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Vitamins in Germination

DETERMINATION OF FREE AND COMBINED INOSITOL IN GERMINATING OATS

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The earliest record of the occurrence of inositol in plant materials appears to be that of Vohl (1856), who isolated inositol from haricot beans. It has subsequently been found in all plants examined, and its combined form phytin, a calcium, magnesium and potassium complex of inositol phosphoric acids, also appears to be ubiquitous in the plant world. Inositol has been found in phospholipids of soya bean (Klenk & Sakai, 1939; Woolley, 1943); of ground nut (Hutt, Malkin, Poole & Watt, 1950); and maize (Scholfield, McGuire & Dutton, 1950). Hawthorne & Chargaff (1954) isolated, from soya bean phosphatides, sugars in combination with inositol monophosphate.

Quantitative studies of inositol have been limited hitherto by the lack of satisfactory analytical techniques and by the difficulty of distinguishing it from its isomers and congeners. The microbiological method is the most sensitive and accurate method at present available for the assay of *meso*-inositol, and in the following studies we have used *Schizosaccharomyces pombe* as assay organism on the lines developed by Northam & Norris (1952), with modifications introduced by Norris & Darbre (1956).

The study of vitamins of the B group in the germination of seeds necessarily waited on the growth of knowledge of the group and the development of satisfactory methods of assay. In the last 10–15 years the changes in content of a number of B vitamins in seeds during germination have been investigated by several workers. The literature has been reviewed by Norris (1947, 1950). In general, the reported changes, whether of increase or decrease of a particular vitamin after germination, have tended to become smaller as time went on. This is due largely to improvements in assay techniques, but also to greater care in planning experiments and assessing results. Burkholder (1943) reported an increase of inositol from 630 μg./g. dry wt. to 1290 μg./g. after 5–6 days' germination of oats. Taking into account the loss in dry weight of the seed on germination, from data given by Burkholder, we have calculated a real increase from 12·2 to 19·1 μg. of inositol/seed. This increase is in sharp contrast with the decrease reported in the present paper. Differences may be due in varying degrees to much higher temperatures and humidities in America than are normal in this country. The analytical procedure is open to question as it involved digestion with a papain–Taka diastase mixture, a method found to result in incomplete liberation of inositol (Jones, 1951).

The literature offers a wide choice of conditions for the liberation of inositol from phytin, whether by chemical (Bartow & Walker, 1938) or enzymic means (Peers, 1953). We believe that the sealed-tube method with N-HCl described below gives a

maximum release of inositol. Hydrolysis by added phytase releases inositol to an extent closely approaching that by acid, but has the disadvantage that extremely lengthy periods of incubation are necessary.

The lipid-inositol fraction in oats is very small, and it is not certain whether acid hydrolysis releases all the inositol in this combined form. McKibbin & Taylor (1949) discuss methods of hydrolysis of lipids, and for the subsequent assay of lipid inositol we have found their method the most satisfactory (Taylor & McKibbin, 1953).

It is hoped to follow this contribution on inositol in the germinating oat seed with a further study of inositol in the germinating bean. Folic acid in these seeds is also under investigation.

EXPERIMENTAL

Germinated oats. Two samples of oats, S. 147 (1951 and 1947), designated batch I and II, were available from previous studies. The seeds of batch I were germinated in Petri dishes, stacked and separated, in large vessels. A slow stream of air, passed through cotton-wool filters, bubbled through water at the bottom of the vessels. The dark-room temperature was constant at 25°, and humidity in the vessels also constant. Batch II had been germinated in bulk on the malting floor in a commercial maltings. The samples were not kiln-dried, but were dried in air, finely ground and stored in tightly stoppered bottles. In the present paper a designation batch I, 2, will indicate a sample withdrawn from batch I after 2 days' germination, and so on.

General methods

Moisture content. The Fischer drier was used with boiling $CHCl_3$ (61°) and P_2O_5 *in vacuo*. The individual samples, weighing about 100 mg., were placed in porcelain weighing boats, and from 8 to 24 hr. was required for them to attain constant wt. All results were calculated to this dry wt. basis.

