Application of Incoherent Inelastic Neutron Scattering in Pharmaceutical Analysis: Relaxation Dynamics in Phenacetin

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ABSTRACT: This study centers on the use of inelastic neutron scattering as an alternative tool for physical characterization of solid pharmaceutical drugs. On the basis of such approach, relaxation processes in the pharmaceutical compound phenacetin (p-ethoxyacetanilide, $C_{10}H_{13}NO_2$) were evidenced on heating between 2 and 300 K. By evaluating the mean-square displacement obtained from the elastic fixed window approach, using the neutron backscattering technique, a crossover of the molecular fluctuations between harmonic and nonharmonic dynamical regimes around 75 K was observed. From the temperature dependence of the quasi-elastic line-width, summed over the total Q range explored by the time-of-flight technique, it was possible to attribute the onset of this anharmonicity to methyl group rotations. Finally, using density functional theory-based methods, we were able to calculate the lattice vibrations in the harmonic approximation. The overall spectral profile of the calculated partial contributions to the generalized density of states compares satisfactorily to the experimental spectra in the region of the lattice modes where the intermolecular interactions are expected to play an important role. This study contributes to understanding the relationships between intermolecular hydrogen bonds, intramolecular dynamics, and conformational flexibility in pharmaceuticals on a molecular level, which can help in evaluating phase stability with respect to temperature variations on processing or on storage, and is related to control of polymorphism and pseudopolymorphism.

KEYWORDS: methyl dynamics, activation energy, density functional theory-based methods, neutron scattering
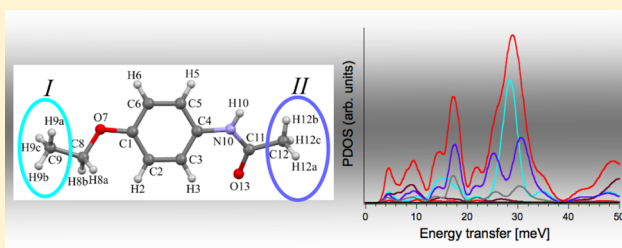
INTRODUCTION

Phenacetin (p-ethoxyacetanilide, $C_{10}H_{13}NO_2$) and paracetamol (N-acetyl-p-aminophenol, $C_8H_9NO_2$) are both derivatives of acetanilide (N-phenylacetamide, C_8H_9NO). In 1886, A. Cahn and P. Hepp introduced acetanilide into medical practice[1] under the name of antifebrin, which presented both analgesic and antipyretic activities. However, acetanilide's unacceptable toxic effects promptly stimulated the search for safer aniline derivatives. A number of compounds were tested; phenacetin and paracetamol, which were introduced into therapy in 1887, being the most satisfactory ones.[2] Paracetamol was quickly abandoned in favor of phenacetin that became one of the most prominent pain-relieving compounds, often in the form of APC (aspirin−phenacetin−caffeine). However, in 1983, phenacetin was removed from the market,[3] after long-term studies suggesting that it is carcinogenic when ingested over long time periods. Paracetamol was then rediscovered.[4] Up to date, paracetamol is the only survivor of the so-called aniline derivatives or aniline analgesics. In contrast to phenacetin, it shows polymorphic behavior, the known forms differing in stability, compressibility, and solubility.[5] This example is just one among many encountered in pharmaceutical science and biopharmaceutics, where too often the characterization of the therapeutic agents that are administrated as solid dosage forms are mainly based on chemical purity only, while little attention is given to the physical properties of the solids.[6,7] At the same time, a minor change in the molecular structure can cause pronounced effect both on toxicity and on the ability to give polymorphs differing in properties.[8−10] The interaction substrate−receptors, which are important for medicinal effects,[11] the drug metabolism in a cell, which is important for toxicity, and the phase stability of a solid form can be related, among other factors, to the dynamics of molecular fragments and the properties of hydrogen bonds in solution or in the solid state.[12−14]

