PROSTHETIC GROUPS IN ALGAL BILIPROTEINS

by

Kenneth Turner Salmon, B.Sc.

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To MY WIFE, ELIZABETH AND MOTHER.

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SUMMARY

The Red alga, Porphyridium cruentum was successfully grown in an artificial sea-water medium. 20-litre cultures were aerated with air containing 5% carbon dioxide and the alga was ready for harvesting after 6-8 weeks growth.

B-phycoerythrin was extracted from the Porphyridium cruentum by subjecting the alga to ultrasonic oscillation followed by freezing and thawing. This treatment brought about cell rupture with consequent release of the biliproteins into solution. The crude biliprotein solution was subjected to a series of purification techniques including centrifugation, celite filtration, ammonium sulphate precipitation, tricalcium phosphate-celite chromatography, and fractional ammonium sulphate precipitation. The final purification stage resulted in the formation of large crystals of B-phycoerythrin and several types of crystal shape are illustrated. The variation in crystal shape is discussed and lends support to the postulation of Bouillene-Walrand and Delarge (1937) that variation in crystal shape may be due to differences in pH in the crystallizing biliprotein solution. Repeated recrystallization of the B-phycoerythrin did not produce any significant alteration in the visible absorption spectrum.

Amino acid analysis of B-phycoerythrin indicated that 80% was recoverable as amino acids after acidic hydrolysis. In common with most amino acid analyses of biliproteins, the content of acidic amino acids is

considerably greater than the content of basic amino acids.

Enzymic digestion of the crystalline biliprotein with pepsin and trypsin produced several chromopeptide fragments. The techniques used to purify the chromopeptide material included celite partition chromatography, molecular sieve gel chromatography, ion-exchange column chromatography, high voltage electrophoresis and thin layer chromatography on Silica Gel plates. Amino acid analyses of the separated chromopeptides indicated a core peptide with the following composition: Asp_2 Thr Ser_2 Glu Pro Gly_2 Ala_2 Val_2 Ile Leu_2. There is also evidence to suggest that serine or aspartic acid residues are the N- or C- terminal residues of this core chromopeptide. The purification techniques used did not allow complete isolation of the chromopeptides and the reasons for various peptide contaminations are discussed.

One of the major chromopeptide fragments was subjected to treatment with lithium and sodium borohydride in an attempt to determine the nature of the chromophore-apopeptide linkage. A decrease in the amount of threonine present in the chromopeptide after borohydride treatment and acidic hydrolysis indicated that there may well be a threonyl ether linkage present in the chromopeptide. This postulation is supported by the appearance of quantities of α-amino butyric acid in the acid hydrolysate.

Borohydride treatment also produced a decrease in

the quantity of aspartic acid residues present and this would indicate a second chromophore-apopeptide linkage between the ω-carboxyl group of aspartic acid and a hydroxyl group on the chromophore.

INTRODUCTION

Description and Occurrence of the Biliproteins

Photosynthetically active red and blue biliproteins, called phycoerythrins and phycocyanins, respectively, have been isolated only from algae. Their prosthetic groups or chromophores are tetrap yrroles known as phycobilins. Unlike the chlorophylls, phycobilins are not readily released from associated proteins; consequently it is the biliproteins rather than the free phycobilins which have been most generally studied. Algal biliproteins, which are of general occurrence in the Rhodophyta (Red Algae), Cyanophyta (Blue-green Algae), and Cryptophyta (Cryptomonad Algae), have also been reported in one or two members of the Chlorophyta. Table 1 lists some of the algae whose biliproteins have been studied. The first algal biliprotein to be studied was in 1836 by von Esenbeck, who reported the appearance of a blue colouring matter on autolysis of an Oscillatoria species. A suggestion that phytochrome (a chromoprotein of higher plants) was related to the algal biliproteins, particularly in the similarity of their prosthetic groups, was confirmed by Siegelman et al. (1965).

The biliproteins are localized in the lamellae of chloroplasts of the Red Algae (Haurowitz, 1958; Brody and Vatter, 1959.); electron micrograph studies indicate that Blue-green Algae also contain chloroplast - like structures (Elbers et al., 1957), and it seems likely that the

chromoproteins are located in their lamellae, rather than in free chromatophores or in the cytoplasm (Thomas and de Rover, 1955.). The chloroplasts are the photosynthetic structures of the algae and the biliproteins are in fact accessory pigments in photosynthesis.

Nomenclature

Several terms have been used to describe phycoerythrins and phycocyanins in general. The terms included "phycochromoproteids" (Kylin, 1910), "bilichromoproteins" (Haxo et al., 1955), "tetrapyrryl proteins" (Haurowitz, 1958), "biliproteins (O'hEocha, 1958) and "phycobiliproteins" (Bogorad, 1965). The term "biliprotein" is now the most generally accepted; the prefix "bili-" indicates the relationship of the chromophore groups of these proteins to the bile pigments.

The system of naming the individual biliprotein members originated with Svedberg and Katsurai (1929). At that time only four biliproteins were known and these phycoerythrins and phycocyanins were designated with an R- or C- prefix according to their origin from a Red (Rhodophyta) or Blue-green (Cyanophyta) alga, respectively. More recently other spectrally different biliproteins were recognised. A phycoerythrin from Smithora naiadum (Bangiales) was termed B-phycoerythrin by Airth and Blinks (1956). The name "P-phycocyanin" was given to a biliprotein occurring in many Red and Blue-green algae (Blinks, 1954; Haxo et al., 1954.) and the prefix - an abbreviation of "pan" - was

TABLE I OCCURRENCE OF VARIOUS BILIPROTEINS

ALGAL GROUP AND SPECIES	BILIPROTEINS	REFERENCES
RHODOPHYTA		
CLASS : BANGIOPHYCEAE		
ORDER : PORPHYRIDIALES		
Porphyridium cruentum	B-PE; R-PC; Allo-PC.	Haxo et al (1955) O'hEocha (1958)
ORDER : BANGIALES		
Porphyra tenera	R-PE; C-PC; Allo-PC.	Hattori and Fujita (1959,c)
Porphyra perforata	R-PE; R-PC; Allo-PC.	Jones and Blinks (1957)
Smithora naiadum	B-PE; C-PC; Allo-PC.	Airth and Blinks (1956)
CLASS : FLORIDEOPHYCEAE		
ORDER : NEMALIONALES		
Rhodochorton rothii	R-PE; PC	O'hEocha (1958)
Rhodochorton floridulum	B-PE; PC	O'hEocha and O'Carra (196[illegible]
ORDER : GIGARTINALES		
Plocamium pacificum	R-PE	O'hEocha (1958)
ORDER : CRYPTONEMIALES		
Grateloupia sp	R-PE; C-PC; Allo-PC	Hattori and Fujita (1959,c)
ORDER : RHODOMENIALES		
Rhodomenia palmata	R-PE; R-PC; Allo-PC	O'hEocha (1960)
ORDER : CERAMIALES		
Ceramium rubrum	R-PE; R-PC; Allo-PC	Svedberg and Katsurai (1929)
Polysiphonia urceolata	R-PE; C-PC; Allo-PC	Hattori and Fujita (1959,c
CYANOPHYTA		
Tolypothrix tenius	C-PE; C-PC; Allo-PC	Hattori and Fujita (1959,c
Arthrospira maxima	C-PC; Allo-PC.	O'hEocha (1958)
Phormidium ectocarpi	C-PE	O'hEocha (1960)
Anabaena cylindrica	C-PC; Allo-PC	Halldal (1958)
CRYPTOPHYTA		
Hemiselmis virescens	PC	Allen et al (1959)
Cryptomonas ovata	PE	Haxo and Fork (1959)
Sennia sp	PE; PC.	O'hEocha and Raftery (1959)
Cynidium caldarium	C-PC	Allen et al (1959)
P.E. ≡ phycoerythrin		P.C. ≡ phycocyanin

chosen as an indication of its wide-spread distribution. This biliprotein was later renamed "allo-phycocyanin" by Haxo et al. (1955) when they suggested that this phycocyanin was probably identical to the allophycocyanin of Lemberg and Bader (1933), who had assumed that this was not a naturally occurring biliprotein, but an artifact produced by the denaturation of C-phycocyanin during extraction. In 1959 three authors reported finding biliproteins in members of the division Cryptophyta (Cryptomonad Algae) (Allen, Dougherty and McLaughlin, 1959; Haxo and Fork, 1959; O'hEocha and Raftery, 1959) and five years later O'hEocha et al. (1964) reported three phycoerythrins and at least three phycocyanins from various Cryptophyta. These biliproteins were all spectrally distinct from those encountered in the Red and Blue-green Algae. It became apparent that the occurrence of any given biliprotein was not limited to any one division and the designations of prefixes were applied instead to spectral varieties regardless of the algal group from which they were isolated. Figs. 1 and 2 illustrate typical absorption spectra of phycoerythrins and phycocyanins and ultraviolet and visible spectra are discussed later in the introduction (see Physical Properties of the Biliproteins).

Factors Influencing Growth of Algal Biliproteins

Studies performed on a large number of algae indicated that various factors influenced their growth rate and biliprotein yield. These factors included growth media, temperature, carbon dioxide supply and nature of illumination.

For marine algae these conditions obviously cannot be controlled, and differences in biliprotein content is likely to be due to a combination of factors affecting growth. In naturally occurring algae these factors can be classified as seasonal variations e.g. in Ceramium rubrum the biliprotein content accounted for 1.9% of the dry weight in December and January, but in March this yield was approximately halved (Lemberg, 1928). The proportions of biliproteins in marine algae are affected by ecological factors as well as seasonal variations e.g. the phycoerythrin content of deep growing algae is greater than in species growing in intertidal or upper sublittoral levels (Kylin, 1937; Jones and Blinks, 1957). This difference can probably be attributed to the nature of the light available at these greater depths. The green radiation in sunlight penetrates more deeply than the red or blue radiation due to the selective absorption and scattering of the light by marine plankton and suspended matter. Hence, as phycoerythrins are more efficient absorbents of green radiation the proportion of phycoerythrins to phycocyanins is found to increase with depth.

Growth factors in cultured algae have been examined in much greater detail than in marine algae. Brody and Emerson (1959) reported that phycoerythrin production in Porphyridium cruentum was stimulated at low intensities more by green light than by blue light. These results partially supported the observations made by Engelmann (Engelmann, 1883; Engelmann and Gaidukov, 1902) who

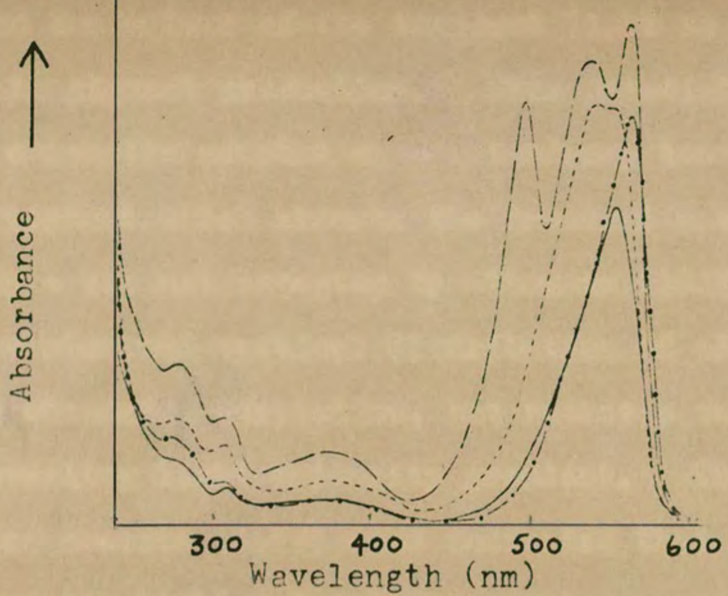


FIG.1. Absorption spectra of Phycoerythrins

----- R-phycoerythrin (Ceramium rubrum)

········ B-phycoerythrin (Porphyridium cruentum)

-·-·- C-phycoerythrin (Phormidium persicinum)

_____ Cryptomonad phycoerythrin (Hemiselmis rufescens)

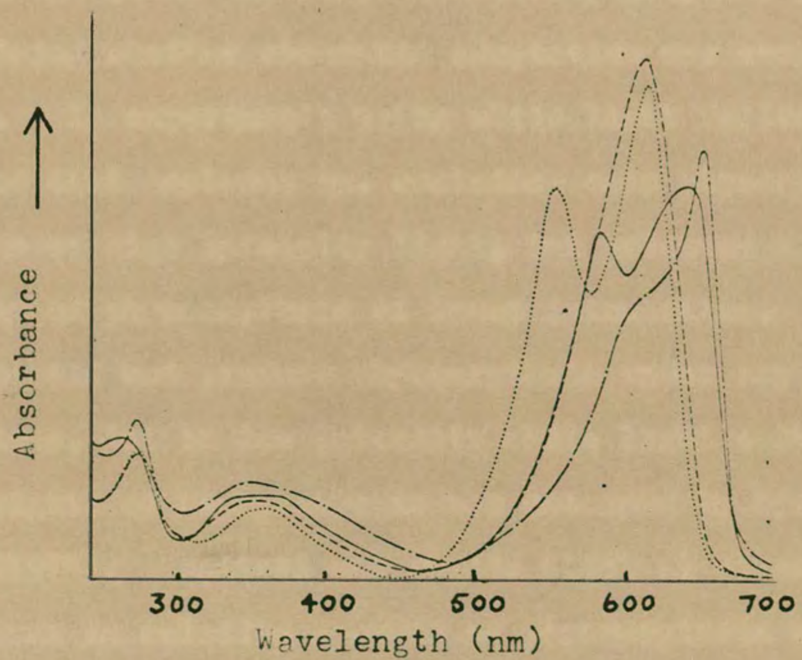


FIG.2. Absorption spectra of Phycocyanins

..... R-phycocyanin (Porphyra laciniata)

------ C-phycocyanin (Nostoc muscorum)

-·--·- Allo-phycocyanin (Nostoc muscorum)

_____ Cryptomonad phycocyanin (Hemiselmis virescens Droop)

regarded the colour of the incident light as a significant factor in pigment production. This principle of complementary chromatic adaptation - a tendency towards formation of the pigment which absorbs the incident wavelengths most strongly - was not accepted by Oltmanns (1923) who argued that the observed vertical distribution of algae did not involve chromatic adaptation, but rather a response to the prevailing light intensity gradient. The results of Brody and Emerson (1959) also indicated that at high monochromatic light intensities counter - complementary chromatic adaptation took place, i.e. high intensity green light decreased the proportion of phycoerythrin in Porphyridium cruentum cells, whereas high intensity blue light increased the proportion of phycoerythrins. These authors concluded that light intensity played a part in determining whether chromatic adaptation was complementary or not. In experiments using white light Halldal (1958) reported that Anabaena sp. contained only phycocyanin when grown under high intensity light, but produced a small quantity of phycoerythrin at lower intensities. Anacystis nidulans did not produce phycoerythrin under any light conditions, but in low intensity white light a 24% yield (dry weight) of C-phycocyanin was obtained by Myers and Kratz (1955). Hattori and Fujita (1959 a) observed variations in relative amounts of phycoerythrin and phycocyanin in Tolypothrix tenuis depending on whether the alga was grown under fluorescent or incandescent light. These authors investigated other factors involved in

biliprotein formation in Tolypothrix tenuis. Pre-illuminated nitrogen - deficient cultures of this alga formed biliproteins in the dark when nitrate was added to the medium, and the proportions of the biliproteins formed were determined by the wavelength of light used for pre-illumination (red light favoured phycocyanin, green favoured phycoerythrin production). In cultures grown heterotrophically in darkness, phycocyanin but not phycoerythrin was formed. On the other hand, if after heterotrophic growth in darkness in the absence of a nitrogen source the unpigmented algae were exposed briefly to green light, phycoerythrin was formed when nitrogen was supplied.

These experiments performed by Hattori and Fujita (1959,b) also serve to illustrate the essential nature of nitrogen requirement in biliprotein formation. Heterotrophic growth in Tolypothrix tenuis was accomplished using ammonium sulphate or a mixture of amino acids as nitrogen source (Kiyohara et al., 1960) Nitrate, ammonium sulphate and L-asparagine were all found to be suitable nitrogen sources for growth of Porphyridium cruentum (Jones, Speer and Kury, 1963). The Blue-green Alga, Anabaena cylindrica assimilated elementary nitrogen and the rate of nitrogen assimilation was found to be temperature dependent (Fogg and Than-Tun, 1960). Several other examples exist of temperature effect on algal growth rate or yield, and Garnier (1962) observed that in Oscillatoria subbrevis temperatures >35°c caused an inhibitory effect on algal growth. The cryptomonad alga

Cyanidium caldarium was one of several algae cultured by Hoogenhout and Amesy (1965) with a view to comparing the growth rates over a range of temperature. They concluded that the optimum temperature for growth was 39 - 41°c. However, this was not in agreement with Allen (1959) who claimed that the optimal temperature for growth of Cyanidium was in the range from 45 - 50°c.

Since the length of the exponential growth phase of Porphyridium cruentum seemed to be limited by the lack of carbon dioxide Jones et al. (1963) prolonged the initial growth rate by aerating the medium with 1% carbon dioxide in air. A suggestion was made by Bidwell and Craigie (1963) that certain marine algae could only utilize bicarbonate for photosynthesis, but McLachlan (1963) argued that the kinetics for the conversion of carbon dioxide to bicarbonate in an alkaline medium were such that bicarbonate could not limit growth provided sufficient carbon dioxide was present. However, the initial growth phase in many algae may be delayed until a sufficient concentration of bicarbonate is formed to induce rapid photosynthesis. A stream of air enriched with 5% carbon dioxide was used by several workers as an efficient method of aeration and agitation of culture media (e.g. Hoogenhout and Amesy, 1965; Paterson, 1967).

Phosphorus is one of the major mineral requirements for normal algal growth, but variation in quantity does not seem to affect the relative amounts of biliproteins formed. However, deficiences in either sodium or molybdenum decreased the yield of phycocyanin in Blue-

green Algae (Fogg, 1952; Allen and Arnon, 1955). Fogg (1963) suggested that molybdenum was involved in nitrate reduction to ammonia in the nitrogen fixation process and this is likely to be one reason for trace requirements of this element. Iron deficiencies also give rise to a reduction in biliprotein yield (Boresch, 1921). Wide variations in the magnesium-to-calcium ratio has little effect on growth in algae examined by McLachlan (1963), except for Porphyridium sp. which has a high magnesium requirement. On the other hand, this alga has no requirement for potassium. Various other metals are required in trace amounts e.g. manganese, vanadium, cobalt, zinc, copper and boron, but no correlations, linking these metals with the relative proportions of biliproteins produced, have been obtained.

In this laboratory vitamin B_{12} was found to increase the growth rate of Porphyridium cruentum and it is probably the case in general that optimum quantities of trace nutrients affect the growth rate rather than the yield or relative proportions of biliproteins. In the Blue-green Alga, Anabaena cylindrica, optimum concentrations of the various micro nutrients induced a two hundred-fold increase in the growth rate.

From the observations made above, it is apparent that growth, in terms of rate, yield and relative biliprotein content, varies considerably according to the nutrients present in the medium as well as depending on light conditions, aeration and temperature of culture.

The optimum growth conditions for any given alga can only be obtained by careful variation of all the above mentioned growth factors.

Extraction and Purification of the Biliproteins

To release the biliproteins from their intracellular locations it is necessary to rupture the cell walls of the algae. Cell breakdown, with consequent release of the biliproteins into the surrounding aqueous medium, has usually been brought about by mechanical methods. Grinding with an abrasive agent or maceration in a blender have been used to achieve this in the larger and more pliant forms of algae. The most practical methods of cell rupture in smaller species have been ultrasonic oscillation and repeated freezing and thawing. The fluorescing biliprotein solution is usually centrifuged to remove larger cell fragments before further purification.

Early methods of purifying this crude biliprotein solution involved fractional precipitation with ammonium sulphate. Svedberg and Lewis (1928) separated phycocyanin from phycoerythrin in Ceramium rubrum by this method. However, this was a long and tedious process and was eventually replaced by adsorption chromatography on columns of tricalcium phosphate (Swingle and Tiselius, 1951). Prior to this great advance Tiselius (1948) described a method whereby proteins and substances of high molecular weight were often adsorbed quite strongly on some adsorbents at moderately high salt concentrations. To separate phycoerythrin from Ceramium rubrum Tiselius

used filter paper as adsorbent and varied the concentration of ammonium sulphate, but the need for a more efficient adsorbent, which could be adapted to column chromatography, was recognised. The following year Svensson and Brattsten (1949) developed an apparatus for continuous electrophoretic separation on glass powder with phosphate buffers. This principle was also adopted on columns of glass powder by Haglund and Tiselius (1950), who separated a mixture of phycoerythrin and phycocyanin by this method.

In 1951 Swingle and Tiselius showed that biliproteins could be separated by adsorption chromatography on columns of tricalcium phosphate gel and this technique is still in everyday use (e.g. Haxo, O'hEocha and Norris, 1955; Hattori and Fujita, 1959; Brody and Brody, 1961.) (see Experimental section on tricalcium phosphate chromatography). Crude biliprotein solutions were applied to the column and adsorbed onto the tricalcium phosphate. Development of the column with phosphate buffers of increasing phosphate concentration resulted in separation of the various biliproteins. Buffer and protein solutions containing 1% sodium chloride were observed to increase the protein adsorption on the column, and generally phycoerythrins were eluted before phycocyanins.

Other techniques in biliprotein purification include the use of ion-exchange columns (Boman, 1955). This author demonstrated the separation of phycoerythrin from phycocyanin on the anion exchange resin Dowex - 2 using tris - HCl buffers, pH 7.2, at 4°c. Proteins were

adsorbed at low ionic strength and eluted by a step-wise increase in concentration of the buffer. In 1958 another method of separation appeared due to Hjerten based on free zone electrophoresis principles and performed in a horizontal, rotating tube. Molecular sieve chromatography of proteins was introduced by Porath and Flodin (1959). The method was based on a column procedure in which the stationary phase comprised a new type of gel consisting of cross-linked hydrophilic chains. These gels were prepared by cross-linking dextran and were hydrophilic by nature of their high concentration of hydroxyl groups. Aqueous protein solutions filtered rapidly through the gel, larger molecules migrating without retention but particles of low or intermediate molecular weight penetrate the gel to an extent determined by their molecular dimensions and the degree of cross-linking of the gel. (see Experimental section on Gel Chromatography). Nultsch (1962) used this method to purify biliproteins from Phormidium autumnale, and observed that phycocyanins migrated faster than phycoerythrin in this alga. A similar column material was developed by Hjerten and Mosbach (1962) and several proteins were chromatographed successfully on elution with 0.05M sodium phosphate buffer, pH 7.5. A recent development in purification of algal biliproteins by molecular-sieve chromatography involved the use of the weak anion exchanger, DEAE cellulose (diethyl aminoethyl-cellulose), which allowed ion exchange and molecular size fractionation on the same column (Eriksson and Halldal, 1965). These authors separated

phycoerythrin from phycocyanin by eluting the biliprotein solution with 0.01M tris-HCl buffer, pH 7.2, containing a straight line gradient increase in sodium chloride concentration up to 0.5M NaCl. Another technique recently described by Fujimori and Pecci (1967) involved a two-phase liquid system of n-butanol and water in which the biliproteins were partitioned into the aqueous phase.

Apart from ammonium sulphate precipitation and tricalcium phosphate chromatography, most other techniques have limitations in preparative scale purification and are best regarded as a means of purity investigation.

Crystallization of the Biliproteins

Crystalline biliproteins have been obtained by ammonium sulphate precipitation of sufficiently purified algal extracts. Before the introduction of tricalcium phosphate chromatography Svedberg and Lewis (1928) crystallized R-phycoerythrin and R-phycocyanin from Ceramium rubrum by fractional precipitation with ammonium sulphate. Another method using ammonium sulphate and rivanol fractionation was put forward by Fujiwara (1955) and proved successful in crystallizing R-phycoerythrin and C-phycocyanin from Porphyra tenera. Phycoerythrin crystallized as needles or prisms, phycocyanin as needles or platelets. Airth and Blinks (1957) did a series of experiments on Smithora naiadum and made the surprising discovery that fresh preparations of B-phycoerythrin could not be crystallized by ammonium sulphate precipitation, but if the protein solutions were allowed to "age" at room temperature, then

crystallization readily occurred. This suggested a change in the protein portion of the phycoerythrin molecule. Hattori and Fujita (1959 a) used a combination of tricalcium phosphate chromatography and ammonium sulphate precipitation to obtain crystalline preparations of C- and allo-phycocyanin and C- phycoerythrin from Tolypthrix tenuis. Butanol extraction and fractional precipitation was employed by Leibo and Jones (1963) to obtain crystalline B- phycoerythrin from Porphyridium cruentum, and they estimated that four litres of this cultured alga yielded approximately 50mgs. of purified phycoerythrin. R-, B-, and C- phycoerythrin and R- and C- phycocyanin were isolated and purified on a preparative scale by tricalcium phosphate chromatography, ammonium sulphate fractionation and crystallization in 1965 by O'Carra. O'hEocha and Raftery (1965) isolated and purified R- phycoerythrin from Ceramium rubrum in a very similar manner and as a further criterion for purity starch gel electrophoresis was carried out. When the ratio of the extinction of the main peak in the visible region to that of the protein peak at 278nm was greater than 4, the biliproteins were considered highly purified. This provides a useful, if somewhat arbitrary, criterion for purity in biliprotein solutions. Table 2 lists a selection of biliproteins crystallized from various algal sources and an interesting feature is the difference in crystal shapes of the biliproteins. These differences were first noted by Bouillene-Walrand and Delarge (1937) who suggested that crystal structure variations could be the result of pH differences in the crystallizing biliprotein solution.

Physical Properties of the Biliproteins

(a) Colour and fluorescence

The colour of the algae containing the biliproteins is either red or blue-green and the protein pigments themselves contribute to this colour. This effect can be demonstrated by observing the correlation between the absorption spectra of intact algal cells and biliprotein solutions.

In vivo biliproteins show very little fluorescence, but when they are released into aqueous solution by rupture of the algal cell, they are brilliantly fluorescent (McLindon and Blinks, 1952). The phycoerythrins form red solutions which emit orange fluorescence, with the maximum at 578 to 580nm (French et al., 1956). Aqueous solutions of phycocyanins are blue and they emit red fluorescence. The allo-phycocyanin fluorescence maximum was reported to be at 663nm while the maximum for C-phycocyanin from Smithora naiadum lay at 637nm (French and Young, 1956; French et al., 1956). However, Duysens (1952) obtained a fluorescence maximum of 650-5nm for C-phycocyanin from Oscillatoria sp., while Fujimori and Quinlan (1963) reported the maximum of this phycocyanin from Anacystis nidulans to be at 640nm. In a study by Berns, Crespi and Katz (1963) various maxima between 637 and 680nm were observed for the phycocyanins. The fluorescence maxima of several cryptomonad biliproteins were examined by O'hEocha, O'Carra and Mitchell (1964) and phycoerythrin isolated from Hemiselmis rufescens gave a fluorescence peak at 580nm; phycocyanin from Chroomonas

TABLE 11 - Examples of Crystalline Structures of Biliproteins.

ALGAL SOURCE	BILI-PROTEIN	CRYSTAL SHAPE OR STRUCTURE	REFERENCE
Porphyra tenera	R-PE	needles or prisms	Fujiwara (1955)
Tolypothrix tenuis	C-PE	thin needles	Hattori and Fujita (1959,a)
Porphyridium cruentum	B-PE	orthorhombic	Leibo and Jones (1963)
Porphyra laciniata	R-PE	broad red needles	O'Carra (1965)
Ceramium rubrum	R-PE	hexagonal prisms	O'Carra (1965)
Rhodochorton floridulum	B-PE	sheaves of fine needles	O'Carra (1965)
Porphyra tenera	C-PC	needles or platelets	Fujiwara (1955)
Tolypothrix tenuis	C-PC	thin platelets	Hattori and Fujita (1959,a)
Tolypothrix tenuis	Allo-PC	thin platelets	Hattori and Fujita (1959,a)
Porphyra laciniata	R-PC	rhombohedric plates	O'Carra (1965)
Nostoc muscorum	C-PC	hexagonal plates	O'Carra (1965)

PE = phycoerythrin ; PC = phycocyanin.

sp. had a maximum at 660nm while the fluorescence peak for phycocyanin from the Plymouth strain of Hemiselmis virescens was at 637nm.

These and other observations on fluorescence maxima revealed similarities within the phycoerythrin and phycocyanin pigments and indicated common prosthetic groups.

(b) Visible and Ultraviolet Absorption

As stated previously, the various algal biliproteins are distinguished on the basis of differences in their absorption spectra. There is evidence, which will be mentioned later, that biliproteins isolated from the same algal source can display spectral variations depending on the method of preparation used. However, to avoid confusion, these spectral variations will be overlooked in the grouping of biliproteins below.

In the Rhodophyta and Cyanophyta the phycoerythrins (red) and phycocyanins (blue) are designated as R-, B-, C- and R-, C-, allo- according to the number and position of their absorption maxima. Phycoerythrin biliproteins have from one to three absorption maxima in the visible region, and in addition three maxima in the ultraviolet region (275 to 280 nm, 305 to 310nm, and 370nm). Phycocyanins have either one or two absorption maxima in the visible region and only two peaks in the ultraviolet (270 to 280nm, and 350nm). Typical absorption spectra for phycoerythrins and phycocyanins are given in figs. 1 and 2, and visible absorption maxima values are as follows:

Biliprotein	Wavelengths (nm)
C-phycoerythrin	565
B-phycoerythrin	565; 545; shoulder at 495-500
R-phycoerythrin	565; 545; 495
C-phycocyanin	615
R-phycocyanin	615; 553
allo-phycocyanin	650; shoulder at 620

C-phycoerythrins, found only in the Cyanophyta, have a single absorption maximum in the visible region and this is attributed to their prosthetic groups having a specific interaction with the apoprotein (that part of the protein chain immediately adjacent to the chromophore group). Variations in the maximum value reported for this biliprotein range from 560 to 563nm (e.g. Nultsch, 1962; O'Carra, O'hEocha and Carroll, 1964).

Airth and Blinks (1956) were first to isolate and name B-phycoerythrin from Smithora naiadum, the two-peaked phycoerythrin involved in the present investigation. These authors observed that the peak at 565nm disappeared when the biliprotein was recrystallized several times at its isoelectric point (pH 4.5). B-phycoerythrin has been reported from other members of the Rhodophyta including Porphyridium cruentum (Haxo, O'hEocha and Norris, 1955) and Rhodosorus marinus (Giraud, 1959). There is general agreement on the position of the main absorption at 545nm in Porphyridium cruentum, but the second peak appears to be variable e.g. a definite maximum at 565nm was reported by Fujimori and Quinlan (1963), while Haxo, O'hEocha and

Norris (1955) found a plateau ranging from 550 to 557nm.

R-phycoerythrin solutions give the most complex visible spectra which is probably due to the chromophore groups being located in two different protein environments and the existence of more than one type of chromophore group (O'hEocha and O'Carra, 1961; O'Carra, 1965). This biliprotein is of wide occurrence in the Rhodophyta (see Table 1) and its spectral variations generally involve the absorption maximum at 545nm.

The spectral differences in the above biliproteins were attributed to variations in extraction and purification procedures, algal source, pH, and contamination by other biliproteins. Spectra for phycocyanins from the Red and Blue-green Algae were subject to similar variations.

As mentioned earlier, cryptomonad biliproteins were first isolated from Cryptophyta in 1959. Three phycoerythrins and three phycocyanins which are spectrally distinct from the Red and Blue-green algal biliproteins are now recognised. On the basis of their spectral characteristics, cryptomonad phycoerythrins were subdivided into three categories (O'hEocha, O'Carra and Mitchell, 1964) according to their visible absorption maxima:

Cryptomonad phycoerythrins	Wavelength (nm)
Type a	$544^{\pm}1$ (in some species there is also a shoulder at 560-570
Type b (e.g. Cryptochrysis sp.)	$555^{\pm}2$
Type c (e.g. fresh water cryptomonas ovata var. palustris)	565-8

Cryptomonad phycoerythrins contain phycoerythrobilin as their prosthetic group (O'hEocha and Raftery, 1959) and results from five different cryptomonad algae indicated that two dissimilar types of prosthetic groups were present in the phycocyanin biliproteins (O'hEocha, O'Carra and Mitchell, 1964). The phycocyanins purified to date are also grouped on the basis of their visible absorption characteristics as follows:

Cryptomonad phycocyanins	Wavelength (nm)
Type (1) (e.g. Hemiselmis virescens Droop	583; 645; shoulder at 620-5
Type (2) (e.g. Plymouth strain No.157	585; 615
Type (3) (e.g. Cryptomonas cyanomagna	583; 625-30.

Ultraviolet spectra of the cryptomonad biliproteins have not been investigated thoroughly, but it appears that they are similar to the spectra of the Red and Blue-green Algae in that spectral variations between different biliproteins are much more pronounced in the visible region. However, the aromatic amino acids of proteins absorb in the region of 280nm and so the ultraviolet spectra can be used in determining the purity in any biliprotein solution. In the case of B-phycoerythrin the spectral ratio is calculated by dividing the absorption at 545nm by the absorption at 280nm. A low value indicates contamination by other species (e.g. colourless proteins, aromatic amino acids, other biliproteins). This ratio is mentioned frequently in the experimental section, where a value of four or greater is considered to indicate high purity.

