Fat Utilization in Germinating Douglas Fir Seed [1, 2]

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In contrast to angiosperm seeds, little is known regarding the metabolic pattern of formation, quiescence, and germination in gymnosperm seed (9, 19). The major food reserve in Douglas fir seed was found to be fats (5, 7) and an increase of carbohydrates accompanying a rapid decrease of glycerides was observed during germination of the seed (6, 7). The quantitative changes of lipid fractions with germination, and various fatty acids in free form, in glycerides, and in acetone insoluble phospholipids will be reported in this paper.

Methods and Materials

Materials. A large quantity of mature seed of Douglas fir (*Pseudotsuga menziesii* Franco) was collected in September 1961 from a single tree located at an elevation of 200 ft near Corvallis. The average weight of the seed was 13 mg and the moisture content was 5 %. After 3 weeks' stratification at 4° the seed was germinated at alternating daily temperatures of 30° for 16 hours with 200 ft-c fluorescent light and 20° for 8 hours in the dark. Chemical analyses were conducted at 6 germination stages: A, nongerminated, mature seed; B, stratified seed; C, seed with emerging radicle (length, 1-10 mm); D, seed with emerging radicle (length, 11-24 mm); E, seed with completely emerged radicle and emerging cotyledons; F, completely emerged seedling with detached seed coat and exhausted endosperm.

The average number of days required to reach stages C, D, E, and F were 5, 7, 10, and 14 days after the commencement of germination.

The study was conducted twice, once in October 1961 (experiment 1) and the other in May 1962 (experiment 2) with duplicate samples for each stage. The fresh weight of samples varied between 4 g for stage A and 35 g for stage F, so that the total extracted lipids (1.3 g) from each sample was approximately constant in weight.

Two separate sets of material comparable to those for chemical analyses were used for determination of individual weight and moisture content at the various stages.

Chemicals. All the chemicals were reagent grade; diethyl ether was further distilled with metallic sodium chips to remove peroxides, and hexane with potassium permanganate.

The liquid phase for gas chromatography was diethylene glycol succinate purchased from 2 different sources, Applied Science Laboratories, Inc., State College, Penn. and Wilkens Instrument and Research, Inc., Walnut Creek, Calif. The inert support was chromosorb W, acid and alcoholic base washed, 90 to 100 mesh (Analytical Engineering Laboratories, Inc., Hamden, Conn.). The liquid phase and support were used without further processing. Column packing was made by stirring a weighed amount of support into a large quantity of acetone containing a known percent of the liquid phase. The coated support was filtered, dried in a vacuum oven at room temperature overnight, and finally weighed again to calculate the percentage of liquid phase (15).

Aluminum utility tubing of 6 mm OD was used for columns, and the length varied from 275 to 450 cm. Column packing was facilitated by vacuum and vibration, and 2 g of packing material per foot of column was generally used. Each column was conditioned for 38 to 48 hours at 220° and 30 psi of helium prior to use for analysis. These columns had a resolution factor for stearic and oleic acids always greater than 1. Figure 1 illustrates the resolution obtainable with these columns.

Pure fatty acids or their methyl esters were obtained from the Hormel Institute, University of Minnesota and the Applied Science Laboratories. Quantitative mixtures of fatty acids were kindly provided by the Metabolism Study Section, Division of Research Grants, National Institutes of Health.

Methods of Extraction and Fractionation. Weighed fresh material was homogenized in 100 ml of boiling isopropanol for 10 minutes at high speed in a Waring blendor to stop enzymatic activity (18), and break lipid-protein bonds (14). The homogenate was filtered with the aid of vacuum, and the blendor and residue were washed twice with boiling isopropanol. The residue was further extracted twice with 100 ml of ether-isopropanol (2:1 (v/v) at room temperature for 4 hours each with occasional stirring. The mixture was filtered and the combined filtrates were dried to a small volume in a rotary vacuum evaporator at 50°. The crude fat extract was dissolved in 50 ml of ether and washed dropwise

