FURTHER STUDIES ON THE CAROTENOPROTEIN, CRUSTACYANIN, AND THE ROLE OF THE PROSTHETIC GROUP, ASTAXANTHIN.

a Thesis presented by

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ABSTRACT

Carotenoproteins, i.e. those proteins containing carotenoid in stoichiometric amounts have been found in many forms of life. Most analytical work however, has been confined to the carotenoproteins occurring in crustacea, and in particular to crustacyanin, the blue carapace chromoprotein of the lobster, Homarus gammarus.

Crustacyanin is a protein of large molecular weight, composed of 8 purple sub-units (β-crustacyanin). Each of these sub-units contains 2 molecules of astaxanthin. The binding of carotenoid to protein results in a bathochromic shift in the spectrum of approximately 150 nm. A hypsochromic shift of almost 50 nm occurs when α-crustacyanin dissociates into β-sized sub-units. Many reasons have been proposed to account for these and other similar spectral shifts, and are discussed in Section 1.

The present thesis has been mainly concerned with analysis of the protein moiety of crustacyanin, with the aim of finding those parts of the molecule necessary to produce a blue or purple colour on combination with carotenoid, and the relationship between the various forms of coloured and colourless derivatives. A modified form of the extraction procedure has been developed to cope with the large quantities of material required for sub-unit preparation. The 5 sub-units that are obtained on removal of carotenoid with acetone or 6 M-urea have

been isolated in a homogeneous state. The relationship between these sub-units and their ability to combine with carotenoids, have been investigated. These results have been compared with various derivatives formed directly from α-crustacyanin without prior removal of the carotenoid. No direct evidence has been obtained about the particular amino acid residues in contact with the carotenoid, and it is felt that much useful work could be performed in this area.

CONTENTS

4. EXPERIMENTAL

SYMBOLS

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. 1970).

$E^{280}/_{580}$	:	The ratio of the optical densities at 280 nm and 580 nm.
$E^{1.0\%}_{280}$	:	The optical density of an 0.1% solution at 280 nm.
$f/_{fo}$	:	Frictional coefficient.
H	:	Average hydrophobicity. (Bigelow, 1967).
(P)	:	Phosphate buffer. ($Na_2HPO_4 + KH_2PO_4$).

1. INTRODUCTION

1.1 Nature of carotenoproteins

The wide variety of colours occurring in crustacea, were at one time divided into three categories (Pouchet, 1873):

Melanique - brown and black colours.

Xanthique - reds and yellows, concentrated in chromatophores.

Cyanique - blues and greens, diffused throughout the tissues in soluble form.

This classification ceased when it became evident that the blue and green pigments could be transformed into a red pigment, zöoerythrin, by the action of physical agents such as heat and extremes of pH (Merejkowsky, 1883). The blue pigment from the carapace of the lobster, Homarus gammarus, was thought to be a combination of a red lipochrome with an unstable organic base. A protein fraction, although always associated with the pigment, was assumed to be a contaminant (Newbigin, 1897, 1898). This was later proved to be incorrect (Verne, 1923, 1926, 1927). Zöoerythrin was found to be a mixture of carotenoids that, on combination with protein, can give rise to blue, green or red pigments. Suggestions that the carotenoids were adsorbed onto the protein surface, or dissolved in the lipid of a lipoprotein (Toumanoff, 1926; Palmer & Eckles, 1914), although true in some cases, were not thought to apply to the entire range of complexes. The term "caroténoïde-protéide" was originally proposed (Lwoff, 1925)

as a general term to cover all degrees of binding, but it now excludes proteins with carotenoids non-specifically bound. Some characteristics which define a carotenoprotein have been proposed, and are listed below (Karrer & Jucker, 1950; Cheesman et al., 1967).

1) Soluble in water or dilute salt solutions.
2) Insoluble in neutral lipid solvents.
3) Production of a red/orange colour typical of free carotenoids on addition of acetone, or lipid solvents in combination with protein denaturants.
4) A reproducible minimum molecular weight based on carotenoid content.

For reviews see Verne, 1926; Lederer, 1935; Cheesman et al., 1967; Ceccaldi, 1968.

Although many of the carotenoproteins recorded in the literature (Ball, 1944; Goodwin, 1952; Lee, 1966a, 1966b; Herring, 1965; Gilchrist, 1968, amongst others) are undoubtedly true carotenoproteins as outlined above, only a few have been extensively purified and shown to exhibit a stoichiometric relationship between carotenoid and protein. These latter include ovoverdin, the green protein of lobster eggs (Kuhn & Sørensen, 1938; Stern & Salomon, 1937, 1938; Zagalsky, 1964; Ceccaldi et al., 1966), ovorubin, the red chromoprotein from the eggs of the prosobranch Pomacea canaliculata (Cheesman, 1958; Norden, 1962), and crustacyanin, the blue carotenoprotein from the carapace of the lobster (Wald et al., 1948; Jencks & Buten, 1964; Cheesman et al., 1966; Kuhn & Kühn, 1967; Jencks & Buchwald, 1968b), as well

as several other crustacean carotenoproteins (Zagalsky et al., 1970). All these chromoproteins contain astaxanthin as their prosthetic group.

Ovoverdin (λ_{max} 660, 476, 278 nm. $E^{280}/_{660+476}$ = 1.7, see fig. 1.) has both a large lipid prosthetic group (22%), and a smaller carbohydrate component (4.8%) (Zagalsky, 1964). It has a mol. wt. of 300,000 (Wyckoff, 1937; Ceccaldi et al., 1966), and a min. mol. wt. of 140,000 based on the astaxanthin content (Zagalsky, 1964), which corresponds to 2 molecules of astaxanthin per molecule of ovoverdin.

Ovorubin (λ_{max} 510, 278 nm. $E^{280}/_{510}$ = 4.5, see fig. 1) has been purified by alumina gel chromatography (Cheesman, 1958). It is a glycoprotein consisting of 20% carbohydrate. The molecular weight of 330,000 is equal to the min. mol. wt. based on the astaxanthin content, indicating one molecule of carotenoid per molecule of protein.

Crustacyanin (λ_{max} 632, 278 nm. $E^{280}/_{632}$ = 0.3, see fig. 1) on the other hand, has neither lipid nor carbohydrate, and from the ratio of min. mol. wt. to mol. wt. contains 16 molecules of astaxanthin per mol. wt. of 360,000.

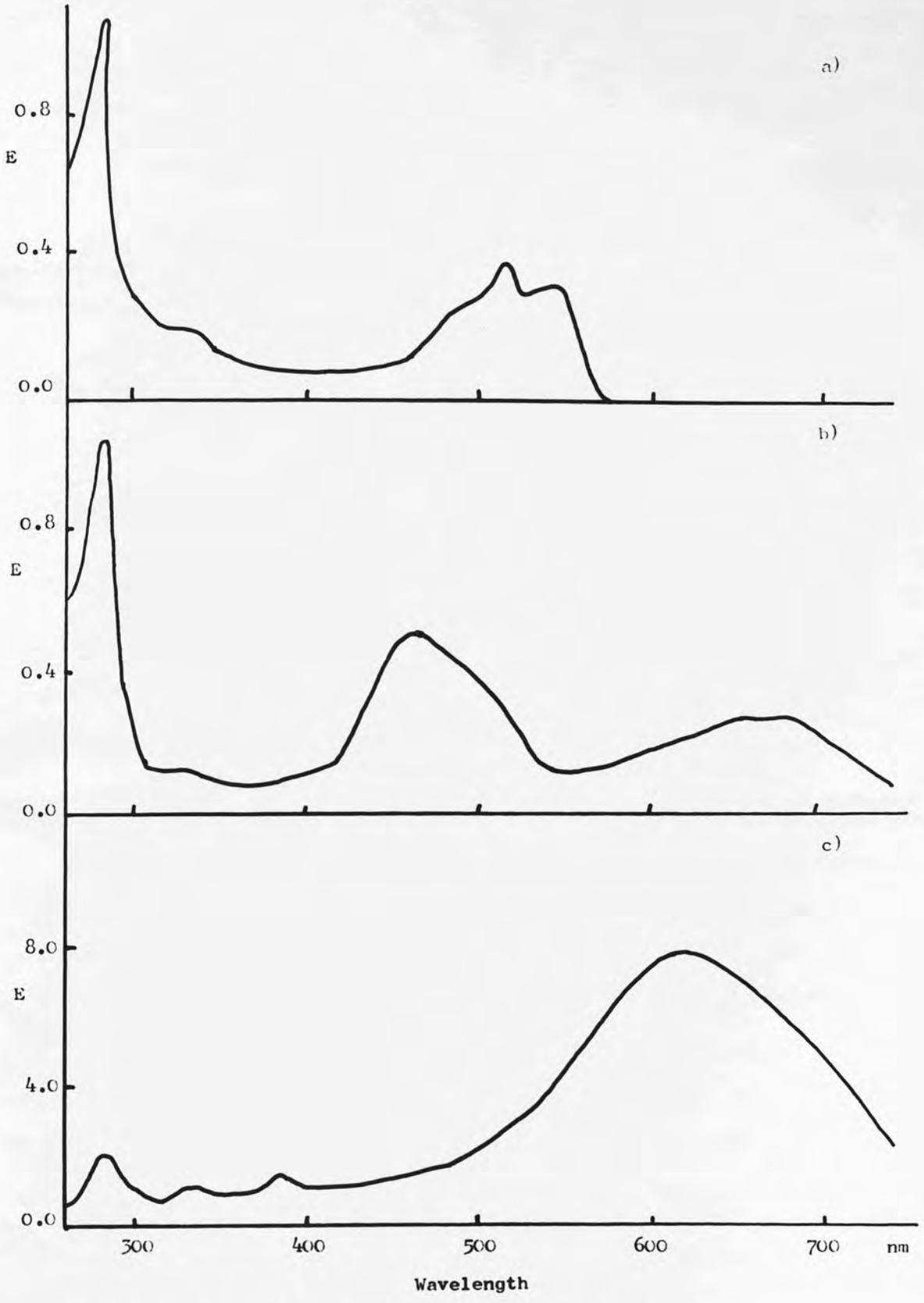


Fig 1. Absorption Spectra of three carotenoproteins.

a) Ovorubin (1mg/ml) in water (after Cheesman, 1958).
b) Ovoverdin (1mg/ml) in 0.05M phosphate buffer, pH 7.0 (after Zagalsky, 1964).
c) Crustacyanin (1mg/ml) in 0.02M phosphate buffer, pH 7.0 (after Cheesman et al., 1966, corrected for revised $E^{1\%}$ value.

1.2 Crustacyanin

The blue pigment crustacyanin occurs in the inner layers of the lobster carapace, and decalcification of the shell is required to bring the protein into solution. Several methods of solubilisation have been used.

Incubation of the shell with NH_4Cl, or dilute HCl (Newbigin, 1897); or of the finely ground shell with citrate buffer (Wald et al., 1948; Zagalsky & Cheesman, 1963); with $(NH_4)_2SO_4$ (Ceccaldi, 1964); calgon (Kuhn & Kühn, 1967); EDTA, (Jencks & Buten, 1964). Methods using the ground shell were found to give satisfactory results.

Purification of the pigment has been effected by adsorption onto $Ca_3(PO_4)_2$ gel, followed by stepwise DEAE-cellulose chromatography (Zagalsky & Cheesman, 1963); fractional precipitation with $(NH_4)_2SO_4$, followed by ultracentrifugation (Kuhn & Kühn, 1967); and gradient elution from DEAE-cellulose collumns (Jencks & Buten, 1964). In all cases the protein, α-crustacyanin (Jencks & Buten, 1964), was electrophoretically pure. It is a simple protein as indicated by its nitrogen content of 15.7%, and has no lipid other than carotenoid, no carbohydrate, or heavy metals. (Cheesman et al., 1966; Jencks & Buchwald, 1968b).

Physical studies have shown it to be a large protein of 300,000 - 380,000 mol. wt. (see Table 1). The main

source of discrepancy is in the value taken for the partial specific volume v, but the value of 0.66 (Kuhn & Kühn, 1967) seems low compared with that calculated from the amino acid content (Jencks & Buchwald, 1968b).

On removal of salts from a dilute solution of α-crustacyanin, α'-crustacyanin (λ_{max} 595 nm) is formed (Cheesman et al., 1966). This readily reforms α-crustacyanin if the ionic strength is increased. In contrast, sub-units of similar mol. wt. (41,000) but with $_{max}$ 585 nm, β-crustacyanin, are formed from α-crustacyanin on standing, or by treatment of α-crustacyanin with mild denaturing agents such as 3M-urea, 10% dioxan, heat, or alterations in pH. (Jencks & Buten, 1964; Cheesman et al., 1966). A certain amount of the β-form is always present during the preparation of α-crustacyanin, but is easily separated by virtue of its lower mol. wt. and reduced affinity for DEAE-cellulose. (Jencks & Buten, 1964; Cheesman et al., 1966; Kuhn & Kühn, 1967). It is probable that an equilibrium exists between the α- and α'-forms, and some of the latter is irreversibly converted into β-crustacyanin.

$$\alpha \rightleftharpoons 8\alpha' \longrightarrow 8\beta$$

Estimation of the minimum mol. wt. of α- and β-crustacyanins gives values of about 27,000 and 22,000 respectively (Table 1) which suggests that β-crustacyanin contains two astaxanthins per molecule, and α-crustacyanin contains 16 molecules of astaxanthin

TABLE 1

Physical constants of the crustacyanins and their apo-units

Partical size	Method	$(\bar{v})$	$E^{1\%}_{1cm}$ 278nm	λ_{max} / λ max	axial ratio ~~f/fo~~ (p)	(o)	Author
α-crustacyanin							
320,000	s.v.	0.66	19.4	38.4	10/1	12/1	K
307,000	l.s.						K
380,000	s.v.	0.76	14.0	46.4			J
630,000	g.f.		11.5	37.4			C
300,000	o.						G
333,000	s.v.	0.77			9/1	10/1	G
450,000	g.s.	0.77					G
370,000	s.v.	0.77					G
340,000	d.f.						G
Minimum molecular weight							
27,000							J
27,000 (corr.)							C
19,000							K.
β-crustacyanin							
39,000	o.						K
36,000	s.v.	0.66					K
49,000	s.v.	0.76	13.0	54.0			J
36,500	g.f.						C
46,000	s.v.	0.76					G
43,000	g.s.	0.76					G
Minimum molecular weight							
21,000							J
γ-crustacyanin							
400,000	s.v.	0.76		47,0			J
Minimum molecular weight							
24,000							J

TABLE 1 (contd).

apo-crustacyanin			
20,000	s.v.	0.67	K
20,000	g.f.		C
21,000	s.v.	0.73	G
20,500	s.e.		G
19,500	g.s.		G

l.s. = light scattering
s.v. = sedimentation velocity
o. = osmometry
g.s. = gel filtration & sedimentation
s.v. = sedimentation and viscosity
d.f. = diffusion and viscosity
s.e. = sedimentation equilibrium
g.f. = gel filtration

K Kuhn & Kühn, (1967)
J Jencks & Buchwald, (1968b)
C Cheesman et al., (1966)
G. Gammack et al., (1971).

and consists of 8 β-sized molecules. This size of 22,000 corresponds to the value obtained for the mol. wt. of apo-crustacyanin, prepared by the removal of the carotenoid prosthetic group with acetone. (Cheesman et al., 1966; Jencks & Buchwald, 1963b). The values for the mol. wt. of the apo-protein calculated from Sephadex gel filtration are, in contrast to measurements for α-crustacyanin, in agreement with those from sedimentation and diffusion, thus indicating a more nearly spherical molecule for the sub-units. However, complete agreement on the mol. wt. of the apo-protein has not been reached by all workers (see Table 1).

On the addition of various denaturing agents, e.g. acetone or 6M-urea, the spectrum of α-crustacyanin alters either to that of astaxanthin in the particular solvent used (λ_{max} 480 nm), or to approximately λ_{max} 400 nm (Jencks & Buten, 1964). On removal of the reagent the characteristic spectrum of α-crustacyanin can usually be regained, although if drastic methods have been used β-crustacyanin is often the reconstituted product. From specificity studies with various carotenoids, both 3,3'-hydroxy groups and 4,4'-keto groups are required for the formation of α-crustacyanin, although β-sized molecules can be formed from carotenoids with only the 4,4'-keto groups (Lee & Zagalsky, 1966).

Crude preparations of α-crustacyanin, while containing some of the β-form, also contain two other pigments.

One of these, γ-crustacyanin (λ_{max} 625 nm), has approximately the same mol. wt. as α-crustacyanin (400,000) and a similar minimum mol. wt. based on the astaxanthin content (25,000), (Jencks & Buchwald, 1966b and Table 1). Since it is excluded from Sephadex G.200 (Cheesman et al., 1966), and has a high intrinsic viscosity (Jencks & Buchwald, 1966b), it would appear to be less symmetrical than α-crustacyanin, and could possibly be a slightly denatured form. The other contaminating pigment, yellow in colour (λ_{max} 400 nm), has a spectrum similar to the product formed by the action of "hydrophobic type" denaturing agents on crustacyanin. The minimum mol. wt. based on the astaxanthin content was found to be 4,400, and it appears to contain 20 molecules of astaxanthin per mol. wt. of 90,000 (Jencks & Buten, 1964; Jencks & Buchwald, 1966b).

Amino acid analyses have been performed on α-crustacyanin and its derivatives. The analyses indicate a relationship between the blue pigments, which appear distinct from the yellow protein. The amino acid content is, in general, similar to that of other blue crustacean carotenoproteins. (Zagalsky et al., 1970, and Table 11).

In order to account for the 10 : 1 axial ratio of the α-protein, it has been proposed that 8 of the fully stretched astaxanthin molecules (each 30 Å long) could fit linearly into one molecule of α-crustacyanin, the length of which has been estimated at 300 Å. Each sub-unit of α-crustacyanin would contain two parallel astaxanthin molecules, embedded in the protein.

TABLE 2.

Amino acid composition of the crustacyanins, the yellow lobster shell pigment and two average proteins

	α	β	γ	yellow	ave.car.	ave.pro.
arg	3.5	3.3	3.5	5.1	3.5	4.0
lys	5.5	5.2	5.6	5.1	6.5	5.9
his	1.8	1.3	1.4	2.4	2.7	1.8
asp	13.5	13.4	13.8	1.5	13.1	9.0
glu	9.6	9.2	9.3	14.4	10.1	9.5
thr	6.2	6.5	6.7	7.3	6.9	5.2
ser	6.0	6.3	6.0	5.5	10.1	5.8
pro	5.6	4.5	4.5	6.7	6.3	4.4
gly	4.9	4.7	4.6	8.0	8.9	6.8
ala	7.5	8.3	7.8	8.6	8.6	7.6
val	6.5	7.3	7.3	7.7	5.8	6.0
ile	4.5	4.4	4.5	7.0	3.5	3.8
leu	5.0	4.4	4.7	8.6	4.6	7.4
cys	2.8	2.8	3.0	2.7	0.6	1.0
met	0.5	0.5	0.4	0.9	0.1	1.3
tyr	8.4	8.5	8.6	3.6	4.7	3.2
phe	8.2	8.1	8.5	5.1	5.0	3.4

Values are given in residues per 100 residues.

α- β- γ-crustacyanins and the yellow lobster shell pigment from Jencks & Buchwald (1968b).

Average carotenoprotein (ave.car.) from Zagalsky et al., (1970.

Average protein (ave.pro.) from Smith (1966).

Values for tryptophan were not given.

(Kuhn & Kühn, 1967). A disc shaped molecule has also been envisaged (Cheesman et al., 1966) in which the number of sub-units would be determined by the radius of curvature of the disc, the astaxanthin molecules being directed towards the centre to form a hydrophobic core.

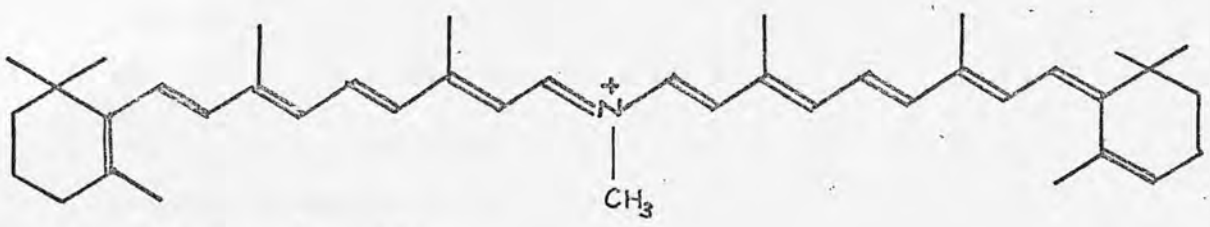
1.3 Spectral changes brought about by the binding of polyene to protein.

Polyenes have an absorption maximum in the visible range which is the result of their long conjugated double bond structure. The longer the conjugated system, the more of the lowest energy orbitals are filled, the easier it is for a π electron to be excited, and consequently the longer is the λ_{max}.

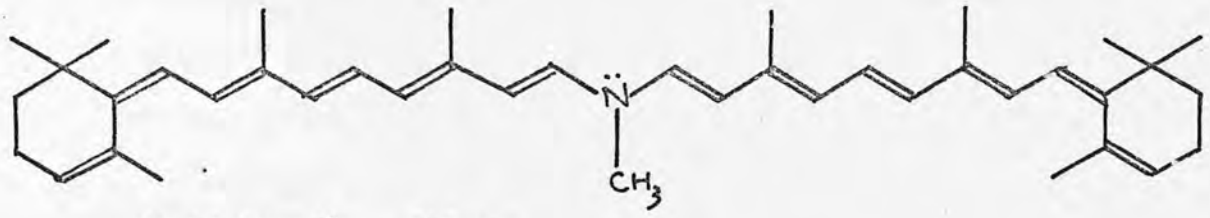
On combination with protein the λ_{max} changes, usually towards the red end of the spectrum, i.e. a bathochromic shift, varying from 40 nm for ovorubin, to 100 nm for the visual pigments and crustacyanin. Although rarer, hypsochromic shifts have also been noted, as for the yellow pigment of the lobster shell.

The most intensively studied polyene-protein complexes are the visual pigments. These consist of derivatives of vitamin A in combination with a protein. The main pigment of the rods in mammals, rhodopsin, has received most attention since it can readily be extracted (Tansley, 1931), and can be obtained in a pure form (Heller, 1968b; Schichi, 1970). It contains 11-cis retinal as its prosthetic group (Hubbard & Wald, 1952), and has λ_{max} 498 nm. This represents a bathochromic shift of 118 nm as compared with the spectrum of 11-cis retinal in hexane (λ_{max} 380 nm).

Indicator yellow, formed on bleaching of rhodopsin, has an absorption maximum at 365 nm in alkali, and 440 nm in acid (Lythgoe, 1937). Synthetic adducts of methylamine and retinaldehyde show a similar spectral change on alteration of pH, and the following formulae were proposed for the acidic and basic forms, providing for two groups of 6 conjugated double bonds in the former, and one group of 12 in the latter. Similar structures were proposed for indicator yellow in which the amino side chains on the protein would take the place of the methylamine (Collins & Morton, 1950).



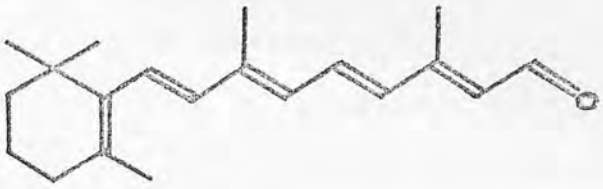
acid form λ_{max} 440 nm.



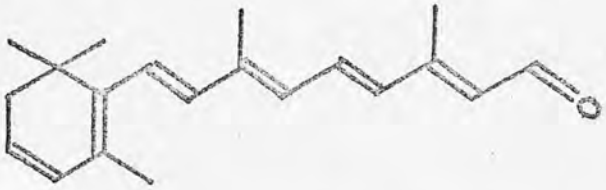
alkali form λ_{max} 365 nm.

A similar 'double vitamin A' structure had already been proposed to account for the bathochromic shift of rhodopsin (Wald, 1949). Porphyropsin, the corresponding pigment in many invertebrates and marine fishes, has retinal$_2$ as its prosthetic group (Wald, 1937). This has one more conjugated double bond

than retinal$_1$, and it therefore absorbs at longer wavelengths. If two molecules were so aligned in porphyropsin the difference in absorption maxima between the two visual pigments should be twice as great as that between the two retinals.



all-trans retinal$_1$ λ_{max} 380 nm.



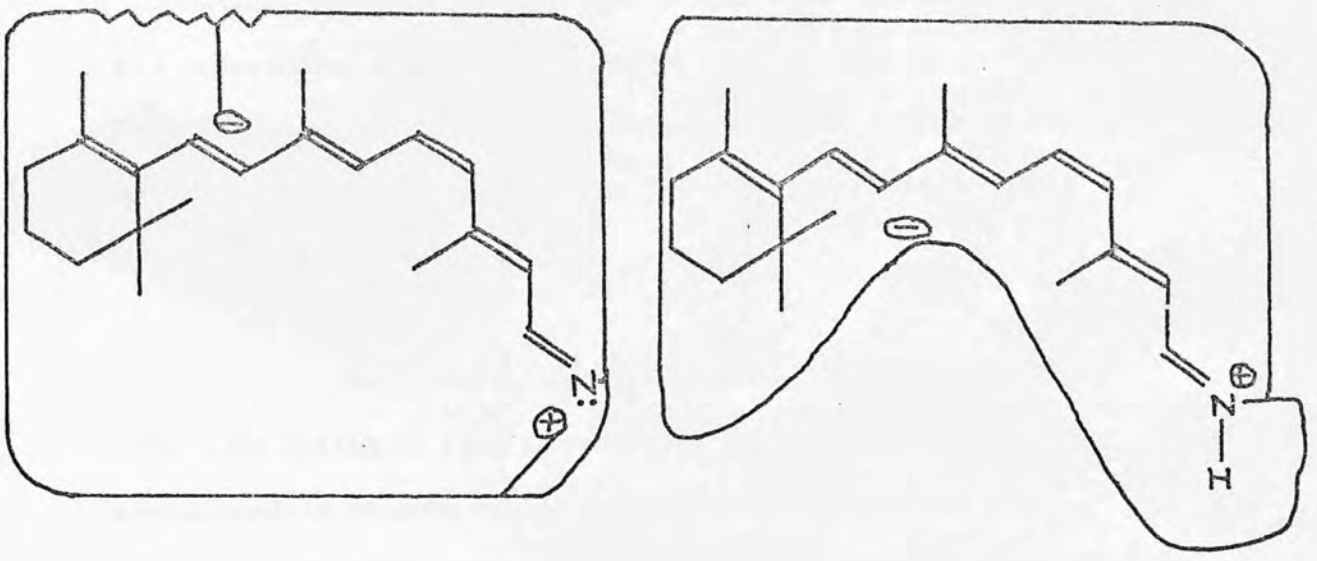
all-trans retinal$_2$ λ_{max} 405 nm.

This is not the case however, so that only one molecule of retinal can be involved as prosthetic group. Attachment of the retinal to an amino group of the protein through a Schiff's base, proposed by Collins (1953), has recently been substantiated in the bleached pigment (Akhtar et al., 1968; Poincelot et al., 1969). Evidence for the presence of a single molecule of ~~astaxanthin~~ retinal as prosthetic group has recently been obtained by analysis of rhodopsin (Heller, 1968b).

Since the absorption maxima of the various visual pigments cluster about different positions between 430 nm and 662 nm (Dartnall & Lythgoe, 1965), the lipoprotein backbone evidently exerts considerable influence on the spectrum. It has been suggested that charged groups on the protein induce permanent dipoles in the polyene chain, the position of the absorption maxima then depends on the length of the chain polarised. The

electronic state of the nitrogen atom of the Schiff's base remains unresolved, and two possibilities have been proposed.

The 'lock and key' theory (Dartnall, 1957), later modified (Dartnall & Lythgoe, 1965), assumes an unprotonated form, supported by the data of Bridges (1962), a bathochromic shift being brought about as a result of polarisation of the chain by two oppositely charged groups on the protein.



(Abrahamson & Ostroy, 1967).

However, since unprotonated retinylidene-Schiff's bases absorb at shorter wavelengths than the free aldehyde (cf. indicator yellow), Morton & Pitt (1955) have

proposed that in rhodopsin the Schiff's base is in the protonated form. In this theory it is proposed that a further bathochromic shift in the absorption spectrum is a result of polarisation of the polyene chain by negative charges on the protein. (Kropf & Hubbard, 1958; Hubbard, 1958). Poincelot et al., (1969) (Kimbel et al., 1970) have suggested that in rhodopsin the 11-cis retinal is involved in a Schiff's base linkage with the amino group of phosphatidyl ethanolamine, the retinal being situated in a hydrophobic pocket. In this case internal protonation of the Schiff's base could occur from the phosphoric acid group of phosphatidyl ethanolamine, as such protonations can be produced in a non-aqueous environment in model systems, (Daemen & Bonting, 1969). Polarisation of the adjacent hydrophobic groups on the protein would also help stabilise the protonated Schiff's base (Irving et al., 1970).

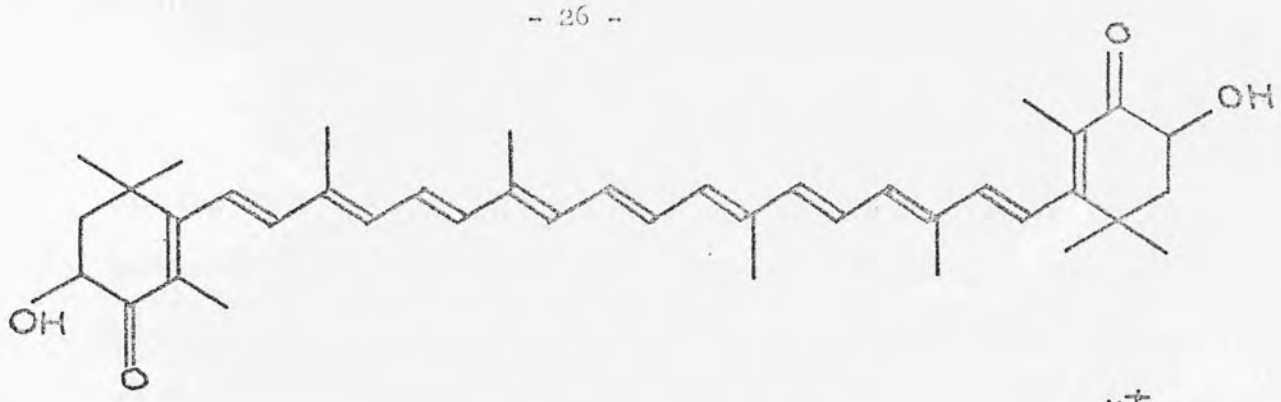
Some workers (Heller, 1968a; Hall & Bacharach, 1970) have failed to find phosphatidyl ethanolamine in stoichiometric amounts (which would be prerequisite for a retinal-phosphatidyl ethanolamine linkage) in purified rhodopsin. Hirtenstein & Akhtar, (1970) have reported that although the reduction of rhodopsin (prior to the formation of meta-rhodopsin II) with $NaBH_4$ does yield some N-retinyl-phosphatidyl ethanolamine, this is not the product in all preparations of the pigment. Also, under conditions where the spectral integrity of rhodopsin itself is intact, reduction with $NaBH_4$ can yield retinal bound in a Schiff's base linkage to various hydrolytic breakdown products of phosphatidyl ethanolamine. They suggest, therefore, that the

linkage of retinal in rhodopsin is to an amino group in a hydrophobic region of the protein, and that it can readily exchange with phosphatidyl ethanolamine during extraction. The possibility that N-retinyl-phosphatidyl ethanolamine is a means of storing retinal in the 11-cis form has also been envisaged (Anderson, 1970).

