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# *N*-Acylphosphatidylethanolamine, a Phospholipid that is Rapidly Metabolized during the Early Germination of Pea Seeds

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1. A phospholipid that rapidly disappears from pea seeds during the early stages of germination has been isolated and shown to be *N*-acylphosphatidylethanolamine. 2. Chromatographic evidence for the presence of the same phospholipid in oats, soya beans and spring (tick) beans has been obtained, and its loss during early germination measured. 3. A scheme for the stepwise degradation of the phospholipid with alkali and acid is presented.

A recent study on the fate of pea-seed phospholipids during germination revealed that only one phospholipid, comprising about 5% of the total fraction, showed a substantial change in concentration during the first 24hr. (Quarles & Dawson, 1969). The phospholipid concerned did not correspond to any of the usual ones found in plants and the present paper describes its isolation and characterization as APE.* Evidence is also presented that the phospholipid is present in soya and spring beans and also in oat and turnip seeds, and this, together with its previous isolation from wheat flour (Bomstein, 1965), suggests that it may be a widely occurring component of plant seeds. A preliminary report of some of this work has been published (Quarles, Clarke & Dawson, 1968).

## EXPERIMENTAL

*Extraction of lipids.* 150g. of dry peas (*Pisum sativum* L.) was ground in a mill (Lee Attrition Mill 9A) and the flour was extracted with 2l. of chloroform–methanol (2:1, v/v) at 50° for 20min. The chloroform–methanol supernatant was decanted from the settled residue and filtered through glass wool. The residue was re-extracted with 1l. of the same solvent for 10min. at 50°. The combined extracts were shaken with 0·2vol. of 0·9% NaCl, and the lower chloroform-rich phase was separated. It was washed twice with 0·3vol. of 'theoretical upper phase' (45ml. of methanol, 47ml. of 0·9% NaCl, 3ml. of chloroform) and evaporated to dryness in a rotary evaporator. The residue was dissolved in 30ml. of chloroform, yielding a green solution containing 69·3mg. of lipid P.

*Isolation of APE.* A sample of the total pea lipids, prepared as given above, containing 25mg. of lipid P was applied to a silicic acid column (40g., activated overnight at 110°; Mallinckrodt Chemical Works, St Louis, Mo., U.S.A.) in chloroform. The column was eluted with 500ml. of chloroform. The eluting solvent was changed to chloroform–methanol (19:1, v/v) and 250ml. collected. Monitoring by t.l.c. (see below) showed that this procedure eluted APE together with phosphatidic acid and cardiolipin. The phospholipids in this fraction were run as a band on a silica gel H plate [E. Merck A.-G., Darmstadt, Germany; solvent chloroform–methanol–water–aq. $NH_3$ (sp.gr. 0·88) (130:60:6:3·4, by vol.)] in a saturation chamber (Parker & Peterson, 1965). Marker strips were sprayed on either side of the chromatogram (Dittmer & Lester, 1964) and the fastest-running band ($R_F$ 0·63) was scraped off and eluted twice with 5ml. portions of chloroform–ethanol–water (3:10:2, by vol.). Better separations were obtained by using layers of analytical thickness with a loading of 15$\mu$g. of lipid P/cm., rather than with thicker layers and heavier loadings. The eluted APE solution was separated from the silicic acid by centrifugation, and evaporated to dryness in a rotary evaporator. The residue was extracted into 5ml. of chloroform, insoluble material being discarded. The yield was approx. 750$\mu$g. of lipid P.

*Preparation of partially deacylated APE.* A sample of total pea lipids (30mg. of P) was incubated for 80min. at 37° in 100ml. of methanolic 0·1M-NaOH. Ethyl formate (15ml.) was added to neutralize the NaOH and the mixture evaporated to dryness in a rotary evaporator. Ethanol–water (1:1, v/v, 10ml.) and 20ml. of light petroleum (b.p. 40–60°) were added. After the mixture had been shaken, the lower phase was separated and the upper phase (light petroleum) washed twice with two 10ml. portions of ethanol–water (1:1, v/v). The combined aqueous-ethanolic extracts were then extracted three times with chloroform. The chloroform extracts were evaporated to dryness and redissolved in 5ml. of chloroform, yielding a practically colourless solution containing approx. 1mg. of P.

