Spectroscopy of Biological Molecules

Theory and Applications – Chemistry, Physics, Biology, and Medicine

edited by

Camille Sandorfy

and

Theophile Theophanides

Department of Chemistry, University of Montreal,
Montreal, Quebec, Canada

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SPECTROSCOPY OF PLANT TETRA-PYRROLES IN VITRO AND IN VIVO

Hugo Scheer

Botanisches Institut der Universität
Menzinger Straße 67
8ooo München 19   F.R. Germany

ABSTRACT

The spectroscopy of chlorophylls and biliproteins is dis-
cussed with examples selected from recent investigations.
These include the structure elucidation of the phyto-
chrome Pfr chromophore and the current status of the work
on chlorophyll-RC I, work on the biosynthesis of chloro-
phylls by non-invasive techniques, studies on the in situ
structure of chlorophylls and biliprotein chromophores,
energy transfer studies in photosynthetic antenna systems.

Abbreviations:

Chl = Chlorophyll, Bchl = Bacteriochlorophyll, Phe = Pheo-
phytin, Bphe = Bacteriopheophytin, Mephe = Methylpheophor-
bide, Bmephe = Bacteriomethylpheophorbide, PC = Phycocyanin
Pr and Pfr = Phytochrome in the red and far-red absorbing
form, respectively, uv-vis-nir = absorption in the ultra-
violet, visible and near infrared spectral range, CD =
circular dichroism, FDMR = fluorescence detected magnetic
resonance, NMR = nuclear magnetic resonance, ESR = electron
spin resonance, ENDOR = electron nuclear double resonance,
HPLC = high performance liquid chromatography.

1a: R = vinyl

2a: R = ethyl
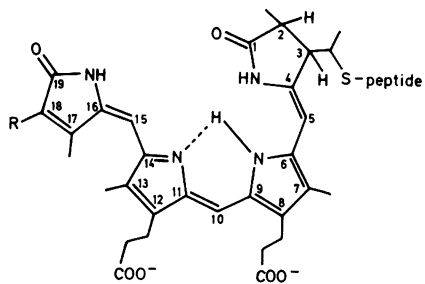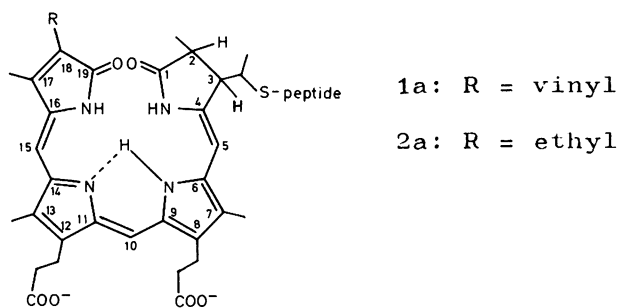
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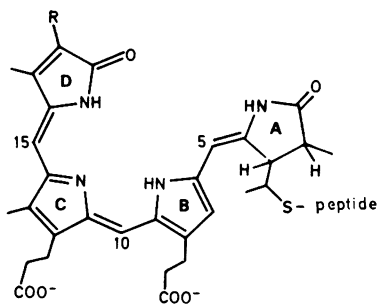
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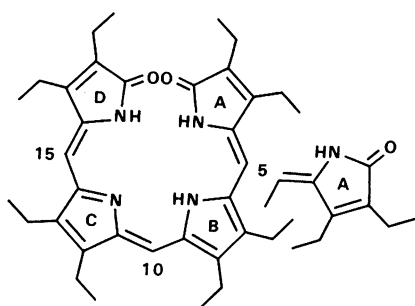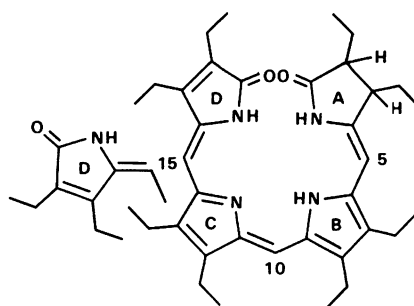
3a   3b   4b   4a

A : INTRODUCTION

Spectroscopic techniques have become increasingly important
in biological studies. This is mainly due to great improve-
ments in their sensitivity and selectivity, but also to
advancements in the underlying theories which made less
ambigous interpretations possible. The technical details of
these improvements are beyond the scope of this talk and
have in part been dealt with by other authors of this
summer school. In view of the vast amount of material on
the spectroscopy of plant tetrapyrroles, I have also not
tried to give a complete review, but have rather focused
on comparatively few examples which are meant to illustrate
the current approaches, potentials and limitations of some
selected methods. I apologize for any undue bias in this
selection.

Plant tetrapyrroles comprise the chlorophylls and the bili-
proteins serving as photoreceptor pigments, the cytochromes
functioning in the electron transport of photosynthesis and
respiration, the sirohemes and cobalamine(s) acting as co-
factors in several important enzymes, and also the bio-
synthetic precursors and degradation products of all these
pigments. I shall only deal with recent investigations on
chlorophylls, biliproteins and metabolically related
structures. The chosen examples focus on two   pects.
The first is the structure elucidation of isolated or at
least enriched compounds (section B). The second is the
spectroscopy of increasingly complex systems up to whole
organelles or organisms (section C). It has been used
study the in situ structure of the pigments, but also to
study their biosynthesis and to investigate the inter-
relations among the different pigment molecules.

B: SPECTROSCOPY OF ISOLATED PIGMENTS

Improved separation techniques have recently allowed the
isolation of many new plant tetrapyrroles and provided
evidence for an even larger number of pigments present
only in small amounts besides the major chlorophylls, e.g.
Chl a and Chl b in plants and Bchl a, b, c and d in bac-
teria. Optical spectroscopy, in particular uv-vis-nir
absorption, is the most sensitive and rapid technique to
classify among the different pigments. Useful structural
hints can be obtained from comparison with pigments of
known structure, but the method is unable to provide more
details. The two major methods for structure elucidation
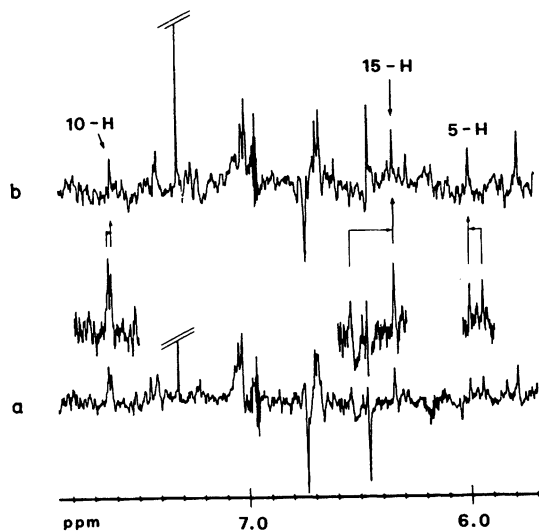
Fig. 1: ^{1}H-NMR-spectra (500MHz) of phytochrome chromo-
peptides in the methine regions. (a) 1:1-
mixture of Pfr (1b) and Pr (1a) peptide ob-
tained from the photoequilibrium mixture of
Pfr. (b) The same sample after irradiation
with white light. The pairs of lines asso-
ciated with the two forms collapse to a
single line of the Z,Z,Z-peptide (Pr=1a),
the largest shift is observed at the site
of isomerisation (C-15).
From Thümmler et al. (7).

5b   5a   5c   6
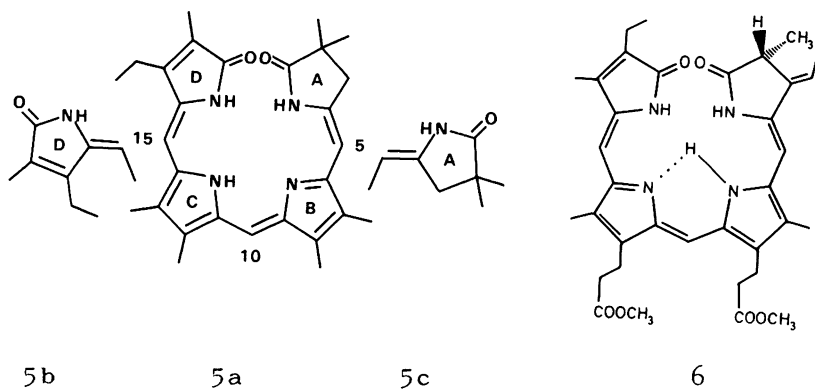
are NMR and mass spectroscopy, which are both well estab-
lished, and have been considerably improved recently. The
limiting factor in NMR spectroscopy is its sensitivity.
It is now sufficiently increased to allow work in the /ug
range, which puts stringent requirements on the sample
preparation and purity. The limiting factor of mass
spectroscopy has been the ionisation without alteration.
It has also been considerably improved recently by new
ionisation techniques, which now allow the direct
analysis of e.g. underivatized chlorophylls. Both methods
are most useful in conjunction with suitable chemical
correlation methods. Two structures are chosen to
illustrate their potentials.

