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THE EFFECTS OF A NATURALLY-OCCURRING FACTOR (FROM THE LEAVES OF *RICINUS COMMUNIS*) ON THE LIGHT REACTIONS OF PHOTOSYNTHESIS: MODEL SYSTEMS FOR THE ACTION OF THIS FACTOR

by

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ABBREVIATIONS USED

ASC - ascorbate

ADP - adenosine diphosphate

BSA - bovine serum albumin

CCCP - carbonyl cyanide m-chlorophenylhydrazone

CMU - 3 (3-chlorophenyl)-1, 1-dimethylurea

DCMU - 3 (3,4-dichlorophenyl)-1, 1-dimethylurea

DCPIP - 2,6-dichlorophenol indophenol

FCCP - carbonyl cyanide p-trifluoromethoxyphenylhydrazone

Fd - ferredoxin

FeCN - potassium ferricyanide

FMN - flavin mononucleotide

HEPES - N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid

MES - 2-(N-morpholino) ethanesulfonic acid

MV - methyl viologen

NADP - nicotinamide adenine dinucleotide phosphate

nm - namometers (10^{-9}M)

O.D. - optical density

PMS - phenazine methosulfate

SDBS - sodium dodecyl benzene sulfonate

SDS - sodium dodecyl sulfate

Tricine - tris (hydroxymethyl) methylglycine

Tris - tris (hydroxymethyl) aminomethane

UV - ultraviolet

INTRODUCTION

The concept that photosynthesis, as it occurs in oxygen-liberating organisms (*i.e.*, in algae and higher plants), involves two different chlorophyll *a*-containing systems arose from early work on the requirement for quanta of different energies. The experiments of Emerson and co-workers (Emerson *et al*, 1956, 1967; see also reviews by Emerson, 1958, and Smith and French, 1963) indicated that at long wavelengths, but still within the red absorption band of chlorophyll *a*, *in vivo*, the quantum yield of photosynthetic oxygen evolution drops sharply. It was also observed that the yield of photosynthesis in the region of the "long wavelength decline" was greater with supplementation by shorter wavelengths than without; these findings implied that "inefficient" far-red light became more effective when coupled to shorter wavelengths. Although Emerson *et al* (1957) visualized the accessory pigments (which absorb at shorter wavelengths than the red band of chlorophyll *a*) as making some necessary contribution to photosynthesis (*i.e.*, playing a role beyond energy transfer), Franck (1958, see also Franck, 1955) suggested that maximum yield would be realized only when approximately equal numbers of molecules of chlorophyll *a* are in different excited states--these being achieved with excitation by far-red light and light of shorter wavelengths.

In 1961, Duysens *et al* noted that cytochrome f oxidation

is driven by far-red light; with shorter wavelength light the cytochrome is reduced. Also, they observed that the effects of the shorter wavelengths could be blocked by the addition of the herbicide DCMU--known to be an inhibitor of oxygen evolution. On the basis of these experiments, and earlier ones which involved studies of energy transfer between accessory pigments and chlorophyll a (Duysens, 1952), Duysens (1961) formulated a scheme in which the two chlorophyll a-containing systems are connected by a chain of electron carriers (in a pattern similar to that suggested earlier by Hill and Bendall, 1960). Duysens called the long wavelength (i.e., short wave number) pigment system "system I" and the short wavelength (i.e., long wave number) pigment system "system II." The functions of these systems (e.g., Clayton, 1965) are thought to be as follows: energy absorbed ($\lesssim$ 680 nm) by the antennae (light-harvesting) chlorophylls of system II--or preferentially transferred to them from accessory pigments (including chlorophyll b and phycobilins)--mediates the oxidation of H_2O to molecular oxygen and reduction of cytochrome (or plastocyanin); energy absorbed at long wavelengths ($\gtrsim$ 680 nm) directly by chlorophyll a's--or preferentially transferred to them (from carotenoids or, indirectly, from system II)--mediates the oxidation of cytochrome and the reduction of pyridine nucleotide. Both pigment systems are thought to contain reaction centers* in

*Franck (Franck and Rosenberg, 1964) has proposed a second hypothesis, namely that one reaction center alternatively services each pigment system.

which specialized chlorophyll a's participate in the conversion of light energy to chemical energy. Furthermore, two types of photophosphorylation are thought to occur; one associated with non-cyclic electron flow between the two pigment systems and the other associated solely with system I.

In Fig. 1 is shown a recent formulation (redrawn from Boardman, 1968) of Duysen's earlier scheme. Note the following: the sites at which specific artificial substances accept or donate electrons, and the sites at which substituted dimethylureas (electron transport inhibitors) are thought to act.

The red absorption band of chlorophyll a, in vivo, is broad compared to the red band chlorophyll a in organic solvents. Many workers have interpreted this as suggestive of chlorophyll a, in vivo, being composed of more than one form of chlorophyll a. Various workers (Albers and Knorr, 1937; Krasnovskii and Kosobutskaja, 1952, 1955; French, 1958; Brown and French, 1961) resolved two absorption maxima under the red band of chlorophyll a, in vivo; these major forms of chlorophyll a have peaks at 670 nm and 680 nm.

The action spectrum for the Emerson "enhancement" effect (the latter being the proportional increase in yield of photosynthesis) when far-red light is supplemented with shorter wavelength light, exhibits a shoulder at 650 nm (associated with chlorophyll b, in vivo) and a peak at 670 nm (Govindjee and Rabinowitch, 1960). This observation

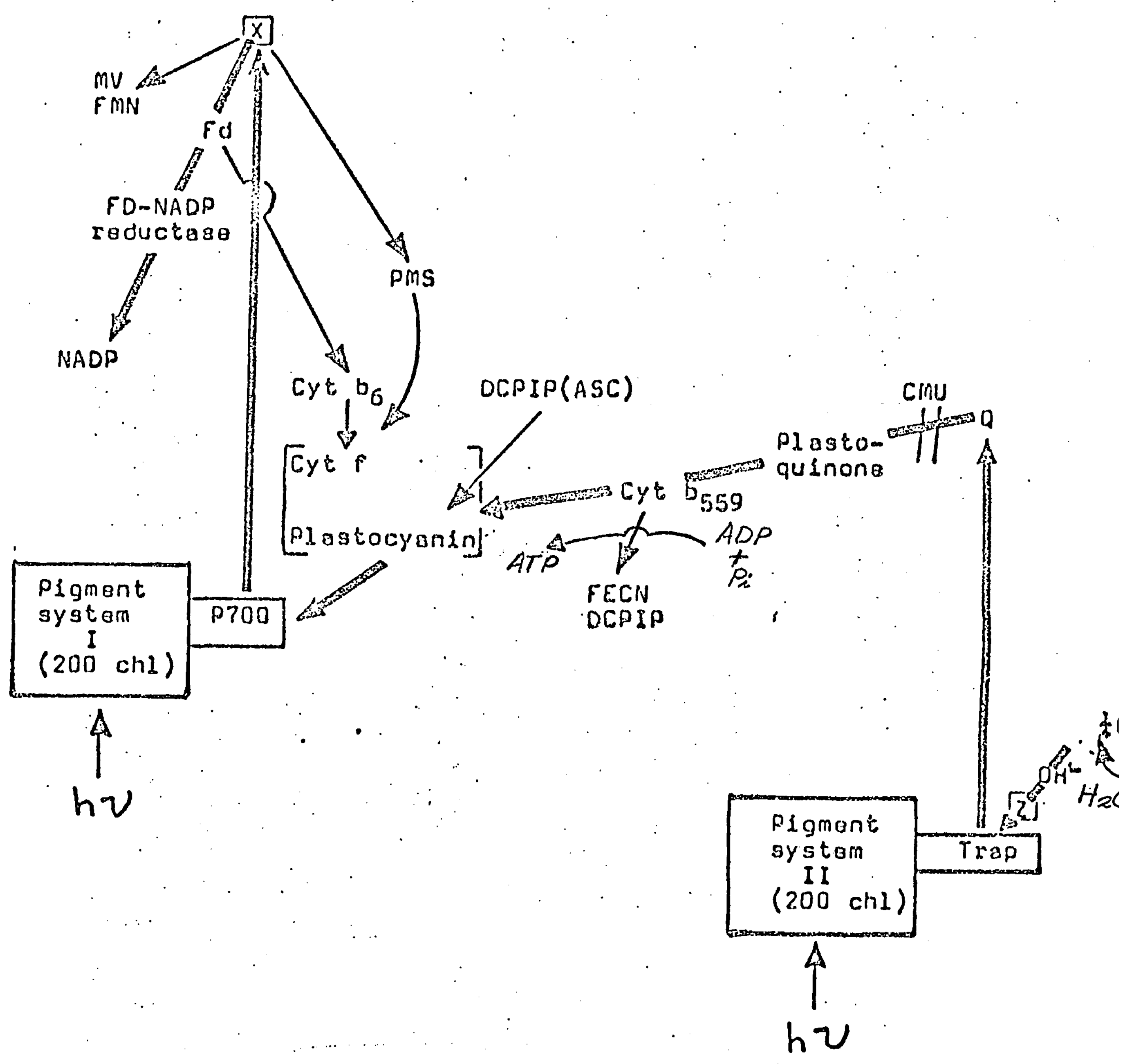


Figure 1. Photo-induced electron flow in chloroplasts. The main path of flow is denoted by the thicker lines; the direction of flow is indicated by arrows. Redrawn from Boardman (1968).

led to the assignment of Ca670* and Cb650 to system II. A dye reduction action spectrum for system I alone has been determined using DCMU to inactivate system II (Kelly and Sauer, 1965). This action spectrum also had a shoulder at 650 nm as well as a peak at 680 nm. From these experiments Ca680 has been placed in system I (and a contribution to this system by chlorophyll b has been noted).

In addition to Ca670 and Ca680, several other forms of chlorophyll a have been discovered. They include Ca690 (Doring et al, 1967); Ca695 (Brown and French, 1961); P700 (Kok, 1959); Ca705 (S. Brody, 1958, and Butler, 1961). (Whether all of these forms exist as separate entities, or whether two or more are identical is still undecided; see reviews by Butler, 1966, and M. Brody, 1968.) The presence of these other forms has been determined on the basis of several techniques, including low-temperature absorption spectroscopy, derivative-absorption spectroscopy, light-induced absorption changes, and low-temperature fluorescence excitation spectroscopy. These techniques, as well as others, e.g., use of electron transport inhibitors, has permitted assignment to either system II or system I; Ca695, P700, and Ca705 have been assigned to system I, whereas Ca690 has been assigned to system II. P700 has been estimated to be present (Kok, 1965) in a concentration

*In conformance with popular usage, the various pigments, in vivo, will be abbreviated as follows: C (to stand for chlorophyll) followed by the subscript a or b (for the two predominant forms of chlorophyll in higher plants) and the wavelength of the absorption maximum in nm.

of 1 per 300 to 400 light-harvesting chlorophyll _a_'s. It is thought (Kok, 1966) to be located at the reaction center of system I, on the basis of light-induced redox changes; system I light oxidizes P700, whereas system II light reduces it.

At room temperature, the fluorescence yield of chlorophyll _a_ (in algae) is approximately 3% (Latimer _et al_, 1957); the main emission band is at 685 nm with a satellite shoulder at about 730 nm. When algae or chloroplasts are cooled to the temperature of liquid nitrogen (-196°C), three bands are observed. The major emission band is at long wavelengths located at 710-730 nm (referred to as F720*) in algae and at 730-740 nm (referred to as F735) in higher plant chloroplasts (however, see M. Brody _et al_, 1965, where it was shown that the location of the long wavelength band maximum is a function of effective chlorophyll concentration, _i.e._, the concentration of chlorophyll on the lamellae). In addition to the band at 685 nm ("F685"), a band is also seen at 698 nm ("F698").

Although other interpretations have been offered (see reviews by French, 1967, and M. Brody, 1968) much evidence suggests that the forms of chlorophyll _a_ fluorescing at 710-740 nm are aggregated species of chlorophyll (see review by S. and M. Brody, 1963). Among the evidence is the finding that concentrated solutions of chlorophyll _a_

*In analogy with the abbreviation used for the various absorbing forms of chlorophyll _a_, the prefix F will be used to denote the various emitting forms.

in organic solvents have long wavelength emission at 715 nm--as well as the usual band at 685 nm (S. Brody, 1958); dilute solutions of chlorophyll a in these solvents have only the band at 685 nm. If chlorophylls are specially prepared (see below), the 698 nm band, as well as the 685 band, may be seen at -196°C (S. and M. Brody, 1963; Broyde and S. Brody, 1966).

The correspondence between the absorption band at 670 nm and fluorescence at 685 nm was recognized long ago, on the basis of fluorescence observations at early stages of greening (Krasnovskii and Kosobutskaja, 1952; 1953). Long wavelength emission, at 710-740 nm, has been associated with Ca683 and Ca705 on the basis of action spectra for exciting this emission in greening algae (S. and M. Brody, 1963; 1965; M. and S. Brody, 1966) and greening bean leaves (Butler, 1965). For further discussion of these assignments, see the review by M. Brody (1968).

The existence of the emission band with maximum at 698 nm has been reported for many algae and higher plants at -196°C (Litvin et al, 1960; M. Brody and H. Linschitz, 1961; Bergeron, 1963; S. and M. Brody, 1963; Govindjee, 1963; Kok, 1963; Goedheer, 1964). Some of the evidence which supports the assignment of F698 to system II is the following. The accessory pigments and Ca670 contribute preferentially to its excitation (fluorescence excitation spectra determined with whole algae and chloroplasts; see Murata et al, 1966; Bergeron and Olson, 1967; Goedheer, 1968). The

use of detergents followed by differential centrifugation (Boardman *et al*, 1966; Kok and Rurainski, 1966; Ke and Vernon, 1967) has permitted a separation of pigment systems I and II--into light (~ 100,000 xg) and heavy (~ 10,000 xg) fractions respectively. With these fractions, it was observed that emission at 685 nm and 698 nm is associated with the heavy subchloroplast particle while longer wavelength emission is associated with the light particle.

In 1963 (Bergeron, 1963, and Govindjee, 1963) it was proposed, *ad hoc*, that the pigment giving rise to emission at 698 nm was the reactive site for photochemistry in system II. In this same work, S. and M. Brody (1963, see also Broyde and S. Brody, 1966) reported that fluorescence at 698 nm, *in vitro** (and *in vivo*), could be quenched by the addition of small amounts of plastoquinone; since plastoquinone is one of the first system II acceptors in the electron transport chain (see Fig. 1), it was inferred that F698 was involved in the primary photochemical steps of system II. Experiments by Cho and Govindjee (1966) and Cho *et al* (1966) on the effect of temperature on emission from reaction centers or "energy traps" reveal that F698 behaves in system II in a fashion analagous to P700 in system I.

Since a number of electron transport inhibitors, e.g. DCMU (Duysens and Sweers, 1963), phenyl mercuric acetate (Vredenberg and Duysens, 1965), FCCP (Bannister, 1967), etc.,

*Whether F698 *in vivo* corresponds to F698 *in vitro* has not been established; however, it is interesting to note that the temperature dependence of fluorescence yield is similar *in vivo* (Broyde and Brody, 1966) and *in vitro* (Goedheer, 1964).

have pronounced effects on the variable yield of fluorescence (*i.e.*, that fluorescence observed during transient illumination at high light intensities)--which is presumed to arise primarily from system II (Lavorel, 1964)--it was decided to see if these substances might be capable of enhancing the level of steady-state fluorescence emitted by the reaction center of system II, *i.e.*, F698.

While these studies were being made, other experiments--in collaboration with Miss B. Nathanson and Dr. M. Brody--revealed that chloroplasts of the castor-oil plant (*Ricinus communis*) underwent time-dependent changes in fluorescence emission (as determined at -196°C). One of the major changes observed with increasing time of incubation of these chloroplasts was an intensification of emission at 698 nm.

The aim of the present investigation was to determine: 1) the nature of this substance present in *Ricinus*, 2) how the spectral changes are brought about, 3) the underlying molecular mechanism of the change, and 4) whether the spectral changes are associated with other modifications of chloroplast function (e.g., electron transport or ultrastructure).

In the course of these studies, a number of substances were examined as possible model systems for the action of the substance present in *Ricinus*. These include the electron transport inhibitors noted above, long-chain unsaturated fatty acids and derivatives, proteolytic and lipolytic enzymes, and detergents. In addition, treatments which might alter the spectral properties of chloroplasts were examined, *i.e.*, UV irradiation or ageing.

MATERIALS AND METHODS

I. PREPARATION OF CHLOROPLASTS AND CHLOROPLAST FRAGMENTS

Several different genera of higher plants were used in the preparation of chloroplasts. Spinach (*Spinacia oleracea*) was obtained from local markets, whereas corn (*Zea mays*), radish (*Raphanus*, sp.), tobacco (*Nicotiana*, sp.), and castor-oil plant (*Ricinus communis*, variety Baker 296, or *Ricinus zanzibarensis*, a mixed variety) were grown from seed in the greenhouse. Chloroplasts were usually prepared by one of the two following methods. Leaves were immersed in running tap water and illuminated for one hour. The lamina were de-petiolated and the midribs removed.

A. Method of Jagendorf and Avron (1958).

50 grams of lamina were homogenized in a Waring Blender with 150 ml of 0.35 M NaCl-0.04M Tris-HCl (pH 7.8), to be referred to as NaCl-Tris, for a total of 15 seconds (5 seconds at 50% of line voltage [110 V] and 10 seconds at 100% of line voltage). The resulting brei was filtered through 4 layers of cheese cloth (Curity Cheese Cloth #60, Kendall Co., New York) and the filtrate was centrifuged for 90 seconds at 500 xg in a Sorvall RC-2 refrigerated centrifuge. The pellets (containing cell debris) were

discarded and the supernatant was centrifuged for seven minutes at 1,000 xg. The resulting pellets were combined and resuspended in a small volume of NaCl-Tris with the aid of a tissue homogenizer.

B. Method of Bertsch et al (1969).

50 grams of lamina were homogenized, as above, with 200 ml of the following medium (in mM): Sucrose, 350; Tricine-NaOH or Tris-HCl, 20 (pH 7.4); NaCl, 1; $MgCl_2$, 1. The homogenate was filtered through 16 layers of cheese cloth and the filtrate was centrifuged for one minute at 7,000 xg. The pellets were combined and the chloroplasts osmotically broken by resuspension in 1 mM NaCl and 1 mM $MgCl_2$.

Broken chloroplasts were also prepared by osmotic shock, utilizing the chloroplasts obtained by method (A); this was accomplished by resuspending them in 0.035M NaCl-0.002M Tris-HCl, pH 7.8 (Wasserman and Fleischer, 1968).

When chloroplasts with intact outer membranes were used, they were prepared according to the method of Spencer (1967). Leaves were chopped into fine pieces with a razor blade, the resulting brei being filtered through four layers of cheese cloth before differential centrifugation. These procedures were carried out in a medium containing (in mM): Sucrose, 400; Tris-HCl, 50 (pH 7.8); NaCl, 10 (this medium to be referred to as STN).

Subchloroplast particles were prepared by sonicating spinach chloroplasts for 45 seconds at 20 KC, using a MSE model #3000 sonerator. After a preliminary centrifugation (6,000 xg for 10 minutes) to remove unbroken chloroplasts, the subchloroplast fragments were sedimented at 104,000 xg for one hour. These procedures were conducted according to the method of McCarty (1968).

System II and system I subchloroplast fractions were prepared by detergent treatment according to Anderson and Boardman (1966). Spinach chloroplasts were fragmented by incubation in 0.5% digitonin for 60 minutes at $0^{o}C$. The subchloroplast fragments were then collected by differential centrifugation; system II, 10,000 xg for 30 minutes; system I, 144,000 xg for 60 minutes.

Unless otherwise noted, all operations in the preparation of chloroplasts were performed in an ice bath, and the chloroplasts were usually used unwashed.

II. GROWTH OF ALGAE

Euglena gracilis strain Z was cultured in the light (daylight fluorescent lamps, light intensity=5 x 10^3 ergs/cm^2-sec) on Difco Euglena Broth for five days ($25^{o}C$). *Scenedesmus obliquus* wild type and two mutant strains, #11 (O_2^-) and #8 (CO_2^-), were grown heterotrophically in the dark at $23^{o}C$ for six days on Bishop's (1964) *Scenedesmus* medium. Prior to use in experiments, the cells were collected by centrifugation and resuspended as follows, *Euglena* in 0.1 M potassium phosphate buffer pH 7.0, and

Scenedesmus ins *Scenedesmus* medium minus glucose and yeast extract.

III. PREPARATION OF LEAF AND CHLOROPLAST EXTRACTS

Crude extracts of *Ricinus* were prepared according to modification of the method of McCarty and Jagendorf (1965). Usually 50 grams of leaf blade tissue were homogenized (in a Waring Blender) with 150 ml of NaCl-Tris. After homogenization, the brei was filtered through four layers of cheese cloth and centrifuged for 20 minutes at 17,000 xg ($0^{o}C$); the precipitate was discarded. The supernatant (crude extract), sometimes used in this form will be henceforth referred to as "RLE." In some cases, RLE was subjected to another centrifugation (104,000 xg for one hour in a Spinco ultracentrifuge, model L-2) to remove small chloroplast fragments. Additionally, in some preparations, RLE and the 104,000 xg supernates were dialyzed against 50-100 volumes of NaCl-Tris overnight.

RLE was sometimes chromatographed on G-25, coarse grade (Pharmacia Fine Chemicals, Piscataway, N.J.) which had been equilibrated at $0^{o}C$; NaCl-Tris was also used as the eluting buffer. The bed volume of the column was 280 cm^3 (3 cm x 40 cm). 50 ml of RLE was applied to the column; the flow rate was approximately 2 ml per minute, and 10 ml fractions were collected. This preparation will be referred to as G-25 *Ricinus* protein.

Extraction of *Ricinus* chloroplast proteins was conducted

according to a procedure suggested by Dr. R. E. McCarty (personal communication) which had been used for the preparation of chloroplast proteins from *Phaseolus vulgaris*.

Ricinus leaves were washed with cold tap water and stored in the dark at 4°C for 2-3 hours, so that starch grains might be reduced in size. 250 grams of leaves, whose petioles and midribs had been removed, were blended in 500 ml of 0.4M Sucrose-0.02M potassium phosphate buffer, pH 6.0 (in 50 gram batches); chloroplasts were isolated according to method (A). The chloroplasts were resuspended in a minimal volume of isolation medium and the chlorophyll (a+b) concentration (see below) was adjusted to approximately 3 mg/ml. The chloroplast suspension was then added (dropwise) to 17 volumes of acetone (-10°C) with rapid stirring. The resultant wet acetone paste was then worked with a spatula (25°C) until all traces of acetone were removed. The acetone paste was then extracted, three times, with a total of 200 ml of 0.05M potassium phosphate buffer (pH 7)-0.002M EDTA* (to be referred to as "KP_i-EDTA"). After each extraction, insoluble material was removed by centrifugation (30,000 xg for 10 minutes). The pooled extracts were heated in a water bath, at 65°C, for two minutes, and then plunged into an ice-water bath. The extract was stored overnight, at 4°C, after which, precipitated material was removed by centrifugation (30,000 xg for 10 minutes). Crystalline ammonium sulfate was added to the supernatant to 22% saturation, and the mixture was

*EDTA - ethylenediamine tetracetic acid

mechanically stirred for 30 minutes to insure complete precipitation. The precipitate was removed by centrifugation (30,000 xg for 10 minutes), and discarded, and ammonium sulfate was added to the supernatant to 60% saturation. The centrifugation step was repeated, except that in this case, the pellets were saved, and the supernatant was discarded. The pellets were combined and resuspended in approximately 5 ml of KP_i-EDTA and dialyzed overnight against 300 volumes of the same buffer. The dialysate was then applied to a G-100 Sephadex column (1.5 cm x 70 cm, bed volume 125 cm^3), which had been previously equilibrated at 25°C. The eluting buffer was KP_i-EDTA and the flow rate was approximately 10 ml per hour; fractions of 4 ml were collected.

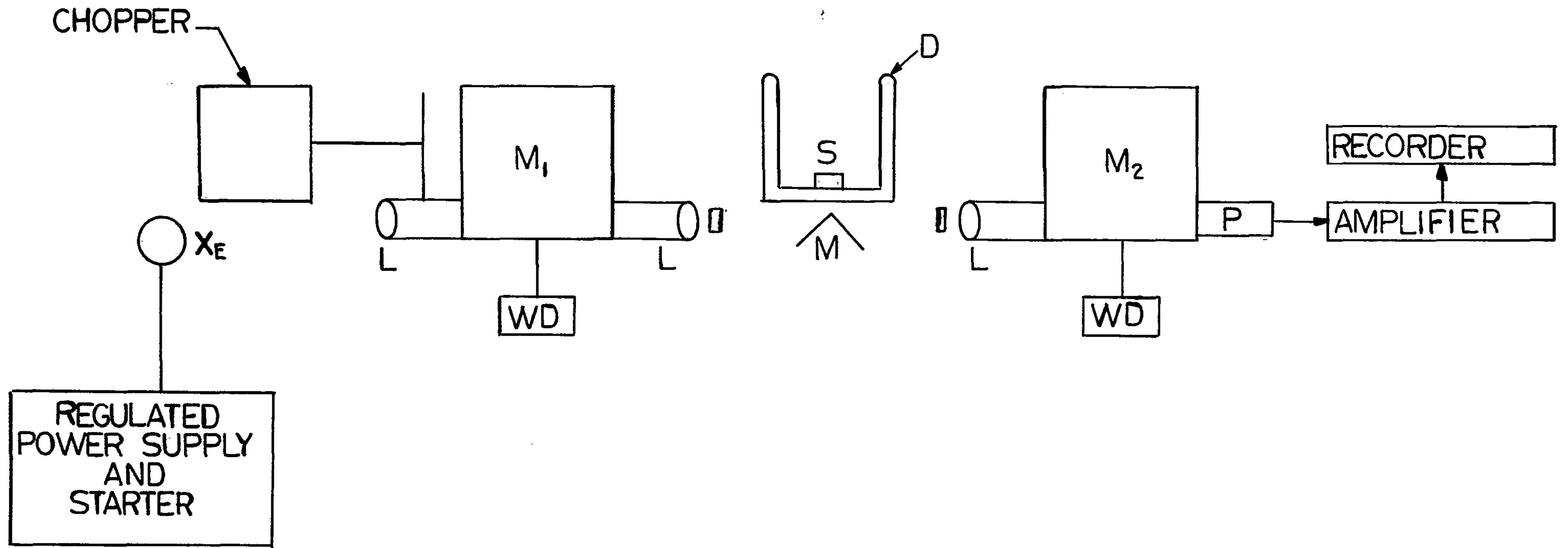
Extracts of primary leaves of <u>Phaseolus multiflorus</u> (scarlet runner bean) were prepared according to Sastry and Kates (1964).

IV. SPECTRAL MEASUREMENTS

A. <u>Fluorescence Emission</u>.

Emission spectra of plant material at room temperature or the temperature of liquid nitrogen were determined with the recording spectrofluorimeter shown in Fig. 2 (M. Brody <u>et al</u>, 1965). The instrument utilizes a 1800 watt Xenon arc lamp, whose output passes through a Bausch and Lomb monochromator (grating blazed at 400 nm). Excitation was generally with light having a maximum at 436 nm. A blue glass filter Corning C55-56 was used to further purify the light. The incident light intensity was 741 $ergs/cm^2$-sec as determined

Figure 2. Block diagram of the apparatus used to measure and record fluorescence emission spectra. D-flat bottom dewar. L-lens. M-mirror. M_1 and M_2-monochromators. P-photomultiplier tube. S-sample. WD-wavelength drive. Xe-Xenon lamp. Redrawn from M. Brody _et al_ (1965).


CHOPPER
D
S
M_1
M_2
RECORDER
P
AMPLIFIER
X_E
L
L
M
L
WD
WD
REGULATED
POWER SUPPLY
AND
STARTER

with a calibrated Eppley thermopile. The sample, contained in a 5 ml beaker, was placed in a mirrored dewar having an optically-clear bottom surface. Front-face fluorescence was collected through a second Bausch and Lomb monochromator (grating blazed at 800 nm) and detected by a Dumont #6911 (S-1 response) photomultiplier tube (operated at 1100 volts). A glass filter was used to eliminate second-order diffracted exciting light (CS 2-62). The slit widths of the exciting and analyzing monochromators were 3 and 1.5 nm, respectively (half bandwidth=4.8 nm). Spectra were not corrected for the spectral response of the equipment.

B. Delayed Light Emission.

Delayed light emission, from 1 to 20 msec after the center of a flash of white exciting light was measured, as previously described (Bertsch *et al*, 1967), with a modified Becquerel phosphoroscope (Fig. 3) in the laboratory of Dr. W. Bertsch.

C. Cytochrome Changes.

Light-induced absorption changes in the cytochrome region of the spectrum were measured with J. M. Olson's double-beam spectrophotometer (which is shown diagrammatically in Fig. 4) at the Brookhaven National Laboratory.

For cytochrome f oxidation, the actinic beam passed through a combination of a 700-nm interference filter (full bandwidth=12 nm), a Corning CS 2-64 glass filter, and 2 cm of H_2O. For cytochrome b_6 reduction, the actinic

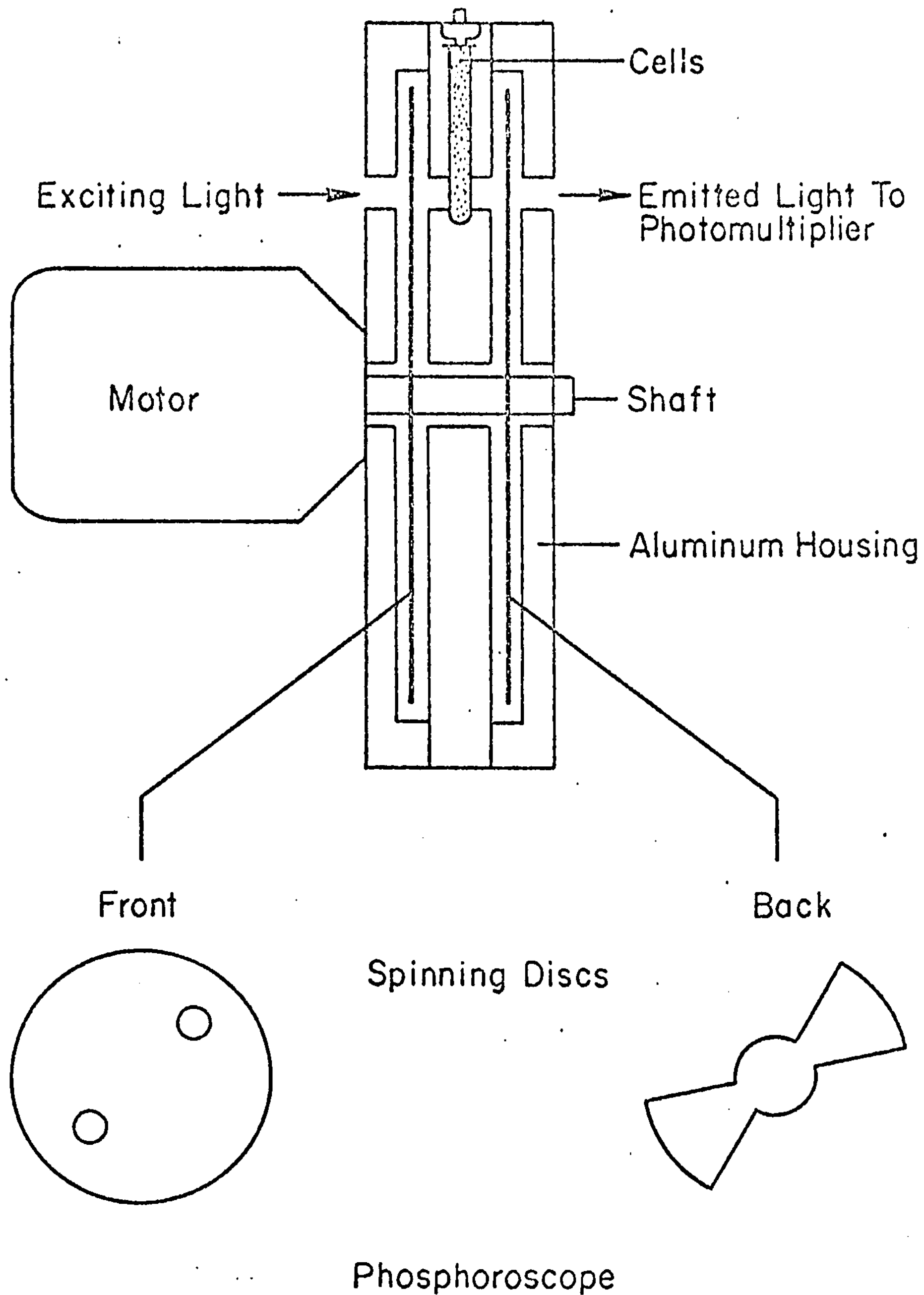


Figure 3. Block diagram of mechanical phosphoroscope used to measure delayed light emission in the millisecond time range. From Bertsch et al (1967).

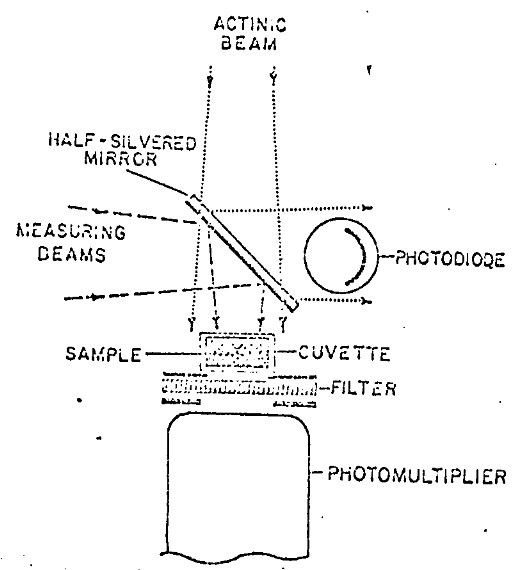


Figure 4. Optical arrangement of sample, measuring beams and actinic beam in the dual-wavelength spectrophotometer. From Morita et al (1965).

beam was passed through a combination of a 670 nm interference filter (full bandwidth=11 nm), a Corning CS 2-61 glass filter, and 2 cm of H_2O. The reference wavelength in both cases was 575 nm. The light intensity incident on the sample was 3.5×10^4 ergs/cm^2-sec (measured with calibrated RCA photodiode).

