Plant Physiol. (1972) 50, 69–72

Sterol Changes during Germination of *Nicotiana tabacum* Seeds[1]

Received for publication November 16, 1971

PARSHALL B. BUSH[2] AND C. GRUNWALD
Department of Agronomy, University of Kentucky, Lexington, Kentucky 40506

ABSTRACT

The identity, composition, and concentration of the total, free, esterified, and glycosidic sterol fractions were determined during germination of tobacco seeds. The total, free, and esterified sterols increased, with stigmasterol and campesterol accounting for most of the increase. Steryl glycosides decreased during germination, and stigmasteryl and sitosteryl glycosides showed the largest decrease. During germination, sitosterol was the major sterol in all fractions but stigmasterol and campesterol showed the greatest changes. The fatty acid composition of the steryl esters and acylated steryl glycosides most closely resembled the di- and triglycerides.

Four sterol forms have been isolated from higher plants; namely free sterol, steryl ester, steryl glycoside, and acylated steryl glycoside (2, 5, 11). The physiological function of these various sterol forms have never been established. It has been postulated that the free sterols are an integral part of plant cell membranes (8, 15), in a manner similar to cholesterol in mammalian membranes; and that these sterols may influence cell permeability (10, 11). From *in vitro* enzyme studies with soybean seeds Hou *et al.* (12) have suggested that the steryl glycosides may serve in the storage of sterols. Least defined is the role of the steryl esters; however, analogous with the cholesteryl ester in animals, it might be possible that the steryl esters are involved in the transport of sterols.

A number of higher plants at various physiological stages have been analyzed for sterols in the hope of explaining the physiological functions of the different sterol forms (2, 5, 12, 13, 15, 16); however, these experiments have brought little success. If free sterols are an integral part of plant membranes and steryl glycosides are involved in the storage of sterols, then germinating seeds should increase in free sterols as a consequence of increased membrane and organelle production, and decrease in steryl glycosides with the utilization of the stored sterols. Only very limited information is available concerning sterol changes during germination. Ingram *et al.* (13) studied the relative changes of total sterol during germination of Cruciferae seeds, however, they did not report the absolute sterol values. The only other report related to this question is a study by Kemp *et al.* (16). They examined the free and esterified sterol changes in 4- to 14-day-old corn seedlings. On a per seedling basis they found an increase in the free sterol content with seedling development; however, they did not isolate the steryl glycosides. Our study was undertaken to characterize the free, esterified, and glycosidic sterols during seed germination.

MATERIALS AND METHODS

Germination of Seeds. Tobacco seeds (*Nicotiana tabacum* L. var. Burley 21) were germinated on Whatman No. 3 filter paper in covered transparent plastic trays. Both ends of the filter paper were submerged in distilled water to insure constant moisture. Seeds were germinated at 27 C, relative humidity of 90 to 100% and a 14-hr day produced with cool-white fluorescent lamps. By visual inspection seeds germinated uniformly, with radicle protrusion occurring between 2 and 2.5 days.

Chlorophyll Determination. Tobacco seedlings were homogenized with an Omni-Mixer in 80% (v/v) acetone and approximately 4 g of fine glass beads (0.05–0.45 mm). The acetone extract was centrifuged at 20,000*g* for 10 min. The supernatant was made to 100 ml with 80% acetone and the absorbancies were measured at 663, 645, and 480 nm with the Hitachi Perkin-Elmer 139 spectrophotometer. The chlorophyll concentration was determined from the nomogram of Kirk (17). Changes in carotenoid content were estimated by correcting the absorbancy at 480 nm for contributions of chlorophyll using the equation derived by Kirk and Allen (18).

