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THE INDUCTION OF TRIGLYCERIDE METABOLISM IN THE GERMINATING WHEAT GRAIN

R. J. A. TAVENER and D. L. LAIDMAN

Department of Biochemistry and Soil Science, University College of North Wales, Bangor, Caernarvonshire, Wales

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Abstract—During germination, the triglyceride reserves of wheat are progressively broken down. Breakdown in the embryo axis, starchy endosperm and the bran starts within 12 hr of imbibition. In the scutellum, it does not commence until after the second day of germination. In the bran, the triglyceride reserves appear to exist in at least two parts. The catabolism of one part is induced by a factor from the starchy endosperm, probably a cytokinin. This induction is prevented by inhibitors of protein synthesis. Catabolism of the other part can be induced by indole acetic acid together with hydroxylamine. Catabolism of the triglyceride reserves of the starchy endosperm can be induced by indole acetic acid and hydroxylamine.

INTRODUCTION

PREVIOUS investigations into the changes in triglyceride levels during germination have been concerned, in most cases, with the oleagenous seeds.[1,2] In all of these cases triglycerides in the endosperm disappeared during germination. A similar situation has been described for the proteinaceous seed of soybean, *Glycine max*.[3]

Although the major nutrient reserve of wheat is starch, the whole wheat grain contains 2–4% of lipid, about half of which is triglyceride.[4] The triglyceride contents of the germ and aleurone tissues are considerably greater than this value. Since these tissues do not contain polysaccharide reserves, the triglycerides may provide important reserves of energy and carbon early during germination. We have, therefore, studied the patterns of triglyceride metabolism in the various tissues of the germinating wheat grain. By studying triglyceride metabolism in excised storage tissues from the grain we have endeavoured to characterize some of the factors that initiate the metabolism during germination. In view of the known effects of gibberellic acid in regulating metabolism in the storage tissues of germinating cereal grains,[5] we have paid particular attention to the possible roles of this and other phytohormones in controlling triglyceride metabolism.

RESULTS

Triglyceride breakdown was detected very early during germination in the bran, starchy endosperm and embryo axis (Fig. 1). The breakdown in the starchy endosperm during the first two days of germination was particularly rapid. No breakdown occurred in the scutellum until after the second day of germination.

The triglyceride contents of the tissues of incubated endosperm halves are shown in Fig. 2. Surprisingly, the level in the bran underwent a sudden fall of 22% within the first

[1] R. DESVAUX and M. KOGANE-CHARLES, *Annls. Inst. Natl. Rech. Agron., Paris* **A3**, 385 (1952).
[2] W. M. CROMBIE and E. E. HARDMAN, *J. Exptl Bot.* **9**, 247 (1958).
[3] B. E. BROWN, E. M. MEADE and J. R. BUTTERFIELD, *J. Am. Oil Chem. Soc.* **39**, 327 (1962).
[4] D. K. MECHAM, in *Wheat: Chemistry and Technology* (edited by I. HLYNKA), p. 353, American Association of Cereal Chemists, Minnesota (1964).
[5] P. FILNER, J. L. WRAY and J. E. VARNER, *Science* **165**, 358 (1969).

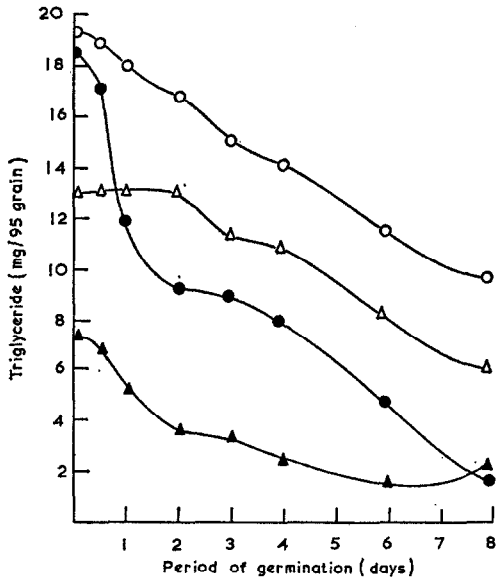

FIG. 1.

