THE ASSOCIATION OF CAROTENOIDS WITH PROTEIN IN CERTAIN INVERTEBRATES

A

THESIS

PRESENTED BY

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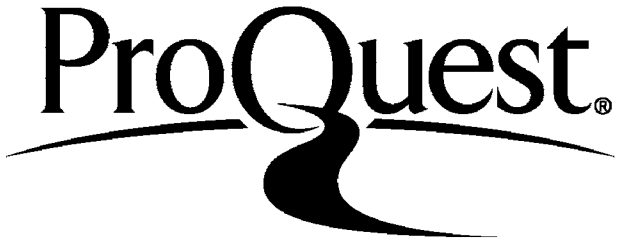


ABSTRACT

Crustacyanin, the blue carotenoprotein of the lobster carapace was obtained in a state homogeneous in cellulose acetate electrophoresis. It was crystallised and shown to be a globulin of large particle size, free from lipid and carbohydrate. The minimum molecular weight of the protein, calculated on the carotenoid content, was found to be one-tenth of that expected from its size.

Reversible changes in spectrum occurring on removal of salt were shown to be attended by dissociation into subunits of the size expected from the minimum molecular weight.

Apocrustacyanin was shown to be of the subunit size and heterogeneous in cellulose acetate electrophoresis. Native crustacyanin was reconstituted from the apoprotein and astaxanthin.

Possible modes of binding of the carotenoid are suggested to account for the spectral changes occurring in urea. Electrophoretic similarities between crustacyanin treated with urea and apocrustacyanin are discussed in relation to the number of different subunits.

It is suggested that crustacyanin is composed of a specific geometrical association of several small subunits, possibly different, and that the carotenoid stabilises the

subunits in a configuration essential for the association and possibly also assisting in the binding.

Ovoverdin, the green carotenoprotein of lobster ovary, was obtained homogeneous in cellulose acetate electrophoresis and shown to be a glycolipoprotein, precipitating at low ionic strength but soluble in distilled water. The possibility of two molecules of carotenoid being associated with each molecule of protein has been investigated.

Astaxanthin-proteins were purified from the carapaces of Eriphia spinifrons, Carcinus maenas, Astacus astacus and Palinurus vulgaris. Their absorption spectra and properties are compared with those of crustacyanin and ovoverdin.

Glycolipoproteins containing a number of carotenoids have been purified from the ovaries of Carcinus maenas, Pecten maximus and Palinurus vulgaris, and from the eggs of Palinurus vulgaris. Their properties have been investigated and the mode of association of the carotenoids is discussed.

CONTENTS

ABBREVIATIONS

The abbreviations used, apart from the few given below, are those recommended by the Biochemical Society in the booklet "Suggestions and Instructions to Authors" (Biochem.J. (1964) 90 1).

E 280/460 = the ratio of the optical densities at 280 mμ and 460 mμ.

$E_{280}^{0.1\%}$ = the optical density of an 0.1% solution at 280mμ.

Δ 280 (cf. saturation curves Figs. 6, 43, 50) = the change in optical density at 280mμ between consecutive readings.

In referring to parts of the thesis the following notation is used:

Chapter I, Section B, subsection (ii), part (a) is indicated as $I.B_{(ii)}.a.$

CHAPTER I

INTRODUCTION

A. (i) Survey of the early literature on polyene-protein complexes

In 1923 Verne suggested that the blue colours occurring widely in the eggs, hypodermis, and carapace of crustacea /Palaemon, Astacus, Galanthea, Portunus, Homarus (carapace)/ were due to the combination of carotenoids with protein; such complexes had previously been suggested to occur in butterfly larvae (Poulton, 1885), cow serum (Palmer & Eckles, 1913) and in plants (Lubimenko, 1921). By virtue of the combination the lipid-soluble carotenoids were rendered water-soluble (in eggs and hypodermis) and were no longer extractable with neutral lipid solvents (e.g. ether) unless liberated from the combination by denaturation of the protein (with heat, acid, alkali or alcohol) - this resulting in the appearance of the red colour of the free carotenoid; the polyene character of the carotenoid was masked in the combination, being no longer oxidised nor reacting with iodine. Some of these properties had been noted early by Merejkowsky (1883) and Newbigin (1897, 1898) for green and blue solutions obtained from a number of crustacea and by Palmer (1922) for the protein-bound carotenoids in cow serum.

Verne named these complexes carotenoalbumines, since they precipitated at ammonium sulphate saturation; he considered that the various shades of colour occuring in crustacea (green in the eggs of Homarus and Nephrops, red in the carapace of Palinurus) were similarly due to the presence of carotenoalbumines, the binding in these cases being less firm. The blue birefringent crystals, first observed by Pouchet (1873), in the hypodermis of Galanthea and Astacus were also considered to be carotenoalbumines (Verne, 1926); this has been questioned by Kuhn and Sörensen (1933) but reaffirmed by Lederer (1935).

Lwoff (1925) proposed the term "carotenoprotein" 'pour les combinaisons, non-chimiquement définies, d'un carotinoïde et d'un protide'. Verne (1923) suggested that their occurrence was widespread in nature, and this has received support from the numerous preparations which have since been obtained (Table I).

(ii) Discussion of the early literature

In the numerous cases reported (Table I) of the occurrence of carotenoproteins, one of the following criteria has been taken as evidence of their existence:

1. The obtaining of coloured aqueous extracts, precipitatable by ammonium sulphate, from which the carotenoid could not be extracted with ether, and

TABLE I

Reported occurrence of carotenoproteins

s = obtained in aqueous solution
λmax = absorption maximum (mμ) of aqueous solution
infl. = inflexion
—— = nature of carotenoid undetermined
N = purified by $(NH_4)_2SO_4$ precipitation

I. Invertebrates

A. Coelenterata

Species	Organ	Carotenoprotein	Carotenoid	Reference
i. *Clava squamata*	Eggs	deep grey	——	Teissier, 1925
ii. *Actinia equina*	Body tissue	blue and green	——	Abeloos-Parize, 1926; Lederer, 1933
iii. *Vellela lata*	Mantles	s, purple, λmax=585-8	astaxanthin)	Fox & Haxo, 1958
	Tentacles	s, blue, λmax=610	astaxanthin)	

B. Echinodermata

1. *Holothuria polii* (et al.)	Gonads and ovaries	s, red, N. s, blue, N.	——	Toumanoff, 1926
Cucumaria plancia (et al.)	do.	do.	——	do.
ii. *Henrica*, *Porania*, *Solaster*, *Hippasteria*	Skin	s, red, max recorded	xanthophylls	Euler et al., 1934i

Species	Organ	Carotenoprotein	Carotenoid	Reference
iii. *Asterias rubens*	Dorsal skin	s, blue, precipitated by 20-40% NH_4Cl, reconstituted	astaxanthin	Euler et al., 1934i, iia
iv. Various *Holothuria*, *Ophiuriodea* and *Crinoidea*	Skin	s, red and blue	—	Lönnberg, 1933
v. *Porania pulvilla*	Skin	s, red-violet, λmax 458, 492	xanthophylls	Lederer, 1935
vi. *Pisaster giganteus*	Skin	purple	metridine	Fox & Scheer, 1941
vii. *Crossaster papposus*	Skin	s, red, λmax500, N(30-50%)	astaxanthin	Willstaedt, 1944
viii. *Marthasterias glacialis*		green or violet		Fox & Vevers, 1960
Asterias gibbosa	Skin	green	—	
		C. *Arthropoda*		
		a. *Crustacea*		
i. *Gebbia*, *Palaemon*	Hypodermis	s, blue	—	Merejowsky, 1883
ii. *Homarus vulgaris*	Eggs and ovary	s, green	—	Newbigin, 1897
	Eggs	s, green, alumina gel purified	astaxanthin	Kuhn & Sörensen, 1938
	Eggs	s, green, λ max=476,660	astaxanthin	Goodwin, 1949
	Carapace	s, blue	—	Newbigin, 1897
	Carapace	s, blue, λmax=625, named crustacyanin	astaxanthin	Wald et al., 1948
iii. *Homarus americanus*	Eggs	s, green, N(50-100%), λmax=470,640, named ovoverdin	astaxanthin	Stern & Salomon, 1938
iv. *Idya furcata*	Retina, eggs	s, blue	—	Lwoff, 1925, 1927

Species	Organ	Carotenoprotein	Carotenoid	Reference
v. *Pandalus borealis*	Eggs, ovary	s, blue	—	Lönnberg, 1933
vi. *Spirontocharis lilljeborgi*	Ovary	s, green	—	Lönnberg, 1933
vii. *Lepas anatifera*, *Lepas fascicularis*	Eggs	s, blue, λ max=600, N(25-42%)	astaxanthin(?)	Ball, 1944
viii. *Nephrops norwegicus*	Carapace	s, blue	astaxanthin	Goodwin, 1949
ix. *Idothea viridis*	Blood, eggs	s, green	—	Fox, 1955
x. *Notostracea trios*, *Conchostraca caenestheria*	Eggs	pink	—	Fox, 1955
xi. *Hippolyte varians*	Carapace	green	—	Fox, 1955
xii. *Macrobrachium rosenbergi*, *Diaptomas vulgaris*	Carapace	blue	—	cf. Fox & Vevers, 1960
xiii. *Astacus pallipes*, *Pandalus borealis*, *Palinurus vulgaris*, *Carcinus maenas*	Carapace	green-brown red red green and orange-red	—	cf. Fox & Vevers, 1960
xiv. *Eupagurus bernhardus*	Eggs	s, purple	astaxanthin ester	Cheesman & Prebble, 1964
xv. *Daphnia magna*, *Cladocera*, *Simophphalus*, and 22 other species Cladocera	Eggs (occasionally blood) Eggs Blood	s, green s, blue or green green	astaxanthin	Green, 1957

b. *Insecta*

i. *Locusta*, *Tettigonia*, *Meconium*, *Sphinx*	Blood, skin	s, brown-red, named insectoverdin, contains bile pigment	lutein	Junge, 1941

Species	Organ	Carotenoprotein	Carotenoid	Reference
ii. Dixippus morosus	Blood, skin	s, yellow-brown, N(0-50%)	β-carotene	Junge, 1941
iii. Oedipoda	Wings	s, blue, red and yellow	astaxanthin	Okay, 1949
iv. Schistocera, Locusta solitaria and gregaria	Wings	s, brown	astaxanthin	Goodwin & Srisukh 1949, 1950, 1951
	Haemolymph	yellow or green (bile pigment)	β-carotene	Goodwin, 1952
solitaria	Integument	s, green (bile pigment)	astaxanthin and β-carotene	
v. Orthoptera	Wings	s, yellow-orange	astaxanthin	Goodwin, 1952
vi. Nomadacris septem	Wings	red	astaxanthin	Goodwin, 1952
D. Mollusca				
i. Volsella modiolus	Gonads, ovary	red	xanthophylls	Euler et al., 1934ii(b)
ii. Pomacea canaliculata	Eggs, ovary	s, red, N.	5 carotenoids	Comfort, 1947, 1949
	Eggs	alumina gel purified λmax 510/545.inf.330,485	astaxanthin derivative	Cheesman, 1954, 1956, 1958
iii. Patella vulgata Patella depressa	Ovary	olive-green	—	Goodwin & Taha, 1950
2. Vertebrates				
Pisces				
i. Scomber, Botryllus, Clavellina	Skin	green or yellow	—	Lönnberg, 1931
ii. Dendrodoa grossularia	Skin	s, brown-orange	astaxanthin	Lederer, 1938i
iii. Crenilabrus pavo	Skin	blue	astaxanthin	Abolins, 1957i
iv. Labrus mixtus	Skin	blue	astaxanthin	Abolins, 1957ii

in which the carotenoid spectrum lies at longer wavelength as compared to that in hexane. /A.(iii); B.(i-v, vii); C.a.(i-ix, xiv, xv); C.b.(i-vi); D.(ii) in Table I_I./

2. Non-extractibility of carotenoids <u>invivo</u> by neutral lipid solvents /D.(i, iii) in Table I_I./

3. Change in colour on heating or addition of ethanol to the red characteristic of free carotenoids /A(i, ii); B.(vi, viii); C.a.(x-xiii) in Table I_1; (i, iii, iv) in Table I_2./

Karrer and Strauss (1938) showed that serum albumin was able to form water-soluble complexes with colloidal solutions of carotenoid in which the carotenoid was un-extractable with ether, precipitated by ammonium sulphate, and in which the spectrum was shifted to longer wavelength by up to 35 mμ (as compared to the carotenoid in hexane) depending on the degree of dispersion of the colloidal carotenoid. The complexes changed from bright red to orange on treatment with ethanol. Other workers (Drummond & MacWalker, 1935) have shown that it is not always possible to extract carotenoids from aqueous colloidal solution, and even when the attempt is made under anhydrous conditions (Na_2SO_4/ether, D.(i, iii) in Table I_I) non-extractibility does not necessarily imply binding to protein; thus bile-

carotenoid complexes (Drummond & MacWalker, 1935) and lecithin-vitamin A complexes (Dingle, 1964) are known to occur. Also, water-soluble complexes may be formed during the extraction purely by non-specific adsorption of the carotenoid on the protein surface (cf. Verne, 1923).

The three criteria listed above are therefore insufficient as evidence of specific binding of carotenoid to protein. Stoichiometry of combination must also be established, and this has only been demonstrated for ovoverdin, the carotenoprotein of lobster eggs (Stern & Salomon, 1938; Kuhn & Sörensen, 1938).

Nevertheless, in the case of the blue and green carotenoproteins the very great bathochromic shift in spectrum (100 mμ or more when compared to the free carotenoid in hexane) represents a large energy change, indicating strong interaction between the carotenoid and its partner. Strong interaction is also indicated when the form of the spectrum is completely altered, e.g. from a single to a three-peaked spectrum as in the case of ovorubin (D.(ii) in Table I_I). In these cases, with one exception (B.(vi) in Table I_I), the carotenoid has been found to be astaxanthin or a derivative of astaxanthin, so that astaxanthin-proteins may be considered to represent a distinct group of carotenoproteins.

Carotenoids can also exist *in vivo* dissolved in the lipid of lipoproteins. Such carotenolipoproteins (envisaged by Toumanoff, 1926 and Lwoff, 1927) might be specific, in the sense of a particular lipoprotein dissolving a particular carotenoid, but not necessarily stoichiometrically, or non-specific, the lipoprotein carrying a whole range of carotenoids. The spectrum would be shifted to longer wavelengths (compared to that in hexane) not only through dissolution in the lipid (Wald, 1943), but also through intermolecular interactions with components in the lipid (Rabinowitch, 1945). Plant and bacterial carotenoproteins are of this nature (see next section); so also may be many of the red carotenoproteins listed in Table I, but none of these have been purified sufficiently for this to be established.

The visual pigments, rhodopsin, porphyropsin, iodopsin and cyanopsin are quite distinct from the other two groups (astaxanthin-proteins and carotenolipoproteins) of polyene-protein combinations. Rhodopsin and iodopsin occur in the retinal rods and cones respectively of marine and land vertebrates; porphyropsin and cyanopsin occur in the rods and cones respectively of freshwater fishes (Wald, 1953). The prosthetic group, vitamin A_1 aldehyde (retinene$_1$), in rhodopsin and iodopsin and vitamin A_2 aldehyde (retinene$_2$)

in porphyropsin and cyanopsin (Ball, Goodwin and Morton, 1948; Wald, 1939), is bound to the protein by covalent linkage (Dartnall, 1957)

B. Carotenolipoproteins

(i) Animal tissues

Recent work on the occurence of vitamin A (C_{20}-polyene) vitamin A ester, β-carotene and xanthophylls in mammalian blood and liver suggests that these are carried separately by specific lipoproteins (Oncley, Gard & Melin, 1950; Krinsky, Cornwell & Oncley, 1958; Ganguily, Krinsky, Mehl & Deuel, 1952; Krinsky and Ganguily, 1953; Cornwell, Kruger & Robinson, 1962). Ashworth and Green (1963) have suggested that the polyene is dissolved in the lipid core of the lipoprotein.

Wallace (1963) showed that the carotenoids in the platelets of Rana pipiens eggs are associated with a lipoprotein having a molecular weight of 420,000. He estimated that one out of twenty lipoprotein molecules carried carotenoid and showed that at least three different carotenoids were associated with the lipoprotein, a xanthophyll predominating (90%).

(ii) Plants

Carotenoids and chlorophylls are concentrated in the lamellae of the granae within the chloroplast (cf. Goodwin,

1962). Non-uniform, macromolecular lipoproteins containing the chloroplast pigments have been obtained by many workers (Smith, 1940; Rabinowitch, 1945) by breakdown of these structures. Uniform pigmented particles having a molecular weight of 265,000 extracted with detergents (Smith, 1940) possibly represent specific units of the photosynthetic apparatus.

Recently, photosynthetically-active pigment lipoprotein particles (quantosomes), believed to be subunits of the chloroplast lamellae, have been isolated from spinach (Lichenthaler & Calvin, 1964). These contain a number of different carotenoids (as well as chlorophyll and quinones) in non-stoichiometric proportions. Similarly uniform lipoprotein macromolecules having a molecular weight of 700,000 and containing carotenoids (and protochlorophyll) in non-stoichiometric proportions have been isolated from dark-grown seedlings (Smith, 1963).

Recently (Nishimura & Takamutsu, 1957), the preparation of a carotenolipoprotein from spinach leaves has been reported. It was shown to contain 33% lipid, with β-carotene as sole chromophore. Under the electron microscope the preparation was demonstrated to consist of uniform birefringent particles, having an estimated molecular weight of eight million; each particle was

calculated to contain 3×10^3 molecules of β-carotene. The spectrum of β-carotene in the complex was shifted by 50 mμ to longer wavelength (as compared to that in hexane). It is known that the spectrum of carotenoids can be shifted to much longer wavelengths by polarisation effects (Platt, 1959), so that the large bathochromic shift observed in the complex may be due to interaction between carotenoid molecules dissolved in the lipid component, rather than to specific binding to protein.

(iii) Bacteria

In the photosynthetic bacterium Chlorobium, carotenoids (and bacteriochlorophyll) are associated with photosynthetically-active, uniform lipoprotein macromolecules having a sedimentation constant of 50 S (Bergeron & Fuller, 1961; Fuller, Conti & Mellin, 1963). Similarly, in two species of non-photosynthetic Corynebacterium, the carotenoids are associated with uniform macromolecules having a sedimentation constant of 35 S (Saperstein & Starr, 1955). Non-pigmented particles of similar size occur in non-photosynthetic bacteria (e.g. E.Coli) (Schachman, Pardie & Stanier, 1952) so that these are probably definite lipoprotein molecules, rather than structural chromatophores, in which the pigments are concentrated in the lipid core. [cf. Fuller, Conti & Mellin, 1963]

C. The Visual Pigments

Most of the work on these pigments has been carried out with the rod pigment, rhodopsin, the properties of which may probably be taken as characteristic of this group of chromoproteins.

(i) Structure

Rhodopsin has been obtained in solution only with the aid of solubilisers, such as digitonin or bile salts. Exposure to light of the solution results in the isomerization of the prosthetic group from 11-cis to all-trans retinene followed by progressive rearrangement and loosening of the protein structure, resulting finally in the liberation of all-trans retinene and free protein (opsin) (Hubbard & Kropf, 1959). An intermediate of this process, indicator yellow, behaves as an acid-base indicator with absorption maximum at 440 mμ in acid and 380 mμ in alkali (Hubbard & St. George, 1956). Ball et al. (1949) showed that Schiff base compounds of retinene with amine compounds showed a similar shift, from 360 mμ to 440 mμ in acid. The further shift in rhodopsin (α-peak, 500 mμ) was suggested by Hubbard and Kropf (1959) to be due to interaction between the conjugated side chain of retinene and opsin, close steric fit permitting a high degree of interaction between the two, enhancing resonance,

and lowering the energy of the excited state. Interaction of this kind is supported by the fact that the polyene chain is no longer available for reaction with potassium borohydride or lipooxidase (Wald & Hubbard, 1960). The synthetic compounds of Ball et al. (1949) show a marked inflexion between 350 and 380 mμ, where rhodopsin shows a small (β) peak, further evidence that retinene may be linked to an amino group of opsin to give a Schiff base.

Hubbard (1954) calculated, from ultracentrifuge studies, a molecular weight of 40,000 for rhodopsin in the rhodopsin-digitonin complex and concluded, utilising the value of the molar extinction coefficient of visual purple (per mole equivalent of retinene) (Wald and Brown, 1953), that rhodopsin carried a single retinene molecule.

(ii) Role of the polyene

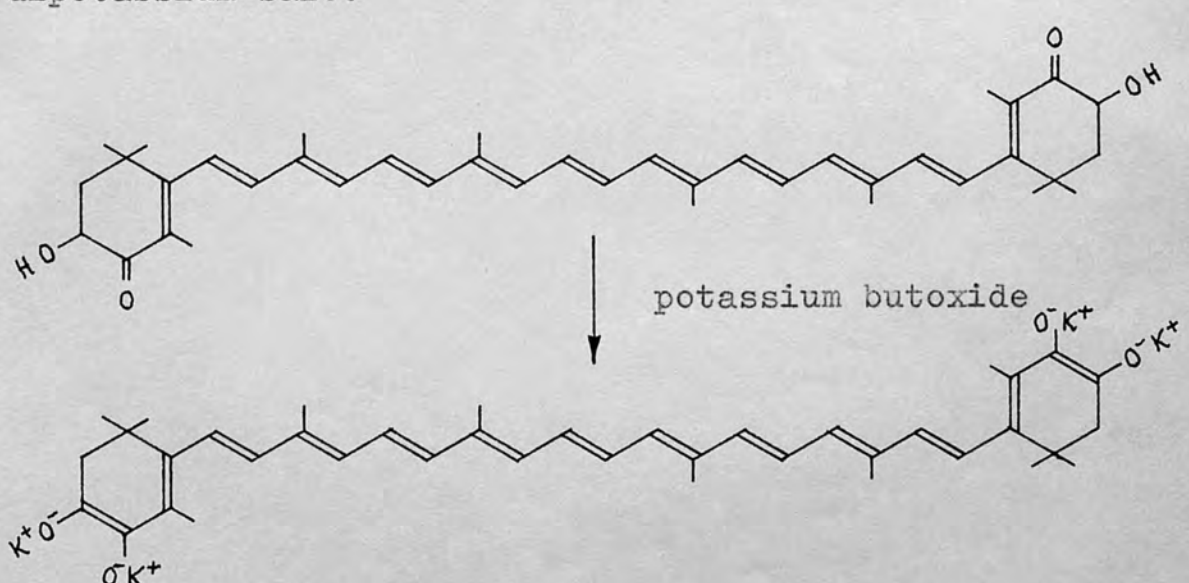
The combination of the labile, sterically hindered 11-cis retinene with opsin, holds the latter in a specific configuration, which results in greater stability of both the opsin and retinene (Wald and Hubbard, 1960). Thus, free opsin is less stable than rhodopsin to heat (Hubbard, 1958) and to the action of acid and alkali (Radding and Wald, 1955); the structure of the protein becomes randomised on detachment of the polyene, as seen by the greater entropy of activation for the denaturation of rhodopsin, (Hubbard, 1958) by the change in ultraviolet spectrum,

(Takugi, 1963) and by the exposure of new groups (two sulphydryl and one acid-binding group) (Wald and Brown, 1953; Radding and Wald, 1955). It has been suggested that the opening-up of the rhodopsin structure on bleaching is a decisive event in visual excitation (Wald, 1958).

D. Astaxanthin-proteins

(i) Structure

Kuhn and Sörensen (1938) showed that astaxanthin, treated with potassium butoxide in the absence of air, turns blue due to enolisation followed by the formation of the dipotassium salt:

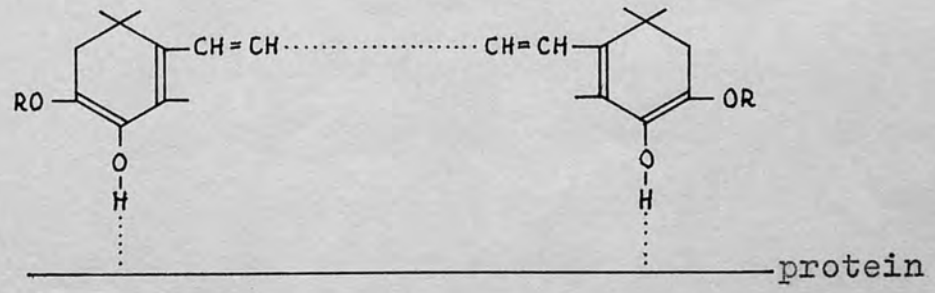


They suggested that the blue astaxanthin-proteins of lobster shell (crustacyanin), the green astaxanthin-protein of the eggs (ovoverdin) might similarly arise from enolisation followed by salt formation, with amino groups of the protein

taking the place of potassium. Also, since astaxanthin in combination with protein, unlike the dipotassium salt, was not autoxidisable, they suggested that some form of (unspecified) secondary linkage was also involved.

A similar structure for the blue astaxanthin-protein of goose-barnacle (Lepas) was proposed by Ball (1944); and the same type of salt-linkage can be envisaged for the blue and green astaxanthin-proteins of Asteria, Vellela, Crossaster, Crenilabrus, Labrum, Nephrops, and Oedipoda (Table I). The salt-like linkage is supported by the ease with which the carotenoid is released from the combination with acetone.

Cheesman (1958) has suggested that in ovorubin, the tautomeric isomer of the astaxanthin-derivative is stabilised by hydrogen bonding to protein:



The change in the resonance structure, arising from the extension of conjugation in the enol form and overlap of the π-orbital of the carotenoid with the electronic orbitals

of the protein, results in alteration in shape of the absorption curve and a bathochromic shift in spectrum; the single-peaked spectrum of the carotenoid (absorption maximum at 490 mμ in pyridine) becomes multiple-peaked (absorption maxima at 510 and 545 mμ with inflexions at 485 and 330 mμ). The inflexion at 330 mμ in the ovorubin spectrum represents the characteristic frequency of one quarter of the conjugated chain (Cheesman, 1958), suggesting a stabilisation of this vibration in the combination by interaction of the conjugated polyene chain with polar groups of the protein.

Ovoverdin, on heating rapidly to 70° in the presence of salt turns red, but reverts to green if rapidly cooled (Stern and Salomon, 1938); crustacyanin (Wald et al., 1948) and the Velella astaxanthin-protein (Fox and Haxo, 1958) likewise reversibly change colour on rapid heating and cooling. Acidification of the goose-barnacle astaxanthin-protein results in the formation of a red product, which reverts to blue on neutralisation (Ball, 1944). Adjustment of ovorubin solutions to pH 1 with mineral acid gives rise to product having a single-peaked spectrum with its maximum at 482 mμ, the original spectrum being restored on neutralisation. Possibly in all these cases, the salt-linkage between the astaxanthin and protein is reversibly

severed, while the secondary linkages involving the polyene chain still bind the carotenoid to the protein.

(ii) General properties

In contrast to the light-sensitive rhodopsin, ovorubin (Cheesman, 1958) and crustacyanin (Wald et al., 1948) are relatively stable to bright sunlight, while ovoverdin (Stern and Salomon, 1938) and the goose-barnacle protein (Ball, 1944) fade slowly; the latter also fade on extensive dialysis. Irreversible changes in colour to the red of the free carotenoid with protein denaturants (acid, alkali or alcohol), and non-extractibility of the carotenoid by ether is characteristic of these proteins; ovorubin is an exception in that it is remarkably stable even to boiling, between pH 4 - 7.

In the cases of the Asterias rubens astaxanthin-protein (Euler et al., 1934 (i)) and ovorubin (Cheesman, 1958), the carotenoid has been separated from the protein and the chromoprotein reconstituted.

(iii) Purification

Few of the astaxanthin-proteins have been purified extensively; some (Asterias, Crossaster, Lepas, etc., see Table I) have been partially purified by salt fractionation. Ovoverdin has been partially purified by repetitive adsorption and elution from alumina gel until a constant nitrogen

to astaxanthin ratio was obtained for the preparation (Kuhn and Sörensen, 1938), but the purity of the preparation was not tested electrophoretically. Ovorubin, purified by alumina gel or carboxy-cellulose chromatography, is the only protein of this group to have been obtained electro-phoretically homogeneous (Cheesman, 1958; Norden, 1962).

(iv) Nature of protein component

Chemical investigations into the nature of the protein moiety have only been performed with ovorubin. This was shown to be a glycoprotein, of which the carbohydrate portion, representing 20% of the protein, consisted of galactose, mannose, fucose and glucosamine. The normal 18 amino acids were shown to be present (Norden, 1962).

(v) Molecular weight

Only in the cases of ovorubin and ovoverdin has an estimation of molecular weight been attempted.

The minimum molecular weight of ovorubin, estimated from dry weight and astaxanthin content, has been determined as 330,000 (Cheesman, 1958). A molecular weight of 300,000 for ovoverdin was suggested from ultracentrifuge studies. Minimum molecular weight determinations have indicated that ovoverdin contains one (Stern and Salomon, 1938) or two (Kuhn and Sörensen, 1938) molecules of astaxanthin per molecule.

(vi) Function of the carotenoid

Ovorubin solutions are difficult to spread as unimolecular films at a water surface. However, if the salt-linkage, joining the carotenoid to the protein, is modified (by short heating with acid or alkali) solutions spread readily, the degree of spreading dependent on the degree of modification of the linkage (Cheesman, 1956, 1958). Preparations of ovorubin in which the join of the polyene chain to protein has been broken (by treatment with ether or strong sunlight) as shown by the disappearance of the 330 mμ inflexion with only minor changes in the visible region of the spectrum, are more susceptible to bacterial and tryptic action. It appears that alteration of either linkage leads to an opening up of structure and decreased stability (Cheesman, 1958), just as in the case of rhodopsin. Cheesman (1958) has suggested that stabilisation of the configurations of protein molecules might be one of the roles of carotenoids in nature. In the eggs of Pomacea (Cheesman, 1958), Daphnia (Green, 1957), Homarus (Goodwin, 1931), Idya (Lwoff, 1927), Clava (Teissier, 1925) and Lepas (Ball, 1944), carotenoproteins occur as storage proteins, the carotenoid-protein linkage being cleaved during development or soon after hatching. It is possible that the role of the carotenoid (found to be astaxanthin or

a derivative when identified) in these proteins is to fix the storage protein in an inert structural form, release of the carotenoid allowing its utilisation.

E. Conclusion

There is evidence for two major groups of carotenoproteins, carotenolipoproteins and astaxanthin-proteins; earlier reports on the occurrence of other types of carotenoprotein need substantiation. The visual pigments, typified by rhodopsin, represent a separate group of polyene-proteins in which the prosthetic group (C_{20}-polyene) is attached to the protein covalently.

Only in the case of ovorubin has any systematic investigation been undertaken into the nature of the carotenoproteins.

The purpose of the present work was to obtain a number of carotenoproteins in an electrophoretically homogeneous state, and to make preliminary investigations into their composition and structure.

CHAPTER II

MATERIALS

All chemical reagents were of Analar grade and solvents were redistilled before use.

DEAE-cellulose (DE 50), ECTEOLA-cellulose (ET 30), CM-cellulose (CM 70) and cellulose (non-ionic, medium grade): Whatman, England.

Sephadex G25 (fine and medium grade), G75 (medium grade), G100 and G200: Pharmacia, Uppsala, Sweden.

Cellulose acetate electrophoretic strips: Oxoid, Ltd., London.

Hydrolysed starch for electrophoresis: Connaught Medical Research Laboratories of the University of Toronto, Canada.

Carbowax 20M: Light & Co., Ltd., Bucks.

Silica gel G; Merck : Anderman & Co., Ltd., London

Calcium phosphate gel was prepared by the method of Keilin and Hartree (1937).

A suspension containing 9.0g. calcium phosphate/500ml. distilled water was used.

Alumina C was prepared by the method of Bertho and Grassman (1938).

Hydroxyapatite was prepared by the method of Polis and Smukler (1953).

Phosphate buffers used in many of the experiments were prepared by mixing solutions of KH_2PO_4 and Na_2HPO_4.

Live lobsters (Homarus vulgaris), crabs (Carcinus maenas, red variety) and scallops (Pecten maximus) were provided by MacFisheries, Marylebone High Street, London.

A few shells of Palinurus vulgaris, Eriphia spinifrons, Astacus astacus and Carcinus maenas (green variety) were collected while on holiday in Antibes, S. France (1962).

The eggs and ovary of Palinurus were provided by Dr. D. Watts, University College, London.

CHAPTER III

GENERAL METHODS

A. Spectroscopic Measurements

Absorption spectra were recorded in a Uvispek spectrophotometer at 5mμ intervals (2mμ intervals near maxima) against appropriate solvent blanks; silica cells of 1 cm. path length and 2.5 cm. capacity were used.

B. Protein Chromatography using Cellulose Ion-Exchangers

Chromatography on cellulose ion-exchangers was performed in the manner suggested by Peterson and Sober (1956, 1962).

Before use, DEAE-cellulose and ECTEOLA-cellulose were washed successively with normal NaOH distilled water, normal HCl, distilled water, and finally methanol; CM-cellulose was similarly washed before use, except that a mixture of equal volumes of 0.5 M-NaCl and 0.5 M-NaOH was used in the place of N-NaOH to avoid its swelling in alkali. Large amounts of impurities which would otherwise have interfered with spectral measurements were removed by the washing. The ion-exchangers were regenerated after use by the same procedure.

The purified exchangers were stirred into distilled water, fines decanted, and the slurry poured into columns

(1.5cm. x 20cm.) connected to a water pump; the bed of the exchanger was packed as hard as possible under suction; the column was never allowed to run dry and the top of the bed (15cm. in length) was protected with a filter paper disc. The column was washed thoroughly with the most concentrated buffer to be used until the effluent attained the same pH as the Buffer and showed no ultraviolet (280 mμ) absorption. After the column had been washed with distilled water, the pressure was removed and the protein to be adsorbed passed through in distilled water or dilute buffer. Passage of the solution was discontinued when 10% of the bed volume, in the case of gradient elution experiments, and 40% for stepwise elution experiments, was taken up with protein; this was easy to judge with the coloured proteins used.

Elution of the adsorbed proteins was carried out by increasing the ionic strength of the perfusing buffer in stages (stepwise elution) or by the passage of a solution of gradually increasing ionic strength (gradient elution). The gradient was obtained with an apparatus consisting of two vessels (mixer and reservoir) of equal diameter joined by a narrow tube. The mixer was joined to the column and stirred with an automatic stirrer: this gave a slightly convex gradient (Wren, 1963). In either case, 2.5 ml. fractions were collected with the aid of a fraction collector

and analysed spectrophotometrically at suitable wavelengths.

Tight packing of the adsorbent resulted in the concentration of the eluted proteins into the smallest possible volume and the slower flow rate so obtained (less than 2 ml./mm.) gave greater resolution.

C. Salting-out Curves

Salting-out curves of protein solutions at constant temperature and pH were obtained by the variable solvent solubility test (Butler, Blatt and Southgate, 1935).

A series of tubes were taken, each containing 5ml. of the protein in 0.2M-phosphate buffer, pH7. Varying amounts of either saturated ammonium sulphate solution or solid ammonium sulphate (in the case of albumins) were added to each tube and the volumes adjusted to 10ml. with distilled water. The tubes were allowed to stand for 2 hours, centrifuged at 12,000 r.p.m. for 30 mins. and the supernatant analysed spectrophotometrically. All operations were carried out at constant temperature (4°).

By an analysis of plots of optical density of the supernatant against the ammonium sulphate concentration for each tube, the number of components in the protein solution and their precipitation range were determined.

D. Preparation of Protein Samples for Electrophoresis

Protein samples for electrophoresis were obtained in

the desired buffer by passage through a Sephadex G25 column equilibrated with buffer (III.C.), and then concentrated to about 5 mg./ml. by placing in a piece of opened out dialysis tubing resting on powdered polyethylene glycol (Carbowax 20M). When freeze-dried preparations of protein were available these were dissolved directly in the appropriate buffer at the required concentration.

B. Electrophoresis

(i) Cellulose acetate electrophoresis

Cellulose acetate electrophoresis was carried out according to the method of Kohn (Smith, 1960).

Cellulose acetate strips, 2.5cm. in width and 10cm. in length were used. 1-5 µl. of the protein solution (containing about 0.3 mg.) in an appropriate buffer was applied as a narrow band with a micropipette to the moist cellulose acetate strip during passage of current. Electrophoresis was continued for 4-7 hours using a current of 2mA per strip. After the run, the strips were dried in an oven at 80° and cut into two pieces, one of which was stained for protein (nigrosine stain, Smith, 1960), the other for carbohydrate (Schiff stain, Smith, 1960). The wet strips were photographed by reflected light.

Buffers used:

(i) 0.05M- phosphate buffer $[Na_2HPO_4-KH_2PO_4]$, pH7

(ii) 0.05M - acetate buffer [NaAc-HAc], pH4.5

(iii) 0.07M - barbitone buffer [5,5-diethyl barbituric acid-sodium barbitone], pH8.6

(ii) Starch gel electrophoresis

(a) Slow

Horizontal starch gel electrophoresis was performed by the method of Smithies (1955).

The gels were prepared in perspex trays (9cm. x 23cm. x 4mm.) as described by Smith (1960) using 12.8 g. hydrolysed starch per 100 ml. of buffer solution, containing in some experiments 6M. urea. Protein samples were applied by inserting rectangular pieces of Whatman 3MM. paper (4mm. x 8mm.) soaked in protein solution (5mg./ml.) into vertical slots in the gel. Up to 6 samples per tray were applied and sealed with a microscope slide smeared with vaseline. The gels were covered with parafilm to within 30mm. of each end to prevent evaporation, and the electrophoresis continued for 16-24 hr. at a potential gradient of 7 volts/cm.

(b) Rapid

Micro-electrophoresis on starch gel (Lagnado, 1962) was carried out by a similar method to that developed for agar (Wieme, 1959).

Coverslides (7.6cm. x 2.5cm.) or thin glass plates (11cm. x 8 cm.) were used to hold the gels, cut out from

the perspex trays (III.E.(ii).a.). Contact with the electrode vessels was made with buffer soaked 3MM. paper, cooling being effected with petroleum ether (40-60°b.p.). Electrophoresis was carried out at a potential gradient of about 20 volts/cm.; 3-4 hr. runs were employed in most cases, but with phosphate buffer it was found necessary to use overnight runs. After the runs, the gels were stained for 1 hr. with a 1% solution of amidoschwartz 10B in glycerol/water/acetic acid (50/50/20, v/v) and washed with several changes of the solvent over a number of days to remove excess dye. They were finally left in glycerol until transparent, and photographed by transmitted light.

Buffers used:

(i) pH8.6. Discontinuous buffer system of Poulik (1957)

0.07M-tris (hydroxymethyl)aminomethane (tris) -0.005M- citric acid (with or without urea for the gels.

0.03M- boric acid-0.05M- NaOH in the electrode vessels.

(ii) pH7. Phosphate buffer.

0.03M- Na_2HPO_4-KH_2PO_4 for gels (with and without 6M. urea) and for electrode vessels.

(iii) pH4.5. Acetate buffer.

0.05M- NaAc-HAc for gels and electrode vessels.

(iv) pH3.1. Formate buffer (Poulik, 1960)
0.05M - formic acid - 0.01M - NaOH for gels (with or without urea) and electrode vessels.

F. Molecular Weight Estimation of Proteins using Gel Filtration

(i) Introduction

Estimations of molecular weight of unknown proteins have been obtained by comparing their gel filtration behaviour on columns of cross-linked dextran (Sephadex) with that of proteins of known molecular weight (Andrews, 1962, 1963, 1964; Lathe & Ruthven, 1956; Burke & Ross, 1964).

Fractionation occurs when a mixture of proteins is passed through a column of Sephadex gel particles because diffusion into the gel pores is more or less restricted according to their size; they pass down the column at rates inversely proportional to the fluid volume accessible to them and thus appear in the effluent in order of decreasing molecular size (Porath & Flodin, 1963). Size and molecular weight are closely related for a homogeneous series of macromolecules (Andrews, 1964) and it has been found for a large series of globular proteins on gel filtration that the plot of effluent volume (Ve), corresponding to maximum concentration, against the logarithm of

the molecular weight gives a smooth, almost linear curve over the molecular weight range for which the gel functions optimally (Granath & Flodin, 1961; Andrews, 1962, 1963).

Serum (bovine and human) separates into three fractions on passage through columns of Sephadex G200. The first fraction represents protein completely excluded from the gel particles (α_2- and β_2-macroglobulins), the second γ-globulin, and the third serum albumin (Killander & Flodin, 1962). Sephadex G200 functions optimally for globular protein molecules between the molecular weights of 20,000 and 500,000, and almost negligible separations are obtained with globular proteins having a greater molecular weight than 500,000. Considering the first fraction to represent protein of molecular weight 500,000, and taking a molecular weight of 67,000 for the albumin fraction (Phelps & Putnam, 1960) and 160,000 for the γ globulin fraction (Phelps & Putnam, 1960), the three serum (bovine) fractions should give points on plotting Ve against log.mol.wt. which lie on a straight line. In the method described (next section) this was shown to be so, and molecular weights of proteins were estimated by measuring their elution volumes and comparing their values to those of the three serum fractions.

(ii) Method

Columns of Sephadex G200 were prepared as recommended

by Flodin (1962). Powdered Sephadex was stirred into a 1% salt solution and allowed to swell for 48 hr. Columns (1.9cm. x 45 cm.) were packed, after decantation of fines, the surface of the Sephadex protected by a filter paper disc, and equilibrated for 24 hr. with the buffer to be used in the experiment. A 1.5 - 3.0ml. sample, containg about 1 mg. of the protein dissolved in bovine serum, was applied to the top of the filter paper disc with a pipette. The column was developed at a flow rate of 10 - 12 ml./hr. with the buffer and 2.5ml. fractions collected and analysed spectrophotometrically.

In preliminary experiments it was verified that the $\alpha_2 - \beta_2$ macroglobulin fraction was completely excluded from the gel particles. The elution volume for a sample of India ink, far too large to enter the gel matrix, was found to be identical to that of the $\alpha_2 - \beta_2$ macroglobulin fraction.

(iii) Discussion

Both size and shape influence the behaviour of molecules on gel filtration (Andrews, 1962), so that molecular weight estimates obtained for proteins differing in shape from the globular standards are less accurate (Andrews, 1964). Thus, linear protein molecules are more slowly eluted and give rise to a low molecular weight estimate (Lathe & Ruthven, 1956), and proteins with a large

carbohydrate prosthetic group (or other strongly hydrated proteins), having a more expanded structure, are more rapidly eluted and give a high molecular weight estimate (Andrews, 1964). The size of some proteins too depends greatly on the pH and ionic strength; thus the molecular weight of parathyroid hormone calculated by gel filtration was found to vary from 38,000 to 85,000 depending on the experimental conditions (Rasmussen & Craig, 1962). Interaction between solute macromolecules (Andrews, 1962) or retardation of strongly basic and exclusion of strongly acid proteins by the acid groups of the gel (Andrews, 1964) would also lead to misinterpretation. However, by performing experiments in fairly high salt concentration (0.25M-NaCl) adsorption is negligible (Lathe & Ruthven, 1956) and electrostatic interaction is depressed or eliminated (Porath, 1962). Also, by repeating the experiments at a number of different pH and ionic strengths alteration in protein structure can be detected.

Molecular weight estimates obtained by the method used in the present investigations are not taken as absolute values, but rather as an indication of molecular size. The molecular weight is that of a globular protein having the same elution volume, and therefore the same effective size, as that of the protein being investigated.

(iv) Thin-layer gel filtration

A method of thin-layer gel filtration with Sephadex G200 was developed to avoid the possibility of solute interactions, and for use in cases where only small quantities of protein were available. It differs in many aspects to that developed by Johansen and Rymo (1963) for Sephadex G25 and has the advantage that the Sephadex can be recovered. Recently, somewhat similar methods have been published (Johansen & Rymo, 1964; Morris, 1964); in these also the Sephadex is not recovered.

A slurry of Sephadex G200, prepared by allowing a suspension of sephadex in buffer to settle for an hour and paring off excess liquid, was used to prepare thin layers of sephadex (0.75 mm. thick) on glass plates (20 cm. x 20 cm.) using a Desaga spreader. A flow of solvent through the layer, resting horizontally, was obtained by having two buffer reservoirs 3 cm. above and below the plate and connected to it by means of Whatman 3 MM. paper wicks. The system was allowed to equilibrate for several hours in the cold to obtain a steady flow rate through the layer. Samples (up to six) of 0.5-2.0 µl. of protein solution representing about 30 µg. of protein (or less with coloured proteins), were then applied in a straight line 2 cm. long from the paper wick as a small (3mm. diameter) spot. After

6-8 hr., by which time completely excluded protein had travelled 10-15cm., the plates were removed and covered with a piece of Whatman 3MM. paper. The paper was pressed down lightly with a glass rod, pealed off, blotted with dry 3MM. paper to remove adhering sephadex, and the coloured protein spots marked with a pencil. It was then dried at 80°, stained with a1% solution of amidoblack in MeOH/HAc/H_2O (50/20/50, v/v) for 10 mins., and washed free from excess stain with several changes of solvent.

It was later found that a more even development could be obtained using perspex trays (9cm. x 23cm.) 0.7mm. deep, and spreading the Sephadex with a glass rod.

The method was used to check the results of the column work. The serum standard and protein sample were applied as separate spots on the thin layer, eliminating the possibility of protein interaction.

Serum separated into three fractions as on columns. As noted previously, the plot of the log.mol.wt. of the three serum fractions against Ve gives a straight line curve. Ve varies inversely as the reciprocal of the distance travelled down the column and it was found empirically for the thin layers that log.mol.wt. of the three serum fractions plotted against the reciprocal of the distance travelled (measured from the centre of the spot) gave a linear plot; molecular weights of proteins estimated from

the plot agreed with those obtained on columns.

Thin layers of Sephadex G25 (medium grade), G75 (medium grade) and G100 were prepared and used in a similar manner.

A certain amount of spreading of the protein spots on obtaining the filter paper impression was unavoidable, but the centre of the spot could easily be distinguished by transmitted light.

G. Desalting and buffer changing

Columns of Sephadex G25 (medium and bead form) and G75 (medium grade) were used for desalting protein solutions and changing buffers (Flodin, 1962).

The columns were prepared as for Sephadex G200 and equilibrated with several bed volumes of distilled water (for desalting) or buffer before use. For complete desalting or changing buffers sample volumes of up to 30% of the bed volume can be employed (Flodin, 1962); protein samples of 10% of the bed volume were used to allow for imperfect packing of the columns.

H. Protein Composition

(i) Protein nitrogen

Protein samples (1-3mg.) were heated to constant weight at 105° (16 hr.), cooled in a desiccator over sulphuric acid and rapidly weighed. The nitrogen in

the sample was determined, after thorough digestion with concentrated sulphuric acid (selenium catalyst), by estimating the ammonia formed using the ninhydrin method of Jacobs (1959). A calibration curve was obtained with dried ammonium sulphate.

(ii) Amino acid composition

The protein was oxidised with performic acid by the method of Grassman (as described by Doesburgh & Hawinga, 1964) to convert methionine into methionine sulphone, and cysteine into cysteic acid, this being necessary for the thin layer separation described below.

1 volume of 30% H_2O_2 and 9 volumes of 99% formic acid were mixed and allowed to stand at room temperature for 20 min. 2ml. were then added to 11mg. of protein and to a standard mixture of 18 amino acids (5mg. each). After 15 min. the performic acid was destroyed by adding 5ml. distilled water, and the solutions freeze-dried. The standard amino acids were taken up in 0.2ml. 0.1NHCl.

The oxidised protein was hydrolysed by refluxing with 50ml. HCOOH-HCl (1/1, v/v) (Bailey, 1962). The formic and hydrochloric acids were removed by repeatedly taking to dryness on a rotary evaporator and the amino acids taken up in 0.2ml. N/10 HCl.

Amino acids were separated by two-dimensional chromatography on thin layers of silica gel g. (Fahmy, Niederwieser,

Pataki & Brenner, 1961). Plates were prepared as for the separation of lipids (III.I(iv).) and allowed to stand in the atmosphere for 1hr. before use. The mixture of standard amino acids was run at the same time as the unknown mixture, 0.5 µl. being applied to corners of the plates. Development was carried out for 15 cm. in each dimension in a tank lined with filter paper saturated with solvent.

Solvent systems;

1st direction: $CHCl_3$-MeOH-17%NH_3 (2/1/1, v/v, cleared with a few drops of methanol).

2nd direction: phenol-water (75/25, w/w) containing 20mg. of NaCN/100ml. solvent.

The plates were dried at 110° for 10 min. after development in each direction. The amino acid spots were revealed by spraying with a modified ninhydrin reagent (Moffat & Lytle, 1959), followed by gentle heating, and identified by comparison with the plate of standard amino acids and the diagrams of Fahmy et al. (1961).

Leucine and isoleucine were not separated by the solvent system used.

(iii) Tyrosine and tryptophan content

Tyrosine and tryptophan in proteins were determined spectrophotometrically by the method of Goodwin and Morton (1946).

Known amounts of protein were dissolved in 0.1N NaOH (about 0.5mg./ml.) and the absorption measured at 280 mμ and 294.4mμ . The tyrosine and tryptophan content were calculated from the equations:

$$Mtyr = (0.592\ K_{294.4} - 0.263\ K_{280})\ 10^{-3}$$

$$Mtry = (0.263\ K_{280} - 0.170\ K_{294.4})\ 10^{-3}$$

where $K_{294.4}$ and K_{280} are the extinction coefficients of the protein in 0.1N-NaOH at 294.4mμ and 280mμ, and Mtyr. and Mtry. are the gm.moles of tyrosine and tryptophan in 1gm. protein. The absorption at 280 and 294.4mμ were corrected for irrelevant absorption by extrapolating linearly to these values from between 340 and 370mμ (Goodwin & Morton, 1946).

In cases where the freeze-dried protein was not soluble in 0.1N-NaOH, solutions containing known amounts of the protein (in water or salt) were diluted with an equal volume of 0.2N-NaOH.

(iv) Protein-bound carbohydrate

(a) Hexose

Protein bound hexose was determined by the orcinol method of Winzler (1955) as modified by Johansen, Marshall and Neuberger (1960).

Lipid was removed from the protein samples by extraction in a Soxhlet apparatus with $CHCl_3$ - MeOH (2/1, v/v) for 4hr.

2-7mg. lipid-free protein were dissolved in 1ml. 0.1N-NaOH, 8.5ml. orcinol-sulphuric acid reagent (7.5 vols. 60% H_2SO_4 added with cooling to 1 vol. 1.6% orcinol in distilled water) were added and the solution heated at 80° for 15 min. After cooling the extinction was measured against a reagent blank, at 505 mμ. Protein-bound hexose was estimated as mannose from a calibration curve obtained with a standard solution of mannose (0.05-0.3 mg./ml.)

Protein blanks, with the orcinol omitted from the reagent were included with each determination.

The maximum amount of colour possible with glucose is only half developed under the above conditions (François, Marshall & Neuberger, 1963), so that if glucose was present in the protein samples, the value obtained for protein-bound hexose would be low.

(b) Protein-bound hexosamine

10-26mg. lipid-free protein were hydrolysed under reflux with 3ml. 4N.HCl. for 4 hr. to liberate the hexosamine (Johansen, et al., 1960), neutralised on a pH meter with 4N-NaOH and made up to 10ml. The solution was assayed for hexosamine by Winzler's (1955) modification of the Elson-Morgan reaction.

1ml. aliquots were mixed with 1ml. Elson-Morgan reagent (1ml. acetylacetone in 50ml.N.Na_2CO_3), heated for 15 min.

100°, cooled, 5ml. absolute alcohol added, followed by 1ml. Ehlich reagent (0.8g. p-dimethylaminobenzaldehyde in a mixture of 30ml. absolute alcohol and 30ml. conc.HCl.), and made up to 9 ml. with absolute alcohol. They were read at 530mμ after 30min. against a reagent blank. A standard curve obtained with glucosamine (0.03-0.15mg.) was used to calculate the protein-bound hexose.

In these cases investigated a 6 hour hydrolysis period resulted in no further liberation of hexosamine.

Randle and Morgan (1955) have shown that high values for hexosamine are obtained by the Elson-Morgan reaction in the presence of many amino acids (especially lysine) and hexose. Immers and Vasseur (1952) have shown that the chromogen formed by lysine and glucose condenses with Ehlich reagent to give a coloured product with maximum absorption at 570 mμ in the absence of acetylacetone (and at 530mμ in its presence). Two parallel determinations with each sample were therefore carried out, in one of which the acetylacetone was omitted from the Na_2CO_3. In no case encountered was there any significant absorption at 570mμ when the acetylacetone was not included.

(v) Lipid content

The total lipid present in freeze-dried protein samples was estimated by direct weighing of the extracted lipids (Sperry, 1955).

0.2-0.3g. freeze-dried protein was extracted for 4 hr. in a soxhlet apparatus with 100ml. $CHCl_3$-MeOH (2/1, v/v). The lipid extract was washed once with 20% of its volume of water to remove water-soluble substances, and evaporated to dryness several times on a rotary evaporator, taking up the lipid in $CHCl_3$-MeOH-H_2O (64/32/4, v/v) to decompose proteolipid (Folch and Lees, 1951). The final lipid residue was taken up in $CHCl_3$-MeOH (2/1, v/v), transferred quantitatively to a weighed specimen tube, and the solvent evaporated on a water pump. The specimen tube was transferred to a desiccator and evacuated to 0.1mm. Hg. pressure over sulphuric acid to remove any traces of water; constant weight was attained after 24hr. The gain in weight of the specimen tube represented the extracted lipid from the given weight of freeze-dried protein.

Washing of the lipid extract was carried out with water rather than dilute salt solution (Folch, Lees and Sloane-Stanley, 1957), as it was thought that the salt might accompany the lipid to some extent during the various stages, giving rise to a possible source of error in the final weighings. Also, phosphatides form stable complexes with salt which might be lost in a salt wash (Marinetti, 1962). Lysolecithin, being appreciably water-soluble, would be partially lost in the water wash (Marinetti, 1962).

I. Lipid Composition

The lipid extracts (from III.H(v).) were taken up in a known volume of $CHCl_3$-MeOH (2/1, v/v) and samples assayed for nitrogen, phosphorus and cholesterol. Samples were also used for thin layer investigation of the lipids present. The extracts probably contained considerable amounts of lipid degradation products, formed by heating and oxidation during the extraction procedure (III.H(v)).

(i) Phosphorus content

The phosphorus content of samples of lipid extracts (containing 0.02-0.08mg. phosphorus) were estimated by the method of Allen (1940), after evaporating off the solvent, thoroughly digesting with 70% perchloric acid (2ml.) on a Kjeldahl rack, diluting, and heating in a water bath for 7 min. to hydrolyse pyrophosphate.

An estimation of the percentage content of phospholipid was obtained by multiplying the percentage of lipid phosphor-us by 25 (Davenport, 1963).

(ii) Nitrogen content

The nitrogen content in lipid samples (containing 0.01-0.1mg. nitrogen) was determined, after evaporation of the solvent and thorough digestion with concentrated sulphuric acid (selenium catalyst) by the ninhydrin method of Jacobs (1959).

Choline does not give ammonia quantitatively by the above procedure (Marinetti, 1962), so that nitrogen values obtained would be low if this were present.

A nitrogen/phosphorus ratio close to unity was taken to indicate that proteolipid was not present in the lipid extracts.

(iii) Cholesterol

The amount of cholesterol and cholesterol esters present in lipid samples (containing 0.04-0.2mg. cholesterol) was determined by the method of Vahouny, Borja and Weersing (1963).

The lipid solvent was evaporated off, 2ml. glacial acetic acid added, followed by 2ml. $FeCl_3.6H_2O$ in concentrated sulphuric acid (1mg./ml.). After heating in a water bath for 3min., followed by cooling in an ice bath for 30min., the absorption at 540mμ was measured and compared with that obtained from a known amount of cholesterol.

(iv) Thin-layer chromatography

Thin-layer experiments were performed as described by Truter (1963).

Glass plates (20cm. x 20cm.) were coated to a depth of 0.25mm. with silica gel G, applied in a suspension of 30g. gel in 60ml. distilled water with a Desaga spreader. The plates were activated by drying at 100° for 30min., and stored in a dessicator over $CaCl_2$.

1-10 µl. quantities of lipid extracts (containing 1-5mg. lipid) were spotted onto the plates in the conventional manner and allowed to develop in equilibrated tanks (lined with solvent-saturated filter paper) until the solvent front had travelled 10cm. The plates were dried in the atmosphere and sprayed to detect the lipids present.

Solvents: (a) for phospholipids

(i) $CHCl_3$-MeOH-H_2O (80/30/3, v/v). This is a modification (Widdas, 1963) of the volumes used by Vogel (1962) and gave a better separation of the lipids encountered.

(ii) $CHCl_3$-MeOH-HAc-H_2O (25/15/4/2, v/v), (Skipski, Peterson and Barclay, 1964).

With both the above solvents neutral lipid travelled at the solvent front.

