squalene by the S_{10} preparation could be *completely* inhibited by I_{PSQ}. In contrast, there was no inhibition of squalene biosynthesis from presqualene pyrophosphate at comparable I/MEVAL ratios by I_{GER}, I_{FAR}, I_{DMA}, or I_{IPT}, evidence that effective inhibition requires a close correspondence of substrate and inhibitor carbon structure. The same results were obtained with the microsomal liver preparation[14] (referred to herein as MLP) which effects squalene biosynthesis from farnesyl or presqualene pyrophosphates but not from C_5 or C_{10} precursors, both with regard to inhibition of squalene biosynthesis from presqualene pyrophosphate by I_{PSQ} and lack of inhibition by the other phosphonophosphates.

Incubation of 50 nmol of tritiated mevalonate, 25 nmol of unlabeled presqualene pyrophosphate, and 500 nmol of I_{PSQ} with sufficient S_{10} enzyme[15] to convert 12% of the mevalonate to squalene in the absence of I_{PSQ} yielded *no* tritiated squalene but showed a 9% conversion (*75% of expected maximum*) of mevalonate to tritiated presqualene pyrophosphate. For identification the labeled presqualene pyrophosphate was purified by thin layer chromatography (silica gel, *n*-propyl alcohol–11 N ammonium hydroxide 1.5:1, R_f identical with that of unlabeled presqualene pyrophosphate) and reincubated separately with both S_{10} enzyme and MLP enzyme to afford in each case tritium labeled squalene. Labeled squalene was identified unambiguously by chromatographic data and also by conversion to the crystalline thiourea complex which could be recrystallized to constant specific radioactivity. Further, characterization of the tritiated presqualene pyrophosphate produced in the above experiment was obtained by reduction with lithium aluminum hydride to labeled presqualene alcohol, chromatographically identical with authentic material (R_f 0.27 on silica gel plates using 2:1 pentane–ether for development). These experimental data indicate that I_{PSQ} can completely turn off squalene biosynthesis from mevalonate or presqualene pyrophosphate and also that presqualene pyrophosphate is formed and accumulated *under normal conditions of squalene biosynthesis from mevalonate* if I_{PSQ} is present. Given these facts and the specific inhibition of the presqualene pyrophosphate to squalene conversion by only I_{PSQ}, there seems to be no way to avoid the conclusion that presqualene pyrophosphate is an *essential intermediate* in squalene biosynthesis in liver; that is, there is no pathway from mevalonate to squalene which does not go through this intermediate.[16]

It seems apparent that the study of phosphonophosphate analogs can be helpful in the elucidation of biosynthetic pathways to terpenoids.[17]

References and Notes

(1) See R. Bentley, "Molecular Asymmetry in Biology", Vol. II, Academic Press, New York, N.Y., 1970, Chapter 4.
(2) Prepared by addition of 1.1 equiv of *n*-butyllithium to dimethyl methylphosphonate in THF at −78° under argon and further reaction at −78° for 0.5 h; see E. J. Corey and G. T. Kwiatkowski, *J. Am. Chem. Soc.*, **88**, 5654 (1966).
(3) The structures assigned to substances reported herein were confirmed by infrared and NMR spectroscopy (^1H and ^{31}P) using chromatographically homogeneous samples. Mass spectral data, obtained for all substances except for salts, also were in accord with designated structures.
(4) L. Zervas and I. Dilaris, *J. Am. Chem. Soc.*, **77**, 5354 (1955).
(5) R. H. Cornforth and G. Popják, *Methods Enzymol.*, **15**, 382 (1969).
(6) Typically 0.5 g of crude trilithium salt was chromatographed on a column of 50 g of EMS silica gel 60, 70–230 mesh using *n*-propyl alcohol–11 *N* ammonium hydroxide (1.5 to 1) for elution. The trilithium phosphonophosphates used in this study showed in the ^{31}P NMR spectra (D_2O solution) the expected pair of doublets at +6.08 and +5.47 ppm (J = 24.6 Hz) and −19.15 and −18.55 ppm (J = 24.3 Hz), relative to an external standard of orthophosphoric acid (Varian XL-100 instrument at 40.5 MHz field).
(7) (a) H. C. Rilling and W. W. Epstein, *J. Am. Chem. Soc.*, **91**, 1041 (1969); (b) H. C. Rilling, C. D. Poulter, W. W. Epstein, and B. Larsen, *ibid.*, **93**, 1783 (1971); (c) G. Popják, J. Edmond, and S.-M. Wong, *ibid.*, **95**, 2713 (1973).
(8) Prepared according to L. J. Altman, R. C. Kowerski, and H. C. Rilling, *J. Am. Chem. Soc.*, **93**, 1782 (1971).
(9) Prepared from $Bu_3{}^+PCH_2PO(OPh)_2Cl^-$ by sequential treatment with 1 equiv of potassium *tert*-butoxide and 2 equiv of sodium methoxide; see J. G. Moffatt and G. H. Jones, U.S. Patent 3 583 974; *Chem. Abstr.*, **75**, 130091q (1971).
(10) G. Popják, *Methods Enzymol.*, **15**, 438 (1969).
(11) See T. T. Tchen, *Methods Enzymol.*, **6**, 509 (1963), for method of incubation. No dispersant (e.g., Tween 80) was used; inhibitor (or substrate in the case of presqualene pyrophosphate) was deposited as a film in the incubator tube by evaporation from benzene solution and mixed with the enzyme solution by agitation using a vortex mixer. Labeled squalene was purified by preparative thin layer chromatography on a silica gel plate (0.25 mm thickness of layer, 15 cm length) using 2% ether–98% petroleum ether for development (R_f 0.60 for squalene).
(12) C. A. West, *Methods Enzymol.*, **15**, 481 (1969).
(13) See, for example, J. W. Cornforth, *Chem. Soc. Rev.*, **2**, 1 (1973); I. Schechter and K. Bloch, *J. Biol. Chem.*, **246**, 7690 (1971).
(14) See ref 10, pp 450–453.
(15) In this and all other experiments with the S_{10} system, NADPH, Mg^{2+}, and all other necessary cofactors had been added in the usual amounts.
(16) For other recent papers relevant to the role of presqualene pyrophosphate in the biosynthesis of squalene, see (a) F. Musco, J. P. Carlson, L. Kuehl, and H. C. Rilling, *J. Biol. Chem.*, **249**, 3746 (1974); (b) G. Popják, H. Ngan, and W. Agnew, *Bioorg. Chem.*, **4**, 279 (1975).