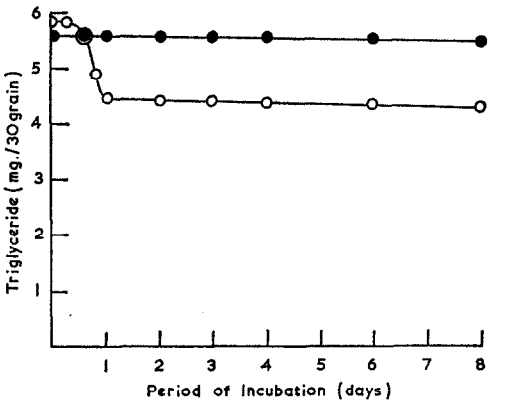

FIG. 2.

FIG. 1. CHANGES IN THE LEVELS OF TRIGLYCERIDES IN THE TISSUES OF GERMINATING WHEAT GRAIN.
●—starchy endosperm; ○—bran; ▲—embryo axis; △—scutellum.
FIG. 2. TRIGLYCERIDE LEVELS IN THE BRAN AND STARCHY ENDOSPERM OF INCUBATED ENDOSPERM HALVES.
●—starchy endosperm; ○—bran.

24 hr of incubation and thereafter remained constant. No triglyceride breakdown occurred in the starchy endosperm of the endosperm halves under these experimental conditions. When the bran from quiescent grain was incubated alone for up to 6 days no change in its triglyceride content occurred.

The results described above showed clearly that the removal of the embryo and the starchy endosperm has profound effects on the metabolism of triglycerides in the bran. In particular, the complete lack of triglyceride breakdown in the excised, incubated bran suggested that factors from the starchy endosperm are responsible for initiating the metabolism of at least a part of the bran triglyceride. Bran was therefore incubated in the presence of extracts of the starchy endosperm. Both water and the propan-2-ol–chloroform extracts effected a 22% drop in triglyceride content exactly the same as that occurring in the bran of incubated endosperm halves (Fig. 3). This result shows that the '22% metabolism' in the bran of incubated endosperm halves is induced by a factor or factors emanating from the starchy endosperm. The active principles were not destroyed by autoclaving the extracts.

The probable nature of the endosperm factor was revealed when excised bran was incubated in the presence of various growth regulatory compounds. Most important, the inductive effect of the endosperm extracts was mimicked exactly by the cytokinins kinetin and benzyl adenine at 'hormonal' concentrations (Fig. 3 and Table 1). Indole acetic acid was able to induce a smaller metabolism of the bran triglycerides, but it could not increase the efficacy of kinetin. Indole carboxylic acid and indole propionic acid were less active than indole acetic acid. It is noteworthy that the cytokinins and indole acetic acid were most

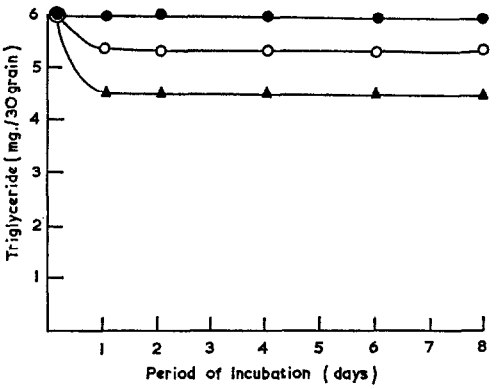

FIG. 3.

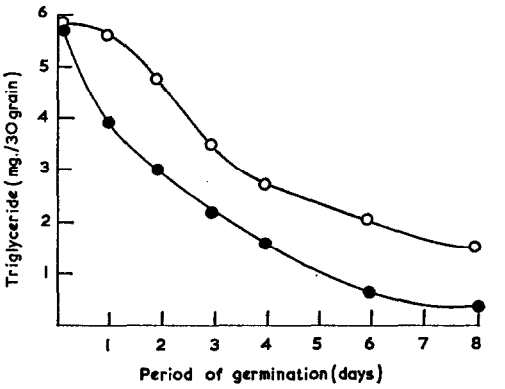

FIG. 4.

