DISCUSSION

Phycobiliproteins, the major photosynthetic accessory proteins of cyanobacteria and red algae, are assembled in phycobilisomes (Gantt, 1980), structurally complex particles that are bound to the outer surface of the thylakoid membrane. They can be detached from the membranes in the presence of mild detergents such as Triton X-100 in concentrated phosphate buffers at pH 7.0-8.0 (Gantt et al., 1979). The spectroscopic properties of isolated phycobilisome particles show that they are functionally intact in transducing radiant energy. The kind of phycobiliprotein present in phycobilisome depends upon the source organism, but in any case they are quantitatively the principal component, devoid of chlorophylls. In cyanobacteria, C-phycocyanin (A_max 620 nm) and allophycocyanin (A_max 650 nm) are universally found, with allophycocyanin B (A_max 670 nm) detectable in many species. Cyanobacterial phycobilisomes also contain several polypeptides in addition to phycobiliproteins. These non-pigmented polypeptides are integral components of the particle, though they are quantitatively minor.

Electron microscopy of cyanobacterial phycobilisomes has shown that they have two morphologically different substructures when seen in "face-view". In one type there is a
core of two contiguous objects appearing disc-like in the face view projection, from which rods radiate in a hemispherical array of up to six, composed of a few stacked discs. In the other type, the core consists of three contiguous elements arrayed in an equilateral triangle with the arrangement of the rods quite similar to the first type.

Isolation and Characterization of Phycobilisomes

The aim of the present study, therefore was to characterize the supramolecular organization of the biliproteins in the phycobilisomes of *Spirulina platensis*.

The study showed that phycobilisomes can be isolated from *Spirulina platensis* using CTAB, a cationic detergent. It is an improvement over the original procedure and its modifications (Gantt and Lipschultz, 1972; Gray and Gantt, 1975; Koller et al., 1977; Ley et al., 1977; Tandeau de Marsac and Cohen-Bozire, 1977; Searle et al., 1978). The main criterion used in assessing well coupled phycobilisome is the fluorescence emission at 678 at 20°C.

Conditions suitable for cell disruption, solubilization of membrane and subsequent phycobilisome fractionation were
determined empirically for *Spirulina platensis*. The effectiveness of the two detergents was tested under identical conditions. The extent of release of phycobilisomes from the thylakoid membranes was measured by absorption of phycocyanin at 615 nm in the soluble fraction of the homogenate, $S_{35}$ (Fig. 2). Results indicated that phycobilisomes released by CTAB treatment were almost free from chlorophyll $a$ as no absorption was detected at 440 and 675 nm. However, small amount of chlorophyll $a$ was invariably observed in Triton X-100 treated homogenate ($S_{35}$). Phycobilisomes were further purified on sucrose density gradient and then their structural and functional characterization were done. Similarity was observed with respect to absorbance (Fig. 3) and fluorescence characteristics of the phycobilisomes (Fig. 5). Phycocyanin represents the major peak with absorption at 618 nm and allophycocyanin with a shoulder at 650 nm. The 678 nm emission of intact phycobilisomes at 20°C arises from far-red-emitting APC forms. One of these, designated as APC-B, was isolated from *Spirulina* in the later part of study. The polypeptide composition of the phycobilisomes isolated by either method was studied using SDS-polyacrylamide gel electrophoresis. Similarity in the mobility of polypeptides in SDS-polyacrylamide gel electrophoresis were also observed (Fig. 10). Phycobilisome is exclusively made up of proteins. SDS-polyacrylamide gel electrophoresis of phycobilisomes showed the presence of
biliproteins (MW 15-20 kD) which were only partly resolved in gel electrophoresis. Phycobiliproteins are made up of $\alpha$ and $\beta$-subunits of the biliproteins, which are identified by their intrinsic colour before staining. Polypeptides of higher molecular weights (70, 38, 30, 26 and 24 kD) were visible on the gel only after staining with Coomassie brilliant blue. These colourless polypeptides accounted for approximately 15 per cent of the total stainable material on the gel. These polypeptides are responsible for the assembly of the biliproteins into disc-shaped aggregates, that serve as the building blocks of the phycobilisome and for the inter-discs interactions (Glazer and Hixon, 1977; Lundell et al., 1981; Yu et al., 1981; Lundell and Glazer, 1983b,c).

New procedures were devised for the isolation and purification of phycobilisome from _Spirulina platensis_. Partial lysis of the cells and concomitant release of the phycobilisomes from the thylakoids, were obtained by incubating the cells with 0.01 per cent CTAB for 40 seconds. The harvested cells suspended in 0.75 M K-Phosphate buffer, pH 7.0, were subjected to complete lysis by physical disruptive method using ultrasonic disintegrator. The released phycobilisomes were purified by using sucrose density gradient centrifugation. In previous isolation procedures (Gantt and Lipschultz, 1972; Gray and Gantt
1975; Koller et al., 1977; Ley et al., 1977; Tandeau De Marsac and Cohen-Bizare, 1977; Searle et al., 1978) the disrupted cells were incubated with Triton X-100 at 20°C for 45 minutes to one hour. Incubation at 20°C for such a long period may result in proteolysis of phycobiliproteins and colourless polypeptides. This can be easily avoided by using CTAB method. The purity, homogeneity and integrity of isolated phycobilisomes, using CTAB method, were comparable to Triton X-100 method. The purity of the fractions is shown by the absorption and fluorescence emission spectra and SDS-polyacrylamide gel electrophoresis. The homogeneity of isolated phycobilisomes is determined by a uniform migration behaviour in sucrose density gradient centrifugations. The structural integrity of the phycobilisomes is confirmed by a functional test, energy transfer ability after excitation with 580 nm light. Thus the present procedure has distinct advantages over the existing method: (1) it requires cell incubation with CTAB for only 40 seconds compared to 40-60 minutes in the Triton X-100 procedure, (2) it prevents the chances of proteolysis of both the phycobiliproteins and colourless polypeptides, (3) it requires an extremely low amount of cationic detergent, CTAB, almost 100 times less than normally used Triton X-100. It has been shown that high concentration of detergent affects the spectroscopic property of phycobiliproteins (Glazer and Hixon, 1975), and (4) it reduces the contamination with thylakoids.
(Chlorophyll \textsubscript{a}) in the early stage of isolation i.e. at $S_{35}$ stage.