V. ASSAY OF PHOTOCHEMICAL ACTIVITY

A. Ferricyanide Hill Activity.

Ferricyanide Hill activity was assayed by three different techniques:

(1) RESIDUAL FERRICYANIDE (method of Jagendorf and Smith, 1962). The following reaction mixture, contained in 3 ml, was used in this assay (in mM): NaCl, 23; Tris-HCl, 13 (pH 7.8); $MgCl_2$, 3.3; potassium ferricyanide, 0.67 and chloroplasts equivalent to 2-3 x 10^{-5}M chlorophyll (a+b). Saturating* red light was obtained using a 500-watt (tungsten filament lamp) slide projector, Leitz Pradovit or Cavalier Performer. A Corning CS 2-62 glass filter was used to cut off wavelengths shorter than 600 nm; a battery jar containing 5 cm of H_2O was employed as a heat filter. The reaction was terminated by adding 0.3 ml of 20% trichloroacetic acid. The acidified samples were centrifuged in an International centrifuge for 10 minutes at 1,500 xg,

*Light intensity was measured with a Yellow Springs Model #65 Bolometer and saturation was found to be 2×10^5 ergs/cm^2-sec.

and the optical density of the supernatant was measured, at 420 nm, with a Hitachi Perkin-Elmer Spectrophotometer. The amount of ferricyanide which had been reduced was determined by measuring residual ferricyanide (*i.e.*, oxidized form) absorption and comparing with a standard curve.

(2) OXYGEN EVOLUTION (method of West and Hill, 1967). A Clark-type oxygen electrode (Rank Bros., Bottisham, England) was used for measurements. The 3 ml reaction mixture contained (in mM): Tricine-NaOH, 13 (pH 7.8); $MgCl_2$, 3.3; potassium ferricyanide, 0.67; chloroplasts equivalent to 2-4 x 10^{-5}M chlorophyll (*a*+*b*). The illumination set up was similar to method (1). Although rates of evolution were found to be linear with time up to four minutes, the data that was used in calculations were those obtained during the second and third minutes of illumination.

(3) DIRECT SPECTROPHOTOMETRIC METHOD. 3 ml of a reaction mixture, either the same as (2), or as follows (in mM): Tricine-NaOH, 50 (pH 8.0); NaCl, 23; $MgCl_2$, 3.3; potassium ferricyanide, 0.33; NH_4Cl, 2.5; chloroplasts equivalent to 1-2 x 10^{-5}M (chlorophyll *a*+*b*). Two identical aliquots (containing the complete reaction mixture) were balanced at 420 nm in an Aminco-Chance Dual-Wavelength Spectrophotomer. The sample cuvette was then illuminated for three minutes with saturating red light, as above, and the O.D. at 420 nm was measured. The difference in O.D. between the illuminated and non-illuminated samples was

used in calculating the amount of ferricyanide reduced. A molar absorptivity of 980 for potassium ferricyanide (Vernon and Shaw, 1965) was employed in the calculations.

B. DCPIP Hill Activity.

Reduction of DCPIP was measured according to Anderson and Boardman, 1966, in a 3 ml reaction mixture containing (in mM): NaCl, 23; Tris-HCl, 13 (pH 7.8); $MgCl_2$, 3.3; DCPIP, 0.02; chloroplasts equivalent to 0.8-1.0 x 10^{-5}M chlorophyll (*a*+*b*). Conditions of illumination were the same as the ferricyanide Hill reaction, except that time of illumination was one minute. After illumination, the O.D. of the sample was measured at 620 nm with a Spectronic 20 colorimeter; there was no change in the O.D., at 620 nm, of a reference cuvette, identical to the test cuvette, but kept in the dark. The amount of DCPIP which had been reduced was calculated with aid of a standard curve.

C. NADP Hill Activity.

Reduction of NADP was measured in a 3 ml reaction mixture containing (in mM): Tricine-NaOH, 13 (pH 7.8); $MgCl_2$, 3.3; NADP 0.25; saturating* amounts of ferredoxin from the blue green alga *Phormidium luridum* (a gift from Dr. H. W. Siegelman), or from spinach (Type 3, Sigma Chemical Co.); chloroplasts equivalent to 2-2.5 x 10^{-5}M

*Saturation, in this case, is defined as that amount of ferredoxin, which yields the maximal rate of NADP reduction; additional ferredoxin did not increase the rate.

chlorophyll (a+b). Determination of the amount of NADP reduced was similar to the direct spectrophotometric procedure for ferricyanide reduction with the following exceptions: the illumination period was two minutes, increase in O.D. at 340 nm was used in the determination of the amount of NADP reduced (a molar absorptivity of 6.22×10^3 for NADP was employed in the calculations). In some instances O.D. measurements were made with a Cary 14R recording spectrophotometer.

D. System I NADP Reduction.

When NADP reduction is measured with chloroplasts in which system II has been previously blocked by the addition of an oxygen evolution inhibitor, and an artificial electron donor couple (ascorbate-DCPIP) is made available, system I activity alone may be observed. The reaction mixture for system I NADP reduction was the same as for NADP Hill activity with the following additions (in mM): sodium ascorbate, 2; DCPIP 0.0067; CMU, 0.08. The amount of NADP reduced was determined as above.

E. System I Methyl Viologen Reduction.

System I activity was also measured using methyl viologen as the electron acceptor. Reduced methyl viologen is autoxidizable, therefore its reduction can be monitored by measuring stoichiometric oxygen consumption (Izawa, 1967). Oxygen consumption was measured using the oxygen electrode described above. The reaction mixture contained

in 4 ml (in mM): NaCl, 23; MES-NaOH, 12.5 (pH 7.0); $MgCl_2$, 3.3; DCPIP, 0.01; neutralized ascorbate, 5; methyl viologen, 0.1; chloroplasts equivalent to 2 x 10^{-5}M chlorophyll (a+b). In this case, system II activity was inhibited by "aging" the chloroplasts in 0.8M Tris-HCl (pH 8.0) for 10 minutes, according to the method of Yamashita and Butler (1968).

F. Cyclic Photophosphorylation.

This assay was done according to the method of Hill and Walker (1959) with the following reaction mixture, contained in 5 ml (in mM): NaCl, 23; Tricine-NaOH, 13 (pH 7.8); $MgCl_2$, 3.3; sodium isoascorbate, 3.3; ADP, 6; Na_2HPO_4, 5; PMS, 0.12; chloroplasts equivalent to 3 x 10^{-5}M chlorophyll (a+b). Samples were illuminated for 10 minutes with red light (10^5 ergs/cm^2-sec) and the disappearance of inorganic phosphate was determined with a molybdate color test. The level of disappearance of inorganic phosphate was less than 0.1% in the dark.

G. Cytochrome Changes.

The reaction mixture employed in the measurement of cytochrome changes was the following (in mM): Tricine-NaOH, 13 (pH 7.8); $MgCl_2$, 3.3; neutralized ascorbate, 10 (for the reduction of cytochrome f in the dark); NH_4Cl, 5; FMN, 5; chloroplasts equivalent to 10^{-4}M chlorophyll (a+b). Illumination conditions were as described in the section on spectral measurements.

VI. ULTRAVIOLET IRRADIATION OF CHLOROPLASTS

Chloroplasts were suspended in the reaction mixture (13 mM Tricine-NaOH, pH 7.8 and 3.3 mM $MgCl_2$) at 10^{-5}M chlorophyll (a+b) and were irradiated in a thin layer (1 mm) at a distance of 8 cm from a G.E. G8T5 8-watt germicidal lamp. The chloroplast suspension was mechanically stirred during the period of irradiation and was maintained at 4°C using an ice-water mixture. The incident light intensity was 1.7×10^4 ergs/cm^2-sec.

VII. ELECTRON MICROSCOPY

Specimens for electron microscopy were fixed with 2% glutaraldehyde for 2 hours and post fixed with osmium tetroxide for two hours according to the techniques of Kellenberger *et al* (1958). After dehydration in a graded series of alcohols they were embedded in epon. Sectioning was with a Porter-Blum MT2 ultramicrotome; in general sections were 500 Å in thickness as judged by refractive color. The sections were stained sequentially with uranyl acetate and lead citrate (according to Reynolds, 1963), and were examined with an RCA EMU-3H electron microscope.

VIII. CHEMICAL ASSAYS

Protein content was determined by the method of Lowry *et al* (1951) using crystalline bovine serum albumin as the standard. Concentration of chlorophyll (a+b) was determined by the method of Arnon (1949). Inorganic phosphate was determined by the method of Allen (1940),

as modified by Hill and Walker (1959). The release of fatty acids was determined colorimetrically using the copper soap method of Mahadevan _et al_ (1969); linolenic and oleic acids were used as standards.

IX. ASSAYS OF ENZYME ACTIVITY

A. _Lipase Activity_.

(1) SUBCHLOROPLAST PARTICLES AS SUBSTRATE. The reaction mixture contained the following (in 0.5 ml): subchloroplast particles equivalent to 9.6×10^{-5}M chlorophyll (_a_+_b_); 50 mM Tricine-NaOH, pH 8.5; 5 mM $MgCl_2$; 0.1 ml of _Ricinus_ protein fraction.* Samples were incubated for one hour at 28°C. The reaction was terminated by the addition of 1 ml of 95% ethanol followed by 0.1 ml of 0.1N HCl. The release of fatty acids was determined colorimetrically as described above.

(2) GALACTOLIPID AS SUBSTRATE. The reaction mixture contained the following (in 0.5 ml): monogalactosyl diglyceride equivalent to 0.41 ueq of acyl ester, or digalactosyl diglyceride equivalent to 0.60 ueq of acyl ester; 25 mM MES-NaOH, pH 7.2; 5% methanol; 5% Triton X-100; 0.1 ml of _Ricinus_ protein fraction. The samples were incubated for one hour, at 30°C, and fatty acid release was measured as above.

(3) PHOSPHOLIPID AS SUBSTRATE. A reaction mixture similar to the one used for galactolipid was used, except that the

*This term is used to designate active protein during any stage of purification between RLE and _Ricinus_ chloroplast protein.

substrate was 0.45 mg of lecithin (dipalmitoyl phosphatidyl choline). Fatty acid release was measured as above.

(4) SULFOLIPID AS SUBSTRATE. The reaction mixture contained (in 0.5 ml): ^{35}S-sulfolipid equivalent to 0.5 ueq of acyl ester; 50 mM Tricine-NaOH, pH 8.5; 2.5 mM $MgCl_2$; 3.3% methanol; 0.1 ml of Ricinus protein fraction. The samples were incubated for one hour at 30°C; the reaction was terminated by the addition of 2 ml of methanol followed by 1 ml of chloroform. After 20 minutes one more ml of chloroform was added followed by 3 ml of water. The mixture was then mechanically agitated followed by centrifugation (2,000 xg for 5 minutes). An 0.6 ml aliquot of the methanol-water phase was then assayed for released ^{35}S, using a dioxane counting mixture (Bray, 1960). Radioactivity was measured with a Beckman Mark I scintillation counter.

B. Protease Activity.

Protease activity was assayed by the method of Nelson et al (1961). In this assay activity is determined by incubating an insoluble dye-protein (Congo Red Hide Powder) with the enzyme; proteolysis is associated with release of the dye in a soluble form (measured as an increase in O.D. at 550 nm). The reaction mixture for the assay contained (in 1 ml): 4 mg of Congo Red Hide Powder; 100 mM Tricine-NaOH, pH 8.5; 0.2 ml of Ricinus protein fraction. Samples were incubated for 90 minutes at 30°C.

X. PREPARATION OF ENZYMES, SOLUTIONS SUBSTRATES, ETC.

All chemicals were reagent grade, and aqueous solutions

were prepared in glass distilled water.

Pronase (Calbiochem), Wheat Germ Lipase (Sigma), Trypsin (Sigma), Lipoxidase (Sigma), and Phospholipase D (Boerhinger) were prepared in either .05M potassium phosphate buffer, pH 7.0, or in .05M Tris-HCl, pH 7.4. Pancreatin extract (Sigma) was prepared in 10% NaCl as described by Sastry and Kates (1964).

Crystalline bovine serum albumin (Sigma) was defatted by exposure to activated charcoal (Norit A, Sigma) at low pH (3.0) according to the method of Chen (1967).

Galactolipids were extracted from spinach chloroplasts by the method of Zill and Harmon (1962); they were further purified according to the method of Sastry and Kates (1964). Lecithin was obtained commercially from General Biochemical Co. ^{35}S-sulfolipid, isolated from Chlorella, was a gift from Dr. A. A. Benson.

Fatty acids (Sigma) and fatty acid derivatives (Sigma) were dissolved in absolute ethanol* immediately before use. CMU (a gift from Dr. H. Todd of Dupont) and DCMU (a gift from Dr. S. S. Brody) were dissolved in absolute ethanol or in a mixture of ethylene-glycol-ethanol (v/v). CCCP and ICI 47776 were gifts from Dr. R. Gregory.

ADP was purchased from P&L Laboratories, Milwaukee, Wisconsin; hydroquinone from Aldrich Chemical Co., Milwaukee, Wisconsin; digitonin and PMS from Sigma Chemical Co., St. Louis, Missouri; glutaraldehyde, methyl viologen,

*The final concentration of organic solvents did not exceed 2% in the reaction mixtures where they were employed, unless otherwise specified.

semicarbazide, sodium dodecyl sulfate, sodium docecyl benzene sulfonate from K&K Laboratories, Plainfield, New York; Tricine, HEPES, MES from Calbiochem, Los Angeles, California; Triton X-100 from Rohm and Hass, Philadelphia, Pennsylvania; osmium tetroxide from United Mine and Chemical Corp., New York, New York.

RESULTS

I. ALTERATIONS IN THE FLUORESCENCE EMISSION SPECTRUM (-196°C) OF *RICINUS* CHLOROPLASTS

When chloroplasts of *Ricinus* are isolated according to method (a) and incubated in NaCl-Tris, at 37°C, large changes with time of incubation are observed in the low temperature (-196°C) fluorescence emission spectrum (experiments in collaboration with Miss B. Nathanson and Dr. M. Brody. In contrast to the control (taken to be chloroplasts stored at 4°C), chloroplasts incubated at 37°C undergo the following changes in the three-banded emission spectrum. The shoulder designated F685 seems to disappear as a discrete entity after about 5 minutes of incubation; this disappearance is accompanied by an apparent increase in F698. After 60 minutes the long-wavelength emission at 735 nm has diminished to such an extent that only fluorescence from F 698 is in evidence.* These effects are shown in Fig. 5.

Glydenholm and Whatley (1968) noted that addition of 10 mg/ml of bovine serum albumin to the isolation and resuspension media for bean chloroplasts (*Phaseolus*) yields chloroplasts which are biochemically stable. In

*That these yield changes are absolute (not just relative) has been shown by comparison with an inert "internal" fluorescence standard (M. Brody and S. Brody, 1970).

Figure 5A,B. Fluorescence emission spectra (-196°C) of <u>Ricinus</u> chloroplasts incubated at 37°C. Curves A and B are for 0 and 5 minutes respectively.

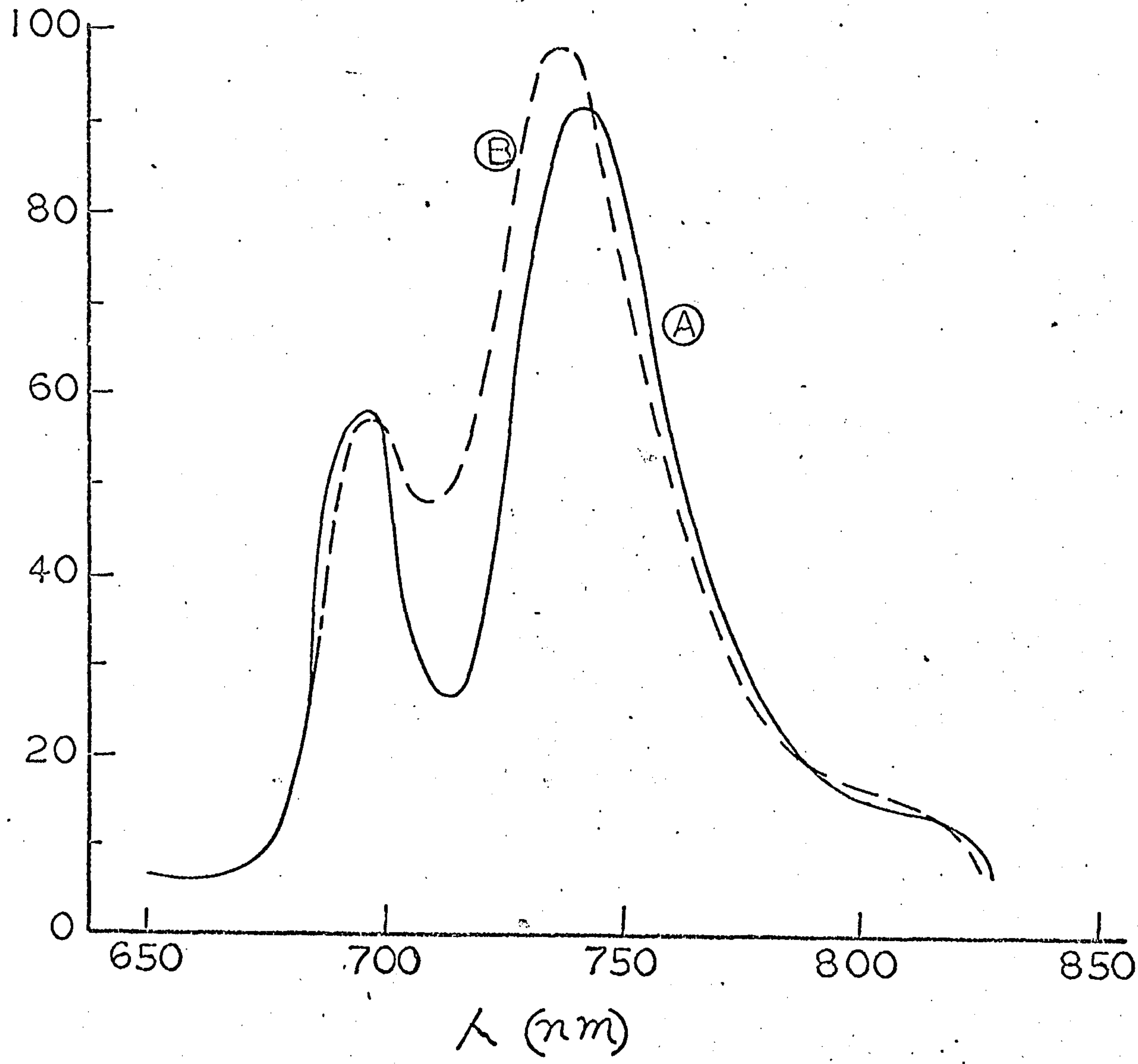
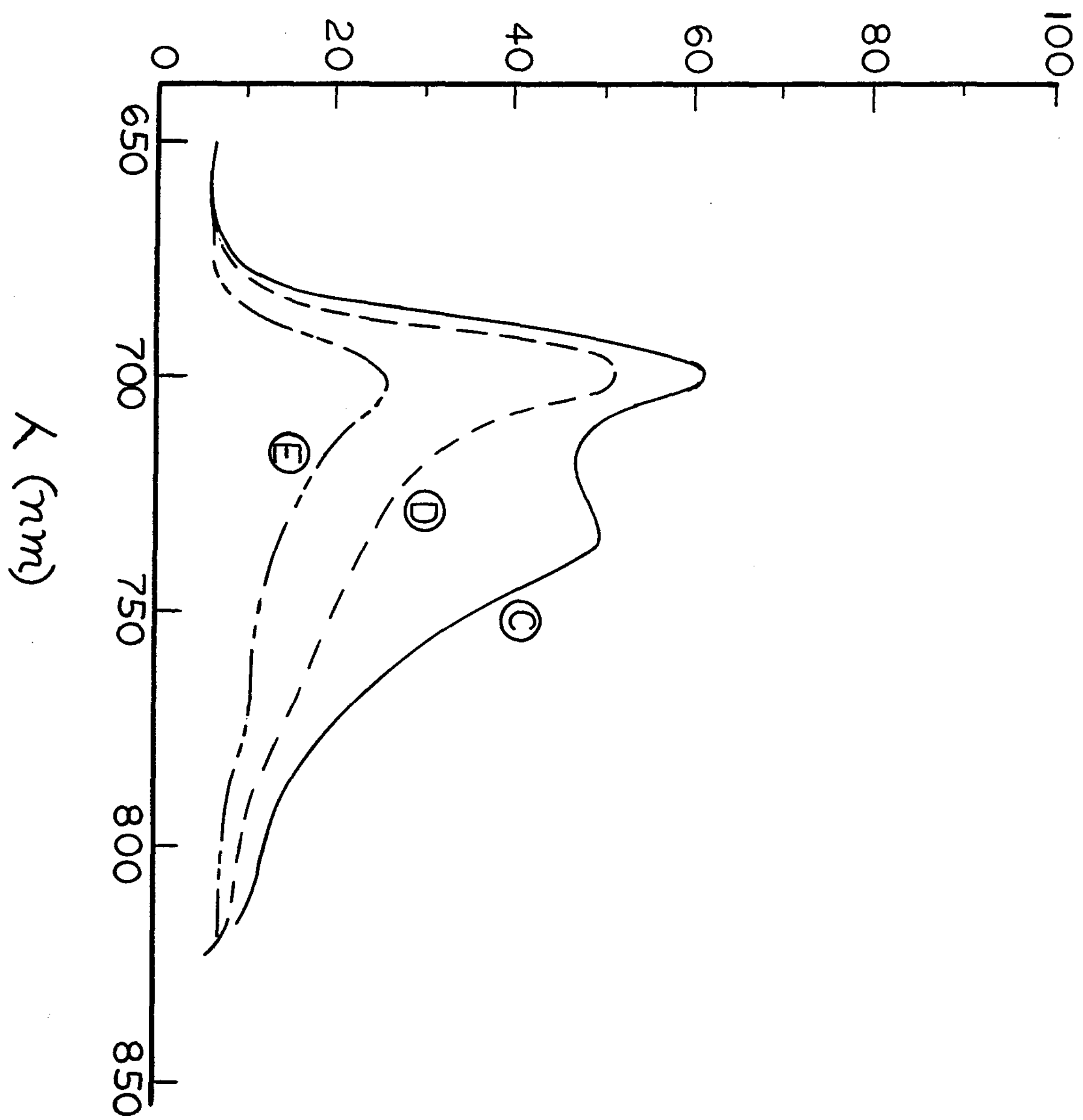

FLUORESCENCE EMISSION INTENSITY
(IN RELATIVE UNITS)
100
80
60
40
20
0
650
700
750
800
850
λ (nm)
B
A

Figure 5C,D,E. Fluorescence emission spectra (-196°C) of <u>Ricinus</u> chloroplasts incubated at 37°C. Curves C, D, E are for 10, 20, and 60 minutes respectively.


FLUORESCENCE EMISSION INTENSITY
(IN RELATIVE UNITS)
0
20
40
60
80
100
650
700
750
800
850
λ (nm)
C
D
E

the present work it was observed that isolation and resuspension of *Ricinus* chloroplasts in NaCl-Tris--supplemented with 10 mg/ml of bovine serum albumin--did not prevent the fluorescence changes from occurring.

If one isolates and resuspends *Ricinus* chloroplasts in STN (in which organelles are presumably whole*), one also notes fluorescence changes similar to those above.

On the supposition that the agent causing the fluorescence changes might be involved in the release of a tannin-like compound (Clendenning, 1957), *Ricinus* chloroplasts were isolated in a medium which protects chloroplasts from the action of these compounds. Such a medium is STN supplemented with 6% Carbowax 4000 and 5 mM reduced gluathione (Miflin and Hageman, 1963); again this medium did not prevent the spectral changes.

Fluorescence changes were not observed to occur when *Ricinus* chloroplasts were isolated by method (a) in a low pH medium consisting of 0.4M sucrose-0.02M potassium phosphate buffer, pH 6.0; the fluorescence spectrum was stable at this pH for 75 minutes at 25°C. The various isolation media which were employed in the preparation of *Ricinus* chloroplasts are summarized in Table I; as well as the extent of spectral change.

*Chloroplasts isolated in media containing sugars or sugar alcohols at concentrations of approximately 0.4M usually have outer envelopes, whereas those isolated in salt media at these concentrations usually do not (Walker, 1966).

TABLE I

VARIOUS MEDIA EMPLOYED IN THE PREPARATION OF *RICINUS* CHLOROPLASTS[a]

	Medium	Emission Spectrum Unaltered	Emission Spectrum Altered
1.	0.35M NaCl-Tris-HCl, pH 7.8, 0.04M	-	+
2.	0.35M NaCl-0.04M Tris-HCl, pH 7.8 + 10 mg/ml BSA	-	+
3.	0.4M Sucrose-0.05M Tris-HCl, pH 7.8-0.01M NaCl	-	+
4.	0.4M Sucrose-0.05M Tris-HCl, pH 7.8-0.01M NaCl-6% Carbowax 4000-0.005M Glutathione	-	+
5.	0.4M Sucrose-0.02M Potassium Phosphate, pH 6.0	+	-

[a]Chloroplasts were isolated, resuspended and incubated in the same medium. Spectral alterations were determined by examining fluorescence emission as a function of incubation time (25°C) for periods up to 120 minutes.

II. THE EFFECTS OF RLE ON ISOLATED CHLOROPLASTS

A. Fluorescence Emission Spectra at -196°C.

In an attempt to determine the nature of the active agent(s) involved in the emission changes, RLE was prepared as described in MATERIALS AND METHODS and the ad hoc assumption was made that the active material was being released from *Ricinus* chloroplasts in a soluble form. Since it was not possible to test this hypothesis using *Ricinus* chloroplasts (because of the presence of endogenous factor) chloroplasts from other sources were used. Incubation of spinach chloroplasts in RLE (Fig. 6) resulted in spectral changes essentially identical to those observed with *Ricinus* chloroplasts (see Fig. 5). In Fig. 7 the ratio of fluorescence intensities at 735 nm and 698 nm (-196°C), *i.e.*, F735/F698 (henceforth to be referred to as *R*) is plotted as a function of incubation time at 25°C. From Fig. 7 it may be seen that these changes do not occur in the absence of the extract.

In addition to spinach chloroplasts, this action has been observed with chloroplasts of other higher plants, *i.e.*, corn, radish and tobacco, and with chloroplast fragments from *Chlamydomonas reinhardtii* (strain 6270C). On the other hand, no such changes were observed with the whole cells of *Chlamydomonas*.

B. Fluorescence Emission Spectra at 20°C.

Room temperature fluorescence emission spectra were

Figure 6A,B. Fluorescence emission spectra (-196°C) of spinach chloroplasts incubated in RLE at 25°C. Curves A and B are for 0 and 60 minutes respectively.

FLUORESCENCE EMISSION INTENSITY
(IN RELATIVE UNITS)

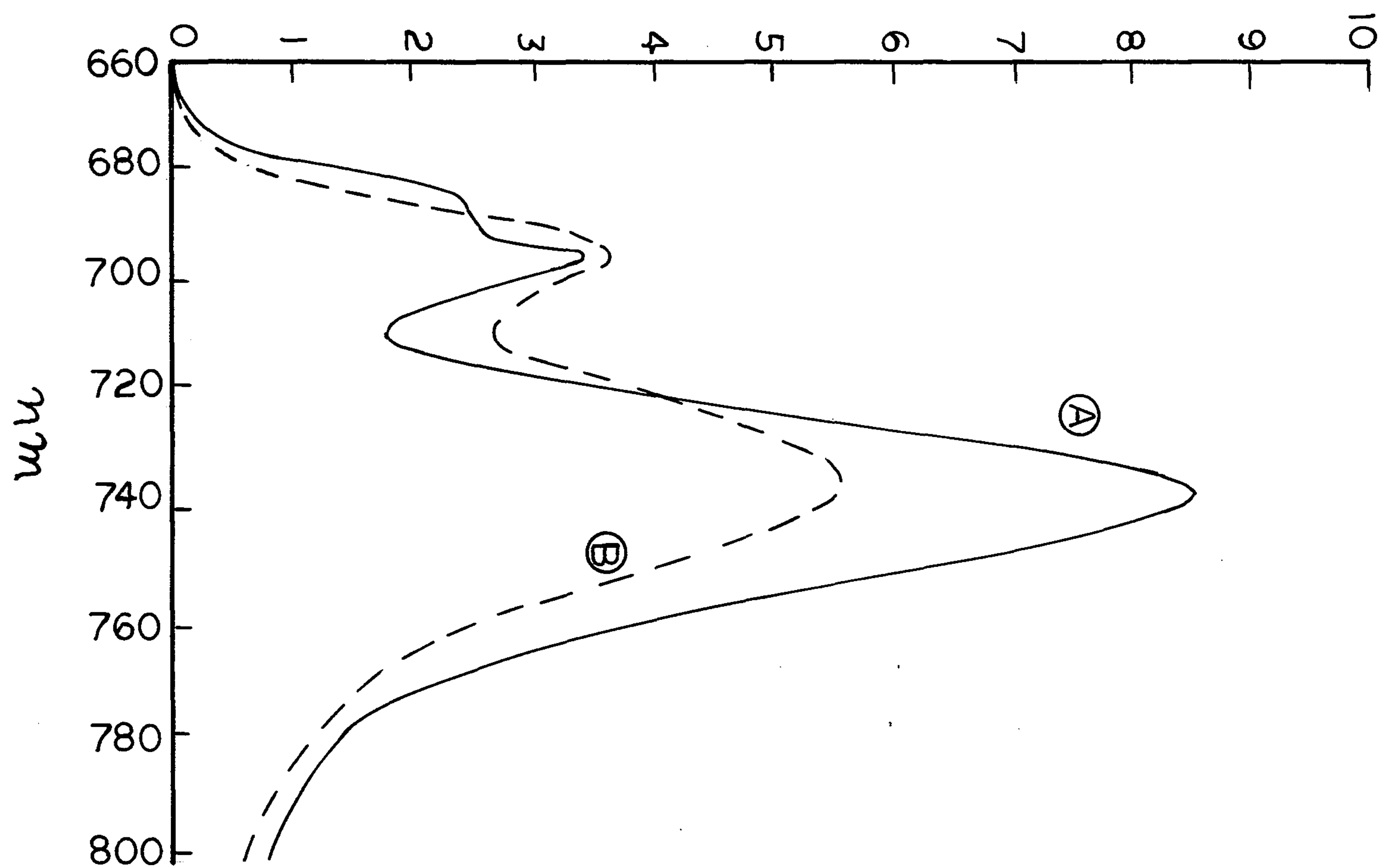
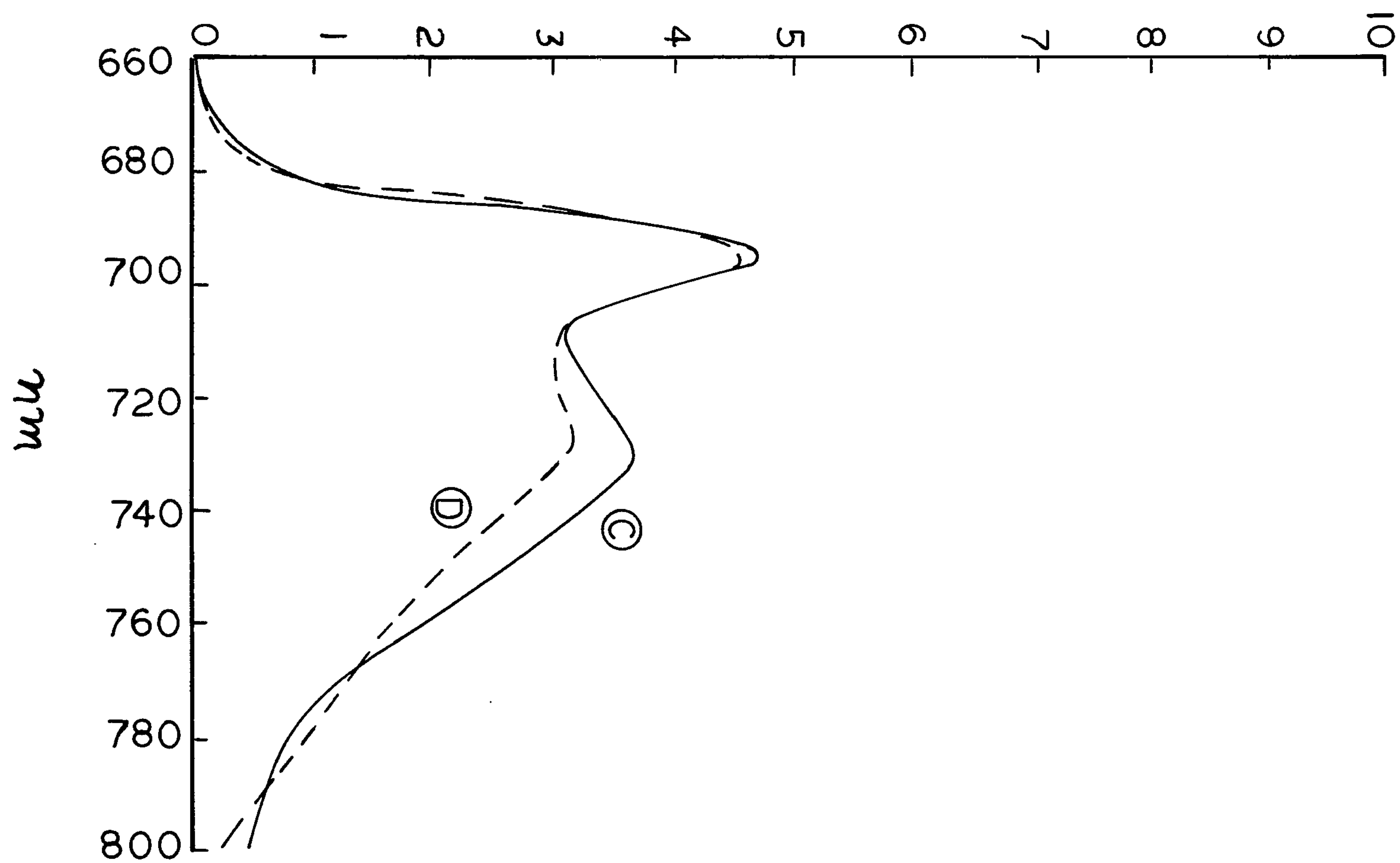


Figure 6C,D. Fluorescence emission spectra(-196°C) of spinach chloroplasts incubated in RLE at 25°C. Curves C and D are for 120 and 180 minutes respectively.

