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THE INDUCTION OF LIPASE ACTIVITY 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—The induction of lipase activity in the storage tissues of the germinating wheat grain is dependent upon factors emanating from the embryo. In endosperm halves, lipase activity can be induced in the starchy endosperm by one of several nitrogenous compounds, in particular glutamine. Lipase activity in the bran of endosperm halves is induced by indole acetic acid in the presence of the nitrogen source. Both of these induction processes are inhibited by inhibitors of energy metabolism, RNA synthesis and protein synthesis, and by azaserine, a glutamine analogue. The action of indole acetic acid and the nitrogenous compound in inducing lipase activity in isolated bran appears to require the prior action of a cytokinin and some other unidentified factor.

INTRODUCTION

TRIGLYCERIDE reserves in the various tissues of the wheat grain are mobilized during germination.[1] The pathways by which these reserves are metabolized are not known with certainty, but it is anticipated that the lipase-catalysed hydrolysis of triglycerides is the first reaction in at least some of the pathways. Thus, lipase probably occupies a cardinal position[2] in the metabolic pathway from the triglycerides and it is a probable locus for any hormonal control of the pathway. In the experiments reported here, therefore, we have not only studied the patterns of lipase activity during germination, but also attempted to characterize some of the most important mechanisms for its control.

Previous studies on lipase activity in germinating seeds are rare, and are restricted to a few species; e.g. cotton seed,[3] castor bean,[4] barley[5] and wheat.[6] Furthermore, some of these studies have been complicated by the problems associated with the determination of lipase activity in plant tissues. The literature relevant to lipases is confusingly entangled with that of esterases in general; lipase exhibits a low substrate specificity but requires precisely defined conditions for action on its true substrate.[7] With this situation in mind we have used a method for lipase estimation based upon the titrimetric method of Desnuelle, Constantin and Baldy[8] and involving corrections for non-lipase acid production.

RESULTS

Preliminary experiments revealed that there was no correlation between lipase and lipoxidase activities in the tissues of the germinating grain. In fact, lipoxidase activity fell rapidly to zero early during germination while lipase activity increased in all of the tissues.

The patterns of lipase activity during germination show several interesting features

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(Fig. 1). In the starchy endosperm lipase activity appeared within 12 hr of imbibition and increased rapidly up to the sixth day of germination. In contrast, in the bran there was no activity until after the first day of germination. The scutellum and embryo axis of the quiescent grain contained very low levels of lipase which did not increase until after the second day of germination.

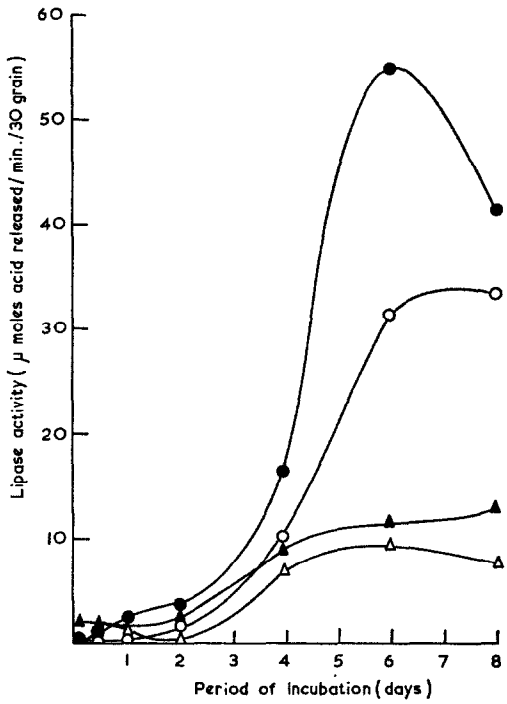

FIG. 1. LIPASE ACTIVITIES IN THE TISSUES OF GERMINATING WHEAT GRAIN.
●—starchy endosperm; ○—bran; ▲—embryo axis; △—scutellum.