B.a: CHROMOPHORE OF PHYTOCHROME IN THE Pfr FORM

The biliprotein, phytochrome is the only photomorpho-
genetic pigment in plants of which some structural details
are known. It is a photoreversibly photochromic pigment,
with one form, Pr, absorbing maximally around 660 nm, and
the other, Pfr, around 730 nm in the long-wavelength
spectral region. Phytochrome occurs only in small amounts
and concentrations in plants, and its photochromic pro-
perties have been used as analytical tool during the
isolation. The similarities in the optical absorption spec-
tra of Pr and the readily accessible phycobiliprotein,
phycocyanin (PC), were substantial in assigning a bile pig-
ment structure, too, to the chromophore of the former (see
RÜDIGER and SCHEER (1) for leading references). The de-
tailed structure analysis of phytochrome was complicated
by the rather stable thioether bond between the chromo-
phore and the apoprotein, which precluded a chromophore
cleavage without alterations of the chromophore, and also
by the poor accessibility of phytochrome. The same chemical
degradation techniques, developed with PC and then applied
to phytochrome (2) provided together with studies on model
pigments like 3, 4 (3) and with the total synthesis of
bile pigments related to the native pigments (e.g. 5,
refs. 4) the structures 1 a and 2 a for the chromophores of
Pr and PC, respectively. The evidence for the 18-vinyl
group in 1 was, however, only circumstantial (5).

The same structures and a proof of the latter substituent
have been arrived at by proton NMR of bilipeptides from
the two pigments (6). The NMR spectrum of linear tetra-
pyrroles covers the spectral range between δ = 1 and 8 ppm.
There is a considerable overlap with the NMR lines of the
peptide moiety of these chromopeptides. The structure
analysis was nonetheless possible by the use of high-field
NMR spectrometers (360 MHz in the particular case). This

Table 1: Incremental chemical shifts (Δ) of the methine
proton NMR signals in Z,E-isomeric bilindiones.
Δ (in ppm) is given as $\delta_E - \delta_Z$.

Compound	5-H	15-H	10-H	solvent	ref.
phytochrome chromophore (1b minus 1a)	-0,057	+0,195	+0,012	acetone-d_6/ H_2O/CF_3COOH	(7)
phycocyanin chromophore (2b minus 2a)	-0,056	+0,195	+0,018	acetone-d_6/ H_2O/CF_3COOH	(7)
octaethyl-bilindione (3b minus 3a)	0	+0,27	0	$CDCl_3$/CF_3COOH	(8)
15E A-dihydro-octaethyl-bilindione (4b minus 4a)	0	+0,23	0	$CDCl_3$	(8)
15E A-dihydro-bilindione (unsubstituted Ring A)	-0,07	+0,23	-0,01	$CDCl_3$	(4b)
15E 3-dihydro-7,8,12,13,18-methyl-2-di-methyl-17-ethyl-bilindione (6b minus 6a)	-0,09	+0,20	-0,03	$CDCl_3$	(9)
4E 3-dihydro-7,8,12,13,18-methyl-2-di-methyl-17-ethyl-bilindione (6c minus 6a)	+0,43	+0,08	+0,26	$CDCl_3$	(9)

did not only allow for sufficient resolution to separate
the overlapping lines from the peptide and the chromophore,
but gave also the necessary increased sensitivity required
for the work with phytochrome. An assignment of all chromo-
phore proton signals in the PC-peptide was achieved by
comparison with a synthetic peptide of the identical se-
quence, but lacking the chromophore. This was in turn the
basis for the assignment of the chromophore derived signals
(including those of the 18-vinyl group), in the Pr peptide,
leading independently to structure 1 a.

The structure elucidation of the Pfr chromophore posed
additional problems. Pfr peptides revert spontaneously to
the Pr form. This reversion is pH-dependent and sufficient-
ly retarded only at moderately low pH. The preparation of
Pfr peptides required therefore peptic digestion and sub-
sequent work-up under conditions which maintained a pH ≈ 3
throughout. This work-up can be done in protonated solvents,
but an exchange with deuterated solvent at the last
chromatographic step is necessary for NMR (7). Unfortunate-
ly, at least one (5-H) of the three methine protons is
readily exchangeable, too, with deuterium under acidic
conditions. Such an exchange was untolerable, because the
methine signals were essential to differentiate between
the discussed mechanisms in the Pr - Pfr interconversion,
e.g. a redox mediated substitution and a Z,E-isomerisation.

The extensive tests to circumvent these obstacles made
again use of the readily accessible PC and of synthetic
bilidiones like 3 and 4 as models for phytochrome, which
were used to optimize the measuring conditions. It could
be shown, that a mixture of deuterated acetone with protic
water and protic trifluoroacetic acid (85:10:5, v/v) was
appropriate. It led to only partial exchange of the 5-H,
which at the same time facilitated its assignment, and it
also fulfilled another spectroscopic requirement. The
presence of a considerable amount of protons from the
solvent ($\rangle$ 10^4 fold molar excess with respect to the Pfr
peptide) leads to a very strong solvent line in the NMR
spectrum. This can principally be coped with by using a
computer with a sufficiently high dynamic range, but only
if this line is well separated from the resonance lines of
the solute. Since the chemical shift of water is strongly
increased in the presence of acid, the amount of acid
added to the system can be used to adjust the water line to
a position where it does not interfere with the solute
lines of interest.

With all the conditions adjusted accordingly, it was then
possible to obtain the NMR spectrum of a Pfr chromopeptide
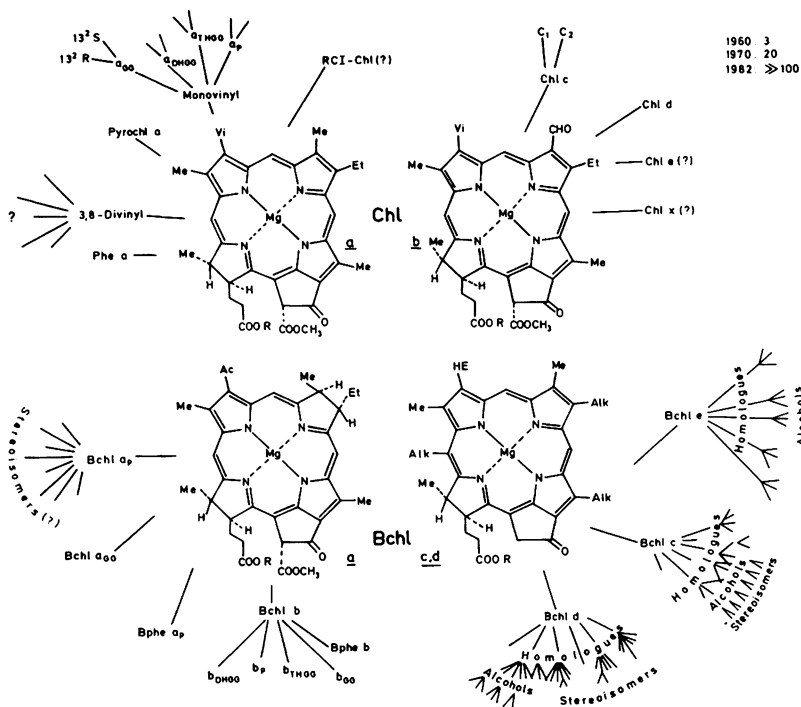
Fig.2: The "chlorophyll explosion". The time table
in the upper right gives the number of
reasonably well established structures.
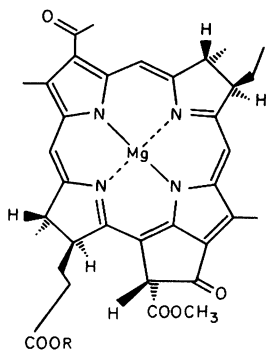
7: R = phytyl

at the first shot (7). Phytochrome accumulated over a
period of several months was phototransformed to Pfr, de-
graded and purified over night to the peptide by the bio-
chemist, taken over by the NMR crew and rushed in the early
morning hours to the 500 MHz NMR spectrometer 300 km away
in the laboratories of the BRUKER company. After measuring
first the spectrum of the Pfr peptide, the latter was
photochemically converted in the NMR tube to the Pr
peptide by irradiation with white light.

The analysis of the spectra clearly established the Pfr
chromophore as the 5Z, 10Z, 15E-isomer 1b of the all-Z
Pr chromophore 1a. This structure follows mainly from the
analysis of the methine proton signals. Studies with the
Z,E-isomers of PC and of the dihydrobilindiones 4a,b (8)
and 6a,b,c (9) have shown, that the chemical shifts of the
methine protons are somewhat solvent dependent. However,
the incremental shifts upon Z, E-isomerisation are solvent
independent, and characteristic for the site of isomeri-
sation (table 1). The largest incremental shift is al-
ways observed at the site of isomerisation. In the Pfr
chromopeptide, the position of the methine signals of
the chromophore agree reasonably well with those of the
5Z, 10Z, 15E-isomers 2b and 4 b (fig. 1). There is a
second set of lines corresponding to the Pr chromophore,
which is always present and continously formed in Pfr
peptides and amounts to ≈ 50 % in the actual sample. This
second set of lines increases accordingly upon photo-
conversion at the expense of the first set,
thus proving directly the relationship of the two species.
The largest incremental shift is observed for the 15-H
signal, and the Pfr chromophore must then contain the
15E-isomer 1b rather than the 5E-isomer 1c (or the for
other reasons unlikely 10 E-isomer) (7).

