Polarized two-photon fluorescence excitation studies of jet-cooled indoles
by David Michael Sammeth

A thesis submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Chemistry
Montana State University
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Abstract:
The first polarized two-photon fluorescence excitation spectra of jet-cooled indole, 3-methylindole, 3-trideuterio-methylindole, and 5-methylindole were obtained. The 1Lb and previously unidentified 1La state were distinguished for the first time under vacuum excitation conditions, at medium resolution (≈.2 cm-1) by measuring the ratio of signal intensity for circularly and then linearly polarized light.

A number of transitions to both states were classified for these compounds. The transitions classified as 1La by two-photon excitation were not identified in earlier one-photon excitation experiments, because of fast internal conversion (< 1 ns) to the 1Lb state prior to fluorescence.

Methyl rotor structure was apparent in one-photon excitation spectra for 3-methylindole and 5-methylindole, and was used to help assign some weak transitions as 1La or 1Lb based on a calculated fit of the methyl rotor. Three new transitions were documented in the spectrum of 3-methylindole. Two-photon data for 3-methylindole also exhibited methyl rotor structure that was helpful in making assignments.

Evidence is presented which supports the assignment of +455 - 480 cm'1 transitions of indole as a split 1La origin. The lowest 1La transition of 5-methylindole is observed 1,424 cm-1 above the 1Lb origin, and is proposed as the 1La origin. Two-photon experiments performed on the 3-methylindole water complex, along with bare 3-methylindole, suggest that there is an avoided-crossing between the 1La and 1Lb potential surfaces. The 1La origin of 3-methylindole appears to split into a number of transitions in the region of +334 - 450 cm-1, possibly a result of the avoided-crossing.

Also included in this work are the polarized two-photon excitation spectra of indole and 3-methylindole in perfluorohexane, and indole vapor at 25°C 1 atm N2. Both states were observed in these experiments.

This work is significant to protein spectroscopy because indole has been studied as a model to help understand the spectroscopy of the amino acid tryptophan. Indole’s 1La and 1Lb states are sensitive to the local environment, and may be useful as probes to protein structure. The detailed information of this work will improve the possibility of using tryptophan as a probe.

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David Michael Sammeth

This thesis has been read by each member of the thesis committee and has been found to be satisfactory regarding content, English usage, format, citations, bibliographic style, and consistency, and is ready for submission to the College of Graduate Studies.

April 10, 1992
Date

Patrick R. Callis
Chairperson, Graduate Committee

Approved for the Major Department

April 10, 1992
Date

John R. Quires
Head, Major Department

Approved for the College of Graduate Studies

April 14, 1992
Date

Henry J. Parsons
Graduate Dean

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Date 10 April 1992

In memory of my grandfather, Donald C. Anderson, who shared his "hands on" approach to life with me.

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ABSTRACT

The first polarized two-photon fluorescence excitation spectra of jet-cooled indole, 3-methylindole, 3-trideuterio-methylindole, and 5-methylindole were obtained. The ^1L_b and previously unidentified ^1L_a state were distinguished for the first time under vacuum excitation conditions, at medium resolution (≈.2 cm^{-1}) by measuring the ratio of signal intensity for circularly and then linearly polarized light.

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This work is significant to protein spectroscopy because indole has been studied as a model to help understand the spectroscopy of the amino acid tryptophan. Indole's ^1L_a and ^1L_b states are sensitive to the local environment, and may be useful as probes to protein structure. The detailed information of this work will improve the possibility of using tryptophan as a probe.

CHAPTER 1

INTRODUCTION

General

Proteins play a crucial role in virtually every biological process. The significance and remarkable scope of their functions include immune protection, enzymatic catalysis, and the generation and transport of nerve impulses. The broad range of functions mediated by proteins results from the diversity and versatility of the amino acids from which they are made. Only by carefully studying the physical properties of the amino acids, will it be possible to begin to understand the chemistry of proteins.

The characteristics of a given protein are governed by the sequence in which the amino acids are linked and the overall conformation assumed by this chain. The procedures for determining the amino acid sequence of a protein have become commonplace (1). Yet, just the opposite is true for ascertaining the conformational aspects of this chain. An understanding of the role conformation plays in protein chemistry is being explored in a number of ways: NMR, molecular modeling and dynamics, crystallography, and laser spectroscopy. Of all the aforementioned areas, laser spectroscopy is a particularly promising area in that *in vivo* experiments are possible, and as such could possibly help reveal the relationship between protein

chemistry and conformational dynamics in a living system.

