LOW TEMPERATURE SPECTROSCOPY OF ALGAE

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THESIS

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I. INTRODUCTION

This investigation is concerned with a systematic study of the absorption, fluorescence and fluorescence excitation spectra of algae at different temperatures ($4^{o}K$ to $295^{o}K$). The purpose of this investigation is twofold: firstly, to study the temperature dependence of the efficiency of energy transfer between pigment molecules so as to identify the preferred mechanism for energy transfer in photosynthetic organisms; secondly, to study the fluorescence bands under conditions where internal conversion is greatly decreased and steady state photosynthesis is stopped (i.e. at low temperatures). Under such conditions, fluorescence from the trap levels may increase and become detectable. In the following paragraphs, the evidence for energy transfer, different possible mechanisms of energy transfer and the theory of fluorescence from the trap will be reviewed briefly.

A. Evidence of Energy Transfer in Photosynthetic Organisms:

The problem of energy transfer in photosynthetic organisms was first brought to attention in 1936 when Gaffron and Wohl (1) interpreted the results of Emerson and Arnold's (2) flashing light experiment. Gaffron and Wohl suggested the possibility of energy transfer from the bulk pigment molecules to particular sites in the cell where photochemical processes took place. In 1943, Dutton *et al.* (3) discovered the evidence for energy transfer from the accessory pigment (fucoxanthol) to chlorophyll *a* (Chl *a*) in diatoms. They found that when light was absorbed by

either fucoxanthol (a carotenoid) or Chl a, fluorescence emitted by Chl a could be detected. This result suggested that energy is transferred from the accessory pigments to Chl a. Similar results were reported by Wassink and Karten (4) in 1946. Based on the same principle of sensitized fluorescence, further studies of energy transfer in different algae were carried out by Duysens (5, 6), French and Young (7), Brody and Brody (8), Ghosh and Govindjee (9) and others. Duysens showed that the efficiency of energy transfer from chlorophyll b (Chl b) to Chl a is almost 100% in Chlorella (6). It will be shown in chapter VI that the transfer efficiency from Chl b to Chl a is almost 100% even at $4^{\circ}K$.

The transfer of excitation energy among the pigment molecules has been demonstrated by several methods other than sensitized fluorescence. Arnold and Meek (10), Goedheer (11) and Teale (12) showed that when polarized light was absorbed by photosynthetic organisms, the fluorescence light was highly depolarized. Since chlorophyll is believed to be imbedded in the protein and lipid structures in vivo, molecular rotations are believed to be small. These authors suggested that the concentration depolarization phenomenon (first discussed by Perrin (13)), caused by the transfer of excitation energy between randomly oriented molecules, may be responsible for the observed depolarization of the fluorescence.

Another line of evidence for energy transfer is suggested by the high efficiency of oxidation and reduction of P700 (a pigment absorbing near 700 nm which is believed to be the reaction center of pigment system I) (14). P700 constitutes only about 0.3% of the "bulk" chlorophyll

molecules, but the quantum requirement for photo-oxidation of P700 sensitized by Chl a is estimated to be one quantum per P700 molecule (15). This high efficiency of oxidation suggests that energy is transferred from the bulk Chl a to P700 molecules.

Having presented the evidence for the existence of energy transfer in photosynthetic organisms, the mechanism of energy transfer can now be discussed.

B. The Mechanism of Energy Transfer:

Along with the discovery of the energy transfer phenomenon in photosynthetic organisms, several possible mechanisms have been advanced. Gaffron and Wohl (1) suggested that energy transfer might be accomplished either by the migration of energy-rich particles generated by each chlorophyll molecule or by the transport of energy quanta among chlorophyll molecules. In the former model, the particles require time to diffuse to the reaction center, and the transfer rate would be too slow to explain the available data on the rate of oxygen production. The latter model, transport of energy quanta, is generally accepted and has been studied by many workers.

One of the proposed mechanisms for energy transfer is that the optically excited electrons and the corresponding positive holes might migrate among the chlorophyll molecules in the same manner as in the photoconduction phenomenon observed in semiconductors. This possibility was first suggested by Szent Gyorgyi (16) in 1941. The possibility

of migration and recombination of excited electrons and holes as a means of energy transfer was elaborated by Katz (17). This idea was further developed by Arnold (18), Arnold and Sherwood (19), Arnold and Clayton (20), Calvin (21) and Tollin et al. (22). Their results provided evidence for some semiconductor properties in dried spinach chloroplasts and dried algae. For instance, Arnold and Sherwood found that the electrical resistance of a film of dried chloroplasts varied with temperature like a semiconductor ($\log R = C_1 + C_2 \frac{\Delta E'}{T}$, where R is the resistance of the film, C_1 and C_2 are constants, $\Delta E'$ is the energy gap between the conduction and the valence bands, and T is the absolute temperature). They also found that dried chloroplasts exhibit thermoluminescence; that is, when dried chloroplasts were illuminated and then stored in the dark for a few hours, they could still emit light upon heating. Other effects like photoconductivity and change of dielectric constants upon illumination have also been found. Unfortunately, most of these results were obtained in dried chloroplasts and dried algae. There is a lack of evidence that such properties operate efficiently enough in vivo to account for the high quantum yield of photosynthesis. Calvin (21) reviewed the studies on electron spin resonance measurements in wet chloroplasts and discussed evidence for the presence of light induced unshared electron pairs at room temperature. However, direct measurements of photoconductivity effects in wet chloroplasts have not been reported.

Another proposed mechanism for energy transfer is the migration of excitons. The possibility of exciton migration in a photosynthetic unit

was first discussed by Frank and Teller (25). They assumed that chlorophyll molecules were arranged in a one-dimensional crystal to which CO_2 molecules (or reaction centers) were attached at the ends of the chain. Light quanta absorbed in any molecule of the crystal must migrate to the end of the chain before energy can be utilized for chemical reactions. However, these authors found that the one-dimensional model could not be reconciled with the available absorption and fluorescence data due to the following reason. For the one-dimensional model, the mean number of steps, $\langle n \rangle$, is proportional to N^2, where N is the number of lattice sites. In a photosynthetic unit ($N \simeq 300$), the exciton must traverse approximately 10^4 to 10^5 molecules before reaching the end of a one-dimensional molecular chain. In order to accomplish this number of transfers within 3×10^{-10} sec (calculated from the fluorescence yield and fluorescence lifetime), the transfer time must be smaller than 3×10^{-15} sec. This fast transfer rate would require a strong interaction energy between the molecules and thus one would expect a considerable red-shift in the *in vivo* absorption spectrum when compared to that of Chl *a* in solution (for example, the absorption band of Chl *a* micro-crystals when compared to that of Chl *a* dissolved in ether is shifted by 70 nm (26)). Experimental results show that the red-shift is too small to explain the one-dimensional model (the absorption band shift of Chl *a* *in vivo* compared to that of Chl *a* dissolved in ether is 25 nm (27)).

Calculations for the two- and three-dimensional models of the photosynthetic units were reported by Pearlstein (28). It turns out

that the number of transfer is much smaller for two- ($\langle n \rangle \alpha N \ln N$) or three-dimensional ($\langle n \rangle \alpha N$) crystals than that for the one-dimensional case because the number of nearest neighbors increases for higher dimensions. Therefore, the slower transfer rate (resonance transfer) in a two-or three-dimensional crystal can still satisfy the requirements for low fluorescence yield and high photosynthesis quantum yield.

Pearlstein's calculations were based on the assumptions that the dipole-dipole interaction was predominant and that exciton migration was caused mainly by the slow resonance transfer (transfer time $\tau_t \simeq 3 \times 10^{-12} - 3 \times 10^{-8}$ sec (ref. 15, p. 113)), the transfer rate was proportional to R^{-6} (R was the intermolecular distance). Pearlstein's assumptions were questioned by Robinson (27) who pointed out that the fast transfer rate ($\tau_t \simeq 3 \times 10^{-14}$ sec) and the first order approximation of the interaction energy (proportional to R^{-3} when one only considers dipole-dipole interaction) are more appropriate for a system where identical molecules are nearest neighbors and the intermolecular coupling energy is strong (but not necessarily strong enough to cause disagreement with the absorption spectrum as pointed out by Franck and Teller (25)). According to Robinson, this model describes a photosynthetic unit when energy is transferred among the Chl a molecules. R^{-6} dependence (slow, resonance transfer) is however more applicable to a system where the interaction energy is weak, and the molecules are not identical because the transfer rate in this case depends on the overlap between the fluorescence band of the donor and the absorption band of the acceptor (30).

Therefore, for nonidentical molecules, a strong band overlap can be obtained in some cases. For example, R^{-6} dependence is generally found in sensitized fluorescence of dyes in a dilute solution. For identical molecules, however, the amount of band overlap is limited by the "Stoke's shift". Pearlstein argued that large organic molecules like Chl a may act as nonidentical molecules because of the instantaneously differing thermal environment (28). Therefore, resonance transfer is possible. However, Robinson (29) estimated the intermolecular coupling energy (V) and found that it is strong enough to cause a faster transfer rate than that of resonance transfer. From a fluorescence lifetime of 1.5 nsec, Robinson calculated the oscillator strength, 0.47, and the transition dipole length, 1.73 Å. With these values he calculated the approximate range of intermolecular coupling energy (V) between chlorophyll molecules (assuming that they are about 1.5 to 2.0 nm apart). He found that V in a photosynthetic unit should be of the order of 100 cm^{-1}, (the exact value depending on the intermolecular distances, the relative orientation of the dipoles and the influence of the higher-poles). Robinson pointed out that a value of V much higher than 100 cm^{-1} would lead to a disagreement between the calculated and the observed band width in the absorption spectrum, whereas a value much lower than 100 cm^{-1} is also unlikely because an unreasonably large intermolecular distance or a very unfavorable relative orientation between the dipoles would be needed. Furthermore, the interaction energy contributed by higher-poles (e.g. quadra-pole) will maintain the value of V to be about 100 cm^{-1}. Calculating from the uncertainty principle,

$t \simeq \hbar/V$, Robinson found that the transfer time was about 5×10^{-14} sec, which is much faster than the resonance transfer rate (3×10^{-8} to 3×10^{-12} sec).

Whether the first order (R^{-3}, fast transfer) or the second order (R^{-6}, slow transfer) is a closer approximation to the energy transfer rate in photosynthetic organisms has not yet been settled. One of the purposes of this investigation is to study this problem. Robinson's approximations are reasonable but exact calculations of V contributed by the dipoles and higher-poles cannot be obtained because the precise intermolecular distance and the orientations of the molecules in vivo are not certain at present. However, the two approximations (slow and fast transfer rate) have different temperature dependence. This temperature dependence was suggested by Förster (31). He proposed that for a strong coupling limit, where the interaction energy was larger than the Franck-Condon energy ΔE (resulting from the difference between the nuclear equilibrium configurations of the ground and the excited states), the transfer rate would be independent of temperature and proportional to R^{-3}. For medium coupling case where the interaction energy was smaller than ΔE, but larger than the spread of a vibrational band $\Delta\epsilon$, the transfer rate would be slightly temperature dependent and proportional to R^{-3}. For a weak coupling limit where the interaction energy was smaller than ΔE and $\Delta\epsilon$, the transfer rate would be strongly temperature dependent and proportional to R^{-6}. Therefore, by studying the transfer rate as a function of temperature, one can determine two important aspects of

energy transfer; namely, whether the coupling energy between the molecules is strong or weak, and whether the transfer rate is fast (transfer time $\tau_t \leq 3 \times 10^{-14}$ sec), intermediate ($\tau_t \simeq 3 \times 10^{-14}$ -3×10^{-12} sec), or slow ($\tau_t \simeq 3 \times 10^{-12}$ -3×10^{-8} sec) (ref. 15, p. 113). In this investigation, the temperature dependence of transfer efficiencies are studied by a systematic measurement of the absorption, fluorescence and fluorescence excitation spectra at different temperatures (4°K to 77°K). It is found that the transfer efficiency from Chl b to Chl a (670 and 678 nm bands) and that from Chl a (670) to Chl a (678) approaches 100%, and they are independent of temperature between 4°K and 77°K. The transfer efficiency from Chl a (678) to longer wavelengths Chl a forms and that from phycocyanin to Chl a increases upon warming from 4°K to 77°K. These results provide information leading toward solving the above questions. Furthermore, the spectra measured provide information on the trap levels at low temperatures.

C. Fluorescence from the Trap Level:

The high quantum yield of photosynthesis leaves only a small fraction (2.7% (32)) of the absorbed light quanta for fluorescence. However, merely stopping the process of photosynthesis (by adding a chemical compound) will not cause all the absorbed light quanta to be emitted as fluorescence because internal conversion is also an effective energy quenching process. It is generally found that upon addition of 3-(3,4-dichlorophenyl)-1, 1-dimethylurea (DCMU), which blocks

photosynthesis, the fluorescence yield increases by two to three fold (33). At room temperature (295°K), the energy transferred to the reaction center is utilized for chemical reactions or it may "boil" back to the bulk Chl a within the lifetime of fluorescence. Hence, the fluorescence from the trap is not easily detectable in cells growing at room temperature. Even upon stopping the process of photosynthesis (e.g. with DCMU) at this temperature, not all the energy used for photosynthesis would emit as fluorescence energy from the trap. This is because the thermal energy at 295°K is sufficient to "boil" most of the trapped energy quanta back to the bulk of Chl a before fluorescence takes place. At low temperatures, however, the thermal energy is greatly reduced, and steady state photosynthesis is stopped, fluorescence from the trap is therefore possible.

Calculations of the thermal energy at different temperatures are shown below:

kT at room temperature = $295 \times 1.38 \times 10^{-16} = 408 \times 10^{-16}$ erg

kT at 77°K $= 104 \times 10^{-16}$ erg

kT at 4°K $= 5.5 \times 10^{-16}$ erg

Assuming that the 698 nm fluorescence band is caused by the trap and the 687 nm fluorescence band is caused by the bulk of Chl a that absorbs near 678 nm (these assumptions will be confirmed in chapter VI). Furthermore, assuming that the ground states of these transitions have the same energy, the energy gap between the excited states can be calculated as:

$$E_{gap} = E_1 - E_2 = h\nu_1 - h\nu_2 = hc \frac{\lambda_2 - \lambda_1}{\lambda_1 \lambda_2} = 495 \times 10^{-16} \text{ erg}$$

Without knowledge of the exact shape of the Franck-Condon potential energy well, one cannot calculate the precise value of the trap depth; however, considering that it has the same order of magnitude as the E_{gap} between the two excited states, the above calculations show that the trap depth is rather shallow at room temperature. At 77°K, the trap depth is about five times greater than kT. Moreover, at 4°K, the trap depth is almost ninety times "deeper" than kT. It is difficult for a trapped energy quantum to gain sufficient thermal energy to "boil" back to the bulk of Chl a at 4°K. Therefore, fluorescence from the trap levels can be expected to increase tremendously.

A large amount of work in the area of the fluorescence of photosynthetic systems has recently been done at low temperatures. When fluorescence spectra are measured at 77°K for a number of different photosynthetic organisms (algae, leaves), the single main fluorescence maximum of around 685 nm, which is observed at room temperature, is replaced by three bands, located at around 685, 696 and 720 nm respectively. The broad 720 nm band was discovered in Chlorella by Brody (34) in 1958; in 1963, Govindjee (35), Bergeron (36), Brody and Brody (37), and Kok (38) independently noted the 696 nm band. Among these authors, Govindjee (35) and Bergeron (36) suggested that the F718-720 and F696-700 bands may be emitted from the trap levels (or energy sinks) of pigment system I (SI) and pigment system II (SII), respectively.

Most of the recent low temperature work in vivo were carried out in the 295°K to 77°K temperature range (39-50). As mentioned above, the trap depth is relatively shallow in this temperature range. In order to increase the probability of fluorescence from the trap level, measurements in this study has been extended to 4°K. The purpose of this study is to investigate the fluorescence bands from the trap levels and to study the transfer efficiency from the bulk of Chl a to the trap level as a function of temperature. Our results confirm the earlier suggestion (35) that F698 is a fluorescence band emitted from a trap level, which is estimated to absorb near 686-688 nm. Furthermore, the transfer efficiency from Chl a (678) to this trap level increases upon warming from 4°K to 77°K.

II. MATERIALS AND METHODS

A. Culturing of the Algae

Chlorella pyrenoidosa and Anacystis nidulans are the organisms used in this investigation. Chlorella is a green alga containing Chl a, Chl b and carotenoids. The average diameter of the cell is approximately 5μ. Although Chlorella is a unicellular organism, it has spectroscopic characteristics similar to that of higher plant cells. Anacystis is a blue-green alga containing Chl a, phycocyanins and carotenoids. Its mean projection area is $(0.92 \pm 0.01\mu) \times (2.67 \pm 0.82\ \mu)$ (36). Anacystis is considered to be a primitive photosynthetic organism because it is prokaryotic (without nuclear membrane) and lacks chloroplast membrane. Both algae have fast growth rates. Chlorella population has been estimated (51) to increase 40 fold within three days. The growth conditions are outlined as follows.

Culture media for Chlorella and Anacystis were described by Govindjee (51). 100 ml of medium was placed in a 300 ml erlenmeyer flask with special gas inlet and outlet tubes, and autoclaved for 15 min. at 15 lbs of pressure per square inch. After cooling, a single strain pure culture was inoculated into each flask using sterile technique. The top of the flask was blocked with a cotton stopper, sealed by parafilm and a rubber cap, and then a mixture of gases (95% air, 5% CO_2) or air was passed through the flask. The gas mixture was introduced at the bottom of the culture at a rate of about 100 bubbles per minute. The turbulence produces an optimum growth condition for the algae. Temperature for

Chlorella growth was 20 ± 2°C, and that for Anacystis was 27 ± 2°C. The light sources were tungsten and white fluorescent lamps. Cells were grown under continuous illumination. Detailed growth conditions were described by Govindjee (51). The Chlorella strain #3 used in this study was isolated by R. Emerson. The Anacystis strain was obtained from J. Myers at the University of Texas.

B. Absorption Measurements

Absorption spectra of Chlorella and Anacystis were measured at 295°K, 77°K and 4°K. The spectrophotometers used were the following.

1. Room Temperature Absorption Measurements by Spectrophotometer

The absorption spectra at room temperature were measured with a Spectronic 505 spectrophotometer built by Bausch and Lomb, Inc. (Rochester New York. Model. 33-28-04). This instrument had a half bandwidth (1/2 B. W.) of 5 nm. The accuracy of the wavelength was within 0.5 nm, and that of the transmitted intensity was within 0.5%. An integrating sphere was attached to the spectrophotometer. This sphere was used to measure the absorption spectra of particles which caused scattering of the incident light (e.g. algae). The inside of the sphere was coated with magnesium oxide to provide uniform and strong reflection of the scattered light, so that this light would still be detected by the photomultiplier. However, sieve and detour effects (52) could not be corrected when absorption measurements are made by this method.

2. Low Temperature Absorption Measurements by Means of Light Guides

The absorption instrument consisted of four main parts: a monochromatic light source, a low temperature compartment, a system for analyzing and recording the spectra and a temperature sensing device. These parts will be discussed separately. (a) Monochromatic light source: monochromatic light was obtained from a monochromator built and described by Cederstrand (53). The light source was a ribbon filament lamp (General Electric Co., Nela Park, Cleveland, Ohio, Model 18A/T10/IP-6V) operated at 6 volt D.C., 18 amperes provided by a voltage-regulated power supply (NJE Corp., Kenilworth, New Jersey, Model CR 18-30). The monochromator, consisting of collimating lenses and a diffraction grating (Bausch and Lomb Optical Co., Rochester, New York, Model 33-53-08-26, 600 grooves/mm), gave a linear dispersion of 1 nm per 0.28 mm of exit slit width. Half bandwidths of 7 nm were employed for the absorption measurements. (b) Low temperature compartment: Light from the monochromator was introduced into the low temperature compartment (Fig. 1) by a fiber glass light-guide (American Optical Co., Southridge Massachusetts, Model GM-5). The low temperature compartment consisted of three parts: a Dewar flask (H. S. Martin & Co., Evanston, Illinois, Model M20312) with inside diameter of 135 mm and depth of 450 mm, a liquid nitrogen jacket made of styrofoam, and a metal sample holder (see Fig. 1). The sample consisted of a paste of algal cells spread between two glass coverslips (2 x 2 x 0.01 cm^3, each). With slight pressure, the paste could be made to spread evenly

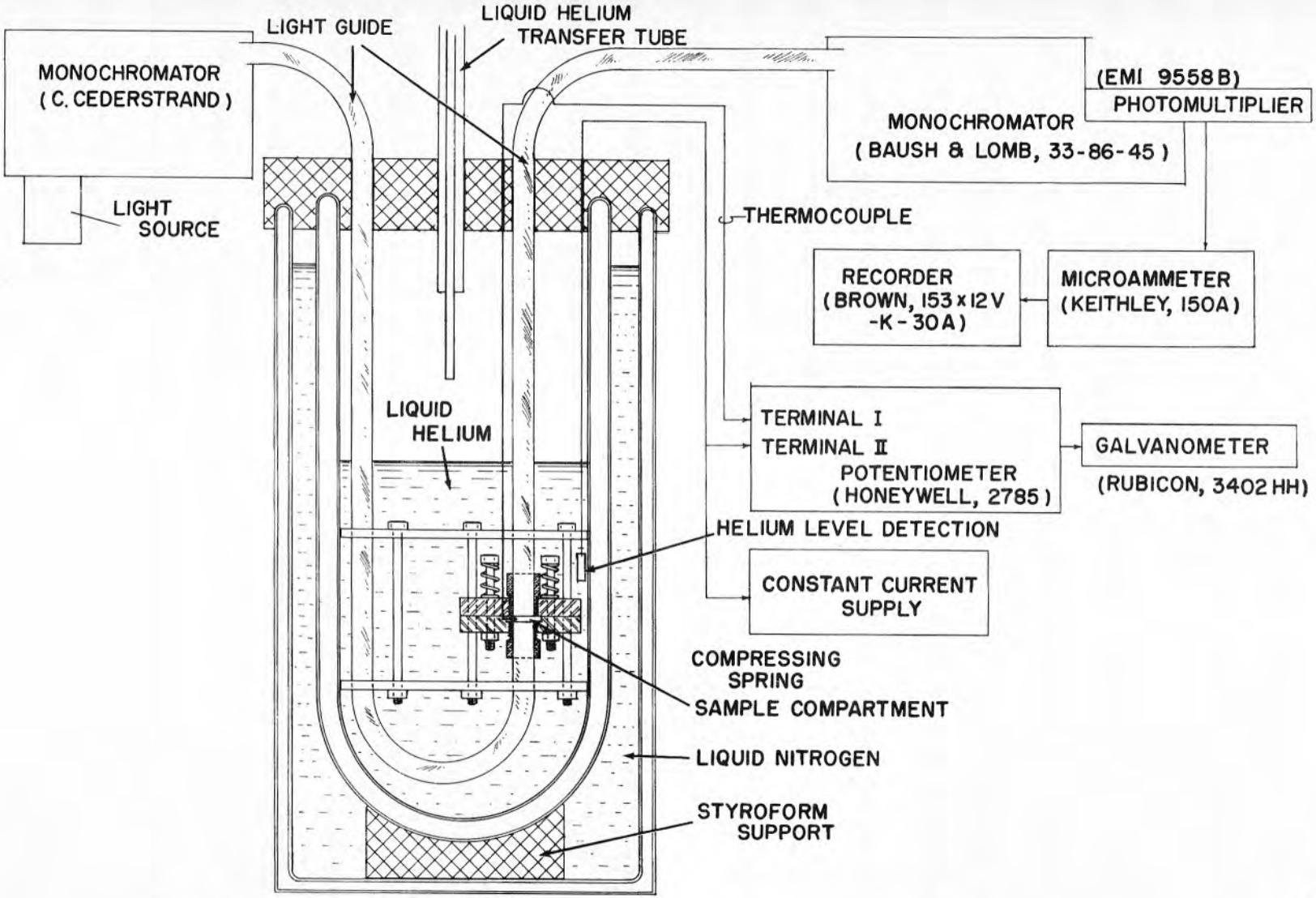


Fig. 1. Apparatus for the low temperature absorption measurements.

between the coverslips. The total thickness (glass-cells-glass) was about 0.022 to 0.023 cm. The sample "sandwich" was clamped with springs between the ends of the two light guides. Because of the spring loading, lowering of temperature would not break the contact between the light guides and the sample "sandwich". This arrangement permitted collection of a large solid angle ($\simeq 2\pi$) of transmitted light and thus minimized scattering losses. The fiber glass light-guides could successfully withstand low temperatures. Calibration showed that the absorption and scattering losses of the light-guides varied by less than 2% upon cooling from room temperature to 77°K. In experiments at 77°K, liquid nitrogen was poured into the Dewar flask directly. In experiments at 4°K, the external styrofoam container was first filled with liquid nitrogen, and liquid helium was then transferred into the Dewar flask through a transfer tube. This was done by applying pressure to the liquid helium container using compressed helium gas. After the transfer, several layers of cotton were used to cover the top of the Dewar flask. The level of helium, which was higher than the sample level, was detected by a solid state temperature sensor (Phylatron Corp., Columbus, Ohio, Model CD-5004), mounted 1" above the sample. When a temperature rise was detected, additional liquid helium was added to the Dewar flask. (c) Temperature sensing device: The temperature of the sample was measured with a thermocouple (made of Advance #34 and Chromel #34), mounted beside the sample "sandwich". The thermocouple, calibrated

against a platinum thermister, gave a potential change of 1.07 mv when the temperature changed from 4^{o}K to 77^{o}K. This potential change was measured by a potentiometer (Honeywell, Denver, Colorado, Model 2785) and a null detecting galvanometer (Rubicon Co., Philadelphia, Pennsylvania, Model 3402HH). (d) Analyzing and recording system: A light-guide conducted light transmitted by the sample to an analyzing monochromator (Bausch and Lomb, Rochester, New York, Model 33-86-45) with a dispersion of 3.3 nm half bandwidth per mm slit width. In absorption measurements, slit widths of 2 mm were used. The purpose of the analyzing monochromator in the absorption measurements was to eliminate the error introduced by fluorescence of the sample. This error is especially prominent at low temperatures at which the fluorescence yield is high ($>$ 30%). A photomultiplier tube (RCA, Harrison, New Jersey, Model C70007A, or by Electra Megadyne Inc., New York, New York, Model 9558B) was placed at the exit slit. This tube was operated at 1250V, which was maintained by a regulated voltage supply (Northeast Scientific Corp., Acton, Massachusetts, Model RE-3002). The output current of the photomultiplier was amplified with a Microvoltammeter (Keithley Instruments, Cleveland, Ohio, Model 150A). The amplifier output, after attenuation by a variable resister, was registered on a potentiometric recorder (Brown Instrument Division, Minneapolis-Honeywell Reg. Co., Philadelphia, Pennsylvania, Model 153 x 12 V-K-30A).

Data for calculating an absorption spectrum at a given temperature were obtained as follows. First, with water as sample (or with cells bleached by 80% acetone as sample), incident intensities $I_o(\lambda)$ were measured throughout the desired spectral range. The "blank" was then removed, the algal sample was inserted, and the transmitted intensities $I(\lambda)$ were recorded. Both $I_o(\lambda)$ and $I(\lambda)$ were measured at the same temperature and with all other conditions held constant. Absorption spectra were calculated according to the formula:

$$\text{O.D.}(\lambda) = \log_{10} \frac{I_o(\lambda)}{I(\lambda)} - \frac{I_o(\lambda')}{I(\lambda')}$$

The second term corrected for any apparent absorbance at wavelength (λ') (e.g. 750-800 nm) at which the extinction coefficient of the alga approached zero. This formula was valid strictly in the wavelength region where the index of reflection and the coefficient of back scattering of the system remained the same as that at λ'. It was assumed that this region included the range 400-800 nm. This assumption was not exactly correct because Latimer (32) found that selective scattering varied as a function of absorption wavelength. However, he also showed that back scattering was only a few per cent of the total scattered light and since the reflection caused by cell wall was almost independent of wavelength, this assumption could still be justified.

C. Fluorescence and Fluorescence Excitation Measurements

Fluorescence and fluorescence excitation spectra were measured by a spectrofluorometer which was designed and built by Govindjee and

Spencer. This system consists of an exciting and an analyzing monochromator (Bausch and Lomb Optical Co., Rochester, New York, Model 33-86-45), a sample compartment (which allows for low temperature (77°K) measurements), a photomultiplier (Electra Megadyne Inc., New York, New York, Model 9558B) and a recording system. The instrument was described in detail by Govindjee (54) and Shimony et al. (55).

For measurements from 4°K to 77°K, some modifications of the low temperature compartment and the temperature measuring device have been made. The modifications include using a Dewar flask (height = 32 cm) having an optically clear and rounded glass bottom, inside which a plunger made of teflon was used for pressing the sample material to the bottom surface of the Dewar. The temperature of the sample was determined by the thermocouple and potentiometer system described in section B.2 of this chapter. The modified instrument is diagrammed in Fig. 2.

The experimental conditions and procedures were as follows. For measuring fluorescence spectra from 4°K to 77°K, 2 ml of thin algal culture (O.D. = 0.1 at 675 nm, 0.3 cm path length) was first transferred to the bottom of the Dewar flask, four layers of cheese cloth were placed on top of the sample, and then the teflon plunger was put on top of the cheese cloth. These were done to prevent the sample from shattering and floating during and after the process of helium transfer. Liquid helium was transferred to the bottom of the Dewar at a fast rate (transfer procedure was described in section B.2), and continued

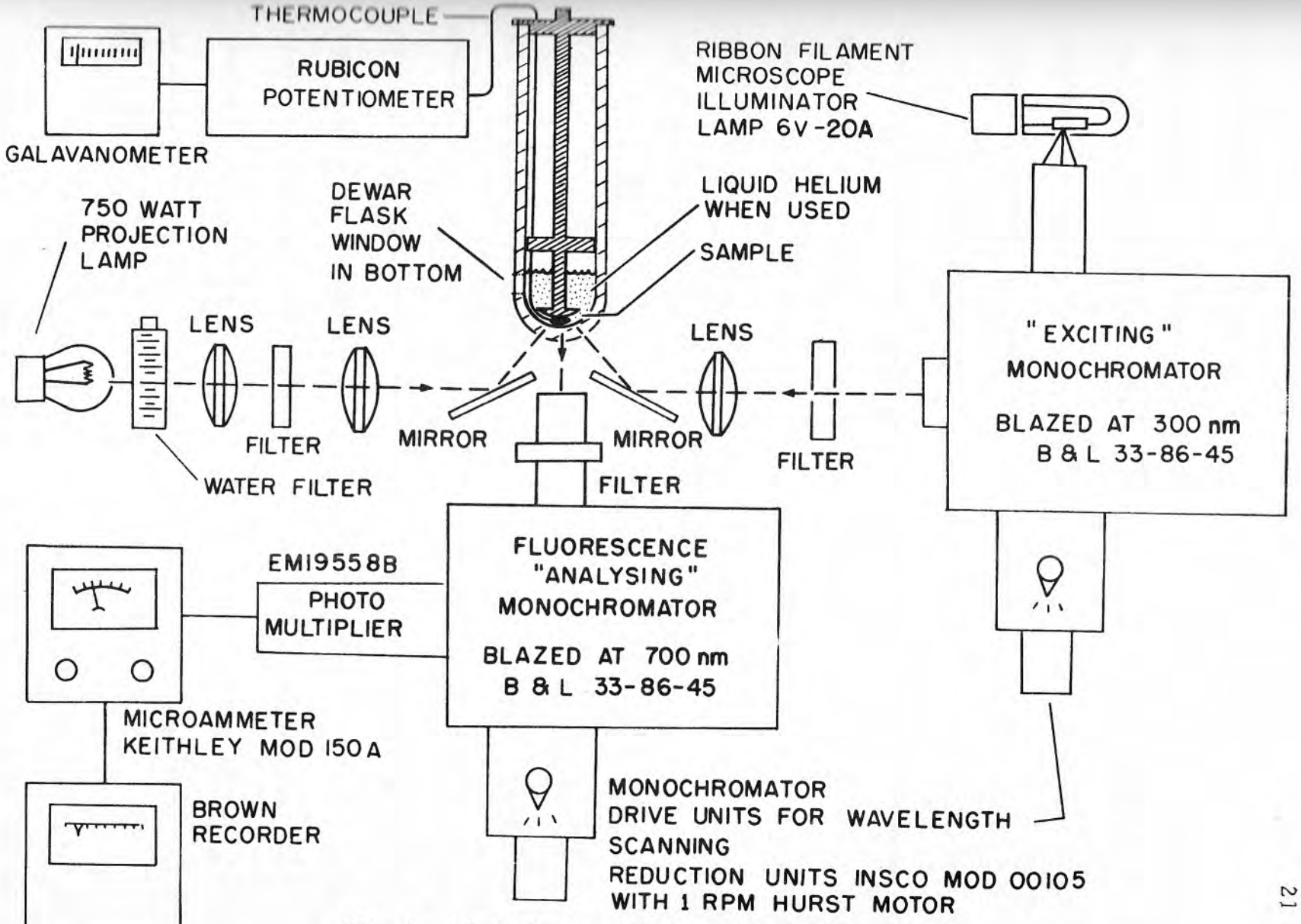


Fig. 2. Block diagram of the spectrofluorometer.

until shortly after ($\simeq$ 1/2 min) the thermocouple potential had reached 9.73 mv (with 0°C at the reference junction of the thermocouple), and then data at 4°K were recorded. After helium transfer had been stopped, the liquid gas in the Dewar flask completely evaporated in about 5 minutes, the sample began to warm up, and the data at the intermediate temperatures were obtained.

