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Blood, Sweat, and Tears. Toward a Rehabilitation of the INADEQUATE Experiment

Debra L. Mattiello and Ray Freeman[1]

Varian NMR Instruments, 3120 Hansen Way, Palo Alto, California 94304

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The double-quantum-filtered carbon–carbon correlation experiment (INADEQUATE) can be accelerated significantly through a reduction in the spin–lattice relaxation times by dissolving oxygen gas in the solution. The effect is enhanced by lowering the temperature and by pressurizing the sample tube with oxygen. This offers a fourfold reduction in the relaxation times of the carbon-13 resonances in the 125-MHz spectrum of methyl salicylate. The addition of perfluorotertiarybutanol (related to the artificial blood substitutes) increases the amount of oxygen that can be dissolved, so that without oxygen pressurization, similar reductions in the relaxation times can be achieved. The nuclear Overhauser enhancements are only slightly reduced by addition of oxygen. Polarization transfer from the directly attached protons (INEPT) further increases the sensitivity if at least one of the two coupled carbon sites is protonated, principally because the *proton* spin–lattice relaxation times of oxygenated samples are shortened by the relaxation agent. These modest improvements in sensitivity are in general complementary to existing enhancement schemes.

Key Words: **artificial blood; carbon-13; double-quantum filtration; INADEQUATE; INEPT; methyl salicylate; Overhauser effect; oxygen; perfluorotertiary butanol; relaxation agent; spin-lattice relaxation.**

INTRODUCTION

The determination of the connectivity of the carbon skeleton of an organic molecule by two-dimensional correlation spectroscopy (INADEQUATE) (*1–5*) has yet to fulfil its true potential. Although it is perhaps the most direct and unambiguous way to attack a structural problem by NMR, it suffers from an intrinsic lack of sensitivity for samples with the natural abundance of carbon-13. Several techniques have been proposed to alleviate this problem, exploiting polarization transfer from protons to carbon (*6*), from carbon to protons (*7*), or round-trip transfer proton–carbon–proton (*8–12*). These schemes falter or may even fail completely where quaternary carbon sites are involved, yet these are the very sites that are difficult to detect because of slow spin–lattice relaxation due to the absence of protons in the immediate vicinity of the carbon site. This results in protracted experiments and failure to detect correlations involving quaternary sites. Methyl groups also exhibit unusually slow relaxation, attributable to the fast rotational motion of these groups, which reduces the fluctuating local fields at the Larmor frequency. Fortunately methyl carbons are less crucial for the determination of the basic molecular skeleton.

Addition of paramagnetic relaxation agents (*13–15*), such as chromium acetylacetonate, brings relaxation times down to more tractable values but is often unacceptable on chemical grounds. This method carries the additional danger of exceeding the optimum "doping" concentration, leading to signal loss by quenching the nuclear Overhauser effect (*13–15*) and through spin–spin relaxation during the pulse sequence. We therefore sought an alternative paramagnetic agent that could be easily removed from the sample after the measurement, and which was mild enough that adequate relaxation rates could be achieved without fear of excess. The substance would need to be readily available and easily handled in a routine preparative chemistry laboratory. Note that by accelerating relaxation in this manner we also degrade the nuclear Overhauser enhancement to a certain extent by introducing a leakage path that competes with the intramolecular dipole–dipole relaxation.

It is well known that dissolved oxygen shortens spin–lattice relaxation times, so we have investigated its effect quantitatively for a simple test molecule, methyl salicylate (Scheme 1), which has four aromatic protonated carbon sites, three quaternary carbons, and a methoxy group. The liquid was mixed with deuterochloroform in a standard (nonspinning) 5-mm sample tube. Carbon-13 spectra were recorded at 11.7 T in a Varian Inova 500-MHz spectrometer with continuous WALTZ-16 decoupling of the protons (*16*). The one-dimensional spectrum is illustrated in Fig. 1. Spin–lattice relaxation times were measured by the standard inversion-recovery technique (*17*) and evaluated by a three-parameter exponential fitting routine. Estimated errors were always below ±10%. The Overhauser enhancements were determined by the standard gated decoupler method.

[1] On leave from the Department of Chemistry, Cambridge University, Cambridge, U.K.

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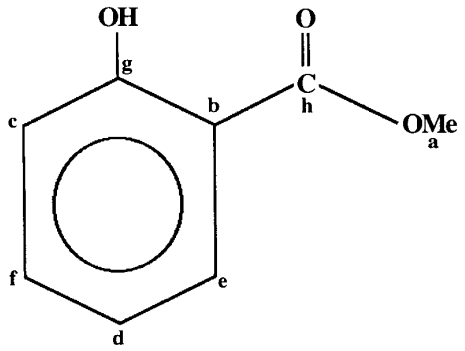

SCHEME 1

EFFECT OF DISSOLVED OXYGEN

Sample A contained 50% by volume of methyl salicylate in deuterochloroform. No attempt was made to degas the sample; this represents the "normal" situation for high-resolution spectroscopy with a dissolved oxygen concentration characteristic of samples in equilibrium with air (21% oxygen). This sample was examined at a probe temperature $T = 25°C$. The quaternary carbons gave T_1 values near 20 s (Table 1), while the protonated sites had T_1 around 4 s. Clearly this sample would require long sessions of data accumulation for the INADEQUATE experiment.

The next sample (B) involved slowly bubbling oxygen gas through the solution inside a standard 5-mm sample tube for an extended period (about 10 min.) followed by closure with the usual plastic cap and cling film to prevent escape of oxygen. This corresponds to equilibration with essentially 1 atm of gaseous oxygen. This sample was also examined at a probe temperature $T = 25°C$. The spin–lattice relaxation times of the quaternary sites were reduced to around 13 to 15 s, while the protonated sites were slightly reduced (Table 1). Although the concentration of methyl salicylate may have been slightly altered by bubbling oxygen through the solution, this is unlikely to have influenced the spin–lattice relaxation times significantly. Clearly this rudimentary procedure would be useful but not decisive; higher concentrations of dissolved oxygen are indicated.