FIG. 3. THE INDUCTION OF TRIGLYCERIDE METABOLISM IN INCUBATED WHEAT BRAN.
●—bran incubated alone (control); ○—bran incubated with 10 μM indole acetic acid; ▲—bran incubated with 1 nM kinetin or with endosperm extract.

FIG. 4. LEVELS OF TRIGLYCERIDE IN ENDOSPERM HALVES INCUBATED WITH 1 mM HYDROXYLAMINE + 10 μM INDOLE ACETIC ACID.
●—Starchy endosperm; ○—bran.

TABLE 1. THE INDUCTION OF TRIGLYCERIDE METABOLISM IN EXCISED WHEAT BRAN

	Triglyceride metabolized (mg/30 grain)		Triglyceride metabolized (mg/30 grain)
Control (bran incubated alone)	0	1 nM kinetin +1 nM GA_3	0·3
10 mM kinetin	0	100 nM kinetin + 100 nM GA_3	0
10 μM kinetin	0·1	1 nM kinetin + 100 nM GA_3	0
100 nM kinetin	1·2	10 μM kinetin + 100 nM GA_3	0
1 nM kinetin	1·4	1 nM kinetin + 10 μM IAA	1·4
10 μM benzyl adenine	0·7	100 nM kinetin + 100 nM IAA	1·4
100 nM benzyl adenine	1·1	1 nM kinetin + 1 nM IAA	1·4
1 nM benzyl adenine	1·4	10 μM IAA + 10 μM GA_3	0·5
10 mM IAA	0·2	10 μM IAA + 100 nM GA_3	0·8
100 μM IAA	0·7	10 μM IAA + 1 nM GA_3	0·6
1 μM IAA	0·5		
10 nM IAA	0·2		
1 μM indole carboxylic acid	0·3		
1 μM indole propionic acid	0·1		
GA_3 (10 mM; 100 μM, 1 μM 10 nM)	0		

Bran samples from 30 grain were incubated for 4 days in the presence of the specified growth regulatory compound and then analysed for their triglyceride contents. The initial triglyceride content of the bran was 5·9 mg/30 grain. GA_3, gibberellic acid; IAA, indole acetic acid.

effective at 1 nM and 100 μM respectively, typically physiological concentrations. Gibberellic acid was ineffective in inducing triglyceride metabolism. On the contrary, it completely inhibited the action of the cytokinin, though not that of indole acetic acid.

The question remained as to whether or not the starchy endosperm acts as an energy source for the induction process in the bran. In order to answer this, bran was incubated in phosphate buffer containing 1 mM sucrose, glucose or maltose. None of these substrates induced any reduction in the bran triglyceride levels.

Table 2 shows the effects of various metabolic inhibitors on the kinetin-induced triglyceride metabolism in the bran. The inhibitors of DNA synthesis were, without exception, ineffective in inhibiting the induction process. On the other hand, acridine orange and proflavin inhibited the process strongly; both of these compounds inhibit the DNA-dependent synthesis of RNA. Actinomycin D, either in the presence or the absence of potassium bromate, was, however, without effect. The effects of several inhibitors of RNA-dependent protein synthesis and inhibitors of energy metabolism presented a more consistent picture; most of the compounds that were tested inhibited the induction strongly.