Fluorescence spectroscopy was first used as a method of quantitative analysis at its inception approximately 50 years ago, and it has since proven useful for a variety of other spectroscopic applications. The interaction of electromagnetic radiation with matter creates a window with which to view the fundamental physics governing the interactions of atoms and molecules. The phenomenon in which matter absorbs resonance energy and then loses it through fluorescence or phosphorescence contains a wealth of information concerning molecular structure and chemical reactivity. Since the observed fluorescence of aromatic amino acids reflects the interaction of balanced forces within the protein, it is extremely sensitive to the local environment of the chromophore within the protein (2,3,4). Spectroscopic measurements of fluorescence parameters such as lifetime and polarization data yield information which is indicative of a chromophore's local environment and thus, contains information relating to the shape or conformation of the protein.

This thesis presents a study of tryptophan's two ($\pi^* \leftarrow \pi$) electronic transitions known as ^1L_a and ^1L_b (S_1 and S_2, respectively, if ^1L_a is lowest) using Platt's nomenclature (5). These two transitions are analyzed with one-photon fluorescence excitation spectroscopy (OPE) and a relatively new application of polarized two-photon fluorescence excitation spectroscopy (TPE) (6). A recent advancement in the interpretation of Ω, the ratio of circular to linear transition intensities, for a TPE spectrum of the indole chromophore is used to analyze the first TPE spectra of jet-cooled indole, 3-methylindole (3MI), 3-trideuterio-methylindole (d_3-3MI), and 5-methylindole (5MI). In preparation for the jet-

cooled experiments, the first TPE spectra of both indole and 3MI solvated with perfluorohexane are measured, as well as the TPE spectrum of indole vapor at room temperature. These experiments are done in conjunction with high resolution jet-cooled OPE experiments of all of the above-mentioned compounds, with this being the first reported jet-cooled spectrum of d_3-3MI.

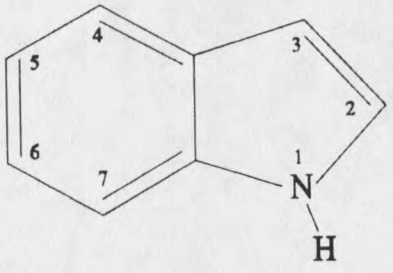

Figure 1. The indole molecule and its ring numbering system.

By measuring the ratio of circular to linear fluorescence intensities obtained from a two-photon excitation process, it is possible to ascertain which of the two possible $\pi^*\leftarrow\pi$ transitions must have taken place; either $^1L_a\leftarrow S_0$ or $^1L_b\leftarrow S_0$ (see Figure 2). The results of OPE spectroscopy are used to help interpret the TPE data as well as providing new insights into the photophysics of indole.

Because the excitation energy necessary to reach either the ^1L_a or ^1L_b excited state depends on the local environment of tryptophan's chromophore, i.e. the relative energy of these states is a function of the protein's conformation in their locale, tryptophan is a candidate for use as a probe to help unravel the relationship between protein chemistry and conformation. Therefore, a complete understanding of indole's photophysics as a function of various perturbations is essential before its spectroscopic behavior in a

protein can be helpful in understanding the local environment of the protein, and thus its conformation.

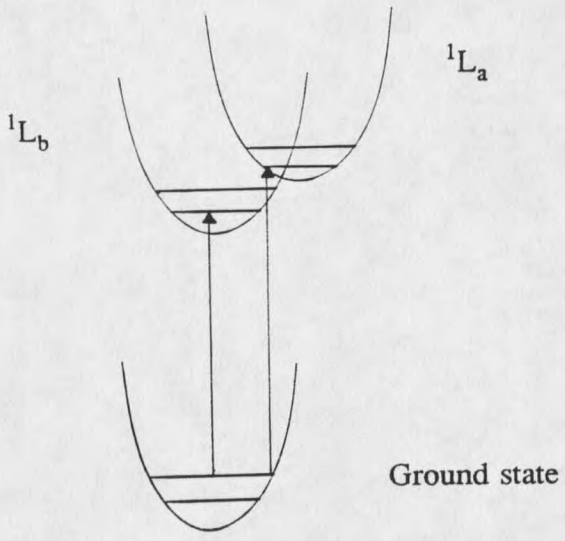

Figure 2. A simple depiction of indole's two overlapping $\pi^* \leftarrow \pi$ electronic transitions.