Mean weight of a seed. The mean wt. of seeds at each stage of germination was determined by counting out at least nine sets of seeds, each set containing varying numbers of seeds from about 50 to 120. Each set was weighed to the nearest 0·1 mg. and the mean wt. of one seed in each set determined. The combined means were then averaged; the standard deviation was normally of the order of 3% and was not considered significant in relation to later procedures involved. The mean wt./seed was calculated to dry wt. after moisture determination.

Assay of inositol. The method used was described by Norris & Darbre (1956). The test organism was a strain of *S. pombe*. Minor modifications in the original method of Northam & Norris (1952) were made as a result of stringent experimental tests. The most important of these were: (*a*) the elimination of asparagine from the medium, since asparagine had no apparent effect on the growth of the organism at 24, 48 and 72 hr.; (*b*) the concentration of biotin was doubled to ensure an adequate supply to the organism. Otherwise the medium corresponded to 'Medium BMI Supp.' of Northam & Norris (1952). Computation of the results was normally made by direct reading from a standard curve. More detailed computations (Norris & Darbre, 1956) were carried out as a check from time to time. The method is, in general, accurate within ±3%.

Total inositol

The choice of hydrolysing agent is influenced by the effect on the assay organism of the nature and amount of the salts formed when the hydrolysate is brought to pH 4·8 for assay purposes. Tests were carried out to study the effect of increasing concentrations of salts such as NaCl, KCl, NH_4Cl, Na_2SO_4, K_2SO_4 and $(NH_4)_2SO_4$, which would be formed when neutralizing the various acids and alkalis used. Provided that the amount of salt added was less than 4 mg./tube, the organism was not detectably inhibited. Inhibition by salts also depended to some extent on the concentration of inositol present. Thus with 1 μg. of inositol/tube the organism was more sensitive to salts than with 2 μg./tube. In general, the concentration of salts should be low enough to ensure that there is no inhibition of growth at the levels of inositol under test.

Alkaline hydrolysis, whether with sodium, potassium or ammonium hydroxide, resulted in low yields of inositol and was abandoned. Sulphuric acid up to 10N was used in attempts to shorten the time of hydrolysis, but neutralization of concentrations higher than N with sodium, potassium or ammonium hydroxide produced amounts of salts which inhibited the organism. Removal as $BaSO_4$ involved losses by adsorption of inositol on the precipitate. Although Gregory (1935), using a much less sensitive method of estimation, stated that inositol was not adsorbed by barium sulphate under acid conditions, Needham (1923) and Bartow & Walker (1938) reported difficulties due to adsorption on insoluble precipitates. Sulphuric acid was found to be less effective than HCl at all concentrations except 0·5N for the same heating period.

Hydrochloric acid appeared to be the most satisfactory hydrolysing agent and the possibilities were examined in detail. Hydrolysis by refluxing was not satisfactory owing to the black sublimate which always formed in both water and air condensers; moreover, the length of time necessary was inconvenient when large numbers of samples were involved.

A more promising method involved heating up to 200 mg. of the test material with 2 ml. of hydrolysing agent in a sealed Pyrex tube at 123° (equivalent to 15 lb./in.²) in an electric oven. Table 1 shows results with N-HCl.

Similar results with a rather broad maximum around 48 hr. were obtained with other materials such as soya-bean flour, barley, etc. (Norris & Darbre, 1956). The peak values obtained with N-HCl could also be reached by using 0·5N-HCl (96 hr.), 2N-HCl (30 hr.), 3N-HCl (24 hr.), 4N-HCl (18 hr.), 5N-HCl (12 hr.) and 6N-HCl (8 hr.). These periods of heating could be considerably extended without significantly affecting the values of inositol obtained. Inositol was stable under these conditions. For example, 250 μg. of

Table 1. *Liberation of inositol from oats by acid*

A sample of batch II, 2, was heated in a sealed tube with N-HCl, at 123°.

Time of heating (hr.) ...	18	24	36	48	60	69
Inositol liberated (μg./g.)	2340	2430	2610	2630	2620	2550

inositol was heated with 2 ml. of N-HCl for as long as 88 hr. at 123°, and 247 μg. was recovered; this was a complete recovery within the experimental limits involved.

Use of the more concentrated acids necessitated removal of the bulk of acid under diminished pressure before neutralization and making up to volume. By the use of 2 ml. of N-HCl, and neutralizing without distillation and making up to 100 ml., a solution with which no inhibition of the organism was detectable was obtained. In practice the dilution for assay purposes is much greater than this and no effect of salt concentration need be feared.

