Long-Lived States of Magnetically Equivalent Spins Populated by Dissolution-DNP and Revealed by Enzymatic Reactions**

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Abstract: Hyperpolarization by dissolution dynamic nuclear polarization (D-DNP) offers a way of enhancing NMR signals by up to five orders of magnitude in metabolites and other small molecules. Nevertheless, the lifetime of hyperpolarization is inexorably limited, as it decays toward thermal equilibrium with the nuclear spin-lattice relaxation time. This lifetime can be extended by storing the hyperpolarization in the form of long-lived states (LLS) that are immune to most dominant relaxation mechanisms. Levitt and co-workers have shown how LLS can be prepared for a pair of inequivalent spins by D-DNP. Here, we demonstrate that this approach can also be applied to magnetically equivalent pairs of spins such as the two protons of fumarate, which can have very long LLS lifetimes. As in the case of para-hydrogen, these hyperpolarized equivalent LLS (HELLS) are not magnetically active. However, a chemical reaction such as the enzymatic conversion of fumarate into malate can break the magnetic equivalence and reveal intense NMR signals.

Introduction

Spin hyperpolarization by dissolution dynamic nuclear polarization (D-DNP) has become a major area of research in NMR. This emerging method provides a way of boosting the sensitivity of NMR experiments by enhancing the intrinsically low nuclear spin polarization dictated by Boltzmann’s law. Thus, 13C NMR signals of small molecules have been enhanced by up to four orders of magnitude.[1] In a typical D-DNP experiment, the frozen sample is initially polarized at low temperatures and moderate fields, and the signals are subsequently measured in solution in a separate detection apparatus operating at room temperature. This implies that the polarized frozen sample needs to be dissolved and transferred rapidly. This transfer, sometimes poetically called voyage, can be performed either manually or by means of a pneumatic system. During the voyage, the hyperpolarized molecules experience low magnetic fields (sometimes as low as the earth’s field, or even lower), which have detrimental effects on the enhanced polarization. This is one of the reasons why D-DNP has been most useful for nuclear spins with long T1, such as the isolated low-gamma quaternary 13C spin in 1-13C pyruvic acid.[2] On the other hand, apart from some exotic experiments,[3] 1H spins have hardly been exploited by D-DNP, as their short T1 relaxation times mean that the hyperpolarization is driven back rapidly toward Boltzmann equilibrium. However, we have shown recently that 1H can be polarized very efficiently and rapidly up to P(1H) = 91% with a buildup time constant as short as T1DNP(1H) = 150 s at B0 = 6.7 T and T = 1.2 K.[4] One possible strategy for taking advantage of this large 1H hyperpolarization consists in storing the magnetization in the form of long-lived states (LLS)[5] with extended lifetimes.

In recent years, several successful studies combining D-DNP with LLS have shown that hyperpolarized magnetization can be converted into LLS with extended lifetimes T1LLS > T1. In a pair of equivalent spins 1/2, the singlet state S0 = (|αβ⟩ + |βα⟩)/√2 is largely disconnected from the triplet states T1 = |αα⟩, T2 = (|αβ⟩ + |βα⟩)/√2 and T3 = |ββ⟩ because relaxation mechanisms that are symmetric with respect to spin exchange (such as the dipole–dipole interaction between the two spins) cannot induce singlet–triplet transitions.[7] Therefore, if a triplet–singlet population imbalance (TSI) is prepared by any means, it is likely to be long-lived. We use the expression TSI in analogy to the A/E imbalance (AEI) recently described for
methyl groups by Benno Meier et al.[8] Both TSI and AEI refer to a difference between the average populations of spin states belonging to different irreducible representations of the spin permutation group, that is, \( \Gamma_a \) and \( \Gamma_e \) in methyl groups and \( \Gamma_g \) and \( \Gamma_u \) (or triplet and singlet states) in pairs of equivalent spins. The excitation and detection of an LLS involving a pair of equivalent spins is challenging because the magnetic equivalence needs to be lifted during both excitation and detection but preserved during storage. In this context, two possible scenarios are: 1) In most experiments described so far, the symmetry is imposed on an otherwise inequivalent two-spin system during the storage period only, or 2) in this work, the symmetry of an inherently equivalent two-spin system is broken during both excitation and detection.

