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THE USE OF SOLID ^{13}C NUCLEAR MAGNETIC RESONANCE FOR THE CHARACTERIZATION OF CHOLESTEROL AND BILIRUBIN PIGMENT COMPOSITION OF HUMAN GALLSTONES

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SUMMARY: The new magic-angle spinning and cross polarization techniques for obtaining a ^{13}C solid NMR spectrum are applied to the characterization of human cholesterol, mixed cholesterol-pigment, calcium bilirubinate and bilirubin pigment gallstones. The stones divide into two general classes: the cholesterol stones and the bilirubin pigment stones. The cholesterol stones are very similar to each other whereas the bilirubin pigment stones exhibit considerably more variation in spectral features. The importance of other material and polymerization in the bilirubin pigment stones is discussed.

INTRODUCTION

Human gallstones occur in a variety of shapes, sizes, and crystalline to amorphous structures and range in color from creamy white to yellow, to brown, to black. They are classified by their gross morphology as cholesterol stones, mixed stones or pigment stones (1). Chemical analysis has confirmed the accuracy of the visual classification for the major constituents of stones (2). X-ray diffraction techniques have been used to determine the crystalline organic and inorganic constituents (3,4). However, chemical analysis fails when much of the stone material is insoluble and x-ray analysis fails for noncrystalline or amorphous stones. Unfortunately pigment stones are often difficult to dissolve (5,6) and they are often amorphous.

Recently, Trotman et al (7) showed that some pigment stone constituents could be quantitated by infrared spectroscopy, but they were able to account

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for a mean of only 63% of the constituents in pigment stones and only 50% of the organic portion of the pigment stone. Therefore, there is yet no totally satisfactory method to document completely gallstone composition, especially in those stones which are insoluble and/or amorphous (8). The pigment stone is more common in humans than had been recognized until recent prospective studies found a prevalence of 22-33% (9-11). Calcium bilirubinate is a major constituent of bilirubin pigment stones (2), but studies have shown that up to 20% of pigment stones contain bilirubin in another form (11-14). The incidence of pigment stones increases with age. The effect of diet on human pigment stone formation is uncertain. However, normal pound dogs fed a diet of normal foodstuffs, but high in carbohydrate, low in protein and with added cholesterol, produce black pigment stones with regularity (15). These stones contain some free bilirubin, cholesterol and trace amounts of porphyrins. However, they are amorphous and 40-60% of the stone is insoluble in acids and organic solvents. A material isolated from human pigment stones recently (8, 12) may be similar to this black, insoluble material of dog stones. This material is found in variable amounts in human pigment stones and may reach 70% by weight of the stone (16). Its constituents are not known but the material is likely polymeric. A similar type of pigment stone occurs naturally in 14% of dogs from local dog pounds (17) and in a long term colony of randomly bred beagle dogs maintained for radiobiology studies (17,18).

We wish to report a new spectroscopic method for characterizing gallstones. It is used on solids to detect the naturally abundant ^{13}C isotope which occurs in nature at 1.1% and could be used also in solids enriched with ^{13}C, biosynthetically. The method uses both magic angle spinning (MAS) (19) and proton cross polarization (CP) (20) techniques to provide a powerful way for eliminating the spectral broadening (21) arising in solid samples from dipolar interactions and anisotropic chemical shielding. The ^{13}C-H dipolar interactions in solids can produce line widths up to 40 KHz in the ^{13}C resonance, while shielding anistropies at

the field strength used in this study can broaden ^{13}C signals by up to an additional 6-7 KHz depending upon the types of carbon being investigated at the magnetic field strengths employed in this study. This is to be contrasted with the line widths of 0.1 Hz or less observed in many liquid samples. The CP method removes the dipolar broadening while the MAS technique will eliminate broadening due to chemical shielding anistropy providing the spinning rate exceeds the broadening in Hz. The MAS also modulates the dipolar interaction and compliments the CP method in reducing dipolar broadening. The importance of both interactions and their effect on line widths has been discussed (21). Relatively high resolution spectra with line widths of only 10 to 50 Hz may be obtained routinely using such spectral techniques depending on the morphology of the sample (22).

