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CHEMICAL CHARACTERIZATION OF PIGMENT GALLSTONES USING ^{13}C NUCLEAR MAGNETIC RESONANCE ANALYSIS

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SUMMARY The unique ability of Carbon-13 nuclear magnetic resonance analysis with cross polarization/magic angle spinning techniques to investigate chemical structures of solids is used to probe the chemical characteristics of several gallstone types. New pulse program techniques are used to distinguish various carbon atoms in studying the polymeric nature of the black bilirubinoid pigment of pigment gallstones. Evidence for the involvement of the carboxyl group and noninvolvement of vinyl groups of bilirubinoids in the polymeric bond formation is presented. Conjugated bilirubin structures are found to be present in some solid residues from pigment stones extracted with acidic methanol/chloroform.

INTRODUCTION Much of the current research being performed on gallstones has, for several reasons, been centered around pigment gallstones including so called "black pigment" stone and the insoluble black residue from pigment stones (1). One reason is that the incidence of pigment gallstone formation is higher than previously believed (1-3). But most importantly, it has been proposed that insoluble pigmented residues may act as nucleation sites (4) for the deposition of cholesterol and may be involved in the formation of the more prevalent cholesterol gallstone as well as the pigmented gallstone. The chemical structure and pathology of the black pigmented residues have been very hard to analyze because of the insolubility of the black material (1,5,6,7). Using infrared and chemical degradation techniques, Suzuki (5) and Wosiewitz (8) respectively showed that the black pigment material of pigment gallstones contain polymers of bilirubinoids; however, very little is known

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about the type of polymer bands or bilirubinoid structure and attached chemical groups.

The use of cross polarization/magic angle spinning (CP/MAS) in the detection of carbon-13 nuclear magnetic resonances in solids offers great advantages for nondestructive analysis and chemical structure determination of gallstones and some of their insoluble residues (9).

Pigment gallstones are subclassified into two general types; the earthy type which are brown in color and contain mostly calcium bilirubinate and the black type believed to contain polymers of tetrapyrrolic substances (10,11). The purpose of this article is to elucidate some of the polymeric structure of the black pigment gallstone and insoluble black residue using solid ^{13}C-NMR analysis.

MATERIALS AND METHODS Gallstone samples from the gallbladder were analyzed using both the modified Varian XL-100 spectrometer previously described (9) and a new Bruker CXP-100 instrument prepared for CP/MAS solid work. Samples (50-100 mg) were powdered and spun at the magic angle in carefully prepared rotors at approximately 4 kHz. All chemical shift values are reported externally referenced to the carbon-13 signal of tetramethylsilane (TMS). The actual reference used was the high field signal of adamantane which is reported in the literature (13) to be at 28.6 ppm from TMS. A rotor of the reference material is periodically placed in the probe of the spectrometer between sample runs to reference the carbon-13 pulse frequency used. This procedure seems to be sufficient to guarantee an accuracy of $\pm$ 1 ppm. The cross-polarization contact time between proton and carbon spins was 3.0 msec. The cycle times for repetitive pulse sequences was 3.0 sec. This was found to be sufficient for the magnetization to return to equilibrium. Improved probe design allowed higher proton decoupler power levels which in turn improved the resolution previously obtainable on the XL-100 instrument. All ^{13}C spectra involve proton decoupling during the signal acquisition period.

The bilirubin reference sample was obtained from Sigma Chemical Company, Saint Louis, Missouri and the cholesterol reference sample was from Baker Chemical Company, Phillipsburg, New Jersey. The number of transients or free induction decay signals needed to produce an accumulation relatively free of noise varied from 5,000 to 25,000 depending upon the sample size and type. Cholesterol samples produce better signal-to-noise levels and thus require fewer scans. Suzuki (12) has reported that black pigment polymerization parallels amounts of complexed copper and iron and the presence of these paramagnetic ions could explain why the pigment stones tend to exhibit poorer signal-to-noise. Paramagnetic metal ions would provide shorter relaxation times, thus producing broadened carbon-13 peaks.