B.b.: NEW CHLOROPHYLLS

The number of chlorophylls has been rather limited until
recently, when an "explosion" of new chlorophyllous pig-
ments raised their number well beyond into account. It
becomes still larger if all suggested or suspected
structural variations are counted (fig. 2). A sound
structure analysis of even the minor pigments seems not
only urgent, but also possible with the recent advances
in spectroscopy. It should help to separate the chloro-
phylls proper from metabolites and artifacts, and to
provide a structural basis for their functional studies.

There are two general approaches for the structure
elucidation of chlorophylls. The first is the direct
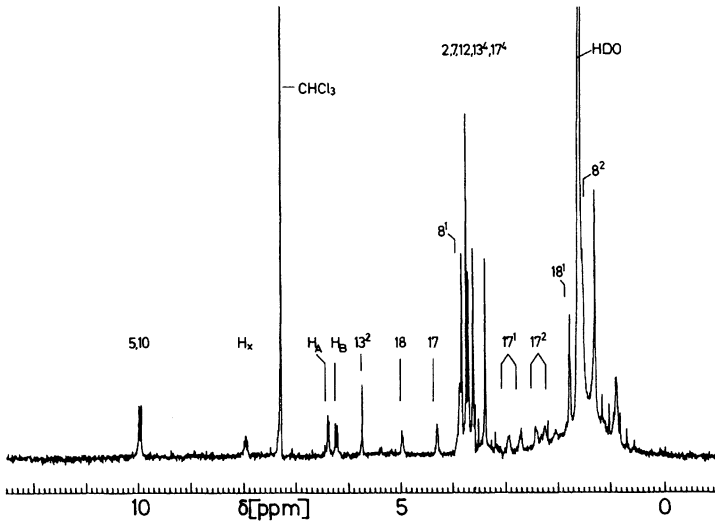
Fig. 3: ^{1}H-NMR-spectrum (500 MHz) of methylpheophor-
       bide-RC I (40 µg in CDCl$_3$). The spectrum is
       rather similar to that of Mephe a (3) at the
       same concentrations, but the 15-H is lacking
       and the 13^2-H is shifted to higher field.
       The spectrum is compatible with structure 11
       for Mephe-RC I.

            10   R = CH$_3$ 11
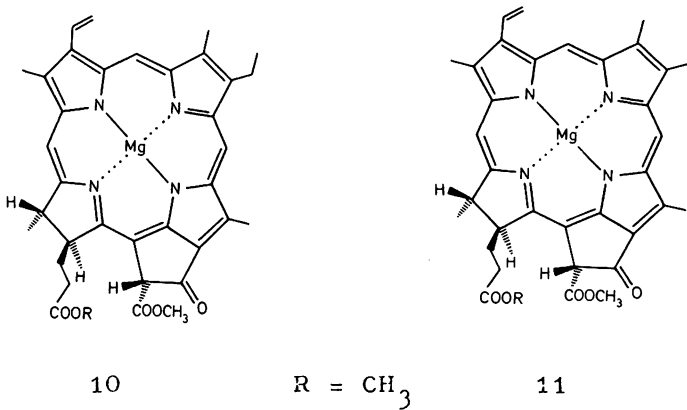
analysis of the isolated pigments. It can be limited by the
instability of the pigments, but is always necessary if the
molecule cannot be modified without extensive and
potentially unknown structural changes (see Bchl b as an
example (10). The second is the controled conversion to
derivatives like the methylpheophorbides which are more
stable and thus amenable to a broader variety of analytical
techniques, and which also provides already a means of
chemical structure relations (see e.g. the identification
of pyropheophetin as a degradation product (11) or of
"divynil" Chl a as a biosynthetic precursor (12), NMR
spectroscopy can usually be   performed with both the
genuine pigments and their derivatives. It gives a wealth
of information and is interpreted in a straightforward
fashion due to the inherent magnetic properties of the
tetrapyrrole macrocyle (13). The latter serves as a built-
in shift reagent which spreads the proton NMR spectrum over
a range from δ =-6 to 12 ppm. The only major source of
error is the pronounced solvent and concentration de-
pendence, which is related to the facile aggregation of
these pigments. The second key method, mass spectroscopy,
has been widely used for the demetalated pigments (14)
but not been suitable for the chlorophylls proper, viz.
magnesium complexes of pheophorbides. The latter cannot
be analyzed by conventional mass spectroscopy with electron
impact ionization. Several different ionization techniques
have now been applied successfully to circumvent this
problem. BRERETON et al. (15) have used the "in beam"
ionization technique to determine the structure of the
allomerization product 7 of bacteriochlorophyll a (8).
KATZ and others (15,16) applied field and ^{252}Cf plasma
desorption as well as fast atom bombardment to a variety
of the "conventional" chlorophylls.

The latter mass spectroscopic technique has also been
applied to one of the new chlorophylls, e.g. Chl-RCI (17).
This pigment occurs only in trace amounts in organisms
capable of oxygenic photosynthesis, where it has been
quantitatively related to the contents of P700, the
reaction center of photosystem I(PS I) (17). The latter
is generally believed to contain the "normal" chlorophyll
a(9) in a special environment and/or aggregation state (see
section Cd). The suggestion that the red shift and other
changed properties of P700 may be due to a modified mole-
cular structure (viz. Chl-RCI, is therefore of consider-
able interest.

Although the structure of Chl-RC I is at present not yet
fully elucidated, the known data are included here as an
example for the general approach to new structures in the

chlorophylls which are accessible only in small quantities.
DOERNEMANN and SENGER (17), who first described and iso-
lated the compound from a Scenedesmus mutant enriched in
PS I, used the first aforementioned approach and studied
the pigment proper. They obtained a satisfactory 252Cf
plasma desorption mass spectrum, which gave a molecular
ion which is 35 mass units higher than that of Chl a
(9). They also obtained NMR evidence, that the C-20
methine proton is missing. With a sample provided to us
by the authors, we could show that the red-shift (as
compared to the respective derivatives of 9) is main-
tained when Chl-RC I is demetalated to its pheophytin and
transesterified to the methylpheophorbide, Mephe-RC I.
The uv-vis-nir absorption of the latter is furthermore
characteristic of a pigment similar to methypheophorbide
a(10) but carrying a substituent other than a hydrogen
atom at C-20. This interpretation was also supported by the
CD spectrum. It showed an increased ratio of the ellipti-
cities of the red and the near-uv ("SORET) band, which
is characteristic for steric hindrance due to substitution
at methine position(s). The convertibility of CHl-RC I
to its methylpheophorbide without an apparent loss of the
important structural variations was then used by us to
obtain larger ($\approx$ 100 μg) amounts of methylpheophorbide-
RC I from a different organism. The blue-green alga,
Spirulina geitleri is available commercially as a spray-
dried powder (Behr, Bonn, FRG). The material is extracted
exhaustively with methanol, and the crude extract con-
taining mostly Chl a is immediately demetalated. The
resulting pheophytin mixture is chromatographed on silica
and transesterified to the methylpheophorbides. Further
repeated chromatography of the latter on silica, and
finally HPLC on a reverse phase adsorbent yielded the
pure Mephe-RC I. The 500 MHz proton NMR spectrum (fig. 3)
was again obtained at the BRUKER facilities. It fully
supported the substitution of C-20, and showed otherwise
all signals present in Mephe a (10). Variations of the
chemical shift can be accounted for by the low concen-
tration of the former, with the possible exception of the
rather large shift of the 13^2-H signal. The "conventional"
electron impact mass spectrum gives a molecular ion which
is 50 mass units higher than that of 10 and shows an
unusally intense (M + 2) ion, which may relate to the
presence of Cl. Taken together with chemical and chromato-
graphic evidence not discussed here, these results are
compatible (but not proof yet) of the 13 -oxy-20-chloro-
structure 11 for Mephe-RC I. Recent field desorption data
support this structure.

