthin treated embryos.

Before chromatography, sterols were removed from the sample by sterol precipitation and the digitonin reaction.

The LiChrosorb system was used to resolve the carotenoids. The h.p.l.c. elution profile is shown in Fig. 67. It was evident that dihydroxycarotenoids were the major components of the extract. Fractions were collected every 30 s except for the peaks which were collected as individual fractions. Fraction 19 and 20 were identified, by spectrophotometry, as lutein (0.202 µg) and zeaxanthin (0.154 µg), respectively. Absorption spectra of F24 and F26 did not aid in their identification (F24 λ_{max} in ethanol at 424.5 447.5 475 nm; F26 λ_{max} in ethanol at 424 445 469.5 nm).

Each fraction was prepared for liquid scintillation counting by normal methods. The results are shown in Fig. 67. Over 36% of the radioactivity is located in the zeaxanthin fraction (F20) clearly showing the deposition of zeaxanthin in feathers.



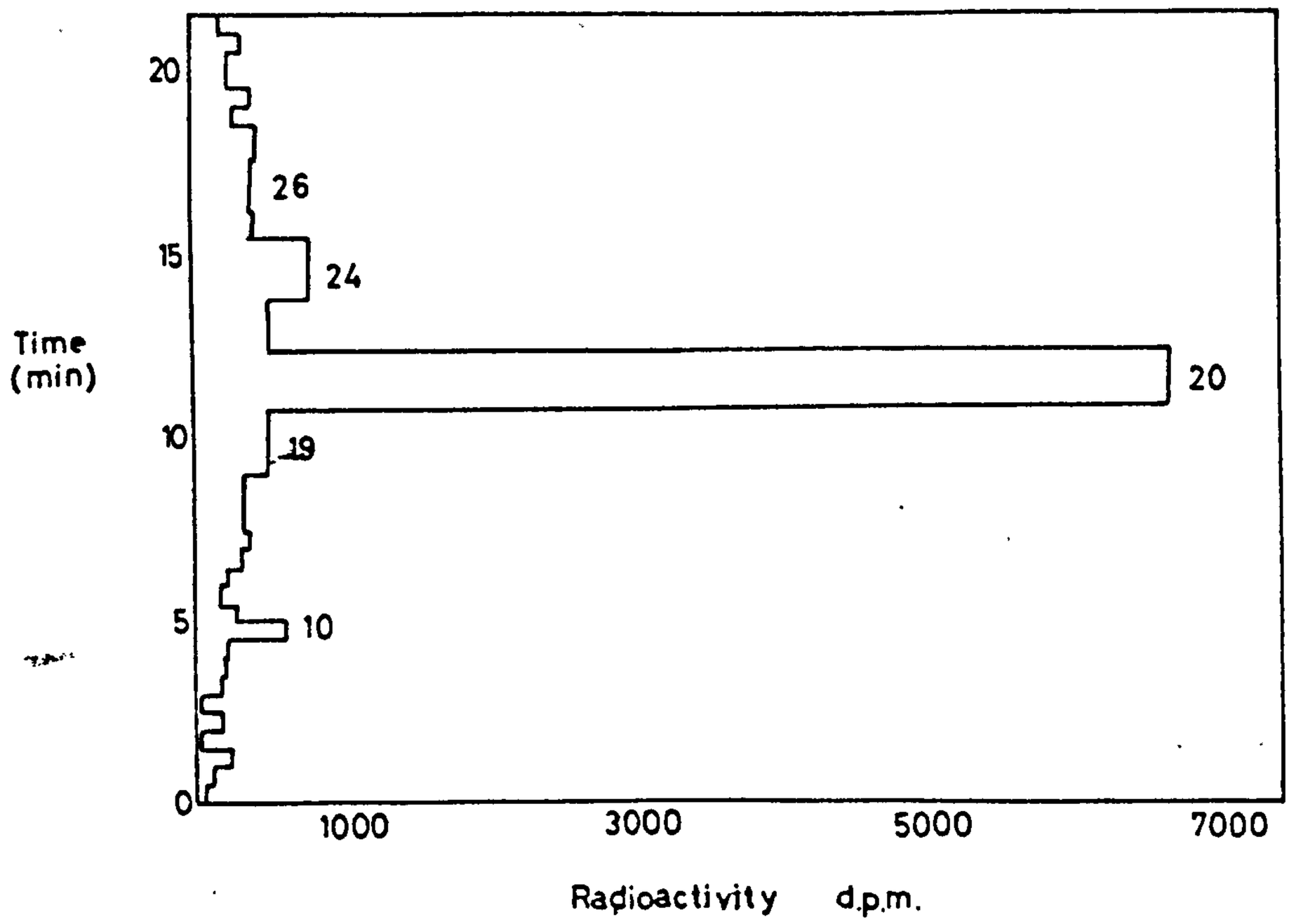


Fig. 67.

H.p.l.c. elution profile and corresponding radioactivity elution profile of the feather extracts from chick embryos developed in the presence of [$^2H_2$]zeaxanthin. Column: LiChrosorb Si-60 (250 mm x 4.5 mm); solvent: hexane/propan-2-ol/CH_2Cl_2 at 1.5 ml/min.

ii. [^{14}C]zeaxanthin treated embryos.

The results are largely the same for this experiment except that the effect is not as obvious due to lower specific activity of the [^{14}C]zeaxanthin administered to the embryos. Approximately 13% of the total radioactivity (196 d.p.m.) was present in the fraction corresponding to zeaxanthin.

4.35 Analysis of chick tissues from embryos developed in the presence of radiolabelled MVA.

The survival rates of chick embryos developed in the presence of [2-^{14}C]MVA were significantly higher than for embryos developed with [$^{3}H_2$]- or [^{14}C]zeaxanthin. This is a result of the method of administration of the MVA. The need to inject radiolabelled zeaxanthin into the egg yolks increased the risk of infection in those experiments.

The four extracts from the MVA treated embryos were heavily contaminated with sterols. Consequently, several sterol precipitations and digitonin reactions were carried out.

Table 28a and b summarise the results of spectrophotometric estimation and liquid scintillation counting of a known aliquot of each of the retinal, hepatic, intestinal and feather extracts.

After classical l.c. on Alumina (Section 4.22), the extracts were chromatographed in two dimensions on Silica Gel G. The retinal extract (neutral carotenoids only) was 'spiked' with 'cold' carotenoids from an adult chick retinal extract and the feather extract was 'spiked' with standard β-carotene and zeaxanthin. The intestinal and hepatic extracts were not 'spiked'. Figures 68-70, are diagrammatic representations of the retinal, intestinal and feather results. The figures show the position of carotenoid zones as well as zones showing fluorescence under

TABLE 28.

Summary of quantitative estimations of amounts of carotenoid present and incorporation into each tissue of embryos developed in the presence of [2-^{14}C]MVA. The data are for tissue extracts of 11 embryos which reached an acceptable stage of development and correspond to a total administration of 22 µCi of [2-^{14}C]MVA.

Tissue	Total volume (ml)	Volume counted (ml)	d.p.m.	Total d.p.m.	µCi	Percentage absorption
Feathers	10	0.1	81	8 100	0.00368	0.016
Intestine	10	0.1	292	29 200	0.0133	0.06
Liver	10	0.1	6124	612 400	0.278	1.26
Retina	10	0.1	53	5 300	0.0024	0.01

Tissue	Total volume (ml)	Absorbance at λ_{max}	Wavelength maxima (in acetone nm)			Total µg	Specific activity µCi/mg
Feathers	1	1.025	(425)	446	469	4.1	0.898
Intestine	2	1.463	(417)	442	470	11.7	1.137
Liver	20	0.616	285 (424)	296 445	325 467	49.28	5.641
Retina	10	0.478	394	421	442 469	19.12	0.125

$A^{1\ \%}_{1\ cm} = 2500$

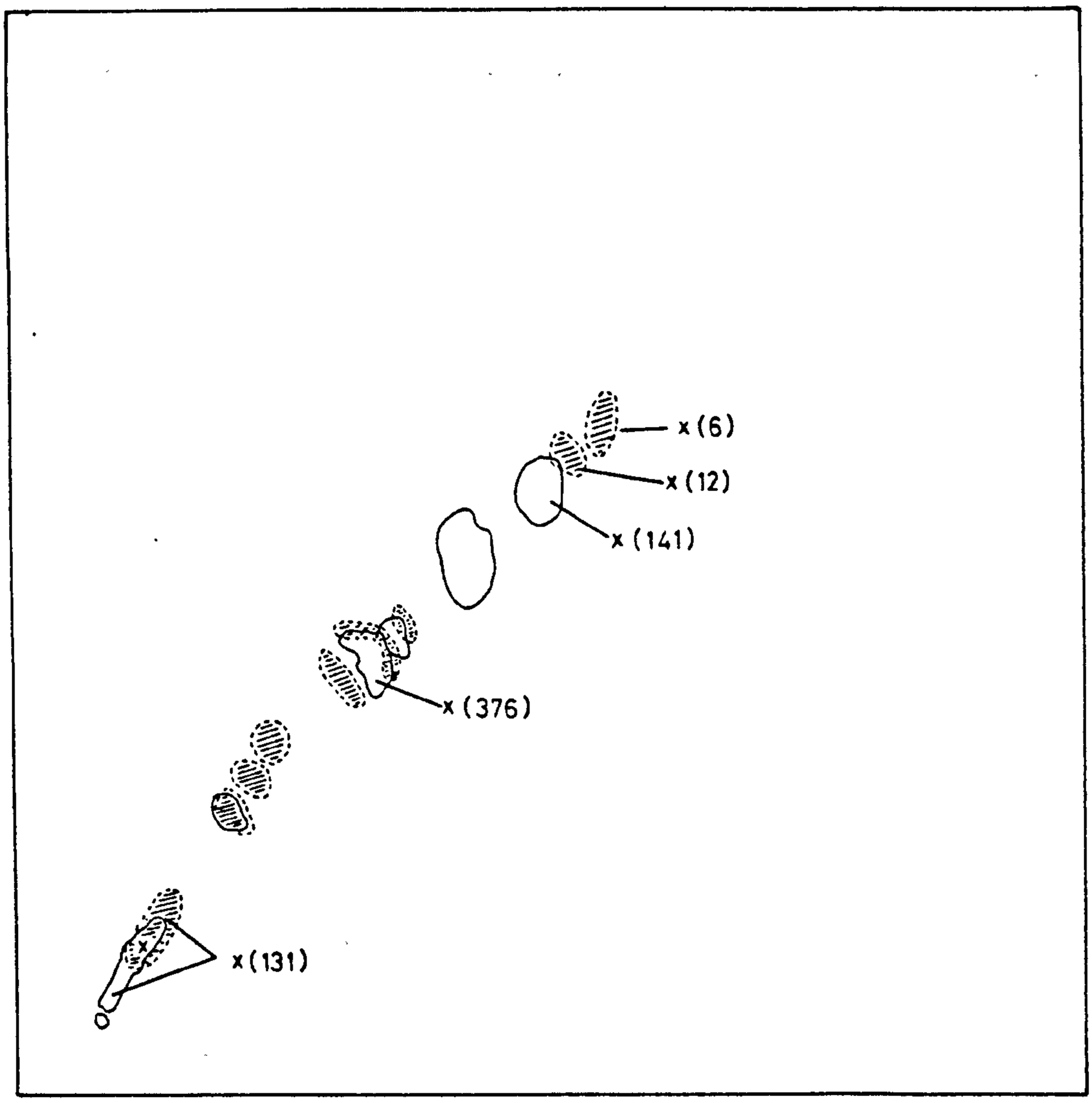


Fig. 68.

Diagrammatic representation of t.l.c. of retinal carotenoids from chick embryos developed in the presence of [2-^{14}C]MVA. Radioactivities per zone, as labelled, are given in brackets alongside the appropriate zone.

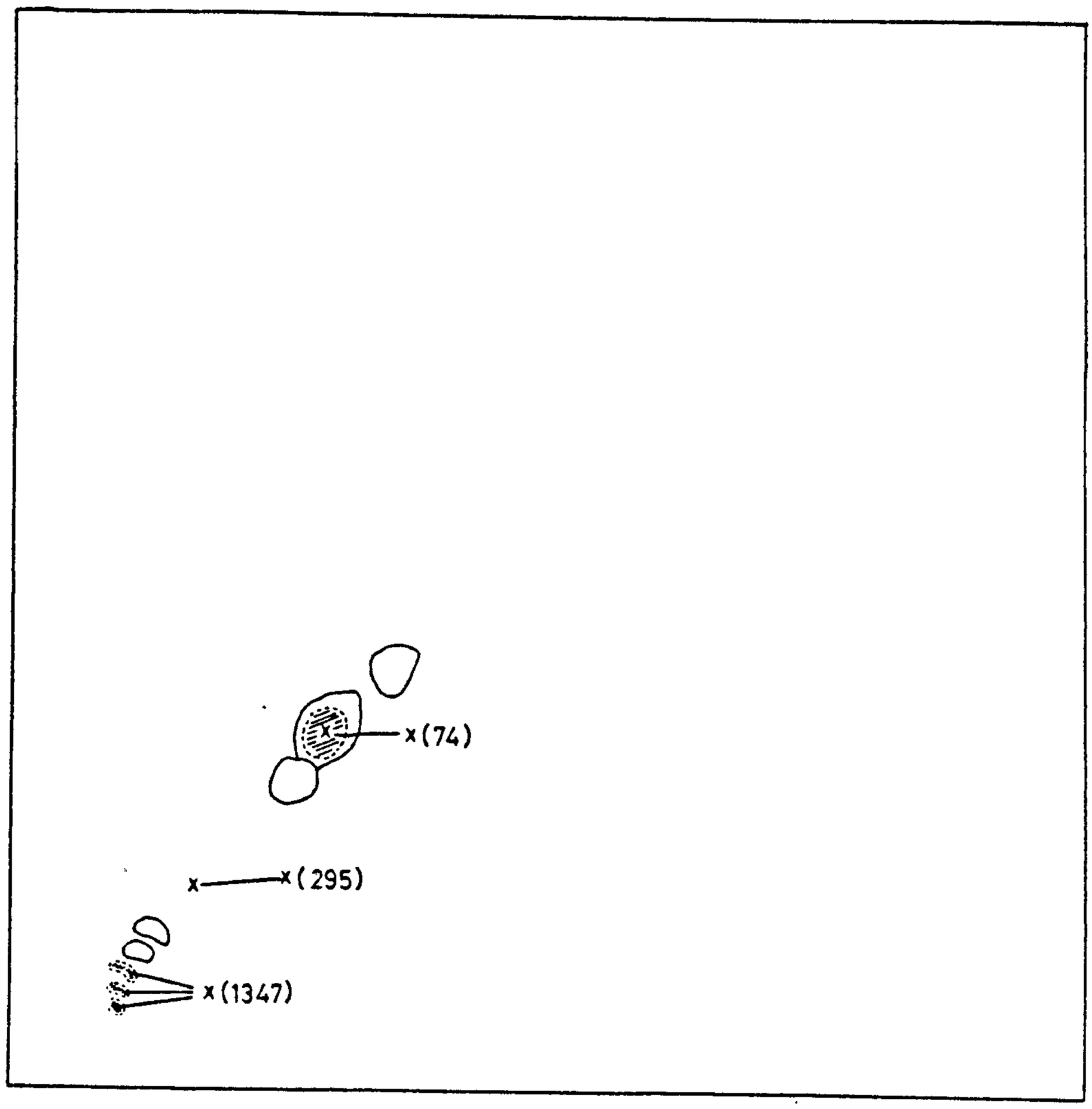


Fig. 69.

Diagrammatic representation of t.l.c. of intestinal extracts from chick embryos developed in the presence of [2-^{14}C]MVA. Radioactivities per zone, as labelled, are given in brackets alongside the appropriate zone.

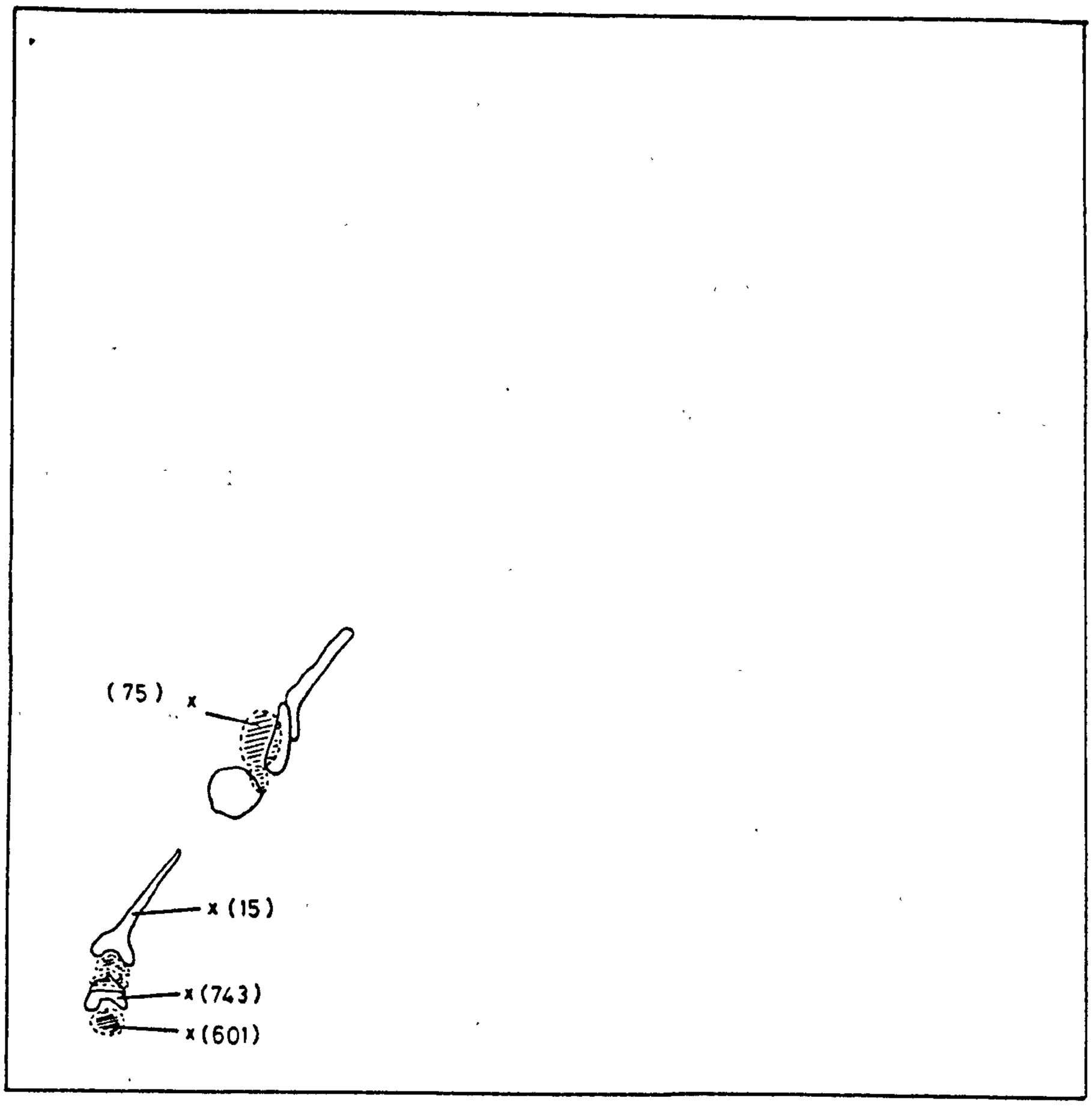


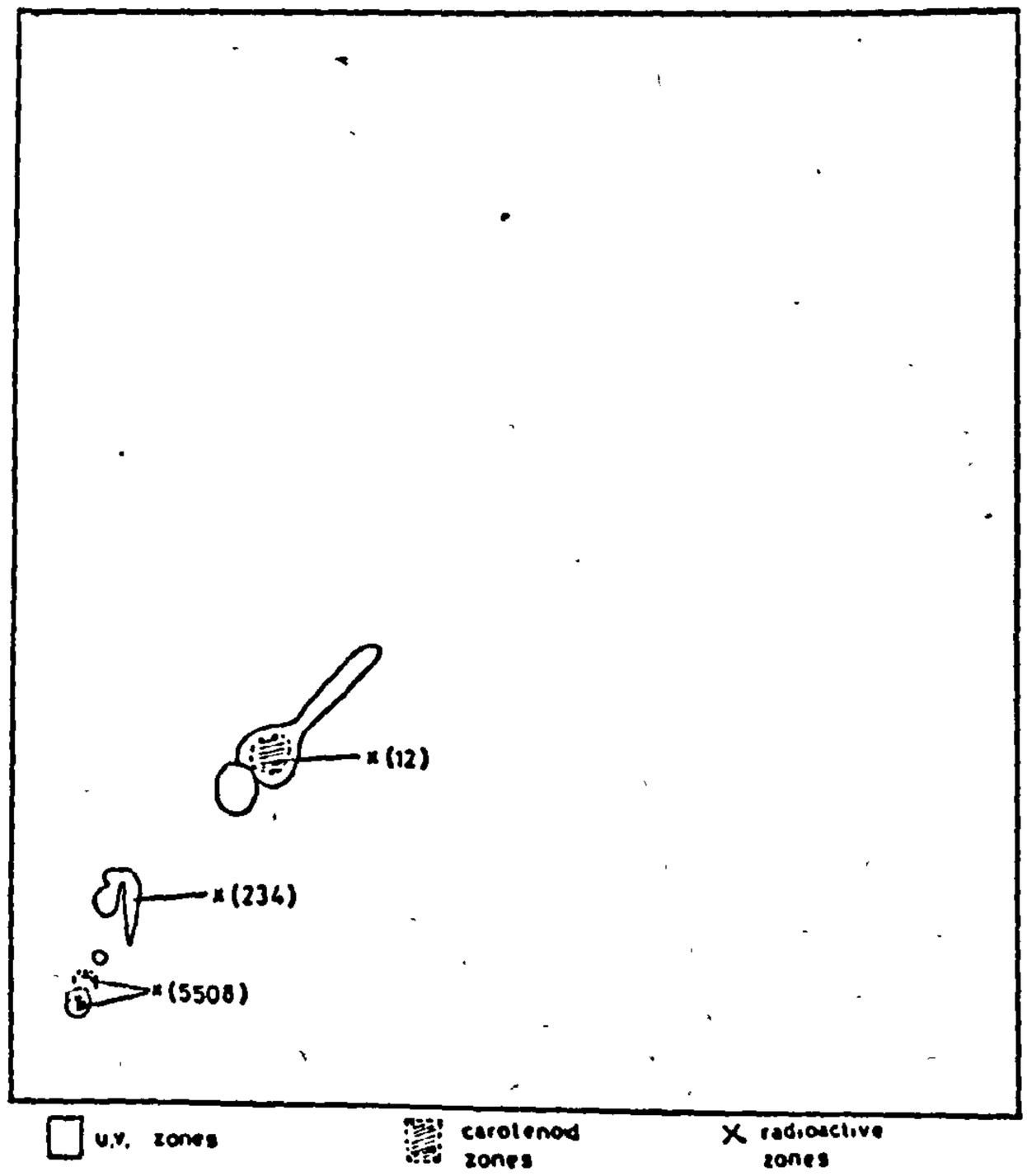
Fig. 70.

Diagrammatic representation of t.l.c. of feather carotenoids from chick embryos developed in the presence of [2-^{14}C]MVA. Radioactivities per zone, as labelled, are given in brackets alongside the appropriate zone.

long wavelength u.v. light. The zones were marked on the t.l.c. plate very carefully prior to radioautography. Following development of the X-ray film, the radioactive zones on the t.l.c. plates were scraped off, eluted and counted. The radioactivities per zone are given in brackets in an appropriate position in Figures 68-70. A photograph of the radioautogram of the t.l.c. from the liver extract of chick embryos developed in the presence of [2-^{14}C]MVA is given in Fig. 71. A diagrammatic representation of the chromatography of liver extract is superimposed onto the radioautogram in the second case to demonstrate the co-incidence of the radioactivity zones with zones corresponding to carotenoids.

The radioautograms from extracts of all four tissues showed that the carotene zone in each case closely corresponded to radioactive zones. It is difficult to determine whether the radioactivity is associated with the carotene component or with something that co-chromatographs with this fraction. Removal of squalene by the Alumina column (Section 4.22) should eliminate the possibility of contamination of carotene with this MVA metabolite. The t.l.c. separation system should be adequate to resolve β-carotene and squalene so that any residual squalene could not interfere with the interpretation of the results.