RESULTS AND DISCUSSIONS Figure 1 shows solid CP/MAS spectra of stones classified as either "cholesterol" (A) or "mixed" (B and C). Spectrum A is within experimental error identical with that of a cholesterol sample, indicating that stone A is predominantly cholesterol. Smaller, variable amounts of bilirubinoid structure become apparent in spectra for stones B and

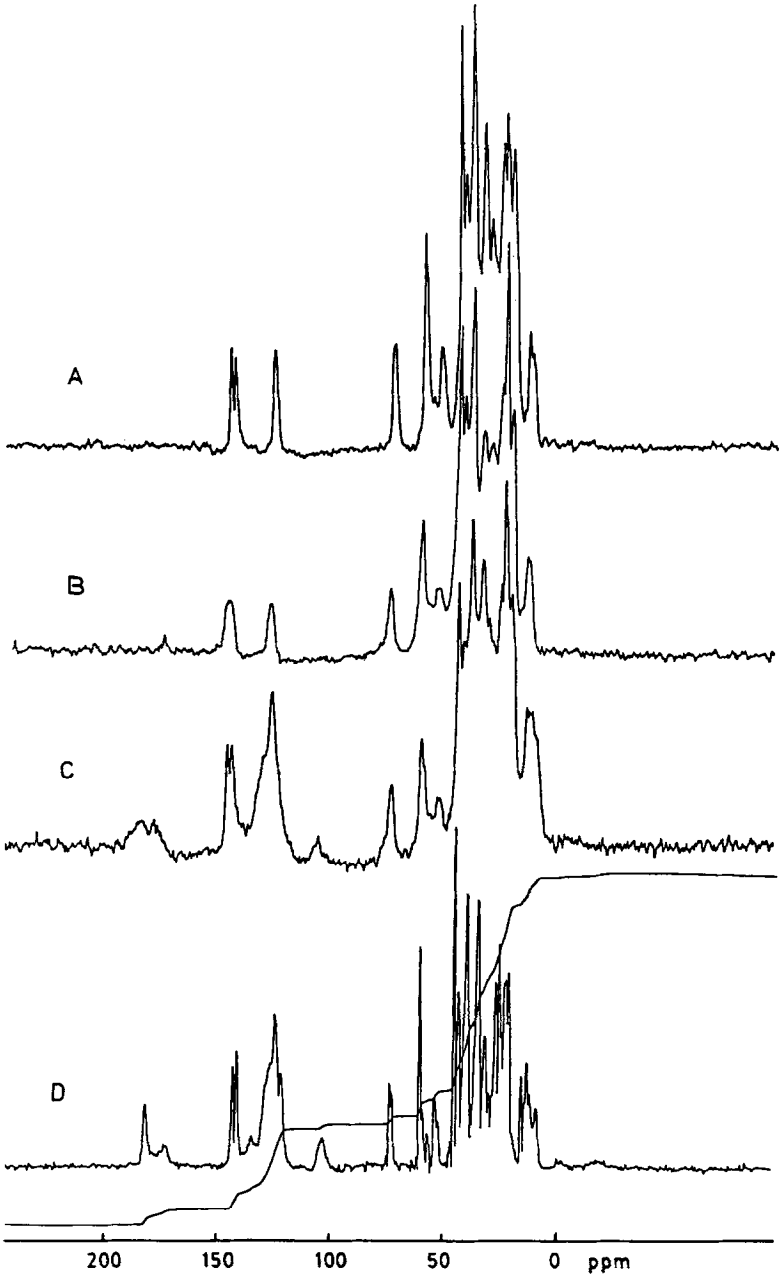
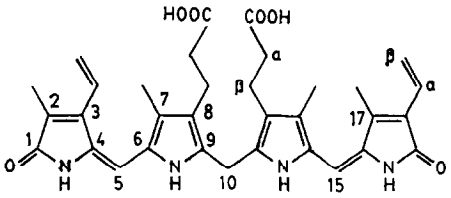


FIGURE 1. Three examples of ^{13}C solid state NMR spectra of the cholesterol or mixed type gallstones (A), (B) and (C) compared to that of the equal weight cholesterol/bilirubin reference spectrum (D). Note the evidence of varying amounts of bilirubin structure as shown by pyrrole ring carbons in the 120-140 ppm ranges.

