previously thought. The recognition of the existence of deep convection of meteoric water during regional deformation and metamorphism has important implications for models of the behaviour of fluids in the crust.

Synorogenic fluid pressure (P_F) often is assumed to equal lithostatic pressure (P_L), the pressure generated by the weight of the overlying column of rock (*1, 8*). In order for surface waters to convect, P_F must be approximately equal to hydrostatic pressure (P_H), the pressure generated by the weight of a column of water (*2*). Since P_H is always less than P_L, then under hydrostatic conditions, P_F is less than P_L. At a depth of 10 km, $P_H = 0.1$ GPa and $P_L = 0.3$ GPa, a substantial difference between fluid and rock pressures, reflecting the differences in density of water and rocks.

In metamorphic petrology, the assumption of $P_F = P_L$ is widely applied in the calculation of metamorphic phase equilibria (*1, 25*). In areas where $P_F \approx P_H < P_L$, such calculations would have to be revised to account for the lower P_F. Low fluid pressures will result in devolatilization reactions occurring at lower temperatures than if $P_F = P_L$. Consequently, under such conditions, isograds based on devolatilization reactions are indicative of lower regional metamorphic temperatures than are typically assumed.

High fluid pressures decrease the effective stress in a rock unit thereby facilitating faulting (*1*). It has been argued that in order for thrust faulting to occur, P_F must equal P_L (*1*). This concept is consistent with our data in that in the thrust faulted Rocky Mountains, there is good evidence for metamorphic fluids dominating the fluid regime, a situation which is compatible with $P_F = P_L$. However, in the strike-slip and extensional faulted terranes, where the geochemical data indicate that there was significant influx of meteoric water, P_F was most likely substantially less than P_L and hence effective stresses were higher than has been generally assumed.

Quartz ± carbonate veins in the Canadian Cordillera and around the world occasionally host gold mineralization. Most researchers studying these deposits have regarded the deposits as forming from metamorphic fluids (*21, 26*). In the Canadian Cordillera, inclusion fluids from gold-bearing quartz ± carbonate veins consistently have low δD values, indicating that this style of mineralization is a product of the meteoric water convection process. Significant gold mineralization is absent in those areas of the Canadian Cordillera that were dominated by metamorphic fluids (*19*).

The results of this study have significant implications to large-scale crustal processes. The convection of meteoric water in the continental crust should have a significant impact on heat flow and cooling of the crust. In addition, recent suggestions of the interrelations of the hydrosphere and seismic activity have been proposed (*27*). Recognition of the variability in the crustal fluid regime may aid in understanding any linkage between seismic activity and crustal fluids.

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A Rotationally Resolved Fluorescence Excitation Spectrum of *all-trans*-1,4-Diphenyl-1,3-butadiene

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Band 1 in the jet-cooled one-photon $S_1 \leftarrow S_0$ fluorescence excitation spectrum of *all-trans*-1,4-diphenyl-1,3-butadiene has been rotationally resolved with a molecular beam laser spectrometer. Both the orientation of the optical transition moment and the rotational constants of the two vibronic levels have been measured. The molecule shows no evidence of being significantly distorted from a C_{2h} geometry when it is low in the vibrational manifolds of either of the two electronic states.

FEW CLASSES OF COMPOUNDS HAVE attracted more attention in the scientific literature than the linear polyenes. From the first demonstration of the

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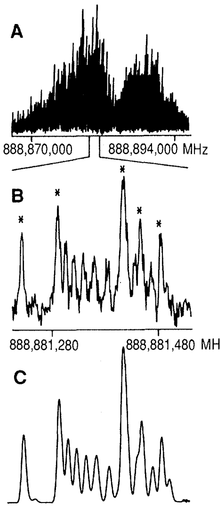


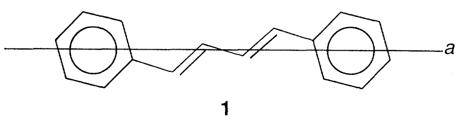
Fig. 1. (**A**) The central ~30 GHz of the rotationally resolved fluorescence excitation spectrum of band 1 in the $S_1 \leftarrow S_0$ one-photon transition of *all-trans*-1,4-diphenyl-1,3-butadiene at ~337 nm, recorded in the collision-free environment of a molecular beam. (**B**) The most congested portion of the Q branch that is observed upon horizontal-scale expansion of the data in (A); this region spans ~300 MHz. (**C**) A computer simulation of the data in (B) based on the parameters in Table 1. The dominant pattern in (B) and (C) is a Q-branch progression in rotational quantum number J with $K_{-1} = 6$, beginning with $J = 6$. Overlapping features are indicated with asterisks and include P-branch lines. In addition, each line in this portion of the spectrum is an unresolved K_{-1} doublet.