The solubility of gases in organic liquids is controlled by the

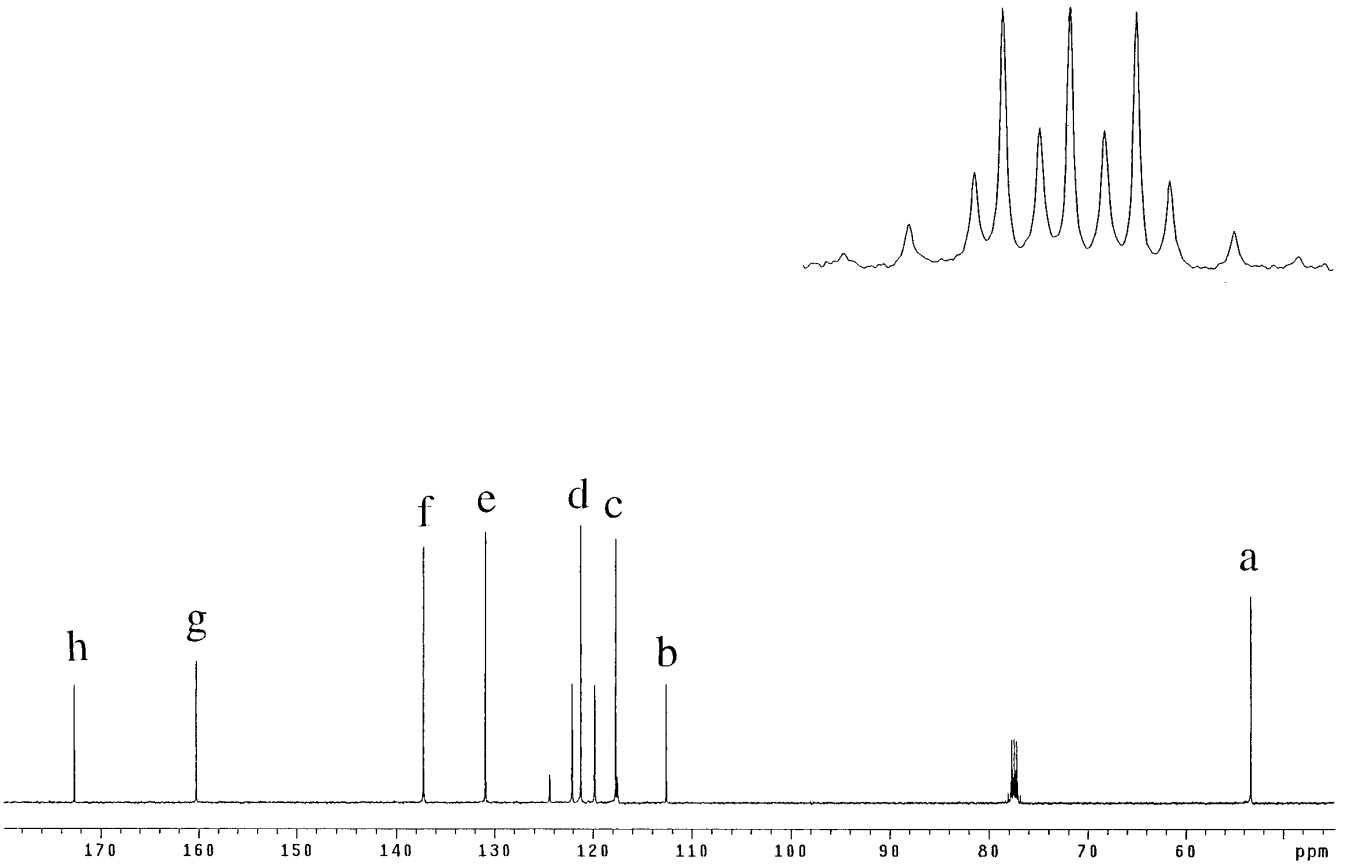

FIG. 1. The 125-MHz carbon-13 spectrum of 20% methyl salicylate in deuterochloroform with 40% added perfluorotertiarybutanol. The assignment of the methyl salicylate resonances is indicated in Scheme 1. The 1:3:3:1 quartet of perfluorotertiarybutanol falls at 121 ppm, and there is also an unusual multiplet at 77.5 ppm (inset, expanded). This turns out to be the superposition of the well-known 1:1:1 triplet of deuterochloroform ($J_{CD} = 32.0$ Hz) and the 10-line multiplet from the quaternary carbon of perfluorotertiarybutanol ($J_{CCF} = 31.1$ Hz). The extreme outer lines are not detected at this level of sensitivity.

TABLE 1

Spin–Lattice Relaxation Times (in seconds) of Carbon-13 Sites of Methyl Salicylate in Deuterochloroform for 8 Different Modes of Preparation of the Sample

	A	B	C	D	E	F	G	H
Quat. (h)	24.4	14.3	13.4	9.9	6.3	9.0	6.6	4.9
Quat. (g)	18.4	13.2	12.1	9.0	5.9	7.2	5.4	4.2
CH (f)	3.5	2.9	2.5	1.9	1.5	1.4	1.1	0.9
CH (e)	3.6	3.2	2.8	2.3	1.9	1.7	1.3	1.1
CH (d)	6.3	4.0	3.0	2.6	2.1	1.7	1.4	1.2
CH (c)	4.7	3.7	3.1	2.2	2.0	1.8	1.4	1.2
Quat. (b)	21.0	15.7	14.2	9.5	6.4	8.4	6.5	4.7

