FORMATION OF GALACTOLIPIDS BY CHLOROPLASTS

Elizabeth F. Neufeld and Clara W. Hall

National Institute of Arthritis and Metabolic Diseases
National Institutes of Health, Bethesda, Maryland
and
Department of Biochemistry, University of California
Berkeley, California

Received December 16, 1963

Chloroplasts of higher plants contain relatively large amounts of galactolipids (Wintermans, 1960). These have been shown to be α, β diglycerides (predominantly dilinolenin), with either one or two D-galactosyl residues attached to the third hydroxyl group of glycerol (Benson et al., 1958; Carter et al., 1961; Sastry and Kates, 1963a). In the present investigation it was found that isolated spinach chloroplasts catalyze the transfer of galactose from UDP-D-galactose-C^{14} to an endogenous acceptor, yielding alkali-labile products which are similar, though not identical, to the galactolipids isolated from plant material.

Preparation of Chloroplasts. Spinach leaf chloroplasts were prepared in 0.5 M sucrose - 0.01 M phosphate buffer, pH 7.4. The chloroplasts obtained from 50 g of leaves were suspended in 2.5 ml of 0.1 M Tris buffer, pH 7.4, and this stock solution (chlorophyll content, 3.8 - 4.5 mg/ml) was either used immediately or after storage at -10° for several weeks.

Measurement of Galactosyl Transfer. A routine assay was based on the formation of radioactive products extractable into chloroform. A suspension of chloroplasts (usually diluted five-fold from the stock in 0.1 M Tris buffer, pH 7.4) was incubated with 25 µl of UDP-D-galactose-C^{14} (0.02 µc, of specific activity 6 µc/µmole) in a total volume of 100 µl. After 45 min at 37°, the mixture was boiled for 1 min, diluted with 1 ml of water and vigorously shaken with 1 ml of $CHCl_3$. The emulsion was broken by centrifugation at low speed and 0.5 ml of the dark green chloroform phase was

pipetted into a plastic planchet, dried and counted with a Geiger-Müller tube. (The extraction with chloroform is incomplete; higher values may be obtained by extracting with 2:1 chloroform-methanol.)

As shown in Fig. 1, the transfer of C^{14} to chloroform-soluble material is a function of time and of chloroplast concentration.

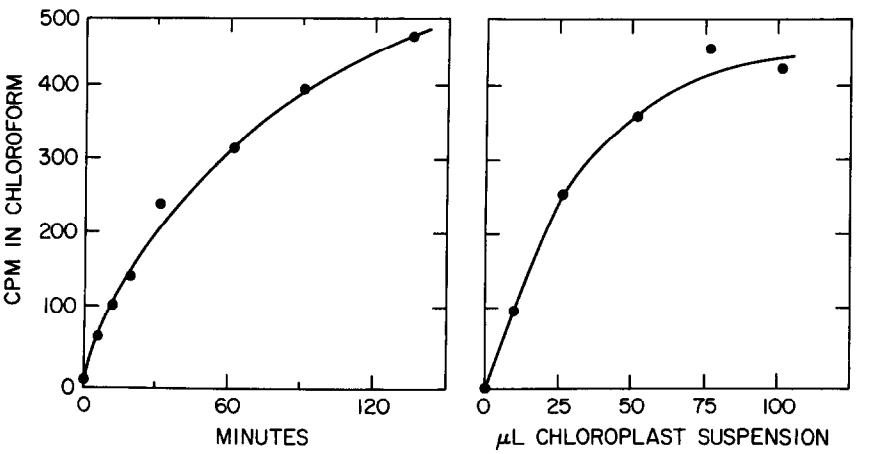

Fig. 1. (a) Rate of galactosyl-C^{14} transfer into lipid products; 2560 cpm of UDP-D-galactose used as substrate. (b) Effect of increasing chloroplast concentration; 1100 cpm of UDP-D-galactose-C^{14} used.