Table 1. Inertial parameters of the vibrational levels of the ground (1^1A_g) and first excited singlet ($\sim 2^1A_g$) states accessed in band 1 of the fluorescence excitation spectrum of *all-trans*-1,4,-diphenyl-1,3-butadiene at ~337 nm. The band origin is at 29650.994 ± 0.002 cm^{-1}. A'', B'', C'', κ'', and $\Delta I''$ are the rotational constants, asymmetry parameter, and inertial defect of the zero-point rotational level of ground-state DPB, respectively; and ΔA, ΔB, ΔC, and $\Delta\kappa$ refer to changes in these parameters that occur upon electronic excitation of DPB to the vibronic level of the $\sim 2^1A_g$ state; $\Delta I'$ is its inertial defect.

	1^1A_g		$\sim 2^1A_g$
A'' (MHz)	2502.7 ± 0.1*	ΔA	−97.0 ± 0.1
B'' (MHz)	145.1 ± 0.1*	ΔB	1.3 ± 0.1
C'' (MHz)	137.5 ± 0.1*	ΔC	1.0 ± 0.1
κ''	0.9936	$\Delta\kappa$	−0.0006
$\Delta I''$ (amu Å^2)	−10.3 ± 0.2	$\Delta I'$	−11.3 ± 0.2

*Calculated values are 2456, 147, and 139 MHz, respectively, based on the equilibrium geometry of Pierce and Birge (*7*).

relation between color and chemical constitution to the more recent discovery of high electrical conductivity in doped conjugated polymers, the polyenes have served as paradigms in the development of our present understanding of molecular orbital and valence bond theories of electronic structure, cis-trans photoisomerization, and the visual process (*1*). Despite this work, little is known about the equilibrium geometries of these compounds and how they change upon electronic excitation. Rotationally resolved optical spectroscopy can address this need. We report the successful observation of resolved rotational structure in the electronic spectrum of a polyene, *all-trans*-1,4-diphenyl-1,3-butadiene (DPB, **1**). An analysis of this structure provides rotational constants for the ground (S_0) and first excited singlet (S_1) states, their inertial defects, and the orientation of the $S_1 \rightarrow S_0$ transition moment in the molecular frame, from which key structural information about the properties of both states of DPB may be derived.



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The experimental apparatus has been described previously (*2*). Briefly, DPB (Aldrich) was heated to 513 K, seeded into 440 torr of Ar, and expanded through a 150-μm quartz nozzle to a pressure of ~10^{-5} torr. The molecular beam (collimated by skimmers 2 and 20 cm from the nozzle) was crossed 100 cm downstream by a tunable ultraviolet (UV) dye laser beam (operating in the continuous-wave mode with a full-width at half-maximum <<1 MHz), producing excitation wavelength-dependent fluorescence that was collected with spatially selective optics and detected with single-photon counting techniques. The resulting fluorescence excitation spectrum (FES) was calibrated in relative frequency with the use of markers from a thermally stabilized, mode-matched, confocal interferometer (free spectral range = 299.7520 MHz) and in absolute frequency with the I_2 absorption spectrum, both simultaneously recorded with the FES. All data were processed and analyzed with a MASSCOMP computer system and specially designed software.

The rotationally resolved FES of band 1 (Fig. 1) was previously recorded at lower resolution in a He supersonic jet (*3, 4*) and estimated to lie 37.2 cm^{-1} to the blue of the two-photon origin of the S_1 state at 29,615.3 cm^{-1} (*4, 5*). The spectrum spans ~30 GHz (1 cm^{-1}) and exhibits more than 2000 lines. Single lines exhibit widths of ~4 MHz, which are presumably Doppler limited since $\tau_f = 63$ ns (corresponding to a homogeneous width of 2.4 MHz) for the upper-state vibronic level accessed in this band (*4*). Despite the apparent lack of a central Q branch, band 1 is a parallel-type transition polarized entirely (>95%) along the a-axis of the near prolate top. All lines were fit to a rigid-rotor Hamiltonian for both vibronic levels with the program ASYROT (*6*). The standard deviation of the fit is 3.1 MHz and is limited mainly by nonlinear effects during the scan. The derived values of the band origin, the rotational constants of both levels, and their inertial defects are listed in Table 1. The rotational temperature of the fit is 5 K.