TABLE 2. THE EFFECTS OF METABOLIC INHIBITORS ON KINETIN-INDUCTED TRIGLYCERIDE METABOLISM IN EXCISED WHEAT BRAN

	Triglyceride metabolized (mg/30 grain)	Percentage inhibition
Controls:		
Bran incubated alone	0	—
Bran incubated with 10 nM kinetin	1·4	—
Inhibitors of DNA synthesis:		
Mitomycin, 100 μg/ml	1·4	0
100 mM hydroxyurea	1·4	0
5 mM chloroquine	1·4	0
Inhibitors of RNA synthesis:		
Acridine orange, 20 μg/ml	0·7	50
Proflavin, 25 μg/ml	0	100
Actinomycin D, 50 μg/ml + 1 mM bromate	1·4	0
Inhibitors of protein synthesis:		
Streptomycin, 100 μg/ml	0·9	35
Chloramphenicol, 20 μg/ml	1·4	0
Mikamycin, 100 μg/ml	0·6	55
Cycloheximide, 3 μg/ml	0·1	95
Puromycin, 50 μg/ml	0·1	95
Inhibitors of energy metabolism:		
10 mM sodium azide	1·4	0
100 μM sodium cyanide	0	100
100 μM sodium arsenite	0	100
10 mM sodium fluoride	0·2	85
100 μM 2,4-dinitrophenol	0	100
Oligomycin, 2 μg/ml	0	100
Chloramphenicol, 500 μg/ml	0·8	45
Antimycin A, 30 μg/ml	0·3	80

Bran samples from 30 grain were incubated for 6 hr in the presence of the specified inhibitor. Kinetin was then added to 10 nM and the incubation continued for 4 days. The triglyceride contents of the bran were then determined.

Although the cytokinins and, to a lesser extent, the auxins induced metabolism of a part of the triglyceride reserves of incubated bran, neither these phytohormones nor gibberellic acid, either singly or in combination, were able to initiate triglyceride metabolism in the starchy endosperm of incubated endosperm halves. At this juncture in the work it had been found that lipase activity in the starchy endosperm could be induced by hydroxylamine, glutamine or one of several other nitrogen-containing compounds.[6] Furthermore, lipase activity in the bran of incubated endosperm halves was induced by hydroxylamine or glutamine together with indole acetic acid.[7] Therefore, endosperm halves were incubated in the presence of hydroxylamine and indole acetic acid and the triglyceride contents of their tissues were determined. The results are presented in Fig. 4. As expected, the induction of triglyceride metabolism occurred in the starchy endosperm. In the bran, triglyceride metabolism proceeded beyond that due to cytokinin action. Furthermore, the pattern of metabolism in this tissue was then much more like that in the bran of intact, germinating grain (Fig. 1).

In order to check that the lipase induced in endosperm halves by embryo diffusates[6] is also accompanied by triglyceride metabolism, the triglyceride contents of these induced endosperm halves were determined. It can be seen (Table 3) that the embryo diffusate induced triglyceride metabolisms in both the starchy endosperm and the bran similar to those induced by a mixture of hydroxylamine and indole acetic acid. Triglyceride metabolism in the bran again proceeded beyond that due to cytokinin action.

TABLE 3. THE INDUCTION OF TRIGLYCERIDE METABOLISM IN ENDOSPERM HALVES BY EMBRYO DIFFUSATES

	Triglyceride content (mg/30 grain)	
	Bran	Starchy endosperm
Unincubated endosperm halves (0 day control)	5·9	5·7
Endosperm halves incubated alone (4 day control)	4·6	5·5
Endosperm halves incubated with embryo diffusate	3·6	3·5

30 endosperm halves were incubated for 4 days in the presence of the embryo diffusate and then dissected into bran and starchy endosperm for analysis.

DISCUSSION

Like the oleaginous seeds, the wheat grain mobilizes its reserves of triglycerides during germination. While these reserves are probably not quantitatively important with respect to the whole grain, they are major nutrient reserves in some of its tissues. The triglycerides in the embryo axis, for example, are probably acting as a substrate and energy source for that tissue early during germination before nutrients are mobilized from the endosperm. Triglyceride metabolism in this tissue proceeds rapidly at first but continues more slowly after about the third day, when the metabolism of the scutellar triglycerides commences. This 3-day period corresponds approximately to the time for which an isolated embryo can support itself when cultured in a nutrient-free medium. The commencement of triglyceride metabolism in the scutellum correlates closely with the appearance of lipase activity in that

[6] R. J. A. TAVENER and D. L. LAIDMAN, *Biochem. J.* **113,** 32P (1969).
[7] D. EASTWOOD, R. J. A. TAVENER and D. L. LAIDMAN, *Biochem. J.* **113,** 32P (1969).

