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THE INFLUENCE OF RICINUS LEAF PROTEIN (AND
LONG-CHAIN UNSATURATED FATTY ACIDS) ON THE
STATES OF CHLOROPHYLL IN CHLOROPHYLLOUS
SYSTEMS HAVING DIFFERENT LEVELS OF STRUCTURAL
COMPLEXITY.

The City University of New York, Ph.D., 1973
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(AND LONG-CHAIN UNSATURATED FATTY ACIDS)
ON THE STATES OF CHLOROPHYLL IN
CHLOROPHYLLOUS SYSTEMS HAVING DIFFERENT
LEVELS OF STRUCTURAL COMPLEXITY.

by

BRINA NATHANSON

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ABSTRACT

The techniques of circular dichroism, absorption and fluorescence spectroscopy were used to demonstrate that the effect of either a naturally occurring protein factor isolated from the leaves of Ricinus communis, or a model system which duplicates its action (long-chain unsaturated fatty acids), is to bring about deaggregation of chlorophyll in chlorophyllous systems of various levels of organization. There are two different mechanisms of action depending upon whether the chlorophyll is (1) in solution, (2) part of a chlorophyll-protein complex, or (3) in a chlorophyll-protein complex organized into a membranous array. In the first case (chlorophyll a in CCl_4 or CCl_4 -hexane) deaggregation occurs solely by direct action on the chlorophyll; in the second case, in addition to this direct action, deaggregation occurs by an indirect means; in the third case, deaggregation is predominantly by the indirect mechanism. From C.D. studies in the U.V., it was determined that the indirect mechanism involves a preliminary unwinding of the protein to which the chlorophyll chromophore is attached. Evidence for partial reversal of deaggregation by this indirect mechanism was observed by post-treating linolenic acid-incubated subchloroplast particles with BSA. That fatty acids could act on other chromoproteids was confirmed by the experiments with linolenic acid-treated Porphyridium or Anacystis-in this case, changes similar to those in isolated chlorophyllous systems were observed.

The changes in C.D. of control particulate chlorophyll-containing systems was interpreted on the basis of the C.D. bands observed in simpler systems (i.e., chlorophyll a in solution and as part of chlorophyll-protein complexes). It was noted that there is a reversal in the polarity of the C.D. spectra of chlorophyll a in particulate systems (e.g., sub-

chloroplast particles and chloroplasts) as compared with less complex systems; this reversal was ascribed to organization of chlorophyll-containing protein into membranous arrays.

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INTRODUCTION

Since the late 1950's, photophysiological experiments have indicated that not only do different forms of chlorophyll a exist in plants, but also that these forms have different functions. In particular, the observations of Emerson and co-workers (Emerson et al., 1956; Emerson, 1958; and Brody and Emerson, 1959) established that on the long wavelength side of the absorption band of chlorophyll a in vivo, the quantum efficiency of O₂ evolution declines. Since in the presence of shorter wavelengths of light, the photosynthetic efficiency of the long wavelengths was much increased, it was concluded that two photoacts occur. Although Emerson et al. (1957) attributed the observed enhancement by shorter wavelengths, to absorption by accessory pigments, other workers attributed it to absorption by a form of chlorophyll a different from that absorbing at longer wavelengths. S. Brody (1958), S. and M. Brody (1961) suggested that, in vivo, chlorophyll a exists in monomeric and aggregated forms (absorbing at shorter and longer wavelengths, respectively) and that excitation of both is necessary for higher plant photosynthesis.

The experimental data of Duysens et al. (1961) seemed to fit well with the Hill-Bendall model (1960) for electron transport between the two photosystems (Duysens and Amez, 1962). Duysens et al. (1961) had observed the differential effect of red and far-red light on the state of oxidation of cytochrome f: in the presence of red light it remains in the reduced state, while far-red light causes its oxidation. Duysens and Amez (1962) proposed that the "first" or shorter wavelength light reaction (light reaction II) involves the reduction of cytochrome f and oxidation of water to molecular oxygen, and the second light reaction (light reaction I) leads to reoxidation of cytochrome f and reduction of

TPN. Duysens and Ameer further suggested that electron flow between systems II and I is accompanied by the (photo)phosphorylation of ADP to ATP^{and}*; also postulated was a scheme whereby light absorbed by "bulk" (the light-harvesting) chlorophyll a's of either system I or II is transferred to the respective reaction centers of that system and used in its primary photochemical reaction.

Associated with these two photosystems** are various accessory pigments -- carotenoids (carotenes and xanthophylls), phycobilins, and chlorophyll b -- which transfer the energy they absorb to the bulk chlorophyll a's. Duysens had much earlier (1951) demonstrated (using the "sensitized fluorescence" technique introduced by Dutton and Manning, 1941) that light absorbed by phycobilins of red or blue-green algae is transferred to "chlorophyll a". Additionally, he found that in P. cruentum, light absorbed by phycoerythrin caused reduction of cytochrome f, while oxidation of the cytochrome was brought out by illumination with "system I-light" (680 nm) (1961). Fluorescence experiments by other groups of workers established the ^{nature of the} specific photosystems with which the other accessory pigments are associated: energy from chlorophyll b (Rabinowitch and Govindjee, 1961) and phycobilins (Duysens, 1961; Duysens and Ameer, 1962) is transferred preferentially to system II while energy from caro-

* In recent years, another model for photosynthetic electron transport involving three light reactions (Knaff and Arnon, 1969; Arnon et al., 1970, 1971) was proposed, i.e., that there are two light reactions in system II -- these two operating in series, and one light reaction in system I operating parallel to the system II light reactions. This scheme was based on evidence that chloroplast fragments which were able to photoreduce NADP with water as an electron donor, had no detectable P 700. However, Esser (1972) has shown that the failure to detect P 700 was due to the experimental set-up itself (for example, the preparative procedure which they used washed out plastocyanin necessary for reduction of P 700).

** An updated "Z" scheme of the type first proposed by Hill and Bendall, and modified to include subsequently determined electron transport intermediates is given in Fig. A (Clayton-1971).

Figure A. Series scheme for the two systems of light reactions in the photosynthesis of green plants. [After Clayton(1971).]



SYSTEM II

SYSTEM I

tenes is transferred preferentially to system I (Goedheer, 1969). In general, light absorbed by xanthophylls is not transferred to chlorophyll (Goedheer, 1969). However, in the case of diatoms and brown algae, which contain fucoxanthin, sensitization of photosynthesis ^{by this pigment} is efficient (Tanada, 1951). Recently, Goedheer (1970) has found that light absorbed by fucoxanthin (in Fucus) is transferred to chlorophyll of system II.

Concomitant with studies on association of accessory pigments with the two photosystems were investigations of the many forms and functions of the chlorophyll a's belonging to systems I and II. The techniques utilized in these studies were conventional (or integral) absorption spectroscopy (at room and low temperature), derivative absorption spectroscopy (at room and low temperature), ^{of light induced changes} absorption spectroscopy, and (principally low temperature) fluorescence emission and excitation spectroscopy.

In 1937, Albers and Knorr were able to resolve two separate maxima, at 670 and 680 nm., in the broad red absorption band of chloroplasts, using photographic densitometry. Work by Krasnovskii and his associates also lent support to the concept that there exists more than one type of chlorophyll a in vivo. In 1952, Krasnovskii and Kosobutskaja were successful in duplicating the in vivo bands of bacteriochlorophyll by drying solutions of (single-banded) bacteriochlorophyll on glass slides. In the same year, Krasnovskii and Kosobutskaja, studying chlorophyll formation in extracts of dark-grown ^Kbean leaves, found that there was, upon greening, a shift in the absorption maximum of chlorophyll a from 673 to 678 nm. Their work suggested to them that chlorophyll in vivo exists in two different forms: a monomeric form absorbing at 670 nm., and an aggregated form absorbing at 680 nm. Later (1955), while studying chlorophyll formation in extracts of dark-grown bean leaves, they found that the 670 and 680 nm. components (comprising a band with maximal absorption at 678 nm.) could be

affected in different ways by environmental conditions -- for example they observed the differential effects of photobleaching of these two forms.

French and his associates have utilized the method of derivative spectroscopy to analyze the contribution of the various absorbing forms of chlorophyll a to total in vivo absorption in the red region of the spectrum (1957 - 1961). These groups of workers (1958) confirmed the existence of the two bands with maxima at 670 and 680 nm and further observed a special long wavelength band at 695 nm. in Euglena cells. These bands were attributed to chlorophyll a in different physical states.

Three interpretations have been proposed to explain why chlorophyll a, homogeneous and single-banded in (dilute) solutions, exists in different forms in vivo. French and his associates have proposed that these different forms may be attributed to the binding of chlorophyll to dissimilar proteins (Mayers and French, 1960; Brown and French, 1961). A second interpretation -- that different forms of chlorophyll arise because of various states of aggregation of the chromophores -- was given by the Brody's, and based mainly on their fluorescence work (see below). This second interpretation is supported by the above-cited work of Krasnovskii et al. in 1952. A third proposal is that R. Olson and his associates (1962, 1964), who interpreted their findings as indicating that the absorption properties of some of the chlorophyll a in vivo is due to crystalline orientation. Recently, French, Brown, Wiessner and Lawrence (1971) have used a computer program to analyze some of their derivative spectroscopy data. By fitting the data with variously modified Gaussian bands, they concluded that there are four, rather than two, major forms of chlorophyll a, and that these forms essentially maintain constant wavelengths and half-widths, varying in proportion to one another. These forms have been designated Ca 662 and Ca 670 (this

double band to replace that formerly considered single at 670 nm.) and Ca 678 and 684 (this double band to replace that formerly considered single at 680 nm.). These forms are supplemented by other forms of chlorophyll a, having long wavelength bands in the spectral region 690 - 720 nm.; ~~the latter~~ long wavelength absorbing forms, according to French and co-workers, are present in some, but not all photosynthetic organisms.

Kok and co-workers (1959, 1961) observed light-induced absorbance changes at 705 nm. and attributed these changes to a distinct form of chlorophyll a. Actually, Kok (1959) and Kok and Hoch (1960) had found small negative bands at 650 and 680 as well as at 705 nm. to be reversibly bleached by light, but although the bleaching of the first two bands could be ascribed to fluorescence yield changes (induced by high intensity light) the change at 705 nm. could not. Kok assumed that this latter pigment represented the photochemically active center of system I (1962) because this pigment, named P 700 by him, was reduced by system II-light and oxidized by system I-light. Butler (1960, 1961) using the techniques of low temperature integral and low temperature derivative absorption spectroscopy, observed a band having a maximum at this wavelength (i.e. at 700 nm.). This band appeared as a shoulder in the direct low temperature absorption spectrum, and as a distinct band in the low temperature derivative spectrum.

Many groups of workers have sought to associate the various fluorescence emission bands of chlorophyll with their corresponding absorption bands. There has been little disagreement about assigning the fluorescence band with maximum at 685 nm. to the form of chlorophyll having an absorption peak at 674 nm. (the monomeric form of chlorophyll a), since this is the first fluorescence band observed in early stages of greening in etiolated plants, in which the only absorbing form of chlorophyll a is

the 670 nm. one. Also, dilute solutions of chlorophyll have a fluorescence band in this region. S. Brody (1958) using low temperature fluorescence spectroscopy, had established that, in organic solvents (such as CCl_4), highly concentrated solutions of chlorophyll a have both emission at 685 nm. (also seen in dilute solution) and a long wavelength emission band at 715 nm. Similar emission patterns were observed with Chlorella cooled to 77°K, but in this case, the long wavelength band peaked at 720 nm. He proposed that the long wavelength emission band corresponds to the form of chlorophyll absorbing maximally at 705 nm. Butler in 1961 confirmed Brody's proposal when he demonstrated that the action spectrum for low temperature fluorescence emitted at 720 nm. in greening bean leaves had a maximum at 705 nm. Further work by S. and M. Brody (1963) with dark-grown Euglena, demonstrated that the maximum of long wavelength emission shifts ^{further} towards the red region as greening progresses. Emission from the aggregate is initially at 715 nm; with increasing time in light there is a progressive shift to longer wavelengths, so that after 80 hours in the light, the long wavelength maximum is at 732 nm. This long wavelength shift was attributed by the Brody's ^{to} changes in populations of various sizes of aggregates with increasing "age" (time in light) of the organism. They determined, by means of matrix analysis, the corresponding absorption and fluorescence maxima of populations of aggregates ^{corresponding to} particular "effective size". The absorption band at 682 nm. was also attributed to aggregated chlorophyll by S. and M. Brody (1961, 1963). They found that excitation spectra for fluorescence at long wavelengths in vivo and in vitro contain maxima at both 682 and 705 nm. Also, it was observed by S. and M. Brody (1961) and Brody et al. (1968) that, in vitro, aggregated chlorophyll has two absorption bands which overlap in the red region of the spectrum.

Evidence for a fluorescence emission band with maximum at 696 nm (at 77°K) was first obtained by Litvin et al. in 1960. They observed that greening bean leaves had this third band in addition to the bands at 685 and 735 nm. A similar low temperature maximum (at 695 nm) was noted in Ochromonas danica by Brody and Linschitz (1961). In 1963, Brody and Brody, Bergeron, Govindjee, and Kok reported that this low temperature band, "F 698", (variously reported at 697 - 698, 700, 696, 696 - 698 nm, respectively) could be observed in many other organisms, having different and various types of accessory pigments. At first, there was disagreement about the particular photosystem and absorption band to which this emission band should be assigned. However, the weight of experimental evidence has indicated its association with system II: F 698 can be quenched by plastoquinone or other quinone-like substances (Brody and Broyde, 1963; Broyde and Brody, 1965); there is preferential sensitization of F 698 by the pigments of system II (Govindjee, 1963); there is light-induced exponential build-up of F 698 at 77°K (Kok, 1963); there is emission of excess energy (at 77°K) at this wavelength upon excitation of system II pigments (Krey and Govindjee, 1966).^{*} These ^{series of} last four _^ experiments further suggest that fluorescence at 698 arises from the chlorophyll a system II reaction center.

In an effort to determine the precise system ^{with} which F 698 is associated and if it can be attributed to the reaction center for

* Originally, Goedheer (1964, 1965) assigned the pigment having a fluorescence maximum at 696 nm to photosystem I, and to the form of chlorophyll absorbing maximally at 683 nm. More recently, (1968), Goedheer has re-assigned this pigment to system II. This was based upon his study of the effect of heating on the selective destruction of pigment systems, as observed by means of fluorescence excitation and absorption spectroscopy. Murata et al. (1966), and Bergeron and Olsen (1967), in contrast to Goedheer, also concluded that F 698 belongs to photosystem II, based on their findings that fluorescence at 696 nm in Anacystis is preferentially excited by phycobilins, which are associated with system II.

system II(its corresponding absorption maximum is not known), a number of higher plants were grown from seed in order to obtain leaves which exhibited a large quantity of this substance(as evidenced by fluorescence emission at 698 nm in the low temperature emission spectrum). One variety of plant gave a large amount of fluorescence at 698 nm--Ricinus communis(the castor oil plant)---in marked contrast to what had been observed with genera such as Spinacea, Raphanus, etc.. Especially interesting was the finding that with increasing time of incubation of leaves in isolation medium, emission from the band with maximum at 698 nm increases relative to that of the band with maximum at 735 nm. Additionally, it was observed that introduction of chloroplasts from other species into extracts of Ricinus leaf resulted in similar phenomena(Brody et al., 1969; Nathanson and Brody, 1970). Concomitant low temperature action spectra for exciting fluorescence at 735 nm revealed that, with increasing time of incubation, contribution of the 682 and 705 nm bands diminishes while that of the 674 nm band increases(Brody et al., 1969; Nathanson and Brody, 1970). The active factor in the Ricinus leaf extract(RLE) was found to be proteinaceous in nature(Brody et al., 1969, Brody, 1969; Cohen et al., 1969) and capable of disrupting normal lamellar structure and electron transport(Cohen et al., 1969; see also Cohen, 1970 dissertation). Since relative decrease in F 735 and increase in F 698 could result from: (a) energy transfer from system II to system I diminishing or (b) if the form of chlorophyll giving rise to F 735 was converted into a form contributing to fluorescence at 698 nm, an answer to the question "Which process is occurring?" was sought. To be described in this dissertation are the experiments that led up to the posing of this question, and to the finding that possibility (b) above, obtained [leading secondarily to possibility (a)] because of conversion of aggregated into monomeric chlorophyll.

Since it was determined, in conjunction with other workers in this laboratory (Cohen et al., 1969), that exogenous long-chain unsaturated fatty acids act as model systems for the action of RLE protein, and since such fatty acids lend themselves to investigations which can be made more quantitative than those utilizing only partially purified Ricinus protein, they were extensively employed in the present investigation.

Besides the experiments on low temperature fluorescence spectroscopy, from which it was originally inferred that deaggregation was occurring, it was deemed advisable to use a second, possibly more direct, technique, for verification of this phenomenon. The technique that was chosen was that of circular dichroism (C.D.) spectroscopy, since this technique allows ^{better resolution of} aggregate and monomer bands (Dratz et al., 1966). Because the configurational changes seen with the electron microscope (Cohen et al., 1969) could reflect conformational changes (Murakami and Packer, 1969) occurring in fatty acid (or Ricinus extract) in treated chloroplasts, circular dichroism studies were further extended into the U.V. region of the spectrum. Indeed, such experiments revealed that more than one type of mechanism for deaggregation might prevail in membranous chlorophyll-containing systems. In addition (or alternatively) to the direct deaggregation effect on chromophore-chromophore interaction (as observed to occur in solution; see below), deaggregation can evidently be brought about indirectly, by effect on the protein portion of the chloroplast lamellae, secondarily promoting changes in interaction between chlorophyll chromophores.

In the course of the present work, a comprehensive investigation was made of the C.D. spectra of chlorophyll systems of various levels of structural organization. With increasing complexity, there were observed changes in polarity of C.D. bands, interconversion of negative and positive

ellipticities, and strong shifts in band peaks. Interpretations are given for these changes based on (1) organization of protein into membranous systems, (2) augmentation of light scattering with increasing frequency of exciting light and (3) integrity of structural organization.

MATERIALS AND METHODS

I. HIGHER PLANTS: SOURCE OF MATERIALS AND PREPARATIVE PROCEDURES FOR OBTAINING CHLOROPLASTS, SUBCHLOROPLAST PARTICLES, AND NON-MEMBRANOUS CHLOROPHYLLOUS SYSTEMS.

A. Source of Plant Materials.

Ricinus communis (castor oil plant) either variety sanzibariensis or Baker 296, Raphanus sativus (radish), Helianthus annuus (sunflower), Zea mays (corn), Sorghum vulgare (sorghum), Triticum sativum (wheat) and Phaseolus vulgaris (bushbean) were grown from seed in a greenhouse. R. communis var. Baker 296 was a gift of Dr. R. Ory, Southern Regional Research Laboratory, New Orleans, La. All other seeds were obtained from Burnett Brothers, N.Y.C. Spinacea oleracea ^(spinach) was obtained from a local market.

B. Preparative Procedures.

1. PREPARATION OF CHLOROPLASTS WITH NON-INTACT OUTER ENVELOPES.

The method used for chloroplast isolation was a slightly modified version of that of Jagendorf and Avron (1958). The procedure was essentially as follows: fifty grams of lamina, which had been prewashed and depetiolated, were homogenized in a Waring blender with 150 ml of ice-cold 0.35 M NaCl - 0.04 M Tris-HCl, pH 7.8, for a total period of 15 seconds (three 5 second blendings at high speed) in a 15°C cold room. The homogenate was filtered through four layers of cheesecloth and the brei centrifuged at 4°C for 90 seconds at 500 x G in a Sorvall RC-2 refrigerated centrifuge.* This pellet was discarded and the resulting supernatant was centrifuged for seven minutes at 1,000 x G. The pellets obtained from this second

* Except where otherwise indicated, all centrifugations were carried out at 4°C in a Sorvall RC-2 refrigerated centrifuge.

centrifugation were resuspended in a small volume of the above-cited isolation medium. Unless otherwise stated, spectral investigations with higher plant chloroplasts employed those prepared according to this method.

Since the active protein factor in Ricinus chloroplasts begins to exert its effects immediately upon isolation, special precautions had to be taken to retain the "control" condition (see below, section II-A-1). Prior to homogenation, the Waring blender jar was chilled in the freezer compartment of a refrigerator. The isolation medium which was used had also been pre-chilled to the point of ice crystal formation.

2. PREPARATION OF CHLOROPLASTS WITH INTACT OUTER MEMBRANES.

Spinach chloroplasts with intact outer membranes (80% whole, as judged by electron microscopy, courtesy of James E. White) were prepared according to the method of Spencer (1967). Five grams of lamina which had been prewashed, depetiolated, and deveined, were chopped for 10 minutes in a petri dish (with several changes of razor blades) in 10 ml of medium containing 0.4 M sucrose, 0.05 M Tris-HCl, pH 7.8, 0.01 M KCl. The chopping was done in a cold room maintained at 15°C. The resultant brei was filtered through four layers of cheesecloth and the filtrate spun at 500 x G for 90 seconds. The supernatant of this first centrifugation was recentrifuged for 3 minutes at 1,000 x G and the resultant pellet was resuspended in a minimal amount of the medium used for isolation.

3. PREPARATION OF SUBCHLOROPLAST PARTICLES.

a. Subchloroplast Particles Obtained by Means of Sonication

These subchloroplast particles (containing both photosystems I and II) were prepared according to the method of McCarty (1968). Spinach

chloroplasts prepared as in I-B-1 were suspended in medium containing 0.02 M Tris-NaOH, pH 8.0, 0.01 M NaCl, 0.04 M sucrose, and exposed to sonication for 45 seconds at 0°C. The stainless steel beaker which contained the chloroplasts had been pre-chilled and placed in ice during the period of sonication. The sonication was performed with an MSE (Measuring and Scientific Equipment Co.) Ultrasonicator, Model #3000, operated at 20 kilocycles.

Large chloroplast fragments were removed by centrifugation at 6,000 x G for 10 minutes. The supernatant was pipetted off and subjected to a further centrifugation for one hour in a Beckman Model L-21, refrigerated ultracentrifuge, at 104,000 x G. The resultant pellet contained the subchloroplast particles.

b. Subchloroplast Particles Obtained through the Use of Digitonin

Two types of digitonin-isolated particles were prepared according to the method of Anderson and Boardman (1966); one contained system I and the other, both systems I and II. Spinach chloroplasts were isolated as in section I-B-1 using the Anderson and Boardman medium of 0.03 M sucrose, 0.01 M KCl, 0.05 M Tris-HCl, pH 7.8. They were then incubated in 0.5% digitonin (weight per volume of Anderson medium) for 60 minutes at 0°C, with stirring. The particles containing system I, and those containing both systems, were isolated by means of differential centrifugation in a Beckman Model L-21, refrigerated ultracentrifuge: systems I and II (heavy) particles were contained in the pellet produced by centrifugation at 10,000 x G for 30 minutes; system I particles were contained in the pellet produced by centrifugation at 144,000 x G for 60 minutes.

The investigation of the effects of ^{defatted fatty acid-treated} BSA on digitonin-isolated particles containing systems I and II was done in the following way: particles

containing systems I and II were divided into three batches; to the first sample was added a given volume of isolation medium; to the next two samples were added the same volume of medium but containing linolenic acid at a concentration of 2×10^{-4} M (a ratio of ^{Fatty acid to chlorophyll} (FA/Chl) of 10:1). After one minute, crystalline defatted BSA (to a final concentration of 20 mg/ml) was added ^{to the third tube}. One minute after the addition of BSA

all three samples were spun down (in a Beckman ultracentrifuge) at 30,000 x G for 10 minutes. ^{Supernatants were discarded and} The samples in the test tubes were resuspended in isolation medium and then C.D. spectra were determined in the following order: control; linolenic acid-treated; linolenic acid post-treated with BSA. The time for the determination of each spectra was ten minutes; the specimens not under examination were kept on ice at 0°C.

4. PREPARATION OF CHLOROPHYLL-PROTEIN COMPLEXES.

a. HP 700 Chlorophyll-Protein Complexes

HP 700 chlorophyll-protein complexes were isolated by the method of Yamamoto and Vernon (1969). Spinach chloroplasts, containing a total chlorophyll (a and b) content of 5 mgs of chlorophyll (see Section IV-A for procedure for chlorophyll determination) were isolated as in Section I-B-1, using the medium of Yamamoto and Vernon: 0.4 M sucrose, 0.01 M NaCl, 0.01 M Tricine, pH 8.8. In general, in contrast to Yamamoto and Vernon, I utilized non-lyophilized chloroplasts in my studies.* These were extracted seven times, by means of a Vortex homogenizer, using a total volume of 1 liter of 15% acetone in hexane (to remove lipids and most of the bulk chlorophyll). After each extraction, the chloroplasts were again pelleted by centrifugation at 1,000 x G for 10 minutes. After the last extraction, the chloroplasts were washed in 100% hexane, re-

* Lyophilization results in loss of C.D. bands at 740 - 760 nm, characteristic of normal "wet" chloroplasts; see Section IV-C-2-b below.

centrifuged, and the residual hexane removed from the pellet by evaporation under nitrogen. The extracted chloroplasts were mixed with 0.05% ^{aqueous} Triton X-100 by means of agitation with a glass rod, and were spun at 39,000 x G for thirty minutes; this treatment with detergent removes residual lipids, which remain in the supernatant. The pellet resulting from this centrifugation was resuspended in 5% Triton X-100 in 0.01 M NaCl, 0.01 M Tricine, pH 7.8, and centrifuged at 39,000 x G for thirty minutes (to bring down any remaining bulk chlorophyll a in the pellet). Three-ml portions of the supernatant were layered on a 2-20% discontinuous sucrose gradient and spun at 131,000 x G for 20 hours in a refrigerated Beckman Model L-21 ultracentrifuge. HP 700 was obtained from the 8% sucrose layer by pipetting off the upper layers.

HP 700 chlorophyll-protein complexes were also prepared from lyophilized chloroplasts (as in the published procedure of Yamamoto and Vernon, 1969) to determine the effects of lyophilization on C.D. of chloroplasts.

b. System I Chlorophyll-Protein Complexes

System I chlorophyll-protein complexes, isolated from the blue-green alga Phormidium (and prepared according to the method of Thornber, 1969) were very kindly given to us by Dr. R. Gregory of the Dept. of Biochem., University of Manchester, Manchester, England.

5. CRYSTALLINE CHLOROPHYLL A.

Crystalline chlorophyll a was a gift from Dr. S. S. Brody (Dept. of Biology, N.Y.U., New York, N.Y.). Its isolation and purification is described by Broyde and Brody (1966).

II. ALGAE: SOURCE OF MATERIALS, CULTURE METHODS AND PREPARATIVE PROCEDURES FOR OBTAINING CHLOROPLASTS.

A. Source of Materials.

Chlorella pyrenoidosa chick (Emerson's strain 3) and Porphyridium cruentum (originally from Dr. R. Lewin, Scripps Inst. of Oceanography, La Jolla, California) were obtained from Dr. S. S. Brody, Dept. of Biology, N.Y.U., New York, N.Y. Anacystis nidulans (from the Starr collection, Indiana University, Bloomington, Indiana) was obtained from Dr. T. E. Jensen, Lehman College, C.U.N.Y., Bronx, N.Y.

B. Cultivation of Algae.

Porphyridium cruentum was cultured for seven days at 25°C, in 250 ml Ehrlenmeyer flasks, containing 150 ml of media prepared according to Jones et al. (1963). The cultures were bubbled with 0.5% CO₂ in air. Chlorella pyrenoidosa was cultured for three days at 25°C in 250 ml Ehrlenmeyer flasks containing 150 ml of media prepared according to Pirson and Ruppel (1962). Again, the flasks were bubbled with 0.5% CO₂ in air. Both Chlorella and Porphyridium were grown over a bank of 8 20-watt Sylvania cool-white fluorescent lamps yielding, on the bottom of the flasks, a light intensity of 8.5×10^3 ergs / cm² / sec. Anacystis nidulans was cultured for four days at 25°C, on a Burrell Wrist-Action shaker, Burrell Corp., Pittsburgh, Pa., in 250 ml Ehrlenmeyer flasks containing 150 ml of medium (Medium C) prepared according to Kratz and Meyers (1955). For this blue-green alga, 2 Sylvania 40-watt daylight fluorescent lamps yielding an intensity of 5×10^3 ergs / cm² / sec (incident on the side of the flasks) was used. Light intensity was measured with a Yellow Springs Radiometer, Precision Thermistor, Model #65, Yellow Springs Instrument Company, Yellow Springs, Ohio.

Prior to experiments, algal cells, in the log phase of growth (as determined by cell counts) were collected by centrifugation at 1,000 x G for 10 minutes, and resuspended either in fresh growth medium or in RLE (see Section III-A).

It was deemed desirable to determine the effects of fatty acids or RLE on the fluorescence of lamellar fragments as well as on intact cells of blue-green and red algae. Since phycobilins readily leach out during preparative procedures, the method of Ludlow and Park (1969) involving pre-fixation of phycobilins to the lamellar membranes was used prior to isolation of lamellar fragments from A. nidulans.^{*} A comparison of control and pre-fixed cells of A. nidulans in the present work (see below) indicates that neither the spectral properties, nor the response to fatty acid treatment is strongly modified by the process of pre-fixation. (Ludlow and Park had previously shown that such pre-fixed cells are capable of performing the Hill reaction.) The procedure used for cell fixation is as follows: cells were centrifuged out of their growth medium and resuspended in 1.5% formaldehyde in 0.20 M phosphate buffer, pH 7.4, for ten minutes. They were then washed twice in phosphate buffer and pelleted by centrifugation at 1,000 x G for 10 minutes.

C. Preparation of Lamellar Fragments from Algae.

Lamellar fragments of Porphyridium and Chlorella were prepared only from unfixed cells; in the case of Anacystis, however, both fixed and unfixed cells served as sources. Lamellar fragments were prepared according to the method of Murano and Fujita (1967). Cells which had been spun down and resuspended in a minimal amount of 0.2 M phosphate buffer, pH 7.4, were sonicated continuously: three minutes in the case of Porphyridium

^{*} This pre-fixation was not used in the case of P. cruentum. As will be seen below, even in the case of pre-fixation, only some of the phycobilins are retained. Because excitation of phycobilins results in much stronger emission at long wavelengths (from the aggregate) in the case of Anacystis than in the case of Porphyridium (see Section below, in Results) and since I was interested in determining the effects of RLE or fatty acid on phycobilins as well as the relationship between the phycobilins and chlorophyll, I concentrated my efforts on Anacystis.

and Chlorella; ten minutes in the case of Anacystis (with the MSE sonic oscillator as described in Section I-B-3-a above). Lamellar fragments were collected by centrifugation at 27,000 x G for 90 minutes.

III. PREPARATION OF RICINUS LEAF EXTRACT (RLE).

RLE was prepared according to a modification of the method used by McCarty and Jagendorf (1965) in making their extract of Phaseolus (bushbean) leaves. Fifty grams of pre-washed, pre-cooled, depetiolated and deveined Ricinus leaves were homogenized in a Waring blender, with 150 ml of ice-cold 0.35 M NaCl-0.04 M Tris-HCl, pH 7.8 for a total period of 90 seconds. The homogenate was strained through four layers of cheesecloth and the brei was spun at 17,000 x G for 20 minutes. At this point, McCarty and Jagendorf dialyzed their extract for four hours, but in the present procedure, the non-dialyzed supernatant was spun at 104,000 x G in a refrigerated Spinco Model L-25 ultracentrifuge for one hour in order to bring about the most rapid possible separation of RLE from some of the other possible contaminants (e.g. small chloroplast fragments). The supernatant was pipetted off and stored at -80°C in a Revco freezer (Revco Inc. Industrial Products, Deerfield, Michigan). Protein concentration in mg/ml was determined as in Section IV-C (below).

IV. METHODS FOR DETERMINING CONCENTRATIONS OF PIGMENTS AND PROTEIN.

A. Concentration of Chlorophyll.

Concentrations of chlorophyll a and/or b were determined for chloroplasts of higher plants and for algae by the method of Arnon (1949). Concentration of chlorophyll extracted from algal cells was determined by the method of MacKinney (1941). Concentrations of chlorophyll a in

solution were obtained by weighing out crystalline samples. In the case of chlorophyll in CCl_4 , where the molar extinction coefficient is known (Sauer et al., 1966), chlorophyll concentration was also determined by use of the extinction coefficient. (With the solvent pair, CCl_4 -hexane, for which there is no published extinction coefficient, chlorophyll concentration was also determined by weighing out samples.)

B. Phycocyanin Concentration.

After removal of phycocyanin from Anacystis cells by means of sonication and subsequent solubilization in the supernatant, its concentration was determined from the published molar extinction coefficient (Troxler and Lester, 1968).

C. Protein Concentration.

In the present study, protein concentrations were determined according to the method of Lowry et al. (1951).

V. SOURCES OF CHEMICALS.

Salts used in algal culture media were reagent grade, and solutions were prepared using glass-distilled water. Fatty acids, crystalline bovine serum albumin, and digitonin were purchased from Sigma Chemical Company (St. Louis, Mo.). Defatted, crystalline bovine serum albumin was a gift from Dr. W. S. Cohen (Dept. Biology, Hunter College, New York, N.Y.). Its preparation is described by Chen (1967). Spectral gradees of ethanol, acetone, hexane, and CCl_4 were obtained from J.T. Baker Chem. Co. (Phillipsburgh, N.J.). Tricine was purchased from Calbiochem, Los Angeles, California. Triton X-100 was obtained from Rohm and Hass, Philadelphia, Pa.

Immediately before use, digitonin was recrystallized twice from boiling ethanol by cooling to 4°C .

Solutions of fatty acids were prepared immediately before use. Solvents were either ethanol or CCl_4 , depending upon the particular experiment.

VI. INSTRUMENTATION.

A. Absorption Spectroscopy.

1. DIRECT (INTEGRAL) ABSORPTION SPECTROSCOPY: ROOM AND LIQUID NITROGEN TEMPERATURES.

Absorption spectra were determined with a Cary 14 R spectrophotometer. The source of illumination was either a hydrogen or tungsten lamp, depending upon the spectral region under investigation.

Measurements of samples of chloroplasts at the temperature of liquid nitrogen were made in the laboratory of Dr. S. S. Brody, Dept. of Biology, N.Y.U., according to the following procedure. A drop of highly concentrated suspension of Ricinus chloroplasts* was placed between two quarter inch thick optical glass plates which were then pressed together to spread the drop into a film; low melting point paraffin wax was used to seal the edges of the glass plates. The cuvette so formed was placed in a brass cuvette holder which was, in turn, inserted into a small, partially silvered Dewar. The Dewar was then filled with liquid nitrogen to such a depth that the cuvette so formed was completely covered. For low temperature studies, path length was 10^{-4} cm, as estimated by Brody and Broyde (1968). Dr. S. S. Brody's Cary Model 14 R recording spectrophotometer was equipped with a scattered transmission accessory. To obtain differences in absorption of control and treated material, spectra were normalized at 560 nm.

2. DIRECT (INTEGRAL) ABSORPTION DIFFERENCE SPECTROSCOPY: ROOM TEMPERATURE

Changes in absorption of spinach chloroplasts incubated at room temperature in buffer (control) or in ELE or in linolenic acid were monitored with an Aminco-Chance Dual-Wavelength Spectrophotometer, kindly made available for use by Dr. Walter Bertsch. Two types of experiments

* Highly concentrated suspensions were obtained by pelleting the chloroplasts out of buffer and then resuspending them in minimal amounts of isolation buffer.

were made. In both cases, the instrument was used in the split-beam mode. The procedure for the first type is as follows. For the control situation, cuvettes, containing isolation medium were placed in the apparatus: one in the sample and one in the reference position. The cuvettes were then balanced against each other at 800 nm. Chloroplasts were injected into the sample position cuvette; a total of four spectra were recorded within ten minutes in the wavelength region 400-800 nm. No change in spectra were observed to occur during this time interval. For the experimental situation, the cuvette in the sample position was replaced by another cuvette, either containing linolenic acid in isolation medium or containing RLE; these were balanced against each other at 800 nm. An aliquot of chloroplasts (identical to that in the control) was injected into the sample cuvette (and an equal volume of isolation medium was injected into the reference position cuvette). Again, a total of four spectra were recorded within an interval of ten minutes.

To determine time-dependent changes within a series (e.g. those occurring in the case of chloroplasts in RLE), spectra obtained at a later time, t , were subtracted from that obtained at time zero, t_0 , (i.e., the time of the first measurement).

In the second type of experiment the change in absorption at a particular wavelength was monitored as a function of time. The wavelengths chosen for such monitoring were those at which maximal changes in absorption had been observed to occur in the first type of experiment. To monitor changes brought about by linolenic acid, the following procedure was used: identical aliquots of chloroplasts were injected into both the reference and sample cuvettes (each containing isolation medium), and the cuvettes were balanced against each other at the chosen wavelength. A specific volume of isolation medium was injected into the

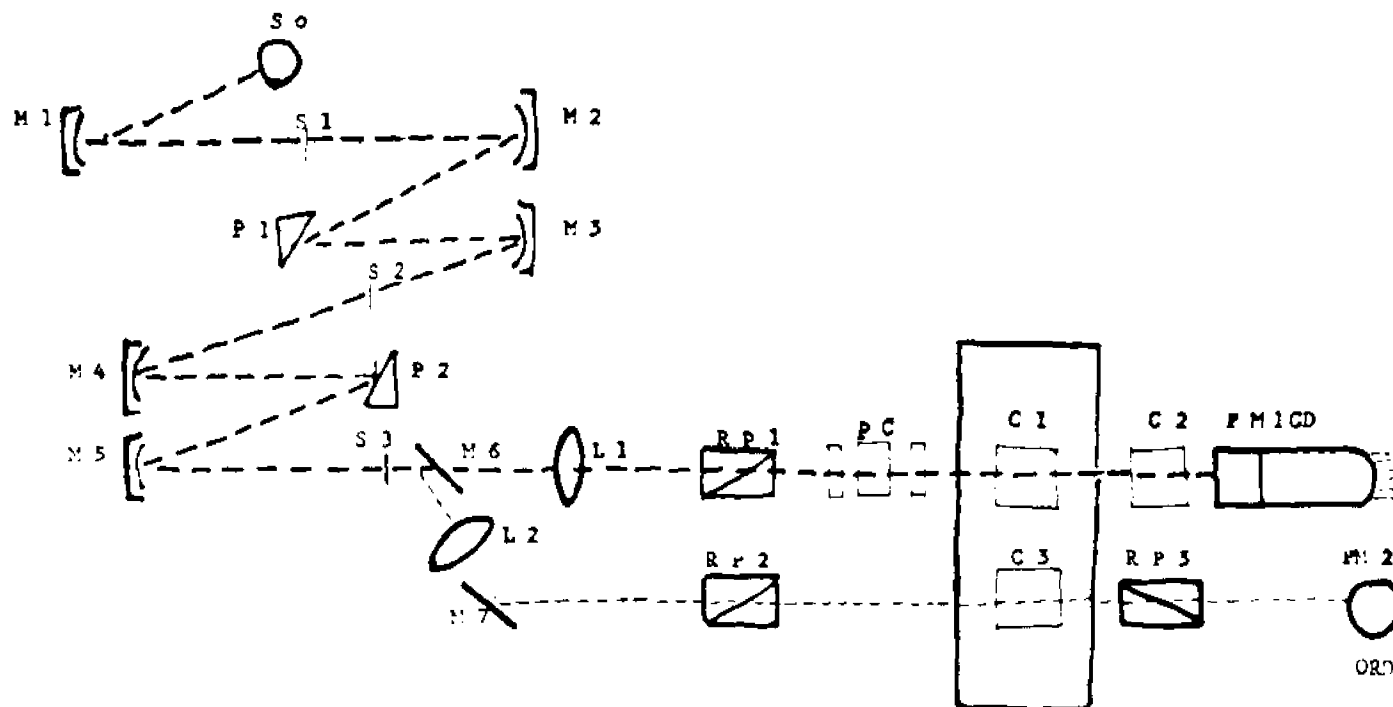
the reference cuvette, and an identical volume of linolenic acid (of known concentration) was injected into the sample cuvette. Changes in absorption were then continuously monitored at the chosen wavelength. In the case of experiments involving RLE, the procedure was as follows: the sample cuvette containing RLE, was balanced, at a particular wavelength, against the reference cuvette, containing isolation medium. Identical aliquots of chloroplasts were injected into both the reference and sample cuvettes, and changes in absorption were then continuously monitored at the chosen wavelength.

3. CIRCULAR DICHROISM SPECTROSCOPY.

The C.D. data in the present work were obtained with a Durrum-Jasco Spectropolarimeter, Model J-20 (Durrum Instrument Corp, Palo Alto, Calif.). Since so few of these particular instruments are in use in the United States at the present time, a block diagram (modified from their booklet #54) is given below (see Fig. B).

The light beam from the monochromator is passed through a Rochon prism which results in the generation of linearly polarized light. By means of a Pockels cell, this linearly polarized light is converted to circularly polarized light. ^{an alternating (A.C.)} electric field is placed across the Pockels cell, in order that left and right hand circularly polarized light may alternately be produced. If there is no difference in the absorption by the sample of the left and right hand circularly polarized light, both components reach the photomultiplier ^(P.M.) in equal amounts. If there is a difference in absorption between the left and right hand circularly polarized light, there is modulation of the A.C., the phase and amplitude of which is equal to that of the circular dichroism absorption. The ratio of A.C. to D.C. signal is amplified and transmitted to the recorder, whose output is calibrated in degrees

Figure B. Block diagram of the Durrum-Jasco Spectropolarimeter (From Booklet # 54, Durrum Instrument Corp., Palo Alto, California.)



So Xenon source
M1 Source mirror
S 1,2,3 Monochromator slits
P 1,2 Monochromator prisms
M 6 Retractable ORD mirror
M 7 Plane mirror
L 1 CD lens
L 2 ORD lens

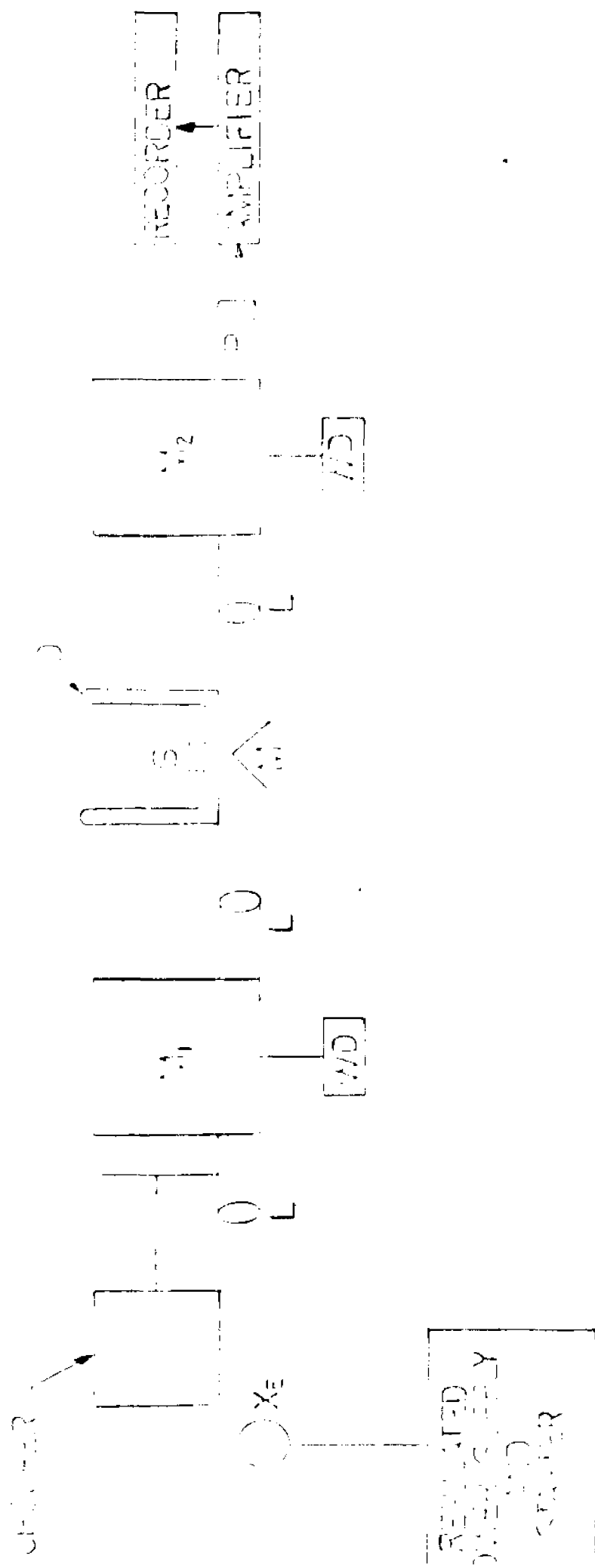
RP 1 CD polarizer (Rochon prism)
RP 2 ORD polarizer(Rochon prism)
RP 3 ORD analyzer (Rochon prism)
PC Pockels cell
C1 CD cell position
C2 CD cell position
C3 ORD cell position
PM 1 CD photomultiplier
PM 2 ORD photomultiplier

of ellipticity as function of wavelength. In most of our experiments, a red-sensitive PMT (S-20 response) was used as a detector. The noise level (in millidegrees, i.e., m^0 ellipticity) generally ranged from 0.2 to 2. The signal to noise ratio, for example, in the experimental data illustrated in Fig.38 (HP 700 particles in buffer and in the presence of linolenic acid) was 12 at 740 nm and 21.5 at 666 nm. Silica cuvettes of 0.05 mm path length were used. In all cases, cuvettes were placed in position C₂, closest to the photomultiplier tube.

B. Fluorescence Spectroscopy.

Fluorescence emission and excitation spectra were determined with the apparatus shown in block diagram (modified from Brody et al., 1965) in Fig. C. Illumination is provided by a high pressure 1800 watt Xenon arc lamp (Hanovia Corp., Newark, N.J.). The output of the lamp (chopped at 450 c.p.s. by a segmented disc) falls upon a Bausch and Lomb grating monochromator (having a grating blazed at 400 nm and a dispersion of 16 Å/mm). In general, for emission measurements, I used exciting light having a maximum at 436 nm. A Corning glass filter # C.S. 5-60 was used in conjunction with the exciting monochromator to exclude second order light. At a slit of 3 mm width (see below) the incident light intensity (in the absence of the blue filter) was 741 ergs/cm² sec; the blue filter reduced the intensity to 505 ergs/cm² sec. In the few experiments involving phycobilin excitation, I (additionally) used exciting light having maxima at 545 and 578 nm for exciting fluorescence of Porphyridium and Anacystis, respectively. The exciting light with maximum at 545 nm was obtained by using Corning glass filters #4-72 and 3-70 in conjunction with the exciting monochromator, and exciting light having a maximum at 578 nm was obtained by using Corning glass filters #3-67 and 4-94. The light intensity at 545 and 578 nm, in the presence of these filters was 146 and 208 ergs/cm² sec, respectively. This was determined with an Eppley thermo-

Figure 6. Schematic diagram of the apparatus used to measure and record fluorescence emission spectra. L-flat bottom dish. L-lens. M-mirror. K_1 and K_2 -rotachroic discs. P-photomultiplier tube. S-sample. WD-wavelengths drive. Xe-Xenon lamp. Redrawn from G. Brady et al. (1965).



pile (Eppley Lab Inc., Newport R.I.), calibrated by the National Bureau of Standards, Wash. D.C. Light from the monochromator used for excitation falls on a mirrored prism and is directed upward to impinge on the optically clear bottom surface of a mirrored Dewar. A 5 ml pyrex beaker, containing the sample, is placed on the bottom of the Dewar. For experiments done at 77°K, liquid nitrogen is used to fill the Dewar. Fluorescence emitted from the sample falls on a second mirrored prism, and is deflected through an optical train to a second Bausch and Lomb monochromator (having a grating blazed at 800 nm and a dispersion of 33 Å per mm). Contamination of fluorescence by stray light is avoided by the use of a Corning glass filter placed on the second mirrored prism. Generally, a red cut-off filter CS-2-63 was used. Fluorescence was detected with a Dumont 6911 photomultiplier tube (S-1 response), the output of which was fed into an amplifier and phase-sensitive detector (Baird Atomic) and recorded on a Varian G-11 recorder. The slit widths of the exciting and analyzing monochromators were 3 mm and 1.5 mm, respectively, yielding halfband widths of 4.8 nm.

VII. CALCULATIONS.

A. Fluorescence Emission Spectra.

Fluorescence emission spectra were not corrected for the spectral response of the instrument.

B. Fluorescence Excitation Spectra.

Fluorescence excitation spectra were corrected for the combined spectral characteristics of the Xenon lamp and the exciting monochromator in the following way:

- a) The energy output of the Xenon lamp, after passage through the monochromator, was determined at 2 nm wavelength intervals by placing an

Eppley thermopile (having a calibration factor of $0.058 \mu\text{v}/\mu\text{watt}$, as certified by the National Bureau of Standards) at the output of the exciting monochromator (i.e., after L_1 in Diagram C). The thermopile was connected to the input of a Beckman Breaker-Amplifier (Model 14, Beckman Instruments Inc., Fullerton, California). The output of the amplifier was in turn connected to two resistors in series ($1,000\Omega$ and 100Ω , respectively). To calibrate the amplifier, test signals of $1\mu\text{v}$ and $3\mu\text{v}$ were given, and the heights of these signals -- in mm on the chart paper of the recorder -- was noted at each gain setting of the phase-sensitive amplifier. Thus, it was calculated, for example, that a displacement of 140 mm on the chart paper is equal to a 10 mv signal. Although the output of the lamp was monitored as a function of wavelength over the range of monochromator slit widths of 0.5 to 10 mm, in the experiments reported here, a slit width of 3 mm was generally used. The Xenon lamp output at two nm wavelength intervals (using a 3 mm slit setting on the exciting monochromator) was calculated in the following formula:

- 1) The height of the signal on the recorder, at each 2 nm interval, in millivolts, "O", was divided by the amplification factor of the test signal, "A". $\underline{A}$, in the case of a slit setting of 3 mm, was found to be equal to $0.675 \text{ mv}/\mu\text{w}$. The quotient of O/A was divided by the thermopile calibration factor "c" equal to $0.058 \mu\text{v}/\mu\text{w}/\text{cm}^2$. The value so obtained (in $\mu\text{w}/\text{cm}^2$) was multiplied by a factor of 10, in order to obtain units of $\text{ergs}/\text{cm}^2/\text{sec}$. Thus "E" (the energy output in $\text{ergs}/\text{cm}^2/\text{sec}$) = $10(O / (A \times C))$. For example, at 646 nm:

$$E = 10(2.47/0.675 \times 0.058) = 630 \text{ erg}/\text{cm}^2/\text{sec}.$$

- 2) " E_p ", the energy (in ergs) per photon, at each wavelength, was calculated from the formula: $E_p = hc/\lambda = \frac{6.6 \times 10^{-27} \text{ erg. sec.} \times 3 \times 10^{10} \text{ cm/sec}}{(\text{in cm})}$

At, for example, a wavelength of 646 nm:

$$E_{p646} = \frac{6.6 \times 10^{-27} \times 3 \times 10^{10}}{6.46 \times 10^{-5}} = 3.06 \times 10^{12} \text{ ergs/photon}$$

- 3) To convert the energy output of the lamp at a particular wavelength (in this case, 646 nm) to photons/cm²/sec, the numerical value obtained in (1) is divided by that obtained in (2), i.e. at 646 nm the corrected energy output " E_{c646} " = $630/3.06 \times 10^{12} = 205.7 \times 10^{12}$ photons /cm²/sec.
- b) The relative amplifications at the various (non-linear) gain settings of the spectrofluorimeter's amplifier and phase-sensitive detector (Baird Atomic Incorporated, Cambridge, Mass.) were determined in the following way:

Both the exciting and analyzing monochromators were set at 680 nm, with slit widths of 0.6 and 0.3 nm, respectively. When necessary, incident light intensity was attenuated by using 2" by 2" pre-calibrated wire screen. The mm height of the signal on the Varian recorder at any given indicated combination of coarse and fine gain settings was first determined, and a straight line was drawn from the signal values for indicated gain settings of 1.3, 2.3, and 3.3 (see Fig. D). The reason that the straight line was drawn between these indicated gain settings was that it was observed, for example, at fine gain settings of less than 0.3 in the coarse gain setting of 3, that there was overlap between the fine gain setting of 0.3 on the coarse gain setting of 2 (see indicated brackets on Fig. D). Three horizontal lines were drawn through the points, from which a straight line was generated (i.e. 1.3, 2.3, and 3.3). On these horizontal lines, vertical lines were dropped from the generated line at measured signal heights so as to give points of intersection with the horizontal line then being equivalent to the actual amplification values. With such a gain calibration curve, one can read directly for any gain setting on any of the three horizontal lines, a corresponding calibration. For example, to

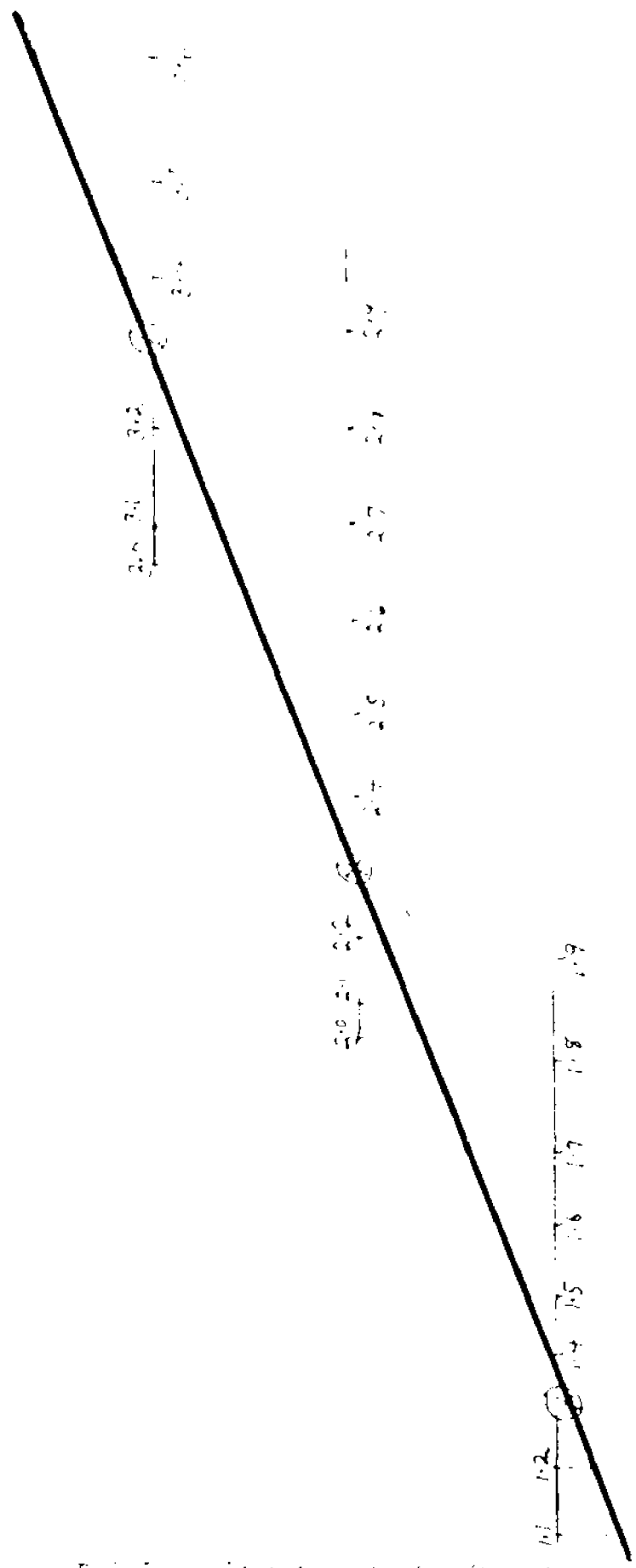
Figure D. Gain calibration curve for the Baird-Atomic Amplifier.

1.0 1.2 1.4 1.6 1.8 2.0 2.2 2.4 2.6 2.8 3.0 3.2 3.4 3.6 3.8 4.0 4.2 4.4 4.6 4.8 5.0 5.2 5.4 5.6 5.8 6.0 6.2 6.4 6.6 6.8 7.0 7.2 7.4 7.6 7.8 8.0 8.2 8.4 8.6 8.8 9.0 9.2 9.4 9.6 9.8 10.0

1.2 1.4 1.6 1.8 2.0 2.2 2.4 2.6 2.8 3.0 3.2 3.4 3.6 3.8 4.0 4.2 4.4 4.6 4.8 5.0 5.2 5.4 5.6 5.8 6.0 6.2 6.4 6.6 6.8 7.0 7.2 7.4 7.6 7.8 8.0 8.2 8.4 8.6 8.8 9.0 9.2 9.4 9.6 9.8 10.0

1.2 1.4 1.6 1.8 2.0 2.2 2.4 2.6 2.8 3.0 3.2 3.4 3.6 3.8 4.0 4.2 4.4 4.6 4.8 5.0 5.2 5.4 5.6 5.8 6.0 6.2 6.4 6.6 6.8 7.0 7.2 7.4 7.6 7.8 8.0 8.2 8.4 8.6 8.8 9.0 9.2 9.4 9.6 9.8 10.0

1.2 1.4 1.6 1.8 2.0 2.2 2.4 2.6 2.8 3.0 3.2 3.4 3.6 3.8 4.0 4.2 4.4 4.6 4.8 5.0 5.2 5.4 5.6 5.8 6.0 6.2 6.4 6.6 6.8 7.0 7.2 7.4 7.6 7.8 8.0 8.2 8.4 8.6 8.8 9.0 9.2 9.4 9.6 9.8 10.0



obtain the gain calibration factor for coarse setting of 1 and fine setting of 0.9, one drops a vertical line from the lowest horizontal line to the generated line, and from the point of intersection, another horizontal line is dropped onto the ordinate, giving a gain calibration of 56.

c) Excitation spectra were corrected for the different incident energies at the various wavelengths by taking the mm height of the recorder signal obtained at any particular wavelength (i.e. the relative intensity of emission) and dividing it by the product of the gain calibration factor and the energy output of the Xenon lamp at the particular wavelength used for excitation. For example:

Wavelength used for excitation (nm)	relative intensity of emission at 735 nm (in mm)	Gain calibration factor for coarse setting of 1 and fine setting of 0.9	Xenon lamp output at 646 nm photons $\times 10^{12}$
646	77	56	208.7

$$\text{Relative Intensity of Excitation} = \frac{77}{(56)(208.7)}$$

C. Energy of Activation.

Calculations of energies of activation for reactions between spinach chloroplasts and RLE or linolenic acid were based on the rate of change of F 735/F 698 with time of incubation (as observed at 77°K), at 8 different temperatures ranging from 0° to 45°C. The rate of change (K) of F 735/F 698 at 10' time - 0' was plotted (in terms of ln K) as a function of 1/RT.* The slopes of the resultant curves equalled the energies of activation of the two reacting systems (RLE or linolenic acid). The rates of change of F 735/total fluorescence and F 698/total fluorescence (total area under the fluorescence envelope) were also plotted as functions of time and temperature of incubation, and energies of activation were also obtained

* R is in calories/°K mole

for these processes using these data.

In order to obtain F 736 or F 698 as functions of "area under the curve", traces of the data sheets were made and the fluorescence curves were replotted, on the basis of wavenumber, rather than wavelength. The contribution of F 698 or F 736 to total fluorescence emission were obtained in two different ways. In the first method, a bandwidth of 20 cm^{-1} to either side of the wavenumber maximum under consideration was traced with a planimeter (Keuffel and Esser Corp., Long Island City, N.Y.) and this area was divided by the total area under the fluorescence emission curve. In the second method, the contributions of each band to total fluorescence was made by weighing out strips of paper corresponding to the appropriate areas (in wavenumber plot) on an analytical balance (Mettler Instrument Corporation, Hightstown, N.J.). The weight, in mg, was then divided by the weight of the total fluorescence curve. In this case also, a bandwidth of 2 nm to either side of the maximum was used for F 698 and F 736.

D. Method of Calculating Rates of Change of Fluorescence Yields.

Since it was observed that there are marked changes in fluorescence yield upon incubation of spinach chloroplasts in linolenic acid, I attempted to estimate the change in fluorescence yield not only of total fluorescence (area under the fluorescence envelope) but also in the major components (i.e. F 735 and F 698). In order to obtain rates of change, a) the area under the fluorescence ^{envelope} and b) the areas 20 cm^{-1} to either side of the fluorescence maximum at 698 nm ($14,330\text{ cm}^{-1}$ in wavenumber) and the fluorescence maximum at 735 nm ($13,610\text{ cm}^{-1}$ in wavenumber) were estimated according to one of the methods given above for calculation of energy activation, i.e. the method in which the fluorescence emission curves (replotted on the basis of wavenumber) were cut out and weighed.

This method of cutting out areas and weighing them was applied to
 a) control chloroplasts at 0 time, b) control chloroplasts incubated for
 any period of time t_n , c) chloroplasts to which linolenic acid had just
 been added and d) chloroplasts incubated in the presence of linolenic
 acid for any period of time t_n .

Rates of change were then calculated in the following way, taking
 incubation time = 1 minute as an example:

Total area

$$\begin{array}{lcl} \text{Rate of change of} & & \\ \text{total fluorescence} & = & \text{fluorescence yield of} \\ \text{yield at time} & & \text{incubated chloroplasts}_{t=1} \\ & & \text{minus} \\ & & \frac{\text{fluorescence yield of}}{\text{control chloroplasts}_{t=1}} \quad \text{fluorescence} \\ & & \text{yield of in-} \\ & & \text{cubated chlo-} \\ & & \text{roplasts}_{t=0} \\ & & \hline & & \text{fluorescence} \\ & & \text{yield of control} \\ & & \text{chloroplasts}_{t=0} \end{array}$$

The rates of change so calculated were plotted against their appro-
 priate t_n 's. (In the case chosen as an example, $n = 1$ minute.)

RESULTS

I. CHANGES IN FLUORESCENCE EMISSION RATIOS OF LEAVES OF HIGHER PLANTS WITH AGE OF THE PLANT.

In Fig. 1 and Table I are shown changes in the low temperature ratio of F_{735}/F_{698} with the "age" of the plant for: R. communis, P. vulgaris, S. oleracea, S. vulgaris, Z. mays and T. sativum. The term "age" is used to designate number of days after the aerial portion of the seedling has broken through the soil. In all cases, measurements were carried out on the first (or first pair of) true leaves. It was generally observed that as age of the plant increases, the low temperature ratio decreases. The ratio in the grasses, initially higher than in the dicots, remains higher throughout the period of investigation. Among the dicots, Ricinus had the lowest ratio of F_{735}/F_{698} , i.e., it shows the relatively strongest emission at 698 nm. For this reason, I decided to study the spectral properties of this plant.

Fluorescence emission spectra of leaves from mature (1 - 4 years old) Ricinus communis plants were compared with corresponding fluorescence emission spectra of isolated chloroplasts. The 77°K fluorescence emission spectrum of such a Ricinus leaf is shown in Fig. 2. It is seen that the (corrected) ratio of F_{735}/F_{698}^* is 4.2. At low temperature, there is observed a three-banded emission spectrum which has peaks at 735 (to 739 nm), 698 and 685 nm. (In the insert to Fig. 2, the low temperature emission of this Ricinus leaf is shown at a higher gain, so that the structure of the shorter wavelength bands may be observed.) The room temperature emission spectrum (Fig. 3) of the leaf exhibits only two

* It was assumed that any apparent fluorescence at 650 nm was due to scattering of light. Therefore, intensities of fluorescence at other wavelengths (e.g. 735 or 698) were measured and the baseline value at 650 nm subtracted, before ratios were taken. All fluorescence emission data, including those given in Table I and Fig. 1, were corrected in this manner.

Figure 1. Ratio of low temperature fluorescence intensities, F_{735}/F_{698} , of primary (or seed) leaf as a function of "age". Age is given in days after aerial portion of plant appears above soil. Curve A, Zea mays; Curve B, Sorghum vulgaris; Curve C, Triticum sativum; Curve D, Ricinus comunis; Curve E, Phaseolus vulgaris; Curve F, Spinacea oleracea. Fluorescence excited at 436 nm and measured at -196°C .