FLUORESCENCE EMISSION INTENSITY
(IN RELATIVE UNITS)



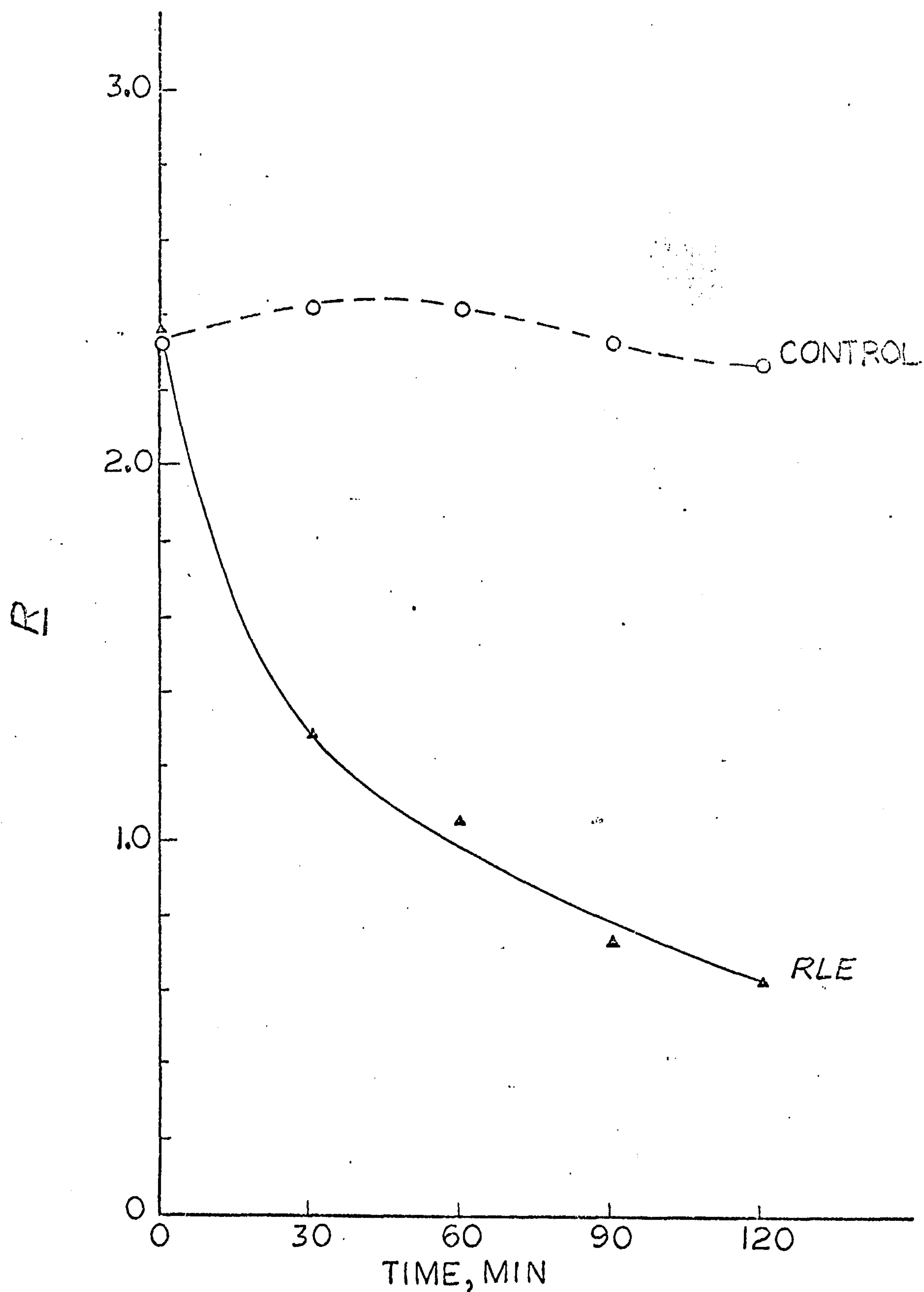


Figure 7. R as a function of time of incubation for spinach chloroplasts suspended in RLE at 25°C. Control chloroplasts were suspended in NaCl-Tris; fluorescence measured at -196°C.

measured only in the case of spinach chloroplasts incubated in RLE, and in this system spectral changes were also noted to occur as shown in Fig. 8.

C. Hill Activity.

The following experiments were performed to determine if the spectral modifications of chloroplasts are accompanied by changes in their photochemical activity. Spinach chloroplasts were incubated in dialyzed 104,000 xg extracts and assayed for ferricyanide Hill activity; see Fig. 9. Even at zero time (*i.e.*, the time the first measurements could be made--approximately 3 minutes after chloroplasts and extracts were mixed) activity was found to be inhibited to 15% of the control. On the other hand, *R* decreased rapidly (*i.e.*, within 15 minutes) to 70% of its control value, and then fell more slowly, reaching 50% of its control value after about one hour of incubation. These results suggested that the only effect of RLE on electron transport was an inhibitory one. However, with a five-fold dilution of the extract, it was observed that the inhibitory phase is preceded by a stimulatory phase (possibly a result of uncoupling phosphorylation from electron transport). Although at the lower concentration of extract the change in *R* still shows biphasic kinetics *R* has only fallen to 88% of the control value after 15 minutes of incubation, and the subsequent decrease is even slower (after one hour of incubation *R* is still 80% of its control value). Thus it appears that the

Figure 8. Effect of RLE on the room temperature fluorescence emission spectrum of spinach chloroplasts. Curve A, chloroplasts suspended in NaCl-Tris; Curve B, chloroplasts suspended in RLE for 120 minutes. Temperature of incubation, $20^{o}C$.

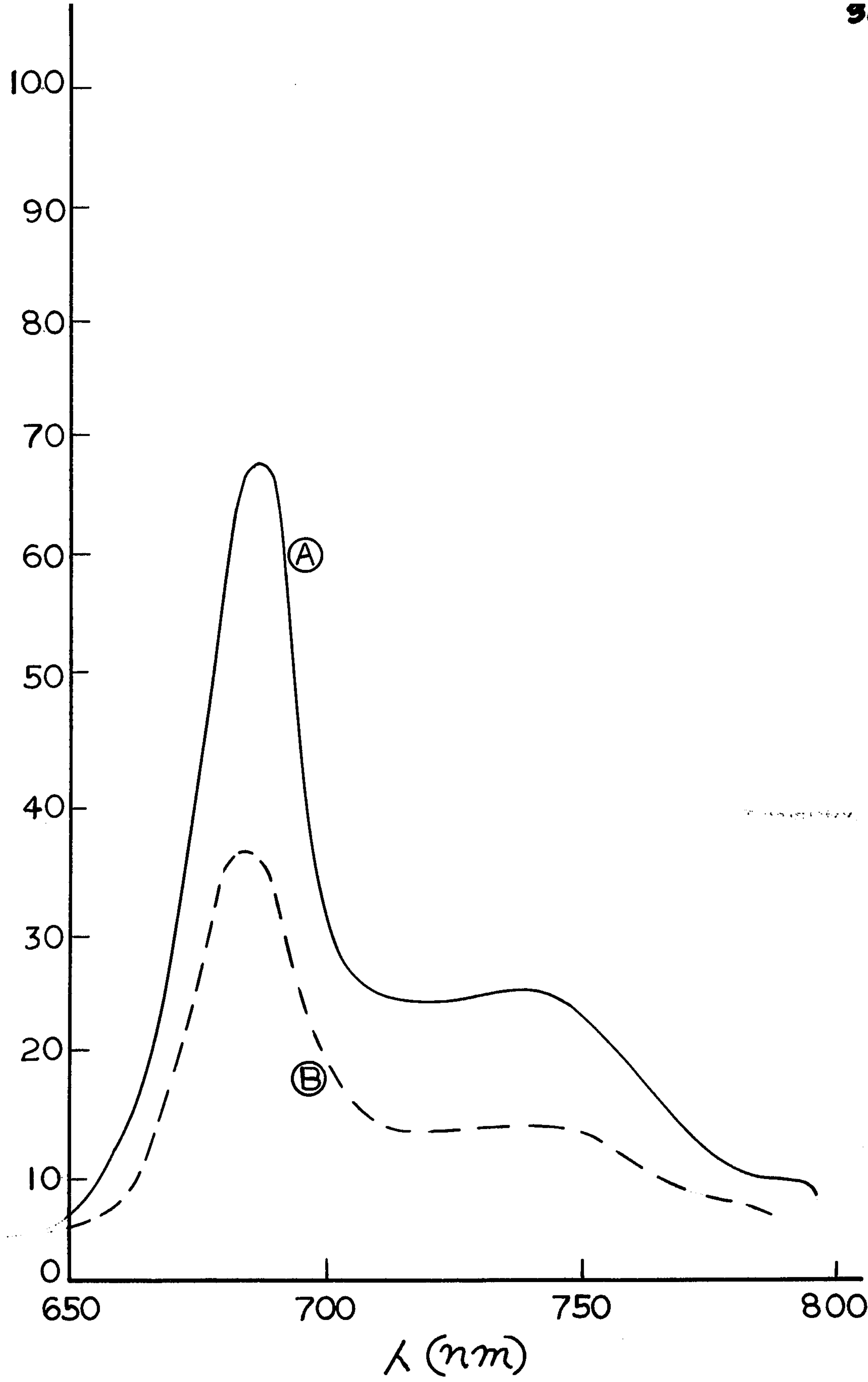
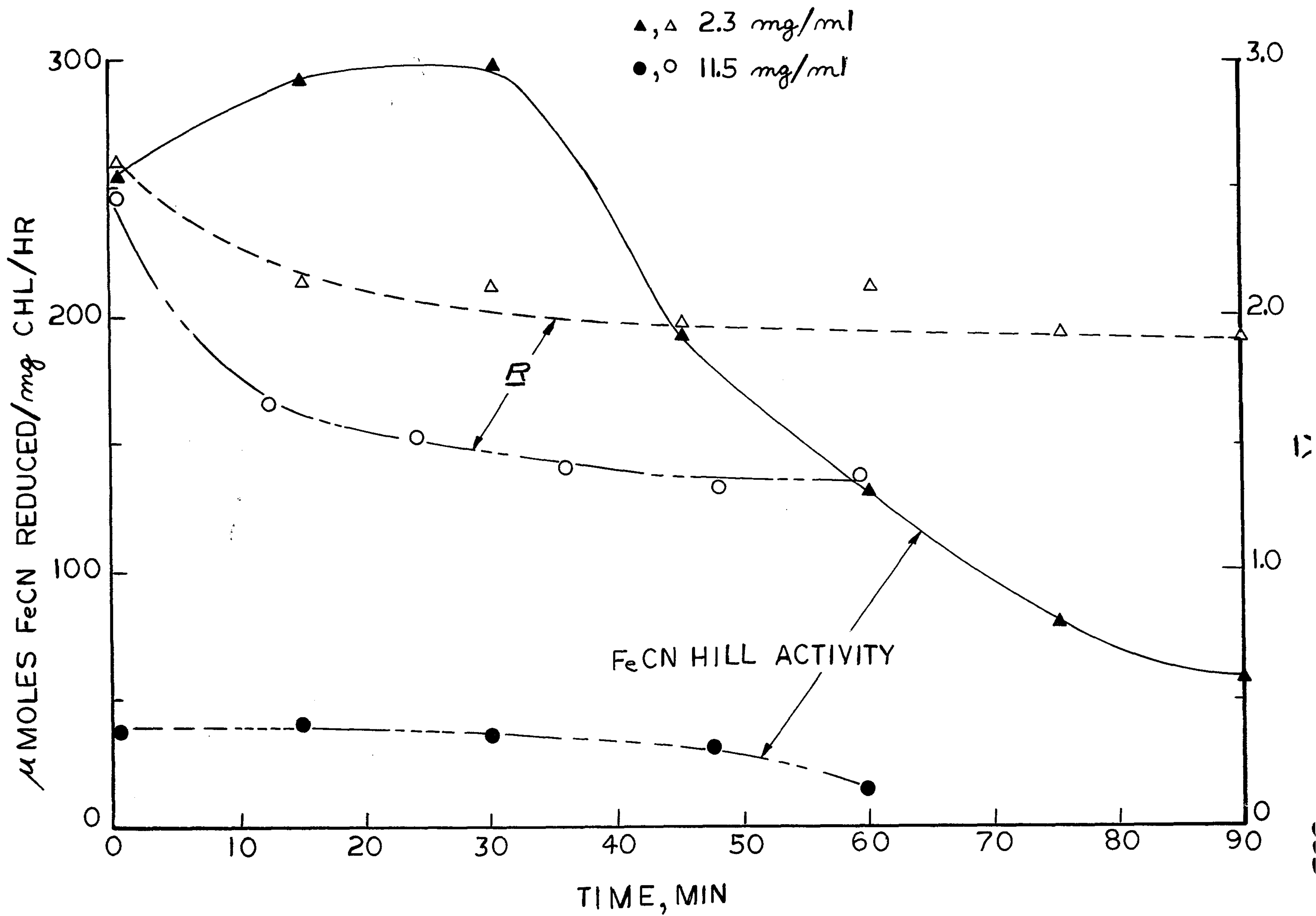

FLUORESCENCE EMISSION INTENSITY
(IN RELATIVE UNITS)
0
10
20
30
40
50
60
70
80
90
100
650
700
750
800
λ (nm)
A
B

Figure 9. The effect of RLE concentration on FeCN Hill activity and R. Incubation temperature, $24^{\circ}C$.


▲, △ 2.3 mg/ml
●, ○ 11.5 mg/ml
μ MOLES FeCN REDUCED/mg CHL/HR
300
200
100
0
3.0
2.0
1.0
0
R
FeCN HILL ACTIVITY
0
10
20
30
40
50
60
70
80
90
TIME, MIN

effect of concentration of extract is largely on the initial fall in *R*. Somewhat similar results were obtained with DCPIP as the Hill oxidant as shown in Fig. 10; however, in this case, even with undiluted extract there was a prolonged increase in Hill reaction activity prior to inhibition. (Uncoupling effects of indophenol dyes have been previously reported (Gromet-Elhanan and Avron, 1964), and with DCPIP there may have been some direct uncoupling effect.)

Incubation of chloroplasts in RLE containing up to 30 mg/ml of bovine serum albumin did not protect against the action of the factor. This was the case, too, for chloroplasts preincubated for 30 minutes in BSA and then added to RLE or RLE plus serum albumin. Attempts to wash out the active factor--after incubation in RLE--with NaCl-Tris supplemented with 5 mg/ml of BSA (five washes followed by resuspension in fresh medium) were also unsuccessful.

III. ATTEMPTS TO ISOLATE THE ACTIVE FACTOR

A. The Protein Nature of the Factor.

The active factor(s) in the crude extract appears to be temperature sensitive. The emission changes do not occur if the extract is boiled prior to incubation (Fig. 11), or if the chloroplasts are isolated and incubated in the extract at $0^{o}C$. The active factor can be precipitated from the crude extract with ammonium sulfate and the removal of dialyzable material from the precipitatedextract does not result in loss of activity. In addition, treatment

Figure 10. The effect of RLE on DCPIP Hill activity and *R*. Protein concentration was 11.5 mg/ml; incubation temperature, 22°C.

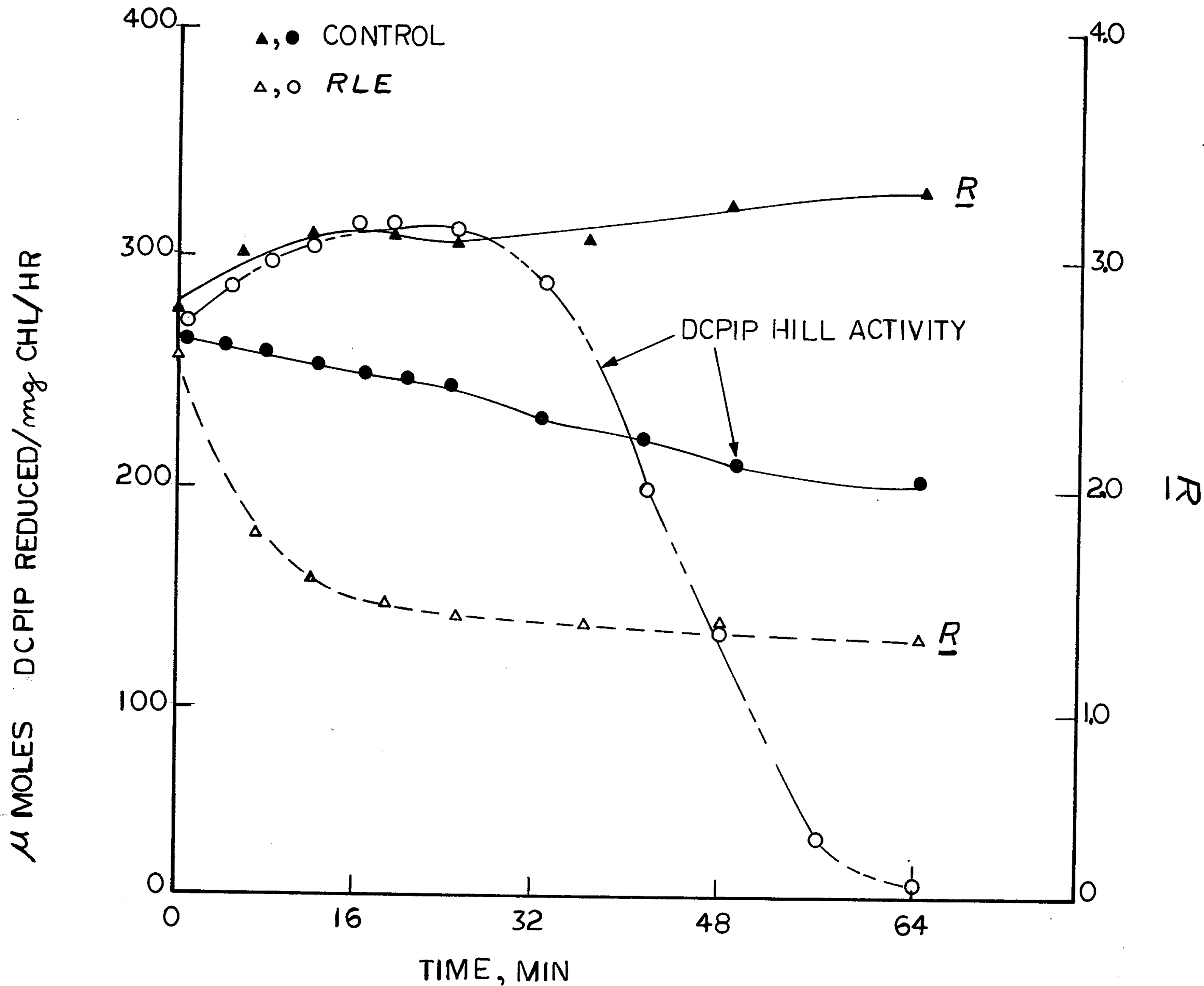
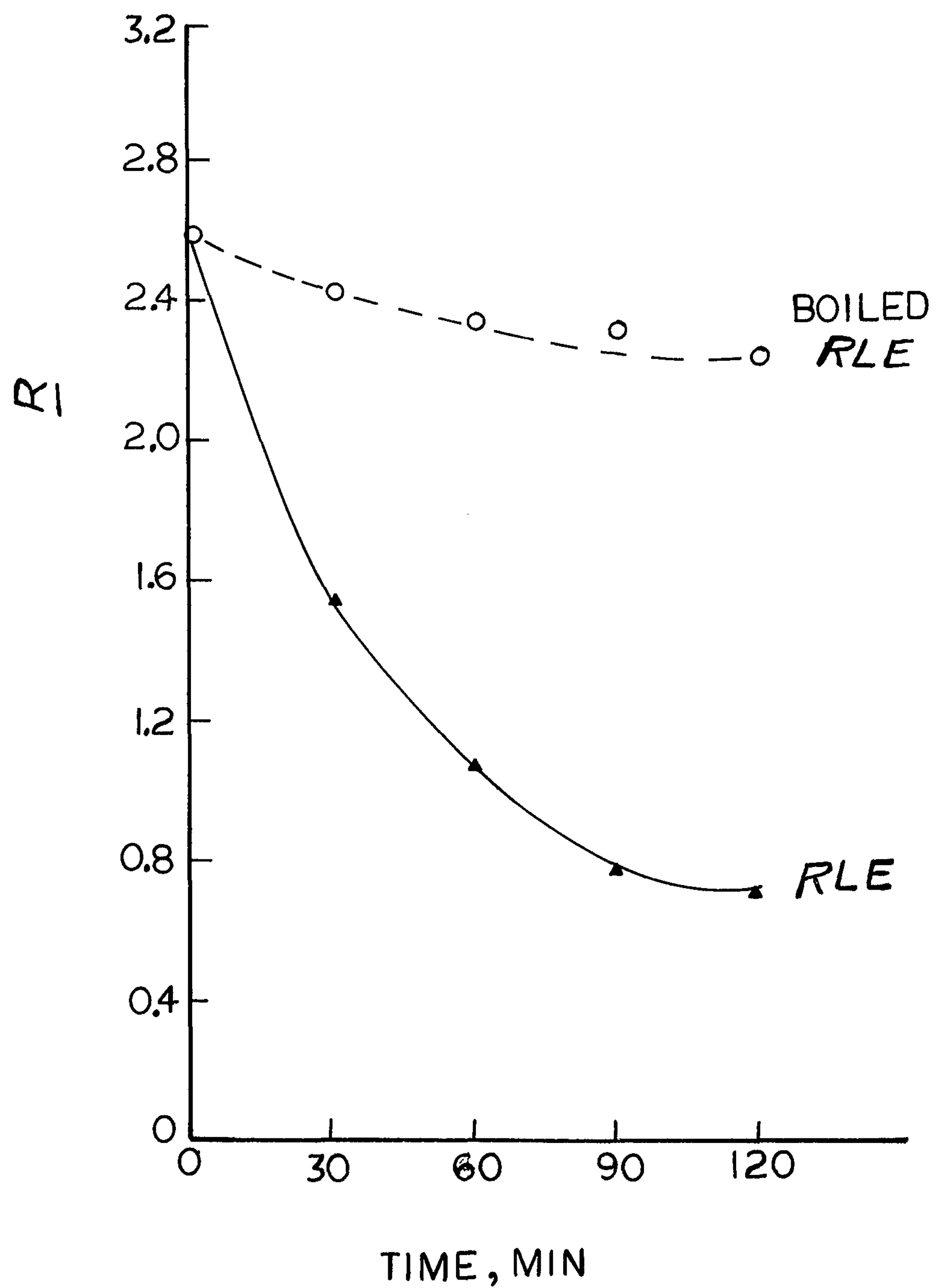

▲,● CONTROL
△,○ RLE
400
300
200
100
0
4.0
3.0
2.0
1.0
0
R
R
DCPIP HILL ACTIVITY
μ MOLES DCPIP REDUCED/mg CHL/HR
R
0
16
32
48
64
TIME, MIN

Figure 11. The inactivation of RLE by boiling (for 5 minutes). Activity was measured as the ability to change *R*. Chloroplasts were incubated in either boiled or untreated RLE at 25°C.


3.2
2.8
2.4
2.0
1.6
1.2
0.8
0.4
0
R1
BOILED
RLE
RLE
0
30
60
90
120
TIME, MIN

of the extract with 10 μg/ml of pronase (30°C) for two hours resulted in a complete loss of ability to induce the spectral change. Dialysis of the "degraded extract" against a small volume of buffer did not result in the release of active material outside the dialysis bag; the material inside the bag was also without activity.

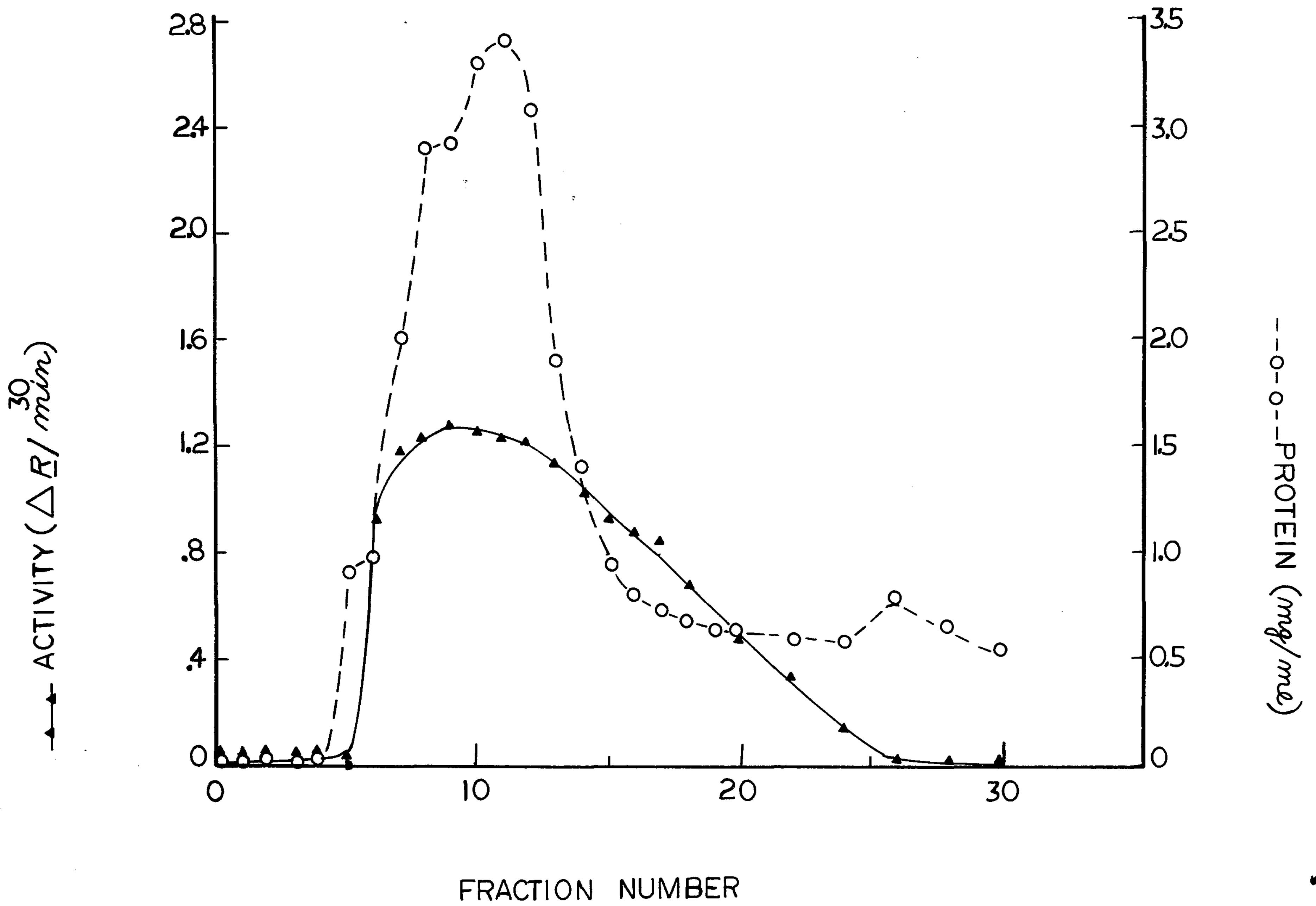
B. Fractionation of RLE.

These results suggested that the active factors were proteinaceous in nature and therefore might be purified by classical methods. Attempts to fractionate the crude extract (or the dialyzed 104,000 xg extract) with ammonium sulfate were unsuccessful for the following reasons: a) there always appeared to be a scattering of activity throughout the various fractions (*i.e.*, 0-35%, 35-55%,...); b) the increase in specific activity (measured as the change in *R*/30 minutes/mg protein) in the most active fractions was only two-fold; c) there was a 30-40% loss of total activity. Similarly unrewarding results were obtained when acetone (at -5°C) was used as the precipitating agent. Attempts to fractionate the crude extract with 45% Carbowax 6000, according to the method of Schopfer and Siegelman (1968), were unsuccessful.

C. G-25 Sephadex Chromatography.

The next efforts, to separate the protein factor from other components in the leaf extract, were based on the

Figure 12. Effluent from the G-25 Sephadex chromatography of crude RLE. Activity was measured as the ability to change $\underline{R}$. Spinach chloroplasts were incubated with the various fractions for 30 minutes at 26°C.



32B

principle of exclusion chromatography. Crude RLE was chromatographed on G-25 Sephadex according to M. Brody (1969); the main peak of activity appeared with the turbid protein fractions excluded by the column. Smaller amounts of activity appeared in a number of subsequent fractions (Fig. 12). Increase in specific activity was variable, in some preparations as high as five-fold, and in others only one- or two-fold (as in Fig. 12).

In addition to the protein fraction, M. Brody reported the presence in *Ricinus* leaf extracts of a smaller, yellow molecule which was completely retarded by the G-25 Sephadex. This molecule appeared to be responsible for "fast changes" in fluorescence induction in extract-treated chloroplasts. A spectroscopically similar fraction (absorption maximum in the UV at $\sim$ 275 nm and visible absorption maximum at $\sim$380 nm) was eluted from the Sephadex with large quantities of .001M Tris-HCl, pH 7.8 after the protein fractions were collected. This yellow fraction did not seem to have any effect on steady-state fluorescence, in confirmation of M. Brody's findings.

D. G-100 Sephadex Chromatography.

Since steady-state fluorescence changes could be observed both with *Ricinus* chloroplasts and with other chloroplasts suspended in RLE, it was suspected that the source of the factor(s) was the *Ricinus* chloroplast. Dr. R. E. McCarty of Cornell University suggested a method for extracting the protein factor directly from the chloroplasts

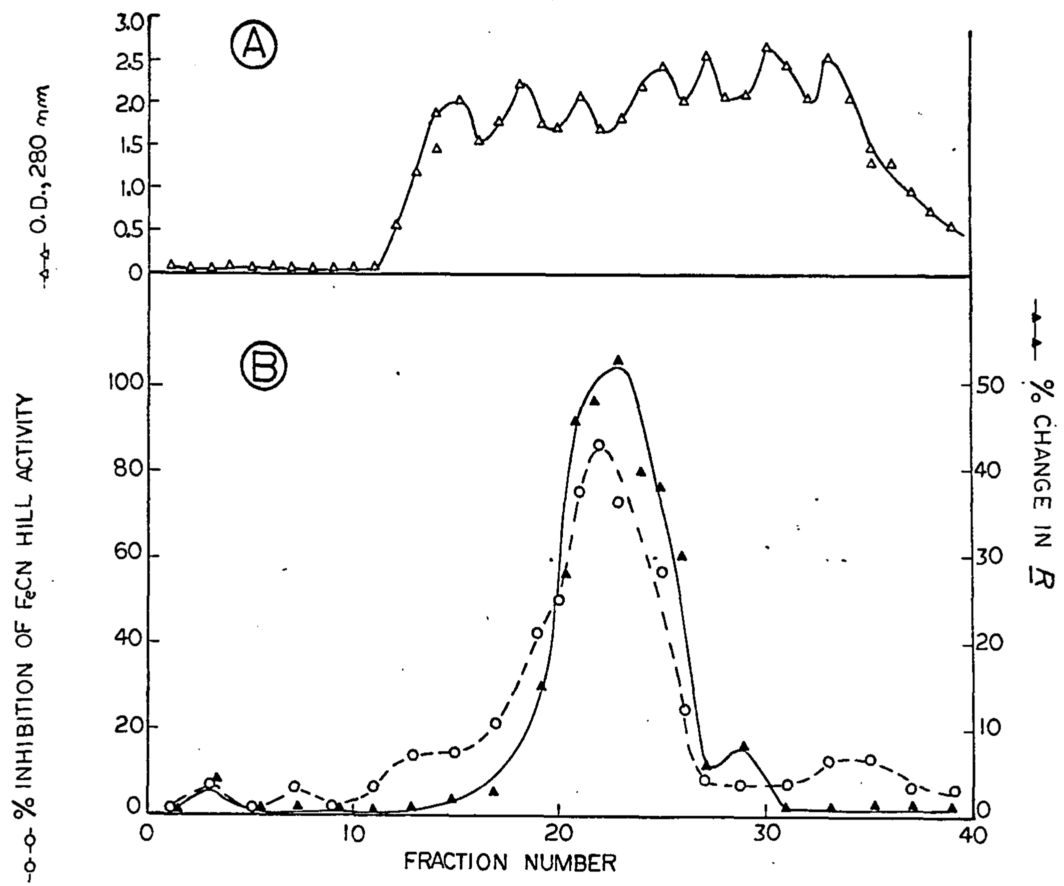
rather than from leaf homogenates. The detailed procedure for this extraction is presented in the section on MATERIALS AND METHODS. Fluorescence changes similar to those brought about by the crude extract of the leaves, resulted from utilization of a particular fraction of the protein extracted by this method.