Biochemical and medicinal aspects of amides have been the subject of constant research.[15−17] Vibrational spectral inves-

tigations as well as quantum chemistry calculations of secondary amide derivatives have been used to understand the force field of proteins and polyamino acids, where compounds such as acetamide,[18,19] formamide,[20] thioamide,[21] and their derivatives served as simple models for investigating the nature of secondary amide functions. Also along these lines, in order to understand the dynamics of individual molecular fragments and to model the dynamics of biopolymers, the compounds of the acetanilide family, Figure 1, have attracted
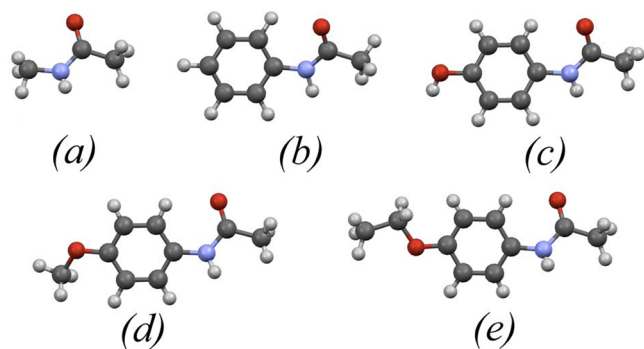
Figure 1. Molecular structures of *N*-methylacetamide (a), acetanilide (b), paracetamol (c), methacetin (d), and phenacetine (e). The color codes for the atoms are as follows. C-atoms are represented in gray, N-atoms in blue, O-atoms in red, and H-atoms in white.

enormous attention. Particular focus has been given to the comparison between the dynamics of methyl groups belonging to the acetamido groups in acetanilide and paracetamol[5,22,23] and those in *N*-methylacetamide.[24−26]

Interestingly, the crystalline molecular packing architectures for acetanilide[27,28] and phenacetin[29,30] are quite similar. As shown in Figure 2, both molecules are connected by two N−H···O hydrogen bonds, with D···A distance of ∼2.9 Å, forming infinite chains, where, within one chain, the molecules are connected head to tail. The layers are stacked with weak interactions, while the infinite chains are also connected to each other by weak interactions (there are no H-bonds!) forming undulating and flat layers in acetanilide and phenacetin, respectively. However, while crystalline acetanilide has been studied in detail by means of spectroscopy methods, such as IR[31] and Raman[32] spectroscopy, NMR,[33] and inelastic neutron scattering,[34] less attention has been paid to methacetin and phenacetin. To the best of our knowledge, only a single, combined IR−ab *initio* quantum chemical calculation of equilibrium geometry and normal vibration frequencies above 50 meV has been reported for the latter.[35] Furthermore, and of main interest, it was recently shown[23] that the dynamics of the methyl groups in the polymorphs of paracetamol can be directly related to the strength and disorder of the hydrogen bonds formed by the molecules with their immediate environment. This observation was related to the difference in relative stability of the two crystalline polymorphs, which might also influence the binding of the drug molecules to the active sites during biochemical processes. The energy required to change a drug molecule to its required active conformation might be obtained from the energy released when the drug molecule binds to its receptor or target.[36] In some cases, the rotation energy barriers are too high, making the drug molecule inactive, i.e., it will not bind. In other cases, however, the energy of binding is sufficient, and the energy barrier is overcome. These ideas are consistent with the concept that binding of a
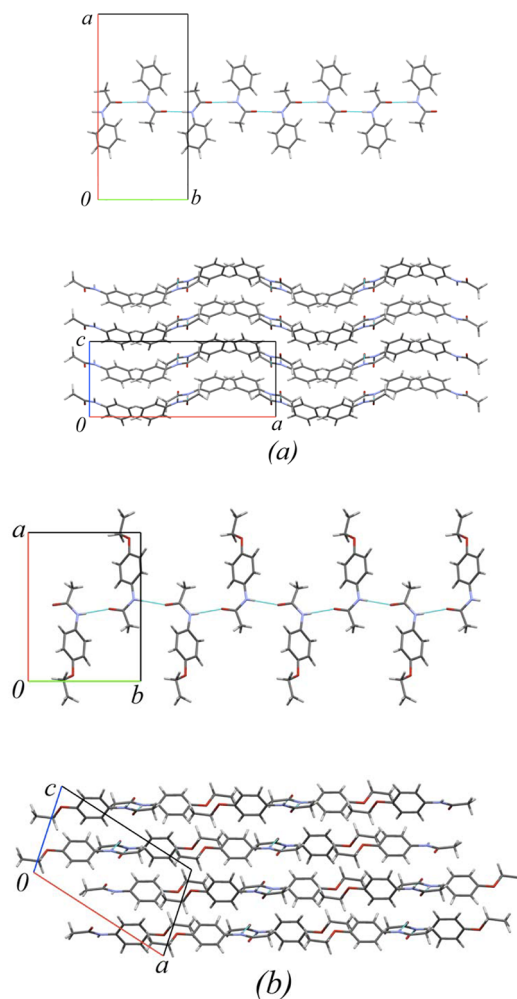