C. A 50:50 mixture of cholesterol and bilirubin gave spectrum D which is remarkably similar to spectrum C once allowance is made for spectral broadening through either polymerization or paramagnetic metal present in stone C. The carboxyl region (170 - 185 ppm) and the unsaturated bridgehead

peak at 102 ppm provide the most distinctive spectral features for documenting the presence of bilirubin structures in the mixed gallstones, although the pronounced spectral broadening in the aromatic region around 130 ppm and subtle changes in the aliphatic region of the spectrum also reveal the presence of bilirubin structural moieties. It is known that different ^{13}C NMR pulse programs using different contact periods and recycle times will generate different intensity responses in the carbon-13 peak by preferentially suppressing carbons of a particular bond type. Furthermore, polarization rates vary in different molecules, and thus the pulse program used to analyze the gallstone materials might preferentially enhance the spectral intensity of one of the compounds. A mixed cholesterol/bilirubin sample was prepared and subjected to the same spectral procedures as was used on the mixed and cholesterol stone materials (Figure 1, Spectrum D). It was observed that there is a preferential spectral response for cholesterol depending on which peaks are chosen for the comparison. Equal weights of cholesterol and bilirubin give a mole ratio of 1.51 cholesterol-to-bilirubin, yet using the peak produced by the unsaturated bridgehead carbons (5 and 15) of bilirubin (I)



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found at 102 ppm and the peak produced by the ring hydroxy carbon of cholesterol found at 71 ppm, a mole ratio of 2.91 cholesterol-to-bilirubin is found from the integration. Moreover, if one compares the carbonyl and carboxyl carbon peaks of bilirubin found near 180 ppm with the cholesterol peak at 71 ppm, a mole ratio of 2.30 cholesterol-to-bilirubin is obtained. Other comparisons produce similar results. Varying the cycle time of the pulse program has little effect on the more favorable response of the cholesterol peaks.

Other possible means of investigating the structure of gallstone materials arise from the fact that chemically different carbon atoms respond differently to a given pulse sequence. In Figure 2, three different pulse sequences are used to differentiate among the carbon atoms of bilirubin. Spectrum C of Figure 2 is produced by employing the normal CP/MAS pulse sequence which uses a proton-carbon contact time of 3 milliseconds under proton spin-locked Hartmann-Hahn conditions (14). This spectrum contains lines representative of all carbon atoms in the structure. The contact time of 3 milliseconds is considered optimally long enough to polarize all carbon atoms having protons within four bonds. It is possible then to discriminate against those carbon atoms which have no directly bonded protons by using very short contact times. Spectrum A of Figure 2 is that of the bilirubin reference run under pulse conditions employing a short contact time of only 32 microseconds. Notice the absence of all pyrrole ring carbon as well as carboxyl and carbonyl carbon peaks. It is also possible to use a pulse sequence having quite the opposite effect as is illustrated in spectrum B of Figure 2. In this spectrum, peaks from carbon atoms with directly bonded protons are suppressed. The absences of all methylene, vinyl and olefin bridgehead carbon peaks is to be noted here. This is accomplished by using a small delay period after the step involving the proton-carbon cross polarization and before the step involving the acquisition of the free induction decay. This 40 microsecond delay allows just enough time for dephasing of carbon atoms which are directly bonded to protons via strong dipolar interactions (15). Thus, those carbon atoms directly attached to one or two protons do not produce peaks in such spectra. Spectra resulting from the short contact time and spectra from the use of the dipolar dephasing delay are not mutually exclusive, however. This is because the methyl group carbons will always produce lines in any of these experiments. The freely rotating methyl groups move fast enough even in solids to suppress the strong dipolar carbon-proton interactions needed for dephasng during the delay-period of the dipolar dephasing pulse sequence (15). This feature is useful since it enables us to

