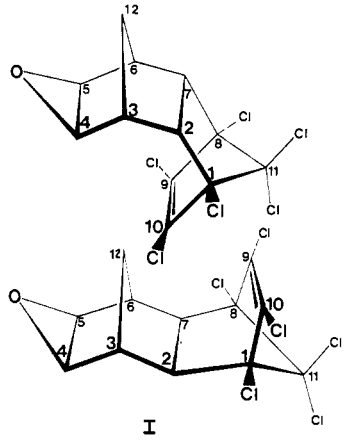
Carbon-13 Fourier Transform Nuclear Magnetic Resonance with Eu(fod)$_3$ for Configurational Confirmation of Polychlorinated Pesticides, Endrin and Dieldrin

The structures of endrin, hexachloroepoxyoctahydro-*endo,endo*-dimethanonaphthalene, and dieldrin, hexachloroepoxyoctahydro-*endo,exo*-dimethanonaphthalene, widely used chlorinated pesticides, were confirmed by natural abundance carbon-13 Fourier transform nmr. Configurational confirmation was accomplished with the aid of a paramagnetic chemical-shift reagent tris(1,1,1,2,2,3,3-heptafluoro-7,7-dimethyl-4,6-octanedionato)europium(III), Eu(fod)$_3$. Assignments were confirmed on the basis of the magnitudes of the chemically induced shifts, the carbon-13 proton coupling constants, and nuclear Overhauser enhancements (NOE). The loss of NOE, observed in the carbon-13 spectra upon successive additions of the chemical-shift reagent, was cited as a diagnostic for making structural assignments. The marked advantages offered by these techniques over existing methods, for pesticide chemistry, are discussed and information regarding the point of attachment of shift reagents to such substrates is observed.

Of the vast number of pesticides currently in use, those of the chlorinated polycyclodiene derivatives, such as endrin, hexachloroepoxyoctahydro-*endo,endo*-dimethanonaphthalene, and dieldrin, hexachloroepoxyoctahydro-*endo,exo*-dimethanonaphthalene (I), are among the most



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stable. Since these pesticides are of such widespread usage, considerable concern is being generated in regard to their being potentially hazardous environmental contaminants. An important consequence of the increasing concentrations of these species lies in the possibility of the formation of detrimental by-products through reactions with other environmental contaminants or naturally occurring substances.

To study adequately the nature of any such reactions, and/or the subsequently generated by-products, one must be able to monitor every possible reactive site of the molecule. In a recent review article, Keith and Alford (1970) clearly elucidated the tremendous potential that nuclear magnetic resonance (nmr) spectroscopy holds for purposes of identifying pesticide metabolites or other such degradation products. Damico *et al.* (1968) and Feil *et al.* (1970) have studied the nature and structures of various dieldrin metabolites by combining a host of instrumental techniques, such as mass spectrometry, infrared (ir), Raman, and proton magnetic resonance (pmr) spectroscopy, with various chemical methods of structural determination. Of the techniques used, pmr seems to be the most widely applicable in pesticide chemistry from a conformational and structural elucidation point of view. Even this technique, however, powerful though it may be, has a marked disadvantage in studies involving highly chlorinated pesticides of the type under investigation here (endrin and dieldrin) in that pmr can only monitor the portion of the molecule that contains protons. For pesticides that have carbon atoms devoid of protons (such as the reactive chlorinated end of endrin or dieldrin) the pmr technique alone is clearly inadequate even though it has been widely used for this purpose (Parsons and Moore (1966), Marchand and Rose (1968), Bukowski and Cisak (1968), McCullock *et al.* (1969), and Keith *et al.* (1970)). Keith (1971) and McKinney *et al.* (1972), in an effort to study degradation reactions of some pesticides, have implemented the use of pmr spectroscopy by employing additional techniques such as proton spin decoupling and a chemical-shift reagent, Eu(dpm)$_3$, tris(dipivalomethanato)europium(III). These studies, while accurately describing the structural features of that portion of the molecule containing protons, yielded no information whatever concerning the crucial chlorinated section of the molecule. Since the chlorinated carbons of dieldrin have been shown by Keith (1971) to participate in a photolyzed degradation reaction involving the breakage of a carbon–carbon double bond, it appears quite evident that other degradative or associative reactions may also take place solely on this portion of the molecule.

