Studies of the Phospholipids, Glycolipids and Sterols of Wheat Endosperm,[1,2]

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Abstract

The phospholipid-glycolipid mixture, isolated chromatographically from the "free" (hexane soluble) and "bound" (hexane insoluble, water-saturated butanol extractable) lipid of wheat endosperm, was fractionated by column and thin-layer silicic acid chromatography. The components were identified by hydrolysis followed by thin-layer or paper chromatography of the products. They included a sterol-containing glycolipid, hitherto unreported in wheat. The fatty acid and sterol compositions of the phospholipid-glycolipid components were determined by gas-liquid chromatography. Differences were found between varieties and between components.

Introduction

THE IMPORTANCE of lipids in flour has long been recognized and studies of the composition of wheat lipids have received increasing attention (1). Lipid classes have been separated by chromatography (2,3) and by solvent fractionation (4). The phospholipid fraction has been studied by counter current extraction (5) and by chromatography (6) and Carter has made an extensive study of glycolipids (7). Sitosterols and other plant sterols (8) as well as sitosterol glucoside (7) have been reported. A study of wheat endosperm lipid classes (2) was made in this laboratory and the complexity revealed in this earlier study showed the need for a more detailed investigation of the phospholipid-glycolipid mixture. This paper presents the results of this investigation.

Materials and Methods

Three wheat varieties, Thatcher, Lemhi and Mindum, representing Hard Red Spring, Soft White Spring and Amber Durum respectively were used in this investigation. The embryos were removed and the degermed kernels milled to 65–70% extraction in a laboratory micro mill (2). The free lipids were extracted from the flour with hexane and the bound lipids released by a further extraction with water-saturated butanol (9). Solvent was removed in a rotary evaporator at temp below 30C and an atmosphere of nitrogen was maintained over the lipid at all times.

Silicic Acid Chromatography. Preparative separations were made by a combination of column and thin-layer chromatography (TLC). Approx 200 mg free lipid or 150 mg bound lipid, representing 24 g flour or 30 g wheat, were fractionated on a 2.0 cm. O.D. column containing 20 g 325 mesh silicic acid (Bio-Rad Laboratories, 32nd & Griffin, Richmond, Calif). Sterol esters were eluted with hexane, triglycerides with 5% diethyl ether in hexane and the free sterols with chloroform. The phospholipid-glycolipid mixture was then separated by a five step increase in the concn of methanol in chloroform 2% (100 ml), 5% (75 ml), 10% (75 ml), 50% (75 ml) followed by methanol (75 ml). The individual components of the fractions were further separated on a preparative scale by means of TLC; 20 x 20 cm plates coated with 0.25 mm layers Silica Gel G (Merck, Darmstadt). Four solvent systems were used: a) hexane-diethyl ether-acetic acid, 90:10:1 (10); b) diisobutyl ketone-acetic acid-water, 40:25:5 (11) c) chloroform-methanol 95:5 and d)chloroform-methanol-water, 65:25:1 (12). System "a" was used to separate the lipid classes and "b" for most of the phospholipid glycolipid components. In the latter separation "b" was supplemented by "c" for the high Rf components and "d" to separate phosphatidyl choline of bound lipid from an unknown component. Iodine vapor (13) was used as a general detecting agent. Specific spray reagents, ninhydrin (14) for the amino group, Dragendorff reagent (15) to detect choline containing lipids and molybdate (16) to detect phospholipids, were also used. Different types of lipids reacted with characteristic color when sprayed with 20% aqueous perchloric acid (17). Less than 1 μg lipid/spot was detected by iodine vapor. However, because of limitations of sensitivity of some sprays and differences in the proportion of components, it was necessary to apply 100 μg mixture at the origin. In preparative separations a guide strip was exposed to iodine vapor while the main portion of the plate was protected with Saran® wrap (18). Lipid corresponding to the guide spots was removed from the plate and the guide strip sprayed with specific reagents for further identification. Separations for identification were made by means of two-dimensional TLC (17) using system "d" in the x-direction, followed by system "b" in the y-direction.

