Dr. rer. nat. habil. Heiko Lokstein
UNIVERZITA KARLOVA
MATEMATICKO-FYZIKÁLNÍ FAKULTA
KATEDRA CHEMICKÉ FYZIKY A OPTIKY

Ke Karlovu 3
121 16 Praha 2
Czech Republic

Tel. 95155 1396
Fax 22191 1249
lokstein@karlov.mff.cuni.cz

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Assessment of the Ph.D. thesis:

Excited state processes in linear conjugated systems

by Mgr. **Václav Šebelík**

The PhD thesis of Mgr. Vaclav Šebelík covers investigations of excited state processes in carotenoids and other linear conjugated organic molecules (polyenes, which can be considered as model systems for carotenoids), using ultrafast laser spectroscopy. Carotenoids are a class of biogenic pigments that occur in virtually all known life-forms. Carotenoids are particularly important in photosynthetic organisms, both, oxygenic and anoxygenic ones. One major function of carotenoids is to provide efficient photoprotection of the photosynthetic apparatus(es). The chromophore of all carotenoids is a (more or less linear) conjugated C=C double bond system, which can be formally derived from linear polyenes. Thus, the excited states of carotenoids (and other polyenes) are conventionally described and annotated by analogy to C_{2h} symmetric molecules.

The investigation of carotenoid photophysics, in particular, in pigment-protein complexes, is particularily challenging, since several excited states, among them the (functionally very relevant) lowest singlet excited state (S_1, $2^1A_g^-$), are optically forbidden ("dark"), i.e., transitions between the ground state (S_0, $1^1A_g^-$) and them are forbidden by symmetry selection rules.

The thesis is overall well structured. The current state of research is succinctly covered in the "Introduction", so that the objectives of the investigations can be easily derived. The quoted literature shows no noticeable bias.

The first chapter of the thesis provides a vivid introduction into the - indeed - fascinating world of carotenoids, from historical/geological, biochemical and spectroscopic perspectives.
This reviewer, however, does not completely agree with all of the presented views: The "first chapter" of the "carotenoid story" was certainly written well before the mentioned 2 billion years (by) ago: Namely, at about 3.5 by before present first cyanobacterial-like fossils and oxydation of sediments can be found in Western Australian sedimentary rock strata. One can hardly imagine that these early oxygenic photosynthetic organisms were devoid of carotenoids.

The second chapter describes the spectroscopic techniques used in the current thesis. The methods are adequate to tackle the problems to be addressed and their information contents as well as the data analyses are sufficiently described. Different methodologies of ultrafast laser spectroscopy were used: Transient absorption (TA) spectroscopy, including TA following two-photon excitation (2PE), and pump dump-probe experiments. Additionally, the Z-scan technique and its information contents are described.

The following chapters (papers I-IV) are written in the form of **published/accepted** papers in renowned peer-reviewed international scientific journals, indicating the high significance of the described research.

Paper I. V. Šebelík, M. Fuciman, R. G. West, T. Polívka, Time-resolved two photon spectroscopy of carotenoids. Chem. Phys. 522 (2019) 171–177.
2PE is commonly assumed to be a suitable technique to assess dark states of carotenoids, in solution and in pigment protein complexes. The author has developed an experimental set-up to measure TA spectra of carotenoids following 2PE to excite the dark S_1 ($2^1A_g^-$) directly. 2PE and one-photon excitation (1PE) data of three carotenoids in solution, lycopene, β-carotene, and neurosporene, were compared. TA spectra of all three carotenoids are broader after 2PE as compared to 1PE, suggesting larger conformational disorder following direct excitation of S_1. Slightly longer S_1 lifetimes after 2PE were assigned to an increased S* signal. 1PE and 2PE were assumed to excite different subsets of conformers.

Question (Q): Wouldn't one rather expect a larger conformational disorder following 1PE (into S_2, with following relaxation, depositing excess excitation energy into the system) than after direct excitation of S_1 by 2PE?