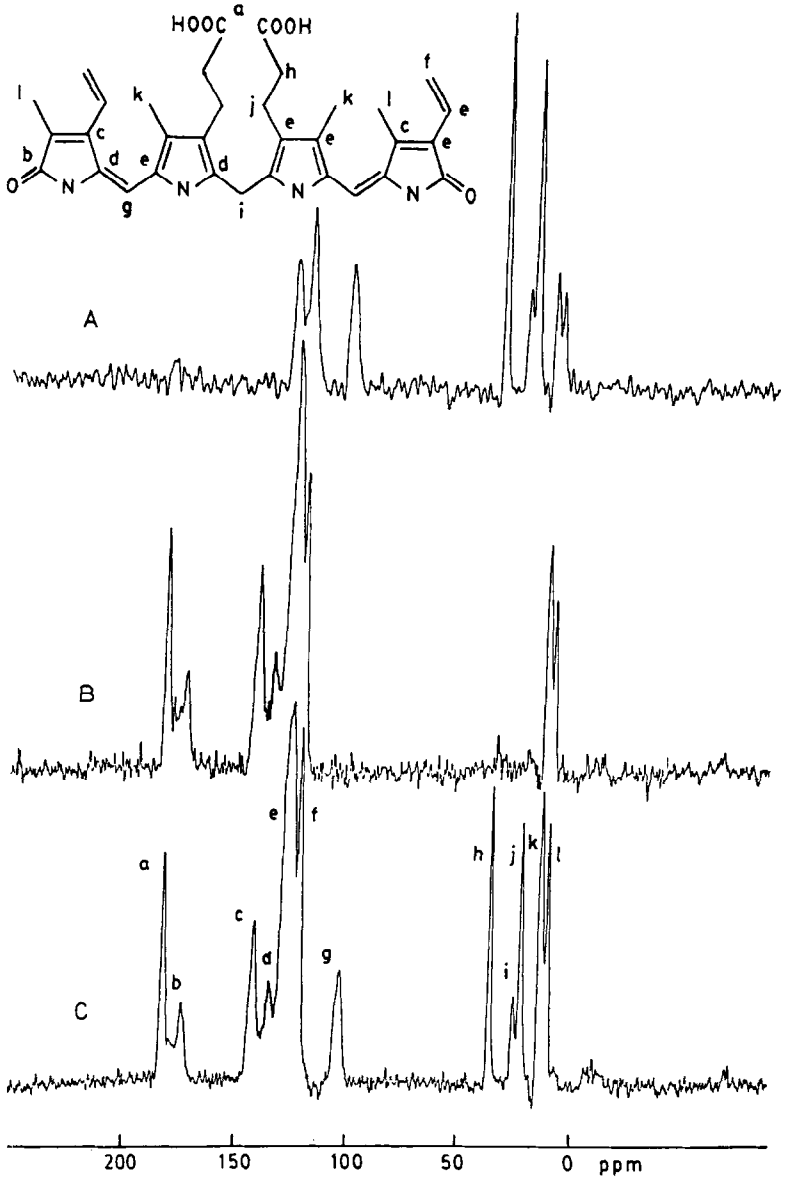

FIGURE 2. Three ^{13}C solid state spectra of the bilirubin reference compound taken with different pulse programs. (A) spectrum resulting from a short proton-carbon contact time of 32 μsec, (B) spectrum resulting from the use of a 40 μsec delay before signal acquisition, and (C) the normal CP/MAS spectrum with 3 msec proton-carbon contact time.

identify methyl carbon peaks. The assignments of the carbon atoms in the solid ^{13}C NMR spectrum of bilirubin have been made with the help of such pulse sequences and the closely related liquid ^{13}C NMR spectrum (16).

