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Historical corner

Carotenoid-sensitized photosynthesis: Quantum efficiency, fluorescence and energy transfer

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Abstract

The observation in the early 1940s that the quantum efficiency of photosynthesis in a diatom was almost the same whether incident light was absorbed by chlorophyll *a* or by fucoxanthol sparked subsequent investigations of the variety of chloroplast pigments and in a diversity of photosynthetic organisms. Subsequent fluorimetric measurements provided the first relevant observation on the existence of excitation energy transfer in photosynthesis. These and some other experiments prior to the classical work of Arnold and Oppenheimer [(1950) J Gen Physiol 33: 423–435] and of Duysens [(1952) Doctoral thesis, State University of Utrecht, the Netherlands] are reviewed here.

Abbreviation: HPLC – high performance liquid chromatography

Introduction

One valuable spin-off of the controversy in the 1930s on the minimum quantum requirement of oxygen evolution [whether 3 or 4 quanta in a 'perfect world' according to Otto Warburg or 8 and greater in an imperfect world of Robert Emerson and others (see Rabinowitch and Govindjee 1969; Huzisige and Ke 1993)] was the equipment and methodology made available for further studies of photosynthesis. At the University of Wisconsin (Madison), attention turned to the role of accessory chloroplast pigments. Perhaps this was because of the interest on the campus in the newly established science of limnology pioneered by the then President, E. A. Birge (1918–1925) and Prof. C. Juday (see Sellery 1956) and their interest in the theory of 'Chromatic Adaptation' in submerged aquatic plants (see group photograph in Figure 1). This theory first suggested by Engelmann (1883) was based on his observations and those of Orsted (1844) on the color stratification of large marine algae. According to this theory, the color distribution of these algae represents an adaptation to the difference in spectral composition of solar energy penetrating to different depths. Differences in color enabled them to more readily absorb dominant rays of solar radiation at various depths (see Dutton and Juday 1943).

In ingenious laboratory experiments with green, red, and brown algae, Engelmann (1883) projected the visible spectrum onto the stage of a microscope and used motility of bacteria as indication of oxygen evolution (see Kamen 1986a). He observed that maximal photosynthetic activity coincided with that portion of the spectrum where maximum absorption of light occurred. In 1936, Monfort, using crude measurements based on transmissions of extracted pigments and relative efficiencies, concluded that light absorbed by carotenoids of marine algae is used in photosynthesis.

By 1940, a combination of methodologies had become available to photosynthesis research: 1) the dropping mercury electrode for oxygen determination, 2) monochromatic light sources of high intensity, 3) chromatography for pigment separations and identifi-

Figure 1. A group photograph of the 1938 staff of the laboratory of the UW Trout Lake Station in northern Wisconsin. Mentioned here in the text and labeled in the front row are Drs Winston Manning, C. Juday and E. A. Birge; behind them with most recent addresses are R. Pennak (University of Colorado), R. Juday (son of C. Juday – University of Montana) and G. Prescott, noted phycologist (Michigan State University). Not identified are many others in this photograph who were to make important contributions to science.

cations, and 4) spectrophotometry for evaluating the absorption of light by the various pigments. Accordingly, the quantum efficiency of oxygen evolution in the diatom *Nitzschia closterium* (now *Pheodactylum tricornutum*) was determined with monochromatic sources (i.e., the action spectrum) to assess the photosynthetic activity of chloroplast pigments (Dutton and Manning 1941). About the same time, Emerson and Lewis (1941, 1942, 1943) determined quantum efficiencies, with Warburg manometry, in the cyanobacterium *Chroococcus* and the green alga *Chlorella vulgaris*.

Measurement of oxygen

Even up to the 1950s the method of choice for determining oxygen exchanges in biological systems was the Warburg manomometer. In its differential form it could measure oxygen in the presence of carbon dioxide and thus being applicable to photosynthesis research.