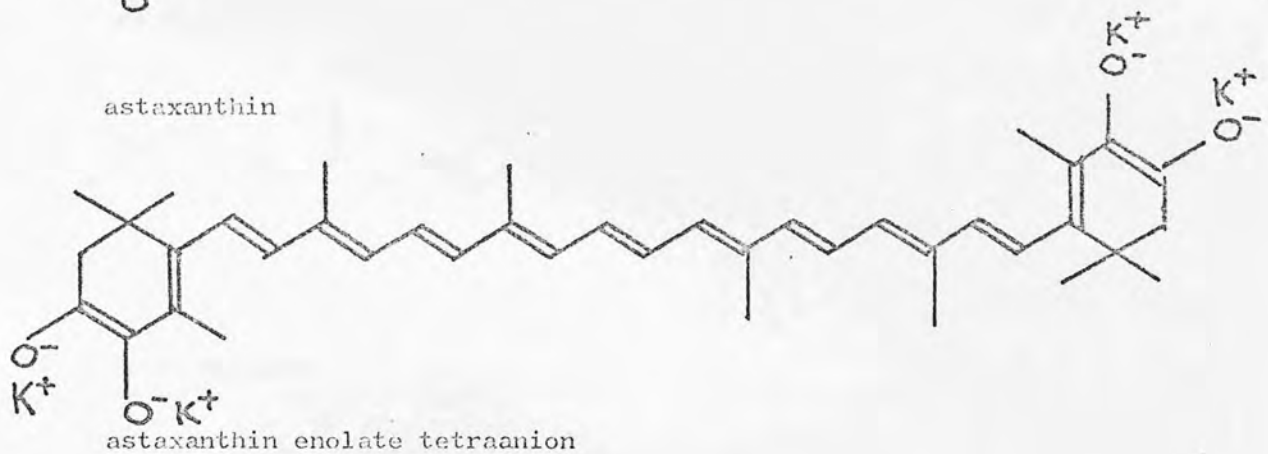
Although the exact nature of the amino group involved in the original Schiff's base is not known, it is probable that it is protonated and present in a hydrophobic environment. On the transition of rhodopsin from the meta I (λ_{max} 478 nm) to meta II (λ_{max} 380 nm) stage, the polyene chain becomes exposed to the aqueous environment, the final Schiff's base is therefore unprotonated and consequently has a lower absorption maximum.

In contrast, the chromophores of the carotenoproteins so far investigated are not covalently linked to the protein, being easily extracted with acetone. The spectral shifts must therefore be explained in terms of hydrophobic, ionic and other non-covalent interactions.

Astaxanthin turns blue in the presence of potassium butoxide (and the absence of air), owing to the formation of the potassium salt of the enolate tetraanion.

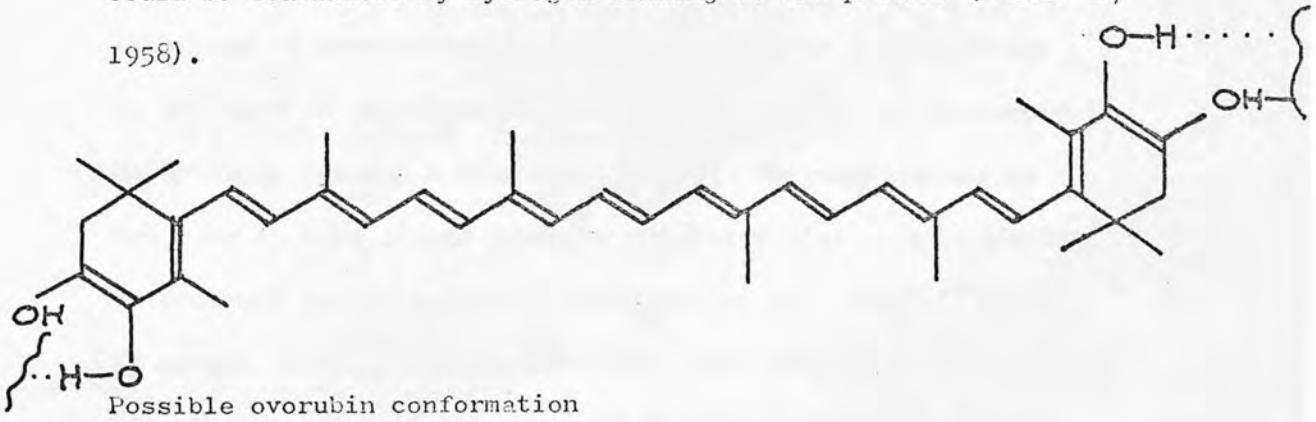


astaxanthin



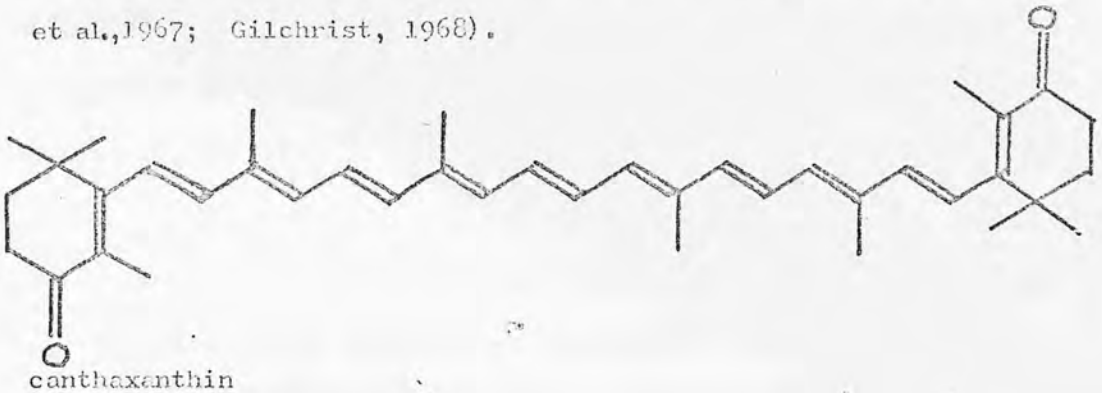
astaxanthin enolate tetraanion

It was suggested that replacement of the K^+ by positive charges on the protein could account for the bathochromic shift in the spectrum of many carotenoproteins. (Kuhn & Sørensen, 1938; Ball, 1944). In explanation of the smaller bathochromic shift and increase in the fine structure observed in the absorption spectrum of ovorubin, it was proposed that the enolate structure could be stabilised by hydrogen bonding to the protein (Cheesman, 1958).



Possible ovorubin conformation

Similar theories have been invoked to explain the spectrum of canthaxanthin-protein complexes, the only other carotenoid at present known to be involved in natural linkages with protein. (Lee et al.,1967; Gilchrist, 1968).



canthaxanthin

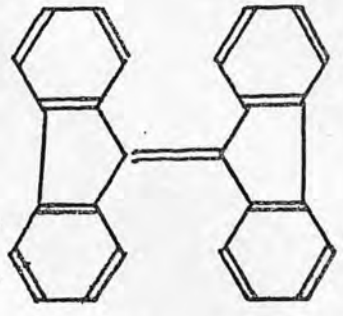
Formation of charge transfer complexes, by interaction with donor (D) and acceptor (A) molecules at the ends of the polyene chain would increase the number of resonance structures, and therefore the absorption maximum (Platt, 1959).

This type of interaction has been suggested as a possibility in the case of rhodopsin (Akhtar, 1968; 1970)*, but is regarded as unlikely (Jencks & Buchwald, 1968b). Astaxanthin has no tendency to form charge transfer complexes with acid tryptophan, in contrast to the retinenes (Ishigami et al., 1966). Also, in normal charge transfer complexes, the spectrum of the complex is superimposed upon the spectrum of the individual components, which is not the case for the majority of carotenoproteins.

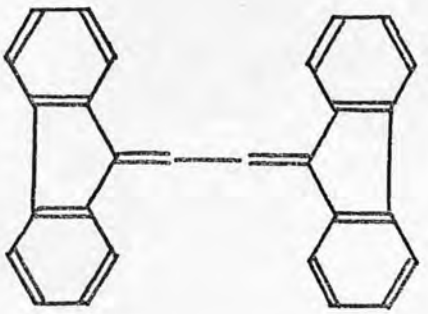
* Akhtar et al., 1968; Akhtar, 1970.

The fact that in crustacyanin and other carotenoproteins the shape of the absorption spectrum is the same as that of astaxanthin, but shifted in position, also tends to invalidate the possibility of formation of an enolate tetraanion, as such a compound would be expected to produce a narrowing of the absorption bands (Jencks & Buchwald, 1968b).

Since polarisation, charge transfer, or carbonium ion formation cannot account for the spectrum of polyene-protein complexes (Jencks & Buchwald, 1968a; 1968b), a theory which more adequately explains their spectral characteristics has recently been proposed. If the polyene chain were twisted about the double bonds, this would have the effect of making the double and single bonds more equivalent, thereby raising the energy of the ground state, lowering the energy required to promote an electron to the first excited state and consequently shifting the absorption maximum to longer wavelengths. For example, in spite of the fact that dibiphenylenebutadiene has a longer conjugated double bond system than dibiphenyleneethylene, it has a lower absorption maximum. In the latter case the steric hindrance of the bulky aromatic residues causes partial rotation about the double bond and thus a decrease in excitation energy required to promote the first excited state.



dibiphenyleneethylene



dibiphenylenebutadiene

If the methyl groups of the polyene chain were immobilised, e.g. by hydrophobic interactions, (Jencks & Buchwald, 1968b), and the β-ionone rings then twisted out of the plane of the polyene chain by ionic binding of the 4,4'-keto groups, the strain would be localised in the double bonds. The advantage of this theory is that it can account for the various absorption maxima both of the carotenoproteins and the visual pigments and their bleaching products, as the degree of twisting about the double bonds of the polyene chain would alter the position of the absorption maximum (Jencks & Buchwald, 1968b).

1.4 Possible functions of carotenoproteins.

The wide variety and distribution of carotenoproteins has naturally led to the development of many theories concerning their function. Perhaps the most obvious is that of protective colouration. As described in the previous section, the combination of polyene with protein can lead to a considerable alteration in the spectrum. The presence of both free carotenoid and carotenoprotein will therefore provide absorption over a large part of the spectrum, and enable organisms to blend with their backgrounds (Wieser, 1965). This has been suggested as a possible function of the blue pigments of the otherwise transparent organisms of the sea surface (Herring, 1965). Variations in relative concentration of the free and bound carotenoid will effect a colour change. Such an alteration has been studied in the maring isopods Idothea granulosa and I. montereyensis, and has been shown to be correlated with an alteration in habitat. (Lee, 1966a, 1966b, 1967). These organisms occur in three colour varieties, red, green and brown, blending with the colour of the algae on which they feed. In Monterey, they exist in two distinct populations; an offshore, exposed adult colony, green in colour, provided with plenty of food and a smaller, red colony, which being more sheltered, harbours the young animals. On migrating from the inshore to the offshore colony the isopods alter in colour from red to green. The red colour, present in the chromatophores, is due to free carotenoid, while the green pigment, consisting of a canthaxanthin-

protein with lutein dissolved in the lipid prosthetic group, occurs free in the cytoplasm.

Another protective function, that of absorbing harmful radiation, has been proposed for the blue pigmentation of many pelagic invertebrates. (Heinrich, 1960; Fox et al., 1967). Although the red light which they absorb has a low power of penetration, it may be significant in the top four inches of sea water where they preponderate (Herring, 1965). This is similar to the proposed function of ovorubin in the eggs of the tropical prosobranch, Pomacea canaliculata (Cheesman, 1956). These are laid out of water, and in this case the carotenoid is known to stabilise the protein against heat denaturation. Protection of the protein by structural means rather than by preferential absorption of harmful radiation may indeed be an important function of the carotenoid. Crustacyanin and ovorubin are known to be much more stable than their apo-proteins, which spread more easily at air-water interfaces (Cheesman, 1958; Cheesman et al., 1966). The greater stability of the polyene complexes over the individual protein components, has also been noted for the visual pigments (Hubbard, 1958; Radding & Wald, 1955; Wald & Hubbard, 1960). It has been found that retinene may only protect the groups on the protein with which it is in contact, allowing the rest of the molecule to partially denature. On prolonged storage, even though the spectrum of the pigment remains unchanged, the retinine will not then recombine with the apo-protein (Abrahamson & Ostroy, 1967).

Carotenoproteins also appear to be concerned in development, since they are present in the eggs and ovaries of many different species, and can often be seen to undergo alterations during hatching and growth (Teissier, 1925; Goodwin, 1950; 1951; Ball, 1944; Green, 1957). It has been suggested that the complex may be important either as a source of proteins or of astaxanthin. In both cases the linkage would stabilise both the entities. The carotenoproteins of Idya, Pomacea and Homarus are the major nitrogen source of the eggs (Lwoff, 1927; Cheesman, 1958; Ceccaldi, 1965), and, in the case of the former, when the carotenoid is split from the protein it is concentrated in the naupliar eye.

The presence of carotenoids, notably astaxanthin, in the eyes of several species, has led to the belief that they may act as photoreceptors in place of retinene which is often lacking* (Goodwin, 1949; Wald & Zussman, 1937; Wald, 1943), and that they could also be involved in other functions normally mediated by vitamin A, such as membrane stability (Scheer, 1940).

Although many of the functions tentatively ascribed to carotenoproteins may indeed be the 'Raison d'etre', they are not universally applicable. In some organisms, including Daphnia magna, specimens grown on a carotenoid free diet and so reputedly entirely devoid of carotenoproteins did not appear to suffer any deleterious effect, with regard either to growth or fertility. (Herring, 1968; Wallace et al., 1967).

* Goodwin & Srisukh, 1949.

2. MATERIALS

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

Urea was recrystallised from hot methanol.

The following materials were used for protein purification :

DEAE-cellulose (DE 32), CM-cellulose (CM 52) and cellulose (non-ionic, medium grade), Whatman, England.

Sephadex G-25, (medium grade), G-75, G-100 and G-200 Pharmacia, Uppsala, Sweden.

Pevikon C. 870, Shandon, London.

Cellogel strips and blocks (gellatinised cellulose acetate) Chemetron, Italy.

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

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

Alumina (Aluminium Oxide G. nach Stahl,) Merck, Anderman & Co. Ltd., London.

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

Lyphogel, Gelman, Hawksley & Sons, London.

Phosphate buffers were prepared by mixing solutions of KH_2PO_4 and NaOH.

Lobsters (Homarus vulgaris), dead, and condemned as unfit for human consumption were kindly provided by Billingsgate fish market.

3. GENERAL METHODS.

3.1. Spectroscopic measurements.

Absorption spectra were recorded on a Beckman S.P. 400 recording spectrophotometer, against appropriate solvent blanks; silica cells of 1 cm light path length and 3.5 cm capacity were used.

3.2. Chromatography.

3.2.1. Ion-exchange chromatography on DEAE- and CM-celluloses.

Columns of various dimensions were prepared according to the method of Peterson & Sober (1962). Elution of the adsorbed proteins was carried out by increasing the ionic strength of the perfusing buffer in stages (stepwise elution), or gradually (gradient elution). Buffers used were normally phosphate (KH_2PO_4-Na_2HPO_4) pH 6.8, with concentrations up to 0.5 molar. Tris buffers of comparable ionic strengths were also employed. Two gradient systems were used, and both were found to give satisfactory results.

The first system consisted of two equally sized vessels, a reservoir and a mixing chamber, connected by a narrow tube, the mixer being stirred continuously. (Parr, 1954; Bock & Ling, 1954). This system gives a linear gradient when the two

densities are equal, but slightly convex when :

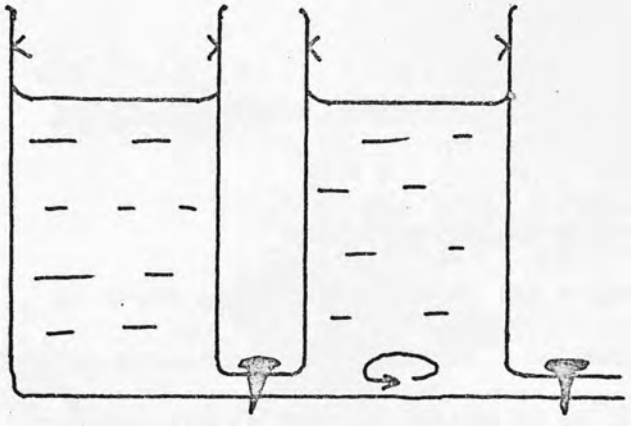
$$p_m d_r^2 > p_r d_m^2$$

The gradient is then given by :

$$d_r/d_m = p_r/p_m \times -1/\log 2 \times \log(c_r - c/c_r - c_m)$$

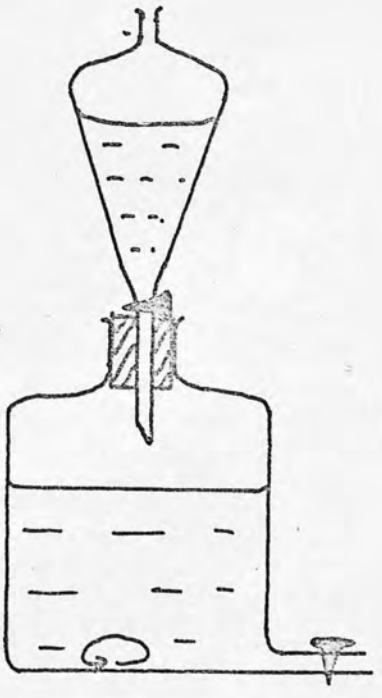
System 2 was formed from a separating funnel, containing the more concentrated buffer, fitted into an aspirator containing the starting buffer, which is stirred by means of a magnetic stirrer. This gives a logarithmically increasing gradient :

$$c_1 - c/c_1 - c_2 = e^{-v/v_2}$$ (Alm et al., 1952; Kocent, 1960).



$$c = c_2 - (c_2 - c_1) \cdot (1 - {}^{v}/_{v_r + v_m})^{\,p_m d_r^2 / p_r d_m^2}$$

System I



$$\frac{c_1 - c}{c_1 - c_2} = c^{-v/v_2}$$

System II

where p_1 = density of starting buffer

p_2 = density of final buffer

c_1 = molarity of starting buffer

c_2 = molarity of final buffer

c = concentration required

v = elution volume

v_2 = volume of starting buffer

d_r = diameter of reservoir

d_m = diameter of mixer

v_r = volume of reservoir

v_m = volume of mixer

3.2.2. Thin-layer chromatography.

Thin-layer experiments were performed as described by Truter (1963). Carotenoids were identified by chromatography on Al_2O_3 plates (30 mg/60 ml H_2O) and developed with pet-ether acetone (75/25 v/v), appropriate standards being run concurrently.

3.2.3. Molecular sieve chromatography.

Molecular weight estimations have been performed on cross linked dextrans of the Sephadex series, according to the method of Flodin (1962). Columns were coated with dimethyl-silane (1% in benzene heated to 60°C) to prevent edge effects, and run in 0.1M-phosphate buffer pH 6.8.

When a mixture of proteins is passed through a column of Sephadex, fractionation occurs, since diffusion into the gel matrix is more or less restricted, depending on the size of the macromolecule. The components of the mixture are therefore eluted in order of decreasing molecular size. (Porath & Flodin, 1963). The method can more accurately be regarded as providing an estimate of the Stokes radius (Andrews, 1964), since retardation by the gel depends on shape as well as size (Ackers, 1967). For globular proteins a correlation between elution volume and mol. wt. has been demonstrated, and a graph of elution volume plotted against the log. mol. wt. has been shown to be almost linear over the range of optimal gel function. (Andrews, 1962; 1964). The molecular weight of a simple globular protein can therefore be estimated by comparison of its elution volume with those of similar proteins of known molecular weight (Andrews, 1965).

A better correlation between elution volume and Stokes radius, for the protein under investigation, can be obtained by comparing the molecular radii of the standards with the inverse error function complement of their partitition coefficient in the bed medium (Ackers, 1967).

3.3. Electrophoresis.

3.3.1. Starch gel electrophoresis.

Horizontal starch gel electrophoresis was performed

by the method of Smithies (1955). Gels (13 - 16% starch), with or without 6M-urea were prepared in perspex trays (9 x 23 x 0.5 cm) as described by Smith (1968). Gels containing 0.005M-dithioerythritol or 0.05M-mercaptoethanol, in addition to 6M-urea were made in a similar manner (Poulik, 1960), these reagents being added to the gel after deaeration, in order to prevent evaporation losses. Samples were applied by inserting pieces of Whatman 3MM filter paper (2 x 0.5 mm), soaked in protein solution (approx. 5 mg/ml) into vertical slits in the gel. The gels were covered with Parafilm to prevent evaporation, and run at a potential gradient of 4 - 7 v/cm for a period of 16 - 36 hr. Long runs were carried out in the cold (4°C). Gels were sliced horizontally and stained with Amido-Schwarz (1% in glycerol - water - acetic acid, 5 : 5 : 1) or with Coomassi blue (1% in the same solvent) for one hour, the background being removed by several washes in the same solvent, and cleared by soaking in glycerol.

Buffer systems :

a) Borate buffer, pH 8.35 (Smithies, 1955)
0.3M in buffer compartments, 0.04M in gels

b) Phosphate buffer, pH 7.0 (KH_2PO_4 - Na_2HPO_4)
0.05M in buffer compartments, 0.025M in gels

c) Formate buffer, pH 3.5 (Poulik, 1962). 0.05M formic acid + 0.01M NaOH, both for gels and electrode vessels

d) Tris-citrate-borate discontinuous system pH 8.35 Poulik, 1957) 0.076M-tris + 0.005M-citric acid for gels, 0.03M-borate buffer, pH8.35 in the electrode vessels

e) Tris-EDTA-borate discontinuous system pH 8.35 (Smith, 1968) 0.07M-tris + 0.003M-EDTA, adjusted to pH 8.35 with boric acid for the gels and 0.03M-borate buffer pH 8.35 in the buffer compartments.

In all cases less alkali was required to obtain the desired pH in the gels containing 6M-urea.

3.3.2 Cellogel electrophoresis.

Electrophoresis on cellogel strips was carried out as described in the Chemetron information sheet according to Del Campo. The strips were soaked in water to remove the methanol, and then in buffer overnight. 2 - 5 µl samples were applied in a thin line 1/3 along the strip and electrophoresed in an enclosed tank at 0.8 ma / cm, 13 v / cm for 1.5 - 2 hr. The strips were run in veronal buffer I = 0.05, pH 8.6. The stain used was lissamine green (0.5% w/v in methanol - water - acetic acid, 5 : 4 : 1) decolourised in 5% acetic acid, and rendered transparent in water - methanol - acetic acid - diacetone alcohol 50 : 37 : 5 : 8 plus 1 - 2 drops glycerol. 1% Amido-schwarz, in the same solvent system was also similarly employed.

Preparative electrophoresis on cellogel blocks 6 x 17 x 0.6 cm was also performed. Holes were made with a needle along the origin before application of the sample in order to ease

penetration and prevent spreading. 2 - 8 ml of material were applied, (2-5 mg/ml). The blocks were run in veronal buffer as for the strips, at 5 - 6 ma/cm and 10 - 12 v/cm at 4°C for 18 - 36 hr.

3.3.3. Preparative electrophoresis in Pevikon.

Preparative electrophoresis in Pevikon (a vinyl copolymer) was performed according to the method of Foxwell et al., (1968). The block was prepared in 0.04M-borate buffer, pH 8.35, in a perspex tray 25 x 50 x 0.5cm. The bridge buffer being 0.3M-borate buffer, pH 8.35. A slit 2 mm wide was cut in the block across the tray, and the Pevikon sucked out with a pasteur pipette. This Pevikon was mixed with the sample and pipetted back into the slit, filter paper soaked in buffer placed 2 - 3 cm on either side of the slit to reduce spreading of the sample. These blocks were run at 0.5 ma/cm and 7 v/cm, at 4°C for 24 hr. Samples were eluted by cutting out the appropriate bands and stirring in 0.05M-phosphate buffer pH 6.8.

3.3.4. Preparative electrophoresis on the Porath column.

Electrophoresis on the Porath column was carried out on an LKB apparatus (Svensson, 1954), as described by Bloemendal (1963).

Both Sephadex and cellulose were tested as supporting medium. Cellulose was found to give greater resolution, and

consequently used in all experiments. Of the buffers tested only the following were found to give successful results.

a) 0.1M-tris + 0.004M-EDTA + 0.015M boric acid pH 8.0

b) 0.1M-tris + 0.1M-maleate + 0.1M-NaOH pH 7.2

Phosphate buffers were tried but produced spreading of the bands. The size of the sample varied, but was never more that 15 ml, containing up to 10 mg protein/ml. The experiments were performed at 40 ma, 200 v for 40 hr, the buffer being normally renewed once during this time. Water at 4^{o}C was circulated in the cooling jacket. Samples were eluted in the buffer of the experiment at 7 ml/hr and analysed for protein spectrophotometrically.

3.4. Immunodiffusion and electrophoresis.

Antibodies to the major apo-units (A_2 & C_1) were kindly prepared from rabbits by Dr. G. Darlow, Department of Physiology, Bedford College. Two standard methods were used:

a) Six intravenous injections of antigen (a total of 10 mg in 1 ml of normal saline) were given at 3 day intervals, and left for 10 days before bleeding.

b) Injections of antigen (a total of 10 mg in 1 ml of normal saline) plus equivalent amounts of Freunds complete adjuvant, were given subcutaneously, at 6 sites in the posterior neck region. 5 mg. of antigen were given intravenously as in 'a'.

Immunodiffusion and electrophoresis was attempted in agar gels (3%) on microscope slides, and cellogel strips (Smith, 1968). These systems were tested using serum albumin, ovalbumin and their respective antibodies.

3.5. Preparation of samples.

Buffer changes and removal of salts were effected either by dialysis or gel filtration on Sephadex G.25 columns previously equilibrated with the desired solvent.

Concentration of the proteins was usually achieved by ultrafiltration (Leggett-Bailey, 1967) or by removal of salt followed by lyophilisation. Samples containing urea, however, were a problem, since the dialysis tubing containing the urea solution tended to swell and burst under pressure. These samples were therefore concentrated by adding the required amount of dried lyphogel (a polyacrylamide hydrogel), and filtering after 5 hr. Concentration was also performed by forcing solutions through diaflo ultrafiltration membranes, either by hand syringe or under gas pressure (50 lbs/sq. in.).

3.6. Enzyme digests.

Enzymic digestion with trypsin, or chymotrypsin was carried out using the insoluble form of the enzymes bound to

Sephadex (Pharmacia Ltd.), 1.0ml of slurry contains the equivalent of 0.2 mg enzyme units, for trypsin and 0.1 mg for chymotrypsin. Experiments were performed in NH_4HCO_3 0.1M, pH 8.6 at 27°C, for 4 hr and 16 hr. Protein/enzyme ratios were 1 : 100 a second addition being made after 4 hr to the overnight samples, i.e. final ratios were 1 : 50 in these experiments. After incubation the enzyme was removed by filtering through cotton wool, the digested material was then either submitted to gel filtration, or concentrated and dialysed for electrophoresis.

3.7. Composition of apo-units.

Amino acid analyses were kindly performed by Dr. R. Daumas at the Station marine d'Endoume, Marseilles, on a Beckman amino acid analyser, according to the method of Spackman et al., (1958). Samples (0.2 - 1.0 mg) were hydrolysed in constant boiling HCl in vacuo for 24 hr at 105°C. Determinations of cysteine and cystine were performed on samples of the protein that had been oxidised by performic acid (Hirs, 1956). Errors were in the range $\pm$ 0.2 - 0.3 % residue weight.

The amino acid content is expressed in two ways:

1) As g anhydro amino acid per 100 g of protein (% residue weight).

2) As number of residues per 100 residues (moles %).

Expression of the composition in terms of mole % allows a more meaningful comparison between proteins, since the number of residues contained in 100g of protein will depend on the particular residues involved. The individual apo-units were compared to the 'average protein' by expressing the occurrence of each amino acid in terms of the standard deviation of that amino acid calculated for the average protein (Smith, 1966). In this manner it can be easily seen which amino acids are present in amounts differing from 'average' by more or less than one standard deviation. In calculating the standard deviations for the amino acids in the 'average protein', Smith considered 80 proteins, and it is probable that as this list is increased the standard deviation for the occurrence of each amino acid will also increase. This will have the effect of reducing the numbers of amino acids apparently occurring in abnormally large or small amounts. Typical proteins had 13 - 15 amino acids within one standard deviation of the mean.

Values for the average hydrophobicity (H) were calculated from the amino acid analyses (Bigelow, 1967). The number of residues of each amino acid present being multiplied by the free energy obtained from transferring one mole of the amino acid from aequeous solution to ethanolic solution taken from Tanford, (1962) $F_e = -RT\ln N_{EtOH}/N_{HOH}$ where F_e = free energy

change, R = gas constant, T = temperature, N_{EtOH} & N_{HOH} are solubility of the amino acid in ethanol and water respectively). The sum of the values obtained divided by the total number of residues gives the average hydrophobicity (H). This figure can range from 440 - 2020 cal/residue and is an indication of the stabilisation energy available to the protein through hydrophobic interactions. Most globular proteins have H values in the region of 980 - 1200.

A maximum value for the fractional charge has also been calculated (Bigelow, 1967; Welscher, 1969), from the ratio of the sum of amounts of glutamic and aspartic acids; histidine, lysine and arginine to the total number of amino acid residues, assuming zero amidation of acidic residues. The total number of residues for each apo-unit was estimated using the molecular weights derived from gel-filtration studies.

