Hexose Transfer from UDP-hexose in the Formation of Steryl Glycoside and Esterified Steryl Glycoside in Leaves[1]

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Several types of glycosylations using UDP-hexose as hexose donor are known to be catalyzed by enzymes from plants. In these reactions hexose acceptors were found to be fructose (Leloir and Cardini, 1953), anthranilic acid (Jacobelli et al., 1958), phenols (Yamaka and Cardini, 1960), starch (Leloir et al., 1961) and quercetin (Barber, 1962). In 1963 Benson suggested UDP-gal could be the hexose donor also in the biosynthesis of leaf galactolipids (Butt and Beevers, 1966).

The incorporation of hexose from UDP-hexose into lipids of leaves and chloroplasts has been studied in different laboratories. Neufeld (1963) and Neufeld and Hall (1964) found that isolated chloroplasts from spinach could catalyze the transfer of hexose from UDP-glucose-C^{14} or UDP-galactose-C^{14} to an "endogenous acceptor". The products were similar to, but not chromatographically identical with, the natural

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galactolipids. It is significant to note that the labeled products found by Neufeld and Hall were alkaline-labile.

Newman (1966) made the interesting observation that UDP-glucose apparently enhances the incorporation of acetate-2-C^{14} into linolenate of lettuce leaf tissue and plastids. To determine the nature of the enhancement due to UDP-glucose we incubated lettuce leaf discs with UDP-glucose-C^{14} and found that mono- and digalactolipids were labeled very slowly, in contrast to what was expected, and that two different substances having different polarities than those of mono- and digalactolipids were very actively labeled (Newman, 1967). The two labeled are identified as steryl glycoside (SG) and esterified steryl glycoside (ESG).

Steryl glycosides were detected in plants very early (Euler and Nordenson, 1908; Power and Salway, 1913). Others have found them in many species and their different organs (Karrer, 1958; Carter et al., 1961; Nichols, 1963; Thirkell and Tristrom, 1963). Esterified steryl glycoside has been found by Lepage (1964a). Both, SG and ESG, seem to be widely distributed among plants and within plant cells. However, chloroplasts seem to contain only a minor amount of the steryl glycosides present in the cell (Eichenberger and Menke, 1966).

Methods

Plant Material and Lipid Extraction

Leaf discs of lettuce plants (Lactuca sativa L., Burpee's Ruby Red) grown in a plant-growth chamber were used. One hundred discs (7 mm in diameter) were incubated for 24 hours, under illumination and in a petri dish which was sterilized, with 5 ml of a buffer solution (0.2 M phosphate, pH 7.4; 0.03 M $NaHCO_3$; 0.03 M Na-acetate). The medium also contained 2.5 µc of uridine diphosphate glucose-C^{14} (glucose U.L.; specific activity, 100 mc per mmole) which was obtained from New England

Nuclear. The lipids were extracted by boiling the tissues in a mixture of chloroform-methanol (2:1, v/v); they then were transferred to diethyl ether. The ether-soluble lipids were chromatographed on TLC plates (Silicagel G "Merck") in two dimensions (chloroform-methanol-water, 65:25:4, v/v; diisobutylketone-acetic acid-water, 80:50:10, v/v; Lepage, 1964b). Radioautograms from these plates exhibited several spots, but most of the radioactivity was found to be localized in four spots (A to D, Fig. 1).

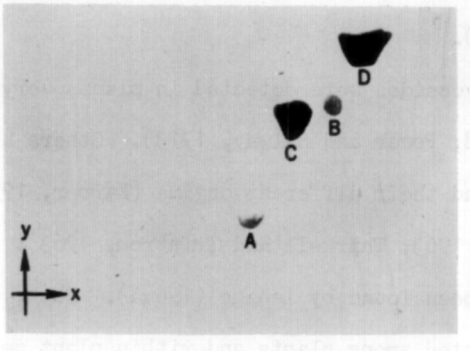

Fig. 1. Radioautograms of chromatographed lipids from lettuce leaf discs incubated with UDP-galactose-C^{14} -- thin layer of Silicagel G "Merck". The chromatogram was developed with chloroform-methanol-water 65:25:4, v/v (x-direction) and with diisobutylketone-acetic acid-water 80:50:10, v/v (y-direction).

Identification of Labeled Compounds

Lipids A and B were identified as digalactosyl diglyceride (A) and monogalactosyl diglyceride (B) by their R_f-values, by their colors, and by their relative intensities on plates sprayed with 20% $HClO_4$ at 100° C. Lipids C and D showed a behavior different from that of lipids A and B.