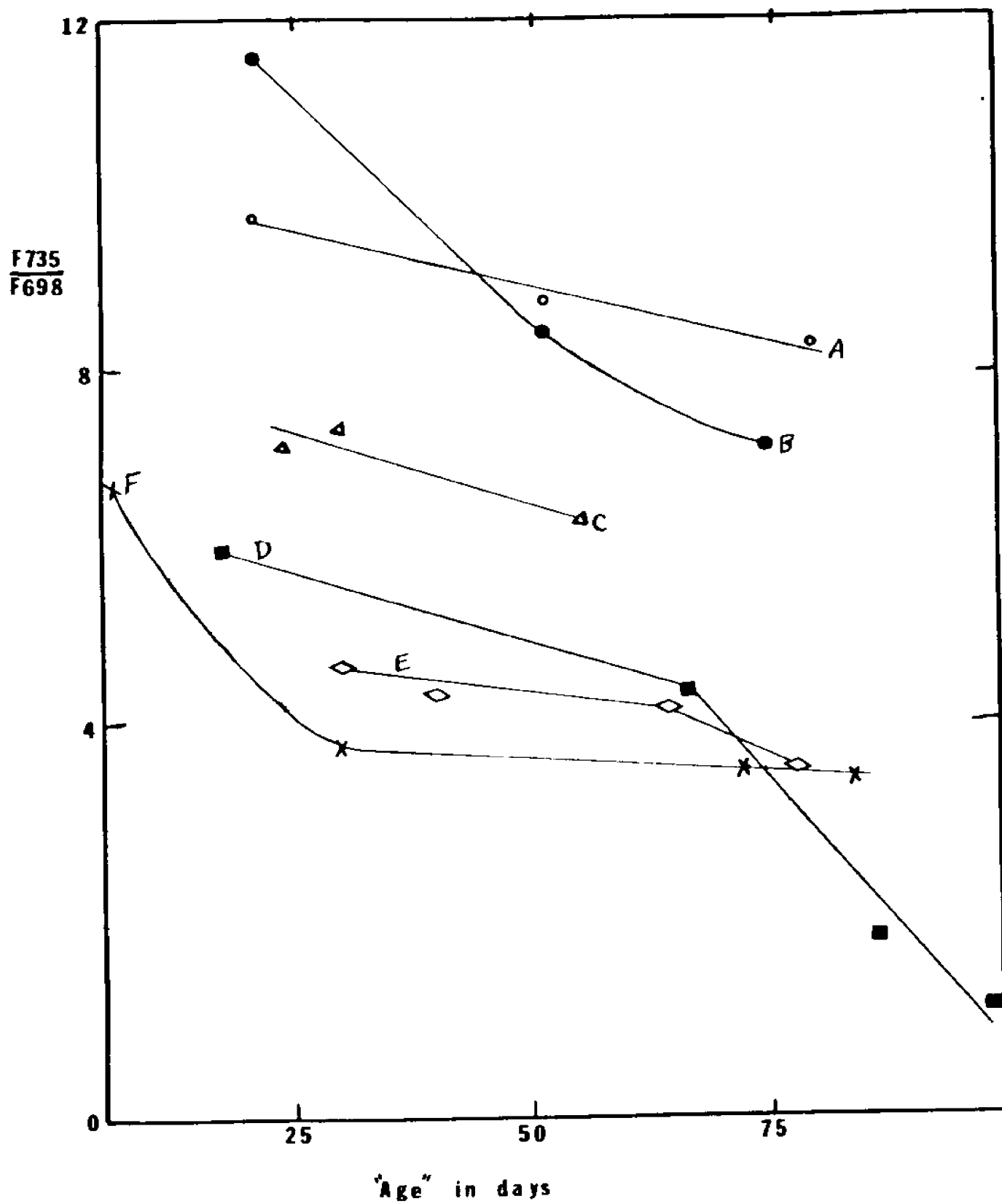


Figure 2. Fluorescence emission of Ricinus leaf and of Ricinus chloroplasts at -196°C . Chloroplasts incubated for indicated number of minutes (see section I: H 2, for definition of minutes of incubation). Spectrum for leaf has been reduced to 1/3 of its original size relative to spectra of chloroplasts, for ease of comparison. Insert: Emission of Ricinus leaf (at higher gain) in the spectral region 683 to 714 nm. Fluorescence excited at 436 nm.

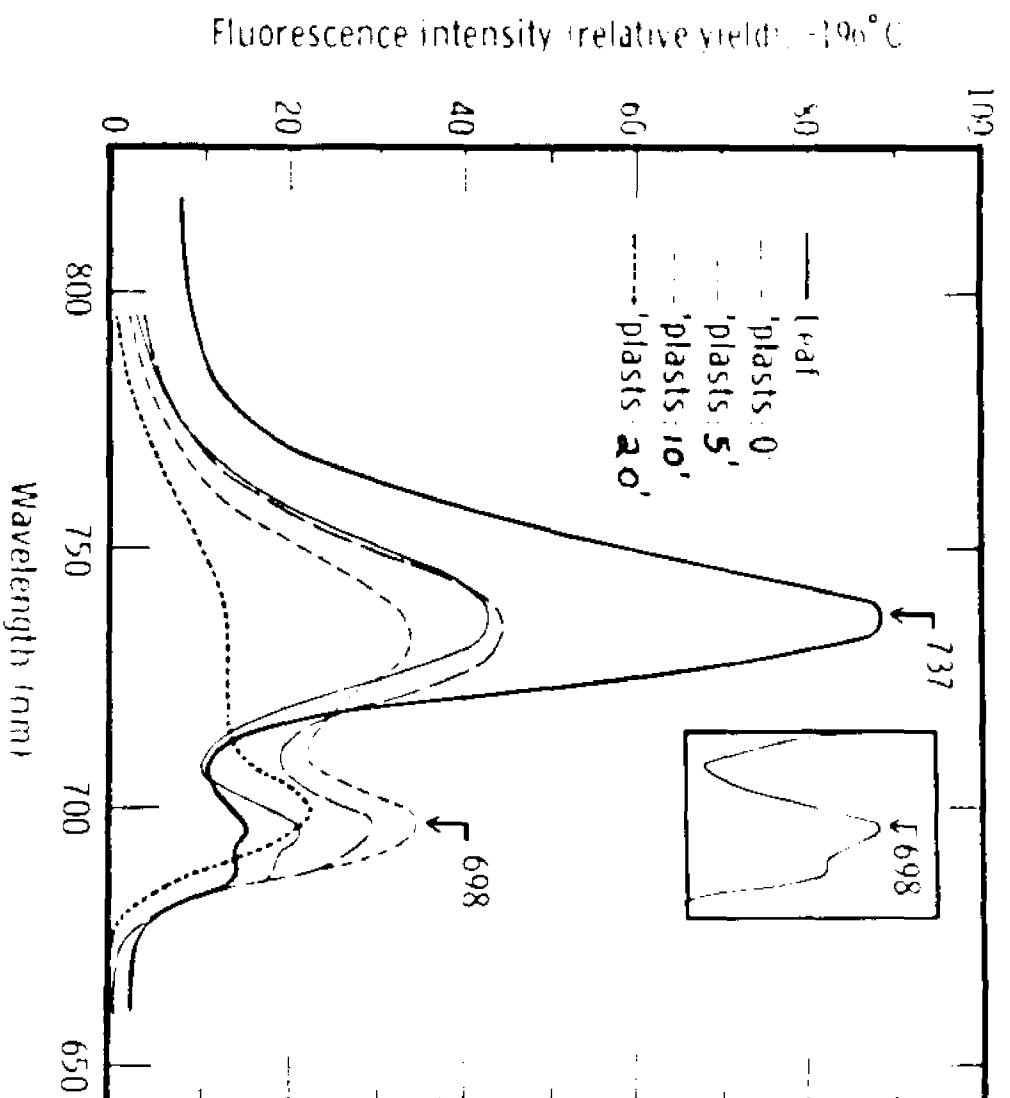


Figure 3. Fluorescence emission of Ricinus leaf and of Ricinus chloroplasts at room temperature. Chloroplasts incubated in suspension medium at 30°C, for indicated number of minutes. Excitation with light of 436 nm.

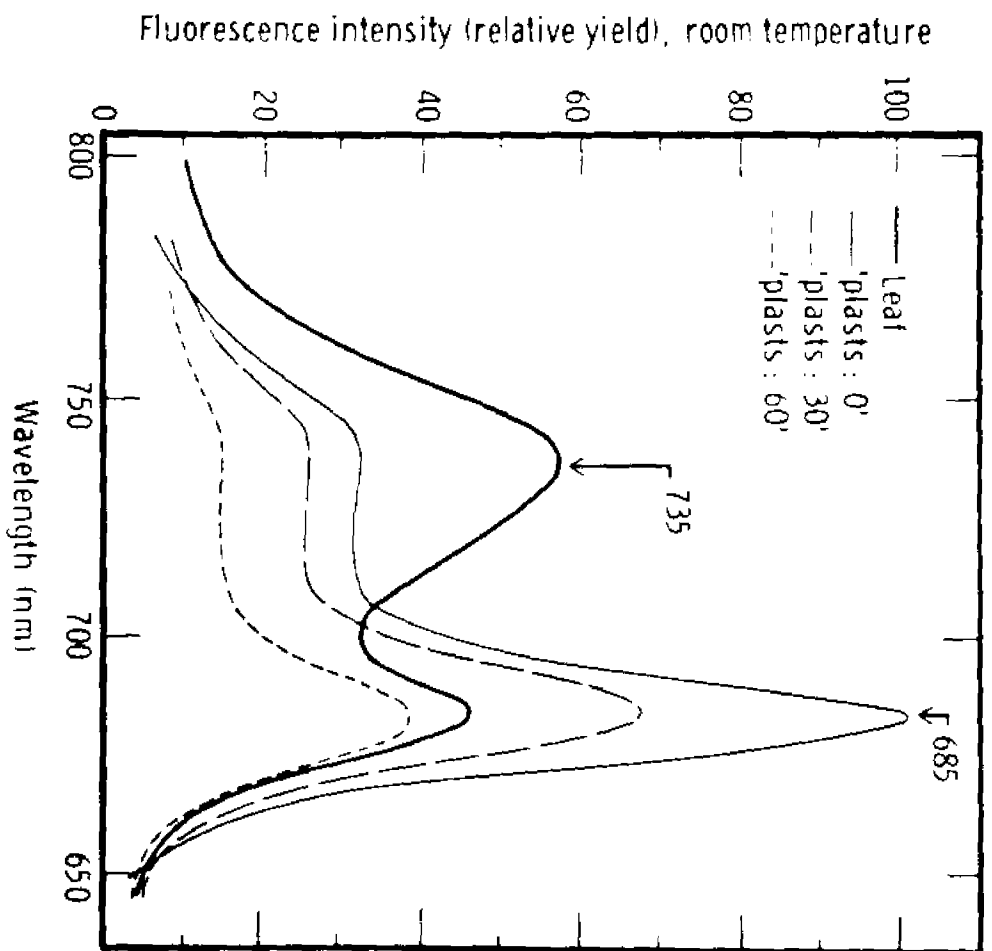


TABLE I

CHANGE IN F 735/F 698 WITH "AGE" OF PLANT

<u>Species of Plant</u>	<u>"Age" of the Plant</u>	<u>F 735/F 698 (spectrum of primary leaf)</u>
<u>Ricinus communis</u>	17	6.00
	66	4.44
	86	1.48
	99 (yellow)	0.66
<u>Phaseolus vulgaris</u>	30	4.65
	40	4.35
	64	4.22
	78	3.52
<u>Spinacea oleracea</u>	6	6.67
	30	3.70
	72	3.50
	84	3.40
<u>Sorghum vulgaris</u>	21	11.63
	51	8.45
	74	7.16
<u>Zea mays</u>	21	9.75
	51	8.82
	84	8.33
<u>Triticum sativum</u>	24	7.16
	30	7.41
	55	6.36

bands -- with maxima at 735 and 685 nm. The peak seen in the low temperature emission spectrum at 698 nm is, in contrast, a valley in the room temperature spectrum. However, F_{735}/F_{698} is similar in both -- at 77°K the ratio is 1.70, while at 293°K the ratio is 1.61.

II. ALTERATIONS IN FLUORESCENCE SPECTRA OF RICINUS CHLOROPLASTS.

A. Time-Dependent Changes in Fluorescence Emission Spectra.

1. CHANGES IN FLUORESCENCE EMISSION SPECTRA MONITORED AT 77°K.

When chloroplasts are isolated from the leaves of R. communis according to Section B-1 of Materials and Methods and their low temperature emission spectrum is observed before incubation (i.e., when they are still in the 0°C isolation medium) it is observed that the low temperature ratio of F_{735}/F_{698} has changed from the situation which obtains in the leaf. In the isolation procedure, the ratio goes from 4.8 (in the leaf) to 1.93 in the chloroplasts.

Upon incubation of such chloroplasts at 37°C in isolation medium (NaCl-Tris), sequential changes in emission occur. When these changes are monitored at 77°K, results depicted in Fig. 2 are obtained. At an incubation temperature of 37°C, the emission band with maximum at 685 nm disappears as a discrete entity after 5 minutes. Concomitant with this change are increases at 698 and 735 nm. With continued incubation, there is progressive diminution of fluorescence at 735 nm relative to increase in emission at 698 nm.* After 20 minutes of incubation, the ratio has changed from about 2 to 0.43. Further incubation results in a decrease in total fluorescence yield (area under the curve); by 60 minutes, the

* That there is an absolute increase in the intensity of the band with maximum at 698 nm, accompanied by an absolute decrease in F_{735} , has been shown in the work of Brody and Brody (1971). These authors employed the use of a fluorescence standard, so that in spite of the different freezing patterns in each sample, emission intensity relative to the standard was determinable for each one of the chlorophyll bands.

fluorescence yield has decreased to 1/3 of that observed in the fluorescence emission spectrum of chloroplasts observed at 20 minutes of incubation. It can also be seen that the (relative) decrease at 735 nm is associated with a hypsochromic shift of the long wavelength maximum from 738 to 728 nm.

2. CHANGES IN FLUORESCENCE EMISSION MONITORED AT ROOM TEMPERATURE.

The room temperature emission spectrum (Fig. 3) of freshly isolated chloroplasts (before incubation, i.e. still in 0°C isolation medium) also differs greatly from that of the leaf. While both exhibit only two fluorescence emission bands -- at 685 and 735 nm -- the ratio 735/685 is 1.68 in the leaf and only 0.33 in the chloroplasts. When such Ricinus chloroplasts are incubated at 30°C, and are monitored at room temperature, time-dependent changes in emission are observed (see Fig. 3). With increasing time of incubation at 30°C, ^{the} fluorescence at 735 nm takes on the appearance of a shoulder on the 685 nm band. There also occurs a large decrease in total fluorescence (area under the curve), and a further small decrease in the ratio of F_{735}/F_{698} (from 0.571 in 0' chloroplasts to 0.500 in chloroplasts incubated for 45 minutes). If the fluorescence emission spectrum is taken after 5 minutes of incubation at 20°C, and compared to that obtained with non-incubated chloroplasts, i.e. at 0' (see Fig. 4, lower curve), the resulting difference spectrum (5' - 0') exhibits minima at about 738 and 685 nm, and a maximum at 700 nm (see Fig. 4, upper curve).

3. CHANGES IN FLUORESCENCE EMISSION RATIOS (MONITORED AT 77°K) AS FUNCTIONS OF TIME AND TEMPERATURE OF INCUBATION.

In Fig. 5 is shown the change in low temperature ratio of F_{735}/F_{698} with time of incubation, at incubation temperatures ranging from 0° to 45°C. Since the rate of decrease of this ratio was found to be incubation temperature-dependent (i.e. speeding up with increasing temperature), these

Figure 4. Fluorescence emission spectra of Ricinus chloroplasts at 20°C. Lower section: curve given by solid line: 0 minutes incubation; curve given by broken line: 5 minutes incubation. Upper section: difference spectrum of curves in lower section (5 minutes minus 0 minutes). Excitation with light of 436 nm.

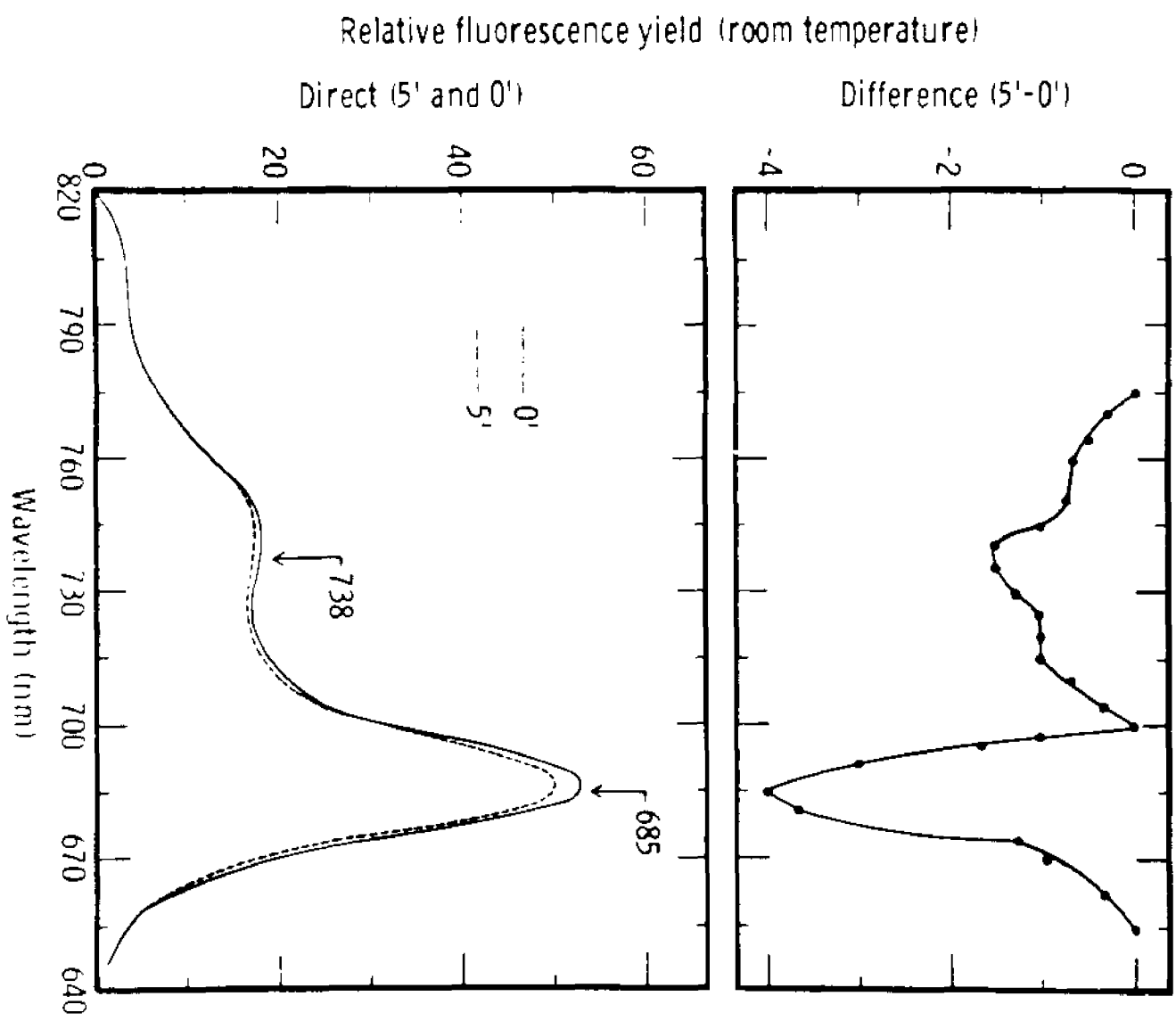
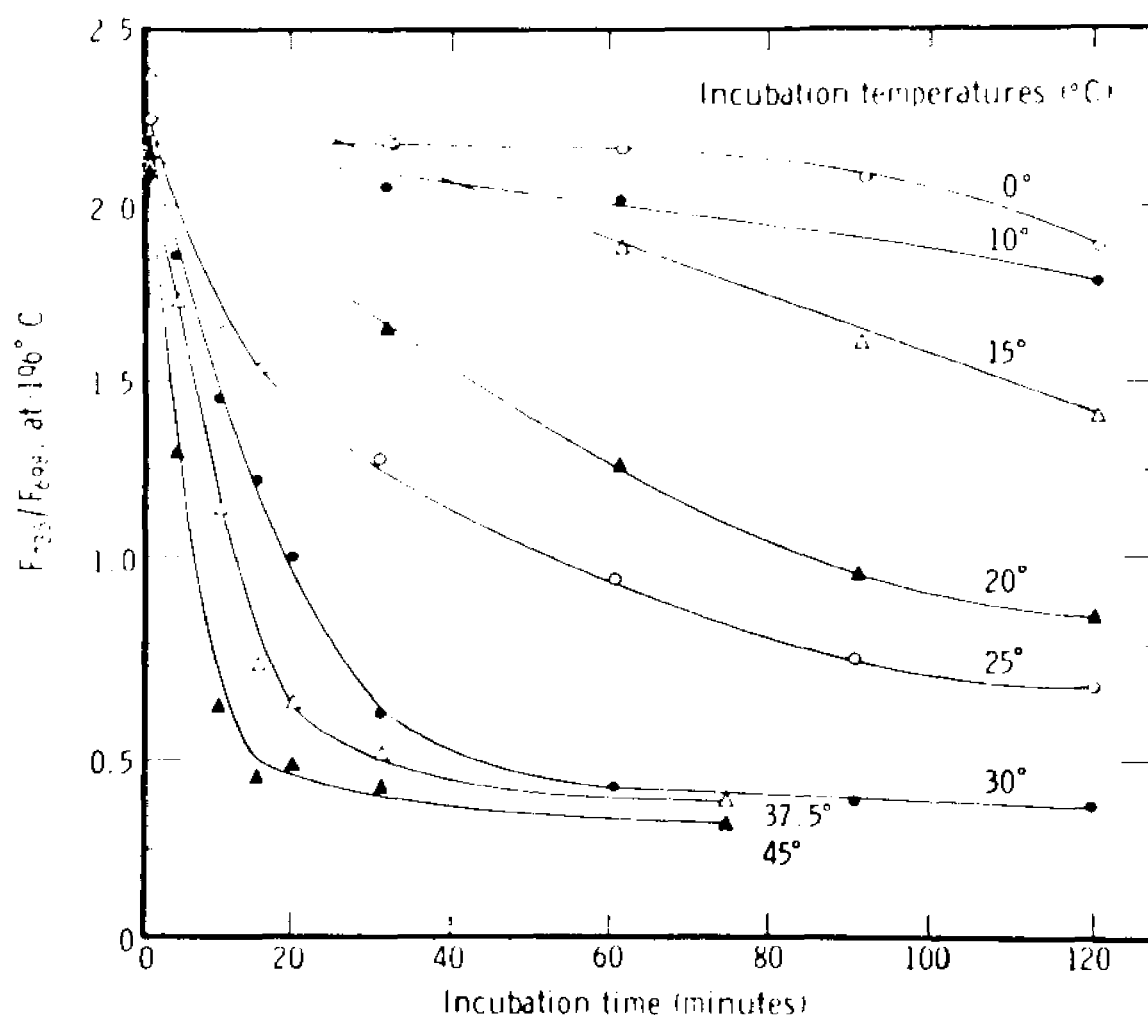


Figure 5. Ricinus chloroplasts: F 735/F 698 (at -196°C) as a function of time, at indicated temperatures of incubation. Fluorescence excited at 436 nm.



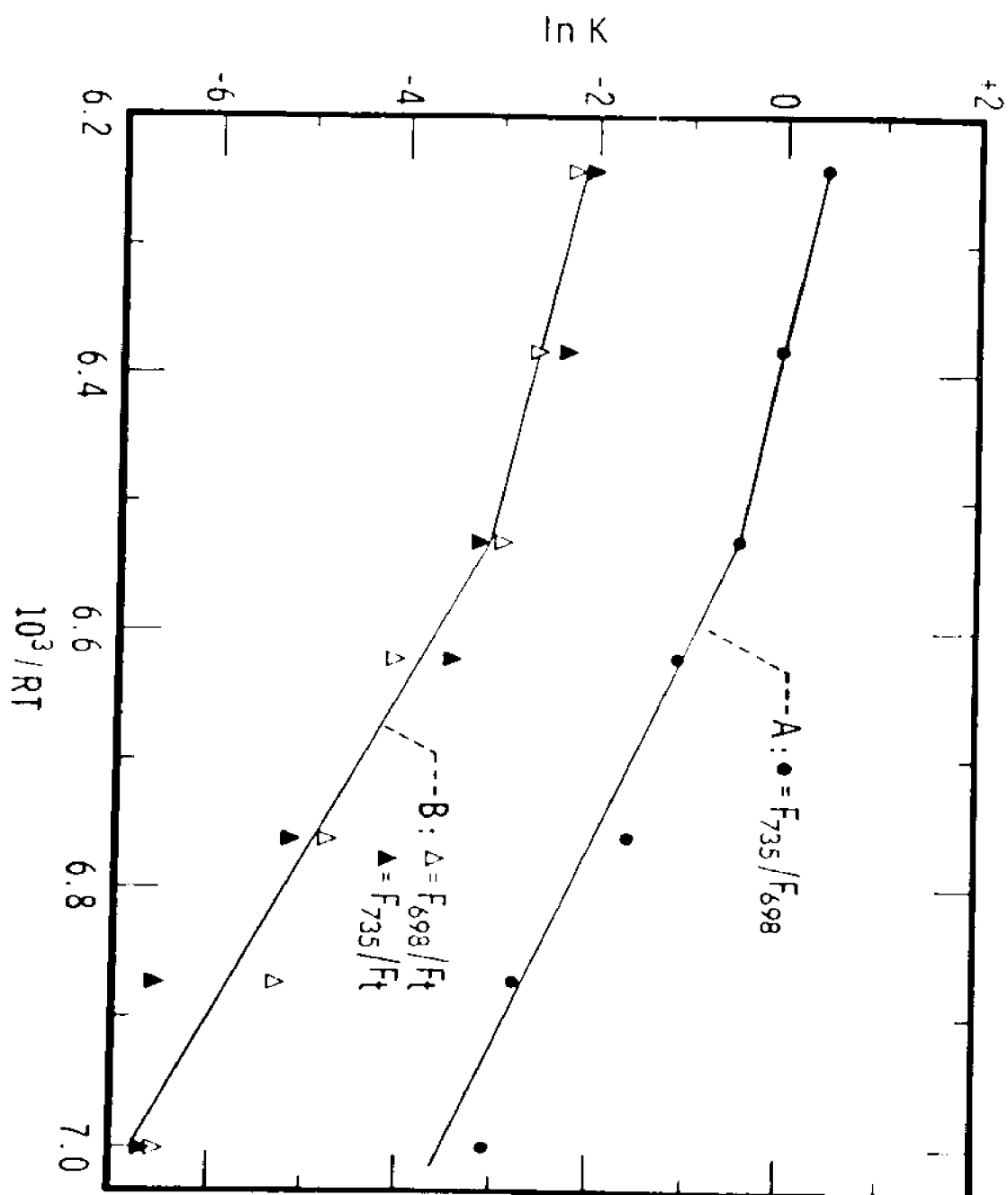
data were replotted in terms of the Arrhenius equation (see Fig. 6). Such a plot yields ¹¹ energies of activation per reaction rate ¹² (F_{735}/F_{698} at 10 minutes of incubation minus F_{735}/F_{698} at zero time) of 8 Kcal and 3 Kcal for the low (10° to 25°C) and high (30°C to 45°C) temperature ranges, respectively (see Fig. 6, curve A). In Fig. 6 curve B is shown the change in F_{698} or F_{735} (at $10' - 0'$). This function was obtained by measuring with a planimeter the fraction that the particular band contributes to the total area under the curve. A band width of 20 cm^{-1} to either side of the maxima was isolated. In both these cases, a bi-phasic curve was obtained, parallelling the one in curve A. That the points for F_{698}/F_t coincide with the points for F_{735}/F_t suggest a close relation between the two processes. The significance of these results will be considered later in this dissertation.

B. Time-Dependent Changes in Fluorescence Excitation Spectra of Ricinus Chloroplasts.

The observation that the decrease in fluorescence at 735 nm is accompanied by an increase in fluorescence at 698 nm can be interpreted in a number of ways. Among the interpretations for these effects are: (a) energy transfer from system II to system I is no longer occurring, (b) the form of chlorophyll giving rise to F_{735} is being destroyed, or (c) the form fluorescing at 735 nm is being converted into a form contributing to fluorescence at 698 nm. It was therefore necessary to determine the correspondence between fluorescence emission and fluorescence excitation changes in Ricinus chloroplasts, and furthermore to compare the excitation changes with absorption changes.

Before such time-dependant excitation studies with chloroplasts were undertaken, a comparison was made of the fluorescence action spectrum of chloroplasts and the intact leaf in order that the effects of isolation

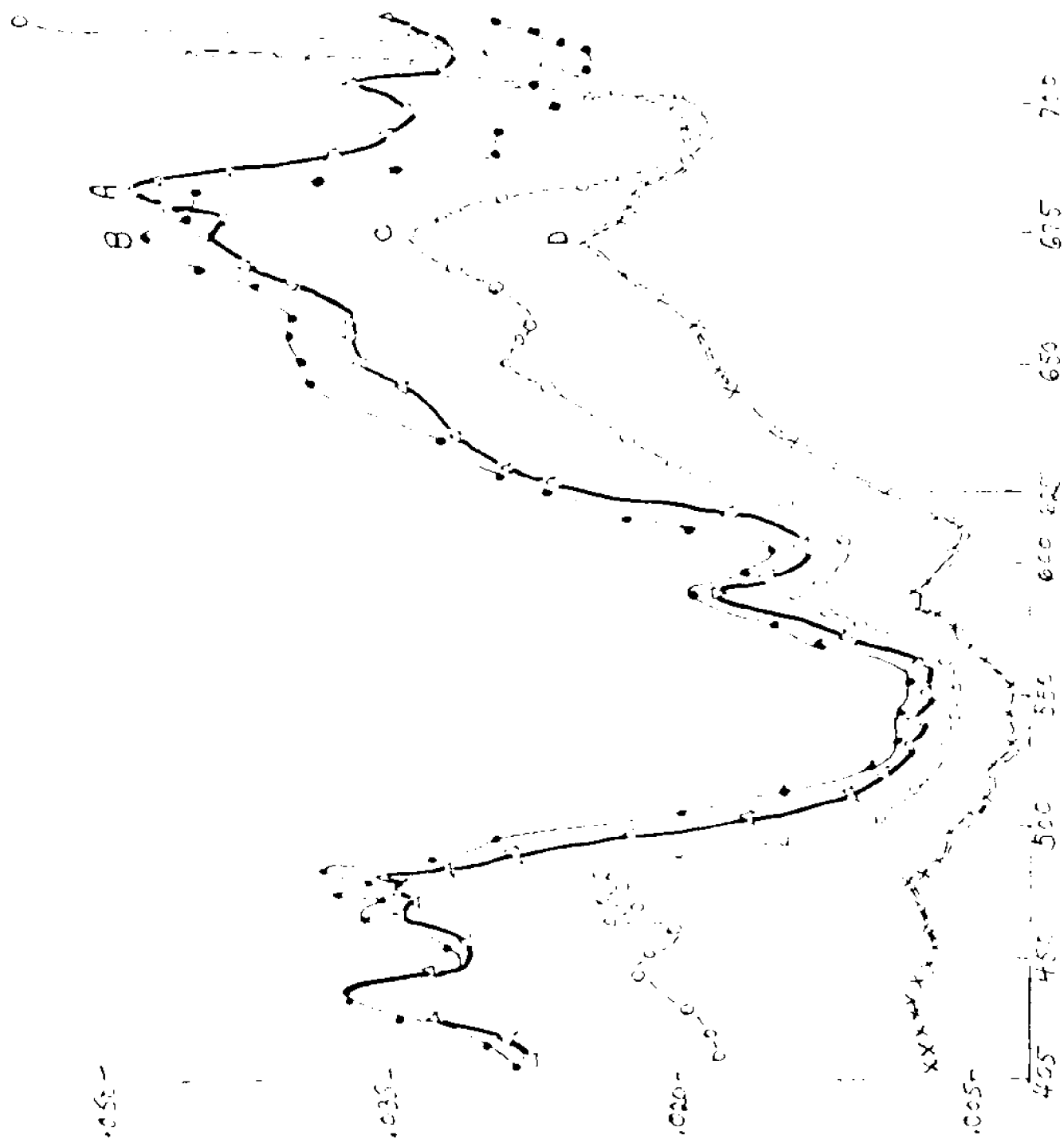
Figure 6. Ricinus chloroplasts: Arrhenius plot($\ln K$) of F_{735}/F_{698} (at -196°C) at 10 minutes of incubation minus 0 minutes of incubation, as a function of $10^3/RT$; data taken from Figure 5. Curve A: F_{735}/F_{698} . Curve B: F_{698}/F_t given by open triangles; F_{735}/F_t given by solid triangles. (For definition of these functions, and methodology employed, see Materials and Methods, section VII-C, page 27 and Results, section III-A-1, pages 39-40.)



could be determined. In Figs. 2 and 3, the fluorescence emission spectrum of Ricinus leaf at 77°K and at room temperature was compared with that of Ricinus chloroplasts which were freshly isolated and then incubated in NaCl-Tris. In Fig. 5.7^{1 and 7} are shown the action spectra for exciting 735 nm fluorescence* (measured at 77°K) for Ricinus leaf and for its isolated chloroplasts (see Fig. 2 for emission curves). The low temperature action spectrum for exciting fluorescence at 735 nm in the leaf has a discrete band with maximum at 636 nm and another band with maximum at 656 nm (chlorophyll b). The 672 nm band (monomer) appears only as a small peak. The predominant band at 694 nm (with a possible shoulder at 705 nm) suggests efficient sensitization of 735 nm fluorescence by the species of chlorophyll (aggregate) absorbing at this wavelength. If we now look at the action spectrum for exciting fluorescence in newly-isolated (i.e. 0') Ricinus chloroplasts, the following difference may be noted: the 636 and 656 nm bands in the leaf have become less pronounced and there is now present a band at 650 nm. Furthermore, the 674 nm band has become more intense and there is now clearly evident a predominant band with max at 682 nm. In the minimum in the spectral region formerly¹ large 694 nm excitation band, there can be seen a small band at 705 nm (which presumably had previously been incorporated into the fluorescence excitation band at 694 nm in the leaf).

* In all of the work that follows (both with RLE and fatty acids), only action spectra for exciting emission at 735 nm are given for the following reason. Perusal of Fig. 7 A shows that the 735 nm action spectrum is capable of revealing more information about the bands that contribute to fluorescence than are the action spectra for excitation of fluorescence at shorter wavelengths. Because of the limitations of the fluorescence spectrofluorimeter it is impossible, for example, to detect contributions of bands with maxima beyond 650 nm to fluorescence at 685 nm; in the case of excitation at 700 nm, it is difficult to go much beyond the 674 nm contributing form.

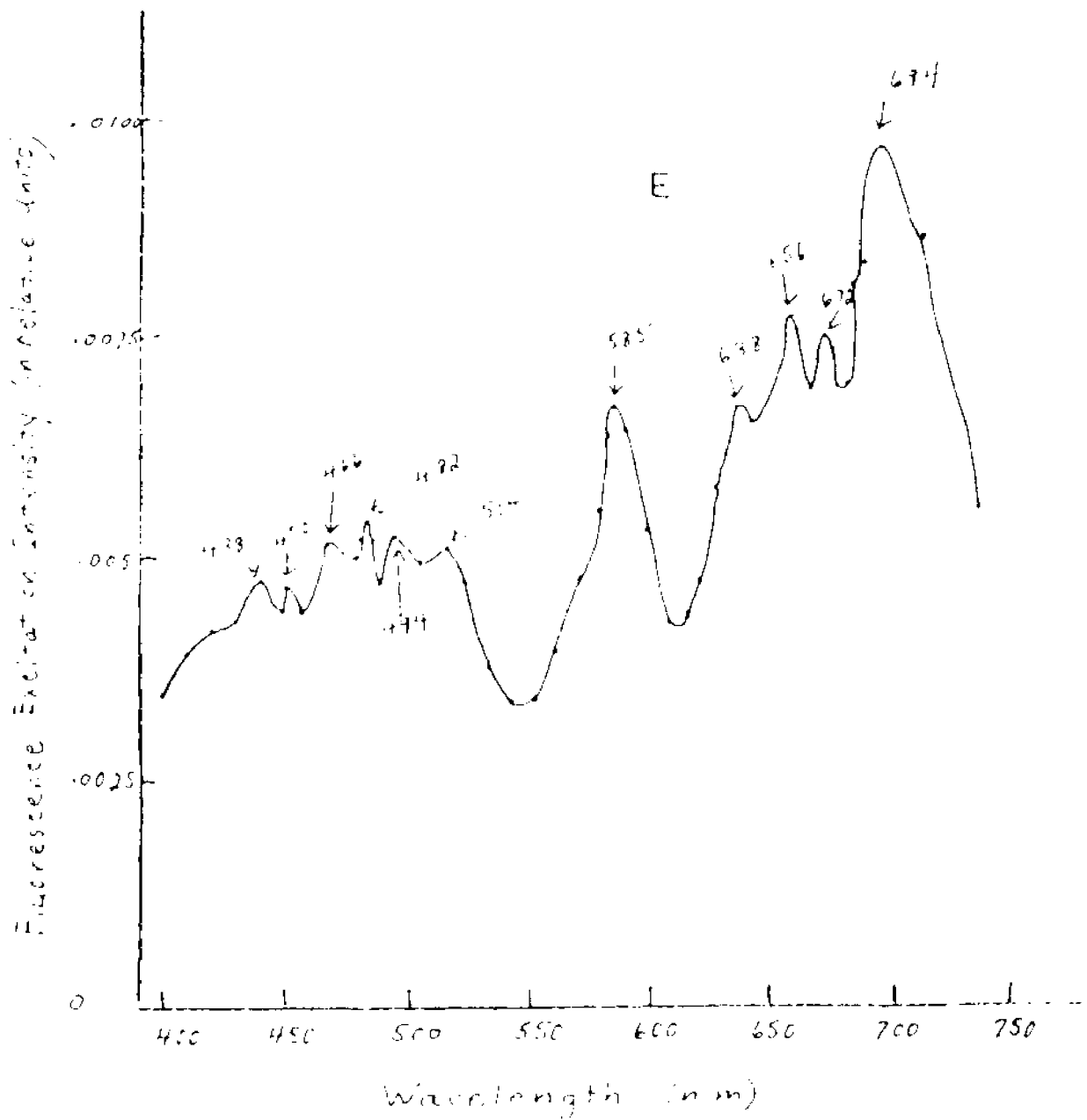
Figure 7. Action spectra for exciting fluorescence, at 735 nm, from Ricinus chloroplasts (at -196°C) as a function of time of incubation at 37°C . Curves A, B, C, and D are for incubation of chloroplasts at 0 (see section II-A-2 for definition of 0 minutes of incubation), 5, 10, and 20 minutes, respectively. Double vertical lines indicate change in units on abscissa, i.e. nm. Spectra have been corrected for equal number of incident quanta.



Waveform (mV)

Figure 3. Waveform (mV) in response to 100 Hz stimulation (100 Hz)

Figure 7'. Action spectrum for exciting fluorescence, at 735 nm, from Ricinus leaf (at -196°C). Spectrum has been corrected for equal number of incident quanta.



The comparison between the "control" Ricinus chloplasts i.e., 0' and the Ricinus leaf suggests that the changes associated with isolation procedure either disrupts energy transfer to the 694 nm band, or destroys the species of chlorophyll absorbing at this wavelength. (It should be recalled (refer to Fig. 2) that this isolation procedure also results in considerable reduction in the intense emission band at 735 nm seen in the leaf.) If now we compare the chloroplasts in the "control" situation with incubated chloroplasts, we note the following changes. The predominant 682 band seen in the 735 nm fluorescence excitation spectrum of "control" chloroplasts is greatly diminished in the chloroplasts incubated at 37°C for 5 minutes. There is also a strong decrease in intensity of the excitation band at 705 nm. These fluorescence excitation changes, it may be recalled, are occurring concurrently with the disappearance of the 685 nm shoulder, and increases at 698 and 735 nm in the fluorescence emission spectrum. (The increases in the latter band are only transient.) As can be seen clearly with chloroplasts incubated for 5 minutes, the diminution in the 682 nm band is accompanied by intensification of the 674 nm band (the latter becoming the predominant one in incubated chloroplasts). Upon further incubation of Ricinus chloroplasts at 37°C, there is a decline in total fluorescence yield, but the contribution of the 674 nm band to emission at 735 nm does not change. With increasing time of incubation, the small excitation band at 650 nm (chlorophyll b?) diminishes in intensity.

III. ALTERATIONS IN THE SPECTRA OF CROSS-REACTING SYSTEMS INCUBATED IN AN AQUEOUS EXTRACT OF RICINUS LEAVES (RLE).

In order to define more closely the nature of the active factor in Ricinus leaves, and in order to obtain better controls, an aqueous extract from Ricinus leaves ("RLE") was prepared (as explained in the Materials and Methods), and chloroplasts from other higher plants and from algae

were incubated in this extract. The changes elicited in these cross-reaction systems are described in this section.

A. Changes in the Low Temperature Fluorescence of Cross-reaction Systems.

1. Fluorescence of Higher Plant Chloroplasts Incubated in RLE.

a. Fluorescence emission changes

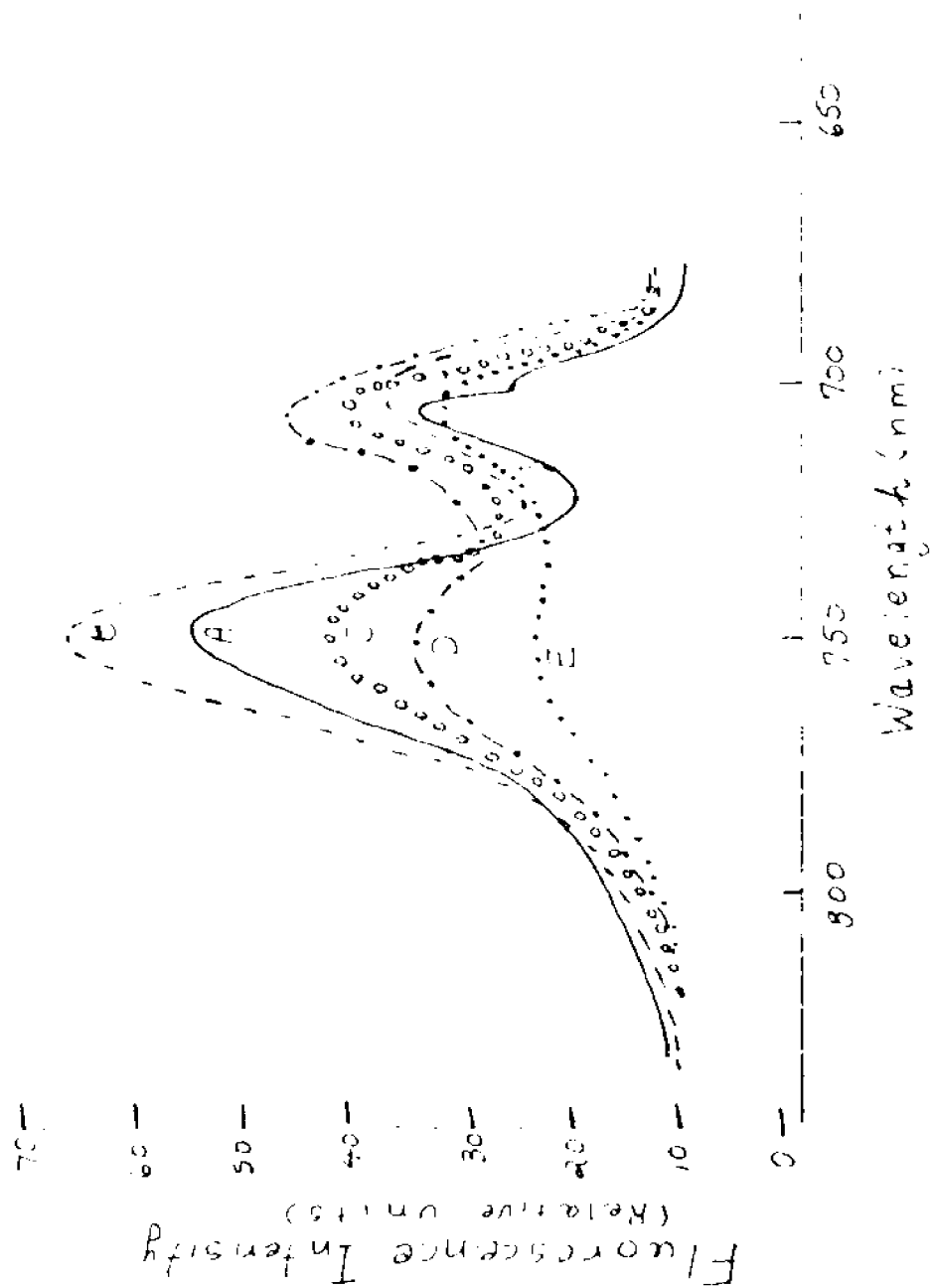
1) Time-dependent changes in fluorescence emission spectra

When chloroplasts from spinach are incubated in RLE, there result time-dependent changes in emission spectra which are similar to those obtained with Ricinus chloroplasts incubated in buffer. The control chloroplasts of spinach (i.e. freshly isolated and suspended in NaCl-Tris buffer) have a similar emission spectrum to the "control" chloroplasts of Ricinus; one difference is that with spinach, the 685 nm band is distinct and not just a shoulder, as it is in Ricinus. In Fig. 8 A is seen the fluorescence emission spectrum of control spinach chloroplasts (suspended in buffer). Upon incubation at 30⁰C, the 685 nm band merges with the intensifying 698 nm band (Fig. 8 B) and upon longer incubation there occur time-dependent changes virtually identical with those described above (Fig. 8 C-E). At 30⁰C, the one-banded emission form (with maximum at 696 nm) is reached after 60 minutes in this particular preparation of RLE.

2) Changes in Fluorescence Emission as a Function of the Total Protein Concentration of RLE.

Investigations in collaboration with other workers in this laboratory (Cohen et al., 1969; see also Cohen, Ph. D. dissertation, 1970), have revealed the protein nature of the active factor of RLE. In the present

Figure 8. Fluorescence emission of Spinacea chloroplasts at -196°C . Chloroplasts incubated in Ricinus leaf extract ("RLE") at 30°C . Curve A: control Spinacea chloroplasts suspended in Tris-NaCl, pH 7.8). Curves B-E: incubation of chloroplasts at zero, 20, 40 and 60 minutes, respectively. Excitation with light of 436 nm.



work, I observed with RLE, that the change in ratio of F 735/F 698 is dependent upon the concentration of protein in the extract.* In Fig. 9 is plotted the effect of total RLE protein concentration, ranging from 0.1 mg protein per ml to 6 mg protein per ml, on spinach chloroplast preparations containing 30 μ g chlorophyll/ml. It appears that for such chloroplasts, the change in F 735/F 698 upon incubation at 37°C for the five minute period (5' minus 0' of incubation) is proportional to total protein concentration of RLE up to about 0.5 mg protein/ml. Beyond this concentration of RLE, the rate of change in the ratio falls off, much as one might anticipate under enzyme-substrate conditions in which the substrate becomes limiting. Therefore, a study was undertaken to determine the interaction between a given concentration of RLE and an incrementing chlorophyll (chloroplast) concentration.

3) Changes in Fluorescence Emission as a Function of Chlorophyll

Concentration of Spinach Chloroplasts Incubated in RLE.

In Fig. 10 is seen a plot of the rate of change of F 735/F 698 as a function of chloroplast concentration, for spinach chloroplasts incubated in RLE at 30°C. The RLE concentration is 0.50 mg of total protein per ml, i.e. a concentration falling on the linear part of the curve in Fig. 9, above. At ~~an~~ chlorophyll concentration of $\sim 50 \mu$ g per ml, the rate falls off (Fig. 10). If a Lineweaver-Burke plot of enzyme activity as a function of chloroplast concentration is made, the apparent K_M , as

* It is recognized that in the crude extract of RLE, the amount of "active" protein may vary from preparation to preparation, and that the total amount of protein as determined by the method of Lowry (see Materials and Methods) is only a very crude approximation to the concentration of "active" protein, since a very large (and perhaps inconsistent) number of other proteins are probably present.

Figure 9. Change of F 735/F 698 (at -196°C) at 5 minutes of incubation at 30°C - 0 minutes of incubation at 30°C, for Spinacea chloroplasts in preparations of RLE containing differing concentrations of total protein. Blue light (436 nm) used for excitation.

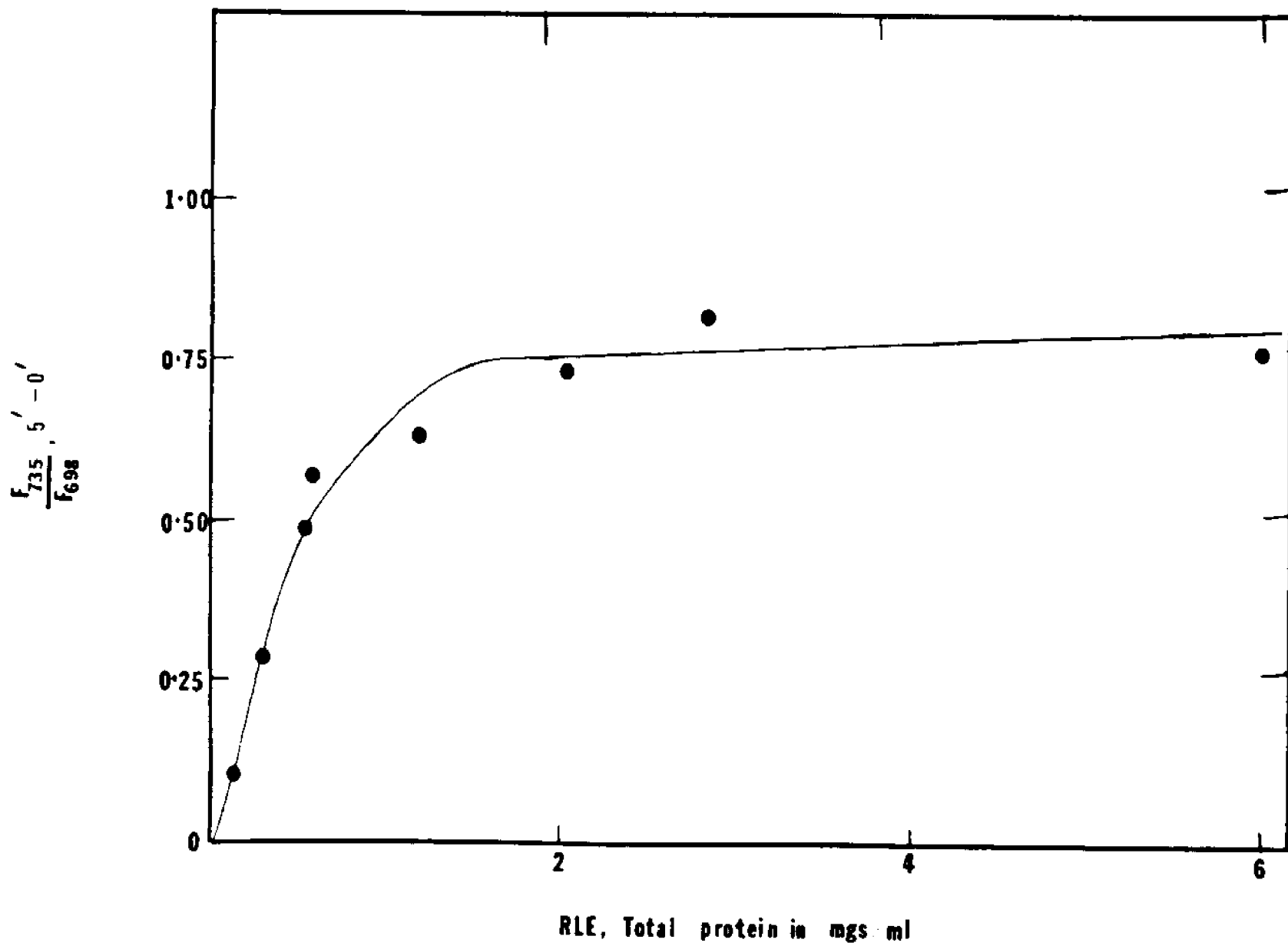
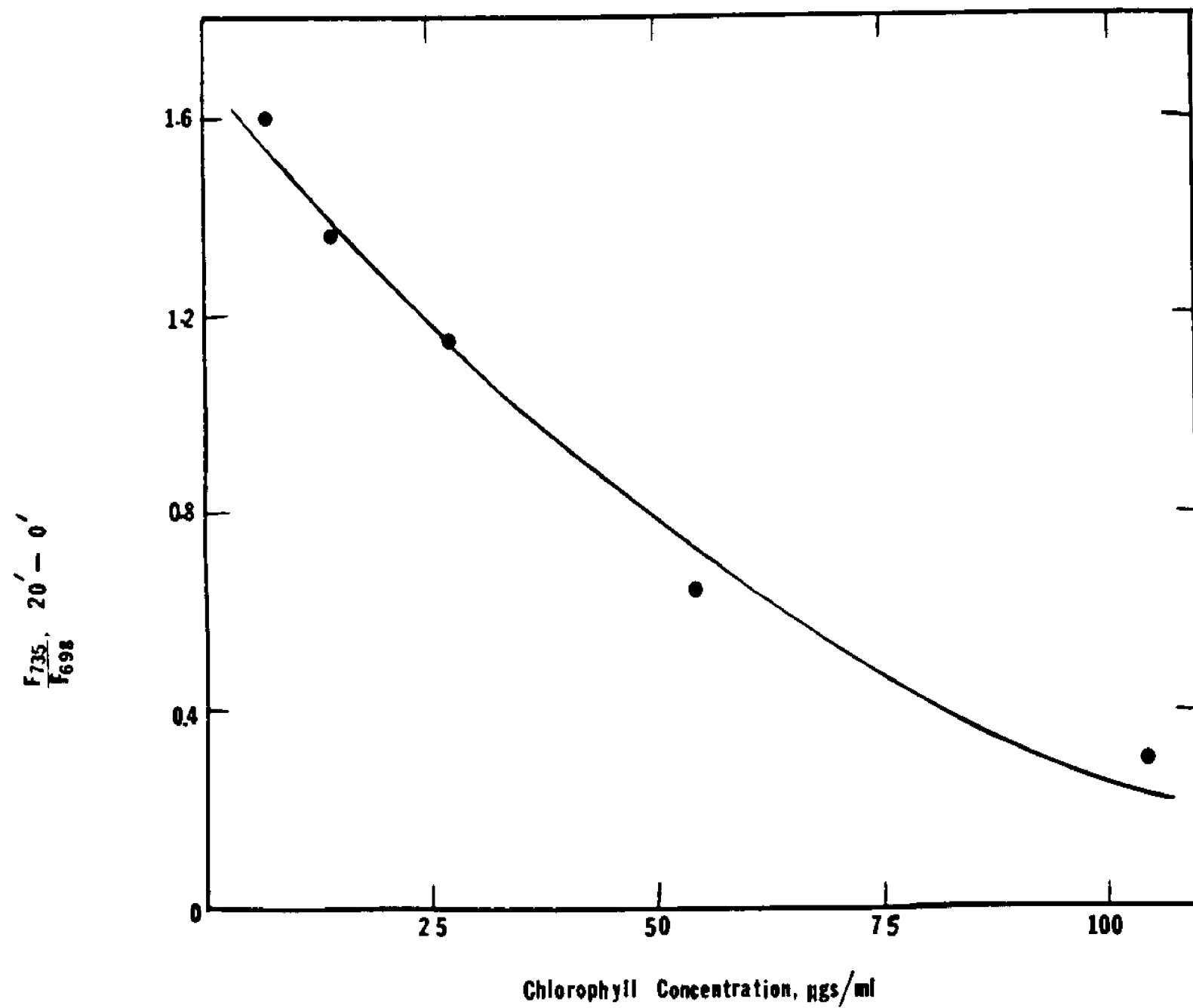


Figure 10. Change of F_{735}/F_{698} (at -196°C) as a function of chloroplast concentration in suspensions of Spinacea chloroplasts incubated at 30°C for 20 minutes in 0.50 mgs. total protein/ml RLE. Excitation with light of 436 nm.



determined from this plot, gives a value of $K_M = 40 \mu\text{g/ml}$, which suggests a high affinity of the active RLE protein for the chloroplast (see Fig. 11). The non-linear portion of the Lineweaver-Burke curve is given by two widely spaced points, and perhaps one cannot safely attach much significance to this curvature; however, possible interpretations are given in the discussion.

In order to determine that the changes seen in fluorescence emission spectra of chloroplasts incubated in RLE did not depend upon chloroplast concentration, all fluorescence spectra were obtained using chloroplasts suspended in undiluted (i.e. saturating amounts) of RLE (in which total protein was in excess of 5 mg/ml).

4) Changes in Fluorescence Emission Ratios (Monitored at 77°K) as Functions of Time and Temperature of Incubation.

If Spinacea chloroplasts are suspended in RLE and incubated at temperatures ranging from 0° to 45°C for various lengths of time, the changes observed in the low temperature ratio of F 735/F 698 are identical with those obtained for Ricinus chloroplasts suspended in buffer. Spinacea chloroplasts suspended in Tris-NaCl buffer (control) and incubated under these same conditions, do not exhibit decreases in the ratio of F 735/F 698 but rather, show increases in this ratio (see Fig. 12). When the changes in ratio of F 735/F 698 for Spinacea chloroplasts suspended in RLE are corrected for the changes in ratio of the controls ("C"), and the change ($10^3 \frac{R_{C-RLE}}{C-RLE} - 0^3 \frac{R_{C-RLE}}{C-RLE}$) is plotted according to the Arrhenius Equation (see Fig. 13), there is obtained a monophasic Arrhenius curve, yielding an energy of activation of 8 Kcal/reaction rate. Since a biphasic curve (similar to the one observed above with Ricinus chloroplasts incubated in buffer) is obtained when the data for Spinacea chloroplasts incubated in RLE are not corrected for effects of temperature, it is probable

Figure 11. Lineweaver-Burke plot of the data given in Figure 10.

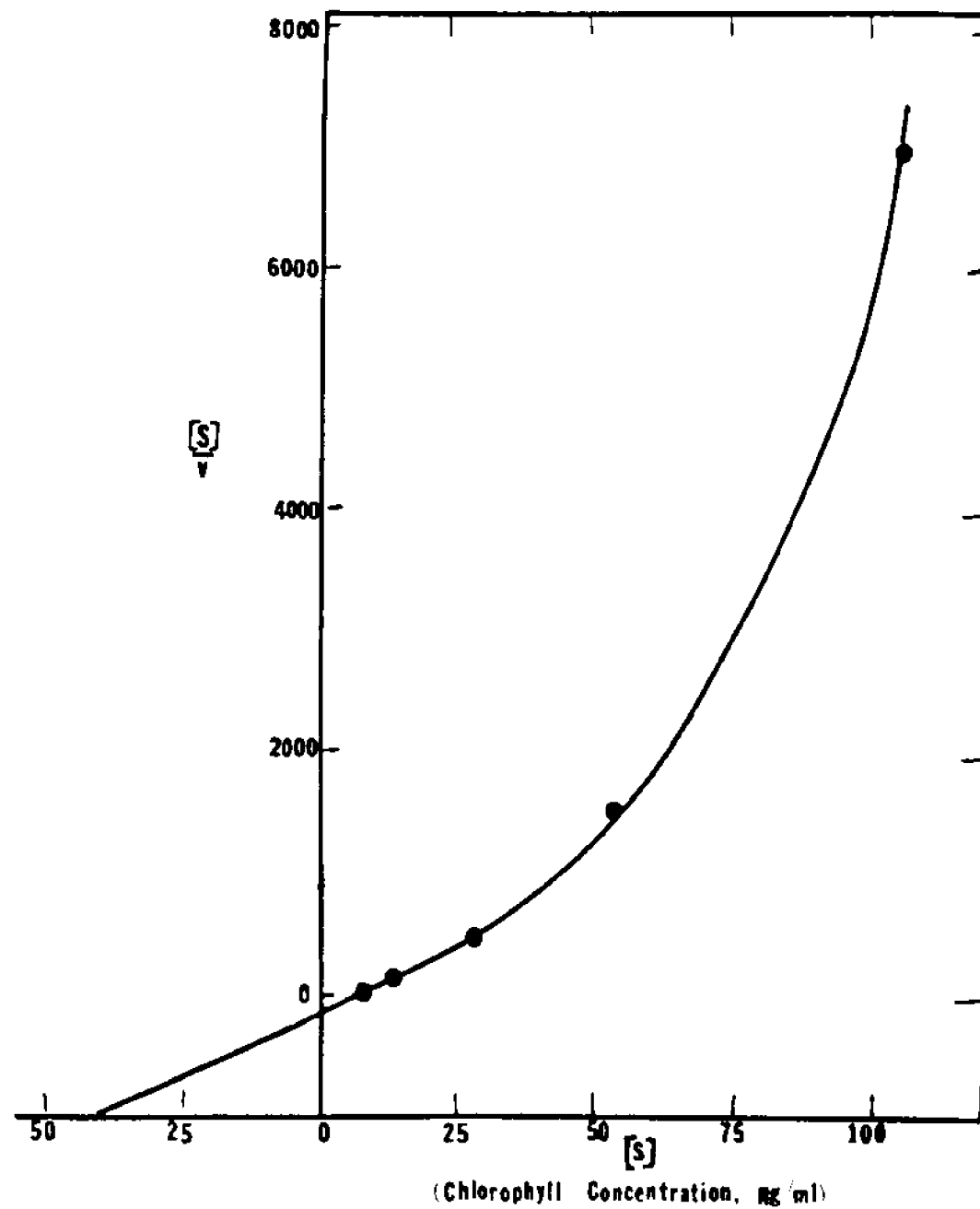


Figure 12. Spinacea chloroplasts suspended in NaCl-Tris HCl buffer, pH 7.8. F_{735}/F_{698} (at -196°C) as a function of time, at indicated temperatures of incubation. Excitation with light of 436 nm.

NEW YORK STATE
 1900
 1400
 0° C
 20° C
 30° C
 40° C
 50° C

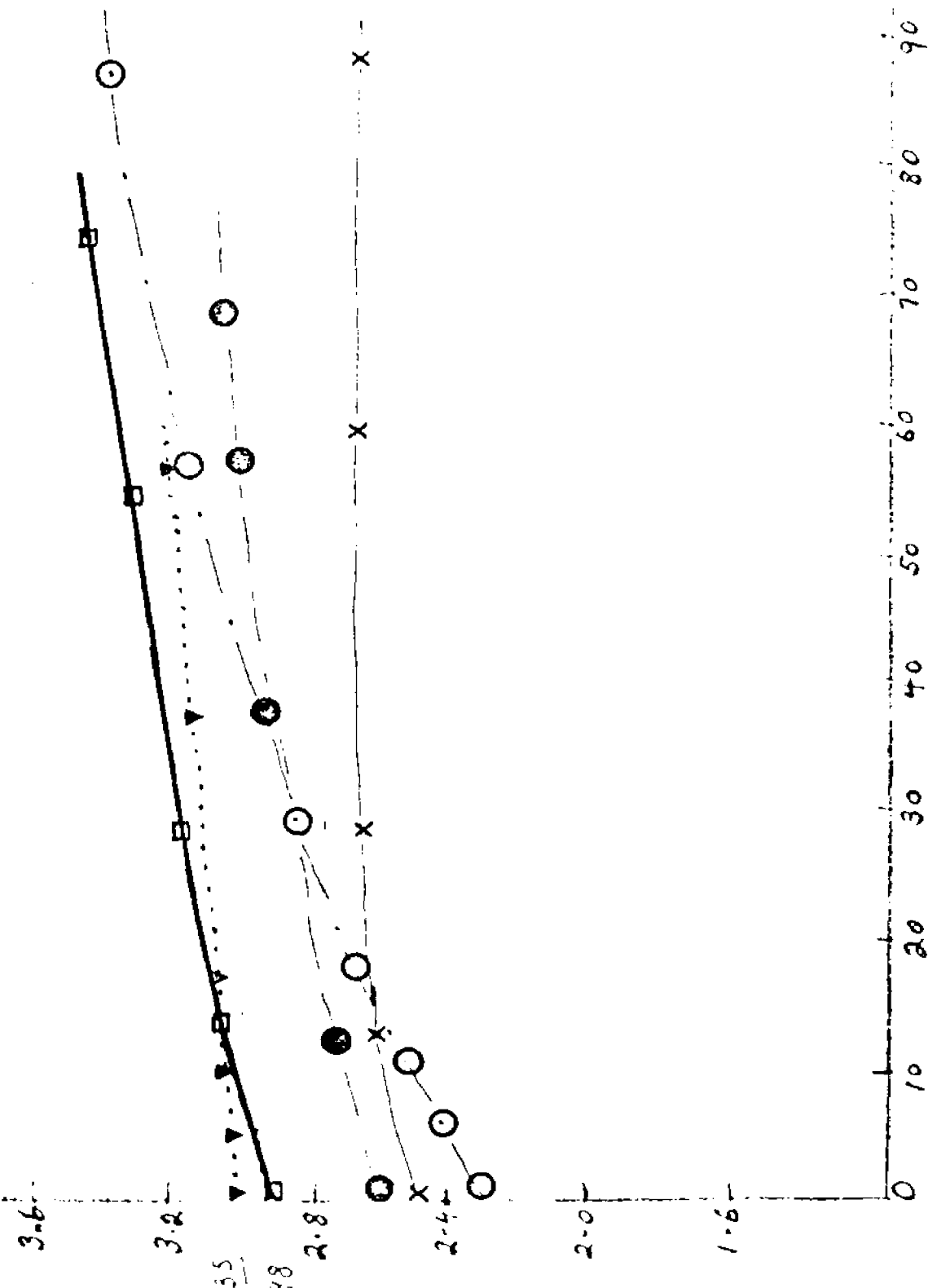
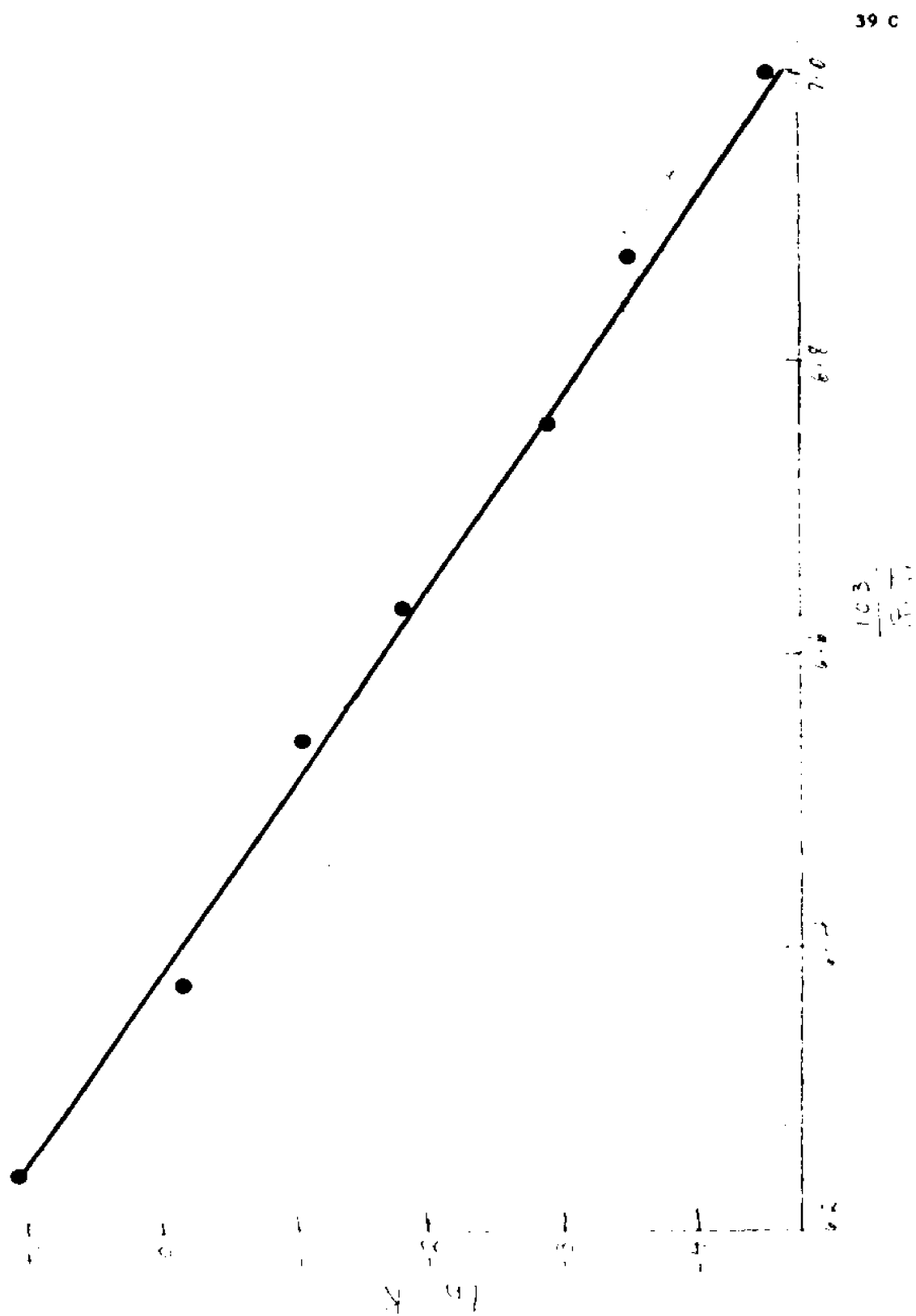


Figure 13. Spinacea chloroplasts incubated in RLE:Arrhenius plot($\ln K$) of F_{735}/F_{698} (at -196°C) at 10 minutes of incubation minus 0 minutes of incubation.(Fluorescence ratios corrected for changes in the controls.)



that even in the case of Ricinus chloroplasts the apparent biphasic nature of the curve results from the effect of temperature on spectral properties as well as the effect of RLE-active protein on spectral properties.