The slit widths of the exciting and the analyzing monochromators were set at 2 mm (half bandwidth, 1/2 B.W. $\mp$ 6.6 nm) for measuring the fluorescence spectra, unless stated otherwise. For fluorescence excitation measurements, the exciting monochromator slit widths were 1.4 mm (1/2 B.W. $\mp$ 4.6 nm), and those of analyzing monochromator were 2 mm, unless stated otherwise.

Several filters were used to prevent the excitation beam from getting into the fluorescence analyzing monochromator. These filters were made by Corning Glass Works (Corning, New York). For measuring fluorescence spectra, CS-4-94 was placed between the exciting monochromator and the sample, whereas CS-2-73 (or CS-3-66) was placed between the sample and the analyzing monochromator. For measuring excitation spectra, CS-7-69 was placed between the sample and the analyzing monochromator when measuring fluorescence at 725 nm (F725) or longer wavelengths, whereas CS-2-64 was used for measuring F687 and F698.

The wavelength settings of the monochromators were calibrated regularly with a mercury-arc lamp. The intensities of the exciting beam

were measured at different wavelengths by a thermopile (Eppley Laboratory, Inc., Newport, Rhode Island). These results formed a set of correction factors (by taking the inverse of the number of excitation light quanta at different wavelengths) for the excitation spectra and accounted for the wavelength dependence of the excitation light. The characteristics of the analyzing system (the analyzing monochromator and the photomultiplier) was calculated from the ratio of the number of excitation quanta and the output current of the photomultiplier caused by this excitation. The inverse of this ratio at different wavelengths formed a set of correction factors for the fluorescence spectra, and accounted for the wavelength dependence of the analyzing system.

III. LOW TEMPERATURE ABSORPTION SPECTRA OF ALGAE

A. Introduction

Low temperature (77°K) absorption spectrum of photosynthetic organisms was first reported by Butler (56) in 1960. In that study, a new absorption band near 705 nm was observed. Butler proposed that this band was caused by a Chl a form which obtained excitation erergy from the bulk Chl a through resonance transfer, and emitted fluorescence at 720 nm. In 1961, Butler (39) reported the absorption and fluorescence excitation spectra of bean leaf measured at 77°K, and he proposed that the 705 nm band was caused by a form of Chl a molecule (Chl a (705)) which acted as the "reaction center" in photosynthetic apparatus. This idea was supported by the differences in the absorption spectra of certain Scenedesmus mutants (which lacked a "reaction center") and the normal cells. (41). However, Kok (38) and Butler (57) later found that Chl a (705) did not correspond to the "reaction center" P700 (see p. 2 of this thesis) because P700 can be bleached by light at 77°K whereas Chl a (705) cannot, and because the concentration of Chl a (705) is much higher than that of P700. They suggested that P700 might be a part of Chl a (705) which was connected to the photosynthetic electron transport chain.

In 1963, Butler and Baker (40) reported the absorption and fluorescence excitation spectra of spinach and Chlorella chloroplast fragments which were obtained by breaking the cells by sonic oscillations and collected by differential centrifugation. They found that Chl a (705)

was not resolved in Chlorella fragments even at 77°K. However, they observed bands at 670 nm and 678 nm. In order to improve the resolution of the band structure, Frei (42) studied the low temperature (93°K) derivative absorption spectra of algae and leaves. He reported that the absorption spectrum of Chlorella (including the 650 nm, 670 nm and 678 nm absorption bands) was greatly sharpened upon cooling. However, when compared to the room temperature spectrum, no new component was found. The 705 nm band was not observed in this derivative absorption spectrum of Chlorella, but was found in the spectra of some other algae such as Scenedesmus and Polyedriella. In summary, the literature reviewed emphasized the study of the nature of Chl a (705) and the absorption bands of different Chl a forms. The temperature range for the measurements was 295°K to 77°K.

In this study, low temperature absorption spectra of Chlorella and Anacystis were measured at 77°K and 4°K, with emphasis on the spectral comparison at different temperatures. This information, in conjunction with the data of excitation spectra, is essential for the calculation of energy transfer efficiency as a function of temperature. Since the absorption bands are better resolved at low temperatures, one would expect the results of this study (measured at 4°K) to provide better resolution of the spectrum than those in previous studies (measured at 77°K). Another improvement in the present measurement is that an analyzing monochromator is used to eliminate the fluorescence of the sample. This correction is important because the fluorescence yield is high at the low temperatures.

B. Experimental Results

Absorption spectra of Chlorella pyrenoidosa and Anacystis nidulans have been studied in the 4°K to 295°K temperature range. The results for the two algae will be discussed separately.

1. Absorption Spectra of Chlorella pyrenoidosa

In order to show that there are no significant systematic errors in instrumentation and technique, two room temperature absorption spectra of Chlorella were measured by the light-guide method (Fig. 3), one using a water blank and the other using bleached cells as reference. The similarity of the two spectra suggests that any errors caused by scattered light are small. The shape and the peak and shoulder positions are similar to those obtained by the oiled filter-paper technique (58), but this spectrum has a relatively higher 680 nm band than that obtained by the integrating sphere technique (59). In Fig. 4, spectra of Chlorella at 77°K with water and bleached-cell blanks are compared. Again, scattering errors are found to be negligible. Fig. 5 shows absorption spectra of Chlorella measured at 4°K and 77°K. Reproducibility of these spectra was confirmed by more than five independent measurements. The two curves shown were obtained with a single sample. The spectra at 4°K and 77°K are very similar, the maximum deviation at the major peaks being less than 5%. This near constancy of the absorbance in this temperature range is important because it simplifies the calculation of energy transfer efficiency as a function of temperature. Both spectra show three major bands in the red region -- namely at 649.5, 670 and 677.5 nm. The Chl a

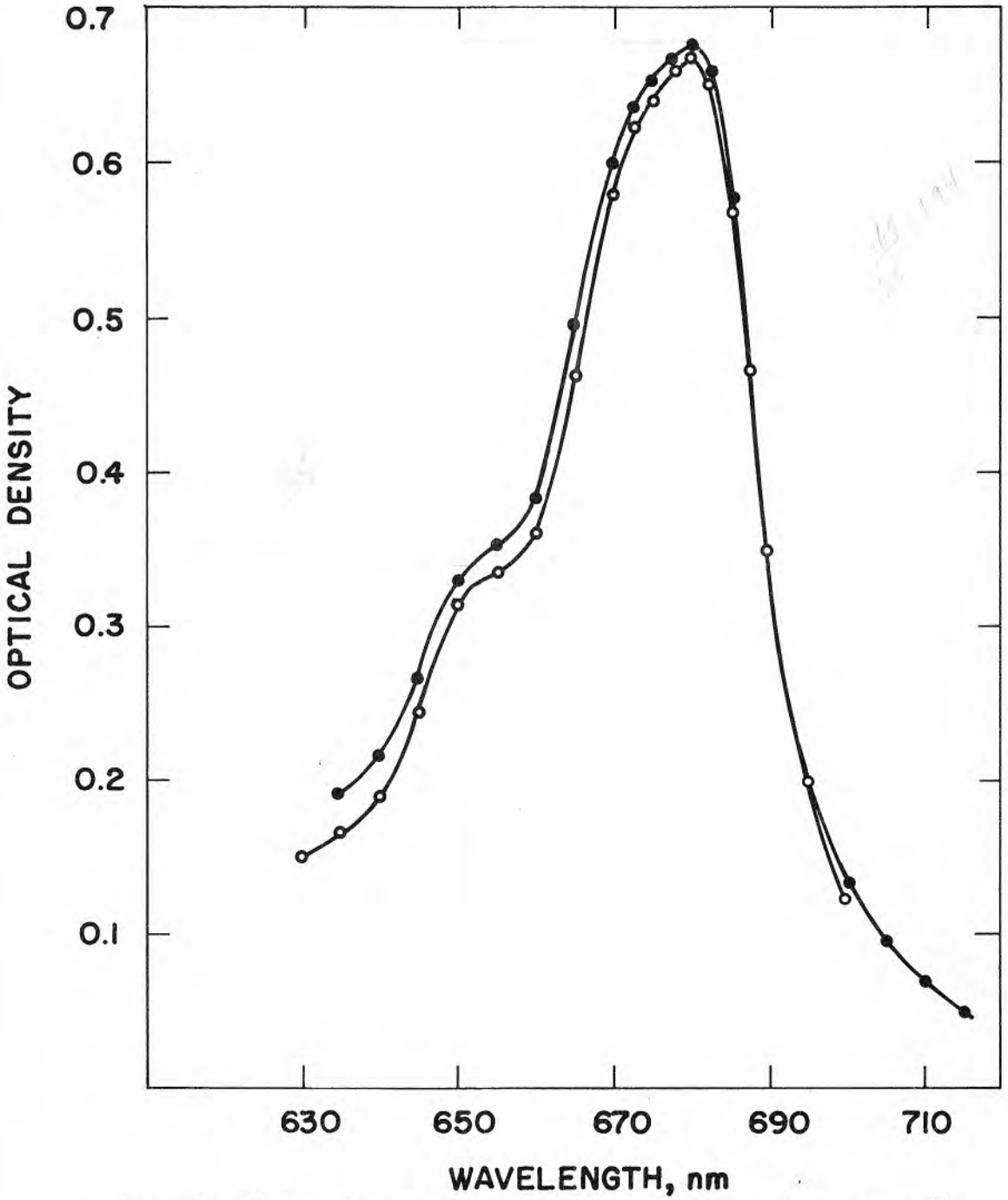


Fig. 3. Absorption spectra of Chlorella pyrenoidosa at 295°K, using water •—•—• and bleached cells o—o—o as references. To obtain the base lines with negligible absorbance near 750 nm, 0.4 and 0.1 are subtracted from the absorption spectra calculated with water and bleached cells and references, respectively.

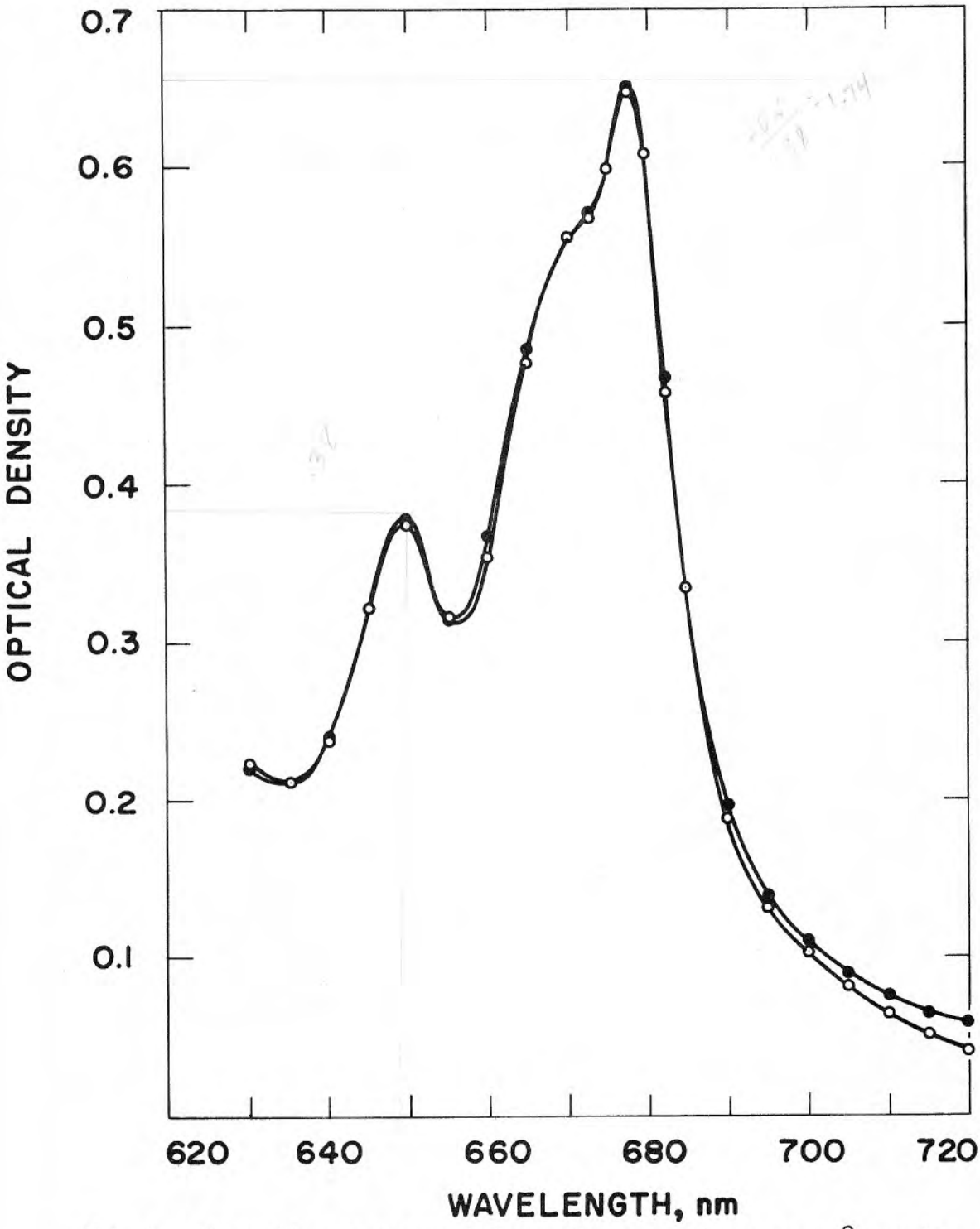


Fig. 4. Absorption spectra of Chlorella pyrenoidosa at 77°K, using H_2O o–o–o and bleached cells •–•–• as references. To obtain the base lines with negligible absorbance near 750 nm, 0.97 and 0.65 are subtracted from the absorption spectra calculated with water and bleached cells as references, respectively.

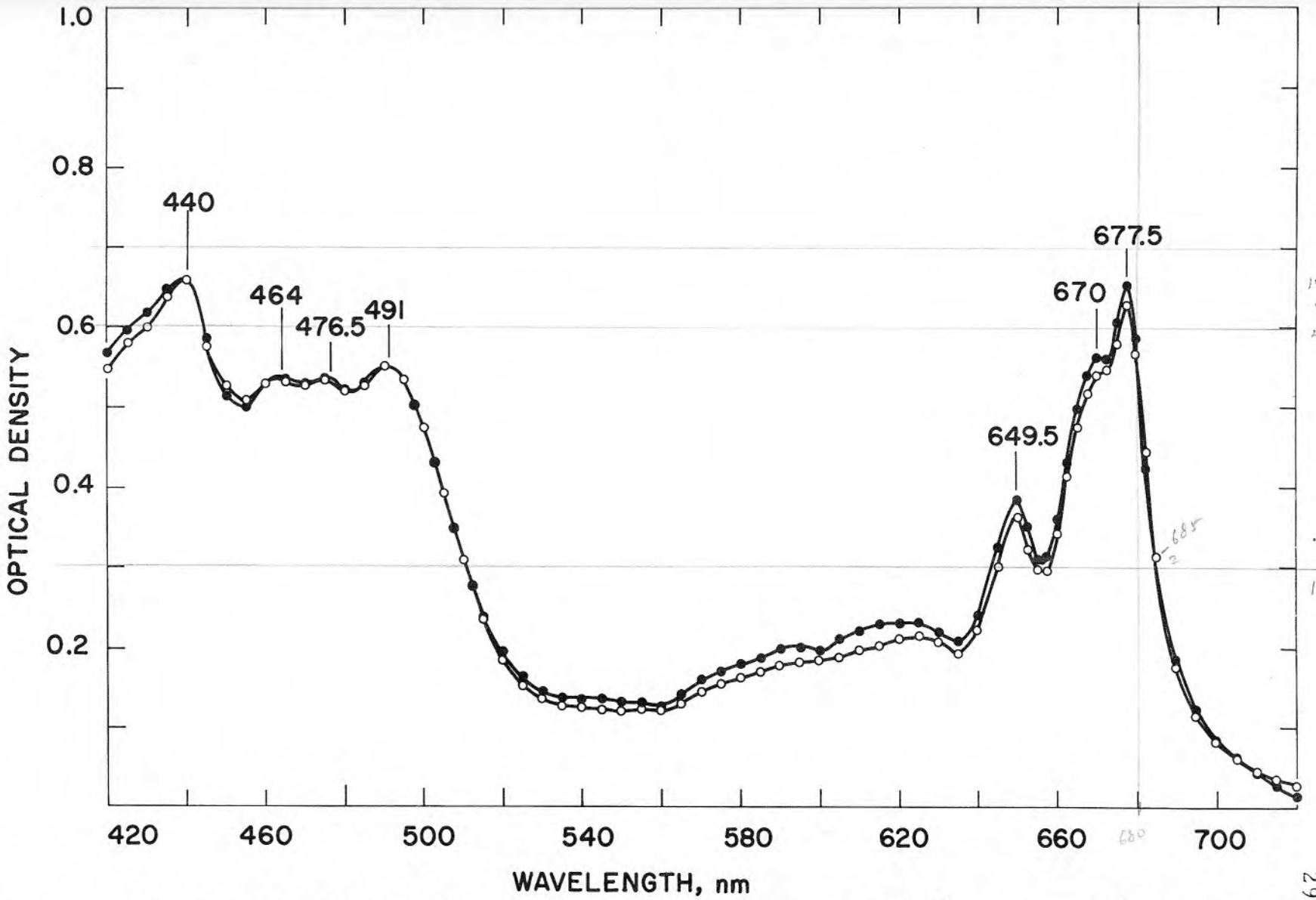


Fig. 5. Absorption spectra of Chlorella pyrenoidosa measured at 4°K ●—● and 77°K o—o—o. H_2O is used as reference. To obtain the base line with negligible absorbance near 750 nm, 0.9 is subtracted from both spectra.

absorption band at 670 nm was only a shoulder at 77°K, but was resolved into a peak at 4°K. Several minor bands beyond 677.5 nm inferred from fluorescence excitation spectra at low temperatures are not resolved in the absorption spectra. In the blue region, four major bands at 440, 464, 476.5 and 491 nm are found. The former three bands are mainly caused by Chl a, carotenoids and Chl b respectively. However, the overlap between the bands is strong. The fourth band is caused by the combined effects of all three pigments, but mainly by the carotenoids. In comparison with room temperature absorption spectra (58, 59), the 677.5 nm and 491 nm bands are much better resolved and the heights of these two bands relative to the other bands increased at 4°K. The room temperature absorption spectra of Chl a, Chl b and carotenoids of Chlorella have previously been resolved by several workers using the curve fitting technique (51, 60, 61). These analyses are in good agreement with the results of this study and the values of the peak positions are shown in Table I.

2. Absorption Spectra of Anacystis nidulans

In Anacystis, the 4°K and 77°K absorption spectra are very similar (deviation is less than 5%) except that the bands at 4°K show slightly better resolution (Fig. 6). The locations of the peaks at room temperature and those at low temperatures are shown in Table II.

In comparison with the room temperature spectrum, the 4°K spectrum shows the following differences: (a) The broad spectrum of carotenoids is resolved into two bands at 465 nm and 502 nm.

Table I. The Absorption Peaks of Chlorella at Low Temperatures and the Analyzed Peak Positions At Room Temperature

in vivo	Analyzed Peak Positions at Room Temperature	Measured Peak Positions at Low Temperatures	
	295°K	77°K	4°K
Chl *a* (red region)	668 nm** 683 nm**	670 nm (shoulder) 677.5 nm	670 nm 677.5 nm
(blue region)	440 nm*	440 nm	440 nm
Chl *b* (red region)	650 nm**	649.5 nm	649.5 nm
(blue region)	480 nm*	477 nm	477 nm
Carotenoids	460 nm* 500 nm*	463-467 nm 491 nm	463-467 nm 491 nm

The analyzed components (at room temperature) are obtained from Govindjee (51)* and Cederstrand *et al* (61)**.

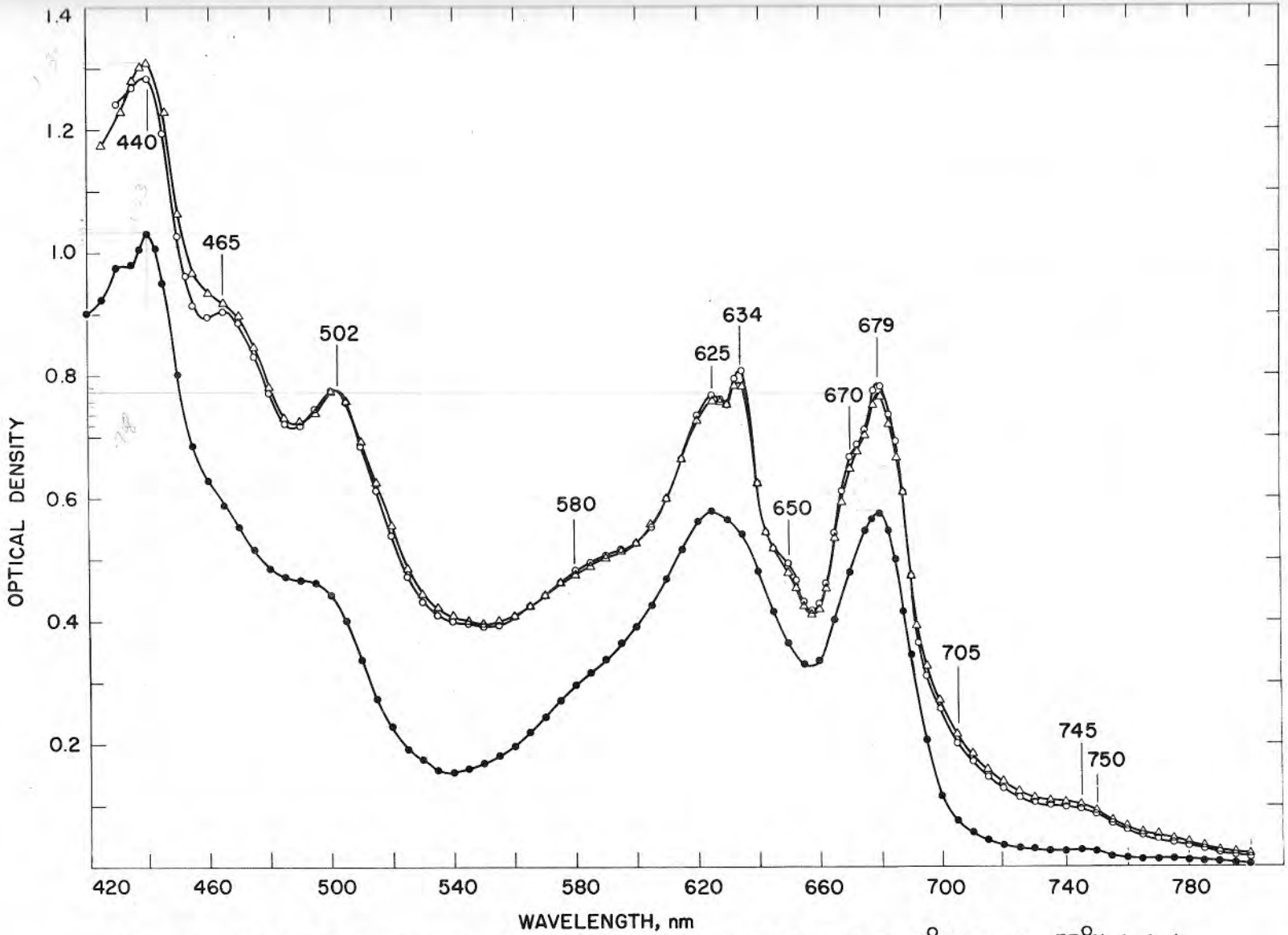


Fig. 6. Absorption spectra of Anacystis nidulans measured at 4°K o—o—o, 77°K Δ—Δ—Δ and 295°K ●—●—●. To obtain the base lines with negligible absorbance near 800 nm, 0.44, 0.40 and 0.08 have been subtracted from the calculated spectra at 4°K, 77°K and 295°K, respectively.

Table II. The Absorption Peaks of Anacystis at Different Temperatures

S = Shoulder, P = Peak

in vivo	295°K	77°K	4°K
Chl a (red region)	679 nm (P; broad)	670 nm (S) 679 nm (P) 686 nm (S) 705 nm (S)	670 nm (S) (slightly) 679 nm (P) sharper than 686 nm (S) at 77°K 705 nm (S)
	750 nm (S)	745 - 750 nm (S)	745 - 750 nm (S)
(blue region)	440 nm (P)	440 nm (P)	440 nm (P)
Phyco cyaning	635 nm (S; not very clear) 625 nm (P) 580 nm (S; not very clear)	650 nm (S; due to allophyco cyanin) 634 nm (P) 625 nm (P) 580 nm (P)	650 nm (S; due to allophyco-cyanin) 634 nm (P) (slightly sharper than 625 nm (P) at 77°K) 580 nm (P)
Carotenoids	490 - 505 nm (S) 460 - 465 nm (S)	502 nm (P) 465 - 470 nm (S)	502 nm (P) 465 nm (P)

(b) The phycocyanin band "splits" into two bands at 625 nm and 635 nm; a shoulder in the 580-600 nm range is also observed. A band at 650 nm, perhaps caused by allophycocyanin (62), is also observed. (c) The Chl *a* band is resolved into two peaks at 670 nm and at 679 nm. In addition, two shoulders at 686 nm and 705-710 nm are observed. A third shoulder at 750 nm, first reported by Govindjee *et al.* (63) at room temperature, is also observed.

C. Discussion

At room temperature, the absorption spectrum for Chlorella has a broad band (1/2 B.W. $\simeq$ 30 nm) at 675 nm and a shoulder near 650 nm in the red region. The former is considered to be an envelope of bands caused by at least two forms of Chl *a* whereas the latter is generally accepted as the Chl *b* absorption band *in vivo*. Several lines of evidence for the multiplicity of Chl *a* forms *in vivo* have been reviewed by Govindjee *et al.* (64). That the 675 nm band is mainly caused by two Chl *a* forms which absorb near 668-672 nm and 677-683 nm is suggested by the derivative absorption spectrophotometry (42, 60, 65, 66), analysis of room temperature absorption spectra (51, 61, 67), low temperature absorption spectra (40), action spectra of the Emerson enhancement effect (68, 69), fractionation and separation of pigment complexes (70, 71) and excitation spectra of Chl *a* fluorescence at low temperatures (44, 72). The evidence that Chl *a* exists in other forms which absorb at longer wavelengths (690-705 nm), is suggested by Chl *a* dichroism (73), action

spectra of the quenching of fluorescence by far-red light (74) and excitation spectra of Chl a fluorescence at low temperatures (46, 75). (Also see chapter V of this thesis.) Some indirect evidence of several Chl a forms in vivo is suggested by the fluorescence yield studies (6) and the fluorescence lifetime analysis (64). Results reported in this chapter confirm the presence of Chl a forms absorbing at 670 nm and 677.5 nm (Chl a (670) and Chl a (677.5)) by direct measurements of the spectra at $4^{\circ}K$, at which temperature these two bands become clearly resolved. This study also confirms the results of Frei (42) and Butler and Baker (40) on Chlorella at $77^{\circ}K$. However, these authors only reported the results in the long wavelength region (600 nm - 720 nm). In the short wavelength region (420 nm - 520 nm), this study shows the resolution of Chl a (440 nm), Chl b (476.5 nm) and the carotenoids (465 nm and 491 nm) absorption bands in Chlorella. The 476.5 nm band is not found in Anacystis because it does not contain Chl b. The carotenoids (465 nm and 491 nm) show lower efficiencies than Chl a and Chl b for sensitized Chl a fluorescence.

Low temperature absorption spectra of Anacystis are not found in the literature. This study shows that it has two bands at 670 nm and 679 nm and shoulders near 686 nm (very weak) becoming more obvious in the excitation spectra shown in chapter VI), 705 nm and 750 nm in the red region. The 686 and 750 nm bands were first observed by Govindjee et al. (63) at room temperature, and they become more obvious at low

temperatures. In the short wavelength region, Chl a at 440 nm and carotenoids at 465 nm and 502 nm are resolved into distinct bands at $4^{o}K$. A shoulder at 650 nm, which is not resolved at room temperature, becomes obvious at $4^{o}K$ and $77^{o}K$. This band might be caused by allophycocyanin (62).

The low temperature absorption spectra of Chl a and Chl b in solution (20% n-propyl ether, 40% propane and 40% propene by volume) were reported by Freed and Sancier (76-78). The absorption bands of both solutions were shifted by 20-25 nm upon cooling from room temperature to $75^{o}K$. The authors interpreted these shifts as solvation effects. This kind of band shift is not found in Chlorella and Anacystis (see Table I and II), suggesting that molecular bondings remain unchanged upon cooling *in vivo*.

In this study, the main purpose of measuring the absorption spectra is to determine the change of absorbance as a function of temperature, which, in conjunction with the data on excitation spectra, enables one to determine the temperature dependence of energy transfer efficiency. This calculation is shown in chapter VI. The absorption data are also used for calculating the relative fluorescence yields at different temperatures. This is done in chapter IV.

In summary, the absorption bands of different chlorophyll components, namely, Chl b (649.5), Chl a (670) and Chl a (678) bands are clearly resolved at $4^{o}K$. The peak positions of these chlorophyll components in the blue region and that of the carotenoids are shown in

Table I and II. The absorption spectra of both algae show very little change upon warming from 4°K to 77°K. The deviations at the main bands are less than 5%.

IV. LOW TEMPERATURE FLUORESCENCE SPECTRA IN ALGAE

A. Introduction

In 1958, Brody (34) discovered a low temperature fluorescence band at 720 nm (F720) in Chlorella. Five years later, Govindjee (35), Bergeron (36), Brody and Brody (37) and Kok (38) independently discovered another fluorescence band in algae near 696 nm (F696) at low temperature ($77^{\circ}K$). Since then many other papers have been published on the low temperature fluorescence spectra of pigments *in vitro* and *in vivo*. These reports may be divided into two categories. The first category is concerned with the low temperature fluorescence spectra of aggregated Chl *a* molecules in different solutions. These spectra were compared to and identified with the long wavelength fluorescence spectra observed in algae (34, 37, 43 and 50). The second category deals with the low temperature fluorescence bands and their excitation spectra *in vivo*. The origin of these bands in terms of the pigment systems and the energy traps of the pigment systems have been investigated by many workers (35, 36, 43-49). Among them, Govindjee (35) and Bergeron (36) were the first to propose that F696 and F720 were emitted from the energy traps (or energy sinks) of system II (SII) and system I (SI), respectively. A brief review of these reports relevant to this study is presented as follows.