Note. Samples: A, 50% solution at equilibrium with air, no additional oxygen, $T = +25$°C; B, 50% solution bubbled with oxygen at 1 atm at +25°C, $T = +25$°C; C, 50% solution at equilibrium with air, no additional oxygen, $T = +5$°C; D, 50% solution bubbled with oxygen at 1 atm at 0°C, $T = +5$°C; E, 50% solution bubbled with oxygen at 3 atm at 0°C, $T = +5$°C; F, 80% solution at equilibrium with air, no additional oxygen, $T = +5$°C; G, 80% solution bubbled with oxygen at 1 atm at 0°C, $T = +5$°C; H, 80% solution bubbled with oxygen at 3 atm at 0°C, $T = +5$°C (T indicates the probe temperature.).

entropy effect, and therefore increases with decreasing temperature. Furthermore, the rate of spin–lattice relaxation also increases with a lowering of the temperature because, as viscosity increases, the rate of molecular reorientation is slowed. We therefore set out to investigate the effect of a relatively modest reduction in temperature (20°C) for an oxygenated sample. A probe temperature of +5°C was maintained by means of a temperature regulation unit that first cooled nitrogen gas to −40°C and then heated it to the control temperature, monitored by a thermocouple just below the receiver coil. Reducing the probe temperature from +25 to +5°C (sample C) tested the effect of increasing the viscosity, showing spin–lattice relaxation slightly faster than that of sample B which had increased oxygen content (Table 1). Sample D was used to test a combination of both effects. Oxygen was bubbled through the sample at 0°C (ice water bath) and the relaxation times were then measured at a probe temperature of +5°C. The relaxation times of the quaternary sites were reduced below 10 s while the protonated sites had relaxation times of about 2 s.

The next test used a special "high pressure" sample tube (Wilmad #528-TR-7) having a screw cap and a butyl rubber septum. Sample E was prepared by slowly bubbling oxygen gas through the solution at 0°C in the open sample tube. Then the pressure cap was firmly attached and additional oxygen gas was introduced through the septum from a 5-ml plastic syringe, reaching a final oxygen pressure of about 3 atm, judged from the ratio of the volumes. (There is always a certain danger from a combination of an inflammable organic liquid with oxygen under pressure, so the appropriate precautions should be taken in handling such samples.) Equilibration with the higher partial pressure of oxygen was achieved by repeatedly inverting the closed sample tube, always maintained at 0°C. The spectrum was then recorded at a probe temperature of +5°C. The quaternary sites showed relaxation times of approximately 6 s and the protonated sites relaxation times of about 2 s (Table 1). It was observed that the spin–lattice relaxation times increased only slowly over a period of several days, indicating that the septum retained the initial pressure of oxygen very satisfactorily. This very slow leakage rate would have little effect on a typical two-dimensional INADEQUATE overnight run, but might become significant if the sample were stored for several weeks without repressurization.

Since viscosity accelerates spin–lattice relaxation by increasing the spectral density at the Larmor frequency, a new sample was prepared containing 80% of methyl salicylate by volume to evaluate this effect. This sample F had no added oxygen and was examined at a probe temperature of +5°C. Slowing the molecular tumbling in this manner has an advantageous effect, reducing the relaxation times of the quaternary sites to between 7 and 9 s, and the protonated sites below 2 s. Further progress was made (sample G) by bubbling oxygen through this solution at 0°C and recording the spectrum at a probe temperature of +5°C. This reduced the spin–lattice relaxation times of the quaternary sites to approximately 6 s, and those of the protonated sites below 1.5 s.

Finally this 80% sample was placed in a Wilmad pressure tube and equilibrated with oxygen at about 3 atm at 0°C, then studied at a probe temperature of +5°C (the same preparation as the 50% sample E apart from the increased viscosity). In this sample H all the quaternary sites had relaxation times below 5 s, and the protonated sites had T_1 of roughly 1 s.

Cooling, viscosity, and increased oxygen pressure clearly have a significant influence on relaxation. Naturally, when the experiment is limited by the solubility of the sample rather than by the quantity, lowering the temperature would be counterproductive, and simple pressurization alone would be preferable.

A reduction of the spin–lattice relaxation times permits the experiment to be speeded up, and hence increases sensitivity by the square root of the ratio of the repetition rates. The degree to which the INADEQUATE experiment can be accelerated is determined by the relaxation time of the slowest-relaxing site (carbon h). Between samples A and H, the relaxation rate at site h is increased by a factor of 5, equivalent to a sensitivity advantage of 2.24. However, it is important to ensure that this gain is not negated by "quenching" the nuclear Overhauser enhancement by a relaxation mechanism that short circuits dipolar cross relaxation (*13–15*). Because quaternary carbons normally exhibit Overhauser effects much weaker than those of protonated carbons, this risk is not high for the sites of principal concern.

The Overhauser enhancements ($1 + \eta$) were measured by comparing intensities with and without presaturation of the proton signals, and are listed in Table 2 for samples A through H. They indicate that the enhancements measured for sample H are on average only 80% of those measured for sample A. The

TABLE 2

Nuclear Overhauser Enhancement Factors for the Carbon-13 Sites of Methyl Salicylate in Deuterochloroform for the 8 Different Modes of Preparation of the Sample Listed in Table 1

	A	B	C	D	E	F	G	H
Quat. (h)	1.34	1.17	1.29	1.24	1.15	1.33	1.28	1.08
Quat. (g)	1.65	1.46	1.63	1.53	1.28	1.70	1.54	1.15
CH (f)	2.65	2.53	2.62	2.45	2.49	2.53	2.54	2.29
CH (e)	2.72	2.54	2.65	2.53	2.35	2.64	2.51	2.00
CH (d)	2.67	2.50	2.63	2.52	2.30	2.63	2.57	2.34
CH (c)	2.75	2.57	2.70	2.61	2.47	2.73	2.63	2.39
Quat. (b)	1.36	1.25	1.32	1.25	1.14	1.39	1.26	1.03

initial sensitivity advantage of 2.24 is therefore reduced to 1.80. Acceleration of spin–lattice relaxation thus significantly outweighs the effect of quenching the Overhauser effect, and a net advantage is achieved. We shall see below that the effect on proton spin–lattice relaxation is even more marked, a significant feature in polarization transfer experiments of the INEPT type (*18*).