Figure 2. Fragments of crystal structures of acetanilide (a) and phenacetin (b).

molecule to its receptor is a dynamic process and that conformational change due to flexibility of both the receptor and the ligand is particularly important.[37,38] Therefore, in drug design, it is most important to realize that ligands, i.e., small molecules, such as neurotransmitters, hormones, pharmaceutical drugs, or toxins, are recognized not exclusively by their molecular structure but to a large extent by their dynamic properties. This implies that analogues, similar to the compounds described here, can exert similar interactions with the functional groups of the binding site and have similar affinities, even if their chemical structures are quite different. Moreover, most reactions of organic solids, including pharmaceutical solids, occur via bonding mechanisms common to solution chemistry: hydrogen bonding, dipole−dipole and dipole−induced dipole interaction, charge-transfer complexation, or covalent bonding.[4,6] Consequently, the understanding of the dynamics of the functional groups, herein the methyl group, in relation to hydrogen bonds formed by adjacent molecular fragments is of general interest in drug design.[39−42]

Here, we studied the vibrational spectra of crystalline phenacetin using inelastic neutron scattering (INS), in order to illustrate that using INS as a tool for characterizing the dynamics of hydrogen atoms in a solid drug has several advantages. The main focus was on the spectral region 0−50 meV, which gives information on the alkyl and peptide methyl

group rotations, methyl group I and methyl group II, respectively, as depicted in Figure 3. The reason for studying
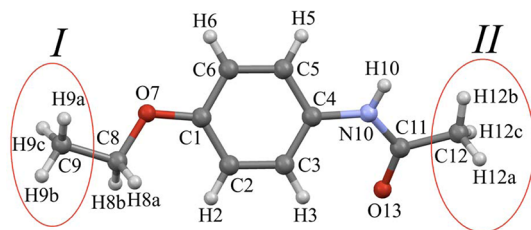
Figure 3. Molecular structure and atom numbering scheme of phenacetin, where the different methyl groups are highlighted by red ellipses.

the low energy spectrum is that these are the modes that can be activated under physiological, i.e., ambient conditions, and in the case of phenacetin, we were able to demonstrate that the main flexibility is associated with methyl group II. Other reasons to use inelastic neutron scattering for such studies are as follows. Although infrared spectroscopy is widely used for the study of pharmaceutical solids as well as to study the water hydration in such crystalline lattices, this methodology is qualitative rather than quantitative. Dielectric relaxation spectroscopy, another common technique of choice that is increasingly being recognized as a tool for materials characterization, including pharmaceutical analysis, is unable to give information on methyl group reorientation motions as they are inactive in such measurements.[43,44] Finally, solid-state NMR spectroscopy, another technique that has been used more and more for the analysis of pharmaceutical solids within the past decade and has provided unique data for a variety of pharmaceutical compounds,[45] does not always produce high-quality spectra and does not reveal the correlations in space and time that are present in the dynamic structure factor measured with neutrons.

The experimental vibrational spectrum was interpreted by means of the solid-state DFT calculations. A number of codes are available for performing density functional theory (DFT) calculations, which allow evaluating the INS spectra. Here, we have used DMol3.[46] The vibrational spectrum was calculated in the harmonic approximation using the direct method to obtain the interatomic forces.

■ EXPERIMENTAL DETAILS

Phenacetin Samples. Phenacetin (commercial powder samples, pure for analysis grade, Kursk Drug Plant, Russia) was purified additionally by recrystallization from an ethanol solution and its purity controlled by chromatography and X-ray diffraction.

Neutron Scattering Measurements. Flat aluminum sample holders were used, and a sample thickness of 0.4 mm was chosen to achieve sufficient, total scattered intensities while avoiding multiple scattering. An orientation angle of 135° with respect to the incident neutron beam direction was used for all samples, including vanadium.