As can be seen from Figs. 68-71 the levels of radioactivity detected in the zones coinciding with carotenoid zones are very low and thus the significance of these findings is questionable. Of the total 22 µCi [2-^{14}C]MVA administered to the 11 chick embryos which survived to hatching, only 0.29 µCi was recovered from the four tissues studied. The majority of the radioactivity from the extracts was removed during preparative steps of sterol precipitation and sterol digitonide formation. This demonstrates the active pathway of sterol formation. The radioactivity remaining in the carotene fraction corresponds to a very minute fraction of the 22 µCi [2-^{14}C]MVA administered, and to a fraction of 1% of the radioactivity detected in the tissues of the newly


x (12)
x (234)
x (5508)
u.v. zones
carotenoid zones
X radioactive zones

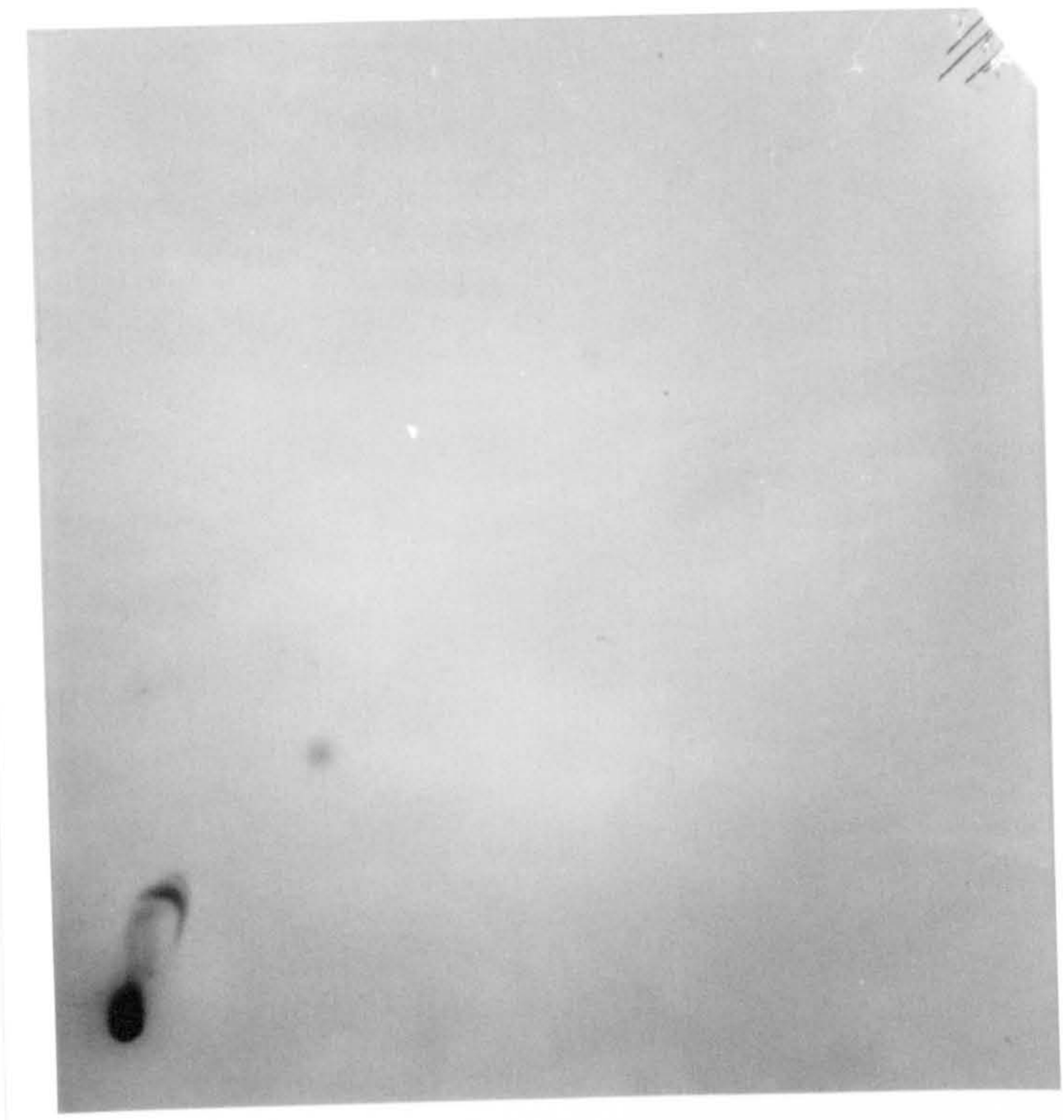
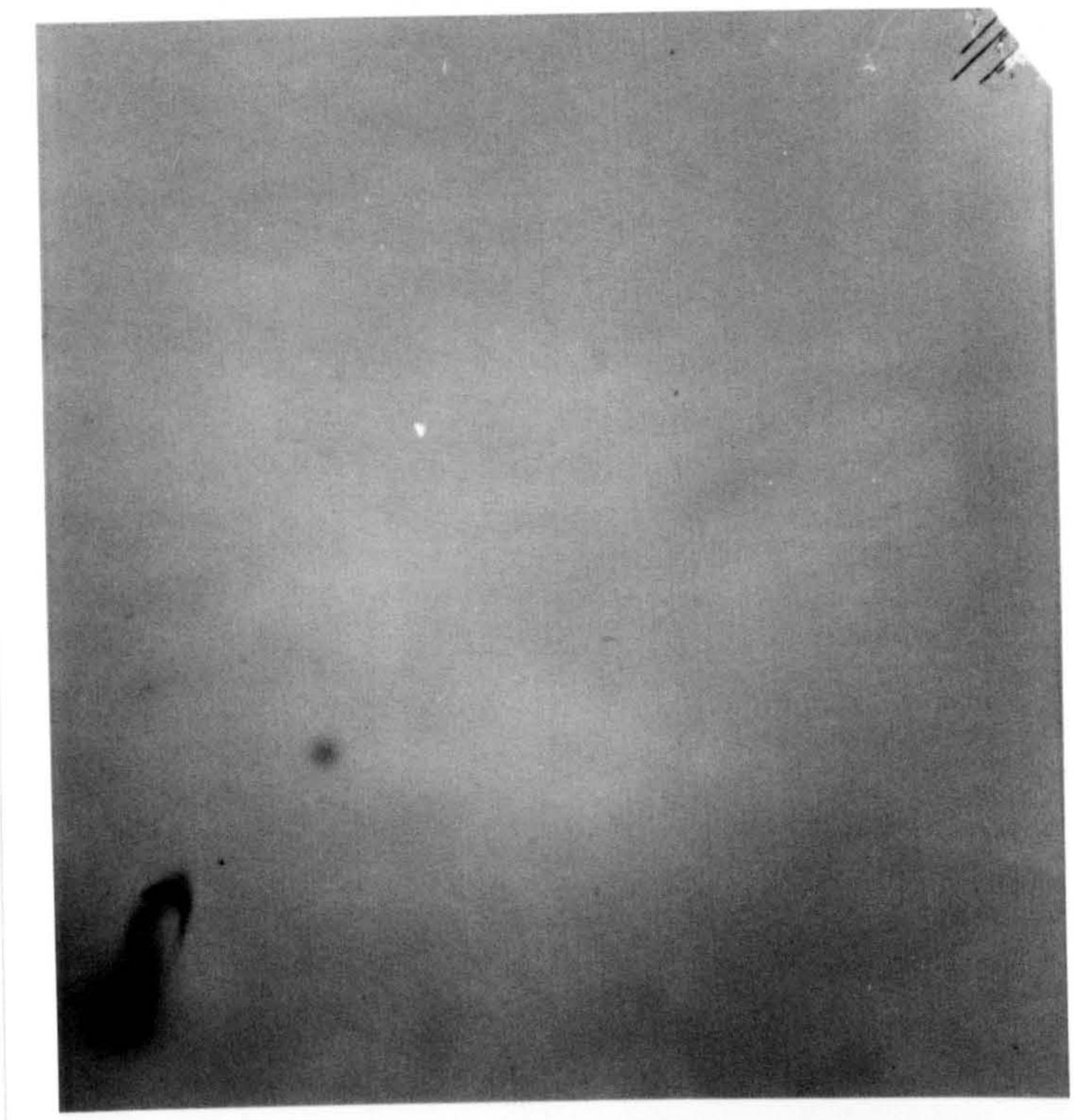


Fig. 71.

Radioautograms of t.l.c. of liver extracts from chick embryos developed in the presence of [2-^{14}C]MVA. Radioactivities per zone, as labelled, are given in brackets alongside the appropriate zone.



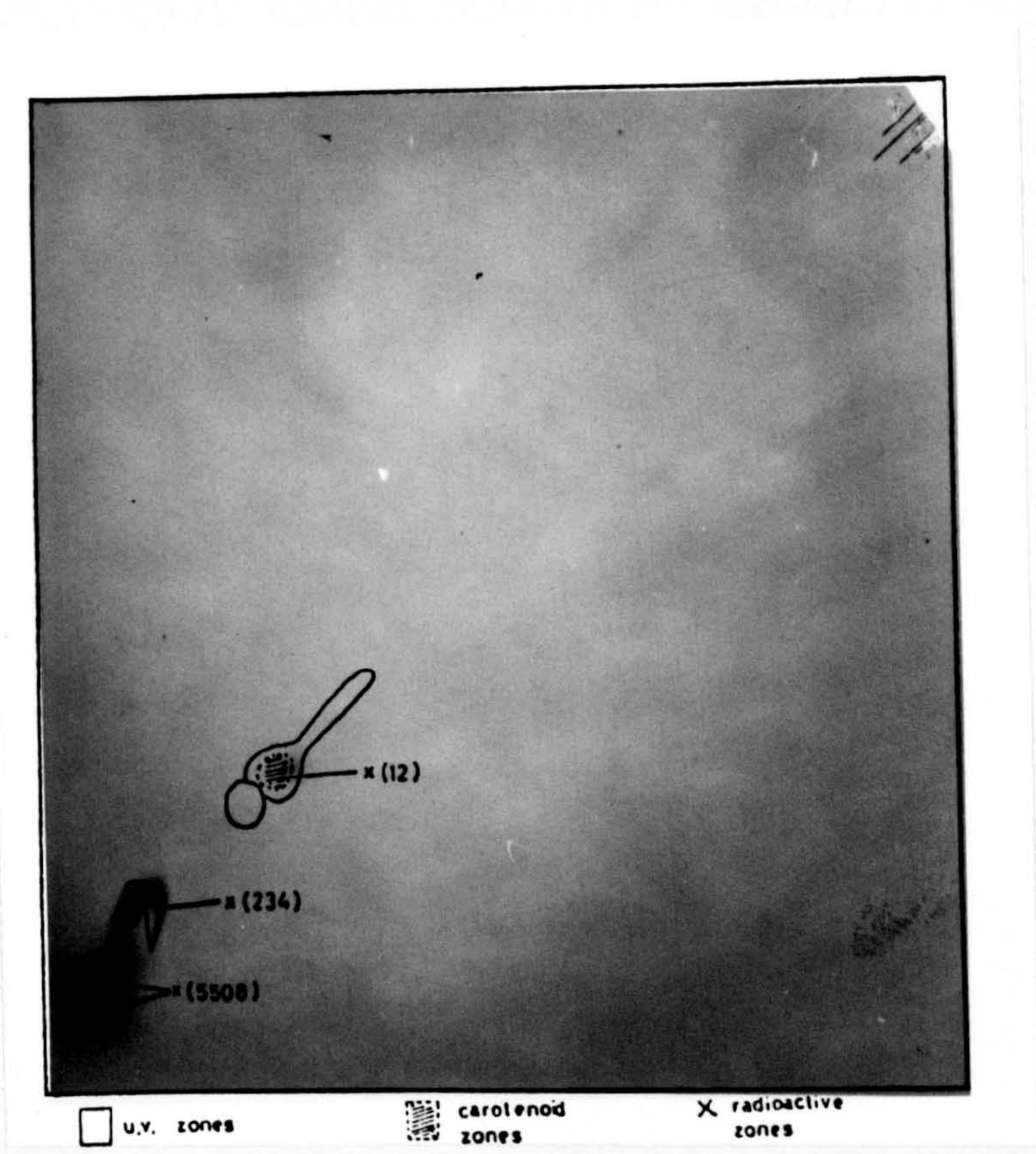


Fig. 71.

Radioautograms of t.l.c. of liver extracts from chick embryos developed in the presence of [2-^{14}C]MVA. Radioactivities per zone, as labelled, are given in brackets alongside the appropriate zone.

hatched chicks.

The only conclusion to be drawn is that very low levels of radioactivity are associated with zones that coincide exactly with carotenoid zones.

4.36 Microspectrophotometric studies (MSP).

A. Fresh tissue.

Eyes were used within 36 h of slaughtering of the animal. Longer periods of storage resulted in marked losses of carotenoids, as seen by the much paler oil droplets.

On dissection of fresh eyes, the retina could be seen as a distinct layer. In fresh tissues the retina was invariably intact and could be removed fairly easily from the other tissues with the aid of forceps. The oil droplets were readily visible under a Zeiss microscope, although the intensity of colour was not as high as anticipated. The 'red' droplets were very pale and more orange in colour than expected. The yellow droplet types were not easily distinguishable. This emphasised the problem of identification of oil droplet types and illustrated the need for a classification system not dependent on visual appearance of droplets. The system of Goldsmith et al., (1984) is based on size, retinal position as well as visual appearance but there are still major problems with its application.

In fresh tissue, the visual cells were intact and it was fairly easy to see oil droplets associated with their cells. Double cones (chief and accessory members) were distinguishable as were single cones. The association of oil droplets with different cell types eased the problem of oil droplet identification.

Oil droplets were measured fairly randomly but wherever possible, droplets associated with cone cells were used. After many spectra of the same type of oil droplet had been collected, a process of averaging was carried out by the computer. All the spectra shown are averages of the different oil droplet types found.

Figure 72 shows five droplet types measured in fresh turkey retina. The absorbance of these droplets were far lower than observed in other species e.g. in chicken retina all four 'coloured' droplet types produced cut-off spectra (Bowmaker and Knowles, 1977). The absorbance at 100% is 0.6.

Curve 1 is the spectrum of the red oil droplet, which occurs in single cones. Dilution of red droplets gave the spectrum of Figure 73. Contrary to expectation, this spectrum was not indicative of a pure carotenoid. Previous reports (Goldsmith et al., 1984) have led to the general belief that red droplets contain pure astaxanthin. This is evidently not the case in turkey retina.

Curve 2 is of the yellow droplet. This has absorption maxima at 430, 450 and 480 nm approx. This is clearly a mixture of at least two carotenoids. The yellow droplets are again found only in single cones.

Curve 3 is a spectrum of the pale droplet type. This has absorption maxima at 415, 430 and 480 nm. Again, this is not indicative of a pure carotenoid. The shape of the spectrum resembles that of a galloxanthin type of carotenoid. These types of droplets are restricted to the chief members of the double cones.

Curve 4 is a spectrum of the 'clear' oil droplet. It is feasible that this oil droplet contains a pure carotenoid. The absorption maxima are at approximately 410 and 430 nm. These droplets were found in single

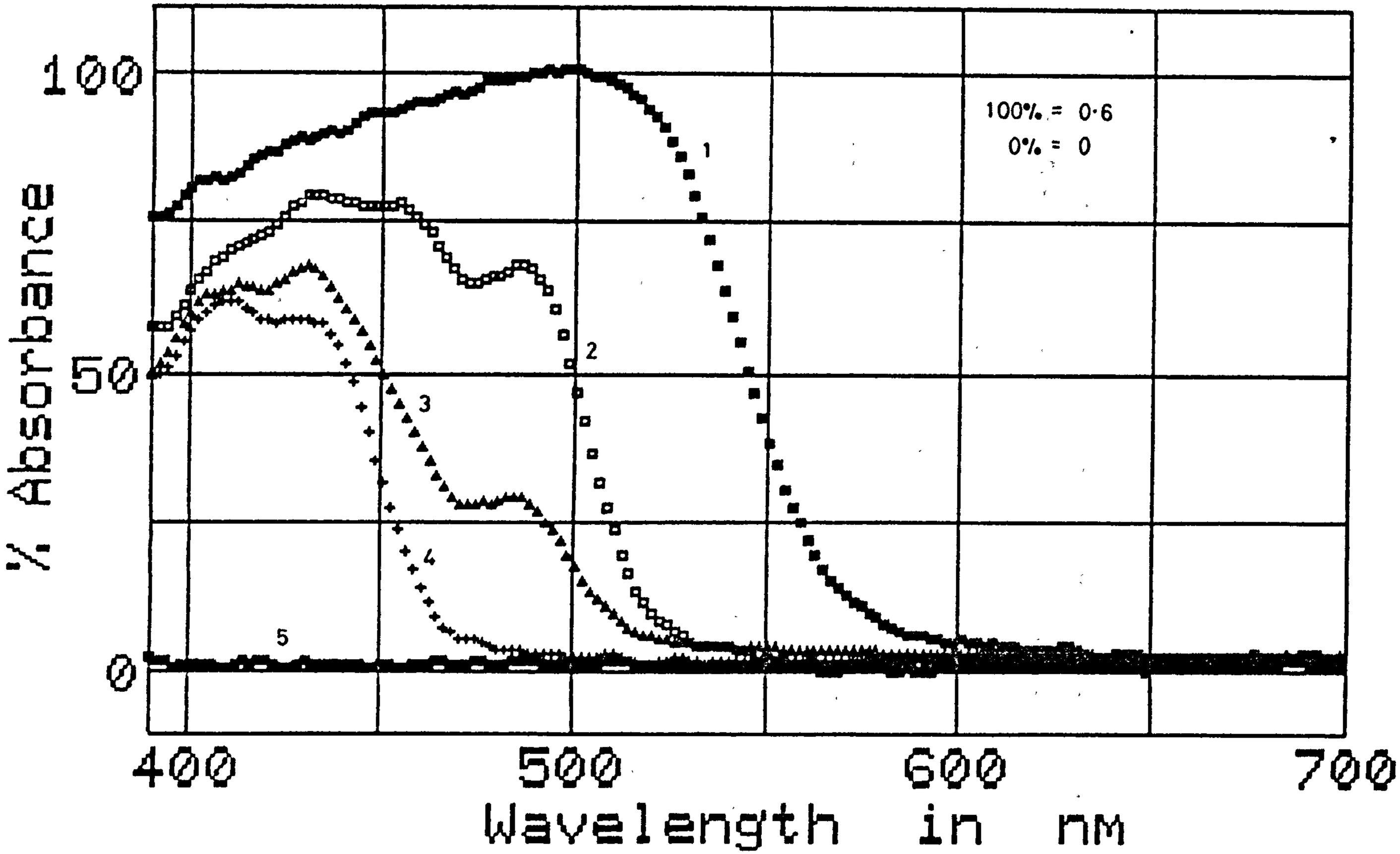


Fig. 72.

The absorption spectra of five of the oil droplet types measured in fresh turkey retina. Curves 1-5 correspond to red, yellow, pale, clear and transparent oil droplets respectively.

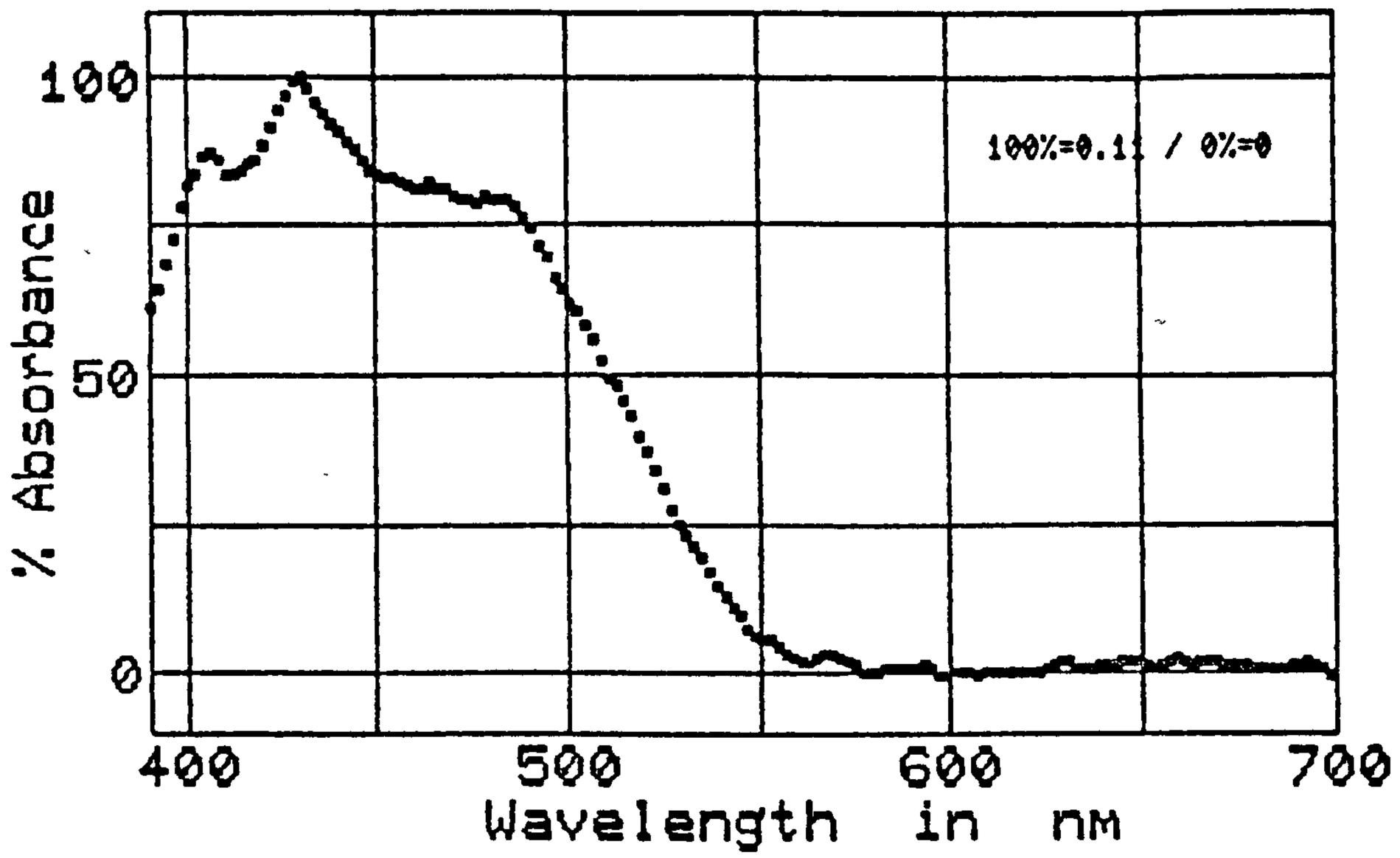


Fig. 73.

The absorption spectrum of the diluted red droplets.

cones only.

Curve 5 is the spectrum of a transparent droplet. These droplets are only present in single cones.

All of the above droplets are routinely seen and identified by Goldsmith et al., (1984).

One other type of oil droplet was identified in the turkey retina. In some accessory members of double cones there were very small oil droplets present. These were measured even though some of the droplets appeared to be colourless. Conversely, in some accessory members there was clear evidence of coloration of the cell but no indication of an oil droplet as a structural feature. These were also measured. The spectra of the two types were identical and the average spectrum is shown in Fig. 74. The spectrum is indicative of a very pure carotenoid. The absorption maxima are at 430, 450 and 480 nm. The shape of the spectrum is consistent with a carotenoid structure such as lutein or even ε,ε-carotene. This type of spectrum was measured in the accessory members of double cones of chicken retina (Bowmaker and Knowles, 1977).

The relative numbers of each type of oil droplet in turkey retina compares well with that found in chicken. The double cones comprised about 50% of the total number of cones with the red and yellow accounting for approximately 20% each. The remaining 10% of droplets were comprised of the clear and transparent droplets in roughly equal proportions.