Force-field (FF) calculations (*7*) predict a C_{2h} geometry for the ground state of DPB. The agreement between the measured S_0 rotational constants and those calculated from the QCFF/PI equilibrium internal coordinates (Table 1) is remarkably good. Thus, although three inertial parameters are clearly insufficient to determine the structure of a molecule possessing 30 atoms, and more sophisticated calculations are possible, it seems reasonable to conclude that DPB is a planar, centrosymmetric species in the zero-point vibrational level of its S_0 state.

A similar conclusion is reached about the S_1 vibronic level accessed in band 1 (Table 1). The ~4% decrease in the A rotational constant that occurs on electronic excitation indicates a slight expansion of the molecule in directions perpendicular to the long axis. Significantly smaller increases in B (0.9%) and C (0.7%) also occur, indicating that a still smaller shortening of the molecule occurs along a. Simple geometry calculations suggest that all of these changes can be accommodated by a model for the S_1 state (*8*) that has equal contributions from single and double excitations with just greater than one-half of the optical electron density being localized on the phenyl groups, with nodes along the bonds in the phenyl rings that are most perpendicular to a. A 0.03 to 0.04 Å increase in these bond lengths is required to match the observed change in A. The model also predicts a significant amount of bond-order reversal primarily localized on the polyene moiety, which may be responsible for the small changes in B and C and may also contribute to ΔA. Rotation of the phenyl groups with respect to the polyene plane produces ΔB and ΔC values of opposite signs, a result that is inconsistent with experiment. Thus, our results show that no large conformational distortion occurs on excitation of band 1 of DPB. The nonzero (negative) inertial defects (Table 1) of both levels may indicate some relatively large out-of-plane vibrational amplitude. (We calculate $\Delta I \sim -12$ amu Å^2 for a 10° torsional rotation of the phenyl groups relative to the polyene plane.) All of these conclusions are consistent with the observed vibrational activity in the optical spectrum of DPB (*1*).

It is currently believed that the polarization of the one-photon–allowed S_2 (1^1B_u) $\leftarrow S_0$ (1^1A_g) transition is along the main-chain axis in the linear polyenes (*8*), and that the two-photon–allowed S_1

$(2^1A_g) \leftarrow S_0$ transition in DPB gains its one-photon oscillator strength through vibronic coupling with S_2, involving a b_u in-plane bending mode (*1*). Our observation that the molecule is essentially planar in both states, and that band 1 is parallel polarized, confirms this interpretation, at least for the vibronic levels accessed in the present experiment (*9*).

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Identification by ENDOR of Trp^{191} as the Free-Radical Site in Cytochrome c Peroxidase Compound ES

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The chemical identity of the amino acid free-radical site that represents one of the two oxidizing equivalents stored in the H_2O_2-oxidized intermediate (compound ES) of the mitochondrial heme enzyme, cytochrome c peroxidase (CcP) has been sought for almost a quarter of a century. Site-directed mutagenesis alone cannot yield this answer. Low-temperature 35-gigahertz (Q-band) electron nuclear double resonance (ENDOR) spectroscopy was used to examine compound ES prepared from proteins containing specifically deuterated methionine or tryptophan, as well as the amino acid replacement $\mathrm{Trp}^{51} \rightarrow \mathrm{Phe}$. The results definitely identify the site of the radical in compound ES as tryptophan, most likely Trp^{191}.