Figure 2 shows a typical two-dimensional INADEQUATE spectrum (sample H containing 80% methyl salicylate, pressurized with oxygen at 3 atm at 0°C, and investigated at a probe temperature of +5°C). It was recorded using an autoswitchable probe optimized for carbon-13 detection. The recycle time was 6.5 s (1.3 times the longest carbon-13 spin–lattice relaxation time) and the entire experiment took 3 h. A noticeable increase in signal-to-noise was achieved by artificially extending the signal in the evolution dimension by linear prediction, permitting the application of a less severe weighting function. All seven correlations are quite evident.

Artificial Blood

Perfluorinated organic molecules are known to dissolve surprisingly large amounts of oxygen, typically 40% by volume, and because they are chemically inert, emulsions of perfluorochemicals have been used to replace blood during surgery and in several other situations where transfusion of normal blood would be undesirable. Unfortunately they are poor solvents and are immiscible with many other liquids. We investigated whether a suitable perfluorochemical could be found that would mix with chloroform and increase the concentration of dissolved oxygen so that carbon-13 spin–lattice relaxation could be accelerated. Ideally, this compound should be rather volatile so that it could be easily removed from the NMR sample at the end of the experiment if necessary.

Perfluorotertiarybutanol (boiling point 45°C) was found to be miscible with chloroform, and we restricted our studies to this material. Figure 1 shows the 125-MHz carbon-13 spectrum

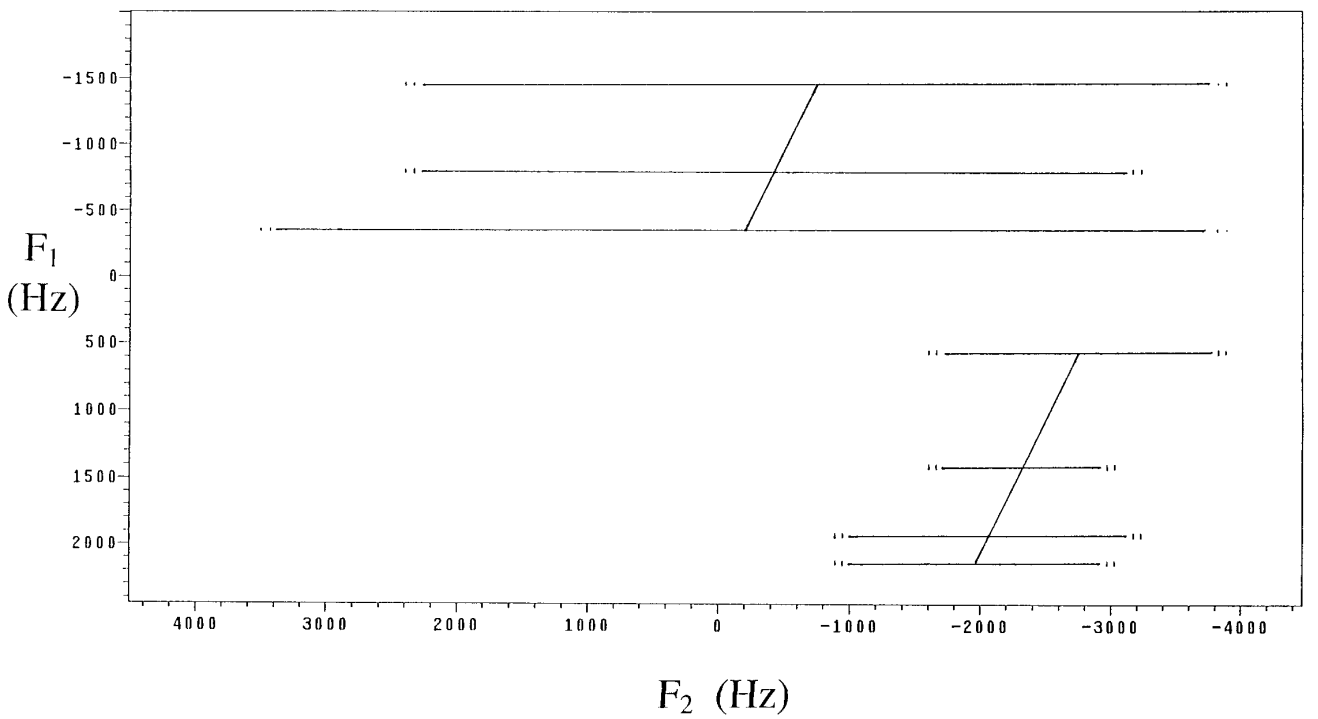

FIG. 2. Two-dimensional INADEQUATE spectrum from sample H, an 80% solution of methyl salicylate and 20% deuterochloroform, bubbled with oxygen at 0°C, then pressurized at 3 atm, and investigated at a probe temperature of +5°C. An autoswitchable probe was used, optimized for carbon-13 detection, with a 7-μs 90° pulse for carbon-13, 50 increments in the evolution interval, extended to 256 points by linear prediction. The recycling interval was 6.5 s and the experiment took 3 h. Folding has been permitted in the F_1 dimension. The skew diagonal ($\Delta F_1 = 2\Delta F_2$) is similarly folded. Seven distinct correlations are evident.