Extraction and Fractionation of Sterols. Tobacco seedlings were homogenized in acetone with an Omni-Mixer for 5 min at full speed. Fine glass beads were added to facilitate homogenization. The resulting homogenate was extracted in a Soxhlet apparatus with acetone for 24 hr. The acetone extract was taken to dryness under reduced pressure, taken up in 50 ml of 95% ethanol and partitioned against an equal volume of *n*-hexane. The partitioning was repeated three times and the *n*-hexane fractions were pooled. The *n*-hexane fraction contained the free sterols and steryl esters. The alcohol fraction contained the steryl glycosides. The free sterols and steryl esters were separated according to the method of Goodman (7). To correct for any losses standard cholesterol-7α-^{3}H (2 μc) and cholesteryl-4-^{14}C palmitate (0.01 μc) were added. The column (1.8 × 7 cm) was packed with 5 g of silica gel (70–325 mesh) as a slurry in *n*-hexane and the sterols were applied to the column as a solution in *n*-hexane. Serial elution was carried out as follows: 50 ml of *n*-hexane followed by 50 ml of 10% benzene in *n*-hexane (discarded), 100 ml of 40%

[1] This work was part of Ph.D thesis of Parshall B. Bush and was supported in part by Crops Research Division, Agricultural Research Service, United States Department of Agriculture Contract 12-14-100-9559 (34). This paper (No. 71-3-30) is part of a project of the Kentucky Agricultural Experiment Station and is published with approval of the Director.

[2] Present address: Department of Horticulture, Michigan State University, East Lansing, Mich. 48823.

benzene in *n*-hexane (steryl esters), 50 ml of benzene (discarded) and 100 ml of chloroform (free sterols). The steryl esters and steryl glycosides were hydrolyzed, extracted as described previously (2), and precipitated with digitonin (14).

Analysis for total sterols was carried out by a modified method of Stedman and Rusaniwskyj (24). A measured aliquot of acetone extract was taken to dryness and refluxed for 12 hr in 25 ml of 95% ethanol containing 0.13 ml H_2SO_4. The mixture was cooled, 15 ml of 10% KOH in 95% ethanol were added and refluxed for 30 min. The hydrolysate was neutralized with dilute HCl, extracted three times with *n*-hexane, and back washed twice with methanol.

The digitonide precipitates were dispersed with pyridine containing cholestane as the internal standard and quantitatively analyzed by gas chromatography (9). The GLC[3] system consisted of an F & M model 402 gas chromatograph equipped with a flame ionization detector, effluent sample splitter and a Hewlett Packard integrator model 3370 A. The column was a 1.80 m U-shaped glass column packed with Anakrom ABS 80/90 mesh coated with 5% OV-101. Corrections were made for differences in detector response.

Extraction, Fractionation, and Analyses of Fatty Acids. Tobacco seedlings were homogenized and extracted with acetone as described above. Silica gel (2 g) was added and the extract was taken to dryness under reduced pressure, and transferred to a 5-g silica gel (70–325 mesh) column as outlined previously (2). The serial elution was as follows (7): 50 ml of *n*-hexane and 50 ml of 10% benzene in *n*-hexane (discarded), 100 ml of 21% benzene in *n*-hexane (steryl esters), 50 ml of benzene followed by 100 ml of chloroform (triglycerides, partial glycerides, free sterols, free fatty acids), 100 ml of 2% methanol in chloroform (acylated steryl glycosides), and 100 ml of methanol (polar lipids). The benzene-chloroform fraction was taken to dryness under reduced pressure and the resultant oil was made up in a small volume of *n*-hexane and transferred to a 12-g Florisil column for further fractionation (3). The lipids were eluted as follows: 20 ml of *n*-hexane followed by 50 ml of 5% ether in *n*-hexane (discarded), 75 ml of 15% ether in *n*-hexane (triglyceride), 60 ml of 25% ether in *n*-hexane (free sterol), 60 ml of 50% ether in *n*-hexane (diglycerides), 75 ml of 2% methanol in ether (monoglycerides), and 75 ml of 4% acetic acid in ether (free fatty acids).

The methyl esters of the fatty acids were prepared by the boron trifloride-methanol method of Morrison and Smith (21). The fatty acid methyl esters were analyzed by GLC in a Barber Coleman gas chromatograph equipped with a flame ionization detector and a Varian electronic integrator Model 480. The GLC column consisted of a 3 m × 0.6 cm stainless steel column packed with 12% diethylene glycol succinate on 80/90 mesh ABS. The fatty acids were identified by relative retention.