We report here the application of carbon-13 nmr (natural abundance, Fourier transform spectroscopy) as a means of establishing the complete molecular configuration of polychlorinated pesticides. This technique is clearly superior to pmr in that it monitors the entire skeletal structure of the pesticide in question (or its metabolites) including the chlorinated portions of the molecule that are most likely to participate in chemical reactions. Natural abundance, Fourier transform carbon-13 nmr does have the disadvantage, though, that concentrations below 0.12 *M* are difficult if not impossible to detect.

A Varian Associates XL-100 nmr spectrometer with a 15-in. magnet (operating at 25.2 MHz in the ^{13}C mode), a pulse unit, broad band random noise ^1H decoupler, and deuterium lock was used in this study. In ~18 min 3000 pulses of 30-μsec duration were applied with 0.4-sec accumulation time between pulses. The range of 5000 Hz was covered by 4096 addresses in the Fourier transform spectrum. All other variables in the system were held constant. Off-resonance decoupling was accomplished by setting the decoupler bandwidth at zero and keeping all other variables constant. The endrin and dieldrin samples were obtained from Shell Chemical Co. and were recrystallized prior to use. The shift reagent was obtained from Alpha Products Corporation and was dried over P_2O_5 in a vacuum desiccator.

Each molecule shown in I possesses a plane of symmetry which bisects the molecule lengthwise passing through the epoxide oxygen and through carbon atoms labeled 11 and 12. This symmetry plane causes carbons labeled 4 and 5 to be identical. The other pairs of identical carbons are labeled 3 and 6, 2 and 7, 1 and 8, and 9 and 10.

Configurational assignments were made on the basis of ^{13}C chemical shifts, the magnitudes of ^{13}C–^1H coupling

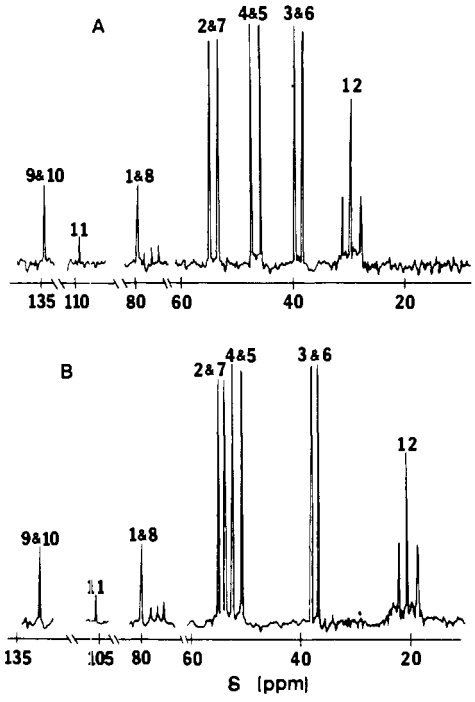


Figure 1. (A) Endrin sample: natural-abundance carbon-13 Fourier transform nmr spectrum with off-resonance decoupling at 36° and 25.2 MHz of chloroform-*d* solution (0.8 *M* and 3000 scans); deuterium lock, spectral offset 32,205 Hz; (B) dieldrin sample, otherwise same as for spectrum A.