(b) for neutral lipids

pet.ether (40-60)/diethylether/glacial acetic acid (60/40/1, v/v). This is a modification (Widdas, 1963) of the volumes used by Morgan (1963)

Lipid detecting reagents (Skipski et al., 1962)

(a) for phospholipids

(i) unsaturated lipids were detected by placing the plates in a closed box containing iodine crystals. The unsaturated lipids stained yellow.

(ii) phospholipids with a free amine group were revealed as red-violet spots by spraying plates with ninhydrin (0.2% in butanol saturated with water) and heating for 5 min. at 100-105°.

(iii) all lipid material was shown up as blue spots by spraying with ammonium molybdate-perchlorate reagent followed by heating at 80° for 10min.

The three detection methods above were used consecutively.

(b) for neutral lipids

(i) iodine stain (as above).

(ii) cholesterol and cholesterol esters were revealed as red spots by spraying with phosphotungstic acid and heating (Randerath, 1963).

(iii) ammonium-molybdate perchloric acid spray (as above).reagents

Reagents (i), (ii) and (iii) were used consecutively.

CHAPTER IV

CRUSTACYANIN, THE CAROTENOPROTEIN OF LOBSTER CARAPACE

Introduction

The blue lobster shell pigment was first extracted by Newbigin in 1878 with dil.HCl. and NH_4Cl. solutions. She noted its precipitation by $(NH_4)_2SO_4$, changes in colour on treatment with alcohol, acetone, acids and alkalis, and the "contamination" of the extracts with protein. She believed the pigment to be "the result of combination between a complex organic base and a lipchrome".

Kuhn and Sörensen (1938) proposed that the blue colour of the shell arose through binding of the red astaxanthin to protein, but they failed to extract it. Later, extraction of the pigment with citric acid (Wald et al., 1948) and dil.HCl. (Goodwin and Srisukh, 1949) was reported.

Wald et al. (1948) named the pigment crustacyanin and showed that it was precipitated at 40% $(NH_4)_2SO_4$ saturation, had an absorption maximum at 625mμ, and had an isoelectric point below pH4.5. They also demonstrated reversible changes in spectrum on heating solutions in barbitone buffer at 60° (absorption maximum, 460mμ) and on lowering the pH below 4.3 (absorption maximum, 412mμ).

Near the conclusion of the present studies, it was brought to my notice that Jencks and Buten (unpublished observations, 1964) had fractioned EDTA extracts of lobster shell by DEAE-cellulose salt gradient chromatography into yellow (absorption maximum at 410mμ), purple (absorption maximum at 590mμ), and blue (absorption maximum at 320, 370 and 630 mμ, in salt solution and 590mμ in water), the last two being named α- and β-crustacyanin respectively. They have shown that α-crustacyanin undergoes denaturation to a red product (absorption maximum at 460mμ) in the presente of "hydrophobic" denaturants and to a yellow product (absorption maximum at 390mμ) in the presence of denaturing agents of the urea-guanidinium class; that the effects with urea are time and concentration dependent, and reversible (if not in contact with urea too long) on dialysis; that acids and bases cause reversible denaturation to yellow and red products, and that non-ionic detergents only altered α-crustacyanin in the absence of salt. They have suggested that "the observed changes probably involve changes in the interaction of the carotenoid with protein, which result from changes in conformation and/or the state of aggregation of the apoprotein induced by denaturing agents", and believe α-crustacyanin to be high molecular.

Experimental

A. (i) Extraction procedures

Lobsters, about 1 lb. in weight, were killed by freezing at -15° in the deep freeze and the shell removed. The blue coloured parts of the shell were washed free from hypodermis and the adhering chitinous membranes pealed off. After blotting dry, the shell was ground to a powder in a coffee-grinder and passed through a 100-mesh sieve.

In early experiments powdered shell was extracted with dilute citrate buffer (Wald, et al., 1948). It was later found that the pigment could be extracted more simply and with less degradation by EDTA.

All extraction and purification procedures were performed in the dark at 4°.

(a) 50g. powdered shell was extracted with stirring with 250ml. cold 0.2M-citrate buffer pH5.5. Extraction was allowed to continue until the pH of the solution reached 6.8 (about 15min.). The shell was then centrifuged down at 3,000 r.p.m. and re-extracted with further 250ml. portions of citrate buffer until the supernatant on centrifugation was almost colourless. A total of about 2l. citrate buffer was required. Solid $(NH_4)_2SO_4$ was added to the pooled extracts to 50% saturation, and the precipitated chromoprotein collected on a Buchner funnel

over a layer of supercel. After thorough draining, the protein was washed through with 100-200ml. 0.05M- phosphate buffer, pH7.

(b) 50g. powdered shell was extracted with 2l. 10% EDTA, pH7.5, overnight with continuous stirring, and the extracted chromoprotein obtained in 0.05M- phosphate buffer, pH7, as in the previous extraction procedure.

(ii) Spectrum of extract

The absorption spectrum of the solution (Fig.I) showed maxima at 630, 410, 370 and 280mμ with inflexions at 320 and 605mμ. The magnitude of the peak at 410mμ and the inflexion at 605mμ varied with different preparations, being more marked in the citrate extracts than the EDTA extracts. The value of E280/630 varied from 0.8 to 3.0 for different preparations.

(iii) Separation of chromoproteins

Method DEAE-cellulose chromatography was employed using a salt gradient (III B.), with 90ml. 0.05M-phosphate buffer, pH7, in the mixer and 90ml. 0.05M-phosphate buffer containing 8% NaCl in the reservoir. The DEAE-cellulose column was thoroughly equilibrated with 0.05M-phosphate buffer, pH7, before passage of the citrate extract (from IVA$_{(ii)}$a.). Optical densities of the fractions were measured at 280, 410 and 630mμ. Cellulose acetate and starch gel electrophoresis were carried out as described in (IIIE).

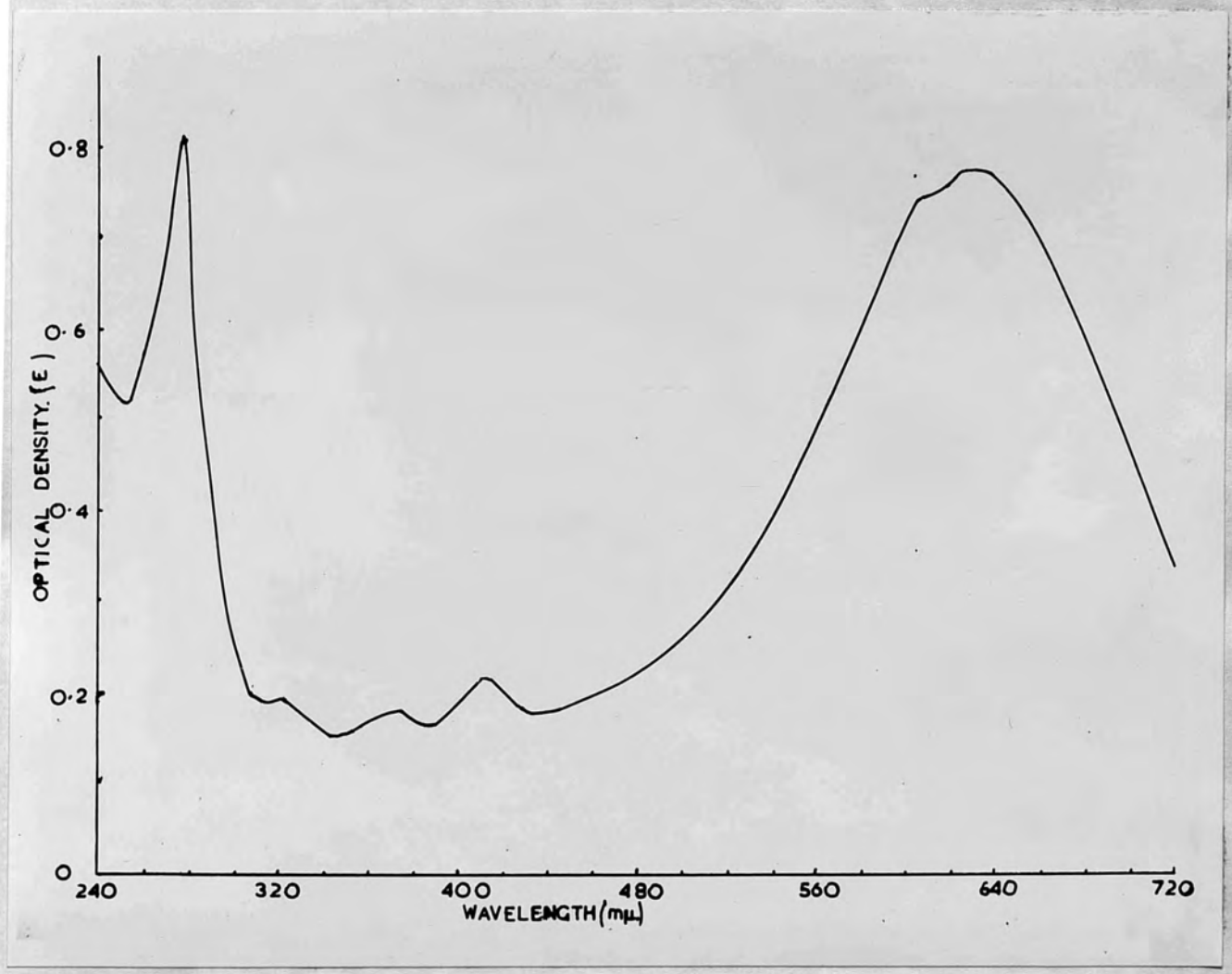


Figure 1. Spectrum of the chromoproteins obtained from a citrate extract of lobster shell, precipitated by 50% $(NH_4)_2SO_4$, and dissolved in 0.2M-phosphate buffer, pH7.

Results The shell extract was resolved into purple, yellow, blue and colourless proteins (Fig. 2).

A purple fraction with maxima at 278, 360 and 585mμ with an inflexion at 315mμ, and a value of $E_{280/585}$ (Fig.3) passed straight through the column in the 0.05M. phosphate buffer. It was heterogenous in cellulose acetate electrophoresis at pH7, giving two purple, barely separated components and a fainter, faster migrating purple component (Fig. 4). At least four purple components were also evident in starch gel electrophoresis at pH7. The purple material, identical with the β-crustacyanin of Jencks and Buten (1964) represented about 15% of the total protein extracted with citrate, but less than about 5% of that extracted with EDTA.

The remainder of the chromoprotein was adsorbed on the DEAE-cellulose column as a blue zone beneath a more strongly held yellow zone. Elution with the salt gradient resulted in the separation of at least 6 components (numbered 1 to 6 in Fig. 2). Components 4 and 5 were yellow and had the spectrum shown in Fig.3., with maxima at 412 and 630mμ, and an inflexion at 480mμ. The yellow chromoproteins were obtained on EDTA extraction.

Component 2, the α-crustacyanin of Jencks and Buten (1964), had the spectrum shown in Fig. 14. with maxima at

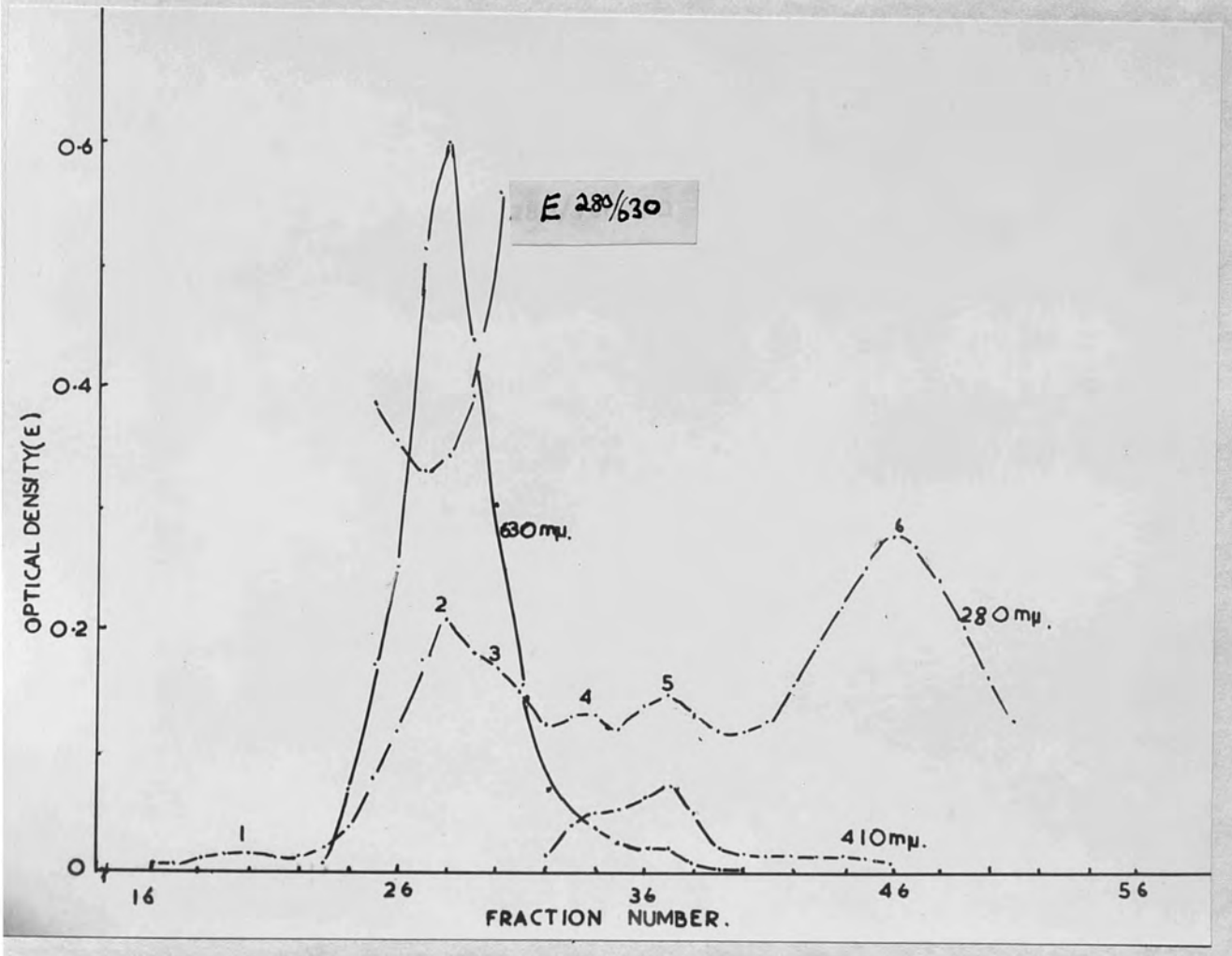


Figure 2. DEAE-cellulose salt-gradient chromatography of a citrate extract of the carapace of Homarus vulgaris.

Bed volume: 13.0 cm. x 1.5cm.
Flow Rate: 1.5ml./min.
Gradient: reservoir, 0.05M-phosphate buffer, pH7 containing 8% NaCl.
mixer, 0.05M-phosphate buffer, pH7.
Hydrostatic pressure: 15cm. buffer.
Fraction volume: 2.5ml.

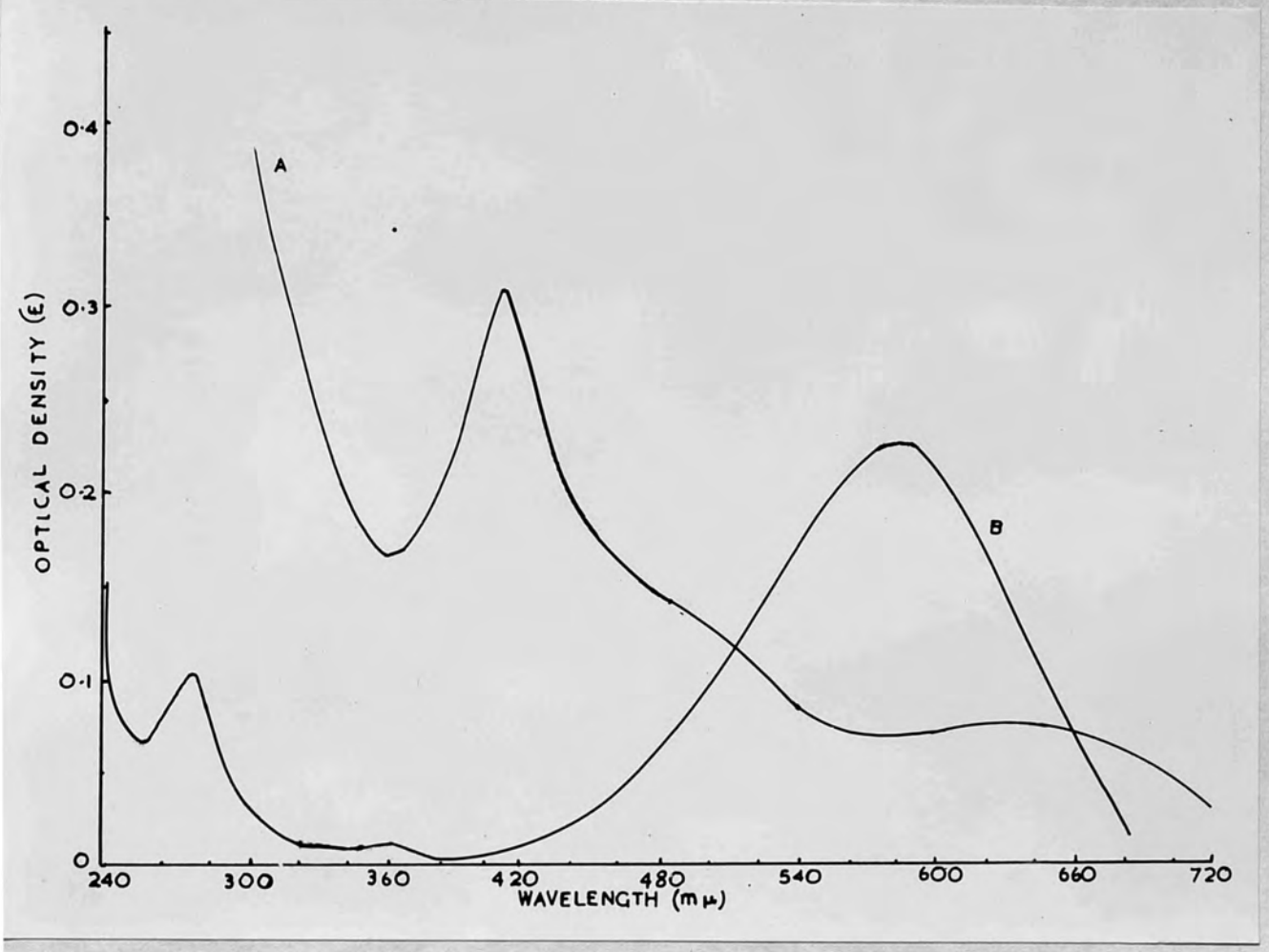


Figure 3. Spectrum of A. yellow chromoprotein and B. β-crustacyanin separated from citrate extracts of lobster shell by salt-gradient DEAE-cellulose chromatography.

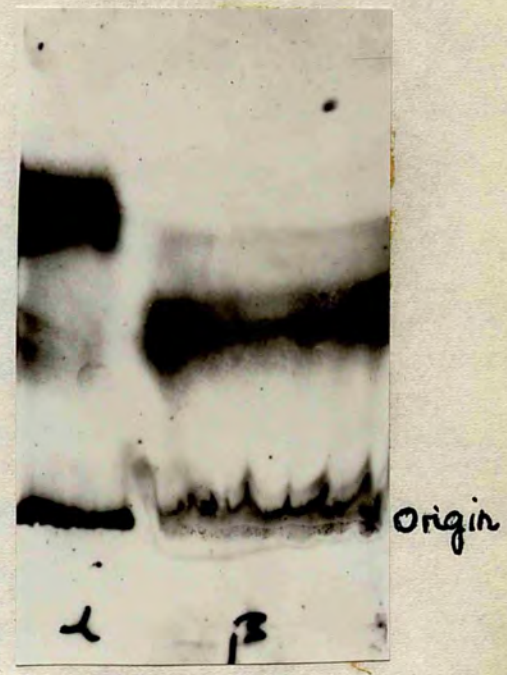


Figure 4. Cellulose acetate electrophoresis

Rt.-hand column, β-crustacyanin

lft.-hand column, purified α-crustacyanin, desalted using Sephadex G75, and freeze-dried.

Buffer: 0.05M-phosphate, pH7.

Current: 0.2mA/5cm. width strip

Time of run: 7 hr.

Figure 5. Cellulose acetate electrophoresis

α-crustacyanin purified by DEAE-cellulose salt gradient chromatography. Fractions 29-31 in Figure 2.

278, 320, 370 and 632.5mμ, and barely noticeable inflexion at about 600mμ. It was not completely separated from component 3 as the value of $E_{280/630}$ was not constant over the fractions in which it was eluted (Fractions 25 to 31). The pooled fractions showed the presence of a colourless protein (Fig. 5) in cellulose acetate electrophoresis. The value of $E_{280/630}$ for the pooled fractions was found to be 0.34, so that pure preparations of α-crustacyanin must give a value less than this.

B. α-Crustacyanin (Jencks and Buten, 1964)

(i) Purification

α-crustacyanin was obtained in a state homogeneous to cellulose acetate electrophoresis by a combination of calcium phosphate gel chromatography, fractional ammonium sulphate precipitating and stepwise elution from DEAE-cellulose. The purpose of the first two procedures was to remove as much impurity as possible, so that the DEAE-cellulose chromatography could be performed more efficiently. Fractional ammonium sulphate precipitation without prior gel adsorption did not result in a homogeneous protein.

(a) Calcium phosphate gel chromatography

The crude crystacyanin solution from $IVA_{(i)}$.b. was made up to 250ml. with distilled water to dilute the

ammonium sulphate present. 10ml. calcium phosphate gel suspension were added with stirring, the pH adjusted to 5.5 with dilute acetic acid, and the gel centrifuged down at 3,000 r.p.m. for 5 min. The gel, coloured bluish-green, was discarded and a further 80ml. gel suspension added to the supernatant with readjustment of the pH to 5.5. Further gel suspension (10ml. at a time) was added with readjustment of pH, until the supernatant on allowing the gel to settle was no longer blue. The gel, coloured deep blue, was centrifuged down and the supernatant, coloured light purple (with a spectrum identical with that of β - crustacyanin) was discarded. The gel was washed with 250ml distilled water on the centrifuge and the crustacyanin eluted from the gel by incubation for 20min. with an equal volume of 0.2M. phosphate buffer, pH7, followed by centrifugation. Two such elutions removed the greater part of the crustacyanin from the gel.

The $E_{280/630}$ value of the eluted material lay between 0.5 and 0.8 for different experiments, and could be slightly decreased by a repetition of the purification procedure; this was found to be of no advantage in the further purification steps employed.

(b) Ammonium sulphate fraction

Method Salting-out curves (III.C.) on the calcium

phosphate gel eluant were used to test the homogeneity of the preparation. The supernatant was examined spectrophotometrically at 280, 460 and 630mμ.

Results Ammonium sulphate fractionation revealed the presence of two major fractions (Fig. 6). The first, brought down between 22.5 and 32.5% saturation, partially redissolved and had the spectrum shown in Fig. 7. It represented 15-20% of the original 280mμ adsorption and evidently contained altered material in which the prosthetic group had become partially detached from the protein, as well as some α-crustacyanin and colourless protein.

The second fraction, precipitating between 32.5 and 45% $(NH_4)_2SO_4$ saturation contained the α-crustacyanin. The value of $E_{280/630}$ for this fraction lay between 0.4 and 0.5, so that it was still appreciably contaminated with other proteins.

The following procedure was therefore adopted for the fractionation of the material eluted from the calcium phosphate gel: A saturated $(NH_4)_2SO_4$ solution was added to the crude crustacyanin solution in 0.2M- phosphate buffer to bring it to 32.5% saturation; after standing for an hour, the solution was centrifuged at 13,000 r.p.m. for 30min. and the precipitate discarded. Further saturated

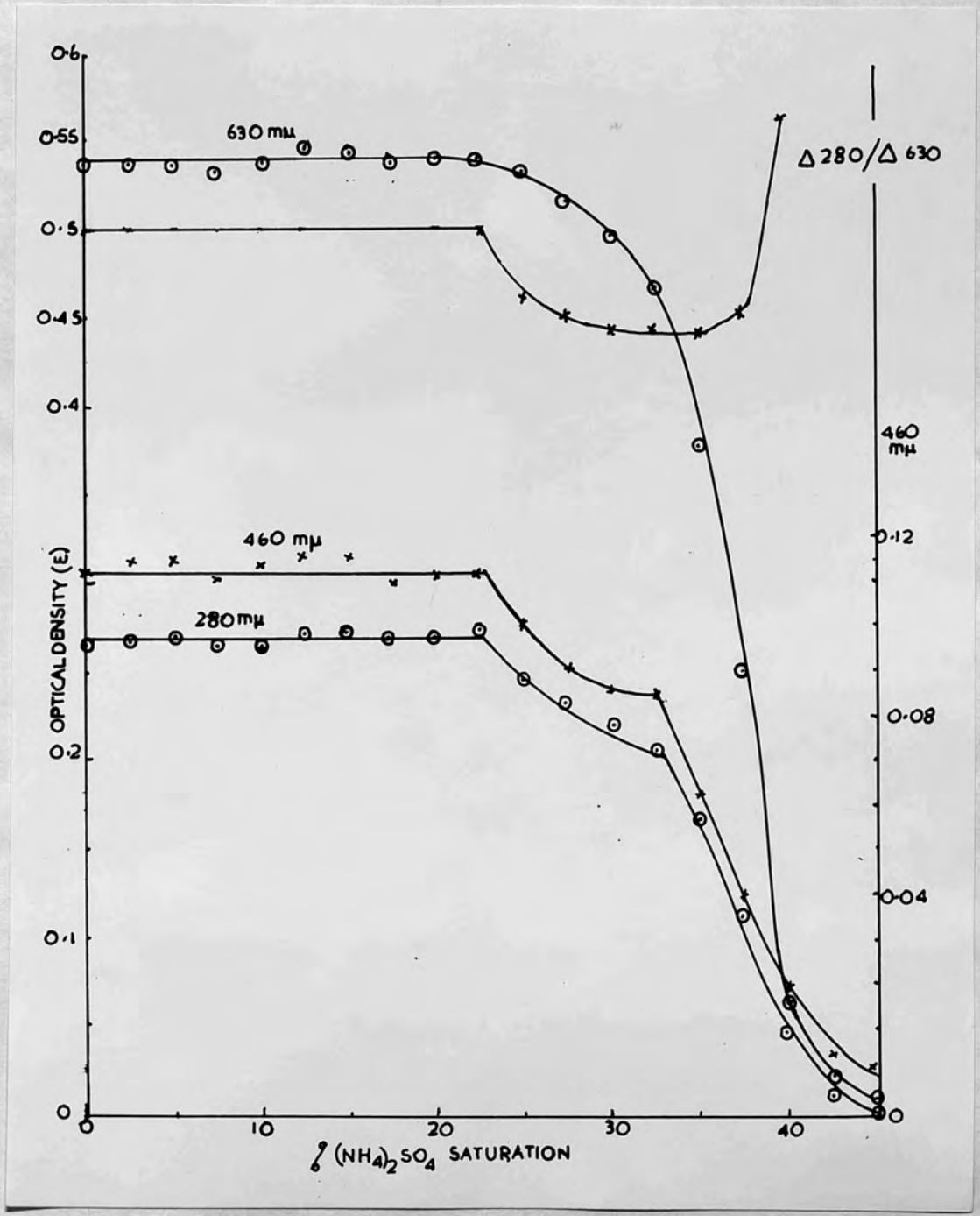


Figure 6. $(NH_4)_2SO_4$ saturation curve of crustacyanin, purified by calcium phosphate gel chromatography.

Temperature: 4°
Buffer: 0.1M-phosphate buffer, pH7.
Concentration: 0.15mg./ml.

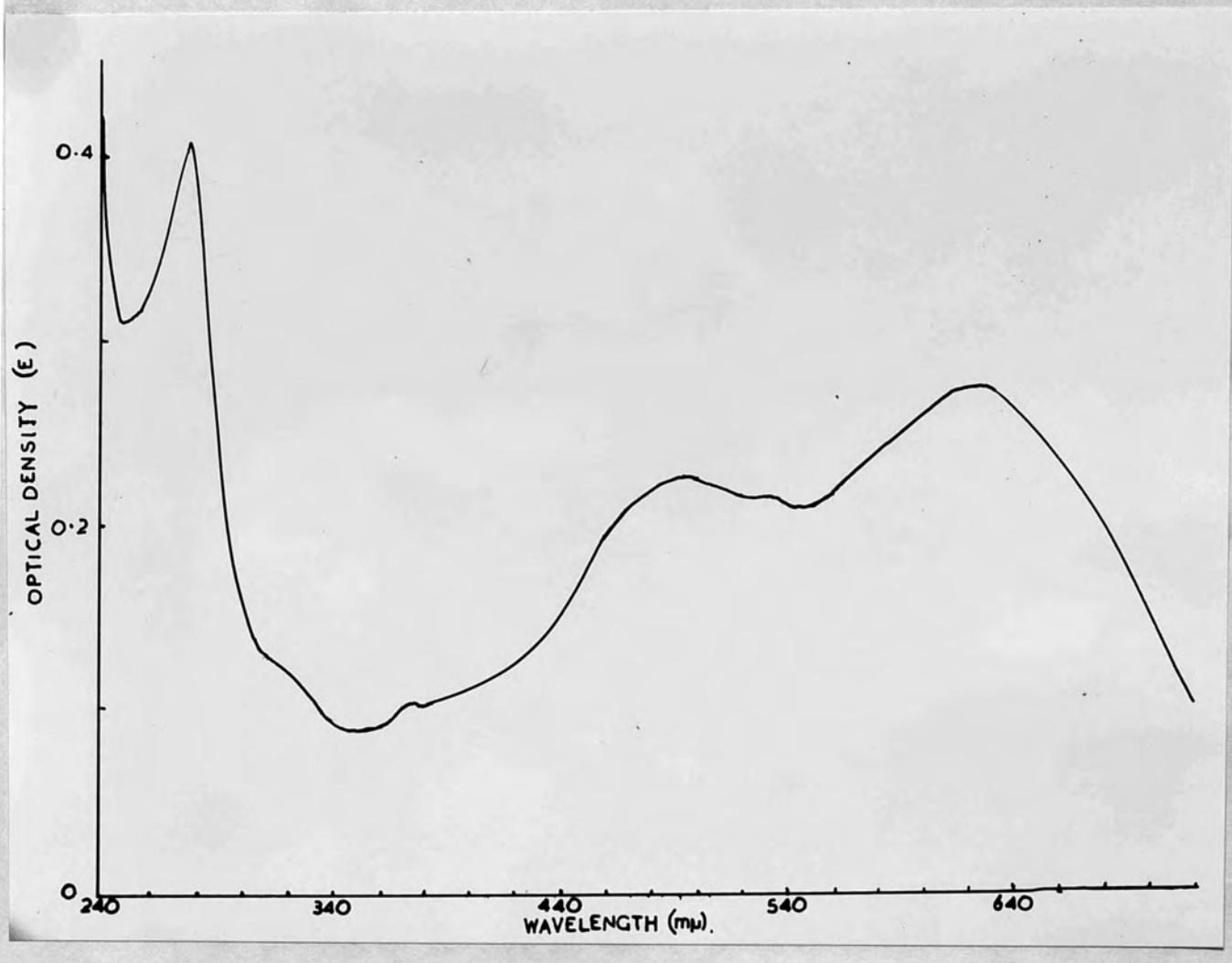


Figure 7. Spectrum of the chromoproteins of lobster carapace in 0.2M-phosphate buffer, pH7, purified by calcium phosphate gel chromatography and precipitated between 0-32.5% $(NH_4)_2SO_4$ saturation.

ammonium sulphate solution was added to the supernatant to bring it to 45% saturation, the solution centrifuged, and the precipitated crustacyanin taken up in 50ml. 0.05M-phosphate buffer, pH7.

(c) DEAE-cellulose chromatography

Method Stepwise salt-gradient elution from DEAE-cellulose was carried out as described previously (III.B.). The crude crustacyanin was adsorbed onto DEAE-cellulose from the 0.05M-phosphate buffer, the column washed with 150ml. of 0.15M-phosphate buffer, pH7, and the α-crustacyanin eluted with 0.25M.phosphate buffer, pH7.

Results A small amount of β-crustacyanin (Fig.3) passed straight through the column in the 0.05M-phosphate buffer. Washing with 0.15M-phosphate buffer removed further impurities. This treatment decreased the capacity of the ion-exchanger and the blue crustacyanin spread out into a wide band.

The α-crustacyanin (Fig.14) was eluted as a narrow band, leaving the top of the column coloured red. Fig.8a shows the result of a typical experiment; the value of $E_{280/630}$ was constant at 0.31 over the fractions (3 to 5) in which the greater part of the crustacyanin was eluted. The fractions in which the tail of the crustacyanin band was eluted had increasing $E_{280/630}$ values, as a colourless

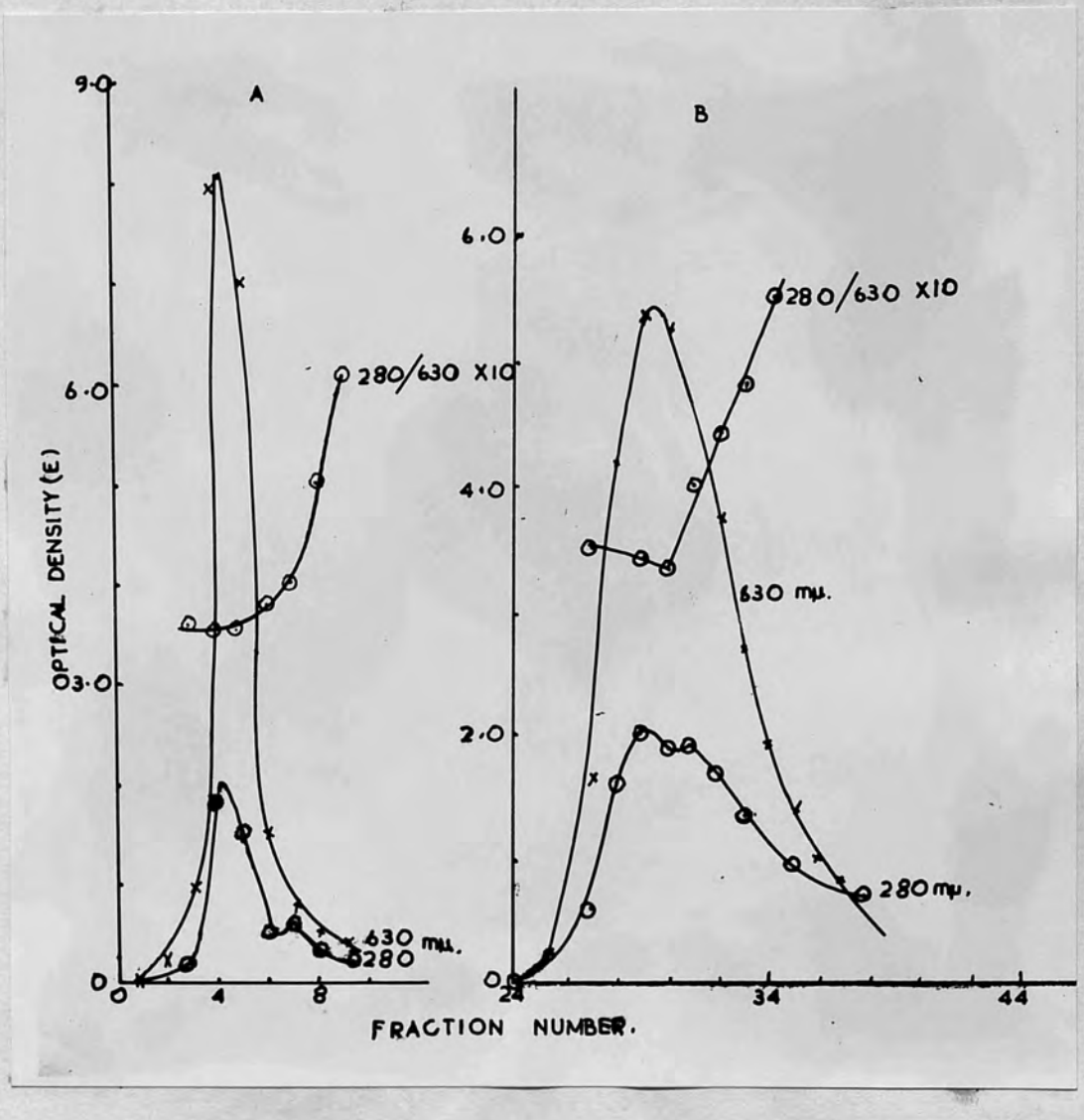


Figure 8. DEAE-cellulose chromatography of α-crustacyanin, purified by calcium phosphate gel chromatography and fractional $(NH_4)_2SO_4$ precipitation.

Flow rate: 2.0ml./min.
Fraction volume: 2.5ml.
A. Stepwise elution
developing solvent 0.25M-phosphate buffer, pH7.
B. Salt-gradient elution.
Gradient: reservoir, 0.05M-phosphate buffer, pH7 containing 4%NaCl.
mixer, 0.05M-phosphate buffer, pH7.

protein band moving down the column in finite adsorption equilibrium was eluted. Repetition of the chromatographic procedure with the pooled fractions (3 to 5) gave no further decrease in the $E_{280/630}$ value, but resulted in the formation of further β-crustacyanin.

Stepwise elution using a fairly long bed length (15-20cm.) was found to be more satisfactory than the more laborious gradient elution. Figure 8b. shows the results of the same experiment but performed with a salt gradient. The α-crustacyanin was eluted over a large number of fractions and there was no significant separation from the colourless impurity, although a flatter salt gradient might have given a better resolution.

(ii) Homogeneity

Method Cellulose acetate and starch gel electrophoresis were used to test the homogeneity of preparations (III.E.).

Results α-crustacyanin purified as above, with $E_{280/630}$ = 0.31, was homogeneous in cellulose acetate electrophoresis at pH4.5, 7 and 8.6 (Fig. 9a, b and c). The cellulose acetate strips did not give a positive stain for carbohydrate.

Similarly, such preparations showed a single component in micro-electrophoresis on starch gel at pH7 (Fig. 10a). At pH4.5 the crustacyanin moved very slowly into the gel but no heterogeneity was observed. The discontinuous

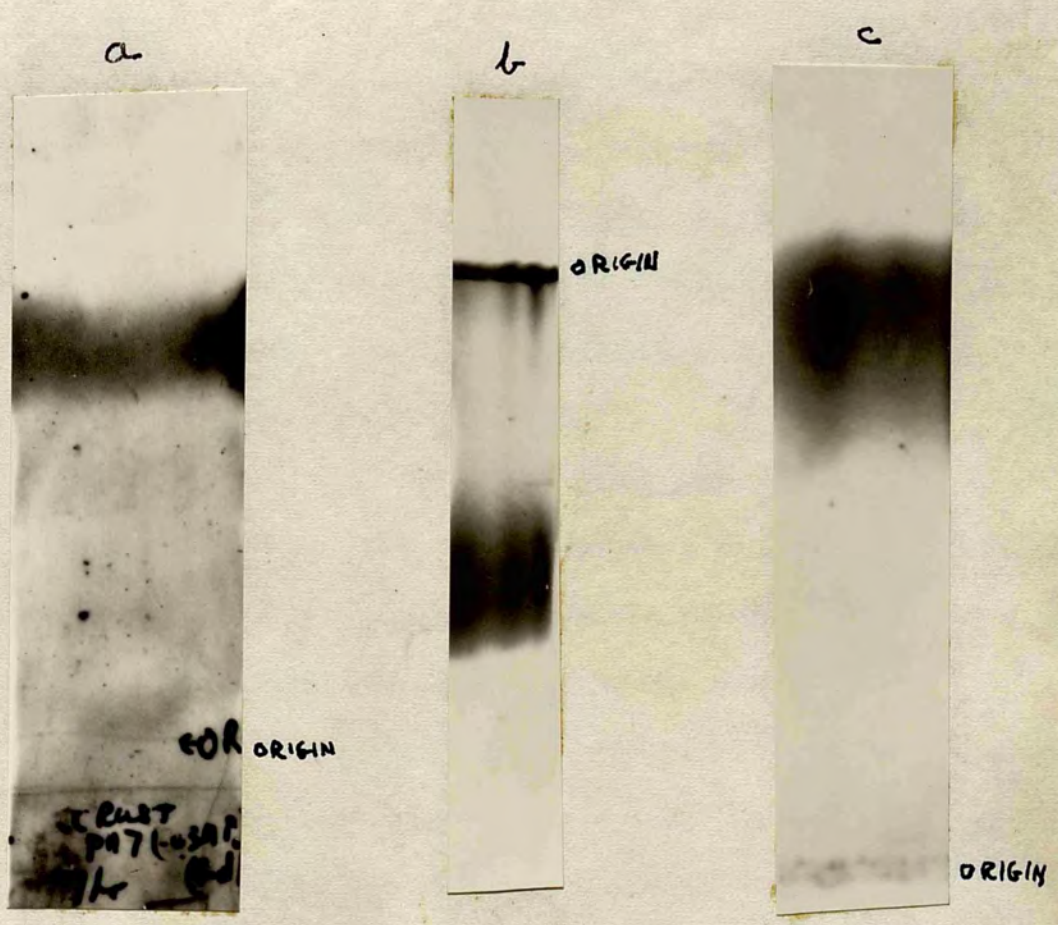


Figure 9 Cellulose acetate electrophoresis of crustacyanin.
0.2mA/5cm. width strip
(a) 0.05M-phosphate buffer, pH7. 7hr. run.
(b) 0.05M-acetate buffer, pH4.5. 5hr. run.
(c) 0.07M-barbitone buffer, pH8.6. 5hr. run.

buffer system of Poulik (1960) caused partial breakdown of α-crustacyanin and gave a number of faster moving purple or purple-red bands besides the blue α-crustacyanin band (Fig.10c). An overnight run in the same buffer system gave the same pattern (Fig. 10b), with diffuse spreading of the main band in the direction of migration.

(iii) Isolectric point

Method The pH between which the direction of migration of the crustacyanin was reversed were determined by cellulose acetate electrophoresis (III.$E_{(i)}$.). Five hour runs were carried out using a series of 0.05M-acetate buffers with pH between 4 and 5. A sample of a dilute solution of Carbowax was applied adjacent to the crustacyanin sample to show up electro-osmotic flow; on drying the cellulose acetate strip, the Carbowax showed up as a shining white band.

Results The direction of migration of α-crustacyanin was reversed between pH4.3 and 4.7. The isoclectric point therefore lies between these two pH values in 0.05M-acetate buffer.

(iv) Preparation of freeze-dried protein

(a) After removal of salt by dialysis

Method A solution containing 10-20 mg. of crustacyanin, homogeneous in cellulose acetate electrophoresis, was dialysed against 5l. cold distilled water for 4 days, with

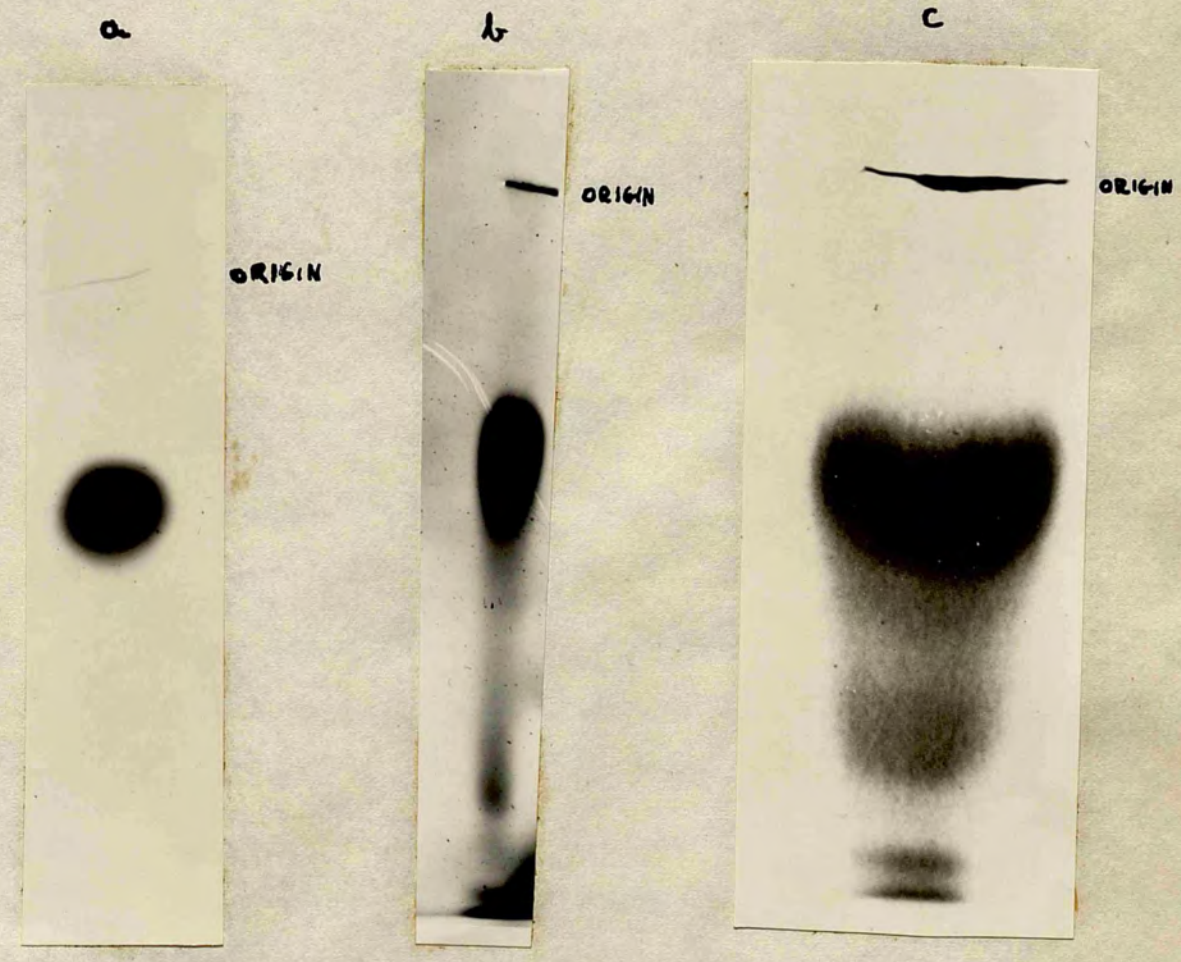


Figure 10 Starch gel electrophoresis of α-crustacyanin.

(a) 0.03M-phosphate buffer, pH7. 20V/cm. 16hr run

(b) Tris-citrate-borate, discontinuous buffer system, pH8.6. 7V/cm. 16hr. run.

(c) Tris-citrate-borate, discontinous buffer system, pH8.6. 20V/cm. 3hr. run.

changes of water twice daily; the solution was then placed in a beaker, frozen at -15°, and freeze-dried in a dessicator at 0.2mm. Hg.pressure over conc.H_2SO_4 containing a spatula end of $K_2Cr_2O_7$. Light was rigorously excluded during the freeze-drying. The homogeneity of the freeze-dried preparations were tested by cellulose acetate electrophoresis (III.$E_{(i)}$.).

Results Dialysis resulted in a change of colour of the crustacyanin solution from blue to purple, and the spectrum became identical to that of β-crustacyanin (on prolonged dialysis).

The reddish freeze-dried preparations were heterogeneous to cellulose acetate electrophoresis at pH7. Four purple bands, before staining, were observed and the faster moving α-crustacyanin was absent or faintly visible (Fig. 11). The relative intensity of the purple bands varied from preparation to preparation depending on the extent of dialysis and on the concentration of the solution dialysed. In thoroughly dialysed preparations the least mobile purple component was the main component, but where complete breakdown of α-crustacyanin had not occurred the most rapid component was as intensely stained. (Fig. 11, column I).

Figure 11 Cellulose acetate electrophoresis.

0.05M-phosphate buffer, pH7, 5 hr. run.

3 different preparations of α-crustacyanin dialysed for 4 days against distilled water, and freeze-dried.

Figure 12 Cellulose acetate electrophoresis.

0.05M-phosphate buffer, pH7. 6½ hr. run.

Column 1 α-crustacyanin, freeze-dried after desalting using Sephadex G75.

Column 2 α-crustacyanin, desalted using Sephadex G75, allowed to stand for 12 hr. and freeze-dried.

(b) <u>After removal of salt by gel filtration</u>

<u>Method</u> Homogeneous crustacyanin preparations were desalted by passage through columns of Sephadex G75 (III.G.), frozen immediately and freeze-dried as in section a.

<u>Results</u> The crustacyanin band changed from blue to purple as it moved down the Sephadex column; its spectrum became similar, but not identical, with that of β-crustacyanin (Fig.14) (see later IV.$B_{(ix)}$a.).

The freeze-dried crustacyanin still showed traces of the four purple bands in electrophoresis at pH7 (Fig. 12, column 1). The purple bands corresponded in position to those obtained with β-crustacyanin (Fig.4). When the desalted solution was allowed to stand at room-temperature overnight, only the four purple components were evident on electrophoresis. (Fig. 12, column 2) and the relative intensities of the bands were different from those of the thoroughly dialysed preparation. Two of the purple bands predominated.

In later experiments Sephadex G25 were used to desalt protein solutions. It was found that part of the blue crustacyanin precipitated after passing a few centimetres into the gel while a small purple fraction remained in solution and moved rapidly through the gel. Two bands

were thus formed, a blue precipitated band behind a purple band. As the blue band moved down the column it spread out and went back into solution, turning purple at the same time. Two purple fractions, identical in spectrum (Fig. 14), were recovered in the eluant.

(c) From ammonium acetate

Method Freeze-dried preparations were obtained from a volatile buffer. Samples of crustacyanin were obtained in ammonium acetate solutions pH6.5 of varying molarity (0.001-0.1M) using Sephadex G25 (fine grade) (III.G.). They were then frozen and freeze-dried over conc.H_2SO_4 and powdered NaOH. The homogeneity of the freeze-dried preparations were tested by cellulose acetate electrophoresis (III.$E_{(i)}$.).

Results Freeze-dried preparations obtained from the blue, more concentrated NH_4Ae solutions (0.025-0.1M.) showed the presence of two more slowly migrating purple bands besides the main blue α-crustacyanin band. Only faint traces of these two components were obtained from the less concentrated purple NH_4Ae solutions (0.005-0.01M). The freeze-dried preparations obtained from 0.003M-NH_4Ae were homogeneous in cellulose acetate electrophoresis; this method was used in further preparations of the freeze-dried protein (cf. Fig. 13).

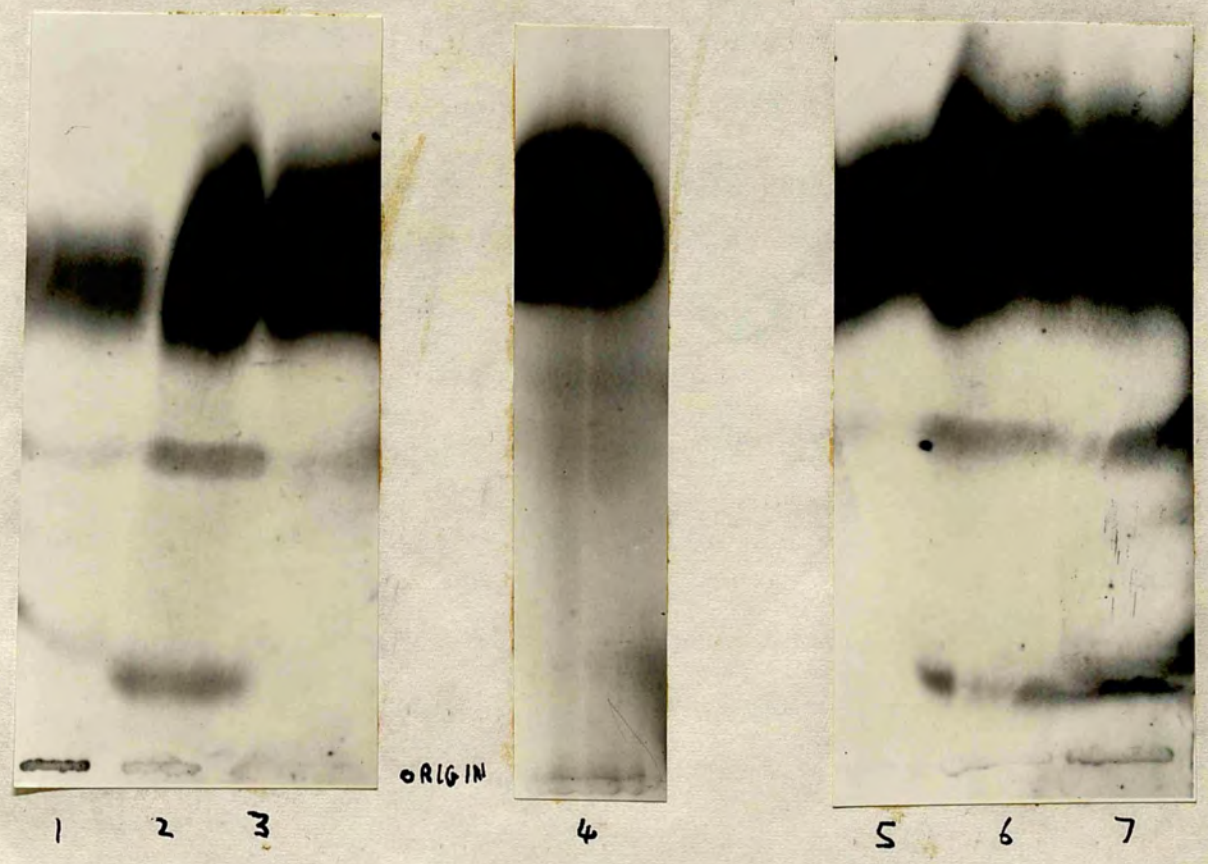


Figure 13 Cellulose acetate electrophoresis
0.2mA/5cm. width strip. 6 hr. run.

α-crustacyanin, freeze-dried from NH_4Ac:

1. 0.033M
2. 0.1M
3. 0.01M
4. 0.003M
5. 0.005M
6. 0.025M
7. 0.05M

The α-crustacyanin was precipitated on columns equilibrated with NH_4Ac solutions of 0.001M. and less.

(v) Properties of the freeze-dried protein

The fluffy purple-red freeze-dried protein dissolved completely in water to give a purple solution (spectrum, Fig.14). It dissolved in 0.05M. salt solution to give α-crustacyanin with unaltered absorption spectrum (Fig.14), having a value of $E^{0.1\%}_{280}$ and $E^{0.1\%}_{632.5}$ of 1.15 and 3.7 respectively.

It was unchanged on storage at 4° over anhydrous $CaCl_2$ in a dark bottle after several months. After exposure to bright sunlight for a week at room-temperature the protein only partially dissolved forming a purple-red solution. The freeze-dried preparation was hygroscopic taking up about 7% moisture in 5 min., and 12% within 30min. on exposure to the atmosphere.

A total yield of 1mg. freeze-dried protein per g. powdered shell was obtained.