The isolation procedure described here also allowed us to isolate structurally intact and functionally active phycobilisomes from \textit{Nostoc muscorum} which grows on low salt (data not presented). Inclusion of salts in the isolation medium (i.e. 1 per cent NaCl in the culture medium before treatment with CTAB) is advisable for those cyanobacteria normally grown on low salts, viz., \textit{Nostoc}, \textit{Anabaena}, \textit{Anacystis} etc. High ionic strength possibly facilitates the detachment of phycobilisome from membrane, by inducing changes in membrane surface charges.

Electron microscopic studies revealed that the overall appearance of phycobilisomes isolated from \textit{Spirulina platensis} is very similar to the phycobilisomes reported from other cyanobacteria (Bryant \textit{et al.}, 1979; Glazer \textit{et al.}, 1979). It has two distinct components - a central triangular core of discs and peripheral arms composed of stacks of discs. The principal variation in phycobilisomes, from the cyanobacteria examined thus far, is a difference in the number of discs in the peripheral arms. The number of discs in the core have not been clearly established for all species. In the model proposed by Bryant \textit{et al.} (1979), the core contains six discs. Only three discs are
apparent in the *Spirulina platensis* phycobilisome shown here. However, this does not preclude the occurrence of six discs. These phycobilisomes were oriented in face-view, and it was not possible to determine the number of layers of discs or the thickness of the discs. Glazer *et al.* (1979) observed phycobilisomes from a strain of *Synechococcus* in which only two discs were visible in the core.

Fluorescence excitation polarization spectra of intact phycobilisomes (Fig. 12), measuring the emission through APC at 678 nm, showed that there was essentially complete depolarization of the excitation beam. Fluorescence polarization determinations of phycobilisomes and their constituent phycobiliproteins (Fig. 22 and 28), showed that phycobilisomes are energetically effective macromolecular structures. Energy migration within the phycobilisome, from PC to APC (the longest wavelength absorbing and emitting phycobiliprotein) was indicated by predominant APC fluorescence emission, which was independent of the phycobiliproteins excited. The high efficiency of the energy migration inside the phycobilisome was reflected by the low polarized fluorescence. Excitation of phycobilisomes in the region of major absorption (500-650 nm) resulted in a degree of fluorescence polarization value between +0.03 and -0.01, whereas these values were 2 to 15 times greater in isolated
phycobiliproteins. This is in agreement with in vitro aggregation studies on PC carried out by Goedheer and Birnie, (1965) and Vernotte (1971). They showed that higher aggregation states exhibited the lowest polarized fluorescence. The low value of fluorescence polarization in intact phycobilisomes has been interpreted to represent efficient energy migration in phycobilisomes. The fluorescence excitation spectra of phycobilisomes at 20°C, Fig. 7, shows the efficient energy transfer among the biliproteins. The excitation spectrum is almost identical with the absorption spectrum, with the exception of a small shoulder at about 600 nm. Excitation peak at 625 nm is due to phycocyanin, and another peak at 650 nm is due to allophycocyanins. The shoulder at 600 nm is due to the presence of biliviolinoid chromophore in phycobilisomes, which has recently been identified from few cyanobacteria (Bishop et al., 1987). In the absorption spectra of phycobilisomes at 20°C, the biliviolinoid chromophore was not resolved. In the latter part of this study the presence of biliviolinoid chromophore in the phycocyanin has been confirmed. The presence of biliviolinoid chromophore was further confirmed by absorption spectra at liquid nitrogen temperature (Fig. 4). Thus energy absorbed primarily by phycocyanin was effectively transferred to allophycocyanin, and all the pigments were contributing to the emission at approximately 678 nm.
The circular dichroism spectra of intact phycobilisomes, gave us more detailed information about the phycobilisomes (Fig. 13). Intact phycobilisome showed two positive CD bands: 655 nm attributed to allophycocyanin and 626 nm arising from phycocyanin. One small, negative, broad peak at 345 nm was due to prosthetic group of the biliproteins.

The CD band corresponding to the phycocyanobilin chromophores is displaced significantly to longer wavelength (\( \lambda_{\text{max}} \) at 626 nm) relative to the absorption band (\( \lambda_{\text{max}} \) at 618 nm). The red shift, and enhancement of the CD band relative to the absorption band, arises because of conversion of monomer to higher aggregation state. The higher aggregation states result in strong interaction of the phycocyanobilin chromophores of \( \alpha \beta \) monomer with those of another in the aggregate (Glazer et al., 1973). The second CD band at 655 nm is due to the allophycocyanin and its presence indicates the interaction of phycocyanin with allophycocyanin.