An estimate of the helical content was obtained from the amounts of serine, threonine and proline, using data provided by Havsteen, (1966). He suggested that there is an inversely proportional relationship between the amounts of these amino acids and $-b_o$ (from the Moffit & Yang equation). $-b_o$ can be related to the helical content by $-b_o$ = (% helix) x 630 (Urnes & Doty, 1961). This relationship holds in proteins where no other ordered structures, e.g. the β-form, make a significant contribution to b_o.

Relatedness among the apo-units was tested for using the $S\Delta Q$ method of Marchalonis & Weltman, (1971). $S\Delta Q = \sum (X_{ij} - X_{kj})^2$ where subscripts i and k refer to different proteins and j to a particular amino acid, X being in residues /100 residues. In their study of over 100 proteins these workers found that 98% of unrelated proteins had $S\Delta Q$ values greater than 100, while most related proteins had values of less than 50 although these figures may be expted to converge as the test samples increase.

The partial specific volumes of the proteins were also calculated from the amino acid content, assuming an additive relationship (Schachman, 1957). $W_i V_i / W_i = V_p$ where W_i = % by weight of the amino acid V_i is specific volume of the residue, V_p is the partial specific volume of the protein.

4 EXPERIMENTAL

4.1 Preparation of α-crustacyanin

4.1.1 Extraction procedure

The extraction procedure employed, was a modification of that used by Zagalsky & Cheesman (1963). Since large quantities of protein were required, and the supply of lobsters was unlimited, grinding the shell in a coffee grinder became impracticable, and a rotary ball mill was used (capacity 5 l). This ground the shell efficiently, but unfortunately could not be used dry as the finely ground shell became packed into 'cement'. The shell (500 g) was therefore ground in the cold in 4 l of 0.3M-boric acid brought to pH 6.8 by the addition of solid tris. The presence of a buffer was necessary in order to prevent elevation of the pH by strongly basic substances liberated during the grinding, (when ground in distilled water the pH rose above 11). The borate buffer also had the advantage over phosphate buffer in that it did not react with compounds in the shell (possibly calcium salts), and consequently did not solubilise the coloured proteins. This step acted as an initial purification since low molecular weight, ultraviolet absorbing, contaminants were brought into solution.

The ground shell was then filtered and incubated with 10% EDTA, pH 7.5, as previously described (Jencks & Buten, 1964; Cheesman et al., 1966). The extracted protein was precipitated

from the solution by the addition of 350 g/l of $(NH_4)_2SO_4$ (50% saturation). The shell was reground and the procedure repeated until most of the blue colour had been extracted. The pooled ammonium sulphate precipitates were centrifuged, dissolved in water and dialysed against 0.2M-phosphate buffer, pH 7.0, to remove the more strongly adsorbed impurities. This method was found to be a simple, more efficient preliminary purification than adsorption onto calcium phosphate gel (Zagalsky & Cheesman, 1963), as the initial borate extraction procedure removed most of the low molecular weight contaminants.

The crude protein which came straight through the DEAE-cellulose column was concentrated, and further purified by the following methods.

4.1.2. Gradient elution from DEAE-cellulose

Gradient elution from DEAE-cellulose was performed as described in section 3.2.1. Columns of 1.8 x 40 cm and samples of protein containing no more than 500 mg were used. Gradients were of the logarithmic type $c_1-c/c_1-c_2 = e^{-v/v_2}$ where v_2 = 1 litre, c_1 = 0.005M-(P), c_2 = 0.05M-(P). After 400 ml c_2 was changed to 0.25, or 0.5M-(P). A typical spectrum is shown in fig. 2, and spectral values for the individual peaks in Table 3.

4.1.3 Electrophoresis on the Porath column

Electrophoresis on the L.K.B. Porath column was

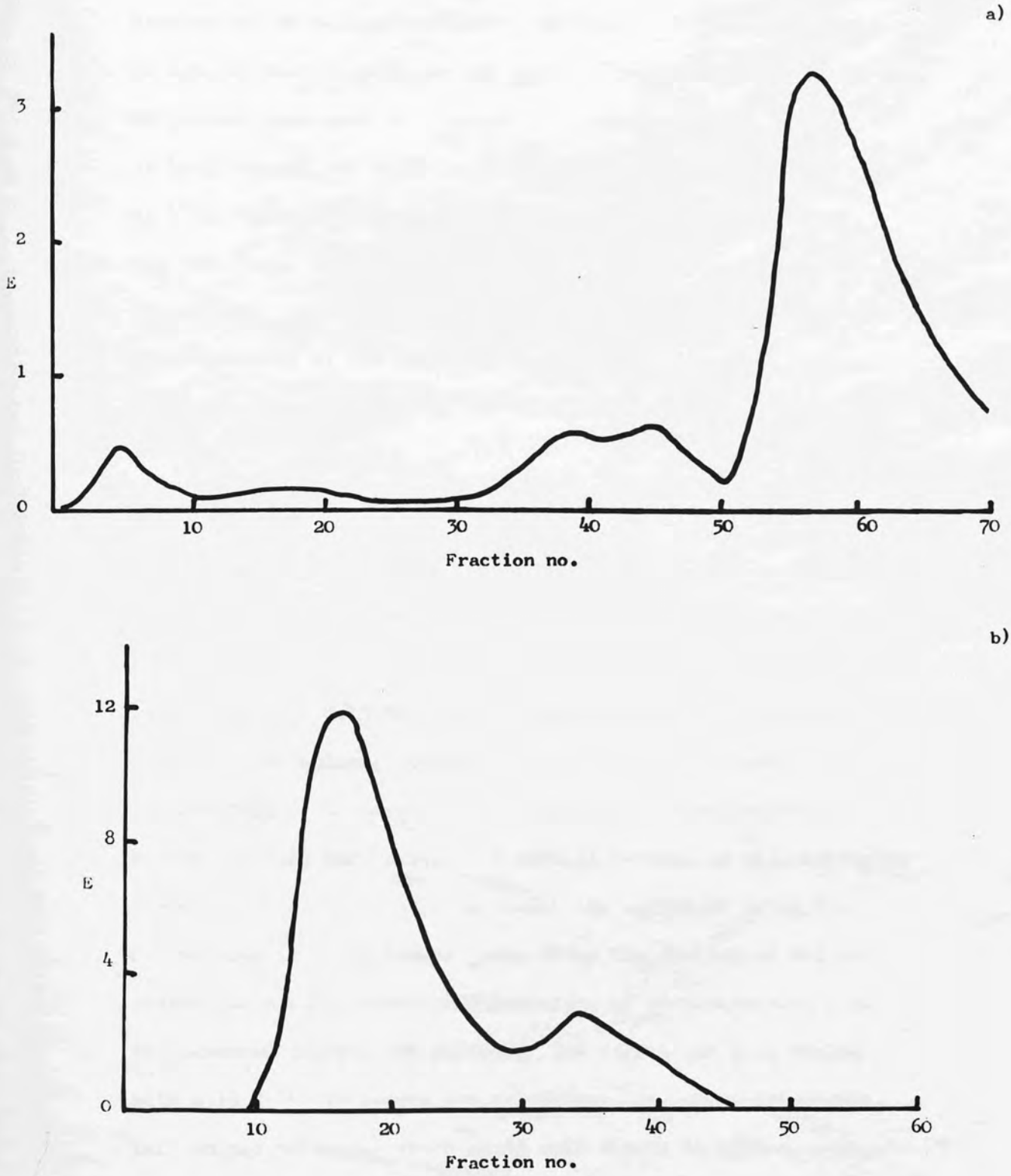


Fig 2. Purification of α-crustacyanin.

a) Purification of α-crustacyanin by gradient elution from DEAE-cellulose. Buffer - phosphate pH 7.0, 7ml/hr. Fraction vol = 5ml

b) Purification of α-crustacyanin by column electrophoresis. Buffer - tris/borate pH 8.3, 200v, 50ma, 36 hr. Fraction vol = 3ml.

carried out in 0.1M-tris-maleate, pH 7.2, or 0.1M-tris-borate, pH 8.3, as described in section 3.3.4. Samples containing 150 mg protein were used in a volume not exceeding 15 ml, and run at 40-50 ma and 200 v for 36-48 hr, the cooling water being kept at 4°C. The column was eluted in the same buffer at 7 ml/hr the rate being controlled by a peristaltic pump at the top of the column. As it took some time to settle to this rate of flow, some spreading of the bands was inevitable. A typical elution pattern is given in fig. 2, and tabulated in Table 3.

4.1.4 Stepwise elution from DEAE-cellulose

Stepwise elution from DEAE-cellulose was employed for large amounts of material (see section 3.2.1). Columns were used so that in 0.15 M-(P) pH6.5 the α-crustacyanin saturated 50-70% of the column. Columns of 3.5 x 30 cm can comfortably support 0.5g of protein. Samples were applied in 0.05M-(P) buffer, pH 7.0, and washed with several volumes of this buffer to remove any β-crustacyanin. Although the cellulose below the α-crustacyanin layer became paler after the elution of the γ-crustacyanin band, continuous formation of β-crustacyanin from the adsorbed protein was evident. The column was then washed with 0.15 M-(P) to remove the γ-crustacyanin. Some colour was left on the cellulose which could only slowly be eluted, even in 0.5 M-(P), but the majority could be brought into solution if let overnight in 0.5 M-(P). Losses of this sort were possibly

up to 20%, and increased with the amount of time the protein was adsorbed onto the DEAE-cellulose. Typical results are given in Table 3.

TABLE 3

Comparative results of the three purification procedures :

α		β		γ		methods
λ_{max}	E^{280}/λ_{max}	λ_{max}	E^{280}/λ_{max}	λ_{max}	E^{280}/λ_{max}	
632	0.32	595	0.50	625	0.32	Porath column
628	0.39	690	0.54	605	0.52	gradient elution
632	0.34	580	0.90	612	0.45	stepwise elution

4.1.5. Comparison of the different procedures

Preparations varied greatly in the relative proportions of the different forms of the crustacyanins. The largest amounts of α-crustacyanin were found in preparations using the tail segments and legs only, and the smallest, using sections of shell from the abdomen. These often appeared brownish-red, especially in some of the lobsters which had died some time before freezing. Degradative enzymes would be present in these preparations, and may have helped to disproportionate the ratio in favour of the β- and γ-forms.

From the data in Table 3 it would appear that the α-crustacyanin prepared by column electrophoresis is the most pure, but the values listed for this method, as with that of gradient elution are those of the pooled peak tubes, whereas those for the stepwise method are those of the whole fraction. Stepwise elution was found to be the most reliable method of initial purification and gave the best recovery, especially for large amounts of material, and was routinely employed.

The α-crustacyanin was usually stored in this state of purity under 50% $(NH_4)_2SO_4$ and further purified either by column electrophoresis or gradient elution, before use. Since the order of elution of the three proteins is different in the two methods, being α-,β-,γ- on the Porath column and β-,γ-,α- on DEAE-cellulose, the choice of method was dictated by the particular contaminant present.

4.1.6 Homogeneity of preparation

α-crustacyanin, prepared as outlined above, had similar characteristics to that previously recorded in the literature i.e. λ_{max} 632 nm, $E^{280/632}$ 0.30 and moved as a single band on cellogel electrophoresis at pH 7.0 and pH 8.35, although at pH 8.35 especially on overnight runs in starch gels, it was often contaminated with some β-crustacyanin, presumably formed as

a result of the high pH.

The protein was tested for homogeneity by comparing samples taken from the leading and trailing sections of the α-crustacyanin band from (a) Sephadex G-200, and (b) DEA-cellulose gradient elution in phosphate buffer pH 7.0, and (c) column electrophoresis in tris-maleate buffer pH 7.2. The results are shown in fig. 3. These samples were applied to starch gels, pH 7.0 and 6 M-urea-starch gels, pH 8.35. The gels were stained with amido-schwarz and cleared as described in section 3.3.1. The urea-gels were scanned and the proportion of each sub-unit estimated by integration of the area under each peak. No separation of α-crustacyanin into proteins of different mobility was observed, and their sub-unit composition was identical.

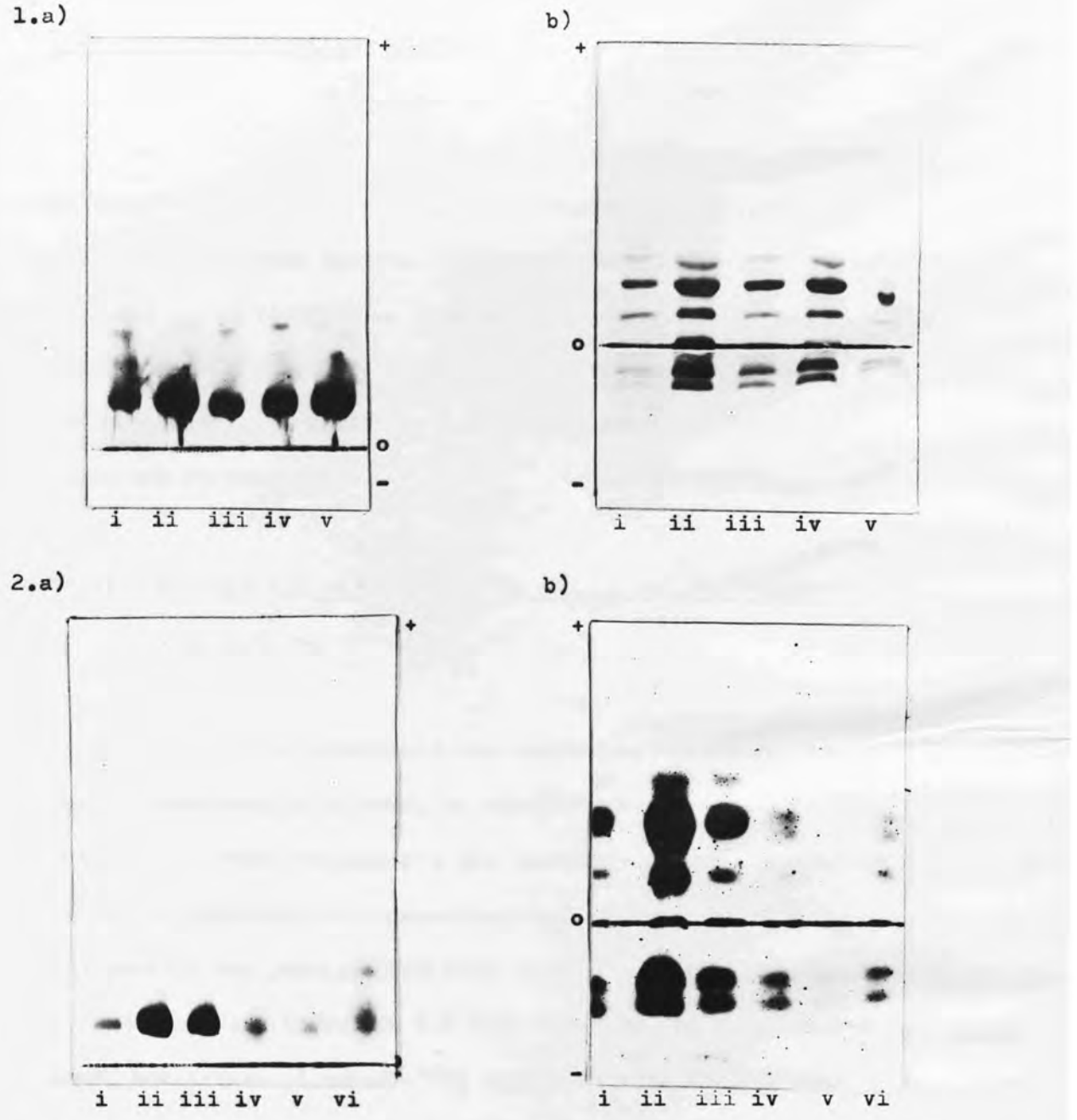


Fig 3. Homogeneity of α-crustacyanin preparations.

1. Electrophoresis on a) starch gel, and b) 6M-urea-starch gel of 6 fractions (i-leading, to vi-trailing) of α-crustacyanin obtained by electrophoresis on a Porath column.
0.04M-borate buffer, pH 8.35, 10ma/gel, 300v, 4hr, with pet-ether cooling.

2. Electrophoresis on a)starch gel, and b) 6M-urea-starch gel of i-leading, ii-middle, & iii-trailing fractions of α-crustacyanin from gradient elution on DEAE-cellulose, and, iv-leading, v-middle, & vi-trailing fractions of α-crustacyanin from gel filtration on Sephadex G.200.
0.04M-borate buffer, pH 8.35, 3ma/gel, 110v, 16hr.

4.2. Preparation of apo-units.

A heterogeneous mixture of sub-units is obtained on incubation of α-crustacyanin in 6 M-urea, or on removal of the carotenoid with acetone. Electrophoresis of this mixture normally gives five bands, 3 anionic and two cationic at pH 8.35. (Cheesman et al., 1966 and fig. 3.1b). Separation of these five sub-units can be achieved by a combination of chromatography on DEAE- and CM-celluloses.

4.2.1. Preparation of apo-units by removal of the carotenoid with acetone.

The carotenoid was removed by treatment with acetone made 0.05 M in KCNS, as described by Cheesman et al., (1966). In order to preserve the integrity of the apo-protein as far as possible, all operations were carried out at 0°C, and the protein was precipitated with acetone only three times. 0.02 M phosphate buffer pH 6.8 was also used, in place of the KCNS, but traces of apo-protein were lost into the solvent. The precipitated protein was centrifuged down, and dissolved in 0.005 M-phosphate buffer, and the acetone was removed, either by dialysis against the same buffer, or by gel filtration on Sephadex G. 25, previously equilibrated with 0.005 M-phosphate buffer. In later experiments dialysis was used although taking longer since, if present in large amounts, acetone altered the characteristics of the Sephadex and caused irreversible adsorption of the protein. The apo-protein was concentrated by ultrafiltration before chromatography.

Chromatography on DEAE-Sephadex and DEAE-cellulose was attempted. The cellulose exchanger was preferred as the Sephadex gave very low flow rates in the low ionic strength buffers required, and therefore incurred greater losses by irreversible adsorption. Both tris, and phosphate buffers were tried initially for the elution of the protein. Phosphate buffers were found to give better separations and were subsequently used. The gradients employed were of two types:

a) linear from 0.005 M to 0.07M in a total effluent volume of 1 litre, and then to 0.25 M in another litre

b) logarithmic, starting with 1 litre of 0.005 M buffer in the mixer and 500 ml of 0.1 M and then 0.3M in the reservoir. (see section 3.2.1).

Similar results were obtained using both methods. Four peaks were eluted, as shown in fig. 4a), in the approximate ratio i : ii : iii : iv = 4 : 2 : 4 : 1.5. This ratio varied slightly depending on the length of the column and the age of the preparation. When the protein from each peak was pooled and concentrated, and submitted to starch gel electrophoresis in borate buffers pH 8.35 (section 3.3.1) the results were as shown in fig.4b) The first peak to be eluted (i) which was unadsorbed by the DEAE-cellulose in 0.005 M phosphate buffer, consisted of the

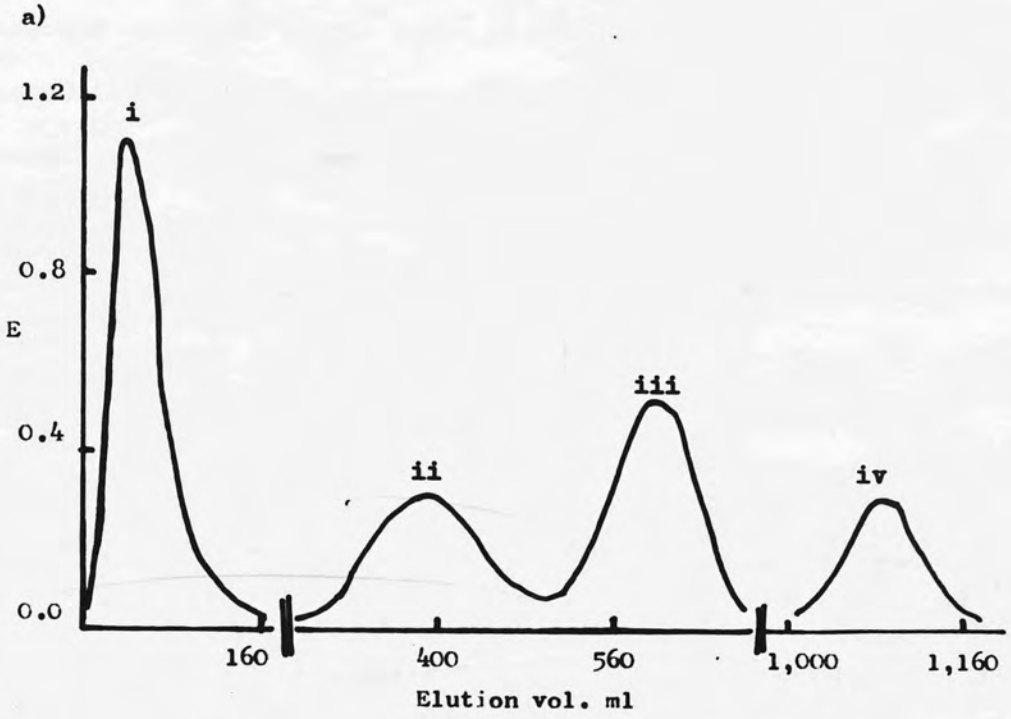


Fig 4. Separation of apo-units 1.

a) Separation of apo-units by gradient elution from DEAE-cellulose after treatment of α-crustacyanin with acetone.

b) Electrophoresis of fractions obtained in a) on starch gel. 3 ma, 110v, 16hr.

two cathodic fractions, C_1 & C_2. The other peaks (ii), (iii), (iv) were eluted by approximately 0.015 M, 0.03 M and 0.05 M phosphate buffer respectively. These were electrophoretically pure and consisted of the three anodic sub-units A_1, A_2 and A_3 respectively. In some preparations the A_2 fraction split into two components. These have been partially separated by gradient elution from DEAE-cellulose using the normal gradient for separation of apo-units as outlined above, and are assumed to be conformational isomers, perhaps formed in preparation of α-crustacyanin on ageing. (fig 3 2b).

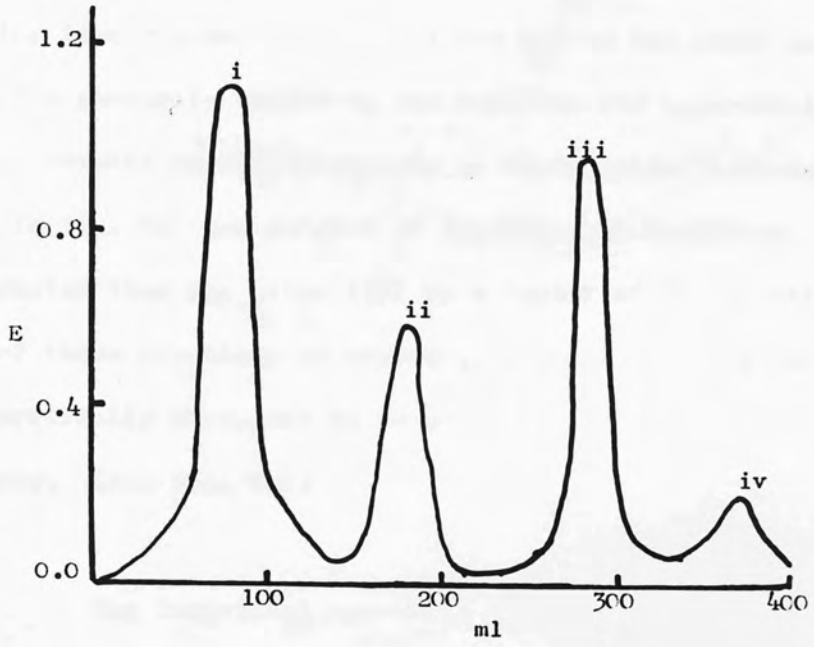
4.2.2. Preparation of apo-units by treatment with 6 M-urea.

The sub-units have also been prepared by incubation of α-crustacyanin with 6 M-urea. These can be separated on DEAE-cellulose with the same buffer gradients as above (4.2.1), except that 6 M-urea must be included in all buffers so that the protein is kept in solution. The astaxanthin is adsorbed at the top of the column. Four peaks are eluted, which, when subjected to electrophoresis in starch-6M-urea gels, appeared to consist of the same apo-units prepared by the removal of the carotenoid with acetone. The results are shown in fig 5.

4.2.3. Separation of the cationic apo-units.

The first fraction eluted from DEAE-cellulose was concentrated, dialysed against water and applied to CM-

a)



b)

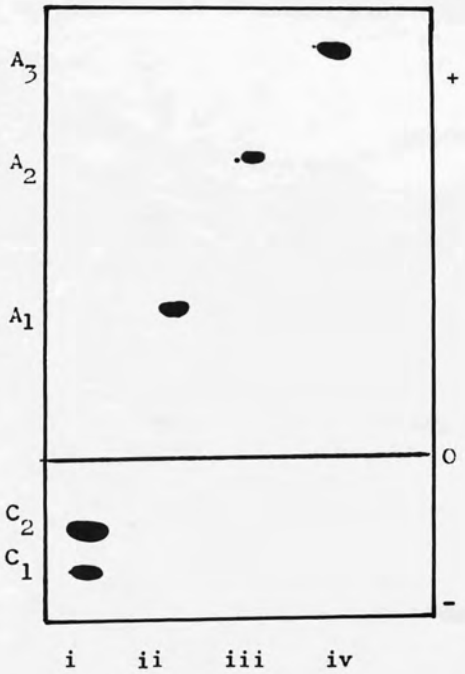


Fig 5. Separation of apo-units 2.

a) Separation of apo-units by gradient elution from DEAE-cellulose after treatment of α-crustacyanin with 6M-urea.

b) Electrophoresis of fractions obtained in 'a') on urea starch gel. 2ma, 130v, 17hr.

cellulose column. A fairly steep gradient was employed - 500 ml H_2O in mixer and 500 ml 0.07 M phosphate buffer in the reservoir for linear gradients, or 1 litre H_2O in the mixer and 500 ml 0.15 M phosphate buffer in the reservoir for logarithmic gradients. Results of chromatography on CM-cellulose columns are shown in fig. 6a and consist of two separate fractions, one (i) greater than the other (ii) by a factor of 3. Electrophoresis of these fractions on starch gels showed them to be electrophoretically pure, and to be composed of C_1 & C_2 respectively. (see fig. 6b).

The individual apo-units have been named C_2, C_1, A_1, A_2 & A_3, as indicated in the following diagram:

A_3 Anode

A_2

A_1

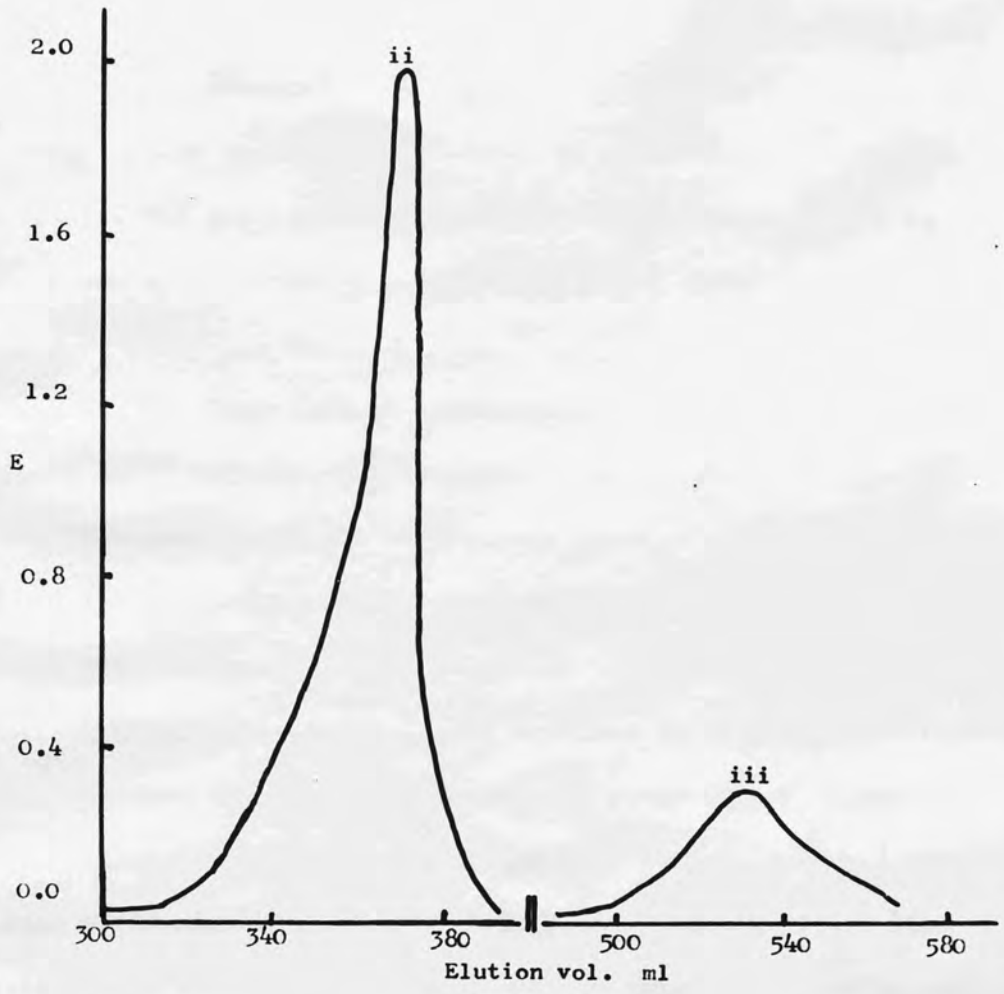
——— Origin

C_1

C_2 Cathode

The sub-units present in greatest amounts are C_1 & A_2 and are referred to as the major apo-units.

a)



b)

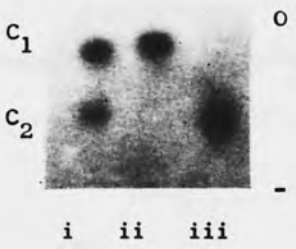


Fig 6. Separation of cathodic sub-units.

a) Separation of apo-unit fraction not adsorbed onto DEAE-cellulose by gradient elution from CM-cellulose.

b) Starch gel electrophoresis of fractions obtained in 'a'), plus
i = sample before separation.
3 ma, 110v, 16hr.

4.2.4. Preparation of apo-units by alterations in pH.

Attempts were also made to disrupt the carotenoid-protein linkage by altering the pH. On the addition of acid or alkali, the absorption maximum of α-crustacyanin alters to 480 or 400 nm respectively, (Jencks & Buten, 1964).