The final step in the protein extraction of the chloroplasts is chromatography on a G-100 Sephadex column. In Fig. 13A, one sees the elution pattern from this column. In Fig. 13B are plotted ferricyanide Hill activity and % change in R as functions of fraction number. That fraction of protein (tubes 20-25) which inhibits the Hill reaction also seems to be responsible for the fluorescence changes. It will be referred to as "Ricinus chloroplast protein." Since G-100 Sephadex excludes proteins of M.W. 150,000 and since the active protein is somewhat retarded on the column (being eluted at approximately 25% of the bed volume), it consists mainly of molecules larger than 10,000 M.W. and smaller than 150,000 M.W.

IV. CHARACTERISTICS OF THE RICINUS CHLOROPLAST PROTEIN

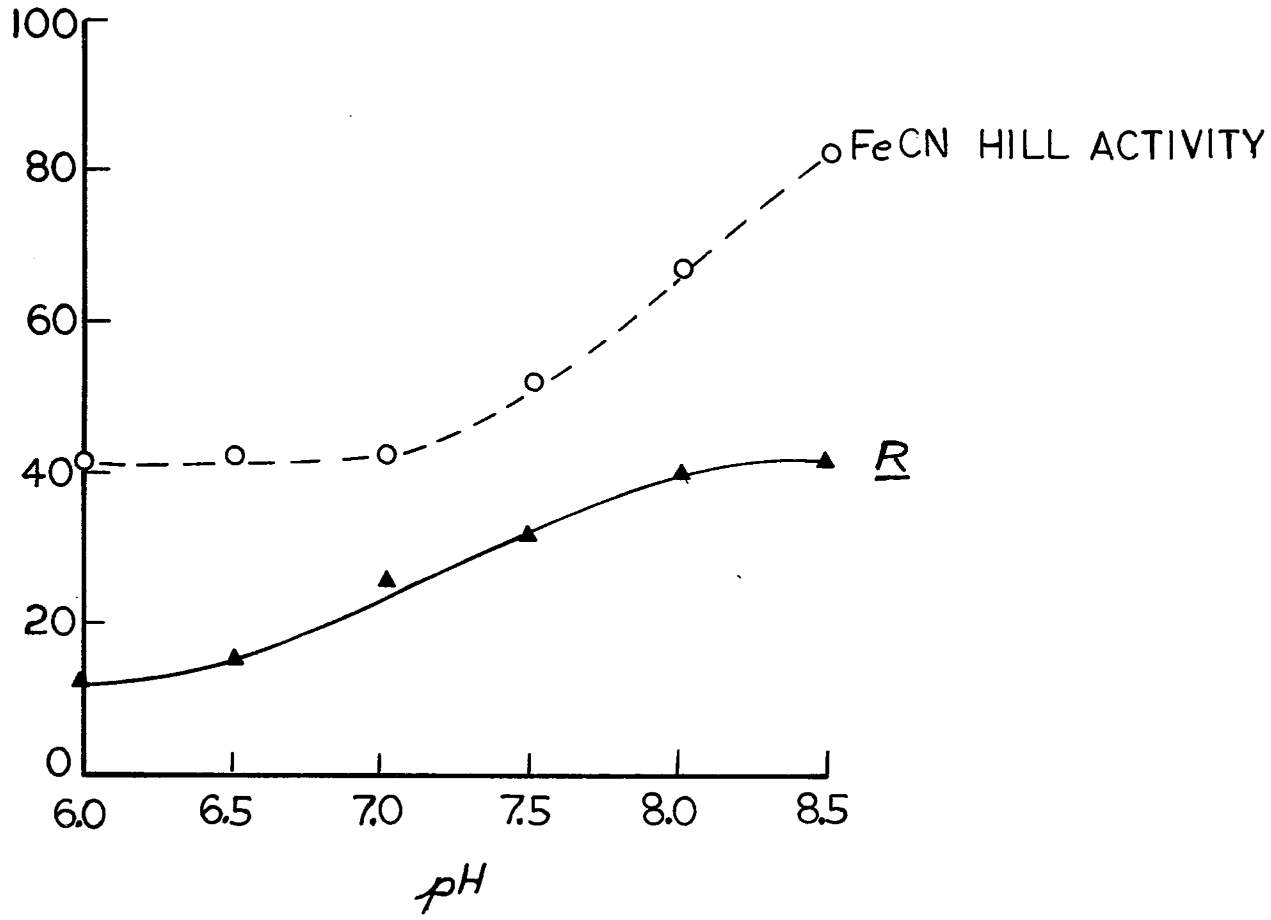
In Fig. 14, one sees that the pH dependence for both the protein's ability to inhibit ferricyanide Hill activity and induce the change in R is somewhat similar, both increase up to about pH 8.5. The reason that pH's above 8.5 were not used is that these higher pH's alone have been shown to result in loss of Hill activity (Punnett, 1959).

Figure 13. Effluent from the G-100 Sephadex chromatography of *Ricinus* chloroplast proteins. A. Protein concentration as a function of fraction number. B. Inhibition of FeCN Hill activity and change in *R* as functions of fraction number. Control rate of FeCN reduction (μmoles/mg chl/hr)=70; control value of *R*=2.44. Incubation temperature, 30°C.



A
B
O.D., 280 mm
% INHIBITION OF FeCN HILL ACTIVITY
% CHANGE IN R
FRACTION NUMBER

Figure 14. pH dependence of *Ricinus* chloroplast protein activity (inhibition of FeCN Hill activity or change in *R*). The assay mixtures contained .104 mg/ml protein. The samples were incubated for 5 minutes for FeCN Hill activity and 60 minutes for the change in *R* at 30°C. The reaction mixtures contained the following buffers: pH 6.0-6.5, MES-NaOH; pH 7.0-7.5, HEPES-NaOH; pH 8.0-8.5, Tricine-NaOH. Control rates of FeCN reduction (μmoles/mg chl/hr): pH 6.0=460; pH 6.5=460; pH 7.0=488; pH 7.5=432; pH 8.0=442; pH 8.5=469. Control values of *R*: pH 6.0=2.21; pH 6.5=2.26; pH 7.0=2.35; pH 7.5=2.42; pH 8.0=2.27; pH 8.5=2.59. Fluorescence measurements were made in the absence of the electron acceptor. FeCN Hill activity was measured by method (3) in the presence of NH_4Cl.



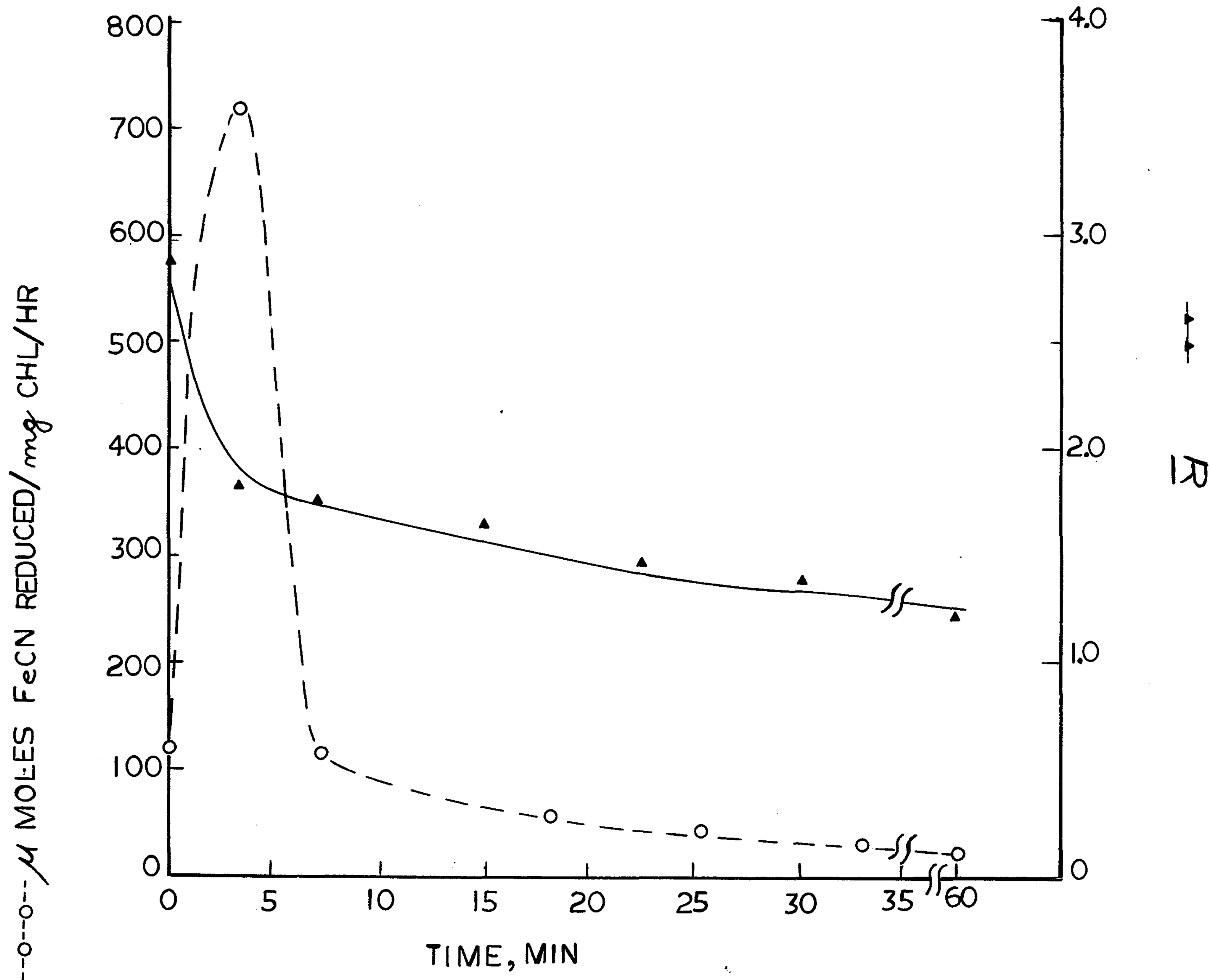
% INHIBITION OF HILL ACTIVITY
OR % CHANGE IN R
100
80
60
40
20
0
6.0
6.5
7.0
7.5
8.0
8.5
pH
FeCN HILL ACTIVITY
R

In Fig. 15 are plotted ferricyanide Hill activity and R for broken spinach chloroplasts concomitantly measured as functions of incubation time in Ricinus chloroplast protein. Decrease in Hill activity to 50% occurs at 15 minutes, whereas the 50% attenuation of R occurs at 35 minutes. This more highly purified preparation (on the basis of its specific activity=42) still exhibits for the change in R biphasic kinetics similar to those observed in RLE.

In another experiment, it was noted (see Table II) that while incubation of spinach chloroplasts in the Ricinus chloroplast protein for 15 minutes results in a considerable loss of Hill activity, there is little diminution of system I activity.

In the case of Ricinus chloroplast protein, attempts to use BSA to "protect" against the induction of the spectral changes were unsuccessful. In addition, if BSA, which had been defatted by the method of Chen, was added together with Ricinus protein the temporal sequence of spectral changes was accelerated rather than prevented (Fig. 16). The final spectrum was "one-banded," however in this case the resulting band maximum was at 687 nm, rather than at 698 nm. Addition of defatted BSA (up to 25 mg/ml), alone, did not have any pronounced effect on control chloroplasts.

Figure 15. The effect of *Ricinus* chloroplast protein on FeCN Hill activity and *R* as a function of time of incubation at 28°C. Protein concentration was .033 mg/ml. Fluorescence measurements were made in the absence of the electron acceptor.



--o--o-- μ MOLES FeCN REDUCED/mg CHL/HR
800
700
600
500
400
300
200
100
0
0
5
10
15
20
25
30
35
60
TIME, MIN
4.0
3.0
2.0
1.0
0
R

Figure 16. The effect of *Ricinus* chloroplast protein (in the presence and absence of defatted BSA) on the fluorescence emission spectrum (-196°C) of spinach chloroplasts. Curve A, chloroplasts incubated in 10 mM Tricine, pH 8.0 plus .05 mg/ml *Ricinus* chloroplast protein for 90 minutes. Curve B, plus 10 mg/ml of defatted BSA and incubated for only 30 minutes. Incubation temperature, 30°C.

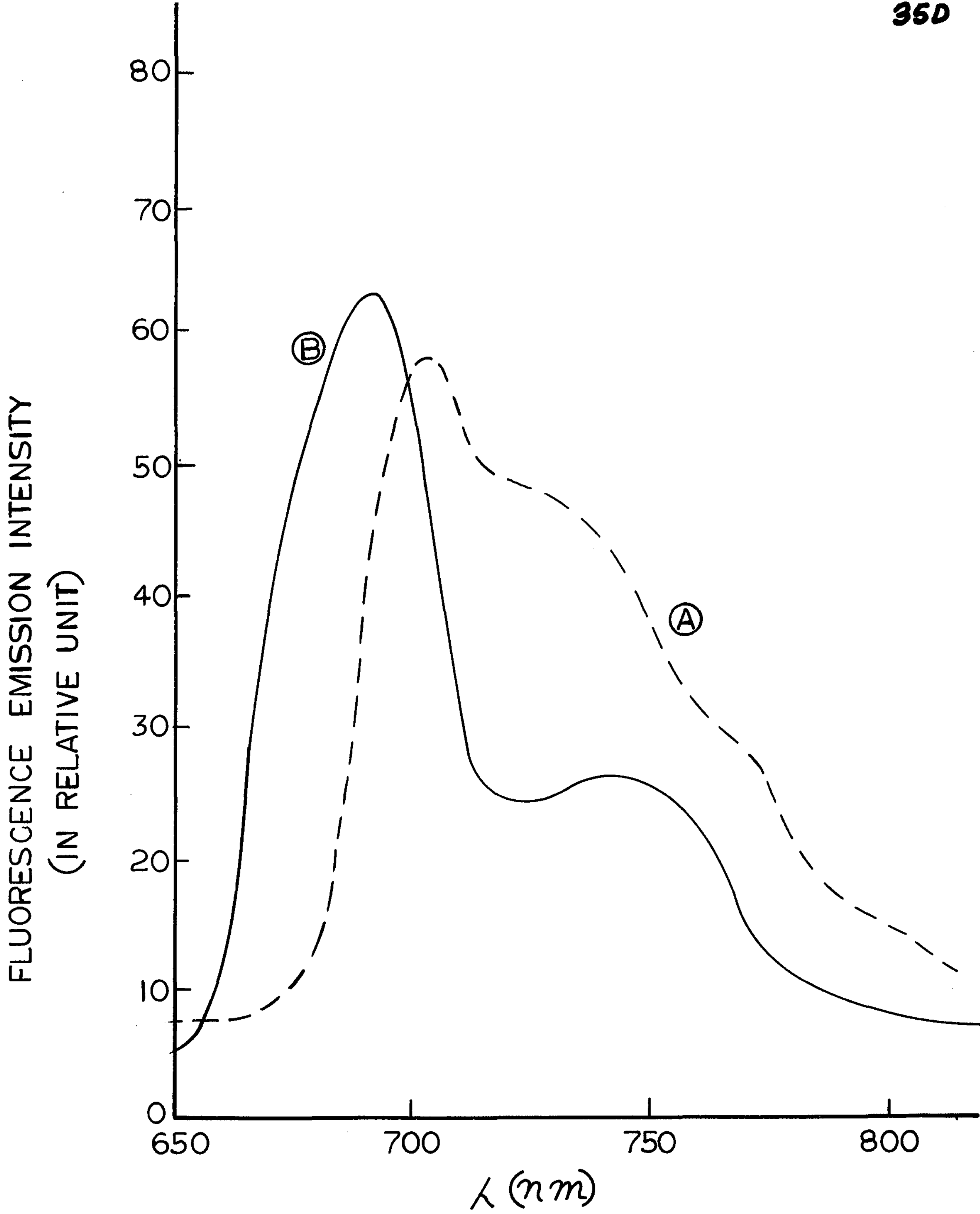

FLUORESCENCE EMISSION INTENSITY
(IN RELATIVE UNIT)
80
70
60
50
40
30
20
10
0
650
700
750
800
λ (nm)
Ⓑ
Ⓐ

TABLE II

THE EFFECT OF *RICINUS* CHLOROPLAST PROTEIN ON NADP REDUCTION[a]

Protein Concentration ($\mu g/ml$)	Electron donor	
	H_2O	DCPIP-ascorbate + DCMU
0	100	100
58	46[b]	90
100	16	80

[a]Spinach chloroplasts were incubated (at 26°C) in 25 mM Tricine-NaOH, pH 8.3 + 6.6 mM $MgCl_2$. NADP reduction was assayed as described in MATERIALS AND METHODS. The control rates of NADP reduction were (μmoles reduced /mg chlorophyll /hr): from H_2O = 18, from ASC-DCPIP = 7.

[b]Data are expressed as percent of control.

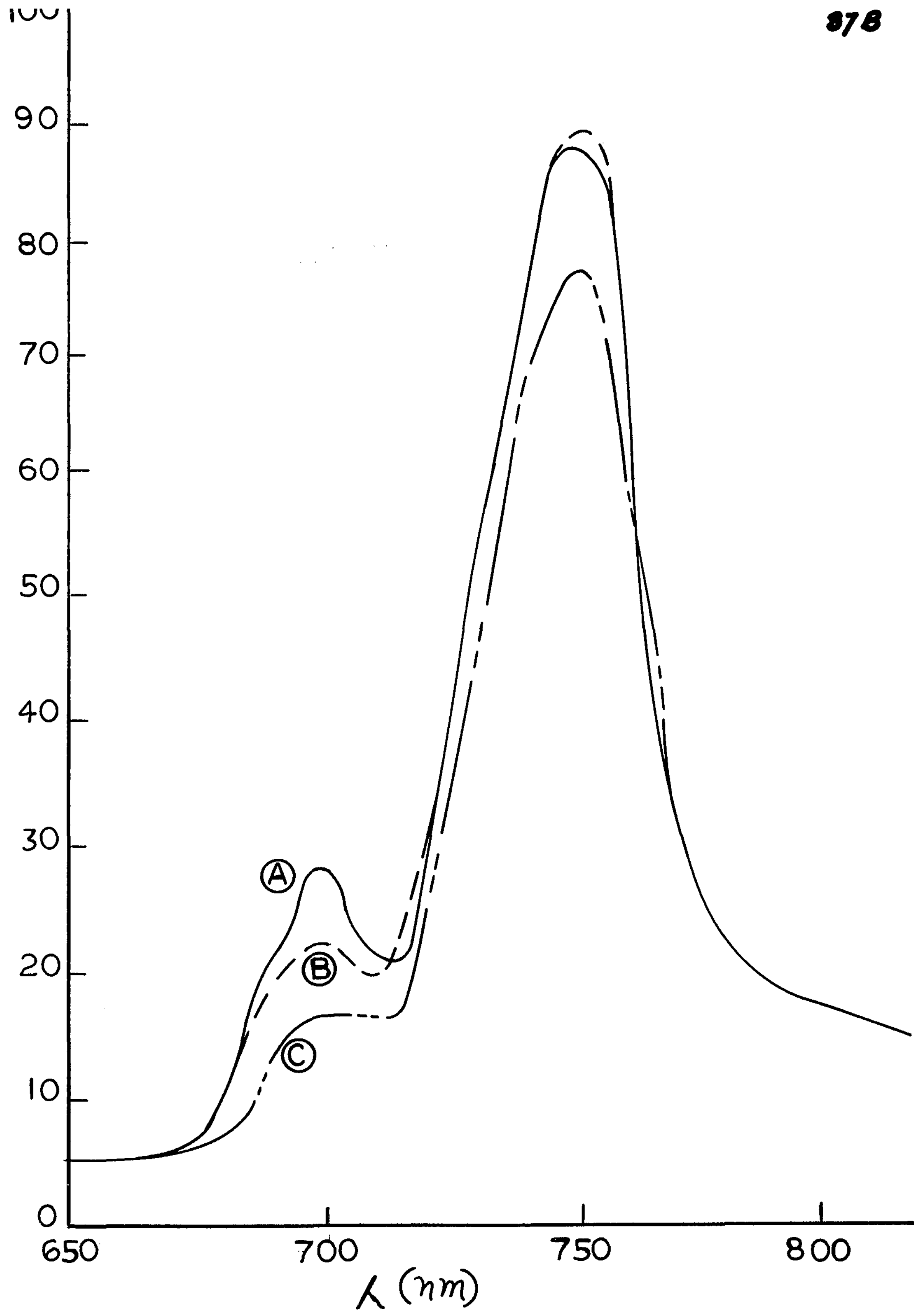
V. MODEL SYSTEMS FOR THE ACTION OF THE RICINUS CHLOROPLAST PROTEIN

A. Proteolytic and Lipolytic Enzymes.

Attempts were made, with a number of substances, to duplicate the ability of RLE (or *Ricinus* chloroplast protein) to induce the steady-state fluorescence changes. Broken spinach chloroplasts (20 μg/ml) were incubated with lytic enzymes, e.g., Trypsin (100 μg/ml); Pronase (50 μg/ml); Wheat germ lipase (100 μg/ml); Lipoxidase (100 μg/ml); or Phospholipase D (100 μg/ml) for periods up to 2 1/2 hours. With both the proteolytic enzymes, Trypsin or pronase, alterations in the steady-state emission spectrum were observed--as shown in Fig. 17. However, in both cases there was a small increase in *R* rather than the large decrease in *R* found after incubation in RLE. The effect of these enzymes appears to be on the forms of chlorophyll which emit at 685 and 698 nm--both of which have been associated with system II; these bands are converted from an inflection and a peak respectively in the control to single broad shoulder. Okayama (1967) also has noted a large decrease in the fluorescence yield at 685 nm of chloroplasts treated with trypsin; he additionally noted that trypsin is a potent Hill reaction inhibitor when employed to treat chloroplasts in the range 2-5 μg/μg chlorophyll (*a*+*b*). The above lipases did not appear to alter steady-state emission.

Incubation of spinach chloroplasts in pancreatin did

Figure 17. Fluorescence emission spectra (-196°C) of spinach chloroplasts incubated in pronase. Curve A, control chloroplasts incubated in 13 mM Tricine, pH 7.8 and 3.3 mM $MgCl_2$. Curve B, plus 0.5 μg pronase/μg chlorophyll (a + b). Curve C, plus 5.0 μg pronase/μg chlorophyll (a + b). Incubation temperature, 33°C.

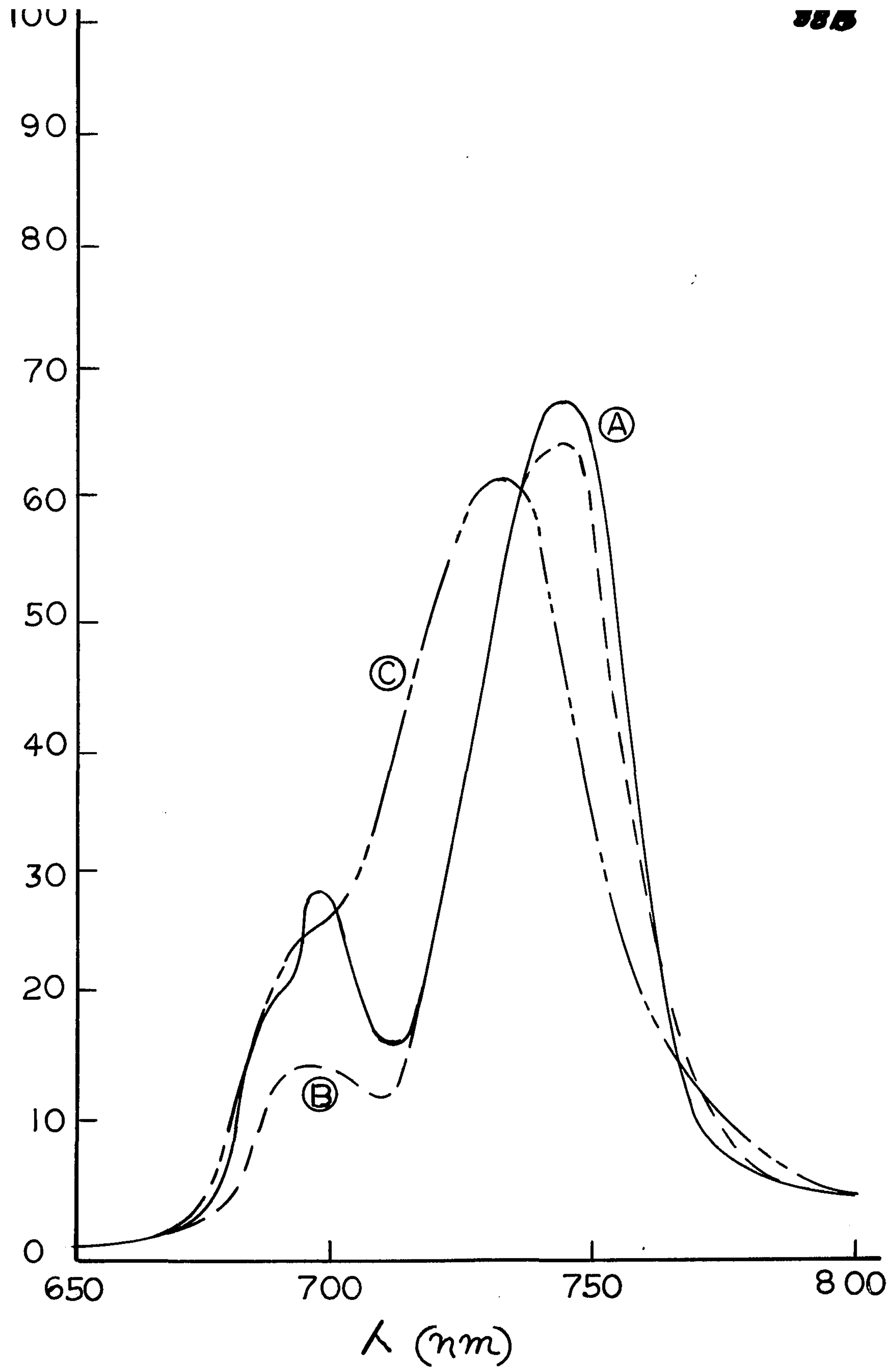


FLUORESCENCE EMISSION INTENSITY
(IN RELATIVE UNITS)
100
90
80
70
60
50
40
30
20
10
0
650
700
750
800
λ (nm)
A
B
C

lead to fluorescence changes somewhat similar to those brought about by RLE. After 5 minutes of incubation at 25°C, R has increased from 2.57 in the control to 4.0. This change is followed by a slower decrease in R, so that after about 60 minutes of incubation, R is back to its control value (note however the difference in the shape of the spectrum, Fig. 18); in 140 minutes of incubation R has decreased to 1.0. Pancreatin is known to have both proteolytic and lipolytic activity, i.e., galactolipase, phospholipase and sulfolipase (Sastry and Kates, 1964; Bishop, 1966; Yagi and Benson, 1963). Since it was observed in the present work (see above) that trypsin and pronase both bring about spectral changes which resemble those occurring initially in pancreatin, it was inferred that the subsequent decrease in R was brought about by the lipolytic activity of pancreatin. It was also noted that if RLE was added to chloroplasts previously incubated in pancreatin--and in which R was observed to be decreasing--the rate of decrease was markedly accelerated.

To test whether lipid-hydrolyzing activity was responsible for the observed changes in fluorescence (i.e., the decrease in R), spinach chloroplasts or subchloroplast particles were incubated either a) in an extract of Scarlet runner bean leaves, which is known to be rich in galactolipase activity, or b) in a purified galactolipase (from chloroplasts of *Phaseolus vulgaris*, var. Kentucky Wonder). When incubation in Scarlet runner

Figure 18. Fluorescence emission spectra (-196°C) of spinach chloroplasts incubated in pancreatin (see body of paper). Curve A, control (in NaCl-Tris). Curve B, pancreatin for 5 minutes. Curve C, pancreatin for 60 minutes. Incubation temperature, 26°C.


FLUORESCENCE EMISSION INTENSITY
(IN RELATIVE UNITS)
0
10
20
30
40
50
60
70
80
90
100
650
700
750
800
λ (nm)
A
B
C

bean extract was carried out at pH 8, no decrease in R was observed. However, incubation at pH 6 did produce the spectral changes. This is in contrast to the findings with Ricinus (chloroplasts, chloroplast protein, or leaf extract) where maximal spectral change occurred at pH 8. Incubation in the purified galactolipase was carried out, at pH 7.0, close to the previously determined (R. E. McCarty, personal communication) pH optimum of 6.5 and yielded fluorescence changes similar to those produced by the Ricinus protein.

B. Long-Chain Unsaturated Fatty Acids.

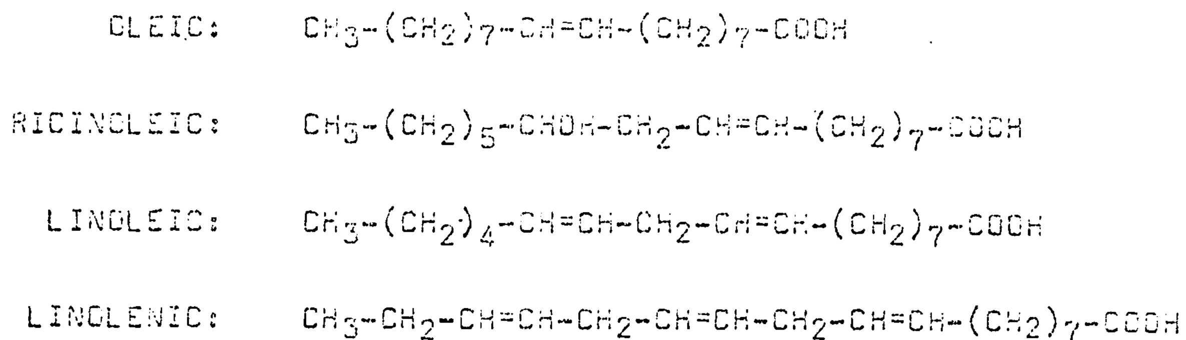
Since the action of lipases (which is similar to the later action of pancreatin; see Fig. 18 above) brought about fluorescence changes like those induced by the Ricinus protein, it seemed worthwhile to investigate the direct effect of exogenous fatty acids. Although fatty acids are only one of the categories of and products of lipolytic activity, it had been reported on several previous occasions that they had pronounced effects on several chloroplast parameters. The inhibitory action of fatty acids on Hill reaction was described by a number of workers (Spikes et al, 1955; Krogmann and Jagendorf, 1959; McCarty and Jagendorf, 1965; Molotkovsky and Zheskova, 1966; Constantopoulo and Kenyon, 1968; Katoh and San Pietro, 1968).

Molotkovsky and Zheskova (1966) additionally reported that such inhibition is accompanied by light-scattering changes--interpreted by them to arise from swelling of chloroplasts. Fatty acids have been shown, also, to be

inhibitors of pyocyanine--catalyzed cyclic photophosphorylation, and uncouplers of ferricyanide-mediated non-cyclic photophosphorylation (McCarty and Jagendorf, 1965).

(1) EFFECTS ON FLUORESCENCE EMISSION (-196°C). Addition of long-chain (C_{18}) unsaturated fatty acids (linolenic, linoleic, oleic or ricinoleic, see Fig. 19 for their structures) to chloroplasts or subchloroplast particles leads to changes in fluorescence emission, which are identical to those induced by RLE; this is shown in Fig. 20. In Fig. 21A is shown R as a function of oleic acid concentration; note that the decrease in R does not begin until the fatty acid to chlorophyll (a+b) (on a mole/mole basis)--to be referred to as "FA/CHL"--reaches 5. In Fig. 21B is plotted R as function of time of incubation; 23°C, for chloroplasts incubated in 10^{-3}M oleic acid. Note that the chlorophyll (a+b) concentration was the same in both cases.