RESULTS AND DISCUSSION

Tobacco seeds germinated uniformly, radicle protrusion occurring after 2.0 to 2.5 days. Chlorophyll and carotenoid synthesis began after approximately 2 to 3 days of germination and increased rapidly through the 6th day at which time there was a decline (Fig. 1).

Germinating tobacco seeds were analyzed by GLC and four major peaks were found which corresponded to authentic stigmasterol, sitosterol, campesterol, and cholesterol. The above four GLC peaks were collected and analyzed by mass spectrometry. The mass-to-charge or m/e values corresponded to those obtained with the corresponding authentic sterol and the published data by Knight (20). Therefore, germinating tobacco seeds have the same 3-β-sterols as reported for mature tobacco leaves (8, 14); however, the quantitative composition was quite different.

The total sterol content, as determined by the method of Stedman and Rusaniwskyj (24), was always slightly higher than the summation of free, esterified and glycosidic sterols (Figs. 2 and 3). A similar observation has been reported for green and etiolated barley shoots (2). During germination, tobacco seeds increased in the total sterols and this increase paralleled the increase in free sterols. The steryl esters also increased during this period but to a lesser extent. The steryl glycoside content was relatively low throughout the experimental period, and decreased during germination. The levels of free sterols reported here are much higher than those reported by Grunwald (8) in mature tobacco leaf tissue; however, the steryl esters and glycosides are of the same order of magnitude.

On a dry weight basis, the total stigmasterol and campesterol content increased with germination, while the sitosterol and cholesterol content remained fairly constant (Fig. 2). During the early stages of germination stigmasterol accounted for 10 to 15% of the total sterol content, while after 6 days stigmasterol accounted for 30 to 35%. These changes in the total sterol composition were paralleled by similar changes in the free sterols (Fig. 3). A number of physiological functions have been postulated for the free sterols (10). Ingram *et al.* (13) have found that stigmasterol was evident in *Brassica napus* only when flowering. Furthermore, it has been reported that floral induction in *Xanthium pennsylvanicum* (6), *Pharbitus nil* (6, 22), and *Lolium temulentum* (4) was suppressed with inhibitors of steroid biosynthesis. Bae and Mercer (1) reported that in *Solanum andigena* sterol changes occurred at the time of tuber formation. These observations have been interpreted to mean that steroids may serve as flower inducing or tuber forming plant hormones; however, this cannot be the case in the present experiments. Secondly, it has been suggested that free sterols stabilize the cellular membranes by interacting with the phospholipid (8, 10, 11, 15, 16). If the latter hypothesis is correct then actively growing tissues, such as germinating seeds and developing seedlings, should accumulate free sterols as a result of increased membrane synthesis. The large increase in free sterol content during germination supports this hypothesis (Fig. 3A).

Comparing the composition of the free, esterified and glycosidic sterol fraction shows quantitative differences, thus indicating that the various sterol forms are not in a simple equilibrium. The major free sterol was sitosterol. Stigmasterol, which was low initially, started to increase after 3 to 4 days of germination and after 7 days had increased to the point where it was approximately equal to the sitosterol content. The free campesterol and cholesterol content also doubled during this period. It is suggested that the large increase in free stigmasterol is a light stimulated process occurring in conjunction with the development of shoots, because the increase was concurrent with initiation of chlorophyll and carotenoid synthesis (Fig. 1). Further evidence in support of this suggestion is found in the reports that the free stigmasterol content increased with greening of etiolated barley shoots (2), and was absent in dark grown barley roots (11) but is synthesized on exposure to light (Bush, unpublished data). These data suggest that the biosynthesis of free stigmasterol can be stimulated by light, while the biosynthesis of stigmasteryl esters is light insensitive.