Commercial instrumentation which uses MAS/CP methods is only just now becoming available, but the ease of data collection and optimization of operation are still somewhat limited. Most of the published work to date has been carried out on either modified commercial units or homemade spectrometers. To our knowledge this study on human gallstones reports the first MAS/CP results in this area of medical research. Four representative samples of different classes of human stones have been selected for this exploratory study.

EXPERIMENTAL

The MAS/CP spectra on the gallstones were obtained on a single-coil, double tuned probe similar in design to that reported earlier (23) except as it has been developed for the electromagnet of the Varian XL-100-15 system. A power activated diode switch was added between the receiver and the probe to convert to single coil operation. The probe of our own construction (24) uses a D_2O external lock and a special rotor and stator assembly designed to provide the MAS. The carbon-13 (25.16 MHz) and proton (100.06 MHz) radio frequency fields are 17 G and 12 G, respectively for 90 watts of power. The ^1H spin locking pulse of 90° may be varied in terms of length and amplitude while the amplitude of the ^{13}C irradiation can be controlled to within 0.1 db to match the Hartmann-Hahn (25) condition. The isolation between the two channels is in excess of 60 db.

The MAS/CP spectra were obtained using a single contact sequence (20). A contact time of 3.0 msec and a cycle time of 5.0 sec were used in these experiments. The spectral intensities obtained on the commercial samples of bilirubin and cholesterol appeared to be satisfactory under these conditions, even though the common intensity distortions associated wth such solid spectra were encountered.