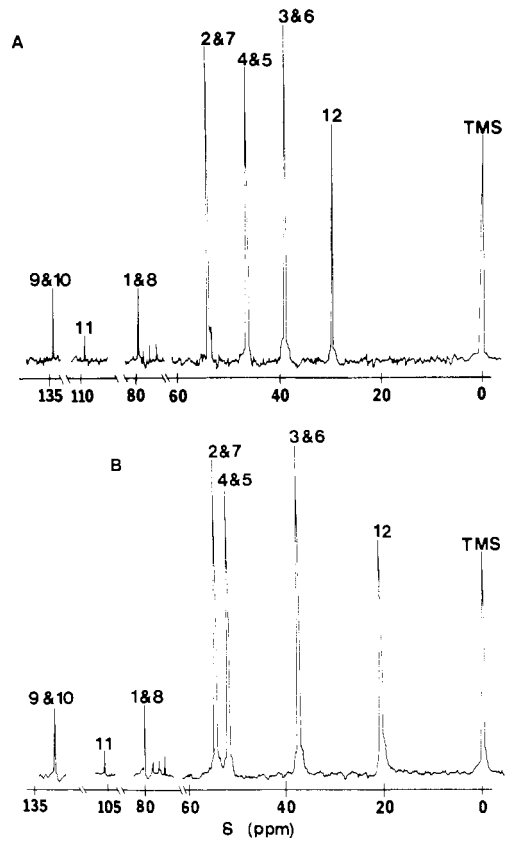


Figure 2. (A) Endrin sample: same as Figure 1A but with broadband proton decoupling (offset 45,545 Hz); (B) dieldrin sample, otherwise same as spectrum A.

constants (J_{C-H} in undecoupled spectra), and changes in ^{13}C chemical shifts induced by the addition of a paramagnetic chemical-shift reagent. By systematically increasing the concentration of chemical-shift reagent, one can simultaneously observe both the changes in magnitudes of the paramagnetically induced chemical shifts as well as the preferential reduction of Overhauser enhancements of each carbon atom peak in the ^{13}C nmr spectrum. This technique alone provides not only structural information about the molecule but also considerable information about the position of attachment of the shift reagent relative to the molecule under investigation.

Figures 1A and 1B show the off-resonance decoupled ^{13}C nmr spectra of endrin and dieldrin. Each spectrum consists of three low-field singlets of low intensity that arise from carbon atoms in the chlorinated portion of the molecules (see I). The high-field peaks (Figures 1A and 1B), from left to right, are attributed to the protonated carbon atoms 2 and 7, 4 and 5, 3 and 6, and 12, and show spin multiplicities of 2, 2, 2, and 3, respectively, arising from their respective proton couplings. The ^{13}C spectrum of C-12 appears as a triplet resulting from spin coupling to two protons. This multiplicity clearly establishes the resonant position for C-12. The first-order ^{13}C–^{1}H coupling constants, obtained from undecoupled spectra, for C-2, -3, -6, -7, and -12 are all approximately equal (J_{C-H} = 145 ± 5 Hz). Carbons-4 and -5, however, contain a considerable amount of sp^2 character (as a result of the epoxide linkage) and exhibit a larger coupling constant, J_{C-H} = 190 Hz. Thus, the resonant position for C-4 and -5 is indicated.

Figures 2A and 2B show the ^{13}C nmr spectra of endrin and dieldrin with complete proton decoupling. C-2–C-7 (and C-12, high field) are clearly distinguished from the resonances of the chlorinated carbon atoms (C-1 and C-8–C-11, low field) by virtue of Overhauser enhancements. Some enhancement, however, might be expected for C-1 and -8 (chlorinated carbons) by virtue of its proximity to protonated carbon atoms, C-2 and -7, and in fact, is observed. The resonant position for C-1 and -8 may further be distinguished from C-9 and -10 and C-11 by chemical shifts and peak intensities. C-9 and -10 are expected to resonate at a lower field position than either C-1 and C-8 or C-11 due to paramagnetic screening effects (as discussed by Ramsey (1950)) imposed by the carbon–carbon double bond (see I) and is so assigned. The intensity of the C-11 resonance is expected to be considerably less than those of either C-1 and -8 or C-9 and -10 by virtue of symmetry (one carbon atom for C-11 and two carbon atoms for each of the other pairs) as well as a sharpened resonance line which results from an increased relaxation time of the carbon atom by virtue of an additional attached chlorine atom.