*Preparation of* N-*stearoylphosphatidylethanolamine.* Phosphatidylethanolamine (25mg.) prepared from eggs by the method of Dawson (1963) was dissolved in 10ml. of chloroform that had previously been freed from ethanol added as a preservative by shaking twice with equal volumes of water and then drying over anhydrous $Na_2SO_4$. To the solution was added 1ml. of triethylamine and 25mg. of stearoyl chloride (recently redistilled *in vacuo*). After 2hr.

* Abbreviation: APE, *N*-acylphosphatidylethanolamine.

at room temperature the solution was washed by being shaken very well with 2vol. of saturated $NaHCO_3$ soln. The acylated product was separated from phosphatidylethanolamine by preparative t.l.c., as described above for the preparation of APE from pea seeds. Its i.r. spectrum was identical with that of the synthetic *N*-acylphosphatidylethanolamine prepared by Bomstein (1965).

*Thin-layer chromatography.* T.l.c. was carried out at 4° on silica gel H layers (Merck) in a saturation chamber (Parker & Peterson, 1965). Either an acidic (chloroform–methanol–water–acetic acid, 130:50:4:1, by vol.) or an ammoniacal [chloroform–methanol–water–aq. $NH_3$ (sp.gr. 0·88) (130:60:6:3·4, by vol.)] solvent was used. $I_2$ vapour was used for the non-specific localization of lipid. Amino lipids were located by a spray of 0·25% ninhydrin at 100°. Phospholipids were made visible by a molybdate spray (Dittmer & Lester, 1964; Vaskovsky & Kostetsky, 1968) and measured by scraping them off the gel, oxidizing with $HClO_4$ and determining inorganic P by the method of Bartlett (1959).

*Fatty acids.* These were determined either by titration (Dawson, 1956) or with a Pye 104 gas chromatograph (polyethyleneglycol adipate column).

## RESULTS

The isolated APE ran as a homogeneous spot on t.l.c. in acid or ammoniacal solvents (on silica gel H) and was distinguishable from the usual plant phospholipids by its fast-running properties in both systems. The spot did not react with ninhydrin. On acid hydrolysis (2M-hydrochloric acid for 3hr. at 100°) APE liberated 2·86moles of titratable fatty acid/mole of P present. On g.l.c. of these fatty acids after methylation, the corresponding ratio found was 2·9moles of fatty acid/mole of P, with an internal standard of arachidic acid and assuming that the peak area on the chromatogram was proportional to molecular concentration. Analysis for glycerol after total acid hydrolysis (Hanahan & Olley, 1958) showed a glycerol/P molar ratio 0·97:1. Acetolysis of APE with acetic acid–acetic anhydride mixtures (at 145° for 4·3hr.) and the separation of the chloroform-soluble products by t.l.c. (on silica gel H, with light petroleum–diethyl ether, 4:1, v/v) indicated that a diglyceride acetate had been formed (Renkonen, 1965). Paper ionophoresis of the water-soluble fragments (Dawson, Hemington & Davenport, 1962) showed that $P_i$ was a major product.

Mild alkaline methanolysis of APE (Dawson *et al.* 1962) yielded, after 20min. at 37°, two lipoidal phosphorus-containing products as well as methyl esters of fatty acids. On examination by t.l.c. in the ammoniacal solvent, the major phosphorus-containing product ran at $R_F$ 0·17 and a minor phosphorus-containing product was visible at $R_F$ 0·48; the original APE had $R_F$ 0·62 in this solvent. After alkaline methanolysis for 1hr., only the slower-running major product (dAPE) was visible. It seems probable that the intermediary product has a monoglyceride (lyso) structure; such compounds are known to be transient intermediaries in the alkaline methanolysis of phospholipids (Marinetti, 1962). It was found that the final product (dAPE) could be conveniently separated from total pea lipids by direct alkaline methanolysis and separation by a simple solvent fractionation (see the Experimental section). This relies on the observation that chloroform can extract dAPE from a solution in aqueous ethanol; rather surprisingly chloroform will not extract it from a suspension in water unless ethanol or hydrochloric acid is added. The product appeared to be homogeneous on t.l.c. although sometimes its solution showed a faint greenish-yellow tinge, suggesting the carrying-over of traces of plant pigments. Its properties were the same as those of the deacylated product prepared from isolated APE and it was used for most subsequent experiments.