(vi) Crystallisation

Method A solution of freeze-dried crustacyanin (2mg./ml.) in 0.2M-phosphate buffer, pH7, containing 10^{-3}M NaF to inhibit bacterial growth, was brought to 30% $(NH_4)_2SO_4$ saturation by the addition of a sat.$(NH_4)_2SO_4$ solution. 5ml. were placed in small specimen tubes, 2cm. in diameter

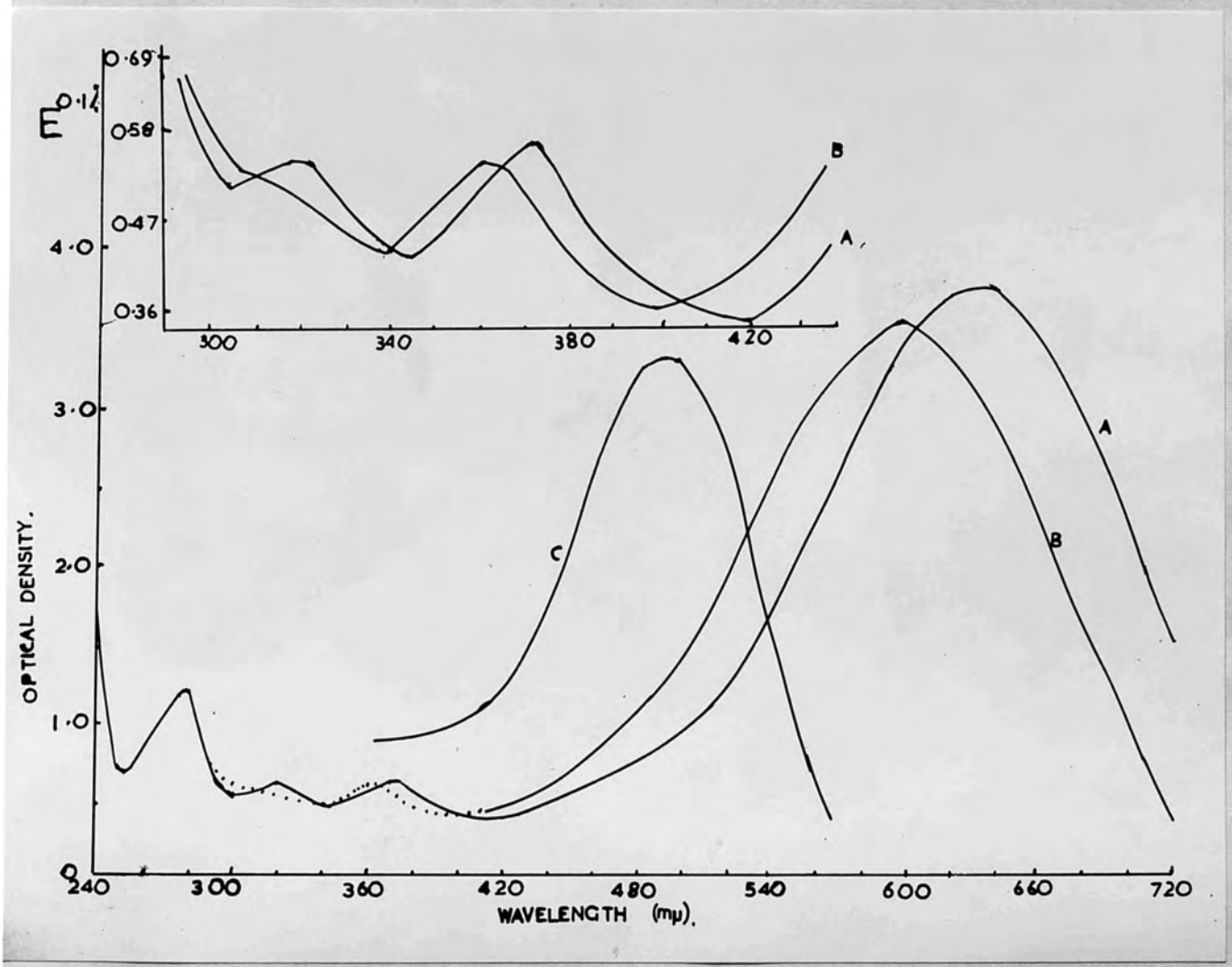


Figure 14. Spectrum of α -crustacyanin.

A. In 0.2M-phosphate buffer, pH7.
B. In distilled water.
C. In 90% pyridine.

and 4.5cm. high and allowed to stand for several days in a dark cupboard at room-temperature.

Results Microscopic, dark blue, hexagonal plates were formed after standing for about 5 days. In some attempts at crystallisation, the crystals tended to form three-dimensional clusters of plates joined along one edge, which were difficult to photograph. The crystals were extremely fragile, movement of the cover-slip distorting their shape; the corners of the hexagon tended to become rounded (Fig.15).

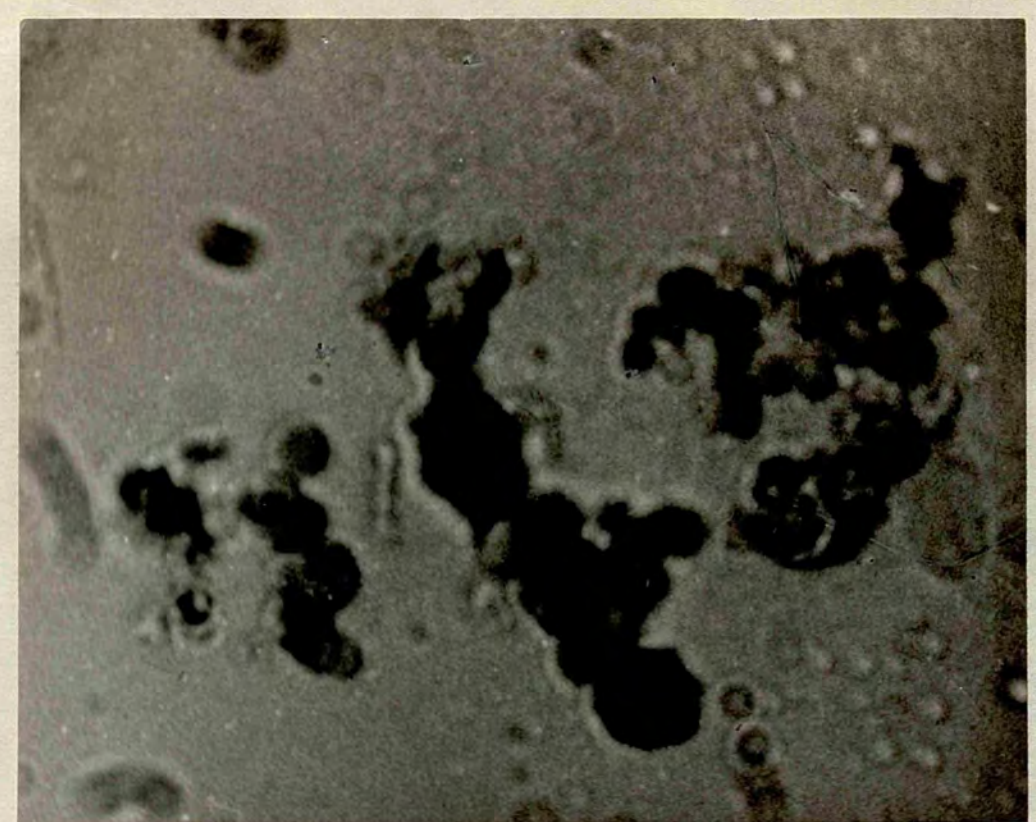
Crystallisation was also attempted by dialysis against a 40% sat.$(NH_4)_2SO_4$ solution in the cold, but this was unsuccessful.

Attempts to crystallise β-crystacyanin failed.

(vii) Minimum molecular weight

Method The minimum molecular weight of crustacyanin was determined by the method of Cheesman (1958). In this method the amount of astaxanthin in a known weight of protein is determined spectroscopically. The molar extinction coefficient (base 10) of astaxanthin is taken as 115,000 cm^2.

10mg. freeze-dried curstacyanin was dissolved in 10ml. distilled water. 1ml. samples were diluted with pyridine to 10ml. this dissociates the astaxanthin from the protein but keeps the latter in solution. The



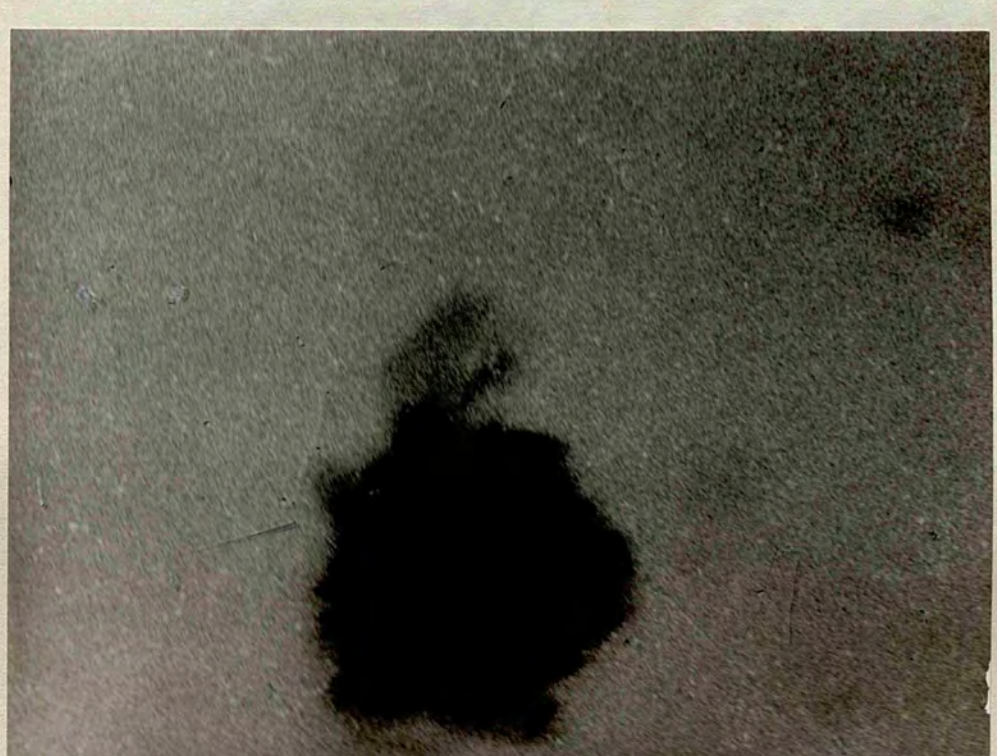


Figure 15. Crystals of α-crustacyanin
Magnification 3,600.

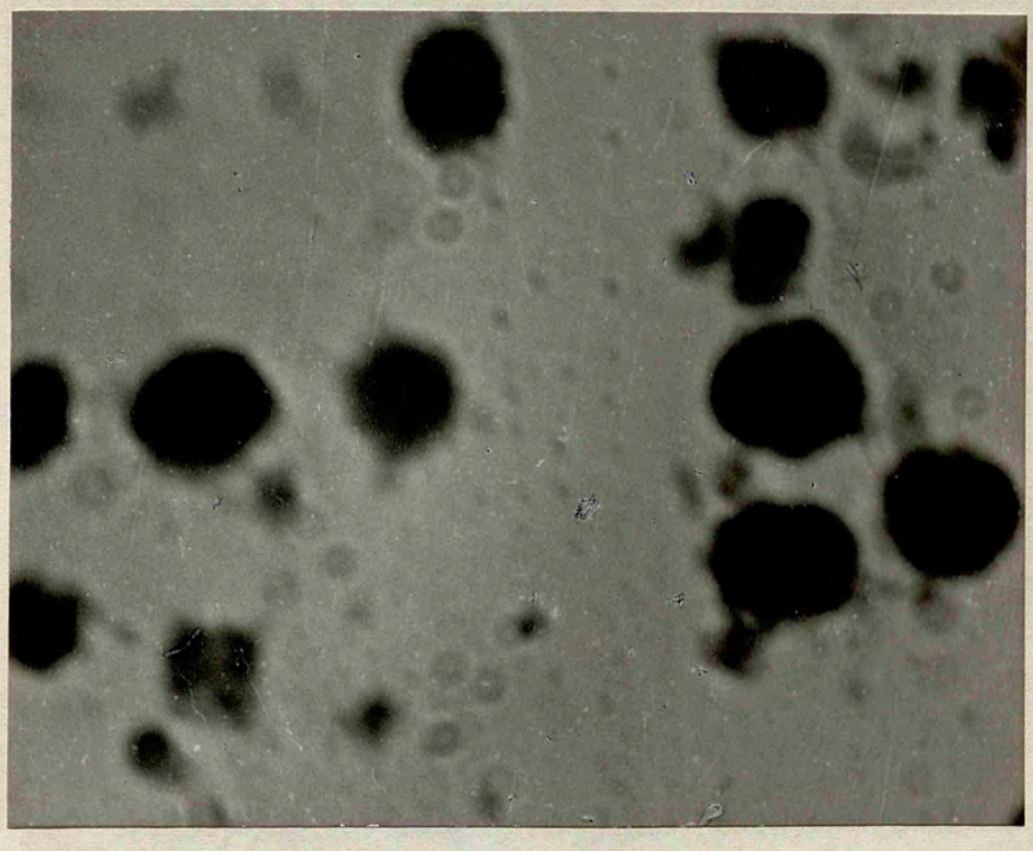


Figure 15. Continued.

solution was mixed and read immediately against 90% pyridine at 495mμ.

Results The minimum molecular weight of crustacyanin was calculated as 35,700 ± 500 from nine determinations using three freeze-dried preparations.

(viii) Molecular size estimation of α- and β-crustacyanin

Method An estimate of the molecular weight of α- and β-crustacyanins were obtained by comparing their gel filtration behaviour on sephadex G200 columns and Sephadex G25, G75, G100 and G200 thin-layers with the bovine serum proteins (IIIF).

Thin-layers were developed with salt solutions (0.1-1.0M-NaCl) at a number of pH (6-8) and columns were developed with 0.02M-phosphate buffer, pH7, containing 1M-NaCl. Fractions were analysed at 280, 590 and 630mμ.

Results α-crustacyanin moved with the solvent (α_2-β_2 globulin) front on thin-layers of Sephadex G25 (exclusion limit 5,000; Flodin (1962)), G75 (exclusion limit 110,000; Andrews (1964)) and G100 (exclusion limit 300,000; Andrews (1964)).

On thin-layers of Sephadex G200 α-crustacyanin moved behind β-globulin but well in front of γ-globulin. Its relative position to these spots was the same under all experimental conditions used (0.1-1.0M-NaCl, pH6-8).

Fig.16 shows the result of a typical experiment. The molecular size of α-crustacyanin corresponded to a globular protein with a molecular weight of 349,000 ± 23,500 (seventeen determinations).

Fig. 17 shows a typical result obtained on gel filtration with Sephadex G200 columns. The molecular size of α-crustacyanin corresponded to a globular protein having a molecular weight of 348,500 ± 21,000 (six determinations).

β-crustacyanin moved behind serum albumin on Sephadex G75, G100 and G200 thin-layer gel filtration as a single spot. It moved with the solvent (β-globulin) front on Sephadex G25. Fig. 18 shows the results using Sephadex G200. The molecular size of β-crustacyanin corresponded to a globular protein of 41,000 ± 300 molecular weight (three Sephadex G200 thin-layer determinations).

Fig.19 shows the result obtained on Sephadex G200 for β-crustacyanin, α-crustacyanin being included in the sample. The molecular size of β-crustacyanin corresponded to a globular protein of 46,000 in this experiment.

Thoroughly dialysed preparations of crustacyanin showing only the four pruple bands on cellulose acetate electrophoresis (Fig.11) gave a single purple spot on Sephadex G75, G100 and G200 thin-layer filtration, moving the same distance as β-crustacyanin. Preparations

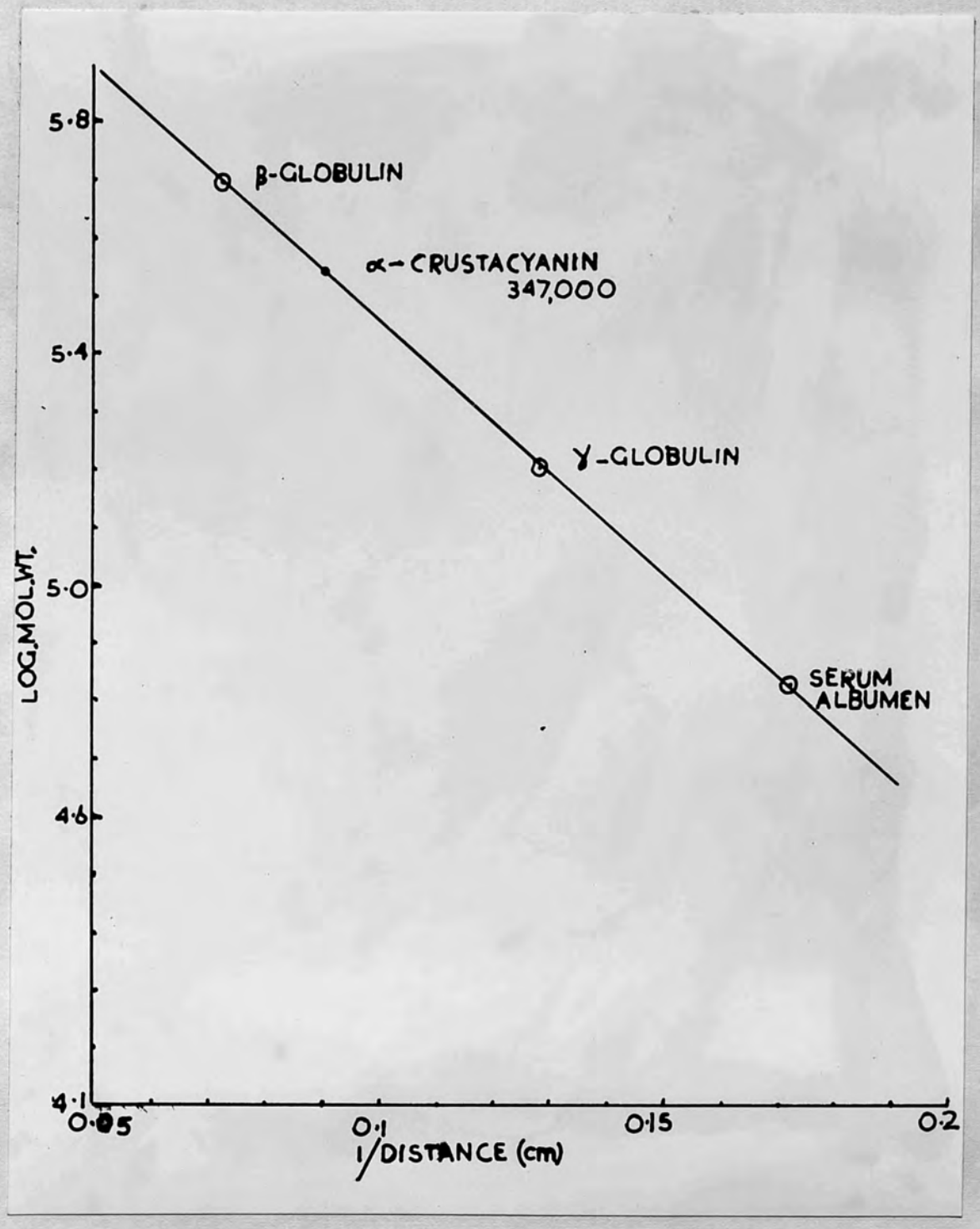


Figure 16. Results of a typical thin-layer gel filtration experiment on Sephadex G200. Plot of log.mol.wt against the reciprocal of the distance travelled for the bovine serum standards.

Samples 1. Bovine serum.
2. α-crustacyanin.

Developing solvent: 0.02M-phosphate buffer, pH7, containing 1M-NaCl.
Running time: 8 hr.

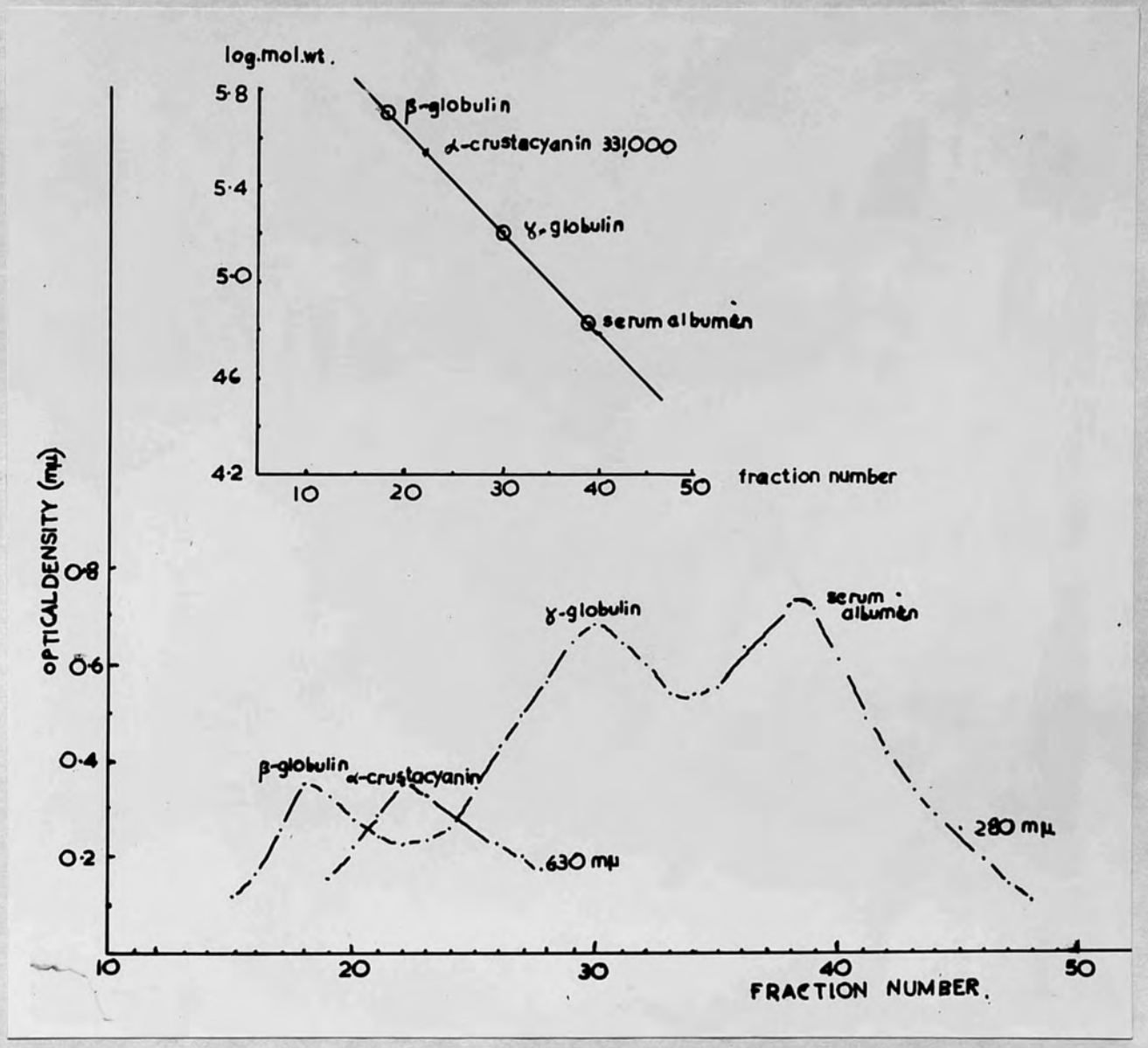


Figure 17. Gel filtration on Sephadex G200.

Sample: 1.5ml. bovine serum containing 0.2mg. α-crustacyanin.
Bed volume: 1.9cm. x 41.0cm.
Flow rate: 10ml./hr.
Fraction volume: 2.5ml.
Developing solvent: 0.02M-phosphate buffer, pH7, containing 1M-NaCl.

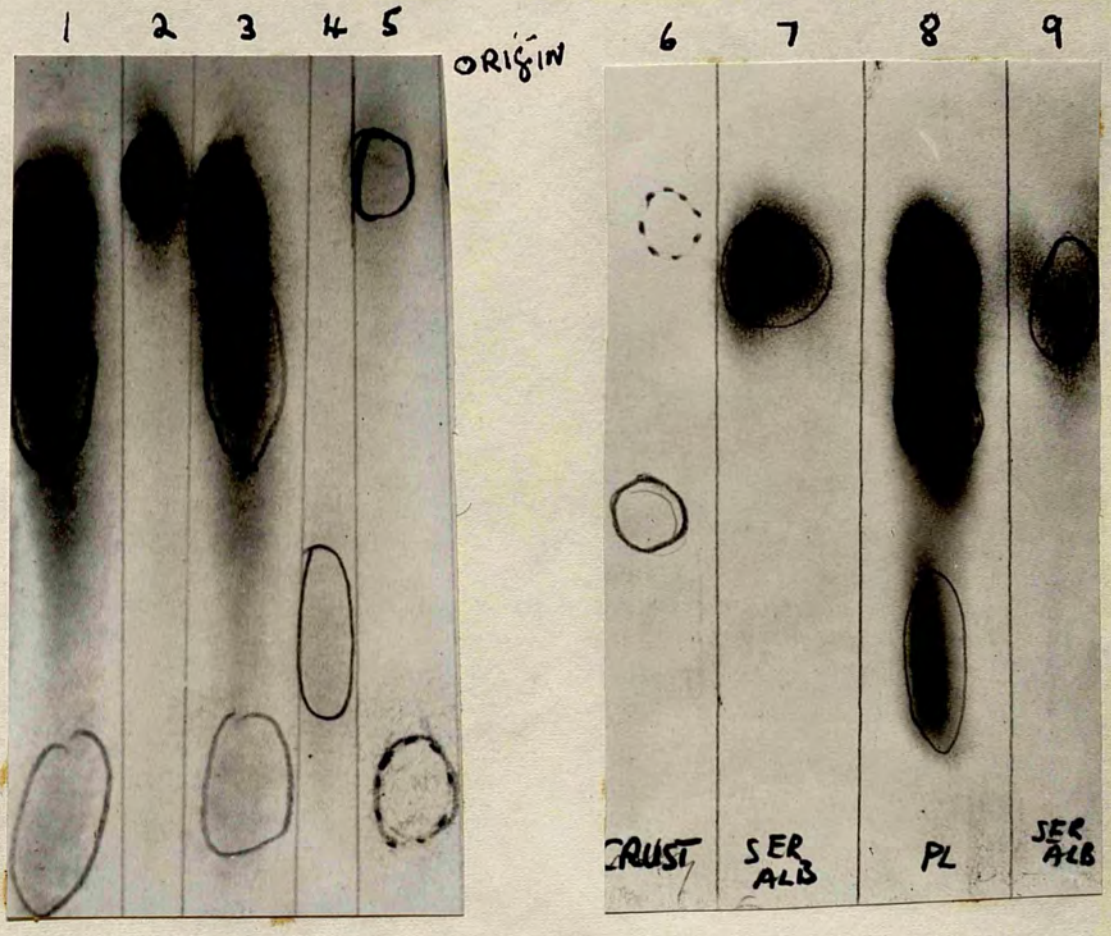


Figure 18. Thin-layer Gel Filtration on Sephadex G200
Columns 1, 3 and 8 Bovine serum
2 β-crustacyanin
4 α-crustacyanin
5 Crustacyanin treated with 6M-urea
6 α-crustacyanin, dialysed against distilled water (2 days)
7 and 9 Bovine serum albumin

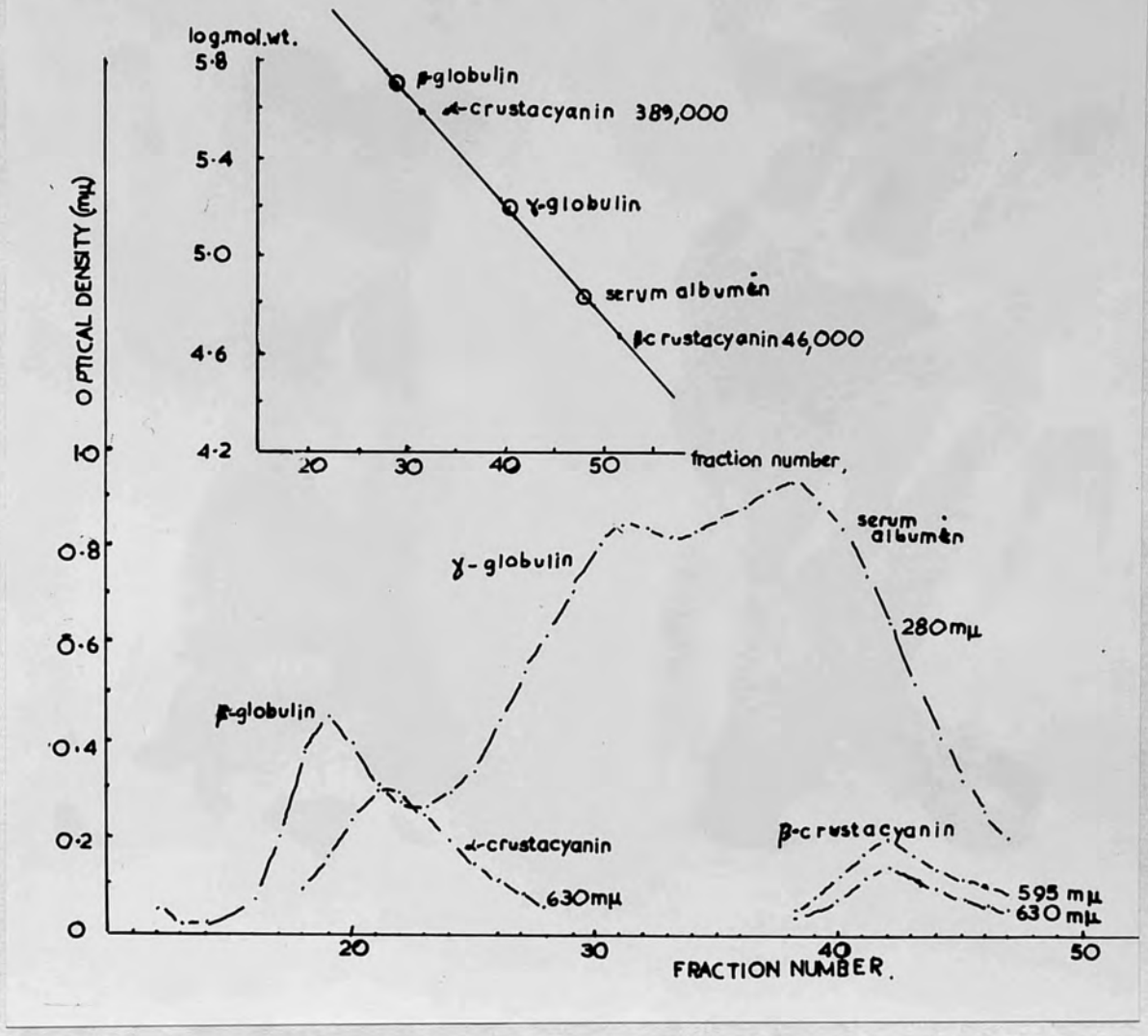


Figure 19. Gel filtration on Sephadex G200.

Sample: 1.5ml. bovine serum containing 1 mg. α-crustacyanin + 0.5ml. β-crustacyanin in 0.05M-phosphate buffer, pH7 (1mg./ml.)

Developing solvent: 0.02M-phosphate buffer, pH7, containing 1M-NaCl.

Flow rate: 10ml./hr.

Fraction volume: 2.5ml.

Bed volume: 2cm. x 50 cm.

containing partially degraded crustacyanin gave the purple component as well as the unaltered α-crustacyanin spot (Fig.18).

(ix) "Salt effect"

(a) Spectral changes on desalting

When a solution of α-crustacyanin in 0.2M-phosphate buffer, pH7, was desalted by gel filtration using Sephadex G75 (IIIG) or by dialysis against two changes of distilled water, it became purple; the maximum at 632.5mμ was displaced to 595mμ, that at 370mμ to 362mμ, while that at 320 became an inflexion at 315mμ (Fig.14); the extinction coefficients = ($E^{0.1\%}$) at the maxima were also decreased.

Leaving the Sephadex-desalted protein in the cold for 48 hr., or dialysing a salt solution of protein for a long period (IV.$B_{(iv)}$.a.), resulted in a change of spectrum to that of β-crustacyanin (Fig.III) with maxima at 585 and 360mμ and an inflexion at 315mμ.

(b) Reversal of spectral changes

The spectral shift to 595mμ in the absence of salt, was completely reversed by addition of NaCl to 0.1M; the solution immediately turned blue and the original spectrum was fully regained on standing overnight in the cold, being exactly superimposable on the original α-crustacyanin spectrum. Other salts ($NaNO_3$, KCl, $MgCl_2$, Na_2SO_4, etc.)

similarly reversed the spectral changes.

The spectrum of the purple material with absorption maximum at 585mμ was not altered by the addition of salt; standing for two weeks in the cold in 1M.NaCl or in 0.2M-phosphate buffer, pH7, containing 0.1M-cysteine had no effect upon the spectrum.

Partial reversal of the spectral shift was observed on intermediate times of dialysis or standing; for example, the Sephadex-desalted material on standing for 3 hr. at room temperature attained a maximum at 627mμ on addition of salt, and had a large inflexion at about 600mμ.

(c) Relation between ionic strength and the spectral changes

Method 30ml. of a solution of crustacyanin (0.1mg./ml.) in 0.2M-phosphate buffer, pH7, was dialysed against 5l. distilled water for two days at 4°, the water being changed daily. The solution then had an absorption maximum at 595mμ and showed full reversion to the original spectrum on addition of 0.1M-NaCl.

3ml. of the dialysed solution were pipetted into test tubes and 1ml. of NaCl. solutions of various concentrations added; the tubes were allowed to stand at 4° in the dark overnight. The absorption at 690mμ, where maximum difference in the spectrum of α-crustacyanin and its salt-free form exists, was recorded and plotted against ionic

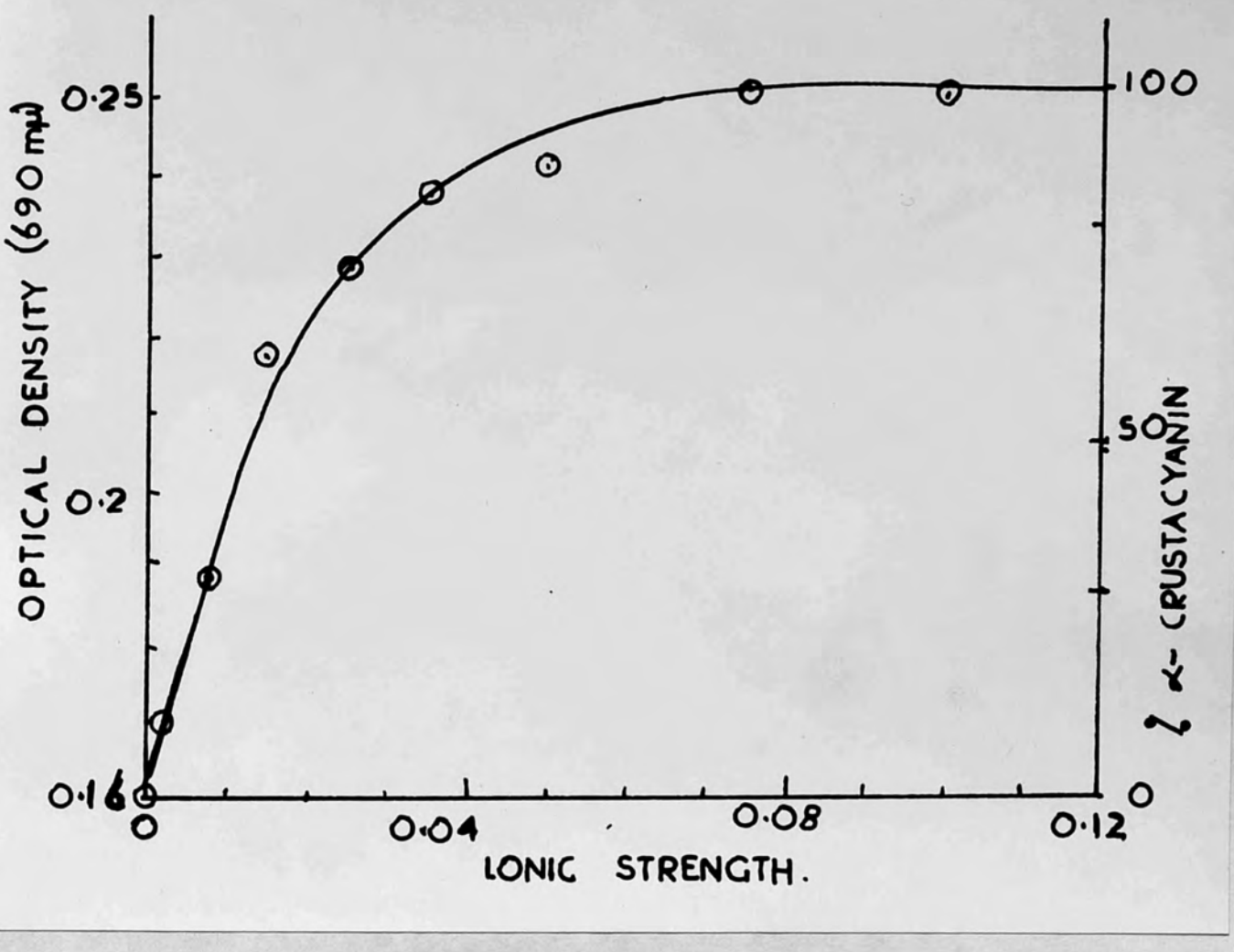


Figure 20. Variation of 690mμ absorption of an α-crustacyanin solution (0.7mg./ml.) with ionic strength (NaCl).

strength. Minor errors in pipetting were corrected by adjusting the absorptions of each tube to the same value at 605mμ, the isosbestic point for α-crustacyanin and its salt-free form (Fig.14).

Results The original spectrum was completely restored at ionic strength (for NaCl.) greater than 0.075 (Fig.20); the value of the 690mμ, absorption at this ionic strength was taken as representing 100% recovery of α-crustacyanin, while the value for the salt-free solution was taken as 0% recovery (100% purple material with absorption maximum 595mμ). Changes in the 690mμ absorption value on addition of salt could then be stated as a percentage recovery of α-crustacyanin (Fig.20).

No recovery of α-crustacyanin was observed below an ionic strength of 2×10^{-4}. A 10% and 50% recovery of α-crustacyanin was attained at ionic strengths of 0.002 and 0.012 respectively; the absorption maxima occurred at 602 and 620mμ in these cases.

(d) Relative stabilities of α-crustacyanin in the presence and absence of salt

(i) Towards heat

Method A crustacyanin solution in distilled water (0.1mg./ml.) was divided into two equal parts. Solid NaCl was added to one part to a concentration of 1 molar. The two solutions were placed in a water bath at 66° and the times taken for them to turn red (indicating complete

dissociation of the carotenoid) noted.

Results No difference in stability towards heat of the two forms of the protein was detected. The time taken for the complete dissociation of the carotenoid was the same for α-crustacyanin in the presence and absence of salt.

(ii) Towards light

Method A crustacyanin solution in distilled water (0.2mg./ml.) was divided into 2 equal parts. An equal volume of 0.2M-phosphate buffer, pH7, was added to one part, and distilled water to the other. The solutions were allowed to stand in bright sunlight for several days and the changes in the extinction at the maxima (630mμ and 595mμ) measured.

Results No significant difference was detected in the stability of the two forms of the protein to light. A decrease of about 3% per day in the absorption at the maximum occurred both in the presence and absence of salt.

(iii) Towards detergents

Method 5ml. samples of crustacyanin solution (0.1mg./ml.) in distilled water and in 0.3M-phosphate buffer, pH7, were mixed with volumes (0.1-1.0ml.) of a 5% deoxycholate solution in water and the colour changes noted.

Results The astaxanthin prosthetic group was more rapidly dissociated from the salt-free form of the protein.

Salt-free crustacyanin solutions turned red-purple in

0.1% sodium deoxycholate and orange in 0.2% sodium deoxycholate. In the presence of 0.05M-phosphate buffer the crustacyanin solutions retained their original blue colour up to 1% sodium deoxycholate concentration.

(e) Ionic strength and gel filtration behaviour

Method Columns of Sephadex G200 were equilibrated overnight at 4° with phosphate buffers, pH7, of ionic strength between 0.003 and 0.12 or with distilled water. 5ml. samples of crustacyanin solution (1-3mg./ml.) were dialysed overnight against 5l. of the buffers. 1.5ml. samples were placed on the columns and these developed with the appropriate buffer. 2.5ml. fractions were collected and analysed at 280, 595 and 630mμ.

After the runs a 1.5ml. sample of diluted India ink was placed on the column; its elution volume was taken to correspond to a protein having a molecular weight of 500,000 (III.F.). The elution volume corresponding to serum albumin was also determined using a 1.5ml. sample of crystalline (bovine) serum albumin (1mg./ml.) or in some experiments by adding 1mg. of the protein to the crustacyanin sample. Rough molecular weight estimates of the crustacyanin fractions were obtained by comparing elution volumes to those of India ink and serum albumin.

Results The change in spectrum at low ionic strength was found to be associated with the dissociation of α -

crustacyanin into subunits having a similar size to β-crustacyanin and as that expected from the minimum molecular weight.

Figs. 21, 22 and 23 show the results obtained for columns developed with phosphate buffers having ionic strengths 0.024, 0.012 and 0.003 respectively. The samples separated into a fast moving blue band and a much slower moving purple band.

The blue component was identical in spectrum and molecular size to α-crustacyanin, while the purple component was identical in spectrum to the salt-free form of α-crustacyanin (Fig.14). The purple component was of the same molecular size as β-crustacyanin.

The proportion of each omponent was estimated roughly from the area under each curve. The results are given in Table II. The figures in brackets represent the percentages of α-crustacyanin present in a NaCl solution of the same ionic strength (read from Fig.20).

Table II. Relative proportions of purple and blue components present in α-crustacyanin solutions at low ionic strength

Ionic Strength	% α-crustacyanin (Blue Component)	% Purple Component	Absorption Maximum of Sample (mμ)
0.024	62 (70)	38	625
0.012	49 (50)	51	615
0.003	17 (12)	83	600

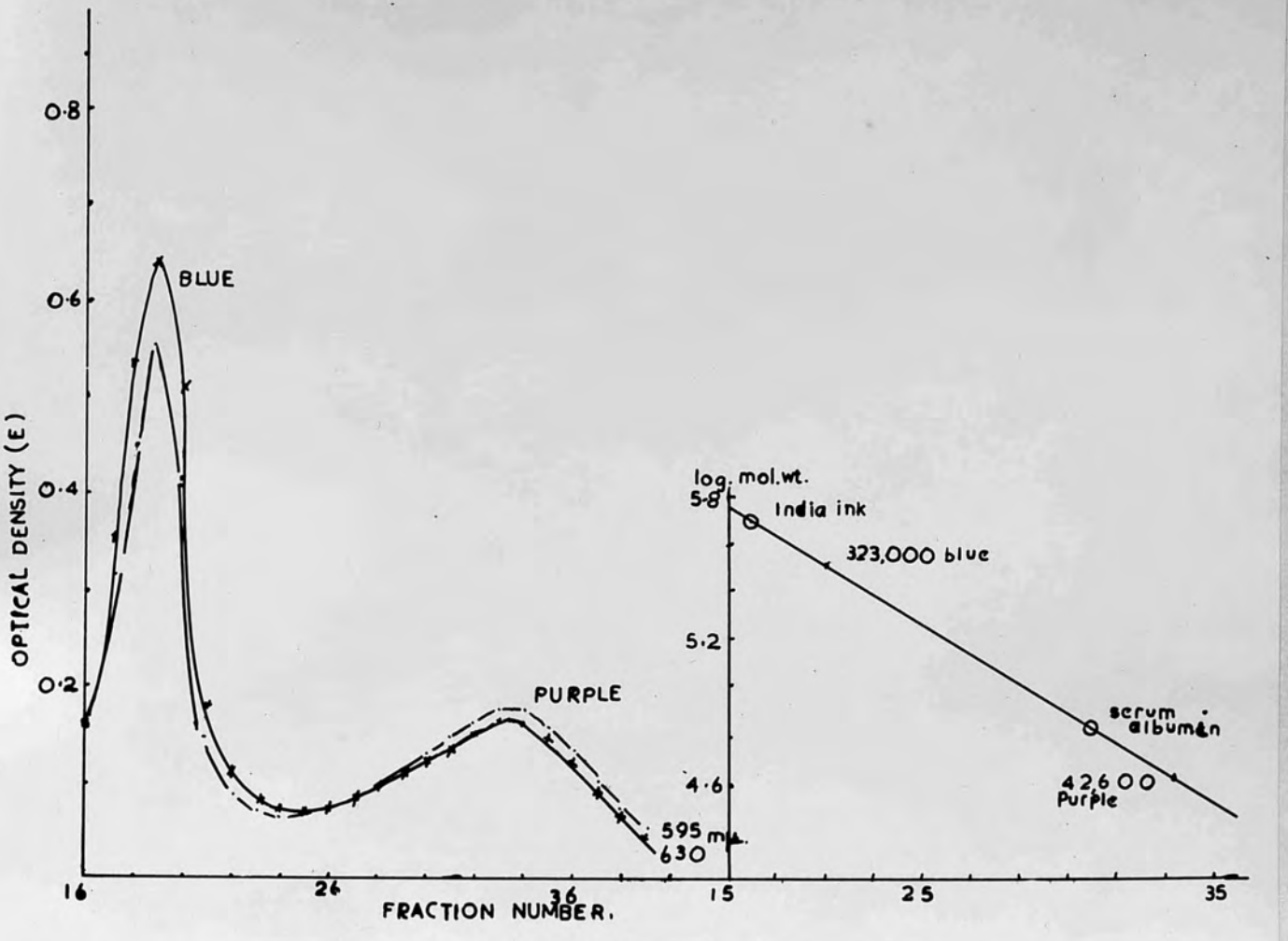


Figure 21. Gel filtration on Sephadex G200.
Sample: 1.5ml. α-crustacyanin (3mg./ml.) in pH7-phosphate buffer, 0.024 ionic strength.
Developing solvent: pH7-phosphate buffer, 0.024 ionic strength.
Bed volume: 1.9cm. x 41.0cm.
Flow rate: 10ml./hr.
Fraction volume: 2.5ml.

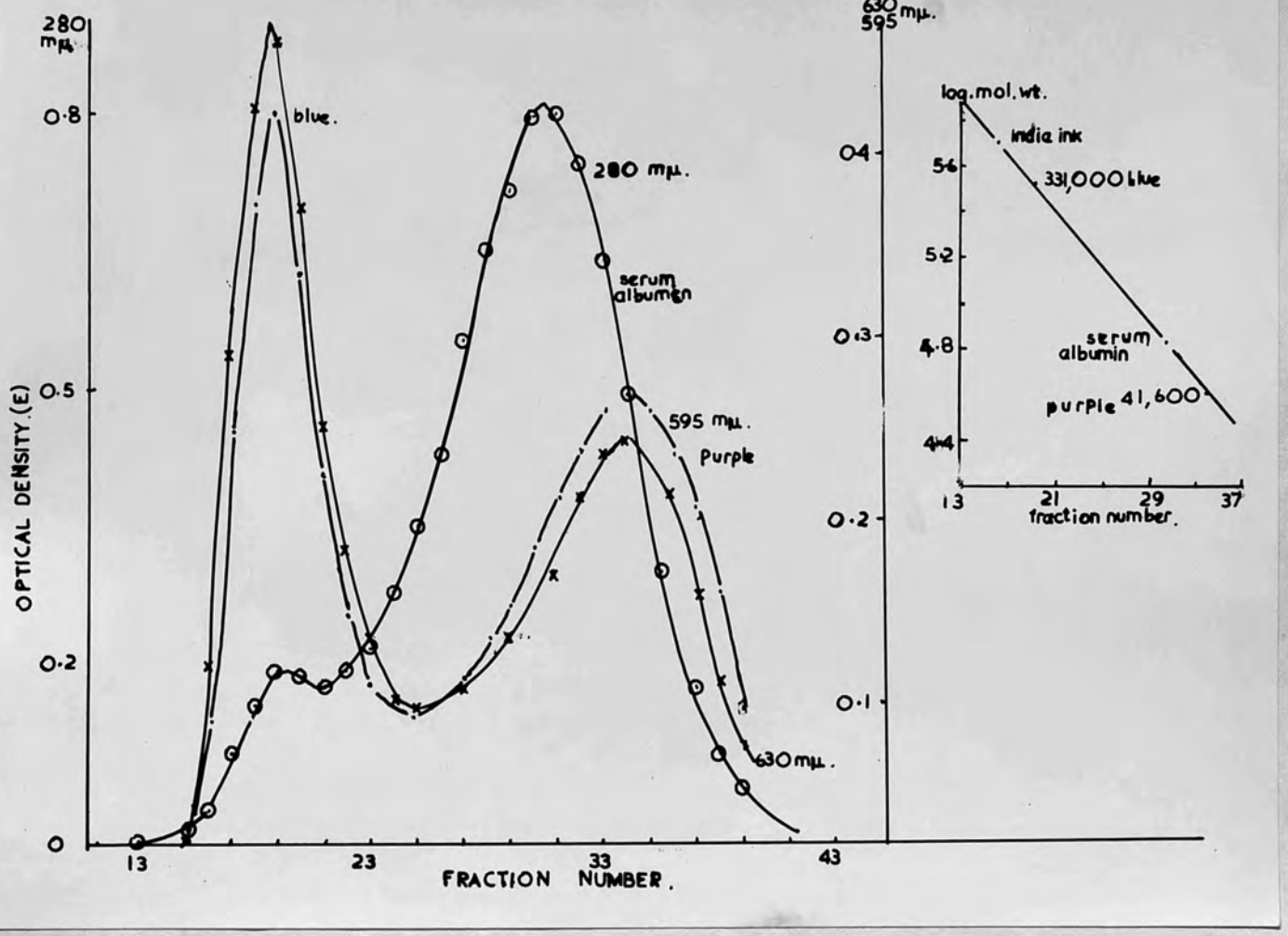


Figure 22. Gel filtration on Sephadex G200.
Sample: 1.5ml. α-crustacyanin (2mg./ml.) in pH7-phosphate buffer, 0.012 ionic strength, containing 2mg. bovine serum albumin.
Developing solvent: pH7-phosphate buffer, 0.012 ionic strength.
Bed volume: 1.9cm. x 40.5cm.
Fraction volume: 2.5ml.
Flow rate: 10ml./hr.

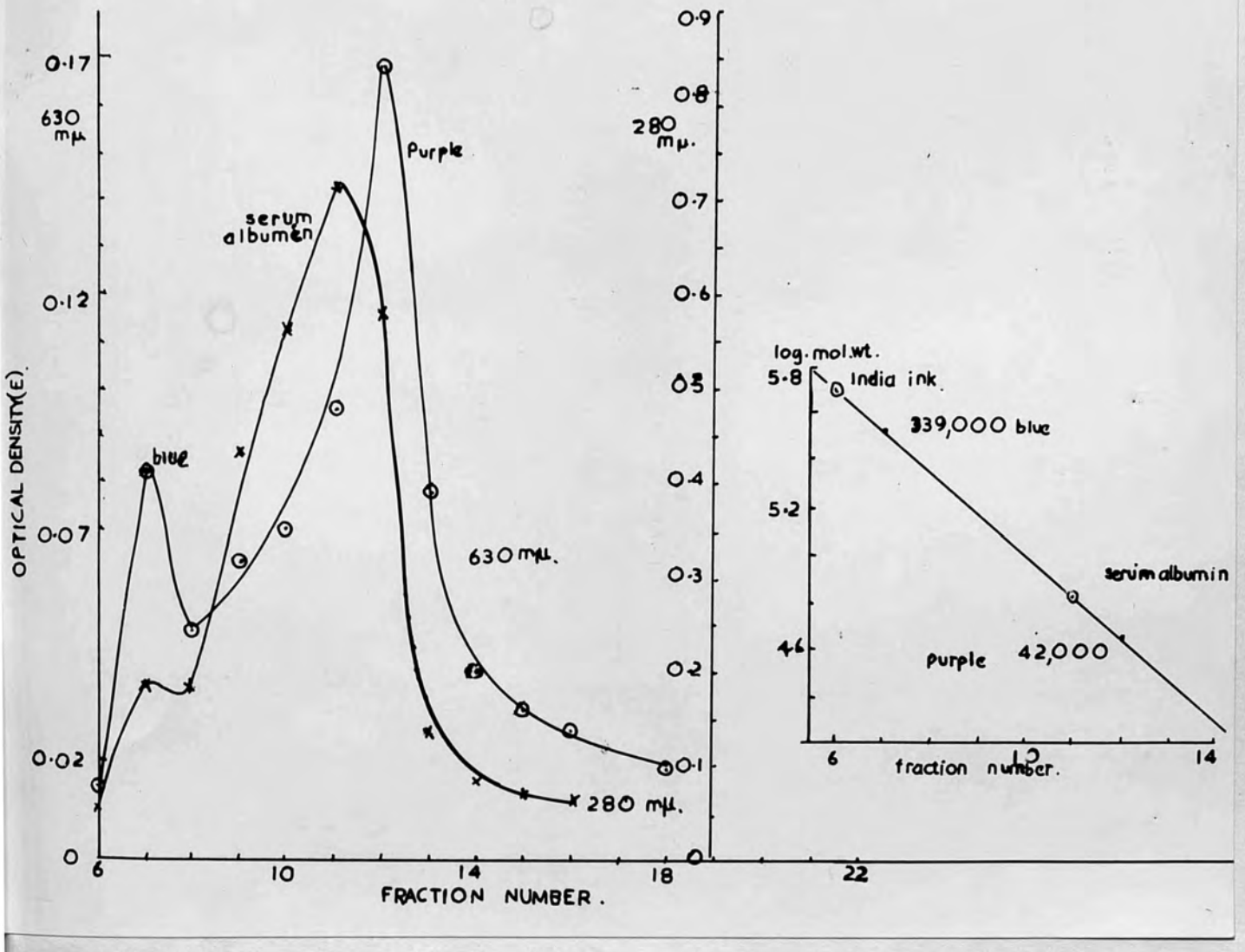


Figure 23. Gel filtration on Sephadex G200.
Sample: 1.5ml. α-crustacyanin (1mg./ml.) in pH7-phosphate buffer, 0.003 ionic strength, containing 2 mg. bovine serum albumin.
Bed volume: 1.4cm. x 35.0cm.
Developing solvent: pH7-phosphate buffer, 0.003 ionic strength.
Fraction volume: 2.5ml.
Flow rate: 10ml./hr.

The absorption spectrum of the blue component altered on standing to that of the original sample; the reversion was slow even at room temperature, being complete after 24hr. Two bands, in about the same proportions as previously observed, were again obtained on refiltering the solution on its respective Sephadex column.

The absorption spectrum of the purple component remained unaltered on standing, and the addition of 1M-NaCl to the solution brought no change in the spectrum. Presumably it had been altered by passage through the column, since the spectra of the original sample before passage through the gel reverted fully to that of α-crustacyanin. The spectrum of the purple component altered to that of β-crustacyanin (Fig.3) on precipitating with ammonium sulphate and dissolving in 0.2M-phosphate buffer, pH7.

Freeze-dried samples of α-crustacyanin dissolved in distilled water and developed on columns equilibrated with distilled water only showed the presence of the purple component, and samples in phosphate buffer of 0.12 ionic strength gave only the blue component when filtered through Sephadex columns equilibrated with this buffer.

(x) Preparation of apoprotein

Method The apoprotein was prepared by a modification of the method used for ovorubin (1958). On account of the alteration of α-crustacyanin occurring at low ionic

strength (IV.B$_{(iv)}$ and$_{(ix)}$.) it was considered advisable to include 0.05M-KCNS in all the steps.

About 20mg. freeze-dried crustacyanin ($E_{280/630}$=.31) were dissolved in 2ml. ice-cold 0.05M-KCNS solution. 50ml. ice-cold acetone containg 0.05M-KCNS were added in small portions with vigorous stirring, keeping the solution cold in an ice-water mixture. The precipitated protein was centrifuged down, dissolved in 2ml. ice-cold 0.05M-KCNS solution and the procedure repeated a number of times. The protein was dissolved finally in 4ml. 0.05M-KCNS solution and dialysed against 5l. of the same solution overnight.

Results The addition of acetate gradually turned the solution purple and at 50% concentration brought it out of solution; further addition of acetone dissociated the carotenoid from the protein.

The majority of the carotenoid was removed after 5 acetone treatments, but the last remnants were difficult to remove. After seven acetone treatments 3% of the original carotenoid was still attached to the protein.

The protein redissolved almost completely after the acetone precipitations but some denatured material appeared on dialysis, there being more of this the greater the number of acetone treatments.

About 80% of the original protein (estimated from the

280mμ absorption) was recovered after five acetone treatments.

(xi) Reconstitution

Method Recombination of the carotenoid and apoprotein was carried out by a modification of the method used for ovorubin (Cheesman, 1958).

Apoprotein ($E_{280/630}$= 8.1) was prepared using five acetone treatments, as in the previous section.

A solution of 2 mg. apoprotein (estimated from the 280mμ absorption) in 1ml. 0.05M-KCNS solution was treated at 6° with a solution of the carotenoid in 1ml. 60% acetone containing 0.05M-KCNS, added in small portions with stirring. The amount of carotenoid used was 25% in excess of that derived from 2mg. crustacyanin. The mixture was diluted with 10ml. cold 0.05M-KCNS solution and the acetone removed by dialysis against 5l.0.05M-KCNS overnight. The reconstituted crustacyanin was purified by DEAE-cellulose chromatography (IV.$B_{(i)}$.c.).

Results 75% of the apoprotein was recovered as reconstituted α-crustacyanin. A small amount of purple protein (β-crustacyanin) eluted from the DEAE-cellulose column with 0.15M-phosphate buffer, pH7, was also formed.

The carotenoid from ovorubin combined equally well with apocrustacyanin to give α-crustacyanin with unaltered absorption spectrum. Likewise astaxanthin derived from

α-crustacyanin combined with apoovorubin to give ovorubin with its characteristic absorption spectrum.

(xii) Thin-layer gel filtration of apoprotein and reconstituted α-crustacyanin

Method The apoprotein and reconstituted protein were prepared as in Sections x and xi, except that the reconstituted protein was not purified by DEAE-cellulose chromatography. The proteins were precipitated by saturating with $(NH_4)_2SO_4$, taken up in 0.2ml. 0.2M-phosphate buffer, pH7, and 1 µl. applied to thin-layers of Sephadex G75, G100 and G200. The layers were prepared and used as described (III.$F_{(iv)}$.), being developed with 0.02M-phosphate buffer, pH7, containing 1M-NaCl.