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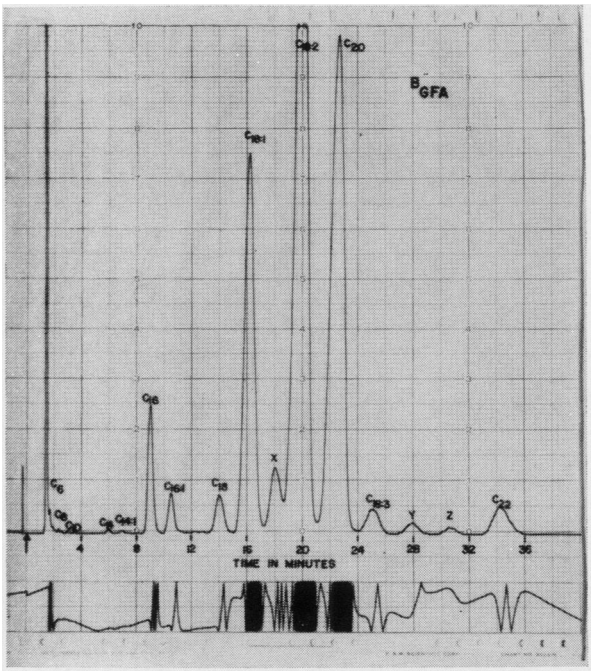

FIG. 1. Chromatogram of fatty acid mixture from glycerides fraction extracted from stratified seed with the addition of arachidic acid (C_{20}) showing analytic characteristics of the mixture and the result of co-chromatographic technique. The mixture was separated by a 360 cm, 6 mm OD column of 12 % diethyleneglycol succinate on chromosorb W/90/100 mesh, with a hydrogen flame ionization detector at column temperature 215°, injection port 195°, and detector 190°; flow rate of air 300 ml/minute, hydrogen 35 ml/minute, and helium 100 ml/minute, detector sensitivity 64 × 10^{-10} amps; sample size 0.6 μl.

with a large quantity of water in a separatory funnel until the washing was clear. The washings were discarded and the clear fat layer and emulsion were dried with vacuum at 50°. The weight of the washed fats was designated as total lipids (TL)[1].

The TL were dissolved in 20 ml of acetone, filtered through a tared filter paper, then washed twice with 50 ml of acetone. The filtrate and washing and the residue were dried in a vacuum at 50° and weighed as acetone soluble and acetone insoluble phospholipids (P).

The acetone soluble fraction was dissolved in 50 ml of ether and extracted 3 times with 1 % Na_2CO_3. The aqueous soap solutions were combined, then acidified and extracted 3 times with hexane. The hexane extract contained mostly free fatty acids; however, each sample was thoroughly washed to reduce contamination and to prevent the polymerization of diazomethane used for methylation of fatty acids for gas-liquid chromatography (GLC). The hexane extract was dried and weighed as free fatty acids (FFA). The ether layer consisting mostly of glycerides, some sterol esters and unsaponifiables were dried and weighed, then saponified with 10 % NaOH in 75 % ethanol for 6 hours. After removing the ethanol with a stream of nitrogen, the unsaponifiable materials (US) was partitioned into ether, and the fatty acids from glycerides (GFA) were recovered from the soap solution with the same procedure used for FFA.

The acetone insoluble phospholipid fraction was hydrolyzed with 4 % NaOH in 50 % ethanol for 16 hours. The unsaponifiables were removed and fatty acids from phospholipids (PFA) were recovered as described for FFA.

All the FA mixtures were methylated at room temperature in a screw-cap vial in the dark with an excess of an ether solution containing diazomethane generated from a precursor, EXR-101, (Provided by E. I. duPont deNemours and Company). The methylation was usually completed in 20 minutes. At this time nitrogen bubbles ceased to emerge upon releasing the screw-cap. The mixture was then concentrated to a 50 to 90 % solution with a stream of nitrogen, and analyzed by an F and M temperature programed gas chromatograph equipped with both thermal conductivity and hydrogen flame ionization detectors. At least 2 analyses were performed on each mixture immediately after methylation; a 10 μl sample was analyzed by a 275 to 450 cm column containing 16 to 20 % liquid phase with thermal conductivity detector and 1 μl or smaller sample was separated by a 275 to 360 cm column containing 6 to 12 % liquid phase with the ionization detector.

