On the other hand, if one considers the formation pathway of the radicals from the various compounds which have been presented as the intermediate products in an early stage of the sugar–amino acid reaction system, the following pathway seems probable; the pyrazine derivative possessing 1,4-diamino acid residues and 2,5-disugar residues would be formed by condensation of two molecules of the enaminol product, e.g., that of N-substituted 1-amino-1-deoxyketose in glucose–amino acid system, and subsequent elimination of the subsitutents of sugar residues by C–C scission will give the proposed pyrazine radical products. However, there are many problems with this postulation, since the presence of such 1,4-disubstituted pyrazine drivatives as the reaction product is hardly reported and, moreover, possibility of the following C–C scission process remains obscure.

To see the possibility of this mechanism, the following experiment was carried out as a preliminary investigation. 1-Alanino-1-deoxy-D-fructose, prepared according to Anet and Reynolds (1957), was heated in alkaline solution, with D-glucose in distilled water, or with β-alanine in distilled water, at 90 °C for 15 to 30 min. In either case no detectable ESR signal with characteristic hyperfine structure was observed.

In any event, it is not at present possible to decide whether the free radical products formed necessarily from glycolaldehyde or from other unknown pathways as presented above. Further investigation is necessary to explain fully this interesting observation.

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Reconstitution of Petroleum Ether Soluble Wheat Lipopurothionin by Binding of Digalactosyl Diglyceride to the Chloroform-Soluble Form

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Lipopurothionins, which are lipid–protein complexes extracted with petroleum ether from the endosperms of wheat and other Gramineae, are converted to a petroleum ether insoluble, chloroform-soluble form by extraction with acetone. It has been found that digalactosyl diglyceride (DGDG) is the only component of the acetone extract that is able to restore petroleum ether solubility when added back to the chloroform-soluble form.

Purothionins are high sulfur, basic polypeptides from wheat that inhibit papain (Balls et al., 1942b), have antimicrobial (Stuart and Harris, 1942; Fernandez de Caleya et al., 1972; Hernandez-Lucas et al., 1974) and uterus contracting properties (Coulson et al., 1942), and are toxic to small animals when injected intravenously or intraperitoneally (Coulson et al., 1942). They were first discovered as lipid–protein complexes (lipopurothionins) in petroleum ether extracts from the endosperm of hexaploid wheat, *Triticum aestivum* L. (Balls et al., 1942a). Analogous complexes were later found in barley (Redman and Fisher, 1969) and in 22 species of the *Aegilops–Triticum* group (Carbonero and García-Olmedo, 1969).

Although the protein moieties of these complexes have been well characterized (Redman and Fisher, 1969; Nimmo et al., 1968; Fisher et al., 1968; García-Olmedo et al., 1968; Nimmo et al., 1974; Fernandez de Caleya et al., 1976) and even sequenced in some cases (Othani et al., 1975; Mak and Jones, 1976), the characterization of the lipid components has been only partially successful because it has not been possible to obtain petroleum ether soluble complexes free of other lipid–protein associations (Balls et al., 1942a; Redman and Fisher, 1968; Hoseney et al., 1970; Fisher, 1976). Here, we present evidence that digalactosyl diglyceride [DGDG; 2,3-di-*O*-acyl-1-*O*-β-(6-*O*-α-D-galactopyranosyl-D-galactopyranosyl)-D-glycerol] is a component of the complex which is essential for its solubility in petroleum ether.

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MATERIALS AND METHODS

Kernels from *Triticum aestivum* L. cv "Aragon 03" and from *T. durum* Desf. cv "Senatore Capelli" were milled without preconditioning in a Brabender Quadrumat mill, and 60–65% yields of milled endosperm (flour) were obtained.

Extractions of flour were carried out with five volumes of the appropriate solvent in a glass column (2 × 15 cm). The solvent was evaporated in vacuo and the extract redissolved in a small volume of the same solvent.

For quantitation purposes, purothionins were precipitated from the extracts with three volumes of ethanolic 1 N HCl, separated by centrifugation (3,000 *g*; 15 min), and redissolved in a known volume of 0.015 M aluminum lactate–lactic acid buffer, pH 3.2, 3 M urea (100 μL/10 g of original flour). Quantitation was carried out after electrophoresis as previously described (Fernandez de Caleya et al., 1976).