*Acid hydrolysis of dAPE.* Strong-acid hydrolysis of dAPE (2M-hydrochloric acid for 3hr. at 100°) liberated 0·96 mole of titratable fatty acid/mole of P, indicating that the mild alkaline methanolysis involved in its preparation had freed two molecules of fatty acid esterified to glycerol, leaving a third in a combined form which was more stable to alkaline conditions. This suggested the possibility of one of the fatty acids being in a fatty amide linkage. This was confirmed by the i.r. spectra of smears of APE and its partially deacylated derivative (dAPE), with strong absorption bands at 1645cm.$^{-1}$ and 1540cm.$^{-1}$, which are indicative of amides and which were seen in a similar spectrum of sphingomyelin.

Acid hydrolysis of dAPE (2M-hydrochloric acid at 100°) for shorter periods and distribution of the products between chloroform and water showed that after 12min. its phosphorus had become entirely water-soluble. The phosphorus-containing product was identified as $\alpha$-glycerophosphoric acid ($M_{P_i}$ 0·82) by paper ionophoresis at pH3·6 and paper chromatography (with phenol saturated with water–ethanol–acetic acid, 50:6:5, by vol.), when it had $R_F$ 0·33.

Examination of the lipoidal component of the hydrolysate at this stage (12min.) by t.l.c. (with ammoniacal solvent) showed predominantly two products, a major one (I) with $R_F$ 0·64 that did not react with ninhydrin and a minor one (II) that ran slightly faster, with $R_F$ 0·67, and reacted with ninhydrin. These lipoidal products were more readily separated with the acidic solvent, when compound (I) had $R_F$ 0·69 and compound (II) $R_F$ 0·40. Isolation of the two products was carried out by preparative t.l.c. with the ammoniacal and acidic solvents successively. This ensured that they were freed from traces of free fatty acid also

liberated during the hydrolysis of dAPE, which ran close to compound (I) in the acidic solvent. It was noticed that the compound (II), after elution, readily reverted to compound (I) so that a mixture of the two forms was obtained on rechromatography of compound (II). Both compounds (I) and (II) broke down on further acid hydrolysis (2M-hydrochloric acid for 1hr. at 100°) yielding fatty acid and a water-soluble ninhydrin-reacting substance. The latter compound was identified as ethanolamine by paper ionophoresis, by paper chromatography (with butan-1-ol–acetic acid–water, 12:3:5, by vol.) and ion-exchange chromatography in an amino acid analyser. The latter indicated a semi-quantitative ratio of 1·1 moles of ethanolamine/mole of P in the original compound.

*Alkaline hydrolysis of dAPE.* When partially deacylated APE was hydrolysed with alkali (1M-sodium hydroxide at 100°) there was a slower release of α-glycerophosphate (this being complete in about 1hr.) than during acid hydrolysis. At this point the main lipoidal product behaved on t.l.c. exactly like the lipoidal product obtained from dAPE by acid hydrolysis. On acid hydrolysis it again liberated ethanolamine and fatty acid. It was identified as *N*-acylethanolamine by: (1) co-chromatography with synthetic *N*-oleoylethanolamine (prepared by the method of Roe, Scanlan & Swern, 1949) in thin layers of silica gel in both ammoniacal and acidic solvent systems; (2) an i.r. spectrum identical with that of the synthetic compound.

*Acid hydrolysis of* N-*oleoylethanolamine.* It seemed possible from the above results that during the acid hydrolysis of dAPE acyl-group migration had occurred in part of the released *N*-acylethanolamine with the production of *O*-acylethanolamine. Thus the ninhydrin-reacting lipoidal compound (II) formed from dAPE on acid hydrolysis would be produced from the initially liberated *N*-acylethanolamine by rearrangement. When synthetic *N*-oleoylethanolamine was subjected to acid hydrolysis (2M-hydrochloric acid for 15min. at 100°) t.l.c. showed the formation of a ninhydrin-reacting lipoidal product which ran identically on t.l.c. with compound (II).

*Comparison of isolated APE and* N-*stearoylphosphatidylethanolamine.* On t.l.c. isolated APE and partially synthesized *N*-stearoylphosphatidylethanolamine ran to identical positions in both solvent systems and this was also true of their partial deacylation products, dAPE and *N*-(stearoyl)glycerylphosphorylethanolamine. The i.r. spectra of both compounds (as smears on rock-salt plates) were virtually identical (Fig. 1).

*Fatty acid composition.* The relative proportions of fatty acids in the *N*-acyl and *O*-acyl positions of *N*-acylphosphatidylethanolamine are given in Table 1. In both positions the fatty acids are predominantly $C_{18}$ and unsaturated, presumably oleic acid and linoleic acid. However, the fatty acids of the *N*-acyl esters are somewhat more saturated, there being a higher proportion of palmitic acid and particularly of stearic acid.