The first application of the dropping mercury electrode to photosynthesis was made at Wisconsin in a cooperative project between Botany and Chemistry Departments involving W.M. Manning, J.F. Stouffer, B.M. Duggar and F. Daniels (Manning et al. 1938; see also Petering et al. 1939) (see photograph of the Wisconsin group, Figure 2). This sensor preceded the now

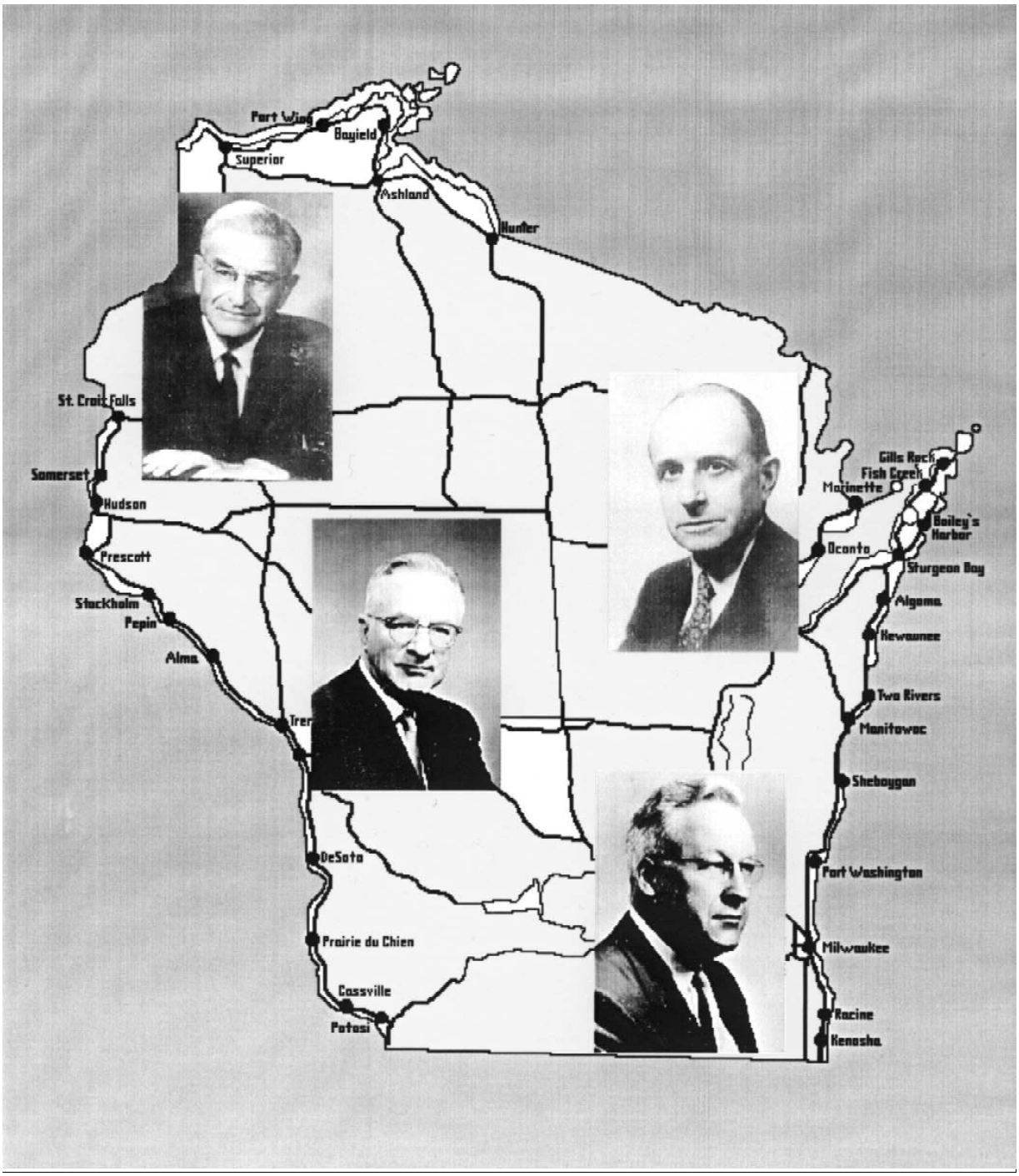


Figure 2. Wisconsin photosynthesis research team (from top to bottom)-Prof. Farrington Daniels, Physical Chemist, author and co-author of the ubiquitous text, Getman and Daniels 'Outlines of Theoretical Chemistry', Prof. B.M. Duggar, Prof of Plant Physiology (and after retirement, discoverer of aureomycin at Lederle Laboratories), Dr Winston Manning, U.W., Carnegie Institution of Washington, Argonne National Laboratory, and the author.