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Effect of Photoselection on Fluorescence-Detected Circular Dichroism

Sir:

In a recent study Turner et al.[1] have proposed that the circular dichroism, CD, of a fluorescent chromophore can be measured by detecting its fluorescence upon excitation by right-handed and left-handed circularly polarized light. The underlying assumption is that the excitation spectrum of a fluorescent chromophore parallels its absorption spectrum, i.e., that the measured fluorescence intensity of the chromophore depends exclusively on the amount of light absorbed by it. It was pointed out that such studies may be advantageous for the specific measurement of the CD of the fluorescent chromophores in biopolymers, thus eliminating contributions from nonfluorescent chromophores with overlapping absorption bands, which are often also present in the macromolecules.[1]

While the proposed method for measuring CD via emitted fluorescence intensity is promising and of much interest, it may be in serious error when applied to chromophores when rotatory Brownian motion is frozen (or restricted) during the lifetime of the excited state of the chromophore. This restriction may apply, for example, to a variety of native chromophores in biopolymers. The physical reason behind the complication which arises in frozen systems is as follows. The light absorbed by the system under study does not excite equally molecules of different orientations, since the probability of light absorption by a specific molecule depends on the orientations of its electric and magnetic dipole as well as electric quadrupole transition moments relative to the vector potential and direction of propagation of the light wave.[2a] In the case of circularly polarized light, the probability of excitation of a specific molecule thus depends on the sense of polarization. If rotatory Brownian motion does not randomize molecular orientations before light emission, different anisotropic populations of excited molecules contribute to the fluorescence upon excitation with right-handed or left-handed circularly polarized light. The observed intensity of fluorescence depends not only on the number of excited molecules, but also on the distribution in space of