In 1963, Govindjee (35) reported the fluorescence spectra of the blue-green algae *Anacystis nidulans* at $77^{\circ}K$. He speculated that F718 and F696 may originate from the energy traps of the two pigment

systems, system I and system II, respectively. This speculation was supported by the fluorescence excitation data; that is, F718 was preferentially excited by system I light (436 nm) when compared to F696, which was mainly excited by system II light (605 nm). F718 was believed to be emitted from the reaction center P700, on the basis of Stokes shift, which predicts that fluorescence is generally shifted 10-15 nm to a longer wavelength with respect to the absorption band. Bergeron (36) reported on the low temperature fluorescence spectra of the same alga, and made a suggestion similar to Govindjee's concerning the sources of F718 and F696. Results parallel to those mentioned above were also found in the red alga Porphyridium cruentum at 77°K by Krey and Govindjee (47).

An important experimental proof showing that SI emits mainly at the long wavelength ($\simeq$ 735 nm) whereas SII emits at 685, 698 and 735 nm was reported by Cederstrand and Govindjee (79) and later independently by Boardman et al. (45). These authors isolated two types of photochemically active particles from the spinach chloroplasts using the method developed by Boardman and Anderson (80). These particles were obtained by incubation of the chloroplasts with digitonin followed by differential centrifugation. It was found (80, 81) that particles sedimenting at 10,000 x g showed properties of pigment system II. The ratio of Chl a to Chl b (2.27) in these particles was lower than that of the chloroplasts (2.83), and they were active in Hill reaction (produce oxygen when TCIP* or ferricyanide was added as oxidant). The particles which

*TCIP: trichlorophenoindophenol

sedimented at around 144,000 x g appeared to represent pigment SI. These particles had a high ratio of Chl a to Chl b (5.34); they were inactive in the Hill reaction but they photoreduced $NADP^+$ * when the electron-donor couple (sodium ascorbate and dichlorophenol indophenol) and both ferridoxin and NADP reductase were added. The fluorescence spectra of the two types of particles were measured at 77°K (45, 79). It was found that the particles which demonstrated system II properties emitted fluorescence at around 685 nm, 698 nm and 735 nm, whereas the fluorescence bands of the "system I" particles were rather weak at around 685 nm but very strong at 735 nm. The ratio of F735 to the total "SI" fluorescence was 0.97 and this ratio for the "SII" fluorescence was 0.6, suggesting that the long wavelength fluorescence (F735) was preferentially excited by SI whereas F685 was preferentially excited by SII. F698 showed higher Chl b to Chl a, (SII), dependence when compared to that of F735. These results favored the interpretations made by Govindjee and Bergeron, who suggested that the 698 nm fluorescence band originated from system II.

Govindjee and Yang (46) reported the fluorescence spectra as a function of temperature (77°K to 298°K). The three bands (F685, F696 and F738) were believed to orignate from three different molecular species, since their intensities varied differently as a function of temperature. In the same paper, the fluorescence spectra were analyzed by Weber's "matrix" method (measurement of the relative intensities of the fluorescence at a set of wavelengths when the cells were excited by

*$NADP^+$: nicotinamide adenine dinucleotide phosphate

another set of wavelengths). The results showed that possibly four molecular species were involved in the fluorescence spectra at low temperature.

The relationship between the fluorescence bands (F698 and F720) and the reaction center was studied by Kok (38). He obtained a mutant strain #8 of Scenedesmus from Bishop; the mutant had no P700. When Kok compared the low temperature (77°K) fluorescence spectrum of the mutant with that of the normal Scenedesmus, he found that the fluorescence band around 725 nm was intense for both samples, but this band was shifted by 5 nm toward the long wavelength region in the mutant. Moreover, F698 was not observed in the mutant sample. This mutant was capable of performing the quinone Hill reaction (SII reaction), although at a lower rate than the normal cells, but was not capable of completing photosynthesis and "photoreduction" (SI reaction). Based on these results, Kok suggested that F698 was related to the pigment system I traps (P700). The absence of P700 might have caused the apparent band shift of the 725 nm fluorescence band, because the fluorescence component near 715 nm was absent.

In summary, most of the experiments performed at low temperatures (except that of reference 38) suggest that F685 and F698 are emitted from SII whereas F718 740 is mainly emitted from SI. It has been proposed (35, 36) that F698 and F718 are emissions from the energy traps. However, the relations between these traps and the chemical reaction centers have not been firmly established.

In this chapter, new information concerning the low temperature fluorescence spectra are found. The measuring temperature has been extended from 77°K to 4°K; furthermore, the spectra at several intermediate temperatures are measured. With the new information, several analyses are made; namely, the fine structure of the complex fluorescence band in the long wavelength region, the increase of the fluorescence yield at low temperatures, the temperature dependence of the transfer efficiency from the bulk of Chl a to the trap, and finally, the evidence of slow and fast transfer rate in photosynthetic organisms.

B. Experimental Results

1. Fluorescence Spectra of Chlorella pyrenoidosa from 4°K to 295°K

It is difficult to obtain consistent results for low temperature fluorescence measurements because peak positions are influenced by the overlap of the neighboring bands, and the relative intensities of these bands vary depending on the concentration of the cells, the rate of cooling and the growth conditions of the culture. Therefore, the peak positions may vary between different experiments by $\pm$ 0.5 nm. The relative heights among F687, F698 and F717 may change many folds by varying the rate of cooling and the concentration of the sample (see Fig. 7). In this experiment, the dilute and the concentrated samples have optical densities of 0.1 and 0.8, respectively, at 675 nm. Quick cooling is obtained by dipping the coverslip spread with Chlorella cells into liquid nitrogen. 77°K is reached within seconds. Slow cooling is obtained by

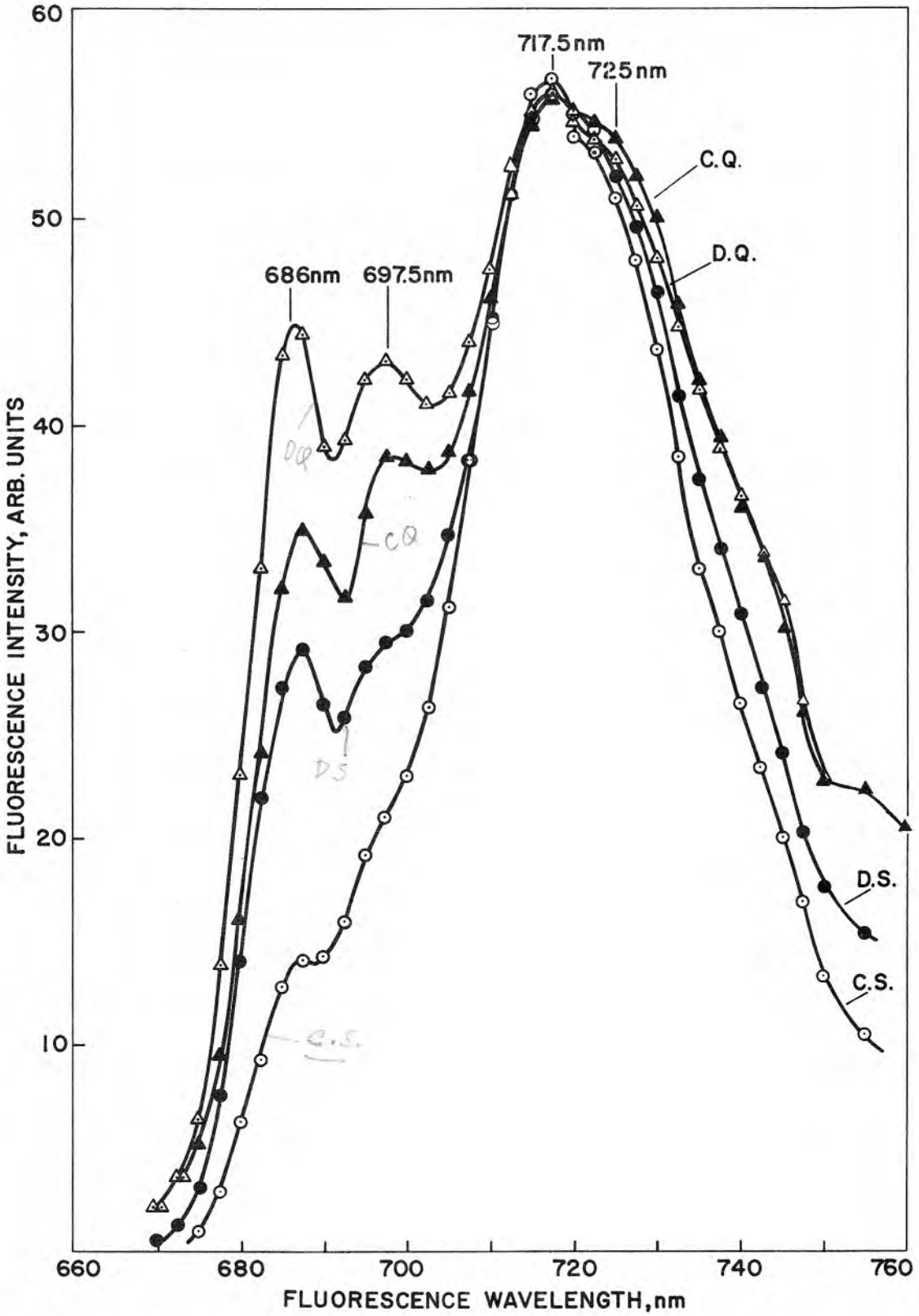


Fig. 7. Fluorescence spectra of Chlorella pyrenoidosa measured at 77°K with two different concentrations and cooling rates; dilute, quick cool (D.Q.) Δ–Δ–Δ, dilute, slow cool (D.S.) ●–●–●, concentrated, quick cool (C.Q.) ▲–▲–▲, and concentrated, slow cool (C.S.) ⊙–⊙–⊙. Excited at 485 nm.

blowing cold air (evaporating liquid nitrogen) onto the sample; freezing is obtained in 1-2 minutes. These spectra indicate how the ice crystal effect and the reabsorption of the fluorescence can influence the fluorescence measurements. These effects will be discussed in more detail in chapter V, where the analysis shows that quick cooling and dilute sample are the appropriate experimental conditions.

Low temperature spectra are measured by first cooling the cells to $4^{\circ}K$ (see chapter II, part C). The spectra at different temperatures are measured as the cells are warming up. The rate of warming is between 5° - $10^{\circ}K$ per minute depending on the temperature range. At $100^{\circ}K$, the rate is approximately $8^{\circ}K$/min. The change of the spectrum caused by the temperature variation during the time of measurement is then corrected by assuming that the fluorescence change as a function of temperature is a continuous function.

Fig. 8 shows the fluorescence spectra of Chlorella from $4^{\circ}K$ to $77^{\circ}K$. The excitation wavelength is at 485 nm. At $4^{\circ}K$, the main fluorescence peak is at 689 nm, with a shoulder at 697.5 nm and a broad complex band near 725 nm. When the temperature is increased to $22^{\circ}K$, F689 (fluorescence at 689 nm) is greatly reduced, and the shoulder at 697.5 nm becomes more obvious. The broad F725 band shows a component near 715-717 nm, and another component near 730 nm. At $36^{\circ}K$, the F697.5 shoulder is resolved into a peak. The rate of decrease of F689 is faster than F697.5 in the $4^{\circ}K$ to $50^{\circ}K$ range. At $51^{\circ}K$, the F697.5 band, which was a shoulder at $4^{\circ}K$, has now become a higher peak than F688.

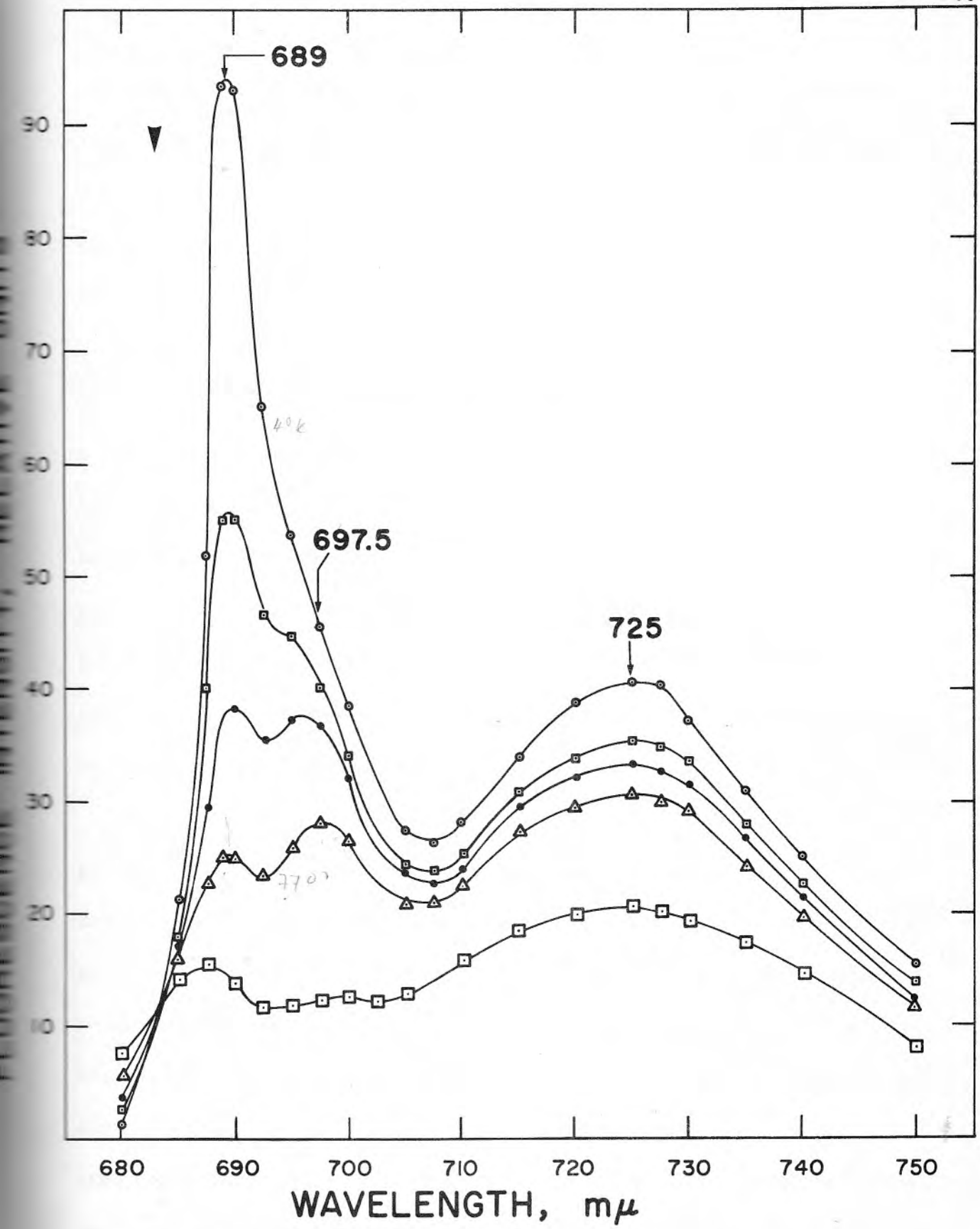


Fig. 8. Fluorescence spectra of Chlorella pyrenoidosa measured at 4°K o—o—o, 22°K ▫—▫— , 36°K •—•—•, 51°K Δ—Δ—Δ and 77°K —⊡— . Excited at 485 nm.

Fig. 9 and 10 show the fluorescence spectra from 77°K to 295°K. At 77°K, which is the boiling point of liquid nitrogen, bands at 686 nm, 697.5 nm, 717 nm and 725 nm are found. Similar band positions (685, 696 and 738 nm) in spinach chloroplasts were reported earlier by Govindjee and Yang (46). Between 80°K and 155°K, there is a sharp decrease of F697.5 and a shift of the 686 nm band to 684.5 nm. The intensity of F685 shows a slight increase ($\simeq$ 5%) while warming up from 80°K to 155°K. From 155°K to room temperature, F685 decreases rapidly as the temperature increases. F725 decreases faster than F717 as the temperature rises. At 155°K, the main fluorescence bands are at 684.5 nm, 714 nm, and 740 nm. The F697.5 and F730 bands are hardly observable at this tmperature. Spectra measured at 187°K, 235°K, 249°K and 295°K show similar relative band heights (i.e. similar normalized spectrum), but the total fluorescence decreases by 6-7 fold as the temperature increases from 187°K to 295°K.

The above figures (8, 9, 10) show that some bands appear as distinct peaks only within a certain temperature range, but are shoulders or are hardly resolvable at other temperatures. This pattern of change is due to the fact that the rate of change of fluorescence intensity as a function of temperature is different for various bands. Therefore the degree of band overlap is also a function of temperature. (Two strongly overlapping bands show a shift of the peaks toward each other, and when one band is much stronger than the other, the latter generally appears as a shoulder.) The long wavelength broad fluorescence band (F725) shows peaks at 715-717 nm,

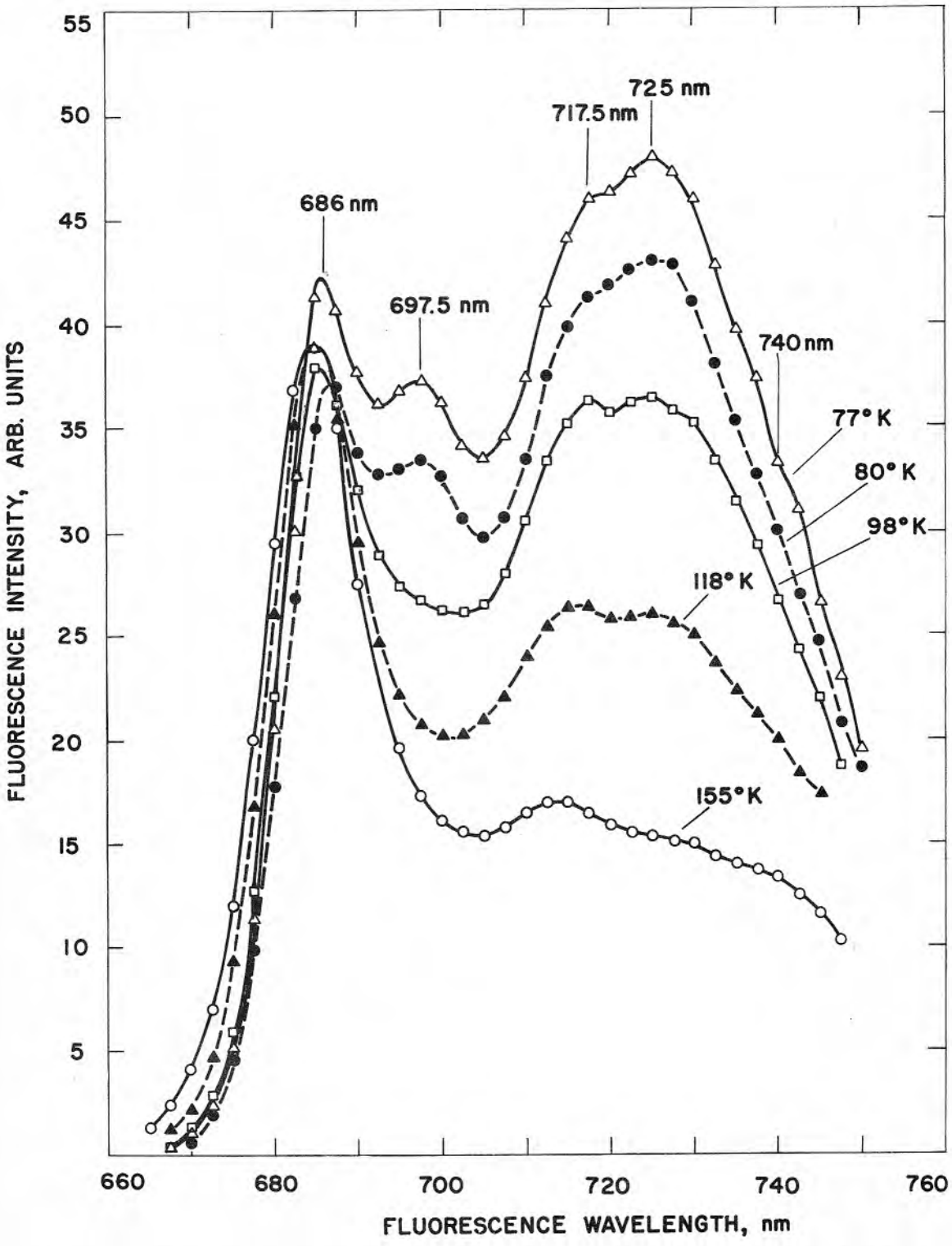


Fig. 9. Fluorescence spectra of Chlorella pyrenoidosa measured at 77°K, 80°K, 98°K, 118°K and 155°K, excited at 485 nm.

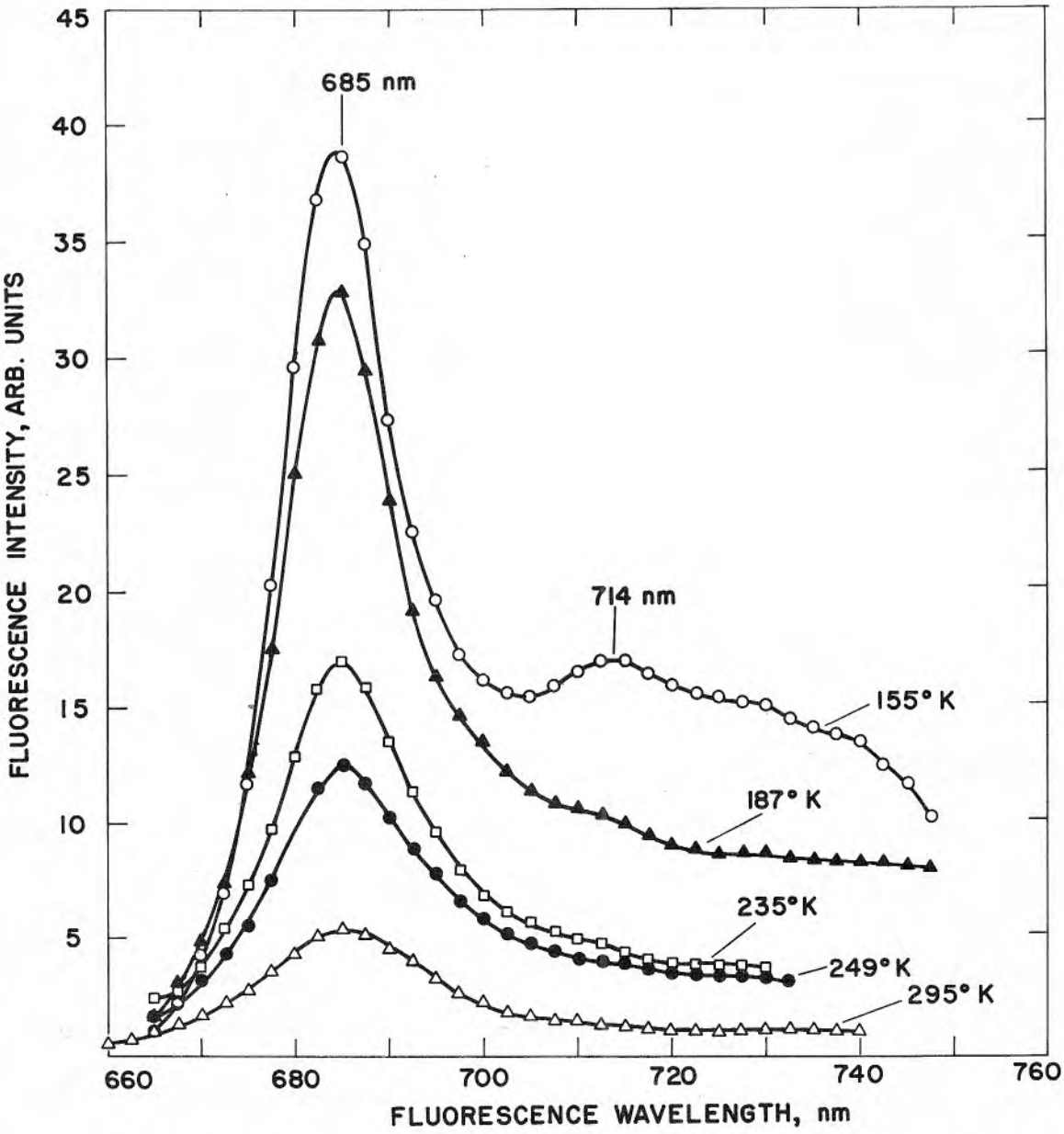
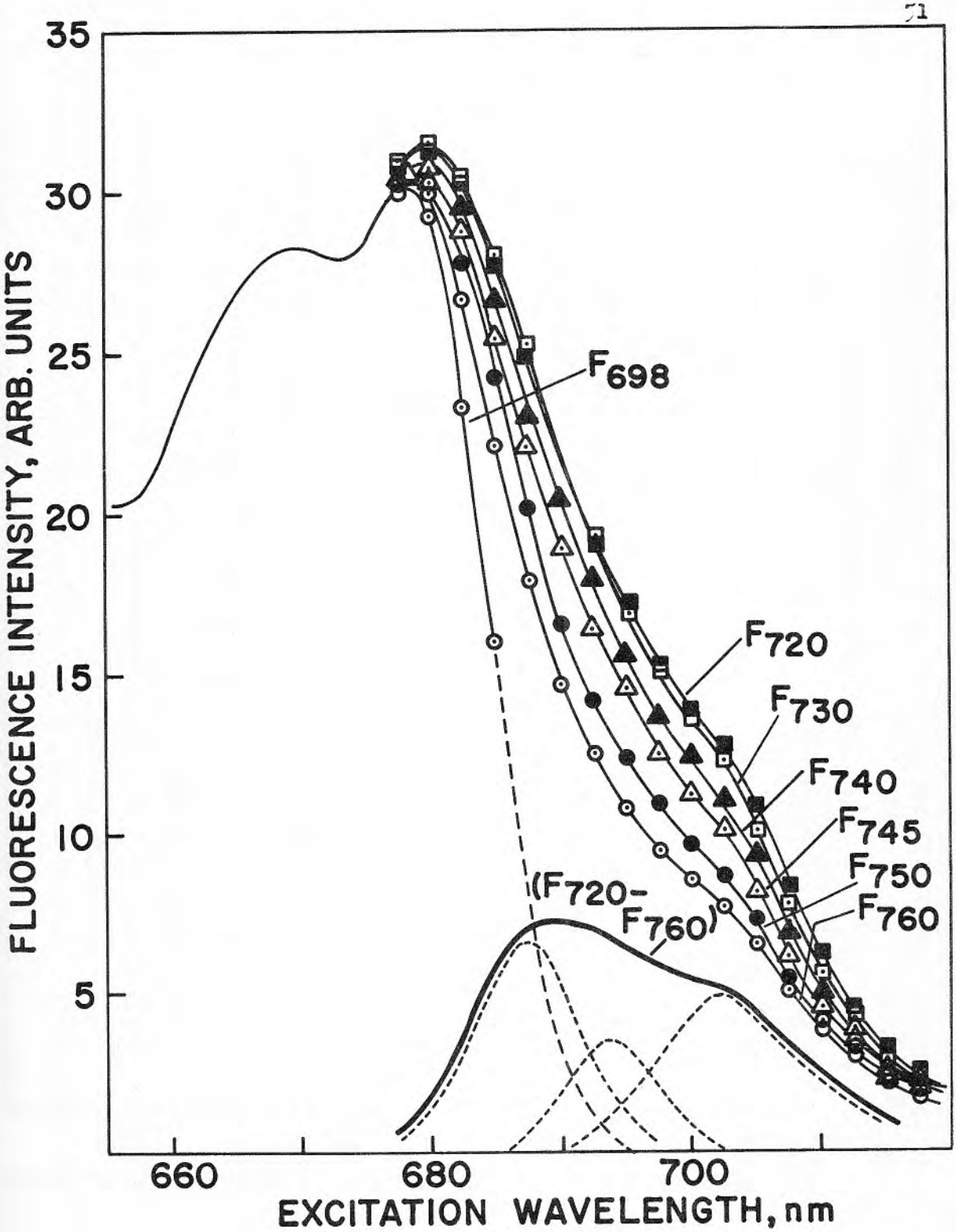


Fig. 10. Fluorescence spectra of Chlorella pyrenoidosa measured at 155°K, 187°K, 235°K, 249°K and 295°K, excited at 485 nm.

725-730 nm and 740 nm at various temperatures. In order to show that all these bands are present at the same temperature ($4^{\circ}K$), the fluorescence excitation spectra of F720 to F760 have been measured at 5 nm intervals (Fig. 11). The analysis of these spectra indicates that all these bands are present at $4^{\circ}K$. Excitation at 650 nm, 670 nm, 675 nm and 680 nm produce almost identical normalized fluorescence spectra in the long wavelength region (720-760 nm). This is shown by the fact that the ratio of fluorescence intensities excited at 650 nm and that excited at the other three wavelengths remain constant for the fluorescence wavelengths between 720 nm and 760 nm. The fact that normalized long wavelength fluorescence spectra are independent of the excitation wavelength within the range of 650 to 680 nm suggests that energy is first transferred from Chl b to Chl a and from Chl a (670) to Chl a (678) before it is further transferred to the longer wavelength Chl a forms. Excitation of longer wavelength Chl a forms, however, causes variation in the fluorescence ratio (see Fig. 12). Excitation at 685 nm causes a sharp decrease of fluorescence in the 750 nm to 760 nm range. Excitation at 695 nm, 700 nm, 705 nm and 710 nm also produce this drop of long wavelength fluorescence. These results suggest that the fluorescence at 750 nm to 760 nm is emitted from a band which obtains excitation energy more efficiently from Chl b, Chl a (670) and Chl a (678) than from the longer wavelength (685-710 nm) chlorophyll forms. Excitation of the broad 683 to 715 nm band is more effective in causing 725 nm fluorescence than causing 760 nm fluorescence. These results suggest that F750 - 760

Fig. 11. Fluorescence excitation spectra of Chlorella pyrenoidosa measured at 4°K. Fluorescence are measured at 698, 720, 730, 740, 745, 750, and 760 nm. The difference spectrum of F760 and F720 can be resolved into three bands with peaks at 687, 694 and 703 nm.