After spraying with 20% $HClO_4$ or with acetic anhydride-concentrated H_2SO_4- absolute ethanol (1:1:10, v/v) -- a modified Liebermann-Burchardt reagent prepared by cooling in ice water -- reddish spots appeared. Alkaline hydrolyzation by refluxing 1 hour with 5% (w/v) KOH in 90% (v/v) methanol did not alter lipid C, but converted lipid D to lipid C (Fig. 2).

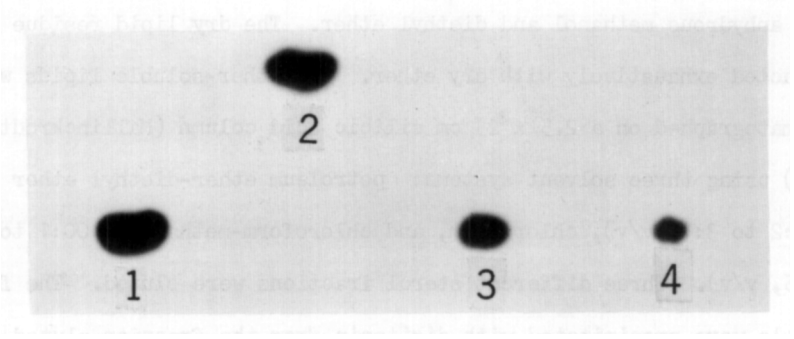

Fig. 2. Radioautograms of unchanged and alkaline hydrolyzed lipids C and D which were chromatographed on Silicagel G "Merck". The thin layer chromatogram was developed with chloroform-methanol-water 65:25:4, v/v. 1-lipid C; 2-lipid D; 3-lipid C hydrolyzed; 4-lipid D hydrolyzed.

Acid hydrolysis by refluxing 16 hours with 1 N H_2SO_4 yielded an ether-soluble, non-radioactive aglycone which was chromatographically identical to free sterols from lettuce leaves. These samples were chromatographed on Silicagel G and developed with benzene-ethyl acetate (4:1, v/v). The aqueous phase after acid hydrolysis, which was found to be radioactive, was chromatographed on Whatman no. 1 paper in a descending direction. The spots were detected by spraying with a

mixture of 1% $KMnO_4$- 2% Na-periodate (1:2, v/v; Sastry and Kates, 1964). The radioactive spots detected by radioautography were found to co-chromatograph exactly with inactive glucose.

To confirm the suggestion that lipids C and D were identical with SG and ESG, these lipids were isolated from lettuce leaves. The isolation has been carried out predominantly by methods described earlier (Lepage, 1964a; Eichenberger and Menke, 1966). Sixty grams of freeze-dried and finely powdered lettuce leaf material was extracted with anhydrous methanol and diethyl ether. The dry lipid residue was extracted exhaustively with dry ether. The ether-soluble lipids were chromatographed on a 2.5 x 25 cm silicic acid column (Mallinckrodt 100 mesh) using three solvent systems: petroleum ether-diethyl ether (100:2 to 3:7, v/v), chloroform, and chloroform-methanol (100:1 to 100:5, v/v). Three different sterol fractions were eluted. The free sterols were precipitated with digitonin from the fraction eluted with petroleum ether-diethyl ether (1:1, v/v). ESG was isolated after further purification of the petroleum ether-diethyl ether (3:7, v/v) fraction on TLC plates -- Silicagel G developed with chloroform-methanol-water (65:25:4, v/v). SG was crystallized directly from the fractions eluted with chloroform-methanol (100:2 to 100:5, v/v) and was further purified by recrystallizations in pyridine-ether.

Radioactive compounds C and D were co-chromatographed in two dimensions on a TLC plate and the spots detected subsequently by radio-autography and spray reagent. An exact coincidence of spots was found on both, plate and X-ray film.

Ten milligrams of inactive SG were added to lipid C and crystallized 5 times in pyridine-methanol. The specific radioactivity of fractions 2-5, determined by a D 47 Gas Flow Detector (Nuclear Chicago), was found to be constant (Table 1).

Table 1. Specific radioactivity of fractions of steryl glycoside from lettuce leaves, recrystallized in pyridine-methanol.

Fraction	Specific radioactivity (cpm per mg)
2	332 ± 8
3	358 ± 11
4	339 ± 8
5	341 ± 4

The same lipid components were found to be labeled when leaf discs were incubated with UDP-galactose-C^{14} (1 µc per experiment and incubated with the label for 24 hours) as a precursor in place of UDP-glucose. For these experiments we also used leaves of spinach (Spinacia oleracea L., Burpee Nobel), bean (Phaseolus vulgaris L., Burpee Stringless Greenpod), yellowwood (Cladrastis lutea Koch) and Norway Maple (Acer platanoides L.). Spinach and beans were grown in a plant-growth chamber, while the tree leaves came from park trees.