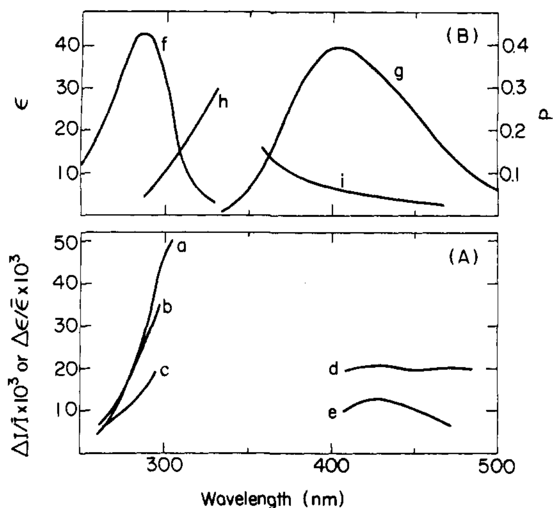

Figure 1. Spectroscopic properties of D-camphor. A. Circular dichroism, presented as $\Delta\epsilon/\bar{\epsilon}$, and difference in fluorescence intensity upon excitation by circularly polarized light of opposite sense, presented as $\Delta I/\bar{I}$. (a) $\Delta\epsilon/\bar{\epsilon}$ in methanol or cyclohexane (concentration: $\sim 10^{-2}$ M); (b) $\Delta I/\bar{I}$ in methanol or cyclohexane as a function of absorption wavelength (emission wavelength: 425 nm); (c) $\Delta I/\bar{I}$ in glycerol as a function of absorption wavelength (emission wavelength: 425 nm); (d) $\Delta I/\bar{I}$ in methanol or cyclohexane as a function of emission wavelength (absorption wavelength: 290 nm); (e) $\Delta I/\bar{I}$ in glycerol as a function of emission wavelength (absorption wavelength: 290 nm). $\Delta I/\bar{I}$ was measured with camphor concentration of $\sim 10^{-2}$ M (optical density 0.08/2 mm), using cuvettes of 2.0-mm optical path. The fluorescence was detected at 0° to the excitation beam. A Schott UG11 and a 2 N solution of $NaNO_2$ (1.0-cm light path) were used as filters on the excitation and emission sides, respectively. Temperature, 22 °C. B. (f) absorption spectrum in glycerol; (g) fluorescence spectrum (in arbitrary units) in glycerol; (h) excitation linear polarization in glycerol (emission wavelength: 365 nm); (i) emission linear polarization in glycerol (excitation wavelength: 300 nm).

their emission transition dipole and quadrupole moments. Under such circumstances, the observed difference in emission intensity upon excitation by light of opposite sense does not reflect in a simple manner the difference in absorption of light of opposite sense by the system studied. This is analogous to the case treated previously[2b] in which the fluorescence excitation spectrum in viscous media does not in general superimpose the corresponding absorption spectrum.

The intensity and the extent of polarization of fluorescence when photoselection takes place in frozen solutions have been treated theoretically in a recent study.[3] By the formalism developed, the intensity of the fluorescence emitted by a frozen system in a given direction can be evaluated as a function of the electric and magnetic transition dipole moments associated with the absorption and emission processes. (Quadrupole contributions have not been treated but may be similarly included.) Analogously to the examples worked out,[3] the following expression is obtained for the fluorescence intensity upon excitation by circularly polarized light. The fluorescence is assumed to be collected in the direction of the excitation beam:[4]

$$\frac{\Delta I}{\bar{I}} = \frac{I^l - I^r}{(I^l + I^r)/2} = 4\frac{3P_1^{ab}\tilde{M}_1^{ab} + 3P_2^{ab}\tilde{M}_2^{ab} + 4P_3^{ab}\tilde{M}_3^{ab}}{3(P_1^{ab})^2 + 3(P_2^{ab})^2 + 4(P_3^{ab})^2} \quad (1)$$

In eq 1, I^l and I^r are the fluorescence intensities emitted by the sample upon excitation by left-handed and right-handed circularly polarized light, respectively; $\mathbf{P}^{ab}$ and $\tilde{\mathbf{M}}^{ab}$ (with components P_i and $\tilde{M}_i$) are the electric and magnetic (absolute value, assuming real wave functions)[3] transition dipole moments, respectively, associated with the light absorption. Quadrupole transition moments have been omitted for the sake of simplicity; in practice their magnitude may not be negligible and they may add to the effect of photoselection. The frame of reference was chosen so that $\mathbf{P}^{em}$, the electric transition moment associated with the emission transition, points in the z direction. Equation 1 was obtained for dilute solutions where the attenuation of the excitation beam is negligible. Equation 1 exposes the complications that arise in the study of fluorescence-detected CD when photoselection takes place. I^l and I^r were assumed by Turner et al.[1] to be proportional in dilute solutions to ϵ_l and ϵ_r, respectively (ϵ_l and ϵ_r being the molar extinction coefficients for left-handed and right-handed circularly polarized light, respectively). The left-hand side of eq 1 should thus be equal to the absorption anisotropy factor g_{ab}, defined as $g_{ab} = \Delta\epsilon/\bar{\epsilon} = (\epsilon_l - \epsilon_r)/[(\epsilon_l + \epsilon_r)/2]$.[2] Equation 1 shows, however, that this is not always the case, since $g_{ab} = 4\mathbf{P}^{ab}\cdot\tilde{\mathbf{M}}^{ab}/|\mathbf{P}^{ab}|^2$ as was shown long ago by Condon et al.[2] Thus, unless $P_3^{ab} = 0$ (i.e., $\mathbf{P}^{ab}$ is perpendicular to $\mathbf{P}^{em}$) or $P_1^{ab} = P_2^{ab} = 0$ (i.e., $\mathbf{P}^{ab}$ is parallel to $\mathbf{P}^{em}$), $(I^l - I^r)/[(I^l + I^r)/2]$ is not equal to $(\epsilon_l - \epsilon_r)/[(\epsilon_l + \epsilon_r)/2]$. $\mathbf{P}^{ab}$ is parallel to $\mathbf{P}^{em}$ for the long-wavelength absorption band of the chromophore involved (provided it involves a strong transition,[5]) but for other transitions $\mathbf{P}^{ab}$ may be neither parallel nor perpendicular to $\mathbf{P}^{em}$ and $\Delta I/\bar{I}$ may not yield g_{ab} for these transitions.