35
30
25
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15
10
5
FLUORESCENCE INTENSITY, ARB. UNITS
660
680
700
EXCITATION WAVELENGTH, nm
F698
F720
F730
F740
F745
F750
F760
(F720-F760)

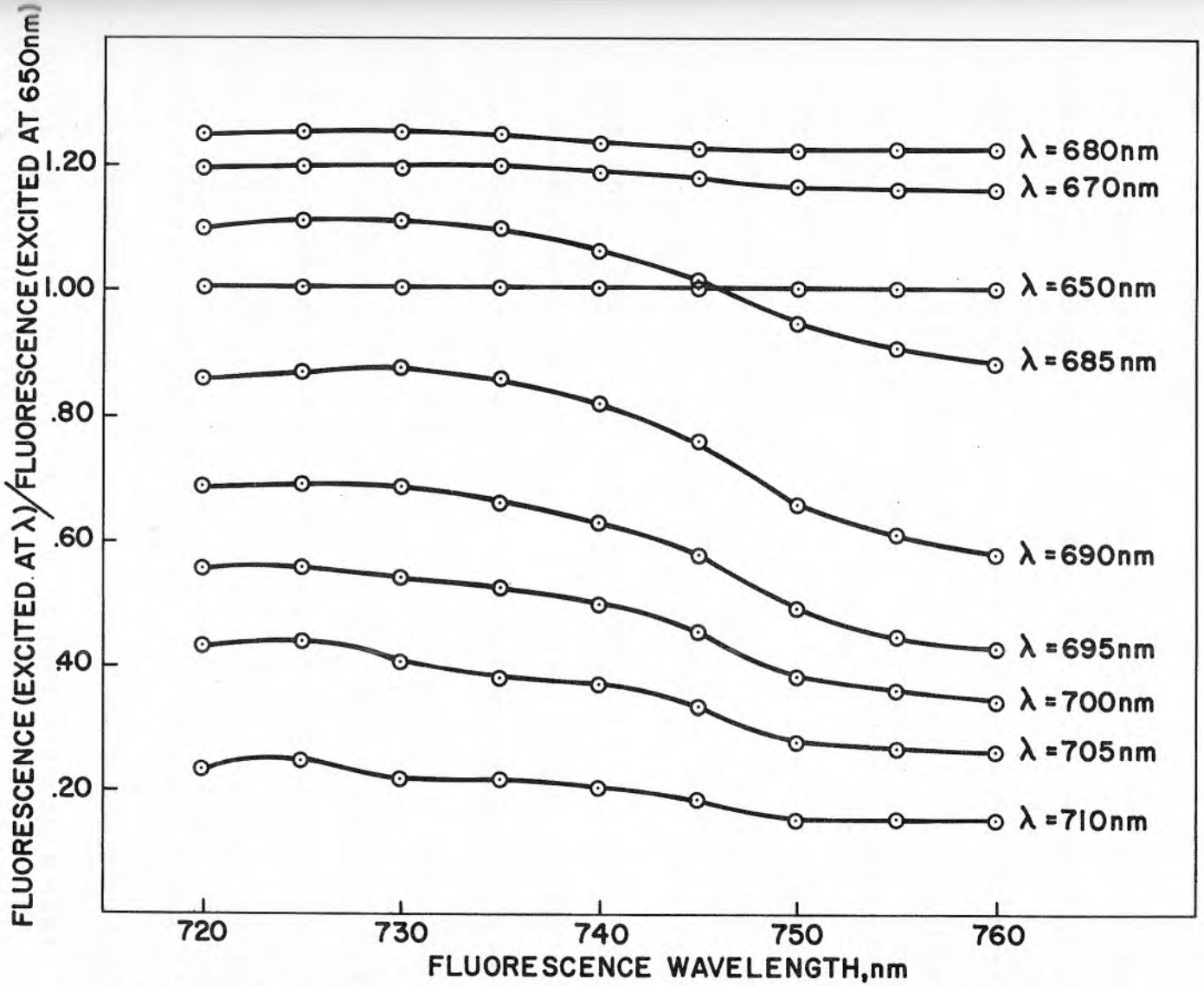

Fig. 12. Ratio of the long wavelength (720-760) fluorescence excited at various wavelengths (λ), and that excited at 650 nm. The data are obtained from the excitation spectra of Chlorella pyrenoidosa measured at 4°K.

nm is partially related to SII, whereas F725 is mainly related to the broad SI absorption band (685-710 nm). Excitation at 705 nm and 710 nm show another plateau in the 730 nm to 745 nm range (Fig. 12). This plateau, indicating a lower fluorescence yield for F740 than for the F725 band when Chl a (705-710) are excited, suggests that F740 is also a distinct fluorescence band. This band is also observable in the spectrum measured at 155°K. In summary, these long wavelength fluorescence bands which become obvious at different temperatures do exist simultaneously at 4°K.

The total fluorescence at 4°K is about 2.2 times of that at 77°K and about 14 times of that at 249°K and about 35 fold of that at room temperature. The absorption increase upon freezing has not been taken into account for the above calculations, but an estimate can be made: According to the absorption measurement of thin sample, the absorption at 485 nm increases by 20% upon cobling from 295°K to 77°K, and increases by 2% from 77°K to 4°K. Latimer (32) reported that the quantum yield of fluorescence ($\bar{\Phi}$) of Chlorella at room temperature is 2.7%. Combining the information of fluorescence and absorption increases at low temperatures and the known value of $\bar{\Phi}$ at room temperature, $\bar{\Phi}$ at low temperature can be calculated. At 77°K, $\Phi \simeq 32\%$; at 4°K, $\Phi = 78\%$. These values are reasonable when compared to the value of Φ of Chl a in ethanol, which has been reported by S. B. Broyde and S. S. Brody (50) to be 83-85% at 4°K. $\bar{\Phi}$ of spinach chloroplasts was reported by Boardman et al. (45) to be 20% at 77°K. Govindjee and Yang (46)

earlier reported a 20 fold increase of fluorescence upon cooling spinach chloroplasts from 295°K to 77°K. The increase of Φ for Chlorella obtained here is 12 and 29 fold for 77°K and 4°K, respectively.

To summarize the above analyses, F685 band of Chlorella at room temperature becomes a very strong band at 4°K and is shifted to 689 nm. At low temperatures, F697.5 and the broad F725 bands are found. Because the fluorescence intensity of different bands varies differently as a function of temperature, the fluorescence spectra measured at different temperatures provide good resolutions for different bands. The broad F725 band can be resolved into 715-717 nm, 725-730 nm and 740 nm bands. An additional band at 760 nm is shown to be present by the comparison of different excitation spectra of the long wavelength fluorescence. The fluorescence quantum yield of Chlorella is 32% at 77°K and 78% at 4°K when 2.7% at 295°K is taken as reference.

2. Fluorescence Spectra of Anacystis nidulans from 4°K to 295°K

The fluorescence spectra of Anacystis are very different depending upon the excitation wavelength. At room temperature, excitation at 560 nm (phycocyanin) causes a broad phycocyanin fluorescence band at 653 nm and a shoulder near 680-685 nm (caused by Chl a). Excitation at 435 nm (Chl a) produces a very weak fluorescence band near 685 nm.

When the cells are cooled to 77°K, the fluorescence intensity increases, and additional bands at 696 nm and 715 nm are found. These results have previously been reported by Govindjee (35), Bergeron (36)

and by Bergeron and Olson (49). They are in fair agreement with those of this study with the exception that the relative heights of the bands are different, probably because of differences in cell concentration and the rate of cooling.

Fig. 13 shows the fluorescence spectra of Anacystis excited at 560 nm. When the cells are cooled to $4^{\circ}K$, the fluorescence intensity is increased. The broad F715 band becomes a peak at 712 nm, with a shoulder near 720 nm. (The 712 nm band was earlier observed by Krey and Govindjee (47) in Porphyridium at $77^{\circ}K$). The fluorescence intensity increases 4-5 times upon cooling from $295^{\circ}K$ to $4^{\circ}K$. However, since the absorption increases by approximately 100% at 560 nm, the net increase of fluorescence yield is about 2 - 2.5 times. Fig. 14 shows the low temperature fluorescence spectra of Anacystis when excited at 435 nm. The fluorescence at 712-715 nm increases tremendously upon cooling. At room temperature, these fluorescence bands are very weak, a 15-17 fold increase of fluorescence intensity at 715 nm is found when the cells are cooled from $295^{\circ}K$ to $77^{\circ}K$, and a 70 fold increase at this wavelength is found when the cells are cooled from $295^{\circ}K$ to $4^{\circ}K$. The total fluorescence intensity (including F687 and F698) increases about 17 fold upon cooling from $295^{\circ}K$ to $4^{\circ}K$; after correction for absorption change at 435 nm, a 13-14 fold increase of fluorescence yield is obtained.

The irregular changes of the fluorescence spectrum caused by the phase transitions of ice crystals (see chapter V) are also found in Anacystis. Near the temperature where ice changes from cubic to

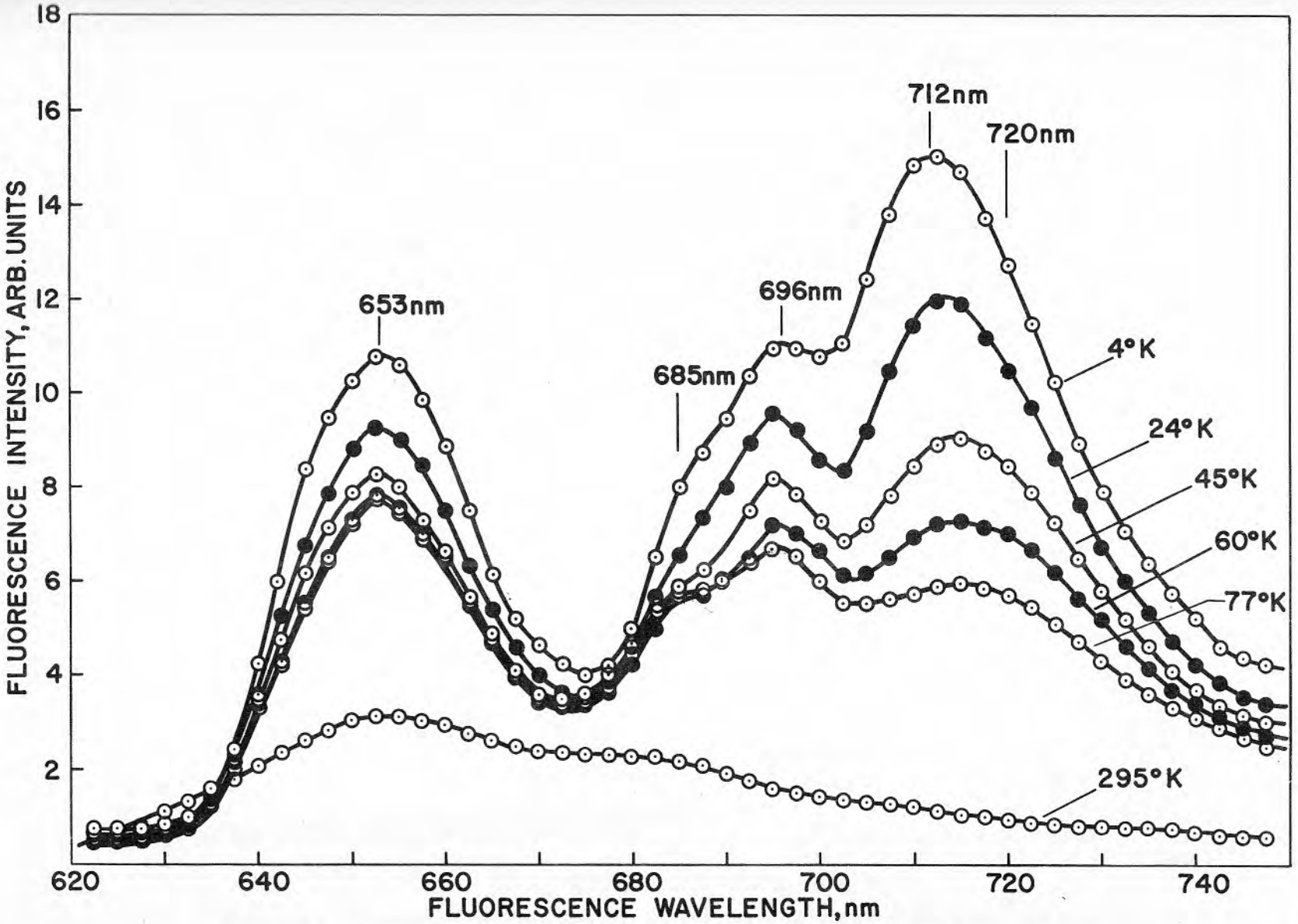

Fig. 13. Fluorescence spectra of Anacystis nidulans excited at 560 nm, measured at 4°K, 24°K, 45°K, 60°K, 77°K and 295°K.

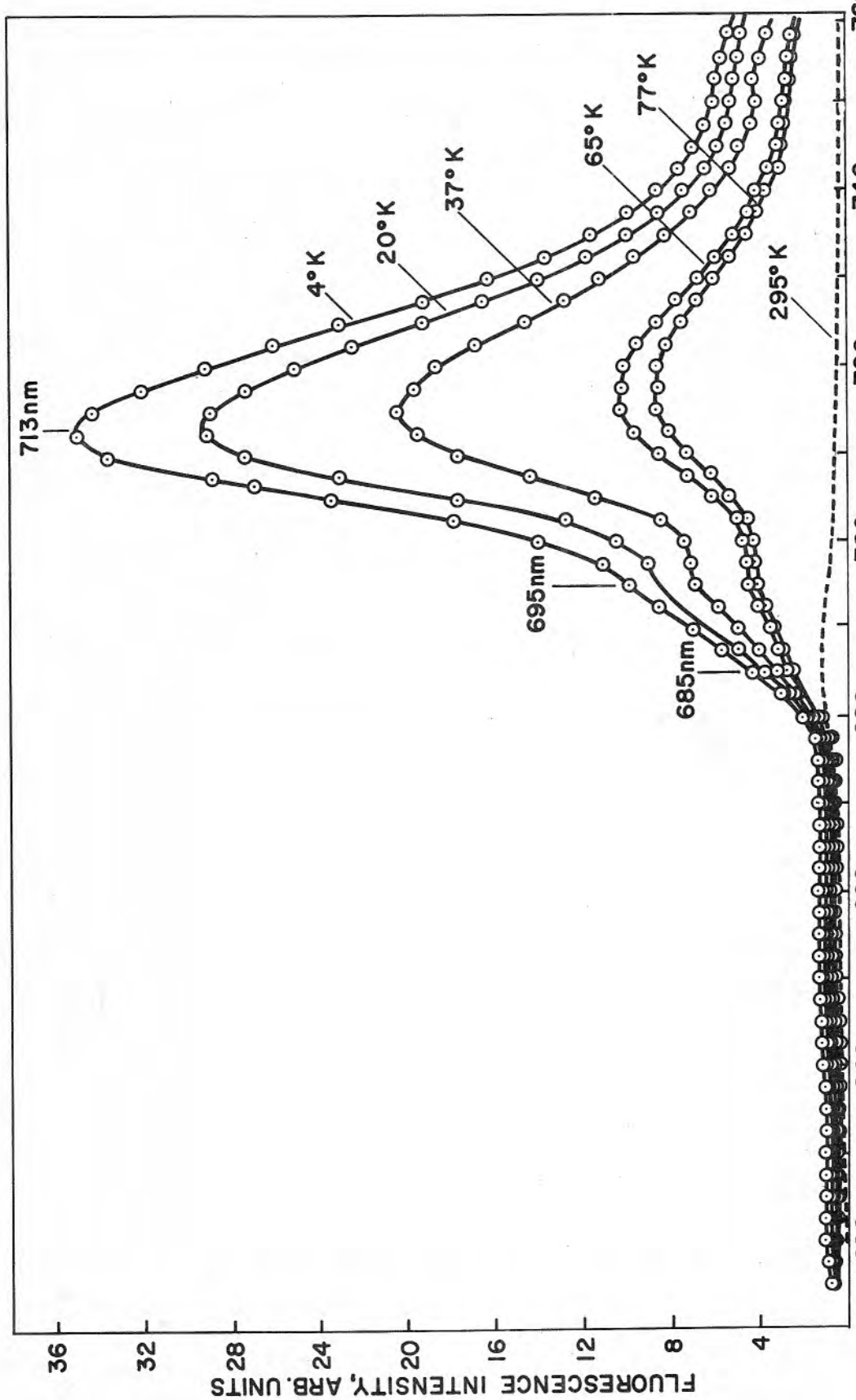

Fig. 14. Fluorescence spectra of Anacystis nidulans excited at 435 nm, measured at 4°K, 20°K, 37°K, 65°K, 77°K and 295°K.

hexagonal phase (200-220°K), the fluorescence intensity at 653 nm begins to increase upon further warming. This increase of fluorescence near 190°K - 220°K was also found in Porphyridium by Krey and Govindjee (47). Since ice changes phase only in the temperature range from 113°K to around 220°K (chapter V), spectrum in the temperature range of 4°K to 77°K is not complicated by ice changing phase. Furthermore, the spectrum in that temperature range shows higher fluorescence yield, therefore, the following discussion will be centered on the measurements in the 4°K to 77°K range.

C. Discussion

The fluorescence spectra in Fig. 8 and Fig. 13 show variations in band intensity and relative band heights at different temperatures. In order to understand these variations, one must first consider all the possible reasons that can cause a fluorescence change. The observed results can be one or a combination of several of the following factors.

1. Quenching of Fluorescence by Thermal Agitation:

Quenching of fluorescence is different at different temperatures depending on the quenching processes. There is no competition between fluorescence and photosynthesis for quenching energy from 4°K to 77°K since most of the "steady state" photosynthesis has stopped in that temperature range. Change of quenching rate due to ice changing phase is not important in the 4°K to 77°K range. Quenching of fluorescence due to collision or molecular vibration should be the main factor which causes

fluorescence change. As the temperature rises, the molecular vibration increases, and this motion enhances the rate of internal conversion. Therefore the fluorescence yield decreases. The experimental result of this study shows a 2.2 fold decrease of the total fluorescence yield of Chlorella (excited at 485 nm) when the cells are warmed from 4°K to 77°K, and a 1.6 fold decrease for Anacystis (excited at 560 nm) in that same temperature range. Unfortunately, one cannot determine the fluorescence yield of individual molecular species on the basis of the total fluorescence yield, because the transfer efficiency between different species may vary as a function of temperature, and the collision and vibrational quenching efficiencies are generally different for different molecular species (82).

2. The Effect of Transfer Efficiency on Fluorescence Spectrum:

As mentioned above, the change of transfer efficiency may cause a difference in the relative heights of the fluorescence bands. The results from excitation spectra (see chapter VI) measured at different temperatures indicate that the transfer efficiencies from Chl b to Chl a and from Chl a (670) to Chl a (678) are independent of temperature from 4°K to 77°K in Chlorella. However, the transfer efficiency from Chl a (678) to longer wavelength Chl a forms (686-700 nm) increases upon warming. The fluorescence spectra of Chlorella as a function of temperature (Fig. 8) also show evidence that the efficiency of energy transfer from Chl a (678 nm) to the species emitting at 698 nm is temperature dependent. The evidence is based on the fact that F698 and F687 change in a complementary manner. At 77°K, the F698 band has a lower intensity than the

F687 band, but the former increases faster as the temperature is lowered. At 51°K, the relative height of the bands has reversed, the F698 band becomes a stronger band. This fast increase of the F698 band can be due to the increase of fluorescence yield when the thermal energy kT is lowered. It has been speculated (35) that F698 is an emission band from the trap (or energy sink). When kT decreases, the chance of having the excitation energy in the trap level which "boils" back to the bulk is decreased, and causes an increase in the trap fluorescence. However, at still lower temperatures, the fluorescence from the bulk chlorophyll (F689) increases sharply while that from the trap (698) increases slowly. At 36°K, the relative height of the bands is reversed, the F689 band becomes almost twice as high as the F698 band at 4°K. This sharp increase of the bulk fluorescence, accompanied by a slowing down in the change of the trap fluorescence, suggests a decrease of energy transfer from the bulk chlorophyll to the trap level. This result can be considered as an experimental evidence of the theoretical prediction of Robinson (29), who pointed out that energy transfer between heterogeneous molecules should obey the resonance transfer model which was developed by Perrin (83) and carefully derived by Förster (30, 31). This model shows that the rate of energy transfer is generally temperature dependent. This is so because the transfer rate is proportional to the overlap between the fluorescence spectrum of the donor and the absorption spectrum of the acceptor molecules. Because of the band sharpening at low temperatures, the transfer rate generally decreases upon cooling, or if there is a perfect

overlap, then there would not be any change upon cooling). In this case, the trap level, which is created by a different molecular species than the bulk molecules, can be expected to have a change of transfer rate upon cooling. This change is proportional to the band overlap between the fluorescence band of the bulk, i.e. F687-F689 band, and the absorption band of F698. The studies on excitation spectra (chapter VI) show that F698 is probably emitted from a species of very low concentration around 683-688 nm, which is determined on the basis of a 10-15 nm Stokes shift. The decrease of band overlap upon cooling can explain part of the observed effects. Another experimental evidence showing that the transfer efficiency between heterogeneous molecules decreases at very low temperature is demonstrated by the transfer from phycocyanin to Chl a in Anacystis. This result and its analysis is presented in chapter VI. The temperature dependence of the transfer efficiency from Chl a (678) to the longer wavelength Chl a forms and that from phycocyanin to Chl a suggests that unless the transfer rate between these heterogeneous molecules are in the order of magnitude of the lifetime of fluorescence, the transfer rate among Chl a (678) and that among the phycocyanin molecules would also be temperature dependent. This is so because transfers between heterogeneous molecules are likely to be a single irreversible step, whereas there are numerous transfer steps (see chapter 1, part B) between homogeneous molecules (Chl a (678)). Therefore, if the transfer rate between heterogeneous molecules is slow (say 10^{-10}-10^{-12} sec) but is much faster than the fluorescence lifetime ($\simeq 3.1 \times 10^{-9}$ sec, see ref. 87), then this step

would not affect the transfer efficiency. Only when the transfer time of this step is in the same order of magnitude as the fluorescence lifetime would it affect the transfer efficiency; otherwise, the change of transfer efficiency would have been caused by the temperature dependence of transfer efficiencies among the Chl a (678) molecules. Additional causes for the observed sharp rise of the F689 band in Chlorella near $4^{\circ}K$ are discussed in parts 3 and 4.

3. The Effect of Half Bandwidth on the Peak Height of a Fluorescence Band:

The peak height will increase when the half bandwidth is decreased even if there is no change in transfer efficiency and fluorescence yield. The increase of the peak height is roughly proportional to the decrease of the half bandwidth, i.e. the product of the half bandwidth and the peak height remains almost constant at different temperatures when there is no change in fluorescence yield and transfer efficiency. Under such conditions, a two to three fold increase of the F689 peak intensity is expected when the half bandwidth decreases from 12-15 nm (at $77^{\circ}K$) to 4-7 nm (at $4^{\circ}K$).

4. The Effect of Decreasing Intermolecular Distance upon Transfer Rate:

Considering only the dipole-dipole interaction, Förster (30, 31) derived the relation between transfer rate and intermolecular distance (R). For weak interaction (resonance transfer), the $\frac{1}{R^6}$ dependence of transfer rate is expected, whereas for strong interaction, $\frac{1}{R^3}$ dependence is expected. Goedheer (44) suggested that there could be a strong increase of transfer rate as a result of structural shrinkage at low temperatures. The

effect of this shrinkage will be estimated.

Weber (33) found evidence that Chl a in vivo do not show extensive rotation, possibly because they are quite rigidly bound to the lipid (or lipoprotein) environment. Therefore, the shrinkage of the lipid can be used as an approximation for the change of the intermolecular distance between the chlorophyll molecules. The coefficient of linear expansion of wax is $4.86 \times 10^{-4}/^{o}C$, that of glycerine is $1.62 \times 10^{-4}/^{o}C$, and that of olive oil is $2.27 \times 10^{-4}/^{o}C$ (84). Since these values for the molecules with chemical structures related to the lipids are all in the same order of magnitude, it is reasonable to assume that the coefficient of linear expansion of the lipid in the grana is also in that order of magnitude. The following calculation assumes that this coefficient is about $3 \times 10^{-4}/^{o}C$. The equation for linear expansion is $L = L_o (1 + \beta T)$, where L, L_o are the linear dimensions of the material at temperatures equal to $T^{o}C$ and $0^{o}C$, respectively, β is the coefficient of linear expansion. Since the intermolecular distance (R) is directly proportional to L, the ratio of the $R_{77^{o}K}$ (R at $77^{o}K$) and $R_{4^{o}K}$ can be calculated as the following:

$$\frac{R_{77^{o}K}}{R_{4^{o}K}} = \frac{R_o (1 - 3 \times 10^{-4} \times 196)}{R_o (1 - 3 \times 10^{-4} \times 269)} = 1.024$$

Assuming no change of band overlap and no change of dipole orientations of the molecules due to cooling, an estimate of the change of transfer rate caused only by the variation of the intermolecular distance is shown as follows. The ratio of the transfer rate (T.R.) for resonance transfer at the two temperatures are:

$$\frac{\text{T. R. } (4^{\circ}\text{K})}{\text{T. R. } (77^{\circ}\text{K})} = \left(\frac{R_{77^{\circ}\text{K}}}{R_{4^{\circ}\text{K}}} \right)^{6} = 1.153$$

The calculation shows that when R is decreased due to cooling from 77°K to 4°K, an increase of about 15% of transfer rate is expected (This is not true when the decrease in transfer rate caused by the changes in band overlap and possible changes of the dipole orientation is present). A range of the values of β from 1×10^{-4} to $6 \times 10^{-4}/^{\circ}$C will provide the ratio from 1.045 to 1.355, i.e., the change of transfer rate would possibly be between 4.5% to 35.5% depending upon the exact value of β.

For the case of strong coupling (29, 31), T.R. $\alpha \frac{1}{R^3}$, the decrease of intermolecular distance has less effect on T.R. than in the weak coupling case (T.R. $\alpha \frac{1}{R^6}$). With the same assumptions as those mentioned above, the ratio of the transfer rate for a strong coupling case becomes:

$$\frac{\text{T. R. } (4^{\circ}\text{K})}{\text{T. R. } (77^{\circ}\text{K})} = \left(\frac{R_{77^{\circ}\text{K}}}{R_{4^{\circ}\text{K}}} \right)^{3} = 1.007$$

When the cells are cooled from 77°K to 4°K, a range of β from 1×10^{-4} to $6 \times 10^{-4}/^{\circ}$C will cause an increase of transfer rate from 2.3% to 16.4%. For $\beta = 3 \times 10^{-4}/^{\circ}$C, the increase of T.R. is 7.4%. (These calculations only account for the changes caused by the intermolecular distance, the possible change of the dipole orientation or the effect of quadrapole have been neglected.) The transfer rate has generally been considered to be independent of temperature in the strong coupling case (31) because the dipole-dipole interaction energy

(proportional to $\frac{1}{R^3}$) is assumed to remain constant at different temperatures. As shown above, this assumption is only approximately correct.

In the previous paragraphs, the change of transfer rate (T. R.) as a function of intermolecular distance has been discussed. However, transfer efficiency (T. E.) rather than transfer rate is the factor that bears a direct effect on the fluorescence intensity. A qualitative relation between T. E. and T. R. is discussed below.

Because there is a direct competition among fluorescence, internal conversion and T. E., the orders of magnitude of the rates of these processes are important. When T. R. is much faster than that of internal conversion and fluorescence, energy is transferred long before the other two processes can occur. Therefore, T. E. approaches 100%. (Duysens (6) showed that the transfer efficiency from Chl b to Chl a is about 100%.) In that case, small change of transfer rate and internal conversion rate will not affect the transfer efficiency significantly as long as T. R. $\gg$ internal conversion rate and fluorescence. When T. R. is in a comparable order of magnitude as internal conversion or fluorescence, a decrease of T. R. or an increase of internal conversion rate can cause a decrease in T. E.

Results in chapter VI show that T. E. from Chl b to Chl a is independent of temperature and T. E. in Chlorella is very high ($\simeq$100%) in the temperature range of 295°K to 4°K. This observation can be explained if T. R. is much faster than the internal conversion and

fluorescence processes. Unfortunately, the constancy of T.E. as a function of temperature does not predict small changes in T.R. when transfer efficiency is high. However, a decrease of T.E. as the temperature is lowered does suggest that T.R. has decreased. This is so because the rate of internal conversion has slowed down at low temperatures,* and in order to obtain a decrease in transfer efficiency, the only obvious solution is to have a lower transfer rate. The decrease of transfer efficiency from phycocyanin (and allophycocyanin) to Chl a in Anacystis and that from Chl a (678 nm) to the "trap" in Chlorella suggests temperature dependence of the transfer rate, which is in agreement with the theoretical prediction (29, 31, 85) of resonance transfer model between heterogeneous molecules.

The time required to transfer a quantum of energy from Chl b to Chl a (678) (i.e. the total amount of time required for a quantum of energy to transfer among the Chl b molecules until it is transferred to Chl a (670), then transferred among the Chl a (670) molecules until it is finally transferred to Chl a (678)) is much shorter than the lifetime (τ) of fluorescence even at low temperatures (τ at 77°K = 3.1 nsec, ref. 85). This is shown by the high transfer efficiency ($\simeq$ 100%) and the lack of fluorescence from Chl b and Chl a (670)** throughout the 4°K to

*The high fluorescence yield ($\simeq$78%) in Chlorella at 4°K shows that the rate of internal conversion is slow when compared to that of fluorescence. At room temperature, the internal conversion rate is high even when there is no photosynthesis (poisoned cells); the fluorescence yield is lower than 10% (33). These facts indicate that the rate of internal conversion decreases as the temperature is lowered.

**F687 is generally (43, 86) believed to be an emission of Chl a (670), but this belief is shown to be wrong in chapter VI.

77°K temperature range. This result, in addition to the finding that transfer efficiencies from Chl b to Chl a and from Chl a (670) to Chl a (678) are independent of temperature (see chapter VI), favors the suggestion of Robinson (27) which predicts fast transfer rate and strong coupling energy among the Chl b molecules and among the Chl a molecules, provided that the number of transfers among the Chl b and Chl a (670) molecules is large. However, the number of transfers between heterogeneous molecules is not yet certain. There is a possibility that Chl b molecules are distributed among the Chl a molecules such that there is no (or very few) transfer among Chl b molecules. Therefore, even if the transfer rate from Chl b to Chl a is slow, the transfer efficiency can still be independent of temperature and approach 100%. This is so because in this case, only one (or a few) transfer within the lifetime of fluorescence is required to obtain a high transfer efficiency. There is little doubt that the transfer from Chl b to Chl a and that from Chl a (670) to Chl a (678) (transfers among the heterogeneous molecules) are irreversible, otherwise the high transfer efficiencies ($\simeq$100% shown in chapter VI) cannot be explained.

In summary, the peak intensities of different fluorescent bands vary as the temperature rises. The variations may be caused by the increasing collisional quenching (or internal conversion), band broadening, changes in transfer rate caused by thermal expansion, and by the temperature dependence of the overlap integral (30). The complexity caused by these factors makes the precise calculation of the transfer

efficiency as a function of temperature very difficult by studying the fluorescence spectrum alone. However, the complementary manner of changes of F687 and F698 suggests that the energy transfer efficiency from the bulk Chl a to the trap level decreases upon cooling. This finding, in addition to the same conclusion obtained from the excitation measurements in chapter VI, confirms the theoretical prediction of Robinson (29) who proposed that the transfer rate between different molecular species (like the bulk Chl a and the trap) are temperature dependent. Furthermore, unless the transfer rate between the heterogeneous molecules are in the order of magnitude of the lifetime of fluorescence, the transfer rate among Chl a (678) and that among the phycocyanin molecules would also be temperature dependent. Two lines of evidence are found in support of the fast (or intermediate) transfer rate between Chl b and Chl a (670) provided the number of transfers among the Chl b molecules and that among the Chl a (670) molecules are large. If Chl b and Chl a are nearest neighbors, or the number of transfer is not large, the resonance transfer model may also be used to explain the observed results.

V. ICE CRYSTAL EFFECTS ON FLUORESCENCE SPECTRA IN VIVO

A. Introduction

In recent years, a number of investigators have studied the structure of ice crystal at low temperatures using methods such as X-ray diffraction (88), electron diffraction (89) and thermal analysis (90). It is generally agreed that there are three possible phases of ice at low temperatures, namely, vitreous (or amorphous), cubic and hexagonal.

