RHAMNOSYL TRANSFER FROM TDP-L-RHAMNOSE CATALYZED BY A PLANT ENZYME*

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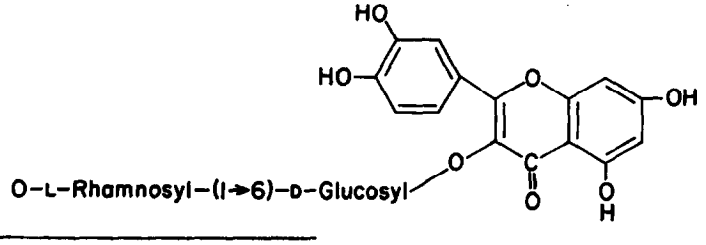
In the last two years several sugar nucleotides containing thymidine diphosphate (TDP) have been identified in microorganisms (Baddiley and Blumson, 1960; Baddiley et al., 1961; Okazaki, 1960; Strominger and Scott, 1959). Recently the enzymes responsible for the synthesis of TDP-L-rhamnose and its precursor, TDP-D-glucose, have been isolated from bacteria (Kornfeld and Glaser, 1961; Glaser and Kornfeld, 1961; Pazur and Shuey, 1961). The formation of TDP-D-glucose has also been reported to take place in extracts from seedlings of alfalfa and soya bean (Pazur and Shuey, 1961).

By analogy to the role of other sugar nucleotides, TDP-L-rhamnose would be expected to be a donor of L-rhamnose to polysaccharides or other glycosides of which rhamnose is a constituent. An instance of such a reaction is reported in this communication. We have been able to show that the L-rhamnosyl moiety of TDP-L-rhamnose is transferred to 3-quercetin-D-glucoside by a soluble enzyme preparation from mung bean leaves to form rutin (3-quercetin-O-L-rhamnosyl-(1→6)-D-glucoside), a ubiquitous plant glycoside the structure of which is shown below.



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Preparation of substrates. C^{14}-labeled α-D-glucose 1-phosphate (37 μc/μmole) was prepared by the action of sucrose phosphorylase (Wolochow et al., 1949) on C^{14}-labeled sucrose. C^{14}-labeled TDP-D-glucose of the same specific activity was synthesized from thymidine triphosphate (Sigma) and radioactive α-D-glucose 1-phosphate with a TDP-D-glucose pyrophosphorylase from Pseudomonas aeruginosa ATCC 7700 (Kornfeld and Glaser, 1961). It was purified by paper electrophoresis at pH 3.6 and subsequently converted to C^{14}-labeled TDP-L-rhamnose in the presence of TPNH, DPN, and an enzyme preparation from the same microorganism (Glaser and Kornfeld, 1961). The sugar nucleotide mixture finally isolated contained 60% TDP-L-rhamnose and 40% TDP-D-glucose and was contaminated by unreacted TPNH.

A mixture of L-rhamnose 1-phosphate and D-glucose 1-phosphate (60/40) was prepared by hydrolysis of the C^{14}-labeled TDP sugars with the venom of Crotalus adamanteus, which contains a nucleotide pyrophosphatase. The sugar phosphates were purified by paper electrophoresis at pH 3.6. It is presumed that the configuration of the glycosidic linkage in these sugar 1-phosphates is the same as that in the sugar nucleotides (Glaser and Kornfeld, 1961)--i.e., β-L-rhamnose 1-phosphate and α-D-glucose 1-phosphate.

Samples of 3-quercetin-D-glucoside were kindly provided by Prof. T. A. Geissman, University of California, Los Angeles, Dr. R. M. Horowitz, U. S. Department of Agriculture, Pasadena, California, and Prof. R. F. Dawson, Columbia University, New York City. Since this compound is only slightly soluble in aqueous solvents, it was used as a 1% suspension in 0.5 M Tris buffer, pH 7.5.

Preparation of the L-rhamnose transferring enzyme. Mung beans (Phaseolus aureus) were germinated and grown in soil in a greenhouse for three weeks. The green leaves (160 g) were removed from the young plants, washed, and ground in a chilled mortar with 35 g of sea sand and 200 ml of 0.1 M Tris/0.01 M mercaptoethanol buffer, pH 7.3. The homogenate was squeezed through two layers of cheesecloth, and particles and debris were removed by centrifugation at 20,000 X G for 30 minutes. Proteins in the supernatant solution

were then fractionated as follows with a saturated solution of ammonium sulfate adjusted to pH 7.0 with NH_4OH. The solution was first made 50% saturated in respect to ammonium sulfate, and the precipitate was separated by centrifugation and resuspended in 32 ml of 0.1 M Tris/0.01 M mercaptoethanol buffer, pH 7.3. A slight green residue was discarded. The 25-50% ammonium sulfate fraction was collected from that solution, suspended in a few ml of 0.025 M Tris/0.01 M mercaptoethanol buffer, pH 7.3, and dialyzed overnight against 2.0 l of the same buffer. The dialyzed protein was subjected to precipitation with an equal volume of the saturated ammonium sulfate solution and redissolved in buffer. After this procedure was repeated three more times, the precipitate was suspended in 1 ml of buffer and dialyzed as before. These steps were taken to remove endogenous quercetin glycosides present in the original ammonium sulfate precipitate. No loss of activity was apparent after storage of the enzyme for several weeks at -20°.