Methods involving the autoclave would be more convenient than the sealed-tube method, but the long periods necessary for complete liberation of inositol render the autoclave method impracticable. Jones (1951) liberated inositol from yeast by autoclaving samples with increasing concentrations of HCl for 1 and 2 hr., and with H_2SO_4 for 2 hr. This method was shown to be inferior to the sealed-tube method for yeast (Norris & Darbre, 1956) and for oats (Table 2).

Method adopted. About 100 mg. of sample was heated with 2 ml. of N-HCl in a sealed Pyrex tube at 123° for 48 hr. After cooling, the tube was opened, the hydrolysate filtered with numerous washings into a measuring flask, the pH adjusted to 4·8 with NaOH and the solution made up to volume. If the solution had to be kept a short time before assay it was heated to 90° in a water bath and after cooling was stored at 0–4°.

Free inositol

To obtain accurate values for pre-existing free inositol: (*a*) extraction from the sample must be as complete as possible; (*b*) enzyme action and hydrolysis during extraction must both be prevented; (*c*) the method of extraction must be suitable in relation to the subsequent method of assay.

Table 2. *Liberation of inositol from oats by autoclaving with acid*

Samples of batch II, 10, were autoclaved for 2 hr. at 15 lb./in.²

Concn. of acid	Inositol liberated (μg./g.) HCl	H_2SO_4
N	370	380
2N	410	—
3N	410	390
5N	650	420

Content by sealed-tube method: 2400 μg./g.

Table 3. *Extraction of free inositol from oats (batch II, 2)*

A sample (1 g.) was homogenized with 10 ml. of extractive for 2 min. at 2000 rev./min., the suspension made up to 25 ml. and allowed to stand at room temperature for the times indicated.

Extractive	Inositol extracted (μg./g. dry wt.) 10 min.	30 min.	3 hr.	36 hr.
Water	92	99	136	239
N-HCl	96	96	95	96
0·04N-HCl	88	91	89	86

(*a*) To ensure complete extraction the samples were treated in a stainless-steel homogenizer of the type suggested by Potter & Elvehjem (1936).

(*b*) The use of various extractives for varying periods was studied and typical results are shown in Table 3. The larger and increasing figures for water extraction indicate clearly that autolytic enzymes were active. With N-HCl slightly higher figures were given than with 0·04N-HCl, probably attributable to a slight degree of hydrolysis by the more concentrated acid. The figures for 0·04N-HCl may be regarded as satisfactory, additional experiments having shown that the autolytic enzymes are completely inactivated by 0·04 or 0·02N-HCl and -NaOH. For example, there was no additional liberation of inositol when the 0·04N-HCl was neutralized and the sample incubated at pH 5·4 and 37° for as long as 600 hr.

(*c*) The low concentration of NaCl produced by neutralization of the 0·04N-HCl and subsequent dilution had no effect on the assay organism.

Method adopted. A portion (1 g.) of the sample was homogenized with 10 ml. of 0·04N-HCl at 2000 rev./min. for 2 min. The difference between the diameters of the homogenizer plunger and tube was 0·9 mm. No special precautions were taken to counteract rise in temperature, which never exceeded 4–5°. The mixture was then transferred with numerous small washings to a 50 ml. centrifuge cup. After standing, with occasional stirring, for at least 10 min., the extract was separated by centrifuging and the residue washed four times; three washings removed all detectable inositol and the fourth represents a safety limit. The combined extracts were adjusted to pH 4·8 (bromocresol green used externally) and autoclaved at 15 lb./in.² momentarily. The precipitate was removed by centrifuging (or filtering through Whatman no. 1 paper if only small in amount) and a further four washings were carried out, after which the extract was made up to volume. Tests showed that inositol was not adsorbed on filter paper between pH 4·0 and 8·2. If not required immediately the solution was heated as described for total inositol and stored at 0–4°.

Inositol liberation by autolysis

The major portion of the inositol in cereal seeds occurs in combination with phosphoric acid. Inositol may be liberated from such phosphoric esters by the autolytic enzymes present, largely phytase, or by added phytase.