To confirm the above assignments and to establish resonant positions for C-3 and C-6 and C-2 and C-7, the paramagnetic chemical-shift reagent tris(1,1,1,2,2,3,3-heptafluoro-7,7-dimethyl-4,6-octanedionato)europium(III), Eu(fod)$_3$, was employed. This shift reagent has been shown by Rondeau and Sievers (1971) to associate with lone pairs of electrons on oxygen atoms and to induce large chemical shifts of peak positions in proton magnetic resonance spectra. These shifts have been described as being pseudocontact in nature by Eaton (1965) and Birnbaumand and Moeller (1969) and, therefore, should create greater shifts for those substituents closest to the epoxide oxygen of either the endrin or dieldrin molecule. The magnitudes of these chemically induced shifts are a function of the interatomic distances from the paramagnetic europium nucleus (associated with the epoxide oxygen) to each carbon atom. This effect has been shown to be operative in the ^{13}C nmr spectrum of cholesterol by Smith and Davenport (1972).

Figures 3A and 3B show that the greatest shift in the ^{13}C resonance positions for endrin and dieldrin, upon the addition of Eu(fod)$_3$, occurred for C-4 and -5 as expected.

Table I. Relative Chemical Shifts (ppm) and Loss of NOE[a] for Endrin and Dieldrin

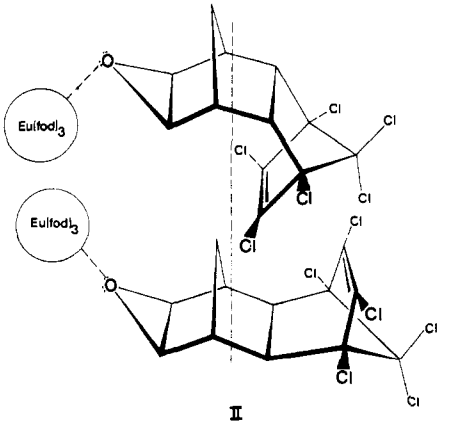
	Endrin		Dieldrin	
Carbons	Chem shift[b]	Loss of NOE[c]	Chem shift	Loss of NOE
4 and 5	22.4	7.8	26.9	10.8
3 and 6	8.0	5.5	6.8	3.1
2 and 7	3.8	1.2	3.5	2.2
12	7.2	1.6	7.6	4.0
1 and 8	3.2	1.0	1.8	1.0
9 and 10	3.2		2.4	
11	1.6		1.1	

[a] Samples were made up at 0.8 *M* in pesticide and 0.344 *M* in $Eu(fod)_3$. [b] Chemical shifts are measured in change of chemical shift relative to the original chemical shift. [c] Relative integrated intensity loss, in arbitrary units, all ± 1.5.

The order of decreasing chemical shifts was found to be: (for endrin) C-4 and -5 > C-3 and -6 > C-12 > C-2 and -7 > C-9 and -10 ≃ C-1 and -8 > C-11; (for dieldrin) C-4 and -5 > C-3 and -6 ≃ C-12 > C-2 and -7 > C-1 and -8 ≃ C-9 and -10 > C-11. This establishes the resonant positions for C-3 and -6 and C-2 and -7 as well as confirming all of our previous assignments. The original chemical shifts for all carbon atoms (Figures 2A and 2B) are: (for endrin) C-4 and -5, 47.2; C-3 and -6, 39.5; C-12, 30.1; C-2 and -7, 54.8; C-1 and -8, 79.5; C-9 and -10, 133; and C-11, 107 ppm; (for dieldrin) C-4 and -5, 50.9; C-3 and -6, 37.0; C-12, 19.8; C-2 and -7, 53.2; C-1 and -8, 80.2; C-9 and -10, 131; C-11, 105 ppm (all *vs.* Me_4Si standard).

It is noteworthy that the sequence of decreasing chemical shifts is the same as that found for the decrease in NOE (for protonated carbon atoms). Upon successive additions of $Eu(fod)_3$ the ^{13}C resonances for the carbon atoms closest to the epoxide suffered the greatest loss in NOE while those farthest away showed very little intensity change (see Table I). The effect of NOE suppression, we feel, will prove to be useful in other structural elucidations.