B. Frozen tissue (-20°and -80°C).

The retinas were not intact as a result of freezing. Detachment of retina from other layers was difficult. There did not appear to be any appreciable loss of carotenoids due to the freezing but, unfortunately distinguishable cone cells were not present. Identification of the pale

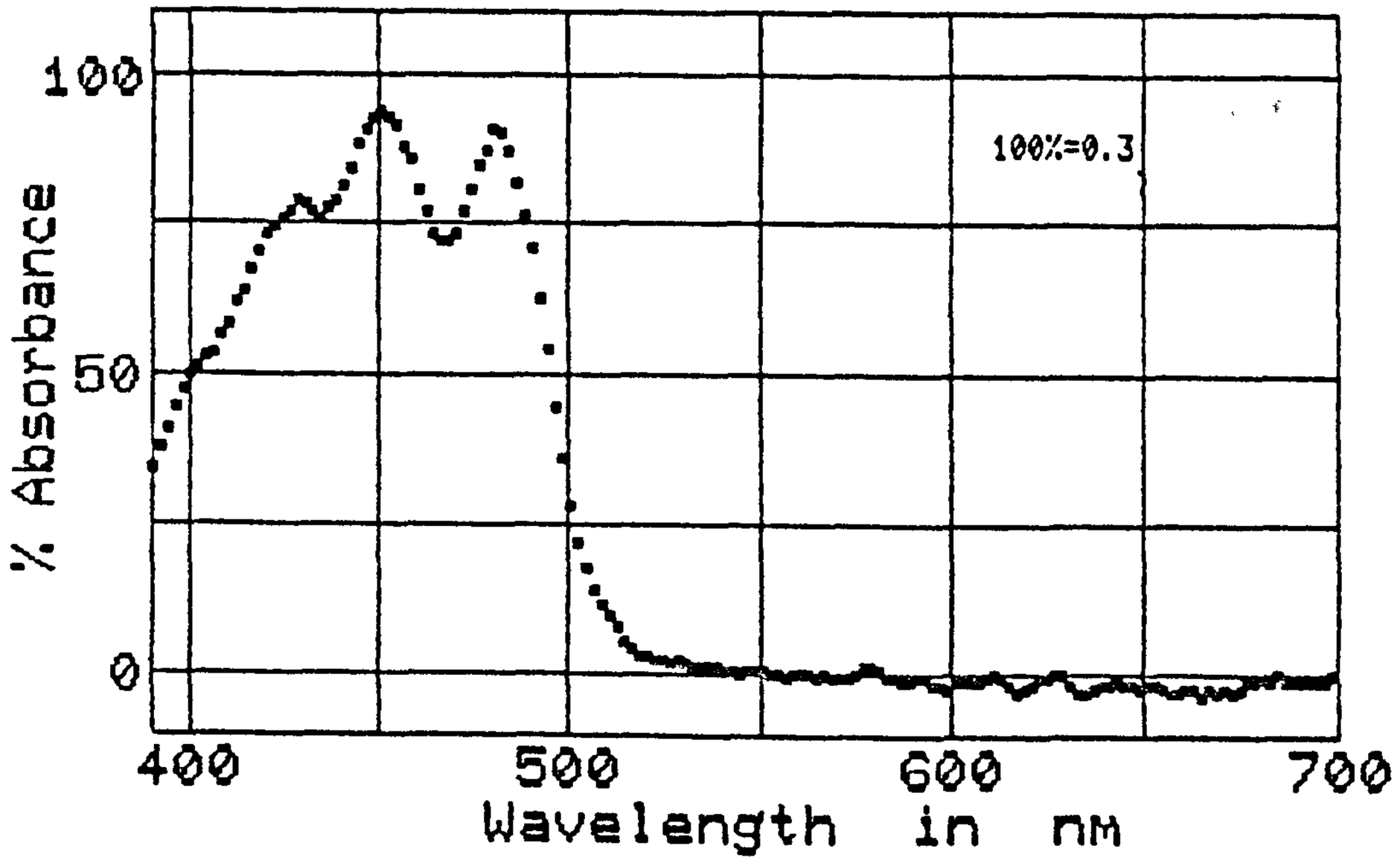


Fig. 74.

Absorption spectrum of oil droplets from the accessory member of double cones.

droplets was very difficult.

Identical spectra were obtained for the frozen tissue as for the fresh tissue, the only exception being the absence of the oil droplet of the accessory member of the double cone. Figures 75 and 76, show the spectra for the tissue stored at different temperatures. On the basis of the classification system (Goldsmith et al., 1984), the curves 1 to 5 represent red, yellow, pale, clear and transparent oil droplets, respectively in each case. Figure 77 shows the absorption spectrum red diluted droplet.

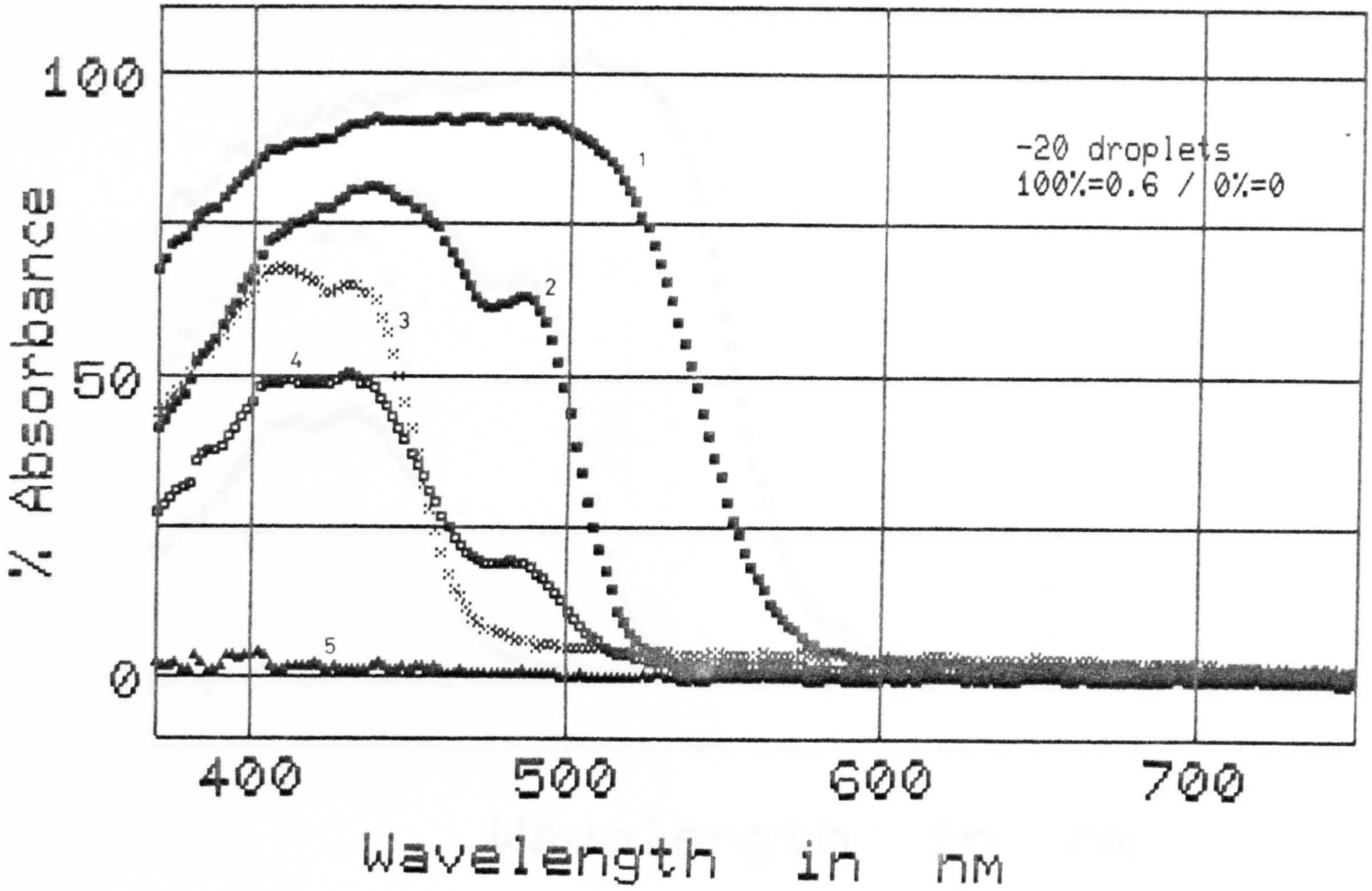


Fig. 75.

Absorption spectra of oil droplets from turkey retinal tissue stored at -20 °C. Curves 1-5 correspond to red, yellow, pale, clear and transparent oil droplets respectively.

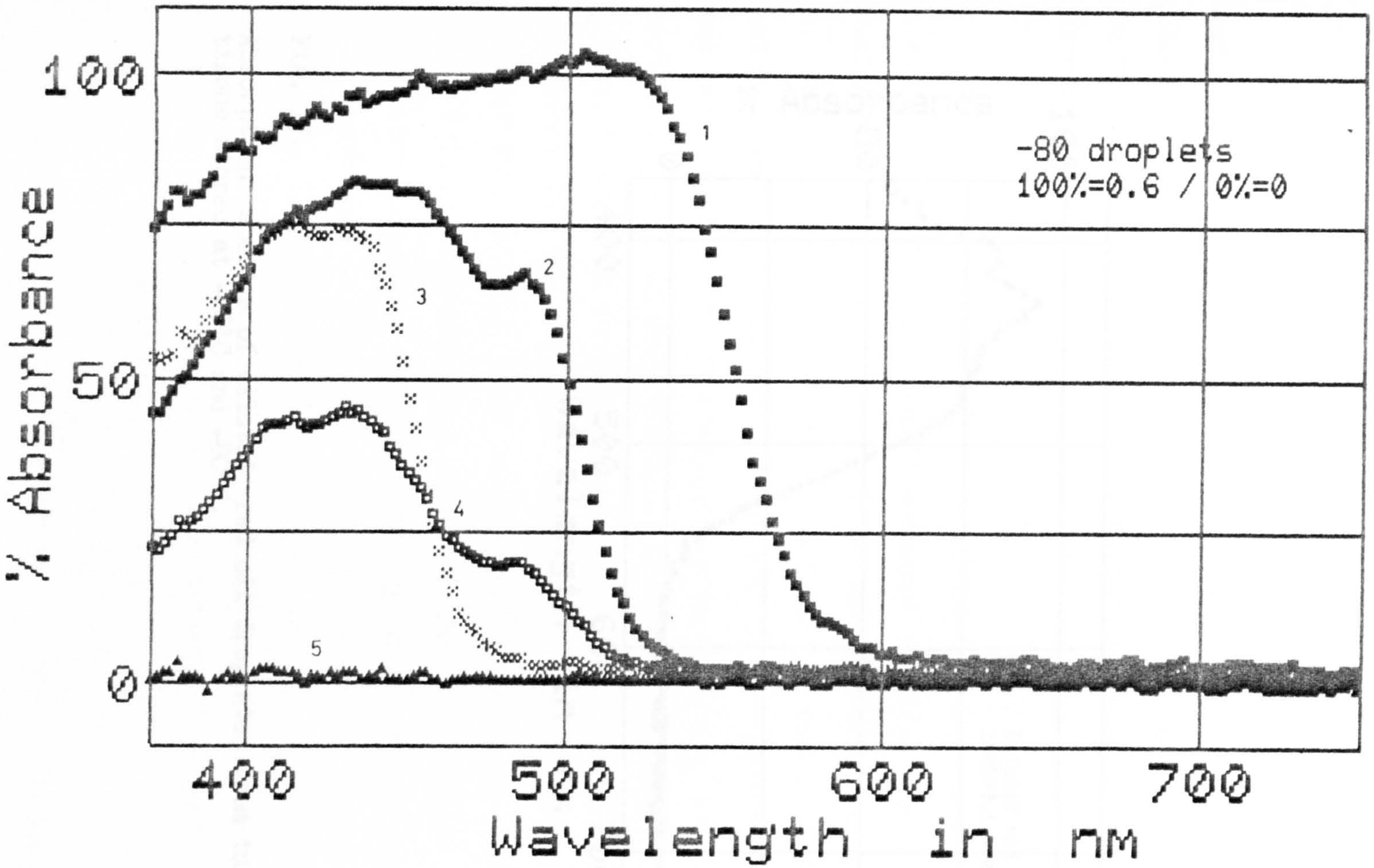


Fig. 76.

Absorption spectra of oil droplets from turkey retinal tissue stored at -80 °C. Curves 1-5 correspond to red, yellow, pale, clear and transparent oil droplets respectively.

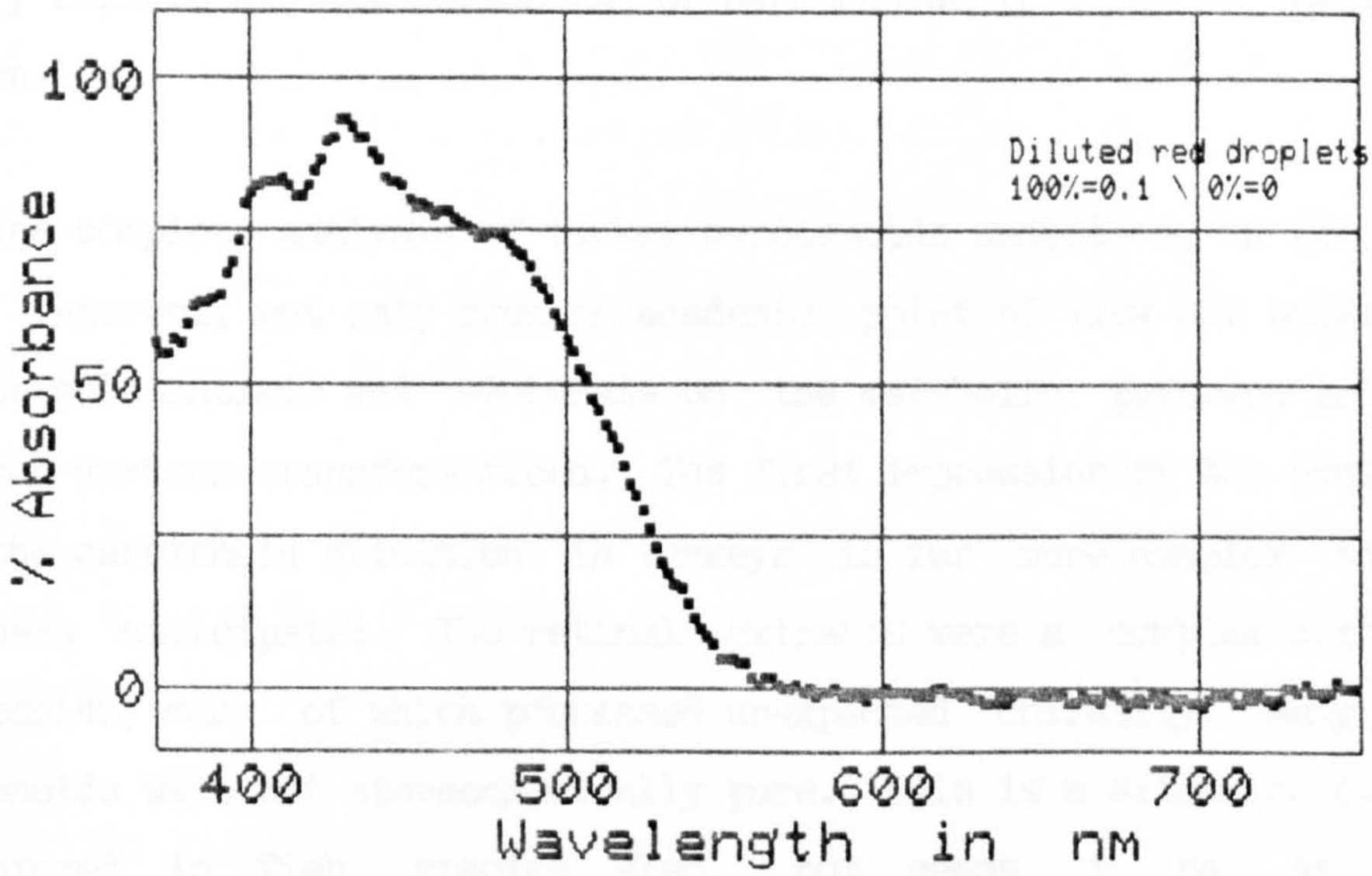


Fig. 77.

Absorption spectra of diluted red oil droplets from turkey retinal tissue stored at -20° C and -80° C.

DISCUSSION

It is obvious from the introduction to this chapter that there is very little carotenoid literature about birds. Except for the pioneering work of Fox on flamingos and the commercially important poultry experiments the metabolism of carotenoids in birds is relatively unknown.

The complete analysis of turkey carotenoids undertaken in this study is of interest, not only from an academic point of view but because the results substantiate and elaborate on the metabolic pathways predicted for the various transformations. The first impression of the results is that the carotenoid situation in turkeys is far more complex than may have been anticipated. The retinal extracts were a complex mixture of carotenoids, many of which possessed unexpected chirality. Many of the carotenoids were not stereochemically pure. This is a situation commonly encountered in fish species also, but seems to be an animal characteristic in that plant carotenoids are normally stereochemically pure. Another interesting and unusual feature is the high proportion of carotenoids containing ε-rings. The cryptoxanthin and sole carotene of the extract have α- and ε-chromophores, respectively. The structure of the newly identified carotenoid possesses an ε-end group. The identification and definition of absolute stereochemistry of the carotenoids of turkey retina is of importance from both metabolic and physiological considerations.

Throughout the course of this study it has been assumed that a direct comparison can be made between the turkey and chick retinal carotenoids. Certainly from a point of view of the same carotenoids being present this is acceptable. However, the chiralities of the chick retinal carotenoids have not been investigated. It is possible that there may be inconsistencies between the two species. Ideally, a similar

analysis of the chick retinal carotenoids should be undertaken to eliminate this possibility. Any differences would be unlikely to affect the conclusions drawn from the metabolic experiments in this presentation.

The analysis of the feed extracts is very interesting. By c.d. analysis the lutein and zeaxanthin of a typical poultry feed have been shown to be (3R,3'R,6'R)lutein and (3R,3'R)zeaxanthin. As anticipated, the carotenoids of the diet are of plant origin. This is in marked contrast to the carotenoids of the turkey retina, clearly demonstrating that metabolism of carotenoids must be occurring in turkey tissues to account for these differences.

The presence of retinol and dehydroretinol in chicken livers inspired the analysis of poultry feed for the presence of the two forms of vitamin A. It is well known that poultry feeds are supplemented with fish meal. The addition of marine fish meal as opposed to freshwater fish meal would be anticipated. Consequently, it was not surprising that retinol could be detected in the turkey diet. However, no dehydroretinol was detected. The absence of dehydroretinol can be accounted for in one of two ways. Firstly, there is a possibility that only very small amounts of dehydroretinol were present, which would be the case if the marine fish meal was the only source of vitamin A in the feed. It has been shown that marine fish only have small amounts of dehydroretinol as compared to freshwater fish (Lambersten and Braekken, 1969). Retinol and dehydroretinol were not detected in turkey egg yolk but the metabolic experiments clearly showed the presence of the two vitamins A in newly hatched chicks. This brings us to the second possibility. Can the presence of retinol and dehydroretinol in chick liver be accounted for by the modification of carotenoids available to it (i.e. yolk carotenoids)? It is well documented that chicks are able to convert β-carotene into retinol. Details of the vast experimental data for this are given in the review by Bauernfeind et al., (1981). One interesting paper reports that

in young chicks β-carotene can be converted to retinol by the first week of life or earlier. These results certainly suggest that this conversion is possible during development of the embryo. Thus the explanation of the presence of retinol is straightforward. A report by Budowski and Gross (1965) that anhydrolutein can be converted to dehydroretinol in chicks could account for the presence of dehydroretinol in newly hatched chicks. Anhydrolutein is not considered to be of plant origin so it may be assumed that it is derived from the large amounts of lutein present in the egg yolk. The absence of retinol and dehydroretinol from turkey yolk seems inconsistent with the role of vitamin A in ensuring normal development and growth of embryos.

The most suitable method to investigate carotenoid metabolism in chick retina is by undertaking a series of embryonic experiments. There are two reasons for this. Firstly, the embryonic environment controls the intake of carotenoids to the animal, yolk carotenoids being the only source. Secondly, it has been proposed by Meyer et al. (1971) that the turnover of carotenoids in oil droplets is very slow as demonstrated by the very slow depletion, if at all, of carotenoids from droplets of quail maintained on a carotenoid-free diet.

For this type of experiment high specific activities of carotenoid substrates were required. With the low uptakes and limited metabolism anticipated, detection of metabolites would be difficult. For this reason the [$^{3}H_2$]zeaxanthin experiments were carried out despite the fact that the [$^{3}H_2$]zeaxanthin was a 50:50 mixture of (3S,3'S)- and (3R,3'R)zeaxanthin. The absence of (3S,3'S)zeaxanthin from hens egg yolk and turkey retina suggests that it does not play any role in chicken carotenoid metabolism. As the absolute configurations of most of the retinal carotenoids is known, any metabolism occurring can be identified and substantiated on stereochemical grounds. The possibility of exchange between peripheral hydrogen atoms and the media precipitated the [^{14}C]zeaxanthin experiments. The low specific activity of the

[^{14}C]zeaxanthin caused problems but substantiated the results from the [$^{3}H_2$]zeaxanthin experiments. Neither experiment alone would be satisfactory.

The results of the metabolic experiments unequivocally demonstrate the ability of chick embryos to metabolise zeaxanthin. In the retina the conversion of zeaxanthin to α-cryptoxanthin, ε-galloxanthin, galloxanthin and astaxanthin is obvious for both the [^{14}C]- and [$^{3}H_2$]zeaxanthin experiments. In neither experiment is it clear whether or not radiolabelled zeaxanthin is being metabolised to ε,ε-carotene. Based on the work of Schiedt et al. (1981b) and a more recent publication, Matsuno et al. (1986), several new metabolic pathways of (3R,3'R,6'R) lutein (lutein A) and (3R,3'R)zeaxanthin in hens egg yolk have been proposed. Schiedt et al. (1981b) demonstrated in laying hens that lutein A was converted into (6S,6'R)-ε,ε-carotene-3,3'-dione via (3R,6'R)-3-hydroxy-β,ε-caroten-3'-one, and (3R,3'R)zeaxanthin was converted to (6S,6'S)-ε,ε-carotene-3,3'-dione via (3R,6'S)-3-hydroxy-β,ε-caroten-3'-one. Matsuno et al. (1986) also detected the above metabolites in hens egg yolk, in addition to (6R,6'R)-ε,ε-carotene-3,3'-dione and 3 new carotenoids, (6R,3'R,6'R)-3'-hydroxy-ε,ε-carotene-3-one, (6S,3'R,6'R)-3'-hydroxy-ε,ε-caroten-3-one and (6'RS)-β,ε-caroten-3'-one. These carotenods were present only in very small quantities, typically 0.2-1.5% of the total carotenoid in hens egg yolk. The major carotenoid constituent of egg yolk was lutein A (40%), with relatively high levels of (3R,3'R)zeaxanthin (19.8%), canthaxanthin (17.9%) and 3R-β-cryptoxanthin (17.3%).

Taking into consideration the metabolic pathways of carotenoid metabolism in chicken proposed by Schiedt et al. (1981b), stereochemical aspects and individual carotenoids present, Matsuno et al. (1986) have confirmed the metabolic pathways of Schiedt et al. (1981b) and proposed the following new pathways.

(i) Lutein A to (6R,6'R)-ε,ε-carotene-3,3'-dione via (6R,3'R,6'R)-3'-hydroxy-ε,ε-caroten-3-one and/or (3R,6'R)-3-hydroxy-β,ε-caroten-3'-one.

(ii) (3R,3'R)Zeaxanthin to (6S,6'R)-ε,ε-carotene-3,3'-dione via (3R,6'R)-3-hydroxy-β,ε-caroten-3'-one and/or (3R,6'S)-3-hydroxy-β,ε-caroten-3'-one.

(iii) 3R-β-Cryptoxanthin to (6'RS)-β,ε-caroten-3'-one.

Essentially, the above demonstrates that lutein of hens egg yolk is of the incorrect chirality to be the natural precursor of the (6S,6'S)-ε,ε-carotene structure present in chicken retina. The only carotenoid present in hens egg yolk of the appropriate chirality to give rise to (6S,6'S)-ε,ε-carotene is (3R,3'R)zeaxanthin.

