compound 1-octen-3-ol has been observed in mold ripened cheese (Adda and Dumont, 1974; Groux and Moinas, 1974) and this compound can be produced by *Penicillium* species (Kaminski et al., 1974). At high concentrations this compound possesses a musty, mushroomy flavor. Ney et al. (1975) found that the inclusion of octenol in a simulated blue cheese flavor improved the overall flavor quality. This compound can be generated from linolenic acid via autoxidation (Forss, 1972). Conceivably the levels of linolenic and possibly linoleic acid in mold mycelium may influence the amount of octenol produced and thereby influence the flavor quality of cheese. Because aged mycelium contains much greater levels of polyunsaturated acids, aged mold-ripened cheeses may have a greater tendency to generate octenol.

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Lipids in the Exterior Structures of the Hen Egg

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Studies are reported on the composition of lipids of shell with cuticle (SC) and shell membrane (SM) from hen egg. Total lipids were approximately 0.045% of SC and 1.35% of SM. The ratios of neutral lipid to polar lipid in SC and SM were 5:1 and 6:1, respectively. The neutral lipid fractions of SC and SM were found to contain mono-, di-, and triglyceride, cholesterol, cholesteryl ester, and free fatty acid as well as fairly large amounts of bis(2-ethyl hexyl) phthalate. The major neutral lipid (excluding the phthalates) was cholesterol, and the levels of triglyceride were very low in the neutral lipids of SC and SM. The polar lipid fractions of SC and SM were found to contain very low levels of phosphatidylethanolamine and phosphatidylcholine. The predominant phospholipid were sphingomyelin. Significant amounts of ceramide mono- and dihexoside were also detected in the polar lipid fractions of SC and SM. At least 17 different fatty acids were present in SC and SM lipids. The level of linoleic acid was higher in SM than in SC neutral lipid. The fatty acid distributions of polar lipids of SC and SM were similar.

The exterior structures of the hen egg (inner membrane, outer membrane, shell, and cuticle) have significant chemical, biological, and mechanical roles in preservation (Baker and Balch, 1962). Little has been reported about the nature of the lipids in these structures (Hasiak et al., 1970a,b; Maesso et al., 1974). One report (Hasiak et al., 1970b) on the fatty acid composition of lipid in these structures included up to 67.7% of nonadecenoic acid and up to 24.0% of heneicosanoic acid. Recently, Pascal and Ackman (1976) have pointed out that these odd numbered

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acids were probably confused with diisobutyl and di-*n*-butyl phthalate esters which were extracted from conventional screw caps on storage vials. In this paper we present new quantitative data on the lipid and fatty acid compositions of the egg exterior structures.

MATERIALS AND METHODS

Eggs. Three-hundred and fifty 1-day old infertile eggs from White Leghorn fowl on a chicken farm were used for this study. The ages of the hens from which the eggs were obtained ranged from 40 to 43 weeks. Eggs were carefully collected from the steel cages using fat-free cotton gloves to prevent contamination. The gloves were cleaned previously by being extracted with ethanol and hexane. Any processing which leads to local contamination of phthalates was not applied to the eggs in the battery area.

Separation of Structures. The eggs were broken in half, the contents removed, and the shells washed in running tap water. The shell structures were manually separated into shell with cuticle (abbreviated as SC) and shell membrane (abbreviated as SM). Care was taken to prevent contamination of these structures with the lipids from hands and equipment.

Solvents and Apparatus. All solvents were of reagent quality and were redistilled in glass before use. The absence of phthalates was verified by gas–liquid chromatography (GLC). Polyvinyl vacuum tubing was used for application of the vacuum to a rotary evaporator, but a glass bulb of 95 mm diameter was connected between the driving part and the evaporating flask in order to prevent back contamination. The solvents were distilled under reduced pressure with an aspirator. All apparatus was glass or porcelain and with a stainless steel homogenizer (Nihon Seiki) was washed with acetone before use.

Extraction of Lipids. Grinding and homogenization were carried out, respectively, by use of a porcelain mortar with a pestle and in a stainless steel homogenizer. Lipids in the egg SC and SM structures were extracted separately with chloroform–methanol (2:1, v/v). In the first extraction, the coarse-ground SC and SM was stirred and homogenized with 5 vol (v/w) of chloroform–methanol (2:1, v/v). After standing at room temperature, the solution was filtered by used of Toyo No. 2 filter paper. The residues were reextracted twice with 2 vol of the same solvent mixture. The extracts were combined and most of the nonlipid contaminants were removed by shaking the combined solvents with 0.2 vol of 1.0% NaCl solution. The lower chloroform phase was evaporated to dryness below 40 °C. The lipids obtained were dried under nitrogen, weighed, and redisolved in dry chloroform and stored at −20 °C for further analysis.

