Phytochemistry, Vol. 25, No. 10, pp. 2255–2259, 1986.
Printed in Great Britain.

0031-9422/86 $3.00 + 0.00
Pergamon Journals Ltd.

EFFECT OF TEMPERATURE ON GLYCEROL METABOLISM IN MEMBRANES AND ON PHOSPHOLIPASES C AND D OF GERMINATING PEA EMBRYOS

LILIANA DI NOLA and ALFRED M. MAYER

Department of Botany, The Hebrew University of Jerusalem, Jerusalem, 91904, Israel

(*Revised received* 3 *February* 1986)

Key Word Index—*Pisum sativum*; Leguminosae; imbibition; temperature; glycerol; choline; phosphatidyl choline; phospholipase C; phospholipase D; plasma membrane; endoplasmic reticulum.

Abstract—The effect of temperature of imbibition on the synthesis and turnover of membrane phosphatidyl choline was studied. Pea seeds (*Pisum sativum* cv. Alaska) were imbibed in [U-^{14}C]glycerol and then germinated. Seeds were kept constantly either at 5° or 25°, or were imbibed at one temperature and then germinated at the other one. Glycerol incorporation into phosphatidyl choline in the ER and the plasma membrane, obtained from the embryonic axes after germination, and the glycerol pool were measured. Embryos from seeds kept constantly at 25° showed a rapid incorporation of glycerol into membranes followed by a loss of label; in embryos from seeds kept at 5° incorporation was much lower. Embryos from seeds transferred from 25° to 5° behaved as if continuously kept at 25°, while the behaviour of the embryos from seeds transferred from 5° to 25° resembled embryos from seeds maintained at 5°. The glycerol content of the axes rose during imbibition and fell thereafter. The activities of phospholipases C and D also responded to the initial temperature of imbibition, but the two activities changed differently. The results are discussed in relation to the effect of transient exposure to temperature changes in the seed membranes and the possible way in which such changes are sensed.

INTRODUCTION

The response of seeds to the ambient temperature is important in controlling the onset of germination, both in cultivated crops and in seeds of wild species. In the former the temperature response may determine the initial seedling stand and therefore eventual productivity. In the latter the temperature response is a crucial factor in determining the distribution of germination over time and hence species survival. The problem of how temperature is sensed has been discussed [1–10]. In two papers [11, 12] we reported on the phospholipid metabolism of pea seeds with special emphasis on the phosphatidyl choline component of the phospholipid and the effect of temperature on its metabolism. These papers showed that phospholipid is metabolized with great rapidity during germination. It was also shown that imbibition temperature had a considerable effect on subsequent metabolism during conditions suitable for germination. In particular choline incorporation into phosphatidyl choline was strongly affected by the temperature of imbibition. The temperature of germination influenced choline metabolism only after 24–48 hr of germination. Since this aspect of germination has been very little explored, we wished to extend these studies. In the present paper we report on incorporation of glycerol into phosphatidyl choline in response to temperature during imbibition and also on the changes in activity of phospholipases in response to the temperature treatments.

RESULTS

Since the temperature of imbibition of pea seeds affected the metabolism of phosphatidyl choline in membranes, it was important to determine whether another precursor of phosphatidyl choline, glycerol, was similarly influenced. Seeds were imbibed for 5 hr at 25° or for 6 hr at 5° in the presence of [U-^{14}C]glycerol, and then germinated for various periods of time at different temperatures. Some 60–65% of the applied glycerol was taken up by the seeds at both temperatures. At the end of the germination time, the embryonic axes were removed, membrane fractions enriched in ER or plasma membranes isolated and phospholipids extracted. The incorporation of radioactive glycerol into phosphatidyl choline was then determined. Since we wished to compare the behaviour of glycerol to that of choline [12] we selected only three times for measurement, after imbibition (5 hr) and after 20 and 48 hr of germination, which had shown clear differences in the study using choline. The results are shown in Tables 1 and 2.

The embryos of seeds maintained at 25° showed a rapid incorporation of glycerol into both ER and plasma membranes during the first 5 hr, i.e. during imbibition. Incorporation continued in the subsequent period, up to 20 hr. Between 20 and 48 hr label was lost from the membranes presumably due to turnover and breakdown.

Embryos of seeds imbibed at 25° and germinated at 5° behaved very similarly to those kept constantly at 25°. Surprisingly, the incorporation was even greater, the differences becoming more evident when the results are expressed on a fresh weight basis. In contrast, in the axes of seeds maintained at 5°, and particularly in those of seeds transferred from 5° to 25°, glycerol incorporation was much lower, in both membrane fractions.