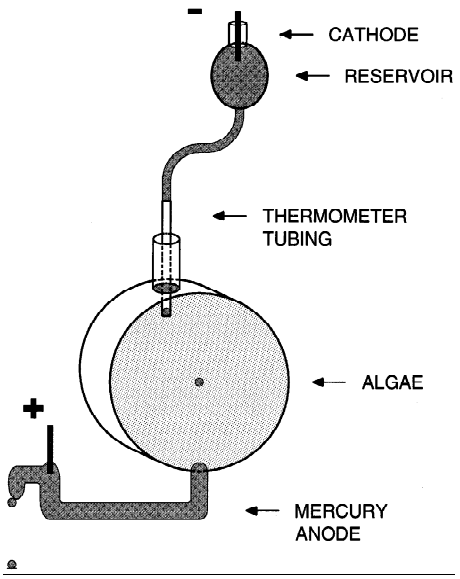


Figure 3. Radiation cell with dropping mercury electrode and mercury overflow.

ubiquitous Clark electrode and had all of the dangers inherent to 'mercury spills'. At that time, the toxicity of mercury was either unknown or ignored. In a closed, constant temperature room, the sins of all users of mercury electrodes, past and present, were disguised under a slatted wood floor from which the day long experimenters were exposed to finely divided mercury dust. The toxicity of metallic mercury was inadvertently but tangibly demonstrated by a photochemist who cleared quartz capillary tubes for reuse in the mercury lamps (to be described later) by boiling off the mercury with a gas-oxygen torch. Fortunately, his symptoms were detected soon enough to avert total disaster. Nevertheless, the dropping mercury electrode constituted a breakthrough for photosynthesis research because of its selective quantitation of dissolved oxygen (Figure 2).

Monochromatic sources

The ever present need for high intensity 'monochromatic' light, required for quantum yield action spectra of photosynthesis, was met with high-intensity mercury and filament sources and with specifically designed Corning glass filters. The homemade quartz capillary mercury lamps mentioned above constituted a major advance for meeting the need of photochemists for high-intensity monochromatic light. These lamps consisted of 1 mm bore quartz tubes 15 cm long, with two bulbs blown at the center and ca. 15 mm apart to localize the arc. Mercury-filled, with electrodes at each end and sealed with Dekotinsky cement, lamps were operated at 500 V DC, in a water bath. With one hand holding a micro-Bunsen burner to momentarily vaporize the column in the middle section and with the other hand on the water valve, the mercury arc either struck or exploded! But this high-intensity 1 by 15 mm lamp was invaluable and particularly adapted for the use before the slit of a monochromator. Alternatively, followed by Corning glass filters, the principal mercury lines of 405, 436, 546 and 577 nm could be readily isolated.

Unfortunately, mercury has no intense line in the red for selective chlorophyll activation. High-intensity filament lamps followed by narrow band liquid filter systems (9 cm water, 4.5 cm N/20 copper sulfate solution) and Corning glass #243 (now #2434) was one of the designs. Further, mercury does not have a strong line in the area of highest carotenoid absorption. Here again, a filter system of relatively low transmission was designed to work with filament lamps with its peak intensity at 475 nm as shown in Figure 4.

In contrast to these sources, Emerson and Lewis (1942, 1943), and later Tanada (1951), used an elegant grating monochromator and a 1000 watt tungsten lamp to provide radiation throughout the visible spectrum. At half width band 5–7 nm, intensities were 1.0–3.0 einsteins per cm^2. The distinct advantage of this system was that complete action spectra were enabled.