Results The apoprotein was found to have the same molecular size as β-crustacyanin.

Apoprotein preparations separated into three distinct spots on Sephadex G200 thin-layers, the main component moving behind serum albumin. A faint blue spot of unaltered α-crustacyanin, and a spot of denatured (or aggregated) protein moving at the solvent front were also present; the intensity of the latter was greatest in those preparations for which a large number of acetone treatments had been used. Preparations obtained by three acetone treatments contained little of this component, but contained 10-15% of unchanged α-crustacyanin. The

samples used in the separations shown in Fig.24 were obtained using five acetone treatments; they contained 3-5% unchanged α-crustacyanin.

The molecular size of the apoprotein corresponded to a globular protein having a molecular weight 38,300 ± 700. (Seven determinations.)

Similarly, on Sephadex G75 (Fig.24c) and G100 (Fig. 24b) thin layers the apoprotein gave a major component moving well behind serum albumin, with a light blue spot of unchanged α-crustacyanin and denatured protein moving at the solvent front.

The reconstituted α-crustacyanin preparation showed only traces of the purple low-molecular component (Fig. 24 a, b and c), consisting mainly of native α-crustacyanin; the denatured material was still present at the solvent front.

(xiii) Electrophoretic studies on apoprotein and reconstituted protein

Method Cellulose acetate and micro-electrophoresis on starch gel were employed as described (III.$E_{(i)}$., III.$E_{(ii)}$. b.).

Samples for electrophoresis were obtained as follows, ice-cold solutions being used throughout.

2ml. 0.05M-KCNS solution of the apoprotein (2mg./ml.) were treated with 2ml. 60% acetone containing 0.05M-KCNS and excess of carotenoid required for reconstitution. At

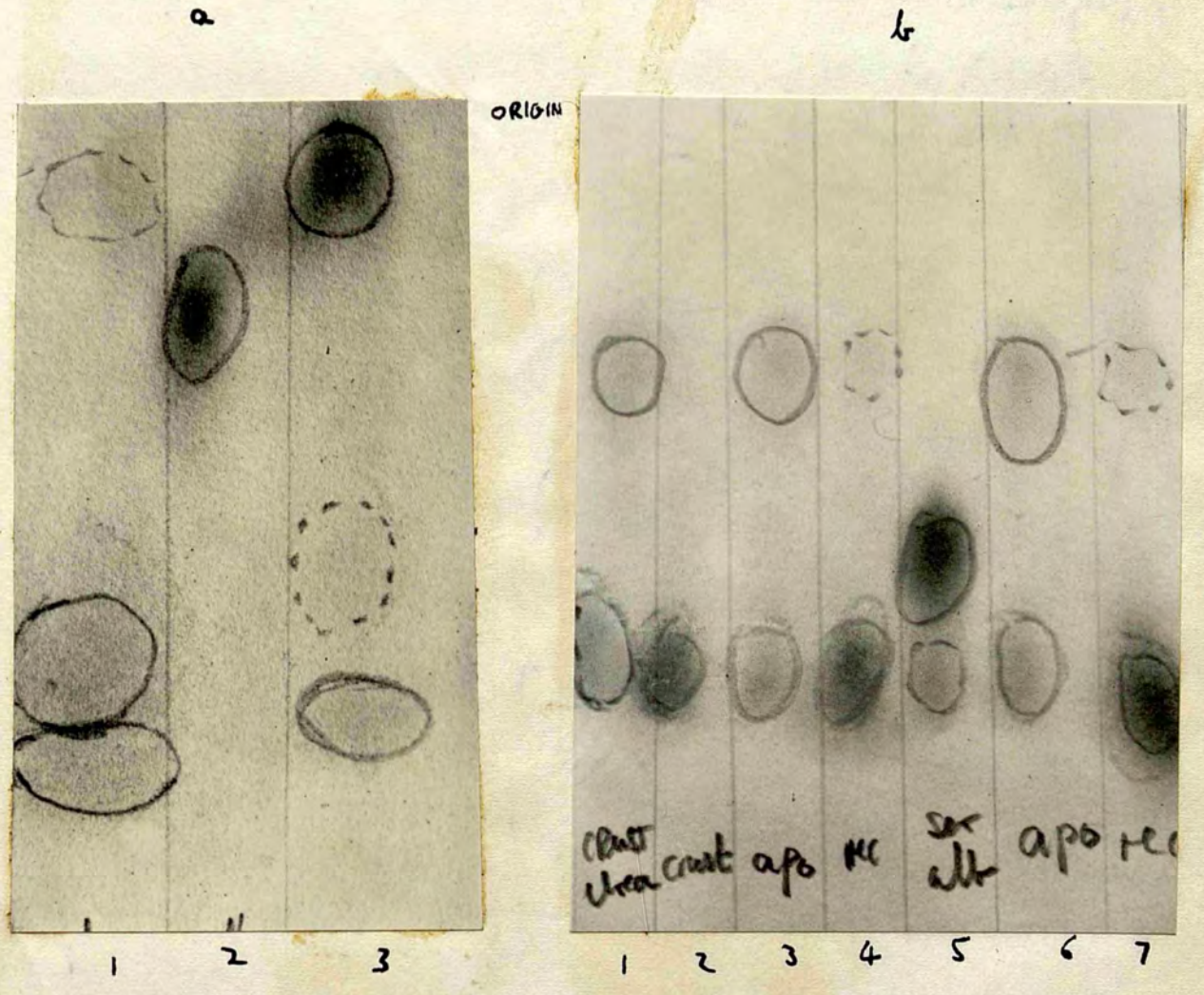


Figure 24 Thin-layer gel filtration on Sephadex.

(a) Sephadex G200. 7hr. run. Plates developed with 1M-NaCl/0.02M-phosphate buffer, pH7.

Column 1 Reconstituted α-crustacyanin
2 Bovine serum albumin.
3 Apocrustacyanin (five acetone treatments)

(b) Sephadex G100. 8hr. run. Plates developed with 1M-NaCl/0.02M-phosphate buffer, pH7.

Column 1 α-crustacyanin treated with 6M-urea in 0.05M-phosphate buffer, pH7, for 2hr.
2 α-crustacyanin
3 and 6 Apocrustacyanin (five acetone treatments)
4 and 7 Reconstituted α-crustacyanin

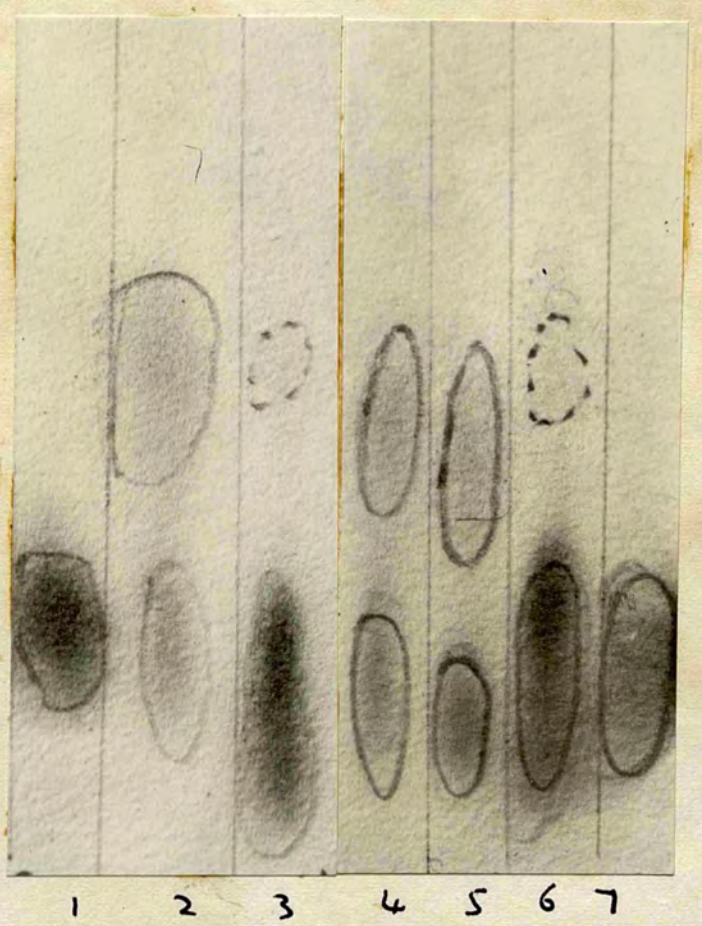


Figure 24(c)

Thin-layer gel filtration on Sephadex G75. 6hr. run.
Plates developed with 1M-NaCl/0.02M-phosphate buffer, pH7.

Column 1 Bovine serum albumin.
2 and 4 Apocrustacyanin (five acetone treatments)
3 and 6 Reconstituted α-crustacyanin
5 Crustacyanin treated with 6M-urea in 0.05M-phosphate buffer, pH7, for 2hr.
7 α-crustacyanin.

the same time another 2ml. of the apoprotein solution were treated identically, but with the carotenoid excluded from the acetone solution. The solutions were then dialysed against 2l. of the electrophoretic buffer for 4hr. with continual agitation. They were then concentrated to a small volume against Carbowax (III.D.).

Results The apoprotein preparation was heterogeneous in electrophoresis, while the reconstituted protein preparation gave the native α-crustacyanin as the main component.

Fig.25 shows a typical result obtained at pH7 on cellulose acetate. The apoprotein preparation (five acetone treatments) showed four main bands besides the unchanged α-crustacyanin band, two more intensely staining, of lower mobility than α-crustacyanin. The reconstituted protein preparation gave mainly the native α-crustacyanin band, but a small amount of a more slowly moving purple component was also present. In both cases a fair amount of denatured protein remained at the origin.

Similarly, the apoprotein preparation was resolved into a number of bands in micro-electrophoresis on starch gel at pH7. In this case, three major bands besides unchanged α-crustacyanin could be distinguished (Fig.26).

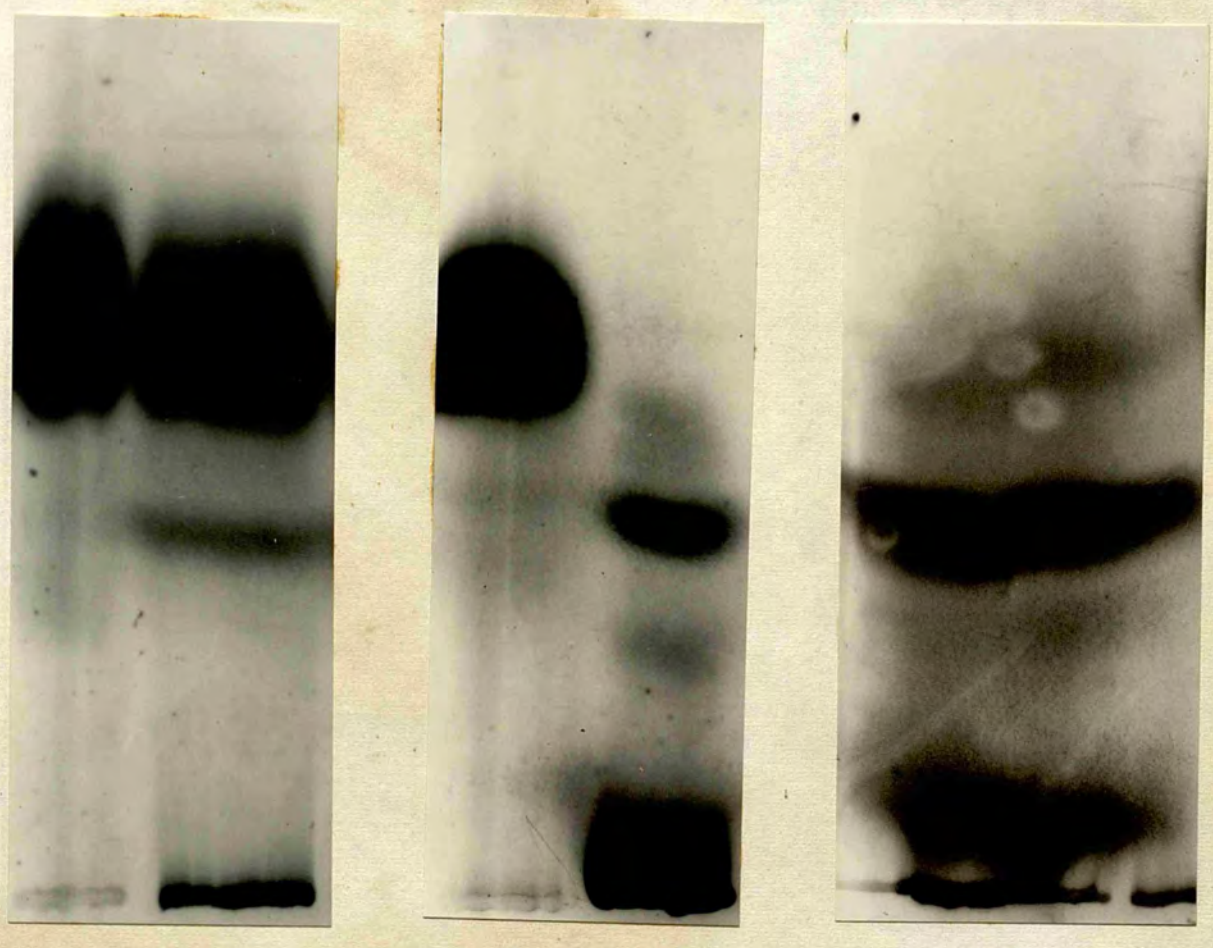


Figure 25 Cellulose acetate electrophoresis. 0.05M-phosphate buffer, pH7. 2mA/5cm. width strip. 7hr. run.

Column 1 and 3 α-crustacyanin
2 α-crustacyanin treated with 6M-urea in 0.05M-phosphate buffer, pH7, for 2hr.
4 Reconstituted α-crustacyanin
5 Apocrustacyanin (five acetone treatments)

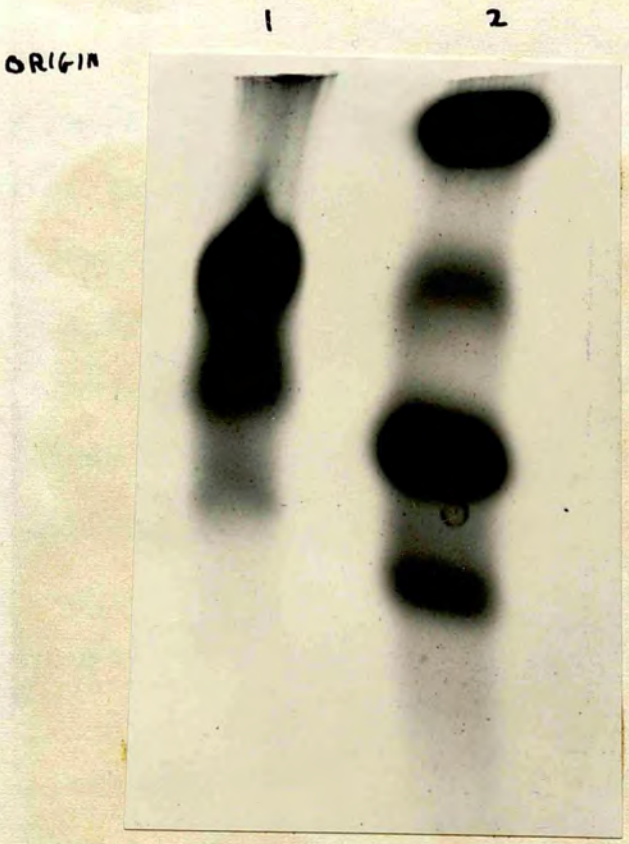


Figure 26 Micro-electrophoresis using starch gel 0.03M-phosphate buffer, pH7. 20volts/cm. 16hr. run.

Column 1 Reconstituted α-crustacyanin

2 Apocrustacyanin (four acetone treatments)

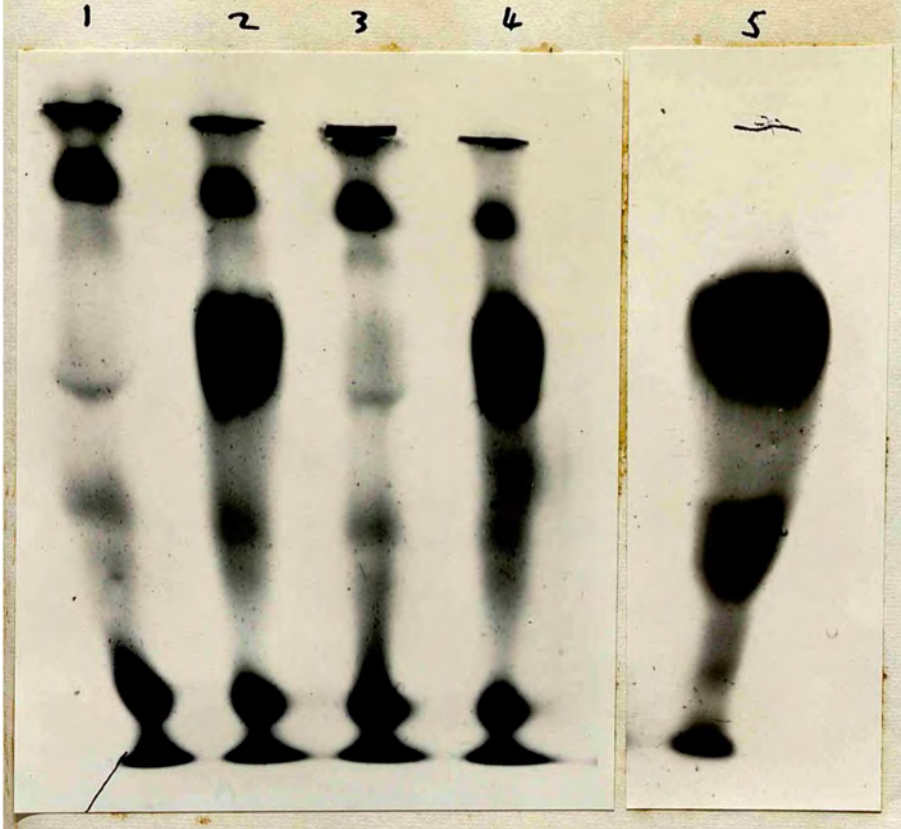


Figure 27
Micro-electrophoresis on starch gel. Trs-citrate-borate, discontinuous buffer system, pH8.65. 20volts /cm. 3hr. run.

Column 1 and 3 Apocrustacyanin (five acetone treatments)

2 and 4 Reconstituted α-crustacyanin

5 α-crustacyanin

In contrast to the cellulose acetate electrophoresis two of these components had a greater mobility than α-crustacyanin. The major band of the reconstituted preparation was that of the native α-crustacyanin, the main slower moving component was absent and the faster moving (now coloured purple) components still present but less intense.

Three major (and several minor) components were observed in the apoprotein preparations at pH8.6 using micro-electrophoresis on starch gel with Poulik's discontinuous buffer system (Fig.27). Two of the major components were carried close to the citrate front, suggesting these might be low-molecular. The reconstituted protein preparation still showed small amounts of these components, but the band of α-crustacyanin predominated. Freeze-dried α-crustacyanin, homogeneous to cellulose acetate electrophoresis, also showed traces of the faster moving components using this buffer system.

(xiv) Relative strengths of binding of carotenoid in ovorubin and crustacyanin

The strengths of carotenoid binding in ovorubin and crustacyanin were compared by combining the apoproteins with carotenoid under conditions in which they competed for the latter. The $E^{0.1\%}_{510}$ value for ovorubin (Cheesman, 1958) is about a tenth of the values of $E^{0.1\%}_{630}$ for

crustacyanin (IV.$B_{(v)}$.). Ten times the amount of apoovorubin as apocrustacyanin was used in the recombination so that, for equivalent amounts of binding (per mg. dry weight), the extinctions at 510 and 630mμ of the reconstituted ovorubin and crustacyanin respectively should be about the same.

Method Ovorubin and apoovorubin were prepared by the method of Cheesman (1958) using four acetone treatments.

2ml. apooverubin (1mg/ml.) in 0.05M-KCNS was mixed with 0.2ml. of apocrustacyanin (1mg./ml.) in 0.05M-KCNS. The mixture was recombined with the carotenoid derived from 0.18mg. crustacyanin (or 1.8mg. ovorubin) as in section xi., dialysed against 0.05M-KCNS and adsorbed onto DEAE-cellulose. The reconstituted crustacyanin and ovorubin were eluted together with 0.25M. phosphate buffer, pH7.

Results The affinity of apocrustacyanin for the carotenoids derived from ovorubin and crustacyanin was much greater than that of apoovorubin.

About 65% of the apocrustacyanin was recovered as α-crustacyanin (as estimated from the 630mμ absorption). The spectrum of the reconstituted mixture showed only traces of the ovorubin spectrum (510 and 545mμ maxima); faint inflexion at 520 and 545mμ were no greater than expected from the amount of unchanged ovorubin present in the apoprotein preparation (Fig.28).

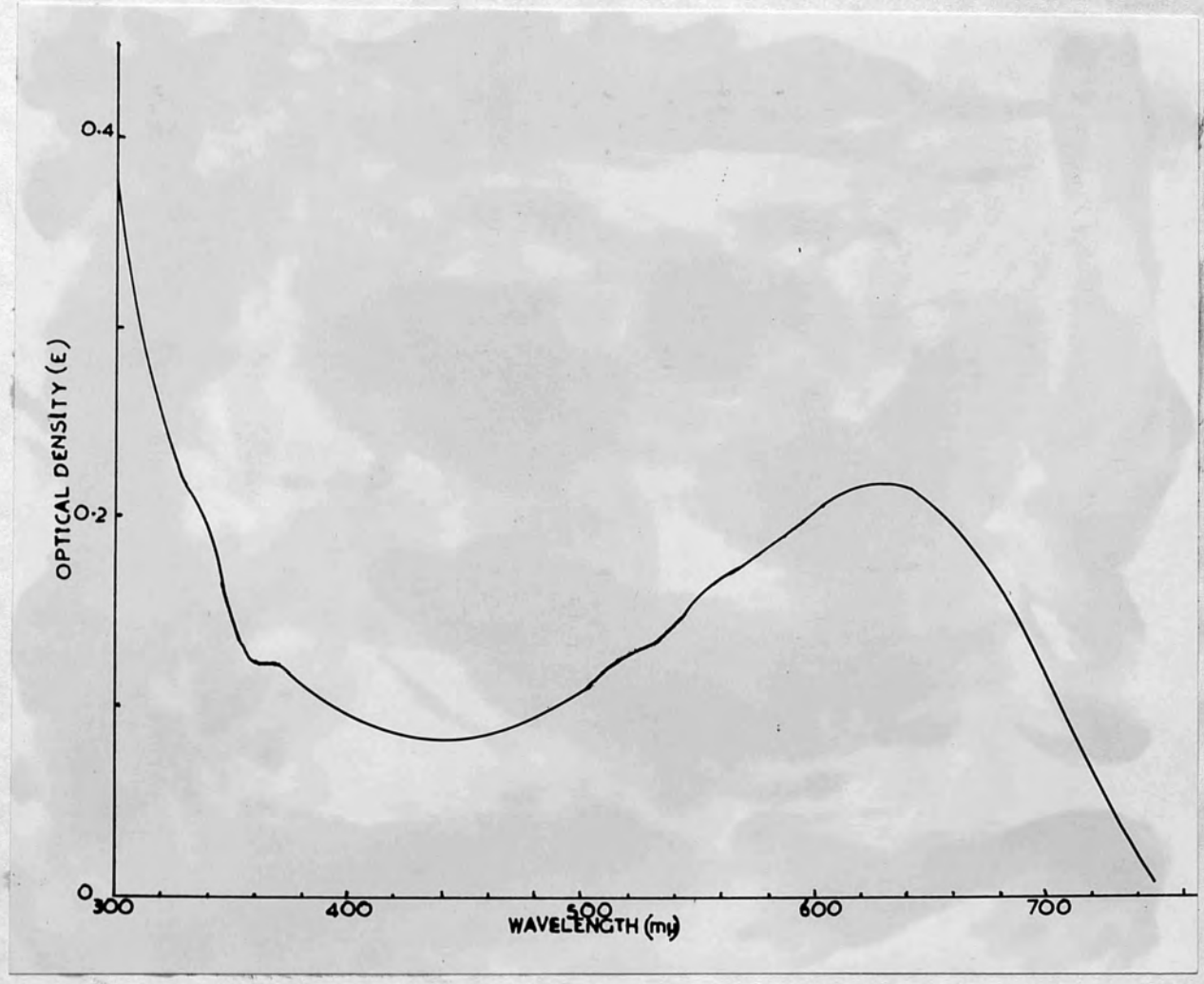


Figure 28. Recombination of apocrustacyanin and apoovorubin with astaxanthin.

(xv) Dissociation of α-crustacyanin on partial disruption of the carotenoid-protein linkage

Method Freeze-dried α-crustacyanin (0.2mg./ml.) was dissolved in cold 10% acetone containing 0.05M-KCNS and allowed to stand for 30min. in the cold. The behaviour on gel filtration of this solution and of a solution of α-crustacyanin in 0.05M-KCNS was investigated on short columns (15cm.) of Sephadex G200 equilibrated with cold 0.05M-KCNS and with 10% acetone containing 0.05M-KCNS. 1ml. samples were used.

Results Alteration of the carotenoid-protein linkage with acetone was accompanied by the dissociation of α-crustacyanin into subunits.

α-crustacyanin was purple in 10% acetone solution. Passage into the Sephadex column equilibrated with 0.05M-KCNS resulted in the separation of two bands. A small blue band (unchanged α-crustacyanin) moved rapidly down the column, while a more intense purple band was strongly retained. The front of the purple component gradually turned blue, spreading out into a wide band. The purple band had completely changed to blue by the time it was halfway down the column, and both bands on emergence had an identical spectrum to that of α-crustacyanin (Fig.14).

A single band was obtained on passage of the 10% acetone solution of crustacyanin into the 10% acetone

equilibrated Sephadex column. The front of the band was initially blue but turned purple on further passage into the gel. The emergent purple material had absorption maxima at 560 and 580 mμ.

When crustacyanin in 0.05M-KCNS solution was passed into the 10% acetone equilibrated Sephadex column a single, fast moving, blue band was initially obtained. This rapidly turned purple, starting at the front of the band, as it moved down the column and concentrated into a very narrow band strongly retained by the gel.

The purple component, using the 10% acetone solution of crustacyanin moved down the acetone equilibrated column at about a third of the rate of India ink, indicating a molecular size similar to that of the apoprotein (see Fig. 24(a)).

(xvi) Spectral changes in 6M- urea

Method Freeze-dried crustacyanin (0.3mg./ml.) was dissolved in cold 6M- urea containing buffers of different pH. Spectra were recorded after standing in the cold for 2 and 24hr. Samples were dialysed, after standing for 2hr., against 51. 5% NaCl for 2 days.

Results The changes in spectrum occurring in the presence of 6M- urea depended on the pH of the buffer used and time of incubation.

At pH4.5 the crustacyanin solution became yellow with a single absorption maximum at 405mμ after 2 and 24 hr. The maximum shifted to 400mμ on removal of the urea (Fig.29).

When an alkaline buffer (pH8.65) was used, the colour changes slowly in two hours from blue to purple-brown. The spectrum had maxima at 395 and 450mμ with an inflexion at 450mμ. The maxima were at 395, 465 and 540mμ after 24hr. standing. Dialysis resulted in partial reversal of these changes and the reappearance of the 632mμ α-crustacyanin peak. (Fig.30).

At pH7 maxima were at 395, 460 and 540mμ after two hours standing, and at 395 and 560mμ after 24hr. The 460 and 540mμ peaks disappeared on dialysis and the 632mμ peak of α-crustacyanin was partially restored (Fig.31).

The maximum in the 400mμ region irreversible formed at all pH faded on standing, especially in light, and upon dialysis.

(xvii) Gel filtration of urea treated protein

Method 5mg. freeze-dried crustacyanin was dissolved in 0.02ml. of (i) 0.03M-phosphate buffer, pH7, containing 6M- urea:

(ii) 0.07M-tris-0.005M. citrate buffer, pH8.6, containing 6M- urea:

(iii) 0.05M. formica acid-0.01M. NaOH, pH3.1, containing 6M- urea.

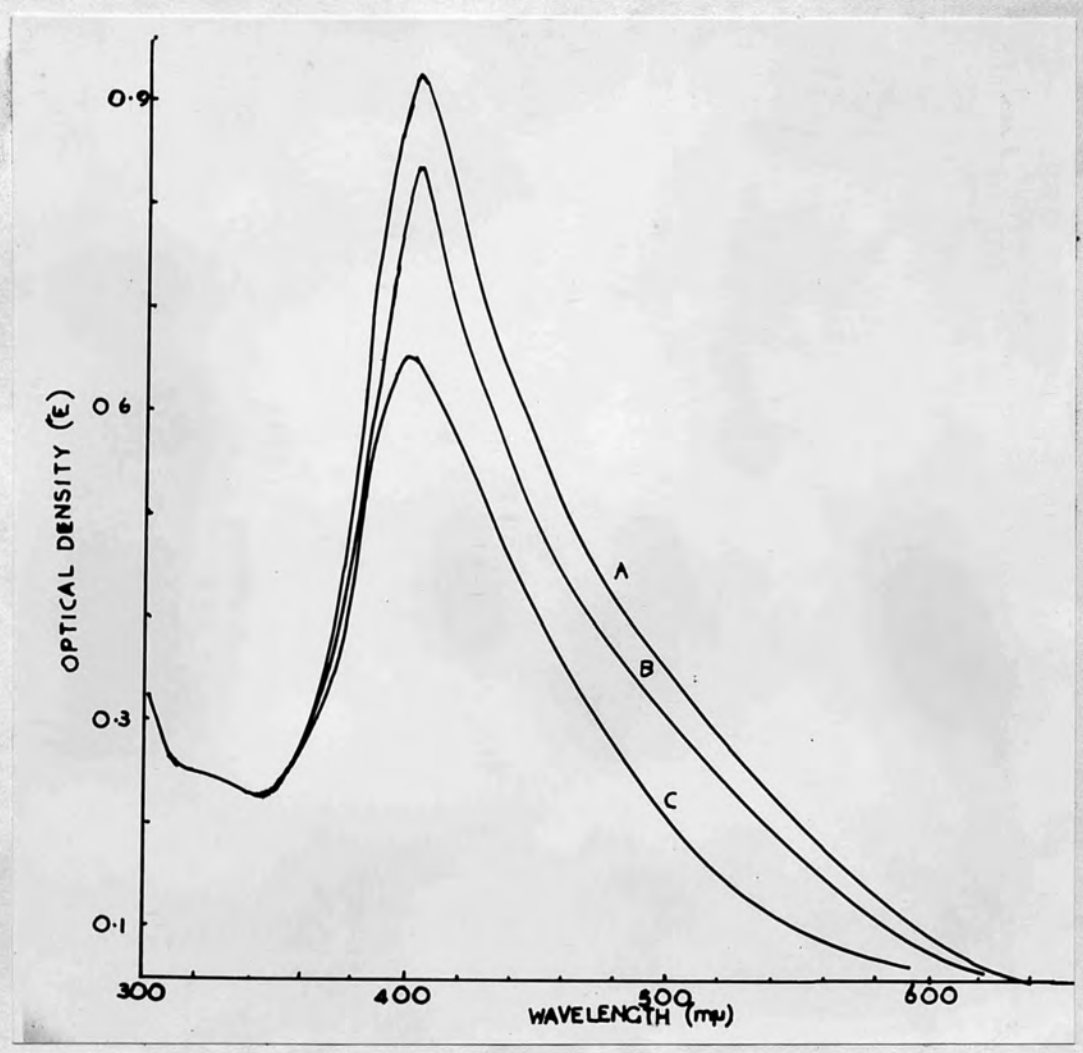


Figure 29. Spectrum of α-crustacyanin in 0.05M-acetate buffer, pH4.5, containing 6M- urea.

A. 2hr. incubation.
B. 24hr. incubation.
C. 2hr. incubation followed by dialysis against 5% NaCl for 24 hr.

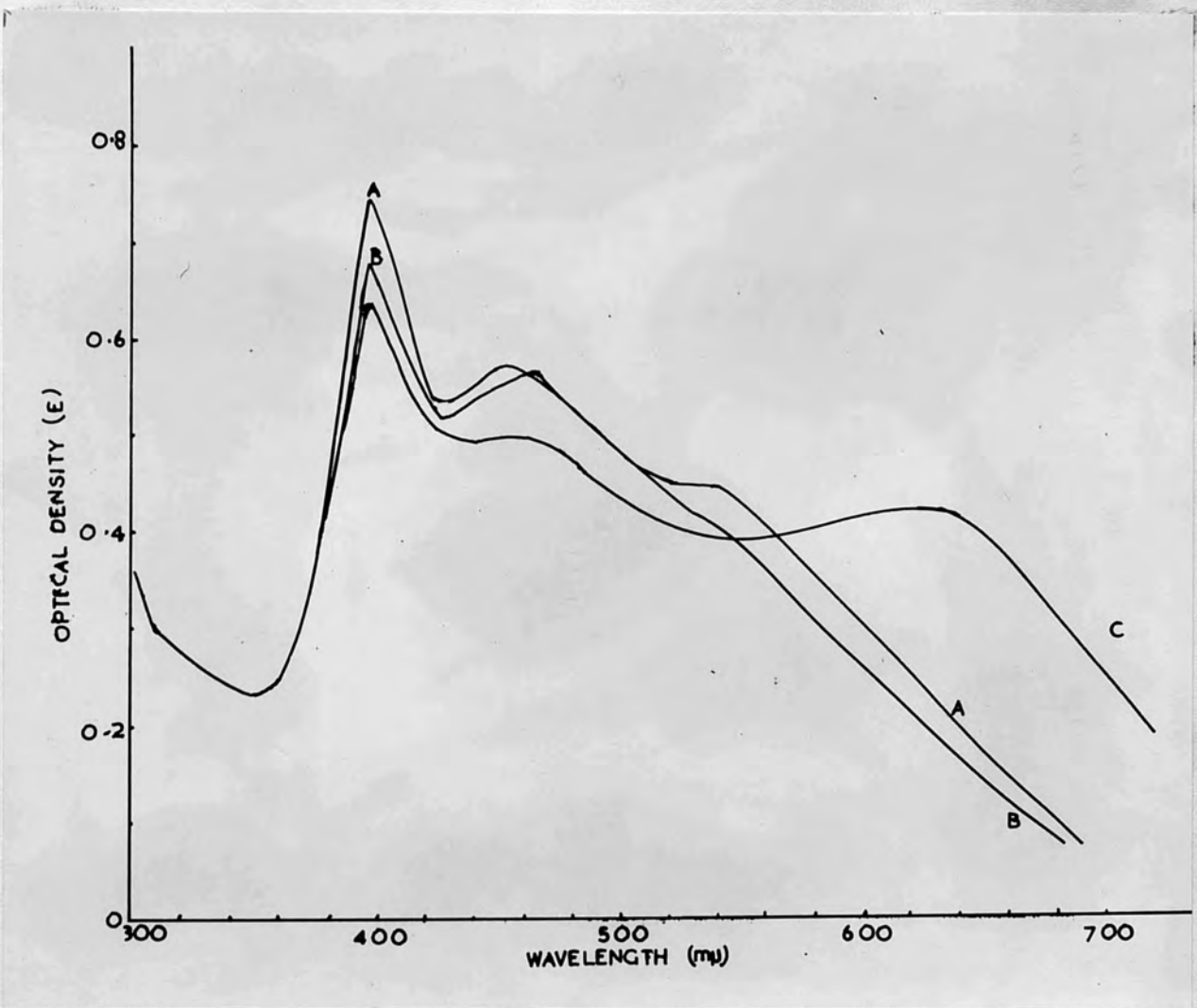


Figure 30. Spectrum of α-crustacyanin in 0.7M- Tris-0.005M-citric acid buffer, pH8.6, containing 6M- urea.

A. 2hr. incubation in the cold.
B. 24hr. incubation in the cold.
C. As in A, followed by dialysis against 5% NaCl for 24 hr.

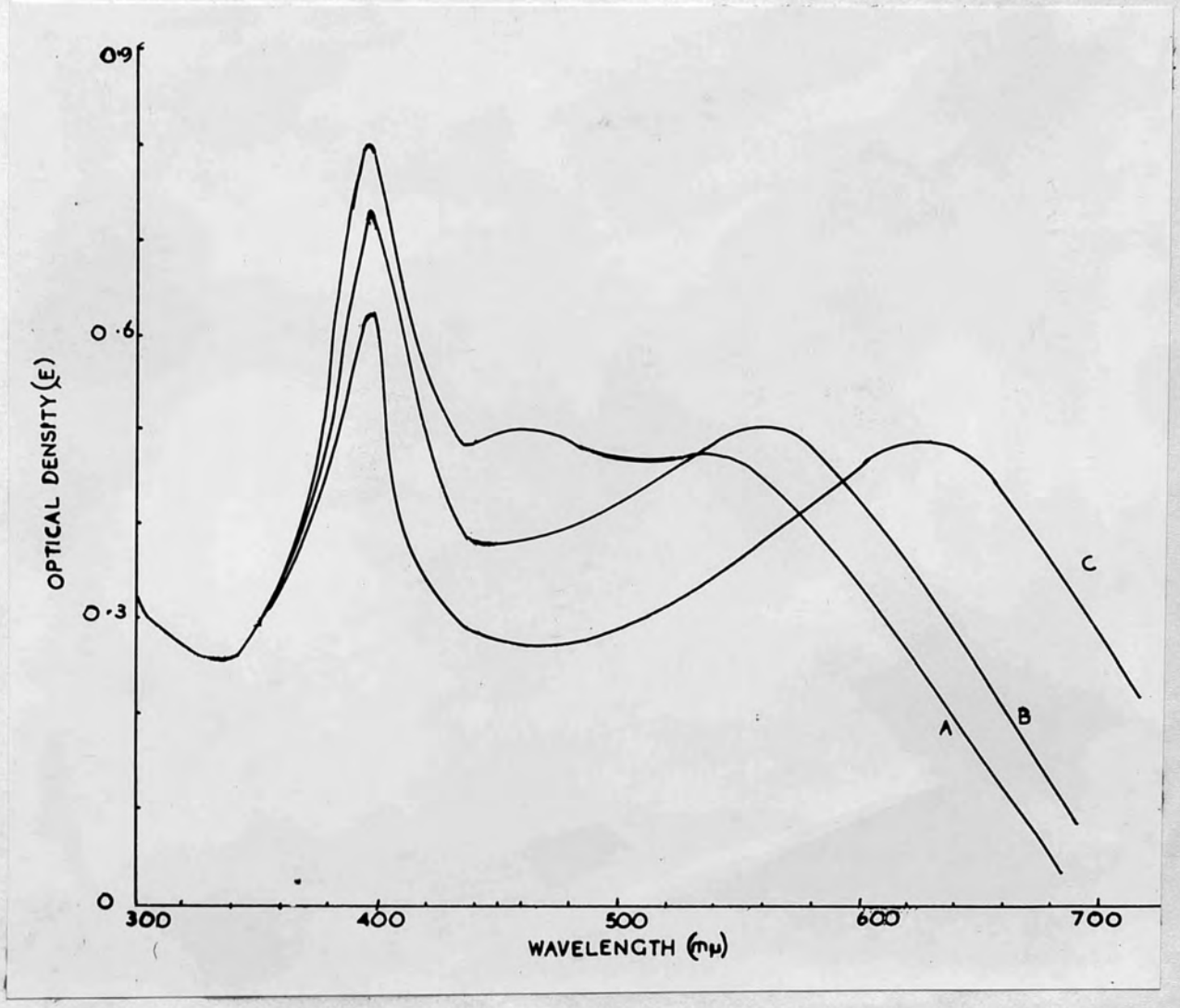


Figure 31. Spectrum of α-crustacyanin in 0.03M-phosphate buffer, pH7, containing 6M. urea.

A. 2hr. incubation in the cold.
B. 24hr. incubation in the cold.
C. As A, followed by dialysis against 5% NaCl for 24 hr.

The solutions were allowed to stand for 2hr. at room temperature and then subjected to thin-layer gel filtration (III.$F_{(iv)}$.) on Sephadex G75, G100 and G200. The layers were developed with 0.02M-phosphate buffer, pH7, containing 1M-NaCl. Short columns (15cm.) of Sephadex G200 were also used, India ink being employed to determine the elution volume of completely excluded protein.

Results Two components were observed at all three pH values in thin-layer gel filtration. One component (coloured faintly purple at pH7 and 8.6) was retained more strongly than serum albumin and the other, coloured yellow, was completely excluded from the Sephadex gels. There was little or no material having the molecular size of α-crustacyanin. The results obtained with crustacyanin treated with 6M-urea at pH7 are shown in Figs. 18 and 24b, c.

The strongly retained component, was of the same size as apocrustacyanin and β-crustacyanin (Figs. 18 and 24) with an estimated molecular weight of 39,000 $\pm$2,000 (three determinations).

On Sephadex G200 columns equilibrated with buffers (i) and (ii) above, the urea-treated crustacyanin gave a yellow band having the same elution volume as India ink and a faint purple band moving down the column at about 0.35 times the rate of India ink. The yellow component had a

sharp absorption maximum at 395mμ and did not revert to α-crustacyanin on standing. The purple band faded greatly during its passage through the column owing to removal of the carotenoid by the Sephadex.

(xviii) Electrophoretic studies

Method Crustacyanin was treated with urea, reduced and reduced-alkylated by a method similar to that used by Poulik (1960).

5-10mg. crustacyanin was dissolved and allowed to stand for 2hr. in:

(i) 1ml. electrophoretic buffer alone, and containing 6M- urea or 1N- mercaptoethanol.

(ii) 1ml. 0.07M-tris-0.005M- citric acid, pH8.6 containing (a) 6M- urea and 0.05M- iodoacetamide (alkylation)

(b) 6M- urea and 0.05M- mercaptoethanol (reduction).

The reduced-alkylated protein was prepared by making solution (ii)b 0.05M. with respect of iodoacetamide, followed by a further 2hr. standing at room temperature.

Samples were subjected to electrophoresis in starch gels made from buffer alone, from buffer containing 6M- urea, and from buffer containing 6M- urea-0.05M- mercaptoethanol. (III.$E_{(ii)}$.) cellulose acetate electrophoresis at pH7 was also used with the samples treated with urea.

Results Urea-treatment cleaved α-crustacyanin into a number of components, and reduction and reduction-alkylation

had profound effects upon the electrophoretic pattern obtained.

(a) Protein treated with 6M- urea

The electrophoretic pattern obtained at pH8.6 and pH7 in micro-electrophoresis on starch gel (the gels made without urea) and in cellulose acetate electrophoresis at pH7, was identical with that obtained for apoprotein preparations (Figs. 32, 33 and 25). A large amount of yellow material did not enter the gels and remained at the origin of the cellulose acetate strips. The main α-crustacyanin band was replaced by slower and faster migrating purple or purple-red components in starch gel electrophoresis. Some carotenoid was removed from the protein as it migrated into the gel, and remained adsorbed to the surfaces of the gel adjacent to the filter paper used to insert the sample. Overnight runs gave the same components as the shorter runs (Figs. 35a. and 32).

Shorter incubations with urea (15min.) before electrophoresis resulted in no decrease of the number of components observed (Fig.35a).

Starch gels containing 6M- urea gave essentially the same electrophoretic pattern, the faster moving components being more crowded together at pH8.6 (Fig.34).

The same electrophoretic pattern was obtained by inserting the crustacyanin sample into the urea gels without pretreatment (Figs. 35b, 36).

Fig. 32

Fig. 33

Figures 32 and 33 Micro-electrophoresis on starch gel.

Figure 32. Tris-citrate-borate, discontinuous buffer system, pH8.6. 20 Volts/cm. 3½hr. run.

Column 1 α-Crustacyanin treated with 6M- urea in electrophoretic buffer for 2hr.
2 Apocrustacyanin.

Figure 33. 0.03M-phosphate buffer, pH7. 20 Volts/cm. 16hr. run.

Column 1 α -crustacyanin treated with 6M- urea in 0.03M-phosphate buffer, pH7, for 2 hr.
2 Apocrustacyanin (5 acetone treatments)

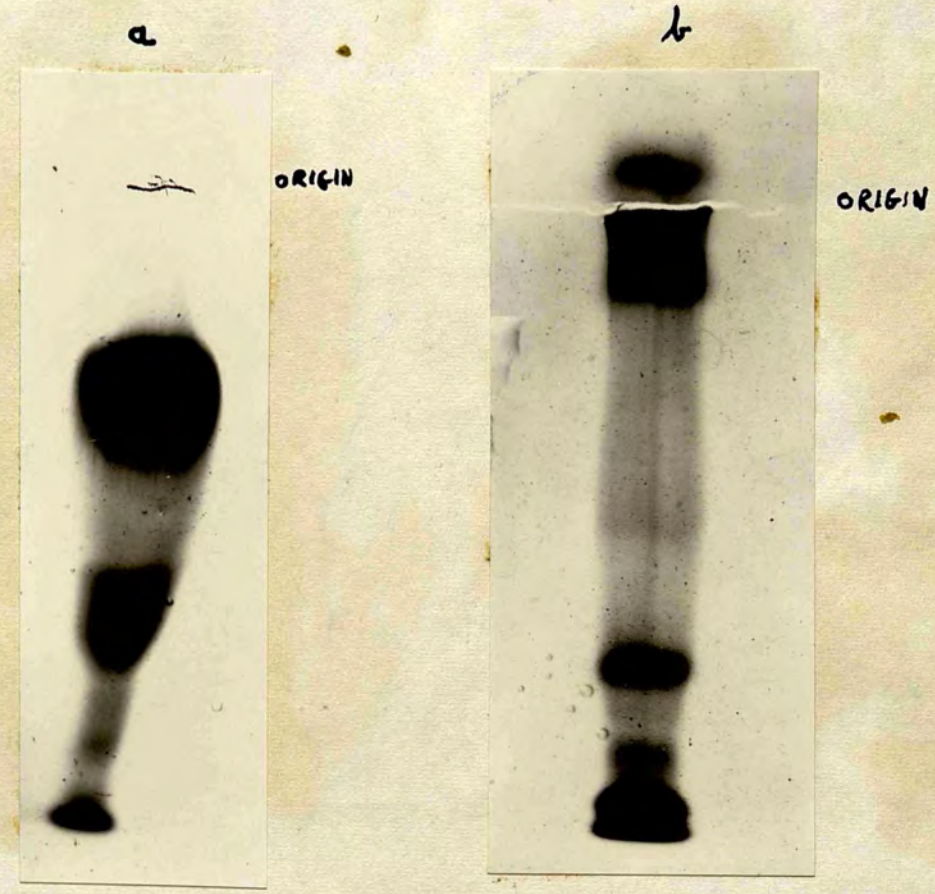


Figure 34. Micro-electrophoresis on starch gel.

Tris-citrate-borate, discontinuous buffer system, pH8.6. 20 Volts/cm. 3½hr. run.

(a) α-crustacyanin.

(b) Starch gel containing 6M- urea. α-crustacyanin treated with 6M- urea in electrophoretic buffer for 2hr.

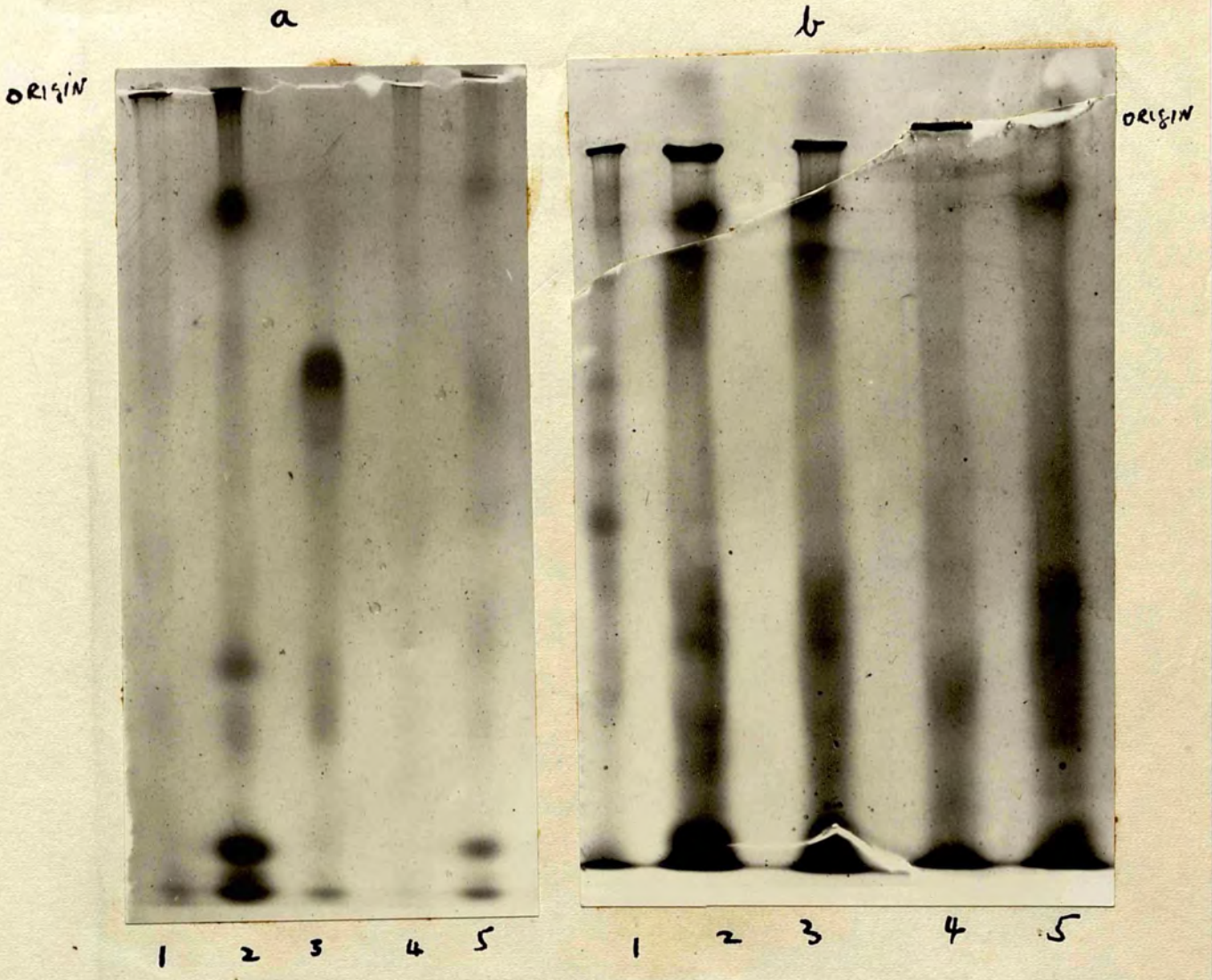


Figure 35 Starch gel electrophoresis.

Tris-citrate-borate, discontinuous buffer, pH8.6.
7 Volts/cm. 16hr. run.

(a) Starch gel

Column 1 Reduced-alkylated crustacyanin
2 α-crustacyanin treated with 6M- urea in electrophoretic buffer for 15min.
3 α-crustacyanin
4 Reduced crustacyanin
5 as 2, but incubated for 2hr.

(b) Starch gel containing 6M- urea

Column 1 Reduced-alkylated crustacyanin
2 α-crustacyanin
3 Alkylated crustacyani
4 Reduced crustacyanin
5 α-crustacyanin treated with 6M- ure in electrophoretic buffer for 2hr.

Fig.37(a) shows the results obtained using a starch gel made with formate buffer, pH3.1. The same pattern was obtained for the crustacyanin dissolved in formate buffer alone and for that containing 6M.- urea. A single colourless band, possibly consisting of a number of closely migrating bands, moved into the gel. A large amount of yellow material remained near the origin. In 6M.- urea-formate starch gels a number of slowly migrating bands were observed (Fig.37(b)); crustacyanin inserted directly into the urea gel was slightly less heterogeneous.

(b) Alkylated protein

Alkylated crustacyanin gave an electrophoretic pattern essentially similar to that for crustacyanin treated with urea (Fig. 35(b)), suggesting that free sulphydryl groups were absent from the components formed by treatment with urea.

(c) Reduced protein

α -crustacyanin, crustacyanin treated with 6M.- urea and reduced crustacyanin behaved similarly on electrophoresis in 6M.- urea-mercaptoethanol starch gels (Fig.36). The pattern was quite different from that obtained with crustacyanin treated with 6M.- urea in 6M.- urea starch gels, indicating that the components contained disulphide bands. There was no reduction in the number of components but the more slowly and more rapidly migrating components were

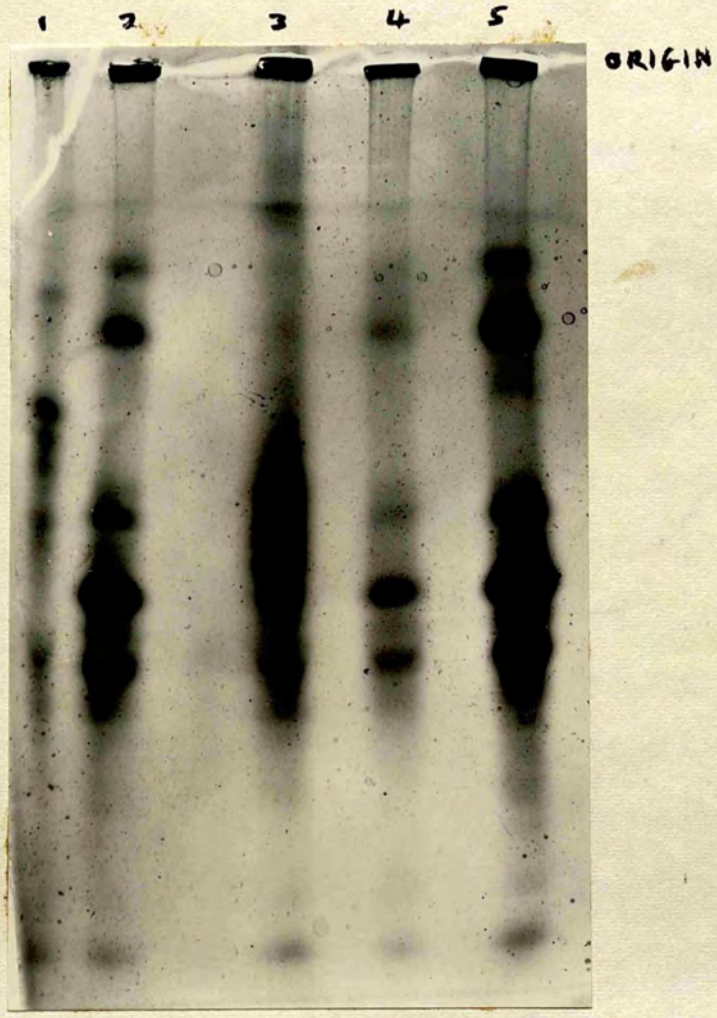


Figure 36 Starch gel electrophoresis.

Tris-citrate-borate, discontinuous buffer, pH8.6.
7 Volts/cm. 16hr. run.

Column 1. Reduced-alkylated crustacyanin.
2. α-crustacyanin.
3. Alkylated crustacyanin
4. Reduced crustacyanin.
5. α-crustacyanin treated with 6M- urea in electrophoretic buffer for 2 hr.

replaced by components of intermediate mobility.

Little of the reduced protein entered urea-starch gels made without mercaptoethanol (Fig.35(b)), and only the fastest moving component and a more slowly moving, diffuse component were observed.

The reduced protein gave a single component in urea-formate starch gels moving slowly into the gel. A large amount of protein did not enter the gel, as indicated by the strong staining at the origin.

With tris-citrate (Fig.35(a)) and formate starch gels (Fig.36(a)) most of the protein did not enter the gels. Similarly, in cellulose acetate electrophoresis little of the protein moved from the origin (Fig. 38).

Mercaptoethanol treatment in the absence of urea did not alter the electrophoretic behaviour of crustacyanin in cellulose acetate electrophoresis (Fig. 38).

(d) Reduced-alkylated protein

The reduced-alkylated protein gave separations distinct from the reduced protein and from crustacyanin treated with urea in both urea and urea-mercaptoethanol starch gels. A number of components were still observed. The major part of the protein did not enter the gels (Figs. 35(b), 36).