The benefits of using pulse programs in the study of pigment structures lie in the ability to resolve line widths observed in solid ^{13}C NMR which

are much larger than found in liquid ^{13}C NMR. Many of the solid peaks thus overlap in the normal CP/MAS spectrum obscuring information. The two bilirubinoid vinyl peaks, for example, found at 120 and 128 ppm (Figure 2A) are obscured by the pyrrole ring carbon peaks in the same chemical shift region in the normal spectrum of the compound (Figure 2C).

Figure 3 presents the spectra obtained from a solid black residue of extracted black pigment stone subjected to the three pulse sequences as in Figure 2. The black pigment stone was extracted (soxhlet) with a chloroform/methanol mixture (2:1, v/v) until the extract was colorless and most of the soluble components removed. As in Figure 2A, the broad peak at 120-130 ppm of Figure 3A corresponds to the four vinyl carbons of the bilirubin moiety. In comparing the relative area of this peak with that of the peak from the two bridgehead olefin carbons numbers 5 and 15 (I) at 102 ppm, one concludes that little vinyl polymerization is occurring since the areas of these two peaks should be at least 2:1 if both structural groups were to remain unaffected during the black pigment formation process.

In spectrum C of Figure 3, the two methyl group peaks of the bilirubin structure are now one broader peak at 10 ppm. This is indicative of a conformational change of the bilirubin itself. The liquid ^{13}C NMR spectrum of bilirubin (16) shows the four methyl resonances to all be very near to 9 ppm (one at 9.38, one at 9.16 and two at 9.04 ppm). The reason why two of the methyl resonances are at 6.7 ppm and two are at 9.7 ppm in the solid spectrum (Figure 2) is explained by the crystal structure of bilirubin (17). The two upfield peaks (6.6 ppm) are from the exomethyls which find themselves adjacent to nonrotating, conformationally fixed vinyl groups at the ends of a V-shaped molecule sandwiched between others of the same intrahydrogen bonded species. The polymeric bilirubinoid structure in the black pigment would undoubtedly show these methyl shifts no matter what the polymer bonds are. The five peaks in the aliphatic region of the bilirubin reference spectrum (Figure 2) are at 7, 10, 18, 21 and 32 ppm. Spectrum C of Figure 3 also exhibits two new rather large peaks at 39 ppm and 23 ppm indicated by the asterisks. These two peaks,