Paper II. V. Šebelík, M. Kloz, M. Rebarz, M. Přeček, E.-H. Kang, T.-L. Choi, R. L. Christensen,T. Polívka, Spectroscopy and excited state dynamics of nearly infinite polyenes. PCCP, *in press.*
Excited state properties of polyenes and carotenoids strongly depend on the the number of conjugated double C=C bonds (N). Steady-state and TA spectra were obtained for two conjugated polymers with $N > 200$ in solution. The polymers exhibit similar decay pathway as observed for polyenes with N = 11-19 and zeaxanthin homologues with N = 11-23. TA spectra with polydiactylene and a much longer, more disordered polyene polymer (Poly(DEDPM)) show that the S_2, S_1, and S* lifetimes of the polymers are almost identical. The S* signals in the polymers are assigned to absorption from vibrationally excited S_0. The observed limit for the $S_0 \rightarrow S_2$ transitions would be consistent with the conservation of bond length alternation in S_0 and a HOMO-LUMO band gap in polyenes with $N > 200$. Coincidence of the $S_0 \rightarrow S_2$ origins and the convergence of the excited

state lifetimes point to a previously proposed common, "nearly infinite" polyene limit.

Q: Regarding the S* state: If S* is a hot S_0 wouldn't one expect **slower** vibrational cooling in larger (or close to infinite) conjugated systems? … in particular with regard to the fact that S* is observed only for carotenoids with N > 13?

Paper III. V. Šebelík, R. G. West, E. Kuthanová Trsková, R. Kaňa, T. Polívka, Energy transfer pathways in the CAC light-harvesting complex of *Rhodomonas salina*. BBA, *in press.*
Photosynthetic organisms have evolved a large variety of light-harvesting complexes (LHCs). Cryptophyte algae combine membrane-extrinsic (phycobiliproteins) and -intrinsic LHCs. Cryptophytes contain membrane-bound Chl *a/c* LHCs (CACs). TA spectroscopy was used to study EET in CAC of *Rhodomonas salina*. The major carotenoid in CAC, alloxanthin, is cryptophyte-specific. Alloxanthin is the only naturally occurring carotenoid with two C triple bonds. Three excitation wavelengths were chosen to excite different pigments. Upon excitation of Chl *c* at either 590 or 640 nm EET between Chl *c* and Chl *a* is demonstrated. Excitation of alloxanthin at 505 nm suggests EET from alloxanthin S_2 to both, Chl *a* and Chl *c,* with an efficiency around 50% and an ~ 1:1 branching ratio. EET from S_1 to the Chls is rather inefficient. 57 ps-EET to Chl *a* suggests a ~25% efficiency of the S_1 route. This low efficiency also renders overall carotenoid-Chl EET efficiency low, according to the authors indicating a regulatory role of alloxanthin.

Q: This reviewer thinks that the low efficiency of the S_1 EET from alloxanthin to Chls is not necessarily indicative of a regulatory role. Wouldn't a regulatory role (in response to changed external or internal conditions) require some change in carotenoid composition (or conformation) such as provided, e.g. by the so called xanthophylls cycle in higher plants?

Comment: The author(s) should apply **standard** Chl nomenclature/abbreviations in their manuscript(s), i.e. Chl *a* (*a* italicized and without hyphen).

The contributions of the author(s) are clearly discernible in the above manuscripts: VŠ performed the TA measurements, analysed the data, participated in writing and revision of the first three manuscripts.

In the last paper/chapter VŠ assisted in developing the population dynamics model:

Paper IV. R. G. West, M. Fuciman, H. Staleva-Musto, **V. Šebelík,** D. Bína, M. Durchan, V. Kuznetsova, T. Polívka, Equilibration dependence of fucoxanthin S_1 and ICT signatures on polarity, proticity, and temperature by multi-pulse femtosecond absorption spectroscopy. J. Phys. Chem. B 122 (2018), 7264–7276.

Some (strongly) substituted carotenoids, including fucoxanthin, feature intramolecular charge transfer (ICT) states, the relationship of which to the S_1 state is still being debated. The data indicate that lowering polarity and proticity of the solvents, as well as of the temperature, delay S_1/ICT equilibration. Using a two-state model, co-developed by the author of the thesis, it is suggested that the data are consistent with the concept that S_1 and ICT are on the same potential energy surface.

Q: Does the the concept, that S_1 and ICT are on the same potential energy surface, also apply to other carotenoids with ICT states?
Q: What do you think might be the functional significance of ICT states of carotenoids in organisms?

The author of the thesis has shown that he can successfully tackle a challenging research topic on the forefront of the field of photo(bio)physics. The current thesis is certainly a significant scientific contribution to the field.

This reviewer strongly recommends acceptance of the thesis by the Faculty of Science, University of South Bohemia.