This research is concerned with understanding the behavior of these two excited states in the absence of any external perturbations on the chromophore. It is important that the photophysics of the bare molecule be studied, so that the effects of solvation can be better understood. The necessary experimental conditions are obtained by using a free-expansion jet system. By directing a carrier gas (He) entrained with sample through a small orifice at high pressure ($\approx$4 atm) into a region of low pressure ($\approx 10^{-6}$ torr), the entrained molecules are cooled down to near their ground vibrational and

rotational states. This cooling process occurs through a collisional energy exchange; the random molecular motion of the gas is converted into directed energy flow of the expansion. Thus, a greatly simplified excitation spectrum of the isolated molecule is created since all transitions must start from approximately the same ground state energy.

Historical

Protein luminescence was probably first observed by Becchari in the early eighteen century. He observed the glow of his own hand in a dark room after exposure to sunlight (7). Yet, it was not until the middle of the twentieth century that the molecular substrate responsible for this phenomenon was discovered.

The fluorescence of various biological moieties began to be reported in the latter half of the nineteenth century. Regnault in 1859 and Schenov in 1859 observed fluorescence of the skin, and in 1883 Soret described the violet fluorescence of myosin. Fluorescence of vertebrate eyes was observed by Hemholtz in 1886; Hess then observed it in the eyes of crustaceans in 1911. The violet, blue, or yellowish-blue color fluorescence observed in albumin, casein, and elastin proteins was investigated by Svedberg (1925), Liesegang (1926), Pringshein (1928), Tiselius (1930), and Teichler (1931) (8). In most cases this fluorescence was due to impurities or chemically altered components of proteins.

By 1950, it was well understood that the observed ultraviolet fluorescence from various biological samples was due solely to proteinaceous material within the various samples. In 1953, Weber postulated that the ultraviolet fluorescence of proteins could

be attributed to the aromatic amino acids (9). This hypothesis was supported by his observations that the band shapes and absorption maxima of proteins were very similar to those observed for the aromatic amino acids. In fact, Weber correctly predicted an important new area of spectroscopy. "A study of the ultraviolet fluorescence of aromatic amino acids and proteins would no doubt provide interesting details as regards the interaction of the aromatic residues in the protein molecules as well as help in the interpretation of the absorption spectra themselves" (8). Three years later, Shore and Pardee were the first to report the measured fluorescence of purified proteins and demonstrate that this fluorescence was due solely to the three aromatic amino acids: phenylalanine, tyrosine, and tryptophan (9). These results were further supported by Duggan and Udenfriend in 1956, and were expanded upon in 1957 when Teale and Weber reported the measured fluorescence of free aromatic amino acids in solution (10,11).

Of the three amino acids possessing an aromatic ring, tryptophan exhibits the most intense fluorescence in the native state and, as a result, almost all of the observed fluorescence of most proteins can be attributed to tryptophan residues. Therefore, studies focusing on the fluorescence of isolated tryptophan molecules are an important step in understanding the spectroscopy of proteins and thus, proteins themselves.

Background

The indole chromophore of the amino acid tryptophan is important in spectroscopic studies of proteins because its spectral characteristics are environment-dependent.

Indole's spectral sensitivity is due to the existence of two overlapping, $\pi\pi^*$, electronic transitions, designated ^1L_a and ^1L_b, using the nomenclature developed by Platt (5,12). Given that excitation energies to these two quasi-degenerate states depend on the molecule's environment, the usefulness of the indole chromophore as a probe of protein structure clearly depends on a thorough understanding of these two states. To this end, a great deal of research has been performed on indole and its analogues, and therefore, the characterization of the ^1L_a and ^1L_b states has slowly developed over time.

In 1960, Weber stated that the polarization spectrum of indole can only be explained by the existence of at least two electronic states (13). After performing fluorescence polarization experiments on indole and tryptophan in rigid propylene glycol, Valeur and Weber concluded that the longest wavelength-absorption (290 nm) band consisted of two independent electronic transitions (14). They resolved these band quantitatively by analyzing the anisotropy of the emission spectrum. These two transitions have been identified as ^1L_a- and ^1L_b-type transitions, in accordance with the absorption theory of aromatic hydrocarbons (13). The anisotropy measurements showed ^1L_a to be a broad band with its absorption maximum at higher energy and its origin at lower energy than the narrower, structured ^1L_b band. These differences are ascribed to their dissimilar Franck-Condon factors (14,15,16,17).