B.c.: BIOSYNTHESIS OF TETRAPYRROLS

Radioactive labeling is the most sensitive method to study
metabolic pathways, but it has several shortcomings which
can be overcome by heavy labeling with stable isotops. The
latter are detected by either mass or NMR spectroscopy. A
particular advantage is the easy distinction of double
labeling within a subset of molecules from a statistical
distribution at two or more sites. BROWN et al. have used
^{17}O labeling in conjunction with masspectroscopy to estab-
lish a twofold oxygenation with O_2 during bile pigment form-
ation from heme in red algae. Spin-spin coupling in NMR gives
even a more detailed history on the biosynthetic pathway, as
demonstrated elegantly for the sequence of reactions involved
in the cyclisation of four porphobilingens to uroporphyrin
III (21a).
^{13}C labeling in conjunction with NMR has recently also been
applied to plants, where it provided conclusive evidence for
the predominance of the so-called C_5-pathway for chlorophyll
formation in maize (21 b,c). The key intermediate δ-amino-
levulinate (Ala) is formed in this pathway from C_5 compounds
like glutamate or ketoglutarate, rather than from succinate
and glycine. With the ^{13}C-NMR spectrum of chlorophyll a fully
assigned, the lable in any of the 35 C-atoms ofthe macro-
cycle can be quantitated in a single experiment. The
technique requires, however, a substantial incorporation of
^{13}C above the 1.1% level of natural abundance. Since the
uptake of organic substrates by plants is low, and their
metabolism more complex than that of animals, it may be
difficult to attain such incorporation economically, ^{14}C
incorporation can then be used to test the feasibility of the
experiment. PORRA et al. incorporated after such tests 15oo
excised primary leaves of maize seedlings with selectively
labeled ^{13}C-glutamate. The contribution of the C_5-pathway to
the chlorophyll biosynthesis in maize is from these experi-
ments $\geqslant$95%, and similar data are obtained for beans by
OHAMA et al. The only position labeled by 2-^{13}C glycine is
C-13^4, the methylester group at the isocyclic ring. Feeding
with 1-^{13}C glutamate yieled a label in all eight positions
originating from C-5 of Ala, and feeding 5-^{13}C-glutamate brings
the lablel to the two ester group C-13^4
Labeling in some other positions can furthermore be accounted
for quantitatively by a partial conversion of 5-^{13}C to
1-^{13}C-glutamate via the citric acid cycle.
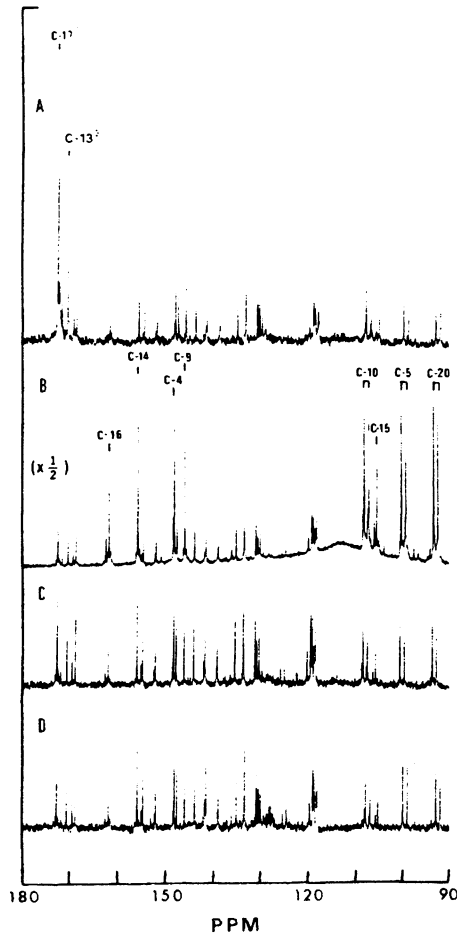
Fig. 3 A: Proton-coupled ^{13}C-NMR spectra from 90 ppm to
180 ppm of chlorophyll a produced by illumination of
excised etiolated maize leaves in the presence of 5-^{13}C
glutamate (A). 1-^{13}C glutamate (B) and 2-^{13}C glycine (C).
Spectrum D is the natural-abundance ^{13}C-NMR spectrum of
chlorophyll a. The vertical axes of spectra A. C and D
are expanded twofold relative to that of spectrum B. From
PORRA et al. (21b).

C: TETRAPYRROLES IN COMPLEX SYSTEMS

The functions of tetrapyrroles can only partly be unter-
stood on the basis of their molecular structure. The
properties of e.g. the biliproteins are quite different
(absorption, fluorescence) from what might be expected
from the structures of the chromophores. Also, a particular
pigment e.g. Chl a serves often rather different functions
(light harvesting, electron donor, electron acceptor) in
different environments. These changes are brought about
by chromophore-protein and chromophore-chromophore inter-
actions. To unterstand the function of tetrapyrroles, their
structure has thus to be known in a more general sense
which encompasses these interactions. These investigations
are generally most effective by the combination of bio-
chemical and spectroscopic techniques. The former allow a
study of the photosynthetic apparatus by topological stu-
dies and the isolation of functional subunits. Spectros-
copic techniques serve as a control to ensure that these
subunits have retained their properties, and as a means for
their structure analysis in the aforementioned general
sense. Neither one of the two standard methods for the
analysis of molecular structures is currently applicable
to complex systems like tetrapyrrole-protein complexes,
whole organelles or organisms, or just larger aggregates.
Proton NMR can generally not cope with the line broadening
caused by the comparably slow tumbling of these systems,
and mass spectroscopy fails for the difficulties involved
in the ionisation process. Furthermore are both methods
principally difficult to apply to mixtures. The need for
both selectivity and sensitivity favors applications of
two- or more dimensional methods, of which only three
shall be discussed here. The first is fluorescence spec-
troscopy, which permits among others the analysis of
complex mixtures and of energy transfer. The second is
RAMAN resonance spectroscopy (RR), which adds a second
dimension to the conventional absorption spectroscopy,
and which is furthermore accessible to rather precise
computer modeling. The third of the selected methods is
ENDOR and related magnetic double resonance techniques,
which are selective for paramagnetic species, and also
well suited for theoretical analysis.

C.a: BIOSYNTHETIC STUDIES USING FLUORESCENCE SPECTROSCOPY

Fluorescence is, within the limits of photostability of
the investigated structures, a non-invasive technique and
thus applicable to complex systems. By recording the
excitation spectra for a number of excitation wavelengths
(or vice versa), it is possible to obtain a minimum set of

Fig.4: Branched pathway for the biosynthesis of
Chl a and homologues, as suggested by REBEIZ
et al. (ref.10). The classical pathway is the
second one from the right. The structure of
divinyl-Chlorophyll a (12,boxed) has been con-
firmed by NMR, MS and chemical correlation
(12,22).
From REBEIZ (ref.20).

12: R = phytyl

components contributing to the fluorescence. The excitation
spectra are furthermore similar to the absorption spectra
of the individual components, if no energy transfer among
the pigments (section C.b.) or other nonlinear effects take
place. The two major limitations are the necessity of
sufficient fluorescence, which has been improved e.g. by
photon counting, but also the difficulty to establish
a full structure from the absorption spectrum alone.

The metabolism of chlorophylls has long been known to
involve a number of cyclic tetrapyrroles which are fluore-
cent (19). REBEIZ and coworkers (20) have exploited the
fluorescence analysis of greening tissue to obtain more
details on the biosynthetic pathway. In addition to the
previously known chlorophylls and their metabolites they
were able to characterize both in vivo and in extracts a
variety of pigments with shifted absorption fluorescence
spectra. From a careful comparison of the excitation spectra
with the absorption spectra of known pigments, they suggested
in particular a branched pathway leading to chlorophylls
which carry not only the common vinyl group at C-3, but
have also still kept the second one at C-8 all the way from
protoporphyrin IX (fig. 4). The major argument for these
structures was, that their excitation spectra showed like
semisynthetic 3,8-divinyl-pigments, a considerably (≈ 10nm)
red-shifted SORET-, but only minimally shifted (2-3 nm)
long wavelength band. This proposed structural principle
has now been proved for at least few of these pigments by
the more informative methods described in section B. BAZZAZ
et al. (21) have isolated 8-deethyl-8-vinyl-chlorophyll a
(12) from a mutant accumulating large amounts of this
pigment, and established its structure by NMR and mass
spectroscopy, and REBEIZ et al. (22) have furthermore per-
formed chemical correlations with this and other related
pigments.

It is nonetheless desirable to increase the information
content of the in situ fluorescence analysis, because the
necessary reference material may not always be at hand. One
such possibility is its extension in the form of fluores-
cence detected magnetic resonance (FDMR). WOLF et al., and
others (23) have recently applied this technique to study.
the pigments in photosynthetic bacteria. The biosynthetic
pathway of bacteriochlorophyll a has been known in many
details from studies with mutants (19). The conventional
fluorescence analysis of Rhodopseudomonas spheroides and
other photosynthetic bacteria made now most of these pig-
ments visible in whole cells, and indicated that some
additional ones are present. In FDMR, the fluorescence at
a selected pair of excitation/emission wavelengths is

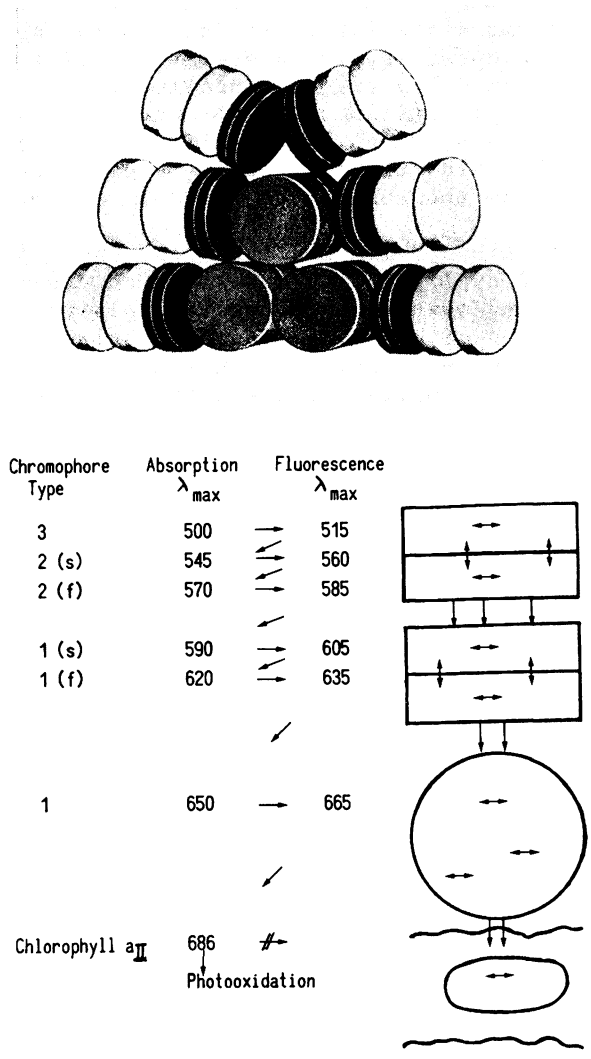| Chromophore Type	Absorption λ_{max}	Fluorescence λ_{max}
3	500	515
2 (s)	545	560
2 (f)	570	585
1 (s)	590	605
1 (f)	620	635
1	650	665
Chlorophyll a$_{II}$	686	