There is a specific experimental setup in which the effect of photoselection on fluorescence detected CD vanishes. Using the theoretical approach described previously,[3] it can be readily shown that if the angle θ between the direction of the excitation beam and the direction of detection of the fluorescence is 54.74° or 125.26° (i.e., $\cos^2\theta = 1/3$), then $\Delta I/\bar{I}$ will yield exactly the absorption anisotropy factor g_{ab}. It should be pointed out that the above calculation was carried out including quadrupole contributions.

The effect of photoselection on the measured fluorescence intensities upon excitation by right-handed and left-handed circularly polarized light is illustrated experimentally for D-camphor in Figure 1. The fluorescence was measured at 0° to the excitation beam on an instrument similar to the one described for the study of circular polarization of fluorescence,[6] except that the modulator was placed in front of the cell holder and the polarizer was placed in front of the modulator. As is evident from Figure 1, similar results were obtained for $\Delta I/\bar{I}$ when the measurements were conducted in two different fluid solvents, methanol and cyclohexane, and both were in good agreement with g_{ab} measured with a Cary 60 instrument. (These were in good agreement with previously measured spectra of D-camphor.[7,8]) The measured value of $\Delta I/\bar{I}$ was, however, significantly different when the viscous solvent glycerol was used, although the CD measured in glycerol solution was similar to that measured in the fluid solvents methanol and cyclohexane.

The variation of g_{ab} across the spectrum deserves a comment. It is expected to be approximately constant across a single electronic band[9] for an allowed transition. The marked variation of this quantity across the absorption spectrum is no doubt a reflection of the weakness of the transition involved and indicates presence of elements of "forbidden" character in this transition. This is also indicated by the marked variation of the linear polarization across the absorption and emission spectra (see Figure 1b).

In summary, when Brownian rotatory motion is frozen or restricted during the lifetime of the excited state, the difference in the fluorescence intensity of a chromophore upon excitation by left-handed and right-handed circularly polar-

ized light does not necessarily represent the circular dichroism of the chromophore in isotropic solution. Discrepancies between the two may occur if the electric dipole transition moments in the absorption band and in the emission band are not parallel or perpendicular to each other. Whether or not such complications occur in a system under investigation may be checked by measurement of the linear polarization of the fluorescence. It may be noted that CD is often used in biochemistry as a diagnostic tool for conformation without an attempt at a physical interpretation of the data, as such discrepancies between CD and fluorescence-detected CD may be of no consequence. In spectroscopic studies of chiral molecules, however, such discrepancies should not be ignored. It is of interest to note that the study of CD by fluorescence in frozen systems has the elements of measuring CD of a nonisotropically distributed molecular assembly; discrepancies between such data and CD obtained conventionally may therefore in principle yield molecular parameters not obtainable from CD alone.[10]

References and Notes

(1) D. H. Turner, I. Tinoco, Jr., and U. Naestre, *J. Am. Chem. Soc.*, **96**, 4340–4342 (1974).
(2) (a) E. U. Condon, W. Altar, and H. Eyring, *J. Chem. Phys.*, **5**, 753–775 (1937); (b) A. H. Kalantar, *ibid.*, **48**, 4992–4996 (1968).
(3) I. Z. Steinberg and B. Ehrenberg, *J. Chem. Phys.*, **61**, 3382–3386 (1974).
(4) If the fluorescence is collected at 90° to the excitation light, the following expression is obtained:

$$\frac{\Delta I}{I} = 4\,\frac{7P_1^{ab}\tilde{M}_1^{ab} + 7P_2^{ab}\tilde{M}_2^{ab} + 6P_3^{ab}\tilde{M}_3^{ab}}{7(P_1^{ab})^2 + 7(P_2^{ab})^2 + 6(P_3^{ab})^2}$$

