LETTERS TO THE EDITOR

On the 3.35 ppm Singlet Resonance in Proton NMR Spectra of Brain Tissue: *Scyllo*-Inositol or Methanol Contamination?

A reliable identification of cerebral metabolites is fundamental to applications of *in vivo* nuclear magnetic resonance (NMR) spectroscopy to neurochemical research and clinical diagnosis. Although extensive knowledge has been compiled in the literature, complications still arise from (i) species differences including man; (ii) alterations of *in vivo* metabolic profiles by tissue selection and *in vitro* preparation; and (iii) the use of variable NMR field strengths, signal types, and relaxation effects. Taking these aspects into account, we previously assigned the singlet resonance at 3.35 ppm in proton NMR spectra of human gray matter to *scyllo*-inositol (*scyllo*-Ins) both under *in vitro* conditions and *in vivo* (1). Moreover, the concentration of *scyllo*-Ins was found to be correlated with that of *myo*-inositol (*myo*-Ins). These observations were confirmed in high-resolution proton NMR studies of hepatic encephalopathy (2), multiple sclerosis (3), and breast carcinomas (4).

More recently, however, the *scyllo*-Ins assignment was questioned by Preece *et al.* in a paper entitled "Experimental encephalomyelitis modulates inositol and taurine in the spinal cord of Biozzi mice" (5). The authors noticed that the 3.35 ppm resonance in their proton NMR spectra of perchloric acid (PCA) extracts of brain tissue did not correlate with alterations of *myo*-Ins in experimental allergic encephalomyelitis. They concluded that the observed resonance was due to a preparation artifact resulting from methanol in the swabbing solution. Although the additional presence of *scyllo*-Ins could not be ruled out, a methanol contamination was proven by investigations of the used swabbing solution.

The purpose of this Letter is to confirm our previous identification of *scyllo*-Ins in human, bovine, and sheep gray matter. Figure 1 shows two high-resolution 7.0 T proton NMR spectra of PCA extracts of bovine medulla in the 3.0–4.0 ppm chemical shift range. Spiking with methanol clearly reveals two separate singlet resonances shifted by 1.2 Hz and thus allows an unambiguous distinction of methanol (at 3.354 ppm) and *scyllo*-Ins (at 3.350 ppm).

In general, Preece *et al.* correctly point out that methanol may give rise to an artifactual signal contribution that may be mistaken for *scyllo*-Ins in proton NMR spectra of brain tissue. Control experiments should involve resonance "spiking" with either *scyllo*-Ins or methanol and extended to chemicals and solutions used for extract preparation. In addition, investigators should be aware of species dependencies as relatively high concentrations of cerebral *scyllo*-Ins have been detected in man, bovine, and sheep *but not* rats (1). Thus, the putative absence of *scyllo*-Ins in mice may further explain the failure of Preece *et al.* to resolve its proton NMR resonance aside of methanol.

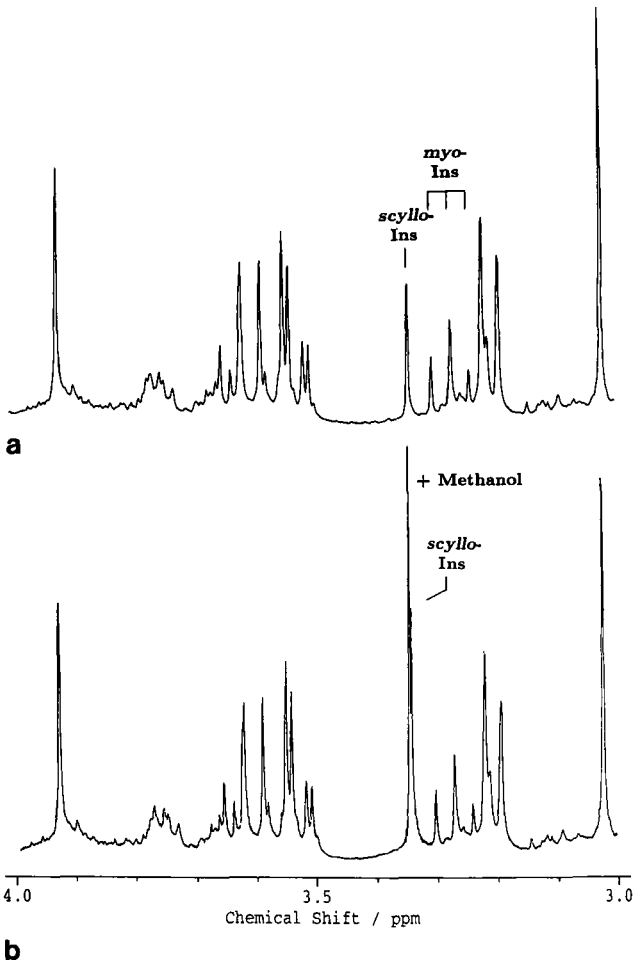

FIG. 1. High-resolution proton NMR spectra (7.0 T, fully relaxed, 3.0–4.0 ppm section) of bovine medulla (a) before and (b) after adding methanol to the perchloric acid extract. Contamination of the *scyllo*-Ins singlet at 3.350 ppm with methanol can be ruled out as the latter singlet resonates at 3.354 ppm and therefore may be clearly discerned from *scyllo*-Ins.

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RESPONSE

We welcome the additional data regarding the unambiguous observation of *scyllo*-inositol (*scyllo*-Ins) in the brains of larger mammals now provided by Michaelis and Frahm, not least because their figure (after methanol "spiking") graphically demonstrates the potential for error inherent in the NMR assignation of singlets in extract and biofluid studies, let alone those studies performed *in vivo*. We remind your readers that in our paper, we did not question the specific findings of Michaelis *et al.* We simply pointed out that in our own study of Biozzi mouse spinal cord the prominent singlet seen at 3.35 ppm could be attributed to methanol contamination, although we could not rule out the additional presence of *scyllo*-Ins. The closeness of the two resonance frequencies serves to emphasize the care that needs to be taken when assigning singlet peaks such as these, and we agree completely with Michaelis and Frahm about the importance of "spiking." Regarding the diminished or absent *scyllo*-Ins signal seen in rodent brain, we have had limited success in detecting *scyllo*-Ins in rat, mouse and guinea-pig brain, despite the original retrospective analysis of literature data reported by Michaelis *et al.*, which claimed to reveal the presence of *scyllo*-Ins in three earlier key studies of rat brain (see (1) for refs).

In general with an insensitive technique such as NMR spectroscopy, intense singlets (particularly those from co-resonant protons) become most attractive for detection, because the strong signal is not split by J-coupling. Unfortunately when a well resolved singlet is the only prominent signal from the metabolite, as is the case with *scyllo*-Ins (and certain N-trimethyl compounds), the absence of coupling effects, either in 1- or 2D experiments means that assignments must be made only on the basis of the chemical shift. This is particularly troublesome in ^{1}H NMR because of its narrow chemical shift range, and means that considerable care needs to be taken with spectral assignments. When the chemical shift of the singlet is close to one of the regular contaminants which plague biological NMR samples such as formate and acetate, or in this case methanol, the need for additional "spiking" experiments becomes paramount.

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