Lipids of the Major Histological Components of Rice Studied by ^{13}C Nuclear Magnetic Resonance Spectroscopy[1]

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ABSTRACT

^{13}C Nuclear magnetic resonance (NMR) spectroscopy of rice and its histological components allowed the determination of 1) the amount of lipid in each component (22% in endosperm, 56% in aleurone cells plus grain coat, and 22% in embryo), 2) the amount of aleurone cells plus grain coat plus embryo (9.5%) in brown rice, 3) the ratio of saturated to unsaturated fatty acids in the embryo, endosperm, and aleurone cells plus grain coat (7.5, 6.1, and 5.4, respectively), and 4) the fact that enzyme lipase is located in the aleurone cells plus grain coat. The amount of lipid in brown rice is 3.6%, in endosperm 0.87%, in aleurone cells plus grain coat 31%, and in embryo 31.7%. The ^{13}C NMR spectrum of lipid extracts of brown rice studied in solution was much sharper than that obtained from the solid material and allowed full evaluation of the lipid composition. The NMR results agreed with those obtained by gas chromatography and averaged, for triglyceride esters: 16:0, 25.9%; 18:1, 39.2%; and 18:2, 34.9%. At 67.89 MHz, small ^{13}C NMR resonances were observed and were assigned to phosphatidyl choline, phosphatidyl ethanolamine, lysophosphatidyl choline, and lysophosphatidyl ethanolamine.

The lipids in rice occur mainly in the spherosomes (Bradbury et al 1980b) which are largely located in the aleurone cells and the embryo. Lipids also occur in the membranes surrounding organelles and in association with protein bodies (Little and Dawson 1960, Mitsuda et al 1967) and starch granules (Mano and Fujino 1975). The lipid in the spherosomes has sufficient mobility to produce a high-resolution ^{13}C nuclear magnetic resonance (NMR) spectrum, as observed previously for soybean, corn, and other seeds by Shoolery (1973) and Schaefer and Stejskal (1974, 1975), but the lipids located in cell membranes and the other solid material (carbohydrate and protein) have insufficient mobility to give rise to a high-resolution spectrum. Thus the ^{13}C NMR spectrum arises only from the mobile lipid present in the spherosomes.

Because of the recent development of methods for the clean separation of the histological components of rice (Bradbury et al 1980a, 1980b), study of the lipid content and composition of these separate components using ^{13}C NMR techniques was considered useful. This work was also extended to a study of lipid extracts in solution, because the much sharper spectra obtained in this way allowed detailed analysis of the lipid composition.

MATERIALS AND METHODS

Materials

The rice (*Oryza sativa* L.) samples, obtained from B. O. Juliano of the International Rice Research Institute (IRRI), consisted of the IRRI variety IR32 and the high protein selection IR480-5-9. Chemicals and solvents were reagent grade and were used without further purification, unless specifically stated otherwise.

Preparation of Histological Components

The isolation of aleurone cells plus grain coat followed the first method described by Bradbury et al (1980b), in which cut grains were softened in water for three days and the starchy endosperm subsequently removed by scraping with a scalpel, except that the aleurone cells plus grain coat component was not washed in a graded alcohol-water series. This method was also used to obtain preparations of starchy endosperm. The embryo was separated from the whole grain by hand-dissection with a needle. The brown rice and starchy endosperm samples were finely crushed to pass through a 36-mesh (420-μm) sieve.

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Extraction of Lipids

Two different but related methods were used.

Method 1. Brown rice (IR480-5-9) or its histological components were crushed and passed through a 36-mesh (420-μm) sieve. A 5-g sample was shaken with 12 ml of chloroform:methanol (2:1, v/v). The mixture was filtered and the residue extracted five more times with 12-ml portions of the chloroform-methanol solution. Fifteen milliliters of 0.2*M* $MgCl_2$ solution was added to the combined lipid extract, and the mixture was allowed to separate for 24 hr. The lower phase, containing lipid, was evaporated to dryness in vacuo (Lee et al 1965).

Method 2. Fifty grams of brown rice (IR480-5-9) was passed through a Waring Blendor, and the powder was stirred in 1,000 ml of chloroform:methanol (2:1, v/v) for 8 hr. After separation, the residue was extracted for 1 hr with 400 ml of butanol saturated with water. The extracts were combined and evaporated to dryness in vacuo. The residue was dissolved in a small volume of chloroform-methanol; one fifth of its volume of water was added, and the mixture was shaken and allowed to separate overnight. The upper phase was removed and the lower layer washed twice with water equilibrated with chloroform:methanol (2:1, v/v). The chloroform-methanol fractions were combined; a little methanol was added to make one phase; and the solution was evaporated to dryness in vacuo at 40°C (Folch et al 1957).