The initial incorporation of glycerol during the first 5 hr was markedly higher than that observed for choline [12],

Table 1. Incorporation of [U-^{14}C]glycerol into phosphatidyl choline in plasma membrane during germination of embryonic axes of peas

		g dry wt			g fr. wt		
Length of germination (hr)		5	20	48	5	20	48
Temperature treatment							
Imbibition	Germination						
25°	25°	0.1	0.25	0.07	0.045	0.066	0.003
25°	5°	0.1	0.34	0.075	0.045	0.15	0.03
5°	5°	0.05	0.12	0.08	0.022	0.052	0.032
5°	25°	0.05	0.1	0.11	0.022	0.033	0.008

Results in nmols glycerol/g dry wt* or fr. wt.
*S.D. was less than 0.03.

Table 2. Incorporation of [U-^{14}C]glycerol into phosphatidyl choline in endoplasmic reticulum during germination of embryonic axes of peas

		g dry embryo			g fresh embryo		
Length of germination (hr)		5	20	48	5	20	48
Temperature treatment							
Imbibition	Germination						
25°	25°	0.66	0.78	0.2	0.3	0.2	0.01
25°	5°	0.66	0.75	0.24	0.3	0.32	0.10
5°	5°	0.06	0.48	0.15	0.03	0.21	0.06
5°	25°	0.06	0.32	0.4	0.03	0.1	0.03

Results in nmols glycerol/g dry wt* or fr. wt.
*S.D. was less than 0.05.

but subsequent incorporation was of the same magnitude as for choline.

The differences in glycerol incorporation could be due to changes in the endogenous pool of glycerol in the embryonic axes. We therefore measured the endogenous glycerol content of the embryonic axis during germination (Fig. 1). The results showed a drop in the endogenous pool of glycerol during the first 20 hr of germination in all the treatments, the decrease being greater in seed embryos germinated at 25°, independently of temperature of imbibition. As germination proceeded, at 25° a further decrease in endogenous glycerol occurred, whereas in seed embryos kept at 5°, or transferred from 25° to 5°, the drop was stopped, probably because growth ceased. When glycerol incorporation (Tables 1 and 2) is compared with the change in the glycerol pool, it is clear that the changes in incorporation induced by the temperature treatment cannot be accounted for by the changes in the glycerol pool. We have no information on the possible compartmentation of glycerol.

The free glycerol content of the axis after 5 hr of imbibition, in all treatments, is surprisingly high. The source of the large glycerol pool is not clear. No comparable data appear in the literature. It is probable that the loss of glycerol between 5 and 20 hr is due to its utilization in respiratory metabolism, but this point has not been checked.

There is considerable similarity between the behaviour of incorporation of choline [12] and glycerol into phosphatidyl choline in membranes of pea embryonic axis. It seemed important therefore to determine whether the enzymes involved in phospholipid breakdown were affected by the different temperature treatments. Two key

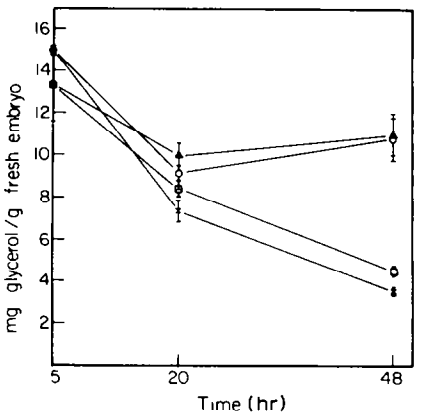

Fig. 1. Effect of temperature on the endogenous free glycerol content in embryonic axes of germinating peas (results in mg/g fr. wt). x - - - - - x, Imbibition and germination at 25°; O - - - - - O, imbibition at 25°/germination at 5°; ▲ - - - - - ▲, imbibition at 5°/germination at 5°; □ - - - - - □, imbibition at 5°/germination at 25°.

enzymes, phospholipase C, which cleaves phosphorylcholine from the phospholipid, and phospholipase D were therefore studied.

Phospholipase D exists in plant cells as soluble and membrane bound forms [13, 14]. We found most of the activity of phospholipase C in the supernatant after centrifugation at 20000 *g* and no activity in the mitochondrial fraction. We therefore measured the activity of the two enzymes in the soluble fraction after centrifugation at 20000 *g*.

The activity of phospholipase C was quite high in dry axes, increased after imbibition at 25°, and then decreased during subsequent germination at 25°. If seeds were transferred to 5°, the drop in activity was much slower (Table 3). Imbibition at 5° resulted in reduced enzyme activity, which then rose again, particularly in seed embryos maintained at 5°, but even in those transferred to 25°. Germination at 5° resulted in high enzyme activity even after 48 hr, regardless of imbibition temperature, whereas germination at 25° caused a gradual decrease. It is evident that the imbibition temperature has an important initial role in determining enzyme activity.