Acetone extracts were fractionated by preparative thin-layer chromatography in silica gel GF_{254} (Merck) using chloroform–methanol–water (65:25:4 v/v/v) as solvent system (system I). The recovered DGDG was further purified by a second chromatographic run using chloroform–methanol–acetic acid–water (170:25:25:4 v/v) as solvent system (system II). Fractions were visualized under

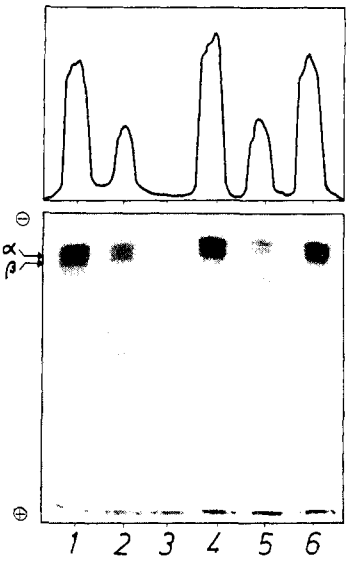

Figure 1. Yield of purothionins from *T. aestivum* cv "Aragon 0.3": purified α- and β-purothionin (1, 6); extracted with petroleum ether from acetone-defatted flour to which the acetone extract has been added back (2); from acetone-defatted flour (3); from whole flour to which acetone extract has been added (4); from whole flour (5). The amount of lipid added is that extracted from an equivalent amount of flour. Aliquots corresponding to the same amount of original flour were inserted in the different slots. Densitometric scanning perpendicular to electrophoretic movement.

UV light or by spraying iodine in chloroform in longitudinal bands, and recovered with chloroform–methanol (2:1). Identification of water-soluble products of alkaline and acid hydrolysis of the fractions was carried out by descending chromatography on Whatman No. 1 paper in 1-butanol–propionic acid–water (151:75:100 v/v/v) (system III).

Fractions from the acetone extract, or the whole extract, were redissolved in a small volume of petroleum ether (bp 50–70 °C) and added back to acetone defatted flour or to whole flour; then the flour was extracted with petroleum ether and the purothionins quantitated as described above.

Chloroform-soluble lipopurothionin was obtained from the petroleum ether extract by precipitation with eight volumes of acetone (Fisher, 1976). Reconstitution of the petroleum ether soluble form was achieved by adding DGDG in chloroform to a chloroform solution of the above precipitate and eliminating the solvent in vacuo.

A mixture of the two genetic variants present in hexaploid wheat, designated α- and β-purothionins, was obtained by preparative electrophoresis as previously described (Hernandez-Lucas et al., 1974).

RESULTS AND DISCUSSION

It has been previously observed that lipopurothionins are not extracted with acetone or with petroleum ether after acetone (Fisher, 1976). Addition of acetone to the petroleum ether extract precipitates purothionin in a form that is soluble in chloroform but not in petroleum ether (Fisher, 1976). Results shown in Figure 1 indicate that when the acetone extract is added back to the acetone-defatted flour, petroleum ether soluble lipopurothionins are reconstituted.

In a previous paper (Fernandez de Caleya et al., 1976), we have demonstrated that only a fraction of total purothionin is extractable with petroleum ether. We now show that addition of acetone extract from an equivalent amount of flour to whole flour practically doubles the yield of petroleum ether soluble lipopurothionin (Figure 1).

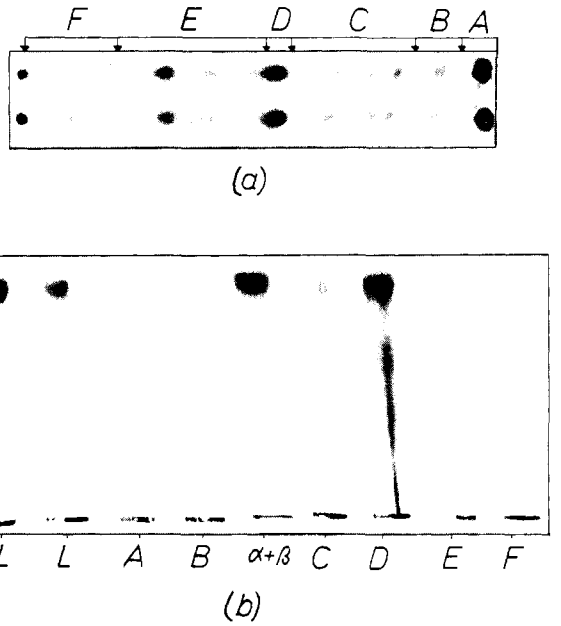

Figure 2. Fractions A–F from the acetone extract obtained by preparative thin-layer chromatography as indicated in (a), were added back to acetone-defatted flour. Yields of purothionins are shown in (b). Purothionins extracted with chloroform from acetone-defatted flour (CL); extracted with petroleum ether from acetone-defatted flour to which the whole acetone extract has been added back (L); purified purothionins (α and β). Amounts about 30% in excess to those originally present in flour were added back.

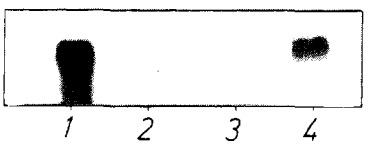

Figure 3. Purothionins obtained by acetone precipitation from the petroleum ether extract of flour: (1) purothionins remaining in the insoluble residue when the precipitate is extracted with petroleum ether; (2) purothionins in the petroleum ether extract of the precipitate; (3) purothionins in the insoluble residue when the extraction is carried out with petroleum ether + DGDG; (4) purothionins extracted from the precipitate with petroleum ether + DGDG. The amount of DGDG added was 50% in excess to that present in the petroleum ether extract from which the precipitate was obtained.