Separation of Lipid Classes. A 2.0-cm diameter chromatography column containing 20 g of silicic acid (Mallinckrodt, 100 mesh) was employed for separation of total lipid into neutral and polar lipid fractions. Lipid in chloroform solution was applied to the column. The neutral lipids were eluted from the column with 500 mL of chloroform and the polar lipids were eluted with successive applications of 300 mL of chloroform–methanol (1:1, v/v) and 200 mL of absolute methanol. The quantities of neutral and polar lipids were determined gravimetrically. The neutral and polar lipids were separated into various classes by thin-layer chromatography (TLC) on glass plates coated with silica gel H (Merck, Darmstadt, West Germany). Prior to use, the TLC plates were activated at 120 °C for 30 min and then cooled in a desiccator. The neutral lipids were separated by successive developments with *n*-hexane–diethyl ether–acetic acid (80:20:1, v/v) and *n*-hexane–diethyl ether (95:5, v/v). The polar lipids were separated by development with chloroform–methanol–water (65:25:4, v/v) for one-dimensional separation, and chloroform–methanol–28% ammonia (65:35:5, v/v) and chloroform–acetone–methanol–acetic acid–water (4:2:1:1:0.5, v/v) for two-dimensional separation. Individual compounds were identified by cochromatography with known lipids and by reference to standard compounds in the same TLC systems. The polar lipid spots were visualized with different analytical detection sprays. Phosphorus, amino groups, and the choline of phospholipids on TLC were identified, respectively, by spraying with Dittmer, ninhydrin, and Dragendorff reagent. Glycolipids were identified by spraying with anthrone reagent. Neutral lipids were made visible by spraying the plate with 50% sulfuric acid and heating at 110–120 °C for 10 min.

Table I. Lipids in Shell with Cuticle (SC) and Shell Membrane (SM)

	SC	SM
Amount of lipids (% dry wt)	0.045	1.350
Neutral lipids[a] (% total lipids)	83	86
Polar lipids (% total lipids)	17	14

[a] Substance X is included in the neutral lipids.

Analytical Methods. Each of the phospholipids separated by TLC was extracted with methanol, dried, and determined by assaying for lipid phosphorus. Phosphorus was determined by the method of Harris and Popat (1954). Plasmalogens were identified by the method described in our previous report (Nakanishi and Suyama, 1969). The cholesterol and cholesteryl ester separated by TLC were extracted with diethyl ether, dried, and determined by the Liebermann–Burchard reaction. Optical densities were determined 30 min after initiating the assay on a Hirama spectrophotometer (Hirama) at 660 nm.

GLC Analysis. The GLC analyses were carried out with a Hitachi Model 063 gas chromatograph equipped with dual flame ionization detectors. Stainless steel columns (2 m in length × 3 mm i.d.) were packed with Diasolid L 80–100 mesh coated with 10% diethylene glycol succinate polyester and operated isothermally at 190 °C. The detector and inlet were at 230 °C, and the carrier gas was N_2.

The fatty acid compositions of the neutral and polar lipid fractions were determined in the form of the methyl ester derivatives by GLC. The methyl esters were prepared by interesterification with methanolic hydrogen chloride (3%) at 98 °C for 4 h in sealed glass tubes containing 1 atm of nitrogen. Tentative indentifications of the methyl esters were through comparisons of retention times with those of methyl ester standards and through equivalent chain length values (Hofstetter et al., 1965). In some case, methyl esters were hydrogenated and analyzed for chain length composition to assure identification and quantification. Relative amounts of component were calculated on the basis of peak areas.