In the vitreous phase, molecular arrangement is random, or amorphous. This phase occurs at temperatures below 113°K (88) and changes irreversibly to the cubic phase upon warming. The easiest way to obtain ice in vitreous phase is to deposit water vapor on cooled surface below 113°K. Luyet (91) claimed that quick cooling of a thin film of water by dipping it into liquid air was also successful, but Dowell and Rinfret (88) were unable to repeat this experiment. Between 113°K and 153°K, the vitreous phase changes to cubic phase. There is a mixed vitreous-cubic ice in this temperature range. The rate of change is exponentially dependent on the temperature. With the warming rate of 5 degrees per minute (close to the rate of warming of the present studies), the conversion appears to take place suddenly near 143°K (88). (Different authors obtain different temperatures $\pm$ 10°K for this phase change.) In cubic phase, oxygen atoms are located in a diamond cubic lattice, the calculated density at 88°K is 0.923 gm/cc. (This density is identical with that of the hexagonal phase within experimental error.) Cubic

phase changes irreversibly to hexagonal phase upon warming from 143°K to 213°K. The rate of change is again exponentially dependent on temperature. With the rate of warming of 5°K/min., the conversion takes place rather quickly near 213°K (88). (The exact temperature for this phase change again deviates by ± 10°K (92).) Owston (93) reviewed studies on the structure of hexagonal ice. This form can be considered as being built up by layers of oxygen and hydrogen atoms, where the oxygen atoms form a network of hexagonal rings. This is the most stable form of ice since conversion to this phase is irreversible.

There seems to be a relation between the change of the fluorescence spectrum and the phase transitions of ice crystals. As mentioned above, vitreous ice changes to the cubic phase near 143°K (or from 123°K to 158°K according to a review by Blackman and Lisgarten (92)). There are several changes in the Chlorella fluorescence spectrum near 140°K to 175°K. These changes are as follows:

1) F680 increases upon warming from 4°K to 140°K. After reaching a maximum at around 140-175°K, the fluorescence intensity of F680 declines upon further warming (see Fig. 8 and 15).

2) The intensity of F698 as a function of temperature shows a sudden change of slope near 140-175°K (see Fig. 15). This change is also found in spinach chloroplasts (46).

3) As the temperature is increased from 80°K to 155°K, the fluorescence peak at 687 nm is shifted to 684.5 nm. There is also a large change of slope of F687 as a function of temperature at 160°K (see Fig. 9 and 15).

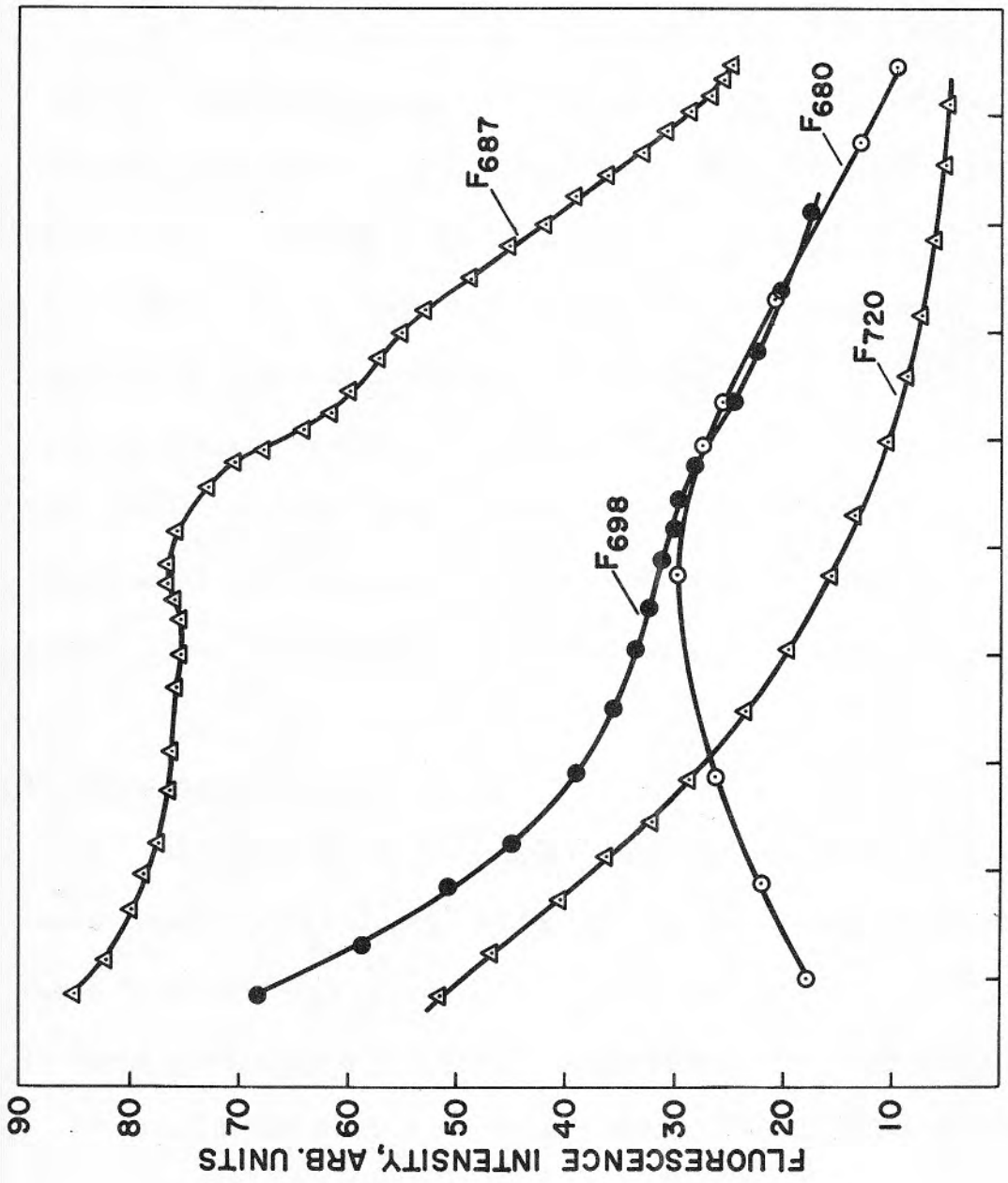

Fig. 15. Fluorescence intensities of F680, F687, F698 and F720 in the temperature range between 80°K and 240°K. The fluorescence is emitted from Chlorella pyrenoidosa, excited at 485 nm.

4) In the temperature range of 200-220°K, cubic phase changes to hexagonal phase, a relative increase of the 670 nm excitation band is observed (see Fig. 16).

5) From 120°K to 200°K, the half bandwidth (1/2 B. W.) of F687 shows no significant change. This temperature range is similar to the temperature range at which ice exists in cubic form (see Fig. 17). (The 1/2 B. W. is determined by $2(\lambda_{peak} - \lambda_{1/2\ height})$.)

The change of slope of F698 and the maximum intensity of F680 occur at the same temperature for each experiment, but the exact temperature deviates between experiments (140°K to 175°K). The similarity between the temperature of ice changing phase and that for the fluorescence spectrum change leads to a series of experiments to study their relationships. These experimental results will be discussed as follows.

B. Experimental Results

The effects of ice crystals on the fluorescence spectrum are studied mainly with Chlorella cells. These effects are studied from three different approaches:

1. The Repeatability of the Fluorescence Spectrum at Low Temperatures

This experiment is designed to examine whether the fluorescence spectrum at 4°K and 77°K can be repeated after warming up to 260°K, at which temperature most of the phase change has been completed. The purpose is to check if there is any relationship between the phase transitions of the ice crystals and the changes of fluorescence spectrum. The

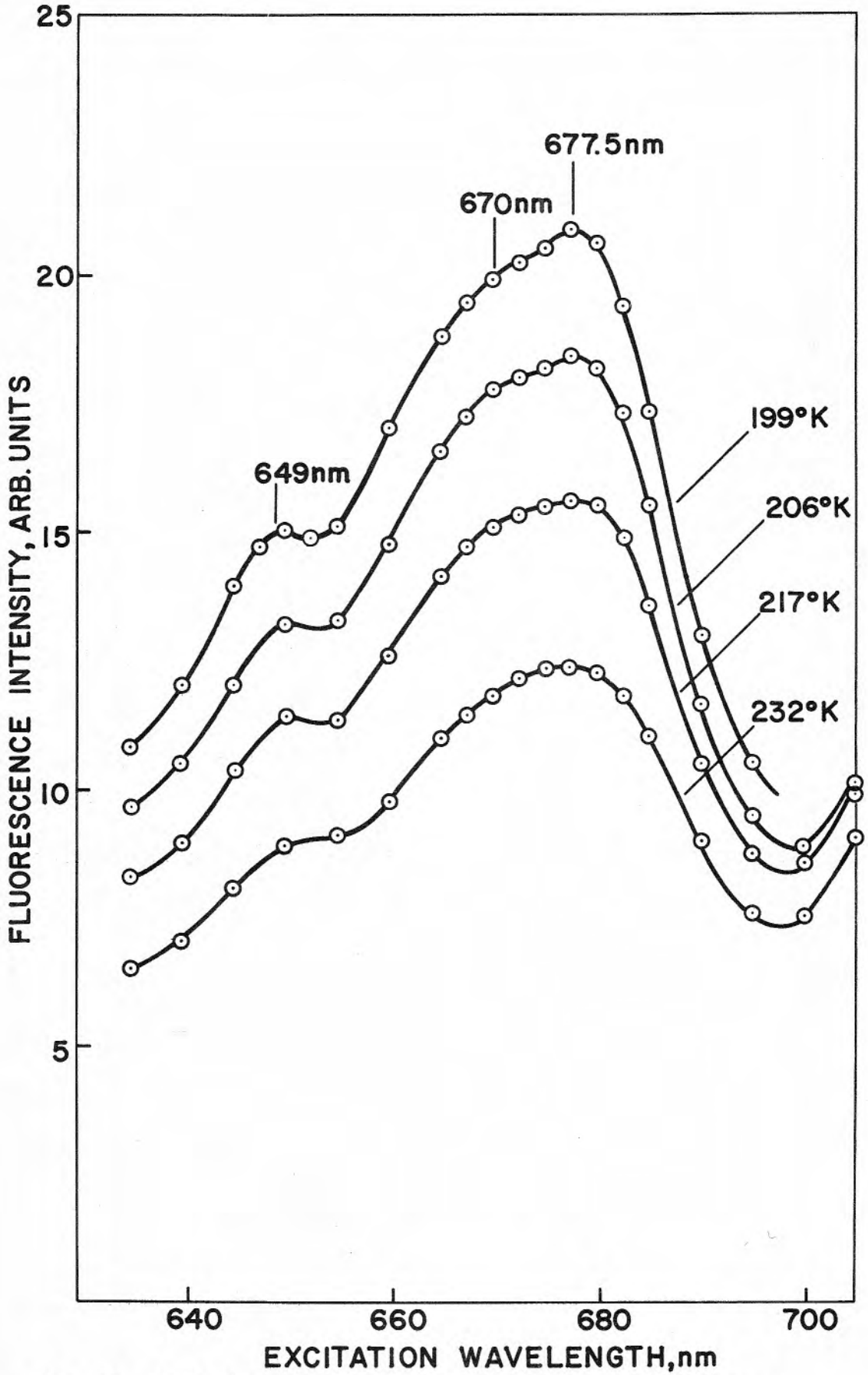

Fig. 16. Excitation spectra of F725 of Chlorella pyrenoidosa measured at 199°K, 206°K, 217°K and 232°K.

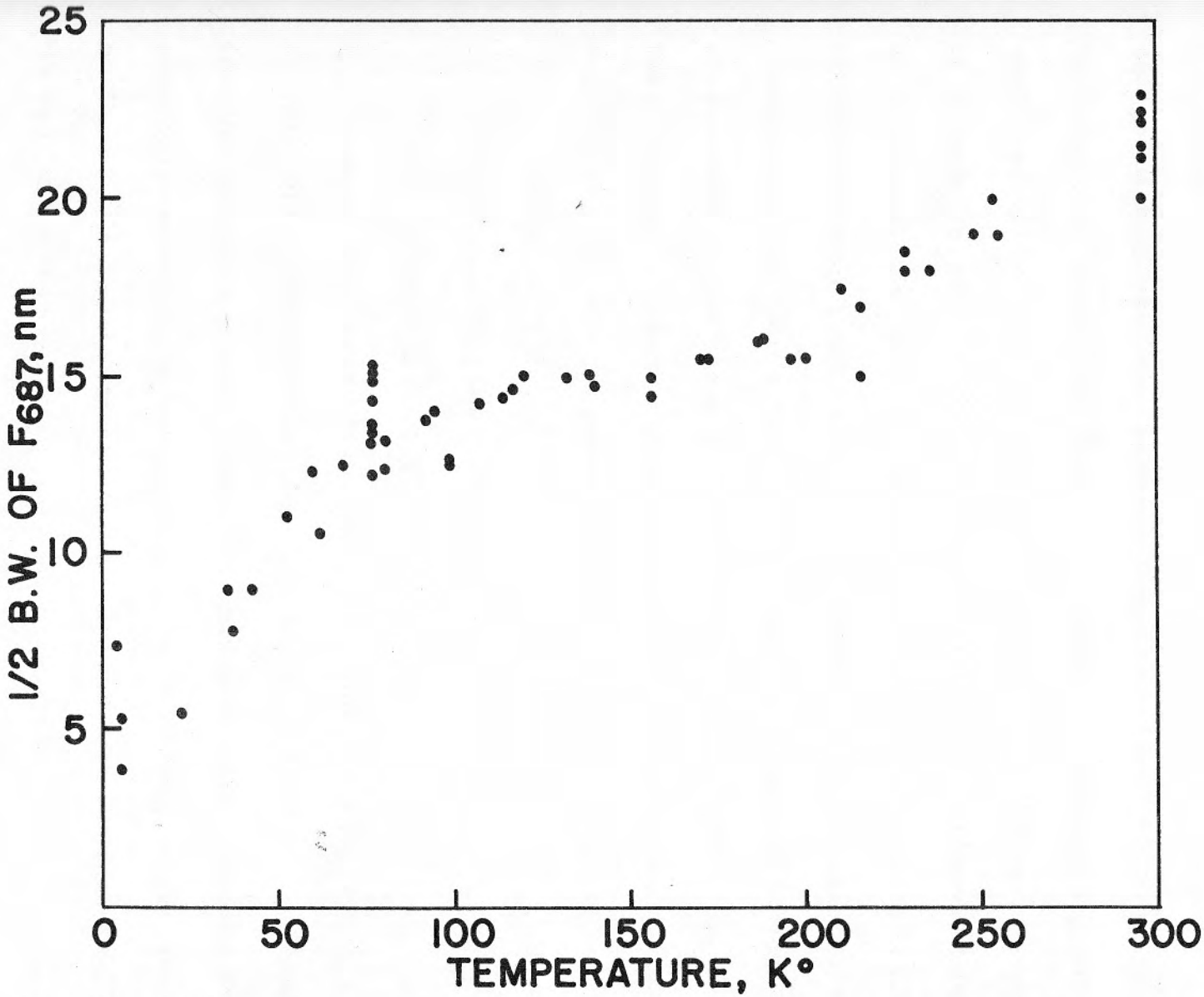

Fig. 17. Half bandwidth (½ B.W.) of the F687 band of Chlorella pyrenoidosa measured from 4°K to 295°K. ½ B.W. is calculated according to $2(\lambda_{peak} - \lambda_{½\ height})$.

phase transitions from vitreous to cubic and then from cubic to hexagonal are irreversible. If these changes are directly related to the sudden changes of the fluorescence spectrum, one would expect the change of the latter to be irreversible also. Experimental results (see Fig. 18) confirmed this relation. Furthermore, from $4^{\circ}K$ to $130^{\circ}K$ where there is no phase transition of ice crystals, there is no sign of irreversibility of the fluorescence spectrum. Most of the irreversibility occurs in the temperature range above $130^{\circ}K$. On may ask if the irreversibility of the fluorescence spectrum is caused by the rupturing of the cells during phase transition, and has no direct bearing on the particular phase of the ice crystal. This possibility was studied by comparing the spectra at $77^{\circ}K$ after the cells had gone through the following cooling processes:

i) $295^{\circ}K \longrightarrow 77^{\circ}K$

ii) $295^{\circ}K \rightarrow 77^{\circ}K \longrightarrow 260^{\circ}K \longrightarrow 77^{\circ}K$

iii) $295^{\circ}K \rightarrow 77^{\circ}K \longrightarrow 295^{\circ}K \longrightarrow 77^{\circ}K$

Fig. 18 shows the results. If the decrease of F687 and F698 is directly caused by the phase transition, one would expect (ii) to be very different from (i), because ice cannot change from hexagonal back to vitreous or cubic phase by cooling (before melting). This is shown to be the case in Fig. 18. If the phase transitions of ice or the process of freezing the cells to low temperature did not rupture the cells severely, one would expect the spectrum of (iii) to be very similar to that of (i). This is again found to be true. However, repeated freezing and warming more than two times generally causes a shift of the bands, and the spectrum

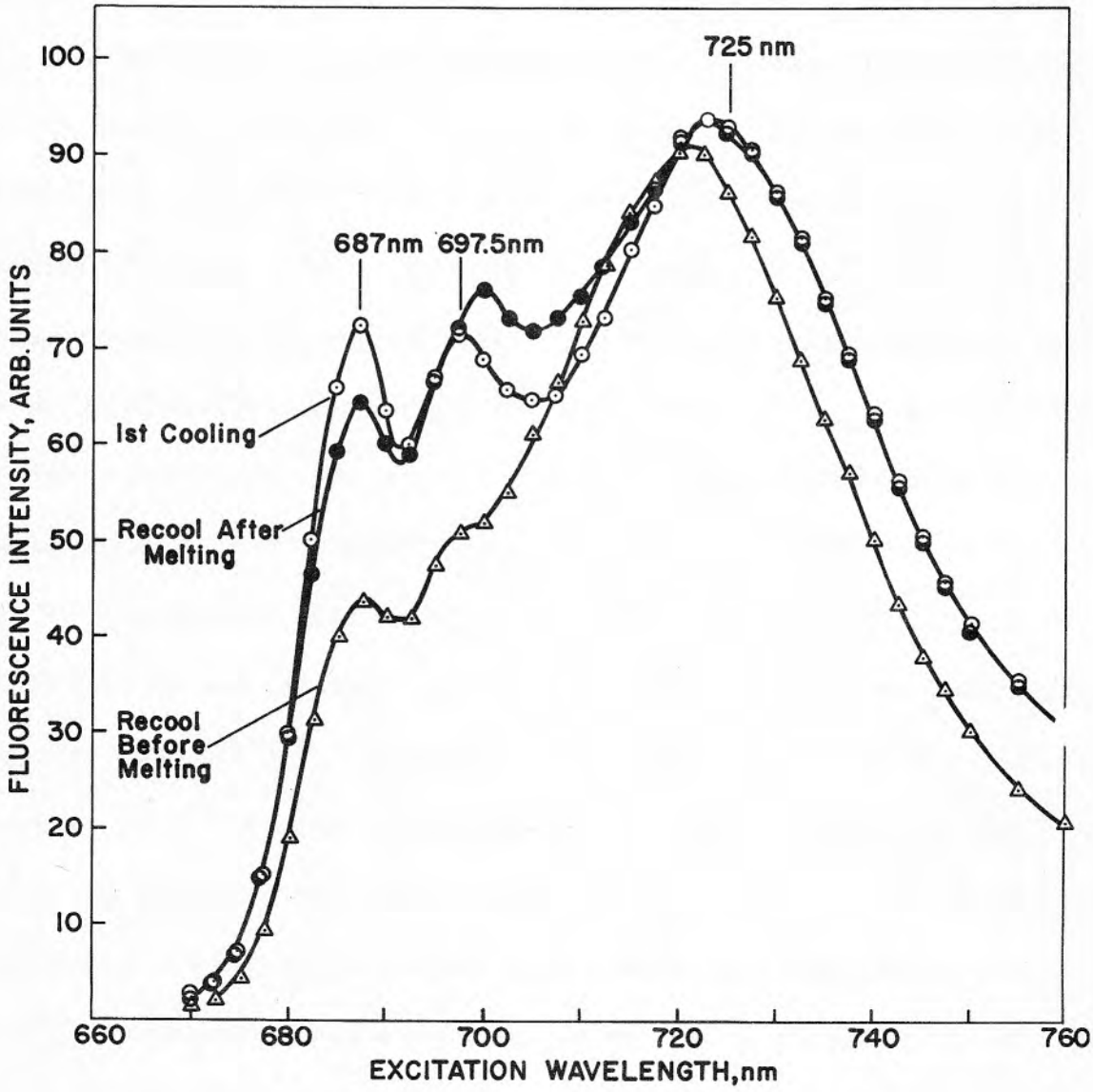

Fig. 18. Fluorescence spectra of Chlorella pyrenoidosa measured at 77°K. 1st cooling --- cooling from 295°K to 77°K. Recool before melting --- warming up from 77°K (after 1st cooling) to 260°K, then recool to 77°K. Recool after melting --- warming up from 77°K (after 1st cooling) to 295°K, then recool to 77°K.

of (i) can no longer be repeated. These results confirm that the change of phase of ice has a direct effect on the fluorescence spectrum.

One obvious interpretation for the reduction of F687 and F698 bands when the ice is in hexagonal phase is that there is an increase in the internal reflection when ice changes phase. Consequently F687 and F698 should be reabsorbed more due to their band overlap with Chl a (678) absorption band. Unfortunately, this interpretation causes difficulties in explaining many other results. For instance, if there is a large increase in internal reflection, one would also expect an increase in the total absorption of the exciting light, which will in turn cause an increase in the long wavelength fluorescence band where no reabsorption takes place. Experimental result shows a decrease of the long wavelength (F730) fluorescence (Fig. 18). Fluorescence excitation spectrum of F725 shows steady decrease of long wavelength excitation when cells are warmed up to 230^{o}K suggesting no increase of absorption in the overlap region when ice is in hexagonal phase. Absorption at 680 nm is 2.2 times stronger than that at 687 nm. If decrease of F687 is caused by increasing internal reflection and reabsorption, one would expect the F680 to be suppressed by a much larger ratio (i.e. about 2.2 times more decrease than that of F687), but the ratio of suppression of F680 is less than that of F687. This argument is further verified by using samples of different concentrations. The concentrated sample shows the effect of reabsorption. Its F687/F680 ratio is much higher than that of the dilute sample. All these results confirm that there is a

decrease of fluorescence intensities at 687, 698, and 730 nm (but not at 717 nm), as ice changes to hexagonal phase, and this fluorescence decrease is not caused by the increase of reabsorption.

2. Fluorescence Spectrum of Dehydrated Chlorella at 295°K and 77°K

It has been discussed in the last section that the change of phase of ice has a direct effect on the fluorescence spectrum. It is of interest to find out the fluorescence spectrum when ice crystal is absent (when the cells are dehydrated). The experiment is as follows: a thin layer of wet Chlorella is placed on a glass coverslip which is then transferred to a vacuum dessicator for 24 hours. The fluorescence spectra of such treated cells are shown in Fig. 19. F698 and F720 bands found in the normal cells are not resolved in these dried cells. Instead, a strong band at 707 nm is found. Upon warming up, the intensity of F707 decreases, and it becomes a shoulder near 705 nm at room temperature. The F698 band was not resolved throughout the 77°K to 295°K temperature range. The fluorescence spectrum at 77°K can be repeated after warming and recooling, i.e., irreversible changes caused by ice changing phase are not found in the dried cells.

3. Fluorescence Spectrum of Chlorella Treated with 10% Dimethylsulfoxide

In order to study the effect of ice crystals on fluorescence, dimethylsulfoxide (DMSO), which is believed to have strong affinity for water (95), is added to the cells. This compound is expected to prevent the formation of large ice crystals which will rupture the cells and cause artifacts on the fluorescence spectrum. Chlorella cells pretreated with 10% DMSO are found to be viable after freezing to 77°K.

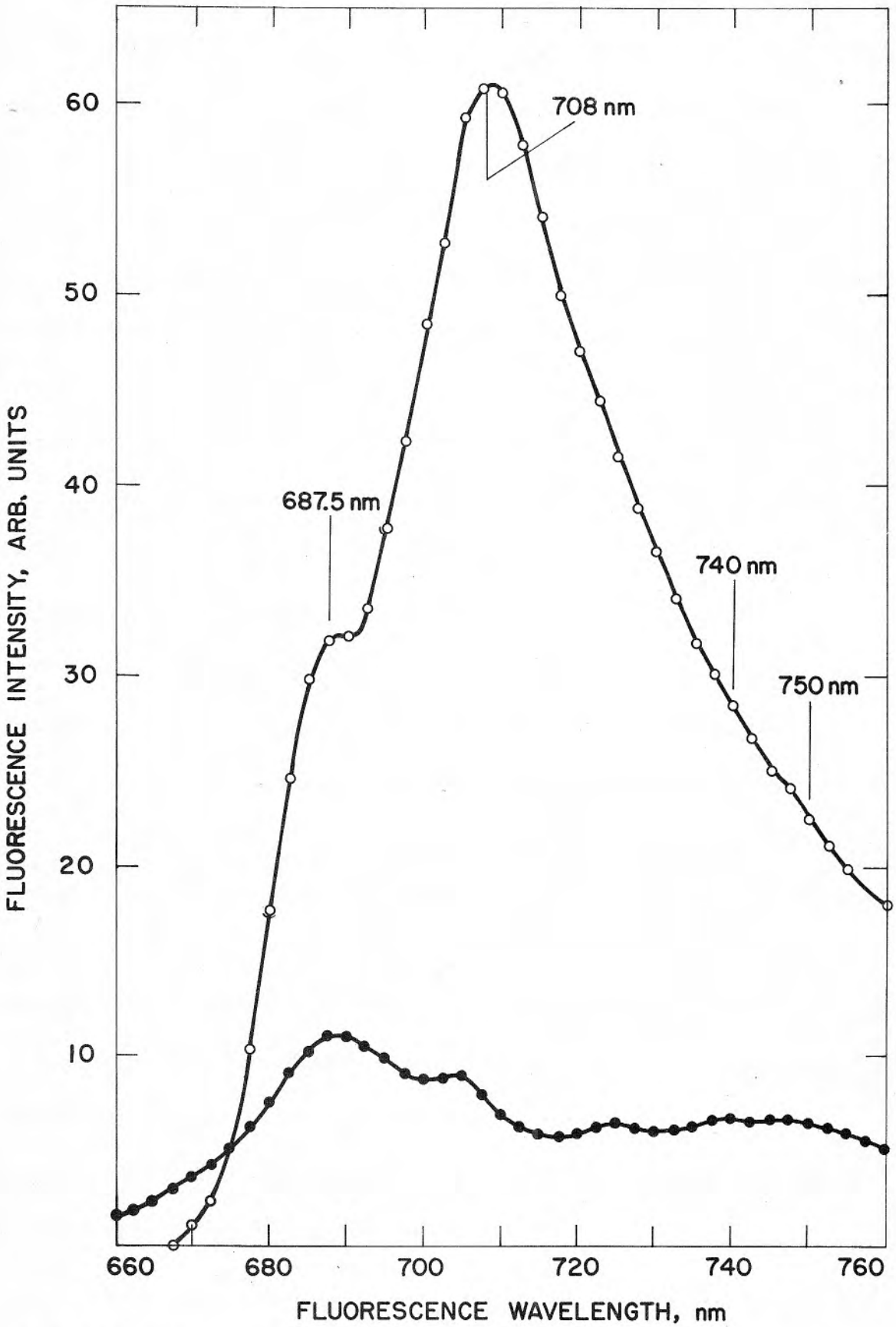

Fig. 19. Fluorescence spectra of dehydrated Chlorella pyrenoidosa measured at 77°K -o-o-o- and 295°K •-•-•, excited at 485 nm.

The experimental procedures are the following. In order to prevent the exothermic (heating) effect of DMSO on the cells, DMSO was first diluted 1:4 with water. This 20% DMSO solution was then added to an equal volume of Chlorella culture. After 30 minutes of equilibration, the cells were cooled down at the rate of $9^{\circ}K$/minute to $250^{\circ}K$, followed by quick cooling to $77^{\circ}K$. After remaining at this temperature for 30 minutes, the cells are warmed up to room temperature by dipping the cell container in water.

Previous attempts by both slow cooling and fast cooling have failed to get any viability after freezing. The protective effect of DMSO is found to be better than glycerol probably because the former can penetrate the cells more readily (94). The mechanism of the protection is not clear; however, it is believed to be caused by its affinity for water (95). The viability of these cells after thawing suggests that the internal structure of the cells are not severely distorted or damaged during freezing to $77^{\circ}K$. It is interesting to find that the fluorescence spectra of these cells (Fig. 20) also show normal F687, F698 and F720 bands. One noticeable difference is that the F698 band is much better resolved in the DMSO treated cells when compared to the normal cells with such slow cooling rate. In conclusion, these results suggest that the low temperature fluorescence bands are not due to artifacts caused by ice crystals which may rupture the cells.

C. Discussion

At the temperatures at which ice changes phase, sudden changes

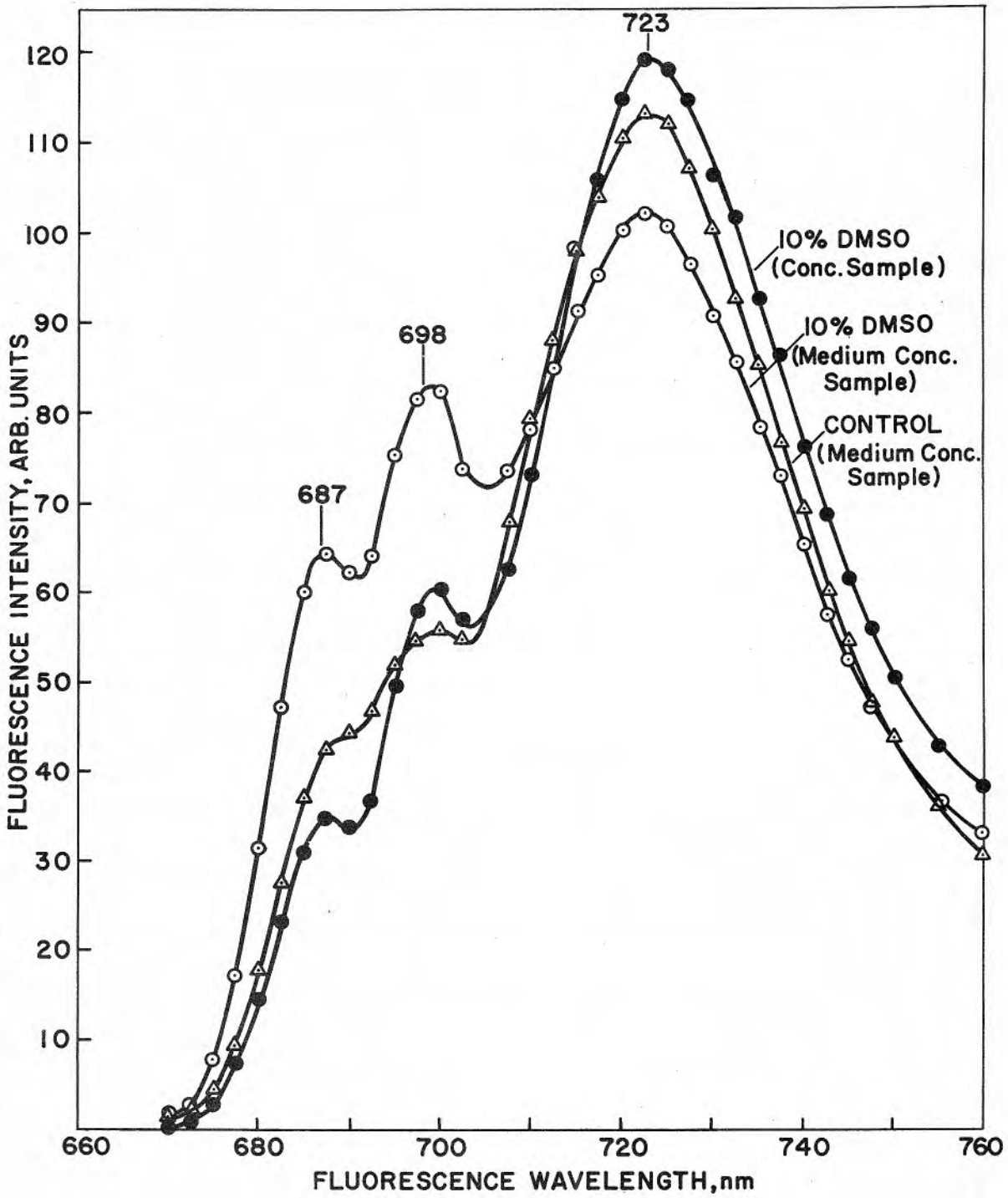

Fig. 20. Fluorescence spectra of Chlorella treated with 10% dimethyl-sulfoxide (DMSO). Concentrated sample --- O.D. ≈ 1.5 at 675 nm. Medium concentration --- O.D. ≈ 0.8 at 675 nm. Cooling rate is discussed in the text.

in the fluorescence spectrum are also observed. The phase transitions of ice crystals are irreversible, so are the changes of the fluorescence spectrum. The irreversibility of the fluorescence bands are not caused by structural damage of the cells because this spectrum can be repeated after melting followed by refreezing. The temperature range of irreversibility of fluorescence spectrum corresponds to that of ice changing from cubic to hexagonal phase.

