STUDIES ON THE
POLYMERIZATION OF
PHYTIC ACID

By

Jagan Nath Ahuja

AN ABSTRACT OF A THESIS

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

Department of Chemistry

1971

ABSTRACT

STUDIES ON THE BIOSYNTHESIS OF PHYTIC ACID

by Jagjit Singh Maloja

The biosynthesis of phytic acid has been studied by following the incorporation of carbon-14 from myo-inositol-U-C^{14} into maturing pea pods. Solutions of labelled myo-inositol were injected into the interior spaces of immature pea pods, and the pods were allowed to develop under various conditions of time and light and dark periods.

After excision from the plant, the pods were homogenized and extracted with 80% ethanol and then with dichloroethylene; the residue was further extracted with acetic acid or trichloroacetic acid and the phytic acid precipitated with barium.

The five fractions (ethanol, "lipid", "barium soluble", phytate and "residue") were counted at various times after injection of C^{14}-inositol. In most of the experiments, the lipid fraction was rapidly labelled and then lost its label rather rapidly. The increase in counts in barium phytate was very slow but steady over the maximum 16-day study period. The relative lack of labelling in the final residue probably reflects the relative metabolic inactivity of myo-inositol.

The ethanol fraction was further separated to show inositol, glucose, sucrose, xylose, glycerol and an organic phosphate ester as components.

The phosphate ester was found to be rapidly and highly labelled, in contrast to the low and essentially constant level of C^{14} in the sugars. The phosphate ester was not completely characterized, but gave an vigorous

and hydrolysis glucose, inositol and inorganic phosphate.

Some of the results may be summarized as follows:

1) The injected C^{14}-inositol appears in the barium phytate fraction only after 24 hours and further this incorporation increases with time.

2) The labelling of the lipid fraction reached a maximum at the end of the first 24 hours and then starts to decrease, with considerable loss of labelling being observed between one to four days.

3) Slow and constant incorporation of C^{14}-inositol into the supernatant solution remaining after the precipitation of barium phytate occurs in the period up to four days after injection, followed by a sudden loss of label between four and eight days and then a more gradual decrease.

4) The total recovered label in the 80% ethanol fraction decreases with time, reflecting losses from inositol and from the phosphate ester compound.

STUDIES ON THE BIOSYNTHESIS OF PHYTIC ACID

By

Jagan Nath Ahuja

AN ABSTRACT OF A THESIS

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

Department of Chemistry

1971

ACKNOWLEDGMENTS

The author expresses his deep appreciation to Dr. Carlon L. Kilgour, not only for his patient guidance and thoughtful criticism, but also for the encouragement received during the course of this study.

Thanks are also due to the National Institute of Health for providing funds in support of this work.

Born, November 12, 1935, Rawalpindi (West Pakistan)

Undergraduate studies:

Delhi university, Delhi (India), 1950-1952
Agra university, Agra (India), 1952-1954
(B.Sc., 1954, Science).

Graduate studies:

Agra university, Agra (India), 1954-1957
(M.Sc., 1956, Zoology)
Michigan State University, 1959-1961.

Research Scholar, Department of Zoology, Government College, Ajmer (India), 1956-1957.

Special Graduate Research Assistant, Department of Chemistry and Biochemistry, Michigan State University, 1959-1961.

Member of the Sigma Xi.

To My Parents

(Mr. and Mrs. C.D. Araujo)

TABLE OF CONTENTS

	Page
INTRODUCTION	1
EXPERIMENTAL	10
I. Changes in orthophosphate and phytin content in growing pea plants	
Germination of the pea seeds	10
Preparation of the samples for analysis	10
Determination of total phosphate	11
Determination of phytin phosphorus	15
II. Feeding experiments	
Preparation of myo-inositol-U-C^{14}	17
Feeding experiments with myo-inositol-U-C^{14}	21
III. Isolation and partial characterization of labelled sugars, lipids and phosphorylated inositols from pea pods fed C^{14}-inositol	
Isolation and identification of components of 80% ethanol fraction	30
Isolation of the lipid fraction	34
Hydrolysis of the lipid fraction	34
Extraction of phytic acid	37
IV. Chromatographic separations	
Column chromatography	40
Paper chromatography	42
RESULTS AND DISCUSSION	59
LITERATURE CITED	76
APPENDIX	79

LIST OF TABLES

Table — Page

1. The changes in orthophosphate in relation to phytic formation in developing pea plants 10

2. Incorporation of C^{14} from inositol injected into maturing pea pods (Preliminary studies) 93

3. Recovery of labelled carbons of inositol-U-C^{14} seven and ten days after injection into maturing pea pods (Experiment I and II) 95

4. Recovery of labelled carbons of inositol-U-C^{14} at various times after injection into maturing pea pods (Experiment III to VII) 97

5. (a) Effect of dark period on the incorporation of C^{14}-inositol in the maturing pea pods. (Experiment VIII) . . 98

   (b) Effect of dark period on the incorporation of C^{14}-inositol in the maturing pea pods (Experiment IX) 99

6. (a) Fractionation of 80% ethanol extract obtained from pea pods fed C^{14}-inositol (Experiments I, VIII and IX). . 32

   (b) Fractionation of 80% ethanol extract obtained from pea pods fed C^{14}-inositol (Experiments I, VIII and IX). . 33

LIST OF FIGURES

Figure			Page
I	(a)	Changes in "bound" inositol during growth of peas	14
	(b)	Changes in phytin and total phosphorus during growth of pea plants	15
	(c)	Changes in phytin phosphorus during growth of pea plants	16
II	(a)	Chromatogram of the organic phosphate ester hydrolysate in the solvent system methanol: formic acid: water (30:15:5)	33
	(b)	Chromatogram of one organic phosphate ester in hydrolysate in the solvent system methanol: formic acid: water (30:15:5)	36
III	(a)	Chromatogram showing separation of C^{14}-inositol from other components of 80% ethanol extract in the solvent system n-butanol: ethanol: water (10:1:2)	43
	(b)	Chromatogram of 80% ethanol extract showing resolution of C^{14}-inositol and other components in the solvent system n-butanol: pyridine: water (3:2:1)	44
	(c)	Chromatogram showing separation of labelled inositol from the rest of the labelled sugar components of the 80% ethanol extract in the solvent system pyridine: amyl alcohol: water (7:7:3)	45
	(d)	Chromatogram showing separation of C^{14}-inositol from other components of the 80% ethanol extract in the solvent system propanol: ammonia: water (7:3:1)	46
IV	A picture of a chromatogram showing separation of components of the 80% ethanol extract of Experiment VIII in the solvent system pyridine: amyl alcohol: water (7:7:3)		49
V	(a)	Chromatogram of lipid hydrolysate in the solvent system isopropanol: acetic acid: water (9:1:1) . . .	51

Page

(b) Chromatogram of lipid hydrolysate in the solvent system pyridine: amyl alcohol: water (7:7:6) 52

VI (a) Chromatogram of the isolated phytic acid from mature peas in one solvent system t-butanol: HCl: water (30:4 ml: 20) 54

(b) Chromatogram showing separation of phytic acid isolated from the peas in the solvent system n-propanol: ammonia: water (5:4:1) 55

(c) Chromatogram showing separation of phytic acid of mature peas in the solvent system isobutyric: ammonia (10:6) 56

(d) Chromatogram of phytic acid of peas in the solvent system t-butanol: formic acid: water (6:1:3) 57

(e) Chromatogram of phytic acid isolated from mature peas in the solvent system isopropanol: ammonia: water (7:1:2) 58

VII Incorporation of C^{14}-inositol into the lipid and the 80% ethanol fractions in the maturing pea pods at various time intervals 60

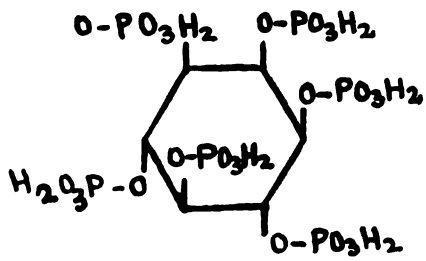
VIII Incorporation of label into components of the 80% ethanol extract obtained from pods fed C^{14}-inositol (Experiment VIII). 68

IX Distribution of label at various times after injection of C^{14}-inositol into maturing pea pods (Experiment VIII) . 70

X Incorporation of C^{14}-inositol into the barium phytate and barium supernate fractions at various time intervals in the maturing pea pods 72

INTRODUCTION

Phytic acid is a hexaphosphate derivative of myo-inositol, one of the water soluble vitamins. Phytic acid is probably the only biologically-produced molecule containing six phosphate ester groups, and certainly the only known compound having six such groups on adjacent carbon atoms. On the basis of the rather limited evidence available, the structure shown below is the one most generally accepted for the compound:



Phytic acid

Phytic acid is universally present in the plant kingdom in the form of a mixed calcium-magnesium salt called "phytin". Phytin was first described by Palladin (1) and later was shown to be an inositol compound by Winterstein (2) after its extraction from seeds of Indian mustard (*Sinapsis nigra*) by acetic acid and precipitation by ammonia.

Phytic acid has assumed considerable importance in nutritional studies, especially because of its effect on the availability of metals such as calcium, magnesium, iron and zinc, and also in commerce, where it is used for the removal of iron from wines (3) and copper from brandies (4).

Phytic acid has been shown to have an inhibitory effect on the peptic digestion of ovalbumin and elastin (5); this effect is believed to be related to its ability to form insoluble combinations with proteins below their isoelectric points i.e. at a pH which corresponds to the optimum for the action of pepsin.

Metal ions, especially calcium and magnesium, are bound very strongly by phytic acid. The high concentration of phytic acid in cereals renders calcium unavailable and is thereby one of the causes of rickets in infants. This is certainly of great concern and importance in parts of the world where unrefined cereals form a considerable portion of the daily diet. Mellanby and Harrison (6) gave evidence that the rachitogenic action of certain cereals containing phytic acid is normally due to the inhibition of calcium absorption from the alimentary canal and not due to the unavailability of phytic acid phosphorus and further that the rachitogenic action of cereals can be overcome by adding extra calcium to the diet. Eddy et.al. (7) found that when phytate replaced potassium phosphate in a non rachitogenic or slightly rachitogenic diet, rats developed typical rachitic symptoms; phytate phosphorus failed to give protection even when its use doubled the amount of dietary P. The appearance of rachitic symptoms could be explained on the basis of the fact that the metal ions such as calcium and magnesium present in the diet are bound strongly by the phytic acid molecule, making them unavailable. The appearance of rickets due to calcium deficiency has been confirmed by Mellanby and Harrison (6). Further, bound phytic acid is not readily hydrolyzed to supply inorganic phosphate and the resulting phosphate deficiency could be an allied factor contributing to the appearance of rickets.

Roberts and Yudkin (8) have shown that phytic acid is responsible for magnesium deficiency in albino rats fed 1-1½ sodium phytate in their diets. The rats fed sodium phytate in their diets became severely ill and addition of calcium or inositol to the diets brought about no improvement, hence eliminating calcium or inositol deficiency as reasons for the illness. Addition of magnesium to the diets resulted in improvement

in the condition of the rats. These studies stress the importance of the binding of metal ions by phytic acid in animal nutrition.

Sholl (10) has stated that phytin was readily soluble in gastric juice and was absorbed without hydrolysis, but had to be split in order that the bound calcium be released and utilized in bone formation.

Nobuji Onishi (11) described the anticalcification effect of rice. These studies of anticalcification effect were carried out using rats which were fed brown or polished rice. The anticalcification appeared to involve the precipitation of calcium by phytic acid. The addition of 0.6-1 % calcium carbonate to the brown rice diet produced better calcification than did the polished rice diet, but on the other hand the brown rice diet gave poorer calcification in the absence of the exogeneous source of calcium carbonate. This clearly shows that the anticalcification effect of phytate can be counteracted by suitable addition of calcium, to overcome the calcium deficiency due to precipitation by phytic acid.

Little is known about the role of phytic acid in plants, where it is found widely distributed and in considerable concentrations. Cleldelin and Lamb (12) concluded from their studies that the phytates in plants act as a store of phosphorus which may be utilized by the plants during sprouting. This opinion was further supported by the fact that the concentration of free inositol increases in several different type of seeds during germination.

Neilands (13) has suggested that phytates serve as carriers for trace metals needed to insure normal plant growth.

The biosynthesis of phytic acid still remains unexplored and in fact no direct approaches have been made to the elucidation of this problem.

Most of the work in this general area has been confined either to the determination of total phytin content or to degradative studies on the phytic acid molecule. A number of studies have however, been concerned with the inositol phosphatides. Their isolation and characterization in various plant species has been extensively worked out.

Anderson (14,15) and Wensel (16) carried out determinations of phosphoric acid esters of inositol in wheat bran and potato leaves respectively. Further studies on phytate phosphorus determinations in various plant products such as corn, oats and certain other cereals were performed by Schormuller and Hurlig (17), Rather (18) and Ashton and Williams (19).

The studies on amounts of phytate phosphorus in the leguminous plants, especially the pea, which is of particular concern in the present work, were done by Holt (20), Fowler (21) and Bogoroditskaya (22).

The problem of biosynthesis of phytic acid is an intriguing one for a number of reasons; prominent among them is the unique position of phytic acid as the most highly phosphorylated compound known in nature. It is possible to conceive of several biosynthetic pathways for formation of the phytic acid and the other inositol polyphosphates.

The most obvious scheme would involve a stepwise phosphorylation of myo-inositol in the presence of a series of inositol and phosphorylatol-inositol kinases. One would then expect the mono-, di-and triphosphates of inositol to function as intermediates in the biosynthesis of phytic acid.

In spite of a number of attempts in different laboratories no one has been able to find any reproducible "inositol kinase" activity, i.e.

$$\text{Inositol} + \text{ATP} \longrightarrow \text{inositol-monophosphate} + \text{ADP}.$$

Hoffmann-Ostenhoff et.al. (23) have reported finding some evidence for the enzymatic phosphorylation of myo-inositol using a crude hexokinase preparation from yeast.

Hübscher and Hawthorne (14,15) reported that crude rat liver extracts were capable of catalyzing the formation of inositol monophosphate from ATP (adenosine triphosphate) and inositol. These are the only references in the available literature concerning the direct enzymatic synthesis of inositol monophosphate.

Since work in other laboratories has failed to corroborate either of these findings, it is quite possible that inositol monophosphate is not formed by a kinase reaction but is in actuality an artifact arising from the hydrolysis of either inositol phosphatides or other phosphorylated inositols.

It should be mentioned here that inositol monophosphate, along with various inositol polyphosphates, is also formed by the enzymatic hydrolysis of phytin by the "phytases" present in some plants, but this is a degradative pathway rather than a biosynthetic pathway. Studies on the hydrolytic products of phytic acid have been carried out by Anderson (6), Fleury and Courtois (7), McCormick and Carter (18) and Desjobert and Fleurent (9).

Another possible biosynthetic route would be one in which inositol monophosphate is formed in the system by the hydrolytic cleavage of phosphomonoinositide and this inositol monophosphate is incorporated into inositol polyphosphates. This would appear to be a good possibility, since free inositol can be incorporated enzymatically into inositol phosphatide and further this would help explain the formation of inositol monophosphate in the absence of inositol kinases.

A number of investigators have been actively engaged in the isolation, characterization and biosynthesis of phosphoinositides.

A soybean phosphoinositide has been prepared and partially characterized by Hooley (30), Folch (31), Carter et. al. (32), Hawthorne and Chargaff (33) and Schofield and Dutton (34).

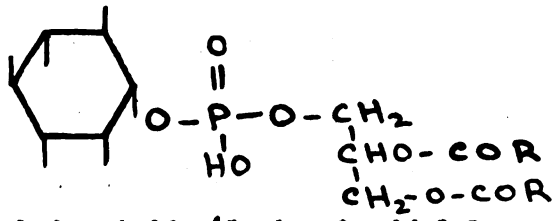
Phosphoinositides have been isolated from cotton seed by Olcott (35), wheat germ by Morelec-Coulon and Faure (37), rat liver by Macpherson and Lucas (37), horse and dog liver by McMibbin (38). A diphosphoinositide from brain was reported by Folch (39) and a triphosphoinositide from beef brain by Tomlinson and Ballou (40) and Brockerhoff and Ballou (41).

Wagenknecht et.al. (42) obtained a phosphoinositide from lyophilized frozen peas and reported that the compound is a monophosphoinositide and probably the only phosphoinositide present in the peas.

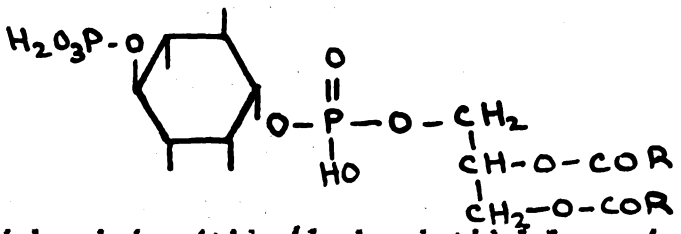
The structure of monophosphoinositide has been reported by Wagenknecht et.al. (42). Tomlinson and Ballou (40) and Brockerhoff and Ballou (41) also carried out structural studies on the phosphoinositide complex of beef brain. Their studies have confirmed the presence of phosphomonoinositide in beef brain along with di- and triphosphoinositides, and their proposed structure for the monophosphoinositide agrees with the one worked out by Wagenknecht et.al. (42).

Tomlinson and Ballou (40) and Brockerhoff and Ballou (41) have also attempted to elucidate the structure of di-, and triphosphoinositides of the beef brain, mainly after deacylation, since deacylation leads to products which are more amenable to separation and structural analysis than are the intact lipids. The lipids were deacylated with hydroxylamine and the resulting mixture of deacylated phosphoinositides was separated by ion exchange chromatography.

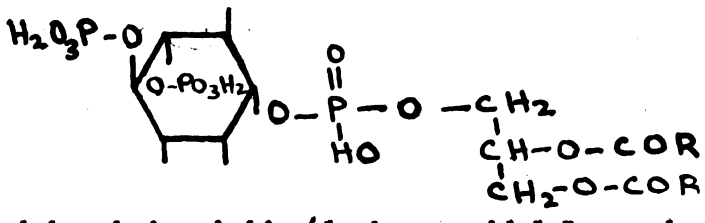
These deacylation studies indicated the presence of glycerol myo-inositol phosphate, glycerol myo-inositol diphosphate, as well as glycerol myo-inositol triphosphate. Further these studies also indicate that the three compounds are inter-related in such a way as to support a stepwise phosphorylation of monophosphoinositide in the biosynthesis of di- and triphosphoinositide. The structure of the three phosphoinositides are shown below:



Monophosphoinositide (1-phosphatidyl-L-myo-inositol)



Diphosphoinositide (1-phosphatidyl-L-myo-inositol 4-phosphate)



Triphosphoinositide (1-phosphatidyl-L-myo-inositol 4,5-diphosphate)

The enzymatic synthesis of inositol monophosphatide has been described by Agranoff et al. (43), Thompson et al. (44) and Hokin and Kennedy (45,46).

An enzymatic reaction in which incorporation of free inositol into inositol monophosphatide is stimulated by the addition of a number of cytidine nucleotides, including cytidine-5' -monophosphate and cytidine

diphosphate choline was first observed by Agranoff *et al.* (1) and later confirmed and clarified by Thompson *et al.* (14) and Paulus and Kennedy (15,16). To explain the effect of CDP-choline a mechanism was postulated by Agranoff *et al.* (1) as follows:-

L-α-glycerophosphate + 2 RCO-SCoA ⟶ phosphatidic acid

phosphatidic acid + CDP-choline ⟶ CDP-diglyceride + P-choline

CDP-diglyceride + inositol ⟶ inositol phosphatide + CMP

Recently Paulus and Kennedy (17) offered evidence that the enzymic synthesis of inositol monophosphatide in liver preparations proceeds through the following reaction scheme:-

L-α-glycerophosphate + 2 RCO-SCoA ⟶ phosphatidic acid

phosphatidic acid + CTP ⟶ CDP-diglyceride + PP_i

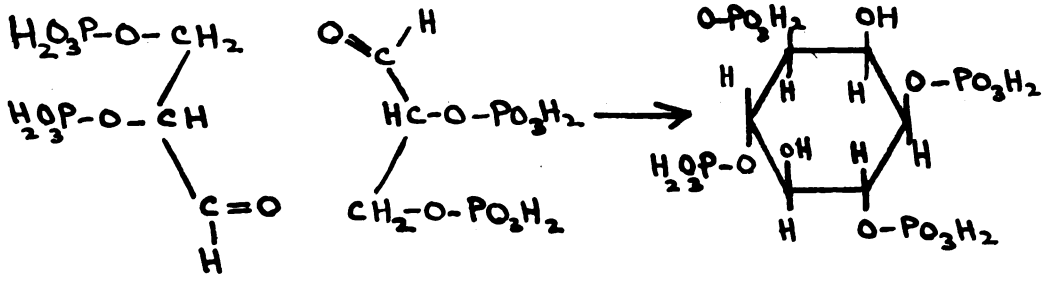
CDP-diglyceride + inositol ⟶ inositol monophosphatide + CMP

These observations indicate the incorporation of free inositol into monophosphatide without the prior formation of inositol monophosphate.