Solutions of α-crustacyanin (10 mg in 3 ml), were brought to pH 3.5 with 0.2 M-acetic acid, and kept at 30°C for 1 hr, (a milder treatment of dialysis against 0.2 M-acetate buffer pH 3.5 in the cold, overnight, resulted in a purple product - mainly β-crustacyanin). The solutions turned a brownish red λ_{max} 440 nm, but the carotenoid remained in solution, presumably still attached to the protein. In this state the astaxanthin was only partially removed by adsorption onto DEAE-cellulose (normally a good astaxanthin adsorbant), $CaCO_3$, or supercel. On neutralisation, the effluent from these columns regained a blue colour, with a broad adsorption maxima from 600 - 635 nm. A yellow band which remained attached to the top of a column of supercel was eluted with 0.1 M-NaoH and had a λ_{max} of 400 nm. When concentrated and electrophoresed on starch gels this band stained only faintly with amido-schwarz.

In order to raise the pH. 0.1 N-NaOH was added slowly to solutions of α-crustacyanin (10 mg/3 ml), until the pH reached 10. In some experiments the pH was taken to 10 by the addition of solid tris. After 2 hr at 37°C the λ_{max} of the solutions

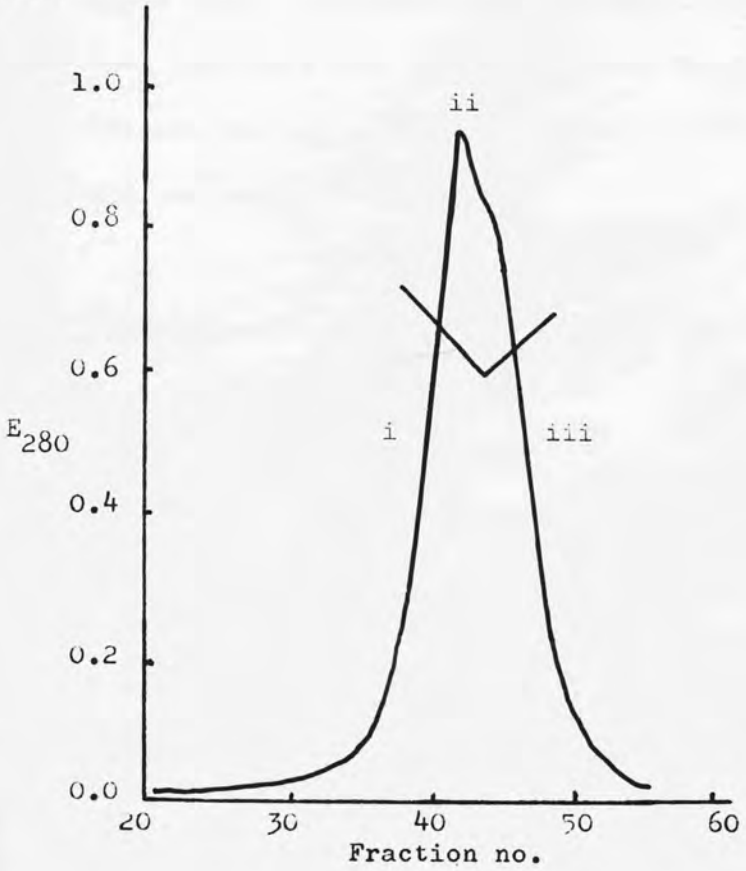
was 400 nm but with a shoulder at 580 nm. The carotenoid was not adsorbed by $CaCO_3$, alumina or supercel. On neutralisation, concentration and analysis in urea-starch gels, it was apparent that the five apo-units had all been formed.

4.2.5. Gel filtration studies on the apo-units.

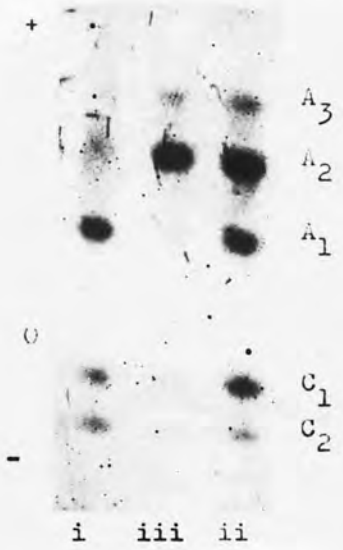
The molecular weight estimation for the apo-fraction as estimated by gel filtration on Sephadex G.75, is similar to that found by sedimentation and diffusion of 27,000 (Cheesman & Zagalsky, 1966; Jencks & Buchwald, 1968b). The band eluted from Sephadex G.75, however, if divided into three fractions, is found to partially fractionate the apo-units. When concentrated and subjected to analysis on starch gels, the leading section is found to be composed mainly of apo-units C_1, C_2 & A_1, and the trailing section contains mainly A_2 & A_3, as shown in fig. 7a.

The apo-units were therefore subjected to gel filtration separately in order to achieve an estimation of the individual molecular weights. The results, given in fig. 7b, are the average of 3 estimations. Each sample was run with

1.a)



b)



2.

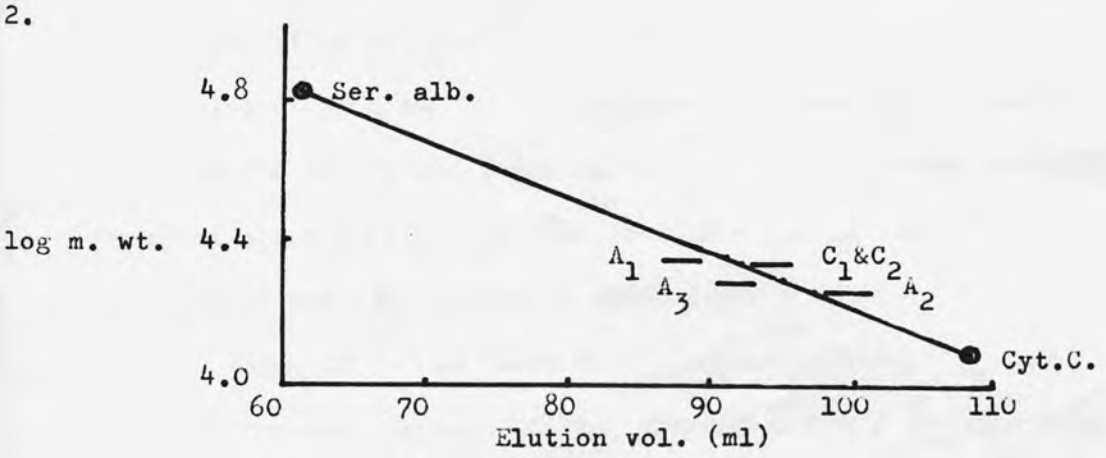


Fig 7. Gel filtration studies on apo-units using Sephadex G.75.

1.a) Crude fractionation of the apo-units. 0.1M-phosphate buffer pH 6.8, fraction vol. 2.5ml, bed vol. 1.8 x 98cc, flow rate 7ml/hr.

b) Samples, numbered as indicated in 'a', subjected to urea-starch gel electrophoresis. 0.04M-borate buffer pH 8.35, 3ma. 110v. 16hr.

2. Gel filtration estimate of molecular sizes of apo-units. 0.1M-phosphate buffer pH 6.8, fraction vol, 2.5ml, bed vol. 1.8 x90cc, flow rate 7ml/hr. Standards: cytochrome C. and serum albumin

serum albumin as standards. Estimates of molecular sizes have been made from the plot of log. mol. wt. against elution volume, and are given below; they are the averages of five estimations -

apo-unit	C_1	C_2	A_1	A_2	A_3
mol. wt.	22,015 ± 800	22,015 ± 800	22,750 ± 800	18,215 ± 800	19,820 ± 800

4.2.6. Amino acid analyses of the apo-units

Amino acid analyses of the individual apo-units were performed as described in section 3.7. The results are listed in Table 4, in terms of a) g amino acid per 100 g of protein and b) residues per 100 residues (cf. section 4.2.5). The compositions of C_1 & A_2 are graphically compared in fig. 8, the ordinate being the amount of amino acid in moles %, and the abscissa the individual amino acids, from left to right in descending order of occurrence in A_2. The 'relatedness' of the sub-units is found by the $S\Delta Q$ method of Marchalonis & Weltman, (1971) (cf. section 3.7.). In Table 6, the composition of the apo-units has been compared to that of the 'average protein' (Smith, 1966) in terms of the standard deviation of their amino acids (section 3.7). Proteins are coded e.g. $10^{2,1,5}_{1,2}$ where 10 refers to the number of amino acids occurring within one standard deviation of the mean. The superscripts refer to the number of amino acids occurring with a frequency greater than the mean plus one standard deviation,

TABLE 4

Amino acid composition of the sub-units.

	C_2		C_1		A_1		A_2		A_3	
	a	b	a	b	a	b	a	b	a	b
arg	4.8	3.5	4.6	3.4	4.0	3.0	4.3	3.2	4.2	3.1
lys	8.8	7.7	8.0	7.1	7.1	6.3	6.3	5.6	5.4	4.8
his	1.7	1.4	1.2	1.0	1.3	1.1	2.4	2.0	2.2	1.8
asp	14.1	13.5	15.2	14.7	16.3	15.9	12.4	12.0	12.9	12.5
glu	10.6	9.2	10.9	9.6	10.0	9.2	10.6	9.6	11.7	10.3
thr	4.2	4.5	4.8	5.2	5.1	5.5	7.9	8.6	7.3	7.8
ser	6.5	7.9	6.9	8.5	5.8	7.2	6.4	7.9	6.1	7.5
pro	4.4	4.9	4.6	5.2	4.8	5.4	5.0	5.6	5.4	6.0
gly	3.8	6.3	3.6	6.2	3.2	5.5	2.9	5.0	3.4	5.8
ala	6.3	9.0	5.3	7.7	4.9	7.2	6.9	10.0	7.5	10.9
val	4.5	4.9	5.3	5.9	4.7	5.2	6.9	7.6	7.0	7.7
ile	4.9	4.8	4.2	4.2	4.4	4.4	3.6	3.6	3.5	3.4
leu	7.1	6.9	6.5	6.4	6.8	6.8	2.5	2.5	2.5	2.5
cys	2.7	2.2	1.8	1.9	1.7	1.8	1.8	1.9	1.8	1.9
met	0.6	0.5	0.2	0.2	tr	tr	0.5	0.4	0.7	0.8
tyr	7.8	5.5	9.2	6.6	11.2	8.1	9.9	7.1	9.0	6.4
phe	8.3	6.4	7.9	6.2	8.3	6.5	8.7	6.8	8.9	6.9

tryptophan was not determined

a. g amino acid per 100 g protein

b. residues per 100 residues

figures are an average of two preparations

TABLE 5

Relatedness of the apo-units

	C_2	C_1	A_1	A_2	A_3
C_2	0				
C_1	8	0			
A_1	9	23	0		
A_2	57	46	57	0	
A_3	52	50	64	4	0

figures are SΔQ values (Marchalonis & Weltman, 1971 & section 3.7).

TABLE 6

Comparison of the amino acid composition of the apo-units to the 'average protein' (Smith, 1966)

	-2	-1	0	+1	+2	+3	Code
C_2	met	leu, val, gly, thr, glu, his, arg	cys, ile, ala, pro, ser, lys	phe, tyr, asp			13^{3}_{1}
C_1		leu, val, gly, his, met, arg	cys, ile, ala, pro, ser, thr, glu, lys	phe, tyr, asp			13^{3}_{1}
A_1		leu, val, ala, gly, glu, his, met, arg	cys, ile, pro, ser, thr, lys	phe, tyr, asp			13^{-3}_{1}
A_2		ile, gly, glu, met, leu, arg	cys, val, ala, pro, ser, lys, asp, his	tyr, thr	phe		$12^{2,1}_{2}$
A_3		met, ile, gly, lys, leu, arg	cys, val, ala, pro, ser, glu, his	tyr, thr, asp	phe		$13^{3,1}_{1}$

Histograms of amino acid composition, numbers refer to standard deviations. For further details see text. (Smith, 1966).

TABLE 7

	C_2	C_1	A_1	A_2	A_3
Total no. of residues	172	171	175	139	154
Mean residue weight	128	129	130	132	129
Average hydrophobicity (H)	1100	1100	1130	950	840
Fractional charge	0.35	0.36	0.36	0.32	0.32
% helix	25.3	23.8	24.5	19.5	20.6
Partial specific volume (v)	71.2	72.3	71.8	71.6	71.3

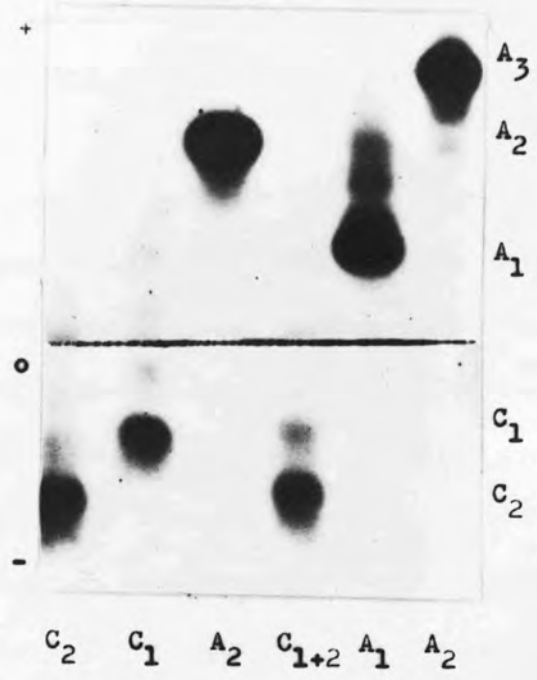
Fig. 8. Comparison of the amino acid composition of the two major apo-units

——— A_2 - - - - - - C_1 Numbers are in moles %.

e.g. 2 between 1 & 2 standard deviations, 1 between 2 & 3 standard deviations and 5 between 3 & 4. The subscripts are similar but refer to numbers of amino acids occurring with a frequency of a certain number of standard deviations less than the mean. (Smith, 1956 and section 3.7). Table 7, shows the total number of amino acids in each sub-unit, calculated using the molecular weight derived from gel filtration (section 4.2.5) and the average residue weight. Values for the helical content, calculated from the serine, threonine and proline content (Havsteen, 1966 and section 3.7), the average hydrophobicity and fractional charge (Bigelow, 1967), and partial specific volume (Schachman, 1957) as described in section 3,7, are given also.

The apo-units were also subjected to electrophoresis in 6M-urea-starch gels containing 0.05M-mercaptoethanol. The tris-EDTA-borate discontinuous system, pH 8.35 gave the best results of those buffers tested (section 3.3.1). Good separations were obtained at this pH, although the apo-units moved with less mobility than in 6M-urea-starch gels containing no mercaptoethanol, the C_1 & C_2 fractions also tended to precipitate out into the gel slots. Formate buffers were therefore tried, as no precipitation occurs at pH 3.5, (Poulik, 1960), but the apo-units are not sufficiently separated at this pH. The results, shown in fig. 9, show the separations obtained in a) a 6M-urea-starch gel and b) a 6M-urea-starch gel containing 0.05M-mercaptoethanol. The apo-units remain as unique entities under the above conditions, and have no tendency either to isomerise or to split into smaller sub-units.

a)



b)

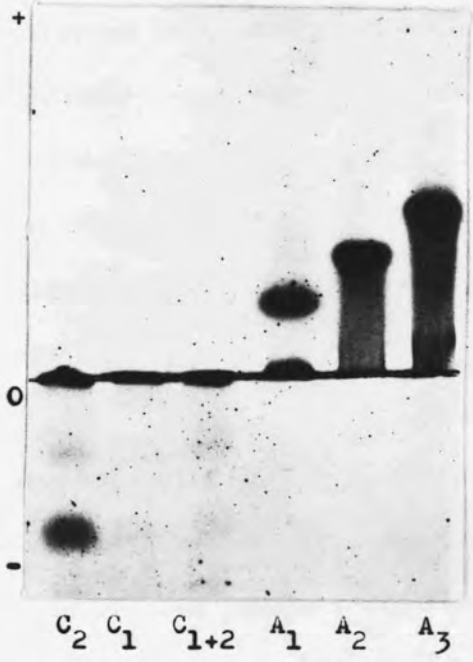


Fig 9. Electrophoresis of Apo-units.

a) 6M-urea-starch gel. Tris-EDTA-borate discontinuous buffer pH 8.35, 110v. 3ma. 17hr.

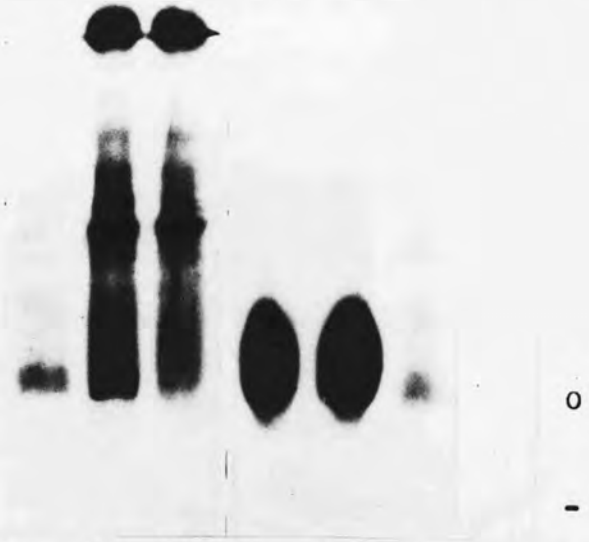
b) 6M-urea-starch gel containing 0.02M-mercaptoethanol. Apo-units incubated with 0.005M-dithiothreitol for 4hr before electrophoresis. Tris-EDTA-borate discontinuous buffer pH 8.35, 110v. 3ma. 17hr.

4.3.7 Tests for relationship between the apo-units by immunological techniques.

Antigens to the major apo-units (C_1 & A_2) were prepared from guinea pigs as described in section 3.4. The serum from three animals (100 ml) was pooled and the γ-globulin fractions were purified on DEAE-cellulose (section 3.4) and contentrated to 2 ml. Electrophoresis of the serum before and after purification of the γ-globulin fraction is shown in fig. 10a. Two extractions with DEAE-cellulose can be seen to remove all other fractions. Very little γ-globulin is lost during this purification as washing the DEAE-cellulose with more buffer does not recoup much. No precipitin lines could be observed in simple diffusion experiments in agar gels when the antibodies were tested against pure apo-units at various concentrations. Serum prepared against B.S.A. gave clear precipitin lines when tested with B.S.A. Diffusion experiments were also attempted in cellogel strips, as this is a more sensitive method, but no precipitation could be detected.

The molecular weight of the apo-units has been estimated to be approximately 20,000, which may be too small to form precipitin lines, even though a soluble antigen-antibody complex may be formed. In order to determine the formation of such complexes the apo-units and γ-globulin fractions both separately and mixed, were subjected to electrophoresis in cellogel strips, using veronal buffer pH 7.6. If any reaction were to take place between the antigen and the γ-globulin, the mixture should have a band which migrates

a)



b)

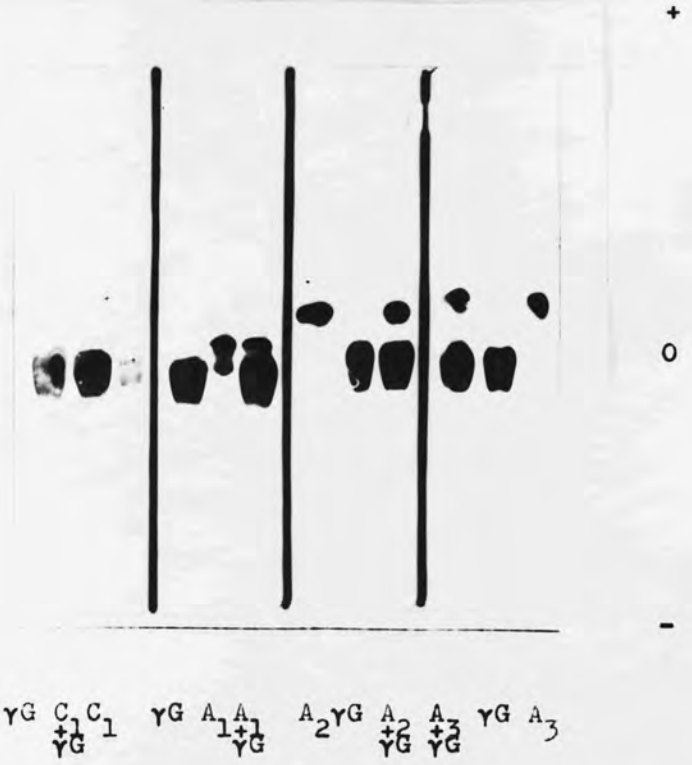


Fig 10. Immunoelectrophoresis in cellogel strips.

a) Electrophoresis of whole serum and purified γ-globulin fraction. ii & iii whole serum, vi γG fraction, initial purification, v γG 2nd purification, i & vi DEAE washings. 0.05I-veronal buffer pH 8.0, 0.8ma/cm. 13v/cm. 1.5hr.

b) Electrophoresis of γ-globulin fraction and individual apo-units.

with a different mobility to the individual components. This was not found to be the case with the anodic sub-units, as shown in fig. 10 b). A decision on the ability of the cathodic sub-units to complex with antibody was more difficult to assess, as they migrate with the same mobility as the γ-globulin fraction at this pH, and the latter being such a diffuse band might obscure any newly formed bands. Alteration of the pH of the experiment was not feasable as reaction between the antigen and antibody normally only occurs between pH 7.0 & 8.0.

4.3. Recombination experiments.

4.3.1. Recombinations with astaxanthin.

Recombination of apo-protein with astaxanthin was attempted using the method of Lee & Zagalsky (1966). The good yields (90%) of α-crustacyanin reported by these workers were not, however, obtained. Most of the apo-protein did recombine (95%), but the majority (60 - 70%) was not adsorbed onto DEAE-cellulose in 0.05 M-phosphate buffer pH 7.0. Gel filtration on Sephadex G.75 showed the product to be mainly of β-crustacyanin size, with an absorption maximum of 590 nm. On electrophoresis in starch gels, the β-crustacyanin formed by this procedure appeared to contain all the normal components of this fraction (see section 4.4.). In an attempt to obtain greater yields of α-crustacyanin the procedure was varied in several ways :

Ice-cold α-crustacyanin (10 mg/ml) was precipitated with a 25 fold excess (v/v) of ice cold acetone, containing either 0.05M-KCNS or 0.05M-phosphate buffer pH 6.8 as described in section 4.2. After centrifugation, the protein was redissolved in the salt and reprecipitated with acetone solution. This precipitation procedure was repeated not more than twice to avoid denaturation, although approximately 10% of the carotenoid still remained attached to the protein. The acetone was removed from the final solution either by dialysis overnight, or by passage through a Sephadex G.25 column previously equilibrated

with 0.05M-(P). The residual α-crustacyanin was removed by adsorption onto DEAE-cellulose in 0.1, or 0.05M(P). The final volume at this stage was approximately 10 ml. Recombination experiments were carried out in the following manner :

Astaxanthin dissolved in acetone, containing either 0.05M-phosphate buffer pH 6.8 or 0.05M-KCNS (20 mg/ml) was added (1 ml) either to the protein solution obtained above, i.e. in 10 ml or, alternatively the protein was concentrated to 1 ml prior to the addition of the carotenoid. In the latter case the solution was immediately diluted to 10 ml with 0.05M-(P) and dialysed against the same buffer. Until all the acetone had been removed the dialysis was carried out at $0^{o}C$, after which the protein was dialysed either at $4^{o}C$ or at room temperature. The higher temperature might be expected to encourage the formation of hydrophobic bonds, which presumably hold the α-crustacyanin molecule together. None of these variations had any effect on the amount of α-crustacyanin formed, which remained in the region of 10 - 30% of the coloured product. Good yields 80 - 90% could, however be obtained if the 10% residual astaxanthin remaining after the apo-preparation was not removed by DEAE-cellulose chromatography prior to recombination.

4.3.2. Recombinations of apo-crustacyanin with synthetic carotenoids.

Although the ability of the apo-preparation to combine with astaxanthin was unaltered, only its ability to

polymerise to α-crustacyanin being diminished, it was thought to be suitable for recombination experiments with synthetic carotenoids. It is realised that if these carotenoids possess a limited ability to polymerise to α-crustacyanin sized molecules, this would possibly go undetected in these experiments. However in similar experiments using naturally occurring carotenoids, only astaxanthin was found to give rise to an aggregated product (Lee & Zagalsky, 1966).

Recombinations were initially performed with the carotenoids dissolved in acetone, as described for astaxanthin (section 4.3.1.). This was found to be unsatisfactory in many cases as several of the carotenoids were only sparingly soluble in this solvent. All were soluble in $CHCl_3$ and CH_2Cl_2, and the following variation was introduced using these two solvents.

The solvent ($CHCl_3$ or CH_2Cl_2) was first washed with NaCl to remove any free HCl, and dried over anhydrous Na_2SO_4. The carotenoids (0.1 - 2 mg) were dissolved in 1 ml of the solvent and spread onto filter paper which had been previously soaked in EDTA, washed with distilled water and dried. The solvent was completely evaporated under a stream of nitrogen, leaving the carotenoid spread in an even layer on the surface of the paper. The apo-protein solution was added (approximately 5 mg in 5 - 10 ml 0.05M-phosphate buffer

pH 7.0). The filter paper was mashed with a glass rod and the mixture allowed to stand in the cold, with intermitent shaking, for 24 - 48 hr. The coloured samples were then passed through $CaCO_3$-supercel (1/1,w/w) columns, equilibrated with 0.1M-(P), in order to remove free carotenoid. The solutions were then concentrated and applied to columns of Sephadex G.75. The effluent pattern obtained from these experiments is given in figs 11 & 12. The amount of apo-protein remaining gives an estimate of the degree of recombination that has occurred. The results of the recombination experiments are given more fully in Table 8.

There appeared to be no differences in the products produced by recombinations performed by the two methods for the majority of the carotenoids, except that yields were better for the $CHCl_3$ method, presumably due to increased availability of the carotenoid to the apo-protein. Astaxanthin, however, when tested by this method gave rise only to β-crustacyanin. On removal of the carotenoid with acetone and analysis by thin layer chromatography on alumina (section 3.2.2) the carotenoid appeared to be 90% astacene. Recombinations with hydroxyl containing carotenoids were therefore performed with the polyene dissolved in acetone.

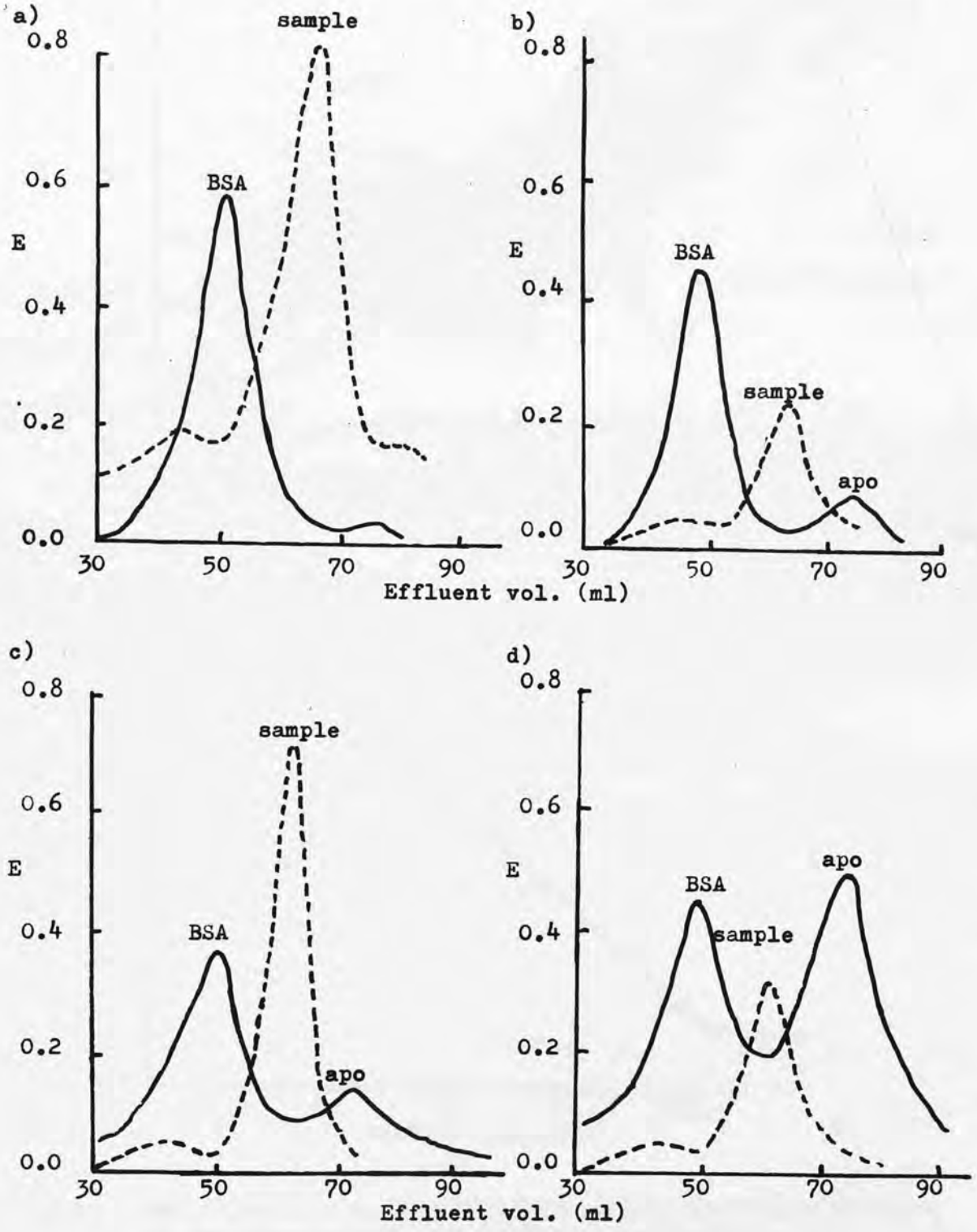


Fig 11. Gel filtration, on Sephadex G.75, of the recombined products of apo-crustacyanin and synthetic carotenoids.

All samples were run in 0.1M-phosphate buffer, pH 6.8 with bovine serum albumin (BSA), in a total vol of 1ml. Fraction vol. 2.5ml, bed vol. 1.8x80cc, flow rate 7ml/hr. — 280nm --- 580nm.
a) astaxanthin b) astacene c) canthaxanthin d) astaxanthin methyl ether.