The irreversibility of the fluorescence spectrum is indicated by the lowering of F687, F698 and the broad F730 bands (but not the F717 band). As discussed in section B-1 of this chapter, it is evident that changing of scattering property (or reabsorption of fluorescence) is not the main cause for the lowering of these bands. Indirect mechanical stress is not likely to be the cause either since ice in cubic and hexagonal phase occupies about the same volume (they have the same density, 0.923 gm/c.c. at 88°K). We also notice that each ice phase has a different quenching effect on the fluorescence. These results are consistent with the interpretation that water may bind directly to some SII chlorophyll molecules in vivo. It seems that F698 is emitted from a molecular species greatly influenced by the water environment because it is sensitive to the change of phase of ice, dehydration, and the addition of polar molecules. If this species is a trap of system II (35), it agrees with the current theory of photosynthesis that system II performs oxidation of water. However, the results do not conclusively prove that water is absent in SI even though its fluorescence is hardly affected by phase

transitions of ice crystals. This is so because the long wavelength fluorescence of SI may be emitted from an aggregated Chl a form, and one would not expect to find much change in this form as a result of ice changing phase since the aggregate is already in a "closely packed" form.

The spectra reported by the former authors resembled the spectrum of a very concentrated sample of Chlorella; it was difficult to distinguish between the major and the minor bands. Furthermore, the Chl a bands in the red region were not reported. However, their results showed good resolution of the bands in the 500-620 nm range. Spectra reported by Goedheer (44) resembled the spectrum of a thin sample (concentration was not mentioned in that report). The band positions were around 440, 470, 490-492, 580, 625, 651, 672 and 682 nm as compared to the 434, 460, 490, 505, 530, 550, 590, 620 and 650 nm reported by Brody and Brody (97). Goedheer reported the excitation bands indicating the presence of different Chl a forms, namely Chl a (670), Chl a (680) and Chl a (695), which are also found in this study. However, new evidence is found in this thesis (see section B-1-b of this chapter) suggesting that F687 (fluorescence at 687 nm) is emitted by Chl a (678) instead of Chl a (670), which contradicts the earlier interpretation of Goedheer (44).

Govindjee and Yang (46) reported the 77^{o}K excitation spectra (F760) of spinach chloroplast fragments, and found an additional band near 700-715 nm. These bands are also observed in Chlorella in this investigation. The 705 nm band was earlier reported by Butler (56) in the low temperature absorption measurements of leaves and algae.

The low temperature fluorescence excitation spectrum (F811) of Anacystis was reported by Govindjee (35). The spectrum showed an additional excitation band near 750 nm. The phycocyanin bands are similar to those reported in this thesis except that the relative heights

among the bands are different. These differences can be explained by the variations in the concentrations and the cooling rates of the samples.

This investigation is different from the previous works in the following ways:

1. In this study, the fluorescence excitation spectra of Chlorella and Anacystis are measured at different temperatures. With these results, the temperature dependence of energy transfer efficiencies among different molecular species are estimated.

2. Experiments of the present investigation are done in a lower temperature range ($4^{o}K$ to $77^{o}K$), which provides better fluorescence yield, better spectral resolutions and the results are not influenced by the phase transitions of the ice crystals.

3. The excitation spectra of different fluorescence bands are compared in this study. These comparisons clearly show the presence of several long wavelength (686- 715 nm) absorption bands.

In spite of the different purposes between the present and the previous works, there is some overlap of experimental results showing good agreement between the two.

B. Results and Discussion

The experimental results of Chlorella and Anacystis are discussed separately in this section.

1. Chlorella pyrenoidosa

The excitation spectrum of F725 (fluorescence at 725 nm) of

Chlorella measured at room temperature is shown in Fig. 21. In the long wavelength region, a peak at 675 nm and a shoulder near 650 nm are found. In the short wavelength region, peaks at 437.5 and 477.5 nm are observed. By comparing the absorption and the excitation spectra, one finds a lower fluorescence yield in the short wavelength region. This result is caused by the carotenoids which transfer only a fraction of the absorbed energy to the chlorophyll molecules that are active in fluorescence (6).

The fluorescence spectra from $4^{o}K$ to $77^{o}K$ show F687, F698 (fluorescence at 687 nm and 698 nm, respectively) and the broad F725 bands (see Fig. 8). The excitation spectra of these bands are measured from $4^{o}K$ to $77^{o}K$. Fig. 21 shows the excitation spectra of F725. The peak positions are 440, 477.5, 491, 580, 625, 649, 672.5 and 679 nm. These wavelengths are in good agreement with those reported by Goedheer (ref. 44 and also see the introduction of this chapter) except the 477.5 nm band. In this study, if the cutoff filter (C.S. 7-69) is not used (i.e. using only the exciting and the analyzing monochromators), the 477.5 nm band is shifted to 471 nm, which is close to that reported by Goedheer. Therefore, the disagreement was probably caused by leakage of light.

The relative heights of the main bands (except the complex 685-710 nm band) remain constant from $4^{o}K$ to $77^{o}K$. The implication of this constancy is discussed in section C.

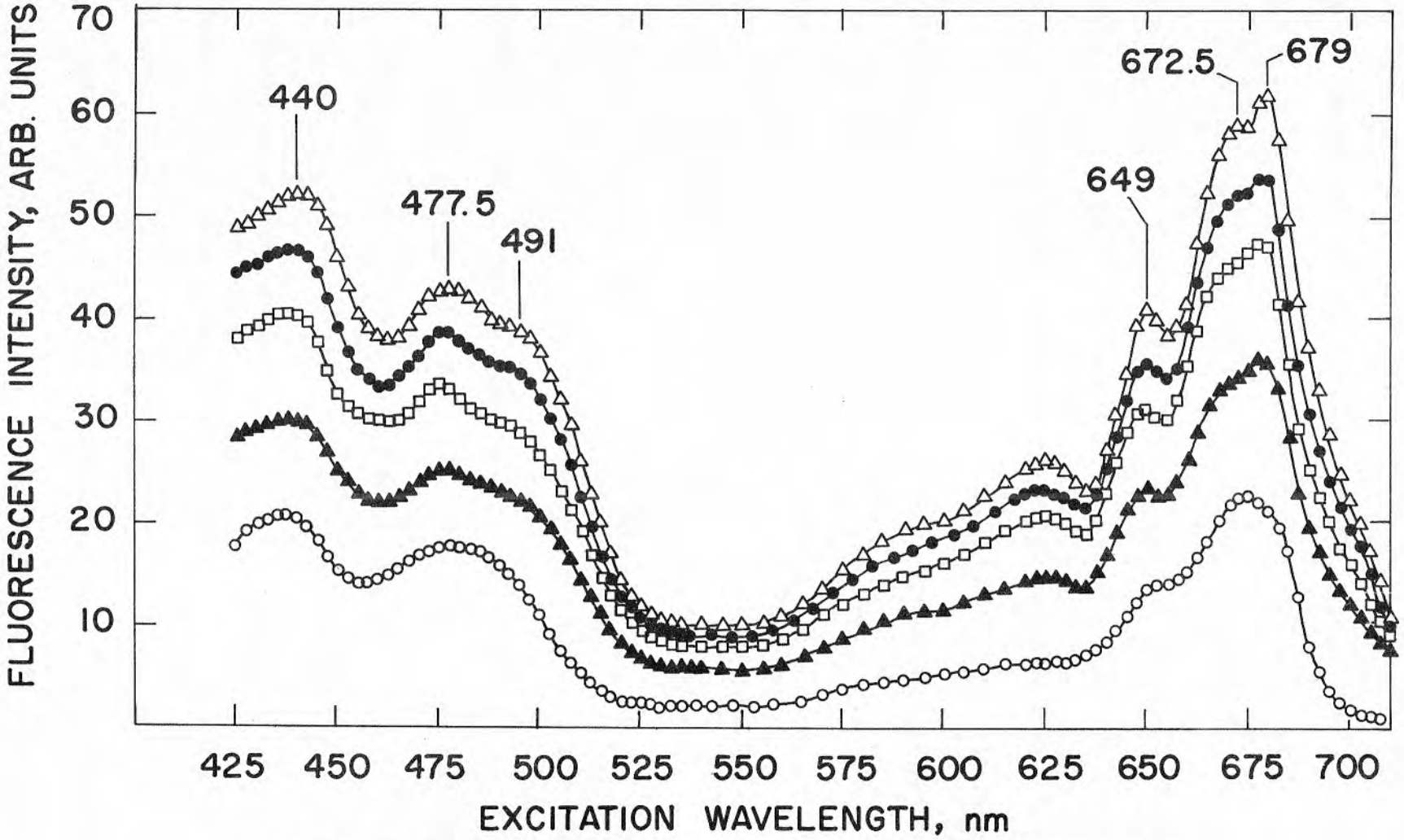

Fig. 21. Excitation spectra of F725 of Chlorella pyrenoidosa measured at 4°K -Δ-Δ-Δ, 37°K ●-●-●, 58°K □-□-□, 77°K ▲-▲-▲ and 295°K o-o-o.

Fig. 22 shows the excitation spectra of F698. The peaks in the long wavelength region are located at 649, 670 and 678 nm. The last two bands are shifted by 2.5 nm and 1 nm, respectively, when compared to that of F725. The apparent shifts are probably caused by the additional bands (686-715 nm) in the F725 excitation spectra. In the short wavelength region, the peaks are located at 437.5 and 485 nm. The relative heights among the bands remain constant upon warming from 4°K to 77°K. Fig. 23 shows the excitation spectra of F685. The peak positions and the relative heights among the bands are very similar to that of F698, suggesting that these two fluorescence bands are emitted from the same pigment system. In the following paragraphs, detailed analyses of these results are presented. In part (a), the 4°K absorption bands, especially in the 686-715 nm region, will be studied; in part (b), the molecular origin of the F725, F698 and F687 bands will be discussed; in part (c), the SI and SII fluorescence at the low temperature will be discussed.

a. Analyses of the Weak Absorption Bands

Excitation spectra provide a means to detect the weak absorption bands. This is done by comparing the excitation spectra of different fluorescence bands. Another method to improve the band resolution is to lower the measuring temperature. In this study the two methods are combined, and the weak bands beyond Chl a (678) are observed.

Fig. 24 shows the excitation spectra of F725 and F698 measured at 4°K, and normalized at 664 nm. This wavelength is chosen for normalization because Chl a (670) in this region is 14 nm away from

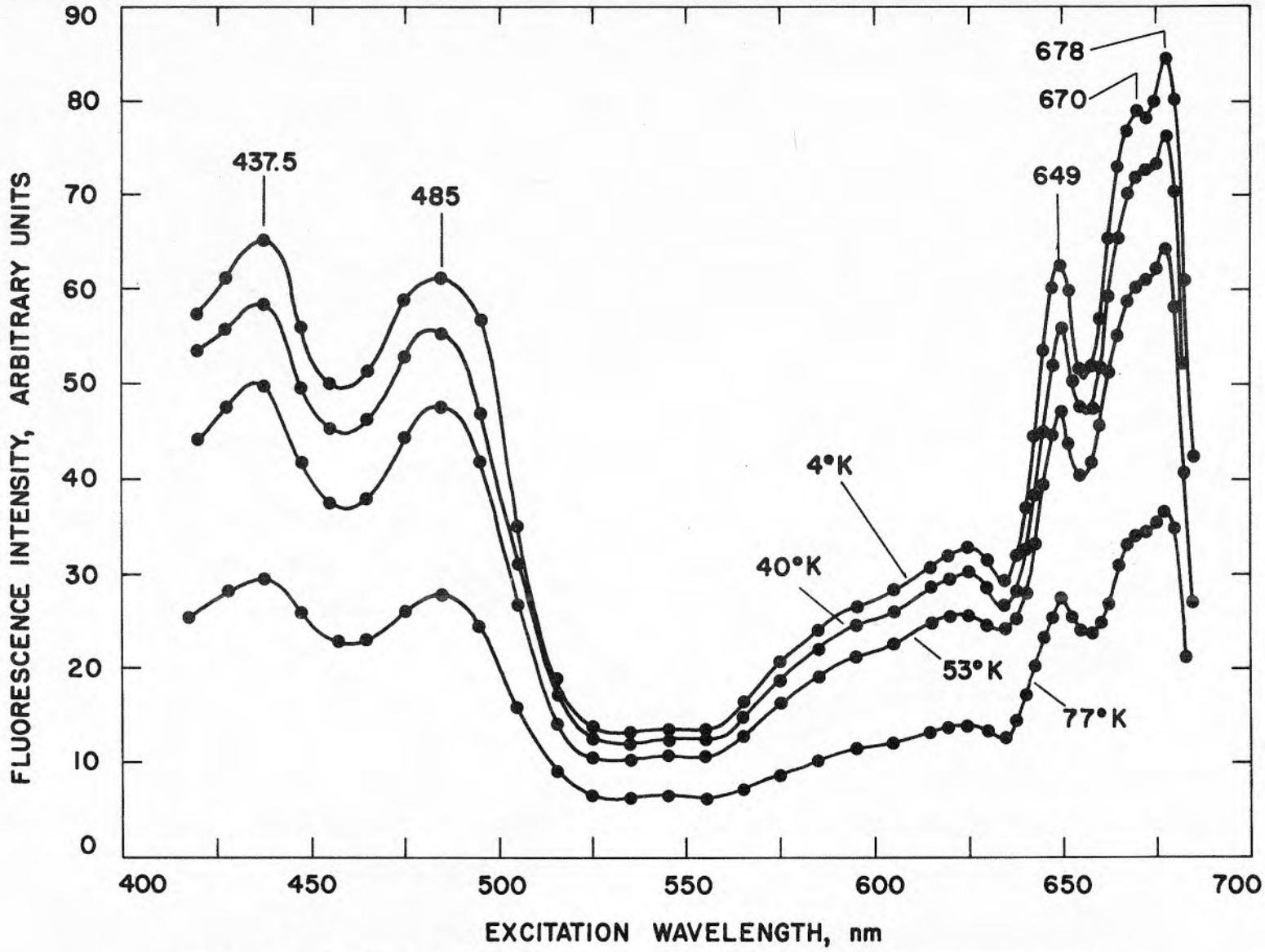

Fig. 22. Excitation spectra of F698 of Chlorella pyrenoidosa measured at 4°K, 40°K, 53°K and 77°K.

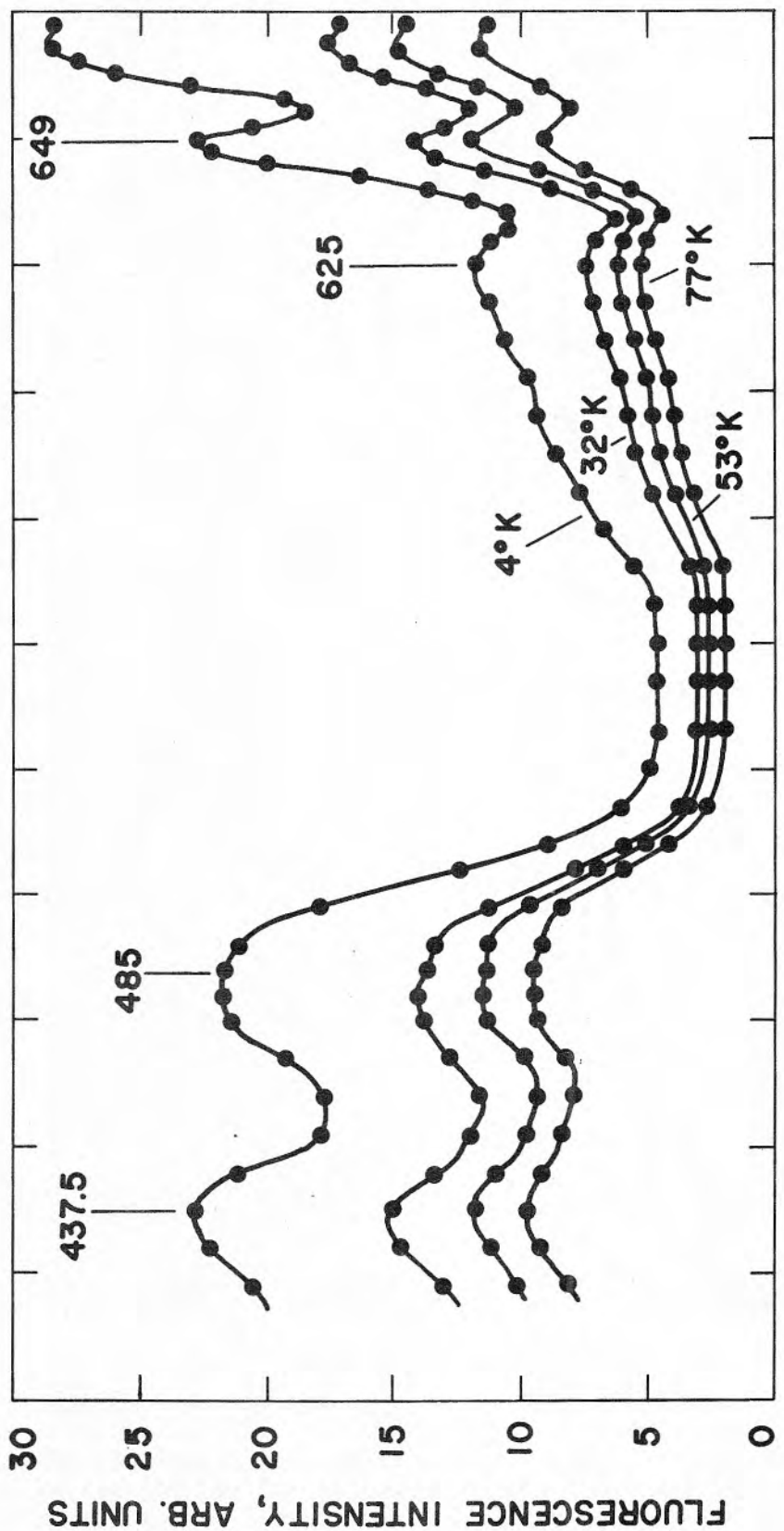

Fig. 23. Excitation spectra of F685 of Chlorella pyrenoidosa measured at 4°K, 32°K, 53°K and 77°K.

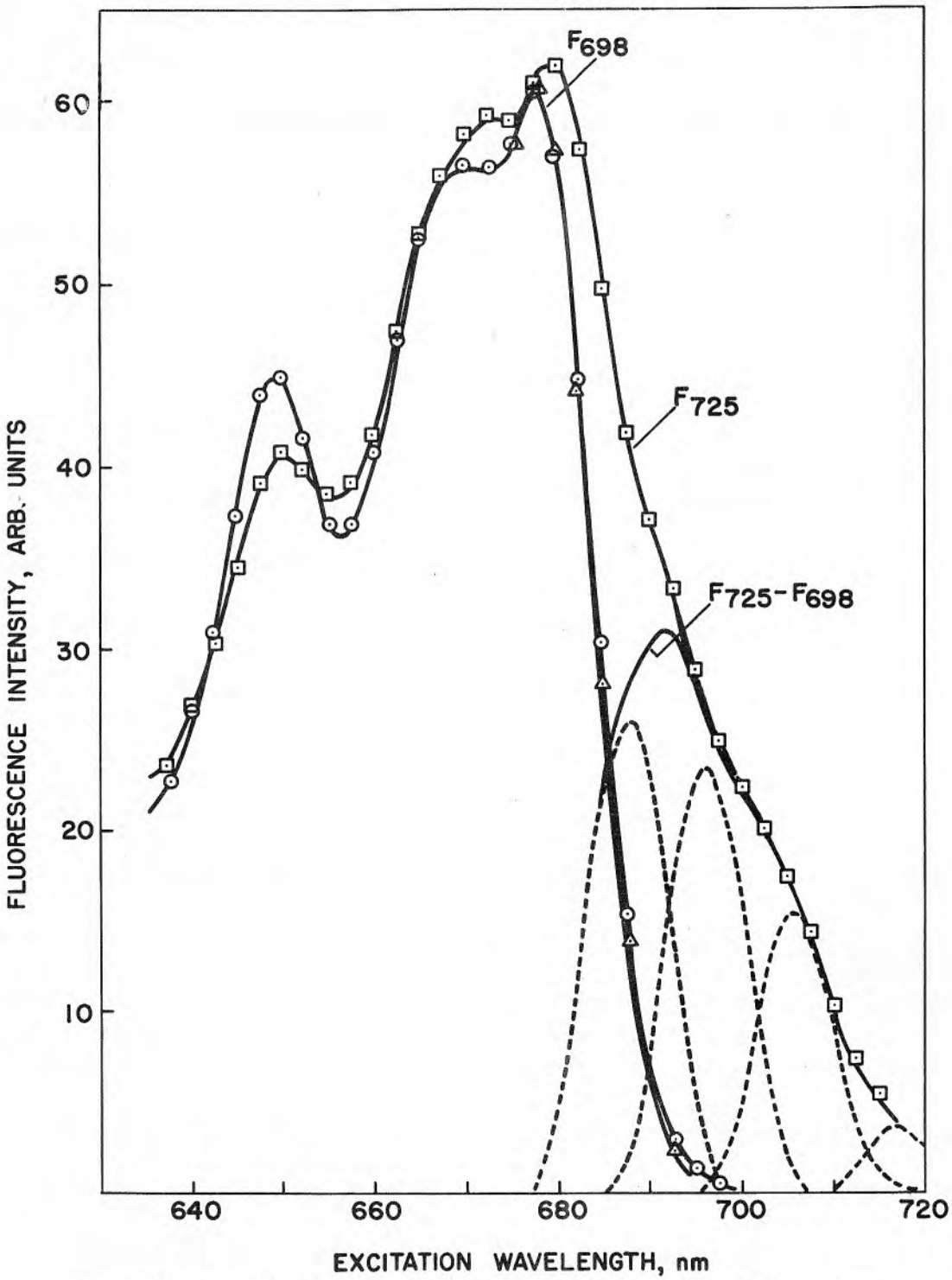

Fig. 24. Excitation spectra of F725 and F698 of Chlorella, measured at 4°K. The two spectra are normalized at 664 nm. The excitation spectra of F698 beyond 687 nm are obtained by correction for the leakage of light Δ—Δ—Δ and by Gaussian extrapolation o—o—o. The difference spectrum of the excitation spectra of F725 and F698 are resolved into four bands with peak positions at 688, 696, 705 and 717 nm.

both Chl b (649) and Chl a (678), therefore the effect of band overlap is minimal. True fluorescence of F698 caused by excitation beyond 687 nm is obtained by subtracting the leakage of light from the measured curve. Leakage of light is measured with cheesecloth permeated with water. This result is shown in Fig. 24. Another method to estimate the excitation spectrum beyond 687 nm is to assume that Chl a (678) follows a Gaussian distribution (53). From the measured excitation spectrum of F698 at 4°K, the peak is found at 678 nm and half height at 685 nm (half bandwidth = 14 nm). When a Gaussian distribution is extrapolated for Chl a (678) in the region beyond 687 nm, one finds that the result is in good agreement with the spectrum which is obtained by correcting for the leakage of light.

The difference of normalized spectra (Fig. 24) shows a broad band with a peak at 692 nm and a shoulder near 705 nm. This broad difference band can be resolved into four components with half bandwidth of 10 nm and peaks near 686-688, 694-696, 703-705 and 717 nm. These components are obtained by curve fitting; the solution is not unique. However, these bands are also found in the difference excitation spectrum of F760 and F725 (see Fig. 11), in which case the first three bands are more obvious. Another evidence for the presence of the 686-688 nm band is provided by the absorption spectrum of Anacystis (ref. 63 and Fig. 6 of this thesis). A similar broad difference absorption band (maximum at 698 nm) was obtained by Das and Govindjee (98). This was obtained by comparing the room temperature absorption spectra of aerobic

(at pH 4.6) and anaerobic (at pH 7.8) sonicated Chlorella cells. The presence of the 705 nm band has been reported by several workers (see a recent review, 64); but the difference spectrum method used in this study seems to provide the best resolution of the bands in the 686-715 region.

There may be more absorption bands in the wavelength region beyond 717 nm, even though these bands are too weak to be observed in the absorption spectrum of Chlorella. It has been shown in chapter IV that there is evidence for the presence of the fluorescence bands near 735-740 nm and 750-760 nm. Litvin et al. (100) suggest another fluorescence band near 810-825 nm. These fluorescence bands could be caused by energy levels other than Chl a (705) and Chl a (717), because the Stokes shift would be too large for these bands to emit at 760 nm or 810 nm (i.e. there would be no band overlap between the fluorescence and the absorption bands). In low temperature solution works, Broyde and Brody (50) assigned the long wavelength fluorescence bands to be the emission of different Chl a forms (aggregates and monomers). An absorption band near 750 nm is found in Anacystis (ref. 63 and see Fig. 6), confirming the presence of long wavelength absorption bands in this blue green alga. It is possible, however, that some of the long wavelength absorption and fluorescence bands are caused by the transition between different vibrational levels of the bulk of Chl a.

In summary, the bands at 649, 670 and 678 nm are directly observable in the excitation spectra at $4^{\circ}K$, the bands near 687-688, 693-696,

702-707 and 717 nm are obtained by analyzing the difference excitation spectrum of F698 and F725 and that of F760 and F725. Other absorption bands may be present and responsible for the fluorescence near 760 and 810 nm; however, they are too weak to be observed in the absorption spectrum of Chlorella.

b. The Molecular Origins of F687, F698 and F725

From the absorption and fluorescence maxima of Chl a in different solutions (ether, alcohols, hydrocarbons), the Stokes shifts are found to be in the range of 4.5 nm to 10 nm (27). Based on this argument, Rabinowitch (personal communication) suggested that the F685 band in vivo is emitted from the Chl a (680). It is difficult to confirm this result experimentally at room temperature because the fluorescence intensity is low and the bands are not well resolved. At low temperatures, the fluorescence yield is greatly increased in vivo, and the absorption bands due to Chl a (670) and Chl a (678) are distinct; therefore, the conditions are suitable to check the above suggestion. Fig. 25 shows the excitation spectra of F698, F690 and F687. The half bandwidth (1/2 B.W.) of the exciting and analyzing monochromators are 2.7 nm. With such narrow 1/2 B.W., the excitation light can be scanned to within 5-6 nm of the analyzing wavelength without noticeable deflections caused by the leakage of light. The spectrum clearly shows that F687 obtains excitation energy from Chl a (678), and energy is transferred efficiently from Chl a (670) to Chl a (678). These results confirm the suggestion made by Rabinowitch. It will be shown in section C that this finding is important to the study of energy transfer efficiency in vivo.

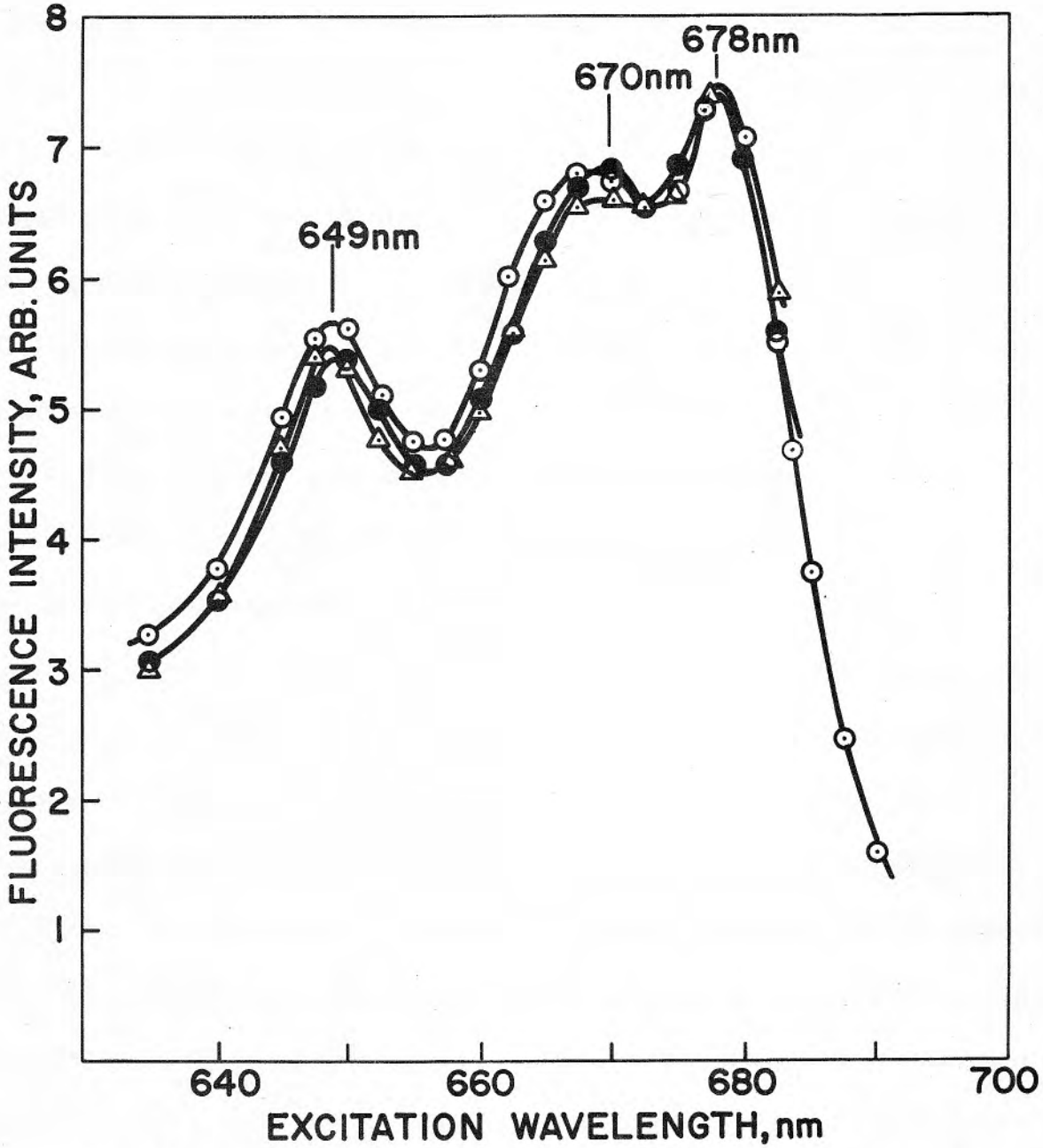

Fig. 25. Excitation spectra of F687, F690 and F698 of Chlorella, measured at 77°K. Slit widths for both exciting and analyzing monochromators are 0.8 mm (½ B.W. = 2.64 nm). Filter C.S. 2-64 is used to cut off the leakage of light.