The metabolic pathway of Fig. 78 depicts another conversion pathway from zeaxanthin to ε,ε-carotene via dehydrolutein, lutein and α-cryptoxanthin. As α-cryptoxanthin is undoubtedly a metabolite of zeaxanthin, it is quite possible that the other two steps to ε,ε-carotene are feasible. The long route from zeaxanthin to ε,ε-carotene demands an extremely high specific activity of zeaxanthin for detection of metabolites. Perhaps, a similar embryonic experiment using, for example, α-cryptoxanthin as substrate would confirm the results of this experiment. Given that α-cryptoxanthin is radiolabelled and is a metabolite along a pathway leading to ε,ε-carotene, it seems reasonable to interpret the results such that the indication of metabolism of zeaxanthin to ε,ε-carotene is a real observation, metabolism not being obvious due to the presence of other more highly labelled metabolites. Thus, it can be concluded that zeaxanthin is central to formation of retinal carotenoids in chicks. Figures 79-81 depict pathways for the formation of astaxanthin, galloxanthin and ε-galloxanthin based on the results of both the metabolic and analytical

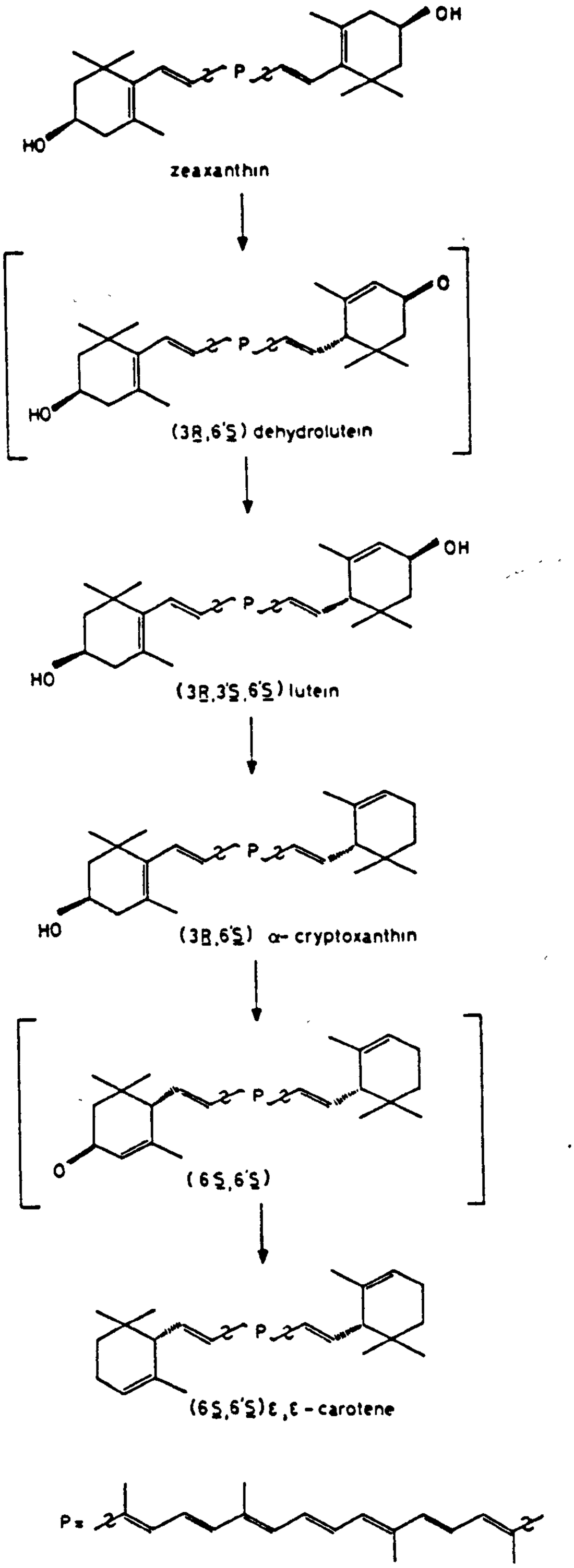


Fig. 78.

Metabolic pathway proposed for the conversion of zeaxanthin to ε,ε-carotene in chick embryos.

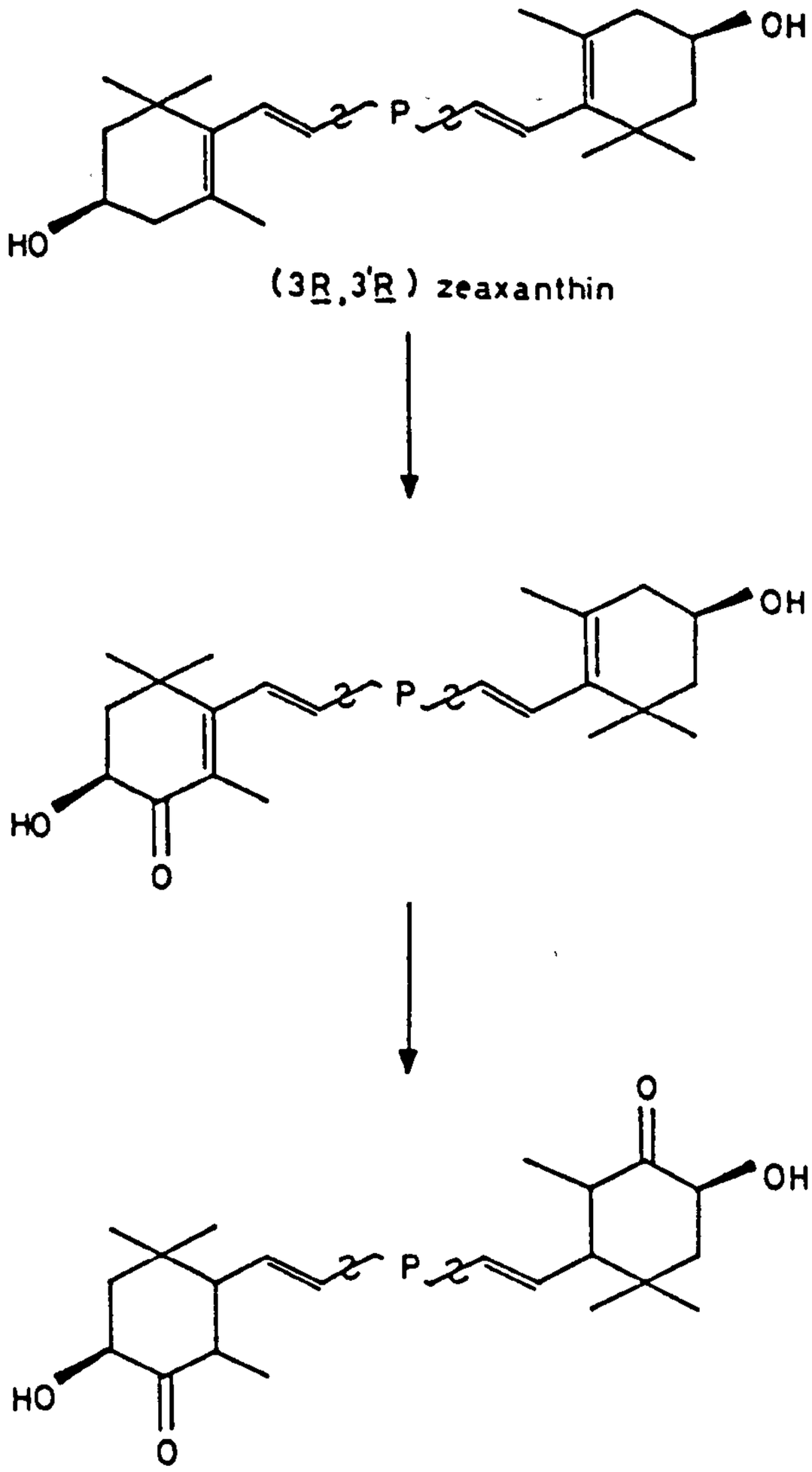


Fig. 79.

Metabolic pathway proposed for the formation of astaxanthin from zeaxanthin in chick embryos.

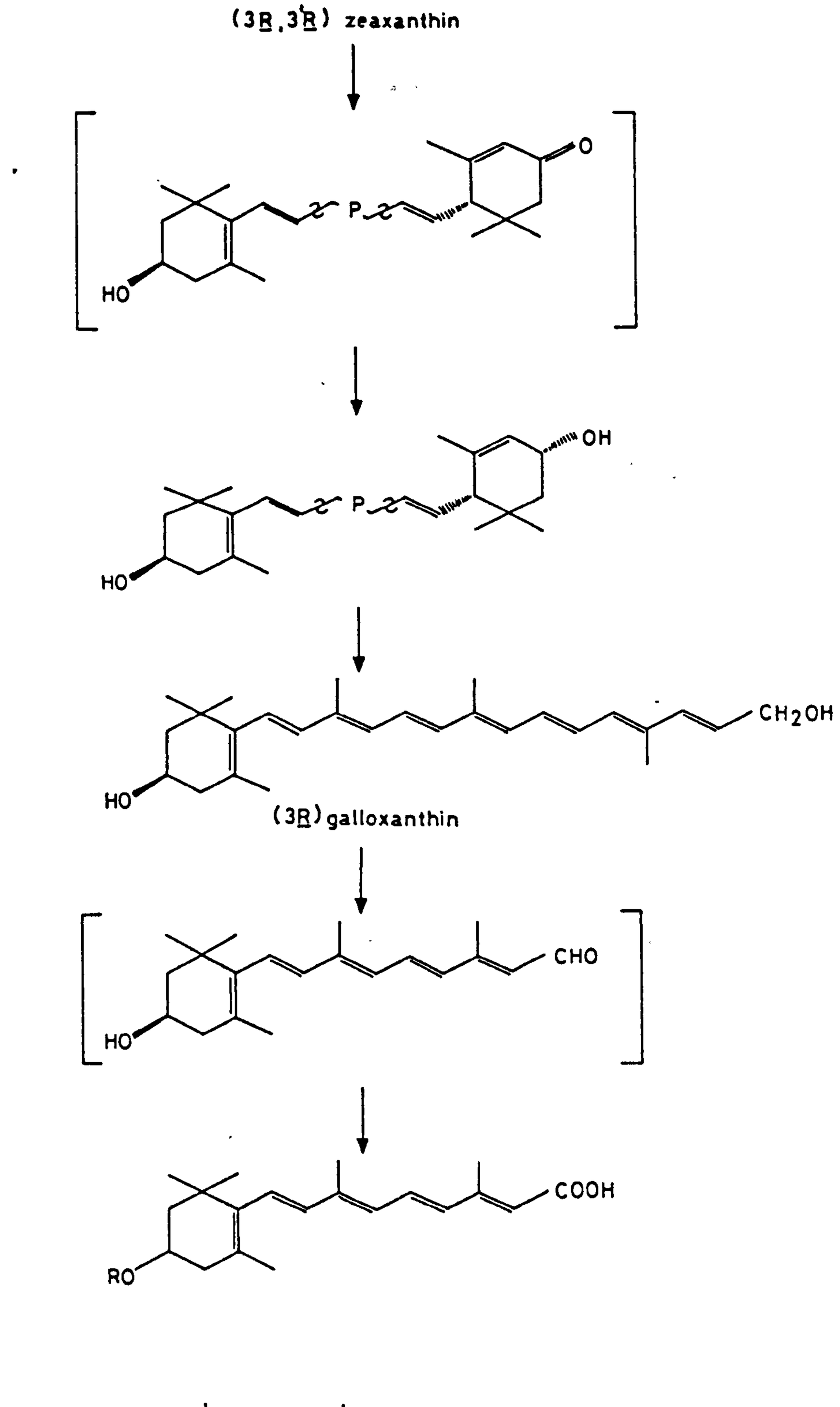


Fig. 80.

Metabolic pathway proposed for the formation of galloxanthin from zeaxanthin in chick embryos.

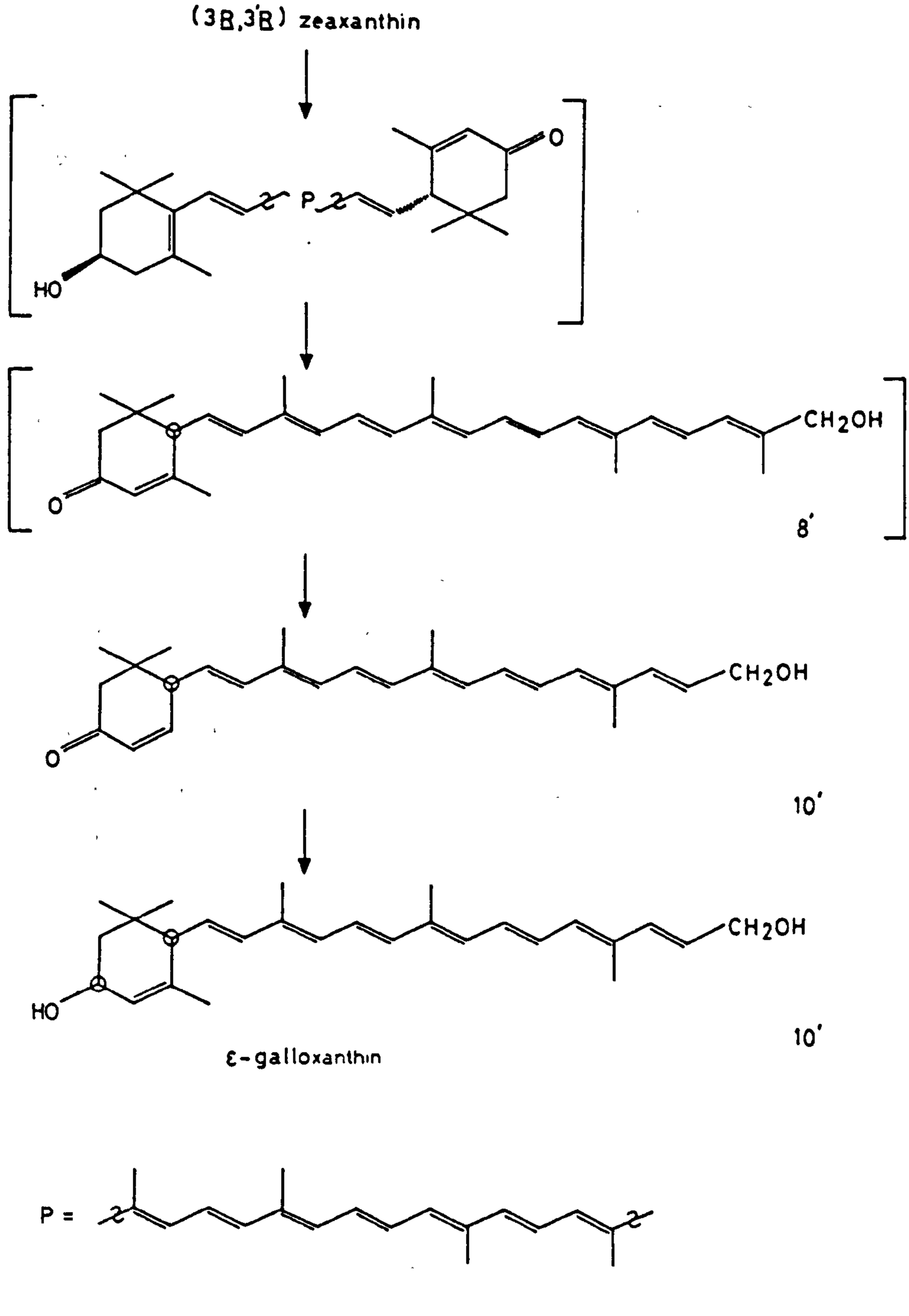


Fig. 81.

Metabolic pathway proposed for the formation of ε-galloxanthin from zeaxanthin in chick embryos.

studies.

The results of the MVA experiments do not provide conclusive evidence for de novo synthesis of carotenoids in chick embryo tissues. Radioactivity from [2-^{14}C]MVA was incorporated into the total lipid extract and was most probably associated with sterols to a very large extent. However, zones of radioactivity corresponding exactly with carotenoid zones were present. On these grounds alone, it would not be appropriate to suggest that de novo synthesis of carotenoids was occurring. Further work is required to clarify this observation.

For the other tissues, the major conclusion to be drawn is that zeaxanthin is readily deposited in the tissues with relatively little modification taking place. From hepatic and intestinal tissue extracts results it can be confirmed that zeaxanthin is not the precursor of the vitamins A. This correlates with the non-activity of xanthophylls as vitamin A precursors in species other than fish.

It is evident that the observed metabolism must be occurring in the retinas of the chicks. This can be concluded because some carotenoids are absent from all the tissues except the retina. These include astaxanthin and galloxanthin. ε-Carotene has been detected in chick blood (Stransky and Schultze, 1977) but not astaxanthin and galloxanthin, therefore, these carotenoids cannot be formed in the liver and then transferred to the retina via the blood. The retina is a highly metabolically active site and is well suited to dealing with such metabolic conversions in that there is a very efficient and prolific blood supply and large numbers of mitochondria close at hand.

Having demonstrated metabolism in the chick retina perhaps the next stage should be the establishment of incubation systems in an attempt to isolate and characterise the enzymes involved with the transformations. There is no doubt that this technically would be a very difficult

project. Difficulties have been encountered with enzyme preparation systems from tissues such as liver and corpus luteum, so it is probable that chick embryo retinal preparations would be extremely problematic.

From microspectrophotometry (MSP) it has become clear that deposition of carotenoids into the oil droplets is not a random process. MSP has established that the droplets do not contain single pure carotenoids as was first proposed. In other avian species e.g. chicken and pigeon, the spectra of red droplets was indicative of pure astaxanthin (Bowmaker and Knowles, 1977; Bowmaker, 1977). This is not the case in turkey retina where the diluted red oil droplets give a spectrum most definitely of a mixture of at least two carotenoids. A possible explanation of this observation is that there is considerably less astaxanthin in turkey retinas than for chicken (Table 20). The comparative paleness of the oil droplets in turkey retinas may be a reflection of the diet and considerable domestication of the turkey.

It is quite remarkable that the same absorption spectrum is recorded for every oil droplet of a particular type, especially as the oil droplets involve complex mixtures of carotenoids. This implies that there is an overall control of the carotenoids deposited in each oil droplet type and hence, each cone type. This supports the statement of Johnston and Hudson (1976) that "there may be a selective membrane transport mechanism specific to each cone type".

As far as the physiologist and photochemist is concerned, the carotenoid composition of the oil droplets may be significant in assessment of the effective spectral sensitivities of each cone type.

There is still no real evidence to favour any of the roles postulated for oil droplets. There is evidently a tightly controlled mechanism for metabolism, uptake and deposition of carotenoids into a

particular cone type. It seems astonishing that energy is used in this way just for the purpose of producing cut-off filters for light. A more random distribution of carotenoids within oil droplets would probably be adequate to produce the same effect. The chiral nature of the carotenoids adds an even more complex factor.

It is feasible that the oil droplets represent a very sophisticated optical system. With such high carotenoid concentrations it is unlikely that the carotenoid molecules would be randomly orientated in the oil droplet. Experiments on the isolation and composition of the carotenoid containing oil droplets from cone photoreceptors of chicken (Johnston and Hudson, 1976) revealed the presence of large amounts of polyunsaturated fatty acids. In the acylglycerol and free fatty acid fraction from the oil droplet, linoleic and arachidonic acid together represented 52 - 83% of the total polyunsaturated fatty acid present. These workers observe that the unsaturated lipid mixture present in the oil droplet appears to act as an excellent solvent for the high concentrations of carotenoid. They also suggest that a different mixture of lipid composition of the oil droplet would perhaps allow for different carotenoid mixtures and hence cone types. It may be envisaged that intermolecular association between molecules such as hydrogen bonding of the hydroxyl functions of the di- and monoacylglycerols and carotenoid could account for the cohesiveness of the oil droplet. This may allow for a high degree of molecular organisation within the oil droplet. It may even be possible that the carotenoids in oil droplets exist as liquid crystals and display some of the unusual properties associated with these materials. Many biological systems are liquid crystals e.g. the bilayer membrane (Brown and Wolken, 1979). A review of the structures and properties of liquid crystalline materials is given by Brown (1977). An important class of liquid crysyals in this context is the cholesteric-nematic structure. The molecular organisation in a cholesteric liquid crystal is such that there is a unidirectional alignment of molecules within a given layer. The individual layers are arranged so that the direction of the long axes

of molecules in one layer is displaced slightly from the direction of the long axes of the adjacent layer. A helical structure is formed which may be either left- or right- handed depending on the nature of the compound. This system will display unique optical properties. To form the cholesteric-nematic phase the molecules concerned must be chiral. Cholesteric-nematic liquid crystals exhibit a very strong optical rotatory power and selectively reflect a band of visible radiation.

If the carotenoids were existing in a form of liquid crystal as above this would result in a very complex optical system. It would mean that the whole phase of the oil droplet would display a o.r.d. as well as the effect of the individual carotenoid chiralities. It would be very interesting to measure the o.r.d. of individual oil droplets within the retina. Should any differences be detected this may indicate that the oil droplets not only function by absorbing light but also by rotating and reflecting light.

The annual migration of birds has always intrigued the biologist and, indeed, many theories have been put forward as to how birds achieve this remarkable navigational feat. Papi (1982) suggested that birds (with particular reference to homing pigeons) could navigate by a memorised map of familiar smells, in combination with sun and magnetic compasses. For night flight, compasses based on stars and the moon would allow for navigation. Obviously, bird navigation and its mysteries is a broad area and covered extensively in a book by Baker (1984). Also mentioned in this book are other feasible explanations of the navigational phenomena.

In the present context one theory of interest mentioned is that birds may make use of polarisation patterns. Due to atmospheric scattering, the light from blue sky is linearly polarised and the plane of polarisation is closely related to the position of the sun. There is a band of maximum polarisation (Brines, 1980) which moves across the sky

during the day maintaining a fixed relationship to the position of the sun. As a result, the detection of polarisation patterns in blue sky would provide a useful compass, either in its own right or as a back up in conditions of patchy cloud. It has been known for some time that under such conditions bees use polarised light for orientation (von Frisch, 1967) and over 90 species of invertebrates have been found to respond to such light. It appears that birds also are sensitive to polarised light (Kreithen and Keeton, 1974; Delius et al., 1976). The mechanism of detection of polarised light in birds is not known but it is probably detected in their eyes. In Salamanders, this is not the case, the polarised light being detected by a receptor system located under the bones of the skull (Alder and Taylor, 1973).

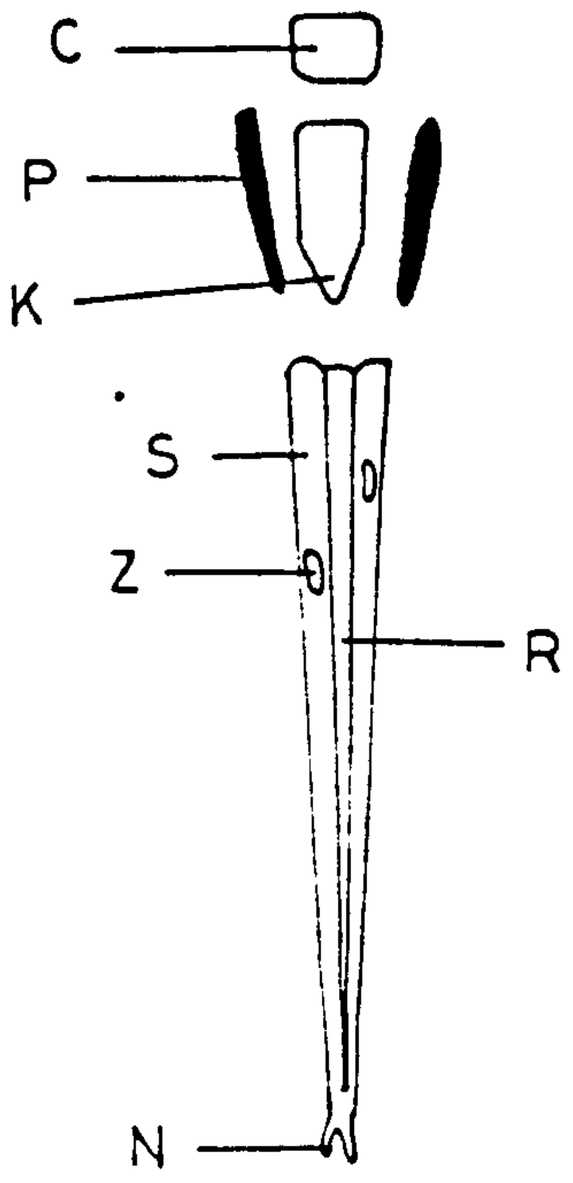
In fact, early experiments using a light source close up to the pigeon suggested that homing pigeons were insensitive to polarised light (Montgomery and Heinemann, 1952). Kreithen (1978) realised the significance of this test when he considered the visual processes of the pigeon. Unlike humans, the pigeon does not focus all images on the fovea. Pigeons use the peripheral retina, with red oil droplets, for near objects and the central and lower part of the retina, with yellow droplets, for distant objects. The polarisation patterns of the sky, being both distant and above, would therefore fall on the 'yellow' region of the retina. Whereas Montgomery and Heinemann (1952) had presented their light source to the red field of the retina, Kreithen and Keeton (1974) had illuminated the yellow region. The birds responded in these circumstances. Experiments with sparrows have indicated a role for polarisation patterns in navigation during twilight as described in Baker (1984).