(c) Molecular Weights and Dissociations of the Biliproteins

Svedberg and co-workers (Svedberg and Lewis, 1928; Svedberg and Katsurai, 1929; Svedberg and Eriksson, 1932) applied their ultracentrifugation technique to a study of the molecular weights of biliproteins under various conditions. They found that molecular weights varied with pH, the greatest stability occurring at the isoelectric point of the protein. Changes in pH of the biliprotein solution can cause dissociation of the molecule into subunits. Eriksson-Quensel (1938) used the ultracentrifugal technique to determine the molecular weight of R-phycoerythrin, and the reported value of 291,000 is in close agreement with that found by Nolan and O'hEocha (1967) who performed molecular weight determinations by gel filtration on the same biliprotein from Ceramium rubrum. This phycoerythrin was stable over a pH range of 3 to 10, but the molecule broke down into smaller units in more alkaline media. Eriksson-Quensel (1938) also recorded a molecular weight of 273,000 for R-phycocyanin over the more limited pH range of 2.5 to 6.0 and observed reversible splitting into half molecules at pH 7.0 to 8.5. Airth and Blinks (1956) isolated B-phycoerythrin from Smithora naiadum and reported a molecular weight of 290,000. B-phycoerythrin from Smithora naiadum and Rhodochorton floridulum gave the same molecular weight result when determined by gel filtration procedure (Nolan and O'hEocha, 1967), but a value of 268,000 was reported for B-phycoerythrin from Porphyridium cruentum using the ultracentrifugal method (Brody and Brody, 1961). The first molecular weight estimation of 208,000

±5,000 at pH 4.7 for C-phycocyanin was given by Svedberg and Katsurai (1929) and these authors noticed that one third of the biliprotein existed as half molecules at pH 6.8. Subunits, one sixth of the original molecular size, were observed on ultracentrifugation when the pH was further increased to 12. In common with other workers at the time, these authors did not correct the sedimentation constants for the density and viscosity of the solvents and later adjustments for other biliproteins indicated that the true value for the molecular weight of C-phycocyanin at pH 4.7 was in the region of 270,000-290,000 (Eriksson-Quensel, 1938). However, the observed dissociation effects of high pH values made at that time were of great importance and later confirmed by several authors (e.g. Hattori and Fujita, 1959; Berns et al., 1963; Hattori et al., 1965). Hattori and Fujita (1959) reported the molecular weight of C-phycocyanin to be 138,000, but this value was determined at pH 7.2 and it is probable that the value nearer the isoelectric point would be double i.e. 276,000. This view was supported by the observed sedimentation constants of 10.9×10^{-13} at pH 5.2 and 6.1×10^{-13} at pH 7.2. The recent work by Hattori, Crespi and Katz (1965) on the dissociation reactions of C-phycocyanin have not only confirmed the dependence on pH, but have also shown a dependence on ionic strength, temperature and protein concentration. These and other authors (e.g. Scott and Berns, 1965) made extensive use of deuterated phycocyanin (produced by cultivating the alga in heavy water) in which all the hydrogen is replaced

by deuterium. The dissociation effects were very similar, but occurred to a much less marked degree than for normal phycocyanin. This was slightly unexpected as the dissociation effects can be explained on the basis of electrostatic interaction, i.e. the phycocyanin molecules are negatively charged in these pH regions where dissociation occurs and as the hydrogen ion concentration increases this charge will reduce, and association rather than dissociation will be observed. Similarly, increasing the ionic strength of the solution will reduce interactions. However, if this was the only explanation, deuteriophycocyanin and normal phycocyanin would be expected to exhibit association-dissociation to the same extent and this was not observed to be the case. The postulated explanation is that in addition to the electrostatic interactions hydrophobic side-chains are involved in an important way in the union of subunits; deuteration reduces the extent of this interaction between the hydrophobic side-chains of individual units and hence deuteriophycocyanin shows less association - dissociation than normal phycocyanin.

Investigations performed by Bergeron (1963) showed a relationship between pH and absorption and fluorescence spectra. He found that C-phycocyanin from Anacystis nidulans had a lower visible extinction maximum at pH 7.5 than at pH 5.7. The spectrum at pH 5.7 was taken to represent C-phycocyanin dimer (M.Wt. 276,000) and that at pH 7.5 as the phycocyanin monomer (M.Wt. 138,000). The fluorescence spectrum of the monomer displayed only one maximum at 680nm, but the dimer showed an additional

maximum at 660nm. When the C-phycocyanin was aggregated by addition of ammonium sulphate only the maximum at 680nm was observed. The fluorescence of Anacystis nidulans cells, from which chlorophyll was extracted with 80% acetone, had maxima at 660 and 680nm. The implication of Bergeron's study is that C-phycocyanin is partly aggregated in vivo. Further studies by Berns and Edwards (1965) on C-phycocyanin from Plectonema calothricoides using an electron microscope, indicated a hexamer structure with a central hole. At higher magnification this structure appeared to consist of six globular monomer units arranged approximately at the vertices of a regular hexagon. The hexamer structure idea for C-phycocyanin received further support from the work of Scott and Berns (1965) mentioned previously. In their investigations on sedimentation velocities with varying pH, ionic strength, temperature and buffers, they postulated a monomer ↔ trimer ↔ hexamer ↔ dodecamer equilibrium system on the basis of all the evidence. Similar types of equilibrium were observed in the phycoerythrins and in this laboratory dissociation of B-phycoerythrin from Porphyridium cruentum was studied using molecular sieve chromatography (Mieras and Wall, 1967). The results indicated a definite dissociation of the protein in aqueous solution into three components of differing molecular weight. Spectral differences between these were also apparent and a certain degree of re-association after precipitation was also observed. The dissociation - association system postulated was monomer ↔ dimer ↔ polymer.

In addition to the above types of dissociation breakdown of protein bonding, both covalent and non-covalent, by chemical reagents has been investigated. Treatment of a biliprotein with p-chloromercuribenzoate, a sulphydryl blocking reagent, resulted in spectral changes and it was usually possible to separate several subunits (Jones and Fujimori, 1961; Fujimori and Quinlan, 1963). Other compounds can be used to break the non-covalent bonds in biliproteins without affecting the primary structure of the protein itself e.g. urea, succinic anhydride and guanidine hydrochloride solutions. The non-covalent bonds are considered to be hydrogen bonds between the chains in addition to the electrostatic interactions between NH_3^+ and COO^- residues. The covalent bonds in polypeptide chains are the disulphide bonds and several methods of breaking these bonds are known. Sanger (1945) used performic acid but this reagent had the major disadvantage of affecting the individual amino acids, in particular tryptophan. B-mercaptoethanol is an effective reagent without any of these disadvantages, but a more efficient disulphide bond breaker is dithiothreitol (Cleland, 1964) which was used in this laboratory in conjunction with guanidine hydrochloride solution (Mieras, 1969).

It is clear therefore that dissociation of the biliproteins can be brought about in a variety of ways and no molecular weight estimations can be carried out without bearing in mind possible dissociation effects. The following table lists some of the molecular weights

reported, specifying the pH ranges over which these hold or have been measured.

Biliprotein	Mol.Wt.	pH range	Reference
R-phycoerythrin	291,000	3-10	Eriksson-Quensel (1938) Nolan and O'hEocha (1967)
B-phycoerythrin	290,000 268,000 290,000	4.3-4.5	Airth & Blinks(1956) Brody & Brody(1961) Nolan and O'hEocha (1967)
C-phycoerythrin	226,000	5.2-7.2	Hattori and Fujita (1959)
R-phycocyanin	273,000	2.5-6.0	Eriksson-Quensel (1938)
C-phycocyanin	276,000	4.7	Hattori and Fujita (1959)
Allo-phycocyanin	134,000	7.2	Hattori and Fujita (1959)
	138,000	7.5 and 5.4	Nolan and O'hEocha (1967)
Cryptomonad phycoerythrin e.g. Hemiselmis cyclopea	24,300-27,800		Nolan and O'hEocha (1967)
Cryptomonad phycocyanin e.g. Cryptomonas cyanomagna	37,300		Nolan and O'hEocha (1967)

(c) Denaturation

Denaturation occurs when the conformation of a protein is altered from that of its native state. The conformation of a protein is defined by its secondary and tertiary structures, which describe the spatial relationship of near neighbours along the peptide chain and the gross folding of that chain. Hence, denaturation disturbs the

spacial arrangement of the native protein without necessarily breaking any covalent bonds.

In the algal biliprotein the most obvious effect of denaturation is the loss of fluorescence in addition to spectral changes in the ultraviolet and visible regions. Obviously denaturation will occur whenever biliproteins are subjected to chemical attack but prolonged heat and light can also bring about denaturation. Chemical reagents known to bring about protein denaturation include acids or alkalis, organic solvents, concentrated solutions of urea or guanidine hydrochloride, but proteins show a wide difference in their sensitivity to any one of these denaturants e.g. the protein enzyme ribonuclease is relatively stable to heat, and trypsin is stable at acid pH values. In some cases, if the treatment is not prolonged unduly, the denaturation may be reversed by restoring the conditions at which the protein is stable. O'hEocha and O'Carra (1961) found that acidification to pH3 of aqueous solutions of phycoerythrins brought about immediate reversible quenching of their fluorescence; irreversible quenching occurred on long standing, or in more concentrated acid. These authors also observed that the visible absorption spectra of B- and R- phycoerythrin were altered by denaturation with dilute acid and 8M urea solution. The intensity of the peaks in the visible region were reduced and the longest wavelength absorption removed. It was suggested that the longest wavelength maximum in the native state was due to hydrogen-bonded fluorescent chromophores, and denaturation resulted in disruption of

hydrogen bonds, quenching of fluorescence and disappearance of the longest wavelength maximum.

A widely held belief is that the denaturation process is associated with an unfolding of tightly coiled peptide chains, leading to the disorganization of the internal structure of the protein. Since denatured proteins are usually more susceptible to the attack of proteolytic enzymes than the native proteins, it was concluded that the unfolding of the native protein makes the peptide bonds more accessible to enzyme action (Linderstrøm-Lang, 1949). Another indication of the disorganization of the internal structure by denaturation is the fact that denatured proteins cannot be crystallized, and thus fail to exhibit the phenomenon most obviously associated with the establishment of an ordered array of molecules in a definite geometrical pattern.

Precautions against denaturation were taken in all stages of the preparation of purified B-phycoerythrin; this usually involved handling the biliprotein in the cold (0-5°c) and shielding the biliprotein solution from direct light.

Analysis of the Biliproteins

(a) Elementary Composition

Crystalline samples of C-phycoerythrin, C-phycocyanin, and allo-phycocyanin, all isolated from Tolypthrix tenuis, were found to give similar elementary analyses i.e. about 49% carbon, 7.5% hydrogen and 15% nitrogen. The two phycocyanins also contained 0.65% sulphur but no ash, while

C-phycoerythrin had only 0.1% sulphur and 0.37% ash (Hattori and Fujita, 1959). The sulphur content of R-phycoerythrin and R-phycocyanin has been reported as high as 1.6% (Akabori and Fujiwara, 1958; Raftery and O'hEocha, 1965). The latter workers purified R-phycoerythrin from Ceramium rubrum without using ammonium sulphate and accounted for about two thirds of the sulphur content as sulphur-containing amino acids. However, they reported the complete recovery of elementary sulphur as sulphur-containing amino acids in C-phycocyanin from Nostoc muscorum. Kimmel and Smith (1958) failed to account for the total sulphur content of R- and C-phycocyanin from Porphyra tenera.

Clendenning (1954) obtained a 4% yield of the tetrapyrrole chromophore group from C-phycocyanin and this figure indicated about sixteen chromophore groups per molecule. This was supported by Brody and Brody (1961) who used a non-destructive assay method based on particle weight, specific extinction coefficient, fluorescence lifetime and fluorescence yield. If the chromophore group has an approximate molecular weight of 590, then C-phycocyanin (M.Wt. 276,000) should have about 3.4% chromophore by weight. The latter authors suggest that while different biliproteins may be capable of binding different numbers of chromophore groups, it is also possible that the availability of the chromophore in the cell determines the number of prosthetic groups per biliprotein molecule.

Amino acid residues make up the bulk of the protein mass and accounted for virtually all the nitrogen of R-phycoerythrin from Porphyra tenera. Kimmel and Smith (1958) suggested that non-nitrogeneous compounds were at least partly responsible for this discrepancy, and earlier work by Fujiwara (1955) indicated that carbohydrates were present in some biliproteins. She later (Fujiwara, 1961) observed that R-phycoerythrin contained 4.8% carbohydrate and that this protein, isolated from Porphyra tenera, was a glycoprotein. This was later confirmed by Raftery and O'hEocha (1965) who used Ceramium rubrum as their source of R-phycoerythrin. Carbohydrates were also identified in some of the chromopeptide material obtained from peptic digests of R-phycoerythrin (Fujiwara, 1960). C-phycoerythrin was reported to contain at least seven sugars, including xylose, mannose, glucose and galactose, when a sample of the biliprotein from Porphyra tenera was hydrolysed (Sasaki and Tsuchiya, 1961). More recently other workers have detected carbohydrates in some biliproteins; R-phycoerythrin from Rhodomenia palmata contained 5% bound carbohydrate (Heard, 1965); the carbohydrate content of B-phycoerythrin from Porphyridium cruentum was found to be less than 2% (Vaughan, 1963; Paterson, 1967) and a similar figure was estimated for C-phycocyanin from Anabaena cylindrica (Lang, 1968).

(b) Amino Acid Composition

Determination of amino acid composition is a necessary first step towards establishing the primary, or covalent structure of a protein. Since amino acid residues account

for the bulk (between 75 and 89%) of the total biliprotein weight, an efficient analysis technique was required. Identification of the amino acids in a protein hydrolysate may be carried out by two-dimensional paper chromatography, but the quantitative accuracy of this technique is not high and at present the most satisfactory method for quantitative separation and analysis of amino acid mixtures utilizes chromatography on columns of ion-exchange resins (see Experimental Section G.T.(d)). The ion-exchange method was employed in analyses of a number of biliproteins (Berns, Crespi and Katz, 1963; Kimmel and Smith, 1958; Raftery and O'hEocha, 1965) and the general picture to emerge from these analyses was that the dicarboxylic acid amino acids were present in greater amounts than the basic amino acids, and there is also a high content of amino acids having hydrophobic side-chains. An estimate of the iso-electric point of C-phycocyanin from its amino acid composition, showed good agreement with that reported from electrophoretic studies i.e. pH 4.76 (Kimmel and Smith, 1958). A similar calculation based on the content of amino acid and ammonia residues of R-phycoerythrin from Ceramium rubrum indicated an isoelectric point in reasonable agreement with that established by electrophoresis i.e. pH 4.3 (Raftery and O'hEocha, 1965). However, this did not apply in the case of R-phycoerythrin from Porphyra tenera (Kimmel and Smith, 1958) and so any conclusion drawn from the amino acid content of biliproteins should be made with caution.

Amino acid analysis figures can also be used to

estimate the minimum molecular weights of biliproteins. The minimal molecular weight unit of C-phycocyanin from four species of Cyanophyta was calculated at about 30,000 on the basis of their content of least abundant amino acids (cystine and histidine). This value agrees well with ultracentrifugal determinations by Berns, Scott and O'Reilly (1964). On the basis of the only available analysis of C-phycoerythrin from Phormidium persicinum a minimum molecular weight of about 61,500 was assigned to this biliprotein (O'hEocha, 1965). However, this value assumed one cystine residue per integral unit and since cystine residues tend to give low yields on direct hydrolysis of proteins, this estimate is likely to be high. On the other hand, minimum molecular weights of 14,600 (Kimmel and Smith, 1958) and 19,100 (Raftery and O'hEocha, 1965) were found for R-phycoerythrin from different algal sources. These values, based on one histidine residue per integral unit, were lower than ultracentrifugal estimates at pH greater than 11.4. The 19,000 molecular weight subunit of R-phycoerythrin from Ceramium rubrum was estimated to contain two cystine residues or their equivalent.

(c) End-Group Analysis

Only a part of the structural information contained in a protein is given by its amino acid composition. Since there are factorial N possible linear arrangements of N different amino acid residues it is necessary to establish not only amino acid composition but also amino

acid sequence. Several end-group analyses have proved valuable in protein structural determination, and this required two types of analyses; one for N-terminal amino acids and the other for C-terminal amino acids. Dinitrophenylation (Sanger, 1945) is a method for determining the N-terminal residue and utilizes the fact that 1-fluoro-2,4-dinitrobenzene reacts with a terminal α-amino group to form an N- (2,4-dinitrophenyl) derivative. The determination of the C-terminal amino acid residue has usually involved treatment of the protein with anhydrous hydrazine (e.g. Raftery and O'hEocha, 1965), which results is solvolysis of peptide bonds and formation of aminoacyl hydrazides. The carboxyl group of the C-terminal amino acid is not attacked and this residue is released as the free amino acid.

Qualitative N-terminal analyses on R-, B-, and C-phycoerythrins showed that methionine was the sole N-terminal amino acid residue (O'Carra and O'hEocha, 1962). Quantitative N-terminal analyses have been reported for R-phycoerythrin from Ceramium rubrum using different techniques. In one analysis, nine methionine residues were obtained per unit of 290,000 molecular weight (Vaughan, 1963). Using the Sanger method, O'Carra (1965) found fourteen N-terminal methionine residues per molecular weight unit of 290,000 in the same protein, a value in better agreement with available C-terminal and total amino acid analyses. O'Carra also reported eight methionine residues per molecule for C-phycoerythrin. The phycoerythrins thus appear to have only one N-terminal

amino acid residue (viz. methionine) although Vaughan (1963) did report finding some N-terminal aspartic acid from R-phycoerythrin and so the assumption that all subunits of R-phycoerythrin are identical may not be the case.

C-phycocyanin from Nostoc muscorum was reported to contain two N-terminal threonine and four C-terminal serine residues per molecular weight unit of 276,000 (O'Carra, 1965; Raftery and O'hEocha, 1965), but results from amino acid analyses and sedimentation experiments indicate a minimum molecular weight for C-phycocyanin of about 30,000 (Berns, Scott and O'Reilly, 1964). This value is substantially lower than those indicated by the above end-group analyses experiments, and it was suggested that C-phycocyanin contains masked or non-amino acid terminal groups. However, Crespi et al. (1967) found N-terminal methionine for C-phycocyanin in addition to threonine. Similarly in R-phycocyanin both threonine and methionine were found to be N-terminal amino acids (O'Carra, 1965), which suggested the possibility that R-phycocyanin might contain two types of subunit.

Alanine was found to be the only C-terminal amino acid residue in R-phycoerythrin from Ceramium rubrum (Raftery and O'hEocha, 1965). Their result indicated twelve alanine residues per unit of molecular weight (290,000). The values of the minimum molecular weight of R-phycoerythrin based on total amino acid and C- and N- terminal analyses agree fairly well and the biliprotein appears to contain about fourteen subunits. The number

is about twice that found by Svedberg and Katsurai (1929) by ultracentrifugation; they reported that R-phycoerythrin is partially decomposed into six fragments at pH 11.4.

The Phycobilins

The coloured prosthetic groups of algal biliproteins are known as phycobilins due to the structural similarity observed between these compounds and the bile pigments of animals (Lemberg, 1928; Lemberg, 1930; Lemberg and Bader, 1933). These bile pigments are open chain tetrapyrrolic structures and a few examples are given in table III.

The precise structures of the native phycobilins is still not known with certainty. The main reasons for this being the general lability of the chromophore groups, its strong attachment to the apoprotein and low yields on cleavage from the biliprotein.

Lemberg and Bader (1933) obtained coloured pigments from R-phycoerythrin and C-phycocyanin on hydrolysis with 30% methanol - HCl at 80°c. These authors believed that these pigments, in addition to being the native prosthetic groups (phycoerythrobilin and phycocyanobilin) of their respective biliproteins, were identical with mesobilirhodin and mesobiliviolin, respectively. They also considered phycocyanobilin to be an oxidised form of phycoerythrobilin, but when later studies by Siedel (1935) indicated that synthetic mesobiliviolin and mesobilirhodin were prototropic isomers, Lemberg and Legge (1949) then assumed the same to be true of the phycobilins. It is

now believed that the pigments isolated by Lemberg were artifacts formed during hydrolysis. O'hEocha (1958) used a method of hydrolysis somewhat less drastic than that of Lemberg, in an attempt to minimise alteration of the native chromophore. Acetone - denatured bili-protein samples were treated at room temperature for 30 minutes with concentrated HCl, from which he succeeded in obtaining chloroform - soluble phycobilins. On the basis of spectral evidence, he concluded that neither phycocyanobilin nor phycoerythrobilin (the isolated pigments of phycocyanin and phycoerythrobilin, respectively) were identifiable with any known bile pigment apart from obvious similar structures. The pigment he obtained from C-phycocyanin was spectrally different from the pigment material obtained by Lemberg (1930), but it was converted to a mesobiliviolin type of pigment when subjected to Lemberg's experimental conditions. These pigments were referred to in terms of their absorption maxima in the visible region: Lemberg's mesobiliviolin type compound was referred to as phycobilin 608 while O'hEocha's phycocyanobilin was termed phycobilin 630. The latter author also concluded that all the phycoerythrins which he examined contained the same chromophore.

It appeared that the pigments obtained depended largely on the conditions used for their release. The severity of these hydrolytic conditions led to doubts as to whether or not the pigments released were identical to those in the native biliproteins. Using spectral studies Rabinowitz (1951) indicated that the spectral

TABLE 111 Structure of typical bile pigments.

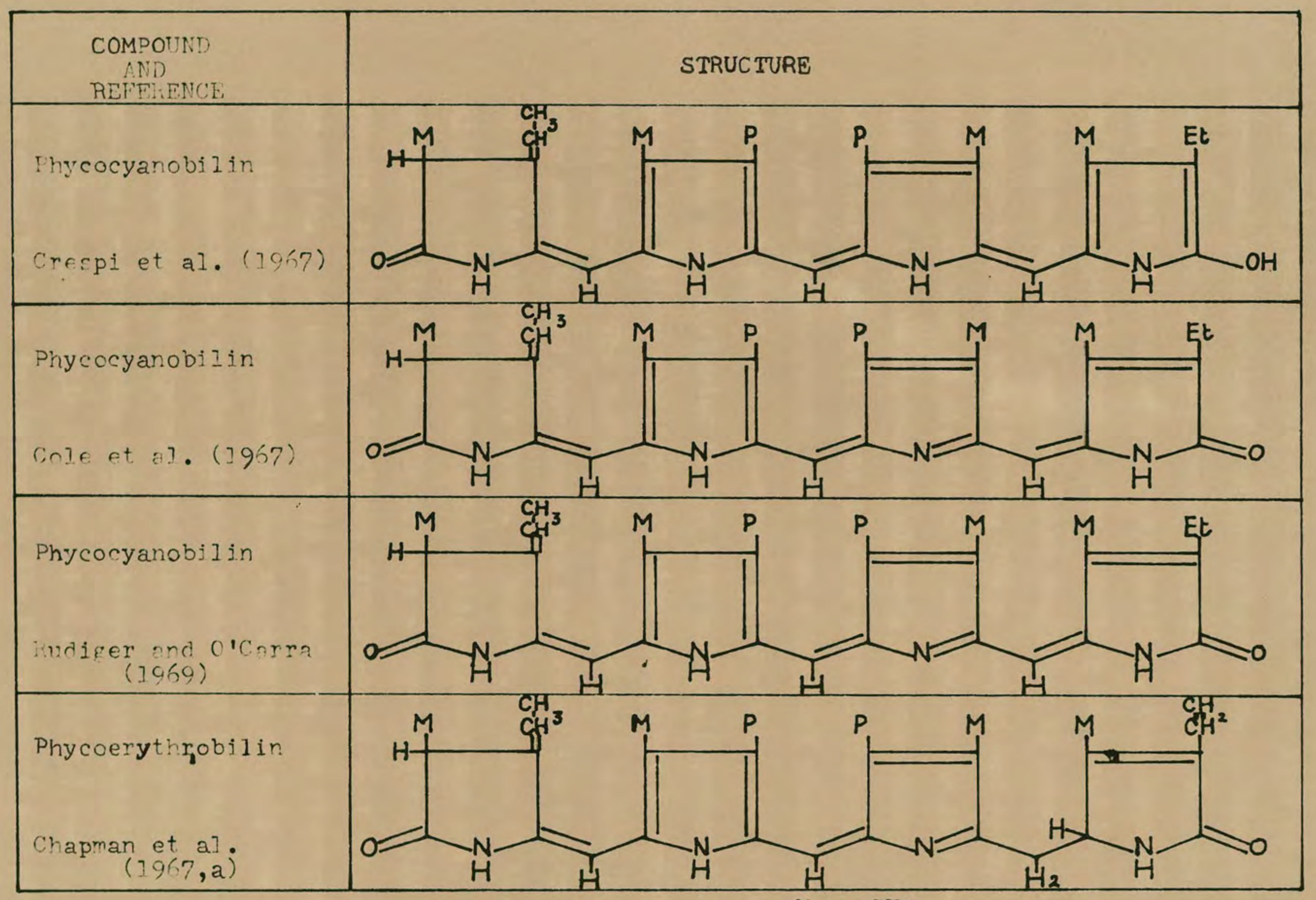
NAME	CLASS	STRUCTURE
BILIVERDIN	BILITRIENE I	
MESOBILIVERDIN	BILITRIENE II	
MESOBILIVIOLIN	BILIDIENE III	
MESOBILIRHODIN	BILIDIENE IV	
MESOBILIRUBIN	BILIDIENE V	
i- UROBILIN	BILENE VI	
STERCOBILIN	BILENE VII	

$M= -CH_3$; $P= -CH_2-CH_2-COOH$; $R= -CH_2-CH_3$ or $-CH=CH_2$

properties of the mesobiliviolinoid phycobilin 608 and C-phycocyanin were such that phycobilin 608 could not be the prosthetic group in the native biliprotein, whereas the absorption maximum of the free base of phycobilin 630 (612nm in neutral chloroform) was very close to that of the native phycocyanin (615nm). This evidence supported O'hEocha's contention that phycobilin 630 was the native phycocyanin pigment (phycocyanobilin). Until recently, the methods available for the isolation of the phycobilins were merely modifications of Lemberg's rather drastic hydrolysis technique with concentrated hydrochloric acid. In 1963, however, Fujita and Hattori were able to isolate pigments from the Blue-green Alga Tolypothrix tenuis by refluxing the intact alga or its biliproteins in methanol with 1% ascorbic acid. This technique has been used by several authors (e.g. Crespi et al., 1967; Cole et al., 1967) although O'Carra and O'hEocha (1966) showed that the purple and blue pigments obtained by this procedure were not the native prosthetic groups as previously claimed (Fujita and Hattori, 1963). However, when dissolved in 10 N-HCl at room temperature the purple and blue pigments were converted to pigments whose spectral properties were virtually identical with those of phycoerythrobilin and phycocyanobilin, respectively. Crespi et al. (1967) used a similar method to obtain a 40% yield of phycocyanobilin which they subjected to N.M.R. and mass-spectral studies. They deduced the structure shown in Table IV, but indicated that their

distribution of side chains was arbitrary, and did not claim that the pigment they had isolated occurred as such in the native protein. Cole et al. (1967) reported the isolation of a similar material, by first denaturing the native biliprotein with trichloroacetic acid; the denatured protein was then refluxed with methanol and esterified by treatment with boron trifluoride. The resultant crystalline material was investigated by N.M.R. and mass spectrometry and shown to have an almost identical structure to that proposed by Crespi et al. (Table IV).

Degradative studies on phycobilins have been limited by the difficulties involved in isolating and purifying sufficient material for classical degradation and analysis. This has recently been overcome by the development of the chrōmic acid microdegradation technique of Rudiger (1967). This technique requires as little as 0.1 mg. bilin and is not affected by the presence of protein, and may therefore be used for direct degradation of phycobilins while still attached to the protein. Oxidation of C-phycocyanin by this method resulted in the same oxidation products as phycobilin 630 and the blue pigment released by prolonged refluxing of C-phycocyanin in methanol. The structure suggested by the separated oxidation products was the same as that proposed by Cole et al. (1967). More recently, Chapman et al. (1968 a) cleaved phycocyanobilin from C-phycocyanin by several procedures and by comparison of the crystalline dimethyl



COMPOUND AND REFERENCE	STRUCTURE
Phycocyanobilin Crespi et al. (1967)	
Phycocyanobilin Cole et al. (1967)	
Phycocyanobilin Rudiger and O'Carra (1969)	
Phycoerythrobilin Chapman et al. (1967,a)	

TABLE IV. Some postulated prosthetic groups

M= - CH3
Et= - CH2·CH3
P= - CH2·CH2·COOH

esters prepared, showed that methanolic hydrolysis and acidic hydrolysis both gave the same pigment. The previous year these authors (Chapman et al. 1967 a) proposed a structure for phycoerythrobilin. This pigment was isolated from C-phycoerythrin, esterified to form the dimethyl ester of phycoerythrobilin, then examined by N.M.R. spectroscopy. The structure proposed is shown in Table 1V and was later confirmed by other workers (Rudiger and O'Carra, 1969; Crespi and Katz, 1969). Chapman et al. (1968 b) considered the possibility of a third phycobilin pigment, phycourobilin, which was thought to occur in some phycoerythrins. R- and B- phycoerythrin both show a distinct absorbance maximum or shoulder respectively at 498-500nm. and this maximum was attributed to the chromophore, phycourobilin, since the bilene urobilins all show absorbance maxima in this region. The failure to eliminate this maximum even from subunits and chromopeptides encouraged the theory of a third chromophore group. However, Chapman et al. (1968 b) were unable to isolate this chromophore and postulated that a specific protein - phycoerythrobilin complex resulted in absorbance properties similar to urobilin. In support of this contention, the fluorescence spectrum of R-phycoerythrin was suggestive of only one chromophore - a single emission maximum at 578nm, corresponding to phycoerythrobilin (Vaughan, 1965), whereas R-phycocyanin, which contained phycocyanobilin and phycoerythrobilin (Chapman et al., 1967 b) was reported to have two maxima in the fluorescence emission

spectrum, corresponding to the two chromophores (O'hEocha, 1965).

As mentioned above Rudiger and O'Carra (1969) confirmed the structure of phycoerythrobilin and also proposed a structure for phycocyanobilin (see Table IV) which was isomeric with that proposed for phycoerythrobilin. When treated with 1N-KOH phycoerythrobilin gave a mesobiliviolin - type conjugated system, while phycocyanobilin yielded mesobiliverdin. This alkaline isomerization was explained as follows:

$$\xrightarrow[\text{100°c, 5 MINS.}]{\text{1N KOH}}$$

Enzymatic cleavage of phycocyanobilin from C- and allo-phycocyanin and phycoerythrobilin from C- and R-phycoerythrin was accomplished by Siegelman et al. (1967). Assuming the chromophore content of the biliprotein to be 5%, a 16% yield of phycocyanobilin was cleaved from C-phycocyanin when digested with Nagarse at pH 7.0 for 16 hours. However, enzymatic cleavage of this type is not the general rule, and enzymes are more often used in the preparation of chromopeptides.

The Chromophore - apoprotein linkage

The first suggestion as to the nature of the linkage between the chromophore and the apoprotein came from Lemberg (1930). He reasoned that since concentrated hydrochloric acid was at that time the only known reagent which would cleave the chromophore from the biliproteins, the linkage must be a peptide type involving the propionic acid side-chains of the chromophore and free amino groups in the protein. However, this was disputed by O'hEocha (1960) when he found that all the ε-NH_2 groups and N-terminal groups in R-phycoerythrin were free to react with dinitrofluorobenzene. O'Reilly and Berns (1963) however, claimed that after treating C-phycocyanin with dinitrofluorobenzene, there remained an unaltered lysine residue. With this evidence they postulated a peptide-type linkage, but the relatively high rate of hydrolysis of the phycobilin linkage from the apoprotein as compared with rates of hydrolysis of peptide bonds suggested that this particular linkage was too labile to be a peptide-type bond (O'Carra, 1962). The relatively acid-labile nature of the prosthetic linkage led O'Carra and others (O'Carra, O'hEocha and Carroll, 1964) to favour an ester-type linkage from one or both propionic acid side chains of the chromophore to hydroxyl groups of the protein residues (e.g. serine, threonine and tyrosine). Another possible type of ester linkage was between a carboxyl group of aspartic or glutamic acid and a hydroxyl group on the chromophore, formed by enolisation of a ring keto-group. The ease of cleavage of phycocyanobilin

from C-phycocyanin by potassium hydroxide solution (Lemberg and Bader, 1933) also suggested that an ester linkage was involved. Siegelman et al, (1967) examined the effectiveness of several proteolytic enzymes for cleaving the chromophore from C-phycocyanin. Only Nagarse digestion released the free chromophore from the protein in sufficient yield (16%) to establish that the released pigment was identical to that released by methanolic hydrolysis. Rudiger and O'Carra (1968) developed a chromic acid oxidation technique which resulted in controlled degradation of bilins to imide products. As mentioned previously, this led to the elucidation of the structures of the phycobilins, and also gave the clearest evidence to date regarding the points of attachment of the chromophore group to the apoprotein. Phycoerythrobilin and purple pigment were prepared by hydrolysis of R-phycoerythrin in 12 N hydrochloric acid for 30 minutes at 18°c, and refluxing the phycoerythrin in methanol, respectively. Conversion of phycoerythrobilin and purple pigment to their respective imides by chromic acid oxidation at 20°c, yielded identical products. The same mixture of products was obtained by subjecting R- and C- phycoerythrin to this oxidation technique at 100°c. These authors also compared the results of oxidative degradation of the phycoerythrins at 20°c and 100°c, and found that after oxidation at 20°c only two of the four pyrrole rings were released from the biliproteins as the imides. The other two remained attached until the temperature was raised to 100°c for 1 hour in 2 N sulphuric

acid. The results indicated that these rings - ring A (carrying the ethylidene group) and one of the middle rings - were firmly bound to the apoprotein through hydrolysable bonds. Furthermore, initial treatment of R-phycoerythrin with hot alkali for 5 minutes appeared to break the linkage of ring A to the protein so that this ring was released (in isomerised form) with the other two free rings on chromic acid oxidation at 20°c. Under these conditions, however, the attached middle ring remained so and was only released by hydrolysis with 2 N sulphuric acid. Rudiger and O'Carra, concluded that the two rings were bound independently through bonds which exhibited different stability characteristics. They supported the theory that the middle ring was attached to the chromophore through its propionic acid side chain, since linkage through the nitrogen group (the only other alternative) would prevent the formation of metal complexes by the still attached phycobilin. Such complexes were recognised by previous workers when the biliproteins were treated with denaturants (e.g. guanidine - HCl or dilute HCl) in the presence of zinc acetate (O'hEocha and O'Carra, 1961). On the other hand, if the linkage of the propionic acid side chain by an ester-type linkage is to a hydroxyl group on the apoprotein, then this linkage displayed unusual stability in alkali. In addition they could not explain the release of phycobilins by hot methanol without esterification of the propionic acid side chain, but suggested that neighbouring regions of the apoprotein might facilitate the release of pigments under these conditions.

The linkage in ring A was postulated to be either through the oxygen atom, in the lactim form, or through the nitrogen atom, perhaps linked to a carboxyl group in the apoprotein.

Subsequent investigations by Killilea and O'Carra (1968) on chromopeptides from R-phycoerythrin indicated that glutamic acid, serine, aspartic acid, threonine, glycine and alanine predominated in the chromopeptide mixture. This was consistent with the results of Rudiger and O'Carra (1968) mentioned above, in that serine and glutamic acid could be involved in the chromophore linkages.

Crespi and Smith (1970) examined the chromophore - protein bonds in phycocyanin from Phormidium luridum, by digestion of the biliprotein with Nagarse. These authors were unable to eliminate cystine from their chromopeptides and postulated that this amino acid was linked to the chromophore by the sulphur atom.

EXPERIMENTAL SECTION

(1) General Techniques

Before giving a detailed experimental account of procedures involved in this investigation it will be convenient to describe here general techniques which occur frequently in the text. This will prevent repetition and lengthy experimental details later on.