TABLE 3

Effect of Oxygen on the Spin–Lattice Relaxation Times (in seconds) of Methyl Salicylate in a 50:50 Mixture of Methyl Salicylate and Perfluorotertiarybutanol

	I	J	K
Quat. (h)	10.1 [1.5]	6.9 [1.3]	4.0 [1.2]
Quat. (g)	8.3 [1.5]	5.6 [1.4]	3.3 [1.4]
CH (f)	1.4 [2.6]	1.2 [2.5]	0.7 [2.4]
CH (e)	1.4 [2.6]	1.2 [2.6]	0.8 [2.4]
CH (d)	1.9 [2.2]	1.5 [2.2]	0.9 [2.3]
CH (c)	1.6 [2.6]	1.3 [2.6]	0.8 [2.4]
Quat. (b)	9.4 [1.3]	6.8 [1.2]	4.0 [1.1]

Note. The corresponding nuclear Overhauser enhancements are indicated in square brackets [ ]. Samples: I, at equilibrium with air, no additional oxygen, probe temperature +25°C; J, bubbled with oxygen at +25°C, probe temperature +25°C; K, bubbled with oxygen at 0°C, probe temperature +5°C.

of a stock solution of 20% methyl salicylate in deuterochloroform to which perfluorotertiarybutanol was added to make 40% by volume of the latter. The assignments of the methyl salicylate resonances are indicated in Scheme 1. The added perfluorotertiarybutanol generates an easily recognizable 1:3:3:1 quartet at 121 ppm (J_{CF} = 289 Hz) from the CF_3 groups, with some partially resolved fine structure due to long-range CF couplings. In one of those bizarre coincidences that sometimes enliven high-resolution NMR spectroscopy, this spectrum exhibits a very strange-looking multiplet at 77.5 ppm (Fig. 1, inset) which at first caused some confusion. It so happens that there is an exact degeneracy of two chemical shifts and an apparent equality of two splittings. There are in fact two superimposed multiplets—one from chloroform and the other from the quaternary carbon of perfluorotertiarybutanol. This interpretation was confirmed by the observations that the spin–lattice relaxation times differ, that there is a slight difference in coupling constants (J_{CCF} = 31.1 Hz, J_{CD} = 32.0 Hz), and that coherent decoupling of fluorine collapses the 1:9:36:84:126:126:84:36:9:1 decet.

We studied a stock solution of 50% perfluorotertiarybutanol and 50% methyl salicylate with a trace of deuterochloroform for lock purposes. Sample I was measured at a probe temperature of 25°C without adding any oxygen (Table 3). The fact that the relaxation times were roughly half those of sample A may be ascribed partly to increased viscosity but mainly to a higher oxygen concentration caused by the presence of perfluorotertiarybutanol, which dissolves a high level of oxygen from the air. The spin–lattice relaxation times of the quaternary sites were further reduced (below 7 s) when oxygen gas was bubbled through the solution at +25°C and the spectra were recorded at the same temperature (sample J). These quaternary sites had relaxation times reduced to 4 s or less by bubbling oxygen through the solution at 0°C and recording the spectra at a probe temperature of +5°C (sample K). These relaxation times are appreciably shorter than the shortest values reported in Table 1 for the pressurized oxygen sample H at the same temperature. Note that the nuclear Overhauser enhancements are not greatly affected by these procedures, so the sensitivity gain due to faster repetition is scarcely degraded.

A second series of experiments was performed to establish the effect of addition of perfluorotertiarybutanol in a more quantitative fashion. A procedure was devised to avoid the effect of evaporating the volatile perfluorotertiarybutanol while oxygen was being bubbled through the solution. A stock solution of 20% methyl salicylate in deuterochloroform was saturated with pure oxygen at a temperature of 0°C (ice bath) where the solubility is known to be appreciably higher than at +25°C. The appropriate volumes of this solution were introduced into a set of five NMR tubes, to which carefully measured volumes of perfluorotertiarybutanol were added to give 0, 10, 20, 30, and 40% solutions of the blood substitute. These samples were then allowed to warm up slowly to +25°C while the tubes were closed with cotton plugs (to discourage ingress of air) but no plastic caps. The assumption was made that there would be sufficient oxygen still available at +25°C to assure that all the samples were saturated, the amount retained in solution being determined by the effect of the added perfluorotertiarybutanol. The measured carbon-13 spin–lattice relaxation times are plotted in Fig. 3 as a function of the concentration of perfluorotertiarybutanol. Spin–lattice relaxation times decrease according to similar smooth curves for all three quaternary sites, and the protonated sites follow a similar pattern. Even 10% by volume of perfluorotertiarybutanol provides a useful reduction in the T_1 values.

It seems clear that the addition of perfluoro–organic compounds and oxygen can significantly enhance the spin–lattice relaxation of carbon sites. It may well be that other perfluoro compounds will prove even more effective, but perfluorotertiarybutanol appears to be a useful additive; it is miscible with chloroform and is volatile enough to be removed at the end of the experiment.

Setting-up Procedure

Because of its intrinsic insensitivity, the two-dimensional INADEQUATE experiment is difficult to set up, since it usually requires an overnight accumulation before reasonable signal-to-noise ratios are obtained. We have confirmed the usual finding that unless a one-dimensional spectrum can be obtained with reasonable signal-to-noise in a single scan, the corresponding INADEQUATE spectrum will not have acceptable sensitivity.