In urea-formate starch gels a single band with mobility similar to that of the reduced crustacyanin slowly moved

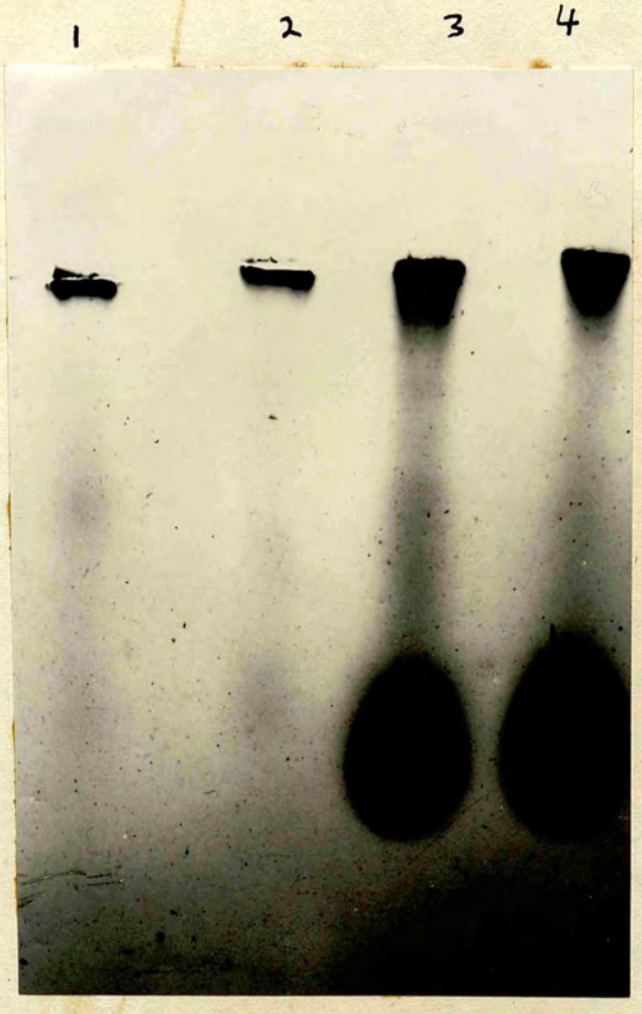
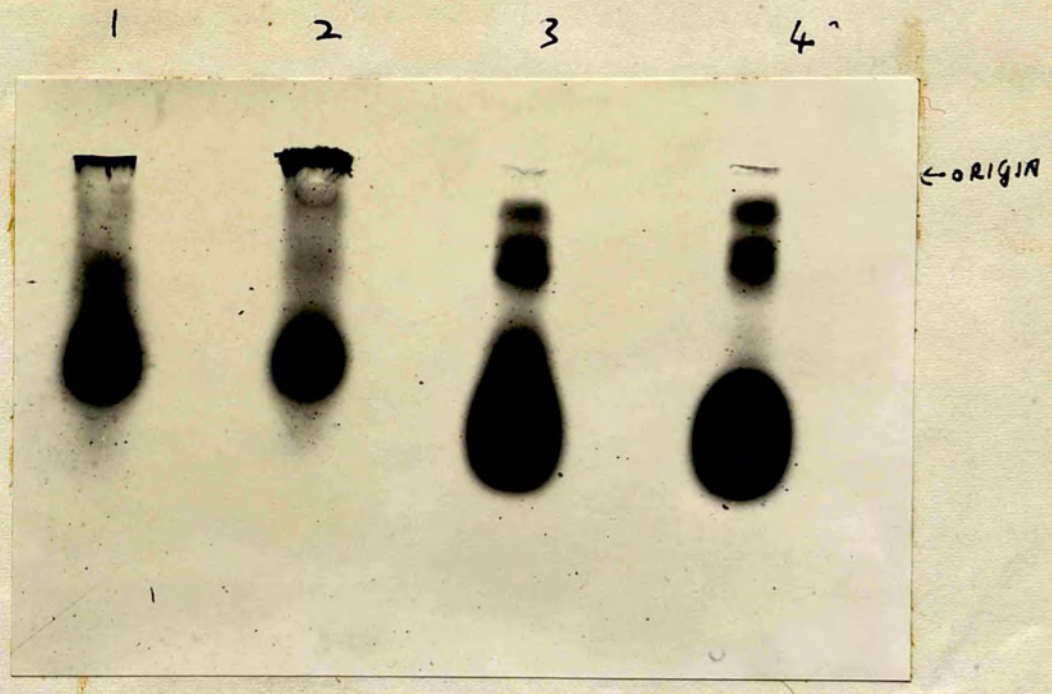


Figure 37 Starch gel electrophoresis
0.05M-formate buffer, pH3.1.
7 Volts/cm. 16hr. run.

(a) Starch gel.
(b) Starch gel containing 6M- urea.

Column 1 Reduced-alkylated crustacyanin
2 Reduced crustacyanin
3 α-crustacyanin treated with 6M- urea in electrophoretic buffer for 2hr.
4 α-crustacyanin.



into the gel. A considerable portion of the protein remained at the origin (Fig. 37(a)). Scarcely any of the reduced-alkylated protein moved into tris-citrate or formate gels made without urea (Figs. 35(a), 37(a)). The protein remained at the origin in cellulose acetate electrophoresis.

(e) Abnormalities

On one occasion, not since repeated, electrophoresis in urea starch gels using Poulik's discontinuous buffer system gave a greater number of components (Fig. 39). The reason for this was never elucidated.

Cyanate is in equilibrium with urea at alkaline pH, the equilibrium being most rapidly attained at higher temperatures (Stark, Stern & Moore, 1960). It is possible that overheating of the gel occurred during the run, resulting in the rapid attainment of the cyanate-urea equilibrium and carbamylation by the cyanate of free amino and sulphydryl groups of the protein. The great number of bands observed could then be components carbomylated to different degrees.

(xix) Composition

(a) Nitrogen content

Method III.H$_{(i)}$.

Result The nitrogen content was determined as 15.7 ± 0.3% (six determinations on two preparations).

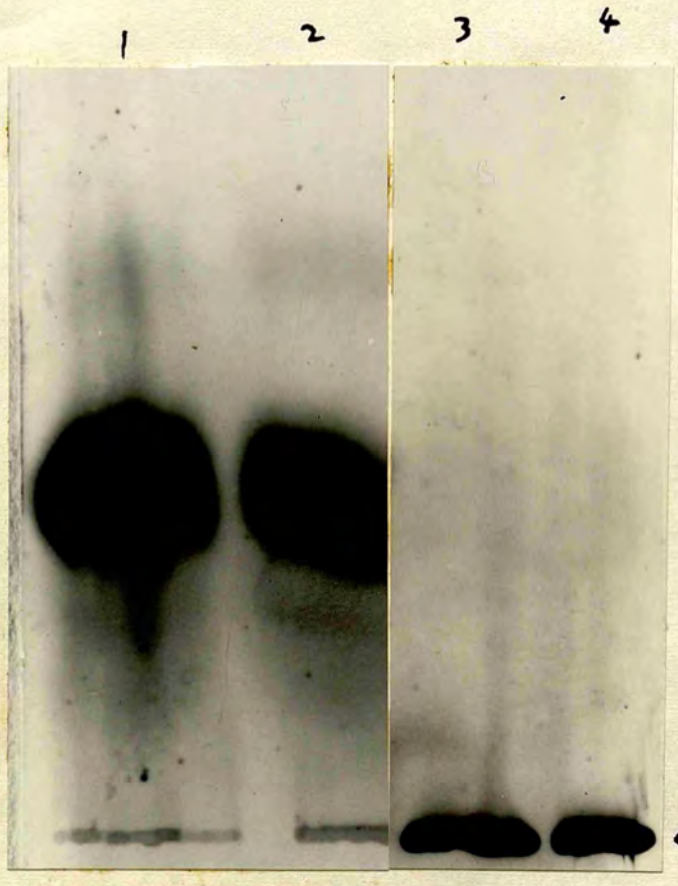


Figure 38 Cellulose acetate electrophoresis

0.2mA/cm. width strip. 5hr. run.

Column 1 α-crustacyanin
2 α-crustacyanin treated with 1M.-mercapto-ethanol in electrophoretic buffer for 2hr.
3 Reduced crustacyanin
4 Reduced-alkylated crustacyanin

Figure 39 Starch gel electrophoresis

Tris-citrate-borate-6M.-urea, pH8.6, 7 Volts/cm. 16hr. run.

3 different α-crustacyanin preparations

(b) Amino acid components

Method III.H$_{(ii)}$.

Results 16 amino acids were identified (Fig.40): glycine, leucine, tyrosine, serine, threonine, glutamic acid, aspartic acid, alanine, phenylalanine, lysine, arginine, histidine, valine, proline, methionine sulphone, cysteic acid, leucine-isoleucine.

(c) Tyrosine and tryptophan content

Method III.H$_{(iii)}$. Apocrustacyanin was prepared as in IV.A$_{(x)}$.) using five acetone precipitations. The protein concentration was estimated from the 280m absorption and the $E_{280}^{0.1\%}$ value (IV.A$_{(v)}$.). The solution was diluted with an equal volume of 0.2N-NaOH for the determinations.

Results The contents of tyrosine and tryptophan were found to be:

Tyrosine	5.6 ± 0.3%	(six determinations using two apoprotein preparations)
Tryptophan	1.2 ± 0.1%	

(d) Protein-bound carbohydrate

Method III.H$_{(iv)}$.

Sephadex was not employed in the preparation of the freeze-dried crustacyanin, as it has a slight solubility in water (Flodin, 1962) and would thus lead to errors in the determinations. 3mg. crustacyanin sample

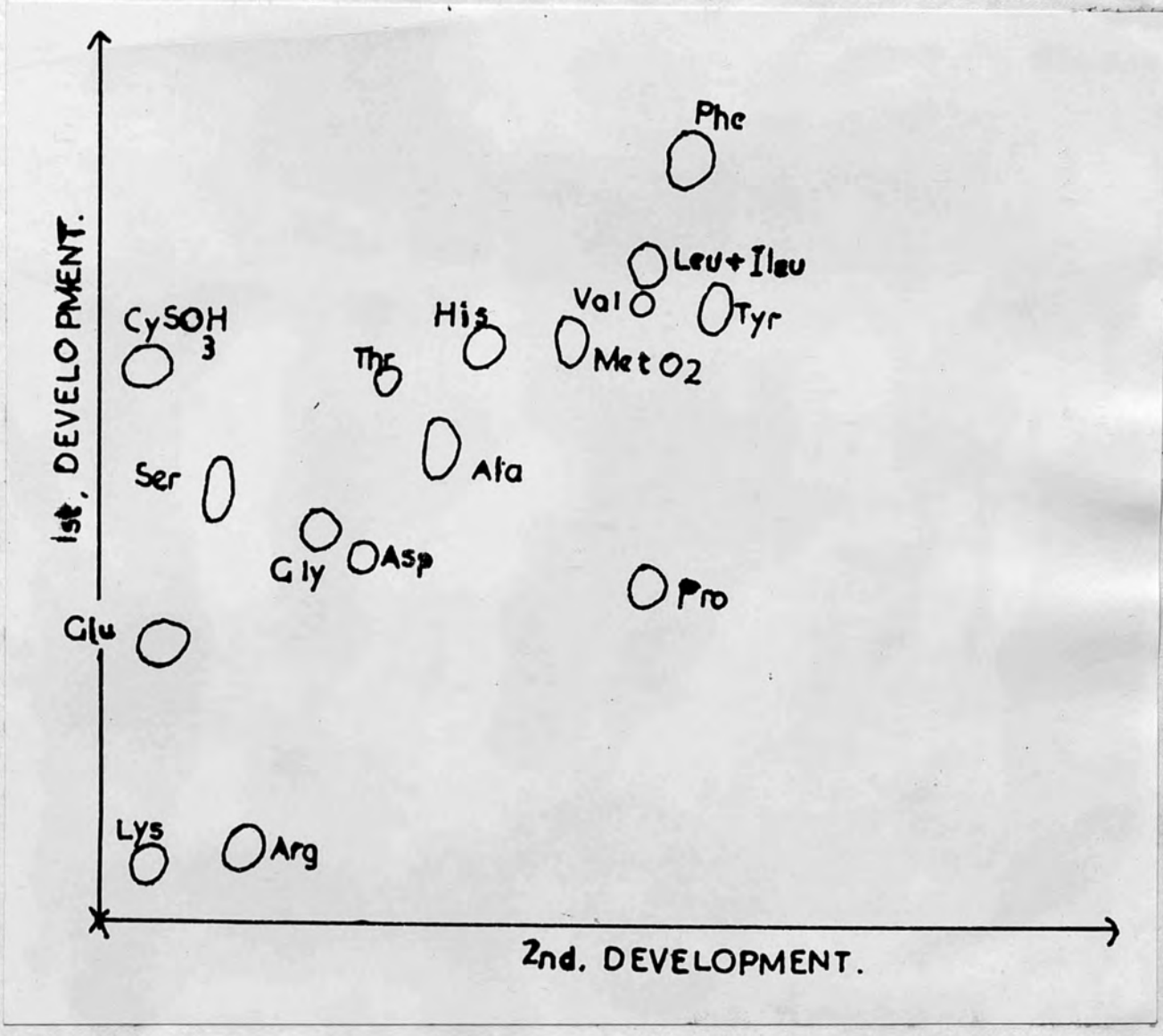


Figure 40. Amino acid composition of an acid hydrolysate of ∝-crustacyanin. Thin-layer chromatography on silica gel G.
Solvents. 1st Development. $CHCl_3$/MeOH/17% NH_3 (2/2/1, v/v) to 15cm.
2nd Development. Phenol/H_2O (3/1, w/w) to 15cm.

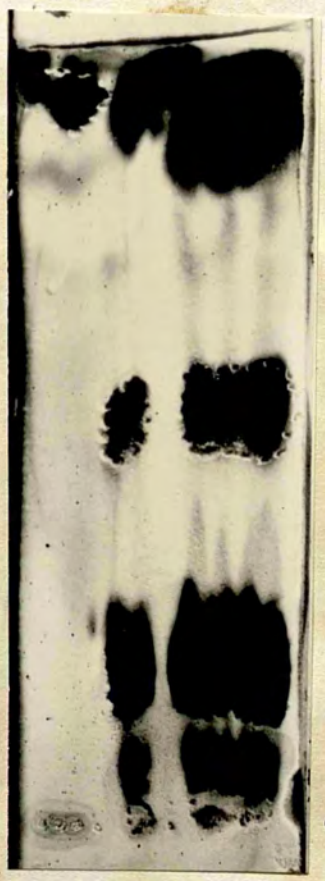


Figure 41 Thin-layer chromatography on plates of silica gel G. Solvent $CHCl_3$-MeOH-H_2O (80/30/3, v/v). Development distance 10cm. Sprays (i) Ninhydrin(ii) Ammonium molybdate-perchloric acid

Column 1. Lipid extract of α-crustacyanin

2 and 3. Hen's egg phospholipid

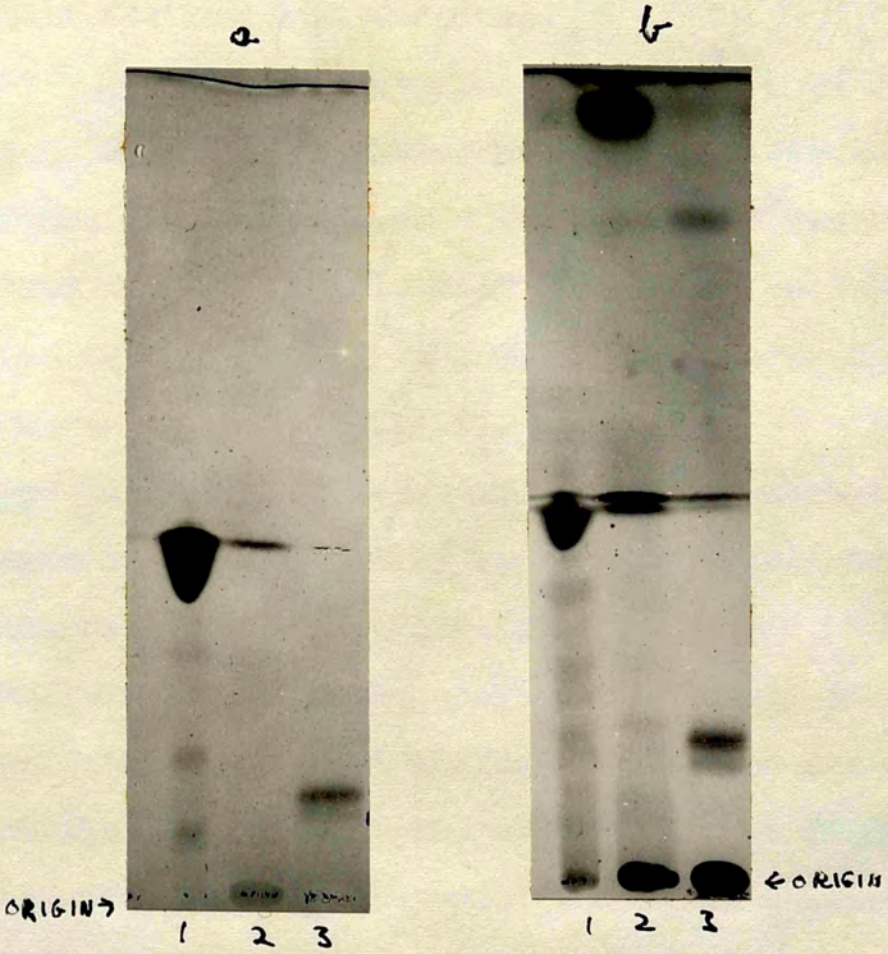


Figure 42 Thin-layer chromatography on plates of silica gel G. Solvent: Petroleum ether/ether/acetic acid (60/40/1, v/v). Development distance 10cm.

(a) Sprayed for cholesterol and cholesterol esters.
(b) Sprayed with ammonium molybdate-perchloric acid.

Column 1. Cholesterol (commercial preparation).
2. Lipid extracted from the carotenoprotein of the ovary of C.maenas.
3. Lipid of α-crustacyanin.

were extracted with $CHCl_3$-MeOH (2/1, v/v) to remove the carotenoid.

Results Crustacyanin contained less than 0.05% hexose and less than 0.02% hexosamine (approximate limits of detection for the 3mg. samples).

(e) Lipid content

Method 20mg. freeze-dried crustacyanin was used for the determination (III.$H_{(v)}$.). The extracted material was dissolved in 0.1ml. $CHCl_3$-MeOH (2/1, v/v) and 20 µl. analysed for phospholipids and neutral lipids using thin-layer chromatography. (III.$I_{(iv)}$.).

Results About 1% (0.2mg.) of lipid was extracted, corresponding approximately to the carotenoid content (1 mole. astaxanthin/35.700g. protein).

Thin-layer chromatography revealed only traces of phospholipid and neutral lipid. Using the phospholipid solvent the carotenoid moved at the solvent front, a faint phospholipid staining component moving just below it (Fig.41). With the neutral lipid solvent a trace of cholesterol (Fig.42(a)) and neutral lipid (Fig.42(b)) was revealed; two carotenoid bands moved near the origin.

Assuming that the phospholipid and neutral lipid spots contained 1 µg. of lipid (limit of detection, Truter (1962)) it was calculated that the crustacyanin preparation contained about 0.05% lipid (besides the carotenoid).

(f) Prosthetic group

Previous workers have identified the prosthetic group of crustacyanin as astaxanthin (Kuhn & Sörensen, 1938; Wald et al., 1949; Goodwin & Srisukh, 1949; Jencks & Buten, 1964). Jencks & Buten (1964) have shown that the prosthetic group of the yellow and purple chromoproteins (IV.$A_{(iii)}$.) is astaxanthin.

Method A solution of α-crustacyanin (or of the yellow or purple chromoproteins) in 0.2M-phosphate buffer, pH7, was treated with 4 vols. acetone and 1 vol. petroleum ether; on addition of water the dissociated carotenoid passed into the upper phase. The petroleum ether layer was washed four times with distilled water and dried over an -hydrous $CaCl_2$. The chromatographic behaviour on columns of $CaCO_3$ and $Ca(OH)_2$ was investigated.

Results The carotenoid was hypophasic in the system 90% MeOH/petroleum ether and had the single banded spectrum of astaxanthin, with maximum at 476mμ in petroleum ether and 500mμ in CS_2.

The carotenoid behaved as astaxanthin on chromatography. Thus it was dated from calcium carbonate as a single component on development with petroleum ether containing 2% MeOH, and was only eluted from calcium hydroxide with acidifed methanol.

(xx) General properties

α-crustacyanin was precipitated quantitatively from solution in 0.2M-phosphate buffer, pH7, with 5% T.C.A. or 0.2M-$HClO_4$. Treatment with organic solvents (e.g. acetone or alcohol) liberated the carotenoid, turning the solution first purple then red. Ether had no immediate effect on the freeze-dried protein, but on standing in the cold for several weeks the protein turned red and small amounts of the carotenoid were extracted into the ether.

Loss of carotenoid occurred on dialysis, and on freeze-drying if light was not rigorously excluded.

The protein could be adsorbed and eluted from alumina gel and hydroxyapatite. It was not adsorbed onto $CaCO_3$, with which it is in close contact within the shell.

ATPase activity, recently found for rhodopsin (McConnell & Scarpelli, 1963), was not associated with the purified crustacyanin.

The absorption of α-crustacyanin remained unaltered between pH4.5 and 9.0. Below pH4 it turned yellow, fading rapidly in the light. The change was reversed by immediate neutralisation but not if the solution were allowed to stand. A solution in 1% NaCl adjusted to pH1 at room temperature by the addition of conc. HCl recovered 95% of the 630mμ absorption when neutralised with 0.2M-

phosphate buffer pH7 within 1min.; 40% of the 630mμ absorption was recovered after 10min. standing and after 30min. the change was completely irreversible. Above pH9 crustacyanin turned irreversibly orange-red.

Solutions of crustacyanin in 0.2M-phosphate buffer, pH7, turned red on heating at 66°, giving a single absorption maximum at 460mμ with slight inflexions at 530 and 330mμ.

Exposure of a 0.2M-phosphate buffer solution (0.1mg./ml.) resulted in slow fading. After 36hr. about 4% of the extinctions at 630 and 360mμ were lost; the peak at 320mμ became an inflexion and an inflexion at 610mμ appeared.

CHAPTER V

OVOVERDIN, THE CAROTENOPROTEIN OF THE LOBSTER OVARY AND EGGS

Introduction

As mentioned in the introduction (I.D.), Newbigin (1897, 1898) was first to extract the green lobster pigment from the eggs and ovary of lobster (Homarus vulgaris) and to investigate its properties. Further studies on the pigment have been carried out by Kuhn & Sörensen (H.vulgaris, 1938), Stern & Salomon (H.americanus, 1938) and Goodwin (H.vulgaris, 1951). The molecular weight of ovoverdin, its properties, spectrum, and structure, have been discussed (I.D.).

Experimental

A. Preparation and purification

Ovoverdin was obtained in a form homogeneous in cellulose acetate electrophoresis by removal of interfering protein with cellulose ion-exchangers, followed by calcium phosphate gel chromatography, fractional ammonium sulphate precipitation and dialysis against dilute phosphate buffer. It was found essential to perform all operations in the dark and cold. Where possible, solutions of ovoverdin were kept under nitrogen.

50g. ovary was washed thoroughly with distilled water and homogenised for 15sec. in a Waring blendor with

300ml. 0.05M-phosphate buffer, pH7. The mixture was allowed to stand for 20min., centrifuged at 13,000r.p.m. for 20min., and the yellow lipid layer at the surface removed. The green supernatant was stirred successively for 10min. with 10g. DEAE-cellulose and 10g. CM-cellulose, followed by centrifugation at 13,000r.p.m. for 10min.

75ml. calcium phosphate gel was added to the supernatant with stirring and the pH adjusted to 5.5 with silute HAc. More gel was added, if necessary to adsorb all the chromoprotein. The gel was washed four times with 5% NaCl and eluted twice with 0.2M-buffer pH7, exactly as for crustacyanin (IV.$A_{(i)}$.a.).

Ammonium sulphate salting-out curves (III.C.) revealed the presence of two fractions having an identical spectrum in the protein purified by chromatography on calcium phosphate gel. The first precipitated between 36 and 44% $(NH_4)_2SO_4$ saturation, and the second between 44 and 55% saturation (Fig.43); points between 50 and 55% $(NH_4)_2SO_4$ saturation were not accurate as the carotenoprotein did not sediment readily between these concentrations.

Sat. $(NH_4)_2SO_4$ solution was added to the material eluted from the calcium phosphate gel to bring it to 44% saturation. The solution was allowed to stand for 15min.

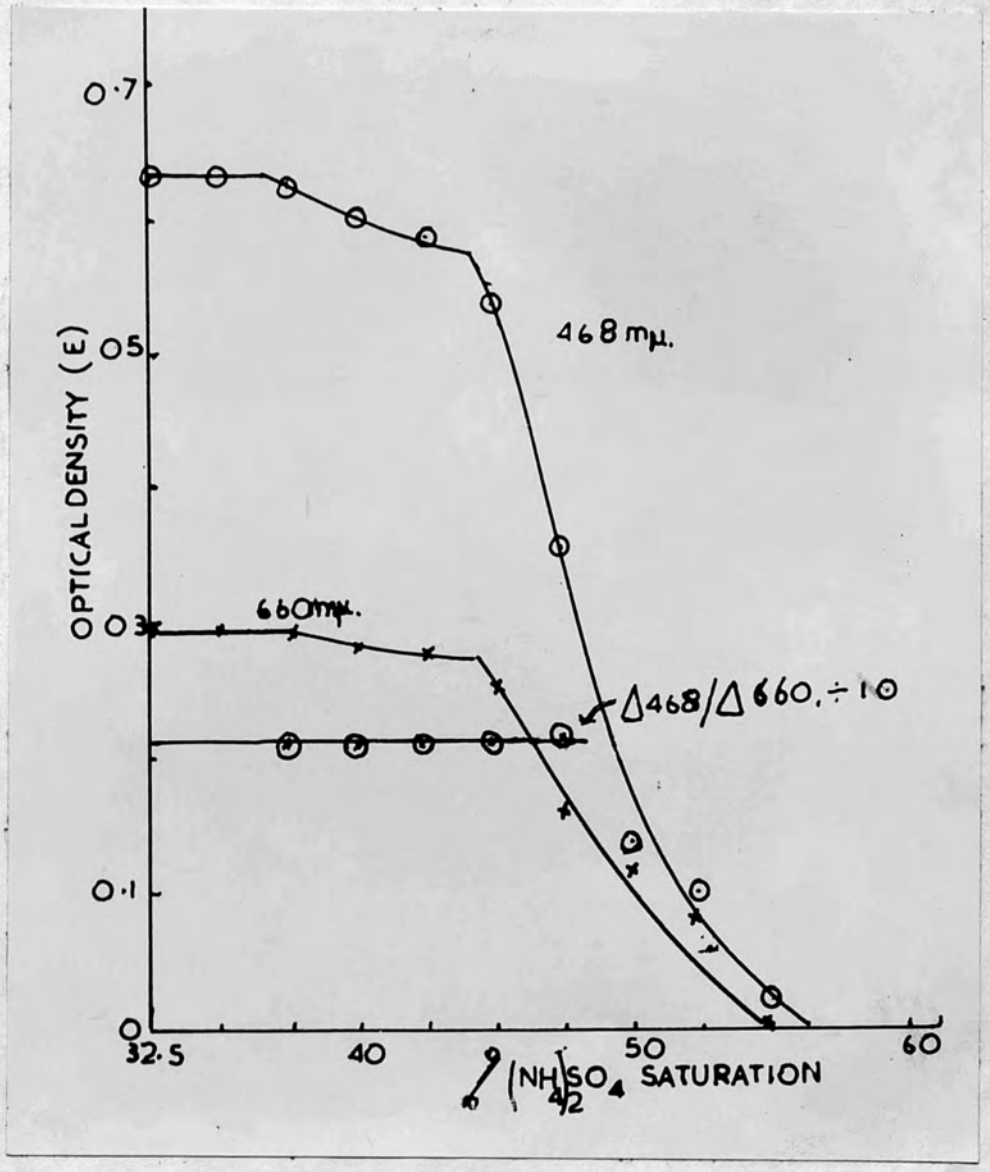


Figure 43. $(NH_4)_2SO_4$ saturation curve of ovoverdin, purified by negative DEAE- and CM-cellulose chromatography and by calcium phosphate gel chromatography.
Temperature: 4°
Buffer: 0.1M-phosphate buffer, pH7
Concentration: 0.14mg. ovoverdin/ml.

centrifuged at 13,000r.p.m. for 20min. and the precipitate discarded. Further solid $(NH_4)_2SO_4$ was added to the supernatant to bring it to 55% saturation, and the solution centrifuged for 30min. at 13,000r.p.m. The chromoprotein was removed from the surface, dissolved in 5-10ml. 0.02M-phosphate buffer, pH7, and dialysed for four hours with continual agitation against 0.00125M-phosphate buffer, pH7. Only a trace of ovoverdin remained in solution. The precipitated ovoverdin was centrifuged down and washed with 20ml. 0.00125M-phosphate buffer, pH7. Samples of the precipitate were dissolved in an appropriate buffer and the homogeneity tested (V.B.). 10ml. distilled water were added to the remainder of the precipitate and the suspension dialysed against 5l. distilled water for two days (two changes daily); considerable fading of the solution occurred. The carotenoprotein turned red on freeze-drying over conc. H_2SO_4 (IV.$A_{(iv)}$.a.) and did not redissolve.

A yield of 2.8g. freeze-dried ovoverdin per 100g. ovary was obtained.

On four occasions the tips of the lobster claws were found to be coloured green. A green chromoprotein with a spectrum identical with that of ovoverdin was extracted with 0.05M-phosphate buffer.

B. Homogeneity

Method Cellulose acetate electrophoresis was employed (III.$E_{(i)}$.).

Results Ovoverdin, prepared as above, was found to be homogeneous in cellulose acetate electrophoresis at pH7, 8.6 and 4.5 (Fig.44). At pH4.5 the protein slowly denatured, resulting in trailing of the band. The ovoverdin band stained positively for carbohydrate. The presence of lipid in the protein was indicated by the appearance of an opaque line in the position of the protein on drying the cellulose acetate strips (1960).

C. Absorption Spectrum

The spectrum of ovoverdin solutions were recorded after the fractional $(NH_4)_2SO_4$ precipitation step of the purification procedure.

In the ultraviolet region of the spectrum there was a maximum at 278mμ and an inflexion at about 330mμ.

Two strong absorption bands were evident in the visible region. The lower wavelength band had an absorption maximum at about 466mμ with inflexions at about 440 and 500mμ while the higher wavelength band consisted of an almost smooth curve with its centre in the 660mμ region.

The absorption spectra of different preparations,

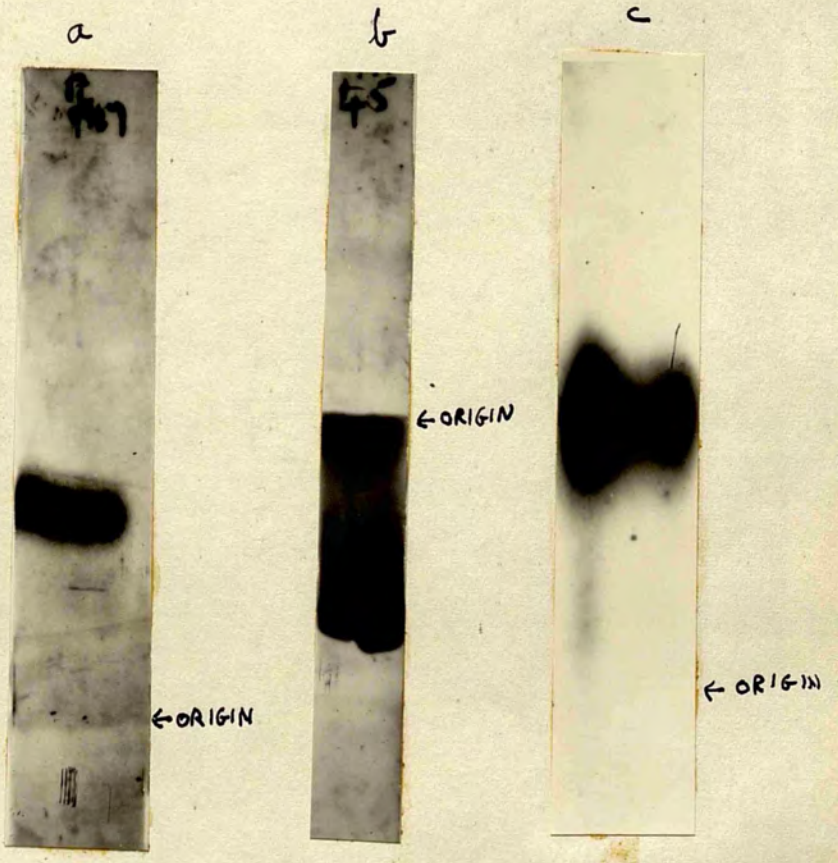


Figure 44 Cellulose acetate electrophoresis of ovoverdin.

(a) 0.05M-phosphate buffer, pH7. 5hr. run.
(b) 0.05M-acetate buffer, pH4.5. 3½hr. run.
(c) 0.07M-barbitone buffer, pH8.6. 4hr. run.

0.2mA/5cm. width strip.

TABLE III

Preparations of Ovoverdin

Date of Preparation	Material	E280/466	E466/660	E280/466+660
1963 April	Frozen for 1 month	2.28	2.39	1.53
May	Frozen for 2 months	1.91	2.18	1.32
May	Frozen for 2 months	2.06	2.22	1.42
1964 Jan.	Frozen for 2 weeks	2.00	2.14	1.36
Jan.	Fresh	2.3	1.48	1.41
May	Frozen for 5 days	1.54	2.81	1.46

compared by adjusting the extinctions at 466mμ to the same value, were not superimposable. Large differences were apparent in the values for E466/660 and E280/466. The values of E280/466+660 were about the same (1.42±.08) for preparations made at different times of the year (Table III).

Minor differences were observed in the 460mμ region of absorption; curves were almost, but not quite, superimposable. The maximum varied between 464 and 468mμ for different preparations. The spectrum in the 660mμ region varied considerably for different preparations. In some cases the curve had a maximum at 650 mμ with a large inflexion at 670mμ ; in others it was smooth with maximum at 670mμ or 660mμ and in one preparation an inflexion was observed at 690mμ (Figure 45).

The value of $E_{280}^{0.1\%}$, determined by measuring the 280mμ absorption of a known volume of the ovoverdin solution, freeze-drying, and weighing, was found to be 1.05.

D. Minimum Molecular Weight

Method The minimum molecular weight was calculated from the protein and astaxanthin content of purified preparations.

1ml. samples of an ovoverdin solution (25mg./ml.) (purified as in section A as far as the fractional

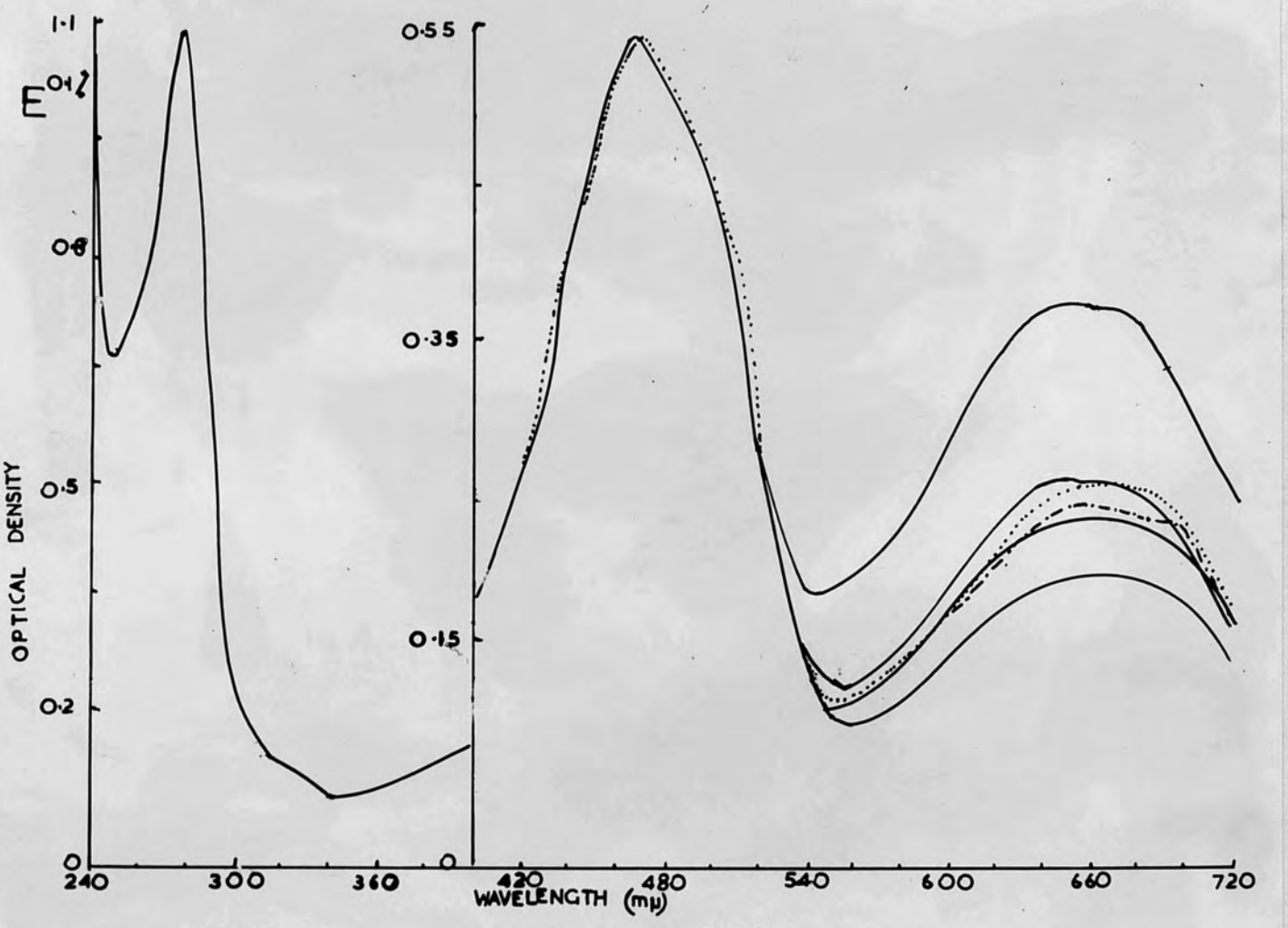


Figure 45. Spectra of ovoverdin preparations in 0.05M-phosphate buffer, pH7. Spectra adjusted to the same optical density at the 472mμ maximum. The curve in the 240-400 mμ region corresponds to the dotted curve in the 400-720mμ region.

$(NH_4)_2SO_4$ step) in 0.05M-phosphate buffer, pH7, was diluted to 10ml. with pyridine, the protein centrifuged down and the extinction at 495mμ measured. The astaxanthin content was estimated assuming a molecular extinction coefficient of 115,000 for astaxanthin (Cheesman, 1958). Another 1ml. sample was diluted to 10ml. with water and the extinctions at 280, 466 and 660mμ measured.

The concentrating of protein in the ovoverdin solution was estimated as follows: the ovoverdin preparation was thoroughly dialysed against distilled water, the extinction at 280mμ measured, a known volume (containing 0.75g. ovoverdin) freeze-dried, and the protein weighed. The value of $E_{280}^{0.1\%}$ was calculated and the amount of protein in the ovoverdin preparation determined from the 280mμ absorption.

Results The minimum molecular weight of ovoverdin was found to be 168,000 ± 4,000 (four determinations).

Table IV shows the result of addition of pyridine to the chromoprotein solution. The extinction value at 495mμ is about 90% of the sum of the extinctions at 466 and 660mμ for the aqueous solution.

TABLE IV

1ml. Ovoverdin solution diluted to 10ml. with A. Water and B. Pyridine

	A.			B.
Wavelength (mμ)	466	660	466+660	495
Extinction	0.53	1.17	1.7	1.53±.03 (4 dets.)

E. Molecular Size

Method The molecular size of ovoverdin was compared with the three bovine serum fractions on a thin layer of Sephadex G200 (III.$F_{(iv)}$.). The layer was developed with 0.02M-phosphate buffer, pH7, containing 1M-NaCl. Mixtures of ovoverdin and crustacyanin were separated on short (15cm.) Sephadex G200 columns (III.$F_{(ii)}$.).

Results Ovoverdin moved behind crustacyanin on thin-layer and column gel filtration; it moved ahead of the γ-globulin serum fraction on thin-layers (Figure 46).

The chromoprotein corresponded in size to a globular protein molecule with a molecular weight of 280,000 ± 16,000 (Seven determinations).

F. Composition

(i) Nitrogen content

Method III.$H_{(i)}$. The nitrogen content of freeze-dried ovoverdin and lipid-free ovoverdin (III.V.) were determined.

Results Ovoverdin was found to have a nitrogen content

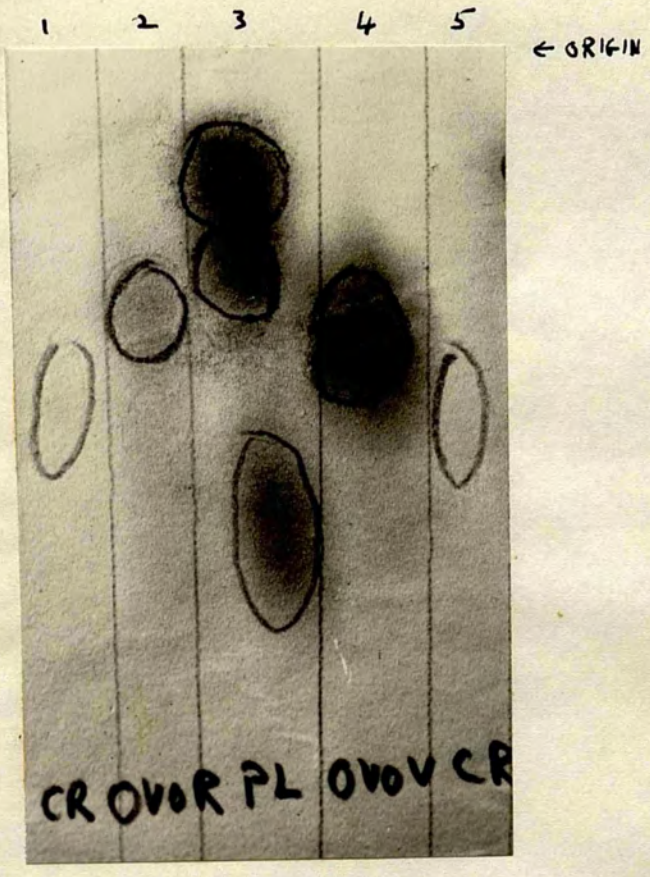


Figure 46. Thin-layer gel filtration on Sephadex G200. Plates developed with 1M-NaCl/0.02M-phosphate buffer, pH7, for 6hr.

Columns 1 and 5 α-crustacyanin
2 degraded ovorubin
3 bovine serum
4 ovoverdin

of 11.3 ± 0.2% (seven determinations on three preparations).

The lipid-free protein had a nitrogen content of 15.7 ± 0.4% (four determinations from two preparations).

(ii) Lipid content and composition

The lipid content was determined as described earlier (III.V.). The lipid extracts were analysed for cholesterol, nitrogen and phosphorus (III.$I_{(i)}$,(ii),(iii).).

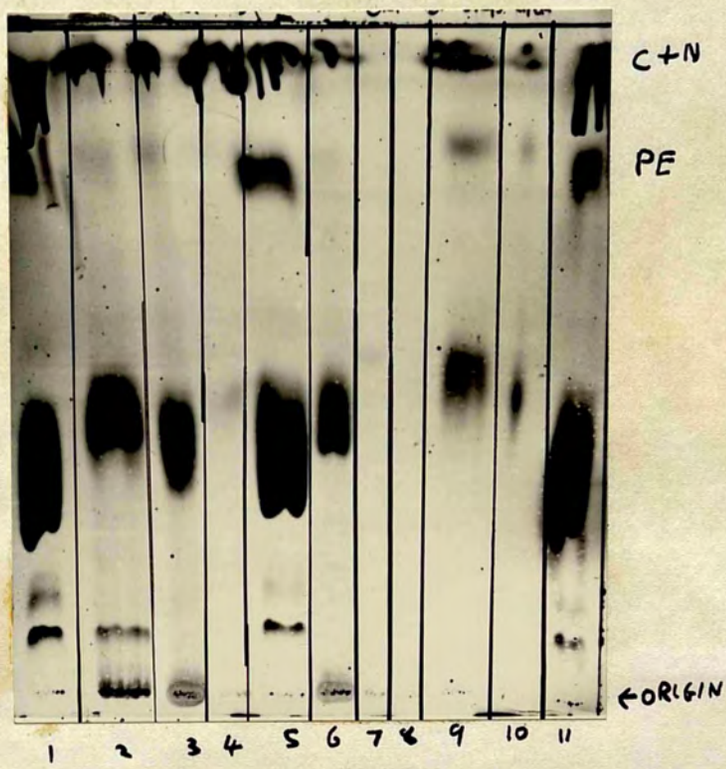
0.2mg. ovoverdin was extracted in a Soxhlet apparatus successively with ether and $CHCl_3$-MeOH (2/1, v/v) and the extracts examined for lipids by thin-layer chromatography (III.$I_{(iv)}$.).

Results Ovoverdin was found to contain 22 ± 3% lipid (three determinations on three preparations).

The extracted lipid was found to contain: 19.0 ± 1.5% cholesterol and cholesterol esters (six determinations on three preparations); 2.57 ± .09% phosphorus, corresponding to a phospholipid content of 65 ± 2.5%; 1.16 ± 0.06% nitrogen and thus an n/P ratio of 1.05/1.

A number of phospholipids and neutral lipids were separated by thin-layer chromatography of the lipid extracts (Figs. 47, 48 and 49).

Phosphatidyl ethanolamine was identified using the phospholipid solvent to develop the plates (Figs. 47 and 48). Additional phospholipids, occurring also in the hen's egg, were present. The neutral lipid and carotenoid moved as



C = carotenoid: PE = phosphatidyl ethanolamine: N = neutral lipid

Figure 47. Thin-layer chromatography on silica gel G.

Solvent: $CHCl_3$-MeOH-HAC-H_2O (25/15/4/2, v/v).

Development distance: 10cm.

Sprays: (i) Ninhydrin.
(ii) Ammonium molybdate-perchloric acid.

Columns 1,5 and 11. Hen's egg phospholipid
4. Ether extract of ovoverdin
2 and 3. $CHCl_3$-MeOH extraction of ether-extracted ovoverdin.
6. $CHCl_3$-MeOH extract of ether-extracted C.maenas (ovary) carotenoprotein.
7. Ether extract of C.maenas (ovary) carotenoprotein.
8. Ether extract of scallop (ovary) carotenoprotein.
9 and 10. $CHCl_3$-MeOH extract of ether-extracted scallop carotenoprotein.

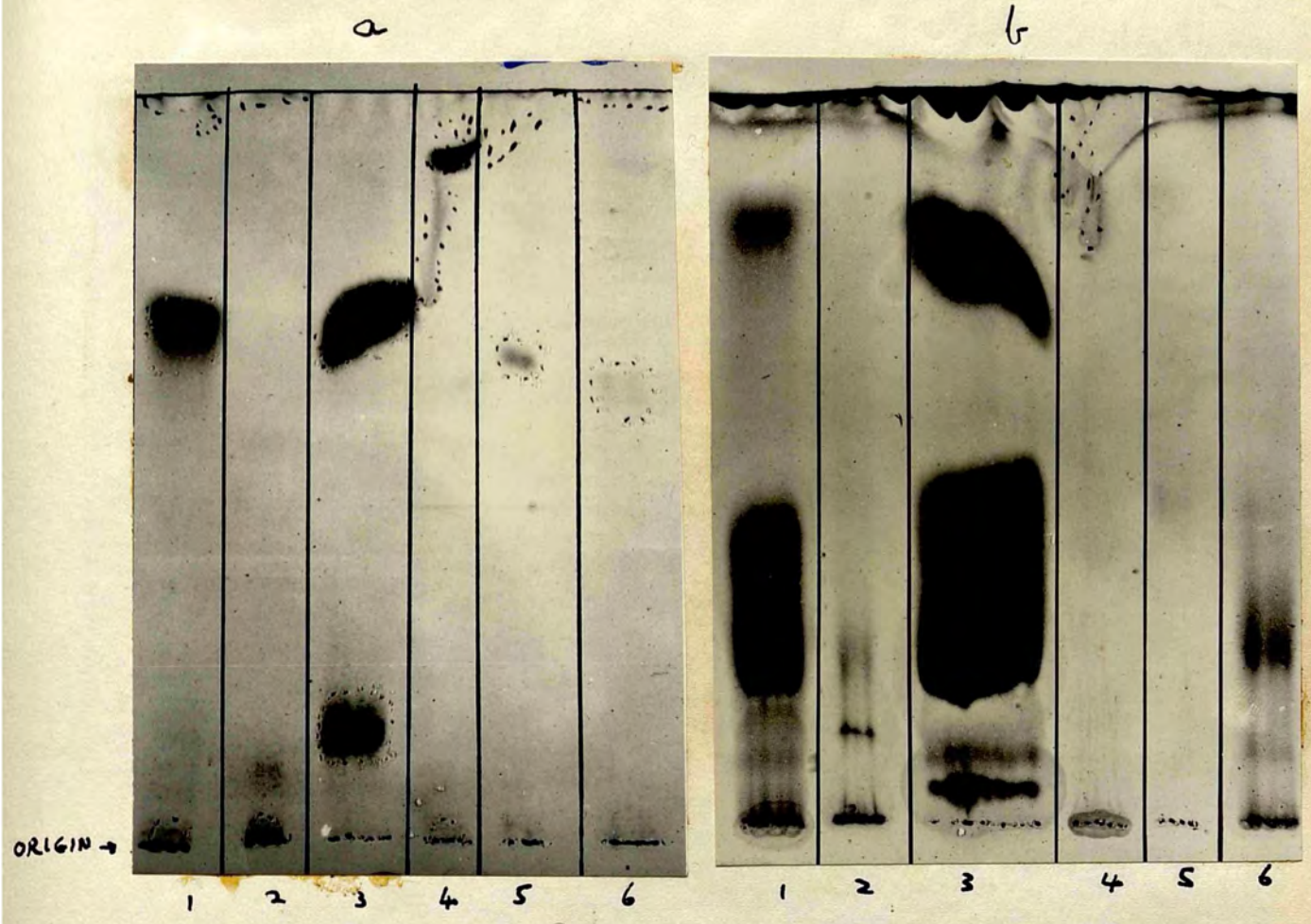


Figure 48. Thin-layer chromatography on silica gel G.
Solvent: $CHCl_3$-MeOH-H_2O (80/30/3, v/v).
Development distance: 10cm.
(a) Sprayed with ninhydrin.
(b) Sprayed with ammonium molybdate-perchloric acid.

Column 1. $CHCl_3$-MeOH extract of C.maenas (ovary) carotenoprotein.
2 & 6. $CHCl_3$-MeOH extract of ovoverdin.
3. Hen's egg phospholipid.
4. $CHCl_3$-MeOH extract of crustacyanin (carotenoid trails near solvent front).
5. $CHCl_3$-MeOH extract of scallop (ovary) carotenoprotein.

C = Carotenoid: PE = phosphatidylethanolamine:
LPE = lysophosphatidylethanolamine.

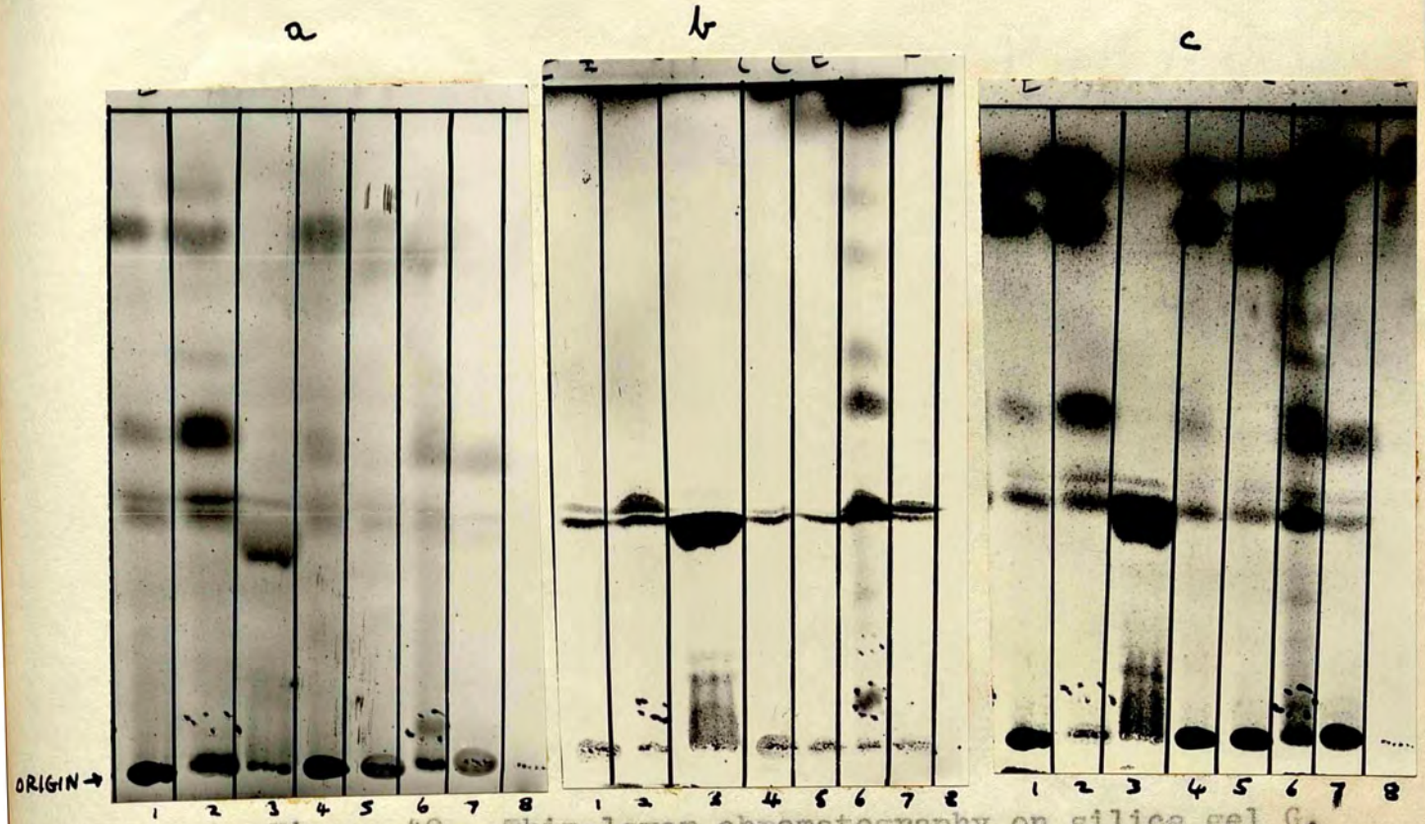


Figure 49. Thin-layer chromatography on silica gel G.
Solvent: petroleum ether/ether/clacial acetic acid (60/40/1, v/v).
Development distance: 10cm.
(a) Stained with iodine
(b) Sprayed for cholesterol and cholesterol esters.
(c) Sprayed with ammonium molybdate- perchloric acid.

Column 1. $CHCl_3$-MeOH extract of ether-extract C.maenas (ovary) carotenoprotein.
2. Ether extraction of C.maenas (ovary) carotenoprotein.
3. Cholesterol standard.
4 & 5. $CHCl_3$-MeOH extract of ether-extracted ovoverdin
6. Ether extract of ovoverdin.
7. $CHCl_3$-MeOH extract of ether-extracted scallop (ovary) carotenoprotein.
8. Ether extract of scallop (ovary) carotenoprotein.

a streak near the solvent front.

Cholesterol and cholesterol esters were separated using a neutral lipid solvent to develop the plates. Other saturated and unsaturated lipids were also present (Fig. 49). The phospholipids remained at the origin, staining darkly with ammonium molybdate-perchloric acid.

Ether extracted most of the neutral lipid and carotenoid, but little phospholipid, from the protein. The $CHCl_3$-MeOH extraction, following the ether extraction, removed the phospholipid and the remainder of the neutral lipid.

(iii) Protein-bound carbohydrate

Method Protein-bound hexose (estimated as mannose) and hexosamine (estimated as glucosamine) were determined on defatted protein samples (III.$H_{(iv)}$.).

Results The lipid-extracted protein was found to contain: 3.2 $\pm$ 0.2% Hexose; 1.63 $\pm$ 0.02% Hexosamine (nine determinations on three preparations).

(iv) Carotenoid components

Method 10ml. of a solution of ovoverdin (25mg/ml.) in 0.2M-phosphate buffer, pH7, was mixed with 2 vols. acetone and 1 vol. petroleum ether. The liberated carotenoid was passed into the petroleum ether layer by dilution with 4 vols. distilled water. The upper layer was evaporated to dryness in a rotary evaporator, and the carotenoids

dissolved in 0.2ml. $CHCl_3$.

2g. ovary was extracted with acetone and the carotenoid obtained in petroleum ether in the same manner.

All operations were conducted in the dark with cold solutions, storing under nitrogen where possible.