RESULTS AND DISCUSSION

The amounts of total lipids in SC and SM are shown on a dry weight basis, together with those of neutral lipid and polar lipid as percents of the total lipid, in Table I. The quantities of total lipid were in close agreement with the results reported by Hasiak et al. (1970a,b). They found that the amounts of the lipid were 0.01–0.09% of SC and 0.5–4.0% of inner and outer membranes. However, in our study, the neutral lipid to polar lipid ratios (N/P ratio)

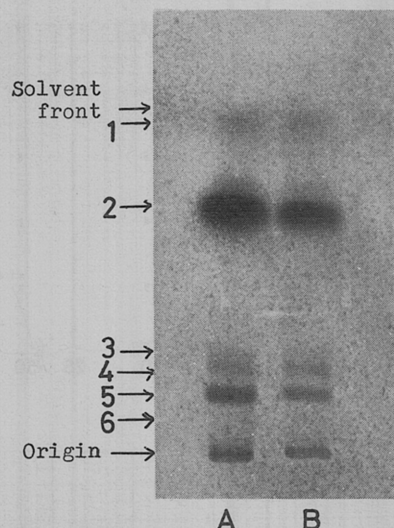

Figure 1. Thin-layer chromatogram of the neutral lipid fractions of SC and SM: A, SC; B, SM; 1, cholesteryl ester and wax; 2, triglyceride and X; 3, free fatty acid; 4, diglyceride; 5, cholesterol; 6, monoglyceride; solvent system, *n*-hexane–ethyl ether–acetic acid (80:20:1, v/v); visualization, 50% sulfuric acid.

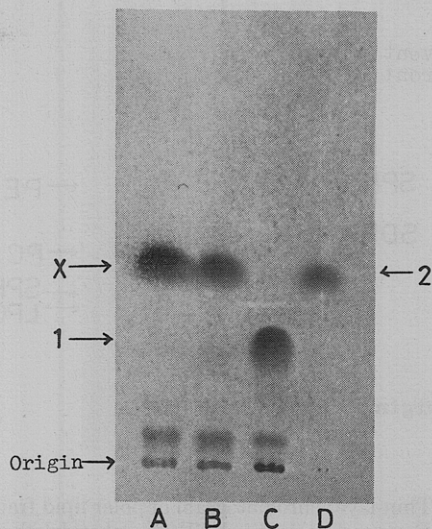

Figure 2. TLC separation of substance X from triglyceride in the neutral lipid fractions of SC and SM: A, SC; B, SM; C, egg-yolk lipid; D, bis(2-ethyl hexyl) phthalate; 1, triglyceride; 2, bis(2-ethyl hexyl) phthalate; X, substance X; solvent system, duplicate development in *n*-hexane–ethyl ether (95:5, v/v); visualization, 50% sulfuric acid.

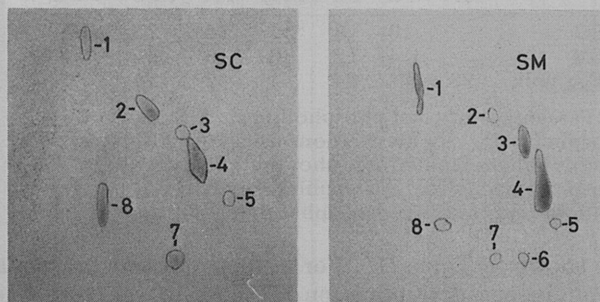

Figure 3. Two-dimensional thin-layer chromatography of phospholipid fractions of SC and SM: 1, unidentified phospholipid; 2, phosphatidylglycerol; 3, phosphatidylcholine; 4, sphingomyelin; 5, lysophosphatidylcholine; 6 and 7, unidentified phospholipids; 8, phosphatidic acid; solvent system, *Y* direction (first), chloroform–methanol–28% ammonia water (65:35:5, v/v); *X* direction (second), chloroform–acetone–methanol–acetic acid–water (5:2:1:1:0.5, v/v); visualization, Dittmer reagent.

in the total lipid of SC and SM were 5:1 and 6:1, respectively, whereas, in their study, the N/P ratio of total lipid was 2:1. This discrepancy could be ascribed to the fact, as described later, that a considerable amount of bis(2-ethyl hexyl) phthalate was included in the lipid extracted from the structures used in these experiments. Thus, it seems reasonable to assume that the contaminant was responsible for elevating the ratio of neutral lipid amounts.