The spectroscopic data on intact phycobilisomes, support the view that phycocyanin is present in the \( (\alpha\beta)_6 \) form in these particles. The CD spectrum of intact phycobilisome and of hexameric phycocyanin are almost similar to each other (Glazer et al., 1973).
The CD spectra of dissociated phycobilisomes is shown in Fig. 13. The difference in the intact and dissociated phycobilisomes were observed by CD spectra. The absorption spectrum of dissociated phycobilisome showed a red shift of 3 nm since the $A_{\text{max}}$ shifted from 618 nm in undissociated phycobilisome to 615 nm in dissociated phycobilisomes. But, the long wavelength, positive CD band, in the CD spectrum was altered. A new CD band at 568 nm appeared, upon dissociation of phycobilisomes. The loss of 655 nm CD band was also observed. The changes in CD spectra of the intact and dissociated phycobilisomes, can be attributed to variation in the aggregation of phycobiliproteins. The CD spectrum of dissociated phycobilisomes exhibits a molecular ellipticity at 615 nm, which is evidently characteristic of the monomer of the phycocyanin. The CD band of intact phycobilisomes, in the region of 626 nm reflects the proportions of higher aggregates of phycocyanin (Glazer et al., 1973). The interactions among the subunits of the phycocyanins, affect the interaction of phycocyanin with allophycocyanin. The complete loss of 655 nm CD band of allophycocyanin confirms this data. The appearance of new band at 568 nm is due to the biliviolinoid chromophore of the phycocyanin. It shows its molecular ellipticity in monomeric conditions. The biological function of phycocyanin is to act as an energy transducer, transmitting the absorbed light energy to
allophycocyanin and thence to chlorophyll a. It is pertinent to consider, the spectroscopic changes associated with the aggregation of the monomer to the hexamer, in relation to this function. Considerable evidence is available in support of the view that the hexamer form \((\alpha \beta)_6\) is an intermediate in phycobilisome assembly (Berns and Edwards, 1965; Berns, 1971; Edwards and Gantt, 1971). Hexameric form \((\alpha \beta)_6\) absorbs energy more efficiently and has greater spectral overlap with the acceptor. Further, the CD changes associated with hexamer formation, indicate intermolecular interaction between the chromophores. It is possible that, this geometry of the hexamer could have been designed, to orient the chromophores with respect to those in allophycocyanin, which results in high efficiency of energy transfer.

Fluorescence emission spectrum of time course dissociation of phycobilisomes is shown in Fig. 14. In intact phycobilisomes, light absorbed by phycocyanin is funnelled to allophycocyanin, with a predominating fluorescence emission at 678 nm. Suspension of intact phycobilisomes in lower concentration phosphate buffer (200 mM K-phosphate buffer, pH 7.0) results in an uncoupling of phycobiliproteins from the energy transfer. Uncoupling, as measured by fluorescence, results in a decrease in energy transfer among the pigments, with
a diminution of fluorescence peak at 678 nm and an increase in the relative fluorescence emission peaks of individual pigments (645 nm phycocyanin and 660 nm allophycocyanin). Uncoupling of phycobilisomes can occur between two or more pigments, and rate being affected by the susceptibility of the pigments to lowered ionic strength. Examination of fluorescence emission of dissociated phycobilisomes has revealed multiple peaks, which suggests uncoupling, at one or several points. The following emission forms and uncoupling points can be noted

\[
\begin{align*}
PC & \rightarrow PC \rightarrow APC \rightarrow APC-B \\
& a \rightarrow b \rightarrow c \\
642 & \rightarrow 648 \rightarrow 664 \rightarrow 678
\end{align*}
\]

The dissociation pattern is characterized by the initial shift in emission from 678 to 664 nm which represents the uncoupling of APC-B. This uncoupling is observed within 10 minutes. By two hours of incubation in low salt concentration, a substantial uncoupling had occurred, as suggested by the appearance of shoulder at 648 nm and an increased peak at 642 nm of phycocyanin emission. This sequence of dissociation when carried out in low concentration phosphate buffer, revealed that the uncoupling of c occurred first, followed by b and lastly a. The 678 nm emission, designated as APC-B has been isolated from the \textit{S. platensis}. The interpretation of the dissociation pattern
is partly based on the analysis of *Porphyridium cruentum* phycobilisomes (Gantt *et al.*, 1976), which revealed a sequential physical release of phycobiliprotein, with concomitant uncoupling as confirmed through fluorescence emission.

The effect of denaturing agent on phycobilisomes was studied with different concentrations of urea. The result as shown in Fig. 15, indicates that the ratio of $A_{\text{visible}}/A_{\text{near UV}}$ of the oscillator strength was $>4$ for native phycobilisomes in 0.75 M K-phosphate buffer, pH 7.0, and this ratio decreased to 0.6 in presence of 6 M urea. With the increase in the molarity of urea, the visible bands declined steadily and there was a corresponding hyperchromicity in the near-ultraviolet absorbance.