A comparison of the data obtained for the two compounds, endrin and dieldrin, given in Table I, reveals important and quite unexpected information concerning the apparent point of attachment of the chemical-shift reagent to these substrate molecules. For endrin, C-3 and -6 are shifted further downfield than is C-12, while in dieldrin, C-12 is shifted further downfield than are C-3 and -6. The portion of each molecule including C-4 and -5, -3 and -6, and -12 has the same conformation (see I). Therefore, the only fact that can account for this reversal in the magnitudes of chemical shift for these corresponding carbon atoms is the position of the chemical-shift reagent. Structure II shows the placement of the chemical-shift reagent in relation to the part of each molecule to the left of the dashed line. The circle encompasses the area of maximum probability for the location of chemical-shift reagent. To the right of the dashed line the two molecules differ in conformation. The data from Table I show that for endrin C-9 and -10 are shifted the same amount as are C-1 and -8. In dieldrin, C-9 and -10 are shifted further downfield than are C-1 and -8. The complete structure of both molecules has been previously assigned (by Keith (1971)). From these data we are able to justify the previous assignment of the position of the chemical-shift reagent. It is interesting to note that the position of the chemical-shift reagent appears to be somehow affected by the position of the double bond. Such an effect has not previously been noted.



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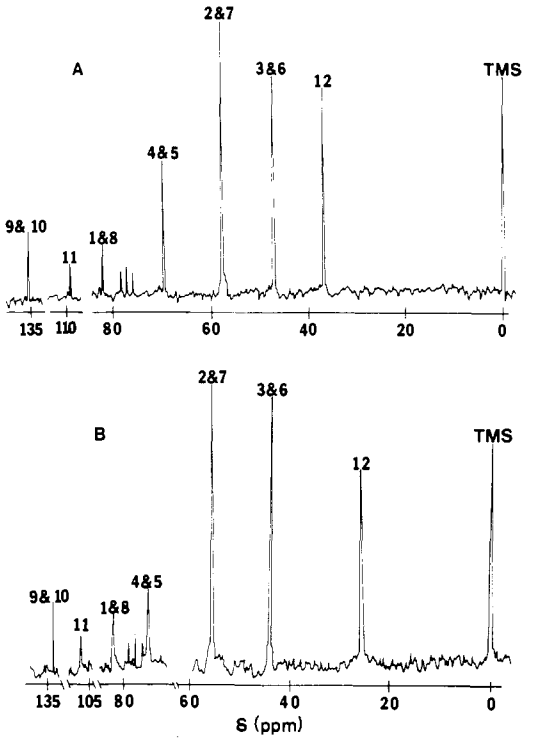

Figure 3. (A) Endrin sample: same as Figure 2A but with 0.344 *M* $Eu(fod)_3$ added; (B) dieldrin sample, otherwise same as spectrum A.

All of the above data are consistent with the structures given previously for the portions of the molecules containing protons (Keith (1971)) and clearly establish the chlorinated portion of the molecules as given in I. Any other molecular geometry would produce a different sequence of paramagnetically induced resonant shifts and Overhauser enhancement changes. The ^{13}C nmr techniques and chemical-shift data reported here may well be useful in ^{13}C investigations of other polychlorinated pesticide derivatives for either mechanistic purposes or structural studies.

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Thermal Gelation of Pig-Skin Tropocollagen

Comparisons of thermal gelation curves for untreated pig-skin tropocollagen with identical samples treated with either pepsin, trypsin, chymotrypsin, or elastase were made. In general, the enzyme treatments altered the shape of the thermal gelation curves by increasing the lag phase and the time required for maximum gelation. However, each of the enzymes affected gelation differently, indicating that cleavage of the tropocollagen molecule occurred at different sites.

