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THE EXTRACTION AND ANALYSIS OF WHEAT PHOSPHOLIPIDS

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Abstract—The extraction of total phospholipids from wheat grain tissues requires more vigorous conditions than for vegetative plant tissues. Water saturated *n*BuOH was the most efficient extracting solvent for wheat grain and extraction with *iso*-PrOH and $CHCl_3$ was greatly inferior. The use of water saturated *n*-BuOH, however, gave rise to artifacts due to phospholipase D and transphosphatidylase activities during extraction. These artifacts could be avoided by denaturing the tissue by heat before extraction. Evidence was obtained that water saturated *n*-BuOH extraction can, even then, give rise to small quantities of lysophospholipid artifacts by non-enzymic hydrolysis.

INTRODUCTION

The course of our main studies on the hormonal control of germination in wheat [1] led us to consider the development of membranes in the aleurone cells. This has necessitated the development of accurate methods for the determination of phospholipids which are good markers for following membrane morphogenesis [2]. In the wheat grain, lipids form 8–15% of the germ, about 6% of the bran and 1–2% of the starchy endosperm [3]. The phospholipids are a minor proportion of these lipids except in the starchy endosperm where they account for about 50% of the total [4]. The composition of wheat lipids is, however, very complex. Most analyses have been performed on wheat flour or wheat starch [5–7], and few data are available on the bran and embryo as separate fractions. The majority of these analyses utilized H_2O satd *n*-BuOH as the lipid extractant, but some investigators have preferred procedures using solvents [6,8] which have been more usually used to extract animal and vegetative plant tissue. We have, therefore, compared various solvent systems for the extraction of wheat phospholipids and we discuss some of their limitations.

Abbreviations: DGDG, digalactosyldiacylglycerol, DPG, diphosphatidylglycerol, LPA, lysophosphatidic acid; LPC, lysophosphatidylcholine, LPE, lysophosphatidylethanolamine, LPG, lysophosphatidylglycerol; LDPG, lysodiphosphatidylglycerol, MGDG, monogalactosyldiacylglycerol, *N*-acyl GPE, *N*-acyl glycerylphosphorylethanolamine, *N*-acyl LPE, *N*-acyl lysophosphatidylethanolamine, *N*-acyl PE, *N*-acyl phosphatidylethanolamine, PA, phosphatidic acid, PB, phosphatidylbutanol, PC, phosphatidylcholine, PE, phosphatidylethanolamine, PG, phosphatidylglycerol; PI, phosphatidylinositol, PS, phosphatidylserine, SG, steryl glycoside

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RESULTS

Extraction of total phospholipids. Extraction successively with *iso*-PrOH, *iso*-PrOH–$CHCl_3$ (1:1) and $CHCl_3$ gave yields of lipid phosphorus that were very reproducible for both ungerminated and germinated seeds (Table 1). However, when samples were dissected into bran, starchy endosperm and embryo prior to extraction, the yields of lipid phosphorus were more variable, being as much as $\pm 22\%$ of the mean in some cases. Re-extraction of the residues left from the extractions of the bran and starchy

Table 1 The comparison of various solvents in the extraction of total lipid phosphorus from wheat

	Total lipid phosphorus (μg P × 95 grains)			
	Whole grain	Bran	Starchy endosperm	Embryo
Extraction with *iso*-PrOH, *iso*-PrOH–$CHCl_3$ (1 1) and $CHCl_3$				
Ungerminated grain	404 ± 7 (3)	90 ± 20 (3)	218 ± 13 (2)	98 ± 3 (2)
Grain germinated 4 days	848 ± 20 (3)	150 ± 10 (2)	183 ± 3 (2)	520 ± 55 (2)
Extraction with *iso*-PrOH–H_2O (8 2), *iso*-PrOH–$CHCl_3$ (1 1) and $CHCl_3$				
Ungerminated grain	880 (1)	125 (1)	nr	nr
Extraction with H_2O satd *n*-BuOH				
Ungerminated grain	1150 ± 29 (8)	180 ± 8 (4)	960 ± 60 (3)	127 ± 4 (3)