Para-hydrogen[9] offers the best example of nuclear singlet order in a molecule with two equivalent spins. The singlet state of \( H_2 \) can be produced at low temperatures (typically 40 K) in the presence of a paramagnetic catalyst, which allows singlet-triplet interconversion by lifting the symmetry of \( H_2 \) near the catalytic surface. The singlet spin state of \( H_2 \) has the lowest energy, primarily determined by the quantization of its rotational state, and therefore, is predominantly populated at low temperatures. This leads to the creation of a large TSI compared to \( H_2 \) in Boltzmann equilibrium at room temperature. Para-\( H_2 \) is not magnetically active, and therefore cannot be observed directly by NMR, but it can be converted into observable signals through an asymmetric hydrogenation reaction by which the two protons stemming from \( \text{dmeA}_{\text{2}} \) are averaged out, so that the spins of dimethyl acetylene dicarboxylate to produce dimethyl maleate.[10] The preserved para state can subsequently be rendered accessible to NMR by another chemical reaction that lifts the symmetry of the molecule.

If one starts with an inequivalent two-spin system, a precursor state, that is, a state that acquires a long-lived property as soon as the two spins are made equivalent during the storage interval, can be prepared by using suitable radio frequency sequences, by adiabatic transport to low fields, or by chemical reactions.[9] Alternatively, a compromise can be found by using systems containing nearly equivalent spins[10] in which the singlet and triplet states are only weakly mixed, but with an admixture that can be augmented by suitable pulse sequences to induce a singlet-to-magnetization (STM) conversion.

Most experiments in which D-DNP is combined with LLS rely on radio frequency sequences to prepare the LLS, usually after the transfer of the hyperpolarized sample to the detection magnet. As a result, extensive relaxation occurs during the transfer. However, Tayler and co-workers[10] have shown that LLS order can be populated directly before the transfer by D-DNP for the two inequivalent \(^{13}\)C spins in 1,2-\(^{13}\)C\(_2\)-pyruvic acid. They pointed out that the polarization of the singlet state \( P_1 \) (= \( P_{20} \), vide supra) is proportional to the square of the spin Zeeman polarization \( P_z \) (i.e., \( P_{20} = P_z = -\frac{1}{2} P_z^2 \)). Therefore, provided a high spin polarization can be reached by D-DNP, say \( P_z = 50\% \), a significant amount of singlet order, in this example \( P_{20} = 8.33\% \), can be created directly without any radio frequency pulses. In the case where \( P_z = 91\% \) can be attained, one obtains \( P_{20} = 28\% \). Such high levels of polarization can indeed be prepared directly by DNP for \( ^1H \) at \( B_0 = 6.7 \) T and \( T = 1.2 \) K and indirectly for \(^{13}\)C or other nuclei through cross polarization from protons.[4]

In this work, we demonstrate that a TSI can be efficiently populated by D-DNP for the pair of magnetically equivalent \(^1H \) spins in fumarate, and that this is preserved in the liquid state after dissolution for a long time \( T_{\text{relax}} \) which was estimated to be of the order of 50 s. We refer to this type of LLS as hyperpolarized equivalent long-lived states (HELLS). We show how HELLs can be readily "revealed" by allowing fumarate to undergo a biologically relevant enzymatic conversion into malate.

