Enzymic Glycosylation of Quercetin to Rutin*

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A compound chromatographically indistinguishable from the flavonol glycoside rutin has been synthesized by the enzymic glycosylation of quercetin. The process takes place in two steps catalyzed by enzyme preparations from the leaves of *Phaseolus aureus*. D-Glucose is first transferred to quercetin from thymidine diphosphate D-glucose or uridine diphosphate D-glucose to form 3-quercetin-β-D-glucoside. Both sugar nucleotides are about equally effective as D-glucosyl donors. Rutin is then synthesized by a transfer of L-rhamnose to the glucoside from thymidine diphosphate L-rhamnose. Further evidence for the identity of the product was obtained by liberation of its disaccharide component, rutinose, with rhamnodiastase from leaves of *Rhamnus californica*.

The flavonol glycoside rutin [3-quercetin-*O*-α-L - rhamnosyl - (1 → 6) - β - D - glucoside][1] is found widely distributed in plants. Although it is sometimes present in remarkably high concentrations (2–3% of the dry weight), its physiologic function is unknown (Stoll and Jucker, 1958). The structure of rutin is shown in Figure 1.

In a preliminary communication from this laboratory enzyme preparations from *Phaseolus aureus* leaves were shown to transfer L-rhamnose from the sugar nucleotide thymidine diphosphate L-rhamnose to 3-quercetin-β-D-glucoside to form rutin (Barber and Neufeld, 1961). This paper represents a more detailed description of experiments designed to elucidate this process. The formation of the rhamnosyl acceptor, 3-quercetin-β-D-glucoside, has also been found to be catalyzed by an enzyme in similar leaf preparations. In this system D-glucose is transferred from thymidine diphosphate D-glucose or uridine diphosphate D-glucose to quercetin to form 3-quercetin-β-D-glucoside. By combining the reactants of the two systems, the complete enzymic glycosylation of quercetin to rutin has been effected.

Experimental Procedure

Methods.—Paper electrophoresis was carried out in an apparatus similar to that of Crestfield and Allen (1955) on Whatman No. 1 filter paper washed with 1% oxalic acid. Radioactive compounds on paper were located by autoradiography (Kodak no-screen x-ray film). Unless otherwise noted, partition paper chromatography was performed in a descending direction in 15 × 45 cm Pyrex cylinders at room temperature or radially at 37° in chambers constructed of two Pyrex baking dishes 24 cm in diameter. The following chromatographic solvents were used: 1-butanol–acetic acid–water (52:13:35); water-saturated phenol; 1-propanol–ethyl acetate–water (7:1:2); 95% ethanol–1 M ammonium acetate (7:3). Radioactive materials were dried on polyethylene planchets and counted with an end-window Geiger-Müller tube and a conventional scaler. Reducing sugars, used as reference standards on chromatograms, were located with the *p*-anisidine phosphate reagent (1 g *p*-anisidine phosphate in 140 ml 70% ethanol).

Substrates and Reference Compounds.—C^{14}-labeled α-D-glucose 1-phosphate (37 μc/μmole) had been previously prepared in this laboratory by the action of sucrose phosphorylase on C^{14}-labeled sucrose (Wolochow *et al.*, 1949). C^{14}-labeled TDP-D-glucose[2] of the same specific activity was synthesized from thymidine triphosphate (Sigma Chemical Company) and radioactive α-D-glucose 1-phosphate with TDP-D-glucose pyrophosphorylase from *Pseudomonas aeruginosa* ATCC 7700 (Kornfeld and Glaser, 1961). Since the $(NH_4)_2SO_4$ fraction in which this activity occurred was found to vary from preparation to preparation, fractions were first tested for the presence of the enzyme by a small-scale adaptation of the procedure given below.