Neutral Lipid Fractions. Figure 1 shows the TLC of the neutral lipid fractions of SC and SM. The triglyceride spots, after spraying with 50% sulfuric acid and heating at 120 °C, gave purple colors. No spot with the color was detected in an analytical blank which was carried out with cholesterol added to the solvents at the extraction stage. As triglyceride does not generally develop a purple color heated with sulfuric acid on TLC, the spot would appear to contain other material in addition to triglyceride. Hasiak et al. (1970a,b) also observed this color, but they did not report the separation and identification of this material. The separation of the material (X) from the triglyceride on TLC was performed successfully by duplicate developments of all of the neutral lipids with a solvent mixture of *n*-hexane–ethyl ether (95:5, v/v) as shown in Figure 2. Furthermore, it is evident from these figures that in the neutral lipid fractions of the two structures cholesterol, cholesteryl ester, and diglyceride were predominant with minor amounts of free fatty acid, triglyceride, and monoglyceride. Triglyceride, especially, was virtually absent from SC. Other minor constituents were not investigated. This composition as a pattern for the neutral lipid fractions appears to be almost unique for lipid in animal tissues. An experiment with egg-white lipid (Negishi et al., 1975) indicated that the triglyceride of egg-white lipid was a minor component, though Sato et al. (1973) showed that triglyceride of egg-white lipid was the predominant component. Our investigation gave results similar to that of the composition described by Negishi et al. for egg-white lipid. Total cholesterol contents were 12.1 and 7.6% in neutral lipids of SC and SM, and the ratios of free cholesterol to cholesteryl ester were about 4:1 and 2:1, respectively, in SC and SM, but no other qualitative or quantitative differences were observed in neutral lipid fractions of SC and SM.

The spot for X from SC lipid was scraped from the TLC plate, extracted with diethyl ether, and evaporated to dryness. The X residue was a viscous liquid and the R_f value on TLC was in good agreement with that of bis(2-ethyl hexyl) phthalate as shown in Figure 2. The IR spectrum of X was also in good agreement with that of bis(2-ethyl hexyl) phthalate. Negishi et al. (1975) detected small amounts of phthalic acid ester in one case of egg-white lipid, and suggested that the egg-white lipid contained phthalic acid ester as a contaminant. Recently, Pascal and Ackman (1976) have claimed that the presence of isobutyl phthalate in egg membrane lipids originated in the extraction of the phthalate by diethyl ether from the liners of conventional screw caps on storage vials used. However, no plastic capped vessels were used throughout the course of our experiment. Thus, our neutral lipid results indicate that the SC and SM were contaminated with the phthalate in the original stage of the egg production.

Polar Lipid Fractions. Two-dimensional and one-dimensional TLC separations of the polar lipid fractions of SC and SM are shown, respectively, in Figures 3 and 4. The components in total phospholipid of SC and SM

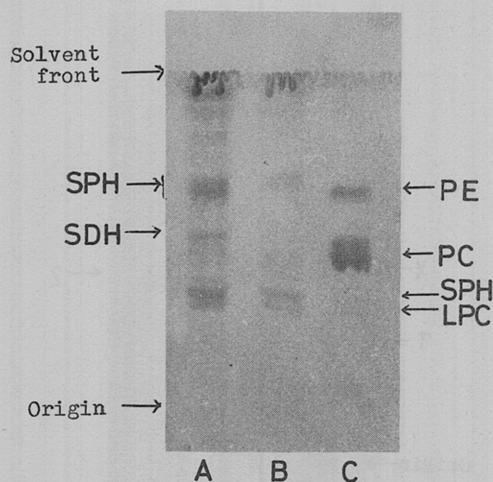

Figure 4. Thin-layer chromatogram of polar lipid fractions: A, SC; B, SM; C, egg-yolk polar lipid; PE, phosphatidylethanolamine; PC, phosphatidylcholine; SPH, sphingomyelin; LPC, lysophosphatidylcholine; SMH, seramido monohexoside; SDH, seramido dihexoside; solvent system, chloroform–methanol–water (65:25:4, v/v); visualization, anthrone reagent.

Table II. Distribution of Phospholipids in SC and SM[a]

	PE[c]	PG	PC	SPH	PA	LPC	O-PL[b]
SC		10	9	57	14	2	8
SM		4	12	63	6	2	13
Egg yolk	23		64	4	2	5	2

[a] Percentage of total phospholipids. [b] Sum of other phospholipids. [c] Abbreviations used are: PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PC, phosphatidylcholine; SPH, sphingomyelin; PA, phosphatidic acid; LPC, lysophosphatidylcholine.

are shown in Table II. For purposes of comparison, a simultaneous determination of those in egg yolk was carried out and the result is shown in the same table. Small differences in concentration between the structures were observed as illustrated in Figure 3. Phosphatidylethanolamine was a minor component in the SM phospholipids but could not be detected in the SC. Phosphatidylcholine, which is a major component in phospholipids of almost all animal tissues, was a minor component in these two egg structures. On the other hand, it is notable that sphingomyelin was the major component (>50%), and relatively large amounts of phosphatidylglycerol and phosphatidic acid were present in each case. No choline or ethanolamine plasmalogen was detected. The unusual characteristics of the two phospholipid compositions became apparent when compared with that of egg-yolk lipids. The lipids of the egg yolk contained far less sphingomyelin, and had phosphatidylcholine and phosphatidylethanolamine as major components.