Elastic incoherent neutron scattering (EINS) measurements on heating from 2 to 300 K were performed on the backscattering spectrometer IN10 at the Institute-Laue-Langevin (ILL, Grenoble, France), using incident wavelength λ = 6.27 Å and resolution ΔE of about 1 μeV (fwhm), over 24 h. IN10 allows access to motions from 100 ps to 1 ns on the Å

length-scale. Neutrons scattered incoherently by the hydrogen atoms in phenacetin dominate the total scattered intensity, thus one can assume that only the self-dynamics of these atoms are being probed.[47] The elastic intensity was analyzed to extract the mean square displacements (MSDs), which will allow for the observation of dynamical transitions in the sample, characterized by a change of the MSDs as a function of temperature due to the onset of anharmonic motions.[48] Moreover, to examine low energy modes, such as reorientational tunneling[22] and jumps,[49] quasi-elastic (QE) spectra were recorded for 6 h between 2 and 250 K.

Motions with characteristic times faster than 10 ps were probed using the time-of-flight spectrometer IN6 also at the ILL with an incident wavelength λ = 5.12 Å, resulting in an elastic resolution of 70 μeV (fwhm) at the elastic line. Raw data were corrected for empty-can subtraction and detector efficiencies as well as for self-shielding effects due to the slab geometry of the samples. After removing the detectors corresponding to Bragg peaks, the remaining spectra were summed and converted into the dynamical structural factor $S(Q, \omega)$ and then into the generalized density of states, GDOS, $G(\hat{\theta},\omega)$:[50]

$$G(\hat{\theta}, \omega) = \frac{S(\hat{\theta}, \omega)}{Q^2(\hat{\theta}, \omega)} B(\omega, T) \tag{1}$$

where $\hat{\theta}$ refers to the averaged scattering angle, $S(\hat{\theta},\omega) = (1/(N_D))\sum_i S(\theta_i,\omega)$ in the summation ranging from the first to the last detector considered, and θ_i is the angle corresponding to this detector. N_D is the total number of detectors, and $S(\hat{\theta},\omega)$ is the scattering vector, which is calculated at each energy transfer for the averaged scattering angle. $B(\omega,T)$ is a function accounting for the population of the vibrational modes with temperature. As the GDOS is obtained after an averaging over the scattering angles considered in the experiment, it improves the statistics but smooths out the coherence effects of the scattering. This is the so-called incoherent approximation. It is important to indicate here that the GDOS can be compared to the phonon density of states (PDOS) predicted by the calculation, but one must consider that the real PDOS can only be extracted from the measurement of the dispersion curves using large single crystals. In the case of powder samples, the GDOS is strictly equal to the PDOS only for cubic Bravais lattices at low temperatures. Figure 4 shows the static structure factor, $S(Q)$, of the sample at selected temperatures.

Computational Details. All calculations were performed using the DFT code, Dmol3, as part of the Materials Studio software suite.[51] DMol3 is an accurate and reliable density functional theory (DFT) quantum mechanical code for research in the chemicals and pharmaceutical industries. It uses numerical, localized atomic orbitals of the hydrogen atom to construct the electron density. In this work, a high precision basis set, double numerical with polarization (DNP), with an orbital cutoff of 3.7 Å, was applied. The GGA functional of Perdew, Burke, and Enzerhof (PBE) was used to describe exchange and correlation contributions to the total energy. The k-point sampling was (2,3,3). Geometry optimization, prior to the normal mode calculation using the direct method, was limited to the atomic coordinates, with initial values for the coordinates and cell parameters being taken from X-ray diffraction data at 113 K;[30] lattice constants a = 13.3236 Å, b = 9.6159 Å, c = 7.7331 Å, β = 103.992°, and space group $P2_1/c$ (No. 14). The dynamical matrix was calculated only for the Γ
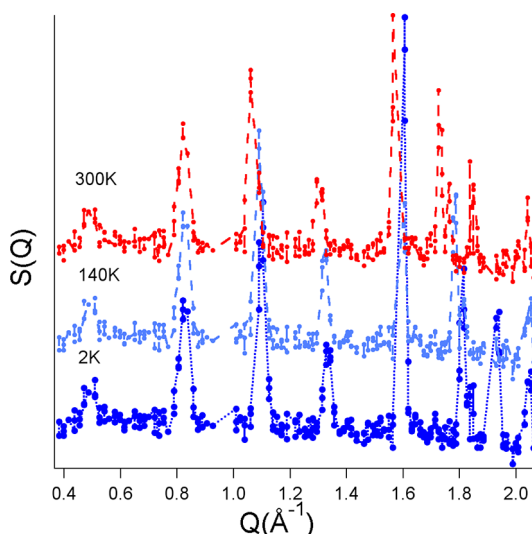
Figure 4. Diffraction patterns of phenacetin at selected temperatures measured with λ = 5.12 Å at IN6. Neither a phase transition nor a significant change in the structure is observed.

point, giving one set of normal modes from which the hydrogen atom density of states was extracted for comparison with the experimental data.

