Nuclear Magnetic Resonance Studies of Plant Biosynthesis. A Bacteriochlorophyll Isotope Mirror Experiment[1]

Sir:

It is frequently difficult to decide whether the biosynthesis of a complex natural product can proceed by more than one pathway. We now report results indicating that the pathway for the biosynthesis of bacteriochlorophyll may not be unique, and that it may be altered by environmental factors.

In an accompanying communication,[2] the isotopic composition of bacteriochlorophyll derived from the photosynthetic bacterium *Rhodospirillum rubrum* grown in H_2O on succinic acid-d_4 as the only reduced carbon source is reported. We have now examined the isotopic composition (by nmr) of bacteriochlorophyll extracted from *R. rubrum* grown in a medium[3] in which the hydrogen isotopic composition is exactly inverted, *i.e.*, D_2O and succinic acid-h_4. We term such a pair of experiments in which the isotopic compositions of the growth media are exactly reversed a hydrogen isotope mirror experiment, and we refer to a pair of products as isotope mirror products. Results of the experiment with D_2O and succinate-h_4 are given in Table I. Comparative results with H_2O and succinate-d_4 are summarized in the accompanying report.[2]

Table I. Relative Areas of Proton Resonances in Methyl Bacteriopheophorbide[a]

Position[b]	Methyl bacteriopheophorbide (D_2O medium, ordinary succinic acid-h_4)	Computed isotope mirror values[e]
α	0.9 ± 0.05[c]	0[c]
δ	0.9 ± 0.08[c]	0[c]
β	1.0 ± 0.1[c]	0[c]
10	0.8 ± 0.05[c]	0[c]
$8 + 3^0$	0.0 ± 0.1	0.0
$7 + 4^0$	0.0 ± 0.1	0.0
11	0.4 ± 0.97	0.4
12	3.0 ± 0.1	3.0
$5 + 1$	2.7 ± 0.2	2.9
2	3.0 ± 0.2[d]	1.6[d]
$7' + 7''$	2.4 ± 0.2	4.0
$4'$	0.6 ± 0.09	1.0
$8' + 3$	2.8 ± 0.2	2.0
$4''$	0.6 ± 0.1	2.0

[a] Nmr measurements carried out as described in ref 2. Values are the averages of four successive 2500-sec scans. The integrals were obtained by integration with an Ott planimeter and internally standardized. The errors are standard deviations. Duplication of the complete experiment gives closely concordant results. [b] See Table I, ref 2 for proton numbering. [c] The bacteriochlorophyll from these organisms showed no absorption in the methine region; these hydrogens were introduced by exchange during the preparation of the methyl pheophorbide derivative. The 10 position of the bacteriochlorophyll was also exchanged during the extraction and isolation procedure. [d] Exchange occurs during conversion of bacteriochlorophyll to the pheophorbide by reaction wih methanolic HCl. [e] Calculated from Table I, ref 2. The isotopic composition of bacteriochlorophyll grown in H_2O on succinic acid-d_4 (column 1 or 2 of Table I, ref 2) is subtracted from the number of hydrogen atoms at the different hydrogen sites in ordinary bacteriochlorophyll (column 3, Table I, ref 2).

If a unique pathway to bacteriochlorophyll exists in *R. rubrum*, a reversal in the isotopic composition of the growth medium should produce an exact reversal of the isotopic distribution pattern of the resultant bacteriochlorophyll for all reactions that do not involve random exchange of carbon-bound hydrogen with the medium.[4] The primary and secondary kinetic isotope effects in reactions that involve rupture or formation of a carbon–hydrogen *vs.* a carbon–deuterium bond will change the rate of a particular reaction and also the rate of the entire sequence, but if the substrates for the reaction are homogeneous (all C–H or all C–D bonds), isotope rate effects will not change the final product composition. The loss of hydrogen (or deuterium) in a particular hydrogen-transfer reaction (*e.g.*, conversion of a propionic acid side chain to vinyl) must occur regardless of relative rates if the reactions in the two media follow the same path. If isotope reversal affects the reaction sequence, it will be expected to change the isotopic composition of the product, and we interpret a deviation from the expected isotopic composition as evidence for the nonunique character of a reaction scheme. The expected isotope mirror values based on the H_2O–succinic acid-d_4 experiment are listed in the last column of Table I.

The requirement for interpretation of the experiment that the carbon substrate be isotopically homogeneous was satisfied in this case by the demonstrated inability of the organism to grow in D_2O in the absence of succinate. Extensive nutritional experiments have confirmed the inability of the organism to produce endogenous succinic acid in D_2O.