Photooxidation

Fig.5: Models for the Rhodella violacea Phycobilisome structure (a, from refs.24c) and energy transfer scheme (b, from refs.3). The outer "plates" (λ max=500-570 nm, light) in (a) contain B-PE, the intermediate ones (dark in (a), λ max=590-640 nm) contain C-PC, and the inner core (gray in (a), λ max=650-670 nm) contains APC.

monitored while a microwave field is swept, which gives the
zero-field splitting parameters of the pigments in their
triplet states. When applied to the bacteria, these para-
meters could be obtained for most of the pigments previ-
ously identified by fluorescence alone, and they agreed
well with the expected values. The full potential of this
technique is probably not yet exploited. First examples
have demonstrated its use where unusual zero-field
splittings can be expected. This does not only include
chlorophylls complexes with unusual metals and biosynthetic
precursors of the lesser investigated (bacterio)chloro-
phylls, but also perturbations due to the particular envir-
onment of a given pigment, viz. to the study of pigment-
protein and pigment-pigment interactions.(23 a, b d)

C.b.: ENERGY TRANSFER STUDIES USING FLUORESCENCE SPECTROS-
COPY

Another important application of fluorescence spectroscopy
has been the investigation of energy transfer processes.
The majority of plant tetrapyrroles is involved in the
light collection process of photosynthesis. They are bound
to proteins and arranged in large light harvesting arrays.
Light energy captured by one of the chromophores is
efficiently transferred to neighboring ones and by
repetition eventually migrates to the reaction center(s),
in which the conversion to redox energy takes place. Sever-
al such antenna systems have been studied,but probably none
of them in such detail as the phycobilisomes. These are
electron microscopically visible aggregates consisting
mainly of biliproteins, which are attached to the outer
surface of the photosynthetic membranes of blue-green and
red algae. Their participation in the light harvesting pro-
cess to photosystem II had been inferred from the classical
biochromatic experiments of EMERSON and coworkers, and
detailed studies became possible with the successful
isolation of entire phycobilisomes and their more or less
dissociated components (24). Phycobilisomes contain at
least two, generally three chromoproteins bearing bile
pigment chromophores. These are the green allophycocyanins
(APC), the blue phycocyanins (PC) and -optionally- the red
phycoerythrins (PE) (see fig. 5 for the spectral data).

All phycobiliproteins are highly fluorescent. Fluorescence
studies of intact phycobilisomes had shown, however, that
only the minor (generally 5 %) and red-most absorbing APC
is responsible for the fluorescence, irrespective of PE,
PC or APC being the absorber. The energy is thus trans-
ported with high efficiency from the higher energetic (PC,
PE) to the lower energetic component (APC). This transfer
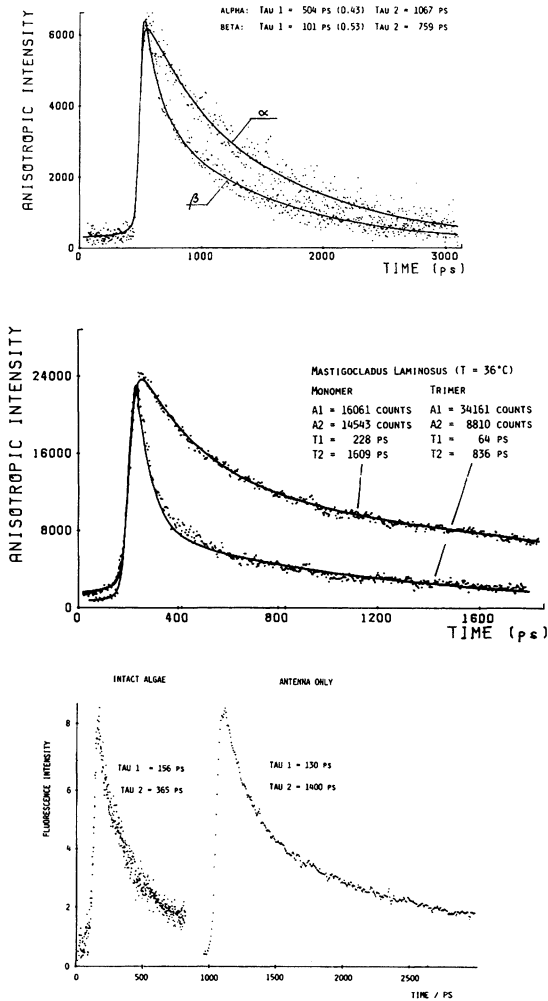
Fig.6: Fluorescence decay curves (points) and best fit
(Marquardt algorithm) for a biexponential
decay (solid lines). The maximum amplitudes
and the time constants are given in the insets.
(a) α-and β-subunits of PC from Mastigocladus
laminosus.(b) The same pigments in its tri-
meric state (α_3, β_3) and in its monomeric state
(α_1, β_1)induced by dissociation of the trimer
with KSCN. (d) Phycobilisomes from Mastigo-
cladus laminosus (right) and whole cells
(left). Figs.(a) and (b) give only the aniso-
tropic component of the fluorescence.Exci-
tation at 580 nm, pulse intensity 10^{14}photons
.cm^{-2}, repetition rate 80 MHz.

is uncoupled after the dissociation of the phycobilisomes,
a process which can be monitored by the gradual increase
of the fluorescence of the fragments. The biochemical and
fluorescence analysis of the fragments yielded a model
in which the morphology of the pigments as well as their
absorptions and emissions are optimized for an efficient
energy transfer (fig. 5). Most if not all of the chromo-
phores are weakly coupled and the energy is transfered
by a Förster type process, which is also supported by
circular dichroism of the isolated pigments (25).

Whereas this static analysis is well suited to study the
energy transfer between chromophores with well separated
absorptions, the individual transfer steps between
neighboring and more alike chromophores required more
sophisticated methods. Fluorescence depolarization revealed
a further subdivision of the chromophores (26). Even in
pigments like PC and many of the PE's bearing up to six
chromophores of the same molecular structure, is the
absorption (and fluorescence) of the individual chromo-
phores fine-tuned for an efficient energy transfer. The
three chromophores of C-PC absorb, for example, around
590, 610 and 620 nm, which allows a graded energy trans-
fer even on this level. The static fluorescence experiments
have been complemented by time-resolved studies. PORTER
et al (27) monitored the small amount of leakage
fluorescence emitted by PE, PC, and APC in intact algae.
After excitation with green light absorbed mostly by PE,
the PE fluorescence rises first, followed by the fluores-
cence from PC and finally from APC. Excitation with laser
pulses of varying photon fluence has also been used to
study the migration of excitation energy via singlet-
singlet annihilation, which gives information on the
number of coupled chromophores (28). It could e.g. be
shown, that   bacterial/antenna preparations with different
detergents may have rather different numbers of coupled
chromophores, in spite of their identical absorptions
(28 b). These studies showed on the other hand, that
rather low fluence rates are required to prevent inter-
ference from the latter process on the fluorescence decay
rates obtained by picosecond time-resolved spectroscopy
(see ref. 29 for a critical discussion).
Picosecond time-resolved spectroscopy has now been applied
by several groups to investigate the energy transfer both
in intact phycobilisomes and their componenents. (41,43).
The is rather complex . Each one of the applied methods of
measurement and data handling has certain advantages and
shortcomings, which are complicated by preparative problems.
A consistent and detailed model can thus not yet be given and
will require considerably more data.

Table 2: Fluorescence decay (in psec) of phycocyanin
from Spirulina platensis

		Trimer		Monomer	
	T (°C)	τ_2 (%)	τ_1 (%)	τ_2 (%)	τ_1 (%)
Isotropic	18.6	1300(47)	320(53)	2200(51)	300(49)
	39.2	1500(49)	270(51)	2200(54)	290(46)
	51.8	1600(36)	260(64)	3200(51)	400(49)
Anisotropic	18.6	–	70(100)	1050(37)	150(63)
	39.2	–	70(100)	1000(38)	250(62)
	51.8	350(54)	70(46)	3500(47)	380(53)

The isotropic decay curves are calculated from $I_{\parallel} + 2I_{\perp}$,
the anisotropic ones from $I_{\parallel} - I_{\perp}$. In a different set of
experiments with C-PC from Mastigocladus laminosus, the
calculated isotropic decay curve has been shown to agree
satisfactorily with the experimental curve obtained with
the emission polarizer adjusted to 55°.