The figures in parentheses refer to the number of analyses ± standard deviation nr No results

endosperm yielded further large quantities of lipid phosphorus, and further experiments showed that the variation in lipid phosphorus extracted by these solvents was due to water carried over with the dissected tissues into the first extraction solvent (unpublished data) By adding 20% H_2O to the *iso*-PrOH used in the first extraction, larger quantities of lipid phosphorus were extracted from both whole seed and dissected bran (Table 1). When the amount of H_2O was increased to 50%, however, so much non-lipid material was extracted that the Sephadex purification chromatography became overloaded with consequent erroneously high values for lipid phosphorus The use of $CHCl_3$–MeOH–H_2O (5·10 4) was also unsuccessful, because the extracts contained suspended material which could not be filtered. H_2O satd *n*-BuOH, on the other hand, proved to be the superior extractant both for whole seeds and for dissected bran (Table 1) It also gave reproducible results, although re-extraction of the residues yielded a further 8% of lipid phosphorus. Nevertheless, because of its superiority over the other methods tried, H_2O-satd *n*-BuOH was adopted as the extracting solvent in all subsequent experiments.

Identification and determination of the individual phospholipids Chromatography of the purified total lipid on columns of silicic acid and then on two-dimensional TLC separated 11 phosphorus-containing spots. The identities of the spots are presented in Table 2. *N*-acyl LPE, PG,

Table 2 The identification of the phospholipids from whole ungerminated wheat

	I_2 vapour	Ninhydrin	Zinzadze reagent	Dragendorff reagent	R_f 1st Dimension	R_f 2nd Dimension
Phospholipids						
1 *N*-acyl PE	+	–	+	–	0 84	0 96
2 *N*-acyl LPE	+	–	+	–	0 66	0 82
3 DPG	+	–	+	–	0 50	0 86
4 PG	+	–	+	–	0 46	0 71
5 PE	+	+	+	–	0 49	0 59
6 PA	+	–	+	–	0·01	0 77
7 *N*-acyl GPE	+	–	+	–	0 21	0 42
8 PC	+	–	+	+	0 37	0 28
9 LPE + PS	+	+	+	–	0 19	0 34
10 PI	+	–	+	–	0 09	0 39
11 LPC	+	–	+	+	0 13	0 09
Other lipids						
a MGDG	+	–	–	–	0 75	0 96
b SG	+	–	–	–	0 60	0 87
c DGDG	+	–	–	–	0 28	0 65
x Unknown	+	–	–	–	0 66	0 94

Table 3 Replicate determinations of the phospholipids from ungerminated wheat using cold H_2O satd *n*-BuOH as the extracting solvent

	Phospholipid content (% total lipid P)					
	Bran				Starchy endosperm	
	Expt 1	Expt 2	Expt 3	Expt 4	Expt 1	Expt 2
N-acyl PE	9·0	27·3	5·9	15·0	7·8	9·5
N-acyl LPE	3·7	2·9	3·6	3·8	6·4	7·3
DPG	1·6	1·7	1·7	1·9	nd	nd
PG	2·1	2·5	2·2	2·4	nd	nd
PE	3·7	2·9	5·2	3·5	0·5	0·5
PA	5·9	4·5	4·0	4·3	nd	nd
N-acyl GPE	0·6	2·1	1·4	2·0	4·9	4·6
PC	34·1	14·1	28·4	24·4	2·9	3·2
LPE + PS	2·4	2·5	4·6	3·4	8·6	8·2
PI	11·6	7·5	11·2	11·7	1·0	0·8
LPC	24·5	28·9	30·4	26·0	66·6	64·1
Origin	0·7	3·3	1·1	1·5	1·2	1·3
Total lipid P (μg/95 grain)	187	174	170	189	1030	910

nd not detected

PE, PA, PC, PI and LPC were identified with reasonable certainty. *N*-acyl PE (spot 1) was never clearly separated from MGDG, but this did not interfere with the quantitative determination of the former by phosphorus analysis. DPG (spot 3) chromatographed close to a compound which did not contain phosphorus and which gave a reddish colour with H_2SO_4 containing sprays. This compound was possibly SG. Spot 9 occasionally resolved itself into two components, but never clearly. Both components contained phosphorus and reacted positively with ninhydrin. By comparison with published R_f data the spot was thought to be a mixture of LPE and PS. It is known that these compounds are difficult to separate [3]. Spot 7 was suspected to be a new phosphorus-containing lipid In view of the existence of *N*-acyl derivatives in wheat flour [7], it was suspected that it may be *N*-acyl GPE. When this was synthesized from egg yolk PE, it was indeed found to co-chromatograph with the compound from spot 7 in both basic and acidic solvent systems. Spot 7 was, therefore, tentatively identified with this compound.