Although in the case of intact algae, steady-state fluorescence changes are not brought about by incubation in RLE, they are observed with fatty acids. For example, treatment of light-grown *Euglena gracilis* strain Z with 10^{-3}M linolenic acid results in a change in R from 150 to 1.40. Under similar conditions R, in *Scenedesmus obliquus* wild type, changed from 1.46 to 1.15. The *Scenedesmus* mutants #8 (CO_2) and #11 (O_2^-) did not show any significant change in fluorescence emission. In addition, it was



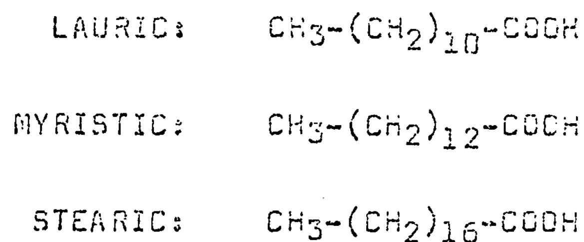
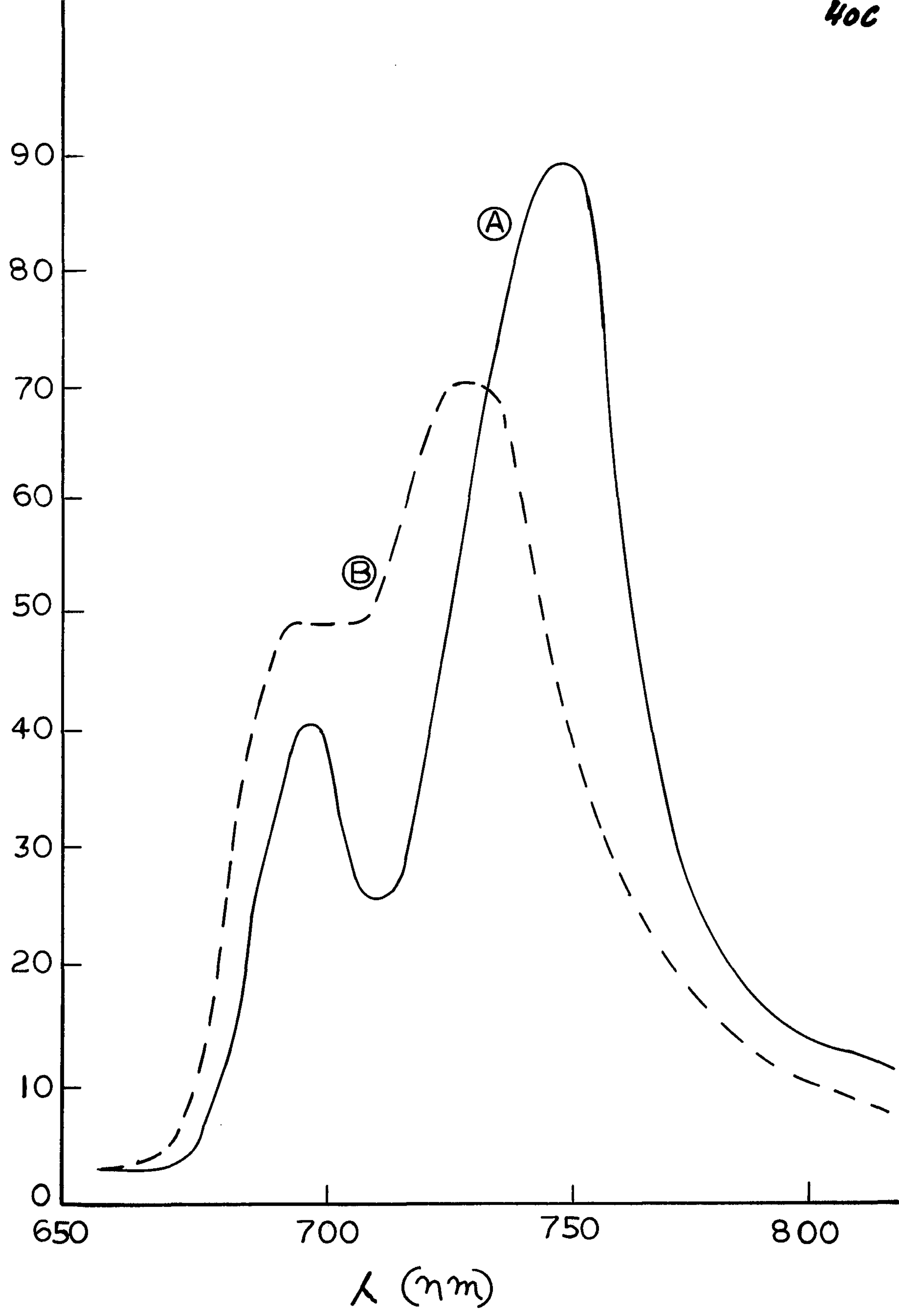


Figure 19. Structural formulae of the various long-chain fatty acids used in the present investigation.

Figure 20A,B. Fluorescence emission spectra (-196°C) of spinach chloroplasts incubated in the presence of 10^{-3}M oleic acid. Curves A and B are for 0 and 5 minutes respectively. Incubation temperature, 23°C.



FLUORESCENCE EMISSION INTENSITY
(IN RELATIVE UNITS)
0
10
20
30
40
50
60
70
80
90
650
700
750
800
λ (nm)
Ⓐ
Ⓑ

Figure 20C,D. Fluorescence emission spectra (-196°C) of spinach chloroplasts incubated in the presence of 10^{-3}M oleic acid. Curves C and D are for 30 and 60 minutes respectively.

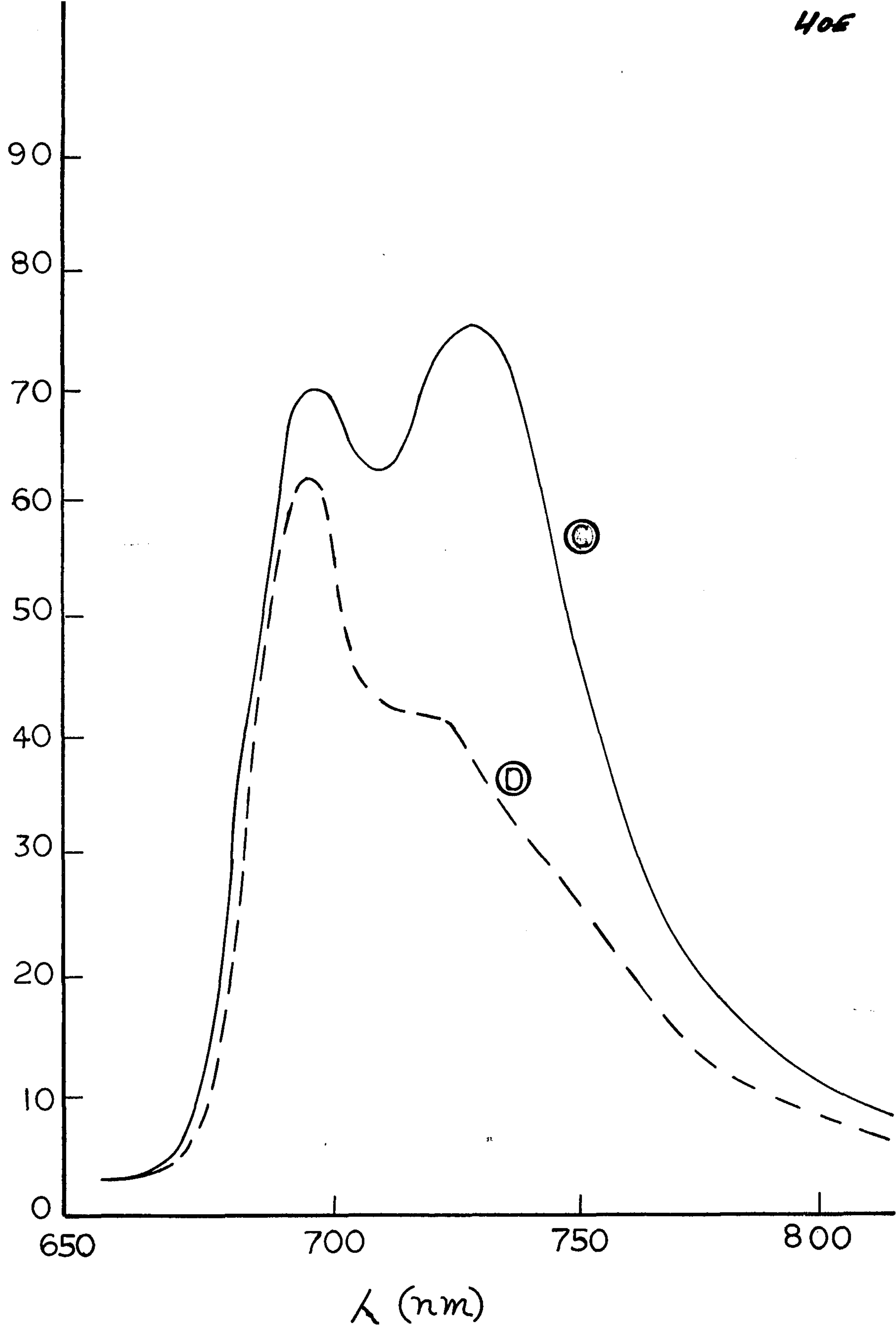
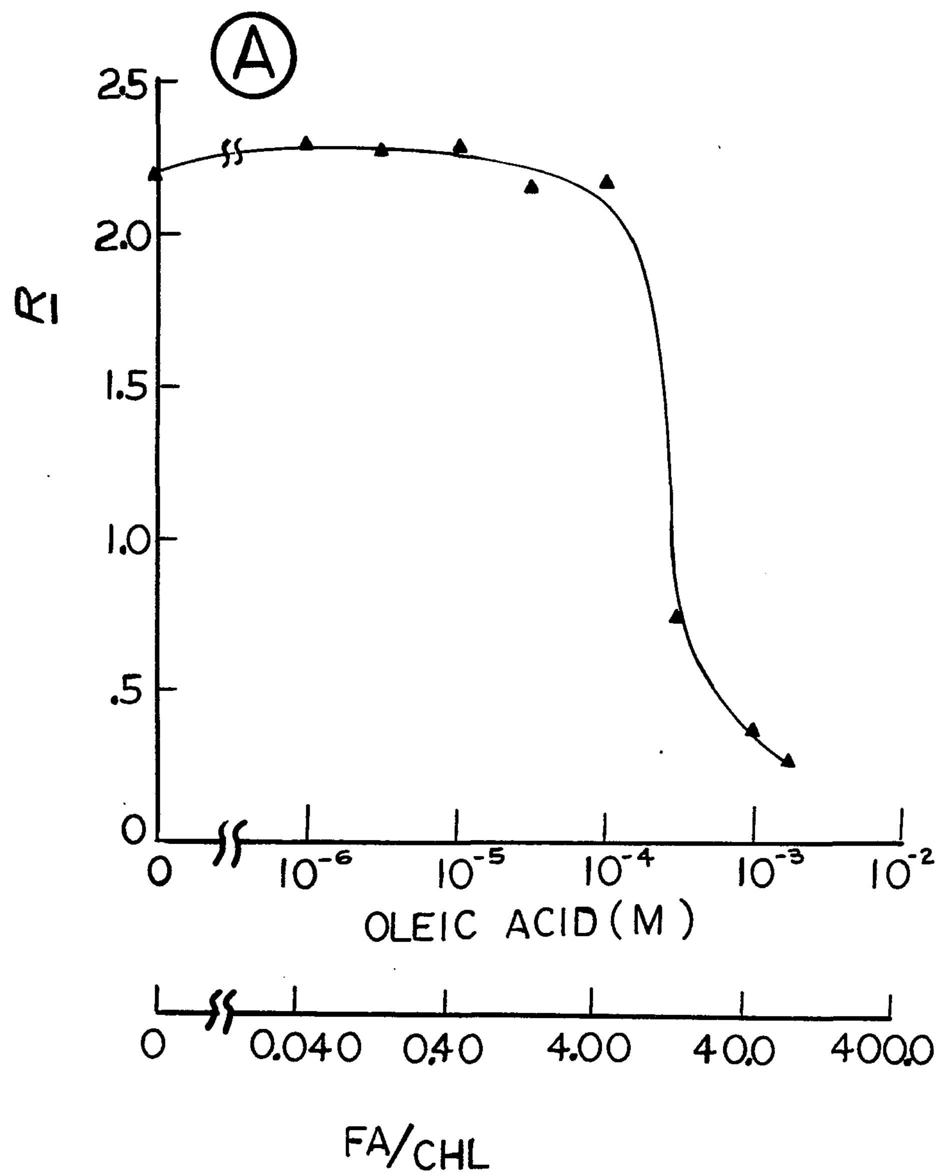
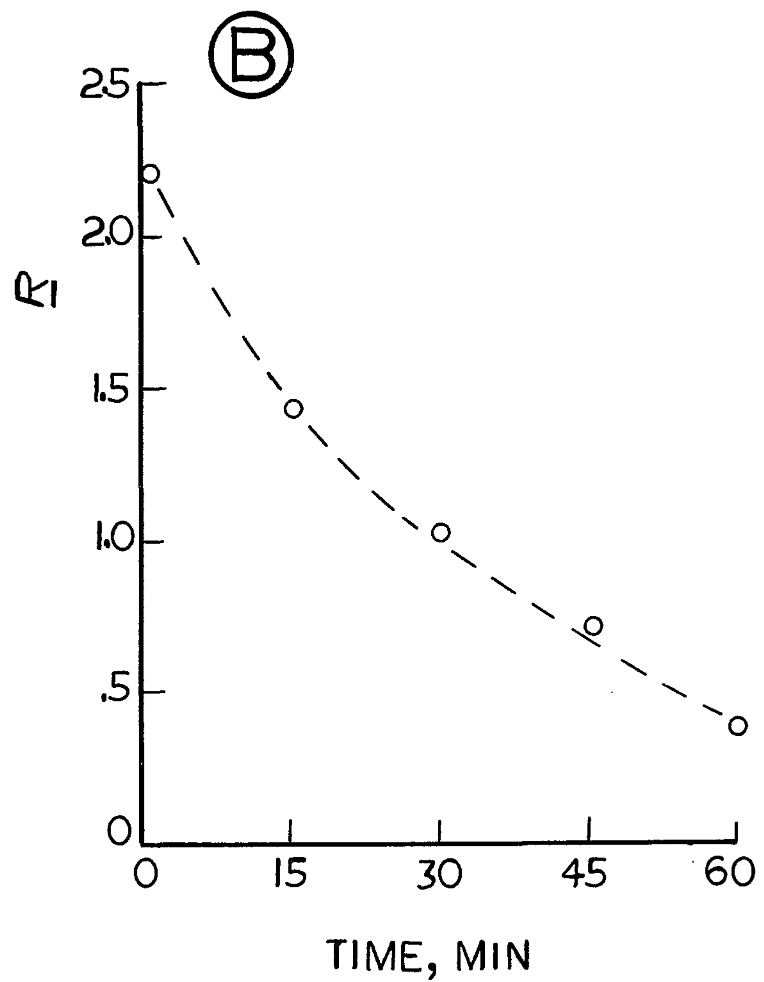

FLUORESCENCE EMISSION INTENSITY
(IN RELATIVE UNITS)
0
10
20
30
40
50
60
70
80
90
650
700
750
800
λ (nm)
C
D

Figure 21. The effect of oleic acid on R. A) as a function of increasing concentration, B) as a function of time of incubation in 10^{-3}M oleic acid. Incubation temperature, 23°C.


A
2.5
2.0
1.5
1.0
.5
0
R1
0
10-6
10-5
10-4
10-3
10-2
OLEIC ACID (M)
0
0.040
0.40
4.00
40.0
400.0
FA/CHL


B
2.5
2.0
1.5
1.0
.5
0
R1
0
15
30
45
60
TIME, MIN

noted that the O_2^- mutant (untreated) appears to be lacking F698; see Fig. 22 which is a difference emission spectrum (wild type-#11, normalized at 725 nm).

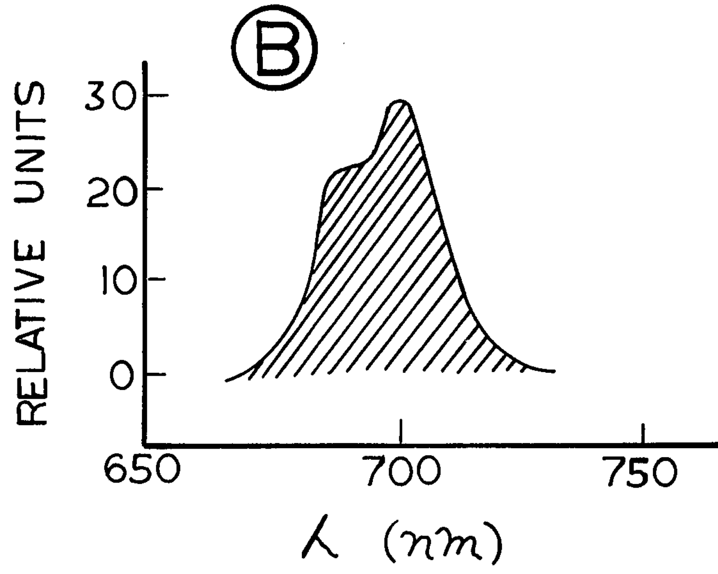
If chloroplast fragments are prepared from this O_2 mutant, and they are treated with fatty acids, a band at ~ 695 nm can be observed. Treatment of chloroplast fragments prepared from the wild type and CO_2 mutant with fatty acid also led to spectral changes similar to those observed with higher plant chloroplasts.

Addition of the saturated fatty acids lauric or stearic to spinach chloroplasts led to no spectral change; only myristic acid produced a very slight change in R, of about 15%, at FA/CHL ~ 100 (at 30°C).

(2) EFFECTS OF BSA AS A PROTECTIVE AGENT. Prior addition of crystalline BSA to spinach chloroplasts preparations (to yield a final concentration of 5 mg/ml) prevents fatty-acid induced fluorescence changes from occurring; introduction of BSA after the addition of fatty acids does not "protect." From Fig. 23, it may be seen that addition of defatted BSA (20 mg/ml) to chloroplasts--which, after treatment with linolenic acid, had become "one-banded" (Curve B)--results in a strong reversal of the spectral change. As a matter of fact this spectrum is more three-banded (*i.e.*, prominent 685 nm band) than the control. However this situation is transitory and with time the emission at 685 disappears (Curve D).

During the course of the experiments in which fatty acids were used there was utilized a variety of chloroplast preparations, *i.e.*, whole chloroplasts, broken chloroplasts, subchloroplast particles (prepared by sonication or with detergents),

Figure 22. A) Fluorescence emission spectra (-196°C) of whole cells of *Scenedesmus obliquus* wild type and mutant #11. B) Difference fluorescence emission spectrum (wild type-#11) normalized at 725 nm.


B
RELATIVE UNITS
30
20
10
0
650
700
750
λ (nm)

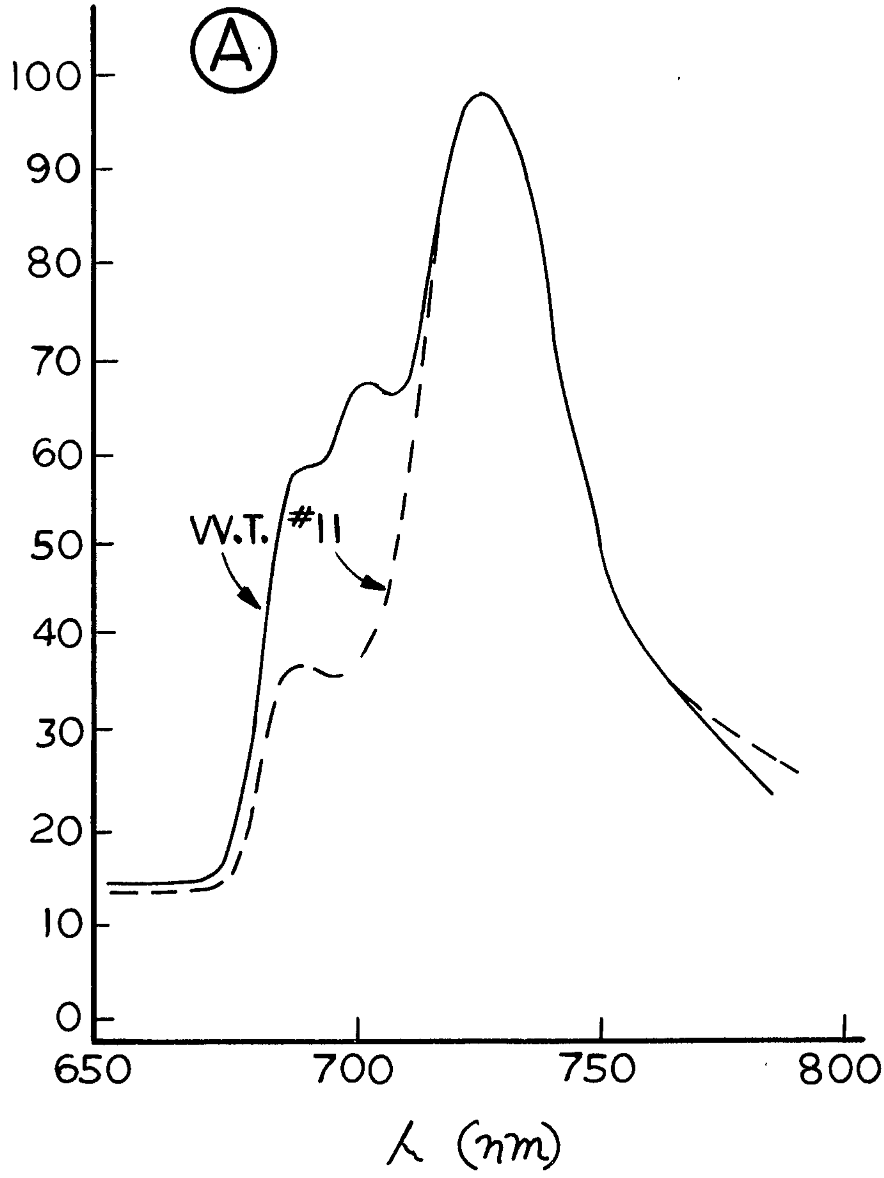
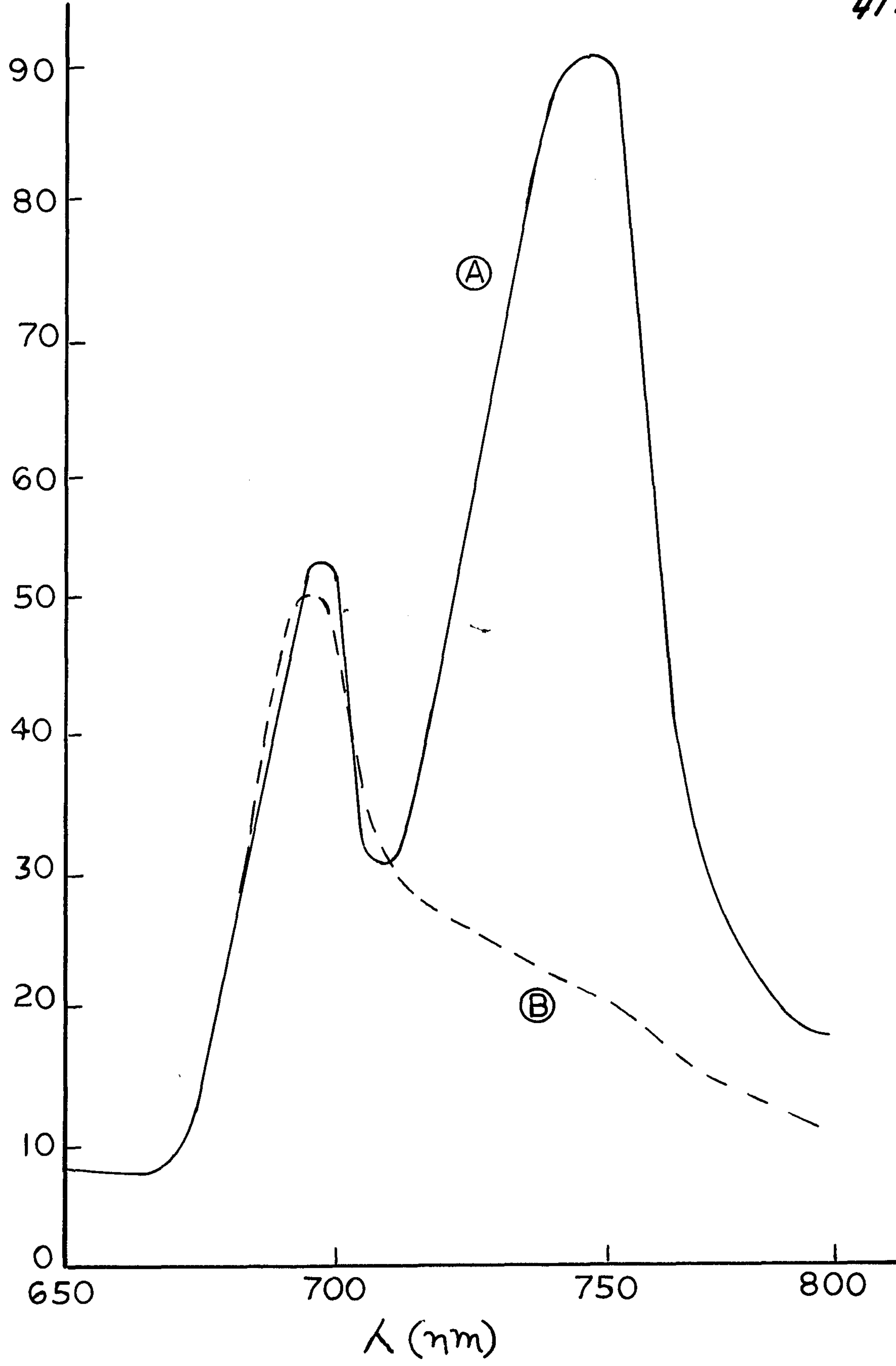
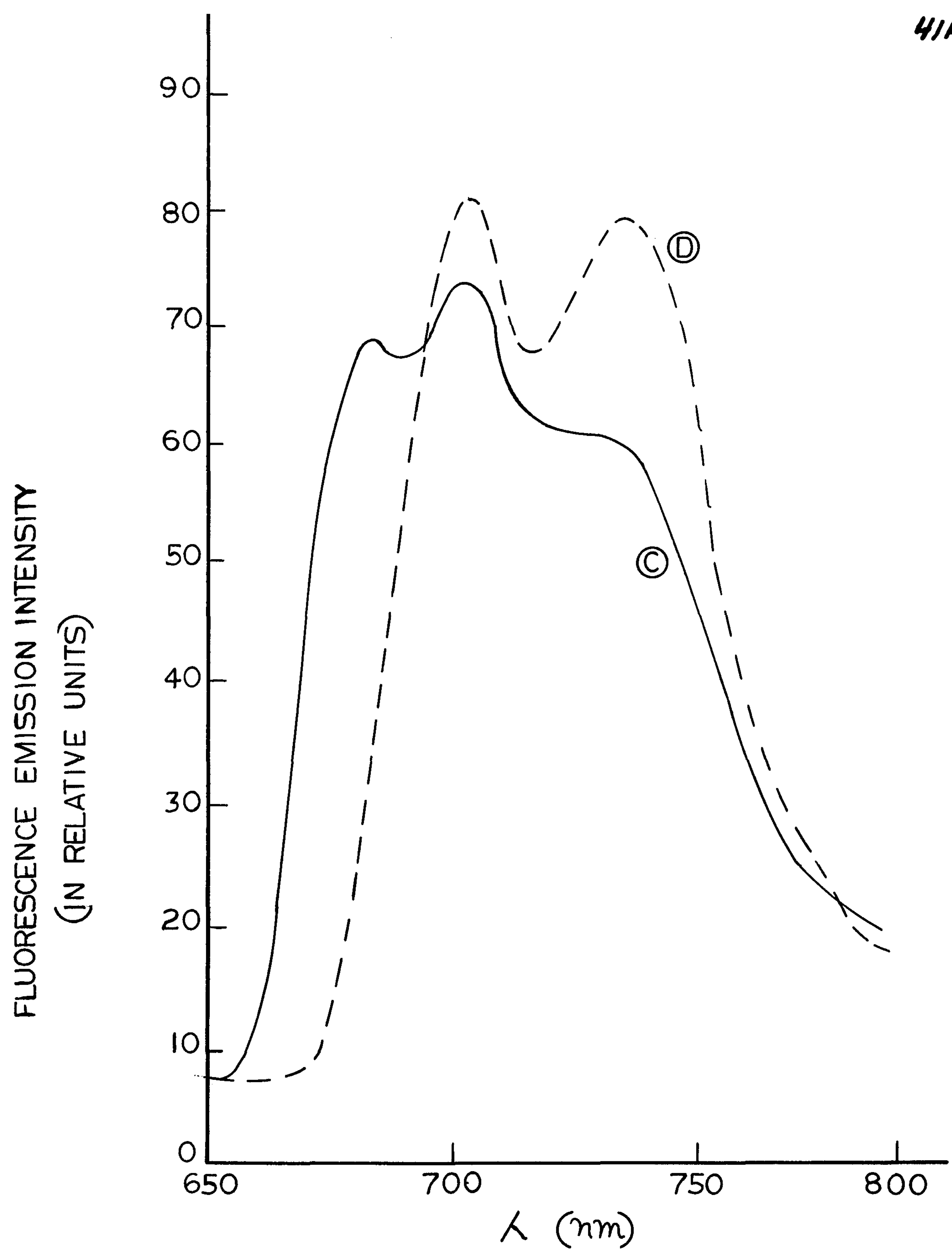

A
FLUORESCENCE EMISSION INTENSITY
(IN RELATIVE UNITS)
100
90
80
70
60
50
40
30
20
10
0
W.T.
#11
650
700
750
800
λ (nm)

Figure 23A,B. The effect of defatted BSA on the fluorescence emission spectrum of linolenic acid-treated spinach chloroplasts. Curve A, control (in the absence of linolenic acid). Curve B, plus 5 x 10^{-4}M linolenic acid for 60 minutes. Incubation temperature, 26°C.



FLUORESCENCE EMISSION INTENSITY
(IN RELATIVE UNITS)
90
80
70
60
50
40
30
20
10
0
650
700
750
800
λ (nm)
Ⓐ
Ⓑ

Figure 23C,D. The effect of defatted BSA on the fluorescence emission spectrum of linolenic acid-treated spinach chloroplasts. Curve C, linolenic acid treatment for 60 minutes then addition of 20 mg/ml of defatted BSA for 5 minutes. Curve D, linolenic acid treatment for 60 minutes then 20 mg/ml of defatted BSA for 20 minutes. Incubation temperature, $26^{\circ}C$.


FLUORESCENCE EMISSION INTENSITY
(IN RELATIVE UNITS)
90
80
70
60
50
40
30
20
10
0
650
700
750
800
λ (nm)
D
C

and variety of fatty acids, with these it was observed that R never fell below ~ 0.20, regardless of the value of R in the control situation (which was as high as 16 in the system I Boardman and Anderson particles from spinach). This lower limit or "saturation plateau" may represent the zero value for F735 emission, i.e., when there is no fluorescence from F735, R ~0.20 represents the ratio of intensities of F698's tail, at 735 nm, to its maximum, at 698 nm. It is interesting to note that R for F698 *in vitro* (the 698 nm-fluorescing form in acetone) is 0.19 (Broyde and S. Brody, 1966).

(3) EFFECTS ON CHLOROPHYLL A IN SOLUTION. If linolenic acid is added to monomeric chlorophyll a in ethanol (~ 10^{-5}M), there is no effect on the emission spectrum at -196°C. This is also true, if the chlorophyll in ethanol is mixed directly with the fatty acid. When chlorophyll a in ethanol is added to 0.1M phosphate buffer (pH 7) and then linolenic acid is added to the mixture, there is a strong increase in long-wavelength fluorescence (~ 740 nm). In the case of chlorophyll a in CCl_4 (a solvent in which aggregates of chlorophyll can exist at low chlorophyll concentrations, Broyde and S. Brody, 1967) addition of linolenic acid results in a decrease in long-wavelength fluorescence at ~ 745 nm, probably due to a de-aggregation of chlorophyll.

(4) EFFECTS ON HILL ACTIVITY AND R. In the case of fatty acids (as above with RLE) the attempt was made to correlate the steady-state fluorescence changes with inhibition of

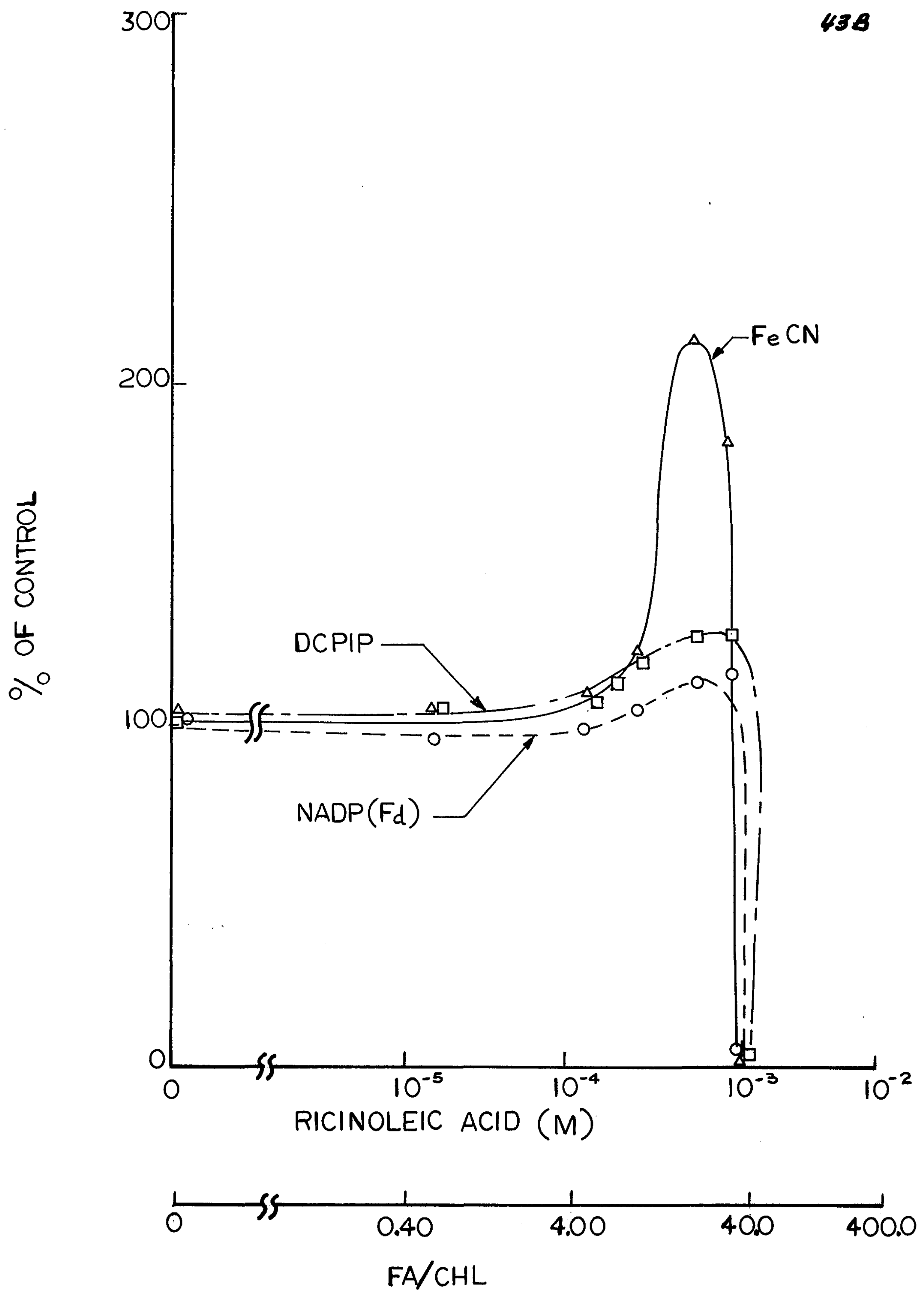
electron transport. In Fig. 24 is given data from an experiment which indicated that regardless of the particular Hill acceptor (ferricyanide, DCPIP or NADP) the FA/CHL (for ricinoleic acid) which inhibited Hill reaction to 50% of the control level was the same. Ferricyanide was chosen for monitoring system II activity, and NADP for monitoring system I and II together.