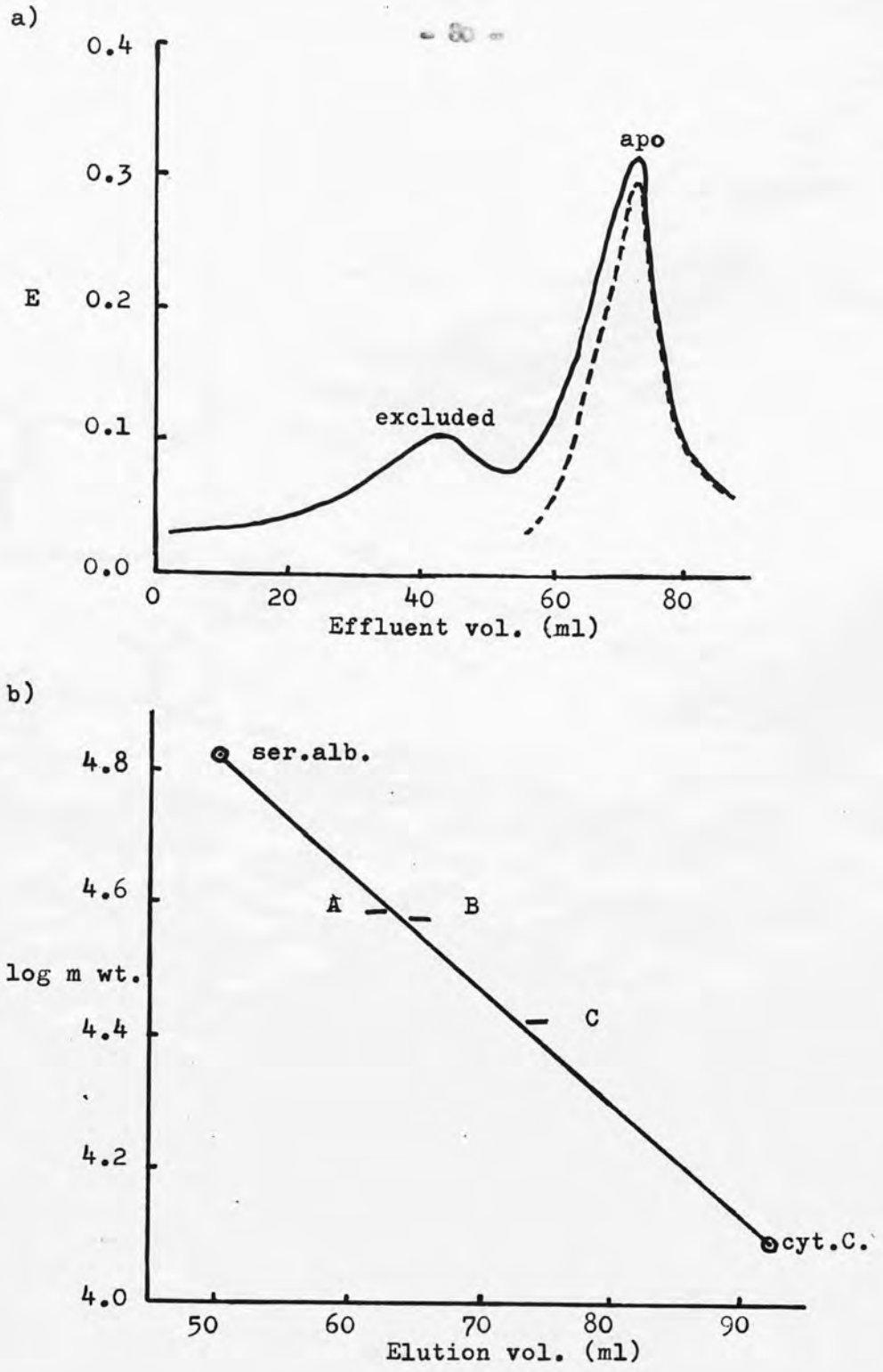


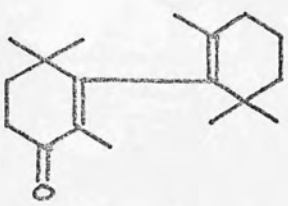
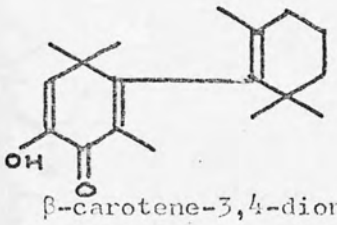
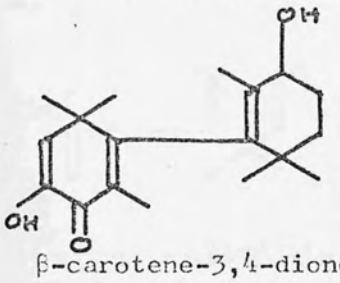
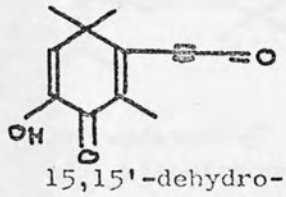
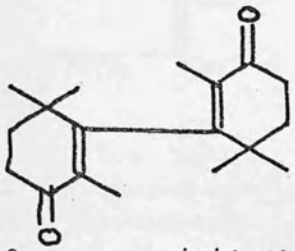
Fig 12. Gel filtration on Sephadex G.75 of the recombined products of apo-crustacyanin and synthetic carotenoids.

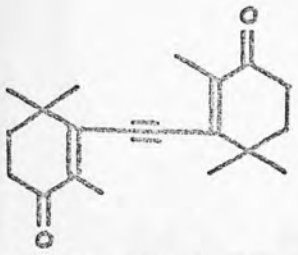
a) 15,15'-dehydro-β-apo-8'-carotenal-3,4-dione, conditions as in fig 11. run without serum albumin.

b) gel filtration estimates of molecular sizes of products. recombined astacene-protein, canthaxanthin-protein & astaxanthin methylether-protein A, and recombined astaxanthin-protein B. Recombined 15,15'-dehydro-β-apo-8'-carotenal-3,4-dione-protein C.

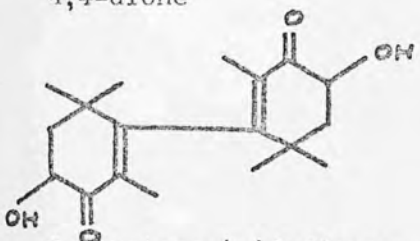
TABLE 8

Recombination of apo-crustacyanin with synthetic carotenoids.

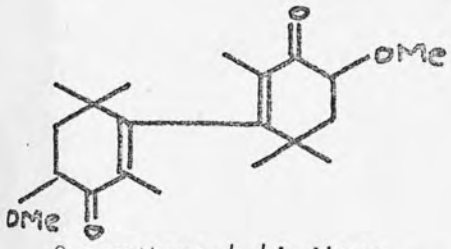
carotenoid	recombination	size of chromo-protein	max of product nm.
 β-carotene-4-one	-	-	-
 β-carotene-3,4-dione	-	-	-
 β-carotene-3,4-dione-4-ol	-	-	-
 15,15'-dehydro-β-apo-8'-carotenal-3,4-dione	fair	apo-size	500
 β-carotene-4,4'-dione (canthaxanthin)	good	β-size	575



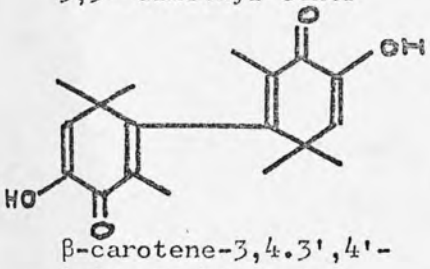
15,15'-dehydro-β-carotene-4,4-dione | - | - | -



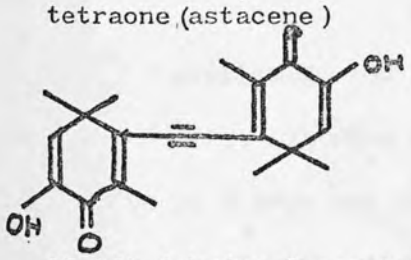
β-carotene-4,4'-dione-3,3'-diol(astaxanthin) | good | α-size 20%
β-size 80% | 630
590



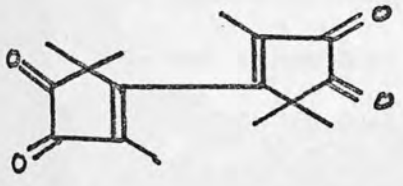
β-carotene-4,4'-dione-3,3'-dimethyl ether | good | β-size | 565



β-carotene-3,4.3',4'-tetraone (astacene) | good | β-size | 575



15,15'-dehydro-β-carotene-3,4,3',4'-tetraone | - | - | -



2,2'-bis nor-β-carotene-3,4,3',4'-tetraone, violerythrin | ?? | - | -

4.3.3. Recombination of individual apo-units with astaxanthin.

The individual apo-units, after separation on DEAE-cellulose (sections 4.2.1. and 4.2.3) were tested for their ability to recombine with astaxanthin individually, and in pairs. The results of the recombinations, together with the absorption maxima of the coloured product where applicable, and the estimated molecular size after gel-filtration on Sephadex G.75 are given in Table 9. The amounts of apoprotein used were fairly small (1.0 - 3.0 mg) of each and under these circumstances the preferred product was a β-sized molecule composed of non-identical sub-units. Where one apo-unit only was used, very little recombination occurred and then mainly to an apo-sized red product. (λ_{max} 560 nm).

Where sufficient quantities of apo-protein were available, the products from gel filtration on Sephadex G.75 (fig. 13) were concentrated and analysed by electrophoresis on starch and 6M-urea starch gels. The results, which are shown in fig. 14, indicate that β-sized molecules composed of identical sub-units can be formed from freshly prepared apo-units though not as readily as those composed of two different sub-units. In the case of the A_3 combination, the apo-protein had been freeze dried and stored for some time. Sample spectrums of a β- and poo-sized product are given in fig. 15.

Table 9.

Recombinations of individual apo-units with astaxanthin

apo-unit	recombination	size of coloured product	absorption maximum of product
A_3 + A_2	poor	apo-size	545
A_3 + A_1	fair	β-size	580
A_2 + A_1	good	β-size	575
A_3 + C_1	fair	β-size	580
A_3 + C_2	fair	β-size	575
A_2 + C_1	good	β-size	580
A_2 + C_2	fair	β-size	580
A_1 + C_1	fair	β-size	570
A_1 + C_2			
C_1 + C_2	poor	β-size	
A_3	fair	apo-size	555
A_2	poor	apo-size	545
A_1	trace	apo-size	
C_1) C_2)	not attempted		

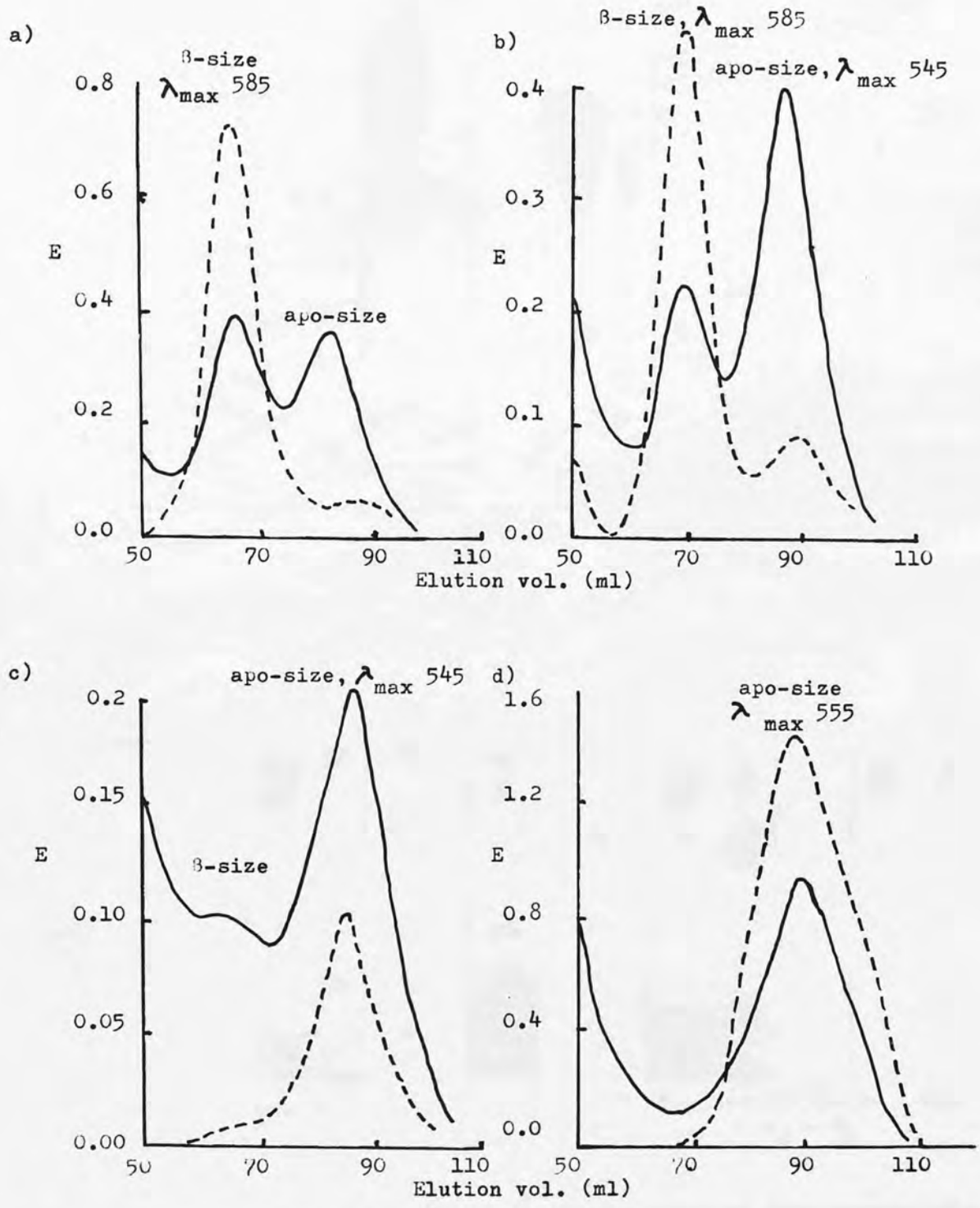


Fig 13. Sephadex G.75 gel filtration of recombined products of astaxanthin with individual apo-units.

Eluant buffer: 0.1M-phosphate pH6.8, fraction vol. 2.5ml, bed vol. 1.8 x 90cc, flow rate 7ml/hr. — 280nm. - - - 560nm.
Apo-units: a) $A_1 + A_2$ b) $A_2 + C_1$ c) A_2 d) A_3.

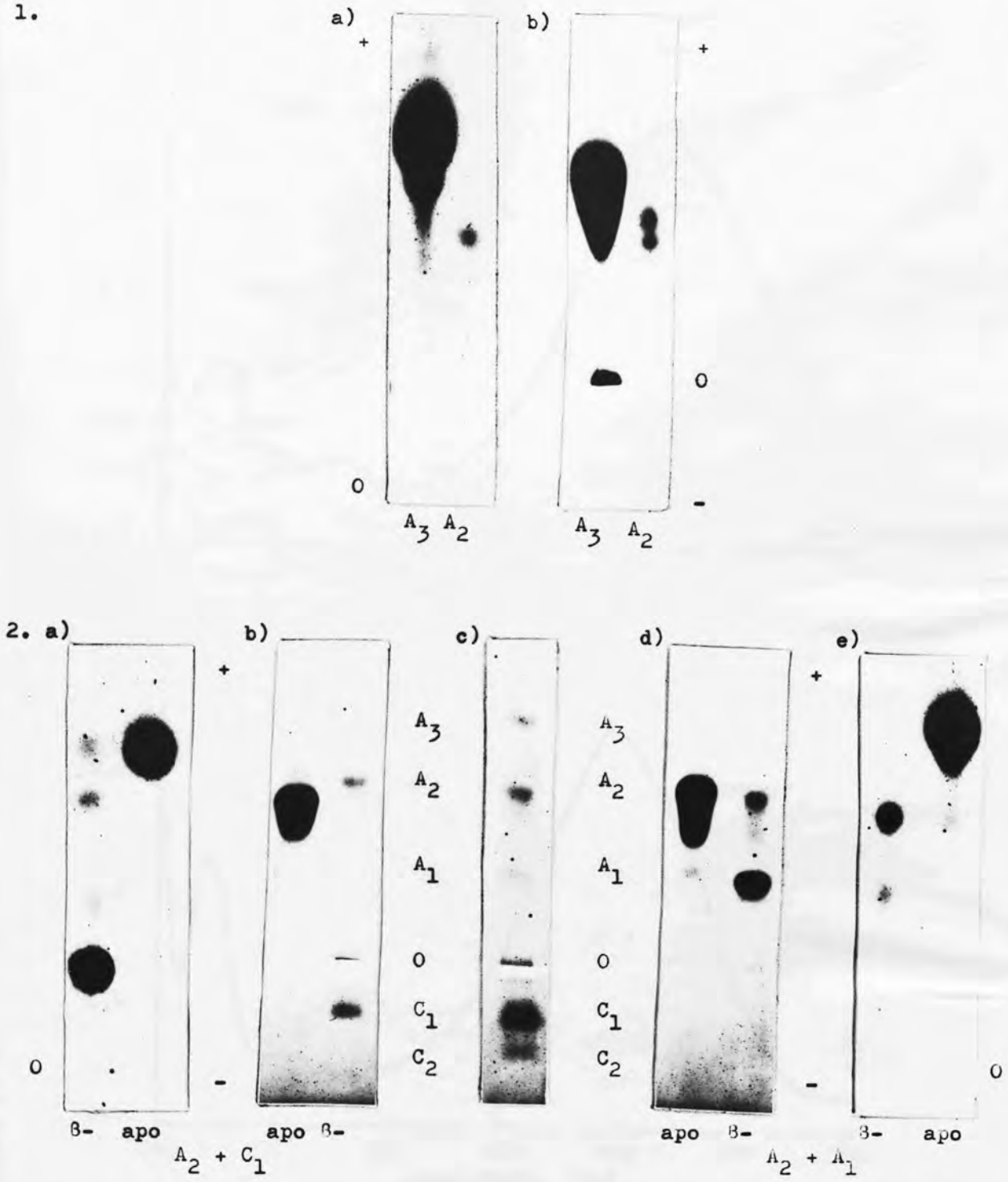


Fig 14. Electrophoresis of recombined fractions obtained from astaxanthin and apo-units after gel filtration on Sephadex G.75.

1. Starch gel (a) and 6M-urea-starch gel (b) electrophoresis of pink (apo-sized) products of recombination of astaxanthin with: A_3 and A_2. 0.04M-borate buffer pH 8.35, 3ma/gel, 110v, 17hr.

2. Starch gel (a) & (e) and 6M-urea-starch gel (b) & (d) electrophoresis of ß- and apo-sized products of recombination of astaxanthin with: $A_2 + C_1$ (a) & (b): $A_2 + A_1$ (d) & (e), (c) standard apo-units in 6M-urea-starch gel.
0.04M-borate buffer pH 8.35, 3ma/gel, 110v, 17hr.

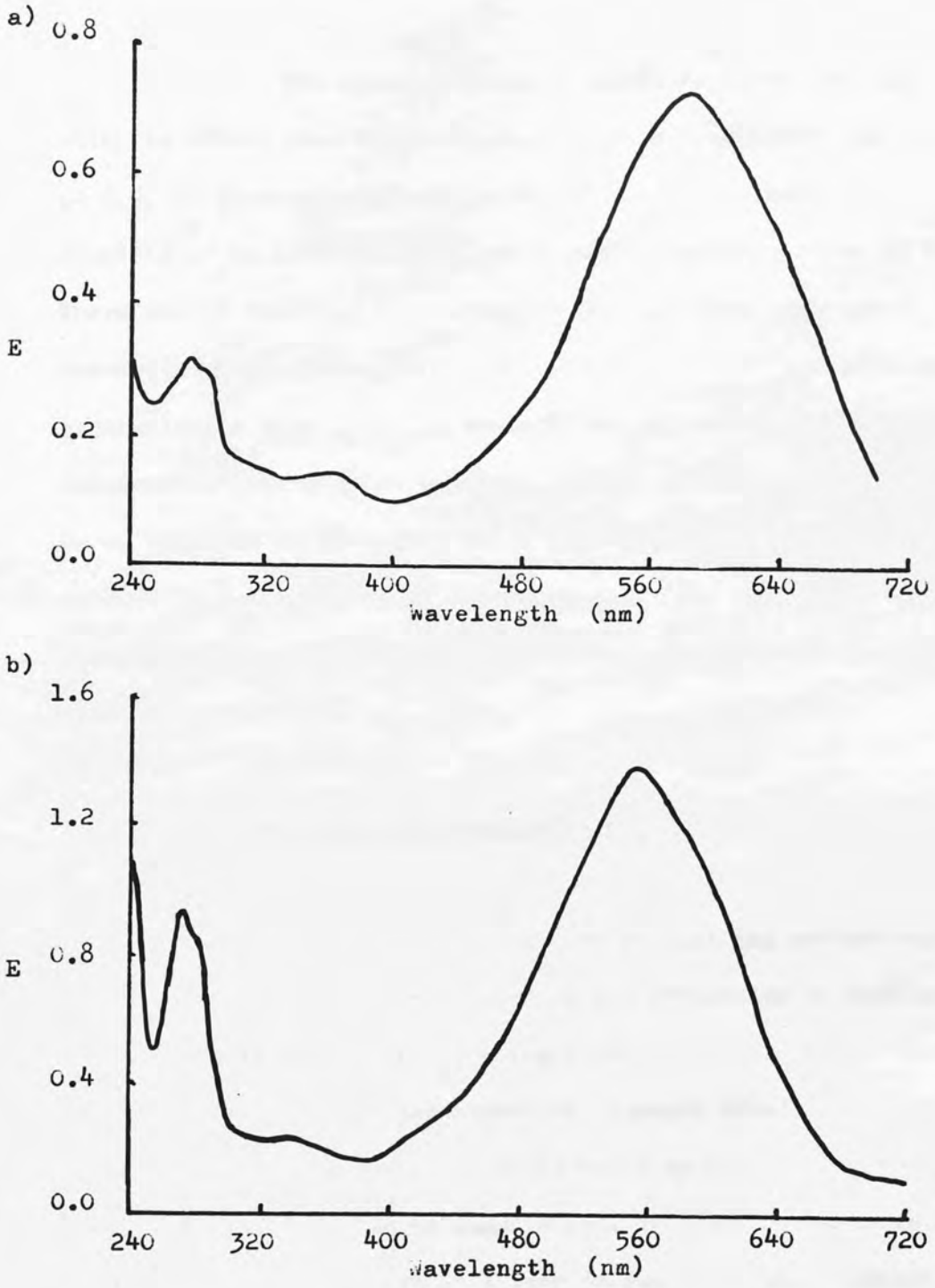


Fig 15. Spectrum of the products resulting from the combination of astaxanthin with different sub-units.

a) Recombination of A_1 + A_2 with astaxanthin - B-sized fraction in 0.1M-phosphate buffer pH 6.8.

b) Recombination of A_2 with astaxanthin - apo-sized fraction in 0.1M-phosphate buffer pH 6.8.

4.4. Preparation of the β-crustacyanins

The α-crustacyanin fraction (mol. wt. 36,000) which is eluted from DEAE-cellulose in 0.05 M-phosphate buffer pH 6.8, is heterogeneous in starch gel electrophoresis. It consists of at least 4 major and 2 minor coloured bands at pH 8.35. There are 15 possible dimers that could be formed from the 5 apo-units of α-crustacyanin. At least 10 of these can be formed synthetically from recombinations of the apo-units in pairs with astaxanthin (see section 4.3.3.). It was therefore thought to be of interest to determine which combinations of the apo are present in preparations of β-crustacyanin from the carapace, and also to see if electrophoretically pure β-crustacyanin species are interconvertible.

4.4.1. Separation of the β-sub units on Sephadex G.75.

It has already been shown that the apo-protein fraction can be crudely separated by gel filtration on Sephadex G.75, apo-units C_1, C_2 & A_1, being eluted before A_2 & A_3, (section 4.2.5 and fig. 7). It was therefore thought likely that a similar crude separation of the β-sub units could be made by the same means. However, as can be seen in fig. 16 β-crustacyanin is eluted from Sephadex G.75 in a very narrow band, which appears to be composed of approximately the same proportions of β-crustacyanins throughout.

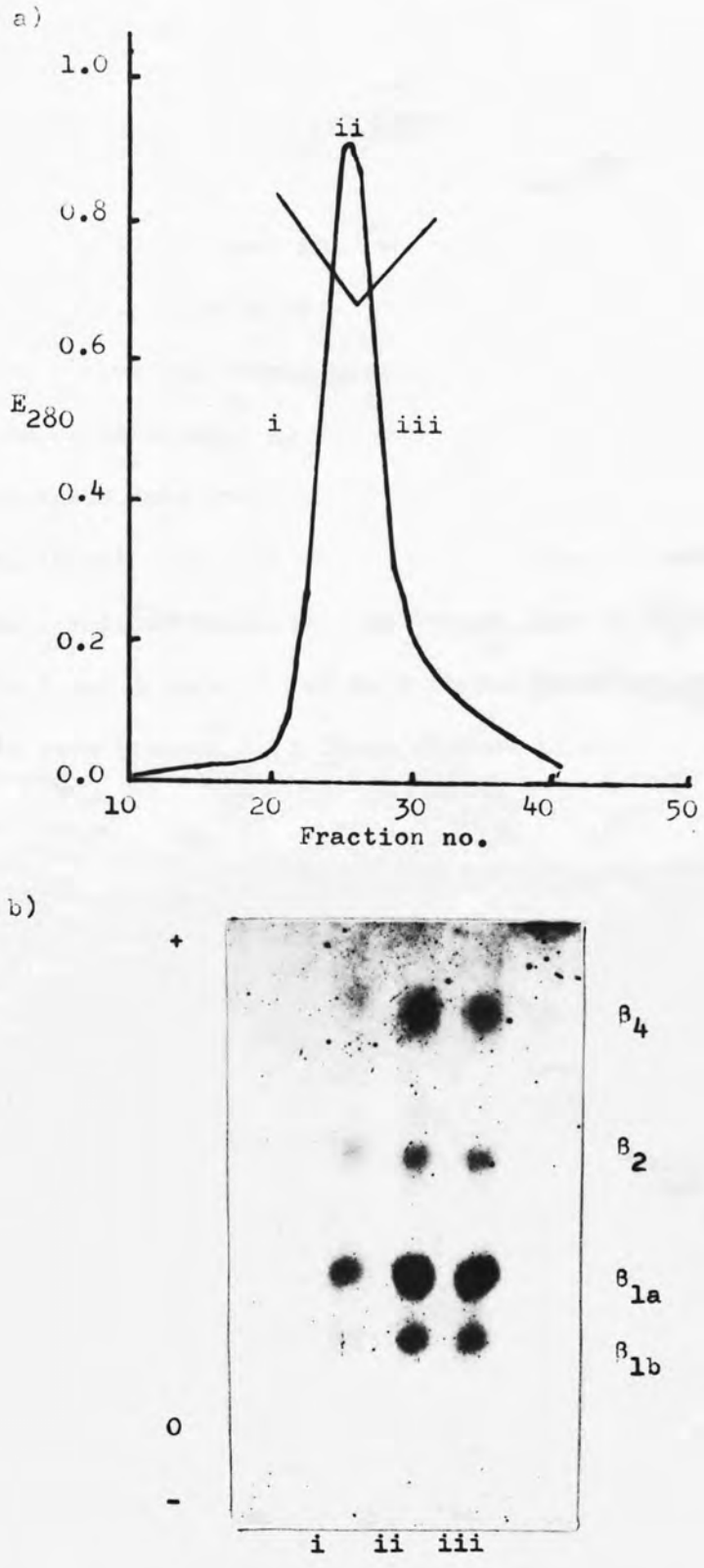


Fig 16. Gel filtration of the β-crustacyanins.

a) Elution pattern of β-crustacyanin following Sephadex G.75 chromatography. 0.05M-phosphate buffer pH 6.8, fraction vol. 2.5ml, bed vol. 1.8 x 90 cc, flow rate 7ml/hr.

b) Starch gel electrophoresis of i-leading, ii-middle & iii-trailing fractions following Sephadex G.75 chromatography (a above). 3ma, 120v, 17hr.

4.4.2. Separation of the β-sub-units by column electrophoresis.

Since the various β-crustacyanins can be distinguished electrophoretically it was hoped to be able to separate them by preparative electrophoresis on the Porath column (section 3.3.4.). A complete resolution was not obtained however, the sample being separated into two fractions, both of which were heterogeneous in starch gel electrophoresis. The elution pattern obtained, and the resulting separation in starch gels is given in fig. 17, β's 1 and 2 were eluted in a narrow fraction, while the remaining β's were present in a large diffuse band.