Transesterification

Lipid (0.082 g) was placed in a 50-ml round-bottomed flask; 5.6 ml of benzene (dried over sodium wire) and 0.4 ml of 2,2-dimethoxypropane (redistilled, bp 76-78°C) were added. A methanolic HCl solution was prepared from dry methanol—dried over $Mg(OMe)_2$ and then distilled—through which was then bubbled dry HCl gas (dried by bubbling HCl through concentrated H_2SO_4). The amount of HCl in dry methanol (10%) was determined by titration with standard NaOH. The methanolic HCl (2 ml, 10%) was added to the solution of the lipid, and the mixture was allowed to stand at room temperature for 22 hr. The solution was neutralized with 0.08 g of anhydrous $NaHCO_3$:Na_2CO_3:Na_2SO_4 (2:1:2, w/w), and the supernatant liquid was injected into the gas chromatograph.

Gas Chromatography

A Packard 824 gas chromatograph with a flame ionization detector was used with a Carbowax 20M column at 200°C and helium carrier gas. The reference compound (Pufa-2) contained the methyl esters of the following carboxylic acids: 14:0 (myristic), 16:0 (palmitic), 18:1 (oleic), 18:2 (linoleic), 18:3 (linolenic), 20:3, 20:4, 20:5, 22:4, and 22:5.

^{13}C NMR Spectroscopy

Spectra were normally obtained at 20 MHz using a Varian

CFT-20 ^{13}C NMR spectrometer. A Brüker HX-270 NMR spectrometer operating at 67.89 MHz for ^{13}C was used to obtain increased sensitivity in several cases. The time between pulses was 1 sec for all solid samples and 4 sec for all solutions. Each solid rice sample was placed in a standard sample tube having a 10-mm diameter and fitted with a long capillary tube, which contained D_2O for locking and dioxan as a chemical shift reference (δ67.4) and which was inserted through the cap of the sample tube.

Because the intensity of the NMR resonance (area under the peak) is related to the number of carbon atoms giving rise to the resonance, the total amount of lipid in the solid sample can be measured. The area under the total ^{13}C NMR spectrum was measured by cutting out the peaks and weighing them. Comparisons between different samples run at different times and on different spectrometers was possible by using, as a reference standard, the height of the ^{13}C dioxan peak from the dioxan in the capillary tube. Differences in the density of packing of various solid samples were allowed for by measuring the weight of sample per centimeter of height of the NMR tube. The results were normalized, using the value obtained for the whole grain. Thus the areas under the total ^{13}C NMR spectrum were corrected for variations in the spectrometer and also for the density of packing of the solid material. These areas are proportional to the total amount of lipid in the rice components, providing that complete relaxation of the nuclei occurs between pulses (this requires a recycle time greater than $5T_1$) and that the nuclear Overhauser enhancements (NOE) of the nuclei used in the calculations are essentially the same. The T_1 (spin lattice relaxation time) values of the C_4–C_{13} carbon atoms of the palmitic acid chain in dipalmitoyl lecithin were found to be 0.6 sec (Levine et al 1972); hence, the recycle time of 4 sec for solution studies was sufficient. For solid samples, the T_1 values are much smaller, as shown by the increased breadth of the resonances (Figs. 1 and 2), and a recycle time of 1 sec was considered adequate (Schaefer and Stejskal 1974, 1975). The NOE values for the different ^{13}C nuclei in the lipids vary, but because we were only concerned with making a comparison between 1) similar spectra obtained from different solid histological components or 2) similar solution spectra, NOE differences were expected to be minimal.