In Fig. 25 is shown both ferricyanide Hill activity and R concomitantly measured as functions of linolenic acid concentration. It may be seen that Hill activity is totally inhibited at FA/CHL of 2-3. At 50% of the concentration which completely inhibits Hill activity, there is maximal stimulation of electron flow; this stimulation has previously been attributed to an uncoupling of phosphorylation (McCarty and Jagendorf, 1965). In other experiments, in the present work, it was noted that complete uncoupling by ammonium chloride, followed by addition of fatty acid, did not yield additional uncoupling--the reverse situation yielded identical results.

In turning our attention to the relationship between Hill activity and changes in the fluorescence emission spectrum, we note that the diminution in R does not begin until Hill activity is largely inhibited.

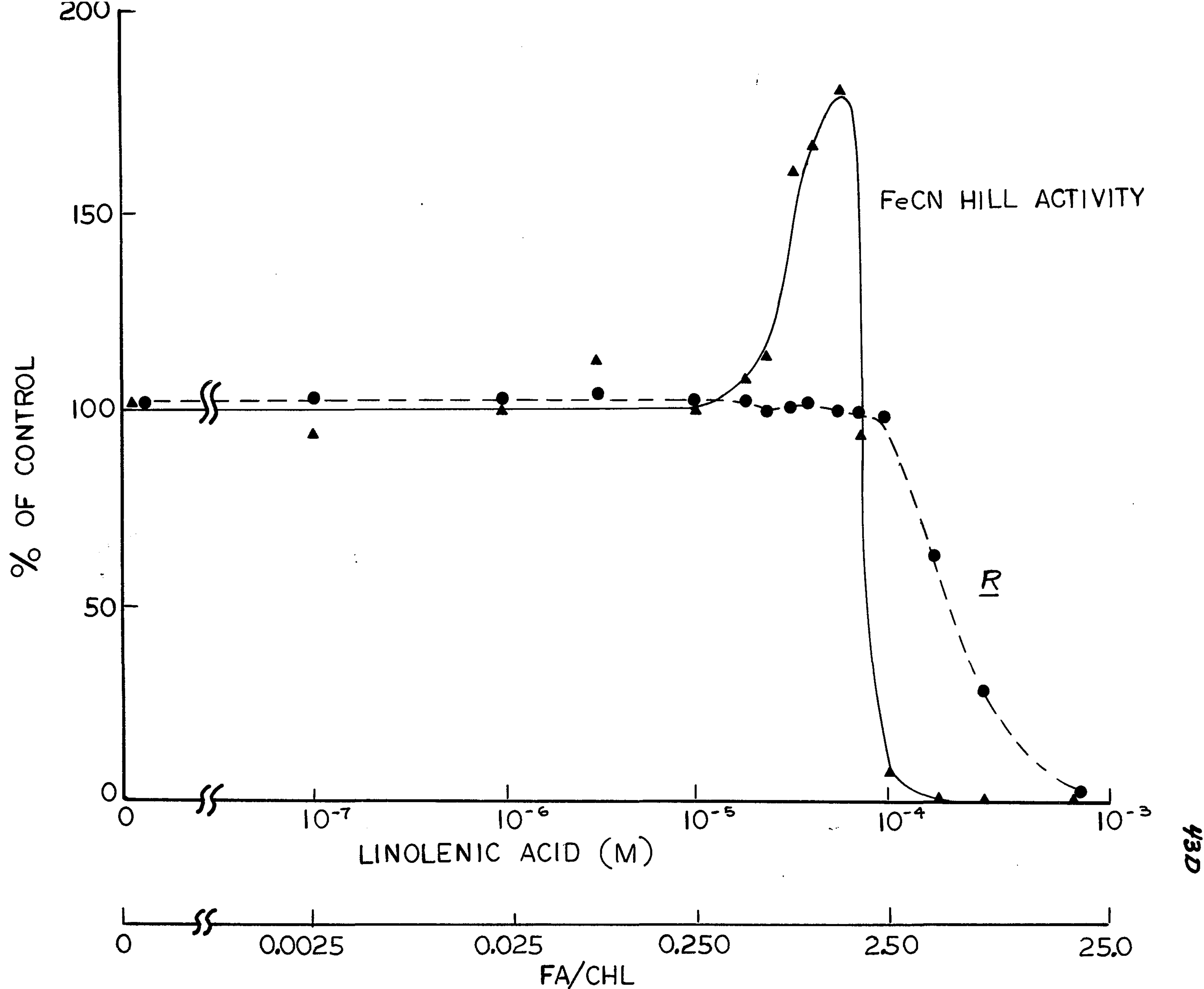
Since one might have tentatively concluded from Fig. 25 that in order for fatty acids to exert their influence on R, they must previously have effected Hill activity--this possibility was investigated. Chloroplasts lacking system II

Figure 24. Effect of ricinoleic acid on Hill activity with FeCN, DCPIP and NADP as electron acceptors. Control rates of reduction (µmoles/mg chl/hr): FeCN, 139; DCPIP, 192; NADP, 42. Incubation temperature, 23°C.



300
200
100
0
% OF CONTROL
Fe CN
DCPIP
NADP(Fd)
0
10^{-5}
10^{-4}
10^{-3}
10^{-2}
RICINOLEIC ACID (M)
0
0.40
4.00
40.0
400.0
FA/CHL

Figure 25. Effect of linolenic acid concentration on FeCN Hill activity and R. Control rate of FeCN reduction (μmoles/mg chl/hr)=150; control value of R=2.17. Incubation temperature 23°C. Fluorescence measurements were made in the absence of the electron acceptor.



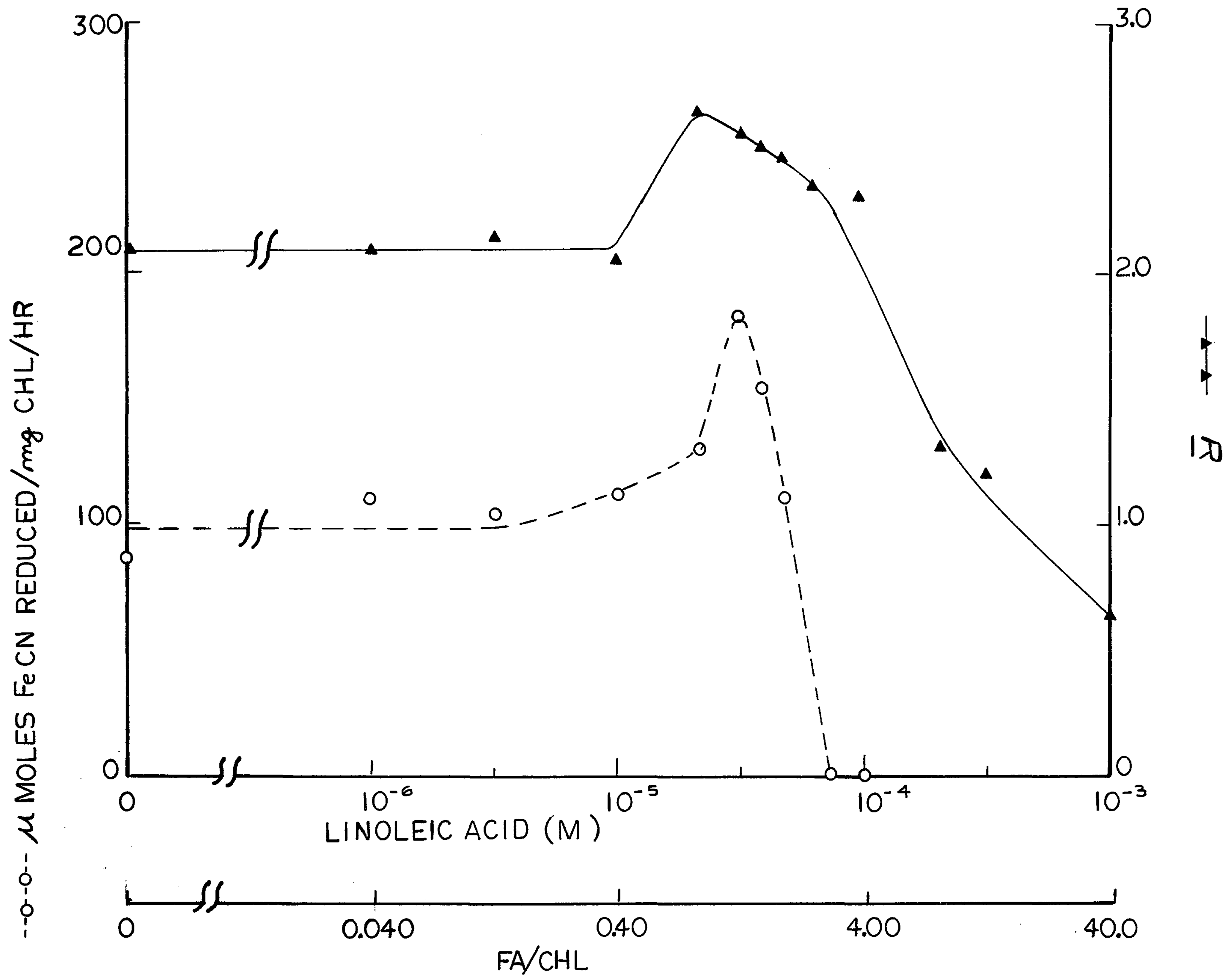
43D

activity (as a result of DCMU treatment or tris aging (see below), or as a result of being isolated from algal mutants incapable of evolving oxygen) were treated with fatty acids. In these cases the diminution in R, associated with fatty acid treatment, was also observed.

It was found that attenuation of R to 50% of the control level occurs at FA/CHL $\sim$ 9, or approximately 3-4 times the concentration which inhibits Hill activity to 50% of the control. An increase in R of as much as 133% of the control has sometimes been observed at fatty acid concentrations just slightly lower than those which produce the decrease in R (Fig. 26). The reasons for this increase are unclear at the present time. The relationship between the number of fatty acid molecules per chlorophyll required to attenuate R and Hill activity to 50% of the control level is summarized in Table III for a number of unsaturated fatty acids.

(5) EFFECTS ON DELAYED LIGHT EMISSION. Measurement of intensity of delayed light emission, in the millisecond range, and ferricyanide Hill activity indicates that linolenic acid causes a marked decrease in intensity of delayed light (see Fig. 27). Fig. 28 shows that this decrease occurs at a lower concentration of linolenic acid than that which promotes inhibition of Hill activity. This lower concentration is in the range (or slightly below that) at which uncoupling is normally found (see above).

Figure 26. Effect of linoleic acid concentration on FeCN Hill activity and *R*. Incubation temperature, 23°C. Fluorescence measurements were made in the absence of the electron acceptor.



44B

TABLE III

THE EFFECT OF UNSATURATED FATTY ACIDS ON R AND ON FERRICYANIDE HILL ACTIVITY

FATTY ACID	50% INHIBITION OF HILL ACTIVITY[a]	50% ATTENUATION OF R[a]	50% LEVEL OF R / 50% LEVEL OF HILL
Linolenic	2.25	9.00	4.0
Linoleic	3.00	19.50	6.5
Oleic	3.50	20.00	5.7
Ricinoleic	27.00	100.00	3.7

[a]Data are expressed as the ratio of fatty acid to chlorophyll on a molar basis.

Figure 27. Effect of linolenic acid (4×10^{-5}M) on the FeCN-induced modulation of delayed light emission in the millisecond range. Incubation temperature, 23°C.

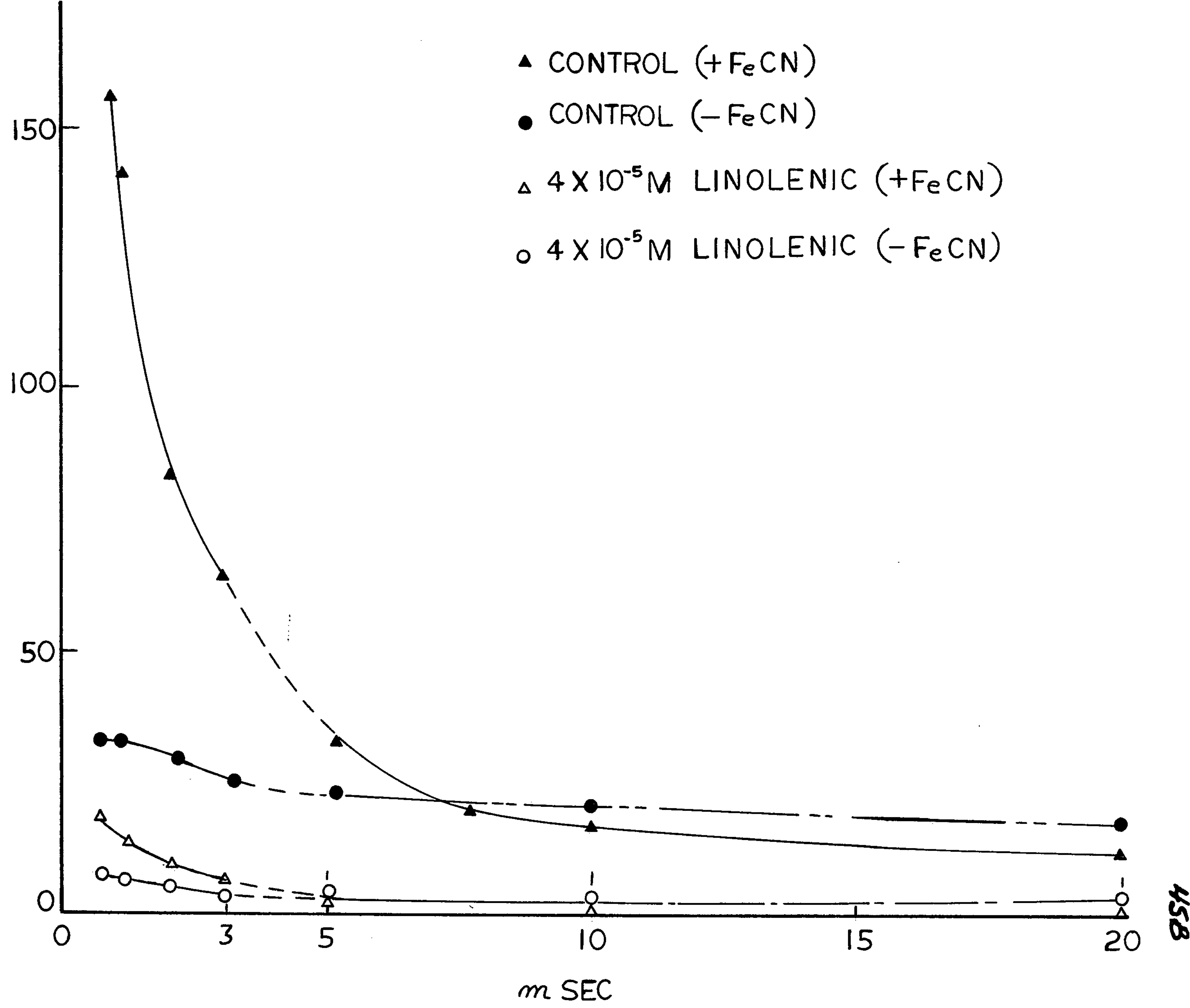
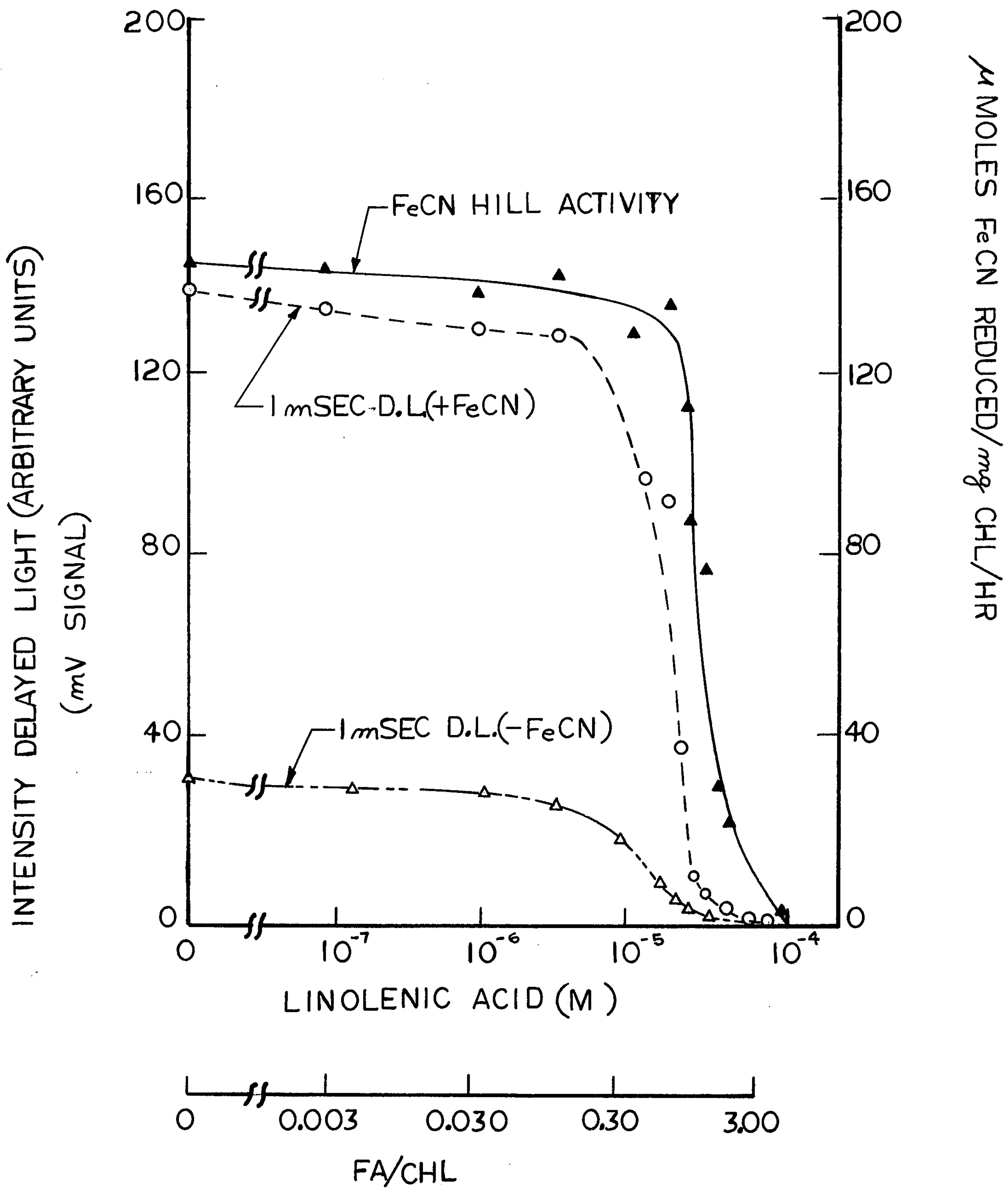

INTENSITY DELAYED LIGHT (ARBITRARY UNITS)
(mV SIGNAL)
150
100
50
0
0
3
5
10
15
20
m SEC
CONTROL (+FeCN)
CONTROL (−FeCN)
4 X 10^{-5}M LINOLENIC (+FeCN)
4 X 10^{-5}M LINOLENIC (−FeCN)

Figure 28. Effect of increasing linolenic acid concentration on FeCN Hill activity and on 1 msec delayed light emission in the presence and absence of FeCN.

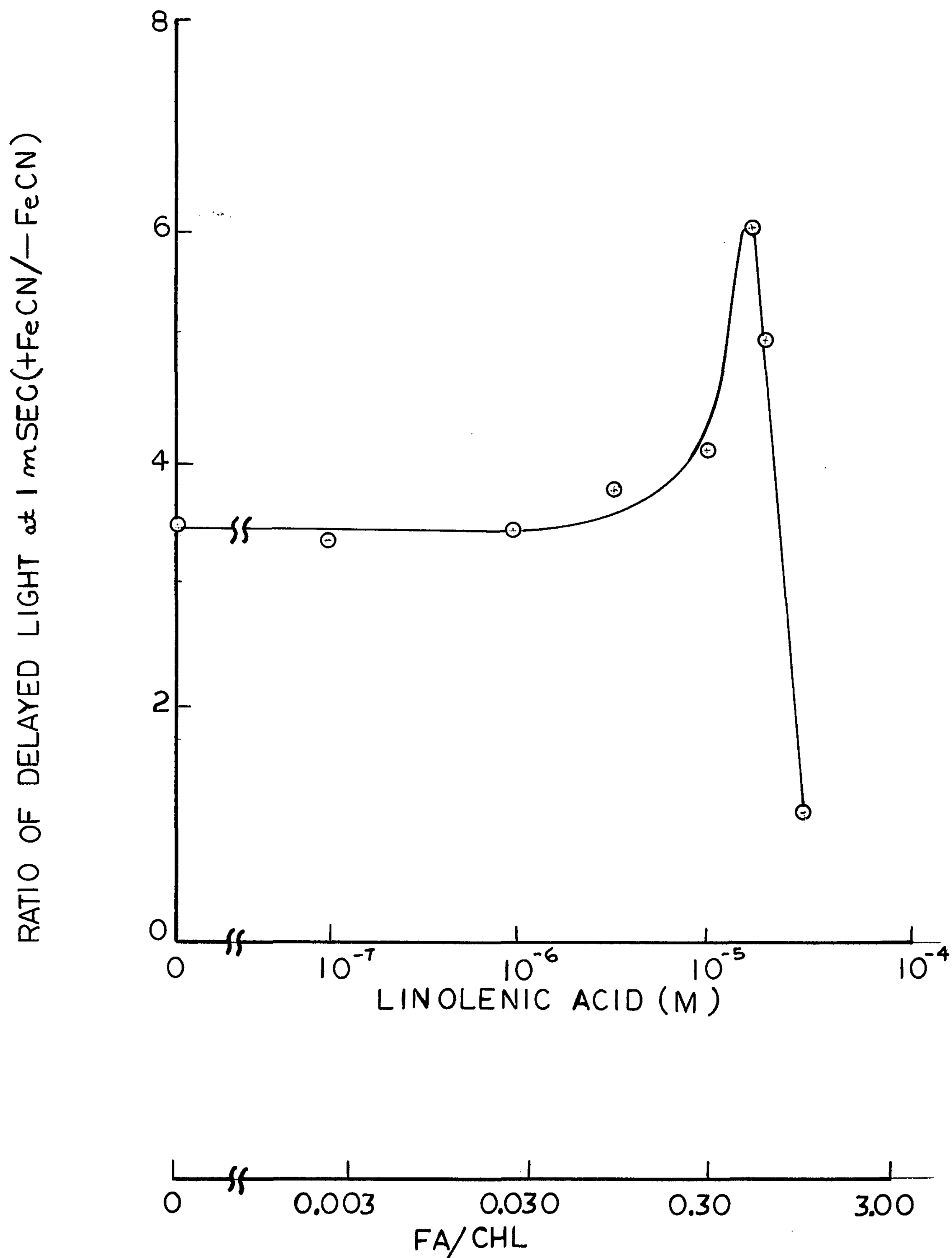

200
160
120
80
40
0
200
160
120
80
40
0
FeCN HILL ACTIVITY
1 mSEC-D.L.(+FeCN)
1 mSEC D.L.(-FeCN)
INTENSITY DELAYED LIGHT (ARBITRARY UNITS)
(mV SIGNAL)
μ MOLES FeCN REDUCED/mg CHL/HR
0
10^{-7}
10^{-6}
10^{-5}
10^{-4}
LINOLENIC ACID (M)
0
0.003
0.030
0.30
3.00
FA/CHL

However, it is likely that in the case of the particular chloroplasts used in these experiments, uncoupling had already occurred, since addition of neither fatty acid nor ammonium chloride led to the expected stimulation of electron flow.

Fig. 29 shows the ratio of 1 msec delayed light emission in the absence and in the presence of ferricyanide, as a function of linolenic acid concentration. Note that in the control (i.e., the zero value on the abscessa), addition of ferricyanide stimulates the intensity of emission 3-4 fold. In the present study it was observed that at a linolenic acid concentration of 2.5×10^{-5}M, the stimulation increases to 7-fold. Above this concentration only a 1-2 fold stimulation is noted upon addition of ferricyanide. At concentrations of linolenic acid above 5×10^{-5}M it is hard to discriminate between signal and noise levels.

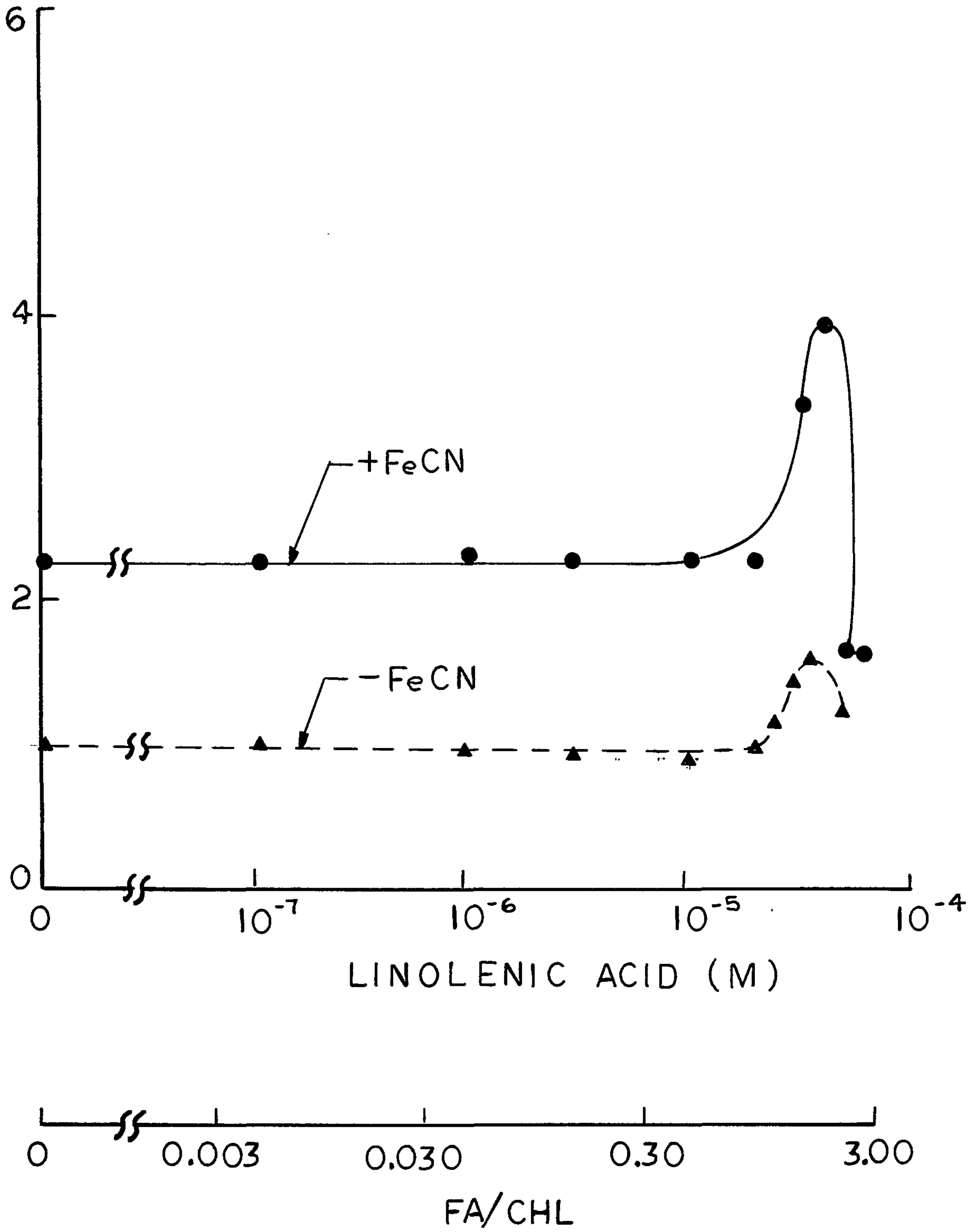
The intensity of delayed light emission at 1 msec was measured in the presence and absence of ferricyanide as a function of fatty acid concentration; the same was done for emission at 3 msec. Fig. 30 gives a plot of the ratio of intensities at 1 msec and at 3 msec (1 msec/3 msec) as a function of concentration. This is done to indicate changes in kinetics of delayed light dark decay. A high value of this ratio indicates rapid dark decay, while a low value indicates slow decay. At concentrations of linolenic acid up to $\sim 10^{-5}$M there are no changes in the shapes of the (+ or - ferricyanide) decay curves. At about 5×10^{-5}M, the decays are accelerated; above this concentration, again, it was hard to discriminate between

Figure 29. Effect of increasing concentration of linolenic acid on the ratio of delayed light at 1 msec in the presence of FeCN to delayed light at 1 msec in the absence of FeCN.



8
6
4
2
0
RATIO OF DELAYED LIGHT at 1 mSEC(+FeCN/— FeCN)
0
10^{-7}
10^{-6}
10^{-5}
10^{-4}
LINOLENIC ACID (M)
0
0.003
0.030
0.30
3.00
FA/CHL

Figure 30. Effect of increasing linolenic acid concentration on the ratio of delayed light at 1 msec/3 msec in the absence and presence of FeCN.



46D

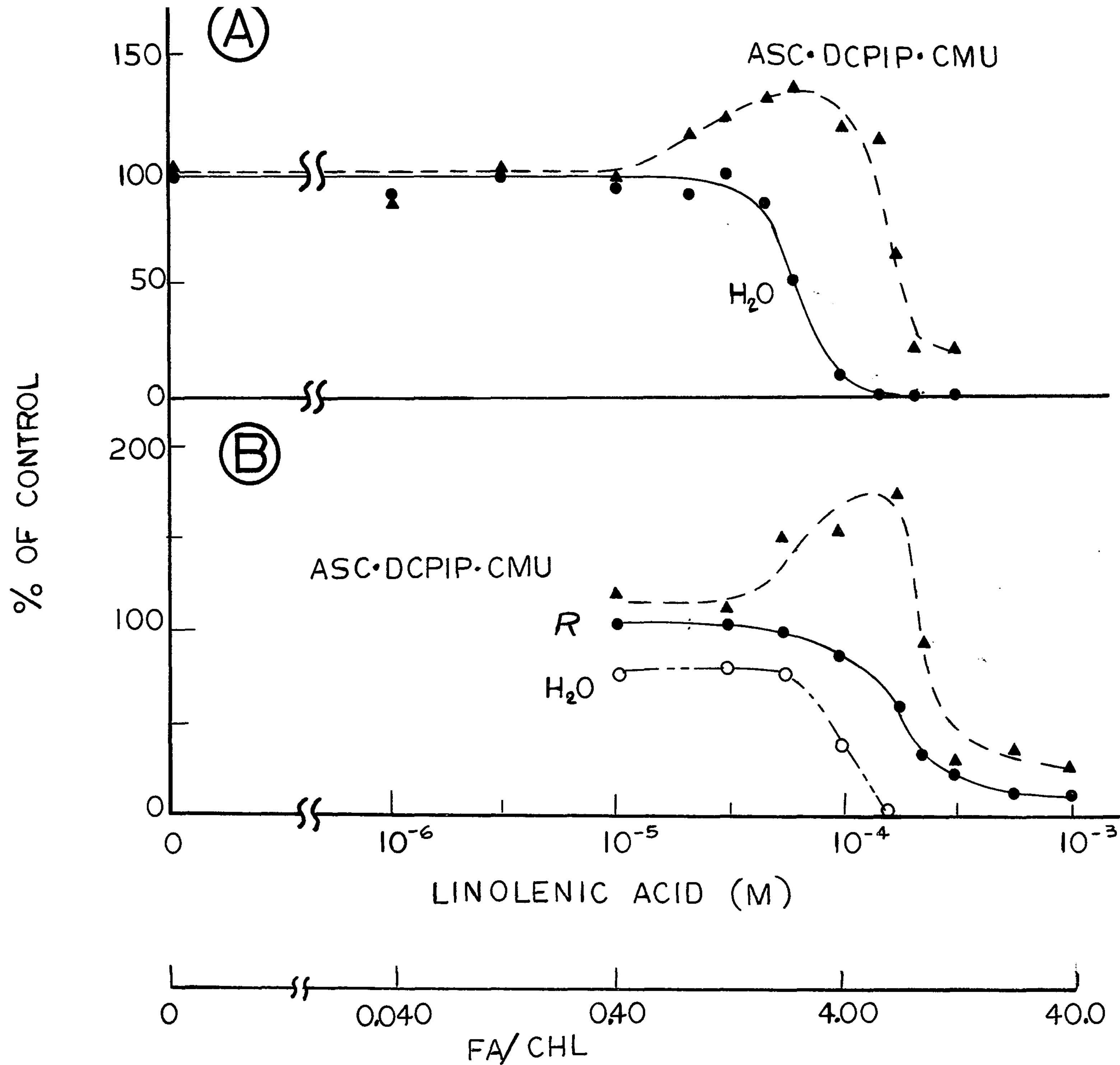
signal and noise levels.

(6) DIFFERENTIAL EFFECTS ON SYSTEM II AND SYSTEM I. Fig. 31A plots NADP reduction occurring either at the expense of electrons from water, or from reduced DCPIP. Inhibition of these reactions by linolenic acid is also shown. Inhibition occurs in the $H_2O \rightarrow$ NADP reaction (systems I and II) in the same FA/CHL range as in the ferricyanide Hill reaction (system II). (Recall Fig. 24, *i.e.*, the parallel situation in which ricinoleic acid was used.) The fatty acid-induced stimulation of electron flow which is noted when ferricyanide is used as Hill oxidant is not observed when NADP is used as oxidant; Izawa and Good (1968) have ascribed this latter lack of stimulation to the existence of rate-limiting steps in the transfer of electrons from ferredoxin to NADP.