Quantum chemical calculations and model-compound data on tetrapyrroles, suggest that the ratio of the oscillator strengths in the visible to near-ultraviolet absorption bands, is indicative of their degree of extension (Burke *et al.*, 1972; Blauer and Wagniere, 1975; Chae and Song, 1975; Suzuki *et al.*, 1975; Scheer, 1981). Higher the ratio, more extended are the chromophores. For phycobilisomes, the visible band is decreased, and the near-ultraviolet band becomes hyperchromic, as the protein is denatured. Our data shows that phycocyanobilins become more cyclic as the protein is denatured, and the bilins
can presumably assume a more stable conformation. It is known that the function of the polypeptides in the native biliprotein, is to hold these chromophores in an extended conformation, in which their light-harvesting and excitation energy transfer abilities are maximized.

The effects of a cationic detergent, CTAB (1 percent) were studied in vitro. The fluorescence emission spectra is shown in Fig. 16. When phycobilisomes were excited at 580 nm, an increase in fluorescence intensity and shift of fluorescence emission peak, towards shorter wavelength, were observed with time. The increase in the fluorescence intensity may be interpreted as change in the conformation of biliproteins, leading to exposure of more number of buried chromophores. The shift of the peak from 678 to 646 nm indicates the loss of interaction between phycocyanin and allophycocyanin.

**Characterization of PC Subcomplexes**

One of the objective of the present study was to develop a procedure for the isolation of well-defined complexes of phycocyanin and linker polypeptides and to examine their spectroscopic properties and assembly of various components in such complexes. To isolate substructures of the phycobilisomes,
these particles were dissociated by transferring them into 0.01 M K-phosphate buffer followed by column chromatography on hydroxylapatite. Unbound phycocyanin, characterized by absorption property (Fig. 17a), was fractionated on a linear sucrose density gradient. Three coloured bands were resolved (Fig. 18) on sucrose density gradient and were further characterized by spectrophotometric and SDS-polyacrylamide gel electrophoresis.

Electron microscopic study of in vitro rod assembly in *Synechococcus* 6301 indicates that trimer \((\alpha\beta)_3\)-X is present in rod assembly (Lundell *et al.*, 1981), where \(\alpha\) represents the phycocyanin monomer and X is the linker polypeptides. The three complexes isolated by sucrose density gradient indicate the three aggregation states of the rod assembly. Similarities in the basic constituents of chromophores were observed by measuring absorption and fluorescence emission spectra of the phycocyanin complexes at liquid nitrogen temperature. The difference in the aggregation state of the complexes were analysed by absorption and fluorescence spectra at room temperature, CD spectra and SDS-polyacrylamide gel electrophoresis. At room temperature, the PC I and PC II showed similar absorption spectra \((A_{\text{max}} = 618 \ \text{nm})\), while PC III showed one shoulder at 600 nm (Fig. 19a). The similarity in the absorption spectra of PC I and PC II was interpreted as almost similar aggregation state of these
complexes. The fluorescence emission study of PC I at room temperature indicates maximum red-shift with $F_{\lambda_{\text{max}}}$ at 652 nm. PC II and PC III showed fluorescence emission at 644 and 640 nm respectively. Fluorescence property of PC II complexes matches with PC trimer from *Anabaena variabilis* (Isono and Katoh, 1983). SDS-polyacrylamide gel electrophoresis showed the association of 30 and 26 kD linker polypeptides with the biliproteins. PC II and PC I were free from colourless polypeptide.

Studies on phycocyanin aggregation indicate that purified phycocyanin may exist in an equilibrium between monomer, trimer and hexamer forms. The particular species present and their proportion have been shown to vary with the pH and ionic strength.

Our result indicates that 30 and 26 kD polypeptides form a part of rod sub-structure and are absent from the core component. They play a role in rod subassembly. In PC I and PC II, the biliproteins are in trimeric form, which serve as a template for the formation of $(\alpha\beta)_6$ X complex. The result is supported by *in vitro* experimental result of assembly process by Yu and Glazer (1982), Lundell *et al.* (1981) and Gingrich *et al.* (1981). The increased $F_{\lambda_{\text{max}}}$ of PC I is due to the interaction of linker polypeptides with biliproteins. The PC III is the monomeric form of phycocyanin. The differences in the monomeric
form (PC III) and trimeric form (PC II) were established with CD spectra and fluorescence excitation polarization spectra. The CD spectra of PC III differs from PC II, because of the presence of CD band at 568 nm in the former case. The appearance of new band at 568 nm is due to the biliviolinoid chromophore of the phycocyanin. It shows its molecular ellipticity in monomeric condition. The monomeric condition is further indicated by the increased fluorescence excitation polarization value of PC III (Fig. 22). The lower polarization of PC II in comparison to PC III suggests a greater amount of aggregation. The increase in polarization for phycocyanin is due to dissociation to monomer (MacColl et al., 1971). Sedimentation coefficient study is required to know further the aggregation state accurately.

Thus, the study of PC complexes indicates that pure biliproteins differ from their assembled form within the intact phycobilisome, with respect to their aggregation state and conformation. Consequently, their spectroscopic properties such as circular dichroism, absorption spectra, fluorescence spectra are altered to some degree. Relative to pure biliproteins, under the same conditions, biliprotein-linker polypeptide complexes show red shift in their fluorescence emission spectra.