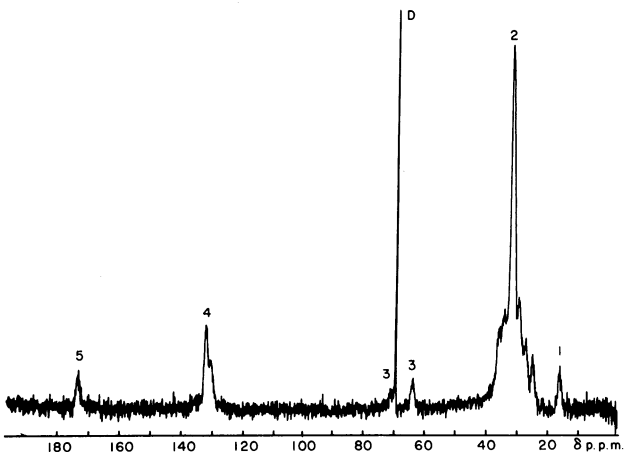


Fig. 1. ^{13}C Nuclear magnetic resonance spectrum at 20 MHz of a sample of embryo from IR32 rice (15,000 transients, recycle time 1 sec). **D** = dioxan external standard. Resonance regions are as follows: **1,** CH_3; **2,** CH_2s of fatty acid chains; **3,** CH_2, CH of glyceryl esters; **4,** olefinic C atoms of oleic and linoleic acids; **5,** carboxyl carbons from ester linkages.

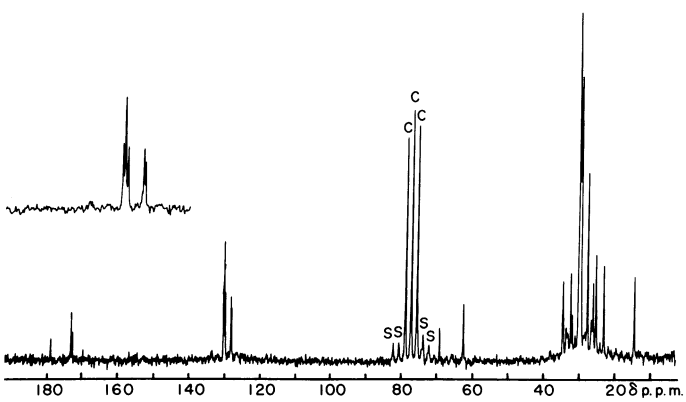


Fig. 2. ^{13}C Nuclear magnetic resonance spectrum at 20 MHz of lipid dissolved in $CDCl_3$ and extracted from brown rice (IR480-5-9) by chloroform:methanol, 2:1, v/v (16,000 transients, recycle time 4 sec). **C** = triplet resonance from the solvent $CDCl_3$; **S** = its sidebands. Inset is an expansion of the olefinic region at δ128–130.

RESULTS AND DISCUSSION

NMR Spectra with Solid Samples

Figure 1 shows a typical ^{13}C NMR spectrum obtained from the embryo. The sums of the areas of peaks 1–5 for the various histological components (after normalization for variations in spectrometer performance using the dioxan standard and for variations in the packing density of the solid material) were compared with the brown rice (IR32) taken as 1.0. Results were as follows for brown rice: whole grain area (A_{WG}) = 1.0, endosperm area (A_{endo}) = 0.228, aleurone cells plus grain coat area (A_{al}) = 8.39, embryo area = 8.42. Because the lipid content is proportional to these areas, the lipid content of the aleurone cells plus grain coat fraction is the same, within experimental error, as that of the embryo, which is much greater than that of the endosperm. This allows a simple check of the amount of these bran components in brown rice by using the equation

$$A_{WG} = x\ A_{al} + (1-x)A_{endo},$$

where x is the fraction of the grain consisting of aleurone cells plus grain coat and embryo (the bran fraction). Substitution in the equation gives x = 0.095, which agrees reasonably well with the midpoint of the range of proportions cited by Juliano (1972)—endosperm = 0.91, aleurone plus grain coat = 0.065, and embryo = 0.025; hence x = 0.090.

Using these values for the proportions of the histological components and the relative amounts of lipid in each component (the area measurements given above), we calculated the percentage of lipid in each component: endosperm, 22%; aleurone cells plus grain coat, 56%; and embryo, 22%. Juliano's values (1972) are ~20, ~54, and ~26%, respectively. The agreement between our results, obtained on clean histological components using NMR, and those obtained on milling fractions by chemical methods is reasonable.