The reaction mixture contained 0.67 μmole D-glucose-C^{14} 1-phosphate (25 μc), 4 μmoles TTP, 0.1 mg crystalline inorganic pyrophosphatase, and 0.15 ml of the bacterial protein fraction precipitating between 40 and 60% $(NH_4)_2SO_4$ concentra-

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[1] Rutinose, the disaccharide portion of rutin, was first synthesized by Zemplen and Gerecs (1935), who assigned the β-L configuration to the rhamnosyl linkage. Gorin and Perlin (1959) have since demonstrated that this conclusion was in error and the configuration is α-L.

[2] The abbreviations used are: TDP-D-glucose, thymidine diphosphate D-glucose; TDP-L-rhamnose, thymidine diphosphate L-rhamnose; UDP-D-glucose, uridine diphosphate D-glucose; ADP-D-glucose, adenosine diphosphate D-glucose; GDP-D-glucose, guanosine diphosohate D-glucose; TTP, thymidine triphosphate; Tris, tris(hydroxymethyl)aminomethane–HCl; NAD, nicotinamide-adenine dinucleotide; NADP, nicotinamide-adenine dinucleotide phosphate; $NADPH_2$, reduced nicotinamide-adenine dinucleotide phosphate.

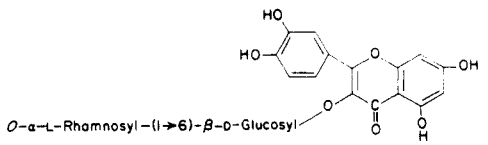

FIG. 1.—Structure of rutin.

tion (*ca* 3.0 mg protein) in 0.05 M Tris–0.01 M $MgCl_2$–0.001 M versene buffer, *p*H 8.0, in a total volume of 0.28 ml. After incubation on a planchet for 1 hour at 37° the mixture was subjected to paper electrophoresis in 0.2 M ammonium formate buffer, *p*H 3.6, at 30 v/cm. TDP-D-glucose moves slightly ahead of a picrate standard in this system. That section of the paper containing C^{14}-labeled TDP-D-glucose was cut out and left overnight in a vacuum desiccator over NaOH flakes and concentrated H_2SO_4 to remove ammonium formate. TDP-D-glucose was then eluted from the paper with water and the solution stored at −10°.

TDP-L-rhamnose labeled in the rhamnose moiety was prepared from C^{14}-labeled TDP-D-glucose with the same crude enzyme preparation from *Pseudomonas aeruginosa* (Glaser and Kornfeld, 1961). The reaction mixture contained 0.35 μmole TDP-D-glucose-C^{14} (12 μc), 5 μmoles $NADPH_2$, 0.08 μmole NAD, 0.2 ml of the bacterial protein fraction precipitating between 40 and 60% $(NH_4)_2SO_4$ saturation (*ca* 4.0 mg protein) in 0.05 M Tris–0.01 M $MgCl_2$–0.001 M versene buffer, *p*H 8.0, in a total volume of 0.3 ml. After incubation for 1.5 hours at 37° the mixture was subjected to paper electrophoresis and the sugar nucleotide isolated as before. Since TDP-L-rhamnose is inseparable from TDP-D-glucose by electrophoresis or chromatography in the systems tested, the yield of TDP-L-rhamnose was determined indirectly. An aliquot of the sugar nucleotide eluate was hydrolyzed for 20 minutes (1 N HCl, 100°), C^{14}-labeled L-rhamnose and D-glucose were isolated from the hydrolysate chromatographically, and the proportion of the total radioactivity in each monosaccharide was determined. More than 90% of the radioactivity of the hydrolysate was found to be in L-rhamnose. The TDP-L-rhamnose initially isolated from the reaction mixture was contaminated with $NADPH_2$, since the two compounds were not completely separated by electrophoresis. To remove $NADPH_2$ the eluate was chromatographed on acid-washed paper with ethanol–ammonium acetate. The section of the paper containing TDP-L-rhamnose was cut out and left in a vacuum desiccator as before to remove residual ammonium acetate. The TDP-L-rhamnose–TDP-D-glucose mixture was then eluted from the paper with water and stored at −10°.