*Presence of APE in other seeds.* Lipid extracts were obtained from a number of other seeds by the same method as used for the pea. These included oats (*Avena sativa* L.), soya bean (*Glycine soja* Sieb et Zucc.), spring bean (*Vicia sativa* L.) and turnip (*Brassica rapa* L.). The lipid extracts were loaded on to silicic acid columns and fractions eluted with chloroform–methanol (19:1, v/v). On examination by t.l.c. in both the acid and the ammoniacal solvents these fractions gave prominent phospholipid spots that ran to the same position as pea *N*-acylphosphatidylethanolamine. On deacylation with alkali all of them gave a lipoidal

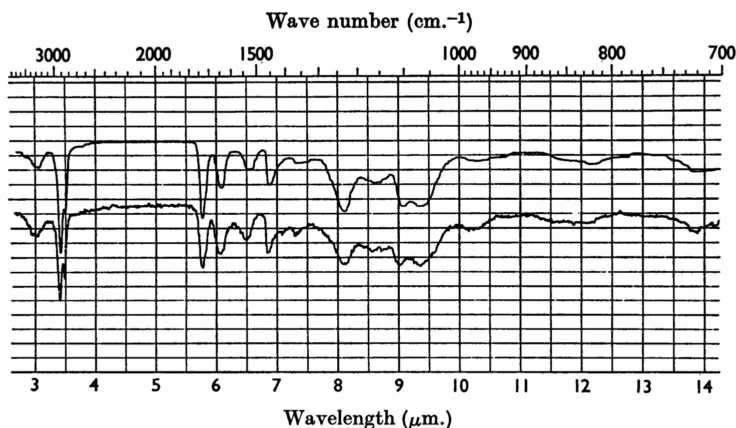



Fig. 1. Infrared spectra of phospholipid isolated from pea seeds (upper trace) and *N*-stearoylphosphatidylethanolamine (lower trace).

Table 1. *Fatty acids in* N-*acyl and* O-*acyl positions of APE*

The compositions are computed as the area of the peak of fatty acid methyl ester as a percentage of the total area of peaks on the chromatogram.

| | Whole phospholipid (after acid methanolysis) | *O*-Acyl fatty acids (alkali-labile) | *N*-Acyl fatty acids (alkali-stable) |
|---|---|---|---|
| $C_{16:0}$ | 14·7 | 11·5 | 19·7 |
| $C_{18:0}$ | 6·1 | 3·5 | 11·3 |
| $C_{18:1}$ | 38·9 | 40·0 | 34·6 |
| $C_{18:2}$ | 37·5 | 42·8 | 32·0 |
| Unknown | 1·1 | — | 0·9 |

Table 2. *Amount of APE in various seeds and its metabolism during early germination*

N.D., Not determined.

| Seed | Time of hydration (hr.) | Increase in weight (%) | Total $\mu$g. of lipid P/seed | Amount of APE/seed ($\mu$g. of P) | APE P as % of total lipid P |
|---|---|---|---|---|---|
| Pea (*Pisum sativum* L.) | 0 | | 125 | 6·0 | 4·8 |
| | 16 | N.D. | 133 | 1·5 | 1·1 |
| | 144 | N.D. | 100 | 1·6 | 1·6 |
| | 0 | | 166 | 10·8 | 6·5 |
| | 24 | 141 | 128 | 2·3 | 1·8 |
| Spring bean (*Vicia sativa* L.) | 0 | | 98 | 2·8 | 2·9 |
| | 48 | 98 | 108 | 1·9 | 1·7 |
| Soya bean (*Glycine soja* Sieb et Zucc.) | 0 | | 83 | 2·9 | 3·5 |
| | 48 | 137 | 81 | 1·2 | 1·5 |
| Oat (*Avena sativa* L.) | 0 | | 3·1 | 0·41 | 13·3 |
| | 24 | 25 | 3·4 | 0·385 | 11·4 |
| | 0 | | 4·1 | 0·48 | 11·6 |
| | 60 | 48 | 4·65 | 0·43 | 9·3 |
| Turnip (*Brassica rapa* L.) | 0 | | | | 0·9 |
| Carrot (*Daucus carota* L.) | 0 | | | | 1·1 |
| Radish (*Raphanus sativus* L.) | 0 | | | | 0·5 |