The presence of diphosphoinositide and triphosphoinositide in the brain indicates that it is possible to have more than one phosphate group attached to inositol while it is still in the form of an inositide. It is not known whether this inositol is split from the di- or triphosphoinositide in the form of a monophosphate or a polyphosphate (di or tri). If it is split as a polyphosphate, it would be well along the path towards the biosynthesis of phytic acid.

In any consideration of possible pathways for biosynthesis of the inositol polyphosphates, however, sequences in which free inositol as such is not involved must be included. One such pathway could involve the direct condensation of a compound such as glyceraldehyde 3-phosphate to yield a poly-phosphorylated inositol (in this case, an inositol

tetraphosphate):-



While any suggestion of a direct connection would be purely speculation, it is interesting to note that phytic acid is found to occur in about the same range of concentration in nucleated erythrocytes as 2,3-diphospho-glyceric acid does in non-nucleated erythrocytes.

The decision to study first the incorporation of C^{14}-inositol into phytic acid was made on two bases.

First, inositol does not appear to be rapidly destroyed in plant tissues, permitting a good recovery of labelled carbon. Secondly, it would yield some information on whether inositol is in fact incorporated into phytic acid as an intact molecule. In this case, also, it would help pin-point intermediates in the biosynthetic pathway.

The choice of developing pea pods as a biosynthetic system followed from the work of Richardson and Tolbert (51), who studied inositol and phytin concentrations in a number of plants at various stages of development. Peas were shown to have a relatively high content of free and "bound" inositol at all times and to produce large quantities of phytin during seed formation.

EXPERIMENTAL

1) Changes in orthophosphate and phytin content in growing pea plants.

Germination of the pea seeds:- The pea seeds used in these experiments were of the dwarf variety "Little Marvel" and were obtained from the U. Atlee Burpee company. Before planting, the seeds were washed with distilled water two or three times and then soaked in water overnight. The seeds were then planted in 4 inch deep wooden flats in Vermiculite (commercially available heat-expanded mica). Five to seven days were required for production of edible sprouts, following which the seeds were watered every second day with a mineral solution (1 gm. $Ca(NO_3)_2 \cdot 4H_2O$, 0.25 gm. KH_2PO_4, 0.25 gm. K_2SO_4, 0.25 gm. $MgSO_4 \cdot 7H_2O$ in one liter of water), and were left to grow in a Sherer growth chamber with daytime temperature 75°F and night temperature 70°F. Light was maintained for 12 hours each day at half intensity.

Preparation of the samples for analysis:- The seedlings from the flats were removed at the desired time intervals, the unused seed portions removed and discarded and the plants washed with distilled water. After washing, the plants were dried at room temperature and then cut into small pieces in a 400 ml. beaker; 300 ml. of hot 95% ethanol was poured into the beaker and the plant material boiled for 15 minutes on a hot plate. The ethanol was then decanted off and the plant material dried in a forced-air oven at 75°C. This material was then stored for later analysis of total phosphorus and phytin phosphorus content. The method for the preparation of samples for analysis in case of mature pea seeds is somewhat different than the one described above. In this case the seeds were soaked overnight in water and in the morning were broken into small pieces in a mortar

boiled in 95% ethanol, dried and stored under ethanol for future analysis.

Determination of total phosphorus :- The ethanol-preserved samples were dried in the oven at 55°C for about 12 hours and 0.3 gm. of the dried sample was taken in a 100 ml. beaker. Duplicate samples were taken in all the determinations carried out.

The samples were oxidized by a nitric-perchloric acid method as follows:- to each sample was added 10 ml. of concentrated nitric acid and the mixture was then stirred thoroughly for about 15 minutes with a glass rod. The glass rods were removed and washed with a minimum quantity of distilled water. About 15 minutes was enough for the nitric acid to oxidize the easily oxidizable portion of the sample. Ten ml. of distilled water was then added to each sample slowly from the sides of the beaker so as to form a separate layer beneath the acid sample mixture. The addition of distilled water was followed by a slow addition of 10 ml. of 70% perchloric acid in the same manner and the beakers were then covered with watch glasses and boiled gently on a hot plate until all organic matter was destroyed (about one hour was required). The samples were allowed to cool and the material from the watch glasses was washed into the beakers with distilled water. The beakers were then returned to the hot plate once again and the contents evaporated to dryness. Following this oxidation, the residue in the beaker was taken up in 25 ml. of 5% nitric acid and heated on a steam bath for about half an hour. This was done in order to achieve the conversion of metaphosphoric acid to orthophosphoric acid. The samples were cooled once again and then treated separately for the colorimetric determination of total phosphate.

One ml. aliquots of the sample were placed in 50 ml. graduated cylinders. To each sample was added two drops of acid sol phenol indicator. The solution was then neutralized by the dropwise addition of 10% sodium

ammonium hydroxide solution 1:1 (v/v) and the addition of excess hydrochloric continued until the color of the solution changed to pink. The sample was then diluted to 30 ml. with distilled water. To this diluted solution was added 10 ml. ammonium molybdate- hydrochloric acid reagent followed, after thorough mixing, by another 5 ml. of dilute stannous chloride solution (details as to the preparation of these reagents appear in the Appendix). After allowing five minutes for the color to develop, the absorbance was read at 500 mμ in a Coleman spectrophotometer.

The above procedure was followed with mature seeds, 5, 10, 15, 20, 25 and 30 day old plants. The results of these experiments appear in Table I and in Figure I.

TABLE I

The changes in orthophosphate in relation to phytin formation in developing pea plants.

Time interval	Phosphorus per gram of plant material.		Phytin phosphorus as percentage of total phosphorus.
	Total (Mg./gm)	Phytin (Mg./gm)	
Mature seeds	5.91	1.15	20.1%
5-day old plants	1.50	0.[illegible]	7.7%
10-day old plants	[illegible]	[illegible]	[illegible]
15-day old plants	[illegible]	[illegible]	[illegible]
20-day old plants	[illegible]	[illegible]	[illegible]
25-day old plants	[illegible]	[illegible]	[illegible]
30-day old plants	[illegible]	[illegible]	[illegible]

Determination of the phytin phosphorus:- These determinations were also carried out on mature seeds, 8, 12, 16, 20, 24 and 28 day old plants.

The ethanol- preserved samples were dried in the forced-air oven at 55°C for 12 hours. Two grams of the sample was taken and extracted with 80% ethanol in a Soxhlet extractor to remove free sugars. The sugar-free sample was then extracted by shaking for three hours with 1.2% hydrochloric acid containing 10% anhydrous sodium sulphate. The suspension was then filtered and the residue discarded. A 25 ml. sample of the above filtrate was diluted with 25 ml. of distilled water and to this solution was added 15 ml. of a 0.2% ferric chloride solution in 0.6% hydrochloric acid with slow stirring. The solution was allowed to stand overnight in order to allow the complete precipitation of ferric phytate. The precipitate was filtered off using a Gooch crucible, washed 3-4 times with 0.6% hydrochloric acid-sodium sulphate solution, to remove traces of non-phytin phosphorus compounds. The precipitate in the Gooch crucible was moistened with 4 drops of 50% magnesium nitrate solution and dried in the oven at 100°C for half an hour, after which it was ignited overnight in the electric furnance at 950°C. After cooling, the precipitate was dissolved in 10 ml. of concentrated hydrochloric acid, diluted, filtered into a 200 ml. volumetric flask and made up to volume with distilled water.

For the colorimetric determinations of phytic acid phosphorus, 10 ml. aliquots of the above solution were taken and the procedure followed was the same as described for the total phosphorus determinations.

The results of these experiments are also shown in Table I and Figure I.

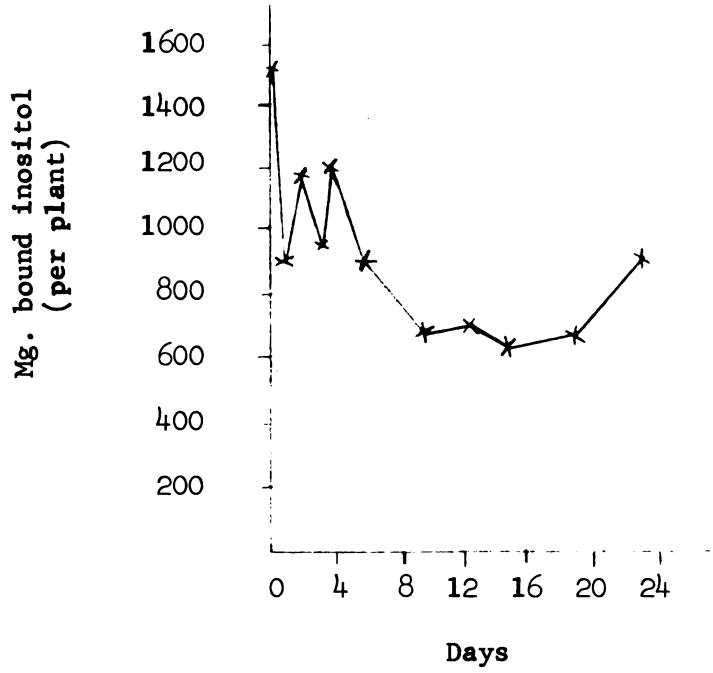


Figure 1 (a):- Changes in bound inositol during growth of peas.

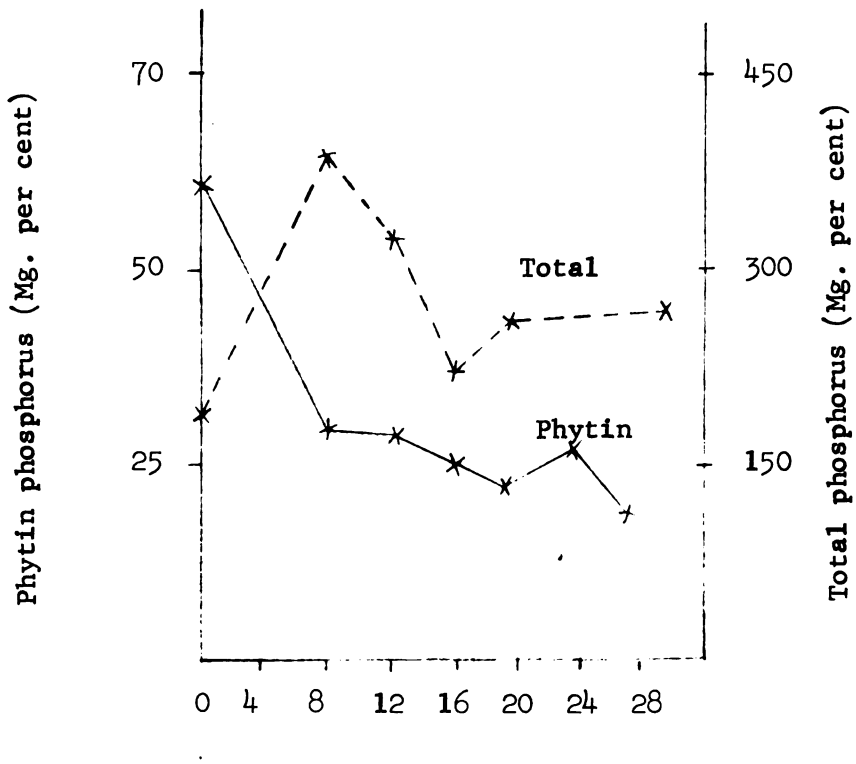


Figure 1 (b):- Changes in phytin and total phosphorus during growth of pea plants.

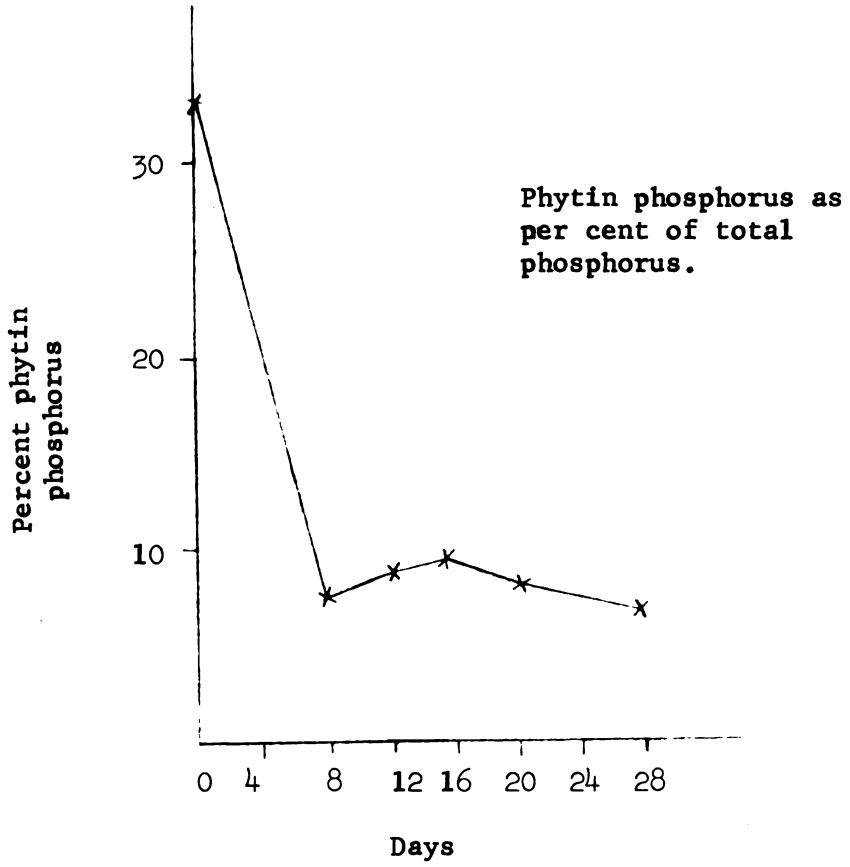


Figure 1 (c):- Changes in phytin phosphorus during growth of pea plants.

2) Feeding experiments.

(A) Preparation of myo-inositol-U-C^{14}:- The preparation of of myo-inositol-U-C^{14} was achieved by feeding $C^{14}O_2$ to pea pods or plants for varying lengths of time, after which they were sacrificed and the C^{14}-inositol isolated and purified by paper chromatography. In these studies both the maturing pea pods and young pea plants were used for the preparation of C^{14}-inositol. Typical procedures followed are described below:-

a) This method involved the feeding of $C^{14}O_2$ to pea plants which were approaching maturity. In a typical case two pea plants about 40-day old were removed from the flats in the growth chamber, where they were growing. Each of these plants was flowering and each had one 4 cm long pod attached to it, in which the seeds were just beginning to form. The plants were removed from the flats along with sufficient vermiculite to protect the roots. The vermiculite was held in place by two layers of cheese cloth. The plants were kept in a stoppered jar which was connected at one end to a filter flask in which a total of 0.5 millicuries of $C^{14}O_2$ was generated, and at the other end to a tube containing sodium hydroxide for trapping excess $C^{14}O_2$. The plants were kept under light only during the day and were supplied with $C^{14}O_2$ as follows:-

1st day- the plants were fed $C^{14}O_2$ for five hours during the day and the feeding of $C^{14}O_2$ was stopped in the evening.

2nd day- the $C^{14}O_2$ feeding was started again in the morning and continued for 12 hours after which it was stopped again.

3rd day- the plants were supplied $C^{14}O_2$ only for six hours. At this point the plants were sacrificed and C^{14}-inositol isolated.

b) This case involves the feeding of $C^{14}O_2$ to enclosed pods from 40-day old plants in a Thunberg tube. In one study two pea pods, each 5 cm long and

containing six immature peas, were used. The pods were removed from the plants in such a way that a short portion of the stem was still attached to them. These pods were placed in a Thunberg tube with the stem portion dipping in water in the bottom of the tube. The Thunberg tube was then evacuated and connected to another Thunberg tube containing 5N sulfuric acid and 0.1 millicurie of $Na_2C^{14}O_3$ in the side arm. The $C^{14}O_2$ was generated by mixing the contents of this tube, and the pod-containing tube was opened and the system allowed to attain equilibrium. The pods were fed $C^{14}O_2$ for 70 hours, after which they were sacrificed and C^{14}-inositol extracted.

c) This experiment is a modification of the one described above. Here six pea pods (from 40-day old plants) were placed in two different Thunberg tubes, with the stem portion of the pods immersed in water at the bottom of these tubes. Both tubes were evacuated and then connected to a third Thunberg tube by means of a T-joint. This third Thunberg tube contained 0.5 millicuries of $Na_2C^{14}O_3$ and had excess 5N sulfuric acid in the side arm. The $C^{14}O_2$ was generated by pouring the acid in the side arm into the tube, and the Thunberg tubes containing the pods were opened and the $C^{14}O_2$ allowed in. After a feeding period of 5 days one of the Thunberg tubes was disconnected and the pods were sacrificed and worked up for the isolation of C^{14}-inositol.

The pods in the second tube were fed $C^{14}O_2$ for eight days, after which time they were also sacrificed and C^{14}-inositol isolated.

d) In this procedure 40-day old pea plants and pods were fed $C^{14}O_2$ for eight days in a vacuum dessicator. Here the apparatus used for generating $C^{14}O_2$ was somewhat different from the one already described; also, lactic acid and $BaC^{14}O_3$ were used for generating $C^{14}O_2$ instead of sulfuric acid and $Na_2C^{14}O_3$. A separatory funnel was connected to the evacuated dessicator

containing the plants and the pods. This separatory funnel contained 5 ml. of lactic acid. In a small beaker in the dessicator was placed a suspension of $BaC^{14}O_3$. The lactic acid from the separatory funnel was allowed to drip into the beaker containing $BaC^{14}O_3$ and evolve $C^{14}O_2$, which was taken up by the plants. The pods and the plants were sacrificed after eight days and used for the isolation of C^{14}-inositol.

e) In this case, young pea pods were used for the preparation of C^{14}-inositol. In one experiment three immature pea pods (about 4-5 cm. long from 40-day old plants) with a small section of stem still attached, were placed in a Thunberg tube in such a way that the ends of the stem were immersed in water at the bottom of the tube. The tube was then evacuated and connected to another Thunberg tube in which was placed 105 microcuries of $BaC^{14}O_3$, with lactic acid in the side arm. The $C^{14}O_2$ was released by mixing the contents of this tube, the evacuated pod-containing tube was then opened and the system permitted to come to pressure equilibrium. The pod-containing tube was then closed, CO_2-free air was permitted to enter the generator tube, and the two tubes were reconnected and opened to draw more $C^{14}O_2$ into the pod tube. This whole process was repeated five times, conveying most of the available $C^{14}O_2$ into the pod tube. After three days the pods were removed from the tube, and worked up for the isolation of C^{14}-inositol.

f) In this experiment pea plants 12-16 days old were used for the preparation of labelled inositol. In a typical experiment 10 plants were removed from the flats in the growth chamber, where they were growing. The vermiculite from the roots was removed and the roots rinsed with distilled water. The roots of each plant were then placed in a 125 ml. erlenmeyer

flask containing about 50 ml. of a nutrient solution**. The flasks were placed in a dessicator and the dessicator evacuated. The evacuated dessicator was connected to a Thunberg tube, in which had been placed 1.0 millicurie of $BaC^{14}O_3$, with lactic acid in the side arm. The $C^{14}O_2$ was generated by mixing the contents of the Thunberg tube and the evacuated dessicator containing the plants was opened and the available $C^{14}O_2$ swept out of the Thunberg as indicated in (e) above. The dessicator was then kept under constant fluorescent and incandescent light for 10 days, after which the plants were sacrificed and C^{14}-inositol was extracted.

Isolation of C^{14}-inositol:- The plants or the pods fed $C^{14}O_2$ were cut into small pieces and homogenized in 80% ethanol in a Virtis homogenizer. The homogenate was extracted with 80% ethanol by refluxing in a Soxhlet extractor for 24 hours. The 80% ethanol extract was evaporated to near dryness under vacuum, redissolved in water, decolorized with charcoal and filtered. The filtrate was deionized with Amberlite IR 120 (H) and IRA 400 (OH) resins. The solution was then evaporated to a small volume in a stream of air under a spotlight and chromatographed. The C^{14}-inositol was purified by paper chromatography.

Five different solvent systems were used, the details of which appear in the section on paper chromatography.

The purification of the C^{14}-inositol samples was followed by running radioautograms.

Typical radioautograms were run by spreading about 500 microliter of

---

** The standard nutrient solution contains 1 gm. $Ca(NO_3)_2 \cdot 4H_2O$, 0.75 gm. KNO_3, 0.25 gm. $NH_4H_2PO_4$, 0.25 gm. $MgSO_4 \cdot 7H_2O$ in one liter of water; this solution was diluted 1:5 in this case.

the labelled samples in the form of a band on Whatmann 3 mm paper and using pyridine (7): amyl alcohol (7): water (6) solvent system. After 24 hours the papers were air dried and then exposed to X-ray films for 2-3 days, after which the films were developed. The pure inositol samples appear as one clear band, whereas the presence of labelled impurities in the C^{14}-inositol samples was indicated by the appearance of more than one band on the X-ray film.