Measurements of the ^1L_a and ^1L_b permanent dipoles show that the ^1L_b dipole is very similar to that of the ground state, while the ^1L_a has a dipole is over two times larger than that of the ground state. The ^1L_a dipole moment was calculated to be 5.4 debye. This analysis was based on values obtained from solvent-induced shifts measured from

absorption spectra (18). A measurement of the vacuum Stark effect yielded a dipole moment of 2.3 debye for the ^1L_b state, which is very close the calculated ground state value of ≈2 debye (19,20).

One-photon absorption studies by Strickland and co-workers illustrate how substitutions and solvent perturbations of the indole chromophore can differentially shift these two states (16,17,21). The ^1L_a is much more sensitive to solvent polarity, as compared to the ^1L_b state. This is a result of the ^1L_a state having a larger permanent dipole moment than the ground state. By utilizing the ^1L_a state's sensitivity to solvent perturbations, Strickland came to the following conclusions regarding indole, 3MI, and 5MI: the ^1L_a origin lies above ^1L_b for indole in vapor and methylcyclohexane, and for 5MI in cyclohexane. Both the ^1L_a and ^1L_b origins of 3MI occur at approximately the same energy in methylcyclohexane, while the ^1L_a origin is seen to shift further to the blue than the ^1L_b origin in vapor.

Callis and co-workers presented a direct method for observing the locations of the ^1L_a and ^1L_b states with polarized two-photon absorption spectroscopy (6,22,23). Their studies focused on how a polar versus a non-polar solvent would affect the positions of the ^1L_a and ^1L_b bands in indole, 3MI, 5MI, and 2,3-dimethylindole (2,3MI). The TPE spectra yielded several important results. First, there was a reasonably close correspondence of the TPE with OPE in all cases. Secondly, all studies are consistent with the premise that a polar solvent causes substantial red-shift of the ^1L_a band relative to the ^1L_b, and that the ^1L_b is more structured, compared with the broader ^1L_a band. Using a non-polar solvent, cyclohexane, Callis and Rehms found the ^1L_a absorption to

be to the blue of the ^1L_b for indole and 5MI, whereas the red edge is predominately ^1L_a absorption in 3MI and 2,3MI. However, using a polar solvent, butanol, all compounds studied revealed that the ^1L_a absorption had red-shifted relative to the non-polar environment of cyclohexane. These results are in agreement with Strickland's work and others (24). The technique of polarized two-photon excitation spectroscopy supplied valuable information which is independent and complementary to that given by conventional OPE spectroscopy.

Early work performed by Hollas and by Mani and Lombardi on the gas phase absorption spectrum of indole identified one electronic transition (25,26). This transition has been assigned to the ^1L_b state (25,26,27,28). Fluorescence excitation spectroscopy in a free-expansion jet has identified many ^1L_b transitions, including the origins, for indole, 3MI, and 5MI, but the ^1L_a state had yet to be observed for any of these compounds when either in a buffer gas at 1 atm or in a free-expansion jet (27,28,29,30, 31,32,33,34,35,36). A possible ^1L_a origin for 3MI in a supersonic jet has been proposed by Hays and co-workers based on comparisons between the relative intensities of the P, Q, and R rotational branches (31). By locating the ^1L_a state of indole and 3MI in methylcyclohexane and following its shift when the solvent is changed to perfluorinated hexane, Strickland and co-workers extrapolated the results to the gas phase in order to locate the ^1L_a origin in the vapor at 274 nm (21). One-photon excitation experiments by Illich showed that the quantum yield of indole vapor increased when the pressure of the buffer gas, argon, was increased from 80 to 800 torr (37). The excitation spectrum at this higher pressure resembles the ^1L_a absorption spectrum, thus suggesting that

fluorescence from the ^1L_a state was observable at higher pressures, but not at lower pressures due to the state becoming dissociative. Therefore, a pressure dependent dissociative mechanism was offered as a explanation for the absences of observed ^1L_a fluorescence under vacuum excitation conditions (40).