The β-sub units have been numbered as indicated in the following diagram:

β_4 Anode

β_3

β_{2a}

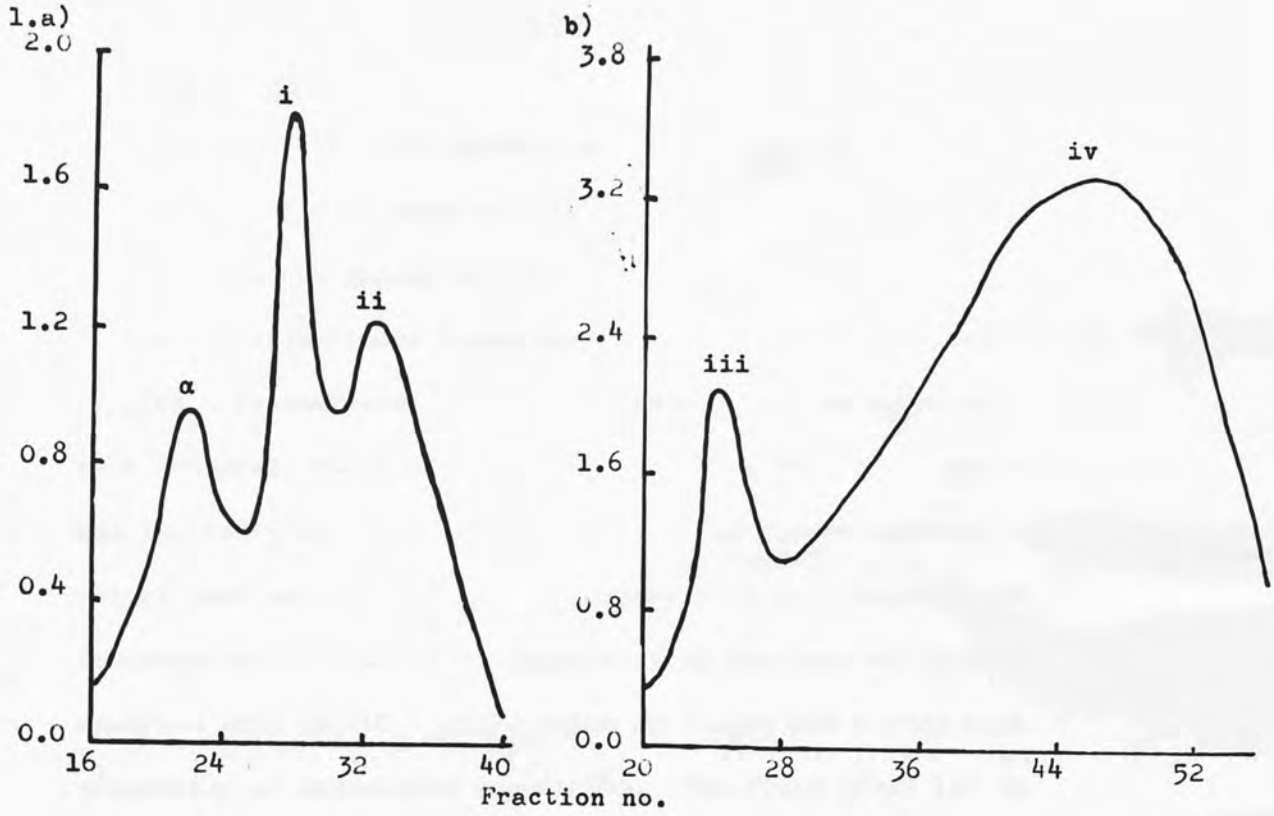
β_{2b}

β_{1a}

β_{1b}

Origin

Cathode



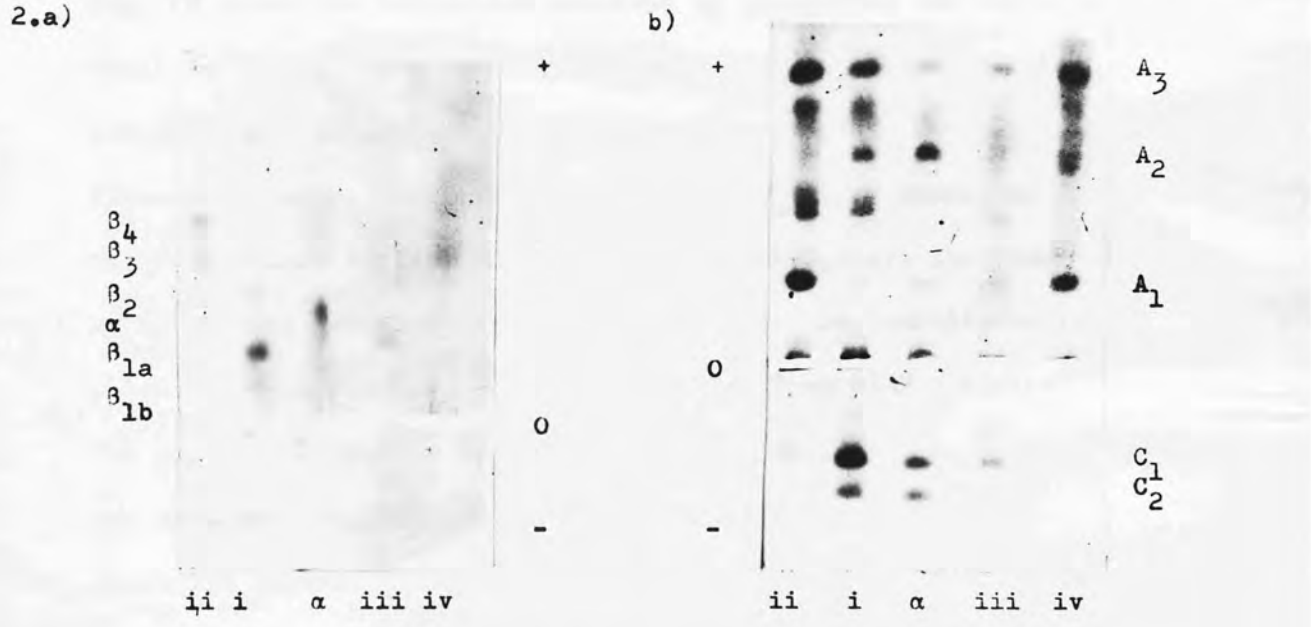


Fig 17. Separation of β-crustacyanin by column electrophoresis.

1. Separation of a) crudeextract after purification on $Ca_3(PO_4)_2$ gel, and b) fraction not adsorbed onto $Ca_3(PO_4)_2$ gel.
   0.2M-tris-EDTA-borate buffer pH 8.3. 400v, 48hr.

2. Electrophoresis of samples obtained in '1', on a) starch gel, and b) 6M-urea-starch gel.
   0.04M-borate buffer, 3ma/gel, 150v, 16hr.

4.4.3. Separation of the β-sub-units by DEAE-cellulose chromatography.

The β-crustacyanin fraction can be resolved by gradient elution on DEAE-cellulose. Column sizes were varied according to the amount of material present. Gradients of both linear and logarithmic types were used, with equally satisfactory results. β-crustacyanin fractions from different sources have been compared, and the results are shown in fig. 18. The protein was initially purified on Sephadex G.75 to remove low molecular weight contaminants. This preliminary step was necessary in order to obtain good separations especially in the case of β- (not adsorbed onto $Ca_3(PO_4)_2$gel), which initially has a very high proportion of colourless impurities. The first graph (a) in fig. 18 shows the β-fraction obtained by incubating the residual shell (after incubation with EDTA) with ice cold 1 M-KCNS, which extracts any remaining colour. This is immediately diluted ten times with water, precipitated with 50% $(NH_4)_2SO_4$, dissolved in 0.5 M-phosphate buffer, pH 7.0, and dialysed against the same buffer. This fraction is composed of α-, β- and γ-crustacyanin which can be separated by stepwise elution from DEAE-cellulose. The fraction eluted in 0.05 M-phosphate buffer, pH 7.0, is the one separated in fig. 18(a). The graph shown in fig. 18 (b) shows the separation of the β-crustacyanin fraction that remains unadsorbed on calcium phosphate gel under the conditions used for the purification of α-crustacyanin. (See section 1.2). The third graph (fig. 18 (c)), illustrates the elution pattern obtained by chromatography of the β-fraction eluted from DEAE-cellulose in 0.05 M-(P) during the preparation of α-crustacyanin (section 1.2).

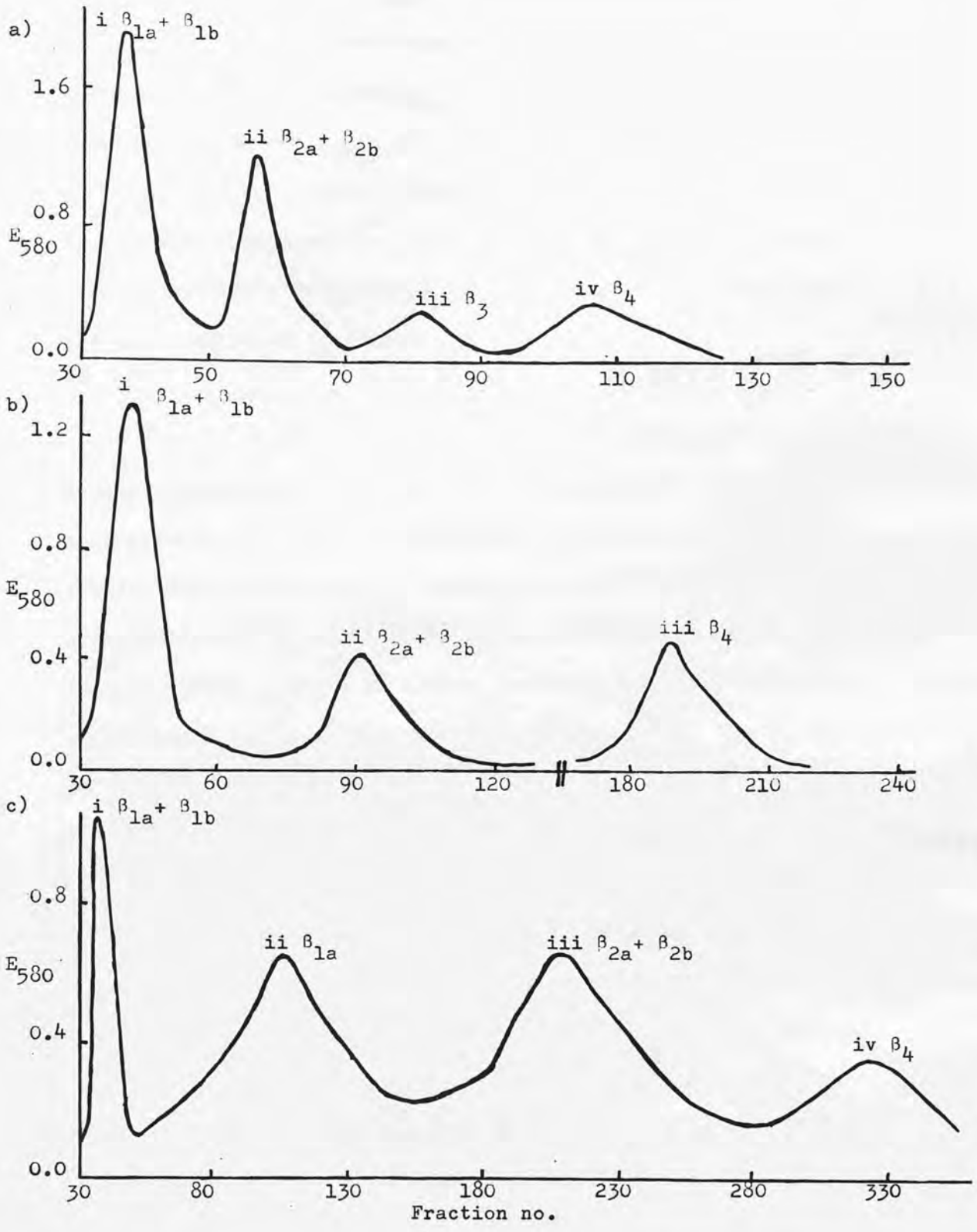


Fig 18. Resolution of β-crustacyanin by ion exchange chromatography on DEAE-cellulose in phosphate buffer pH 6.8.

a) β-fraction obtained by treatment of shell residue with 0.1M-KCNS. Column 1.8 x 12 cm, gradient systeml, 500ml H_2O + 500ml 0.1M-(P).

b) β-fraction not adsorbed onto $Ca_3(PO_4)_2$ gel. Column 1.8 x 53 cm. Gradient, system 11, 1litre 0.005M-(P) + 500ml 0.1M-(P), then 500ml 0.25M-(P).

c) β-fraction not adsorbed onto DEAE-cellulose in 0.05M-(P). Column, 1.8 x 45 cm. Gradient, system 11, 1litre 0.005M-(P) + 500ml 0.05M-(P), then 150ml 0.1M-(P), then 500ml 0.2M-(P).

The proteins obtained from these separations were concentrated and analysed by electrophoresis in starch and urea-starch gels. These results are shown in fig. 19 and it is evident that, except for an additional fraction obtained from β-(KCNS), the same β-fractions are obtained from all three preparations. Two heterogeneous fractions containing β_{1a} & β_{1b} and β_{2a} & β_{2b} respectively, and pure samples of β_3 (β-KCNS) and β_4.

β-crustacyanin fractions have also been prepared from α-crustacyanin by a) prolonged dialysis ~~and~~ against water, and b) incubation with 3 M-urea. These were also separated by gradient elution from DEAE-cellulose, and the resultant fractions subjected to electrophoresis in starch and 6 M-urea-starch gels as above. The results, given in figs. 20 and 21, show that the same β-fractions predominate from whatever source they are prepared. The β_3 fraction is present in 3 M-urea.

It was thought that it might be possible to separate β's and 1 and 2 into their two components by using a shallower gradient. However, this was unsatisfactory owing to the increased time during which the protein was bound to the cellulose, and the consequent large losses of material by irreversible adsorption. The initial purification outlined above was therefore used routinely and β's 1 and 2 were further purified as follows.

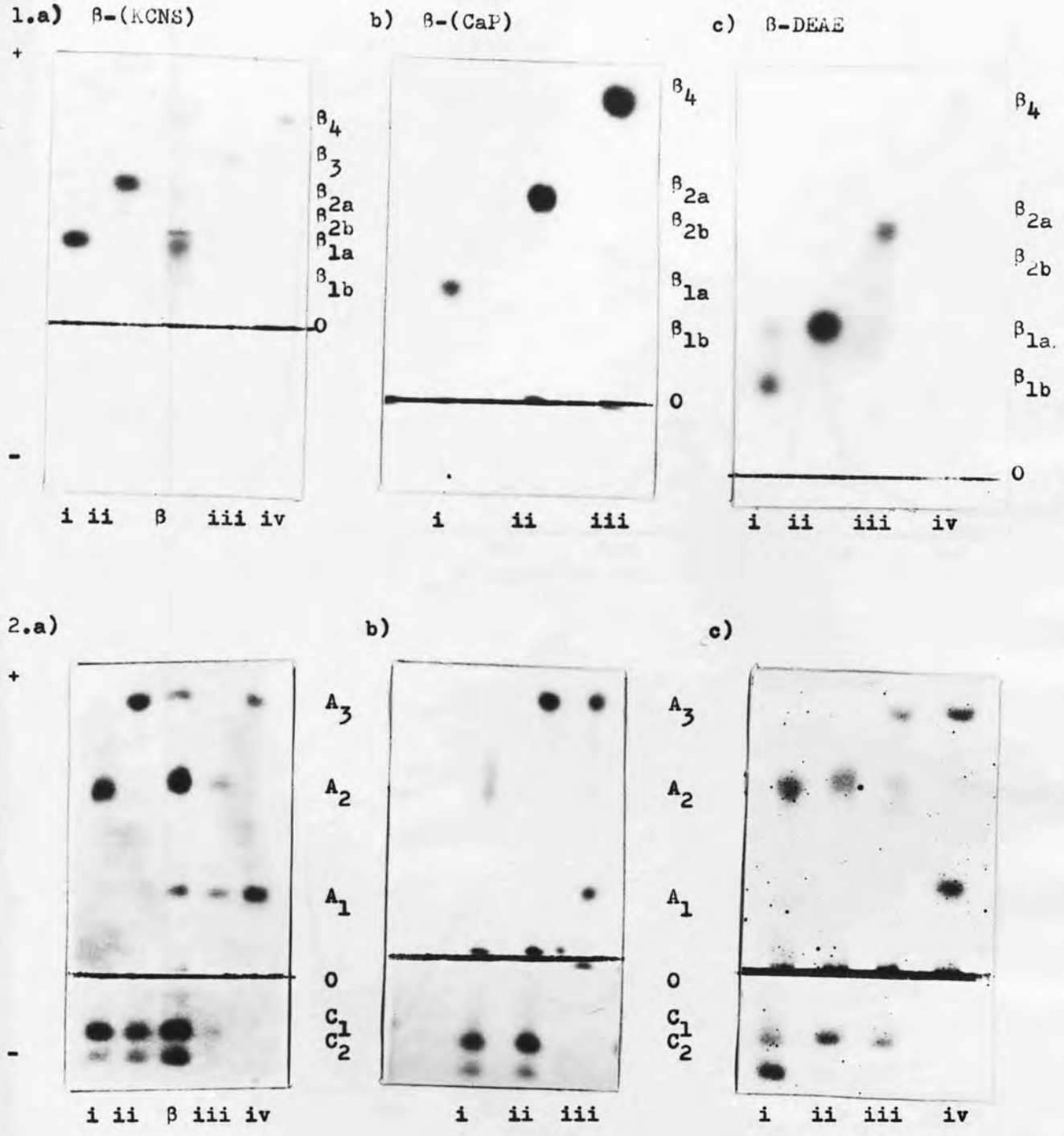


Fig 19. Electrophoresis of resolved β-crustacyanin fractions.

Electrophoresis of samples obtained by gradient elution from DEAE-cellulose, as shown in fig 18.
1 - starch gels, 2 - 6M-urea-starch gels.
a) β-fraction obtained by treatment of the shell residue with 0.1M-KCNS.
b) β-fraction not adsorbed onto $Ca_3(PO_4)_2$ gel.
c) β-fraction not adsorbed onto DEAE-cellulose in 0.05M-(P).
1.b) photograph of unstained gel.

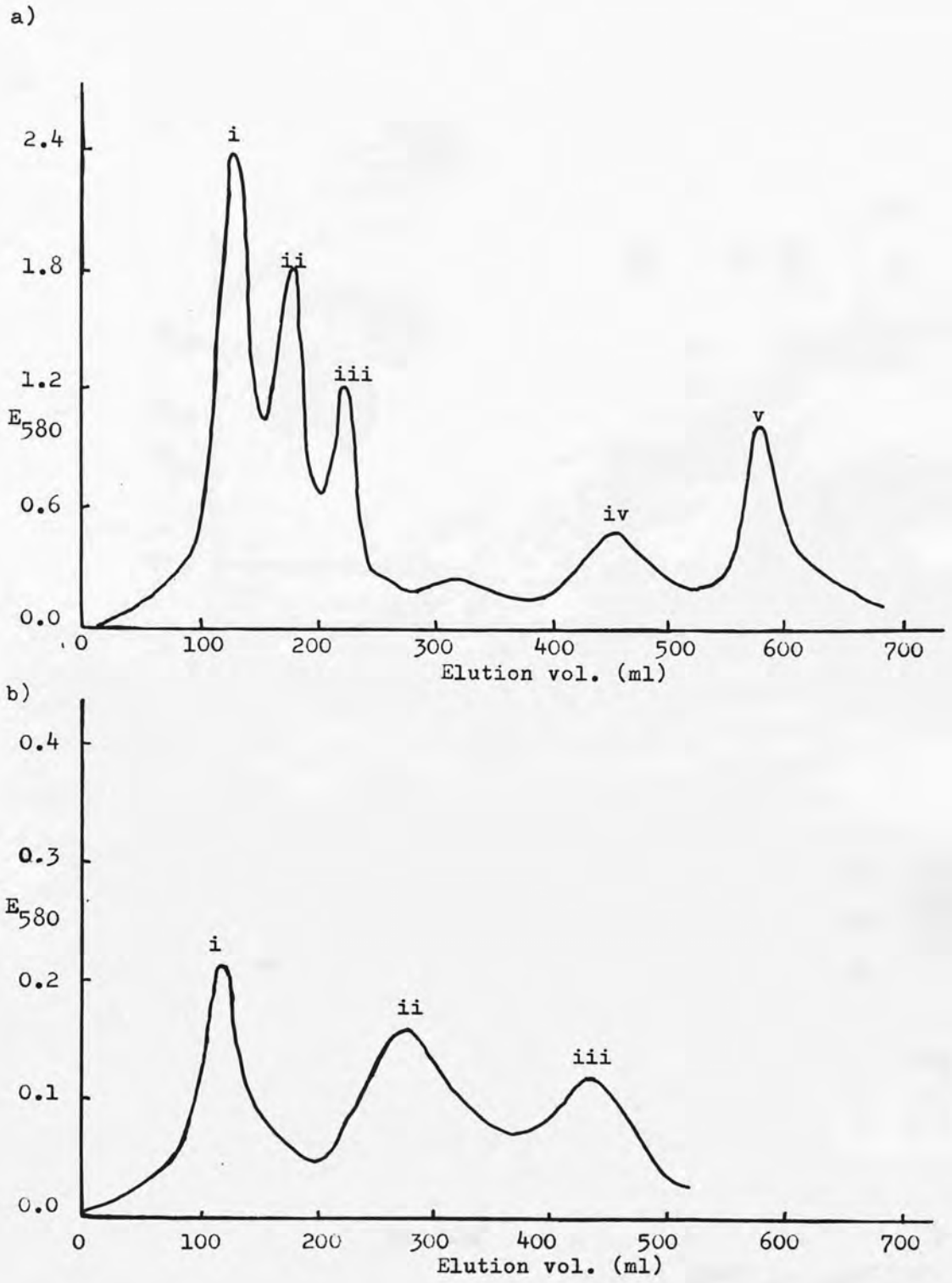


Fig 20. Resolution of β-crustacyanin by ion exchange chromatography on DEAE-cellulose in phosphate buffer pH 6.8.

a) β-crustacyanin prepared by extensive dialysis of α-crustacyanin against distilled water. Column 1.8 x 20 cm. Gradient, system 11 llitre 0.0005M-(P) + 340ml 0.1M + 100ml 0.25M-(P) + 200ml 0.5M-(P).

b) β-crustacyanin prepared by incubation of α-crustacyanin in 3M-urea. Column 1.8 x 15 cm. Gradient system 11 0.005M-(P) + 450ml 0.15M-(P) + 100ml 0.3M-(P). All buffers contained 3M-urea.

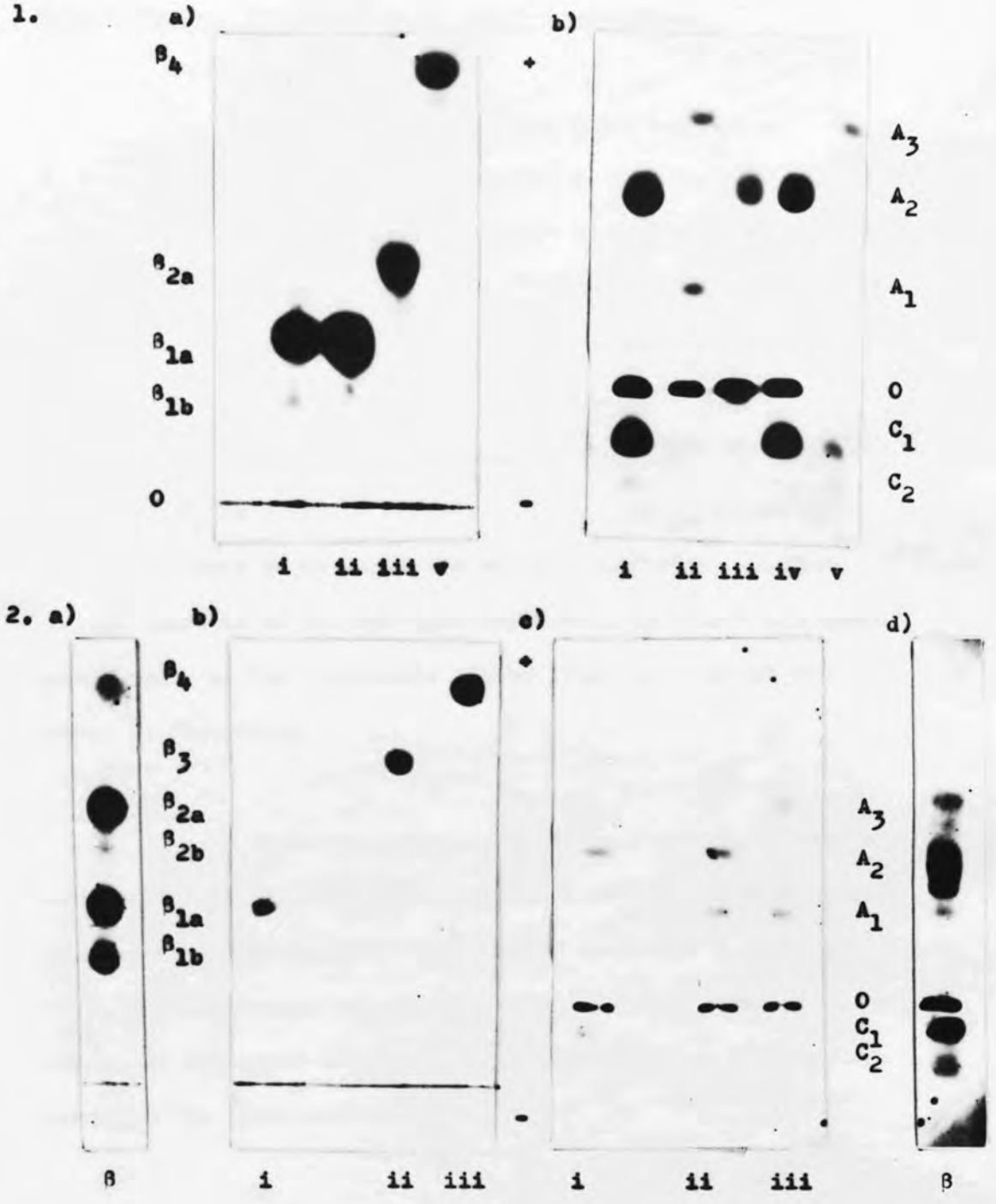


Fig 21. Electrophoresis of resolved β-crustacyanin fractions.

Electrophoresis of samples obtained by gradient elution from DEAE-cellulose as shown in fig 20.

1. **β-fraction obtained by prolonged dialysis of α-crustacyanin against distilled water.** a) starch gel b) 6M-urea-starch gel. 0.04M-borate buffer pH 8.35, 3ma/gel 110v, 16hr. **Numbers as in fig 20.**
2. **β-fraction obtained from α-crustacyanin by incubation in 3M-urea.** b) starch gel c) 6M-urea-starch gel , numbering as in fig 20. a) & d) a crude β-fraction run as standard. 0.04M-borate buffer pH 8.35, 3ma/gel 130v, 16hr.

4.4.4 Further purification of the β-sub-units.

As described in section 3.3.3 samples of β_1 (a + b) and β_2 (a + b) were applied to Pevikon blocks and subjected to electrophoresis for 24 hr in the cold. Fig. 22(b) shows the results obtained after electrophoresis of β_2 (a + b), their elution and further analysis in 6M-urea-starch gels.

Concentrated samples (10 mg/ml) of the impure fractions β_1 (a + b) and β_2 (a + b) were also submitted to electrophoresis in cellogel blocks as described in section 3.3.2. Results of further electrophoresis in starch and urea-starch gels of the components eluted from the cellogel are shown in fig. 22(a).

Although separations of the β-fractions can be achieved by the above methods losses occur, due to heating of the blocks and trailing of the leading samples. β_1a and β_2a were, in this manner obtained in a homogeneous state and their component sub-units determined. β_1b and β_2b were not readily purified by these methods.

Concentrated samples of β_1 (a + b) fractions were also rechromatographed on small (1.8 x 12 cm) columns of DEAE-cellulose. The gradients used were linear, 0.005 M-(P) to 0.025 M-(P) in 1 litre, and 0.009 M-(P) to 0.035 M-(P) in

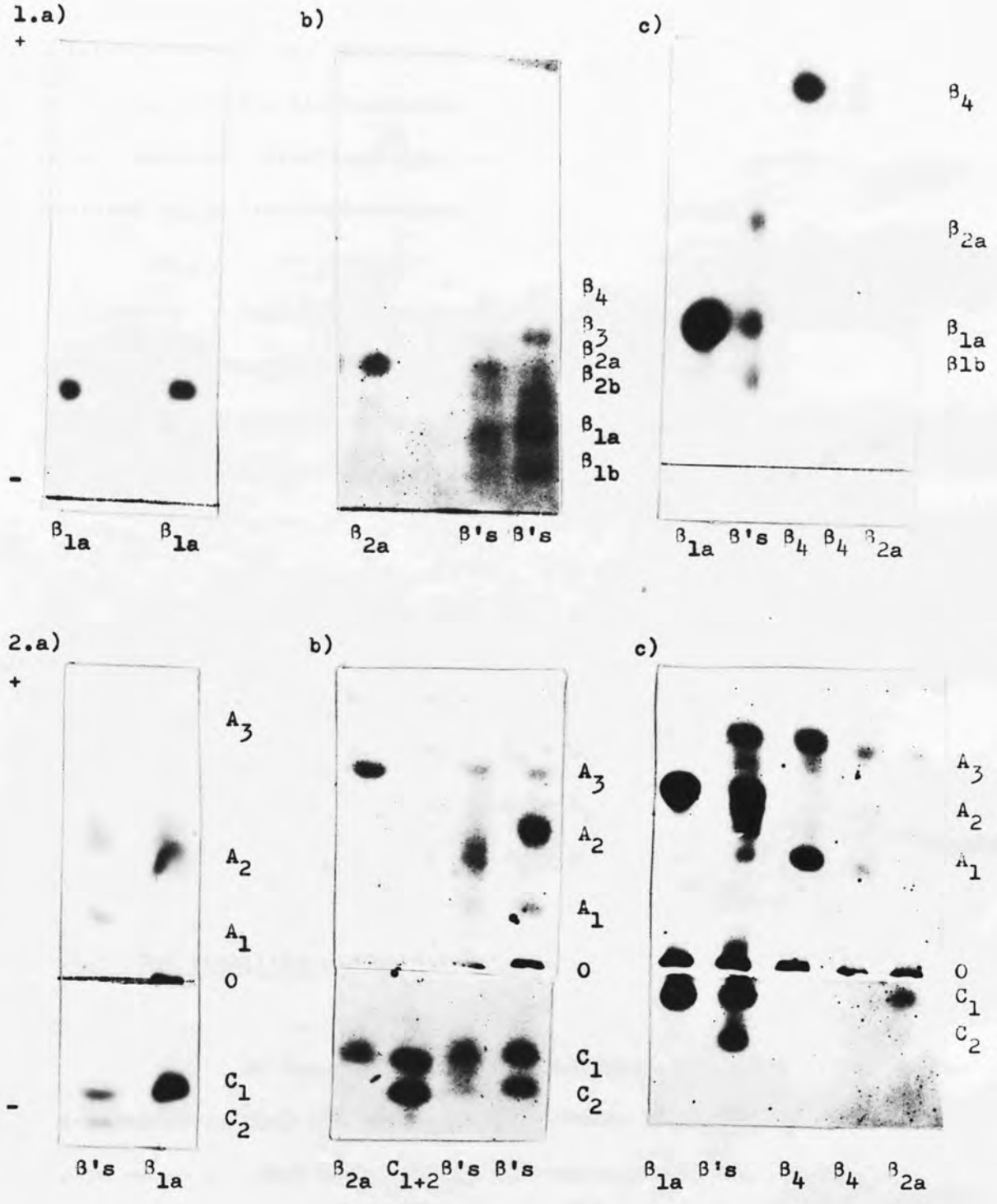


Fig 22. Purification of β-crustacyanins (β_{1a} & β_{2a}).

Electrophoresis on 1, starch gels and 2, 6M-urea-starch gels of β-crustacyanins.

a) β_{1a} purified by electrophoresis on cellogel.

b) β_{2a} purified by electrophoresis on Pevikon.

c) β_{1a} and β_{2a} prified by rechromatography on DEAE-cellulose.