(B) Feeding experiments with myo-inositol-U-C^{14}:- A number of experiments were performed in which radioactive inositol was fed to developing pea pods. Two different types of feeding experiments were carried out:

1) Feeding C^{14}-inositol through the stems of excised pods:- In this case the pods were removed from the plants with about one inch of the stem still attached to them. These pods were placed, stem down, in beakers containing a C^{14}-inositol solution.

In a typical example, three pods of about 4-5 cm length were removed from 40-day-old plants and placed in a small beaker in such a manner that the stem was immersed in 5 ml. of water containing the radioactive inositol. The beaker with the pods was then placed in a large dessicator containing a tray of water to prevent excessive evaporation from the pod-containing beaker. The beaker contained an amount of inositol equal to 6×10^5 counts per minute. After three days the pods were found infected with some kind of mould or fungus growth. Some of the less infected portions were homogenized with 5% hydrochloric acid, plated on a planchet and counted, but showed negligible incorporation of label.

The same experiment was repeated in the open air, where water had to be added from time to time to replace that which evaporated out of the inositol solutions. The pod-containing beaker had inositol equal to

2.2 x 10^4 counts per minute. After a week the pods were removed and homogenized with 2% hydrochloric acid. To the homogenate was added 20 ml. of 10% trichloroacetic acid and the mixture was allowed to stand for about an hour to complete the precipitation of the proteins. The proteins were filtered off and the filtrate was heated on a hot plate, then treated with 10% aqueous ammonia solution until the solution was alkaline to litmus. An aliquot of this solution, containing mostly organic phosphate esters, as evidenced by paper chromatography, was plated and counted in order to determine the extent of incorporation of C^{14}-inositol. The total incorporation was found to be less than one-half of one per cent of the added counts and because of these very low incorporations this type of feeding experiment was abandoned.

c) Feeding C^{14}-inositol by injection to pods while still attached to the plants:- This method consisted in injecting C^{14}-inositol solution into the interior space of the pods, by means of a 50 λ Hamilton microsyringe. The pods were left attached to the plants in order that they might continue to develop naturally. Variations of the following methods were followed:-

a) Pea pods attached to four different 55-day-old plants were injected with the following volumes of a C^{14}-inositol solution:

Pod I - 5 microlitres
Pod II - 10 microlitres
Pod III - 7 microlitres
Pod IV - 15 microlitres

The injected C^{14}-inositol had 9.5 x 10^3 counts per minute per 10 microlitres and the total labelled material fed to the four pods contained 3.5 x 10^4 counts per minute. The pods were left attached to the plants and allowed to develop in the laboratory under constant fluorescent and incandescent light. After a week the pods were removed from the plants, cut into small pieces and homogenized in 15 ml. of 95% ethanol in a

Virtis homogenizer. An aliquot of the homogenate was plated and counted to determine the total recovery of label (Table II), the suspension was centrifuged and the supernatant discarded. The precipitate was air dried to remove traces of ethanol, and then stirred for three hours with 10 ml. of 10% trichloroacetic acid to extract acid-soluble phosphates. The extract was made basic with 0.2N barium hydroxide, chilled for several hours and centrifuged. The precipitate was dissolved in 2% hydrochloric acid and aliquots used for chromatography and counting. (Table II).

TABLE II

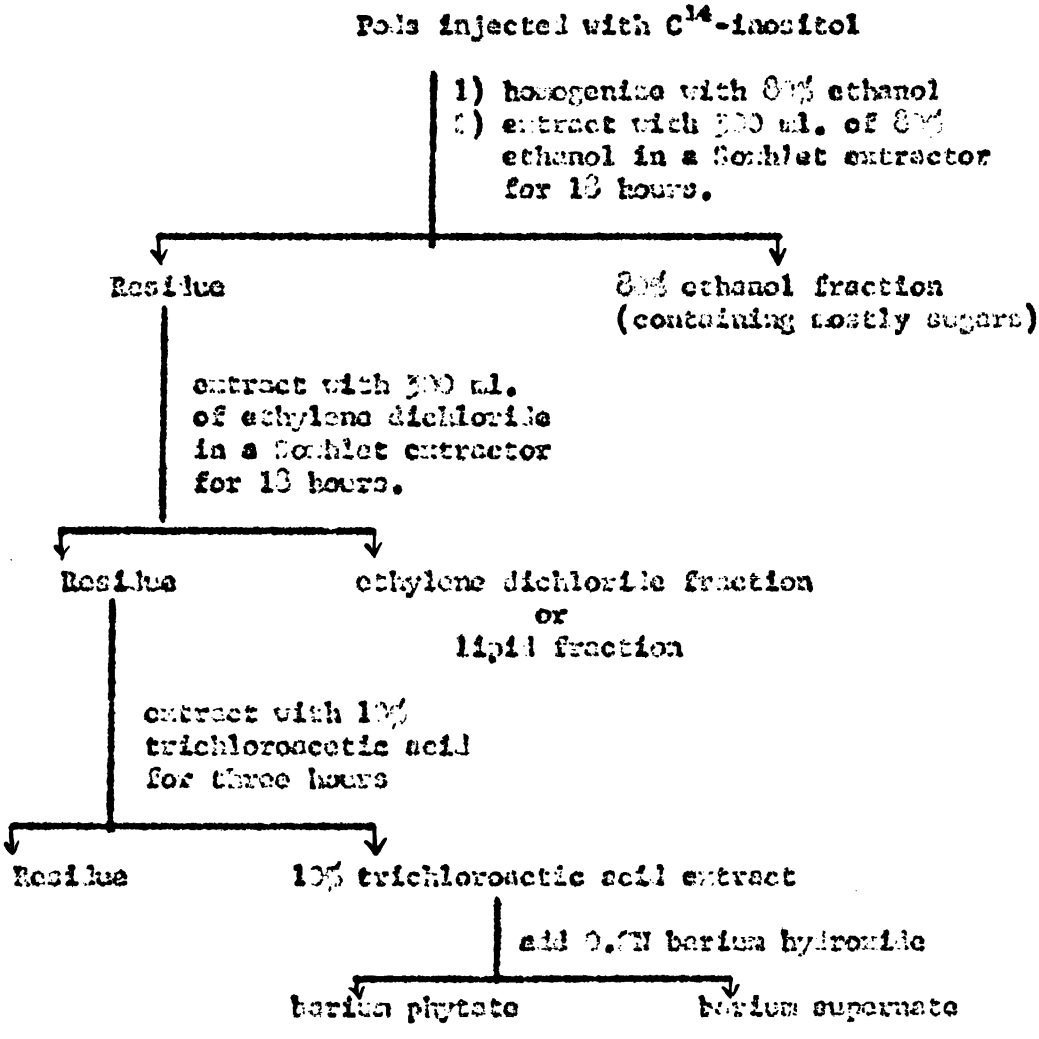
Incorporation of C^{14} from inositol injected into maturing pea pods.

Total counts**	
Fed to 3 pods	35,000
Total recovered in homogenate	30,100
Residue after TCA extraction	4000
Supernate from barium precipitation	5400
Barium precipitate	810

**All figures are for count rates corrected only for background, no correction has been made for counter geometry; all were infinitely thin plates.

b) This experiment was run to determine the extent of incorporation of C^{14}-inositol into phytic acid with increasing time periods. Seven different experiments were performed in which the pods were fed varying amounts of C^{14}-inositol and then sacrificed at different time intervals, and the incorporation of the label into various fractions studied.

The following scheme shows the procedure followed in isolating the sugar, lipid and barium phytate fractions from the pods after injection of C^{14}-inositol:



In experiment I, two pea pods from 40-day old plants were injected with C^{14}-inositol equal to 9.5×10^3 counts per minute. After a week the pods were removed from the plants, cut into small pieces and homogenized in 80% ethanol in a Virtis homogenizer. The homogenate was extracted with 80% ethanol to get the sugar fraction. The residue from this treatment was dried and extracted again with ethylene dichloride to get a lipid fraction, and was further treated for the extraction of phytic acid. Aliquots of these fractions were plated and counted. (Table III).

In Experiment II, three pods were injected with C^{14}-inositol equal to 8.4×10^3 counts per minute. After ten days the pods were sacrificed, homogenized with 80% ethanol and the labelled sugars, lipids and phytic acid fractions isolated as stated earlier and the extent of labelling determined. Results appear in Table III.

Experiments III to VII started with the injection of 20 microliters of C^{14}-inositol (equal to 4.2×10^3 counts per minute) into each of ten pods. The pods were sacrificed in pairs at 15 minutes, 24 hours, 4,8, and 12 days and were cut into small pieces and homogenized with 80% ethanol in a Virtis homogenizer. The homogenate was processed as indicated earlier for the isolation of sugars, lipids and phytic acid fractions and the extent of labelling determined in these fractions by plating aliquot and counting. Results are shown in Table IV.

c) This experiment was devised in such a manner that the incorporation of C^{14}-inositol could be studied under conditions of continuous light and also under conditions where light was discontinued at night for about six to seven hours.

TABLE III

Recovery of labelled carbons of inositol-U-C^{14} seven and ten days after injection into maturing pea pods.

Experiment number	Total C^{14}-inositol injected	Recovery in homogenate	Per cent recovery	C^{14} in individual fractions (counts/minute)				Individual fractions as per cent of total.			
				80% EtOH	Lipid	Barium phytate	Residue	80% EtOH	Lipid	Barium phytate	Residue
I (7 days)	9540	8400	88.05%	3834	4040	390	136	45.52%	48.0%	4.64%	1.61%
II (10 days)	8480	5227	61.63%	3224	1050	735	118	63.57%	20.0%	14.0%	2.27%

All count rate figures are corrected for background only; all were infinitely thin plates.

TABLE IV

Recovery of labelled carbons of inositol-U-C^{14} at various times after injection into maturing pea pods.

Experiment number	Time after injection of C^{14}-inositol	Amount of C^{14}-inositol fed	Total per cent recovery	Recovered in individual fractions.				
				80% EtOH	Lipid	Barium phytate	Barium supernate	Residue
III	15 minutes	8480	78.17%	4050	1460	0	133	199
IV	24 hours	8480	81.9%	2295	3773	360	440	373
V	4 days	8480	69.3%	2495	1966	406	690	533
VI	8 days	8480	60.5%	1876	1000	502	392	763
VII	12 days	8480	52.3%	1360	460	460	341	925

TABLE V (a)

Effect of dark period on the incorporation of C^{14}-inositol in the maturing pea pods. (Experiment VIII)

Time	80% Ethanol	Lipid	Phytic acid	Barium supernate	Residue
2 days	3902	2098	856	571	307
4 days	4018	673	1427	843	637
8 days	3331	361	1654	752	855
12 days	2801	346	2046	542	997
16 days	2276	460	2387	532	1010

In this experiment the light was turned off for 6-7 hours each night.

TABLE V (b)

Effect of dark period on the incorporation of C^{14}-inositol in the maturing pea pods. (Experiment IX).

Time	80% Ethanol	Lipid	Phytic acid	Barium supernate	Residue
15 mts	20,056	9054	0	206	200
1 hr.	19,565	9500	0	315	275
6 hr.	17,146	10,128	0	595	292
24 hr.	12,260	13,990	175	605	356
2 days	12,115	10,245	363	745	453
4 days	10,394	8430	695	985	535
8 days	10,575	5750	1275	495	675
12 days	11,150	3050	1505	315	795

In this experiment the light supply was continuous.

Experiment VIII was performed under conditions where light was discontinued at night for six to seven hours. Ten pods from 40-day-old pea plants were used and in each case the pods were injected with inositol equal to 2.1 x 10^4 counts per minute (specific activity 2.0 x 10^6 counts per minute per millimole). These pods were sacrificed in groups of two after 2, 4, 8, 12 and 16 days. The pods were cut into small pieces, homogenized in 80% ethanol in a Virtis homogenizer and the homogenate processed for the isolation of various labelled fractions, for each of which the extent of labelling was determined. (Table V).

In experiment IX, the C^{14}-inositol-injected pods were kept under constant light. Here 18 pea pods were injected with C^{14}-inositol equal to 2.1 x 10^4 counts per minute (specific activity 2.0 x 10^6 counts per minute per millimole). The pods were sacrificed two at a time at intervals of 15 minutes, one hour, six hours, 24 hours, 2, 4, 8, 12 and 16 days. The pods once again were cut into small pieces, homogenized in 80% ethanol in a Virtis homogenizer and the sugar, lipid and phytic acid fractions isolated and counted. Results are shown in Table V.

3) Isolation and partial characterization of labelled sugars, lipids and phosphorylated inositols from pea pods fed C^{14}-inositol.

a) Isolation and identification of components of 80% ethanol fraction:-

The 80% ethanol homogenate of the pods fed C^{14}-inositol was filtered, and the residue extracted with about 300 ml. of 80% ethanol for 18 hours. The filtrate and the extract were then combined. The 80% ethanol fraction thus obtained contains mostly the sugars and inositol. The residue was further treated for the extraction of lipids.

The 80% ethanol extract was evaporated to a small volume under vacuum, treated with Norite and filtered. The filtrate was deionized with IR 120

(H) and IRA 400 (OH) resins. The deionized solution was evaporated to a small volume under a spot light in presence of a continuous stream of air. An aliquot of this extract was plated and counted to determine the extent of total labelling in the 80% ethanol extract. The results of Experiments I to IX are shown in Tables III to V.

The 80% ethanol extract was chromatographed in the pyridine (7): amyl alcohol (7): water (6) solvent system and showed the presence of six components: inositol, sucrose, glucose, xylose, glycerol and an organic phosphate ester.

In order to find out the extent of labelling in these six components, a portion of the 80% ethanol extract was run in the form of a strip on Whatman 3 mm paper. Reference spots were run along with standards and the position of the various components located by spraying the reference portion of the paper with ammoniacal silver nitrate. The strips were cut and the six components eluted from the strips with water. The water extract was evaporated to a small volume, plated and counted.

These determinations were carried out only in experiments I, VIII and IX. The results are shown in Table VI.

It has not been possible to identify the organic phosphate ester present in the 80% ethanol extract, despite a number of attempts. This compound appears as a blue spot on paper chromatograms sprayed with the Axelrod and Bonlursky (50) modification of Hanes and Isherwood molybdate spray. However, on hydrolysis in 3N hydrochloric acid for 3 hours at 100°C, this organic phosphate ester though not completely hydrolysed, showed the presence of inositol, glucose and inorganic phosphate. Another spot trailing the glucose spot appeared on the paper chromatograms; this is probably due to the presence of hydrochloric acid in the hydrolysate.

TABLE VI(a)

Fractionation of the 80% ethanol extract obtained from pea pods fed C^{14}-inositol

Exp. No.		Total* Counts	Inositol	Sucrose	Glucose	Xylose	Glycerol	Organic phosphate ester
I		3834	442	437	416	438	132	1324
VIII	A	3922	1339	139	416	269	127	1085
	B	4218	2664	128	180	280	68	464
	C	3331	1125	87	108	165	96	1380
	D	2801	761	73	83	192	57	1334
	E	2276	720	48	72	92	48	789
IX	A	20256	6689	708	1522	443	1257	8677
	B	19565	6230	257	1050	400	832	6274
	C	17146	4197	276	1116	777	450	7665
	D	12660	1748	210	400	321	653	5781
	E	12115	1608	1008	1562	680	1215	4789
	F	10394	1789	832	1773	1231	1070	3185
	G	10575	2240	448	656	472	1016	3480
	H	11150	1907	589	673	458	901	2879

* All Counts are corrected for background only; the plating was infinitely thin.

TABLE VI (b)

Fractionation of the 80% ethanol extract from pea pods fed C^{14}-inositol.

Exp. No.		Components as per cent of the total					
		Inositol	Sucrose	Glucose	Xylose	Glycerol	Organic P-ester
I		11.52%	11.39%	10.85%	11.42%	3.70%	34.53%
VIII	A	34.14%	3.54%	10.64%	6.85%	3.23%	26.38%
	B	62.92%	3.03%	4.26%	6.63%	1.63%	11.0%
	C	33.77%	2.63%	3.24%	4.95%	2.88%	41.42%
	D	27.16%	2.60%	2.96%	6.89%	2.03%	47.62%
	E	32.07%	2.10%	3.20%	4.04%	2.10%	35.10%
IX	A	33.03%	3.50%	7.51%	2.19%	6.1%	42.34%
	B	31.84%	1.31%	5.87%	2.04%	4.25%	32.34%
	C	24.47%	1.60%	6.50%	4.53%	2.63%	44.70%
	D	13.8%	1.65%	3.16%	2.52%	5.15%	21.96%
	E	14.0%	8.32%	12.06%	5.62%	10.0%	39.52%
	F	17.21%	8.0%	17.05%	11.93%	10.39%	30.64%
	G	21.18%	4.23%	6.20%	4.46%	9.6%	32.9%
	H	17.1%	5.28%	6.05%	4.1%	8.08%	25.82%

The results of the chromatography of the hydrolysate are presented in Figure (II).

b) Isolation of the lipid fraction:- The residue left after 80% ethanol extraction was dried and extracted with 300 ml. of ethylene dichloride for 10 hours in a Soxhlet extractor. The ethylene dichloride extract containing the lipids was evaporated down to a small volume. The residue from the lipid extraction was stored for the extraction of phytic acid. An aliquot of the lipid extract was plated and counted; results on the incorporation of label in the lipid fraction of Experiments I to IV appear in Tables III to V.

Hydrolysis of the lipid fraction:- The lipid fraction was hydrolysed and some of the components in the hydrolysate were determined by paper chromatography. The method followed was essentially the one described by Wagenknecht et.al. (40). The lipid fraction of Experiments III to VII were pooled together, concentrated to a small volume and hydrolysed.

Mild acid hydrolysis:- Mild acid hydrolysis was carried out in a sealed tube in an Abderhalden pistol. In the sealed tube was placed 1 ml. of concentrated lipid extract and 0.5 ml. of 0.5N sulfuric acid. The hydrolysis was carried out for 24 hours using water as a refluxing solvent. The hydrolysate was adjusted to pH 4 by stirring with Dowex-2 (HCO^-) resin. The resin was filtered off and the filtrate was taken to dryness by lyophilisation. The dried residue was taken up in distilled water and chromatographed.

Strong acid hydrolysis:- Another 1 ml. of the lipid extract and 0.5 ml. of 6N hydrochloric acid was placed in a sealed tube in the Abderhalden pistol. The sample was hydrolysed for one hour using dimethylcellosolve as the refluxing solvent. The hydrolysate was adjusted to pH 4 with Dowex-2 (HCO^-) resin. The resin was filtered off and the filtrate taken

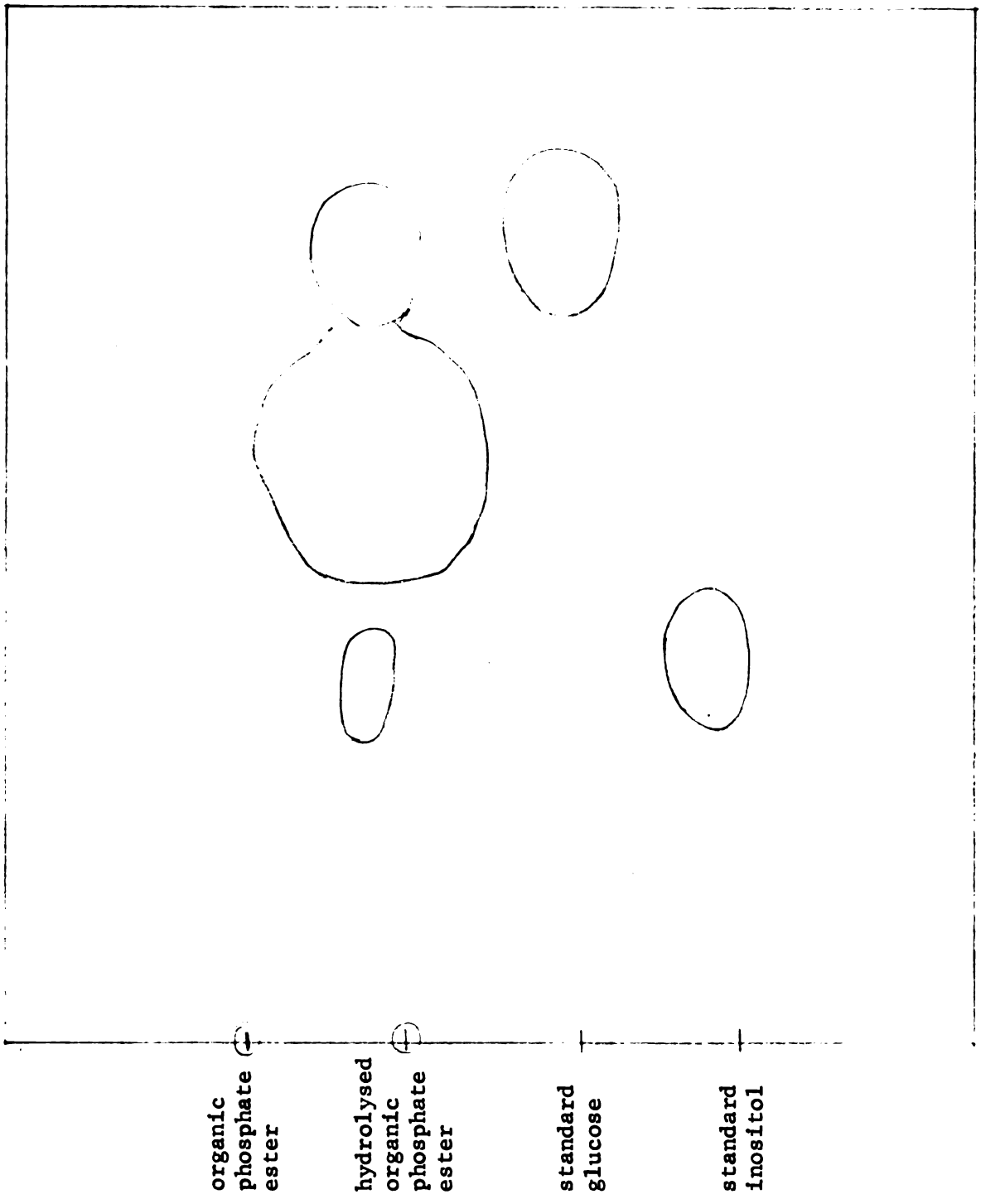


Figure II (a):- A typical paper chromatogram of the organic phosphate ester hydrolysate in Methanol: formic acid: water (80:15:5) solvent system. The paper was sprayed with sodium periodate-benzidine spray.