The optimal pH was found to be about 7, in 0.1 M Tris or 0.1 M phosphate buffers. The reaction proceeded equally well in light and in darkness. 6×10^{-3} M EDTA and cysteine had no effect; nor did 3.8×10^{-3} M $MgCl_2$. At that concentration, Mn^{++}, Co^{++}, Fe^{++}, and Ni^{++} were markedly inhibitory, while Zn^{++}, Fe^{+++}, Ce^{++++}, and Cu^{++} were completely so. UTP at a concentration of 5×10^{-3} M almost completely abolished the reaction, whereas 5×10^{-3} M ATP, CTP, ITP, GTP, dTTP, or dUTP had little effect.

Specificity of Glycosyl Donors. Of a number of sugar nucleotides tested, UDP-D-galactose and UDP-D-glucose were the only glycosyl donors in the system described (Table I). The transfer from UDP-D-glucose may probably be attributed to UDP-D-galactose epimerase activity in the chloroplasts.

Table I

Specificity of Glycosyl Donor

Substrate	% Incorporation*	Substrate	% Incorporation*
UDP-D-galactose	30	UDP-D-glucose	15
dTDP-D-galactose	0.5	UDP-D-glucuronic acid	<0.5
dUDP-D-galactose	0.5	UDP-D-galacturonic acid	<0.5
ADP-D-galactose	<0.5	UDP-pentose (mixture of D-xylose and L-arabinose)	<0.5
α-D-galactosyl phosphate	<0.5		

*Percentage of radioactivity transferred from the substrate to chloroform-soluble products.

Dissociation of Enzyme and Acceptor. An acetone powder of chloroplasts was prepared by squirting 5 ml of stock chloroplast suspension into 30 ml of acetone at -10°. The mixture was stirred with a glass rod and filtered with suction. Suction was continued for about an hour at -10° to dry the powder. The filtrate was freed of acetone at room temperature, under a stream of N_2, and frozen. The coarse green precipitate which appeared upon thawing was collected by low-speed centrifugation, suspended in 1.5 ml of H_2O, and dispersed before use by sonication for 3 min. As can be seen from Table II, neither this preparation (designated "filtrate" for convenience) nor the acetone powder had transferring activity when incubated separately but were active in combination. The nature of the galactosyl acceptor is now being investigated with the aid of the acetone-extracted enzyme.

In the dissociated system, a slight stimulation by Mg^{++} (up to two-fold) became apparent. Neither 0.1 M glycerol, nor 0.01 M α-L-glycerophosphate or β-glycerophosphate interfered with the reaction.

Identification of Products. Naturally occurring galactosyl diglycerides may be deacylated by alkaline transesterification (Benson and Maruo, 1958). The water-soluble products resulting from this treatment are 1-O-β-D-galactosyl-D-glycerol and O-α-D-galactosyl-(1→6)-O-β-D-galactosyl-(1→1)-D-glycerol.

Table II

Dissociation of Enzyme and Acceptor

Conditions	Cpm in Organic Phase
Complete	915
Filtrate omitted	67
Acetone powder omitted	0
Mg^{++} omitted	634

The complete system included 5800 cpm of UDP-D-galactose, 50 µl acetone filtrate, 1.6 mg acetone powder, 0.5 µmole $MgCl_2$, 5 µmole Tris buffer, pH 7.4, in 130 µl. After 45 min at 37°, the missing component was added, the mixture boiled, diluted with 0.1 ml H_2O, and the lipids extracted with 1.0 ml of 2:1 chloroform-methanol.

A quantity of radioactive galactolipid sufficiently large for deacylation and identification of the carbohydrate moiety was prepared by incubating 200 µl of chloroplasts with 2 µc of UDP-D-galactose. After 3 hr at 37°, the product formed was subjected to paper electrophoresis in 0.2 M ammonium formate at pH 3.7. The neutral area was eluted with water and saved for further examination. Radioactive material which had not been removed from the paper by water was exhaustively eluted with methanol.