0.04M-borate buffer pH 8.35, 3ma/gel, 120v, 16hr.

1 litre respectively. The results are shown in fig. 22(c). As in the previous electrophoretic methods it is difficult to obtain the minor fractions pure, but β_1a and β_2a are readily obtained in an electrophoretically homogeneous state.

From these experiments it is obvious that, as a result of the breakdown of α-crustacyanin, only 6 of the 15 possible β-forms predominate. Their sub-unit composition, evident from analyses on urea-starch gels is given below :

$$\beta_1\ b = A_2 + C_2$$

$$\beta_1\ a = A_2 + C_1$$

$$\beta_2\ b = A_3 + C_2$$

$$\beta_2\ a = A_3 + C_1$$

$$\beta_3 = A_2 + A_1$$

$$\beta_4 = A_3 + A_1$$

4.4.5 The stability of the β-crustacyanins.

In these experiments β_1 a and β_4 only were used as examples as they are the easiest to obtain pure, and in fairly large amounts. They also contain between them four out of the five apo-units. They were stored under $(NH_4)_2SO_4$ for three months, and then divided into three fractions, each of 1 ml, containing 5 mg of protein. They were treated in the following manner :

a) 30 ml of ice cold acetone was added to the protein, the resulting apo-protein was centrifuged, dissolved in 0.005 M-(P) buffer pH 7.0. Astaxanthin dissolved in acetone was added dropwise and the mixture dialysed (cf section 4.2.1).

b) The sample was made 6 M with respect to urea and incubated at room temperature for 3 hr. The resultant red solution was then dialysed against 0.005 M-(P) buffer pH 7.0 in the cold for 24 hr.

c) The sample was made 0.05 M with respect to mercaptoethanol to encourage S - S interchange, then dialysed against 0.005 M (P) buffer pH 7.0.

All these samples were then concentrated and submitted to starch gel electrophoresis, pH 8.35, the results of which are shown in fig. 23. No isomerisation of any of the β-fractions appeared to occur under these conditions.

4.4.6 Treatment of β-crustacyanin with 6 M-urea.

Incubation of β-crustacyanin (5 mg/ml) with 6 M-urea for two hours at room temperature results in a dark brownish solution. This can be resolved into two fractions by gel-filtration on Sephadex G-75 (previously equilibrated with

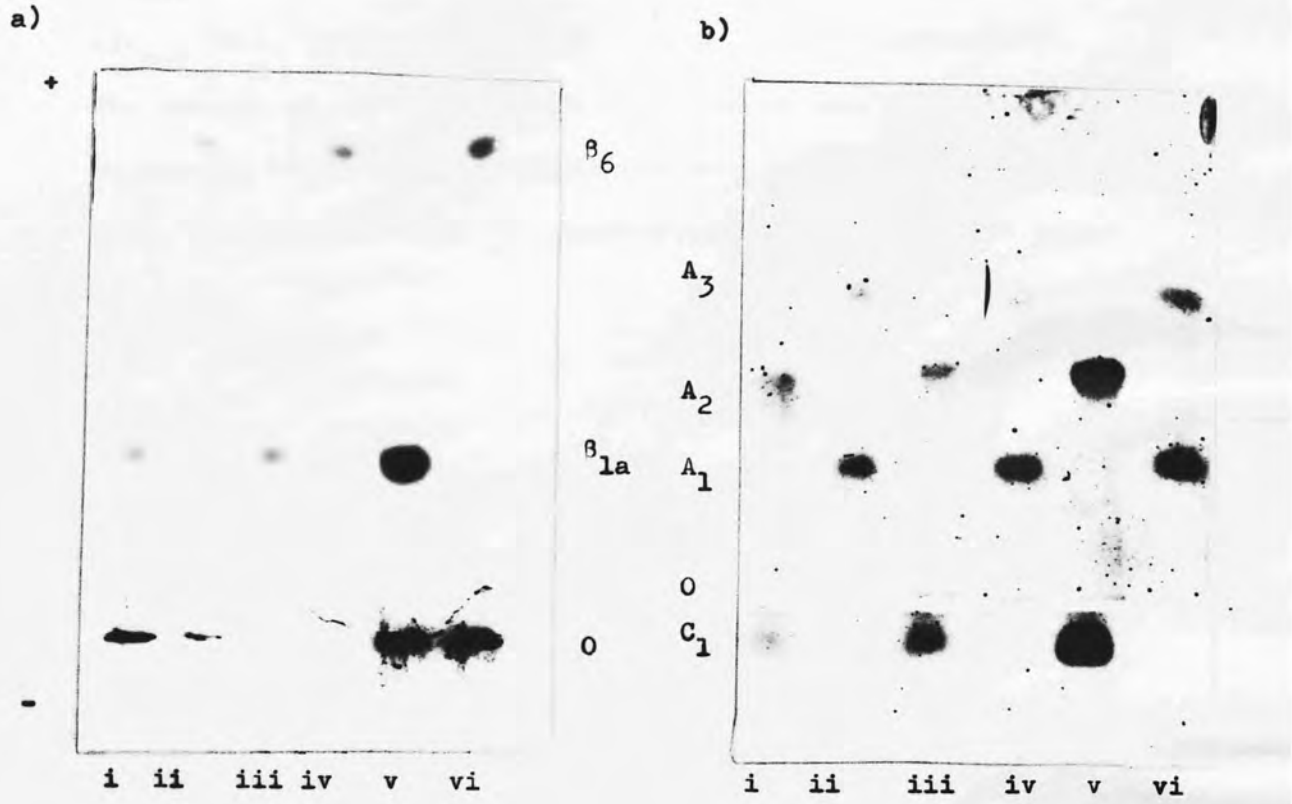


Fig 23. Stability of β-crustacyanins (β_{1a} & β_4).

Electrophoresis of β_{1a} & β_4 on a) starch and b) 6M-urea-starch gels.
i & ii after treatment with 6M-urea.
iii & iv after reduction in mercaptoethanol.
v & vi after destruction of the carotenoid-protein linkage with acetone.
All samples were dialysed against 0.05M-phosphate buffer overnight, and then against water before electrophoresis.
0.04M-borate buffer, 3ma/gel, 120v, 16hr.

6 M-urea / 0.05 M-phosphate buffer pH 7.0): a yellow protein (λ_{max} 400) which is excluded from the gel, and a pink component (λ_{max} 560), with the same elution volume as apo-crustacyanin. The spectra of the two proteins are given in fig. 24. Complete separation is difficult to achieve since, using long columns of G-75, the yellow pigment is adsorbed onto the Sephadex and cannot be eluted.

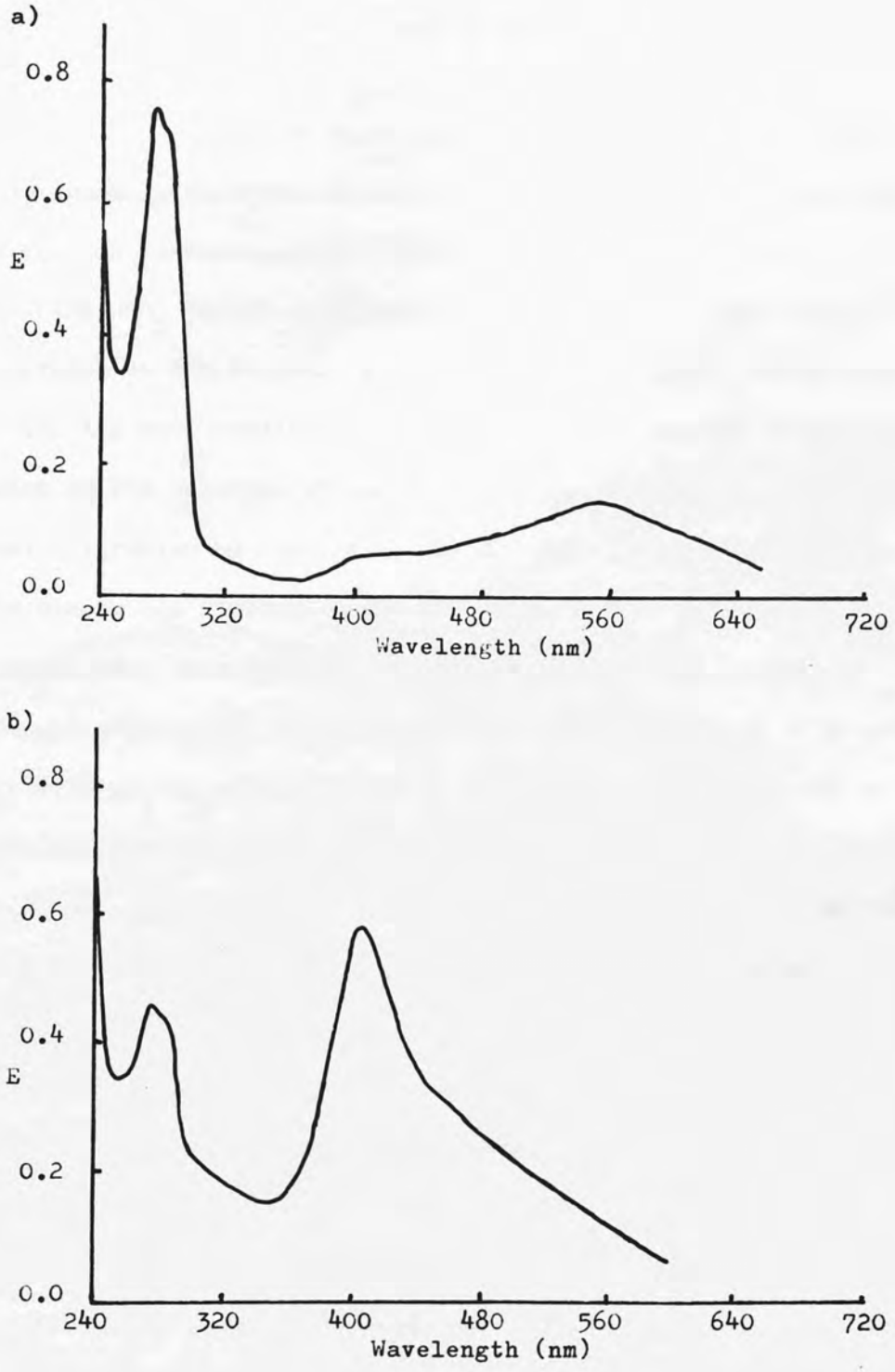


Fig 24. Spectrum of fractions eluted from Sephadex G.75, following filtration of β-crustacyanin dissolved in 6M-urea.

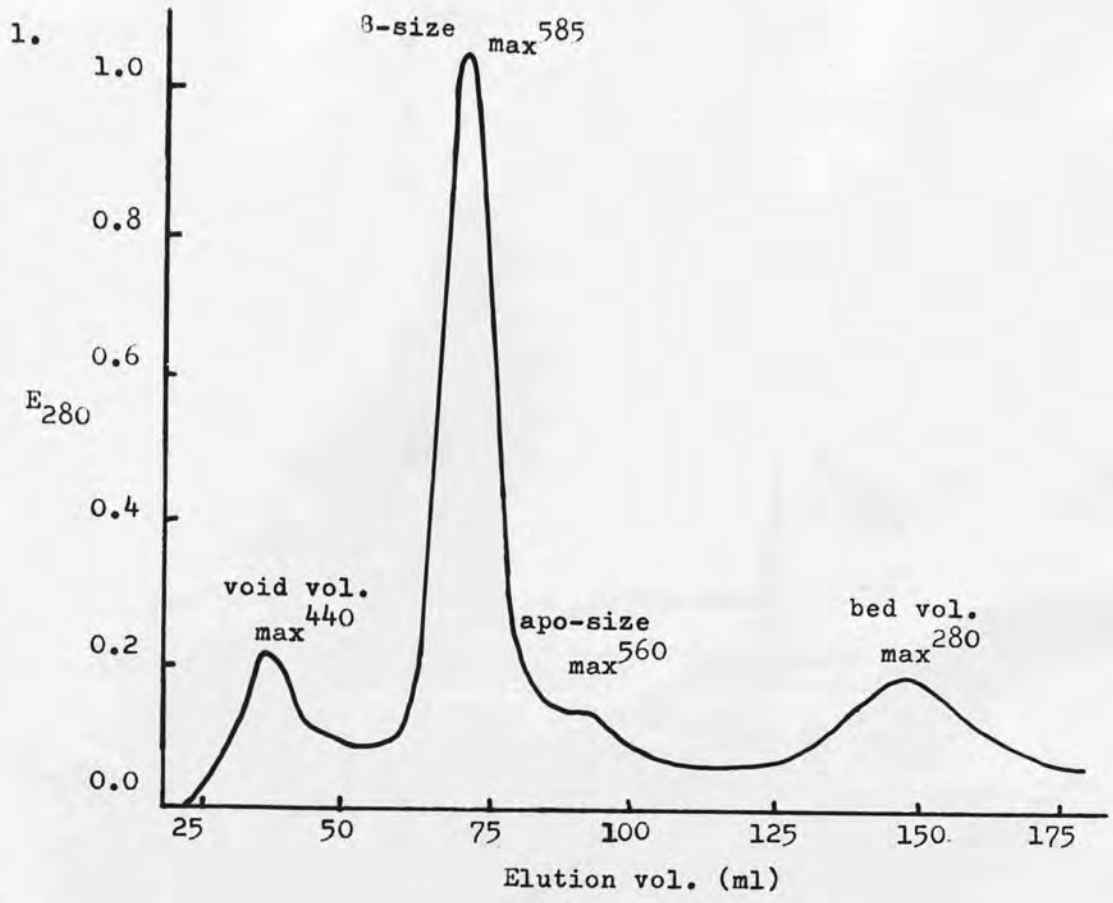
a) Pink fraction eluted in the apo-crustacyanin elution volume

b) Yellow fraction eluted in the exclusion volume.

Samples were dissolved in 0.05M-phosphate buffer pH 6.8, containing 6M-urea.

4.5. Enzymic digestion of the crustacyanins.

Enzymic digestion of α- and β-crustacyanin was performed as described in section 3.6. Neither enzyme had much effect on α-crustacyanin. After incubation at 26° in 0.1M-NH_4HCO_3 pH 9.0 for 16 hr. α-crustacyanin becomes partly degraded to the β-form. Incubation with trypsin or chymotrypsin under the same conditions does not have much further effect as seen by the relative proportions of the different peaks after gel filtration on Sephadex G.75, and also following electrophoresis in starch and 6M-urea-starch gels (fig 25.2). β-crustacyanin is slightly more degraded by these enzymes. The pattern of β-crustacyanin (10 mg) incubated with chymotrypsin (1 : 50 w/w) in 0.1M-NH_4HCO_3 at 26°for 16 hr is given in fig 25.1. The result after incubation with trypsin under identical conditions is similar. The composition of the β-fraction however, is different. Incubation with trypsin does not appear to alter the composition of the β-fraction as can be seen from starch and 6M-urea-starch gel electrophoresis (fig 25.2). Chymotrypsin, on the other hand produced at least 2 new bands. These appear to be formed from β_{1a} and β_4 respectively each having a slightly greater mobility at pH 8.35 (fig 26.1). Unfortunately in this experiment soluble chymotrypsin was used and was not removed before electrophoresis, consequently there were no bands in the urea gel, as further degration of the apo-protein could possibly have taken place after removal of the carotenoid in the urea. In the experiment recorded



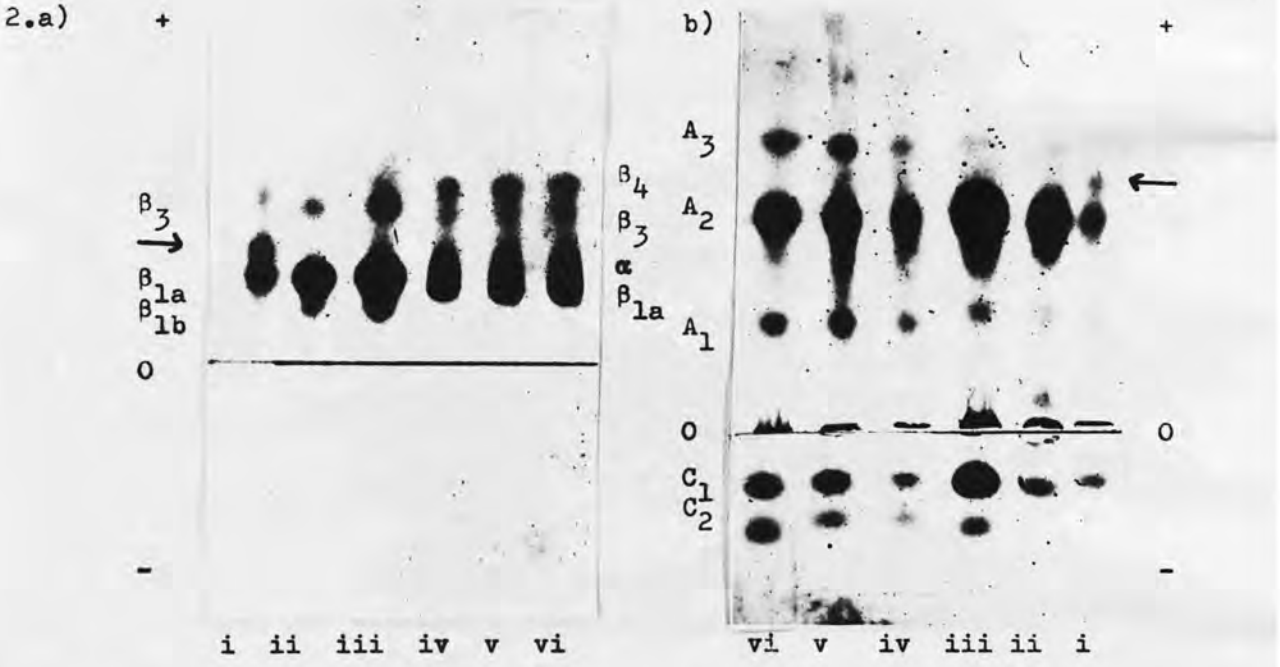


Fig 25. Enzyme digests of the crustacyanins.

1. Sephadex G.75 gel filtration of β-crustacyanin following incubation for 16hr with chymotrypsin 1 : 50 (w/w) in 0.1M-NH_4HCO_3 pH 9.0, 26°. Phosphate buffer pH 6.8, fraction vol. 2.5ml, bed vol. 1.8 x 93cc, flow rate 7ml/hr.

2. a) starch gel and b) 6M-urea-starch gel electrophoresis of β (i - iii) and α (iv - vi) crustacyanins. iii & iv, incubated with 0.1M-NH_4HCO_3 pH 9.0; ii & v, with 0.1M-NH_4HCO_3 pH 9.0 + 1 : 50 (w/w) trypsin; i & iv, with 0.1M-NH_4HCO_3 pH 9.0 + 1 : 50 (w/w) chymotrypsin. Arrows indicate the appearance of new bands.

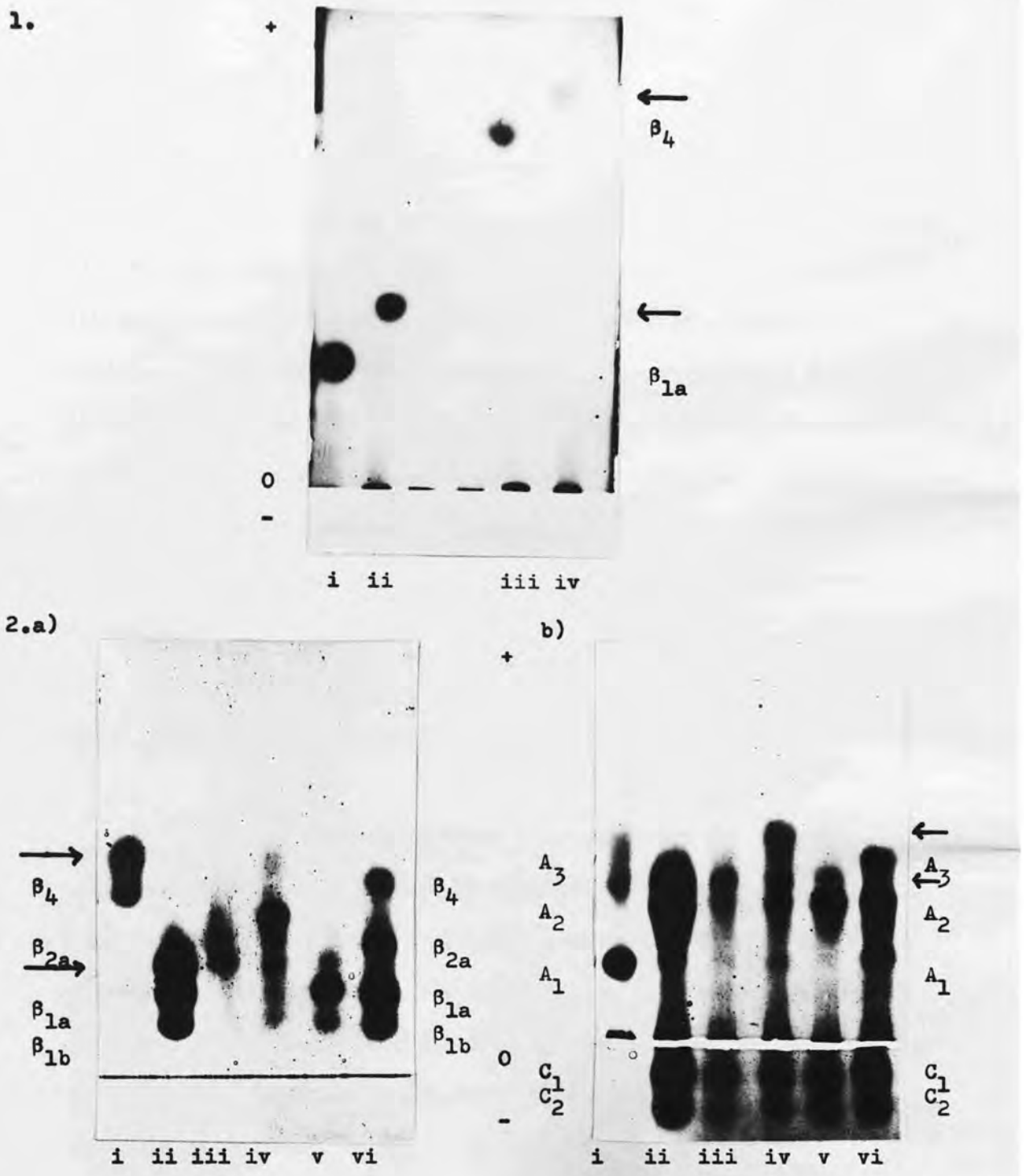


Fig 26. Starch gel electrophoresis of chymotrypsin digests of β-crustacyanin.

1. Starch gel electrophoresis of i, β_{1a} and iii, β_4 after incubation at 26° with 0.1M-NH_4HCO_3 pH 9.0 for 16hr; ii, β_{1a} and iv, β_4 after incubation at 26° with 0.1M-NH_4HCO_3 pH 9.0 + chymotrypsin i : 50 (w/w) for 16hr. 0.04M-borate buffer pH 8.35, 3ma, 130v, 17hr.

2. a) starch and b) 6M-urea-starch gels of vi, whole β-fraction; i, ii, iii, iv & v rough fractionation of β-crustacyanin after incubation with chymotrypsin 1 : 50 (w/w). The reaction being carried out in 0.1M-NH_4HCO_3 pH 9.0 at 26° for 16hr. 0.04M-borate buffer pH 8.35, 3ma/gel, 110v, 16hr. Arrows indicate new bands.

in fig 26.2, in which solid chymotrypsin was used and removed prior to electrophoresis, new apo-protein bands appear to correspond with the new β-forms. In this experiment the crude β-crustacyanin (10 mg) was fractionated by gradient chromatography on DEAE-cellulose after incubation overnight with chymotrypsin (1 : 50 w/w) in 0.1M-NH_4HCO_3 pH 9.0. A complete separation was not obtained, although small quantities of one new β-form were obtained almost pure. From 6M-urea-starch gel electrophoresis this appears to consist of C_{1+2} and a new apo-unit with a mobility in between A_2 and A_3.

4.6. Preparation of γ-crustacyanin.

As previously shown γ-crustacyanin can be separated from α-crustacyanin by chromatography on DEAE-cellulose (Jencks & Buchwald, 1968a), or by chromatography on the Porath column (cf section 4.1.3). In neither case, however, is it free from β-crustacyanin, after electrophoresis on starch gels at pH 8.35. It did appear homogeneous when subjected to electrophoresis in cellogel strips at pH 8.3 for 1.5 hr (fig 27). γ-crustacyanin λ_{max} 625 does appear less stable than α-crustacyanin, being more easily broken down into the β-form. In 6M-urea starch gels it can be seen to consist of the five apo-crustacyanin units in the same relative proportions as found in α-crustacyanin.

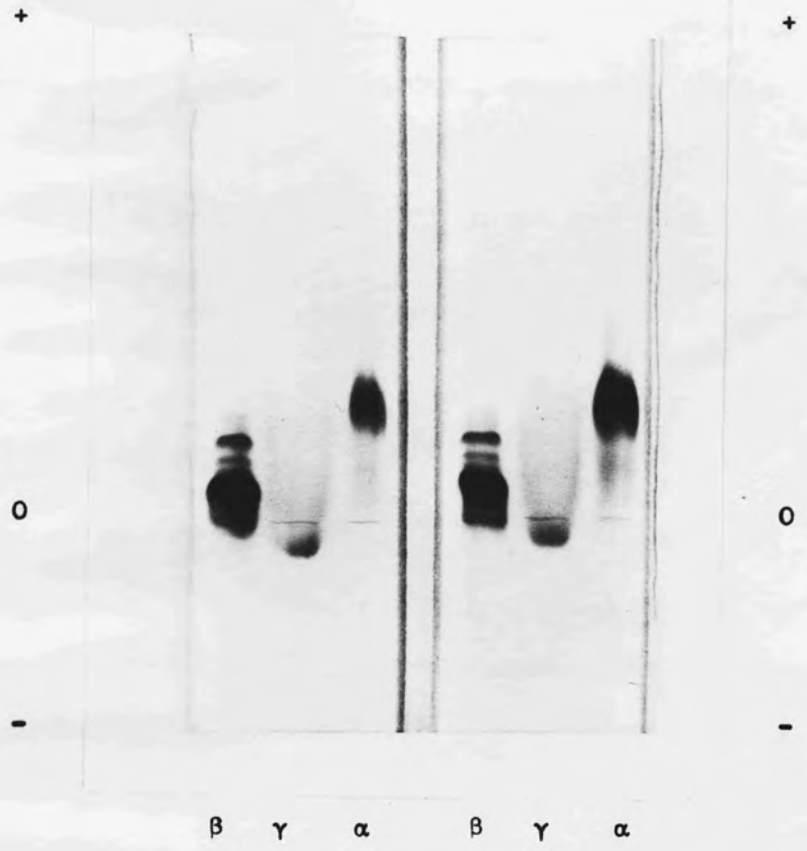


Fig 27. Electrophoresis of the crustacyanins on cellogel strips.

Electrophoresis of β- γ- & α-crustacyanins on cellogel strips.
0.05M-veronal buffer pH 8.6, 2ma/strip, 13v/cm, 2hr.

5. DISCUSSION

5.1. Preparation of α-crustacyanin.

Modifications in the preparation procedure of α-crustacyanin, described in section 4.1. reduced the losses incurred by over heating, during grinding and irreversible adsorption onto $Ca_3(PO_4)_2$ gel obtained by previous methods when dealing with large quantities of lobster shell. No decrease in the purity of the product was observed as judged by an $E^{280}/_{632}$ value of 3.

The parts of the carapace used, however, did influence the proportion of the different chromoproteins. Greatest yields of α-crustacyanin were obtained from the tail segments and legs, while large amounts of γ-crustacyanin were found in preparations using the thoracic sections of carapace. These segments, especially from lobsters that had been dead for some time before freezing, appeared red-purple in colour, rather than bright blue, and were less brittle than those of freshly killed lobsters. It is therefore possible that the pigments of these lobsters were partially denatured in situ.

5.2. Recombinations.

In most cases the α-crustacyanin had been stored under 50% $(NH_4)_2SO_4$ for one or more months, also the lobsters from which it was prepared had been kept in the deep freeze for periods

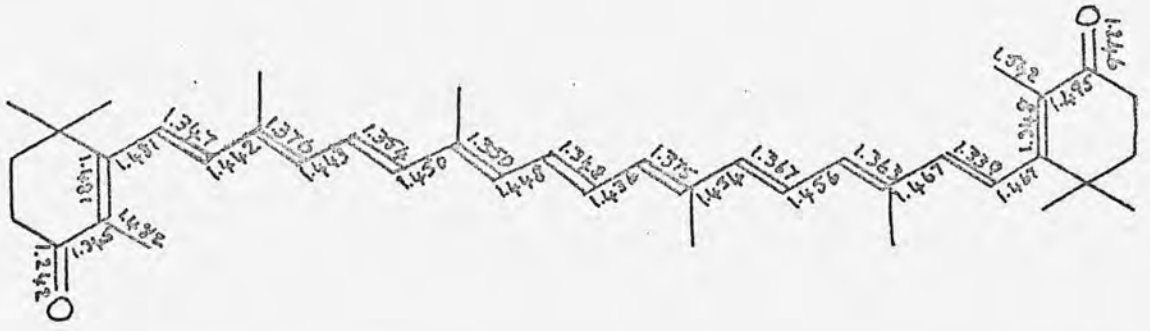
of up to 2 years. It has been suggested, in the case of rhodopsin, that the polyene chain only protects the amino acid residues in the protein with which it is in contact, allowing the remainder of the molecule to partially 'denature'. In aged preparations, therefore, although the spectrum of rhodopsin cannot be distinguished from that of a fresh preparation, on removal of the prosthetic group, conformational changes take place so that the ability to recombine is lost (Abrahamson & Ostroy, 1967). It is therefore possible that the same may be true of α-crustacyanin and that ageing of the protein may render it slightly altered so that although the apo-protein retains the ability to recombine with astaxanthin to form β-crustacyanin, the ability to polymerise to α-crustacyanin is diminished.

Similar results for the carotenoids canthaxanthin, astacene, euglenanone and echinenone, were found to those reported by Lee & Zagalsky (1966). Interestingly, although astaxanthin dipalmitate and astaxanthin diacetate will not recombine with apo-protein (Lee & Zagalsky, 1966), astaxanthin dimethyl ether shows fair recombination (section 4.3.2). Therefore the only permissable substitutes found so far for positions C_3 & C_3' of the β-ionone rings are H, OH and OCH_3, $OCOCH_3$ and larger groups presumably prevent binding of carotenoid to apo-protein by steric hindrance. The present results also confirm that keto functions on C_4 & C_4' are essential for binding to occur.