The behaviour of phospholipase D (Table 4) showed some unusual features. Imbibition, no matter at what temperature, reduced enzyme activity. This decrease was bigger than can be accounted for simply by the increased weight of the embryos. For details of growth of the embryos see ref. [12]. A low activity was maintained in seeds kept throughout at 25°. Seeds either transferred to 5° after 5 hr or kept continuously at 5° showed an increased activity between 5 and 24 hr, and activity was still high after 48 hr. Seeds transferred from 5° to 25° behaved initially as if kept at 5° and only between 24 and 48 hr did the drop associated with the higher temperature occur. It can also be noted that the phospholipase D activity detected was much greater than that of phospholipase C.

DISCUSSION

Imbibition of dry seeds is a critical event in the germination process, and many authors have related it to the reorganization of cellular membranes and to the restructuring of subcellular organelles [1, 2, 4, 15–19]. In the aleurone of barley and wheat the formation of ER starts during imbibition of the seeds [20].

In the cotyledons of soybean seeds, during imbibition, integrity of the plasma membrane is re-established, ER is formed, and mitochondria recover their normal configuration [2, 17]. Exposure to low temperatures during imbibition changes the reorganization of the membranes and their phospholipid content in these seeds.

Chilling has been reported to decrease the level of phosphatidyl choline (PC) and phosphatidyl ethanolamine (PE) in cucumber and cotton leaves [21], and the physical properties of the membranes were partially ascribed to relative quantities of head groups in polar lipids, which could be responsible for a lower or higher transition temperature in membranes [22–24]. The phase transition at chilling temperatures increases leakage [4, 19], alters the normal functions of membranes [2, 23, 25, 26] and changes the activity of some soluble and membrane-bound enzymes [27–30].

In wheat tissues exposed to freezing temperatures there was a rapid degradation of polar lipids, due to phos-

Table 3. Effect of temperature on phospholipase C activity in pea seed embryos during germination

Length of germination (hr)		0	5	24	48
Temperature treatment					
Imbibition	Germination				
25°	25°	0.33 ± 0.0	0.41 ± 0.01	0.12 ± 0.01	0.03
25°	5°	0.33 ± 0.0	0.41 ± 0.01	0.2 ± 0.01	0.11 ± 0.02
5°	5°	0.33 ± 0.0	0.11 ± 0.01	0.19 ± 0.01	0.11 ± 0.02
5°	25°	0.33 ± 0.0	0.11 ± 0.01	0.16 ± 0.01	0.03

Results in enzyme units*/g fresh embryo.
*1 unit liberates 1 μmol phosphoryl choline from PC/min.

Table 4. Effect of temperature on phospholipase D activity in pea seed embryos during germination

Length of germination (hr)		0	5	24	48
Temperature treatment					
Imbibition	Germination				
25°	25°	18.0 ± 1	5.7 ± 1	8.5 ± 0.04	2.2 ± 0.02
25°	5°	18.0 ± 1	5.7 ± 1	23.3 ± 2.0	14.0 ± 0.06
5°	5°	18.0 ± 1	5.5 ± 1	23.0 ± 2.0	18.0 ± 0.02
5°	25°	18.0 ± 1	5.5 ± 1	14.4 ± 0.5	4.3 ± 1.0

Results in enzyme units*/g fresh embryo.
*1 unit liberates 1.0 μmol choline from PC/min.

pholipase D activity [31]; freezing also enhanced the activity of this enzyme in soybean cotyledons [32].

Despite the many reports on the effect of temperature on membrane structure and function, very little is known about the effects of transient exposure to low temperature on the subsequent metabolism of seeds, and on the underlying sensing mechanism.

In a previous paper we reported that the incorporation of choline into PC in ER and in the plasma membrane of germinating pea seed embryos was strongly affected by temperature of imbibition: thus, while incorporation was very great in embryos of seeds constantly kept at 25°, it was much reduced in seed embryos maintained continuously at 5°, or transferred from 5° to 25°, as well as in those transferred from 25° to 5°. The exposure to low temperature during imbibition apparently determines subsequent choline metabolism, and delays germination, while the exposure to favourable temperature during imbibition is not enough to start germination, since metabolic processes are then stopped by transfer to 5°.