Attempts to restore system II activity in linolenic acid-treated chloroplasts (in which system I was functional) using electron donors, *i.e.*, hydroquinone (in the presence of ascorbate) or semicarbazide, were unsuccessful. Yamashita and Butler (1968) had previously determined that these compounds supply electrons between water and photosystem II and could restore Hill activity to tris-aged or UV-irradiated chloroplasts in the present work.

Fig. 31A also shows the reaction of NADP, in which system II has been previously blocked by the addition of CMU (as determined by monitoring oxygen evolution), and an artificial electron couple (ascorbate-DCPIP) is made

Figure 31. Effect of linolenic acid on NADP reduction and <u>R</u>. Control rate of NADP reduction (μmoles/mg chl/hr): A, from H_2O=57, from ASC-DCPIP (CMU)=39; B, from H_2O=39, from ASC-DCPIP (CMU)=18. Control value of <u>R</u>=2.07. Incubation temperature, 23°C. Fluorescence measurements were made in the absence of electron acceptors or donors.



(A)
ASC·DCPIP·CMU
H2O
(B)
ASC·DCPIP·CMU
R
H2O
% OF CONTROL
150
100
50
0
200
100
0
0
10-6
10-5
10-4
10-3
LINOLENIC ACID (M)
0
0.040
0.40
4.00
40.0
FA/CHL

available (i.e., only system I is functional). If one refers back to the first curve of Fig. 31A, it may be seen that within the FA/CHL range in which system II is beginning to be inhibited, there occurs a stimulation of electron flow in system I. The FA/CHL which inhibits system I to the 50% level is $\sim$ 10, or about 3-4 times that which inhibits system II to the 50% level. Complete inhibition in the system I reaction was never noted. (The reasons for this are unclear at the present time; however, they may relate to the limit of solubility of fatty acids in aqueous solution, or, alternatively, they may indicate a basal NADP reduction which still occurs in the presence of fatty acids).

System I activity measured using methyl viologen as the electron acceptor also indicated a stimulation of electron flow at a FA/CHL $\sim$ 4-5. In this case there was complete inhibition of activity; this occurred at a FA/CHL $\sim$ 30.

In Fig. 31B is shown the results of an experiment in which concomitant measurements were made of NADP reduction (using water or the ascorbate-DCPIP couple) and steady-state fluorescence (-196°C) as a function of linolenic acid concentration. Note that the onset of the decline in R occurs at FA/CHL concentrations which are inhibitory to system II electron flow but stimulatory to system I flow.

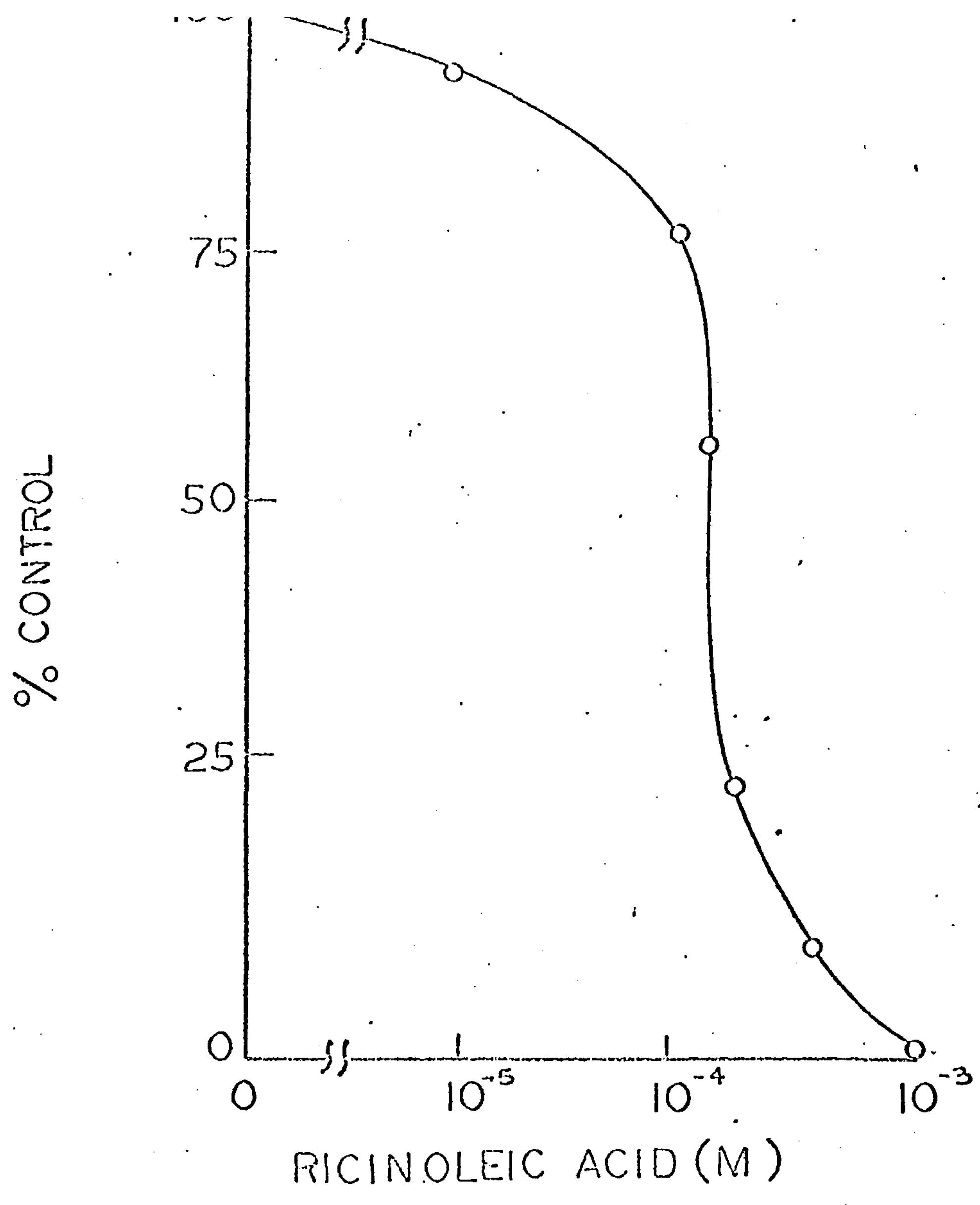
(7) EFFECTS ON CYCLIC PHOTOPHOSPHORYLATION. An examination of cyclic photophosphorylation (with PMS as co-factor) in the presence of ricinoleic acid indicates that phosphorylation is completely inhibited at FA/CHL concentrations which are stimulatory to system I electron flow (Fig. 32). The effects of ricinoleic acid on phosphorylation (cyclic) are in agreement with the earlier findings of McCarty and Jagendorf (1965), who used linolenic acid. While stimulation of electron flow in the non-cyclic case may be readily interpreted as resulting from uncoupling, the stimulation of flow in system I is not as easily interpreted. Perhaps this stimulation in system I is due to the removal of a rate-limiting step in the cyclic pathway.

VI. EFFECTS OF FATTY ACIDS AND RICINUS PROTEIN ON LIGHT-INDUCED CYTOCHROME CHANGES

To determine the site of action of fatty acids and the Ricinus protein in the photosynthetic electron transport chain, it was decided to use the sensitive technique of double beam spectrophotometry to examine the effects of these agents on light-induced absorbance changes in the cytochrome region of the spectrum.

Fig. 33 shows recorder tracings of light-induced cytochrome f absorbence changes in the presence and absence of 3 x 10^{-4}M linolenic acid and G-25 Ricinus protein. A stimulation of the on-response (oxidation) is noted in the case of either agent. Note that the off-response (reduction in the dark) is markedly accelerated in both. In addition,

Figure 32. Effect of ricinoleic acid on cyclic photophosphorylation with PMS as co-factor. The control rate of phosphorylation (μmoles P_i esterified/mg chl/hr) was 312. Incubation temperature, 24^oC.


% CONTROL
75
50
25
0
0
10^{-5}
10^{-4}
10^{-3}
RICINOLEIC ACID (M)

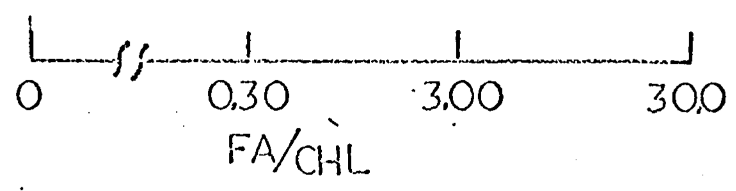
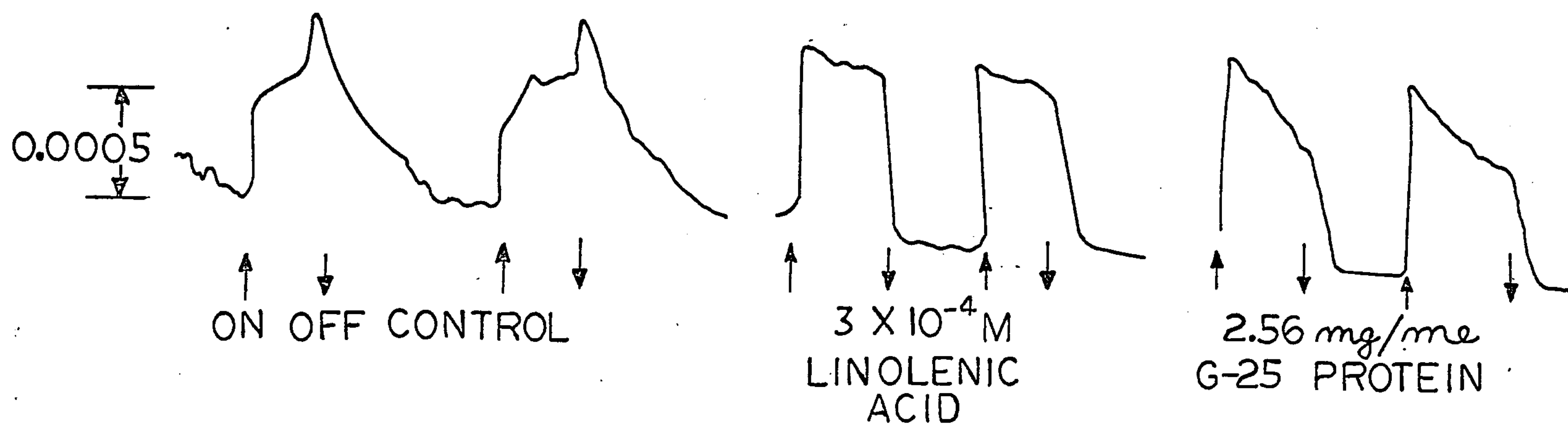

0
0.30
3.00
30.0
FA/CHL

Figure 33. The light-induced (700 nm) oxidation of cytochrome f in the presence of 3×10^{-4}M linolenic acid and 2.56 mg/ml of G-25 *Ricinus* protein. Upward arrows indicate actinic light on; downward arrows indicate actinic light off.

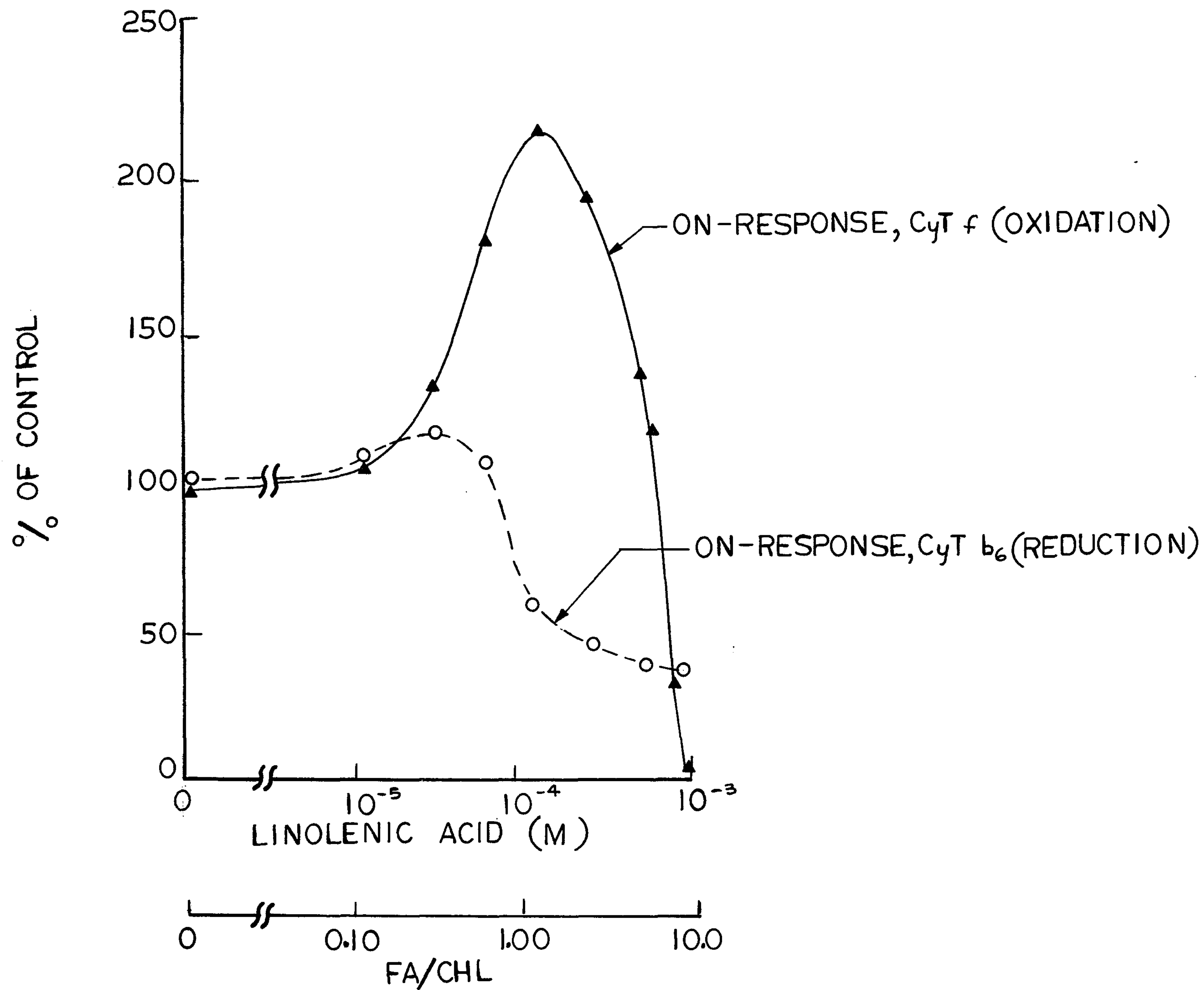

λ554 — λ575
Δ ABSORBANCE
0.0005
ON OFF CONTROL
3 X 10⁻⁴ M
LINOLENIC
ACID
2.56 mg/ml
G-25 PROTEIN
Time
30 Sec

note that in the control situation, when the actinic beam is turned off, there is a small, further, decrease in absorbance. Absorption changes were also monitored as a function of concentration of exogenous linolenic acid. This is shown in Fig. 34. Stimulation of cytochrome f oxidation is maximal at FA/CHL $\sim$ 1; at concentrations of FA/CHL $\sim$ 10 cytochrome f oxidation is completely inhibited. The on-response for cytochrome b_6 (reduction), in the presence of linolenic acid, indicates a small initial stimulation followed by a progressive inhibition. At 10^{-3}M, the on-response is observed to decrease to 35% of the control level (see Fig. 34). One would expect that when electrons are no longer flowing through cytochrome f, reduction of cytochrome b_6 (a system I reaction--Hind and Olson, 1966) would also be inhibited. No explanation can be offered for the differential inhibition of the system I cytochromes.

During the interval of time monitored ($\sim$10 minutes), treatment of chloroplasts with a concentrated preparation of _Ricinus_ protein (2.56 mg/ml) yielded only the stimulation phase of the cytochrome f oxidation response and a decrease of the cytochrome b_6 response to 50% of the control.

Treatment of chloroplasts with either fatty acids or _Ricinus_ protein strongly inhibited the absorption change at 518 nm (using system I or system II actinic light). These results are similar to those found by S. Brody _et al_ (1969) and M. Brody _et al_ (1970).

Figure 34. Effect of increasing linolenic acid concentration on cytochrome f oxidation and cytochrome b_6 reduction. The reference wavelength in both cases was 575 nm. Temperature=22°C.



250
200
150
100
50
0
% OF CONTROL
ON-RESPONSE, CyT f (OXIDATION)
ON-RESPONSE, CyT b6 (REDUCTION)
0
10^-5
10^-4
10^-3
LINOLENIC ACID (M)
0
0.10
1.00
10.0
FA/CHL

VII. ELECTRON MICROSCOPIC STUDIES OF RLE- AND FATTY ACID-TREATED CHLOROPLASTS

Incubation of chloroplasts in RLE for a period of time sufficient to produce a one-banded fluorescence spectrum results in morphological forms which scarcely resemble the original chloroplasts as shown in Fig. 35. In the treated chloroplasts the membranes are greatly twisted and myelin-like in appearance.

Since a similar effect on fine structure was seen with fatty acids, the sequence of events which led to the gross configurational changes were studied using the simpler model system.

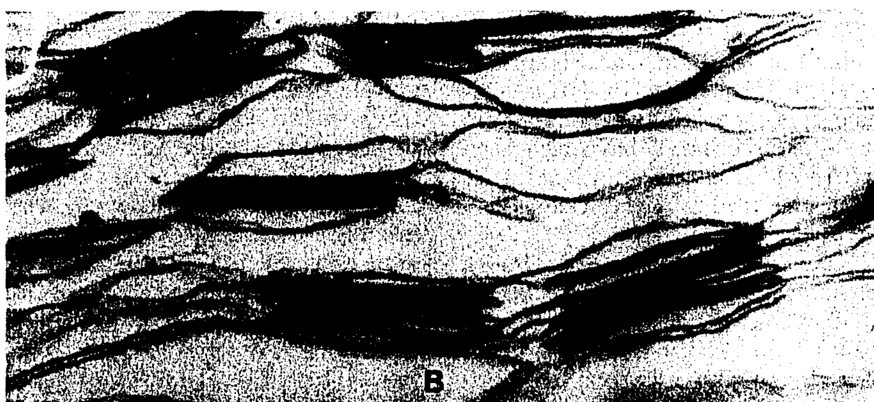
Investigations with whole spinach chloroplasts reveal that with increasing concentration of linolenic acid (i.e., in the FA/CHL range 0.001 to 0.5), intact chloroplasts progressively become naked lamellae, until at concentrations of FA/CHL $\gtrsim$ 1, no intact chloroplasts are observed. As these changes in the chloroplast envelope are occurring, the lamellar membranes are also being affected. Sequentially, the stromal (intergranal) membranes are the first to separate (FA/CHL 0.0001 to 0.01)--as in Fig. 36A; at higher concentrations (FA/CHL $\sim$ 0.1) the grana discs have become swollen--as in Fig. 36B; still at higher concentrations (FA/CHL $\gtrsim$ 1) there seems to be a separation of the fusion layers of the grana thylakoids--as in Fig. 36C. The myelin figures seen with RLE-treated chloroplasts appear in linolenic acid-treated chloroplasts at a FA/CHL $\gtrsim$ 1. The

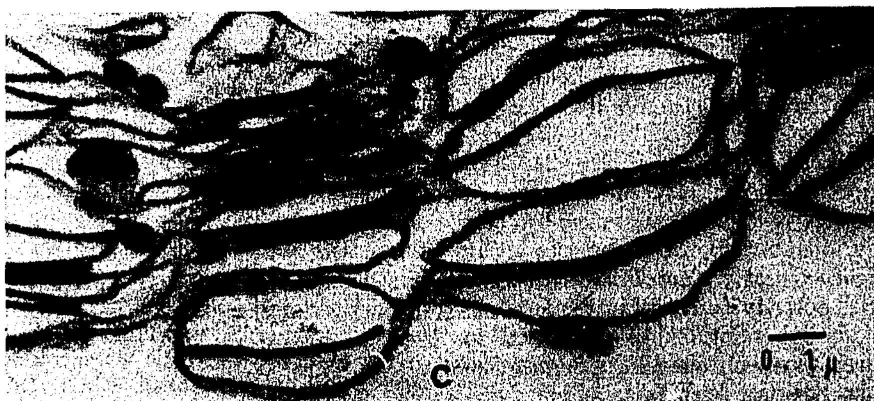
Figure 35. Electron micrographs of control and RLE-treated spinach chloroplasts. A, control; B, plus RLE for 60 minutes. x 36,000.

A

B
0.1μ

Figure 36. Electron micrographs of whole spinach chloroplasts incubated in linolenic acid. A, separation of stromal thylakoids (FA/CHL=0.001 to 0.01); B, swelling of granal thylakoids (FA/CHL∼0.1); C, separation of granal thylakoids (FA/CHL∼1). x 72,000.


B


C
0.1 μ

membrane changes associated with a particular concentration were typical of 65-70% of the microscopic fields examined.

For ease of comparison, the various parameters described above, which were measured as functions of FA/CHL (for linolenic acid) have been summarized in Table IV.

VIII. ENZYMATIC ACTIVITY OF RICINUS PROTEIN FRACTIONS.

A. Lipase Activity.

To determine if the Ricinus chloroplast protein induced changes in fluorescence emission and electron transport were associated with the release of fatty acids, the following experiments were performed. If subchloroplast particles, prepared from spinach, are incubated at 30°C for one hour with 0.9 mg/ml of Ricinus chloroplast protein, the amount of fatty acid released is very small < 2 nmoles/0.5 ml or $< 10^{-6}$M final concentration.* Under similar conditions the ferricyanide Hill reaction was inhibited to 80% of the control value in 10 minutes. If Ricinus protein fractions (RLE, G-25 protein, or the purified chloroplast protein) are incubated with purified lipids of the type predominantly found in the chloroplast membrane (Benson, 1963), i.e., galactolipid, phospholipid, or sulfolipid, one cannot detect lipase activity, either as release of free fatty acid, or, in the case of radioactive sulfolipid, as release of ^{35}S. One can also say that the Ricinus

*Under similar conditions, the (.025 mg/ml) purified galactolipase from Phaseolus liberated 50 nmoles of fatty acid from subchloroplast particles.

TABLE IV

THE EFFECT OF LINOLENIC ACID ON VARIOUS CHLOROPLAST PARAMETERS[a]

FA/CHL	R	DYE REDUCTION System II FeCN	DYE REDUCTION System II NADP	DYE REDUCTION System I NADP	DYE REDUCTION System I MV	CYTOCHROME f OXIDATION	CYTOCHROME b_6 REDUCTION
2-3	100	50	50	150	250	180	50
10	50	0	0	50	180	0	35

[a]Data are expressed as per cent of control.

protein fractions contain negligible amounts of fatty acids, since assays of these fractions for free fatty acids were negative.

B. Protease Activity.

In addition it also appears that the protein fractions have no detectable protease activity, since results of the Congo Red Hide Powder assay were negative. Under similar incubation conditions, *i.e.*, temp., protein content, etc., treatment of the substrate with trypsin resulted in an increase in O.D. (550 nm) of .426 in 10 minutes, whereas treatment with *Ricinus* chloroplast protein resulted in an increase in O.D. (550 nm) of only .01 after 90 minutes.

IX. EFFECTS OF OTHER CHEMICAL AND PHYSICAL TREATMENTS ON FLUORESCENCE EMISSION (-196°C)

A. Fatty Acid Derivatives.

The changes which occur in the fluorescence emission of fatty acid-treated chloroplasts are also observed to occur upon incubation of chloroplasts or subchloroplast particles in fatty alcohols or in methyl esters of fatty acids. A comparison of the rate of change of *R*, in chloroplasts treated with equimolar concentrations of linolenic acid, or the methyl ester of linolenic acid, or linolenoyl alcohol, indicates that the order of potency is acid alcohol methyl ester. In the case of ricinoleic acid the free fatty acid and the methyl ester appear to be equally potent. Phytol (fatty alcohol tail) of chlorophyll also brings about the fluorescence changes.

B. Detergents.

Incubation of chloroplasts (for periods up to one hour) with detergents in the concentration range generally used to inhibit Hill activity (Okayama, 1967; Vernon and Shaw, 1965), e.g., 0.004% SDS or 0.02% Triton X-100 leads to low-temperature fluorescence changes similar to those produced by trypsin or pronase (*i.e.*, decrease in fluorescence yield at 685 nm and 698 nm). Incubation at much higher detergent concentrations--0.1% SDS, 0.1% SDBS, 0.1% Triton X-100 or 0.5% digitonin (in the concentration range normally used to fractionate chloroplasts)--results in temporally-ordered spectral changes similar to those produced by *Ricinus* chloroplast protein or fatty acids. (The effect of high detergent concentrations does not appear to be a combination of a low detergent effect and then a reversal of this phenomenon.) The "one-banded" spectrum produced by the detergents had the following peak maxima: 688 nm for SDS; 693 nm for SDBS; 683 nm for Triton; 685 nm for digitonin.

Similar maxima were observed in the present work with the 144,000 xg supernatant of digitonin-fractionated chloroplasts (and also by Ke and Vernon, 1967, for the 144,000 xg supernatant of Triton-fractionated chloroplasts). This material probably represents detergent-chlorophyll-protein micelles; the temporal parameter involved in the production of these maxima may be indicative of a progressive conversion of the chlorophyll-protein complexes

(of the lamellar membranes) into detergent micelles.

One very important distinction between treatment of chloroplasts with detergents and with *Ricinus* protein or linolenic acid is that the latter substances do not disrupt lamellar membranes; this conclusion is based on the observation that centrifugation of chloroplasts treated in the latter fashion (10 minutes at 30,000 xg) resulted in a colorless supernatant and a green pellet (*i.e.*, no components have been released).

C. Electron Transport Inhibitors.

(1) CMU, DCMU. Anderson and Thorne (1968) have reported that addition of 10^{-5}M DCMU to spinach chloroplasts results in an increase in *R* from 2.3 (control) to 2.9. It is not clear from their paper whether the organic solvent used to dissolve the DCMU was added to the control chloroplasts. Their experiments were repeated in the present work using several concentrations of DCMU and CMU with the precaution taken of adding the organic solvent (absolute ethanol or ethanol-ethylene glycol, v/v) to the control. This study revealed that if the organic solvent is added at 1% final concentration, there seems to be no effect of DCMU (Table V). But if the solvent concentration is 5 or 10%, there appears to be both a solvent effect (lowering of *R* in the control) and a DCMU effect (apparent nullification of the solvent effect). In any event, it is of interest to note that treatment with DCMU or CMU (up to 10^{-3}M) results in an increase in *R*, rather than a decrease.

TABLE V

THE EFFECTS OF DCMU ON STEADY-STATE FLUORESCENCE (-196°C)[a]

DCMU CONCENTRATION (M)	FINAL ORGANIC SOLVENT[b] CONCENTRATION (%)	R
0	1	2.06
	5	1.62
	10	1.69
10^{-4}	1	2.08
	5	2.11
	10	2.07

[a]Experiments with broken spinach chloroplasts.

[b]Organic solvent used was ethanol-ethylene glycol (v/v) in 13 mM Tricine-NaOH (pH 8) + 3.3 mM $MgCl_2$.

(2) PHENOL. Neumann and Drechsler (1967) have reported that phenol at high concentrations (20-50 mM) blocks Hill activity and weakly inhibits system I activity. Incubation of chloroplasts in 50 mM phenol, for periods up to 90 minutes, resulted in fluorescence changes similar to those induced by fatty acids; phenol unlike detergents does not disrupt chloroplast membranes. In contrast to the fatty acid case, $\underline{R}$ never fell below 0.70.

(3) CCCP AND ICI 47776. Gregory (1969) has reported that CCCP (like fatty acids) sequentially inhibits system II and I as a function of increasing concentration. The same is true for ICI 47776 (Gregory, personal communication). Incubation of spinach chloroplasts at concentrations which inhibit both photosystems (i.e., 1.6×10^{-4}M for ICI 47776 or 6.65×10^{-4}M for CCCP) for up to 120 minutes did not result in any effect on steady-state emission.

D. UV Irradiation.

Preliminary experiments indicate that irradiation with ultraviolet light also results in changes in steady-state fluorescence similar to those produced by fatty acids. With chloroplasts frozen immediately (to -196°C) after 30 minutes of irradiation $\underline{R}$ was found to decrease from 1.57 (control) to 0.62. In addition, it was observed that 20 minutes of illumination with white light (filtered through 5 cm of water) following 10 minutes of UV irradiation (which reduced $\underline{R}$ from 1.57 to 1.11) did not reverse the spectral change. An identical sample kept in the

dark for the 20 minute post-illumination period did not show any further spectral change.

E. Ageing.

Ageing of spinach chloroplasts, which leads to a progressive loss of photochemical activity, has been attributed by McCarty and Jagendorf (1965) and Constantopolous and Kenyon (1968) to increasing release of endogenous fatty acids. (However, Wasserman and Fleischer, 1968, could not find any increase in the free fatty acid level in aged chloroplasts.) When spinach chloroplasts were aged according to the procedure of McCarty and Jagendorf, *i.e.*, in unbuffered 0.35M NaCl at 20°C for 2 1/2 hours or at 0°C for 24 hours, no changes in emission were found to occur. Again, negative results were found for chloroplasts incubated at 37°C for two hours in 6.7 mM KCl + 20 mM Tris-HCl, pH 8.0, according to the method of Constantopolous and Kenyon; therefore, if fatty acids are being released, perhaps their final concentration is too low to affect the pigment-pigment interaction, although it may be high enough to affect electron transport.

DISCUSSION

I. THE ACTION OF FATTY ACIDS ON CHLOROPLASTS

A. Differential Effect on the Two Photosystems.

In the present investigation the sequential inhibition of system II- and system I-associated electron flow has been demonstrated as a function of increasing concentration of exogenous linolenic acid. Differential inhibition of the two photosystems by linolenic acid had been implied earlier by Katoh and San Pietro (1968), although it was not explicitly demonstrated (see their paper, Fig. 6). A similar sequential inhibitory pattern was observed with linolenic acid by S. Brody *et al* (1969), who used light-induced absorption changes to monitor the activities of chlorophylls a_{II} and a_{I}. This differential effect of fatty acids on the two systems is interesting, since it had been previously observed for only a limited number of compounds, e.g., Triton X-100 (Vernon and Shaw, 1965), CCCP (Gregory, 1969), and ICI 47776 (Gregory, personal communication).

(1) SYSTEM II. The molar ratio (number of molecules of linolenic acid per chlorophyll) required to inhibit Hill activity to the 50% level with ferricyanide and NADP was found to be 2.25 in the present work. About the same magnitude of FA/CHL has been reported for inhibition of other system II-associated phenomena, *i.e.*, FA/CHL=3 to attenuate variable fluorescence to 50% of the control value (M. Brody,

1969); FA/CHL=1.6, to attenuate the light-induced absorption change (fast component at 690 nm) of chlorophyll a_{II} to 50% of the control level (S. Brody _et al_, 1969). The latter was found with both chloroplasts and purified (Boardman and Anderson) system II particles.

The effects of fatty acids on delayed light emission--in the millisecond range--appear to be different from the effects of other system II inhibitors. a) The additional stimulation which ferricyanide elicits in the presence of 2.5×10^{-5}M linolenic acid is not observed, for example, when DCMU is used as the inhibitor [at any concentration] (Bertsch, personal communication). b) Also, the acceleration of the dark decay observed with fatty acid is not seen with DCMU. One similarity that was observed with both is that linolenic acid and DCMU substantially reduce the intensity of delayed light prior to any significant inhibition of Hill activity.

S. Brody _et al_ (1969) have also noted differences in the effects of DCMU and linolenic acid on light-induced absorption changes of chlorophyll a_{II}; they found that if the fast component ($t_{\frac{1}{2}}=2 \times 10^{-4}$ sec) of this absorption change is inhibited, using DCMU, it cannot be regenerated by the addition of ferricyanide--whereas, if inhibition is effected using linolenic acid, ferricyanide is partially active in restoration ($\sim$50%).