Statement of the Problem

In previous research, there had not been any positive identification of the ^1L_a state for either indole, 3MI, or 5MI when the fluorescence excitation spectra had been measured in a buffer gas at one atmosphere or in a free-expansion jet when performing one-photon excitation experiments, even though ^1L_b states have been identified under these same conditions. This led to the notion that the ^1L_a state must have solvent stabilization in order for it to be observed. In fact, the fate of the ^1L_a state in a vacuum has been much debated, and as such, non-radiative pathways have been implicated in an effort to explain the missing ^1L_a fluorescence. Apparently, these pathways are so fast for the ^1L_a state, that they cause the quantum yield to drop below experimental detection, as opposed to ^1L_b excitation, which has measurable fluorescence. The two most common proposals used to explain the missing ^1L_a fluorescence are internal conversion to the ground state, and a model where excitation to the ^1L_a state leads to dissociation, presumably through the scission of the nitrogen hydrogen bond (18,30,36,39,40). As support for the dissociation model, Evleth and co-workers proposed a simple correlation diagram for co-planar and non-planar N-H bond rupture based on CNDO/S calculations (41). Other non-radiative pathways that have been

implicated to explain the missing ^1L_a fluorescence are intersystem crossing to the triplet state and photoejection (42,43,44). However, the simple possibility that the ^1L_a state has not been observed due to lack of resonance, i.e., the correct excitation energy has not been used, must not be overlooked.

The goal of the research presented here was to determine the fate of the ^1L_a state upon excitation under vacuum conditions. Because there was evidence that the fluorescence quantum yield of the ^1L_a state was pressure-dependent, the approach was to first study indole at 1 atm with nitrogen as the buffer gas. This experiment was used to determine whether or not the ^1L_a state was observable in the gas phase at 1 atm. Since the results of this two-photon excitation experiment positively identified both the ^1L_a and ^1L_b states of indole, the next experiment was to measure the two-photon fluorescence excitation spectrum of jet-cooled indole. The identification of indole's ^1L_a and ^1L_b states under vacuum excitation conditions open the door for a complete study of these states in indole, 3MI, d_3-3MI, and 5MI.

Technique

The technique developed by Callis and co-workers which utilizes the photoselection imposed by a one-color two-photon absorption process was used. This technique involves the absorption of two visible photons to reach the same excited state that one photon of ultraviolet energy will reach. Since the molecules are motionless in the time interval of two-photon absorption, photoselection occurs in the ground state, which means that any observed photoselection is dependent only upon the *initial* excited state

reached, and therefore information regarding this state is available whether or not it is the same state that fluoresces (45). These authors have shown that polarized two-photon fluorescence excitation spectroscopy offers a direct method of identifying the ^1L_a and ^1L_b states of the tryptophan chromophore (6,22).

Photoselection dependents on the polarization of the photons. Therefore, states which reflect this dependence can be distinguished based on the polarization of photons used (46). Indole's ^1L_a and ^1L_b states were determined to have preferential absorption depending on the polarization of the exciting light (6,22,47,48). This dependency can easily be observed when the polarization ratio, Ω, the ratio of two-photon absorptivities for circularly polarized versus linearly polarized light ($\Omega = \delta_{cir}/\delta_{lin}$), is plotted versus wavelength. A high polarization ratio ($\Omega \approx 1.5$) is associated with the ^1L_b state, while a low polarization ratio ($\Omega \approx 0.5$) is associated with the ^1L_a state. These experimental results are in harmony with the theoretical calculations. Callis and co-workers have computed the two-photon properties of the ^1L_b and ^1L_a states of indole using an INDO/S method. Theoretical results predict the polarization ratio to be approximately 1.5 for the ^1L_b state and 0.35-0.7 for the ^1L_a state (22,49).

Experimentally, it has been shown that the polarization dependencies of the ^1L_a and ^1L_b states of indole are not changed significantly by methyl substitution or solvent perturbations. Thus, a positive identification of the regions of ^1L_a and ^1L_b absorption of methylated indoles is possible. This technique has been utilized successfully in studies of solvent, substitution, and charge effects on methyl indole's ^1L_a and ^1L_b states (6,23).

CHAPTER 2

MOLECULAR TWO-PHOTON SPECTROSCOPY

Two-photon Cross-section

The possibility of two-photon absorption was first outlined theoretically by Goeppert-Mayer in 1931 (50). This possibility occurred to her when she was working with Dirac's dispersion theory. Dirac had already realized that the first-order perturbation theory of the effect of light on matter yielded terms describing the absorption and emission of a single photon, and that second-order perturbation theory gave terms which represented transmission and scattering events. But when she studied these second-order terms, she realized that these terms also described the simultaneous absorption or emission of two non-resonant photons (whose sum energy is resonant) by matter (51).