Experiments

For efficient D-DNP, the samples usually consist of frozen glassy solids containing typically 10–50 mM polarizing agents such as TEMPO in addition to the molecules of interest. In our experiment, the molecule of interest shall possess two spins \( I \) and \( S \) that are magnetically equivalent in the liquid phase, but inequivalent in the frozen state and in moderate magnetic fields because they are exposed to slightly different environments and therefore experience different chemical shifts because of chemical shift anisotropies (CSAs) and different inter- and electron–nuclear dipolar couplings. Given that freezing to low temperatures lifts the equivalence, the energy levels are better expressed in the product basis (PB). At \( T = 1.2 \) K and \( B_0 = 6.7 \) T, the proton Boltzmann polarization without DNP is \( P_z = 0.57\% \). Therefore, the deviations of diagonal elements from the demagnetized state \( \Delta \sigma = \sigma - \bar{E} \) will be \( \Delta \sigma_{\text{origin}, \text{iso}} = \Delta \sigma_{\text{iso}, \text{iso}} \), \( \Delta \sigma_{\text{iso}, \text{iso}} = \frac{1}{4}(2P_z^2 - P_z^2 - P_z^2 - 2P_z^2 + P_z^2) \). Assuming, for simplicity, that DNP could confer a Zeeman polarization \( P_z = 100\% \), only the lowest energy level \( |\text{iso}|-DNP \rangle \) would be populated by hyperpolarization, so that \( \Delta \sigma_{\text{iso}, \text{iso}} = \Delta \sigma_{\text{iso}, \text{iso}} = 0.75 \), \(-0.25 \), \(-0.25 \), \(-0.25 \). (See Figure 1a: TSI Preparation). As soon as the polarized sample is heated and dissolved to the liquid state, CSAs and dipolar couplings are averaged out, so that the spins \( I \) and \( S \) become magnetically equivalent. The density operator can therefore better be expressed in the singlet–triplet basis (STB). In our case, as \( n_{\text{iso}} = n_{\text{iso}} \) (and hence \( \Delta \sigma_{\text{iso}, \text{iso}} = \Delta \sigma_{\text{iso}, \text{iso}} \)), it is easy to see that \( \sigma_{\text{STB}}(PB) \) [and hence \( \Delta \sigma_{\text{STB}}(PB) \)] with the following diagonal elements: \( \Delta \sigma_{\text{origin}, \text{iso}, \text{iso}} = \Delta \sigma_{\text{iso}, \text{iso}} = \Delta \sigma_{\text{iso}, \text{iso}} = \Delta \sigma_{\text{iso}, \text{iso}} \). Hence, \( \Delta \sigma_{\text{iso}, \text{iso}} |_{\text{PB}} = \Delta \sigma_{\text{iso}, \text{iso}} |_{\text{PB}} - \Delta \sigma_{\text{iso}, \text{iso}} |_{\text{PB}} = -P_z^2/3 \). The TSI will thus result from the depletion of \( n_{\text{iso}} \) by hyperpolarization (Figure 1b). The spins are equivalent, so the TSI can be stored differently in a low or high magnetic field (in a magnetic path for example). During the storage period, the populations of the three triplet states will equilibrate, that is, the deviations of the population of the three triplet levels will average out to give \( \Delta \sigma_{\text{iso}, \text{iso}} = (\Delta \sigma_{\text{iso}, \text{iso}}) = \frac{1}{4}(\Delta \sigma_{\text{iso}, \text{iso}} + \Delta \sigma_{\text{iso}, \text{iso}} + \Delta \sigma_{\text{iso}, \text{iso}}) = -P_z^2/3 \). The singlet should not be affected by dipole–dipole relaxation, so the TSI in principle remains equal to \( P_{20} = -P_z^2/3 \).
Enzymatic reactions are not instantaneous, and do not necessarily lead to complete conversion into the product. Figure 2 shows an example of the conversion of fumarate into malate by fumarase under conditions that can be combined with D-NPR. The steady-state concentrations are only reached after 25 min. This has important implications for our experiment. In (c), the full density matrix is given to show the off-diagonal elements.

(See Figure 1 b: TSI storage). The sample is then transferred to the NMR or MRI magnet for detection. The system of two equivalent spins can then be transformed (chemically or enzymatically) into a system of two inequivalent spins, so that the "sealed" hyperpolarization can be "revealed" by conversion into observable magnetization. If the reaction is fast and goes to completion, one can convert \( \Delta \sigma \) from the STB back to the PB by using a suitable base transformation (see Ref. [15]). \( \Delta \sigma (PB) \) resulting from this transformation can be expressed as a superposition of longitudinal two-spin order and zero-quantum coherence since \( \Delta \sigma (PB) = P_2^Z/\delta (2, S_z + Z Q_z) \). (See Figure 1 c: TSI revelation).

Enzymatic reactions are not instantaneous, and do not necessarily lead to complete conversion into the product. Figure 2 shows an example of the conversion of fumarate into malate by fumarase under conditions that can be combined with D-NPR. The steady-state concentrations are only reached after 25 min. This has important implications for our experiment. In fact, a highly polarized state \( \Delta \sigma = 2I_S + 2ZQ \), is indeed produced instantaneously in malate whenever fumarate molecules carrying a TSI undergo an enzymatic conversion, but the ZQ_z term immediately starts evolving under the difference of chemical shifts, and therefore rapidly dephases and averages to zero as the reaction proceeds. Furthermore, the hyperpolarized TSI of fumarate, once it is transferred to malate, will tend to relax to thermal Boltzmann equilibrium.