THE FULLY OXIDIZED STATE OF CcP, called compound ES, contains an unusual free-radical species whose identity has long been sought (*1*). The H_2O_2-dependent oxidation of ferrocytochrome c is catalyzed by CcP (*2, 3*) and, like horseradish peroxidase (HRP) and catalase (*4*), CcP can store two oxidizing equivalents after treatment with peroxide. In this fully oxidized state, called compound I in the case of HRP and catalase, one of the oxidizing equivalents is stored as the oxy-ferryl [Fe(IV) = O] state of the heme iron with spin $S = 1$ (*4, 5*). In compound I the second equivalent is stored as the π-cation radical of the porphyrin macrocycle (*4, 6*), but in ES it exists as a reversibly oxidized amino acid residue that gives an electron paramagnetic resonance (EPR) signal whose axial g-tensor [$g_{\parallel} = 2.04$ and $g_{\perp} = 2.01$ (*7–10*)] is unlike organic radicals composed of first-row elements. The ES state of CcP is one of only four known instances in which an enzyme makes use of a stable amino acid radical. The others are ribonucleotide reductase (*11*), prostaglandin H synthase (*12*), and signal II of photosystem II (*13*), each of which contains a Tyr π-radical.

Several species have been proposed as the free-radical site (R·) of ES. In the high-resolution crystal structures of CcP (*14*) and ES (*15*), the indole ring of Trp^{51} lies about 3.6 Å above and parallel to the heme pyrrole ring II. On this basis, it was suggested that R· is the Trp radical formed by hydrogen atom abstraction from the nitrogen of Trp^{51} (*14*). The single-crystal EPR study of Hori and Yonetani (*10*) further implicated Trp and implied that the residue must be either Trp^{51} or Trp^{191}; the latter was first noted as a possible site in (*3*). However, paramagnetic resonance data [g-values observed in EPR (*6*); hyperfine splittings obtained from ENDOR spectra (*7, 16*); and the linear electric field (LEFE) response of the signal (*17*)] were interpreted to be inconsistent with R· being an isolated aromatic free radical, and magnetic circular dichroism (CD) measurements detected no effects attributable to radical formation at Trp (*18*). As an alternative, we proposed that the EPR signal is sulfur-based and is associated with a nucleophilically stabilized Met radical. Met^{172}, which is located two residues away from the proximal iron ligand, His^{175}, and is in contact with the proximal heme face, seemed an ideal candidate to carry at least some of the spin density associated with the ES EPR signal. A pair of Met residues, 230 and 231, also were considered as an alternative radical site (*15*), in consonance with our initial proposal that R· is a dimeric thioether radical cation $(R_2SSR_2)^+$ in which two proximate Met sulfurs share the charge created by the loss of one electron (*8*).

Recent site-directed mutagenesis studies in which Met^{172} (*19*) was replaced by Ser and Trp^{51} (*20, 21*) or Trp^{191} (*22*) were replaced by Phe have been used to probe the identity of the radical. Unfortunately, the mutagenesis approach has given definitive answers to the wrong question. A site-specific modification of CcP that leaves the radical site intact may be taken to prove that the site in question is not associated with the radical, which indeed is the case for Trp^{51} and Met^{172}. However, if modification of a site changes or abolishes the radical EPR signal, this in no way constitutes a proof that the radical resides at the site in question—it merely shows that the enzyme in the ES state has been perturbed. An example of this type is the modification of Trp^{191}, which lies perpendicular to the heme and in contact with the His ligand to iron. Replacement of Trp^{191} by Phe decreased, but did not eliminate, enzymatic activity and eliminated the radical signal (*22*). It appears unlikely that continued mutagenesis studies could identify the location of the radical with logical certainty.

To unambiguously determine the identity of the radical site, we used a structurally nonperturbative technique, isotopic labeling plus ENDOR spectroscopy, to probe the radical site. The enzyme containing Met-Lys-Thr on the amino terminus, CcP-(MKT), was produced in *Escherichia coli* by the plasmid pLACCCP under control of the *lac* promoter (*23*). Purified protein made by this method exhibited normal functional properties, and gave optical and EPR spectra in the native and compound ES states that were unaltered from those of the protein isolated from yeast. Auxotrophic strains of *E. coli* deficient in Trp or Met biosynthesis were used to produce enzyme selectively deuterated at Trp or Met; these samples also were subjected to H-D solvent exchange. This approach was augmented by examination of the $\mathrm{Trp}^{51} \rightarrow \mathrm{Phe}$ mutant. The protein samples used in this analysis were:

1) ES(H,H), perprotonated (natural isotopic abundance) enzyme, CcP(MKT), in H_2O buffer;

2) ES(d_3-Met,H), prepared in H_2O buffer from the methionine auxotroph grown on L-methionine-d_3 (CD_3-methionine);

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