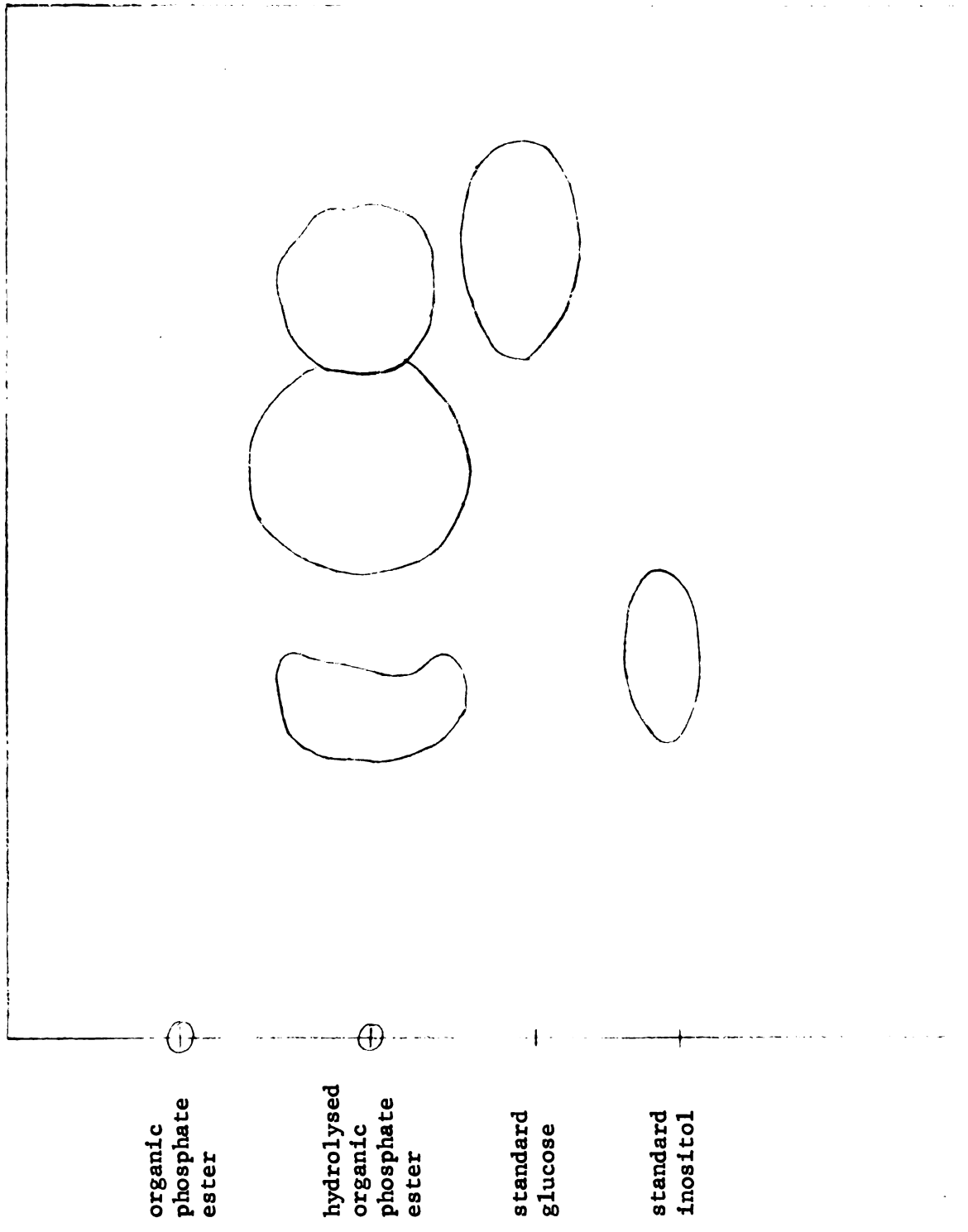


Figure II (b):- A representative paper chromatogram of the organic phosphate ester hydrolysate in one solvent system methanol: (80);formic acid (15); water (5). Chromatogram sprayed with ammoniacal silver nitrate.

to dryness by lyophilisation. The dried residue was dissolved in distilled water and chromatographed.

Alkaline hydrolysis:- Alkaline hydrolysis was carried out in methanolic potassium hydroxide. One ml. of the lipid extract was dissolved in moist benzene and to this was added one ml. of 1.43N methanolic potassium hydroxide. The hydrolysis was carried out at room temperature for 24 hours with occasional shaking. The reaction mixture was filtered and the residue was washed with six 5 ml. portions of benzene-methanol (3:1). The residue was then dissolved in distilled water, the pH adjusted to 3 with acetic acid, and the solution then extracted with petroleum ether. The ether layer was discarded and the aqueous layer filtered through Celite 535. The filtrate was extracted three times with ethyl ether to remove acetic acid and was then lyophilised. This hydrolysate was dissolved in water and chromatographed.

The chromatographic details of lipid hydrolysates appear in the section on paper chromatography.

c) Extraction of phytic acid:- A variety of methods were tried for the extraction of phytic acid.

During the early stages of this study when C^{14}-inositol was prepared by feeding $BaC^{14}O_3$ to the pods and also when C^{14}-inositol was fed to the maturing pods, the procedure for the isolation of phytic acid began by homogenizing the pods with 5% hydrochloric acid.

In a typical case, two pods from 40-day-old plants, which were fed $BaC^{14}O_3$ in a vacuum desiccator for ten days, were homogenized with 5% hydrochloric acid. To the 5% hydrochloric acid extract was added 15 ml. of 10% trichloroacetic acid with continuous stirring. After one hour the precipitated proteins were filtered off and the filtrate was warmed on a

hot plate, then treated with 1% aqueous ammonia solution until the solution was slightly alkaline to litmus. The solution was heated for a short time, allowed to cool and then concentrated under vacuum. On evaporation white crystals appeared. These crystals were dissolved in 10 ml. of 1% acetic acid and heated for a short time. After cooling an excess of ammonia and 95% ethanol was added and the solution was chilled overnight. A white suspension appeared and the solution was chromatographed.

Nine different solvent systems were used for phytic acid chromatography, the details of which are mentioned in the paper chromatography section.

In the later studies variations on the two main methods of extraction were tried. The first method entails the use of 1% acetic acid (55) and the second method consisted in a trichloroacetic acid extraction followed by precipitation of barium salts at pH 8.2 (56).

These methods were first employed for the extraction of phytic acid from mature pea seeds in an attempt to devise an adequate method for further extractions.

Preparation of seeds for extraction:- Ten grams of the pea seeds were soaked in water, broken into small pieces and then stirred with 150 ml. of ether for three hours. The ether was decanted off, and the peas were oven-dried until the last traces of ether had been removed. The weight of the dried seeds was 3.2 grams and these seeds were used for phytic acid extraction.

Acetic acid extraction:- 1.6 grams of ether-dried seeds were stirred with 25 ml. of acetic acid for three hours. After filtration the extract was concentrated and chromatographed.

Trichloroacetic acid extraction:- 1.6 grams of the ether-dried seeds were treated with 25 ml. of 10% trichloroacetic acid and mechanically stirred for three hours. The supernatant liquid was collected and 0.2N barium hydroxide solution was added to a pH of 8.0 to 8.5. It was then set aside for an hour and the precipitate, which usually formed immediately, was allowed to settle. The precipitate was then spun down in a centrifuge and washed first with distilled water, then with 95% ethanol and finally with distilled water again. The precipitate was dried in a vacuum dessicator overnight in the centrifuge tube. Half of the precipitate was suspended in 0.2N hydrochloric acid and the other half in 10% acetic acid. The precipitate was allowed to stand for about an hour, the undissolved portion was removed by centrifugation, and the solutions were used for paper chromatography.

For the extraction of phytic acid from pea pods injected with C^{14}-inositol, the "barium extraction" method was followed. The pod residue left after lipid extraction was extracted with 10% trichloroacetic acid for three hours and the procedure described above was followed. The barium precipitate was taken up in 2% hydrochloric acid and acetic acid for paper chromatography, (details in the section on paper chromatography). An aliquot of the sample was plated and counted to find out the labelling in phytic acid. Results are shown in Tables II to V.

The supernatant solution left after phytic acid precipitation by barium was concentrated to a small volume under a spot light. A portion of this solution was plated and counted.

The final residue left was suspended in water, plated and counted.

4) Chromatographic separations.

A) Column chromatography.

Column chromatography of the barium-soluble fraction:- Attempts were made to identify the components of the supernatant fraction left after the precipitation of the barium phytate.

Two methods were used for separating the barium supernate components. The first method entails the use of varying concentrations of lithium chloride as the eluting solvent (41), while the second method utilizes ammonium formate-formic acid as the eluting solvent (45).

(a) Lithium chloride separation:- The supernatant fraction was evaporated down to a small volume, at which point a white precipitate started to appear. The suspension was then treated with IRA 120 (H) resin and the precipitate went back into solution. The resin was filtered off and the filtrate was made strongly basic with cyclohexylamine and evaporated to dryness under vacuum.

The residue was dissolved in hot absolute ethanol and the insoluble mass (which appeared to contain no organic phosphate) was filtered off. The filtrate was taken to dryness, the residue was dissolved in 15 ml. of distilled water, and this solution was subjected to column chromatography and paper chromatography.

The ion exchange resin used was Dowex-1 chloride (100-200 mesh); column size 36 cm by 1.5 cm, rate of flow was adjusted to 1 ml. per 2.5 minutes.

After packing, the column was loaded with 10 ml. of the sample and the column washed with 50-60 ml. of distilled water. A modified gradient elution technique was used, first keeping 250 ml. of water in the mixing chamber and 0.2M lithium chloride in the reservoir. After collecting 90

fractions of 15 ml. each, the concentration of the eluting solvent in the reservoir was changed to 0.4M lithium chloride without changing the contents of the mixing chamber and another 75 fractions were collected.

The total phosphate determinations of these fractions were carried out according to the method of Barley and Deturk (49).

The column fractions were then evaporated down to a small volume and subjected to paper chromatography.

(b) Formate separation:- The supernatant fraction after treating with IRA 120 (H) resin was extracted three times with 40 ml. of ether. The water layer was reduced in volume and put on the column.

The ion exchange resin used was Dowex-2 (100-200 mesh, formate); column size 1.5 cm by 12 cm; rate of flow 1 ml. per minute.

The resin was first washed with 3N hydrochloric acid and then with 3N ammonium formate until free from chloride. The excess of ammonium formate was removed by washing with water.

After loading, the column was washed with 300 ml. of distilled water and then eluted with the following solvents:-

a) 400 ml. of a solution made 0.01N in formic acid and 0.03 molar in ammonium formate; 35 ml. fractions were collected.

b) 400 ml. of 0.01N formic acid - 0.09M ammonium formate; 35 ml. fractions were collected.

These fractions were concentrated to a small volume and subjected to paper chromatography.

Column chromatography of the 80% ethanol extract from pea pods fed C^{14}-inositol:- The procedure followed was the same as mentioned for the chromatography of barium supernatant fraction (95).

The eluting solvent system was formic acid - ammonium formate. The 80% ethanol extract was concentrated to a small volume and put on the column. The column was washed with about 100 ml. of distilled water to remove the sugars, and after that the rest of the procedure is the same as mentioned under (B) above. The water wash and the other fractions were taken to a small volume and chromatographed.

B) Paper chromatography.

Purification of C^{14}-inositol by paper chromatography:- A variety of solvent systems were used for the isolation and purification of C^{14}-inositol from the 80% ethanol extract.

In most cases ascending chromatograms were run in chromatographic tanks for 24 hours on unwashed Whatman # 1 paper. Each jar was covered with a polystyrene cover or a cardboard box in order to protect it from air currents and condensation.

The following solvent systems were tried in order to find a solvent system which would give the best separation of the components of the 80% ethanol extract.

1) n-butanol (10): ethanol (1): water (2)
2) n-butanol (4): pyridine (1): water (1)
3) pyridine (7): amyl alcohol (7): water (6)
4) n-propanol (6): ammonia hydroxide (3): water (1)
5) n-butanol (5): acetic acid (1): water (?)

Four of these solvent systems (numbers 1, 2, 3 and 4) gave a reasonably good separation. Typical results of these separations are shown in Figure III.

For the visualization of spots, the papers were first air dried and then sprayed with the ammoniacal silver nitrate spray of Ballou and

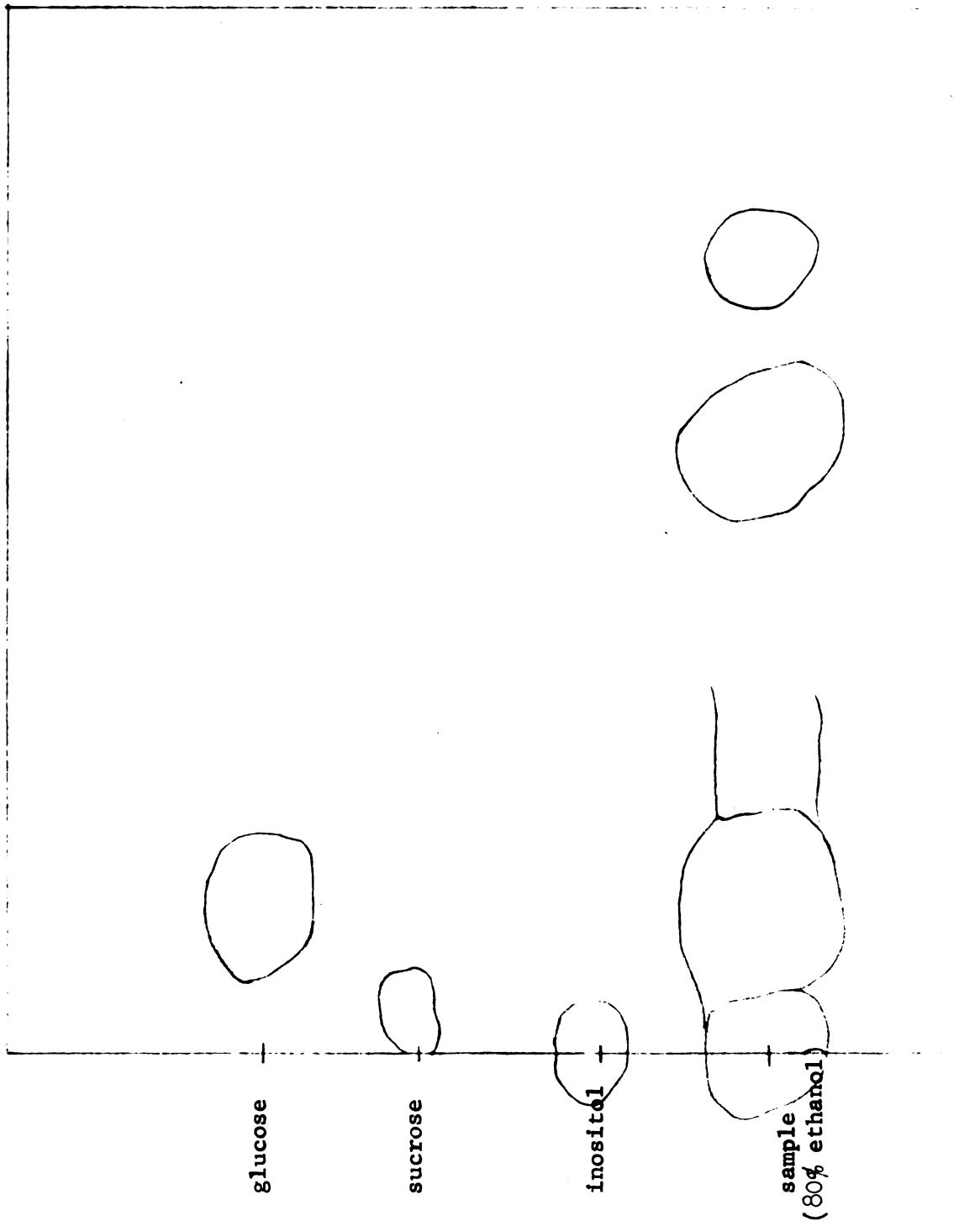


Figure III (a):- A typical chromatogram showing separation of C^{14}-inositol from other components of 80% ethanol extract in the solvent system n-butanol: ethanol: water (10:1:2).

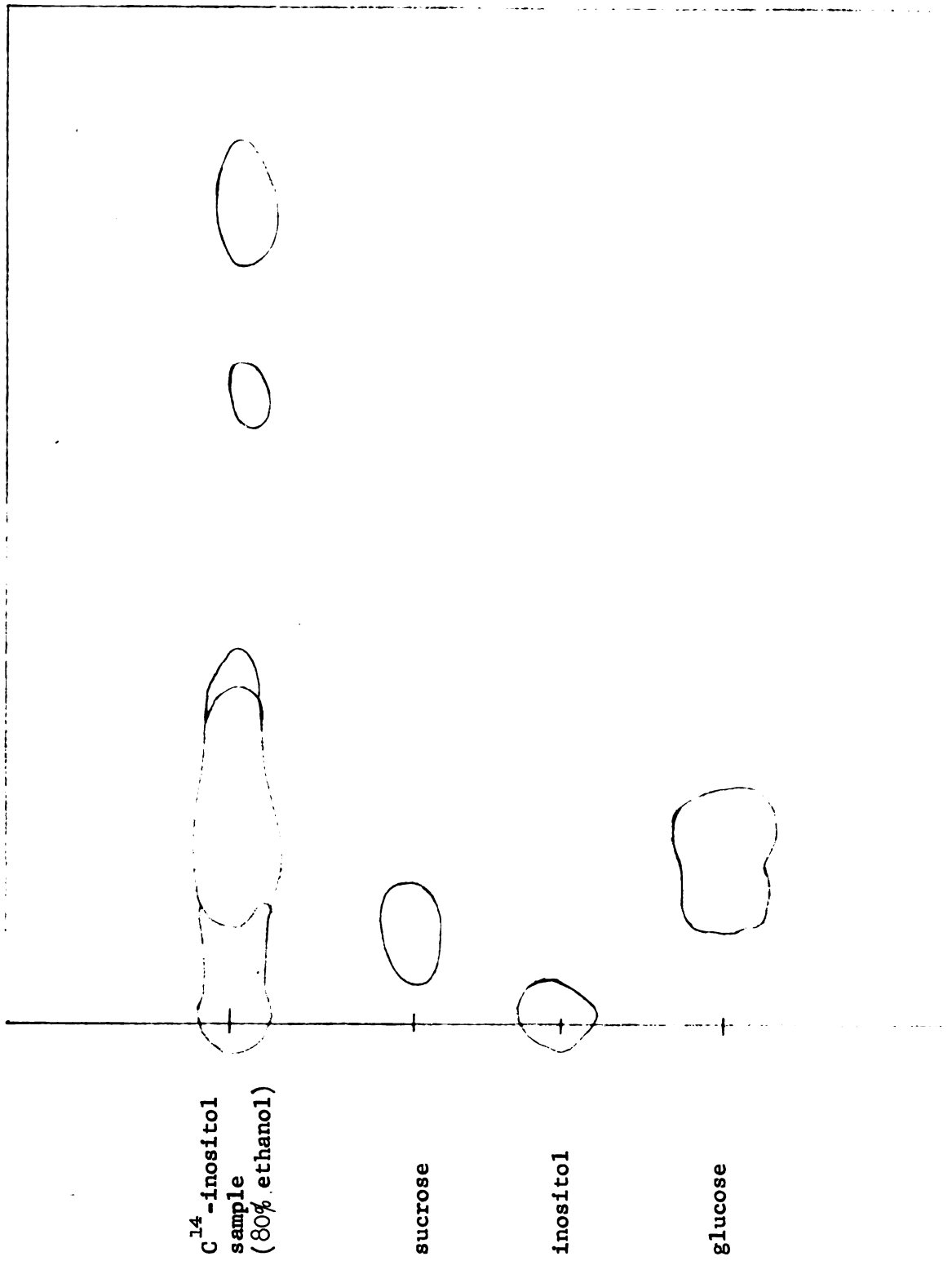


Figure III (b):- A paper chromatogram showing resolution of C^{14}-inositol and other components of the 80% ethanol extract in the solvent system n-butanol: pyridine: water (8:2:2).