We have recently started a systematic investigation of
isolated phycobiliproteins and their aggregates by time-
resolved fluorescence depolarization. These studies use a
repetitive streak camera for the detection,which allows for
reduced photon fluence in the individual pulses. In a study
with PC from two different algae, it could be shown that
there is always a complex decay of fluorescence, which can
be fit satisfactorily by a biexponential (fig. 6). The
analysis of the components in the isotropic and anisotropic
fluorescence yielded different amplitues and rate constants,
but the slow component was always less pronounced and in the
aggregated pigments even absent in the polarized fluorescence
(table 2). Since rotational depolarization can be excluded
for proteins of this size on the picosecond time-scale, this
depolarization has been assigned to energy transfer among
the individual chromophores. It is accordingly more pronounced
in the aggregates and appears to be absent in the $\propto$-subunit
bearing only a single chromophore (fig. 6a). The two rather
long-lived components are here assigned to two species, the
shorter lived being probably an artifact from the denaturation
-renaturation necessary to separate the subunits. Additional
support for the interpretation of the short lived component
to reflect energy transfer comes from the increased difference
between isotropic and anisotropic fluorescence with increasing
aggregation (table 2). The decreased lifetimes in whole algae
(fig. 6c) are finally understandable on the basis of a
further transfer to the chlorophylls which acts as an effi-
cient quencher for the biliprotein fluorescence. It should
be pointed out, however,that a quantitative analysis of this
process is at present rather difficult and depends not on
the signal-to-noise ratio of the data, but also critical on
the model. It was thus not possible to distinguish a biex-
ponentialfrom a higher exponential or a more complex decay
law (e.g. $e-\sqrt{t}$), which might be expected for a FOERSTER
type energy transfer.

High-resolution spectroscopy in the time domain can also
be supplemented by high-resolution spectroscopy in the fre-
quency domain. Hole-burning experiments have been performed
with chlorophylls and biliproteins (fig. 7) to yield high-
resolution absorbtion spectra (42). The process involves
site-selective photochemistry at liquid helium temperatures,
most likely protein transfer reactions, which leave a "hole"
in the absorbtion band corresponding to the natural line-
width as determined by the kinetics of the process. In the
case of C-phycoerythrin, these holes are acompanied by
slightly wider satellite holes at the long-wavelenght side
of the primary hole. They may be related to energy transfer
between the different chromophores in this pigment (42a).
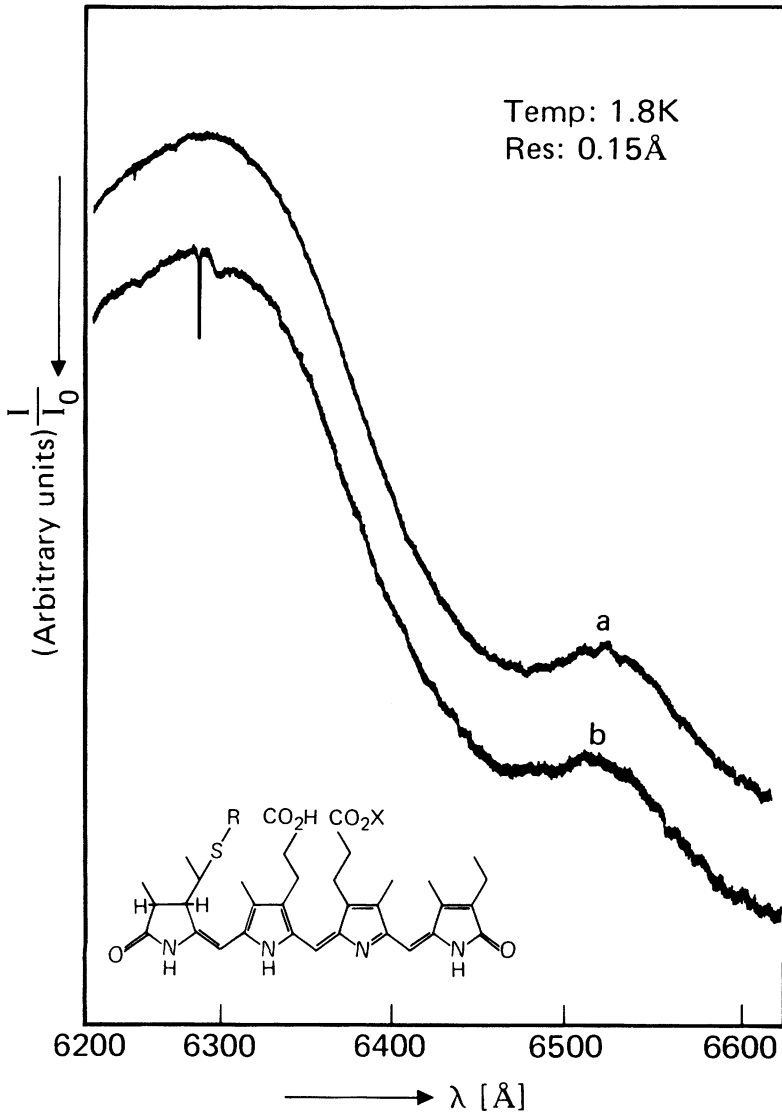
Fig.7: Hole burning experiment with a mixture of
       C-<u>PC</u> and <u>APC</u> from <u>Spirulina platensis</u>.
       Absorption spectrum at 1.7 K before (a) and
       after irradiation (b) with a narrow-band
       laser. The sharp hole develops at the laser
       frequency. It is accompanied by a broader
       phonon side-band.

C.c: NATIVE STATE OF CHROMOPHORES

The properties of tetrapyrole chromophores are usually considerably changed when they are bound to the native apoproteins. Spectroscopy has become in many ways a mediator between the native structures and models mimicking certain aspects of this state . Chlorophyll aggegation has played an important role to evaluate its donor-acceptor properties for coordinative bonding (44). Although the self-aggregation of chlorophylls appears currently less important in vivo, the interactions with the protein are governed by essentially the same factors. It is also well possible, that the forme. have been an older evolutionary feature which has later on been replaced by the latter, which may be suggested from the antenna system of green bacteria.

In the biliproteins, conformational changes have been made most likely as an important factor determining the properties of the native state. The uv-vis spectral differences between native and denatured biliproteins, e.g. PC or Pr, can be accounted for by an extended and cyclic conformation,respectively, of the chromophore (3). This has been supported both by the properties of synthetic bile pigments with restricted conformational freedom, and by MO calculations. The comparable large red-shift of native P_{fr} can, however, not been rationalized on this basis alone. There are only two experimentially studied processes leading to similarly large red-shifts, e.g. deprotonation and cation radial formation. Since the latter is unlikely from ESR data, the extended anionic 15-E structure 1c has been suggested for native P_{fr} (1,3).More recently,we have obtained evidence that the high fluorescence of native biliproteins can be explained in terms of a restricted conformational freedom. This interpretation comes from the high fluorescence yield of rigid bilirubins, which we investigated in cooperation with A.R. Holzwarth from Mülheim (Isr. J. Chem., in press).

1c

Structure 10a   Structure 10b
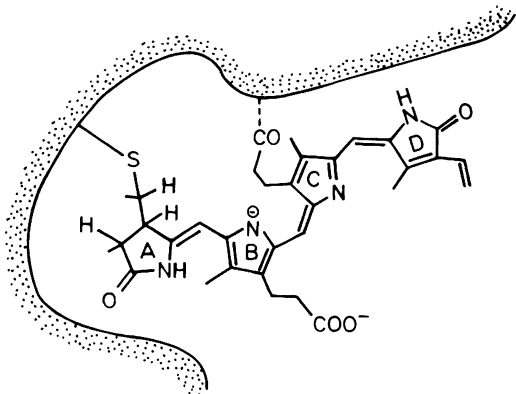
Fig.8: Suggested geometry for the __PC__ chromophore in
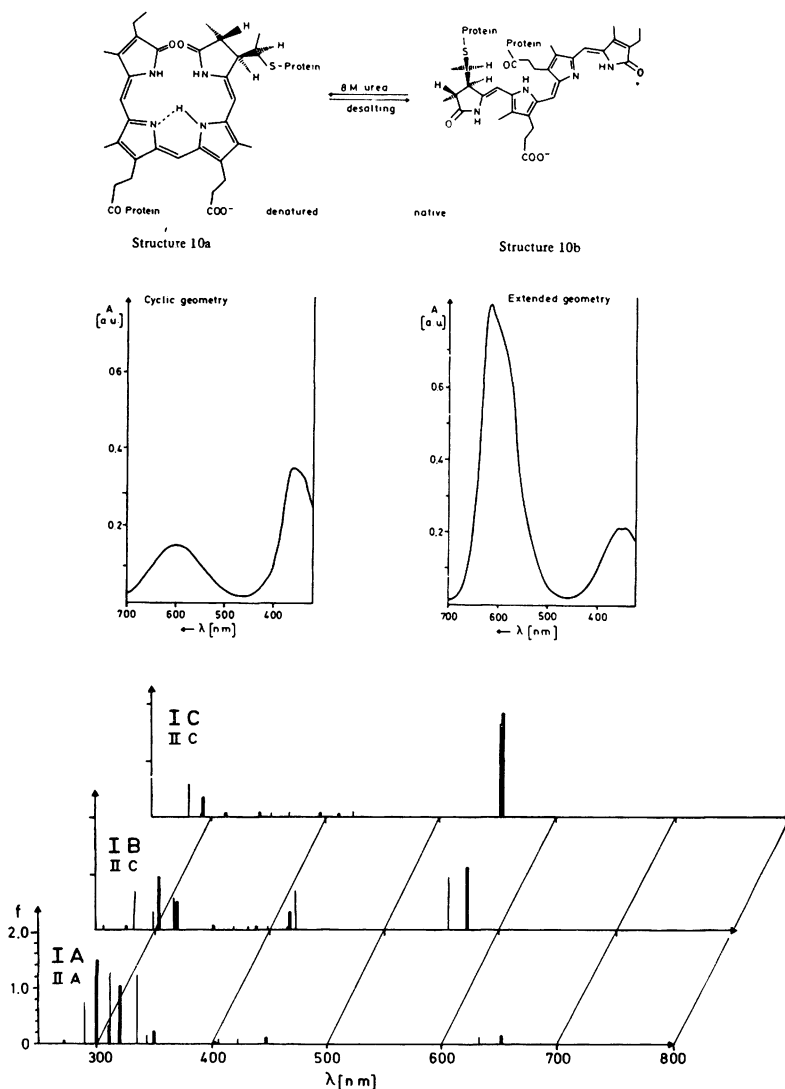   its native (right) and denatured state, to-
       gether with the corresponding experimental
       absorption spectra (bottom of (a)) and the
       calculated spectra (I,II A,C, respectively).
       I and II denote the tautomer, carrying a
       proton at N_{22} and N_{23}, respectively. B is
       the calculated spectrum for a semi-open
       geometry.