The major steryl ester was sitosterol and, with the exception of cholesterol, all steryl esters increased during germination (Fig. 3B). The stigmasteryl ester content was very low prior to radicle protrusion, but increased thereafter. This pattern

[3] Abbreviation. GLC: gas-liquid chromatography.

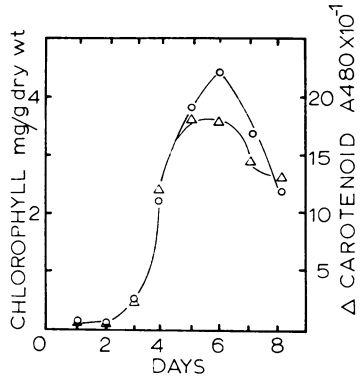

FIG. 1. Changes in chlorophyll and carotenoid content during germination of tobacco seeds. Chlorophyll (○) and carotenoid (△).

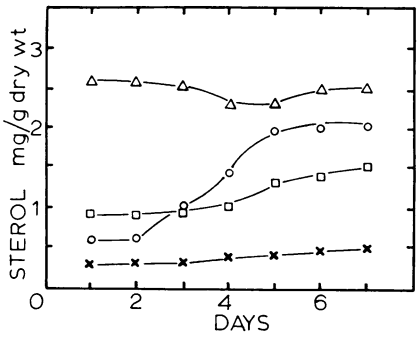

FIG. 2. Changes in the composition of total sterols during germination of tobacco seeds. Total sterols were isolated as described by Stedman and Rusaniwskyj (26). Sitosterol (△), stigmasterol (○), campesterol (□), and cholesterol (×).

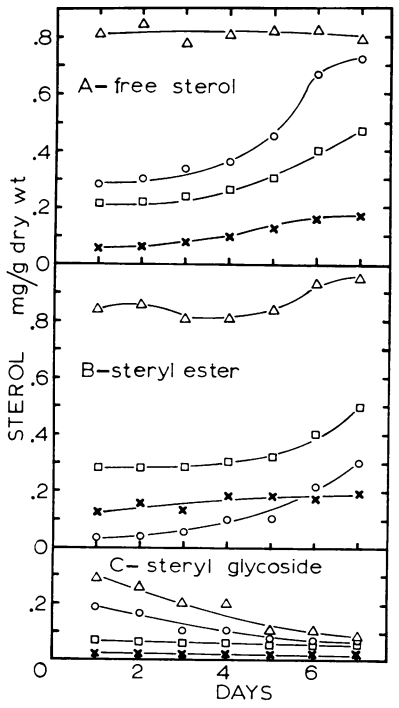

FIG. 3. Changes in the composition of free (**A**), esterified (**B**), and glycosidic (**C**) sterols during germination of tobacco seeds. Sterol isolation was by partition and column chromatography. Sitosterol (△), stigmasterol (○), campesterol (□), and cholesterol (×).

Table I. *Long Chain Fatty Acids of Various Lipid Fractions Isolated from 6-Day-Old Tobacco Seedlings*

Amounts are expressed as a percentage of C-16 and longer fatty acids normalized to 100.

Lipid Fraction	Fatty Acid						
	16:0	18:0	18:1	18:2	18:3	20:0	>20
	%						
Steryl esters	11.6	1.8	7.4	79.2	trace		
Triglycerides	15.3	4.8	18.9	54.1	trace	5.1	1.8
Diglycerides	12.0	5.0	12.2	70.8	trace	trace	
Monoglycerides	36.5	16.6	12.4	29.4	5.1	trace	
Free fatty acid	33.4	30.0	9.8	13.4	13.4	trace	
Acylated steryl glycosides	27.9	11.3	16.0	32.2	2.5	3.8	6.3
Polar lipids	31.4	6.4	3.1	39.9	19.2	trace	trace