UDP-D-glucose labeled uniformly in the glucose moiety (37 μc/μmole) was synthesized by Dr. Elizabeth Neufeld by methods previously described (Feingold *et al.*, 1958).

L-Rhamnose 1-phosphate labeled with C^{14} was prepared by hydrolysis of C^{14}-labeled TDP-L-rhamnose with the venom of *Crotalus adamanteus*, which contains a nucleotide pyrophosphatase. A mixture of 20 μl of TDP-L-rhamnose-C^{14} (0.15 μc), 4 μl of a 1% solution of *C. adamanteus* venom in 0.1 M glycine buffer, *p*H 8.9, and 2 μl 0.05 M $MgCl_2$ was incubated for 1 hour at 37°. L-Rhamnose 1-phosphate was separated from the mixture by electrophoresis on paper.

Samples of 3-quercetin-β-D-glucoside were gifts of Dr. T. A. Geissman, Dr. R. M. Horowitz, and Dr. R. F. Dawson.

A mixture of authentic rutinose, D-glucose, and L-rhamnose was prepared by hydrolysis of rutin in 10% acetic acid (Arakawa, 1956).

Salicin, amygdalin, and *o*-coumaric acid-β-D-glucoside were donated by Dr. E. E. Conn.

Enzymes.—Crystalline inorganic pyrophosphatase (Kunitz, 1952) was obtained through the courtesy of Dr. K. K. Reddi.

Rhamnodiastase (Bridel and Charaux, 1925), an enzyme which hydrolyzes the disaccharide rutinose from rutin, was prepared as follows. Leaves of the native shrub *Rhamnus californica* were collected at the Botanical Garden of the University of California through the courtesy of Mr. Wayne Roderick. Thirty grams of the leaves were chopped with scissors and ground in a chilled mortar with washed sand and 50 ml of 0.1 M Tris–0.01 M β-mercaptoethanol buffer, *p*H 7.5. The gummy homogenate was squeezed through two layers of cheesecloth, and particulate material was removed by centrifugation at 20,000 × *g* for 20 minutes in the cold. Proteins in the supernatant solution were fractionated with a saturated solution of $(NH_4)_2SO_4$ adjusted to *p*H 7.0 with NH_4OH. Fractions precipitating between 0–25, 25–50, and 50–75% $(NH_4)_2SO_4$ saturation were dissolved in minimal volumes of 0.025 M Tris–0.01 M β-mercaptoethanol buffer, *p*H 7.5, and dialyzed overnight against 2.0 liters of the same buffer. The enzyme fractions were tested for their ability to hydrolyze rutin by incubating aliquots with suspensions of rutin at *p*H 4.8, 5.8, or 7.5 for 4 hours at 37°. Portions of the suspensions were then applied directly to radial chromatograms and developed with propanol–ethyl acetate–water at 37°. A mixture of authentic rutinose, D-glucose, and L-rhamnose was chromatographed on the same paper for comparison. Sugars were detected with the *p*-anisidine phosphate reagent and the relative degree of hydrolysis was estimated visually. The 50–75% $(NH_4)_2SO_4$ fraction at *p*H 4.8 was found to constitute the most active system. Only rutinose and quercetin were detected as products of the enzymically catalyzed hydrolysis.

Mung bean (*Phaseolus aureus*) seedlings were grown in soil, or in sand watered with Hoagland's solution, in a greenhouse for from 2 to 5 weeks. The leaves, mostly primary at that stage, were removed, washed, and ground in an equal weight of 0.1 M Tris–0.01 M β-mercaptoethanol buffer, *p*H 7.5, in a chilled mortar with acid-washed sea