The FA mixtures obtained at later stages of germination were often contaminated with pigments and other foreign substances. The contaminants frequently damaged the column for separation and poisoned the catalyst for hydrogenation of the mixtures. It was found that activated alumina was very effective in removing these contaminants (22) and it was therefore used routinely for mixtures of fatty acids isolated at stages D, E, and F.

Analysis of Fatty Acids. Matching of relative retention time or volume of known fatty acids with unknowns, co-chromatographic technique of authentic fatty acids with the unknown mixtures, and comparing analytical data of the original mixtures with hydrogenated mixtures were the 3 general methods employed for identification (11, 17).

Two methods were used for quantitative analysis of the fatty acids; weighing the peaks cut out from a chromatogram (experiment 1), or counting the signals of a disc integrator (experiment 2) recorded on the chromatograms. The percentages were corrected after the column was calibrated against the known mixtures obtained from the National Institutes of Health.

[1] Abbreviations: TL, total lipids; P, phospholipids; FFA, free fatty acids; GFA, fatty acids from glycerides; PFA, fatty acids from phospholipids; GLC, gas-liquid chromatography; US, unsaponifiables; FA, fatty acids; G, glycerides.

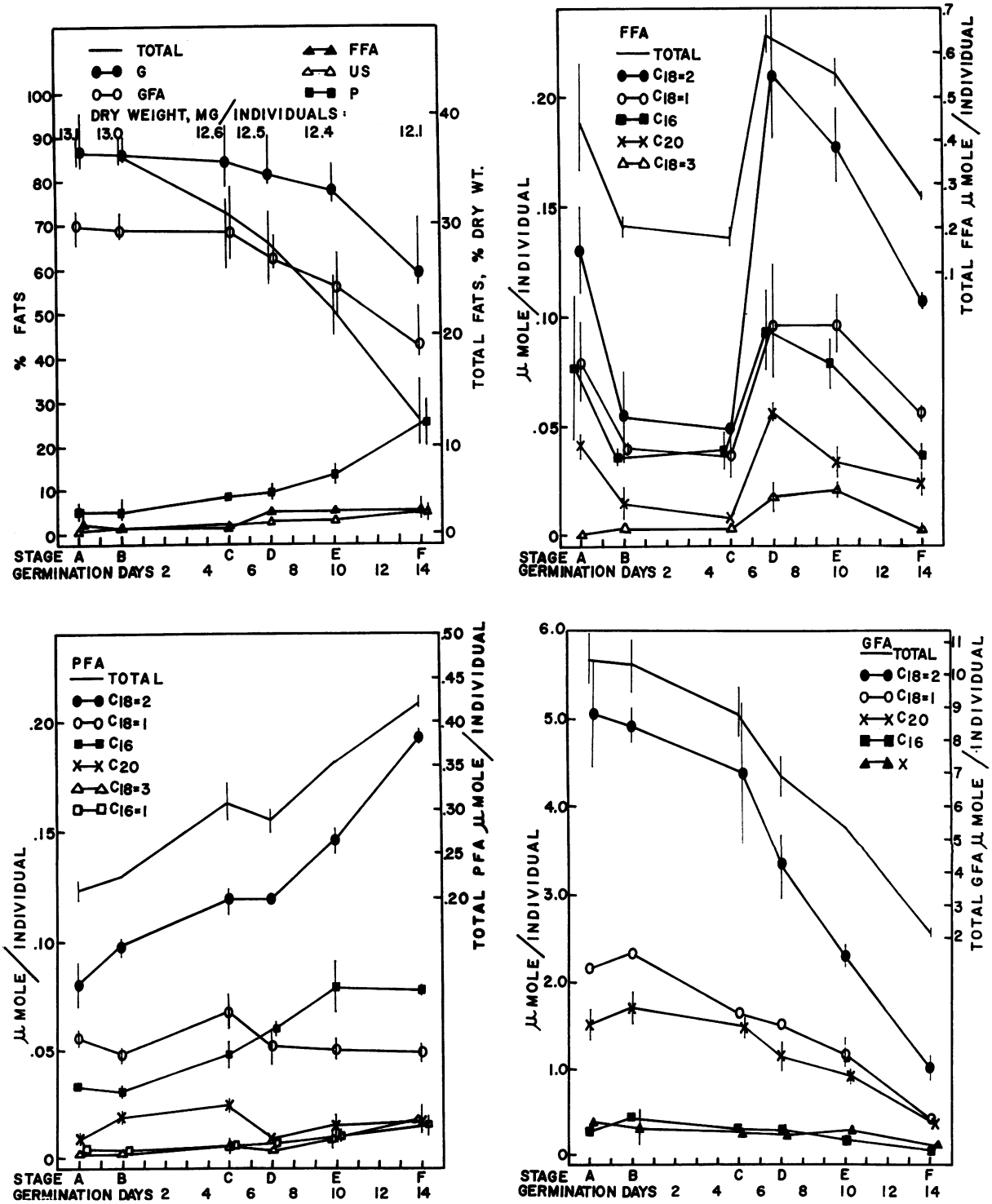

FIG. 2 (*upper left*). Changes of dry weight, total fats in percentage of dry weight, glycerides (G), fatty acids in glycerides (GFA), phospholipids (P), free fatty acids (FFA), and unsaponifiables (US), in percentage of fats at different stages of germination. Vertical lines show range of variation among duplicated samples of the 2 experiments.

FIG. 3 (*upper right*). Changes of total free fatty acids and specific fatty acids as μmole per individual at different stages of germination. Vertical lines show range of variation between 2 experiments.

Results and Discussion

Changes in Lipid Fractions During Germination. The TL as percent of dry weight in seeds at different stages of germination and the percentage distribution of "glycerides", GFA, P, FFA and US in total lipids are summarized in figure 2. The variation between the 2 experiments for each mean figure is shown by the respective vertical lines. The average dry weights of the materials are also presented here. Four general trends are evident: (1) a rapid reduction of TL from 36 % to 12 % of dry weight during germination; (2) a gradual diminution of "glycerides" from 86 % to 59 % of the total lipids; (3) a steady increase of P from 5 % to 25 % of total lipids with advancing germination, and (4) a small accumulation of US from 1 % to 5 % of the total lipids.