In order to identify which factors from the acetone extract were limiting solubility in petroleum ether, the extract was fractionated by preparative thin-layer chromatography, as indicated in Figure 2a, and the fractions added back to acetone-defatted flour. Only fraction D was effective in reconstituting petroleum ether soluble lipopurothionins (Figure 2b). Characteristics of this fraction closely matched those published for DGDG, a lipid class first discovered by Carter et al. (1956) in wheat endosperm and then found to be ubiquitous in plants. R_f values for fraction D in thin-layer chromatography were 0.60 with system I (DGDG R_f 0.62; Lepage, 1964) and 0.35 in system II (DGDG R_f 0.36; Galliard, 1968). Spraying with 50% sulfuric acid (heating at 110 °C) and with modified Dragendorff reagent gave brown red and yellow colors, respectively (Lepage, 1964). The infrared spectrum was practically superposable with that of DGDG (Allen et al., 1966; Galliard, 1969). Alkaline methanolysis yielded a petroleum ether soluble fraction that cochromatographed with fatty acid methyl esters and a water-soluble product with an R_f value of 0.10 in system III (digalactosylglycerol R_f 0.10, Galliard, 1969; R_f 0.13, Ferrari and Benson, 1961). Acid hydrolysis of the deacylated product yielded galactose

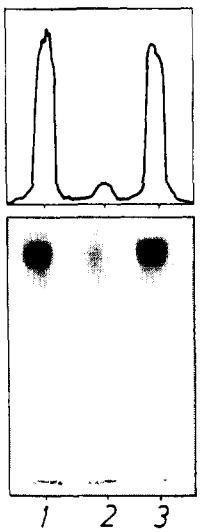

Figure 4. Yield of purothionins from *T. durum* cv "Senatore Capelli": whole flour extracted with petroleum ether + DGDG (1); whole flour extracted with petroleum ether (2); purified α- and β-purothionins (3). The amount of DGDG added was that of an equivalent weight of *T. aestivum* flour. Densitometric scanning perpendicular to electrophoretic movement.

and glycerol (Galliard, 1969).

As shown in Figure 3, when purified DGDG was added to acetone precipitated purothionins, complete solubilization in petroleum ether was achieved. Monogalactosyl diglyceride (MGDG), DGDG, phosphatidyl ethanolamine (PE) and phosphatidyl choline (PC) have been previously found to be present in lipopurothionin preparations in which the presence of other lipid–protein complexes was not unequivocally excluded (Redman and Fisher, 1968; Hoseney et al., 1970; Fisher, 1976). The chloroform-soluble purothionins preparations obtained by acetone precipitation contains unidentified lipids, PC, PE, and some DGDG, but no MGDG. Acetone removed all of the MGDG and most of the DGDG present in the petroleum ether extract, but only the addition of DGDG permits recovery of petroleum ether solubility by the precipitated purothionins.

A mixture of purified α- and β-purothionins (free of other protein and of lipids) was not solubilized by 60 mM DGDG in petroleum ether but did bind about 5 mol of DGDG/mol of protein, as estimated by extraction of the undissolved purothionin with acetone, fractionation of the extract by thin-layer chromatography, and densitometry. This result confirms the ability of DGDG to bind purothionin and indicates that at least some of the other lipids present in chloroform-soluble purothionin (PC, PE, etc.) are also required for solubility in petroleum ether.

We have previously reported that the yield of petroleum ether soluble lipopurothionin is considerably higher in hexaploid wheat, *Triticum aestivum* L. (genomes AABBDD), than in tetraploid wheat, *T. durum* Desf. (AABB), although the level of total purothionin is similar in both species (García-Olmedo et al., 1968; Fernandez de Caleya et al., 1976). This difference in yield was traced to a gene (or genes) located in the short arm of chromosome 5D and we postulated that this gene was probably controlling the synthesis of a lipid factor responsible for the petroleum ether solubility of lipopurothionins (Fernandez de Caleya et al., 1976). The fact that addition of an appropriate amount of DGDG to *T. durum* whole flour increased th yield of lipopurothionin to the level of that of *T. aestivum* (Figure 4) points to DGDG as the lipid factor responsible for the different yield between the two wheat species. The appropriate genetic study has fully confirmed that the short arm of chromosome 5D controls the higher level of DGDG in hexaploid wheat (Hernandez-Lucas et al., 1977).

The possibility that petroleum ether soluble lipopurothionins are extraction artifacts has been previously considered, based on the fact that they represent only a fraction of total purothionins (Fernandez de Caleya et al., 1976). The present results indicate that ether-soluble lipopurothionins can be formed during extraction. However, Hoseney et al. (1970) have demonstrated the presence of both purothionin and DGDG in the 2% NaCl extract of flour, which suggests that the complex does exist as such in the endosperm.

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