It is, however, possible to "sustain" the LLS of malate by so-called "high-field" methods, [7c, 12, 15b] for example, by applying an rf irradiation halfway between the two chemical shifts (either continuous-wave (CW), or, if desired, by applying a WALTZ-16 pulse train), [16] thus preserving the full \( \Delta \sigma = 2I_S + 2ZQ \) state. This strategy allows one to slow down relaxation of \( 2I_S \) and prevent dephasing of ZQ_z. For the two inequivalent protons in malate, we thus determined \( T_{\text{tsi}} = 6 \text{ s} \) at \( B_0 = 7 \text{ T} \) and \( T = 298 \text{ K} \). Moreover, the use of WALTZ-16 pulse trains has the advantage of wiping out any single-quantum magnetization that would not arise from HELLS. A conventional LLS detection sequence, for example, the second half of the "Sarkar sequence" [15b] (Fig...
ure 3), can then be used to transform $\Delta \sigma = 2I_{S_2} + 2ZQ_{e}$ into observable magnetization.

The lifetime of the LLS of malate ($T_{LLS}^{M} = 6$ s at 300 MHz if the rf amplitude of the CW field is $\nu_r = 3$ kHz) is short compared to the enzymatic transformation, so the time $T_{LLS}$ (see Figure 3) allocated for the LLS to accumulate in malate before it is converted into observable signals needs to be optimized carefully. The concentrations $[F]$ and $[M]$ of fumarate and malate can be described by pseudo first-order kinetics as shown in Equation (1), in which $[F](t)$ and $[M](t)$ are the concentrations of fumarate and malate, $k_{FM}$ and $k_{MF}$ are the apparent kinetic constants of the overall enzymatic conversion of fumarate into malate and vice versa, without considering the details of the Michaelis–Menten mechanism.

\[
\frac{d[F](t)}{dt} = -k_{FM}[F](t) + k_{MF}[M](t)
\]
\[
\frac{d[M](t)}{dt} = -k_{MF}[M](t) + k_{FM}[F](t)
\]

The temporal evolution of the expectation value $P_{LLS}^{M}$ in malate arising from the conversion of fumarate can be obtained by solving numerically the rate equations [Eq. (2)], in which $P_{TSI}^{F}$ and $P_{LLS}^{M}$ are the expectation values of the TSI in fumarate and of the LLS in malate, and $R_{TSI}^{F}$ and $R_{LLS}^{M}$ are their relaxation rates.

\[
\frac{d[P_{TSI}^{F}](T)}{dt} = -(k_{FM} + R_{TSI}^{F})P_{TSI}^{F}(T) + k_{MF}P_{LLS}^{M}(T)
\]
\[
\frac{d[P_{LLS}^{M}](T)}{dt} = -(k_{MF} + R_{LLS}^{F})P_{LLS}^{M}(T) + k_{FM}P_{TSI}^{F}(T)
\]

The “apparent” rate constants $k_{FM}$ and $k_{MF}$ can be obtained by fitting the signal amplitudes in Figure 2 to the rate equations in Equation (1). One can then calculate the temporal evolution of $P_{TSI}^{F}$ in fumarate in the presence of ten units of enzyme, as well as $P_{LLS}^{M}$ of malate obtained by the conversion of the TSI of fumarate into an LLS of malate that relaxes with $T_{LLS}^{M}$ (Figure 4). These curves were obtained by assuming that $T_{TSI}^{F} = 60$ s for fumarate (on the basis of preliminary observations as discussed below), and using the experimentally determined time constant $T_{TSI}^{F} = 6$ s for malate. According to Figure 4, the optimal delay to maximize the conversion of the TSI of fumarate into the LLS of malate is 10 s. Thus, one should wait $T_{TSI}^{F} = 10$ s while sustaining the LLS by a suitable rf field before attempting to convert the LLS of malate into observable magnetization. The alternation of rf irradiation and signal observation can be repeated $n$ times. During each interval $T_{LLS}$, the LLS on malate will be replenished by the enzymatic conversion of the slowly relaxing TSI of fumarate. The decay of the magnetically silent TSI of fumarate will be reflected indirectly in the decay of the malate signal as $n$ increases. Moreover, it can be seen in Figure 4b that only around 1% of the HELLS of fumarate is transferred to malate during each loop $n = 1, 2, …, N$.