Sincerely yours,

(Heiko Lokstein)

Technische Universität München | Lichtenbergstr.4| 85748 Garching

To whom it may concern
University of South Bohemia
Faculty of Sciene
Institute of Physics

Garching bei München, September 16th 2020

Evaluation of the PhD-Thesis entitled "Excited state processes in linear conjugated systems", by Václav Šebelík

The thesis specified above employs an extended toolbox of ultrafast spectroscopy to describe the energy level scheme and deactivation dynamics of biological relevant molecules and a pigment protein complex. The emphasis lies on systems with an extended π-electron network, more specifically carotenoids and polyenes. Carotenoids are fundamental building blocks of natural light harvesters with notoriously complicated and ultrafast energy deactivation networks. In order to disentangle such complex relaxation dynamics, the vast majority of studies – as convincingly and comprehensively surveyed in the thesis under discussion – employs transient absorption measurements. One way forward is to invoke data analysis of growing complexity, *i.e.* replacing simple sequential decay models or analysis based on evolution associated difference spectra by models allowing branching between the excited states, leading to species associated difference spectra. A recent alternative was given by the so-called vibrational energy relaxation approach (VERA, as discussed in the thesis), in which a simple quantum mechanical model of displaced harmonic oscillators is employed to capture the observed population dynamics as well as spectral line shapes in their time-dependence.
The thesis under discussion follows a different path, namely the use of experimental techniques beyond standard transient absorption. Specifically, the candidate implemented pump dump probe as well as two photon absorption pump – whitelight probe (2PA-TA) experiments to study ultrafast dynamics in carotenoids, polyenes and a light harvesting complex. The results lead to a total of four accepted articles in international peer-reviewed journals. This high level of scientific output alone speaks for a successful dissertation.

When analysing the candidate's contributions in detail, it becomes obvious that he is a well-trained experimental scientist with expertise in data analysis, who is willing to accept experimental challenges such as weak spectroscopic signatures. Having worked with 2PA-TA on carotenoids myself, I know how challenging it is to find trustworthy signals. Despite those hardships, the experimental data presented in the thesis (paper I) is of outstanding quality. Paper II resorts to standard transient absorption spectroscopy and answers the important question of delocalization length in so-called infinite polyenes. Again, the data quality is

Technische Universität München
Fakultät für Chemie
Dynamische Spektroskopien

Prof. Dr. Jürgen Hauer
Lichtenbergstraße 4
85748 Garching

Tel. +49 89 289 13420
Fax +49 89 289 13416

juergen.hauer@tum.de
www.tum.de

Bayerische Landesbank
IBAN-Nr.:
DE10700500000000024866
BIC: BYLADEMM
Steuer-Nr.: 143/241/80037
USt-IdNr.: DE811193231

impressive. The candidate also shows his proficiency in global data analysis. Paper III analyses ultrafast dynamics in the chlorophyll a/c antenna (CAC) of a cryptophyte. The number of studies on this light harvesting system and also its fascinating carotenoid alloxanthin are limited. The respective paper represents a series of important findings, such as carotenoid-to-chlorophyll transfer efficiencies from S_2 and S_1, where the latter is surprisingly low. The main result of this work is a detailed energy relaxation model of CAC, including excited state lifetimes ranging from 100 fs to more than 100 ns. Paper IV is a personal favourite of mine as it combines an advanced spectroscopic method, pump-repump-probe, and elaborate global data analysis to answer a question that is as relevant as simple: is it the ICT- or the S_1 state in fucoxanthin that transfer excitation energy to the chlorophylls? In the thesis, a convincing case for S_1 as the primary donor state is made.

I would like to add the following general comments and questions for the discussion part of the defence:

1. On page 34, the author describes a fundamental dilemma in ultrafast spectroscopy, which rests on Fourier transformation. (the uncertainty principle as stated in the text applies Fourier transform theory on quantum mechanical operators and observables as the results of measurements; in the present context, it is in my opinion sufficient to talk about Fourier transform pairs rather than the properties of operators). Specifically, one would like to excite bands specifically to avoid confusion in data analysis. Narrowband excitation however leads to elongated time-resolution. While this logic is irrefutable, I would argue that it is a fundamental problem only in transient absorption spectroscopy. In recent years, multidimensional ultrafast methods have successfully circumvented this problem. Please discuss such methods briefly and evaluate if the application of such methods to carotenoids is useful.