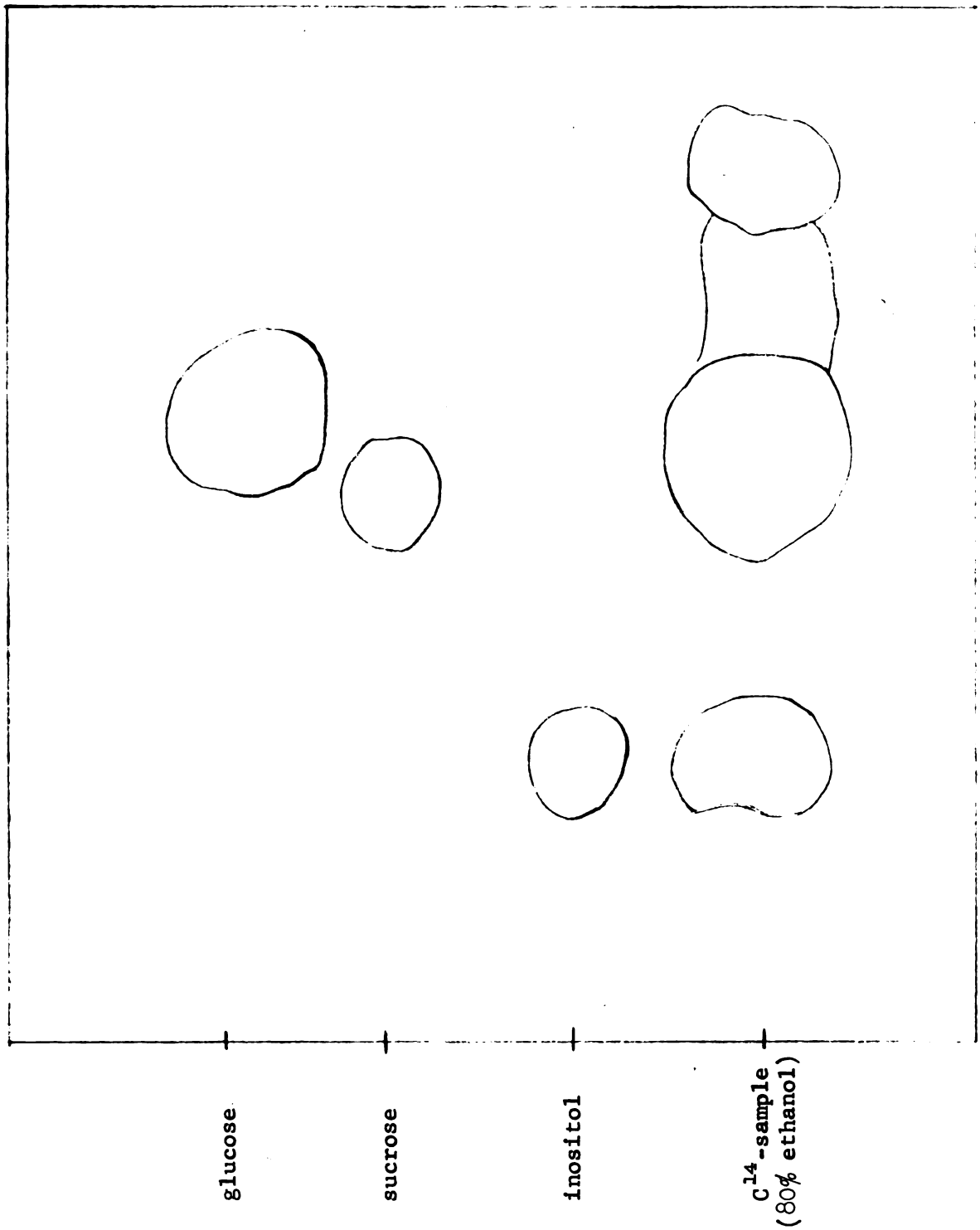


Figure III (c):- A typical chromatographic separation of labelled inositol from the rest of the labelled sugar components of the 80% ethanol extract in pyridine (7): amyl alcohol (7): water (6) solvent system.

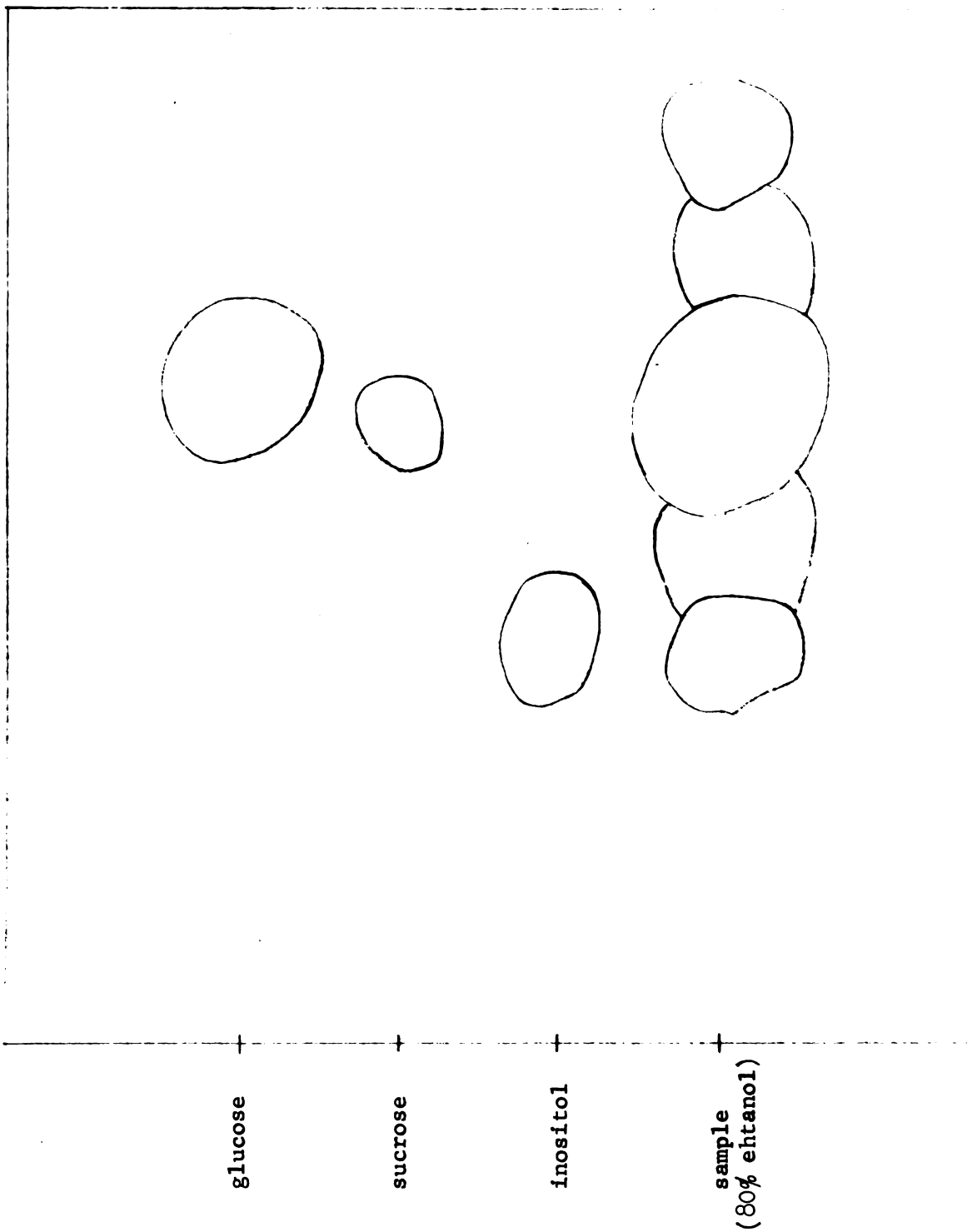


Figure III (d):- Separation by paper chromatography of C^{14}-inositol from other components of the 80% ethanol extract in propanol (6): ammonia (3): water (1) solvent system.

Anderson (53) or the silver nitrate-sodium hydroxide dip reagent of Anet and Reynolds (54), and heated in an oven at 100°C for two to three minutes.

Further chromatographic studies showed that pyridine: amyl alcohol: water solvent system gave the best resolution of the components of the 80% ethanol extract in 24 hours.

For purification purposes the 80% ethanol extract was run in the form of a band on Whatman 3 MM paper. Reference spots were also run along with the standards and the C^{14}-inositol, glucose, sucrose and other labelled components were located by spraying the reference portion of the paper with ammoniacal silver nitrate. The corresponding strips were cut from the paper and the C^{14}-inositol eluted with water. The water extract was evaporated to a small volume in a stream of air in the presence of a spot light and chromatographed again in the same solvent. This procedure was repeated until a chromatographically pure sample of C^{14}-inositol was obtained.

Chromatographic identification of components of 80% ethanol extract from pea pods fed C^{14}-inositol:- For the identification of components of the 80% ethanol extract, ascending chromatograms were run in the solvent systems mentioned earlier for the purification of C^{14}-inositol.

Once again, most of the chromatograms were run in the pyridine: amyl alcohol: water solvent system for 24 hours (ascending) and the spots developed with ammoniacal silver nitrate solution. The results showed the presence of six different components namely:- inositol, glucose, sucrose, xylose, glycerol and organic phosphate ester.

In order to find out the extent of labelling in these components, the 80% ethanol extract was run in the form of a strip on Whatman 3 MM paper in a descending tank for 30 hours in the same solvent system.

Reference spots were run along with the standards and the position of the components was located by spraying the papers with silver nitrate spray. The strips were cut and the six components eluted with water and the extent of labelling in them determined. Results of the chromatographic separation are presented in Figure IV.

Paper chromatography of lipid hydrolysate:- The lipid hydrolysates were chromatographed in four different solvent systems:-

1) isopropanol (3): acetic acid (1): water (1)

2) n-butanol (4): acetic acid (1): water (5)

3) n-propanol (3): acetic acid (1): water (1)

4) pyridine (7): amyl alcohol (7): water (6)

Out of these four solvent systems only number 1 and four above were found to give a clear resolution.

The spots were developed once again by using the silver nitrate spray. Results are presented in Figure V.

Chromatography of phytic acid and inositol polyphosphates:- For the separation of phytic acid and inositol polyphosphates, various solvent systems were tested for both descending and ascending paper chromatography. The nine solvent systems tested were of the following composition:-

1) t-butanol (6): formic acid (2): water (2)

2) t-butanol (8): TCA 4 grams: water (2)

3) t-butanol (8): picric acid 3.5 grams: water (95)

4) methanol (80):formic acid (15): water (5)

5) methanol (6): ammonia (1): water (3)

6) isobutyric acid (10): 0.5N ammonia (6)

7) n-propanol (50): ammonia (40): water (10)

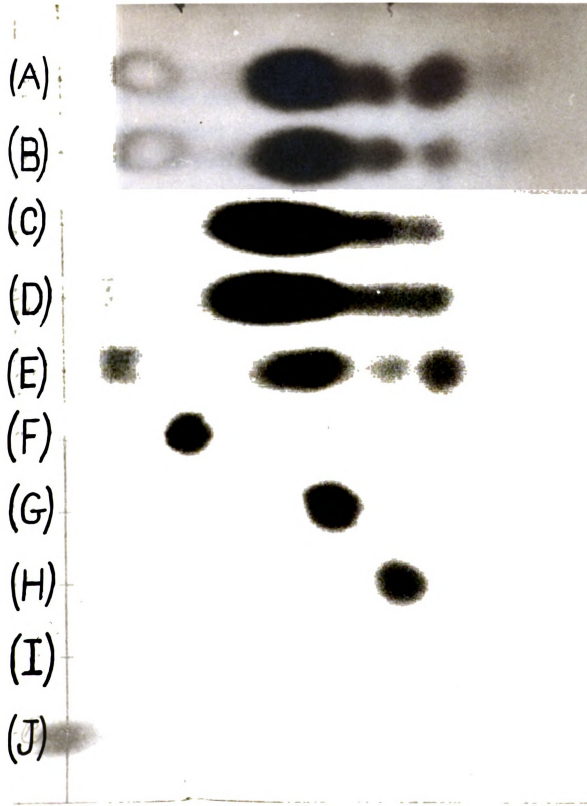


Figure IV

Figure XI:- The picture shows the separation of components of the 80% ethanol extract of Experiment VIII on paper chromatograms run in the pyridine (7): amyl alcohol (7): water (3) solvent system.

(A), (B), (C), (D) and (E) represent samples of the 80% ethanol extract of peas obtained 0, 4, 8, 12 and 16 days (respectively) after injection of C^{14}-inositol.

(F) - standard inositol, (G) - standard glucose, (H) - standard xylose, (I) - standard sucrose, (J) - standard inositol monophosphate.

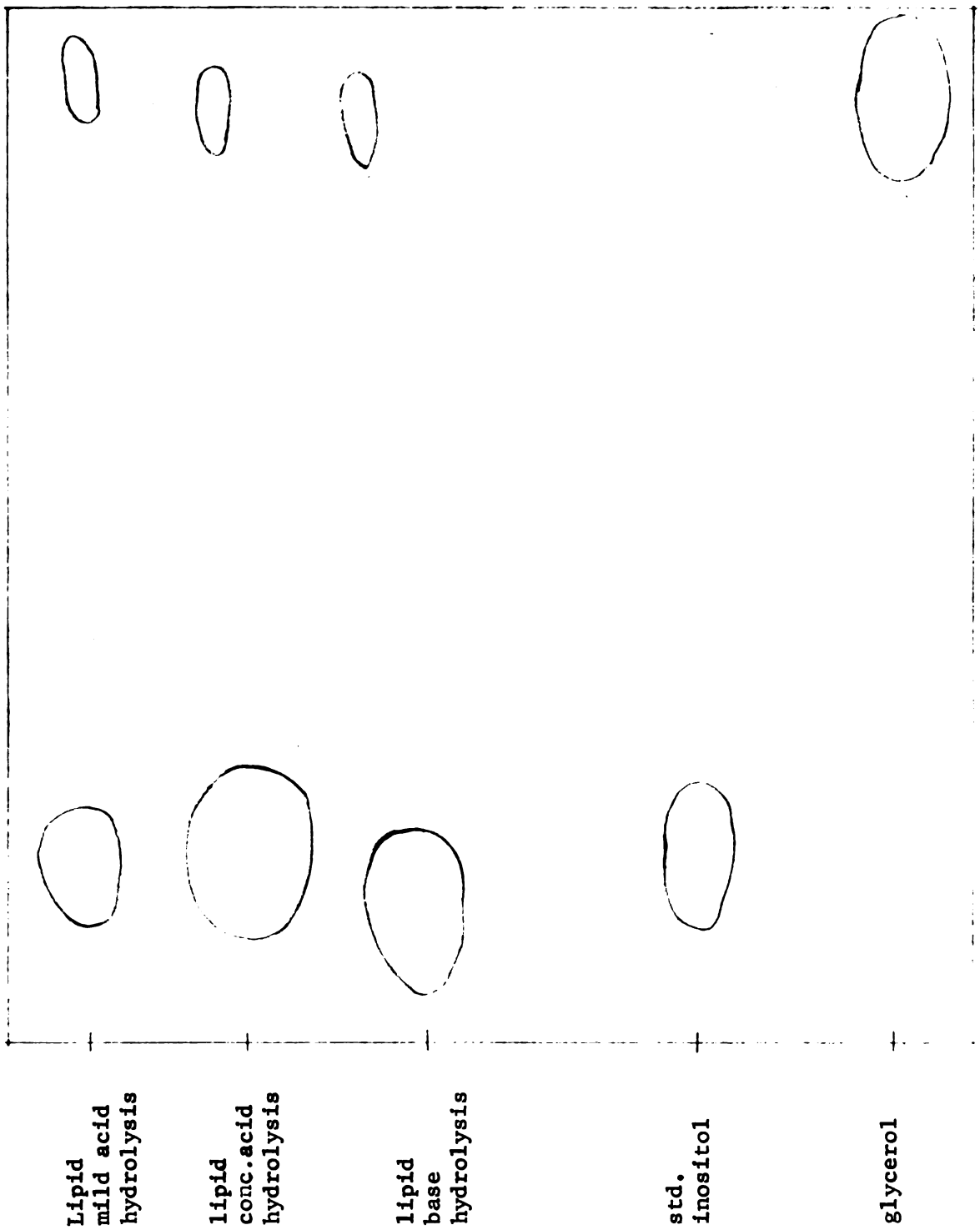


Figure V (a):- A typical chromatogram of lipid hydrolysates in the solvent system isopropanol: acetic acid: water (3:1:1).

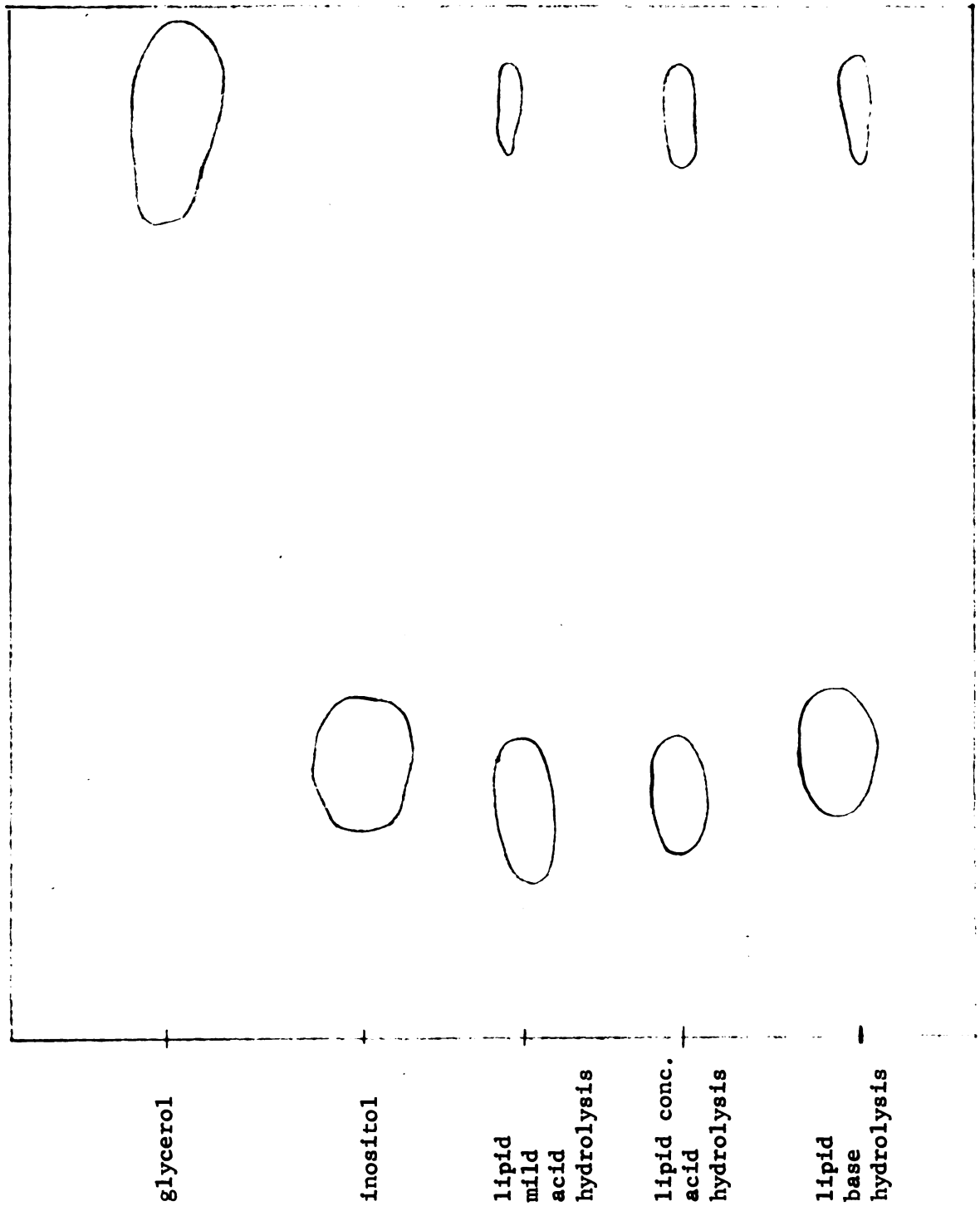


Figure V (b):- A paper chromatogram of lipid hydrolysates in pyridine (7): amyl alcohol (7): water (6) solvent system.