Phospholipase D and transphosphatidylase activities during lipid extraction and in cell-free systems. Replicate experiments in which dissected bran samples were extracted with H_2O satd *n*-BuOH produced surprising inconsistencies in the proportions of individual phospholipids, although the total lipid phosphorus values were consistent within ±5% (Table 3). The percentages of *N*-acyl PE and PC were most inconsistent, but variations in the proportions of other phospholipids were also observed. In contrast, the starchy endosperm showed very consistent phospholipid levels (Table 3). These levels indicated that, in the bran, changes were occurring during extraction leading to elevated levels of *N*-acyl PE at the expense of other phospholipids Experiments not reported here using commercial bran samples (presumed to be dead) gave more consistent values for *N*-acyl PE and PC, suggesting that the discrepancy was associated with a living tissue, and hence that an enzyme was involved.

The only known enzyme of wide occurrence in plants and which attacks phospholipids in the presence of organic solvents is phospholipase D [9]. This enzyme is also capable of transphosphatidylase activity using primary alcohols as acceptors for the phosphatidyl group. One way in which phospholipase D activity could be reconciled with the results of Table 3 would be if the spot identified as *N*-acyl PE was, in fact, a mixture of *N*-acyl PE and PB produced by the reaction of phospholipids with the extracting solvent The known heat lability of phospholipase D [10] allowed this possibility to be tested. The results of this experiment are presented in Table 4. When batches of dissected bran were killed, either in boiling, H_2O satd *n*-BuOH or in a tube standing in a boiling H_2O bath, before extraction, low

Table 4 The effect of extraction conditions on the determination of the phospholipids from wheat bran

	Phospholipid content (% total lipid P)			
	Expt 1	Expt 2	Expt 3	Expt 4
N-acyl PE	33·7	15·3	5·9	3·7
N-acyl LPE	3·6	4·9	4·7	3·3
PE	3·6	4·9	4·1	5·1
PA	10·8	7·8	4·7	5·6
PC	10·5	27·0	23·4	27·0
PI	5·4	10·4	9·5	8·8
LPC	21·7	18·4	37·0	32·0
Others	10·8	11·7	14·4	14·4

Expt 1 Bran frozen, thawed in H_2O satd *n*-BuOH and incubated 2·5 hr before extraction Expt 2 Bran homogenized in cold, H_2O satd *n*-BuOH and incubated 1·5 hr before extraction Expt 3 Bran killed at 100° before extraction Expt 4 Bran killed in boiling *n*-BuOH before extraction

levels of *N*-acyl PE with accompanying high levels of PC were obtained In contrast, bran that had been frozen overnight, thawed in H_2O satd *n*-BuOH and incubated in that solvent before extraction, yielded 34% of its lipid phosphorus in the spot corresponding to *N*-acyl PE. Furthermore, the PA content of this extract was also high. Dissected bran that had been homogenized in cold, H_2O satd *n*-BuOH and then incubated, also yielded large proportions of lipid phosphorus in the *N*-acyl PE spot. (In cases where cold H_2O satd *n*-BuOH was used the temperature of the extraction rose to *ca* 70° during homogenization)

It remained to demonstrate directly, using cell-free preparations, the presence of phospholipase D and transphosphatidylase activities in bran. Preliminary experiments showed that these cell-free preparations had no phospholipase D activity against PC substrate dispersed in acetate buffer, even in the presence of ether [11], and they were only slightly active against ultrasonically dispersed PC However, when PC ultrasonically dispersed in the presence of sodium dodecylsulphate [12] was used as substrate, a rapid hydrolysis occurred producing PA (Table 5) This phospholipase activity was calcium dependent, was inhibited by 0·1 mM *p*-chloromercuribenzoate, and was inactivated by boiling, which are characteristic properties of the enzyme [10]

The active cell-free system also showed high transphosphatidylase activity (Table 5) In the presence of 10 mM *n*-BuOH a new product was formed which co-chromatographed on TLC with *N*-acyl PE in both basic and acid solvent systems. It was tentatively identified as PB. The transphosphatidylase activity was heat labile Appreciable quantities of LPC were also found in the products from the enzyme reactions, but since the controls also contained this compound, it was assumed that it was not a product of the enzyme action. In contrast to the bran, the starchy endosperm had negligible phospholipase D activity.