Fluorescence excitation polarization spectra of all the sub-complexes (Fig. 21) showed that the polarization value is not
constant throughout the absorption band. The complex polarization spectra of the native phycobiliproteins indicate that in the macromolecules different chromophore types are present, those that absorb at shorter wavelength ('s' type) sensitizing those that absorb and emit ('f' type) at longer wavelength. The relative orientation of the 's' and 'f' chromophores and their relative absorption intensities determine the detailed shape of the polarization spectrum. The coincidence of excitation and absorption spectra throughout the visible-absorption range (Fig. 20a and 21) indicates that energy transfer from 's' to 'f' type is very efficient, probably as a consequence of close proximity between donor and acceptor chromophores.

Characterization of APC Sub-complexes

A freshly isolated pool of allophycocyanin, separated from phycocyanin by hydroxylapatite chromatography, fractionates into two bands on linear sucrose density gradient in 0.3 M K-phosphate, pH 7.0 (Fig. 25). These are designated as APC I (15-20%) and APC II (80-85%). The spectrum of APC I shows absorption maximum at 654 nm. Allophycocyanin II has a similar absorption maximum at 653 nm but its relative absorption at 615 nm is more than that of APC I. The fluorescence emission maxima of both the sub-particles are at 664 nm.
By comparing the entire APC population with an estimated 95-100% recovery from dissociated *S. platensis* phycobilisomes, it appears that considerable heterogeneity exists in APC in vitro. Variations within the major biliprotein class are not unprecedented, as has been shown with phycoerythrin (Bennett and Bogorad, 1971; Gantt and Lipschultz, 1974) and phycocyanin (Berns, 1971; Brown et al., 1975; Kao et al., 1975; Scott and Berns, 1965). However, these variations seem to involve the aggregation state at the level of trimers to hexamers. The variations in size among the APC forms reported here are considerably less and differences are more subtle.

Fluorescence polarization spectra of the APC forms (Fig. 28) show that polarization value was not constant across the visible absorption band. Since fluorescence excitation spectra of these proteins coincide with their absorption spectra, the bilins that absorb at shorter wavelength must transfer their excitation energy to others. The shape of polarization spectra indicates the presence of sensitizing "s" and fluorescing "f" chromophores in both the allophycocyanin complexes. SDS-polyacrylamide gel electrophoresis of the complexes (Fig. 29) showed the association of 70 kD polypeptide with APCI. The fast migration of APCI on linear sucrose density gradient is explained on the basis of linker polypeptide association with APCI. The
little difference in the absorption property may be due to association with the linker polypeptide. The location of APCI may be close to the thylakoid where 70 kD polypeptide may play an important role in attachment of phycobilisome to thylakoid.

**Isolation and Characterization of APC-B**

The distinctive chromatographic properties, visible absorption spectrum and fluorescence emission spectrum clearly show that allophycocyanin is hitherto differ from APCI and APCII, described earlier. APC-B was not resolved during sub-particle isolation of allophycocyanin using linear sucrose density gradient centrifugation. The reason may be the presence of APC-B in extremely low amount. It is suggested that APC-B occupies a position between allophycocyanin and chlorophyll a in the energy transfer path from accessory pigments to species of chlorophyll a with absorption maxima at 673 nm. Suggestive evidence comes from examination of the fluorescence emission properties of phycobilisomes. The maximum fluorescence emission peak of *Spirulina* phycobilisome lies at 678 nm. In contrast, the fluorescence emission peak of purified allophycocyanin lies at 660 nm. These observations taken in conjugation with the present study, suggest that the 678 nm fluorescence originate from allophycocyanin-B. Fluorescence excitation polarization spectrum
indicated that APC-B contain 'sensitizing' and 'fluorescing' chromophore.

**Characterization of PC Subunits**

The present investigations have shown that phycocyanin of the *Spirulina platensis*, is composed of two dissimilar polypeptide subunits. The \( \alpha \) and \( \beta \)-subunits of phycocyanin were separated by ion-exchange chromatography on Bio-Rex-70. Peak area in the elution profile and protein measurement indicates the subunits ratio of \( \alpha \) to \( \beta \) subunit 1:2 indicating that these two subunits are not present in equal amount. The quantitative absorption spectra of phycocyanobilin in 8 M urea at pH 3.3 have been determined (Glazer and Fang, 1973; Glazer and Hixon, 1975). Under these conditions, the influence of protein conformation on the absorption properties of the chromophores is abolished and both protein-bound and free phycocyanobilin exhibit absorption maxima at 662 nm. Quantitative absorption spectra determined for the two subunits indicate that ratio of chromophores was 1:2 in the \( \alpha \) and \( \beta \)-subunits respectively. The similarity in the absorption pattern of \( \alpha \) and \( \beta \)-subunits (\( \lambda_{\text{max}} \) at 662 nm) indicates the presence of similar kind of chromophore. Upon refolding of the protein the absorbance in the wavelength range of 500-620 nm increases and the shape becomes more assymmetrical. The shape of the \( \beta \) subunit is similar to phycocyanin, however,
the $\alpha$-subunit differs from these two since in the $\alpha$-subunit the absorbance peak is split into two components. We attribute this splitting of $\alpha$-subunit to the fact that the two chromophores absorb at different energy because of slightly different configurations of the protein environment and/or some change in chromophore conformation. The absorption spectra of the $\alpha$-subunit matches with the absorption spectra of biliviolinoid chromophore (PXB) reported from the *Anabaena variabilis* (Bishop et al., 1987). The $\alpha$-sub-unit of PC carries a biliviolinoid chromophores (PXB) of unknown structure, which exhibits a distinctive visible absorption maximum at 595 nm. The subunit of PC carries two phycocyanobilin chromophores. The fluorescence emission spectra of phycocyanin and both subunits are nearly identical. The polarized excitation spectra show a plateau with nearly constant degree of polarization beyond 500 nm upto 590 nm and a sharp rise from 590 to 630 nm. The plateau results from excitation of "s" chromophore whereas the rise is due to increasing direct excitation of "f" chromophore. The increase of polarization of the $\alpha$-subunit coincides with the long-wavelength absorption maximum. Similar results were obtained with monomeric C-phycocyanin (Vernotte, 1971; MacColl et al., 1971). The polarization is highest for the subunit which has the lowest number of chromophores and is lowest for phycocyanin which has the highest number of chromophores.
Characterization of APC Subunits