Tristram *et al.* (1965) have studied the effects of thermal denaturation upon soluble calf-skin collagen. Rubin *et al.* (1965), Drake *et al.* (1966), and Kühn *et al.* (1966) have shown that proteolytic enzyme treatments alter the aggregation properties and the α- and β-component ratios of calf-skin tropocollagen. Crevasse *et al.* (1969) working in our laboratory verified the effects of proteolytic enzymes upon thermal gelation of calf-skin collagen. They demonstrated that prevention of thermal gelation was greater in the case of elastase followed in order by chymotrypsin, trypsin, and pepsin treatments. Since no information is available on thermal gelation of pig-skin tropocollagen, the present study was undertaken to investigate its thermal gelation properties as altered by treatment with trypsin, pepsin, chymotrypsin, and elastase.

MATERIALS AND METHODS

Acid-soluble pig skin was extracted and purified according to a modification of the procedure utilized in preparation of calf-skin collagen by Rubin *et al.* (1965) as modified by Crevasse *et al.* (1969) with the following exceptions: (1) the precipitate of acid-soluble collagen (fraction 2-A) was redissolved and reprecipitated seven times; (3) the KCl precipitate (fraction 2-B) from the supernatant of the acid-soluble collagen preparation was redissolved and centrifuged for long periods of time and then reprecipitated against 2% NaCl. The precipitate was redissolved in 0.05% acetic acid and centrifuged at 25,000*g* for 24 hr. This was repeated several times, but the supernatant still remained slightly cloudy. After dialysis against 2% NaCl, the sample was frozen and stored.

Enzymatic reactions with pepsin, trypsin, chymotrypsin, and elastase were carried out as outlined by Crevasse *et al.* (1969), except that the substrate concentration was 5.8 mg/ml. Furthermore, the protein was not lyophilized after precipitation, but instead was dialyzed free of salts against distilled water and stored at 4°. Before analysis, the samples were dialyzed against 0.05% acetic acid. Nitrogen determinations were made by the micro-Kjeldahl procedure (Crevasse *et al.*, 1969) and hydroxyproline analysis by the method of Woessner (1961).

Short- and long-term thermal gelation was monitored at 230 nm in a Beckman DU-2 monochronometer using a Gilford automatic cuvette positioner and an absorbance converter connected to a recorder as described in more detail by Crevasse (1967).

RESULTS AND DISCUSSION

Figure 1 shows thermal gelation curves for untreated acid-soluble pig-skin collagen and samples after treatment with pepsin, trypsin, chymotrypsin, and elastase. The untreated sample and the pepsin-treated sample showed similar maximum gelation values. However, untreated collagen had a very short lag phase and maximum gelation had occurred within 10 min. Although pepsin-treated

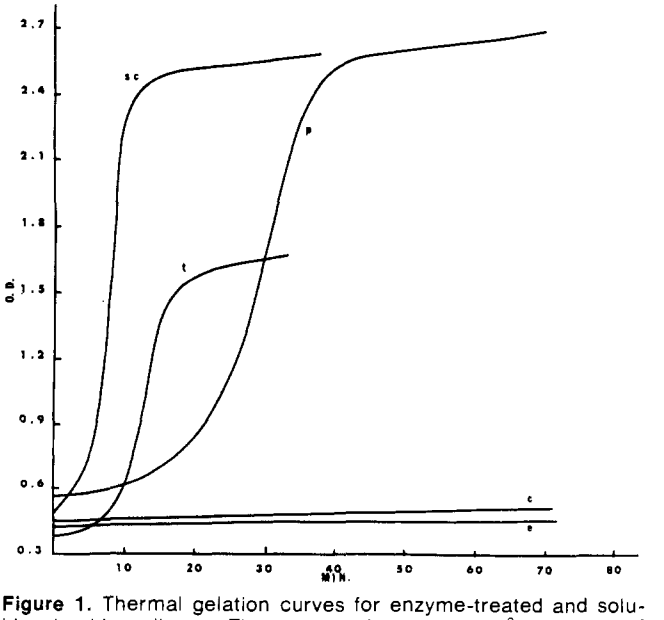

Figure 1. Thermal gelation curves for enzyme-treated and soluble pig-skin collagen. Thermal gelation was at 33° using 0.1 *M* phosphate buffer at pH 7.4: (sc) untreated pig-skin collagen; (p) pepsin treated; (t) trypsin treated; (e) elastase treated; (c) chymotrypsin treated.