The rapid reduction of TL and "glycerides" with germination is a well established fact in fatty angiosperm seed (3, 9, 19, 26) as well as in other lots of Douglas fir seed (6, 7). Apparently it is a common pattern in the germination of seed with fats as the major reserve.

The gradual increase of P in the early stages of germination, a rapid gain at cotyledon emerging stage, and a high content in completely germinated seedling are of interest. The trends seem to follow the genesis of cellular organelles. Since the major components of membrane in cellular organelles are protein and phospholipids (8, 13, 21), the change of P observed here could be accounted for largely by their ontogenetic development. Similar increases were also indicated during germination of watermelon seed (10), rape seed (16, 20), and linseed (20).

Since the unsaponifiable fraction usually includes pigments, waxes, and sterols which are largely associated with chloroplasts, leaf surfaces, and membranes (8, 13, 14, 21), the small increase of US with germination was expected. A twofold increase of sterols in soybean during germination has also been reported (4).

The percentage of FA's in glycerides varied somewhat from stage to stage, 83 % in A, 80 % in B, 85 % in C, 78 % in D, 69 % in E, and 72 % in F. This indicates some mono, diglycerides, glycolipids and/or sterol esters are also present in the fraction. If this fraction consisted mainly of triglycerides, the fatty acids would be more than 90 %, since fatty acids with C_{16}, C_{18}, and C_{20} comprised more than 95 % of the GFA in any stage. The presence of glycolipids and others in seeds has been reported (2, 20, 27). Further study on the detailed identification of lipid fractions with column and thin layer chromatography would be desirable for clarification (14).

The PFA content in P fluctuated between 26 % and 31 % and was not shown in the figure, since the curve would be parallel with P. An investigation to characterize the P in these materials might elucidate the possible biological significance of P in development.