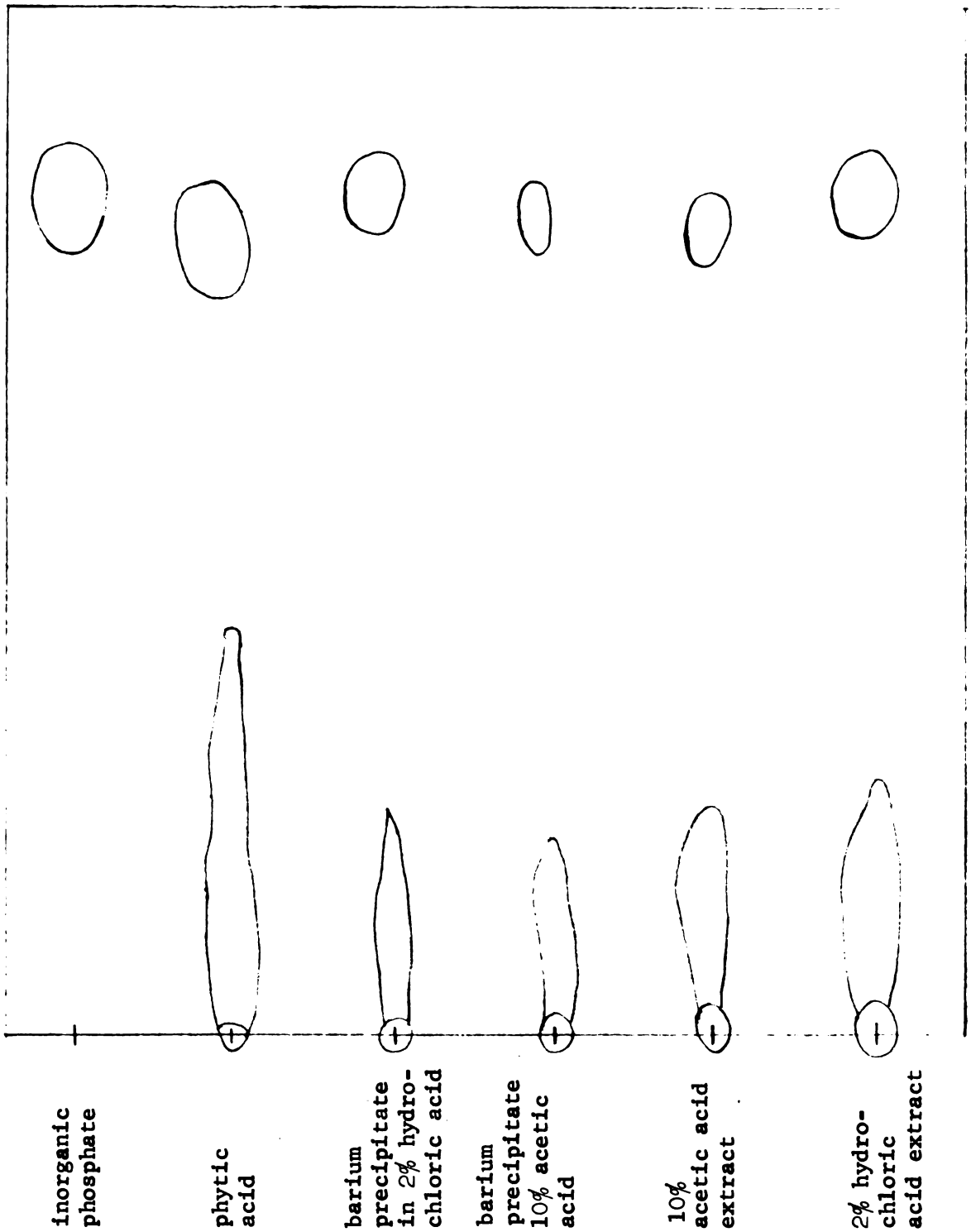


Figure VI (a):- A typical chromatographic separation of the isolated phytic acid from mature peas in t-butanol: TCA: water (80:4 gm: 20) solvent system.

8) isopropanol (40): picric acid 1 gm: water (60)

9) isopropanol (70): ammonia (10): water (20)

Of these solvents six of them (numbers 1, 2, 4, 6, 7 and 9 above) were found to be satisfactory. Some of the typical results are presented in Figure VI.

Most of the chromatographic separations reported here were carried out either in isobutyric acid: ammonia or isopropanol: ammonia: water solvent systems. The inositol phosphates moved very slowly in these solvents and the spots were much more compact and clearer than those obtained in any of the other solvents mentioned above. The solvent also travelled very slowly on the paper and therefore three to four days were necessary for the development of the chromatogram to get a good resolution of the components.

For the detection of spots, the papers were air dried and sprayed with the Amelrod and Blursky (57) modification of the Hanes and Isherwood molybdate spray for the detection of organic and inorganic phosphates. The papers were then heated in the oven for 2-3 minutes at 100°C and exposed to ultraviolet light.

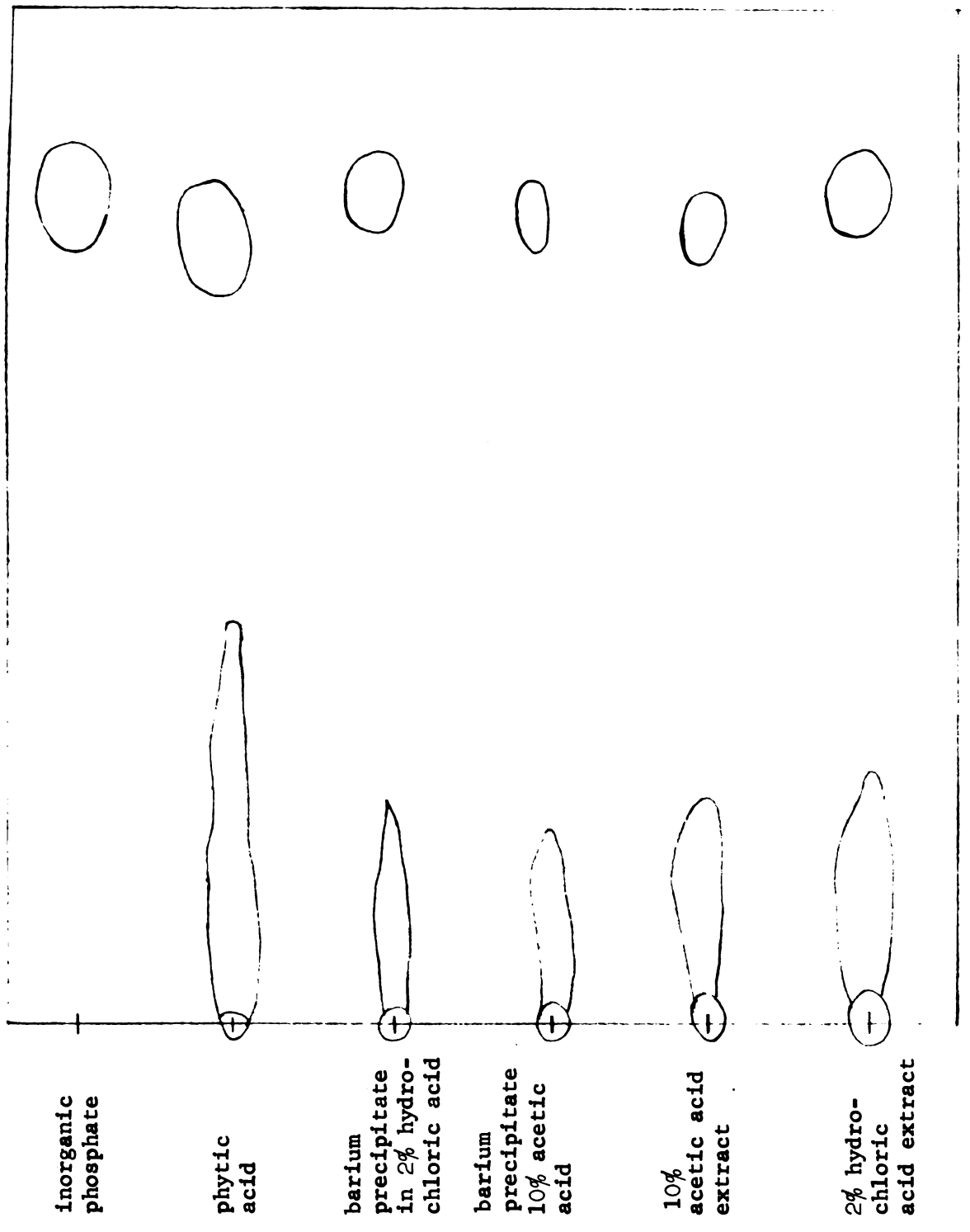


Figure VI (a):- A typical chromatographic separation of the isolated phytic acid from mature peas in t-butanol: TCA: water (80:4 gm: 20) solvent system.

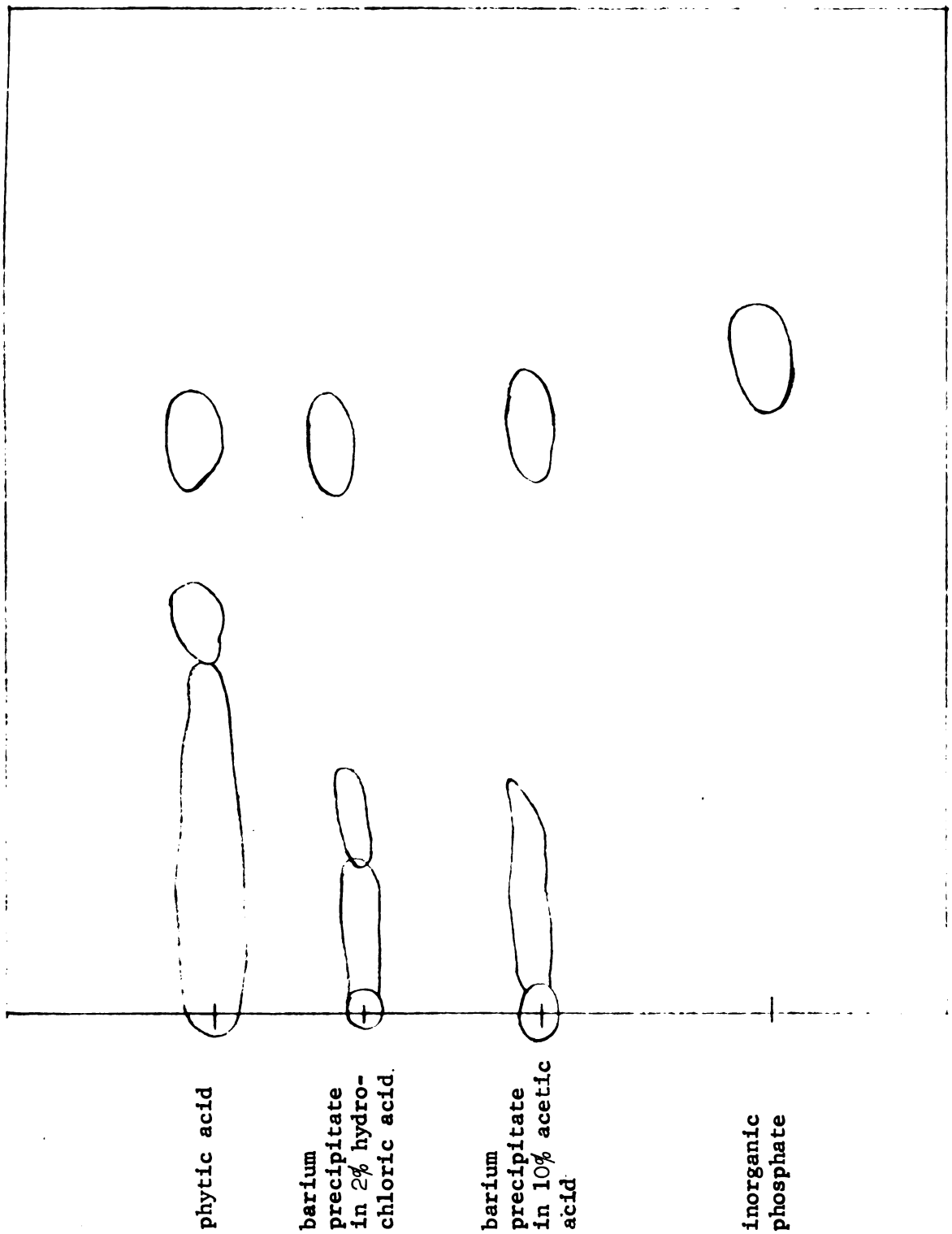


Figure VI (b):- A representative chromatogram showing separation of phytic acid isolated from the peas in the solvent system n-propanol (5): concentrated ammonium hydroxide (4): water (1).

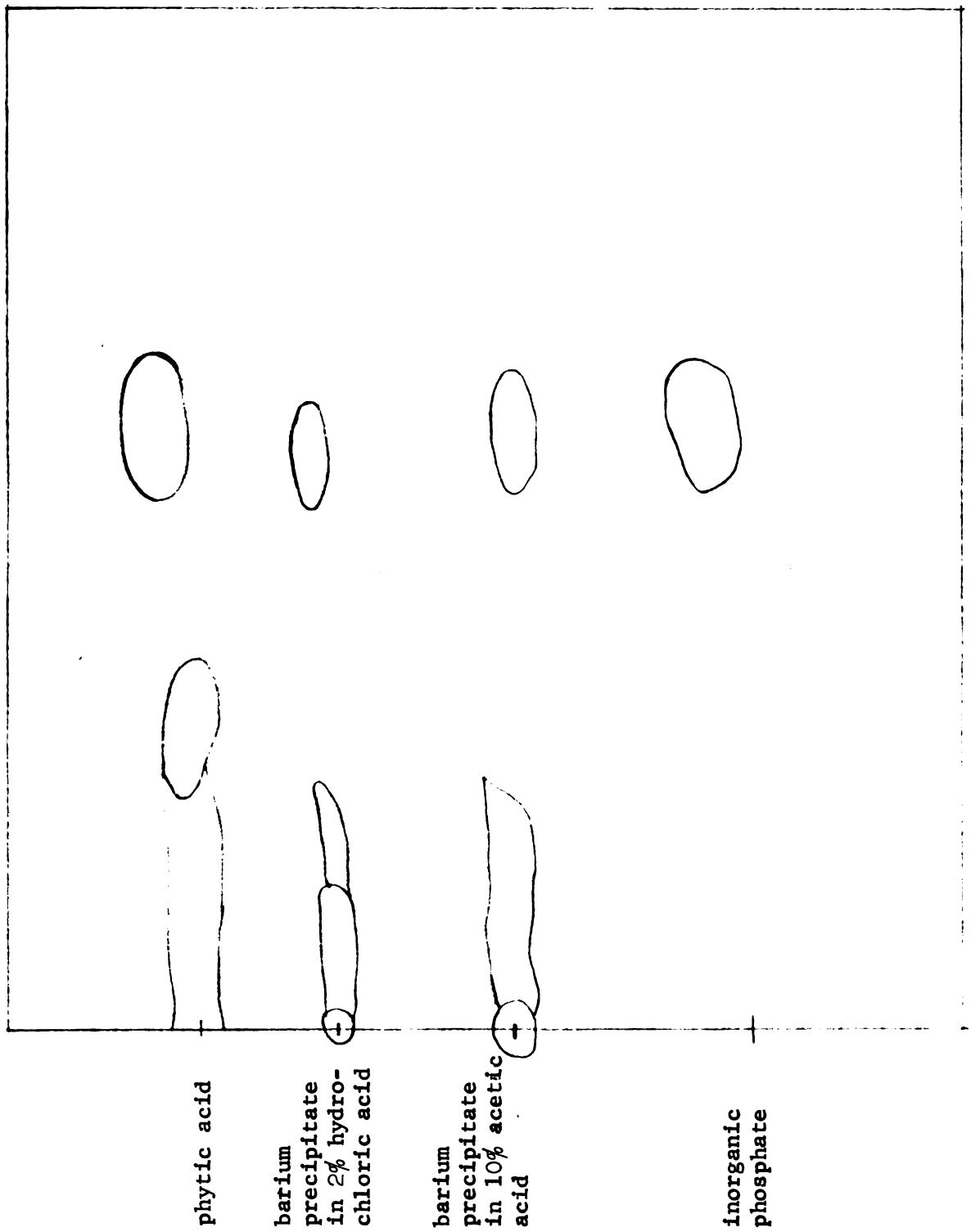


Figure VI (c):- A paper chromatogram showing separation of phytic acid of mature peas in the isobutyric (10): concentrated ammonium hydroxide solvent system.

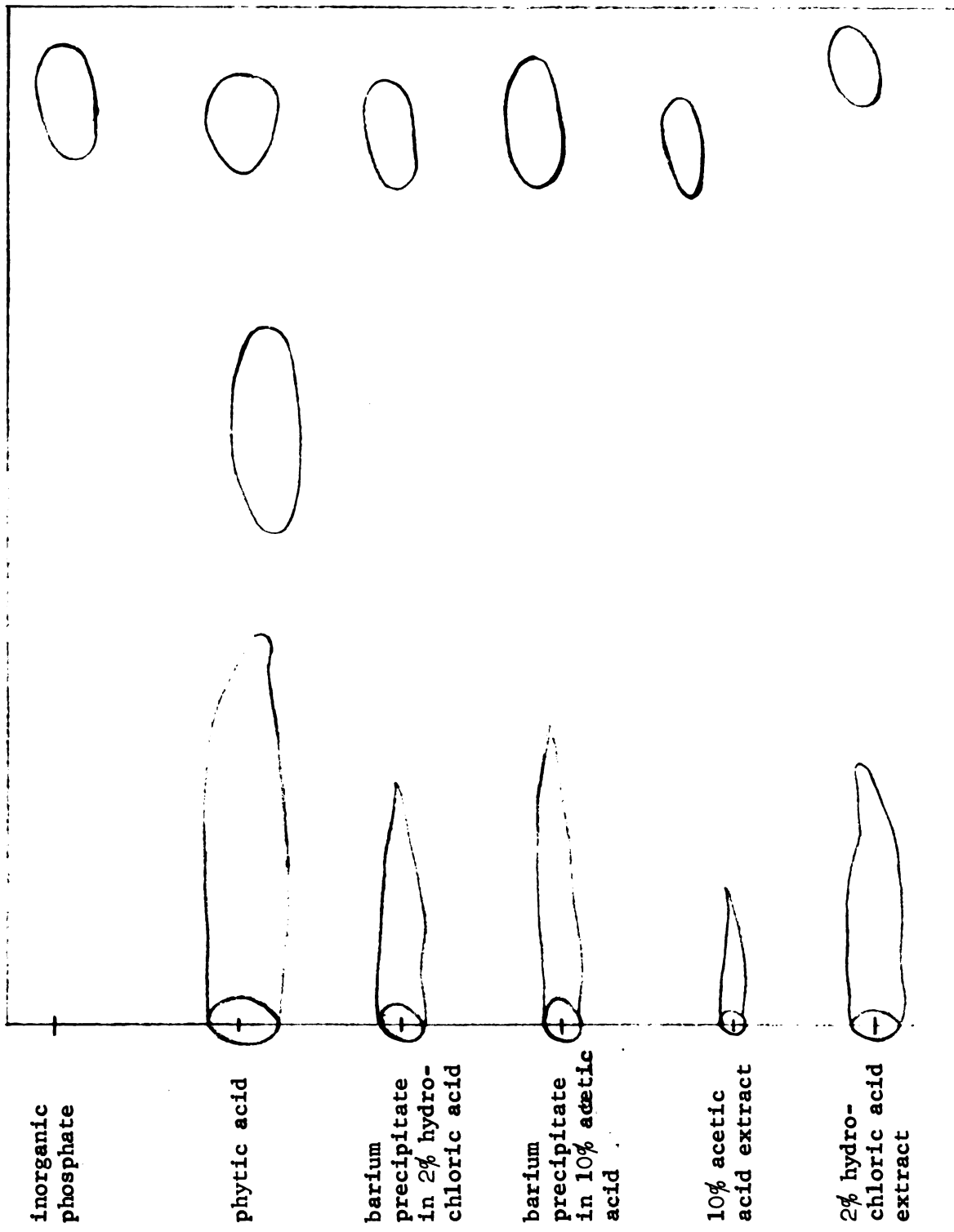


Figure VI (d):- A typical paper chromatogram of phytic acid of peas in the solvent system t-butanol: formic acid: water (6:2:2).