Negishi et al. (1975) have pointed out that sphingomyelin was the predominant lipid of egg white. Hasiak et al. (1970a,b) showed that the phospholipid fraction of the egg exterior structures included approximately 75% phosphatidylcholine and the remainder was made up of lysophosphatidylcholine (5.8–7.4%), phosphatidylethanolamine (13.5–16.6%), and sphingomyelin (2.2–5.2%). The differences between our results and those of Hasiak et al. (1970a,b) are much too large to be readily explained. It is well known that the amino group of phosphatidylethanolamine reacts easily with carbonyls formed via autoxidation of polyunsaturated fatty acids of phospholipids (Lea, 1957) and plasmalogens are very easily hydrolyzed under the atmosphere (Nakanishi and Suyama, 1973) but sphingomyelin is very stable to oxidation

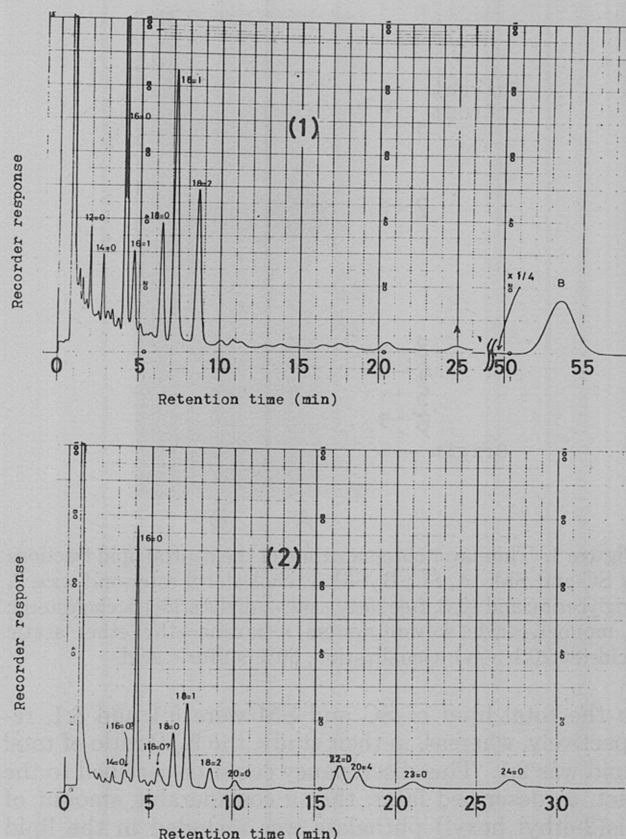

Figure 5. Gas chromatograms of fatty acid methyl esters from neutral (1) and polar (2) lipid fractions of SC: A, di-*n*-butyl phthalate; B, bis(2-ethyl hexyl) phthalate.

(Nakanishi and Suyama, 1967). On the other hand, considerable amounts of ceramide mono- and dihexoside were identified as illustrated in Figure 4. These sphingolipids may play some unknown chemical and biological roles. Other "polar" compounds with similar chromatographic properties were present in both SC and SM, but they were not identified.

Fatty Acid Composition. Typical gas chromatograms of fatty acid methyl esters obtained respectively from the neutral and polar lipid fractions of SC are shown in Figure 5. Methyl esters of lauric ($C_{12:0}$), myristic ($C_{14:0}$), palmitic ($C_{16:0}$), stearic ($C_{18:0}$), oleic ($C_{18:1}$), linoleic ($C_{18:2}$), eicosanoic ($C_{20:0}$), arachidonic ($C_{20:4}$), docosanoic ($C_{22:0}$),tricosanoic ($C_{23:0}$), and tetracosanoic ($C_{24:0}$) acids were identified. Di-*n*-butyl- and bis(2-ethyl hexyl) phthalates which had not been interesterified by the 3% HCl–methanol were found to be present in neutral fractions.