Because of the success of hot, H_2O satd *n*-BuOH in extracting grain tissues, its efficiency with a green tissue was investigated, and compared with the more usual procedure using *iso*-PrOH and $CHCl_3$ In contrast to the situation found with seed tissues, H_2O satd *n*-BuOH was only a slightly superior extractant for the leaf total lipid phosphorus (Table 6). As expected, heat-denaturation of the leaf tissue prior to extraction prevented significant formation of PB

Table 5 The phospholipase D and transphosphatidylase activities of cell-free preparations from wheat bran

	Experiment					
	1	2	3	4	5	6
Reaction conditions						
Lecithin substrate	+	+	+	+	+	+
$CaCl_2$	+	−	+	+	+	+
n-BuOH (10 mM)	−	−	−	+	+	−
p-Chloromercuribenzoate (0·1 mM)	−	−	−	−	−	+
Enzyme extract	+	+	−	+	−	+
Boiled enzyme extract	−	−	+	−	+	−
Phospholipids in reaction product (% total lipid P)						
PC	19·3	84·5	83·2	18·5	83·2	76·0
PA	65·8	3·8	nd	34·6	nd	8·3
PB	nd	nd	nd	32·2	nd	nd
Others (mainly LPC)	14·8	11·5	16·7	14·8	16·9	15·9

nd not detected

Table 6 The comparison of two solvents for the extraction of phospholipids from green pea leaves

	Phospholipid content (% total lipid P)	
	Extraction with *iso*-PrOH *iso*-PrOH $CHCl_3$ (1:1) and CHCl	Extraction with hot H_2O satd *n*-BuOH
N-acyl PE	2·0	4·0
PE	24·6	19·2
PA	2·0	2·3
PC	30·0	26·0
PI	8·0	7·2
LPC	1·5	9·0
PG	*	*
DPG	32·0	32·1
Origin	*	*
Total lipid P (μg 10 g tissue)	1025	1350

as shown by the low values obtained for the *N*-acyl PE spot. There was more firm evidence, however, that the use of hot, H_2O satd *n*-BuOH led to the production of small quantities of LPC.

DISCUSSION

H_2O satd *n*-BuOH has been found to be the most effective solvent for the extraction of total phospholipids from whole wheat grains and their dissected parts. In the bran and embryo, artifact formation due to transphosphatidylase activity can be prevented by dropping the tissue into the boiling extraction solvent The extraction procedure incorporating this step has been in use in our laboratory for some years giving reproducible extraction of total lipid phosphorus and of the individual phospholipids. The absence of detectable phospholipase D activity in the starchy endosperm accounts for the more consistent phospholipid values and the absence of PA in this tissue when extracted without prior heat treatment. Recently, de la Roche *et al.* [13] have reported that boiling *iso*-PrOH followed by $CHCl_3$–MeOH–H_2O extraction was superior to H_2O satd *n*-BuOH. In their studies, however, H_2O satd *n*-BuOH did extract the most material, but considerable losses of fatty acids and lipid phosphorus occurred during the subsequent partition procedure which they used to purify their extracts. They explained the losses as being due to lipoprotein "fluff" at the interface in the partition procedure. Possibly, evaporation of their H_2O satd *n*-BuOH extract to dryness in the presence of $CHCl_3$ and H_2O, to break lipoprotein bonds, followed by purification on columns of Sephadex G-25, would have overcome these difficulties. Most other studies with wheat products such as starch and flour confirm that H_2O satd *n*-BuOH is the superior extractant. Since H_2O satd *n*-BuOH was also the best extractant for bran, the present studies are also relevant to the problems of extracting phospholipids from non-starchy seed tissues and non-starchy seeds generally.