tissue during germination.[8] In the embryo axis however, there is no such correlation. The rapid triglyceride metabolism during the first 2 days in this tissue must be either by a pathway not involving lipase, or it must be catalysed by the relatively low level of lipase that is present in the embryo axis of the quiescent grain.[8]

Triglyceride metabolism in the starchy endosperm is also associated with lipase activity.[8] It is important to appreciate that neither lipase activity nor triglyceride metabolism in this tissue are induced by any of the growth regulators tested, but instead they are induced by certain nitrogen-containing compounds. The case of glutamine is particularly relevant in this respect. It has been known for many years[9] that germinating seeds accumulate considerable quantities of either glutamine or asparagine during germination, although in the ungerminated seed they are present only in very small quantitites. Our own investigations (unpublished data) show that the wheat grain is in the glutamine-producing group.

The storage triglycerides in the bran appear to exist in at least two distinct parts metabolized by different pathways. One part, which constitutes some 22% of the total triglycerides, is metabolized by a pathway that is induced by a hormone emanating from the starchy endosperm. This hormone is almost certainly a cytokinin. Significantly, cytokinins have been isolated from maize endosperm[10] and detected in germinating barley.[11] The nature of the cytokinin-induced metabolism is not known; it occurs in circumstances where no lipase is detectable by our lipase assay procedure.[8] Considered together, the results of the experiments with metabolic inhibitors suggest that the induction by cytokinins is dependent upon both protein synthesis and energy metabolism. The inability of actinomycin D to block the induction is, however, unexpected in view of the high inhibitory activities of proflavin and acridine orange. These results cannot, therefore, be taken to demonstrate the involvement of RNA synthesis in the induction process. It is of interest that actinomycin D also did not inhibit ubiquinone synthesis in the same tissue.[12] The inhibition of the cytokinin action by gibberellic acid suggests that these hormones might form a balanced controlling system during germination.

The metabolism of the second part of the triglyceride reserves in the bran is associated with lipase activity in that tissue[8] and is clearly initiated by factors emanating from the embryo. Although indole acetic acid together with glutamine can substitute for the embryo in this role, the present results do not allow the conclusion that the embryo is the source of these compounds during germination. Further investigations are needed to clarify this situation.

In conclusion, the induction and control of triglyceride metabolism in the storage tissues of the germinating wheat grain appears to be brought about by complex control mechanisms involving three phytohormones and glutamine. This situation is similar to that described for the induction and control of mineral mobilization in the same system,[7,13] but quite different from that described for the mobilization of carbohydrate reserves.[5]

EXPERIMENTAL

Sterilization and incubation of plant tissues. 5 g samples (95 grain) of wheat (var. Cappelle Desprez; 1965 harvest) were sterilized and allowed to germinate at 25° by our routine procedure.[12] Samples were harvested at periods up to 8 days and dissected into their component parts for analysis.

[8] R. J. A. TAVENER and D. L. LAIDMAN, *Phytochem.* **11,** 989 (1972).
[9] A. G. CHIBNALL, in *Protein Metabolism in the Plant*, p. 87, Yale University Press, New Haven (1939).
[10] D. S. LETHAM and C. O. MILLER, *Plant Cell Physiol., Tokyo* **6,** 355 (1965).
[11] H. A. VAN ONKELEN, R. VERBEEK and L. MASSART, *Naturwissenschaften* **52,** 561 (1965).
[12] G. S. HALL and D. L. LAIDMAN, *Biochem. J.* **108,** 475 (1968).
[13] D. EASTWOOD and D. L. LAIDMAN, *Biochem. J.* **113,** 33P (1969).

Endosperm halves were prepared from samples of 30 grain (1·9 g) by removing all scutellar and embryonic tissue with a transverse cut just behind the embryo. The embryo containing portions were discarded. The endosperm halves were sterilized by our routine procedure and incubated at 25° under sterile conditions on moistened filter paper in 9 cm crystallizing dishes fitted with a Petri dish lid. Upon harvesting they were dissected into bran and starchy endosperm for analysis.