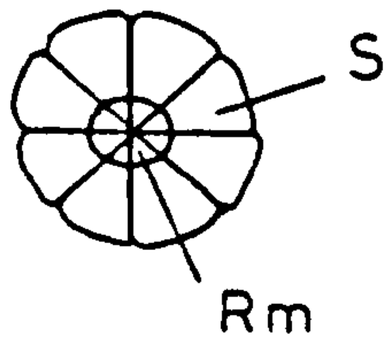
In order to detect the plane of vibration of polarised light an analyser is required, which itself produces polarised light of a definite plane of vibration. If the incident light has the same plane of polarisation as the analyser then it can have a maximum effect. If its

plane is different the light is weakened progressively to a minimum at an angle of 90°.

In bees and other invertebrates, the analyser for the plane of polarised light is thought to be the rhabdomeres of the sensory cells of the compound eye. The rhabdomeres are radially arranged and so detection of polarised light would be possible if it were assumed that the rhabdomeres themselves polarise the light in different directions. Since the receptor cells of the ommatidium (Fig. 82) have individual nerves, there exists a basis for analysis of the brightness patterns, which differ specifically from one another in accordance with the plane of polarisation of the incident light. By electron microscopy the fine structure of the rhabdomeres has been investigated and has produced a basis for the detection of polarised light. Perpendicular to the long axis of the rhabdomere there are very fine, tightly packed tubelets (microvilli). It is postulated that the orderly arrangement of the microvilli in the rhabdomere provides the foundation for an equally orderly arrangement of visual pigment molecules and that the latter functions, through dichroic absorption, as the analyser.

The radial arrangement of the fine structure in the rhabdomeres has been confirmed over a very broad range of animals that are capable of detecting polarised light, from the lowest to the most advanced insects, in spiders, octopuses and in squids. The connection between the fine structure described and the ability to detect polarised light is very convincing as the absence of this structure correlates well with an inability to analyse polarised light. This is true of the vertebrate where the outer ends of the rods and cones are packed with very fine double-walled sacs that are parallel to one another and are perpendicular to the direction of incident light. There is, however, no radial arrangement which would seem to be the critical factor in the detection of polarised light.





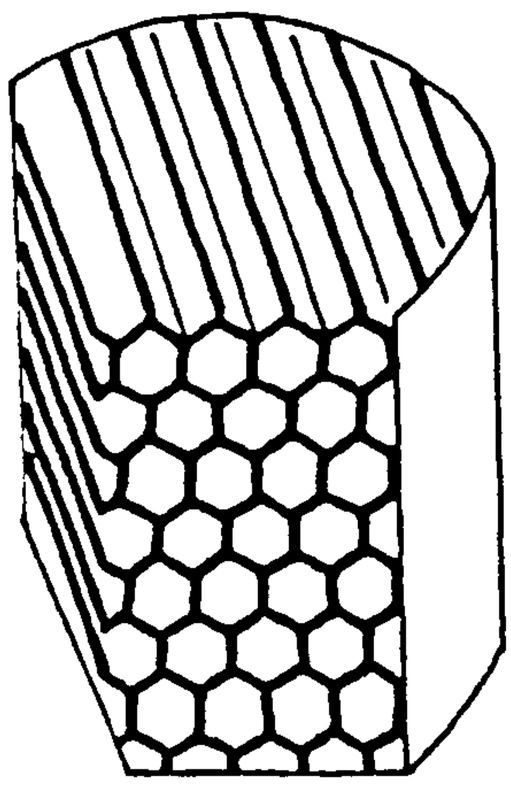
A. B.

Fig. 82.

Diagrammatic representation of the visual receptor cells of insects.

An individual eye (ommatidium) from an insects compound eye: A) longitudinal section; B) cross section, at higher magnification, through the sensory cell.
S-sensory cells; Z-cell nucleus; N-nerve fibre;
C-corneal lens; K-crystalline cone; P-pigment.

On its internal face each sensory cell forms a visual rod (rhabdomere, Rm); the rhabdomeres may unite to form a rhabdom (R).



C.

Small segment of a rhabdomere of a fly's eye as determined by electron microscopy. The tiny tubelets are perpendicular to the direction of transmission of light.

Kreithen and Keeton (1974) explain their observation of detection of polarised light by pigeons on the basis of the position of the photoreceptor in the eye. Vertebrate photoreceptors illuminated at angles different to that of their main axis respond differently to varying planes of polarised light. It may be possible for light to strike a photoreceptor cell at an angle on the sloping walls of the fovea. However, the mechanism of detection of polarised light is not understood in the pigeons nor, indeed, the bees and other invertebrates.

The spherical oil droplets with their high degree of molecular organisation may possibly possess the characteristics required to detect polarised light. A simple experiment could be envisaged to test this in pigeons. The response of carotenoid-depleted pigeons (i.e. birds reared to possess only colourless droplets) to polarised light should be tested and compared to pigeons with 'normal' oil droplets.

The only real conclusion that can be drawn from the MSP studies is that the composition of carotenoids in the oil droplets is complex, both in terms of mixtures of carotenoids being present and that the carotenoids themselves are complex with regards to chirality.

CHAPTER 5

INTRODUCTION

5.1 Phaffia rhodozyma.

Phaff et al. (1972) were the first to isolate 10 strains of this red yeast from slime fluxes of deciduous trees from Japan and North America. Initially this organism was incorrectly referred to as Rhodozyma montanae. However, Miller et al. (1976) described, by standard methods of yeast taxonomy (van der Walt, 1970), a new genus of yeast, Phaffia, to accommodate the single species Phaffia rhodozyma. This organism was unusual in that it both fermented sugars and produced carotenoids. Non-fermenting yeasts are quite common, especially in the Deuteromycete and Basidiomycete orders.

P. rhodozyma is a unicellular organism with ellipsoid cells, that may be found singularly, paired or in short chains. Reproduction is by budding, a sexual cycle not having been demonstrated for this organism. On solid media P. rhodozyma forms glossy to semi-dull colonies, which are soft and broadly convex in cross-section. The borders were lobulate to nearly entire, of an orange to salmon red colour. In liquid media, pellicles formed and the organism became deposited in thin rings, with a little sedimentation.

The characteristic orange-pink coloration is due to the formation of astaxanthin, its principal carotenoid. Torulene and torularhodin, the typical red yeast carotenoids, are not present in P. rhodozyma (Miller et al., 1976).

5.2 Distribution of carotenoids in fungi.

The carotenoid distributions in fungi, including yeasts, have recently been reviewed (Goodwin, 1980; Liaaen-Jensen, 1979). It is common to encounter C_{40} aliphatic, mono- and bicyclic carotenes and carotenoids and also apocarotenoids amongst fungal carotenoids. Notably, many fungi, in particular the Phycomycetes, synthesise only carotenes but β-carotene, although widely distributed and sometimes the major carotene on a quantitative basis, is by no means the only carotene synthesised. In fact, γ-carotene is widely distributed and in some cases e.g. Cladochytrium replicatum, β-carotene is absent but replaced by γ-carotene (Fuller and Tavares, 1960). In red yeasts particularly, but not exclusively, a carotene (torulene) is found which has an additional double bond at C-3'. Pigments with a 3,4 double bond in a ψ-end group are rare in higher plants and algae, but are characteristic of photosynthetic bacteria (Andrewes and Liaaen-Jensen, 1972). Apocarotenoid acids e.g. neurosporaxanthin, are characteristically found in fungi.

No carotenoids with an α-chromophore have been positively identified in fungi and also rare in fungi are the typical xanthophylls of higher plants e.g. β-cryptoxanthin, violaxanthin and neoxanthin. However, carboxylic acids such as torularhodin and neurosporaxanthin are common. Many of the Discomycetes are characterised by unique xanthophylls e.g. phillipsiaxanthin from Phillipsia carminea (Arpin and Liaaen-Jensen, 1967a), plectaniaxanthin from Plectania coccinea (Arpin and Liaaen-Jensen, 1967b) and esterified aleuriaxanthin in Aleuria aurantia (Arpin et al., 1973).

Ketocarotenoids found in algae and higher plants are also encountered in fungi e.g. echinenone and astaxanthin in Peniophora aurantica (Arpin et al., 1966), canthaxanthin in Cantharellus cinnabarinus (Haxo, 1950) and astaxanthin in Phaffia rhodozyma (Andrewes

et al., 1976). Goodwin (1980) details the distribution of carotenoids in the various orders of fungi.

In terms of stereochemistry, the absolute configuration of β,γ-carotene, astaxanthin and plectaniaxanthin are of especial interest. The (6'S)-β,γ-carotene from Caloscypha fulgens (Hallenstvet et al., 1977), (3R,3'R)astaxanthin from P. rhodozyma (Andrewes and Starr, 1976) and (2'R)-plectaniaxanthin (Rønneberg et al., 1982) are noteworthy as these are of the opposite chirality to analogous carotenoids from bacteria and algae.

5.3 Functions of fungal carotenoids.

Functions of carotenoids in fungi can be broadly divided into two areas, namely photoresponse and reproduction.

Carotenoids in fungi take part in photoprotection but the mechanism of this action appears to be different to that in photosynthetic tissues. Few experiments have been carried out to investigate the mechanism of this photoprotection, although the effect is well-documented. Schrott (1985) and Goodwin (1980) describe recent developments in carotenoid photoprotection in fungi.

That carotenoids are indirectly involved in reproduction has become apparent due to their conspicuous accumulation in reproductive organs of many species, both plants and animals. An involvement of carotenoids in fungi has been demonstrated repeatedly. It has been suggested that in Mucorales, β-carotene is the precursor of the sex hormone trisporic acid. Trisporic acid was identified as the sex hormone extracted from Blakeslea trispora and Mucor mucedo. This is extensively reviewed by Gooday (1974). β-Carotene has also been proposed as the precursor of sporopollenin, which is the resistant protective material in the outer wall of the zygospore (Gooday et al., 1973).

5.4 Carotenoid metabolism in fungi.

The isolation of mutants of carotenoid synthesis for several fungal species meant that much of the early investigations of carotenoid synthesis were carried out using fungi. Mutants in which carotenoid synthesis has been blocked at various stages have been isolated for Neurospora crassa (Haxo, 1956), Phycomyces blakesleeanus (Meissner and Delbrück, 1968 and Cerdá-Olmedo and Torres-Martinéz, 1979) and Epicoccum nigrum (Gribanovski-Sassu et al., 1970). Cell-extracts from these fungi, P. blakesleeanus and N. crassa especially, have been used extensively to study carotenogenic enzymes.

Much of the work carried out on P. blakesleeanus can be attributed to Bramley and his colleagues. The carotenoid (car) mutants of P. blakesleeanus have been used to prepare cell-free systems for the investigation of the carotene pathway (Bramley and Davies, 1975; Bramley et al., 1977; Sandmann et al., 1980 and de la Concha et al., 1983). Subcellular fractionation of crude cell extracts has shown that a membrane fraction is required for β-carotene production (Riley and Bramley, 1982). To learn more about the carotenogenic enzymes of P. blakesleeanus the enzymes must firstly be solubilised and then purified without loss of their activity. The progress made in this area is reported by Bramley (1985).

Carotenoid synthesis in N. crassa has also been much investigated, in particular by Rau and his co-workers. Recent reviews of their work are given by Rau and Mitzka-Schnabel (1985) and Mitzka-Schnabel (1985). As with P. blakesleeanus the carotenogenic enzymes of N. crassa have been localised in the membrane fraction, in particular the plasma membrane and endoplasmic reticulum. The phenomena of photoinduction and photoregulation have also been examined.

General factors controlling synthesis of carotenoids e.g. carbon source, pH, temperature, light and oxygen, are well established and are described by Goodwin (1980).

It is evident that there is a better understanding of carotenoid synthesis in fungi than of animal carotenoid metabolism. Research has progressed to the stage of purification of a crude enzyme fraction with a view to characterise these enzymes involved in carotenoid synthesis.

5.5 Carotenoids of Phaffia rhodozyma.

The relative composition of carotenoids found in P. rhodozyma (Andrewes et al., 1976) is given in Table 29. Unexpectedly, astaxanthin comprises approximately 85% of the total carotenoid content of P. rhodozyma. Astaxanthin is normally considered as an animal carotenoid (Goodwin, 1984). Also unusual is the absence of torulene and torularhodin from the organism.

5.6 Carotenoid metabolism in Phaffia rhodozyma.

The amount of astaxanthin formed per gram of P. rhodozyma was estimated as 50-800 µg (Johnson et al., 1977). This high yield of astaxanthin (10 times greater than shrimp meal) has commercial importance as a source of pigment in fish diets. Astaxanthin from P. rhodozyma was found to be far less effective in pigmentation of American lobster carapace than Artemia salina astaxanthin (Johnson et al., 1977). In the lobster, astaxanthin acts as the prosthetic group of an apoprotein. Since proteins are stereospecific in Nature, the astaxanthin from P. rhodozyma is presumably of the incorrect chirality to associate with the protein.

Andrewes and Starr (1976) determined the chirality of P. rhodozyma astaxanthin as (3R,3'R)astaxanthin. Except for yeasts, the organisms

TABLE 29.

Carotenoid content of Phaffia rhodozyma (Andrewes and Starr, 1976).

Carotenoid	Percentage of total carotenoid
Astaxanthin	83 - 87
Phoenicoxanthin	5 - 7
3-Hydroxyechinenone	3 - 4
Echinenone	2 - 4
β-Carotene	2 - 2.5
3-Hydroxy-3',4'-didehydro-β,ψ-caroten-4-one	0.3 - 0.5
γ-Carotene	0.01
Neurosporene	0.01
Lycopene	0.01

synthesising carotenoids de novo produce optically pure (3S,3'S)astaxanthin only (Schiedt et al., 1985). A typical example is Haematococcus pluvialis which, under nutritional stress, produces (3S,3'S)astaxanthin (Andrewes et al., 1974). Recently, the occurrence of (3S,3'S)-, meso- and (3R,3'R)astaxanthin in marine animals (Matsuno et al., 1984) has posed a difficult biosynthetic question as to the origin of these carotenoids in animals. Also difficult to explain is that astaxanthin diesters from Liliocens lilii were analysed and found to be the optically pure (3R,3'R)isomer. By comparative methods the mite Trombidium sp. was found to possess (3R,3'R) and meso-astaxanthin but no (3S,3'S)astaxanthin (Partali et al., 1984a). L. lilii feeds on leaves of lily and Trombidium on insects so again the metabolic origin of these is difficult to explain.

Recently, evidence has been brought forward to show that the meso- and (3R,3'R)astaxanthin forms of some species are of dietary origin. Firstly, feeding of pure enantiomers and meso- forms of astaxanthin to rainbow trout showed no C-3 epimerisation to give (3R,3'R)astaxanthin (Foss et al., 1984a,b) which preferentially accumulates in the flesh (Schiedt et al., 1985). Secondly, the carotenoids of Daphnia magna, after feeding on a diet of phytoplankton of known carotenoids, were analysed and found to be identical with the carotenoids of the green alga, with a few additional carotenoids present. The results support the general assumption that zooplankton cannot synthesise carotenoids de novo but can metabolise carotenoids from the diet. Astaxanthin was found in the D. magna and was determined to be (3S,3'S)astaxanthin (Partali et al., 1984b). Finally, Storebakken et al. (1984) studied the fauna of Lake Sjøsetervatnet. Three types of crustaceans and three species of fish were caught. In the zooplankton H. gibberum and Bosmina sp. (3S,3'S)astaxanthin was dominant, but it was found that G. lacustris also contained meso- and (3R,3'R)astaxanthin. G. lacustris is a major part of the diet of Salmo trutta and Salmo alpinus and can be found in the stomachs of these fish. Again this implies that meso- and

(3R,3'R)astaxanthin in fish originate from the diet. The biosynthesis of these must therefore take place in G. lacustris or its feed.

The problem of astaxanthin formation in plants and animals may prove to be a central issue in carotenoid metabolism.

Andrewes et al. (1976) suggested a pathway for the formation of astaxanthin in P. rhodozyma based on the pigments isolated in extracts of the organism (Fig. 83). It was proposed that β-carotene was formed from neurosporene via well known reactions. The β-carotene could then be converted directly into echinenone without passing through the intermediate monool, isocryptoxanthin, which was not present in the extracts. Echinenone may then be converted to hydroxyechinenone. The absence of canthaxanthin from P. rhodozyma extracts is consistent with this introduction of a C-4 keto group followed by immediate hydroxylation of C-3. It is, of course, feasible that the conversion of canthaxanthin to phoenicoxanthin is very rapid and efficient, resulting in the apparent lack of canthaxanthin. The final step is the direct ketonisation of C-4', again without the intermediate hydroxy-carotenoid. Thus astaxanthin, by this pathway, is formed from phoenicoxanthin.

Predicting a metabolic pathway for the formation of different stereochemical forms of astaxanthin is more difficult, but Andrewes and Starr (1976) suggest that the astaxanthins of opposite chiralities might arise by a different order of hydroxylation and ketonisation of β-carotene. They point out that hydroxylation of an unsubstituted β-ring at C-3 always leads to the 3R configuration. Because astaxanthin has two functions on the β-rings, there is scope for optically different astaxanthins to be formed. Figure 84 illustrates the three different possible biosynthetic pathways from the β-end group to the astaxanthin end group. The first possibility is the hydroxylation of a β-group (to give (3R,3'R)zeaxanthin) followed by ketonisation of C-4. Due to the reversal of group priorities, this could account for the formation of

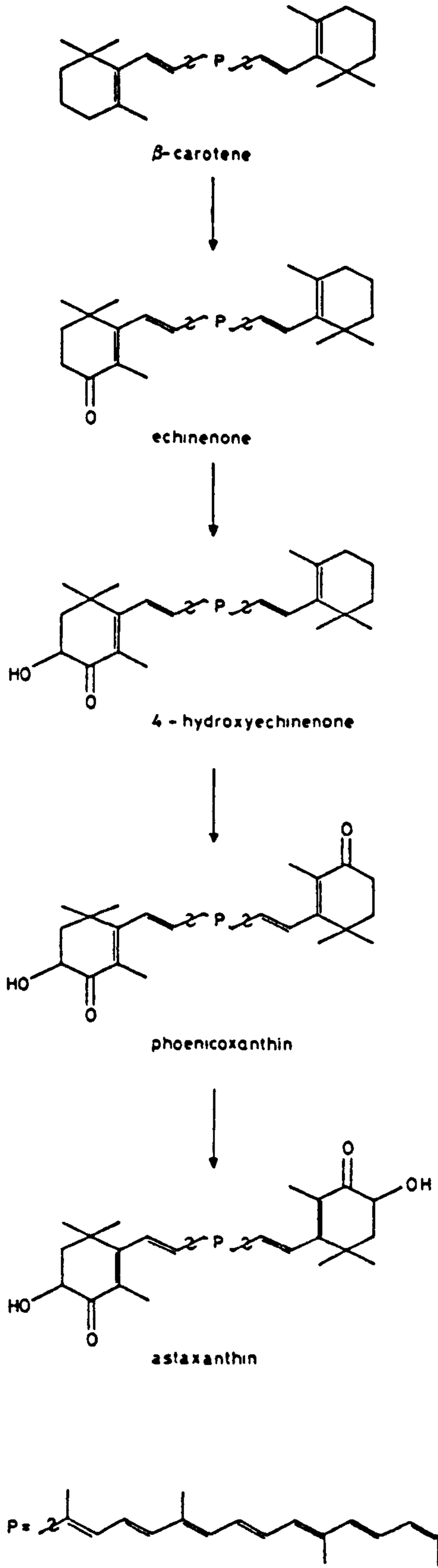


Fig. 83.

Possible pathway of conversion from β-carotene to astaxanthin in Phaffia rhodozyma based on the carotenoids present in this organism.

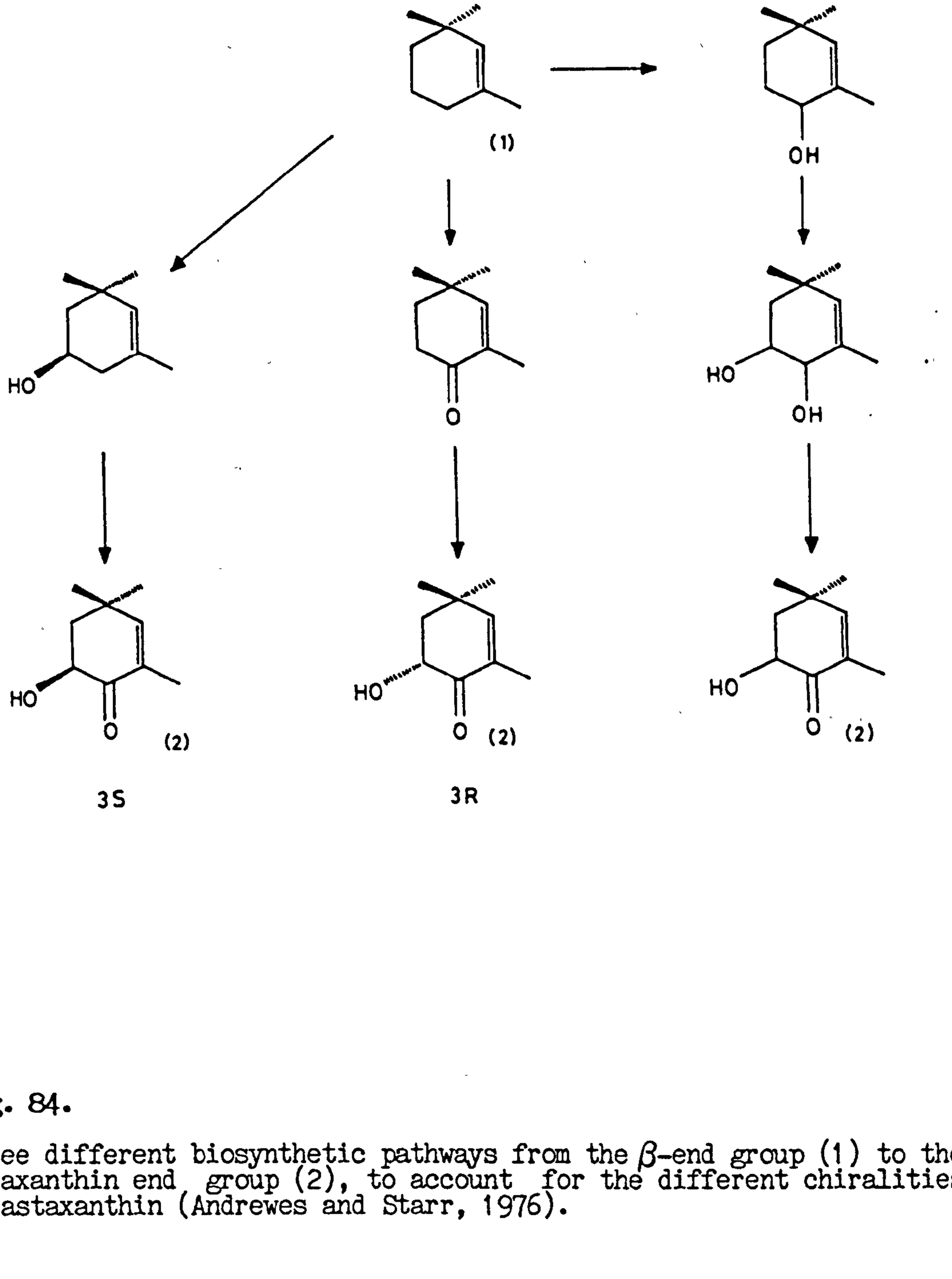


Fig. 84.

Three different biosynthetic pathways from the β-end group (1) to the astaxanthin end group (2), to account for the different chiralities of astaxanthin (Andrewes and Starr, 1976).

(3S,3'S)astaxanthin. However, if a keto function were introduced onto the β-end group first, this may cause the hydroxylation to be 3S-specific, resulting in the formation of (3R,3'R)astaxanthin. The third possible pathway to the astaxanthin end group is successive hydroxylation at C-3 and C-4 followed by oxidation of the C-4 hydroxyl to a keto group. If there are three broad specificity enzymes present as proposed by Davies (1985), the existence of a mixture of (3R,3'R)-, (3S,3'S)- and meso-astaxanthins in the same organism may be explained by all three enzymes working on β-carotene in different orders.