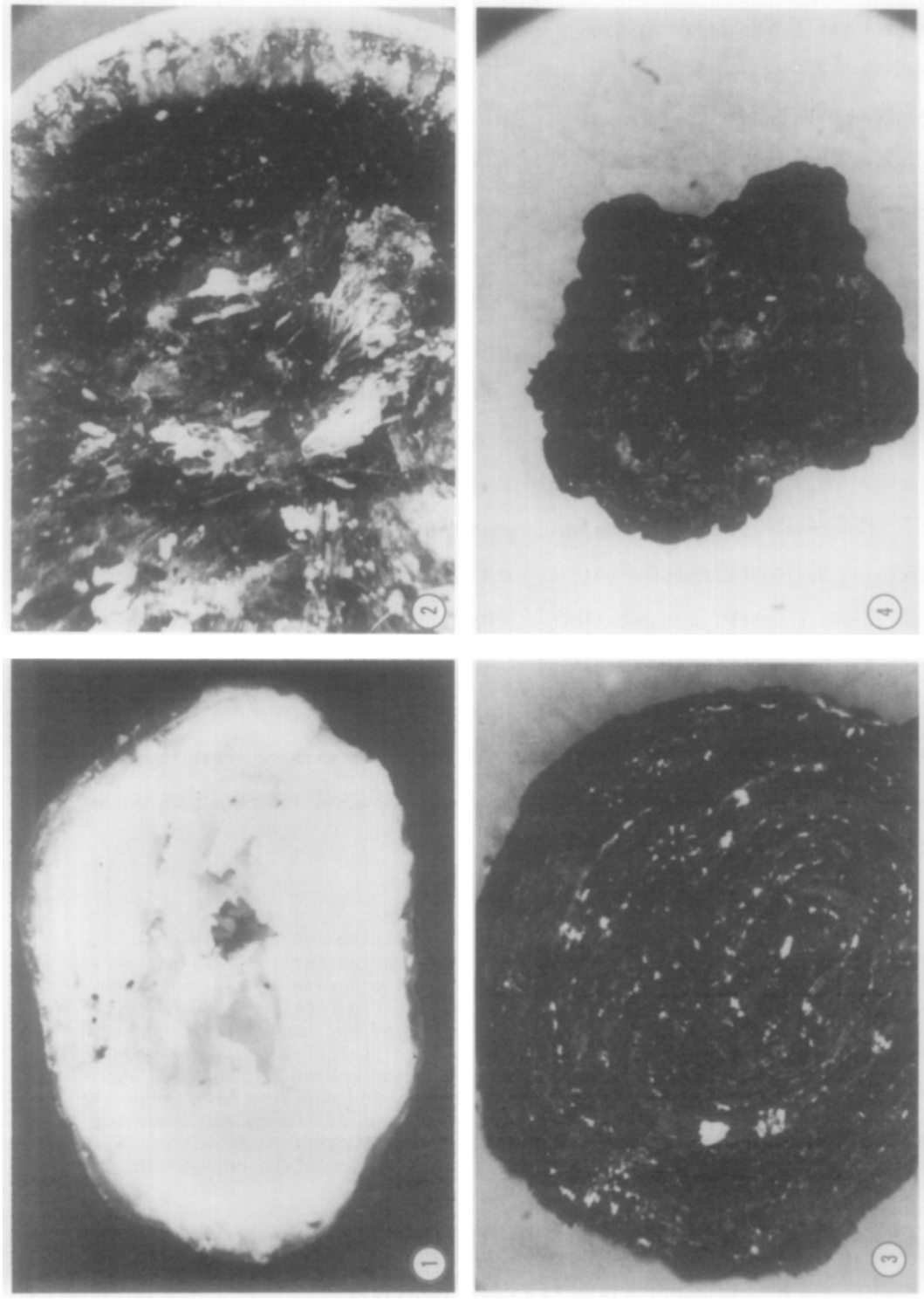

Figure 1: Photographic cross-sections of four characteristic human gallstones: 1) cholesterol stone, 2) mixed cholesterol-pigment stone, 3) calcium bilirubinate stone, and 4) black amorphous pigment stone.

Our high speed magic-angle spinner operated at about 5,000 Rev per sec and was fabricated from Kel-f plastics. The stator assembly, described in detail elsewhere (24), was also constructed of Kel-f which does not produce a ^{13}C background. The magic-angle is adjusted by revolving the stator while monitoring the signal from a rotor containing a standard sample of hexamethylbenezene (HMB). Once the optimal magic-angle has been selected the HMB rotor can be replaced with the sample rotor with no loss of alignment.

The gallstones shown in Fig. 1 and reference compounds were pulverized into finely divided powders, homogenized, and about 80-120 mg packed firmly into the cavity of the conical rotor. For this size of sample a time averaged spectrum with reasonable signal to noise may be obtained in 8,000 repetitions or in approximately 11 hrs. The gallstones selected were of four general types: (1) cholesterol stone, (2) mixed cholesterol-pigment stone, (3) pigment stone usually classified as calcium bilirubinate stone, and (4) amorphous, black pigment stone. These representative stones were taken from human gall bladders obtained at cholescystectomy at either the University of Utah or VA Medical Centers in Salt Lake City, Utah. The four stones provide a representative sample of the typical varieties of human gallstones. The reference samples of cholesterol and bilirubin were obtained from Baker Chemical Co. and Eastman Chemical Co., respectively.

RESULTS AND DISCUSSION

A photograph of the cross-section of the four studied gallstones is given in Figure 1 with the numerical labels corresponding to the numerical ordering of the stones in the previous paragraph. Significant differences in both morphology and color is noted for the four selected stones. The MAS/CP spectra of these four stones and the two reference samples are contained in Figures 2 and 3. Spectroscopically the four stones may be divided into two groups of two stones each based on the similarity of the spectra of the stones with MAS/CP spectra of either solid cholesterol or bilirubin. Thus, the spectra of stones 1 and 2 along with that of solid commercial cholesterol appear in Figure 2 and of stones 3 and 4 and bilirubin in Figure 3. There is essentially no similarity between the spectra of stones 1, 2 and those of stones 3, 4.