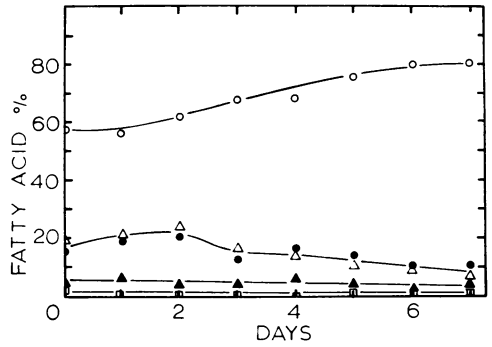

FIG. 4. Changes in fatty acid composition of the steryl esters during germination of tobacco seeds. Linoleic acid (○), palmitic acid (●), oleic acid (△), stearic acid, (▲), and linoleic acid. (□)

is unlike the free (Fig. 3A) and glycosidic sterols (Fig. 3C), where stigmasterol was the second most important sterol. During the early stages of germination the stigmasteryl ester content was below that of the cholesteryl ester content. Steryl esters have been isolated from several higher plant tissues (2, 8, 15, 16). It has been postulated that steryl esters might serve in sterol transport (16). Goad (6) has alternatively suggested that the sterol esters might in some way be associated with the biosynthesis of phytosterols.

Steryl glycosides occur in acylated and nonacylated form. The steryl glycosides of germinating tobacco seeds constituted only a small fraction of the total sterol content and from preliminary experiments it was found that only about 20% of the steryl glycoside was in the acylated form. It was, therefore, not readily feasible to separate the acylated and nonacylated steryl glycosides. During germination sitosteryl and stigmasteryl glycosides decreased, while the glycosides of cholesterol and campesterol remained constant (Fig. 4C). Information on the physiology of steryl glycosides is limited. Smith (23) has proposed that in *Mycoplasma* carotenoids or sterols may serve as carriers in the transport of glucose. Alternatively, Hou *et al.* (12) have observed that the steryl glycosides accumulate during seed maturation and they proposed that in seeds the glycosides are a storage form for sterols. Present data (Fig. 3C) support the latter hypothesis, because during germination the total steryl glycoside content decreased markedly, and sitosteryl and stigmasteryl glycosides accounted for most of the decrease.

The fatty acid composition of various lipid fractions isolated

from 6-day-old tobacco seedlings is given in Table I. The major fatty acid in the steryl ester fraction was linoleic acid at 79.2%. Other fatty acids, in order of decreasing content, in this fraction were: palmitic acid (11.6%), oleic acid (7.4%) stearic acid (1.8%), and a trace of linolenic acid. Linoleic acid (32.2%) was also the major fatty acid in the acylated steryl glycoside fraction. Other fatty acids in this fraction were: palmitic acid (27.9%), oleic acid (16.0%), stearic acid (11.3%), arachidic acid (3.8%), linolenic acid (2.5%), and some higher fatty acids. The major free fatty acid, however, was palmitic acid (33.4%). Linoleic acid was the major fatty acid in the diglycerides (70.8%) and triglycerides (54.1%). Changes in the fatty acid composition of the steryl ester fraction during germination of tobacco seeds are presented in Figure 4. Steryl linoleate was the major steryl ester and this ester increased slightly with germination. Change in the other steryl esters was only slight. Kemp and Mercer (15) have also reported that linoleic acid was the major fatty acid of the steryl ester fraction isolated from endosperm, scutellum, and root of corn. There is evidence that in rat liver the cholesteryl esters are constantly turning over and that the turnover rate is influenced by the fatty acid esters (19). In tobacco seedlings the fatty acid composition of the steryl esters most closely approximated the fatty acid composition of the di- and triglycerides (Table I). By analogy with the animal system, therefore, the phytosterol esters may be in a continuous state of change and their turnover may be influenced by the fatty acid esters.