Bywater (1984; unpublished work) has prepared an enzyme system from P. rhodozyma and has carried out preliminary experiments to try and establish whether canthaxanthin or zeaxanthin is the precursor of astaxanthin in this organism. Unexpectedly, both [^{14}C](3R,3'R)zeaxanthin and [^{14}C]canthaxanthin were incorporated into astaxanthin in separate experiments. It may be that both substrates can be used by broad specificity enzymes, one substrate yielding (3S,3'S)astaxanthin and the other (3R,3'R)astaxanthin. If the original view of Andrewes and Starr (1976) is correct then [^{14}C]canthaxanthin should be incorporated into (3R,3'R)astaxanthin and [^{14}C](3R,3'R)zeaxanthin into (3S,3'S)astaxanthin.

AIM OF THIS WORK

To follow the metabolism of [^{14}C]canthaxanthin and [$^{3}H_2$]zeaxanthin in incubations from enzyme preparations of Phaffia rhodozyma, with special emphasis on the analysis of the stereochemical forms of astaxanthin from these incubations.

MATERIALS AND METHODS

5.7 Phaffia rhodozyma; growth, maintenance and extraction.

Although the organism was difficult to grow initially, once established on solid media the problems were diminished. The strain used for this work was P. rhodozyma NYCC 847.

A. Culture conditions.

P. rhodozyma was cultured in the following medium (from Andrewes et al., 1976):

	Grams per litre.
Glucose	20
$(NH_4)_2SO_4$	5
$CaCl_2.2H_2O$	0.1
KH_2PO_4	1
Yeast extract (YM Agar, Difco)	0.5
$MgSO_4.7H_2O$	0.5

The pH was adjusted to 4.5 with HCl.

Stock cultures were maintained on slopes of either the above medium supplemented with agar (3% w/v) or Agar tablet (Oxoid) slopes. After 48h incubation at approximately 22°C in the dark, the slopes were stored at 5°C. Sub-culturing was necessary every 4 weeks for slopes stored at 5°C. Starter liquid cultures (500 ml) were incubated in a shaker (Gallenkamp) for 48 h at 22°C and 120 r.p.m. The starter culture could be used to inoculate 10 l batches of medium. The organism was cultured in 10 l media fermentor tanks (New Brunswick, New Jersey, U.S.A) for 4 days. Thorough aeration was provided by an air supply at a rate of 6000 cm^3/min. Silicone antifoaming agent (BDH Chemicals Ltd., Poole, Dorset) was added (5 ml) to the large cultures.

B. Harvesting.

Cells from the 10 l batches were harvested with an Alfa Laval continuous centrifuge (Alfa Laval Co. Ltd., Brentford, Middlesex) operating at a flow rate of 20 l/h. From a 10 l batch of culture a typical yield of cells was 140 g (wet weight). For smaller batches of cultures (up to 1 l) the cells were harvested by centrifugation (10,000 g for 5 min at 0 - 4°C).

C. Extraction of lipid.

P. rhodozyma is a very difficult organism to extract due to the tough nature of its cell wall. Scharf and Simpson (1968) used a French press to rupture cells whereas Bae et al. (1971) used a Braun homogeniser. Bywater (1984; unpublished work) extracted freeze-dried P. rhodozyma. The fragmented cells were ground to a powder, with acid-washed sand, using a pestle and mortar. Acetone extraction released the most coloration although residual colour was evident in the cell fragments.

Each of the physical methods tried above were adequate to rupture the yeast cells. However, homogenisation of the untreated wet cells in ethanol followed by several homogenisation/extractions with toluene proved to be a very effective method of extraction of P. rhodozyma carotenoids. The efficiency of toluene extraction was used to advantage in the preparation of cell-free incubations (Section 5.9). The toluene and ethanol extracts were bulked, ether and water added until two phases appeared and the resultant ethereal extract washed and dried by normal methods.

5.8 Treatment of P. rhodozyma carotenoids.

The carotenoid of most interest in this work was

(3R,3'R)astaxanthin, therefore the extracts could not be saponified. Saponification of the astaxanthin would result in the loss of chirality of the astaxanthin due to the formation of the artifact astacene.

The initial separation of P. rhodozyma carotenoids was achieved by t.l.c. on Silica Gel G plates. The solvent system used was toluene/ethyl acetate/methanol (75:20:5, v/v). This system could be transferred to h.p.l.c. for further purification of the astaxanthin (Bywater, 1984; unpublished work).

Column:	Nucleosil 50-5 (200 mm x 4.5 mm)
Solvent:	Toluene/ethyl acetate/methanol (75:20:5, v/v)
Flow rate:	1 ml/min
Detection wavelength:	440 nm
Chart speed:	60 cm/h

A fairly pure astaxanthin sample could be obtained by h.p.l.c. of the astaxanthin zone from t.l.c.

The chirality of the astaxanthin from P. rhodozyma was most important. Separation of the (3R,3'R)- form of astaxanthin found in P. rhodozyma, from the (3S,3'S)- and meso-astaxanthin may be achieved either by derivatisation of the astaxanthins to their dicamphanates followed by h.p.l.c., or by h.p.l.c. of the underivatised astaxanthins on a chiral column. Both methods were tried.

A. Preparation and separation of (3RS,3'RS)astaxanthin di-(-)-camphanates.

The method of preparation of (3RS,3'RS)astaxanthin di-(-)-camphanates is described by Müller et al. (1980) and is outlined below. A solution of (3RS,3'RS)astaxanthin (2 mmol) in pyridine (15 ml) was

cooled under nitrogen to 0°C. Camphanic acid chloride (5 mmol; Fluka Chemicals) was added with stirring. The stirring was continued for approximately 10 min. The end of the reaction was determined by t.l.c. on Silica Gel G plates with CH_2Cl_2/ether (9:1, v/v) as developing solvent. Finally water was added (45 ml) in order to precipitate the product, which was collected by filtration. The precipitate was then washed with water (several times) and hexane (twice). The precipitate was dried *in vacuo* at room temperature. The resultant precipitate was a loosely-packed red-brown powder.

Schiedt *et al.* (1981a) found that, for biological samples, the above conditions had to be adapted because the non-carotenoid lipids (usually 90 - 98% of the total lipid) interfered with the esterification. Thus, a solution of 0.5–1.5 mg astaxanthin/20 mg lipid in 0.5 ml dry pyridine was allowed to react with 50 mg (-)-camphanic acid chloride for 10 min. The reaction mixture was adapted to sample size, the lower limit being 5 µg astaxanthin in 0.1 ml pyridine.

Separation of enantiomeric and *meso*-astaxanthin as di-(-)-camphanates was described by Vecchi and Müller (1979):

Column:	LiChrosorb Si-60-5 (250 mm x 4.5 mm)
Solvent:	Hexane/ethyl acetate (75:25, v/v)
Flow rate:	0.7 ml/min
Detection wavelength:	460 nm
Chart speed:	30 cm/h

B. Separation of enantiomeric and meso-astaxanthins by chiral column h.p.l.c.

The chiral column used in this work was prepared in a manner similar to the method of Pirkle and Finn (1981). It was produced by treating γ-aminopropyl silanized silica with (R)-N-(3,5-dinitrobenzoyl)-

phenylglycine. The ionically bonded chiral acid did not leach from the column at significant rates so long as relatively non-polar mobile phases were used. A mobile phase was sought that would adequately resolve the three forms of astaxanthin. The systems tried were based on the mobile phase used by Matsuno et al. (1984) to separate the stereoisomeric forms of astaxanthin from aquatic animals (i.e. hexane/CH_2Cl_2/ethanol 48:15:0.8; column, Sumipax OA-2000; flow rate, 1 ml/min).

The final conditions for separation of enantiomeric and meso-astaxanthin by chiral h.p.l.c. were:

Column:	Chiral column (250 mm x 4.5 mm)
Solvent:	Hexane/CH_2Cl_2/ethanol (48:20:2, v/v)
Flow rate:	0.7 ml/min
Detection wavelength:	440 nm
Chart speed:	15 cm/h

5.9 Incubation procedures.

Guidelines for the incubation experiments were provided by the work of two research groups working on Neurospora crassa (Mitzka-Schnabel and Rau) and Phycomyces blakesleeanus (Bramley and Davies). The approaches of the two groups are quite comparable in that their incubation mixtures are similar, although the procedures of preparation of cell-free systems are different. It is possible that this difference is a function of the two different organisms.

A preliminary investigation of the role of zeaxanthin and canthaxanthin in astaxanthin formation in Phaffia rhodozyma by Bywater (1984, unpublished work) was based on the previous work of Bramley and Davies (1975). However, the tougher nature of the cell wall of P. rhodozyma required more severe treatments for its disruption.

The following sections outline the Bramley and Davies procedure and other methods tried for the preparation of active cell-free systems. In each case the cell-free preparations were used in incubations containing co-factors required for carotenoid synthesis and metabolism. Essentially the same incubation components were used for incubations with MVA, zeaxanthin and canthaxanthin.

A. Preparation of cell-free systems.

i. Freeze dried method (Bramley and Davies, 1975).

After harvesting, the whole cells were freeze dried at -40 °C (Edwards Freeze Dryer Super Modulyo, Crawley, Sussex) until thoroughly dried. The material was then rubbed through a 40-mesh sieve. Two volumes of 0.2M Tris/HCl buffer, pH 8 were added to the powder and the resultant paste ultrasonicated (MSE Soniprep 150 Ultrasonicator) for 3 x 30 s with 1 min intervals. The paste was then centrifuged at 10,000 g at 4 °C for 30 min, and the supernatant used in the incubations immediately. The degree of disruption of the cell wall could be assessed by light microscopy. If necessary, the cell-free preparation (supernatant) could be kept on ice for short periods of time.

ii. Braun homogenisation.

The Braun model MSK mechanical cell homogeniser causes disruption by oscillation of the cells and glass beads. Freshly harvested cells (10 g) were transferred to a Braun bottle (capacity 75 ml) and 12 ml of ice cold buffer added. Two buffers were tested for effectiveness. It was found that 0.2 M Tris/HCl buffer (pH 8) was more effective than 0.1 M K_2HPO_4 with 5 mM $MgCl_2$ and 5 mM N-acetylcysteine (pH 7.4). Glass beads of 0.45 - 0.5 mm diameter were added (28 g). The bottle lid was secured and the flask attached to the homogeniser. The flask was agitated at 4,000 r.p.m. During agitation liquid CO_2 was introduced into the chamber to

cool the suspension. In addition, the flask contents were prevented from overheating by homogenising for 4 x 30 s with 30 s intervals.

The homogenate was centrifuged at 0 - 4°C at 10,000 g for 10 min. The supernatant was used in incubations as soon as possible.

The effectiveness of glucose-6-phosphate and glucose-6-phosphate dehydrogenase in the incubation as a regeneration system of NADPH was tested by carrying out incubations in the presence and absence of these components. The inclusion of glucose-6-phosphate and glucose-6-phosphate dehydrogenase was beneficial (see Table 31).

Longer homogenising periods were tested also. The optimal was established as 4 min homogenisation with appropriate cooling intervals.

iii. French pressure cell.

Mechanical disruption of the cells by French pressure cell was attempted. The French press (French pressure cell press, Aminco, American Instrument Co. Ltd., Maryland, U.S.A.) was operated at 138 MPa. Cells were either treated once (incubation A) or were pressed twice (incubation B). In both cases the homogenates were centrifuged at 10,000 g for 15 min, and the supernatant used in incubations as soon as possible.

iv. Toluene permeabilisation.

The procedure used was based on the method of Murakami et al. (1980). They found that yeast cells could be partially permeabilised by treatment with a small quantity of toluene, probably due to the refractory membrane, thereby permitting examination of yeast enzymes in situ. It was thought that the yeast enzymes remained within the cell wall.

Toluene (15 ml) was added to 10 g (wet weight) of P. rhodozyma cells and the mixture heated to 40 - 42 °C for 5 min with stirring. The suspension was centrifuged at 10,000 g for 5 min. The pellet was resuspended in 20 ml of 0.2 M Tris/HCl buffer (pH 8) and used in the incubations as soon as possible.

v. Triton X-100 treatment.

The procedure was based on the experimental work of Miozzari et al. (1978) on Saccharomyces cerevisiae.

The homogenisation buffer used was 0.1 M Tris/HCl (pH 8). Yeast cells (10 g) were suspended in 20 ml of buffer and Triton X-100 added (0.05% v/v). The mixture was agitated vigorously by means of vortexing (Spinmix, Gallenkamp). The resultant suspension was used in the incubations as soon as possible.

B. Incubation mixtures.

All incubations were carried out aerobically, in a water bath (22°C) with shaking (120 r.p.m.) for 18 h. In all cases, 4 ml of cell-free preparation or enzyme preparation was used. The following co-factors were added:

$MgCl_2.7H_2O$	4 mM
$MnCl_2.4H_2O$	6 mM
ATP	10 mM
Glutathione	20 mM
NADPH	1 mM
NADH	3 mM
Glucose-6-phosphate	3 mM

Glucose-6-phosphate dehydrogenase (Sigma, Type XI from Torula yeast)	2 µl

i. MVA incubation.

The substrate in these preliminary incubations was [2-^{14}C]MVA in the form of its DBED salt. Amounts from 0.5 µCi to 3.5 µCi were added per incubation. From these experiments the effectiveness of a particular enzyme preparation could be assessed prior to undertaking the [^{14}C]canthaxanthin and [$^{3}H_2$]zeaxanthin incubations.

ii. Carotenoid incubations.

Stock solutions were prepared of standard zeaxanthin, standard canthaxanthin, [$^{3}H_2$]zeaxanthin and [^{14}C]canthaxanthin for use in the incubations. Solutions were prepared such that the specific activities of [$^{3}H_2$]zeaxanthin and [^{14}C]canthaxanthin were comparable. To reach this end, the [$^{3}H_2$]zeaxanthin was diluted with unlabelled standard zeaxanthin. In each [$^{3}H_2$]zeaxanthin incubation an equivalent amount of unlabelled canthaxanthin was added and vice versa.

[^{14}C]Canthaxanthin incubations.

[^{14}C]Canthaxanthin (0.336 µCi, 17.55 µg; specific activity 19.15 µCi/mg) and unlabelled zeaxanthin (15.99 µg) was dissolved in 1 ml of a 4% L-α-phosphatidylcholine solution (w/v). This was added to the enzyme preparation resulting in a final volume of 5 ml per incubation.

[$^{3}H_2$]Zeaxanthin incubations.

[$^{3}H_2$]Zeaxanthin (0.44 µCi, 3.65 µg (plus 15.99 µg unlabelled

standard zeaxanthin); final specific activity 22.4 μCi/mg) and unlabelled canthaxanthin (14.31 μg) was dissolved in 1 ml of L-α-phosphatidylcholine and added to the 4 ml incubation to give a final volume of 5 ml per incubation.

Protein estimation.

The protein per incubation was estimated by the biuret method (Layne, 1957) using bovine serum albumen as the standard.

RESULTS

5.10 Separation of P. rhodozyma carotenoids by t.l.c. and h.p.l.c.

The t.l.c. system described in Section 5.8 resulted in a clean separation of the carotenoids of interest in this work. The R_f values of standard zeaxanthin, astaxanthin and canthaxanthin on Silica Gel G with toluene/ethyl acetate/methanol (75:20:5, v/v) as solvent were 0.24, 0.35 and 0.57 respectively.

When this t.l.c. system was transferred to h.p.l.c. (Section 5.8) the retention times of zeaxanthin (peak 3), astaxanthin (peak 2) and canthaxanthin (peak 1) were 4.8 min, 3.4 min and 2.9 min respectively (see Figure 85).

5.11 Separation of the stereoisomeric forms of astaxanthin.

A. Separation of (3RS,3'RS)astaxanthin di-(-)-camphanates.

Numerous attempts at producing the astaxanthin di-(-)-camphanates from P. rhodozyma astaxanthin fractions were unsuccessful. The possible interference from other lipids was eliminated by increasing the molar ratio of astaxanthin to camphanic acid chloride as described by Schiedt et al. (1981a). Extreme care was taken to ensure that the pyridine used was dry. The astaxanthin fraction from P. rhodozyma was dried over P_2O_5 and the camphanic acid chloride sublimed at approximately 65°C at reduced pressure (water pump) but on no occasion was a successful reaction carried out. Attempts at producing the dicamphanates of a standard racemic mixture of astaxanthin were also unsuccessful.

B. Chiral h.p.l.c.

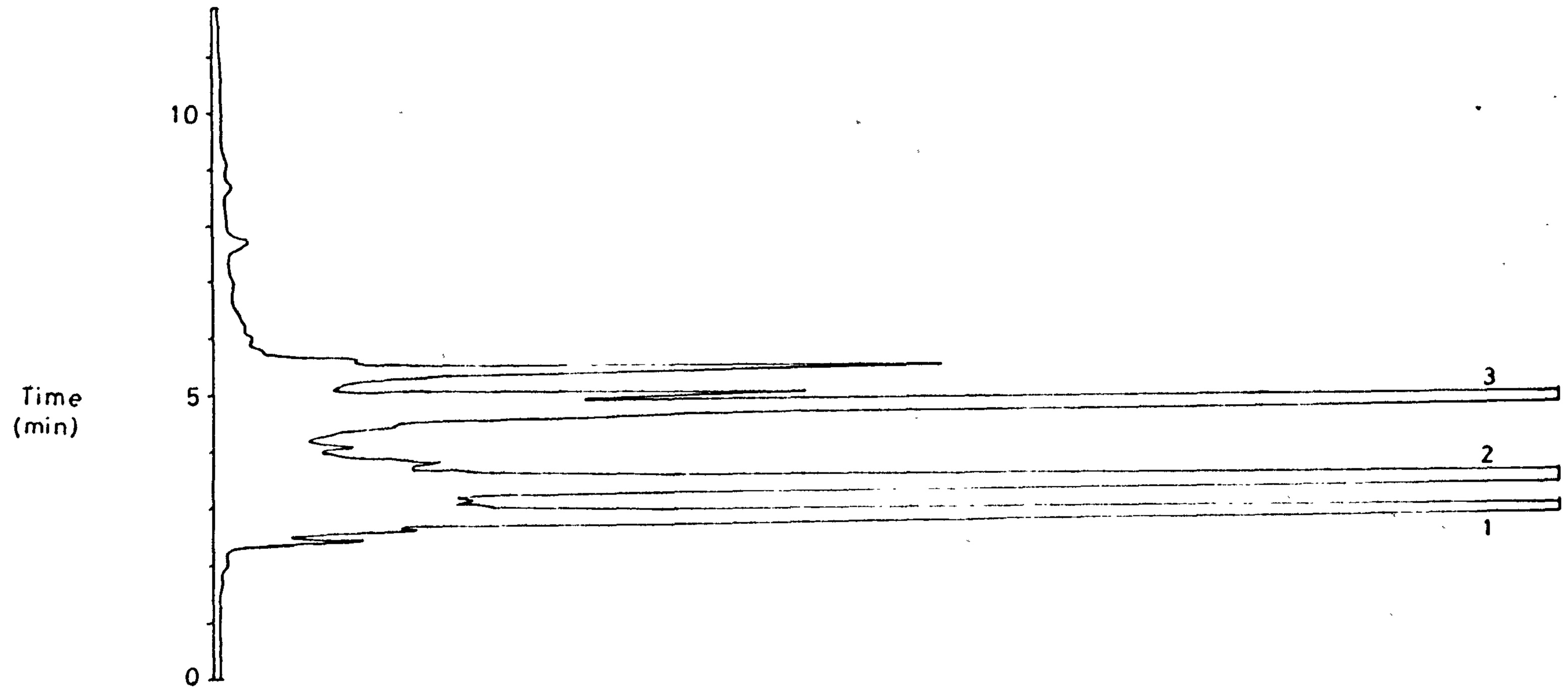


Fig. 85.

H.p.l.c. elution profile of whole extracts of P. rhodozyma on Nucleosil 50-5, solvent toluene/ethyl acetate/methanol (75:20:5, v/v) at 1 ml/min.

Under the final conditions described in Section 5.8B the retention times of (3R,3'R)- (1), meso- (2) and (3S,3'S)astaxanthin (3) were found to be 40.4 min, 42.8 min and 44.8 min, respectively (see Fig. 86). Some variation in the retention times was noted and in later experiments the retention times had increased to approximately 42 min, 44 min and 46 min for (3R,3'R)-, meso- and (3S,3'S)astaxanthins respectively. The three forms were identified by comparison with standards.

5.12 Incubation experiments.

The MVA incubation experiments were used to assess the success of a particular enzyme preparation. For each method of preparation of the enzyme system, the incorporation of radioactivity from [2-^{14}C]MVA into the total lipid extract was used to give an indication of the activity of the system. Only the more successful methods were used for further experimentation, with zeaxanthin and canthaxanthin as substrates.

5.13 [2-^{14}C]MVA incubations.

A. Freeze dried method (Bramley and Davies, 1975).

This method was successful for the incorporation of [2-^{14}C]MVA into total lipid (5.07% and 10.09%). The inclusion of standard canthaxanthin (14.31 µg) in the incubation mixture appears to lower the incorporation of MVA into total lipid but the significance of this is difficult to assess due to the variation of percentage incorporations in the cases of incubations 1 and 4 (Table 30). The effect, if present at all, is very much less when standard zeaxanthin (15.99 µg) is included in the incubation mixture. The percentage incorporation in these experiments is higher than those achieved by Bywater (1984; unpublished work) who observed 2.15% incorporation from [2-^{14}C]MVA into total lipid.

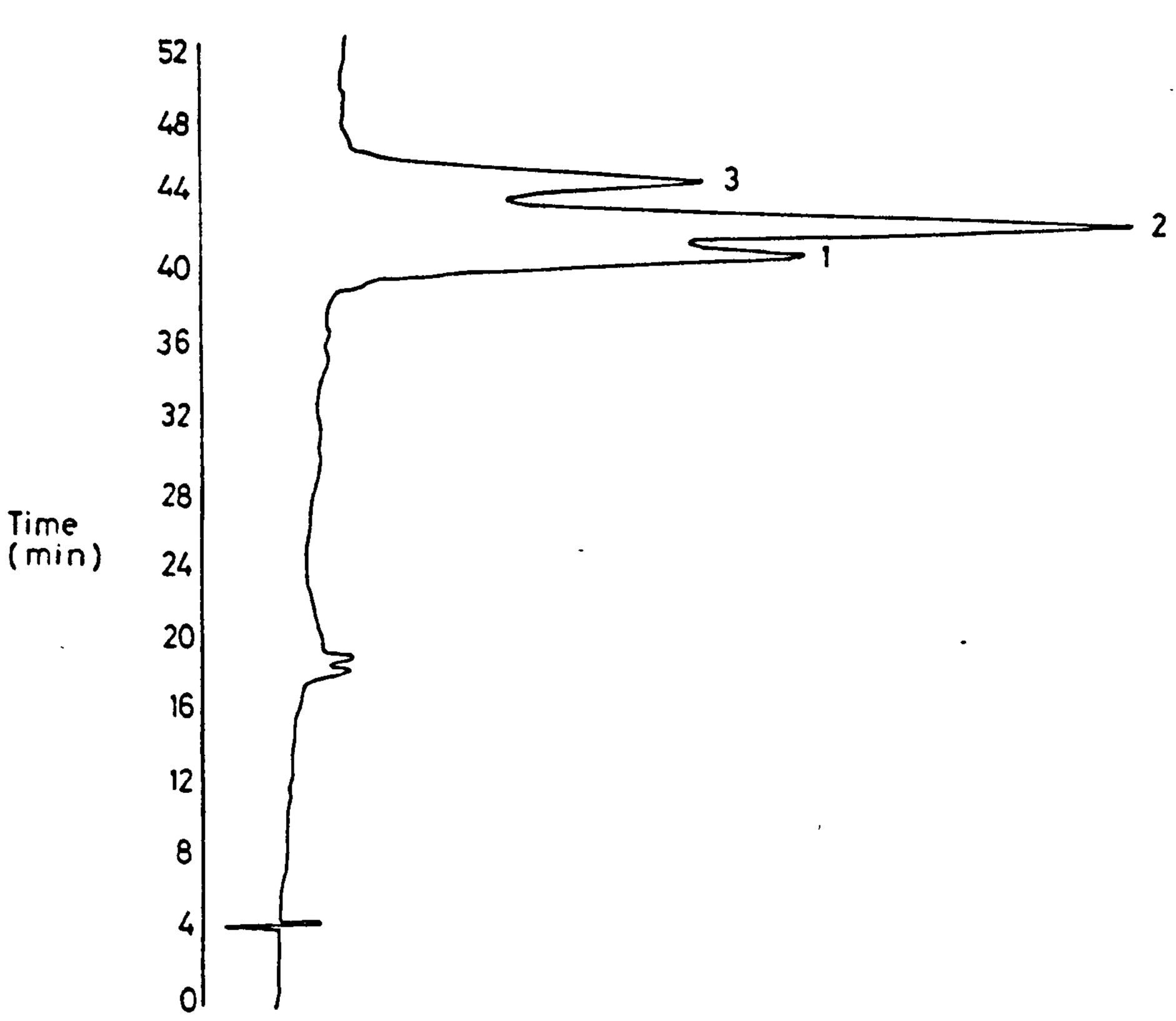


Fig. 86.