Hydrolysis. Mild alkaline hydrolysis was performed in toluene-pyridine, 10:1, using methanolic potassium hydroxide in a modified Dawson procedure (19). Acid hydrolysis was accomplished by refluxing the lipid overnight in a 5% solution of anhydrous hydrogen chloride in absolute methanol. The water-soluble products of mild alkaline hydrolysis were chromatographed on paper using water-saturated phenol as developing agent. The sugar-containing spots were made visible by means of a periodate-benzidine spray (20) and their identity determined by comparison with known Rf values.

Gas-Liquid Chromatography. Fatty acid methyl esters were prepared by heating with hydrogen chloride in absolute methanol (2) and purified by TLC. They were then analyzed on a 4 ft x $\frac{3}{16}$ in. column packed with 10% polyvinyl acetate (w/w) (Union Carbide, type AYAC 8285) on 60–80 mesh "Gas Chrom P" (Applied Science Laboratories, State College, Pa.). These separations were effected on a Research Specialties Series 600 gas chromatograph, operating with a beta ionization detector, a column temp of 185C and an argon flow of 45 ml/min.

The sterols were converted to the trimethyl silyl

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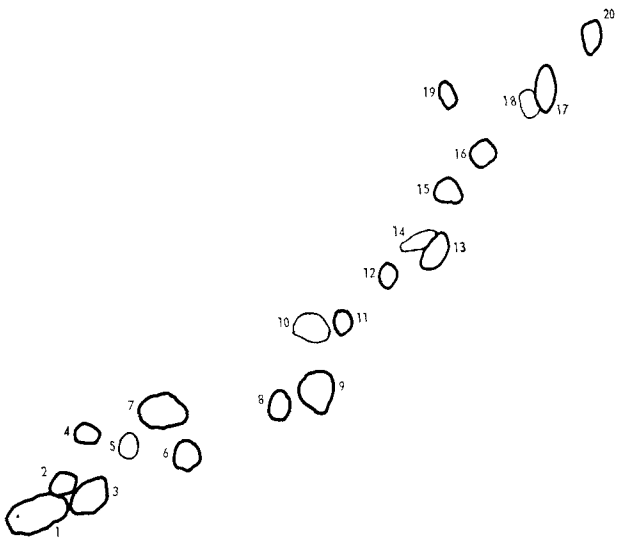

FIG. 1. Two-dimensional thin-layer chromatoplate of endosperm lipid of Thatcher wheat.

1,2,3. Complex mixture including lysophosphatidyl choline
4. Unknown
5. Unknown
6. Phosphatidyl inositol
7. Phosphatidyl choline
8. Unknown
9. Di-galactosyl glyceride
10. Phosphatidyl ethanolamine
11. Unknown
12. Sterol glycoside
13. Mono-galactosyl glyceride
14. Unknown
15. Esterified sterol glycoside
16. Phosphatidic acid
17. Triglyceride
18. Free sterols
19. Unknown
20. Sterol esters

ethers (21) and separated on a 2 ft x $\frac{3}{16}$ in. column containing 3% SE 30 (General Electric, Silicone Products Dept., Waterford, N. Y.) on 80–100 mesh "Gas Chrom P. For sterol derivative separations the Series 600 gas chromatograph was operated with a flame ionization detector, column temp of 218C and nitrogen flow of 83 ml/min. A hydrogen pressure of 7.5 lb/sq in. and air pressure of 12 lb/sq in. proved suitable for the operation of the detector.

Test mixture D distributed by the National Institutes of Health was used as a reference standard for the quantitative determination of the methyl esters, and for the sterol analysis the reference was a derivative mixture prepared from sterols of known identity. Reference mixtures were used daily and no analyses were carried out until the reference mixtures could be analyzed to within 1 unit per cent.

Experimental and Results

Identification. The distribution pattern shown in Figure 1 is characteristic of that obtained with all the wheat endosperm lipid samples analyzed in the present study. Differences in degree rather than kind served to differentiate the samples on a lipid class basis, e.g. the triglycerides, spot 17, the largest component of the free lipid samples, was present in only small amt in the bound lipid. Similarly, free sterols (spot 18) were present in both free and bound lipid; but sterol esters (spot 20) were almost completely absent from bound lipid. The bound lipid samples, however, did contain material of low Rf not found in free lipid.