The carotenoids in the extracts were separated by thin-layer chromatography on silica gel plates, $CHCl_3$ being used as developing solvent. The preparation and development of the plates were identical with that used for lipids (III.$I_{(iv)}$.). After development the carotenoid bands were eluted with 5ml. MeOH, transferred to petroleum ether, and the spectra recorded.

Results Ovoverdin was found to contain all the carotenoids present in the whole ovary (Table V). Astaxanthin greatly predominated in both the ovary and ovoverdin; other hypophasic carotenoids were present only in traces. The unidentified epiphasic carotenoid present in the ovaries in fairly high concentration was present in ovoverdin in traces.

TABLE V

Carotenoid components present in ovoverdin and in the whole ovary of lobster

Rf	Colour	Absorption Maximum (mμ)	Behaviour in 90% MeOH/pet. ether
0.95	Yellow	462, 492	epiphasic
0.59	Red	--	hypophasic
0.33	Red	--	hypophasic
0.27	Red	--	hypophasic
0.22	Red	476	hypophasic (astaxanthin)
0.16	Yellow	--	hypophasic

G. General Properties of Ovoverdin

All experiments were performed in 0.01M-phosphate buffer, pH7.

Ovoverdin came out of colution when solutions were desalted on Sephadex G25 columns. The protein appeared in the effluent in the precipitated state. Most of it redissolved on addition of salt.

When solutions of ovoverdin (10mg./ml.) were dialysed against distilled water overnight, the protein came out of solution. On further dialysis (for two days) it redissolved. Addition of 0.2M-phosphate buffer, pH7, to the dialysed solution to a concentration between 2×10^{-3}M. and 2×10^{-4}M. caused turbidity of the solution; at a concentration of 1.25×10^{-3}M. most of the ovoverdin came

out of solution. No turbidity was evident at higher concentrations of phosphate buffer. Addition of 1% NaCl solution to the dialysed solution to a final concentration between 1×10^{-2} and 5×10^{-3}M. likewise caused precipitation of the protein, this being most evident at 7.5×10^{-3}M.

Solutions of ovoverdin (10mg./ml.) dialysed in the dark and cold lost about 65% of the extinction at 466mμ and 75% of the extinction at 660mμ after four days. After an overnight dialysis, the absorption curve in the 660mμ region became a smooth curve with maximum at 652mμ.

When shaken with ether, ovoverdin solutions turned red and the protein was denatured, forming a layer at the surface; some of the carotenoid entered the ether phase.

60-80% $(NH_4)_2SO_4$ was required to precipitate the protein from salt-free solutions.

Ovoverdin was adsorbed onto CM-cellulose from thoroughly dialysed solutions as a single green band, and eluted with 0.05M- phosphate buffer, pH7.

CHAPTER VI

THE CAROTENOPROTEIN OF THE OVARY OF THE SCALLOP (Pecten maximus)

Introduction

Lederer (1934(i), (ii); 1938(ii)) has investigated the carotenoid composition of the ovaries of the scallop, Pecten maximus. He showed that a number of xanthophylls occurred, of which one, which he named Pectenoxanthin, predominated and was unique. The chemical constitution of Pectenoxanthin has not been fully elucidated; Lederer (1938 (ii)) has suggested that it contains eleven double bonds, two hydroxyl groups, and possibly a ketonic group.

Experimental

A. Extractibility of carotenoids

Method Water was removed from two orange-red scallop ovaries by freeze-drying or by grinding with anhydrous Na_2SO_4. The material was extracted with 50ml. portions of acetone with grinding until no further pigment was extracted, four extractions being sufficient. The residue was extracted three times with 50ml. MeOH which removed the remainder of the carotenoid. The ether and methanol solutions were taken to dryness in a rotary evaporator and the carotenoids dissolved in 30ml. MeOH. The amounts of carotenoid extracted by the two procedures were compared by measuring the extinction of the solutions

at 450mμ.

Results The extinction of the methanol extract was eleven times that of the ether extracts at 450mμ.

The ether extraction removed all but traces of epiphasic (in the system 90% MeOH/petroleum ether) carotenoids from the ovary.

The eluted material absorbed strongly in the ultra-violet and had a maximum at 260mμ, possibly owing to the presence of nucleic acid. This was partially removed by $(NH_4)_2SO_4$ precipitation, the last traces being removed with DEAE-cellulose.

The material eluted from the calcium phosphate gel was brought to 50% $(NH_4)_2SO_4$ saturation by the addition of an equal volume of saturated $(NH_4)_2SO_4$ solution and centrifuged at 13,000r.p.m. for 30 min. The centrifuge tubes were thoroughly drained, the dark red precipitate dissolved in 100ml. 0.2M-phosphate buffer, pH7, stirred for 5min. with 3g. DEAE-cellulose and centrifuged.

$(NH_4)_2SO_4$ salting-out curves (III.C.) on the super-natant revealed the presence of two coloured fractions. One fraction precipitated between 22.5 and 32.5% $(NH_4)_2SO_4$ saturation, the main fraction precipitating between 32.5 and 45% $(NH_4)_2SO_4$ saturation (Fig. 50). The 22.5-32.5% fraction only partially redissolved and had a spectrum in the visible region identical with that of the second fraction.

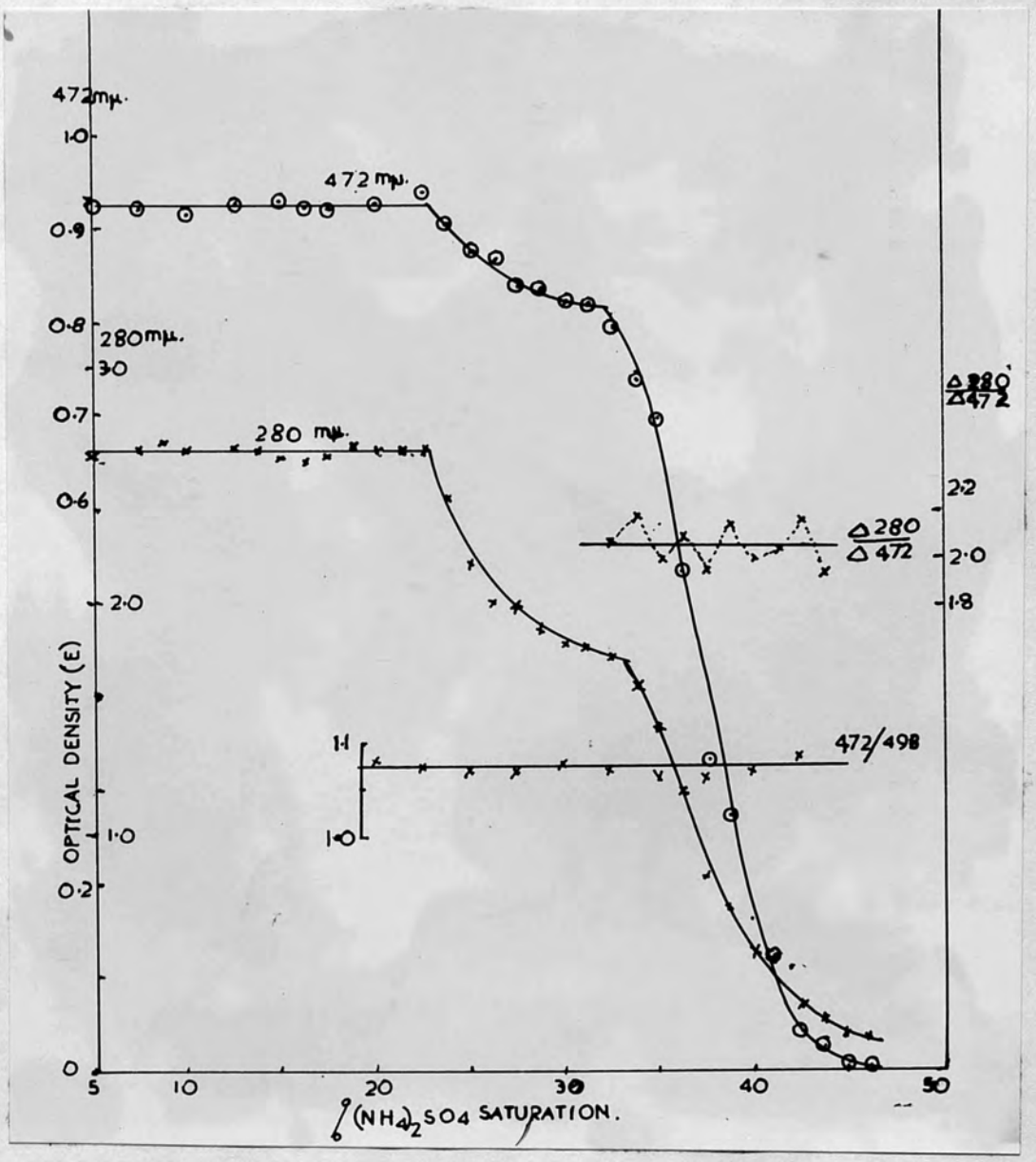


Figure 50. $(NH_4)_2SO_4$ saturation curve of the scallop carotenoprotein, purified by calcium phosphate gel chromatography.
Buffer: 0.1M-phosphate buffer, pH7.
Temperature: 4°.

The solution of the chromoprotein was therefore brought to 32.5% $(NH_4)_2SO_4$ saturation by the addition of a saturated $(NH_4)_2SO_4$ solution, centrifuged after 20min. and the light red precipitate discarded. The supernatant was brought to 45% $(NH_4)_2SO_4$ saturation by further addition of saturated $(NH_4)_2SO_4$ solution, centrifuged, and the dark red precipitate dissolved in about 10ml. 0.2M-phosphate buffer, pH7.

The chromoprotein had an absorption maximum at about 472mμ. The values for E280/472 following each purification step are shown in Table VI.

The purified material gave a value for E280/472 of 2.1±0.1.

B. Extraction and purification of carotenoprotein

The extraction and purification of the carotenoprotein was carried out in the cold and dark, storing the solutions under nitrogen where possible.

The orange-red female reproductive glands were removed from fresh scallops, and dissected clear of the pink male organ. They were then washed with 10% NaCl (1 l./90g. ovaries) on a Buchner funnel to remove the slime layer surrounding them, followed by thorough washing with water. 90g. (from 12 scallops) was homogenised for 15 secs. in a Waring blendor with 300ml. distilled water. After two

hours the mixture was centrifuged at 13,000r.p.m. for 30min. and the supernatent and yellow lipid floating at the surface discarded. The bright red pellet remaining was suspended in 200ml. distilled water and recentrifuged. It was then homogenised for 5 secs. in a Waring blendor with 200ml. 10% NaCl solution, allowed to stand for 1 hour, centrifuged for 30mins. at 13,000r.p.m., and the red supernatant poured off. The residue, still lightly coloured, was re-extracted with 200ml. 10% NaCl solution for a further hour; this removed most of the remaining carotenoid, leaving the residue coloured light orange.

10ml. calcium phosphate gel was added to the pooled supernatants, the pH adjusted to 5.5 with dilute acetic acid, and the gel spun down at 3,000 r.p.m. for 5 min. and discarded. A further 90ml. calcium phosphate gel was added to the supernatant and the pH adjusted to 5.5. Further gel was added if necessary to adsorb all the chromoprotein. The gel was washed on the centrifuge four times with 10% NaCl, and the red chromoprotein eluted twice with an equal volume (50-100ml.) of a solution prepared by mixing volumes of 0.2m-phosphate buffer, pH7, and 10% NaCl solution (3/1, v/v). This could not be improved by a second $(NH_4)_2SO_4$ fractionation step, by alumina gel chromatography, by hydroxyapatite chromatography or by variation in the extraction procedure (Section F).

Table VI

Purification of the carotenoprotein of the scallop ovary.

Purification Procedure	Extinction Ratio E280/472
Calcium phosphate gel chromatography	10 - 15
50% $(NH_4)_2SO_4$ precipitation	3 - 5
Negative DEAE-cellulose adsorption	2.6 - 3.3
32.5-45% $(NH_4)_2SO_4$ precipitation	2.1 ± 0.1

C. Spectrum

The chromoprotein had a maximum at 278mμ and an inflexion at about 315mμ. The spectra (Fig. 51) of different preparations in the visible region were compared by adjusting the extinction at the maximum to the same value. The spectra of preparations from scallops bought at different times were not superimposable. The main maximum was between 470 and 472 mμ for different preparations with an inflexion at about 435mμ. In some preparations there was a second maximum at 496-8mμ, but in others this was an inflexion with centres between 496 and 500mμ. The ratio of the extinctions E472/498 varied for different preparations. Table VII indicates the type of spectrum obtained for preparations made between October and March; the differences in spectrum appeared to be random and not due to any definite seasonal variation.

When ovaries from individual scallops, bought at the same time, were worked up independently, the spectrum of

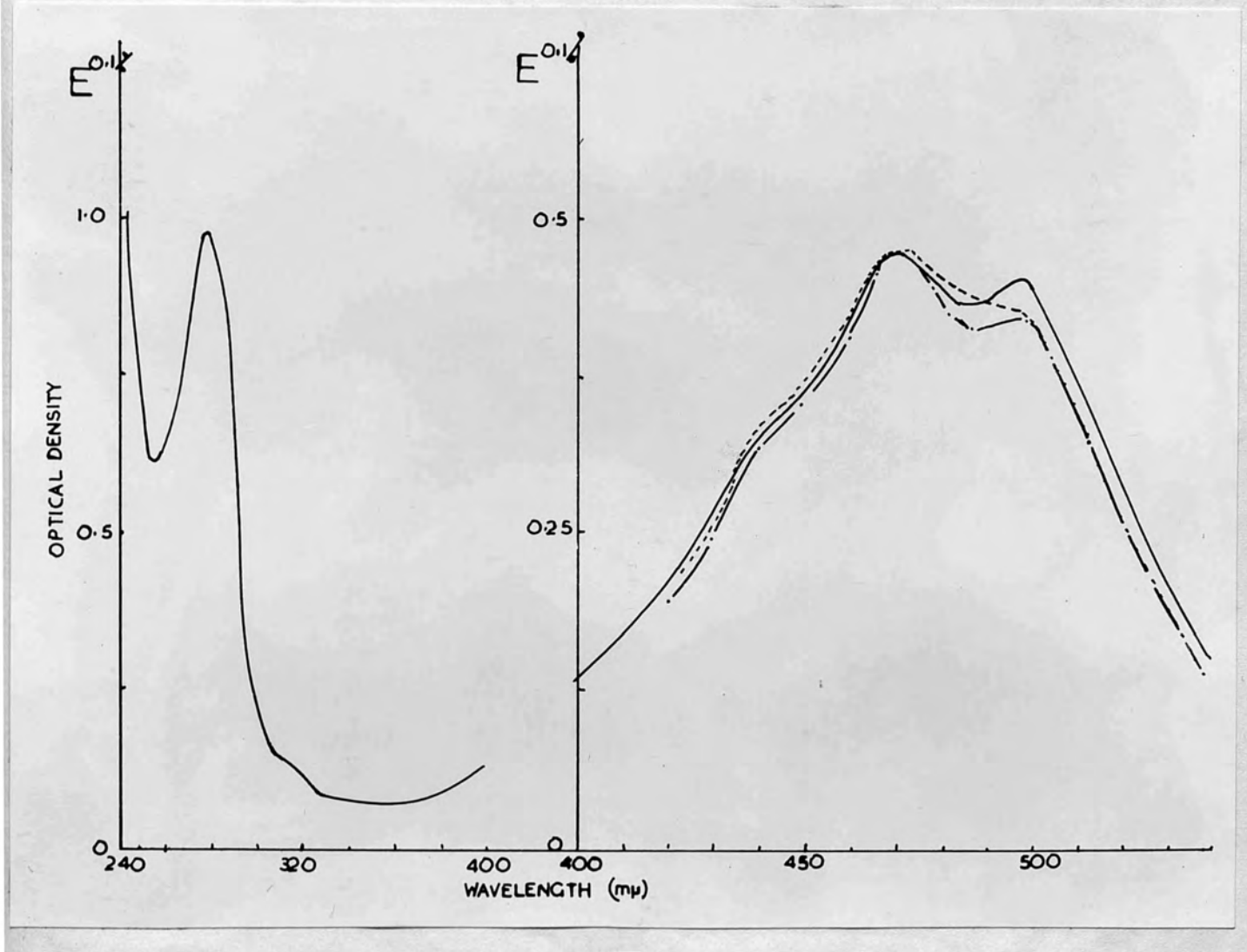


Figure 51. Spectra of preparations of the carotenoprotein of the ovary of Pecten maximus in 0.2M-phosphate buffer, pH7.

the chromoproteins were exactly superimposable; no alteration in the spectrum occurred during any of the purification steps.

Addition of urea to a concentration of 6M. to a solution of the chromoprotein in 0.2M-phosphate buffer, pH7, resulted in a 5mμ hypochromic shift in spectrum and alteration of its shape, as shown in Figure 52.

The maximum of the chromoprotein lay at 15mμ greater wavelength than the carotenoid in petroleum ether derived from it.

D. Dialysis and freeze-drying

Dialysis was performed in the cold and dark and light was rigorously excluded during the freeze-drying.

The chromoprotein came out of solution on dialysing overnight against distilled water or on desalting by passage through Sephadex G25, and only partially re-dissolved in 0.2M-phosphate buffer, pH7. If the dialysed solution was allowed to stand for two days, a negligible amount of the precipitated protein redissolved. The presence of 10^{-3}M-EDTA, pH7.5, in the dialysis water slowed down but did not abolish this denaturation. Dialysis of small volumes (10ml.) of concentrated chromoprotein solutions against 5l. distilled water for four hours with continual agitation, brought the majority of the protein out of solution; it completely redissolved in 0.2M-

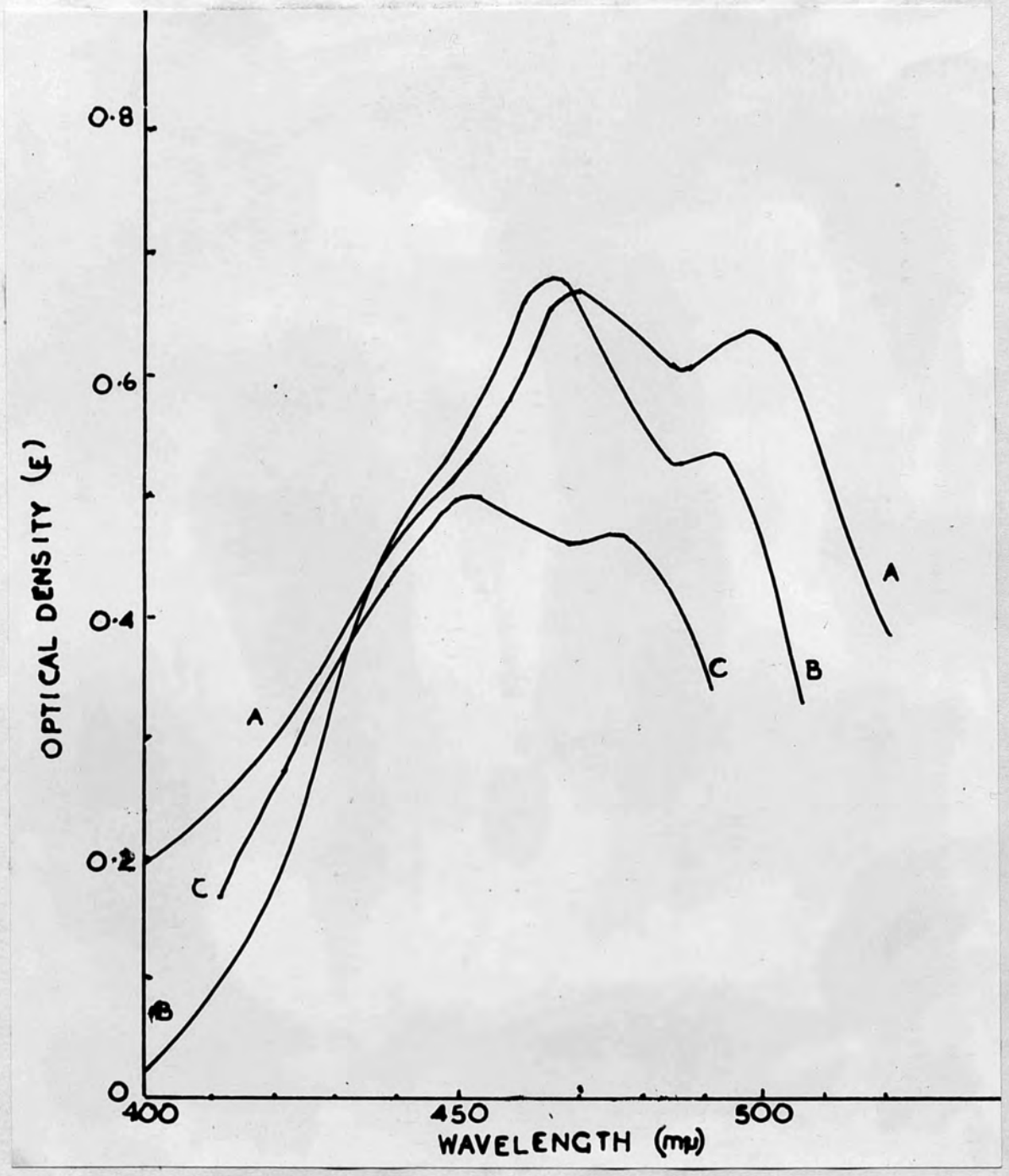


Figure 52. Spectrum of the scallop carotenoprotein.
A. In 0.2M-phosphate buffer, pH7.
B. In 0.2M-phosphate buffer, pH7, containing 6M- urea.
C. Spectrum of the carotenoid in petroleum ether derived from the carotenoprotein.

phosphate buffer, pH7. The chromoprotein had a low solubility in 0.05M-phosphate buffer, pH7.

Freeze-drying from a volatile buffer could not be employed on account of the high buffer molarity (0.3M-NH_4Ac) required to keep the protein in solution at a reasonable concentration.

Table VII

Preparations of the carotenoprotein of the scallop ovary

Date of Purchase		E472/498	Type of Spectrum (number of maxima in the visible region)
1962	Jan.	1.06	2
	March	1.09	2
	Sept.	1.07	2
	Oct.	1.08	1
	Nov.	1.09	2
	Dec.	1.14	1
1963	Jan.	1.07	2
	March	1.14	1
1964	Feb.	1.07	2
	March	1.09	1

A freeze-dried preparation which dissolved in salt solution almost completely, with unaltered absorption spectrum in the visible region was obtained as follows: The purified chromoprotein (March 1962 preparation, Table VII) was dialysed with stirring for four hours against 5l. distilled water containing 10^{-3}M- EDTA, pH7.5. The

precipitate was centrifuged down, washed three times on the centrifuge with cold distilled water, suspended in the minimum quantity of distilled water, frozen, and freeze-dried over conc. H_2SO_4. Slight fading of the solution occurred during the dialysis and the freeze-dried material had a value of E280/472 at 2.25, compared with 2.03 before dialysis.

The freeze-dried protein slowly denatured on storing in a specimen tube in the dark and cold; after about a week none of the protein redissolved.

A yield of 0.3g. freeze-dried protein/90g. washed ovaries was obtained.

The value for $E_{280}^{0.1\%}$ was found to be 0.98.

E. Carotenoid components

Method The carotenoid composition of the chromoprotein was investigated by thin-layer chromatography, as described (V.$F_{(iv)}$.), and compared with those in the ether and methanol extracts of the whole ovary (Section A). The relative amounts of the carotenoids were compared by scraping off the carotenoid bands, eluting the carotenoid by stirring with 5ml. MeOH, and determining the extinction at the absorption maximum. Three developments with $CHCl_3$ to 8cm. were required for sufficient separation of the carotenoid bands, the plates being dried briefly with a stream of nitrogen between runs. The chromatography

was performed in the dark and cold, and the tank was filled with nitrogen. Corresponding bands from the whole ovary and from the chromoprotein were scraped off the plates within a few seconds of each other to minimise errors due to oxidation.

Results The chromoprotein contained all the hypophasic (in the system 90% MeOH/petroleum ether) carotenoid components present in the ovary (Figure 53) and about in the same proportions (Table VIII). A small amount of yellow epiphasic carotenoid, moving at the solvent front in extracts from the whole ovary, was present in the carotenoprotein only in traces. Ether extraction (Section A) of the ovaries removed all but traces of the epiphasic carotenoid. The carotenoid components corresponding to bands 2 and 3 (Figure 53) were not obtained pure by thin-layer chromatography. Band 3, obtained pure by chromatography on cellulose or anhydrous Na_2SO_4, had the spectrum (Table IX) of pectenoxanthin. The carotenoid corresponding to band 4 had identical spectrum to sulcatoxanthin in CS_2 (Karrer & Jucker, 1950), but was not hypophasic in in the system 65% MeOH/petroleum ether.

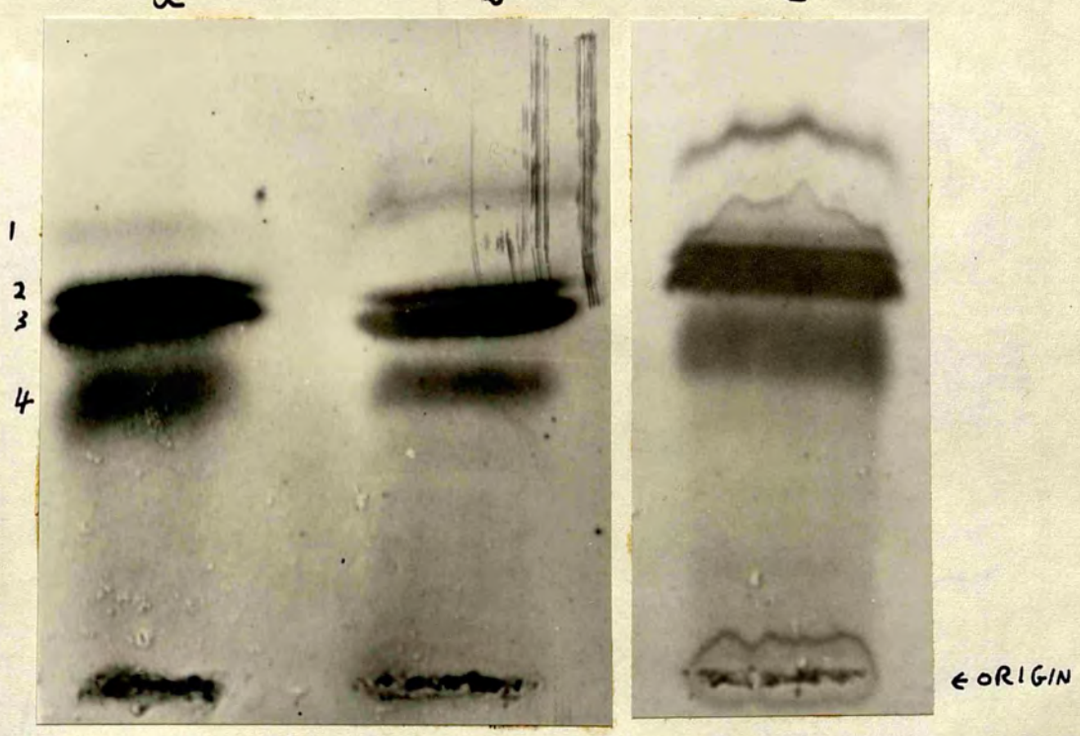


Figure 53. Thin-layer chromatography on silica gel G of carotenoids (a) from scallop (ovary) carotenoprotein using MeOH extraction, (b) from scallop ovary using ether extraction (c) from scallop ovary using MeOH extraction. Plates developed three times to 8 cm. with $CHCl_3$.

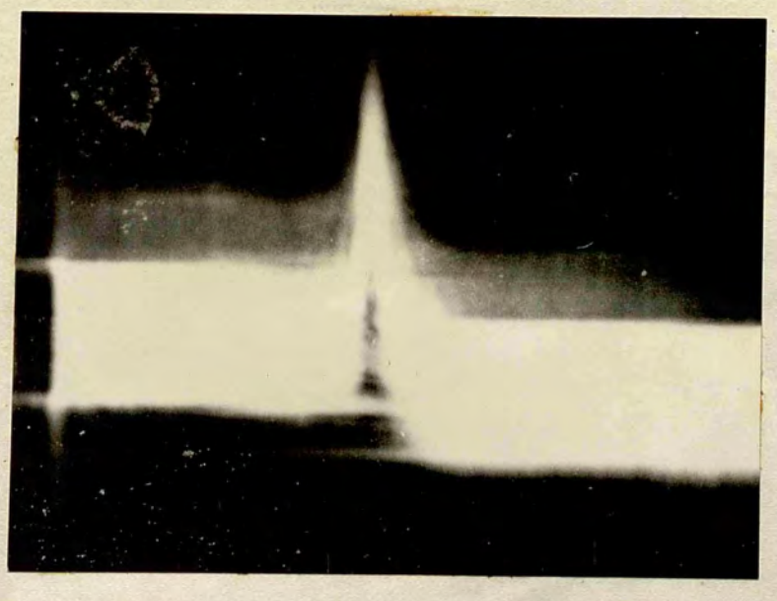


Figure 54. Antweiler microelectrophoresis of the carotenoprotein of the ovary of P.maximus.

0.07M-barbitone buffer, pH8.6, 80 volts, 1.6mA.

After 16 min.

Table VIII

Relative proportions of the carotenoids in the whole ovary and carotenoprotein of *Pecten maximus*

Carotenoid Bands (as numbered in Fig. 53)	Wavelength (mμ)	Carotenoprotein Extinction in 5ml. MeOH	Whole ovary Extinction in 5ml. MeOH
(1) Preparation Oct. 1963			
1	460	0.16	0.27
2	460	0.33	0.34
3	460	1.63	1.63
4	452	1.61	0.98
(2) Preparation Feb. 1964			
1	460	0.13	0.14
2 and 3	460	0.91	0.91
4	452	0.47	0.25

Table IX

Spectrum of carotenoid components of the whole ovary and carotenoprotein of *Pecten maximus*

(a) Epiphasic in the system 90% MeOH/pet. ether (possibly a mixture of pigments)

Solvent	Maxima (mμ)	Inflexions (mμ)
pet. ether	452, 480	432
$CHCl_3$	477, 504	445
CS_2	450, 480	---

(b) Hypophasic in the system 90% MeOH/pet. ether carotenoid components (Fig. 53):

Band 2 Eluted from cellulose columns with 6% acetone in petroleum ether (possibly still contaminated with band 3).

Solvent	Maxima (mμ)	Inflexion (mμ)
pet. ether	464, 484	---

Band 3 Eluted from anhyd. Na_2SO_4 and cellulose with 8% acetone in petroleum ether.

Solvent	Maxima (mμ)	Inflexions (mμ)
pet. ether	458	488
CS_2	488	516

Band 4 Eluted from $CaCO_3$ by 2% MeOH in petroleum ether after passage of 15% acetone in petroleum ether.

Solvent	Maxima (mμ)	Inflexions (mμ)
$CHCl_3$	463, 494	435
CS_2	482, 516	450
pet. ether	448, 474	425
MeOH (not fully investigated)	458	

F. Variation in extraction procedure and purification

Attempts to separate the chromoprotein preparation into a number of carotenoproteins by varying the extraction and purification procedures failed. In all cases the spectra of the aqueous extracts, from scallops bought at the same time, were superimposable. The variations of extraction and purification employed are summarised below:

(i) The ovaries were initially extracted with 0.05M-phosphate buffer, pH7. The carotenoprotein was extracted from the residue with 0.2M-phosphate buffer, pH6-8. The extract was adjusted to pH7, shaken with DEAE-cellulose and the spectrum recorded. The chromoprotein was more readily extracted at neutral or alkaline pH.

(ii) The ovaries were initially extracted with distilled water. The carotenoprotein was extracted from the residue with 30% $(NH_4)_2SO_4$ solution, precipitated at 50% $(NH_4)_2SO_4$ saturation, dissolved in 0.2M-phosphate buffer, pH7 and the spectrum recorded.

(iii) Separation of carotenoproteins by displacement chromatography (Polis and Schmukler, 1953) on 10cm. hydroxyapatite columns was attempted.

A purified (VI.B.) solution (10mg./ml.) of the carotenoprotein in 0.2M-phosphate buffer, pH7 (E280/472= 2.1) was dialysed against 5l. phosphate buffer to remove $(NH_4)_2SO_4$ and passed through the hydroxyapatite column. The carotenoprotein was adsorbed as a uniform band at the top of the column. The passage of the solution was discontinued when four cm. of the column were saturated with the adsorbed protein, and the columns developed with 50ml. 0.2M-phosphate buffer, pH7; the red band remained almost stationary. The column was extruded, the red band divided into the equal parts, and the protein eluted by suspending in 0.5M-phosphate buffer, pH7. The suspension was centrifuged and the spectrum recorded.

(iv) The spectra of fractions of the purified carotenoprotein (VI.B.) in 0.1M-phosphate buffer, pH7 precipitating between 40-42.5% and 42.5-50% $(NH_4)_2SO_4$ saturation were determined.

(v) A solution of the carotenoprotein in 0.2M-phosphate buffer was dialysed against distilled water with stirring until about 30% of the chromoprotein had precipitated (two hours). The spectra of the supernatant and of the precipitate in 0.2M-phosphate buffer were measured.

G. Attempted removal of lipid-dissolved carotenoids from the carotenoprotein

Method Ashworth & Green (1963) showed that the lipid-dissolved carotenoids of serum β-lipoprotein were removed by shaking with celite. This procedure was tried with the scallop carotenoprotein.

20ml. of the carotenoprotein (2mg./ml.) in 0.2M-phosphate buffer, pH7 were shaken mechanically overnight with 0.5g. celite. The celite was centrifuged down, washed twice with buffer solution, shaken with 2ml. MeOH, and centrifuged.

Result The supernatant was colourless, suggesting a stronger binding of the carotenoids in the scallop carotenoprotein than in β-lipoprotein.

H. Homogeneity

Method The purified carotenoprotein (VI.B.) solution was dialysed, centrifuged down and dissolved in an appropriate electrophoretic buffer. Homogeneity was tested by cellulose acetate electrophoresis (III.$E_{(i)}$.) and electrophoresis in the Antweiler micro-electrophoresis

apparatus.

Results High molarities of buffer had to be used for the electrophoresis on account of the low solubility of the protein in electrophoretic buffers of the usual strength. Long times of running had therefore to be employed.

The protein streaked from the origin on cellulose acetate electrophoresis using 0.1M-acetate buffer, pH4.5, and 0.1M-barbitone buffer, pH8.6. At pH7 a single component could be distinguished, streaking still occurred but was less pronounced. The protein band stained positively for carbohydrate and the presence of lipid was revealed on drying (III.$E_{(i)}$.).

A single component was observed on free-boundary electrophoresis at pH8.6 using a saturated solution of the protein in the electrophoretic buffer (Fig. 54). Insufficient protein dissolved at pH4.5 and 7 (0.05M-acetate and 0.05M-phosphate buffer) for this method of electrophoresis.

I. Minimum molecular weight

All carotenoids which have been crystallised have $E^{0.1\%}_{max.}$ values in petroleum ether varying between 1,900 and 3,100, most of the values being around 2,500 (cf. Goodwin, 1954). If a molecular weight of 600 is considered for the carotenoids in the scallop carotenoprotein, and assuming a value

of $E_{472}^{0.1\%}$ carotenoid for the carotenoid mixture bound to the chromoprotein (as an approximation since attachment of carotenoids to protein does not alter the extinction of the carotenoid more than about 10%, cf. Fig. 14), a solution of the carotenoprotein containing 1 mole of carotenoid per l. would have an extinction at 472mμ of 150,000. Using the values of E280/472 and $E_{280}^{0.1\%}$ of the preparation (2.1 and 0.98 respectively, Section A), the minimum molecular weight of the carotenoprotein (for 1 mole of carotenoid) would be about 320,000.

J. Molecular size

Method The molecular size of the carotenoprotein was investigated on thin layers and columns of Sephadex G200 (III.$K_{(ii)}$, (iv).). A partially purified preparation of the carotenoprotein was used (the DEAE-cellulose chromatography and $(NH_4)_2SO_4$ fractionation steps (VI.B.) being omitted).

Results The carotenoprotein moved more rapidly than α-crustacyanin through Sephadex G200 thin layers and columns Figures 55 and 56). A considerable amount of high molecular weight material (possibly denatured protein) completely excluded from the gel, was present in the preparation.

The carotenoprotein was found to correspond in size to a globular protein with a molecular weight of 375,000 ±

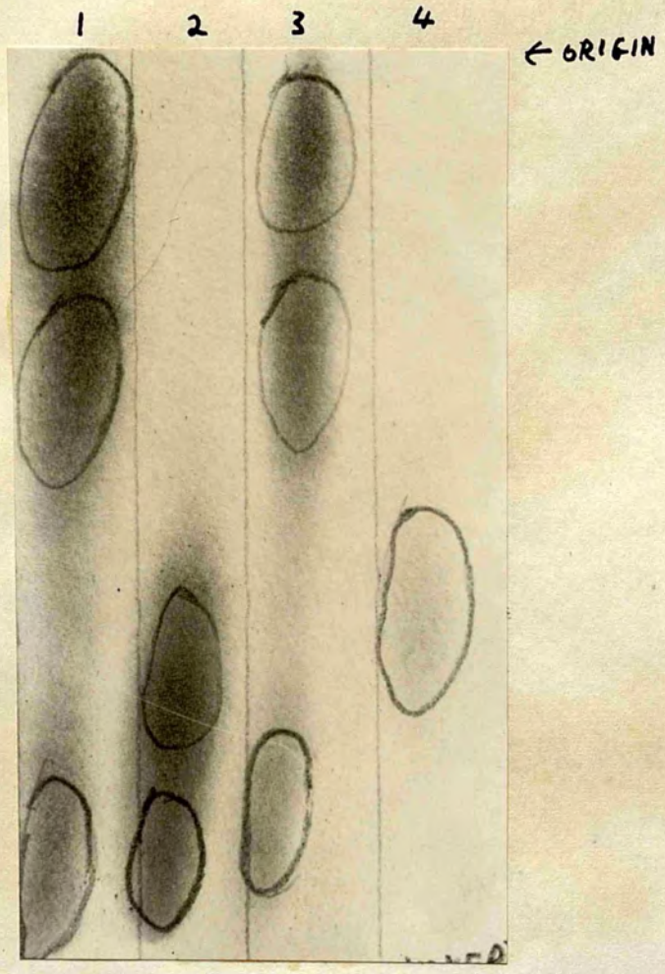


Figure 55. Thin-layer gel filtration on Sephadex G200. Plates developed 1M-NaCl/0.02M-phosphate buffer, pH7. 8hr. run.

Columns 1 and 3. Bovine serum.
2. Crude scallop (ovary) carotenoprotein.
4. α-crustacyanin.

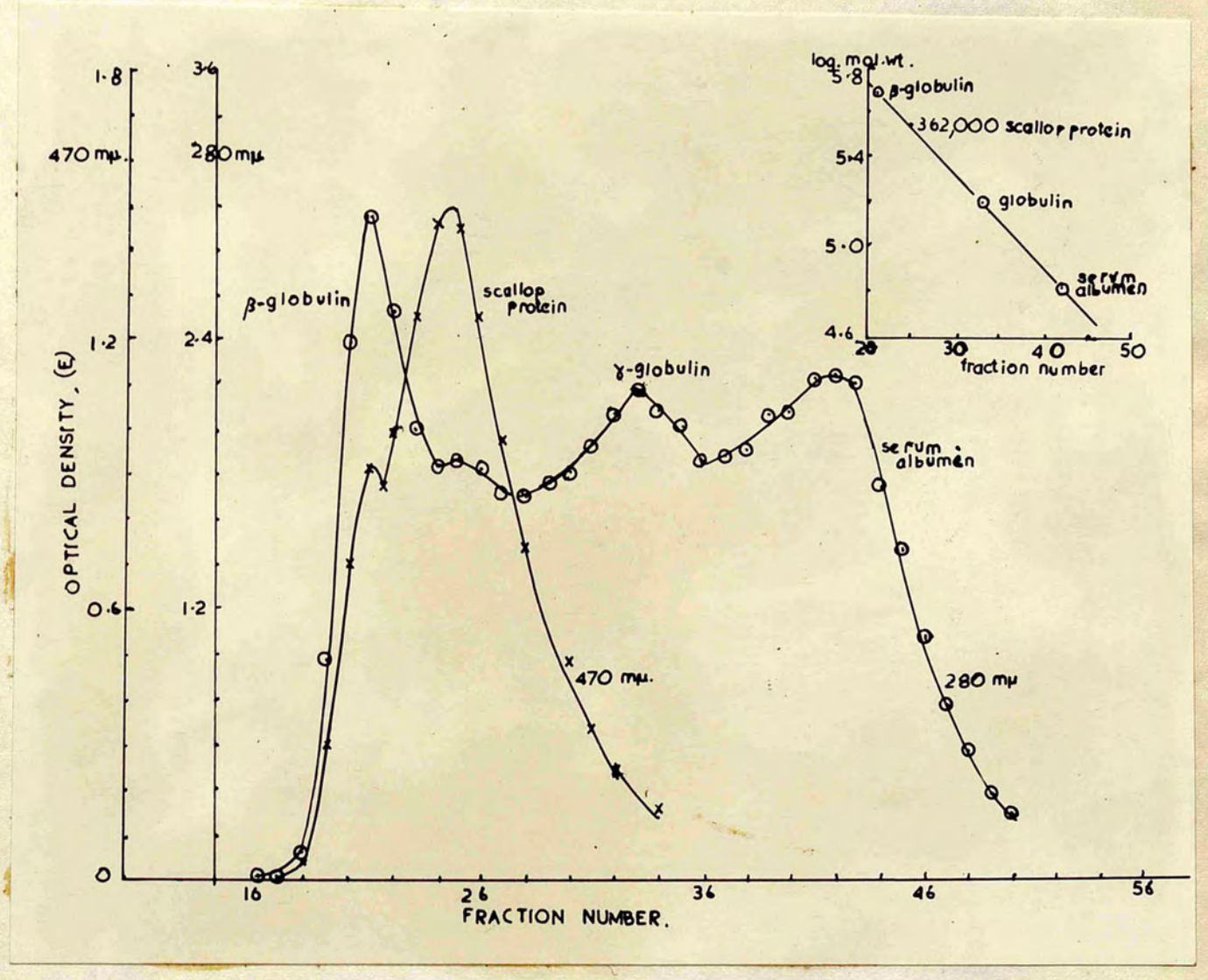


Figure 56. Gel filtration on Sephadex G200.
Sample: 1ml. bovine serum + 1ml. crude scallop carotenoprotein in 0.2M-phosphate buffer pH7.
Bed-volume: 1.9cm. x 41.0cm.
Developing solvent: 0.02M-phosphate buffer, pH7, containing 1M- NaCl.
Flow rate: 10ml./hr.
Fraction volume: 2.5ml.

10,000 by thin-layer gel filtration (two determinations) (Fig. 55) and of molecular weight 360,000 by column gel filtration (one determination (Fig. 56)).

K. Composition

(i) Nitrogen content

Method The nitrogen content of the freeze-dried chromoprotein and of the lipid-free protein (III.$H_{(i)}$.).

Results The nitrogen content of the carotenoprotein was found to be 14.8 ± 0.3% (six determinations on three preparations) and that of the lipid-free protein 15.8 ± 0.2% (two determinations on one preparation).

(ii) Protein-bound carbohydrate

Method III.$H_{(iv)}$.

Results The lipid-free protein was found to contain

0.26 ± 0.03% Hexose (estimated as Mannose)

1.48 ± 0.04% Hexosamine (estimated as Glucosamine)

(four determinations on two preparations).

(iii) Lipid content and composition

Method III.V. and III.I. The protein was extracted with ether for four hours in a Soxhlet apparatus and then with $CHCl_3$-MeOH (2/1, v/v), for two hours and the extracts subjected to thin-layer chromatography.

Results 12.5 ± 2.5% lipid was extracted from the protein (two determinations) with $CHCl_3$-MeOH (2/1, v/v).

The lipid contained 1.12 ± 0.1% phosphorus, and thus 28 ± 2.5% phospholipid.

Ether extracted little phospholipid and neutral lipid and negligible amounts of carotenoid. (Figures 47 and 49). Thin-layer chromatography of the $CHCl_3$-MeOH extracts revealed the presence of a number of phospholipids, including phosphatidyl ethanolamine and neutral lipids, including cholesterol (Figure 49).

L. General properties of the carotenoprotein

All experiments were performed in 0.2M-phosphate buffer, pH7.

Solutions of the carotenoprotein slowly fade in the dark and more rapidly in the light; storing under nitrogen reduces the rate of fading (Table X). Less fading occurred the more concentrated the solution and the greater the ionic strength.

TABLE X

Stability of the carotenoprotein of Pecten maximus dissolved in 0.2M-phosphate buffer, pH7 (1.5mg./ml.)

Treatment	% Loss in 472mμ absorption (approximate)
1. Room temperature for 3 hours	15 - 45
2. Dark and cold overnight	20 - 25
3. As in 2., but stored under nitrogen	5 - 10
4. As in 1., but stored under nitrogen	10 - 20
5. Overnight dialysis	20
6. Chromoprotein (10mg./ml.) in 20% $(NH_4)_2SO_4$; dark and cold.	4

No alteration in spectrum was obtained on mixing the carotenoprotein solution with an equal volume of 40% urea. When this solution was heated on a water bath at 80° for 3 min., followed by rapid cooling in ice-water, the maximum at 472mμ shifted to 464, and that at 498mμ became an inflexion at 493mμ; the value of E472/498 changed from 1.07 to 1.15. No reversal of the spectral changes was observed on allowing the solution to stand in the cold.

CHAPTER VII

THE CAROTENOPROTEIN OF THE OVARY OF CARCINUS MAENAS

A. Extraction and purification

Three preparations of the carotenoprotein were made from ovaries of crabs bought in May, July and September, 1963. Experiments were conducted in the cold and dark, storing solutions of the carotenoprotein under nitrogen where possible.

The orange-red carotenoprotein was extracted with 300ml. 0.05M-phosphate buffer from 50g. ovaries, and purified in the same manner as that used for ovoverdin (V.A.). Two extractions left the residue almost colourless.

Nucleic acid and protein impurities were removed by negative DEAE-cellulose and CM-cellulose chromatography and the carotenoprotein purified by chromatography on calcium phosphate gel. Fractions of the eluted protein precipitating between 30 - 50% and 50 - 60% $(NH_4)_2SO_4$ saturation (obtained by addition of a saturated $(NH_4)_2SO_4$ solution), were dissolved in the minimum volume of water, desalted on Sephadex G25 columns and freeze-dried (July preparation only).

The yield of freeze-dried protein was 1.6 and 2.4g./100gm. ovaries for the 30- 50% and 50 - 60% $(NH_4)_2SO_4$ fractions respectively.

B. Spectrum

Slight differences in the spectrum of the preparations were observed when these were corrected to the same extinction values at the long wavelength maximum (490mμ). (Figure 57).

The spectrum showed maxima at 490 and 278mμ and either a maximum or inflexion at 470mμ; there was an inflexion at 325mμ.

The two $(NH_4)_2SO_4$ fractions had identical spectra with an E280/490 value of 4.4, and an $E_{280}^{0.1\%}$ value of 1.2 ± 1 (four determinations on one preparation).

C. Homogeneity

Method III.$E_{(i)}$.

Results The 30 - 50% and 50 - 60% $(NH_4)_2SO_4$ fractions both showed two orange-coloured components in cellulose acetate electrophoresis at pH 8.6 and 7. The 50 - 60% $(NH_4)_2SO_4$ fraction contained only traces of the faster moving component. Both components stained positively for carbohydrate and showed the presence of lipid on drying the cellulose acetate strips. At pH4.5 the protein streaked from the origin.

D. Carotenoid components

Method The carotenoids present in the carotenoprotein were compared to those present in the whole ovary by thin-layer chromatography (VI.E.).

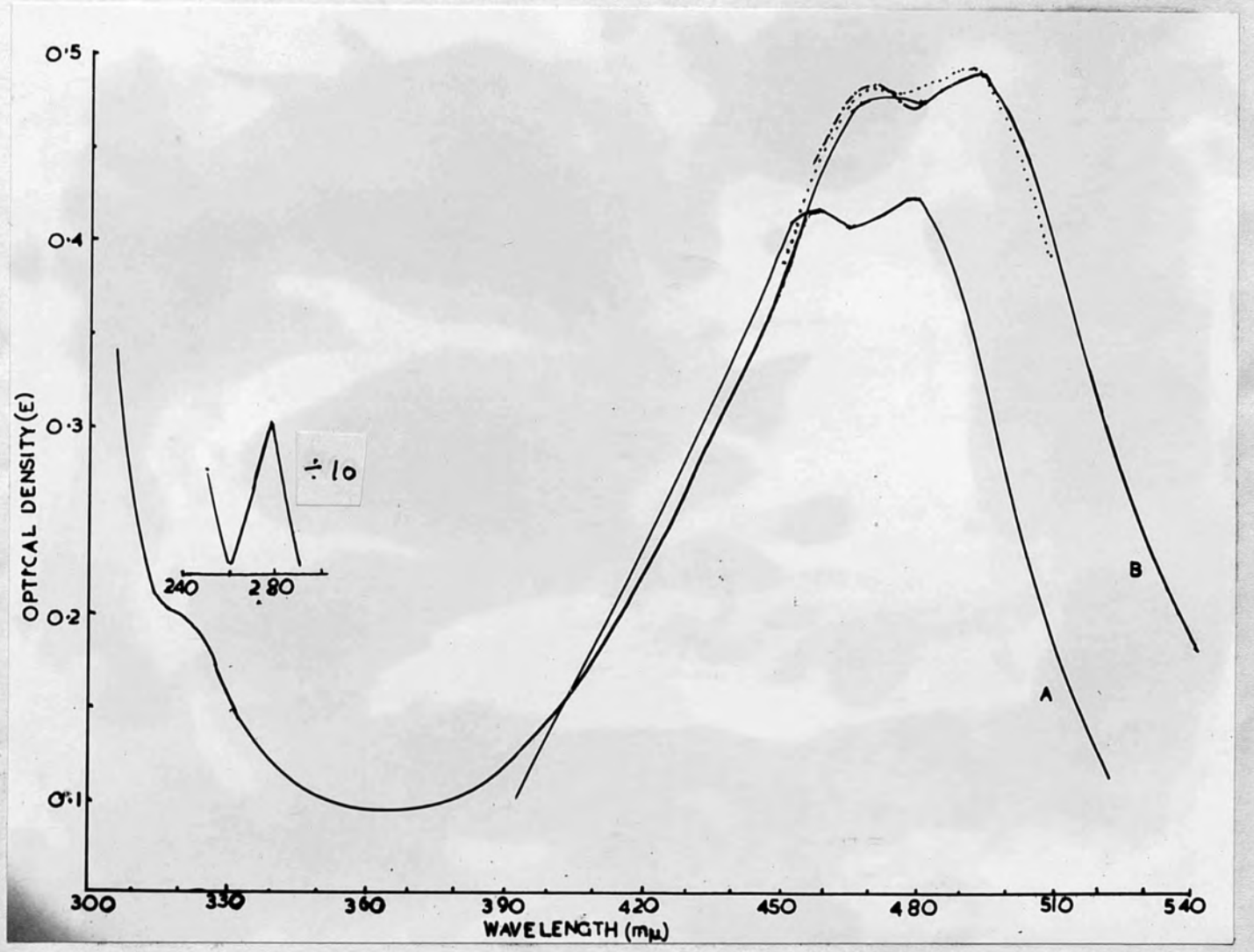


Figure 57. Spectra of preparations of the carotenoprotein of the ovary of Carcinus maenas (Curve B).

Curve A. Spectrum of the carotenoid in petroleum ether, derived from the carotenoprotein.

Results The carotenoprotein contained all the carotenoids present in the ovary (Table XI) in about the same relative proportions (Table XII).

The major component (carotenoid 4 in Table XI) was not separated from astaxanthin (obtained from crustacyanin) on mixed chromatography on thin layers, developing to 10cm. twice with $CHCl_3$, and had a spectrum identical with that of astaxanthin in petroleum ether (Table XIII).

TABLE XI

Thin-layer chromatography of the carotenoids of scallop ovary and carotenoprotein on plates of silica gel G, developed to 10cm. with $CHCl_3$.

Carotenoid	Rf	Colour	Partition in the system 90% MeOH/ petroleum ether
1. (strong)	0.98	yellow	epiphasic
2. (faint)	0.58	orange-red	hypophasic
3. (very faint)	0.45	red	hypophasic
4. (intense)	0.22	red	hypophasic
5. (very faint)	0.19	red	hypophasic
6. (faint)	0.14	yellow	hypophasic

TABLE XII

Estimation of relative proportion of carotenoids in scallop ovary and carotenoprotein using thin-layer chromatography on plates of silica gel G, developing to 10cm. with $CHCl_3$.

Carotenoid	Wavelength (mμ)	Extinction	
		Whole ovary	Chromoprotein
1.	470	0.22	0.19
2 and 6.	450	0.29	0.09
4.	455	1.48	1.48

TABLE XIII

Absorption spectra of carotenoid components listed in Table XI.

Carotenoid	Solvent	Maxima (mμ)	Inflexions
4.	pet.ether	476	
1.	$CHCl_3$	462, 490	410
	pet.ether	450, 480	

E. Composition

Method The composition of the two $(NH_4)_2SO_4$ fractions were determined by the methods discussed (III.H,I.); ether and $CHCl_3$-MeOH (2/1, v/v) extracts were obtained as in (VI.$K_{(iii)}$.) and subjected to thin-layer chromatography.

Results Both fractions appeared to consist of glycoproteins having identical compositions within experimental error. The results are listed below (Table XIV).

TABLE XIV

Composition of the carotenoprotein fractions and their lipid components purified from the ovary of Carcinus maenas by chromatography on calcium phosphate gel.

	30-50% $(NH_4)_2SO_4$ Fraction		50-60% $(NH_4)_2SO_4$ Fraction	
Nitrogen (i) freeze-dried protein	11.7 %	±0.2	11.5 %	± 0.1
(ii) lipid-free protein	15.3	±0.3	15.6	± 0.2
Lipid	22	± 5	24	± 3
Carbohydrate (lipid extracted protein)				
(i) hexose	2.62	±0.3	2.59	± 0.2
(ii) hexosamine	0.25	±0.01	0.25	± 0.01

Table XIV (cont.)

Extracted lipid	%		%	
Nitrogen	1.14	± 0.2	1.08	± 0.05
Phosphorus	2.67	± 0.03	2.57	± 0.1
Phospholipid	66.6	± 5	64.1	± 0.8
cholesterol	20.7	± 1.8	21.0	± 2.0

Ether extraction of the freeze-dried protein removed all the carotenoid and most of the neutral lipid (Fig. 49), but negligible amounts of phospholipid (Fig. 47). A number of neutral lipids were present including cholesterol (Fig. 49). $CHCl_3$-MeOH extracted the phospholipid. This was similar in composition to hen's egg phospholipid (Figs. 47 and 48).

F. Molecular weight and size

Method The minimum molecular weight (for one mole of carotenoid) of the carotenoprotein (50-60% $(NH_4)_2SO_4$ fraction) was estimated in a similar manner to that used for the scallop carotenoprotein (VI.I.). Values of 1.2 and 4.4 were taken for $E^{0.1\%}_{280}$ and E 280/490 respectively.

The extinction at 490mμ for a solution of the carotenoprotein containing 1 mole of carotenoid was taken as 115,000, the molar extinction coefficient of astaxanthin (this carotenoid predominating in the carotenoprotein).

The molecular size was estimated by gel filtration on thin layers of Sephadex G200 (III.$F_{(iv)}$.).

Results The molecular size corresponded to a globular protein with a molecular weight of 200,000 ± 15,000 (three determinations).

The minimum molecular weight was estimated as 420,000.

G. General properties of the carotenoprotein

All experiments were performed in 0.2M-phosphate buffer, pH7.

Chromoprotein solutions faded rapidly in the light and more slowly in the dark. Dialysis against distilled water also resulted in a considerable loss of colour.

The freeze-dried protein slowly denatured on storing in the cold and dark; after three weeks the majority of the protein no longer dissolved.

Shaking solutions of the chromoprotein with ether brought most of the protein out of solution, part of the carotenoid dissociating from the protein and dissolving in ether.

CHAPTER VIII

THE CAROTENOPROTEIN OF THE CARAPACE OF CARCINUS MAENAS

A. Green variety

(i) Extraction and Purification

5g. cleaned shell was ground to a fine powder, passed through a 100-mesh sieve, extracted overnight in the cold and dark with 200ml. 10% EDTA, pH7.5, and centrifuged. The supernatant was yellow, had a main absorption maximum at 390mμ with minor maxima at 460 and 490 mμ, and faded rapidly in light.

The shell residue was suspended in 200ml. 0.05M-phosphate buffer, pH7.5, stirred in the dark and cold for 1 hr. and centrifuged. The green carotenoprotein in the supernatant was purified by calcium phosphate gel chromatography (as used for ovoverdin (V.A.), washing the gel four times with distilled water and eluting the protein with 0.2M-phosphate buffer, pH7. The carotenoprotein was precipitated between 30 and 50% $(NH_4)_2SO_4$ saturation by addition of sat. $(NH_4)_2SO_4$ solution, centrifuged down and dissolved in water.