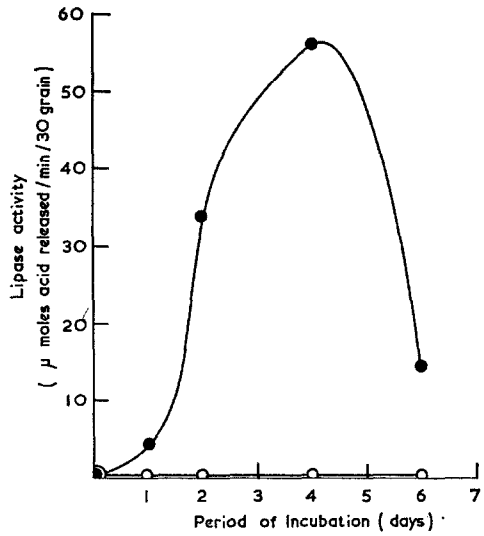

FIG. 2. LIPASE ACTIVITIES IN THE TISSUES OF ENDOSPERM HALVES INCUBATED WITH 1 mM HYDROXYLAMINE.
●—starchy endosperm; ○—bran.

When endosperm halves were incubated alone for periods up to 6 days, no lipase activity could be detected in either the bran or starchy endosperm. Because no lipase activity could be detected in incubated endosperm halves, there is presumably a requirement for a factor, possibly a hormone, from the embryo. Endosperm halves were therefore incubated for 4 days in the presence of various plant growth regulators. Neither gibberellic acid, indole acetic acid, benzyl adenine nor abscisic acid, at a range of concentrations and either singly or in combination, were able to induce any activity.

In contrast to the growth regulators, a number of nitrogenous compounds, at substrate concentrations, were able to induce lipase activity in the starchy endosperm of the endosperm halves (Table 1). None of these compounds induced any activity in the bran. The response to hydroxylamine (Fig. 2) was quantitatively similar to the *in vivo* pattern of lipase development in the starchy endosperm (Fig. 1). Besides hydroxylamine, glutamine and a range of purine and pyrimidine nucleotides were very effective, although adenosine 5′-triphosphate was not effective and actually inhibited induction by the other nucleotides. In contrast to

TABLE 1. INDUCTION OF LIPASE ACTIVITY IN THE STARCHY ENDOSPERM OF INCUBATING ENDOSPERM HALVES

	Lipase activity (μmoles acid released/min/30 grain)
Control (endosperm halves incubated alone)	0
Urea	9
Hydroxyurea	0
Serine	14
Alanine	14
Aspartic acid	0
Asparagine	0
Glutamic acid	0
Glutamine	42
Casein hydrolysate, 1 mg/ml	17
Sodium nitrate	11
Sodium nitrite	16
Ammonium chloride	21
Hydroxylamine	55
Inosinic acid	15
Adenosine 5'-phosphate (AMP)	30
Adenosine 5'triphosphate (ATP)	0
Guanosine 5'phosphate (GMP)	28
Guanosine 5'-triphosphate (GTP)	43
Cytidine 5'phosphate (CMP)	42
Cytidine 5'triphosphate (CTP)	42
Uridine 5'phosphate (UMP)	24
Uridine 5'-triphosphate (UTP)	25
UTP + CTP + GTP + AMP, each at 200 μM	41
UTP + CTP + GTP + ATP, each at 200 μM	24
Papain, 140 μg/ml + cysteine, 1·6 mg/ml at pH 6·0	0

In each experiment 30 endosperm halves were incubated for 4 days in the presence of the specified compound. Except where specified, the compounds were applied at 1 mM concentration.

glutamine, glutamic acid was without effect. In a further experiment, not reported in detail here, it was shown that hydroxylamine could induce lipase activity in the starchy endosperm in the absence of the bran. A papain/cysteine mixture was inactive.

The effects of various metabolic inhibitors on the hydroxylamine-induction of lipase activity are recorded in Table 2. While a number of inhibitors of DNA synthesis were without effect, several inhibitors of RNA synthesis and protein synthesis, and some inhibitors of oxidative energy metabolism inhibited the induction strongly. Azaserine, a structural analogue of glutamine, was also a very effective inhibitor.

The experiments described above revealed that factors other than phytohormones on their own, or nitrogenous compounds on their own, are necessary for the induction of lipase activity in the bran of incubating endosperm halves. This induction proved to require indole acetic acid together with a suitable nitrogen source such as hydroxylamine (Table 3). Gibberellic acid was unable to replace indole acetic acid. Exogenous cytokinins were not tested in this experiment because a cytokinin was assumed to be present in the starchy endosperm.[1] The patterns of induced lipase development in the tissues of the endosperm halves (Fig. 3) were very similar to those in the intact germinating grain (Fig. 1).