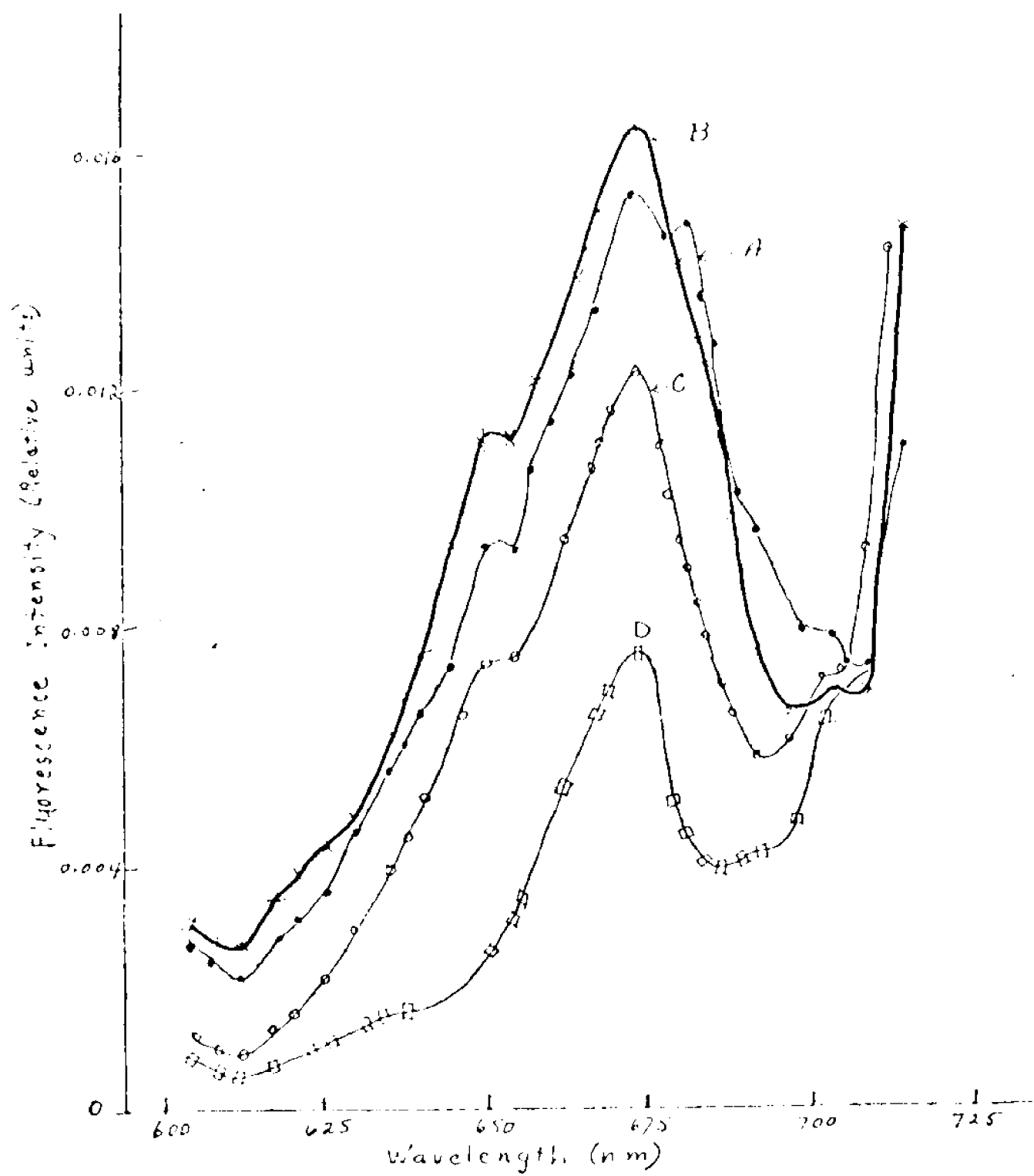
b. Time-Dependent Changes in Fluorescence Excitation of Higher Plant Chloroplasts Suspended in RLE.

The changes elicited in the 735 nm fluorescence excitation spectrum of chloroplasts isolated from the leaves of higher plants and incubated in RLE, were identical to the changes brought about by incubation of Ricinus chloroplasts in buffer (refer back to Fig. 7). For example, in Fig. 14 are shown the changes brought about in Spinacea chloroplasts incubated in RLE at 30°C for increasing periods of time. These spectra were measured with the same material as used in Fig. 8, i.e. Fig. 8 and Fig. 14 represent the simultaneous changes which occur in the emission spectra and action spectra, respectively, as functions of incubation time in RLE. It can be seen in Fig. 14 that the changes in fluorescence excitation in RLE-incubated Spinacea are comparable to those obtained with Ricinus chloroplasts. We note again, that at 0' in RLE (Fig. 14B) compared to the control, there is an increase in contribution of the 674 nm band, and decreases in contributions of the 682 and 705 nm bands, to excitation at 735 nm. It should be recalled (refer again to Fig. 8) that in the cross reaction system, the emission spectrum of such chloroplasts exhibits incorporation of the 685 nm shoulder into the intensifying 698 nm band. Again, upon further incubation, there is decrease in total fluorescence but no further change in contribution of the 674 nm excitation band to 735 nm fluorescence.

2. FLUORESCENCE OF ALGAL CHLOROPLASTS INCUBATED IN RLE.

In order to determine whether the effects observed with RLE on

Figure 14. Action spectra for exciting fluorescence at 735 nm from Spinacea chloroplasts at -196°C . Chloroplasts incubated in Ricinus leaf extract at 30°C . Curve A: Control chloroplasts in Tris-NaCl, pH 7.8. Curves B - B: incubation of chloroplasts at 0, 20, and 60 minutes, respectively.

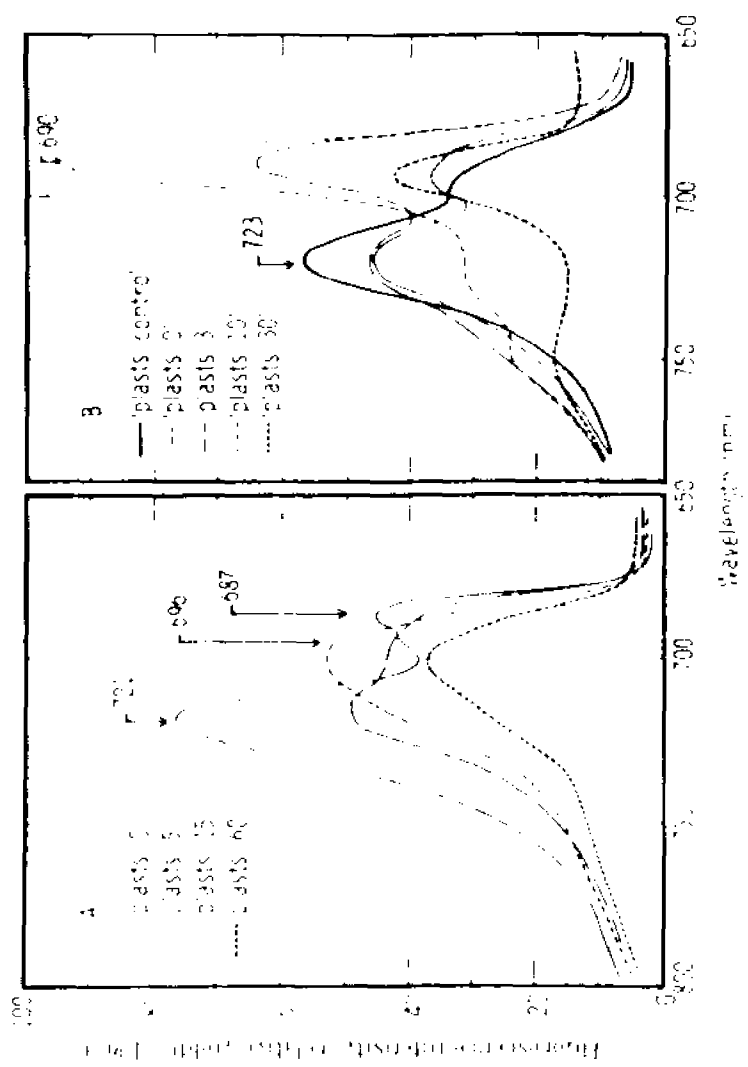


chloroplasts of higher plants also obtain with intact algae cell suspensions of either the green alga C. pyrenoidosa or the red alga P. cruentum (having chlorophyll concentrations of 30 micrograms per ml) were incubated in RLE at 30°C for 120 minutes. No changes were observed in fluorescence emission spectra of these intact algae. If, however, chloroplasts (more correctly - lamellar fragments) from these algae are prepared and incubated in RLE changes such as those seen with higher plant chloroplasts are observed. These time-dependent changes in fluorescence emission are illustrated in Fig. 15. In part A, the effect of RLE on Chlorella is shown; again there is the progression to the one-banded system from the original three-banded one. There is also a slight shift in the emission maximum of the one-banded form, from 696 to 700 nm, with further incubation. In Fig. 15B is shown a fluorescence emission spectrum for Porphyridium chloroplasts incubated in RLE under conditions similar to those for Chlorella. Again there is the decrease in F 735 and increase in F 698. The single resulting band in Porphyridium seems, however, narrower and more intense than that of Chlorella. Also, this predominant single band in Porphyridium exhibits a maximum at 690 nm rather than the usual one at 696 - 698 nm.

In chloroplasts of both Chlorella and Porphyridium, the fluorescence emission changes elicited by RLE proceed at a more rapid rate than those obtained under comparable conditions with chloroplasts from higher plants. The reason(s) for these differences in rate are not fully apparent to me at the present time but perhaps relate to the smaller size of the algal lamellar fragments (which were centrifuged down as a pellet at 7,000 x G for 20 minutes) compared to the chloroplasts of higher plants (which were pelleted at 1,000 x G for 10 minutes).

B. Comparison of Difference Fluorescence Action Spectra with Difference Absorption Spectra for Ricinus Chloroplasts and for Cross Reaction Systems.

Figure 15. Cross reaction systems: A: Chlorella thylakoids suspended in RLE. Fluorescence emission at -196°C , as function of incubation time (at 30°C). Excitation with 436 nm light. B: Porphyridium thylakoids suspended in RLE. Fluorescence emission at -196°C as a function of time of incubation (at 30°C). Excitation with light of 436 nm.



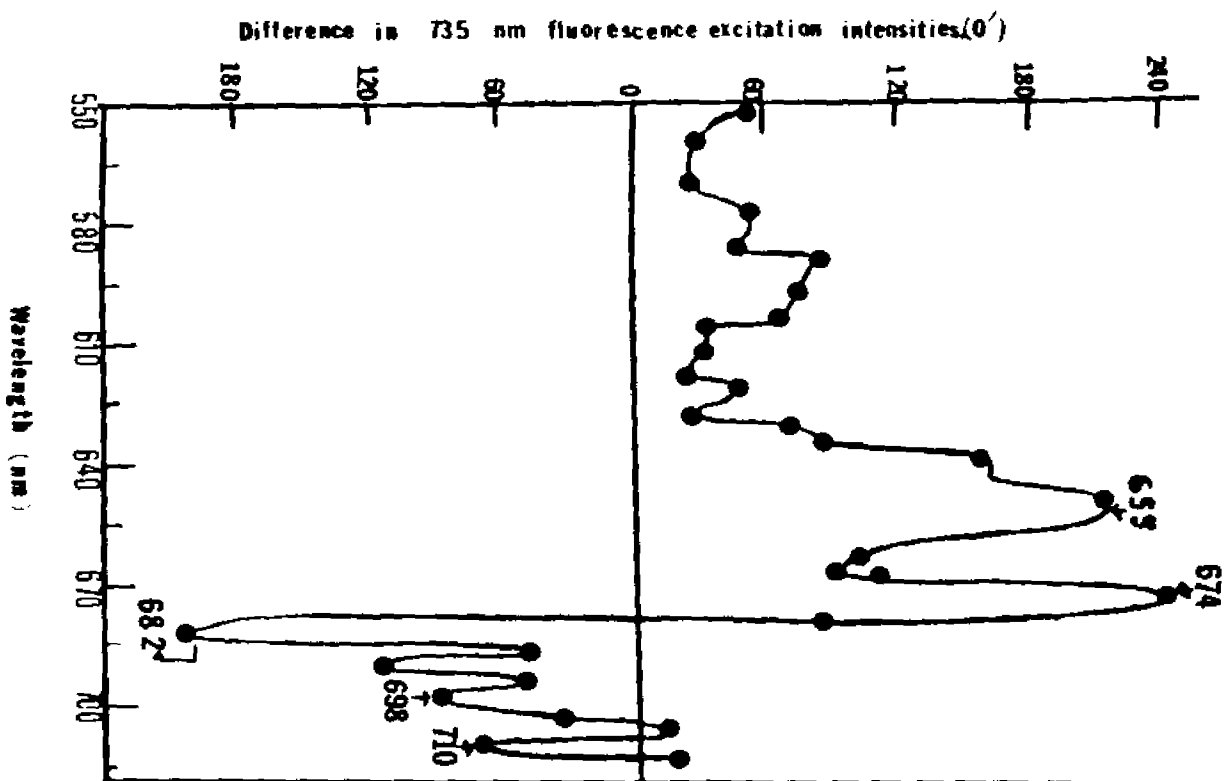
In Fig. 16A is a difference action spectrum for exciting low temperature fluorescence at 735 nm; this spectrum was obtained in the following way: the low temperature action spectrum for exciting fluorescence of control Spinacea chloroplasts has been subtracted from the low temperature fluorescence action spectrum of Spinacea chloroplasts at 0' in RLE at 37°C (see Fig. 14 for the direct fluorescence action spectrum used in this calculation). The following features of Fig. 16 should be noted: there is a strong decrease in the band with maximum at 682 nm, and a slightly larger increase in the band with maximum at 674 nm. The 655 nm band (chlorophyll b) also shows enhanced contribution to emission at 735 nm. It should be kept in mind that under these conditions there is observed in the corresponding emission spectra increase in intensity of both the 698 and 735 nm fluorescence bands. These same phenomena are also observed when iced Ricinus chloroplasts (at 0°C in buffer) are incubated for 5 minutes in a 37°C water bath.

Although it was not possible to determine with precision the changes in fluorescence excitation which occur in the blue region of the spectrum in the case of spinach chloroplasts incubated in RLE (due to strong absorption by crude yellow RLE; see Cohen, Ph. D. thesis, 1969), experiments with Ricinus chloroplasts (suspended in buffer) show that, in addition to the changes observed in the red region of the spectrum, there are increased contributions by carotenoid bands (at 475, 483, and 488 nm).

A comparison can now be made between these data and the corresponding room temperature difference absorption data obtained by subtracting the absorption spectrum of control Spinacea chloroplasts (monitored in a Cary 14 R recording spectrophotometer) from the absorption spectrum obtained at 0' with Spinacea chloroplasts in RLE at 37°C. In this difference absorption curve (Fig. 17), we note that the decreases in O.D. at 685 nm (0.14 O.D. units) and 710 nm (0.04 O.D. units) are larger than the increase at 674 nm

Figure 16. Difference action spectra for exciting low temperature(-196°C) fluorescence at 735 nm from Spinacea chloroplasts:control suspended in Tris-NaCl buffer,pH 7.8 minus chloroplasts suspended in RLE:A at 0 minutes of incubation and B at 45 minutes of incubation at 37°C .Data from Figure 14.

A



B

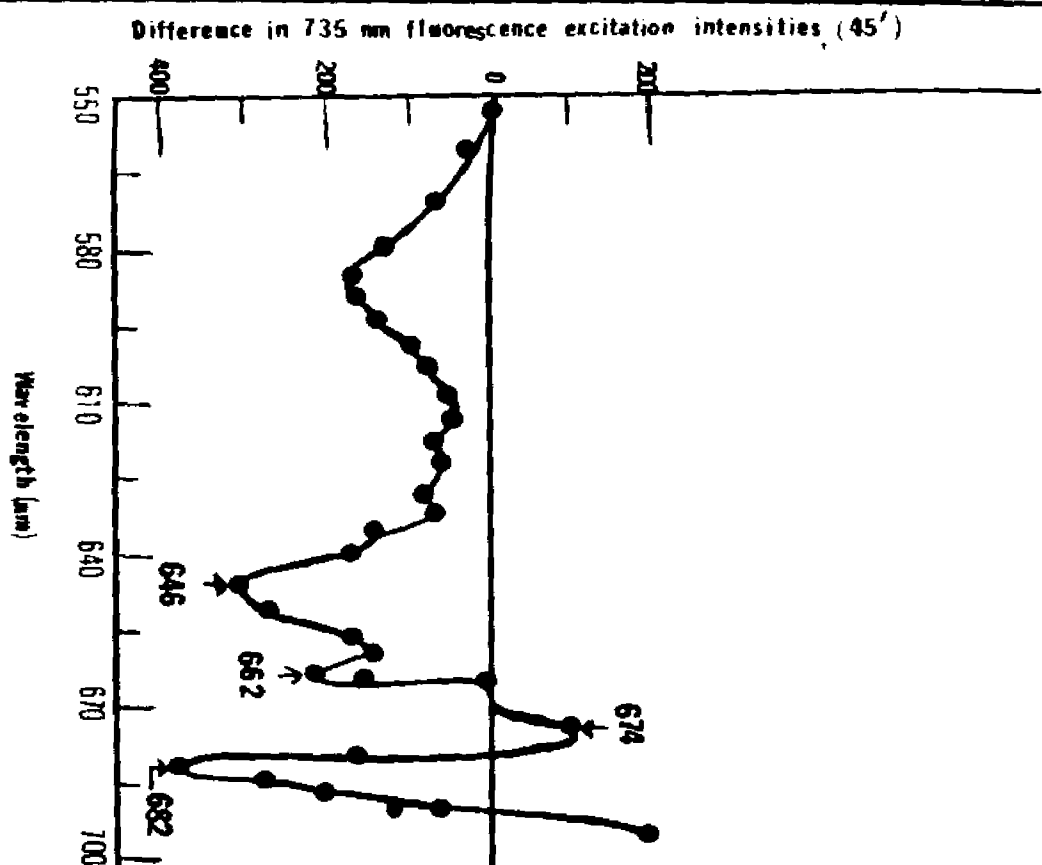
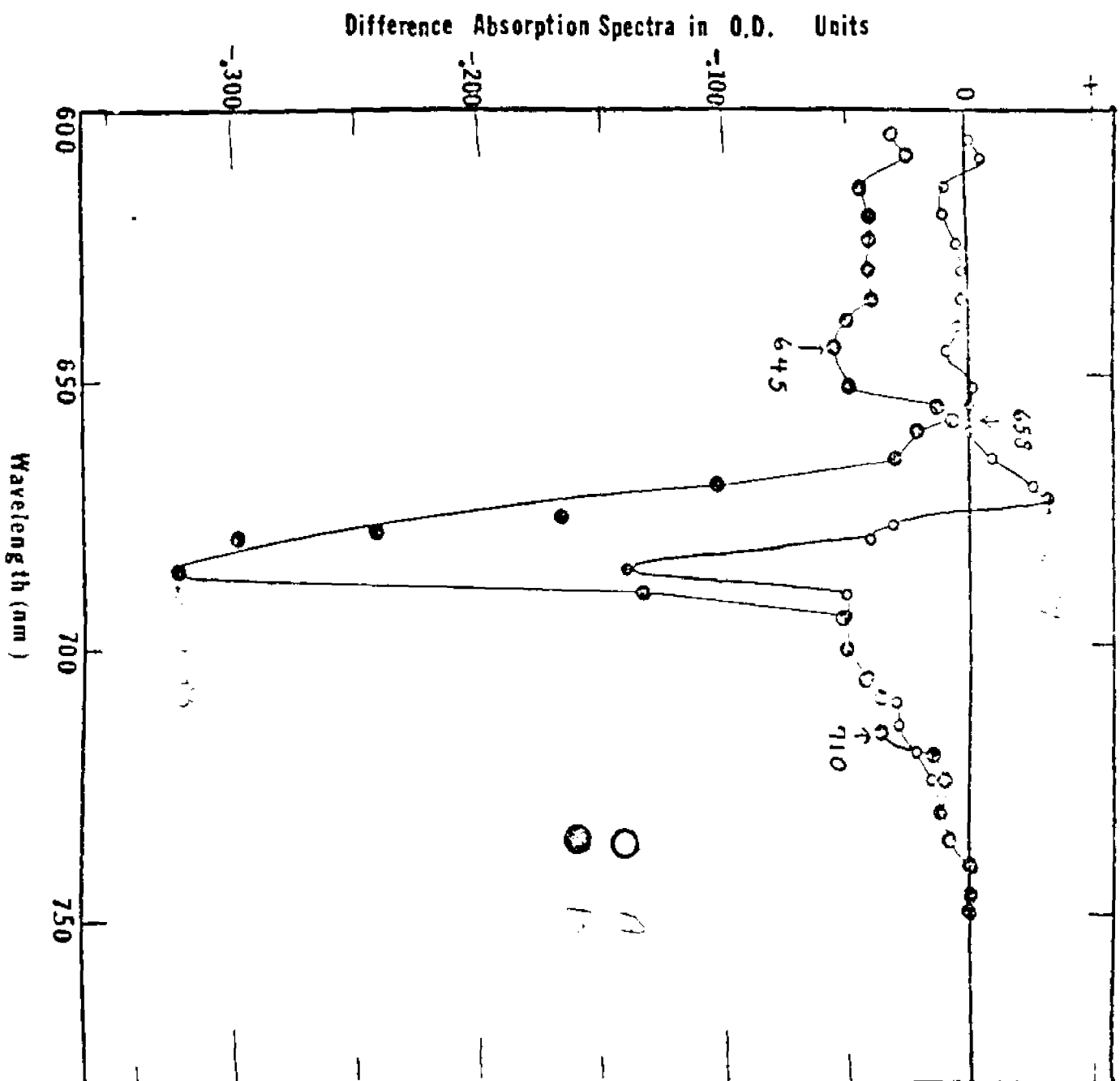


Figure 17. Room temperature difference absorption spectra of Spinacea chloroplasts incubated in RLE at 37°C. Curve A: Spinacea chloroplasts suspended in RLE at 0 minutes - control (Spinacea) chloroplasts (suspended in NaCl Tris-HCl buffer, pH 7.8). Curve B: Spinacea chloroplasts suspended in RLE for 45 minutes - control Spinacea chloroplasts (suspended in NaCl Tris-HCl buffer, pH 7.8).



(0.03 O.D. units). If we keep in mind that the small increase in absorption at 674 nm is occurring while there is a much larger transient increase in the contribution of the 674 nm band to emission at 698 and 735 nm, we must conclude that the fluorescence phenomenon arises only in part from an increase in the form of chlorophyll absorbing at 674 nm. The remaining increase in fluorescence must be due to increased efficiency of energy transfer from the incrementing 674 nm excitation band.

In this same difference absorption spectrum (Fig. 17) we noted just above a rather large decrease at 685 nm; this decrease in the form of chlorophyll absorbing at 685 nm may be sufficient to account for most (if not all) of the reduced efficiency of the 685 nm band in sensitizing fluorescence at 735 nm. It should be recalled however, that during these early stages of incubation, yields of emission at both 698 and 735 nm are increasing. While the enhanced emission at 698 nm may be attributed to both increase in concentration of the 674 nm absorbing form, and increase in efficiency of energy transfer from this form to F 698, the situation in regard to the long wavelength emission band is not so clear. Since data of other workers (see discussion) indicates that the aggregate excitation band at 685 nm contributes strongly to F 735, one would expect that decrease in the 685 nm absorbing form would be accompanied by decrease in intensity of emission at 735 nm. Instead, the opposite is noted in the present work. How then can one explain this seeming paradox? One possible explanation comes to mind, if one looks carefully at the emission spectrum in Fig. 8 - comparing curve A (control) with curve B (5 minutes incubation in RLE). In this comparison, one observes that although the intensity of emission at 735 nm has increased, there has also occurred a short wavelength shift from 738 to 733 nm and a narrowing of the long wavelength band - phenomena possibly due to a conversion of larger

aggregates into smaller ones, the latter emitting at shorter wavelength than the former. It may also be that part of the increase at 735 nm is brought about by enhanced efficiency of transfer from the (intensifying) 674 nm band to F 735 (via F 698). Additionally, in these early stages of incubation, there is enhanced contribution by carotenoids to emission at 735 nm. Any one of these factors, or a combination of them, may explain the above "paradox".

In experiments in which the Aminco-Chance apparatus was used to sequentially determine absorption spectra of spinach chloroplasts during early stages of incubation in RLE at room temperature, it was noted that there were decreases in absorption in the spectral regions around 682 and 705 nm, which were accompanied by absorption increase in the spectral region around 674 nm. When rates of change at specific wavelengths were followed in the early stages of incubation, there were observed a) increases in absorbance from 0.93 O.D. units to 1.03 O.D. units at 674 nm, and b) decreases in absorbance of 0.18 and 0.04 O.D. units (from control values of 0.83 and 0.05) at 682 and 705 nm, respectively. The results in this section suggest that the effect(s) of RLE is to deaggregate chlorophyll, since the regions in which decreases are noted correspond to those at which chlorophyll aggregates absorb (i.e. at 682 - 5 and 705 nm), while the region in which enhancement occurs corresponds to monomer absorption.

With spinach chloroplasts incubated in RLE for longer periods of time (past the stage when the low temperature fluorescence emission spectrum exhibits 1 band with a maximum at 698 nm, i.e. when the total fluorescence yield has begun to decrease) comparisons of difference absorption and difference fluorescence excitation can also be made. In Fig. 16B is seen the low temperature difference action spectrum for exciting fluor-

escence at 735 nm, obtained by subtracting the spectrum of control spinach chloroplasts at 0', 37°C from the spectrum of spinach chloroplasts suspended in RLE for 30' at 37°C. Although with this further incubation there continues to be increase in contribution of the 674 nm band, this increase is much smaller than the concomitantly occurring decrease at 682 nm. That there is decrease in 682 nm absorption (presumably by aggregated species of chlorophyll) without concomitant increase in absorption at 674 nm (i.e. by the monomer) at these later stages of incubation, may be taken to mean that, at this time there may possibly occur not just conversions of aggregate to monomers, but also formation of solubilized chlorophyll or pheophytin (see Discussion and Conclusions). Along with these changes there also occur decreases in the contribution of the 645 and 662 nm bands to emission at 735 nm (later stages of incubation of Ricinus chloroplasts in buffer indicate that there follow, additionally, pronounced decreases in carotenoid bands at 473, 483, and 499 nm).

A comparison of Fig. 16B with the corresponding difference absorption spectrum (Fig. 17B) indicates that, in part, the reduced contribution to fluorescence at 735 nm by the 674 nm excitation band, in these later stages of incubation, is due to decrease in absorption by this (monomeric) form of chlorophyll. (At these later stages of incubation, when the fluorescence yield, i.e. total area under the curve, has become considerably reduced (see Fig. 8), this form of chlorophyll may be altered, or destroyed, or converted into solubilized chlorophyll (see Section IV, dealing with the effects of fatty acids on the circular dichroism of chlorophyll in solution)). The decrease in contribution of the 682 nm band to fluorescence at 735 nm in later stages of incubation seems to be largely (and perhaps completely) due to its destruction, as observed in

the difference absorption spectrum (Fig. 17B).

Although low temperature absorption spectra were not monitored with spinach chloroplasts incubated in RLE, they were monitored with Ricinus chloroplasts incubated in buffer at 37°C for 45 minutes. In Fig. 18 is plotted the low temperature difference absorption spectrum obtained by subtracting the spectrum of Ricinus chloroplasts incubated at 0° for 45 minutes ("control") from the spectrum of Ricinus chloroplasts incubated at 37° for 45 minutes. A comparison of this low temperature difference absorption spectrum with the room temperature difference absorption spectrum of spinach chloroplasts (Fig. 17B) reveals that in both, absorption changes are comparable as regards the 712, 685, and 674 nm bands. (The reader's attention should be drawn to the fact that the method (see Materials and Methods) for making "cuvettes" for low temperature experiments requires normalization, since path length varies from sample to sample. The low temperature difference absorption spectrum in Fig. 18 was normalized at 560 nm - the region of smallest change - and this normalization may explain, in part, the differences seen in this figure and Fig. 17.

IV. ALTERATIONS IN THE SPECTRA OF CHLOROPHYLLOUS SYSTEMS INCUBATED IN THE PRESENCE OF LONG CHAIN UNSATURATED FATTY ACIDS.

Investigations in collaboration with other workers in this laboratory (Brody et al., 1969; Cohen et al., 1969; see also Cohen, Ph. D. dissertation, 1970) have revealed that long chain unsaturated fatty acids can serve as good model systems for the effects of RLE on photosynthetic systems. These effects include changes in system I and system II-associated electron flow (Cohen et al., 1969), fluorescence induction patterns (Brody, 1969), light-induced absorption changes by chlorophylls a_{11} and a_1 (Brody et al., 1971; Brody et al., 1971) and chloroplast ultrastructure (Brody et al., 1969;

Figure 18. Low temperature difference absorption spectrum of Ricinus chloroplasts incubated for 45 minutes (i.e. Ricinus chloroplasts incubated at 37°C ^{minus} Ricinus chloroplasts incubated at 0°C). Curves were normalized at 560 nm before subtraction.



Cohen et al., 1969).

A. Changes in Low Temperature Fluorescence from Chloroplasts of Higher Plants Incubated in the Presence of Linolenic Acid.

Even in very preliminary experiments, I noted that the changes in fluorescence emission which were brought about by fatty acids were almost identical to those brought about by RLE; however, while the spectral changes in the presence of the protein were clearly time-dependent, in the case of fatty acids they were concentration-dependent. Therefore, a study of the dependence of the rate of change in F_{735}/F_{698} (R) as a function of concentration of linolenic acid was made, keeping chlorophyll concentration (in the chloroplasts) constant.

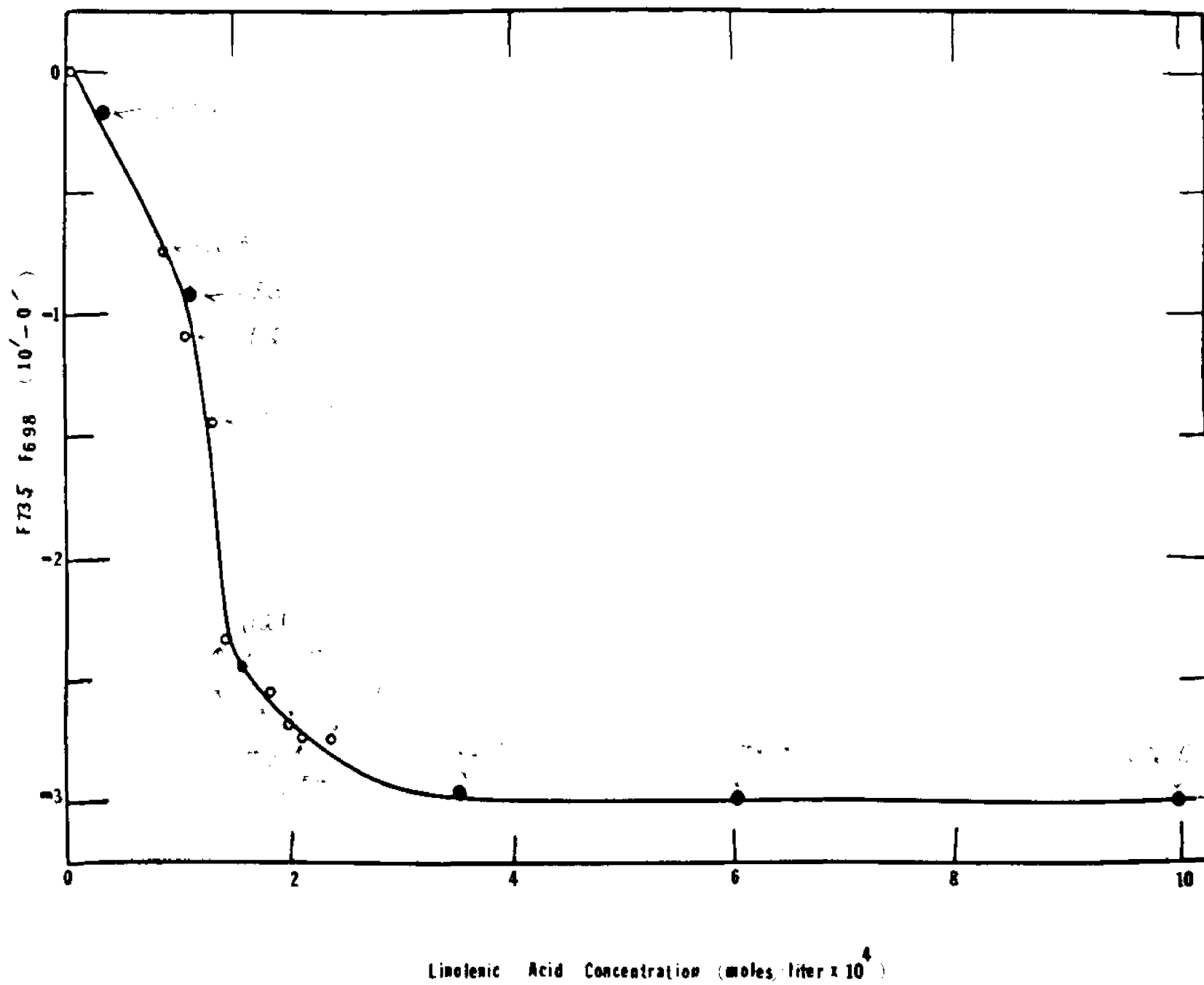
1. CHANGES IN LOW TEMPERATURE FLUORESCENCE EMISSION.

a. Changes in Low Temperature Fluorescence Emission from Chloroplasts of Higher Plants as a Function of Exogenous Linolenic Acid Concentration.

In Fig. 19 is plotted the change in ratio of F_{735}/F_{698} (at 10 minutes of incubation at 30°C minus zero minutes of incubation) as a function of the (molar) concentration of linolenic acid. Chlorophyll (a + b) in these spinach chloroplasts was 13 micrograms per ml. The mole/mole ratio of linolenic acid compared to chlorophyll is indicated for each point on the graph. It can be seen that at FA/Chl ratios between 6.5 and 11, the rate of change of F_{735}/F_{698} is linear with concentration of linolenic acid. Saturation appears to occur at FA/Chl ratios of about 23. From the linear portion of the curve, FA/Chl of 10 was chosen for subsequent experiments, since this ratio gives reasonable rates of change which can conveniently be monitored.

In order to obtain spectra which were relatively free of noise, a higher concentration of chlorophyll was utilized for later experiments.

Figure 19. Spinacea chloroplasts incubated at 30°C. Rate of change (10 minutes - 0 minutes of incubation) of F 735/F 698 (at -196°C) as a function of concentration of exogenous linolenic acid. Blue light (436 nm) used for excitation. Chlorophyll concentration = 1.3×10^{-5} M. Figures in parentheses indicate the mole/mole ratio of linolenic acid to chlorophyll, for each point of the graph. Open circles and closed circles refer to two different sets of experiments.



Since this chlorophyll concentration (in chloroplasts) was chosen to be 30 $\mu\text{g/ml}$, the fatty acid concentration used was $\sim 3 \times 10^{-4} \text{M}$.

b. Fatty Acid-Induced Time-Dependent Changes in the Low Temperature Fluorescence Emission Spectra of Chloroplasts from Higher Plants.

In Fig. 20 are seen the time-dependent changes which occur in the low temperature fluorescence emission spectrum of Spinacea chloroplasts when these are incubated at 30°C in $3.5 \times 10^{-4} \text{M}$ linolenic acid. In the first 5 minutes of incubation there is a transient increase in total fluorescence yield (curve B). With further incubation the now familiar increase in fluorescence at 698 nm and decrease at 735 nm occur (C, D). As can be seen by a comparison of these data with Fig. 8 (Spinacea chloroplasts in RLE), fluorescence emission changes are, for all purposes, identical in the two systems.

1) Effect of an Inorganic Acid on the Low Temperature Fluorescence Emission Spectrum of Spinacea Chloroplasts.

In the above paragraphs, it was observed that exogenous fatty acids exert effects on chloroplasts similar to those induced by RLE. That these effects are not due solely to the acidic properties of these fatty acids (but rather are a consequence of specific action between the fatty acid and the chlorophyll lipoprotein moiety of the chlorophyll membrane) may be deduced from the data given below.

In Fig. 21 is seen the effect of increasing concentrations of HCl on the low temperature fluorescence emission of Spinacea chloroplasts suspended in Tris-NaCl buffer, pH 7.8. (As seen above, the concentration of fatty acids used in the present work fall in the concentration range of 10^{-4} to 10^{-3}M , which yield, respectively, pH's of 7.60 and 7.14 when added to Tris-NaCl buffer of pH 7.8). It may be observed

Figure 20. Fluorescence emission of Spinacea chloroplasts at -196°C . Chloroplasts incubated at 30°C in 3.5×10^{-4} M linolenic acid. Curve A: control Spinacea chloroplasts suspended in NaCl Tris-HCl buffer, pH 7.8; Curves B-D: Spinacea chloroplasts incubated in 3.5×10^{-4} M linolenic acid (in buffer) for 0, 5, and 10 minutes respectively. Chlorophyll concentration - 3×10^{-5} M. Excitation with 436 nm light.

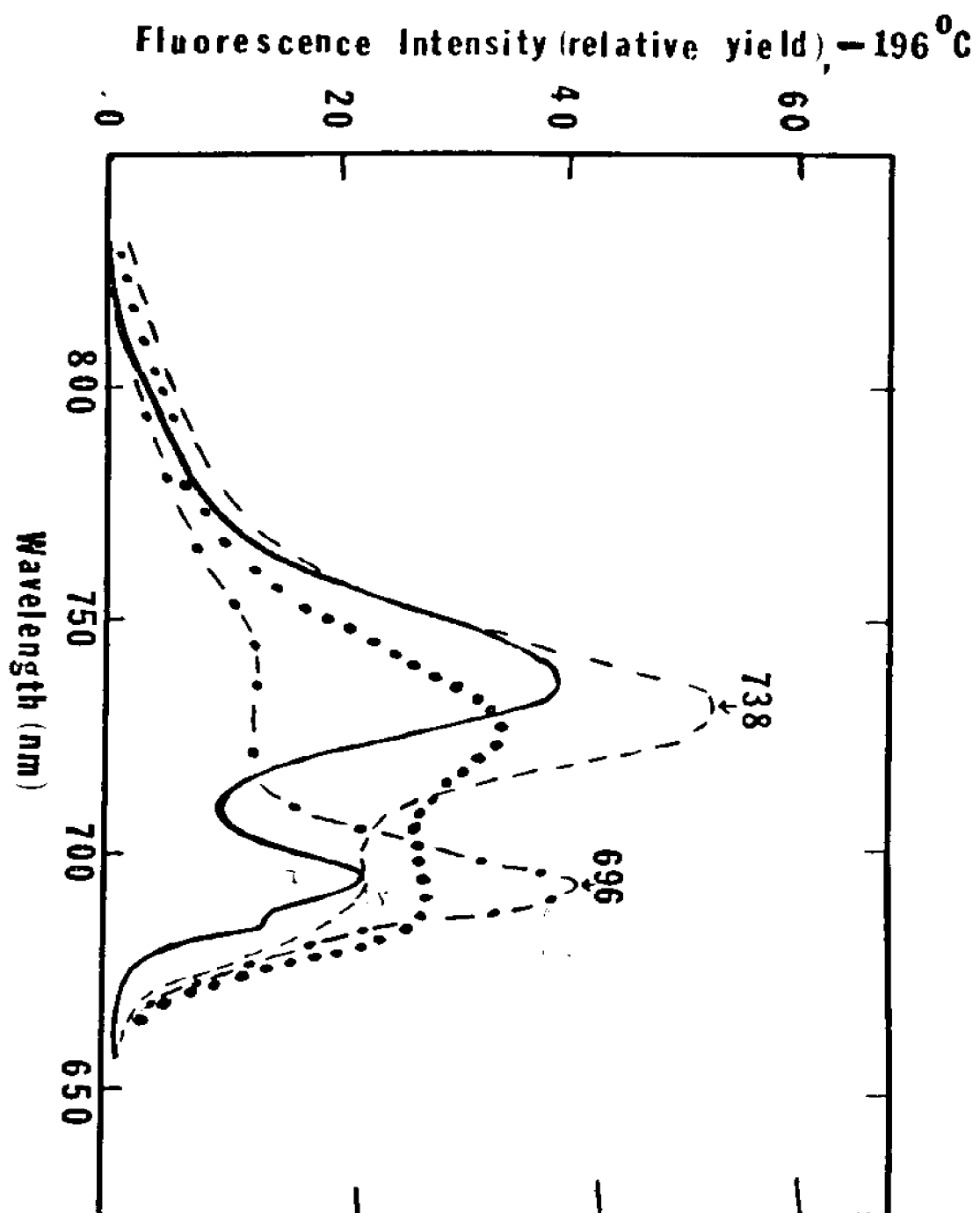
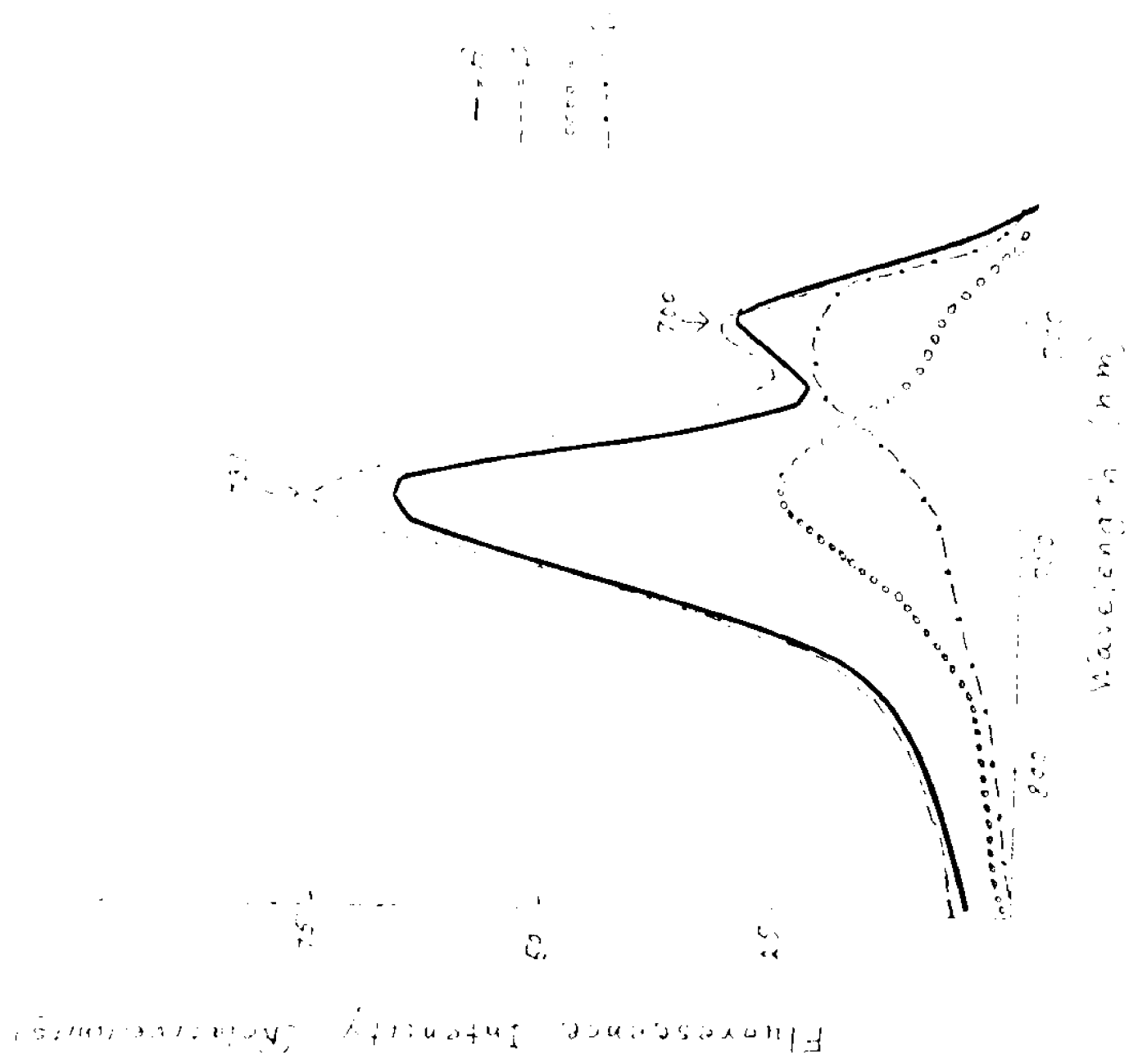


Figure 21. Fluorescence emission of Spinacea chloroplasts at -196°C in the presence of HCl. Chloroplasts suspended in NaCl Tris-HCl buffer, pH 7.8; Curves B - D: Spinacea chloroplasts suspended in buffer to which was added (final molar concentration) 2×10^{-3} M, 2×10^{-2} M, and 2×10^{-1} M HCl, respectively. Chlorophyll concentration = 3×10^{-3} M. Excitation with 436 nm light.



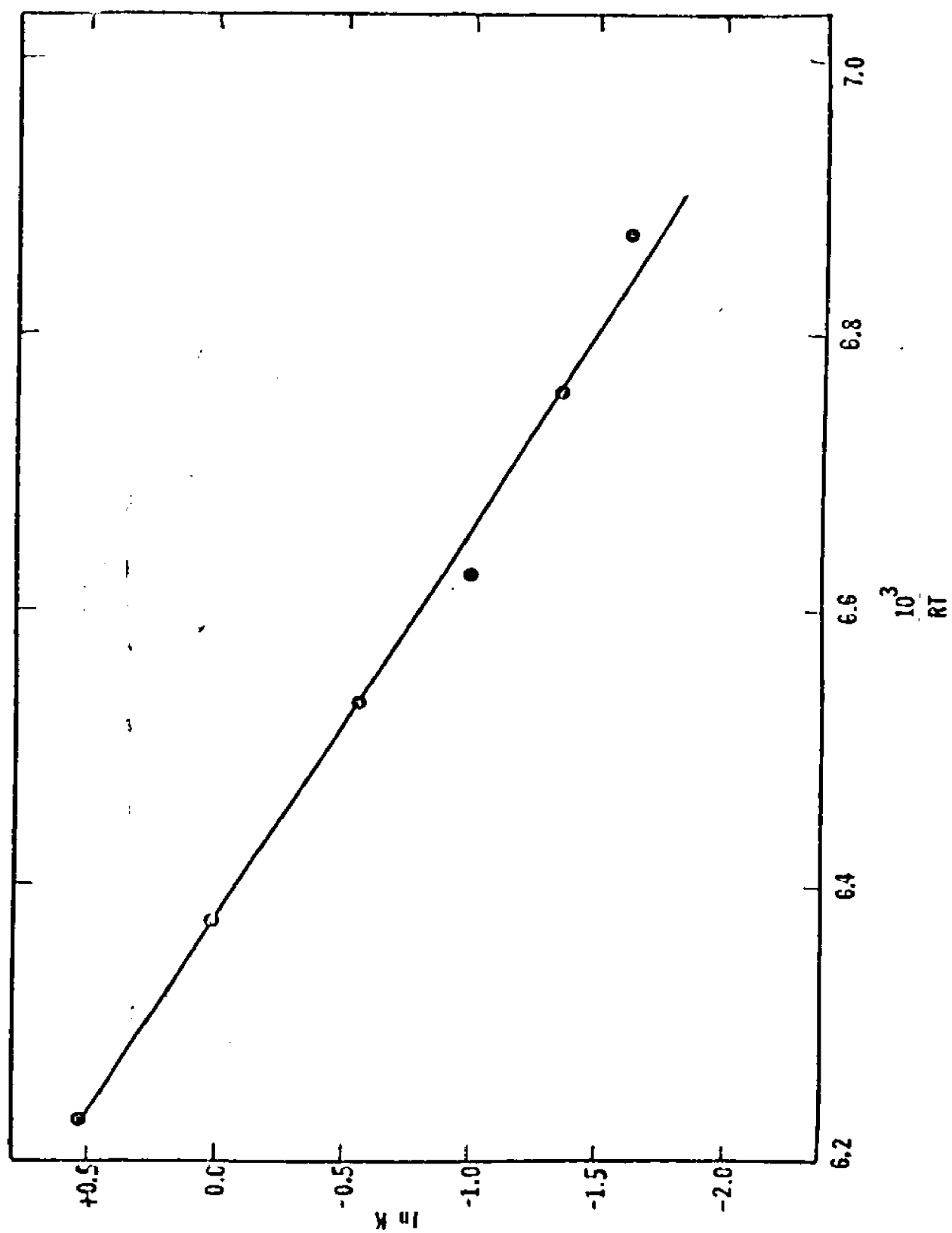
that at an HCl concentration of 2×10^{-3} M (100 X the chlorophyll concentration), incubation of the chloroplast suspension for 1/2 hour, results only in minor changes in the fluorescence emission. This is in contrast to the results obtained with fatty acids, in which incubation, even at a concentration 10 X that of the chlorophyll concentration, results in conversion to the one-banded fluorescence emission form (see Fig. 20). At concentrations of 10^{-2} M HCl (pH 4.0) there is pronounced decrease in fluorescence yield. Finally, at concentrations of $\sim 10^{-1}$ M HCl (pH 3) solubilization of the membrane occurs, and emission is primarily from monomeric chlorophyll (Fig. 21 D). Results similar to these were obtained by Steffen and Calvin (1970) in their studies of the effect of HCl and octanoic acid on the spectral properties of bacterial chromatophores.

c. Changes in Fluorescence Emission Ratios (at 77°K) of Spinacea Chloroplasts Incubated in the Presence of Linolenic Acid as Functions of Time and Temperature of Incubation.

In Fig. 12 were shown the time-dependent changes occurring in control Spinacea chloroplasts incubated in NaCl-Tris, pH 7.8, at temperature ranging from 0° to 45°C for up to 90 minutes. In subsequent experiments, induced changes in emission ratios were corrected for changes observed in these controls.

A series of experiments were made in which linolenic acid was added to Spinacea chloroplasts (FA/Chl ~ 10) and incubation was carried out at several different temperatures (in the range 0° to 45°C). The change in F 735/F 698 (at 10 minutes minus zero minutes incubation) for these temperatures was plotted in terms of the Arrhenius equation (see Fig. 22). This Arrhenius plot turns out to be similar to those for Ricinus chloroplasts in incubation medium and Spinacea chloroplasts in RLE. In all

Figure 22. Spinacea chloroplasts incubated in 3.5×10^{-4} M linolenic acid: Arrhenius plot ($\ln K$) of F 735/F 698 (at -196°C) at 10 minutes 0 minutes of incubation.



three cases, "an energy of activation of 8 ± 0.1 Kcal/reaction rate was obtained from the slope of the curve.

In Fig. 23 is shown the rate of change of F 698, F 735 and also total fluorescence (area under the curve - see Materials and Methods section VII-C) as a function of time of incubation of spinach chloroplasts (at 45°C) in the presence of linolenic acid. Under these conditions, five minutes of incubation has already led to conversion to the single-banded spectrum, with maximum at 700 nm. Although after the conversion, the decrease in total fluorescence yield becomes very apparent, it can be shown that the changes in the area under the fluorescence emission curve occur continuously throughout the incubation period and are only weakly related to the change in F 735/F 698. It can be observed that, although the rates of change for the increase in F 698 and decrease in F 735 with time are very similar, the rate of change of the total area under the curve is different from either of these and, indeed, increases very rapidly with time. (That the diminution in total fluorescence yield is related to actual disruption of intact thylakoids will be considered below, in the discussion.)

2. CHANGES IN LOW TEMPERATURE FLUORESCENCE EXCITATION OF CHLOROPLASTS FROM HIGHER PLANTS INCUBATED IN THE PRESENCE OF LINOLENIC ACID.

In Fig. 24 are shown the time-dependent changes in the low temperature fluorescence excitation spectra of Spinacea chloroplasts incubated in a (final) concentration of 3.5×10^{-4} M linolenic acid in NaCl-Tris buffer, pH 7.8. These spectra were measured with the same material as that used for obtaining the fluorescence emission spectra given above in Fig. 20. In the control (Fig. 20A), there are contributions by both the 682 nm and 674 nm bands to the action spectrum for exciting fluorescence at 735 nm. In the spectrum for fluorescence

Figure 23. Rate of change of F 698, F 635 and total fluorescence emission as a function of time of incubation of Spinacea chloroplasts (at 45°C) in the presence of linolenic acid. Curve A: rate of change of F 698; Curve B: rate of change of F 735; Curve C: rate of change of total area under the fluorescence emission envelope. Chlorophyll concentration = 3×10^{-5} M. Linolenic acid concentration = 5×10^{-5} M.

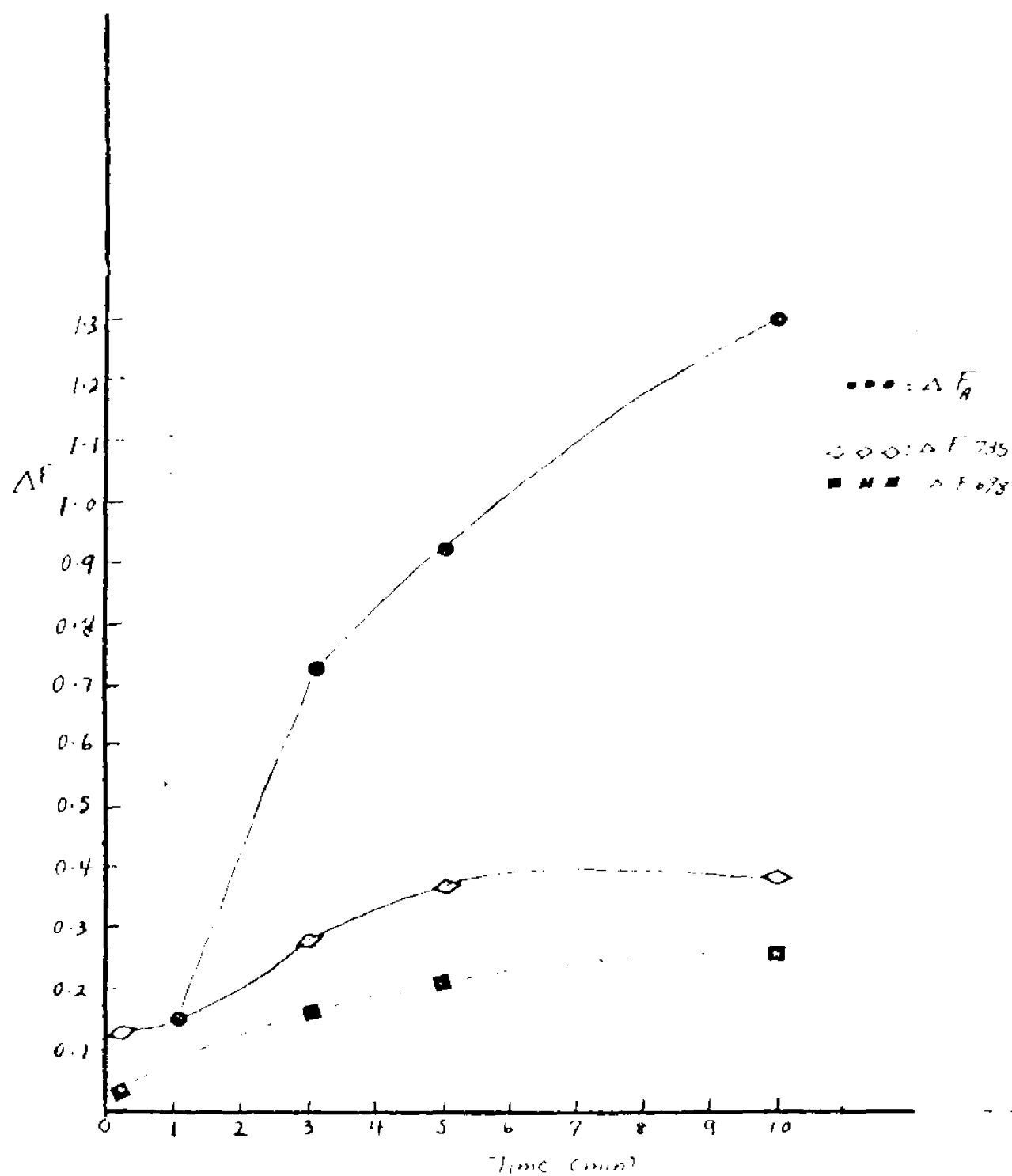
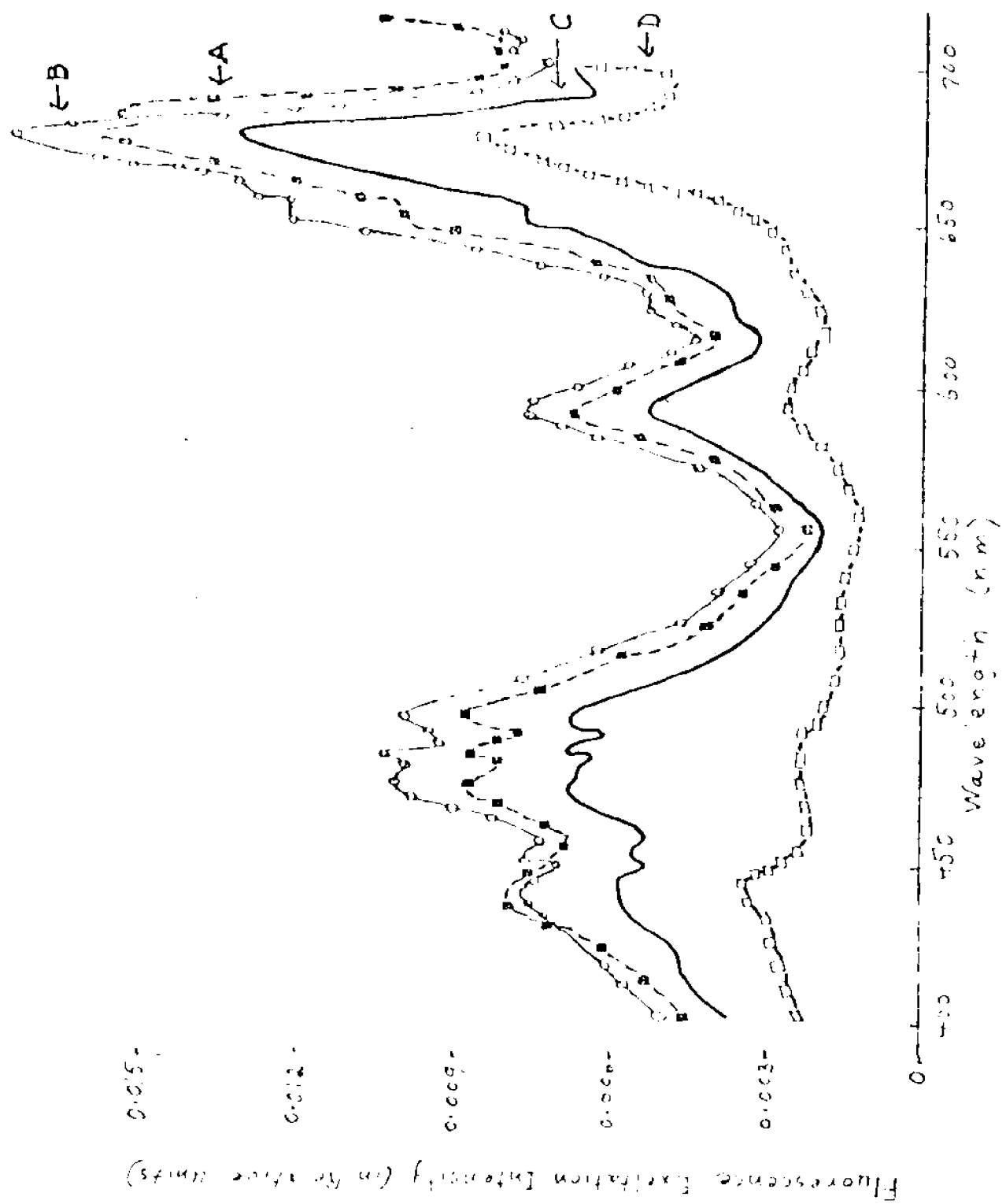


Figure 24. Action spectrum for exciting fluorescence at 735 nm from Spinacea chloroplasts at -196°C . Chloroplasts incubated at 30°C in 3.5×10^{-4} M linolenic acid. Conditions are the same as in Figure 20.



excitation at 0' (i.e. the spectrum examined immediately* upon addition of fatty acid) these effects intensify and are further accompanied by the disappearance of the 705 nm band. All these changes were observed previously - when Ricinus chloroplasts were incubated in NaCl-Tris buffer (refer back to Fig. 7) or when Spinacea chloroplasts were incubated in RLE (see Fig. 14).

Changes in fluorescence emission and fluorescence excitation of chloroplasts from other higher plants (i.e. Raphanus), which occur in the presence of either linolenic or ricinolenic acid ** are comparable to those observed here for Spinacea chloroplasts.

B. Changes in Absorption of Chloroplasts from Spinacea Incubated in the Presence of Linolenic Acid.

From the above paragraphs (IV A 1 and 2; see also Cohen, 1970, Ph. D. thesis; Cohen et al., 1969) it may be concluded that long chain unsaturated fatty acids serve as good model systems for the action of RLE as far as low temperature fluorescence emission and excitation changes are concerned. Since the instantaneous changes in room temperature absorption resulting from incubation in RLE could be followed with ease using the Aminco-Chance spectrophotometer, similar measurements were made with Spinacea chloroplasts incubated in linolenic acid. During the first 60 seconds after introduction of linolenic acid to the chloroplast suspension (chlorophyll content of 20 micrograms of chlorophyll/ml) there is an (110%) increase in absorption at 674 nm of 0.006 O.D. units and

* The time required from addition of fatty acid to final freezing of the experimental sample was in the order of 30 seconds - this is referred to a zero time.

** In the case of ricinoleic acid, the ratio of FA/Chl necessary to bring about changes comparable to those observed in the case of linolenic acid was 20/1, i.e. twice as much ricinoleic acid as linolenic acid was needed in the median range of activity.

decreases in absorption at 682 nm (20%) and 705 nm (20%) of 0.002 and 0.005 O.D. units, respectively.* It can therefore be seen, that, as in the case of spinach chloroplasts suspended in RLE introduction of linolenic acid results in changes in absorption indicative of conversion of aggregated forms of chlorophyll (absorbing at 682 and 705 nm) into monomeric forms (absorbing at 674 nm).

C. Linolenic Acid-Induced Changes in Circular Dichroism of Chlorophyllous Systems** of Increasing Structural Complexity.

1. A COMPARISON OF RLE-INDUCED AND LINOLENIC ACID-INDUCED CHANGES ON THE C.D. OF DIGITONIN-ISOLATED SUBCHLOROPLAST PARTICLES CONTAINING SYSTEMS I AND II.