The methanol eluate, which contained 68% of the total radioactivity, was treated with 0.4 ml of 0.1 N methanolic KOH for 30 min at 37°. The mixture was diluted with 0.5 ml H_2O, passed through a small column of Dowex-50 H^+, dried, and subjected to circular paper chromatography in n-propanol-ethyl acetate-water, 7:1:2. Four bands appeared on a radioautogram, containing radioactivity in the ratio of 100:34:14:4, in order of diminishing R_f. When subjected to descending paper chromatography in 80:20 phenol-water (in which these compounds moved very rapidly) or in 40:11:19 butanol-ethanol-water (in which they moved very slowly) the compounds arranged themselves in a fashion typical of members of a homologous series (French and Wild, 1953).

The fastest moving compound behaved as β-D-galactosyl glycerol in the following systems: (a) chromatography in 80:20 phenol-water, 7:1:2 n-propanol-ethyl acetate-water, and 40:11:19 butanol-ethanol-water; (b) electrophoresis in 0.05 M $Na_2B_4O_7$; (c) liberation of galactose (identified chromatographically) on treatment with β-galactosidase of E. coli, but not with yeast α-galactosidase.

The compound with the next highest R_f had mobilities similar to those of O-α-D-galactosyl-(1→6)-O-β-D-galactosyl-(1→1)-D-glycerol in the systems mentioned. However, only 17% of the terminal galactose was hydrolyzed with α-galactosidase, the remainder being susceptible to β-galactosidase. The inner linkage appears to be entirely β. The linkage configuration of the compounds in the third and fourth bands (presumably tri- and tetra-galactosyl glycerol) has not been determined.

Examination of the intact radioactive galactolipid products (prepared using either whole chloroplasts or acetone powder plus filtrate) by thin layer chromatography on silicic acid, using 40:25:5 diisobutyl ketone-acetic acid-water as solvent, revealed at least 8 radioactive components. Three of these were located in the approximate area of monogalactosyl distearin, and two in the area of digalactosyl distearin. The mixture of lipid products is more complex than had been anticipated, and its composition is being investigated.

Water Soluble Galactosides. The water eluate, which accounted for 7% of the total radioactivity, contained a number of radioactive compounds which, by a combination of the chromatographic, electrophoretic and enzymatic techniques described above, were identified as hexose (24%), β-galactosyl glycerol (16%), digalactosyl glycerol (57%, approximately half of this having a terminal β- linkage), and trigalactosyl glycerol (4%). The soluble galactosides were postulated to be hydrolytic breakdown products of the lipids - a hypothesis which has since been strengthened by the report of the existence of enzymes catalyzing the deacylation of dilinolenin (Sastry and Kates, 1963b).

Acknowledgments

The work done at the Department of Biochemistry of the University of California was supported in part by a research grant (A-1418) from the National Institutes of Health. The support and encouragement of Dr. W. Z. Hassid is gratefully acknowledged. The authors wish to thank Dr. A. A. Benson and Dr. M. Kates for their generous gifts of mono- and di-galactosyl glycerol, and mono- and di-galactosyl distearin, respectively.

References

Benson, A. A., and Maruo, B., Biochim. Biophys. Acta 27, 189 (1958).
Benson, A. A., Wiser, R., Ferrari, R. A., and Miller, J. A., J. Am. Chem. Soc. 80, 4740 (1958).
Carter, H. E., Ohno, K., Nojima, S., Tipton, C. L., and Stanacev, N. Z., J. Lipid Research 2, 215 (1961a).
Carter, H. E., Hendry, R. A., and Stanacev, N. Z., J. Lipid Research 2, 223 (1961b).
French, D., and Wild, G. M., J. Am. Chem. Soc. 75, 2612 (1953).
Sastry, P. S., and Kates, M., Biochim. Biophys. Acta 70, 214 (1963a).
Sastry, P. S., and Kates, M., Abstr. 145th Meeting Am. Chem. Soc. (1963b) p. 2C.
Wintermans, J. F. G. M., Biochim. Biophys. Acta 44, 49 (1960).