The content of FFA in nongerminated seed (A) was 2.4 % of the total lipids, decreased to 1.5 % of the total lipids in stratified seed, (B) and at early germination stage, (C) increased to 5.3 % then maintained in the later stages. The content of FFA at a certain stage of germination is largely dependent upon the difference between the rate of hydrolysis of glycerides and the rate of fatty acid oxidation and utilization for functional cellular structures and energy. Prior to radicle emergence (C), the rate of oxidation for energy and utilization was apparently higher than hydrolysis; thus accounting for the observed reduction. Just after emergence, hydrolysis of reserve glycerides was more rapid, so an increase of FFA was observed at stage D. From then on the depletion of FFA was proportional to the reduction of the TL and a balance was maintained to the completion of germination.

A constant small quantity of FFA has been reported in germinating seed of watermelon and cotton (10, 25) while a small accumulation of FFA was observed in tung, soybean, and other seeds (4, 9, 12). These variations may hinge upon genetic makeup and/or environmental influences, and any changes in FFA should perhaps be considered as individual cases rather than as a general rule.

Percentage Distribution of Fatty Acids in Various Fractions Extracted From Different Stages. The average percentage distributions of the fatty acids in FFA, PFA, and GFA analyzed by GLC are compiled in table I, II, and III respectively. A total of 22 fatty acids were separated by GLC in this seed lot, while 24 peaks were observed in others (5). There were 3 unknown peaks observed in the material: 0 peak apparently is an unsaturated C_{16} acid since it disappeared upon hydrogenation and a correspondent increase of C_{16} was obtained; x and y peaks had identical retention time or volume as C_{19} and C_{21} respectively, but both were found as unsaturated acid by hydrogenation. Their positive identification is in progress.

The qualitative distribution in GFA and PFA in all cases was very similar except quantitatively PFA had 10 or more percent palmitic than GFA, while

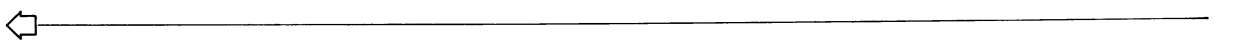

FIG. 4 (*lower left*). Changes of total and specific fatty acids in phospholipids as μmole per individual at different stages of germination. Vertical lines show range of variation between 2 experiments.

FIG. 5 (*lower right*). Changes of total and specific fatty acids in glycerides as μmole per individual at different stages of germination. Vertical lines show range of variation between 2 experiments.

Table I

Percentage Distribution of Fatty Acids in Free Form, at Various Stages of Germination of Douglas Fir Seed

Fatty acids	Relative retention	Germination stages A	B	C	D	E	F
Caproic, C6	0.05	1.6	0.5	0.3	2.1	3.4	1.1
Caprylic, C8	0.08	2.5	1.5	0.5	3.1	2.7	1.1
Capric, C10	0.13	1.8	0.5	0.8	1.2	1.1	0.7
Lauric, C12	0.23	3.5	1.4	3.4	1.6	1.5	1.1
Myristic, C14	0.37	3.1	0.8	1.5	1.5	1.1	0.8
Myristoleic, C14=1	0.44	...	...	...	...	...	...
Palmitic, C16	0.61	17.0	17.6	20.7	14.8	14.2	12.5
Palmitoleic, C16=1	0.72	3.3	5.9	3.8	2.4	3.8	2.1
Unknown 0	0.74	0.5	1.7	1.7	...	...	...
Stearic, C18	1.00	3.3	2.3	4.5	3.9	1.9	2.8
Oleic C18=1	1.16	19.0	22.9	20.8	16.3	19.4	21.6
Unknown X	1.31	2.3	4.6	4.4	3.3	4.1	3.9
Linoleic, C18=2	1.44	31.3	27.8	29.1	35.4	35.2	41.2
Arachidic, C20	1.64	10.6	7.7	5.6	9.2	6.4	9.1
Linolenic, C18=3	1.86	...	1.4	2.2	3.6	4.6	0.8
Unknown Y	2.11	...	...	...	...	...	...
Eicosadienoic, C20=2*	2.34	...	...	...	...	...	...
Behenic, C22	2.64	...	2.8	0.5	1.4	0.6	1.2
Erucic, C22=1	3.20	...	...	...	...	...	...
Docasadienoic, C22=2*	3.86	...	...	...	...	...	...
Lignoceric, C24	4.41	...	...	...	...	...	...
Tetracosenoic, C24=1*	5.36	...	...	...	...	...	...