Dye and other lasers were yet three decades away to provide the required high intensity monochromatic radiation at all visible wavelengths.

Algal culture

The brown fucoxanthol containing diatom, *Pheodactylum tricornatum*, as well as the favorite green alga *Chlorella*, already introduced by Warburg and used by R. Emerson, were grown by continuous culture methods. Previously, monocellular algal species were routinely cultured and maintained by individual batch inoculation in 250 ml Erlenmeyer flasks and these were illuminated from the bottom. Rubber stoppers for the flasks contained cotton-filled filter tubes to introduce and exit the 5% carbon dioxide enriched air. Liquid media were the expression of the art and science of the phycologists. They were evaluated by the rate of growth of algal populations. Our pride was a contin-

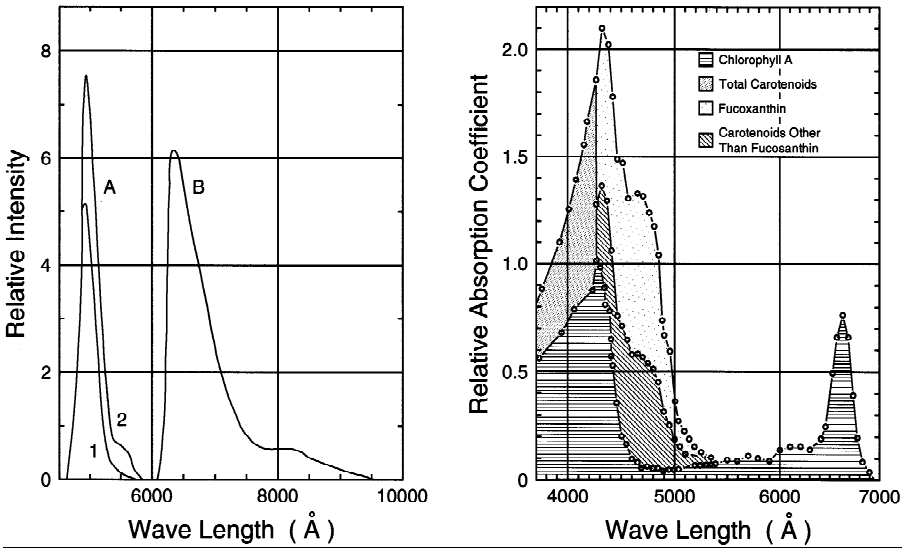


Figure 4. (Left) Relative energy distribution curves. (A) blue green light source with 9.36 mm. thickness of filter #430, (2) 5.0 mm. filter of #430. (B) red light source. (Right) The absorption spectra in acetone, of component pigment fractions in *N. closterium*. The lowest curve is the absorption spectrum for chlorophyll *a*, the intermediate curve is for chlorophyll *a* plus carotenoids other than fucoxanthin, and the uppermost curve is for these pigments plus fucoxanthin, Below 425 nm, measurements were made on two fraction, chlorophyll and total carotenoids. (Dutton et al. 1941).

uous mode of culture for *Pheodctylum* and *Chlorella* by which it was hoped that the daily samples used were of uniform if not optimal growth characteristics. An enriched sea water medium served for *Pheodactylum* and a modified Warburg medium for *Chlorella* (Manning et al. 1938). The continuous culture apparatus for *Pheodactylum* consisted of an 8-l Wolf flask, with entrance holes at the lower side and the center top. Carbon dioxide-enriched air was introduced into the medium through a tube in the side stopper, providing stirring, and was exited from above the media surface by a second glass tube. The top hole held a large test tube-like glass cylinder and was sealed to the neck of the Wolf flask opening by thin rubber tubing. This cylinder contained the U-shaped neon light source which emitted light but minimal heat. The whole system was located in a constant temperature room at 18 °C. The culture was maintained at approximately constant concentration and volume by frequent withdrawal of a part of the suspension and replacement with fresh sterile nutrient.