APC subunits were purified in the similar fashion as PC subunits by using Bio-Rex-70, an ion exchanger. The coloured $\alpha$ and $\beta$-subunits were indistinguishable with regard to difference in charge in Bio-Rex column. The subunits displayed absorption maxima at 662 and 353 nm in 8 M urea. The ratio of the absorption of phycocyanobilin at 662 and 353 nm was approximately 1.0. This indicates that chromophore-protein interaction had been completely disrupted in the separated subunits in 8 M urea. The total area under the 660 nm peak of $\alpha$-subunit and the $\beta$-subunit were in the ratio of 2:3. It indicates that the chromophore ratio of $\alpha$ and $\beta$-subunit is 2:3. The absorption spectra of the APC upon refolding shows slightly different pattern. Since phycocyanobilin is the only chromophore in the phycocyanin and allophycocyanin, as shown in the absorption spectra in 8 M urea, the difference in the absorption spectra of the allophycocyanin subunits may be due to different environment of polypeptide surrounding the chromophore. Fluorescence emission spectra of the $\alpha$ and $\beta$-subunits show nearly similar pattern. The two absorption bands are consistent with fluorescence excitation polarization spectra of $\alpha$ and $\beta$-subunits of allophycocyanin which indicates the presence of sensitizing "s" and fluorescing "f" chromophore in each of the subunit. Thus the native allophycocyanin is the aggregate of combination of sensitizing
and fluorescing chromophore which is required for the efficient energy transfer within the phycobilisomes.

**Trypsin Digestion Study**

This type of study has been done with the aim that analysis of the product of limited trypsin digestion of *Spirulina* phycobilisome could give information on the organization of the complexes within the phycobilisomes. The time course of trypsin digestion and its analysis on SDS-polyacrylamide gel electrophoresis indicates that 70, 38 and 24 kD linker polypeptides are susceptible to trypsin digestion. 38 kD linker polypeptide is cleaved within one minute of digestion. 70 and 24 kD linker polypeptides are less susceptible. The role of 70 and 24 kD polypeptides in the attachment of phycocyanin to allophycocyanin can be envisaged, since the partial cleavage of these polypeptides (10 minutes) leads to partial detachment of the phycocyanins to allophycocyanins as shown by the sucrose density gradient centrifugation and analysis of the isolated complexes by absorption and fluorescence emission spectra. The time course emission spectra also confirms it. Complete loss of interaction among the phycocyanin and allophycocyanin takes place in two hours as revealed by time course fluorescence emission spectra. Since in previous result (characterization of the APC)
the 70 kD polypeptide has been shown to be the component of the core, the 24 kD polypeptide seems to be more important in attachment of rods to core. The cleaving of 38 kD polypeptide within a minute may be interpreted as high accessibility to trypsin. Further work is required to know the detail mechanism of the assembly.

**Dynamics of Phycobilisomes**

Phycobiliproteins which make up a major portion of the soluble cell protein in cyanobacteria (Myers and Kratz, 1955), are not required in the photochemical reactions of photosynthesis (Arnon et al., 1974). Allen and Smith (1969) showed that cellular levels of phycocyanin in the unicellular cyanobacterium *Synechococcus* sp. 6301 decreased markedly when cells were deprived of a source of fixed nitrogen. Later, Lau et al. (1977) showed that the loss of phycocyanin absorbance (usually used as an assay for phycocyanin degradation) is accompanied by a loss of the polypeptide components on SDS-polyacrylamide gels which correspond to the α and β subunits of this protein. On pulse labelling with $^{14}$C-leucine under nitrogen starvation conditions, active incorporation of amino-acid into cell protein was demonstrated, but no incorporation of radioactivity into phycocyanin could be shown (Lau et al., 1977). It appears,
therefore, that during "nitrogen chlorosis" pre-existing phycocyanin is degraded and the synthesis of new phycocyanin is repressed.

The phycobilisome plays a dual role in photosynthesis and cellular nitrogen storage. Such a role is reasonable when one considers the abundance of cellular nitrogen contained in phycobilisomes, and also the low priority for light harvesting by an accessory pigment system during nutrient starvation.