The fact that Chl a (678) emits fluorescence is supported by an analysis of the "red drop" of fluorescence yield. At room temperature, Chl a (670) and Chl a (683) absorb equally at around 675-677 nm (61). If Chl a (683) is non- or weakly fluorescent, while Chl a (670) is fluorescent, then the half height of the fluorescence yield would be around 675-677 nm. However, Duysens (6), Das and Govindjee (98) and Szalay et al. (101) found that the half height of the fluorescence yield was around 690-692 nm. Therefore, it is clear that Chl a (683) is fluorescent. The Chl a forms with absorption bands at 686-687 nm and at 693-696 nm absorb equally near 692 nm, the observed drop of fluorescence yield to its half-height at 690-692 nm can be explained if the 693-696 nm band is inactive in fluorescence. This suggestion has recently been confirmed by Das and Govindjee (98) in sonicated Chlorella.

The excitation spectrum of F698 at $4^{\circ}K$ is shown in Fig. 24. F698 is not emitted from Chl a (678) because we have shown above that Chl a (678) emits at 687 nm. Based on the assumption of 10-15 nm Stokes shift (which is a reasonable assumption when compared to that of F687), the molecular species which emits at 698 nm is likely to absorb near 686-688 nm. The absorption band of this species would have a strong overlap with the fluorescence band of Chl a (678). Therefore, excitation at Chl b, Chl a (670) and Chl a (678) can be transferred efficiently to this species and cause fluorescence at 698 nm. The F698 excitation spectrum (Fig. 24) shows that the concentration of the 686-688 nm species is very low. It can therefore be considered as an energy trap of one of the pigment

systems. These results confirm the suggestion (35) that F698 is emitted from the trap of SII. F698 has been (45) and will be shown (see part c) to be mainly SII fluorescence.

F725 is likely to be emitted from the species that absorbs around 705-715 nm. A broad band from 680 nm to 715 nm is clearly seen in the F725 excitation spectra (Fig. 11 and Fig. 24). Excitation energy absorbed at 650 nm, 670 nm and 678 nm is first transferred to Chl *a* (705) and Chl *a* (715) by way of Chl *a* (693), and then it is emitted at 725 nm. In the aerobic sonicated Chlorella, where Chl *a* (693) is damaged, F725 band shows a drastic decrease (98).

In summary, there are three lines of evidence showing that F687 is emitted from Chl *a* (678). The 5-10 nm Stokes shift of Chl *a* fluorescence in various solutions suggests the order of magnitude of this shift *in vivo*. Secondly, the wavelength at which the half height of fluorescence yield occurs at room temperature can only be explained by assuming that Chl *a* (678) is fluorescent, and that the main fluorescence band at room temperature is at 685 nm. Finally, the direct measurement of the excitation spectrum of F687 shows a peak at 678 nm. The conclusion that F687 is emitted from Chl *a* (678) is important because this fluorescence is emitted from SII (see part c); the result therefore indicates that SII also contains Chl *a* (678), confirming earlier suggestions of Govindjee *et al.* (64). Excitation spectrum of F698 shows that this fluorescence is emitted from a molecular species of low concentration when compared to Chl *a* (678). This species is likely to absorb near 686-690

nm and is suggested to be a trap of pigment system II. F725 is emitted from the species which absorbs near 705 - 715 nm. Some of the energy abosrbed by Chl b, Chl a (670) and Chl a (678) is transferred to this species by way of the species which absorb near 686-705 nm.

c. System I and System II Fluorescence at Low Temperatures

It has been demonstrated (45, 46, 79) that low temperature SI fluorescence is mainly located at the long wavelength region (715-760 nm) whereas that of SII is located at 685-687, 695-698 and the long wavelength region. In this study, additional evidence is found in support of these previous findings and new analyses have been added. The more important earlier works are discussed in the following paragraphs.

Boardman and Anderson (80) found that spinach chloroplasts could be separated into several fractions after incubating the chloroplasts with digitonin. One of the fractions could perform SI reaction (reduction of $NADP^+$ with reduced DCPIP as reductant), and another fraction could perform SII reaction (Hill reaction with DCPIP as oxidant). Cederstrand and Govindjee (79) and Boardman et al. (45) found that this "SI fraction" emitted strongly at 735-740 nm but not at 698 nm, whereas the "SII fraction" emitted strongly at 685, 698, and 735-740 nm at $77^{\circ}K$. Another line of evidence was provided by Govindjee and Yang (46) who found higher Chl a /Chl b excitation ratio for F760 than that for F685 in spinach chloroplasts. These ratios (675 nm/650 nm) were 1.43 and 2.04 for F685 and F760, respectively. Since Chl a/Chl b ratio is higher in SI than in SII, these authors suggested that F685 was mainly

emitted from SII and F760 was mainly emitted from SI (46). In the red alga Porphyridium, excitation of SI and SII (by green light) produced strong fluorescence bands at 685, 696 and 712 nm whereas excitation of SI alone (by blue light) produced a dominant peak at 712 nm at 77°K suggesting that F696 and F712 were emitted from SII and SI, respectively (47).

In this study, when the 4°K excitation spectra of F725 are compared with those of F698 and F685, the former shows a preferential excitation by Chl a. From Fig. 21 and Fig. 22, the ratio of Chl a/Chl b (calculated by Chl a (670) + Chl a (678) / Chl b (650)) is 3 for F725 and 2.6 for F698. These ratios vary according to the culture conditions and the concentration of the sample (46). However, this ratio for F725 is always higher than those for F698 and F687 in the same sample. These results are consistent with the suggestion that F725 is preferentially excited by SI pigments (46).

Another evidence showing that F725 is mainly emitted by SI is the following. It has been shown in part (a) of this section that F725 is emitted by a molecualar species that absorbs near 705-715 nm, and energy is transferred to this species by way of the Chl a (686-705) which mainly belong to pigment SI. Chl a (686-705) were shown to be mainly in SI by the difference photosynthetic action spectrum of the two systems (61, 69, 102). Fig. 11 shows that the 686-705 nm excitation band decreases for F760, and at the same time the relative height of the 650 nm excitation band increases, suggesting that F760 has higher fraction of SII emission

than F725. The F760 *in vivo* is probably the same band as the F755 band analyzed by Broyde and Brody (50). These authors attributed the 755 nm emission band to $\pi - \pi^*$ phosphorescence from unsolvated monomers.

In this study, two additional lines of evidence are found showing that F685 and F698 are emitted from the same pigment system:

i) From $4^{\circ}K$ to $77^{\circ}K$, the excitation spectra of F698 and F685 are very similar. The peak positions are at the same wavelengths and the relative heights among the bands are almost equal. These spectra are different from those of F725 (see Fig. 21-23).

ii) Fig. 26 shows the kinetics of the fluorescence transient of F685, F695 and F725 at $77^{\circ}K$. The kinetics of the former two bands are similar (we recognize that there is overlap between F685 and F695 bands ($\simeq 20-30\%$). These results were obtained by placing the Chlorella cells in the dark for 4 minutes before cooling to $77^{\circ}K$. Blue excitation light (430-500 nm) was then turned on and the fluorescence transient was recorded. The intensity of the excitation light remained constant during the measurements. Kok (38) and Kok *et al.* (103) earlier showed that this low temperature fluorescence transient effect was related to the photoconversion of energy quenching centers of SII. Duysens (104) reported that at $77^{\circ}K$ the "SI fraction" of Boardman and Anderson did not show any light induced fluorescence transient at 730 nm which was observed in the "SII fraction" at the same wavelength. Therefore, from Fig. 26, and from the analysis of Chl *a* / Chl *b* ratio of F725, it is clear that F725 originates from both SI and SII. We now show a calculation

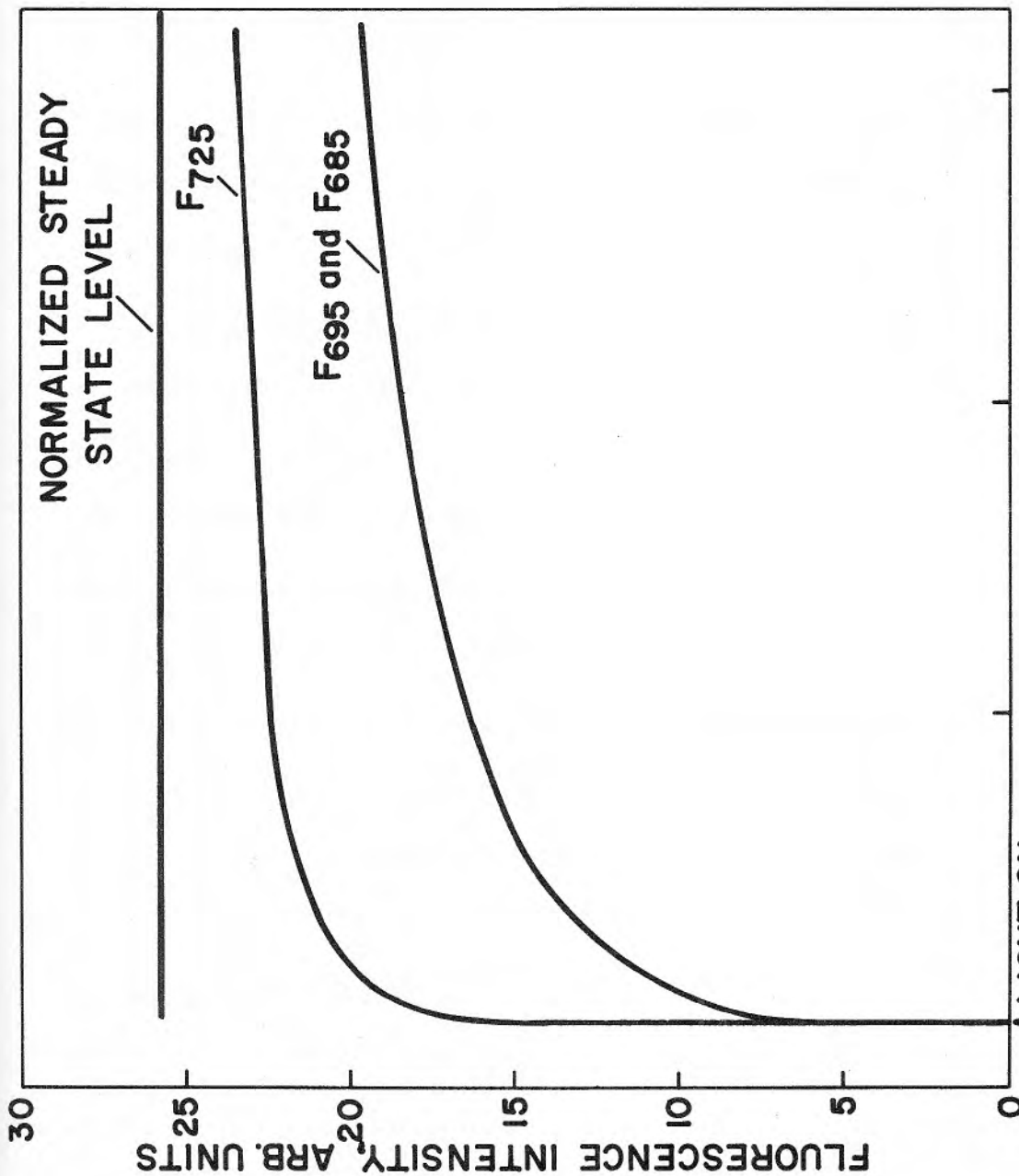

Fig. 26. Low temperature (77°K) fluorescence transient of Chlorella pyrenoidosa, excited with broad band blue light, using C.S. 4-94. 4 minutes dark period before cooling. F685, F695 and F725 are normalized at the steady state levels, obtained after 20 seconds of illumination at 77°K.

of the ratio of SI and SII fluorescence at 725 nm. This calculation is based on two assumptions. Firstly, F725 of SI does not show any transient whereas that of SII does show transient (104). Secondly, all SII fluorescence transients have similar kinetics. The data obtained from Fig. 26 show that F725 is composed of ≃62% SI with no transient component and ≃38% of SII component with the kinetics of transient similar to that of F685.

In summary, on the basis of the similarity between the low temperature ($4^{\circ}K$ to $77^{\circ}K$) excitation spectra and the similarity of the kinetics of the fluorescence transient ($77^{\circ}K$) of F698 and F687, we conclude that these two bands are emitted from the same pigment system. Furthermore, on the basis of the data analyzed here, it becomes obvious that these two bands are emitted from SII, and F725 is mainly emitted from SI (≃62%), whereas longer wavelength fluorescence (F760) shows a higher SII component than that of F725. These conclusions are consistent with the suggestions made by the previous workers (45-47, 79).

2. Anacystis nidulans

The excitation spectra of the main fluorescence bands (F653, F687, F698 and F715) are measured in the $4^{\circ}K$ to $77^{\circ}K$ temperature range.

Fig. 27 shows the excitation spectra of F653 at six different temperatures. In the $4^{\circ}K$ spectrum, bands due to phycocyanin absorption at 580, 623 and 637 nm are observed. The relative heights among these

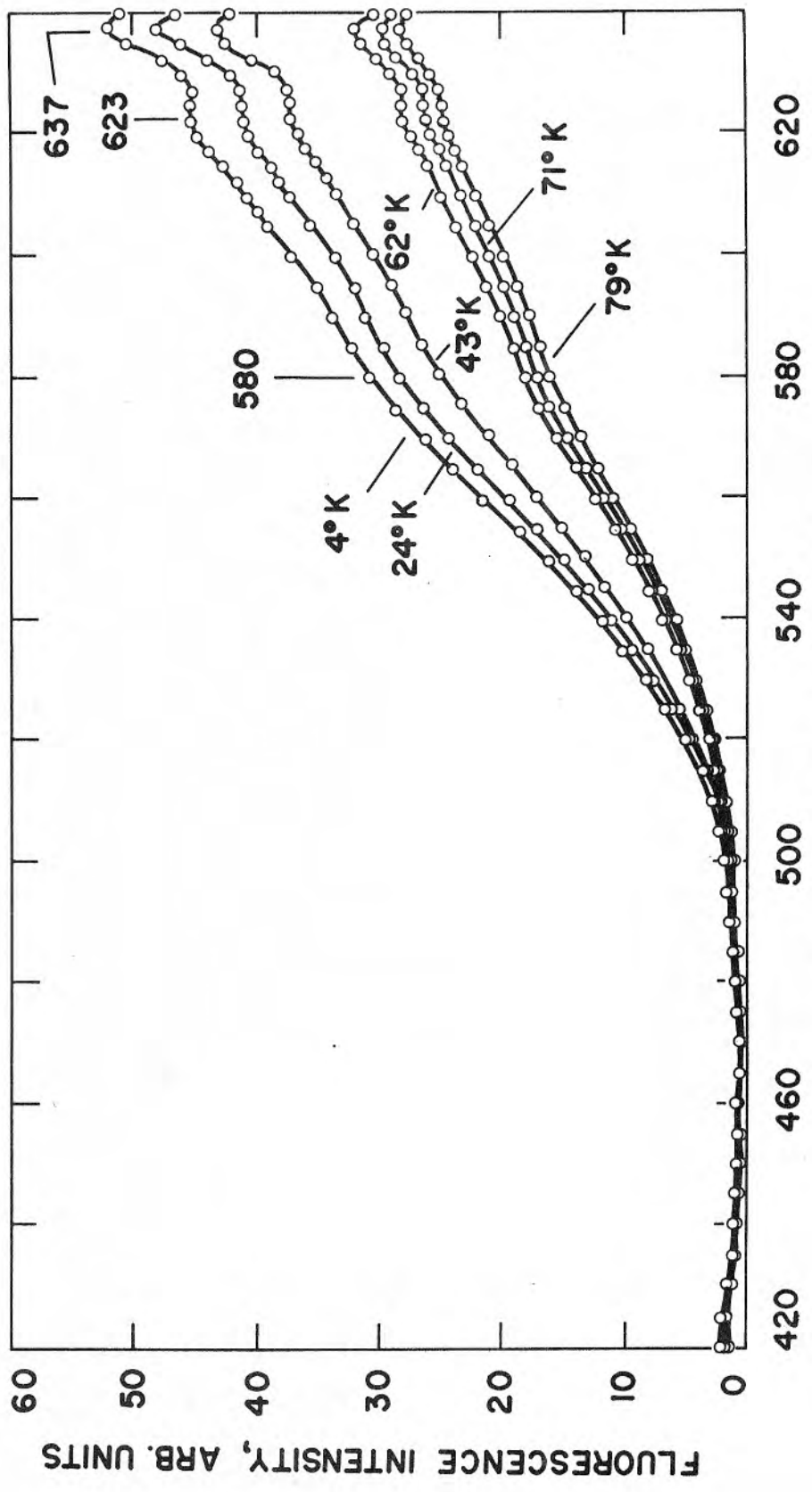

Fig. 27. Excitation spectra of F653 of Anacystis nidulans measured at 4°K, 24°K, 43°K, 62°K, 71°K and 79°K.

bands remain almost constant upon warming from 4°K to 77°K.

Fig. 28 shows the excitation spectra of F687 at six different temperatures. The phycocyanin bands at 580, 623 and 637 nm are again observed; a shoulder near 650 nm is also found. The 650 nm band is believed to be caused by the presence of allophycocyanin because the extract of this pigment shows an absorption band near 650 nm (105, 106). The main difference between the excitation spectra of F687 and F653 in the blue region is that the Chl a band (near 435 nm) is observed in the former but not in the latter. The temperature dependence of the relative intensities among the bands will be discussed in part 3.

Fig. 29 shows the excitation spectra of F698 at five different temperatures. Bands at 436 nm (Chl a), 472 nm and 505 nm (carotenoids) are clearly resolved at 4°K. The relative heights of these bands to the phycocyanin bands are higher in the excitation spectrum of F698 than those of F687. The phycocyanin bands near 580, 623 and 637 nm are again observed. The 650 nm band, however, is weaker than that observed in F687. The Chl a bands in the red region are also observed in these spectra. The relative intensities of Chl a and phycocyanin excitation bands are highly temperature dependent; the analysis is shown in part 3.

Fig. 30 shows the excitation spectra of F715 at five different temperatures. The Chl a excitation bands at 440, 670 and 678 nm are observed. A shoulder which is not clearly resolved is also found at 686 nm. The positions of the bands for carotenoids and phycocyanin

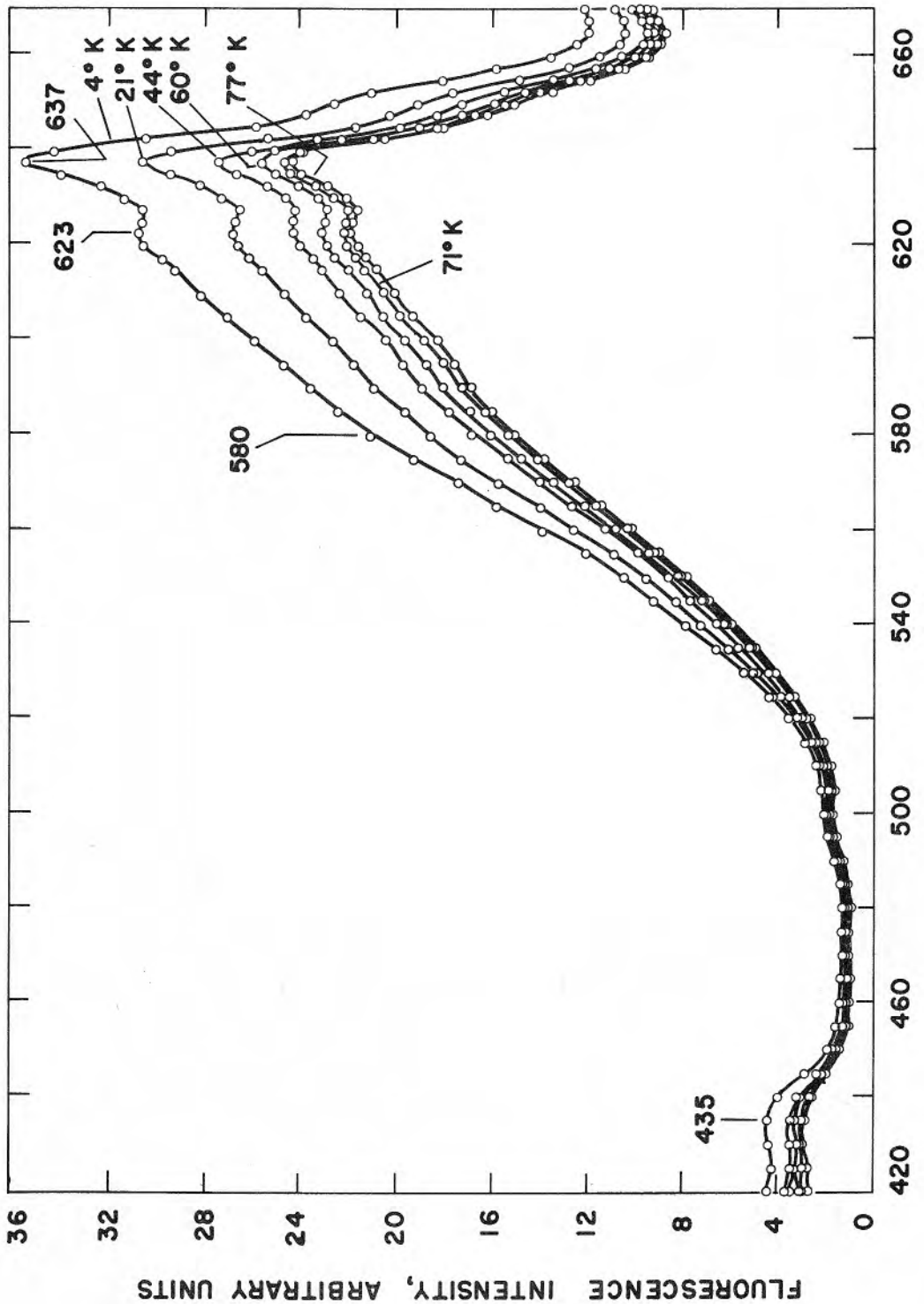

Fig. 28. Excitation spectra of F687 of Anacystis nidulans measured at 4°K, 21°K, 44°K, 60°K, 71°K and 77°K.

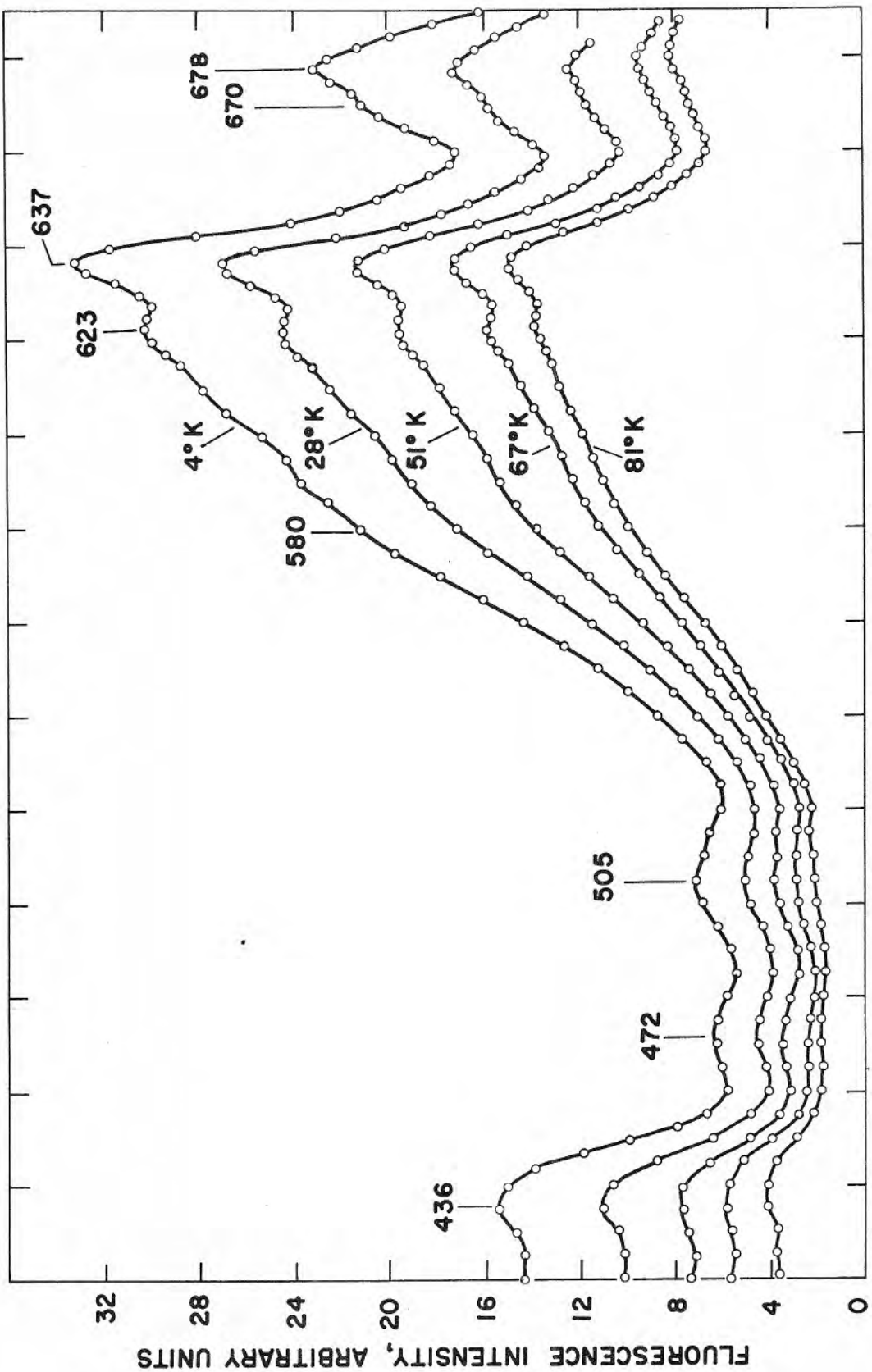

Fig. 29. Excitation spectra of F698 of Anacystis nidulans measured at 4°K, 28°K, 51°K, 67°K and 81°K.

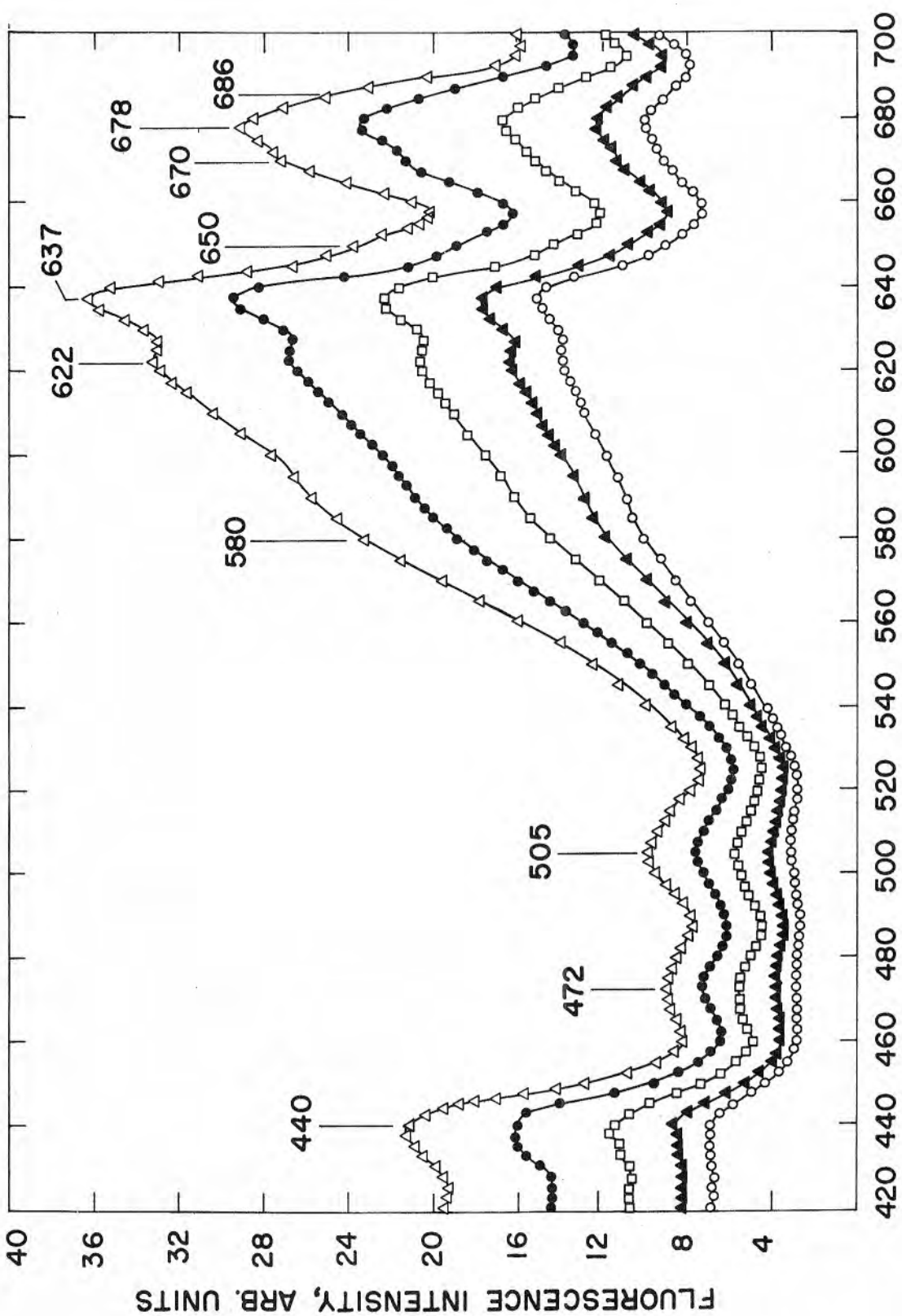

Fig. 30. Excitation spectra of F715 of Anacystis nidulans measured at 4°K Δ—Δ—Δ, 31°K •—•—•, 53°K □—□—□, 68°K ▲—▲—▲ and 79°K o—o—o. Points beyond 690 nm are partly caused by leakage of the exciting light.

are similar to those found in the excitation spectra of F698. At 4°K, the ratio of excitation bands at 435 nm to that at 637 nm is about 20% higher in F715 than that in F698. Higher Chl a excitation band for F715 than F698 have previously been discussed (46, 47, 49). The result suggests that the former band belongs to SI. The relative heights of the excitation bands are highly temperature dependent; analyses will be shown in part 3. The results obtained in the present study is better resolved than those reported (at 77°K) by Bergeron and Olson (49); the 637 nm band found in this study (in some experiments, this band is found at 633 nm) was not reported by these authors, although the general shape of the excitation spectra of the two studies are similar.

In the following paragraphs, the analyses of these spectra will be presented. In part a, the origin of the different fluorescence bands are investigated. In part b, the irreversible energy transfer from phycocyanin to Chl a is discussed.

a. The Molecular Origins of F653 and F680

Fig. 13 shows the fluorescence bands at 653, 685-687, 696 and 712 nm. Additional shoulders at 725, 740 and 755 nm are not clearly resolved. These band positions are similar to those found in Chlorella except the phycocyanin fluorescence band at 653 nm. This band is not found in Chlorella because it does not contain phycocyanin.

F653 is a phycocyanin fluorescence band; this is further indicated by the similarity between the excitation spectrum of F653 (Fig. 27) and that of the extracted phycocyanin which is shown in Fig. 31.