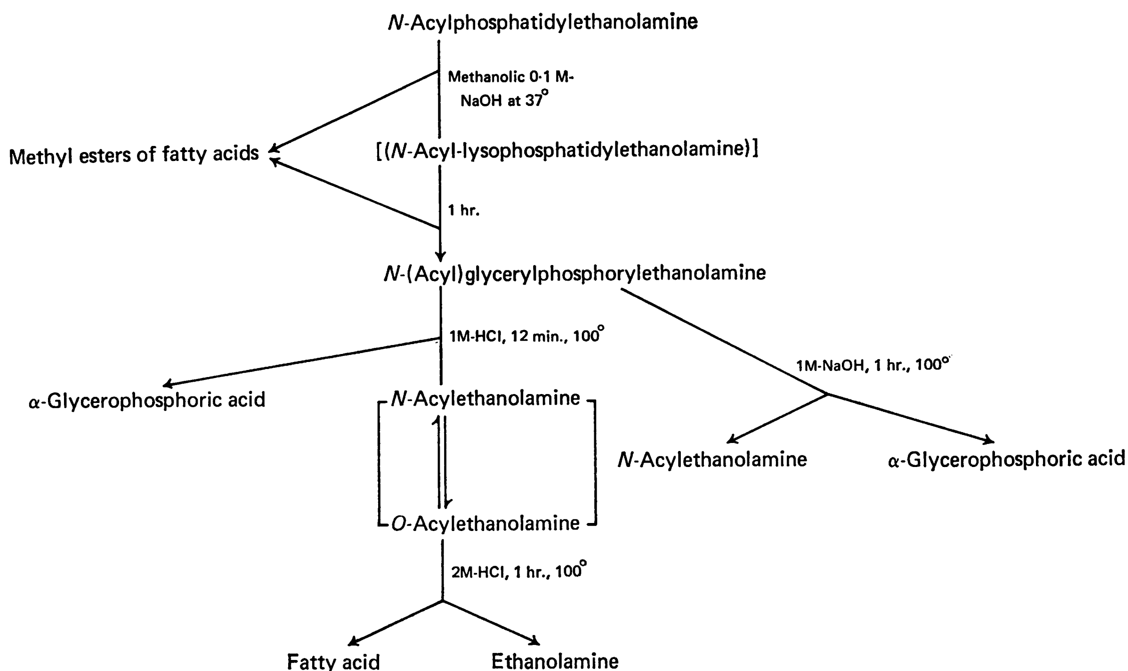
phosphorus-containing product which on t.l.c. ran to the same position as *N*-(acyl)glycerylphosphorylethanolamine.

*Behaviour of APE on germination of seeds.* Quantitative t.l.c. studies were made of the amount of APE in a number of seeds and its behaviour during the early stages of germination (Table 2). The percentage of APE varied considerably, ranging from 13% of the total phospholipids in oats to much lower percentages in radish and turnip seeds. The rapid loss from pea seeds during early germination was confirmed (Quarles & Dawson, 1969). Decreases were also observed in the APE content of soya and spring beans after hydration for 48hr. In contrast, oat seeds, although containing the highest percentage concentration of APE, retained most of it even after germination for 60hr. None of the seeds showed any equivalent loss of total phospholipid P during these early periods of germination.

## DISCUSSION

The present results indicate that the phospholipid in pea seeds which decreases so dramatically in the early stages of germination is an *N*-acylphosphatidylethanolamine. Thus the phospholipid contains fatty acid, glycerol, ethanolamine and phosphate in the molar proportions 2·9:0·97:1·1:1·0, and a diglyceride monoacetate, *N*-acylethanolamine and *N*-(acyl)glycerylphosphorylethanolamine can be demonstrated among the partial-degradation products. Confirmation is provided by the identical behaviour on t.l.c. and the identical i.r. spectra of the isolated phospholipid and authentic *N*-stearoylphosphatidylethanolamine. Scheme 1 shows the sequential chemical degradation of the phospholipid used in the present investigation.