Separate resolution of the olefinic resonances from oleic acid and linoleic acid (Fig. 1, peak 4) is not possible because of the line broadening of the resonances, due to the slowed movement of the ester molecules within the spherosomes (Bradbury et al 1980b) of the embryo tissue. These resonances are not resolved in the solid state at 67.89 MHz but are readily separated in spectra recorded in solution, where considerable line sharpening of all resonances occurs (Fig. 2), as previously observed in related systems (Schaefer

TABLE I
Ratios of Saturated to Unsaturated Carbon Atoms in Lipids of Rice Components

Histological Component	Ratio By ^{13}C NMR[a]	Ratio Calculated[b]
Whole grain	6.05	...
Endosperm	6.1	6.24,[c] 5.74[d]
Aleurone cells plus grain coat	5.4	5.9
Embryo	7.5	5.9

[a] Nuclear magnetic resonance; rice sample IR32.
[b] From Resurreccion and Juliano (1975); rice sample IR20.
[c] Petroleum ether extraction.
[d] Chloroform-methanol (2:1, v/v) extraction.

and Stejskal 1974). However, we calculated the ratio of the areas of peaks 1 and 2 (saturated C atoms) to peak 4 (unsaturated C atoms) for each of the histological components (Table I). The result obtained for endosperm agrees reasonably well with the values calculated from the data of Resurreccion and Juliano (1975). In making this comparison, we note that considerable variation has been found between the amounts of oleic and linoleic acid extracted using different extraction procedures (Lee et al 1965, Lugay and Juliano 1964, Resurreccion and Juliano 1975) and that esters are lost if the transesterification reaction is conducted under reflux (Mason and Waller 1964). On the other hand, the ^{13}C NMR technique will only observe mobile lipid; hence, lipoproteins and lipids (phospholipids, glycolipids, etc.) immobilized in cell membranes would not be observed.

A point of interest not previously observed is that the lipid of aleurone cells plus grain coat is richer in unsaturated fatty acids than is that of the endosperm, which is richer than that of the embryo. The biological significance of this result is not clear. A sample of separated aleurone cells plus grain coat was allowed to stand at room temperature for 120 days before the lipid content was measured by ^{13}C NMR. The lipid signal was only 36% of its original intensity, presumably due to degradation of the lipid by the enzyme lipase (Funatsu et al 1971) and/or possibly by oxidation. This experiment indicates that lipase is probably located in the aleurone cells plus grain coat of rice.

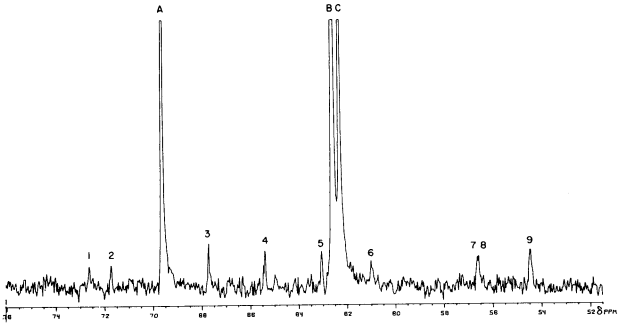
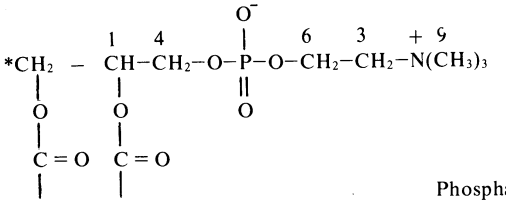
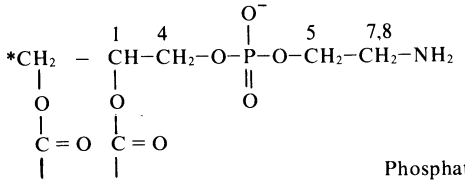


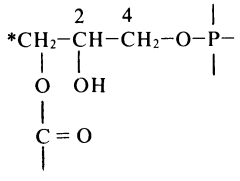
Fig. 3. Partial ^{13}C nuclear magnetic resonance spectrum at 67.89 MHz of the lipid, dissolved in $CDCl_3$-CD_3OD (2:1, v/v) and extracted by $CHCl_3$-CH_3OH (2:1, v/v) followed by water-saturated butanol, from IR480-5-9 brown rice. Recycle time 1 sec, 4,000 transients, chemical shifts referenced to CD_3OD. Resonances **B** and **C** are from the $\underline{C}H_2O$ carbon atoms of glyceryl esters and **A** from the corresponding $\underline{C}HO$ carbon atom. Assignments of resonances 1–9 are based on chemical shifts obtained by Birdsall et al (1972) and from the approximate expected composition of lipids.



