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OXIDATION OF PEA LIPIDS IN MODEL SYSTEMS

by

(C) MUHAMMAD HAYDAR

A THESIS

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research, for acceptance, a thesis entitled OXIDATION OF PEA LIPIDS IN MODEL SYSTEMS submitted by MUHAMMAD HAYDAR in partial fulfilment of the requirements for the degree of Doctor of Philosophy.

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ABSTRACT

The enzymatic and nonenzymatic pathways of lipid oxidation in pea seeds have been investigated. Individual lipids of seeds and of subcellular particles were isolated and fractionated by column and thin layer chromatography and their fatty acid composition was determined.

Lipoxidase distribution in pea seed, its subcellular particles and cytoplasm revealed the absence of compartmentalization of the enzyme. The enzyme was purified from the seed by ammonium sulfate precipitation followed by Sephadex and DEAE cellulose column chromatography.

Disc gel electrophoresis revealed the presence of 3 - 4 isoenzymes while the molecular weight determination in presence of sodium dodecyl sulfate gave a value of 74,000. The pH optimum of the pure enzyme was 7.2 and the Michaelis constant was 2.3×10^{-3} M.

The specificity study of the enzyme showed for pea lipids, a high activity with the free fatty acids, less activity with pure triglycerides and no activity with phospholipids such as phosphatidyl choline, phosphatidyl ethanolamine and diphosphatidyl glycerol. Furthermore, no activity was obtained with mono- or digalactosyl diglycerides.

The nonenzymatic oxidation revealed an induced oxidation of lipids accompanied by a volume change of viable mitochondria in the presence of Fe^{2+}, Fe^{3+}, Cu^{2+} ions, but not in the presence of Mn^{2+} and Co^{2+} ions or ascorbic acid. Swelling per se did not induce lipid oxidation.

Finally, nonenzymatic oxidation of lipids coated on matrices such as carbohydrates and pea proteins was found to depend on both the

the matrix used and the lipid class being oxidized. Upon UV irradiation of the methyl esters of oleic, linoleic and linolenic acids, the free radicals formed were readily detectable by electron spin resonance spectroscopy.

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LIST OF ABBREVIATIONS

ADP	Adenosine diphosphate
ATP	Adenosine triphosphate
BSA	Bovine serum albumin
CMP	Cytidine monophosphate
DEAE-cellulose	Diethyl aminoethyl cellulose
DGDG	Digalactosyl diglyceride
DPG	Diphosphatidyl glycerol
EDTA	Ethylene diamine tetraacetic acid
EGS	Ethylene glycol succinate
EM	Electron microscopy
ESR	Electron spin resonance
FA	Fatty acid/s
FAME	Fatty acid methyl esters
FFA	Free fatty acids
GLC	Gas liquid chromatography
HMDS	Hexamethyldisilazane
LPC	Lysophosphatidyl choline
LPE	Lysophosphatidyl ethanolamine
MGDG	Monogalactosyl diglyceride
NL	Neutral lipids
NMR	Nuclear magnetic resonance
OV-17	Phenyl methyl silicone, 50% phenyl
PA	Phosphatidic acid
PC	Phosphatidyl choline

PE	Phosphatidyl ethanolamine
PG	Phosphatidyl glycerol
PI	Phosphatidyl inositol
PL	Polar lipids
PL	Phospholipids
PS	Phosphatidyl serine
RCR	Respiratory control ratio
SCE	Saturated calomel electrode
SDS	Sodium dodecyl sulfate
SE-30	Methyl silicone
SL	Sulfolipid
SG	Sterol glycoside
TBA	Thiobarbituric acid
TEMED	N, N, N', N' - tetramethyl - ethylenediamine
TES	N-tris (hydroxymethyl) methyl - 2 - aminoethane sulfonic acid
TG	Triglycerides
TL	Total lipids
TLC	Thin layer chromatography
TMCS	Trimethylchlorosilane
Tris	Tris (hydroxymethyl) amino methane

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I. INTRODUCTION

Quality changes in food may result from microbiological, enzymatic and nonenzymatic changes. The latter two are of particular importance in preservation of fresh, enzyme active, and processed, enzyme inactive peas. Deterioration involving off-flavor development in both cases occurs mainly via lipid oxidation. The off-flavors developed in unblanched peas during cold storage were characterized by Bengtsson et al. (1967), and pea lipoxidase was recently studied by Eriksson and Svensson (1970). However, a comprehensive study dealing with pea lipids, the effect of lipoxidase and the involvement of proteins and carbohydrates has not been done. Therefore, this study was undertaken in an attempt to explain the roles of these various components in pea lipid oxidation.

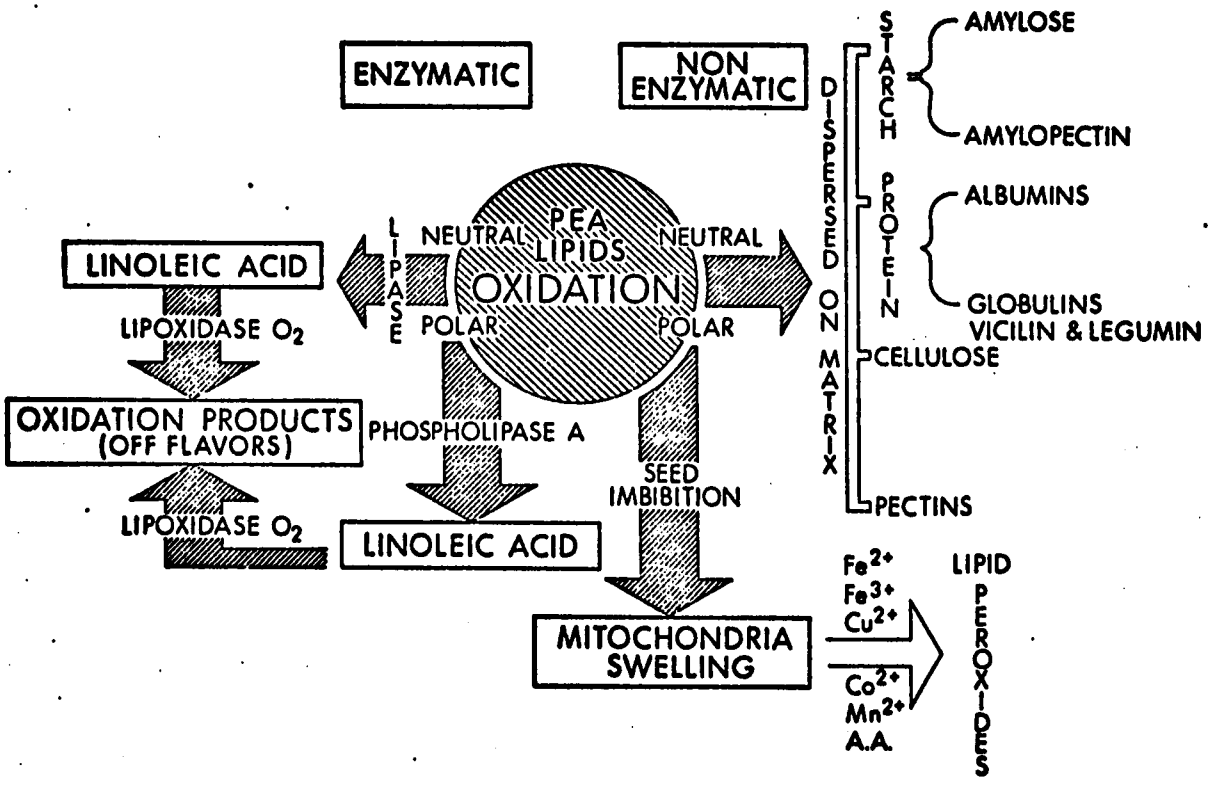
The possible compartmentalization of the lipoxidase enzyme (1.99.2.1) in the subcellar particles of germinated pea seeds and of pea seedlings was investigated. The enzyme was isolated from pea seeds and its purity and molecular weight were determined by gel electrophoresis which also revealed the presence of lipoxidase isoenzymes.

Pea lipids were separated into individual components and their fatty acids were determined. The extent of oxidation of these lipids by the partially purified pea lipoxidase was investigated.

In order to elaborate on the nonenzymatic oxidation pathway, it was necessary to isolate pea albumins and globulins and to determine the mineral composition of peas. Additionally, it required the isolation

of viable mitochondria. Finally, the effect of proteins and carbohydrates as matrices on the oxidation of the isolated neutral and polar lipids was investigated.

The envisaged approaches for this study are illustrated below:



II. REVIEW OF LITERATURE

A. Peas, General Aspects

Garden or canning peas (Pisum sativum, L.) are, besides being among the most nourishing vegetables, a widespread and important constituent in the diet of most races of people (Lynch et al. 1959). They have a high content of carbohydrates and proteins, and provide an excellent source of carotenes, ascorbic acid and thiamine. Both the smooth-seeded and wrinkle-seeded peas are of commercial significance. Due to their importance in food processing, peas were and still are the object of many scientific and technological studies. These include chemical composition, biochemical and physiological studies during growth and post-harvest storage, and changes induced by processing, quick freezing, and dehydration. Related to these are the changes in color, flavor and texture. Since the aim of this study is to elucidate the enzymatic and non-enzymatic pathways of pea lipid oxidation--which may cause the off-flavor production--only those reports related to or indirectly implicated in lipid oxidation will be reviewed.

No data are available about the status of lipids in pea seeds. No studies about the association of lipids with protein or starch bodies or matrices have been reported. However, such data have been reported for wheat. Hess (1954) suggested the presence of large concentrations of bound lipids, high in phospholipid content, surrounding the starch granules. Furthermore, he differentiated between lipid-rich protein

having a fibrillar structure, and the matrix protein which was amorphous. Seckinger and Wolf (1967) using EM and thin sectioning technique, could not prove the presence of a continuous layer of proteins rich in lipids around the starch granule. Small patches of protein on the starch surface havebeen ascribed as being part of the matrix protein. In addition no differentiation could be observed between the "wedge" amorphous protein, filling the space between starch granules and "adhering" proteins. The free lipids were found as dark staining inclusions, but not as distinct bodies, randomly distributed throughout the protein matrix. However, the bound lipids were found in small inclusions throughout the protein suggesting that they are remnants of cytoplasmic structures present in the endosperm cells at the time of maturation.

Contrary to lipids, intracellular distribution of proteins in pea cotyledons has been investigated (Varner and Schidlovsky 1963). Examination by EM revealed the presence of all structures of a normal cell i.e., nuclei, mitochondria, chloroplasts, and starch granules. In addition, a major fraction of the cell volume was occupied by large roughly spherical bodies with no visible internal structure. These bodies preserved their identity after homogenization of whole pea cells and could be isolated by low speed centrifugation. By subsequent separation on a DEAE cellulose column, the authors proved the identity of these bodies as globulins. The bodies appeared in pea seeds at various stages of growth and maturation, but upon germination for several days they gradually disappeared. A surrounding membrane around the protein body was suggested, but unequivocal proof was not provided. Simultaneously, it was concluded that the globulins are the major

proteins of pea cotyledons.

Characterization of pea globulins has been reported by Danielsson (1949, 1950) and Danielsson and Lis (1952). It was demonstrated that globulins consist of two proteins, vicilin (mol. weight 186,000) and legumin (mol. weight 331,000) readily separable by isoelectric precipitation at pH 4.7 (0.2 M NaCl), in which condition legumin precipitates while vicilin remains in solution. Free boundary electrophoresis revealed the homogeneity of both proteins, while the amino acid composition revealed differences between them and from pea albumins.

Vaintraub and Hofman (1961) demonstrated that N-terminal amino acids are aspartic acid and serine, for vicilin, glycine and leucine for legumin. In addition, these authors suggested that vicilin consists of three chains each having a molecular weight of 58,000, while legumin twelve polypeptide chains each of an average molecular weight of 33,000.

In a study related to variability of proteins in pea seeds during ripening Klimenko and Pinegina (1964) reported that at early stages of ripening vicilin prevails over legumin in a ratio 3:1, while in fully ripened seed an increase in legumin and a decrease in vicilin occur reaching nearly an equal ratio.

Basic information on the nature and behaviour of purified starches from peas with respect to their iodine binding capacity, Brabender viscosities, patterns of granule swelling, and solubilization during pasting were reported by Schoch and Maywald (1968). Wrinkled peas had a highly restricted swelling due to the internal rigidity

imparted by high content of associated linear molecules present in the granule. Iodine affinity of 15.2% indicated in starch the presence of at least 75% of amylose. Smooth pea starches showed Brabender curves with no pasting peak and with constant or increasing viscosity during heating at 95^{o}, reflecting also a high degree of crystallinity within the starch granule.

McCready et al. (1950) developed a method for determination of total starch and amylose contents in vegetables and found for wrinkled pea varieties, Stratagem and Laxton, about 33% starch of 70% amylose and for the smooth pea variety Alaska, 44% starch of 36% amylose.

Pectin content of peas and its influence on the texture of the processed peas were partly investigated (Haisman 1962). The relative amounts of water soluble, water soluble but rendered insoluble by Ca^{2+}-ion, and water insoluble pectins were reported for dried and cooked, hard and soft peas. On cooking, the water extractable pectin rose to about 50%, and was due to the release of Ca-bound pectin. Surprisingly, no significant difference in pectin fractions was obtained for hard and soft peas, but the hard peas had a slightly lower degree of esterification than the soft ones. Extensive de-esterification occurred during soaking and even more during cooking. The water soluble pectin was found to be about 70% esterified, while the fraction bound to Ca^{2+}-ion was less than 10%. The intercellular location of pectin in peas both in meristematic cell walls and in parenchyma tissue suggests a spatial segregation of pectin and lipids within the fresh seed, but not after cooking. As reported by Haisman (1962), the cooked peas can be considered as a conglomerate of swollen tissue cells in a gel,

formed by the partial dissolution of the intercellular pectic substances. Hence there is a possibility that pectin may assume the role of a matrix in subsequent oxidation of the available lipids.

It has been known for many years that unblanched peas undergo substantial changes during cold storage. Within several weeks the presence of free fatty acids and their peroxides could be demonstrated and simultaneously, characteristic off-flavors could be detected (Lee et al. 1955). These changes are catalyzed by enzymes and seem not to occur in blanched peas at comparable rates. Data on hydrolytic changes i.e., formation of free fatty acids in lipids of unblanched peas stored between -5 to -20^{0} were presented by Bengtsson and Bosund (1966). The value of Q_{10} in this temperature range was found to be 2.5, while the corresponding value for off-flavor development was 3.0. Analysis by GLC revealed the preference of unsaturated fatty acids hydrolysis, a tendency being particularly evident at lower temperatures. Net change for linoleic and linolenic acids was observed suggesting their substantial breakdown into smaller molecules detectable in storage headspace.

Bengtsson and Bosund (1964) and Bengtsson et al. (1967) applying the headspace analysis technique characterized the off-flavors developed during cold storage of peas. They identified ethanol, acetaldehyde, and hexanal among the volatiles and suggested that the latter is formed either by lipoxidase action or through autoxidation in processed peas. Ethanol appeared only before processing and seemed to be a result of enzyme activity. Furthermore, the authors suggested that the level of hexanal, rather than that of ethanol or acetaldehyde, might be used as a measure of off-flavor development.

Similar findings for frozen green peas were reported by Joslyn and David (1952), David and Joslyn (1953), and more recently by Whitfield and Shipton (1966). However, up to twelve compounds of carbonyls were detected consisting of 2-one, homologous n-aldehydes, alk-2-enals and alk-2,4-dienals, which strongly suggested the involvement of lipids in off-flavor development.

B. Pea Lipids

1. Lipids of the seeds

Wagenknecht (1957a) studied the lipids of green peas and found that they comprise 6% of the dry weight of peas. He fractionated the crude lipids with acetone, and the acetone soluble portion was subjected to countercurrent distribution between n-heptane and 95% methanol. The heptane fraction was found to consist mostly of mixed TG; the methanol fraction was a mixture of TG, PL, sugars and nitrogenous compounds. The acetone-insoluble fraction contained 10% PI and nearly equal amounts of alcohol-soluble and alcohol-insoluble PL. Wagenknecht et al. (1959) isolated Ca-Mg salts of PI from pea lipids and obtained a yield of 5% of the total lipids. PI was obtained from raw peas of all stages of maturity, excluding the dry pea seeds. The occurrence of plasmalogens in lipids of green peas has also been reported (Wagenknecht 1957b).

Quarles and Dawson (1969) reported PC as the major phospholipid in pea seeds followed by PI, PE, and PA. The phosphorus of these lipids was in the ratio of 1:0.32: 0.27: 0.06 respectively. The seeds were found virtually devoid of plasmalogens and sphingomyelin. Other phospholipids; PS, PG, DPG were present in minute amounts. However,

another unidentified phospholipid that was not attacked by phospholipase D, disappeared rapidly during early germination, and had the highest R_f value on TLC among PL, was present to the extent of 5% of total PL. This phospholipid was later identified as N-acyl phosphatidylethanolamine (Quarles et al. 1968; Dawson et al. 1969).

Adhikari et al. (1961) reported that, on dry basis, leaves, pods, and seeds of the common pea contained 10.3, 2.4 and 4.8% lipids respectively. Leaf lipids contained much higher concentration of galactose compared to lipids from pods and seeds. The authors claimed that while TG are the main components of seed lipids, traces of galactolipids may also be present. However, no analytical data were presented to support this conclusion.

The FA composition of pea lipids was reported in few studies. Bengtsson and Bosund (1966) found the following FA, in a decreasing order: linoleic, palmitic, oleic, linolenic and stearic acid. Palmitic acid was higher in PL than in NL while the contrary was true for oleic and linolenic acids. The ratio of oleic to linolenic acids differed considerably from one harvest to another. Both palmitic and linoleic acid ratios decreased slightly with maturity, while linolenic increased and oleic did not change. Great differences were found between FA composition of lipids from pea cotyledons and skins, especially with regard to oleic and linoleic acids. In the cotyledons, these acids occurred in about equal amounts, while in the skins, the amount of linoleic acid was about 9 times as high as that of oleic.

Similar FA composition to that found by Bengtsson and Bosund has also been reported by Lee and Mattick (1961). However, a different

picture has been obtained by Azarova and Olifson (1971). These authors studied FA composition in five pea varieties and reported the presence of 9 to 10 FA ranging from C_{14} to C_{20} in chain length. The concentration of palmitic acid was considerably less than that reported in the earlier studies while that of oleic acid was much higher and that of linoleic acid was comparable.

General conclusions about FA of plant PL have been drawn by Aylward (1956). a) Saturated FA tend to form a greater percentage of PL than of the corresponding glycerides, and the amount of palmitic may be high, up to 20%. b) All acids occurring in the glycerides are found in the corresponding PL. c) Linoleic acid may be regarded as a characteristic PL acid, at least in seeds. d) Small amounts of highly unsaturated long chain acid may occur in seed PL but not in the corresponding glycerides, the highly unsaturated acids being characteristic of animal PL.

2. Lipids of seedlings and subcellular particles

Changes in lipid composition during greening of etiolated pea seedlings have been studied by Trémolières and Lepage (1970). After 7 days of germination in the dark, the cotyledons, stems and young leaves were rich in linoleic acid; but after illumination, a significant increase in linolenic acid was observed in the young leaves, whereas only small variations were noted in the FA composition of the other parts. The increase in linolenic acid resulted from the increase in galactolipid content of the young leaves.

The total PL present in the cotyledons of pea seeds were depleted during germination and growth (Quarles and Dawson 1969).

Of the individual phospholipids, PC and PE showed the same loss in 11 days as the whole PL fraction, whereas PI was decreased to a greater extent and DPG and PS were not decreased. There was no increase of PA as might have been expected if the PL had disappeared through hydrolysis by phospholipase D.

Allen et al. (1964, 1966) reviewed the methodology for the separation of plant lipids and studied the lipids of spinach leaf and chloroplast lamella. They used countercurrent distribution, DEAE cellulose and silicic acid chromatography, TLC and GLC. The presence of 17 lipids in spinach leaf, 8 of which were unknown, was reported. The major lipids were MGDG, DGDG, PG, SL, PC, and PI, while PE, sterol glycosides and cerebrosides could not be detected in chloroplast lamella lipids. The presence of polygalactolipids, tri- and tetra-galactosyldiglycerides in spinach chloroplasts has been reported by Webster and Chang (1969). A phytoglycolipid has been isolated from leaves of green beans by Carter and Koob (1969).

Lipid composition of leaves of 20 photosynthetic species has been reported by Roughan and Batt (1969). The average molar ratios of chloroplast lipids were reported to be approximately, 7, 5, 0.8, 1 for MGDG, DGDG, SL and PG respectively, but the standard deviations were rather large. Pullishy (1971) isolated lipids of Phaseolus vulgaris chloroplast and reported the presence of the above mentioned lipids as the only major ones present. On the other hand, Ongun et al. (1968) reported the presence of PC, PE and PI in chloroplasts isolated by aqueous and nonaqueous techniques. Similar lipid composition was found in both chloroplast preparations.

The function and metabolism of fatty acids and acyl lipids in chloroplasts have been reviewed by Nichols and James (1968). They stated that leaf chloroplasts possess a much simpler acyl lipid composition than the whole cell and contain MGDG, DGDG, SL and PG as the only major acyl lipids. The presence of PC in spinach chloroplast lamella (Allen et al. 1966) was explained to originate from the mitochondrial-microsomal fractions of the leaf. Whole etiolated leaves contain the same lipid classes of green leaves, but in different proportions. However, trans-3-hexadecenoic acid was found in the green leaves but not in the etiolated tissues (Nichols et al. 1965; Nichols 1965). Wintermans (1960) reported more galactolipids in the green than in the yellow leaves of Phaseolus vulgaris. Rosenberg and Gouaux (1967) reported the accumulation of chlorophyl and galactolipids simultaneously at almost linear rates during light-induced formation of chloroplasts in Euglena gracilis.

Leaf chloroplasts contain a high proportion of polyenoic acids, found in combination with galactolipids, and chloroplasts from different plant species differ in their FA composition more than in the corresponding acyl lipid composition (James and Nichols 1966). Palmitic, oleic, linoleic and linolenic acids are the principal constituents of chloroplast FA (Wolf et al. 1966). The FA are not randomly distributed between the different acyl lipids, but show a high degree of specificity for certain lipids (Allen et al. 1966; Nichols 1965). As reported by many authors (O'Brien and Benson 1964; Sastry and Kates 1963; James and Nichols 1966; Van der Veen et al. 1967; Gardner 1968; Roughan 1970) MGDG contains linolenic acid predominantly, as does DGDG. However, the latter contains larger concentration of $C_{16:3}$ than the former. Palmitic acid is

found mainly with PG and SL, whereas trans-3-hexadecenoic acid is found only in PG. The FA composition of the other lipids shows considerable variations.

Surprisingly, the lipids of plant mitochondria have scarcely been investigated compared to those of chloroplast or animal mitochondria. Kahn and Hanson (1959) found that isolated corn mitochondria show a rapid loss of lipid phosphate during incubation in K^+ containing media. They suggested that the loss of lecithin destroys the integrity of the diffusion limiting barrier allowing the leaching out of accumulated K^+ ion. Lecithin breakdown was partially reversed by the addition of glycerophosphate, acetylcholine and CMP. The results indicated endogenous synthesis of lecithin which prevented the back diffusion of K^+ ion.

The FA of sweet potato mitochondria have been reported by Richardson et al. (1962) who pointed out the absence of any detectable amounts of arachidonic acid. The major FA were linoleic, palmitic and myristic in amounts of 32, 28 and 25% of the total FA present. On the other hand Uritani and Yamaki (1969) reported the ratios of these acids as 41, 15 and 3%, and found that lauric acid comprised 12% of the total FA. Furthermore, they reported the presence of a long chain monoenoic fatty acid (22:1) to the extent of 4%. However, the scarcity of data in the literature does not contribute to a clear understanding of the lipids and fatty acids present in plant mitochondria.

C. Nonenzymatic Oxidation of Lipids in Model Systems

1. Effect of solid supports

The removal of water from a food by freeze-drying results in a product that has a sponge-like matrix. The porosity of the food then

permits oxygen ready access to the matrix components of the food thereby initiating oxidative changes in which the autoxidation of the lipids is of major importance.

a. Carbohydrates as matrix

Starch

Commercially prepared starches contain a small amount of lipids in which palmitic, oleic and linoleic acids are the major FA. FA of most starch granules can be made available for extraction with the usual nonpolar lipid solvents, only by preliminary acid, diastatic, or alkaline rupture, and hydrolysis of the granule structure (Gracza 1965). Oleic acid has been reported to be able to form a crystalline complex with the linear fraction of starch. When the cereal starches are dispersed in water and subjected to a direct current potential, a portion of linear fraction electromigrates by reason of the charge imparted by this absorbed FA. Moreover, the linear fraction, isolated by butanol precipitation absorbed FA in preference to iodine. Thus the addition of 7% oleic or 10% palmitic acids to this fraction completely repressed its iodine binding capacity. Similar effect has been observed upon the addition of 2 - 2.5% FA to the raw starches (Schoch and Williams 1944).

As reviewed by Senti and Erlander (1964), amylose forms inclusion compounds that are stable in solution as well as in solid states. This is contrary to many clathrates non-stoichiometric compounds which exist only in the solid state, and in which guest molecules are included in cavities or cages provided by the crystalline structure of the host. Hence, amylose in solution also provides a cavity in which guest molecules such as FA can be bound.

Amylose complexes with long chain FA are microcrystalline. Mikus et al. (1946) have determined unit cells from powder patterns of the lauric, palmitic, stearic and oleic acid complexes. That the FA is located within the amylose helix was further supported by two additional observations: firstly, the FA can be introduced into dried butanol-precipitated amylose by suspension in either alcohol or carbon-tetra-chloride solutions of FA, secondly, on exposure to iodine vapor, most of the FA are displaced from the complex.

A constant composition of the long chain FA complexes might be expected on the weight basis if the FA molecules are packed end to end in the helices. From the data of Mikus et al. (1946) amylose binds 6.5% of FA, which is about 20% less than the capacity of the helix, a discrepancy that could be attributed to the presence of contaminating amylopectin.

After exhaustive extraction of wheat starch granules with methanol or dioxane, Wren and Merryfield (1970) found that the granules still contained measurable yields of lipids. When the granular structure was disrupted by gelatinization in boiling water or by erosion in pure dimethyl sulphoxide, the lipids present were released and consisted mainly of lysolecithin. By the erosion technique they confirmed the distribution of lysolecithin at all depths within the starch granule structure.

Similarly, Acker and Schmitz (1967) obtained lysolecithin from starch granules of wheat rice and maize and gave evidence that this is a natural constituent rather than an artifact from lecithin cleavage during isolation.

Since firmly bound lipids could be released from wheat starches by mild chemical methods, it is most unlikely for lipids to be bound covalently. Lysolecithin was suggested to be held in starch granules in helical complexes by noncovalent forces. A simple laboratory procedure for the formation of a lysolecithin-rice amylose complex has already been reported (Nakamura et al. 1958).

An indication for the existence of amylose-FA complexes in model systems might also be supported by the observed viscosimetric effects of fats and non-ionic surfactants on starch pastes. Contrary to natural and hydrogenated fats where no effects were observed on the gelatinization or on the cooling curves of starch pastes, mono-chain structural surfactants brought about a marked increase in the temperature at which viscosity increased and a marked change in the shape of cooling curves (Osman and Dix 1960).

In a more recent study, interactions between glycolipids and gelatinized wheat flour starch were reported using IR and NMR spectroscopy (Wehrli and Pomeranz 1970). The results obtained by IR suggested that the polar galactosyl moiety of the lipid is bound to the starch by hydrogen bonds, whereas the FA chain is free and existing in an extended trans conformation. The NMR spectra showed that the terminal methyl group of the acid is involved in binding rather than the whole acid chain.

Finally, with the exception of a study by Bishov and Henick (1961) which involved carbohydrates as matrix in model systems for lipid oxidation, where only carbohydrates possessing a charge exhibited a protective action against lipid oxidation, no other study applying

starch or its fractions as matrix, has been reported.

Cellulose

Autoxidation of methyl linoleate in a freeze-dried model system based on microcrystalline cellulose was reported by the same group of authors (Maloney et al. 1966; Labuza et al. 1966; Karel et al. 1966; and Tjhio and Karel 1969). In these reports the FA ester component was thoroughly purified through urea inclusion compound and as such was used with cellulose in a weight ratio 1:6. The initial stage of lipid oxidation was achieved by extracting the freeze-dried system with methanol and measuring the diene conjugation at 233 nm, while later the oxidation was followed manometrically. In such model system it was found that water has an inhibitory effect being most pronounced in the initial stages of oxidation, including the period in which the hydroperoxide decomposition follows the monomolecular kinetics.

In the same model system the effect of water on cobalt catalyzed oxidation was also studied. Again an inhibitory effect of water was demonstrated in the period of monomolecular decomposition and during the hydroperoxide decomposition followed by a bimolecular kinetics. The inhibition by moisture was interpreted as due to a deactivation of the metal catalyst by hydration, and as due to a possible hydrogen bond existence between water and hydroperoxides, which bond could then interfere in subsequent stage of bimolecular decomposition (Labuza et al. 1966).

In a system based again on microcrystalline cellulose the oxidation of methyl linoleate in presence and absence of added amino

acids was investigated. In the absence of water, histidine, lysine and cysteine have shown an antioxidant activity, the nature of which was unique since it prolonged the induction period and affected only the initial rate of oxidation but not the subsequent bimolecular phase of oxidation. As is well known, the commercial antioxidants perform an inhibitory effect in the second phase also (Karel et al. 1966).

A study on the effect of heavy metals possessing multiple valency states, such as transition metals, and of histidine on the autoxidation of methyl linoleate on ash-free filter paper as the solid matrix has been reported by Tjhio and Karel (1969). As earlier, the oxidation was followed by the increase of diene conjugation and manometrically. Thus cobalt and manganese were found to have a pronounced catalytic effect on the lipid oxidation, but only at high and not low values of pH. Histidine enhanced the catalytic effect of manganese but inhibited that of cobalt. The same study has shown that in the presence of manganese, histidine decomposes and the decomposition products actually enhanced the catalytic degradation of lipid.

Nevertheless the above studies do not include investigation of lipid oxidation as influenced by cellulose itself neither do they present a comparison of this matrix with other potential porous sponge-like food matrices such as starch and other carbohydrates. The ash-free cellulose was simply accepted with the assumption of being an ideal inert lipid carrier.

Pectic substances

With the exception of data from Bishov and Henick (1961), that pectin exhibits a protective action against lipid oxidation, which

protection is correlated with methylation degree, or with the residual negative charges of pectin-chain molecules, no other data so far have been reported.

b. Proteins as matrix

Studies of lipid oxidation on model systems involving protein as carrier matrix have shown that on a smooth dry protein surface, such as gelatin, the extent of lipid oxidation was less than one half of that observed on an inert glass surface. Furthermore, the range of oxidation rates on various gelatins was found to correlate with the numerical difference of pI - pH, which difference was considered as a possible measure of the residual charges on the protein surface (Togashi et al. 1961). In addition the correlation suggested that a lipo-protein orientation may account for the reduced oxidation rate of the lipids.

PL were also assayed and found to enhance the protective action of protein against oxidation of refined cottonseed oil. Furthermore, evidence was obtained that the order of layering the lipid and PL on the protein matrix was of importance. The PL prevented the autoxidation only when it was layered on lipid, or when it was sandwiched between the protein and lipid, but not when it was mixed with protein before film formation (Togashi et al. 1961).

The protective effect of PL was more clearly shown in freeze-dried emulsion model systems. Applying carboxymethylcellulose as an emulsion stabilizer and as a relatively inert matrix, it was demonstrated that protein exerts a protective effect. The protection of a mixture of protein and PL was additive (Bishov et al. 1960).

There are few recent studies on oxidation of protein-lipid

systems in which changes in protein after oxidation were investigated. These results indicated an extensive browning accompanied by co-polymerization of oxidized lipids and proteins. Thus Andrews et al. (1965) studied changes in protein after oxidation of gelatin-methyl linoleate system and reported aggregation of proteins.

Zirlin and Karel (1969) studied the same system by incubating it in air at 50^{0}. Such incubation caused a drop in the viscosity of gelatin solutions, an increase in the retention time of gelatin on a Sephadex G-150 column and a reduction in the melting point of gelatin gel. No such changes in gelatin occurred when incubation was performed at a relative humidity of 60%. Experiments using C^{14}-labeled methyl linoleate showed that oxidation caused incorporation of linoleate derived C^{14} into gelatin, which incorporation was greatly suppressed in presence of moisture.

Roubal (1970, 1971a) monitored the free radical concentration in a lipid protein system with a low moisture content, both during and after oxygen absorption by the lipid. The FA used consisting of $C_{22:6}$ and $C_{20:5}$ and the test mixtures were prepared merely by mixing together lipid and protein in a ratio of 1:2 w/w. In addition to a major central ESR signal in g= 2 region in samples containing oxidizing or oxidized lipids, there appeared one and or two additional signals downfield from the central resonance peak, i.e., lipid signal region. Following the signal intensities of the free radicals by ESR, it was demonstrated that a rapid accumulation of radicals occurred during the time when lipid absorbed oxygen. When a marked decrease in radical content was noted, an iminopropene Schiff-base fluorescence was detected, indicative of a

malonaldehyde-amino acid interaction (formation of N,N'-disubstituted 1-amino-3-iminopropene). Amino acid analyses correlated well with time course observations of trapped radicals and fluorescence. These results further strengthen the suggestion first introduced by Roubal and Tappel (1966) that radical and not aldehyde attack on protein is predominantly responsible for protein damage in a protein lipid model system.

To assess thoroughly the rate of lipid oxidation in a protein-lipid model system, a possible lipid protein interaction preceding oxidation should also be considered. There is ample evidence of such interaction in vivo with biological membranes as one example. Some recent data presented evidence of such interaction in vitro using lipid and wheat flour protein model systems. Thus IR spectroscopy indicated a glutenin galactosylglyceride complex in which the lipid was not bound by Van der Waals forces alone. In the complex, the absence of the methylene rocking band suggested a nonpolar interaction. The NMR spectra of galactosylglyceride in D_2O revealed well defined peaks for both methyl- and methylene groups. In the presence of glutenin, the latter was substantially reduced while the former remained unchanged. Since glutenin alone produced no methyl peak, it was concluded only the middle portion of the FA chain is bound to protein, while the methyl terminal is apparently free (Wehrli and Pomeranz 1970).

Galactosylglyceride binding of gliadin was weaker than that of glutenin. Extraction with petroleum ether only reduced but not removed the bands assigned to the ester and C-H stretchings. In addition no methylene rocking band was present, which findings indicated that glycolipid is bound with gliadin by both Van der Waals and hydrogen

bonds (Wehrli and Pomeranz 1970).

Similar results of lipid-gliadin and glutenin interactions have been obtained by analysing their complexes isolated from wheat flour with different lipid solvents. These analyses have shown that lipids bound to glutenin were mainly nonpolar TG and glycolipids bound with their hydrophobic part of the molecules, i.e., petroleum ether soluble. The lipids bound to gliadins were all polar, mainly MGDG and DGDG bound with their hydrophilic part of the glycolipid, i.e., soluble in water saturated butanol (Hoseney et al. 1970).

Interaction of PL, triphosphoinositide and PS with water soluble wheat proteins has also been reported (Fullington 1967). In such model systems, weak interactions were observed using starch gel and free boundary electrophoresis techniques. In presence of Ni^{2+}, Ca^{2+}, or Mg^{2+} ions the interactions were greatly enhanced and the pattern of protein species bound by the lipid-metal complex was dependent on both the PL and the metal ion used. The striking aspect of this observation was the specificity of certain protein components towards forming such complexes.

2. Lipid peroxidation induced by mitochondria swelling

The osmotic swelling-contraction characteristics of plant mitochondria have been studied by Stoner and Hanson (1966); Earnshaw and Truelove (1968); Yoshida and Sato (1968); and more recently by Overman et al. (1970) and Malhotra and Spencer (1970). From these studies, it is well established that the plant mitochondria undergo a respiration independent "large amplitude" swelling in electrolyte solutions. After reaching an equilibrium volume, this process may be

reversed into contraction by the addition of an oxidizable substrate or by ATP.

Plant mitochondria undergo a similar swelling when transferred from a sucrose medium to isomolar solutions of monosaccharides such as glucose or ribose (Lorimer and Miller 1969). The respiration-independent swelling of mitochondria (water influx) in solutions of electrolytes such as KCl has been suggested to be due to dissipation of a high energy bond associated with the relaxation of a contractile mechanism regulating mitochondrial volume (Earnshaw and Truelove 1968). Contrary to animal mitochondria, plant mitochondria swell passively; (substrate independent), while they contract actively; (substrate dependent).

Hoffsten et al. (1962) and Hunter et al. (1963) found that swelling is promoted by ascorbate, reduced glutathione and Fe^{2+}. This induced swelling did not depend on endogenous or added substrates, but it correlated with lipid peroxides formed. They also observed that an extensive lipid peroxidation leads to lysis and disintegration of mitochondria. In addition they found that swelling per se does not result in lipid peroxide formation because swelling induced by phosphate anion, FA, detergents and aging in KCl is not associated with lipid peroxide formation.

Corwin (1962) has shown that lipid peroxide formation may also result from electron transport chain activity if the tissue, from which mitochondria were isolated, is deficient in vitamin E.

Nonenzymatic formation of lipid peroxides in mitochondrial suspensions upon addition of ascorbic acid and iron salts was also reported by Ottolenghi (1959). He found a quantitative relationship existing between the formation of peroxides and the oxidation of ascorbic

acid. Addition of EDTA resulted in total inhibition of peroxide formation in presence of iron salts, whereas oxidation by ascorbic acid was not inhibited.

Yoshida and Sato (1968) observed that mitochondria swell osmotically within the first 10 seconds irrespective of the kind of solute. After that they swell markedly with time in monosaccharide solutions, but scarcely in di-, or tri-saccharide solutions. In inorganic electrolyte solutions such as chlorides of K^+, Na^+, Li^+, NH_4^+, Ca^{2+} or in K^+ salts of different anions, mitochondria swell more rapidly than in sugar solutions.

Lehninger and Remmert (1959) first described an enzymatic formation of "U factor" within mitochondria which could cause swelling. Since then the factor was characterized as an endogenous free FA. Wojtczak and Lehninger (1961) demonstrated protection against "U factor"-induced swelling by the addition of serum albumin, which binds FA.

In a report on relation of mitochondrial phospholipase A activity to mitochondrial swelling, Waite et al. (1969) indicated that the swelling effect is primarily brought about by phospholipase A activity, and that the amount of FA hydrolyzed from endogenous PE and PC during swelling was 20-30 times less than the amount of added FFA that gave comparable swelling.

Earnshaw et al. (1970) in an extensive study on the swelling of Phaseolus mitochondria observed that spontaneous swelling is strongly inhibited by BSA, suggesting that its swelling could be a consequence of endogenous free fatty acids acting as detergents on the membrane. Nevertheless swellings due to these phenomena did not produce lipid

peroxides unless iron or ascorbic acid were present.

Hoffsten et al. (1962) observed that Fe^{2+} induced swelling of mitochondria in KCl medium but not in sucrose, with concurrent peroxidation of lipids. In addition the study indicated that Fe^{3+} alone gave no swelling, while Fe^{2+} ions caused relatively little swelling. On the other hand the presence of both ions induced swelling after only a short lag period. The rate of swelling was related to the amount of lipid peroxide formed.

Cash and Gardy (1965) suggested that the enhanced swelling observed with reduced glutathione is largely accompanied by enhanced lipid peroxidation due to Fe^{2+} ion present as a contaminant. They also noted that this ion is a much more potent swelling agent than Ca^{2+}, Zn^{2+}, and/or Cu^{2+}. However treatment of mitochondria with Fe^{2+} may not always result in swelling. McKnight and Hunter (1966) upon treating dilute suspensions of mitochondria with Fe^{2+} reported lipid peroxidation, extensive fall in turbidity value and loss of 65% mitochondrial protein and 39% of the mitochondrial lipids into the suspending medium.

A recent study on tobacco mitochondria (Lee 1968) has shown an induced swelling due to ozone which could be prevented by reduced glutathione, L-cysteine and ascorbate. Since BSA could prevent ozone induced swelling it was suggested that swelling was caused by lipid peroxides.

Surface active agents such as Na-soaps of oleic and linoleic acids, lauryl sulfate, saponin and gramicidin also caused rapid swelling. But similar to the effect of free fatty acids no lipid peroxides have been formed (Hunter et al. 1963).

Certain antioxidants for lipid oxidation, induced by swelling have also been reported. Vitamin E, selenomethionine, ubiquinone and ubiquinols may function as endogenous antioxidants within mitochondria (Mellors and Tappel 1966; Walter and Roy 1971). ∝-tocopherol and serotonin produced complete inhibition of lipid peroxidation and swelling caused by Fe^{2+} and so did vitamin A, but Vitamin K_1 was without effect. On the other hand Mn^{2+}, a powerful inhibitor of lipid peroxidation, caused nearly a complete inhibition of peroxidation and swelling induced by Fe^{2+} ion (Hunter et al. 1963).

3. Free radicals in lipid oxidation as studied by ESR

The autoxidation of unsaturated fatty acids is almost universally accepted to occur via a free radical mechanism in which light and trace metal ions, among others, play an important role in the initiation step. The best direct method for ascertaining the presence of free radicals is ESR spectroscopy since by its very nature it cannot respond unless unpaired electrons are present. However, little early application of this technique was made in the lipid field (Chapman 1965).

Tappel (1965) investigating the free radicals generated during arachidonate peroxidation in aqueous emulsion was unable to detect them by ESR using the most favorable experimental conditions. ESR studies were performed on hemoglobin catalyzed oxidation of arachidonate which was peroxidizing at a rate of 10^{-5} M/sec. It was concluded that although lipid peroxidation is a well characterized free radical chain reaction, it is difficult experimentally to generate radicals in a steady-state concentration sufficient to be detected by ESR.

Truby et al. (1957) studied free radicals in irradiated tristearin and found that irradiation at -196^{0} gave radical "A" and at -78^{0} radical "B". Irradiation at -78^{0} of tristearin precooled to -196^{0} gave an "A'" free radical identical to a decay product of free radical "A" indicating that these two are associated with a certain crystal structure of tristearin formed at -196^{0}, and stable at -78^{0}, but not formed at -78^{0}. Furthermore, tristearin cooled to -196^{0} and warmed to -78^{0} for 30 sec caused structure alteration so that a subsequent irradiation at -78^{0} formed the new free radical "B". The latter could be transformed irreversibly to type "A" by cooling to -196^{0}.

Bradshaw and Truby (1959) found that accumulation of peroxides in irradiated fat depended on the type of free radicals produced and on their rates of decay relative to their affinity towards oxygen. The type of free radicals produced varied with substrate and temperature of irradiation, and so did the accumulation of peroxides. However, the previous two studies elaborated neither on methodology used nor on the properties of the free radicals which were obtained; e.g., their g values and hyperfine structure.

Leibler et al. (1968) and Wozniak and Krauze (1968) studied by ESR the free radicals of several FA, formed by γ-irradiation and the effect of temperature on these radicals. The concentration of free radicals in FA oxidized by air, irradiated by UV or irradiated at 1-1.2 Mrad was too low to be detected by ESR. Therefore, they used a γ-irradiation dose of 3.5 Mrad and were able to report few types of spectra; a) A spectrum with one line observed at temperatures between -20^{0} and +20^{0}, found in all FA studied and was attributed to the structure

$R-\overset{O}{\overset{\|}{C}}-O^{\cdot}$. b) A set of spectra attributed to three resonance forms (A, B and C) noticed for linoleic acid

$$-CH=CH-\dot{C}H-CH=CH- \qquad A$$

$$-\dot{C}H-CH=CH-CH=CH- \qquad B$$

$$-CH=CH-CH=CH-\dot{C}H- \qquad C$$

A major quartet was assigned to form A, which persisted at low temperature from 80^{o} to -196^{o}, and a quintet, attributed to forms B and C, and resulting from overlapping of two triplets was observed at -80^{o} to -40^{o}, but vanished at higher temperatures. c) In saturated FA, a spectrum assigned to the radical $R-CH_2-\dot{C}H-COOH$ was reported. However, most of the ESR spectra for different FA tested, their characteristics and interpretations were not given by the authors. From the few spectra presented, it was obvious that the interpretations were not without ambiguity.

Roubal (1971a) found that fish proteins containing lipids exhibited characteristic ESR signals when exposed to air. Samples prepared by freezing in liquid nitrogen and then freeze-dried produced resonance in the g = 2 region devoid of hyperfine structure. Some freeze-dried fish samples, though initially devoid of the g = 2 signal soon gave rise to two resonances when exposed to air, and when oxidation has taken place. The signals observed were the central g = 2 resonance and additional one or sometimes two peaks, downfield from the central line, designated by the author as "lipid signal region". Many pure proteins exhibited this signal when coated with a thin film of oxidized lipid. The author suggested that a peroxy radical is responsible for the signal, but neither accurately measured g values, nor a hyperfine

splitting consistent with structure of the suggested radical was presented.

Poole and Anderson (1959) studied the UV irradiated unsaturated alkenes by ESR. For 2-hexene, 2-heptene, and 2-octene, they reported a common spectra with six hyperfine components and hyperfine intervals ranging from 25 to 29 gauss. They could detect neither hydrogen atoms nor methyl radicals resulting from irradiation. This study supports the mechanism in which alkenyl free radicals are formed upon irradiation of alkenes with unpaired electrons being on the α carbon of the double bond.

Fatty acids do not necessarily behave the same way when irradiated by X rays. Gordy et al. (1955) reported somewhat different ESR spectra for acetic, propionic and palmitic acids. The last two had a common quartet with a total spread of 80 gauss which was attributed to the presence of a methyl radical.

Reactions of ozone and nitrogen dioxide with certain FA have also been studied by ESR. Goldstein et al. (1968) detected three ESR signals during ozonization of linoleic acid, which disappeared in less than two minutes after halting ozonization. Estefan et al. (1970) studied the reactions between nitrogen dioxide and unsaturated PL, FA, and hydrocarbons, and detected both transient and stable free radicals.

4. Estimation of lipid oxidation

a. TBA test

The spectrophotometric determination of the TBA red pigment has been used to follow rancidity in a wide variety of food products. These include meat and fish (Turner et al. 1954; Sinnhuber and Yu 1958), milk and dairy products (Patton and Kurtz 1955; Sidwell et al. 1955), cereals (Caldwell and Grogg 1955), and animal and vegetable fats

(Jennings et al. 1955). The TBA test has also been used extensively to follow lipid peroxidation in plant and animal tissues and in subcellular particles such as mitochondria and microsomes (Rhee and Whatts 1966a; Zalkin and Tappel 1960; Fortney and Lynn 1964; Thiele and Huff 1965; Hunter et al. 1964; Wills 1970).

The TBA method has the advantages that the principal reactant, malonaldehyde, is a water soluble compound formed or released upon heating the sample in acidic media, and that the intensity of color formed is so high that the method offers an exceptional sensitivity in detection and measurement of lipid autoxidation (Dahle et al. 1962). An additional advantage is the applicability of the test to samples containing lipids without their prior extraction. However, with unknown mixtures of unsaturated lipids, TBA reaction can have only qualitative and comparative significance (Dahle et al. 1962).

The reliability of the test is not completely clarified, since Tarladgis et al. (1962, 1964) have demonstrated that the TBA reagent itself is unstable in the presence of acid, peroxide and heat, under which conditions it is generally used, and that its degradation products absorb at the same wavelength as the TBA malondialdehyde complex. Furthermore, when the reaction is carried out with tissue homogenates and subcellular particles in acidic medium, protein precipitation, and color absorption on the precipitate interfere in absorption readings. Filtration or centrifugation lengthens the procedure and removes the absorbed color from solution. To avoid such errors, Placer et al. (1966) suggested the use of an alkaline pyridine butanol mixture which dissolves both proteins and lipids.

b. Peroxide determination by iodometry and polarography

Determination of peroxides has been reviewed and discussed by Link and Formo (1961). Common methods are based on iodometric estimation of iodine released by peroxides in acidic media. The two standardized and commonly accepted procedures are that of the AOCS (1964) and that of International Fat Commission of IUPAC (1954). The major limitation of the iodometric method is the transitory nature or instability of peroxides so that the actual state of oxidation is not reflected, and hence the correlation between the two is mostly empirical. In addition, iodine is partly absorbed by the unsaturated bonds present in the sample, and this accounts for 2-8% of the total iodine released (Lea 1962).