The probability of a two-photon absorption process is given by the two-photon cross-section, which is shown below,

$$W_{i\to f} = K\ I^2 \left| \sum_k \frac{(e_1 \cdot \langle f|r|k\rangle)(\langle k|r|g\rangle \cdot e_2)}{w_{kg} - w} \right|^2$$

were K is a set of constants, and I is the intensity of the incident radiation (52). This

equation shows that the probability of a two-photon absorption is proportional to the square of the laser intensity. The expression being summed over k is the 3 x 3 transition tensor for a two-photon absorption process, as opposed to a dipole allowed one-photon process, which has only a transition vector. This tensor represents a two-photon transition from an initial state "*g*" to a final state "*f*", via a set of virtual states "*k*". Absorption of the first photon with a polarization "*e*" changes the state of the system "*g*" to "*k*", and the simultaneous absorption of a second photon with polarization "*e*", changes the system from "*k*" to "*f*". w_{kg} is the energy difference between the initial and virtual state, and w is the laser frequency.

Polarization Dependence

The probability of a two-photon absorption event occurring is not based only on purely molecular properties; the expression for two-photon cross-section contains the polarization vectors e_1 and e_2 (53). These polarization vectors, i.e. the polarization of the incident radiation, can be experimentally manipulated, which allows some limited control over what rotational and vibrational transitions will be allowed (54). This implies that by changing the polarization of the exciting light, new spectroscopic information for a molecule can be accessed.

The two-photon transition tensor can be factored into three independent components which are expressed in the form of the products of the geometrical factors C_J, the molecular factors M_J, and the rotational factors R_J as shown below (45).

$$\langle W_{i\to f} \rangle = C_0 M_0 R_0 + C_1 M_1 R_1 + C_2 M_2 R_2$$

The R_J's are rotational factors determined by rotational selection rules, the M_J's are the molecular factors obtained from the two-photon tensor, and the C_J's are the polarization-dependent geometrical factors. The three above terms are, moving from left to right, known as the isotropic, antisymmetric, and symmetric anisotropic tensors. For one-color experiments (both photons have the same energy) the antisymmetric term goes to zero, and because all experiments presented here are of this type, the antisymmetric term will not be considered further (55).

If a transition is due to only the isotropic component ($C_0M_0R_0$) then rotational selection rules require that ΔJ and $\Delta K = 0$, while a symmetric anisotropic ($C_2M_2R_2$) transition can have ΔJ and $\Delta K = 0, \pm 1, 2\pm$ (53). This results in only the Q rotational branch being allowed for the isotropic tensor, but the symmetric anisotropic tensor can have O, P, Q, R, and S rotational branches. The molecular factors depend on the particular transition occurring. Geometrical factors depend on both the polarization of the laser light and the form of the transition tensor. A transition induced by linearly polarized laser light will have a value of 1/3 for C_0 and 2/15 for C_2. In contrast, circularly polarized light has a geometrical factor of 0 for C_0 terms, and 1/5 for C_2 terms. Thus, if two transitions were measured, one due to only an isotropic tensor, and the other due to a symmetric anisotropic tensor, quite different results would occur, depending on the polarization of the light used to make the transitions. Linear light would show similar intensity for both transitions, but the isotropic one would exhibit all

its intensity in a single dominant Q-branch, whereas the symmetric anisotropic transition would spread its intensity over all rotational branches. In contrast, circularly polarized light would show only the transition due to the symmetric anisotropic tensor since; the C_0 for the isotropic transition is equal to zero.

The two-photon polarization parameter Ω is defined as (56)

$$\frac{\langle W_{i\to f}\ OO\rangle}{\langle W_{i\to f}\ \uparrow\uparrow\rangle} = \Omega$$

The value is bound to the interval $0 \leq \Omega \leq 1.5$. This arises from the fact that an isotropic tensor will have an Ω value of 0, and a symmetric anisotropic tensor has a value of 1.5 for Ω. The character of the tensors making up a given transition will be reflected in the polarization ratio. A transition whose makeup was ≈50% isotropic and ≈50% symmetric anisotropic in character would result in a measured Ω of .75, the average value of these two tensors.