2. In the conclusions of paper I, an inhomogeneous ground state model is invoked to explain 2PA-TA spectra of carotenoids. I would argue that VERA has the potential of describing the observed line broadenings without the need to include more than one ground state species. In *Photosynthesis Research* volume 135, 55–64 (2018), VERA is applied to explain excitation dependent effects in 1PA-TA spectra of β-carotene, *i.e.* after excitation at 430, 450 and 500 nm. I would argue that the differences between 1PA and 2PA-TA in paper I stem from related effects, namely that 1PA-TA populates S_1 in a high lying vibrational state, whereas 2PA creates a (fairly) vibrationless S_1-state. The results are nonetheless counterintuitive, as 2PA creates broader instead of narrower ESA spectral features. To explain this, I would suggest to consider transfer to a high-lying vibrational state on S_0: Initial excitation of a (long-lived) minimum on the S_1-surface may exploit wavefunction overlap between S_1 and hot S_0, which leads to altered (and S*-intense) deactivation dynamics as compared to deactivation via S_2. I look forward to discussing this aspect in detail, knowing that definitive answer can only be reached by a thorough VERA-type study on direct S_1 excitation.

3. An obvious question in relation to paper IV is: Redeckas *et al.* use the same experimental approach, yet arrive at the opposite conclusion, namely that it is the ICT-state that acts as a donor state towards the chlorophyll. Please comment on this obvious difference.

4. I have a series of technical questions, which I would like to summarize here:

   4.1. The experimental setup on page 33 shows a CaF_2-plate for whitelight generation. The material is relatively soft; how did the author avoid photodamage without rotating the CaF_2-plate?

   4.2. In paper I, the solvent response in 2PA-TA is dealt with by subtracting the results from pure solvent. This is less than ideal, as two consecutive measurements need to be referenced and compared. Did the author try to reduce the size of the artefact by employing magic angle polarization between pump and probe?

   4.3. The setup on page 33 shows a 2 mm cuvette for sample handling. Given the high excitation densities in 2PA-TA, would it not have been advantageous to flow or stir the sample? Maybe I missed this point.

In summary, the thesis presented here is of exemplary thoroughness and scientific quality. Besides the high scientific output witnessed by four accepted articles, I especially enjoyed the introduction with its balanced and interesting historic review of the field.

The presented thesis is in all respects sufficient for obtaining a PhD-degree.

Jürgen Hauer

Review of the Ph.D. thesis of Mgr. Václav Šebelík "Excited state processes in linear conjugated systems"

The Ph.D. thesis is devoted to the experimental study of excited states of carotenoids in solvents and light-harvesting antennas. In spite of great progress in the research of carotenoids in the last decades, many problems and questions remain unresolved, particularly those related to the behavior and properties of dark excited states and their roles in the light-harvesting and photoprotection in photosynthetic organisms. The main goal of the thesis is to obtain new knowledge about excited states in carotenoids by means of ultrafast optical spectroscopy.

The work is divided into six chapters. The first two chapters provide a detailed introduction to the topic and experimental methods and data analysis. The key part of the thesis are the next chapters which contain experimental results and discussions, based on published papers.

The author used in the research the different methods of ultrafast spectroscopy. Namely, transient absorption spectroscopy (pump and probe technique), two-photon excitation, and pump-dump-probe spectroscopy. The work has an experimental character with included a corresponding theoretical background.

The work is written in a very good level of English. The text is clear, well arranged, and logically coherent. There is a very small number of typographical and typing errors. The used theoretical and experimental methods and procedures are fully adequate for the studied topics. The work brings very interesting findings that may extend knowledge about excited states in carotenoids.

The obtained results author published in four papers in international peer-reviewed journals. Václav Šebelík is the first author on three papers of them.

I have a couple of questions and comments regarding the theses:

1) You used for detection a two array CCD. Could you describe its type and basis properties?

2) What is a time and spectral resolution your setup in Fig. 2-1?

3) The measurement of z-scan was not successful. The signal of the sample with carotenoids was nearly the same as the signal of the solvent. Do you believe that the origin of the signal is a thermal lens or other? Do you think that z-scan measurement of your samples can be successful in the future?

4) Why is the black solid line representing fitting in Fig. 3-3 above zero before time-zero?

5) In my opinion, the power dependence shown in Fig. 3-S1 should be fitted by $a.Power^2$, not by a general polynomial of the second order. We can see on the figure that the fitting function is growing up under power of 6 mW.

I conclude that Václav Šebelík reached given goals and he presented original results that extend knowledge about excited states and relaxation processes in carotenoids. The Ph.D. thesis "Excited state processes in linear conjugated systems" proves the qualifications of the author for independent creative scientific work. I fully recommend the dissertation for the defense.

Prague, September 10, 2020

doc. RNDr. František Trojánek, Ph.D.
Department of Chemical Physics and Optics
Faculty of Mathematics and Physics
Charles University

Trojánek