Polarography of organic peroxides in non aqueous media has shown the existence of a linear relationship between the results obtained by it and by the iodometric method only in early stages of oxidation (Lewis et al. 1949). Lewis and Quackenbush (1949) obtained different polarograms when autoxidation was carried out at 100^{0} instead of 45^{0}. Various peroxide structures persisted during the oxidation but they could not be distinguished. Willits et al. (1952) using DME observed the reduction of acyl peroxides in a range of 0 to 0.2V, diketones and hydroperoxides at 0.6 to 1.0V and of unsaturated aldehydes and ketones around 1.5V and beyond. These compounds in a mixture could be determined both qualitatively and quantitatively when 0.3M lithium chloride as a supporting electrolyte is used in methanol-benzene (1:1 v/v) as solvent (Willits et al. 1953). Hydroperoxides were identified at a half wave potential of 0.8V using SCE as reference.

Saunders et al. (1955) using polarography showed up to 95% of the peroxides formed during methyl oleate autoxidation as hydroperoxides, beside a small but significant amount of some other peroxides formed concurrently. The authors formulated equations to express the relationship between iodometrically determined peroxides, polarographically determined hydroperoxides and the total oxygen uptake. Kalbag et al. (1955) reported the presence of peroxides in autoxidized soybean oil reducible by DME at a more positive potential, 1.4 to 1.8V, than the hydroperoxides, 0.8V. A classification of various organic peroxides into five groups based on half wave potentials was reported by Kuta and Quackenbush (1960).

Ricciuti et al. (1955) compared iodometric and polarographic methods for peroxide determination and obtained identical results for chemically pure compounds, but for impure samples polarography gave lower values and was more specific and reliable. Niederstebruch and Hinsch (1967) reported the following advantages of the polarographic method: specificity for hydroperoxides, with no influence of their individual structures, high sensitivity up to 10^{-6}M/l and finally the short time needed for analysis.

c. Other methods

The other methods for estimation of lipid oxidation include manometric techniques for determination of oxygen uptake, spectrophotometric methods for measuring diene conjugation and fluorescence, various techniques for isolation and estimation of carbonyl compounds, and the methods used in food industry for stability measurements of fats and oils. It is beyond the object of this study to discuss these methods in detail.

D. Enzymatic Oxidation of Lipids

1. Occurrence and localization of lipoxidase

Lipoxidase in peas has been first studied by Siddiqi and Tappel (1956) who demonstrated that the enzyme preparation from green pea extracts does not possess fatty acid oxidase or dehydrogenase activity, yet oxidizes linoleate but not oleate and behaves like a true lipoxidase. Green peas were found to contain 35% of lipoxidase activity relative to that of soybean and rank behind urd beans and lentils (Siddiqi and Tappel 1957) but ahead of peanuts, navy, red and lima beans, wheat, barley and sunflower seeds (Tappel 1963). The other studies of pea lipoxidase include those of Dillard et al. (1960, 1961), Eriksson (1967), Hale et al. (1969), and Eriksson and Svensson (1970). The lipoxidase enzyme has been found in a wide variety of plants such as alfalfa (Chang et al. 1971), flaxseed (Zimmerman 1966), sunflower seeds (Surrey 1965), corn (Fritz and Beevers 1955), barley (Franke and Frehse 1953), wheat (Irvine and Anderson 1953b; Guss et al. 1967a) and in many plant leaves (Holden 1970). Different wheat varieties have been reported to contain different lipoxidase activities (Irvine and Anderson 1953b).

The distribution of lipoxidase in green peas has been studied by Eriksson (1967) who reported that 80% of the total amount of the enzyme is located in the outer part of the cotyledon, 5 - 8% in the skin part, and 12% in the inner part, while the concentration of the enzyme calculated on dry basis is somewhat higher in the inner than in the outer part of the cotyledons. Mapson and Moustafa (1955) observed the oxidation of glutathione by an ungerminated pea extract in the presence of small amounts of certain alcohols and explained the reaction as a

coupled oxidation of the sulfhydryl compound and an unsaturated fatty acid of the linoleic acid type under the action of a lipoxidase enzyme. These authors claimed the absence of any lipoxidase activity in ungerminated pea seeds or in pea mitochondria, but they found enzyme associated with the soluble part of the cytoplasm.

Holman (1948) found in the case of soybean seedlings that the highest concentration of lipoxidase was in the cotyledons which also contained the highest concentration of substrates. Guss et al. (1968) found that lipoxidase activity in etiolated wheat seedlings grown for 3.5 days was localized primarily in the coleoptile and first leaf of the seedling with moderate activity in the germinated seed and root. The etiolated first leaf contained four times the relative activity (units/mg protein) as that of nonetiolated and about 2.5 times that of the dormant seed. After 7 days growth, the distribution of the enzyme within the plant did not change markedly while the total activity decreased. The same authors reported a marked change in the relative intensities of the four lipoxidase bands representing isoenzymes after germination of wheat. In her studies of lipoxidase activity in leaves, Holden (1970) reported that leaves of etiolated cereal seedlings were up to three times as active as green leaves of the same age. Much of the activity was associated with the plastid fraction of etiolated wheat leaves and could be extracted with either SDS or Triton X-100, the latter being more effective. The residue after two extractions with Triton still had far greater activity than appeared to be in the starting sample. She also reported that leaf extracts lost their activity after boiling for 2 minutes and that the pH optimum for peroxidation

varied slightly between species but was always between pH 6-7. Fritz and Beevers (1955) found that lipoxidase substrate system accounts for a major portion of oxygen absorption at pH 5.0 of homogenates from 2½ days corn seedlings, but at pH 7.2 other enzyme systems are contributing to that absorption. Cyanide ion functioned as an inhibitor at pH 7.2 (50% inhibition) but not at pH 5.0.

2. Isolation and purification of lipoxidase

A pioneering contribution in the preparation of pure lipoxidase came from the classical work of Theorell et al. (1947 a, b) on soybean. Their method is even today widely used in commercial scale preparation of the crystalline enzyme, and many of its features are applied in other methods developed later. Holman (1955) recommended using light petroleum ether for defatting plant tissues before enzyme extraction, and stated that some other fat solvents might inactivate the enzyme.

The extraction media commonly used include water, sucrose solution, buffers of acetate, phosphate, and Tris-Cl. The buffer molarity mostly used is 0.1 M, but the pH ranges from 4.5 to 7.2. The pH of the extraction medium has been reported to influence the yield of the enzyme. The highest yield of the enzyme from soybean was obtained by water extraction but unbuffered water offers the hazard that the extracts from different sources may have widely different pH values (Theorell et al. 1947a). The extraction at pH 4.5 gave more reproducible conditions, a purer enzyme preparation and was recommended despite the somewhat lower total yield (Holman 1955).

Pea lipoxidase has been isolated by Siddiqi and Tappel (1956) by a similar method to that of Theorell et al. (1947b), but they reported

that purification by ammonium sulfate fractionation resulted in a considerable loss of total activity. Hale et al. (1969) extracted lipoxidase from ground fresh peas by cold 12.5% sucrose solution for 30 min under nitrogen. The slurry after straining through cheesecloth was centrifuged at a speed of 62,000 x g for 30 min. Pea seeds were de-fatted by a cold acetone treatment before enzyme extraction. Eriksson and Svensson (1970) extracted pea lipoxidase from powder de-fatted by cold acetone and diethyl ether treatment, with Tris-Cl buffer pH 7.2. The crude enzyme was then purified by ammonium sulfate fractionation, Sephadex column, and DEAE-cellulose ion exchange chromatography.

Purification of lipoxidase has been achieved by a variety of means among which ammonium sulfate fractionation constitutes the first step. Stevens et al. (1970) employed gel filtration on Sephadex and chromatography on DEAE-cellulose. Christopher et al. (1970) added another purification step consisting of chromatography on a hydroxylapatite column. Graveland (1970) used ultracentrifugation at 300,000 g for twenty minutes, followed by chromatography on Sephadex and then on a CM-Sephadex. Catsimpoolas (1969) used isoelectric focusing in a sucrose gradient column in a pH range of 5 - 8, and obtained a 230-fold concentration of the enzyme. The final preparation was found homogeneous by disc electrofocusing, disc electrophoresis and by immunoelectrophoresis using several anti-soybean whey protein sera.

3. Enzyme assay methods

Measurement of lipoxidase activity has been reviewed by Holman (1955). The early methods depended on colorimetric determination of carotenoid destruction based upon a coupled oxidation by lipoxidase.

These methods depend on a secondary reaction of which the proportion to the primary reaction is empirical. Furthermore, the linear relationship between enzyme concentration and carotene discoloration exists over only a narrow range. The ferric thiocyanate method used for measurement of the peroxide formed (Sumner 1943; Koch et al. 1958; Dillard et al. 1959) suffers from the disadvantage that the red color produced is quite sensitive to enzyme concentration changes, not easily reproducible and is unstable (Holman 1955; Mitsuda et al. 1967).

Manometric methods for direct measurement of the absorbed oxygen have been used by several authors (Theorell et al. 1944; Tappel et al. 1953; Tookey et al. 1958; and Tappel 1962). However, it was pointed out that in some instances, the oxygen uptake was not proportional to the amount of enzyme extract (Theorell et al. 1944). Furthermore, by this method several minutes are needed to obtain accurate results, and changes in rate over a period less than ½ min cannot be followed (Allen 1968).

The spectrophotometric method employing the increase in ultraviolet absorption at 230 - 234 nm due to absorbance by diene conjugation of linoleate hydroperoxides was developed independently by Holman and Burr (1945) and by Bergström (1945). This method was later modified by several authors (Tappel et al. 1953; Tappel 1962; Surrey 1964; and Ben-Aziz et al. 1970). Nevertheless, the method encounters a problem in obtaining an optically clear and homogeneous substrate solution. This is usually achieved by running the assay at a rather non physiological pH of 9, where linoleate forms a true solution, or by removing aliquots and diluting them with ethanol until the solution is clear. Surrey (1964)

introduced Tween 20 to the reaction mixture for solubilization of linoleic acid a development which extended the use of the method to a wide range of pH. Using Surrey's technique Ben-Aziz et al. (1970) claimed that optically clear solutions were obtainable only in alkaline media and hence they modified the procedure so as direct spectrophotometric readings on the reaction mixture could be followed also in acidic media. However, their substrate medium was clear only up to 10^{-3}M of the substrate concentration. Nevertheless, the spectrophotometric method at best is still subject to inherent difficulties in the presence of any substances which interfere with absorption readings at 230 - 234 nm.

Lipoxidase activity has been measured polarographically by Rhee and Watts (1966a), Mitsuda et al. (1967), and by Eriksson and Svensson (1970). Rhee and Watts used a Beckman Oxygen Analyzer and large volumes of enzyme extract and substrate, 10 and 50 ml respectively. The enzyme and the substrate were mixed and then transferred to the reaction flask before inserting the sensor. This procedure appears to introduce errors due to a) the presence in the mixture of some air bubbles that could replenish part of the oxygen consumed by lipoxidase and give rise to erroneous readings, and b) the fact that a time has elapsed before the actual tracing is recorded and hence the rate of oxygen consumption is not actually the initial rate. Eriksson and Svensson used smaller volumes of substrate, 6.35 ml as well as that of enzyme, 0.5 ml. The reaction took place in a closed liquid system in a thermostatically controlled vessel. Although these authors seem to have overcome the difficulties mentioned earlier, they did not elaborate on the procedure they used.

Mitsuda et al. (1967) described their polarographic procedure in

detail and specified the advantages of the method for measuring lipoxidase activity such as the proportionality of the rate of oxygen uptake to the enzyme concentration, the continuous and direct measurement of oxygen, the lack of restriction being imposed by the presence of contaminants or added substances. Furthermore, the method permits the use of small volumes of enzyme, 0.05 - 0.10 ml, and of substrate, 2.90 - 2.95 ml. However, the authors did not comment on the possible cathodic reduction of certain compounds at the potential of 0.6 V used.

4. Enzyme properties

Soybean lipoxidase was extensively investigated compared to that of peas. The early studies of Theorell et al. (1947a) reported the mol. wt. using thin-layer gel filtration technique of 10.2×10^4 and the isoelectric point of pH 5.4. A recent study by Stevens et al. (1970) reported a mol. wt. of 10.8×10^4 determined by the sedimentation equilibrium method. These authors reported dissociation of the enzyme when treated with guanidine hydrochloride or with SDS and suggested the presence of two subunits each of 5.4×10^4 mol. wt. Catsimpoolas (1969) obtained an isoelectric point of 5.65 for purified soybean lipoxidase. The pH optimum was found by Surrey (1964) to range from 5.5 - 7.0 depending on the substrate. Koch et al. (1958) obtained a pH optimum of 8 for soybean linoleic acid lipoxidase. Ames and King (1966) found that the pH profile is considerably altered by changes in the ionic strength of the reaction medium, but the changes in the nature of the buffer did not affect the rate of the reaction. A shift in optimal pH from 9 - 7 has been reported by Ben-Aziz et al. (1970) at high concentrations of linoleate and Tween 20. Optimal pH of 6.6 and 9.5 have been reported

for two soybean lipoxidase isoenzymes (Christopher et al. 1970), while different values (7.5 and 9.0) have been reported by other workers (Yamamoto et al. 1970).

Pea lipoxidase was found to have a pH optimum of 6.9 (Siddiqi and Tappel 1956). Dillard et al. (1960) obtained two peaks in the pH activity curve with green pea lipoxidase and trilinolein as substrate; the first at 5.5 and the second at 7.5. The maximum activity for linoleic acid as substrate was observed at pH 7.5. Eriksson and Svensson (1970) calculated the mol. wt. of purified pea lipoxidase and reported 7.2×10^4 on the basis of amino acid analysis and 6.7×10^4 by ultracentrifugation. The enzyme preparation proved homogeneous by ultracentrifugation but separated into two main and narrow fractions on isoelectrofocusing with pI 5.80 - 5.82.

Lipoxidase does not contain metal prosthetic groups (Tappel 1963). However, conflicting reports are present concerning thiol groups. Holman et al. (1950) found no thiol groups in soybean lipoxidase, while Stevens et al. (1970) obtained four residues of free sulfhydryl groups and four residues of half-cystine per enzyme molecule although these groups are not available for reaction, which may explain the lack of enzyme inactivation by thiol reagents. Yamamoto et al. (1970) reported four half cystine residues in soybean lipoxidase while Eriksson and Svensson (1970) reported seven half cystine residues in pea lipoxidase. Siddiqi and Tappel (1957) suggested the participation of essential thiol groups in lipoxidase catalyses in urd bean and mung bean but not in soybean, pea, wheat, or peanut. Irvine and Anderson (1955) reported as much as 63% inhibition of wheat lipoxidase by 5×10^{-3} CN^- ion. The

maximum inhibition occurred when the enzyme was incubated with CN^- for 15 min and the inhibition depended on the enzyme concentration and on the total volume of reaction media used.

Calcium ions have been reported to activate linoleic acid oxidation by ground navy bean lipoxidase at pH 7.5 but not at pH 5.5 (Koch 1968). Oxidation of trilinolein was not activated at either pH. Deoxycholate increased the reaction rate of the Ca^{2+} ion stimulated system which effect was explained by increased availability of the substrate, which was brought about by its emulsification. Yamamoto et al. (1970) found that most divalent ions including Ca^{2+} exert a rather inhibitory effect on the well defined soybean lipoxidase "A" isoenzyme but a marked stimulatory effect on the "B" isoenzyme. In addition, they observed that high concentration of substrate has an inhibitory effect on lipoxidase "B", an effect readily reversible by Ca^{2+} ion. The inhibitory effect of Tween 20 on lipoxidase catalysis has been recorded by Surrey (1964) and by Ben-Aziz et al. (1970). Dillard et al. (1961) observed an inhibitory effect of Triton X-100 on legume lipoxidases. Graveland (1970) found that wheat lipoxidase is absorbed reversibly on glutenin and that the absorption is inhibited when glutenin lipids are removed. The effect of the most important group of lipoxidase inhibitors, i.e., lipid antioxidants of the chain reaction terminating type, has been summarized by Tappel (1963) and more recently studied by Yasumoto et al. (1970). According to them the general inhibition by the polyphenolic antioxidants is one of the criteria for establishing the identity of true lipoxidases. Rhee and Watts (1966b) found that the effect of various antioxidants on lipoxidase activity

differed considerably in model systems from that in pea slurries.

The rate of lipoxidase catalyzed reaction is dependent on the concentration of linoleate and oxygen but proportional to the enzyme concentration. Michaelis constants of 1×10^{-3} M (Toshio 1952, Franke and Frehse 1953) and 1.35×10^{-3} M (Holman 1947) have been reported for linoleate with soybean lipoxidase. Barley lipoxidase has for linoleate a Km of 1×10^{-3} M (Franke and Frehse 1953), while that of wheat is 5×10^{-6} M (Irvine and Anderson 1953a). On the other hand, for oxygen a Km of 3×10^{-5} M was reported at 3.6×10^{-4} M linoleate while at 7.2×10^{-3} M linoleate, it was 3×10^{-4} M (Tappel et al. 1952). Ben-Aziz et al. (1970) reported a Km value of 2.5×10^{-5} M and Allen (1968) reported a value of 2.4×10^{-5} M.

5. Lipoxidase isoenzymes, specificity and mechanism of action

The existence of more than one form of lipoxidase has been suggested and demonstrated by several authors. Koch et al. (1958) gave evidence for the existence of two lipoxidase isoenzymes, the first utilizing linoleic acid and the second trilinolein. Dillard et al. (1960) reported the occurence of these two isoenzymes in peas and other legumes and found for the triglyceride activity two distinct peaks on the pH curves. Guss et al. (1967a) devised a specific staining procedure for lipoxidase on acrylamide gels that reveals the enzyme as brown to blue bands. The procedure involved incubation of gels containing starch with linoleate substrate, followed by treatment of the gels with acidic potassium iodide. Using this procedure, they reported the presence of three to four bands in crude aqueous extract of soybean and two to four bands in the extract of wheat milling fractions, while mung beans and

purified commercial soybean lipoxidase showed one band each. Hale et al. (1969) demonstrated the presence of two to three isoenzymes in fresh green peas and pea seeds, but one to two bands in fresh green beans and green bean seeds and four bands in wheat.

Christopher et al. (1970) isolated a lipoxidase isoenzyme from soybean that was distinct from Theorell enzyme by its elution profile from DEAE-Sephadex, disc gel electrophoresis, pH activity profile, substrate specificity and heat stability. The authors reported that both lipoxidase isoenzymes were active on linoleic acid and trilinolein and claimed that there is no reason to assume the existence of separate specific linoleic acid and trilinolein lipoxidases. Yamamoto et al. (1970) separated an isoenzyme designated as lipoxidase "B", from soybean by fractional precipitation with ammonium sulfate followed by ion exchange chromatography. This isoenzyme was substantially stimulated by Ca^{2+} ion and had a pH optimum of 7.5 when compared to the well defined Theorell enzyme which was inhibited by Ca^{2+} ion, had a pH optimum of 9.0, and had a different electrophoretic mobility.

The substrates most commonly available to lipoxidase are the essential FA, linoleic, linolenic and arachidonic, all of which contain the necessary cis, cis - 1, 4 - pentadiene system. These FA are oxidized at equal rates in the presence of lipoxidase, whereas the autoxidation increase in dependence on their unsaturation (Tappel 1963). Other FA such as saturated, monoenes, and trans polyenes are either unreactive or competitive inhibitors.

The main initial product of lipoxidase catalysis was first shown by Privett et al. (1955) to be an optically active cis, trans-

conjugated monomeric hydroperoxide. Dolev et al. (1967a) and Gardner and Weisleder (1970) suggested that there are at least two FA lipoxidases, one specifically oxidizing linoleic acid at C_{13} and the other at C_9. A high specificity of flaxseed lipoxidase for attack at C_{13} was claimed by Zimmerman and Vick (1970) who explained the formation of the C_9 isomer as a result of autoxidation. However Veldink et al. (1970) demonstrated the enzymatic formation of the C_9 isomer by soybean lipoxidase. Chang et al. (1971) found a 1:1 ratio of the C_9 and C_{13} isomers produced by alfalfa and a 3:7 ratio of the hydroperoxides produced by soybean lipoxidase respectively. A marked specificity for oxygen introduction into polyunsaturated FA was reported by Hamberg and Samuelsson (1965). They proved as a pre-requisite the presence of two sites in a diene system in order to be attacked by the enzyme. The first being a diene system starting at C_9 counted from the methyl terminal (ω-6) irrespective of the presence of a double bond in position ω-3, and the second, a diene system beginning with a C_9 counted from the carboxyl end (C-9). They substantiated these generalizations by the finding that docosa -8, 11, 14 - trienoic acid was not oxidized by lipoxidase though it contains the 1, 4-pentadiene system. In addition, these authors obtained during oxidation 70 - 80% isomer C_{13} and 20 - 30% isomer C_9.

Richardson et al. (1961) found that added lipoxidase did not catalyze oxidation of lipids present in mitochondria and concluded that the unsaturated FA are so tightly bound in the lipoprotein matrix as to be unavailable to added lipoxidase. In another study, neither mono - nor dilinolein acted as a good substrate for either wheat or soybean lipoxidases (Guss et al. 1967b). The wheat extract exhibited constant but low activity

toward all glycerides as substrates, while soybean extract showed decreasing activity with increasing polarity of the glycerides. Oxygen uptake with trilinolein as substrate was greatly reduced in the presence of 1% albumin with both soybean and wheat extracts, and it was suggested that albumin may form a complex with trilinolein rendering it unavailable for oxidation by the enzyme. The same authors found no catalytic activity on DGDG by either soybean or wheat extracts, however the addition of trace amounts of linoleic acid did enhance the activity of soybean lipoxidase but not that of wheat. Graveland (1970) found that wheat lipoxidase is absorbed by glutenin and their complex then oxidises linoleic acid to hydroxy-epoxy acids that are rapidly further hydrolyzed to trihydroxy acids. The activity of lipoxidase on phospholipids has not been reported in the literature.

The mode of action of lipoxidase has been suggested by Tappel (1963) as an initial abstraction of one hydrogen atom from a methylene group between two cis double bonds, followed by an attack of one molecule of oxygen. This results in the formation of a hydroperoxide with two conjugated double bonds, where the isomerized double bond has attained the trans configuration. Walker (1963) demonstrated by ESR the existence of free radicals during the course of linoleate oxidation by lipoxidase. Dolev and co-workers (1967a, b) proved that the oxygen incorporated into the hydroperoxide product is derived from dissolved molecular oxygen and not from water. These authors reported the exclusive formation of 13 hydroperoxyoctadeca - 9, 11 - dienoic acid by the action of lipoxidase on linoleic acid and proposed the following mechanism:

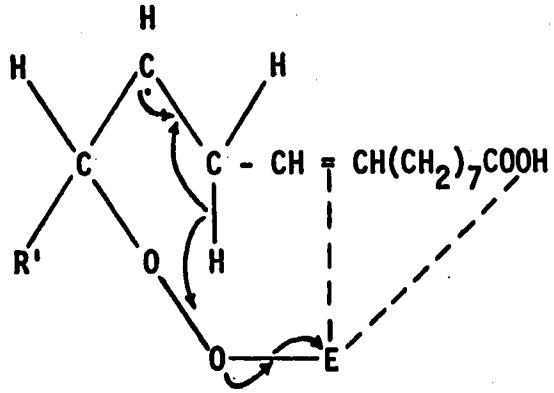
Activation of the enzyme by oxygen forms a free radical (I) that adds

to the substrate rather than abstracts hydrogen from it.

The free radical reacts with linoleic acid (II) by addition at C_{13}, and is probably attached to the substrate via the double bond at C_9 and/or the carboxyl group.

A series of electron shifts, indicated by arrows, then takes place in intermediate (III) and results in establishment of a new trans double bond at C_{11}, a transfer of hydrogen atom to oxygen to form the hydroperoxide group and liberation of the enzyme perhaps as a free radical E·:

$$\underset{\text{I}}{EOO^{\cdot}} + \underset{\text{cis}}{R'CH = CHCH_2CH} = \underset{\text{cis II}}{CH(CH_2)_7COOH}$$



III

$$E^{\cdot} + R'\underset{\displaystyle OOH}{\underset{|}{CH}} - \underset{\text{trans}}{CH = CH} - \underset{\text{cis}}{CH = CH(CH_2)_7COOH}$$

6. Lipoxidase induced oxidation in presence of lipases, phospholipases and other factors

The presence of lipase in peas has been established by Wagenknecht et al. (1958) who isolated the enzyme and found it active on blanched pea slurries and also in degrading chlorophyll to pheophytin. Frankel and Garber (1965) examined the esterases in extracts from germinating seeds of twelve pea varieties by starch gel electrophoresis and showed a maximum of six detectable esterases, of which the presence of two is possibly governed by monogenic differences. Pea was also found to possess six esterases, five of them were classified as carboxylesterases and one classified as aryl esterase (Norgaard and Montgomery 1968).

Specificity of lipase from several seeds was studied by Berner and Hammond (1970) who reported that oat lipase favored the hydrolysis of linoleyl groups but discriminated against stearoyl groups. Positional specificity was absent in the seeds examined, except one seed which had a tendency to hydrolyze preferentially the acyl groups of TG in positions 1 and 3. Drapron et al. (1969) in their study of the development and distribution of wheat lipase activity during germination found the activity to be mainly localized in the coleoptile. The activity increased by increased germination temperature and it was lower in light than in darkness.

Lee and Wagenknecht (1958) isolated partially purified forms of lipoxidase, lipase, catalase and peroxidase from raw peas, added them to blanched pea slurries and studied their effects on quality during prolonged storage. They reported that color changes were brought about

by the four enzymes with the greatest change produced by lipoxidase and lipase. The greatest change in flavor score was associated with added catalase, but lipoxidase and lipase produced pronounced off-flavors while peroxidase produced a mild change in flavor which was not objectionable. In another study the same authors (Wagenknecht and Lee 1958) observed that samples of macerated blanched peas containing added lipoxidase and lipase showed a considerable oxygen uptake, an effect which was not apparent either when lipoxidase was alone or when catalase and/or peroxidase were present along with lipoxidase. From acidity determination they also concluded that considerable amounts of TG were split by the added lipase. Furthermore, during the progressive development of off-flavors in frozen raw peas during storage, the increase in acid number invariably preceded the development of peroxides detectable in lipids (Lee et al. 1955). It was concluded that lipase may serve as an adjunct to lipoxidase in off-flavor development and that the FFA are apparently more readily utilizable as lipoxidase substrate than the intact TG (Wagenknecht and Lee 1958).

There is little information about the occurence of phospholipases A and/or B in plants (Barron 1964), although phospholipase B has been reported in rice grains (Contardi and Ercoli 1933) and more recently in barley malt (Acker and Geyer 1969). In the latter, the enzyme was found to split the FA of lecithin indiscriminately from positions 1 and 2. Galliard (1970) reported the rapid formation of FFA and their oxidation products from phospho - and galactolipids in potato tuber homogenates. He also described the phospholipid and galactolipid hydrolase activities which, together with lipoxidase, were responsible

for such a breakdown of endogenous lipids. A high activity of phopholipase D in pea seeds, and an appreciable activity had been reported to be retained in pea seeds for at least one year after drying (Quarles and Dawson 1969).

Large losses in the FA of TG and PL in unblanched peas during cold storage have been reported by several authors (Lee and Mattick 1961; Pendlington 1962; Bengtsson and Bosund 1966). Breakdown of PL was comparable to that of NL at very low temperatures, but was higher at -5 and -8^{o} (Bengtsson and Bosund 1966). The change in PI was negligible, while that in PC and PE was considerable. The authors suggested that the resistance of PI to breakdown is probably due to the inability of phospholipase A to attack it.

In their studies on substrate specificity of phospholipase A, Van Deenen and De Haas (1963) found that D-α-PL were not attacked, neither PE in which the hydroxyl group was protected. They also reported that only one FA ester bond located adjacent to the phosphoryl-alcohol linkage is necessary for phospholipase A action. Glycol derivatives were suitable as substrates and so was DPG whereas sphingomyelin resisted phospholipase A.

Some enzymes other than lipoxidase have been reported to participate in lipid oxidation. Blain and Barr (1961) presented evidence that linoleate hydroperoxides arising from lipoxidase activity are subsequently decomposed by an enzyme in soybeans. A similar hydroperoxide-decomposing enzyme from alfalfa seedlings were reported by Gardner and Clagett (1965). Oxidation of linoleic acid by a sequential enzyme system, lipoxidase and linoleate hydroperoxide isomerase, in corn

extract has been described by Gardner (1970). Buckle and Edwards (1970) in their study of model systems with pea and soybean extracts indicated that chlorophyll loss and lipid oxidation during storage were not likely caused by lipoxidase but by a lipohydroperoxide breakdown factor, present in crude extract. A specific lipohydroperoxidase is known to occur in soybean extract (Gini and Koch 1961).

III. MATERIALS AND METHODS

Chemicals

Mannitol was obtained from Aldrich Chem. Co. (Milwaukee, Wisc.), Rhodamine 6 G from Allied Chem. Corp. (New York, N.Y.), Trimethylchlorosilane from Applied Science Lab. Inc. (State College, Pa.), Tween-20 from Atlas Powder Co. (Brantford, Ont.), DEAE-cellulose from Baker Chem. Co. (Phillipsburg, N.J.), ADP from BDH Chemicals Ltd. (Poole, England). BSA fatty acid poor, cis-aconitic acid, dithiothreitol, reduced glutathione, and thiamine-pyrophosphate were obtained from Calbiochem (Los Angeles, Calif.). Chromosorb P 100/120 mesh, EGS, SE-30 were from Chromatographic Specialties (Brockville, Ont.), Taurocholate-Na from Difco Lab. Inc. (Detroit, Mich.). Acrylamide, N-methylene-bis-acrylamide, TEMED, 2', 7'-dichlorofluorescein, guaiacol, 2-mercaptoethanol and thiobarbituric acid were obtained from Eastman Organic Chem. (New York, N.Y.). EDTA, Glycine, Hyflo Super Cel, K-malonate, mineral salts, A.G., reagent grade HNO_3, SDS, TRIS, sucrose, anhydrous D-glucose and D-galactose certified A.C.S., were obtained from Fisher Scientific Co. (Fair Lawn, N.J.). Pectin (geno-pectin slow set) was from Food Products Ltd. (Montreal, Que.). $HClO_4$, 70%, double distilled, was from G. Frederick Smith Chem. Co. (Columbus, O.). OV-17 was from Hewlett Packard (Avondale, Pa.). Linoleic acid and fatty acid methyl esters high purities, and phospholipid standards, were from Hormel Institute (Austin, Minn.). Silicic acid chromatography grade, 100 mesh was from Mallinckrodt (New York, N.Y.). Soybean

lipoxidase liophylized, was from Mann Res. Lab. (New York, N.Y.). Anthrone, BF_3, Cysteine and Na-deoxycholate from Matheson, Coleman and Bell (East Rutherford, N.J.). Pyrogallol and Silica gel G (according to Stahl), were from Merck AG. (Darmstadt, Germany). Amylopectin, BSA, crystalline, Gum acacia, wheat germ lipase were obtained from Nutritional Biochemicals Corp. (Cleveland, O.). Hexamethyldisilazane from Pierce Chem. Co. (Rockford, Ill.). Sephadex G-25, G-150 and LH 20 were obtained from Pharmacia (Uppsala, Sweden). Triton X-100 was from Rohm and Haas (Philadelphia, Pa.). Certified protein standards: Albumin (bovine) cryst., Chymotrypsinogen-A (beef pancreas), Cytochrome C (horse heart), Myoglobin cryst. (sperm whale) salt free, and Ovalbumin (2 x cryst.) and Coomassie brilliant blue were obtained from Schwarz and Mann (Orangeburg, N.Y.). Amylose, beef liver catalase, α-ketoglutarate, phospholipase A_2 (EC 3.1.1.3) from Ophiophagus Hannah snake venom, K-succinate, and TES (N-tris [Hydroxymethyl] methyl-2-aminoethane Sulfonic Acid) were obtained from Sigma Chem. Co. (St. Louis, Mo.). Carbon celite absorbent (Nuchar attaclay) was obtained from Varian Aerograph (Walnut Creek, Calif.).

Equipment

Centrifuges used were a) Sorvall SS-1 Superspeed Angle Centrifuge, Ivan Sorvall Co., Inc. (Norwalk, Conn.), b) Spinco LC 2-65B ultracentrifuge with rotors SW - 27 and a fixed angle type 40, Beckman Instr. Inc. Spinco Div. (Palo Alto, Calif.). Electrophoresis equipment was from Buchler Instruments (Fort Lee, N.J.) with destainer from Canalco (Rockville, Md.). Gas chromatograph used was a Bendix Model 2500, Bendix Instruments Div. (Ronceverte, W. Va.). Oxygen uptake measurements were performed by a) Gilson differential respirometer

Model GR 20, Gilson Medical Electronics, Inc. (Middleton, Wisc.) and b) Biological Oxygen Monitor Model 53, equipped with a Clark electrode, Yellow Springs Instruments (Yellow Springs, O.), which was connected to a Beckman 100 mV potentiometric recorder, Beckman Instruments, Inc. (Fullerton, Calif.). Irradiation sources used were for gamma irradiation a Co^{60} gamma cel 220, designed by the Atomic Energy of Canada Ltd. (Chalk River, Ont.), and for UV, a 500 W PEK, model 915 L.H. mercury arc lamp (Sonnyvale, Calif.). Gamma doses applied were measured by a Fricke-Miller dosimeter. The spectroscopy equipment used were: Atomic absorption spectrometer, Perkin Elmer Model 303, Perkin Elmer Corp. (Norwalk, Conn.). ESR was performed by using a Varian (V-4500-10 A) X band spectrometer, Varian Aerograph (Walnut Creek, Calif.) utilizing 100 Kc/sec modulation. The spectrometer was fitted with a Varian (V-4547) temperature controller, a Fieldial (V-FR 2503) magnetic field regulator and an Alpha (M 3093) Digital NMR Gaussmeter. Infra red spectra were recorded by using a Perkin Elmer Model 21 spectrophotometer and for visible and UV spectra a Beckman Model DBG grating spectrophotometer and a Spectronic 20, Bausch & Lomb Inc. (Rochester, N.Y.), were used. Other general equipment used were: fraction collector ISCO model 327 from Instruments Specialties Co. Inc. (Lincoln, Neb.), freeze-drier, RePP, manufactured by the Virtis Co. Inc. (Gardiner, N.Y.), a Beckman Expandomatic SS-2, pH meter, peristaltic pump was a LKB Perspex pump, LKB Produkter, AB (Bromma, Sweden), a Sargent polarograph, Model XV, E.H. Sargent & Co. (Chicago, Ill.), and a Raytheon sonicator, 250 W, 10 Kc Sonic Oscillator , Raytheon Manuf. Co. (Waltham, Mass.).

Methods

A. Isolation of subcellular particles

All isolation steps, unless otherwise stated, were performed at 2 - 4°.

1. Mitochondria and peroxisomes

The cotyledons of etiolated pea seedlings, var. Homesteader, were used. Certified seeds were obtained from Seed Centre Ltd., Edmonton, and were stored at 0° until use. The seeds were soaked in tap water for 4 h and then planted over a 3" layer and under 3/4" layer of vermiculite and grown at 27° in dark at 100% RH. The tissue was designated one day old, 24 h after the start of soaking. Cotyledons were collected by removing the seed coat, hypocotyl and epicotyl. They were rinsed with distilled water, blotted dry, weighed, and then gently ground for 3 min with a pre-chilled mortar and pestle in a grinding medium (3 ml/g cotyledons) consisting of 0.5 M mannitol, 2 mM EDTA, 0.3% (w/v) BSA, 0.5% (w/v) Cysteine and 5 mM TES, pH 7.4 to obtain the cell free homogenate, which was then centrifuged at 2,500 x g for 10 min. The pellet was discarded and the mitochondria were sedimented from the supernatant by centrifuging at 20,000 x g for 10 min. The resulting supernatant and the light colored material surrounding the dark brown mitochondrial pellet were removed by suction. The pellet obtained was suspended in suspension medium (0.5 ml/g cotyledons) consisting of 0.3 M mannitol, 3.0 mM $MgCl_2$, 0.3% BSA (w/v), 50 mM TES, pH 7.2 (Tris, at 0°). A uniform suspension was prepared by

gently sucking the solution up and down a wide bore pipette and then the suspension was centrifuged at 10,000 x g for 10 min. The mitochondria pellet was finally suspended in a small quantity of the suspension media without BSA (2.0 ml/100 g cotyledons).

The crude mitochondria was either used at this stage or further purified by sucrose density gradient centrifugation. The discontinuous gradient consisted of 5 ml 2.75 M sucrose and 7 ml each of 1.4, 1.3 and 0.73 M sucrose respectively. Approximately 2 ml of the crude mitochondria was layered on the gradient and the separation into bands was accomplished by centrifugation at 25,000 rpm (average 115,000 x g). Each of the four layers separated was collected and suspended in dilution buffer pH 7.2 to a final sucrose concentration of 0.3 M. After centrifuging at 6,000 x g for 16 min, the pellets were suspended in suspension buffer without BSA and assayed for protein content and lipoxidase activity. Protein determinations throughout this study were performed according to Lowry et al. (1951). A standard curve (Fig. 1) was prepared by using crystalline BSA. The flow diagram for isolation procedure used is given on Fig. 2.

The integrity of the isolated mitochondria was examined by studying their swelling and contraction properties, measuring their respiratory control ratio (RCR) and by ADP/O value. Volume changes were studied by reading the absorbance at 520 nm, at which wavelength the decrease in absorbance indicated swelling, and increase a contraction. Mitochondrial suspension, 50 - 100 ml (about 100 mg protein) was added at 0 time to a cuvette containing 2.9 ml of 0.1 M KCl in 0.02 M Tris-Cl buffer, pH 7.5. Readings were taken at time intervals adequate

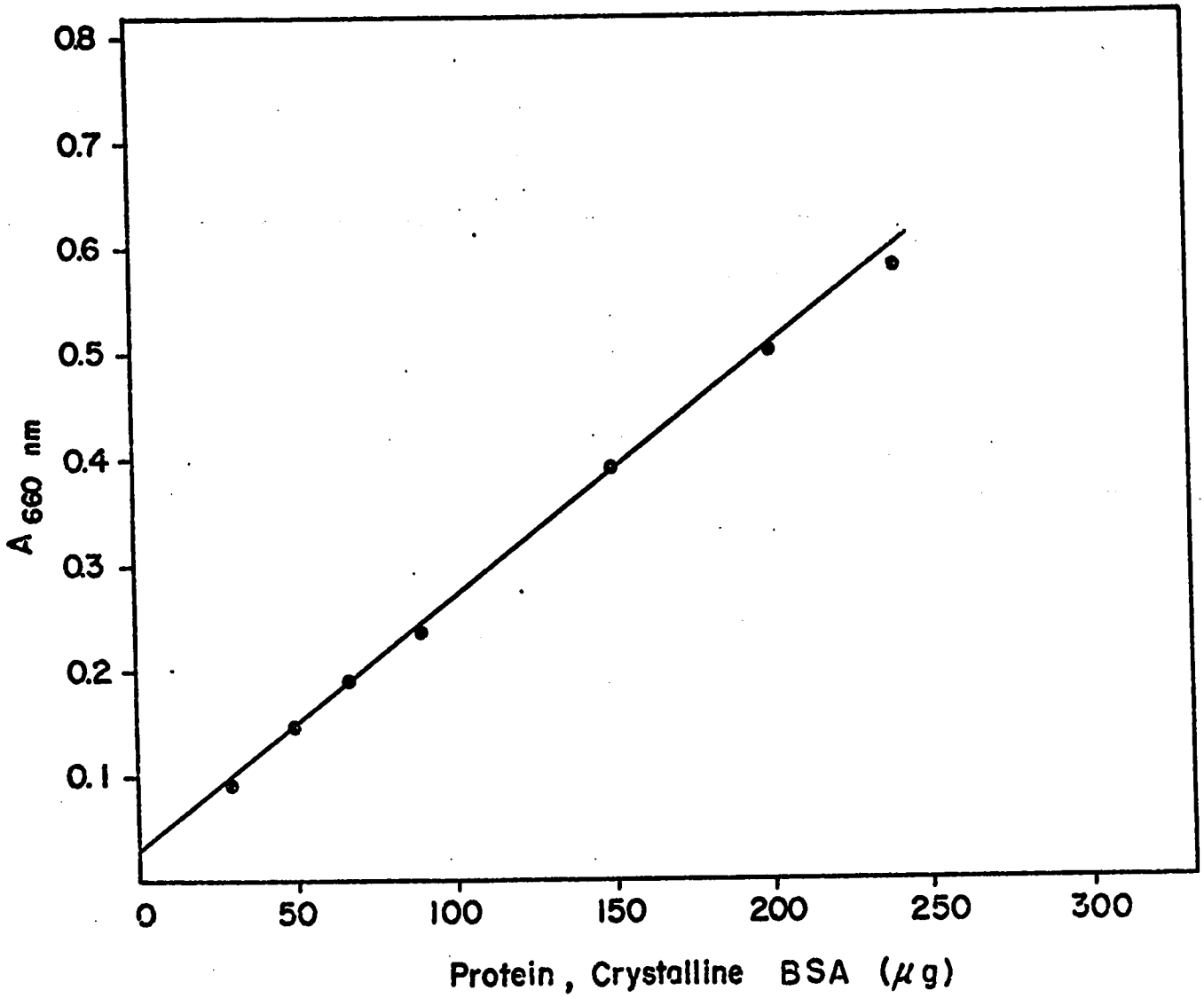


Fig. 1. Calibration Curve for Protein Determination. The method was that of Lowry et al. (1951). Readings were taken in 1 cm cell using a Beckman DBG Spectrophotometer.

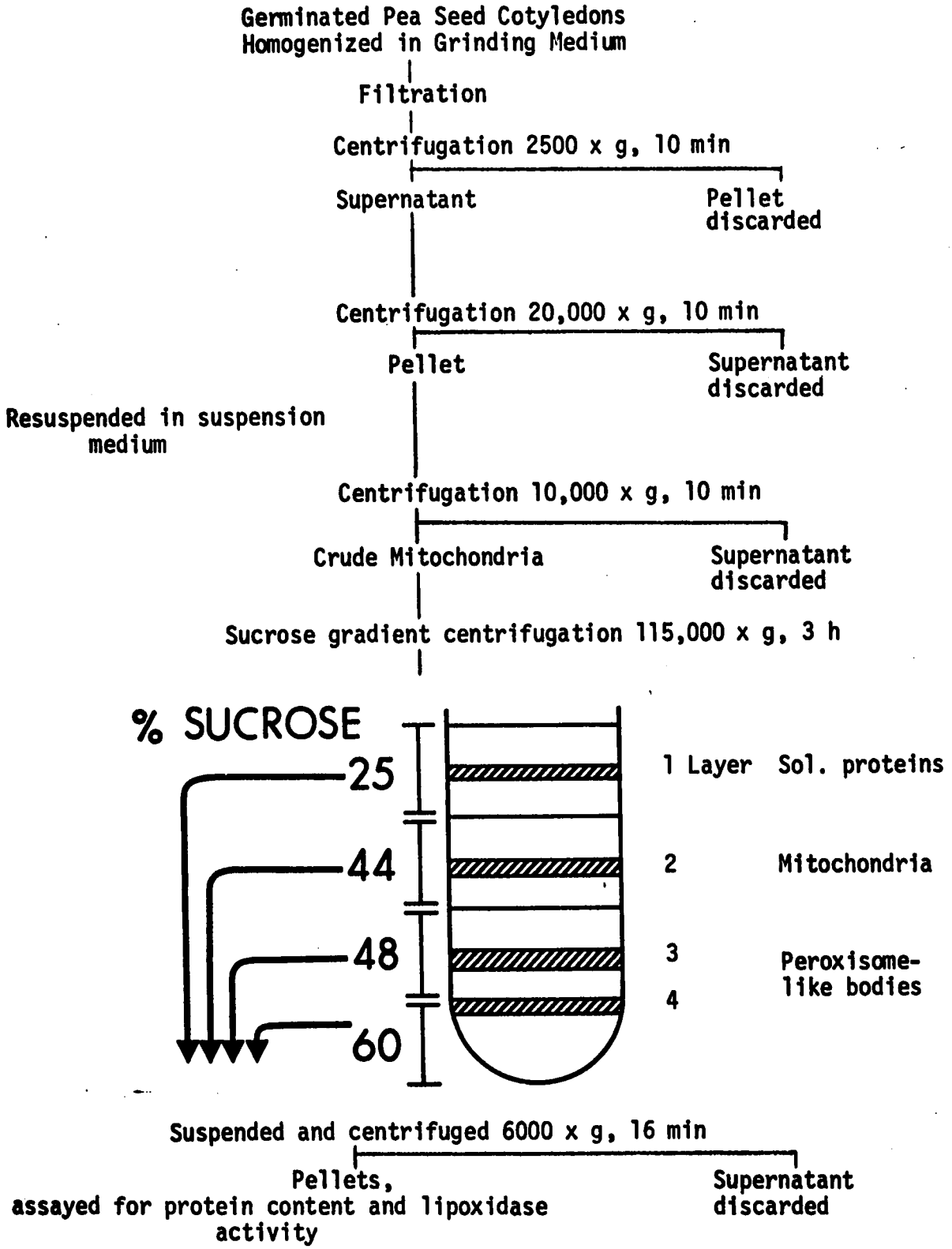


Fig. 2. Flow Diagram for Mitochondria and Peroxisomes Isolation.

to establish the trend of the curve (1 - 10 min). After completion of swelling indicated by levelling off the curve, contraction was initiated by the addition of 10 µl of potassium succinate to a final concentration of 2 mM and readings were taken till maximum contraction was attained.

The value of RCR was determined from the ratio of the respiration rates in state 3 (phosphate, oxygen, substrate and ADP in excess) and state 4 (ADP limiting). ADP/O values were obtained by measuring the amount of oxygen consumed during state 3. The assay media consisted of 0.3 M mannitol, 4 mM $MgCl_2$, 5 mM H_3PO_4, 0.075% BSA (w/v), 50 mM TES, pH 7.2 (Tris, at 25^0) with either a mixture of 8 mM α-ketoglutarate, 5 mM malonate, and 0.07 mM thiamine pyrophosphate, or 8 mM succinate. The assay medium, 2.9 ml, was placed in the oxygraph cell and stirred for 3 min for air saturation and temperature equilibrium. The electrode was then immersed in the reaction mixture by a rotation movement in order to exclude the air bubbles present. A voltage of 0.8 was then applied and the amplifier set to read the full scale 100%. Then, 100 µl of mitochondria suspension was added to the cell through the slit on the probe using a Hamilton microsyringe equipped with a thin extension tube made of teflon. A known amount of ADP was added (0.3 mM to succinate media and 0.6 mM to α-Keto glutarate media) at least twice to record more than one cycle for RCR and ADP/O values. The solubility of oxygen in the above assay media was 225 µM as determined by Winkler's method (Solomos et al. 1972).