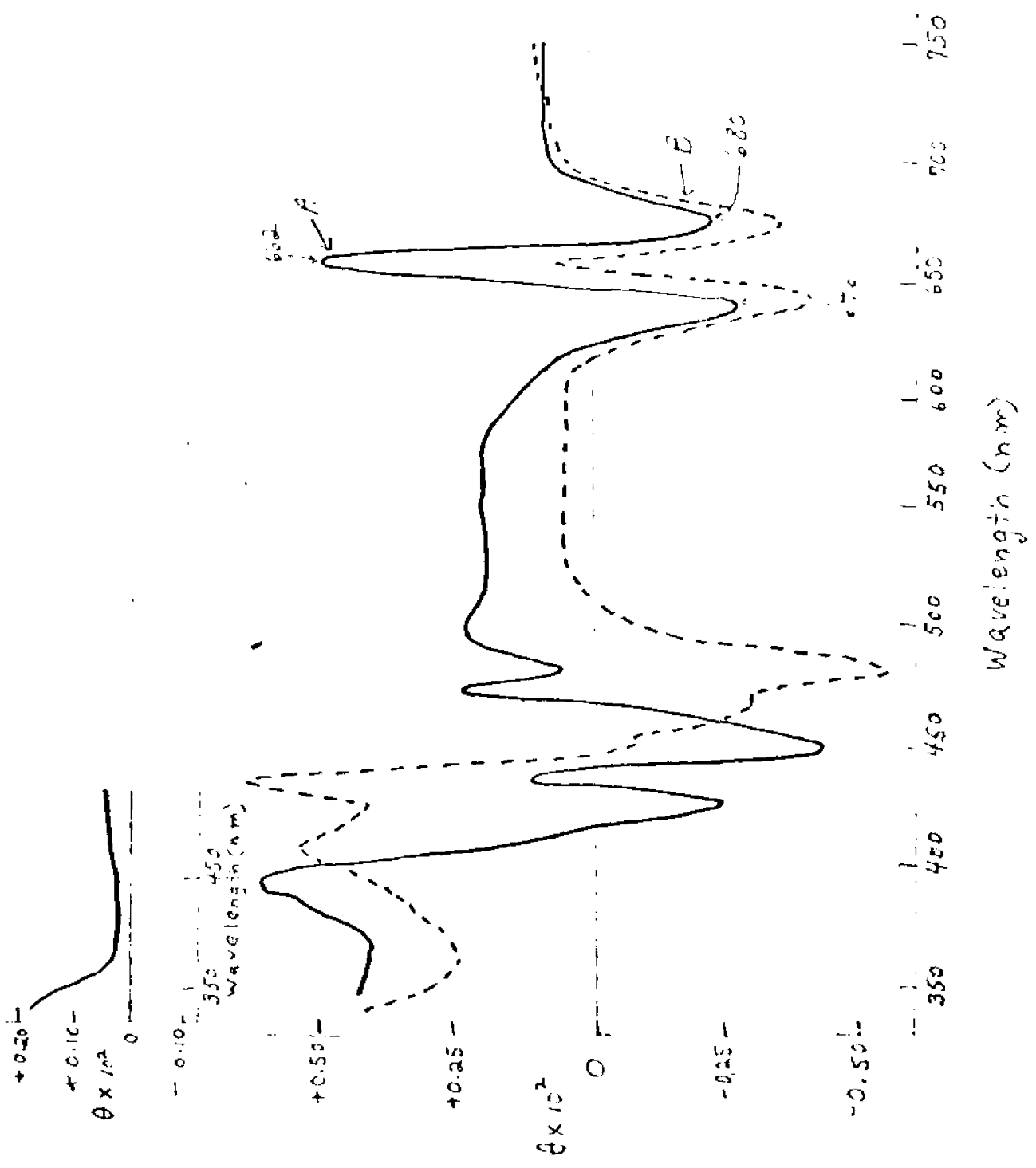
The absorption and fluorescence emission and excitation data obtained with chlorophyll-containing systems incubated in RLE leads to one possible interpretation that one action of RLE is to cause de-aggregation of chlorophyll via fatty acids liberated from the chloroplast membrane, i.e. RLE might have a proteolytic or lipolytic effect on chloroplasts, with subsequent liberation of fatty acids. This would be one possible explanation for the similar effects seen on chlorophyllous systems in the presence of either RLE or fatty acids.

Since Dratz (1966) and Houssier and Sauer (1970) had shown that aggregate and monomer bands of chlorophyll in vivo and in vitro, could be resolved separately in circular dichroism spectra, I turned to the use of this technique to determine whether the supposition about the changes in fluorescence and absorption having their origin in deaggre-

* The O.D.'s of control chloroplasts at 674, 682 and 705 nm were 0.86, 0.71, and 0.03 respectively.

** derived from spinach leaves

Figure 25. Circular dichroism(C.D.)spectra of digitonin-isolated sub-chloroplast particles containing systems I and II incubated at 30°C. Curve A: control(particles suspended in KCl-Tris buffer,pH 7.8). Curve B:particles incubated in RLE for thirty minutes.Chlorophyll concentration = 1×10^{-5} M. Cuvette path length = 1 cm. Insert to Figure 25:C.D. spectrum of RLE,cuvette path length = 1 cm.

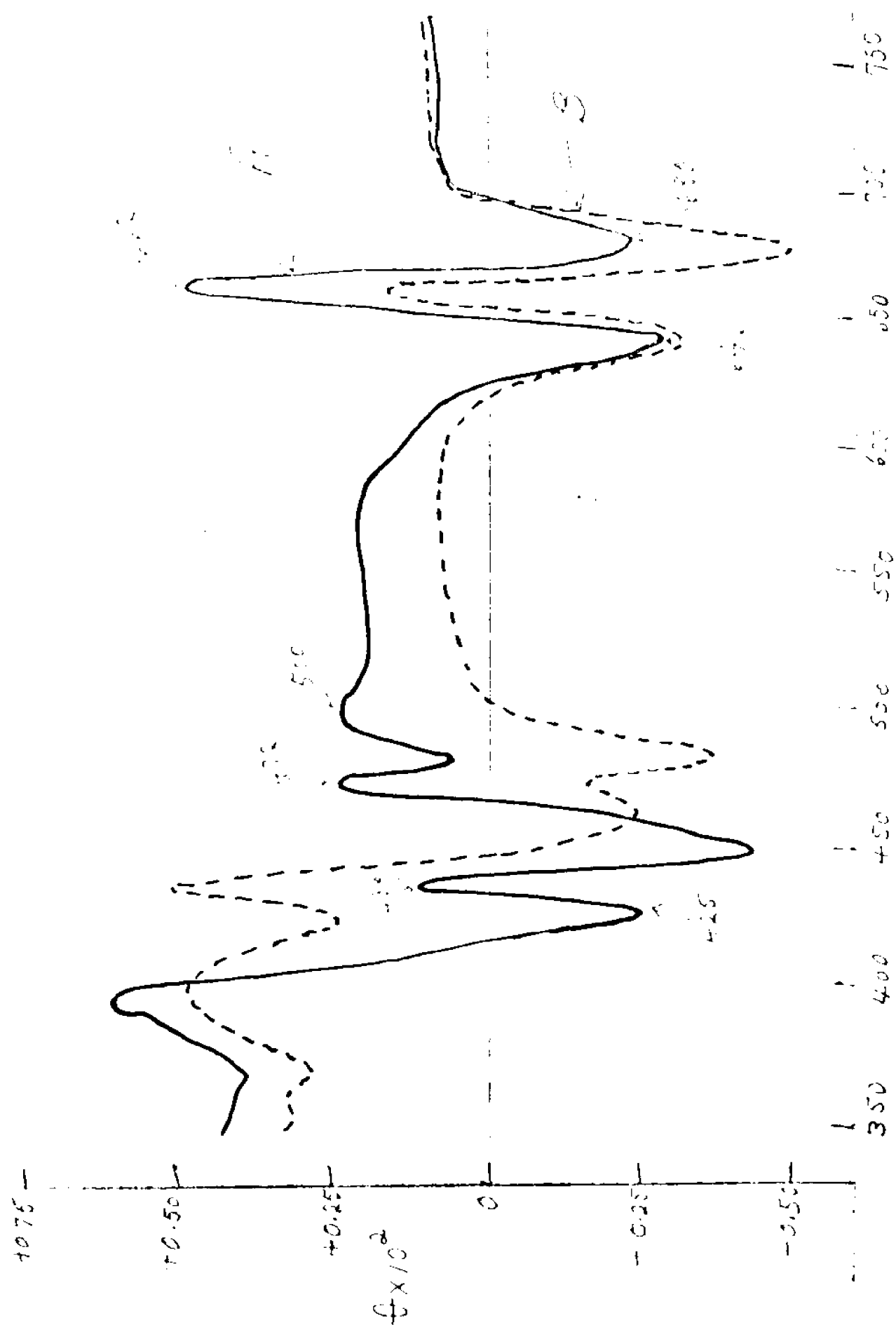


gation is a tenable one. In Fig. 25 is shown the C.D. spectrum of digitonin-isolated subchloroplast particles containing systems I and II, suspended in KCl, Tris buffer, pH 7.8. In the control are observed chlorophyll a dimer bands at 679 and 660 nm (as per Dratz et al., 1966). Upon incubation in RLE for 30 minutes at 30°C, changes in intensities of these two bands occur. These changes continue with further incubation (for one hour) in the presence of RLE. The contribution of RLE, itself, to C.D. structure at wavelengths above 425 - 430 nm is negligible (see insert to Fig. 25).

In Fig. 26 may be seen the effects of fatty acids (linolenic acid) on the C.D. structure of digitonin-isolated particles containing systems I and II. Note that the changes which occur in the presence of exogenous linolenic acid are the same as those observed with RLE, i.e. the former also act as good model systems for the action of RLE in regard to circular dichroism changes. I decided to continue my studies using fatty acids rather than RLE, for a number of reasons. One particular advantage of using fatty acids as model systems for the action of RLE is that the amount of "active" protein (see page 38 above) remains unknown (at the time of this writing, the active protein is still incompletely characterized). The molecular weights of the commercially-obtained fatty acids are known, and therefore it is possible to express the data obtained in the present work in terms of mole/mole ratios of fatty acids to chlorophyll.

Initial C.D. work (with both control spinach chloroplasts and fatty acid-treated spinach chloroplasts) convinced me that, in order to understand these intricate spectra, it would be necessary to investigate the simplest possible systems, i.e. chlorophyll in solution, and then work my way up through more complex systems - such as subchloroplast particles - finally to intact chloroplasts. A fortuitous consequence of this system-

Figure 26. Circular dichroism(C.D.)spectra of digitonin-isolated sub-chloroplast particles containing systems I and II, incubated at 30⁰C. Curve A: control.Curve B:particles incubated in 2×10^{-4} M linolenic acid for thirty minutes.Chlorophyll concentration = 1×10^{-5} M,Cuvette path length = 1 cm.



atic study was the opportunity to interpret some of the C.D. bands of the more structurally complex systems in terms of their occurrence in the simpler systems (i.e. chlorophyll in solution). Another outcome of this systematic investigation was the recognition that deaggregation can occur by two different mechanisms: one, by direct action on the chromophore; and, two, by an indirect (preliminary) action on the (chromophore-bearing) protein.

2. INFLUENCE OF LINOLENIC ACID ON THE STATES OF CHLOROPHYLL IN SYSTEMS HAVING A LOW DEGREE OF ORGANIZATION.

a. Effect of Linolenic Acid on Chlorophyll a in Solution.

1) Effect of Ethanol on the State of Chlorophyll in Solution.

In Fig. 27 A (control) is shown the C.D. spectrum of 10^{-3} M chlorophyll in CCl_4 . In this control, there is seen a large positive band with maximum at 676 nm and a small negative band with maximum at 656 nm. These two bands were assigned the status of a doublet dimer of chlorophyll a* (Dratz, 1966; Houssier and Sauer, 1970). At the chlorophyll concentration used in this experiment, up to 90% of the total chlorophyll may be in the dimeric form (Brody and Broyde, 1968).

If 10^{-3} M chlorophyll a is dissolved in the solvent pair 60% hexane - 40% CCl_4 , there are observed two additional bands in the C.D. spectrum. Based upon their work with I.R. and N.M.R., Katz et al. (1963 a and b) concluded that solvent pairs such as hexane - CCl_4 favor aggregates of higher order than dimers. The two additional bands seen in the C.D. of this solvent pair (see Fig. 28 A) consist of a small, positive band at 760 nm and a very large negative band at 740 nm. I have tenta-

* The assignment of the 656 - 678 nm bands to a double dimer is based upon (1) the similarity of behavior of these bands to perturbing forces (i.e. deaggregation by ethanol; see Houssier and Sauer, 1970) and (2) the fact that the cross-over point between these bands (i.e. the zero point) occur near the red absorption maximum of dimeric chlorophyll.

Figure 27. C.D. spectra of a) chlorophyll a dissolved in CCl_4 (solid line) and b) chlorophyll a dissolved in CCl_4 to which had been added 0.02% ethanol (broken line). Chlorophyll concentration = 3×10^{-3} M; cuvette path length = 0.005 cm. Insert: C.D. spectrum of chlorophyll a dissolved in ethanol. Chlorophyll concentration = 3×10^{-3} M; cuvette path length = 0.005 cm.

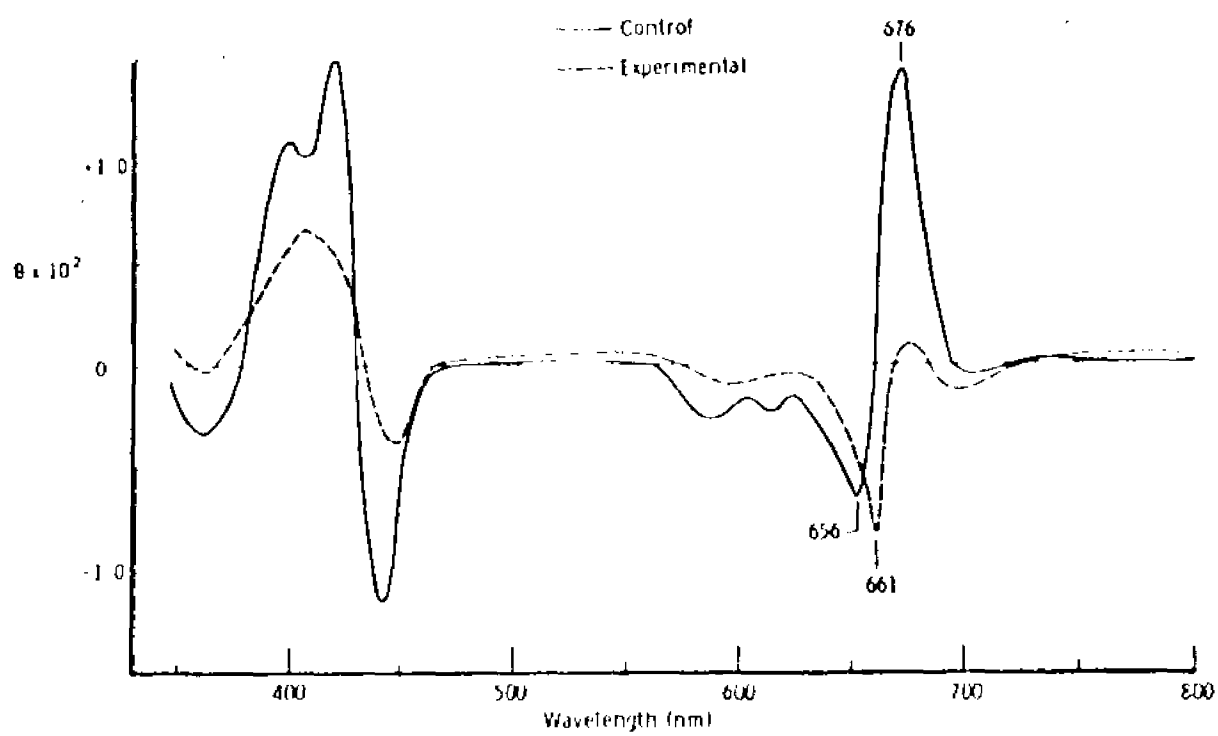
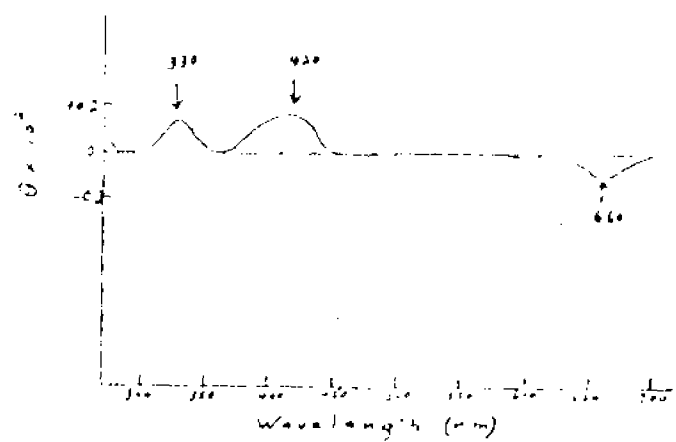
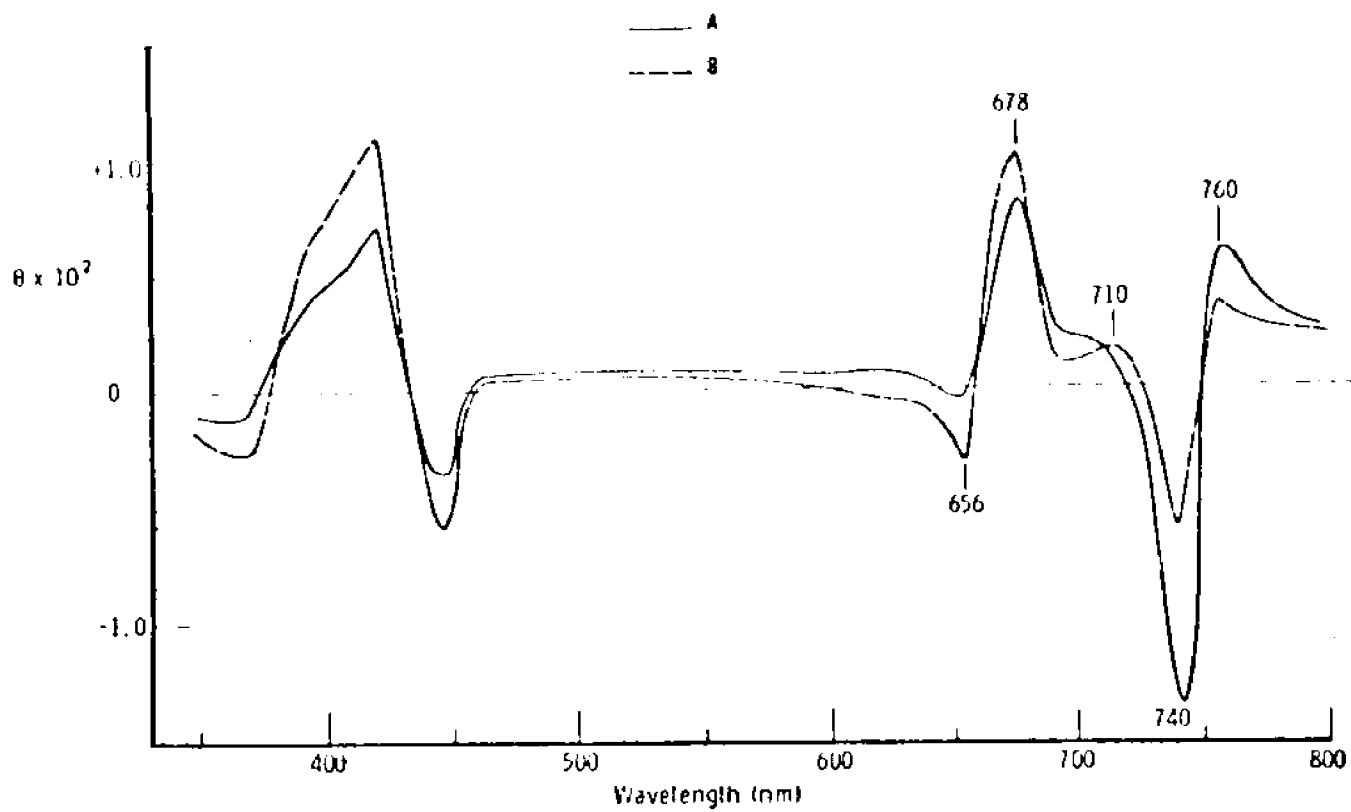


Figure 28. C.D. spectra of a) chlorophyll a dissolved in 40% CCl₄-60% hexane (solid line) and b) chlorophyll a dissolved in 40% CCl₄-60% hexane, to which had been added 0.02% ethanol (broken line). Chlorophyll concentration = 3×10^{-3} M; cuvette path length ~ 0.005 cm.



tively assumed that these two bands represent the doublet of a highly aggregated form of chlorophyll. If one compares the C.D. spectra obtained here with those obtained by Dratz et al. (1966) for microcrystals of chlorophyll a in isooctane, one notes that, in the latter, there is a large negative band at 761 nm and a small positive band at 718 nm. In addition to the dimer doublet bands and these long wavelength bands at 740 and 760 nm, there can also be seen in the control, a shoulder at around 700 nm. In analogy with the results of Dratz et al., we may assign the 700 nm band to the vibrational band of chlorophyll a aggregates and the 750 - 760 nm bands to the actual C.D. absorption of these aggregates. Although Dratz et al. did not observe a positive band at wavelengths greater than 751 nm, it is possible that any positive absorption could not be readily observed since even in our spectra the positive 760 nm peak is barely discernible. Therefore, the 10 nm shift in wavelength (from 740 to 750 nm) could be explained if we presume that the chlorophyll in CCl_4 - hexane is highly aggregated and approaches the size of the microcrystals as seen in the spectra of Dratz et al. (1966).

If one compares absorption spectra of chlorophyll dissolved in these two solvent systems, one notes that chlorophyll a in CCl_4 exhibits a large band with maximum at 668 nm (see Fig. 29 A) while in the case of chlorophyll a dissolved in the solvent pair (see Fig. 30 A) the peak (at 667 nm) is much reduced, and an additional, small, absorption band with maximum at around 745 nm is present.

If one adds 0.02% ethanol to chlorophyll a dissolved in CCl_4 , one observes in the C.D. spectrum (Fig. 28 B) a large decrease in the positive band with maximum at 678 nm. There is an increase in negative ellipticity (120%) and a shift in the negative maximum from 656 to 661 nm. This single negative peak corresponds to the C.D. absorption maximum of the monomeric form of chlorophyll a - as shown by Houssier and Sauer, 1970 -

Figure 29. Absorption spectra of samples shown in Figure 27.

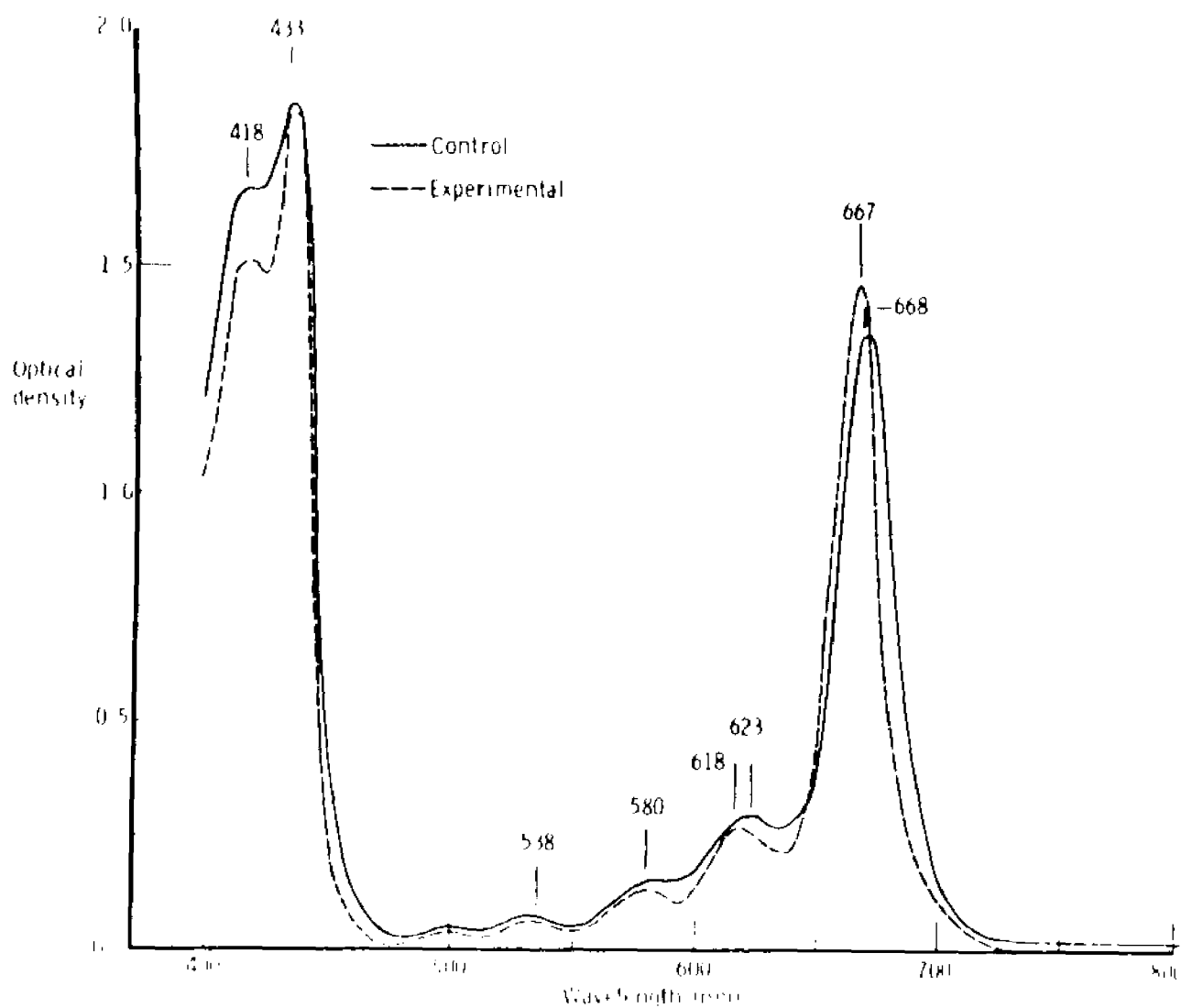
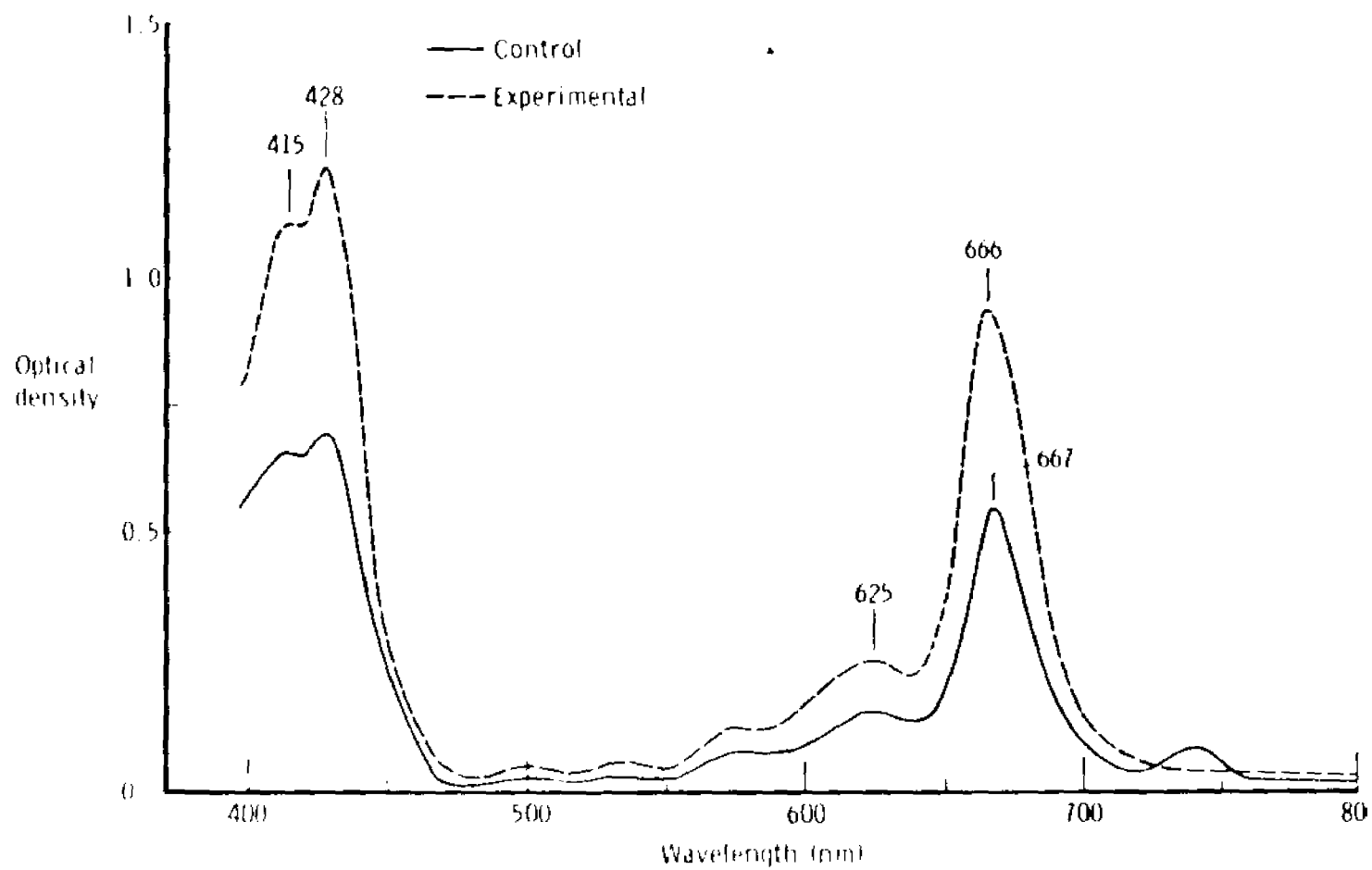


Figure 30. Absorption spectra of samples shown in Figure 28.



for chlorophyll a dissolved in solvents such as ether (in which monomer chlorophyll predominates). See also insert to Fig. 27, in which is shown the C.D. of 10^{-3} M chlorophyll a in ethanol (in which monomeric chlorophyll is the predominant form). That ethanol can cause deaggregation of chlorophyll was shown by Houssier and Sauer (op. cit.) and also in the I.R. and N.M.R. work of Katz et al (1963 a and b), both groups having used ethanol as concentrations an order of magnitude larger than used in the present work*. In the absorption spectrum of chlorophyll a dissolved in CCl_4 to which 0.02 % ethanol was added (i.e. 5×10^{-3} M ethanol), there is seen (compared to the control) an increase near 667 nm (the monomer band) and a decrease in absorption near 682 nm (see Fig. 29 B). The reason that there is residual absorption near 682 nm in the (ordinary) absorption spectrum, while there appears to be total deaggregation in the monomeric form, as seen in the C.D. of Fig. 27 B could be attributed to contribution by the monomer to absorption at that wavelength (Sauer et al., 1969).

If ethanol at a concentration of 0.02 % is introduced into the solvent pair 60 % hexane-40 % CCl_4 , containing 10^{-3} M chlorophyll a, there are observed in the C.D. (see Fig. 28 B) in addition to the small shift in the vibrational band from 710 to 700 nm, decreases in the bands corresponding to the highly aggregated form (at 760 and 740 nm) with concomitant increases in the dimer doublet absorption at 656 and 678 nm. In the corresponding absorption spectrum, there is observed (compared to the control), a disappearance of the 745 nm peak accompanied by marked increase in absorption at 666 nm (see Fig. 30 A and B). Therefore, ethanol has the

* In the present work, we did not take special care to ensure that the reagents used (CCl_4 and hexane) and chlorophyll a were completely free of water, in contrast to the procedure of Houssier and Sauer (op. cit.). Since Katz et al. (1968) have shown that the presence of water can influence the state of chlorophyll aggregation, it is possible that deaggregation could occur at lower ethanol concentrations in the presence of trace amounts of water.

ability to cause conversion of highly aggregated chlorophyll to chlorophyll dimers.

It has been assumed in the present work, that when effects similar to those brought about by ethanol on C.D. and direct absorption are produced with other reagents, they may also be attributed to deaggregation.

2) Effect of Linolenic Acid on the States of Chlorophyll in Solution.

Upon addition of 1.3×10^{-3} M linolenic acid to chlorophyll a in CCl_4 (i.e. a mole/mole ratio of 1:2), there is seen in the C.D. spectrum a strong decrease at 678 nm (see Fig. 31 B). However, in contrast to the results obtained when ethanol was added to chlorophyll in CCl_4 (at a mole/mole ratio of 2:1), there is no increase in the negative C.D. band, but rather a slight decrease at 656 nm. Although in the corresponding absorption spectrum (Fig. 32 B) there is decrease at 682 nm (chlorophyll dimer) upon addition of fatty acid, there is also a decrease in absorption at 667 nm (monomer) which is accompanied by the appearance of pheophytin bands with maxima at 508 and 538 nm. The addition of ethanol, as seen above, did not result in pheophytin formation but only in deaggregation of chlorophyll.

If one examines I.R. spectra of (a) chlorophyll a in CCl_4 , (b) chlorophyll a in CCl_4 to which had been added 0.02 % ethanol and (c) chlorophyll a in CCl_4 to which had been added 1.3×10^{-3} M linolenic acid (see Fig. 33 a, b, and c), one observes the following: the 1650 cm^{-1} band, which has been attributed to the dimeric form of chlorophyll (by Katz et al. in their I.R. work of 1963) disappears upon addition of either ethanol or linolenic acid. In spectrum (b) (addition of ethanol to chlorophyll a in CCl_4) it can be clearly seen that concomitant with the disappearance of the 1650 cm^{-1} band, there is increase in absorption

Figure 31. C.D. spectra of a) chlorophyll a dissolved in CCl_4 (solid line) and b) chlorophyll a dissolved in CCl_4 , to which had been added 1.3×10^{-3} M linolenic acid (broken line). Chlorophyll concentration = 2.5×10^{-3} M; cuvette path length = 0.005 cm.

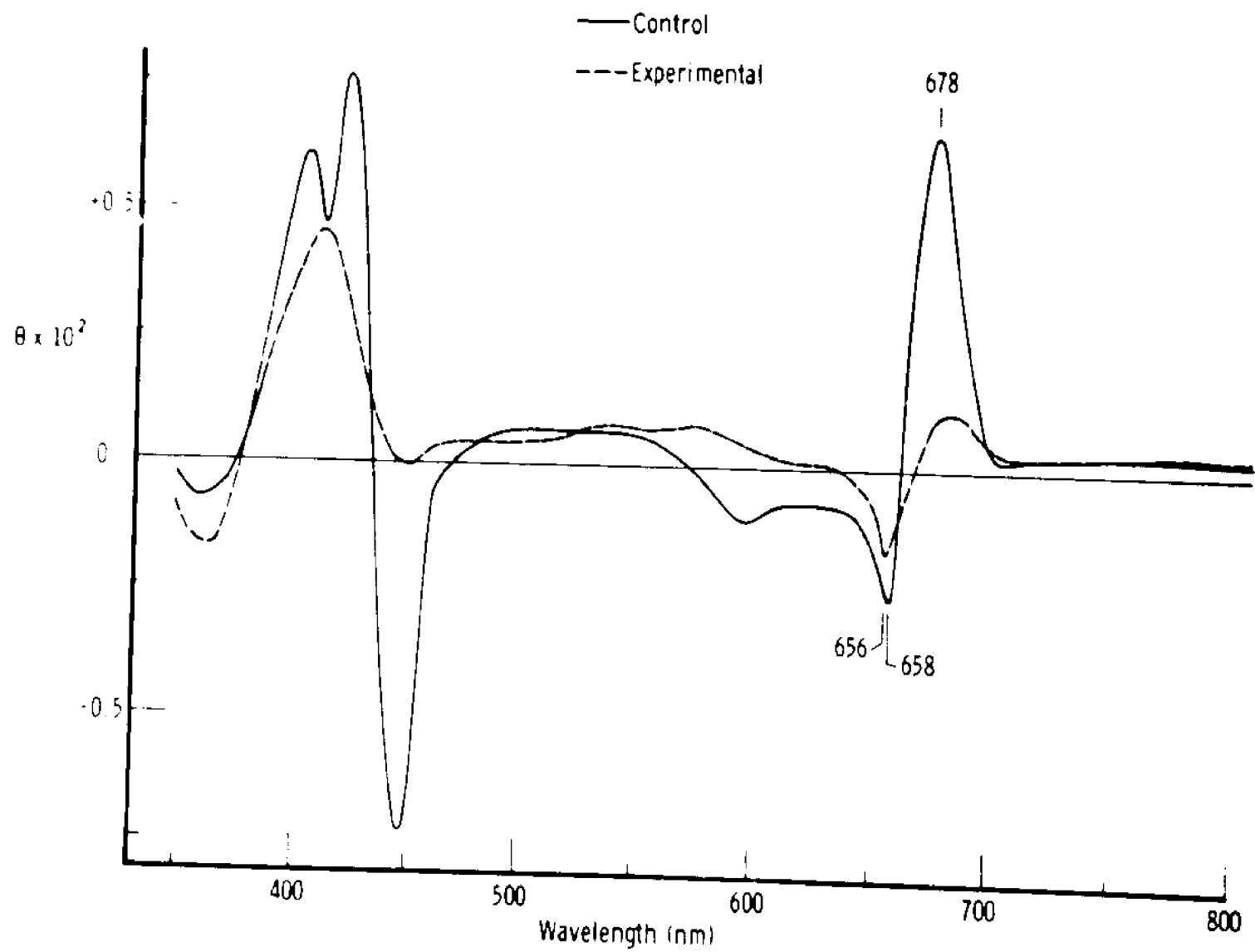


Figure 32. Absorption spectra of samples shown in Figure 31. Insert:
Difference absorption spectrum(Chlorophyll a dissolved in CCl_4 minus
Chlorophyll a dissolved in CCl_4 to which had been added $1.3 \times 10^{-3} \text{ M}$
linolenic acid.)

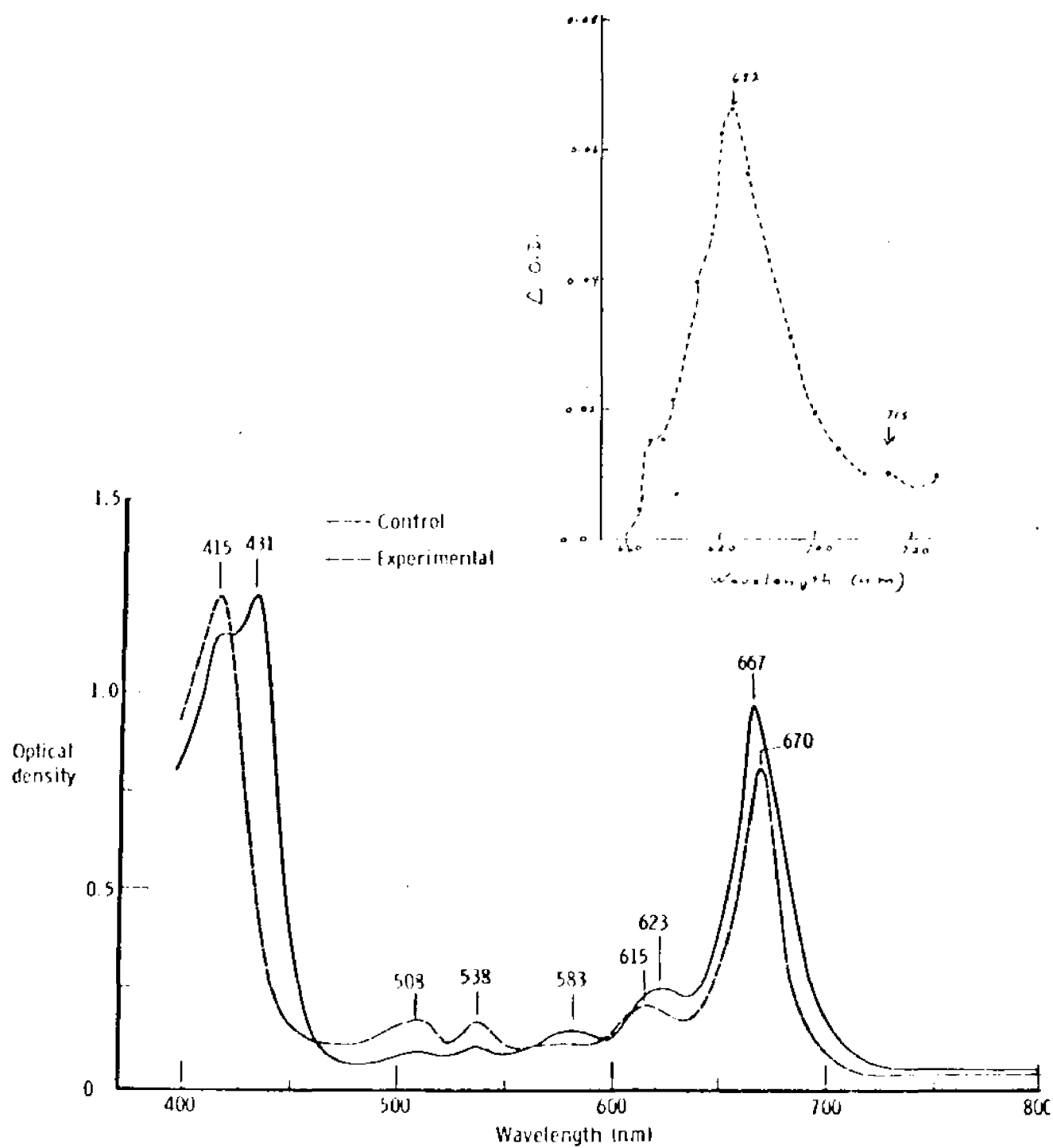
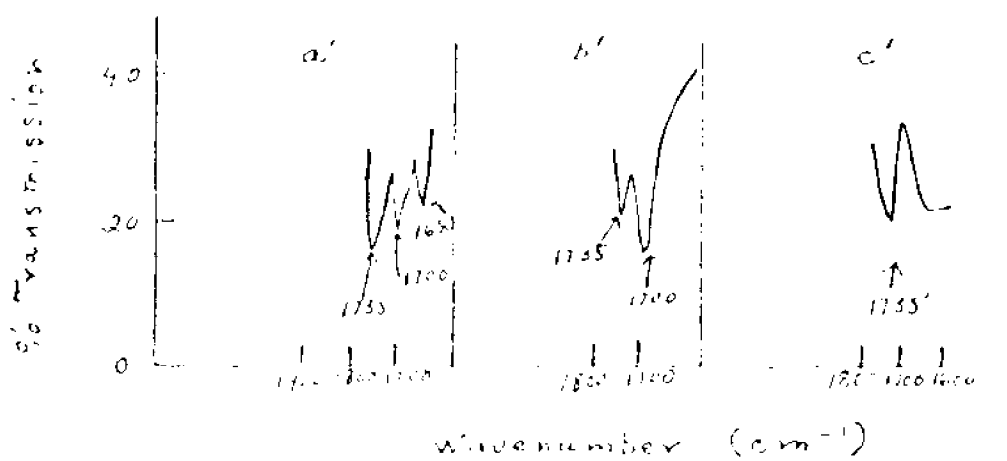
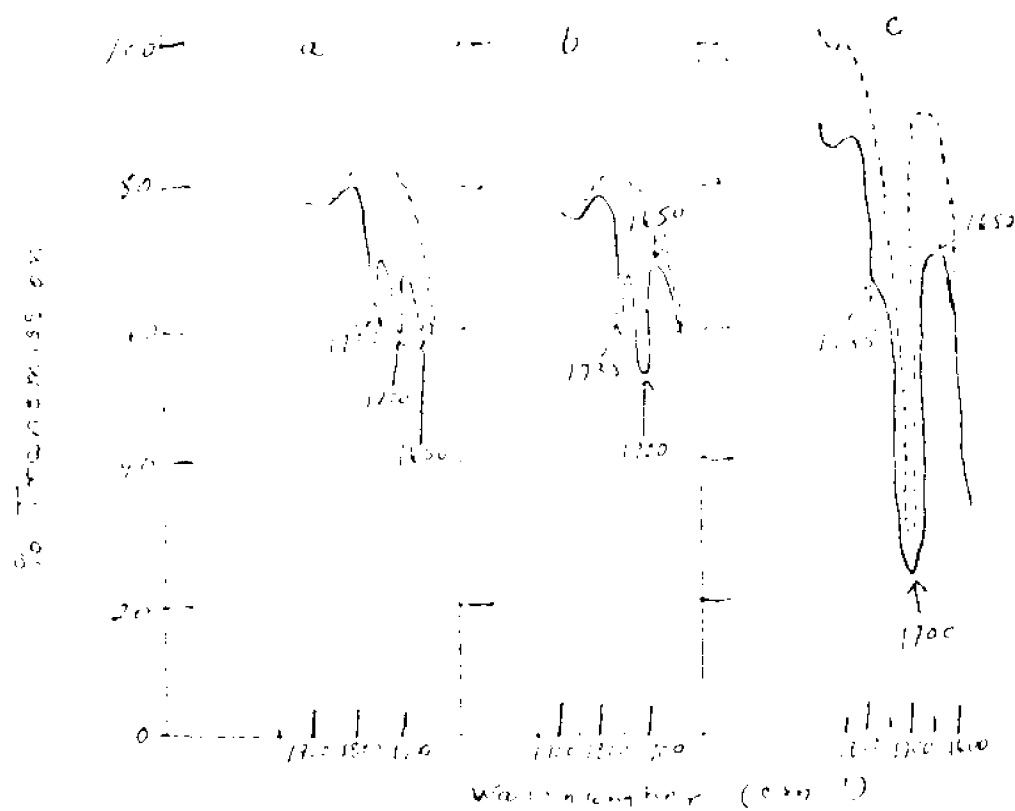


Figure 33. Infrared (I.R.) spectra of a) chlorophyll a dissolved in CCl_4 , b) chlorophyll a dissolved in CCl_4 , to which had been added 0.02% ethanol, and c) chlorophyll a dissolved in CCl_4 , to which had been added 1.3×10^{-3} M linolenic acid. Spectra labelled a', b' and c' have been corrected for the contribution of the solvents (CCl_4 , CCl_4 + ethanol, CCl_4 + linolenic acid, respectively) to I.R. absorption.



at 1700 cm^{-1} . This latter band, which has been assigned by Katz *et al.* (*op. cit.*) to the ketone of ring V of chlorophyll a, has been found by these latter workers to increase either in polar solvents or when deaggregation has occurred. In Fig. 33 C, in which is represented the I.R. spectrum of chlorophyll a in $\text{CCl}_4 + 1.3 \times 10^{-3}\text{ M}$ linolenic acid, the 1700 cm^{-1} band is less clearly evident, due to absorption at that wavelength by linolenic acid itself.

The observation that pheophytin is formed when linolenic acid is added to chlorophyll a in CCl_4 , poses several questions. Do fatty acids (a) deaggregate chlorophyll dimers into chlorophyll monomers, (b) convert chlorophyll a dimers into pheophytin dimers or (c) deaggregate chlorophyll a dimers into chlorophyll monomers - some (or all) of which become converted to pheophytin monomers? A choice between (a) and (c) involved the use of experimental apparatus not available. Norris *et al.* (1970) have shown that pheophytin aggregates have a direct absorption band at 695 nm; since such a band was not apparent in our spectra, possibility (b) is not a strong one. However, that deaggregation of higher aggregates of chlorophyll into dimers and monomers does occur upon addition of fatty acids is shown in the direct absorption spectrum of chlorophyll a in the solvent pair CCl_4 -hexane.

In Fig. 34 is seen the C.D. of chlorophyll a in CCl_4 - hexane, (A), and chlorophyll a in CCl_4 - hexane to which had been added $1.3 \times 10^{-3}\text{ M}$ linolenic acid. It can be seen that addition of linolenic acid results in conversion of part of the highly aggregated form into chlorophyll a dimers (absorbing at 678 and 656 nm). The formation of chlorophyll a monomers, which occurs upon addition of fatty acids, cannot be seen in the C.D. because of overlap with the 656 nm component of the dimer band. However, examination of the corresponding direct absorption spectra, Fig. 35, show that, upon addition of linolenic acid(B) to the control (A),

Figure 34. C.D. spectra of a) chlorophyll a dissolved in 40% CCl₄-60% hexane (solid line), and b) chlorophyll a dissolved in 40% CCl₄-60% hexane, to which had been added 1.3×10^{-3} M linolenic acid (broken line). Chlorophyll concentration = 2×10^{-3} M; cuvette path length = 0.005 cm.

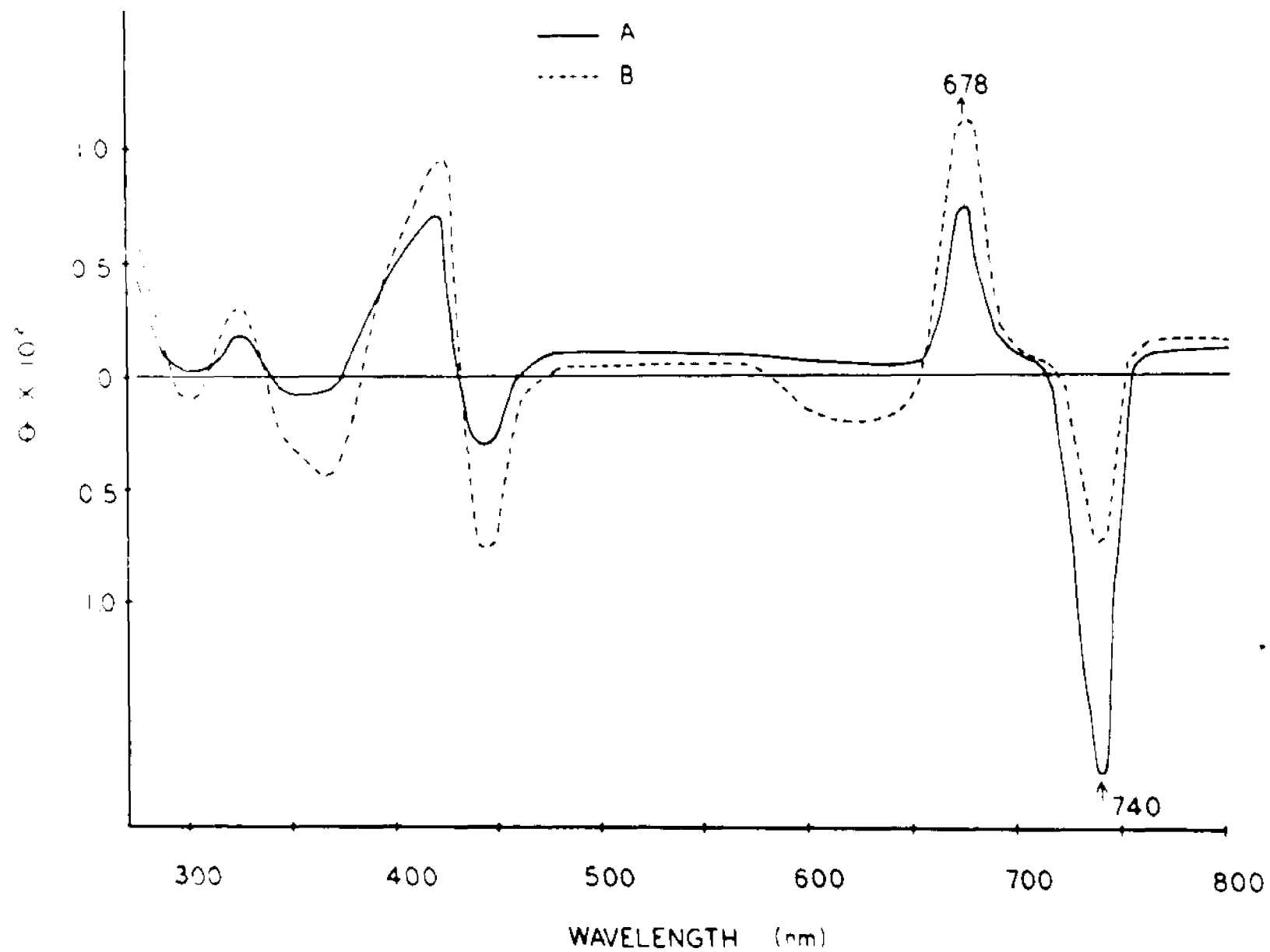
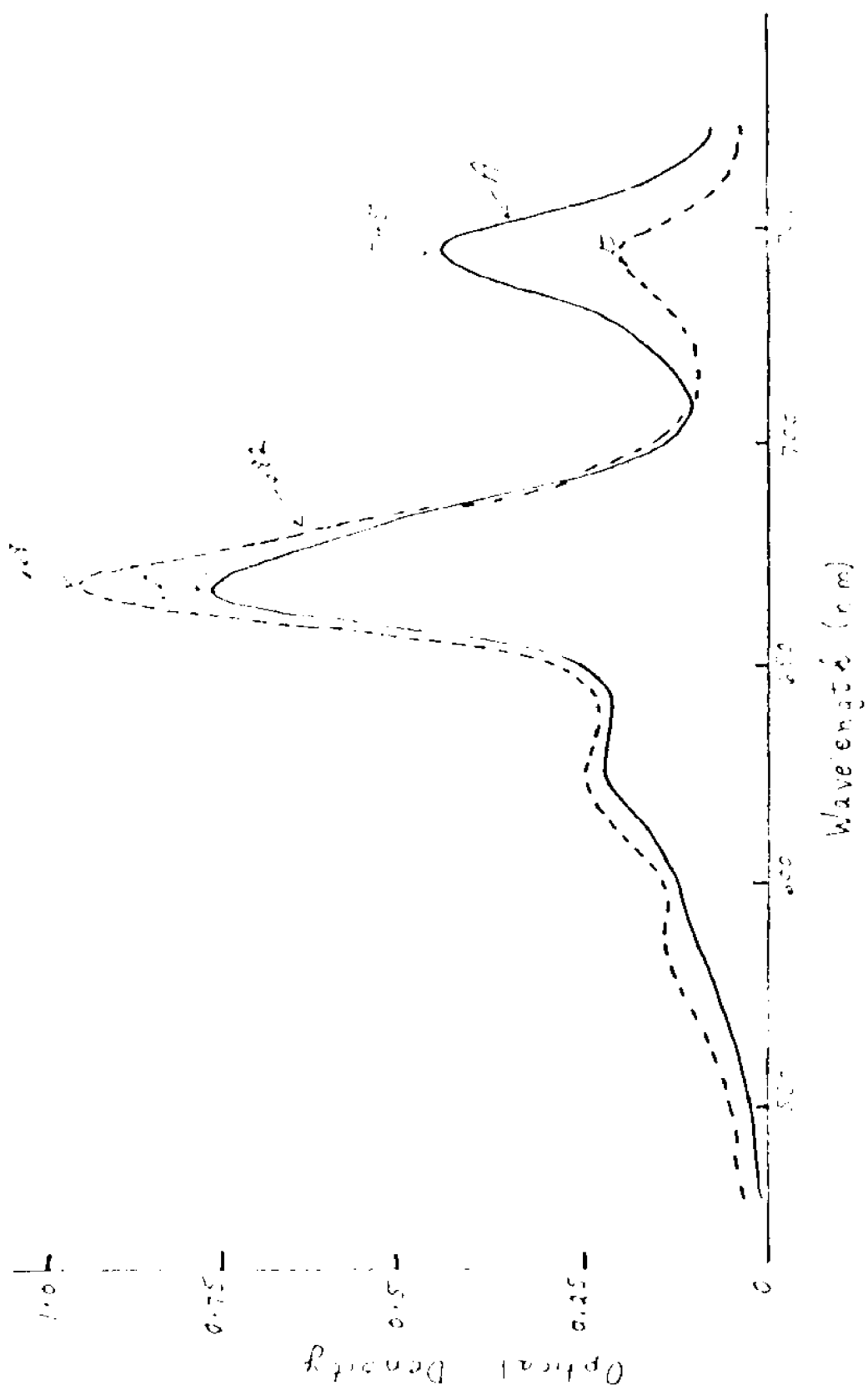


Figure 35. Absorption spectra of samples given in Figure 34.



there is (in addition to the decrease at 745 nm) an increase in absorption at 667 (monomer) and (possibly) 682 nm (the dimer band) as well as pheophytin formation (as evidenced by appearance of a band with maximum at 585 nm). Compare these direct absorption spectra with those in Fig. 28 B - the result of addition of ethanol to chlorophyll a in the solvent pair. The latter yield similar results with the exception that no pheophytin band appears at 535 nm.

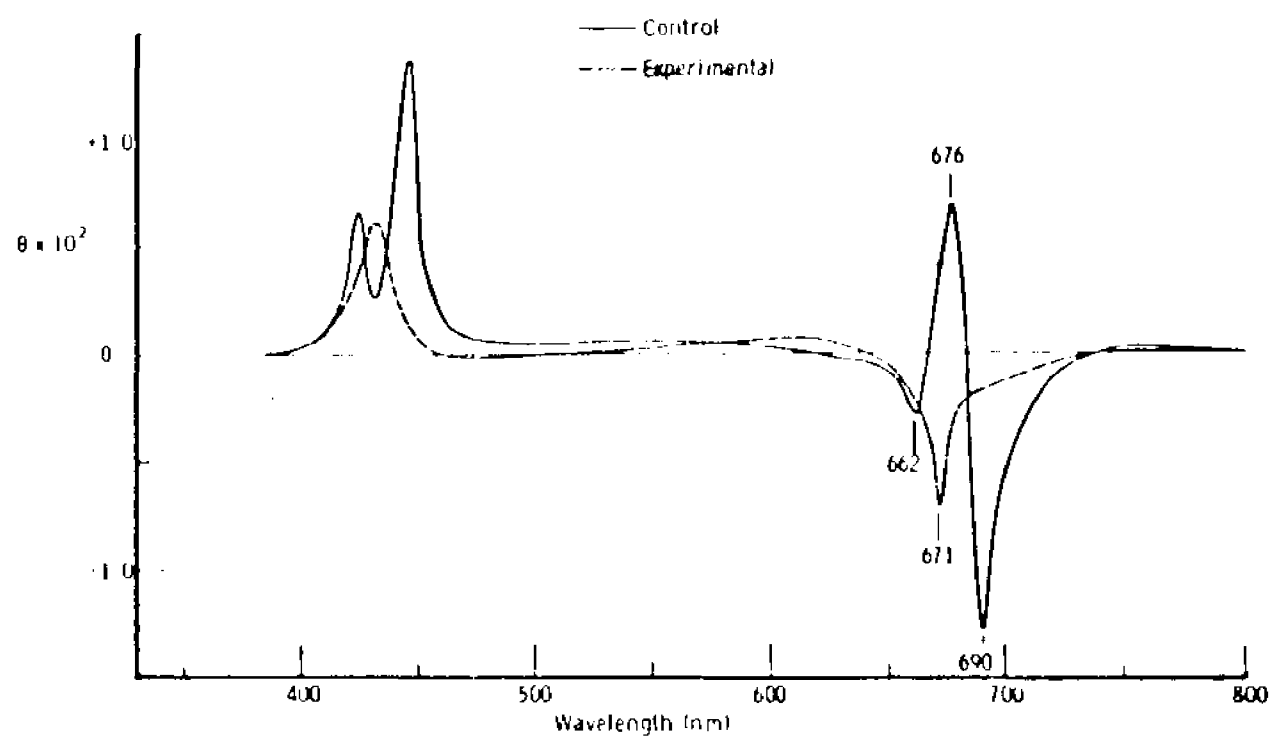
b. Effect of Linolenic Acid on System I Chlorophyll-Protein Complexes.

1) System I Complexes Isolated from Phormidium.

In Fig. 36 may be seen the C.D. spectrum of control system I chlorophyll-protein complexes suspended in 0.05 M Tris buffer, pH 8.0, containing ethanol at a final concentration of 0.5%. The chlorophyll concentration, as calculated using the extinction coefficient of Thornber (1969), was 1.5×10^{-5} M. In this control there are observed positive bands at 676 and 690 nm and a negative band at 662 nm; the 662 and 676 nm bands presumably corresponding to the dimer doublet seen previously with chlorophyll a dissolved in CCl_4 - the 690 nm band perhaps belonging to a different species of aggregate.* In this regard, it is interesting to note that French (1970) has analyzed the absorption spectrum of chlorophyll-protein complexes isolated from Phormidium, and has resolved the spectrum into components having maxima at 665, 672, 678, 688, 695, and 708 nm. In Fig. 36 B is shown the C.D. spectrum resulting when lino-

* There is another possibility - namely that the 676 - 690 bands belong to one type of aggregate and the 662 nm band corresponds to C.D. absorption by a monomer. However, as seen in Fig. 35, upon addition of linolenic acid a one-banded form, having a maximum at 671 nm, appears, replacing the spectrum of the control, in which there are three bands with maxima at 662, 676, and 690 nm. The 662 and 676 nm bands have maxima at wavelengths corresponding to the dimeric form of chlorophyll a, as seen earlier in this work, and in the work of Dratz et al. (1966). Furthermore, the 670 nm band is at a wavelength corresponding to one of the monomer absorption peaks, as resolved by French et al. (1970).

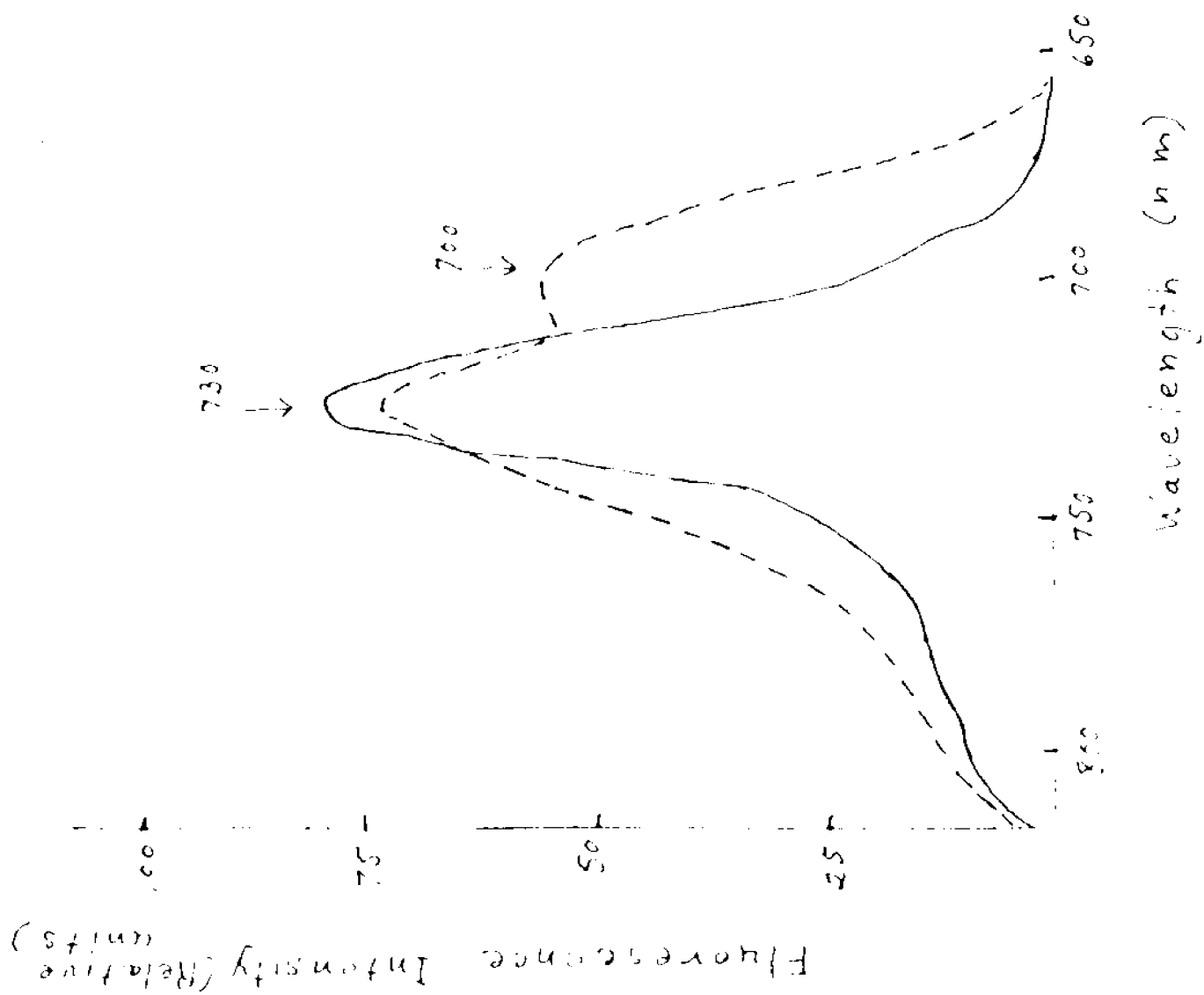
Figure 36. C.D. spectra of a) control system I chlorophyll-protein complexes (from Phormidium) suspended in 0.05 M Tris-HCl buffer, pH 8.0 (solid line) to which had been added 0.5% ethanol, and b) chlorophyll-protein complexes suspended in Tris-HCl buffer to which had been added 1.5×10^{-4} M linolenic acid (broken line). Chlorophyll concentration = 2.5×10^{-5} M; cuvette path length = 1 cm.



lenic acid (in ethanol) was added to these system I chlorophyll-protein complexes to yield a final fatty acid concentration of 1.5×10^{-4} M (and a final concentration of ethanol of 0.5%); there results a disappearance of the bands at 662 and 676 nm, and also a marked decrease of the band at 690 nm. A negative band with maximum at 671 nm remains. This band may actually represent monomer absorption at 670 nm with a shoulder at 660 nm, corresponding to absorption by 660 monomer (see footnote, page 59) or it may represent residual absorption at 690 superimposed upon absorption by monomer at 660 nm - the resultant band having absorption at 671 nm.