## ◼ EVIDENCING DIFFERENT DYNAMICAL ENVIRONMENTS OF THE METHYL GROUPS IN PHENACETIN

Even if phenacetin presents a rather large number of Bragg peaks allowing for a relatively limited analysis of the momentum transfer range, Q ($Q = 4\pi \sin \theta/\lambda$, where 2θ is the scattering angle), the analysis of the elastic scattered intensity, $S_{elastic}(Q \rightarrow 0, T, \omega \approx 0)$, obtained using IN10, in the framework of the Gaussian approximation was possible and is presented in Figure 5. In this approach, the following is considered:

$$\left\langle u^2 \right\rangle = -6 \frac{d(\ln(S(Q, T, \omega \approx 0)))}{d(Q^2)}\bigg|_{Q \rightarrow 0} \quad (2)$$

In Figure 5a, the pronounced drop in elastic intensity between 60 and 140 K corresponds mainly to QE scattering, due to a local dynamical process, emerging from the elastic line and then becoming essentially a flat background at high temperature. The continuing decay in the elastic intensity at higher temperature is generally due to the Debye–Waller effect.[52]

The evaluated MSDs, $\langle u^2 \rangle$, are shown in Figure 5b. The fast increase in $\langle u^2 \rangle$ around 60 K confirms the onset of anharmonic dynamics as the temperature is increased, while around 160 K, the model of eq 2 breaks down due to the onset of underlying fast diffusion motion.

To verify the dynamic origin of the observed transitions, the quasi-elastic signal observed using IN10 was evaluated, and the results are shown in Figure 5c. From this figure, a QE broadening is evident between 90 and 150 K. At 220 K, the QE signal might be characterized by a small amplitude but a large width, which is not covered by the dynamic range of IN10, and hence hardly detectable. At IN6, however, the QE signal can be identified up to the maximum temperature studied, as shown in Figure 6a,b.
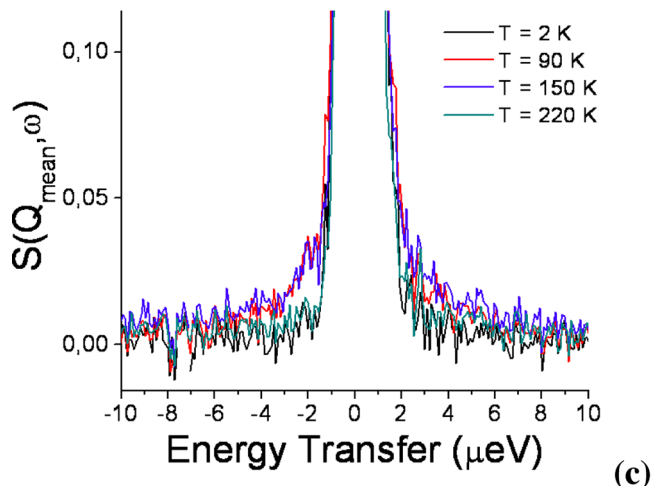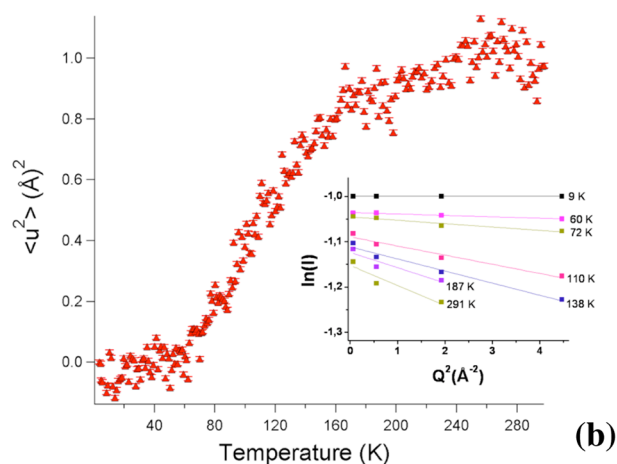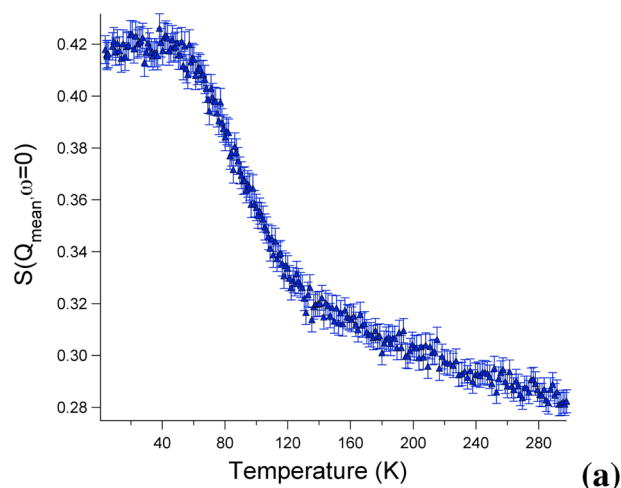
Figure 5. (a) Elastically scattered intensity for phenacetin obtained using IN10 binned over the explored Q range as a function of temperature. (b) Evaluated MSDs, $\langle u^2 \rangle$, deduced by analyzing the elastically scattered intensity $S(Q, T, \omega = 0)$ using the Gaussian approximation. The inset shows the log of the intensity vs Q^2 at selected temperatures. The solid lines result from the fit using eq 2. (c) Corresponding spectra at 2, 90, 150, and 220 K, showing a QE component at temperatures between 90 and 150 K.