2. Plastids

a. Chloroplasts

Pea seeds, var. Homesteader, were planted in vermiculite and grown for 16 days in growth chambers at 21°, 60% RH, and an illumination of 1400 ft-candles. For chloroplasts isolation, leaves were chilled, cut and minced in a mortar and pestle with 0.6 M sucrose in 10 mM Tris-Cl buffer, pH 7.5 (2:1 v/w). The resultant slurry was pressed through 8 layers of cheesecloth and the cell free homogenate obtained was then centrifuged at 800 x g for 10 min. The supernatant was recentrifuged at 3,000 x g for 10 min and the chloroplast pellet collected was then suspended in the buffer (7 vol. buffer:1 vol. closely packed chloroplasts). The suspension was recentrifuged at 3,000 x g for 10 min to yield the washed chloroplasts. The latter was suspended in a small volume of the buffer (2 ml for chloroplasts yield from 100 g fresh leaves) and purified by centrifugation on a discontinuous sucrose density gradient. The gradient consisted of 7 ml 2.5 M sucrose, 10 ml each of 2.0, 1.5 and 1.0 M sucrose respectively. Washed chloroplast pellet, 2 ml, was layered on top of the gradient and the contents were centrifuged at 25,000 x g for 40 min. Broken chloroplast and intact chloroplast layers (see Fig. 3) were collected and diluted with buffer without sucrose to 0.6 M sucrose concentration. They were then centrifuged at 5,000 x g for 10 min, washed and sedimented as previously, and the purified pellets collected were then used for further studies.

b. Etiolated plastids

Pea seeds were germinated as described for mitochondria

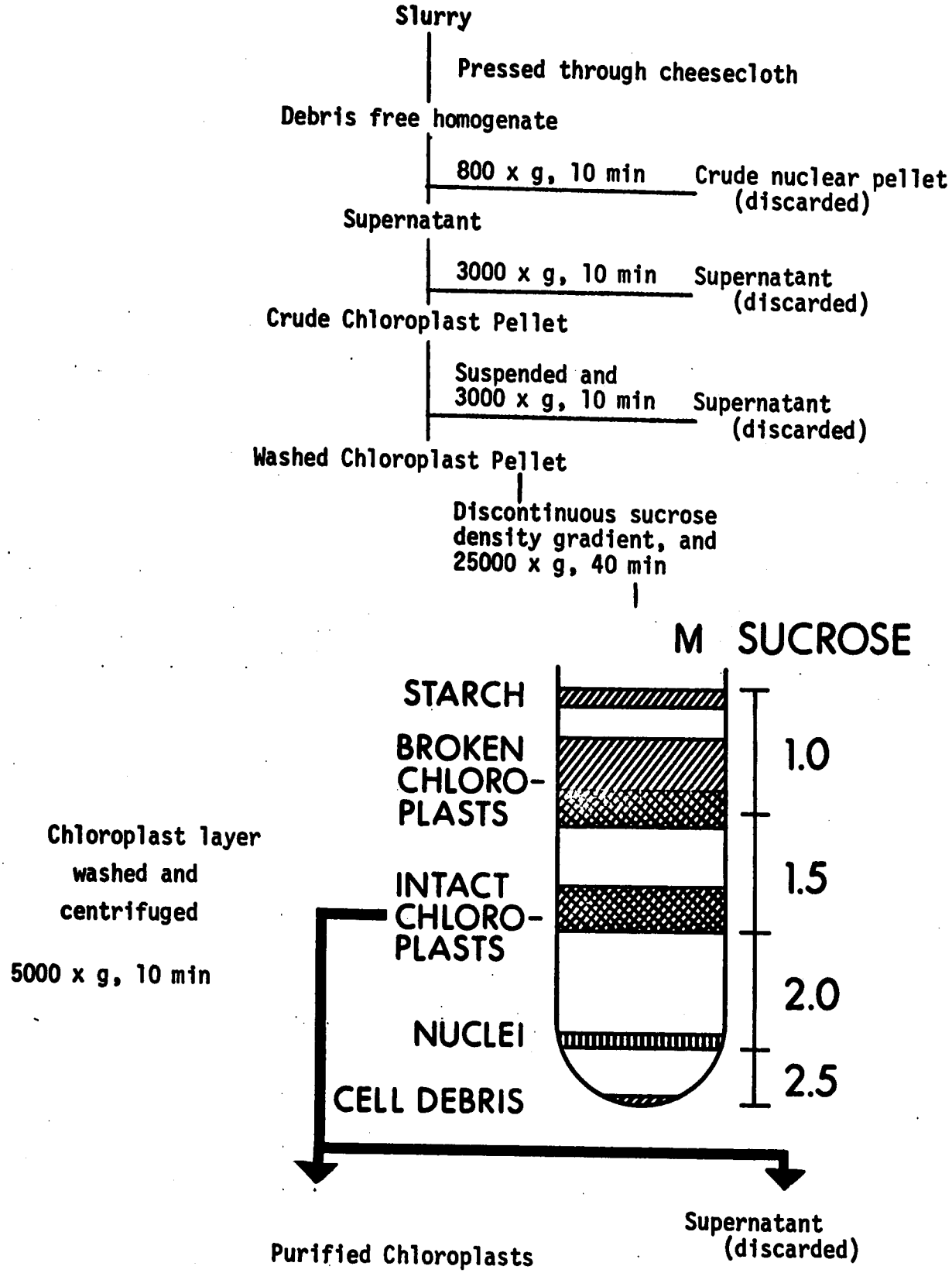


Fig. 3. Flow Diagram for Chloroplast Isolation

isolation. Etiolated, 5-day-old, pea leaves (50 g) were ground in 200 ml of 0.1 M phosphate buffer, pH 7.0 using a mortar and pestle. The slurry was filtered through 4 layers of cheesecloth and the collected homogenate was further centrifuged at 500 x g for 10 min. The supernatant was collected and centrifuged at 3,000 x g for 10 min. The pellet consisting of etiolated plastids was suspended in 2 ml of the grinding buffer and assayed for protein content and lipoxidase activity. The supernatant of the 3,000 x g centrifugation (see Fig. 4) was recentrifuged at 20,000 x g for 10 min and the pellet containing the mitochondria and peroxisome-like bodies was discarded. The supernatant was further centrifuged at 115,000 x g for 2 h. The supernatant representing the ribosome free cytoplasm was assayed for protein content and lipoxidase activity.

c. Plastids disruption

The pellet of etiolated plastids was washed with 0.1 M phosphate buffer pH 7.0 (3:1, v/v) and centrifuged at 20,000 x g for 10 min. The resulting pellet was divided into two portions (1.6 ml each) and mixed on a Vortex for 5 min with 2 ml of either 0.5% Triton X-100 or 0.5% SDS. After centrifugation at 20,000 x g for 30 min, the detergent treatment and centrifugation steps were repeated and the final residues suspended each in 1 ml of the phosphate buffer. The first and second extracts as well as the residues were assayed for their protein content and lipoxidase activity.

Chloroplasts were treated with Triton X-100 and with SDS in a similar way. In addition, chloroplasts were disrupted either by an osmotic shock or by ultrasonication. A volume of 1 ml of closely

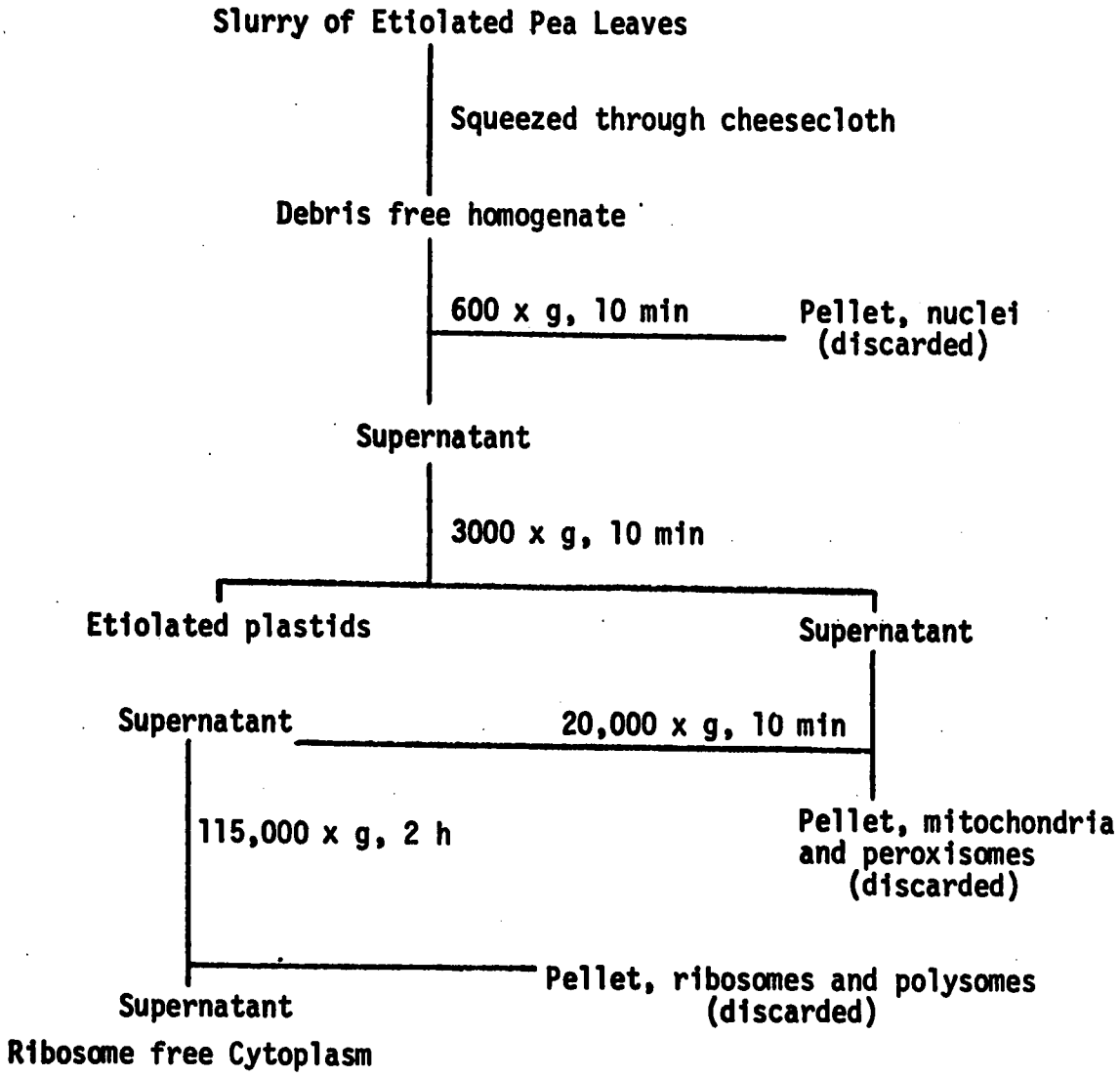


Fig. 4. Flow Diagram for Etiolated Plastids and Cytoplasm Isolation

packed chloroplasts was shaken with 9 ml deionized water for 10 min and checked for lipoxidase activity. In ultrasonication, the chloroplasts volume suspended in a tenfold volume of sucrose Tris-Cl buffer was sonicated for 3 min using a Raytheon 250 W, 10 K Cycle Sonic Oscillator tuned to 1.2 amp. The sonicated chloroplasts were then assayed for lipoxidase activity.

B. Lipid analysis

1. Isolation and purification

Three varieties of peas, Homesteader, Laxton, and Stratagem were analyzed. Crude lipids were extracted from 100 g samples by refluxing the ground seeds with 4 - 6 volumes of chloroform methanol (2:1, v/v) for 5 min. After filtration, the residue was re-extracted with hot fresh solvent mixture and the procedure repeated until a colorless residue was obtained. The extracts were combined and the solvent evaporated at room temperature using a vacuum flash rotary evaporator.

Non lipid contaminants were removed by either the technique of Folch et al. (1957) or that of Williams and Merrillees (1970). In the first, the crude lipid extract (≈ 1 ℓ) was mixed thoroughly in a separatory funnel with 0.2 its volume of an aqueous solution of 0.58% sodium chloride. The mixture was kept at 4^{0} until two phases separated. The upper phase was then removed by a Pasteur pipette, and the remaining interface was rinsed twice with small amounts of upper phase pure solvent. The lower phase was evaporated to dryness and the resulting residue was then dissolved in anhydrous, ethanol free chloroform prepared by refluxing chloroform over calcium chloride for 2 - 3 h. The

lipid solution was then flushed with nitrogen and stored in darkness at -20°.

In the second purification procedure, to the crude lipid extract about 15 g of SephadexG 25 was added and the solvents evaporated until no liquid remained in the flask. Then, the Sephadex was resuspended in 300 ml of anhydrous chloroform and dried down once more, after which it was again suspended in chloroform and poured into a chromatographic column (4.5 cm I.D.). The lipids were eluted with 200 ml anhydrous chloroform, concentrated, and stored as mentioned earlier. Using the same isolation and purification steps, total lipids were obtained from mitochondria, germinated pea cotyledons, and chloroplasts.

2. Lipid fractionation

a. Neutral and polar lipids

Total lipids were separated into NL and PL on a 2.2 x 40 cm silicic acid column. Silicic acid was washed with hot methanol followed with hot acetone, and then dried at 105° for 2 h. Batches of Hyflo Super-Cel were also treated in the same way. A mixture of 15 g silicic acid and 5 g of Hyflo Super-Cel was suspended in anhydrous chloroform and poured into the column. When the chloroform was drained, the TL were applied in the ratio of 30 mg/g silicic acid. The NL were eluted with 250 ml chloroform and the PL with 250 ml chloroform methanol (1:1, v/v). Solvents were evaporated and the remaining lipids were dissolved in anhydrous chloroform and stored until further use.

For TLC, Silica gel G according to Stahl was used as an adsorbent. A slurry of 30 g gel and 60 ml water was spread on 20 x 20 cm

plates. The thickness of the layer was 200 μ or 500 μ in the preparative plates. The plates were dried at room temperature, activated at 110° for 1 or 2 h depending on thickness of the layer, and stored in a desiccator until use. After applying samples, the chromatograms were developed in chambers well lined with filter paper saturated with the solvent. The following solvent systems were used: Ethyl ether - petroleum ether b.p. 40 - 60° (20:100, v/v) for NL and (10:100, v/v) for sterols. One of two solvent systems was used for two dimensional development of PL. The first system was that of Nichols and James (1964) using in the first dimension chloroform - methanol - 7N ammonia (65:25:4, v/v) and in the second dimension chloroform - methanol - acetic acid - water (170:25:24:4, v/v). The second system was that of Rouser et al. (1970) in which the first solvent is chloroform - methanol - 28% aqueous ammonia (65:25:5, v/v) and the second chloroform - acetone - methanol - acetic acid - water (3:4:1:1:0.5, v/v). In two dimensional TLC the first solvent was evaporated usually under a vacuum of 28" at room temperature for 20 min.

Spots were visualized by spraying the plates with 50% sulfuric acid and charring at 100° for 10 - 15 min. Rhodamine 6 G or 2', 7' - dichlorofluorescein were used for non destructive detection of the spots. The first was prepared on the day of use by mixing 1 ml of stock solution of 0.1% (w/v) dye in methanol with 25 ml of 2 N ammonia. Plates were sprayed with this solution or with 2', 7' - dichlorofluorescein (0.5% in 95% ethanol) and viewed while still damp under UV light.

The identity of the spots was confirmed by comparison with authentic samples and by specific detection methods. Glycolipids

appeared as violet or green spots after spraying with 0.2% (w/v) anthrone in concentrated sulfuric acid followed by heating at 70° for 20 min (Rosenberg et al. 1966). Spraying the plates with 0.2% ninhydrin in water saturated butanol followed by heating for several minutes at 105° in an oven saturated with moisture, resulted in red violet spots for lipids containing free amino groups.

The reagent prepared as described by Vaskovsky and Kostetsky (1968) was used for detection of PL. Ammonium molybdate 16 g was dissolved in 120 ml of water to give solution I. Concentrated sulfuric acid 40 ml and 10 ml of mercury were shaken with 80 ml of solution I for 30 min to give after filtration, solution II. Concentrated sulfuric acid 200 ml followed by solution II was added carefully to the rest of solution I. The cooled mixture was diluted with water to 1 ℓ. Phospholipids were detected immediately after spraying at room temperature as blue spots on a white background.

For detection of PC, the plate was dried at 110° for 15 min to remove traces of water and was sprayed while hot with 0.25% solution of cis-aconite anhydride in acetic anhydride. After the developments of red spots, the background was removed by spraying the plate with distilled water. The background disappeared and PC showed as a bright red violet spot (Vaskovsky and Suppes 1971). Cis-aconite anhydride was prepared by heating the acid at 140° under a vacuum of 5 - 10 μ for 15 min. The residue was treated with hot benzene, cooled, and the crystallised anhydride recovered by filtration using a Buchner funnel.

From the plates, spots were recovered by scraping with razor blades and extracted with chloroform methanol 1:1. Silicic acid was

then removed by low speed centrifugation and the individual lipids obtained were used for further studies.

The sugar identity in the isolated galactolipids and sterol glycoside lipids was confirmed by the procedure of Sweeley et al. (1963). A sample of approximately 5 mg of the lipid was placed into a screw cap vial fitted with a teflon liner in the cap, and then 4 ml of dry 0.5 N methanolic - HCl was added. The vials were placed in an oven at 75^{o} for 24 h. Then, the reaction mixture was cooled and treated with an equal volume of n-hexane to remove the FAME formed from the lipids during methanolysis. The methanolic layer containing sugars was collected and evaporated to dryness in a water bath with a stream of nitrogen. Trimethylsilyl derivatives were prepared by the addition of 0.5 ml mixture (10:2:1, v/v) of dry pyridine, hexamethyldisilazane and trimethylchlorosilane. After 15 min, 1 - 5 µl of the reaction mixture was injected into a GLC column 6 ft, ¼" O.D. packed with 2% SE-30 or with 3% OV 17 on chromosorb W, HP. The operating conditions were: injector temperature 250^{o}, detector temperature 270^{o}, nitrogen carrier gas flow rate 30 ml/min, and programmed column temperature 100 - 160 at 2^{o}/min or 120 - 200 at 4^{o}/min.

b. Galactolipids

Neither MGDG nor DGDG were obtained pure from the chloroplast by two-dimensional TLC, and hence, other attempts have been made for their isolation. The procedure described by Van der Veen et al. (1967) which employs column chromatography on carbon celite adsorbent (Nuchar Attaclay) followed by silicic acid column chromatography did not yield

pure galactolipid fractions. The final fractions still contained residual pigments. In another attempt, chloroplast lipids were fractionated into neutral and polar lipids using silicic acid column chromatography followed by precipitation of PL from the polar fraction by cold acetone treatment. The filtrate was chromatographed on a column of Sephadex LH-20 as described by Helmsing (1967). Both MGDG and DGDG fractions obtained were still contaminated with residual pigments and PL. Finally, the procedure described by Rosenberg et al. (1966) and by Gardner (1968) was adapted and modified for preparative scale.

Silicic acid, 150 g, was slurried in n-heptane and poured into 4.5 x 50 cm column. Heptane was drained and the column was washed with 1 ℓ chloroform. The crude chloroplast lipid extract, 7 ml, (520 mg lipid) was applied to the column and eluted with 1 ℓ chloroform and then with 500 ml chloroform-acetone (1:1, v/v) and then with 500 ml acetone followed by 500 ml methanol. The separation was performed at 2 - 4^{o} in a column protected from light. Both chloroform acetone, and acetone effluents were collected in 11 ml fractions (46 fractions each). The individual fractions were examined by TLC and their phosphorous and galactose content determined. Fractions containing MGDG and DGDG were combined separately and subjected to one dimensional TLC using 500 μ layer of Silica gel G. Acetone-acetic acid-water (10:2:1, v/v) was used as developing solvent.

Phorphorus determination was carried out on 0.5 ml aliquots of the lipid solution, (1 - 5 μg P). The aliquots were evaporated to dryness, mixed with 0.5 ml 10 N sulfuric acid and heated at 150 - 160^{o} for 3 h. Several drops of 30% hydrogen peroxide were added and the solution reheated for an additional hour. To the digest 4.6 ml of 0.22% ammonium molybdate and 0.2 ml of Fiske-Subba Row reagent were added and

thoroughly mixed. The tubes were covered and heated for 7 min in a boiling water bath. The absorbance of the color developed was read at 830 nm (Bartlett 1959). The concentration of phosphorus was then calculated from a standard curve prepared in a similar way (Fig. 5).

Sugars were determined in 1 ml aliquots (10 - 80 µg galactose) of the lipid fraction. The aliquots were evaporated to dryness and then hydrolyzed for 1 h at 100° with 2 ml of 3 N sulfuric acid. After cooling, 1 ml of 2% aqueous phenol and 4 ml of concentrated sulfuric acid were added. The contents were then mixed with a Vortex, kept at room temperature for 15 min, and the absorbance read at 490 nm (Dubois et al. 1956). Galactose concentrations were calculated from the standard curve shown in Fig. 6.

3. Saponification, esterification and FA analysis

Saponification was achieved by refluxing under nitrogen for 30 min an aliquot of pea seed total lipids with 5% methanolic potassium hydroxide. The unsaponifiable material was removed with hexane and the remaining solution was acidified with an excess of 10 N sulfuric acid. The FA released were extracted with hexane and esterified with 14% boron trifluoride in methanol by heating at 100° for a few minutes.

For transesterification, NL (10 - 20 mg) were gently refluxed in 2 ml of 1% (w/v) sulfuric acid in methanol for 2 h, while PL (10 - 20 mg) were esterified under nitrogen with 2 ml of 2% (w/v) sulfuric acid in methanol in sealed ampules at 65° overnight. After esterification the contents were diluted with 3 ml water and the FAME were extracted twice with hexane. Extracts were combined, washed once with

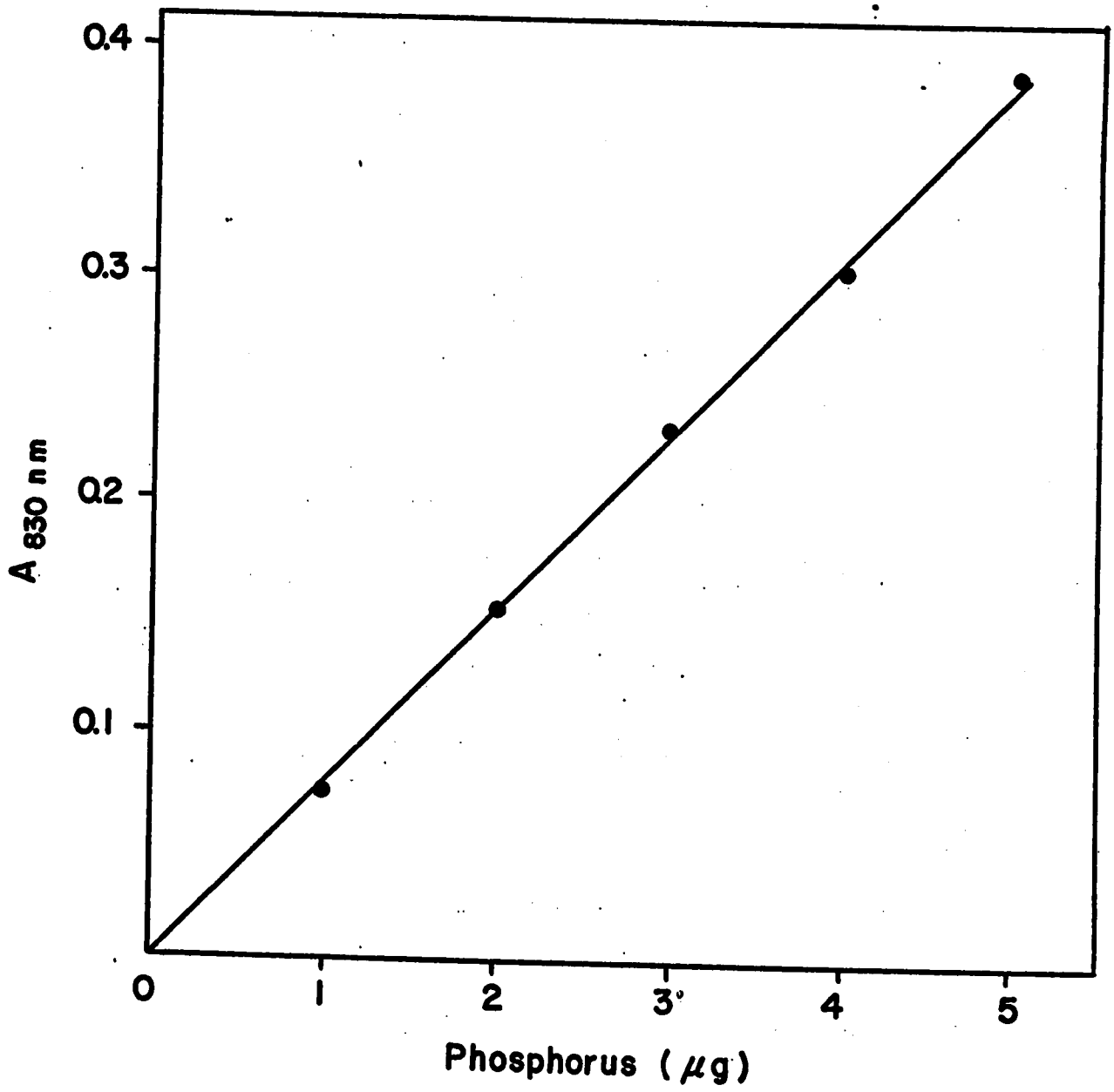


Fig. 5. Calibration Curve for Phosphorus Determination. The method was that of Bartlett (1959). Readings were taken in 1 cm cell using a Bausch and Lomb Model 20 Spectrophotometer.

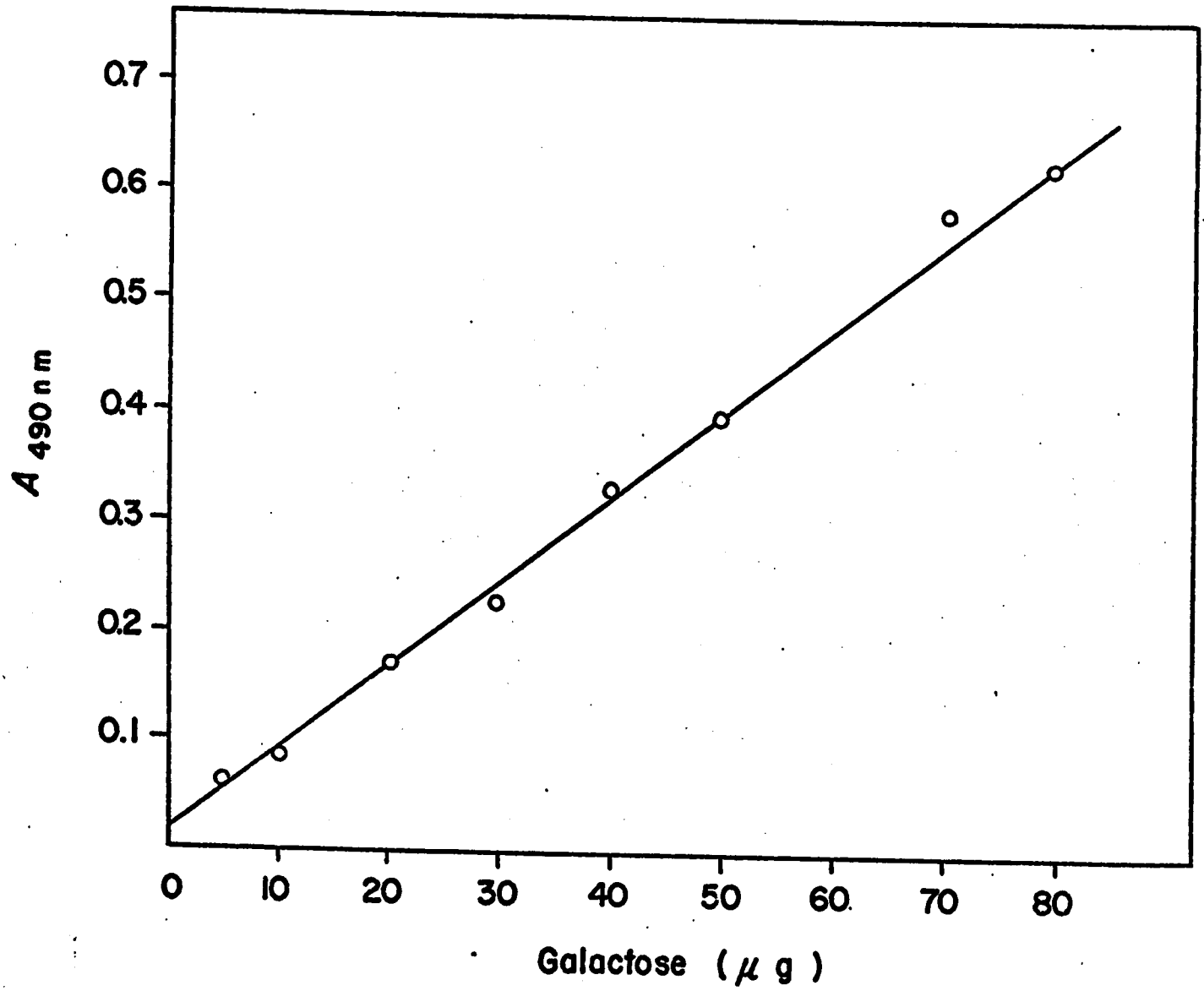


Fig. 6. Calibration Curve for Galactose Determination. The method used was that of Dubois et al. (1956). Readings were taken in 1 cm cell using a Beckman DBG Spectrophotometer.

3 ml of 0.2N potassium carbonate and then twice with 3 ml of water. The residual water was removed by anhydrous sodium sulfate, and the methyl esters were concentrated and used directly for GLC.

For GLC, a flame ionization detector, and U shaped glass columns (6 ft x 1/8") packed with 15% EGS coated on AW 100/120 mesh chromosorb P were used. The operating temperatures were for columns 185^{0}, injector ports 200^{0}, detectors 220^{0}. For nitrogen as carrier gas, the flow rate was 60 ml/min. The FA have been identified by means of their retention times and comparison with known standards. The calibration curve representing retention times versus equivalent chain length of FA is shown in Fig. 7.

C. Lipoxidase

1. Isolation and purification

Pea seeds var. Homesteader, 300 g, were ground with dry ice in a Waring blendor. The powder was extracted by stirring with 1.0 ℓ of 0.1 M Tris - Cl buffer, pH 7.2 at 2 - 4^{0} for 16 h. The slurry obtained was filtered through 4 layers of cheesecloth and centrifuged at 20,000 x g for 15 min. The supernatant was recentrifuged at an average of 115,000 x g for 2.5 h. The supernatant was subjected to 25% ammonium sulfate precipitation for 30 min and then centrifuged at 20,000 x g for 15 min. The supernatant was collected and then precipitated with 50% ammonium sulfate for 30 min and centrifugation repeated. The pellet was collected, dissolved in 90 ml of 0.05 M Tris - Cl buffer, pH 7.2 and dialyzed against the same buffer for 15 h with several changes of buffer. The resultant lipoxidase extract (dialyzate) was further

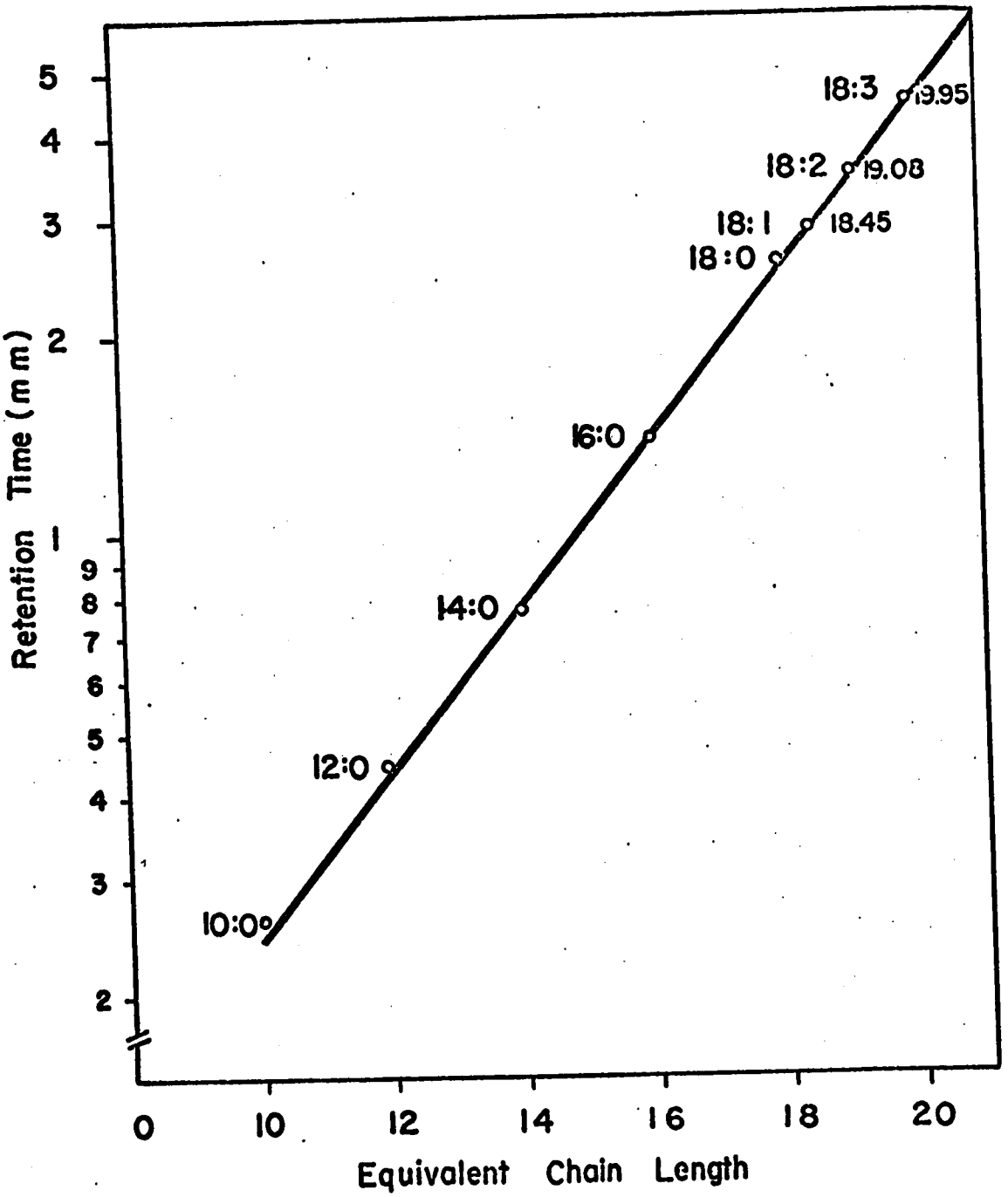


Fig. 7. Retention Time of Standard FAME vs Equivalent Chain Length Using EGS as Liquid Phase. Gas chromatograph Bendix 2500; isothermal separation 185°, N_2 flow rate 60 ml/min. Pure samples of FAME were from Hormel Institute, Austin, Minn.

purified by Sephadex and DEAE Cellulose column chromatography. The isolation and purification steps (Fig. 8) were performed according to the procedure described by Eriksson and Svensson (1970) except that the acetone defatting step was omitted and an ultracentrifugation step was introduced.

Sephadex G-150, 26 g, were allowed to swell in excess water by heating on a water bath for 5 h. The swollen Sephadex was cooled and poured into a chromatographic column (1.5 x 90 cm) avoiding air bubble formation. The water was drained off and the Sephadex column settled to about 80 cm in height. The column was then equilibriated with 0.05 M Tris-Cl buffer pH 7.2. The dialyzate (7.5 ml, 412 A_{280} units) was applied to the column and eluted with the same buffer. The elution rate was kept constant with a peristaltic pump, and fractions of 4.5 ml were collected every 15 min by means of a time operated fraction collector. The absorbance at 280 nm of each fraction was determined, after which all fractions having UV absorbance were assayed for lipoxidase, peroxidase, and catalase activity. The fractions containing lipoxidase were combined and the enzyme precipitated by 50% ammonium sulfate saturation at 0^{o} and sedimented at 15,000 x g for 20 min. The precipitate obtained was dissolved in a few ml of 0.01 M Tris-Cl buffer (pH 6.5) containing 2 mM $CaCl_2$ and 1 mM 2-mercaptoethanol (Tris-$CaCl_2$ - SH). The solution was dialyzed against the same buffer for 15 h with several changes of buffer.

DEAE-Cellulose was pretreated by soaking in 0.1 N HCl containing 0.5 N NaCl, twice, each time for several hours. The suspension was poured in a chromatographic column (2.5 x 45 cm) filled with the Tris-$CaCl_2$-SH

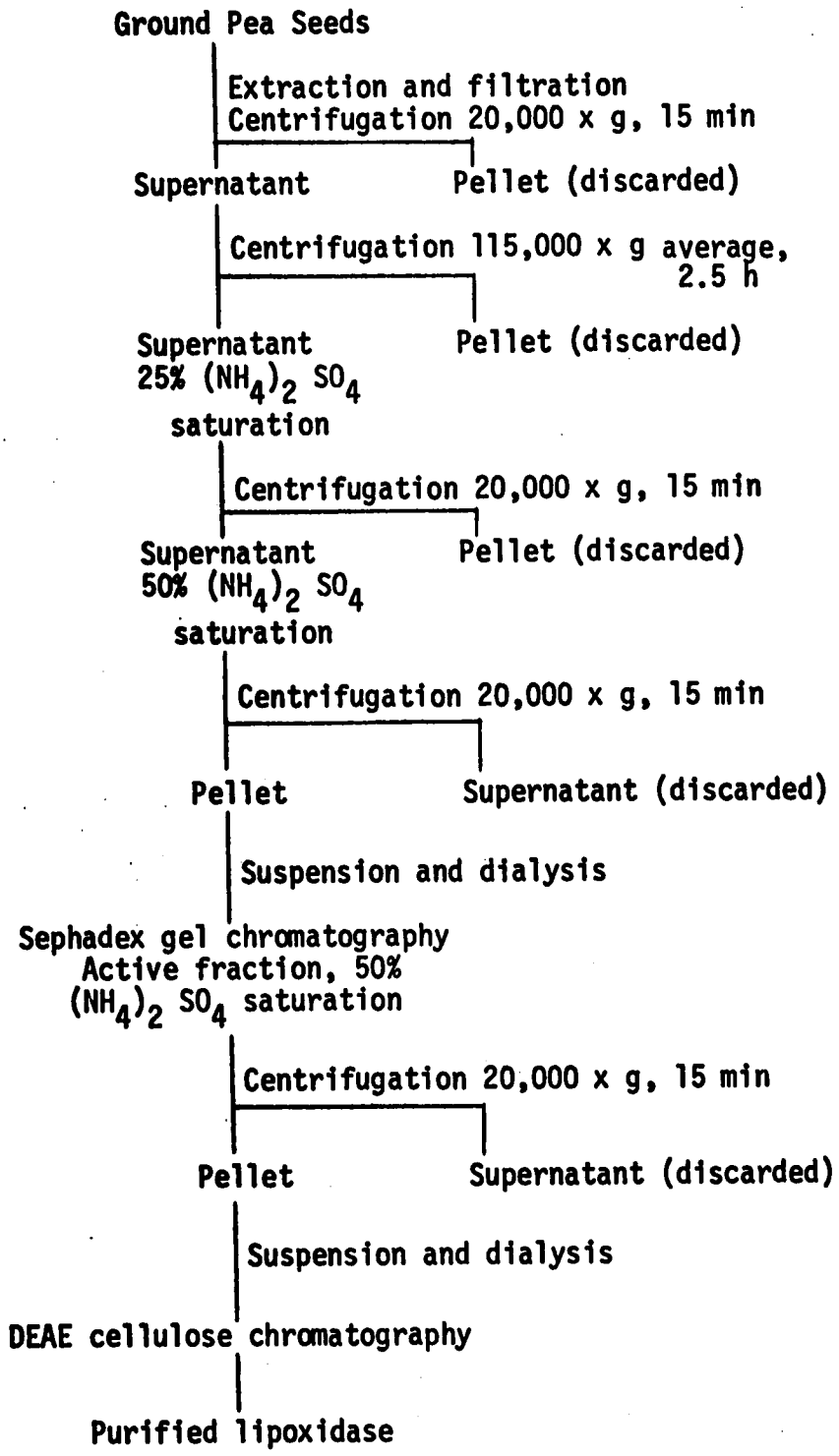


Fig. 8. Flow Diagram of Lipoxidase Isolation and Purification

buffer and allowed to settle. The column was then washed with 1 ℓ of the same buffer and the dialyzed lipoxidase solution usually obtained from two separations on the Sephadex column (8 ml, 450 A_{280}) was applied. Elution was performed by applying a linear salt gradient ranging from 0.01 - 0.61 M NaCl in a total volume of 500 ml Tris-$CaCl_2$-SH buffer. Fractions of ≈5 ml were collected every 12 min and assayed for UV absorption and lipoxidase activity. Lipoxidase fractions were assayed for catalase and peroxidase activity, pooled, concentrated and dialyzed as previously to yield the final purified lipoxidase.

The activity of lipoxidase was determined at 25° by polarography using as substrate 7.5 x 10^{-3} M linoleic acid in 0.1 M borate buffer, pH 7.0, containing 2.5 ml Tween 20 per litre. The emulsion was freshly prepared to avoid high initial peroxide values. One unit of the enzyme was ascribed to the initial reaction rate involving 1 μM O_2 per min at 25°. The relationship between enzyme concentration and initial oxygen uptake was studied and so was the effect of pH on enzyme activity. For the latter study Kolthoff's borax phosphate buffer (Kolthoff 1925) was used. When CN^- inhibition was studied, 5 x 10^{-3} M potassium cyanide was included in the substrate solution.

Catalase activity was determined by the oxygen cathode method of Goldstein (1968) which is similar to that of lipoxidase determination. Initially, the amplifier was set to read 50% of full scale with the electrode in air-saturated distilled water at 25°. Then, 6.0 ml of 0.033 M sodium perborate in 0.05 M phosphate buffer, pH 7.0, were equilibriated in the reaction cell at 25° for 3 min and then 10 - 50 μl of the enzyme solution added. The oxygen concentration rose rapidly at

a constant rate for at least 30 sec before the rate decreased or bubbles formed. The results were expressed in micromoles of oxygen evolved per min per ml of column effluent collected.

Peroxidase activity was determined spectrophotometrically at 470 nm using as substrate freshly distilled guaiacol 7×10^{-3} M in 7×10^{-3} M phosphate buffer at pH 7.0 (Hackett 1964). The reaction mixture, 2 ml substrate and 1 ml enzyme solution, were placed in the cuvette and at 0 time 20 µl of 10 mM hydrogen peroxide was added. The mixture was stirred with a teflon rod and the absorbance of the pinkish color developed was read at 470 nm against the substrate as blank after 30 and 60 sec. The activity of the enzyme was calculated from the linear part of the curve obtained from the time versus changes in optical density plot and was reported as ΔA_{470} per min per ml.

The effect of thiol group on catalase activity was studied using 2-mercaptoethanol, reduced glutathione and cysteine at concentrations of 1, 2, 5 and 10 mM in 0.05 M phosphate buffer pH 7.0. At these concentrations, the effect of incubation time on the enzyme activity was studied at time intervals of 0, 1, 3 and 5 h. For this study, beef liver catalase was used in concentrations of 0.05 and 0.1 mg per ml of 0.05 M phosphate buffer pH 7.0.

2. Disc gel electrophoresis

Analytical disc gel electrophoresis was performed as described by Davis (1964). The stacking gel pH 6.7 (0.2 ml, 2.5%) was carefully layered over 0.25 ml of 40% sucrose in vertical tubes of 7.0 cm in length and 0.5 cm I.D., and polymerized by exposure to light for 20 min. The tubes were then filled with the separating gel pH 8.9 (1.8 ml, 7%)

solution and the gel allowed to polymerize in the dark for 30 min. The sample, about 100 µg protein, was applied in 40% sucrose, total volume 100 - 200 µℓ, and the electrophoresis conducted for about 1 h at 3 mA per tube. After completion of the electrophoresis, the gels were stained for 1 h with 0.2% (w/v) Coomassie blue in an aqueous solution containing 46% methanol and 9.2% glacial acetic acid. The excess dye was removed by washing with 7.5% acetic acid in 50% methanol and the gel was placed in a fresh 7.5% acetic acid solution without methanol for viewing and storage.

For molecular weight estimation of lipoxidase, the purified enzyme, 1 - 2 mg protein/ml, was incubated with dithiothreitol, 0.5 mM final concentration, and SDS, 1% final concentration, at 37^{o} for 30 min. The sample was then mixed with an equal volume of 60% glycerol and applied to a 5% polyacrylamide gel pH 7.1 prepared as mentioned earlier except that the gel contained 0.1% SDS and 10% of 0.5 M phosphate buffer pH 7.1. The stacking gel was excluded and the electrode buffer consisted of 0.05 M phosphate buffer pH 7.1 containing 1% SDS. Electrophoresis was conducted for 3 h at 5 mA per tube. The following proteins have been used as standards:

Protein	Molecular Weight
Cytochrome C (horse heart)	12,400
Myoglobin Cryst. salt free (sperm whale)	17,800
Chymotrypsinogen - A - beef pancreas	25,000
Ovalbumin (2 x cryst.)	45,000
Albumin (Bovine) Cryst.	67,000

Ovalbumin was included with the lipoxidase sample as an internal standard.

The presence of isoenzymes of pea lipoxidase was investigated by the starch iodine staining method of Guss et al. (1967a). Soybean lipoxidase was applied for comparison. The stacking gel was excluded and the separating gel contained 1% amylose. Relatively large amounts of the lipoxidase extract had to be applied (200 - 500 µℓ). After electrophoresis with a current of 4 mA per tube, the gels were removed from the tubes, placed in 10 x 100 mm test tubes and were completely covered with substrate solution. This was prepared by ultrasonically dispersing linoleic acid, greater than 99% purity, in 20 ml distilled water. Ultrasonification was carried out for 3 min using the sonic oscillator tuned to 1.2 amp. The dispersed linoleic acid was then diluted with 0.05 M Tris-Cl buffer pH 8.3 to yield a final concentration of 2×10^{-3} M linoleate. The gels were kept in the substrate solution for 30 min at room temperature with frequent inversion to assure adequate aeration. In the inhibition test, potassium cyanide at 10^{-3} M was added to the substrate prior to incubation.

After substrate incubation, the gels were removed from the tubes, thoroughly rinsed with distilled water and placed in test tubes filled with a staining solution consisting of 5 ml freshly prepared potassium iodide solution per 100 ml of 15% acetic acid. Violet brown colored activity bands appeared within 5 - 10 min and were optimally developed within 30 min. To prevent background staining due to autoxidation of the potassium iodide, the acetic acid solution was degassed by the application of vacuum prior to and after the addition of

potassium iodide.

D. Pea mineral analysis

Pea seeds of the three varieties were separately ground in a mortar and pestle and dried in an oven at 105^{0} till constant weight, (2.5 - 3 h). Of each variety 0.5 g was digested for 1 - 2 h with 5 ml of 70% perchloric acid containing less than 0.1 ppm Fe, and a few ml of nitric acid containing less than 0.2 ppm Fe. The clear digest was made up to 25 ml with double distilled deionized water and used for analysis, by atomic absorption, of the following minerals: K, Na, Ca, Mg, Fe, Co, Ni, Cu, Mn, and Zn. Prior to calcium analysis, La^{3+} ion was added to the sample to a final concentration of 1500 ppm. Results were then calculated for corresponding calibration curves. These curves were prepared for each element assayed by using standard salt solutions of different ppm levels.

E. Lipid oxidation in model systems

1. Enzymatic oxidation of lipid substrates

The enzymes used in this study were the purified pea seed lipoxidase, wheat germ lipase, and snake venom phospholipase A_2. Pure TG and NL were obtained from pea seed total lipids. The individual PL such as PC, PE, PG, and DPG were isolated from pea mitochondria while the galactolipids, MGDG and DGDG, were isolated from pea chloroplasts. Pure PL were emulsified ultrasonically in 0.1 M borate buffer pH 7.0 in the presence of 0.04% deoxycholate and 2.5×10^{-3} M calcium acetate. On the other hand, NL were emulsified in 0.1 M borate buffer pH 7.4 containing 0.4 M NaCl, 0.005 M $CaCl_2$ and 2 mg sodium taurocholate and

3 mg gum acacia per ml of buffer.

Preliminary work on the activity of lipoxidase in the media for lipase and phospholipase proved that the media do not affect lipoxidase activity. Furthermore, a preliminary study of the kinetics of the enzyme showed a Michaelis-Menton constant Km = 2.3×10^{-3} M when linoleic acid was used. Also, the polarographic method of lipoxidase activity was satisfactorily sensitive to as low as 10^{-4} M linoleic acid in the above media. Hence, suitable amounts of the individual lipids had been included in the media to give a concentration of bound linoleic acid ranging between 10^{-4} and 2.3×10^{-3} M. Thus for instance, pea mitochondrial PC as found by GLC analysis contains 58% of its total FA as linoleic acid, and has a mol. wt. of approximately 790. The ratio (w/w) of PC to its linoleic acid content will be 790 ÷ (2 x 280 x 58/100) = 2.4, and hence PC concentration used in the substrate medium is ranging between 2.4×10^{-4} and 6.5×10^{-3} M i.e., 0.2 and 5.2 mg/ml. The same calculation was applied to all lipids used.

The substrate was divided into two aliquots, 3 ml each, one of which was incubated with the enzyme while the other was used as a control. Substrate with PL was incubated with 1 mg of phospholipase A by shaking at 37^{o} for 1 h while NL containing substrate was incubated with 10 mg lipase at 25^{o} for 3 h after which the pH of 7.4 was readjusted to 7.0 - 7.2 by few drops of 0.5 N HCl. Both controls and incubated samples were checked for lipoxidase activity. Since this study is of a qualitative nature, no further analyses were done to determine the degree of lipid hydrolysis after incubation.

2. Nonenzymatic oxidation

a. Oxidation induced by mitochondria swelling

To clarify the parameters that affect pea mitochondria swelling the effects of KCl concentration in the suspension buffer, germination time, and storage of mitochondria were investigated. Therefore, swelling was studied for mitochondria isolated from pea cotyledons germinated for 1, 2, 3, 4, 5, 6 and 7 days, and for the isolated mitochondria after storage for 24 h at 2 - 4^{0}. The swelling was studied in 0.2 M Tris-Cl buffer, pH 7.5 containing 0, 0.05, or 0.1 M KCl. From the results obtained, germination time of 1 day and KCl concentration of 0.1 M, were chosen for subsequent oxidation study. In addition, the isolated mitochondria were used within 1 h after their preparation.