TABLE I

Sterol Composition[a]
Weight %

	β-Sitosterol[a]		
	Thatcher	Lemhi	Mindum
Sterol esters			
Free lipid	80.8	81.8	73.8
Bound lipid	77.3	73.9	76.0
Free sterols			
Free lipid	75.0	73.5	72.3
Bound lipid	79.5	76.4	75.9
Esterified sterol glycoside			
Free lipid	85.2	85.2	82.0
Bound lipid	84.4	82.6	76.4
Sterol glycoside			
Free lipid	84.9	77.4	80.1
Bound lipid	84.5	83.6	75.9

[a] Percentage of γ-sitosterol = 100 minus % of β-sitosterol.

Some of the components could be identified satisfactorily on the initial chromatoplate by means of their reaction with specific reagents. Other compounds required more elaborate treatment and these were prepared in mg quantity by fractionation on a silicic acid column followed by further separation on thin-layer plates. Through this procedure, low Rf spots and lysophosphatidyl choline in particular, were resolved in greater detail.

Lipid material, corresponding to spots 13 and 9, was separated in this way and subjected to mild alkaline hydrolysis. The water-soluble products were chromatographed on paper in phenol-water as the solvent system, using galactose as a marker and periodate-benzidine as spray reagent. These products were found to have the same Rf values as monogalactosyl glycerol and di-galactosyl glycerol respectively.

Component 15 proved to be of particular interest; this component, as well as 12, showed a characteristic sterol color when the plate was sprayed with aqueous perchloric acid. After milk alkaline hydrolysis of 15, a product chromatographically identical with 12, and, in addition, fatty acids were found. Acid hydrolysis of these chromatographically identical compounds liberated the same free sterols and sugar, probably glucose. Component 12 was therefore identified as a sterol glycoside and 15 as an esterified sterol glycoside, previously unreported in wheat lipids. This esterified sterol glycoside was chromatographically identical with that isolated from potatoes and characterized by Lepage (22), who has shown that the linkage between fatty acid and sterol glycoside is through the carbon-6 of the sugar.

Component 6, corresponding to phosphatidyl inositol in its Rf value, gave a color characteristic of sugar when sprayed with aqueous perchloric acid. Compo-

TABLE II

Fatty Acid Composition of Sterol Esters and Esterified Sterol Glycosides
Weight %

	14:0	16:0	16:1	18:0	18:1	18:2	18:3
Sterol esters							
Thatcher, free lipid	0.6	70.9	1.1	2.0	4.9	20.6	0
bound lipid	0	68.5	4.9	10.3	5.5	10.8	0
Lemhi, free lipid	0.6	75.6	0.5	0.7	2.8	19.8	0
bound lipid	0	76.5	2.8	2.3	7.5	10.9	0
Mindum, free lipid	1.8	42.9	1.0	1.8	12.2	40.3	0
bound lipid	0	46.3	13.2	2.9	8.2	29.4	0
Esterified sterol glycoside							
Thatcher, free lipid	0	31.7	0	0	5.9	61.3	1.1
bound lipid	0	50.2	1.1	0	8.2	40.5	0
Lemhi, free lipid	0	36.4	0	0	5.0	58.6	0
bound lipid	0	67.5	1.5	0.8	17.0	13.3	0
Mindum, free lipid	0	57.6	0.4	3.7	7.4	30.9	0
bound lipid	0	46.9	2.7	2.6	8.1	39.6	0