If one examines low temperature fluorescence emission and excitation spectra of system I chlorophyll-protein complexes, corresponding to the above control, and to the situation in which linolenic acid was added to these complexes, the following are noted: in the fluorescence emission spectrum of the control (see Fig. 37 A) only a single, broad emission band, with maximum at 730 nm, is apparent. In the action spectrum for exciting emission at 730 nm in these control complexes, the action spectrum bands with maxima at 674 nm and 682 nm are of equal intensity. The fluorescence emission spectrum of the linolenic acid-treated complexes, (see Fig. 29 B) has, in addition to the 731 nm band, a new band with maximum at 700 nm, 4/5 the intensity of the 730 nm band. The corresponding excitation spectrum show an increase in the 674 nm band and a reduction in the contribution of the 682 nm band to fluorescence excitation at 730 nm. Thus, it would appear that fatty acids can cause deaggregation in both the cases of chlorophyll in solution and chlorophyll attached to protein. That the fatty acids can act on the protein portion of the chlorophyll-protein complexes (as well as on the chromophore, as shown above in solution) is demonstrated below, in the section dealing with the effects of linolenic acid on HP 700 chloro-

Figure 37. Fluorescence emission spectra of samples in Figure 36. Fluorescence excited at 436 nm and measured at -196°C .



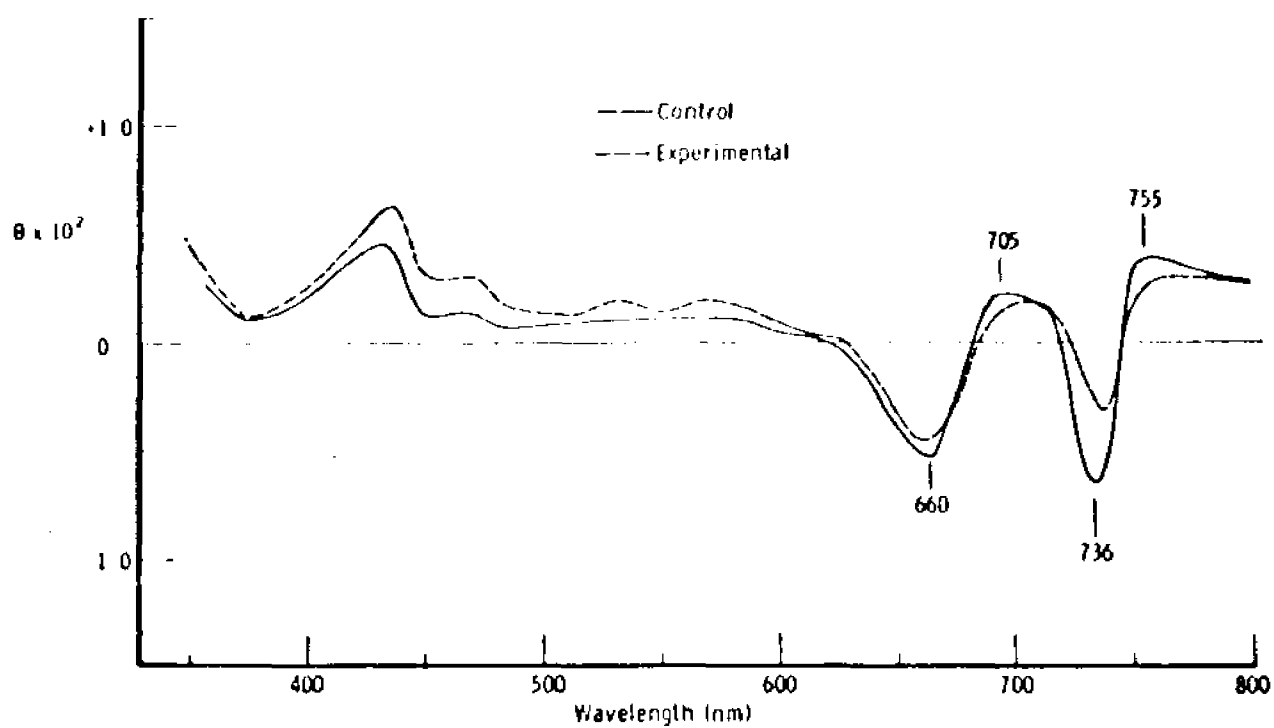
phyll-protein complexes.

2) Effect of Linolenic Acid on HP 700 Chlorophyll-Protein Complexes.

In Fig. 38 ^{isolated line} is shown the control C.D. spectrum of HP 700 chlorophyll-protein complexes (chlorophyll concentration = 2.5×10^{-5} M) suspended in STN buffer, pH 8.0. There is observed in the red region of the spectrum 4 bands. The small positive band at 755 nm and the large negative band at 736 nm can be assigned to the doublet of highly aggregated chlorophyll, based upon the position of these bands in relation to those observed with chlorophyll a dissolved in CCl_4 -hexane. The broad, positive band with maximum at 695 - 700 nm can be assumed to be another type of aggregate band (compare with Fig. 28 A, i.e. chlorophyll a in CCl_4 -hexane), while the large negative band with maximum at 660 nm can be assigned to the chlorophyll monomer, the latter again based upon previous work involving the C.D. spectra of dilute solutions of chlorophyll a. If HP 700 is prepared from lyophilized chloroplasts, or, if HP 700 complexes are lyophilized (and then re-suspended), the bands corresponding to aggregated chlorophylls (at 736 and 755 nm) disappear, along with concomitant disappearance of the band with maximum at 705 nm, indicating the likelihood of the involvement of water in formation of these particular aggregates.

The observation that linolenic acid could cause an apparent deaggregation of chlorophylls in chlorophyll-protein complexes (i.e. system I chlorophyll-protein complexes isolated from Phormidium) raised the possibility that deaggregation could be brought about as a secondary process, resulting from an initial effect on the protein to which the chlorophyll chromophores are attached - for example, an unwinding or an increase in random coil formation. In order to test this possibility, I investigated the effect of linolenic acid-treatment on the

Figure 38. C.D. spectra of a) control HP 700 chlorophyll-protein complexes suspended in STN buffer, pH 8.0 (solid line), and b) HP 700 chlorophyll-protein complexes in STN buffer to which had been added 2.5×10^{-5} M linolenic acid (broken line). Chlorophyll concentration = 2.5×10^{-5} M; cuvette path length = 1 cm.

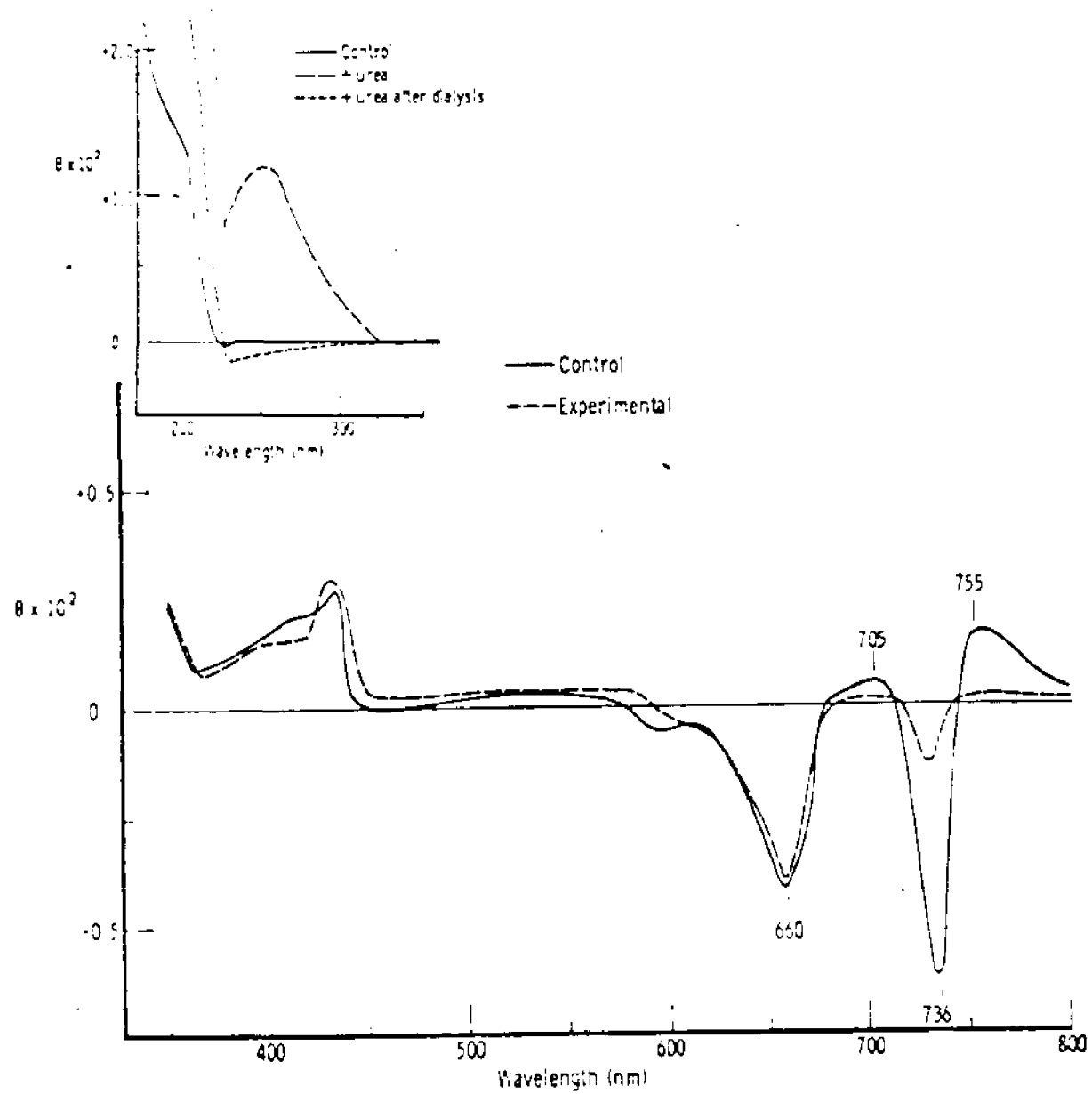


C.D. of HP 700 complexes in the U.V. region of the spectrum, as well as the red region of the spectrum. In Fig. 38 (broken line) is shown the C.D. (in the red region of the spectrum) for linolenic acid-treated HP 700 chlorophyll-protein complexes. There is observed a large decrease at 736 nm and decreases at 755 and 695-700 nm. Preliminary to these changes, there is seen in the U.V. portion of the spectrum a large increase at 190 nm (protein absorption) and at 280 nm (absorption by tyrosine residues). Cellulose sac dialysis did not result in a return to the control spectrum, either in the U.V. or in the red region of the spectrum.

In Fig. 39 is shown the spectrum of control HP 700 complexes (solid line) and complexes in the presence of 8.0 M urea (broken line). As mentioned above, linolenic acid may be bringing about deaggregation of HP 700 indirectly, by acting firstly on the protein portion of the HP 700 complexes. Since these changes were not found to be reversible, it was decided to see whether a substance known to effect changes in protein conformation, namely urea, could act in a similar manner. Also, in the case of urea, changes in protein conformation can often be reversed (Tanford, 1969). As in the case of linolenic acid, treatment with 8 M urea resulted in disappearance of aggregate bands, with preliminary changes in the U.V. region (see insert to Fig. 39). These latter changes were also observed in the case of 4 M urea (although changes in the red region of the spectrum were not observed at this urea concentration). Cellulose sac dialysis (against buffer) did not always result in a return to the control situation (even a partial one) in the red region of the spectrum, although the ultraviolet region showed good reversibility (see insert to Fig. 39, line given by short dashes).

There was no pheophytin detectable when fatty acids were added to chlorophyll-protein complexes (or, indeed, to any membranous systems), in contrast to the results observed with chlorophyll in solution. Steffen and Calvin (1970) have postulated that the protein of the chlorophyll-

Figure 39. C.D. spectra of a) control HP 700 chlorophyll-protein complexes suspended in STN buffer, pH 8.0 (solid line), and b) HP 700 chlorophyll-protein complexes in STN buffer to which had been added 8 M urea (broken line). Insert: Ultraviolet C.D. spectra of : control HP 700 complexes suspended in STN buffer(solid line);suspended in STN buffer containing 8 M urea(line given by long dashes);suspended in the latter and dialyzed 1/2 hour(line given by short dashes.Chlorophyll concentration = 2.5×10^{-5} M;path length = 1 cm.



lipo-protein complexes in membranous systems can protect against pheophytinization of chlorophyll.

The observation that there was no increase in absorption in the (monomer) C.D. at 660 nm, although there was disappearance of aggregate bands, (with no accompanying pheophytin formation), can be explained with the assumption that the extinction coefficient for monomeric chlorophyll in the C.D. is very small. That is, there is little intrinsic dichroism of absorption due to the structure of the chlorophyll chromophore itself, but much of the structure seen in the C.D. of the dimer is due to interaction between the two chromophores of the dimer.

3. INFLUENCE OF LINOLENIC ACID ON THE STATES OF CHLOROPHYLL IN SYSTEMS HAVING A HIGH DEGREE OF ORGANIZATION (MEMBRANOUS SYSTEMS).

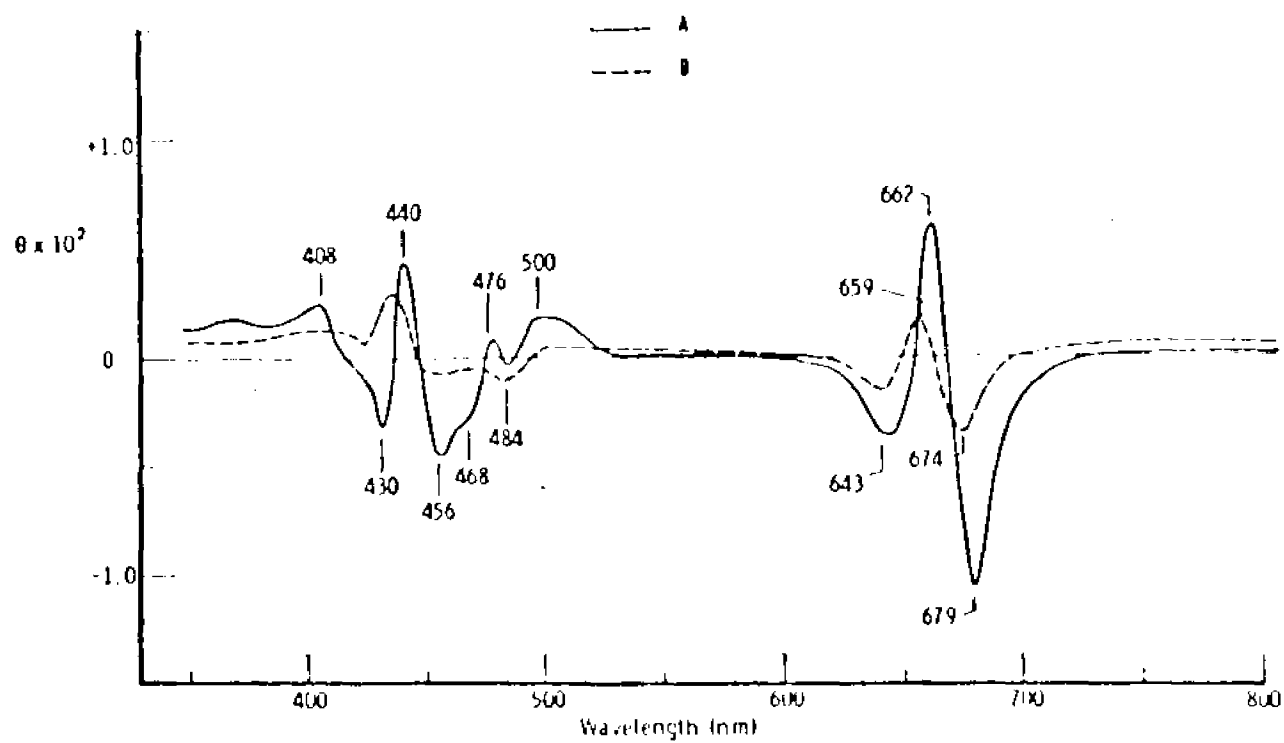
a. Effect of Linolenic Acid on Digitonin-Isolated System I Particles.

In Fig. 40 A is seen the control C.D. spectrum of digitonin-isolated system I particles suspended in 0.01 M KCl, 0.05 M Tris-HCl buffer, pH 7.8. The negative band at 643 nm can be attributed to chlorophyll b.

As Dratz et al. (1966) have shown, chlorophyll b in CCl_4 has dimer C.D. bands at 638 (negative) and 654 (positive) nm; these workers compared quantosomes* from normal and a chlorophyll b-free mutant of barley - in contrast to the latter, which has only chlorophyll a bands at 666 and 682.5 nm - the normal, i.e. chlorophyll b-containing barley quantosomes have C.D. bands at 653, 671 and 682.5 nm. These workers attribute the double C.D. bands in the chlorophyll b region to chlorophyll-chlorophyll interaction. Since chlorophyll b in these digitonin-isolated particles has

* A quantasome, containing about 300 chlorophyll molecules, is presumably the primary photosynthetic unit (Clayton, 1965).

Figure 40. C.D. spectra of a) control digitonin-isolated particles containing system I and suspended in 0.01 M KCl, 0.05 M Tris-HCl buffer, pH 7.8 (solid line) and b) the above particles after 10 minutes of incubation in buffer to which had been added linolenic acid to yield a final concentration of 4.5×10^{-5} M (broken line). Chlorophyll concentration = 2×10^{-5} M; cuvette path length = 1 cm.



only a single band, at 643 nm, it most probably represents a monomeric state.

From my work, I would like to suggest that the C.D. bands at 679 nm (negative) and 662 nm (positive) represent the same kind of chlorophyll a dimer doublet as I observed with chlorophyll a in CCl_4 -hexane, in which the (slightly shorter wavelength) bands * occur at 676 (positive) and 656 (negative) nm (see preceding section on C.D. of chlorophyll a in solution). This inference is different from that of Dratz et al. (1966) who interpreted the chlorophyll a bands they observed in quantosomes as originating from a crystalline state; for further consideration, see the Discussion section, below.

As seen in the work of Dratz et al. (op. cit.) and also, more extensively, in the present work, there is a reversal in C.D. sign in membranous as compared with non-membranous systems. The reason(s) why organization into membranous systems brings about the observed reversal in polarity (as will be considered in greater detail in the Discussion section) is not obvious. However, Goedheer (1957) in experiments in which he made monolayers of chlorophyll and oleic acid (and also by theoretical calculations) showed that reversal in polarity may be brought about by differences in refractive index $n_{\parallel}$ and $n_{\perp}$ by orientation of chlorophyll in the lipo-protein lamellae.

The chlorophyll concentration of the digitonin-isolated system I particles shown in Fig. 39 A is 2×10^{-5} M, and the chlorophyll a/b ratio (as determined from the extinction coefficients of the extracted chlorophyll in acetone) was 5.19.

* Kasha (1960) has shown that as the solvent polarity is increased, there is a shift in the absorption maxima of chlorophyll to longer wavelengths.

If linolenic acid at a final concentration of 4×10^{-5} M is added to these particles in buffer, and they are incubated for a period of 30 minutes (see Fig. 40 B), there is a reduction in intensity of all three bands in the red region of the spectrum, accompanied by shifts in maxima from 662 to 659 nm and from 679 to 674 nm. There is no shift in the chlorophyll b maximum.

If one examines the direct absorption spectra for system I particles incubated in the presence of linolenic acid for 10 to 30 minutes (Fig. 41 B and C) there is seen a strong decrease in the major red and blue bands compared to the control (Fig. 41 A), and a shift in the red absorption maximum from 668 to 675 nm. That deaggregation is occurring is confirmed by the low temperature fluorescence emission spectrum (at 77°K) see Fig. 42. In the control, one observes a strong aggregate peak at 740 nm, and small bands at 685 and 700 nm. After 10 minutes of incubation in fatty acid at a FA/Chl of 2, there is a slight increase in fluorescence at 740 nm (this is accompanied by increase in contribution of the 674 nm band and decrease in contribution of the 682 nm band in the action spectrum for exciting fluorescence at 740 nm). After 30 minutes of incubation, fluorescence at 740 nm has fallen to 50% of its original intensity and the long wavelength maximum has shifted to 730 nm (conversion of larger to smaller size aggregates). These phenomena are accompanied by a concomitant increase in fluorescence (6 fold) at 695 nm.

With increasing concentration of linolenic acid (to 10 and 100 fold that of the chlorophyll concentration of the digitonin-isolated system I particles) there is noted at 0° (see Fig. 43) transition to a C.D. spectrum approaching that of the chlorophyll a monomers in CCl_4 (see Fig. 27 A). In the corresponding absorption spectra (Fig. 44 A, B, and C)

Figure 41. Absorption spectra of samples given in Figure 40; additionally, an absorption spectrum is given for 30' of incubation.

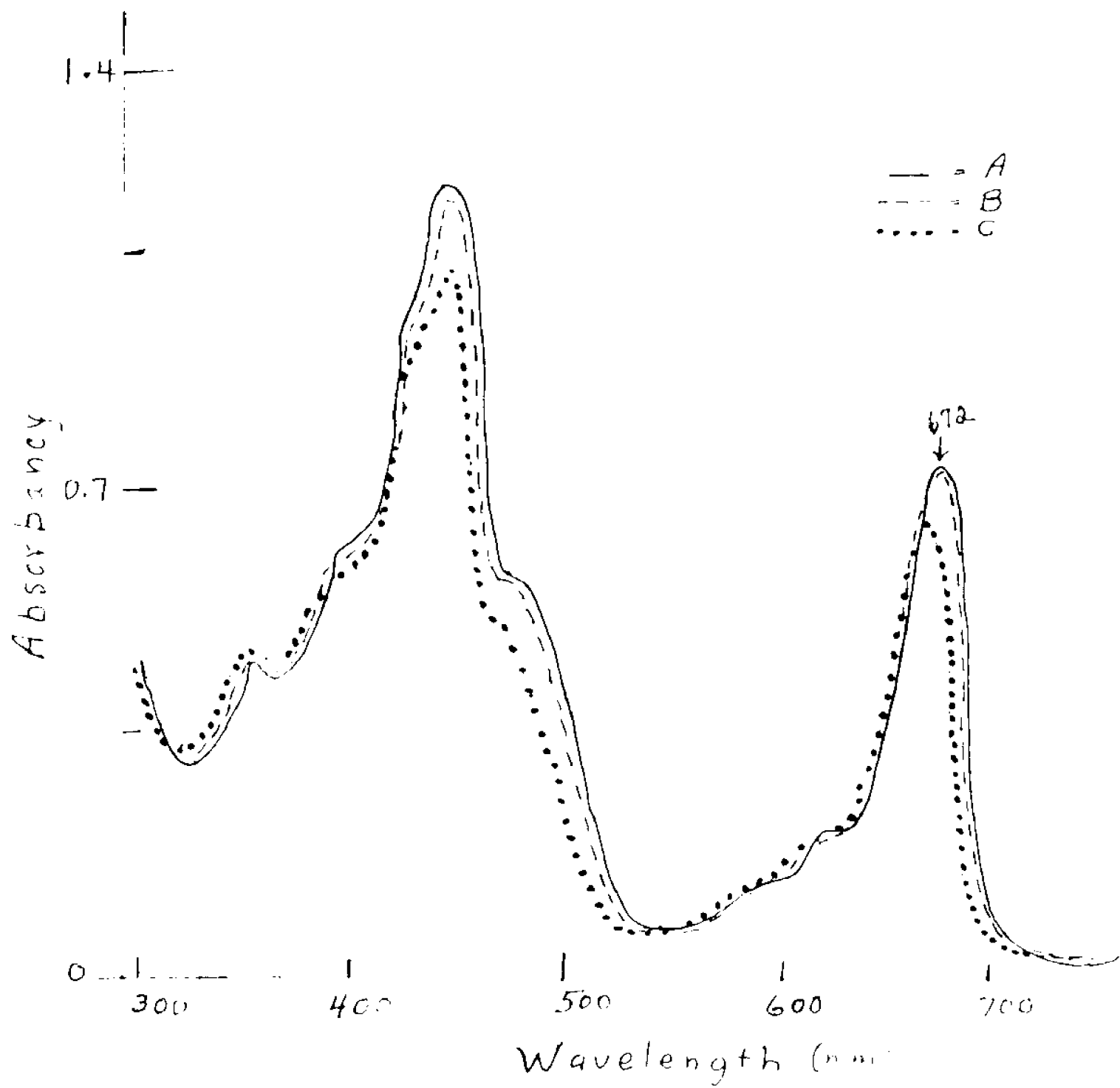


Figure 42. Fluorescence emission spectra of samples in Figure 40; additionally, an emission spectrum is given for 30 minutes of incubation. Fluorescence excited at 436 nm and measured at -196°C .

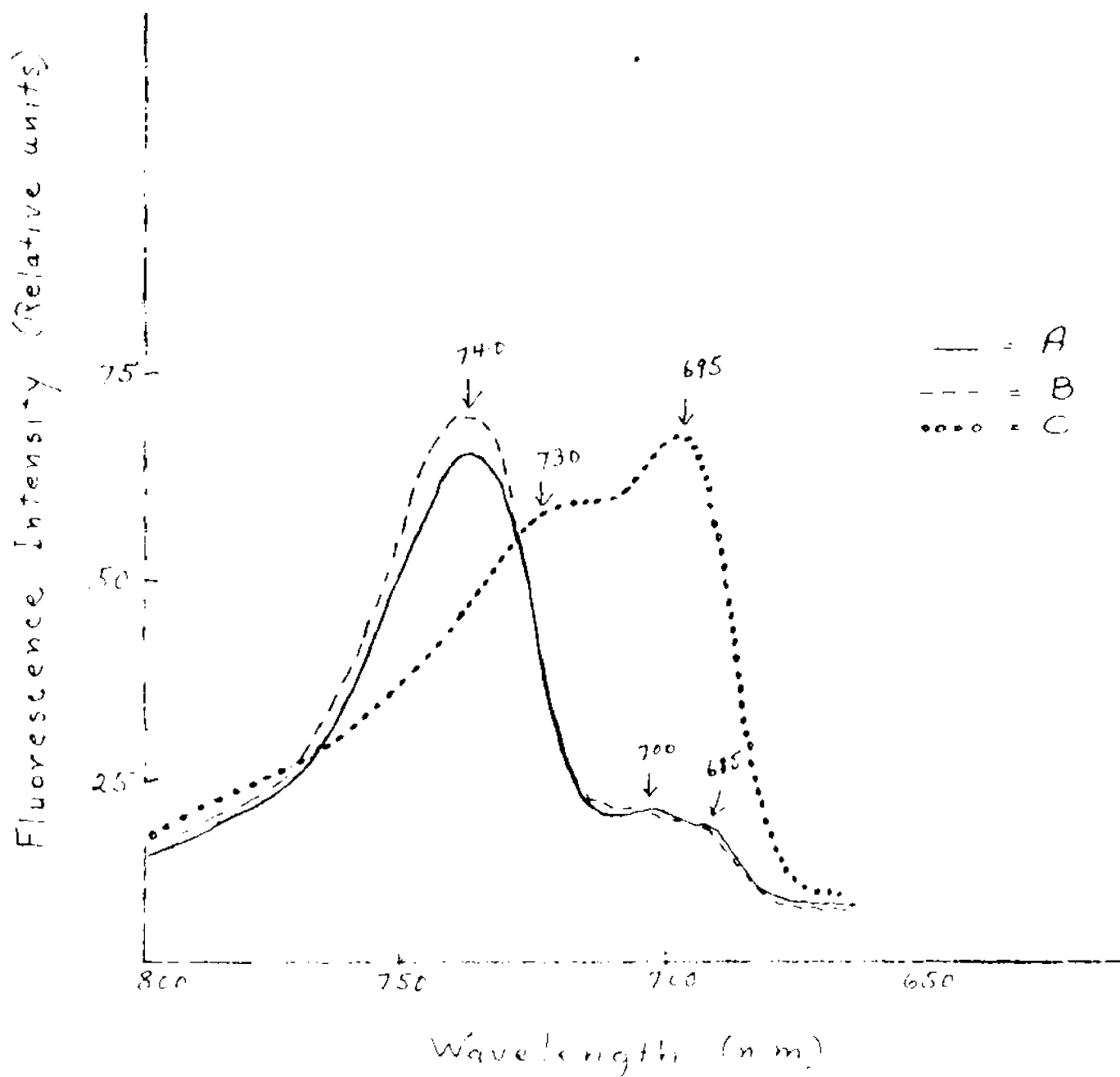


Figure 43. C.D. spectra of a) control digitonin-isolated particles containing system I and suspended in 0.01 M KCl, 0.05 M Tris-HCl buffer, pH 7.8 (solid line), b) particles after addition of 1×10^{-4} M linolenic acid and c) particles after addition of 1×10^{-3} M linolenic acid. Chlorophyll concentration = 2×10^{-5} M. Cuvette path length = 1 cm.

$\theta \times 10^3$
 $\theta \times 10^3$
 $\theta \times 10^3$

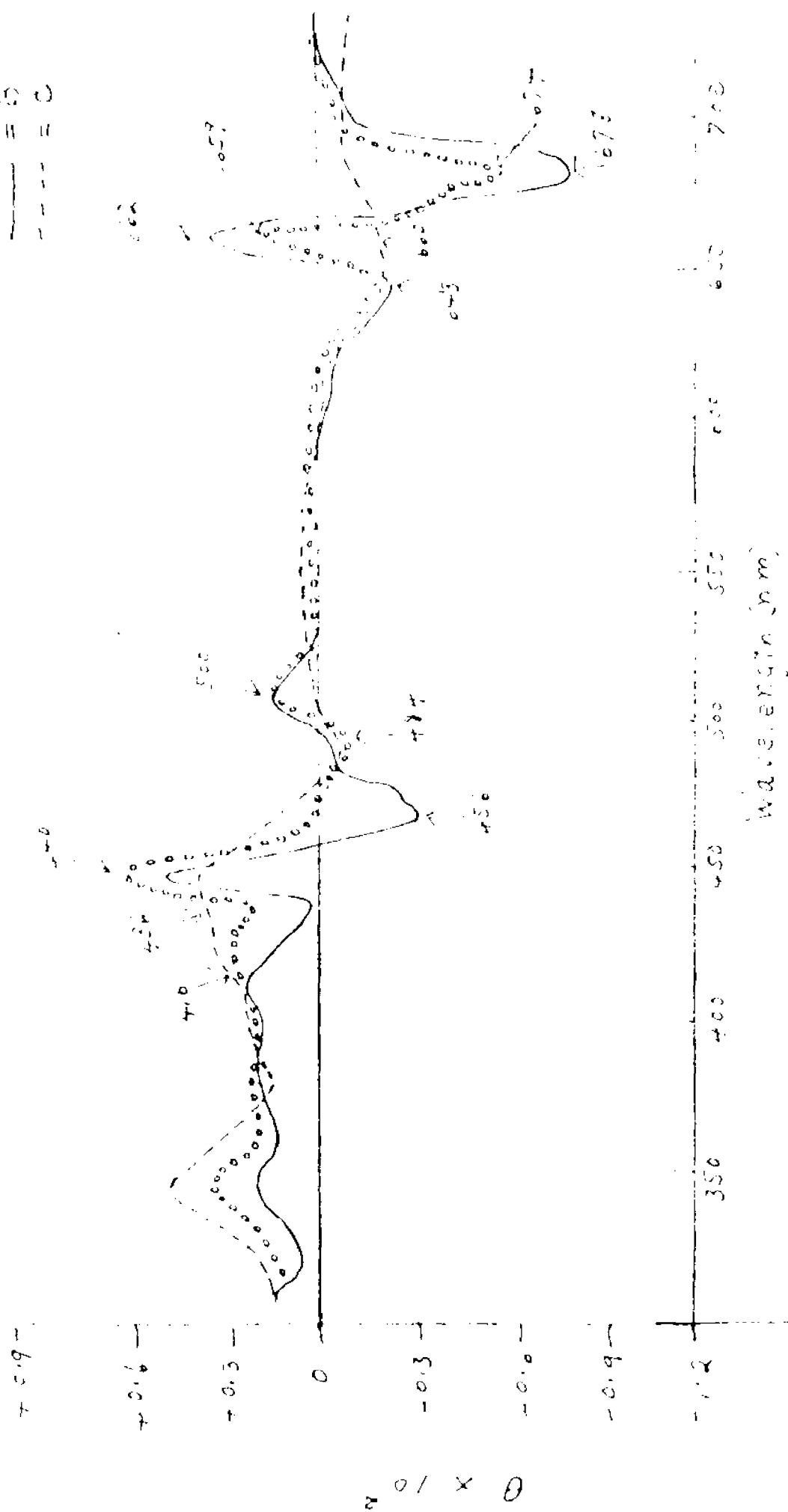


Figure 44. Absorption spectra of the samples given in Figure 43.

Absorbancy

0 -

0.30 -

0.60 -

0.90 -

1.20 -

750 700 650 600 550 500 450



--- = C
 ... = B
 — = A

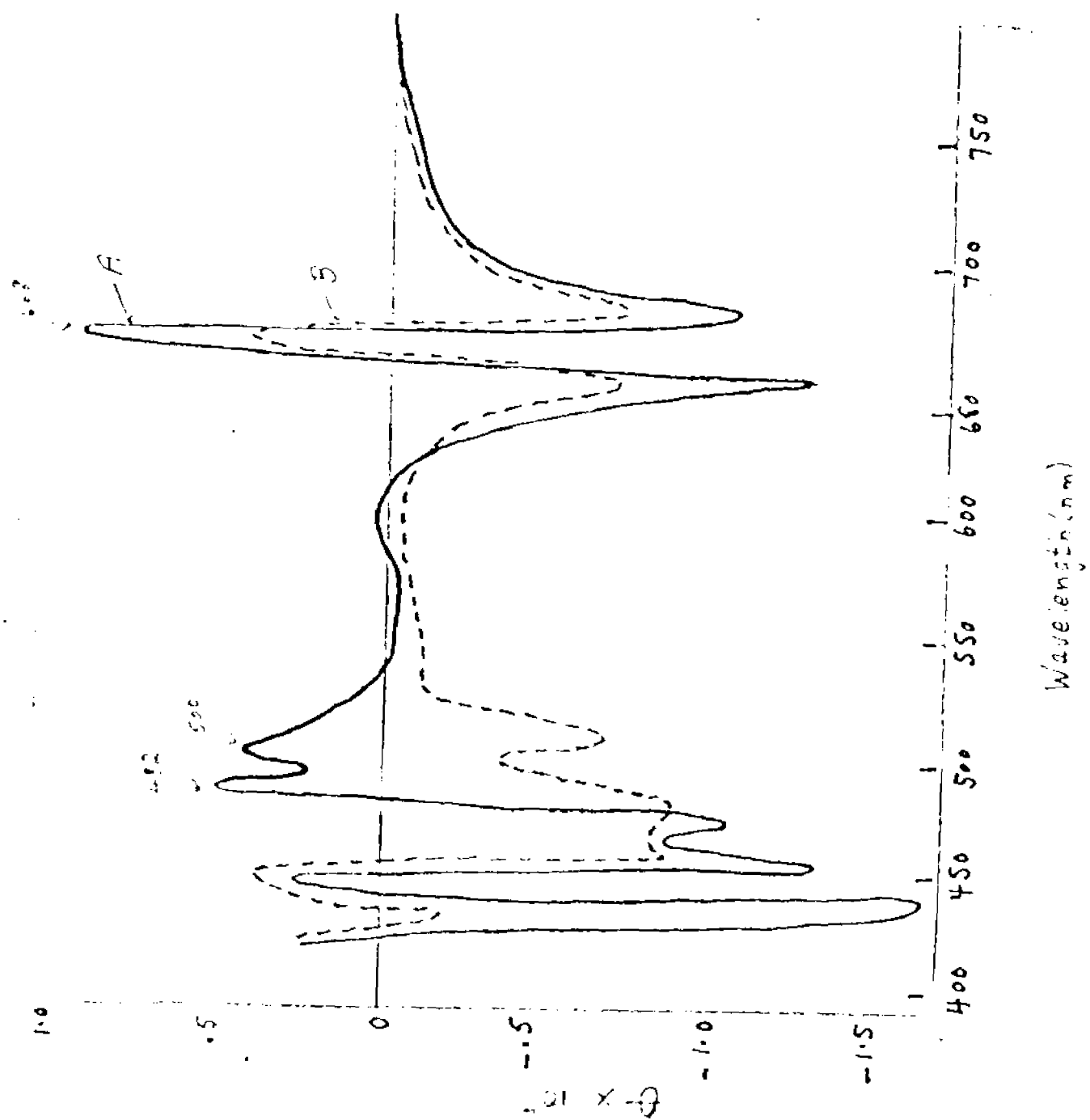
there is a shift in the red absorption maximum from 672 nm in the control to 658 nm in the case of particles treated with 2×10^{-3} M linolenic acid. The FA/Chl ratio of 10 changes R_{λ} (at 77°K) from 5.0 in the control to 0.33; in the case of particles treated with 2×10^{-3} M linolenic acid (FA/Chl = 100) the ratio was reduced to 0.24. It was observed that at a FA/Chl ratio of 10, there were considerable changes in the (77°K) fluorescence emission and excitation spectra but only small changes in direct absorption and C.D. The latter are probably brought about by conversion of aggregate to monomer whereas the former results additionally from changes in energy transfer (presumably brought about by fatty acid-induced changes in protein conformation). A similar conclusion was reached in comparison of the magnitude of fluorescence emission and excitation, and absorption changes in Section III-B, above.

b. Effect of Linolenic Acid on Particles Containing Systems I and II.

1) Effect of Linolenic Acid on Digitonin-Isolated Particles Containing Systems I and II.

In Fig. 45 A is seen the control C.D. spectrum of digitonin-isolated particles containing systems I and II (10,000 x G pellet) suspended in 0.01 M KCl, 0.05 M Tris-HCl buffer, pH 7.8. If this control is compared to the control of digitonin-isolated particles containing system I only, it can be seen that in the former, the chlorophyll b band is much stronger in relation to the chlorophyll a doublet (i.e. about 1.2 x the intensity compared to 0.33 the intensity as seen in the case of particles containing system I). The chlorophyll a/b ratio of the particles containing systems I and II is 3.32 (in contrast to 5.19 for the fraction containing only system I). In the blue region of the spectrum, the bands at 482, 486 and 500 nm are positive - compare this to the more negative bands in the digitonin-isolated particles containing system I; the

Figure 45. C.D. spectra of a) control digitonin-isolated particles containing systems I and II, suspended in 0.01 M KCl, 0.05 M Tris-HCl buffer, pH 7.8 (solid line) and b) particles after 30 minutes of incubation in buffer to which had been added 2×10^{-4} M linolenic acid (broken line). Chlorophyll concentration = 2×10^{-5} M; cuvette path length = 1 cm.

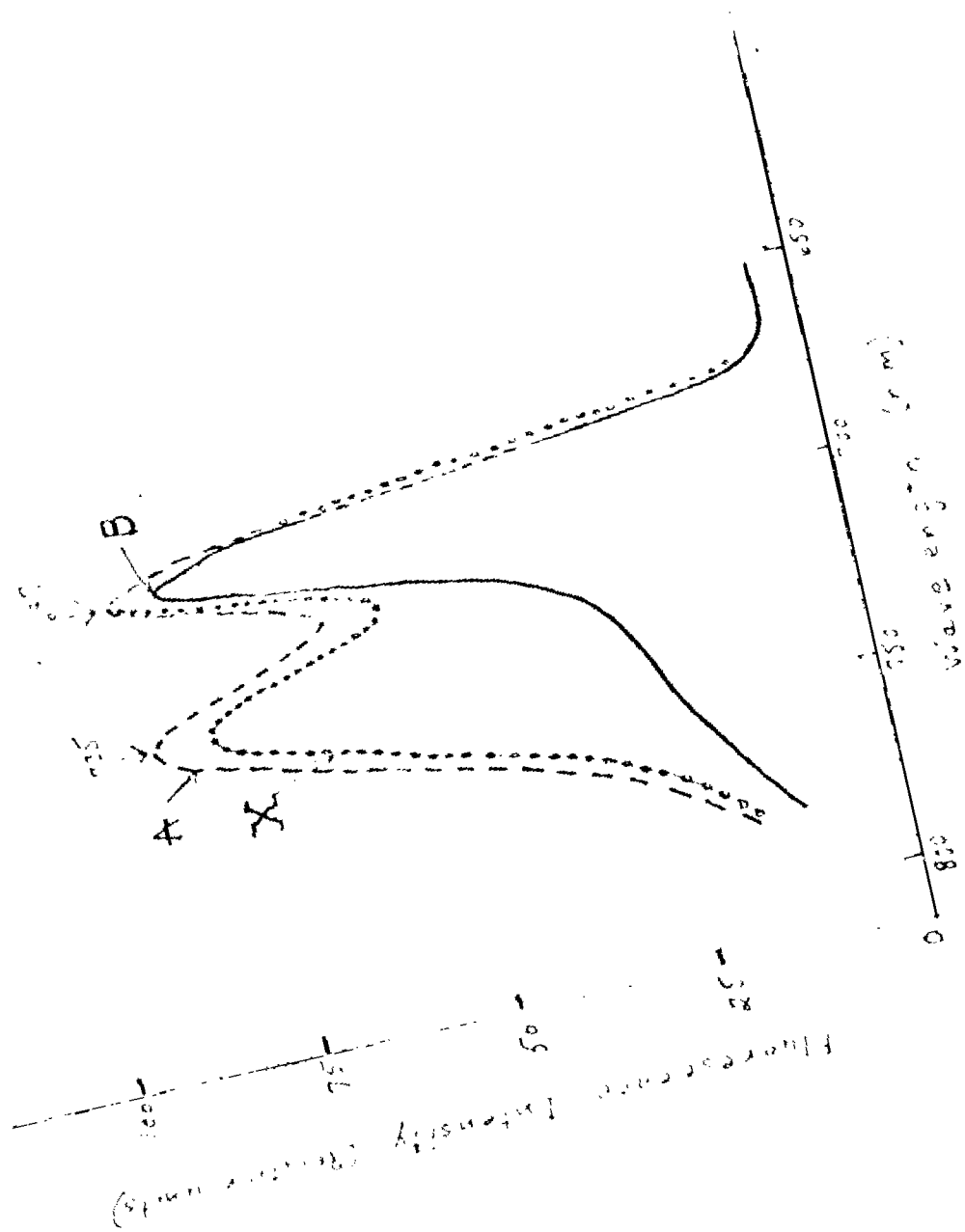


latter, of course, are much smaller in size. If linolenic acid at a concentration of 2×10^{-4} M (10 x the ^{amount of} chlorophyll _a) is added to these particles and they are incubated for 30 minutes, there occur strong decreases in the bands at 668 and 680 nm (chlorophyll a doublet) and the chlorophyll b band at 652 nm. In the blue region of the spectrum there is an apparent reversal in sign of the bands at 482 and 500 nm. This "reversal" will be considered below, in connection with the study of the effect of linolenic acid on subchloroplast particles isolated by means of sonication.

The corresponding fluorescence emission spectra for the control and for the linolenic acid-treated particles, again give evidence for the deaggregating effect of linolenic acid. There is apparent in the control a two banded emission spectrum (measured at 77°K) with maxima at 735 nm and 697 nm (see Fig. 46 A). Incubation for 30 minutes in the presence of linolenic acid results in conversion to a one-banded form, with maximum at 697 nm (in the fluorescence excitation spectra, there ^{are} observed concomitantly a decrease in contribution of the 682 nm band and ^{an} increase in the 674 nm band in the 735 nm fluorescence action spectrum).

As observed above, with HP 700 complexes, treatment with linolenic acid or urea (8 M) resulted in disappearance of C.D. aggregate bands in the red region of the spectrum. These changes were preceded by changes in the U.V. region of the spectrum, indicative of changes in protein conformation. Partial removal of urea (by cellulose sac dialysis or Sephadex chromatography) did not always result in a return to the control spectrum (for reasons not apparent at the present time). At no time, however, did dialysis result in a return to the control spectrum when deaggregation had been brought about by linolenic acid.

Figure 46. Fluorescence emission spectra (measured at -196°C of the samples given in Figure 45; additionally, an emission spectrum is given for 5 minutes of incubation.) Fluorescence excited with light of 436 nm.



Although cognizant of the ability of defatted bovine serum albumin to (a) protect against (McCarty and Jagendorf, 1965; Cohen et al., 1969; Cohen, Ph. D. dissertation, 1970; see Discussion and Conclusions) and (b) reverse (Cohen, Ph. D. dissertation, 1970) changes induced by fatty acids, I made no attempt to use BSA in conjunction with these HP 700 complexes for purposes of achieving reversal, since it would have been virtually impossible to separate the BSA from HP 700 within a "reasonable" length of time: that is, an amount of time amenable to further spectroscopic studies. For this reason, the experiments begun with HP 700 were extended to digitonin-isolated particles containing systems I and II - for these particles (which can be pelleted at $10,000 \times G$ for 30 minutes) lend themselves to ready separation from BSA.

In Fig. 47 are shown C.D. spectra in the visible region (U.V. region in insert) of digitonin-isolated particles containing systems I and II; A: control; B: linolenic acid-treated; and C: linolenic acid-treated + BSA (see Materials and Methods, page 14 for procedure). The time elapsed between spectral recordings was ten minutes, during which time samples were kept on ice. Curve B's visible spectrum is very similar to that observed in Fig. 45 B, in that both exemplify linolenic acid-induced deaggregation. Curve C is of interest because it demonstrates that 20 mg/ml of defatted BSA strongly reverses the linolenic acid-induced changes: i.e. can ^{permeabilization of} reaggregate the chlorophyll previously deaggregated by linolenic acid.

The attention of the reader is called to Fig. 48, in which ultra-violet C.D. spectra for random coil, for α helix and for β extended-chain of polyamino acids in aqueous solution are given, for purposes of comparison with the present work.

Figure 47. C.D. spectra of a) control digitonin-isolated particles containing systems I and II, suspended in 0.01 M KCl, 0.05 M Tris-HCl buffer, pH 7.8 (solid line), b) particles after 1 minute of incubation in buffer to which had been added 2×10^{-4} M linolenic acid (broken line), and c) particles after 1 minute of incubation in buffer to which had been added 2×10^{-4} M linolenic acid, followed by 1 minute incubation in the presence of 20 mg/ml defatted BSA (dotted line).

Insert to Figure 47:

ultraviolet spectra of the aforementioned particles (see Materials and Methods for exact details of procedure).

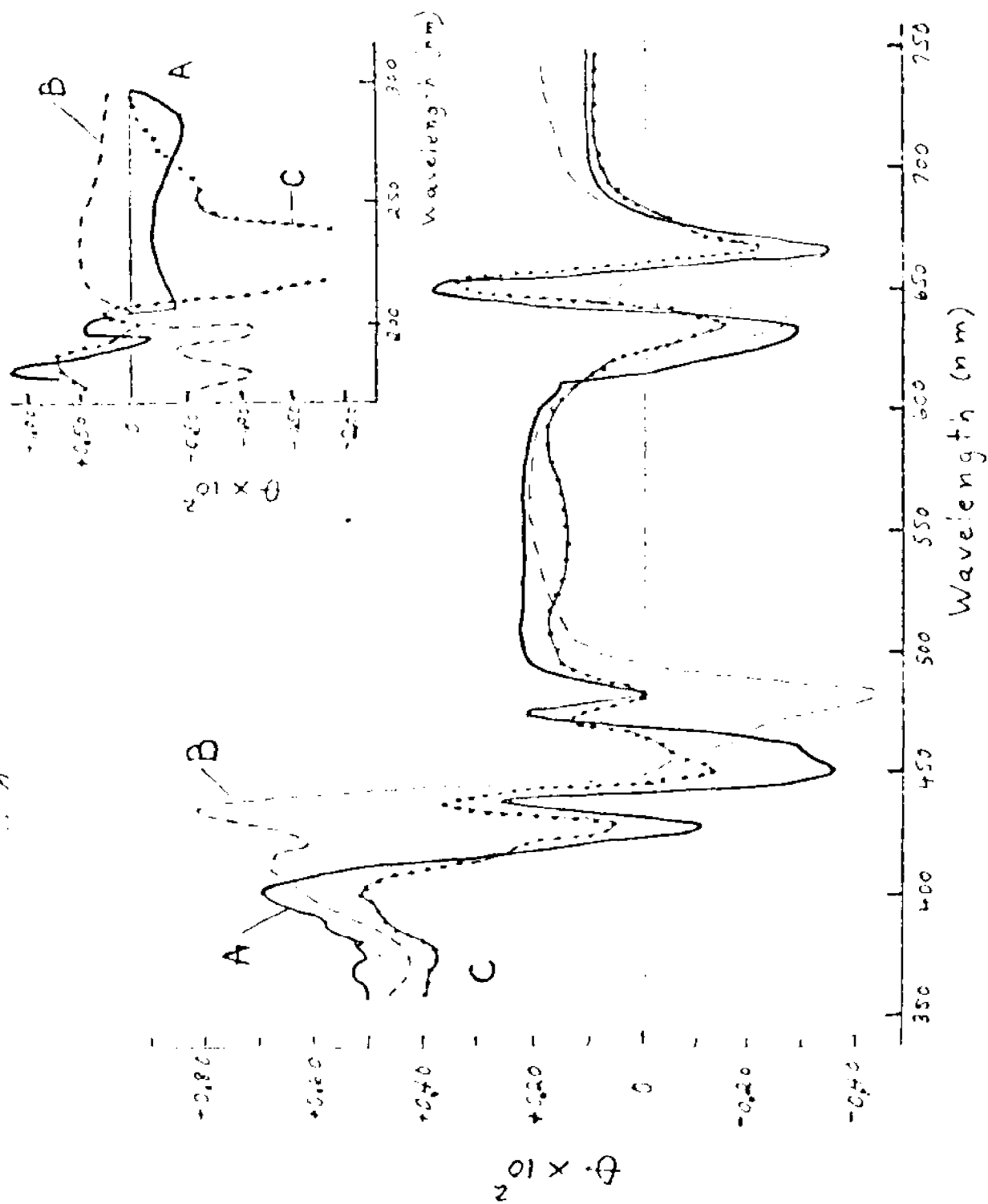
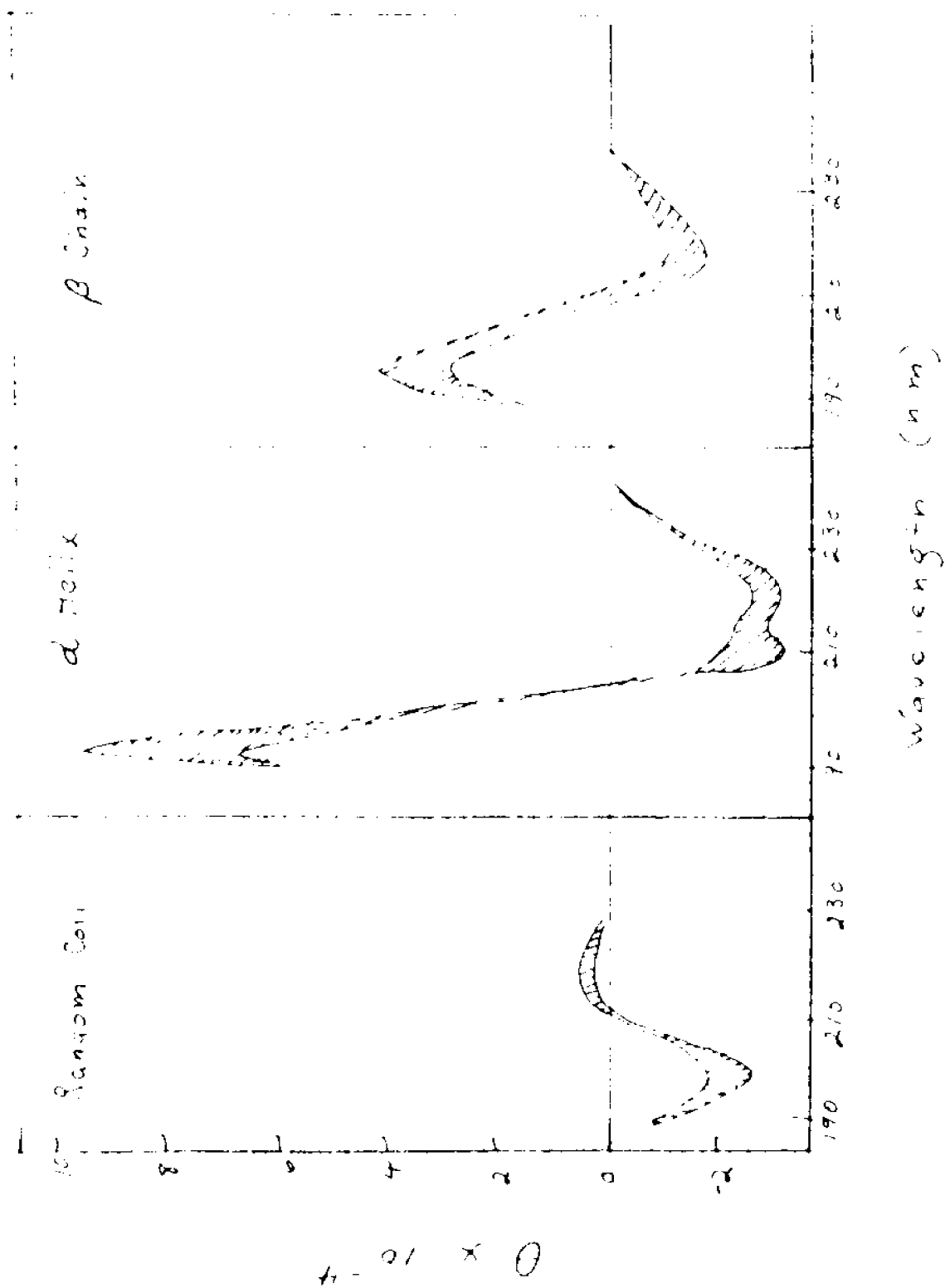


Figure 48. Ultraviolet C.D. spectra of a) random coil, b) alpha helix and c) beta extended chain of polyglutamic acid in aqueous solution. Curves redrawn from Gratzer and Lowburn (1966).



From the insert in Fig. 47, it may be seen that control particles yield a C.D. spectrum having primarily α helix attributes. The sharp positive peak with maximum at 190 nm is characteristic of alpha coil, as is the negative maximum at 210 nm. The slightly positive inflection, with maximum at 215 nm - also attributable to α helix structure - is observed in the control to be only a shoulder on the increasingly steepening slope of the long wavelength side of the 210 negative maximum (the basis of this steep slope in the present work is not understood).

The characteristics of the random coil are such that it can be readily recognized - compare published curve with curve B - obtained when particles are treated with fatty acids. There is a negative band with maximum at 198 nm which rises to a broad, slightly positive plateau from 210 - 220 nm. The addition of BSA* to the linolenic acid-treated particles gives C.D. structure much more characteristic of a helix rather than a random coil. The spectrum contains (a) a broad, slightly positive band with maximum beginning at about 210 (and extending to shorter wavelengths) having a shoulder at 220 nm, and (b) a pronounced negative maximum at 230 nm rising to a broad plateau beginning at 250 nm and is unlike any C.D. spectrum¹ I have seen described in the literature. Although the nature of this helix cannot be identified with certainty at the present time, it is interesting to note, in this connection, that Gratzer and Cowburn (1966) have stated "Whereas the characteristics of the alpha helix and random state are quite universal, the β form is variable, probably because of the fairly generous possibilities of geometric variation within this general type of structure".

* BSA itself gives a C.D. spectrum of an α helix.

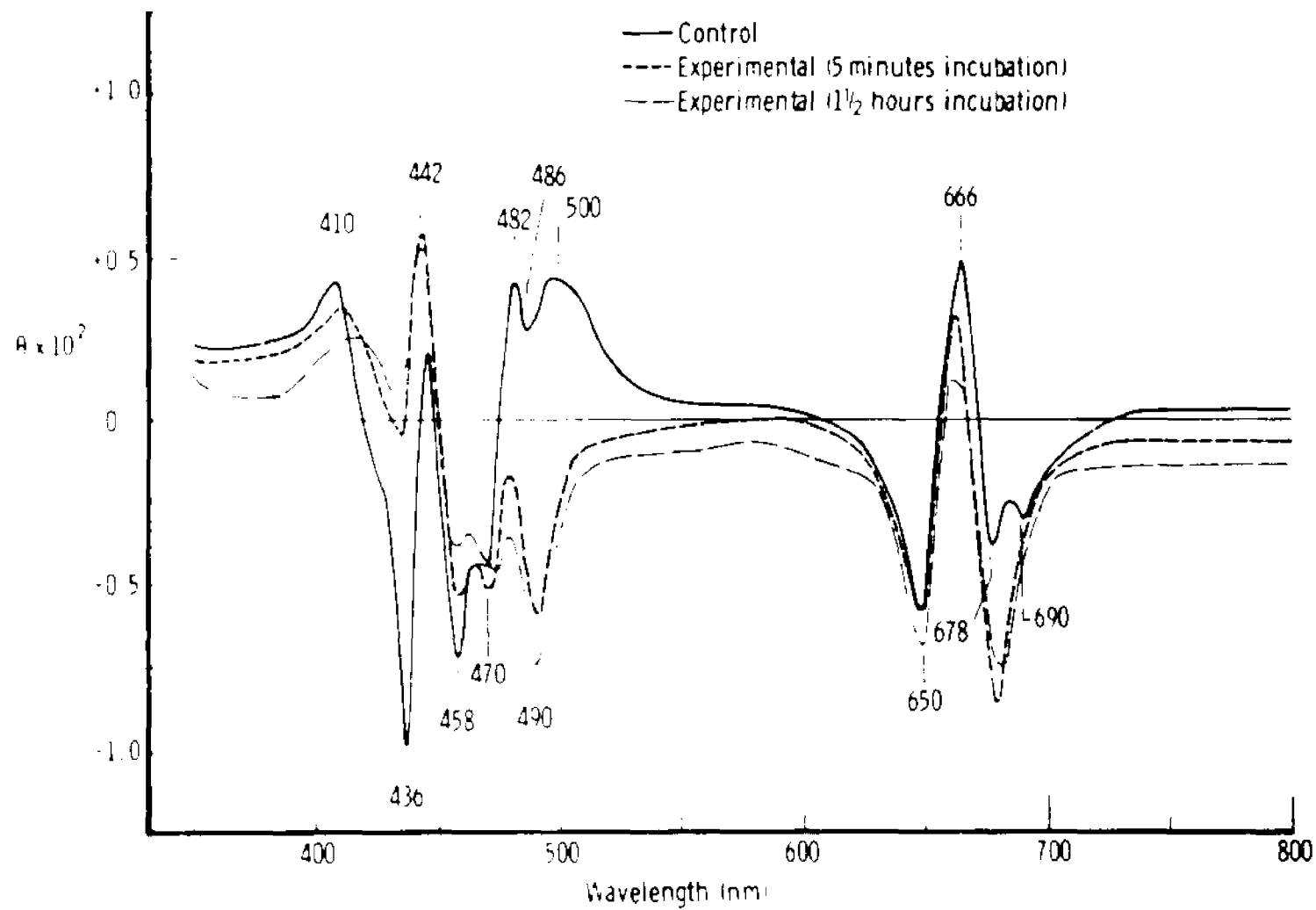
2) Effect of Linolenic Acid on Subchloroplast Particles, Containing Systems I and II, and Isolated by Means of Sonication.
(Solid line)

In Fig. 49 A is seen the C.D. spectrum for control subchloroplast particles suspended in STN buffer, pH 7.8, according to the method of McCarty (1968). The chlorophyll concentration of these particles was 2.1×10^{-5} M and the Chl a/b ratio was 2.52. There is observed in the red region of the spectrum, a negative chlorophyll band with maximum at 650 nm (probably a dimer), a positive band at 666 nm, and two negative bands at 678 and 690 nm, these latter two bands forming peaks around a more positive trough, itself having a peak at 683 nm. Interpretation of this latter peak will be considered in the section dealing with the effect of linolenic acid on intact spinach chloroplasts.

If one examines the blue region of the spectrum, one notes (as in the case of the digitonin-isolated particles containing systems I and II) that the band at 486 nm has positive ellipticity, in contrast to the negative ellipticity found at 486 nm in the digitonin-isolated system I particles. Moreover, if a comparison is made between the spectra obtained with these subchloroplast particles, and the C.D. spectra of barley quantasomes as observed by Dratz et al. (1966), it can be seen that the bands at 482, 486, and 500 nm are positive in our preparation and negative in theirs (Dratz et al., op. cit.). These differences in sign (not mirror image*) may be related to the greater structural complexity of these particles than of quantasomes - the structural complexity of the former perhaps confirming increase in light scattering.

* By mirror image is meant the same positioning of the C.D. bands, but with a difference in sign. For example, the mirror image of a band having a positive peak at 482 nm would be a band having a negative peak at 482 nm. In the case of Dratz et al., there is not a negative peak at 482 nm but rather a displacement of the entire region to a negative direction.

Figure 49. C.D. spectra of a) control subchloroplast particles (isolated by means of sonication) suspended in STN buffer, pH 7.8 (solid line), b) particles after 5 minutes of incubation in STN buffer to which had been added 2.1×10^{-4} M linolenic acid (line given by short dashes) and c) particles after $1 \frac{1}{2}$ hours of incubation in STN buffer to which had been added 2.1×10^{-4} M linolenic acid (line given by long dashes). Chlorophyll concentration = 2.1×10^{-5} M; cuvette path length = 1 cm.

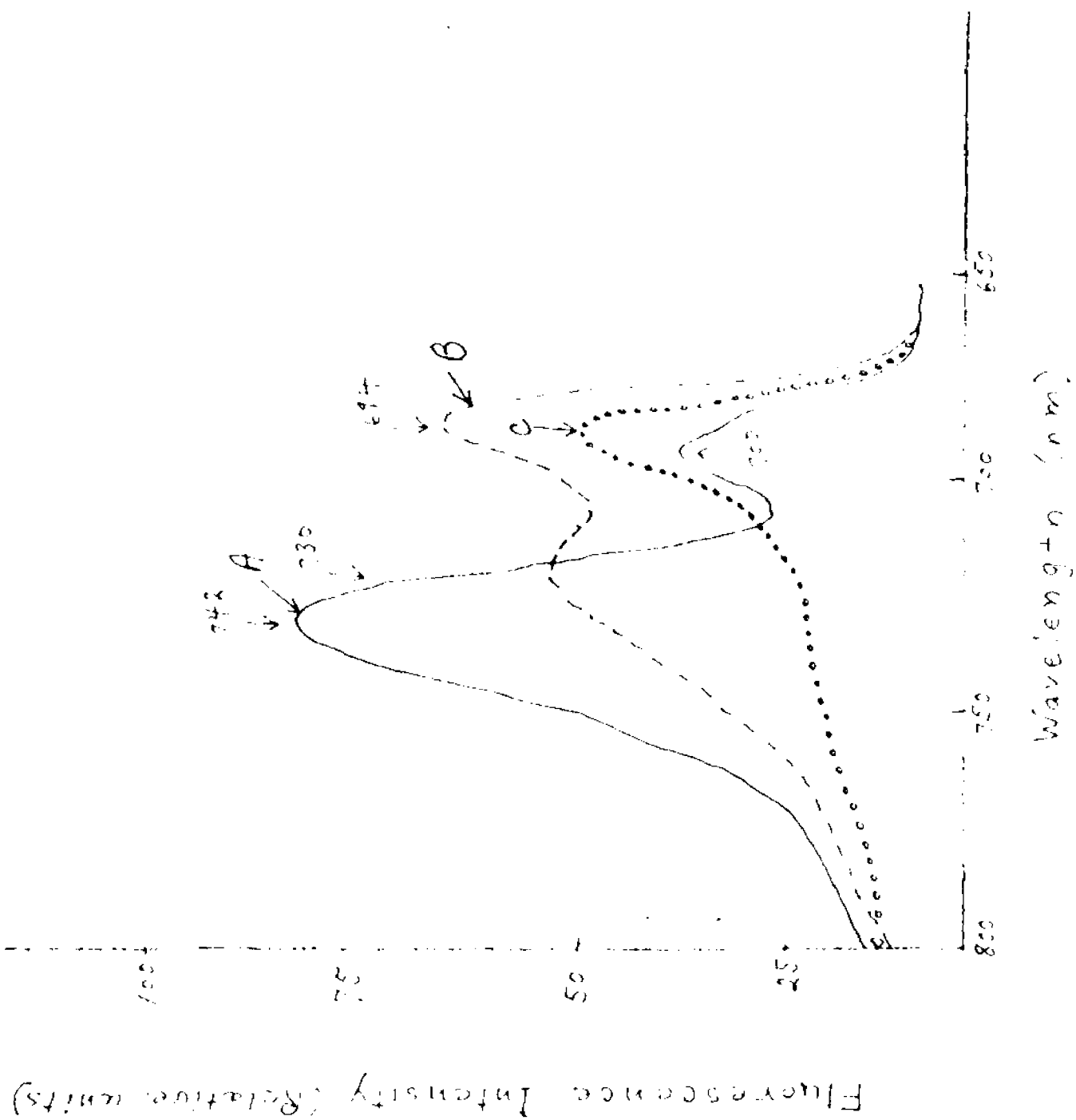


In the course of the present work, some preliminary experiments were made in which talc was introduced into suspensions of system I digitonin-isolated particles suspended in buffer. Along with the increase in light scattering (absorbancy at 800 nm increased to 0.500 O.D. units compared to control values of 0.060 O.D. units) there were observed shifts in band positions, as well as the change from negative to positive ellipticity in the blue region of the spectrum. Increases in light scattering were also observed, if the concentration of the chromophore-bearing material was increased from the control value.