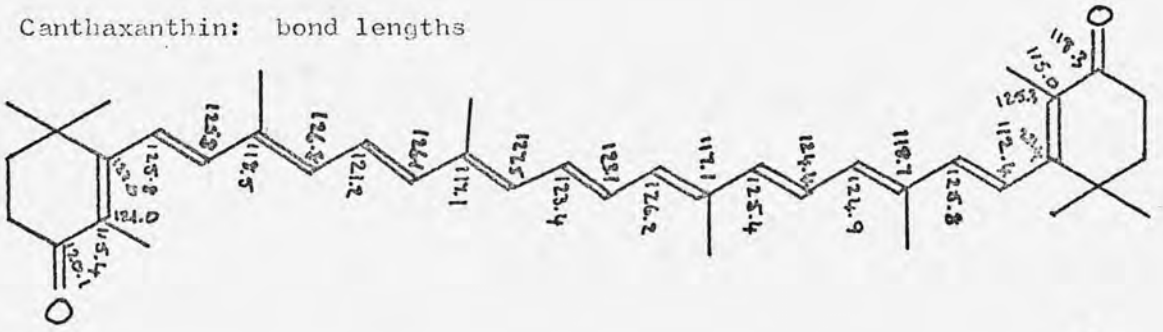
Carotenoids containing a keto group on only one of the β-ionone rings, e. g. euglenanone, echinenone and β-carotene-3,4-dione 4'ol do not appear to bind. In these carotenoids the inability of the β-ionone ring not containing the requisite keto groups, to bind to the appropriate amino acid residue on the protein possibly prevents the correct alignment of apo-protein and polyene so that binding of the other β-ionone ring, although theoretically possible does not take place. A carotenoid lacking a second β-ionone ring, 15 ,15'-dehydro-β-apo-8'-carotenal-3,4-dione, however did combine to give an apo-sized red product. In this case the β-ionone ring containing a C_4 keto group, presumably binds to the normal groups on the protein. The shortened polyene chain would not span the distance between the two ring binding sites, and evidently the incorrectly positioned C_8' keto function does not inhibit interaction. It is possible that in this case the polyene chain does not bind onto the normal site as dimerisation does not occur. Unfortunately results of recombination experiments with violerythrin (which has 2 5 membered rings each possessing 2 oxygen functions) were not conclusive as only minute amounts of this carotenoid were available. These results could be expected to give further information on the required stereo-specificity of the ring binding sites.

Further indications of the critical spatial limitations of the carotenoid binding site are given by the fact that neither 15,15'-dehydro-β-carotene-3,4,3',4'-tetrone, or 15,15'-dehydro-β-carotene-4,4-dione would combine, although

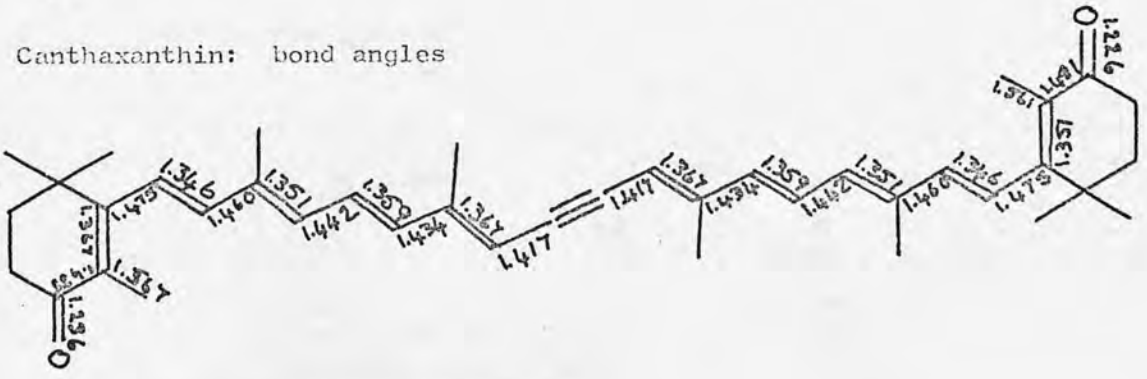
In the acetylene derivatives so far studied there is a decrease in alternate nature of the single and double bond toward the centre of the molecule, which in the case of dehydro canthaxanthin results in a considerable shortening of the 14 - 15 single bond. The dehydro derivatives also possess a centre of symmetry about the triple bond and exhibit an extended trans configuration about the 3 colinear central bonds (Sly, 1964). Another significant difference between the two carotenoids is the dihedral angle between the chains and the rings which, in canthaxanthin is 43° and in dehydrocanthaxanthin is 28°



Canthaxanthin: bond lengths

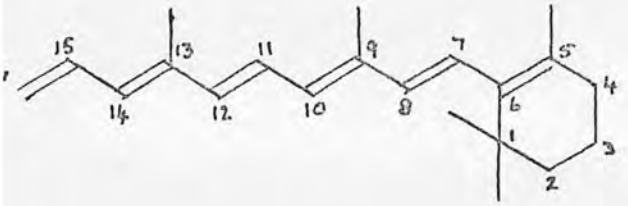


Canthaxanthin: bond angles

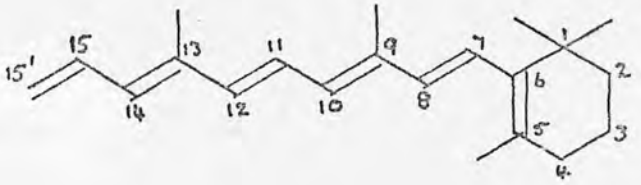


Dehydrocanthaxanthin: bond lengths.

containing the required keto functions on C_4, $C_{4'}$. The molecular structures of canthaxanthin and dehydrocanthaxanthin have been studied by X-ray crystallography (Bart & Macgillavry, 1968a, 1968b). In these carotenoids as in β-carotene (Sterling, 1964) the β-ionone ring does not continue the all trans arrangement from the polyene chain but has an S-cis configuration about the C(7)-C(6) bond.

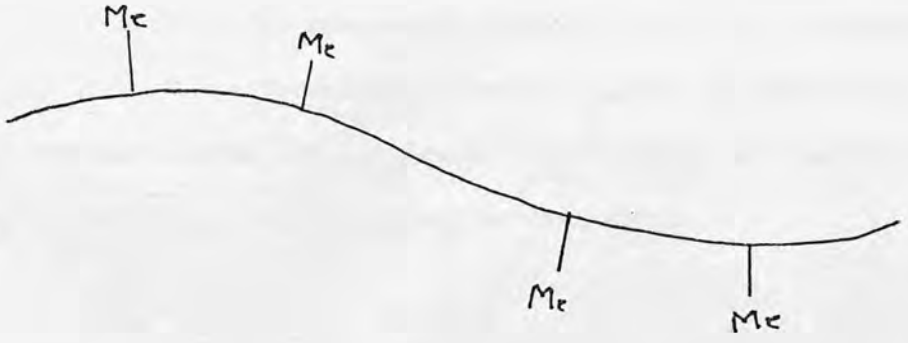


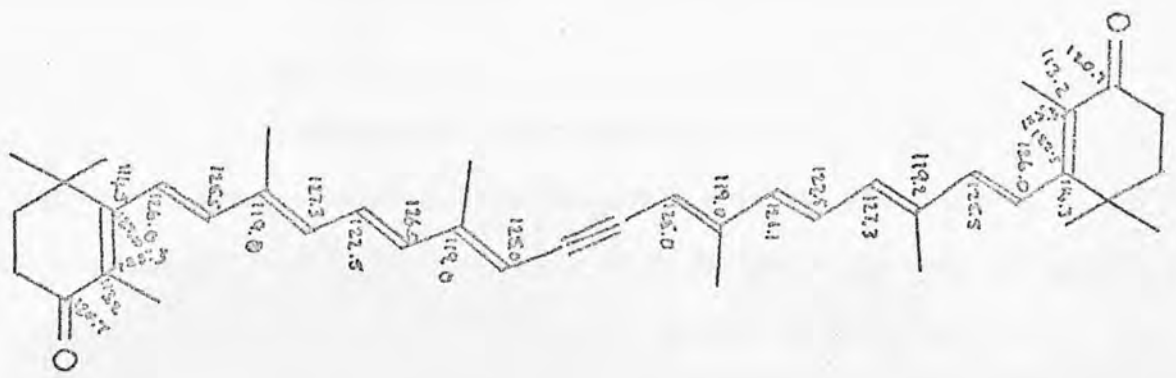
S-trans about the C(7) - C(6) bond.



S-cis about the C(7) - C(6) bond.

Although the polyene chain was originally thought to be planar. The molecule takes up an S-configuration in which the methyl groups project from the convex side. The binding is partly caused by the non-bonding interactions of the methyl groups with the hydrogen atoms at C_{11} & C_{15} and might therefore be expected to exist in solution.



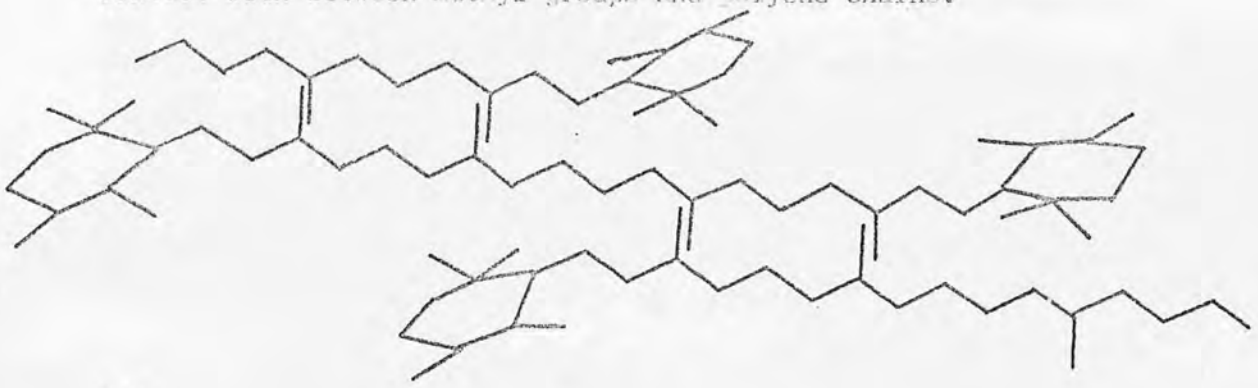


Dehydrocanthaxanthin: bond angles

(Bart & Macgillavry, 1968a; 1968b)

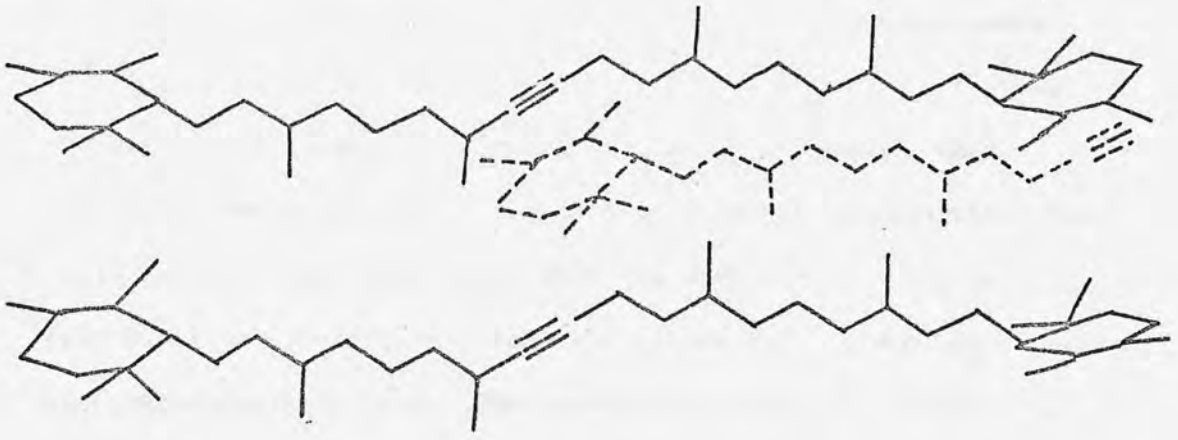
An interesting factor which is probably significant in determining the ability of carotenoids to bind to apo-crustacyanin, can be calculated from a knowledge of the bond lengths and angles. This is the interatomic differences between the 4,4' oxygen atoms. In canthaxanthin the two oxygen atoms are 28.18Å apart while in the acetylene derivative they are only 26.81 Å apart. The two oxygen atoms, previously shown to be essential for binding of carotenoid to protein are therefore 1.57 Å closer together in dehydrocanthaxanthin than in canthaxanthin. This distance is of the same order as a double bond and is approximately 5% of the total length of the carotenoid molecule, and might therefore, together with the difference in dihedral angles, be expected to be significant within the obviously narrow limits of specificity required in the carotenoid-protein interactions.

Canthaxanthin and dehydrocanthaxanthin also differ in crystal packing. Canthaxanthin crystals are formed from closely packed rows, in which there is intermolecular contact both between methyl groups and polyene chains.



(a)

In dehydrocanthaxanthin crystals however, there is overlap between the ring of one molecule and the chain of another, and no contact between backbones.



(b)

(a) Canthaxanthin. (Bart & Macgillavry, 1968b).

(b) Dehydrocanthaxanthin. (Bart & Macgillavry, 1968b).

Although it is realised that these are the preferred interactions that occur under conditions of high concentration and would not necessarily be found in solution, they might nevertheless indicate the preferred conformations of each carotenoid where carotenoid-carotenoid interactions take place. As such interactions possibly occur in crustacyanin (Jencks & Buchwald, 1966b), their different stacking properties may be partly responsible for the fact that dehydrocarotenoids are unfavourable prosthetic groups of crustacyanin.

The specificity studies outlined above give added weight to the explanation offered by Jencks & Buchwald, (1966b) for the spectral shifts evident in carotenoid-protein interaction. Thus for the dimer β-crustacyanin, if the oxygen atoms (at C_4 & C'_4) of the β-ionone rings, 28.8 Å apart were to bind firmly to the apo-proteins so that the methyl groups of each carotenoid could interlock rigidly providing good contact between polyene chains, and helping to localise the strain in the double bonds, then the absorption maxima would be shifted to longer wavelengths. This would help to explain the fact that the spectrum of apo-size recombination products (sections 4.3.2. and 4.3.3.) was not greatly bathochromically shifted. The increased bathochromic shift found when β polymerises to α-crustacyanin may be considered as increased twisting or enhanced interactions brought about by conformational changes in the protein.

5.5. The uniqueness of the apo-units.

The appearance of five subunits after the removal of the carotenoid was noted by Cheesman et al., (1966). Since two of these (C_1 & A_2) always predominated these authors suggested that the other three arose by secondary reactions.

Wisse et al., (1966) working on α-crystallin found that multiple subunits of this protein were apparant on electrophoresis in starch gels containing 6M-urea. They concluded that isomerisation of a single species occurred in the urea. This does not appear to be the case with α-crustacyanin however, as the same five subunits appear if the carotenoid is removed with acetone and the apo-units subjected to electrophoresis in the absence of urea. Moreover after separation by gradient elution from DEAE-cellulose they show no tendency to isomerise in urea (with the possible exception of the C_1 & C_2 fractions which are difficult to prepare free from each other). Minor bands often do appear in urea gels, but these have not been classed as apo-crustacyanin-units. (Section 4.2.2.). The apo-units also appear as distinct entities after electrophoresis in gels containing 6M-urea and mercaptoethanol, indicating that they are not isomers formed by disulphide interchange, (section 4.2.6.)

It was also thought possible that α-crustacyanin was itself a series of similar proteins, each composed of one or two sub-units. Examination of the α-crustacyanin band from gel

filtration on Sephadex G.200, column electrophoresis or gradient elution from DEAE-cellulose, failed to resolve the protein into separate entities. (section 4.1.6.).

The proportion of the five apo-units remains approximately constant from preparation to preparation, as seen from densitometer readings of 6M-urea-starch-gels, as well as calculations of the amounts of each obtained after separation, (section 4.2.). Jencks & Buchwald, (1966b) suggested that the products of the action of 6M-urea on α-crustacyanin (yellow protein and colourless protein) were different from those of acetone on α-crustacyanin (carotenoid and apo-protein). They found that both the yellow protein and colourless protein were required in stoichiometric amounts to reform α-crustacyanin. Recombinations of carotenoid and apo-protein also yield α-crustacyanin. However, from analysis in 6M-urea-starch gels the composition of the apo-units appear identical no matter how the carotenoid is removed, (i.e. by action of acetone, 6M-urea or extremes of pH section 4.2.). α-crustacyanin nevertheless cannot be reformed from mixtures of the purified apo-units and carotenoid. It was possible therefore that another protein moiety, which remained undetected after electrophoresis, besides the five apo-units was necessary for the formation of α-crustacyanin, and that this protein remained attached to the carotenoid in 6M-urea to give the yellow protein. However, β-crustacyanin in the presence of 6M-urea also gives a yellow protein and the apo-units (section 4.4.6.). The purified individual apo-units

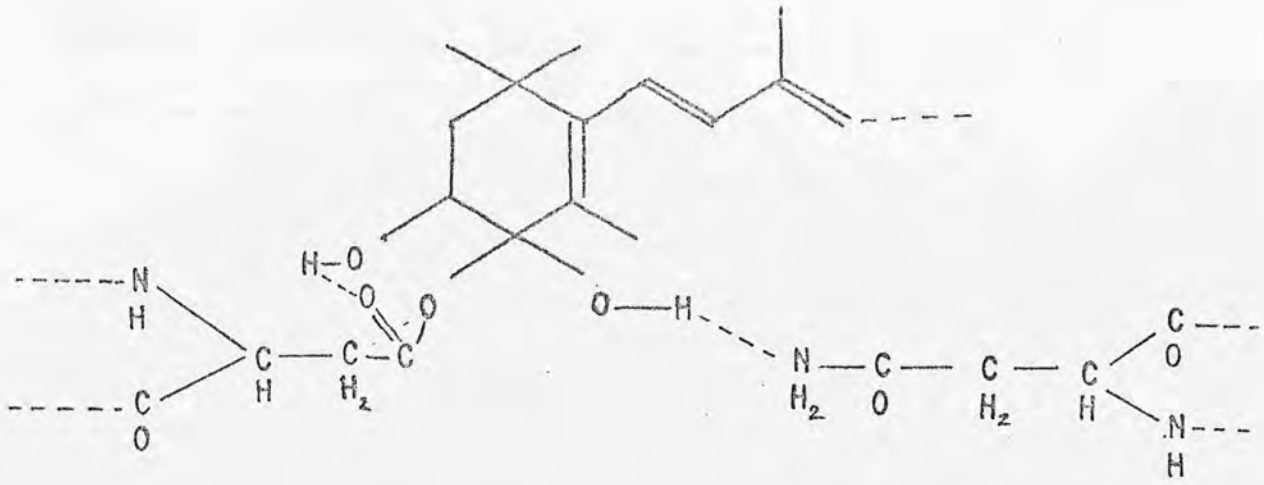
when recombined in pairs with astaxanthin do give rise to β-crustacyanin, the presence of the yellow protein being unnecessary. It is thought likely therefore, that the 'yellow protein' formed in 6M-urea is a non specific attachment of carotenoid to denatured apo-protein and that the five apo-units found in 6M-urea-starch gels are the only proteins necessary for the formation of α-crustacyanin. This of course does not mean that the sub-units are present as such in α-crustacyanin as they may have been formed at constant rates from the native sub-units on their disintegration.

A study of the amino acid content of the apo-units (section 4.2.6.), indicates a possible identity between components A_2 & A_3. This premise is upheld by the $S\Delta Q$ value which is 4. (Table 5). Marchalonis & Weltman, (1971), found that the $S\Delta Q$ value from amino acid analysis of different preparations of the same protein could have a value of 4 units. Their behaviour on Sephadex G. 75 is also similar and distinct from the C-units and A_1. Their different electrophoretic mobility at pH 8.35 and apparent difference in molecular size could simply be accounted for by a conformational alteration, although the former could be accounted for by a difference in aspartic acid content, if significant.

The amino acid compositions of C_1, C_2 & A_1 are also similar as can be seen from their behaviour in Sephadex G. 75, and the $S\Delta Q$ values given in Table 5. The possibility of their

being conformational isomers cannot therefore be overlooked, although the difference in lysine and aspartic acid may account for the difference in electrophoretic mobility.

The parameters of all sub-units, relating to gross three dimensional structure, such as % helix, fractional charge (maximum), average hydrophobicity and partial specific volume (Table 7) are similar as might be expected from proteins with a binding site for astaxanthin. These values all lie within the range for carotenoproteins (Zagalsky et al., 1970). From Table 6 it can be seen that the majority of the amino acids occur in normal amounts (i.e. within one standard deviation of the mean value from 100 proteins, Smith, 1966 and section 3.7). Phenylalanine, tyrosine and aspartic acid (+asparagine) however, consistantly appear in greater than average amounts. The occurrence in high amounts, of phenylalanine and tyrosine although common, is not general among carotenoproteins, but large amounts of aspartic acid seem to be characteristic of those so far studied (Zagalsky et al., 1970). In this context it is interesting to note that aspartic acid has been implicated in the bathochromic shift in rhodopsin, either in creating an acid environment around the prosthetic group (Kito et al., 1968) or enabling the Schiff's base to be protonated (Irving et al., 1970). It has recently been postulated that in crustacyanin aspartic acid and asparagine have a direct role in binding the carotenoid, as shown in the following diagram (Zagalsky & Herring, 1971) -



Tryptophane was not determined, but in view of the value of 2.5g/100g obtained by Kuhn & Kühn, (1967) would not be expected to significantly alter the above results.

It was hoped that relationships among the apo-units might be revealed by immunoelectrophoresis (section 4.2.7). Unfortunately no antibody formation could be detected by the methods used. It is thought that more decisive results may be obtained using the technique of anaphylactic shock, as greater amounts of antibody are produced in this state.

It is interesting to note that all five apo-units retain the ability to bind carotenoid and to dimerise to β-crustacyanin when recombined in pairs (section 4.3.3). These β-species are also stable once formed, and show no tendency to isomerise in the presence of 6M-urea, 0.05M-mercaptoethanol,

acetone, or storage under $(NH_4)_2SO_4$ (section 4.4.5). The β's obtained most readily from recombination (A_2 & A_3 with C_1, C_2 & A_1) are also those present in greatest amounts in preparations from α-crustacyanin. Homologous β's (e.g. C_1C_1 & A_2A_2) can be formed in recombination experiments from freshly prepared apo-units (section 4.3.3). These β's have not been isolated as such but their makeup can be supposed in that more than one blue β-sized product is formed from the combination of two apo-units with astaxanthin. In the few attempts of recombination with a single sub-unit (e.g. A_2 & A_3) no β-sized product was formed, only pink apo-sized proteins, which in the case of A_3 had $\lambda_{555} > \lambda_{280}$ so that a fair proportion of the apo-unit must have reacted. These sub-units however, had been stored in a freeze-dried condition and must therefore have undergone some conformational change which prevented dimerisation on combination with carotenoid. It is possible of course, that in these instances the carotenoid has not bound to its normal site but is less firmly attached to other apo-protein groups on the protein.

It seems possible therefore that there are only two real apo-units and that α-crustacyanin consists of 8 identical β-sub-units which are composed of one A_2/A_3 type sub-unit plus one $C_1/C_2/A_1$ type sub-unit. The amino acid composition of α-crustacyanin (an average of values published by Kuhn & Kühn, 1967;

Jencks & Buchwald, 1968b and Zagalsky et al., 1970) is compared to that of two theoretical α-crustacyanins; one a straightforward average of the two major apo-units (C_1 & A_2) and the second a mean of an average cathodic sub-unit ($C_1 + C_2 + A_1/3$) and an average anodic sub-unit ($A_2 + A_3/2$). The results are given in Table and the low S Δ Q value of under 4 might lend support to this supposition.

5.4. Enzyme digests.

Both α- and β-crustacyanin appear relatively stable to trypsin, α-crustacyanin especially being degraded to only a small extent. No high molecular weight coloured intermediates appear to be formed, since the breakdown products are colourless and not fractionated on Sephadex G.75 (mol. wt. below 10,000).

Chymotrypsin, while producing only small amounts of low molecular weight products does produce blue β-sized proteins with altered mobility in starch gels pH 8.35. Chymotrypsin breaks the peptide bonds adjacent to the amino acids phenylalanine and tyrosine, both of which occur in above average amounts in all the apo-units (Table 6, section 4,2,6). It is possible that they may be involved at the carotenoid binding site, providing for π-orbital overlap with the polyene chain, as suggested for the serum retinal binding protein by Kanai et al., (1968). Further investigation of the chymotrypsin digests of individual β-crustacyanins could possibly provide information on the binding site for carotenoid, and experiments in this line are being continued.

TABLE 10.

	apo 1	α	apo 2
arg	3.3	3.5	3.2
lys	6.4	5.6	6.1
his	1.5	1.8	1.6
asp	13.6	13.1	13.7
glu	9.6	10.2	9.7
thr	6.9	6.8	6.7
ser	8.2	7.8	7.8
pro	5.4	6.3	5.5
gly	5.6	5.2	5.7
ala	8.8	8.2	9.2
val	6.7	6.5	6.5
ile	3.9	4.2	4.0
leu	4.4	4.9	4.6
cys	1.9	1.7	2.0
met	0.3	0.4	0.5
tyr	6.9	6.9	6.8
phe	6.6	6.9	6.7

SΔQ values

apo 1	0		
α	3.4	0	
apo 2	0.7	3.2	0
	apo1	α	apo 2

Values for α-crustacyanin are an average of three published results :

Kuhn & Kühn, (1967); Jencks & Buchwald, (1968b) and Zagalsky et al., (1970).

apo 1 is an average of the two major apo-units $(C_1 + A_2/2)$.

apo 2 is an average of all the apo-units $(C_1 + C_2 + A_1/3) + (A_2 + A_3/2)/2$.

Values are in moles %

SΔQ values calculated from Marchalonis & Weltman (1970).

5.5. γ-crustacyanin.

γ-crustacyanin (λ_{max} 625) as seen from 6M-urea starch gels contains the same apo-units as α-crustacyanin. It breaks down more easily to β-crustacyanin and is therefore suggested as being a slightly denatured form. Although it is excluded from Sephadex G.200 (Cheesman et al., 1966) its mol. wt. is similar to that of α-crustacyanin (Table 1; Jencks & Buchwald, 1968b) it must consequently be a more asymmetrical molecule. If α-crustacyanin is, as has been suggested (Cheesman et al., 1966), a circular molecule of 8 β-units then, γ-crustacyanin might be envisaged as a linear molecule of 8 β-units. This could explain both the low stability and high asymmetry of the molecule.

SUMMARY

1. A modification procedure for coping with large quantities of lobster carapace has been developed for the purification of crustacyanin. The characteristics of the protein are indistinguishable from those previously reported in the literature.

2. Different methods for the separation of α-crustacyanin from β- and γ-crustacyanin have been compared.

3. α-crustacyanin appears to be a homogeneous fraction, not a series of closely related proteins.

4. Five apo-units have been prepared, in a state homogenous to electrophoresis, from α-crustacyanin, by gradient elution from DEAE-cellulose, after removal of the astaxanthin by acetone or 6M-urea. A numbering system (C_2, C_1, A_1, A_2, & A_3) has been given.

5. The five apo-units are also present when the carotenoid is removed by extremes of pH.

6. The apo-protein is not homogeneous to gel filtration on Sephadex G. 75. C_1, C_2 & A_1 being eluted in front of A_2 & A_3.

7. The amino acid composition of each apo-unit has been studied, compared to that of the 'average protein', and their relatedness estimated. The possible significance of the high tyrosine, phenyl-alanine and aspartic acid contents is mentioned, and the likelihood of the five apo-units being conformational isomers of two proteins is discussed.

8. The apo-units remain as unique entities following electrophoresis in 6M-urea-starch gels containing 0.05M-mercaptoethanol. The possibility that the isomers could be formed by disulphide interchange is therefore discounted.

9. It was hoped to be able to test the relationships between the apo-units by immunological techniques, but unfortunately no antibody formation could be detected.

10. Recombinations of the apo-proteins with various synthetic carotenoids were attempted. C_4 & C_4' oxygen functions were essential for the formation of a dimer; confirming earlier results. The 15 - 15' dehydrocarotenoids did not recombine, and the possible reference this may have to the binding site is discussed.

11. The apo-units show no tendency to dimerise in the absence of carotenoid. Preferred β-crustacyanins are formed between C_1 C_2 of A_1 and A_2 of A_3 in the presence of astaxanthin. Other dimers can be formed, although only with freshly prepared apo-units.

12. In contrast the apo-crustacyanin, β-crustacyanin is homogeneous following gel filtration on Sephadex G. 75.

13. β-crustacyanin can be separated into two fractions by preparative column electrophoresis. Both are heterogeneous on starch gel electrophoresis.

14. Four β-crustacyanin fraction are obtained by gradient elution from DEAE-cellulose, two of which are homogenous to electrophoresis and have been termed β_3 & β_4, and two of which are heterogeneous, showing two components in electrophoresis: β_{1a+b} and β_{2a+b} respectively.

15. These six β-forms are present in the various methods of preparation of β-crustacyanin.

16. The two heterogeneous fractions were separated into their components by a) Pevikon electrophoresis b) electrophoresis in cellogel blocks and c) rechromatography on DEAE-cellulose. In all cases the major fractions (β_{1a} & β_{2a}) were easily prepared in a pure form, but only small quantities of β_{1b} and β_{2b} are obtained.

17. The sub-unit composition of each β-fraction has been demonstrated by dissociation and electrophoresis in 6M-urea-starch gels.

18. The β-fractions β_4 & β_{2a} are not interconvertible in 0.05M-mercaptoethanol, or after removal of the carotenoid in 6M-urea, or acetone.

19. In 6M-urea two protein fractions are produced from β-crustacyanin after gel filtration on Sephadex G.75. A yellow fraction (λ_{max} 440) which is excluded from the gel and a pink (λ_{max} 550) apo-sized fraction.

20. Chymotrypsin and trypsin do not extensively degrade α- and β-crustacyanin. Chymotrypsin produced coloured fractions which are eluted in the same elution volume as β-crustacyanin from Sephadex G.75, but migrate with a different mobility in starch gel electrophoresis. This work is being continued.

21. γ-crustacyanin has been prepared homogeneous to cellogel electrophoresis. It has a similar composition in 6M-urea-starch gels to α-crustacyanin, but breaks down to the β-forms more easily.

22. The relationship of the apo-units to α-, β- & γ-crustacyanins is discussed.

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