H.p.l.c. separation of (3R,3'R)-, meso- and (3S,3'S)astaxanthin on the chiral column, solvent hexane/CH_2Cl_2/ethanol (48:20:2, v/v) at 0.7 ml/min.

TABLE 30.

Percentage incorporation of radioactivity into total lipid for incubations prepared by the freeze dried method.

Incubation	Carotenoid added to incubation µg C	Z	µg carotenoid extracted from incubations	Percentage incorpration of MVA into total lipid
1	-	-	1.5	10.09
2	-	15.99	20.4	5.08
3	14.31	-	8.6	2.42
4	-	-	1.5	5.07

C = canthaxanthin

Z = zeaxanthin

TABLE 31.

Percentage incorporation of radioactivity from [2-^{14}C]MVA into total lipid for incubations prepared by Braun homogenisation of P. rhodozyma cells.

Incubn	G-6-P	Buffer	Time (min)	mg protein per incubn	µg carotenoid extracted per incubation	% incorpn in total lipid
1	-	Tris/HCl	2	80.4	14.17	0.3
2	+	Tris/HCl	2	80.4	16.8	0.87
3	-	K_2HPO_4	2	80.4	13.56	0.53
4	+	K_2HPO_4	2	80.4	13.35	0.76
5	+	Tris/HCl	4	86.7	13.8	6.68

"+ and - G-6-P" refers to the presence or absence, respectively of G-6-P and G-6-P dehydrogenase

time refers to the period of homogenisation

B. Braun homogenisation.

As can be seen from Table 31, doubling the homogenisation period from 2 to 4 min is very effective in releasing the enzymes associated with carotenoid and other lipid synthesis. It is evident that 0.2 M Tris/HCl (pH 8) buffer is slightly more effective than 0.1 M K_2HPO_4 (pH 7.4) buffer. The inclusion of glucose-6-phosphate and glucose-6-phosphate dehydrogenase in the mixture was also beneficial.

All subsequent incubations were carried out in 0.2 M Tris/HCl buffer with glucose-6-phosphate and glucose-6-phosphate dehydrogenase included in the incubation mixture.

C. French pressure cell.

Table 32 summarises the results of incubations from cell-free systems prepared using the French pressure cell. There was very little carotenoid present in either incubation. The second passage through the French pressure cell seems to have a detrimental effect on enzyme activity.

D. Toluene permeabilisation.

Table 33 summarises the results of the MVA incubation experiments. In experiments 1-4, the enzyme preparation was derived from the pellet and thus contained cell fragments. In experiments 5-7 the supernatant was used (i.e. toluene) as the enzyme system and thus the protein estimation was not carried out. As can be seen from Table 33 the percentage incorporation of MVA into total lipids was comparable with that in the best Braun experiments and some of the freeze dried preparation experiments. The simplicity of the system was attractive and further experiments were carried out using this method. Table 34 shows the percentage incorporation into carotenoids for incubations prepared by

TABLE 32.

Percentage incorporation of radioactivity from [2-^{14}C]MVA into total lipid for incubations prepared by the French pressure cell.

Incubation	Pressure MPa	mg protein per incbn	µg carotenoid extracted per incubation	Percentage incorporation into total lipid
1	138	78.12	0.575	7.56
2	138 x 2	86.6	0.955	5.09

TABLE 33.

Percentage incorporation of radioactivity from [2-^{14}C]MVA into total lipid for incubations prepared by toluene permeabilisation of P. rhodozyma cells.

Incubn	Supernatant	Pellet	mg protein per incbn	µg carotenoid extracted per incubation	Percentage incorporn into total lipid
1	-	+	62.36	13.17	4.3
2	-	+	62.36	16.08	3.7
3	-	+	n.d	16.04	1.84
4	-	+	n.d	13.2	0.7
5	+	-	n.d	14.87	7
6	+	-	n.d	n.d	3.46
7	+	-	n.d	n.d	1.07

n.d = not determined

TABLE 34.

Percentage incorporation of radioactivity from [2-^{14}C]MVA into carotenoids from incubations prepared by toluene permeabilisation of P. rhodozyma cells.

Incubation	Carotenoid	Percentage incorporation from MVA into carotenoid
1	zeaxanthin	0.046
1	astaxanthin	0.27
1	canthaxanthin	0.13
2	zeaxanthin	0.11
2	astaxanthin	0.048
2	canthaxanthin	0.18
3	zeaxanthin	0.167
3	astaxanthin	0.054
3	canthaxanthin	0.011

TABLE 35.

Percentage incorporation of radioactivity from [2-^{14}C]MVA into total lipids from incubations prepared by Triton X-100 treatment of P. rhodozyma cells.

Incubation	µg carotenoid extracted per incubation	Percentage incorporation of MVA into total lipid
1	7.2	0.95
2	9.68	0.46

the toluene permeabilisation method. Incorporation into carotenoids is a factor of 10 higher in these experiments than for the Braun method.

E. Triton X-100 treatment.

As can be seen from Table 35, this method was not particularly successful and not further investigated as a possible method for releasing the enzymes involved with carotenoid biosynthesis.

5.14 Carotenoid incubations.

Three major experiments were carried out using carotenoids as enzyme substrates. Two of the experiments used enzyme preparations from toluene permeabilised cells and the third made use of cell-free preparations from freeze-dried cells.

In each experiment, the incubation components were the same. Likewise, the treatment of the carotenoids from each incubation was identical. An initial separation of the major carotenoids (astaxanthin, zeaxanthin and canthaxanthin) was achieved by t.l.c. (Section 5.8). The crude astaxanthin fractions from each incubation were then subjected to chiral h.p.l.c. (Section 5.8). Fractions were collected into scintillation vials every 1 min along the chromatogram, the solvent removed and scintillation fluid added (10 ml). The radioactivity per vial (and per minute) was then determined by liquid scintillation counting.

It was not possible to prepare [^{14}C]zeaxanthin for these experiments and so [$^{3}H_2$]zeaxanthin (from F. Hoffmann-La Roche and Co. Ltd.) was used. The chirality of this zeaxanthin was defined as a mixture of RR : RS : SS, 1 : 2 : 1. Although not ideal, any metabolism occurring can be identified and, on stereochemical grounds, assigned to the appropriate enantiomers of zeaxanthin.

5.15 Freeze dried method.

The method of Bywater (1984; unpublished work) was used to generate a cell-free enzyme preparation sufficient for eight different incubations. Four of the incubations had [2-^{14}C]MVA (2.5 µCi active isomer) as substrate, and two of each of [$^{3}H_2$]zeaxanthin (0.44 µCi) and [^{14}C]canthaxanthin (0.336 µCi). The incubations were carried out as normal. Table 30 shows the percentage incorporations into total lipid from the MVA experiments. The results of the canthaxanthin and zeaxanthin experiments are shown in Table 36. T.l.c. of each of the P. rhodozyma extracts (incubations 1-8) cleanly separated astaxanthin from the other carotenoids present. The astaxanthin fractions were further analysed by h.p.l.c. on the chiral h.p.l.c. system (Section 5.8B). In each case, fractions were collected into scintillation vials (every 1 min) during the chromatography. The h.p.l.c. solvent was removed by evaporation on a hot-plate, under a stream of nitrogen. Scintillation fluid (10 ml) was added to each vial and the radioactivity per vial determined by liquid scintillation counting.

A. MVA incubations.

In each of the four incubations, the major incorporation (over 90%) was detected at the early stages of h.p.l.c. (approximately 5-7 min). This corresponds to a very non-polar carotenoid. There was no indication of incorporation into astaxanthin (any of the three stereochemical forms) except in incubation 4, where 107 d.p.m. was detected in fraction 41 (i.e. 41 min). It is feasible that this radioactivity may be associated with (3R,3'R)astaxanthin. However, 107 d.p.m. represents 0.84% of the total radioactivity of the crude astaxanthin fraction and 0.002% of the [2-^{14}C]MVA included in the incubation.

TABLE 36.

Percentage incorporation of radioactivity into total lipid from [^{14}C]canthaxanthin and [$^{3}H_2$]zeaxanthin incubations prepared by the freeze dried method (Section 5.15).

Incubn	µCi [$^{3}H_2$] zeax	µCi [^{14}C] canth	µg zeax	µg canth	µg carotenoid extracted per incubn	percentage recovery
5	-	0.335	15.99	-	32.4	71.83
6	-	0.336	15.99	-	25.8	72.31
7	0.44	-	15.99	14.31	30.0	84.9
8	0.44	-	15.99	14.31	26.5	35.7

zeax = zeaxanthin

canth = canthaxanthin

TABLE 37.

Percentage incorporation of radioactivity into total lipid from [^{14}C]canthaxanthin and [$^{3}H_2$]zeaxanthin incubations prepared by the toluene permeabilisation method (Toluene expt 1; Section 5.16)

Incubn	µCi [$^{3}H_2$] zeax	µCi [^{14}C] canth	µg zeax	µg canth	µg carotenoid extracted per incubn	Percentage recovery
SC1	-	0.336	15.99	-	27.8	89.2
SC2	-	0.336	15.99	-	25.0	87.5
SZ1	0.44	-	15.99	14.31	34.8	79.6
SZ2	0.44	-	15.99	14.31	19.0	78.2
PC1	-	0.336	15.99	-	58.0	79.5
PC2	-	0.336	15.99	-	51.8	84.3
PZ1	0.44	-	15.99	14.31	69.5	76.1
PZ2	0.44	-	15.99	14.31	62.0	81.3

zeax = zeaxanthin

canth = canthaxanthin

B. Canthaxanthin incubations.

In neither incubation was there any indication of incorporation of radioactivity into (3R,3'R)astaxanthin as was hoped to be the case. However, in incubation 5 there was heavy incorporation into fractions 9-11 (77.6% of the total), which probably corresponds to canthaxanthin. In incubation 6, there was slight incorporation (5.12% of the crude astaxanthin fraction and 0.085% of the total [^{14}C]canthaxanthin included in the incubation) into a fraction which coincides with (3S,3'S)astaxanthin (Fig. 87). Bywater (1984; unpublished work) demonstrated an incorporation of 1.48 and 2.2% of radioactivity from [^{14}C]canthaxanthin into astaxanthin.

C. Zeaxanthin incubations.

Incorporation into (3S,3'S)astaxanthin was anticipated, however, this was not demonstrated. If incorporation occurred at all, it was into the (3R,3'R)astaxanthin but this was not certain due to low levels of radioactivity.

5.16 Toluene permeabilisation (experiment 1).

P. rhodozyma cells (40 g wet weight) were permeabilised with toluene (20 ml) as described previously (Section 5.9A). The resultant pellet and supernatant were both used in subsequent incubations. The pellet, on resuspension in 20 ml buffer, was used in 4 incubations, 2 of each of [^{14}C]canthaxanthin and [$^{3}H_2$]zeaxanthin (Section 5.9B). Similarly, the supernatant was used for 2 [^{14}C]canthaxanthin and 2 [$^{3}H_2$]zeaxanthin incubations. Table 37 summarises the percentage incorporations into each total extract at the end of the incubation period. As can be seen, losses were quite high, in some cases only 76% of the initial radioactivity being recovered.

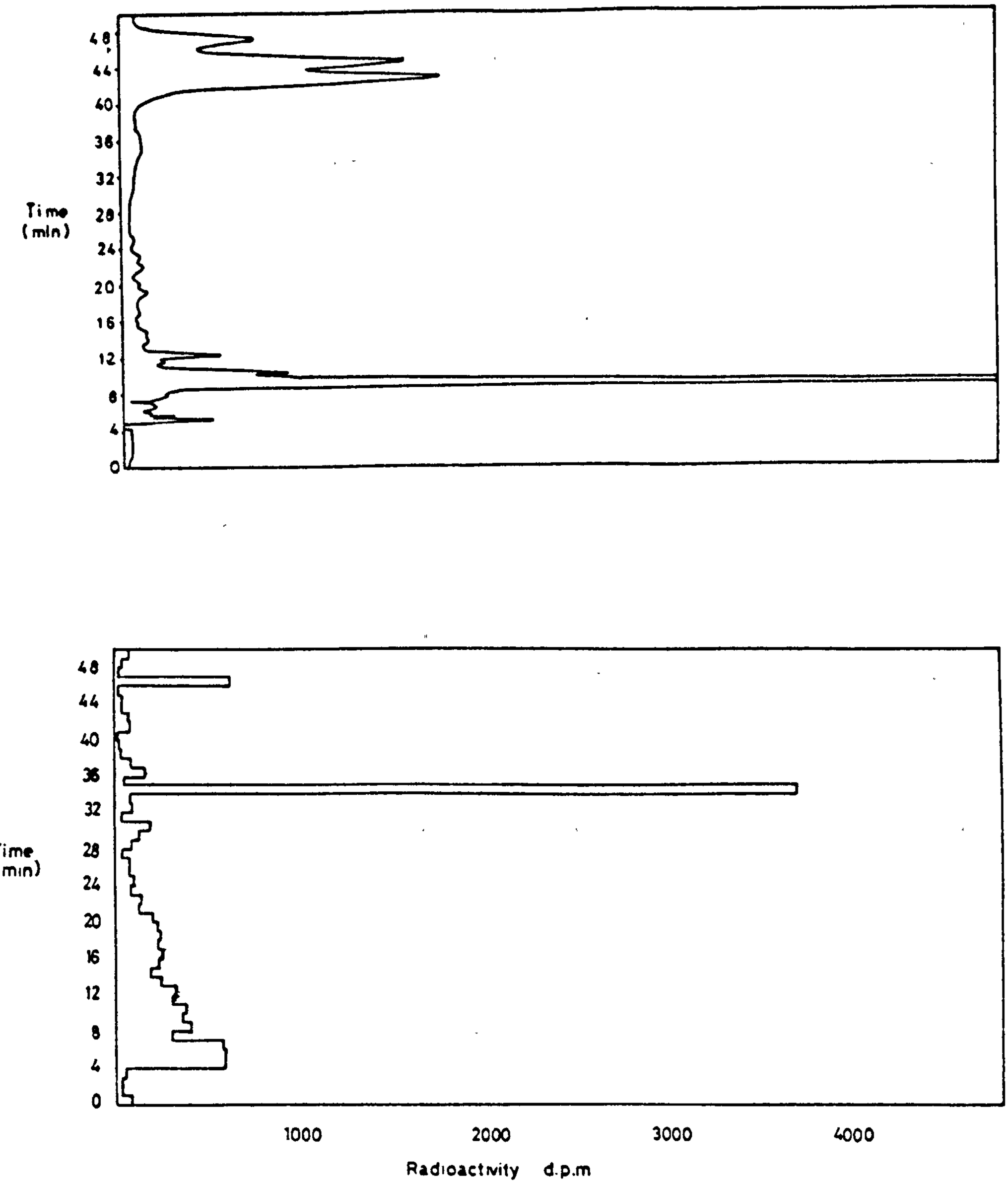


Fig. 87.

H.p.l.c. elution profile and corresponding radioactivity elution profile of the astaxanthin fraction from the freeze dried [^{14}C]canthaxanthin incubation. Column: chiral; solvent: hexane/CH_2Cl_2/ethanol (48:20:2, v/v) at 0.7 ml/min.

Each of the extracts from the pellet incubations were analysed by t.l.c. and the astaxanthin zones scraped off the plate and eluted. An aliquot from each astaxanthin fraction (PC1A, PC2A, PZ1A and PZ2A) was withdrawn and total incorporation into the crude astaxanthin fraction determined by liquid scintillation counting. The results are shown in Table 38.

One-fifth aliquots of the total astaxanthin from two of the above incubations (PZ1A and PC2A) were then purified by the h.p.l.c. system devised by Bywater (1984; unpublished work, section 5.8). In both cases, the astaxanthin fractions collected were radiolabelled. The results are shown in Table 39.

On chromatography of PZ1A, PZ2A, PC1A and PC2A on the chiral h.p.l.c. system, the incorporation into astaxanthin as shown by the Bywater method could not be substantiated. Again, one-fifth of each of the fractions was used and fractions collected every min (Section 5.9), but no significant incorporation into astaxanthin of any stereochemical form could be detected. A random distribution of radioactivity was observed.

One explanation of these contradictory results, is that the Bywater (1984; unpublished work) purification method was inadequate and that the radioactivity detected was trace amounts of the radioactive substrate in the incubation. The retention time of the astaxanthin on this system is short and not very different from that of canthaxanthin and zeaxanthin (Section 5.10).

5.17 Toluene permeabilisation (experiment 2)

P. rhodozyma cells (40 g wet weight) were permeabilised with toluene (20 ml; section 5.9). The pellet, on resuspension in 20 ml of buffer, was used in four incubations, two had [2-^{14}C]MVA (3.5 µCi active isomer)

TABLE 38.

Percentage incorporation of radioactivity into astaxanthin zones from t.l.c. for toluene experiment 1.

Incubation	Total µg carotenoid	Total d.p.m. per zone	Percentage incorporation
PC1A	25.11	83 125	11.24
PC2A	17.5	14 962	2.02
PZ1A	21.14	54 425	5.635
PZ2A	21.82	71 725	7.41

TABLE 39.

Percentage incorporation of radioactivity into astaxanthin purified by the method of Bywater (1984; unpublished work) for toluene experiment 1.

Incubation	d.p.m. per 1/5 th aliquot	Total d.p.m. in astx	% incorpn into astx	% incorpn into astx (Bywater)
PC1A	982	4 910	0.66	1.48 2.20
PZ1A	7855	39 275	4.05	2.33 18.75

astx = astaxanthin

as substrate and one of each of [^{14}C]canthaxanthin (0.336 μCi) and [$^{3}H_2$]zeaxanthin (0.44 μCi). Astaxanthin, canthaxanthin and zeaxanthin standards in L-α-phosphatidylcholine solution were added to the MVA incubations. The incubations were carried out as described in Section 5.9B.

Percentage incorporations of radioactivity into total lipid for each of the incubations are shown in Table 40. The incorporations observed from the MVA incubations (A and B) are higher than those achieved by Bywater (1984; unpublished work) but not as high as in some of the previous experiments.

Total extracts from all four incubations were analysed by t.l.c. The zones corresponding to canthaxanthin, zeaxanthin and astaxanthin were scraped off the plates, eluted and the amount of carotenoid and radioactivity per zone estimated by spectrophotometry and liquid scintillation counting, respectively. The results for incubations B,C and D are shown in Table 41. T.l.c. of total extract from incubation A was not as expected, due to the absence of zeaxanthin and astaxanthin. Only one band was seen, which may be tentatively identified as canthaxanthin, although the R_f was rather higher than normal.

The astaxanthin fractions from incubations B,C and D were analysd by chiral column h.p.l.c. The MVA incubation clearly demonstrated that the enzyme preparation was active, although no incorporation of radioactivity into astaxanthin was detected. However, less polar carotenoids (i.e. at the start of the chromatography) were heavily radiolabelled . Astaxanthin from the canthaxanthin incubation was not radiolabelled in these experiments, which is in contrast to the freeze-dried experiment (Section 5.15). Very high levels of radioactivity were present from time 8-15 min. Radioactivity was detected (several 100 d.p.m.) in each vial from time 15 min to the end of the chromatography. Consequently, it was not appropriate to try and associate any radioactive fraction with any of the

TABLE 40.

Percentage incorporation of radioactivity into total lipid for incubations prepared by the toluene permeabilisation method (toluene experiment 2; Section 5.17).

Incubn	µCi [$^3H_2$] zeax	µCi [^{14}C] canth	µCi [2-^{14}C] MVA	µg carotenoid extracted	Percentage recovery
A	-	-	3.5	49.76	2.2
B	-	-	3.5	122.30	3.9
C	-	0.366	-	87.3	85.6
D	0.44	-	-	167.7	81.4

zeax = zeaxanthin

canth = canthaxanthin

TABLE 41.

Percentage incorporation of radioactivity into carotenoid fractions separated by t.l.c. for the incubations B,C and D (from Table 40) as prepared by the toluene permeabilisation method (Toluene experiment 2; Section 5.17).

Incubation	Carotenoid	µg carotenoid per zone	Percentage incorporation
B	canthaxanthin	25.09	0.047
B	astaxanthin	n.d	0.069
B	zeaxanthin	32.74	0.123
C	canthaxanthin	7.28	43.62
C	astaxanthin	6.6	15.25
C	zeaxanthin	5.13	4.85
D	canthaxanthin	71.34	1.25
D	astaxanthin	15.52	2.73
D	zeaxanthin	56.6	26.84

n.d = not determined

stereochemical forms of astaxanthin.

Problems were also encountered in the analysis of the [$^{3}H_2$]zeaxanthin incubation. A histogram of the radioactivity per fraction along the chromatogram is shown in Fig. 88. The h.p.l.c. elution profile is also shown. There is an indication of incorporation into fractions 49 and 50. As can be seen from Fig. 88 these fractions coincide exactly with (3S,3'S)astaxanthin. However, the % incorporation into these fractions corresponds to 1.1% of the radioactivity in the crude astaxanthin fraction and to only 0.023% of the total radioactivity in the incubation. Bywater (1984; unpublished work) observed 2.33% and 18.75% incorporation of ^{14}C from zeaxanthin into astaxanthin. The significance of the low levels of radioactivity detected in the (3S,3'S)astaxanthin is questionable.

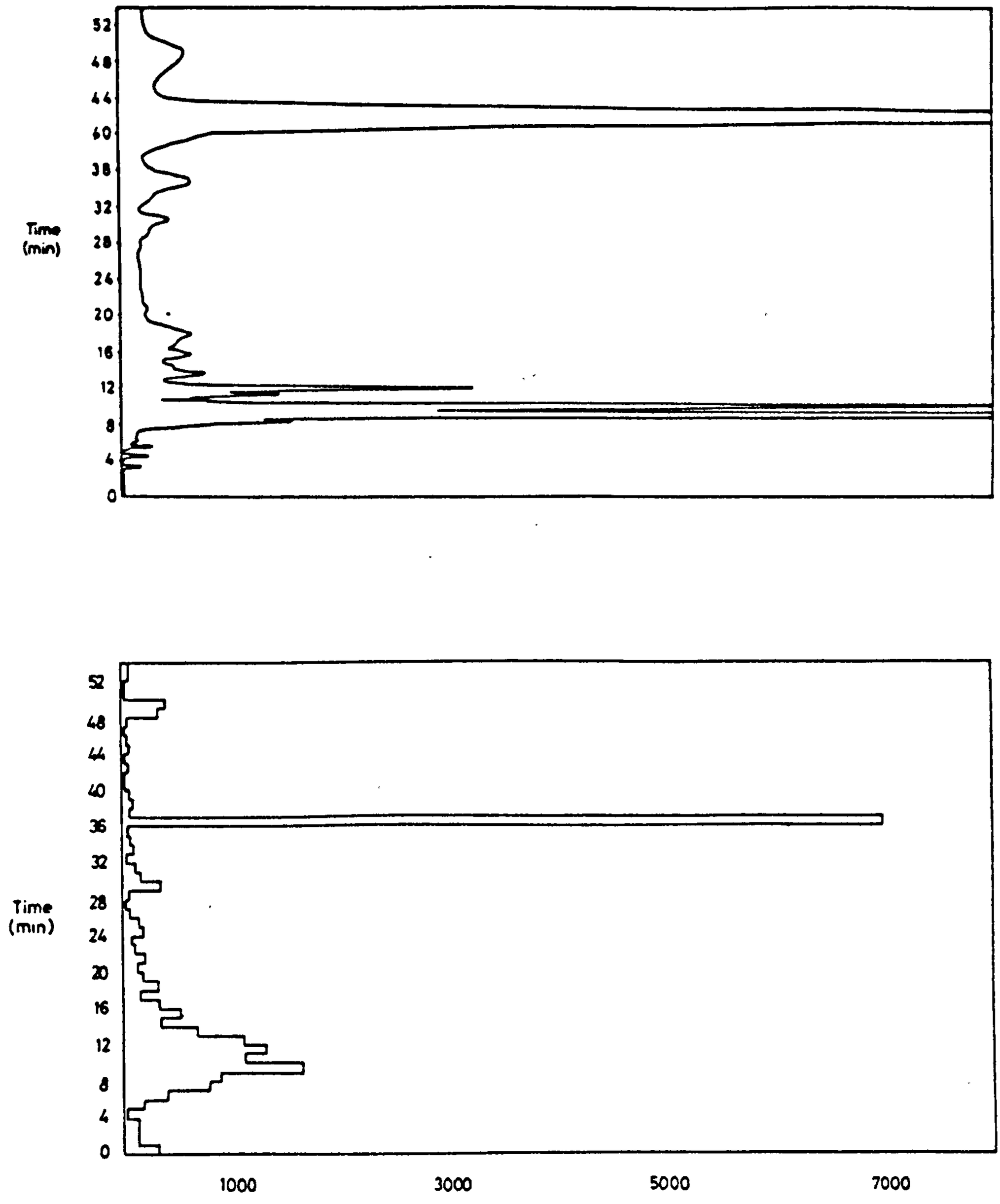


Fig. 88.