(a) Centrifugation

The apparatus used was on M.S.E. "Refrigerator" centrifuge model "P" (M.S.E. Ltd., London, England), capable of centrifugal fields up to 6,000g with a maximum capacity of 1,500mls. For most purposes the use of two heads (angle or swing-out) was adequate. Large volume samples (e.g. cultured algae) were centrifuged in the swing-out head which carried six 250ml glass tubes up to speeds of 3,000 r.p.m. (2,300g) at 2°c. Greater speeds using this head resulted in a rise in the bowl temperature due to air friction. Greater speeds (up to 6,000g) could be attained, however, by using the streamlined angle head, which held eight 50ml. glass tubes in a head specially designed to cut down excessive air turbulence. If required, even greater "g" values could be obtained by fitting a Superspeed Unit to the centrifuge motor. This unit can take four 25ml. stainless steel tubes up to 10,000g.

(b) Dialysis

Dialysis is basically a molecular sieving through a semi-permeable membrane. It was used in the present

investigations to remove small molecules (mainly salts) from aqueous solutions of high molecular weight substances (mainly proteins). The usual procedure was to wash dialysis tubing (Cellulose or Visking) thoroughly with distilled water, knot one end of the tubing, and then introduce the sample dissolved in the minimum of buffer or distilled water. Air was forced out of the tubing, after which the other end was knotted securely and placed in the dialysis medium. Only half the capacity of the closed tubing was filled with solution to allow for expansion by osmosis. Dialysis was usually performed in a magnetically stirred 2 litre conical flask containing buffer solution or distilled water. Stirring prevented a build up of solute molecules immediately adjacent to the membrane to avoid "clogging" of the membrane pores. These experiments were carried out in a darkened cold room at 2°c to reduce the possibility of protein denaturation by heat or light. Four or five changes of dialysis medium over a period of about 48 hours were normally sufficient to free the solution of most smaller molecules.

(c) Ultrasonication

1. Cell rupture.

This technique has been used for several years in this laboratory as a means of releasing the biliprotein from the intact algal cell. A suspension of the algal cells was subjected to ultrasonic vibration; and this process ruptured cell walls and allowed release of the biliprotein into the surrounding medium. The source of

ultrasonic vibration was a Dawes Soniprobe (type 1130A) supplied by Dawes Instruments Ltd., London, England.

The algal suspension was cooled to about 5°c in a 1 litre tall beaker and surrounded by an ice-bath before sonication to prevent heat denaturation of the biliprotein. This treatment was continued for twenty minutes and was always followed by freezing the suspension in a deep-freeze overnight, then thawing out at room temperature the following day. The freezing and thawing process in the aqueous medium encouraged further cell rupture.

2. Slurry Preparation.

When preparing cellulose plates for T.L.C. (G.T."J") the slurry was sonicated for 1 minute prior to layering. This procedure removed aggregates of cellulose powder which could lead to non-uniformity of layer thickness.

(d) Amino Acid Analyses

Analyses for the amino acids were done on a Technicon Automatic Amino Acid Analyser (Technicon Instruments Company Ltd., Chertsey, Surrey, England.) Basically the technique was the same as that used by Spackman, Stein and Moore (1958) as modified by Benson and Paterson (1965). For most purposes two columns were used - a long column for the separation of acidic and neutral amino acids and a short column for basic amino acids. The resins used were Amberlite C.G.120 (type III) and Technicon Chromobeads type "A" (Dowex 50 x 8). Both materials (8% cross-linked sulphonated polystyrenes), one being a pulverized resin (Amberlite),

the other a spherical resin, were classified into fractions possessing the desired ranges of particle size by the hydraulic method of Hamilton (1958). The particle size found to be most suitable for the present purposes and conditions was the particle having Martin's diameter 25 ± 5 microns. Sodium citrate buffers were used to elute the amino acids from the cationic exchange resins and the experimental conditions are given in the table (Table V). After elution from the column the amino acids were mixed with a fixed quantity of ninhydrin-hydrindantin solution. Colour was developed by passing the mixture through a heated coil immersed in an oil bath heated to 95°c, and measured by photoelectric colourimeters which record the results on a moving chart. The total analysis time using this system was 4hrs. 25mins. (3hrs. 15mins. for acidic and neutral amino acids, and 1hr. 10mins. for basic amino acids).

Samples were dissolved in a predetermined quantity of sodium citrate buffer (pH 2.2; 0.066M w.r.t. sodium citrate) before analysis. This buffer contained the internal standards norleucine (NOL) and L-α-amino-B-guanido-propionic acid hydrochloride (AGPA) in a concentration of 0.1 μM per 0.5ml. Sample (0.5ml) was applied to the column using a micro-pipette or automatic dispenser and forced into the resin by nitrogen pressure (2 Kg/cm^2). The sample was washed into the column with three applications of 0.2ml of sodium citrate buffer, using nitrogen pressure with each application. The column was then topped up with wash buffer, the starting

buffer pump line attached and the analysis run commenced. The concentration of each amino acid present in the sample was measured by the integration of the area of the peak representing it on the chromatogram. This was assessed by the height/width method which entailed multiplying the peak height in terms of absorption value, by its width, in terms of dotted points, at half that height. This value was termed the peak area, Aa, of the amino acid involved (see specimen table of amino acid analysis). To calibrate the instrument, standard runs were performed at intervals. This standardisation procedure involved dissolving 2mls. of an Eel standard amino acid mixture, containing 1μmole of each amino acid per ml. (supplied by Evans Electroselenium Ltd., Halstead, Essex.) in 8mls sodium citrate buffer (pH 2.2; 0.066M w.r.t. sodium citrate) containing the internal standard NOL and AGPA, to a final concentration of 0.1μmole per 0.5ml.

An amino acid analysis was performed on this standard mixture and areas obtained from the resulting chromatograph for the individual amino acids. Since each amino acid was present in equal concentration, by dividing the internal standard area by the amino acid standard area, the NOL and AGPA equivalents were obtained

i.e. $$\text{NOL EQUIVALENT} = \frac{\text{NOL PEAK AREA}}{\text{AMINO ACID STANDARD PEAK AREA}}$$

The NOL and AGPA equivalent values were an indication of the colour yield of each amino acid under the given experimental conditions. Hence, in any amino acid analysis the number of micro moles of any given acid was

TABLE V - Amino Acid Analyser Details

	Long Column		Short Column
Resin	Amberlite C.G. 120 ("100")	Technicon A ("100")	Amberlite C.G. 120 ("100")
Column dimensions	62x0.636cms.	59x0.636cms	6.5x0.636cms
Jacket temp.	52°c	54°c	52°c
Sodium concentration	0.2N	0.2N	0.35N
Starting buffer pH	3.28±0.02	3.28±0.02	5.28±0.02
Second buffer, pH	4.25±0.02	4.25±0.02	-
Buffer flow rate	30mls./hr.	30mls./hr.	30mls./hr.
Back pressure	180 p.s.i.	100 p.s.i.	110. p.s.i.
Buffer timer change	63 mins.	68 mins.	-
Wash buffer, pH	3.28+10%MeOH	3.28+10%MeOH	5.28

determined as follows:

$$\text{Micro moles acid} = \frac{Aa \times E}{As} \times 0{\cdot}1$$

, where Aa is the peak area of the amino acid involved, E is the NOL or AGPA equivalent value, and A_s is the peak area of the internal standard.

(e) Evaporations

Evaporations were normally carried out using a rotary evaporator. Two techniques were used depending on the solvent to be removed. For low boiling solvents a Buchi "Rotavapor" Rotary Vacuum Evaporator, used in conjunction with a water-pump was found to be satisfactory. For higher boiling solvents, however, when undue heating of the solution was undesirable, it was necessary to use a Jones and Stevens Type V.W. vacuum evaporator which had the advantage that it could be used in conjunction with an Edwards "Speedivac" High Vacuum oil-pump. The flasks containing solution to be evaporated were heated on a water bath whose temperature was maintained below 40°c.

(f) Measurement of pH

All pH measurements were made using a Radiometer pH meter (type TTT 1c) in conjunction with a scale expander (Radiometer, Copenhagen, Denmark). With a combination glass electrode (type No.27 D.R. Activion, Fife, Scotland) readings were reproducible to two decimal places.

(g) Acidic Hydrolyses

Protein and peptide samples were hydrolysed with

6N HCl in sealed tubes at 105°c. Approximately 2mgs. of sample were weighed on an electrobalance (Cahn Instruments Co., Paramount, California, U.S.A.), and then transferred to a 27cm. pyrex hydrolysis tube. 6mls. of 6N constant boiling HCl (redistilled) were added to the tube and the contents frozen in liquid nitrogen before evacuation on a water pump for 15 minutes. The tube was sealed by fusion and then allowed to come to room temperature; and finally it was placed in an oven thermostatically controlled at 105 ± 0.5°c. The heating time was 24 hrs. unless otherwise stated in the text; hydrolysates were cooled and frozen in liquid nitrogen before opening, and then were evaporated to dryness on a Buchi rotary evaporator. A few mls. of de-ionised water were added to the hydrolysate and the solution was again taken to dryness to remove the last traces of HCl. This procedure was repeated three times, and the dry samples were stored in a vacuum desiccator over sodium hydroxide and phosphorus pentoxide.

(h) Freeze-Drying

Freeze-drying was used for efficient drying of protein and other solutions. The main advantage of this technique over others is that it involves no undesirable heating which would lead to complete and irreversible denaturation of protein solutions.

The freeze-driers used were the Centrifugal Freeze Dryer Model 30P1/599 (Edwards High Vacuum Ltd., Crawley, Sussex, England) and the Manifold Freeze Dryer type M.F.D. 3/R (N.G.N. Ltd., Accrington, England). The solution to

be freeze-dried was frozen as a thin film inside a round-bottomed flask by immersion in liquid nitrogen with constant rotation. Solvent was removed from the solute by sublimation at high vacuum (in the region of 0.02 torr).

(i) Column Chromatography

Several types of column chromatography were employed for the purposes of protein and peptide separation and purification. The physico-chemical phenomena involved in the different types of chromatography will be grouped separately for convenience, but it should be emphasized that no one type of phenomena can ever be said to be taking place exclusively in any given example of chromatography.

(1) Adsorption Chromatography with Tricalcium Phosphate

Swingle and Tiselius (1951) demonstrated that phycoerythrins and phycocyanins could be separated by adsorption chromatography on columns of tricalcium phosphate gel. This technique was a great advance in the separation of proteins and is still in widespread use.

Non-adsorbent $CaHPO_4.2H_2O$ was prepared by the method of Siegelman, Wieczovek and Turner (1965), and this was converted into reactive hydroxyl apatite, $Ca_5(PO_4)_3OH$, as described by Levin (1962).

The final adsorbent was stored in 0.001M sodium phosphate buffer (pH 6.5); but for column usage most workers have found it advantageous to mix the finely divided hydroxyl-apatite with a five-fold volume of celite to give usable flow rates.

A column (60 x 6cms.) was half filled with .0025M sodium phosphate buffer of pH 6.5 and a plug of glass wool wedged in the base of the column. Celite, which had previously been stirred, settled and decanted four times in a three-fold volume of the same buffer to remove fines, was then poured into the column in a thin slurry and allowed to settle to a depth of 3cms. The mixture of tricalcium phosphate and celite (1:5) was similarly slurried and poured into the column. After an initial 2cms. had settled, the remainder of the column was uniformly packed to a height of 20cms. by opening the outlet valve. A final layer of celite, 2cms. thick, was allowed to settle on top of the packed bed and acted as a filter, removing denatured protein and any other insoluble material. When all the protein had been adsorbed onto the column, the top celite layer could be stirred into suspension and removed, giving an improved flow rate.

Protein material precipitated with 35% ammonium sulphate was dialysed in the dark at 2°c with four changes of 2 litres of 0.01M sodium phosphate buffer, pH 6.5, over a period of 2 days (see G.T.(b)). The strongly fluorescing solution was then loaded onto the tricalcium phosphate column, which had previously been equilibrated with 2 litres of 0.0025M sodium phosphate buffer (pH 6.5). The column was then developed by stepwise elution with sodium phosphate buffers (pH 6.5) of increasing molarity (0.005M —— 0.1M).

The biliproteins are adsorbed by the tricalcium

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phosphate at the lower phosphate concentration. When the phosphate concentration is increased the column capacity for an adsorbed protein species is decreased, and at a certain phosphate level, a given species will be desorbed and eluted. Using step-wise elution, the R_f of a given species can increase from zero to 1 in one buffer change, giving a clean-cut elution band without any "tailing" effects.

(2) Molecular Sieve Chromatography (Gel Filtration)

Two types of molecular sieve material were used for the purposes of fractionating proteins and peptides, viz. Sephadex (Pharmacia Fine Chemicals, Uppsala, Sweden) and Bio-Gel P (Bio-Rad Laboratories, Richmond, California, U.S.A.).

Sephadex is a modified dextran of microbial origin, in which the linear dextran chains are cross-linked within well defined limits to give a three dimensional network of polysaccharide chains. As a result of the high proportion of hydroxyl groups present, these chains are strongly hydrophilic and in aqueous solutions form swollen gels, which are able to "sieve" or exclude molecules over a certain size. Generally, the exceptions being noted below, the size of molecule being excluded can be correlated with molecular weight and for any given gel this molecular weight is termed the exclusion limit. Large molecules, (i.e. those exceeding the exclusion limit of the gel) which cannot diffuse into the gel interstices, move through the column faster, and hence are separated

from smaller molecules. The greater the degree of cross-linking in the gels, the lower the swelling of the gels in aqueous solution, resulting in a lower exclusion limit.

Bio-Gel P is a cross-linked co-polymer of acrylamide and N, N^1- methylene bisacrylamide produced in the form of spherical beads. By varying the cross-linking and the concentration of the two basic ingredients, gels of different pore sizes are produced, resulting in properties very similar to Sephadex.

As stated above, separations using these gels do not depend solely on molecular size, since adsorption and stereochemical effects must also be taken into account. For example, linear molecules tend to be eluted faster than globular molecules of the same molecular weight; aromatic and heterocyclic compounds tend to be retarded; on Sephadex, basic compounds are adsorbed and acidic compounds excluded when very small quantities are introduced and eluted with distilled water, due to a very low carboxyl content in the Sephadex. Bearing in mind the above anomalies, the following table lists the various types of Sephadex and Bio-Gel P available and gives their approximate exclusion limits:

Type	Exclusion Limit	Type	Exclusion Limit
Bio-Gel P-2	2,600	Sephadex G-10	700
P-4	3,600	G-15	1,500
P-6	4,600	G-25	5,000
P-10	12,000	G-50	10,000
P-30	30,000	G-75	50,000
P-60	60,000	G-100	100,000
P-100	100,000	G-150	150,000
P-150	150,000	G-200	200,000
P-200	200,000		
P-300	300,000		

All Bio-Gel P and some Sephadex gels are further subdivided into particle size ranges, giving variations in flow rate and resolution. As a general rule, when the substances to be fractionated are close together in molecular weight, a fine particle size gel is chosen; when they are relatively far apart large bead sizes can be used. Fine particle size gives minimum band spreading with a corresponding sacrifice in flow rate. Large particle sizes give rapid flow rates, easier column packing and an increase in band spreading.

The technique used for gel preparation and column packing was identical for Bio-Gel P and Sephadex, but as the working conditions were widely varied (e.g. column dimensions and buffers), only a general outline of the method will be detailed here, fuller details being given in the text.

The dry gel beads were hydrated by slow addition,

with stirring, to a large volume of buffer and allowed to swell for the recommended hydration time. Entrapped air bubbles were removed by degassing the swollen gel and excess buffer in a vacuum flask attached to a water pump. The chromatographic column, clamped vertically, and equipped with a capillary outlet and wide-necked filter funnel, was filled with degassed buffer. If necessary, fines were removed from the gel suspension by repeated decantation before pouring the slurry carefully down the sides of the funnel. The stirred gel was allowed to settle to a depth of a few centimetres before the outlet valve was opened and the rest of the column packed under an even flow of buffer. During packing, a rising horizontal surface of gel in the column indicated proper uniformity in packing. To stabilize the column, the elution medium selected for the experiment was allowed to run through the column for several hours under a constant head of water, or more usually, using a peristaltic pump. All eluting buffers were degassed before use.

(j) Thin Layer Chromatography (T.L.C.)

This technique has evolved rapidly in the last twenty years, particularly since the commercial availability of adsorbents and apparatus. The adsorbents MN-Cellulose powder 300 and MN-Silica Gel G-HR (Macherey, Nagel and Co., Duren, Germany.) were selected as most suitable for peptide chromatography.

Glass plates (20 x 20cms. and 5 x 20cms.) were cleaned before use with chromic acid, detergent solution,

acetone, and finally de-ionised water.

Two techniques were used for spreading according to whether the experiment was qualitative or quantitative or preparative. Plates to be used in qualitative experiments were prepared using the Desaga spreader (C. Desaga, Heidelberg, Germany.) which covered five 20 x 20cm plates or an equivalent number of 5 x 20cm plates in one operation. Any variations in the thicknesses of the glass plates used would give rise to non-uniformity of spreading. The thickness of layers prepared could therefore only be given as approximately 0.25mm.

The second, and most frequently used technique, was the Shandon thin layer apparatus (Shandon Scientific Co. Ltd., London.) which included an adjustable spreader and leveller. The leveller overcame the problem of variations in glass plate thickness by incorporating an inflatable air bag which raised the glass plates until their upper surfaces were in one plane. This apparatus spread the same number of plates as the Desaga, but since the layers were thicker (generally 0.6mm), differing quantities and proportions of adsorbents were used, as indicated by the following methods of preparation:

(a) Cellulose: MN-Cellulose powder 300 (15gms.) was mixed with de-ionised water (70mls.) and ethanol (10mls.), then sonicated for 1 minute to ensure proper dispersion.

(b) Silica Gel: (Desaga) MN-Silica Gel G-HR (30gms.) was shaken with de-ionised water (70mls.) for 90 seconds.
: (Shandon) MN-Silica Gel G-HR (80gms.) was shaken with de-ionised water (120mls.) for 90 seconds.

The prepared slurries were used immediately and the plates were allowed to stand for 30 mins. after spreading, before being transferred to a chromaplate rack to equilibrate overnight at room temperature with atmospheric moisture. Before the cellulose plates could be used it was necessary to remove an impurity by washing the plate in the appropriate solvent.

Material was applied to the plates with a fine glass capillary and runs were generally in the region of 2-4 hours, giving a solvent front movement of 10-15cms. in pre-saturated chromatography tanks. Developed plates were examined under U.V. light and bands removed as soon as possible to prevent oxidation or breakdown of the peptide material. After chromatography, the separated chromopeptide bands were scraped off the plate and washed through a small quickfit sintered glass funnel with methanol and very dilute acetic acid, aided by a vacuum water pump. The filtrate was collected in a 50ml. peak-shaped flask. When the adsorbent was free of peptide material, the contents of the flask were reduced to dryness on a Buchi rotary evaporator and stored in a vacuum dessicator Solvents:

(1) Butanol : Acetic acid : Water (3:1:1 by volume)

(2) Toluene : Acetic acid : Water (10:10:1 by volume)

(3) Phenol : Acetic acid : Water (1:1:1 by volume)

(k) <u>High Voltage Paper Electrophoresis</u>

The apparatus used was the Miles Hivolt Electrophoresis Unit Mark III, an instrument designed to deliver D.C. voltage up to 10K.V. at 500mA.

The material for electrophoresis was applied to the Whatman paper (15 x 55cms.) as a thin band across the centre line and separations were carried out with the paper placed between two water cooled (8 litres per minute; room temperature) electrically insulated aluminium alloy plates. Thin polythene sheets (0.05cm.) were used for electrical insulation between the plates and the paper. Six paper wicks (Whatman 3MM maintained contact between the paper strip and the polythene buffer reservoirs, which each contained between six and seven litres of electrolyte. The electrodes were platinum wires with gold contacts. Before the commencement of any run the levels in each of the buffer vessels were equalled using a siphoning device to prevent any siphoning effects during the actual run.

On completion of a run the electrophoretogram was allowed to dry in air, then viewed under U.V. light to determine the position of any fluorescent bands. Amino acids were detected by spraying with ninhydrin solution (a 0.5% solution of indanetrione hydrate in water-saturated butanol) and drying in an oven at 80°c for 10 minutes to develop the characteristic purple colour.

The grades of paper used included Whatman Nos. 1,4, 54, the buffers used were:

(1) Pyridine : Acetic acid : Water (6:4:290 by volume), pH 5.0.

(2) Pyridine : Acetic acid : Water (1:10:289 by volume), pH 3.6.

(2) Culture of Porphyrium cruentum

Porphyridium cruentum, first described by Naegeli (1849), is a unicellular red alga which grows on soil, damp walls and in various marine environments. It is a primitive member of the Rhodophyta, order Porphyridiales (Table 1), containing a relatively high proportion of the biliprotein of the present investigation, B-phycoerythrin, together with smaller quantities of R- and allo-phycocyanin.

Early attempts to culture the alga on solid media met with no success until Pringsheim and Pringsheim (1949) discovered that relatively good growth could be obtained in solid or liquid media if natural sea-water was included. This suggested that the alga was of marine origin, a postulate which was later confirmed when several authors isolated Porphyridium cruentum from marine environments (e.g. Starr (1960), Allen (1960) and Reith (1961)). Cultured alga had usually been grown successfully only in media containing natural sea-water and soil extracts until Brody and Emerson (1959) grew the alga in an artificial inorganic medium. This advance did not allow large scale culture, but this difficulty was overcome by Jones, Speer and Kury (1963) with the development of an artificial sea-water medium (A.S.W.) A slight modification of this medium was used to grow Porphyridium cruentum in this laboratory. Porphyridium cruentum (Agardh) Naegeli Cambridge Culture Collection No.1380A/1A; Vischer 1935; No. 107, Switzerland, was originally obtained in bacteria-free culture from the "Culture Collection of Algae and

Protozoa," the Botany School, Cambridge, England.

Culture Medium

Cultures were grown in an A.S.W. medium containing the following salts:

27gms. per litre of NaCl
6.6 " " " " $MgSO_4.7H_2O$
5.6 " " " " $MgCl_2.6H_2O$
1.5 " " " " $CaCl_2.6H_2O$
1.0 " " " " KNO_3
0.07 " " " " KH_2PO_4
0.04 " " " " $NaHCO_3$

This solution was buffered with 20mls. per litre of 1M Tris (2-amino-2-(hydroxymethyl)-propane-1,3-diol, i.e. $NH_2.C(CH_2OH)_3$) - HCl buffer, pH 7.6 to which was added 1.0ml per litre of a chelated iron solution (240mgs. $FeCl_3.4H_2O$ per 100mls. 0.05M Na_2E.D.T.A., pH 7.6) and also 1.0ml. per litre of a trace metal solution of the following composition per litre:

40mgs. $ZnCl_2$
600mgs. H_3BO_3
15mgs. $CoCl_2.6H_2O$
40mgs. $CuCl_2.2H_2O$
400mgs. $MnCl_2.4H_2O$
370mgs. $(NH_4)_6Mo_7O_{24}.4H_2O$

The medium was completed by the addition of 0.01ml. per litre of vitamin B-12 solution (5mgs. cyancobalamin in 100ml. distilled water). Analar grade chemicals and distilled water were used throughout. The Tris-HCl

buffer, chelated iron, trace metal and vitamin B-12 solutions were kept in dark bottles in a refrigerator and even after long periods of storage showed no signs of deterioration in appearance or function.

Stock Cultures

Stock cultures of *Porphyridium cruentum* were maintained in Pyrex screw-capped test-tubes and conical flasks containing sterilised culture medium with 1.5% Difco Bacto-Agar added. These cultures, however, were rarely used and were retained more as a precautionary measure than for inoculation purposes.

Culture Vessels

Two twenty litre aspirators were found to be the most practical vessels for culturing the alga in reasonable quantities. Before use, the vessels were thoroughly cleaned with chromic acid, hot detergent solution and finally rinsed with distilled water.

Sterilisation and Inoculation

The culture vessel containing culture medium and covered with a brown paper hood was placed in the autoclaver together with any other apparatus requiring sterilisation (e.g. 50ml. pipette, standard condenser tubing, air filter loosely packed with cotton wool, rubber stoppers and sintered glass filter stick.) Autoclaving for 20 minutes at $1Kg/cm^2$ pressure was sufficient for sterilisation, after which the contents of the autoclaver were allowed to return to atmospheric

conditions by standing overnight.

Inoculation was then carried out under aseptic conditions by transferring about 100mls of material from a previously grown culture using a sterile pipette. The inoculated medium was supplied with a 1 litre per minute flow of air through a sintered glass filter stick which extended to the bottom of the culture flask. This current of air, enriched with 5% carbon dioxide and passed through sterilised tubing and cotton wool filters, also agitated the media and according to previous workers (e.g. Paterson, 1967), this mixing enhanced the growth rate of the alga. The inoculated culture vessels were continuously illuminated from one side by two Philips "Cool White" fluorescent lamps (M.C.F.E.; 80w/33). The culture room temperature was not thermostatically controlled but the room dimensions and lack of ventilation, coupled with the heat produced by the lighting, gave rise to only small fluctuations in temperature ($22 \pm 2^{o}c$).

Six weeks was the optimum growth period, after which the algal cells were ready for harvesting. If left much beyond this time cell lysis and consequent protein leaching into the surrounding medium began.

(3) Harvesting and Extraction of B-Phycoerythrin from Porphyridium cruentum

After the optimum growth period of six weeks in twenty litre aspirators the culture medium was dense with dark red cells of Porphyridium cruentum ready to be harvested. Alga adhering to the sides of the aspirator was scraped off and the suspension of cells transferred

to 250ml. centrifuge tubes. Centrifugation was performed for 30 minutes at 1,500g in the cold (2°c). The very pale pink supernatant was discarded, while the combined residues were transferred to a 1 litre tall beaker with a minimum of distilled water. This algal suspension was then placed in an ice-bath and subjected to sonic oscillation for 20 minutes (G.T.(C)). For further rupture of the cell walls the mixture was placed in a deep-freeze overnight and then allowed to thaw out at room temperature. The release of biliproteins from the algal cells was evidenced by the brilliantly fluorescing solution.

The crude biliprotein solution was separated from solid material (unruptured cells and cell fragments) by centrifugation for 30 minutes at 2,300g again at about 2°c. When large quantities of alga were harvested it was found that one extraction of biliprotein by sonic oscillation, freezing and thawing was not sufficient to release all biliprotein from the cells. The supernatant was removed and the combined residues were slurried in water then re-subjected to the extraction procedure as above until the residue from centrifugation was no longer a dull red colour. Cell debris which was too fine to be sedimented by centrifugation was then removed by filtering the combined supernatants (crude biliprotein solution) though a celite pad. A quantity of celite was sedimented several times in distilled water and the smaller particles decanted. A bung of glass wool was fitted into the bottom of a large column (30 x 5cms.)

and a slurry of celite poured into the column to give a settled bed depth of about 6cms. The crude biliprotein solution was then passed through the celite column and collected in an ice-cooled flask. The top of the celite bed was slurried several times to facilitate passage of the solution through the column, and pressure was also applied to the top of the column to increase the flow rate.

The filtered biliprotein solution was then precipitated by adding finely powdered ammonium sulphate to a final concentration of 30% (weight/volume). Ammonium sulphate was added slowly to the fluorescent biliprotein solution, which was cooled, magnetically stirred and shielded from direct light during this procedure. The solution was allowed to stand for several hours (usually overnight) in a cold room (2°c) to precipitate the biliproteins and these were collected by centrifugation at 2,300g for 30 minutes at 2°c. The supernatant was normally discarded, but in some cases was still coloured red indicating that some biliprotein remained in solution. This was found to be the case in the more dilute crude biliprotein solutions and in these instances, an additional 5% (W/V) ammonium sulphate was added to the supernatant to complete the biliprotein precipitation. The combined precipitates were collected and dissolved in the minimum of distilled water and then transferred to dialysis tubing. The solutions were dialysed against distilled water overnight, and then against four changes of 2 litres of 0.01M sodium phosphate buffer (pH 6.5) (see G.T.(b)). The dialysed

biliprotein solution was centrifuged at 2,700g for 20 minutes at 2°c before visible and ultraviolet spectra were run (fig. 3.). The visible absorption spectrum of this impure B-phycoerythrin solution indicated that phycocyanin was present as an impurity. Also, the spectral ratio (the absorption at 545nm divided by that at 280nm) was generally in the region of 2, confirming the low purity state of B-phycoerythrin.

Chromatography on tricalcium phosphate - celite columns (25 x 6cms.) was the next stage in the purification of B-phycoerythrin. Columns were prepared and eluted as described previously (see G.T. (1)). The centrifuged biliprotein solution (approximately 300mls.) was loaded onto the column which had previously been equilibrated with 2 litres of 0.0025M sodium phosphate buffer (pH 6.5). B-phycoerythrin was found to be eluted with sodium phosphate buffers of molarities 0.01 - 0.05, concentrations which were not sufficient to desorb the bulk of the phycocyanins. The eluted biliprotein solutions were collected in approximately 100ml fractions, samples of which were subjected to visible and ultraviolet absorption spectroscopy, and fractions having similar spectra and spectral ratios were combined. Initially B-phycoerythrin was eluted on its own, but subsequent spectra indicated that R-phycocyanin was also being eluted, although the spectral ratios of these latter solutions were frequently greater than 4. The other phycocyanin found in Porphyridium cruentum, allo-phycocyanin, remained adsorbed until the phosphate buffer concentration was increased to 0.2M,

when the spectral ratio was found to be less than unity. Figs. 4a and 4b illustrate the separation achieved by tricalcium phosphate chromatography. Fig. 4a shows the spectrum of the major fraction with visible absorption maxima at 545nm, 558nm and a shoulder at 495nm. In the ultraviolet region there are maxima at 278-80nm, 312nm and 370-80nm. These maxima correspond to those of B-phycoerythrin. Fig. 4b shows the spectrum of a later fraction and is very similar to fig.4a except that a small peak at 618nm is present. This is attributed to trace amounts of R-phycocyanin.

Columns could be regenerated after use by washing through residual material with 0.25M sodium phosphate buffer, then washing the columns with distilled water. However, in this investigation all columns were packed with fresh adsorbent for each run. As in the case of celite filtration, it was advisable to stir up the top celite layer at intervals to improve the flow rate. Also, the elution was performed under pressure and all the previously mentioned precautions were taken against denaturation of the biliprotein by heat or light. Previous workers in this laboratory (Paterson, 1967; Mieras, 1969) added 1% (W/V) sodium chloride to the biliprotein solution prior to loading and also to the eluting buffers. Swingle and Tiselius (1951) reported that sodium chloride promoted adsorption of phycoerythrin, but in this investigation no appreciable affect on the adsorption of B-phycoerythrin was noted and so the sodium chloride was omitted.

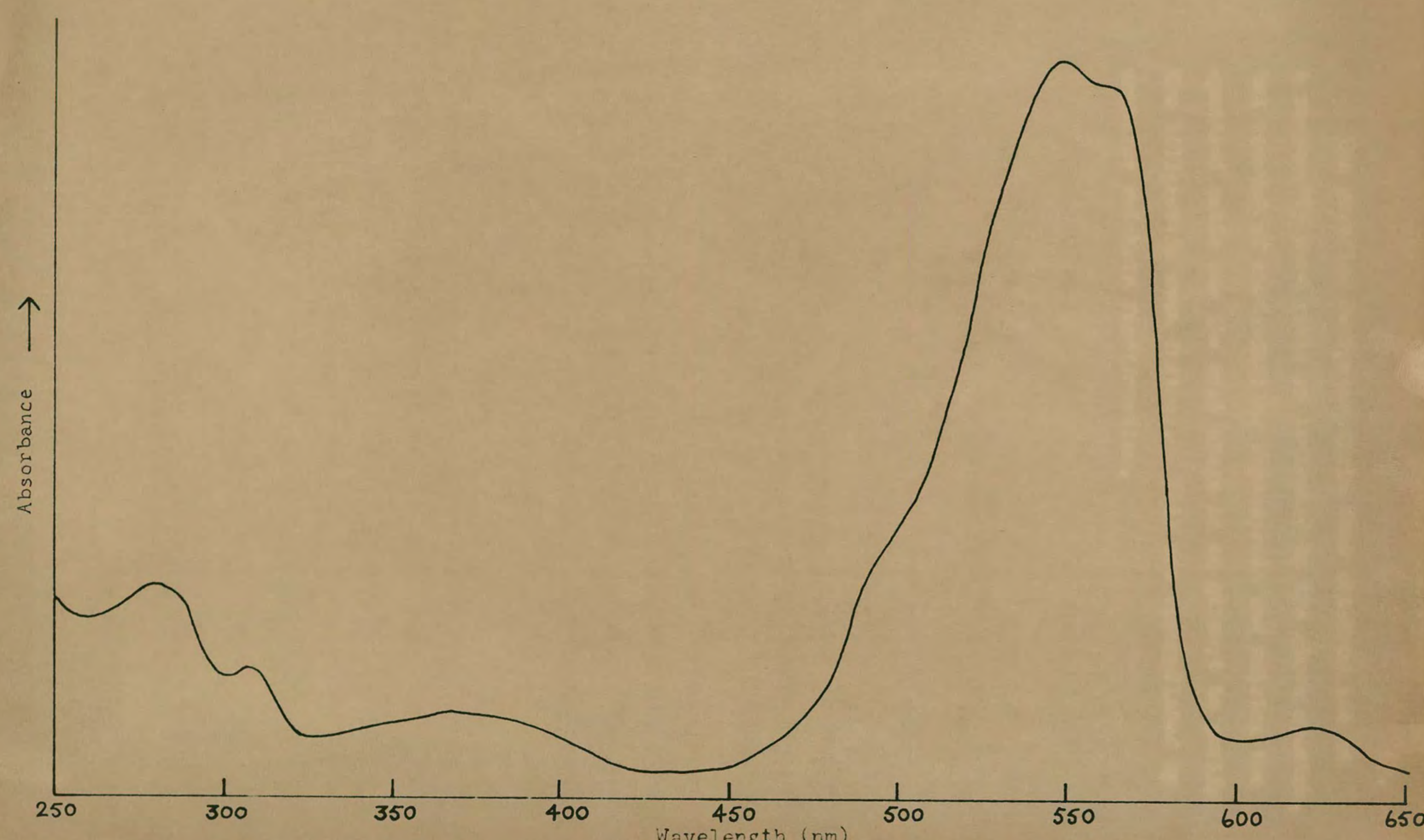


FIG.3. Ultraviolet and Visible Absorption spectrum of B-phycoerythrin after Celite filtration and 30% precipitation.

The purified B-phycoerythrin thus obtained was in a reasonably pure state as indicated by the above spectra, but removal of contaminating phycocyanin and raising the spectral ratio was the next stage in an attempt to purify the biliprotein even further. This was accomplished by ammonium sulphate fractionation.