After 5 minutes of incubation in the presence of 2.1×10^{-4} M linolenic acid (see Fig. 49 B) there appears to be an increase and narrowing of the C.D. band at 678 nm and concomitant disappearance of the 683 nm inflection and the 690 nm band (however, see the paragraphs below). In the blue region of the spectrum there is observed a change in sign of ellipticity of the 482, 486, and 500 nm bands from positive to negative. This change in sign of ellipticity probably results from a reduction in light scattering by linolenic acid - which is in concert with the results of Molotkovsky and Zheskova (1966), who found that treatment of non-intact chloroplasts with fatty acids results in decreased (visible) light scattering. Longer incubation (60 ') in the presence of linolenic acid results in no further changes in peak positions but merely decreases in intensities of the C.D. bands (see Fig. 49 C).

In the absorption spectra associated with these C.D. changes, there is observed a decrease in, and shift of, the peak position in the maximum of the red region of the spectrum, from 680 to 675 nm, indicating the deaggregating effect of linolenic acid. This is confirmed in the 77°K fluorescence emission spectra (Fig. 50) in which disappearance of the (control) 742 nm long wavelength band is accompanied by increase

Figure 50. Fluorescence emission spectra of the samples given in Figure 49.
Fluorescence excited with light of 436 nm.



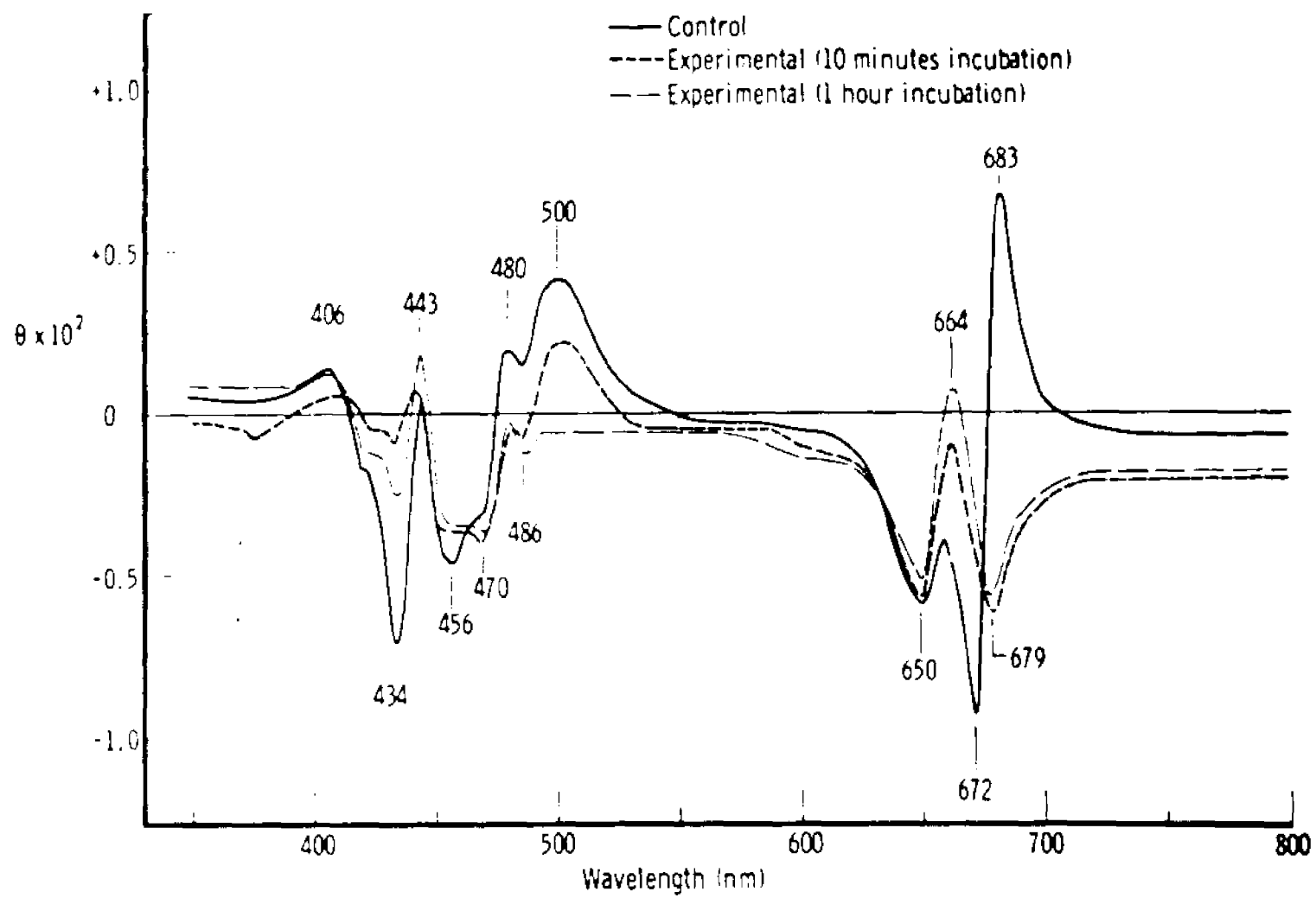
in fluorescence at 694 nm. In the fluorescence emission spectrum (at 77°K) for subchloroplast particles incubated with linolenic acid for 1 and 1/2 hours, there remains only one band with maximum at 695 nm.

c. Effect of Linolenic Acid on Intact Chloroplasts.

In Fig. 51 A is seen the C.D. spectrum of intact (i.e. envelope-surrounded) spinach chloroplasts suspended in STN buffer, pH 7.8; note the chlorophyll b band (probably dimer) at 650 nm. In these particles there are also observed bands at 663 nm and 672 (rather than 679) nm. Predominant in the spectrum is a large positive band seen at 683 nm. (This band explains 683 inflection between the "troughs" at 678 and 690 nm in broken (i.e. subchloroplast particle) isolated by means of sonication; refer back to Fig. 49). The "troughs", thus are formed by the superimposition of the residual (positive) 683 nm absorbing form of chlorophyll on a negative minimum somewhere between 678 and 690 nm. The occurrence of the band at 672 rather than 679 nm may be explained if one postulates that the 672 nm band is associated with the 683 nm band, as part of a chlorophyll a dimer doublet different from that of the dimer doublet at 664 - 679 nm. In any case, the 679 component of the latter would be hidden by the 672 and 683 nm bands.

Evidence for the existence of this distinct species of aggregate having C.D. bands at 672 and 683 nm is obtained when "intact" spinach chloroplasts are incubated for 10 minutes at 22.5°C in the presence of linolenic acid at FA/Chl concentration of 10 (see Fig. 51 B). It is seen that the C.D. bands at 672 and 683 nm disappear, the 664 nm band becomes more positive, and the 679 nm band is revealed. That the 664 and 679 nm bands belong to one species of chlorophyll, and the 672 - 683 nm bands belong to another species, is in agreement with the recent data obtained by French et al. (1971) in which derivative absorption

Figure 51. C.D. spectra of a) control chloroplasts suspended in STN buffer, pH 7.8 (solid line), b) chloroplasts after 10 minutes of incubation in STN buffer to which had been added 1.1×10^{-4} M linolenic acid (line given by short dashes) and c) chloroplasts after 1 hour of incubation in STN buffer to which had been added 1.1×10^{-4} M linolenic acid (line given by long dashes). Chlorophyll concentration = 1.3×10^{-5} M; cuvette path length = 1 cm.



spectra have revealed the presence of these four bands in chloroplasts of higher plants.

When the number of chloroplasts in suspension is increased, so that the final chlorophyll concentration is 3×10^{-5} M (rather than 1.3×10^{-5} M, as in Fig. 51) there are shifts in C.D. band maxima (from 672) to 676 - 678 nm and (from 683) to 688-9 nm.

Incubation of intact chloroplasts in the above concentration of linolenic acid for 1-1/2 hours results only in further changes along the lines observed with shorter incubation time.

B. Concentration Dependence of the Linolenic Acid-Induced Change in F 735/F 698 as a Function of the Structural Complexity of Chlorophyll-Containing Systems.

From the previous section on C.D., it is apparent that with increasing structural complexity (of the chlorophyll-containing system under investigation), the ratio of linolenic acid to chlorophyll, required to bring about equivalent change in ellipticity, also increases. A similar parallelism was noted between increasing structural complexity and the amount of linolenic acid needed to reduce R (the F 735/F 698 at 77°K) to one half its control value, (Changes in R, it was observed, occurred in complex chlorophyllous systems at much lower concentrations of fatty acid than did changes in C.D.; in the latter case, limits of solubility of fatty acid sometimes made it impossible to obtain data on the complex systems comparable to those on the simpler systems, and therefore, computations were limited to changes in R.)

In Table II may be seen a tabulation of the FA/Chl ratio required to bring about a change in F 735/F 698 to one half its initial value, as measured at 77°K. As can be seen from this table, as the level of structural complexity increases, the ratio of FA/Chl necessary to bring

TABLE II

Change in F 735/F 698 (at 30°C for five minutes of incubation in linolenic acid) in chlorophyllous systems of increasing structural complexity.

Material Under Investigation	Chlorophyll Concentration M/L	Linolenic Acid Concentration M/L	(1) F 735/F 698 Control	(2) F 735/F 698 5 Minutes of Incubation	Protein Concentration μ g/ml.	(2)/(1)	Fa/Chl
1) Chlorophyll <u>a</u> in CCl_4	2×10^{-3}	1×10^{-3}	44	30	0	0.70	0.50
	"	2×10^{-3}	"	15	"	0.50*	0.90*
2) System I Particles (digitonin-isolated)	2×10^{-5}	4×10^{-5}	1.15	1.03	150.3	0.90	2.00
	"	1×10^{-4}	"	0.50	"	0.53	5.00
	"	2×10^{-4}	"	0.35	"	0.50** 0.30	6.00** 10.00
3) Particles Containing Systems I and II (Isolated by sonication)	2×10^{-5}	1×10^{-4}	2.34	2.22	74.8	0.95	5.00
	"	1.5×10^{-4}	"	1.50	"	0.64	7.50
	"	2.0×10^{-4}	"	0.80	"	0.50*** 0.35	8.70*** 10.00
4) Spinach Chloroplasts	2×10^{-5}	2×10^{-4}	1.70	0.96	73.6	0.57	10.00
5) <u>Chlorella</u> Cells	2×10^{-5}	2×10^{-3}	1.90	0.90	211.5	0.47	100.00
6) <u>Euglena</u> Cells	2×10^{-5}	2×10^{-3}	30.00	17.00	206.4	0.57	100.00

*, **, *** obtained by interpolation

about the specified changes also increases from 1 (in structurally simple systems such as chlorophyll a in CCl_4) to 100 (intact algal cells). From the section above (Section IV-C) dealing with the effects of linolenic acid on the C.D. of chlorophyll-containing systems, it was noted that linolenic acid induces changes in the protein portion of BP 700 chlorophyll-protein complexes, preliminary to its effect on chlorophyll chromophores. Indeed, there is evidence (effect of fatty acids on mitochondria, Packer et al., 1968; effect of fatty acids on the chloroplast envelope, Cohen et al., 1969) to suggest that fatty acids act not only on the protein portion of the chlorophyll chromoprotein, but also with extra-chloroplast protein. As the structural complexity of the chlorophyll-containing system increases, the amount of "non-specific" protein also increases, and consequently, there is an increment in the ratio of FA/Chl necessary to bring about changes (in fluorescence emission) at rates comparable to those systems which are structurally simpler.

It is interesting to note that, in the case of system I digitonin-isolated particles, in which the protein concentration is quite high, the required ratio of fatty acid/chlorophyll to reach $3_{1/2}$ is small compared to the other chlorophyllous systems. One possible interpretation is that the protein in these particles is fairly specific, i.e. mostly that to which chlorophyll is attached, whereas in the case of intact cells, much of the fatty acid is being absorbed by non-specific protein.

V. EFFECT OF LINOLENIC ACID ON LOW TEMPERATURE (77°K) FLUORESCENCE OF ALGAL CELLS AND THYLAKOIDS.

A. Effect of Linolenic Acid on the Fluorescence Emission of Algal Cells and Thylakoids.

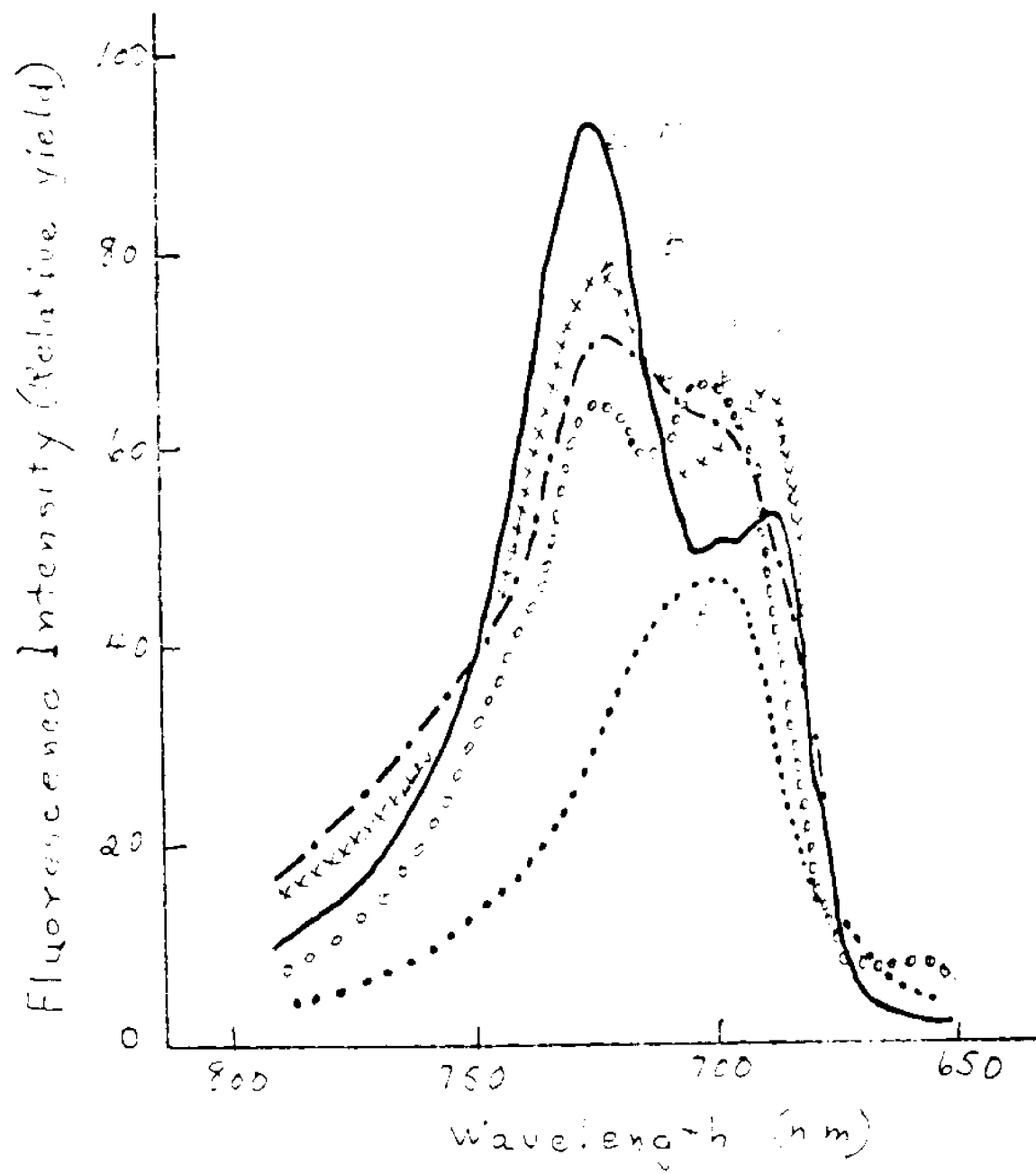
In Section III, investigations of the effects of linolenic acid on

spinach chloroplasts, and fractions derived thereof, demonstrate that the addition of this fatty acid results in decrease of the low temperature fluorescence emission at 735 nm accompanied by increase in emission at 698 nm. In part 1 of the present section, I present results of investigations of the effects of linolenic acid on intact cells and thylakoids of a green photosynthetic alga (Chlorella), comparing these results with those obtained with spinach chloroplasts, and then (in parts 2 and 3) contrasting these results with those obtained from investigations with Porphyridium and Anacystis organisms containing accessory pigments (namely phycobilins) other than chlorophyll b.

1. EFFECT OF LINOLENIC ACID ON THYLAKOIDS AND CELLS OF CHLORELLA PYRENOIDOSA.
 - a. Effect of Linolenic Acid on Fluorescence Emission of Thylakoids Derived from Chlorella Cells.

In Fig. 52 are shown the fluorescence emission spectra (excited with light of 536 nm) which result when linolenic acid is introduced into a suspension of Chlorella thylakoids (obtained by sonication). In the control, given in Fig. 52 A, is seen a predominant long wavelength band with maximum at 723 nm. The position of this long wavelength maximum differs from that seen in spinach chloroplasts - in the latter, the maximum occurs at 735 nm (refer back to Fig. 2 A); however, both have been ascribed to aggregated forms of chlorophyll (Brody and Brody, 1963). In Fig. 52 A there are also evident bands having emission maxima at 698 and 687 nm. After addition of linolenic acid, at a mole/mole ratio of 10 FA/1 Chl. (Fig. 52 B), there is seen in the spectrum (incubation at 30°C for 0 minutes) an increase in emission at 698 nm, and a strong decrease in emission and shift in long wavelength maximum from 723 to 719 nm - the latter probably indi-

Figure 52. Fluorescence emission spectra (measured at -196°C) of Chlorella thylakoids suspended in 0.04 M Tris-0.01 M NaCl buffer, pH 7.8 at 30°C . Curve A: control thylakoids suspended in buffer; Curves B - E: thylakoids incubated in 3×10^{-4} M linolenic acid (in buffer) for 0, 10, 15, , and 60 minutes respectively. Chlorophyll concentration = 3×10^{-5} M. Excitation with light of 436 nm.

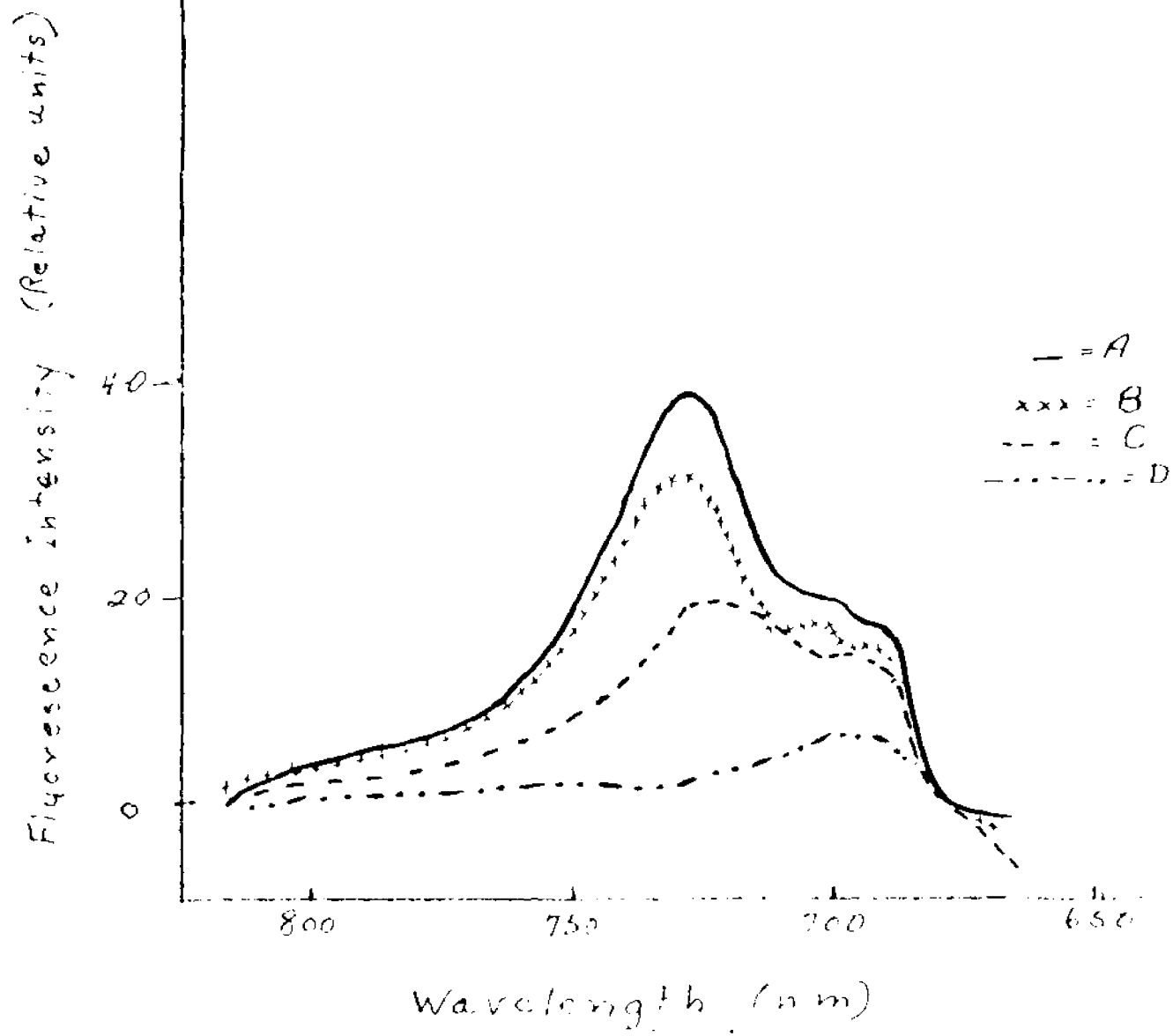


cating conversion of larger to smaller size aggregates with further incubation (for 15 minutes) there is observed very large increase in fluorescence at 698 nm. Longer incubation times result in narrowing of the band(s) and decreases in total fluorescence yield (see Curves C - E).

b. Effect of Linolenic Acid on Fluorescence Emission of Intact Chlorella Cells.

If a comparison is made between the effects of linolenic acid on thylakoids isolated from Chlorella and on intact Chlorella cells, the following is observed. In the control (see Fig. 53 A), the ratio of F_{725}/F_{698} is 1.75 in Chlorella thylakoids compared to 1.90 for Chlorella cells (in both cases, the chlorophyll concentration was 2×10^{-5} M). The fluorescence emission at 687 nm is, on the other hand, somewhat less in the cells than in the thylakoids (i.e. F_{698}/F_{687} is 1.13 compared with 0.90). Upon incubation in linolenic acid (at FA/Chl ratio of 100, as compared with FA/Chl of 10 in the case of thylakoids; see above paragraph) the (considerable) decrease in fluorescence emission at 725 nm is accompanied by decrease at 698 nm. However, as in the case of Chlorella thylakoids incubated in the presence of linolenic acid there is a decline in F_{725}/F_{698} with time of incubation; i.e. both bands decrease in intensity, but the long wavelength band decreases more rapidly. The reason for the lack of increase in F_{698} in cells, as compared with thylakoids, may be related to the relatively rigid cell wall in the former. Although Molotkovsky and Zheskova (1966), and Cohen et al. (1969), showed that fatty acids can induce swelling in spinach chloroplasts, it is possible that the degree of swelling needed to convert aggregates to monomers simply does not occur when a pressure restriction such as that offered by an intact cell wall is present. In

Figure 53. Fluorescence emission spectra (measured at -196°C) of Chlorella cells suspended in 0.04 M Tris-0.01 M NaCl buffer, pH 7.8 at 30°C . Curve A: control cells suspended in buffer; Curves B - D: cells incubated in 2×10^{-3} M linolenic acid (in buffer) for 0, 3, and 10 minutes, respectively. Chlorophyll concentration = 3×10^{-5} M. Excitation with light of 436 nm.



this regard, it is interesting to note, however, that with both intact cells and chloroplasts of Euglena, linolenic acid appears to induce no increase in fluorescence at 698 nm, although there is a decline in intensity of long wavelength fluorescence.

While it is conceivable that the pellicle in Euglena is sufficiently rigid to explain this observation, one must look elsewhere for an explanation in regard to the chloroplast envelope. One possible explanation for this lack of increase in F 698 might be a strong difference in kinetics of the decrease in total fluorescence emission (area under the curve), compared with those obtained for the rate of change of the individual fluorescence bands; recall Section IV-A-1c, Fig. 23. Perhaps in Euglena chloroplasts (and even intact cells) the rate of change of total fluorescence is so rapid that any increase in F 698 is overridden by the decrease in total area under the curve.

2. EFFECT OF LINOLENIC ACID ON CELLS AND THYLAKOIDS OF PORPHYRIDIUM ORIENTUM.

In Figs. 54 and 55, respectively, are shown the fluorescence emission spectra of intact Porphyridium cells in which system I pigments (presumably chlorophyll a + carotenoids) are excited by 436 nm light, and system II pigments (presumably phycoerythrin) by 546 nm light, in the absence and presence of linolenic acid. If we turn our attention to Fig. 54, we note that, in the control, excitation with 435 nm light results in a three-banded emission - at 696, 712 and 740 nm - only when the organism is very young - see for example Krey and Govindjee (1966). The single-banded emission spectrum, such as that shown in the present work, results from intense emission from that form of chlorophyll which is preponderantly in the aggregated form, in older organisms. When excitation is with 546 nm light (phycoerythrin sensitization) we note that in the control,

Figure 54. Fluorescence emission spectra (measured at -196°C) of Porphyridium cells suspended in 0.04 M Tris-0.01 M NaCl buffer, pH 7.8 at 30°C . Curve A: control cells suspended in buffer; Curves B - C: cells incubated in 2×10^{-3} M linolenic acid (in buffer) for 10 and 20 minutes, respectively. Chlorophyll concentration = 2×10^{-5} M. Excitation with light of 436 nm.

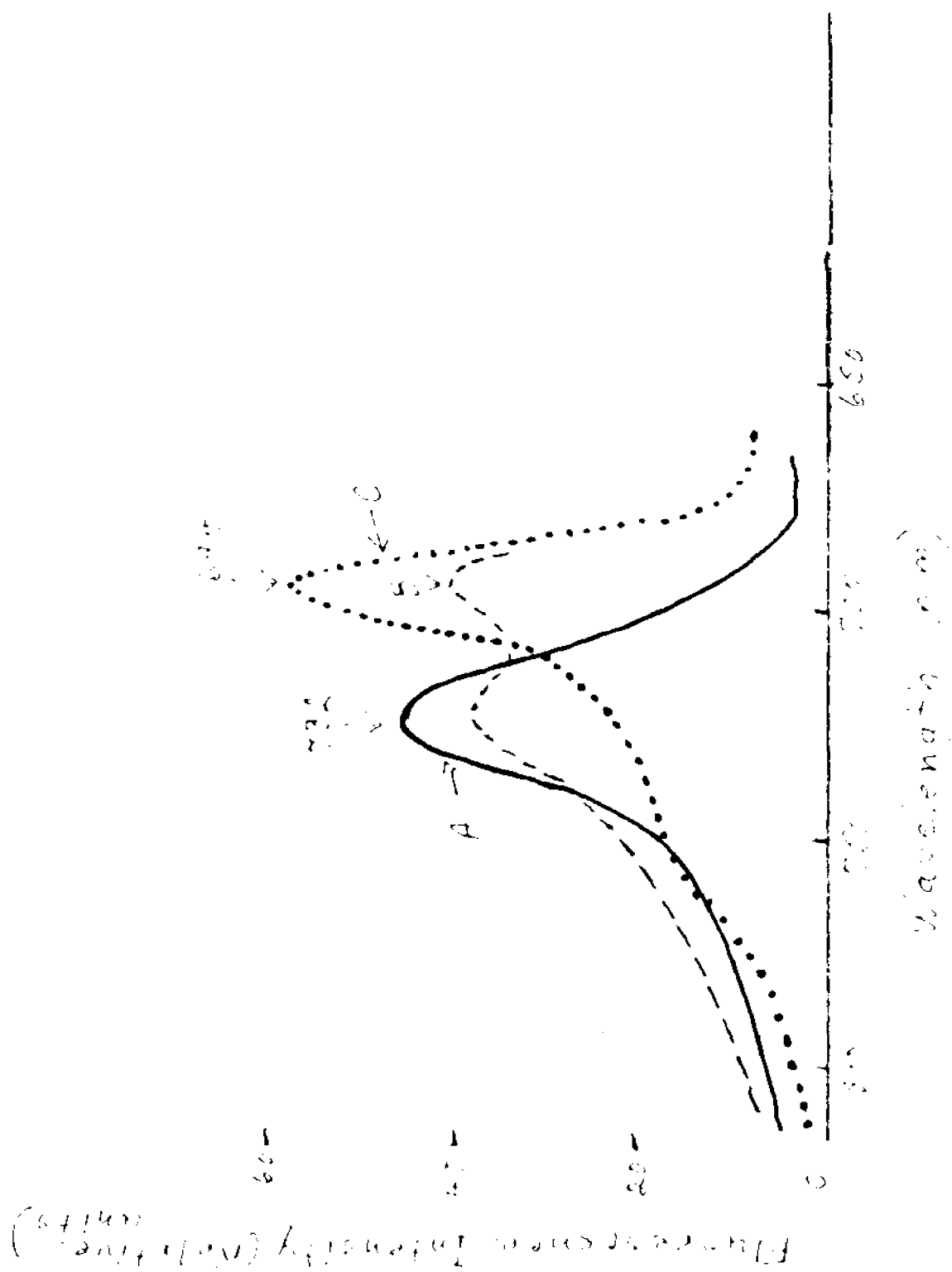
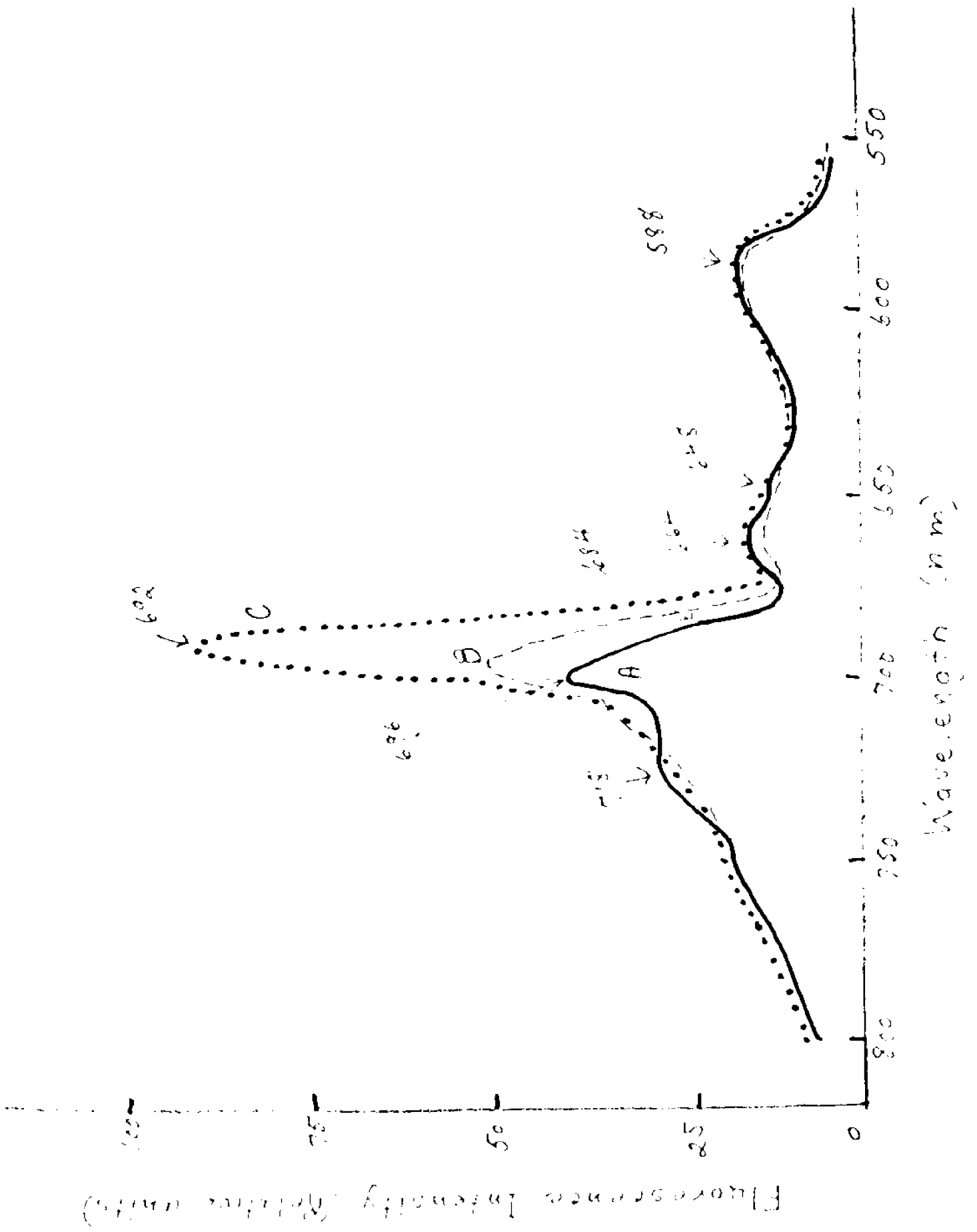


Figure 55. Conditions for fluorescence emission curves as in Figure 54; however excitation was with light of 545 nm.

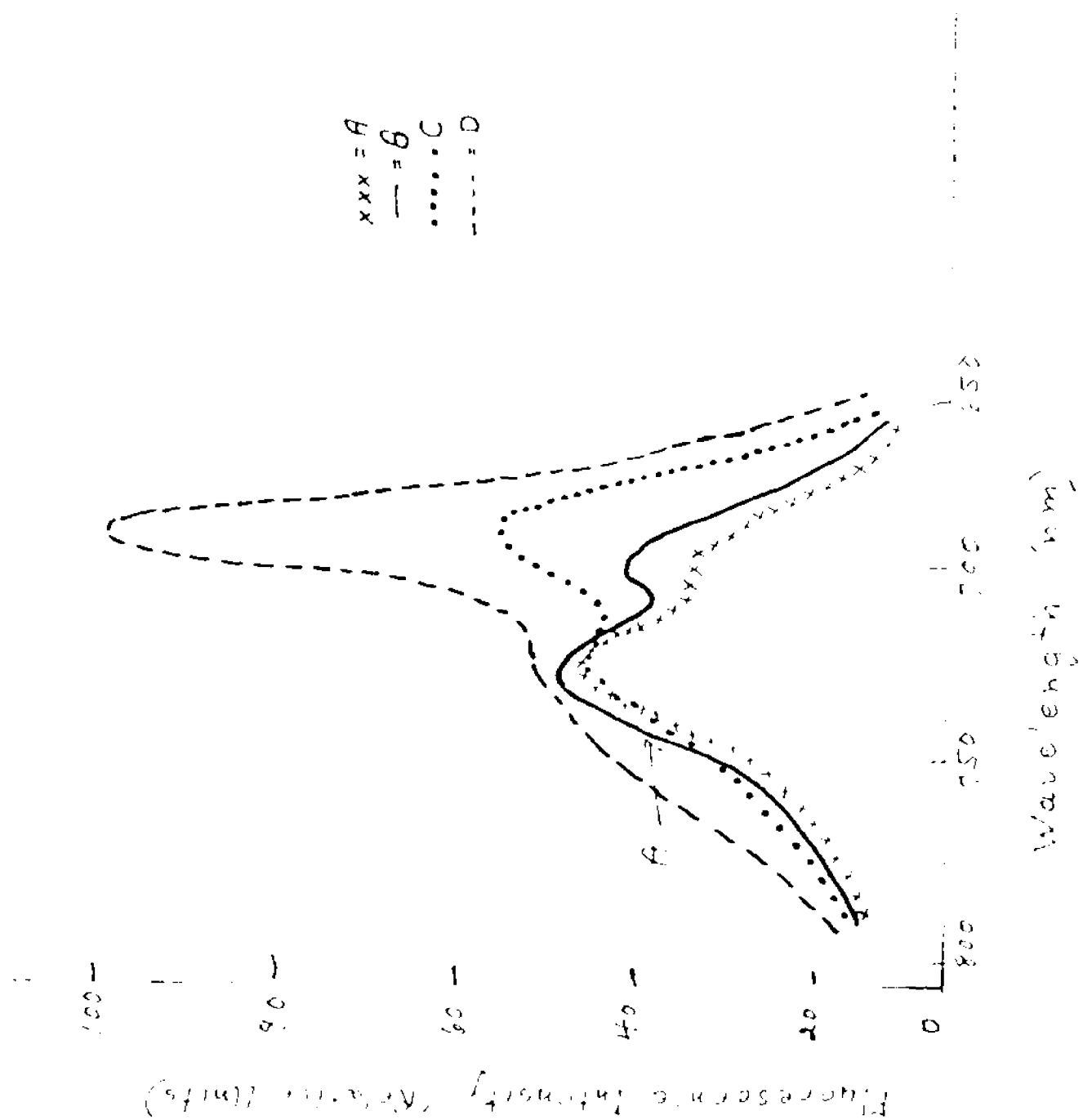


there are evident two (probably) phycocyanin bands at 648 and 664 nm and four chlorophyll bands with maxima at 685, 696, 718 and 740 (?) nm. Only weak phycoerythrin fluorescence may be observed to result from phycoerythrin excitation at 588 nm - showing the tight coupling of energy transfer between the two photosystems. As might be expected, excitation of system II results in much more intense emission of system II chlorophylls, at 684 and 696 nm, than does indirect sensitization by 436 nm light.

With the introduction of linolenic acid, excitation with 436 nm light reveals the same sort of phenomena observed with green photosynthetic organisms. The single band with maximum at 728 nm is converted into a single band with maximum at 694 nm (monomer). Although in green organisms, the monomer band has a maximum at 693 nm, in the phycobilin-containing organisms (see also Anacystis, below) the monomer band is at slightly shorter wavelengths. In Fig. 55 we note that with addition of fatty acid, all bands at 740, 718, 696 and 684 nm lose their discreteness and become converted (and/or enveloped) into the intensifying monomer band (maximum at 692 nm). Indeed, in this case, it seems as though the relative fluorescence yield has actually increased. (While no data can be offered in support of the following contention at the present time, it may be that this increase in yield is based on the aggregated forms of chlorophyll in Porphyridium having a lower yield than the monomeric form(s).) As far as emission from phycobilins is concerned, there seems to be little, if any, effect of fatty acids. In this case, the general question about the effect of fatty acids on water-soluble pigments will be considered in relation to C.D. of Anacystis (see below).

From a comparison of 436 nm excitation of control thylakoids and intact cells (Fig. 56 A), we note that isolation already results in

Figure 56. Fluorescence emission spectra (measured at -196°C) of Porphyridium thylakoids suspended in 0.04 M Tris-0.01 M NaCl buffer, pH 7.8 at 30°C . Curve A: control thylakoids suspended in buffer; curves B - D: thylakoids incubated in 2×10^{-4} M linolenic acid (in buffer) for 0, 5 and 10 minutes, respectively. Chlorophyll concentration $\sim 2 \times 10^{-5}$ M. Excitation with light of 436 nm.



some structural changes, because there can be observed, in addition to a band with maximum at 725 nm, a monomeric band(s) with maximum at 692 nm. Further indication of the disruptive influence of isolation may be obtained by comparing the 546 nm excitation of control thylakoids (Fig. 57 A) with that of intact cells. In the control thylakoids, there is virtually no emission at wavelengths corresponding to aggregated forms of chlorophyll, there being only a single band with maximum at 690 nm. Additionally, phycobilin fluorescence has become predominant, resulting from an uncoupling of energy transfer from system II to system I, with subsequent re-radiation of light absorbed by the phycoerythrin.

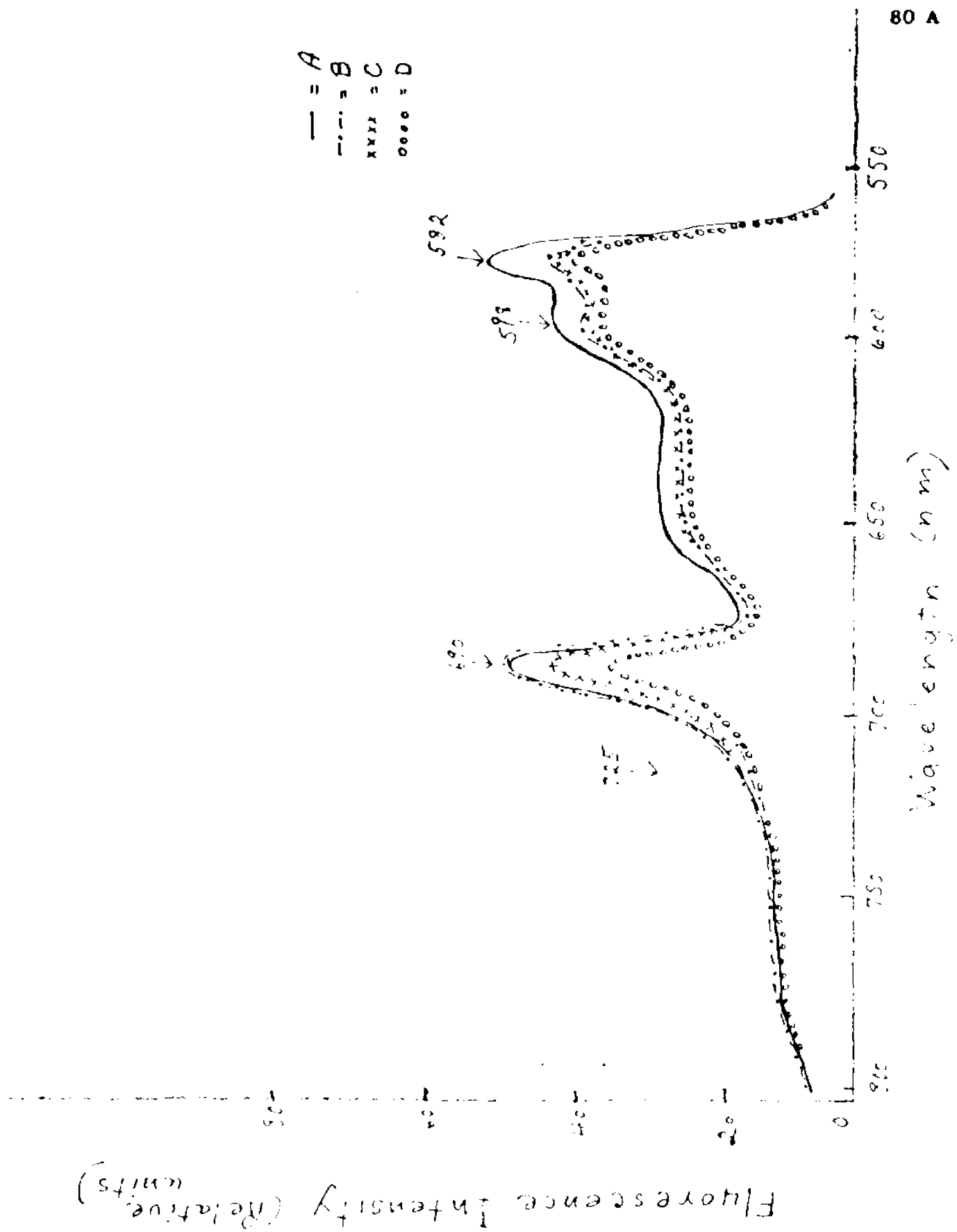
Excitation with 436 nm light reveals that addition of fatty acids to thylakoids results in further conversion of aggregated to monomeric chlorophyll, see Fig. 56 B-E (as it does also in the case of whole cells). Excitation with 546 nm light reveals that addition of fatty acid to thylakoids results in the same sort of fluorescence emission changes as occur in intact cells of Porphyridium. However (as also observed with Chlorella), the concentration of linolenic acid necessary to induce a similar rate of change is 10 fold in the case of Porphyridium cells, compared to thylakoids.

3. EFFECT OF LINOLENIC ACID ON THYLAKOIDS AND CELLS OF ANACYSTIS NIDULANS.

a. Effect of Linolenic Acid on Intact and Formaldehyde-Treated Cells of A. nidulans.

Phycoerythrin, the major phycobilin constituent of the blue-green alga A. nidulans, has absorption bands at 580, 625, and 634-637 nm (Cho and Govindjee, 1970), while the minor constituent, allophycocyanin (also present in Porphyridium), has no absorption maximum at 650 nm. Energy transfer between these phycocyanin bands and chlorophyll is more tightly coupled than is energy transfer between phycoerythrin and chlorophyll in Porphyridium (the absorption maximum of phycoerythrin is at 545 nm

Figure 57. Conditions for fluorescence emission curves as in Figure 56; however, excitation was with light of 545 nm.



in Porphyridium; Brody and Emerson, 1959), in major part, because of greater spectral overlap, and to some lesser degree, (probably) because of closer physical proximity (spatial relationship).

As a consequence of the tight coupling between the phycocyanin pigments and chlorophyll a_{II}, and between chlorophyll a_{II} and chlorophyll a_I, excitation with light absorbed at 578 nm results in considerable emission from the aggregate at 728 nm (see Fig. 58) in contrast to the situation that obtains with system II, sensitization of Porphyridium (see Fig. 55, control cells). In addition to this emission from the aggregate, there is also slightly more intense emission from the monomer (system II) at 692 nm, as well as weak phycocyanin emission at 660 nm (see Fig. 58 A). Excitation of control, intact, Anacystis cells with 436 nm light (see Fig. 59 A) results primarily in long wavelength emission at 728 nm, a condition similar to that which obtains when Porphyridium is excited with blue light.

Since I observed with Porphyridium, that the procedure for isolation of thylakoids results in some loss of water-soluble pigments and uncoupling of energy transfer between system I¹ and system I, and since it had been reported by Ludlow and Park (1969) that glutaraldehyde (or formaldehyde) fixation results in stabilization of phycobilin proteins on the chlorophyll-bearing thylakoids*, I decided to prepare Anacystis thylakoids from such formaldehyde-treated cells because I wanted to determine whether fatty acids could, on their own, affect the states of phycobilins (as well as chlorophyll). Inasmuch as formaldehyde-treated cells were to be used for preparation of thylakoids, the effect of such treatment on the fluorescence emission of Anacystis cells, both in the absence and presence of linolenic acid, was investigated.

* Additionally, Ludlow and Park (op. cit.) showed that such formaldehyde-treated (or glutaraldehyde-treated) cells were still active in the Hill reaction.

Figure 58. Fluorescence emission spectra (measured at -196°C) of Anacystis cells suspended in 0.04 M Tris-0.01 M NaCl buffer, pH 7.8 at 30°C . Curve A: control cells suspended in buffer; Curves B - D: cells suspended in 1.6×10^{-3} linolenic acid (in buffer) for 0, 10, and 60 minutes, respectively. Chlorophyll concentration = 1.26×10^{-5} M. Excitation with light of 578 nm.

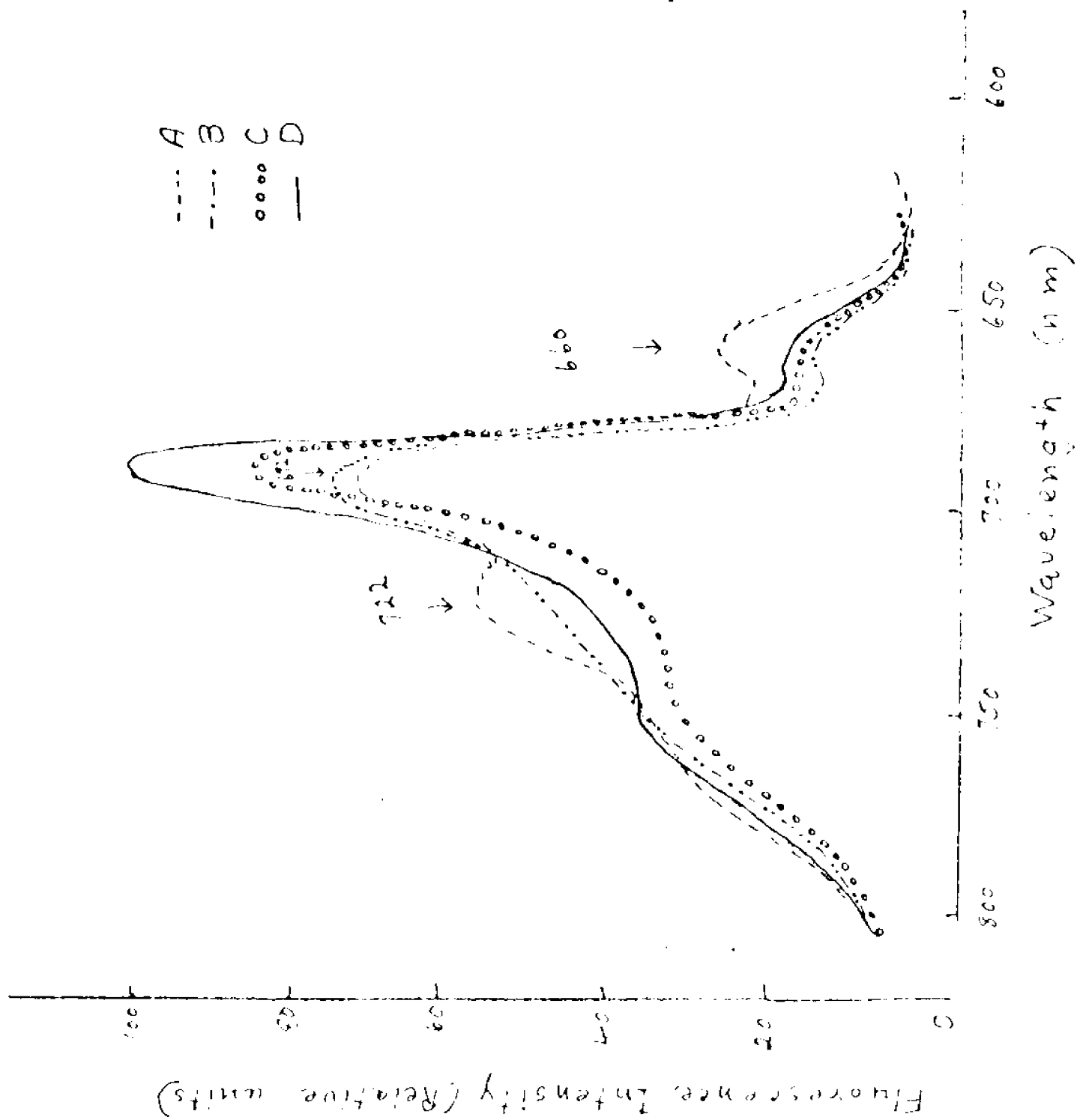
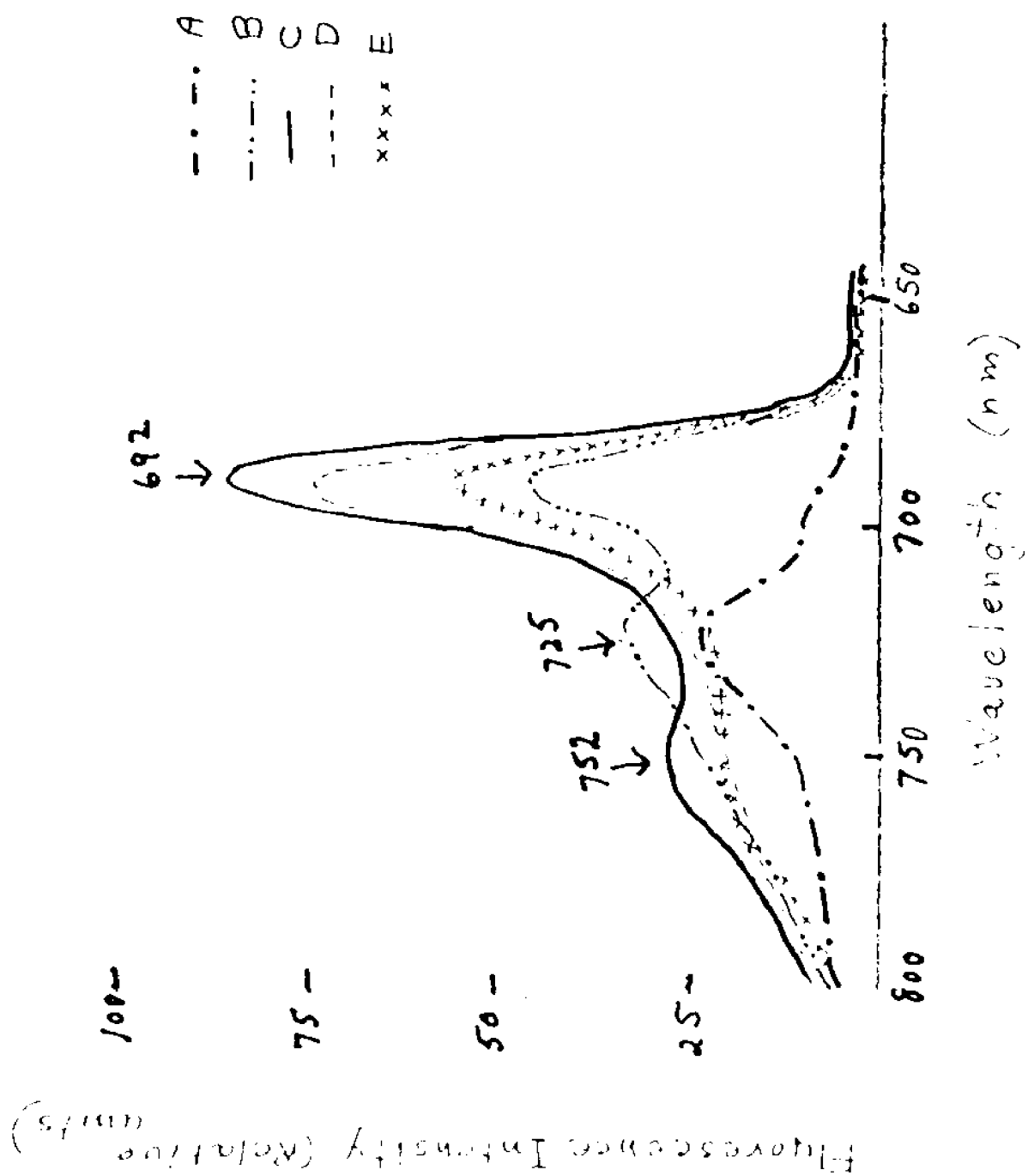


Figure 59. Conditions for fluorescence emission curves as in Figure 58 (additionally, an emission spectrum is given for 30 minutes of incubation); however, excitation was with light of 436 nm.



In Figs. 60 and 61 are shown the fluorescence emission spectra of formaldehyde-fixed Anacystis cells, excited with 578 and 436 nm light, respectively. The effect of linolenic acid treatment is also shown in these figures. If the emission spectrum, excited by 578 nm light, is compared between "control" formaldehyde-treated cells and control unfixed cells (see Figs. 60 A and 58 A, respectively), one notes that treatment with formaldehyde has resulted in reduction in intensity of the 692 nm band, and increase in intensity of the 728 nm (aggregate) band. A comparison of the controls of fixed and unfixed cells excited with 436 nm light, can also be made (see Figs. 61 A and 59 A); excitation with system I light results in emission spectra which appear to be identical in the two cases (a single emission band with maximum at 728 nm), in contrast to the situation which obtains when such cells as illuminated with 578 nm light. Thus it would seem that formaldehyde is affecting only the phycobilin chromoproteid (or transfer between the phycobilins and chlorophyll), but not the chlorophyll chromoproteid.

If one now looks at the effect of linolenic acid treatment on fixed and unfixed Anacystis cells, it is seen that essentially similar changes occur. In Figs. 58 B-D are shown the sequential changes resulting when Anacystis, treated with linolenic acid, is excited with light of 578 nm; there is noted a decrease in aggregate fluorescence (at 728 nm) and an increase in fluorescence at 692 nm. These changes are also observed in the case of formaldehyde-fixed Anacystis cells subsequently treated with linolenic acid (Fig. 60 B-D). In both the fixed and non-fixed cells, there is noted a decrease in the fluorescence of the phycocyanin band at 660 nm. Also, a comparison of Figs. 59 and 61 B-E (excitation with 436 nm light) reveals that changes induced by linolenic acid in both fixed and unfixed cells are essentially similar; there is an

Figure 60. Fluorescence emission spectra (measured at -196°C) of Anacystis cells fixed in formaldehyde and suspended in 0.04 M Tris-0.01 M NaCl buffer, pH 7.8 at 30°C . Curve A: control formaldehyde-treated cells suspended in buffer; Curves B - D: cells suspended in 1.6×10^{-3} M linolenic acid (in buffer) for 0, 10 and 60 minutes, respectively. Chlorophyll concentration = 1.6×10^{-5} M. Excitation with light of 578 nm.

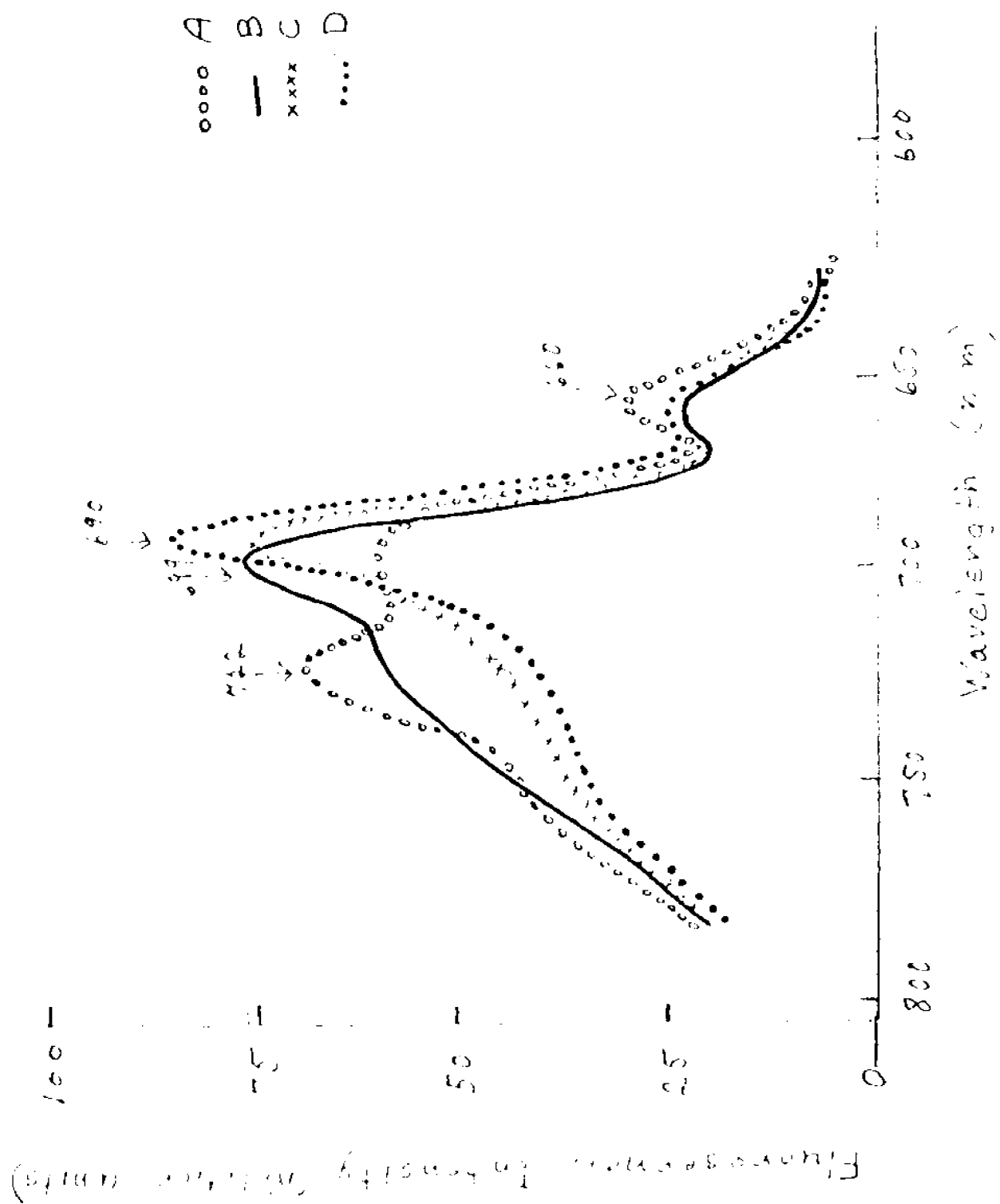
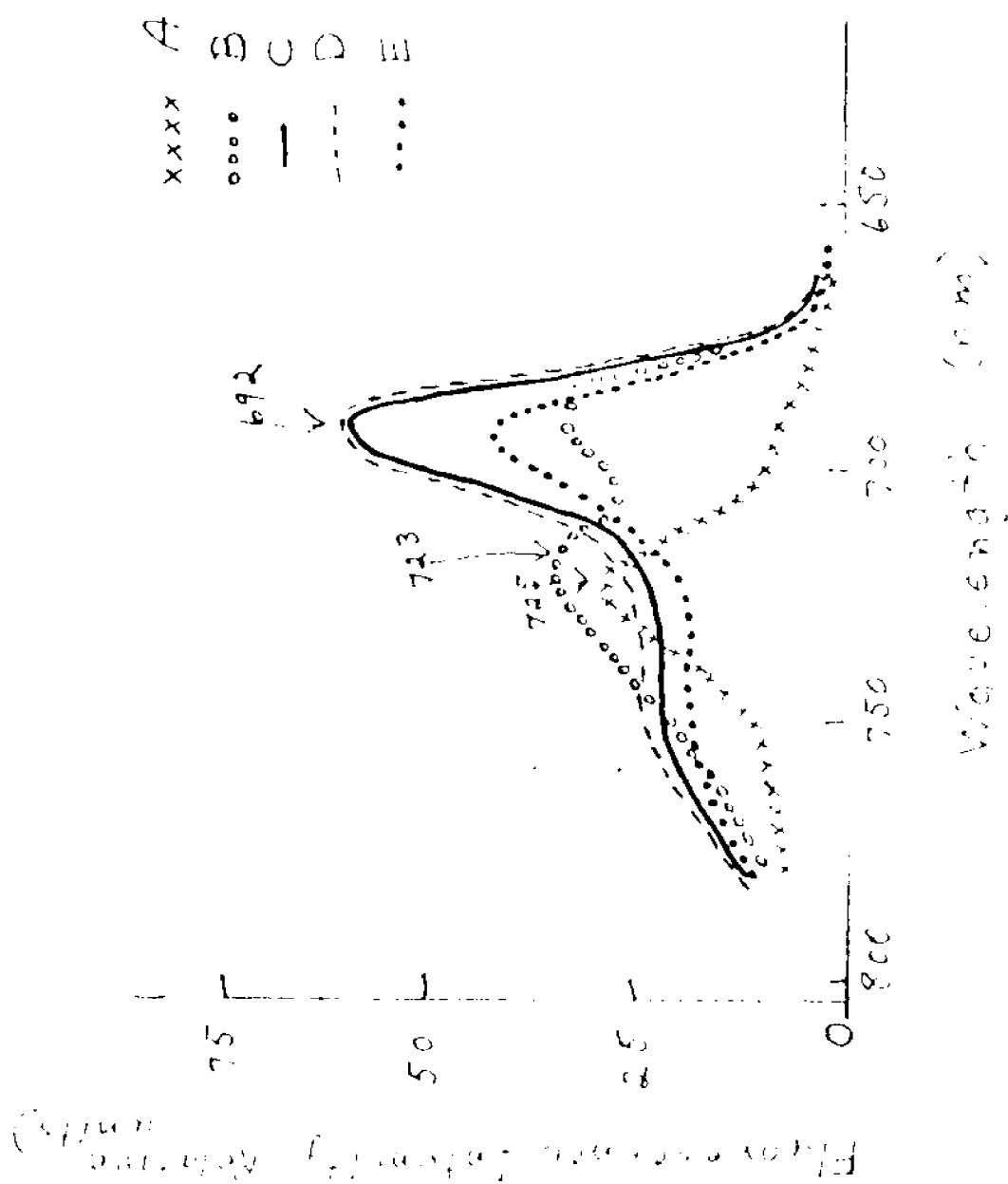


Figure 61. Conditions for fluorescence emission curves as in Figure 60; however, excitation was with light of 436 nm. (Additionally, an emission spectrum is given for 30 minutes of incubation.)



increase in fluorescence at 692 nm and a decrease in fluorescence at 692 nm and a decrease in fluorescence of the aggregate (at 725 nm).

That the decrease in fluorescence of the phycocyanin band (660 nm) which occurs upon introduction of linolenic acid to both fixed and unfixed cells is not solely due to a change in energy transfer, but to an effect on the phycocyanin chromoproteid(s) itself, may be seen in Fig. 62 - the O.D. of unfixed Anacyctis cells. In the control (Fig. 62 A), are observed bands at 679, 670, 440 and 436 nm (chlorophyll a), 550 (shoulder), 439, 465 and 436 nm (carotenoids), 650, 630, 628 and 617 nm (phycocyanin and allo-phycocyanin) (see Cho and Govindjee, 1970, for these assignments). Upon treatment with linolenic acid, at a concentration of 2×10^{-3} M (i.e. FA/Chl of 100, and FA/P.C. of 20), there are observed decreases in the O.D. of bands corresponding to absorption by phycocyanin (at 615, 628 and 634 nm) and allophycocyanin (650 nm) with appearance of the phycocyanin band at 593 nm. Due to the necessity of using such high concentration of fatty acids with algal cells, there is considerable increase in light scattering, with consequent effect on the O.D. especially in the blue region of the spectrum, leading to an apparent increase in absorption at 502 - 434 nm. This problem, as well as the presence of carotenoids, makes it impossible to detect changes in the core bands of chlorophyll. For a discussion of the effect of scattering on O.D., see section IV-C-3-b(2), above.

b. Effect of Linolenic Acid on Thylakoids Isolated from Intact and Formaldehyde-Treated Cells of A. nidulans.