Comparison of the spectra in Figure 2 indicates that even though both stones 1 and 2 vary greatly in color, they are predominately (80-90% or more) cholesterol with only minor spectral differences noted in the 20-40 ppm range. Aliphatic and substituted aliphatic carbons normally appear in this spectral region. It is not surprising that the white crystalline material of stone 1, would match in almost every detail the commercial cholesterol solid

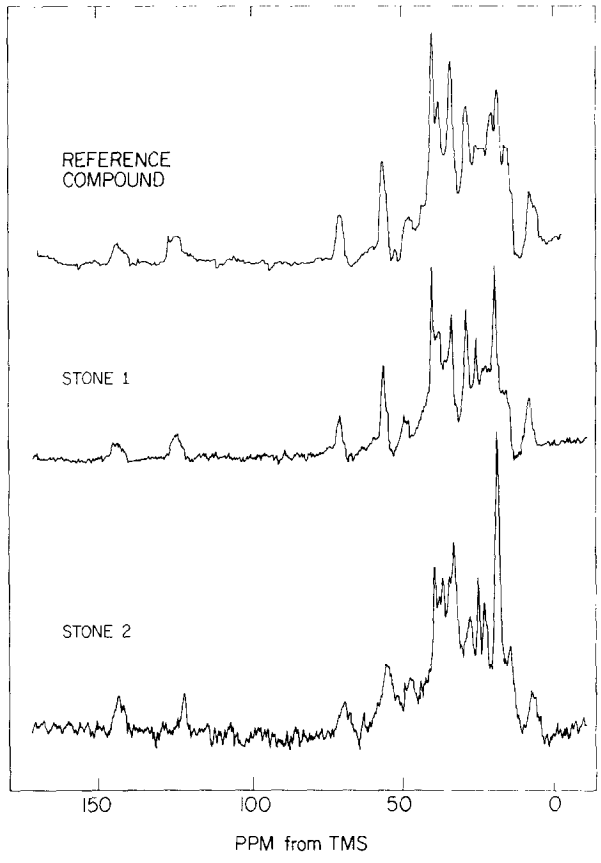

Figure 2: MAS/CP solid ^{13}C spectra of cholesterol and cholesterol-related stones, cholesterol stone (#1) and mixed cholesterol-pigment stone (#2).

spectrum. However, the extensive coloring found in stone 2, a mixed stone, does not greatly affect the MAS/CP spectrum of this stone. The minor spectral difference between the spectra of commercial cholesterol and stone 2 could easily be accounted for by materials in the stone which do not exceed 10% of carbon appearing in the spectrum. If the pigment in stone 2 is associated with free radicals or paramagnetic metal ions then the resonance lines due to carbon in the pigment material may be inordinately broadened, and the estimates of less than 10% pigment material could be in error. This point will need future attention. Based on the MAS/CP spectra, however, one must conclude that stones 1 and 2 are both predominately cholesterol stones. This agrees with the established etiology of these types of stones. The