H.p.l.c. elution profile and corresponding radioactivity elution profile of the astaxanthin fraction from a toluene permeabilisation (experiment 2) [$^2H_2$]zeaxanthin incubation. Column: chiral; solvent: hexane/CH_2Cl_2/ethanol (48:20:2, v/v) at 0.7 ml/min.

DISCUSSION

The novel explanation of Andrewes and Starr (1976) for the formation of astaxanthin of opposite chiralities in any one species stimulated this investigation. The groundwork and initial observations of Bywater (1984; unpublished work), that ^{14}C was incorporated from both [^{14}C]canthaxanthin and [^{14}C]zeaxanthin into astaxanthin was encouraging and presented an exciting opportunity to study the process of astaxanthin formation.

Typically, the percentage incorporation of radioactivity from [2-^{14}C]MVA into total lipid achieved by Bywater (1984; unpublished work) was low. Davies (1973) consistently observed 40-50% incorporation of [2-^{14}C]MVA into the unsaponifiable fraction using cell-free preparations of P. blakesleeanus prepared in the same manner. This large difference may be attributed to the very tough nature of the cell wall of P. rhodozyma. Other factors resulting in low incorporations may be a function of lower enzymic activities. The age of the culture was important in this respect, an old culture not being as active, as indicated by lower percentage incorporations of radioactivity from [2-^{14}C]MVA into the unsaponifiable fraction. In P. blakesleeanus a feedback process was found to inhibit carotenoid synthesis (Davies, 1973) and it is feasible that a similar mechanism operates in P. rhodozyma. Excess carotenoid levels used in these experiments may, therefore, have posed a problem, but unfortunately this was unavoidable.

At one time, solubilisation of carotenoids with detergents, such as the Tweens, was thought to seriously affect the incorporation of radioactive precursors into carotenoids (Bramley and Davies, 1976). Subsequent work (Bramley, 1985) suggests that some detergents (Tweens included) can solubilise the carotenogenic enzymes without loss of biological activity. Nonetheless, the use of Tweens was avoided and L-α-phosphatidylcholine used as a successful alternative.

The same cofactors and ions were used in this work as by Davies (1973), Bramley and Davies (1975), Bramley et al. (1977) and Sandmann et al. (1980). It is probable that the incubation components would be adequate and certainly not limiting for the incorporation of [2-^{14}C]MVA into total unsaponifiable lipid. Optimisation of conditions of pH and temperature were attempted (Bywater, 1984; unpublished work) and were found to be very similar to those of P. blakesleeanus. The inclusion of compactin in the incubations may be used to increase the level of incorporation from [2-^{14}C]MVA into the total unsaponifiable lipid, by preventing the endogenous formation of MVA by the yeast.

If further work is to be carried out on P. rhodozyma, a thorough optimisation of growth and incubation conditions should be the first priority. Davies (1973) emphasises the sensitivity of the activity of P. blakesleeanus enzymes to the time of harvesting the culture. A similar study of growth characteristics of the cultures, including enzyme activities in relation to time should be pursued for P. rhodozyma.

The poor incorporation of [2-^{14}C]MVA into total unsaponifiable lipid may also have been due to the ineffective release of enzymes because of inefficient disruption of P. rhodozyma cell walls. Consequently, other methods were investigated in an attempt to improve disruption of P. rhodozyma cells, without causing damage to carotenogenic enzymes. A selection of mechanical and other methods were tried.

Amongst the mechanical methods tried were the French press and Braun homogeniser. A review of the use of French presses and their efficiency in the disruption of cells, including yeasts, is given by Hughes et al. (1971). A discussion of disruption of yeast cells by an agitator mill, comparable to the Braun homogeniser, is given by Currie et al. (1972). The paper specifies conditions of increased disruption efficiency e.g. higher agitator speeds, greater loading of glass beads and lower rates of upward recycle of yeast suspension through the mill. An increase in

temperature from 5 to 42°C was accompanied by a reduction in disruption efficiency of approximately 20%. The Braun homogeniser was used successfully to disrupt cells of the fungus Puccinea graminis (Rust) by Rick et al. (1966). Neither the French press nor the Braun homogeniser have been used with respect to carotenogenic enzymes in the past. For P. rhodozyma both these methods were as effective as sieving freeze dried material. Other physical methods of disruption are by osmotic shock, temperature shock (freeze-thawing technique) and ultrasonic treatment (Felix, 1982).

More recently, methods have been developed to permeabilise cells, without lysis of cells or destruction of the whole inner organisation. In most cases the plasma membrane is damaged in such a way that the morphology of the cells remains intact, yet low molecular weight molecules can freely enter and leave the cell. The enzymes are believed to remain at their original place and hence, under ideal conditions, reactions can be measured under conditions which closely resemble the in vivo situation. Felix (1982) reviews all the methods available e.g. organic solvents, antibiotics, detergents, chelating agents and limited digestion of cell walls. The choice of method depends on the organism and the composition of the cell wall and membrane. The kind of enzyme reaction to be investigated is also important.

The method of Miozzari et al. (1978) was investigated as it was shown to be very effective in the permeabilisation of Saccharomyces cerevisiae. The method had been optimised for the tryptophan biosynthetic enzymes, but it had been applied to other yeast enzymes as well. The method of Murakami et al. (1980) was used initially for S. cerevisiae. Toluene treatment was found to make yeast cells completely permeable to exogenous substrates, and intracellular enzymes did not leak out of the treated cells. Electron microscopic examination of toluene-treated cells indicated that they were essentially intact. Several enzymes e.g. hexokinase, phosphofructokinase, pyruvate kinase,

alcohol dehydrogenase and AMP deaminase, were assayed. Other methods of yeast permeabilisation e.g. limited digestion of the cell wall with glusulase (Yee et al., 1976) were not attempted although reputed to be very successful. None of the methods used in this study improved incorporation of [2-^{14}C]MVA into total unsaponifiable lipid to any significant degree.

The use of (3RS,3'RS)[$^{3}H_2$]zeaxanthin in these experiments did not pose any problems. If incorporation of radioactivity into any of the stereochemical forms of astaxanthin had occurred, the precursor (i.e. (3R,3'R)- or (3S'3'S)zeaxanthin) could be identified on stereochemical grounds, assuming that no C-3/3' epimerisation was taking place.

The results of this investigation could not substantiate the findings of Bywater (1984; unpublished work). There are two possible explanations. Firstly, the apparent lack of conversion of canthaxanthin into astaxanthin and zeaxanthin into astaxanthin in this work may be a result of poor enzyme systems. This seems unlikely because the MVA incubations indicate active enzyme preparations. The second possibility is that the purification of astaxanthin by Bywater is not adequate. This is more probably the case, and was partially verified in Section 5.16. Bearing in mind that neither canthaxanthin nor zeaxanthin is present in P. rhodozyma extracts, perhaps it is not altogether surprising that these carotenoids are not involved in astaxanthin formation.

It may be useful to carry out P. rhodozyma enzyme incubations with [^{14}C] β-carotene as substrate. A pathway of formation as proposed by Andrewes et al. (1976) may be followed i.e. via echinenone, 3-hydroxy echinenone etc.

The theory put forward by Andrewes and Starr (1976) as to the formation of astaxanthins of opposite chiralities may still apply for some organisms, although this was not demonstrated for P. rhodozyma. For

the Andrewes and Starr (1976) theory to operate, the enzymes involved must be of broad specificity. It is suspected that animal carotenogenic enzymes fulfil this requirement; however, it is possible that yeast enzymes are more specific as illustrated by the optically pure carotenoids they produce and the relatively simple complement of carotenoids present. If this is the case, there may not be enzymes available for the required transformations of canthaxanthin to astaxanthin and zeaxanthin to astaxanthin. Further work is needed to investigate these possibilities.

CHAPTER 6 : CONCLUDING DISCUSSION

CHAPTER 6

A series of comparative studies was undertaken to investigate the metabolism of carotenoids (particularly the xanthophylls, lutein and zeaxanthin) in both animal and yeast systems. From Chapters 3-5 it is evident that differences exist between these systems.

The situation in animals would appear to be far more complex than that in yeast systems. The complexity was displayed in terms of both the variety of carotenoids present, and in terms of the absolute configurations of these carotenoids. The origin of some of these carotenoids was uncertain, although many could be attributed to dietary intake i.e. the food chain. Since the food chain for goldfish and chickens is essentially of plant origin, the carotenoids from these animals would be expected to be of plant nature. Optically pure carotenoids are a characteristic feature of plant carotenoids and so the occurrence of carotenoids in animals of a mixture of enantiomers or of opposite chirality to that expected for plants, poses a problem as to their origin. It is assumed that these 'unusual' carotenoids in animals are a result of modification of dietary carotenoids, rather than de novo synthesis in the animal.

The results of Chapters 3 and 4 demonstrate carotenoid metabolism in animals. From t.l.c. and h.p.l.c. of both goldfish tissues and chick embryo tissues after administration of radiolabelled carotenoids, it became apparent that the metabolism was far more complex than anticipated. This very much supports the theory of Davies (1985), that the enzymes involved with carotenoid metabolism are of broad specificity. Consequently, animals are very versatile in that they can make use of different carotenoid substrates to produce their full complement of carotenoids. Although, metabolism has been unequivocally demonstrated, there still remain the fundamental questions as to (a) why a particular mixture of carotenoids is produced, (b)how their production and

maintenance is regulated and (c) what their function is in the animal?

Although useful as a preliminary investigation of carotenoid metabolism, whole animal feeding experiments have their problems. Low levels of absorption of dietary carotenoids, low specific activities of radiolabelled carotenoids administered and low rates of carotenoid turnover demand sensitive techniques to detect metabolism, if it occurs at all. The combination of t.l.c. and h.p.l.c. followed by liquid scintillation counting is an appropriate method to analyse extracts from animal feeding experiments. Once metabolism has been demonstrated, the next step should be the preparation of working enzyme systems from the relevant animal tissues. As yet, little progress has been made in this area, the notable exception being the intestinal system used to demonstrate β-carotene to retinal cleavage. Tissue slices from bovine corpora lutea have been shown to form terpenoids under the correct conditions (Austern and Gawienowski, 1969) and could therefore be used as the basis of other tissue preparations. Liver preparations from rats are used to investigate sterol biosynthesis, and modification of these methods may provide a grounding for the preparation of liver systems to examine carotenoid metabolism. Until such systems have been prepared and used, only relatively basic information about carotenoid metabolism will be generated.

The understanding of carotenoid synthesis, and the enzymes involved, in yeasts is far greater than for animals. Many cell-free preparations are in routine use to study aspects of carotenoid biosynthesis and enzymology. Nevertheless, problems were encountered during this work on the metabolism of carotenoids in Phaffia rhodozyma. Bywater (1984; unpublished work) noted the conversion of canthaxanthin to astaxanthin and zeaxanthin to astaxanthin. The failure to demonstrate these conversions in this study may be a result of the experimental protocol or may be a true observation. If zeaxanthin and canthaxanthin are not metabolised to astaxanthin this may reflect a difference between animal

and yeast systems. It can be envisaged that the enzymes in yeast are not as versatile in their substrate specificity and are under a tighter mechanism of control. Feedback systems have already been demonstrated for Phycomyces blakesleeanus (Davies, 1973).

The importance of the role of the organic chemist in carotenoid biochemistry has adequately been illustrated during the course of these studies. The research on chick embryo carotenoid metabolism, in particular, emphasises the need for detailed analysis of carotenoid structures. Without absolute configurations of carotenoids, the assignment of carotenoid transformations occurring in animal systems would be dangerous, if not impossible.

There is much to be done in this difficult field of biochemistry. Although many technical problems exist, a concerted effort by the biologist, chemist and biochemist is needed to solve the many problems of carotenoid synthesis, metabolism and function.

APPENDIX 1

A.1 F. Hoffmann-La Roche and Co. Ltd., analysis of turkey retinal carotenoids.

A lipid extract from 1200 turkey eyes prepared in this work was sent to Mrs. K. Schiedt at F. Hoffmann-La Roche and Co. Ltd., Basel, Switzerland for analysis of the astaxanthin fraction. Facilities for the determination of the chirality of the astaxanthin were readily available e.g. anaerobic saponification apparatus, the relevant chemical expertise for the preparation of astaxanthin dicamphanates and their subsequent resolution by h.p.l.c. In addition to the analysis of the astaxanthin fraction, the Roche research group carried out a comprehensive analysis of all the carotenoids present in the extract.

The following sections outline the Roche analysis of the carotenoids of turkey retina. Details of h.p.l.c. analyses and the appropriate h.p.l.c. equipment are given and similarly, the details of c.d., m.s. and n.m.r. instruments are included.

A.2 H.p.l.c. instruments.

Several instruments were used during the analysis. All instruments were assembled with a combination of Kontron pumps, detectors and recorders, in conjunction with Varian Integrators (Vista System CDS 401). The pumps used were either Kontron LC pump 414-T or the newer Kontron h.p.l.c. pump 420. The detectors were Kontron Uvikon LCD 725 and Kontron Uvikon 720-LC, both of which facilitated the recording of uv/vis spectra by means of the stopped-flow technique. The recorders were Kontron W and Kontron W1100.

The h.p.l.c. packing materials used were LiChrosorb Si-60 (Merck, Darmstadt, Germany), Nucleosil 50-5 (Macherey Nagel, Düren, Germany),

Spherisorb 5S-W (Phase Separations Ltd., Queensferry, Wales) and ODS Hypersil (Shandon, Runcorn, England).

A.3 C.d. spectroscopy.

C.d. spectra were recorded on a modified Dichrographe Mark II (Iobin Yvon) in conjunction with an on-line DEC 11/23 computer, which facilitated background subtraction, smoothing and spectra accumulation. The solvent used was EPA (diethyl ether/propan-2-ol/ethanol, 5:5:2 v/v). The concentration of carotenoid and cell pathlength used for each spectrum is given in the appropriate section of Chapter 4. Spectra were recorded at room temperature (approximately 22°C).

A.4 Mass spectrometry.

The mass spectra were obtained on an AEI-MS 9 mass spectrometer, updated with a ZAB-Console by VG (Vacuum Generators, Altrincham, England), and in combination with a data system SS 200 (Finnigan MAT, Bremen, Germany).

The sample was introduced into the chamber on the tip of a glass rod and positioned close to the electron beam of the ion source. The ion source temperature was 250°C and the electron energy 70 eV.

A.5 N.m.r. spectroscopy.

A Bruker WM-400 spectrometer with Aspect 2000 computer was used for all n.m.r. spectra recorded. The reference solvent in each case was $CDCl_3$.

A.6 Turkey retinal extract.

The carotenoid present in 1200 turkey retina (7.88 mg) accounted for

a small proportion of the total lipid extract (weight of extract was 12.44 g).

Partitioning of the carotenoids between hexane and DMSO revealed that there was practically no free hydroxyl carotenoids. The distribution was such that 95.3% of the carotenoids were in the hexane and 4.7% in the DMSO. Consequently, the two fractions were pooled.

A.7 Procedure of analysis.

Chromatography of the total turkey retina lipid extract on Alumina (Brockmann Grade V; 12% water, v/w) yielded 8 fractions. Each fraction was treated in the manner detailed below.

FRACTION 1.

This fraction was eluted with 100% light petroleum. The fraction was comprised of xanthophyll diesters and accounted for 50% of the total lipid extract (3.98 mg).

The fraction was saponified and an absorption spectrum recorded (λ_{max} 283 314 326 (379) 399 419 468 nm). Rechromatography of the saponified carotenoids on alumina gave 7 fractions.

a. ε,ε-carotene.

From the uv/vis absorption spectrum this fraction was identified as ε,ε-carotene. H.p.l.c. analysis confirmed the ε,ε-carotene structure. The h.p.l.c. system used was as follows:

Column:	Nucleosil 50-5 (500 mm x 6 mm)
Solvent:	n-Hexane - 0.05% (v/v) N-ethyl diisopropylamine
Flow rate:	2 ml/min

The ε,ε-carotene structure (24% cis- and 76% trans-ε,ε-carotene) was also confirmed by m.s. and n.m.r., and c.d. analysis confirmed the (6S,6'S) chirality.

b. Dehydroretinal ?

This fraction was tentatively identified as dehydroretinal although the absorption maximum was rather high (λ_{max} 378 nm). Another fraction showed similar characteristics and so no further analysis of this fraction was undertaken.

c. Fraction 3.

This fraction was composed of two elements.

i. Cryptoxanthin.

The absorption spectrum was consistent with α-cryptoxanthin (λ_{max} (398) 419 442 471 nm). This was confirmed by h.p.l.c. of cryptoxanthin carbamates. Separation of the cryptoxanthin carbamates was carried out by the same method used for the carbamates of zeaxanthin and other xanthophylls (Rüttimann et al., 1983). M.s., n.m.r. and c.d. analyses of the fraction were also carried out.

ii. Retinol.

The total amount of retinol in this fraction was 2.24 mg. Absorption spectra and h.p.l.c. analysis verified the identity of the retinol.

d. Fraction 4.

Unidentified carotenoid (λ_{max} 395 417 469 nm).

e. Fraction 5.

Unidentified carotenoid (λ_{max} 356 377 397 nm).

f. Fraction 6 and 7.

Fractions 6 and 7 were pooled after t.l.c. and spectral comparisons. By h.p.l.c. of the free carotenoids it was seen that galloxanthin, zeaxanthin, epilutein and lutein were present. Two h.p.l.c. systems were available for the separation of zeaxanthin, lutein, epilutein and galloxanthin.

System I

Column:	LiChrosorb Si-60 (500 mm x 3.1 mm)
Solvent:	n-Hexane - 6.5% (v/v) dichloromethane -0.25% (v/v) propan-2-ol - 0.1% (v/v) N-ethyldiisopropylamine
Flow rate:	1 ml/min

System II

Column:	Spherisorb 5S-W (500 mm x 3.1 mm)
Solvent:	as for system I
Flow rate:	1 ml/min

An aliquot of the pooled fraction was acetylated (see Section 4.25 A viii). The diacetates of the xanthophylls were separated preparatively on Silicagel 60 (Merck 5729) t.l.c. plates with developing system ethyl acetate/hexane (1:2, v/v). The galloxanthin diacetate was purified by h.p.l.c.

Column:	Nucleosil 50-5 (250 mm x 4 mm)
Solvent:	n-Hexane - 0.125% (v/v) propan-2-ol
Flow rate:	1.5 ml/min

The pure galloxanthin diacetate was submitted for n.m.r., m.s. and c.d. analysis.

Epilutein, lutein and zeaxanthin were converted to their dicarbamates and their absolute configurations determined by h.p.l.c. and c.d. as appropriate (Rüttimann et al., 1983; see Chapter 4).

FRACTION 2.

This fraction was eluted with 10% ether in light petroleum. Again the fraction was comprised of diesters, accounting for 19% of the total carotenoids (1.42 mg).

The fraction was saponified and an absorption spectrum recorded (λ_{max} 277 (357) (379) 398 420 472 nm). Further chromatography of the free carotenoids on Alumina gave 4 fractions.

a. Fraction 1.

This fraction was a mixture of dehydroretinal (λ_{max} (hexane) 275 385 nm) and the two retinal homologues described in Chapter 4 (Section 4.25 A).

b. Fraction 2 and 3.

This fraction was found to be a mixture of lutein, epilutein, zeaxanthin and galloxanthin. Galloxanthin was by far the predominant component. H.p.l.c. systems I and II (see Fraction 1, part f) were used.

c. Fraction 4.

Unidentified carotenoid (λ_{max} 274 (330) (357) (378) 400 420 468 nm).

FRACTION 3.

This fraction was eluted with 10% ether in light petroleum. The fraction constituted 8% of the total carotenoids (640 µg).

The major component of this fraction was the retinal homologue $C_{23}H_{32}O$ (51%). The other components were not conclusively identified but may be a mixture of 3-hydroxyechinenone (18%), echinenone (11%), adonixanthin (11%), xanthophyll (5%) and others (4%). These identifications were on the basis of uv/vis absorption spectra and t.l.c. only.

FRACTION 4.

This fraction was also eluted with 10% ether in light petroleum. This fraction was fairly large (20% of the total carotenoids) and was comprised entirely of astaxanthin diester. The fraction was accidentally saponified in the presence of air to give astacene and consequently could not be used to determine the chirality of astaxanthin.

FRACTIONS 5, 6 and 7.

These fractions were eluted with 20% ether in light petroleum, 50% ether in light petroleum and 2% methanol in ether, respectively. Together they comprised 3% of the total carotenoids. Fortunately, these fractions were identified as astaxanthin mono- and diesters with trace amounts of 3'-dehydrolutein, lutein and other unidentified carotenoids.

Following anaerobic saponification of the fractions and derivatisation of the astaxanthin to its dicamphanates, h.p.l.c. showed the presence of (3R,3'R)astaxanthin (4%), meso-astaxanthin (30%) and (3S,3'S)astaxanthin (66%).

FRACTION 8.

This fraction was eluted with 3% methanol and 3% glacial acetic acid in ether and constituted 2% of the total carotenoid (181 µg). The

TABLE 42.

Chromatography of turkey retina lipid extract on neutral Alumina (Brockmann Grade V).

Fraction	Solvent		Carotenoid components
1	Hexane	50%	Xanthophyll diesters ε,ε-Carotene Retinal Retinol α-Cryptoxanthin Galloxanthin Zeaxanthin Lutein Epilutein
2	10% E/hexane	19%	Diesters Retinal Galloxanthin Unidentified fraction
3	10% E/hexane	8%	Retinal
4	10% E/hexane	20%	Astaxanthin diester
5	20% E/hexane	1%	Astaxanthin diester
6	50% E/hexane	1%	Astaxanthin diester Astaxanthin monoester
7	2% MeOH/E	1%	Astaxanthin diester Xanthophylls
8	3% MeOH/E	2%	Astacene, semiastacene Retinoic acid

E = ether
MeOH = methanol

fraction was found to be a mixture of astacene, semiastacene esters and retinoic acid.

Table 42 summarises the chromatography of the turkey retina lipid extract as undertaken by the Roche group.

A.8 Analysis of poultry feeds.

The dihydroxycarotenoid fraction from each feed was analysed separately in the first instance. H.p.l.c. system II allowed the separation of lutein, zeaxanthin and epilutein.

Column:	Spherisorb 5S-W (500 mm x 3.1 mm)
Solvent:	n-Hexane - 6.5% (v/v) dichloromethane - 0.25% (v/v) propan-2-ol - 0.1% (v/v) N-ethyldiisopropylamine
Flow rate:	1 ml/min

The compositions of the dihydroxycarotenoid fractions are summarised in Table 43. The ratio of lutein to zeaxanthin for each feed was approx. 9:1.

The five dihydroxycarotenoid fractions were bulked before preparation and analysis of the dicarbamates of the lutein and zeaxanthin (Rüttimann *et al.*, 1983). The lutein (95.65% (3*R*,3'*R*,6'*R*)-*trans*-lutein and 3.64% *cis*-lutein) and zeaxanthin (99.88% (3*R*,3'*R*)zeaxanthin) were stereochemically pure.

TABLE 43

Composition of dihydroxycarotenoid fraction from 5 feeds constituting a typical poultry feeding programme.

Feed	Wt in g of sample extracted	Total µg of carotene diol	Percentage composition				
			trans lutein	trans zeaxanthin	trans epilutein	cis isomers	unidentified
Starter crumbs	220.7	156	70.29	5.67	2.68	17.73	3.6
Mini-pellets	320.75	258	70.80	6.59	2.00	16.86	4.7
Grower no. 1	182.5	72	65.63	8.5	2.09	20.32	3.5
Grower no. 2	523.7	52	63.77	13.91	-	13.05	9.3
Finisher	444.25	460	67.7	10.45	0.4	19.52	2.6

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