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"Spin-Labelling" with the *Second* Hydrogen Radioisotope: Radicals Formed from Squalene by Muonium Addition

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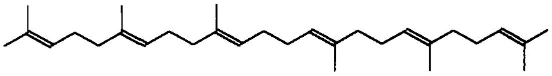
By radio-labelling with muonium (the *second* hydrogen radioisotope), a free radical species has been clearly identified in squalene (2,6,10,15,19,23-hexamethyl-2,6,10,14,18,22-tetracosahexaene) with a muon–electron hyperfine coupling of 240.7 MHz, as measured by the muon spin rotation (MuSR) technique. The radical is undetectable in pure squalene due to its high viscosity and the large molecular size which leads to extreme line broadening, but its signals may be resolved on reducing the viscosity of the medium by dilution with diethyl ether. The potential of the MuSR method is thus demonstrated as a means for spin-labelling radical species such as are formed from cellular antioxidants; ESR spectroscopy is unsuitable for studies of radicals formed from this molecule, due to extreme line-broadening and spectral complexity.

Keywords: Radioisotope, muonium, squalene, antioxidant, free radical, membrane, dietary supplement

INTRODUCTION

Squalene (2,6,10,15,19,23-hexamethyl-2,6,10,14,18,22-tetracosahexaene) (I), is a key intermediate



(I)

in the biosynthesis of steroids.[1] Recently, it has received attention as a dietary supplement with antioxidant properties, and as such is proposed to be a potential anti-cancer agent.[2] It is also proposed to protect the skin against oxidative stress, and there is evidence that it can lower cholesterol and triglyceride levels.[2] Chemical studies have shown that squalene is capable of quenching singlet oxygen in n-butanol solution as a function of its relatively low ionisation potential,[3] and its free radical scavenging properties have been demonstrated in an ESR study of the radiation

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chemical yield of radicals formed in poly(methylmethacrylate).[4]

Direct studies of free radicals formed from squalene by ESR spectroscopy have not, to our knowledge, been reported, and we have ourselves been unsuccessful in observing ESR spectra arising from H-atom abstraction from squalene by photochemically generated tert-butoxyl radicals. We believe that such H-atom abstractions must certainly occur, but, in accord with our later discussion, the viscous nature of a medium rich in squalene is expected to cause extreme line-broadening, and along with the multiline complexity of the ESR spectra of the resulting radicals is probably the reason for our failure to observe them.

In contrast, we have now utilised the Transverse Field Muon Spin Rotation (TF-MuSR) technique which as we show is capable of detecting squalene derived radicals, this time formed by H-(muonium) atom addition to squalene, the positive muon acting as a radioactive spin-label. Thus our initial aim, that relatively large free radical molecules (squalene molecular mass 411 Da) can be made detectable by spin-labelling using positive muons, is met and so, potentially, both molecular dynamic and kinetic data can be measured for them.

MATERIALS AND METHODS

Squalene, 3-ethylpent-3-ene, 2,4,4-trimethylpent-2-ene and diethyl ether were purchased from Aldrich/Fluka, and used as supplied. Solutions of the pure liquids and of pure squalene or its solution in diethyl ether were sealed in 35 mm diameter, thin-walled glass ampoules and were transported to the Paul Scherrer Institute (Switzerland) where they were irradiated with positive muons of momentum 85 MeV/c at the MuE1 beamline, while simultaneously applying an external magnetic field of 2 kG; for the squalene/diethyl ether sample, a total of 325 million good decay events were accumulated to obtain good signal-to-noise with the very broad spectral lines; full details of the MuSR method and the data analysis have been given previously in this journal,[5] but, most importantly, muons (μ^+) are detected by virtue of their radioactive decay (emitting positrons, e^+, so μ^+ is a "β-emitter") and having spin ($I = 1/2$) are magnetic, and so may also reveal details of hyperfine coupling constants in radicals where they are present as "labels".