Table 4 records the effects of metabolic inhibitors on the induction of lipase activity in

TABLE 2. THE EFFECTS OF METABOLIC INHIBITORS ON THE HYDROXYLAMINE INDUCTION OF LIPASE ACTIVITY IN INCUBATING ENDOSPERM HALVES

	Lipase activity (μmoles acid released/min/30 grain)	Percentage inhibition
Control (no inhibitor)	55	—
Inhibitors of DNA synthesis:		
Mitomycin, 100 μg/ml	56	0
100 mM hydroxyurea	55	0
5 mM chloroquine	55	0
Inhibitors of RNA synthesis:		
Proflavin, 25 μg/ml	0	100
Actinomycin D, 20 μg/ml	0	100
Glutamine analogue:		
1 mM azaserine	6·2	89
Inhibitors of protein synthesis:		
Streptomycin, 100 μg/ml	53	4
Chloramphenicol, 20 μg/ml	0	100
Mikamycin, 100 μg/ml	0	100
Cycloheximide, 3 μg/ml	0	100
Puromycin, 50 μg/ml	0	100
Inhibitors of energy metabolism:		
1 mM sodium azide	2·1	96
100 μM sodium cyanide	0	100
100 μM sodium arsenite	54	2
10 mM sodium fluoride	1·4	97
Oligomycin, 2 μm/ml	55	0
Chloramphenicol, 500 μg/ml	0	100
Antimycin A, 30 μg/ml	3·5	94
100 μM 2,4-dinitrophenol	0	100

Batches of 30 endosperm halves were incubated for 12 hr in the presence of the specified inhibitor. Hydroxylamine was then added to 1 mM and the incubation continued for 4 days. The whole endosperm halves were analysed.

the bran of endosperm halves. The induction was inhibited strongly by inhibitors of RNA synthesis, protein synthesis, energy metabolism and by azaserine.

Embryo diffusates induced lipase activity in both the starchy endosperm and the bran of incubating endosperm halves, thus demonstrating that, in the germinating grain, the lipase-inducing factors emanate from the embryo. In this experiment the responses of the endosperm half tissues to the diffusate (Fig. 4) were markedly less than those to the hydroxylamine–indole acetic acid mixture (Fig. 3), although they followed similar development patterns. This discrepancy was presumably due to a concentration effect. The inhibitions of this diffusate-induction of lipase activity by various metabolic inhibitors (unpublished data) were essentially the same as their inhibition of the hydroxylamine–indole acetic acid induction.

Hydroxylamine with indole acetic acid failed to induce any lipase activity in bran that had been excised from the quiescent grain (Table 5). When the bran was pre-incubated with a cytokinin, however, hydroxylamine and indole acetic acid were then able to induce a low but definite lipase activity in the tissue. This activity was less than 20% of the maximum level found in the bran of the germinating grain. In an attempt to improve the induction, the experiments were repeated with the bran incubated on filter paper in the manner described for endosperm halves. No improvement was, however, achieved. Because of the poor induction, no attempt was made to study the effects of metabolic inhibitors in this system.

TABLE 3. INDUCTION OF LIPASE ACTIVITY IN THE BRAN OF INCUBATING ENDOSPERM HALVES

	Lipase activity (μmoles acid released/min/30 grain)	
	Bran	Starchy endosperm
Control (endosperm halves incubated alone)	0	—
3 mM hydroxylamine	0	—
3 mM hydroxylamine + 100 nM GA_3	0	—
3 mM hydroxylamine + 10 μM GA_3	0	—
3 mM hydroxylamine + 10 μM IAA	24	46
3 mM hydroxylamine + 100 nM IAA	15	48

In each experiment 30 endosperm halves were incubated for 4 days in the presence of the specified compounds.

GA_3, giberellic acid; IAA, indole acetic acid.