The tentative fatty acid compositions of neutral and polar lipid fractions of SC and SM are shown, with the phthalates excluded, in Table III. In the neutral lipid fractions, palmitic, stearic, and oleic acids were present in the highest concentrations. Minor differences were observed between the fatty acid composition of neutral lipid fraction of SC and that of SM. The SM contained higher levels of linoleic acid and unsaturated fatty acids. Linoleic acid, which ranged between 5.3 and 13%, was more than about 1% in the data recalculated from the results of Hasiak et al. (1970a,b). The difference may be due to loss of polyunsaturated acids by autoxidation during the drying of their sample, as suggested by Pascal and Ackman (1976). The gas–liquid chromatograms clearly show that the substance X of A and B corresponds to the mixture of di-*n*-butyl- and bis(2-ethyl hexyl) phthalates of unknown origin. The nonadecenoic acid and heneicosanoic acids reported by others were not observed.

Table III. Fatty Acid Analysis of Neutral and Polar Lipid Fractions of SC and SM[a]

	Neutral lipids		Polar lipids	
	SC	SM	SC	SM
12:0[b]	tr	tr	1.0	1.0
14:0	2.7	1.1	1.0	1.2
15:0	0.3	tr	tr	tr
i-16:0	tr	tr	2.2	3.8
16:0	48.7	43.8	25.9	27.7
16:1	1.9	0.3	1.1	0.7
17:0	tr	0.4	0.3	0.8
i-18:0	tr	tr	1.5	0.9
18:0	16.0	13.9	12.4	12.5
18:1	14.9	16.4	15.3	15.2
18:2	5.3	13.0	4.4	3.5
20:0	0.6	1.2	2.3	1.0
20:4	1.3	3.2	13.4	13.2
22:0	0.5	tr	12.3	12.2
23:0	1.1	0.3	1.5	0.8
24:0	1.3	1.3	4.8	1.9
26:0	tr	tr	1.0	0.4

[a] Percent of total fatty acid. [b] Shorthand designation of fatty acids; carbon chain length:number of double bonds; tr = trace, less than 0.2%.

The patterns of fatty acid compositions of polar lipid fractions of SC and SM were similar. Palmitic, stearic, oleic, eicosanoic, docosanoic, tetracosanoic, and arachidonic acids were present in the highest concentrations. Peaks for unknown fatty acids were minor quantities as illustrated in Figure 5.

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Localization of Iron in *Vigna sinensis* L. and *Zea mays* L.

Jacqueline W. Jacobs*[1] and Richard B. Walker

With the aid of histo- and cytochemical examinations, and elemental x-ray analyses of maize, *Zea mays* L. var. Idahybrid 216, iron was found to be highly concentrated in the outer cell layers of the scutellum and in the aleurone layer. It is associated with roughly spherical structures identified as protein bodies. Analyses of the blackeyed pea, *Vigna sinensis* L., seeds indicate that iron is distributed throughout the cotyledon; however, the most intense concentration is at the periphery where the protein bodies are quite numerous. The histo- and cytochemical tests were made with the aid of the Prussian Blue reaction (acidified potassium ferrocyanide) and the Ferrozine reagent. The x-ray analyses were made with the aid of the elemental x-ray analysis method (EXAM) using a Model 707A energy dispersive x-ray analyzer(EDAX), and a scanning electron microscope (SEM).

The study of iron absorption and incorporation into food plants has in itself intrinsic interest, and as well can contribute substantially in the area of iron nutrition of man and animals. The plant may serve as the link between soil (the source) and man (the consumer).

Some studies by Layrisse (1970) and Layrisse et al. (1969) have suggested that iron from animal sources is more available than iron from vegetable sources. When values of food iron absorption were compared to those of absorption from iron salts, the iron from foods proved to be less well absorbed.

The literature contains few data on the localization and/or form(s) of iron in seeds and grain. This problem has seldom been studied, even though knowledge concerning the storage forms of iron in seeds and grain should help to explain the difficulties which the human organism encounters in utilizing iron from nutritional products derived from plants.

Therefore, this study deals primarily with locating the highly concentrated iron sites in seeds and grain. This knowledge should aid in future studies dealing with identification and isolation or iron-containing compounds.

BACKGROUND

Protein bodies, or aleurone grains, were discovered by Hartig (1865) and have since then been studied in great detail by other investigators. There is general agreement that protein bodies occur widely in the cotyledons and endosperm of both starch-bearing and oil-bearing seeds, that some of the protein bodies contain crystalline inclusions of inorganic salts, that they are probably surrounded by membranes, that they contain most of the cellular protein but none of the oil, that their formation commences during the later stages of ripening of the seed

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