**Results**

A sample comprising ten frozen pellets of 10 μL each of 0.5 mM fumarate with 50 mM TEMPOL was hyperpolarized by microwave irradiation at $B_0 = 6.7$ T and $T = 1.2$ K for about 20 min. The sample was then dissolved, together with ten frozen pellets of 10 μL each of 3 mM sodium ascorbate in D,O, with 5 mL D,O at 400 K and 1.0 MPa, and transferred in 4.5 s to a holding chamber just above the magnetic center of a 7 T NMR (300 MHz) spectrometer, where the static field is $B_{ho} > 6.5$ T. After a preinjection delay $1 < T_{PI} < 60$ s, which allows one to assess the lifetime $T_{TSI}$ of the TSI $P_{TSI}$ of hyperpolarized fumarate in the holding chamber, the solution was injected into a 5 mm NMR tube containing fumarase to start the conversion of fumarate into malate, and to transfer concomitantly the TSI of fumarate into an LLS on malate. The latter was sustained by a WALTZ-16 pulse train with an rf amplitude $\nu_r = 3$ kHz. The sequence of Figure 3 was then used to convert the LLS of malate into observable magnetization.

Figure 5d shows four spectra of malate acquired at 7 s intervals ($N = 4$ loops, each comprising a sustaining interval $T_{LLS} = 6$ s and an acquisition time of 1 s) after the injection of hyperpolarized fumarate into the NMR tube containing fumarase. In this case, the preinjection delay $T_{PI} = 1$ s during which the fumarate was kept in the holding chamber was negligible compared with $T_{TSI}$. The enzymatic conversion is relatively slow, so the signals in Figure 5d arise from the conversion of a small fraction of fumarate into malate ($\approx 1\%$ every 7 s, according to
A time constant $T_{\text{TSI}}$ reflects 1) the decay of the inaccessible TSI of fumarate with chemical properties that can be addressed by HELLS. As fumarate plays a crucial role in the Krebs cycle, it may be of interest for lifelong research since we observed that the remaining signal of malate after the glass-forming mixture D$_2$O:[D$_6$]ethanol (60:40 v/v) were added drop by drop to avoid precipitation. The solution was then sonicated for 10 min. Ten frozen pellets of 10 m each containing 3 m ascrobate (Sigma–Aldrich) in D$_2$O to scavenge the radicals after dissolution. [17] We have shown that a pure TSI can be created readily by D-DNP in a system that contains two magnetically equivalent spins in solution. Once dissolved, this imbalance displays a lifetime $T_{\text{TSI}}$ that is much longer than the longitudinal relaxation time $T_1$. We believe this to be the first proof of principle of the creation of hyperpolarized long-lived states for equivalent spins (HELLS) by D-DNP. Such a long-lived spin order can be used readily to monitor a slow enzymatic process of biochemical relevance, but may find applications in other areas of magnetic resonance such as imaging (MRI), for which hyperpolarization by D-DNP has become a technique of choice to enable metabolical imaging, and in which short lifetimes of hyperpolarized molecules are usually a major limitation. The HELLS methodology will be applied to more challenging molecules containing magnetically equivalent pairs of spins, such as CH$_3$RR', CH$_2$Cl$_2$, and possibly H$_2$O. We are currently investigating molecules with interesting lifetimes and interesting chemical or biochemical properties that can be addressed by HELLS. As fumarate plays a crucial role in the Krebs cycle, it may be of interest for in vivo studies as it has been demonstrated to be a probe for cellular necrosis.[18]