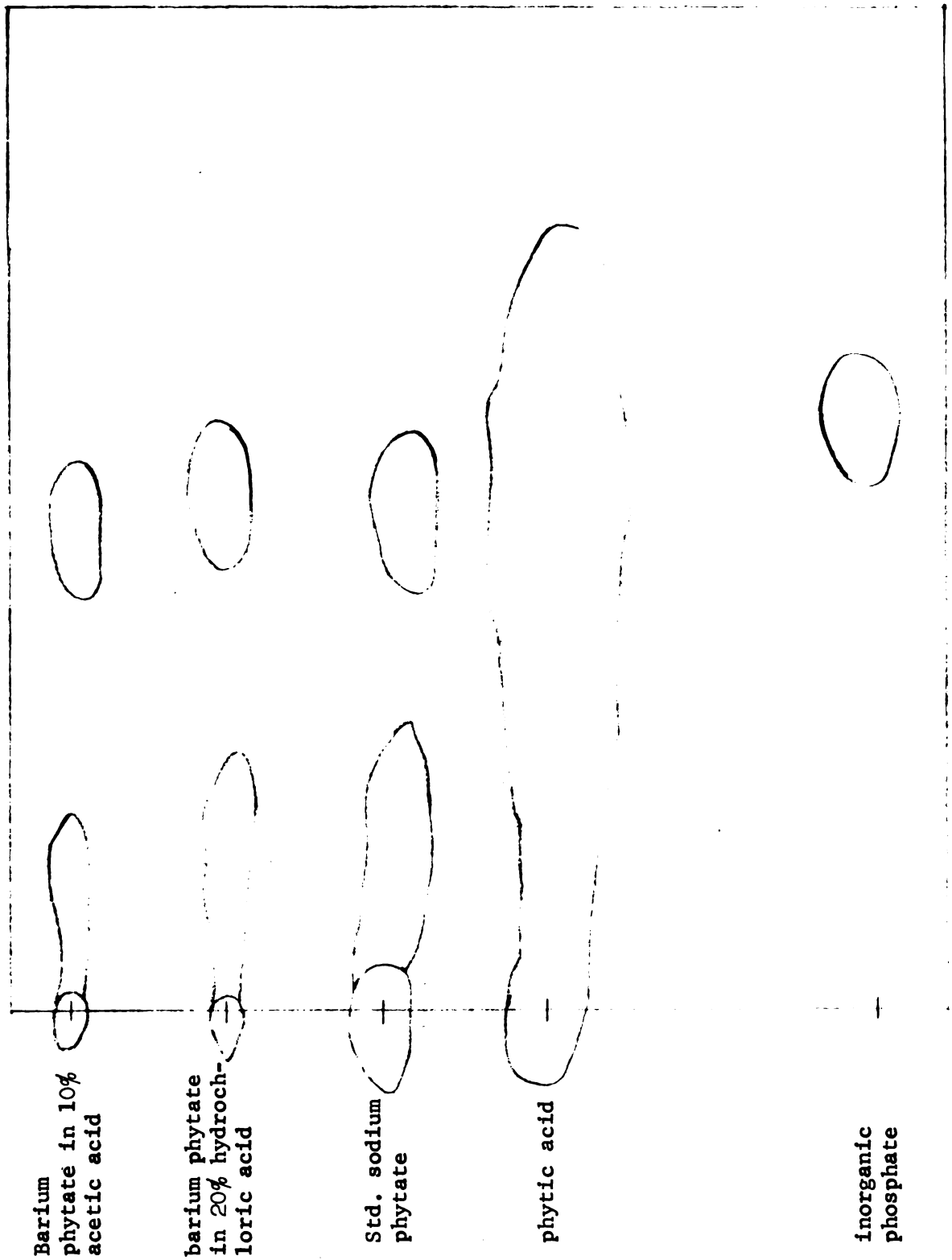


Figure VI (e):- A typical separation of phytic acid of peas in the solvent system isopropanol: concentrated ammonium hydroxide: water (7:1:2).

RESULTS AND DISCUSSION

In the present biosynthetic study, prior to doing any tracer feeding experiments, studies were carried out on the formation of phytin in the growing pea plants, with the intention of obtaining some information regarding the time at which phytic acid synthesis approaches a maximum rate. Previous investigators (29, 31, 40, 48) used a variety of methods to determine the phytin phosphorus concentration in plants, ranging from the diminution in color of a ferric thiocyanate solution (29) (which by a suitable choice of concentration of acid and of the reactants can be made almost proportional to the phytate concentration over a limited range), or separating phytin and orthophosphate chromatographically on paper by use of suitable solvent systems and then estimating the spot area or spot lengths after development of the chromatograms (48), and/or the standard colorimetric determinations of inorganic phosphate before and after hydrolysis of the phytin phosphate (49).

1) Changes in phytin phosphorus in relation to inorganic orthophosphate.

These determinations were carried out according to the method of Earley and Deturk (49), and the colorimetric determinations of phosphate were done according to the procedure of Dickmann and Bray (50). This particular method of Earley and Deturk has advantages over the methods devised by Holt (29) and Fowler (48). In the method of Holt, where phytin concentration was measured by the diminution in color of a ferric thiocyanate solution, certain anions (notably orthophosphate) are found to interfere and the orthophosphate concentration during the period of study here is quite high. Similarly, the method of Fowler has the disadvantage that the estimation of phytin phosphorus and orthophosphate by measuring the spot area or spot length and or measuring the density of the spots on

paper chromatograms is more a qualitative procedure than quantitative and will give lower concentration values.

Richardson and Axelrod (51) have shown in a number of higher plants that "bound" inositol decreases sharply after germination, remains more or less constant for a while and then slowly increases as the plant approaches maturity and seed production. Similar studies here on the changes in total phosphorus and phytin phosphorus content of the pea plants indicate that phytin concentration drops sharply during germination and remains at an essentially constant low level until maturity. Typical results are shown in Table I and Figure I. It is apparent from figure I that the results of Richardson and Axelrod (51) and this study are in agreement i.e. the decrease in "bound" inositol during and just after germination does in fact represent largely breakdown of phytin phosphorus. It is worthwhile to mention here that studies of Richardson and Axelrod were concerned with the total "bound" inositol, whereas in the present case only the phytin phosphorus was determined, but the results are still in agreement.

These results indicate a great difference in phytin concentration in the flowering pea plants and the matured pea pods, where the concentration is relatively higher, giving indications of its synthesis during seed formation. Therefore, it was decided to carry out tracer feeding experiments with the immature pea pods, where the seeds are just begining to form. Because of ease in handling a dwarf variety "Little Marvel" was selected for tracer studies.

C) Feeding experiments.

(A) Preparation of myo-inositol-1-C^{14}

For the reasons mentioned in the introduction, it was decided to use

first myo-inositol-C^{14} as a possible precursor of phytic acid. This compound is not obtainable commercially, and several methods of preparation had to be tested before a suitable procedure was developed.

The studies of Richardson and Tolbert (51) indicated labelling of inositol both in 10-day old pea plants and in excised maturing pea pods when exposed to a $C^{14}O_2$ atmosphere. A number of procedures were followed in order to devise a method adequate for the isolation of C^{14}-inositol and which would give good yields.

Initial studies, where the flowering pea plants were exposed to a $C^{14}O_2$ atmosphere in a jar, gave very poor results due to a very little time exposure of plants to $C^{14}O_2$ atmosphere and also to more than adequate exposure of plants to light resulting in water depletion from the plants and condensation of this water on the walls of the jar. This also resulted in the wilting of plants. Overall result of this experiment was a poor incorporation of $C^{14}O_2$ into the inositol fraction, which was due in part to the feeding time period and to the fact that maturing pea plants are slow to incorporate $C^{14}O_2$ into inositol. The small amount of $C^{14}O_2$ incorporated into these plants went largely into the glucose and sucrose fractions.

In early studies where the $C^{14}O_2$ was formed and fed to pods within a single Thunberg tube, the extent of labelling over a period of 3-5 days was mostly confined to the glucose and sucrose fractions, and those fractions were also poorly labelled, whereas the labelling in the inositol fraction was negligible.

In later cases, where young pea pods were exposed to an atmosphere of $C^{14}O_2$ in a partially evacuated Thunberg tube for three days and where lactic acid was used for generating $C^{14}O_2$, somewhat better results were obtained,

but not good enough to be used as a routine method for the isolation of C^{14}-inositol. Feeding periods of one to three days were unsatisfactory, since the major labelled components corresponded to sucrose and glucose, with inositol a lesser component. Five days gave better yield of inositol. From one five day experiment in which 1.0 millicurie of $C^{14}O_2$ was used, a total of 1.0 microcurie of purified inositol activity was obtained; at the same time the combined glucose plus sucrose fraction was also eluted and gave 7.3 microcuries.

In later experiments the procedure was modified to obtain maximum amount of labelling in the inositol fraction rather than in the glucose and sucrose fractions. In this modified procedure 12 to 16-day-old plants were used; they were exposed to an atmosphere of $C^{14}O_2$ in a vacuum desiccator for a period of 10 days. In a typical case 1.0 millicurie of $C^{14}O_2$ was used to feed 10 plants and yielded 26.0 microcuries of purified inositol. Not only does this represent a great improvement in yield, but also the ease of purification of the inositol was greatly increased. In all the cases where the young pea seedlings were used, total incorporation of $C^{14}O_2$ into other sugar fractions was less than one-third of the incorporation into inositol.

From the experiments described above it appeared that the best procedure for the isolation of C^{14}-inositol consisted in feeding of $C^{14}O_2$ to 12 to 16-day-old plants for a period of 10 days in a vacuum desiccator. This became the standard procedure and was used for the preparation of C^{14}-inositol whenever needed.

The C^{14}-inositol was isolated and purified from the 80% ethanol extract by paper chromatography and its purification was followed by means of radioautograms. A number of solvent systems were used for effecting a

good separation of inositol from the other sugar components present in the 80% ethanol extract. Out of the five different solvent systems tried, two of them, namely n-propanol: concentrated ammonium hydroxide: water (6:3:1) and pyridine: amyl alcohol: water (7:6:3), seem to separate the components very readily, but the latter solvent was used most often in the preparative separations. It gave a better resolution of the whole 80% ethanol extract and the spots were more compact and clearer than those obtained in any of the other solvents used.

(B) Feeding experiments with myo-inositol-U-C^{14}:-

Various attempts were made to study the incorporation of C^{14}-inositol in the developing pea pods. These studies gave a clear indication that the best way to feed C^{14}-inositol to the maturing pea pods and study its incorporation consisted in injecting the C^{14}-inositol solution into the interior space of the pods by means of a microsyringe.

Variation of two main methods was tested for effectiveness. In the first case, where maturing pea pods were removed from the plants and placed in beakers containing the inositol solution, very poor incorporation resulted. This type of feeding, both in evacuated desiccator and in the open air, resulted in fungus growth on the pods in less than three days and negligible incorporation of label into the pods.

In one case the pods were allowed to grow for six days in beakers containing a C^{14}-inositol solution. After six days the pods were showing infection by mold and were sacrificed. Incorporation in each pod was found to be less than one-half of one per cent of the added counts. (2.2×10^4 counts per minute of inositol).

This type of feeding was abandoned due to low incorporation and repeated contamination of the pods by molds or fungus growths.

In the second type of feeding experiments, C^{14}-inositol was injected into the interior space of the pods and the pods were left attached to the plants in order that they might continue to develop naturally. Mold or fungus growth was not observed in connection with this type of feeding and this enabled the study of incorporation of label at various time intervals at least up to sixteen days, without major complications.

In a typical case where four immature pods of about 3 cm length were injected with a total of 3.5×10^4 counts per minute, a seven day incorporation study showed a 30% incorporation of the injected inositol in some non-alcohol-extractable form in the pods. Also, about 8% of the incorporated C^{14}-inositol could be located in the barium phytate fraction as shown in Table II. These results were encouraging and it was anticipated that sufficient incorporation for precursor studies could be obtained by feeding higher levels of C^{14}-inositol.

Later studies of incorporation of C^{14} from inositol into phytic acid fractions with increasing time indicated that about 25-30% of the incorporated inositol could actually be located in the barium phytate fraction after a maximum feeding period of 16 days. Also, labelled carbon recoveries in the phytic acid fraction were very low between two to four days and in fact there was no incorporation at all into phytic acid within the first twenty-four hours after feeding C^{14}-inositol. These percentage incorporation values were calculated on the basis of the total counts recovered from the pod homogenates and not on the basis of the total counts fed as C^{14}-inositol.

Based on these observations, further attempts were made to locate the label in components other than the Barium phytate fraction. For this purpose, the pod homogenates were extracted with 80% ethanol to get a

sugar fraction. (Figure VII) The residue was further extracted with ethylene dichloride which gave a crude lipid fraction.

The 80% ethanol extract (Figure VII) when spotted on chromatograms indicated the presence of six different components, namely: inositol, glucose, sucrose, xylose, glycerol and an organic phosphate ester. All of these six components had incorporated label, with considerable labelling in the organic phosphate ester and the inositol. The labelling of the rest of the sugars and the glycerol was less significant and the variations were very slight for as long as the studies were carried out. Typical results of Experiment VIII showing the distribution of activity in these components are presented in Figure VIII.

The results indicate a high incorporations of label in the organic phosphate ester. The identity of this organic phosphate ester is still not known, despite various attempts. However, some of the products of hydrolysis of this compound have been identified by paper chromatography. The hydrolysis was carried out in 6N hydrochloric acid for 3 hours at 100°C and the hydrolysate showed the presence glucose, inositol and inorganic phosphate.

The ethylene dichloride extract containing the lipids showed rapid and extensive incorporation of C^{14}-inositol, which is probably due to the incorporation of C^{14}-inositol into the phosphoinositide, since this is the only inositide reported in peas (47). This is clearly shown in Figure VII. The incorporation of label into the lipid fraction (shown in Figure VII) was found to be very rapid immediately after injection of inositol, with a later lowering in level and concomitant rise in the incorporation of label into the phytic acid fraction.

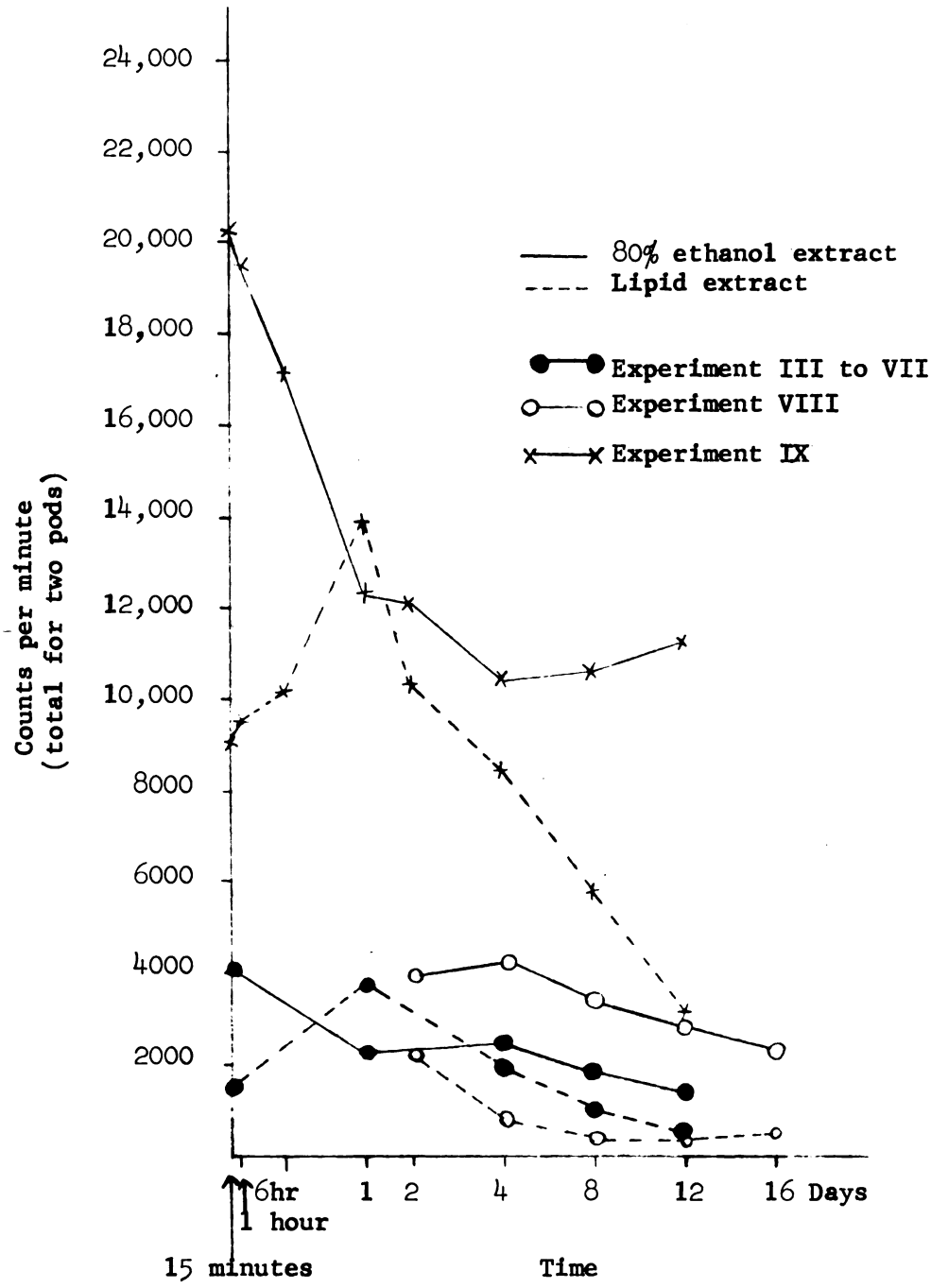


Figure VII:- Incorporation of C^{14}-inositol into the lipid and the 80% ethanol fractions in the maturing pea pods.

An acid hydrolysis of the lipid fraction was carried out in an attempt to determine the labelled component in the inositide.

The lipid hydrolysate on chromatography showed the presence of inositol and glycerol, but only the inositol of the hydrolysate appeared to be labelled. This was determined from the results obtained on running a paper chromatogram through a special paper-strip counter.

Later feeding experiments were run on a comparative basis in order to study the C^{14}-inositol incorporation with increasing time.

In Experiments I and II, the results of which are presented in Table III, the feeding period varied from a week to ten days.

The results show that seven day feeding period in Experiment I gave a considerable increase of label in the lipid fraction, where as the incorporation of label into the phytic fraction is quite low.

The increase of the feeding time to 10 days in Experiment II, gave higher yields of incorporated label in phytic acid, whereas the labelling in the lipid fraction declined. There is further an increase of label in the 80% ethanol extract, which could possibly be due to the breakdown of the phosphoinositide present in the lipid extract or of some other inositol derivative.

In Experiments III to VII, where pois were fed a total of 4.2×10^3 counts per minute and sacrificed at 15 minutes, 24 hours, 4, 8, and 12 day intervals, a steady increase in the incorporation of label into the phytic acid was observed, starting with zero incorporation after 15 minutes and increasing to a maximum of 10.3% of the total incorporation after 12 days. The results are presented in Table IV.

The recovery of the total label in the homogenates is also decreased with increasing time, which may be a result of the translocation of the

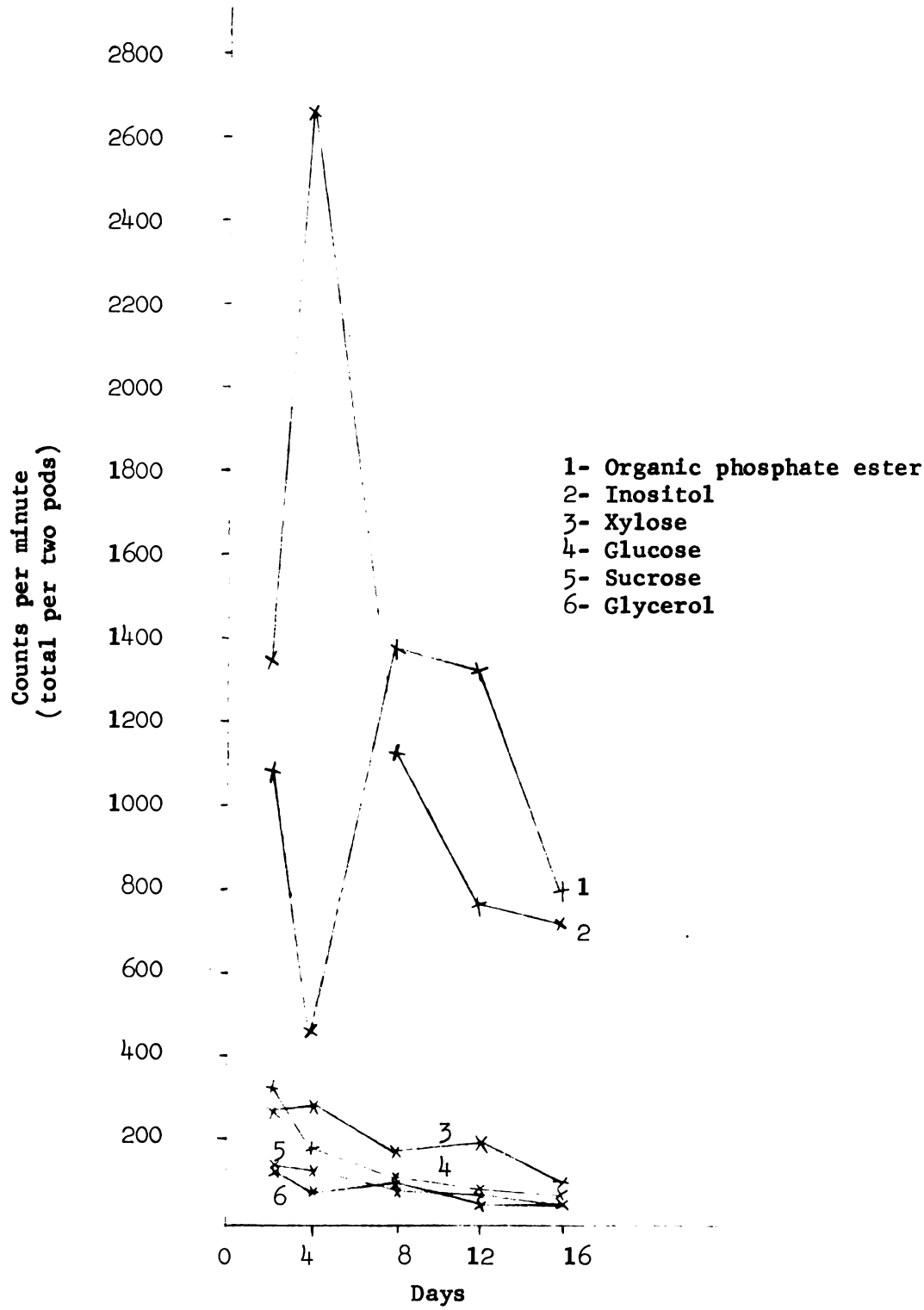


Figure VIII:- Incorporation of label into components of the 80% ethanol extract obtained from pods fed C^{14}-inositol (Experiment VIII).

fed C^{14}-inositol to other parts of the plant, or may represent losses due to metabolic oxidations to CO_2 and water.