DISCUSSION

The results of the experiments on endosperm halves show clearly that a factor or factors from the embryo are responsible for the induction of lipase activity in the storage tissues of the germinating wheat grain. The nature of these factors are not yet known. In the case of lipase induction in the starchy endosperm it is clear that the responsible factor cannot be identified with any of the known plant hormones. On the other hand, the compound immediately responsible for this induction is probably glutamine, since this compound

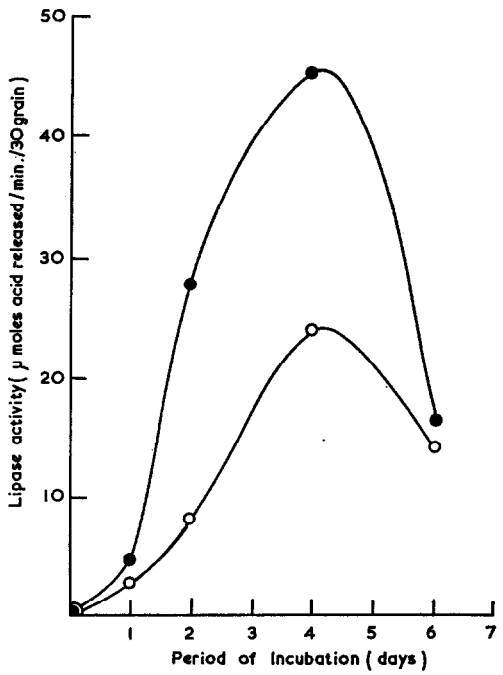

FIG. 3. LIPASE ACTIVITIES IN THE TISSUES OF ENDOSPERM HALVES INCUBATED WITH 1 mM HYDROXYLAMINE + 10 μM INDOLE ACETIC ACID.
●—starchy endosperm; ○—bran.

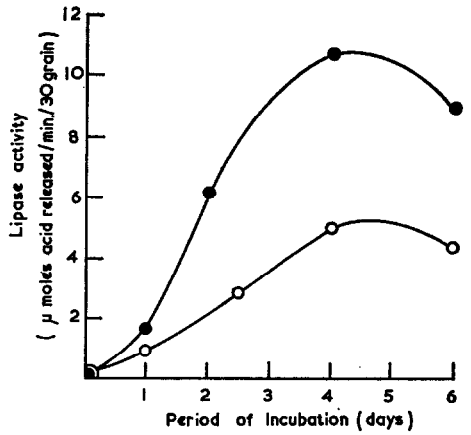

FIG. 4. LIPASE ACTIVITIES IN THE TISSUES OF ENDOSPERM HALVES INCUBATED WITH EMBRYO DIFFUSATE.
●—starchy endosperm; ○—bran.

TABLE 4. THE EFFECTS OF METABOLIC INHIBITORS ON THE INDUCTION OF LIPASE ACTIVITY IN THE BRAN OF ENDOSPERM HALVES

	Lipase activity (μmoles acid released/min/30 grain)	Percentage inhibition
Control (no inhibitor)	9·5	—
Inhibitors of DNA synthesis:		
Mitomycin, 100 μg/ml	8·9	6
5 mM Chloroquine	9·0	5
Inhibitors of RNA synthesis:		
Proflavin, 25 μg/ml	1·1	88
Actinomycin D, 20 μg/ml	0·7	93
Glutamine analogue:		
1 mM azaserine	1·9	80
Inhibitors of protein synthesis:		
Chloramphenicol, 20 μg/ml	1·4	85
Cycloheximide, 3 μg/ml	1·0	89
Puromycin, 50 μg/ml	0·9	90
Inhibitors of energy metabolism:		
100 μM sodium cyanide	0·9	90
100 μM 2,4-dinitrophenol	1·5	84

Batches of 30 endosperm halves were incubated for 3 days in the presence of 1 mM hydroxylamine and then for 12 hr in the presence of the specified inhibitor and 1 mM hydroxylamine. Indole acetic acid was then added to 10 μM and the incubation continued for a further 2 days. The bran was dissected from the incubated endosperm halves and analysed for its lipase content.

accumulates in appreciable quantities in the germinating grain[1] and it had a high inductive capacity in our experiments. Other nitrogen compounds showed inductive capacity possibly because they were able to donate nitrogen to the amide group of glutamine. Hydroxylamine is particularly interesting in this respect. The enzyme glutamine synthetase catalyses several reactions including the formation of γ-glutamyl hydroxamic acid from glutamic acid and hydroxylamine and a slower conversion of the γ-glutamyl hydroxamate to glutamine by a