Two-dimensional TLC separated eleven phosphorus-containing spots of which ten have been identified with reasonable certainty. The eleventh has been tentatively identified as *N*-acyl GPE With regard to other possible identities, de la Roche *et al.* [13] have reported the presence of cyclic and non-cyclic forms of LPA in whole wheat Our new spot is unlikely to be either of these compounds because they have very different R_f values in both basic and acidic solvent systems [13]. LPA would most likely be produced from LPC under conditions of high phospholipase D activity. This possibility is, however, precluded by the presence of small and consistent amounts of material in our spot 7 both from bran (high phospholipase D activity) and starchy endosperm (low phospholipase D activity) An alternative explanation is that our compound is LPA, formed by the hydrolysis of PA during extraction, but this is also unlikely because PA is present only in small amounts in the bran and embryo and it is absent from the starchy endosperm. The fact remains, however, that our extracts may contain non-cyclic LPA cochromatographing with LPC in our systems. Two other possibilities are that our new spot is LPG or LDPG; certainly they have comparable R_fs in similar TLC systems to those used in our study [14]. This would not be expected, however, since PG and DPG, from which they would most likely be formed, are absent from the starchy endosperm (Table 4).

The large quantity of LPC in the starchy endosperm was almost double that found in commercial wheat flour [7], and a re-examination of the data for bran lipids in Tables 3 and 4 reveals very high and variable LPC values. This raises the question of whether some LPC might have arisen from PC by hydrolysis during extraction. Green leaves apparently do not contain lysophospholipids [15]. The presence of significantly larger quantities of LPC in *n*-BuOH extracts than were found in *iso*-PrOH–$CHCl_3$ extracts from pea leaves (Table 6) suggests, therefore, that the use of the former solvent does indeed lead to acyl hydrolysis of PC. The quantities of LPC arising in this way must, however, be very small since the incorporation of ^{14}C-choline into PC in incubated bran or green leaves yields no detectable radioactivity in the LPC fraction [16]. On the other hand, the observation that the highest LPC values were obtained in extractions involving the prior heat treatment of the bran tissue suggests that hot *n*-BuOH is responsible for artifact formation during preliminary inactivation of the phospholipase D and during subsequent homogenization If this is true then the new phospholipid,

tentatively identified as *N*-acyl GPE, may also be an artifact due to acyl hydrolysis of *N*-acyl PE and *N*-acyl LPE. Obviously, the exact origins of the lysophospholipids extracted from wheat bran by *n*-BuOH are not yet resolved.

EXPERIMENTAL

Chemicals and solvents PG, DPG, PS, PC, PI, MGDG, DGDG and SG were given by Dr B W Nichols, Colworth House, Sharnbrook, Beds, U K *N*-acyl PE and *N*-acyl LPE were given by Dr W R Morrison, University of Strathclyde, U K *N*-acyl GPE was synthesized from egg yolk PE [17]. LPC was purchased from B D H Ltd. $CHCl_3$ for chromatography was washed with H_2O, dried and redistilled. All other solvents for extraction and chromatography were redistilled before use.

Plant material Grains of the soft winter cv Cappelle Desprez were used in batches of 5 g (95 grains). Grains were sterilized and allowed to germinate at 25° by our routine procedure [18]. Ungerminated and germinated grains were either analysed directly or they were dissected into their parts for separate analysis. The bran was washed ×3 with H_2O to remove adhering starch. Pea plants were grown in the greenhouse and their leaves harvested immediately before analysis.

Extraction of total lipids Plant material was extracted by homogenizing it in the extracting solvent using a top-drive homogenizer and filtering through sintered-glass. Three solvent systems were investigated as to their efficiency in extracting phospholipids. (a) Extraction successively with *iso*-PrOH, *iso*-PrOH–$CHCl_3$ (1 1) and $CHCl_3$ was carried out according to our routine procedure for the extraction of neutral lipids [18]. In addition extraction with various *iso*-PrOH–H_2O mixtures followed by extraction with *iso*-PrOH–$CHCl_3$ (1 1) and then $CHCl_3$ was investigated. (b) Extraction ×3 with $CHCl_3$–MeOH–H_2O (5 10 4). (c) Extraction ×3 with H_2O satd. *n*-BuOH. In the procedure finally adopted, the tissue was dropped into boiling H_2O satd. *n*-BuOH, and left for 3 min before homogenizing. Filtrates from extraction were combined and evaporated either to a small vol. (extractions involving $CHCl_3$) or to dryness (extraction with H_2O satd. *n*-BuOH) at 50° in a rotary evaporator. Atm. pres. was reinstated with N_2 and residue was taken up in a small vol. of $CHCl_3$–MeOH (19 1). H_2O (10 drops) was then added and the extract was reduced once more to dryness in order to break lipoprotein associations [19]. The residue was taken up again in a small vol. of $CHCl_3$–MeOH (19 1) and purified by partition chromatography on Sephadex G-25 [20]. The lipid fraction from the column was evaporated to dryness under N_2 and the last traces of solvent removed under vac. Finally the lipid was taken up in 5–10 ml $CHCl_3$ and stored under N_2 at 4° for later analysis.