LITERATURE CITED

1. Bae, M. and E. I. Mercer. 1970. The effect of long- and short-day photoperiods on the sterol levels in the leaves of *Solanum andigena*. Phytochemistry 9: 63-68.
2. Bush, P. B., C. Grunwald, and D. L. Davis. 1971. Changes in sterol composition during greening of etiolated barley shoots. Plant Physiol. 47: 745-749.
3. Carroll, K. K. 1961. Separation of lipid classes by chromatography on florisil. J. Lipid Res. 2: 135-141.
4. Evans, L. T. 1964. Inflorescence initiation in *Lolium temulentum*. Aust. J. Biol. Sci. 17: 24-35.
5. Galliard, T. 1968. Aspects of lipid metabolism in higher plants. I. Identification and quantitative determination of lipids in potato tuber. Phytochemistry 7: 1907-1914.
6. Goad, L. J. 1967. Aspects of phytosterol biosynthesis. *In:* J. B. Pridham, ed., Terpenoids in Plants. Academic Press, Inc., London. pp. 159-190.
7. Goodman, D. S. 1969. Hydrolysis and formation of cholesterol esters in rat liver. *In:* R. B. Clayton, ed., Methods in Enzymology. Vol. XV, Steroids and Terpenoids. Academic Press, Inc., New York. pp. 522-537.
8. Grunwald, C. 1970. Sterol distribution in intercellular organelles isolated from tobacco leaves. Plant Physiol. 45: 663-666.
9. Grunwald, C. 1970. Quantitative analysis of free phytosterols by gas chromatography using stationary phase OV-101. Anal. Biochem. 34: 16-23.
10. Grunwald, C. 1968. Effect of sterols on the permeability of alcohol-treated red beet tissue. Plant Physiol. 43: 484-488.
11. Grunwald, C. 1971. Effect of free sterols, steryl ester and steryl glycoside on membrane permeability. Plant Physiol. 48: 653-655.
12. Hou, C. T., Y. Umemura, M. Nakamura, and S. Funahashi. 1968. Enzymatic synthesis of steryl glycoside by a particulate preparation from immature soybean seeds. J. Biochem. 63: 351-360.
13. Ingram, D. S., B. A. Knights, I. J. McEvoy, and P. McKay. 1968. Studies in the Cruciferae: changes in the composition of the sterol fraction following germination. Phytochemistry 7: 1241-1245.
14. Keller, C. J., L. P. Bush, and C. Grunwald. 1969. Changes in content of sterols, alkaloids, and phenols in flue-cured tobacco during conditions favoring infestation by molds. J. Agr. Food Chem. 17: 331-334.
15. Kemp, R. J. and E. I. Mercer. 1968. Studies on the sterols and sterol esters of intracellular organelles of maize shoots. Biochem. J. 110: 119-125.
16. Kemp, R. J., L. J. Goad, and E. I. Mercer. 1967. Changes in the levels and composition of the esterified and unesterified sterols of maize seedlings during germination. Phytochemistry 6: 1609-1615.
17. Kirk, J. T. O. 1968. Dependence of chlorophyll synthesis on protein synthesis. Planta 78: 200-207.
18. Kirk, J. T. O. and R. L. Allen. 1965. Dependence of chloroplast pigment synthesis on protein synthesis: effect of actidione. Biochem. Biophys. Res. Comm. 21: 523-530.
19. Klein, P. D. and R. A. Martin. 1959. Heterogeneity in the turnover rates of liver cholesterol ester. J. Biol. Chem. 34: 1685-1687.
20. Knight, B. A. 1967. Identification of plant sterols using combined GLC/mass spectrometry. J. Gas Chromatog. 5: 273-282.
21. Morrison, W. R. and L. M. Smith. 1964. Preparation of fatty acid and methyl esters and dimethylacetate from lipids with boron fluoride methanol. J. Lipid Res. 5: 600-608.
22. Sachs, R. M. 1966. Inhibition of flower initiation in *Pharbitis nil* by an inhibitor of steroid biosynthesis is dependent on seedling age. Plant Physiol. 41: 1392-1394.
23. Smith, P. F. 1969. The role of lipids in membrane transport in *Mycoplasma laidlawii*. Lipids 4: 331-336.
24. Stedman, R. L. and W. Rusaniwskyj. 1959. Composition studies on tobacco. V. Free and combined 3-β-sterol of freshly harvested, aged or fermented tobaccos. Tob. Sci. 3: 44-47.