From a comparison of the 578 excitation spectrum of intact cells, either unfixed or fixed in formaldehyde, with that of thylakoids isolated from unfixed cells (Fig. 63), we note that there results, as a consequence of the isolation procedure, a doubling of the intensity of

Figure 62. C.D. of a) control (unfixed) Anacystis cells suspended in 0.04 M Tris-HCl buffer, pH 7.8 (solid line) and b) cells suspended in buffer to which had been added 2×10^{-3} M linolenic acid (broken line). Chlorophyll concentration = 2×10^{-5} M; cuvette path length = 1 cm.

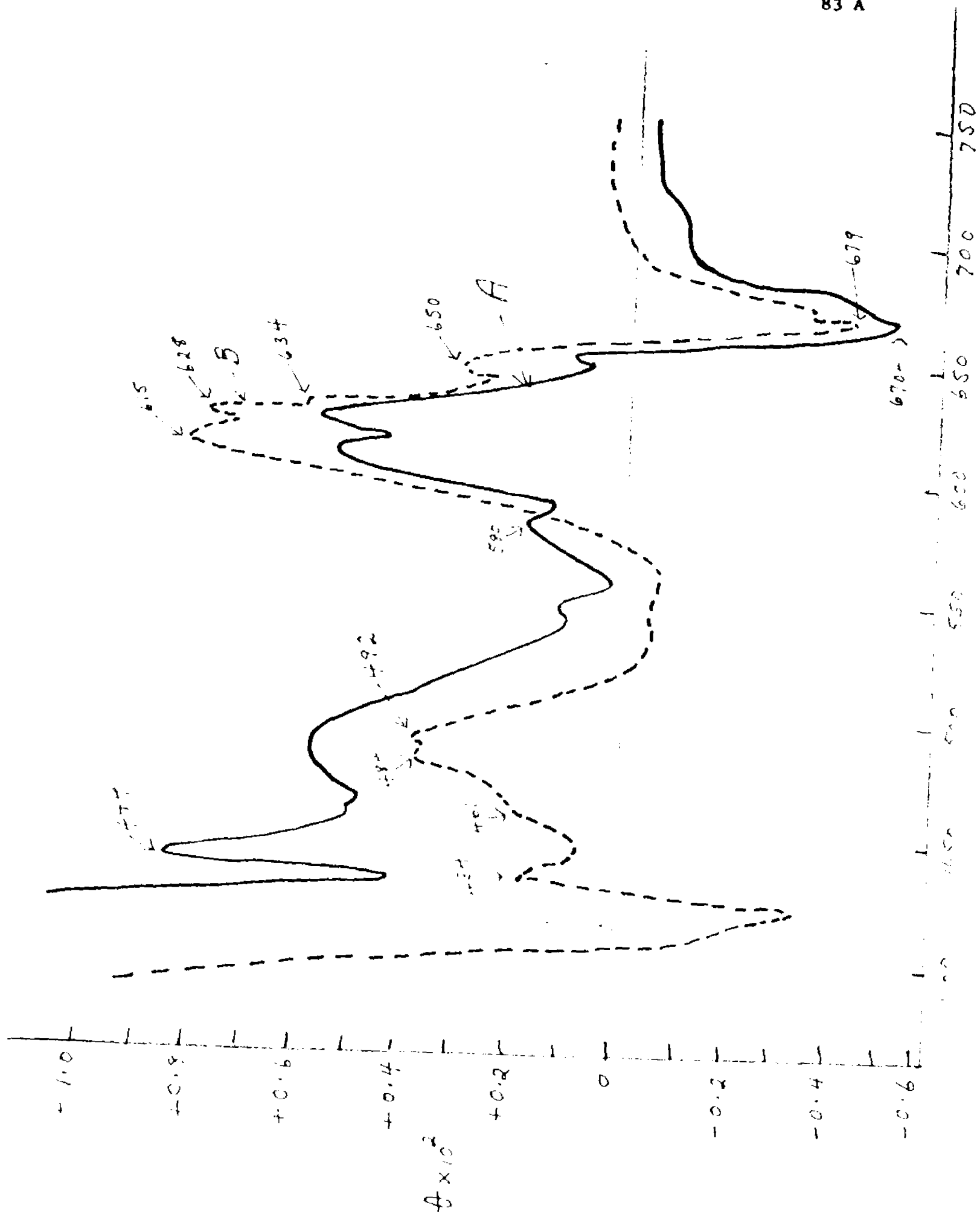
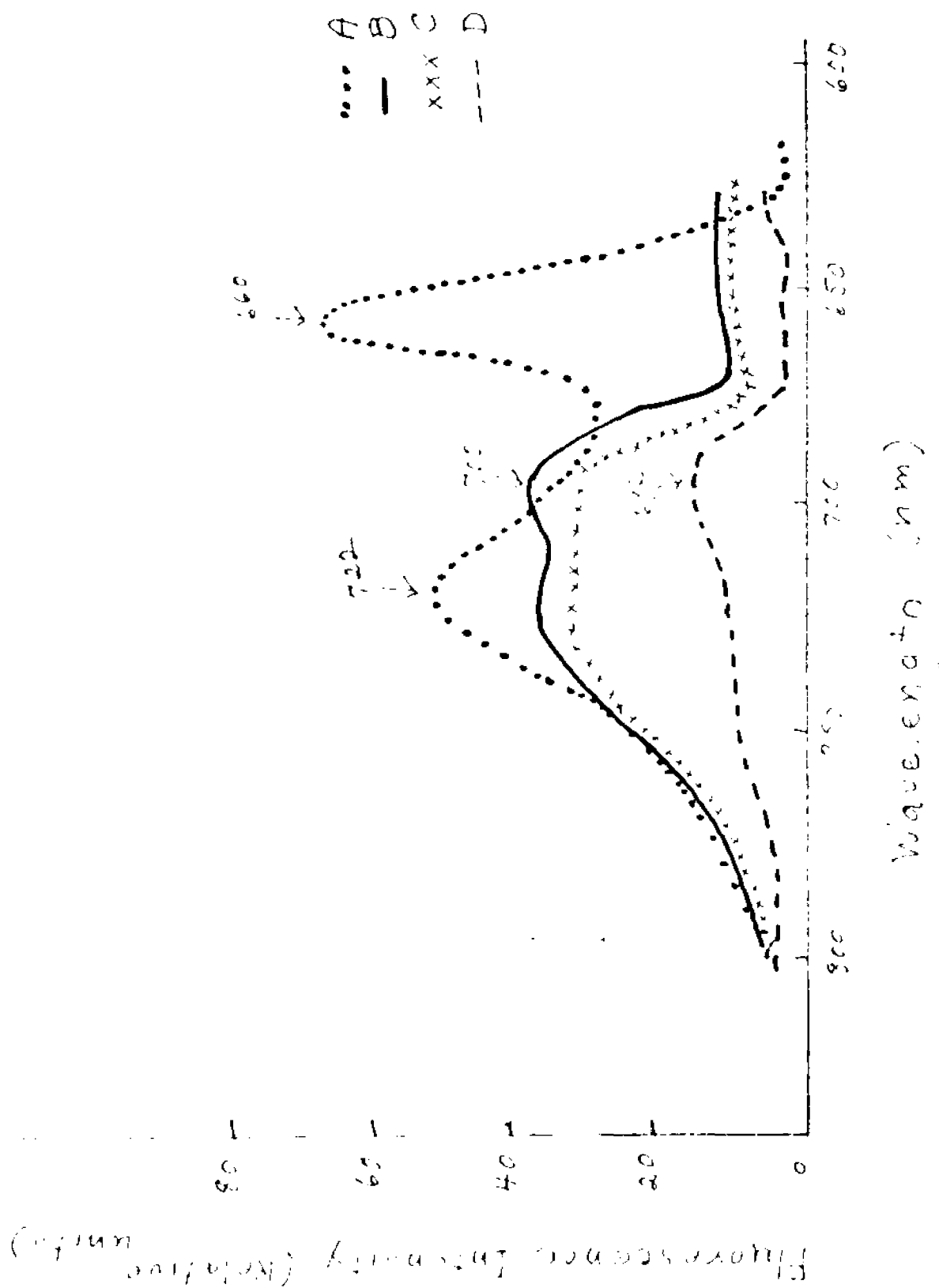


Figure 63. Fluorescence emission spectra (measured at -196°C) of Anacystis thylakoids isolated from non-fixed cells and suspended in 0.04 M Tris-HCl buffer, pH 7.8 at 30°C . Curve A: control thylakoids suspended in buffer; Curves B - D: thylakoids suspended in 1.3×10^{-4} M linolenic acid (in buffer) for 0, 30 and 80 minutes, respectively. Chlorophyll concentration = 1.3×10^{-5} M. Excitation with light of 578 nm.



fluorescence emission from phycocyanin (at 660 nm) as well as decreased emission of the chlorophyll monomer band with maximum at 692 nm. The increase at 660 nm is due to the uncoupling of energy transfer from phycocyanin to chlorophyll a_{II}, as a consequence of the disruptive effects of isolation. The effect of formaldehyde fixation of cells prior to isolation of thylakoids is seen in Fig. 65. Here, the level of phycocyanin fluorescence is comparable to that seen in (either fixed or unfixed) Anacystis cells, supporting the observation (Clement-Metral, 1971) that fixation of cells prior to isolation of thylakoids has a stabilizing effect on the attachment of the phycocyanobilins to the photosynthetic membrane.

If the 436 nm excitation spectrum of control thylakoids isolated from unfixed cells is compared to that obtained with intact cells (see Fig. 65 A), one notes that in both cases, there is only a single emission band, with maximum at 722 nm. The same situation is obtained with thylakoids isolated from fixed cells (see Fig. 66 A).

When thylakoids isolated from either unfixed or fixed cells are incubated in the presence of linolenic acid (see Figs. 65 and 66, respectively) excitation with 436 nm light reveals that, in both cases, there results a conversion of aggregated to monomeric chlorophyll. Excitation with 578 nm light shows that addition of fatty acid to thylakoids results in the same sort of changes as occur in intact cells of Anacystis (see Figs. 63 and 65).

B. Effect of Linolenic Acid on Fluorescence Excitation of Algal Cells and Thylakoids.

In section IV, investigation of the effects of linolenic acid on spinach chloroplasts revealed that the changes induced in the action spectrum for emission at 735 nm (measured at 770K) were decreased contri-

Figure 64. Conditions for fluorescence emission curves as in Figure 63; however, excitation was with light of 436 nm.

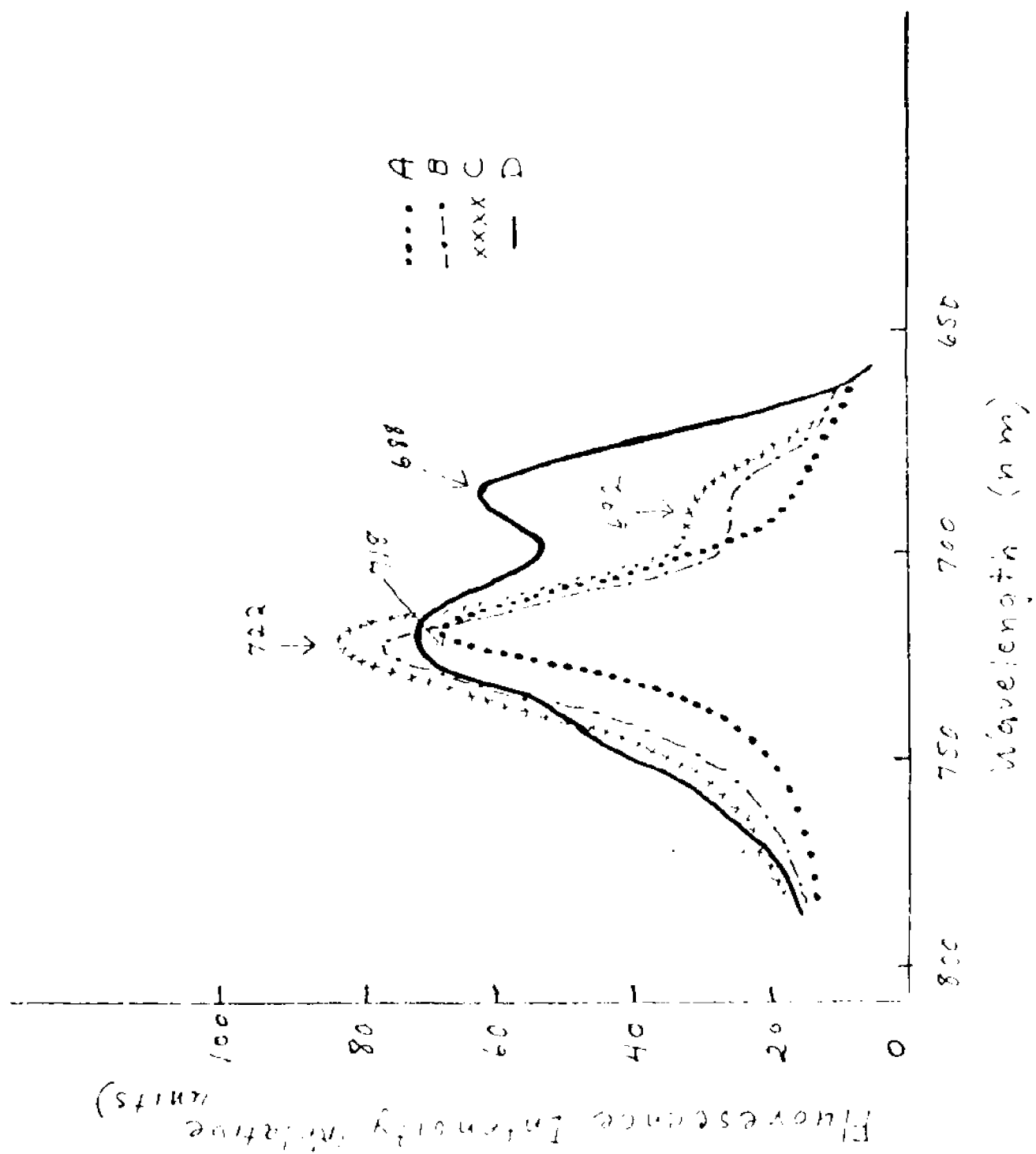


Figure 65. Fluorescence emission spectra (measured at -196°C) of Anacystis thylakoids isolated from formaldehyde fixed cells and suspended in 0.04 M Tris-HCl buffer, pH 7.8 at 30°C . Curve A: control thylakoids suspended in buffer; Curves B - D: thylakoids suspended in 1.3×10^{-4} M linolenic acid (in buffer) for 0, 30, and 80 minutes, respectively. Chlorophyll concentration = 1.3×10^{-5} M. Excitation with light of 578 nm.

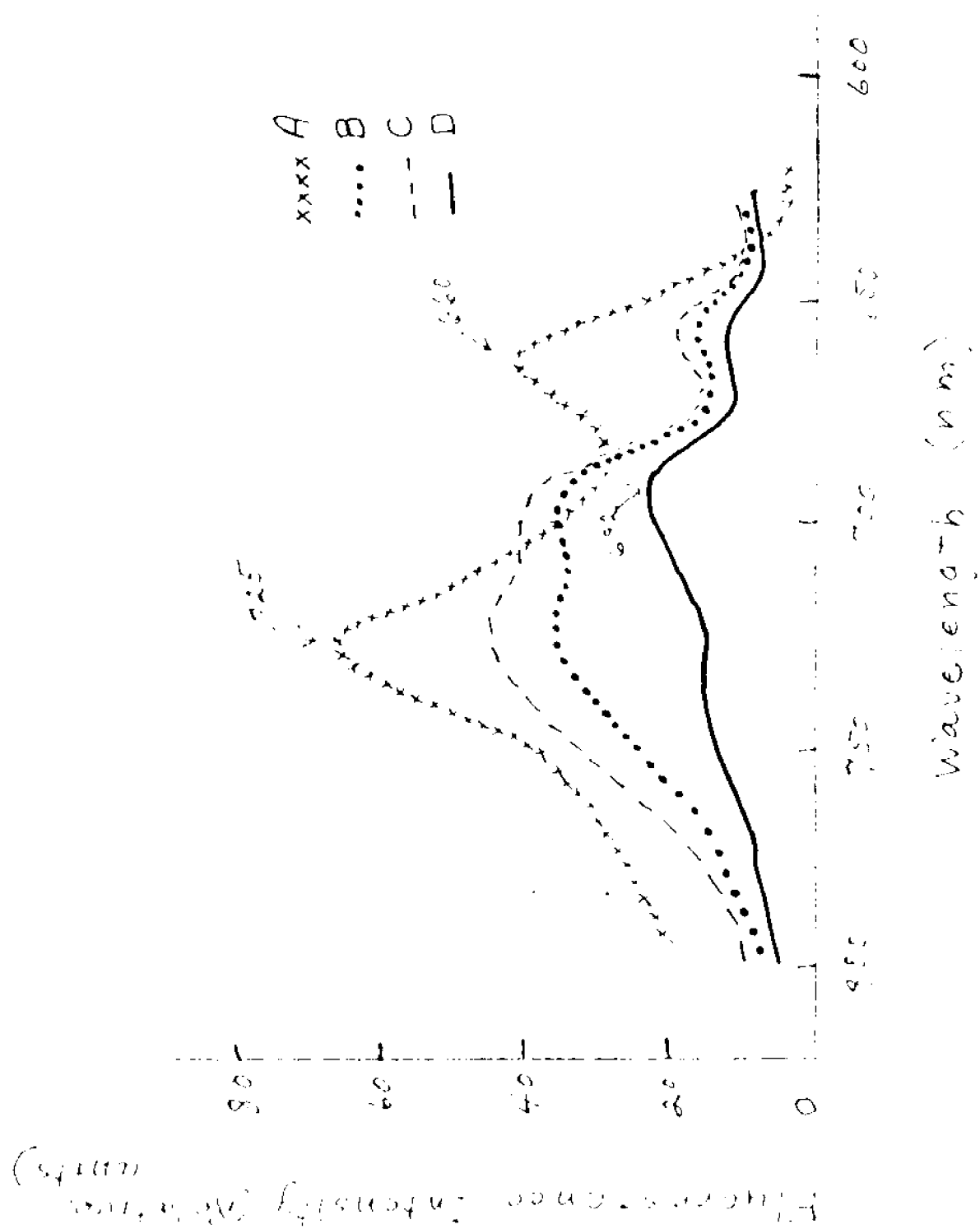
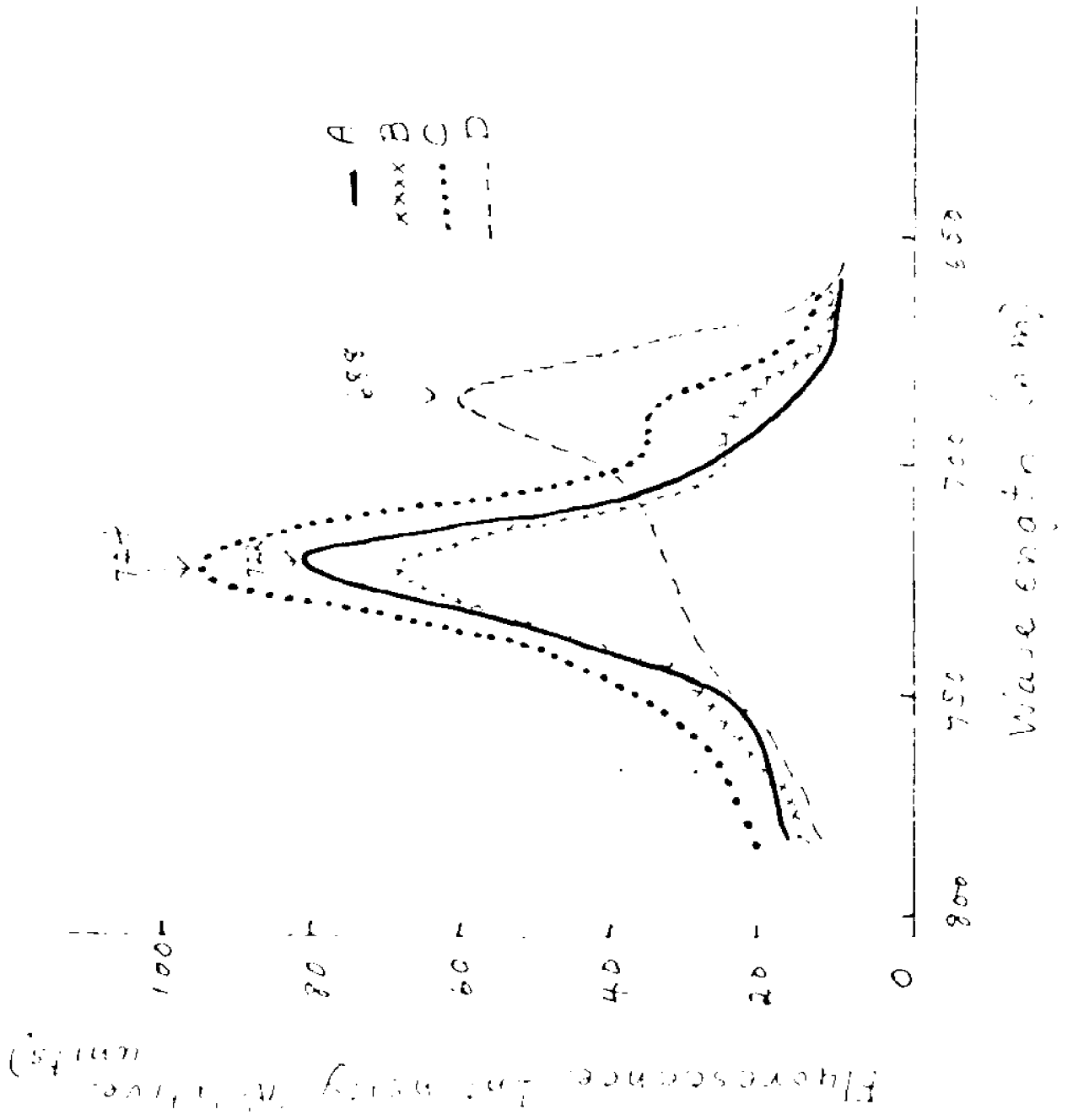


Figure 66. Conditions for fluorescence emission curves as in Figure 65; however, excitation was with light of 436 nm.



bution of the 682 and 705 nm excitation bands and increased contribution in the 674 nm band. However, the effects of linolenic acid on fluorescence excitation of system I⁺ pigments were not reported in this section. What follows below is a study of the effects of linolenic acid on fluorescence excitation of system I (725-30 nm fluorescence) and fluorescence excitation of system II (700 - 705 nm fluorescence*) in the green alga Chlorella. Results of a similar study with Anacystis are also presented and excitation patterns for both organisms are compared. In the latter case, the excitation patterns of system I and system II are more distinctly different from each other than are those obtained in the case of Chlorella.

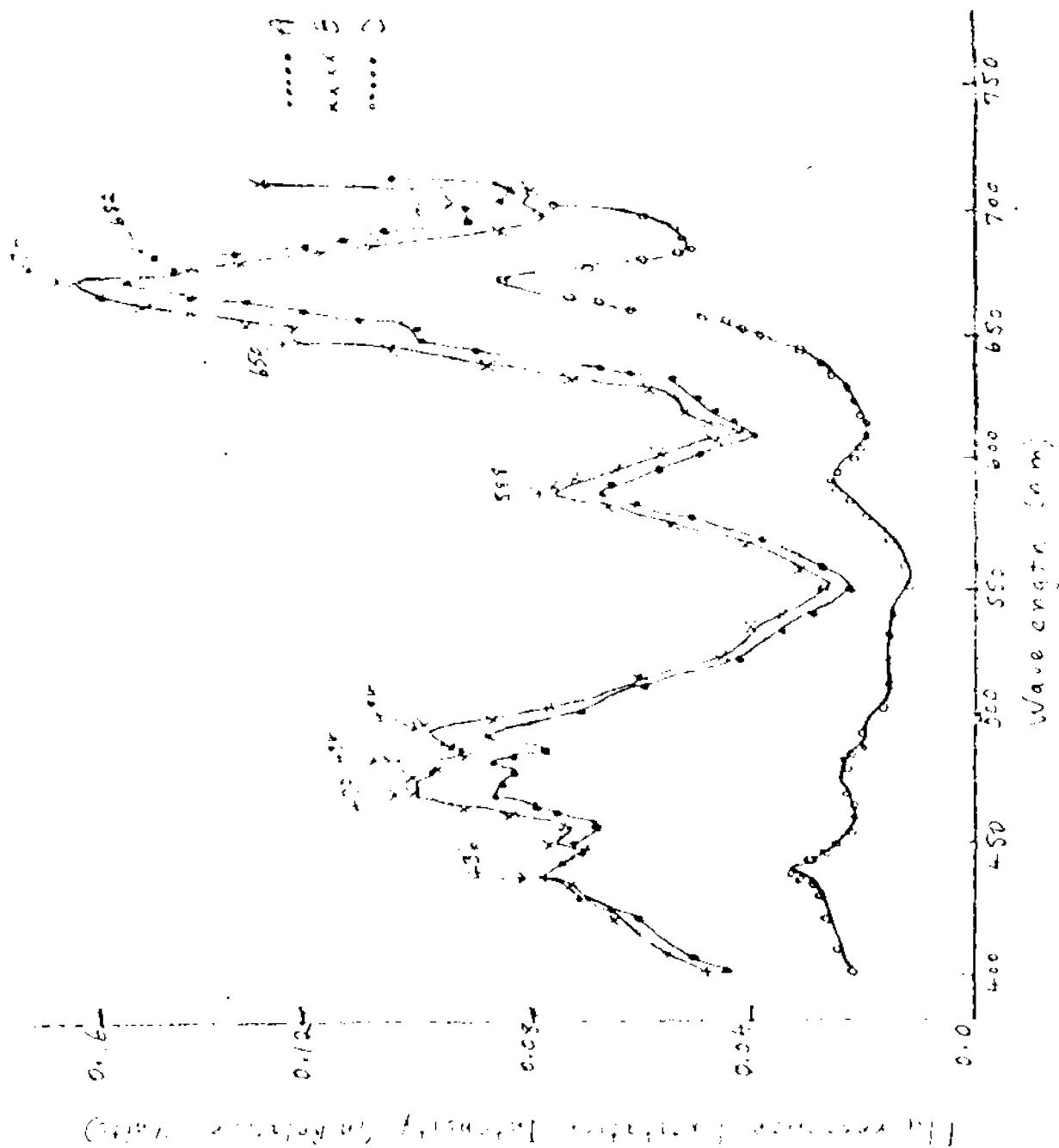
1. EFFECT OF LINOLENIC ACID ON FLUORESCENT EXCITATION OF CHLORELLA THYLAKOIDS.

In Fig. 67 are seen the 730 nm excitation spectra for control Chlorella thylakoids and for Chlorella thylakoids incubated at 0' and 60 minutes (at 30°C) in 2×10^{-4} M linolenic acid. These excitation spectra correspond to the emission spectra given in Fig. 49. There are evident in the control spectrum (Fig. 67 A) bands (for assignments to postulated pigments, see Goedheer, 1966) with maxima at 436 nm (chlorophyll a + carotenoids), 470, 488 and 494 nm (carotenoids), 538 nm** (vibrational band of chlorophyll a), 650 (chlorophyll b), 674 and 682 nm

* Under the experimental conditions used in the present work, it was not possible to determine the action spectrum for fluorescence at 685 nm; since at 77°K, emission from this band is relatively weak to begin with, and disappears (or is enveloped by the interfering band at 693 nm) under the influence of RLE or exogenous fatty acids, the required increase in monochromator slit width imposed a long wavelength limit of 650 nm on the 685 nm excitation band.

** As indicated in the Materials and Methods, excitation spectra are corrected but not for the spectral response of the multiplier phototube (Dumont 6911), which has a maximum (3.1) response at 800 nm.

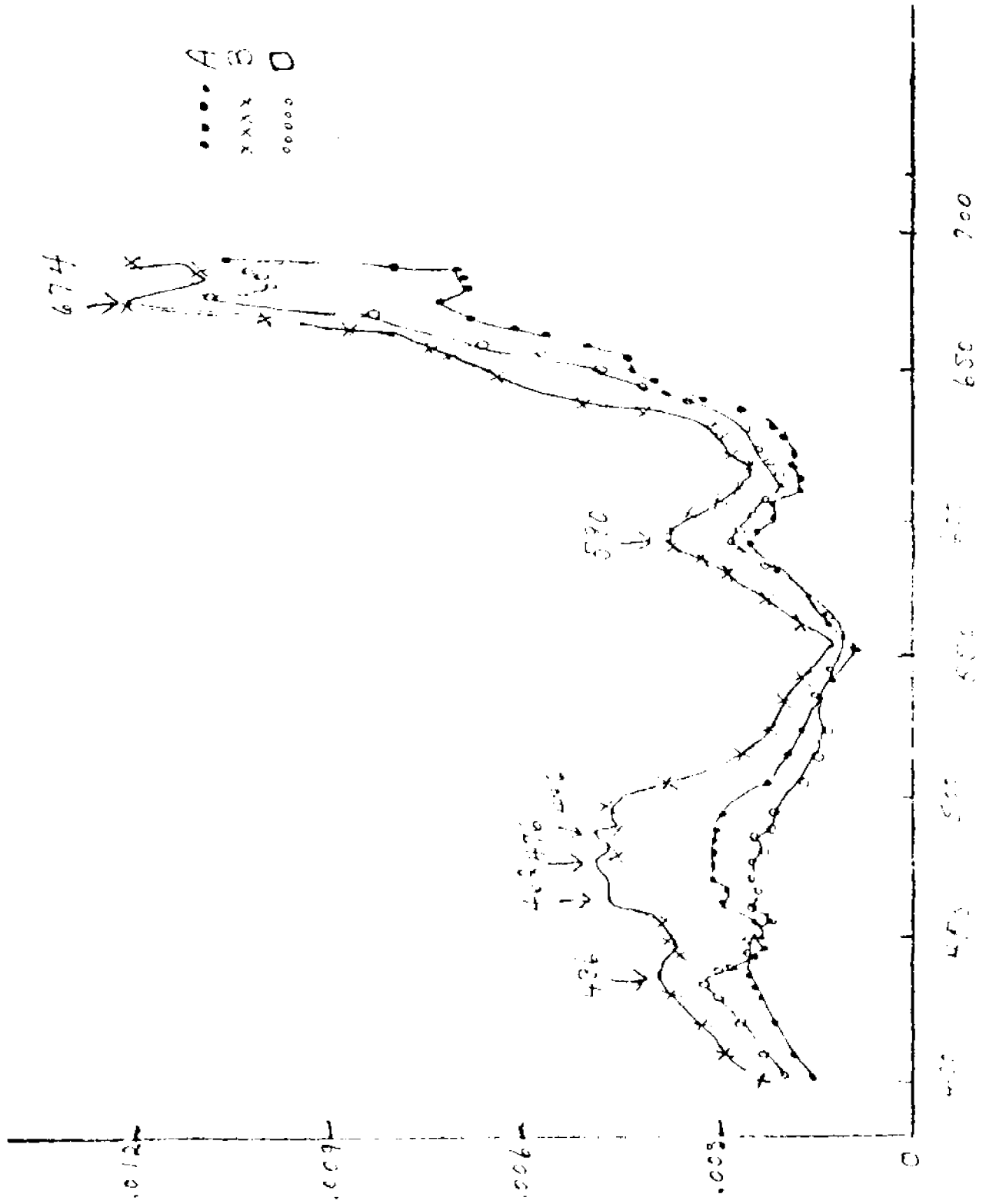
Figure 67. Action spectra (measured at -196°C) for exciting fluorescence at 730 nm in Chlorella thylakoids suspended in 0.04 M Tris-0.01 M NaCl buffer, pH 7.8 at 30°C . Conditions of the experiment correspond to those in Figure 52.



(chlorophyll a), as well as a small chlorophyll a band (aggregate) with maximum at 705 nm. The relative fluorescence excitation intensities of the 436 nm and 478 - 494 nm bands compared to the 675 nm band (i.e. 436/674 and 470 - 494 - 674) is about 0.5. Upon introduction of linolenic acid ($0'$), the contribution of the carotenoid bands increases considerably, as does the 674 nm band (corresponding to the monomeric form of chlorophyll a) so that the ratio of carotenoid (470 - 494 nm) to chlorophyll a (674 nm) increases to 0.67. Thus, in addition to the effect of linolenic acid on chlorophyll a, there is also an increase in excitation of carotenoids and chlorophyll a_I - 730 nm via (an increased) chlorophyll a_{II} (674 nm band). It is also observed that the excitation band corresponding to chlorophyll b (650 nm) increases its contribution to fluorescence at 730 nm upon addition of linolenic acid, perhaps because of tighter coupling between the chlorophyll b and the incrementing chlorophyll a band at 674 nm; incubation in linolenic acid for longer periods of time, not only results in pronounced decrease in yield (recall that fluorescence at 730 nm has all but disappeared at this time), but also results in decrease in contribution of carotenoids to 730 nm fluorescence relative to contribution by the 674 nm band.

In the control of the 705 nm excitation spectrum (Fig. 60) the relative intensity of the band heights in the blue region of the spectrum compared to the 674 nm band are lower than in the case of the control 730 nm action spectrum (i.e. 436/674 = 0.3; 470 - 494/674 = 0.4). However, introduction of linolenic acid ($0'$) does not lead to change in relative contribution of carotenoids to fluorescence at 705 nm, in contrast to the situation observed above for 730 nm excitation in the presence of fatty acid. Note, here too, the increased contribution of the 650 and 674 nm bands to 705 nm fluorescence in the $0'$ spectrum compared to the control.

Figure 68. Action spectra (measured at -196°C) for exciting fluorescence at 705 nm in Chlorella thylakoids suspended in 0.04 M Tris-0.01 M NaCl buffer, pH 7.8 at 30°C . Conditions of the experiments correspond to those in Figure 52.



Wavelength (nm)

Figure 1. Absorption spectra of the polymer films.

2. EFFECT OF LINOLENIC ACID ON THE FLUORESCENCE EXCITATION OF ANACYSTIS CELLS AND ANACYSTIS THYLAKOIDS.

In Fig. 69 is seen the 725 nm excitation spectrum of Anacystis cells fixed in formaldehyde. (The spectra correspond to the emission spectra shown in Figs. 60 and 61.) Note in the control, the relatively small contribution of chlorophyll and carotenoids in the blue region of the spectrum relative to that of the 682 nm excitation band. In contrast to the situation obtained in the case of Chlorella thylakoids, the ratio between the blue and red bands is 1/6 rather than 1/2. (The lower efficiency of energy transfer between the carotenoids and chlorophyll in phycobilin-containing algae was noted as long ago as 1952 by Duysens). Sensitization by phycocyanin (590), and, to a lesser degree, by allophycocyanin (630) are also evident.

Upon addition of linolenic acid, there is seen, immediately, an increased efficiency of energy transfer from the blue region (440, 536 nm) to chlorophyll a_1 , probably via the rapidly augmenting chlorophyll a_{11} band at 674 nm. Although there is very little change in contribution of phycocyanin (590 nm) to emission, the yield of emission of phycocyanin itself has decreased (see Fig. 60). By this time, the 682 nm (aggregate) excitation band has all but disappeared. As expected, total excitation intensity drops by 60 minutes of incubation (when the one-banded chlorophyll fluorescence emission form is achieved).

In the 700 nm spectrum of the control formaldehyde-fixed cells (Fig. 70) the contribution of bands in the blue region of the spectrum relative to the red region, is even less than in the case of 725 nm excitation (i.e. approximately 1/2 of the former). (This is to be expected, since carotenoids transfer even less efficiently to chlorophyll of system II - 705 nm, than to chlorophyll of system I - 725 nm,

Figure 69. Action spectra (measured at -196°C) for exciting fluorescence at 725 nm in Anacystis cells fixed in formaldehyde and suspended in 0.04 M Tris-0.01 M NaCl buffer, pH 7.8 at 30°C . Conditions of the experiment correspond to those in Figures 60 and 61.

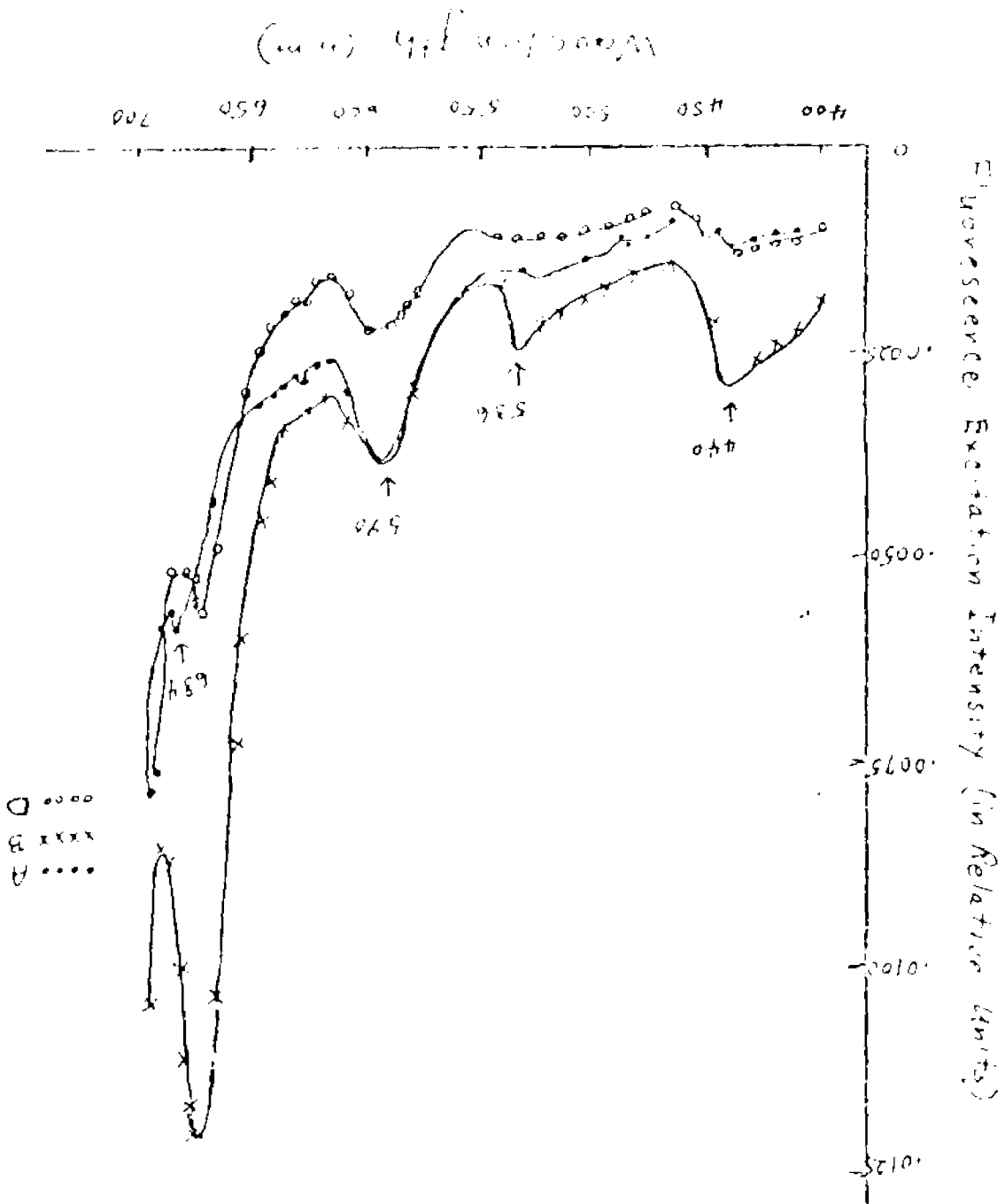
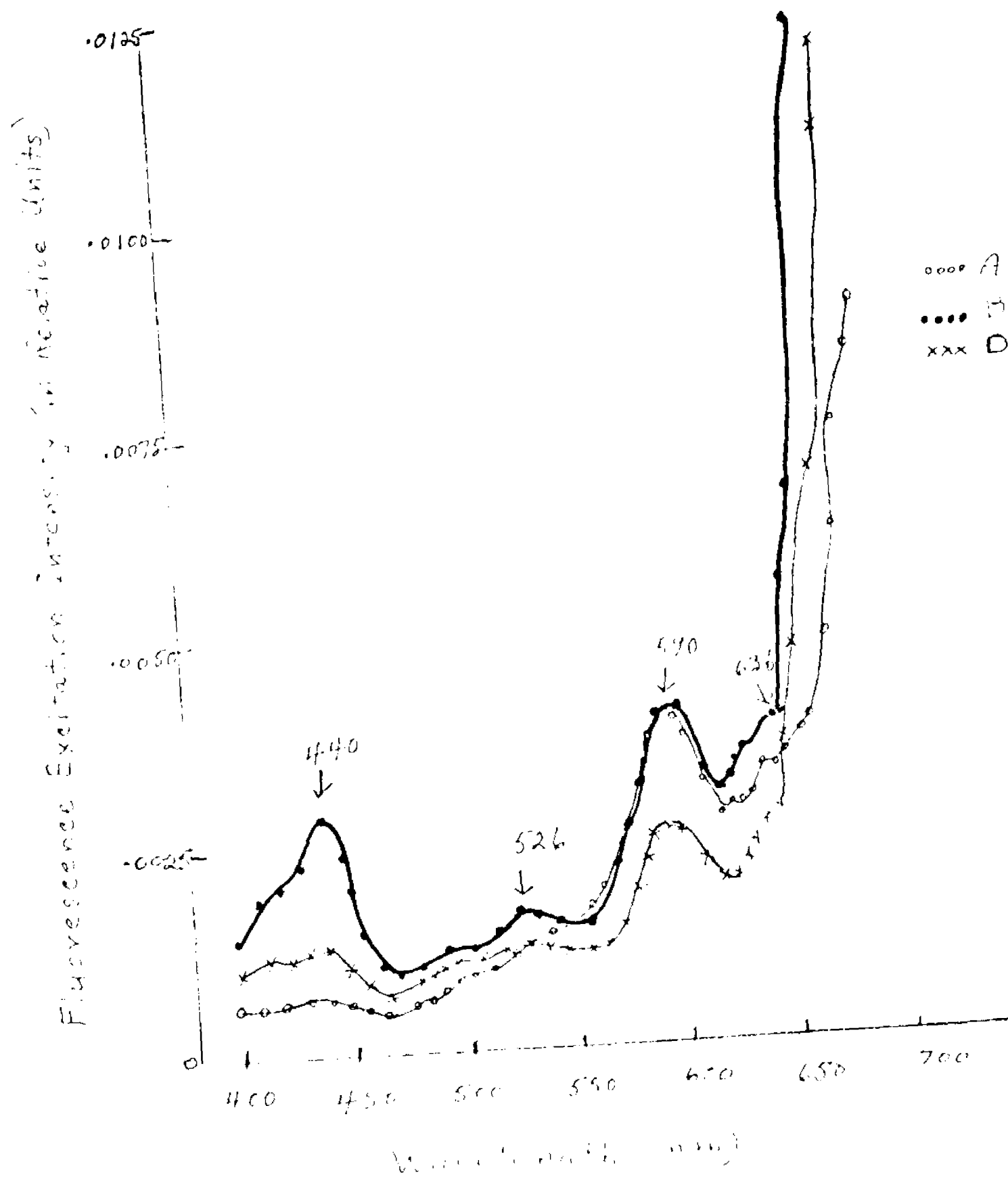


Figure 70. Action spectra (measured at -196°C) for exciting fluorescence at 700 nm in Anacystis cells fixed in formaldehyde and suspended in 0.04 M Tris-0.01 M NaCl buffer, pH 7.8 at 30°C . Conditions of the experiment correspond to those in Figures 60 and 61.



in phycobilin-containing algae). Changes induced in the presence of linolenic acid are similar to those obtained in the case of 725 nm excitation.

In the 725 nm excitation of Anacystis thylakoids isolated from unfixed cells (see Fig. 71), phycocyanin contribution is less than it is in the case of intact cells, perhaps to be anticipated on the basis of the observations that isolation of thylakoids from unfixed cells results in loss of phycocyanin and a pronounced uncoupling of energy transfer to chlorophyll. See Figs. 63 and 64 for the corresponding fluorescence emission spectra. Upon addition of linolenic acid to the thylakoids, the contribution of phycocyanin to 725 fluorescence is increased, perhaps as a result of tighter coupling between the remaining phycocyanin and the incrementing quantity of 674 nm chlorophyll. The remaining changes upon addition of fatty acids are similar to those occurring in Anacystis cells. If one examines the 705 nm excitation spectrum (see Fig. 70) in the control situation and in the presence of linolenic acid, again, observations made with Anacystis cells are also applicable to thylakoids from unfixed cells of Anacystis.

While fixation of Anacystis cells with formaldehyde results in stabilization of the phycocyanobilin to the thylakoids of Anacystis (Ludlow and Park, 1969; Cohen-Bazire, 1970; Clement-Letral, 1971) - see Figs. 64 and 65 - the 725 nm and 705 nm excitation spectra (Figs. 73 and 74, respectively) are very similar to those of the thylakoids isolated from unfixed cells, indicating that the bulk of the phycocyanin which leaches out upon isolation of thylakoids, is not needed for energy transfer to chlorophyll. Changes in energy transfer induced by linolenic acid are also similar to the ones obtained with thylakoids isolated from unfixed cells.

Figure 71. Action spectra (measured at -196°C) for exciting fluorescence at 725 nm in Anacystis thylakoids isolated from non-fixed cells, and suspended in 0.04 M Tris-0.01 M NaCl buffer, pH 7.8. Conditions of the experiment correspond to those in Figures 63 and 64.

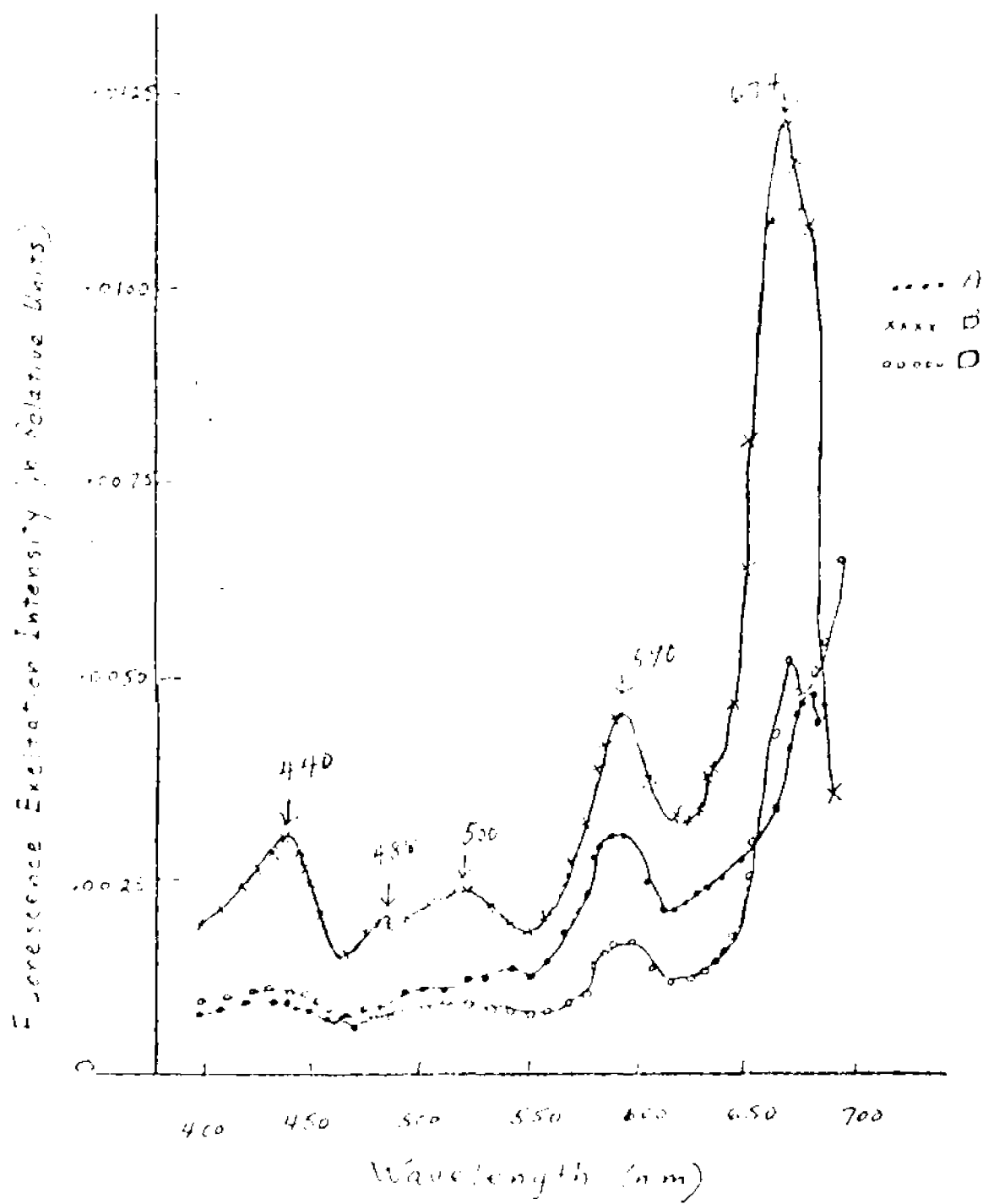


Figure 72. Action spectra (measured at -196°C) for exciting fluorescence at 700 nm in Anacystis thylakoids isolated from non-fixed cells and suspended in 0.04 M Tris-0.01 M NaCl buffer, pH 7.8. Conditions of the experiment correspond to those in Figures 63 and 64.

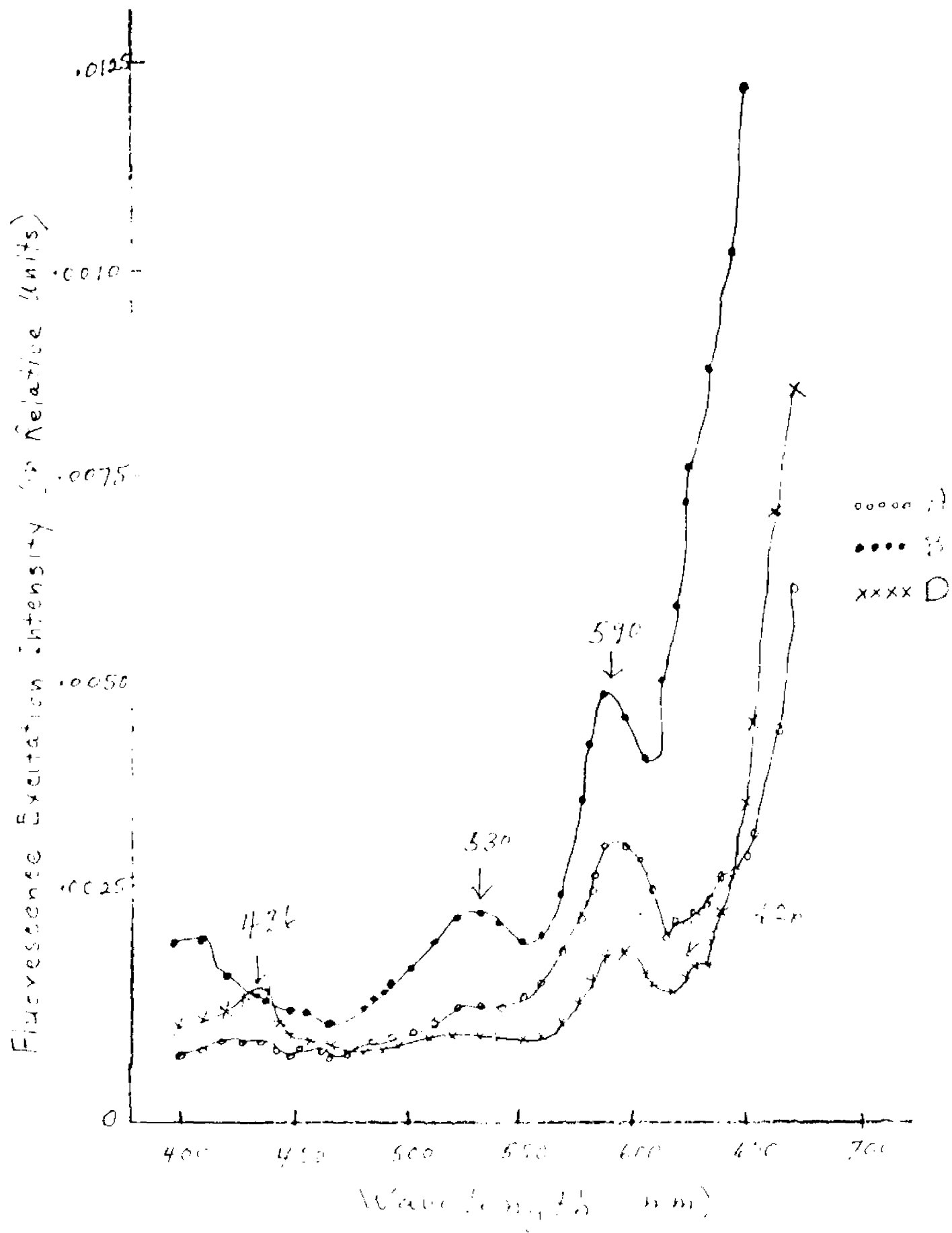


Figure 73. Action spectra (measured at -196°C) for exciting fluorescence at 725 nm in Anacystis thylakoids isolated from formaldehyde-fixed cells and suspended in 0.04 M Tris-0.01 M NaCl buffer, pH 7.8. Conditions of the experiment correspond to those in Figures 65 and 66.

Fluorescence Excitation Intensity in Relative Units

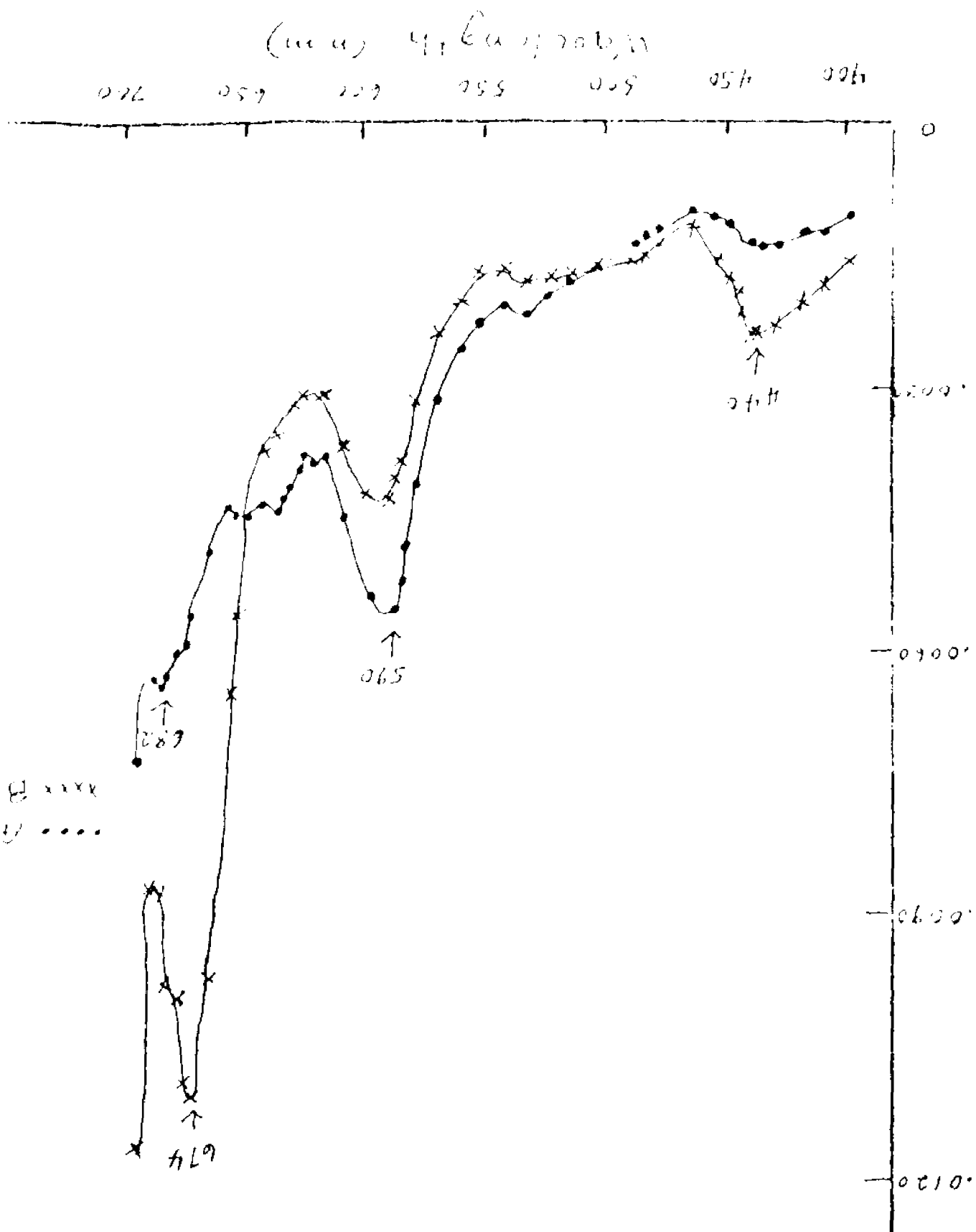
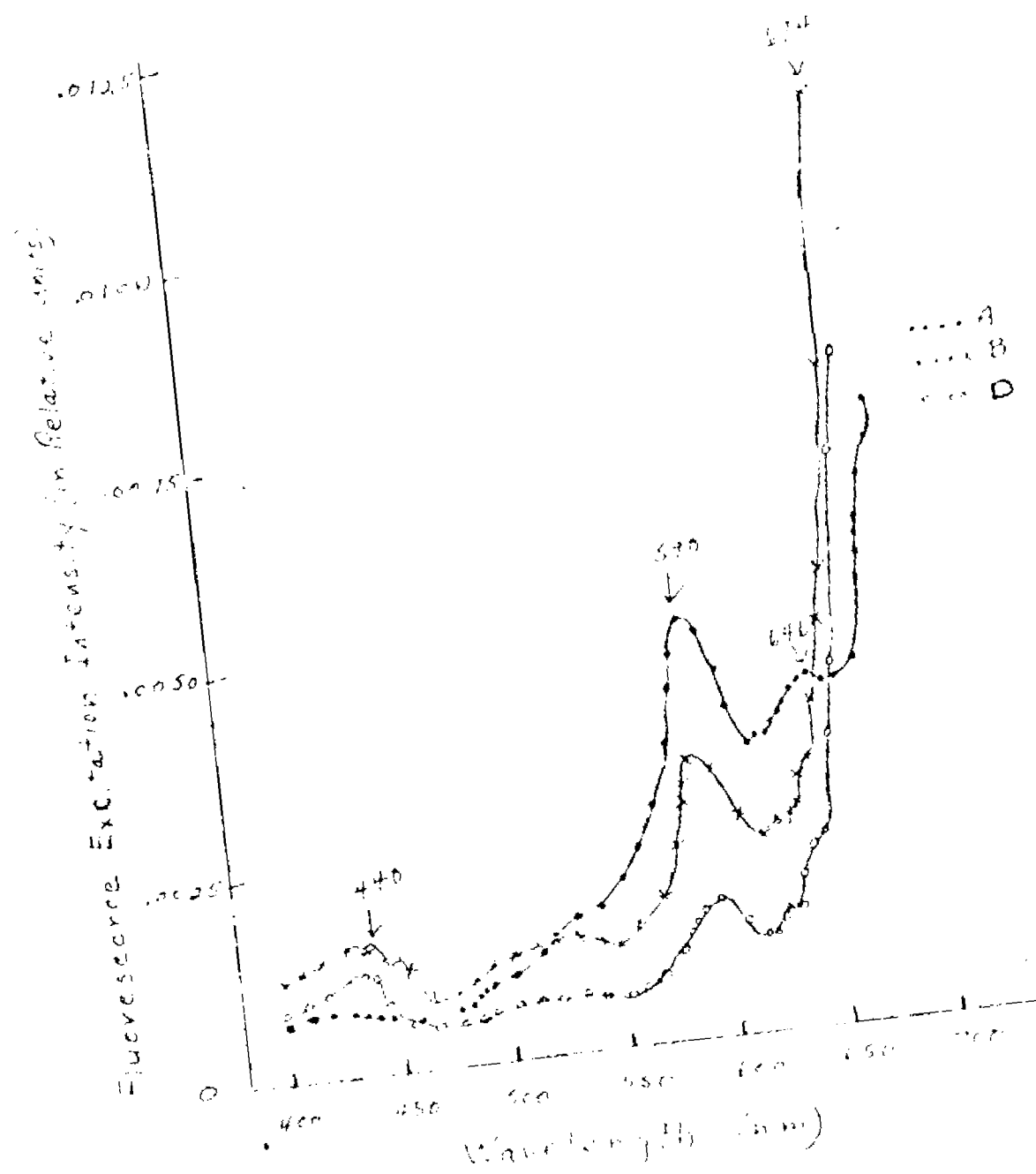


Figure 74. Action spectra (measured at -196°C) for exciting fluorescence at 700 nm in Anacystis thylakoids isolated from formaldehyde-fixed cells and suspended in 0.04 M Tris-0.01 M NaCl buffer, pH 7.8. Conditions of the experiment correspond to those in Figures 65 and 66.



DISCUSSION AND CONCLUSIONS

I. ACTION OF RICINUS LEAF EXTRACT ON CHLOROPHYLL-CONTAINING SYSTEMS

The disappearance of the discrete emission band, with maximum at 685 nm, from the low temperature emission spectrum, takes place during the initial stages of incubation in RLE, at a time when there is increase in the intensities of both F 698 and F 735. In the case of the latter band, its (transient) increase is coupled to a bathochromic shift in maximum. As incubation proceeds, the spectral change that becomes most obvious at low temperatures is the diminution in ratio of long wavelength to short wavelength fluorescence. The room temperature data given above, as well as the low temperature experiments made with fluorescence standards (Brody and Brody, 1971) have shown that this diminution in ratio results from absolute decrease in yield of F 735 and absolute increase in yield of F 698.

It is possible to interpret the decrease in F 735 and concomitant increase in F 698 in at least two ways: the first (1) is that energy transfer from system II to system I is no longer occurring; the second (2) is that there is conversion of the form of chlorophyll which gives rise to fluorescence at 735 nm into a form contributing to fluorescence at 698 nm. To help decide between these two interpretations, it should be remembered that as F 735/F 698 decreases in the emission spectrum, there is observed an increase in relative contribution of the 674 nm excitation band to both the fluorescence bands at 698 nm and 735 nm. At the same time, absorption studies reveal that the concentration of molecules absorbing at 674 nm increases, while the concentrations of molecules absorbing at 682 and 705 nm decreases. Excitation and absorption bands at about 682 and 705 nm in vivo have, it should be recalled, been attributed to aggregated species of chlorophyll, pre-

umably the source of fluorescence at 735 nm (Brody and Brody, 1965; Butler, 1965). On the other hand, it has been observed that the excitation band with maximum at 674 nm is the predominant one in the action spectrum for exciting the special species of chlorophyll designated F 698 (Brody and Broyde, 1963; Broyde and Brody, 1965, 1966; Anderson and Boardman, 1966); this 674 nm excitation band also strongly excites fluorescence (F 685) from the bulk monomer.

Again in regard to interpretation (2), a particular subset of it that is consistent with the data collected in the present work, is that RLE brings about a conversion of aggregated chlorophyll (fluorescing at 735 nm) into a monomeric chlorophyll that contributes to emission at 698 nm. In regard to the first interpretation (1) given above, it is worthwhile to note, that even if RLE had no direct effect on attenuating the efficiency of energy transfer between photosystems II and I, the indirect result of a deaggregation such as that postulated in interpretation (2) would lead to the same outcome.

Although I would like to determine which type of monomer is produced by deaggregation, such a determination is not easy to make. Augmentation of emission at 698 nm might equally well be brought about by deaggregation directly into the special monomer, or by deaggregation into bulk monomer (the source of F 698), followed by increased efficiency of energy transfer to the special monomer. It is also possible that some combination of these phenomena occurs. Production of either monomer could explain the initial apparent disappearance of F 685, since augmentation of F 698 would most likely make difficult the resolution of a discrete F 685 band.

That transformation of larger aggregates into smaller aggregates precedes conversion into a monomeric form, is suggested by the occurrence of a 5 nm bathochromic shift of the long wavelength maximum

(from 738 to 733 nm) during the initial stages of incubation; this shift ^{might} also explain the transient increase in F 735 which occurs during these initial stages of incubation.

In the present work, it has been observed that RLE has a similar effect on F 735 and F 720 (the usual long wavelength maximum ascribed to higher plants and algae, respectively). Brody and Brody (1963) proposed that both these fluorescence bands arise from aggregated species of chlorophyll, the position of the long wavelength maximum changing with greening, increase in size of aggregates, and variations in the populations of larger size aggregates. The similar effects of RLE on both these bands supports the contention of the Brodys' concerning the similarity in nature and function of these "different" long wavelength emission bands in algae and higher plants - i.e., it seems likely that F 735 and F 720 have similar chromophore-chromophore interactions or similar binding to the lipo-protein lamellae.

That the fraction of RLE which brings about the above described changes in steady-state fluorescence is proteinaceous in nature (Cohen et al. 1969), is supported by my observation that such fluorescence changes ^{and hence RLE} are barely detectable when whole algae are treated, but occur very rapidly in the case of lamellar fragments from these algae. RLE protein may be interacting with the chlorophyll-bearing lipo-protein lamellae in any one (or more) of the following ways: (a) by directly deaggregating chromophores or (b) by indirectly deaggregating chromophores, for example, by first inducing a conformational change in the protein portion of the lamellae. Such an indirect effect could be brought about in turn, by liberation of smaller molecules (such as ^{FAA, etc.} ; see below) from the RLE protein or more likely, from the lipo-protein chloroplast lamellae - these smaller molecules could then directly deaggregate chromophores or produce conformational changes secondarily

resulting in deaggregation.