Considerable effort has been directed toward improving the signal-to-noise ratio by data processing (*19–22*), or by detailed consideration of the experimental parameters (*23–26*). Another factor of 2 can be obtained with the ingenious pulse sequence of Nielsen *et al.* (*27*). Cross polarization by hyperpolarized xenon (*28*) promises spectacular enhancements. Where the aim

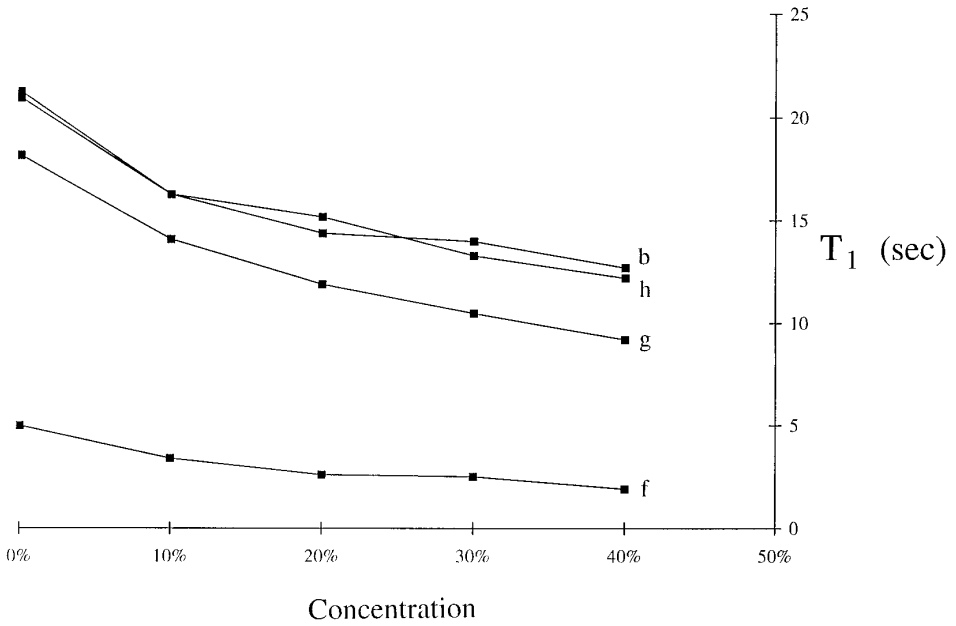

FIG. 3. Experimental carbon-13 spin–lattice relaxation times of sample K made up from a stock solution of 20% methyl salicylate in deuterochloroform (saturated with 1 atm of oxygen) plotted as a function of the concentration of added perfluorotertiarybutanol. The three quaternary carbon sites are shown (b, g, and h) and one representative protonated site (f). All four sites exhibit relaxation times that follow similar curves.

is to obtain an INADEQUATE spectrum from a limited amount of material it may be useful to employ probes specifically designed for very small samples (*29–31*) or superconducting receiver coils (*32, 33*). A second type of limitation is imposed if the solubility is too low. In all cases the probe should be one that has been optimized for detection of carbon-13 spectra, not the more common indirect-detection arrangement where the carbon-13 coil is more remote from the sample. In most of these cases the improvements, though small, are *cumulative* and therefore important.

Clearly it is an advantage to determine the carbon-13 relaxation times from an initial inversion-recovery experiment on isolated carbon-13 sites (neglecting the slight increase in relaxation rate that would be caused by dipole–dipole interaction with an adjacent carbon-13 spin). This experiment has much higher sensitivity and can be completed in a matter of minutes. Calculations of the optimum recycling delay Δ for the highest sensitivity per unit time in the steady-state regime (*34*) indicate that for a flip angle of 90°, the signal-to-noise ratio has a broad maximum centered near $\Delta = 1.3\ T_1$, but shows less than 4% degradation between $\Delta = 0.8\ T_1$ and $\Delta = 2.0\ T_1$. Very little improvement is achieved by setting the flip angle smaller than 90°. For consistency we always employed 90° excitation pulses and a setting $\Delta = 1.3$ times the longest carbon-13 relaxation time in the molecule.

A second problem is that the spin–spin coupling constants are not usually known at this point, so it is difficult to set the timing delays to $1/(4J_{CC})$. One remedy is to employ a sequence that employs coaddition of responses obtained with a variable preparation time τ (*35*). Here we found it useful to perform an initial exploratory measurement in the form of a *one-dimensional* INADEQUATE spectrum, where *all* the correlations fall on a single trace by running the standard two-dimensional INADEQUATE program with $t_1 = 0$. This spectrum indicates the values of the spin–spin coupling constants. For example, the coupling between the carbonyl carbon (b) of methyl salicylate and the nonprotonated aromatic carbon (h) was found to be 74 Hz by this method, which allowed the timings in the final INADEQUATE experiment to be optimized to favor this elusive correlation. Previously the τ delay had been set for $J_{CC} =$ 40 Hz which gave a null response for the (b)–(h) correlation. The use of a rather short τ delay has the advantage that such null conditions are less likely to occur.

The initial test run also indicates the optimum setting of the transmitter frequency to minimize pulse imperfections due to off-resonance effects (most serious for the (b)–(h) correlation). Once the recycling delay Δ and the τ delays are properly chosen, then a definitive two-dimensional INADEQUATE experiment can be undertaken.

Enhancement through Polarization Transfer

It seems clear that the nonprotonated carbon sites represent the main sensitivity challenge owing to their slow spin–lattice relaxation and weak nuclear Overhauser effect. Polarization transfer from protons benefits coupled pairs of carbon atoms if both are protonated, and to a lesser extent when only one is protonated. It is ineffective for two adjacent quaternary sites unless an experiment is specifically designed for transfer through a long-range CH coupling; this is difficult to set up and

TABLE 4

Spin–Lattice Relaxation Times (in seconds) of Proton Sites of Methyl Salicylate in Deuterochloroform for Sample H Which was Pressurized with Oxygen at 3 atm and Examined at a Probe Temperature of +5°C

CH (f)	0.37 ± 0.05
CH (e)	0.37 ± 0.05
CH (d)	0.36 ± 0.05
CH (c)	0.39 ± 0.05

Note. The protons were assigned by a heteronuclear correlation experiment.

there are significant signal losses during the relatively long preparation period $(2J_{CH})^{-1}$.

The methods described above for shortening the carbon-13 relaxation times have an even more powerful influence on proton relaxation (Table 4), permitting the INEPT sequence (*18*) to be repeated at a fast cadence. This provides much more than the nominal enhancement of a factor 4 based on the ratio of magnetogyric ratios. The INEPT sequence is simply tacked onto the front of the INADEQUATE sequence and then two 180° pulses are combined (*6*). Note that the nuclear Overhauser enhancement is no longer involved, so partial quenching by addition of oxygen is no longer an issue in polarization transfer experiments.