The shell residue, still coloured light green, was resuspended in 100ml. 0.05 M-phosphate buffer, pH7, containing 6M urea, and allowed to stand for 1 hr. in the cold. The supernatant was brownish yellow after centrifugation

and had a similar spectrum to the EDTA extract, with maxima at 390, 440, 455 and 620mμ (Figure 58); dialysis of the solution against distilled water resulted in considerable fading, the solution remaining brownish yellow.

(ii) General properties of the carotenoprotein

All experiments were performed in 0.2M-phosphate buffer, pH7.

The preparation had masima at 278, 460 and 625 mμ, with inflexions at 320, 408 and 500mμ, and values of E460/625 and E278/460+625 of 2.1 and 1.6 respectively (Figure 58).

In 6M-urea pH7 the protein turned irreversibly brownish yellow (spectrum in Figure 58).

Solutions faded in the light, and after several days deposited a blue precipitate. Fading also occurred on extensive dialysis, the protein remaining in solution.

The carotenoid derived from the carotenoprotein was hypophasic in the system 90% MeOH/petroleum ether and was identical in spectrum with astaxanthin (obtained from crustacyanin) in petroleum ether and CS_2 (maxima at 476mμ and about 500mμ, respectively). It was strongly adsorbed on calcium hydroxide columns, requiring acidified methanol for elution as characteristic of astaxanthin. Astaxanthin has previously been identified in the carapace (Lenel, 1962).

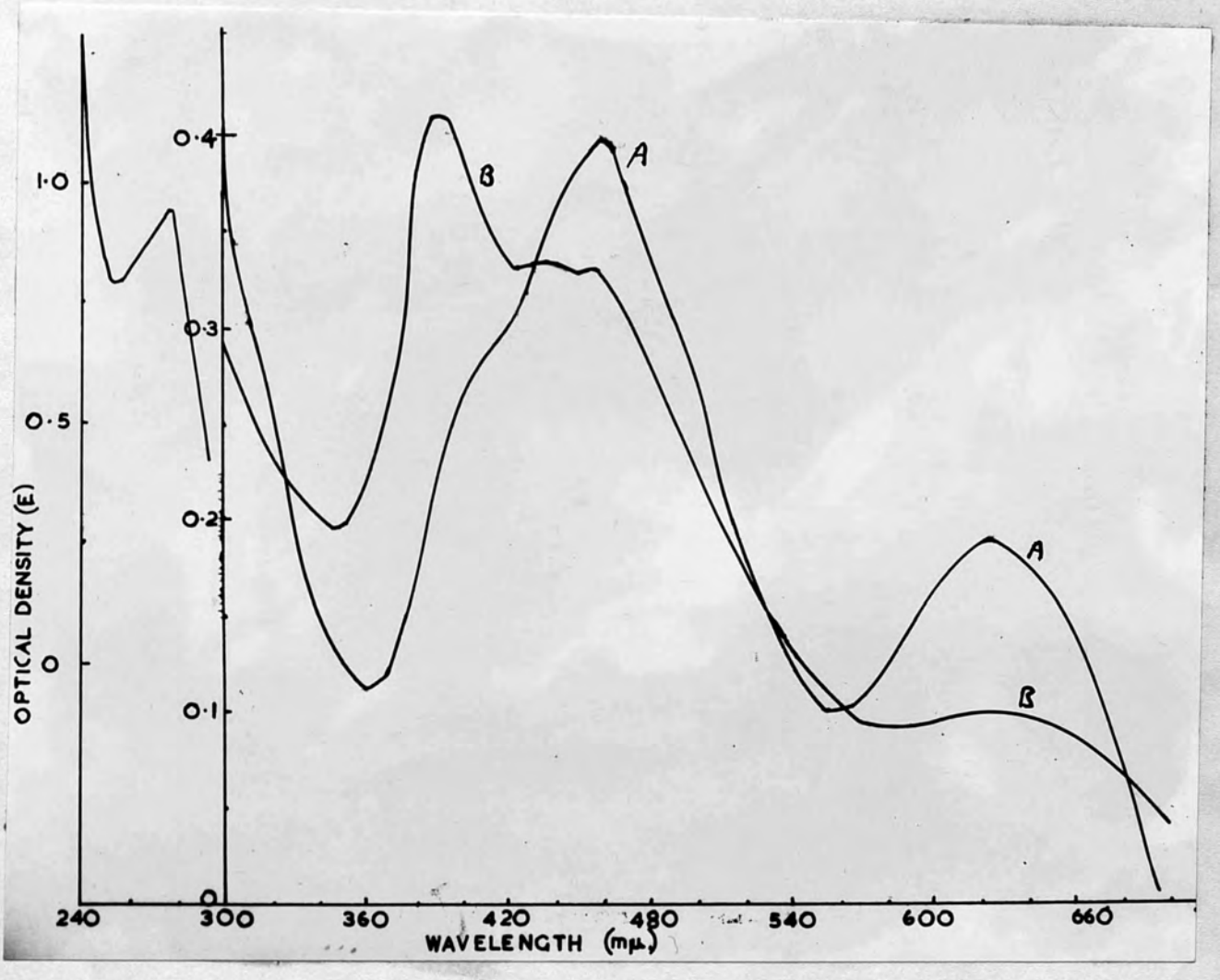


Figure 58. Spectrum of the carotenoprotein of the carapace of Carcinus maenas.

A. In 0.05M-phosphate buffer, pH7.
B. In 0.05M-phosphate buffer, pH7, containing 6M. urea.

The green-yellow colour of the shell is produced by juxtaposition of the colour of the carotenoprotein and the carotenoid, (epiphasic in the system 90% MeOH/petroleum ether).

B. Red variety

10% EDTA pH7.5, 6M- urea at alkaline and acid pH, and glycerol failed to extract the carotenoid in a water soluble form from the powdered shell. Organic solvents (acetone, alcohol, etc.) liberated the astaxanthin from its combination in the shell.

CHAPTER IX

THE CAROTENOPROTEIN OF THE CARAPACE OF ERIPHIA SPINIFRONS

A. Extraction and purification

The reddish-purple carapace was ground to a fine powder and passed through a 100-mesh sieve. 5g. powdered shell was extracted overnight in the cold and dark with 200ml. 10% EDTA, pH7.5. The supernatant after centrifugation was red-brown. The shell residue was suspended in 200ml. 0.05M-phosphate buffer, pH7, and allowed to stand in the cold for 2 hours. The supernatant was purple-red after centrifugation.

The chromoprotein was adsorbed on calcium phosphate gel in the usual manner (IV.$B_{(i)}$.a.), washed four times with distilled water on the centrifuge, eluted twice with an equal volume of 0.2M-phosphate buffer, pH7, precipitated between 0 and 35% $(NH_4)_2SO_4$ saturation by the addition of a sat. $(NH_4)_2SO_4$ solution, and dissolved in 0.02M-phosphate buffer, pH7. The purple-red chromoprotein had an absorption maximum at 532mμ and an E280/532 value at 0.38.

The chromoprotein was further purified by adsorption and stepwise elution from ECTEOLA-cellulose (III.B.). The chromoprotein was adsorbed as a purple-red band below a red band. The column was washed with 0.1M-phosphate

buffer pH7 and the chromoprotein eluted with 0.2M-phosphate buffer, pH7. It gave a value for E280/532 of 0.35.

A further yield of the protein was obtained by suspending the shell residue, still coloured purple, in 0.05M-phosphate buffer, pH7 containing 6M-urea and stirring for 30min. The mixture was centrifuged and the red-purple supernatant dialysed against running tap-water for 5 hrs. The solution turned purple-red. The chromoprotein was purified by ECTEOLA-cellulose chromatography, as above.

B. Homogeneity

Method Cellulose acetate electrophoresis (III.$E_{(i)}$.) was employed. Sufficient material for only one electrophoretic run was available.

Results At pH7 the chromoprotein appeared homogeneous in cellulose acetate electrophoresis.

C. General properties

The chromoprotein showed absorption maxima at 278 and 532mμ with slight inflexions at 310, 340 and 400mμ (Figure 59).

On dialysis against distilled water the chromoprotein came out of solution; it redissolved in 0.02M-phosphate buffer, pH7.

Solutions of the chromoprotein turned first brown then red on heating. No reversion to the purple-red colour occurred when solutions in 0.2M-phosphate buffer, pH7, were

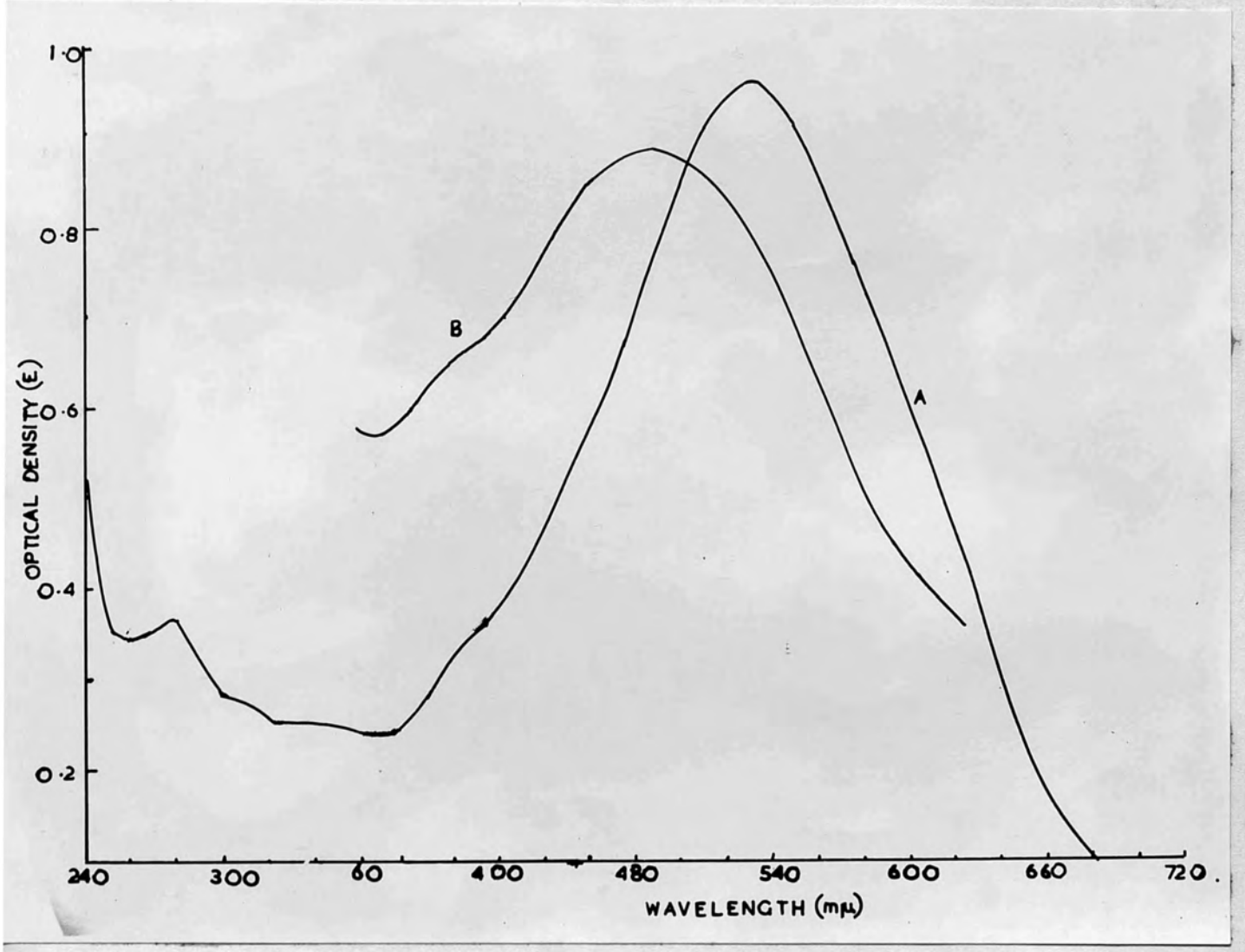


Figure 59. Spectrum of the carotenoprotein of the carapace of Eriphia spinifrons.

A. In 0.05M-phosphate buffer, pH7.
B. In 0.05M-phosphate buffer, pH7, containing 6M-urea.

heated at 80° until brown, and rapidly cooled.

The chromoprotein turned red-brown on prolonged standing in cold 0.05M-phosphate buffer, pH7, containing 6M-urea (spectrum, Fig. 59). Even after 24 hrs. standing in the cold, a considerable amount of the purple-red chromoprotein was reformed on dialysis against distilled water.

No separation of crustacyanin and the purple chromoprotein was observed when mixtures of the two proteins were filtered through 15cm. columns of Sephadex G200 (equilibrated with 0.05M-phosphate buffer, pH7, containing 1M-NaCl) indicating that the protein must have a similar size to α-crustacyanin.

Negligible fading of the chromoprotein in 0.2M-phosphate buffer, pH7, occurred on exposure to bright sunlight (July) for 4 hrs.

The carotenoid derived from the chromoprotein was hypophasic in the system 90% MeOH/petroleum ether and showed a single-peaked absorption spectrum with maximum at 476mμ in petroleum ether and at about 500mμ in CS_2. The carotenoid was not separated from astaxanthin, derived from crustacyanin, on mixed thin-layer chromatography (V.$F_{(iv)}$.), using $CHCl_3$ as the developing solvent. It was strongly adsorbed onto calcium hydroxide, requiring acidifed methanol for elution as characteristic of astaxanthin.

The red-purple colour of the carapace is produced by juxtaposition of the purple carotenoprotein and a yellow xanthophyll (absorption maxima 450 and 475mμ with an inflexion at 425mμ in petroleum ether).

CHAPTER X

THE CAROTENOPROTEIN OF THE OVARY OF PALINURUS VULGARIS

A. Extraction and purification

10g. bright red ovary was homogenised for 15 sec. with 200ml. distilled water, the solution centrifuged at 13,000 r.p.m. for 30 min., and the yellow lipid floating on the surface removed. The red chromoprotein in the supernatant was adsorbed on calcium phosphate gel (as in IV.$B_{(i)}$.a.), washed four times with distilled water and eluted with 0.2M-phosphate buffer, pH7. Solid $(NH_4)_2SO_4$ was added to 50% saturation, the solution centrifuged, and the light red precipitate discarded. Further $(NH_4)_2SO_4$ was added to the supernatant to saturation and the solution centrifuged. The bright red chromoprotein was removed from the surface, dissolved in 100ml. distilled water and adsorbed onto a DEAE-cellulose column (III.B.). The column was washed with water and the chromoprotein eluted with 0.05M-phosphate buffer, pH7.

One extraction removed most of the colour from the ovary, leaving the residue coloured light orange.

B. Absorption spectrum

The chromoprotein had absorption maxima at 466 and 490mμ with inflexions at 325 and 435mμ (Figure 60)

Addition of solid urea (to 6M.) to a solution of the

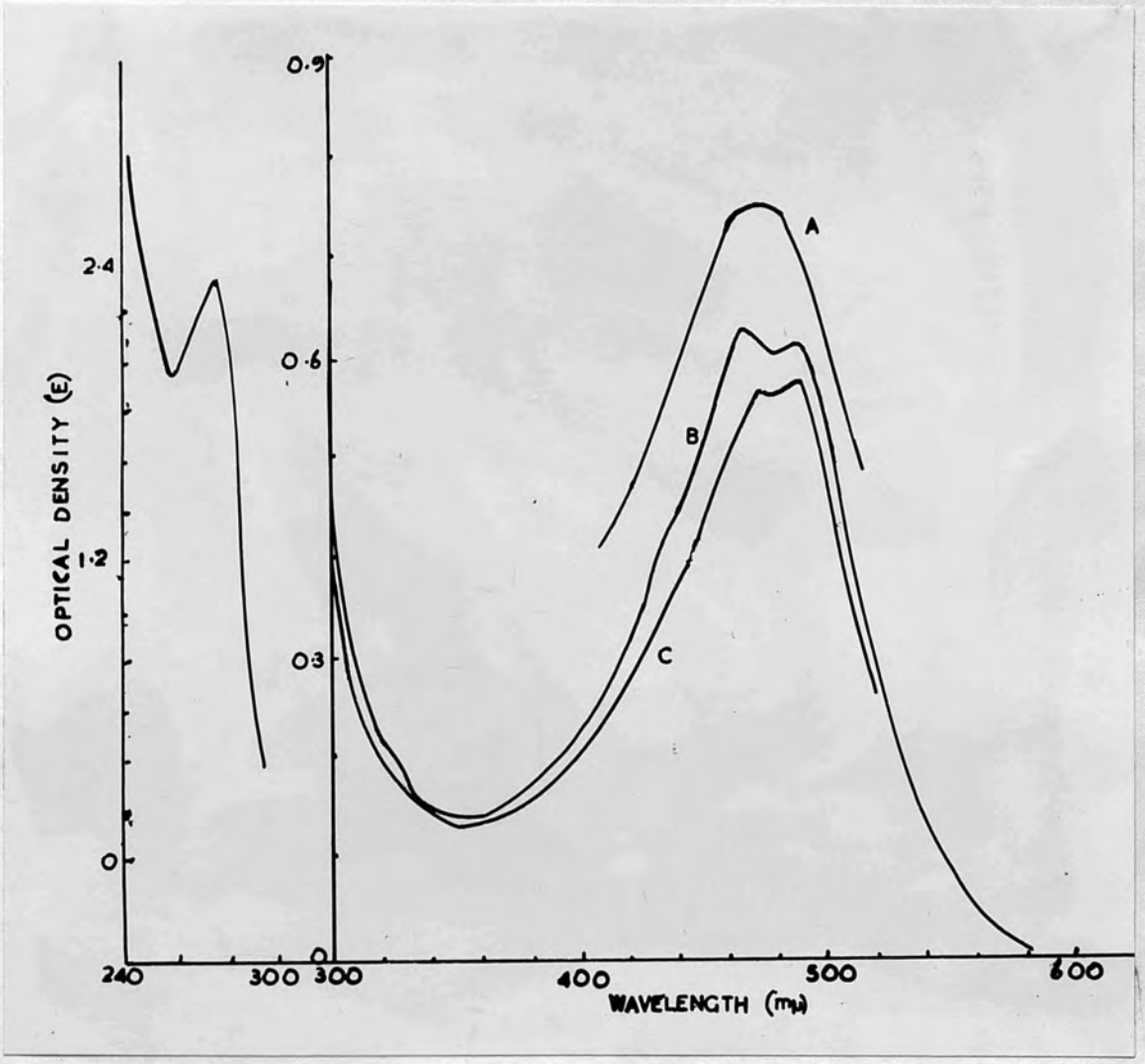


Figure 60. Spectrum of the carotenoprotein of the ovary and eggs of Palinurus vulgaris.

B. In 0.05M-phosphate buffer, pH7.
C. In 0.05M-phosphate buffer, pH7, containing 6M- urea.

Curve A. Spectrum in petroleum ether of the carotenoid, derived from the carotenoprotein.

carotenoprotein in 0.2M-phosphate buffer, pH7, altered the shape of the spectral curve. The 466mμ maximum shifted to 470mμ and the extinction was lowered below that of the 490mμ maximum; the inflexion at 325mμ disappeared. Fading was accelerated in the urea solution.

The value of E280/466 for the preparation was 3.8.

The spectrum of the carotenoid derived from the protein had a maximum at 476mμ with a slight inflexion at 465mμ.

C. Homogeneity

Method III.$E_{(i)}$.

Results At pH8.6 a single orange component was observed in cellulose acetate electrophoresis but at pH7 a colourless band was also observed moving towards the cathode (Fig. 61). At pH4.5 the proteins streaked from the origin in cellulose acetate electrophoresis.

D. Molecular size

Method III.$F_{(iv)}$.

Results The chromoprotein preparation gave a single red spot on gel filtration using thin layers of Sephadex G200. A trace of high molecular weight protein was also revealed on staining (Fig. 62).

E. Carotenoid composition

Method The carotenoid compositions of the chromoprotein

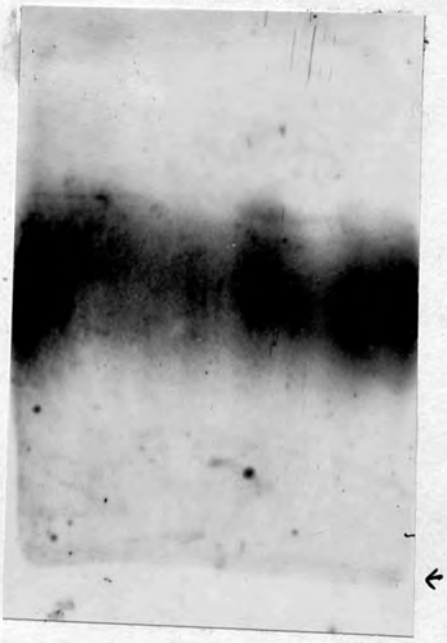


Figure 61. Cellulose acetate electrophoresis of the carotenoprotein of the ovary of P.vulgaris.

0.07M-barbitone buffer, pH8.6, 5hr. run.

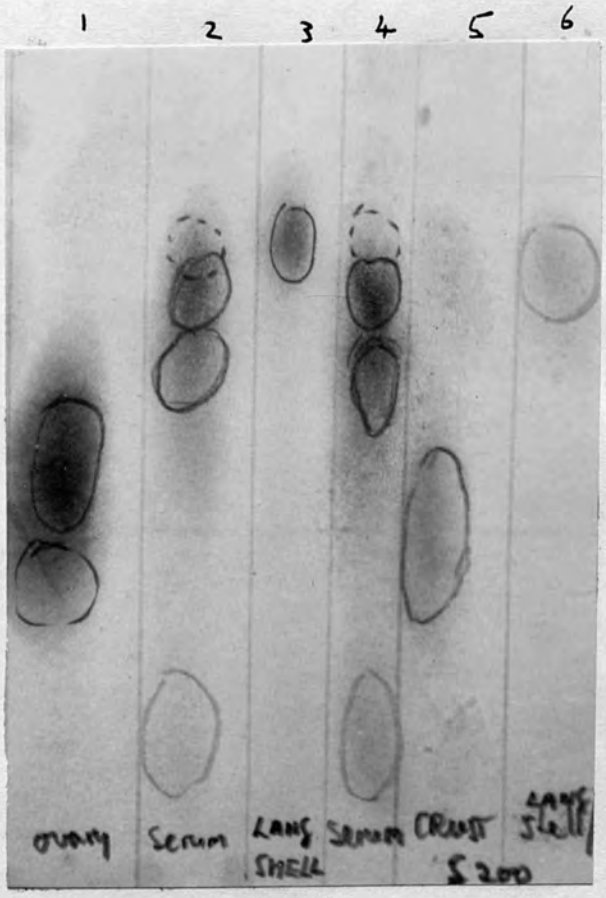


Figure 62. Thin-layer gel filtration on Sephadex G200. Plates developed with 1M. NaCl/0.02M-phosphate buffer pH7.

Column 1. Crude carotenoprotein preparation obtained from the ovary of P.vulgaris.
Columns 2 and 4. Human serum (Haemoglobin indicated by the dotted circle)
Columns 3 and 6. The carotenoprotein of the carapace of P.vulgaris.
Column 5. α-crustacyanin.

and whole ovary were investigated by thin-layer chromatography on plates of silica gel G. The plates were developed to 10cm. with $CHCl_3$.

Results The chromoprotein contained all the carotenoids present in whole ovary. The major carotenoid (No. 1 in Table XV) had a spectrum identical with that of astaxanthin in petroleum ether and CS_2 (maximum at 476mμ and about 500mμ respectively), and was not separated from it in mixed thin-layer chromatography.

TABLE XV

Carotenoids of whole ovary and carotenoprotein of *Palinurus vulgaris*

Thin-layer chromatography on plates of silica gel G, developing to 10cm. with $CHCl_3$.

Carotenoid	Rf	Colour	Partition in the system 90% MeOH/petroleum ether
1, Intense	0.23	red	hypophasic
2, Faint	0.38	red	hypophasic
3, Faint	0.48	yellow	hypophasic
4, Faint	0.98	yellow	epiphasic

F. Lipid content

The chromoprotein preparation (Section A) was first further purified by block electrophoresis using Sephadex G25 (medium grade) as supporting medium (Dose & Krause, 1962).

Method A slurry of Sephadex G25 in 0.05M-phosphate buffer, pH7, was poured into a perspex tray and an even

layer formed by moving a glass rod over the surface. A 0.3cm. band of Sephadex towards the centre of the plate was removed, blotted with filter paper mixed with ½vol. concentrated protein solution in 0.05M-phosphate buffer, and replaced in the slot. Electrophoresis was then carried out as for starch gels (III.E$_{(ii)}$.a.) for 8 hrs. at 10 volts/cm. The red chromoprotein band was dug out, suspended in 4 vols. $CHCl_3$ - MeOH (2/1, v/v) and left in the cold for two days. The Sephadex was then centrifuged down, the extract evaporated to dryness, and the lipid taken up in a few drops of the $CHCl_3$ - MeOH solution. The lipids were then subjected to thin-layer chromatography (III.I$_{(iv)}$.).

Results A filter paper impression (Figure 63) of the tray after removal of the chromoprotein revealed that the impurity observed on cellulose acetate electrophoresis was well separated from the chromoprotein.

Phospholipids and neutral lipids were revealed on thin-layer chromatography (Figures 64 and 65). Phosphatidylethanolamine and other phospholipids present in hen's egg were detected in the phospholipid run (Fig. 64). Cholesterol, cholesterol esters and other neutral lipids were detected in the neutral lipid run (Fig. 65).

G. General properties

Solutions of the chromoprotein in 0.2M-phosphate

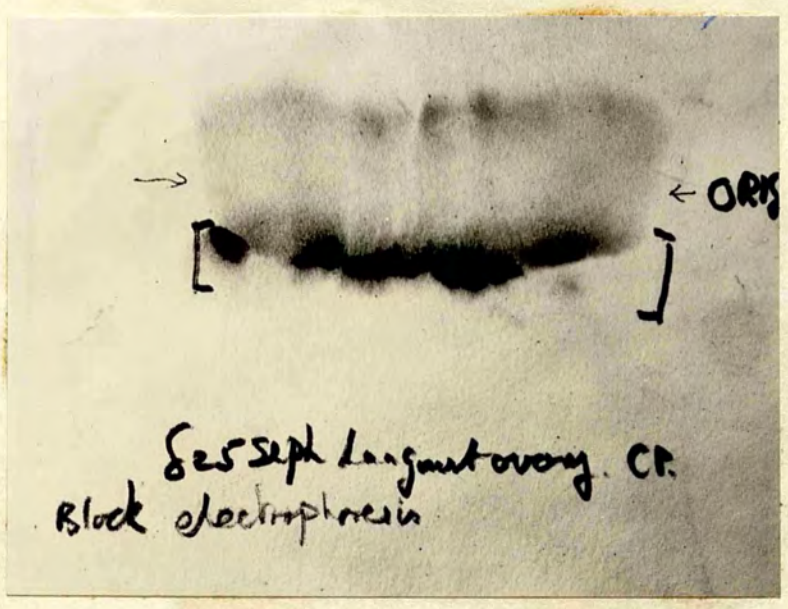


Figure 63 Sephadex G25 (medium grade) block electrophoresis of the partially purified carotenoprotein of the ovary of P. vulgaris. 10 volts/cm. 8 hr. run.

Figure 64. Thin-layer chromatography on silica gel G.
Solvent: $CHCl_3$/MeOH/H_2O (80/30/3 v/v).
Development distance: 10cm.
Column 1. $CHCl_3$-MeOH extract of the carotenoprotein of the ovary of P. vulgaris.
Column 2. Hen's egg phospholipid
Column 3. $CHCl_3$-MeOH extract of Sephadex G25(control).

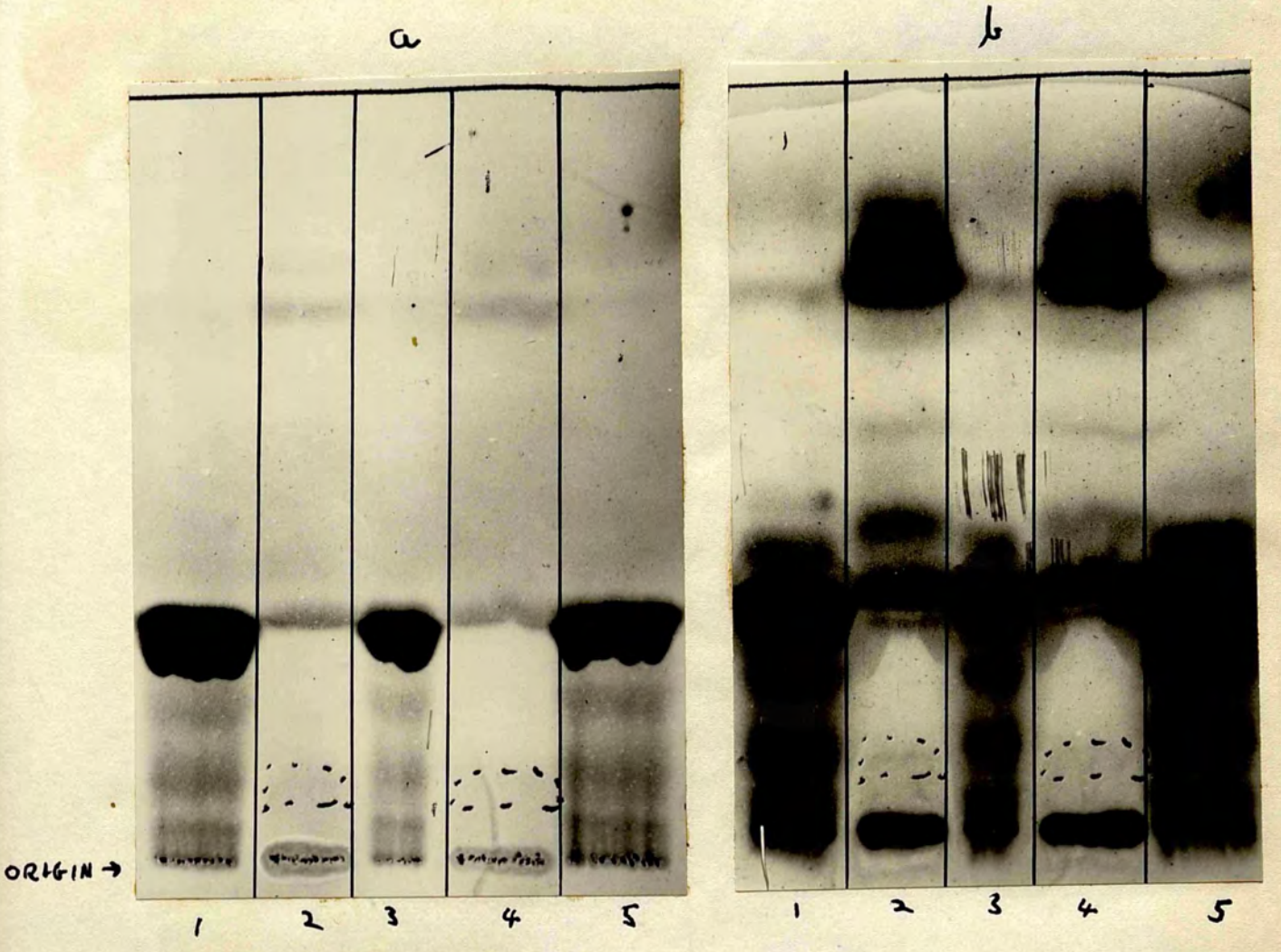


Figure 65. Thin-layer chromatography on silica gel G.
Solvent: petroleum ether/ether/glacial acetic acid (60/40/1, v/v).
Development distance: 10cm.

Columns 1, 3 and 5. Cholesterol standard.
2 and 4. Lipid extract of the carotenoprotein of the ova of P.vulgaris (carotenoid spot is circled).

(a) Sprayed for cholesterol and cholesterol esters.
(b) Sprayed with ammonium molybdate-perchloric acid.

buffer, pH7, faded rapidly in the light, and more slowly in the dark. Fading also occurred on dialysis, the protein remaining in solution.

CHAPTER XI

THE CAROTENOPROTEIN OF THE EGGS OF PALINURUS VULGARIS

A red chromoprotein, identical in spectrum to that of the ovary, was obtained and purified from the eggs by the same method as that used for the ovary chromoprotein (X.A.). The preparation was shown to contain the neutral lipids, plus phospholipids and carotenoids present in the ovary carotenoprotein.

CHAPTER XII

THE CAROTENOPROTEIN OF THE CARAPACE OF PALINURUS VULGARIS

A. Extraction and purification

10g. powdered shell was extracted overnight with 200ml. 10% EDTA, pH7.5, as described (IV.A.). The extract was very faintly blue. The shell residue was shaken with 200ml. 0.05M-phosphate buffer, pH7 for 1 hr. The mauve supernatant had maxima at 560 and 525 mμ and an inflexion at 470 mμ (Fig. 64). The chromoprotein was adsorbed onto DEAE-cellulose, the column washed with 0.1M-phosphate buffer, pH7, and the purple chromoprotein eluted with 0.2M-phosphate buffer, pH7. It was then precipitated at 50% $(NH_4)_2SO_4$ saturation and dissolved in distilled water.

0.05M-phosphate buffer, pH7, containing 6M. urea eluted more of the chromoprotein from the shell residue. The solution turned red within two hrs. On dialysis the purple colour characteristic of the chromoprotein re-appeared, provided the solution was not left to stand for more than a few hours.

B. Absorption spectrum

The chromoprotein had absorption maxima at 278 and 560mμ, with inflexions at 350 and 600mμ. The value of E278/560 was 0.98 (Figure 66).

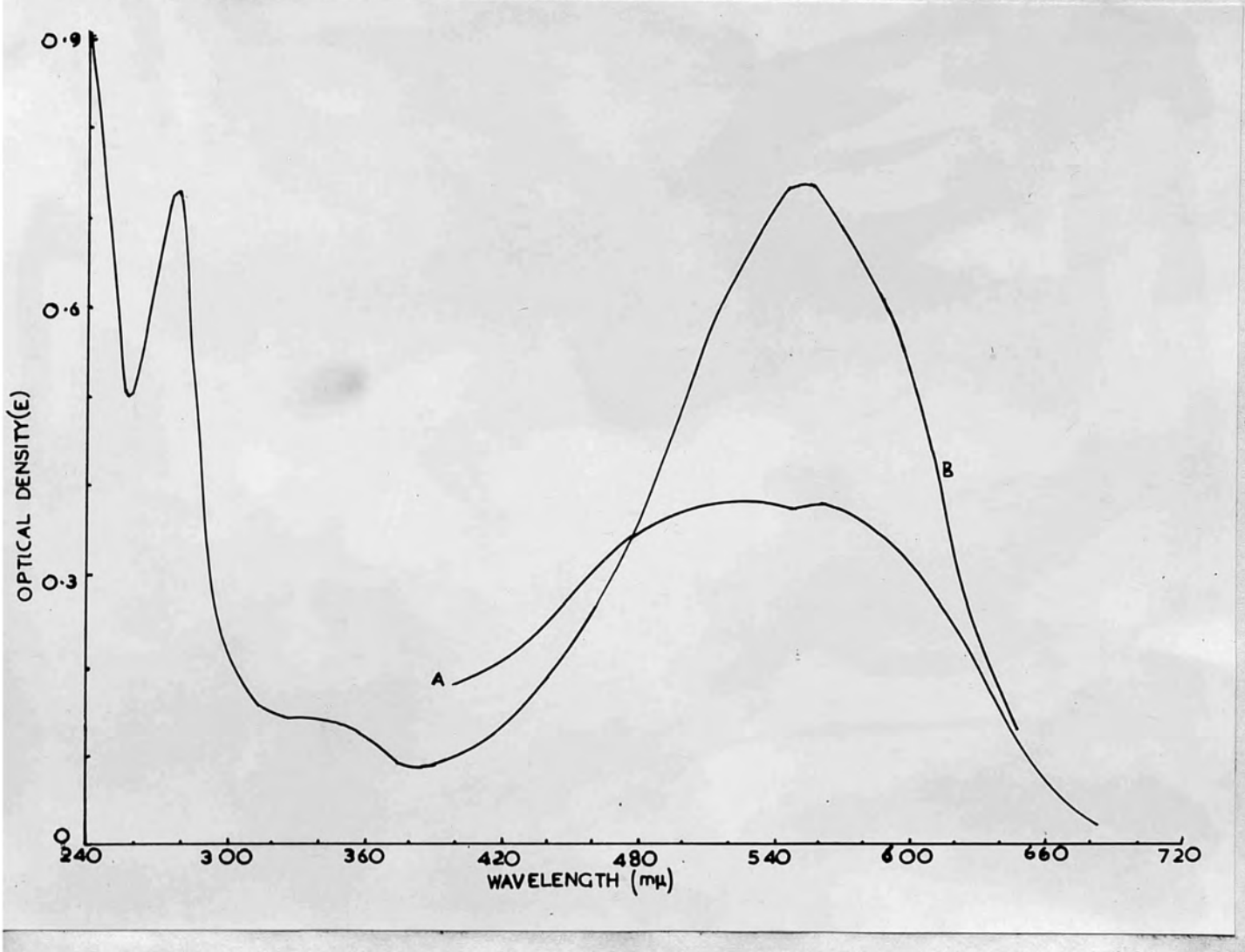


Figure 66. Spectrum of the carotenoprotein of the carapace of Palinurus vulgaris.

A. Initial aqueous extract.
B. Purified preparation.

C. Homogeneity

Method Insufficient material was available to do more than one electrophoretic run. Overnight starch gel electrophoresis using Poulik's discontinuous buffer system was employed (III.$E_{(ii)}$.a.).

Results Two purple bands migrated close to the solvent front, the faster moving band greatly predominating. Slight traces of more slowly moving, colourless proteins were revealed on staining.

D. Carotenoid composition

The carotenoid component of the chromoprotein had a spectrum identical with that of astaxanthin in CS_2 and petroleum ether (maximum at about 500mμ and 476mμ respectively), and was only eluted from $Ca(OH)_2$ columns with acidifed MeOH as characteristic of astaxanthin. Astaxanthin has previously been identified in the carapace (Fabre & Lederer, 1934).

E. Molecular size

Method The molecular size of the protein was estimated by gel filtration on thin layers of Sephadex G200 (III.$F_{(iv)}$.).

Results The chromoprotein moved as a single component behind serum albumin on filtration through the gel. (Fig.62).

The molecular size corresponded to that of a globular protein with a molecular weight of 36,500 ± 2,000 (two determinations).

F. General properties

Solutions of the chromoprotein were fairly stable in light, negligible fading occurring on exposure to sunlight (July) for two hours. The protein remained in solution on dialysis against distilled water. No alteration in spectrum occurred on desalting solutions using columns of Sephadex G25.

CHAPTER XIII

THE CAROTENOPROTEIN OF THE CARAPACE OF ASTACUS ASTACUS

A. Extraction and purification

5g. powdered shell was extracted with 200ml. 10% EDTA, pH7.5 overnight, centrifuged, the green supernatant brought to 50% $(NH_4)_2SO_4$ saturation, centrifuged, and the green precipitate dissolved in distilled water. The chromoprotein was adsorbed onto calcium phosphate gel in the usual manner (IV.$B_{(i)}$.a), the gel washed four times with distilled water and the chromoprotein eluted twice with 0.2M-phosphate buffer, pH7. The pooled eluants were brought to 30% $(NH_4)_2SO_4$ saturation by the addition of a sat. $(NH_4)_2SO_4$ solution, centrifuged, and the light green precipitate discarded. Further sat. $(NH_4)_2SO_4$ solution was added to 50% saturation, the solution centrifuged and the large green precipitate dissolved in distilled water. The supernatant, coloured light blue-green, was saturated with $(NH_4)_2SO_4$, centrifuged, and the small blue-green precipitate dissolved in water.

B. Spectrum

The spectra of the original EDTA extract and the fractions obtained at 30 - 50% and 50 - 100% $(NH_4)_2SO_4$ saturation are shown in Figure 67. The chromoproteins had maxima at 278 and 632 mμ with inflexions at 320, 470

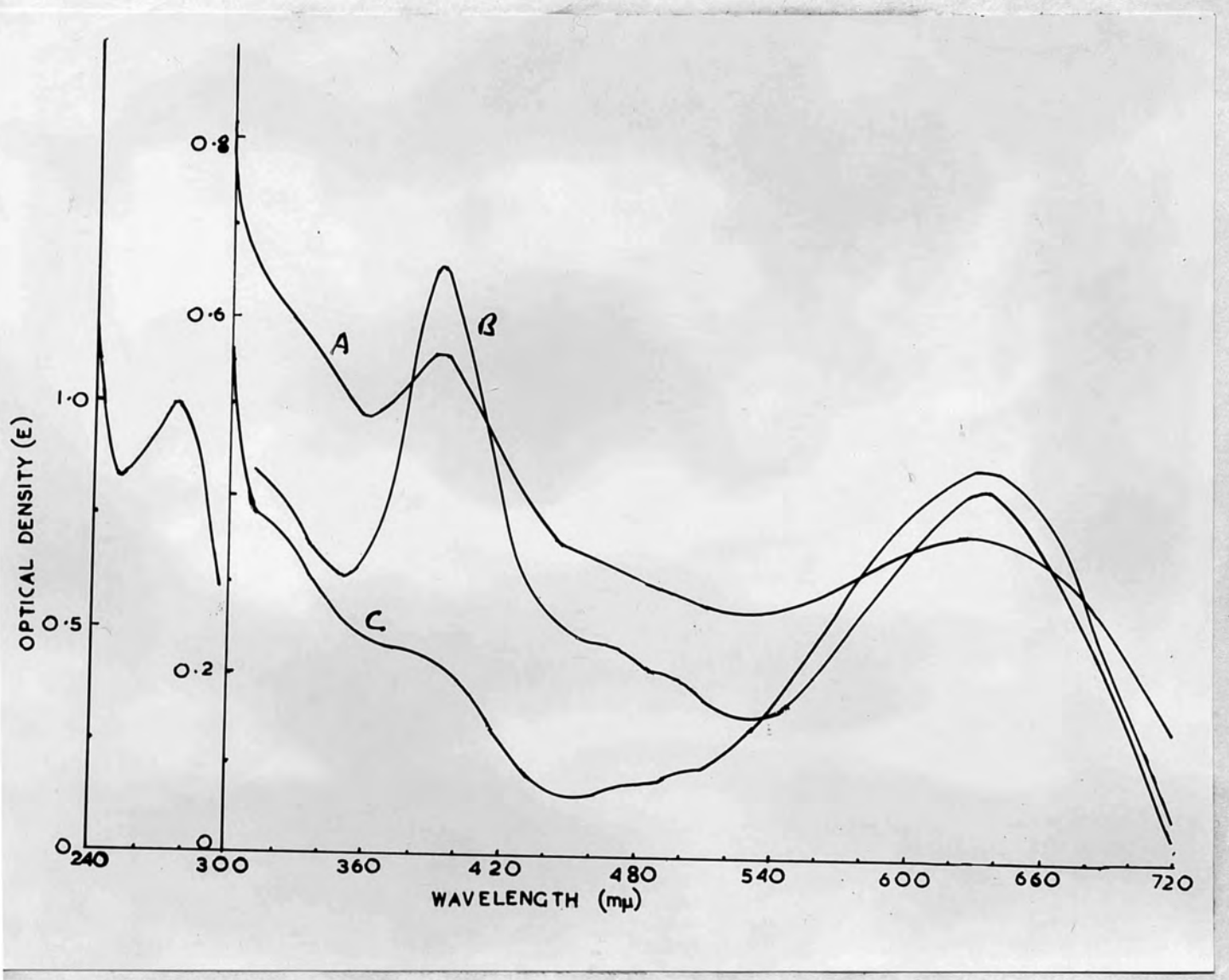


Figure 67. Spectrum of the carotenoprotein of the carapace of Astacus astacus.

A. purified by calcium phosphate gel chromatography.
B. as A, precipitated between 0-50% $(NH_4)_2SO_4$ saturation dissolved in 0.2M-phosphate buffer, pH7.
C. as in A, precipitated 50-100% $(NH_4)_2SO_4$ saturation and dissolved in 0.2M-phosphate buffer, pH7.

and 495mμ. The 50 - 100% $(NH_4)_2SO_4$ fraction had a slight inflexion at about 390 mμ, whereas the original extract and the 30 - 50% fraction had a sharp maximum at 392 mμ. The 30 - 50% fraction gave a value of E278/392 + 632 of 1.1.

C. Carotenoid composition

The carotenoids derived from the chromoprotein were hypophasic in the system 90% MeOH-petroleum ether. A carotenoid of spectrum identical with that of astaxanthin in petroleum ether and CS_2 predominated. It was eluted from $Ca(OH)_2$ columns with acidified MeOH, and from columns of icing-sugar with 4% MeOH in petroleum ether, as characteristic of astaxanthin. Astaxanthin has previously been identified in the carapace (Fabre & Lederer, 1934).

Traces of axanthophyll, eluted from icing-sugar with 2% MeOH in petroleum ether, and from $CaCO_3$ with 10% acetone in petroleum were also present in the chromoprotein preparation. The xanthophyll had maxima at 424, 445 and 476 mμ in petroleum ether and at 440 -450, 474 and 505mμ in CS_2.

D. General properties of the 30 - 50% $(NH_4)_2SO_4$ fraction

All experiments were performed in 0.2M-phosphate buffer, pH7.

The protein remained in solution on dialysis against

distilled water but faded considerably; the solution became bluer on dialysis.

Solutions of the chromoprotein turned first blue, then orange on heating. Considerable fading occurred in the light; thus, the value of E392/632 changed from 1.3 to 1.1 on exposure to sunlight for one hour and the solution became blue after a few hours standing in sunlight.

The chromoprotein moved just behind α-crustacyanin on columns of Sephadex G200 developed with 0.05M-phosphate buffer, pH7, containing 1M - NaCl, but was not completely separated from it on 15 cm. columns (using a 1.5ml. mixed protein sample). It must therefore have a molecular size of a similar order of magnitude to that of α-crustacyanin.

The yellow-green carapace contained, besides the carotenoprotein, considerable quantities of free xanthophylls and epiphasic carotenoids (including possibly astaxanthin ester; maximum 472mμ in petroleum ether). The yellow-green colour of the carapace is produced by juxtaposition of the colours of the free and bound carotenoids.

CHAPTER XIV

DISCUSSION

A. Crustacyanin

(i) Extraction (IV.A.) and purification (IV.B(i)'(ii)'(vi))

Crustacyanin is located in the inner calcified layers of the lobster shell (Verne, 1923), the outer layer (epicuticle) being composed of wax, polyphenol and lipoprotein (Richards, 1951). Fine grinding of the shell exposed the inner layers allowing attach by decalcifying agents, and consequent liberation of the water-soluble pigment; without fine grinding of the shell, extraction of the pigment was slow and the yield poor.

The purple, yellow and blue chromoproteins separated by gradient elution on DEAE-cellulose (IV.A(iii).) all had astaxanthin as prosthetic group (IV.B(xix)f). The absorption maximum of the yellow pigment (412mμ) corresponded to that of acid-denatured crustacyanin (Wald et al., 1948) and was presumably denatured material; less of this component was obtained using EDTA extraction. β-crustacyanin also appeared to be an irreversible denaturation product of α-crustacyanin; repeated DEAE-cellulose chromatography always resulted in the appearance of some β-crustacyanin (also observed by Jencks & Buten, 1964), and it was formed from α-crustacyanin by allowing

salt-free solutions to stand (IV.$B_{(iv)}$b). Changes in proteins on DEAE-cellulose chromatography have been observed in other cases (Broman, 1964).

Although citrate gave the more rapid extraction, considerable amounts of the yellow and purple chromoproteins were formed, and the EDTA extract was preferred.

Chromatography on calcium phosphate gel (IV$B_{(i)}$a) led to the appearance of denatured material in which the carotenoid was dissociated from the protein. This material had an absorption maximum at 495mμ that of free astaxanthin in digitonin solution (Wald et al., 1948). It was not present in the original extracts and must therefore have been formed during the chromatographic procedure. It may represent the denatured, more strongly held, protein layer adsorbed on the gel (Zittle, 1953).

The absorption maxima of α-crustacyanin at 630, 370 and 320mμ represent electronic transitions from the ground state of the protein-conjugated chromophone to the first, second, and third excited states respectively. Slight vibrational fine structure was observed for the first transition, there being a slight inflexion at about 600mμ. This was present in electrophoretically pure preparations and was not thought to be due to traces of β-crustacyanin.

The α-crustacyanin purified by the procedure described

had a low value of E280/630 (0.31) than that obtained by Jencks and Buten (1964), so that their preparation was probably not completely homogeneous. It was obtained electrophoretically homogeneous at three pH values, the only carotenoprotein besides ovorubin to be so purified. Heterogeneity observed using starch gels with the tris-citrate-borate system of Poulik (1957) may be attributed to the partial breakdown of the protein into its subunits in this buffer system; possibly the sudden change in potential gradient as the citrate band moved through the protein causes distortion of the protein with resultant loosening of its structure.

Crystals of α-crustacyanin of microscopic size are readily obtained. The ease of crystallisation suggests that large crystals might be obtained by more refined techniques. The carotenoid, representing a fairly large structural feature of the protein (1 mole/35,700g. protein, IV.$B_{(vii)}$), might be detectable by x-ray crystallography if large enough crystals were obtained; such studies might well throw some light upon the mode of binding of the carotenoid in the combination.

(ii) Complexity of β-crustacyanin

β-crustacyanin obtained from the original shell extract (IV.$A_{(iii)}$) or from thoroughly dialysed preparations of α-crustacyanin (IV.$B_{(iv)}$.a) was shown to consist of at

least four purple components (IV.$A_{(iii)}$). The relative intensity of the components in dialysed preparations of α-crustacyanin varied with the extent of dialysis. In most cases, one or two of the components predominated, and it is possible that the observed heterogeneity arose through alteration of a single component (or perhaps two components). The components might be conformational isomers or chemically altered units (e.g. by amide bond hydrolysis or disulphide interchange). Denaturation with resultant heterogeneity might be expected to arise in the absence of salt when the isoionic point of the protein is low. The isoionic point of the mixture of crustacyanin subunits present in the absence of salt (IV.$B_{(ix)}$e), was not investigated; it probably does not differ greatly from the isoelectric point of α-crustacyanin, which is itself low (IV.$B_{(iii)}$).

β-crustacyanin prepared directly from the shell extracts was as heterogeneous as the material obtained by dialysis and freeze-drying. It seems unlikely, therefore that the complexity of the β-crustacyanin could have arisen during the freeze-drying or from bacterial action.

(iii) Composition (IV.$B_{(xix)}$)

The nitrogen content found for the freeze-dried preparation indicated that it must be essentially protein

in nature; no carbohydrate was detected analytically or on staining cellulose acetate strips and the traces of lipid observed (IV.$B_{(ix)}$.e) were probably unspecifically adsorbed on the protein surface. The proteins contained the usual amino acids occurring in proteins.

The value of $E_{280}^{0.1\%}$, and the tyrosine and tryptophan content were similar to those obtained for ovorubin (Cheesman, 1958; Norden, 1962).

(iv) Molecular sizes of α- and β-crustacyanin (IV.$B_{(vii)}$,$_{(viii)}$)

The molecular size of α-crustacyanin was found to be about nine times that of β-crustacyanin. If we assume that α- and β-crustacyanin are of the same shape as the globular serum protein standards used in the gel filtration, then the molecular weight of α-crustacyanin is nine times that of β-crustacyanin. As the shapes of α- and β-crustacyanin were not investigated, molecular weight estimates obtained by gel filtration must be taken with caution. As discussed (III.$F_{(iii)}$), if α-crustacyanin were found to be a fibrous molecule the molecular weight estimate by gel filtration would be low, whereas if it has an expanded structure it would be high. The molecular weight estimate for β-crustacyanin derived from gel filtration lies within 10% of the minimum molecular weight of α-crustacyanin calculated from the carotenoid content, so that β-crustacyanin appears to represent the caroteno-

protein subunits of which α-crustacyanin is composed. If the molecular weight estimate for α-crustacyanin is correct, α-crustacyanin would consist of about ten of these subunits (possibly different), each binding a carotenoid. It is hoped to confirm the validity of the molecular weight estimation by physical methods; viscosity measurements would establish whether or not α-crustacyanin is globular, as assumed.

β-crustacyanin gave a single component on gel filtration (IV.B$_{(vii)}$), so that the different components of β-crustacyanin must be of roughly the same size.

α-crustacyanin dissociated into its subunits on a number of treatments. Removal of salt (IV.B$_{(iv)}$.a; and IV.B$_{(ix)}$.e) the action of acetone (IV.B$_{(xv)}$), 6M-urea (IV.B$_{(xvii)}$), and possibly acid (IV.B$_{(xiii)}$.a., as discussed in XIV.A$_{(vii)}$.c.) all resulted in the formation of fragments having approximately the same size as β-crustacyanin.

(v) Salt effect (IV.B$_{(ix)}$)

The change in spectrum on removal of salt was found to be associated with the reversible dissociation of α-crustacyanin into subunits (possibly different) of roughly the same size, that of β-crustacyanin (IV.B$_{(ix)}$.e). Below an ionic strength of 0.07 for NaCl (0.1mg. protein/ml.) an equilibrium, slowly established was shown to exist between

α-crustacyanin and its subunits (IV.B$_{(ix)}$.c). At an ionic strength of 0.12 and above, no trace of the subunits were detected. In distilled water α-crustacyanin was completely dissociated into its subunits (absorption maximum 595mμ) but on addition of salt reaggregation occurred; on prolonged standing in the absence of salt, reaggregation no longer occurred and the spectrum of the subunits became identical with that of β-crustacyanin (absorption maximum 585mμ). The difference in spectrum between β-crustacyanin and the subunits was presumably due to alteration in the linkage between carotenoid and protein which prevented reaggregation of the subunits in the presence of salt. The spectrum of the subunits remained unaltered on passage through Sephadex but the ability to aggregate was lost; penetration into the gel particles presumably altered some feature of the configuration of the protein, essential for the association, other than that involved in binding the carotenoid.

It appeared therefore that the subunits in α-crustacyanin were held together by weak forces at a number of points in the peptide chain, alteration of the carotenoid-protein linkage (as in β-crustacyanin) or of other parts of the molecular (as in gel filtration) resulting in the loss of ability to associate.

Protein molecules of size intermediate between that

of α-crustacyanin and the subunits were not detected on columns of Sephadex G200, suggesting an "all or nothing" cleavage into subunits. Since α-crustacyanin has a homogeneous molecular size in gel filtration (IV.$B_{(viii)}$), it must be composed of a definite number of subunits in a highly organised structure.

The reversible dissociation of α-crustacyanin, dependent on ionic strength (IV.$B_{(ix)}$.c) can be depicted as follows:

$$\alpha\text{-} \underset{\text{salt}}{\overset{H_2O}{\rightleftharpoons}} \underset{(\lambda max, 595 m\mu)}{n\, S_{1,2,3..}} \rightarrow {}^{n}\beta_{1,2,3...}\ (\lambda max, 585 m\mu)$$

$$\underset{(\lambda max, 595 m\mu)}{n\, S_{1,2,3..}} \rightarrow {}^{n}\gamma_{1,2,3...}\ (\lambda max, 595 m\mu)$$

where, λmax = the wavelength of the absorption maximum.

$S_{1,2,3..}$ = the subunits, possibly different, but of roughly the same size.

n = the number of subunits formed by dissociation of one molecule of α-crustacyanin, and which could have a value as great as 10.

$\gamma_{1,2,3..}$ = the subunits modified irreversibly by passage through Sephadex.

$\beta_{1,2,3..}$ = β-crustacyanin formed irreversibly from the subunits on standing in the absence of salt.

The equilibrium might depend on protein concentration as well as on ionic strength, but this was not investigated.

Dissociation of proteins with increasing ionic strength (cf. haemoglobin and haemocyanin) is more usual

than the effect observed here. However, analogous behaviour has been observed for hog thyroglolubin (Svedberg & Pedersen, 1940), which dissociates into subunits at low ionic strength, no trace of dissociation being detectable at high salt concentration (0.2M.).

Fortunately, in the electrophoretic experiments performed to test the homogeneity of α-crustacyanin preparations (IV.$B_{(ii)}$), the ionic strength was high enough to prevent dissociation into subunits.