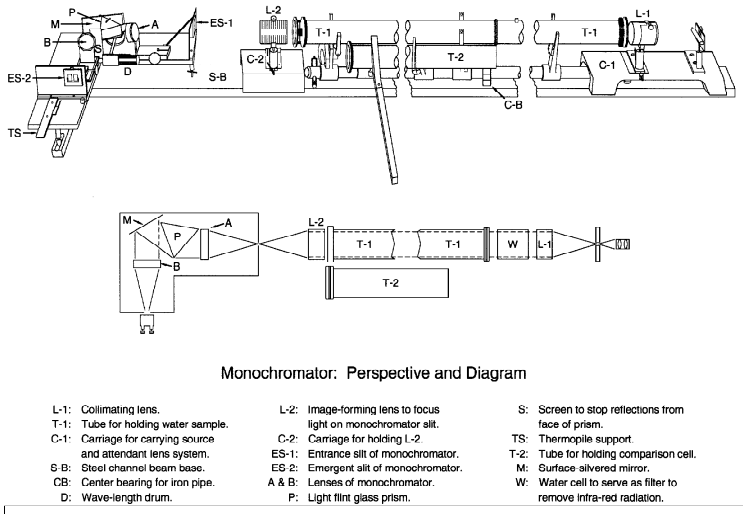
Chromatography

Chromatography for chloroplast pigment separations was just making its way across the Atlantic. Methods were published in Austria (Zechmeister and Cholnoky 1937) and later in the United States (Strain 1942). Originally, they were directed toward pigment separations, as the prefix 'chrom-' would imply. The chromatography of Russian botanist Tswett, who was first to separate pigments on a sugar column (Tswett 1906), had used this technique for qualitative observation. An abstract in 1941 'Chromatographic Adsorption of Plant Pigments as a Limnological Method' captures some of the excitement of this promising new tool in the United States (Dutton 1941). Quantitative separation of individual chlorophyll and carotenoid pigments was made possible on powdered sugar columns; therefore, an estimate of the contribution of each pigment to the light absorption spectrum could be made (Dutton and Manning 1941; Emerson and Lewis 1943).

About this same time, Emerson and Lewis (1942, 1943) had the cooperation of none other than H.H. Strain at the Carnegie Institution of Washington, Stanford in the separation, identification and spectrophotometry of chloroplast pigments.

Spectrophotometry

Absorption spectra at Wisconsin were initially obtained on President (Emeritus) E.A. Birge's spectrophotometer built by the Physics Department (Figure 5) for his limnological studies of light transmission in lakes (James and Birge 1938). The instrument filled a



Monochromator: Perspective and Diagram

L-1:	Collimating lens.	L-2:	Image-forming lens to focus light on monochromator slit.	S:	Screen to stop reflections from face of prism.
T-1:	Tube for holding water sample.	C-2:	Carriage for holding L-2.	TS:	Thermopile support.
C-1:	Carriage for carrying source and attendant lens system.	ES-1:	Entrance slit of monochromator.	T-2:	Tube for holding comparison cell.
S-B:	Steel channel beam base.	ES-2:	Emergent slit of monochromator.	M:	Surface silvered mirror.
CB:	Center bearing for iron pipe.	A & B:	Lenses of monochromator.	W:	Water cell to serve as filter to remove infra-red radiation.
D:	Wave-length drum.	P:	Light flint glass prism.		

Figure 5. Diagram of optics of primitive spectrophotometer built for studies of transmission of lake waters (James and Birge 1937), also used for early light absorption studies of pigments and source of high-intensity monochromatic light for quantum efficiency studies (Manning 1938).

Figure 6. A personal gallery of a few of the world's early researchers on energy transfer in photosynthesis. (Top row, left to right) William Arnold, L.N.M. Duysens, Robert Emerson, C.S. French. (Bottom row, left to right) Govindjee, J.R. Oppenheimer, E. Rabinowitch, Takuma Tanada, E.C. Wassink.

large room. At this time it had a hollow glass prism, 12 cm on an edge filled with ethyl cinnamate, for light dispersion and a thermopile-galvanometer system for light detection. The several meter optical lever arm of the galvanometer was such that Brownian motion fuzzed the image on a meter stick to be read in the darkened room. The design, unfortunately, placed absorption cells before the slit where they received the full radiation of the tungsten source. Fortunately, a visual spectrophotometer with polarizing prisms, which read out directly in optical density, was located in the Dept. of Agricultural Chemistry for repeating the absorption spectra.