Determination of total lipid phosphorus Samples of lipid from Sephadex G-25 were digested first with redist. conc. HNO_3 and then with 70% $HClO_4$ [21] prior to determination of Pi [22].

Chromatographic analysis of polar lipids The total lipid from Sephadex G-25 chromatography was chromatographed on 1·3 g silicic acid prepared according to ref. [23] in a glass column (10 mm int. diam.). Lipid was applied to the column in $CHCl_3$ soln. Neutral lipids were eluted with 30 ml $CHCl_3$, the polar lipids (glycolipids + phospholipids) were eluted with 30 ml MeOH [20]. The separate elution of the glycolipids with Me_2CO and of the phospholipids with MeOH [20] was not possible because the *N*-acyl phospholipids were eluted with the glycolipids. The polar lipid fraction was evaporated to dryness under N_2 and re-dissolved in a small vol. of $CHCl_3$. Separation of the polar lipids into classes was achieved by 2-D TLC on Si gel G. Of a large number of solvent systems investigated, the most successful were $CHCl_3$–MeOH–ammonia (30% w/v) (13 5 1) for the 1st dimension and $CHCl_3$–Me_2CO–MeOH–HOAc–H_2O (6 8 2 2 1) for the 2nd dimension. The 1st solvent was removed under vac., Atm. pres. was reinstated with N_2 and chromatograms were immediately run in the 2nd solvent. Chromatograms were then dried in air and the spots were detected using I_2 vapour.

Identification and determination of individual polar lipids Phospholipids were tentatively identified by comparing their TLC R_f values with published data, by co-chromatography with authentic samples and by their behaviour with various spray reagents. For the latter purpose the developed chromatograms were sprayed with 0·3% ninhydrin–5% lutidine in H_2O satd. *n*-BuOH for amino groups [24], a modified Dragendorff reagent for choline [24] and a modified Zinzadze reagent for phosphate [25]. The use of 0·6% K_2Cr_2O in 55% H_2SO_4 as a general detection reagent [20], also allowed the identification of some non-phosphorus polar lipids. The quantities of individual phospholipids were determined by a modification of the method in ref. [26]. Chromatogram spots that had been located using I_2 vapour and Si gel blanks were aspired directly into 50 ml Kjeldahl flasks containing a liquid trap of 0·7 ml 70% $HClO_4$. The contents of the flask were refluxed at 200° until clear and then cooled. 3·3 ml H_2O, 0·5 ml 2·5% ammonium molybdate, and 0·5 ml 10% ascorbic acid were then added. The mixture was transferred to a reductase tube, and heated for 5 min at 100°. After cooling, the Si gel was removed by a brief centrifugation and the *A* at 800 nm of the supernatant was measured. For large spots the reagent quantities were doubled and for small spots the corresponding areas from two chromatograms were combined for analysis.

Determination of phospholipase D and transphosphatidylase activities Batches of 30 grains were dissected to give bran and starchy endosperm. All subsequent preparations were conducted at 4°. Each tissue was homogenized for 1 min in 5 ml H_2O using a Polytron homogenizer set at its slowest speed. Cell debris was removed by centrifuging 5 min at 3000 ***g*** and the supernatant was used directly in the enzyme assay. PC substrate was prepared by sonicating pure PC with equimolar SDS [27] in H_2O for 5 min at 0° using a 60W sonicator. The enzyme assay mixture contained 1 μmol PC, 1 μmol SDS, 80 μmol NaOAc buffer pH 4·8, 40 μmol $CaCl_2$, and 0·5 ml enzyme extract in a total vol. of 1·1 ml. When activators or inhibitors were added they were included at the concns. given in the results section. The reaction was started by adding the enzyme extract and after incubation for 60 min at 20° 0·1 ml of 0·1 M HCl was added to stop the reaction. The mixture was then extracted with 2 ml EtOH–Et_2O (4 1), and the contents of the Et_2O layer were analysed by two-D TLC.

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