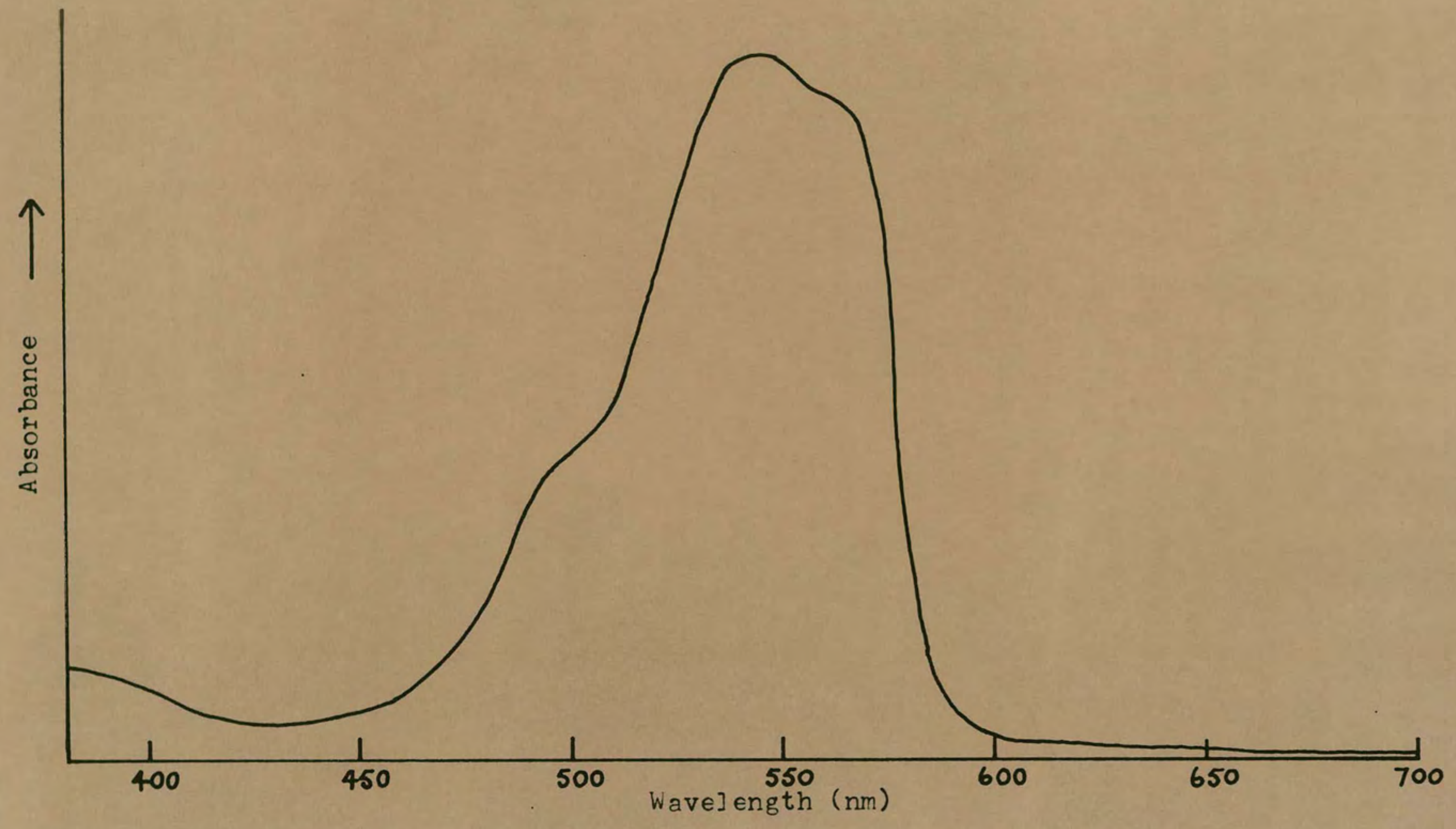


FIG.4.(a) Visible Absorption spectrum of B-phycoerythrin purified by tricalcium phosphate - celite chromatography.

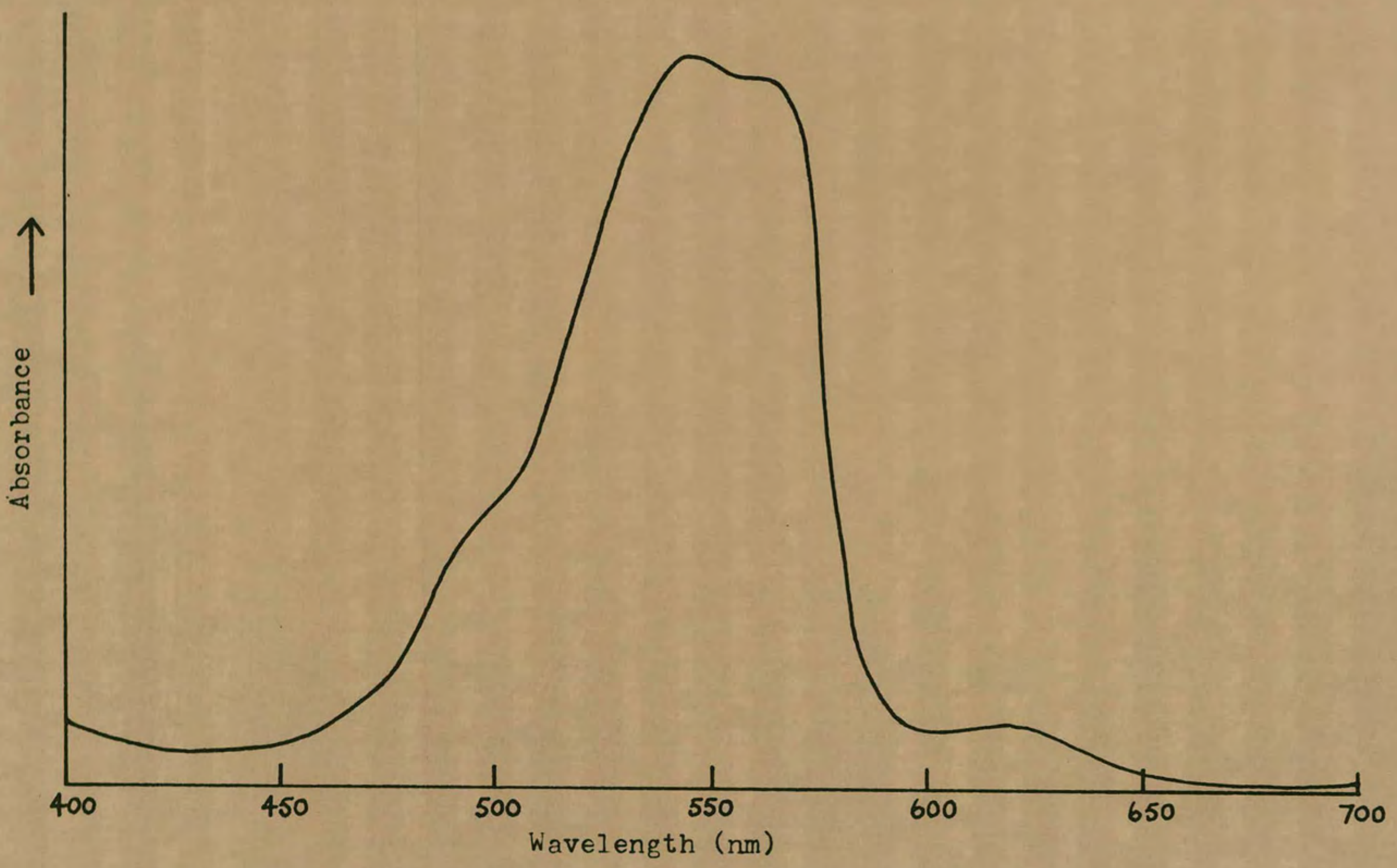


FIG. 4(b) Visible Absorption spectrum of impure B-phycoerythrin.

(4) Crystallization of B-phycoerythrin by Ammonium Sulphate Fractionation.

Ammonium sulphate fractionation over different ranges was found to be an efficient method of purifying B-phycoerythrin to such a high state of purity that it could be crystallized out of solution. However, this method was only used as a purification technique on samples which were already relatively pure as a result of tricalcium phosphate chromatography. This was due to the fact that this method only gave enrichment of the individual biliproteins in the different fractions and not a clear separation.

After tricalcium phosphate - celite column chromatography, biliprotein solutions of similar spectral ratios and visible absorption spectra were combined and precipitated by adding 30% (W/V) finely ground ammonium sulphate. The ammonium sulphate was added slowly to the magnetically stirred and cooled solutions in conical flasks, which were then stored at 2°c in the dark. Several experiments were performed on the resulting precipitated B-phycoerythrin with a view to obtaining the biliprotein in a crystalline form.

Experiment 1 : The 30% precipitated biliprotein solution was centrifuged at 1,500g for 20 minutes at 0°c. The precipitate was collected and dissolved in the minimum of distilled water, then dialysed for 20 hours with three changes of 0.01M sodium phosphate buffer (pH 6.7) at 2°c in a darkened cold room. The fluorescent solution was

then centrifuged as above and the very slight blue precipitate was discarded before the supernatant was precipitated with 20% (W/V) ammonium sulphate. The solution, still strongly fluorescent, was placed in the dark at 2°c for 20 hours to allow complete precipitation to take place, then centrifuged at 1,500g for 30 minutes at 0°c. The supernatant (A) was removed from the precipitate (B) and brought to 30% w.r.t. ammonium sulphate then stored at 2°c in the dark (flask A).

The precipitate (B) was re-dissolved in the minimum of distilled water and dialysed in the dark for 20hrs. at 2°c, using three changes of 0.01M phosphate buffer (pH 6.7). After centrifugation at 2,100g for 30 minutes at 0°c the slight precipitate was discarded and the fluorescent solution again brought to 20% (W/V) with ammonium sulphate, then left overnight at 2°c in the dark. This solution was centrifuged at 2,100g for 30mins. at 0°c and the supernatant brought to 30% w.r.t. ammonium sulphate before adding to flask A. The precipitate from the above centrifugation was again dissolved in water, dialysed for 20hrs. at 2°c against several changes of 0.01M phosphate buffer, and centrifuged at 2,100g for 15mins. at 0°c. The precipitate was again discarded and the solution brought to 15% with ammonium sulphate before standing overnight in a darkened cold room. The slight blue precipitate found on centrifugation under the above conditions, was discarded and the fluorescent solution brought to 20% with ammonium sulphate. This purified solution was placed in flask B and stored in a

refrigerator at 2°c.

Microscopic examination of flasks A and B after three days cold storage indicated that crystallization had occurred. Flask A was found to contain pink, fluorescent, needle-shaped crystals which, apart from certain individual crystals, tended to be small or fragmented. There was also some amorphous material present. The pH of the solution was 5.2. The crystals in flask B were also pink and fluorescent, but not needle-shaped. They appeared to be rectangular and very large crystals were found amongst clusters of small crystals. There was no amorphous material present and the pH of the solution was 5.7.

Experiment 2 : The above experiment was repeated exactly up to the stage where 20% ammonium sulphate was added for the first time. At this point the addition of 20% ammonium sulphate was over a period of 3½hrs. instead of over approximately 1 hour (as in experiment 1) and the resulting fluorescent solution was then allowed to equilibrate for 48hrs. in a covered flask (C) at 2°c. On microscopic examination flask C was found to contain large and uniform pink rectangular-shaped crystals. The pH of this solution was 5.7. A photomicrograph of these crystals indicated that the dimensions of the crystals were 20μ by 3μ.

Experiment 3 : The experiment was repeated but again using a slightly different method. After the pink coloured, fluorescent solution was collected from the

tricalcium phosphate - celite chromatography, it was treated with 20% ammonium sulphate and allowed to stand at 2°c for 3 days. The solution was found to be fluorescent and contained some needle-shaped crystals. This solution was then centrifuged for 50 minutes at 0°c at 2,100g. The precipitate was dissolved in the minimum of distilled water and dialysed against three changes of 0.01M phosphate buffer (pH6.5) for 20 hours at 2°c. After centrifugation at 1,500g for 15 minutes at 0°c, 20% ammonium sulphate was added very slowly and with stirring to the supernatant. This fluorescent solution was stored in a covered flask (D) at 2°c for a week. Crystallization occurred but the pink crystals were very small, and not very numerous. An extra 1% ammonium sulphate was added in an attempt to produce larger crystals. After a week in the dark at 2°c the crystals had grown considerably and were found to be of the same type as C. No amorphous material was present, and the pH of the solution was 5.7.

From the results obtained above, it appeared unnecessary to follow the rigorous purification detailed in experiment 1, especially the initial precipitation with 30% ammonium sulphate after tricalcium phosphate - celite chromatography. This precipitation was only found to be of use when eluted biliprotein fractions were so dilute that 20% ammonium sulphate would not bring about crystallization, and 30% precipitation was a convenient method of concentrating the biliproteins. Hence, initial experiments demonstrated that biliproteins could be

crystallized by addition of 20% finely ground ammonium sulphate to sufficiently concentrated solutions purified by tricalcium phosphate chromatography. Examination of the crystals formed after a few days in the dark at 2°c indicated that the largest and most well defined crystals were obtained from solutions whose pH was in the region of 5.7. In solutions of pH around 5.2 the crystals were generally fewer, smaller and more fragmented in structure. In addition, these latter solutions almost invariably contained a high proportion of amorphous material, which was assumed to be precipitated biliprotein.

A sample of B-phycoerythrin was centrifuged at 2,100g for 30 mins. at 0°c, the supernatant removed, and the crystals dissolved in the minimum of water before dialysing against several changes of 0.01M phosphate buffer (pH 6.5). The fluorescent biliprotein solution was again centrifuged as above and any precipitate was discarded. Ultraviolet and visible spectroscopy indicated that crystallization produced an increase in the purity of the B-phycoerythrin. In the visible region the small maximum at 618nm attributed to R-phycocyanin disappeared completely while the spectral ratio increased to about 5.5. It has been shown therefore, that the major reason for crystallization - to further purify the B-phycoerythrin - has produced the desired results. It should also be noted that on repeated recrystallization of B-phycoerythrin and examination of the spectra, the visible region was found to be unchanged. Airth and Blinks (1956) reported that twice recrystallized

B-phycoerythrin from Smithora naiadum exhibited no 565nm maximum, but this was not found to be the case here.

As mentioned above, the ammonium sulphate was finely ground before addition to the biliprotein solution, and furthermore additions were made over a period of about 4 hrs. If this procedure was not followed, amorphous rather than crystalline biliprotein was formed. There appear to be several factors contributing to this phenomenon. Ammonium sulphate lowers the pH of the solution towards the isoelectric point of the biliprotein (4.4 - 4.5) and consequently the biliprotein becomes less soluble. With the addition of solid ammonium sulphate to the solution, there is probably a considerable drop in the pH in localized regions around the dissolving ammonium sulphate. As a result the B-phycoerythrin is thrown out of solution rapidly and precipitates in the amorphous state. Slow addition of very finely ground ammonium sulphate would minimise these drastic pH variations, and allow formation of the more stable crystalline form. While dissolving, ammonium sulphate even in a finely powdered state, was observed to release tiny bubbles (presumably entrapped air pockets from the ammonium sulphate crystal structure). This constant stream of air is likely to have an inhibiting effect on crystal formation by denaturing the protein at the air - water interface.

In later crystallizations these difficulties were avoided by adding the ammonium sulphate as a concentrated

solution. 430gms. of ammonium sulphate were dissolved in 700mls. of distilled water then filtered through glass wool or filter paper. On the basis of this concentrated ammonium sulphate solution (approximately 61% W/V) a graph was plotted for volume of biliprotein solution against the volume of concentrated ammonium sulphate solution. Using a linear plot, the required volume of ammonium sulphate solution to give a final concentration of 20% with any given biliprotein solution could be determined immediately e.g. the addition of 64mls. of concentrated ammonium sulphate solution to 100mls. protein solution resulted in a 20% ammonium sulphate solution. This proved to be the most satisfactory method of inducing crystallization in B-phycoerythrin solutions, since the above mentioned disadvantages involved in the addition of solid ammonium sulphate were eliminated. Also, the laborious procedure of adding small quantities of ammonium sulphate over a long period can be omitted, since the ammonium sulphate can be added dropwise from a burette.

Photomicrographs were obtained for some of the crystal structures formed and are shown in figs. 5a, b, c and d.

(5) Amino Acid Analysis of B-phycoerythrin.

The amino acid content of B-phycoerythrin was determined using a Technicon Automatic Amino Acid Analyser (G.T. (d)). Protein was usually hydrolysed to a mixture of its constituent amino acids by 24-hour treatment with re-distilled 6N hydrochloric acid at 105°c (see G.T. (g)). Tryptophan is destroyed under these conditions, and can only be determined by alkaline hydrolysis of the protein. The sulphur-containing amino acids are partially or completely oxidised after acid hydrolysis, e.g. cysteine and cystine are converted to cysteic acid; methionine is converted to methionine sulphoxide and methionine sulphone.

Crystalline B-phycoerythrin was centrifuged at 2,100g for 30mins. at 0°c, and the precipitate dialysed as usual against 0.01M sodium phosphate buffer (pH 6.5). The fluorescent solution was finally dialysed against three changes of de-ionised water, then freeze-dried. About 4mgs. of this freeze-dried B-phycoerythrin was weighed out accurately and hydrolysed in constant boiling 6N hydrochloric acid (8mls.) at 105°c for 24 hrs. The protein hydrolysate was then subjected to automatic amino acid analysis and the results shown in Table VI. Other B-phycoerythrin samples were treated similarly and the results indicated that the analyses were reproducible to within ± 3% (the recommended limits of accuracy of the technique).

It was observed that under the above conditions of

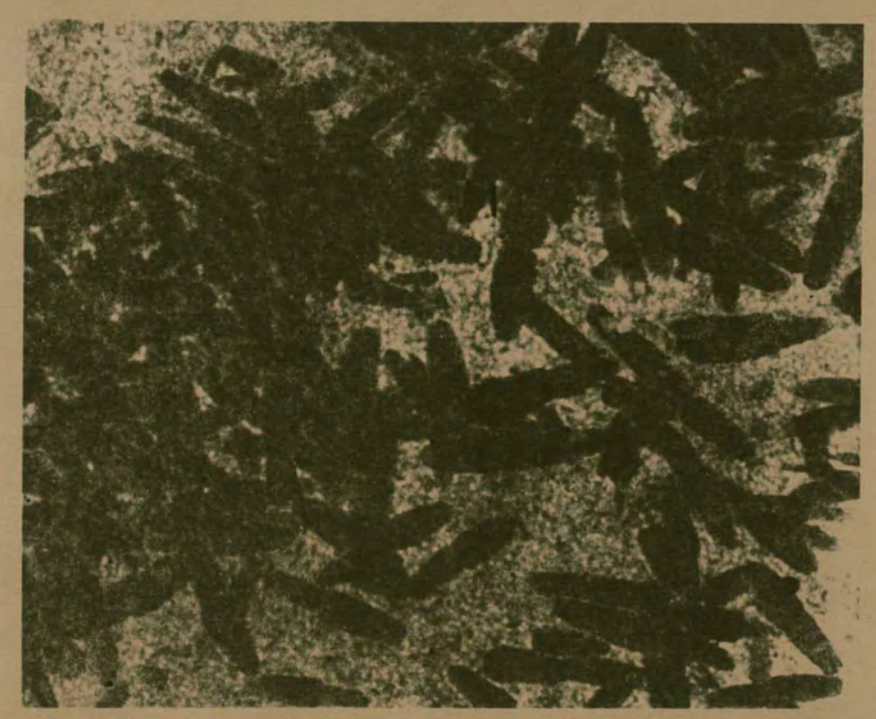


FIG.5(a). Crystalline B-phycoerythrin; precipitated with 20% (W/V) of solid ammonium sulphate; magnification x 400.

FIG.5(b). Crystalline B-phycoerythrin; biliprotein solution made 20% with conc. ammonium sulphate solution; magnification x 400.

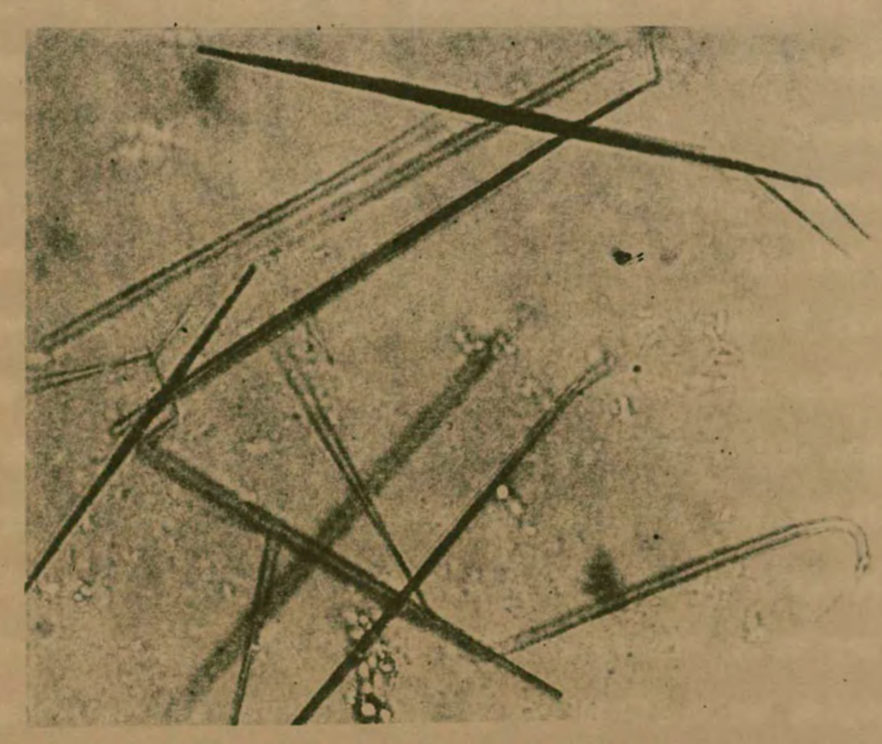


FIG.5.(c). Crystalline B-phycoerythrin; as 5(b).

FIG.5.(d). Crystalline B-phycoerythrin; as 5(b).

hydrolysis, methionine was responsible for a second peak on the amino acid chart due to partial oxidation of some methionine to methionine sulphoxide. Hence to obtain a more accurate value for the methionine content, performic acid oxidation was carried out on samples of freeze-dried B-phycoerythrin using the method of Bidmead and Ley (1958). Performic acid is a powerful oxidising agent which cleaves disulphide bonds. Cysteine and half-cystine residues are converted(almost quantitatively) to cysteic acid which is stable under the conditions of acid hydrolysis. Performic acid treatment also converts methionine almost quantitatively to methionine sulphone.

5mls. of 30% hydrogen peroxide solution was added to 95mls. of 98-100% formic acid together with 0.5ml. methanol. The mixture was kept at room temperature for 2hrs. to allow maximum formation of performic acid and was then cooled to - 10°c. The presence of methanol prevented the mixture from freezing. About 6mgs. of freeze-dried B-phycoerythrin were weighed out accurately and suspended in a few mls. of 98-100% formic acid in a 250ml. round-bottomed flask. The flask was then cooled to - 10°c and 25mls of the cold performic acid reagent were added. The mixture was allowed to react for 2½ hours and then diluted with 2-3 volumes of ice-cold distilled water. Freeze-drying removed the bulk of the performic acid present. A further 25mls. of de-ionised water was added to the freeze-dried material and the freeze-drying repeated to remove virtually all the performic acid. During freeze-drying a lung of glass

TABLE VI - Amino Acid Analysis of B-phycoerythrin

AMINO ACID	μ mole	RESIDUE WT. (mgs.)	MOLES/MOLE HISTIDINE
Cysteic acid	·0257	·96	2
Aspartic acid	·2066	8·56	15
Threonine	·0759	2·76	5
Serine	·1528	4·79	11
Glutamic acid	·1527	7·10	11
Proline	·0456	1·60	3
Glycine	·1450	2·98	10
Alanine	·2855	7·30	20
Valine	·1362	4·86	10
Methionine	·0480	2·27	3
Isoleucine	·0873	3·56	6
Leucine	·1464	5·97	10
Tyrosine	·0853	5·01	6
Phenylalanine	·0464	2·46	3
Lysine	·849	3·92	6
Histidine	·0140	·69	1
Ammonia	·2907	-	-
Arginine	·1189	6·69	8
Moisture	-	10	-
Total	-	80	-

Column 1: μ moles of each amino acid in sample analysed

Column 2: the residue weight/100mgs. biliprotein (moisture content 10%)

Column 3: "rounded off" values for moles of each amino acid relative to one mole of histidine.

wool was used to prevent loss of material from the flask. On completion of the second freeze-drying the glass wool was washed into the 250ml flask with constant boiling hydrochloric acid, and the flask agitated to remove material adhering to the sides of the flask. The contents and washings from the flask were transferred to a hydrolysis tube then hydrolysed as usual at 105°c for 24hrs. Amino acid analyses of samples oxidised as above were generally run on longer ion-exchange columns (64cms.) as this increased the resolution of aspartic acid and methionine sulphone. The results from performic acid oxidation are also quoted in table VI.

(6) Enzymic Hydrolysis of B-phycoerythrin.

One of the principle aims of this investigation was to obtain small chromopeptides (i.e. peptides with the chromophore group still attached) by enzymic digestion. Amino acid analyses of sufficiently purified chromopeptides could then be used to postulate the nature of the chemical bonds which link the tetrapyrrolic chromophore group of B-phycoerythrin to the apoprotein.

Proteolytic enzymes, or proteases, are available from a large number of animal, plant, fungal and bacterial sources. Of the many enzymes capable of splitting internal peptide bonds (endopeptidases), only trypsin, chymotrypsin and pepsin are sufficiently specific in their action for a controlled degradation of proteins. Other enzymes like papain and subtilisin are particularly useful when it is desired to reduce a larger peptide to a small group of di- and tri-peptides. A number of factors control the rates at which these enzymes attack certain peptide bonds. Apart from the spatial arrangement inherent in amino acid sequences, the folding of the chain imposes further structural relationships. Many native proteins are resistant to attack by enzymes, but are readily digested after denaturation or oxidation. The splitting of certain bonds may modify the behaviour of adjacent susceptible bonds and the cleaved products themselves tend to influence the specificity of an enzyme, leading to unexpected cleavage products.

Although enzymes are more stable at low temperatures, digests at 37-40°c allow faster reaction rates. Difficult bonds may be split using the forcing conditions of a high enzyme to substrate ratio, or by prolonging the digestion time. The optimum conditions for the controlled degradation of a protein are best obtained by construction of hydrolysis rate curves.

Two enzymes were utilised in the present work. These were pepsin and trypsin, and a brief description of the properties of these enzymes will be given before the experimental details are described.

Pepsin: Commercial crystalline pepsin is obtained from gastric juice or gastric mucosa and is a mixture of enzymes. It is fairly stable at pH 5.6, is denatured above pH 6.0 and is optimally active on proteins at pH values between 1 and 2. During the formation of pepsin from pepsinogen, a large fragment of the molecule is split off (Vunakis and Herriott, 1956). This polypeptide is a natural inhibitor acting between pH 5 and 6. Pepsin exhibits a very wide specificity, although experiments with synthetic substrates have shown that a preferential attack is made at bonds involving the amino groups of aromatic amino acids. There are however many instances where such bonds are resistant in proteins. The specificity of pepsin is enhanced at low enzyme-substrate ratio by limiting the reaction time to about 30 minutes (Braunitzer, Hilschmann and Müller, 1960)

Trypsin: Trypsin is isolated from bovine pancreatic juice. It is normally used in the pH range 7 - 9, but owing to a strong tendency to autolyse it is not possible to keep the enzyme active for long periods at high pH. The most valuable property of trypsin is its narrowly restricted specificity which limits cleavage exclusively to bonds linking the carboxyl group of a basic amino acid to the amino group of another amino acid or to the hydroxyl group of an alcohol. It has long been known that certain native proteins are resistant to the action of trypsin, but that proteolysis takes place readily after denaturation of the protein. On the basis of amino acid composition it is possible to predict the number of peptides that might be expected in a tryptic digest of a denatured protein.

(6A) Pepsin Digestion of B-phycoerythrin.

The procedure used for the peptic digestion of B-phycoerythrin was basically that of Lemberg (1928).

Pepsin Digest 1.

240mgs. of freeze-dried B-phycoerythrin were dissolved in 50mls. of 0.1N hydrochloric acid containing 50mgs. of crystalline pepsin (from stomach mucosa, 3 x crystallized; Koch-Light Labs. Ltd., Colnbrook, England). The mixture was incubated at 34°c for 24hrs., then a further 10mls. of 0.1N hydrochloric acid containing 50mgs. pepsin were added and the incubation conditions repeated. The digest was centrifuged to remove insoluble material and the supernatant extracted several times with small portions of t-amyl alcohol (Total 100mls.). Centrifugation was always necessary to bring about separation of the two layers. The final extraction by t-amyl alcohol exhibited little coloured material in the organic phase. Ultraviolet and visible spectra were run on a sample of the organic layer (see fig. 6). Two maxima occurred in the visible region - one at 499nm and the other at 546-550nm. The absorption in the ultraviolet region also indicated two maxima at 312nm and 278nm. The aqueous phase was treated with five volumes of ethanol to precipitate the pepsin, and after 3 days the colloidal precipitate was removed by centrifugation. Absorption spectra from a sample of the aqueous ethanolic supernatant indicated that this contained virtually no chromopeptide material. This solution was only slightly pink in

colour and the colloidal precipitate was red coloured, which seemed to indicate that more than pepsin was present.

The t-amyl alcohol layer was reduced to dryness by rotary evaporation and the residue was dissolved in 4mls. of ethyl alcohol. This redish solution contained chromopeptide material and was subjected to the following purification techniques in an attempt to isolate a relatively pure chromopeptide.

(1) High Voltage Electrophoresis

An attempt was made to purify this material by high voltage paper electrophoresis, using pyridine - acetic acid - water (6:4:290) electrolyte (pH 5.0) on Whatman No. 1 paper. A small quantity of the chromopeptide mixture was applied to the Whatman paper (15 x 55cms.) and electrophoresis carried out for 30mins. at 3Kv (i.e. 55 volts/cm.) (see G.T. (k)). On completion of the run, the electrophoretogram was allowed to dry in air then viewed under ultraviolet light to determine the position of any fluorescent bands. Ninhydrin - positive groups were detected by spraying the electrophoretogram with ninhydrin solution (a 0.5% solution of indanetrione hydrate in water-saturated butanol), then drying in an oven at 80°c for 10 mins. to develop the characteristic purple colour. The results are shown in fig. 7. The experiment was repeated using higher voltage viz. 6Kv for 25 minutes, and an almost identical result was obtained. These results indicated that at

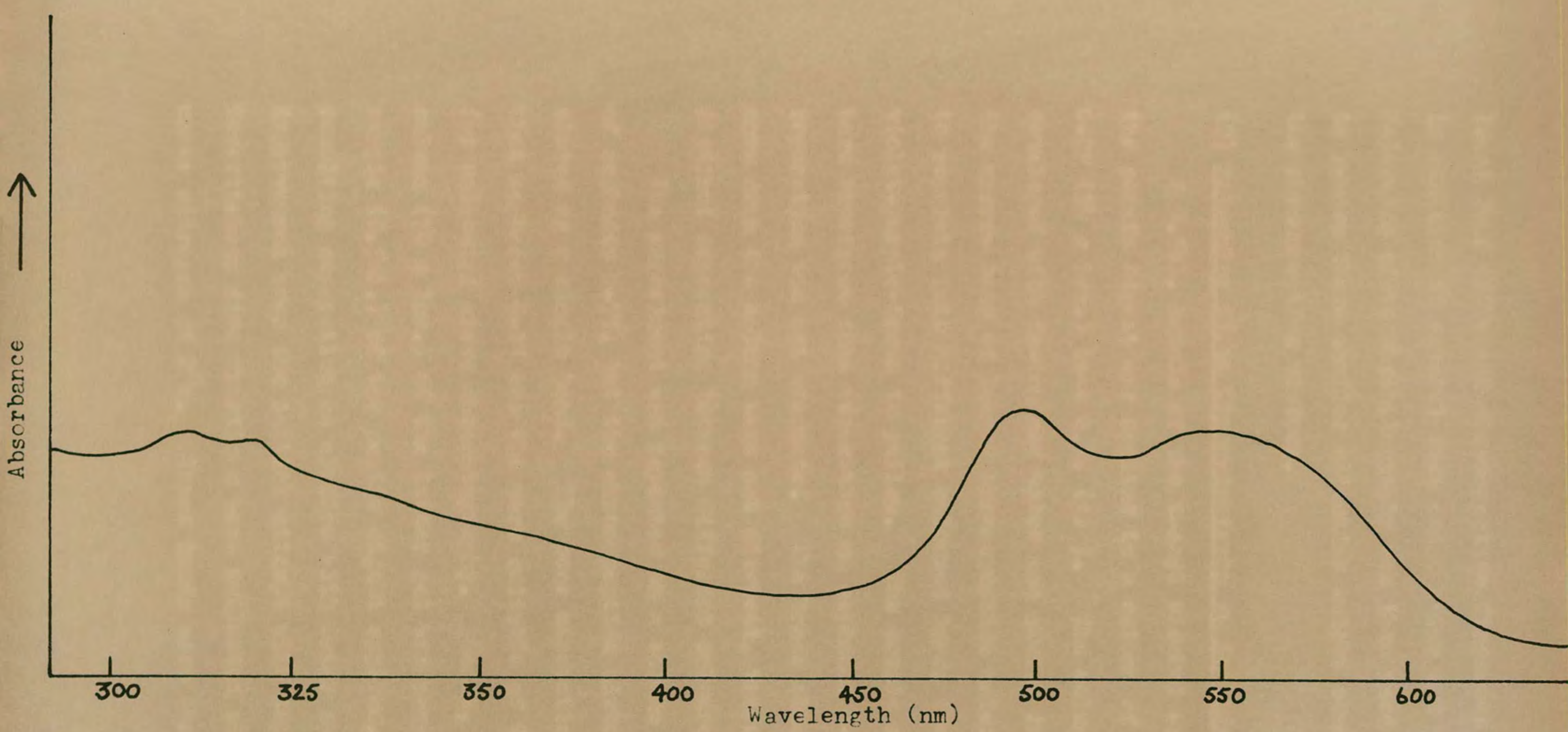


FIG.6. Ultraviolet and Visible absorption spectra of t-amyl alcohol extract of pepsin digest.

least four different chromopeptide fragments were present in addition to several free amino acids and peptides. Molecular sieve chromatography was then used in an attempt to remove these smaller molecular weight compounds from the chromopeptide mixture.