The stabilisation energy associated with the spectral change in the presence and absence of salt, can be calculated from the difference in the energies involved for the electronic transitions from the ground to first excited state in the two cases. The energy of the transition, ΔF, is given by Planck's equation:

$$\Delta F = \frac{Nhc}{\lambda_{max}} \quad \text{i.e.} \quad \frac{2.854 \times 10^7}{\lambda_{max}(m\mu)}$$

where N = Avagadro's number, h = Planck's constant, c = the velocity of light, λ = the absorption maximum

and the stabilisation energy accompanying the addition of salt to the salt-free solution is hence

$$2.854 \times 10^7 \left(\frac{1}{632.5} - \frac{1}{595}\right) = -2.8 \text{Kcals/subunit.}$$

This additional strength of binding between the subunits is thus of the same order as that of hydrophobic bonds

formed between the paraffin chains in micelles of soaps and detergents (0.2 to -1.5Kcal/mole, Haurowitz, 1963(iii)). It is possible that bonding energies of this order of magnitude might be obtained by π-orbital overlap between adjacent carotenoid molecules of different subunits in α-crustacyanin. The subunits (molecular weight 35,700 (IV.B$_{(vii)}$,$_{(viii)}$), if spherical would have a diameter of about 40 Å (Hubbard & Kropf, 1959), the planar carotenoid molecule (about 20 Å in length) would then be a large structural feature of the subunit, and $\pi - \pi$ orbital interaction between carotenoid molecules on the different subunits could reaidly occur on collision. If bonding of this nature were present, the hydrophobic carotenoid molecules might be expected to be located in the internal part of the α-crustacyanin molecule, surrounded by hydrophilic peptide chains. The subunits might then be held together by forces between the carotenoid molecules and on adjacent peptides as well as by Van der Waals' forces between peptide chains.

The suggestion made above is reminiscent of the role proposed for the DPNH prosthetic group of lactic dehehydrogenase in binding together the four subunits and for causing conformational changes in the protein structure (Di Sabato & Kaplan, 1964); in this case however, the mechanism may be quite different.

Two explanations are possible for the aggregation of the subunits with increasing ionic strength. Both involve the rearrangement of protein structure known to occur for some proteins under such conditions, (Rasmussen & Craig, 1962), and stabilisation of protein configuration by the carotenoid, suggested to be the role of the polyene in rhodopsin (I.$D_{(vi)}$.; I.$C_{(ii)}$.):

1. As the ionic strength is increased, reversible alteration in the configuration of the subunits occurs about the point of attachment of the carotenoid. This results in a closer fit between the protein and carotenoid, shifting the spectrum to longer wavelength, and resulting in stabilisation of the specific configuration about this part of the molecule required for association.

2. In both the presence and the absence of salt, the specific configurations of the subunits about the carotenoid-protein linkage, essential for their association, are maintained by the binding of the prosthetic group. Some other part of the subunit structure involved in the association reversibly acquires the necessary configuration for association as the ionic strength increases, and on aggregation, the astaxanthin molecules on adjacent subunits are brought into close proximity; overlap between their extended π-orbitals shifts the spectrum to longer wavelength and results in increased stability of the polymer form.

Interaction between carotenoid and protein or between carotenoids in α-crustacyanin would lower the energy of the excited states of the chromophore. The energy changes for electronic transitions would then be reduced and the probability of their occurrence increased, with resultant bathochromic shift in spectrum and increased extinction at the maximum. Such changes are observed for crustacyanin in the presence and absence of salt (Table XVI):

TABLE XVI

Spectral data for α-crustacyanin in the presence and absence of salt

Electronic transition	Salt-free form		Salt present	
	λ_{max} (mμ)	$E^{0.1\%}_{max}$	λ_{max} (mμ)	$E^{0.1\%}_{max}$
ground → 1st excited state	595	3.50	630	3.72
ground → 2nd excited state	365	0.56	370	0.60
ground → 3rd excited state	Inflex-ion 315	0.50	320	0.56

The firmer binding of the carotenoid to protein in the presence of salt was indicated from the relatively higher stability of the linkage to treatment with detergent (IV.$B_{(ix)}$.d).

(vii) Dialysis and freeze-drying (IV.$B_{(iv)}$)

Thorough dialysis followed by freeze-drying gave a heterogeneous product owing to the irreversible formation of β-crustacyanin in salt-free solutions. Even when

Sephadex columns were used for desalting, small amounts of β-crustacyanin were formed. Possibly the heterogeneity results from a low isoionic point, as discussed in section (ii).

The behaviour of α-crustacyanin during desalting on Sephadex columns (IV.$B_{(iv)}$.b) suggests that it is a globulin. Rapid removal of salt by passage through Sephadex G25 (fine grade) precipitated the α-crustacyanin before it had time to dissociate completely into subunits, giving rise to two bands on the column. Continued removal of salt as the precipitated band moved down the column accelerated the dissociation and the precipitated protein redissolved in the form of its subunits.

When the protein was freeze-dried from a volatile (NH_4Ac) buffer having an ionic strength in which the protein was completely in the α-form heterogeneity was observed on electrophoresis. The denaturation probably arose from the inability to remove the volatile buffer sufficiently rapidly at these concentrations. Freeze-dried preparations obtained from volatile buffers in which the protein was almost completely dissociated into subunits were homogeneous on electrophoresis. Evidently the configurations of α-crustacyanin and $S_{1,2,3...}$ (Section (vi)) were preserved on freeze-drying and no irreversible changes to β-crustacyanin occurred. If the freeze-dried

protein was allowed to become moist, β-crustacyanin was slowly formed.

The purple-red colour of the freeze-dried carotenoprotein suggests that the carotenoid-protein linkage is less firm that in solution; this is indicated by the greater instability of the material to light than in aqueous solution (IV.B$_{(v)}$).

A parallel to crustacyanin is found in the case of caeruloplasmin (Poulik, 1962). This protein is composed of a number of subunits and it irreversibly dissociates into these upon chromatography or on dialysis against buffer of low pH.

(vii) Action of urea

(a) Alteration in size (IV.B$_{(xvii)}$)

The opening up of the protein structure in concentrated urea solution, by exposure of internal peptide and amide groups (Robinson & Jencks, 1963), had two distinct effects upon crustacyanin:

(i) Irreversible formation of a yellow form, completely excluded from Sephadex G200. This was similar in spectrum to acid-denatured crustacyanin (Wald, et al., 1948) and the yellow chromoprotein separated from shell extracts (IV.A$_{(iii)}$; Jencks & Buten, 1964) and represented aggregated (polymeric) material.

(ii) Reversible (for short incubation) dissociation into subunits (monomers) of the same size as β-crustacyanin.

Both the polymer (aggregated) material and monomer were present in urea solutions at both acid and alkaline pH. Whether an equilibrium existed between monomer(s) and polymer, or whether the polymer was formed irreversibly from α-crustacyanin or from the subunits, was not investigated in the present studies.

The polymer may have been formed by disulphide interchange known to occur in concentrated Urea solutions (Wake & Baldwin, 1961; Haurowitz, 1963(iv)), for disulphide groups were shown to be present in the crustacyanin subunits (IV.$B_{(xviii)}$ and XIV.$A_{(vii)}$.c). Alternatively, the disruption by urea of the carotenoprotein linkages might have enabled new linkages to be formed between protein molecules, the carotenoid acting as a cross-link (discussed in Section (viii)).

The formation of the monomer(s) was presumably the result of the unfolding of the peptide chains with consequent disruption of the native configuration of the subunits, essential for association, and the breaking of intersubunit hydrophobic bonds by the penetration of water molecules into the spaces between the unfolded peptide chains (Haurowitz, 1963 (iii)).

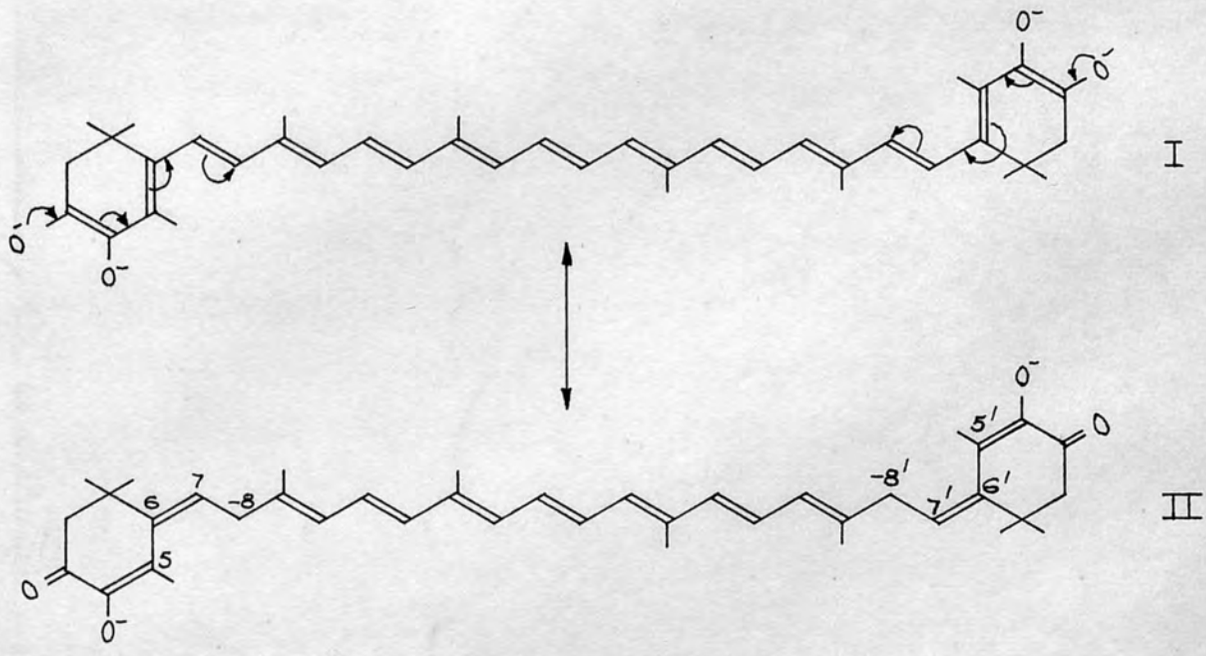
(b) Spectral changes in urea (IV.B$_{(xvi)}$)

Urea altered the spectrum of crustacyanin solutions by disruption (complete or partial) of the carotenoid-protein linkage either by direct action on this linkage or indirectly through the rearrangement of the protein structure.

The rate and extent of structural rearrangements of proteins in urea might well be pH dependent and could explain the different, and time-dependent, changes in spectrum observed with urea solutions at different pH. The maxima in urea solutions at 540mμ (pH7 and 8.6) and at 560mμ (pH 7), removed upon dialysis, are presumably due to disruption of the polyene chain-protein linkage in varying degrees, the salt-linkage remaining intact. The maximum in the region of 400mμ, irreversibly formed in urea solutions at all pH, is difficult to explain. It lies at a wavelength much lower than that of the free carotenoid in aqueous digitonin (absorption maximum 495mμ, Wald, et al., 1948).

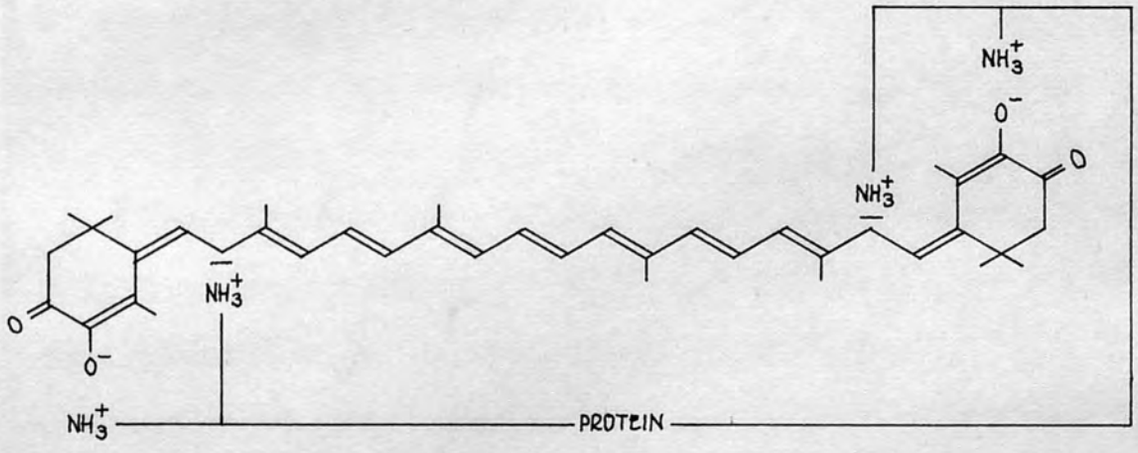
The tetra-anion form of astaxanthin (Formula I) proposed by Kuhn and Sörensen (1938) for the blue potassium salt of astaxanthin, should be presented as a mesomeric mixture of forms in which the negative charges are not only located on the oxygen atoms but also on the C atoms of the polyene chain. Numerous such mesomeric forms such as

Formula II, can be written.



Formula I might be expected to make a large contribution to the structure on account of the greater electroneg-ativity of the oxygen atom. However, the closeness of the negative charges on adjacent oxygen atoms might mean that mesomeric forms such as Formula II would be favoured. In crustacyanin, the close stereospecific fit between the protein and carotenoid brings cationic (NH_3^+) groups of the protein into the vicinity of the oxygen atoms, thus essentially "freezing" the structure of the carotenoid in Formula I (Kuhn & Sörensen, 1938). If the salt-linkages were broken by rearrangement of the protein structure in the urea solutions, it is possible that new salt-linkages

might be formed involving the polyene chain of the carotenoid The C_{7-8} and $C_{7'-8'}$ double bands of carotenoids have the most double-bond and least localisation energy for electrophilic attack (Pullman & Pullman, 1963), so that salt-linkages involving the polyene chain at these positions would be most easily formed:



This would result in loss of coplanarity, the groups about carbon 8 and 8' assuming an almost tetrahedral configuration; p-orbital interaction between carbon atoms 8, 9 and 10 (and 8', 9', and 10') would be greatly diminished, perhaps completely, leaving only the seven central double bonds completely conjugated. The properties of the form of crustacyanin with the 400mμ maximum obtained by acid or urea treatment, are fully explained by such a structure. Thus, the absorption maximum would be expected to be in the 400mμ region (cf. Karrer & Jucker, 1950) and astaxanthin would be liberated unaltered on dissociating the salt-linkage with acetone. On formation of these salt-

linkages, cationic groups of different protein molecules could be involved; limitless polymerisation would then be possible, the carotenoid acting as a cross-link between peptide chains of the subunits.

The 460mμ maximum present in the spectrum of urea solutions at pH7 and 8.6 can be explained by the formation of salt-linkages between the protein and the polyene chain in one half of the molecule only.

(c) Electrophoresis ($IV.B_{(xviii)}$)

The electrophoretic results for crustacyanin treated with urea were complex. They did not allow an unequivocal interpretation as to the number of different subunits present in α-crustacyanin.

The yellow, high-molecular material obtained with urea remained at the origin in both cellulose acetate and starch gel electrophoresis, so that the intensely staining bands represented the small-size protein fraction obtained on Sephadex G200 gel filtration ($IV.B_{(xvii)}$). Some of the fainter bands may have been due to traces of aggregated material not revealed in the gel filtration studies.

About nine bands were observed in starch gel electrophoresis at pH8.6, using Poulik's discontinuous buffer system (1957), of which about four were darkly staining. Three darkly staining bands were distinguished on starch gel and cellulose acetate electrophoresis at pH7.

Size, as well as shape, influences the mobility of proteins in starch-gel electrophoresis (Poulik, 1962). The fact that two of the darkly staining bands move faster than α-crustacyanin in starch gel electrophoresis at pH7, but behind it on cellulose acetate electrophoresis, must mean that these are smaller than α-crustacyanin. Artifacts can arise with Poulik's discontinuous buffer system as is seen from the breakdown of α-crustacyanin (IV.$B_{(ii)}$) and from the case where about fifteen components were separated from crustacyanin treated with urea (Fig. 39). Possibly the observed complexity was due to the formation of stable conformational isomers from a small number, perhaps a single, subunit. It could not have been due to reaction of amino groups or sulphydryl groups of the protein with cyanate, known to be in equilibrium with urea at alkaline pH (Stark, et al., 1960), since treated apoprotein preparations gave identical electrophoretic patterns under the same conditions (IV.$B_{(xiii)}$). Elution and re-electrophoresis of the individual bands would be necessary (Cohen & Porter, 1964) to decide whether the components were conformational isomers or distinct subunits.

A large number of components were still obtained after reduction and reduction-alkylation in electrophoresis at pH8.6 using urea-mercaptoethanol starch gels. The altered electrophoretic patterns obtained indicate that the

structure of the components formed by the action of urea were partially maintained by intramolecular disulphide linkages. The observed complexity of the electrophoretic patterns could not have been due to disulphide-bond interchange, known to take place in urea solutions (Wake & Baldwin, 1961), since reduction and reduction-alkylation would have eliminated this possibility.

The presence of only one band for the reduced and reduced-alkylated proteins in electrophoresis at pH 3.1 using urea-starch gels might seem to suggest the occurrence of just one type of subunit. However, it is possible that the band contained a number of components having similar mobilities at this pH, and the distance migrated during overnight runs was short. The slower moving bands observed with crustacyanin treated with urea were removed by reduction and reduction-alkylation; they were probably aggregates and isomers formed by disulphide bond interchange (Poulik, 1962).

Crustacyanin treated with urea and untreated crustacyanin gave an identical electrophoretic pattern in formate-starch gels at pH3.1. It seems probable therefore that at this pH crustacyanin was dissociated into its subunits, however, this was not investigated further.

Alkylation, without prior reduction, did not alter the electrophoretic pattern so that free sulphydryl groups were probably absent from the subunits.

Scarcely any of the reduced and reduced-alkylated proteins entered starch gels not containing urea; they remained at the origin in cellulose acetate electrophoresis. Reduced-alkylated γ-globulin subunits aggregate and come out of solution when the urea is removed (Poulik, 1962), so that perhaps this could be the explanation for the behaviour of the reduced and reduced-alkylated crustacyanin.

A parallel to crustacyanin is found in ceruloplasmin (Poulik, 1962 a & b). A number of subunits (nine from ceruloplasmin treated with urea, five on reduction-alkylation) were separated by electrophoresis in urea-formate-starch gels. No definite conclusion as to the number of subunits ceruloplasmin contained could be reached. In a number of other cases e.g. casein (Wake & Baldwin, 1961) and α_2-macroglobulin (Poulik, 1960) it has not been possible to determine the number of different subunits present from electrophoretic studies on the protein treated with urea. The problems of interpretation are clearly evident in the case of casein, where twenty bands, moving singly on re-electrophoresis, were obtained for the protein treated with urea using Poulik's discontinuous buffer system and urea-starch gels (Wake & Baldwin, 1961).

Crustacyanin in 3M. urea of pH7.5 has a single maximum at 590mμ (Jencks & Buten, 1964), characteristic of the monomer.

If the protein is dissociated into its subunits at this concentration of urea, electrophoresis in 3M. urea-starch gels might give a simpler electrophoretic pattern; there would be less chance of artifact formation at the lower ionic strength.

A slightly higher concentration of starch in the gels, to decrease the mobility of the faster moving components, might have given less pronounced crowding of the bands at the citrate front with the discontinuous buffer system.

(viii) Apoprotein and reconstitution (IV.B$_{(x)-(xv)}$)

As in the case of crustacyanin treated with urea, thin-layer gel filtration of apocrustacyanin revealed proteins of two sizes; one was smaller than serum albumin, the other of large size being completely excluded from the gel. The excluded material was presumably aggregated material formed by disulphide bond interchange. The strongly retained component had the same size as β-crustacyanin and was therefore the apo form of the carotenoprotein subunits.

α-crustacyanin (and a small amount of β-crustacyanin) was reformed in high yield when the carotenoid was restored to apoprotein preparations. The high molecular weight, aggregated material, was still present, but the apoprotein subunits were almost completely reconverted to α-crustacyanin Evidently the apoprotein subunits were unable to associate

in the presence of salt unless the carotenoid was attached. This must mean either that the specific configuration required for aggregation of the subunits is only maintained when these have the carotenoid attached to them, as previously suggested (Section (v)). It could also mean that a single carotenoid molecule acts as a cross-link between the subunits. This is unlikely, since β-crustacyanin subunits, in which the carotenoid-protein linkage is altered but not cleaved, do not aggregate in the presence of salt. However, the suggestion made previously (Section (vi)) that $\pi - \pi$ interaction between carotenoid molecules might assist in the mutual binding of the subunits may also be true.

Alteration of the carotenoid-protein linkage with acetone (IV.$B_{(xv)}$) also resulted in cleavage into subunits. In this case, however, the original linkage between the carotenoid and protein was restored on removal of the acetone and α-crustacyanin was reformed. The dissociation could be explained by the breaking of hydrophobic bonds, including the possible carotenoid-carotenoid bonds, formed between the subunits, as a result of the greater interaction between solvent and hydrophobic groups in the acetone solution as compared with aqueous solutions. Alternatively it could be explained if alteration of the carotenoprotein linkage resulted in loss of a specific configuration

essential for the preservation of the highly organised, geometrical arrangement of the subunits that must exist in α-crustacyanin.

Electrophoresis of apoprotein preparations (IV.B$_{(xiii)}$) revealed a number of components, greater complexity being observed at pH8.6 than at pH7. A large amount of protein remained at the origin in both starch gel and cellulose acetate electrophoresis. This was presumably the polymeric component present in the preparations (IV.B$_{(xii)}$), being too large to enter the gels and precipitating in cellulose acetate electrophoresis. Strongly staining bands must therefore represent the components of the protein fraction moving behind serum albumin on gel filtration (IV.B$_{(xii)}$). As mentioned previously (Section (viii)c), the reversal in order of migration of two of the strongly staining components compared to α-crustacyanin in cellulose acetate and starch gel electrophoresis must mean that these are of smaller size than α-crustacyanin. The exact similarity in the electrophoretic pattern obtained with apoprotein preparations and crustacyanin treated with urea (IV.B$_{(xviii)}$) must mean either that the components observed were distinct subunits of α-crustacyanin, or that the actions of both urea and acetone resulted in the formation of the same mixture of stable conformational isomers in the same relative proportions from a single subunit or from a small

number of different subunits. The preponderance of two bands in electrophoresis at pH7 of about the same intensity might indicate that two different subunits are present in α-crustacyanin, other minor bands being artifacts formed from these.

The reconstituted protein showed mainly the α-crustacyanin band in electrophoresis, and the components present in apoprotein preparations were either completely absent or present in much reduced amounts (IV.$B_{(xiii)}$). This must mean that these components are able to regain the specific configuration, necessary for aggregation to occur, on re-attachment of the carotenoid.

It is hoped that reconstitution experiments and chemical analysis, using the individual components of the apoprotein preparations separated by electrophoresis, will reveal which of the components are subunits and which are artifacts derived from them.

B. Ovoverdin (V)

(i) Extraction and purification (V.A)

Distilled water extracted only about 60 - 80% of the carotenoprotein from the ovary. 0.05M-phosphate buffer, pH7, removed the remaining pigment from the water-extracted residue. Dilution of the distilled water extract with distilled water resulted in precipitation of part of the ovoverdin; this, after centrifugation was found to re-

dissolve in dilute salt solution. The extracts with distilled water and with 0.05M-phosphate buffer, after purification, had identical spectra with the same values for E280/466, E280/466+660 and E466/660. It was therefore considered that the two fractions were identical. Possibly the salt present in the ovary accounted for the extraction of the pigment with distilled water, since a second water extraction failed to remove further pigment.

The initial extract absorbed strongly in the ultra-violet and had a maximum at 265mμ, possibly owing to the presence of nucleic acid in the preparation. This was not removed by fractional calcium phosphate gel adsorption, or by repetitive $(NH_4)_2SO_4$ precipitation. It was removed quantitatively by stirring with DEAE-cellulose. A negative adsorption step using CM-cellulose was included in the purification to remove protein impurities. A subsequent calcium phosphate gel purification step, followed by fractional $(NH_4)_2SO_4$ precipitation was necessary for the preparation of an electrophoretically pure product.

The unique property of ovoverdin in precipitating at low salt concentration but being completely soluble in distilled water (V.G.) was incorporated in the purification procedure to remove any remaining traces of contaminating protein.

The maximum possible yield of freeze-dried ovoverdin, estimated from the absorptions at 660 and 466mμ of the initial extract and the $E^{0.1\%}_{466+660}$ value found experimentally, was 6.5g./100g. starting material.

Kuhn & Sörensen (1938) purified ovoverdin from the eggs of H. vulgaris by repetitive adsorption and elution from alumina gel till a constant nitrogen to astaxanthin content for the preparation was obtained. However, the homogeneity of the preparation was not tested electrophoretically, and it is quite possible that the preparation contained a number of proteins besides ovoverdin, adsorbing and eluting from the gel under the conditions used. They stated that 240mg. ovoverdin contained 1mg. astaxanthin. The sum of the extinction at 470mμ + 660mμ for a solution of 240mg. ovoverdin per 1. can be estimated by assuming it to be 10% greater than the extinction at 495mμ of 1mg. astaxanthin per 1. ($E^{mol.}_{495}$ = 115,000); the extinction at 280mμ can be estimated by using the $E^{0.1\%}_{280}$ value (1.85, V.C.) found in the present studies. The value of E280/470+660 for the solution would then be 1.8, 25% greater than that found for pure preparations of ovoverdin in the present studies. Kuhn & Sörensen's ovoverdin preparation might therefore have contained about 25% of other proteins, assuming the protein impurities had the same tyrosine and tryptophan contents as ovoverdin.

(ii) General properties

Ovoverdin prepared from the ovary was found to be a glycolipoprotein (V.F.). Its complex composition and high concentration in the eggs (Stern & Salomon, 1938) would make it an ideal storage protein. Before the hatching of the eggs, the carotenoid-protein linkage in ovoverdin is disrupted and free astaxanthin liberated (Goodwin, 1951). Possibly, as suggested for crustacyanin (XIV.$A_{(v)}$), the carotenoid has a role in stabilising the protein in its native configuration, the carotenoid-protein linkage having to be cleaved before the protein can be utilised by the developing embryo.

The precipitation of ovoverdin by very small concentrations of salt is reminiscent of the fibrous protein, myosin (Szent-Györgyi, 1947).

Besides astaxanthin, ovoverdin was found to contain the other carotenoids present in the ovary; this is presumably due to partition of these between the free lipid of the ovary and the lipid component of ovoverdin. Attempts to prepare apoovoverdin failed, the protein no longer redissolving after acetone precipitation.

(iii) Molecular Weight (V.D. and V.E.)

Wyckoff (1937), using a crude 50% $(NH_4)_2SO_4$ solution of ovoverdin obtained from the eggs of H. vulgaris, found that ovoverdin had a sedimentation constant (S_{20}) of

10.3×10^{-3}. He suggested that a protein of about 300,000 molecular weight would have a sedimentation constant of this value. In view of the high lipid content found for ovoverdin ($V.F_{(ii)}$) the molecular weight could be much greater than this. However, since Wyckoff used an impure solution of ovoverdin it is possible that the observed sedimentation constant was not that of ovoverdin but of ovoverdin complexed with other protein or nucleic acid present in the preparation (cf. Haurowitz, 1963 (i)).

Kuhn and Sörensen (1938) assuming ovoverdin to contain one molecule of astaxanthin, derived a molecular weight for ovoverdin, purified by alumina gel chromatography, from its astaxanthin content of 144,000. These workers assumed a nitrogen content of 16.4% in their determinations and considered that their preparation was pure. Assuming their ovoverdin preparations contained 25% of other proteins (Section (ii)) and that ovoverdin has a nitrogen content of 11.3% ($V.F_{(i)}$), then the value for the molecular weight of ovoverdin, correcting Kuhn and Sörensen's results, would be in the region of 160,000.

Stern and Salomon (1938) similarly derived a molecular weight for ovoverdin from its astaxanthin content. These workers, using ovoverdin solutions purified by fractional $(NH_4)_2SO_4$ precipitation, found the molecular weight of ovoverdin to be 300,000. In their calculations they

allowed for 10% protein impurity, suggested from Wyckoff's ultracentrifuge experiments. It is possible that their preparation contained far more than 10% protein impurity. The method used for protein determination was not stated in their work, and so it is not known whether they assumed a nitrogen content of 16.4% for ovoverdin. Stern and Salomon's molecular weight determination cannot therefore be accepted with any confidence. Even if it is correct, the difference from Kuhn and Sörensen's determination might be explained by differences existing in the ovoverdin of the two species of lobster.

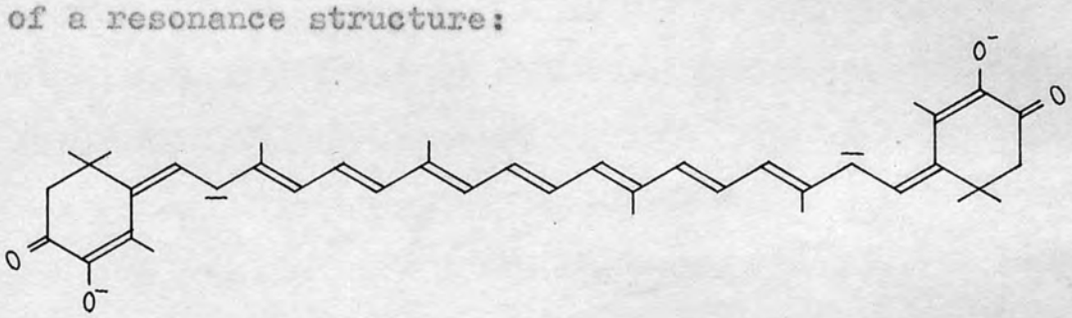
In the present studies the minimum molecular weight of ovoverdin, obtained from H. vulgaris ovaries, was found to be 168,000 (V.D.). The molecular weight estimated from gel filtration (V.E.) was found to be 1.7 times the minimum molecular weight, and would be grater than this if ovoverdin had a high axial ratio (III.F$_{(iii)}$). These results suggest that ovoverdin contains two molecules of carotenoid bound to one molecule of the protein. This possibility was previously suggested by Kuhn and Sörensen from their minimum molecular weight estimates and from Wyckoff's ultracentrifuge experiments. However, conclusions from the gel filtration studies must be viewed with caution until the shape of ovoverdin has been established (III.F$_{(iii)}$).

(iv) Spectrum

Stern and Salomon (1938) reported that ovoverdin solutions, obtained from H. americanus eggs, had absorption bands centred at about 470 and 640 mμ and an E470/640 value of 2.6. Goodwin (1949) showed that ovoverdin solutions from H. vulgaris eggs exhibited a broad shallow band in the red region with maximum at 660mμ and a much sharper band in the blue region with maximum at 476mμ. The differences in the spectra observed by these authors may have been due to differences of the ovoverdin in the two species of lobster.

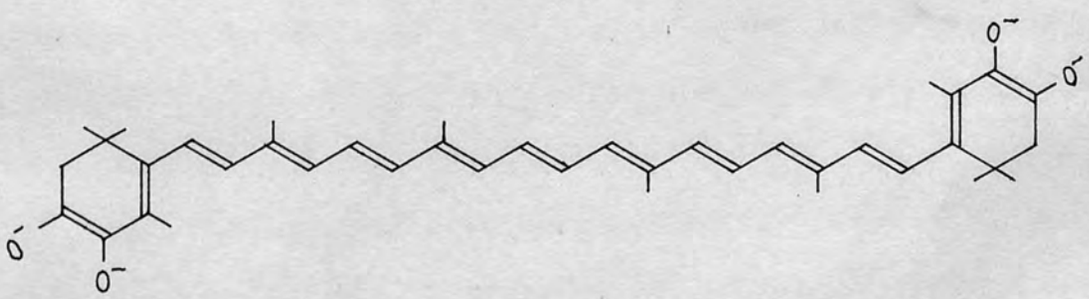
The spectrum of ovoverdin solutions obtained from H. vulgaris ovaries, reported in the present studies (V.C.), differs from that reported for the eggs (Goodwin, 1949). This may possibly be the result of changes in the protein structure on egg formation. Slight differences observed in the position of the 466mμ bond of ovoverdin may have been due to the presence of differing quantities of lipid-dissolved carotenoids in the preparations. Both regions of absorption were shown to have fine structure. The inflexions in the spectrum at 440 and 500mμ were probably due to vibrational changes superimposed on the electronic transition; alternatively they could be due to the presence of carotenoids dissolved in the lipid component of the protein or adsorbed unspecifically on its surface.

The two absorption bands in the visible region probably represent the first electronic transition for two different modes of binding of the carotenoid. The band with maximum at 466mμ can be explained by the predominance of a resonance structure:



I

as discussed previously for crustacyanin (XIV.$A_{(viii)}$.b). The band with maximum in 660mμ region would be due to the predominance of the tetra-enol resonance structure:



II

The variation of the value of E466/660 and the constancy of E280/466+660 might seem to suggest that the protein can bind the carotenoid in two ways, one mode of binding "freezing" the structure in form I above, and the other "freezing" the structure in form II. The variation in spectrum could then be explained by the occurrence of different mixtures of the two carotenoproteins. No separation into blue and red carotenoproteins was achieved by

electrophoresis (V.B.), CM-cellulose chromatography (V.G.), or $(NH_4)_2SO_4$ precipitation (V.A.), so that the attachment of the carotenoid in the two different ways does not result in any major differences in the properties of the protein.

Alternatively, the structure of the carotenoid in the combination might best be described as a mixture of resonance structures, of which the two shown above are made to predominate by the close proximity of anionic groups of the protein to the oxygen atoms and carbon atom 8 (Cheesman, 1964). Slight alteration in tertiary protein structure might then greatly effect the relative contribution of the two resonance forms to the structure, and hence the value of E466/660. The different rates of fading of the two absorption bands (V.G.), the slight alteration in spectrum on dialysis (V.G.) and the different values of E466/660 obtained from different preparations may thus be the result of changes in the tertiary protein structure during egg development. The variations of the spectrum occurring in the 660mμ region are presumably due to differences in the strength of binding of the carotenoid, this affecting the energy levels of the excited states of the chromophore.

It is unlikely that the spectrum of ovoverdin arises through the presence of two carotenoid molecules attached

to one molecule of the protein, each present in the different modes of binding (I and II) discussed above. E466/660 values should then be constant having a value close to unity (since binding of carotenoid to protein does not alter the extinction greatly, cf. crustacyanin in Figure 14). Also, the values of E280/660 and E280/466 should be constant for different preparations.

The two suggestions made above do not exclude the possibility that two carotenoids are attached to a single protein molecule.

C. The carotenoprotein of the ovary of Pecten maximus (VI)

(i) Extraction and purification (VI.B.)

The extraction of only a fraction of the carotenoid content of the ovary with ether (VI.A.) suggested that the carotenoprotein obtained on salt extraction was not an extraction artifact but occurred in vivo.

Ultraviolet absorbing material, present in the initial extracts and still accompanied the carotenoprotein after chromatography on calcium phosphate gel and repetitive $(NH_4)_2SO_4$ precipitation, possibly nucleic acid. The presence of this impurity altered the behaviour of the carotenoprotein to dialysis. Thus when a solution of the chromoprotein, purified by calcium phosphate gel chromatography, was dialysed, the protein first precipitated and then redissolved. When the impurity was removed with

DEAE-cellulose, the protein precipitated on dialysis and did not redissolve unless salt were added.

Scallops stored in the deep-freeze for several months gave much less chromoprotein. Denaturation of the carotenoprotein on dialysis and storage (VI.D.) was presumably due to its lipoprotein nature.

As in the case of crustacyanin (IV.$B_{(i)}$.a), ovoverdin (V.A.), and ovorubin (Norden, 1962) the first $(NH_4)_2SO_4$ fraction obtained from the material eluted with calcium phosphate gel was probably denatured protein.

(ii) Composition

The chromoprotein had a value of $E_{280}^{0.1\%}$ (VI.D.), and hence a combined tyrosine and tryptophan content, similar to that of ovorubin (Norden, 1962). It was shown to be a glycolipoprotein (VI.K.), differing in composition from ovoverdin in containing less hexose and phospholipid.

A large proportion of the hypophasic carotenoid present in the ovary was associated with the carotenoprotein (VI.A., B.).

(iii) Mode of combination

The finding that the carotenoprotein preparation contained all the hypophasic carotenoids present in the ovary (VI.E.) and in about the same relative proportions, must mean either that the preparation consisted of a mixture of carotenoproteins, or that the carotenoids were dissolved

in the lipid component of a lipoprotein. If the latter were true, the E280/472 value should be greater in April than in October when the carotenoid content of the ovaries is about one hundredth of that in the former month (Lederer, 1938). The constancy of the E280/472 value at different times of the year (VI.C.) suggests that the first alternative is correct. The significant alteration in spectrum in 6M. urea (VI.C.), the lack of extractibility of carotenoid from the freeze-dried preparation with ether (VI.A.) and the similarity between the values of the molecular weight derived from carotenoid content (VI.I.) and from gel filtration, (VI.J.), lends further support to this suggestion. The differences in absorption spectrum of different preparations might then be due to differences in the relative proportions of the carotenoproteins.

A single apoprotein is probably involved in the combination with carotenoid, since attempts to separate the preparation into a mixture of carotenoproteins by a variety of methods failed (VI.F, G, H.). The specificity of the binding can therefore not be great. Some specificity does exist however, since the epiphasic carotenoid present in the ovary (VI.E.) was only present in the carotenoprotein in traces. Possibly the ~~main amount~~ trace of epiphasic pigment in the carotenoprotein is due to dissolution in the lipid component of the protein or to

unspecific adsorption to the protein surface.

D. The carotenoprotein of the ovary of Carcinus maenas (VII)

(i) Homogeneity

Two protein fractions were obtained by $(NH_4)_2SO_4$ precipitation of the calcium phosphate gel eluent, and these had identical spectrum and composition (VI.B.E.). It is probable that the first $(NH_4)_2SO_4$ fraction represented material altered during the gel adsorption. A complete separation of the two fractions was not achieved with a single $(NH_4)_2SO_4$ precipitate step (VII.C.).

(ii) Mode of combination

The carotenoprotein preparation contained all the carotenoids of the ovary in about the same relative proportions (VII.D.). The minimum molecular weight was large in relation to the molecular weight determined by gel filtration (VII.F.). These facts, together with the variation in the spectra of different preparations (VII.B.), favour the suggestion that the carotenoids are dissolved in the lipid component (VII.B.) of the carotenoprotein. Only one preparation was fully investigated so that this possibility needs verification. Variation in E280/490 values for preparations made at different times of the year would be expected if the carotenoprotein was of this nature.

Astaxanthin greatly predominated in both the ovaries and eggs (VI.D.). The 490mμ maximum of the carotenoprotein preparation corresponds to that of free astaxanthin

in aqueous digitonin solution (Wald, et al., 1948), so that strong interaction between the carotenoid and protein seems unlikely. The maximum or inflexion at 470mμ is presumably due to the absorption of the other carotenoids present in the carotenoprotein preparation. The removal of the carotenoids (together with neutral lipid) with ether from the freeze-dried protein, not occurring in the cases of crustacyanin (IV.$B_{(xx)}$) and the Pecten maximus carotenoprotein (VI.$K_{(iii)}$), demonstrates the looseness of the combination.

Most of the carotenoid present in the ovary was associated with the chromoprotein (VII.A.).

E. The carotenoprotein of the carapace of Carcinus maenas (VIII)

(i) Extraction (VIII.A.)

The yellow chromoprotein extracted with 10% EDTA represented denatured material in which the carotenoid-protein linkage had been altered. The maximum at 490mμ is similar to that of free astaxanthin in aqueous digitonin (Wald, et al., 1948), and the maxima at 390 and 460mμ to those of crustacyanin treated with urea (IV.$B_{(xvi)}$).

The green chromoprotein, insoluble in the high EDTA concentration was partially eluted from the decalcified shell with dilute phosphate buffer. A considerable amount of the carotenoprotein was adsorbed to the shell residue and

only eluted with 6M. urea, this process altering irreversibly the carotenoid-protein linkage.

(ii) Comparison with crustacyanin

The spectrum of the partially purified green astaxanthin protein (VIII.$A_{(ii)}$) was similar to that of ovoverdin, with absorption maxima in the red and blue regions of the spectrum. The inflexion at 408mμ might have been due to traces of carotenoprotein in the preparation with altered carotenoid-protein linkage; it corresponds in position to the maximum observed for crustacyanin treated with urea (IV.$B_{(xvi)}$). The maximum at 625mμ is close to that of crustacyanin.

The spectrum of the preparation is consistent with a mixture of two carotenoproteins, with absorption maxima in the red and blue respectively, or with the preponderance of two mesomeric structures of astaxanthin in the complex, as described for ovoverdin (Section B.).

The maximum observed at 390mμ in 6M- urea (VIII.$A_{(ii)}$) is characteristic also of crustacyanin in 6M- urea solution (IV.$B_{(xvi)}$).

In contrast to ovoverdin, the absorption band in the 460mμ region of the spectrum faded more rapidly than that in the 600mμ region, indicating that the structure responsible for this absorption is not as greatly stabilised by the protein as that responsible for the 600mμ absorption

F. The carotenoprotein of the carapace of Eriphia spinifrons (IX)

(i) Extraction (IX.A)

As in the case of the carotenoprotein from C. maenas carapace (Section E), the carotenoprotein was not extracted in unaltered form by 10% EDTA solution, and only partially from the decalcified shell with dilute phosphate buffer. It was presumably bound by Van der Waals' forces to a component of the shell residue, from which it was liberated in an altered form, with 6M. urea. Just as in the case of crustacyanin (IV.$B_{(xvi)}$) removal of the urea by dialysis resulted in the recovery of the original spectrum. indicating the restoration of the carotenoid-protein linkage.

(ii) Comparison with crustacyanin

The inflexions at 310 and 350mμ, in the spectrum of the chromoprotein (IX.C.) correspond to the maxima at 320 and 360 mμ in the spectrum of crustacyanin (IV.$B_{(i)}$). The slight inflexion at 405mμ corresponding to that found in lobster shell extracts (IV.$A_{(ii)}$) and crustacyanin treated with urea (IV.$B_{(xvi)}$), was presumably due to the presence of altered material in the preparation. The maximum at 532mμ corresponds to that of crustacyanin heated to 60° in barbitone buffer, pH8.6 (Wald, et al., 1948).

The chromoprotein was shown to be a light-stable globulin, with astaxanthin as prosthetic group, of large

molecular size, with a low 280mμ extinction compared to that of the maximum in the visible region (IX.C). In these respects it compares to crustacyanin.

C. The carotenoprotein of the ovary of Palinurus vulgaris (X)

Mode of combination

Lack of material did not allow extensive investigations into the nature of the chromoprotein to be performed.

The spectrum (X.B) was similar to that of the chromoprotein from C.maenas ovary. Astaxanthin was shown to be the major carotenoid component, but other carotenoids of the ovary were also contained in the carotenoprotein (X.E.). The chromoprotein was shown to be of a lipoprotein nature (X.F), so that it is possible that the carotenoids are dissolved in the lipid component. Thus, the maximum at 490mμ could be accounted for by lipid-dissolved astaxanthin, while that at 466mμ to other lipid-dissolved carotenoids. The slight alteration of the spectrum occurring in 6M-urea (X.B.) does not necessarily indicate specific binding of the carotenoid to protein; the alteration in spectrum could have arisen from changes in lipid-carotenoid interations occurring on alteration of the lipoprotein structure in urea.

H. The carotenoprotein of the eggs of Palinurus vulgaris

The spectrum of the egg chromoprotein was identical

to that of the ovary chromoprotein, and it contained the same carotenoid and lipid components.

I. The carotenoprotein of the carapace of Palinurus vulgaris (XII)

As in the cases of the carapace carotenoproteins of C. maenas (VIII) and E. spinifrons (IX), the carotenoprotein was only partially extracted from the decalcified shell with dilute phosphate buffer, further yield of chromoprotein being obtained with a urea extraction (XII.A) follow-ed by dialysis.

The starch gel electrophoretic studies (XII.C) suggest that the protein has a small size, travelling close to the citrate front (Lagnado, 1963). Gel filtration studies confirmed this, indicating that the protein was of similar size to β-crustacyanin (XII.E.).

The spectral maximum of the chromoprotein at 560mμ (XII.B.) corresponds to that of α-crustacyanin in 10% acetone (IV.$B_{(xv)}$). The maxima at 470 and 525mμ in the dilute phosphate buffer extract (XII.A.) occur near the same positions in the spectrum of crustacyanin treated with 6M. urea at pH8.6 (IV.$B_{(xvi)}$).

J. The carotenoprotein of the carapace of Astacus astacus (XIII)

The green chromoprotein extracted from the carapace was found to have maxima at 390 and 632 mμ respectively;

these maxima correspond in position to those found for crustacyanin (IV.B$_{(i)}$) and crustacyanin treated with urea respectively (IV.B$_{(xvi)}$).

Further investigation is needed to decide whether the chromoprotein consisted of a mixture of two carotenoproteins, one with an absorption maximum at 390mμ and the other at 632mμ, or whether the spectrum is derived from a single carotenoid or two carotenoid molecules bound to the same protein.

The extinction at 390mμ faded more rapidly than that at 632mμ on heating or exposure to light (XIII.D.). The small amount of blue-green material precipitating between 50 and 100% $(NH_4)_2SO_4$ saturation, might therefore have represented altered material rather than demonstrating the presence of a carotenoprotein having the 632mμ maximum only.

The traces of a xanthophyll present in the carotenoprotein could explain the small inflexions at 470 and 495mμ in the spectrum of the carotenoprotein. The 495mμ maximum might also have been due to the presence of astaxanthin adsorbed unspecifically onto the protein surface or to the presence in the preparation of denatured carotenoprotein.

The maximum at 390mμ could arise in the same manner as that suggested to explain the spectrum of crustacyanin treated with urea (XIV.A$_{(vii)}$.b).

K. Concluding remarks

An attempt has been made to obtain a number of carotenoproteins in a high state of purity and to study their composition and properties. With the exception of ovorubin, no detailed study of these proteins has previously been made and crustacyanin is the first protein of this class to be crystallised.

It is apparent from the present work that the polyene prosthetic group of crustacyanin has a decisive function in stabilising the protein conformation. Although such a role has been proposed for the polyene prosthetic groups of rhodopsin and ovorubin, the effect is made more apparent for crustacyanin by the great change in molecular weight accompanying alteration in the carotenoid-protein linkage. It appears that crustacyanin is composed of a large number of subunits each binding a carotenoid molecule, arranged in a highly specific geometry. It is probable that there are several different subunits of about the same size. The integrity of crustacyanin is only maintained when the carotenoid-protein linkage is intact. Removal of the carotenoid or alteration in the carotenoid-protein linkage causes dissociation of the protein. The dissociation of crustacyanin at low ionic strength indicates that the intramolecular forces between the subunits must be weak. It is possible that

the subunits are bound together not only by electrostatic attraction but also by $\pi - \pi$ interaction between carotenoid molecules.

The wide range of coloured astaxanthin-protein complexes purified from the carapaces of various species of crustacea indicates the importance of such complexes in providing characteristic colouration in these species. The advantage of using astaxanthin as chromophore is that any particular shade can be provided when certain of the many mesomeric forms are favoured by attachment to protein. Although no case has so far been reported, rapid changes in colour to suit an alteration in environment could be obtained by modification of the mode of combination of the carotenoid and protein. It remains to be seen whether the astaxanthin prosthetic group in other carotenoproteins has a function similar to that in crustacyanin of stabilising the protein structure.

The ovary carotenoproteins studied are distinct from those of the carapace in that they all contained a high proportion of lipid and a number of different carotenoids. These lipoproteins are present in the ovary in high concentration and are presumably major storage proteins for the developing embryo. Possibly in the case of ovoverdin the carotenoid has a function in maintaining the protein structure, the carotenoid-protein linkage

being cleaved when the protein is utilised. Non-stoichiometry of combination between the carotenoid and protein in the case of the carotenoprotein of Carcinus maenas disproves any specific function for the carotenoids in stabilising the protein structure. The stoichiometry of combination for the scallop carotenoprotein suggests that the carotenoids are specifically bound either to the protein proper or to a component of the lipid portion of the molecule. The carotenoid may well have some function in stabilising the lipid component of the lipoproteins against autoxidation.

L. Tabulation of results

The absorption maxima of the astaxanthin proteins and lipid-containing carotenoproteins isolated in the present studies are tabulated in Table XVII.

TABLE XVII

Tabulation of Results

	Maxima (mμ)	Inflexions (mμ)
(i) Astaxanthin proteins		
Homarus vulgaris		
(1) Carapace		
α-crustacyanin (plus salt)	320, 370, 632.5	590
α-crustacyanin (salt-free)	365, 595	315
β-crustacyanin	360, 585	315
Yellow chromo-protein	412, 630	480
(2) Ovary		
Ovoverdin	466, 650-70	330, 440, 500
Carcinus maenas (green variety)		
Carapace	460, 625	320, 408, 500
Eriphia spinifrons		
Carapace	532	310, 340, 400
Palinurus vulgaris		
Carapace	560	350, 600
Astacus astacus		
Carapace	392, 632	320, 470, 495
(ii) Carotenoproteins containing lipid		
Pecten maximus (ovary)	470-2, 496-8	315, 325
Carcinus maenas (ovary)	470, 490	325
Palinurus vulgaris (ovary and eggs)	466, 490	325, 435
Homarus vulgaris (ovary)	466, 650-70	330, 440, 500

SUMMARY

1. A survey has been made of the literature on polyene-protein complexes (I).

2. Citrate extracts of the blue regions of lobster carapace have been separated on DEAE-cellulose by salt-gradient elution into blue (α-crustacyanin), purple (β-crustacyanin) and yellow chromoproteins. The absorption spectra of these proteins have been recorded. All three pigments have astaxanthin as prosthetic group (IV.A$_{(iii)}$., (IV.B$_{(xix)}$.f).

3. α-crustacyanin has been purified and its properties investigated (IV):

(a) α-crustacyanin has been obtained in an electrophoretically homogeneous state by successive calcium phosphate gel chromatography, fractional $(NH_4)_2SO_4$ precipitation, and DEAE-cellulose chromatography (IV.B$_{(i)(ii)}$). The protein has been obtained in a freeze-dried state, homogeneous to electrophoresis (IV.B$_{(iv)}$), and crystallised (IV.B$_{(vi)}$).

(b) The purified protein has absorption maxima at 280, 320, 370, and 632.5mμ (in the presence of salt). The value of E280/630 is 0.31, and that of $E_{280}^{0.1\%}$ is 1.15. The isoelectric point lies between pH 4.3 and 4.7. (IV.B$_{(iii)}$., IV.B$_{(v)}$).

(c) The protein is composed of the usual 17 amino acids, carbohydrate being absent. Only slight traces of lipid are detectable in freeze-dried preparations (IV.B$_{(ix)}$).

(d) Heterogeneity is induced by calcium phosphate gel chromatography, DEAE-cellulose chromatography, dialysis and electrophoresis in starch gels using Poulik's (1960) discontinuous buffer system (IV.B$_{(i)}$ & $_{(ii)}$).

(e) β-crustacyanin is formed irreversibly from α-crustacyanin, and can be separated into a number of components in electrophoresis (IV.B$_{(i)}$,$_{(iv)}$). The "complexity" of β-crustacyanin is discussed (XIV.A$_{(ii)}$).

(f) The minimum molecular weight, determined from the astaxanthin content is 35,700. (IV.B$_{(vii)}$).

(g) The molecular size of α-crustacyanin corresponds to a globular protein with a molecular weight of about 350,000; that of β-crustacyanin to a globular protein of molecular weight about 39,000. (IV.B$_{(viii)}$).

(h) Reversible changes in the absorption spectrum occurring on the removal of salt are accompanied by the reversible dissociation of α-crustacyanin into subunits. An equilibrium, slowly established, exists between the subunits and α-crustacyanin at low ionic strength; its dependence on the latter has been investigated (IV.B$_{(ix)}$). The relationship between the subunits and β-crustacyanin

is discussed, and a function for the carotenoid in stabilising the protein configuration is proposed (XIV.$A_{(v)}$). The stability of crustacyanin to heat, light, and detergent action has been investigated (IV.$B_{(ix)}$.d.).

(i) The apoprotein has been prepared and α-crustacyanin reconstituted by addition of the carotenoid. (IV.$B_{(x)}$,$_{(xi)}$). The relative affinities of apocrustacyanin and apoovorubin for carotenoid has been investigated (IV.$B_{(xiii)}$).

(j) The apoprotein has a low molecular weight and is heterogeneous to electrophoresis (IV.$B_{(xii)}$,$_{(xiii)}$). The significance of the results in relation to the proposed function of the carotenoid and the structure of α-crustacyanin is discussed (XIV.$A_{(viii)}$).

(k) The effect of 6M- urea on the spectrum at various pH is investigated and the results are discussed in relation to the mode of binding of the prosthetic group (IV.$B_{(xvi)}$, XIV.$A_{(vii)}$.b.).

(l) The molecular size in 6M- urea is similar to that of the apoprotein, crustacyanin treated with 10% acetone, salt-free crustacyanin and β-crustacyanin (IV.$B_{(viii)}$,$_{(ix)}$.e., (xv),$_{(xvii)}$).

(m) Crustacyanin treated with urea, reduced crustacyanin and alkylated crustacyanin have been investigated by

electrophoresis in starch gels in the presence and the absence of urea. The complexity of the separations and the similarity of pattern obtained for crustacyanin treated with urea and apoprotein preparations are discussed (IV.$B_{(xviii)}$., XIV.$A_{(vii)}$.c., XIV.$A_{(viii)}$.).

4. Ovoverdin has been purified from the ovaries of Homarus vulgaris and its properties investigated (V):

(a) The protein has been obtained electrophoretically homogeneous by successive negative DEAE-cellulose and CM-cellulose chromatography, calcium phosphate gel chromatography, fractional $(NH_4)_2SO_4$ precipitation and dialysis (V.A).

(b) The absorption spectra of different preparations are recorded and the results are discussed in relation to the binding of the carotenoid (V.C., XIV.$B_{(iv)}$).

(c) The minimum molecular weight has been determined from the astaxanthin content and an estimate of the molecular size obtained by gel filtration (V.D.,E). The number of carotenoid molecules per protein molecule is discussed (XIV.$B_{(iii)}$).

(d) The carbohydrate, lipid and nitrogen content of preparations have been determined. The nitrogen, phosphorus and cholesterol contents of the lipid extracts have been determined and the phospholipids and neutral lipids separated by thin-layer chromatography (V.F).

(e) The nature and number of the carotenoid components has been investigated (V.$F_{(iv)}$).

(f) The protein has been found to be precipitated by small concentrations of salt (V.G).

(g) The general properties of the protein are recorded. (V.G.)

5. A carotenoprotein has been extracted and purified from the female reproductive gland of Pecten maximus.

(a) The carotenoprotein has been purified by selective extraction, calcium phosphate gel chromatography, negative DEAE-cellulose chromatography and fractional $(NH_4)_2SO_4$ precipitation (VI.B).

(b) The spectra of different preparations are recorded and the variations discussed (VI.C. ; XIV.$C_{(iii)}$).

(c) The carotenoid composition of the carotenoprotein and that of the whole ovary are compared, and the results discussed in relation to the mode of binding of the carotenoids (VI.E. ; XIV.$C_{(iii)}$).

(d) The preparation and properties of the freeze-dried protein are described (VI.D).

(e) The homogeneity (VI.F,G,H.), molecular weight (VI.I.), molecular size (VI.J.), and composition (VI.K.) of the carotenoprotein have been investigated.

6. A carotenoprotein has been extracted and purified from the ovaries of Carcinus maenas and its properties investi-

gated (VII).

(a) The carotenoprotein has been purified by negative DEAE-cellulose and CM-cellulose chromatography, followed by calcium phosphate gel chromatography, and divided into two $(NH_4)_2SO_4$ fractions (VII.A). The composition, homogeneity and spectrum of the two fractions are compared and their nature discussed (VI.C,E. ; XIV.$D_{(i)}$).

(b) The minimum molecular weight of the carotenoprotein has been determined and its molecular size estimated (VII.F).

(c) The carotenoid composition of the carotenoprotein and the whole ovary are compared and the results discussed in relation to the mode of binding of the carotenoids (VII.D. ; XIV.$D_{(ii)}$).

7. A green astaxanthin-protein has been extracted and partially purified from the carapace of Carcinus maenas (green variety); its absorption spectrum and properties are recorded and compared with those of ovoverdin and crustacyanin (VIII. ; XIV.E).

8. A purple astaxanthin-protein has been extracted and purified from the carapace of Eriphia spinifrons. The absorption spectrum and general properties are recorded and compared with those of crustacyanin (IX. ; XIV.F).

9. A water-soluble lipoprotein, containing a number of carotenoids, has been extracted and purified from the ovary

and eggs of Palinurus vulgaris. The absorption spectrum and general properties are recorded (X., XI.). The mode of binding of the carotenoids is discussed (XIV.G).

10. A purple astaxanthin-protein has been extracted and partially purified from the carapace of Palinurus vulgaris. The molecular size and general properties of the protein are investigated and compared with those of crustacyanin (XII. ; XIV.I.).

11. A green astaxanthin-protein has been extracted and purified from the carapace of Astacus astacus. The properties and absorption spectrum of the carotenoprotein are recorded and the mode of binding of the carotenoid compared with that occurring in crustacyanin. The nature of a blue-green carotenoprotein precipitating at a higher $(NH_4)_2SO_4$ saturation is discussed (XIII. ; XIV.J).

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