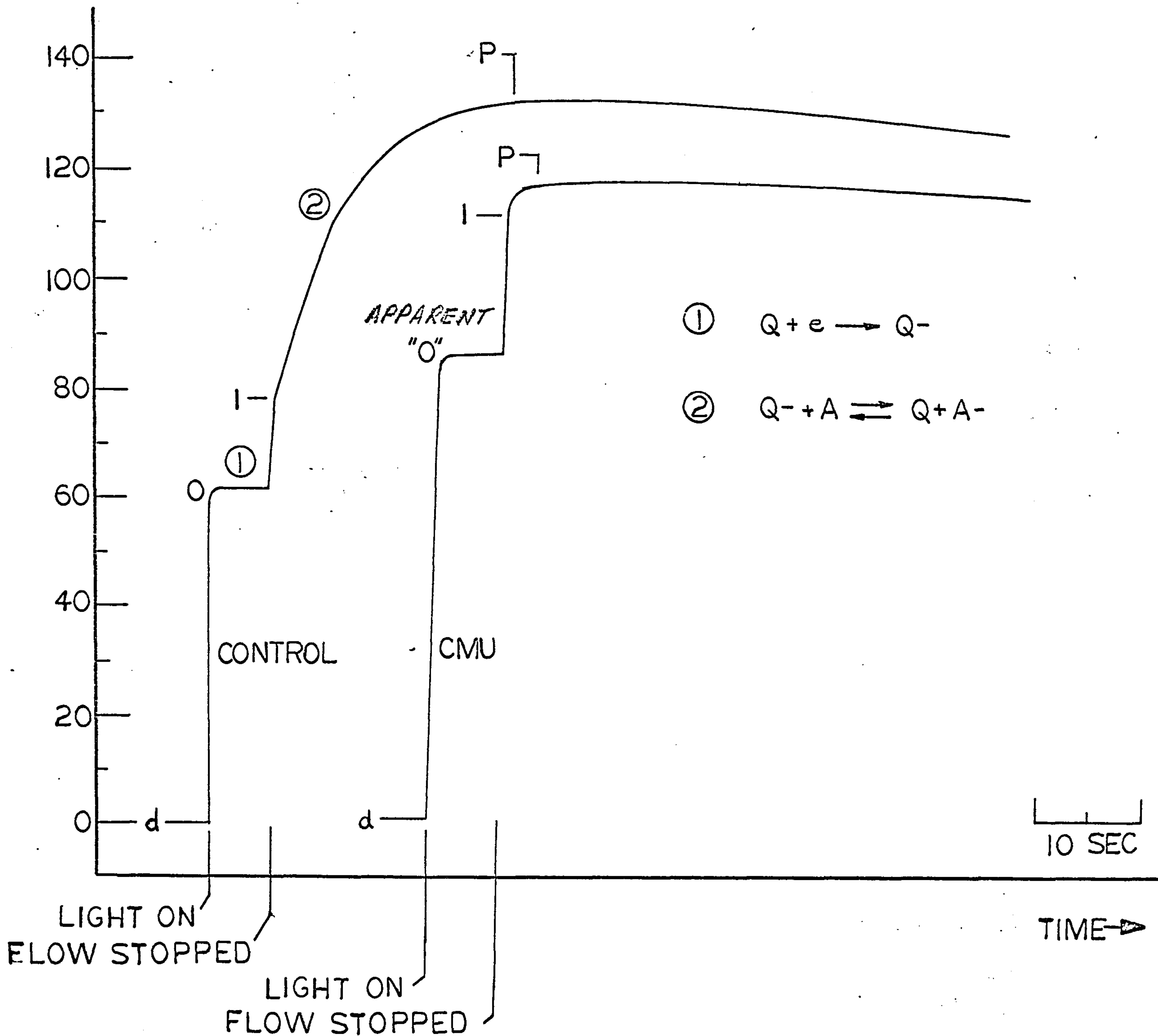
M. Brody (1969) has observed that RLE has dual effects on the fluorescence induction of chloroplasts; those effects that were observed after five minutes of incubation in RLE were fast effects--those observed after 90 minutes were designated as slow effects. The most striking effect of the former was a

speeding of the O I (photochemical) transition (eq. 1); this same phenomenon is noted upon addition of CMU (or DCMU), CMU additionally blocks the slow rise I P (photochemical and non-photochemical, eqs. 1 and 2) as in Fig. 37. The effect of CMU has been interpreted as a block in the re-oxidation of Q by A. The CMU site may be the site at which the fast acting component of RLE acts or the block may be closer to system I.

The slow-acting component (protein) of RLE affects fluorescence induction in about the same way as do fatty acids. They both lead to a diminution of fluorescence yield, particularly the O→P rise. There seems to also be a retardation in the I→P transition. It may be concluded that the slow-acting component of RLE and fatty acids act on system II in some way to modify the amount of Q^- and Q in a manner different from CMU.

In 1968, Katoh and San Pietro proposed that fatty acid-induced inhibition of the Hill reaction results from "destruction" of system II. They based their interpretation on the observation that high concentrations ($> 10^{-2}$M) of ascorbate (which donates electrons between H_2O and photoact II--according to Trebst _et al_, 1963) could not regenerate Hill activity in chloroplasts inhibited by fatty acids, although it could, in acid-incubated or aged or heat-treated chloroplasts. In the present work, attempts were made to restore system II activity using other compounds which donate electrons between H_2O and photoreaction II, e.g. hydroquinone (in the presence of catalytic amounts of ascorbate to keep the hydroquinone reduced) or semicarbazide; these attempts were unsuccessful. Neither ascorbate or the other donors can restore Hill activity in DCMU-treated chloroplasts.

Figure 37. Fluorescence induction pattern of control and CMU-treated *Zea mays* chloroplasts. Redrawn from M. Brody (1969). The redox equations used to represent the various portions of the induction curve are taken from Lavorel and Levine (1968).


FLUORESCENCE INTENSITY (RELATIVE UNITS)
140
120
100
80
60
40
20
0
d
d
O
I
P
P
I
①
②
APPARENT
"O"
CONTROL
CMU
① Q + e → Q-
② Q- + A ⇌ Q + A-
10 SEC
TIME
LIGHT ON
FLOW STOPPED
LIGHT ON
FLOW STOPPED

The questions that one would like to ask about the effects of fatty acids on system II are:

1) are electrons not being delivered to photoreaction II, i.e., do fatty acids produce a block between water and reaction center II?

2) are electrons being delivered to the reaction center but not being utilized for normal photosynthetic pathways (i.e., not resulting in O_2 evolution), and, if so, what are the possible reasons?

In answer to question 1) the data on the use of artificial electron donors may be called upon. These data (as well as those of Katoh and San Pietro) seem to imply if fatty acids are acting between water and reaction center II (see Fig. 1) they must be acting very close to the reaction center, since electron donation by these donors is not possible.

If it is assumed that electrons can get to the reaction center in fatty acid-treated chloroplasts, the question then becomes--what is preventing normal photosynthesis from occurring? One possibility is an actual diminution in the number of reaction centers; such an interpretation has been made by S. Brody (1969) from his amperometric studies with linolenic acid-treated chloroplasts. Also the data from fluorescence induction studies which indicate that increasing concentrations of linolenic acid prevent the rise of variable fluorescence may be used to support the idea that reaction centers are being destroyed; although an alternate hypothesis from this data is that electrons are leaving the intact reaction center, but not getting to Q.

If the electrons are leaving the reaction center, are they by-passing Q--is a new (non-photosynthetic) pathway being established? Two pieces of data support this suggestion. In the experiments on delayed light, it was observed that ferricyanide was able to elicit an additional stimulation of emission and an additional acceleration of the dark decay without an enhancement of oxygen evolution. Perhaps this is indicative of ferricyanide being able to directly accept the electrons which by-pass Q. The data on the light-induced absorption changes of chlorophyll a_{II} in fatty acid-treated chloroplasts also support the idea of an alternate pathway, since introduction of ferricyanide is able to partially restore the absorbance change. While oxygen evolution was not monitored concomitantly with the light-induced absorption changes, absence of stimulation of O_2 evolution in the delayed light studies suggests that the flow of electrons to ferricyanide is cyclic in nature* (the reoxidation of ferricyanide being accomplished by an internal pool of oxidant).

It is of interest to note that in the case of DCMU--in contrast to fatty acids--ferricyanide can "restore" neither the delayed light or the chlorophyll a_{II} absorption change. Again this is consistent with the site of DCMU action being on the system I side of the system II reaction center (see Fig. 1). While the present data do not permit the definitive localization of the action of fatty acids in system II (with respect to DCMU's site), they at least

*Malken and Jones (1966) have also suggested an internal cyclic pathway which diverts electrons from Q, in the case of UV-photoinhibited chloroplasts.

permit the narrowing of the options to two likely ones: a) between the system II reaction center and Q or b) between Q and the DCMU site.

While it is impossible to choose between the two options, the data from the fluorescence induction studies of M. Brody (1969) suggest that there is a greater likelihood of a) obtaining over b). The data indicated that with increasing concentration of fatty acid, variable fluorescence decreased markedly, *i.e.*, there is less and less Q (quencher) in the Q^- (non-quencher) form. This is what is to be expected if fatty acids are detouring electrons away from the reaction center via a cyclic pathway. The reason that b) is less likely is that if electrons do get to Q and are blocked after Q by the fatty acids, most of Q would be in the Q^- form, which would lead to a high yield of variable fluorescence.

(2) SYSTEM I. Although we know less about the action of fatty acids on system I than on system II, some conclusions may be drawn on the basis of the data collected in the present work. As seen above, a strong stimulation* of system I electron flow (with ascorbate-DCPIP as the donor to NADP or methyl viologen) was observed in linolenic acid-

*Electron flow, observed in non-cyclic systems in which phosphorylation is occurring, is stimulated when such flow is uncoupled from phosphorylation, which usually involves a rate-limiting step. It is possible, of course, that a stimulation of electron flow even in cyclic systems may be observed if other rate-limiting steps (not necessarily phosphorylation) are "uncoupled." The same would apply to pseudo-cyclic electron flow (which obtains, for example, when the added electron acceptor is re-oxidized by evolved oxygen, and there is no observable accumulation of reduced acceptor).

treated chloroplasts. With respect to chlorophyll concentration, stimulation was initiated in the FA/CHL range of 1-2, was maximal at 4-5 (at which concentration, both Hill activity and cyclic photophosphorylation are virtually abolished), and was no longer apparent, i.e., crossed the control value, at FA/CHL $\sim$ 8. The degree of stimulation with MV, i.e., 3-fold by fatty acids, is worthy of note, since it is about 2 times that observed with NH_4^+ ion (the most frequently used agent to uncouple non-cyclic electron flow); however, it is less than the 6-fold stimulation observed with methylamine (Izawa et al, 1966). The stimulatory effect of methylamine on system I electron flow is apparently not related to its uncoupling action on non-cyclic electron flow; this may also be true for fatty acid-treated chloroplasts.

The same sort of stimulation of system I electron flow was observed in experiments on cytochrome f oxidation and by S. and M. Brody (personal communication) in experiments on light-induced absorption changes of chlorophyll a_1. In the case of cytochrome f oxidation stimulation was initiated at a linolenic acid to chlorophyll concentration of 0.5, was maximal at 1, and stimulation was no longer apparent at 8. In the case of the light-induced chlorophyll absorption changes (as observed by the Brodys), stimulation was not systematically studied as a function of fatty acid concentration; it was initiated at a FA/CHL of 0.5 and no longer apparent at a FA/CHL of 1-2.

The stimulation observed in the cytochrome f studies may be due to the removal of rate-limiting steps concerned with phosphorylation (cyclic or pseudocyclic)--see footnote p. 65. Since this stimulation is an increased extent of oxidation, one is tempted to locate cytochrome f on the reducing side of the rate-limiting step in the pathway. Unfortunately, all other data (see Fig. 1) point to cytochrome f being on the oxidizing side of the phosphorylation site. How then can the present data be otherwise explained? One explanation consistent with other observations made in the present work (see section on electron microscopy below) is that the stimulation arises because certain components of the electron transport chain are transiently brought into closer proximity by conformational or configurational changes in the lamellar membranes. A similar explanation was offered by Hind (1968) to explain increased extent of cytochrome b_{559} oxidation in Triton-treated (and therefore structurally disrupted) chloroplasts.

B. Effect on the 515 nm Absorbance Change.

The light-induced absorbance change having a maximum in the 515 to 520 nm region of the spectrum, was first discovered by Duysens (1954). That this absorbance change can be sensitized by light of system I or system II was shown by Muller *et al* (1963), Rubinstein and Rabinowitch (1964), Kok *et al* (1963), and Chua and Levine (1969). Witt and co-workers (Junge and Witt, 1968) have associated this change with an electric field across the thylakoid membrane

which promotes phosphorylation-coupled ion (specifically proton) flow. As noted above, both linolenic acid and the *Ricinus* chloroplast protein attenuate the 518 nm change (using system I or system II light). Since other treatments which affect the integrity of lamellar membranes, e.g., osmotic shock or incubation with gramicidin (Junge and Witt, 1968), treatment with detergents (Hind, personal communication) or sonication (Chua and Levine, 1969), greatly inhibit or completely abolish the absorbance change, it is presumed that fatty acids and the *Ricinus* protein have somewhat similar effects on membrane integrity (permeability?). This idea is further supported by the finding that low concentrations of fatty acids inhibit the light-induced pH rise in chloroplasts--which is dependent on membrane integrity (Jagendorf and Neumann, 1965).

C. Effects on Chloroplast Ultrastructure.

Molotkovsky and Zheskova (1966) ascribed the decrease in light-scattering of chloroplasts induced by fatty acids to swelling. Izawa and Good (1966) further correlated the (light-induced) decrease in light-scattering observed with chloroplasts with an alteration of ultrastructure. Configurational changes in thylakoid membranes similar to those of the present work (at intermediate FA/CHL concentrations) were earlier shown to occur in light-induced swelling of chloroplasts [in the absence of an electron acceptor], in chloroplasts incubated in a medium containing 10-20% acetone (Murakami and Nobel, 1967), and in methylamine-uncoupled chloroplasts [illuminated in the presence of ferricyanide] (Izawa and Good, 1966). With these other substances, even in the case where the outer envelope was

intact, treatment did not result in stripping off of the envelope as it does in the case of fatty acids. Perhaps to some extent the degree of swelling is greater in fatty acid treatment, because the stripping off of the outer membrane lessens constraints.

Further treatment of chloroplasts with high concentrations of fatty acids (or RLE) results in the production of myelin-like figures reminiscent of those which result from treatment of (naked) chloroplasts for 30 minutes in the dark in a medium containing 10-20% acetone (Murakami and Nobel, 1967). These figures are most likely due to rupture and recombination of membranes not ordinarily found *in situ*. Although in the present work no data are available, the following *ad hoc* assumption can be made about the role of the fatty acids in producing the swelling changes. Membrane permeability is enhanced (this increased permeability is probably the cause of the attenuation of the 518 nm absorbance change as noted above) so that ions normally kept out of the intradisc space of the grana flow inward with subsequent diffusional flow of water into the discs. It is probably safe to assume, also, that there is stretching in lipid-soluble areas of the membrane, thus allowing greater flexibility of the membrane; this may also be true in the case of acetone-induced swelling.

D. Relative Effectiveness of Closely Related Compounds.

The results of the experiments with fatty acid analogues

indicates that the methyl ester of linolenic acid is rather feeble at producing the spectral changes, when compared to the free fatty acid. In addition, the observation was made that in the case of ricinoleic acid, the free fatty acid and the methyl ester were equally potent. These results may be indicative of the requirement that, for a molecule to interact with the pigment, it must be amphiphilic, i.e., have a hydrophilic moiety and lipophilic moiety. On the basis of this consideration, the methyl ester of ricinoleic acid should be an effective molecule, even though its carboxyl group is blocked with a methyl--it has a hydroxyl on the "12" carbon (see Fig. 19). This explanation is also a tenable one for the fatty alcohols, since they have a hydroxyl in place of the carboxyl of the free fatty acid. The effectiveness of the unsaturated fatty acids is greater than the saturated ones; the reason for this is not known, but may be related to the greater degree of partition of unsaturated compounds versus saturated ones in lipid solvents (the membrane).

E. Effects on the Pigment System.

One would like to be able to answer the question as to what is the underlying mechanism(s) for the steady-state fluorescence changes and associated phenomena. Since configurational changes occur in fatty acid-treated chloroplasts (of the type observed with the electron microscope), it is possible that swelling (stretching) of the membrane

is pulling apart aggregates of chlorophyll *a*. Since the same fluorescence changes were observed upon addition of fatty acids to sublamellar fractions, *i.e.*, Boardman and Anderson system I particles and a chlorophyll-protein complex of the system I type (B. Nathanson, personal communication), it would seem at a finer level of organization that conformational changes are also occurring, which result in de-aggregation.* S. and M. Brody (1961) suggested earlier that conformational changes may be responsible for the de-aggregation of chlorophyll. Finally, that there are direct effects on the chromophores themselves (as well as indirect effects of the fatty acids on the proteins) was observed in experiments in which fatty acids de-aggregated chlorophyll *a* in solution (CCl_4). Trosper and Sauer (1968) have reported that chloroplast lipids, e.g. galactolipids and sulfolipids, can de-aggregate chlorophyll *a* in carbon tetrachloride by competing for chlorophyll-chlorophyll interactions; apparently the chlorophyll-lipid interactions are more stable thermodynamically. It is possible, therefore, that free fatty acids may affect the chlorophyll-chlorophyll interactions in a similar fashion.

*Later spectral experiments by B. Nathanson, who collaborated with the author on some of the earlier aspects of the work reported here, have revealed that the steady-state fluorescence changes have their foundations largely in de-aggregation; her conclusions were based on concurrent determinations of (room temperature and low temperature) fluorescence emission, fluorescence excitation, absorption, and circular dichroism as a function of exogenous fatty acid concentration for chlorophyll *in vivo* and *in vitro*. (Nathanson and M. Brody, 1969)

II. MODEL SYSTEMS

S. Brody *et al* (1966) and Okayama (1967) have previously shown that various substances, e.g. salts, urea, enzymes, detergents, etc., have pronounced effects on the fluorescence emmision spectrum of chloroplasts. However, the studies of S. Brody *et al* were conducted using chloroplast fragments of *Euglena gracilis*, an organism in which F698 is very weak in relation to F735 (-196°C). The investigations of Okayama (1967), in turn, were concerned only with changes in fluorescence yield (he monitored the intensity of the 685 nm fluorescence band--the predominant one at room temperature). In the present study many of the same substances used by S. Brody and Okayama were utilized; in addition, other treatments which affect photoreactions, e.g. UV irradiation, were employed. The present investigation was carried out, in large part, with material cooled to -196°C; it has the additional advantage of employing spinach chloroplasts in which emission at 698 nm is much more intense relative to F735 (-196°C), than it is in *Euglena*.

A. Electron Transport Inhibitors.

With the exceptions of the electron transport inhibitors CCCP and ICI 47776 (which do not change *R*), all of the substances or treatments employed in this investigation result in solubilization, degradation or swelling of chloroplast membranes. On the bases of these two exceptions, it would seem that substances capable of blocking electron transport, but incapable of modifying the "state" of the

membranes, have little influence on pigment-pigment interactions.

DCMU does not fit into either one of the above categories, since it does affect fluorescence emission (elicits an increase in *R*), but does not appear to affect membranes. Although one may offer the plausible explanation--that the observed increase in *R* in the presence of DCMU results from an inhibition of electron flow in system I (the block in chemistry leading to an increase in fluorescence of system I chlorophylls)--this is contrary to experimental finding. Specifically, Izawa (1967) has shown that electron transport driven by system I is completely resistant to concentrations of DCMU (0.1-1 mM), two or three orders of magnitude greater than those which result in complete inhibition of system II.

B. Detergents.

In the present study, it was demonstrated that treatment of chloroplasts with low concentrations of detergents ($\lesssim$ .005-.01%), *i.e.*, concentrations which are used to inhibit Hill activity, results in a decrease of fluorescence intensity at 685 and 698 nm. In contrast, treatment with high concentrations of detergent ($\gtrsim$ 0.1%), *i.e.*, concentrations generally used to fractionate chloroplasts, leads to time-dependent decreases in fluorescence at 735 nm and increase in fluorescence at 685-695 nm. Okayama (1967), whose studies with detergent were limited to SDS, had observed similar concentration-dependent effects on fluorescence yield (at 685 nm) after 60 minutes of incubation.

Effects on fluorescence at high detergent concentrations are perhaps more readily explained than those at low

concentrations. In this respect, Bril (1964) noted that incubation of <u>Rhodopseudomonas</u> <u>spheroides</u> chromatophores in 0.1% Triton or SDS results in decrease in fluorescence of the band with maximum at 895 and increase in the band with maximum at 865 nm. These changes were interpreted by Bril as indicating a spatial separation ("splitting off") of the bacteriochlorophyll form (B890) that emits at 895 nm (presumably the reaction centers), from chromatophores, but leaving attached the bulk form of bacteriochlorophyll (B850) that emits at 865 nm.

In the case of higher plant chloroplasts treated with detergents (as observed in the present work), the decrease in 735 nm emission may be associated with a splitting off of the system I pigment complex (system I is known to be more readily dissociated from the chloroplast lamellae by the action of detergents than is system II; Boardman, 1968) leaving behind a membrane containing system II components. Consequently, the short wavelength "one-banded" spectrum which results from detergent action, may represent in large part increased fluorescence emission from system II components that can no longer transfer their energy to system I.* Such an explanation was offered by S. Brody <u>et al</u> (1965) for the 150,000 xg supernatant of sonicated chloroplasts.

*Since absorption by aggregated chlorophyll in the chloroplast is not readily observable, it is likely that the concentration of aggregates is much lower than that of monomer; the intensity of fluorescence from the aggregates in such chloroplasts must therefore result from a highly efficient transfer of energy from system II (S. Brody <u>et al</u>, 1965).

In the case of the lower concentration of detergent, the microenvironment of system II chlorophylls might be modified, but not to a degree sufficient to disperse the chlorophyll-protein complexes; such modification could bring about the observed decrease in short wavelength fluorescence.

C. Enzymes.

It was also noted that low concentrations of proteolytic enzymes (pronase and trypsin) are similar in action to low concentrations of detergent; they both lead to decreases in fluorescence at 685 and 698 nm. Okayama (1967), see above, also reported a decrease in the fluorescence of short wavelength forms of chlorophyll a in (the same concentration range of) trypsin-treated chloroplasts. In 1968, Michel-Wolwertz reported that treatment of *Chlorella* chloroplast fragments with low concentrations of trypsin or pronase (1-5 µg/µg of chlorophyll) for one hour, at room temperature, results in a preferential destruction of C_a680. If absorption at 680 nm is divided between monomeric and aggregated forms* or if there are two forms of C_a680 (Brown, 1968) it may be that at low concentrations of enzyme, the portion of C_a680 which does not give rise to F720 or F735 is being destroyed first. This would

*In 1963, S. and M. Brody ascribed both specific absorption and fluorescence maxima to numbers of chlorophyll molecules, e.g. one molecule (monomer) to C_a676 and F687, two molecules (dimer) to C_a705 and F719, etc.; from extrapolation it may be seen that absorption at 680 nm is by two forms--the monomer and the dimer.

explain the observation, in the present work, that at low concentrations fluorescence at 685 nm decreases while absorption at 680 nm also decreases. It is interesting to note that at high concentrations of enzyme (one-two orders of magnitude or greater) there is a small increase in short wavelength fluorescence and a larger decrease in long wavelength fluorescence (S. Brody *et al*, 1966). Thus it appears that the concentration-dependent effect of enzymes is similar to the concentration-dependent effect of detergents.

D. *UV*.

From the preliminary experiments on the effects of UV irradiation on the fluorescence spectrum of chloroplasts, it was seen that the intensity of emission from the long-wavelength form decreases, while that of F698 apparently increases. Brown (1968) has observed somewhat similar effects in the low temperature emission spectrum of UV-irradiated cells of *Phaeodactylum tricornutum*; with cells irradiated for 10 minutes, there was a decrease in fluorescence at 710 nm and an increase at 680 nm. Her examination of these cells with the light microscope did not reveal changes in gross morphology; changes in membrane structure such as those reported in the present work are, however, best revealed by electron microscopy.

It is interesting to note that fluorescence induction changes, in some respects similar to those observed by

M. Brody (1969) with chloroplasts suspended in fatty acids (or RLE), are also seen upon UV irradiation of chloroplasts (Malkin and Jones, 1966). In the case of UV irradiation, the decrease in variable yield of fluorescence is accompanied by inhibition of the DCPIP Hill reaction. Furthermore, the addition of DCMU to such UV-irradiated chloroplasts led to an increase in fluorescence yield, in contrast to fatty acid-treated chloroplasts in which DCMU did not increase the yield. Thus, it would seem likely that while fatty acids (or RLE) and UV irradiation both lead to an attenuation in R, the pathways to this end are different.

III. THE RICINUS PROTEIN FACTOR

A. Protein-like Nature.

In the present study, it was determined that the substance responsible for the changes in steady-state fluorescence of *Ricinus* chloroplasts, as well as other chloroplasts treated with extracts derived from *Ricinus* leaves, is proteinaceous in nature. It is heat-labile, non-dialyzable, can be precipitated by ammonium sulfate (and acetone), and is degraded by proteolytic enzymes to a non-active form.

Using the method of McCarty to extract the protein(s) directly from the chloroplast has permitted a partial purification of the protein. Employing the change in R

as a measure of activity, it has been possible to obtain preparations (after G-100 Sephadex chromatography) having specific activities (Δ R/60 min/mg protein) as high as 200 compared to the original KP_i-EDTA extracts of the chloroplasts. Although it is more difficult to compare specific activities of the purified fractions to that of RLE (the activity of the latter most likely varying largely as a function of the release of the active factor from the chloroplasts into the extract), an estimate of 900-fold higher can be made.

As reported in the present research, the protein is excluded by G-25 Sephadex, and slightly retarded by G-100 Sephadex--indicating a molecular weight greater than 10,000, but less than 150,000. Its size may explain why it so poorly penetrates intact algae.

The optimal pH for the protein's action (measured either as inhibition of Hill activity or ability to induce the spectral change) is approximately 8 to 8.5. This explains why Ricinus chloroplasts isolated in a medium buffered at pH 6 undergo little spectral change--at this pH, the activity of the protein is low (50% of the peak value).

B. Effects on Electron Flow.

As noted above, studies on light-induced changes in the cytochrome region indicate that the Ricinus obtained from the G-25 Sephadex chromatography step stimulates the extent of oxidation of cytochrome f (see Fig. 33). Also

TABLE VI

PURIFICATION OF *RICINUS* CHLOROPLAST PROTEIN

	Total Protein (mg)	Specific Activity (ΔR/60 min/mg protein)	Total Activity
Crude leaf extract	33,300.0	0.0198	659.0
KP_i-EDTA extract	4,500.0	0.092	414.0
Heat-treated extract	3,562.0	0.086	285.0
$(NH_4)_2SO_4$ 22-60% fraction	159.0	0.897	142.4
G-100 Sephadex fraction	5.45	21.4	116.6

as noted above, a similar stimulation of system I activity (measured as NADP reduction) is not observed (Table II). This result may be explained in the following way: the cytochrome studies were made using the G-25 protein--which is more heterogeneous in nature than the protein obtained from the G-100 Sephadex chromatography step; the latter was used for the dye reduction studies. Therefore another substance(s) may have been responsible for the observed stimulation of cytochrome oxidation. The dye reduction studies, in which the G-100 protein was utilized, indicate that the protein effects an action initially on system II and secondarily on system I.

The fluorescence induction studies of M. Brody (1969), the experiments on light-induced absorption changes of chlorophylls a_{II} and a_{I} of M. Brody _et al_ (1970), and the amperometric studies of S. Brody (1969) cited above were all made with crude _Ricinus_ leaf extract, _i.e._, RLE. These studies all indicate an initial (fast) action on system I, followed by an action on system II. The apparent conflict between these data and those from dye reduction studies probably arises from the additional presence of another molecule(s) in the crude extract, which has a more rapid effect on system I, than does the _Ricinus_ chloroplast protein. This "additional" molecule(s) may be identical with (or present in the same low molecular weight fraction as) the "yellow molecule" cited above, to which M. Brody (1969) ascribed the "fast" changes in fluorescence

induction pattern of RLE-incubated chloroplasts--in the same work ascribing to "the protein fraction" the "slow changes" in fluorescence induction.

IV. FATTY ACIDS AND THE MECHANISM OF ACTION OF THE *RICINUS* PROTEIN

Although certain of the lipases employed in the present research, *i.e.*, scarlet runner bean extract, pancreatin, or purified galactolipas from *Phaseolus vulgaris*--as well as long chain-unsaturated fatty acids--serve as good models for the action of the *Ricinus* chloroplast protein, the protein itself has lipolytic activity to a degree barely detectable by the assay method used here. As noted above, incubation of subchloroplast particles (or purified lipids of the type predominantly found in the thylakoid membrane, e.g. galactolipids, phospholipids, or sulfolipids) with the *Ricinus* chloroplast protein led to little release of fatty acids. In the case of the subchloroplast particle, for example, the fatty acid molecules released per chlorophyll is $< .01$ (or < 1 fatty acid/100 chlorophylls). This value for FA/CHL (assuming all fatty acid released is linolenic) is approximately 1/200th the number of exogenously added linolenic molecules required to inhibit ferricyanide Hill activity to 50% of the control value, and 1/800th the number required to attenuate *R* by 50%.

Constantopoulos and Kenyon (1968) have noted that when Hill activity is inhibited either by ageing or by addition of exogenous fatty acids--the fatty acids endogenously

released (as a result of ageing)--are 40 times more effective in inhibiting Hill activity to the same level than are fatty acids which are exogenously added. Therefore, one must assume that if fatty acids are the only active factors released by the action of the *Ricinus* chloroplast protein, they are effective at a much lower concentration than that found by Constantopoulos and Kenyon for endogenous inhibition. (The possibility of fatty acids being "carried along" by the *Ricinus* protein is unlikely, since assays--made during the various preparative steps--for free or "loosely bound" fatty acids were negative.)

A possible interpretation for the inability of BSA (at concentrations up to 30 mg/ml) to offer protection against the action of the protein, is that the action does not result primarily from the liberation of fatty acids.

For the observed acceleration of the spectral changes in the presence of defatted BSA, there is still no explanation. In this regard, however, Gyldenholm and Whatley (1968) have noted that high concentrations of BSA (similar to those used here) inhibit photochemical activity, and they have suggested that the action of BSA, at these concentrations, is a physical removal of fatty acids from the lipids of the thylakoid membrane. Such removal of fatty acids by BSA may lead also to configurational changes--possibly permitting greater access of the *Ricinus* protein to its site of action.

The observed maximum (687 nm) for the "one-banded" emission of chloroplasts incubated in the presence of both defatted BSA and Ricinus chloroplast protein (see Fig. 16) is "shifted" 11 nm to the blue from the peak which obtained in the presence of the protein alone (see Fig. 16). (The maxima of control chloroplasts did not change upon addition of BSA.) This maximum may be a reflection of the combined action of the two substances on the local environment of the emitting form.

V. ALTERNATIVE MODELS FOR THE ACTION OF THE RICINUS PROTEIN

A number of alternatives to the interpretation that the spectral changes (and associated phenomena) brought about by the Ricinus chloroplast protein arises solely from the action of released fatty acids are possible.

Perhaps, the values of FA/CHL required for endogenous inhibition, as measured by Constantopoulos and Kenyon, were an overestimation, and smaller amounts of fatty acids can be effective. If amounts such as those found in the present work, e.g. FA/CHL=1/200-1/800 to effect the changes in Hill activity and emission spectra respectively, are effective, then it is possible that the fatty acids are "hitting" special chlorophylls, i.e., reaction centers, which are present in very low concentration (P700 is apparently present at a concentration of 1/400 total chlorophylls). It would also follow that the differential effect on system II and system I electron transport would be explained on the basis of greater sensitivity of the system

II reaction center. The spectral changes--which are interpreted as arising from de-aggregation--may result from a conversion of system I chlorophyll (most likely bulk) into a monomeric form, spectrally similar to the proposed (see INTRODUCTION) reaction center of system II.

Another explanation which may explain why the changes may occur at such seemingly low concentrations of fatty acid is that several components (including fatty acids) derived from the lipid portion of the membrane are effective. That the production of free lipids from the lipiprotein lamellae could have a de-aggregating effect is indicated by the work of Trosper and Sauer (see above).

Another possibility is that these lipids are converted to monoglycerides by loss of a single fatty acid moiety from each molecule. Since monoglycerides ("lyso" compounds) are known to be effective at low concentration ($\sim 10^{-7}$M) [see Condrea et al, 1964], the amount of fatty acid release associated with their formation would be barely detectable. Compounds of this type (monoglycerides) are also known to be surface active (detergent-like) and potent inhibitors of enzyme reactions, e.g., as noted above, low concentrations of detergents (exogenously added) are capable of inducing spectral changes.

Since linolenoyl alcohol can also induce the spectral changes, it is possible that a molecule of this type is being released from the thylakoid membrane as a result of the action of the protein. A likely candidate might be

the phytol tail of chlorophyll itself. (The removal of the phytol to yield chlorophyllide would have no effect on the absorption or emission spectrum *per se*, Rabinowitch, 1956.) Since for each chlorophyll one phytol could be released, in this case, at least, the endogenous concentrations would be extremely high. As noted above exogenously added phytol can bring about the fluorescence change.

Another possible class of molecules which could be involved are the phenolic compounds; these have been implicated as endogenous inhibitors of oxygen evolution in sugar cane chloroplasts (Gross *et al*, 1969). As observed in the present work, phenol itself leads to spectral changes similar to those produced by the *Ricinus* protein. Since it has been previously shown that bovine serum albumin can protect mitochondria against the uncoupling action of simple phenols (Weinbach and Garbus, 1965); the above-noted failure of serum albumin to protect, may lead one to suggest that if phenols are involved, they are either complex in nature, or are derivatives which do not complex with the albumin.

It should also be kept in mind that there may be more than one active type of molecule (perhaps a combination of the above) producing the observed effects.

SUMMARY

1. A protein has been isolated from the chloroplasts of the castor-oil plant (*Ricinus communis*), which has pronounced effects on a number of chloroplast parameters. Incubation of chloroplasts, from higher plants or algae, in the presence of the protein leads to time-dependent changes in steady-state fluorescence emission (-196°C), electron transport, and ultrastructure.

2. Attempts to determine the protein's mode of action using model system compounds have revealed that long-chain unsaturated fatty acids are the best models.

3. In addition, the sequential inhibition of system II-associated and system I-associated electron flow has been demonstrated as a function of increasing concentration of exogenous fatty acid (linolenic).

4. System II phenomena, *i.e.*, system II dye reduction and delayed light emission, are all affected at a linolenic acid to chlorophyll ratio (on a molar basis) of $\sim$2, while system I phenomena, *i.e.*, system I dye reduction, cytochrome f oxidation, attenuation of *R*, are all affected at a linolenic acid to chlorophyll ratio of $\sim$10.

5. The *Ricinus* chloroplast protein has very weak lipolytic activity and no detectable protease activity. A number of alternative models for its action are discussed.

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PUBLICATIONS RESULTING FROM THIS WORK

1. Brody, M., Nathanson, B., and Cohen, W. S. (1969A). The similarity of action of *Ricinus* leaf extract and fatty acids on isolated chloroplasts. *Abstr. Biophys. Soc.* 9, A-121.

2. Brody, M., Nathanson, B., and Cohen, W. S. (1969B). Enhancement of emission from chloroplasts at 698 nm by a naturally-occurring factor. *Biochim. Biophys. Acta* 172, 340-342.

3. Cohen, W. S., Nathanson, B., White, J. E., and Brody, M. (1969). Fatty acids as model systems for the action of *Ricinus* leaf extract on higher plant chloroplasts and algae. *Arch. Biochem. Biophys.* 135 (1-2), 21-27.

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