Lipid peroxidation induced by the presence of transitional metal ions was studied by the DME polarographic method of Niederstebruch and Hinsch (1967). Mitochondria preparation, 1 ml, was added to 2 ml of KCl, Tris-Cl buffer that contained 50 μM of either Fe^{2+} or Fe^{3+}. Stock solutions of iron were freshly prepared from its ammonium sulfate salts in cold unbuffered KCl solution deaerated before use. After incubating mitochondria for the required time, 1 - 60 min, 1 ml aliquot was taken, added to 0.25 ml of 40% TCA, and centrifuged at 2500 rpm for 10 min. The pellet was extracted at 60^{0} for several minutes with 5 ml methanol benzene solvent (1:1, v/v) containing 0.3 M LiCl, which served also as an electrolyte. Then it was centrifuged as above and the supernatant was collected and checked for hydroperoxides.

Polarographic studies have shown that traces of both TCA and dissolved oxygen gave interfering waves while Fe^{2+}, Fe^{3+}, Tris and

traces of water did not interfere. Hence, TCA was omitted and the incubation was stopped instead, with 3 ml of hot chloroform methanol (1:1, v/v). The suspension was centrifuged, the supernatant collected and evaporated to dryness in a stream of nitrogen, and then dissolved in the electrolyte. Oxygen was removed by purging the sample with nitrogen for at least 7 min and by passing the gas over the sample during the polarographic run. Nitrogen was purified from traces of oxygen by passing it through 2.5% pyrogallol in 40% potassium hydroxide solution and the gas was then saturated with the electrolyte solvent before reaching the sample. A cathodic current was applied and scanning was performed from 0 to 1.0 V. The half wave potential of hydroperoxides in this media was found to be -0.65 V. However, by this method no hydroperoxides could be detected in mitochondrial extracts although the method easily detected hydroperoxides formed in butter fat oxidized by heating at 70^{0} for 10 h.

The TBA test was then applied for following mitochondrial lipid oxidation. The common procedure suggested by Dahle et al. (1962), Hunter et al. (1963), Barber (1966), in which mitochondrial residues after the TBA color development are removed by filtration or centrifugation, was found unsatisfactory due to color adsorption on the protein particles removed. Therefore, the method of Placer et al. (1966) was adapted. A 0.8% TBA solution was prepared by dissolving the acid in a small amount of NaOH, then neutralized with 7% perchloric acid. The TBA reagent was prepared by mixing 2 vol. of this stock solution with 1 vol. of 7% perchloric acid. Of this reagent, 1 ml was added to 1 ml of the incubated mitochondrial preparation and the mixture was then heated

at 100° for 10 min. After cooling the contents, 2.0 ml pyridine, n - butanol mixture (3:1, v/v) and 0.7 ml of 1 N NaOH were added and thoroughly mixed by a Vortex. Immediately after mixing, the absorbance was read at 548 nm.

Mitochondrial lipid oxidation was studied in dependence on the following factors: 50 μM Fe^{2+}, 50 μM Fe^{3+}, 50 μM Fe^{2+} and Fe^{3+} (1:4), 20 μM Cu^{2+}, 30 μM Mn^{2+}, 10 μM Co^{2+} and ascorbic acid at two levels, 0.2 and 2 x 10^{-3} M. The concentrations of the transitional metal ions were 4 - 24% of the physiological levels found in peas by atomic absorption spectrophotometry, while those of ascorbic acid were below and above the physiological level reported in peas (26 mg/100 g), Nehring (1968).

b. Matrix effect

The matrix used in this study consisted of cellulose, amylose, amylopectin, pectin, and protein. The latter consisted of albumins and globulins isolated from pea seeds according to Danielsson (1949, 1950) and Danielsson and Lis (1952). Pea seeds var. Homesteader, 100 g, were ground and extracted overnight by stirring with 350 ml of 0.05 M phosphate buffer pH 7.0 containing 0.2 M NaCl. The slurry was filtered through cheesecloth and debris free homogenate was then centrifuged at 10,000 x g for 10 min. Proteins in the superantant were precipitated overnight by 70% ammonium sulfate saturation and centrifuged at 10,000 x g for 10 min. The pellet was dissolved in 40 ml of the extraction buffer and dialyzed against running tap water overnight, followed by distilled water for 5 h and finally against double distilled and deionized water till the absence of traces of Cl^- ions. Isolation steps were performed at 2 - 4° and are presented in Fig. 9.

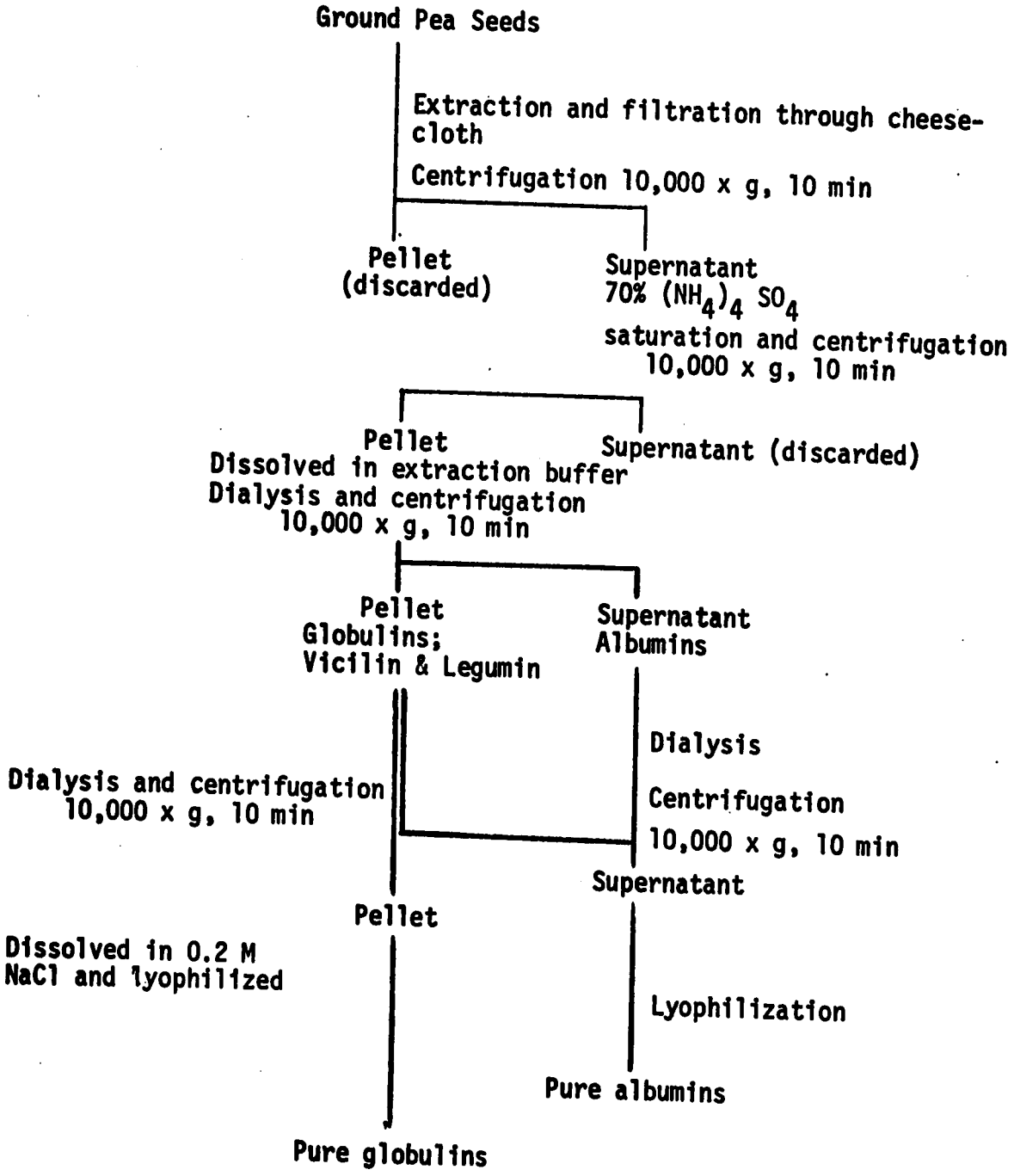


Fig. 9. Flow Diagram for Isolation of Pea Albumins and Globulins

The dialysate with protein suspension was centrifuged at 10,000 x g for 10 min to yield the globulin pellet (vicilin and legumin) and the supernatant with albumins. The latter was dialyzed for an additional 5 h against deionized water and centrifuged at 10,000 x g for 10 min. The supernatant yielded the pure albumins. The pellet was combined with the previous pellet and dialyzed against deionized water for 5 h. The dialysate was centrifuged at 10,000 x g for 10 min and the pellet consisting of pure globulins was dissolved in a small volume of 0.2 M unbuffered NaCl solution. Albumin and globulin fractions were then lyophilized by freezing in dry ice - acetone bath and drying under a vacuum of 10 - 20 μ for 24 h.

The filter paper technique of Karel (1960) was adopted for studying the matrix effect on lipid oxidation. Ash free filter paper discs 3.5 cm in diameter were dipped for 10 sec in 1% aqueous solution of the matrix except for globulin when water contained 0.2 M sodium chloride. The discs were dried in air on a stainless steel wire tray for 10 min and then dried at room temperature for 24 h in a vacuum of 10 - 20 μ. The dehydrated filter papers were weighed, dipped for 10 sec in chloroform solutions containing 10 - 20% NL or PL, and the dehydration procedure repeated. Four filter paper discs were placed in the reaction flask of a Gilson differential respirometer. The oxidation was conducted in air at 50^{0} for 20 - 50 h. To eliminate the effect of oxygen depletion, the flasks were flushed with dry air at suitable intervals, 10 - 15 h at the beginning, and 2 - 5 h near the end of the run. All experiments were done at least in duplicates.

c. Studies applying ESR spectroscopy

Gamma rays and UV light have been used as initiators for the oxidation of methyl esters of oleic, linoleic, and linolenic acids. About 200 mg of FAME was placed in a thin-walled 4 mm O.D. Spectrosil quartz tube and degassed by alternative freezing and warming for several times under high vacuum (5 - 10 μ). The tubes were sealed and irradiated in a Dewar flask filled with liquid nitrogen (-196^{o}). In case of UV irradiation, the Dewar flask was made of quartz. The light from UV lamp was passed through a 2" wide water filter to eliminate infra red light, and then focused on the sample placed about 6" from the lamp. Condensation of moisture on the outer wall of the Dewar flask was prevented by passing a jet of dried air around the flask. At the end of irradiation (≈ 1 Mrad in gamma and 4 - 6 h for UV), the sample tube was transferred to the spectrometer cavity which was precooled to -196^{o} in order to prevent recombination of the free radicals, and the spectra recorded.

IV. RESULTS

A. Pea Lipid Analysis

1. Separation and identification

Pea seeds contain 3 - 4% TL extractable by a hot chloroform methanol mixture (2:1, v/v). Laxton had the highest TL lipid content while Homesteader had the lowest, and Stratagem was in between (Table 1). The NL/PL ratio varied considerably between varieties and was highest for Laxton while that of Homesteader and Stratagem was low and comparable. Since all varieties were extracted and their lipids fractionated in the same way, the differences in the total as well as in the neutral and polar lipids might reflect varietal characteristics. Only the lipids of Homesteader variety were further investigated.

TABLE 1

THE TL PERCENTAGE AND THE RATIO OF NL/PL IN THE INVESTIGATED PEA SEED VARIETIES*

Variety	% Moisture	% TL	NL/PL Ratio
Homesteader	15.4	3.0	1.8
Laxton	17.5	3.9	2.3
Stratagem	16.0	3.4	1.7

*Percentages are calculated on dry basis.

The NL of pea seeds as revealed by one dimensional TLC consist mainly of TG, small amounts of DG, FFA, free and esterified sterols, and

pigments (Fig. 10). The non polar developing solvent, pet. ether-diethyl ether (100:20, v/v) rendered clear separation among the individual lipid components which made possible the isolation of pure TG needed for the subsequent oxidation studies. Pea sterols recovered from the unsaponifiable fraction after alkaline saponification of TL were also complex in composition. One dimensional chromatograms obtained by a slightly less polar solvent than that used for developing NL revealed seven well defined spots within this class of compounds.

The PL of pea seeds were separated by two dimensional TLC using two different solvent systems. Both systems revealed the presence of 9 - 10 clear spots (Figs. 11 & 12) of which PI, PC, PE, and SG (S_1) were the major lipids. Among these, PE appeared as the most intensive spot upon charring the plate with sulfuric acid. Present to a lesser extent were MGDG, DGDG, PG, traces of NL and pigments, a stationary faint spot at the origin, a sterol spot (S_2) and possibly DPG. No spots representing PA or PS were apparent in either solvent system. Using the first solvent system (Fig. 11), two overlapping spots appeared below the spot containing MGDG, but appeared as one spot when the second solvent system was used (Fig. 12).

Among PL, all individual phospholipids gave positive reactions with the phosphorus detecting reagent; the intensity of the blue colored spots being strongest for PE followed by PC and then PI. PE spot gave a distinct red color with ninhydrin, and PC gave a positive reaction with the cholinedetecting reagent. The spots containing galactolipids, MGDG and DGDG, gave a violet color when sprayed with α-naphthol in sulfuric acid before heating. Sterols were revealed by their distinct

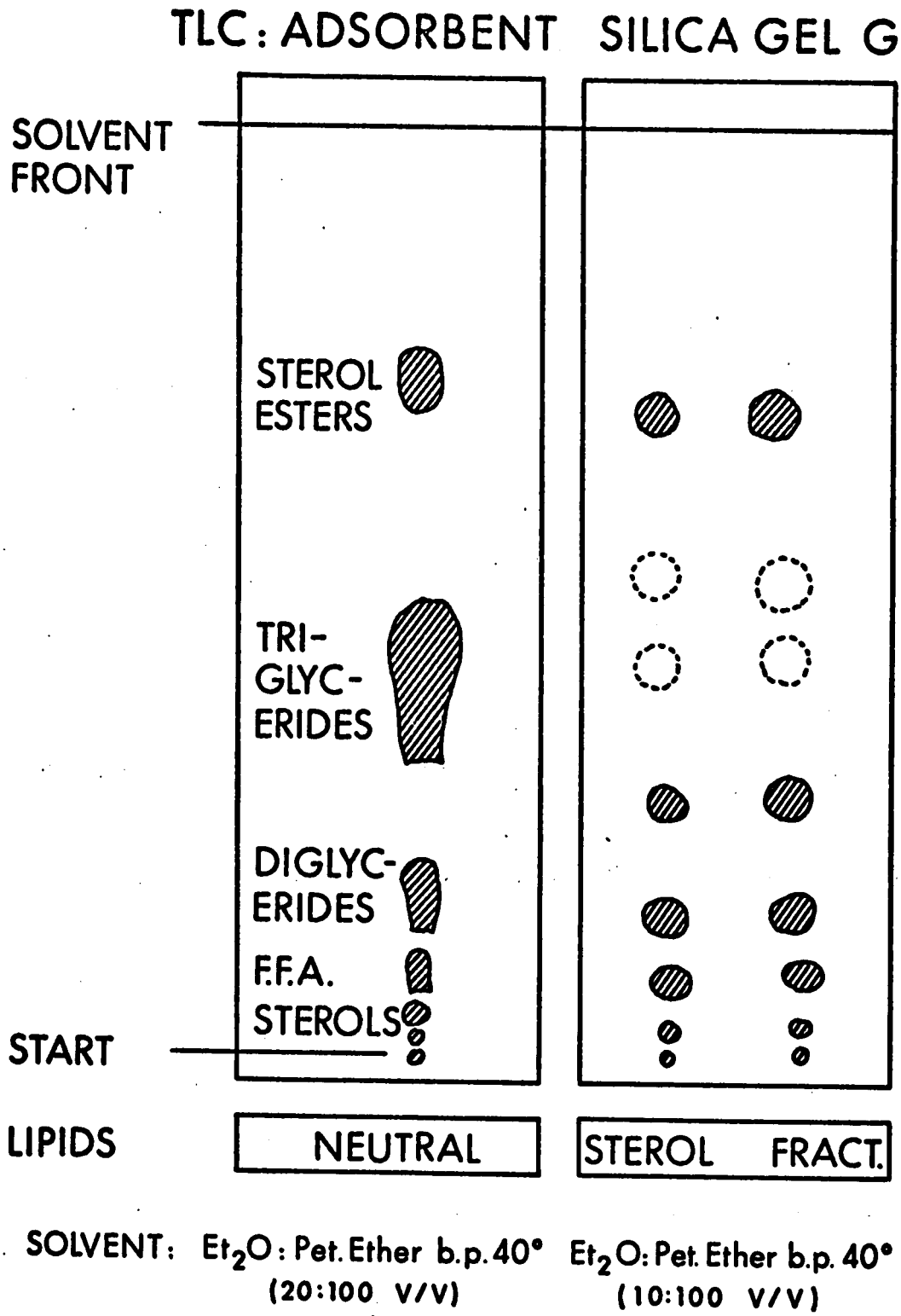


Fig. 10. One Dimensional TL-Chromatograms of Neutral and Nonsaponifiable Fractions of Pea Seed Lipids.

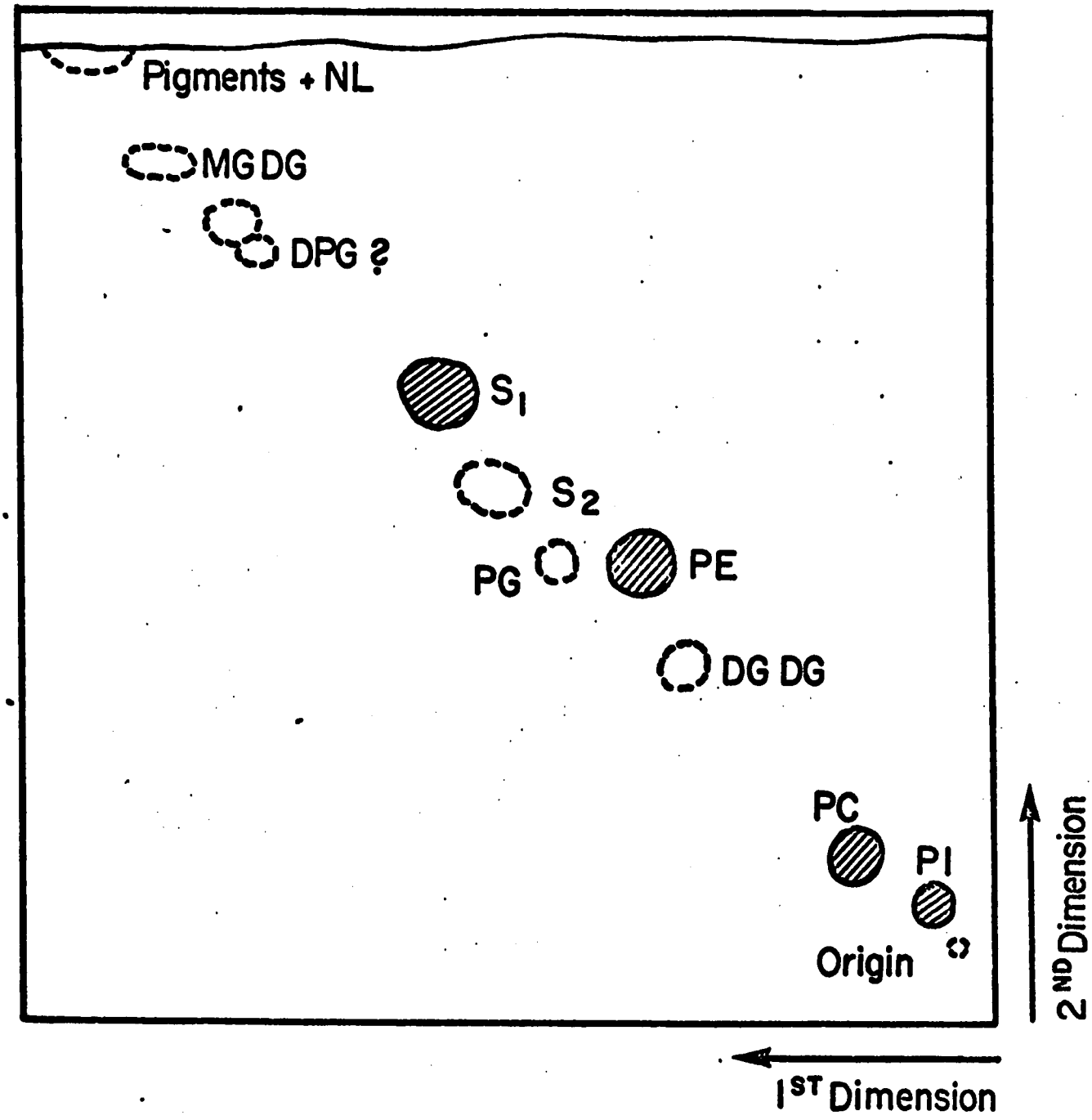


Fig. 11. Two Dimensional TL-Chromatograms of Pea Seeds' PL. The adsorbent layer was 200 μ of Silica Gel G (Merck). Solvent system was chloroform-methanol-7 N ammonia (65:25:4, v/v) in the first dimension, and chloroform-methanol-acetic acid-water (170:25:24:4, v/v) in the second dimension (Nichols and James 1964).

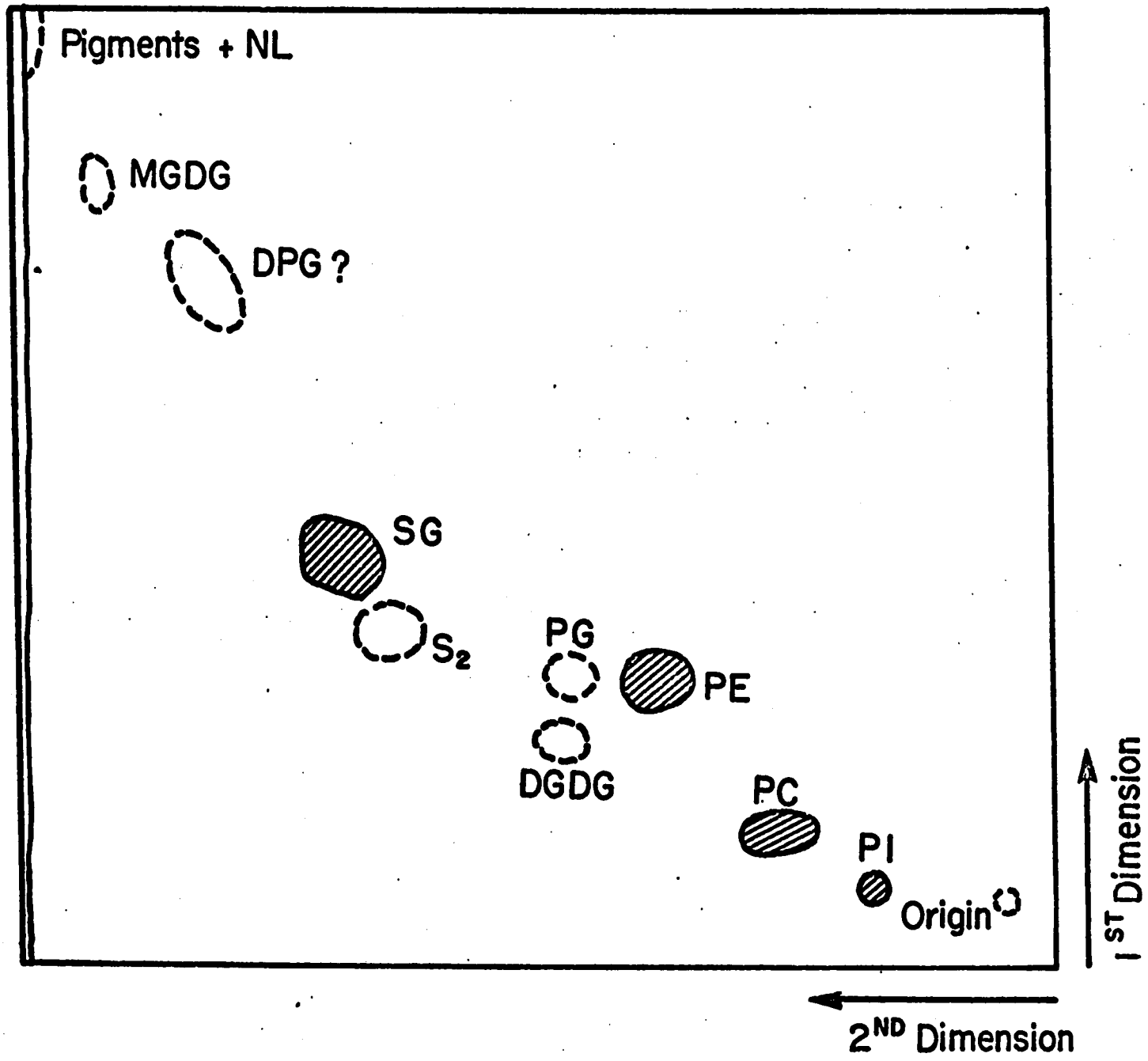


Fig. 12. Two Dimensional TL-Chromatograms of Pea Seeds' PL. The adsorbent layer was 200 μ of Silica Gel G (Merck). Solvent system used was chloroform-methanol-28% ammonia (65:25:5, v/v) in the first dimension, and chloroform-acetone-methanol-acetic acid-water (3:4:1:1:0.5, v/v) in the second dimension (Rouser et al. 1970).

purple color developed at the beginning of charring with sulfuric acid. Besides the use of specific spraying reagents the identity of the major lipids was also confirmed by the migration pattern of identical commercial samples or individual lipid samples prepared in this laboratory (MGDG and DGDG). In addition, IR spectra were used for further confirmation of some lipid compounds.

Spectra of IR absorption for MGDG and DGDG in the solid state (potassium bromide pellet) revealed major absorption bands at 2.9, 3.4, 5.75, and 9.4 μ, the first major peak corresponding to -OH stretching from sugar moiety, the second as a double peak to the symmetrical and non symmetrical stretching of hydrocarbon chain, the third to stretching of C = O in ester group, and the fourth as a wide band resulting from C - O stretching of ester group. The small bands representing unconjugated cis double bond appeared at 3.3 and 6.1 μ reflecting the bending vibration of olefinic hydrogen atoms. Cis double bonds, in addition, were easily detected by a rather broad band at the region of 14 - 15 μ. From the spectra recorded, it appears that none of these bands even that of -OH could be used to distinguish MGDG from DGDG. Nevertheless, the-OH absorption band was reliable in distinguishing galactolipids from some phospholipids such as PE and PC.

Identification of PE and PC in ethanol free chloroform solutions has been carried out by recording their spectra in the region of 8.5 - 11 μ. Absorption patterns related to nitrogenous moieties were reflected by a single peak at 9.3 μ for PE and by a double absorption peak between 9 - 9.7 μ for PC.

Germinated cotyledons and pea mitochondria gave similar TL-chromatograms after two dimensional separation. The fraction of NL and pigments moved along the solvent front in both dimensions leaving behind the separated constituents of PL fraction - glyco - and phospholipids. These chromatograms obtained might be considered similar to those of PL. In comparison to seed PL, the germinated tissue provided a much higher number of individual lipids. Nine to ten spots contained phosphorus with the highest content being present in PC, PE, and in an unidentified spot (P^+?) which was absent in chromatograms of seed PL. On the other hand, PI being a major spot in seed lipids became a minor one in TL of either germinated cotyledons or mitochondria. Furthermore, while SG, MGDG and DGDG were rather major spots in case of germinated cotyledons, DPG and an unidentified spot (u) appeared instead as major spots in mitochondrial PL. The TL-chromatograms of mitochondrial PL are shown for both solvent systems in Figs. 13 & 14. Some minor PL spots were identified from their chromatographic behavior and/or co-chromatography procedure as PA, PS, LPC, and LPE, however an unequivocal identification of these as well as the other minor spots was not established.

Chloroplasts' TL were found to be less complex. In comparison to mitochondria, much smaller number of spots was revealed. Thus chromatograms developed by using the first solvent system revealed the presence of MGDG and DGDG as two major spots, and by spot intensity followed by PG, SL, PC, PI and PE (Fig. 15). Furthermore, two faint spots which were present only in chloroplasts chromatograms, migrated similar to the galactolipids in the first dimension but did not migrate

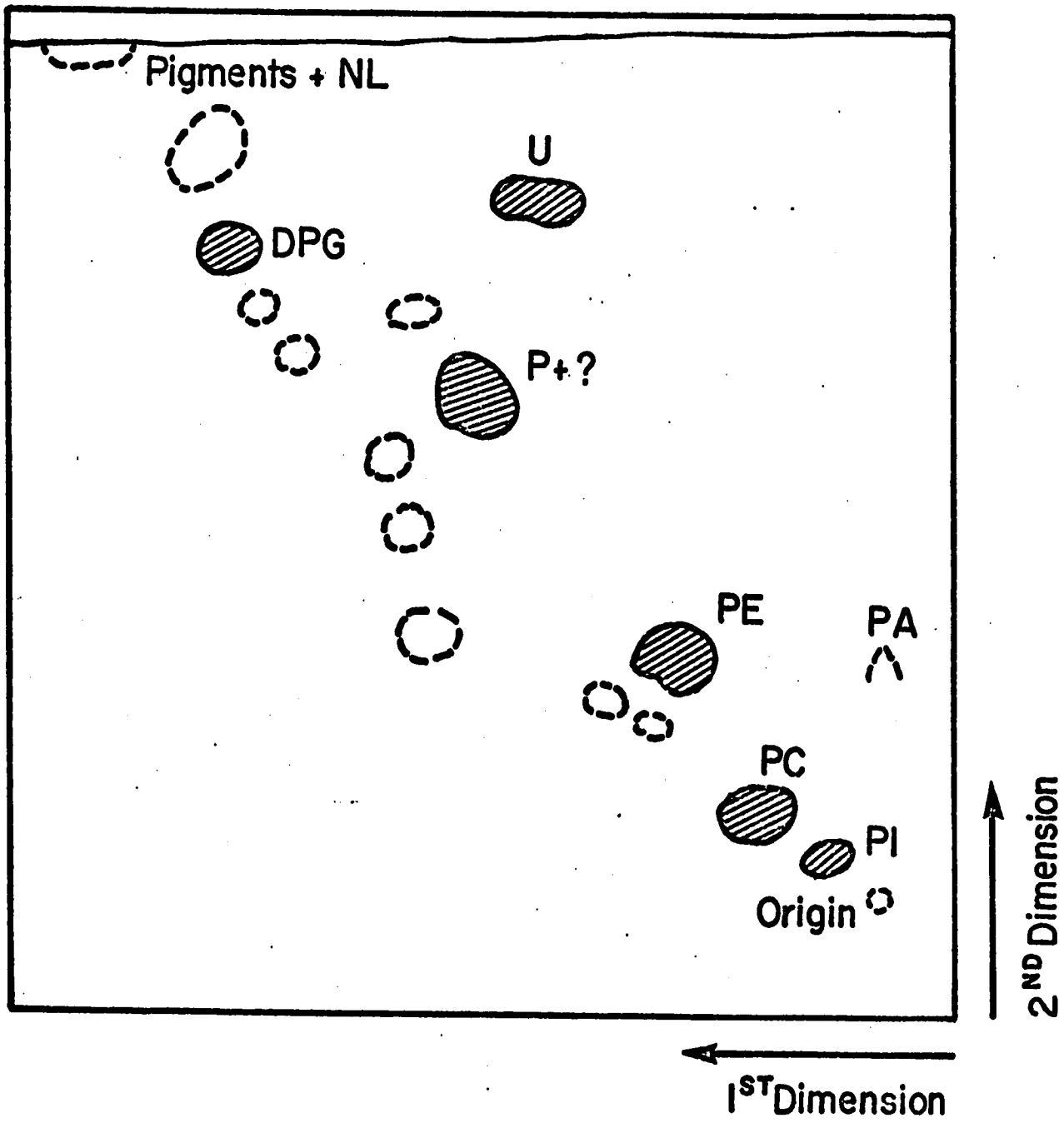


Fig. 13. Two Dimensional TL-Chromatograms of Pea Mitochondrial Lipids. Mitochondria were isolated from cotyledons germinated for 4 days according to Solomos et al. (1972). Separation was performed as in Fig. 11 using the solvent system of Nichols and James (1964).

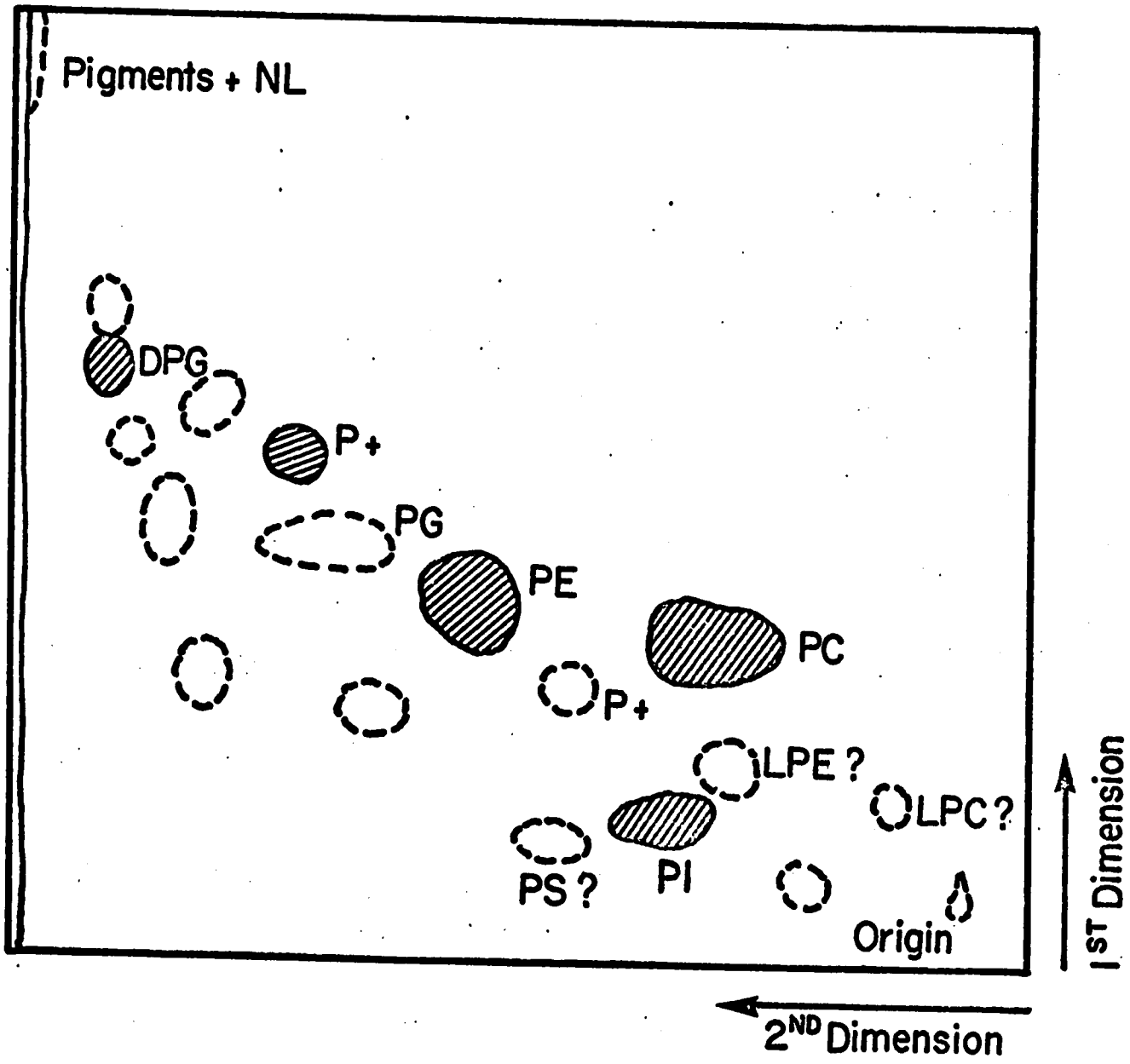


Fig. 14. Two Dimensional TL-Chromatograms of Pea Mitochondrial Lipids. Mitochondria were isolated from cotyledons germinated for 4 days according to Solomos et al. (1972). Separation was performed as in Fig. 12 using the solvent system of Rouser et al. (1970).

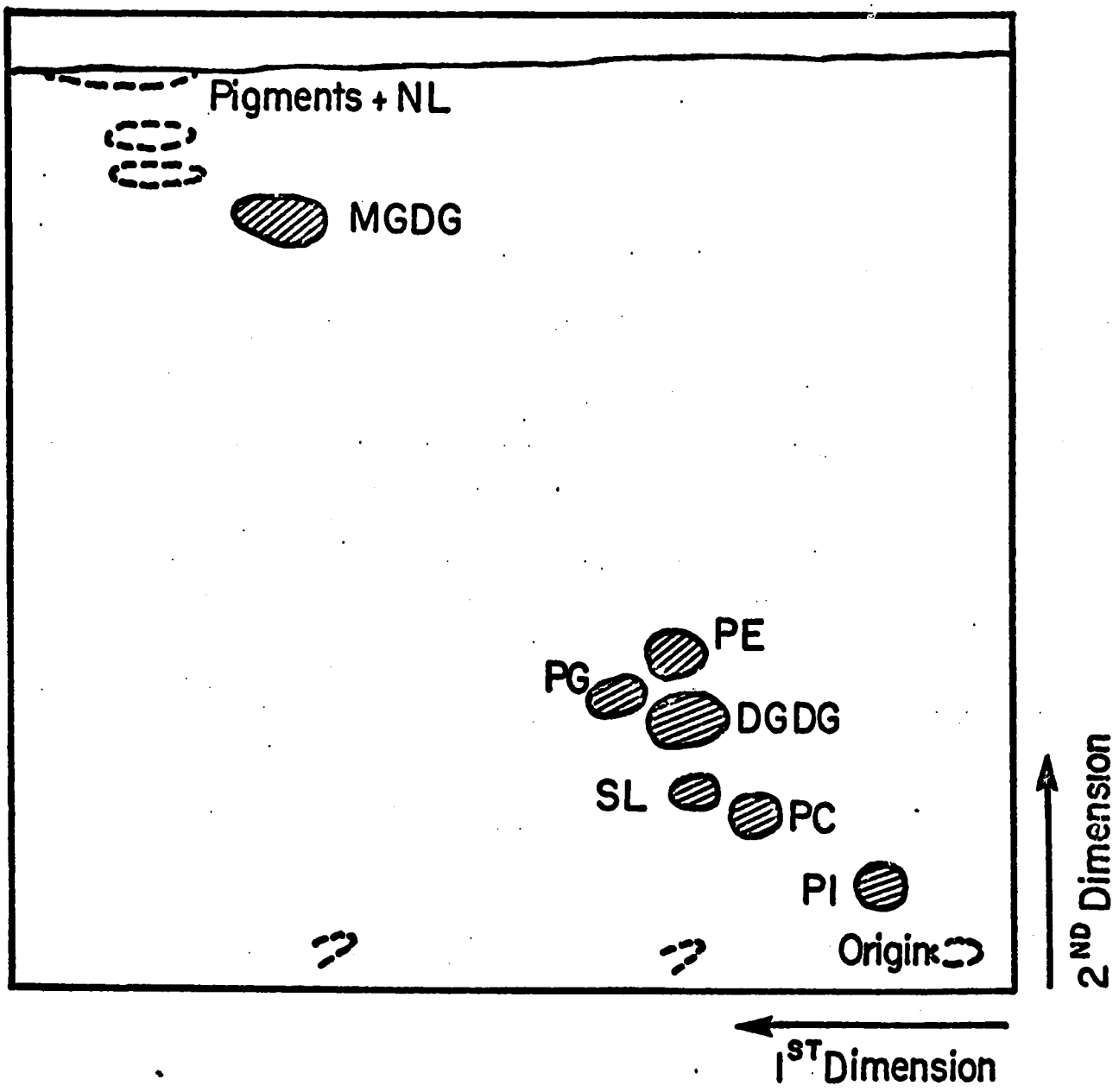


Fig. 15. Two Dimensional TL-Chromatograms of Pea Chloroplasts' Lipids. Chloroplasts were isolated from 16 day old pea seedlings in 0.6 M sucrose in 10 mM Tris-Cl buffer pH 7.5. Separation was performed as in Fig. 11 using the solvent system of Nichols and James (1964).

in the second, were assumed to be degradation products of chloroplasts' MGDG and DGDG. The intensity of these spots increased slightly upon prolonged storage of chloroplasts' lipids even when they were stored as chloroform solution under nitrogen in dark at -20°.

For the oxidation study, sufficient amounts of pure galactolipids could not be recovered directly from two dimensional TL-chromatograms. Hence, other procedures were used for their isolation. Two column chromatography purification steps were applied for chloroplasts' TL, first on silicic acid and then on carbon-celite adsorbant (Nuchar Attaclay) yielding pure MGDG but not DGDG which was still contaminated with chlorophyll and phospholipids. Further drawbacks were in the low yield of both galactolipids and the large elution volumes required for isolation. In another attempt, applying silicic acid column chromatography followed by precipitation of phospholipids by cold acetone treatment, and then by chromatography on a Sephadex LH-20 column, contaminated galactolipid fractions were also obtained. Finally, the procedure involving a preliminary fractionation of chloroplast lipids on a silicic acid column using stepwise elution with eluents such as chloroform, chloroform acetone 1:1, acetone and methanol, followed by a preparative one dimensional TLC, proved to be satisfactory for the separation of pure galactolipids.

The results of the preparative isolation of galactolipids are given in Fig. 16. Monitoring the column effluent by TLC and by simultaneous galactose and phosphorus determinations and using as eluent chloroform-acetone (1:1) MGDG fraction was collected, while with acetone as the next eluent DGDG was collected. Both fractions

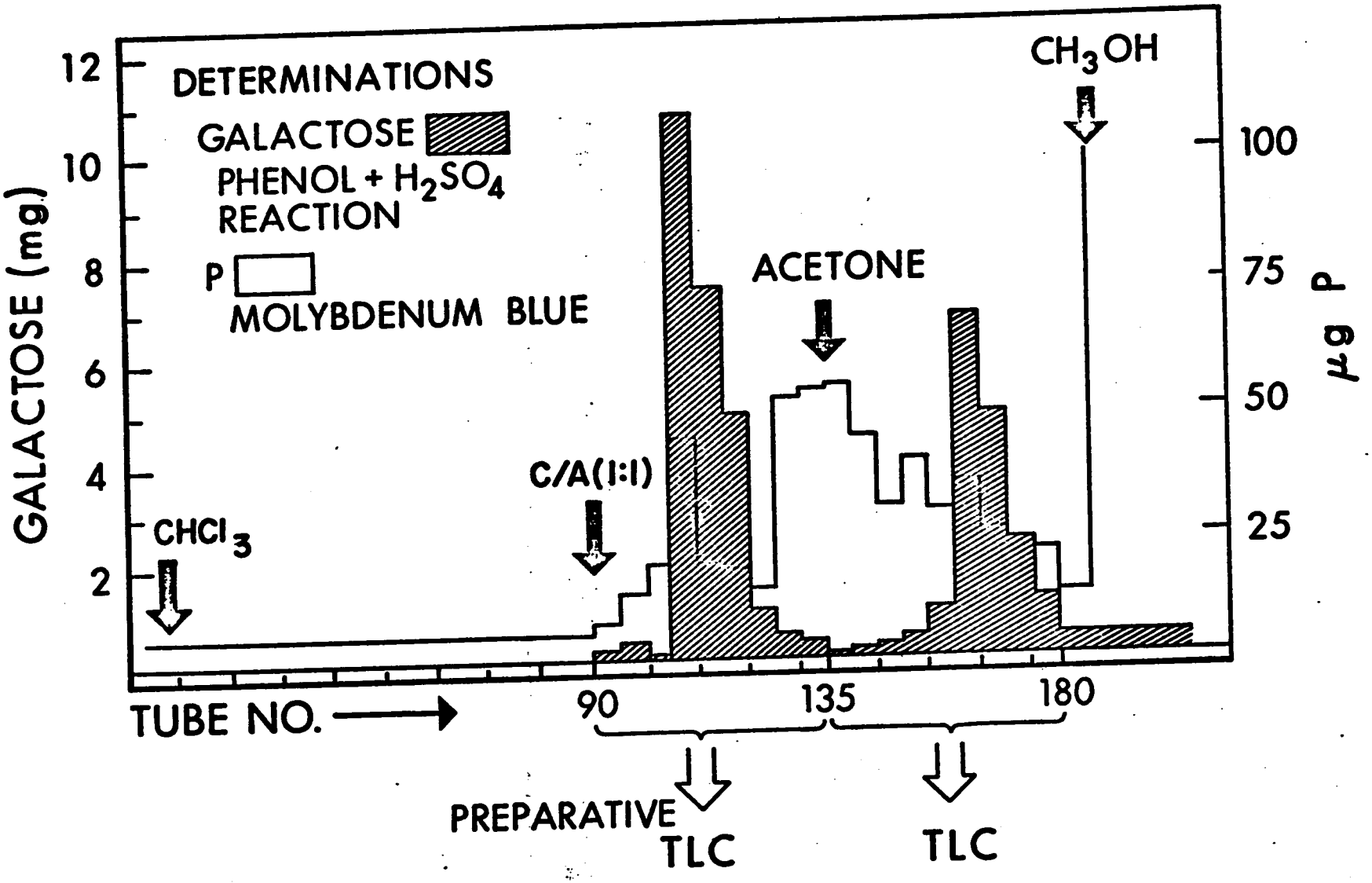


Fig. 16. Separation of MGDG and DGDG from Chloroplasts' Lipids on Silicic Acid Column. For details see text page 98.

contained the galactolipids in high yields but were still contaminated with small amounts of PL and/or pigments. The bulk of pigments was separated by elution with chloroform while the majority of phospholipids was retained in the column until eluted by methanol. The traces of residual pigments and/or phospholipids were readily eliminated by subsequent TLC.

To establish the identity of the sugar moiety in the isolated pure galactolipids as well as in the two sterol spots S_1 and S_2 of pea seed lipids (see Fig. 11), the lipids were subjected to methanolysis, the released sugars silylated and analyzed by GLC. As illustrated in Fig. 17, the sugars in both galactolipids correspond to D -galactose while that in both sterols to D -glucose. On two different liquid phases applied D-galactose gave three peaks attributed to α-, β-, and γ-D -galactose, the first two corresponding to galactopyranosides and having higher retention times than the last one which probably represents galactofuranoside. Despite the clear separation of these peaks it was not yet possible to ascertain the form of galactose present in each lipid. The presence of all three forms of silylated D -galactose might reflect a possible mutarotation of the sugar occurring during methanolysis or silylation steps. Silylated D -glucose gave two peaks corresponding to α- and β-glucopyranosides and both peaks coincided with the sugars recovered from pea sterol lipids. The FAME as the by-products of methanolysis were not readily separated between methanol and hexane phases and thus appeared as small peaks in the chromatograms of silylated sugars.