RESULTS

In Figure 1(a) is shown the resulting TF-MuSR spectrum recorded from a 50% by volume

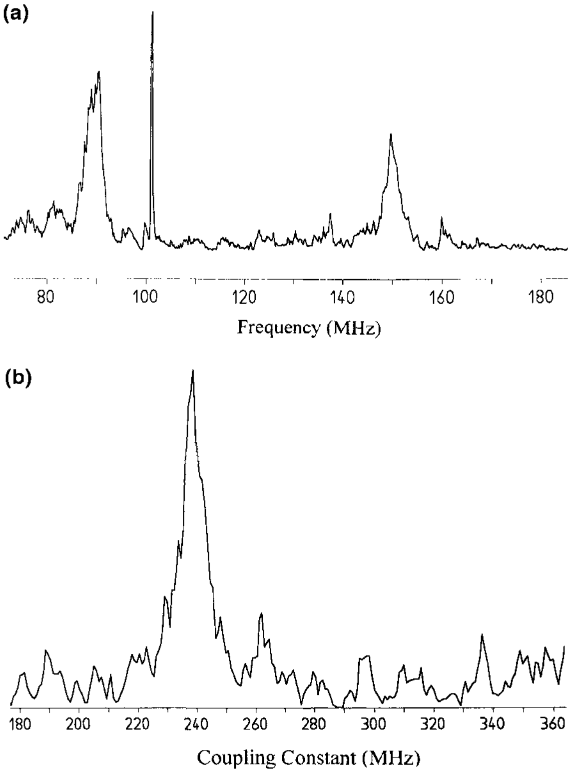

FIGURE 1 (a) TF-MuSR spectrum recorded from a 50 wt% solution of squalene in diethyl ether, showing two broad signals from a mixture of radicals of type RCH$_2$C$^\bullet$(Me)CH(Mu)-CH$_2$R′, resulting from muonium addition to the 3,7,11 (14,18,22) positions of squalene (I), with an almost common coupling (the sharp peak at *ca.* 100 MHz is the first harmonic of the cyclotron frequency, at *ca.* 50 MHz); (b) its correlation spectrum confirming the presence of only radicals with a similar functional identity (the scale is that of *coupling constant* directly).

TABLE I Isotropic muon–electron hyperfine coupling constants (MHz) for muonium adduct radicals formed from squalene and related model compounds

Radical	Coupling constant (MHz)
$Me_2C^\bullet$-CH_2Mu[a]	291.6
$Me_2C^\bullet$-C(Mu)(H)Me[a]	251.4
$Me_2C^\bullet$-C(Mu)(H)Bu^t	251.2
$Et_2C^\bullet$-C(Mu)(H)Me	240.0
$RCH_2(Me)C^\bullet$-C(Mu)-(H)CH_2R' (From squalene)	240.7

[a] Data from Ref. [6].

solution of squalene in diethyl ether; in contrast, no signals were observed when neat squalene was the substrate. The spectrum shows two signals only, which are confirmed to be from a single type of radical species by the correlation spectrum (Figure 1(b)); furthermore, Figure 1(b) confirms that there is just one radical hyperfine coupling present, as there is just one (correlated) peak. The peaks are unusually broad for an organic radical in solution, with an average linewidth of 14 MHz, and correspond to a muon–electron hyperfine coupling for the radical of 240.7 MHz. Coupling constants are given in Table I, for functionally related radicals, which serve as models for the more complex squalene system.

DISCUSSION

The chemical aspect of this work concerns the muonium atom (Mu), which is a hydrogen atom in which a positive muon is the nucleus. Since the muon undergoes radioactive decay (with a mean lifetime of 2.2 μs), muonium may be considered the *second* radioisotope of hydrogen (after tritium). It behaves identically to a normal hydrogen atom (with a proton nucleus), allowing for differences in rates caused by mass effects (Mu has a mass of one-ninth that of a normal hydrogen atom). Therefore, all reactions found for normal hydrogen atoms are to be expected, namely those of abstraction and addition, e.g. (1) and (2):

$$-CH_2-C{=}C- + Mu^\bullet \rightarrow MuH + -CH^\bullet-C{=}C- \tag{1}$$

$$-CH_2-C{=}C- + Mu^\bullet \rightarrow -CH_2-C(Mu)-C^\bullet- \tag{2}$$

However, the TF-MuSR method is highly specific in that it can only detect free radicals which contain a muon, and which may be considered as spin-labelled by it. So radicals formed by addition (2) are the only contributors to the TF-MuSR spectrum recorded from an unsaturated organic compound; those arising by abstraction (1) are invisible to the muon which is lost as (non-radical) muonated dihydrogen.

