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# The Breakdown of Lipid Reserves in the Endosperm of Germinating Castor Beans

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1. Lipid extracts were obtained from castor-bean endosperm tissue at various times during germination and, after purification, the total lipid content was determined. Quantitative measurements of the triglyceride and phospholipid content together with the fatty acid composition were made. 2. The total lipid content of the endosperm rapidly decreased during germination; after 10 days less than 20% of the original weight of lipid remained. In contrast, the phospholipid content (initially less than 0.5% of the total lipid) increased slightly during this time. The fatty acid composition and the relative proportions of the triglyceride species of the total lipid extract remained constant during 10 days of germination. 3. Gibberellic acid (0.3mM) markedly stimulated the rate of lipid breakdown but did not alter either the fatty acid composition or the relative proportion of triglyceride species. 4. The embyro had little effect on lipid metabolism in the endosperm tissue; only after 6 days of germination were differences observed in the rate of fat utilization in the presence and absence of the embryo.

Storage lipid, chiefly triglyceride, is rapidly and effectively converted into sucrose in castor-bean endosperm tissue during germination (Beevers, 1961; Canvin & Beevers, 1961). The triglycerides are stored in fat-droplets (Harwood *et al.*, 1971) and some lipolysis could occur within these globules. However, a substantial part of the metabolic sequences takes place in single membrane-bound organelles, glyoxysomes, which contain the enzymes for $\beta$-oxidation and the glyoxylate cycle (Breidenbach *et al.*, 1968; Cooper & Beevers, 1969*a*,*b*; Hutton & Stumpf, 1969). Muto & Beevers (1974) have demonstrated the presence of an alkaline lipase activity in these organelles.

Glyoxysomes and their characteristic enzymes could not be detected in mature castor-bean seeds (Gerhardt & Beevers, 1970), and it was concluded that the activity of the enzymes and the number of organelles rises and later falls during the germination period, in phase with fat breakdown. Several investigations have been made on the role of metabolites, hormones and the embryo on glyoxysomal-enzyme activities in fat-storing seeds during germination (Marcus & Feeley, 1964; Penner & Ashton, 1967; Lado *et al.*, 1968; Pinfield, 1968*a*,*b*; Scala *et al.*, 1969; Vincenzini *et al.*, 1973). However, since significant amounts of free long-chain fatty acids do not accumulate (Beevers, 1961; Canvin & Beevers, 1961), lipolysis is a possible important control point in the initiation of fat utilization during germination. We have therefore investigated the changes in the storage lipid during germination and the factors which effect its breakdown.

Gibberellic acid is effective in mobilizing the storage reserves in cereal seeds (Filner *et al.*, 1969) and it was decided to study the effect of this hormone on lipid breakdown in oleaginous seeds. Pinfield (1968*b*) found that gibberellic acid increases the activity of the glyoxysomal enzyme isocitrate lyase in germinating hazel seeds, and Younis *et al.* (1971) suggest that conversion of oil into carbohydrates in castor-bean seeds is accelerated by gibberellic acid treatment. The presence of the embryo is important in the breakdown of cereal endosperm reserves (Radley, 1967, 1969) and it was therefore necessary to discover whether a similar control by the embryo exists in castor-bean seeds during germination.

## Methods

### *Materials*

Castor bean (*Ricinus communis* var. Zanzibariensis mixed) seeds were purchased locally (Sanders, Cambridge, U.K.). Gibberellic acid containing not less than 90% gibberellic acid was obtained from BDH (Poole, Dorset, U.K.). Solvents were redistilled before use.

### *Germination of seeds*

All manipulations were carried out under sterile conditions on a sterile air bench. For each time-point, seeds of a similar weight (±0.5g) were

selected and surface-sterilized by immersion in ethanol for 2min. The testa was removed and the seeds were then soaked for a further 2min in Milton solution (Richardson–Merrell Ltd., London, U.K.). After thorough rinsing with sterile water the seeds were placed either intact or with the embryos removed on 60ml of solid agar in polypropylene jars of 700ml capacity. Noble agar (1%) (Difco Laboratories, Detroit, Mich., U.S.A.) in glass-distilled water was used and for some experiments contained 0.3mm-gibberellic acid sterilized by Seitz filtration. The seeds were allowed to germinate in the dark at $26\pm2°C$.