This was long before Arnold Beckman's Model DU spectrophotometer and even longer before Cary's recording instrument were available.

Some results

Despite the primitive equipment it was found that the quantum efficiency of oxygen evolution was as high in *Pheodactlyum* when 90% of the light was absorbed by carotenoid fucoxanthol as when light was absorbed solely by chlorophyll (Dutton and Manning 1941) (see Table 1).

This established that energy from the carotenoid fucoxanthol is transferred highly efficiently (ca. 90%) to chlorophylls .

In the next year, Emerson and Lewis (1942), then at Stanford, CA, expanded our knowledge of the blue green algae *Chroococus* by showing 'light absorbed by phycocyanin is used for photosynthesis with an efficiency closely approximating that of chlorophyll'. Tanada in 1951, using Emerson's grating monochromator and differential manometry equipment (in Urbana, IL), provided an action spectrum for the diatom *Navicula minima* and confirmed the high photosynthetic efficiency of fucoxanthol.

It is of passing interest that this highly efficient 'internal conversion' of energy without radiation was cited by Oppenheimer (1941) (for Arnold's contribution to it, see Knox 1996) before he became 'father of the atomic bomb'. I learned one day from a bulletin board, in 1942 as I remember it, that 'post doc' Martin Kamen (see his perspective Kamen 1986b) was talking about carotenoids and photosynthesis in Oppenheimer's physics seminar at Berkeley. I made it to the back row, though late. It turned out that Martin was reviewing our 1941 paper! I had met Oppenheimer months earlier and when Martin made snide comments, e.g. 'Why was a paper on quantum efficiency published in a botany journal?', 'Oppie' would turn and wink. Oppenheimer introduced me at the end and poor Martin! – Perhaps in penance, after the seminar Martin gave me a specially guided tour through the restricted cyclotron buildings up in the Berkeley hills. Arnold, in cooperation with Oppenheimer, in 1950 expanded further on the theory of internal conversion with application to the cyanobacterium (then called blue-green algae) (Arnold and Oppenheimer 1950; see also Arnold 1991).

Table 1. Summary of quantum yield of photosynthesis and light absorption at different wavelengths

Wavelength (Å)	Percent of absorbed light absorbed by carotenoids	Average quantum yield yield	Average ratio of yields (± standard error)		Number of pairs of experiments
4,047–78	38	0.057	Violet/Red	= 1.08 ± 0.08	6
6650	0	0.052			
4358	49	0.064	Blue/Red	= 1.04 ± 0.05	7
6650	0	0.063			
4960	93	0.059	Blue-green/Red	= 0.75 ± 0.03	9
6650	0	0.080			
5461	48	0.065	Green/Red	= 1.10 ± 0.04	7
6650	0	0.060			
6650					
6650			Red/Red		
		0.059		= 1.01 ± 0.05	7
4358			Blue/Blue		
4358					

In the 1930s, geographical areas of specialization in chemistry existed in the United States, e.g. if one was interested in organic chemistry, the choice was with Roger Adams at Illinois; for biochemistry, it was Steenbock or Hart at Wisconsin or Gortner at Minnesota; if the interest was in physical chemistry, the center of excellence was the West Coast. At Berkeley, the word had it that Prof. G. N. Lewis would not countenance contamination of his department of chemistry with biochemistry. The pioneering, outstanding work of S. Ruben and M. Kamen in photosynthesis gained entry and was rationalized as photochemistry; therefore, conducted in photochemist Roelefson's 'Rat Lab' (see Kamen 1989). It was to the lawn outside the Rat Lab that Ruben was able to walk after the tragic laboratory accident that took his life.