A detailed analysis of the geometry of the observed motion in the QE region can be achieved by analyzing its dependence as a
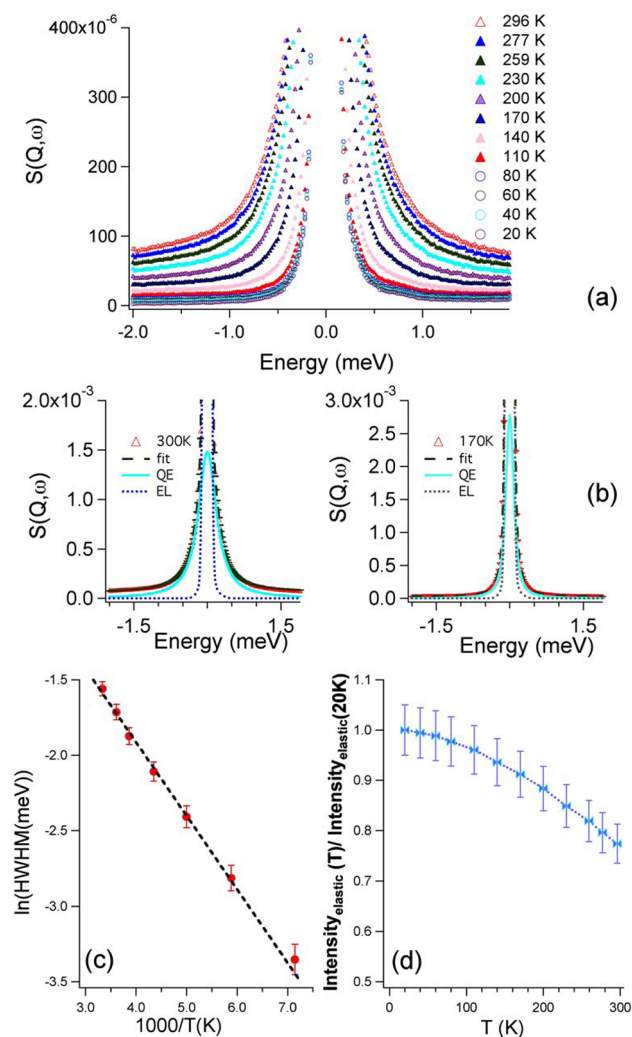
Figure 6. (a) Spectra for phenacetin obtained using IN6 between 20 and 300 K with a resolution of 70 μeV binned over the explored Q range. The low-temperature IN6 data show no quasielastic broadening and thus merely reflect the resolution of the spectrometer. Note that the spectra match the resolution function below 110 K. (b) Examples of the qualitative description of the QE signal summed over the total Q range: the Dirac function represents the EL component, while a single Lorentzian line describes the QE signal. (c) Arrhenius representation of the quasi-elastic width obtained from the fits of phenacetin as described in the text. The line was obtained using the Arrhenius relationship:[49] $\Gamma = \Gamma_0\ e^{-E_{act}/kT}$, with Γ_0 and E_{act} being the attempt frequency and the apparent activation energy related to the motion observed. (d) Temperature dependence of the elastic neutron scattering intensities for phenacetin ($C_{10}H_{13}NO_2$) derived from the QENS data analysis observed using IN6. One note that 77% of the total scattering, mostly from the hydrogen atoms, is seen as immobile. The intensities are normalized to their corresponding values at 20 K.