TABLE 5. INDUCTION OF LIPASE ACTIVITY IN INCUBATING, EXCISED BRAN

	Lipase activity (μmoles acid released/min/30 grain)
Control (bran incubated alone)	0
3 mM hydroxylamine	0
3 mM hydroxylamine + 100 nM GA_3	0
3 mM hydroxylamine + 10 μM IAA	0
100 nM kinetin + 3 mM hydroxylamine	0
100 nM kinetin + 3 mM hydroxylamine + 100 nM GA_3	0
100 nM kinetin + 3 mM hydroxylamine + 10 μM IAA	9·0
100 nM kinetin + 3 mM hydroxylamine + 100 nM IAA	7·6

In each experiment the bran from 30 quiescent grain was incubated for 4 days in the presence of the specified compounds. Where the effects of kinetin were studied, the bran samples were pre-incubated for 24 hr with this hormone before incubation with the specified compounds.

GA_3, gibberellic acid; IAA, acetic acid.

transfer reaction involving ammonia.[9] Indeed, inhibition of hydroxylamine induction by azaserine suggests strongly that hydroxylamine is first metabolized to glutamine. We have, however, been unable to detect glutamine synthetase activity in the endosperm of the germinating wheat grain,[10] and recent studies in other species have demonstrated that, during germination, the amide nitrogen of asparagine is derived from cyanide by a pathway not involving asparagine synthetase.[11,12] Glutamine in the germinating wheat grain may well be produced by a similar pathway.

Lipase induction in the bran is almost certainly located in the aleurone cells and resembles the induction of phytase in that tissue.[13] The sequential action of cytokinin and indole acetic acid in lipase induction parallels the sequential action of cytokinin and gibberellic acid in α-amylase induction in the same tissue.[14] The poor induction of lipase activity in the isolated bran suggests, however, that a further, unidentified factor is also involved.

The powerful action of several inhibitors in preventing lipase induction in the starchy endosperm and in the bran demonstrates the dependence of both induction processes on both RNA and protein synthesis. The situation in the starchy endosperm is particularly interesting in this respect. Glutamine is the preferred nitrogen source for nucleotide synthesis, and it is attractive to speculate that glutamine exerts its inductive effect by donating nitrogen for the synthesis of nucleotides and hence of a ribonucleotide. Further investigations are clearly necessary to test this hypothesis.

The energy relationships of the induction process present problems of interpretation. The proposed induction by glutamine of ribonucleic acid synthesis would place an energy demand on the starchy endosperm, but this is usually considered to be a dead tissue with no detectable mitochondria. The inhibition by several inhibitors of oxidative energy metabolism thus becomes difficult to interpret. The alternative explanation that microbial contamination is responsible for the phenomena that we have observed is also unlikely. Although tests (unpublished data) have revealed the presence of gram-negative rod bacteria within the starchy endosperm of the 'sterilized' grain and endosperm halves, the growth of these organisms did not occur in the presence of chloroquine, streptomycin or chloramphenicol, while they did grow in the presence of cycloheximide. This inhibition pattern was quite different from that for the lipase induction system and effectively rules out a procaryotic organism as the causative agent.[15] Furthermore, we have been unable to detect significant numbers of molds or yeasts in our 'sterilized' grain. If such a eucaryotic micro-organism is responsible for our *in vitro* observations, it is most likely also responsible for the induction *in vivo*.

There are marked qualitative similarities between the induction patterns of lipase activity and aspects of triglyceride metabolism in the storage tissues of the grain.[1] These similarities are suggestive of a cause and effect relationship between the two. The levels of lipase activity do not, however, correlate well with the observed rates of triglyceride metabolism at different times during germination. In particular, the measured lipase activities are very high in comparison with the amounts of triglyceride present, suggesting that lipase-

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substrate interactions are controlled by factors that have not been revealed in the present studies. At this stage of the work, therefore, the idea of a causal relationship between triglyceride metabolism and lipase activity must be advanced with some reservations.

EXPERIMENTAL

Sterilization and incubation of plant tissues. 1·9 g samples (30 grain) of wheat (var. Cappelle Desprez; 1965 harvest) were sterilized and allowed to germinate by our routine procedure.[16] Samples were harvested at periods up to 8 days and dissected into their component tissues for analysis.