The results fully confirm the initial report of Bomstein (1965) that a phospholipid of this structure exists in Nature. This author described the isolation




Scheme 1. Stepwise degradation of APE with alkali and acid.

of an N-acyl-lysophosphatidylethanolamine from soft wheat flour and provided strong evidence for the existence of N-acylphosphatidylethanolamine, although this was not isolated in a state of purity. The present results, showing that small quantities of the phospholipid are present in all five samples of plant seed examined, suggests that the phospholipid may be fairly widely distributed in such plant tissues. Although the possibility of acylation of phosphatidylethanolamine as an artifact by base-catalysed aminolysis during the isolation (Wren & Merryfield, 1965) has to be borne in mind there is no evidence that this has occurred in the present instance, and the phospholipid is found in preparations in which care has been taken to avoid alkaline conditions. Moreover, phospholipids from other sources examined by the same procedure, although containing abundant phosphatidylethanolamine, contain none of the N-acylated derivative. The demonstration that the phosphorus-containing lipoidal products when soya-bean lipids are mildly saponified with alkali contain N-(acyl)glycerylphosphorylethanolamine invites a comparison with the results of Carter *et al.* (1958). These authors prepared, from soya beans by mild alkaline hydrolysis, a complex phospholipid that contained sugars, inositol, glycerol, phytosphingosine and glucosamine. Ethanolamine was also detected chromatographically although it was not believed to constitute part of the molecule and it seems possible that this might arise from the N-(acyl)glycerylphosphorylethanolamine contaminating the phytoglycolipid.

The significance of the marked decrease in amount of APE during early germination of many seeds is not clear. However, many degradative enzymes in seeds are known to be activated during the initial hydration phenomenon (Mayer & Poljakoff-Mayber, 1963). If phosphatidylethanolamine is produced by the catabolism of APE there would be a pronounced change in the physical characteristics of the seed's membranes at this point. Thus the phospholipid would pass from the acidic to the zwitterionic form and at the same time lose a hydrophobic residue. Another possible catabolic product, N-acylethanolamine, has been reported in lipid extracts prepared from seeds (Kuehl, Jacob, Ganley, Ormond & Meisinger, 1957), but since an alkaline saponification was employed in its isolation it is likely that this had been formed as an artifact by base-catalysed aminolysis (Wren & Merryfield, 1965) or through the saponification of APE. However, N-acylethanolamine has been described as an authentic component of mammalian tissues by Bachur, Masek, Melmon & Udenfriend (1965).

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## REFERENCES

Bachur, N. R., Masek, K., Melmon, K. L. & Udenfriend, S. (1965). *J. biol. Chem.* **240**, 1019.

Bartlett, G. R. (1959). *J. biol. Chem.* **234**, 466.

Bomstein, R. A. (1965). *Biochem. biophys. Res. Commun.* **21**, 49.

Carter, H. E., Celmer, W. D., Galanos, D. S., Gigg, R. H., Lands, W. E. M., Law, J. H., Mueller, K. L., Nakayama, T., Tomizawa, H. H. & Weber, E. (1958). *J. Amer. Oil Chem. Soc.* **35**, 335.

Dawson, R. M. C. (1956). *Biochem. J.* **64**, 192.

Dawson, R. M. C. (1963). *Biochem. J.* **88**, 414.

Dawson, R. M. C., Hemington, N. & Davenport, J. B. (1962). *Biochem. J.* **84**, 497.

Dittmer, J. C. & Lester, R. L. (1964). *J. Lipid Res.* **5**, 126.

Hanahan, D. J. & Olley, J. N. (1958). *J. biol. Chem.* **231**, 813.

Kuehl, F. A., Jacob, T. A., Ganley, O. H., Ormond, R. E. & Meisinger, M. A. P. (1957). *J. Amer. chem. Soc.* **79**, 5577.

Marinetti, G. V. (1962). *Biochemistry,* **1**, 350.

Mayer, A. M. & Poljakoff-Mayber, A. (1963). *Germination of Seeds.* Oxford: Pergamon Press Ltd.

Parker, F. & Peterson, N. F. (1965). *J. Lipid Res.* **6**, 455.

Quarles, R. H., Clarke, N. & Dawson, R. M. C. (1968). *Biochem. biophys. Res. Commun.* **33**, 964.

Quarles, R. H. & Dawson, R. M. C. (1969). *Biochem. J.* **112**, 787.

Renkonen, O. (1965). *J. Amer. Oil Chem. Soc.* **42**, 298.

Roe, E. T., Scanlan, J. T. & Swern, D. (1949). *J. Amer. chem. Soc.* **71**, 2215.

Vaskovsky, V. E. & Kostetsky, E. Y. (1968). *J. Lipid Res.* **9**, 396.

Wren, J. J. & Merryfield, D. S. (1965). *Biochim. biophys. Acta,* **98**, 589.