Inspection of the molecular structure of squalene (I) shows that it contains 6 C=C double bonds, and so there are potentially 12 sites at which a muonium atom could add. In such additions, it is normally tertiary radicals which are formed in preference to secondary radicals, and so we expect addition at the 3,7,11 positions, in each case giving a radical of type:

$$RCH_2C^\bullet(Me)CH(Mu)CH_2R'$$

R′ is, in each case, a "large" hydrocarbon group, and the spin-delocalising properties of the RCH_2CMeCH units are expected to be all very similar; therefore, because there is no reason to believe that addition at either positions 3 or 7 or 11 (equivalent by symmetry with positions 14, 18 and 22) predominates over the others, three radicals are expected, but with an almost common coupling constant. The lines are broad, and it seems likely that any small differences in couplings that may be present are masked by this width. At *ca.* 241 MHz, the coupling is typical for (smaller) tertiary radicals of similar structural type (Table I), which may be considered as models for the squalene-muonium adduct radical. In these radicals, the coupling, which is the result of a time-average, is reduced from that in, e.g. $Me_2C^{\bullet}-CH_2Mu$, by a weighting of conformations in which the C–Mu bond overlaps weakly with the unpaired electron orbital.[6]

We have mentioned that we did not detect any signals in neat squalene, and thought that this might be due to the high viscosity of the liquid;[7] the following arguments support this idea. Spectral lines in magnetic resonance tend to increase in viscous media, and indeed provide a probe of the viscosity, as is usually expressed by the formula[8] (3),

$$\tau = 4\pi\eta r^3/kT, \tag{3}$$

which relates τ, the motional correlation (reorientation) time of a molecule, to the viscosity of its medium η, and r is the mean molecular radius. Clearly, molecules reorient more slowly directly in proportion to the viscosity, and large molecules more slowly still.

For fairly small molecules in mobile liquids such as diethyl ether, motional correlation times in the region of 5×10^{-12} s have been measured.[8] Following the approach outlined for TF-MuSR studies of molecular motion in surface adsorbed systems,[9] and using (3) we estimate that this will correspond to a linewidth of 6 kHz, which is negligible compared with the observation timescale of *ca.* 0.5–15 MHz. However, squalene is around 4000 times more viscous than is diethyl ether, which would contribute a linewidth of 24 MHz; therefore, the lines are expected even only on this basis to be extremely broad and difficult to detect, and the large molecular size (r^3) of squalene could provide a broadening up to *ca.* 100 MHz.

On dilution with diethyl ether (50 wt%), the lines are detected (Figure 1) with widths of 14 MHz. Simple inspection of the solution shows that its bulk viscosity is vastly reduced from that of squalene, but it is interesting to note that a linewidth of 14 MHz corresponds to a motional correlation time of *ca.* 10^{-8} s, and so at the molecular level, which is actually probed by magnetic resonance, the reorientation rates are still reduced by a factor of 2000 from normal fluid media containing small molecules,[8] i.e. at this molecular level it is the interactions between the large and relatively slowly moving squalene molecules which are dominant and are not greatly reduced from those occurring in neat squalene.

CONCLUSIONS

The results presented here show that the TF-MuSR technique has promise for the investigation of radicals formed even from relatively large molecules and their media are relatively viscous, as in membranes. The positive muon can act as a spin-label, providing information on molecular reorientation, can identify the type of radical formed on the basis of the muon–electron hyperfine coupling constant, and could further be used to study the kinetics of the reactions of these molecules with cellular components.[10]

Acknowledgements

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