If both coupled carbon-13 sites are quaternary, there is no significant advantage to be gained by polarization transfer from protons. When both coupled sites are protonated, polarization transfer achieves its full effect. Of particular interest here is the intermediate case where only one of the two coupled sites is protonated while the other is a quaternary carbon; then we expect the fourfold enhancement at the protonated site to be "diluted" by a factor 2. This is to be compared with the conventional INADEQUATE experiment, where the enhancement would be the arithmetic mean of the two individual Overhauser effects, one relatively large and the other rather small. Consequently the polarization advantage in INEPT-INADEQUATE may well be roughly matched by the nuclear Overhauser effect in the simple INADEQUATE spectrum, but there should be a further advantage when a relaxation agent is used, because of the appreciably faster proton spin–lattice relaxation, permitting a far higher repetition rate.

INEPT-INADEQUATE measurements were carried out on sample H which had the reduced proton spin–lattice relaxation times (less than 400 ms) set out in Table 4. A short carbon-13 acquisition time (200 ms) was chosen so as to minimize the duty cycle of the decoupler, in order to avoid saturation of the protons. Presaturation of the natural carbon-13 signal was accomplished by a string of 10 hard 90° pulses, separated by intervals of 100 ms, with a 90° phase shift after the first 6 pulses. This suppresses the natural carbon-13 signal, thus improving the rejection of undesirable signals from isolated carbon-13 sites. The recycle time was 1.2 s, roughly three times the proton spin–lattice relaxation times.

The correlations of interest here are the (b–e) and (c–g) connectivities, which both involve a quaternary site coupled to a protonated site. The results from a 1-h INEPT-INADEQUATE experiment are set out in Fig. 4, showing signal-to-

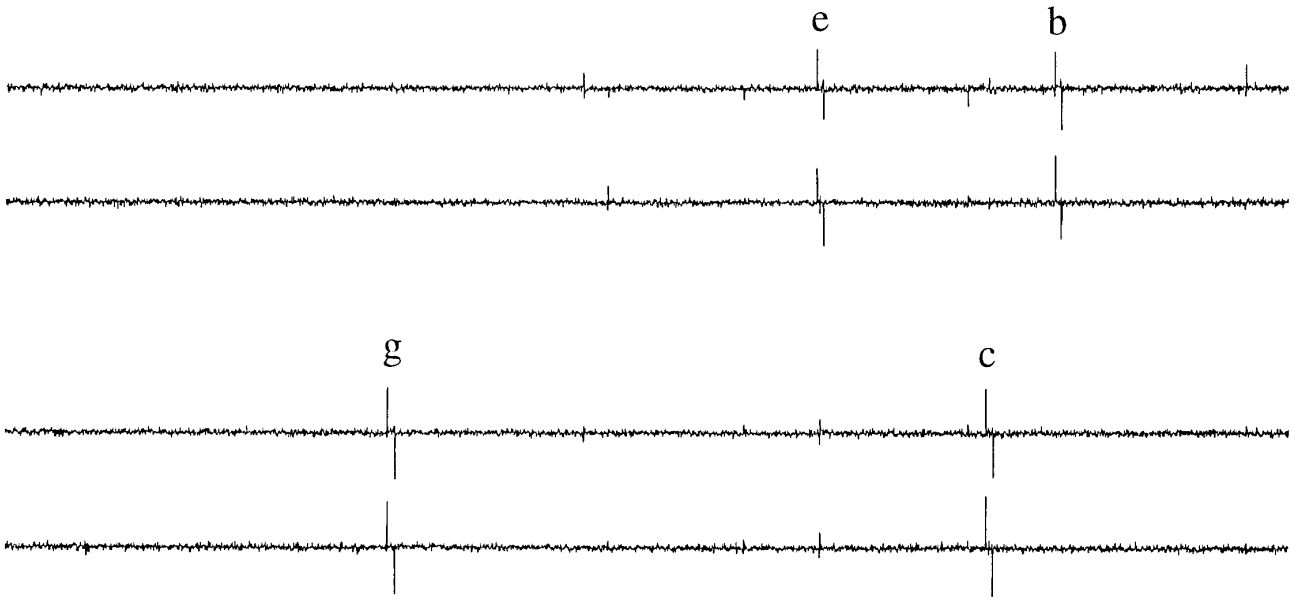

FIG. 4. Traces showing (b)–(e) (top) and (c)–(g) correlation traces (bottom) for methyl salicylate. Each correlation involves one protonated site and one quaternary site. The upper trace of each pair represents a 1-h INEPT-INADEQUATE experiment. The lower trace of each pair corresponds to a 5.8-h INADEQUATE experiment. The signal-to-noise ratios are the same, indicating an almost sixfold time saving with the polarization transfer scheme, which had a high repetition rate determined by the short proton spin–lattice relaxation times.

noise ratios of 11.4 for (b–e) and 13.4 for (c–g). Virtually identical signal-to-noise ratios were recorded for these two traces from a simple INADEQUATE experiment with a recycle time of 6.4 s, equal to 1.3 times the longest carbon-13 spin–lattice relaxation time, requiring a total accumulation time of 5.8 h. In this example of coupling between a protonated and a quaternary site, the theoretical INEPT population advantage is only 2.0, whereas the mean nuclear Overhauser effect in the simple INADEQUATE experiment is 1.5 for (b–e) and 1.8 for (c–g) (Table 2). INEPT-INADEQUATE has thus shortened the experimental time almost sixfold, mainly though the increase in repetition rate. Note however that only the simple INADEQUATE experiment detects correlations like (b–g) and (b–h) where both sites are quaternaries.