(2) Molecular Sieve Chromatography on Sephadex

A 0.5ml. sample of the t-amyl alcohol extract from the pepsin digest was applied to a column of sephadex G-25 (14 x 1cm.) (see G.T. (i)). The column was developed with de-ionised water and almost immediately the narrow band of loaded chromopeptide material began to separate into different coloured bands from pink-violet-purple-brown to yellow. The sample appeared to have undergone some separation on sephadex G-25, but the separation was not adequate. The bands observed to move down the column were not discreet, there being no distinct separation between different coloured bands.

The experiment was repeated on a column (7 x 1cm) of sephadex G-75, but the chromopeptide mixture was eluted as a single band. The poor separation indicated that sephadex G-25 was the more suitable of the two gels for molecular sieve chromatography. Hence, a large column (50 x 1cm.) of the latter gel was prepared and a sample (0.5ml.) of the peptic digest applied. The resulting separation on elution with water showed little improvement over the initial attempt with sephadex G-25. Nevertheless five fractions were collected and stored under nitrogen at 2°c. These bands were then reduced to

CATHODE ANODE

FLUORESCENT MATERIAL

NINHYDRIN POSITIVE MATERIAL

FIG.7. H.V.E. of t-amyl alcohol extract of pepsin digest; pyridine-acetic acid-water (6:4:290), pH 5·0; 30 minutes at 55 volts/cm.

CATHODE ANODE

D C B A

FLUORESCENT MATERIAL

NINHYDRIN POSITIVE MATERIAL

FIG.8. H.V.E. of band 1 from Sephadex G-25; pyridine-acetic acid-water (6:4:290), pH 5.0; 30 minutes at 73 volts/cm.

dryness by rotary evaporation and dissolved in two drops of ethanol. High voltage paper electrophoresis was performed on each of the five collected fractions with a view to determining whether there was any observable difference. In all cases electrophoresis was carried out for 30 minutes at 4Kv (73 volts/cm.) in pyridine - acetic acid - water (6:4:290) buffer, pH 5.0. The electrophoretogram resulting from the first eluted fraction (band 1) is shown in fig.8. with fluorescent components A, B, C and D; it is very similar to that obtained by electrophoresis of the chromopeptide mixture, prior to sephadex G-25 chromatography (see fig.7.). Also, band 2 contains components B and C; band 3 contains component B and another fluorescent component at the origin; bands 4 and 5 contain components B, C and D.

These results appeared to indicate that sephadex G-25 chromatography developed with water was not suitable as a means of separating the chromopeptide mixture.

Pepsin Digest 2:

200mgs. of freeze-dried B-phycoerythrin were dissolved in 20mls of 0.1N hydrochloric acid containing 49mgs. of pepsin. The mixture was digested at 37°c for 24hrs., after which a further 10mls. of 0.1N hydrochloric acid containing 45mgs. pepsin were added and the incubation continued for a further 24 hours. The pepsin digest was centrifuged to remove any insoluble material, then extracted several times with small portions of iso-amyl alcohol. To the amyl alcohol extract (100mls.) seven volumes of petroleum ether were added, and the mixture was

allowed to stand overnight in a separating funnel. The lower phase was separated and the amyl alcohol removed by rotary evaporation with the oil pump. The residue was dried over P_2O_5 in an atmosphere of nitrogen then dissolved in 5mls. of methanol. The coloured solution was centrifuged, but no precipitate of peptone-like material was observed. The chromopeptide solution was stored in the cold prior to attempts at further purification.

(1) Thin Layer Chromatography on Cellulose plates

Thin layer plates were prepared using the Desaga apparatus and cellulose powder MN300 (see G.T. (J)). The chromatographic solvent used was phenol-acetic acid-water (1:1:1) and the plates were washed with this solvent before use. A sample of the chromopeptide mixture in methanol was applied to a plate and allowed to develop for 6 hours. After drying, the plate was viewed under an ultraviolet lamp, then sprayed with ninhydrin solution and heated to 80°c for colour development. Only two bands were observed, one red band at the solvent front which was ninhydrin-positive but non-fluorescent; the other at R_f value 0.9 was ninhydrin-positive and fluorescent.

(2) Ion-exchange Chromatography

The strong cation-exchange resin Amberlite CG-50 (type 111) (Rohm and Hass Co., Philadelphia, U.S.A.) was slurried before use to remove fines. A 1:10 slurry (resin:water) of the sodium form of the resin was allowed to settle for 3hrs. The smallest particles, which remained suspended were decanted. This process was

repeated six times, then the resin, as a 1:5 slurry, was allowed to settle in a two litre graduated cylinder. The upper 30-40% of the resin was removed by suction and discarded.

A column (50 x 1cm.) of this resin was then prepared and equilibrated with 0.2M sodium phosphate buffer (pH 6.50). A dried sample of the red chromopeptide mixture was dissolved in 1.5mls. of the sodium phosphate buffer and applied to the column. The yellow chromopeptide solution was developed with the above 0.1M sodium phosphate buffer, and the eluate monitored automatically at 440nm. The absorption trace indicated at least three peaks but the eluates were not retained as the separation was considered inadequate.

Pepsin Digest 3 :

The pepsin digest and extraction of chromopeptide material was carried out by the same procedure as in Pepsin digest 2. After extraction with petroleum ether (60-80°), the iso-amyl alcohol layer was dried and stored in a vacuum dessicator as before. This material was then used in a series of experiments with sephadex gels in an attempt to find a suitable gel and elution solvent.

(a) Sephadex G-75.

A column (13 x 1cm.) of sephadex G-75 in water was prepared (G.T. (i)) and a sample of the chromopeptide mixture dissolved in 0.5ml. methanol was applied. A very narrow band was observed to move down the column very slowly when developed with water. This band

contained all the coloured material from the chromopeptide mixture, and showed no tendency to be separated into different fractions. Presumably the pore sizes in this gel (exclusion limit 50,000) were so much larger than the various chromopeptides that the chromopeptide components of the mixture were equally retarded by diffusion through the gel.

(b) Sephadex G-50.

A column (13 x 1cm) of sephadex G-50 was prepared as above and a sample of the chromopeptide mixture, dissolved in water, was added to the column. When developed with water, this material was observed to separate into an orange-yellow band followed by a purple band. However, the solute was then seen to precipitate out of solution and formed aggregates on the column gel.

A second column was prepared as described above, but on this occasion the gel was prepared in a 1% acetic acid solution. The chromopeptides were also applied to the column and eluted with 1% acetic acid solution. These conditions resulted in a similar, but less defined separation, as that obtained when water alone was used. The only indication of any separation at all was an orange-yellow colour at the solvent front. On further elution, the solute appeared to be precipitating out on the column, but to a lesser extent than the previous case. Before the bands were eluted from the column they became very diffuse, and as a result were spread over a considerable length of the column.

To prevent the chromopeptide material precipitating out during chromatography, it was decided to examine the effect of using a buffer as the developing solvent. Accordingly, a column (13 x 1cm.) of G-50 was prepared and equilibrated with 0.2M sodium phosphate buffer, pH 6.5. A sample of the chromopeptide mixture was dissolved in 1.0ml. of the phosphate buffer and applied to the column. When the column was developed with this buffer, the solute did not precipitate out on the column, and some separation of the mixture was observed. An orange coloured band preceded a purple band down the column and three fractions were collected. The first fraction contained the orange coloured band, the third fraction contained the purple band, and the second fraction contained the eluate intermediate between these two bands. The fractions were reduced to dryness on a vacuum rotary evaporator, then dissolved in a few drops of methanol. Each of the fractions was subjected to high voltage paper electrophoresis on Whatman no.1 paper for 30 minutes at 4Kv (73 volts/cm.) using pyridine-acetic acid-water (6:4:290) buffer, pH 5.0.

The first fraction from gel chromatography (orange coloured) produced two components on electrophoresis; one about 1cm on the anode side of the origin was fluorescent but not ninhydrin-positive; the other 2-3cms. on the cathode side of the origin was ninhydrin-positive but non-fluorescent. The third fraction from gel chromatography (purple coloured) gave a fluorescent, non ninhydrin-positive band at the origin, a fluorescent non

ninhydrin positive band 3cms. on the anode side of the origin, and a fluorescent ninhydrin-positive band 2cms. on the cathode side of the origin. Electrophoresis of the second fraction from molecular sieve chromatography gave components which indicated that this fraction was a mixture of fractions one and three.

Sephadex G-25.

Column chromatography of the chromopeptide mixture on sephadex G-25 proved to be more successful than any used so far. A column (13 x 1cm.) was loaded with a sample of the chromopeptides and eluted with water. Unlike the previous case, precipitation did not occur and an orange-pink band was observed to separate from the mixture. A mauve coloured band remained at the top of the column long after the orange-pink band was eluted. On further development, this mauve band became fainter and finally disappeared.

This experiment was repeated using a 1% acetic acid solution for column equilibration, loading and elution. The applied material was eluted as a single band with virtually no separation. As a result of these experiments, a separation of the chromopeptides was attempted by using a combination of eluants. A column (13 x 1cm.) was prepared as above and the applied chromopeptide mixture eluted initially with de-ionised water. When the red coloured band had moved half way down the column, the water was replaced by a 1% acetic acid solution. Hence two fractions were collected using this procedure; a red

band was eluted with water followed by a mauve band eluted with 1% acetic acid solution.

<u>Sephadex G-10</u>.

The techniques used for sephadex G-10 were similar to those applied above. A chromopeptide sample was dissolved in 1ml. of water and applied to a column (13 x 1cm.) packed with sephadex G-10. When eluted with water, a rust coloured band appeared to move unretarded through the column and was eluted at the solvent front. The rest of the coloured material remained on the column long after the elution of the first band, but was subsequently removed as a mauve band when eluted with 1% acetic acid. Visible absorption spectra were obtained for the two fractions and indicated differences in absorption maxima. The rust coloured band (A) displayed three maxima in the visible region: one was at 492nm, a smaller one at 588nm and another at 643nm. The mauve coloured band (B) displayed only two maxima; one was at 492nm, the other one about 540-560nm. Both bands were reduced to dryness and subjected to high voltage electrophoresis on Whatman no.1 paper for 30mins. at 4Kv, using pyridine-acetic acid-water (6:4:290) pH 5.0. Band (A) gave rise to one fluorescent component on the cathode side of the origin and three on the anode side. On the other hand, (B) gave no fluorescent material on the anode side of the origin, and only one fluorescent component on the cathode side. Band (B) also left a mauve coloured fluorescent component at the origin. All the fluorescent

components were observed to be ninhydrin-positive. In addition, there was also ninhydrin positive material which did not exhibit fluorescence (presumably free amino acids or peptide fragments).

The above column chromatography on sephadex G-10 was repeated using a 1% acetic acid solution for column equilibration and elution. As might be expected, no separation of the chromopeptide material was obtained and the applied sample moved down the column as a compact band.

Pepsin Digest 4 :

As reasonable quantities of crystalline B-phycoerythrin were now available, it was decided to use crystalline material for subsequent enzymic digests. A sample containing large needle-shaped crystals was centrifuged at 2,100g for 20 minutes at $2^{o}c$, and the crystalline precipitate dialysed for 48 hours against five changes of de-ionised water in a darkened cold room. The contents of the dialysis tubing were then transferred to a 100ml. conical flask and 25mgs pepsin in 12mls. of 0.1N hydrochloric acid added. The pH of the solution was adjusted to 1.6 with 1.0N hydrochloric acid and then the mixture was allowed to digest for 20 hours at $37^{o}c$. A further 30mgs. pepsin in 12mls, of 0.1N hydrochloric acid were added, the pH adjusted to 1.6, and the incubation allowed to proceed for a further 28 hours. The reaction was stopped by adding an equal volume of 20% trichloroacetic acid. After the precipitated protein

had been removed by centrifugation, the supernatant liquid was extracted with iso-amyl alcohol. A seven-fold volume of petroleum ether (60-80°) was mixed with the iso-amyl alcohol extract and the mixture allowed to stand overnight. The lower phase was separated and reduced to dryness on a rotary evaporator. The residue was dissolved in 10mls. of methanol and the small quantity of peptone-like material removed by centrifugation. After reducing the supernatant to dryness as above, the residue was stored in a dessicator over phosphorus pentoxide.

An attempt was made to separate the chromopeptides on a column of "Isopor" De-acidite M-IP resin (SRA 154) (The Permutit Co. Ltd., London, England). A quantity of this weakly basic anion exchange resin was allowed to equilibrate in de-ionised water, then washed with 50% acetic acid in a Büchner funnel. This was followed by washing the resin with de-ionised water, 2.0M sodium hydroxide and finally removing excess hydroxide with de-ionised water. A column (35 x 0.6cm) was prepared and equilibrated with water until the eluate was free of hydroxide. A sample of the chromopeptide mixture was dissolved in 1.5mls. water and applied to the column. The solvents used for elution were 1%, 5% and 10% acetic acid solutions. Initial separation into 3 coloured bands (red, brown and green) was observed shortly after the addition of the first eluant. The effect of the 5% acetic acid solution was to decrease the elution time of the two front bands (red and brown) without seriously

affecting the separation. 10% acetic acid was required to elute the green band from the resin. The three fractions were collected separately, reduced to dryness, and each subjected to high voltage electrophoresis on Whatman no 1 paper (15 x 55cms.) for 30 minutes at 4Kv using pyridine-acetic acid-water (6:4:290) buffer, pH 5.0. The resulting electrophoretogram indicated that each fraction contained several fluorescent ninhydrin-positive components, and that most components were common to all fractions.

From these, and preceeding results it was apparent that the separation of chromopeptide mixtures could not be accomplished by column chromatography alone. Also, the use of ion-exchange columns did not prove to be any more efficient than molecular sieve chromatography.

Pepsin Digest 5:

The peptic digestion of crystalline B-phycoerythrin (600mgs) and extraction of the chromopeptide material were carried out by the same procedure as pepsin digest 4.

Molecular sieve chromatography of the chromopeptide mixture was performed on columns of Bio Gel P-6 (G.T. (i)). A column (13 x 1cm.) of Bio Gel P-6 was prepared and equilibrated with de-ionised water prior to loading with a sample of chromopeptide material dissolved in 0.5ml water. The column was then eluted with water, 1% acetic acid, 5% acetic acid and finally 20% acetic acid solutions. Various coloured fractions were eluted. In order of elution from the column, these ranged from pink-red-

colourless-purple-mauve-colourless to green. Thirteen fractions were collected and their visible absorption spectra recorded, using a Unicam SP/800 spectrophotometer. The main fractions are shown in fig.9.

A similar column (21 x 1.3cms.) of Bio Gel P-6 was prepared in de-ionised water and washed with 6 volumes of de-ionised degassed water. A 2ml. sample of the chromopeptide mixture was loaded onto the column using a syringe, then eluted initially with de-ionised water. The eluant was supplied by a peristaltic pump at 15mls./hour, while the eluate was continuously monitored at 253nm using an L.K.B. Uvicord flow photometer in conjunction with a fraction collector (L.K.B., Stockholm, Sweden.). The Uvicord monitor was connected to a servoscribe potentiometric recorder in such a way that both the absorption of the eluant and each change of tube on the fraction collector were recorded automatically. The absorption trace indicated five maxima (fig. 10) and fractions occurring beneath these maxima were combined and labelled A, B, C, D and E. When visible absorption spectra of these fractions were obtained they were virtually identical to the absorption spectra illustrated in fig.9.

Pepsin Digest 6:

A sample of crystalline B-phycoerythrin was centrifuged at 2°c for 30 minutes (2,700g) and the precipitate dialysed with 0.01M sodium phosphate buffer, pH 6.5, for 48hrs. in a darkened cold room. The fluorescent solution

was then freeze-dried (G.T. (h)) for 20hrs. 50mgs. of this material were dissolved in 50mls. of 0.1N hydrochloric acid before mixing with 20mgs of pepsin dissolved in 10mls. of 0.1N hydrochloric acid. The pH was adjusted to 1.6 and the mixture left to digest at 37°c for 24hrs. when a further 24mgs. of pepsin, dissolved in 0.1N hydrochloric acid, were added. After readjustment of the pH, the digest was incubated for a further 24hrs. at 37°c, then cooled to room temperature. 70mls. of 20% aqueous trichloroacetic acid were added and the precipitate removed by centrifugation. The coloured supernatant was extracted with several small volumes of iso-amyl alcohol then combined with a seven-fold volume of petroleum ether (60-80°) and allowed to stand overnight. The lower layer was removed and reduced to dryness by freeze-drying.

The chromopeptide material was further purified by partition chromatography on celite using the procedure suggested by Dus, Bartsch and Kamen (1962). 12gms. of celite were washed with 50ccs. of 0.5% aqueous acetic acid solution, 50ccs. of de-ionised water and finally with 60ccs. of very dilute ammonia solution (1 part conc. ammonia in 8,000 parts water). The cleaned material was allowed to dry in air then mixed with 4.4gms. of the lower phase of a freshly prepared mixture of n-butanol-acetic acid-water (4:1:5 by volume). Sufficient liquid from the upper phase was added to produce a fluid slurry. A 1.2cm diameter column was filled with the upper phase liquid before pouring the slurry and packing the column.

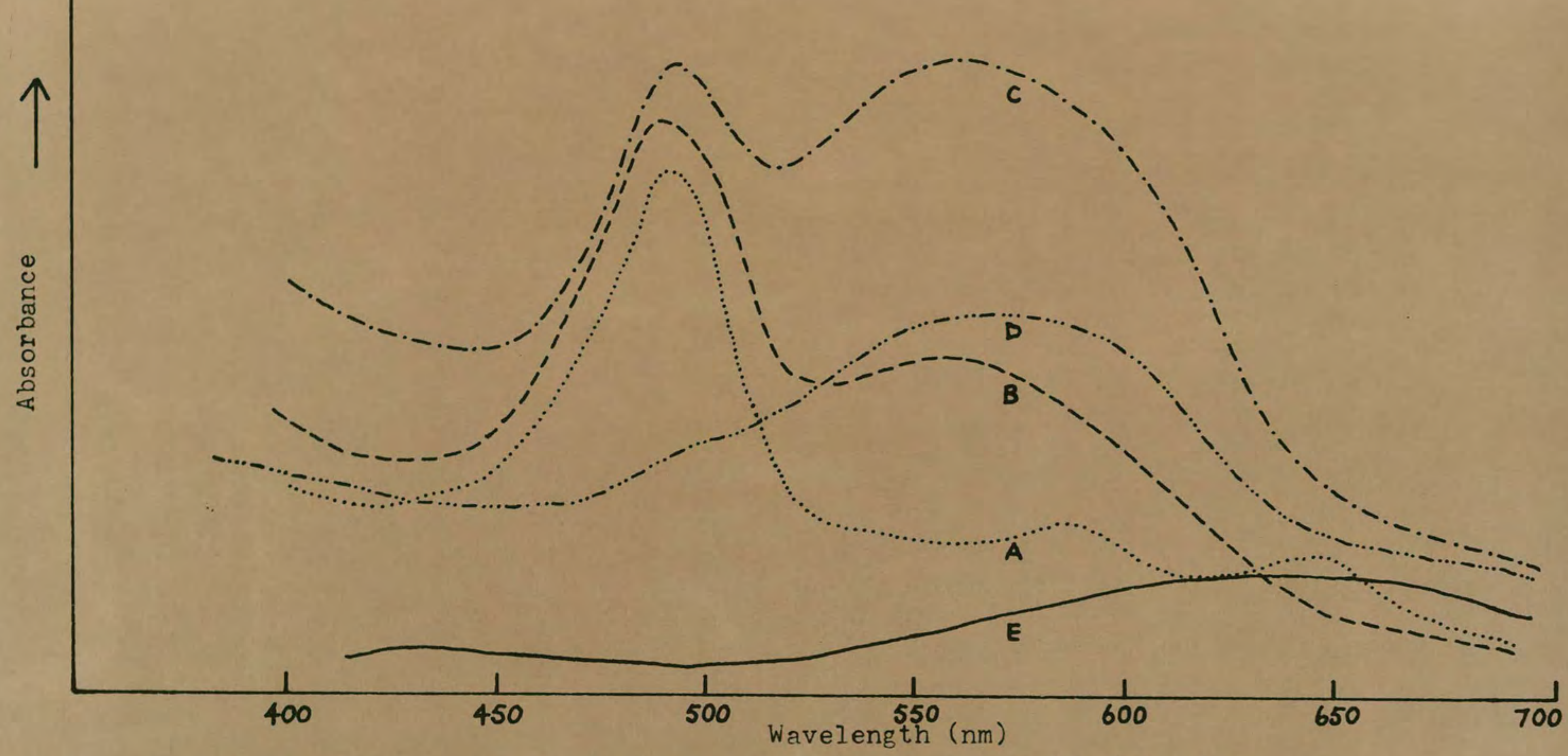


FIG.9. Visible absorption spectra of fractions eluted from Bio Gel P-6 column.

A: Pink band eluted with water
B: Red band " " "
C: Purple band " " 1% acetic acid solution
D: Mauve band " " " " " "
E: Green band " " 20% acetic acid solution

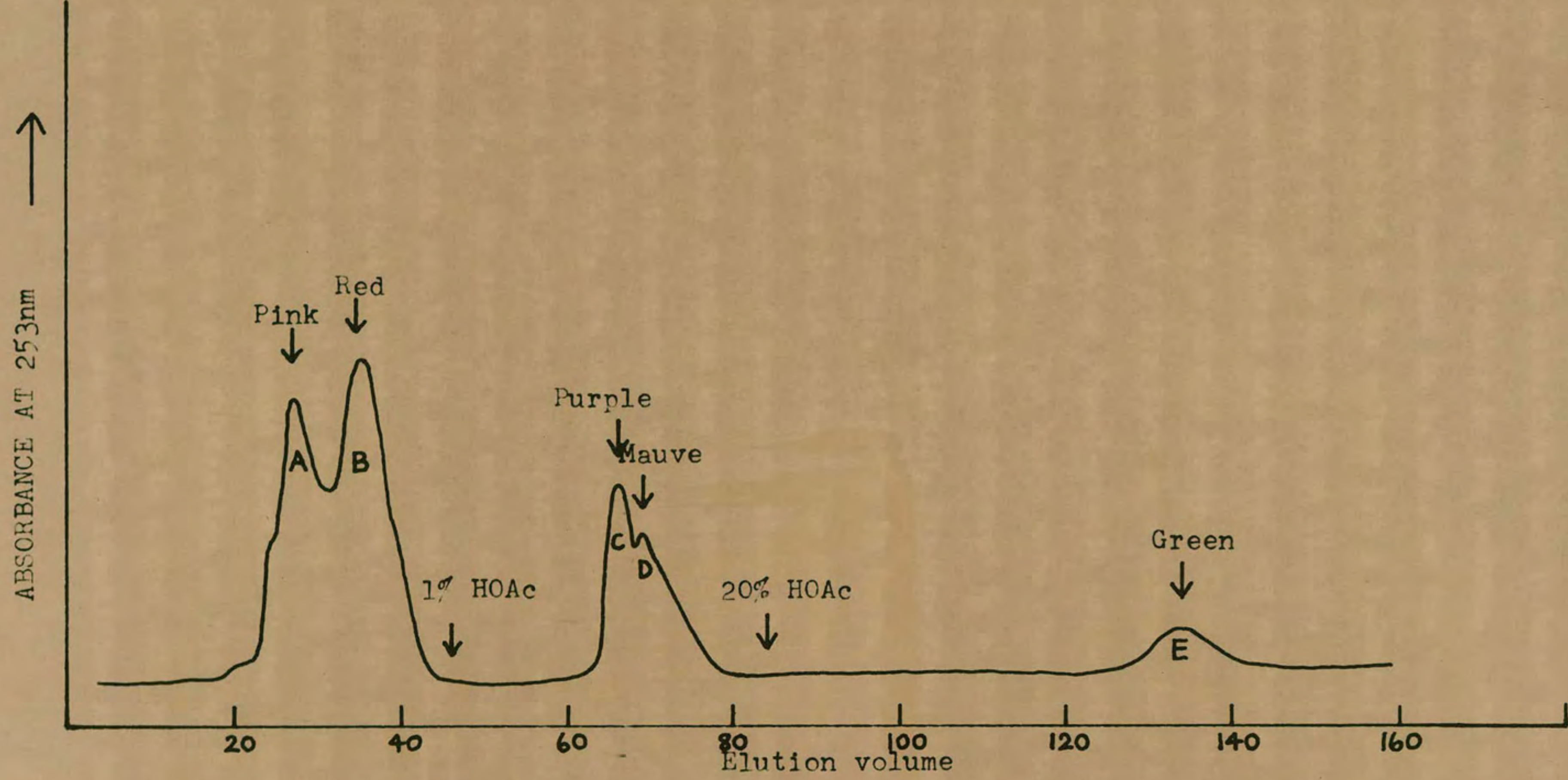


FIG.10. Absorption trace (at 253nm) of chromopeptides eluted from Bio Gel P-6 column.

Slurry addition was carried out by means of a 10ml. pipette, which prevented aggregates of celite entering the column. This procedure also reduced the likelihood of trapping air bubbles in the column. The chromopeptides were dissolved in 1.5mls. of the upper phase liquid applied to the column (25 x 1.2cms.) and elution commenced with the upper phase liquid. The purple chromopeptide mixture was eluted and collected at the solvent front as a single band. This material was then subjected to gel filtration on a column (40 x 0.9cm.) of Bio Gel P-6 using phenol-acetic acid-water (1:1:1 by volume) as eluant. Two bands were observed to move down the column, a purple band followed by a brown band. The front band was collected, freeze-dried, then hydrolysed for 90hrs. at 105°c in 8mls. of constant boiling hydrochloric acid. The prolonged hydrolysis time was considered necessary since the freeze-dried chromopeptide fraction was not completely dissolved on addition to the constant boiling hydrochloric acid. Apart from this, the acidic hydrolysis and amino acid analysis were performed as detailed in G.T. (g) and G.T. (d). The results of amino acid analysis of this chromopeptide sample (A) are shown in Table VII, but arginine was not determined since a large quantity of unidentified material was found at the elution volume for arginine. The second fraction (brown coloured) collected from the gel filtration on Bio Gel P-6 was collected but not subjected to amino acid analysis. This material was subsequently identified as being

derived from chromatography on the celite column, and was absent when the chromopeptide material from the celite column was reduced to dryness by freeze-drying prior to gel filtration.

Pepsin Digest 7 :

The digestion procedure was varied slightly in that biliprotein solution (rather than freeze-dried material) was subjected to enzymic hydrolysis. A sample of crystalline B-phycoerythrin was centrifuged and dialysed as previously (see pepsin digest 6). To determine the quantity of protein digested, a measured sample of the fluorescent biliprotein solution was subjected to visible absorption spectroscopy. From the recorded optical density obtained, the concentration of biliprotein in solution was calculated using the relationship $E^{1\%}_{1cm} = 82.3$ (i.e. the optical density of a 1% (W/V) B-phycoerythrin solution in a 1cm. cell = 82.3). As a check on the value obtained, a measured sample of the biliprotein solution was freeze-dried and weighed. Assuming the biliprotein solution is 0.01M in sodium phosphate, the weight of sodium phosphate present in solution was calculated and subtracted from the weight of freeze-dried material. The value obtained for biliprotein concentration by this method was in good agreement with the more accepted method using optical densities.

The biliprotein solution (63mls.) was estimated to contain approximately 150mgs. of B-phycoerythrin and

TABLE VII - Amino Acid Analysis of Chromopeptides from Peptide Digestion.

AMINO ACID	SAMPLE A	SAMPLE B	SAMPLE C
Cysteic Acid	-	4.1	17.9
Aspartic Acid	17.6	12.4	11.1
Threonine	3.8	3.7	4.7
Serine	8.8	9.5	13.1
Glutamic Acid	11.1	9.8	13.1
Proline	4.5	8.8	3.2
Glycine	13.5	11.6	7.7
Alanine	7.1	8.2	5.9
Valine	5.8	6.7	4.4
Methionine	1.0	1.6	3.9
Isoleucine	9.3	6.8	7.0
Leucine	9.6	10.3	6.4
Tyrosine	5.9	2.8	1.7
Phenylalanine	2.0	3.5	-
Lysine	8.4	N.D.	3.1
Histidine	3.5	N.D.	.6
Arginine	N.D.	N.D.	6.7

The amount of each amino acid residue is expressed as a percentage of the total content of amino acid residues.

N.D. = Not Determined.

TABLE VII (CONTD.) - AMINO ACID ANALYSIS OF CHROMOPEPTIDES FROM PEPTIDE DIGESTION

AMINO ACID	SAMPLE A	SAMPLE B	SAMPLE C
CYSTEIC ACID	-	.0191	.0255
ASPARTIC ACID	.0407	.0584	.0158
THREONINE	.0088	.0172	.0067
SERINE	.0204	.0447	.0187
GLUTAMIC ACID	.0257	.0458	.0186
PROLINE	.0105	.0412	.0045
GLYCINE	.0311	.0546	.0109
ALANINE	.0165	.0385	.0084
VALINE	.0134	.0316	.0063
METHIONINE	.0022	.0077	.0055
ISOLEUCINE	.0214	.0320	.0099
LEUCINE	.0221	.0483	.0091
TYROSINE	.0136	.0132	.0024
PHENYLALANINE	.0046	.0165	-
LYSINE	.0194	N.D.	.0044
HISTIDINE	.0081	N.D.	.0009
ARGININE	N.D.	N.D.	.0096

The amount of each amino acid residue in a sample of the hydrolysate is expressed in μ moles.

was adjusted to pH 1.6 by addition of 1N hydrochloric acid. 50mgs. of pepsin were dissolved in 20mls. of 0.1M hydrochloric acid and digested with the biliprotein solution for 24hrs. at 37°c. A further 50mgs. of pepsin in 20mls. of 0.1M hydrochloric acid were added and the digestion continued for a further 24hrs. at 37°c. The mixture was centrifuged and the residue was re-dissolved in 30mls. of 0.1N hydrochloric acid on warming to 37°c. This material was re-digested with a further 50mgs. of pepsin in 20mls. of 0.1N hydrochloric acid at pH 1.6 for 72 hours. On centrifugation the residue was discarded and the supernatant combined with that from the initial digest. The combined supernatants were extracted with iso-amyl alcohol, petroleum ether and finally the extracted chromopeptide mixture was purified by partition chromatography on a celite column as previously (pepsin digest 6).

This partially purified chromopeptide material was then passed through a column (40 x 0.9cm.) of Bio Gel P-6. Elution was commenced with 10mls. of de-ionised water, followed by a 1% aqueous acetic acid solution. The first pink fraction and a later purple coloured fraction were collected and freeze-dried. These fractions corresponded to peaks A and C in fig.10. The freeze-dried material from the pink fraction was re-chromatographed on a column (40 x 0.9cm.) of Bio Gel P-6 using phenol-acetic acid-water (1:1:1) as eluant. The coloured material was collected and reduced to

dryness by rotary evaporation before acidic hydrolysis and amino acid analysis (see table VII; material (B)). The purple coloured fraction eluted from the Bio Gel P-6 column with 1% acetic acid was similarly reduced to dryness, hydrolysed and subjected to amino acid analysis (table VII; material (C)).

The results of amino acid analyses on chromopeptide material (table VII) indicated that some amino acid residues were present in greater proportions than others. Also, most of the predominating residues were present in the chromopeptide material in approximately the same concentration. However, a relatively large number of frequently occurring amino acid residues were present, (viz. cysteic acid, aspartic acid, serine, glutamic acid, glycine, alanine, iso-leucine, leucine, lysine and arginine) indicating rather large chromopeptide fragments. This observation is in general agreement with Fujiwara (1957) who found fifteen amino acid residues in the main fraction of her chromopeptide mixture after digestion of R-phycoerythrin with pepsin. In an attempt to obtain smaller peptide moieties it was decided to subject the peptic digest to further enzymic hydrolysis with trypsin.

(6B) Enzymic Hydrolysis using Pepsin and Trypsin

Trypsin (from bovine pancreas; 2 x crystallized from ethanol. Sigma Chemical Co., St. Louis, U.S.A.) was selected for use in further enzymic hydrolysis because of its specificity for cleavage of peptide bonds between the carboxyl group of basic amino acids and the amino group of other amino acids. From the amino acid analyses obtained so far it was indicated that the chromopeptide material contained basic amino acid residues which would be susceptible to tryptic hydrolysis.

Pepsin/Trypsin Digest 1 :

Approximately 400mgs. of crystalline B-phycoerythrin were dialysed free of ammonium sulphate by the usual centrifugation and dialysis procedures. The pH of the solution was adjusted to 1.6 using 1N hydrochloric acid before 50mgs. of crystalline pepsin dissolved in 15mls. of 0.1N hydrochloric acid were added. The mixture was incubated for 24hrs. at 37°c, then a further 50mgs. of pepsin in 15mls. of 0.1N hydrochloric acid were added and the incubation repeated. The mixture was then centrifuged and the residue subjected to further incubation at 37°c for 24hrs. with 30mgs. of pepsin in 0.1N hydrochloric acid. The supernatants from the centrifuged digests were combined and extracted with several aliquots of iso-amyl alcohol (total volume, 150mls). To the iso-amyl alcohol extract seven volumes of petroleum ether (60-80°) were added and the mixture was allowed to stand

overnight. The lower layer containing the chromopeptides was separated and taken down to dryness on a rotary evaporator. This material was initially purified by partition chromatography on a celite column (15 x 1cm) with butanol-acetic acid-water (4:1:5 by volume) as detailed previously (see pepsin digest 6). The eluted chromopeptide mixture was further purified by gel filtration on a column (50 x 0.9cms.) of Bio Gel P-6 in phenol-acetic acid-water (1:1:1 by volume). All the coloured material eluted from the gel column was reduced to dryness, dissolved in 100mls. of de-ionised water, and brought to pH 7.6 with very dilute sodium hydroxide solution. 15mgs. of crystalline trypsin were added to this solution before digestion at 37°c. After 24hrs. the digest was centrifuged and the supernatant extracted several times with 20ml. volumes of n-butanol. The butanol extract was taken down to dryness using a rotary evaporator in conjunction with an oil pump, then purified as above by celite partition chromatography and Bio Gel P-6 gel filtration. During this latter chromatography no separation was observed except for a green band which was eluted considerably later than the bulk of the coloured material. This green fraction was collected and subjected to acidic hydrolysis and amino acid analysis (Table VIII; 1-1). The remainder of the coloured material from the Bio Gel P-6 column was reduced to small volume prior to gel filtration on the anion exchanger DEAE Sephadex A-25.

A column (20 x 1cm.) of DEAE Sephadex A-25 was prepared in 0.1M pyridine acetate solution, pH 5·0. On applying the above chromopeptide mixture to the column and eluting with 0.1M pyridine acetate (pH 5·0) no separation was achieved, due to bad streaking down the column. All the coloured material was collected, reduced to small volume by rotary evaporation and desalted on a sephadex G-50 column (20 x 1cm.). The material was successfully desalted and eluted as a single band with water.