function of temperature and Q range. However, because of the very weak QE signal and low counting statistics, all attempts to analyze the QE signal measured on IN10 failed. In the case of IN6, although it was not possible to perform an analysis of the Q-dependence of the motion, we were able to obtain a qualitative description by fitting the QE signal summed over the total Q range measured. Considering that the QE signal is caused by methyl group reorientation only, due to the nonequivalence of methyl groups I and II ,the temperature-dependent spectra should be described as a superposition of

two Lorentzians following Arrhenius dependences, $\Gamma = \Gamma_0\ e^{-E_{act}/kT}$, with different activation energies, E_{act}.[49] However, the IN6 data could be well described by a Dirac function and only one Lorentzian line. The Arrhenius plot of the variation of Γ as a function of temperature is shown in Figure 6c. From this curve, an activation energy, E_{act}, of about 41 meV (or 3.9 ± 0.1 kJ/mol) is found. Therefore, our results indicate either (i) that the activation energies of the rotations for the two methyl groups are well separated in energy or (ii) that the reorientation of the methyl groups are close in energy, and we observe only an average activation energy, $\langle E_{act}\rangle$. To solve this question, the comprehensive description of the excitations originating from each CH_3 group obtained through DFT calculations was considered and is described in the next section.

■ VIBRATIONAL BEHAVIOR OF THE METHYL GROUPS IN PHENACETIN

INS spectra of phenacetin are illustrated in Figure 7,a and the calculated INS spectra using DFT for the crystal are given in
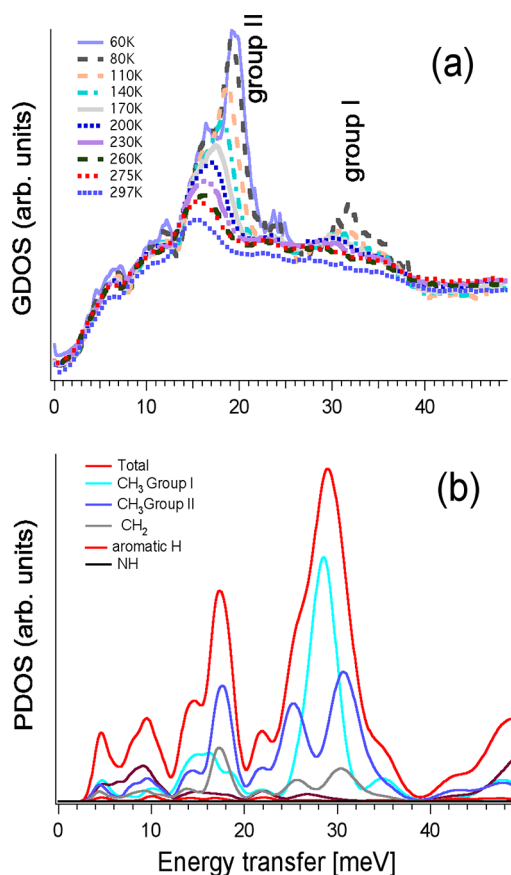
Figure 7. (a) Experimentally determined desnisty of states, GDOS, of phenacetin at selected temperatures. (b) DFT-derived total and partial density of states below 50 meV.