*Extraction and purification of lipid*

At selected time-intervals the endosperm tissue was carefully removed from the cotyledons and the embyro weighed and homogenized in 40ml of hot propan-2-ol by using a MSE homogenizer at top speed for 2min. This gave an initial extract and inhibited lipase activity. After standing for 30min, the suspension was filtered and the residue extracted with $3\times25$ml of chloroform–methanol (2:1, v/v). The first extract was carried out for 18h and the last two for 2h each. All the extracts were combined and dried down by rotary evaporation, the lipid residue was transferred to a graduated tube and made up to 10ml with chloroform–methanol (19:1, v/v, saturated with water). Samples (1ml) of this crude extract were purified by the method of Wells & Dittmer (1963) on a Sephadex G-25 column ($150mm\times10mm$). The purified lipid was dissolved in chloroform to a final volume of 2ml and the total lipid content was assayed gravimetrically. All extractions were carried out under $N_2$ and the lipid extracts were stored in this way at $-18°C$.

*Determination of fatty acid composition*

Samples of the purified lipid extract were evaporated to dryness and fatty acid methyl esters were prepared by the method of Nichols & Moorhouse (1969). Trimethylsilyl ethers of the hydroxy acid methyl esters were subsequently produced (Sweeley *et al.*, 1963).

The derivatives were characterized by g.l.c. with comparison of their retention times with derivatives prepared from standard fatty acids. A Pye series 104 gas chromatograph fitted with a Pye Unicam DP 80 digital integrator was used. Separation was performed on glass columns ($1.5m\times3.3mm$) containing 10% polyethylene glycol adipate adsorbed on acid-washed Diatomite C (100–120mesh) with an argon carrier gas-flow rate of 60ml/min and an oven temperature of 185°C.

*Analysis of triglycerides and measurements of phospholipid*

The phospholipid content of the purified lipid extracts was determined as $P_i$ by the procedure of Chen *et al.* (1956). Triglycerides were separated by t.l.c. into four groups depending on their hydroxylated fatty acid (ricinoleic acid, 12-D-hydroxyoctadec-9-*cis*-enoic acid) content. Samples of the purified extract were applied to prepared t.l.c. plates ($40mm\times120mm$) coated with a 0.25mm thickness of silica gel G (Macherey, Nagel and Co., Düren, Germany). The developing system was diethyl ether–light petroleum (b.p. 40–60°C)–acetic acid (25:25:1, by vol.) (Manjiro & Yamada, 1971) and the individual components were located with iodine vapour. After the yellow colour of the iodine had faded, the areas corresponding to the triglyceride were cut out and eluted 3 times (3ml) with diethyl ether–light petroleum (b.p. 40–60°C) (7:3, v/v). Quantitative determination of triglycerides was done by the method of Van Handel & Zilversmit (1957). A standard calibration curve was made with tripalmitate. This was linear over the range 0–100$\mu$g and the assays were always arranged so that values not exceeding 100$\mu$g were obtained.

Confirmation of the composition of the triglyceride species was carried out by preparative t.l.c., followed by g.l.c. of the fatty acid components.

## Results

*Germination of seeds*

Seeds incubated on agar containing gibberellic acid had a more swollen appearance than equivalent seeds grown on water–agar. The fresh weight of the endosperm tissue from these swollen seeds was higher at each time-point during germination.

*Action of gibberellic acid and the embyro on lipid mobilization*

Fig. 1 shows that at each time-point the endosperm of seeds incubated on water–agar had a higher lipid content than tissue from seeds incubated on agar containing gibberellic acid. This was found in both the presence and the absence of the embryo. Thus gibberellic acid had a stimulatory effect on lipid breakdown. Initially this mobilization in the endosperm was similar in both the presence and the absence of the embryo. However, after approx. 6 days of germination the rate of lipid breakdown was slower in the absence of the embryo.