Endosperm halves and bran from batches of 30 grain were prepared and incubated under sterile conditions.[1] Upon harvesting the endosperm halves were dissected into bran and starchy endosperm for analysis.

Preparation of embryo diffusates. These diffusates were prepared and sterilized by the method described in the accompanying paper.[1]

Determination of lipase (E.C. 3.1.1.3) activity. The tissue samples were placed in McCartney bottles at ice temperature. Using a top-drive micro-homogenizer (Silverson Machines Ltd., London) they were extracted with 3 × 10 ml vol. of ice-cold acetone. The acetone extracts were bulked and centrifuged to pellet any suspended material. This pellet was transferred back to the McCartney bottle. All traces of acetone were removed from the extracted tissues under a stream of nitrogen and finally under vacuum. The resulting acetone powders were stored at −25° until required.

Lipase activity was determined in the acetone powders using a titrimetric method based on that of Desnuelle, Constantin and Baldy.[8] The principal modification was the use of a neutral pH for the determinations, in contrast to the alkaline pH used in the earlier work; pH 7 is nearer to the physiological pH of the grain tissues. In the enzyme-substrate mixture for the determination of lipase activity there are a number of acid producing reactions in addition to lipase activity itself. These are endogenous acid production by the crude enzyme extract, substrate acid production by auto-oxidative processes in the triglyceride-gum acacia emulsion used as substrate, and acid production by the enzymic activity of the enzyme extract on the gum acacia (acaciase). The procedure described below is designed to take these facts into account.

1 ml corn oil (Boots Pure Drug Ltd., Nottingham) was added to 9 ml sterile, 10% gum acacia in a McCartney bottle and the mixture was homogenized for 2 min using the Silverson micro-homogenizer set at the slowest speed. 4 ml of this corn oil suspension were pipetted into the micro-titration vessel of an automatic titrator (Radiometer, A.G., Copenhagen). The titration vessel was maintained at 25°. The pH-stat was set at pH 7·0 and 0·01 N. NaOH was used as the titrant. The reaction rate was measured between 10 and 30 min when the titration curve had become linear. The value obtained was termed the 'Substrate acid production rate'. The corn oil-gum acacia mixture in the pH-stat was then replaced by 4 ml of an aqueous suspension of the tissue acetone powder in a separate titration vessel. This suspension was prepared immediately before use by homogenizing the acetone powder for 2 min in 10 ml sterile, distilled water using the microhomogenizer at its slowest speed. The rate of acid production of this suspension, measured when the titration plot became linear, was termed the 'endogenous acid production rate'. 1 ml of the acetone powder suspension was then added to 4 ml of the substrate, and the rate of acid production was determined. This value was the 'apparent lipase activity'. Finally, 4 ml of 10% gum acacia were titrated alone under the same conditions. This value was termed the 'acacia acid production rate'. 1 ml of acetone powder suspension was then added to this gum acacia solution and the acid production rate again determined—the 'apparent acaciase activity'.

From the data obtained a value for the lipase activity could be calculated, taking into account all the necessary blank determinations:

$$\text{Actual lipase activity} = \text{apparent lipase activity—substrate acid production rate} - \frac{\text{endogenous acid production rate}}{4} \text{—acaciase activity.}$$

$$\text{Where: Acaciase activity} = \text{apparent acaciase activity} - \frac{\text{endogenous acid production rate}}{4} \text{—acacia acid production rate.}$$

n this calculation it is assumed that the acaciase activity is the same both in the presence and in the absence of the corn oil.

Duplicate determinations of lipase activity agreed within close limits.

Determination of lipoxidase (E.C. 1.13.1.13) activity. Lipoxidase activity in the acetone powders might lead to the production of acidic metabolites from the corn oil substrate used for the lipase determination. Because this activity would interfere with the lipase determinations, check lipoxidase assays were carried

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out on selected tissue powders using the method of Surrey.[17] After this work had been completed, our attention was drawn to the presence in some seeds of a hydroperoxide isomerase which removes the chromphore that is the basis of the Surrey method for lipoxidase estimation.[18] If this enzyme is present in our wheat, it could seriously compromise our check for lipoxidase activity.

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