The effect of homogenization of the sample upon the action of the autolytic enzymes was studied, since although homogenization might be expected to enhance the liberation, this might be counterbalanced by some inactivation of the enzymes. A portion (1 g.) of oats, batch II, was homogenized for 2 min. with 10 ml. of water, transferred to a beaker and water added to 40 ml. The pH of this extract was 5·15 (measured with a glass electrode). A volume (4 ml.) of 0·2M sodium acetate–acetic acid buffer, pH 5·15, was added, and the volume finally made up to 100 ml. with water. Another 1 g. of the sample was similarly treated, but with omission of the preliminary homogenization. Both samples were then incubated at 39° under a layer of toluene and assayed at various times up to 48 days. The results are shown in Table 4, the initial content of free inositol being 86 μg./g.

The homogenization appears to decrease the amount liberated in the earlier stages as compared with that for the sample not so treated. At the end of the period the

homogenized sample has slightly overtaken the other. However, the release of combined inositol by the autolytic enzymes is extremely slow, and the amount reached after 48 days was still less than that obtained by acid hydrolysis (2650 μg./g.).

Method adopted. Samples (1 g.) were homogenized with 10 ml. of water for 2 min., made up to 100 ml., including 4 ml. of acetate buffer, pH 5·15, and incubated at 37° under a thin layer of sulphur-free toluene.

Action of phytase

The liberation of inositol by added phytase, prepared from barley by the method of Adler (1915), was carried out after preliminary inactivation of autolytic enzymes with 0·04 N-HCl as described previously.

The results of experiments with acetate buffers to determine the optimum pH for phytase action are indicated in Fig. 1. A convenient working optimum was taken as pH 5·4 [cf. Peers (1953), who proposed pH 5·15]. Since Hoff-Jørgensen & Porsdal (1947) had found that rye phytase was activated by lactate, use of a lactate buffer was compared with that of the acetate buffer. 0·2 M Sodium acetate–acetic acid and 0·2 M sodium lactate–lactic acid buffers (4 ml./100 ml. total volume) were used. No effect was observable with oats, which gave, for example, two results after 260 and 408 hr. of 2340 and 2630 μg./g. with lactate buffer, as compared with 2340 and 2650 μg./g. with acetate buffer.

Table 4. *Liberation of inositol by autolytic enzymes of oats*

Samples (batch II, 2) were mixed with water and incubated at 39°. pH 5·15.

Incubation time (hr.)	Inositol found (μg./g.)	
	Homogenized	Not homogenized
138	370	490
233	630	820
306	870	1100
408	1160	1200
1152	2300	2230

Method adopted. A portion (1 g.) of the sample was homogenized for 2 min. with 10 ml. of 0·04 N-HCl and allowed to stand for at least 10 min. The homogenate was then made up to 100 ml. in a volumetric flask with 4 ml. of 0·2 N acetate buffer at pH 5·4, and 0·025 g. of phytase. The mixture, under a thin layer of sulphur-free toluene, was incubated at 37–39°. Sampling was carried out by taking about 7 ml. of the well-stirred flask contents and centrifuging. A volume (5 ml.) of the supernatant was diluted with water, adjusted to pH 4·8, autoclaved and made up to volume as described for free inositol.

Lipid-combined inositol

This is the most difficult fraction to deal with, being both small in amount and of low inositol content. Different procedures were tested, and although results were not wholly satisfactory, there are points of interest, particularly with regard to the assay organism.

A number of assay organisms are affected by fats or fatty acids. *S. pombe*, however, appeared to be unaffected by their presence, since samples which were pre-extracted two or three times with ether or light petroleum (b.p. 40–60°) gave assay results not significantly different from those for untreated samples. *S. pombe* was unaffected also by choline, commonly present in lipid hydrolysates. Taylor & McKibbin (1952) found that their assay organism for inositol, *Saccharomyces carlsbergensis*, was inhibited to some extent with a molar ratio of choline to inositol of 0·3:1. With *S. pombe* a molar ratio as high as 1450:1 was without effect. In attempting to assay lipid-combined inositol several procedures were tested.

Extraction of lipid material. Two methods were tried. (i) Preliminary treatment with 95% ethanol, followed by continuous extraction with ethanol–ether (3:1) (Bloor, 1929). (ii) Complete extraction with light petroleum (b.p. 40–60°).