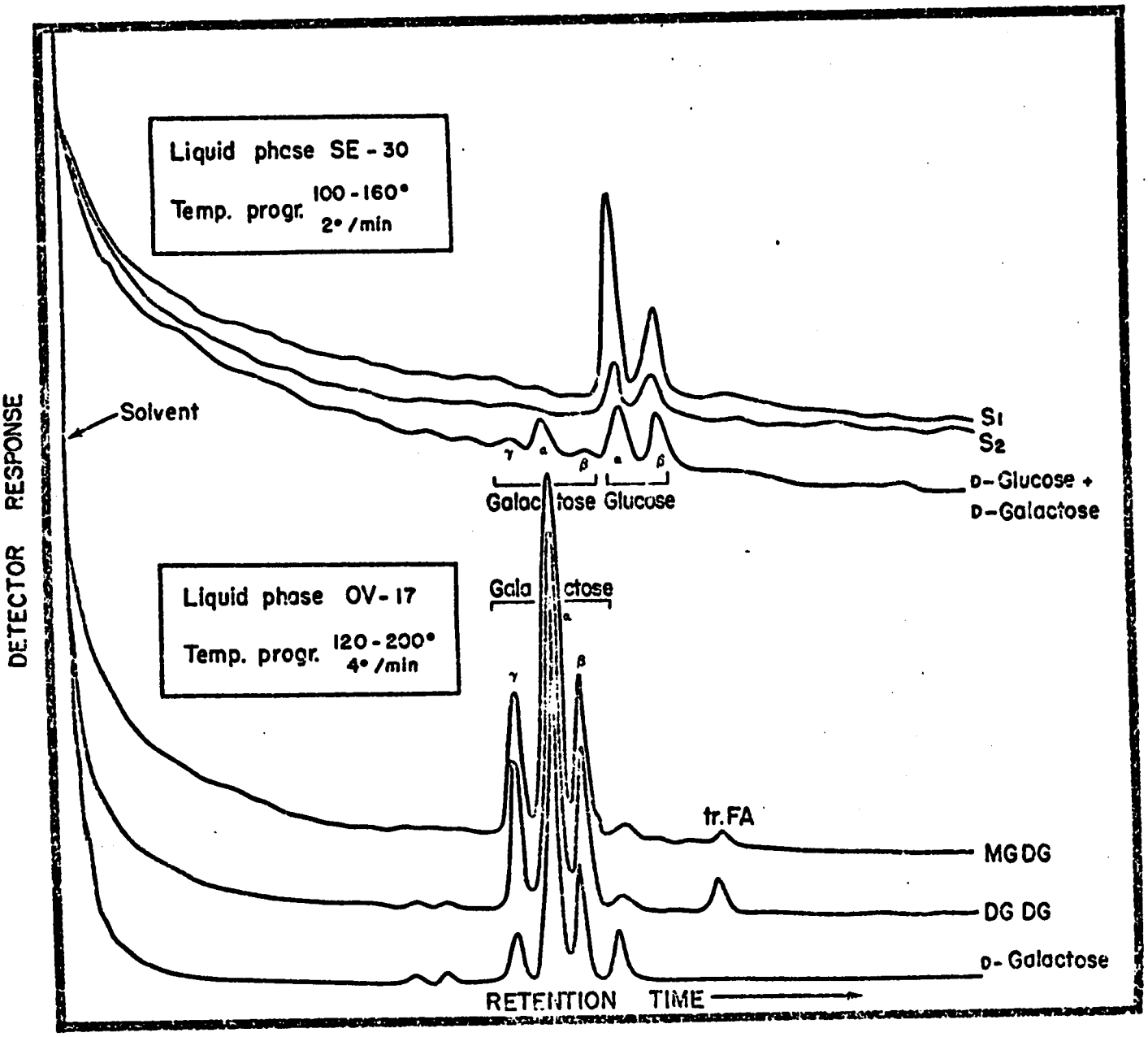


Fig. 17. GL-Chromatograms of Silylated Sugar Moiety of Chloroplasts' Galactolipids and Pea Seeds' Sterol Glycosides. The pure lipids were recovered from TL plates, and after methanolysis, the sugars were recovered and silylated by using a reagent mixture of TMCS and HMDS in pyridine as solvent.

2. The FA composition

The FA composition of total, polar, and neutral pea seed lipids is presented in Table 2. In all cases the presence of about ten FA was apparent. Linoleic acid was the major FA up to 40 - 50% in TL, PL, and NL of pea seed, followed in decreasing order by oleic, palmitic, linolenic and stearic acids. The other FA including the odd carbon number acids were present only in traces. Minor differences in FA ratios were observed in TL among the three varieties. Thus, Homesteader had a slightly higher content of linoleic but a lower content of oleic acid. The ratios of the other FA were comparable. The FA composition of PL revealed, with no exception, a higher content of saturated acids such as palmitic and stearic, but a lower content of linolenic acid, when compared to those of NL. The ratios of linoleic and linolenic acids were comparable in both NL and PL. Among the three varieties, Homesteader had the highest content of linoleic and the lowest content of stearic acid in both lipid fractions, i.e., NL & PL. For PL fraction, the lowest amount of palmitic acid was found in Stratagem. Nevertheless, differences in FA composition of the three varieties investigated were not sufficiently high for varietal characterization. However, the small differences observed could be attributed to the procedures used such as methylation. Thus when FA of TL were esterified by cold and warm BF_3 methylation procedures, minor differences were observed in their ratios as shown in Table 3. In addition, some differences may be obtained even among duplicates treated in the same way. The same minor differences in FA composition appear when the results with BF_3 methylation are compared with those in Table 2 where esterification was achieved by using methanolic sulfuric acid.

TABLE 2

THE FA COMPOSITION OF TOTAL, NEUTRAL AND POLAR PEA LIPIDS

FA*	Homesteader			Laxton			Stratagem		
	TL	NL	PL	TL	NL	PL	TL	NL	PL
12	tr**	tr	tr	tr	tr	tr	tr	tr	0.7
14	tr	tr	tr	tr	tr	tr	tr	tr	0.5
15	tr	tr	tr	tr	tr	tr	tr	tr	0.7
16	11.4	10.1	17.6	11.5	10.9	18.0	11.2	9.9	12.5
16:1	tr	tr	0.7	tr	tr	0.9	tr	tr	3.2
16:2 (or 17)	tr	tr	0.7	tr	tr	0.6	tr	tr	1.8
18	4.0	3.8	5.6	3.4	2.9	3.4	4.5	4.0	4.7
18:1	27.6	26.7	24.8	34.9	29.3	30.6	31.5	29.8	30.9
18:2	48.1	46.4	46.1	41.5	43.4	42.1	45.2	45.5	41.8
18:3	8.3	12.4	3.6	8.3	12.4	3.2	7.4	10.3	2.8

*In this and following tables expressed as % of total FA content.

**tr, less than 0.5%.

TABLE 3

THE FA COMPOSITION OF TOTAL PEA LIPIDS AS INFLUENCED BY BF_3 METHYLATION PROCEDURE

	Homesteader		Laxton		Stratagem	
FA	Cold*	Warm**	Cold	Warm	Cold	Warm
12	tr	tr	tr	tr	tr	tr
14	tr	tr	tr	tr	tr	tr
15	tr	tr	tr	tr	tr	tr
16	10.9	10.6	10.7	11.0	10.6	10.8
16:1	tr	tr	tr	tr	tr	tr
16:2 (or 17)	tr	tr	tr	tr	tr	tr
18	3.9	3.1	2.9	2.2	3.5	2.9
18:1	27.9	28.6	35.4	33.9	33.0	32.3
18:2	48.6	48.1	42.8	44.1	45.7	46.6
18:3	8.4	9.3	8.0	8.5	6.9	7.0

*Esterification at room temp. for 45 min, **at 65^{o} for 2 min

For IR analysis of FA, the peas TL were used. After the unsaponifiables were removed, the FFA were recovered and aliquots methylated by cold and by hot esterification procedures. The latter involved heating FFA at 65^{0} for 2 min in a closed vial with 14% methanolic BF_3. The cold esterification was achieved by allowing the FFA to stand at room temperature for 45 min with 5% methanolic sulfuric acid. The IR recordings in the neat state between 2.5 - 15 μ revealed the characteristic hydrocarbon chain absorptions at 3.4, 6.85, 7.25 and 13.85 μ. No superimposed bands of functional groups such as -OH or $\rangle C = O$ were detected. The ratio of absorbance at 3.3 μ to that at 3.5 μ (= C - H stretching) being for Homesteader 0.285, Laxton 0.319, and 0.311 for Stratagem, revealed a degree of unsaturation equivalent to iodine numbers of 108, 124, and 121 respectively. The absence of a peak at 10.36 μ and its presence at 4 μ region indicated the absence of trans and the presence of only cis unsaturation. Furthermore a high resolution recording in the region of 8.25 - 11.1 μ revealed the absence of any absorption peak due to conjugated dienes. All these findings were the same for FA samples of the three varieties, regardless of the esterification procedure used.

As shown in Table 4 there are great differences in FA composition of the lipids of pea seeds, seedlings and subcellular particles. As seen from the results given for germinated cotyledons, a large increase in the content of palmitic acid and a comparatively smaller increase in stearic and linoleic acids accompanied by a substantial decrease in oleic and linolenic acids occurred when pea seeds were germinated.

TABLE 4

THE FA COMPOSITION OF THE LIPIDS OF SEEDS, SEEDLINGS AND SUBCELLULAR PARTICLES OF PEA VAR. HOMESTEADER

FA	Seed	Germinated Cotyledons	Mitochondria	Green Leaves	Chloroplasts
10	tr	0.5	tr	tr	tr
12	tr	0.6	tr	tr	tr
14	tr	tr	tr	tr	4.5
15	tr	tr	0.5	tr	tr
16	11.4	21.4	13.3	14.4	12.0
16:1	tr	tr	tr	1.1	1.8
16:2 (or 17)	tr	0.9	0.9	tr	tr
18	4.0	6.4	5.8	3.4	2.9
18:1	27.6	14.7	15.9	2.0	1.6
18:2	48.2	51.8	59.2	21.6	13.8
18:3	8.3	3.2	4.1	56.7	64.6

When germinated cotyledons are compared with their mitochondria, then a substantial decrease in palmitic and an increase in linoleic acid is observed in mitochondria. However, in comparison with seeds, mitochondria contains a much lower content of oleic and linolenic acids but a higher content of linoleic acid, while the contents of the other FA are not much affected.

Contrary to linoleic acid as the major acid in the seeds, germinated cotyledons, and mitochondria, linolenic acid was found as the major one in green leaves and chloroplasts (Table 4). This fact may

reflect the stimulated synthesis of linolenic acid rich galactolipids during photosynthetic activity of green pea tissue. Accumulation of linolenic acid is accompanied by a decrease in the content of palmitic, oleic, and linoleic acids, particularly when the FA of green leaves are compared with those of germinated cotyledons.

When chloroplasts are compared with the whole green leaves tissue, they had a higher content of linolenic and myristic acids, but a lower content of linoleic acid. Differences found among other FA were not as substantial.

A notable finding in the FA composition of all the samples analyzed was the absence of any peak after that of linolenic acid even when chromatograms' recordings continued up to twice the retention time of that last peak. Consequently, in subsequent FA analysis, chromatograms were recorded only until linolenic acid peak was obtained. The amounts of longer chain FA in individual lipids, if present, would therefore be negligible. For the FA analysis of major PL, the individual lipids were in most cases recovered from TL-chromatograms developed by the first solvent system (Figs. 11, 13 & 15), due to a superior reproducibility when compared to that of the second solvent system.

The FA composition of major individual PL of seeds, germinated cotyledons, mitochondria, and chloroplasts revealed considerable variations not only among individual lipids from the same tissue, but also in the same lipid compound obtained from different tissues (see Tables 5, 6, 7 & 8). Despite the differences found, some general conclusions could be drawn.

TABLE 5

THE FA COMPOSITION OF MAJOR PL OF PEA SEEDS
VAR. HOMESTEADER

FA	PI	PC	PE	SG	MGDG	DGDG
10	tr	tr	tr	1.1	tr	0.9
12	1.0	tr	tr	3.6	3.2	1.4
14	1.7	0.8	tr	4.8	4.2	1.0
15	0.6	0.6	tr	tr	tr	0.7
16	35.5	41.9	22.4	18.6	21.4	20.5
16:1	1.6	0.9	tr	3.2	tr	tr
16:2(or 17)	1.6	1.4	tr	tr	tr	1.2
18	13.4	3.3	6.9	10.4	12.4	6.7
18:1	34.1	26.1	46.9	47.8	40.4	33.2
18:2	10.0	24.3	23.5	10.5	18.4	34.2
18:3	tr	tr	tr	tr	tr	tr

TABLE 6

THE FA COMPOSITION OF MAJOR PL OF GERMINATED PEA COTYLEDONS

FA	PC	PE	(P^+?)	SG	MGDG	DGDG
10	0.7	1.9	0.6	3.6	3.0	3.8
12	1.0	2.2	0.7	7.2	3.6	7.6
13	tr	tr	tr	3.4	tr	tr
14	0.7	tr	tr	7.6	2.7	5.5
15	1.2	tr	tr	tr	tr	tr
16	38.4	19.2	14.4	37.6	23.3	30.3
16:1	tr	1.0	tr	4.3	tr	tr
16:2 (or 17)	1.2	1.6	0.8	4.6	2.4	5.4
18	8.5	7.0	8.8	15.3	13.2	17.3
18:1	9.8	12.6	18.0	6.0	8.0	10.7
18:2	36.6	52.8	54.9	10.4	33.0	13.4
18:3	1.4	1.7	1.4	tr	8.3	tr

TABLE 7

THE FA COMPOSITION OF MAJOR PEA MITOCHONDRIAL PL

FA	PI	PC	PE	(P^+?)	DPG	Spot U
10	tr	tr	tr	tr	0.6	0.6
12	tr	tr	0.6	0.5	0.8	1.7
14	tr	tr	0.9	0.8	2.0	3.6
15	tr	tr	tr	tr	1.8	0.8
16	24.1	11.2	10.5	2.7	17.0	19.7
16:1	1.0	tr	tr	1.4	3.3	3.9
16:2 (or 17)	0.5	0.6	tr	tr	0.8	1.7
18	9.2	7.3	4.7	2.3	11.3	8.2
18:1	13.3	19.8	12.4	15.8	22.5	26.1
18:2	48.9	58.0	68.2	70.1	36.7	32.1
18:3	2.4	2.4	2.4	5.3	2.8	1.5

TABLE 8

THE FA COMPOSITION OF MAJOR PEA CHLOROPLASTS' PL

FA	PI	PC	PE	PG	MGDG	DGDG
10	tr	tr	tr	tr	tr	tr
12	tr	1.0	1.4	0.9	tr	tr
14	1.5	1.8	2.8	1.5	tr	0.5
15	tr	tr	tr	tr	tr	tr
16	30.5	13.8	15.1	31.5	1.7	5.9
16:1	1.8	1.4	2.5	8.9	0.5	tr
16:2 (or 17)	tr	tr	0.5	0.5	tr	tr
18	8.2	4.7	12.2	5.4	1.0	1.7
18:1	22.5	7.4	28.9	18.0	2.8	2.4
18:2	16.2	32.5	26.7	16.2	11.7	5.4
18:3	18.7	37.0	10.5	17.1	82.0	83.8

As seen from Table 5, all pea seeds' PL analyzed contain high amounts of palmitic and oleic acids but only traces of linolenic acid. Among the PL, PC had the highest content of palmitic and the lowest content of stearic acid, while both SG and MGDG had the highest content of myristic acid with SG having also the highest content of oleic acid. When FA composition of the two galactolipids were compared, MGDG had considerably higher levels of saturated acids such as myristic and stearic, and a higher level of oleic acid but a much lower level of linoleic acid, than those in DGDG. From the FA composition found for the six major constituents of the seed PL analyzed, no FA specificity for a given lipid compound could be ascertained.

The FA composition of germinated pea cotyledons' major PL is presented in Table 6. It is apparent that individual lipids after seed germination do not retain their previous FA composition present in the seed. Thus, a marked increase in stearic and linoleic acids accompanied by a simultaneous decrease in oleic acid occurred in PC. The same trend was reflected in PE except that the level of stearic acid remained unchanged. Large changes in FA composition were also found in galactolipids, which changes were rather irregular and unlike those found in PC and PE. Finally, a high level of linoleic acid in the unidentified phospholipid (P^{+}?) and a high level of palmitic acid in SG were observed in germinated cotyledons.

Table 7 shows the FA composition of the mitochondrial major PL. As seen, PI had the highest level of palmitic acid while DPG had the highest levels of stearic and oleic acids among the phospholipids present. The higher unsaturation degree found for PC, PE, and the

unidentified P^+? reflects the higher unsaturation found previously for FA composition of mitochondrial TL (see Table 4). When these results are compared with those obtained for the germinated cotyledons, a lower content of palmitic and a higher content of linoleic acids in mitochondrial PC, PE, and in the unidentified P^+? are apparent.

The FA composition of chloroplasts' PL is presented in Table 8. Of particular interest were galactolipids which showed a high content, up to 82 - 84%, of linolenic acid, typical of these lipids in photosynthetic tissue. Although DGDG revealed a slightly higher content of linolenic acid than MGDG, the latter had still a higher degree of unsaturation due to the presence of a higher amount of linoleic and a lower amount of palmitic acid. Poly-unsaturated C_{16} was not detectable in either galactolipid. Two typical GL-chromatograms of the FA composition of chloroplasts' DGDG and mitochondrial PC are presented in Fig. 18.

Chloroplasts' major phospholipids found and analyzed were PC, PE, PG and PI. The results showed a rather low degree of unsaturation particularly when compared to chloroplasts' galactolipids and even to the corresponding phospholipids of mitochondria. The major unsaturated FA were oleic for PI and PE, and linolenic for PC. The least unsaturation seems to be present in PG with almost equal amounts of the unsaturated acids of C_{18} series. But contrary to other phospholipids, it had a high content of palmitoleic acid. The major saturated FA for all phospholipids was palmitic acid with a level twice as high in PI and PG when compared to that in PC and PE. However, PE contained the highest amount of stearic acid which was about three times as that found in PC. Finally, with the exception of linolenic acid association

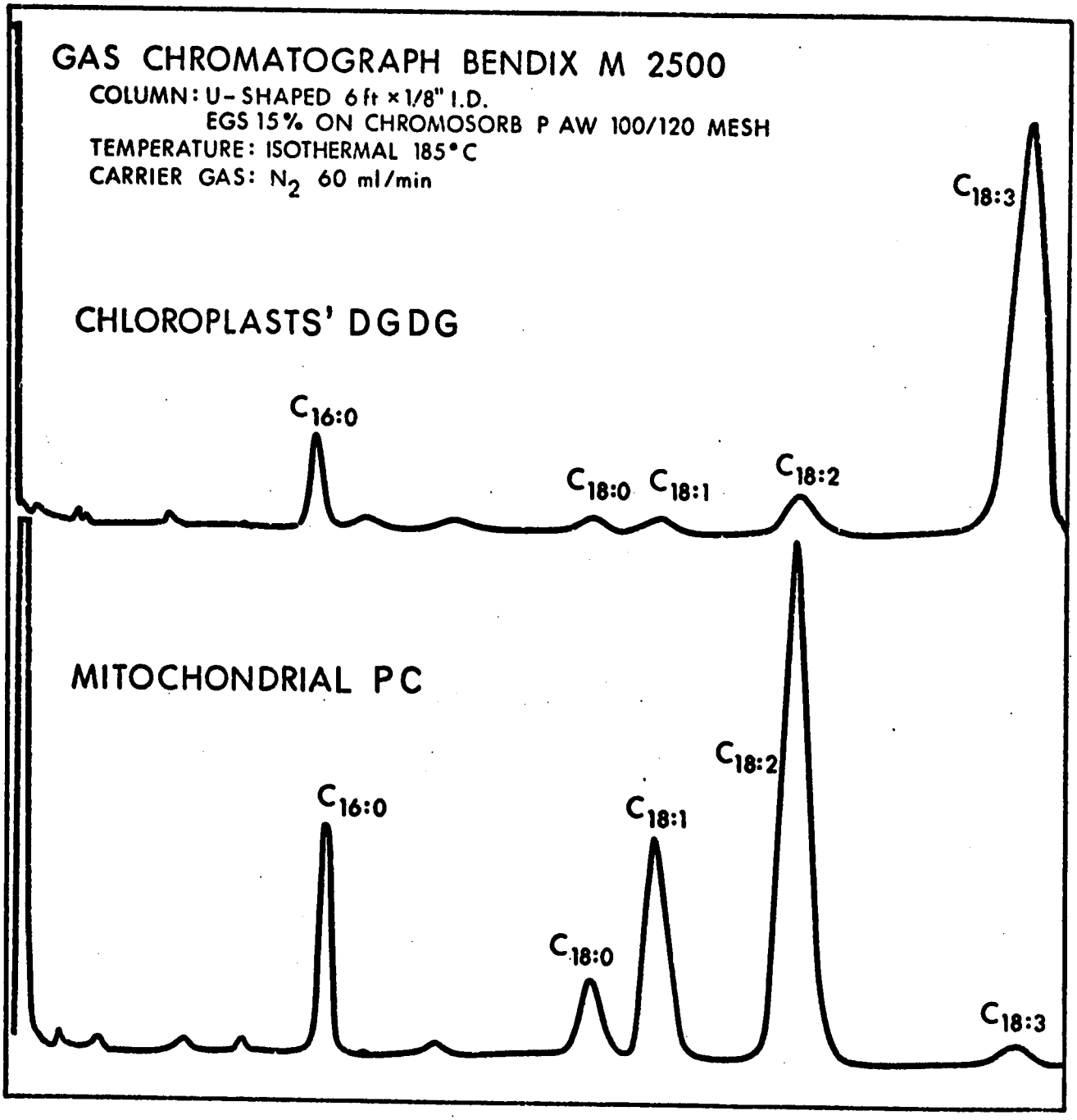


Fig. 18. GL-Chromatograms of the FA of Pea Chloroplasts' DGDG and Mitochondrial PC. Chloroplasts were isolated from 16 day old pea seedlings in 0.6 M sucrose in 10 mM Tris-Cl pH 7.5. Mitochondria were isolated from pea cotyledons germinated for 4 days according to Solomos et al. (1972). DGDG was isolated by column and TL chromatography while PC by two dimensional TLC.

with chloroplasts' galactolipids and the possible specific association of palmitoleic acid to PG, any FA specificity towards individual PL could not be concluded.

B. Pea Lipoxidase

1. Localization of the enzyme

Lipoxidase activity was apparent in the three pea seed varieties investigated. The highest activity was found in Laxton and was about 1.7 times as that found in Homesteader seeds and about 1.4 times as that of Stratagem. This reflects a rather high variation in lipoxidase activity among wrinkled pea varieties. The high degree of enzyme inhibition, up to 40%, by cyanide ion observed for all three varieties indicated that the enzyme extract is not entirely composed of true lipoxidase, but also of heme-containing lipid oxidizing enzyme. As shown by the degree of inhibition, the varietal difference in pea lipoxidase is not only quantitative but qualitative as well. The results are shown in Table 9. The residual activity after heating the enzyme extracts at 100^{0} for 3 min, a treatment simulating the blanching process of vegetables, was similar in the three varieties and was about 0.5% of the original activity. Since pure lipoxidase has a low heat resistance, the residual activity found after the heat treatment further supports the finding obtained by CN^{-} ion inhibition that other lipid oxidizing enzymes are present with lipoxidase in the pea extract.

Lipoxidase presence in pea mitochondria was established in both the crude and the purified mitochondria samples in addition to the soluble proteins and peroxisome-like bodies (Table 10). The highest enzyme activity was found in the purified mitochondria while the lowest

activity was present in the heavier peroxisome-like bodies which formed the fourth layer in sucrose density gradient centrifugation. Since the mitochondria were isolated from germinated seeds of Homesteader variety, the increased enzyme activity as compared to that of the seed must have resulted from an increase in activity due to germination. In some purified mitochondria preparations, the enzyme activity was as high as 1.40 units mg protein, however despite the high activity in mitochondria, the enzyme was not strictly localized in these cell organelles. As seen from Table 10, lipoxidase activity in peroxisome-like bodies is about two-thirds of that present in mitochondria.

TABLE 9

LIPOXIDASE ACTIVITY IN PEA SEEDS
AND ITS INHIBITION BY CYANIDE

Variety	Specific Enzyme Activity (Units/mg Protein in extract)	% Inhibition by CN^- ion
Homesteader	0.72	30
Laxton	1.24	34
Stratagem	1.00	40

When BSA was excluded from mitochondria isolation media, the enzyme activity in mitochondria dropped considerably. This might indicate that BSA which function is to bind mitochondrial FFA, thus preserving the integrity of mitochondria, brings about a simultaneous preservation of lipoxidase activity. Whether the enzyme activity loss is due to mitochondria swelling or to lysis is however not clear.

TABLE 10

LIPOXIDASE DISTRIBUTION IN PEA MITOCHONDRIA DURING ITS PURIFICATION

Lipoxidase Localization	Activity (units/mg Protein)
Crude Mitochondria	0.84
Soluble Proteins (1st layer)	0.86
Purified Mitochondria (2nd layer)	1.06
Peroxisome-like Bodies (3rd layer)	0.90
Peroxisome-like Bodies (4th layer)	0.69
Crude Mitochondria (isolation media without BSA)	0.35

The inhibition effect of cyanide ion on lipoxidase activity of crude and purified mitochondria was high (70 - 77%) and was similar to that of peroxisome-like bodies. The degree of inhibition was considerably higher than that observed for the seed enzyme extract indicating an increase in the heme containing type of lipoxidase during germination. As a further proof of such an increase, the crude mitochondria after heating at 100^{0} for 3 min was found still to retain 3.3% of its original enzyme activity.

During the mitochondrial lipoxidase assay, the initial rate of oxygen uptake versus mitochondrial protein was found to increase linearly up to 300 μg protein after which the rate started to decline as shown in Fig. 19.

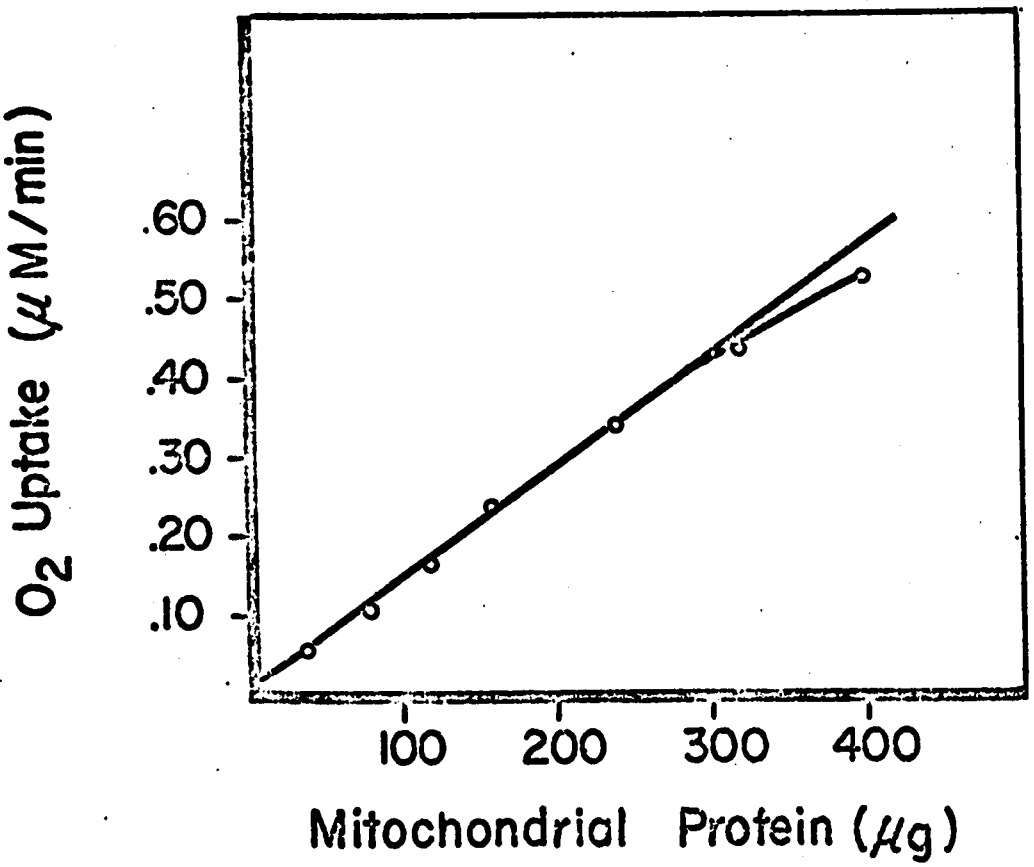


Fig. 19. Initial Rate of Oxygen Uptake by Lipoxidase vs Mitochondrial Protein. Protein was determined according to Lowry (1951); oxygen uptake was measured in 7.5×10^{-3} M linoleic acid in 0.1 M borate buffer pH 7.0 containing Tween 20 as emulsifier; oxygraph used was a Biological Oxygen Monitor, Yellow Springs Instruments.

The activity of lipoxidase in crude and purified chloroplasts is shown in Table 11. The activity expressed per mg protein was considerably lower than that found for mitochondria and was lowest in the intact chloroplasts. However, the enzyme activity does not seem to be an adequate index of chloroplasts purity. Lipoxidase activity in the intact chloroplasts has higher susceptibility to cyanide inhibition than that of the broken chloroplasts collected from their corresponding gradient layer. A considerably higher activity of the enzyme is obtained by Triton X-100 than by SDS treatments of chloroplasts. Whether the higher membrane disrupting or solubilizing ability of the nonionic detergent of Triton X-100 than that of the ionic SDS, or a possible interaction of the enzyme with detergent resulting in activity change is responsible for these findings is not clear. Contrary to detergent treatments, chloroplasts disruption by high frequency sonication or by osmotic shock resulted in a decrease of lipoxidase activity to about 0.13 units/mg protein.

The enzyme activity of plastids isolated from etiolated leaf tissue differed greatly from that of chloroplasts. Etiolated plastids had a higher enzyme activity than either chloroplasts or mitochondria, but had a lower degree of cyanide inhibition. The activity almost doubled when plastids were treated with Triton X-100, which was not the case when SDS was applied. About a five fold increase of enzyme activity was found in plastids' supernatant after mitochondria removal and an even higher activity was obtained for the cytoplasm when ribosomes were removed (Table 12). However, the degree of cyanide inhibition increased by removing ribosomes and approached that found for etiolated plastids.

TABLE 11

LIPOXIDASE ACTIVITY IN CRUDE AND PURIFIED CHLOROPLASTS

Lipoxidase Localization	Specific Enzyme Activity (Units/mg Protein)	% Inhibition by CN^-	Extracted with Triton X-100, % Activity	Extracted with SDS, % Activity
Crude Chloroplast	0.23	20	59	31
Broken Chloroplasts (1st layer)	0.24	38	89	61
Intact Purified Chloroplasts (2nd layer)	0.13	47	87	10

TABLE 12

LIPOXIDASE DISTRIBUTION IN ETIOLATED PEA LEAF TISSUE

Lipoxidase Localization	Specific Enzyme Activity (Units/mg Protein)	% Inhibition by CN^- ion
Etiolated Plastids	1.37	28
Mitochondria free Plastids Supernatant (20,000 x g)	6.54	9
Ribosome free Supernatant (115,000 x g)	8.66	22
Triton X-100 Extract of Plastids	2.42	13
SDS Extract of Plastids	0.44	-

2. Lipoxidase isolation and purification

Enzyme extraction from pea seeds, ultracentrifugation, and ammonium sulfate precipitation steps gave a good yield of a crude, four fold concentrated preparation which maintained its original activity for several months during storage at 0 - 4^{o}. However, the preparation contained considerable catalase and peroxidase activities and therefore had to be further purified. Purification by column chromatography using Sephadex G-150 resulted in three major protein peaks followed by a small peak shouldering the third. A major portion of catalase and peroxidase activity together with lipoxidase was concentrated in the second peak (see Fig. 20). By collecting the narrow band with high lipoxidase activity a three fold purification of the enzyme was achieved, despite

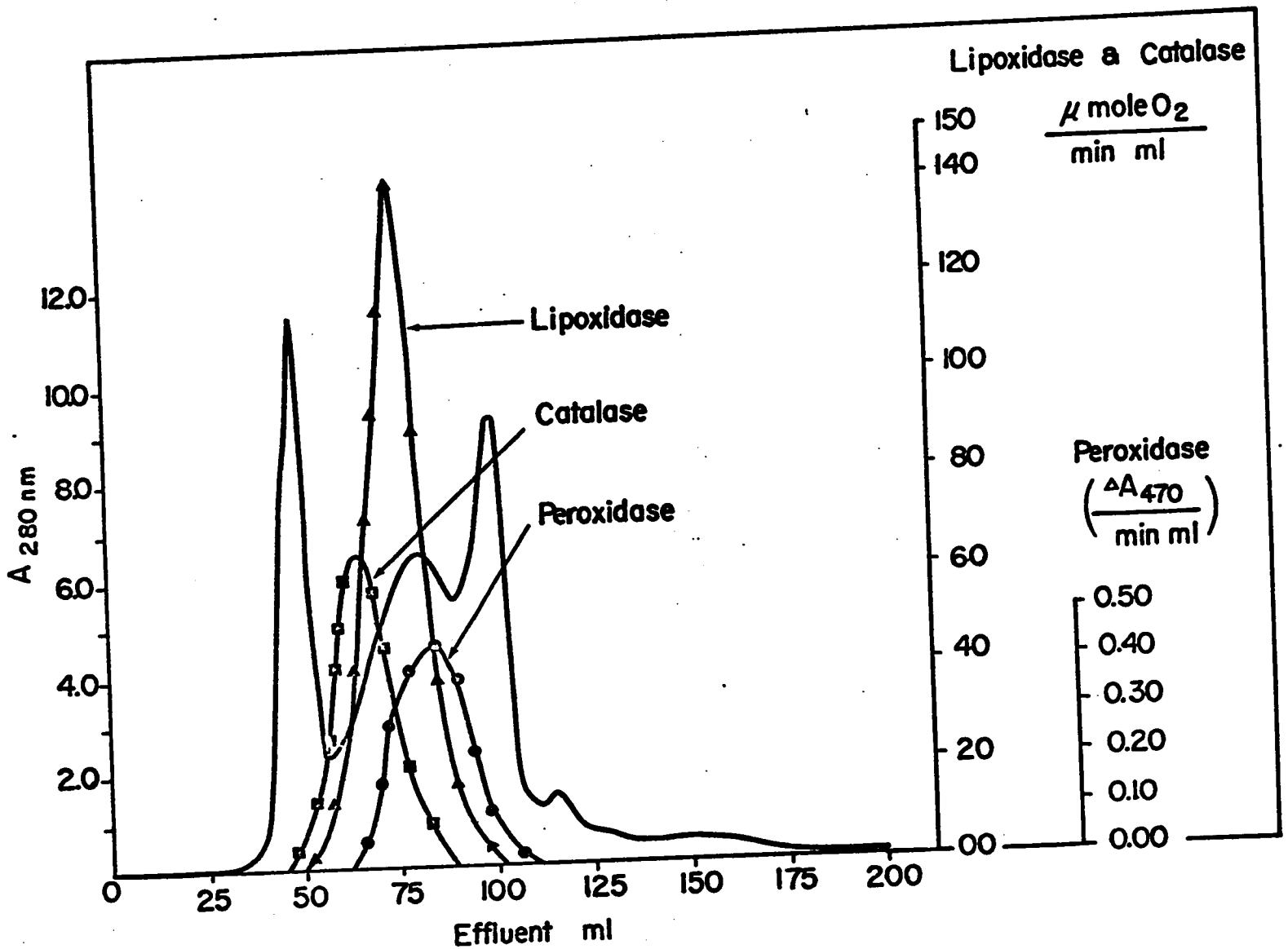


Fig. 20. Separation of Pea Seed Lipoxidase on Sephadex G-150. Column dimensions 1.5 x 90 cm; units of A_{280} applied were 412; eluent used 0.05 M Tris-Cl buffer pH 7.2; lipoxidase activity measured as in Fig. 19; catalase activity measured amperometrically according to Goldstein (1968), and peroxidase activity spectrophotometrically with guaiacol as substrate.

the loss of about one third of the total activity. Further purification on a DEAE-Cellulose column offered two more fold purification and removed few protein contaminants but resulted also in a considerable loss of total enzyme activity. The lipoxidase activity peak corresponded closely with the protein absorption curve of the first elution peak (Fig. 21), was free from any detectable catalase or peroxidase activity, and hence was collected and considered to be a pure enzyme preparation. Lipoxidase activity during isolation and purification steps is presented in Table 13. The stepwise elimination of interfering proteins from lipoxidase during its purification was additionally ascertained by disc gel electrophoresis (see Fig. 22).

TABLE 13

LIPOXIDASE ACTIVITY DURING ISOLATION AND PURIFICATION STEPS (Values Are Given for 100 g of Pea Seeds var. Homesteader)

Purification Step	Total Protein (mg)	Specific Activity (Units/mg protein)	Total Activity (Units)	Degree of Purification	% Recovery
Crude Extract	2290	6.1	13970	1.0	100
Ribosome Free Supernatant	2070	6.7	13870	1.1	99
25% $(NH_4)_2$ SO_4 Supernatant	1790	7.2	12890	1.2	92
50% $(NH_4)_2$ SO_4 Precipitate	490	25.9	12700	4.2	91
Sephadex Effluent	112	80.8	9050	13.2	65
DEAE-Cellulose Effluent	16	143.0	2290	23.4	16

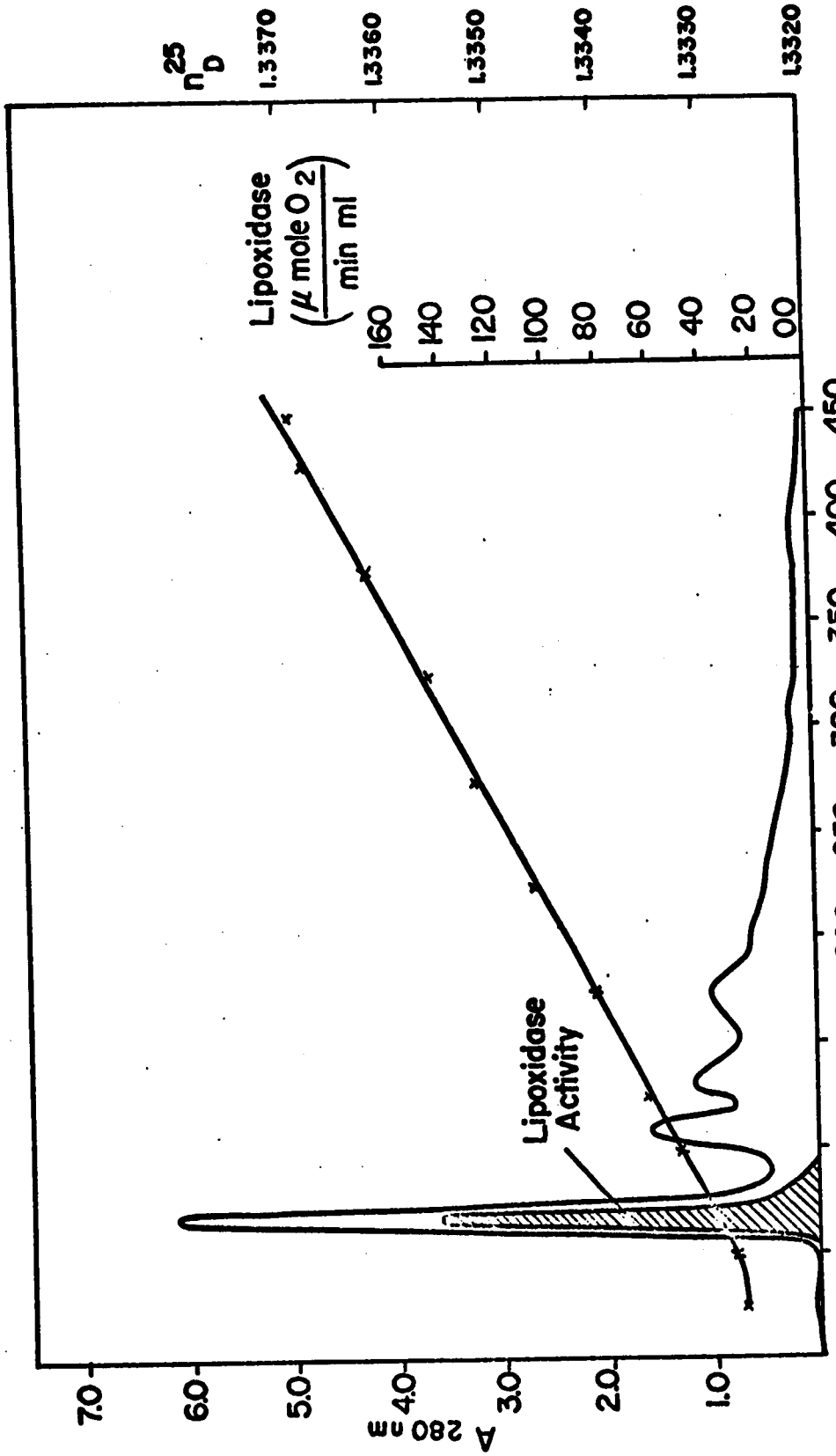


Fig. 21. Purification of Pea Seed Lipoxidase on DEAE-Cellulose. Column dimensions 1.5 x 90 cm; units of A_{280} applied were 450; continuous NaCl gradient from 0.01 to 0.61 M was applied in eluent medium of 0.1 M Tris-Cl buffer pH 6.5 containing 2 mM $CaCl_2$ and 1 mM 2-mercaptoethanol; lipoxidase activity measurement as in Fig. 19.

During purification steps it was observed that the lipoxidase extract after elution from Sephadex, protein precipitation step by 50% ammonium sulfate, dialysis, and solubilization in the -SH containing buffer, lost some of its catalase activity during storage at 0^{o} for 24 h. This reproducible effect led to the investigation of possible inactivation of catalase by cysteine, reduced glutathione and 2-mercaptoethanol as thiol reagents. The results using beef liver catalase as enzyme (Table 14) indicated a definite inactivation of catalase by the three thiol reagents used. It is apparent that the inactivation is enhanced either by increasing the reagent concentration or by increasing the incubation time of catalase with the reagent.

3. Some enzyme properties

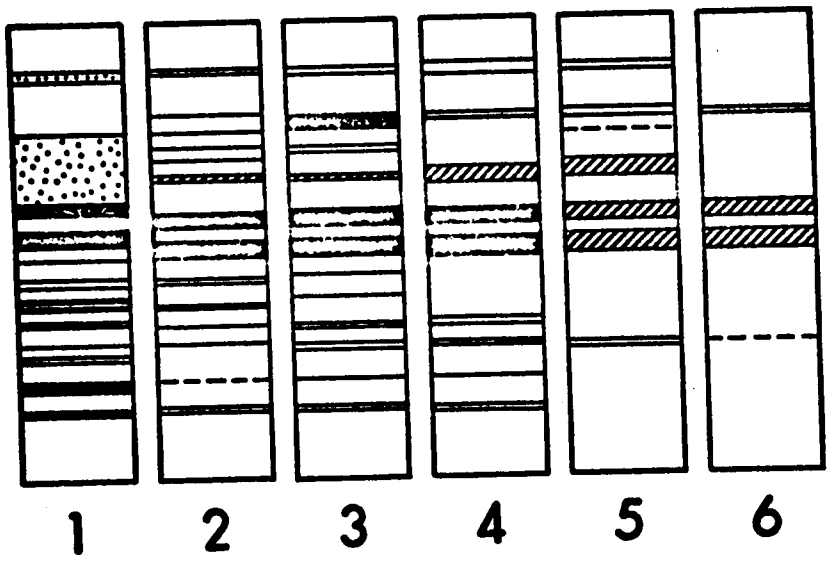
The band patterns obtained by disc gel electrophoresis during isolation and purification steps of lipoxidase are shown in Fig. 22. The lipoxidase specific staining procedure revealed the presence of four enzyme bands for the purified pea enzyme and 2 - 3 bands for a commercial preparation of soybean lipoxidase applied for comparison. The band pattern was similar in both preparations except for a faint band having a high electrophoretic mobility present in the enzyme from pea but absent in that from soybean. Furthermore, the two main bands, the slower moving being the major one, were not clearly separated but were more distinct in the pea zymogram. Addition of cyanide in 5×10^{-3} M concentration to the substrate used for staining had no effect on the band pattern for both preparations.

TABLE 14

CONCENTRATION AND INCUBATION TIME EFFECTS OF THIOL REAGENTS ON CATALASE ACTIVITY
(Values Are % of the Original Activity Present in Control)

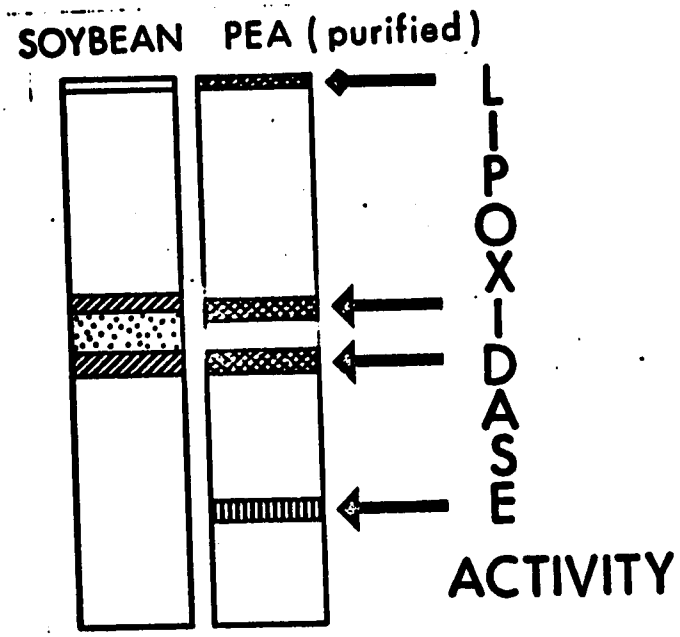
Thiol Reagent Concentration		1 mM		2 mM		5 mM		10 mM	
Enzyme Conc.*		C_1	C_2	C_1	C_2	C_1	C_2	C_1	C_2
Thiol Reagent	Time of Enzyme Incubation with Thiol Reagent, h								
Cysteine	1	70	74	69	68	70	62	49	51
	3	62	55	44	54	42	49	38	41
	5	48	47	36	52	40	43	35	35
Reduced Glutathione	1	75	75	76	78	73	76	69	67
	3	60	60	66	76	60	65	48	51
	5	54	57	57	57	50	48	31	43
2-Mercapto-ethanol	1	69	70	56	57	46	43	40	39
	3	53	53	41	43	33	32	30	28
	5	42	45	31	32	26	25	24	24

*0.05 mg enzyme/ml in C_1 and 0.1 mg/ml in C_2



SEPARATION PROCEDURE
DAVIS (1964).

1. CRUDE EXTRACT
2. ULTRACENTRIFUGATION SUPERNATANT
3. 25% $(NH_4)_2$ SO_4 SUPERNATANT
4. 50% (NH_4) SO_4 PELLET
5. SEPHADEX COLUMN EFFLUENT
6. DEAE COLUMN EFFLUENT



PROCEDURE

DAVIS (1964) EXCEPT FOR 1% AMYLOSE INCORPORATED IN THE SMALL PORE GEL

SPECIFIC STAINING

THE GELS WERE INCUBATED FOR 30 MIN I ULTRASONICALLY DISPERSED 2 x 10^{-3} M LINOLEIC ACID SOLUTION, THEN RINSED AND STAINED IN 15% ACETIC ACID CONTAINING 5 ml SATURATED KI/100 ml SOLUTION.
THE BROWN COLORED BANDS APPEARED AFTER 10 - 30 MIN

ADDITION OF CN^- ION (10^{-3} M) TO THE SUBSTRATE HAD NO EFFECT ON THE BAND PATTERN

Fig. 22. Disc Gel Electrophoregrams of the Crude and Purified Pea Lipoxidase. Top - crude enzyme during isolation steps, bottom - isoenzymes of purified lipoxidase.

The mol. wt. of purified lipoxidase was determined by disc gel electrophoresis using SDS and dithiothreitol. Lipoxidase relative mobility was found to be slightly lower than that of the highest mol. wt. standard used (BSA). Therefore, lipoxidase mol. wt. was calculated by slightly extrapolating the calibration curve, and was found to be 74,000 (Fig. 23).

The effect of pH on the enzyme activity is shown in Fig. 24. The enzyme had a rather typical symmetrical curve with no sign of irregularities. The curve displays a narrow pH range optima (7.1 - 7.5) with the peak at pH 7.2 - 7.3. A very slight increase in the enzyme activity was noticed at pH 9.6 over that at pH 8.6.

By heating the purified enzyme preparation at 100^{o} for 3 min no detectable activity remained while a small residual activity was found for the crude enzyme when similarly treated. Furthermore, in case of purified enzyme when cyanide (5×10^{-3} M) was included in the substrate media, no inhibitory action was detected.

Michaelis-Menton constant was determined for the purified enzyme at pH 7.2 and varying concentrations of linoleic acid (Fig. 25). The least square method was used for this purpose and the results indicated that lipoxidase seems to conform to the Michaelis-Menton equation. The equation of the line was found to be $Y = 5.3 \times 10^{-3} x + 2.26$. The calculated Km was 2.3×10^{-3} M and V max. was 0.44.