C.d: ENDOR SPECTROSCOPY OF THE PRIMARY DONOR IN PHOTO-SYNTHESIS

Electron spin resonance (ESR) is a sensitive and highly selective technique for the investigation of paramagnetic species. It has also a comparably large information content and its theory is well worked out to allow the investigation of structures without too stringent requirements with regard to model compounds. The two major groups of paramagnetic structures in biological systems are heavy metal ions and organic radicals. Both participate - among other functions - in biological oxidations and reductions, and important information has been obtained on these systems by the application of ESR. Major limitations are the overlap of signals from organic radicals, which are usually clustered within a small spectral range and the limited time resolution, which has only recently been extended to the sub-nanosecond time scale.

One of the first studies of ESR in biology showed, that an unstructured line can be observed in photosynthetic tissue upon illumination. This signal (fig. 9a) has later been assigned to the cation radical of the primary donor of bacterial reaction centers, or of photosystemIof green plants (29). The primary step during the photosynthetic energy conversion is the electron transfer from an electronically excited chlorophyll molecule to an acceptor which is probably also a chlorophyll or pheophytin. This leaves the primary donor as a cation radical, which is re-reduced on the μsec or msec time scale. Since the latter process is in most reaction centers rate limiting in strong light, the primary donor can be fully converted to the oxidized form during saturating irradiation. This results in the reversible, light induced formation of a strong ESR signal, and in the concomitant bleaching of absorption bands associated with the primary donor chlorophyll.

The optical changes are usually monitored around 700, 870 and 960 nm in the reaction centers of PS I, Bchl a and Bchl b containing bacteria, respectively. They are rather small, however, in all but highly enriched reaction center fractions, because they overlap with the absorption of antenna pigments present in large excess. Since the latter are ESR inactive, the ESR signal does not suffer from this interference. After the kinetic correlation of the two signals   and its assignment to a chlorophyll cation radical, has ESR therefore been used extensively to monitor and to obtain structural information on the primary donor. The most data have currently been accumulated on bacterial systems, because the chemical identity of its origin is
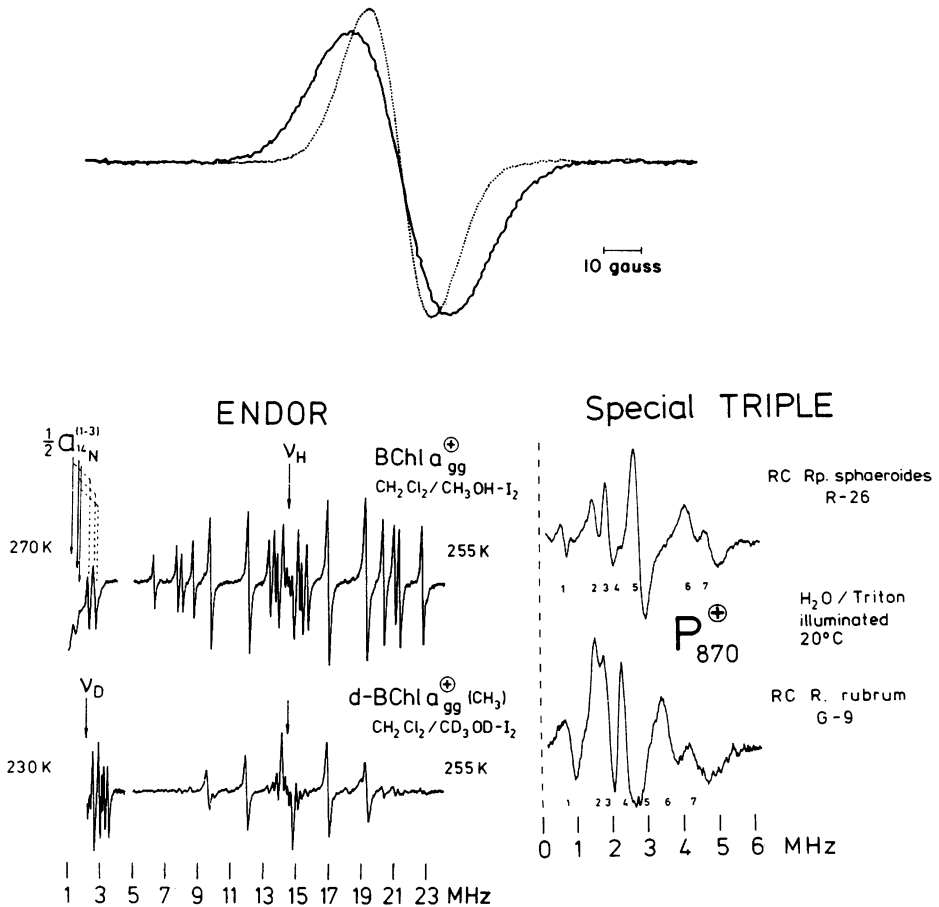
Fig.9: (a) ESR spectrum of the cation radical
Bchl a$^{+\bullet}$ in methanol /CH$_2$Cl$_2$ (heavy line)
and of P 870$^{+\bullet}$ in isolated reaction centers
from Rhodopseudomonas spheroides (thin line).
(b) ENDOR spectrum of fully protonated (upper
trace) and selectively deuterated Bchl a$^+$ in
the same solvent system. (c) Special TRIPLE
spectrum of P 870$^+$ in reaction centers from
Rhodopseudomonas spheroides R26 (upper trace)
and Rhodospirillum rubrum G9. In this tech-
nique, two radiofrequencies are applied
simultaneously which are symmetric to the free
proton frequency (=ν_H in b).

well established (Bchl a or Bchl b depending on the
species), and reaction centers can be isolated which are
essentially free from other components of the photo-
synthetic apparatus (30).

The ESR spectra of chemically oxidized bacteriochloro-
phyll a in solution and of the light induced in situ
signal of the primary donor in Bchl a containing bacteria
are very similar (fig.9a). The only exception is the line
width, which is reduced in the natural systems by a factor
of 1.4, NORRIS and coworkers were the first to relate this
narrowing to aggregation, and arrived at an aggregation
constant of two from a second moment analysis ("special
pair" model) (31). This interpretation was later on
supported by electron-nuclear double-resonance (ENDOR)
spectroscopy. This allowed the resolution of at least
five coupling constants in the inhomogeneously broadened
ESR line. All resolved ENDOR couplings were reduced in the
in situ signal by a factor of about two as compared to the
signal of free Bchl b, which strongly supported the above
model (32).