In order to locate the spots, the lipids were made visible with iodine vapors. The silicic acid was scraped off and finely suspended with Cab-O-Sil in a toluene-BBOT (0.4%, w/v) scintillator. The counting efficiency was about 43%.

Special attention was given to the question, whether leaves provided with UDP-galactose would incorporate galactose into steryl glycosides. SG from spinach leaf discs, incubated with UDP-galactose-C^{14} was hydrolyzed with 1 N H_2SO_4 and neutralized with $Ba(OH)_2$. The aqueous phase was chromatographed with inactive glucose and galactose on TLC plates (Cellulose MN 300; n-butanol-pyridine-water 6:4:3, v/v, in two dimensions; van Wyk, 1966). The spots were detected subsequently with X-ray film and aniline phthalate spray reagent. Radioactivity was detected exclusively in the glucose spot, but not in that of galactose.

Results and Discussion

These data indicate that green plant tissue is able to incorporate hexose from UDP-hexose into the carbohydrate moiety of galactolipids. However, a great part of the provided hexose is combined with sterols in formation of sterol glycosides (SG and ESG). The ratio of radioactivity incorporated into galactolipids to that into steryl glycosides is different in different species. Yellowwood leaves label galactolipids most abundantly, while lettuce leaves incorporate radioactivity mostly into steryl glycosides (Table 2).

Table 2. Ratio of C^{14} incorporated into steryl glycosides to that incorporated into galactolipids. UDP-galactose-C^{14} was used as the precursor and the tissue was incubated for 24 hours.

Plant	Ratio C^{14} $\frac{\text{steryl glycosides}}{\text{galactolipids}}$
Yellowwood	0.23
Bean	1.46
Maple	1.44
Spinach	2.27
Lettuce	56.1*

*Incubated 8 hours with UDP-glucose. Comparison of radioautograms indicates that this value is approximately equal to that obtained for 24 hours incubation with UDP-galactose.

The fact that UDP-hexose is an effective hexose donor also in the biosynthesis of steryl glycosides is interesting because still very little is known about the metabolism and biological function of plant sterols. Whether sterols act as specific acceptors in this reaction, is still under investigation.

Since the radioactive part of steryl glycosides from spinach leaves provided with UDP-galactose-C^{14} was found to be glucose, it is suggested that incorporation of hexose into steryl glycosides may involve a conversion of hexose to glucose. This corresponds with an earlier observation that steryl glycosides contain predominantly glucose but no galactose (Eichenberger and Menke, 1966).

References

Barber, G. A., Biochemistry, 1, 463 (1962).

Benson, A. A., in Proc. 5th Internatl. Cong. Biochem., Pergamon Press, Oxford, Vol. VI, pp. 340-351 (1963).

Butt, V. S. and Beevers, H., in Plant Physiology (F. C. Steward, ed.), Academic Press, New York, pp. 265-414 (1966).

Carter, H. E., Ohno, K., Nojima, S., Tipton, C. L., and Stanacev, N. Z., J. Lipid Research, 2, 215 (1961).

Eichenberger, W. and Menke, W., Z. Naturforschg., 21b, 859 (1966).

Euler, H. and Nordenson, E., Hoppe Seiler's Z. physiol. Chem., 56, 223 (1908).

Jacobelli, G., Tabone, M. J., and Tabone, D., Bull. Soc. Chim. Biol., 40, 955 (1958).

Karrer, W., Konstitution und Vorkommen der organischen Pflanzenstoffe, Birkhäuser, Basel (1958).

Leloir, L. F. and Cardini, C. E., J. Am. Chem. Soc., 75, 6084 (1953).

Leloir, L. F., Fekete, M. A. R. de, and Cardini, C. E., J. Biol. Chem., 236, 636 (1961).

Lepage, M., J. Lipid Research, 5, 587 (1964a).

Lepage, M., J. Chromatog., 13, 99 (1964b).

Neufeld, E., Federation Proc., 22, 464 (1963).

Neufeld, E. F. and Hall, C. W., Biochem. Biophys. Research Communs., 14, 503 (1964).

Newman, D. W., Plant Physiol., 41, 328 (1966).

Newman, D. W., Phytochemistry, 6, 187 (1967).

Nichols, B. W., Biochim. Biophys. Acta, 70, 417 (1963).

Power, F. B. and Salway, A. H., J. Chem. Soc., 103, 399 (1913).

Sastry, P. S. and Kates, M., Biochemistry, 3, 1271 (1964).

Thirkell, D. and Tristrom, G. R., J. Sci. Food Agri., 14, 488 (1963).

van Wyk, D., Z. Naturforschg., 22b, 700 (1966).

Yamaka, T. and Cardini, C., Arch. Biochem. Biophys., 86, 127, 133 (1960).