C. Enzymatic Oxidation of Individual Pea Lipids

The study of pea lipoxide substrate specificity has been carried out on isolated NL, on PC, PE and DPG as the major components of phospholipids, and on MGDG and DGDG as the major components of

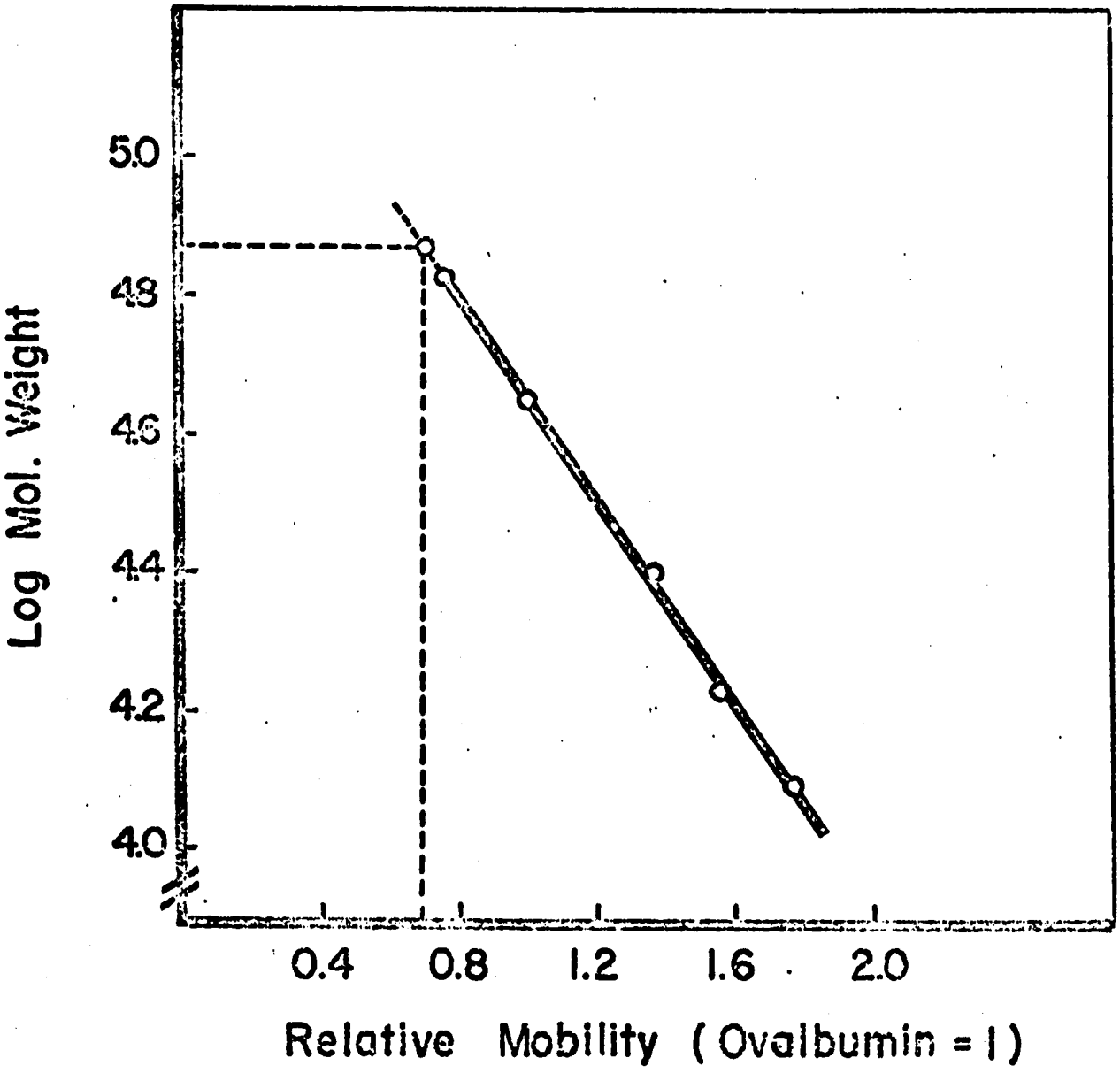


Fig. 23. Calibration Curve for the Estimation of Lipoxidase Mol. Wt. The method used 5% acrylamide gel electrophoresis in presence of 1% SDS. Protein samples before separation were incubated at 37° for 30 min with 0.5 mM dithiothreitol and 1% SDS. The individual points were obtained by using cytochrome C, myoglobin, chymotrypsinogen, ovalbumin, and bovine serum albumin as protein standards.

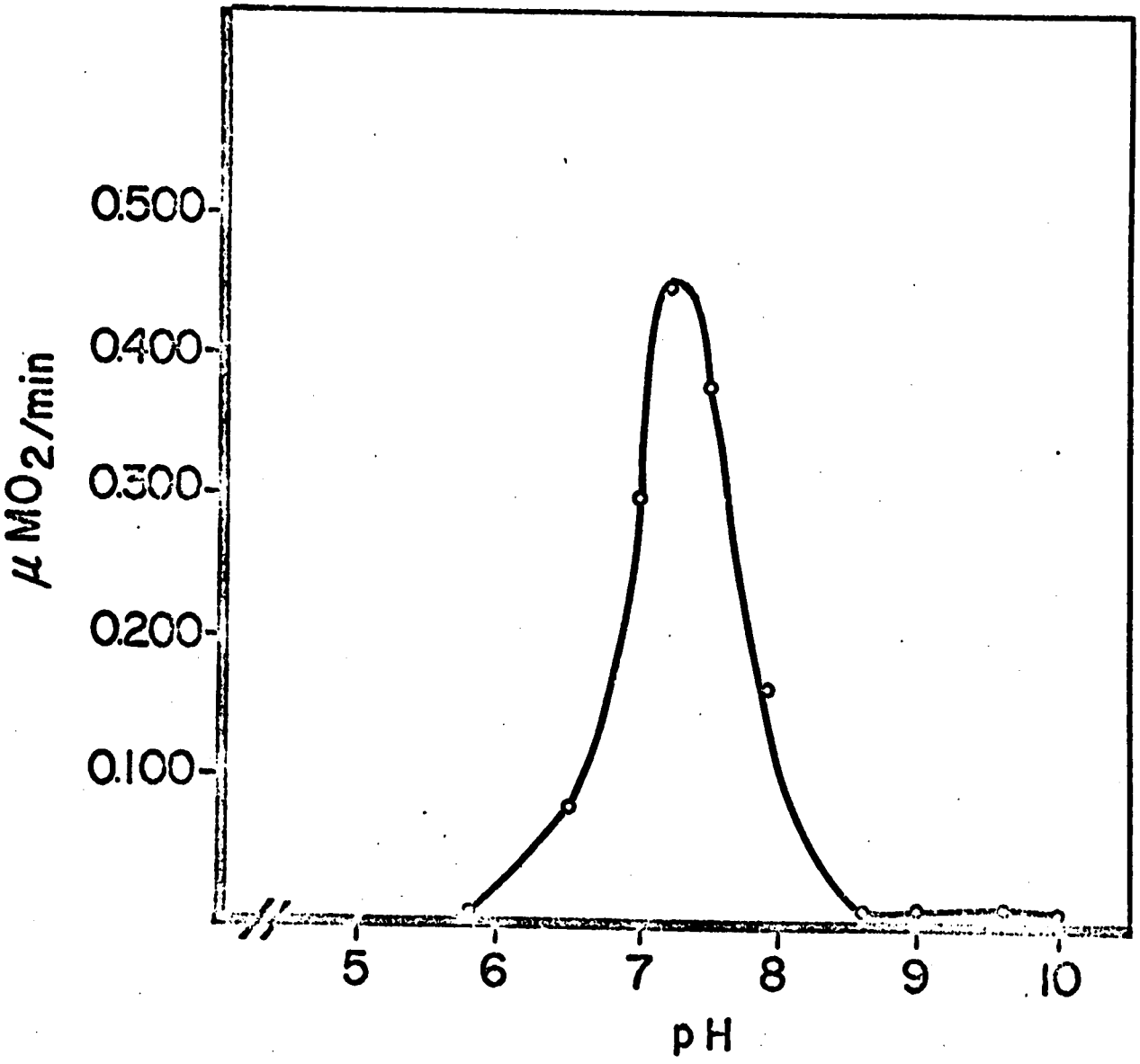


Fig. 24. Activity of Pea Lipoxidase in Dependance of pH. Lipoxidase activity was determined as in Fig. 19, using 0.1 M borax-phosphate buffer (Kolthoff 1925).

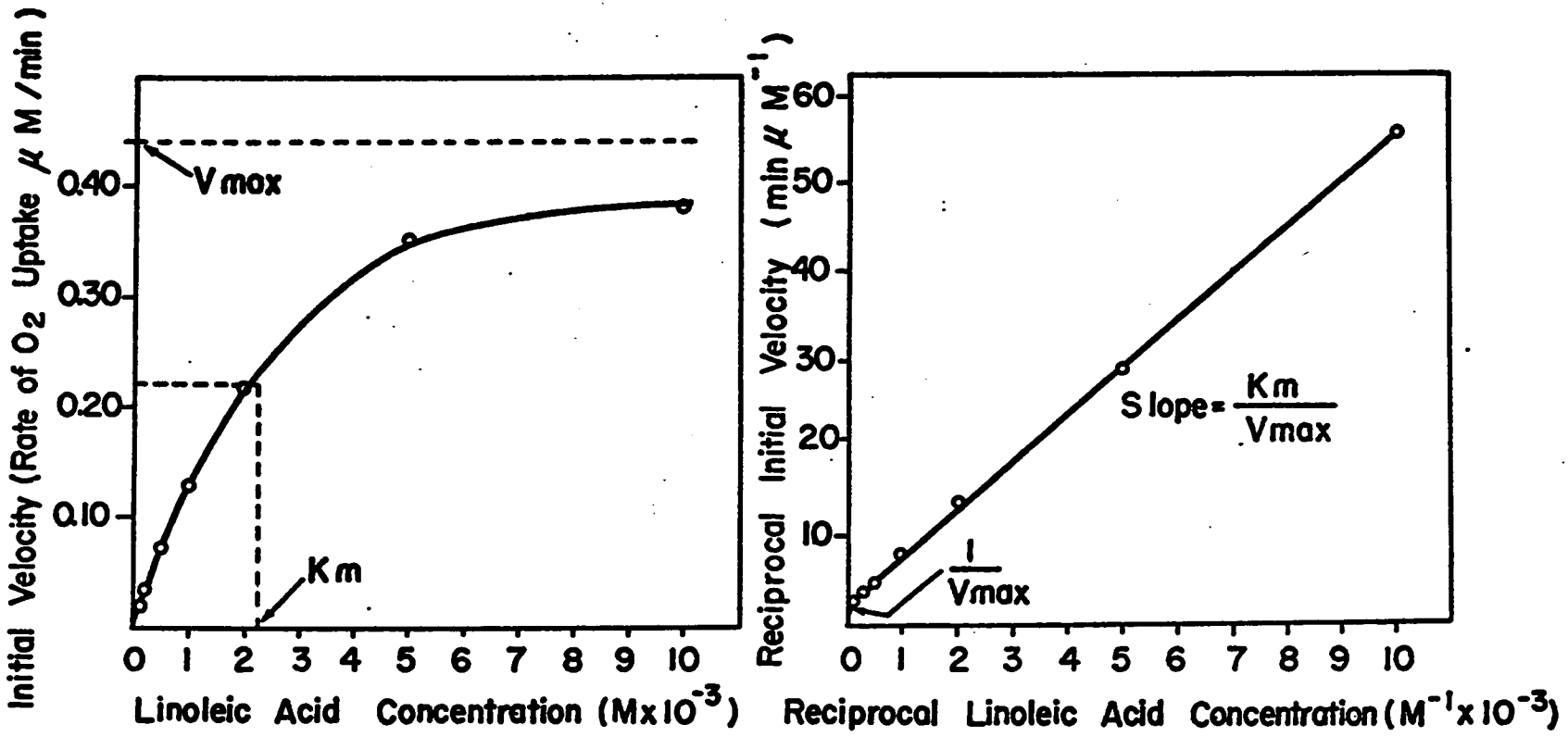


Fig. 25. Some Kinetic Data of Purified Pea Seed Lipoxidase.

galactolipids. Replacing free linoleic acid as substrate by the above lipid samples, the oxidation results obtained with purified pea lipoxidase are presented in Fig. 26 and Table. 15.

TABLE 15

OXYGEN UPTAKE ($\mu M\ O_2$/min) BY DIFFERENT LIPID SUBSTRATES IN PRESENCE OF LIPOXIDASE, PHOSPHOLIPASE A_2, AND LIPASE

	Substrate	Control	+Phospholipase A_2	Activity Increase (times)
PL	PC	0.005	0.209	42
	PE	0.004	0.223	56
	DPG	0.006	0.162	27
	MGDG	0.006	0.382	64
	DGDG	0.007	0.362	52
		Control	+ Lipase	Activity Increase (times)
NL	NL	0.119	-	-
	TG	0.090	0.133	1.5

As seen from oxygraph tracings, oxygen consumption was negligible with galactolipids and all phospholipids but not with NL used as substrate. This lipid fraction gave a many fold higher consumption of oxygen than any of the individual PL tested. To clarify such reactivity, an analysis of NL by one dimensional TLC was performed.

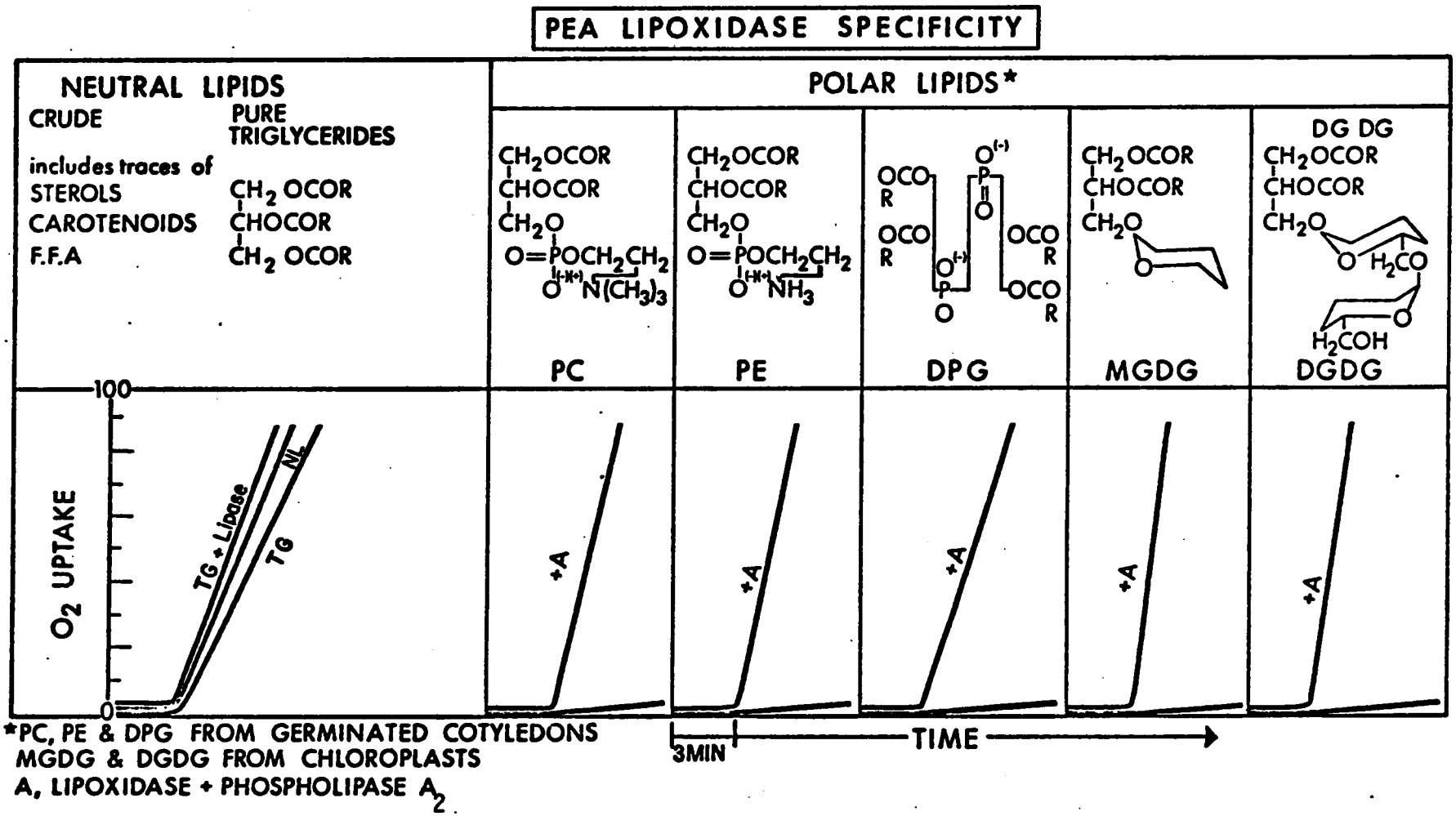


Fig. 26. Lipoxidase Catalyzed Oxidation of Some Individual Pea Lipids with or without Lipase and Phospholipase A_2. Lipids were ultrasonicated in 0.1 M borate buffer containing 0.04% deoxycholate and 2.5×10^{-3} M Ca-acetate in case of PL; 0.4 M NaCl, 0.005 M $CaCl_2$ and 2 mg Na-taurocholate and 3 mg gum acacia/ml buffer in case of NL.

The chromatograms obtained revealed the presence of TG, sterols, mono- and diglycerides, and in addition a considerable amount of FFA. Assuming that the FFA and/or the mono- and diglycerides are responsible for the high substrate reactivity, enzymatic oxidation was repeated with pure TG recovered from TL plates. A decrease in oxidation rate from 0.119 to 0.090 μM O_2/min was recorded. Such a decrease (only 25%) indicated that pure pea lipoxidase prefers FFA containing substrates but does not discriminate TG as a substrate. When pure TG was pre-incubated with lipase, the activity of lipoxidase, due to the release of FFA, increased and exceeded by 12% the activity obtained with the unpurified NL fraction.

Substrate specificity study with phospho- and galactolipids revealed a similar trend. In the absence of FFA, both MGDG and DGDG had a negligible oxygen consumption rate of about 6×10^{-3} μM/min, a rate which changed only slightly when PC, PE, or DPG were used as substrates. This low oxidation rate with individual PL was about 20 times less than that obtained for pure TG.

Although all of the PL investigated contained a high degree of unsaturation and particularly a high content of the 1, 4-pentadiene system required by lipoxidase, they proved to be poor substrates. When enzymatic hydrolysis with phospholipase A_2 was performed, and the same system in the presence of released FFA tested for lipoxidase action, the rates of oxidation became exceptionally high. As seen in Table 15, for PC, PE, and DPG, an increase in oxidation rate to about 200×10^{-3} μM/min was obtained, and this corresponded to a many fold increase in oxygen uptake when compared to that recorded before hydrolysis.

This finding including that for TG indicate the inability of

lipoxidase to oxidize effectively the unsaturated fatty acid moiety in glycerides whenever the α position is being occupied with phosphoryl or sugar compounds. Furthermore, these results showed that after hydrolysis, PC and PE are better substrates than DPG. This result may partly be explained by the FA composition of the three lipids. Though DPG contained 26% less linoleic acid, its unsaturation degree was still comparable to those of PC and PE due to its higher content of palmitoleic and oleic acids. But on the other hand, these acids are not recognized substrates for lipoxidase catalyzed oxidation. In addition, phospholipase A being more specific for hydrolysis of FA at the β position might reflect the lower unsaturation at this position in DGP than in PC or PE.

The large increase of oxidation rate for galactolipids when incubated with phospholipase A was unexpected and somewhat striking. In comparison to the results of oxygen uptake in absence of FFA, there was a 64 fold rate increase for MGDG and a 52 fold increase for DGDG. From these observed increases, it is apparent that like phospholipids, galactolipids are also poor substrates for lipoxidase attack unless hydrolyzed. Simultaneously, it is also obvious that phospholipase A is not specific for hydrolysis of phospholipids only, but it is equally effective in hydrolyzing galactolipids.

D. Nonenzymatic Oxidation of Pea Lipids

Nonenzymatic oxidation of lipids may involve transitional metal ion-induced oxidation of mitochondrial lipids, oxidation of lipids coating the natural solid matrices and the oxidation of free lipids induced by irradiation.

1. Mineral composition of pea seeds

The content of certain metal ions particularly those of transitional trace elements in pea seeds was determined, and the results are presented in Table 16. The highest amount of the elements analyzed was that of K which was about 1.3% of the dry matter, followed by a smaller amount of Ca, while the rest of the elements ranged from less than 0.25 ppm as found for Ni, to about 70 ppm for Mg and Fe.

TABLE 16

MINERAL COMPOSITION OF PEA SEEDS

Element*		Expressed in ppm								
Pea Variety	K %	Na	Ca	Mg	Fe	Co	Ni	Cu	Mn	Zn
Homesteader	1.24	42	775	75	70	2.5	<0.25	9.9	10.8	38
Laxton	1.32	55	1550	40	65	2.5	<0.25	8.2	11.2	41
Stratagem	1.31	39	625	70	53	3.5	<0.25	9.6	7.6	50

*The values are averages of duplicates which do not differ by more than 10%.

Among transitional metal ions, Fe was the most abundant followed in decreasing amounts by Zn, Mn, Cu, and Co. The level of Cu and Mn was about one fourth that of Zn, less than one sixth that of Fe, but about four times that of Co.

The differences among varieties were mostly apparent in the contents of Ca and Mg. While in two varieties, the Mg equivalent ratio of these metals was about 6:1, in the variety Laxton it was 23:1.

Since the analysis was done by a wet ashing rather than by an aqueous leaching technique, the differences found among the elements may not reflect their actual availability as free ions for diffusion during pea seeds' imbibition. This is especially true for transitional metal ions which are usually present in a bound form with proteins or firmly chelated with various enzyme substrates. Nevertheless, it is assumed that a small proportion of their total contents presented in Table 16 would be free, and thus, able to affect the swelling of mitochondria as well as the oxidation of lipids.

2. Viability of pea mitochondria

Mitochondrial integrity was examined by their oxidative phosphorylation and by the ability to swell and contract. The latter is shown in Fig. 27. As seen, swelling was initially rapid, slowed down with time, and was almost complete within 30 min. At this point considerable contraction was obtained by the addition of succinate. Contraction was reflected by immediate rise in the absorbance at 520 nm and reached its maximum within 20 min. The results indicated that the isolated mitochondria exhibit good ability to swell and contract, which was considered as an initial proof to their integrity.

Polarographic traces of mitochondrial activity as reflected by ADP/O and RC values are shown in Fig. 28. The rate of oxygen uptake by endogeneous respiration was very low (state 3), but increased on addition of ADP and decreased after a few min indicating consumption of added ADP. The calculated ADP/O ratio was 1.3 - 1.4 with succinate as substrate and twice as high with α-ketoglutarate. A similar trend was observed for RC value which in the presence of succinate had the lowest

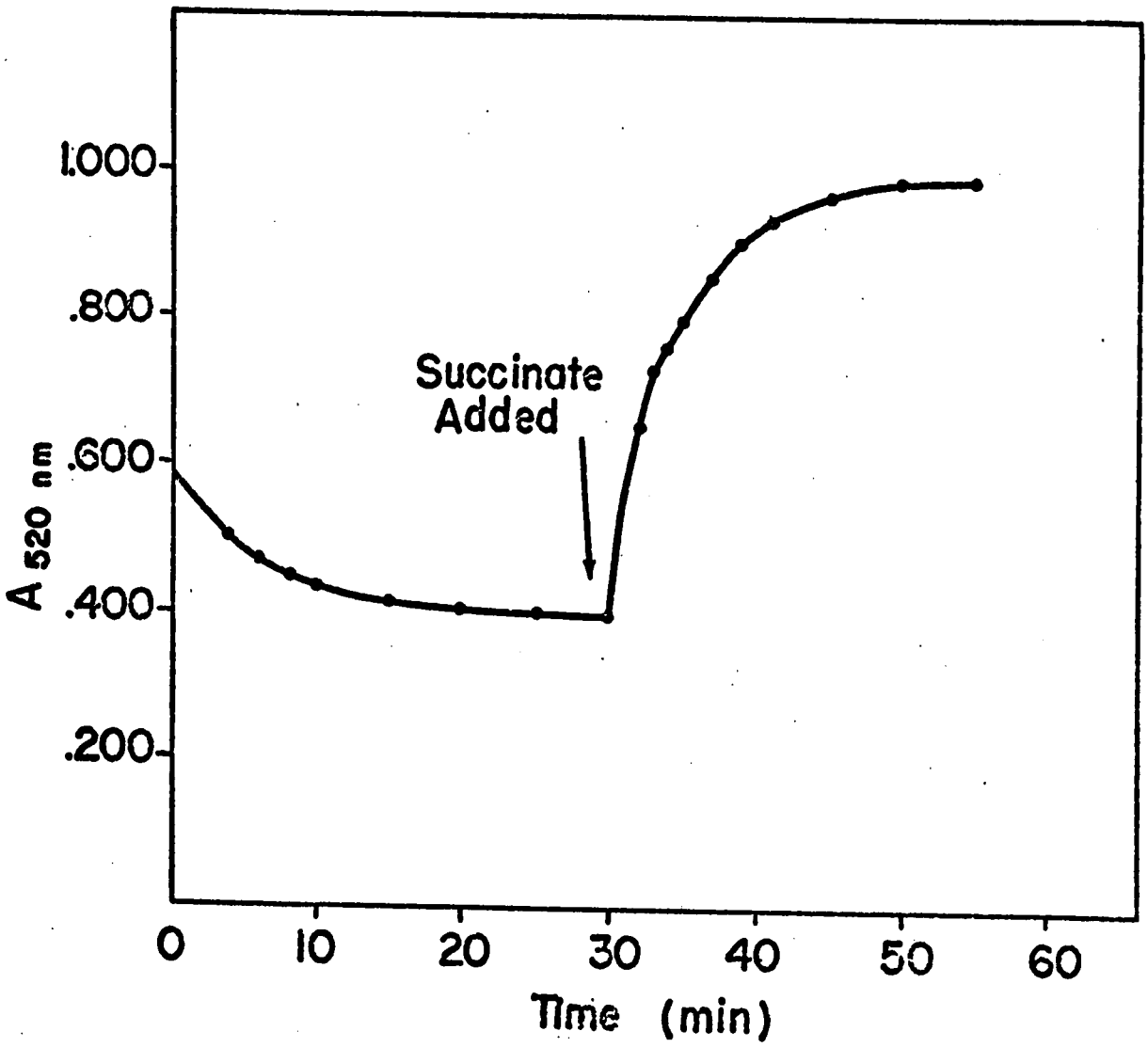
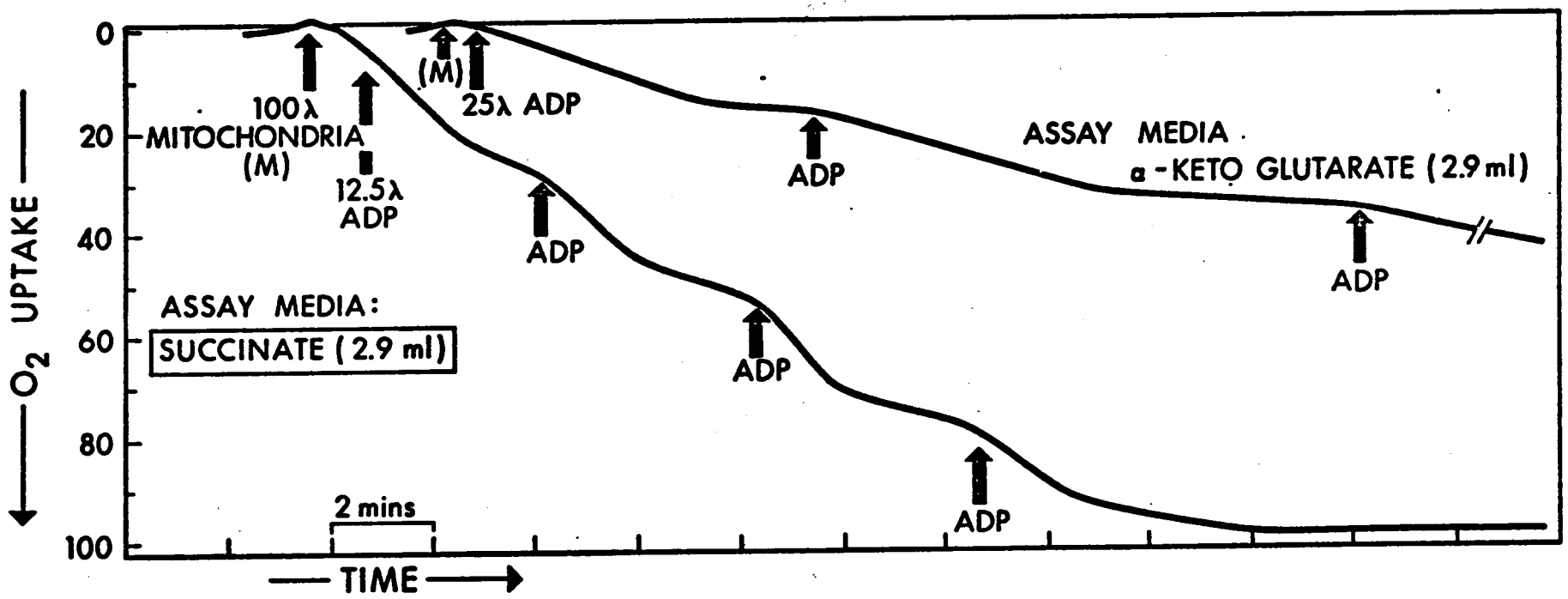


Fig. 27. Swelling and Contraction of Pea Mitochondria. 100 µl of mitochondria (≈ 100 mg protein) were added at 0 time to 2.9 ml of 0.1 M KCl in 0.02 M Tris-Cl buffer pH 7.5; after 30 min 10 µl of K-succinate was added to give a final concentration of 2 mM. Absorbance was read in 1 cm cell using a Beckman DBG Spectrophotometer.



	SUCCINATE		α-KETO GLUTARATE	
	RCR	ADP/O	RCR	ADP/O
CYCLE 1	2.62	1.3	5.32	2.7
CYCLE 2	2.69	1.3	6.30	2.8
CYCLE 3	2.75	1.4		2.8
CYCLE 4	3.49	1.4		2.9

Fig. 28. Amperometric Tracings of Oxygen Consumption by Mitochondria Isolated from Pea Cotyledons after 4 Days of Germination. For instrument used, see Fig. 19.

value of 2.62 in the first cycle, increased values in subsequent cycles and approached the considerably high value of 3.5 in the last fourth cycle. From the oxygraph tracings it is apparent that the rate of oxygen uptake with α-ketoglutarate is lower than that with succinate.

The results of oxidative phosphorylation were in good agreement with the previous results for swelling and contraction, and both were considered as a sufficient proof for biochemical integrity of the isolated mitochondria. However, the degree of swelling was chosen as a convenient index for the subsequent study.

The effects of KCl concentration in the swelling medium, germination time of pea seeds, and in vitro aging of mitochondria, on mitochondria swelling were particularly investigated. It was found that mitochondria swell in hypo- and isotonic solutions of KCl. As seen from Fig. 29, the two chosen concentrations of KCl (0.05 and 0.10 M) excerted similar influence on the degree of swelling. Hence, the isotonic concentration of 0.1 M KCl was chosen for the subsequent study.

Mitochondria swelling as affected by germination time of pea seeds and by one day aging of the isolated mitochondria is illustrated in Fig. 29. Mitochondria isolated from pea cotyledons after one day germination exhibited a considerable degree of swelling, which degree dropped after the 2nd day and rose gradually up to the 5th day when it became close to that of the first day. High swelling amplitude was maintained during the 6th, 7th and 8th day of germination with that of the 7th day being the highest. Apart from the rather low degree of swelling observed in the 2nd day of germination, swelling did not seem to be largely affected by germination time, and at no time during the

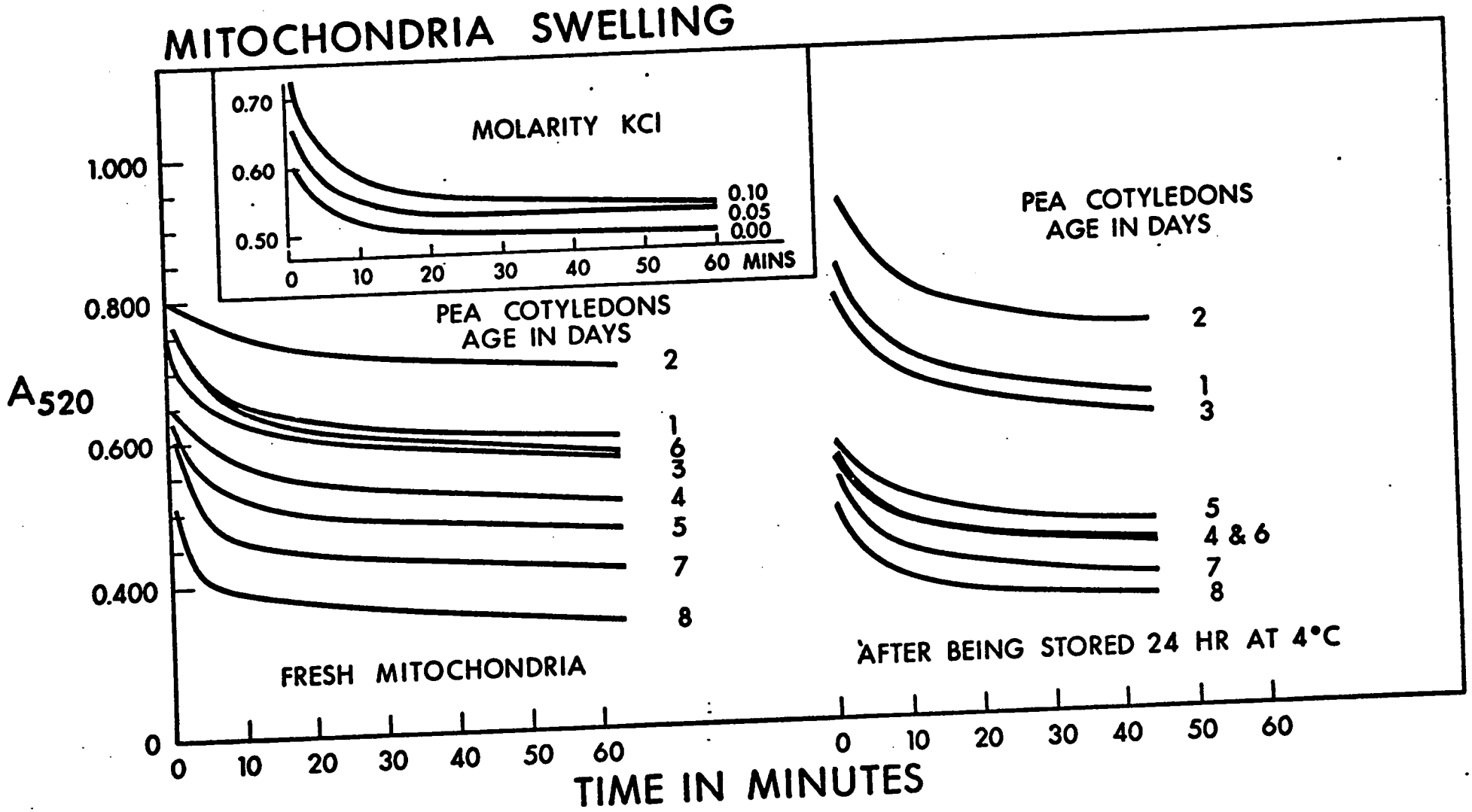


Fig. 29. Swelling Characteristics of Fresh and Aged Mitochondria in 0.1 M KCl in Dependence on Germination Time of Pea Cotyledons. Mitochondria were isolated according to Solomos et al. (1972). Absorbance was read in 1 cm cell using Beckman DBG Spectrophotometer.

eight days assayed did it drop to a negligible degree. Mitochondria stored for one day at 4^{o} retained their ability to swell and in some instances (2-day germination) even exceeded that of the fresh preparations.

During mitochondria swelling assays, it was difficult to obtain the same initial absorbance from different preparations. Due to this difficulty, swelling results were supposed to be better expressed as a relative change in absorbance (i.e., $\Delta A_{520}/A_{520}$) versus time as shown in Fig. 30. However, the interpretation of the resulting curves and their comparison became as difficult as the previous ones; the change in absorbance seems not to increase proportionally with initial increase in absorbance to provide a reliable comparison.

Since one day germination of pea seeds did provide mitochondria with a good swelling ability, this age, being convenient has been chosen for mitochondria isolation and their use in subsequent oxidation assays. In addition, fresh mitochondria were used within 1 h after isolation, though, their ability to swell satisfactorily was maintained even after 24 h.

3. Estimation of lipid oxidation

An attempt was made to determine the lipid peroxides in swollen mitochondria by nonaqueous DME polarography. As seen in Fig. 31, by scanning from -0.2 to -1.0 V, lipid peroxides were readily detected in butter fat oxidized by heating at 70^{o} for 10 h. The half-wave potential was found to be -0.65 V, which potential coincided with that of molecular oxygen. When mitochondrial lipids were analyzed by the same procedure, an interfering wave without a limiting current was

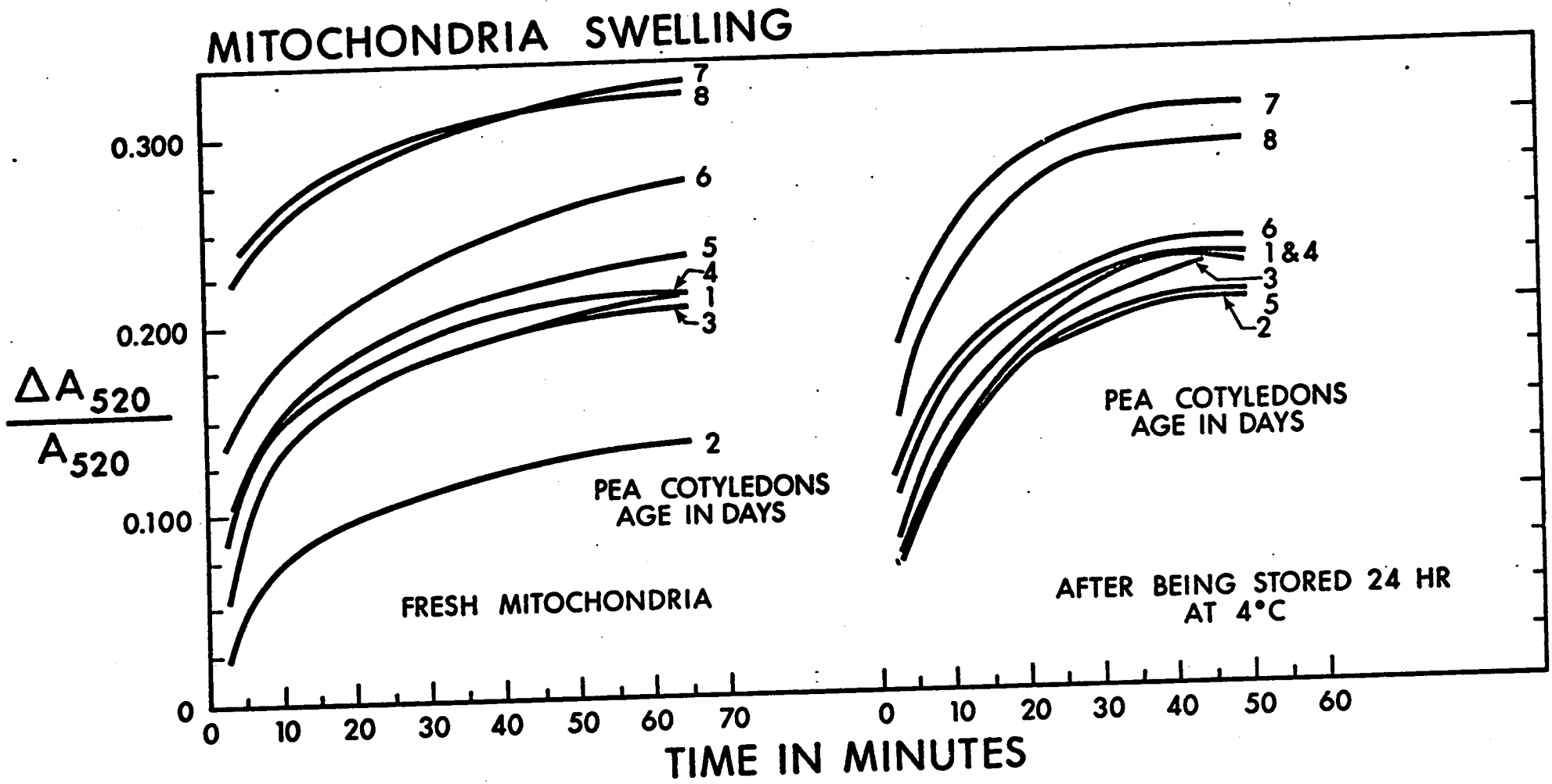


Fig. 30. Swelling Characteristics of Fresh and Stored Mitochondria in 0.1 M KCl in Dependence on Germination Time of Pea Cotyledons. Other details as in Fig. 29.

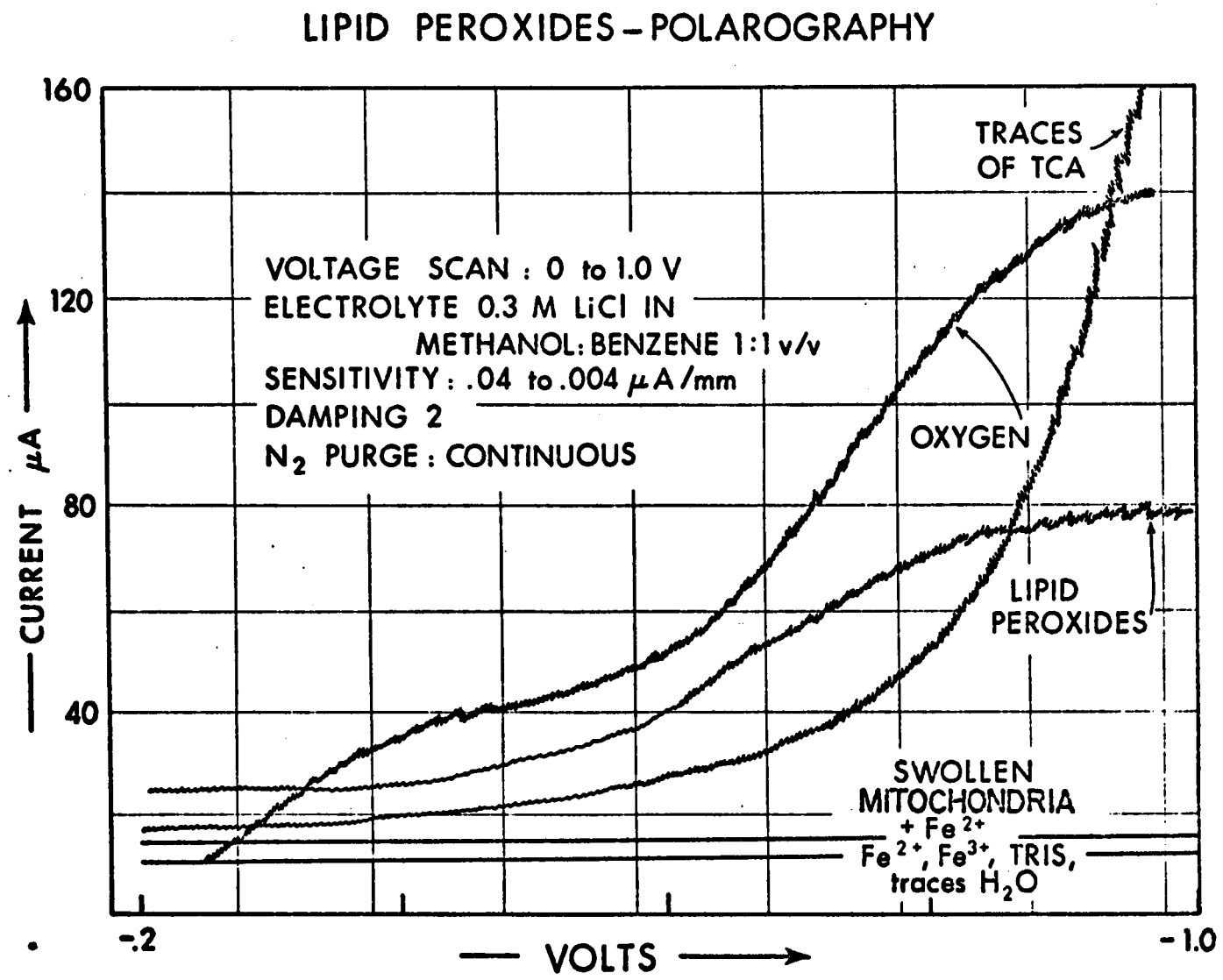


Fig. 31. Polarograms of Lipid Peroxides, Traces of O_2, TCA, and Media Constituents used in Mitochondria Swelling Experiments. Instrument used, Sargent Polarograph, Model XV.

recorded. Suspected interfering substances were those used in the swelling media; Fe^{2+}, Fe^{3+}, Tris, and traces of water, and TCA used for mitochondria precipitation prior to lipid extraction. Only TCA gave a wave which coincided with the interfering one. Hence, it was replaced by a hot mixture of chloroform methanol (2:1). This solvent system precipitated mitochondria, extracted the lipids present, and its traces after evaporation did not produce an interfering wave. When this mixture was applied to mitochondria (up to 8 mg mitochondrial protein) in which lipid oxidation was induced by Fe^{2+}, no peroxide wave was detected. From these assays, it was concluded that even with the highest sensitivity level of 0.004 μA/mm, polarography is not a suitable method for determination of lipid peroxides in mitochondria.

When polarography was substituted by TBA test, the color developed was apparent but was mostly adsorbed on the mitochondrial protein precipitate and thus was removed during filtration or centrifugation. The procedure by which protein precipitate is retained and solubilized by alkaline pyridine butanol mixture proved to be satisfactory. Moreover, TBA did not produce a color detectable by absorbance at 548 nm in presence of any transitional metal ions used at the physiological levels found in peas. Hence, this procedure was adopted for estimation of lipid oxidation in mitochondria.

Matrix effect on pea lipid oxidation was studied by manometric technique since it provides continuous measurement of oxygen uptake. However, when it was used to follow oxidation of lipids distributed on powdered solid matrices, poor reproducibility of oxygen uptake was recorded. Improved results were obtained when the filter

paper technique was applied. Hence, it was adopted for studying matrix effect on pea lipid oxidation.

4. Lipid oxidation in pea mitochondria

By measuring TBA reactants produced in mitochondria during swelling, in presence of Cu^{2+}, Fe^{2+}, and Fe^{3+}, the absorbance of the red pigment produced was found to increase by increasing incubation time (Fig. 32). Since no comparable increase in absorbance was observed in the control sample, the increase was attributed to mitochondrial lipid oxidation induced by these ions. Ferrous ion at a level of 50 μM was considerably more effective in inducing the oxidation than Fe^{3+} when used at the same level and than Cu^{2+} when used at 20 μM. Intermediate values were obtained when a mixture of Fe^{2+} and Fe^{3+} in a ratio of 1 to 4 and at a level of 50 μM was used. On the other hand, neither Mn^{2+} at a level of 30 μM, nor Co^{2+} at 10 μM was effective in inducing lipid oxidation. A slight increase in absorbance was observed in case of Co^{2+} after 10 min of incubation, however, the final change in absorbance was similar to that of the control. Apart from iron which was used at 4% of the physiological level, other ions were used in 12 - 24% of their level found in Homesteader peas.