The changes brought about during the first stage of nitrogen starvation in the physiological and spectroscopic properties of phycobilisomes show a preferential loss of phycocyanin from the structure. The phycobilisomes from partially starved cells sediment at a slower velocity (Fig. 44) and higher allophycocyanin to phycocyanin ratio. The suggested models for the location of the individual phycobiliproteins within membrane-bound phycobilisome (Gantt, 1975; Gantt et al., 1976; Morschel et al., 1977) place phycocyanin in a position peripheral to allophycocyanin and allophycocyanin-B. The known pathway of the transfer of absorbed light energy is Phycocyanin $\rightarrow$ allophycocyanin $\rightarrow$ allophycocyanin-B $\rightarrow$ chlorophyll a (Lau et al., 1977; Glazer, 1977). Consequently, partial loss of phycocyanin need not lead to a decrease in the effectiveness of
the residual structure in energy transfer. This view is supported by the fluorescence emission data, which show sufficient energy coupling between phycocyanin and allophycocyanin-B in phycobilisomes from nitrogen starved cells (Fig. 45b).

Only one colourless polypeptide of 24 kD is lost during one day of nitrogen starvation (Fig. 46). The loss fairly coincides with change in absorption and fluorescence spectra.

Our previous results suggest that the 26 kD polypeptide is associated with phycocyanin in the phycobilisome. This polypeptide may be associated with the peripheral phycocyanin, since the remaining particle from which it is absent shows unimpaired energy transfer (Fig. 45b).

During later stage of nitrogen starvation, e.g. 3 days (Fig. 46), two other polypeptides of 70 and 24 kD are lost. It is at this stage that fluorescence emission of phycobilisome shows a small but distinct shift to shorter wavelength and the structure becomes less stable. Allen and Smith (1969) had shown that the viable cell count of a nitrogen starved culture of Anacystis nidulans reached a minimum around the time when phycocyanin was no longer detectable.
The enzymes responsible for phycobilisome turnover during nitrogen starvation have not yet been adequately identified, nor is the signal which triggers this turnover understood. Several groups have reported phycocyanin-degrading activities in cyanobacteria but in no instance has the enzyme (or enzymes) responsible, been isolated and characterized (Lau et al., 1977; Foulds and Carr, 1977).

Our results show that during the breakdown of phycobilisome, degradation of both phycobiliproteins and non-pigmented polypeptides takes place. The protein present in the cell of Spirulina shows strict specificity for phycobiliproteins. Moreover, phycocyanin is degraded more rapidly than allophycocyanin. This shows that significant kinetic differences exist in the rate of proteolytic degradation of different phycobiliprotein within the cell during metabolic stress.
SUMMARY

A new procedure was devised for the isolation and purification of phycobilisome from *Spirulina platensis*. Partial lysis of cyanobacterium, *Spirulina platensis* and concomitant release of the phycobilisomes from the thylakoids were obtained by incubating the cells with 0.01 percent CTAB for 40 seconds. The released phycobilisomes were purified by using sucrose density gradient centrifugation. The purity, homogeneity and integrity of isolated phycobilisomes by using CTAB method were comparable with Triton X-100 method. The purity of the fraction was confirmed by the absorption and fluorescence emission spectra and SDS-polyacrylamide gel electrophoresis. The homogeneity of isolated phycobilisomes was determined by a uniform migration behaviour in sucrose density gradient during ultracentrifugation. The structural integrity of the phycobilisomes is confirmed by their energy transfer ability after excitation with 580 nm light. Thus the present procedure has distinct advantages over the existing method in that (1) it requires cell incubation with CTAB for only 40 seconds compared to 40-60 minutes incubation period with Triton X-100 needed in the conventional procedures. (2) It prevents the chances of proteolysis of both the phycobiliproteins and colourless polypeptides. (3) It requires an
extremely low amount of cationic detergent, CTAB, almost 100 times less than normally used Triton X-100. It has been shown that high concentration of detergent affect the spectroscopic property of phycobiliproteins. It reduces the contamination with thylakoids (chlorophyll a) at early stage of isolation.

In the absorption spectrum phycocyanin represents the major peak with absorption at 615 nm. The 678 nm fluorescence emission of intact phycobilisomes at 20°C arises from far-red emitting APC forms. The results indicate that phycobilisome is exclusively comprised of proteins. SDS-polyacrylamide gel electrophoresis of phycobilisomes showed the presence of biliproteins (MW 15 - 20 kD), identified by their intrinsic colour before staining. Polypeptides of higher molecular weight (70, 38, 30, 26 and 24 kD) were visible on the gel only after staining the gel with coomassie blue. These colourless polypeptides accounted for approximately 15 percent of the total stainable material on the gel. These polypeptides are responsible for the assembly of the biliproteins into the disc-shaped aggregates that serve as the building blocks of the phycobilisome and for the interaction between these discs.

Electron microscopic studies revealed that the overall appearance of phycobilisome isolated from Spirulina platensis is
very similar to the phycobilisomes reported from other cyanobacteria. They consist of two distinct components - a central triangular core of discs and peripheral arms composed of stacks of discs.

Fluorescence excitation polarization spectra of intact phycobilisomes measuring the emission through APC at 678 nm showed that there was essentially complete depolarization of the excitation beam. It indicates that phycobilisomes are energetically effective macromolecular structures. Energy migration within the phycobilisome to allophycocyanin, the longest wavelength absorbing and emitting phycobiliprotein was indicated by predominant allophycocyanin fluorescence emission which was independent of the phycobiliproteins excited. The high efficiency of the energy migration inside the phycobilisome was reflected by the low polarized fluorescence.