* Tentatively identified by plotting relative retention time (1).

Table II

Percentage Distribution of Fatty Acids in Phospholipids at Various Stages of Germination of Douglas Fir Seed

Fatty acids*	Germination stages A	B	C	D	E	F
C6	T**	T	T	T	T	T
C8	T	T	T	T	T	T
C10	T	T	T	T	T	T
C12	T	0.2	0.1	0.5	0.1	0.1
C14	T	0.3	0.2	0.2	0.2	0.2
C14=1	...	...	0.2	0.2	0.2	0.2
C16	14.3	13.0	15.4	19.7	20.7	16.8
C16=1	2.9	2.4	3.3	3.9	3.3	4.5
O	1.0	0.9	1.0	0.9	1.3	0.5
C18	3.8	3.0	3.2	3.8	3.2	3.0
C18=1	26.3	21.7	22.4	18.9	14.9	13.4
X	2.5	2.3	2.5	1.9	1.9	1.5
C18=2	42.7	44.4	40.3	42.7	42.3	45.4
C20	4.1	8.9	8.4	3.6	4.6	4.4
C18=3	1.0	1.1	1.1	1.4	3.0	5.0
Y	0.3	...	0.7	0.5	1.0	0.1
C20=2	0.4	...	0.3	...	0.1	0.3
C22	0.5	0.3	0.8	0.4	...	2.0
C22=1	...	0.2	...	1.1	2.6	0.8
C22=2	...	0.3	...	...	0.4	1.0
C24	...	0.4	...	...	...	0.3
C24=1	...	...	...	...	...	0.3

* Name and retention data are in table I.
** Trace, less than 0.1 %.

Table III

Percentage Distribution of Fatty Acids in Glycerides at Various Stages of Germination of Douglas Fir Seed

Fatty acids*	Germination stages A	B	C	D	E	F
C6	T**	T	T	T	T	T
C8	T	T	T	T	T	T
C10	T	T	T	T	T	T
C12	T	T	T	T	T	T
C14	T	T	T	T	T	T
C14=1	...	T	...	T	...	...
C16	2.6	3.8	3.1	4.0	3.6	2.6
C16=1	1.0	0.7	1.1	0.9	0.9	0.9
O	...	T	0.1	0.2	T	0.1
C18	1.2	1.2	1.6	1.1	1.3	1.0
C18=1	20.6	21.3	19.2	21.9	22.8	20.1
X	4.0	3.3	3.3	3.5	3.9	4.1
C18=2	48.2	49.9	49.3	48.0	43.4	44.9
C20	14.4	17.0	17.2	16.1	17.7	19.0
C18=3	3.6	1.1	2.5	2.5	2.1	3.5
Y	1.5	0.3	0.9	0.8	0.2	...
C20=2	...	0.1	0.4	0.2	1.1	0.9
C22	2.0	1.2	0.1	...	1.1	1.7
C22=1	...	...	1.0	0.6	1.2	1.0
C22=2	...	...	...	...	...	...
C24	...	...	...	...	...	...
C24=1	...	...	...	...	...	...

* Name and retention data are in table I
** Trace, less than 0.1%.

GFA had 10 or more percent arachidic acid. Such similarity has already been generalized by Lovern (20) and Shorland (23) in rapeseed, peanut, cottonseed, sunflower seed, soybean, and linseed. FFA had more short chain acids than PFA or GFA at any stage; a decrease of short chain acids and saturated acids was also shown with the advancement of germination. The analytic characteristics of these materials by GLC are also illustrated in figure 1. In the chromatogram peak Z was tentatively identified by plotting relative data as eicosadienoic acid ($C_{20:2}$) (1).

Quantitative Change of Major Fatty Acids in Various Fractions of Different Stages. It is more meaningful to compare the changes of fatty acids in terms of moles per individual seed or seedling than by percentages of weight; thus the weights were converted and the results are summarized in table IV as mole percentage distribution in different fractions of the 6 germination stages. A parallel pattern was clearly shown as in the change of weight percentage (fig 2), except that a faster rate of increase in advanced germination was indicated in FFA and PFA and correspondingly a more rapid reduction of GFA.