*Fatty acid composition of the total lipid extract during seed germination*

The fatty acid composition of the endosperm tissue at selected time-points and under different

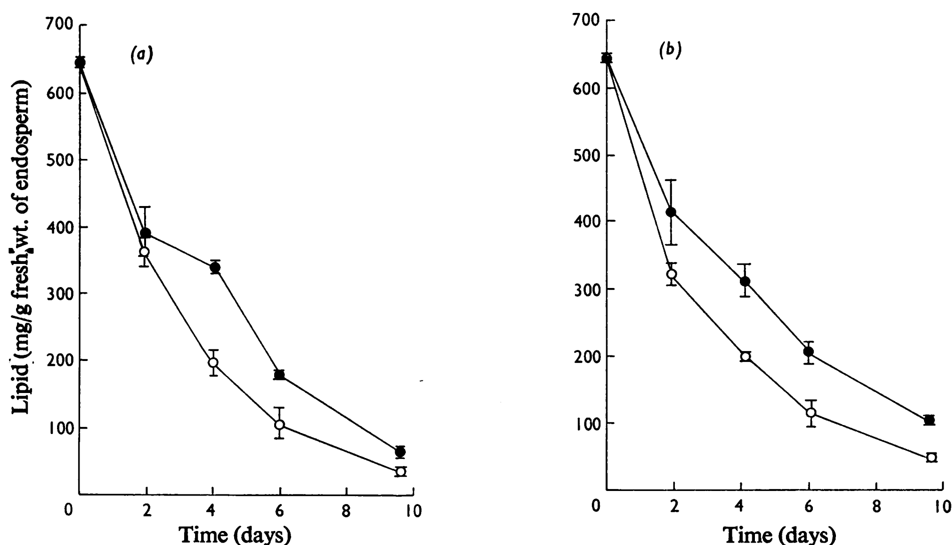


Fig. 1. *Changes in the lipid content of castor-bean endosperm during germination*

Seeds were germinated for different time-periods either intact (*a*) or with the embryo removed (*b*) on either water–agar (●) or on agar containing 0.3mM-gibberellic acid (○). Lipid was extracted from castor-bean endosperms by homogenizing the tissue in hot propan-2-ol and subsequently extracting a further three times with chloroform–methanol (2:1, v/v). The crude extract was purified and the total lipid content assayed gravimetrically. Each value is the mean of three experiments, and the standard deviation is indicated by bars.

Table 1. *Fatty acid composition of the purified lipid extract from castor-bean endosperm at different times during germination*

Fatty acid methyl esters were prepared from samples of the purified lipid extract and trimethylsilyl ethers of the hydroxy acid methyl esters were subsequently produced. The derivatives were characterized by g.l.c. Each value is the mean of triplicate experiments (S.D. ±0.5%).