A second anion exchange column, Bio Rad AG1x2 (200-400 mesh; acetate form), was prepared in 0.05M pyridine acetate buffer (pH 4·8). The above desalted chromopeptide material was applied to a column of this resin (45 x 1.4cms.) and eluted with the 0.05M pyridine acetate buffer. The first reddish-brown band was collected and reduced to small volume; a sample of this material was hydrolysed as usual and subjected to amino acid analysis (Table VIII; 1-2). The remainder was applied to a column (30 x 1.4cm) of the strong cationic exchange resin, Dowex 50 x 8 (25 ± 5μ size). This column had previously been equilibrated with 0.05M pyridine acetate buffer (pH 2.4). On elution with this buffer the chromopeptide material was observed to move down the column as a greenish-brown band. A sample of this material was hydrolysed and examined for amino acids (Table V111; 1-3)

Pepsin/Trypsin Digest 2 : Approximately 150mgs. of crystalline B-phycoerythrin were dialysed free of

TABLE VIII - Amino Acid Analysis of Chromopeptides from Pepsin/Trypsin Digest 1.

AMINO ACID	SAMPLE 1-1	SAMPLE 1-2	SAMPLE 1-3
Cysteic Acid	-	1.2	3.9
Aspartic Acid	6.2	9.1	9.0
Threonine	3.0	4.6	6.1
Serine	5.3	9.2	7.4
Glutamic Acid	4.1	8.3	13.0
Proline	1.0	5.7	5.9
Glycine	3.9	13.9	12.2
Alanine	9.1	8.1	8.1
Valine	10.9	10.5	9.2
Methionine	2.7	1.0	-
Isoleucine	9.5	9.2	6.8
Leucine	24.3	13.2	14.8
Tyrosine	10.2	3.1	1.8
Phenylalanine	10.1	3.2	1.8
Lysine	-	.9	-
Histidine	-	1.1	-
Arginine	-	-	-

The amount of each amino acid residue is expressed as a percentage of the total content of amino acid residues.

TABLE VIII (CONTD.) - AMINO ACID ANALYSIS OF CHROMOPEPTIDES FROM PEPSIN/TRYPSIN DIGEST 1.

AMINO ACID	SAMPLE 1-1	SAMPLE 1-2	SAMPLE 1-3
CYSTEIC ACID	-	.0036	.0079
ASPARTIC ACID	.0433	.0278	.0182
THREONINE	.0208	.0139	.0124
SERINE	.0372	.0280	.0150
GLUTAMIC ACID	.0285	.0253	.0262
PROLINE	.0067	.0173	.0118
GLYCINE	.0271	.0422	.0247
ALANINE	.0640	.0246	.0163
VALINE	.0770	.0319	.0185
METHIONINE	.0188	.0031	-
ISOLEUCINE	.0665	.0280	.0137
LEUCINE	.1708	.0401	.0298
TYROSINE	.0715	.0094	.0037
PHENYLALANINE	.0711	.0098	.0037
LYSINE	-	.0026	-
HISTIDINE	-	.0034	-
ARGININE	-	-	-

The amount of each amino acid residue in a sample of the hydrolysate is expressed in μ moles.

ammonium sulphate and brought to pH 1.6 using 1.0N hydrochloric acid. 25mgs. of pepsin were dissolved in 10mls. of 0.1N hydrochloric acid and added to the acidic protein solution. The mixture was allowed to digest at 37°c for 24 hours, then a further 25mgs. of pepsin in 10mls. of 0.1N hydrochloric acid were added and the mixture again allowed to digest for a further 24hrs. The digest was centrifuged and the supernatant extracted with several small volumes of n-butanol until the butanol layer was no longer coloured red. The butanol extracts were combined and reduced to small volume (2-3mls.) before purification on columns of celite and Bio Gel P-6 as detailed in pepsin digest 6. Following gel filtration on Bio Gel P-6, the chromopeptide material was reduced to dryness by rotary evaporation then dissolved in 50mls. of de-ionised water. The pH of the solution was adjusted to 7.6 with 0.1N sodium hydroxide solution. 15mgs. of trypsin were added and the mixture incubated for 24 hours at 37°c. The chromopeptide digest was brought to pH 3.8 and extracted once with 25mls. of chloroform. The aqueous layer was then extracted with two 25ml. portions of n-butanol. The butanol extracts were then pooled and washed twice with 0.1N sodium hydroxide solution before reducing to dryness by rotary evaporation.

Crespi and Smith (1970) used preparative thin layer chromatography to separate chromopeptides derived from phycocyanin by nagarse digestion. These authors used

preparative T.L.C. on plates coated with Adsorbosil 5 (Anspec). In the present investigation the adsorbents used were MN-Cellulose powder and MN-Silica Gel G-HR. 20 x 20cm. plates were prepared as described previously using the Shandon spreading apparatus (G.T. (J)). Samples of chromopeptide material from the n-butanol layer were dissolved in a few drops of methanol - 25% aqueous acetic acid solution (1:1) and applied to the chromatography plates. When n-butanol - acetic acid - water (3:1:1 by volume) was used as the solvent on 1mm. cellulose plates some separation was obtained after 3 hours. During this time the solvent front travelled 12cms. giving a separation into three coloured bands: a brown band at R_f 0•2, a green band at R_f 0•3 and a second brown band at R_f 0•9, and all were ninhydrin - positive. Using MN-Silica Gel G-HR plates (1mm.) an improvement in separation was observed under identical conditions. Two brown bands were again obtained at R_fs 0•2 and 0•9, but there was also a greenish-brown band at R_f 0•4, a red band at R_f 0•6 and a yellow band at R_f 0•65. The chromopeptide mixture was then subjected to thin layer chromatography using other solvent systems. When toluene - acetic acid - water (10:10:1, by volume) and n-butanol - acetic acid - water (4:1:1, by volume) were investigated as possible solvent systems, the resulting chromatograms indicated poor separations. Hence, n-butanol - acetic acid - water (3:1:1) on Silica Gel adsorbent proved to be the most suitable T.L.C. system; and in addition, an increase in resolution of

the coloured bands was observed when the thickness of the layers was reduced to 0·6mm.

Pepsin/Trypsin Digest 3 : Since the digestion, extraction and purification procedures become rather involved at this stage, a schematic diagram was constructed (see fig. 11).

300mgs. of crystalline B-phycoerythrin were dissolved in de-ionised water and the ammonium sulphate removed by dialysis in ·01M sodium phosphate buffer (pH 6·5). The resulting fluorescent biliprotein solution (spectral ratio = 4·7) was adjusted to pH 1·6 with 1·0N hydrochloric acid and digested with 75mgs. pepsin dissolved in 20mls. of 0·1N hydrochloric acid. After 18 hours at 37°c a further 60mgs. of pepsin were added and the digestion allowed to continue for a further 12 hours. The digest was centrifuged and the precipitate removed. Following extraction of the supernatant with n-butanol at pH 1.6, the alcohol layer was reduced to dryness and chromatographed on a celite column (15 x 1cm.) with the top layer of an n-butanol - acetic acid - water (4:1:5, by volume) system. The material was collected as a single fraction and freeze-dried prior to trypsin hydrolysis. 20mgs. of trypsin were dissolved in very dilute sodium hydroxide and added to an aqueous solution of the pepsin extract. The mixture was brought to pH 7·6 with dilute sodium hydroxide and allowed to digest for 24hrs. at 37°c. On cooling the trypsin digest, the pH was adjusted to 3·6 before vigorous extraction with chloroform. The chloroform extract was taken down to dryness and stored (3-AA).

The aqueous layer was then extracted with n-butanol and the butanol layer reduced to small volume prior to chromatography on a celite column as above. The coloured material eluted as a single band from the column was reduced to dryness by rotary evaporation and subjected to gel chromatography on a column (2·5 x 24cms.) of Bio Gel P-6. The column was eluted with 25% aqueous acetic acid (degassed) in an attempt to purify and, if possible, fractionate the chromopeptides. Considerable separation into different coloured fractions was observed and five fractions were collected and reduced to dryness. In order of elution these fractions were red, pale pink, purple, blue, green and were labelled 3-A, 3-B, 3-C, 3-D and 3-E respectively.

The observations made in the previous experiment (pepsin/trypsin digest 2) indicated that good separation of chromopeptide fractions could be obtained by T.L.C. on MN-Silica Gel G-HR. Hence the chromopeptide material isolated as described above was subjected to T.L.C. on 0·6mm silica gel plates using n-butanol - acetic acid - water (3:1:1, by volume) as the developing solvent.

(a) T.L.C. of chromopeptide material 3-AA (chloroform soluble)

This acid chloroform extract of the trypsin digest gave three coloured bands on ascending T.L.C. as described above: red (R_f 0·75), purple (R_f 0·89) and green (R_f 0·94). Each band was scraped off the plate and transferred to a small sintered glass funnel, where the coloured material

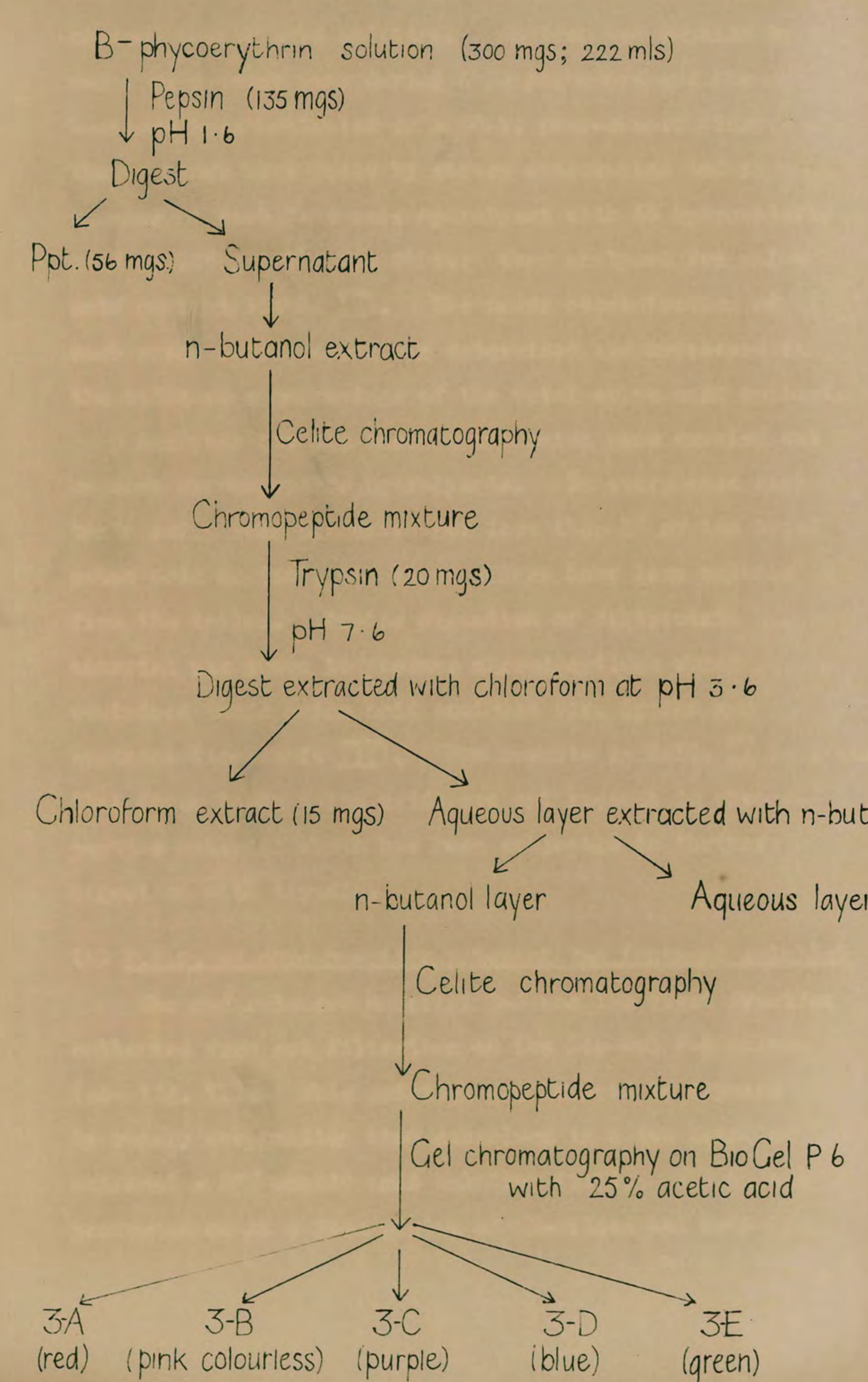


FIG. II SCHEMATIC DIAGRAM FOR PEPSIN|TRYPSIN DIGEST 3
B-phycoerythrin solution (300 mgs; 222 mls)
Pepsin (135 mgs)
pH 1·6
Digest
Ppt. (56 mgs.)
Supernatant
n-butanol extract
Celite chromatography
Chromopeptide mixture
Trypsin (20 mgs)
pH 7·6
Digest extracted with chloroform at pH 3·6
Chloroform extract (15 mgs)
Aqueous layer extracted with n-butanol
n-butanol layer
Aqueous layer
Celite chromatography
Chromopeptide mixture
Gel chromatography on BioGel P 6
with 25% acetic acid
3-A
(red)
3-B
(pink colourless)
3-C
(purple)
3-D
(blue)
3E
(green)

was washed from the silica gel using methanol or 25% aqueous acetic acid solution. Suction was applied by means of a water pump during this procedure and the washings were collected in a 50ml. pear-shaped flask. The washings were dried by rotary evaporation before samples of each band were hydrolysed with 6N hydrochloric acid at 105 ± 0·5°c for 24hrs; then analysed for amino acid content (Table IX). The molar concentration of each amino acid present was determined and expressed in the table as a percentage of the total amino acid content.

Analyses for the basic amino acids were carried out on each chromopeptide hydrolysate. In every case each basic amino acid accounted for less than 1% of the total amino acid content, and these values have been omitted from the tables. Peptic digestion of biliprotein material was shown to produce chromopeptides which contained basic amino acid residues viz. lysine and arginine (see Table VII). The results in Table IX indicate that these basic amino acids are not present in chromopeptide material which has been subjected to further digestion with trypsin.

(b) T.L.C. of chromopeptide material 3-A (Butanol soluble)

The red coloured material 3-A was the first fraction collected from gel filtration on the Bio Gel P-6 column (see fig. 11). This material was reduced to dryness by rotary evaporation then dissolved in a few drops of methanol - 25% acetic acid solution (1:1). T.L.C. was performed on 20 x 20cm plates of MN-Silica Gel G-HR

(0·6mm.). Chromopeptide material 3-A was applied to a plate and developed as above. When the plate was dried and examined under ultraviolet light only one discreet band was observed. This band was coloured red, fluoresced orange under ultraviolet light and was found at R_f 0·29. This red material was removed from the plate using the technique described above, then reduced to dryness by rotary evaporation (band 3-A1). The material remaining on the plate was removed in the usual manner and reduced to small volume. This material was then rechromatographed in an attempt to achieve better separation. A much improved separation was in fact observed and this consisted of three brownish-green bands at R_fs 0.38 (band 3-A6), 0.46 (band 3-A5), 0·51 (band 3-A4), and also a green band at R_f 0·85 (band 3-A3). The separated bands were removed from the plate and reduced to dryness as usual. Samples of each of the five bands were hydrolysed for 24hrs. in 6N hydrochloric acid at 105°c, then analysed for amino acid content. (Table X̄).

(c) T.L.C. of chromopeptide material 3-B

This pink coloured fraction collected from the Bio Gel P-6 column was treated exactly as in the previous case. Chromatography on silica gel plates revealed two well separated bands: an orange band at R_f 0·48 (band 3-B1) and a green band at R_f 0·80 (band 3-B2). As before, samples of both bands were hydrolysed and examined for amino acids. The results are shown in Table X̄, and all bands were seen to contain significant amounts of leucine,

TABLE IX - Amino Acid Analysis of Chromopeptides from Chloroform Extract of Pepsin/Trypsin Digest 3 (i.e. 3-AA)

AMINO ACID	3-AA1 Rf 0·75	3-AA2 Rf 0·89	3-AA3 Rf 0·94
Cy SO_3H	7.9	5.3	5.3
Asp	6.6	5.0	4.9
Thr	5.3	4.6	7.1
Ser	10.1	8.0	10.6
Glu	9.6	6.9	3.1
Pro	-	2.6	1.8
Gly	14.0	10.2	8.8
Ala	10.0	12.3	7.5
Val	13.6	13.5	15.2
Met	-	-	-
Ile	2.5	8.3	5.2
Leu	13.9	18.6	26.4
Tyr	1.6	1.2	1.4
Phe	4.9	3.6	2.7

The amount of each amino acid residue is expressed as a percentage of the total content of amino acid residues.

TABLE IX (CONTD.) - AMINO ACID ANALYSIS OF CHROMOPEPTIDES FROM CHLOROFORM EXTRACT OF PEPSIN/TRYPSIN DIGEST 3 (i.e. 3-AA)

AMINO ACID	3-AA1	3-AA2	3-AA3
CySO3H	.0111	.0074	.0129
Asp	.0093	.0069	.0119
Thr	.0074	.0064	.0171
Ser	.0142	.0111	.0258
Glu	.0135	.0097	.0075
Pro	-	.0037	.0044
Gly	.0197	.0143	.0213
Ala	.0141	.0173	.0183
Val	.0191	.0189	.0369
Met	-	-	-
Ile	.0034	.0116	.0126
Leu	.0196	.0261	.0640
Tyr	.0022	.0017	.0033
Phe	.0069	.0051	.0066

The amount of each amino acid residue in a sample of the hydrolysate is expressed in μ moles.

valine and alanine. Glycine, aspartic acid and serine were present in some bands whereas iso-leucine was only present twice in significant amounts (bands 3-A3 and 3-B2). It should also be noted that in these latter cases leucine was present in concentrations approximately twice that of any other amino acid present.

(d) T.L.C. of chromopeptide material 3-C

When material 3-C was chromatographed as in the above cases at least eight bands were observed. However, only two bands were removed from the plate and the remainder combined and rechromatographed. The two bands, which were well separated and removed individually, consisted of a faint pink band at R_f 0·18 (band 3-C1) and a pinkish-mauve band at R_f 0·53 (band 3-C6). The rechromatographed material gave a further three well separated bands: an orange-yellow band at R_f 0·54 (band 3-C3), a blue-mauve band at R_f 0·62 (band 3-C4) and a brown-mauve band at R_f 0·69 (band 3-C5). As before, coloured material remaining on the chromatography plate after removal of the well defined bands, was rechromatographed. This final separation produced three bands: a pink band at R_f 0·45 (band 3-C2), a pink band at R_f 0·55 (band 3-C7) and a green band at R_f 0·63 (band 3-C8).

Samples of all eight bands were hydrolysed then analysed for amino acid content as previously. The results for the amino acid analysis of the components of fraction 3-C are shown in Table XI. Analysis of

TABLE X - Amino Acid Analysis of Chromopeptides from Fractions 3-A and 3-B.

AMINO ACID	3-A1 Rf.29	3-A6 Rf.38	3-A5 Rf.46	3-A4 Rf.51	3-A3 Rf.85	3-B1 Rf.48	3-B2 Rf.80
Cy SO_3H	2.1	4.0	2.9	3.1	-	1.0	0.6
Asp	10.8	12.7	10.1	10.1	6.2	11.0	3.7
Thr	5.9	5.7	6.5	8.5	4.5	5.1	3.1
Ser	12.3	13.5	11.8	9.7	6.9	14.3	5.1
Glu	6.6	6.1	6.9	7.3	6.9	4.7	3.0
Pro	6.3	6.0	5.7	5.2	4.2	1.7	2.5
Gly	11.9	11.4	12.3	12.5	9.0	6.4	12.6
Ala	10.4	8.9	10.2	9.2	10.3	13.1	10.4
Val	9.4	11.2	11.4	11.5	10.1	12.2	9.8
Met	1.5	-	-	-	2.0	-	0.9
Ile	7.6	8.6	7.0	6.5	10.2	7.5	13.0
Leu	10.6	8.7	10.5	11.9	22.0	11.4	18.6
Tyr	2.4	1.7	2.4	2.2	3.0	7.3	8.9
Phe	2.1	1.4	2.2	2.2	3.8	4.5	7.3

The amount of each amino acid residue is expressed as a percentage of the total content of amino acid residues.

TABLE X (CONTD.) - AMINO ACID ANALYSIS OF CHROMOPEPTIDES
FROM FRACTIONS 3-A and 3-B.

AMINO ACID	3-A1	3-A6	3-A5	3-A4	3-A3	3-B1	3-B2
$CySO_3H$	.0206	.0298	.0135	.0119	-	.0085	.0069
Asp	.1054	.0945	.0474	.0391	.0483	.0916	.0456
Thr	.0580	.0421	.0306	.0330	.0347	.0425	.0384
Ser	.1201	.1002	.0553	.0376	.0534	.1183	.0627
Glu	.0645	.0456	.0322	.0283	.0537	.0393	.0376
Pro	.0616	.0448	.0268	.0200	.0325	.0141	.0307
Gly	.1163	.0846	.0575	.0484	.0698	.0528	.1555
Ala	.1020	.0664	.0478	.0356	.0805	.1085	.1286
Val	.0918	.0830	.0533	.0443	.0790	.1011	.1203
Met	.0144	-	-	-	.0153	-	.0115
Ile	.0745	.0642	.0325	.0251	.0798	.0619	.1600
Leu	.1031	.0646	.0488	.0458	.1713	.0948	.2300
Tyr	.0230	.0128	.0112	.0084	.0233	.0606	.1101
Phe	.0206	.0107	.0101	.0087	.0296	.0372	.0899

The amount of each amino acid residue in a sample of the hydrolysate is expressed in μ moles.

band 3-C1 is not included in the results table since only trace amounts of amino acids could be found in this material.

(e) T.L.C. of chromopeptide material 3-D and 3-E.

Fractions 3-D and 3-E were the blue and green fractions eluted last off the Bio Gel P-6 column (fig.11). When these fractions were reduced to dryness by rotary evaporation, very little coloured material was left. Both fractions were subjected to T.L.C. as above. 3-D appeared to give seven bands between R_fs 0·81 and 0·98, while 3-E gave only two green bands at R_f 0·34 and R_f 0·69. Samples of the two green bands from 3-E were hydrolysed and analysed for amino acids. These analyses indicated little more than trace amounts of amino acids, and the amino acids present in greatest concentration were generally the same amino acids which occurred in greatest concentrations in the analyses of bands from previous fractions (i.e. 3-A, 3-B and 3-C). However, due to the minute quantities of material present no reliable conclusions could be drawn from these results. For the same reason, no amino acid analyses were performed on the bands isolated from fraction 3-D.

Pepsin/Trypsin Digest 4 :

The digestion procedure was similar to that used for digest 3, and again a schematic diagram was constructed for easy reference (fig. 12).

About 300mgs. of crystalline B-phycoerythrin were dialysed free of ammonium sulphate using 0·01M sodium

TABLE XI - Amino Acid Analysis of Chromopeptides from Fraction 3-C.

AMINO ACID	3-C2 Rf.45	3-C3 Rf.54	3-C4 Rf.62	3-C5 Rf.69	3-C6 Rf.53	3-C7 Rf.55	3-C8 Rf.63
Cy SO_3H	3.9	3.4	2.8	1.7	2.5	1.2	0.8
Asp	14.8	13.4	8.9	3.9	5.8	6.3	7.5
Thr	3.9	3.5	5.2	3.0	4.3	2.1	2.7
Ser	11.7	13.1	14.3	7.6	13.1	5.3	8.2
Glu	4.0	5.0	3.9	2.5	4.3	3.7	3.0
Pro	2.2	-	1.1	0.9	1.6	1.3	1.6
Gly	8.9	6.5	6.5	4.4	6.6	6.7	7.1
Ala	8.3	12.3	11.9	8.7	9.3	9.2	8.5
Val	16.1	17.8	12.0	5.5	7.3	6.2	11.0
Met	-	-	0.7	0.4	-	-	-
Ile	5.6	3.4	5.4	4.6	3.7	5.2	5.2
Leu	9.2	5.3	6.2	7.7	7.1	8.2	10.2
Tyr	11.5	16.2	16.6	24.3	23.6	13.9	25.5
Phe	N.D.	-	4.7	24.7	11.3	30.8	8.8

The amount of each amino acid residue is expressed as a percentage of the total content of amino acid residues.

N.D. = Not Determined.

TABLE XI (CONTD.) - AMINO ACID ANALYSIS OF CHROMOPEPTIDES FROM FRACTION 3-C.

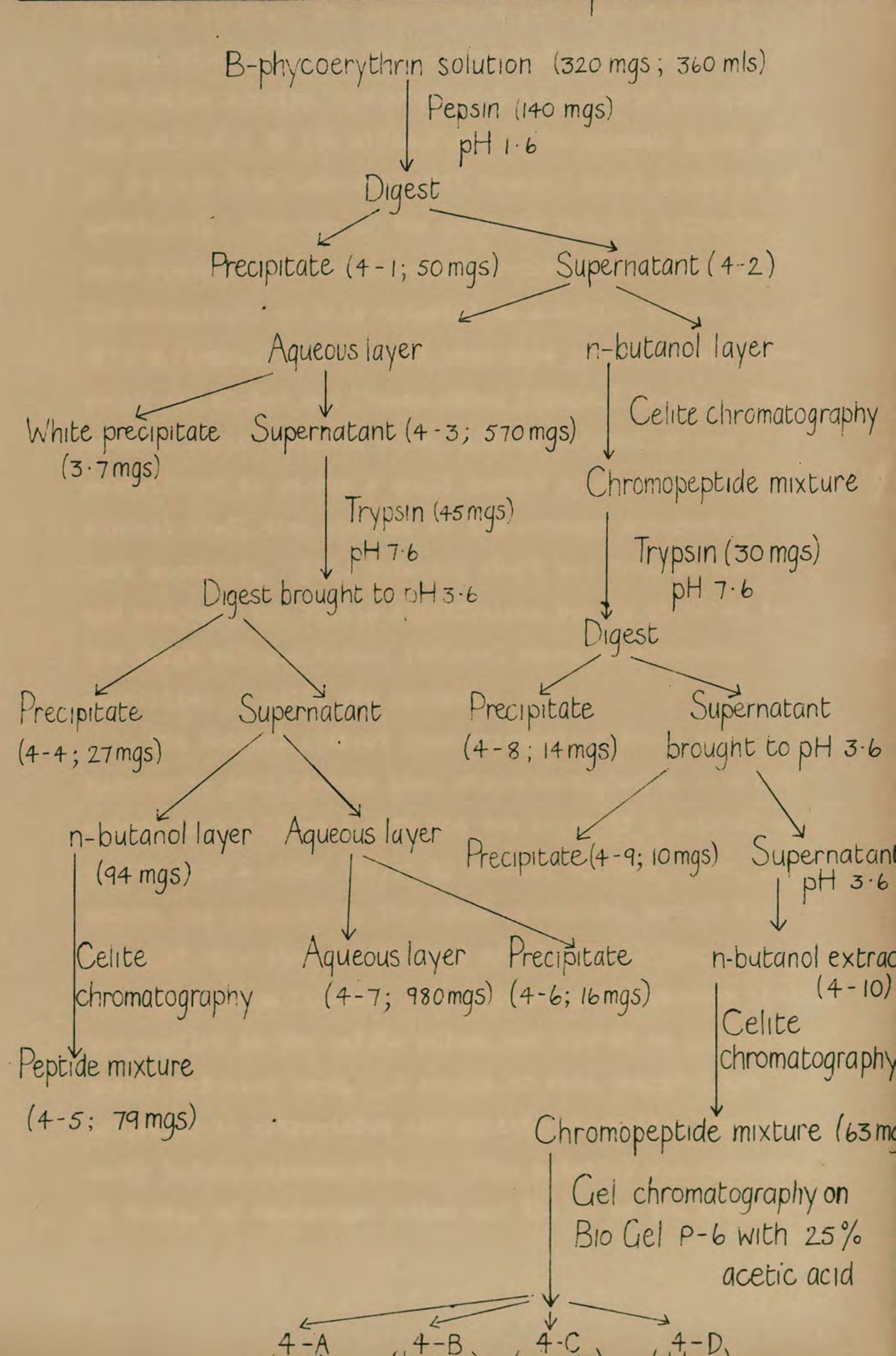
AMINO ACID	3-C2	3-C3	3-C4	3-C5	3-C6	3-C7	3-C8
$CySO_3H$	.0076	.0037	.0184	.0252	.0121	.0110	.0182
Asp	.0292	.0144	.0577	.0586	.0277	.0564	.1730
Thr	.0077	.0038	.0336	.0446	.0209	.0186	.0630
Ser	.0231	.0141	.0930	.1142	.0628	.0474	.1880
Glu	.0078	.0054	.0255	.0370	.0207	.0329	.0698
Pro	.0043	-	.0069	.0132	.0075	.0112	.0356
Gly	.0175	.0070	.0426	.0668	.0315	.0598	.1628
Ala	.0163	.0132	.0773	.1304	.0445	.0826	.1952
Val	.0317	.0191	.0783	.0822	.0353	.0556	.2528
Met	-	-	.0042	.0063	-	-	-
Ile	.0111	.0037	.0351	.0697	.0180	.0466	.1193
Leu	.0180	.0057	.0403	.1160	.0340	.0732	.2354
Tyr	.0227	.0175	.1079	.3668	.1108	.1246	.5860
Phe	N.D.	-	.0303	.3734	.0543	.2754	.2011

The amount of each amino acid residue in a sample of the hydrolysate is expressed in μ moles.

N.D. = Not Determined.

phosphate buffer, pH 6.5. The fluorescing biliprotein solution (360mls.) was then digested with 60mgs. of pepsin. The pepsin was dissolved in 15mls. of 0·1N hydrochloric acid and added to the biliprotein solution, acidified to pH 1·6 with 1N hydrochloric acid. The mixture was digested for 24hrs. at 37°c after which the pH was found to be 1·8. An additional 80mgs. of pepsin in 15 mls. of 0·1N hydrochloric acid were added to the digest and the pH adjusted to 1·6 before incubation at 37°c for a further 18hrs. The final pH of the mixture was found to be 1·6. The material was centrifuged at 2,700g for 30 minutes at room temperature and the residue collected and freeze-dried (approx. 50mgs.) (4-1). Ultraviolet and visible absorption spectra were run on the supernatant (4-2) prior to extraction with small aliquots of n-butanol. Spectra were then run on a sample of the n-butanol extract. This extract was then reduced to dryness using an oil pump. The aqueous layer from the peptic digestion was centrifuged to remove a white precipitate, then absorption spectra of this aqueous extract were obtained. Freeze-drying of this aqueous extract yielded about 570mgs. of material (4-3). This material was dissolved in 50mls. of de-ionised water and the pH of the mixture adjusted to 7·6 with 0·1N sodium hydroxide. 45mgs. of trypsin were added and the mixture digested for 24hrs. at 37°c. On cooling, the digest was adjusted to pH 3·6 with 0·1N hydrochloric acid then centrifuged to remove the small quantity of precipitated

FIG 12 SCHEMATIC DIAGRAM FOR PEPSIN TRYPSIN DIGEST 4



material (4-4). The supernatant was extracted with 20mls. of chloroform, but ultraviolet and visible absorption spectra indicated that no coloured material was extracted by the chloroform. The aqueous layer at pH 3·6 was then extracted with several small aliquots of n-butanol. Spectra of the combined butanol extracts were recorded and the material freeze-dried (94mgs.). Chromatography of this material on a celite column (1·2 x 14cms.) by the usual procedure gave a single band which was eluted and freeze-dried (79mgs.) (4-5). The aqueous layer from the above butanol extraction was centrifuged to remove a small amount of precipitate (16mgs) (4-6) then spectra of the aqueous supernatant were recorded. This material (4-7) was freeze-dried and weighed, but little significance can be attached to the large value obtained (980mgs) since considerable quantities of salts are present.

The butanol extract from supernatant (4-2) was reduced to small volume then chromatographed on a celite column (2·3 x 19cms.) with the top layer of a butanol - acetic acid - water (4:1:5 by volume) system. The column was prepared by the usual procedure, except that 20gms of air-dried celite were mixed with 11·5gms. of the lower phase liquid. The chromopeptide mixture (8mls.) was applied to the celite column and eluted as a single, purple coloured band. A sample of this material was subjected to ultraviolet and visible absorption spectroscopy before freeze-drying. This material was dissolved in 50mls. of de-ionised water and the pH of the mixture

adjusted to 7·6 with 0·1N sodium hydroxide. 30mgs. of crystalline tyrpsin were added and the mixture digested for 48hrs. at 37°c. On cooling, the digest was centrifuged and the pH of the supernatant adjusted to 3·6. The small quantity of precipitate resulting from this change in pH was again centrifuged and removed (4-9). The acidic supernatant was extracted with small aliquots of n-butanol until the butanol layer no longer contained coloured material. Ultraviolet and visible spectra of the combined butanol extracts (4-10) were recorded after reducing the material to small volume. This material was further purified by celite column chromatography as described above and the purple fraction eluted was freeze-dried (63 mgs.). The next stage in the purification of this chromopeptide material involved gel chromatography on Bio Gel P-6.

18gms. of Bio Gel P-6 were slurried in 25% aqueous acetic acid and used to pack a column (2·5 x 24cms.). The chromopeptide material was loaded and eluted with 25% acetic acid. As in the case of pepsin/trypsin digest 3, considerable separation of the purple coloured mixture was observed and four coloured fractions were collected as follows: the initial 45mls. collected were colourless; 27mls. of red coloured material were then collected (band 4-A), followed by 32mls. of brown material (band 4-B), 9mls. of purple material (band 4-C) and finally 33mls. of pale blue material (band 4-D). Spectra were recorded on samples of these bands before they were

reduced to dryness by rotary evaporation. The final purification of the chromopeptide material was achieved by T.L.C. on MN-Silica Gel G-HR, as in the previous experiment.

(a) T.L.C. of chromopeptide material 4-A.

This red coloured material eluted first from the Bio Gel P-6 column was chromatographed on plates of silica gel (20 x 20cms; 0·6mm. thickness) using n-butanol - acetic acid - water (3:1:1 by volume) as the developing agent. Chromatography initially revealed three bands: a pink coloured, orange fluorescent band at R_f 0·10; a rust coloured, orange fluorescent band at R_f 0·46 (band 4-A1) and finally a brownish-green coloured band at R_f 0·73-0·86. Unfortunately, the material at R_f 0·10 could not be analysed for amino acids due to implosion of the hydrolysis tubes. The brownish-green coloured band at R_f 0·73-0·86 was rechromatographed and gave two distinct bands: a rust coloured, pale pink fluorescent band at R_f 0·55 (band 4-A2) and a brown coloured band at R_f 0·90 (Band 4-A3). The separated bands were removed from the plates and reduced to dryness as usual. Samples of each of the three bands were hydrolysed for 24hrs. in 6NHCl at 105°c, then analysed for amino acid content (Table XII).