The sequential character of the biosynthetic pathway to chlorophyll as presently understood[5] provides a suitable basis for the discussion of our mirror experiment. The relative area of the $7' + 7''$ resonance[6] in the methyl bacteriopheophorbide derived from D_2O–succinate-h_4 grown organisms is much smaller than would be expected if only exogenous succinate were used in the bacteriochlorophyll synthesis and no exchange reactions occurred at these positions during the biogenesis.[5] The mirror composition for this experiment expected from the H_2O–succinic acid-d_4 experiment would be 4.0 protons as compared to the observed 2.4 at positions 7′ and 7″. It would thus appear that the hydrogen atoms on the side chain at position 7 were specifically exchanged with the medium during the biosynthetic process. This exchange must have occurred after the formation of porphobilinogen[5] because prior exchange (or, for that matter, the utilization of endogenous succinic acid-d_4) would have also decreased the relative number of protons of the 1, 5, 8′, and 3 groups. Table I shows such a decrease did not occur (see below for further discussion).

In the bacteriochlorophyll derived from bacteria grown on H_2O medium with succinic acid-d_4, the ethyl

(1) Based on work performed under the auspices of the U. S. Atomic Energy Commission.

(2) R. C. Dougherty, H. L. Crespi, H. H. Strain, and J. J. Katz, *J. Am. Chem. Soc.*, **88**, 2854 (1966).

(3) E. Flaumenhaft, S. Bose, H. L. Crespi, and J. J. Katz, *Intern. Rev. Cytol.*, **18**, 313 (1965).

(4) The extent of carbon-bound hydrogen exchange with the medium will be determined by the rate at which equilibrium is established and the rate at which the exchangeable intermediate is converted to a nonexchangeable form. In general, both of these rates in this nonequilibrium system will be subject to primary and secondary isotope effects that are different in the two experiments.

(5) J. Lascelles, "Tetrapyrrole Biosynthesis and Its Regulation," W. A. Benjamin, Inc., New York, N. Y., 1964.

(6) See Figure 1 in ref 2 for nomenclature.

group at position 4 clearly had the composition $-CHD-CHD_2$.[2] A reversal of this substitution pattern would have given an ethyl group with the composition $-CDH-CDH_2$. The observed composition after isotope reversal, however, was $CD_{\sim 1.4}H_{\sim 0.6}-CD_{\sim 2.4}H_{\sim 0.6}$. Further, it was clear that this substitution pattern was not due to the presence of only two chemical species (*e.g.*, $-CD_2CD_3$ and $-CDH-CDH_2$), since the shape of the resonances under deuterium decoupling suggested that the predominant hydrogen species was $-CHD-CHD_2$ that was mixed with several other hydrogen species. One explanation for these nmr observations would be that the enzymes responsible for the transformation of the propionic acid side chain at position 6 into ring V may operate on the side chains, at positions 4 and 7 (and 2) in the deuterio organisms, but not in the protio organisms, to give an acrylic acid type side chain at some stage in the biosynthesis.

The 1 and 5 methyl resonances from the D_2O–succinate-h_4 derived methyl bacteriopheophorbide showed a reversal of the isotope substitution pattern observed for the isotope mirror organisms within experimental error, whereas the 3 and 8′ resonances did not. The number of protons at the 3 and 8′ positions showed an increase over the expected isotope mirror composition. The 3 and 8′ positions must have undergone less exchange in the D_2O medium than in H_2O. The 3 and 8′ areas relative to the 1 and 5 resonances in the pheophorbide derived from the D_2O–succinate-h_4 grown bacteria were virtually the same. This suggests that, in the organisms grown on D_2O and succinate-h_4, the chemical history of the four ring-methyl groups could have been very similar. In the isotope reverse of this experiment, however, there were substantial differences either in the chemical modifications of the methyl groups or in the exchange reactions with the medium.

The methyl ester function at position 11 for the D_2O–succinate-h_4 derived organisms showed a substitution pattern suggesting the synthesis of this group from succinate followed identical courses in the two different growth media.

We believe that the experiments described here illustrate a procedure that may be widely applicable to the study of chemical reactions in living organisms.