| Conditions of germination and incubation | Time after germination (days) | Fatty acids (%, w/w) expressed to nearest 0.25% | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | $C_{16:0}$ | $C_{18:0}$ | $C_{18:1}$ | $C_{18:2}$ | $C_{18:3}$ | Ricinoleic acid | $C_{20:2}$ |
| Dry seeds | | 1.75 | 0.7 | 3.0 | 4.25 | 0.5 | 86.75 | 2.75 |
| Intact seeds on water–agar | 2 | 1.25 | 0.5 | 3.5 | 4.25 | 0.5 | 88.75 | 1.0 |
| | 4 | 1.0 | 1.0 | 3.0 | 5.0 | 0.5 | 88.0 | 1.5 |
| | 6 | 2.0 | 1.0 | 2.5 | 5.5 | 0.5 | 85.0 | 1.0 |
| | 10 | 3.0 | 1.75 | 3.75 | 7.75 | 1.25 | 80.75 | 0.75 |
| Intact seeds on water-agar containing 0.3mM-gibberellic acid | 2 | 1.25 | 0.5 | 4.0 | 5.0 | 0.5 | 87.25 | 1.5 |
| | 4 | 1.5 | 1.0 | 3.0 | 5.0 | 0.5 | 87.5 | 1.0 |
| | 6 | 1.0 | 1.0 | 3.5 | 8.0 | 1.0 | 82.0 | 0.5 |
| | 10 | 3.0 | 1.5 | 4.0 | 9.5 | 1.25 | 79.75 | 1.25 |
| Seeds with embryo removed on water–agar | 2 | 2.0 | 0.5 | 3.25 | 4.25 | 0.75 | 88.25 | 1.25 |
| | 4 | 1.5 | 0.75 | 3.0 | 4.75 | 0.5 | 88.0 | 1.75 |
| | 6 | 1.0 | 1.0 | 3.5 | 5.5 | 0.5 | 85.5 | 0.75 |
| | 10 | 2.25 | 0.75 | 3.0 | 7.0 | 0.75 | 84.5 | 1.0 |
| Seeds with embryo removed on agar containing 0.3mM-gibberellic acid | 2 | 1.5 | 0.5 | 4.5 | 5.5 | 0.5 | 86.0 | 1.5 |
| | 4 | 1.5 | 1.0 | 3.25 | 5.25 | 0.5 | 87.0 | 1.0 |
| | 6 | 1.5 | 1.0 | 4.0 | 6.0 | 0.75 | 84.0 | 0.75 |
| | 10 | 2.25 | 1.0 | 3.75 | 9.25 | 1.25 | 79.0 | 1.0 |

conditions of germination is shown in Table 1. The identity of the fatty acid for which no standard was available and which was present in very small amounts in the total lipid extract was determined by a James plot (James & Martin, 1952) and shown to be possibly $C_{20:2}$ (Table 1). Other workers (Achaya *et al.*, 1964; Sreenivasan, 1967) have reported the presence of approx. 1% (w/w) dihydroxystearic acid in castor oil. However, the fatty acid found in the lipid extract of the endosperm was not dihydroxystearic acid, since no change in the retention time of its methyl ester was obtained on trimethylsilylation.

The proportion of ricinoleic acid in the total lipid varied only slightly during the time-course but the amount of ricinoleic acid/g of endosperm fell dramatically in a manner identical with that of the total lipid (Fig. 1). Gibberellic acid produced a considerable decrease in the total amount of ricinoleic acid. This was expected from its effect on stimulating the depletion of total lipid. However, Table 1 shows that the proportion of ricinoleic acid present in the lipid varied only slightly between the incubations on water–agar and on agar containing gibberellic acid.

During the initial period of germination the proportion of ricinoleic acid was almost constant, but after 10 days of germination this value showed a more significant variation. The percentage (w/w) of ricinoleic acid in the total lipid extract was decreased and this resulted in a corresponding increase in the relative proportions of the other fatty acids, particularly for $C_{16:0}$ and $C_{18:2}$ (Table 1). Thus the incubation of seeds in the presence of gibberellic acid slightly decreased the proportion of ricinoleic acid and increased that of $C_{18:2}$ acid over a period of 10 days. Nevertheless, these variations were small and neither the presence nor the absence of the embryo or of gibberellic acid in the incubation medium had a marked effect on the fatty acid composition of the total lipid of castor-bean endosperm during the germination period.

*Phospholipid content of the seeds during germination*

The phospholipid content of the endosperm of the mature castor bean seeds was very low, less than 0.5% (w/w) of the total lipid extract. It fell slightly at the onset of germination but subsequently increased to a maximum after approx. 6 days and this higher value was maintained at least until 10 days after germination (Table 2). A similar pattern was observed in the absence of the embryo. The effect of gibberellic acid on the phospholipid content was not so clear as its effect on total lipid, probably because the phospholipid values were very low.