Excitation energy transfer and sensitized fluorescence

One further first step forward in the understanding of photosynthesis was made by the use of 'monochromatic sources' and fluorescence detection. At this point in time the question remained whether carotenoid sensitized photosynthesis was a process separate from the chlorophyll process. In their review of 1969, Rabinowitch and Govindjee stated: 'The first relevant observation was made in 1943 by H.J. Dutton, W.M. Manning and B.M. Duggar at the University of Wisconsin. They measured the yield of fluorescence of chlorophyll *a* in a diatom, using monochromatic excitation, and found that this yield was almost the same whether the incident light was absorbed by chlorophyll *a*, or by fucoxanthol.'

By 1941 (see Dutton et al. 1943), a 1000 watt, water cooled, high pressure mercury lamp of commercial design had become available. The G.E. type H-6 was gratefully substituted for the home-made capillaries. For measurements of absorbed and fluoresced light, algal suspensions were placed in a large glass cell of 0.5 cm optical path and equipped for bubbling carbon dioxide enriched air. Absorbed light was calculated after measuring incident and transmitted light with a calibrated thermopile-galvanometer system. A Weston Photronic cell-galvanometer combination was used for fluorescence intensity measurements. Two glass filters (Old Corning numbers 507 and 211) placed between the algal cells and the photocell absorbed practically all of the remaining incident light and transmitted only the fluoresced light longer than 680 nm. For reasons of scattering and reabsorption of fluoresced light, suspensions as thin as possible were used. The results of measurements on *Pheodactylum* and *Chlorella* suspensions and on their acetone extracts are reproduced in Table 2 (Dutton et al. 1943).

It was concluded that the quantum yield of chlorophyll fluorescence in *Pheodactlyum* was found to be constant, within rather large limits of experimental error, for exciting light of wave lengths 436, 470, 578 and 600 nm. Light absorbed by yellow pigments in *Pheodactylum* therefore must reappear as chlorophyll fluorescence. This leads to the conclusion that the previously observed carotenoid-sensitized photosynthesis in *Pheodactylum* takes place principally through the transfer of absorbed energy from carotenoid to chlorophyll molecules with subsequent reactions the same as though chlorophyll molecules were primary absorbers. (In acetone extracts of *Pheodactylum* light absorbed by yellow pigments did not contribute appreciably to chlorophyll fluorescence, indicating that little or no energy was transferred between pigments in acetone solution.)

$$\text{Fucoxanthol} \xrightarrow{90\%\text{transfer}} \text{chlorophyll}\,a \longrightarrow \text{photosynthesis}$$

The first confirmation of these observations came from Wassink and Kersten three years later in 1946. After nine years, French and Young (1952) made application of their newly constructed 'spectrofluorimeter' to the red algae and to the transfer of energy from phycoerythrin to phycocyanin and chlorophyll. This came just about the time of the most influential thesis of Duysens in 1952. Duysens exploited the sensitized fluorescence method to its fullest capability by demonstrating energy transfer from phycocyanin, from phycoerythrin and from carotenoids to chlorophyll *a* in cyanobacteria and algae (see an excellent review on 'Biophysics of Photosynthesis' by Duysens 1964).

In a trip to the Netherlands, I had the pleasure of visiting Duysens and seeing his laboratory. He averred over lunch that my two papers were the starting points for his thesis. It was his good fortune, due to his sharp mind, and his dedication that he developed an international reputation in photosynthesis – while I applied my pigment separation, identification and quantitation expertise to the color and nutrition of dehydrated vegetables and dried eggs for the US Army Quartermaster Corps! However, in the late 1970s, I was able to submit and receive formal approval for the first photosynthesis project within US Department of Agriculture. Among the early successes was the first application of high performance liquid chromatography to plant pigments,