TABLE III
Fatty Acid Composition of Phospholipids and Glycolipids
Weight %

	15:1	16:0	16:1	18:0	18:1	18:2	18:3	20:1
Mono-galactosyl glyceride								
Thatcher, free lipid	2.5	23.0	0.7	1.6	15.0	57.2	0	0
bound lipid	0	8.3	0.4	0.8	50.9	29.5	7.9	2.2
Lemhi, free lipid	0	16.5	0	0	9.0	74.5	0	0
bound lipid	0	16.4	0.7	0.3	9.3	72.6	0.8	0
Mindum, free lipid	0	22.0	0	1.5	10.9	64.9	0.7	0
bound lipid	0	22.7	0	2.2	11.4	63.6	0	0
Di-galactosyl glyceride								
Thatcher, free lipid	0	13.4	0	0	8.5	76.3	1.8	0
bound lipid	0	16.0	0	0.5	8.3	75.2	0	0
Lemhi, free lipid	0	16.2	0	2.4	8.7	70.6	2.2	0
bound lipid	0	17.8	0.3	0.6	7.6	73.0	0.7	0
Mindum, free lipid	0	24.1	0	2.5	10.3	63.2	0	0
bound lipid	0	19.1	1.3	3.0	8.4	65.8	2.5	0
Phosphatidyl ethanolamine								
Thatcher, free lipid	0	26.1	1.0	0	9.7	63.3	0	0
bound lipid	3.0[a]	25.4	0	0	8.3	63.4	0	0
Lemhi, free lipid	0	28.1	0.4	0.8	7.6	61.9	1.2	0
bound lipid	4.3	31.2	1.3	0	8.2	55.0	0	0
Mindum, free lipid	0	27.7	0.9	1.9	11.0	58.6	0	0
bound lipid	0	22.8	0	0	14.0	63.2	0	0
Phosphatidyl choline								
Thatcher, free lipid	0	48.8	0	0	19.3	31.9	0	0
bound lipid	1.5	38.0	0	0	17.1	43.4	0	0
Lemhi, free lipid	1.6	46.5	0.9	0.6	14.8	35.6	0	0
bound lipid	0	20.4	0	0	21.7	55.7	2.2	0
Mindum, free lipid	0	28.2	1.4	3.0	16.7	50.7	0	0
bound lipid	0	25.9	0.6	2.3	16.1	52.9	2.3	0
Lysophosphatidyl choline								
Thatcher, free lipid	0	34.4	0.6	0.7	21.8	42.0	0.7	0
bound lipid	0	45.7	0	1.2	8.0	45.2	0	0
Lemhi, free lipid	0	63.4	0.7	3.6	5.3	27.1	0	0
bound lipid	0	79.2	1.2	3.6	10.0	5.9	0	0
Mindum, free lipid	0	65.0	1.2	5.8	5.4	22.6	0	0
bound lipid	0	47.0	1.3	2.4	6.8	42.5	0	0
Phosphatidyl inositol								
Thatcher, bound lipid	9.1[b]	28.4	0	0	10.9	51.7	0	0
Lemhi, bound lipid	0	9.0	0	0	44.8	35.9	10.3	0
Mindum, bound lipid	0	6.4	0.4	1.1	61.7	21.5	6.3	2.5

[a] Carbon number 15.2.
[b] Carbon number 15.3.

nent 16 corresponded in Rf value to phosphatidic acid and was shown to contain phosphorus.

Similar patterns were observed in all three wheat varieties. The main components of the phospholipid-glycolipid mixture were di-galactosyl glyceride, mono-galactosyl glyceride and phosphatidyl choline. The sterol-containing glycolipids were also present in appreciable quantities. A complex mixture of low Rf material was encountered in the bound lipid. This material contained some free amino acid and some phosphorus, but remained essentially unidentified.

Sterol-Containing Lipids. A comparative study was made of the composition of the sterol-containing glycolipids and the sterol esters and free sterols. Gas-liquid chromatography was used to determine the sterol composition, and, in the case of the esterified components, the fatty acid composition as well. The sterol composition, Table I, showed only minor variation between classes and between varieties. Beta sitosterol was present to the extent of more than 70%. The earlier appearing sterol had the same retention time as the previously reported γ-sitosterol (23). If any other sterols were present, they were in quantities too small to detect (less than 1%).