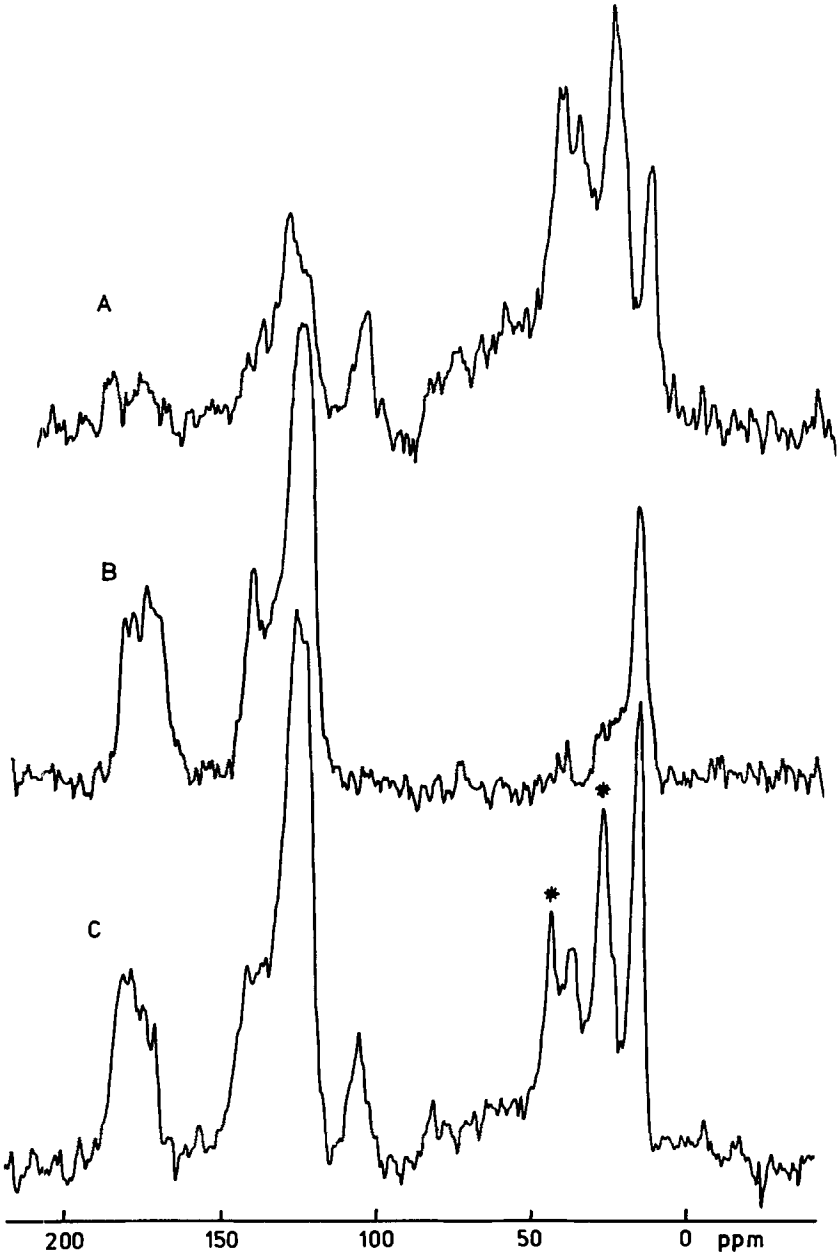

FIGURE 3. Three ^{13}C solid state spectra of an extracted black pigment gallstone residue using the same pulse programs as for the bilirubin reference (Figure 2). The two asterisks in spectrum (C) denote peaks of the aliphatic region not found in the spectrum of the reference.

taken together with the fact that the carboxyl peak at 180 ppm is now quite broad and undefined, lend evidence toward polymer bond formation through the propionic acid group of the endopyrrole rings. Spectrum B of Figure 3 shows that these peaks are most probably methylene or methine in nature. It is