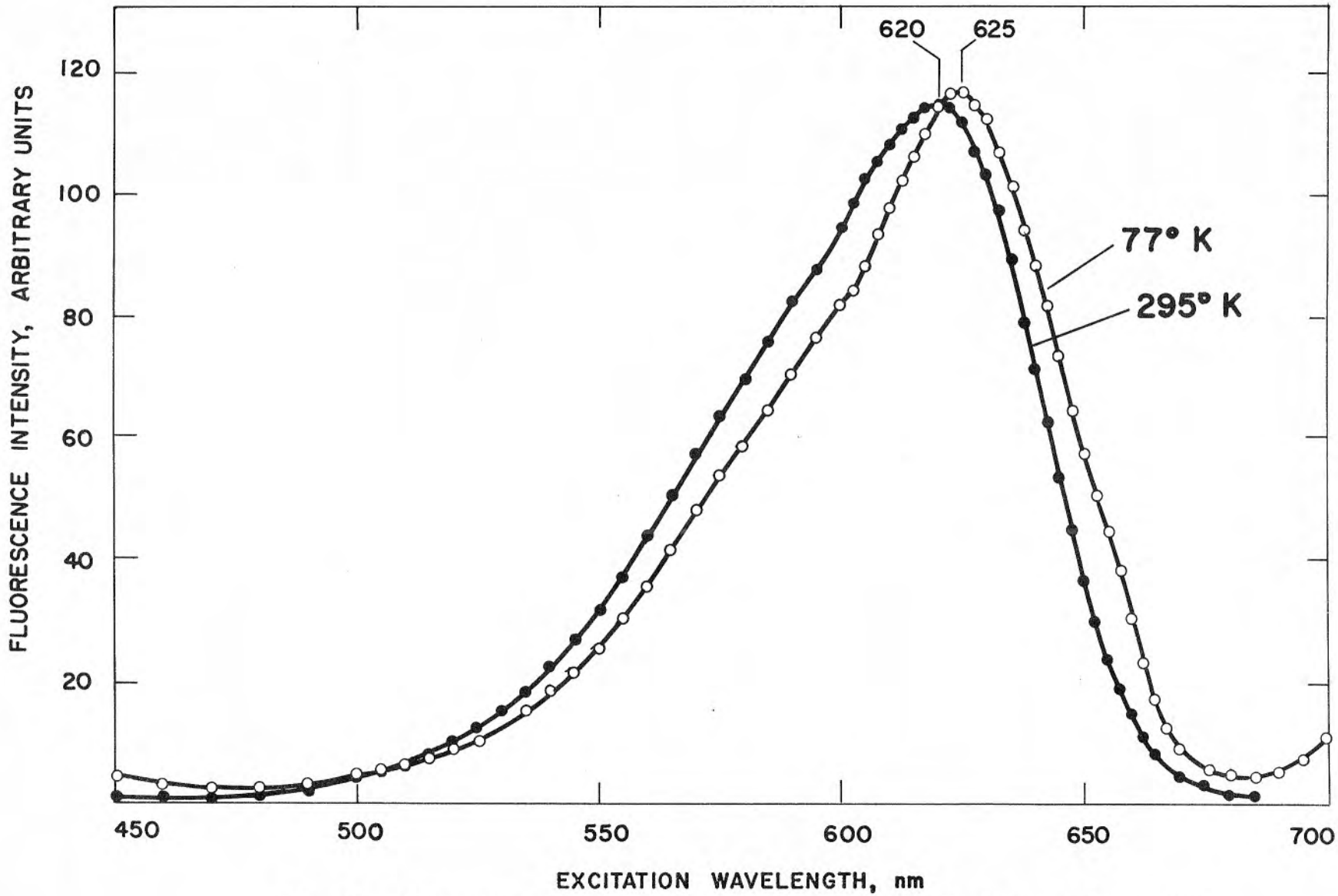

Fig. 31. Fluorescence excitation spectra of phycocyanin (in vitro) measured at 77°K o–o–o and 295°K •–•–•. (Data for this figure were supplied by Dr. G. Papageorgiou.)

The data for this figure were supplied by Dr. G. Papageorgiou; preparation of the sample is described in reference 107. The excitation spectrum of the long wavelength fluorescence of the extracted phycocyanin shows excitation bands at 580, 625 and 650 nm (at 77°K) as compared to the 580, 623 and 637 nm found in Anacystis. The relationship between Chl a molecules and the fluorescence at 687, 696 and 712 nm are studied with Anacystis which has been repeatedly extracted with 80% acetone (the sample was kindly supplied by Mr. Prasanna Mohanty). The absorption spectrum of this sample shows that only about 5-10% of the Chl a molecules still remain in the cells. Fig. 32 shows the 77°K fluorescence spectra of these cells excited at 435 nm and 560 nm. The most obvious difference between these spectra and those of the normal cells (Fig. 13-14) is that the long wavelength (>700 nm) fluorescence is greatly reduced in the Chl a-extracted cells, confirming the earlier suggestions that the long wavelength fluorescence bands are caused by Chl a molecules (37). Furthermore, when the fluorescence spectrum excited at 435 nm is compared to that excited at 560 nm, the former shows a higher F681-760 to F653 ratio, which indicates again, that the fluorescence from 681 nm to 760 nm is caused by the Chl a molecules.

The new fluorescence band at 681 nm may be caused by Chl a (670) that are close to the phycocyanin molecules. Because energy cannot be transferred any further to Chl a (678) after 90% of the Chl a has been extracted, therefore fluorescence from Chl a (670) occurs. This result is consistent with the earlier findings of Cederstrand et al. (70), who used

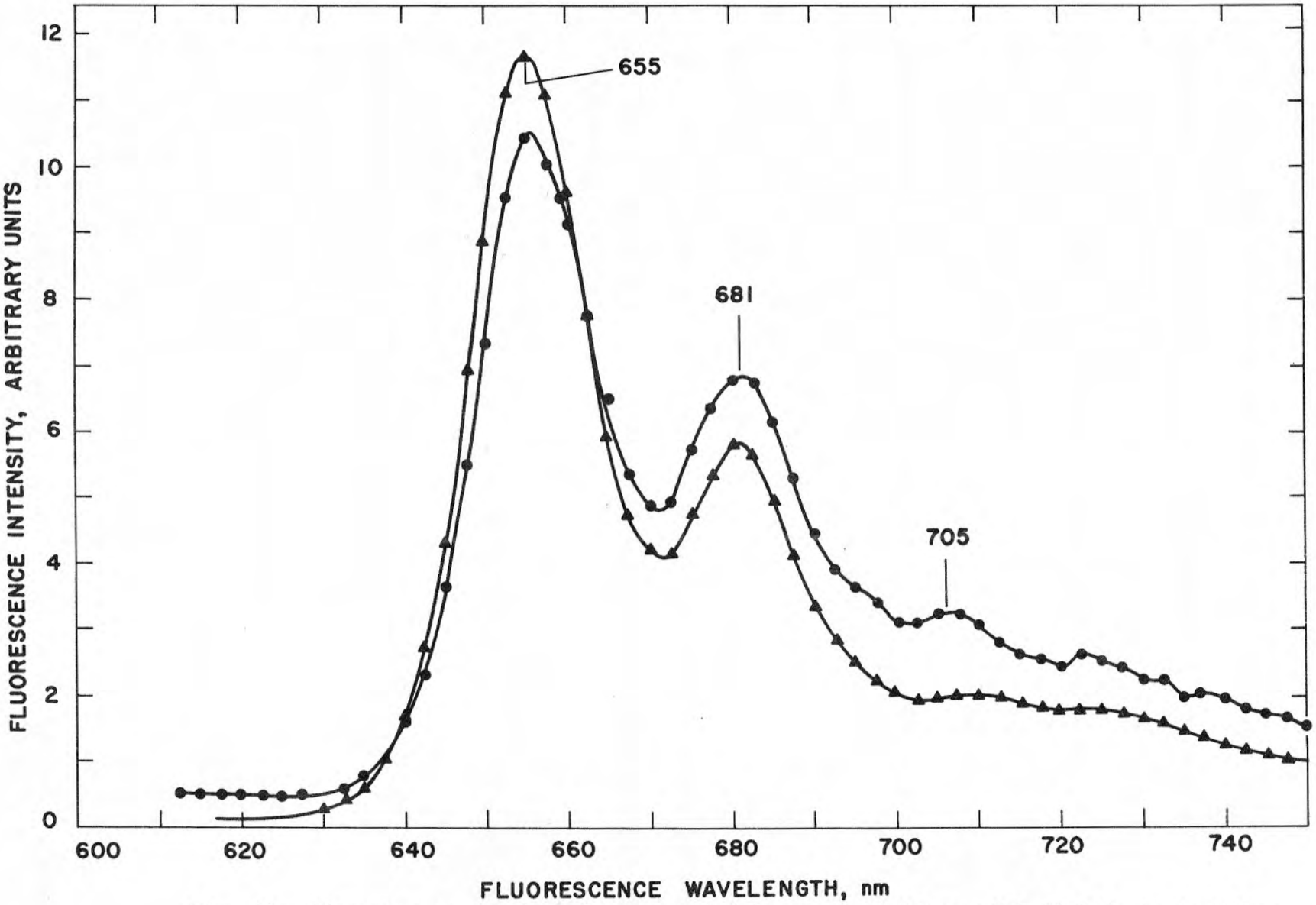

Fig. 32. Fluorescence spectra of resuspended Anacystis nidulans with 90-95% of the Chl a content extracted. The fluorescence spectra are measured at 77°K and excited at 435 nm ●—●—● and 560 nm ▲—▲—▲.

different concentrations of acetone and methanol to extract chlorophyll molecules from spinach chloroplasts. A gradual increase of the F680 band was found in the resuspended chloroplasts as the concentration of the acetone or methanol was raised from 30% to 80%. These authors also found that the absorption peak of the resuspended chloroplasts began to shift towards 670 nm in the same concentration range (30% to 80%). These results confirm the above suggestion that Chl a (670) emits near 680 nm. This conclusion fits in very well with the finding in part 1-b of this section which states that Chl a (678) emits near 687 nm.

In summary this study confirms that F653 is emitted from phycocyanin and F712 is emitted from a Chl a form (35-37, 47). The new conclusion obtained in this study is that Chl a (670) emits near 680 nm.

b. Irreversible Energy Transfer from Phycocyanin to Chlorophyll a -- Resonance Transfer

In Anacystis, excitation of phycocyanin causes fluorescence not only from phycocyanin, but also from Chl a (Fig. 13). This result is a good indication of energy transfer from phycocyanin to Chl a even at $4^{\circ}K$. Similar conclusion at $77^{\circ}K$ was suggested earlier by other authors (35, 36, 49).

When Chl a or (carotenoids) are excited, only Chl a but no phycocyanin fluorescence is found (Fig. 27-30), suggesting that energy can only be transferred from phycocyanin to Chl a, but not in the reverse direction. This irreversible energy transfer pathway satisfies the condition for the resonance transfer model which was discussed in detail by Förster (30, 31) and by Robinson (29). Energy transfer

from phycocyanin to Chl a excitation band in the red region (1st singlet excited state) is energetically favored since the former is at a higher energy level. For the same reason, it should be feasible for the excitation energy of Chl a in the blue region (2nd singlet excited state) to transfer to phycocyanin. However, when the transfer rate is slow (like that of resonance transfer) as compared to the decay rate, the excitation of Chl a in the 2nd singlet excited state decays to the 1st excited state before transfer to phycocyanin can occur (transfer time from phycocyanin to Chl a is 0.3-0.5 nsec (108, 109)). In this case, energy transfer from Chl a (after decaying to the 1st singlet excited state) to phycocyanin becomes energetically unfavorable, and irreversible energy transfer from phycocyanin to Chl a is found. This result therefore indicates that the transfer rate from Chl a to phycocyanin is much slower than the decay rate from the 2nd to the 1st singlet excited state of Chl a. Another reason why the irreversible energy transfer from phycocyanin to Chl a supports the interpretation of resonance transfer is the following. For resonance transfer, the transfer rate is proportional to the overlap of the fluorescence band of the donor and the absorption band of the acceptor. The fluorescence band of Chl a and the absorption band of phycocyanin show very little overlap. Therefore, according to the resonance transfer model energy transfer from Chl a to phycocyanin is rather improbable. This is indeed the observed result. Furthermore, according to this model, the transfer rate may vary as a function of temperature, because the amount of band overlap may change upon cooling as a result of sharpening and shifting

of the bands. It will be shown in the next section that energy transfer from phycocyanin to Chl a indeed obeys this condition.

In summary, the above analyses show that energy transfer from phycocyanin to Chl a in Anacystis satisfies the theoretical predictions for the resonance transfer model discussed by Förster (30, 31) and Robinson (29).

3. Energy Transfer Efficiency and Its Temperature Dependence

a. Chlorella pyrenoidosa

The temperature dependence of energy transfer efficiency of the two pigment systems are determined by comparing the sensitized fluorescence measured at various temperatures. The two systems are considered separately for the convenience of calculations. The derivation of the method is shown as follows.

Let E_{678}^{687} and E_{670}^{687} be the intensities of the SII fluorescence, F687, excited at 678 and 670 nm (subscript), respectively. A_{678} and A_{670} are the number of quanta absorbed in SII at 678 and 670 nm, respectively. Φ_{678}^{687} is the SII fluorescence efficiency of F687 (superscript) when excited at 678 nm (subscript). $R_{670\rightarrow 678}$ is the transfer efficiency (% transferred) from Chl a (670) to Chl a (678) in SII. Then:

$$E_{678}^{687} = A_{678}\,\Phi_{678}^{687} \qquad (1)$$

$$E_{670}^{687} = A_{670}\,R_{670\rightarrow 678}\cdot\,\Phi_{678}^{687} \qquad (2)$$

The ratio of equation (1) and (2) shows

$$R_{670\rightarrow 678} = \frac{E_{670}^{687}}{E_{678}^{687}}\cdot\frac{A_{678}}{A_{670}} \qquad (3)$$

The right hand side of equation (3) is determined by the excitation bands at 670 and 678 nm of F687 (see Fig. 25), and by the ratio of the SII absorption bands at 678 nm and 670 nm which are determined from the absorption data of Fig. 5 and from the action spectra of photosynthesis of the two pigment systems (61, 69, 102). These studies show that the ratios of Chl a (678)/Chl a (670) for the two pigment systems are about equal; therefore, the ratio of Chl a (678)/Chl a (670) of the individual pigment system can be determined from the absorption ratio of the cells at these wavelengths (678 nm and 670 nm). Even though the ratio of Chl a (670)/Chl a (678) for the two pigment systems are alike, the absolute value of this ratio may vary according to the culture conditions. Therefore we use the absorption data provided by Fig. 5 to determine the Chl a (678) / Chl a (670) ratio for the present investigation. $\frac{A_{678}}{A_{670}}$ is determined by the percent of light absorbed at the two wavelengths when the incident intensities at these wavelengths are normalized. However, since the concentration used in the fluorescence excitation measurements are very low (O.D. $\simeq$ 0.1 per cm), $\frac{A_{678}}{A_{670}}$ approaches the ratio of $\frac{678\text{ nm}}{670\text{ nm}}$ of Fig. 5, which is shown in O.D. unit. When values obtained in such a manner are substituted into equation (3), the transfer efficiency from Chl a (670) to Chl a (678) in SII approaches 100% (allowing 5% error) at 77°K. Furthermore, this transfer efficiency shows practically no change (<3% deviation) upon warming the cells from 4°K to 77°K indicating that in SII, the efficiency of energy transfer from Chl a (670) to Chl a (680) is practically independent of temperature.

Assuming that energy absorbed by Chl a (670) is first transferred to the long wavelength Chl a forms*, it can be shown as before that:

$$R'_{670\rightarrow 678} = \frac{E'^{725}_{670}}{E'^{725}_{678}} \cdot \frac{A'_{678}}{A'_{670}} \qquad (4)$$

where $R'_{670\rightarrow 678}$ is the transfer efficiency from Chl a (670) to Chl a (678) in SI, and all the definitions are the same as above except the symbol "prime" stands for SI. When the value of $\frac{E'^{725}_{670}}{E'^{725}_{678}}$ obtained from the excitation spectrum of F725 (mainly SI), and the value of A'_{678}/A'_{670} obtained from the absorption spectrum of SI (61, 69, 102 and Fig. 5), are substituted into equation (4), one finds that the efficiency of energy transfer from Chl a (670) and Chl a (678) in SI to be approaching 100% (allowing 5% error). Furthermore, this value remains constant in the $4^{o}K$ to $77^{o}K$ temperature range.

To analyze the efficiency of energy transfer from Chl b (649) to Chl a (670) and Chl a (678), the following formulae are considered.

Let E^{687}_{649} be the fluorescence intensity at 687 nm, excited at 649 nm. A_{649} is the quanta absorbed by SII at 649 nm. $R_{649\rightarrow 670}$, $R_{649\rightarrow 678}$ are the transfer efficiencies from Chl b (649) to Chl a (670)

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*This assumption is justified by the fact that the emission band of Chl a (670) is at 680 nm (see section 2-a) which has a good band overlap with the absorption band of Chl a (678). Furthermore, the number of Chl a (678) molecules is much higher than those of the longer wavelength Chl a forms; therefore, the probability of energy transfer from Chl a (670) to Chl a (678) is highly favored.

and Chl a (678), respectively, in SII. Φ_{678}^{687} and $R_{670\rightarrow 678}$ are defined the same as before, then:

$$E_{649}^{687} = A_{649} \cdot R_{649\rightarrow 670} \cdot R_{670\rightarrow 678} \Phi_{678}^{687} + A_{649} \cdot R_{649\rightarrow 678} \Phi_{678}^{687} \quad (5)$$

The second term on the right hand side of equation (5) takes into account the direct transfer between Chl b (649) and Chl a (678). (This consideration is necessary because the population of Chl a (678) is high, therefore the probability of having Chl b and Chl a (678) as nearest neighbors are also rather high, which is a favorable condition for energy transfer between these two molecular species.) When equation (5) is divided by equation (2), then:

$$\frac{R_{649\rightarrow 670} \cdot R_{670\rightarrow 678} + R_{649\rightarrow 678}}{R_{670\rightarrow 678}} = \frac{E_{649}^{687}}{E_{670}^{687}} \cdot \frac{A_{670}}{A_{649}}$$

Substituting the value of $R_{670\rightarrow 678}$ obtained from equation (3), where $R_{670\rightarrow 678} \simeq 1$, then:

$$R_{649\rightarrow 670} + R_{649\rightarrow 678} \simeq \frac{E_{649}^{687}}{E_{670}^{687}} \cdot \frac{A_{670}}{A_{649}} \quad (6)$$

Assuming that the pigment content of Chl a and Chl b in each pigment system do not change because of cooling, then the ratios on the right hand side of the equation can be estimated from the excitation spectrum of F687 (or F725 for SI estimation) and from references 61, 69, 102 and

Fig. 5. The product of these ratios again approaches 100% (allowing 10% error). This estimation is not as accurate as that of $R_{670\rightarrow678}$, the reason being the ratios $E^{687}_{649}/E^{687}_{670}$ and $E^{725}_{649}/E^{725}_{670}$ fluctuated to a much greater extent than the ratio $E^{687}_{670}/E^{687}_{678}$. The fluctuations are caused by the concentration and the culture condition of the cells. However, the above conclusion is checked by another method. It is found that the value on the right hand side of equation (6) remains constant between 4°K and 77°K. Furthermore, this value changes less than 10% upon cooling from room temperature to 4°K. Duysens (6), Tomita and Rabinowitch (109) reported earlier that the transfer efficiency from Chl b to Chl a is 100% at room temperature. Since we find that the transfer efficiency at 4°K and that at room temperature varies by less than 10%, the efficiency of energy transfer from Chl b to Chl a must be over 90% even at 4°K.

Energy transfer efficiency in the longer wavelength region (685-715 nm) cannot be calculated because of the complexity of the weak absorption bands in that region. This is so because the overlap between the weak 695 nm and the 687 nm absorption bands causes the estimation of A_{685}/A_{695} for the individual pigment systems to be inaccurate. However, the absorption spectra (Fig. 5) show that the absorbance at these wavelengths remains almost constant from 4°K to 77°K. If we assume that this constancy indicates the constant absorbance of the individual pigment systems also, then, an estimation of the temperature dependence of the transfer efficiency is possible, because A_{685}/A_{695} becomes a

constant at various temperatures and is cancelled out in the calculation.

Assuming that energy absorbed at 685 nm is completely transferred to Chl a (695) before being transferred to other long wavelength Chl a forms, then, an equation similar to equation (4) is obtained; the comparison of the transfer efficiencies at $4^{\circ}K$ and $77^{\circ}K$ becomes:

$$\frac{\left(R'_{685\rightarrow 695}\right)_{4^{\circ}K}}{\left(R'_{685\rightarrow 695}\right)_{77^{\circ}K}} = \left[\frac{E'^{725}_{685}}{E'^{725}_{695}} \cdot \frac{A'_{695}}{A'_{685}}\right]_{4^{\circ}K} \Big/ \left[\frac{E'^{725}_{685}}{E'^{725}_{695}} \cdot \frac{A'_{695}}{A'_{685}}\right]_{77^{\circ}K}$$

$$= \left[\frac{E'^{725}_{685}}{E'^{725}_{695}}\right]_{4^{\circ}K} \Big/ \left[\frac{E'^{725}_{685}}{E'^{725}_{695}}\right]_{77^{\circ}K} = 0.9 \qquad (7)$$

where $R'_{685\rightarrow 695}$ is the SI transfer efficiency, E'^{725}_{685} and E'^{725}_{695} are the SI fluorescence intensities measured at 725 nm, excited at 685 and 695 nm, respectively. A'_{695} and A'_{685} are the SI absorbance at 695 and 685 nm, respectively. The result shows a 10% change of transfer efficiency in the long wavelength region (685 to 695 nm) upon warming from $4^{\circ}K$ to $77^{\circ}K$.

In summary, the analyses show that the efficiencies of energy transfer from Chl b to Chl a (670 and 678), and from Chl a (670) to Chl a (678) in Clorella approaches 100% even at $4^{\circ}K$ and these efficiencies are independent of temperature from $4^{\circ}K$ to $77^{\circ}K$, whereas the transfer efficiency in the long wavelength region (685 nm to 695 nm) increases about 10% upon warming up in that temperature range.

b. Anacystis nidulans

The energy transfer efficiencies and their temperature dependence in Anacystis are analyzed by the same methods as those in Chlorella.

Assuming that energy absorbed by Chl a (670) is first transferred to Chl a (678) and then to longer wavelength Chl a forms and is emitted at 715 nm, then, equation (4) of part (a), with slight modification, is applicable to calculate the transfer efficiency in Anacystis. The modified equation being:

$$R'_{670\rightarrow 678} = \frac{E'^{715}_{670}}{E'^{715}_{678}} \cdot \frac{A'_{678}}{A'_{670}} \qquad (8)$$

where $R'_{670\rightarrow 678}$ is the transfer efficiency from Chl a (670) to Chl a (678) in SI. E'^{715}_{670} and E'^{715}_{678} are the SI fluorescence intensities measured at 715 nm, excited at 670 and 678 nm, respectively. A'_{678} and A'_{670} are the quanta absorbed by SI at 678 and 670 nm, respectively.

Since most of the Chl a of Anacystis is in SI (shown by the action spectrum of photosynthesis of the two pigment systems (110)) and since excitation at the Chl a absorption bands causes a strong fluorescence band at 712-715 nm, and weak bands at 685 and 695 nm (Fig. 14), $E^{715}_{670}/E'^{715}_{678}$ is indicated by the ratio of the excitation band heights of F715 (Fig. 31), whereas A'_{678}/A'_{670} can be determined from the ratio of the absorption band heights of Chl a (678) and Chl a (670) shown in Fig. 6. This is so because the concentration of the sample for measuring the excitation spectrum is very low (O.D. - 0.1 to 0.2 at 675 nm).

therefore, the ratio of the absorbed quanta can be estimated by the ratio of the absorption band heights. The result shows that the transfer efficiency from Chl a (670) to Chl a (678) approaches 100% (allowing 5% error), and this efficiency is independent of temperature from $4^{\circ}K$ to $77^{\circ}K$.

The estimation of the transfer efficiency from allophycocyanin (or phycocyanin) to Chl a is attempted in a similar way as from Chl b to Chl a (see equation 6). Unfortunately the ratio $\frac{A_{670}}{A_{650}}$ for SI and SII are not certain for Anacystis, therefore the transfer efficiencies cannot be obtained. The ratio of the total absorbance at 650 and 670 nm are constant in the $4^{\circ}K$ to $77^{\circ}K$ range. With assumptions similar to those used for the derivation of equation 7, the temperature dependence of the transfer efficiencies can be estimated as follows:

$$\text{SI:}\left[\frac{(R'_{650\rightarrow 670})_{4^{\circ}K}}{(R'_{650\rightarrow 670})_{77^{\circ}K}}\right] = \left[\frac{E'^{715}_{650}}{E'^{715}_{670}}\right]_{4^{\circ}K} \Big/ \left[\frac{E'^{715}_{650}}{E'^{715}_{670}}\right]_{77^{\circ}K} = 0.89 \qquad (9)$$

$$\frac{(R'_{637\rightarrow 670})_{4^{\circ}K}}{(R'_{637\rightarrow 670})_{77^{\circ}K}} = \left[\frac{E'^{715}_{637}}{E'^{715}_{670}}\right]_{4^{\circ}K} \Big/ \left[\frac{E'^{715}_{637}}{E'^{715}_{670}}\right]_{77^{\circ}K} = 0.80 \qquad (10)$$

$$\text{SII:}\ \frac{(R_{650\rightarrow 670})_{4^{\circ}K}}{(R_{650\rightarrow 670})_{77^{\circ}K}} = \left[\frac{E^{698}_{650}}{E^{698}_{670}}\right]_{4^{\circ}K} \Big/ \left[\frac{E^{698}_{650}}{E^{698}_{670}}\right]_{77^{\circ}K} = 0.80 \qquad (11)$$

$$\frac{(R_{637\rightarrow 670})_{4^\circ K}}{(R_{637\rightarrow 670})_{77^\circ K}} = \left[\frac{E^{698}_{637}}{E^{698}_{670}}\right]_{4^\circ K} \Big/ \left[\frac{E^{698}_{637}}{E^{698}_{670}}\right]_{77^\circ K} = 0.77 \qquad (12)$$

Equation (9) and (10) show the increase of SI transfer efficiency from phycocyanin and allophycocyanin to Chl a upon warming from $4^\circ K$ to $77^\circ K$: equations (11) and (12) show the increase of SII transfer efficiency under the same condition. The excitation spectra of F715 and F698 provided data for SI and SII, respectively. An average increase of 20% is found for the transfer efficiency from phycocyanin to Chl a upon warming from $4^\circ K$ to $77^\circ K$.

In summary, the transfer efficiency from Chl a (670) to Chl a (678) in Anacystis approaches 100% even at $4^\circ K$. This efficiency is independent of temperature in the $4^\circ K$ to $77^\circ K$ temperature range. The transfer efficiency from phycocyanin to Chl a, however, increases about 20% upon warming from $4^\circ K$ to $77^\circ K$. The main purpose of analyzing the transfer efficiencies and their temperature dependence is to study the mechanism of energy transfer in the photosynthetic organisms as described in chapter I.

The high efficiency ($\simeq$100%) of energy transfer from Chl b to Chl a and from Chl a (670) and Chl a (678) suggests that the transfer rate is much faster than the lifetime of fluorescence (τ = 3.1 nsec at $77^\circ K$, ref. 87). The fact that this efficiency is independent of temperature between $4^\circ K$ and $77^\circ K$ suggests that the transfer rate among the homogeneous molecules varies by less than a few percent of $\frac{\tau}{\langle n \rangle}$

in this temperature range ($\langle n \rangle$ is the mean number of transfers among the homogeneous molecules); otherwise one would have observed a change in the transfer efficiency. In case $\langle n \rangle$ is large, say $\simeq 10^4$, a change of transfer time of 10^{-14} sec (the change of transfer time $\Delta\tau_t$ must be $\leqslant 10^{-14}$ sec, which is the transfer time for strong coupling model; i.e., if $\Delta\tau_t$ is $> 10^{-14}$ sec, the transfer time would have exceeded the fast transfer limit) would have caused a noticeable (3.3%) change in the transfer efficiency. Since our results show no change of the efficiency of transfer from Chl b to Chl a, therefore the strong coupling, fast transfer model (29) for energy transfer among the homogeneous Chl b molecules and the homogeneous Chl a molecules is possible provided $\langle n \rangle$ is large. In case $\langle n \rangle$ is small, for example, Chl b and Chl a (670) molecules are scattered among the Chl a (678) molecules, then energy can be transferred directly from Chl b to Chl a (678) in which case $\langle n \rangle$ can be as small as unity. In this case, the change of transfer rate can be as large as 10^{-11} sec without noticeable change in the transfer efficiency. This change of transfer rate is in the order of magnitude of resonance transfer rate, therefore weak coupling resonance transfer model can also explain the observed results (see discussion in chapter IV, at the end of section C).

The transfer efficiencies from phycocyanin to Chl a, from Chl a (678) to the trap level, and from the molecular species that absorb at 685 nm to 695 nm are all temperature dependent. These results suggest that energy transfer among the heterogeneous molecules obeys

the resonance transfer model, which is in agreement with the theoretical prediction (29-31).

VII. CONCLUSIONS AND SUMMARY

Absorption, fluorescence and fluorescence excitation spectra of Chlorella pyrenoidosa and Anacystis nidulans have been measured from $4^{\circ}K$ to $295^{\circ}K$.

For both algae, there is little absorbance change when the cells are warmed up from $4^{\circ}K$ to $77^{\circ}K$. The broad 675 nm absorption band at room temperature ($295^{\circ}K$) is resolved into two bands (670 and 677.5 nm) at $77^{\circ}K$, and becomes more clearly resolved at $4^{\circ}K$. Detailed comparisons of the absorption spectra measured at different temperatures are discussed in chapter III.

The phase transitions of ice crystals have direct effects on the relative heights of the fluorescence bands. This effect can be explained neither by the change in scattering and the consequent change in reabsorption of fluorescence, nor by the change of ice volume. Careful study shows that SII fluorescence is irreversibly lowered when ice changes from the cubic to the hexagonal form. The close relationship between the ice changing phase and the decrease of SII fluorescence suggests that SII is related to aqueous environment (although the possibility that SI is also in such as environment cannot be excluded). Since ice changes phase between $113^{\circ}K$ and $220^{\circ}K$, most of the spectral analyses are done in the $4^{\circ}K$ to $77^{\circ}K$ range.

The main fluorescence band at 685-687 nm is shown to be an emission band of Chl a (678), whereas Chl a (670) is found to emit at

680 nm when Chl a (678) is selectively removed.

F698 is emitted from a molecular species which is low in concentration when compared to the bulk of Chl a. This molecular species is considered to form a trap level with estimated absorption band near 686 nm. It obtains excitation energy from the same pigment system as F687, namely, from SII. The intensities of F687 and the trap fluorescence-F698, are sensitive to the form and composition of the aqueous environment.

F725 is a broad band which can be resolved into at least four bands at $4^{\circ}K$; namely, 715-717, 725-730, 735-740 and 750-760 nm. The former two bands show mainly SI characteristics whereas the 750-760 nm band shows more SII characteristics than F725. The F715 band is affected to a much lesser extent by the ice crystal effect than the other fluorescence bands.

The efficiencies of energy transfer as a function of temperature have been studied. The transfer efficiencies from Chl b to Chl a and from Chl a (670) to Chl a (678) approach 100%, even at $4^{\circ}K$. Furthermore, this efficiency is independent of temperature from $4^{\circ}K$ to $77^{\circ}K$. These results support the fast transfer strong coupling model (29) for energy transfer among the homogeneous Chl b and the homogeneous Chl a (670) molecules, provided that the number of transfers among these molecules is large (see section 3 - b of chapter VI). However, if the energy absorbed by these molecules is immediately transferred to Chl a (678) without extensive transfer among Chl b or Chl a (670), the above

results can also be explained by the resonance transfer model. Transfer efficiencies from the bulk of Chl a (678) to the "trap" (the molecular species that emits at 698 nm), from the species that absorbs at 685 nm to that at 695 nm, and from phycocyanin and allophycocyanin to Chl a are temperature dependent. These results support the weak coupling, resonance transfer model for energy transfer between the heterogeneous molecules in vivo.

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