The results of Experiments VIII and IX are shown in Table V. These experiments were performed to study the effect of dark periods on the incorporation pattern. In both cases the pods were fed the same amounts (2.1×10^6 counts per minute) of C^{14}-inositol of specific activity 2.0×10^6 counts per minute per millimole.

In Experiment IX the pods were grown under constant light conditions whereas in Experiment VIII the light supply was discontinued at night for about 6-7 hours. The results once again indicate a steady increase of incorporation of label into phytic acid fractions in both the experiments, although the total recoveries are lower in Experiment VIII than in Experiment IX.

There is an initial rapid incorporation of C^{14}-inositol into the lipid fraction; after 24 hours the label starts to disappear rapidly until the fourth day, after which the decrease of label is slow. Further it is also clear from Table V that the incorporation of C^{14}-inositol into phytic acid in Experiment VIII is greater for the total period than in Experiment IX in terms of total counts per minute.

The result of incorporation of C^{14}-inositol into various fractions of Experiment VIII are shown in Figure IX.

The decreased incorporation of C^{14}-inositol into phytic acid under conditions of continuous light could be explained on the grounds that the pea pods are synthesizing inositol and this synthesis of inositol will be comparatively much greater in the presence of continuous light. This would mean that the injected C^{14}-inositol, though incorporated in the phytic acid, is in competition with the inositol formed in the pods, which accounts

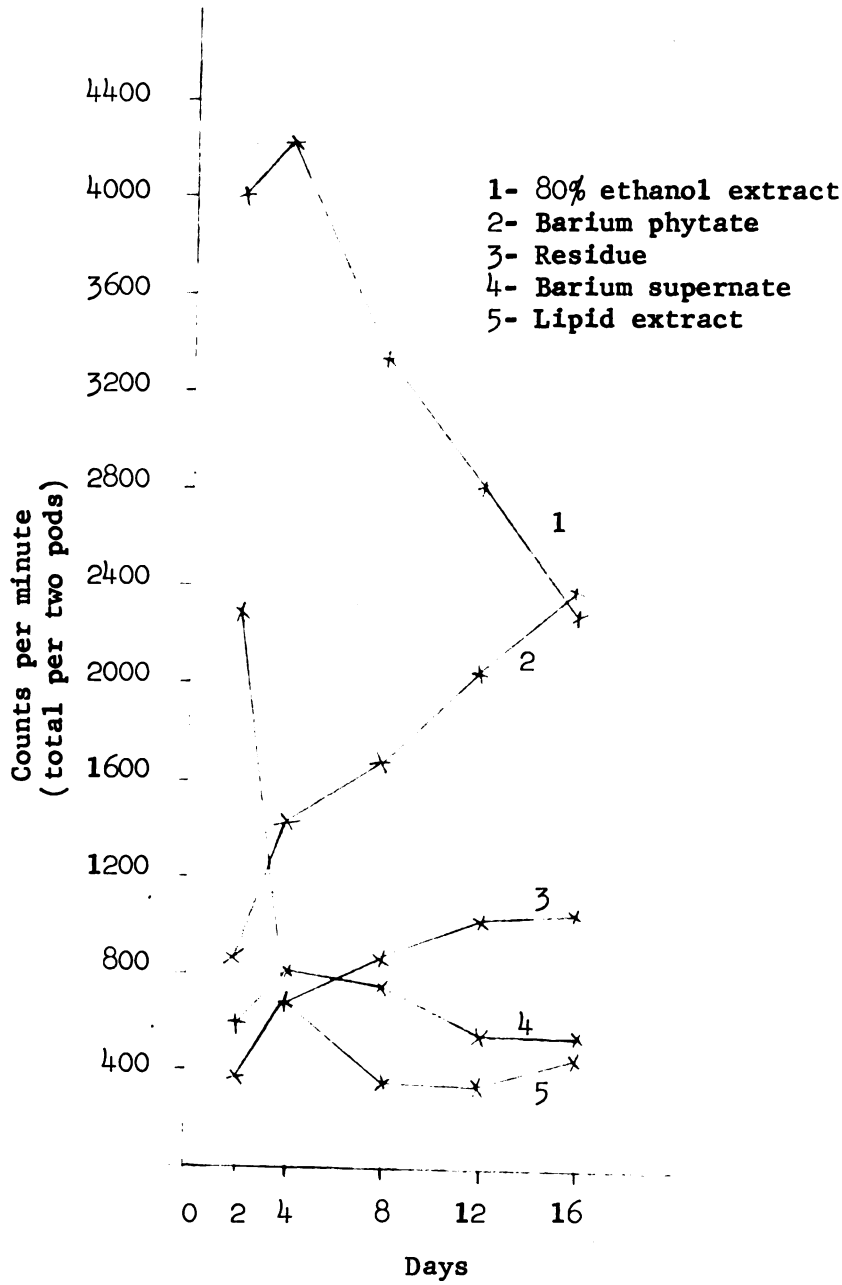


Figure IX:- Distribution of label at various times after injection of C^{14}-inositol into maturing pea pods. (Experiment VIII).

for the lesser incorporation of C^{14}-inositol under continuous light conditions.

Also, the low total recoveries of label in Experiment VIII may be due to the fact that during the dark period, when photosynthesis-linked energy production ceases, the plant systems are metabolizing endogenous materials and one such available compound is the injected C^{14}-inositol. Since it is known that inositol is converted to glucose, xylose and sucrose etc., this could provide a route for its eventual destruction. Translocation into other parts of the plant may also be increased during dark periods. This possibility was not investigated during this work.

A portion of the carbon of the fed inositol is also deposited in the cell walls, which is indicated by a constant increase of label in the final residues with increasing time periods.

Along with the constant increase of C^{14}-inositol incorporation into phytic acid, the supernatant solution left remaining after barium precipitation of phytic acid also showed increase in labelling up to a period of four days and then started to decrease. It is possible that this label lost from the supernantant fraction between four and eight days appears in the barium phytate at eight days, since the results indicate a great increase in the incorporated label in the barium phytate in going from four to eight days. Results appear in Figure X. However, the decrease of labelling in the lipid fraction can also account for such an increase in the barium phytate fraction and this might well be true in Experiment IX, where the drop in the labelling of lipid fractions is considerable in going from four to eight days. In this case, however, all of the decrease cannot be accounted for in the barium phytate fraction. A similiar conclusion cannot be drawn in Experiment VIII, where loss of label from

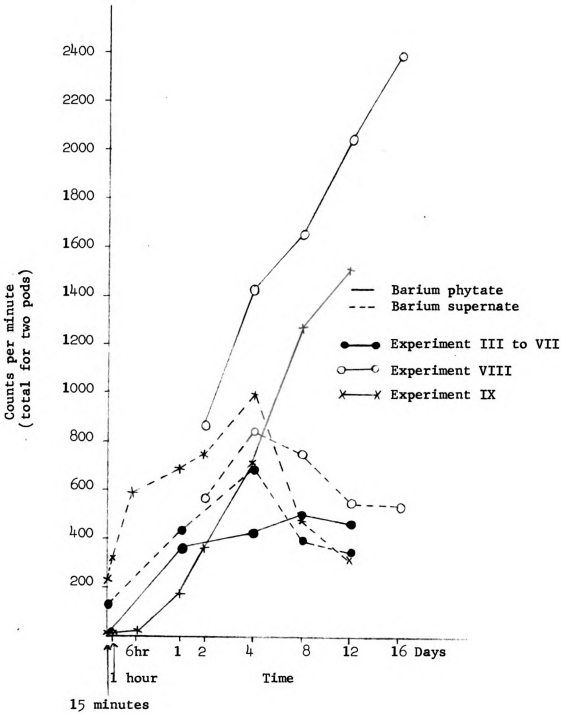


Figure X:- The incorporation of C^{14}-inositol into the barium phytate and barium supernate fractions at various time intervals in the maturing pea pods.

lipid fraction in going from four to eight days is essentially negligible compared to the loss from the barium supernatant fraction and also compared to the increase in the barium phytate. It is possible that the components of both the lipid fraction and the barium supernatant fraction contribute to the barium phytate labelling.

From these observations the following conclusions can be drawn:-

I) That the injected C^{14}-inositol appears in the barium phytate fraction only after 24 hours and further that this incorporation increases with time.

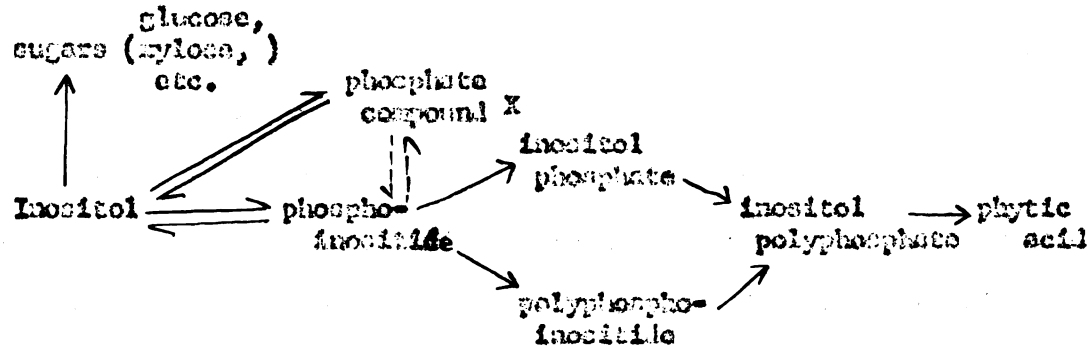
2) The labelling of the lipid fraction reaches a maximum at the end of the first 24 hours and then starts to decrease, with considerable loss of labelling being observed between one to four days.

3) Slow and constant incorporation of C^{14}-inositol in the barium supernatant fraction up to four days, followed by a sudden loss of label from four to eight days and then a gradual decrease.

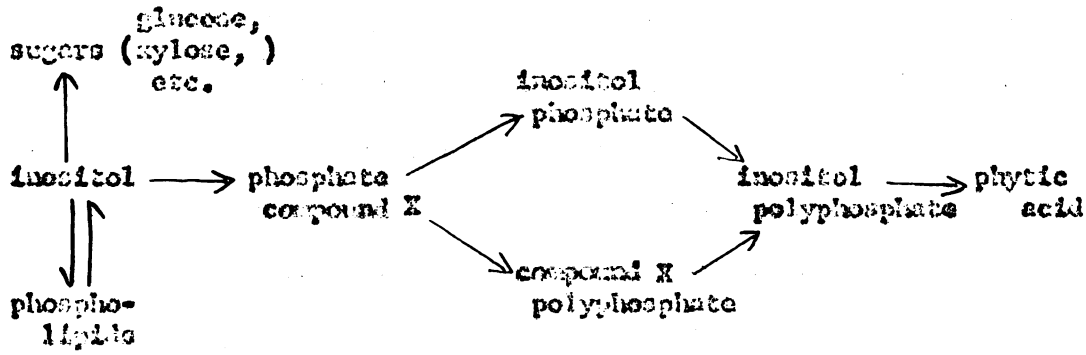
4) The recovered label in the 80% ethanol fraction decreases with time.

The results of these studies are consistent with any one of the following possible pathways for the biosynthesis of phytic acid:

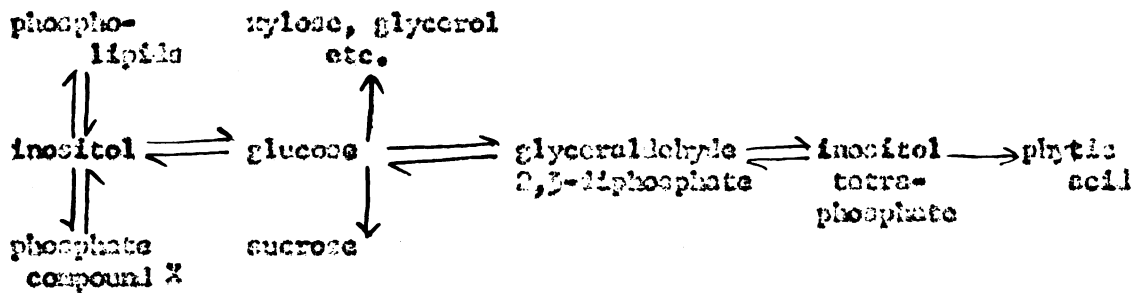
a)



b)



c)



Between the first two pathways, it is not yet possible to make even a tentative choice. Pathway (c), however, seems less likely than either (a) or (b). First, incorporation of C^{14} label into various sugars is relatively small compared to other compounds, especially in view of the steady increase in incorporation into phytic acid. Secondly, since it may be expected that glucose will be built rather rapidly into the cell-wall constituents, if glucose were an active intermediate in phytic acid formation, it should produce a relatively rapid labelling in the residue material after extraction. Actually, this material becomes labelled only very slowly (see Tables III, IV and V).

Whether one or the other of the paths shown, or some combination of them, represents the correct sequence of reactions in formation of phytic acid will have to await further examination of the structures and roles of the "phosphate compound X", the phosphoinositides, and the various compounds present in the "barium supernate".

LITERATURE CITED

1. Palladin, W., Z. Biol., 31, 199 (1895).

2. Winterstein, E., Ber. Dtsch. Chem. Ges., 11, 2299 (1897).

3. Capt, E., (Estab. Federaux essais analyses agr., Lausanne, Suitz) Mitt. Gebiete Lebensm. U. Hyg., 47, 431 (1956).

4. Corlomier, R., Ann. Inst. Natl. Recherche agron., Ser. E., 2, 179 (1954); Chim and Ind. (Paris) 71, 117 (1954).

5. Barrel, R., Ann. Pharm. Franc., 14, 182 (1956)

6. Mellanby, E., and Harrison, D.C., Biochem. J., 33, 1660 (1939).

7. Kiby, W.H., Miller., H.R., and Haft, H.L., Proc. Amer. Soc. Biol. Chem., J. Biol. Chem., 141 50 (1941).

8. Roberts, H.A., and Yudkin, John., Nature 165, 803 (1950).

9. Common, R.H., Nature., (London)., 143, 379 (1939).

10. Shohl, A.T., 'Mineral Metabolism' 1939, P. 175 (New York: Rheinhold Corp.).

11. Nobuji Onishi., Eiyo to Shokuryo 2, 86 (1950).

12. Chalkley, V.H., and Lane, R.L., Proc. Soc. Exptl. Biol. Med., 54, 53 (1943).

13. Weilbein, E.R., Jr., Mellon Inst. Bibliographic Ser. Bull. 6 (1951).

14. Anderson, R.J., J. Biol. Chem., 12, 441 (1914).

15. Anderson, R.J., J. Biol. Chem., 20, 463 (1915).

16. Wenzel, W., Landwirtsch. Forsch., Sonderh., No. 9, 74 (1957).

17. Schormuller, J., and Wurdig, G., (Tech. Univ., Berlin-Charlottenburg) Dent. L benem Rundschau 5, 1-10 (1955).

18. Rather, J.B., J. Amer. Chem. Soc., 39, 1935 (1917).

19. Ashton, W.W., and Williams, P.C., J. Sci. food. Agr. (England) 2, 505 (1950).

20. Holt, R., J. Sci. food. Agr., 6, 136 (1955).

21. Fowler, H.D., J. Sci. Food. Agr., 7, 381 (1956).

22. Bogoroditskaya, V.P., (Nutrition Inst. Acad. Med. Sci., U.S.S.R., Moscow) Voprosy Pitaniya. 14, No. I 55 (1957).

33. Hoffmann-Ostenhoff, O., Jungfarth, C., and Deuel, H.E., (Univ. Vienna), Naturwissenschaften, 40, 685 (1953).

34. Hübscher, G., and Hawthorne, J.N., Biochem. J., 61, 55P (1955).

35. Hübscher, G., and Hawthorne, J.N., Biochem. J., 62, 503 (1957).

36. Anderson, R.J., J. Biol. Chem., 59, 475 (1924).

37. Fleury, P., and Courtois, J., Helv. Chim. Acta., 29, 1297 (1946).

38. McCormick, M.M., and Carter, H.E., Biochem. Preps., 2, 69 (1952).

39. Dangeard, A., and Fleurent, P., Compt. Intern. Biochim., Resumes Communs., Compt. Brussels., 22, (1955).

40. Wooley, D.W., J. Biol. Chem., 147, 581 (1943).

41. Folch, J., Fed. Proc., 6 (1947).

42. Carter, H.E., Celmer, W.D., McCormick, M.M., Nyman, C.O., and Stansbury, F.W., Fed. Proc., 6, 190 (1947).

43. Hawthorne, J.N., and Chargaff, E., J. Biol. Chem., 206, 27 (1954).

44. Scholfield, C.R., and Dutton, H.J., J. Biol. Chem., 208, 461 (1954).

45. Olcott, H.S., Science., 120, 256 (1954).

46. Morelec-Goulon, M.J., and Favre, M., (Paris) Bull. Soc. Chim. Biol., 39, 947 (1957).

47. Hepherson, L.E., and Lucas, C.C., Fed. Proc., 6, 173 (1947).

48. McKibbin, J.M., J. Biol. Chem., 210, 537 (1954).

49. Folch, J., J. Biol. Chem., 177, 505 (1949).

50. Tomlinson, R.V., and Bellow, C.E., J. Biol. Chem., 236, 1910 (1961).

51. Brockerhoff, H., and Bellow, C.E., J. Biol. Chem., 236, 1907 (1961).

52. Wagenknecht, A.C., Lewin, L.M., and Carter, H.E., J. Biol. Chem., 232, 1035 (1958).

53. Ayanoff, B.N., Bradley, T.M., and Brady, R.O., J. Biol. Chem., 233, 1077 (1958).

54. Thompson, W., Strickland, R.F., and Rossiter, R.J., Fed. Proc., 17, 309 (1958).

55. Paulus, H., and Kennedy, E.P., J. Amer. Chem. Soc., 80, 6000 (1958).

56. Paulus, H., and Kennedy, E.P., J. Amer. Chem. Soc., 81, 4856 (1959).

47. Paulus, H., and Kennedy, E.P., J. Biol. Chem., 235, 1303 (1960).

48. Fowler, H.D., J. Sci. Food Agr., 8, 333 (1957).

49. Harley, E.B., and Detwiler, E.E., J. Amer. Soc. Agronomy., 36, 603 (1944).

50. Dickmann, S.R., and Bray, R.H., Ind and Eng. Chem., Anal. Ed., 12, 665 (1940).

51. Richardson, K.E., and Axelrod, B., Plant Physiol., 32, 334 (1957).

52. Axelrod, B., and Bandurski, R.S., J. Biol. Chem., 192, 405 (1951).

53. Ballou, C.E., and Anderson, A.B., J. Amer. Chem. Soc., 75, 648 (1953).

54. Anet, E.F.L.J., and Reynolds, T.M., Nature (London), 174, 909 (1954).

55. Clarke, G., J. Chem. Soc., 105, 939 (1914).

56. Greenberg, D.M., Symposium on phosphorus metabolism; ed. McElroy, W.D., and Glass, B., 1950, Vol. II, P 5-9 (Baltimore: John Hopkins press).

Reagents:-

Ammonium molybdate - HCl reagent:- 15 grams of ammonium molybdate (C.P) were dissolved in 300 ml. of warm distilled water (about 50°C) in a 1 liter volumetric flask. The solution was allowed to cool and 350 ml. of 1:1 hydrochloric acid was added slowly with shaking to this solution. The solution was cooled to room temperature and diluted to one liter. After thorough mixing the solution was stored in an amber colored bottle. This solution is stable for two months.

Stannous chloride stock solution:- Ten grams of $SnCl_2.2H_2O$ were dissolved in 25 ml. of concentrated hydrochloric acid in an amber colored bottle. This solution is also stable for two months.

Dilute stannous chloride solution:- To one ml. of the stannous chloride stock solution was added 333 ml. of distilled water. This solution deteriorates rapidly and had to be prepared fresh daily.

Standard phosphate solution:- Weighed 0.7165 gm. of KH_2PO_4 in a one liter volumetric flask and diluted to volume. This stock solution contained 50 P.P.M. of phosphorus. Fifty ml. of this solution was diluted to 500 ml. to obtain a solution containing 5 P.P.M. of phosphorus.