The purpose of this investigation was to determine whether incorporation of glycerol into phospholipids responded in the same way to temperature transition as the choline moiety. Probably most of the incorporation was into the backbone of the phospholipid, but no doubt part of the glycerol could be recycled and eventually be incorporated into acyl groups of the lipids. The results shown in Tables 1 and 2 clearly show that an initial temperature of imbibition of 5° had a profound influence on subsequent glycerol metabolism resulting in a prolonged delay in glycerol incorporation. Seeds imbibed at 25° responded as if kept at 25° even if transferred to 5°. These results thus support the idea that phospholipid metabolism is part of the sensing mechanism of temperature. It should however be noted that the time course of glycerol metabolism in phospholipid is distinctly different from that of choline. This is not unexpected since presumably different parts of the phospholipid molecule, which arise from different metabolic pools, will be synthesized, degraded and turned over at different rates. The temperature treatment had little effect on the glycerol pool of the seeds, which was however quite large. We have no information whether glycerol was compartmentalized in the cells or whether there is a single glycerol pool. Indeed little attention has been paid to glycerol metabolism in plants except with regards to its function in osmoregulation in algae [33] and to its protective action against freezing [34].

The two enzymes involved in phospholipid degradation, phospholipases C and D, differed in their response to temperature treatment. The activity of phospholipase C was first enhanced and then decreased in embryonic axis of seeds kept constantly at 25°, whereas imbibition at 5° delayed the increase in activity, which was maximal after 24 hr. A very similar activity was shown by axes of seeds transferred from 25° to 5°, while seed axes transferred from 5° to 25° 'remembered' the imbibition temperature longer.

Very little is known about phospholipase C activity in plants, and the documentation in the literature is very poor. From our results, it could be argued that the high activity detected in seed embryos after 5 hr imbibition at 25° could be responsible for an early breakdown of PC at this temperature, and could account for the greater incorporation of choline into PC in axes of seeds constantly kept at 25°.

Although there are relatively few reports on phospholipase D activity in plants it is well known that activity of the enzyme is increased by chilling or freezing of cells [14, 35]. This is thought to be caused by the breakdown of compartmentation which allows the enzyme to come in contact with cellular membranes [32, 36, 37]. Phospholipase D activity is enhanced by Ca^{2+} and inhibited by Mg^{2+} [38]. It is possible that in the germinating seed these two cations also play a role, since both Ca^{2+} and Mg^{2+} are chelated in the dry seed, but become solubilized during germination [39]. However we have no direct evidence on this point.

The results reported here on glycerol metabolism and on phospholipase activity are essentially in agreement with the report for choline [12]. It is quite clear that the temperature of imbibition has a direct effect on subsequent metabolism of phospholipids, at a different temperature. This effect must be mediated by enzymes responsible for synthesis or breakdown. Our results so far indicate that phospholipases are involved in the temperature response. Further studies are now under way to investigate the effect of temperature during imbibition on the composition of the acyl group of the phospholipid, on the degree of unsaturation and on the function of synthetic enzymes.

EXPERIMENTAL

Pea seeds cv. Alaska were obtained from the Ferry Morse Seed Co. Seeds were peeled prior to imbibition. Imbibition and germination were as previously described [11]. For determination of glycerol incorporation, peeled seeds were imbibed for 5 hr in an aq. soln of [U-^{14}C]glycerol (170 mCi/mmol) in a final concn of 5 μCi in 29 ml soln/10 g dry seed, rinsed and transferred to conditions for germination on moist cotton wool, at different temps. Embryonic axes were isolated after suitable periods of time. Membrane fractions, enriched in ER or plasma membranes were isolated by discontinuous gradient centrifugation as described by Yarden and Mayer [11] and Di Nola and Mayer [12]. Characterization of the fraction is described in ref. [11]. Phospholipids were extracted from the membrane fractions, separated by TLC and the zones located [11]. The zones were scraped off the TLC plates and radioactivity determined by scintillation counting.

Endogenous glycerol was measured by the method of Lambert and Neisch [40] in the total soluble fraction of the embryos, obtained after centrifugation at 20000 *g*. The activity of phospholipase C was determined by the method of Krug *et al.* [41] using phosphatidyl choline as substrate, and by measuring the Pi released by the method of Fiske and Subba-Row [42].

One unit of phospholipase C activity was defined as the amount which liberates 1.0 μmol phosphorylcholine from PC per min.

The activity of phospholipase D was determined by the method of Kates and Sastry [43], and the choline formed was measured using the Reinecke salt as described by Jarden and Mayer [11]. One unit of phospholipase D activity was defined as the amount which liberates 1.0 μmol choline from PC per min.