Table 2. Results of the fluorescence measurements

Material	Calculated fluorescent yield ratios					
	Wavelengths compared	Percent of absorbed light absorbed by by chlorophyll	Assuming no energy transfer from carotenoid to chlorophyll	Assuming complete transfer of energy	Observed ratios (geometric mean ± standard error)	Number of pairs of experiments
	(Å)					
Nitzchia closterium	4700 vs. 5780 or 6000	26 95 or 99	0.27	1.0	1.2 ± 0.2	5
	4358 vs. 5780 or 6000	51 95 or 99	0.53	1.0	1.1 ± 0.2	6
Chlorella pyrenoidosa	4700 vs.52 5780 or 6000	0.52 100	1.0	1.05 ± 0.01	2	
	4358 vs. 5780 or 6000	81 100	0.81	1.0	0.93 ± 0.18	4
Acetone extract of *N. closterium*	4700 vs. 6000	19 100	0.19	1.0	0.22 ± 0.02	2
	4358 vs. 5780	40 99	0.40	1.0	0.49 ± 0.07	2
Acetone solution of chlorophylls *a* + *b*	4358 vs. 5780	100 100	1.0[a]	1.0[a]	0.97 ± 0.06	5

[a]The proportion of light absorbed by these two chlorophyll components is nearly equal at 4358 and 5780 Å. Hence, the yield ratio should be approximately 1.0, despite differences in the fluorescence spectra of chlorophylls *a* and *b*.

including those of *Pheodactylum* (Eskins et al. 1977), demonstration in a model system of exclusive energy transfer from chlorophyll *b* to chlorophyll *a*, both pigments adsorbed on a C-18 HPLC support (Eissler and Dutton 1981) and of energy transfer from fucoxanthol to chlorophyll *a* in an isolated, purified non-denatured chromo-protein isolate from *Pheodactylum*, but not after mild heat denaturization (Gugliemelli et al. 1981).

Among the wealth of literature on sensitized spectrofluorimetry that followed its initial applications are the numerous, significant contributions of Govindjee and co-workers. We cite here only some of his extensions to liquid helium temperature (Cho et al. 1966; Cho and Govindjee 1970a,b) that supported the Förester's energy transfer concepts, and, the discovery of a new emission band at room temperature in the red alga *Porphyridium cruentum* (Krey and Govindjee 1964). (For a review of chlorophyll *a* flourescence, see Govindjee 1995.)

Further, on the human interest side of photosynthesis research, it was the late C. Stacy French who told Winston Manning, who told me in the 1930s, of a simple experiment: Dip a frond of *Laminaria* into 60 °C water (or use a cigarette lighter as Stacy told me in the 1970s) and see the sharp color change from brown to olive green. Curiously, it was the late Robert Emerson who told and showed Govindjee in 1956, with a glint in his eyes, how heating the diatom *Navicula minima* changes its color from brown to green. It was this remarkable visual impact that led Govindjee to do the detailed action spectrum of the Emerson enhancement effect in *Navicula minima* leading him to discover that both photosystems of Emerson, the short-wave and long-wave photosystems, were sensitized by chlorophyll *a* of different spectral forms (Govindjee 1960; Govindjee and Rabinowitch 1960). The action spectrum of chlorophyll *a* flourescence of heated and unheated samples of *Navicula minima* done by Govindjee and coworkers some time during 1961–1962 were

never published and data unfortunately has since disappeared from the face of the earth. However, the same simple observation of color change (in Laminaria) also inspired the 1980s studies of energy transfer, color and spectral changes in fluorescence of the isolated chromo-proteins of *Pheodactylum* (Gugliemelli et al. 1981) mentioned above. These two anecdotes, interesting in themselves, may serve to throw light on the mechanism of seemingly simultaneous and independent scientific discoveries; in this instance it may have been casual conversations by one or two astute observers of nature whose 'twice-told tales' sparked the imaginations of two researchers working independently to discover new fundamental information about the photosynthetic process.

An underlying theme, up to now not explicitly articulated, is the demonstration in this research area that progress in understanding was preceded by developments of procedures for quantum efficiency measurements, light sources, detectors, monochromators and spectrofluorimeters. It, therefore, is appropriate to cite the anonymous quotation taken from Zechmiester's 1937 text, 'Die Carotenoide', which seems to have particular relevance to this episode of photosynthesis history:

– 'Jeder wissenschafliche Fortschritt ist ein Fortschritt der Methode'
– (Every advance in scientific knowledge is first an advance in technique)

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