The excitation spectrum of phycobilisomes is almost identical to the absorption spectra, with the exception of the small shoulder at about 600 nm. Excitation peak at 625 nm is due to phycocyanin and other peak at 650 nm is due to allophycocyanins. The shoulder at 600 nm may be due to the involvement of biliviolinoid chromophore in the phycobilisome which has recently been identified from few cyanobacteria.
The CD spectra of intact phycobilisomes showed two positive CD bands, 655 nm attributed to allophycocyanin and 626 nm arising from phycocyanin. One small negative broad peak at 346 nm was due to prosthetic group of the biliproteins. The difference in the intact and dissociated phycobilisomes was observed by CD spectra. A new CD band at 568 nm appeared upon dissociation of phycobilisomes. The loss of 656 nm band of circular dichroism was also observed. The changes in CD spectra of dissociated phycobilisome, in comparison to that of intact phycobilisome, can be attributed to change in the aggregation state of phycobiliproteins.

Fluorescence emission spectra of the time course dissociation of phycobilisome results in an uncoupling of phycobiliproteins from the energy transfer. Uncoupling, as measured by fluorescence, results in a decrease in energy transfer among the pigments with diminution of fluorescence peak at 678 nm and an increase in the relative fluorescence emission peaks of individual pigments.

The effect of denaturing agent like different concentration of urea indicates that phycocyanobilins become more cyclic as the protein is denatured and the bilins can presumably assume a more stable conformation. It is known that
the function of the polypeptides in the native biliprotein is to hold these chromophores in an extended conformation in which their light-harvesting and excitation energy transfer abilities are maximized.

Different components of the phycobilisomes were purified using column chromatography. Phycocyanin and allophycocyanins were separated using hydroxylapatite column. The isolated PC were further fractionated into three complexes using linear sucrose density gradient centrifugation. The three complexes of phycocyanins differ in spectroscopic properties due to aggregation state and association of linker polypeptides as revealed by fluorescence emission and CD spectra. The fluorescence excitation polarization spectra of all the sub-particles showed that the polarization is not constant throughout their absorption band. The complex polarization spectra of the native phycobiliproteins indicate that in the macromolecule different chromophore types are present. Those chromophores which absorb at shorter wavelength ('s' type) sensitizing and those that absorb and emit ('f' type) fluorescing at longer wavelength. The coincidence of the excitation and absorption spectra throughout the visible absorption spectral range means that energy transfer from 's' to 'f' type is very efficient, probably as a consequence of close proximity between donor and acceptor chromophores.
Two allophycocyanin complexes were also isolated using in similar procedure as for phycocyanin. The two APC differ in their spectroscopic properties. SDS polyacrylamide gel electrophoresis of complexes indicated the association of 70 kD polypeptide with APCI. Fluorescence polarization of the two complexes was not constant throughout the absorption bands.

Another species of allophycocyanin was isolated by using chromatographic procedure called APC-B. This species is present in extremely low amount. Fluorescence emission spectra of APC-B showed emission peak at 678 nm, which is the absorption maximum of the chlorophyll.

The subunits of phycocyanins were separated by ion exchange chromatography on Bio-Rex 70. Peak area in the elution profile and protein measurements showed the subunit ratio of subunits 1:2 indicating that these two subunits are not present in equal amount. Quantitative absorption spectra determined for the two subunits indicated that ratio of chromophores was 1:2 in subunits. In 8 M urea both the subunits showed similar absorption maxima at 662 nm. On refolding of protein, the absorbance in the wavelength range of 500-620 nm increases and its shape becomes more assymetrical. The shape of the subunit absorption is similar to that of
native phycocyanine, however, the subunits differ from subunits. Fluorescence excitation polarization indicates the presence of 's' and 'f' types of chromophores in both the subunits.

APC subunits were also purified using Bio-Rex column. Spectroscopic properties of the subunits were studied in detail. The two absorption bands are consistent with fluorescence excitation polarization spectra of the subunits of allophycocyanin, which indicate the presence of sensitizing 's' and fluorescing 'f' chromophore in each of the subunit.

Quaternary interaction in the Spirulina phycobilisome is revealed by partial tryptic digestion. The results indicated that 70, 38 and 21 kD polypeptides are susceptible to trypsin digestion. 38 kD polypeptide is cleaved within one minute of digestion. 70 and 24 kD linker polypeptides are less susceptible. The role of 70 and 24 kD polypeptides in the attachment of phycocyanin to allophycocyanin can be envisaged, since the partial cleaving of these polypeptides leads to partial detachment of the phycocyanins to allophycocyanins.

The phycobilisome plays a dual role in photosynthesis and cellular nitrogen storage. Such role is reasonable when one
considers the abundance of cellular nitrogen contained in phycobilisomes, and also the low priority for light harvesting by an accessory pigment system during nutrient starvation. The changes brought about at the first stage of nitrogen starvations in the physiological and spectroscopic properties of phycobilisomes show a preferential loss of phycocyanin from the structure. The phycobilisomes from partially starved cells sediment at a slow velocity and higher allophycocyanin to phycocyanin ratio. Only one colourless polypeptide of 26 kD is lost during one day of nitrogen starvation. The loss fairly coincides with change in absorption and fluorescence spectra. During later stage of nitrogen starvation, two other polypeptide of 70 and 24 kD are lost. It is at this stage that fluorescence emission of phycobilisome shows a small but distinct shift to shorter wavelength and the structure becomes less stable.