A drawback of earlier ENDOR studies had been their
restriction to low temperatures ($\leqslant$ 77K). The development
of ENDOR at ambient temperatures by MOEBIUS and coworkers
prompted us a few years ago, to study in cooperation with
them bacterial reaction centers in liquid solution (33).
This is not only an advantage with respect to the more
physiological temperatures, but gives also a considerably
increased resolution due to the averaging of dipolar
interactions (fig. 9   ). The ENDOR lines were assigned by
a combination of isotope labeling, chemical correlations
and spectroscopic techniques (fig. 9,b,c) and the results
are summarized in table 3. for reaction centers from two
photosynthetic bacteria, Rhodopseudomonas spheroides and
Rhodospirillum rubrum. There are some distinct and pre-
paration independent differences between the species (fig.
9) but the general picture is identical: All coupling con-
stants in the reaction centers are reduced in comparison
to the respective couplings from oxidized Bchl a in solu-
tion. The reduction factors are, however, considerably
different for different proton couplings, which indicates
a redistribution of the spin densities in the oxidized
primary donor. The average reduction factor is, however,
again close to two, thus indicating again a dimeric
structure for P870. There is furthermore only one set of
lines for each coupling in the in situ species, e.g. the
individual lines are not split. Since such a doubling of
lines would be expected for an unsymmetric spin distri-
bution over the two halves of the special pair, this argues

Table 3: Comparison of isotropic hfc's a (MHz) of
Bchl $\underline{a}^{+}$(255 K) and P_{870}^{+} (20°C) cation radicals

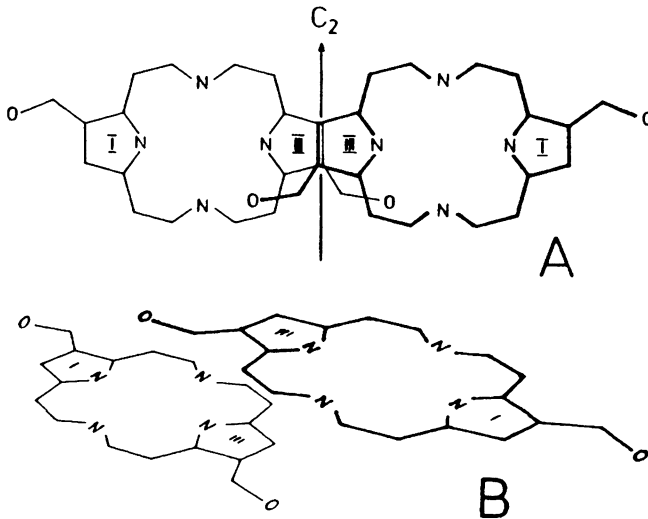| position	Bchl $a_{gg,P}^{+}$ a	R-26		G-9	
		P_{870}^{+} a	RF	P_{870}^{+} a	RF
CH_3 (1a)	+ 4.85	+4.00	1.21	+3.40	1.43
CH_3 (5a)	+ 9.50	+5.60	1.70	+4.85	1.96
ß-H	+11.61	+3.30	3.52	+3.95	2.94
ß-H	+13.00	+4.45	2.92	+5.28	2.46
ß-H	+13.59	+8.60	1.58	+7.50	1.81
ß-H	+16.43	+9.50	1.73	+8.50	1.93

Fig. 10: Proposed geometry for P_{870}.t, the oxidized
primary donor of bacterial (Bchl a)photo-
synthesis. The plane-to-plane distance of the
molecules is 3.5-4A. The fit is obtained from
simulations of the ESR line shown in fig. 9
with the couplings from table 3.

for a dimer with C2 symmetry.

The experimental spin densities can now be used to
simulate the ESR spectrum. A satisfactory fit is again
only obtained under the assumption of a dimeric species
for the oxidized primary donor. Based on these data has
the Berlin group recently started molecular orbital cal-
culations to assess the geometric relationship of the two
molecules in the special pair. The two Bchl a molecules are
treated as a super-molecule in these calculations. The
preliminary results make the model shown in fig.10 most
likely. The two molecules have a C2 axis, and they are
essentially parallel $(\leqslant 5^{\circ})$ to each other at a distance
of 3.5 to 4 A. The two macrocyles are, however, shifted
against each other and overlep only in the region of ring
three. This model fits all the data, it accounts in
particular for the symmetry of the entire system, and
also for the observed spin density redistribution in the
two halves.

Whereas it is rather nicely self-consistent, some con-
straints should be pointed out. It is firstly only
applicable to Bchl a containing reaction centers from the
Rhodospirillales, and the available ENDOR data indicate
a rather different situation for Bchl b containing reaction
centers and for the photosystem I and II of oxygenic
photosynthesis. This is also evident from recent ESR
experiments with organisms highly enriched in both ^{13}C and
^{2}H, which rather support a monomeric structure for the
latter (35), and from the recent indication that a
pigment structually different from Chl a is present in
photosystem I (section B.b). There is finally a suggestion
that severe distortions of the highest occupied molecular
orbital (by mixing in of the energetically close lowest
unoccupied MO), could principally explain the experimental
data also with a monomeric species (36). Although none of
our data give indications that this suggestion is correct,
the model predicts some characteristic features (high
spin density at the central nitrogen and methine positions)
which are at least principally accessible to conclusive and
direct measurements (e.g. ^{15}N-labeling). Such tests are
currently under way.

C.e.: RAMAN RESONANCE STUDIES IN PHOTOSYNTHESIS

RAMAN resonance (RR) is probably one of the most general
techniques for the study of complex pigment systems, be-
cause it is very sensitive, widely applicable, quite
selective, provides an extremely high time resolution and
is accessible to a rather straightforward theoretical
analysis. The technique makes use of the strong enhancement
of the RAMAN spectrum of pigments, if the excitation occurs
in one of its absorption bands. This reduces the background
from ubiquitous components like proteins on water
sufficiently for a selective study. The major problem -
besides technology - is the interference of even low
levels of fluorescence. Unfortunately are most photo-
synthetic pigments highly fluorescent, but this problem
can be circumvented by excitation into higher excited
states, e.g. into the SORET band. Another problem is then,
however, the overlap of the absorption lines of many
pigments in this spectral range, which can partly be
circumvented by appropriate biochemical manipulations (iso-
lation of complexes, redox changes) and by spectroscopic
means (variation of the excitation wavelength). Two
laboratories have concentrated in the past few years on RR
spectroscopy of photosynthetic pigments, in particular
the chlorophylls (37,38). COTTON et al. (38) have begun
with a systematic study of in vitro systems, e.g. free
chlorophylls in different states of aggregation and
oxidation, to obtain an interpretational basis for
photosynthetic complexes. LUTZ et al. (37) have started
with biological systems (whole cells, chlorophyll-protein
complexes), and based their interpretation strongly on
such complexes for which good structural data are avail-
able (e.g. the water-soluble antenna bacteriochlorophyll-
protein from Prostecochlorus aestuarii).

There are two spectral regions in the RR spectra of chloro-
phyll (-protein) which are well accessible to analysis.
One is the carbonyl region, which shows the C=O groups
which are conjugated to the macrocycle π-system. Their
binding state and conformation can be assesed from shifts
and intensity variations, respectively, in the carbonyl
stretching bands. The major conclusion from these data was,
that a differentiation is possible among most of the indi-
vidual carbonyl groups   to be expected from the number
of chlorophylls present in a particular chromoprotein, and
that most if not all carbonyl groups are hydrogen bonded,
probably to the apoprotein. The second most informative
region is around 300 cm^{-1}, which has been assigned to the
central Mg-N vibrations. There is again one general result,
e.g. the central Mg is five coordinated in most of the pro-

tein bound pigments.

Studies with bacterial reaction centers showed changes in
the carbonyl region upon oxidation of the primary donor,
but few changes in other regions. LUTZ has used this as
an argument against any close association among pigments,
and in particular against the special pair model, because
more pronounced changes are expected in aggregated systems
(37a). The full meaning of this discrepancy is currently
not yet clear. ENDOR and RR work at rather different time
scales, which means that a hopping rate in the region of
10^{12} sec^{-1} would be seen as a delocalized electron by the
former, but a localized one by the latter technique.
BOWMAN and (39) NORRIS have recently narrowed this time
gap by spin-echo experiments, but it is still in the range
of two orders of magnitude.

It is also desirable, that the RR method becomes more
selective for the different pigments present in reaction
centers, since only one or two of the six tetrapyrroles
are part of the primary donor. Two promising extensions
of the conventional technique have recently been applied
for the first time to chlorophyll proteins, which may be
useful in this respect. One is the surface enhanced RR,
the complexes are deposited on a silver electrode. COTTON
et al. (38a) have shown with bacterial reaction centers,
that this enhances strongly the sensitivity and quenches
the interfering fluorescence, and that it is also possible
to enhance selectively the RR signal from different pig-
ments within the reaction center by variation of the
potential applied to the electrode. Another technique which
is at least principally related to RR is the coherent anti-
STOKES RAMAN scattering. Its most important advantage over
conventional RR is its greatly reduced sensitivity to
fluorescence, because the vibrational lines are observed at
the high-rather than the low-frequency side of the ex-
citation line. HÖXTERMANN et al. (40) have demonstrated,
that this permits the measurement of chlorophylls in the
red spectral region, which is much more sensitive to
variations in the chlorophyll environment and thus allows
more selective studies. Although the method seems to be
somewhat less sensitive in the carbonyl region, it allowed
like the RR technique a distinction of several bands as-
signed to the carbonyl groups of Chl a and Chl b mole-
cules differing by their different environments. This site-
specific distinction is supported by the analysis of the
more intense spectral region between 1600 and 100 cm^{-1}. It
is dominated by the double bond stretching vibrations of the
the conjugated system, which also yield multiple lines in
the chlorophyll proteins. The decreased interference of

fluorescence in <u>CARS</u> renders its application to
other pigments possible, e.g. the biliproteins.
There is only a limited amount of RR data on their
chromophores (40, and <u>CARS</u> may help to get more
details about their native chromophores.

<u>Conclusions</u>
It has been tried to sketch some recent examples of spec-
troscopic techniques to investigate plant tetrapyrroles,
either isolated or in their more or less intact natural
environment. It should be emphasized that all the selected
examples required the cooperation of spectroscopists and
biochemists although the latter havebeen neglected in this talk.
None can do without the other in such research, which poses
some problems in timing, a multiplied chance of breakdowns,
and last not least sample transfer. The examples show, that
these shortcomings are sometimes compensated by the results,
which have led to an improved picture of the structure and
function of tetrapyroles on the molecular level.

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