Hydrolysis of lipid extracts. The extracts were evaporated *in situ* in a Pyrex tube and hydrolysed with 5 ml. of 4 N-HCl at 123° for 40 hr. (cf. Taylor & McKibbin, 1953).

Treatment of hydrolysate. Three methods were tested.

(i) Direct: the acid was removed under reduced pressure and the residue taken up in water. The pH was adjusted to

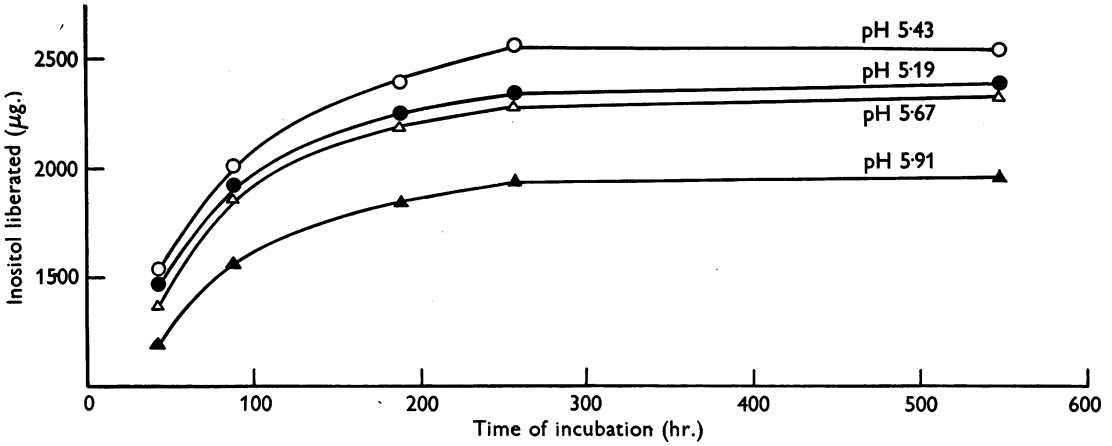

Fig. 1. Inositol liberated from oats by added phytase at pH 5·19–5·91.

4·8, the extract filtered and made up to volume. Assays of this extract were erratic and invalid; there were upward and downward 'drifts' in results obtained by comparison with the standard curve.

(ii) Treatment with charcoal: by addition of a few mg. of activated charcoal to the water extract and boiling for a few minutes, interfering substances could be removed by filtration and the assays were then more satisfactory. Bonner & Dorland (1943) reported adsorption of inositol on charcoal; but with the sample we used ['Charcoal decolorizing powder (activated)', B.D.H. Ltd., London], there was no indication of loss in experiments with standard solutions of inositol.

(iii) Chloroform extraction: this method (Taylor & McKibbin, 1953) gave the most satisfactory results. The acid hydrolysate was transferred to a 50 ml. separating funnel and extracted three times with 15 ml. portions of $CHCl_3$. The aqueous layer was distilled under reduced pressure to remove acid, the residue taken up with water, adjusted to pH 4·8, filtered and made up to volume. Results of assays were examined statistically and were found to be valid in all cases. The first method of preliminary extraction and the above $CHCl_3$ extraction appear to be the most promising procedures.

RESULTS

The results obtained from application of the methods indicated in the previous sections, with oats at various stages of germination, are shown in Tables 5–9.

DISCUSSION

The germination of the two batches followed the same general course, but there were quantitative differences (Table 5). Batch I, germinated in small

Table 5. *Dry weight of oats during germination*

Batch I		Batch II	
Germination time (days)	Dry wt. (mg./seed)	Germination time (days)	Dry wt. (mg./seed)
0	33·3	2	26·5
2	31·3	3	28·0
6	30·5	10	26·3
10	30·4	12	23·3

Table 6. *Total inositol of oats during germination*

Batch I			Batch II		
Germination time (days)	μg./g.	μg./seed	Germination time (days)	μg./g.	μg./seed
0	2690	89·5	2	2650	70·2
2	2760	86·2	3	2640	73·9
6	2770	84·5	10	2400	63·2
10	2630	79·9	12	2330	54·3

Table 7. *Free inositol of oats during germination*

Batch I			Batch II		
Germination time (days)	μg./g.	μg./seed	Germination time (days)	μg./g.	μg./seed
0	85	2·8	2	86	2·3
2	57	1·8	3	98	2·7
6	126	3·8	10	204	5·4
10	300	9·1	12	206	4·8