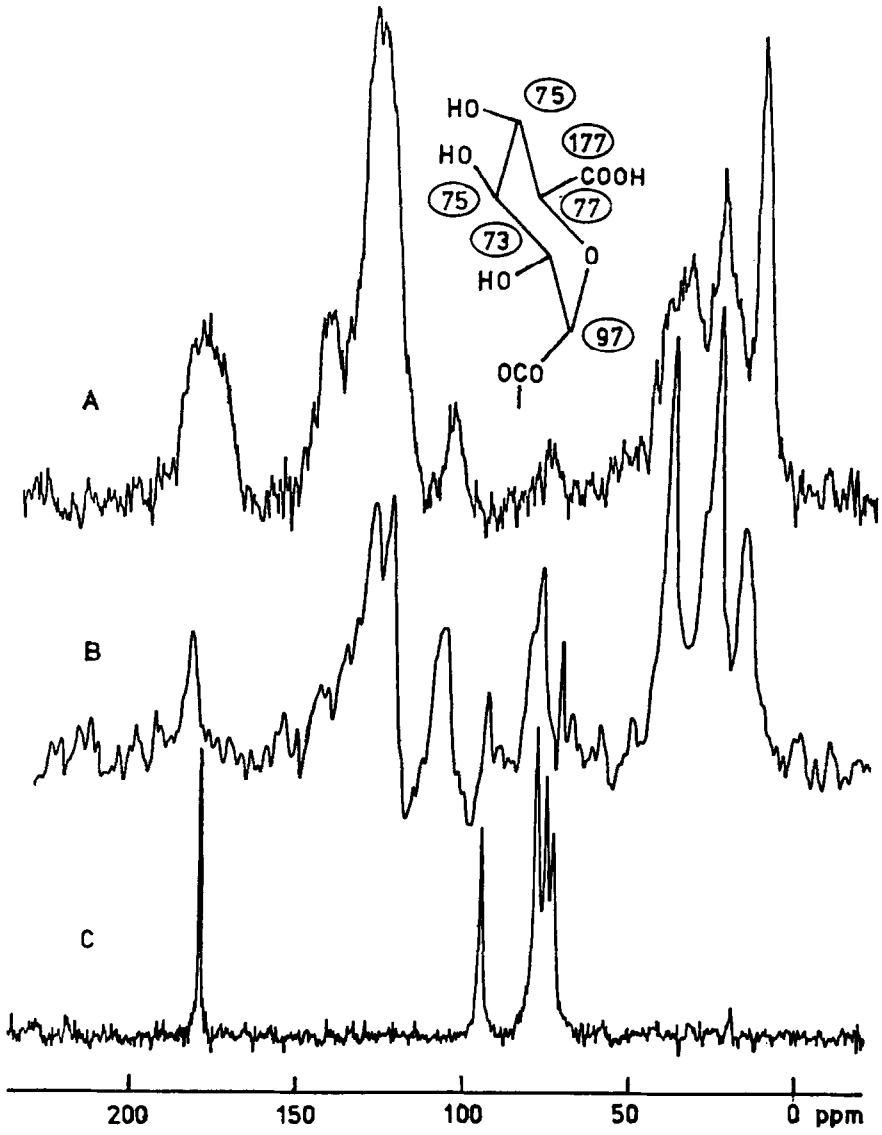

FIGURE 4. ^{13}C spectrum of extracted pigment gallstone residue, (B) compared to that of original unextracted gallstone (A). Peaks in 95-60 ppm range have been interpreted as evidence for conjugation with sugars such as glucuronic acid by esterification of bilirubinoid carboxyl group. ^{13}C solid state spectrum of sodium glucuronate (C).

presently thought that these two peaks arise from the α and β carbons of the proprionate side chain being shifted down field due to the new bond formation of the acid functional group, the α-carbon being affected more than the β as expected.

Figure 4 shows the spectrum (A) of a powdered black pigment gallstone and the solid residue (spectrum B) after 24 hours of extraction with a mixture of

$CH_3OH/CHCl_3/HCl$ (20 parts CH_3OH to 40 parts $CHCl_3$ to 1 part con. HCl v/v at a temperature of 23° C). Spectrum B of Figure 4 shows evidence that acidic chloroform/methanol extractions of black pigment stones change the basic bilirubinoid structure of the black pigment. Note the attenuation of the peak at 140 ppm assigned to the pyrrole ring carbons (number 3 and 17) of the bilirubinoid molecule (I). Also, the peaks near 180 ppm assigned to the carboxyl and carbonyl carbons have drastically changed. Both these changes can be ascribed to a reduction of the carbonyl carbons. Also, the spectrum of the extracted residue (Figure 4B) showed several peaks that were enhanced. The most striking enhancement which gives the evidence of conjugation, are the three peaks at 90, 74 and 66 ppm of Figure 4B. If the extracted residue is the polymer itself, it would seem to be more strongly conjugated than the unpolymerized material which was washed away. For as Figure 4 illustrates, the peaks found between 71 and 78 ppm are those associated with the backbone carbons of sugar molecules $(-CHOH-)_n$ which appear to account for the enhanced peaks in the spectrum of the solid extacted residue. Spectrum C of Figure 4 is that of sodium glucuronate and the structural assignments made at the top of the figure are for glucuronic acid. Unconjugated bilirubin has been suspected of being the primary bilirubinoid in the precipitation of pigment gallstones since the unconjugated structure is much less soluble in bile than the conjugated species (18). The ^{13}C-NMR analysis of the solid black pigment residue indicated that conjugated bilirubinoids are important constituents in this type of pigment stone also; especially in the insoluble black residue of extracted stone.

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