Figure 5. a) Conventional NMR spectrum excited by a 90° pulse 25 min after injection into a solution containing fumarase, when the enzymatic reaction has reached a steady state and the hyperpolarization (both $P_{\text{TSI}}$ in fumarate and $P_{\text{LLS}}$ in malate) has decayed to thermal equilibrium. Note the signals of fumarate, malate, ethanol, and buffer. The HDO peak was attenuated by pre-saturation with a selective pulse with an rf amplitude of 75 Hz and a duration of 5 s. b) Spectrum of malate (without significant stopover in the holding chamber since $\tau_{\text{TSI}}=1\,\text{s} \ll T_{\text{TSI}}$) recorded with the sequence of Figure 3, shortly after injection ($n=1$) into a solution containing fumarase in the 7 T NMR system. c) Spectrum of malate recorded after keeping the hyperpolarized fumarate for $\tau_{\text{TSI}}=50\,\text{s}$ in the holding chamber at $B_0 > 6.5\,\text{T}$ prior to injection into the fumarase solution. d) The first four spectra of malate acquired with $n=1, 2, 3,$ and 4 at intervals of 7 s using the sequence in Figure 3 ($\tau_{\text{TSI}} = 1\, \text{s}$, acquisition time 1 s) showing that $P_{\text{LLS}}$ is replenished through the enzymatic reaction.

Conclusion

We have shown that a pure TSI can be created readily by D-DNP in a system that contains two magnetically equivalent spins in solution. Once dissolved, this imbalance displays a lifetime $T_{\text{TSI}}$ that is much longer than the longitudinal relaxation time $T_1$. We believe this to be the first proof of principle of the creation of hyperpolarized long-lived states for equivalent spins (HELLS) by D-DNP. Such a long-lived spin order can be used readily to monitor a slow enzymatic process of biochemical relevance, but may find applications in other areas of magnetic resonance such as imaging (MRI), for which hyperpolarization by D-DNP has become a technique of choice to enable metabolical imaging, and in which short lifetimes of hyperpolarized molecules are usually a major limitation. The HELLS methodology will be applied to more challenging molecules containing magnetically equivalent pairs of spins, such as CH$_3$RR', CH$_2$Cl$_2$, and possibly H$_2$O. We are currently investigating molecules with interesting lifetimes and interesting chemical or biochemical properties that can be addressed by HELLS. As fumarate plays a crucial role in the Krebs cycle, it may be of interest for in vivo studies as it has been demonstrated to be a probe for cellular necrosis.[18]

Experimental Section

DNP samples

Solutions of 0.5 m dibasic sodium fumarate (Sigma–Aldrich) in the glass-forming mixture D$_2$O:[D$_6$]ethanol (60:40 v/v) were doped with 50 mM TEMPOL (Sigma–Aldrich). Ethanol was added drop by drop to avoid precipitation. The solution was then sonicated for 10 min. Ten frozen pellets of 10 mL each of this mixture were inserted in the polarizer, along with ten frozen pellets of 10 mL each containing 3 m ascrobate (Sigma–Aldrich) in D$_2$O to scavenge the radicals after dissolution.[17]
DNP polarization and dissolution

DNP was performed at 1.2 K and 6.7 T in a home-built polarizer by applying frequency-modulated microwave irradiation\[^{[10]}\] at \( f_{\text{MW}} = 188.3 \text{ GHz} \) and \( P_{\text{MW}} = 100 \text{ mW} \), with a modulation frequency of 10 kHz and modulation amplitude of 50 MHz. The polarized pellets were dissolved in 0.7 s with 5 mL D2O, preheated to \( T = 400 \text{ K} \) at \( P = 1.0 \text{ MPa} \), and transferred in 4.5 s to a 7 T (300 MHz) magnet by pushing with helium gas at 0.6 MPa through a PTFE tube (1.5 mm inner diameter) running through a magnetic tunnel (3 m length).

Enzymatic detection

The hyperpolarized solution was kept at \( B_0 = 6.5 \text{ T} \) in a holding chamber just above the NMR sample tube for a variable delay \( \tau_{\text{TSL}} \) to monitor the relaxation of the TSL of furamate. The sample was then injected in 2 s into an NMR tube containing D2O (200 \text{ mL}) for field-frequency locking, NaCl (200 \text{ mm}) and TRIS buffer (25 \text{ mm}), and furamate (5 \text{ ml}, 5.8 mg/mL, 12.5 units) from porcine heart (Sigma–Aldrich). Finally, the LLS detection sequence described in Figure 3 was applied with \( n \) sustaining delays of \( \tau_{\text{LLS}} = 6 \text{ s} \) each with WALTZ-16 irradiation.

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