Table 2. *Phospholipid content of the purified lipid extract from castor-bean endosperm at different times during germination*

The phospholipid content of the purified lipid extracts was determined as inorganic phosphate. Each value is the mean of duplicate experiments.

| Conditions of germination and incubation | Time after germination (days) | Phospholipid (mg/g of endosperm) |
|---|---|---|
| Dry seeds | | 3.44 (±0.24) |
| Intact seeds on water–agar | 2 | 2.26 (±0.57) |
| | 4 | 3.67 (±0.65) |
| | 6 | 5.66 (±0.26) |
| | 10 | 5.6 (±0.15) |
| Intact seeds on agar containing 0.3mM-gibberellic acid | 2 | 2.75 (±0.60) |
| | 4 | 4.11 (±0.52) |
| | 6 | 7.73 (±0.23) |
| | 10 | 4.95 (±1.15) |
| Seeds with embryo removed on water–agar | 2 | 2.39 (±0.38) |
| | 4 | 2.63 (±0.50) |
| | 6 | 6.10 (±0.39) |
| | 10 | 4.73 (±1.11) |
| Seeds with embryo removed on agar containing 0.3mM-gibberellic acid | 2 | 2.51 (±0.21) |
| | 4 | 2.93 (±1.16) |
| | 6 | 5.07 (±0.30) |
| | 10 | 3.90 (±0.02) |

*Analysis of the triglyceride species during germination*

The t.l.c. system separated four distinct triglyceride species ($R_F$ values: 0.76, type 1; 0.50, type 2; 0.23, type 3; 0.12, type 4) and these were characterized by determination of their component fatty acids. Type 1 contained no ricinoleic acid, type 2 33% ricinoleic acid, type 3 66% ricinoleic acid and type 4 contained only ricinoleic acid.

Table 3 shows that the proportion of each of the four triglycerides did not vary significantly during germination and that neither the absence of the embryo nor the presence of gibberellic acid altered this.

**Discussion**

The results reported here show that the storage lipid in castor-bean endosperm was rapidly metabolized during germination and after 10 days less than 20% of the original weight of the total lipid remained. No initial lag period in breakdown was observed, in contrast with the results of Muto & Beevers (1974), who found that the total lipid content of the seed remained unchanged during the first 2 days of germination. However, part of the initial sharp decline is due to the rapid uptake of water and consequent increase in fresh weight. In our experiments the testa was removed to aid uptake of the hormone and to lessen any problems of sterility, whereas Muto & Beevers (1974) germinated intact seeds.

Table 3. *Triglyceride content of the purified lipid extract from castor-bean endosperm at different times during germination*

Four distinct triglyceride species were separated from the lipid extract by t.l.c. on silica gel G [diethyl ether–light petroleum (b.p. 40–60°C)–acetic acid (25:25:1, by vol.)]. These were type 1, $R_F$ 0.76; type 2, $R_F$ 0.50; type 3, $R_F$ 0.23; and type 4, $R_F$ 0.12. They were eluted and measured quantitatively. Each value is the mean of duplicate experiments.

| Conditions of germination and incubation | Time after germination (days) | Triglyceride (%) | | | |
|---|---|---|---|---|---|
| | | Type 1 | Type 2 | Type 3 | Type 4 |
| Dry seeds | | 11 | 12 | 21.5 | 55.5 |
| Intact seeds on water–agar | 2 | 11 | 12.5 | 24 | 53 |
| | 4 | 8.5 | 8.5 | 21 | 62 |
| | 6 | 10 | 12 | 23 | 55 |
| | 10 | 9.5 | 10.5 | 23.5 | 54 |
| Intact seeds on water–agar containing 0.3mM-gibberellic acid | 2 | 9 | 15 | 24.5 | 50.5 |
| | 4 | 9 | 8.5 | 22.5 | 60.5 |
| | 6 | 9 | 8.5 | 25.5 | 56 |
| | 10 | 10.5 | 13 | 23.5 | 51 |
| Seeds with embryo removed on water–agar | 2 | 9.5 | 12 | 23 | 55 |
| | 4 | 7.5 | 8.5 | 26 | 57.5 |
| | 6 | 5.5 | 9.5 | 25 | 63 |
| | 10 | 9 | 11.5 | 20.5 | 54 |
| Seeds with embryo removed on agar containing 0.3mM-gibberellic acid | 2 | 13 | 12 | 24 | 50 |
| | 4 | 8.5 | 7.5 | 22 | 62 |
| | 6 | 9 | 9.5 | 22.5 | 58.5 |
| | 10 | 10 | 12 | 25.5 | 51.5 |