Differences in the fatty acid composition of both free and bound lipid were observed between Spring and Durum wheat, Table II. In the sterol esters of the Spring wheats, palmitic acid accounted for at least 70% of the fatty acids and linoleic for about 20%. In the Durum wheat the level of saturation was lower; the free lipids were characterized by less palmitic and more linoleic and oleic acids, and the bound lipid contained more palmitoleic as well. In the esterified sterol glycoside of Spring wheats there was much less palmitic and more linoleic acid especially in the free lipid. In the free lipid of Lemhi the ratio of saturated to unsaturated was 3.3 in the sterol esters and only 0.6 in the esterified sterol glycoside, while in Mindum the ratio was essentially one.

Fatty Acid Composition of Phospholipids and Glycolipids. The fatty acids of the phospholipids and glycolipids were mainly linoleic, palmitic and oleic acids, Table III. Differences in proportion were observed between components as well as between varieties. The most highly unsaturated components were the galactolipids in which a saturated/unsaturated ratio of 0.1–0.4 was found in both free and bound lipid of all varieties. An especially interesting feature was the difference in unsaturation of the fatty acids of phosphatidyl choline and lysophosphatidyl choline, those of the former being much more unsaturated, especially in free and bound lipid of Lemhi and Mindum. Nevertheless, the percentage of unsaturated fatty acid in the lysophosphatidyl choline was appreciable.

Discussion and Conclusion

In a study such as this, the possibility of artefacts must be considered and the presence of lysophospholipids raises the question of degradation. Considerable unsaturated fatty acid, however, was found in the lysophosphatidyl choline suggesting that this lyso compound was not primarily a degradation product. This possibility is under investigation.

Positive identification is still lacking for a number of the components of the phospholipid-glycolipid mixture. Some of these have not been completely separated by the solvent systems used, others have not been separated in sufficient quantity for positive identification. In both thin-layer and column chromatography, the high proportion of galactolipids and phosphatidyl choline resulted in some overloading of these components on plate or column, with a consequent decrease in resolution even when the conen of minor components was very low. The object of this study was to determine components present in sufficient quantity to exert an influence on the properties of flour. Thus no attempt was made to achieve the ultimate in separation of minor components.

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REFERENCES

1. Fisher, N. "Recent Advances in Food Science," Vol. 1, Ed. J. Hawthorn and J. M. Leitch, Butterworth's, London, 1962. p. 226–245.
2. McKillican, M. E., and R. P. A. Sims, JAOCS *41*, 340–344 (1964).
3. Wren, J. J., and S. C. Elliston, Chem. Ind. 30–81 (1961).
4. Meredith, P., J. Sci. Food Agr. *11*, 320–329 (1960).
5. Mason, J. L., and A. E. Johnston, Cereal Chem. *35*, 435 (1958).
6. Houston, D. F., Cereal Sci. *6*, 288–300 (1961).
7. Carter, H. E., K. Ohno, S. Nojima, C. L. Tipton and N. Z. Stanacev, J. Lipid Res. *2*, 215–222 (1961).
8. Cookson, M. A., and J. B. M. Coppock, J. Sci. Food Agr. *7*, 72 (1956).
9. Sims, R. P. A., F. B. Johnston, M. E. McKillican and J. C. Mes, in preparation.
10. Mangold, H. K., and D. C. Malins, JAOCS *37*, 383–385 (1960).
11. Marinetti, G. V., J. Erbland and J. Kochen, Fed. Proc. *16*, 837–844 (1957).
12. Wagner, H., L. Horhammer and P. Wolff, Biochem. Z. *334*, 175–184 (1961).
13. Sims, R. P. A., and J. A. G. Larose, JAOCS *39*, 232 (1962).
14. Rouser, G., G. V. Marinetti, R. F. Witter, J. F. Berry and E. Stotz, J. Biol. Chem. *223*, 485–497 (1956).
15. Bregoff, H. M., E. Roberts and C. C. Delwiche, *Ibid. 205*, 565 (1953).
16. Bandurski, R. S., and B. Axelrod, *Ibid. 193*, 405 (1951).
17. Lepage, M., J. Chromatog, *13*, 99–103 (1964).
18. Negishi, T., M. E. McKillican and M. Lepage, J. Lipid Res., in press.
19. Maruo, B., and A. A. Benson, J. Biol. Chem. *234*, 254 (1959).
20. Cifonelli, J. A., and F. Smith, Anal. Chem. *26*, 1132 (1954).
21. Research Specialties Co. Chromatofacts, Sept.-Oct. 1962.
22. Lepage, M., J. Lipid Res., in press.
23. Supina, W. R., Applied Science Laboratories, State College, Pa., private communication.