DISCUSSION

For samples with relatively long spin–lattice relaxation times, the INADEQUATE experiment can be accelerated by dissolving oxygen in the solution, preferably at low temperatures. If necessary the sample tube can be pressurized with a few atmospheres of oxygen. Addition of a soluble perfluorinated organic molecule such as perfluorotertiarybutanol appreciably enhances the solubility of oxygen, and further increases the relaxation rates. A fivefold reduction of the duration of the experiment has been demonstrated for a sample of methyl salicylate. Polarization transfer from protons to carbon can be advantageous when at least one of the coupled sites is protonated, and this technique also benefits from the presence of dissolved oxygen through a reduction in the proton spin–lattice relaxation times. In all these schemes, careful attention to the choice of the appropriate experimental parameters is essential (the sweat and tears aspect of the experiment). Acceleration of spin–lattice relaxation is complementary to most other methods for enhancing the sensitivity of the INADEQUATE technique.

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REFERENCES

1. A. Bax, R. Freeman, and T. A. Frenkiel, *J. Am. Chem. Soc.* **103,** 2102 (1981).
2. A. Bax, R. Freeman, T. A. Frenkiel, and M. H. Levitt, *J. Magn. Reson.* **43,** 478 (1981).
3. R. Freeman, T. Frenkiel, and M. B. Rubin, *J. Am. Chem. Soc.* **104,** 5545 (1982).
4. T. H. Mareci and R. Freeman, *J. Magn. Reson.* **48,** 158 (1982).
5. A. J. Shaka and R. Freeman, *J. Magn. Reson.* **50,** 502 (1982).
6. O. W. Sørensen, R. Freeman, T. A. Frenkiel, T. H. Mareci, and R. Schuck, *J. Magn. Reson.* **46,** 180 (1982).
7. P. Keller and K. Vogele, *J. Magn. Reson.* **68,** 389 (1986).
8. Y. Q. Gosser, K. P. Howard, and J. H. Prestegard, *J. Magn. Reson. B* **101,** 126 (1993).
9. J. Chung, J. R. Tolman, K. P. Howard, and J. H. Prestegard, *J. Magn. Reson. B* **102,** 137 (1993).
10. J. Weigelt and G. Otting, *J. Magn. Reson. A* **113,** 128 (1995).
11. S. W. Sparks and P. D. Ellis, *J. Magn. Reson.* **62,** 1 (1985).
12. B. Reif, M. Kock, R. Kerssebaum, H. Kang, W. Fenical, and C. Griesinger, *J. Magn. Reson. A* **118,** 282 (1996).
13. G. N. LaMar, *J. Am. Chem. Soc.* **93,** 1040 (1971).
14. D. F. S. Natusch, *J. Am. Chem. Soc.* **93,** 2566 (1971).
15. R. Freeman, K. G. R. Pachler, and G. N. LaMar, *J. Chem. Phys.* **55,** 4586 (1971).
16. A. J. Shaka, J. Keeler, and R. Freeman, *J. Magn. Reson.* **53,** 313 (1983).
17. R. L. Vold, J. S. Waugh, M. P. Klein, and D. E. Phelps, *J. Chem. Phys.* **48,** 3831 (1968).
18. G. A. Morris and R. Freeman, *J. Am. Chem. Soc.* **101,** 760 (1979).
19. P. H. Bolton, *J. Magn. Reson.* **68,** 180 (1986).
20. R. Dunkel, C. L. Mayne, R. J. Pugmire, and D. M. Grant, *Anal. Chem.* **64,** 3133 (1992).
21. R. Dunkel, C. L. Mayne, M. P. Foster, C. M. Ireland, D. Li, N. L. Owen, R. J. Pugmire, and D. M. Grant, *Anal. Chem.* **64,** 3150 (1992).
22. T. Nakazawa, H. Sengstschmid, and R. Freeman, *J. Magn. Reson. A* **120,** 269 (1996).
23. J. Buddrus and H. Bauer, *Angew. Chem. Int. Ed. Engl.* **26,** 625 (1987).
24. K. V. Schenker and W. von Philipsborn, *J. Magn. Reson.* **66,** 219 (1986).
25. A. M. Torres, T. T. Nakashima, R. E. D. McClung, and D. R. Muhandiram, *J. Magn. Reson.* **99,** 99 (1992).
26. J. Lambert and J. Buddrus, *J. Magn. Reson. A* **101,** 307 (1993).
27. N. C. Nielsen, H. Thøgersen, and O. W. Sørensen, *J. Am. Chem. Soc.* **117,** 11,365 (1995).
28. E. Brunner, M. Haake, A. Pines, J. A. Reimer, and R. Seydoux, *Chem. Phys. Lett.* **290,** 112 (1998).
29. T. M. Barbara, *J. Magn. Reson. A* **109,** 265 (1994).
30. J. K. Harper, R. Dunkel, S. G. Wood, N. L. Owen, D. Li, R. G. Cates, and D. M. Grant, *J. Chem. Soc. Perkin Trans. 2,* **1,** 91 (1996).
31. D. L. Olson, T. L. Peck, A. G. Webb, R. L. Magin, and J. V. Sweedler, *Science* **270,** 1967 (1995).
32. P. Styles, N. F. Soffe, C. A. Scott, D. A. Cragg, F. Row, D. J. White, and P. C. White, *J. Magn. Reson.* **60,** 397 (1984).
33. W. A. Anderson, W. W. Brey, A. L. Brooke, B. Cole, K. A. Delin, J. F. Fuks, H. D. W. Hill, M. E. Johanson, V. Y. Kutsubo, R. Nast, R. S. Withers, and W. H. Wong, *Bull. Magn. Reson.* **17,** 98 (1995).
34. J. S. Waugh, *J. Molec. Spectrosc.* **35,** 298 (1970).
35. O. W. Sørensen, M. H. Levitt, and R. R. Ernst, *J. Magn. Reson.* **55,** 104 (1983).