(5) A. C. Albrecht, *J. Chem. Phys.*, **33**, 156–169 (1960).
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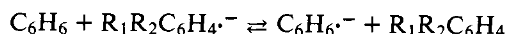
The Relative Stability of Alkyl-Substituted Benzene Anions in the Gas Phase

Sir:

The negative ions of benzene and the alkylbenzenes may be formed in the gas phase by capture of an impacting electron into the low-lying π^* orbitals. The ions, however, are short-lived and decay by ejecting the electron back into the continuum in times of typically 10^{-14} s. Despite the transient character of these ions, their formation is easily observed as a sharp variation, or "resonance", in the cross section for electron scattering from the neutral molecule. The impact energy at which the resonant structure is observed yields the magnitude of the gas phase electron affinity; the sign of the electron affinity is taken by convention to be negative for an unstable anion. In this communication, we have determined the electron affinities of a series of alkyl-substituted benzenes using high resolution electron transmission spectroscopy.[1-3] For a detailed discussion of this method as applied to certain hydrocarbons, we refer the reader to a recent paper by Nenner and Schulz.[2]

The motivation for this study is provided by recent work[4,5] demonstrating that the relative stability of certain anions such as alkoxides may display opposite ordering in the gas phase and in solution. It is of interest to determine whether solvation energies oppose the intrinsic gas phase orderings for other classes of anions. An investigation of the relative stabilities of the benzene anion and its alkyl derivatives is particularly timely since the ordering of these anions in solution has recently been established. Lawler and Tabit[6] have determined the relative electron affinities from equilibrium studies:



$$C_6H_6 + R_1R_2C_6H_4\cdot^- \rightleftarrows C_6H_6\cdot^- + R_1R_2C_6H_4$$

Solutions with known concentrations of benzene and alkylbenzenes were reduced by sodium–potassium alloy in tetrahydrofuran–dimethoxymethane and the ESR spectra taken to determine the equilibrium constants. The measured free energy changes $\Delta G°$ indicate[7] that the introduction of alkyl groups onto the ring makes one-electron reduction more difficult or, equivalently, destabilizes the negative ion in solution. We note that this result is frequently rationalized by "the electron release by the alkyl groups to the ring".

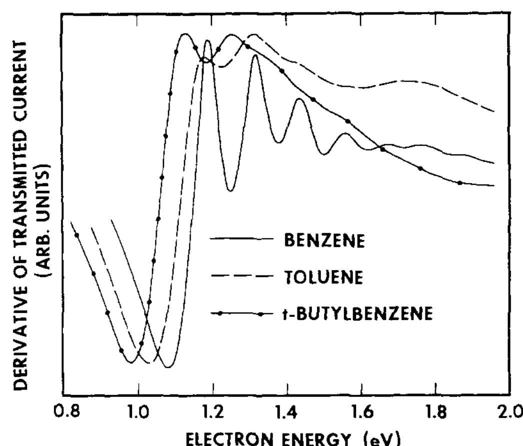


Figure 1. The derivative with respect to energy of the electron current transmitted through the gas cell is plotted as a function of electron energy. The results for benzene and two substituted benzenes, toluene and *tert*-butylbenzene, are shown. These results demonstrate that the anions of the substituted compounds are formed at lower electron energy than that of benzene.

In Figure 1 we present the electron transmission spectra of benzene, toluene, and *tert*-butylbenzene. These spectra are acquired by sending a high resolution electron beam into a cell containing gas at sufficient density to scatter approximately 60% of the electron beam. The *unscattered* or transmitted portion of the beam, which is collected, is related exponentially to the total scattering cross section. To enhance the visibility of the sharp variations in the cross section, the derivative with respect to energy of the transmitted current is plotted in Figure 1 as a function of electron impact energy. The means by which the derivative data are taken is fully described elsewhere.[1]

Our benzene spectrum is in good agreement with those published by Schulz et al.[1,2] The structure corresponds to the capture of an incident electron into the doubly degenerate e_{2u} π^* orbital, yielding the ground electronic state of the benzene anion. The lifetime of the anion is sufficiently long that a progression of vibrational levels of the ν_2 symmetric breathing mode is observed. The spectra of the alkyl-substituted benzenes, two of which are shown in Figure 1, differ from that of benzene in two respects. For the purpose of this communication, the more significant feature is that the anions are formed at *lower* electron impact energies, that is, the alkylbenzene anions are energetically *more* stable than $C_6H_6^-$ in the gas phase. This is opposite to the behavior in solution observed by Lawler and Tabit.[6] A summary of the gas and liquid phase results is presented in Table I.

The visibility of the vibrational structure in the substitut-