The fatty acid content of castor oil has been widely studied (Binder *et al.*, 1962; Achaya *et al.*, 1964; Sreenivasan, 1967) and our analysis of the total lipid extract from mature seeds agrees well with the previous reports. Yamada (1955) suggested that ricinoleic acid was preferentially broken down during germination. The values he obtained for the ricinoleic acid content of the seeds were calculated from measurements of the iodine and acetyl values of the total fatty acids. However, we have shown by characterization and measurement of fatty acids by g.l.c. that the relative proportions of the fatty acids remain constant, and these results are confirmed by the work of Brown *et al.* (1970).

The lipid is stored in the endosperm as triglyceride and little attention has been accorded to the metabolism of this neutral lipid. The triglyceride content of castor oil had previously been analysed by a complex series of crystallizations (Gupta *et al.*, 1951) or by countercurrent distribution (Achaya *et al.*, 1964). Manjiro & Yamada (1971) have shown by t.l.c. followed by g.l.c. that four distinct triglyceride types are present in the endosperm and our work indicates that the proportions of each remains remarkably constant during germination. The triglyceride content of germinating hazel seeds (Shewry *et al.*, 1972) and *Andropogon gayanus* seeds (Williams & Bowden, 1973) have been investigated and in these also there is little change in the relative proportions of triglyceride groups.

Hydrolysis of triglycerides by lipase preparations from castor beans goes to completion, giving fatty acids and glycerol (Ory *et al.*, 1960). Although a positional specificity for the lipase action has been suggested this has recently been questioned (Noma & Borgström, 1971). Nizamudin & Kulkarni (1953) described a selective hydrolysis by the lipase on substrates such as groundnut, safflower and linseed oils, but Savary *et al.* (1958), using cocoa butter, suggested that the positional specificity of *Ricinus* lipase was poor. However, Ory *et al.* (1969), with butane-2,3-diol-dioleate or hexane-2-ol-oleate as substrates stated that the lipase could not bring about the hydrolysis of these secondary esters, so that hydrolysis of the triglycerides involved isomerization of the acyl group of the secondary ester to a primary alcoholic group. Such an isomerization can occur, but Noma & Borgström (1971) have shown that although the primary ester bonds of a triglyceride were preferentially hydrolysed the lipase could hydrolyse the resulting diglyceride equally at the primary or secondary ester groups. Our results on the triglyceride metabolism in the intact endosperm show that the overall action of the endogenous lipase is non-specific, so that the triglyceride components are utilized non-selectively.

In contrast with the total lipid, the phospholipid content of the endosperm increases during germination. A similar increase has been found in hazel seeds (Shewry *et al.*, 1973). In castor beans this may be

due to the proliferation of membranes which occurs on germination and in particular the number of glyoxysomes and mitochondria increases during the first 5 days of germination and declines when fat utilization is complete (Gerhardt & Beevers, 1970). Phospholipid content also reaches a maximum at around 5–6 days after germination.

Gibberellic acid stimulated the rate of breakdown of the lipid reserves in the endosperm of castor bean, but it did not alter the relative proportions of either triglyceride or fatty acid components. It is not known whether gibberellic acid has an intrinsic role in the germination of *Ricinus* seeds. This hormone has no effect on lipid mobilization in germinating wheat grains, in contrast with the breakdown of carbohydrates. However, the lipid metabolism in wheat seeds can be induced by the application of cytokinin and indol-3-yl acetic acid together with hydroxylamine (Taverner & Laidman, 1972).

Surprisingly the presence or absence of the embryo conferred little change in the lipid metabolism of castor-bean endosperm except perhaps during the later stages of germination. Clearly the mechanism whereby the cereal embryo controls the mobilization of storage reserves by production of phytohormones in the early stages of wheat germination is not operative in castor beans. If gibberellic acid or any other factor is necessary for the controlled breakdown of the endosperm tissue then in castor-bean these hormones would seem to be present in the endosperm of the resting seed.

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