Enzymatic L-rhamnosyl transfer. When TDP-L-rhamnose-C^{14}, 3-quercetin-D-glucoside, ATP, and the leaf enzyme were incubated together and the mixture chromatographed on paper, a radioactive compound appeared with the mobility of rutin. This compound could not be eluted readily with water. The rate of its formation is shown in Table I.

Table II shows that the synthesis of this compound has an absolute requirement for 3-quercetin-D-glucoside, the postulated L-rhamnosyl acceptor. It is presumed that ATP protects TDP-L-rhamnose from hydrolysis by serving as a substrate for pyrophosphatases present in the crude enzyme and thus, in effect, stimulates the transrhamnosylation reaction. There is no transfer of L-rhamnose from L-rhamnose 1-phosphate.

Identification of the radioactive reaction product as rutin. The labeled reaction product which migrated with rutin upon chromatography was mixed with 10 mg of rutin carrier, and the rutin was crystallized four times from aqueous methanol. The specific activity of the material remained essentially unchanged. Maintenance of specific activity of a labeled

Table I

Enzymatic incorporation of radioactivity into rutin

The reaction mixtures contained 1800 cpm TDP-L-rhamnose-C^{14} (4×10^{-4} μmole), 1200 cpm TDP-D-glucose-C^{14} (2.7×10^{-4} μmole), 0.4 μmole ATP, 0.001 ml of a 1% suspension of 3-quercetin-D-glucoside, and 0.020 ml of mung bean leaf enzyme containing about 1 mg of protein. Total volume was 0.030 ml. The mixtures were incubated at 37° in sealed capillary tubes. After incubation the content of each capillary and 0.1 μmole of authentic rutin were added to 0.75 ml of warm (50°-60°) methanol and the precipitate which then formed was reextracted with 0.5 ml of the solvent. The combined methanol extracts were allowed to evaporate to a small volume and were chromatographed on paper with butanol-acetic acid-water, 52:13:35. Rutin was located by its fluorescence in ultraviolet light and radioactive compounds by autoradiography. Material in the rutin area was eluted from the paper with methanol, while residual sugar nucleotides and enzymatic breakdown products--hexose phosphates and free hexoses--were eluted with water. Eluates were dried on polyethylene planchets in a vacuum desiccator and their radioactivity estimated with a conventional mica window Geiger counter and scaler.

Time of incubation in minutes	% total recovered radioactivity incorporated into rutin
0	0
15	8
30	14
60	27
120	41

Table II

Requirements for the formation of radioactive rutin

The components of the complete system are the same as those described in Table I, with the addition of 0.2 μmole $MgCl_2$. Changes in this system are indicated in the table. In the last experiment, the TDP sugars were replaced by a mixture of L-rhamnose 1-phosphate (3800 cpm) and D-glucose 1-phosphate (2500 cpm). After incubation at 37° for 60 minutes, the reaction mixtures were treated as described in Table I.

System	% total recovered radioactivity incorporated into rutin
Complete	26
- 3-quercetin-D-glucoside	0.5
- ATP	5
- $MgCl_2$	28
TDP sugars replaced by corresponding hexose 1-phosphates	0

compound through multiple crystallizations with a carrier is ordinarily considered good evidence for its identity. However, this criterion could

not be relied upon in this case, since it was found that an unidentified minor reaction product, separable from rutin upon paper chromatography, could also be repeatedly crystallized with rutin carrier without any change in specific activity.

The radioactive compound formed in the L-rhamnosylation of 3-quercetin-D-glucoside has been tentatively identified as rutin by the exact coincidence of its chromatographic position with that of authentic rutin upon development with butanol-acetic acid-water, 52:13:35, and water-saturated phenol. The only radioactive product produced upon mild acid hydrolysis was a compound inseparable chromatographically from authentic rhamnose in the aforementioned two solvent systems. Thus, TDP-D-glucose-C^{14}, which was always present in the reaction mixtures, does not appear to exchange D-glucose with that present in 3-quercetin-D-glucoside.

Work is currently in progress to determine the immediate precursor of D-glucose in rutin.

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