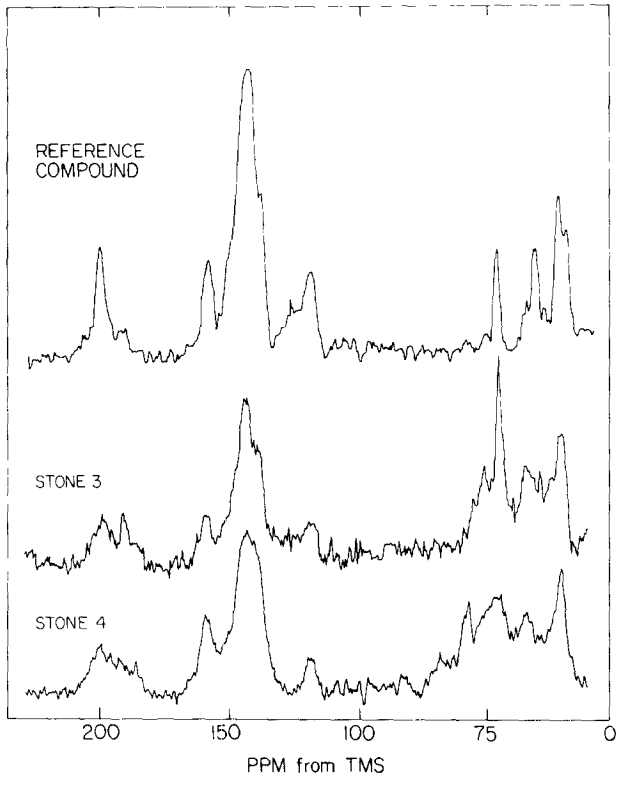

Figure 3: MAS/CP solid ^{13}C spectra of bilirubin and bilirubin-related stones, calcium bilirubinate stone (#3) and black, amorphous pigment stone (#4).

strong pyrrole band at 150 ppm associated with bilirubin spectra (see Figure 3) does not appear in the spectra of stones 1 and 2 with the present technique.

The spectra of bilirubin and related stones (3 and 4) given in Figure 3 on the other hand exhibit considerably more variation than the cholesterol stones even though the characteristic bilirubin spectral features strongly influence the spectral responses of both stones 3 and 4. The aromatic and vinyl region (120-160 ppm) is remarkedly similar in all three spectra. This spectral feature clearly rules out any appreciable polymerization of the bilirubin contained in these two stones through the vinyl groups. The terminal carbon in the vinyl group whose peak appears at about 120 ppm is very strong in the pigment stone (#4). If ethylenic polymerization had been extensive this peak would have disappeared.

The aliphatic region (20-60 ppm) does vary extensively between the three spectra given in Figure 3, and this richness of spectral detail provides a basis for extensive future investigation of the bilirubin related stones. The aliphatic spectral region is very responsive to lipids, sugars, and bile acids, and future studies need to focus on the possible presence of such materials. The methyl band and two prominent methylene peaks appearing in commercial bilirubin are reflected in stones 3 and 4 indicating that tetrapyrrolic pigment is predominant in both, but in the stones considerably more lines are also observed in the aliphatic region and these need to be identified.

The carboxylic region of the spectrum around 200 ppm varies extensively between bilirubin and its related stones. Instead of the reasonably sharp single resonance line found in the reference compound, band structure is found in this spectral region in both of the bilirubin related stones indicating the presence of a variety of carboxyl carbons in the sample. The carboxyl band in stone 3 can be explained by the presence of calcium bilirubinate salts which affect the resonance at the carboxyl position. Some polymerization through ester or amide linkages would also be a possible explanation. In the polymeric pigment stone (#4) the broadening of the carboxyl resonance would be explained if the polymerization took place at the carboxyl group. As ester and amide linkages account for a great variety of both natrually occurring and synthetically produced polymers, the proposal of carboxylic polymerization with alcohols and amines is attractive. Such polymerization would not alter the basic bilirubin spectral features. The molecular moieties cross-linking carboxylic groups need only then have aliphatic type carbons to explain the anomalous peaks falling in this spectral region.

Extensive dispersion of carboxylic resonances in the bilirubin stones indicates that major structural differences exist at these centers in the stones if compared with the commercial bilirubin. This spectral feature

argues for polymerization at the carboxylic position. Such polymerization would account especially for the rubbery character and insolubility of the black pigment stones (#4). The tetrapyrrolic structure of the bilirubin molecule does not otherwise appear to be greatly affected. The vinyl moieties are not altered to any appreciable extent ruling out polymerization through this group.

The MAS/CP nuclear magnetic resonance technique for obtaining relatively high resolution spectra on solids is well suited for studies of gallstones. Distinctive features and a wealth of information characterize the spectra obtained. MAS/CP provides a reasonably rapid method for characterizing human gallstones and presently suffers only from the high degree of sophistication required to secure the spectral information and from limitations associated with a new instrumental field which has not developed totally to the stage where routine instrumentation is available.

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