In the last stages of incubation, a drop in total fluorescence yield (area under the emission envelope) is observed. This decrease in yield is especially evident after formation of the (essentially) one-banded emission spectrum, and is probably related to the disruption of normal lamellar structure, observed - by electron microscopy - to occur after prolonged exposure of chloroplasts to RLE (Cohen, Ph. D. dissertation, 1970).

That other proteins can affect the state of chlorophyll has been shown by Terpetra (1967, 1968), who demonstrated that methanol extracts of chlorophyll from Chlorella, Anacystis and Rhodospirillum could be "bleached" by a protein factor isolated from spinach leaves. Although neither the control methanolic chlorophyll extracts nor the control colloidal chlorophyll preparations (the latter prepared by diluting methanolic extracts with buffer) showed significant bleaching of the red band at 666 or 674 nm, respectively, upon illumination with a 100 W incandescent lamp, in the presence of the (partially) purified protein spinach leaf factor there was a steady decrease in absorbance with increasing time of irradiation. Terpetra also showed that urea could inhibit the bleaching effect brought about by this protein factor, presumably by its denaturation. An effect similar to that observed with chlorophyll was noted with Magnesium Tetraben ~~spor~~phyrin (magnesium-containing, unsubstituted fully unsaturated porphyrin). However, there was observed no significant bleaching of pheophytin (chlorophyll minus magnesium) in the presence of this factor, indicating the necessity of Mg for the bleaching effect to take place. In a continuation of this work, Terpetra (1968) found that the spinach protein factor caused a decrease in the form of colloidal bacteriochlorophyll absorbing at 847 nm, and the appearance of a form absorbing

at 780 nm - he discusses two possible mechanisms for this shift to shorter wavelengths - one, a deaggregation, and two, a conversion to a "new" form. Although I too, observed the direct deaggregation of chromophores by fatty acids (in the case of chlorophyll in solution), I additionally observed an indirect effect in systems in which chlorophyll is attached to protein-system which Terpetra did not consider.

One way to explain the seeming paradox of the high ratio of F_{735}/F_{698} of intact Ricinus leaves, compared to the low ratio one obtains upon isolation of these chloroplasts, is that in chloroplasts in situ the active factor is compartmentalized away from the chlorophyll-bearing lamellae, and that these compartments are opened as a result of isolation procedures. Another possibility is, of course, that in chloroplasts in situ the active protein exists in an inactive (physiologically non-functional) form and that its activation occurs upon contact with a stimulator or co-factor brought into contact with it upon isolation.

The major, if tentative conclusion reached as a result of experiments with RLE is that it acts by causing a change in the state of aggregation of chlorophyll. However, in order to obtain quantification of data and further test this hypothesis, I decided to use a model molecule of known molecular weight and size in place of RLE. A number of different systems have been offered as models for the action of the RLE protein on chloroplasts. Cohen et al. (1969) and Cohen (Ph. D. dissertation, 1970) have shown that a lipid-hydrolyzing enzyme (a galactolipase - either in aqueous extracts of Scarlet Runner Bean leaves, or in partially purified form - see Cohen, Ph. D. dissertation - (personal communication from Dr. R. McCarty) produced the same sort of effect on the steady-state low-temperature fluorescence emission of spinach chloroplasts as did RLE - namely, a decrease in

F 735/F 698. Since one of the classes of end products of lipolytic activity are fatty acids, a study of the efficacy of exogenous fatty acids and their derivatives (fatty alcohols and esters of fatty acids) in bringing about emission changes was also made by these workers. Of the latter three chemical classes, fatty acids proved to be the most effective. Consequently, the particular type of model system which I chose to use extensively in this investigation is the polyunsaturated (9, 12, 15- C_{18}) fatty acid, α -linolenic acid - the predominant fatty acid of lamellar glycolipids of chloroplasts in higher plants (Bensen, 1964; Goedheer, 1957). Its use has many advantages over that of the still relatively undefined active RLE protein. The knowledge of its molecular weight allows determination of effective concentration on a mole/mole basis in comparison with substrate (chlorophyll or protein). Also, while the introduction of RLE is limited to aqueous systems, the polar aspects of the molecular structure of linolenic acid allows interaction with protein (through the carbonyl group) while its long hydrocarbon chain makes for high solubility in organic solvents. Because of the small size of the fatty acid molecule (relative to RLE protein) its use introduces only minimal optical interference (i.e. small scattering effects) in the determination of direct and C.D. absorption. (The absorption bands which the RLE protein exhibits in the blue and near U.V. are, of course, absent in the case of fatty acids.)

II. ACTION OF EXOGENOUS FATTY ACIDS ON CHLOROPHYLL-CONTAINING SYSTEMS.

In this part of my thesis I will consider, in turn, the manifold effects of fatty acids on: (A) low temperature fluorescence emission and excitation of chloroplasts of higher plants; (B) direct light absorption by chloroplasts of higher plants; (C) circular dichroism of chlorophyllous (Spinacea) systems of increasing structural complex-

ity (in this section, control C.D. spectra will be discussed first and in some detail, since many of these systems have not been described in the literature); (D) F 735/F 698 as a function of exogenous linolenic acid concentration in chlorophyllous systems of increasing structural complexity; (E) the low temperature fluorescence emission and excitation, and circular dichroism of control and formaldehyde-fixed cells, chloroplasts and lamellar fragments of various algae.

A. Changes in Low Temperature Fluorescence from Chloroplasts of Higher Plants Incubated in the Presence of ¹Linolenic Acid.

As shown in Section III of the Results (also see Brody et al., 1969; Cohen et al., 1969) the end results of the incubation of chloroplasts in the presence of long chain unsaturated fatty acids (as exemplified by linolenic acid) are spectrally similar to the results obtained upon incubation of these chloroplasts in the presence of RLE; these small molecules act as good model systems for the action of the Ricinus protein. In both cases, the changes that were invoked could be interpreted to result from the deaggregation of chlorophyll. One apparent difference, however, between the action of these substances is that the changes induced by RLE are time-dependent, whereas the changes induced in the presence of fatty acids are concentration-dependent. The origin of this "difference" becomes understandable if the presumed mechanism of action of RLE is considered; if RLE is acting, for example, to liberate from the lipoprotein chloroplasts lamellae, molecules of free fatty acid, then the observed rate of change in fluorescence ratio becomes dependent, in turn, upon the rate of liberation of these fatty acid molecules. Even in the case of addition of exogenous fatty acids, time-dependence of spectral change can be noted; at low concentrations of fatty acid (but above FA/Chl of 4, below which range no spectral

changes are noted) i.e. in the range 4-8 FA/Chl, spectral changes are time-dependent, time decreasing with increasing amount of fatty acids, while at high concentration; i.e. $\approx$ in the range > 15 FA/Chl there is almost immediate conversion to the one-banded form.

From the experiments I made on the changes in low temperature emission of chloroplasts of higher plants as a function of concentration of exogenous linolenic acid, the following need to be considered. At a ratio of < 4 FA/Chl, no changes in $F\ 735/F\ 698$ are observed (even at 30°C for periods up to 60 minutes). This finding is in agreement with Cohen et al. (1969), who observed that $F\ 735$, $F\ 698$ at 25°C , does not change at FA/Chl of 2 - 3, but falls to 50% of its control value at FA/Chl of 10.

In this thesis (see also Nathanson and Brody, 1970), in which I monitored RLE and fatty acid-induced changes in $F\ 735/F\ 698$ for a very large number of different organisms, I found, in agreement with Cohen et al. (1969), that this low temperature emission ratio never fell below ≈ 0.2 - which substantiates the contention that this ratio represents solely emission from F 698. In this regard, it is interesting to note that Broyde and Brody (1966) found $F\ 735/F\ 698$ to be ≈ 0.18 in solution of specially prepared chlorophyll fluorescing at 698 nm (which they called "F 698" in vitro).

With chloroplasts from higher plants I found, in the present work, that in the concentration range of FA/Chl of ≈ 4 to 11, there exists a linear relationship with respect to rate of change of $F\ 735/F\ 698$. I also found with higher plant chloroplasts, that at FA/Chl of ≈ 11 , linearity with rate of change of $F\ 735/F\ 698$ no longer holds - saturation occurring at FA/Chl ≈ 23 . The significance of these FA/Chl ratios relative to rate of change of $F\ 735/F\ 698$ will be considered below, in that portion of the discussion dealing with effects of fatty acids

on chlorophyllous systems of various levels of structural organisation.

One of the questions that arose early in my explorations of the effects of fatty acids on fluorescence emission of higher plant chloroplasts was whether the observed decrease in F 735/F 698 had its origins in a pH effect. First I will consider the qualitative differences and then the quantitative differences between the spectra obtained with hydrochloric acid and linolenic acid. In the case of the inorganic acid, the initial increases in yields of both F 735 and F 698 are similar to those observed with fatty acids; however, the now familiar decrease in F 735/F 698 observed with fatty acids (and RLE) is, instead, observed to be replaced by an increase in F 735/F 698. Even after the occurrence of the HCl-induced decrease in total yield (similar to that observed in the later stages of incubation in fatty acid - and resulting, I believe, from a disruption of normal lamellar structure) there is left a single (?) band dissimilar from the sharp single band (maximum at 698 nm) that I obtained with use of fatty acids, insofar as the HCl induced band is very broad, having a plateau from 688 to 713 nm. As far as quantitative differences are concerned, the following should be considered. I worked with fatty acids in the concentration range 10^{-4} to 10^{-3} M, which in 0.04 M Tris-NaCl buffer of pH 7.8 ^{which} yields measured pH's of 7.60 and 7.14 at each extreme, respectively. It was observed that upon one-half hour incubation at an HCl concentration of 2×10^{-3} M (100 x the chlorophyll concentration on a mole/mole basis) only very minor changes in fluorescence had occurred, while upon one-half hour incubation at a linolenic acid concentration of 2×10^{-4} M (10 x the chlorophyll concentration on a mole/mole basis) the one-banded form (F 698) had been reached - this in spite of the fact that HCl ionizes completely (yield ^{which} a hydrogen ion concentration of 2×10^{-3} M

while linolenic acid has an ionization constant of 1.3×10^{-5} , which on the basis of the observed pH changes indicates a hydrogen ion concentration of between 9.2×10^{-9} and $7.45 \times 10^{-8} M$, respectively. My conclusion from the above is that, with the linolenic acid concentration range within which I worked, increased hydrogen ion concentration played only a minor role in eliciting the observed spectral changes.

An interesting comparison with this aspect of my work is afforded by the studies of Steffen and Calvin (1970, 1971) on the effect of octanoic acid on bacterial chromatophores. This short chain (C_8) fatty acid is saturated and, as previously shown by Cohen et al. (1969; see also W. Cohen, Ph. D. dissertation, 1970), such acids have little or no effect on chloroplasts in the low concentration range in which long chain unsaturated fatty acids are effective. Steffen and Calvin (1970, 1971) found that large pH changes, resulting from their use of 10^{-3} to 10^{-2} M octanoic acid, respectively, brought about pheophytinization of bacteriochlorophyll in isolated chromatophores. It may be recalled that I encountered no pheophytin formation in chlorophyllous systems having structural complexity above that of chlorophyll in solution. Even in solution, I found that with linolenic acid, pheophytin formation occurred at concentrations greater than 10^{-3} M, in which the mole/mole ratio of fatty acid to chlorophyll was 1:1. Steffen and Calvin (1971) also proposed that the presence of protein "protects" chlorophyll from pheophytinization, and suggested (but gave no data in support of) de-aggregation by unfolding of the pigment chlorophyll complex). (Their suggestion is similar to, but 10 years later than, that of S. S. Brody and M. Brody (1961) who first proposed that changes in state of aggregation could be brought about by effects on the protein to which chlorophyll chromophores are attached.)

It may be remembered that Arrhenius curves were plotted for three experimental situations in which the change in F_{735}/F_{698} at $10' - 0'$ incubation was monitored at several different temperatures (in the range 0° to 45°C). These experimental situations were: (a) Ricinus chloroplasts in incubation media; (b) Spinacea chloroplasts in RLE; and (c) Spinacea chloroplasts in linolenic acid. In all three cases an energy of activation of 8 ± 0.1 Kcal per reaction rate was obtained (from the slope of each curve). The experimental finding that the situation involving RLE resulted in a monophasic curve similar to the situation involving the use of fatty acids, was surprising to me, at first - since I was using as my working model for the action of RLE the (possibly lipolytic) liberation of fatty acids from the lipoprotein chloroplast lamellae, with subsequent deaggregation effects on chlorophyll. Therefore, I expected to find at least biphasic Arrhenius curves for these rather more complicated processes. However, my present conclusion is that in the experimental conditions I set up, ^{at 10' - 0'} all I would be monitoring in any case, is the last step in the chain of events, namely the conversion of aggregated chlorophyll into monomeric chlorophyll - independent of the nature of the processes preceeding this deaggregation.

B. Changes in Absorption and Fluorescence Excitation Induced in Spinach Chloroplasts in the Presence of Linolenic Acid.

Since both (a) absorption changes and (b) the changes in low temperature fluorescence excitation are the same whether the agent that induces them is RLE or linolenic acid, and since the significance of these changes has already been considered in Section I, above, they will not be considered further here.

C. Linolenic Acid-Induced Changes in C.D. of Chlorophyllous Systems of Increasing Structural Complexity.

Although, in the Results Sections of this thesis, the C.D. data, IV C were divided into 3 major groupings [IV C 1 (A Comparison of RLE-Induced and Linolenic Acid-Induced Changes on C.D. of Digitonin-Isolated Subchloroplast Particles Containing Systems I and II), IV C 2 (Influence of Linolenic Acid on States of Chlorophyll in Systems Having a Low Degree of Structural Organization) and IV C 3 (Influence of Linolenic Acid on States of Chlorophyll in Systems Having a High Degree of Organization)] the discussion of these data - which follows immediately below - will be given in a somewhat different format, to allow (a) comparisons to be more readily made, and (b) conclusions to be more comprehensively drawn. The arrangement of the discussion will be such so as to permit interpretations of more complex systems in terms of simpler systems, which will already have been discussed. For each level of chlorophyll organization, the control C.D. situation will be examined prior to consideration of the effects of agents (such as ethanol, linolenic acid, urea and RLE) which induce changes in the control.

As seen in the Results section IV C 2 a (1) (the effect of ethanol on the state of chlorophyll in solution) the red C.D. spectrum of the control chlorophyll in CCl_4 consists of a positive band at 676 nm and a negative band at 656 nm. According to earlier C.D. work (Dratz et al., 1966; Housster and Sauer, 1970) these two bands represent a dimeric form of chlorophyll a, the monomers of which interact asymmetrically (or degenerately) with one another to yield a C.D. doublet that crosses zero in the region of the absorption maximum. [(It was earlier estimated that, in the solvent CCl_4 , and at the chlorophyll

concentration used in the present work, up to 90% of the pigment is in dimeric form (Brody and Broyde, 1968)]. Although in this thesis I have made it quite clear that where I have encountered - in a previously unreported system - a C.D. doublet crossing zero in the region of the absorption maximum, I have taken this doublet to represent degenerate interaction between chlorophylls. I have done this with the recognition that even single bands may have their origin in aggregated chlorophyll. The manifestation of a second band is a function of the respective transition moments between two successive chlorophylls in the aggregate (see Schellman (1968) for a theoretical consideration of the factors which control the disposition of aggregate C.D. bands).

I observed that when the same amount of chlorophyll (as dissolved in CCl_4) is dissolved instead in the solvent pair 60% hexane-40% CCl_4 , additional bands appear in the control at 710, 740 and 760 nm. Because of their structure I have assumed that the 740 and 760 nm bands represent (the doublet of) a highly aggregated form of chlorophyll. Although no C.D. work of others is available for interpretation of this system, Katz et al. (1963 a and b) conclude, on the basis of their work with I.R. and N.M.R. experiments, that solvents such as hexane- CCl_4 favor formation of chlorophyll aggregates of higher order than dimers. It is interesting to note that Dratz et al. (1966) in examining the C.D. spectra of microcrystals of chlorophyll a in the "solvent" (more appropriately, carrier) iso-octane, also found C.D. bands similar to those I observed in hexane- CCl_4 ; in their case, however, the bands were shifted to slightly longer wavelengths - a shoulder appearing at 718 nm (rather than at 710 nm) and a strong negative peak appearing at 751 nm (rather than at 740 nm). They postulated that the 718 nm band has its

origin in the vibrational band of aggregated chlorophyll and that the 751 nm band represents the C.D. of the electronic transition in these microcrystals. Since the bands I observed in this dissertation have essentially the same form as observed by Dratz et al. (op. cit.) for microcrystals, but occurring at shorter wavelengths, I assume that these represent chlorophyll-chlorophyll interactions at states of aggregation slightly lower than those given by microcrystals. In contrast to the situation obtained in hexane-CCl₄, where I noted a positive peak at 760 nm, (in addition to the negative maximum at 740 nm), Dratz et al. observed no positive C.D. bands beyond the (negative) C.D. band at 751 nm. In the case of hexane-CCl₄, I would like to suggest that the negative band at 740 is part of a doublet which has a positive band at 760 nm, and furthermore, that the shoulder at 710 nm represents a C.D. absorption of the vibrational band of this (same form of) highly aggregated chlorophyll. I make my suggestion on the basis of the similar reactivity of the 740 and 760 nm bands to treatment by ethanol or linolenic acid (see below). Absorption data (to be described below) also support the observations of the effects of these deaggregating agents. With respect to the 710 nm shoulder which I observed in hexane-CCl₄, and which Dratz et al. observed at 718 nm, *it is* assigned to the vibrational band of highly aggregated chlorophyll, in analogy with the assignment of Dratz et al.

In regard to the 760 nm positive band which I observed in hexane-CCl₄, and for which there was no parallel observation by Dratz et al. in the case of chlorophyll in iso-octane, the following needs to be considered: in the C.D. spectrum for chlorophyll in hexane-CCl₄, the 760 (positive) band is much weaker than the 740 (negative) band. This situation is somewhat different from that observed with chlorophyll in

CCl_4 - in which the doublet C.D. bands of the dimer are somewhat more equal in intensity than is the case for chlorophyll in the solvent pair. This may indicate that the aggregates of chlorophyll higher than dimers have a structure such that the angles between the transition moments of two successive chlorophylls are not similar to those observed for dimeric chlorophyll - but are instead modified by interaction with many more chlorophyll neighbors. I propose that such interactions can lead, successively, with increasing order of aggregation, to reduction in intensity of the 760 nm band (from the unequal situation in what is probably a semi-crystalline array in hexane- CCl_4) to the (possible) absence* of this band in microcrystals of chlorophyll suspended in iso-octane.

My findings that addition of ethanol at a final concentration of 0.02% to chlorophyll a (2×10^{-3} M) in CCl_4 results in marked reduction in intensity of the positive C.D. band with maximum at 676 nm and accompanying shift in the negative C.D. band from a maximum at 656 nm to a maximum at 661 nm, is similar to those reported by Houssier and Sauer (1970) who used, however, a higher final concentration of ethanol (0.5% ethanol) and a lower concentration of chlorophyll (2×10^{-5} M). While the latter workers assumed the 660 nm band which results from addition of ethanol to be a monomer [on the basis of the existence of this band also in ethereal solutions (in which aggregates do not form)], I base my similar conclusion about the 661 nm band being a monomer,

* The reason for my modifying the word "absence" with the term "possible" is that later work by some of the same workers (Houssier and Sauer, 1970) revealed that, in the earlier experiments (Dratz et al., 1966), sensitivity of detection was so low as to make impossible the resolution of the monomer band of chlorophyll in CCl_4 , reported in the same study. Therefore, it is possible that this low resolution would make it difficult to detect an even weaker positive C.D. aggregate band at wavelengths longer than 751 nm.

on my observation that in very dilute solution (10^{-6} M) in CCl_4 this band is also apparent. One of the reasons for my having observed deaggregation at lower ethanol concentration (about 1/25 that which they used) is footnoted in the Results section (page 55) namely, that their reagents were specially dried, and it is possible that in my experiments, trace amounts of water permitted deaggregation to occur at lower concentration of ethanol. Another possible explanation is that given by Tropper and Sauer (1968) who, on the basis of (unpublished) results, concluded that ethanol deaggregates by forming a one:one complex between it and a chlorophyll a monomer. Thus, even in my experiments, there is more than enough ethanol to cause deaggregation (the mole/mole ratio of ethanol to chlorophyll a being 2:1); in the experiments of Houssier and Sauer, the amount of ethanol is excessive (a mole:mole ratio of ethanol to chlorophyll of 125/1). The deaggregating effects on chlorophyll dimers in CCl_4 were also shown in visible absorption studies (Houssier and Sauer-1970) and I.R. studies (Katz et al.-1963). My results agree with these earlier works, in so far as I observed that addition of ethanol (a) brings about diminution of visible absorption at 682 nm and increase at 667 nm and (b) results in decrease at 1650 cm^{-1} and increase at 1700 cm^{-1} in the I.R. spectrum. (The 1700 cm^{-1} band, has been assigned to the ketone of ring V in chlorophyll by Katz et al. (1963); this band has been found to increase with increasing polarity of solvent and also when deaggregation occurs.)

A question arises as to the disposition of the C.D. monomer band. In the control, there is neither a discrete band in the case of chlorophyll a in CCl_4 , nor in the case of the solvent pair hexane- CCl_4 ; the "absence" may reasonably be ascribed to interference (overlap) with dimer bands, since in experiments I made with dilute

solutions of chlorophyll (2×10^{-6} M) in CCl_4 , I observed a single negative monomer C.D. band with maximum occurring at 660 nm. (With 2×10^{-3} M chlorophyll a in ethanol - in which about 98% of the pigment is in the monomeric form (Broyde et al., 1968) - I found a single negative C.D. band to also occur at about 660 nm.) *see insert fig 8'12*

Although upon deaggregation of 2×10^{-3} M chlorophyll a in CCl_4 by 0.2% ethanol, the negative 661 nm band becomes predominant, it does not appear upon deaggregation in the solvent pair hexane- CCl_4 under otherwise similar conditions - the resulting conversion of higher aggregates into dimers probably still obscuring the monomer band. However, when ethanol was added in concentrations sufficient to bring about complete deaggregation (2%) the monomer band becomes apparent.

Throughout my research, I used as a working hypothesis, the assumption that reagents which bring about effects in C.D. (etc.) similar to those brought about by ethanol, are causing deaggregation. Since there are no published results on effects of addition of ethanol to chlorophyll in the solvent pair 60% hexane-40% CCl_4 , in which, in addition to dimers, aggregates of higher order occur, consideration of this system was in order. Although it is well known that the large (monomeric) band with (direct) absorption maximum at 668 nm occludes the dimer band in the case of chlorophyll in CCl_4 , direct absorption of the same amount of chlorophyll a dissolved in the solvent in the solvent pair hexane- CCl_4 has not been reported. The peak, in this case at 667 nm, is reduced to about one-half of what it is in the case of the single solvent, and an additional (small) absorption band, with maximum at about 745 nm, *is present in 20% ethanol* in the case of microcrystals of chlorophyll suspended in iso-octane.

Since I found that introduction of a final concentration of 0.02%

ethanol to chlorophyll in the solvent pair results in disappearance of the 745 nm peak - this disappearance being accompanied by marked increase in the absorption peak at 667 nm - I have assumed that the 745 nm band represents absorption by the higher aggregates and that in the mixed solvent, the chlorophyll monomer in interaction with the dimer absorbs at 667 nm. This direct absorption study is substantiated by the changes that occur in the C.D. spectrum of chlorophyll in hexane-CCl₄, since I observed that, upon addition of 0.1% ethanol there is a small decrease (4 m° less positivity) in ellipticity at 760 nm and a large decrease (less negativity) of 12 m° in the 740 nm band; these decreases are accompanied by increases (in the dimeric form of chlorophyll) at 656 and 676 nm. Thus, in the presence of ethanol, which is known to deaggregate chlorophyll (Katz et al.-1963 a, Houssier and Sauer-1970) there is conversion of highly aggregated chlorophyll ^{to chlorophyll} dimers.

In section IV C 2 a (2), I reported the effects of linolenic acid on the state of chlorophyll a in solution. I found that the change in C.D. which results from addition of linolenic acid to chlorophyll in CCl₄ is similar to that which occurs upon addition of ethanol, in so far as there is strong decrease of the C.D. band at 678 nm, and a weaker decrease in the negative band at 656 nm. However, the anticipated shift to a negative band with maximum at 661 nm (as observed upon deaggregation with ethanol) is not observed upon addition of linolenic acid (a slight inflection in this latter band is seen at about 660 nm, and may possibly represent monomer). An explanation should be sought for why these two bands (which are considered to be a dimer doublet) do not behave the same way as they do upon deaggregation by ethanol. One explanation is based upon the observation that the negative peak of monomeric chlorophyll occurs (in C.D. absorption) at 661 nm. Thus, any decrease in the C.D. band at 656 nm (part of the dimer doublet) would

be obscured by an overlap in absorption at this wavelength by monomeric chlorophyll. Another problem resulted from the difference in the findings obtained with deaggregation in the case of linolenic acid as compared with deaggregation of chlorophyll in the case of ethanol. In the case of the former, it was noted that, although there were decreases in the positive band at 676 nm and in the negative band at 656 nm, there was no shift in the negative maximum at 656 nm, but only a slight inflection at 661 nm. Apparently, only very small amounts of monomeric chlorophyll are formed in the process of deaggregation by linolenic acid. An explanation of these results was obtained with experiments concerning the effect of linolenic acid on direct absorption of chlorophyll a in CCl_4 ; it was found that addition of linolenic acid results in decrease in absorption at 682 nm, a smaller decrease in absorption at 667 nm, and appearance of two discrete bands with maxima at 508 and 538 nm - the latter two bands corresponding to absorption by monomeric pheophytin a. Accompanying these changes, are changes in the I.R., which consist of decrease in the 1650 cm^{-1} band and (very small) increase of the 1700 cm^{-1} band.* I believe that the difference between the deaggregation results obtained with ethanol and with linolenic acid relate to the fact that pheophytin is formed in the latter case, while it is not in the case of addition of ethanol. The formation of pheophytin would explain why there is very little increase in the negative (C.D.) band with maximum at 661 nm.

In contrast to the single solvent (CCl_4) in which it is difficult to determine whether linolenic acid causes deaggregation of dimers into

* Although decrease in 1650 cm^{-1} I.R. band was noted upon addition of linolenic acid, increase at 1700 cm^{-1} is difficult to discern because of strong absorption by linolenic acid at this wavelength.

monomers with resulting pheophytinization of these monomers, or whether deaggregation in the presence of linolenic acid results directly in production of pheophytin, experiments with chlorophyll a in the solvent pair hexane-CCl₄ (in which not only dimers, but aggregates larger than dimers occur) allow one to observe directly that addition of linolenic acid results not only to conversion to pheophytin but also in formation of dimers from higher chlorophyll aggregates. As in the case of deaggregation by ethanol, the introduction of linolenic acid converts the highly aggregated species of chlorophyll (with C.D. bands at 740 and 760 nm) into dimers absorbing at 678 and 656 nm (some of the absorption in this latter band may be due to formation of monomers). Although the C.D. spectra which result from deaggregation in hexane-CCl₄ are similar whether the deaggregation is brought about by ethanol or linolenic acid, visible absorption spectra reveal that (here too, as in CCl₄) deaggregation by linolenic acid produces pheophytin while ethanol does not. (That is, in both solvents, similar direct absorption decreases at 745 nm and increases at 668 nm are accompanied, in the case of linolenic acid-induced deaggregation, by appearance of a pheophytin (monomer) band at 535 nm.) Since there was no band apparent at 690 nm in either C.D. or direct absorption, aggregates of pheophytin were not formed.

In regard to the situation of chlorophyll in solution, clearly deaggregation is brought about by direct action on the chromophores. I will turn my attention now, to the next higher level of organization - the cases in which the chlorophyll chromophores are attached to protein in (non-membranous) chlorophyll-protein complexes. Since the C.D. spectra of the system I complexes isolated from Phormidium (Results, Section IV-C-2 b (1)) and HP 700 chlorophyll-protein complexes (Results, Section IV-C-2-b (2))

were first reported on the basis of work done for this thesis (Brody and Nathanson, 1972), I will consider them in some detail, discussing initially the control situation in regard to these two types of complexes. I will then discuss the effects of linolenic acid on these systems. Since it was with HP 700 chlorophyll-protein complexes that deaggregation by a mechanism other than direct action on chromophores (i.e., a conformational change of protein secondarily bringing about deaggregation) was first observed (see also Brody and Nathanson, 1972), there will follow a discussion of the U.V. and visible C.D. spectra for fatty acid and urea-treated HP 700 complexes.

It should be recalled that system I Phormidium complexes (prepared according to Thronber) have a red C.D. spectrum containing a small negative band at 662 nm, a large positive band at 676 nm, and an even larger negative band with maximum at 690 nm. I made the (tentative) assignment of the shorter wavelength bands to the dimer doublet, in analogy to the doublet observed when chlorophyll is dissolved in CCl_4 . Since a band corresponding to the 690 nm Phormidium band is absent in the C.D. spectrum of chlorophyll a in CCl_4 , I assumed that this band arises from another aggregate, possibly (because of its long wavelength absorption) a different type of dimer, perhaps related to the 688 nm band resolved by French (1968), in the derivative absorption spectrum of Phormidium chlorophyll-protein complexes.

The control C.D. spectrum of HP 700 chlorophyll-protein complexes bears a strong resemblance to the C.D. spectrum of chlorophyll a in the solvent pair hexane- CCl_4 , while the C.D. spectrum of Phormidium system I complexes more closely resembles that of chlorophyll in the single solvent CCl_4 . It is on this basis I concluded that, in HP 700 complexes, there exist very few dimers, but primarily aggregates of higher order.

In the control HP 700, the four bands in the red region of the spectrum were resolved in the following ways. As in the case of chlorophyll a in hexane-CCl₄, I assumed that the long wavelength bands - the small positive band at 755 nm and the large negative band at 735 nm - constitute the doublet of this highly aggregated form of chlorophyll (more about these bands below). The two shorter wavelength bands are: a small rather broad, positive band with maximum at about 695 ---> 700 nm, and a large negative band with minimum at 660 nm. The negative band at 660 nm could either be characteristic of HP 700 monomer or be due to contamination by solubilized chlorophyll. The C.D. bands of the chlorophyll-protein complex at 690 - 705 nm may either represent dimers or (more likely) the vibrational state of the highly aggregated form of chlorophyll.

I wish to go back to a consideration of the nature of the two longer wavelength bands. It may be recalled that the HP 700 complexes which yield the spectrum being described, are prepared in a procedure which is a modification of the method of Yamamoto and Vernon (1968) who used lyophilized chloroplasts. I observed that, when I prepared HP 700 complexes, lyophilized them and resuspended them in buffer, *à la* Yamamoto and Vernon, the two long wavelength bands at 735 and 755 nm were absent. I noted a similar lability of these bands when HP 700 was prepared from lyophilized chloroplasts. I am led to the conclusion that the formation of these higher aggregates* requires water in the case of HP 700, just as it requires hexane in the case of chlorophyll a in CCl₄ - the solubility of chlorophyll being extremely low in either

* That these long wavelength bands are not evident at higher levels of organization may mean that either such aggregates do not exist, or that they exist in very low concentration.

hexane or water.

I prepared HP 700 complexes in yet another modification of the technique of Yamamoto and Vernon. In their method, they used chloroplasts containing 5 mg of chlorophyll homogenized in 16 ml of ^{5%} Triton-X-100. In my (second) modification, I used chloroplasts containing 10 mg of chlorophyll homogenized in 16 ml of ^{5%} Triton-X-100. Such preparations were enriched in the highly aggregated form of chlorophyll; also, there was no evidence of monomer absorption in the C.D. spectrum of these preparations - i.e., the 660 nm band was missing.

A few words concerning the disposition of the monomer band in protein-chlorophyll complexes are in order. Although this band can be seen quite clearly, (a) in the HP 700 complexes prepared according to Yamamoto and Vernon (1968) (as a matter of fact, this is the only band in the red region of the spectrum that appears in lyophilized preparations), and (b) in complexes prepared according to Yamamoto and Vernon but from non-lyophilized chloroplasts (e.g. Fig. 38) in which it is accompanied by C.D. bands of highly aggregated chlorophyll, it cannot be seen in the specially prepared (second method) HP 700 complexes containing high concentrations of highly aggregated chlorophyll, probably because all the chlorophyll in this (second type of) preparation is in the highly aggregated state.

In regard to the broad C.D. band at 690 - 700 nm, which, as I suggested above, represents dimers, the following is pertinent. Two groups of workers, Quinlan (1968) and Sherman and Fujimori (1968) have shown that aggregates larger than dimers occur in solvents such as dioxane and aqueous formamide. (The chlorophyll in aqueous suspension complexed with formamide represents a model of a chlorophyll-protein complex, since the formamide molecules interact via

hydrogen bond formation between them (NH_2) and chlorophyll (COOH).

Both Quinlan, and Sherman and Fujimori have suggested that dimers, absorbing at 705 nm, may pass through intermediate stages of aggregation (with absorption maximum at 715 nm) in the formation of aggregates of higher order (absorbing at 740 nm), (the latter representing the C.D. "cross over point" for the 735 and 755 nm high aggregate doublet). In connection with this, it should be recalled that, in 1963, Brody and Brody attributed the shifting absorption and fluorescence maxima seen in greening Euglena to transitional populations of chlorophyll aggregates of increasing size.

I would now like to consider the effect of linolenic acid on chlorophyll-protein complexes. In sections IV-C-2-b (1) and (2), it was shown that linolenic acid could induce changes in system I chlorophyll-protein complexes from Phormidium and in HP 700 chlorophyll-protein complexes - these changes being indicative of deaggregation of chlorophyll. In the case of Phormidium chlorophyll-protein complexes, it was observed that addition of linolenic acid causes a disappearance of the two bands with maxima at 662 and 676 nm (which I had earlier assumed to be a dimer doublet), as well as a (virtual) disappearance of the band with maximum at 690 nm (the "different" type of dimer). Replacing these bands, upon deaggregation by linolenic acid, was a lone negative band with maximum at 671 nm. This latter band may either represent (a) residual absorption of the 690 nm band superimposed upon absorption of the monomer at 660 nm, or (b) pure monomer absorbing at 671 nm (this second suggestion made in analogy with the situation obtained when ethanol is added to chlorophyll in CCl_4).

In the case of HP 700 chlorophyll-protein complexes, the addition of linolenic acid was found to result in a very large decrease at 735 nm

and also decreases at 695 - 700 and 755 nm. At sufficiently high concentrations of linolenic acid (FA/Chl = 100) only the 660 nm band remains. In 1961 Brody and Brody (see also Brody and Brody, 1963) postulated that chlorophyll dimers and monomers are attached periodically to protein helices, and that conformational changes in the protein could bring about changes in the proportion of these chlorophyll species. Therefore, when I participated in works (M. Brody et al., 1969; Cohen et al., 1969) in which it was observed that addition of fatty acid to chloroplasts brings about configurational modification, I wished to determine whether the source of these modifications were conformational changes in the protein to which the chlorophyll is attached. I began this enquiry with HP 700, which had been reported to be a relatively pure preparation of system I reaction center chlorophyll-protein complexes (Yamamoto and Vernon, 1966). These preparations have an additional advantage (over, for example, Phormidium complexes) in that lipid is removed from HP 700 prior to detergent treatment, by exhaustive washing with acetone in heptane, presumably yielding lipid-free chlorophyll-protein complexes. As reported above, in the presence of 8 M urea, as in the presence of linolenic acid, deaggregation occurs, as judged by now-familiar changes in the red region of the spectrum. These changes in the red region of the spectrum are preceded by increases in the intensities of the near ultraviolet C.D. bands - which in the case of other proteins have been attributed to conformational changes.

Partial removal of urea (by cellulose sac dialysis or Sephadex chromatography) did not always result in a return to the control spectrum, for reasons not apparent at the present time. (However, in this regard, see the section of the discussion, pg 121, concerning the effect of bovine

serum albumin on linolenic acid-induced changes in the C.D. of digitonin-isolated particles containing systems I and II.)

Since the results obtained with linolenic acid in the red region of the spectrum (as well as in the ultraviolet) are virtually identical with those obtained by the use of urea, it seems reasonable to conclude that linolenic acid-induced deaggregation of protein-containing chlorophyllous systems arises primarily from a conformational change in the protein to which chromophores are attached. A second piece of support for the mechanism of deaggregation in protein-containing systems being primarily conformational change rather than direct action on the chromophore, is that pheophytin formation was not detected within the range of fatty acid concentrations generally used in the present work with chlorophyllous systems above the level of chlorophyll in solution.

The next step up in my study of chlorophyllous systems of increasingly complex structural organization is from (non-membranous) chlorophyll-protein complexes to membranous chlorophyll-containing systems (Section IV-C-3 a, b, and c). I will consider these membranous systems in increasing order of complexity, going from (a) digitonin-isolated particles containing only system I, to (b-1) digitonin-isolated particles containing both systems I and II, to (b-2) particles containing systems I and II, isolated by means of sonication, to (c) intact chloroplasts. A comparison of the chlorophyll a C.D. of all of the membranous systems reveals an interesting distinction between these and the chlorophyll a C.D. bands of non-membranous systems. In the non-membranous systems, the chlorophyll a C.D. bands are opposite in sign to the corresponding C.D. bands in membranous systems. The structural

array brought about by organization into membranes seems to confer on chlorophyll a the kind of configuration which results in the observed mirror-image relationship. The change in polarity induced by the incorporation of chlorophyll a into a membranous system holds true, not only for digitonin-isolated particles ^{containing} only system I, but for all membranous systems - from those containing both systems I and II through intact chloroplasts.

That a similar kind of mirror-image relationship is not observed in the case of chlorophyll b (the C.D. bands of the chlorophyll b dimer-doublet in CCl_4 have a negative short wavelength component at 638 nm and a longer wavelength positive component at 654 nm (Dratz et al., 1966)) ^{CP} these bands are in the same direction in quantasomes of normal barley, in which - in interaction with chlorophyll a - they appear at 653 and ≈ 671 nm (ibid.) - suggests that organization into membranes influences the structural array of chlorophyll b less than that of chlorophyll a. (In this regard, it is of interest to note that chlorophyll b is more soluble in polar solvents than is chlorophyll a, and may be attached to a slightly different part of the lipo-protein membrane.)

In dealing with membranous systems I will first consider the control spectrum for digitonin-isolated particles containing system I (refer back to Fig. 40 A), since my report (in conjunction with M. Brody, 1972) of their C.D. structure was the original one in the literature. Although the C.D. bands at 662 (positive) and 679 nm (negative) (primarily a dimer doublet of chlorophyll a, in analogy with the CCl_4 situation) and the C.D. band at 643 nm (negative chlorophyll b band) are fairly easy to identify, assignment of bands in the blue region of the spectrum, i.e., as to their origin being in chlorophylls a or b, or carotenoids,

is very difficult to make. For example, even in the work of Dratz et al. (1966), in which the C.D. of quantasomes prepared from normal barley were compared with quantasomes from a chlorophyll b-less mutant, interpretation of results was limited to their assigning the 470 nm band (lacking in the mutant) to chlorophyll b. (In the absence of the 470 nm band in the mutant, an unidentified band with maximum at 490 nm grows in.) To show the complexity of this situation, it is interesting to note that Garay et al. (1972), in their work with lycopene mutants of maize (in which the lycopene carotenoids are greatly reduced in concentration, while the chlorophyll b content remains normal), have observed that there occurs disappearance of the band with maximum at 470 nm. However, these latter authors offer the possibility that carotenoid deficiency could lead to changes in the 470 nm "chlorophyll b" band (even though the concentration of chlorophyll b is normal) because the structure of this band arises from interaction between carotenoid and chlorophyll b.

Because of its intricacy, the blue region of the spectrum has largely been avoided by investigators in the field. I wish to note, however, that the system I particles with which I worked, not only had a high concentration ratio_{of} chlorophyll a/b (of 5.19) but a similarly high C.D. ratio of 1.8 (662 to 643 nm).

It was seen in the Results section (Fig. 34) that, after 10 minutes of incubation in linolenic acid (FA/Chl = 2), the three bands in the red region of the spectrum underwent diminution and shift. While the changes in the 643 nm band could well represent deaggregation of chlorophyll b into monomer, diminution of the chlorophyll a doublet components was not accompanied by appearance of a chlorophyll a monomer band (encountered at 660 nm in the case of deaggregation of dimers by linolenic

acid in HP 700 and in CCl_4).

It was, however, shown with system I particles, that at high concentrations of fatty acids (FA/Chl = 100), a single negative C.D. band appears at 660 nm. Possible explanations for these phenomena will be given on page 127 of the Discussion and Conclusions.

I now turn to the effect of linolenic acid on complex membranous systems containing both system I and II (subchloroplast particles and chloroplasts). Two methods were used for the isolation of such subchloroplast particles: the first involved the utilization of digitonin, and the second, the use of sonication. (Here too, my report in conjunction with M. Brody (1972) was the original one in the literature.)

First I will discuss the control situation in regard to digitonin-isolated subchloroplast particles containing systems I and II. Comparison of this control with that containing only system I reveals that, in the former, the chlorophyll b band is 1.2 x the intensity of the 666 nm chlorophyll a band, compared with a value of 0.33 in the case of system I particles. This would be expected, since the chlorophyll a/b ratio in these particles is 2.32, compared with a value of 5.19 in system I particles. In the blue region of the spectrum, the bands at 482 and 500 nm are positive, in contrast to the situation which obtains in the case of system I particles - the significance of this will be discussed below, in conjunction with particles containing systems I and II isolated by means of sonication. Although, on the basis of direct absorption measurements with extracted pigment, control subchloroplast particles containing systems I and II, isolated either by means of digitonin or by sonication, appear to have the same chlorophyll a/b concentration ratio, inspection of C.D. spectra reveal that these two kinds of particles may differ in the state of their

chlorophyll in situ. Both particles have about the same ratio of C.D. bands at 663/666 nm (i.e. chlorophyll b/short wavelength component of chlorophyll a doublet); the two differ however, in their ratio of C.D. bands at 643/678 nm (i.e. chlorophyll b to long wavelength component of chlorophyll a doublet). In the case of digitonin-isolated particles containing both systems I and II, the ratio of 666/678 is 1.32; in the case of sonicated particles containing systems I and II, the ratio of 666/678 is 0.75. In the latter, the 678 nm band is accompanied by a band (absent in the digitonin-isolated particles) at 690 nm. The 678 and 690 nm bands form peaks around a more positive trough having an inflection at 683 nm. The significance of this new 683 nm inflection will be considered below, in connection with its greater predominance in the C.D. spectrum of control intact spinach chloroplasts.

In turning to the blue region of the spectrum, one observes that its appearance is virtually identical in both types of subchloroplast particles. A particularly striking difference between these particles, and subchloroplast particles containing only system I, is the positive nature of the blue bands (from 480 to 590 nm) in the former. One possible explanation for this shift from negative to positive ellipticity is the increase in internal light scattering which accompanies the greater structural organization found in particles containing both systems I and II. In regard to this, it should be mentioned that Menke (1970) attributed "distortion" of C.D. bands in the ultraviolet to light scattering by thylakoid suspensions. Urry and Krivacic (1970) have also discussed the effect of light scattering on C.D. spectra of increasing concentrations of poly-L-glutamic acid in suspension. I have found that when the turbidity of suspensions of chlorophyll-containing systems (e.g. digitonin-isolated particles) is enhanced (either by increasing

the concentration of biological material, or by introducing inert material, such as talc) there occurs "interconversion" of negative to positive ellipticities, as well as strong shifts in band peaks.

The deaggregating effects of linolenic acid (in the red region of the spectrum) are similar in the cases of digitonin-isolated particles (decreases in the 643, 666, and 680 nm bands) and sonicated particles (decreases at 650 and 666 nm). In the case of the latter, it should be recalled that there exists additionally, an inflection at 683 nm between the negative 678 and 690 nm bands; upon addition of linolenic acid this inflection disappears, and the 678 and 690 nm "bands" merge into a much stronger negative band with maximum at 678 nm. (Again, these phenomena will be discussed in relation to the 683 nm band predominant in the spectrum of intact chloroplasts.) With increasing time of incubation in the presence of linolenic acid, the 678 nm band decreases slightly (gets less negative). It is interesting to note that the C.D. spectrum I obtain, after addition of linolenic acid to subchloroplast particles, resembles the C.D. spectrum that Dratz et al. (1966) obtained with control Barley quantasomes. (Quantasomes, it should be remembered, are smaller particles obtained by longer sonication than the subchloroplast particles used in the present work.) Although the bands Dratz et al. observed with control quantasomes (at 653, 671 and 682 nm) are also evident in the present linolenic acid-treated subchloroplast particles,* I observed additionally in the control subchloroplast preparations a negative inflection with maximum at 683 nm (which disappears upon addition of linolenic acid). Since control subchloroplast particles have this

* In my preparation, these bands occur at 650, 666, and 679 nm, respectively.

inflection, and Dratz's control quantasome preparations do not, it seems likely that the chlorophyll in my preparations is more highly aggregated than in Dratz's preparations.

In the blue region of the spectrum, similar results with both types of systems I and II subchloroplast particles (i.e., digitonin-isolated and sonicated) are obtained upon addition of fatty acid, namely, a conversion from positive to negative ellipticities in the C.D. bands between 480 and 500 nm. These results are opposite in sense of direction to those obtained with digitonin-isolated system I particles, in which the same bands, negative in ellipticity in the controls, are converted to positive ellipticity upon increase in turbidity via addition of talc. Thus it would seem that the change may be reasonably interpreted to be a consequence of decrease in light scattering - in agreement with the findings of Molotkovsky and Zhasikova, who, in 1966, on the basis of direct absorption measurements, reported that treatment of non-intact chloroplasts with fatty acids yields a decrease in scattering of visible light, ^{at} 666 and 679 nm, respectively.

McCarty and Jagendorf (1965) had earlier reported that "protection" by defatted BSA is afforded against linolenic acid-induced inhibition of Hill reaction. In this regard, Glydenholm and Whately (1968) note that high concentrations (> 3 mg/ml) of defatted BSA inhibit photochemical activity and suggested that the action of BSA (at these high concentrations) is the physiological removal of fatty acids from the thylakoid membrane. Cohen et al. (1969) showed that incubation of chloroplasts in BSA prior to addition of linolenic acid prevent induction of change in steady-state fluorescence. My findings that there are no changes in C.D. of control digitonin-isolated particles upon addition of (concentrated) BSA are in agreement with those of Cohen

(Ph. D. thesis, 1970) who reported that BSA showed no effect on the fluorescence spectra of control chloroplasts. Cohen (Ph. D. thesis) additionally noted that linolenic acid-induced conversion of chloroplast spectra to the one-banded fluorescence emission stage, could be reversed, i.e. brought back to the three-banded stage, by post addition of BSA.

From the work presented in the results section of this thesis, on digitonin-isolated particles containing both systems I and II, it would seem that the primary action of linolenic acid in bringing about ^{on systems I and II isolated from chloroplast protein complexes,} deaggregation, i.e., the conversion of (mostly α) helical protein to random coil, can be almost completely reversed by BSA's removal of fatty acid with attendant rewinding of the helix to restore the aggregated state. If linolenic acid-induced randomization of the coil (and consequent deaggregation) is allowed to proceed (a) in the presence of ^{very} high concentrations of fatty acid, or, (b) for long periods of time (in excess of ten minutes or more) addition of BSA results only in partial restoration of helical structure; this incomplete restoration may be logically ascribed to some of the helix having become so extensively randomized as to be permanently denatured. This same type of denaturation may be the reason for the lack of constancy in being able to recover helical structure (and the aggregated state) after treatment of H P 700 with (4-8 M) urea.

The final portion of this section, on the C.D. of chlorophyll-containing systems of various levels of organization will be a consideration of intact chloroplasts, and the effects on them of linolenic acid. In the control, I observed, in addition to the C.D. bands at 650 (chlorophyll b) and 663 nm (see below), an intense negative band at 672 nm and an intense

positive band at 683 nm.*' It was already suggested in the Results section, that these latter two bands represent an aggregate doublet on the basis of the similarity of their reaction to linolenic acid. The chloroplasts used in the present work were prepared according to the method of Spencer(1967), in which lamellae are chopped with razor blades, resulting in production of chloroplasts $\approx$ 85 % whole (i.e. intact chloroplasts). I observed that when no special precautions were taken to preserve the integrity of the chloroplasts, i.e. when they are isolated by usual means e.g. in a Waring blender, these long wavelength bands of aggregated chlorophyll are absent. When I described the subchloroplast particles prepared by means of sonication, I noted that these have two minimum troughs (at 678 and 690 nm) around an inflection at 683 nm; one might expect such a condition to obtain where there remains only a residual quantity of the form of chlorophyll absorbing at 683 nm, with this absorption being superimposed upon a much stronger band at 679 nm.

The incubation of chloroplasts in linolenic acid results in dis-

* As noted in the Results section of this thesis, wide variations in C.D. spectra of chloroplasts are possible. If, for example, the chloroplast concentration of chlorophyll/unit volume is increased from 13 μ g of chlorophyll/ml (yielding bands with maxima at 683(+) and 672(-) nm) to 30 μ g of chlorophyll/ml, these long wavelength bands are shifted to 683-90 and 676-78 nm, respectively. Although, from this, it would seem that light scattering (the degree of which I have not determined) has a significant effect on the exact location of these maxima, the fact remains that these novel bands are similar in so far as the shorter wavelength one is always negative while the longer wavelength one is always positive.

' The manuscript (Brody and Nathanson, Biophys. J.) in which I reported these novel bands was "received for publication, May 21, 1971". In June, 1971, Gregory et al. presented a paper in which they also reported similar long wavelength bands having maxima at 686(+) and 677(-) nm in barley chloroplasts, and at 690 and 680 nm in "intact" spinach chloroplasts - the degree of the wavelength shift being a reflection of the "intactness" of the chloroplast. They also concluded that breakage of the chloroplast results in redistribution of "matrix" chlorophyll (connecting the two photosystems), with "consequent reduction in dimer content."

appearance of the C.D. bands with maxima at 683 nm and 672 nm, appearance (disclosure) of the 679 nm band, and increase in intensity of the 664 nm band supports my designating the 683-672 nm bands as components of one type of dimeric chlorophyll, and the 664-679 nm bands components of yet another type of dimer (on the basis of the similarity of the last pair with the dimer doublet of chlorophyll a in solution).

6. Concentration Dependence of the Linolenic Acid-Induced Change in P_{735}/P_{690} as a Function of the Structural Complexity of the Chlorophyll Containing Systems.

In section IV D of the results (see page 75) a compilation was made of the mole/mole ratio of fatty acid/chlorophyll required to bring about a 50% reduction of the control value of P_{735}/P_{690} (as measured at 22°C) in chlorophyllous systems having various degrees of structural complexity. It seems reasonable to assume that there exists a positive correlation between quantity of "non-specific" structural protein (i.e. proteins to which chlorophyll is not complexed) and level of organizational complexity. Since exogenous fatty acids were shown in the present work first to interact with chlorophyll-bearing proteins (CP 700 (page 61) and digitonin-isolated particles containing systems I and II (page 66)), and secondarily with 23A (page 68), a similar affinity even for the "non-specific" protein of the photosynthetic apparatus is presumed. On the basis of this presumed affinity, the observed incrementation in μ /mol (for bringing about equivalent deaggregation) in chlorophyllous systems of increasing structural complexity is understandable.

7. Effect of Linolenic Acid on the Thylakoids of Green and Blue-Green Algae.

In this section I would like to make a comparison between the effects

of linolenic acid on green (i.e. chlorophyll a and b-containing) photosynthetic systems (spinach chloroplasts and the thylakoids of the green alga Chlorella) and on photosynthetic systems in which the pigments accessory to chlorophyll a are not chlorophyll b, but rather the water-soluble phycobilins which occur in red and blue-green algae.* Since in these latter types of algae the antennae, or light-harvesting, chromophores of system II are predominantly phycobilins, the small degree of overlap in absorption between the water-soluble pigments (phycoerythrin and phycocyanin) and chlorophyll of system II (see below, page 126) makes it relatively easy to determine the effects of linolenic acid on energy transfer between the two photosystems in these algae.

A good place to be in this discussion is in a comparison of the effects of isolation on the fluorescence excitation spectra of thylakoids of algae (green and blue-green) with that of chloroplasts of higher plants. Action spectra for exciting fluorescence from Chlorella cells are very similar to those obtained upon excitation of these same bands with spinach chloroplasts (the 694 nm band observed in the 735 nm excitation spectrum of the leaf - which disappears upon isolation of spinach chloroplasts - is absent in the 735 nm excitation spectrum of whole Chlorella cells - suggesting that this highly aggregated, but labile, form of chlorophyll a is absent in Chlorella). Upon isolation of thylakoids from Chlorella, there is, compared to the whole cell, an increased contribution of the 674 nm excitation band and decreased contribution of the 682 nm band to fluorescence excitation at 735 nm; these changes, which indicate that isolation procedures themselves

* I used the term accessory pigments in the older, more classical sense, to mean pigments other than chlorophyll a recognizing that this term is used by some workers to cover even the antennae (bott) chlorophyll a's, which transfer energy to reaction center chlorophyll a's.

bring about some degree of deaggregation, are similar to, but more pronounced than, those observed upon isolation of spinach chloroplasts.

Examination of the excitation spectrum for exciting long wavelength fluorescence, at 725 nm, in intact Anacystis (control cells) compared with thylakoids isolated from these cells, indicates that here too, the isolation procedure results in deaggregation (i.e. decrease at 682 nm and increase at 674 nm). In contrast thylakoids prepared from formaldehyde-fixed cells show little or no change in this regard. As far as the phycocyanin is concerned, one notes that preparation of thylakoids ^{from intact cells} results in increase in emission at 660 nm compared to intact cells (very little emission at this wavelength is observed in the case of thylakoids prepared from formaldehyde-fixed cells), this indication of isolation-induced uncoupling of systems I and II is further substantiated by the observation that, with thylakoid preparation, contribution of the 660 nm excitation band to emission at 725 nm is considerably diminished (much less so in the case of thylakoids from formaldehyde-fixed cells). Phenomena similar to those which occurred upon isolation of thylakoids from blue-green algae, occurred upon isolation of thylakoids from the red alga Porphyridium (i.e. deaggregation and decrease in energy transfer between systems II and I).

Upon addition of linolenic acid to either cells or thylakoids of Chlorella, the now well-known pattern of deaggregation, such as that observed in spinach chloroplasts, was observed. Introduction of linolenic acid to either whole Anacystis cells (non-treated or formaldehyde-treated) and whole Porphyridium cells, or thylakoids isolated from these cells results in (a) similar patterns of chlorophyll deaggregation and (b) even more pronounced inhibition of energy transfer between systems II

and I (the latter as noted by decrease in contribution by phycobilin bands to long wavelength emission). The observation that incubation in linolenic acid results in drastic decrease in directly excited phycobilin emission suggests that linolenic acid is also affecting this chromophore-protein complex. Further substantiation of the effect of linolenic acid on the phycocyanin proteid is obtained from the O.D. data which show that the (presumably aggregated*) O.D. bands at 580 and 620 nm decrease in intensity upon addition of linolenic acid to Anacystis cells. (The question as to whether fatty acids are affecting the chromophores directly, or indirectly by acting on the protein to which the chromophore is attached, i.e. as in the case of chlorophyll-protein systems, was not further explored.)

Although I cannot extrapolate the findings with phycobilin-containing organisms - that linolenic acid decreases energy transfer between systems II and I to solely chlorophyllous systems, it seems reasonable to assume that the types of configurational changes observed with such chlorophyllous systems (i.e. swelling of chloroplasts) would also result in decrease in energy transfer.

III. RELATIONSHIP BETWEEN CHANGES INDUCED IN THE O.D. AND FLUORESCENCE OF MEMBRANOUS SYSTEMS BY LINOLENIC ACID OR OIL.

Although there is good parallelism between the (fatty acid-induced) decreases of aggregate bands and increase of monomer bands in both fluorescence emission and excitation of membranous chlorophyll-containing systems, the decrease in intensity of aggregate bands in O.D. spectra is not similarly paralleled by increase in monomer. I have two explana-

* Perci and Fujimori (1967, 1969) have shown that phycobilins may be isolated as aggregates in vitro.

tions to offer for this seeming discrepancy. The first explanation is based upon the assumption that deaggregation of (bulk) aggregate produces a bulk monomer which is optically inactive as far as C.D. is concerned. While it is recognized that the C.D. structure of the monomer in solution arises from the intrinsic asymmetry of this chromophore, it may be that bulk monomer, in attachment to protein, has restraints placed upon this intrinsic asymmetry. However, in the aggregated state, Pratz et al. (1966) have postulated that component bulk monomers would undergo dipole-dipole interactions resulting in C.D. structure. I further assume that the chlorophyll a monomer, which is observed in the C.D. spectrum, arises from the "special" type described in the introduction, i.e. that form of reaction center chlorophyll directly responsible for low temperature fluorescence at 696m μ in vivo and in vitro. It was earlier postulated (Branch and Rosenberg, 1964) that this special monomer is located in a "protected environment", i.e. one different, and less accessible than that of bulk chlorophyll; perhaps it is this particular environment that confers upon the special monomer its C.D. structure.

That modification in the chromophore's environment can have pronounced effects on its C.D. structure was shown also by Henke (1970) who, in his ultraviolet c.d. studies, demonstrated that removal of lipid from spinach lamellar protein causes a partial conversion of alpha to the beta form. Additionally, Fry et al. (1967) on the basis of their observations that, in the case of helical polypeptides, transfer to media of low polarity causes a red shift in the ultraviolet c.d. bands, tentatively concluded that helical segments of membrane proteins normally lie in a hydrophobic environment. Their view was supported by the finding that treatment of membranes with phospholipase a or lysolecithin (Wallach and Gordon, 1967) (which remove the fatty, or hydrophobic, portions of membranes)

results in a blue shift of the ultraviolet C.D. bands.

The alternative explanation which I wish to offer for the observed discrepancy is based on my calculations (see below) that the monomer in solution has a much lower C.D. difference extinction coefficient than does the dimer. Although, in the case of Phormidium chlorophyll-protein complexes, the low temperature linolenic acid-induced decrease of long wavelength fluorescence is accompanied by conversion of the aggregated C.D. spectrum into one characteristic of monomeric chlorophyll, the situation is quite different in regard to the (membranous) digitonin-isolated particles containing system I. In the latter case, even when fluorescence emission has reached the one-banded stage, it is observed that blue shifts are accompanied by only slight decreases in both absorption and C.D. I made calculations of the difference extinction coefficients for the dimeric and monomeric forms of chlorophyll a for both the cases of chlorophyll a in CCl_4 and chlorophyll a in ethanol. At a concentration of 10^{-3}M chlorophyll a in CCl_4 , 95 % of the chlorophyll is in the dimeric form (Brody and Broyde-1968). At a concentration of 10^{-6}M , the predominant form of chlorophyll (i.e. 90 %) is the monomeric one. In the case of 10^{-3}M chlorophyll in ethanol, 98 % of the chlorophyll is in the monomeric form (Brody and Broyde, 1968). Based upon these figures, and using the ellipticities garnered from my data for chlorophyll a in the two solvents, I calculated the difference molar extinction coefficients from the following formula: $\Delta A = \Delta ECL$. (ΔA represents the difference in absorption for left and right circularly polarized light, ΔE represents the difference molar extinction coefficient, C represents the molar concentration of chlorophyll in solution and L represents the path length). Equating 1 millidegree (m^0) of ellipticity with $\Delta A = 3 \times 10^{-5}$ (see booklet # 54, Durrum Instrument Company, Palo Alto, California), I performed the following calculations:

Type of solution	Measured ellipticity of Chlorophyll band (in m°)	$\Delta H \times 10^{-5}$	$\frac{1}{l}$ (cms)	ΔE
1. Chlorophyll in CCl_4 (2×10^{-3} M)	14	42	0.005	42
Chlorophyll in CCl_4 (2×10^{-6} M)	0.6	1.3	1.0	9
2. Chlorophyll in Ethanol (2×10^{-3} M)	2	6	0.005	6

From the above calculation, one can see that the monomer has a much lower (about an order of magnitude) difference extinction coefficient than does the dimer in solution; I assume the same conditions exist when chlorophyll is in a more highly organized state. In combination with the fact that there are still large amounts of dimer present even when fluorescence emission has reached the one-banded state (i.e., 10% intensity of aggregate has only decreased by 1/3, leaving quite a lot of overlap in the anticipated monomer region) it is likely that contribution to 10% by the monomer would be difficult to detect in chlorophyll-containing systems more complex than those *in vitro*.

Another question that remains to be resolved is why only a change (of approximately 10%) in direct absorption and 10% is accompanied by complete conversion to the one-banded emission form. One possible answer is that, while initially there is conversion of (bulk) aggregate to (bulk) monomer (and subsequent energy transfer from this bulk monomer to "special monomer" p. 693) there is also an uncoupling of energy transfer between systems I and II resulting in the large decrease in bulk aggregate fluorescence as well as in the decrease in total fluorescence yield. (Contribution to the two latter phenomena may also be a general breakdown of lamellar structure (Cohen et al., 1969).)

In regard to this entire question it is interesting to note that

Edelman and McClure (1968), in their study of fluorescence probes and the conformation of proteins demonstrated that with increasing temperature, immunoglobulin showed first an initial increase in fluorescence (of the tryptophane residues of the protein, and of the probe-ANS) and then a secondary decrease in fluorescence. They attributed the large initial increase in fluorescence (at the thermal transition point) to unfolding (denaturation) of the protein, and the subsequent decrease to a general breakdown of protein structure.

SUMMARY

The techniques of circular dichroism and absorption and fluorescence spectroscopy were used in determining that both "RLE" (a naturally-occurring proteinaceous factor extracted from the leaves of Ricinus communis) and exogenous fatty acids (which serve as good models for the action of RLE), bring about deaggregation of chlorophyll in chlorophyllous systems of various degrees of structural complexity. Two mechanisms for this deaggregation were uncovered. In the simpler systems (i.e., chlorophyll in solution) the action is solely a direct one on the chromophores; in the more complex systems, deaggregation occurs predominantly through an indirect means, i.e. via conformational changes in proteins to which the chromophores are attached.

Although photosynthetic higher plants normally exhibit three chlorophyll fluorescence bands at 77° K - at 685, 698 and 735 nm (presumably corresponding to emission from "bulk" system II monomer, "special system II reaction center" monomer, and system I aggregate(s), respectively), these bands, in chloroplasts isolated from the leaves of Ricinus communis, undergo time and temperature-dependent transformations to produce an essentially one-banded spectrum (with maximum at 698 nm). Similar changes in emission were found to occur when chloroplasts of other higher plants, or thylakoids of algae, were suspended either in RLE ("cross-reaction" systems) or in buffer to which long-chain unsaturated fatty acids had been added.

That these emission changes resulted primarily from deaggregation was inferred from data obtained in room and low temperature absorption studies in which there were observed concomitant decreases at 689 and 705 nm (bands ascribed to absorption by aggregated chlorophyll) and

increase at 674 nm (maximum of the chlorophyll monomers). Since incubation in either RLE or fatty acid resulted in decreased contribution by the 682 and 705 nm excitation bands and concomitant increase in contribution by the 674 nm band (to fluorescence at 698 or 735 nm), excitation data were found to be in agreement with absorption data.

In the visible region of the spectrum, the separate resolution of C.D. bands of aggregated and monomeric chlorophyll, in vitro and in vivo, made it possible to establish with greater certainty the deaggregation induced by RLE or exogenous fatty acids. Although, in the cases of chlorophyll a in solution (in CCl_4 or in the mixed solvent CCl_4 -hexane) deaggregation occurs by direct action on the chromophore. C.D. studies in the U.V., with systems in which chlorophyll is attached to protein, revealed that the (linolenic acid or urea-induced) changes observed in the visible region of the spectrum are preceded by conformational changes of the protein. Further evidence for this indirect mechanism was obtained with digitonin-isolated subchloroplast particles containing systems I and II. With such particles it was observed that linolenic acid-induced deaggregation could be partially reversed by addition of defatted bovine serum albumin.

Interpretations of the C.D. bands of structurally complex chlorophyllous systems were materially aided by application of the C.D. data obtained earlier with chlorophyll a in solution. For example, C.D. bands observed with chlorophyll a in either CCl_4 or the mixed solvent CCl_4 -hexane were used to interpret spectra of chlorophyll-protein complexes (HP 700 and system I chlorophyll-protein complexes isolated from Phormidium). Resolution of these latter spectra allowed, in turn, interpretation of the more intricate C.D. spectra seen in membranous systems (digitonin-isolated subchloroplast particles con-

taining system I, subchloroplast particles containing both systems I and II - isolated either (by means of digitonin or by sonication, and intact chloroplasts).

It was observed, with controls of the above, that C.D. spectra of chlorophyll a chromophores in membranous systems have a reversed polarity relative to that of chlorophyll a in solution. This reversal was postulated to have its origins in the organization of chlorophyll-containing proteins into membranous arrays.

Two alternative explanations were invoked in regard to the dilemma of why deaggregation in membranous systems does not result in (anticipated) increase in intensity of the monomer C.D. band. One was based on the assumption that, in membranous systems, only a special form of monomeric chlorophyll (i.e., that found in the system II reaction center) has C.D. structure. The other explanation was based on the calculation of the difference extinction coefficient of the C.D. of monomeric chlorophyll in solution being only 1/6 that of the dimeric form; with such a relationship also postulated for the situation in regard to chlorophyll in membranes (and with incomplete deaggregation) little increase in the intensity of the C.D. monomer would be predicted.

It was also shown that fatty acids could affect chromoproteids other than chlorophyllous ones in membranous systems. A study of the linolenic acid-induced changes in fluorescence emission and excitation and C.D. in the red alga Porphyridium and in the blue green alga Anacyctis revealed that the water-soluble phycobiline undergo changes similar to those which occur in the chlorophyll chromoproteid.

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