Figure 7b. The overall agreement between observed and calculated data is reasonable, even in the low-frequency region, where the intermolecular interactions are expected to play an important role. From these results, we can conclude that all vibrational bands up to 50 meV are mixed, involving all parts of the molecule. The vibrational modes located between 8 and 11 meV are related to torsion vibrations of CN, CO, and COC bonds, while the strongest and broad contributions observed between 10 and 22 meV and between 25 and 40 meV are due

to a mixture of the librational modes related to the methyl groups I and II.

The mode assignment can be further substantiated using the accurate numerical relationships characterizing the dynamics of each methyl group given by a potential as described in ref 53 and recently summarized in ref 54. Such relationships associate the rotational potential barrier V_3 describing a rigid methyl group in 3-fold symmetry, the activation energy E_{act}, and the first librational energy E_{01}, as follows: $E_{01}(meV) = 0.47-[V_3(K)]^{0.548}$ and $E_{act}(K) = 0.598[V_3 (K)]^{1.05}$. For an $E_{act} = 41$ meV, one expects a librational mode at 17 meV and $V_3 = 60$ meV, while for a librational mode at 30 meV, one anticipates $E_{act} = 175$ meV (16.6 kcal/mol) and $V_3 = 200$ meV. Therefore, we can conclude that reorientation of the methyl group I in phenacetin is related to the librational mode observed at 30 meV, while the methyl group II libration is located at about 20 meV. Furthermore, it is interesting to note that, based on deuterium nuclear magnetic resonance spectroscopy, it was shown that, in aspirin, the peptide methyl group (group II) undergoes fast thermally activated rotation with an activation energy of 51.5 meV (4.9 ± 0.05 kJ/mol).[55] Moreover, the calculated and observed librational transitions are pretty much in agreement, except for the intensity of the mode at 30 meV. However, from structural analysis, it is known that the ethyl fragment in phenacetin is disordered, in addition, the phenacetin molecule is more flexible than acetanilide and paracetamol.[56] Hence, as in other orientationally disordered crystals,[57,58] the observed experimental intensity of the libration modes are smeared exclusively due to the Debye−Waller effect. In addition, considering that the crystal field effects are not very significant, there is no particular reason why librational modes should show particular temperature dependencies.

Lastly, if one considers that the elastic component of the QENS spectra is attributed to the contribution of the immobile nuclei and that the coherent scattering lengths of C, O, N, and H as well as the incoherent cross-section of C, N, and O are much smaller than the incoherent scattering length of H, the value of the elastic intensity measured on IN6 and displayed on Figure 6d can be ascribed to the fraction of protons that remain static on the time scale corresponding to the spectrometer resolution. This means that only about 30% of all H atoms participate in the fast motion. Consequently, we can infer that our experiments allowed observing mainly the motions related to the peptide methyl (group II). Finally, for the methyl group I, one expects a corresponding tunnel splitting in the neV range, while for the methyl group II, the tunnel splitting should be observed below 1 μeV. In both cases, the splitting cannot be resolved within the experimental resolution of our experiment.

CONCLUSIONS

Understanding the lattice vibrations is a difficult task; however, the application of solid-state density functional theory (DFT) methods can be used as a reliable approach to simulate this part of the vibrational spectra and to reveal the underlying physical nature of the low-energy vibrational motions. Furthermore, it is possible to link the correlation times of the methyl group rotation with deviations of the mean square displacements, $\langle u^2 \rangle$. In this study, we were able to assign the hopping process of the peptide methyl (group II) to the librational band observed in the inelastic part of the spectra, reflecting the differences in the chemical and crystallographic environments of the methyl groups in phenacetin. On the basis of such a behavior, one can conclude that phenacetin can assume different conformations, and knowing this can help the understanding of how chemical reactions occur. In the particular case of biochemistry and molecular biology, such results can help understating the ways molecules interact with each other in living systems. In the future, to follow in detail the effects of crystal packing, the intermolecular hydrogen bonds, and the nature of other molecular fragments adjacent to the acetamide fragment on the dynamics of the peptide methyl, an INS study of related compounds from the same series would be interesting. In particular, the comparison of the dynamics of methyl groups in a series of structurally related compounds could shed light on the role of the alkyl fragments in drug toxicity, a puzzling but well-known question to pharmacologists.[59−61] Another potentially relevant problem is understanding different stability of acetanilide derivatives with respect to polymorphism, as well as the problem of their phase stability on variations of temperature, which may occur on processing or on storage.

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Notes

The authors declare no competing financial interest.

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