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The Stereoelectronic Course of the Diene–Sulfur Dioxide Reaction

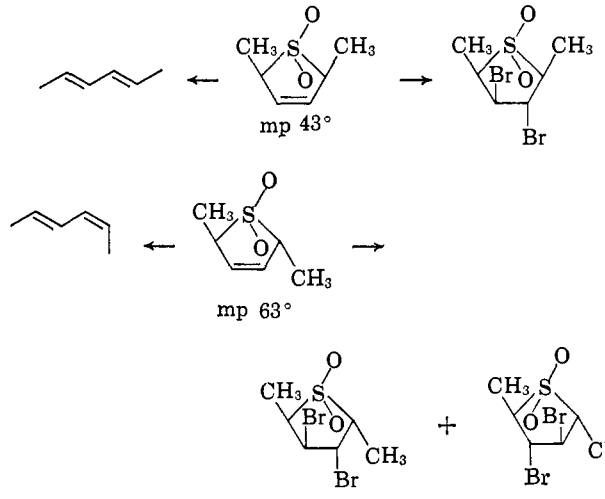
Sir:

We wish to report that the 1,4 addition of sulfur dioxide to conjugated acyclic dienes is a concerted *cis* addition or in the recently introduced terminology[1] is a disrotatory process.[2] This conclusion follows from a study of the retroreaction involving the isomers of 2,4-hexadiene.

(1) R. B. Woodward and R. Hoffmann, *J. Am. Chem. Soc.*, **87**, 395 (1965).

(2) The decomposition in cases involving cyclic dienes is necessarily disrotatory: W. L. Bailey and E. W. Cummings, *ibid.*, **76**, 1936, 1940 (1954).

The known isomer of 2,5-dimethyl-2,5-dihydrothiophene 1,1-dioxide, mp 43° (*Anal.* Found: C, 49.12; H, 6.80), was prepared according to Backer[3] and crystallized from pentane. Chromatography of the mother liquor on alumina (hexane–benzene eluent) provided a second isomer, mp 63–63.5° (*Anal.* Found: C, 49.89; H, 6.67). Upon vapor phase pyrolysis at 200° and 1 mm of argon pressure in a rapid flow system with subsequent trapping and analysis by vapor phase chromatography[4] these substances gave *trans,trans*- and *trans,cis*-2,4-hexadiene, respectively, with greater than 99.9% stereospecificity.



Configurational assignments for the isomeric 2,5-dimethyl-2,5-dihydrothiophene 1,1-dioxides are based on the nmr spectra of their corresponding dibromides. The nmr spectrum of the dibromide, mp 123–124° (*Anal.* Found: C, 23.57, H, 3.45), of the isomer affording *trans,trans*-2,4-hexadiene shows nonequivalent methyl groups (τ 8.47 and 8.51, doublets, J = 6.9 and 7.2 cps in $CDCl_3$) and therefore has methyl groups *cis* to one another, assuming *trans* addition of bromine to the double bond.[5] The other isomer afforded two dibromides in unequal amount, each possessing equivalent methyl groups as shown by nmr (τ 8.47 and 8.49, doublets, both J = 6.7 cps). Hence this isomer has methyl groups *trans* to one another. The more abundant dibromide, mp 88–88.5° (*Anal.* Found: C, 23.97; H, 3.25), was isolated by fractional crystallization from hexane.

The importance of steric factors in this decomposition is revealed by the facts that the *cis*-dimethyldihydrothiophene dioxide gives only *trans,trans*-2,4-hexadiene and no *cis,cis*-2,4-hexadiene[6] and that the decomposition temperature of the *trans*-dimethyldihydrothiophene dioxide is 50° higher in the neat liquid than that of the *cis* isomer (150 *vs.* 100°, vigorous gas evolution). This latter observation cannot be ascribed to destabilization of the *cis* isomer relative to the *trans* isomer due to steric repulsion by the methyl groups since at equilibrium this species predominates. Equilibration was achieved from either isomer in 3–4 days

(3) H. J. Backer, J. Strating, and C. M. H. Kool, *Rec. Trav. Chim.*, **58**, 778 (1939).

(4) L. K. Montgomery, K. Schueller, and P. D. Bartlett, *J. Am. Chem. Soc.*, **86**, 622 (1964).

(5) This conclusion was confirmed by the formation of an osmium tetroxide–pyridine adduct of the olefin in which the methyl groups are equivalent in the nmr spectrum.

(6) Only *trans*-piperyline is obtained from 2-methyl-2,5-dihydrothiophene 1,1-dioxide: D. Craig, *J. Am. Chem. Soc.*, **65**, 1006 (1943).