(b) T.L.C. of chromopeptide material 4-B.

Chromatography of this brown coloured material yielded a rust coloured, pale pink fluorescent band at

R_f 0·47 (band 4-B1) and a brownish-green band at R_f 0·81-0·87. The latter band was rechromatographed to produce a further three bands: a brown coloured fluorescent band at R_f 0·52 (band 4-B2); a brown coloured, green fluorescent band at R_f 0·84 (band 4-B3) and a green coloured band at R_f 0·92 (band 4-B4). As before, samples of these bands were hydrolysed with 6NHCl then subjected to amino acid analysis (Table XII).

(c) T.L.C. of chromopeptide material 4-C.

Three fluorescent bands were observed when this material was subjected to the usual T.L.C. procedure: a greyish band at R_f 0·47 (band 4-C1), a greyish band at R_f 0·85 (band 4-C2) and a pale green band at R_f 0·96 (band 4-C3). All bands exhibited fluorescence under ultraviolet light. The three bands were removed and a sample of each was analysed for amino acid composition. Due to the exceptionally small amounts of amino acids obtained for the analysis of band 4-C3, the results were not included in Table XII. For the same reason, the amino acid analysis results on five bands isolated by T.L.C. of material 4-D were omitted.

TABLE XII - Amino Acid Analysis of Chromopeptides from Fractions 4-A, 4-B and 4-C.

AMINO ACID	4-A1 Rf.46	4-A2 Rf.55	4-A3 Rf.90	4-B1 Rf.47	4-B2 Rf.52	4-B3 Rf.84	4-B4 Rf.92	4-C1 Rf.47	4-C2 Rf.85
Cy SO_3H	1.9	3.4	-	1.5	1.0	0.6	-	3.3	2.1
Asp	8.8	9.6	9.1	9.8	8.9	5.5	5.4	11.4	15.9
Thr	5.3	4.6	4.1	4.3	2.8	3.3	2.8	3.5	1.7
Ser	7.1	6.6	6.4	10.5	6.9	2.5	2.7	7.5	5.7
Glu	13.1	12.1	10.0	9.7	5.9	5.9	5.3	5.3	2.2
Pro	6.7	7.0	5.2	3.8	3.4	1.4	-	-	-
Gly	22.4	17.3	13.0	11.8	11.8	5.9	7.9	10.7	4.4
Ala	8.3	8.3	9.6	12.6	12.5	9.8	6.1	7.9	4.3
Val	9.4	10.0	9.4	11.2	10.6	8.6	7.6	5.8	6.8
Met	0.8	-	1.2	1.8	2.3	0.9	-	-	-
Ile	3.8	5.8	9.5	6.6	8.2	15.2	23.4	2.1	1.9
Leu	10.4	12.7	18.6	11.8	12.5	25.5	27.1	8.0	18.0
Tyr	0.9	1.3	2.0	2.1	4.9	7.2	6.9	27.4	31.5
Phe	1.2	1.3	1.9	2.7	8.4	7.7	4.7	6.1	5.5

The amount of each amino acid residue is expressed as a percentage of the total content of amino acid residues.

TABLE XII (CONT'D.) - AMINO ACID ANALYSIS OF CHROMOPEPTIDES FROM FRACTIONS 4-A, 4-B and 4-C.

AMINO ACID	4-A1	4-A2	4-A3	4-B1	4-B2	4-B3	4-B4	4-C1	4-C2
$CySO_3H$	.0206	.0152	-	.0154	.0100	.0043	-	.0038	.0039
Asp	.0932	.0425	.0228	.1068	.0865	.0389	.0197	.0131	.0301
Thr	.0563	.0204	.0102	.0467	.0269	.0234	.0101	.0040	.0033
Ser	.0753	.0294	.0160	.1152	.0662	.0180	.0100	.0086	.0107
Glu	.1394	.0536	.0249	.1059	.0566	.0416	.0192	.0061	.0041
Pro	.0713	.0311	.0130	.0412	.0327	.0101	-	-	-
Gly	.2388	.0766	.0324	.1292	.1138	.0421	.0290	.0123	.0083
Ala	.0880	.0367	.0240	.1374	.1205	.0694	.0224	.0091	.0082
Val	.1007	.0444	.0234	.1223	.1021	.0607	.0278	.0067	.0128
Met	.0083	-	.0031	.0199	.0227	.0061	-	-	-
Ile	.0403	.0256	.0237	.0717	.0794	.1080	.0854	.0024	.0036
Leu	.1102	.0562	.0465	.1295	.1212	.1812	.0993	.0092	.0342
Tyr	.0093	.0059	.0050	.0234	.0473	.0511	.0253	.0326	.0597
Phe	.0124	.0059	.0047	.0292	.0810	.0547	.0171	.0070	.0104

The amount of each amino acid residue in a sample of the hydrolysate is expressed in μ moles.

(7) Reduction of Chromopeptide material with Borohydride

The possibility of a chromophore-protein ester linkage was investigated using lithium borohydride. Chibnall and Rees (1953, 1958) studied the reaction of insulin methyl ester with lithium borohydride and their later work showed that an eight-fold molar excess of lithium borohydride was sufficient to reduce all ester linkages. A chromophore-protein ester linkage could be formed in several ways depending on the amino acid residue involved in the linkage and the site of attachment on the chromophore. An ester linkage is possible between a carboxyl group of aspartic or glutamic acid and a hydroxyl group on the chromophore, formed by enolisation of a ring keto-group. A second type of ester bond involves a linkage from one of the propionic acid side chains of the chromophore to hydroxyl groups of the protein residues. Acidic hydrolysis of either type of ester bond results in the formation of the free amino acid. However, if the chromopeptide material were initially treated with lithium borohydride, reduced amino acids may be formed after acidic hydrolysis. For example, if the ω-carboxyl group of aspartic acid were involved in the ester linkage, then reduction with lithium borohydride followed by acidic hydrolysis may give rise to homoserine (γ-hydroxy-α-aminobutyric acid) in the hydrolysate. Similarly, glutamic acid could result in the formation of some δ-hydroxy-α-aminovaleric acid. A comparison between acidic hydrolysates of

chromopeptide material treated with lithium borohydride and that of untreated material would then indicate the nature of the linkage. However, if the ester linkage is of the type which involves a hydroxyl group from an amino acid residue and a propionic acid side chain of the chromophore, reduction with lithium borohydride may have no effect on the products of acid hydrolysis.

The solvent used for the reaction was tetrahydrofuran which was dried before use. Commercial tetrahydrofuran was refluxed over calcium hydride, then distilled and stored in a dark bottle. This solvent rapidly develops peroxides and samples stored for longer than two weeks were redistilled before use. Lithium borohydride was purified by dissolving 5gms. in a 250ml. centrifuge bottle with 200mls. of anhydrous ethyl ether. The insoluble impurity was separated by centrifugation and the ether was evaporated in vacuo.

Experiment 1. A sample of chromopeptide material 4-A3 was dried thoroughly in a vacuum dessicator containing P_2O_5. The material was then transferred to a 3-necked 25ml. flat-bottomed flask using 10mls. of tetrahydrofuran. The flask was fitted with a condenser and a soda-lime trap. 10mgs. of the purified lithium borohydride were then introduced into the flask and the mixture refluxed for 24 hours. The reactants were cooled and small aliquots of a methanol/dilute hydrochloric acid solution were added with gentle shaking. This solution neutralized the reaction mixture and converted excess lithium

borohydride to methyl borates and lithium chloride. The mixture was stirred for 1 hr., then evaporated to dryness on a rotary evaporator. A few mls. of de-ionised water were added to the reduced material which was again dried by rotary evaporation. This procedure was repeated to ensure the complete removal of methyl borate and hydrochloric acid. 6mls. of constant boiling hydrochloric acid was added to the dried material and the mixture hydrolysed for 24 hours at 105°c. The hydrolysate was subjected to amino acid analysis and the results are shown in table XIV together with those for the material not treated with lithium borohydride.

Experiment 2. A sample of the chromopeptide material 3-A1 was dissolved in 6mls. of a methanol/25% aqueous acetic acid solution (1:1 by volume). 2mls. were set aside and used in a later experiment (see experiment 3). The remaining 4mls. were reduced to dryness then dissolved in 15mls. of tetrahydro furan containing 25mgs. of lithium borohydride. The mixture was refluxed for 20hrs. as above, then treated with a slight excess of methanolic hydrochloric acid with stirring for 1 hour. After making up the volume to 25mls. with deionised water half the solution was reduced to dryness, then washed twice with deionised water. This material was hydrolysed with 6N hydrochloric acid for 24hrs. at 105°c and the hydrolysate analysed for amino acid content (Table XV, column 2).

TABLE XIV - Amino Acid Analysis of Chromopeptide material 4-A3 treated with Lithium Borohydride.

AMINO ACID	UNTREATED	TREATED
Aspartic acid	9·1	9·5
Threonine	4·1	4·5
Serine	6·4	8·1
Glutamic acid	10·0	10·9
Proline	5·2	5·3
Glycine	13·0	13·9
Alanine	9·6	9·5
Valine	9·4	8·5
Methionine	1·2	-
Isoleucine	9·5	8·2
Leucine	18·6	17·8
Tyrosine	2·0	2.1
Phenylalanine	1·9	1·9

The amount of each amino acid residue is expressed as a percentage of the total content of amino acid residues.

TABLE XIV (CONTD.) - AMINO ACID ANALYSIS OF CHROMOPEPTIDE MATERIAL 4-A3 TREATED WITH LITHIUM BOROHYDRIDE

AMINO ACID	UNTREATED	TREATED
ASPARTIC ACID	.0228	.0305
THREONINE	.0102	.0144
SERINE	.0160	.0261
GLUTAMIC ACID	.0249	.0350
PROLINE	.0130	.0170
GLYCINE	.0324	.0448
ALANINE	.0240	.0305
VALINE	.0234	.0274
METHIONINE	.0031	-
ISOLEUCINE	.0237	.0265
LEUCINE	.0465	.0573
TYROSINE	.0050	.0068
PHENYLALANINE	.0047	.0062

The amount of each amino acid residue in a sample of the hydrolysate is expressed in μ moles.

The remaining 12·5mls. were extracted with three 3ml. portions of chloroform, the third chloroform extract showing virtually no trace of coloured material. The aqueous layer was reduced to dryness, washed twice with deionised water, then hydrolysed as above. Amino acid analysis of the hydrolysate gave the results shown in table XV, column 3.

Experiment 3. The 2ml. aliquot set aside from the previous experiment was reduced to dryness and dissolved in 15mls. of tetrahydrofuran containing 10mgs. of sodium borohydride. The mixture was refluxed for 20hrs, care being taken to exclude moisture from the system. On cooling, a slight excess of methanolic hydrochloric acid was added with shaking. Rotary evaporation reduced the material to dryness and this was followed by two deionised water washes. The dried material was then hydrolysed with 6mls. of 6N hydrochloric acid for 24hrs. at 105°c. The hydrolysate was dried, washed twice with deionised water and again dried; and then the amino acid content was determined. The results are shown in table XV, column 4.

In the three experiments detailed above, no attempt was made toremove contaminating salts derived from lithium and sodium borohydride. Since these decomposition products did not interfere with the amino acid ion-exchange profile, little would be gained by their removal.

TABLE XV - Amino Acid Analysis of Chromopeptide material 3-A1 treated with Lithium and Sodium Borohydride.

AMINO ACID	UNTREATED	$LiBH_4$ TREATMENT	$LiBH_4$ TREATMENT AND $CHCl_3$ EXT^N	$NaBH_4$ TREATMENT
Cysteic acid	2.1	–	–	–
Aspartic acid	10.8	9.5	9.6	8.6
Threonine	5.9	5.5	5.5	4.8
Serine	12.3	12.3	12.6	12.2
Glutamic acid	6.6	7.1	7.7	7.3
Proline	6.3	7.4	5.1	7.3
Glycine	11.9	12.3	12.6	12.6
Alanine	10.4	10.4	10.8	10.8
Valine	9.4	8.9	9.3	9.8
Methionine	1.5	3.7	1.6	3.1
Isoleucine	7.6	7.7	7.8	7.9
Leucine	10.6	10.5	10.4	10.6
Tyrosine	2.4	2.6	2.7	2.7
Phenylalanine	2.1	2.1	2.2	2.3
α-Amino Butyric acid	–	PRESENT	PRESENT	PRESENT

The amount of each amino acid residue is expressed as a percentage of the total content of amino acid residues.

Column 1: Analysis of chromopeptide material
Column 2: Analysis of chromopeptide material treated with $LiBH_4$.
Column 3: Analysis of chromopeptide material treated with $LiBH_4$. and $CHCl_3$ extracts removed.
Column 4: Analysis of chromopeptide material treated with $NaBH_4$.

TABLE XV (CONTD.) - AMINO ACID ANALYSIS OF CHROMOPEPTIDE MATERIAL 3-A1 TREATED WITH LITHIUM AND SODIUM BOROHYDRIDE

AMINO ACID	UNTREATED	$LiBH_4$ TREATMENT	$LiBH_4$ Treatment and $CHCl_3$ EXT^N.	$NaBH_4$ TREATMENT
Cysteic Acid	.0206	-	-	-
Aspartic Acid	.1054	.0587	.0493	.0452
Threonine	.0580	.0338	.0281	.0251
Serine	.1201	.0763	.0645	.0640
Glutamic Acid	.0645	.0436	.0394	.0385
Proline	.0616	.0456	.0378	.0382
Glycine	.1163	.0763	.0647	.0662
Alanine	.1020	.0644	.0554	.0567
Valine	.0918	.0549	.0478	.0511
Methionine	.0144	.0227	.0080	.0162
Isoleucine	.0745	.0477	.0400	.0416
Leucine	.1031	.0648	.0536	.0556
Tyrosine	.0230	.0162	.0138	.0142
Phenylalanine	.0206	.0131	.0114	.0121
α-amino butyric acid		PRESENT	PRESENT	PRESENT

The amount of each amino acid residue in a sample of the hydrolysate is expressed in μ moles.

Column 1: Analysis of chromopeptide material.
Column 2: Analysis of chromopeptide material treated with $LiBH_4$
Column 3: Analysis of chromopeptide material treated with $LiBH_4$ and $CHCl_3$ extracts removed.
Column 4: Analysis of chromopeptide material treated with $NaBH_4$.

(8) Thin Layer Chromatography of Individual Amino Acids

From the tables giving the amino acid analyses of chromopeptide material separated by thin layer chromatography (Tables VII - XV) it appeared possible that certain amino acids might be present as the free amino acids. In order to establish whether or not this was the case, individual amino acids were chromatographed with butanol - acetic acid - water (3:1:1). Fourteen of the more frequently occurring amino acids were subjected to T.L.C. on MN-Silica Gel G-HR plates (20 x 20cms; 0·6mm. thickness) using the above solvent system. The developed plates were removed when the solvent front had travelled approx. 12cms. After air-drying, the plates were sprayed with a ninhydrin solution (G.T. (K)) and placed in an oven at 80°c for 10mins. to allow colour development. The R_f values of each amino acid were then calculated and are shown in Table XIII.

DISCUSSION

Culture of Porphyridium cruentum

The artificial sea-water medium based on that developed by Jones, Speer and Kury (1963) was found to support good growth of the Porphyridium cruentum. Inoculation of sterilised medium was accomplished by using 100ml. aliquots of culture medium which contained alga about to be harvested. Alga was originally cultured in 4-litre conical flasks, but as larger quantities of material became desirable it was discovered that the alga could be cultured quite successfully in 20-litre aspirators. The culture media were aerated with air containing 5% carbon dioxide at the rate of 1 litre per minute. This stream of gas also agitated the media and according to previous workers (e.g. Paterson, 1967), this mixing enhanced the growth rate of the alga. Under continuous illumination by two "cool white" (Phillips N.V.) fluorescent 80W lamps at a temperature of 22 ± 2°c the alga was ready for harvesting after 6-8 weeks growth. Vitamin B_{12} solution was added to the culture media in trace amounts, although the precise effect of its inclusion in the media was not investigated.

Harvesting and Extraction

After the optimum growth period Porphyridium cruentum was harvested by centrifugation at 1,500g. Cell rupture, with consequent release of the biliproteins into solution, was brought about by ultrasonic oscillation

TABLE XIII - R_f Values of Amino Acids

Values calculated on MN-Silica Gel G-HR plates using butanol - acetic acid - water (3:1:1)

AMINO ACID	R_f VALUE
Phenylalanine	.56
Leucine	.53
Tyrosine	.53
Isoleucine	.51
Valine	.40
Aspartic acid	.31
Glutamic acid	.29
Alanine	.28
Threonine	.27
Glycine	.23
Serine	.23
Proline	.19
Cysteic acid	.12
Cystine	.12

followed by freezing and thawing. One such treatment was never sufficient to release all the biliproteins, and five or six extractions were usually required. After the final extraction the remaining algal material was observed to be dark green in colour. The crude biliprotein solution was centrifuged and filtered, then precipitated with 30% ammonium sulphate. In some cases, especially with less concentrated biliprotein solutions, an additional 5% ammonium sulphate was necessary to bring about complete precipitation. This requirement was particularly noticeable in biliprotein solutions derived from algal material which was frozen and thawed several times. Leibo and Jones (1964) found that in B-phycoerythrin from Porphyridium cruentum, repeated freezing and thawing produced a decrease in the concentration of native phycoerythrin and an increase in phycoerythrin sub-units. Also, in dilute aqueous solutions Mieras (1969) observed the dissociation of native B-phycoerythrin into sub-units. The formation of sub-units of B-phycoerythrin during the extraction procedure could well be the reason for the higher concentrations of ammonium sulphate required. The precipitated crude biliprotein material was dialysed and centrifuged prior to tricalcium phosphate-celite chromatography. The absorption spectra before and after chromatography (figs. 3, 4(a) and 4(b)) clearly indicate the separation achieved by this procedure. All biliprotein material was initially adsorbed onto the tricalcium phosphate

and step-wise elution with sodium phosphate buffers (pH 6·5) selectively desorbed the biliproteins. In most cases 0·02M sodium phosphate was required to desorb B-phycoerythrin from the column. However, absorption spectra indicated that some R-phycocyanin (λ max 615nm. and 553nm) was also being eluted with the later fractions of B-phycoerythrin (fig. 4(b)). The other contaminating phycocyanin present in Porphyridium cruentum, allophycocyanin, remained on the column at the above phosphate buffer concentration. In fact desorption of allophycocyanin only occurred when the phosphate buffer concentration was raised to 0·2M. Hence, tricalcium phosphate-celite chromatography was observed to give complete separation of B-phycoerythrin from allophycocyanin and partial separation from R-phycocyanin. It should be noted however, that 0·02M sodium phosphate buffer was not always necessary for B-phycoerythrin desorption; 0·01M buffer was adequate under certain conditions. When large quantities of crude biliprotein were being prepared for column chromatography, incomplete dialysis can result in a considerable quantity of ammonium sulphate being left in solution. Ammonium sulphate has the effect of decreasing the adsorption capacity of the tricalcium phosphate and consequently the biliproteins are displaced at lower phosphate concentrations. Swingle and Tiselius (1951) reported that sodium chloride promoted the adsorption of phycoerythrin on the column. Previous workers in this

laboratory (e.g. Paterson, (1967); Mieras, (1969)) made all buffer and biliprotein solutions 1% (W/V) w.r.t. sodium chloride, but in this investigation no appreciable affect on the adsorption of B-phycoerythrin was noted, and so the sodium chloride was omitted. This observation was in agreement with O'hEocha (1955) and O'Carra (1962). On the basis of spectral ratios, considerable purification of B-phycoerythrin was achieved by tricalcium phosphate-celite chromatography. The ratio prior to chromatography was generally in the region of 1 to 2, as compared with values of 3 to 4 in some samples of chromatographed material. Further increases in the spectral ratio were obtained when these biliprotein solutions were precipitated with ammonium sulphate prior to crystallization.

Crystallization

B-phycoerythrin purified by tricalcium phosphate chromatography was used to obtain crystalline samples of the biliprotein. Initially crystallization was brought about by the addition of solid ammonium sulphate and precipitating the biliproteins present within certain defined ranges. This procedure was adopted for several reasons, the main one being removal of R-phycocyanin from partially purified biliprotein solutions. R-phycocyanin was observed to be more soluble than B-phycoerythrin in a solution which was 30% w.r.t. ammonium sulphate. Hence, the supernatant of a partially

purified biliprotein solution made 30% w.r.t. ammonium sulphate, would be enriched with respect to R-phycocyanin, whereas the precipitate would be enriched with respect to B-phycoerythrin. When this precipitate was centrifuged, dissolved in deionised water and dialysed against 0•01M phosphate buffer, the absorption spectra of the resulting fluorescent solution was an indication of the purification achieved. The increase in spectral ratio and relative decrease in the maximum due to contaminating R-phycocyanin shows the success of this purification.

The three experiments on crystallization detailed in the experimental section illustrate the effect of different ammonium sulphate concentrations on purified biliprotein solutions. Biliprotein material which had been precipitated with 30% (w/v) ammonium sulphate was redissolved and dialysed free of ammonium sulphate. The purified B-phycoerythrin solution was then treated with different percentages of solid ammonium sulphate to obtain solutions with known concentration ranges with respect to ammonium sulphate (e.g. 15 - 20%, 20 - 30%). Several interesting observations were made by comparing the different solutions. No B-phycoerythrin crystals were found in solutions containing less than 15% or more than 30% ammonium sulphate. The addition of 30% ammonium sulphate lowered the pH of the biliprotein solution from 6•5 to 5•2, whereas 20% ammonium sulphate reduced the pH to 5•7. The main difference observed between solutions of different sulphate concentration

was one of crystal shape. Solutions containing 20-30% ammonium sulphate produced needle-shaped crystals, while solutions of lower sulphate concentration (e.g. 15-20%) gave more rectangular B-phycoerythrin crystals. This observation held true over a large number of experiments and when considered with the above remarks on pH changes, lends support to the postulation of Bouillene-Walrand and Delarge (1937) that crystal shape variations in proteins may be due to differences in pH in the crystallizing biliprotein solution. With the addition of ammonium sulphate, crystallization generally occurred within 48 hrs., and an increase in crystal size was observed in solutions which were allowed to "age" at 2°c in the dark. Airth and Blinks (1957) reported that fresh preparations of B-phycoerythrin from Smithora naiadum could not be crystallized by ammonium sulphate precipitation. However, if these solutions were allowed to "age" at room temperature, then crystallization readily occurred. The previous year these authors also reported that the visible absorption peak at 565nm disappeared when the biliprotein was recrystallized several times at its isoelectric point (pH 4.5). In the present investigation B-phycoerythrin was recrystallized several times, without any significant alteration in the visible absorption spectrum. As previously mentioned, however, all crystallizations were obtained in solutions of pH value greater than 5.0, and crystallization at the isoelectric point could bring about changes

in the protein structure which have an effect on the visible absorption spectrum of the biliprotein.

A comparison between 20% and 30% ammonium sulphate biliprotein solutions indicated that when crystallization occurred in these solutions, it was virtually always accompanied by a precipitate of amorphous material in the 30% solution. On the other hand, crystallization of B-phycoerythrin with 20% solid ammonium sulphate rarely produced any amorphous precipitate. The addition of ammonium sulphate lowers the pH of the biliprotein solution towards its isoelectric point. Since the biliprotein is least soluble at its isoelectric point, the more ammonium sulphate dissolved the less soluble the biliprotein becomes. When solid ammonium sulphate additions are made there is probably a considerable drop in the pH in localized regions around the dissolving ammonium sulphate. As a result the B-phycoerythrin is thrown out of solution rapidly and precipitates in the amorphous, rather than the more stable crystalline state. To minimise this effect, ammonium sulphate was always finely ground and additions were made over a considerable time with constant stirring.

A second problem arising due to the addition of solid ammonium sulphate was the release of entrapped air when the ammonium sulphate dissolved. This constant stream of tiny air bubbles was likely to have an inhibiting effect on crystal formation by denaturing the protein at the air-water interface. In later crystallizations

this difficulty was overcome by adding the ammonium sulphate as a concentrated solution. A predetermined quantity of this solution was added dropwise to the stirred biliprotein solution and crystallization normally occurred within 48 hrs. Photomicrographs of a selection of B-phycoerythrin crystals are shown in figs. 5a, b, c and d. A point of interest is that the crystals shown in figs. 5b, c and d were all obtained by adding a concentrated solution of ammonium sulphate to the biliprotein solution until the mixture was 20% w.r.t. ammonium sulphate. Furthermore, all three biliprotein solutions were obtained by elution from a tricalcium phosphate-celite column with 0·01M sodium phosphate buffer. The most obvious difference in these three solutions is one of biliprotein concentration, and this may well be a significant factor in determining the crystal shape of the biliprotein. The crystals illustrated in fig. 5d were the largest obtained and were approximately 250 x 5 μ.

Amino Acid Analysis of B-phycoerythrin.

Crystalline samples of B-phycoerythrin were used to determine the amino acid composition of the biliprotein. The crystalline material was dissolved in phosphate buffer and dialysed against de-ionised water prior to freeze-drying. Moisture determinations carried out on this material showed a moisture content of 10%. Acidic hydrolysis of this material for 24 hrs. at 105 $\pm$ 0·5°c in 6N hydrochloric acid indicated that

80% was recoverable as amino acids. This recovery compares reasonably well with that of Raftery and O'hEocha (1965), who claimed an 85% recovery of amino acids from R-phycoerythrin in Ceramium rubrum. Under the above conditions for acidic hydrolysis cystine was converted to cysteic acid, while methionine residues were converted to methionine with no detectable quantities of methionine sulphoxide or sulphone. However, as a check on the cystine and methionine values obtained from this analysis, a quantity of the freeze-dried bili-protein was subjected to performic acid oxidation prior to amino acid analysis. Cystine residues were again recovered as cysteic acid, but methionine residues were recovered as methionine sulphone. However, by comparing the recoveries of cysteic acid and methionine or meth-ionine sulphone, both methods were found to yield the same result. It was concluded therefore, that acidic hydrolysis had converted cystine residues to cysteic acid, but methionine residues were not oxidised to the sulphoxide or sulphone. The results of a typical analysis are shown in table VI. The first column gives the micromoles of each amino acid residue present in the sample analysed. The second column gives the weight of each amino acid residue present in every 100gms. of biliprotein. When these residue weights were totalled (excluding the NH_3 value) and an allowance made for the moisture content of the bili-protein, it was estimated that 80% of the protein

weight was recoverable as amino acid residues. An estimate of the minimum molecular weight was made by assuming one histidine residue per integral unit, since histidine was the least frequently occurring amino acid residue. The "rounded off" values for each amino acid residue relative to one histidine residue are given in column 3, and from this a minimum molecular weight of 14,000 was derived for B-phycoerythrin.

In common with most amino acid analyses of biliproteins, the content of acidic amino acids is considerably greater than the content of basic amino acids. Satisfactory separation of all the amino acids was achieved on the Technicon Analyser using the conditions quoted in table V.

Pepsin Digestion

Seven peptic digestions are detailed in the experimental section and all were carried out under approximately the same conditions. The substrate : enzyme ratio was in the region of 2 : 1 in all cases, and the digestion conditions were such that maximum enzyme cleavage would be obtained.

In the first pepsin digestion the chromopeptide material was completely extracted with t-amyl alcohol (fig. 6.) and this material was then subjected to a series of experiments designed to produce relatively pure chromopeptides. High voltage paper electrophoresis indicated that at least four different chromopeptide

fragments were present in addition to several free amino acids and peptides (fig. 7.). To eliminate contaminating amino acids and peptides, molecular-sieve chromatography on Sephadex G-25 and G-75 was attempted. However, Sephadex G-75 gave no separation of the t-amyl alcohol extract, presumably because there was insufficient difference in the molecular weights and configurations of the individual components. Sephadex G-25 gave some separation into different coloured fractions and these were subjected to H.V.E. Examination of the developed electrophoretograms indicated that the chromopeptide material had not been completely separated from the free amino acids and peptides (e.g. fig. 8.)

The chromopeptide mixture obtained from the second peptic digestion was subjected to thin layer chromatography on cellulose with phenol-acetic acid-water (1:1:1) as solvent. A red band at R_f 0.9 was observed to be the chromopeptide mixture, while a quantity of ninhydrin-positive, non-fluorescent material at the solvent front was identified as free amino acids and peptides. Hence a measure of separation was achieved by this procedure. The strong cation-exchange resin Amberlite CG-50 gave little or no separation of the chromopeptide mixture. Elution was performed with 0·2M sodium phosphate buffer, pH 6·5, and one reason for the poor separation is the affinity of this type of resin for aromatic compounds. Hence all chromopeptide material is likely to be retained on the column

irrespective of variations in the peptide portions of the individual chromopeptides. Also, for larger molecules like chromopeptides, a low cross-linked resin would be more suitable as this would permit greater penetration of larger molecules into the resin matrix.

The series of experiments using molecular sieve chromatography with a variety of gels and eluants proved to be one of the more successful purification procedures. Sephadex G-75 was found to be unsuitable for chromopeptide separation regardless of the eluant used. Sephadex G-50 gave some separation of chromopeptide material when 0·2M sodium phosphate buffer (pH 6·5) was used as eluant, but high voltage paper electrophoresis indicated that the eluted chromopeptide fractions contained a considerable proportion of peptide material. On the other hand, with Sephadex G-10 a rust coloured fraction moved unretarded through the gel when the chromopeptide mixture was eluted with deionised water. The mauve coloured fraction remaining on the column was subsequently removed by elution with 1% acetic acid solution. However, this latter fraction was found to contain large amounts of non-chromopeptide material. An interesting difference between the rust and mauve coloured fractions is the position of their respective visible absorption maxima. The rust coloured band displayed a maximum at 492n.m. while the mauve coloured band had, in addition, a broad absorption

maximum at 540-560n.m. A similar separation was obtained with Sephadex G-25 on elution with deionised water and 1% acetic acid. The main disadvantage of gel chromatography with Sephadex G-10 and G-25 is that the 1% aqueous acetic acid required to remove the mauve coloured fractions also appeared to elute peptide material.

The attempted separation of a chromopeptide mixture on a column of "Isopor" De-acidite M-IP resin produced three distinct coloured fractions. This weakly basic anion exchange resin was eluted with 1%, 5% and 10% aqueous acetic acid and high voltage paper electrophoresis on the separated fractions indicated that all three fractions contained common components.

When Bio Gel P-6 became available this was found to be the most successful of the molecular sieve chromatography materials. Several different coloured fractions were obtained on elution with 1%, 5% and 20% aqueous acetic acid solution and, furthermore, these fractions showed a separation which had previously been unattainable. Figs. 9 and 10 illustrate the separation achieved when a chromopeptide mixture was chromatographed on a column of Bio Gel P-6. Killilea and O'Carra (1968) isolated and purified chromopeptide material from R-phycoerythrin by celite chromatography and gel filtration on Sephadex G-50. However, these authors made no attempt to isolate different chromopeptide fractions, but merely to separate chromopeptide

material from non-chromopeptide material. In the present investigation a sample of the chromopeptide mixture from pepsin digestion was purified by partion chromatography on a celite column using the procedure suggested by Dus, Bartsch and Kamen (1962). The purple chromopeptide mixture was eluted and collected at the solvent front. This material was then subjected to gel filtration on Bio-Gel P-6 using phenol-acetic acid-water (1:1:1) as eluant. (Synge and Youngson, 1961). This procedure did not fractionate the chromopeptide mixture into different coloured bands and only one purple coloured fraction was eluted. This was subjected to prolonged acidic hydrolysis and the amino acid analysis of this material (Table VII, Sample A) indicates that most amino acids are present. This would suggest the presence of either a large chromopeptide or a mixture of small chromopeptides.

The chromopeptide mixture derived from the seventh pepsin digest was subjected to partion chromatography on a celite column as above, but, since molecular sieve chromatography on Bio-Gel P-6 with phenol-acetic acid-water did not appear to yield any improvement in purification, deionised water and aqueous acetic acid solutions were used as eluants instead. A pink and a purple coloured fraction corresponding to peaks A and C in fig. 10 were eluted and analysed for amino acids. (Table VII; samples B and C respectively). These results do not suggest the presence of a small chromopeptide either, even if an allowance is made for

contamination by free amino acids or peptides in the coloured fractions. In an attempt to obtain smaller chromopeptide fragments it was decided to subject the peptic digest to further enzymic hydrolysis with trypsin.

Trypsin Digestion

The first tryptic digestion was carried out on a peptic digest which had been partially purified by celite partition chromatography and Bio-Gel P-6 molecular sieve chromatography. All chromopeptide extracts from peptic digestion were adjusted to pH 7·6 before further digestion with trypsin. The first tryptic digest was extracted with butanol then purified by celite chromatography and Bio-Gel P-6 gel filtration. During this latter chromatography with phenol-acetic acid-water (1:1:1) no separation was observed except for a green band which was eluted considerably later than the bulk of the coloured material. Amino acid analysis of this green fraction is very interesting (Table VIII; 1-1). The presence of large quantities of the leucines, tyrosine and phenylalanine in this "chromopeptide" fraction is regarded with suspicion. Pepsin is particularly specific in cleaving amide linkages involving the amino groups of aromatic amino acids and amino acid analyses of peptic chromopeptides after chromatography on Bio-Gel P-6 with aqueous acetic acid indicates a very low proportion of the aromatic amino acids tyrosine and phenylalanine (Table VII;

samples B and C). From this it seems reasonable to assume that tyrosine and phenylalanine residues were not present in the chromopeptide. However, their occurrence in the green "chromopeptide" fraction after tryptic hydrolysis and Bio-Gel P-6 chromatography with phenol-acetic acid-water indicates that celite partition chromatography and gel filtration with phenol-acetic acid-water as eluant does not separate tyrosine and phenylalanine (or peptides containing these aromatic amino acids) from the chromopeptides. The solvents used to extract enzyme digests, and the eluants used in partition chromatography were selected on the basis of their ability to extract the aromatic chromopeptides from a mixture of peptides and amino acids. Unfortunately, it appears that these solvents also extract the aromatic and some of the aliphatic amino acids and peptides. The material eluted first from the Bio-Gel P-6 column with phenol-acetic acid-water was split into two portions; one was subjected to anion exchange on Bio Rad AG 1 x 2 (acetate form), the other was subjected to cation exchange on Dowex 50 x 8 (hydrogen form). The first reddish-brown fraction eluted from the anion exchange resin was collected and analysed for amino acids. (Table VIII; 1-2). Similarly, a sample of the greenish-brown material eluted from the cation exchanger was analysed for amino acids (Table VIII; 1-3). In contrast to sample 1-1, these fractions contained very little tyrosine and phenylalanine. Also, these fractions

were almost completely free from basic amino acid residues. This latter observation is the result of the specificity which trypsin shows towards amide bonds between the carboxyl groups of basic amino acids and the amino group of other amino acids. Hence, trypsin appears to have eliminated all the basic amino acid residues from these chromopeptide fragments (c/f Table VII.) In fact, later amino acid analyses indicated an absence of basic amino acid residues from all chromopeptide fragments.