When mitochondria were incubated in a medium containing ascorbic acid at a concentration of 2×10^{-4} M, the increase in absorbance was low and close to that of the control. Even when the concentration was increased ten times and exceeded that reported in peas, no further increase in absorbance was obtained. Therefore, it is evident that ascorbic acid does not induce lipid oxidation in pea mitochondria. The absorbance of TBA reactants vs incubation time of

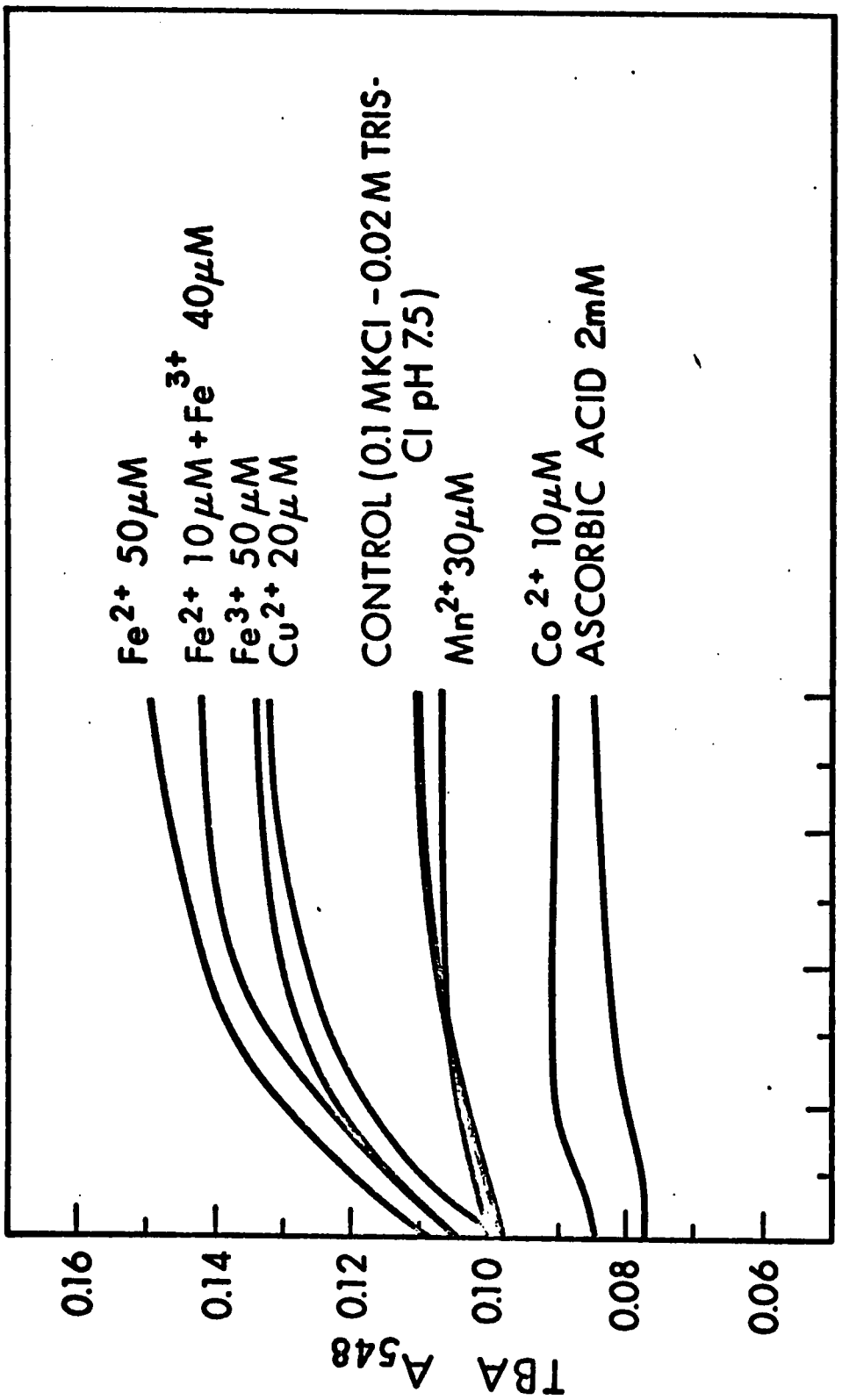


Fig. 32. Oxidation of Pea Mitochondrial Lipids as Influenced by Some Transitional Metal ions and Ascorbic Acid. Oxidation was followed by absorbance readings at 548 nm of TBA reactants in 1 cm cell using a Beckman DBG Spectrophotometer. Mitochondria were isolated from cotyledons germinated for 1 day. Reaction medium is 0.1M KCl in 0.02M Tris-Cl pH 7.5.

mitochondria in presence of ascorbic acid and the various ions used is presented in Fig. 32.

Lipid oxidation in mitochondria could be the result of a possible increase in swelling degree in presence of Fe^{2+} and Fe^{3+}. To elaborate on this assumption, mitochondria swelling assays were performed in presence of these ions, Co^{2+}, and ascorbic acid (Fig. 33). Unexpectedly, the degree of swelling was highest in the control followed in decreasing order by those in the presence of Co^{2+}, ascorbic acid and Fe^{3+}. This finding indicated that induced lipid oxidation in mitochondria does not correlate with the degree of swelling. This was further supported by the finding that Fe^{2+} induced contraction instead of swelling, which contraction was reflected by a considerable increase in absorbance readings at 520 nm. Furthermore, when Fe^{2+} replaced Fe^{3+} to the level of one fifth of molarity, the degree of swelling was lower than that observed with Fe^{3+} alone. Since Fe^{2+} induced the highest degree of lipid oxidation, accompanied by contraction and not by swelling of mitochondria, and since KCl alone in the control medium brought about little or no oxidation yet the largest degree of swelling, lipid oxidation cannot be attributed only to swelling of mitochondria, but also to a volume change which involves contraction.

5. Matrix effect on lipid oxidation

Preliminary experiments with 30 mg of NL and 18 mg of PL, coated on one filter paper disc, and oxidized at 25^{o} and 35^{o} resulted in an uptake of 0 - 2 µl O_2 in the first 48 h, and less than 2 µl for NL, and less than 7 µl for PL in the initial 24 h respectively. Therefore, oxidation experiments were conducted at 50^{o}. At this

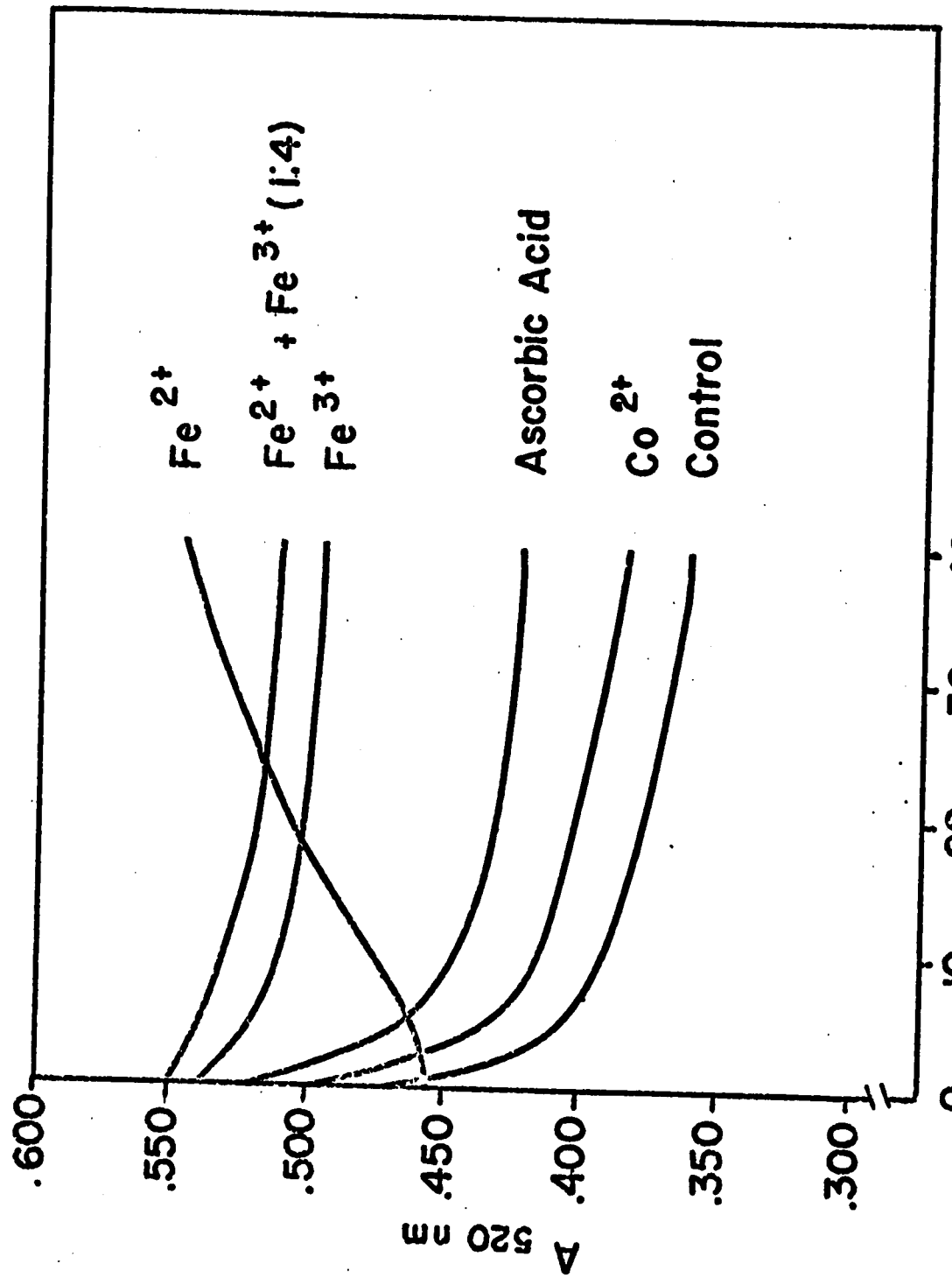


Fig. 33. Mitochondria Swelling as Influenced by Ascorbic Acid and Iron and Cobalt Ions. Swelling media was 0.1 M KCl in 0.02 M Tris-Cl pH 7.5. Absorbance was read in 1 cm cell using a Beckman Model DBG Spectrophotometer.

elevated temperature about 120 mg of neutral and 70 mg of polar pea lipids were found satisfactory for obtaining reliable readings of oxygen uptake when proteins and carbohydrates were applied as matrices. Furthermore, when these amounts of lipids were coated on one filter paper disc, having a surface area of 14 cm^2, by dipping the disc in a 50% chloroform solution of lipids, the amounts of lipids adsorbed were poorly reproducible. Satisfactory results were obtained when 10 - 15% solution of lipids were used and instead of one, four filter paper discs per 15 ml flask of the respirometer. Due to the high gelling property of pectin and to the high retrogradation of pure amylose solutions, these matrices along with those of proteins were prepared in 1% aqueous solutions. The amount of solid support adsorbed per disc averaged 3.0 mg, i.e., 0.215 mg/cm^2. On such matrices the amounts of NL and PL adsorbed reflected a ratio of 1.67 which approximately simulated that found in pea seeds.

The effects of carbohydrates such as amylose, amylopectin, and pectin on the oxidation rate of polar and neutral pea lipids are shown in Fig. 34. The rate of oxidation, being expressed in accumulated actual oxygen consumption vs time, was for NL slow at the first 12 h, after which time it increased and after 40 h reached a value of 1 - 1.5 µl O_2/mg of lipid applied. These results have shown that lipids coated on cellulose disc itself which was not precoated and thus had a role of a cellulose matrix, were oxidized at a higher rate than lipids present on cellulose precoated with other carbohydrates.

Among carbohydrates, the oxidation rate with amylopectin after 40 h was 26% less than that found for amylose, while with pectin

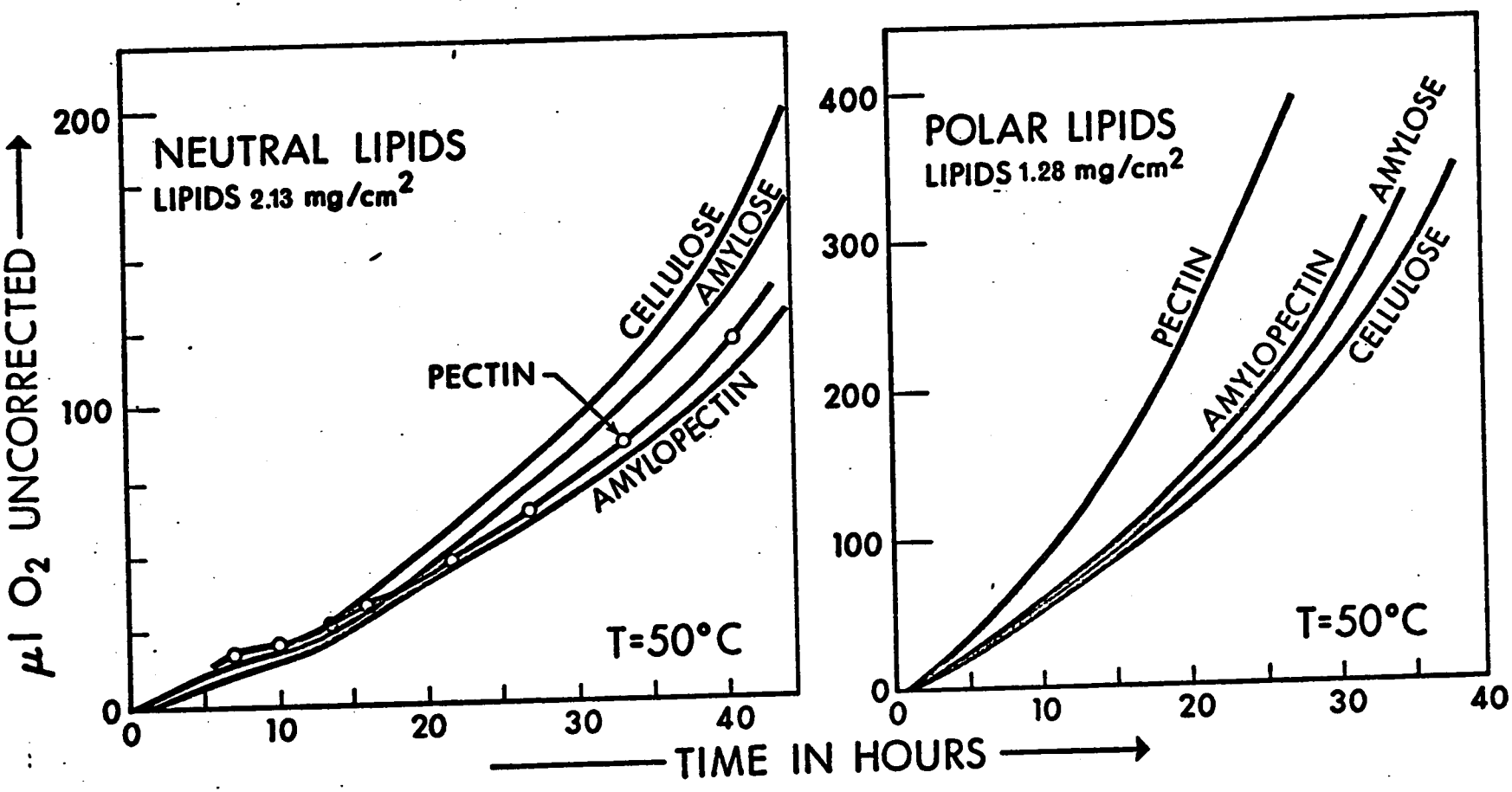


Fig. 34. Oxygen Uptake of Neutral and Polar Pea Lipids as Influenced by Carbohydrates used as Matrices. The filter paper disc procedure and a Gilson Differential Respirometer were applied.

as matrix, the oxidation was highest at the initial 12 h, but decreased by time and after 22 h ranked between the rates for amylose and amylopectin. In comparison to cellulose, the matrix of pectin induced a 26% inhibition in oxygen uptake. When this inhibition is compared to that of amylopectin which had the highest retardation degree of oxidation, was still about 11% less effective.

Contrary to peas' NL, the oxidation rate for PL when expressed per mg of adsorbed lipid was more than four times higher. In these experiments pectin as matrix exerted the highest promoting effect thus giving the highest oxidation rate, while cellulose gave the lowest. The effects of amylose and amylopectin were similar, but in comparison to NL their effects on lipid oxidation were reversed; amylose being the lower. Finally, with the exception of pectin all matrices exerted an equal effect during the initial stage of oxidation, and with all matrices the oxidation proceeded with no apparent induction period.

When recorded oxidation rates and previously given FA composition for peas' NL and PL are compared, it appears that the unsaturation degree is not of primary importance but the polarity of the lipid which is being oxidized.

The effect of proteins such as pea albumins and globulins on the oxidation rate of lipids is shown in Fig. 35. Again there was a smaller effect in promoting oxidation of NL. After 40 h the oxygen uptake for albumin matrix was about 16% less than that of globulins. When compared to cellulose matrix, used in these experiments as control, both proteins were slightly retarding the oxidation rate. After 40 h

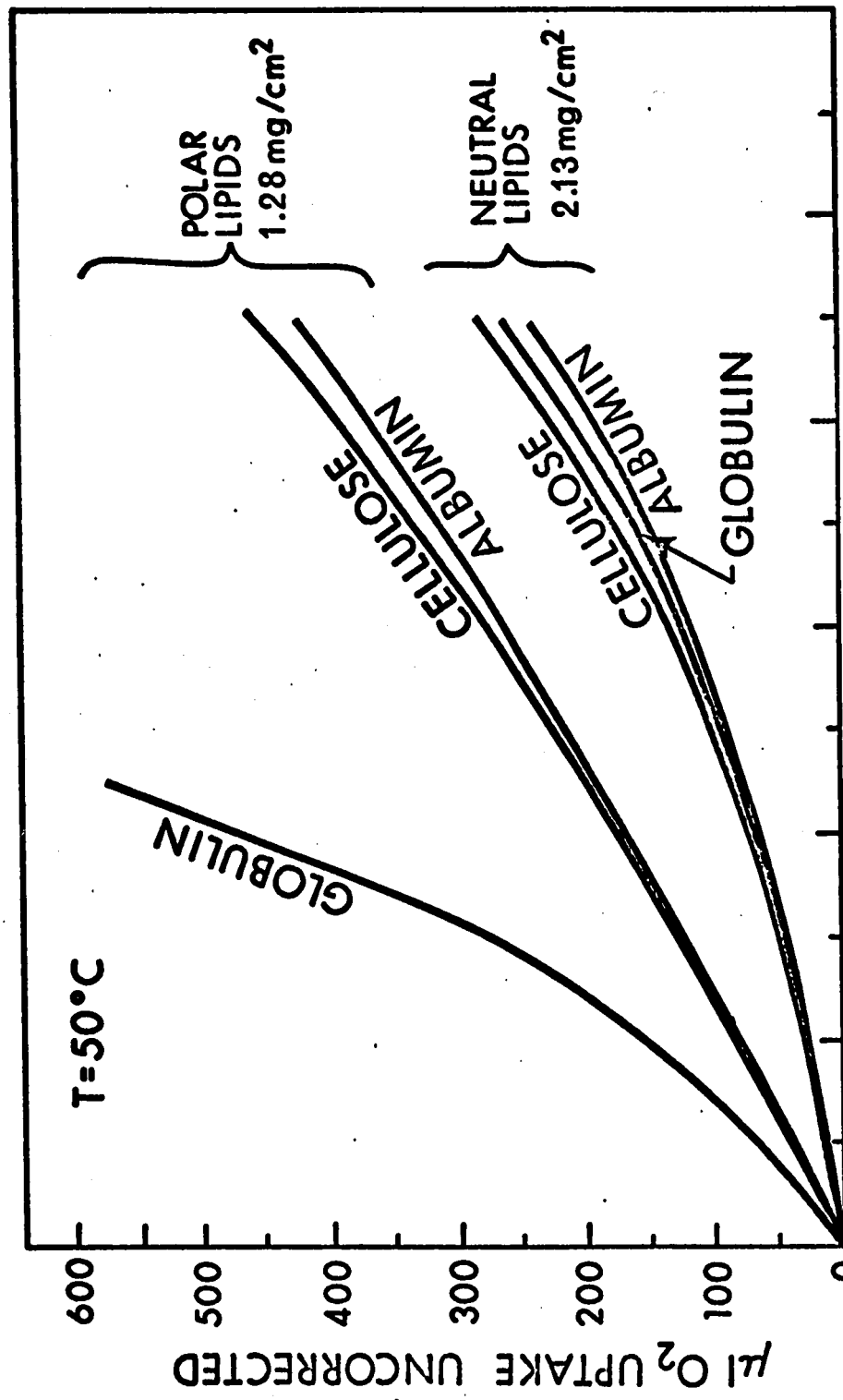


Fig. 35. Oxygen Uptake of Neutral and Polar Pea Lipids as Influenced by Pea Proteins used as Matrices. Other details as in Fig. 34.

of oxidation their retardation degree averaged round 13%, being slightly higher for albumin.

Oxidation rates for peas' PL were very high. This was particularly the case with globulins as matrix, which after 20 h was 2.8 times higher than that with albumin, while after 40 h of oxidation the results were not comparable by direct manometric readings. The albumin matrix effect was similar to that of cellulose but again slightly retarding the oxidation degree. With albumin as matrix, the oxidation rate of PL when expressed per mg lipid and compared to that of NL was 3.4 times higher after 40 h, while with globulin, it was 13 times higher after only 20 h of oxidation. This again suggests that polarity rather than unsaturation degree has the primary influence in lipid oxidation. Furthermore, the results show clearly that globulin and not albumins is the main oxidation promoting matrix.

6. The ESR of FAME free radicals induced by γ and UV irradiation

Methyl esters of oleate, linoleate and linolenate were irradiated in quartz tubes by a γ ray dose of 500 Kv under 10 μ vacuum at liquid nitrogen temperature. The ESR spectra revealed a non symmetrical signal which was identical for the three FAME examined. The composite signal had a g value of approximately 2.038 and spread over approximately 20 gauss. The hyperfine structure was apparent but the shape of the derivative of the line was not characteristic of all radiation doses. Increasing the irradiation dose to 3.5 Mrad resulted in a different hyperfine structure and the same change occurred for the three FAME. When the spectra were recorded at varying temperatures, the

signal deteriorated slightly and decreased in intensity at higher temperature. The major signal component was stable and persisted even at 120^{o} and thus was suspected to be from quartz. This was ascertained by obtaining a similar signal from irradiated quartz. Furthermore, the same signal was also obtained when either cellulose powder or cellulose coated by methyl linoleate, were irradiated in quartz at a dose of 1 Mrad. The signal obtained for quartz and for methyl linolenate is presented in Fig. 36.

When Spectrosil was used and the FAME samples were irradiated with a high intensity UV light being focused on the sample for 1 or 2 h no signals could be detected. After 3 h of irradiation a small signal was detectable in methyl linolenate sample only, but when the irradiation was increased to 4 h the signal was apparent in methyl oleate and linoleate. A further increase in irradiation time brought about a small increase in the signal intensity. Attempts to resolve its hyperfine structure were unsuccessful thus making the interpretation of the spectra difficult. Reducing modulation amplitude or power resulted in almost complete loss of the spectra. It was evident that the signal observed was near the limit of instrument detection. The narrow line width, 2.5 gauss from peak to peak, might partly be attributed to delocalization of the unpaired electron. The apparent g value of the signal was 2.0361 which is close to that found for the composite signal obtained in gamma irradiation of the sample in quartz. The spectra recorded for methyl linoleate after 6 h of UV irradiation is presented in Fig. 37. Due to the low intensity of the signal obtained for pure commercial FAME, to the lack of its hyperfine structure and to the large

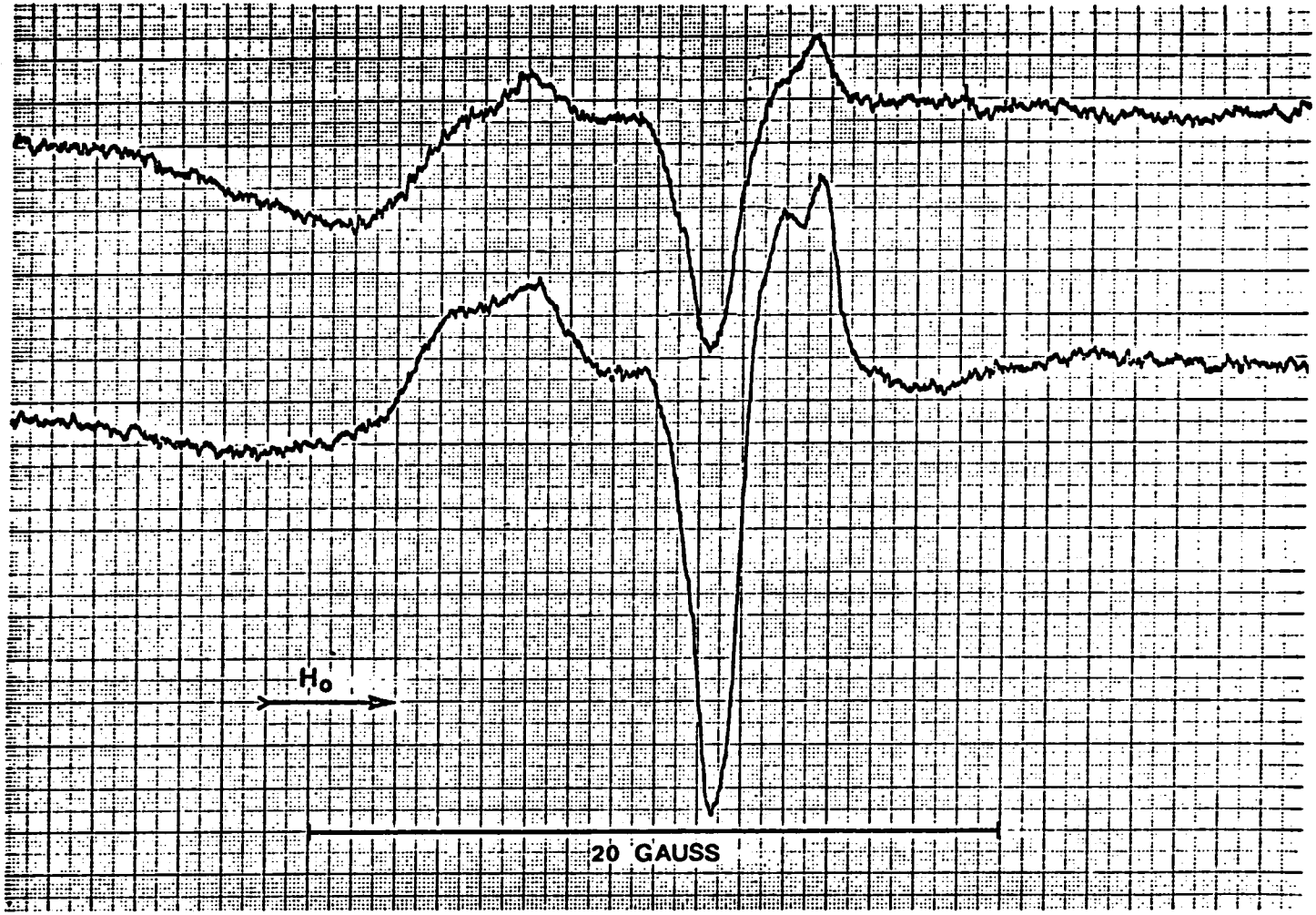


Fig. 36. Tracings of ESR of an Ordinary Grade Quartz Blank (top) and of Methyl Linolenate (bottom) After Irradiation with 0.5 Mrad. Irradiation and recording were performed at -196°; the curve represents first derivative of the actual absorption line. Similar spectra were obtained for methyl oleate and linoleate.

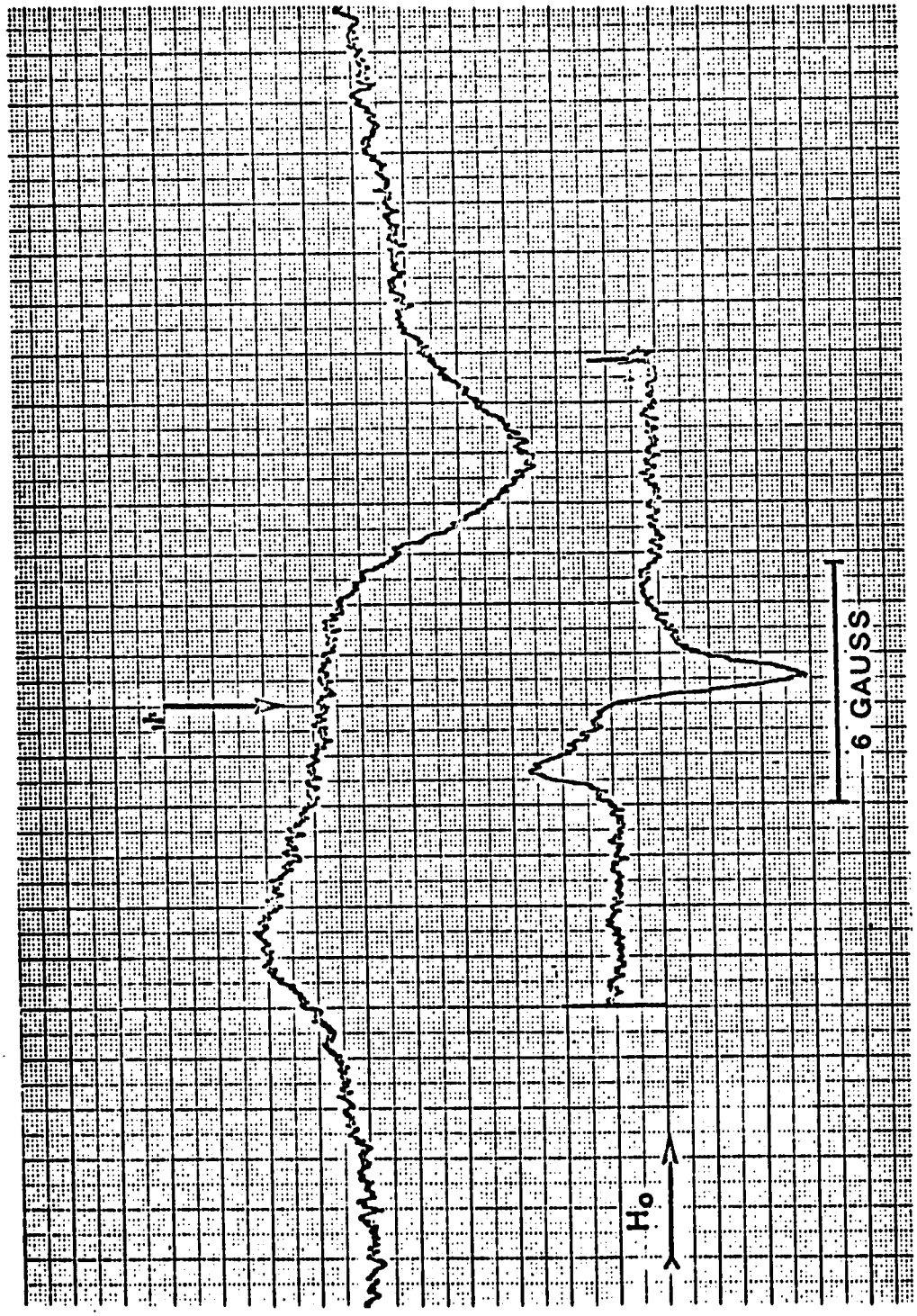


Fig. 37. Tracings of ESR of Methyl Linolenate Irradiated with UV Light for 6 h in a Quartz Spectrosil Grade Tube. Irradiation and recording were performed at -196°; the curve represents first derivative of the actual absorption line; g value of the marker is 2,0361; upper curve is an expanded recording. Identical spectra were obtained for methyl oleate and linoleate.

amounts of lipid samples required, further application of this technique for individual pea lipids was abandoned.

V. DISCUSSION AND CONCLUSION

Pea seeds investigated in this study were found to contain 3.0 to 3.9% lipids on dry weight basis. This amount was considerably lower than that reported by Wagenknecht (1957a), and by Adhikari et al. (1961), who reported values of 6 and 4.8% respectively. In addition, these authors reported different values, 51 and 38%, for the amount of TL soluble in acetone. However, the acetone solubility values cannot be compared with the NL/PL results obtained in the present study.

The PL of pea seeds are more complex than NL but less complex than PL of the germinated cotyledons and mitochondria. This may indicate that NL have a storage function while PL are more engaged in a physiological function required in metabolism and growth. The major seeds' PL found were PE, PC, and PI in decreasing order. These results are not in agreement with those reported in the literature, which also are inconsistent. Wagenknecht et al. (1959) reported the absence of PI in dry pea seeds while Quarles and Dawson (1969) reported PI as a major PL second only to PC. The latter authors reported the presence of PG, DPG, PA and PS in minute amounts, and N-acyl PE in a relatively large amount, however, the last three lipids were not detectable in our study. But, while Adhikari et al. (1961) suggested the presence of galactolipids in pea seeds, merely by galactose determination, our results confirmed the presence of MGDG and DGDG, and established the presence of two other glycolipids that were not reported earlier. These lipids behaved as sterol glycosides containing glucose as the sugar moiety, and

one of them was present as a major component of seeds' PL.

The relative amounts of individual PL changed rather markedly during germination. In agreement with Quarles and Dawson's results PI decreased considerably, but in addition we also observed an increase in DPG, and the presence of PA, PS and possibly LPC and LPE which were not detectable in seed lipids. These results indicate the possible action of phopholipases during germination. Furthermore, the presence of an intense unidentified PL spot in mitochondria and germinated cotyledons was apparent. In comparison to these, the chloroplasts PL were much simpler. The presence of PC, PI and PE was obvious and believed not to be due to mitochondrial contaminations as has been suggested by Nichols and James (1968). This could partly be supported by the different FA composition obtained for mitochondrial PC and chloroplasts' PC. Galactolipids appeared as the usual major chloroplast lipids, but their possible function remains to be elucidated.

Many difficulties were encountered in the isolation and identification of chloroplast galactolipids. Two procedures reported in the literature for their isolation (Van der Veen et al. 1967; Helmsing 1967) proved to be irreproducible, while the third one utilizing column and TL chromatography (Gardner 1968) was satisfactory. In addition, even taking all possible precautions during the isolation such as the use of nitrogen, low temperatures, and protection from light, did not seem to be sufficient against deterioration of chloroplast lipids as evidenced from their TL chromatograms. Furthermore, despite the distinct IR spectra obtained for these galactolipids and the proof of their sugar identity by silylation and GLC analysis, neither approach

was adequate for their differentiation. Thus, the presence of β galactose in DGDG could not be ascertained by either technique.

Although slight variations in FA composition of pea seeds' TL was found between the varieties investigated, large differences from those reported in the literature were apparent. On the average we found in the total percentage of FA content $C_{18:2}$ comprised 45, $C_{18:1}$ 32, C_{16} 11, $C_{18:3}$ 8 and C_{18} 4 compared to 55, 12, 23, 9, and 2 reported by Bengtsson and Bosund (1966) respectively. On the other hand, the FA composition of PL revealed a lower degree of unsaturation than that of NL, which agrees with the early generalizations about seed lipids made by Aylward (1956) and with the results reported by other authors (Lee and Mattick 1961; Bengtsson and Bosund 1966). The IR analysis of FA showed that they are devoid of hydroxy and keto groups as well as diene conjugation and trans unsaturation.

During germination palmitic acid increased on the expense of oleic and linolenic acids. The short chain FA as well as the odd numbered ones increased also but were still present in traces. This finding indicated among others the presence of α oxidation pathway during germination. Mitochondrial FA had a higher degree of unsaturation than the whole cotyledon and higher content of linoleic acid than that of seed, seedlings and chloroplast. However, this finding alone does not justify an assumption of a metabolic role of linoleic acid in mitochondria. Our results differ from those obtained by Richardson and Tappel (1962) for sweet potato mitochondria. These authors reported a two times higher palmitic acid content, but almost only half of linoleic acid content found in this study. Oleic acid was only 0.5%

compared to about 16% found for peas. In addition, they reported the absence of arachidonic acid and the presence of $C_{21:0}$ and $C_{22:1}$, while we did not detect any FA having a longer chain than 18:3. On the other hand, their results do not agree with those of Uritani and Yamaki (1969) who used the same plant material and reported widely different values for the major and the minor FA. Chloroplasts' and green leaves' FA were distinguished from those of the seed, germinated cotyledons and mitochondria by their high content of linolenic acid which is known to be a characteristic chloroplast FA (Wolf et al. 1966) but its role is not fully understood.

The FA of individual PL showed considerable variation in the same tissue as well as for the same lipid compound in different tissues. They may indicate the high turnover of FA in the plant material. The large differences in the FA composition of MGDG and DGDG in pea seeds also indicates that the latter is not likely to be formed by galactosylation of the former. Upon germination, PL seem to acquire a higher degree of unsaturation, which is more apparent in mitochondria.

Lipoxidase was found in all pea tissues and subcellular particles investigated. Its presence in mitochondria was established and was in disagreement with the results of Mapson and Moustafa (1955), who reported the absence of lipoxidase from pea mitochondria. However, while our results are obtained from direct assay of the enzyme, theirs were based on a coupled oxidation of glutathione and an unsaturated fatty acid, which was attributed to the lipoxidase enzyme action. The presence of the enzyme in pea mitochondria is further supported by the higher concentration found in the purified mitochondrial preparation

than that found in the following: the crude mitochondria, the cytoplasm, peroxisome-like bodies, and the seeds themselves. The enzyme activity was proportional to mitochondrial protein up to 300 μg protein. Furthermore, when BSA was excluded from the isolation media of mitochondria, the enzyme activity dropped considerably, which indicated that the enzyme is associated with the particle itself rather than being a contaminant.

The enzyme concentration was low in the crude and purified chloroplasts compared to those of seed or mitochondria but was high in the etiolated plastids. The concentration of the enzyme in the etiolated plastids was about 6 times as that of the green chloroplasts and 2 times that of the seed. Similar data have been reported in the literature by Guss et al. (1968) and by Holden (1970), however, we presented evidence that a major part of the enzyme in the etiolated leaf is concentrated in the cytoplasm. Upon treatment of the etiolated plastids with Triton X-100 the total activity increased unlike that with green chloroplasts. This is also in agreement with the results reported by Holden, but both results cannot be explained. Although lipoxidase enzyme was found in all pea organelles and cytoplasm, its function in the plant kingdom in general is still obscure (Tappell 1963).

As a corollary it was proposed by Tolbert et al. (1969) that in vivo all glycolate pathway enzymes are functionally organized and exclusively contained in peroxisomes. Significant yields of this organelle were obtained for the plant species typical for photo-respiration. Thus, for pea peroxisomes, a high activity of the

following enzymes was recorded: glucolate oxidase, NADH glyoxilate reductase, catalase, malate dehydrogenase, and cytochrome C oxidase. Our study revealed a high amount of peroxisomes in germinated pea cotyledons sedimenting with the crude mitochondria fraction. A further sucrose density gradient centrifugation separated these peroxisomes into two distinct layers which could account either for two species of peroxisomes, or for broken particles and less dense whole organelle. Both layers had lipoxidase activity which could be inhibited by cyanide ion. Nevertheless, this finding suggested, that peroxisomes besides the glycolate pathway enzyme contains heme-enzymes which possess activity similar to that of lipoxidase.

Purification of pea seed lipoxidase was achieved satisfactorily by ammonium sulfate precipitation followed by Sephadex and DEAE cellulose column chromatography. The procedure used was similar to that of Eriksson and Svensson (1970), except for the introduction of an ultra-centrifugation step prior to ammonium sulfate precipitation. After the latter step, we obtained 92% enzyme recovery and 1.2 fold purification compared to 89% and 1.1 obtained by Eriksson and Svensson respectively. It is apparent from comparing the results that the peas we used contained about 2 - 5 times activity as that used by these authors. However, their effluents from Sephadex contained much higher peroxidase activity, 40 times, and lower catalase activity, 10 times, than those we obtained. This comparison leads to the conclusion that these enzymes in peas differ greatly from one place to another. The final lipoxidase preparation obtained was 23.4 fold purified relative to the crude extract, had 16% of the total activity recovered and had a specific

activity of 143 units per mg protein compared to 35 fold purification, 10% recovery, and 84 units per mg protein reported by Eriksson and Svensson (1970). However, these authors used two steps of ion exchange chromatography; while we used one step only. We have established that the absence of catalase activity is not an adequate criterion for lipoxidase purity due to the inactivation of catalase by the thiol reagent used for lipoxidase elution. Therefore, disc gel electrophoresis was used for checking enzyme purity.

The final purified enzyme was found to have a molecular weight of 74,000 as determined by disc gel electrophoresis; a result comparable to that reported by Eriksson and Svensson using ultra-centrifugation. The electrophoresis procedure in the presence of SDS has been reported to give mobilities independent of isoelectric point, or of other protein properties other than the molecular weight (Dunker and Rueckert 1969). The pure enzyme had a narrow pH optima around 7.2 as compared to 6.9 reported by Siddigi and Tappel (1956). Although lipoxidase catalysis is reported to occur via a free radical, we found that it conformed to the Michaelis-Menton equation. The calculated Km for pea lipoxidase was 2.3×10^{-3} M compared to 1.35×10^{-3} M reported for soybean (Holman 1947) and 1×10^{-3} M for barley (Franke and Frehse 1953). The enzyme was found to contain 3 - 4 isoenzymes compared to 2 - 3 for a commercial soybean preparation used in this study while Hale et al. (1969) reported the presence of 2 - 3 isoenzymes in pea seeds.

Despite the presence of 3 - 4 isoenzymes established by disc gel electrophoresis using a specific staining procedure, and the present

evidence in the literature suggesting the presence of lipoxidases specific for certain substrates, we found that purified pea lipoxidase can oxidize both TG and FFA but doesn't oxidize any of the PL investigated. The preferential catalysis of FFA over TG has been suggested by Wagenknecht and Lee (1958), but our results were supported by a direct evidence using purified enzymes rather than acid values. Thus, lipase may not only produce hydrolytic rancidity in peas but it also supplies a good substrate for production of oxidative rancidity by lipoxidase. The adjunct action of lipase and lipoxidase becomes more significant when specificity of lipase from different seeds has been shown to prefer hydrolysis of linoleic acid instead of stearic acid (Berner and Hammond 1970). Our results have not been found to support the presence of any substrate specificity in pea lipoxidase.

Apart from a study by Guss et al. (1967b) who used galactolipids contaminated with cerebrosides as substrates and reported negligible lipoxidase catalyzed oxidation of these galactolipids, no other PL have been studied as substrate. Using purified individual pea lipids and lipoxidase enzyme we found that pea lipoxidase does not oxidize these lipids. However, after their incubation with phospholipase A, a high increase in the rate of oxidation was recorded.

It was repeatedly suggested that a major portion of off-flavor development in unblanched peas is accompanied by breakdown of PL, but the possible additive action of phospholipase and lipoxidase was not investigated. However, this study established that phospholipase action on PL is prerequisite to their oxidation by lipoxidase. Furthermore, it

was found that phospholipase A_2 is capable of hydrolyzing galactolipids, contrary to the requirement of an FA ester bond adjacent to a phosphoryl alcohol linkage reported by Van Deenen and De Haas (1963). The presence of phospholipid and galactolipid acylhydrolase activity has recently been reported in potato tubers (Galliard 1970).

Lipid oxidation induced by volume change of mitochondria was studied in systems involving viable organelles. It was stated by Zeevaart et al. (1968) that the oxidative capacity of pea mitochondria diminishes with the period of germination and falls rapidly after about two days. When the uncoupling factors were eliminated by BSA after 2 and 4 days of germination, the isolated mitochondria had a P/O value of 1.12 and 1.00 respectively. In the present study, the mitochondria had a ratio of 1.3 when isolated from 4 day old or younger cotyledons. This improvement was achieved by following the necessary conditions for isolation of tightly coupled mitochondria outlined recently by Ikuma (1970), which involves gentle tissue disruption, exclusion of contaminating particles from the mitochondrial fraction, and the use of an optimal grinding media containing mannitol, buffer, and BSA. The swelling assays were performed with mitochondria isolated from cotyledons germinated for only 24 h. This choice was motivated by the fact that this time is closer to that imbibition time usually encountered in pea processing. As found by Kolloffel and Sluys (1970), the addition of water to a powder of dried cotyledons produced a detectable oxygen consumption by the mitochondria of such cotyledons with succinate, malate and α ketoglutarate, even after 2 h of germination. In addition, the present study demonstrates that after 24 h of germination, the

mitochondria swelling ability matches those found for longer germination time. Hence, lipid oxidation assays in this study should be considered as still involving highly viable mitochondria.

Pea mitochondria swelling in presence of ascorbic acid did not induce lipid oxidation. This finding is contrary to that documented for animal mitochondria in which case lipid oxidation is correlated with oxidation of ascorbic acid. Furthermore, pea mitochondria swelling in presence of Cu^{2+} induced lipid oxidation. Whether these findings reflect a possible copper ascorbic acid oxidase molecule exchange bringing about an active oxidation of mitochondrial lipids cannot be elucidated. It is even less clear why ascorbic acid oxidase present in pea mitochondria does not bring about the oxidation of its own substrate, i.e., ascorbic acid.

The presence of Fe^{2+} induced the highest degree of lipid oxidation and instead of swelling it induced contraction of pea mitochondria. This finding is also not in agreement with that reported for animal mitochondria. The phenomenon is also not clearly understood since the contraction of plant mitochondria is well known to be substrate dependent, i.e., active swelling.

Mustakas et al. (1969) reported increased peroxide values and rancid odors with increased soaking time of whole soybean in water. These authors attributed the lipid oxidation to a slight lipase activity and to a considerable lipoxidase activity basing their argument solely, on the production of peroxide. However, the present study indicates that this is more likely to be a nonenzymatic oxidation of mitochondrial lipids induced by swelling in presence of beans own

transitional metal ions.

The oxygen uptake by neutral and polar pea lipids as influenced by matrix revealed distinct differences among matrices as well as lipid fractions. The amylopectin NL system consistently performed a retarding action in lipid oxidation. A lipid amylopectin chain interaction is assumed to occur in a way similar to that used to explain the antistaling properties of NL experienced in current baking industry practice. On the other hand, a decrease in the PL oxidation in presence of amylose might strengthen the current informations that PL fatty acid moieties are within amylose helices forming a clathrate nonstoicheiometric compound, thus being protected from oxidation. Enchanced oxidation of PL by matrices carrying a charge such as pectin and proteins suggests an interaction between negatively charged matrix and zwitter-ion molecules present in PL fraction. The basic electrophylic nitrogen of choline and ethanolamine moieties would interact, while the nucleophylic centers released would orient the fatty acid chains to a more sterically exposed conformation, thus increasing their oxidation rate. A particularly high rate of oxidation with globulin as matrix suggest additionally that globulin intracellular aggregation into separate bodies within the pea seeds, observed <u>in vivo</u> from lipid oxidation point of view is well justified. Furthermore, if lipid limiting membrane is assumed to exist, then it should involve NL rather than polar pea lipids. However all these possible explanations are just assumptions which should be strengthened by further experimental work.

In conclusion, the results of the present study demonstrate

that the lipids in peas are of two major fractions. Firstly, NL consisting of TG, sterol lipids and traces of DG, MG and FFA. Secondly, a PL fraction consisting of phospholipids and glycolipids. Both lipids are oxidizable within the pea seed either by enzymatic or nonenzymatic pathways. The requirement for enzymatic oxidation is the enzyme lipoxidase present abundantly in peas. This enzyme is highly active on free fatty acid pool, but less active when TG is present as the substrate. The PL fraction appeared to be a poor substrate unless phospholipase A is present. This is valid for phospholipids as well as for mono- and digalactosyl lipids, the last being the major constituent of glycolipids. The enzymatic oxidation of TG is enhanced in presence of lipase.

Nonenzymatic oxidation pathway involves the oxidation of lipids induced by a volume change of pea mitochondria. Swelling per se did not induce oxidation unless ferrous, ferric or copper ions are present. Manganese and cobalt ions do not influence such oxidation, neither does ascorbic acid. The lipid peroxides formed within mitochondria, up to 8 mg mitochondrial protein could not be detected by DME polarography, but are readily determined colorimetrically using TBA reagent.

Finally nonenzymatic oxidation of lipids coated on matrices such as pea proteins, and carbohydrates such as amylose, amylopectin, cellulose and pectin have confirmed an earlier observation that the oxygen uptake by such systems depend not only on the matrix sterical conformation and/or its charge but also upon the residual charges of lipids being oxidized. As revealed by ESR spectra such oxidations

proceed through a free radical mechanism. On the other hand the spectra themselves do not reveal clearly the fate of the free radicals formed.

VI. REFERENCES

Acker, L., & Geyer, J. (1969). Phospholipase B of barley malt. II. Studies on the function of the enzyme. Z. Lebensmitt. Untersuch. u. Forsh., 140: 269 - 75.

Acker, L., & Schmitz, H.J. (1967). Lipids of wheat starch. II. Identification and isolation of lisolecithin. Stearke, 19: 233 - 9.

Adhikari, S., Shorland, F.B., & Weenink, R.O. (1961). Lipids of the common pea (Pisum sativum L.) with special reference to the occurence of galactolipids. Nature (London), 191: 1301 - 2.

Allen, C.F., Good, P., Davis, H.F., & Chisum, P. (1966). Methodology for the separation of plant lipids and application to spinach leaf and chloroplast lamella. J. Am. Oil Chemists' Soc., 43: 223 - 31.

Allen, C.F., Good, P., Davis, H.F., & Fowler, S.D. (1964). Plant and chloroplast lipids. I. Separation and composition of major spinach lipids. Biochem. Biophys. Res. Commun., 15: 424 - 30.

Allen, J.C. (1968). Soybean lipoxygenase. I. Purification, and the effect of organic solvents upon the kinetics of the reaction. European J. Biochem., 4: 201 - 8.

Am. Oil Chemists' Soc. Official and Tentative Methods. (1964). 2nd ed., Cd 8 - 53.

Ames, G.R., & King, T.A. (1966). The assay of pH profile of lipoxidase. J. Sci. Fd. Agr., 17: 301 - 3.

Andrews, F., Bjorksten, J., Trenk, F.B., Henick, A.S., & Koch, R.B. (1965). The reaction of an autoxidized lipid with proteins. J. Am. Oil Chemists' Soc., 42: 779 - 81.

Aylward, F. (1956). Phospholipids in foods. Chem. & Ind. p. 1360 - 6.

Azarova, M.V., & Olifson, L.E. (1971). Fatty acid composition of pea lipids. Vop. Pitan., 30(1), 67 - 9. (Chem. Abstr., 74: 98458f).

Barber, A.A. (1966). Lipid peroxidation in rat tissue homogenates: Interaction of iron and ascorbic acid as the normal catalytic mechanism. Lipids, 1: 146 - 51.

Barron, E.J. (1964). Phospholipases. In Modern Methods of Plant Analysis, Eds. Linskens, H.F., Samual, B.D., & Tracey, M.V. Vol. 7, p. 454, Springer-Verlag, Berlin.