Phosphatidyl choline (PC)



Phosphatidyl ethanolamine (PE)



Lyso PC or lyso PE

C atoms marked with an asterisk probably give resonances obscured under resonances **B** and **C**. The lack of ^{13}C-^{31}P coupling of resonances 1 and 2, 4 and 3 (and the small coupling of 7 and 8) is unexpected in view of the results of Birdsall et al (1972).

NMR and Gas Chromatography of Extracted Lipids

The NMR spectrum in Fig. 2 shows the same five spectral regions as in Fig. 1 but with much increased clarity, due to the freedom of motion of the lipids dissolved in $CDCl_3$. The three resonances at low field are assigned to the carboxyl carbons from esters as follows: δ178.9, oleic; δ173.3, palmitic; and δ172.9, linoleic acid. The olefinic region of the spectrum shows a doublet that contains two carbon atoms of linoleic acid and a triplet consisting of a pair of overlapping doublets that arise from the other two olefinic carbon atoms of linoleic acid and the two olefinic carbon atoms of oleic acid. The resonance at δ69.0 is due to $\underline{C}HO$ and that at δ62.1 is a singlet in $CDCl_3$ but a doublet in a mixed solvent (Fig. 3) and is due to the $\underline{C}H_2O$ of the glyceryl esters. The large number of resonances at high field have been analyzed by Schaefer and Stejskal (1974). The largest peak (a closely spaced triplet at δ29.7, 29.6, and 29.1) contains 10, 8, and 5 C-atoms from palmitic, oleic, and linoleic acids, respectively. The adjacent resonance at δ27.2 contains two carbon atoms from each of oleic and linoleic acids.

From the areas under the peaks in the olefinic region, the relative amounts of oleic to linoleic acid can be obtained and, similarly, from the areas under the peaks in the high field region, the ratio of palmitic acid to oleic or linoleic acid is obtained. Thus the amounts of each of these lipids can be evaluated for the extracted triglyceride (Table II). The amount of each lipid was also obtained by gas chromatography of the methyl esters, and the results agree, within the experimental error of the techniques. The small differences between our results and those of Choudhury and Juliano (1980), given in Table II, may arise from differences in extraction procedures (they used butanol saturated with water after the chloroform-methanol procedure) and in the transesterification procedure.

TABLE II
Composition of Lipids Extracted from Brown Rice (IR480-5-9)

	Total Lipids		
Methods	**16:0**	**18:1**	**18:2**
^{13}C NMR[a]	24.3	39.8	35.9
Gas chromatography on methyl esters[b]	27.5	38.6	33.9
Mean	25.9	39.2	34.9
Choudhury and Juliano (1980)[c]	23–24	32–37	36–40

[a] Shown in Fig. 2.
[b] Traces of methyl esters of linolenic acid (18:3) and possibly of myristic acid (14:0) were also found.
[c] Ranges of composition for three samples of rice, including IR480-5-9, which all gave similar results.

TABLE III
Lipid Contents and Proportions of Histological Components of Brown Rice

		Component		
	Rice	**Endosperm**	**Aleurone Cells plus Grain Coat**	**Embryo**
Lipid content				
Percent of total lipid	100	22	56	22
Percent of component	3.6[a]	0.87[a]	31.0	31.7[a]
Percent of rice by weight[b]	100	91	6.5	2.5

[a] Compared with 3.5–4, 0.98, and 34–36, respectively (Choudhury and Juliano 1980).
[b] From Juliano (1972).

Extraction of 50 g of IR480-5-9 using method 2 yielded 3.6% lipid. Using this result, the amounts of aleurone cells plus grain coat, embryo, and endosperm in brown rice, and the amounts of lipid in each of these components, we calculated the percent lipid in each component. All results, summarized in Table III, show the high concentration of lipid in the bran components compared to that in the endosperm. A ^{13}C NMR spectrum of the lipid extract at 20 MHz showed no evidence of resonances from phospholipids, but a spectrum run at 67.89 MHz gave clear evidence of such resonances (Fig. 3). From the relative intensities of the small phospholipid resonances, compared to those of the large resonances (A, B, and C in Fig. 3), it is calculated that the amount of phospholipid is about 10% of the total lipid (Choudhury and Juliano 1980).

^{13}C NMR spectroscopy is very useful for the determination of the total amount of lipids in the histological components of rice, and examination of lipid extracts by ^{13}C NMR allowed elucidation of the detailed composition of the lipids in rice.

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