Table 8. *Inositol liberated by autolytic enzymes of oats (batch II)*

Germination time (days) ...	2		3		10		12	
Incubation time (hr.)	μg./g.	μg./seed	μg./g.	μg./seed	μg./g.	μg./seed	μg./g.	μg./seed
144	320	8·4	300	8·5	1480	39·0	1190	27·7
287	540	14·3	570	15·9	1870	49·1	1270	29·5
430	920	24·5	870	24·2	1980	52·0	1500	35·0
546	1190	31·4	1100	30·7	2030	53·5	1590	37·0
714	1600	42·5	1550	43·5	2130	56·1	1670	38·8

Table 9. *Inositol liberated by added phytase from oats (batch II)*

Germination time (days) ...	2		3		10		12	
Incubation time (hr.)	μg./g.	μg./seed	μg./g.	μg./seed	μg./g.	μg./seed	μg./g.	μg./seed
260	2340	62·0	2410	67·4	2160	56·7	—	—
408	2650	70·3	2610	73·1	2380	62·5	2230	51·9

amount in the laboratory, shows a germination loss of dry wt. of 8·7 % at 10 days, which is rather smaller than normal, possibly because of lower temperatures and humidities. The commercially malted batch II shows some irregularities but has a more normal germination loss of 12·1 % between the second and twelfth days. The original, ungerminated sample in this case was not available.

The total inositol (Table 6) shows a real loss during germination, and the differences between the batches are almost certainly a reflexion of the differing germination conditions. By calculating contents per seed the effect of weight loss during germination is automatically allowed for. It will be seen that with batch I there is a real loss of 10·7 % after 10 days' germination; but in batch II there is a real loss of 22·7 % between the second and twelfth days. The full loss from the ungerminated seed over 12 days' germination must be higher still.

On the other hand, there are gains of over 200 % and of over 100 % of free inositol in the respective batches (Table 7). On a weight per seed basis, however, there is in fact an overall loss of inositol liberated during germination. Thus with batch I the bound inositol (difference between free and total) shows a loss of 15·9 μg./seed, which is only partly accounted for by a gain in free inositol of 6·3 μg./seed after 10 days' germination. In the same manner the overall loss in batch II is even more marked. It might be inferred, although present evidence is not conclusive, that inositol liberated from bound forms during germination is lost in the metabolic processes involved.

The release of inositol by autolytic enzymes will be conditioned largely by the circumstances of germination. With the autolytic enzymes (Table 8) it will be seen that the rate of liberation of inositol increases as germination proceeds, but after about 10 days' germination there was a considerable decrease, presumably owing to a fall in enzyme activity. However, the values obtained in this way fall considerably short of those obtained by chemical means. Even after such a prolonged period of incubation as 714 hr. the mean value for the four germinated samples is only 69 % of the chemical value.

Where added phytase was the only operating enzyme (Table 9), no significant increase in inositol liberated was observable after 408 hr., and the phytase-liberated inositol averaged as high as 95 % of that indicated by the chemical method. For estimating total inositol the chemical method is much more speedy than the phytase method; results by the latter indicate that the bound form of inositol is almost exclusively phytin.

It is not possible to do more than indicate results in relation to lipid inositol. In addition to the difficulties already mentioned, it should also be pointed out that whereas inositol is usually regarded as 'insoluble' in ethanol and other fat solvents, it is nevertheless impossible to extract with such solvents without removing some inositol dissolved out by water present. It is necessary to assay before and after hydrolysis to distinguish between 'water-soluble' and lipid inositol. The amount of lipid inositol is less than 1 μg./seed. Further experiments are proceeding with solvents rendered as near absolute dryness as possible.

SUMMARY

1. The assay of inositol by *Schizosaccharomyces pombe* is used in a study of inositol in germinating oats. The organism is unaffected by fats, fatty acids and choline.

2. Methods for assessing total and free inositol are described. Liberation of inositol from bound forms, chiefly phytin, by autolytic enzymes and by added phytase, are studied.

3. During germination there is a net loss of inositol. A decrease in bound forms of inositol is compensated only partially by an increase in free inositol.

4. Lipid inositol may be separated and assayed with some difficulty but is present only in small amount.

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