Table IV

Change of Fatty Acids Content in Various Fractions during Germination of Douglas Fir Seed

Germination stage	Experiment	Mole percentage of total FA's			Total FA's
		FFA	PFA	GFA	μmole/seed
A	1	3.4	1.9	94.7	10.31
	2	4.9	1.8	93.3	12.08
B	1	2.3	2.2	95.5	9.97
	2	1.5	2.0	96.5	11.37
C	1	1.7	3.8	94.5	8.60
	2	1.6	2.9	95.5	10.12
D	1	8.4	4.0	88.6	7.24
	2	8.0	3.3	88.7	8.55
E	1	8.4	5.6	86.0	6.26
	2	9.1	5.5	85.4	6.34
F	1	9.2	13.8	77.0	3.02
	2	9.6	15.5	74.6	2.76

In order to detect quantitative changes of a particular FA in the 3 fractions, the compositions of 5 major FA's in each fraction were plotted (fig 3, 4, and 5). In figure 3, all acids observed showed a similar pattern with the changes in linoleic ($C_{18=2}$) being somewhat accentuated.

A preferential accumulation of linoleic acid and a steady slow increase of palmitic and palmitoleic acids with germination in PFA were indicated in figure 4. Oleic and arachidic acids fluctuated somewhat with germination stages.

A preferential utilization of linoleic acid was shown in GFA with germination (fig 5). The small increase of oleic, arachidic, and palmitic acids in B might indicate some in situ saturation process or shifting of FA's from other fractions during rapid turn over. All the FA's decreased with germination. This diminution is probably common to all tissues with fat reserves.

Studies of this nature reveal quantitative changes during seed germination. The complexity of lipid metabolism and the need of information in plant material have been pointed out by Zill and Cheniae (27) and Stumpf (24), and many of the speculation and unsolved problems mentioned in this paper await further research.

Summary

Douglas fir seeds were collected from one tree located near Corvallis, Oregon and germinated under controlled environment. Lipids from nongerminated and stratified seeds, and from germinated seedlings of 4 size groups were separated into fractions of free fatty acids, acetone-insoluble phospholipids, glycerides, and unsaponifiables. The fatty acids in phospholipids and glycerides were isolated, and the fatty acid mixtures were analyzed by gas-liquid chromatography using diethylene glycol succinate as liquid phase.

Total fats decreased rapidly with germination from 36 % to 12 % of the dry weight, which also decreased from 13.1 mg to 12.1 mg per individual seed. Glycerides were utilized during germination and a diminution of 86 % to 59 % of the total fats was found. Acetone-insoluble phospholipids increased gradually in the early stages, then rapidly at later stages of germination. They increased from 5 % to 25 % of total fats, of which fatty acids comprised approximately 30 % at any stage of germination. A small reduction from 2.5 % to 1.5 % of total fats, then an increase of 5 % was observed in the fraction of free fatty acids during germination. A small but consistent gain from 1 % to 5 % of unsaponifiable material was related to germination. A total of 22 fatty acids was found in these materials. In free fatty acid fraction, 30 % of the acids had carbon chain length less than 17 in nongerminated seed. The percentage of these short chain acids decreased to 19 % in completely germinated material. A decrease of saturated fatty acids was also shown during germination. The major components of free fatty acids were linoleic (27–41 %), oleic (16–23 %), palmitic (14–21 %) and arachidic (6–11 %) acids. The major components in phospholipids were linoleic (40–45 %), oleic (13–26 %), and palmitic (13–21%) acids. A slight increase of shorter chain acids and more saturated acids was indicated with germination. More long chain fatty acids were found in this fraction than in free form and in glycerides. Little change of percentage distribution of various fatty acids in glycerides was observed with germination. The major components in glycerides were linoleic (43–50 %), oleic (19–23 %), and arachidic (14–19 %) acids.

A preferential utilization of linoleic acid in glycerides and a preferential increase of linoleic and palmitic acids in phospholipids were clearly demonstrated.

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