Bran was prepared under sterile conditions from sterilized endosperm halves. The halves were quartered longitudinally and placed with their starchy endosperm resting downwards on moistened filter paper. After 3 hr, the starchy endosperm could be scraped away from the bran with the back of a scalpel blade. The excised bran from 30 endosperm halves was incubated under sterile conditions in 25 ml 0·2 M phosphate buffer, pH 6·0, contained in a 100 ml conical flask. The flask and contents were incubated at 25° with reciprocal shaking.

When endosperm halves or excised bran were incubated in the presence of solutions of tissue extracts or diffusates (see below), growth regulatory compounds or inhibitors, these solutions were sterilized by ultrafiltration immediately before application to the incubating tissues. In the case of incubated bran, the hormones and inhibitors were added as appropriately concentrated solutions to give the desired final concentration in the buffer media.

Preparation of embryo diffusates. 5 g sterilized wheat grain were allowed to germinate for 24 hr in the dark. Their embryos (embryo axis + scutellum) were then dissected from the grain and incubated for 6 hr at 25° in 80 ml sterile distilled water. The embryos were then discarded. The incubation medium was acidified and extracted three times with ethyl acetate. After extraction the aqueous phase was reduced to dryness at 40° in a rotary evaporator. The resulting residue was taken up in 5 ml distilled water, sterilized by ultrafiltration and the solution made up to a standard volume of 10 ml with sterile, distilled water.

Preparation of endosperm extracts. The starchy endosperm from 5 g ungerminated grain was homogenized and extracted three times with distilled water. The bulked extracts were evaporated to dryness at 40° in a rotary evaporator. The residue was taken up in 5 ml distilled water, sterilized by ultrafiltration and made up to 10 ml with sterile, distilled water. Other samples of starchy endosperm were similarly extracted with propan-2-ol–$CHCl_3$ (1:1, v/v).

Determination of triglycerides. Total lipids were extracted from the tissues by hot propan-2-ol and $CHCl_3$ and chromatographed on acid-washed alumina according to our routine procedure.[14] The gravimetric distribution of material in the fractions from chromatography revealed four major peaks at fractions 1–3, 8–10, 12–18 and 28–29. TLC of these fractions on Silica gel G, using authentic compounds as standards, revealed that the peaks corresponded to hydrocarbons, steryl esters, triglycerides and sterols respectively. The triglyceride-containing fractions were then bulked and analysed for triglycerides by the method of Carlson and Wadstrom.[15] In order to check this method, samples of the bulked triglyceride fractions from the alumina chromatography were also subjected to methanolysis,[16] and the resulting mixture of fatty aid methyl esters were analysed quantitatively by gas chromatography. For this purpose a Pye 104 chromatography fitted with flame ionization detectors was used. The stationary phase was 2% polyethylene glycol succinate supported on 100–120 mesh Chromosorb W-AW-DMCS (Johns Manville Products Corp., New York). The identity of the individual fatty acid methyl esters was deduced from a comparison of their retention times with those of authentic compounds and the chromatograph was calibrated individually for each compound. The results of the gas chromatography were confirmed qualitatively by TLC.[17] The results from the gas-chromatographic and the Carlson–Wadstrom analyses agreed within very close limits (±3%) and showed that the bulked triglyceride fractions from the alumina column chromatography contained only small quantities of other compounds.

Towards the end of this work a Pye Liquid Chromatograph, System 2 (Pye–Unicam, Cambridge) became available. This instrument gave a direct, gravimetric analysis of the triglyceride and other major lipid fractions eluted from the alumina column. The machine was calibrated for triglycerides against the Carlson and Wadstrom method, and day to day reproducibility of the triglyceride analyses was achieved by co-chromatographing a known amount of *n*-docosanol as an internal standard. This compound was eluted immediately after the triglycerides and before the sterols. By comparison of the under-peak area of the triglycerides with that of the standard a reproducibility of ±2% was achieved. The results shown in Fig. 4 and Tables 2 and 3 were obtained using this automated method.

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Key Word Index—Triticum aestivum; Gramineae; wheat; germination; triglycerides; hormones.