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Methanesulfonic Acid-Catalyzed Additions IV. Additions to Methyl Linoleate[1]

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Abstract

Varied aromatic compounds including phenol, *o*-chlorophenol, 2-naphthol, resorcinol, methyl salicylate, anisole, phenetole, *p*-toluenethiol, *p*-chlorobenzenethiol and 2-napthalenethiol were added to methyl linoleate. Using methanesulfonic acid as a solvent-catalyst the additions took place as readily as to oleic acid in previous experiments. The recovery of mono-adducts, however, was complicated by the presence of di-adducts and presumably polymeric material.

As observed in the earlier oleic acid studies, evidence for the formation of ether intermediates was obtained. The only identified products from arylthiol additions were thioethers.

Introduction

METHANESULFONIC ACID, like H_2SO_4, has a hydrogen bonded structure, high dielectric constant and, although weaker than H_2SO_4, has high proton availability (1). It does not have the tendency to oxidize organic compounds as readily as H_2SO_4. This latter property suggested its possibilities as a substitute for H_2SO_4 as a carbonium ion producer and catalyst for addition reactions. Recent reports from this laboratory (2,3,4) have described the acid-catalyzed addition of phenols, arylthiols, phenyl ethers and benzoic acid to oleic acid and to cyclohexene with methanesulfonic acid playing the role of both solvent and catalyst for the reaction. It was particular noteworthy that arylthiols could be added in this medium since previous investigators (5) experienced difficulties with this reaction when sulfuric acid was used.

This report concerns the investigation of the addition reaction directed toward linoleic acid as its methyl ester. In this case, both mono-addition and di-addition products are possible. A number of the nucleophilic substances that were successfully added to oleic acid and to cyclohexene were tried similarly with methyl linoleate. In most instances good yields of products were obtained but the recovery of the products was more involved. Methyl linoleate, a more reactive compound than oleic acid, had a greater tendency to form polyaddition products and polymeric substances. The presence of these materials complicated the separation procedures. The results of the addition experiments are summarized in Table I.

In an earlier communication (3), it was noted that previous investigators, particularly Niederl et al. (6, 7,8), had proposed a mechanism for the addition of a phenol to an olefin which involved the formation of an ether intermediate which subsequently rearranged to form the final product. Evidence supporting this mechanism was obtained by us from an IR study of fractions resulting from the methanesulfonic acid-catalyzed addition of *m*-cresol to oleic acid. A similar type of evidence was obtained by IR examination of the product obtained from the methanesulfonic acid-catalyzed addition of 2-naphthol to methyl linoleate. The crude product showed a high degree of absorption at 1250 cm^{-1} characteristic of arylalkyl ethers, and absorption present in the 3300–3700 cm^{-1} region indicated the presence of free hydroxyl groups. These findings suggested that the product contained a mixture of both 2-naphthoxy and hydroxynaphthyl derivatives. Both of the characteristic absorptions were present in the spectra of the early fractions obtained from molecular distillation of the crude product. This indicated that other means were necessary to effect the separation. As expected, the analyses of these fractions agreed well with theory for a mono-addition product in all respects except for hydroxyl content.

By adoption of the procedures found to be successful in our earlier work on oleic acid, it was possible to effect a separation by chromatography. The 2-naphthoxy derivative is less polar than the derivative containing a free hydroxyl group, and the separation of the isomers was made by chromatography on a Florisil column. The early fractions eluted from the column when examined by IR had a strong absorption peak at 1250 cm^{-1} showing the presence of alkylaryl ether. The loss of absorption in the 3300–3700 cm^{-1} region indicated the absence of free hydroxyl groups. The fractions obtained from later

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