Bartlett, G.R. (1959). Phosphorous assay in column chromatography. J. Biol. Chem., 234: 466 - 8.

Ben-Aziz, A., Grossman, S., Ascarelli, I., & Budowski, P. (1970). Linoleate oxidation induced by lipoxygenase and heme proteins. Anal. Biochem., 34: 88 - 100.

Bengtsson, B., & Bosund, I. (1964). Gas chromatographic evaluation of the formation of volatile substances in stored peas. Food Technol., 18: 773 - 6.

Bengtsson, B., & Bosund, I. (1966). Lipid hydrolysis in unblanched frozen peas (Pisum sativum). J. Food Sci., 31: 474 - 81.

Bengtsson, B.L., Bosund, I., & Rasmussen, I. (1967). Hexanal and ethanol formation in peas in relation to off-flavor development. Food Technol., 21: 478 - 82.

Bergstrom, S. (1945). The oxidation of linoleic acid with lipoxidase. Arkiv Kemi, Mineral. Geol., A21, No. 15, 1 - 8.

Berner, D.L., & Hammond, E.G. (1970). Specificity of lipase from several seeds and Leptospira pomona. Lipids, 5: 572 - 3.

Bishov, S.J., & Henick, A.S. (1961). Cited in Koch, R.B. Dehydrated foods and model systems. In Symposium on Foods: Lipids and Their Oxidation, Eds. Schultz, H.W., Day, E.A., & Sinnhuber, R.O. (1962). p. 230 - 51. The AVI Pub. Co., Inc., Westport, Conn.

Bishov, S.J., Henick, A.S., & Koch, R.B. (1960). Oxidation of fat in model systems related to dehydrated foods. Food Res., 25: 174 - 82.

Blain, J.A., & Barr, T. (1961). Destruction of linoleate hydroperoxide by soya extracts. Nature (London), 190: 538 - 9.

Bradshaw, W.W., & Truby, F.K. (1959). Cited in Chipault, J.R. High energy irradiation. In Symposium on Foods: Lipids and their Oxidation, Eds. Schultz, H.W., Day, E.A., & Sinnhuber, R.O. (1962). p. 151 - 69. The AVI Pub. Co., Inc., Westport, Conn.

Buckle, K.A., & Edwards, R.A. (1970). Chlorophyll degradation and lipid oxidation in frozen unblanched peas. J. Sci. Fd. Agr., 21: 307 - 12.

Caldwell, E.F., & Grogg, B. (1955). Application of the thiobarbituric acid test to cereal and baked products. Food Technol., 9: 185 - 6.

Carter, H.E. & Koob, J.L. (1969). Sphingolipids in bean leaves (Phaseolus vulgaris). J. Lipid Res., 10: 363 - 9.

Cash, W.D., & Gardy, M. (1965). Role of contaminants in the mitochondrial swelling activities of reduced and oxidized glutathione preparations. J. Biol. Chem., 240: 3450 - 2.

Catsimpoolas, N. (1969). Isolation of soybean lipoxidase by isoelectric focusing. Arch. Biochem. Biophys., 131: 185 - 90.

Chang, C.C., Esselman, W.J., & Clagett, C.O. (1971). The isolation and specificity of alfalfa lipoxygenase. Lipids, 6: 100 - 6.

Chapman, D. (1965). The Structure of Lipids by Spectroscopic and X-ray Techniques, p. 208 - 19. John Wiley and Sons, Inc., New York, N.Y.

Christopher, J., Pistorius, E., & Axelrod, B. (1970). Isolation of an isozyme of soybean lipoxygenase. Biochim. Biophys. Acta, 198: 12 - 9.

Contardi, A., & Ercoli, A. (1933). Uber die enzymatische Spaltung der Lecithine und Lysocithine. Biochem. Z., 261: 275 - 302.

Corwin, L.M. (1962). Studies on peroxidation in vitamin E - deficient rat liver homogenates. Arch. Biochem. Biophys., 97: 51 - 8.

Dahle, L.K., Hill, E.G., & Holman, R.T. (1962). The thiobarbituric acid reaction and the autoxidations of poly-unsaturated fatty acid methyl esters. Arch. Biochem. Biophys., 98: 253 - 61.

Danielsson, C.E. (1949). Investigations of vicilin and legumin. Acta Chem. Scand., 3: 41 - 49.

Danielsson, C.E. (1950). An electrophoretic investigation of vicilin and legumin from seeds of peas. Acta Chem. Scand., 4: 762 - 71.

Danielsson, C.E., & Lis, H. (1952). Differences in the chemical composition of some pea proteins. Acta Chem. Scand., 6: 139 - 48.

David, J.J., & Joslyn, M.A. (1953). Acetaldehyde and related compounds in frozen green peas. Food Res., 18: 390 - 8.

Davis, B.J. (1964). Disc electrophoresis. II. Method and application to human serum proteins. Ann. N.Y. Acad. Sci., 121: 404 - 27.

Dawson, R.M.C., Clarke, N., & Quarles, R.H. (1969). N-acylphosphatidylethanolamine, a phospholipid that is rapidly metabolized during the early germination of pea seeds. Biochem. J., 114: 265 - 70.

Dillard, M.G., Henick, A.S., & Koch, R.B. (1960). Unsaturated triglyceride and fatty acid lipoxidase activities of navy beans, small red beans, green peas and lima beans. Food Res., 25: 544 - 54.

Dillard, M.G., Henick, A.S., & Koch, R.B. (1961). Differences in reactivity of legume lipoxidases. J. Biol. Chem., 236: 37 - 40.

Dolev, A., Rohwedder, W.K., & Dutton, H.J. (1967a). Mechanism of lipoxidase reaction. I. Specificity of hydroperoxidation of linoleic acid. Lipids, 2: 28 - 32.

Dolev, A., Rohwedder, W.K., Mounts, T.L., & Dutton, H.J. (1967b). Mechanism of lipoxidase reaction. II. Origin of the oxygen incorporated into linoleate hydroperoxide. Lipids, 2: 33 - 6.

Drapron, R., Anh, N.X., Launay, B., & Guilbot, A. (1969). Development and distribution of wheat lipase activity during the course of germination. Cereal Chem., 46: 647 - 55.

Dubois, M., Gilles, K.A., Hamilton, J.K., Rebers, P.A., & Smith, F. (1956). Colorimetric method for determination of sugars and related substances. Anal. Chem., 28: 350 - 6.

Dunker, A.K., & Rueckert, R.R. (1969). Observations on molecular weight determinations on polyacrylamide gel. J. Biol. Chem., 244: 5074 - 80.

Earnshaw, M.J., & Truelove, B. (1968). Swelling and contraction of Phaseolus hypocotyl mitochondria. Plant Physiol., 42: 121 - 9.

Earnshaw, M.J., Truelove, B., & Butler, R.D. (1970). Swelling of Phaseolus mitochondria in relation to free fatty acid levels. Plant Physiol., 45: 318 - 21.

Eriksson, C.E. (1967). Pea lipoxidase, distribution of enzyme and substrate in green peas. J. Food Sci., 32: 438 - 41.

Eriksson, C.E., & Svensson, S.G. (1970). Lipoxygenase from peas, purification and properties of the enzyme. Biochim. Biophys. Acta, 198: 449 - 59.

Estefan, R.M., Gause, E.M., & Rowlands, J.R. (1970). Electron spin resonance and optical studies of the interaction between NO_2 and unsaturated lipid components. Environ. Res., 3: 62 - 78.

Folch, J., Lees, M., & Stanley, G.H.S. (1957). A simple method for the isolation and purification of total lipids from animal tissues. J. Biol. Chem., 226: 497 - 509.

Fortney, S., & Lynn, W. Jr. (1964). Role of ascorbate and cysteine on swelling and lipid peroxidation in rat liver mitochondria. Arch. Biochem. Biophys., 104: 241 - 7.

Franke, W., & Frehse, H. (1953). Zur Autoxydation der ungesattigten Fettsauren. VI. Uber die Lipoxydase der Gramineen, im besonderen der Gerste. Z. Physiol. Chem., 295: 333 - 49.

Frankel, T.N., & Garber, E.D. (1965). Esterases in extracts from germinating seeds of twelve pea varieties. Botan. Gaz., 126: 221 - 2.

Fritz, G., & Beevers, H. (1955). Lipoxidase and the oxygen absorption of homogenates from corn seedlings. Arch. Biochem. Biophys., 55: 436 - 46.

Fullington, J.G. (1967). Interaction of phospholipid-metal complexes with water-soluble wheat protein. J. Lipid Res., 8: 609 - 14.

Galliard, T. (1970). The enzymic breakdown of lipids in potato tuber by phospholipid - and galactolipid - acyl hydrolase activities and by lipoxygenase. Phytochem. 9: 1725 - 34.

Gardner, H.W. (1968). Preparative isolation of monogalactosyl and digalactosyl diglycerides by thin-layer chromatography. J. Lipid Res., 9: 139 - 41.

Gardner, H.W. (1970). Sequential enzymes of linoleic acid oxidation in corn germ: lipoxygenase and linoleate hydroperoxide isomerase. J. Lipid Res., 11: 311 - 21.

Gardner, H.W., & Clagett, C.O. (1965). Sulfur, ethylene, and lipid metabolism. Plant Physiol., 40 (Suppl.): 17.

Gardner, H.W., & Weisleder, D. (1970). Lipoxygenase from Zea mays: 9-D-hydroperoxy-trans-10, cis-12-octadecadienoic acid from linoleic acid. Lipids, 5: 678 - 83.

Gini, B., & Koch, R.B. (1961). Study of a lipohydroperoxide breakdown factor in soy extracts. J. Food Sci., 26: 359 - 64.

Goldstein, B.D., Balchum, O.J., Demopoulos, H.B., & Duke, P.S. (1968). Electron paramagnetic resonance spectroscopy. Free radical signals associated with ozonization of linoleic acid. Arch. Environ. Health, 17: 46 - 9.

Goldstein, D.B. (1968). A method for assay of catalase with the oxygen cathode. Anal. Biochem., 24: 431 - 7.

Gordy, W., Ard, W.B., & Shields, H. (1955). Microwave spectroscopy of biological substances. II. Paramagnetic resonance in X-irradiated carboxylic and hydroxy acids. Proc. Natl. Acad. Sci. U.S.A., 41: 996 - 1004.

Gracza, R. (1965). In Starch Chemistry and Technology, Eds. Whistler, R.L. & Paschal, E.F. Vol. 1, p. 107. Academic Press, New York, N.Y.

Graveland, A. (1970). Modification of the course of the reaction between wheat flour lipoxygenase and linoleic acid due to adsorption of lipoxygenase on glutenin. Biochem. Biophys. Res. Commun., 41: 427 - 34.

Guss, P.L., Macko, V., Richardson, T., & Stahmann, M.A. (1968). Lipoxidase in early growth of wheat. Plant & Cell Physiol., 9: 415 - 22.

Guss, P.L., Richardson, T., & Stahmann, M.A. (1967a). The oxidation-reduction enzymes of wheat. III. Isoenzymes of lipoxidase in wheat fractions and soybean. Cereal Chem., 44: 607 - 10.

Guss, P.L., Richardson, T., & Stahmann, M.A. (1967b). Oxidation of various lipid substrates with unfractionated soybean and wheat lipoxidase. J. Am. Oil Chemists' Soc., 45: 272 - 6.

Hackett, D.P. (1964). Enzymes of terminal respiration. In Modern Methods of Plant Analysis, Eds. Linskens, H.F., Sandwal, B.S., & Tracey, M.V. Vol. 7, p. 684. Springer-Verlag, Berlin.

Haisman, D.R. (1962). Factors controlling the texture of peas. First Intern. Congr. Food Sci. and Technol., Vol. 1, 711 - 8.

Hale, S.A., Richardson, T., Von Elbe, J.H., & Hagedorn, D.J. (1969). Isoenzymes of lipoxidase. Lipids, 4: 209 - 15.

Hamberg, M., & Samuelsson, B. (1965). On the specificity of the lipoxidase catalyzed oxygenation of unsaturated fatty acids. Biochem. Biophys. Res. Commun., 21: 531 - 6.

Helmsing, P.J. (1967). Isolation and separation of mono- and digalactosyldiglycerides from spinach leaves with Sephadex LH-20. J. Chromatog., 28: 131 - 2.

Hess, K. (1954). Protein, Kleber, und Lipoid in Weizenkorn und Mehl. Kolloid-Z., 136: 84 - 99.

Hoffsten, P.E., Hunter, F.E. Jr., Gebicki, J.M., & Weinstein, J. (1962). Formation of lipid peroxide under conditions which lead to swelling and lysis of rat liver mitochondria. Biochem. Biophys. Res. Commun., 7: 276 - 80.

Holden, M. (1970). Lipoxidase activity of leaves. Phytochem., 9: 507 - 12.

Holman, R.T. (1947). Crystalline lipoxidase. II. Lipoxidase activity. Arch. Biochem., 15: 403 - 13.

Holman, R.T. (1948). Lipoxidase activity and fat composition of germinating soybeans. Arch. Biochem., 17: 459 - 66.

Holman, R.T. (1955). Measurement of lipoxidase activity. In Methods of Biochemical Analysis, Ed. Glick, D. Vol. 2, p. 113 - 9. Interscience Pub , New York, N.Y.

Holman, R.T., & Burr, G.O. (1945). Spectrophotometric studies of the oxidation of fats. IV. Ultraviolet absorption spectra of lipoxidase-oxidized fats. Arch. Biochem., 7: 47 - 54.

Holman, R.T., Panzer, F., Schweigert, B.S., & Ames, S.R. (1950). Crystalline lipoxidase. III. Amino acid composition. Arch. Biochem. Biophys., 26: 199 - 204.

Hoseney, R.C., Finney, K.F., & Pomeranz, Y. (1970). Functional (breadmaking) and biochemical properties of wheat flour components. VI. Gliadin-lipid-glutenin interaction in wheat gluten. Cereal Chem., 47: 135 - 40.

Hunter, F.E. Jr., Gebicki, J.M., Hoffsten, P.E., Weinstein, J., & Scott, A. (1963). Swelling and lysis of rat liver mitochondria induced by ferrous ions. J. Biol. Chem., 238: 828 - 35.

Hunter, F.E. Jr., Scott, A., Hoffsten, P.E., Gebicki, J.M., Weinstein, J., & Schneider, A. (1964). Studies on the mechanism of swelling, lysis and disintegration of isolated liver mitochondria exposed to mixtures of oxidized and reduced glutathione. J. Biol. Chem., 239: 614 - 21.

Ikuma, H. (1970). Necessary conditions for isolation of tightly coupled higher plant mitochondria. Plant Physiol., 45: 773 - 81.

International Union of Pure and Applied Chemistry. (1954). In Standard Methods for the Analysis of Fats and Oils (1964). Oils and Fats Division of IUPAC. 5th ed. Butterworths, London.

Irvine, G.N., & Anderson, J.A. (1953a). Kinetic studies of the lipoxidase system of wheat. Cereal Chem., 30: 247 - 55.

Irvine, G.N., & Anderson, J.A. (1953b). Note on the lipoxidase activity of various North American wheats. Cereal Chem., 30: 255 - 7.

Irvine, G.N., & Anderson, J.A. (1955). The inhibition of wheat lipoxidase by cyanide. Cereal Chem., 32: 140 - 3.

James, A.T., & Nichols, B.W. (1966). Lipids of photosynthetic systems. Nature (London), 210: 372 - 5.

Jennings, W.G., Dunkley, W.L., & Reiber, H.G. (1955). Studies of certain red pigments formed from 2-Thiobarbituric acid. Food Res., 20: 13 - 22.

Joslyn, M.A., & David, J.J. (1952). Acetaldehyde and alcohol in raw or underblanched peas. Quick Frozen Foods, 15: 51.

Kahn, J.S., & Hanson, J.B. (1959). Some observations on potassium accumulation in corn root mitochondria. Plant Physiol., 34: 621 - 9.

Kalbag, S.S., Narayan, K.A., Chang, S.S., & Kummerow, F.A. (1955). Polarographic studies of fat oxidation. J. Am. Oil Chemists' Soc., 32: 271 - 4.

Karel, M. (1960). Effects of water and of oxygen on reactions of food components. Ph.D. thesis, M.I.T., Cambridge, Mass.

Karel, M., Tannenbaum, S.R., Wallace, D.H., & Maloney, H. (1966). Autoxidation of methyl linoleate in freeze-dried model systems. 3. Effect of added amino acids. J. Food Sci., 31: 892 - 7.

Klimenko, V.G., & Pinegina, R.I. (1964). Variability of the proteins of pea seeds during ripening. Biokhimiyd,29: 327 - 34.

Koch, R.B. (1968). Calcium ion activation of lipoxidase. Arch. Biochem. Biophys., 125: 303 - 7.

Koch, R.B., Stern, B., & Ferrari, C.G. (1958). Linoleic acid and trilinolein as substrates for soybean lipoxidase(s). Arch. Biochem. Biophys., 78: 165 - 79.

Kolloffel, C., & Sluys, J.V. (1970). Mitochondrial activity in pea cotyledons during germination. Acta Bot. Neerl., 19: 503 - 8.

Kolthoff, I.M. (1925). A new set of buffer mixtures that can be prepared without the use of standardized acid or base. J. Biol. Chem., 63: 135 - 41.

Kuta, E.J., & Quackenbush, F.W. (1960). A polarographic study of organic peroxides. Anal. Chem., 32: 1069 - 72.

Labuza, T.P., Maloney, J.F., & Karel, M. (1966). Autoxidation of methyl linoleate in freeze-dried model systems. II. Effect of water on cobalt-catalyzed oxidation. J. Food Sci., 31: 885 - 91.

Lea, C.H. (1962). The oxidative deterioration of food lipids. In Symposium on Foods: Lipids and Their Oxidation, Eds. Schultz, H.W., Day, E.A., & Sinnhuber, R.O. p. 6. The AVI Pub. Co., Inc., Westport, Conn.

Lee, F.A., & Mattick, L.R. (1961). Fatty acids of the lipids of vegetables. I. Peas (Pisum sativum). J. Food Sci., 26: 273 - 5.

Lee, F.A., & Wagenknecht, A.C. (1958). Enzyme action and off-flavor in frozen peas. II. The use of enzymes prepared from garden peas. Food Res., 23: 584 - 90.

Lee, F.A., Wagenknecht, A.C., & Hening, J.C. (1955). A chemical study of the progressive development of off-flavor in frozen raw vegetables. Food Res., 20: 289 - 97.

Lee, T.T. (1968). Effect of ozone on swelling of tobacco mitochondria. Plant Physiol., 43: 133 - 9.

Lehninger, A.L., & Remmert, L.F. (1959). An endogenous uncoupling and swelling agent in liver mitochondria and its enzymatic formation. J. Biol. Chem., 234: 2459 - 64.

Leibler, K., Wozniak, J., & Krauze, S. (1968). Application of EPR for detection of free radicals in fatty acids. Roczniki PZH, 19: 231 - 8.

Lewis, W.R., & Quackenbush, F.W. (1949). The use of the polarograph to distinguish between the peroxide structures in oxidized fats. J. Am. Oil Chemists' Soc., 26: 53 - 7.

Lewis, W.R., Quackenbush, F.W., & DeVries, T. (1949). Polarographic studies of organic peroxides in nonaqueous solutions. Anal. Chem., 21: 762 - 5.

Link, W.E., & Formo, M.W. (1961). Analysis of autoxidation mixtures. In Autoxidation and Antioxidants, Ed. Lundberg, W.O. p. 367 - 416. Interscience Publ, New York., N.Y.

Lorimer, G.H., & Miller, R.J. (1969). The osmotic behaviour of corn mitochondria. Plant Physiol., 44: 839 - 44.

Lowry, O.H., Rosenbrough, N.J., Farr, A.L., & Randall, R.J. (1951). Protein measurement with the Folin phenol reagent. J. Biol. Chem., 193: 265 - 76.

Lynch, L.J., Mitchell, R.S., & Casimir, D.J. (1959). The chemistry and technology of the preservation of green peas. Advances in Food Res., 9: 61 - 151.

Malhotra, S.S., & Spencer, M. (1970). Changes in the respiratory, enzymatic, and swelling and contraction properties of mitochondria from cotyledons of Phaseolus vulgaris L. during germination. Plant Physiol., 46: 40 - 4.

Maloney, J.F., Labuza, T.P., Wallace, D.H., & Karel, M. (1966). Autoxidation of methyl linoleate in freeze-dried model systems. I. Effect of water on the autocatalyzed oxidation. J. Food Sci., 31: 878 - 84.

Mapson, L.W., & Moustafa, E.M. (1955). The oxidation of glutathione by a lipoxidase enzyme from pea seeds. Biochem. J., 60: 71 - 80.

McCready, R.M., Guggolz, J., Silviera, V., & Owens, H.S. (1950). Determination of starch and amylose in vegetables. Anal. Chem., 22: 1156 - 8.

McKnight, R.C., & Hunter, F.E. Jr. (1966). Mitochondrial membrane ghosts produced by lipid peroxidation induced by ferrous ion. II. Composition and enzymatic activity. J. Biol. Chem., 241: 2757 - 65.

Mellors, A., & Tappel, A.L. (1966). Quinones and quinols as inhibitors of lipid peroxidation. Lipids, 1: 282 - 4.

Mikus, F.F., Hixon, R.M., & Rundle, R.E. (1946). The complexes of fatty acids with amylose. J. Am. Chem. Soc., 68: 1115 - 23.

Mitsuda, H., Yasumoto, K., Yamamoto, A., & Kusano, T. (1967). Study on soybean lipoxygenase. Part I. Preparation of crystalline enzyme and assay by polarographic method. Agr. Biol. Chem., 31: 115 - 8.

Mustakas, G.C., Albrecht, W.J., McGhee, J.E., Black, L.T., Bookwalter, G.N., & Griffin, E.L. Jr. (1969). Lipoxidase deactivation to improve stability, odor, and flavor of full-fat soy flours. J. Am. Oil Chemists' Soc., 46: 623 - 6.

Nakamura, A., Kono, T., & Funahashi, S. (1958). Nature of lysolecithin in rice grains. I. Lysolecithin as a constituent of non-glutinous rice grains. Bull. Chem. Soc. Japan, 22: 320 - 4.

Nehring, P. (1968). Frischgemuse. In Handbuch der Lebensmittelchemie, Ed. Schormuller, J., Vol. 5/2, p. 320. Springer-Verlag, Berlin.

Nichols, B.W. (1965). Light induced changes in the lipids of Chlorella vulgaris. Biochim. Biophys. Acta, 106: 274 - 9.

Nichols, B.W., & James, A.T. (1964). The lipids of plant storage tissue. Fette, Seifen, Anstrichmittel, 66: 1003 - 6.

Nichols, B.W., & James, A.T. (1968). The function and metabolism of fatty acids and acyl lipids in chloroplasts. In Plant Cell Organelles, Proc. Phytochem. Group Symp. Ed. Pridham, J.B. p. 163 - 78. Academic Press, London.

Nichols, B.W., Wood, B.J.B., & James, A.T. (1965). The distribution of trans-Δ^3-hexadecenoic acid in plants and photosynthetic organisms. Biochem. J., 95: 6P.

Niederstebruch, A., & Hinsch, I. (1967). Polarogaphische Bestimmung von Hydroperoxiden in Olen. Fette, Seifen, Anstrichmittel, 69: 637 - 42.

Norgaard, M.J., & Montgomery, M.W. (1968). Some esterases of the pea (Pisum sativum L.). Biochim. Biophys. Acta, 151: 587 - 96.

O'Brien, J.S., & Benson, A.A. (1964). Isolation and fatty acid composition of the plant sulfolipid and galactolipids. J. Lipid Res., 5: 432 - 6.

Ongun, A., Thomson, W.W., & Mudd, J.B. (1968). Lipid composition of chloroplasts isolated by aqueous and nonaqueous techniques. J. Lipid Res., 9: 409 - 15.

Osman, E.M., & Dix, M.R. (1960). Effects of fats and nonionic surface-active agents on starch pastes. Cereal Chem., 37: 464 - 75.

Ottolenghi, A. (1959). Interaction of ascorbic acid and mitochondrial lipids. Arch. Biochem. Biophys., 79: 355 - 63.

Overman, A.R., Lorimer, G.H., & Miller, R.J. (1970). Diffusion and osmotic transfer in corn mitochondria. Plant Physiol., 45: 126 - 32.

Patton, S., & Kurtz, G.W. (1955). A note on the thiobarbituric acid test for milk lipid oxidation. J. Dairy Sci., 38: 901.

Pendlington, S. (1962). Chemical changes in unblanched peas after vining. First Intern. Congr. Food Sci. and Technol. Vol. 4: 123 - 30.

Placer, Z.A., Cushman, L.L., & Johnson, B.C. (1966). Estimation of product of lipid peroxidation (malonyl dialdehyde) in biochemical systems. Anal. Biochem., 16: 359 - 64.

Poole, C.P. Jr., & Anderson, R.S. (1959). Electron spin resonance of ultraviolet irradiated compounds. I. Unsaturated hydrocarbons. J. Chem. Phys., 31: 346 - 54.

Privett, O.S., Nickell, C., & Lundberg, W.O. (1955). Products of the lipoxidase-catalyzed oxidation of sodium linoleate. J. Am. Oil Chemists' Soc., 32: 505 - 11.

Pullishy, G. (1971). Separation and characterization of acyl lipids of bean chloroplasts. M.Sc. thesis, Univ. of Alberta, Edmonton, Alta.

Quarles, R.H., Clarke, N., & Dawson, R.M.C. (1968). Isolation of N-acyl phosphatidylethanolamine from pea seeds. Biochem. Biophys. Res. Commun., 33: 964 - 8.

Quarles, R.H., & Dawson, R.M.C. (1969). The distribution of phospholipase D in developing and mature plants. Biochem. J., 112: 787 - 94.

Rhee, K.S., & Watts, B.M. (1966a). Evaluation of lipid oxidation in plant tissues. J. Food Sci., 31: 664 - 8.

Rhee, K.S., & Watts, B.M. (1966b). Effect of antioxidants on lipoxidase activity in model systems and pea (Pisum sativum) slurries. J. Food Sci., 31: 669 - 74.

Ricciuti, C., Coleman, J.E., & Willits, C.O. (1955). Statistical comparison of three methods for determining organic peroxides. Anal. Chem., 27: 405 - 7.

Richardson, T., Tappel, A.L., & Gruger, E.H. Jr. (1961). Essential fatty acids in mitochondria. Arch. Biochem. Biophys., 94: 1 - 6.

Richardson, T., Tappel, A.L., Smith, L.M., & Houle, C.R. (1962). Polyunsaturated fatty acids in mitochondria. J. Lipid Res., 3: 344 - 50.

Rosenberg, A., & Gouaux, J. (1967). Quantitative and compositional changes in monogalactosyl and digalactosyl diglycerides during light-induced formation of chloroplasts in Euglena gracilis. J. Lipid Res., 8: 80 - 3.

Rosenberg, A., Gouaux, J., & Milch, P. (1966). Monogalactosyl and digalactosyl diglycerides from heterotrophic, hetero-autotrophic, and photobiotic Euglena gracilis. J. Lipid Res., 7: 733 - 8.

Roubal, W.T. (1970). Trapped radicals in dry lipid-protein systems undergoing oxidation. J. Am. Oil Chemists' Soc., 47: 141 - 4.

Roubal, W.T. (1971a). Free radicals, malonaldehyde and protein damage in lipid-protein systems. Lipids, 6: 62 - 4.

Roubal, W.T. (1971b). Nature of free radicals in freeze-dried fishery products and other lipid-protein systems. Fishery Bull., 69: 371 - 7.

Roubal, W.T., & Tappel, A.L. (1966). Polymerization of proteins induced by free radical lipid peroxidation. Arch. Biochem. Biophys., 113: 150 - 5.

Roughan, P.G. (1970). Turnover of the glycerolipids of pumpkin leaves. The importance of phosphatidylcholine. Biochem. J., 117: 1 - 8.

Roughan, P.G., & Batt, R.D. (1969). The glycerolipid composition of leaves. Phytochem., 8: 363 - 9.

Rouser, G., Fleischer, S., & Yamamoto, A. (1970). Two dimensional thin layer chromatographic separation of polar lipids and determination of phospholipids by phosphorus analysis of spots. Lipids, 5: 494 - 6.

Sastry, P.S., & Kates, M. (1963). Lipid components of leaves. III. Isolation and characterization of mono- and digalactosyl diglycerides and lecithin. Biochim. Biophys. Acta, 70: 214 - 6.

Saunders, D.H., Ricciuti, C., & Swern, D. (1955). Reactions of fatty materials with oxygen. XVI. Relation of hydroperoxide and chemical peroxide content to total oxygen absorbed in autoxidation of methyl oleate. J. Am. Oil Chemists' Soc., 32: 79 - 83.

Schoch, T.J., & Maywald, E.C. (1968). Preparation and properties of various legume starches. Cereal Chem., 45: 564 - 73.

Schoch, J., & Williams, C.B. (1944). Adsorption of fatty acids by the linear components of corn starch. J. Am. Chem. Soc., 66: 1232 - 3.

Seckinger, H.L., & Wolf, M.J. (1967). Lipid distribution in the protein matrix of wheat endosperm as observed by electron microscopy. Cereal Chem., 44: 669 - 74.

Senti, F.R., & Erlander, S.R. (1964). Carbohydrates. In Nonstoichiometric Compounds, Ed. Mandelcorn, L. p. 568 - 605. Academic Press, New York, N.Y.

Siddiqi, A.M., & Tappel, A.L. (1956). Catalysis of linoleate oxidation by pea lipoxidase. Arch. Biochem. Biophys., 60: 91 - 9.

Siddiqi, A.M., & Tappel, A.L. (1957). Comparison of some lipoxidases and their mechanism of action. J. Am. Oil Chemists' Soc., 34: 529 - 33.

Sidwell, C.G., Salwin, H., & Mitchell, J.H. Jr. (1955). Measurement of oxidation in dried milk products with thiobarbituric acid. J. Am. Oil Chemists' Soc., 32: 13 - 6.

Sinnhuber, R.O., & Yu, T.C. (1958). 2-thiobarbituric acid method for the measurement of rancidity in fishery products. II. The quantitative determination of malonaldehyde. Food Technol., 12: 9 - 12.

Solomos, T., Malhotra, S.S., Prasad, S., Malhotra, S.K., & Spencer, M. (1972). Biochemical and structural changes in mitochondria and other cellular components of pea cotyledons during germination. Can. J. Biochem., 50: 725 - 37.

Stevens, F.C., Brown, D.M., & Smith, E.L. (1970). Some properties of soybean lipoxygenase. Arch. Biochem. Biophys., 136: 413 - 21.

Stoner, C.D., & Hanson, J.B. (1966). Swelling and contraction of corn mitochondria. Plant Physiol., 40: 255 - 66.

Sumner, R.J. (1943). Lipoid oxidase studies. A method for the determination of lipoxidase activity. Ind. Eng. Chem., Anal. Ed., 15: 14 - 5.

Surrey, K. (1964). Spectrophotometric method for determination of lipoxidase activity. Plant Physiol., 39: 65 - 70.

Surrey, K. (1965). Modification of the relationship between growth and metabolism in seeds by X-irradiation. Radiation Res., 25: 470 - 9.

Sweeley, C.C., Bentley, R., Makita, M., & Wells, W.W. (1963). Gas-liquid chromatography of trimethylsilyl derivatives of sugars and related substances. J. Am. Chem. Soc., 85: 2497 - 2507.

Tappel, A.L. (1962). Lipoxidase. In Methods in Enzymology, Eds. Colowick, S.P., & Kaplan, N.O. Vol. 5, p. 539 - 42. Academic Press, New York, N.Y.

Tappel, A.L. (1963). Lipoxidase. In The Enzymes, Eds. Boyer, P.D., Lardy, H., & Myrback, K. Vol. 8, p. 275 - 83. 2nd ed. Academic Press, New York, N.Y.

Tappel, A.L. (1965). Free-radical lipid peroxidation damage and its inhibition by vitamin E and selenium. Fed. Proc., 24: 73 - 8.

Tappel, A.L., Boyer, P.D., & Lundberg, W.O. (1952). The reaction mechanism of soybean lipoxidase. J. Biol. Chem., 199: 267 - 81.

Tappel, A.L., Lundberg, W.O., & Boyer, P.D. (1953). Effect of temperature and antioxidants upon the lipoxidase-catalyzed oxidation of sodium linoleate. Arch. Biochem. Biophys., 42: 293 - 304.

Tarladgis, B.G., Pearson, A.M., & Dugan, L.R. (1962). The chemistry of the 2-thiobarbituric acid test for the determination of oxidative rancidity in foods. I. Some important side reactions. J. Am. Oil Chemists' Soc., 39: 34 - 9.

Tarladgis, B.G., Pearson, A.M., & Dugan, L.R. (1964). Chemistry of the 2-thiobarbituric acid test for determination of oxidative rancidity in foods. II. Formation of the TBA-malonaldehyde complex without acid-heat treatment. J. Sci. Fd. Agr., 15: 602 - 7.

Theorell, H., Bergstrom, S., & Akeson, A. (1944). Lipoxidase enzymes in soybean. Arkiv Kemi, Mineral. Geol., A19(6), 9.

Theorell, H., Holman, R.T., & Akeson, A. (1947a). Crystalline lipoxidase. Acta Chem. Scand., 1: 571 - 6.

Theorell, H., Holman, R.T., & Akeson, A. (1947b). A note on the preparation of crystalline soybean lipoxidase. Arch. Biochem. Biophys., 14: 250 - 2.

Thiele, E., & Huff, J. (1965). Thiobarbituric reacting substance(s) produced in fractions of normal liver and tumors. Proc. Soc. Exptl. Biol. Med., 118: 689 - 92.

Tjhio, K.H., & Karel, M. (1969). Autoxidation of methyl linoleate in freeze-dried model systems. 4. Effects of metals and of histidine in the absence of water. J. Food Sci., 34: 540 - 3.

Togashi, H.J., Henick, A.S., & Koch, R.B. (1961). The oxidation of lipids in thin films. J. Food Sci., 26: 186 - 91.

Tolbert, H.E., Oeser, A., Yamazaki, R.K., Hageman, R.H., & Kisaki, T. (1969). A survey of plants for leaf peroxisomes. Plant Physiol., 44: 135 - 47.

Tookey, H.L., Wilson, R.G., Lohmar, R.L., & Dutton, H.J. (1958). Coupled oxidation of carotene and linoleate catalyzed by lipoxidase. J. Biol. Chem., 230: 65 - 72.

Toshio, A. (1952). J. Japan. Biochem. Soc., 24: 129. Cited in Tappel (1963).

Tremolieres, A., & Lepage, M. (1971). Changes in lipid composition during greening of etiolated pea seedlings. Plant Physiol., 47: 329 - 34.

Truby, F.K., O'Meara, J.P., & Shaw, T.M. (1957). Cited in Chipault, J.R. High energy irradiation. In Symposium on Foods: Lipids and Their Oxidation, Eds. Schultz, H.W., Day, E.A., & Sinnhuber, R.O. (1962), p. 151 - 69. The AVI Pub. Co., Inc., Westport, Conn.

Turner, E.W., Paynter, W.D., Montie, E.J., Bessert, M.W., Struck, G.M., & Olson, F.C. (1954). Use of the 2-thiobarbituric acid reagent to measure rancidity in frozen pork. Food Technol., 8: 326 - 30.

Uritani, I., & Yamaki, S. (1969). Mechanism of chilling injury in sweet potatoes, Part III. Biochemical mechanism of chilling injury with special reference to mitochondrial lipid components. Agr. Biol. Chem., 33: 480 - 7.

Vaintraub, I.A., & Hofman, Y.Y. (1961). N-terminal amino acids of pea legumin and vicilin. Biokhimiya, 26: 10 - 5.

Van Deenen, L.L.M., & De Haas, G.H. (1963). The substrate specificity of phospholipase A. Biochim. Biophys. Acta, 70: 538 - 53.

Van der Veen, J., Hirota, K., & Olcott, H.S. (1967). Comparison of column chromatographic methods for the quantitative determination of mono- and digalactodiglycerides in fresh alfalfa (Medicago sativa). Lipids, 2: 406 - 10.

Varner, J.E., & Schidlovsky, G. (1963). Intracellular distribution of proteins in pea cotyledons. Plant Physiol., 38: 139 - 44.

Vaskovsky, V.E., & Kostetsky, E.V. (1968). Modified spray for the detection of phospholipids on thin-layer chromatograms. J. Lipid Res., 9: 396.

Vaskovsky, V.E., & Suppes, Z.S. (1971). Detection of choline-containing lipids on thin-layer chromatograms. J. Chromatogr., 63: 455 - 6.

Veldink, G.A., Vliegenthart, J.F.G., & Boldingh, J. (1970). Proof of the enzymatic formation of 9-hydroperoxy-10-trans, 12-cis-octadecadienoic acid from linoleic acid by soya lipoxygenase. Biochim. Biophys. Acta, 202: 198 - 9.

Wagenknecht, A.C. (1957a). The lipids of green peas. J. Am. Oil Chemists' Soc., 34: 509 - 13.

Wagenknecht, A.C. (1957b). Occurence of plasmalogens in lipids of green peas. Science, 126: 1288.

Wagenknecht, A.C., & Lee, F.A. (1958). Enzyme action and off-flavor in frozen peas. Food Res., 23: 25 - 31.

Wagenknecht, A.C., Lee, F.A., & Graham, R.J. (1958). The preparation of lipase from peas and the action of steapsin and pea lipase on crude pea lipid. Food Res., 23: 439 - 45.

Wagenknecht, A.C., Lewin, L.M., & Carter, H.E. (1959). The phosphatidyl inositol of peas. J. Biol. Chem., 234: 2265 - 8.

Waite, M., Van Deenen, L.L.M., Ruigrok, T.J.C., & Elbers, P.F. (1969). Relation of mitochondrial phospholipase A activity to mitochondrial swelling. J. Lipid Res., 10: 599 - 608.

Walker, G.C. (1963). The formation of free radicals during the reaction of soy bean lipoxidase. Biochem. Biophys. Res. Commun., 13: 431 - 4.

Walter, R., & Roy, J. (1971). Selenomethionine, a potential catalytic antioxidant in biological systems. J. Org. Chem., 36: 2561 - 3.

Webster, D.E., & Chang, S.B. (1969). Polygalactolipids in spinach chloroplasts. Plant Physiol., 44: 1523 - 7.

Wehrli, H.P., & Pomeranz, Y. (1970). A note on the interaction between glycolipids and wheat flour macromolecules. Cereal Chem., 47: 160 - 6.

Whitfield, F.B., & Shipton, J. (1966). Volatile carbonyls in stored unblanched frozen peas. J. Food Sci., 31: 328 - 31.

Williams, J.P., & Merrilees, P.A. (1970). The removal of water and non-lipid contaminants from lipid extracts. Lipids, 5: 367 - 70.

Willits, C.O., Riccuiti, C., Knight, H.B., & Swern, D. (1952). Polarographic studies of oxygen-containing organic compounds. Functional groups of autoxidation products. Anal. Chem., 24: 785 - 90.

Willits, C.O., Ricuitti, C., Ogg, C.L., Morris, S.G., & Riemenschnieder, R.W. (1953). Formation of peroxides in fatty esters. I. Methyl oleate. Application of the polarographic and direct oxygen methods. J. Am. Oil Chemists' Soc., 30: 420 - 3.

Wills, E.D. (1970). Effects of irradiation on sub-cellular components. I. Lipid peroxide formation in the endoplasmic reticulum. Int. J. Radiat. Biol., 17: 217 - 28.

Wintermans, J.F.G.M. (1960). Concentrations of phosphatides and glycolipids in leaves and chloroplasts. Biochim. Biophys. Acta, 44: 49 - 54.

Wojtczak, L., & Lehninger, A.L. (1961). Formation and disappearance of an endogenous uncoupling factor during swelling and contraction of mitochondria. Biochim. Biophys. Acta, 51: 442 - 56.

Wolf, F.T., Coniglio, J.G., & Briges, R.B. (1966). The fatty acids of chloroplasts. In Biochemistry of Chloroplasts, Ed. Goodwin, T.W. p. 187 - 94. Academic Press, New York, N.Y.

Wozniak, J., & Krauze, S. (1968). Free radicals in irradiated fatty acids. Roczniki PZH, 19: 317 - 27.

Wren, J.J., & Merryfield, D.S. (1970). 'Firmly-bound' lysolecithin of wheat starch. J. Sci. Fd. Agr., 21: 254 - 7.

Yamamoto, A., Yasumoto, K., & Mitsuda, H. (1970). Isolation of lipoxygenase isozymes and comparison of their properties. Agr. Biol. Chem., 34: 1169 - 77.

Yasumoto, K., Yamamoto, A., & Mitsuda, H. (1970). Effect of phenolic antioxidants on lipoxygenase reaction. Agr. Biol. Chem., 34: 1162 - 8.

Yoshida, K., & Sato, S. (1968). Swelling and contraction of isolated plant mitochondria. I. Passive swelling in sugar and electrolyte solutions. J. Fac. Sci. Univ. Tokyo, 10: 49 - 62.

Zalkin, H., & Tappel, A.L. (1960). Studies of the mechanism of vitamin E action. IV. Lipids peroxidation in the vitamin E-deficient rabbit. Arch. Biochem. Biophys., 88: 113 - 7.

Zeevaart, A.J., Gruber, M., & Van Raalte, M.H. (1968). Oxidative phosphorylation in mitochondria from germinating peas. Acta Bot. Neerl., 17: 349 - 56.

Zimmerman, D.C. (1966). A new product of linoleic acid oxidation by a flaxseed enzyme. Biochem. Biophys. Res. Commun., 23: 398 - 402.

Zimmerman, D.C., & Vick, B.A. (1970). Specificity of flaxseed lipoxidase. Lipids, 5: 392 - 7.

Zirlin, A., & Karel, M. (1969). Oxidation effects in a freeze-dried gelatin-methyl linoleate system. J. Food Sci., 34: 160 - 4.

APPENDIX

Copper:

The hollow cathode lamp specification.

Fill gas	neon
Window	quartz
Operating current	3 mA
Strike & operating voltages	280 & 200 V
The line used	3247.5 Å
Spectral band width	1.7 Å
Sensitivity	0.04 ppm
Burner gas	oxygen-acetylene

Calibration Curve

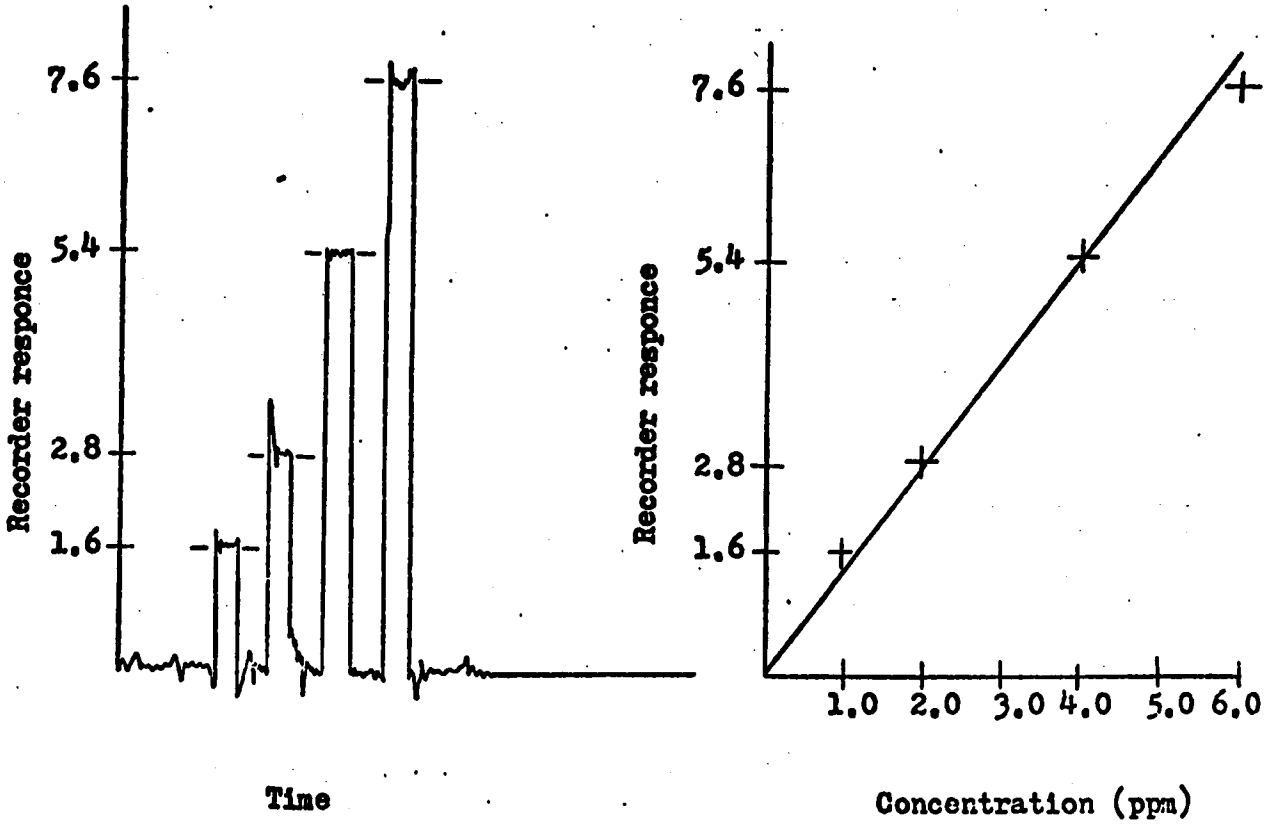


Fig. A1. Hollow Cathod Lamp Specification and Calibration Curve for Copper as Determined by Atomic Absorption Spectrophotometry. Similar calibration curves were obtained for the other elements analyzed.

Calcium:

The hollow cathode lamp specification.

Fill gas	neon
Window	quartz
Operating current	4 mA
Strike voltage	260 V
Operating voltage	150 V
The line used	4226.7°A
Spectral band width	3.34 A
Sensitivity	0.03 ppm
Burner gas	oxygen-acetylene

Calibration Curve

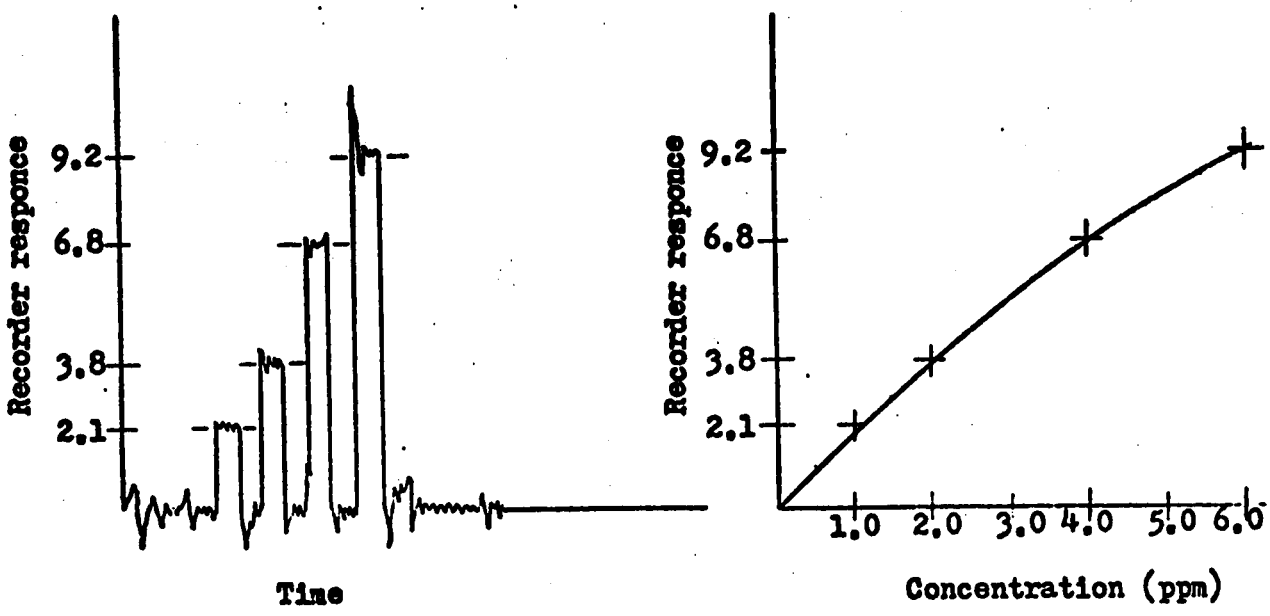


Fig. A2. Hollow Cathod Lamp Specification and Calibration Curve for Calcium as Determined by Atomic Absorption Spectrophotometry. Similar calibration curves were obtained for the other elements analyzed.