The second tryptic extract of chromopeptide material was used to investigate the possibility of T.L.C. as a means of chromopeptide separation. Crespi and Smith (1970) used preparative thin layer chromatography to separate chromopeptides derived from phycocyanin by nagarse digestion. In the present investigation n-butanol-acetic acid-water (3:1:1) on Silica Gel G-HR adsorbent proved to be the most suitable T.L.C. system. The best resolution of the coloured bands was observed when the thickness of the layers was 0·6mm. Separation and purification by this technique was a considerable improvement over high voltage paper electrophoresis, which usually gave inferior separation and resolution. In addition, removal of material after electrophoresis invariably meant loss of material, whereas bands separated by T.L.C. were easily removed by washing with methanol or aqueous acetic acid.

The extraction and purification procedure for the

third tryptic digest is illustrated in fig. 11. and the separated chromopeptide material was successfully chromatographed in the above T.L.C. system. Tables IX, X and XI show the amounts of each amino acid residue present in every isolated band. Table XVI gives the total amount of these amino acid residues present in each band, and also expresses the concentration of each amino acid present relative to one mole of threonine. These concentrations were "rounded off" to the nearest whole number. Table XVI indicates that one of the most abundant chromopeptides is 3-A1 and the composition of this chromopeptide is: Asp_2 Thr Ser_2 Glu Pro Glu_2 Al_2 Val_2 Ileu Leu_2. This material was coloured red and had an R_f value of 0·29. Band 3-A5, which was coloured brownish-green and had an R_f value of 0·46, consisted of exactly the same proportions of amino acid residues. Two other brown coloured bands, 3-A6 (R_f 0·38) and 3-B1 (R_f 0·48) also yield an almost identical residue composition. Band 3-A6 contained 2 residues of isoleucine, while 3-B1 contained no proline and three serine residues. Tyrosine and phenylalanine were also present in this latter material but tables X and XI indicate that relatively large quantities of these amino acids are present in all hydrolysates from fraction 3-B and 3-C, regardless of the R_f value of the band involved. The presence of tyrosine and phenylalanine in such circumstances seems to support the argument that tyrosine and phenylalanine residues are not in fact present in

these chromopeptide fractions, but merely contaminants. Table VIII indicated that the purple coloured chromopeptide material did not contain any significant quantities of tyrosine or phenylalanine, and only fractions with high elution volumes on Bio-Gel P-6 contained large quantities of these amino acids. For this reason it is suspected that fractions 3-B and 3-C are contaminated with varying amounts of their amino acids. Band 3-A4 contains the same residues as band 3-A1 but these residues are present in almost equimolar quantities.

The band containing the largest weight of amino acids was 3-A3, a green coloured band at R_f 0.82 - 0.87. This band appears to have the following amino acid composition: Asp Thr Ser_2 Glu_2 Pro Gly_2 Ala_2 Val_2 Ile_2 Leu_5 Tyr Phe and as such differs considerably from the above chromopeptides. However, this band was found to have a high R_f value in butanol:acetic acid:water (3:1:1) and could be contaminated with free amino acid or peptide material having a high R_f value in this solvent. Table XIII shows the R_f values of individual amino acids on Silica Gel plates using the above solvent system, and phenylalanine leucine tyrosine and isoleucine all have high R_f values. Band 3-A3 may therefore be contaminated by peptides derived from these amino acids, and if so the remaining amino acid residues would correspond almost exactly to the residue composition of band 3-A1. When the same argument is put forward for

TABLE XVI - Amino Acid Analysis of Chromopeptides from Pepsin/Trypsin Digest 3.

AMINO ACID	3-AA1		3-AA2		3-AA3		3-A1		3-A6	
	A	B	A	B	A	B	A	B	A	B
Cy SO_3H	.0445	2	.0596	1	.1545	1	.657	-	.245	1
Asp	.0371	1	.0554	1	.1429	1	3.378	2	.755	2
Thr	.0295	1	.0512	1	.2055	1	1.857	1	.336	1
Ser	.0567	2	.0890	2	.3100	2	3.842	2	.800	2
Glu	.0541	2	.0772	2	.0898	-	2.062	1	.365	1
Pro	-	-	.0294	1	.0530	-	1.972	1	.358	1
Gly	.0787	3	.1144	2	.2560	1	3.722	2	.676	2
Ala	.0564	2	.1380	3	.2190	1	3.262	2	.530	2
Val	.0764	3	.1510	3	.4420	2	2.940	2	.663	2
Met	-	-	-	-	-	-	.462	-	-	-
Ile	.0138	1	.0926	2	.1508	1	2.384	1	.512	2
Leu	.0784	3	.2082	4	.7675	4	3.300	2	.515	2
Tyr	.0089	-	.0136	-	.0395	-	.736	-	.103	-
Phe	.0276	1	.0408	1	.0790	-	.660	-	.086	-

Column A: μ moles of each amino acid residue contained in whole band.

Column B: moles of each amino acid residue relative to one threonyl residue. These values are "rounded off" to the nearest whole number.

TABLE XVI (CONTD.) - Amino Acid Analysis of Chromopeptides from Pepsin/Trypsin Digest 3.

AMINO ACID	3-A5		3-A4		3-A3		3-B1		3-B2	
	A	B	A	B	A	B	A	B	A	B
Cy SO_3H	.216	-	.191	-	-	-	.135	-	.111	-
Asp	.759	2	.626	1	7.73	1	1.465	2	.730	1
Thr	.490	1	.528	1	5.55	1	.680	1	.616	1
Ser	.886	2	.602	1	8.55	2	1.893	3	1.003	2
Glu	.515	1	.453	1	8.60	2	.629	1	.602	1
Pro	.430	1	.320	1	5.19	1	.226	-	.491	1
Gly	.920	2	.775	1	11.18	2	.846	1	2.49	4
Ala	.766	2	.570	1	12.88	2	1.736	2	2.06	3
Val	.855	2	.710	1	12.62	2	1.618	2	1.93	3
Met	-	-	-	-	2.46	-	-	-	.185	-
Ile	.520	1	.402	1	12.78	2	.993	1	2.56	4
Leu	.782	2	.734	1	27.4	5	1.520	2	3.68	6
Tyr	.179	-	.134	-	3.73	1	.970	1	1.765	3
Phe	.162	-	.139	-	4.73	1	.595	1	1.438	2

Column A: μ moles of each amino acid residue contained in whole band.

Column B: moles of each amino acid residue relative to one threonyl residue. These values are "rounded off" to the nearest whole number.

bands 3-B2 and 3-AA2 (R_Fs 0·80 and 0·89 respectively), the same result is obtained. Band 3-B2 was shown to have an especially high proportion of contaminating peptides and this could well be the result of its slow elution from the Bio-Gel P-6 column. Fractions having this slow elution are almost bound to be contaminated with peptide material from enzymic digestion and this peptide material is more likely to contain the amino acid residues with the greatest solubility in organic solvents (e.g. the aromatic and neutral amino acids). Table XI indicates that the bands isolated by chromatography of fraction 3-C contain an even larger proportion of contaminating peptide material; most bands were shown to contain between 30% and 50% of tyrosine and phenylalanine. In addition, the quantities of material removed from the T.L.C. plates were so small that the amino acid analyses were subject to greater errors. Chromopeptide composition determinations as performed on fractions 3-AA, 3-A and 3-B would not, therefore, be justified for fraction 3-C.

The schematic diagram for the pepsin/trypsin digest 4 is shown in figure 12 and fractions 4-A, 4-B, 4-C and 4-D were subjected to T.L.C. Samples of the bands isolated by T.L.C. were acid hydrolysed and their amino acid content determined. Table XII expresses the amino acid residues as a percentage of the total amino acid content. Table XVII gives the total amount of these amino acid residues present in each band, and also expresses the concentration of each amino acid present relative to one mole of threonine. Table XVII shows that fractions

4-B and 4-C contain large amounts of contaminating tyrosine and phenylalanine. This is very similar to pepsin/trypsin digest 3 where the contamination by these amino acid residues began with fraction 3-B. Similarly, all bands with high R_f values (e.g. 4-A3, 4-B3, 4-B4 and 4-C2) show a large concentration of isoleucine and leucine. When the amino acid composition of each band is compared with the basic core chromopeptide previously suggested, i.e., Asp_2 Thr Ser_2 Glu Pro Gly_2 Al_2 Val_2 ILeu Leu_2 a similarity is evident. However these bands appear to represent either larger chromopeptides or increased contamination by peptide materials. Most bands have an increase in the glutamic acid residue content which corresponds to two residues per chromopeptide. There is also a noticeable increase in the glycine, alanine and valine residues, especially in the bands isolated from fraction 4-B. These increases correspond to rather large increases in the isoleucine and leucine residue content of these bands.

From the above observation it appears that the bands separated from pepsin/trypsin digest 4 contain greater amounts of several amino acid residues, when compared with the bands isolated from pepsin/trypsin digest 3. The only variation in the peptic digestion procedure used for both digests was a difference in pepsin concentration. In digest 3 the pepsin concentration was 0•06% compared with 0•04% for digest 4. However, the high enzyme/substrate ratio for both digests (approx. 1:2) should

TABLE XVII - Amino Acid Analysis of Chromopeptides from Pepsin/Trypsin Digest 4.

AMINO ACID	4-A1		4-A2		4-A3		4-B1		4-B2	
	A	B	A	B	A	B	A	B	A	B
Cy SO_3H	.1648	-	.243	1	-	-	.0616	-	.160	-
Asp	.7456	2	.680	2	.730	2	.4272	2	1.38	3
Thr	.4504	1	.326	1	.326	1	.1868	1	.430	1
Ser	.6024	1	.470	1	.512	2	.4608	2	1.06	2
Glu	1.1152	2	.858	3	.797	2	.4236	2	.906	2
Pro	.5704	1	.498	2	.416	1	.1648	1	.523	1
Gly	1.9104	4	1.225	4	1.038	3	.5168	3	1.82	4
Ala	.7040	2	.587	2	.769	2	.5496	3	1.93	4
Val	.8056	2	.710	2	.750	2	.4892	3	1.635	4
Met	.0664	-	-	-	.099	-	.0796	-	.364	1
Ile	.3224	1	.410	1	.760	2	.2868	2	1.27	3
Leu	.8816	2	.900	3	1.489	5	.5180	3	1.940	5
Tyr	.0744	-	.094	-	.160	-	.0936	1	.757	2
Phe	.0992	-	.094	-	.150	-	.1168	1	1.30	3

Column A: μ moles of each amino acid residue contained in whole band.

Column B: moles of each amino acid residue relative to one threonyl residue. These values are "rounded off" to the nearest whole number.

TABLE XVII (CONTD.) - Amino Acid Analysis of Chromopeptides from Pepsin/Trypsin Digest 4.

AMINO ACID	4-B3 A	4-B3 B	4-B4 A	4-B4 B	4-C1 A	4-C1 B	4-C2 A	4-C2 B
Cy SO_3H	.0688	-	-	-	.0152	1	.0624	1
Asp	.622	2	.788	2	.0524	3	.481	9
Thr	.374	1	.404	1	.0160	1	.0528	1
Ser	.288	1	.400	1	.0344	2	.1713	3
Glu	.665	2	.768	2	.0244	2	.0655	1
Pro	.161	-	-	-	-	-	-	-
Gly	.674	2	1.160	3	.492	3	.1328	3
Ala	1.110	3	.896	2	.0364	2	.1312	3
Val	.972	3	1.112	3	.0268	2	.2045	4
Met	.098	-	-	-	-	-	-	-
Ile	1.730	5	3.416	8	.0096	1	.0576	1
Leu	2.900	8	3.972	10	.0368	2	.547	10
Tyr	.818	2	1.012	3	.1304	8	.955	18
Phe	.875	2	.684	2	.0280	2	.166	3

Column A: μ moles of each amino acid residue contained in whole band.

Column B: moles of each amino acid residue relative to one threonyl residue. These values are "rounded off" to the nearest whole number.

compensate for the low enzyme concentration and complete enzymic cleavage of the biliprotein would be expected in both digests. In addition, a digestion period of more than 30hrs. should not result in incomplete cleavage. Fujiwara (1957) reported that complete enzymic cleavage of R-phycoerythrin occurred after 3hrs. with an enzyme/substrate ratio of 1:7 when the substrate concentration was about 0·2%. Hence assuming complete cleavage of the biliprotein in both digests, the reason for increased amounts of amino acid residues being found in the bands isolated from digest 4 may be less efficient purification techniques. Tryptic digest 4 was not subjected to a chloroform extraction step prior to extraction with n-butanol. Crespi and Smith (1970) reported that failure to extract their original digest with chloroform led to difficulties in interpreting the chromatograms of isolated chromopeptide material from phycocyanin. In the present investigation when tryptic digest 3 was extracted with chloroform, the amino acid analysis of bands derived from this extract (3-AA) showed an abnormally high content of cysteic acid, glutamic acid, glycine, alanine, valine, isoleucine and leucine. These are the same amino acid residues which are found in increased amounts in bands derived from tryptic digest 4. On the basis of this evidence, and the findings of Crespi and Smith (1970) it appears that the differences in amino acid composition between chromopeptides derived from pepsin/trypsin digests 3 and 4 are due to contamination

by peptide material; this peptide material can be removed by extracting the digest with chloroform.

The absorption spectra of the chromopeptides obtained by the peptic digestion and extraction procedures are shown in fig. 6 and fig. 9. These spectra are very similar to absorption spectra obtained by Fujiwara (1957). The absorption maxima of material from peptic digestion were observed at 492nm. 550-570nm. 640-650nm. In the visible absorption spectrum of the chromopeptide mixture from peptic digestion (fig.6.) the absorption at 492nm. becomes very conspicuous. Compared with the absorption spectrum of the native biliprotein the absorption in the 492nm. region (assumed to be due to the tetrapyrrole structure of the pigment) is little affected, whereas the absorption at 545nm. and 565nm. is greatly reduced.

Chromopeptide - Chromophore Linkage

The possibility of a chromophore-protein ester linkage was investigated using lithium and sodium borohydride. Band 3-A1 was treated with these reagents in an attempt to reduce any esters present to the corresponding alcohols. The results are shown in Table XV and indicate that neither glutamic acid nor serine is involved in an ester linkage. Had a glutamic acid residue been involved in the ester linkage, a decrease in the amount of glutamic acid present would be expected after treatment with borohydride. This was not found to

be the case and in fact the quantity of glutamic acid present was found to increase after the borohydride treatment. The amount of serine present appeared to be unaffected by this treatment. However, the quantity of aspartic acid residues present was significantly reduced by refluxing the chromopeptide material with borohydride, especially sodium borohydride. This would appear to indicate the possibility of a chromophore-apopeptide linkage between the ω-carboxyl group of aspartic acid and a hydroxyl group on the chromophore. The expected reduction product from such a linkage would be homoserine (γ-hydroxy-α amino butyric acid) and this material would be expected to appear on the amino acid chromatogram just before glutamic acid. No such peak was observed but, as stated above, there was a slight increase in the glutamic acid. This increase may in fact be due to the presence of homoserine beneath the glutamic acid peak. The only other significant change in the amino acid content of the chromopeptide material after borohydride treatment is a decrease in the amount of threonine present. If the chromophore linkage involved an ester linkage between the hydroxyl group of a threonine residue and a hydroxyl group from one of the propionic acid side chains of the chromophore, then reduction of this linkage with borohydride would be expected to give rise to threonine. This being the case, the amino acid analysis of material treated with borohydride would not

be expected to show any significant difference in threonine content when compared with the amino acid analysis of untreated material. However, this was not found to be the case and in addition an extra peak was observed in the chromatogram. This peak emerged prior to valine and corresponded to α-amino butyric acid. This material could only result from β-elimination of a threonine ether. An ether linkage between the hydroxyl group of a threonine residue and a hydroxyl group on the chromophore would result in the formation of free threonine. However, a base catalysed β-elimination reaction would result in the formation of the dehydro product. Reduction and hydrolysis of this product would give rise to α-amino butyric acid. Heard (1965) found tentative evidence for the involvement of threonine in an ether bond in R-phycoerythrin. He used sodium borotritiide instead of sodium borohydride and found, by paper chromatograph methods and by radioactive counter, evidence for α-amino butyric acid incorporating two atoms of tritium. The rather small quantities of α-amino butyric acid found to be present made accurate quantitative determinations difficult. Also, on the chromatogram this peak occurred just before the buffer changeover, which is accompanied by a rise in the baseline. The above factors did not allow any sufficiently accurate x-amino butyric acid determinations to be made. Obviously, the postulated threonine ether linkage has not undergone complete conversion to α-amino butyric acid and the results indicated that sodium borohydride

was more effective than lithium borohydride. In fact lithium borohydride appeared to have little effect on chromopeptide material 4-A3(Table XIV). Fig. 13. illustrates a possible chromophore-apoprotein linkage through threonine and aspartic acid residues. The proposed chromophore-apoprotein linkage is illustrated using the chromophore structure proposed by Rudiger and O'Carra (1969), since these authors present the most conclusive evidence to date.

In concluding it must be admitted that the evidence for an aspartyl and threonyl linkage to the chromophore is not conclusive and would require further justification. The smallest chromopeptide obtained in reasonable quantities consisted of the following amino acid residues: Asp_2 Thr Ser_2 Glu Pro Gly_2 Ala_2 Val_2 Ile Leu_2 and although several amino acid residues occurring in the protein have been eliminated from the chromopeptide, a smaller chromopeptide fragment would have been desirable. Further digestion of purified chromopeptide material with an enzyme of low specificity (e.g. Pronase) would very probably give the desired result. The results of chromopeptide analyses do not indicate the peptide sequence but several chromopeptide fragments show only one serine or one aspartic acid residue which suggests that these residues might be the N- or C- terminal residues.

The difference in absorbance maxima observed in various chromopeptide fractions probably results from the complex nature of chromophore-apopeptide interactions

FIG.13. Proposed chromophore-apopeptide linkage in B-phycoerythrin.

and not as a result of the presence of different chromophore groupings. This contention is supported by the unsuccessful attempts of several authors to isolate a second chromophore from the phycoerythrins (e.g. Chapman, Cole and Siegelman, 1968, b). A further reason for the observed colour differences in chromopeptide material is alteration of the chromophore group. Rüdiger and O'Carra (1969) found that treatment of R-phycoerythrin with 1N-KOH yielded a mixture of pigments, the main component of which was spectrally almost identical with mesobiliviolin. The other main component of the mixture was a green pigment very similar in spectral properties to mesobiliverdin. Oxidative degradation of these pigments indicated that the phycoerythrin ethylidene group had been isomerized as shown on page 38. This isomerization would account for spectral shifts to longer wavelengths. Another reason for the variations in absorption spectra of chromopeptide material is the changes resulting from the chromophore group being exposed to acidic conditions during extraction and purification procedures. O'Carra, O'hEocha and Carrol (1964) demonstrated that the native chromphore group of phycoerythrin, phycoerythrobilin, underwent prototropic isomerization to a mesobiliviolin pigment on acidification. Hence, the variety of coloured chromopeptides obtained may not be solely indicative of variations in the amino acid residue content of these peptides, but merely an indication that the native chromophore group has undergone structural variations. Since bile pigments have been shown to

exhibit different R_f values when subjected to thin layer chromatography in the same solvent systems (Rüdiger and O'Carra, 1969), it is not unreasonable that chromopeptides with the same amino acid residue content and sequence, but differing chromophoric structures may exhibit differences in their R_f values in the same solvent system. Figs. 14 and 15 are designed to illustrate the separation achieved by thin layer chromatography of partially purified fractions from pepsin/trypsin digests 3 and 4. Individual bands have been examined and compared separately earlier in the Discussion, but it may be advantageous to consider the bands collectively in order to explain the apparent heterogeneity of the chromopeptides. The first fractions eluted from the Bio Gel P-6 column were red or brown in colour (i.e. fractions 3-A, 3-B, 4-A and 4-B) and their visible absorption maxima corresponded fairly closely to the visible maxima for denatured B-phycoerythrin. Hence these fractions can be said to contain chromopeptide material in which the prosthetic groups are relatively unaltered. However, fractions eluted much later from the Bio Gel P-6 column were purple, blue and green in colour (i.e. 3-C, 3-D, 3-E, 4-C and 4-D) and their visible absorption maxima had shifted to longer wavelengths, indicating considerable alterations in the chromophore groups. It is possible that the chromophore groups of these later chromopeptide fractions were more susceptible to alterations such as prototropic isomerization, since their slow elution from the molecular-sieve column would

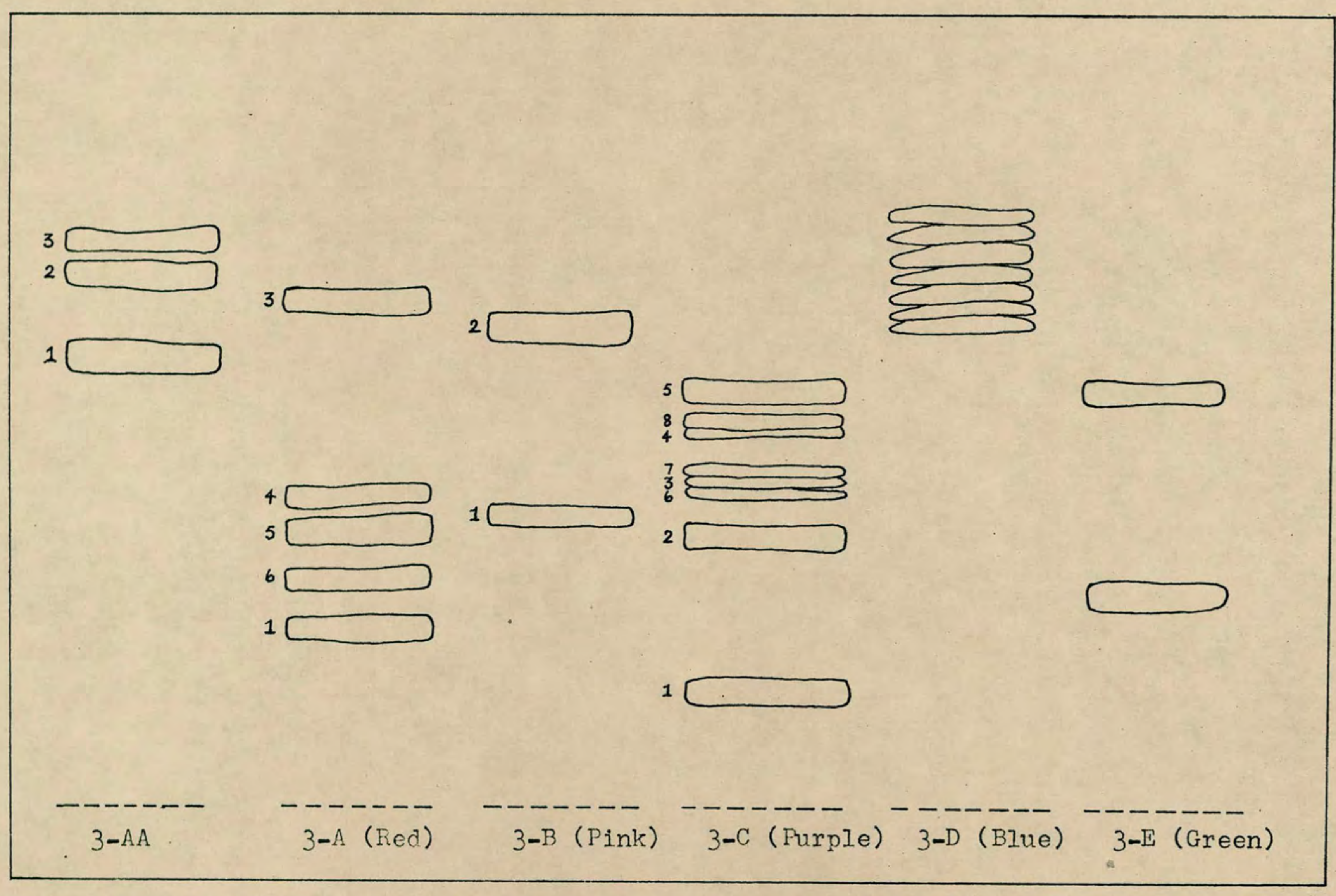


FIG.14. Bands isolated by thin-layer chromatography of fractions separated from pepsin/trypsin digest 3.

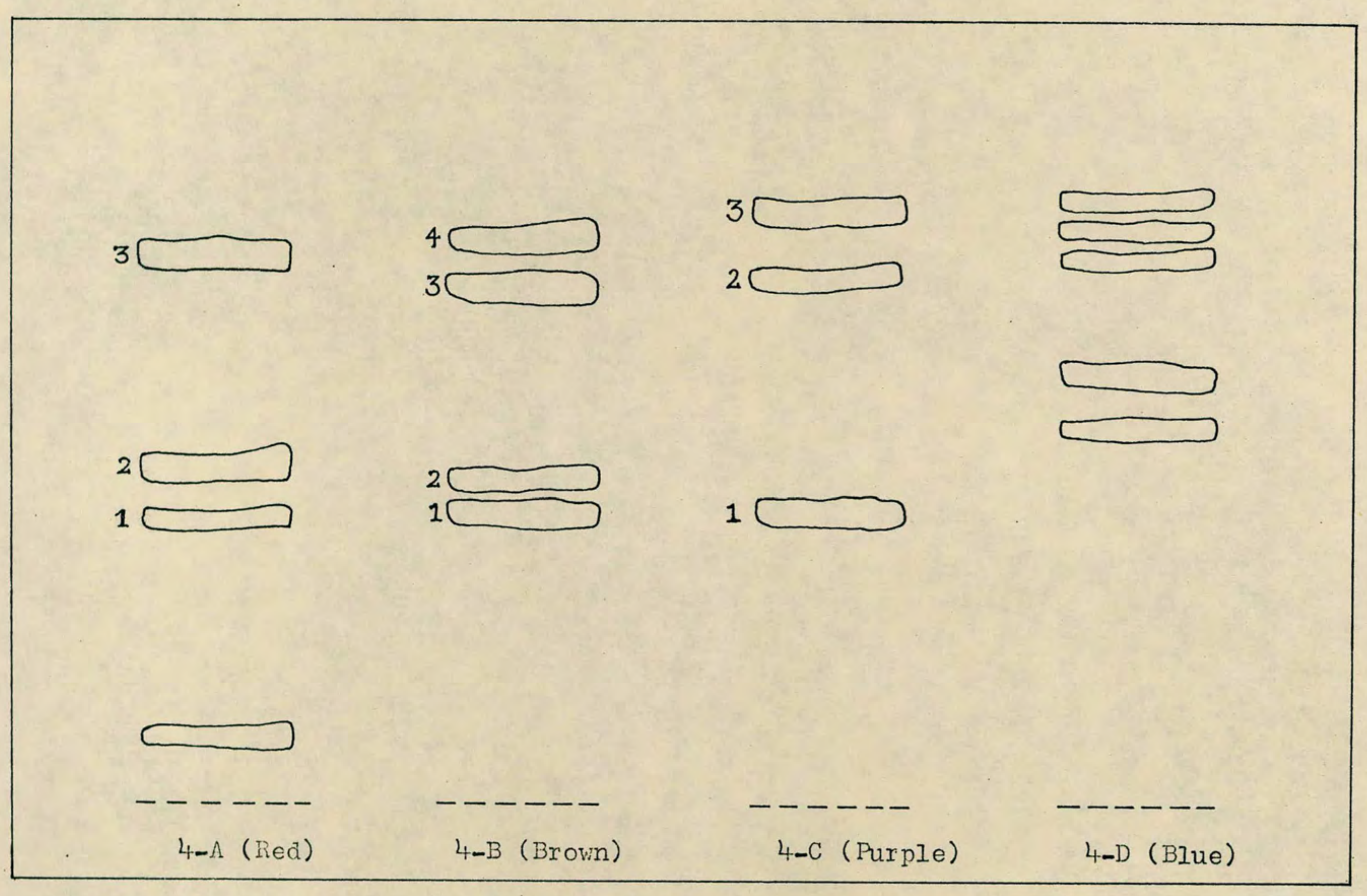


FIG. 15. Bands isolated by thin-layer chromatography of fractions separated from pepsin/trypsin digest 4.

indicate that these fractions probably contain smaller chromopeptides, in which case the chromophore group will receive less protection in the event of chemical attack. However, the slowest moving chromopeptide fractions were found to contain non-chromopeptide material. For this reason fractions 3-A, 3-B, 4-A and 4-B were thought to give more accurate determinations of the amino acid residue content of the chromopeptides. Thin layer chromatography of these red and brown coloured fractions with n-butanol-acetic acid-water (3:1:1) resulted in the separation of several different coloured bands, suggesting that the chromophore groups of some of the chromopeptides had undergone alteration prior to thin layer chromatography. However, in spite of their variations in colour and R_f values, the chromopeptides were found to be remarkably similar in amino acid composition and this investigation proposes a basic core chromopeptide with the following composition:

Asp_2 Thr Ser_2 Glu Pro Gly_2 Ala_2 Val_2 Ile Leu_2

It is also the contention of this investigation that each band separated by thin layer chromatography does not represent a distinct chromopeptide unit with a unique amino acid sequence, but may in fact contain the same apopeptide attached to altered chromophore pigments. An alteration in the chromophore group could impart a change in colour and R_f value to the chromopeptide. Alternatively, a small variation in the composition of the apopeptide chain would result in a change in the R_f value of any

given chromopeptide.

By comparing the chromopeptide material before and after tryptic digestion a significant conclusion can be drawn regarding the position of the basic amino acid residues in chromopeptides derived from peptic digestion. Amino acid analyses of chromopeptide material derived from peptic digestion revealed the presence of lysine, histidine and arginine. However, after tryptic hydrolysis these amino acids were found to be absent from all chromopeptide samples. Since trypsin has a fairly limited specificity which limits cleavage to bonds linking the carboxyl group of a basic amino acid to the amino group of another amino acid, all basic amino acid residues in peptic chromopeptides must be situated between the N-terminal residue and the chromophore group. This is the only situation which could result in the removal of all basic amino acid residues when the chromopeptides are digested with trypsin.

The choice of chromopeptide material for reduction studies was on a three-fold basis;
firstly, the selected material had to be relatively free from contamination by non-chromopeptide material;
secondly, it had to be representative of chromopeptide material as regards the amino acid composition (i.e. it had to contain the basic core chromopeptide postulated above); and
finally, it had to be available in quantities large enough to make the experiments possible and the results significant. Bands 3-A1 and 4-A3 satisfied all three requirements.

As in any study of protein chemistry it was essential to obtain highly purified samples of the compound to be studied, B-phycoerythrin, prior to enzymic digestion or amino acid analyses and several authors have investigated the homogeneity of biliprotein preparations. O'Carra (1965) purified several biliproteins by tricalcium phosphate-celite chromatography and ammonium sulphate precipitation. Examination of the absorption spectra of the purified biliprotein preparations showed that they were free from contamination with other biliproteins or other coloured impurities. When these purified samples were recrystallized and rechromatographed on tricalcium phosphate-celite and Sephadex G-75 there was no change in the extinction coefficients or in the spectral ratios. This suggested the absence of colourless ultraviolet-absorbing impurities. A further indication of the high degree of purity obtained by these purification techniques were the N-terminal analysis results. In each phycoerythrin preparation the only N-terminal residue found was methionine. This again pointed to the absence of protein impurities. Raftery and O'hEocha (1965) performed a series of purification techniques on R-phycoerythrin from Ceramium rubrum prior to amino acid and C-terminal residue determinations. Two chemical tests were used to study the purity of the biliprotein preparations. N-terminal analyses showed that the methods used for the purification of R-phycoerythrin effected the disappearance of all N-terminal groups except methionine. The purified

material was also subjected to starch-gel electrophoresis and stained with nigrosine; no other proteins were detectable. Raftery and O'hEocha also determined the spectral ratio of these purified biliprotein solutions and on the basis of the above tests contended that the biliproteins were highly purified when the spectral ratio was greater than 4·0. Using their purification procedure a spectral ratio of 4.37 was obtained with Ceramium phycoerythrin. In the present investigation spectral ratios in excess of 5·0 were obtained.

In this laboratory Paterson (1967) demonstrated that biliprotein material isolated from Porphyridium cruentum and purified by tricalcium phosphate-celite chromatography and ammonium sulphate precipitation was free from colourless proteins. The technique employed by Paterson was disc electrophoresis on vertical columns of polyacrylamide gel using naphthalene black to stain the protein components after the electrophoretic run. These experiments confirmed the contention of Raftery and O'hEocha (1965) that biliprotein material exhibiting a spectral ratio greater than 4·0 was highly purified.

Over the past forty years several proposals have been put forward as to the nature of the chromophore-apopeptide linkage. Lemberg (1930) suggested a peptide linkage between the propionic acid side-chains of the chromophore and free lysyl side chain amino groups in the protein. As a result of her studies on R-phycoerythrin Fujiwara (1957) proposed sulphur bridges between cysteine

residues and the chromophore, as in the case of cytochrome C. O'hEocha disputed both these possibilities on the grounds that all the free lysyl side chain amino groups in R-phycoerythrin were free to react with dinitrofluorobenzene leaving none which could participate in Lemberg's proposed linkage. O'hEocha also treated R-phycoerythrin with silver sulphate (a mild oxidising reagent used to cleave thioether bonds in cytochrome C) and since no pigment was released he concluded that there were no thioether bonds present. Within the last ten years several authors have suggested ester linkages either between a free carboxyl group on the apopeptide chain and a hydroxyl group on the chromophore, or between the propionic acid side-chains of the chromophore and a free hydroxyl group on the peptide chain (e.g. O'Carra, 1962; Siegelman, Chapman and Cole, 1967; Killilea and O'Carra, 1968; Rüdiger and O'Carra, 1969). However, none of these authors has supplied evidence of their proposed linkages and most conclusions are arrived at by a process of elimination. To date authors suggesting a propionic acid side-chain ester have not put forward satisfactory evidence to account for the release of phycobilins by hot methanol without esterification of the propionic acid side-chain.

Evidence, by no means conclusive, is presented here which would indicate an ester linkage between the ω-carboxyl group of an aspartic acid residue and a hydroxyl group on the chromophore. A second linkage is proposed

involving the hydroxyl group from a threonine residue in an ether linkage with a hydroxyl group on the chromophore.

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