REFERENCES

1. Simon, E. W. (1973) *New Phytol.* **73**, 377.
2. Bramlage, W. J., Leopold, A. C. and Parrish, D. J. (1978) *Plant Physiol.* **61**, 525.

3. Trémolières, H., Trémolières, A. and Mazliak, P. (1978) *Phytochemistry* **17**, 685.
4. Leopold, A. C. (1980) *Plant Physiol.* **65**, 1096.
5. Riley, G. J. P. (1981) *Planta* **151**, 68.
6. Riley, G. J. P. (1981) *Planta* **151**, 75.
7. Givelberg, A., Horowitz, M. and Poljakoff-Mayber, A. (1981) *J. Exp. Botany* **35**, 1754.
8. Yoshida, S. (1984) *Plant Physiol.* **75**, 38.
9. Yoshida, S. and Uemura, M. (1984) *Plant Physiol.* **75**, 31.
10. Mayer, A. M. (1986) *Isr. J. Botany* (in press).
11. Yarden, R. and Mayer, A. M. (1981) *Phytochemistry* **20**, 2669.
12. Di Nola, L. and Mayer, A. M. (1985) *Phytochemistry* **24**, 2549.
13. Heller, M. (1978) *Adv. Lipid Res.* **16**, 267.
14. Yoshida, S. (1979) *Plant Physiol.* **64**, 241.
15. Hallam, N. D., Roberts, B. E. and Osborne, D. J. (1972) *Planta* **105**, 293.
16. Simon, E. W. and Raja Harun, R. M. (1972) *J. Exp. Botany* **23**, 1076.
17. Priestley, D. A. and Leopold, A. C. (1980) *Physiol. Plant.* **49**, 198.
18. Seewaldt, V., Priestley, D. A., Leopold, A. C., Feigenson, G. W. and Zalduondo, F. G. (1981) *Planta* **152**, 19.
19. Senaratna, T. and McKersie, B. D. (1983) *Plant Physiol.* **72**, 620.
20. Mirhabar, R. B. and Laidman, D. L. (1982) *Biochem. J.* **208**, 93.
21. Wright, M. and Simon, E. W. (1973) *J. Exp. Botany* **24**, 400.
22. Yamaki, S. and Uritani, I. (1972) *Plant Cell Physiol.* **13**, 67.
23. Dogras, C. C., Dilley, D. R. and Herner, R. C. (1977) *Plant Physiol.* **60**, 897.
24. Patterson, B. D., Kendrick, J. R. and Raison, J. K. (1978) *Phytochemistry* **17**, 1089.
25. Clarke, A., Coulson, G. and Morris, G. J. (1982) *Plant Physiol.* **70**, 97.
26. Thebud, R. and Santarius, K. A. (1982) *Plant Physiol*, **70**, 200.
27. Raison, J. K. (1973) *Symp. Soc. Exp. Botany* **27**, 485.
28. Lyons, J. M. (1973) *Ann. Rev. Plant Physiol.* **24**, 445.
29. Houslay, M. D. and Palmer, R. W. (1978) *Biochem. J.* **174**, 909.
30. Brasitus, T. A. and Schachter, D. (1980) *Biochemistry* **19**, 2763.
31. Willemot, C. (1983) *Phytochemistry* **22**, 861.
32. Yoshida, S. (1979) *Plant Physiol.* **64**, 252.
33. Hellebust, J. A. (1976) *Ann. Rev. Plant Physiol.* **27**, 485.
34. Luyet, B. J. and Gonzales, F. (1952) *Biodynamica* **7**, 101.
35. Yoshida, S. and Sakai, A. (1974) *Plant Physiol.* **53**, 509.
36. Roughan, P. G. and Slack, C. R. (1976) *Biochim. Biophys. Acta* **431**, 86.
37. Horvath, I., Vigh, L., Belea, A. and Farkas, T. F. (1979) *Physiol. Plant.* **45**, 57.
38. Yoshida, S. (1979) *Plant Physiol.* **64**, 247.
39. Mayer, A. M. and Poljakoff-Mayber, A. (1982) *Germination of Seeds*, 3rd edn. Pergamon Press, Oxford.
40. Lambert, M. and Neisch, A. C. (1950) *Can. J. Res.* **28B**, 83.
41. Krug, E. L., Truesdale, N. J. and Kent, C. (1979) *Analyt. Biochem.* **97**, 43.
42. Fiske, C. H. and Subba-Row, Y. (1925) *J. Biol. Chem.* **66**, 375.
43. Kates, M. and Sastry, P. S. (1969) in *Methods in Enzymology* (Lowenstein, J. M., ed.) Vol. XIV, p. 197. Academic Press, New York.