sand. All subsequent operations were conducted in the cold. The homogenized tissue was squeezed through two layers of cheesecloth, and particulate matter was removed by centrifugation for 30 minutes at 20,000 × *g*. Protein in the supernatant solution was fractionated with a saturated solution of $(NH_4)_2SO_4$ adjusted to *p*H 7.0 with NH_4OH as follows. To the supernatant solution an equal volume of saturated ammonium sulfate solution was added slowly with stirring. The suspension was allowed to stand for 20 minutes, after which the precipitate was collected by centrifugation. The protein was dissolved in a volume of 0.025 M Tris–0.01 M β-mercaptoethanol buffer, *p*H 7.5, equal to about one fourth the volume of the original supernatant solution. From this solution protein precipitating between 25–50% ammonium sulfate saturation was recovered and dissolved in one half the previous volume of the dilute Tris–β-mercaptoethanol buffer. Protein was precipitated again with an equal volume of saturated $(NH_4)_2SO_4$ solution and redissolved in buffer. The same procedure was then repeated two more times. These last steps were taken to remove endogenous quercetin glycosides, which tend to be adsorbed by the protein. Finally the precipitate was taken up in a minimal volume of 0.025 M Tris–0.01 M β-mercaptoethanol buffer, *p*H 7.5, and dialyzed overnight against two 1-liter volumes of the same buffer. The enzyme was stored in small aliquots at −10°. There was no significant loss of activity for at least 4 weeks.

Enzymic Reactions.—Reactions were generally carried out in 1.5-mm capillary tubes sealed in a flame (Porter and Hoban, 1954). To separate C^{14}-labeled rutin or 3-quercetin-β-D-glucoside from the mixtures, the contents of each capillary were mixed in a centrifuge tube with 0.75 ml of methanol and the protein residue removed by centrifugation. The precipitate was washed once with 0.25 ml of methanol and the extracts were combined and allowed to evaporate to a convenient volume on a planchet. The extracts were then applied to acid-washed paper (Whatman No. 1) in 2-cm bands. If paper which had not been acid-washed was used for chromatography, these compounds became difficult to elute and immobile if rechromatographed in ordinary solvents, presumably because they underwent oxidation and polymerization catalyzed by traces of metals in the paper. Chromatograms were developed with butanol–acetic acid–water. Rutin and 3-quercetin-β-D-glucoside when used as carrier or reference compounds were located under ultraviolet light ("Mineralight") by their characteristic brown fluorescence changing to bright yellow upon exposure to ammonia fumes.

Flavonol glycosides were eluted from the papers with methanol in an elution chamber. The material remaining on the chromatograms, consisting of various proportions of rhamnose, glucose, hexose phosphates, and unreacted sugar nucleotides, was eluted with water.

To prepare rutinose from enzymically synthesized rutin, the methanol extract of the reaction mixture was mixed with 0.2 μmole of carrier rutin and chromatographed as before. Radioactive rutin was eluted with methanol and the extracts evaporated to dryness on a planchet. The following materials were then added to the planchet: 10 μl of rhamnodiastase (*ca* 0.15 mg protein), 2.5 μl 1 M Na acetate buffer, *p*H 4.9, and 20 μl of water. The mixture was incubated on the planchet for 2 hours at 37°, applied directly to a radial chromatogram, and developed with propanol–ethyl acetate–water.

Results

Rhamnosyl Transfer.—When a mixture of the mung bean leaf enzyme, C^{14}-labeled TDP-L-rhamnose, and 3-quercetin-β-D-glucoside was incubated, a new radioactive compound appeared which was chromatographically indistinguishable from carrier rutin in the two most effective solvent systems for flavonols: butanol–acetic acid–water and water-saturated phenol. Upon acid hydrolysis of the enzymically formed rutin (1 N HCl, 30 minutes, 100°) the only radioactive product was L-rhamnose. The latter compound was identified by co-chromatography with carrier rhamnose in two dimensions on a 46 × 57 cm sheet of filter paper with butanol–acetic acid–water and water-saturated phenol. Enzymic hydrolysis of this C^{14}-labeled rutin with rhamnodiastase yielded a radioactive compound which was indistinguishable from authentic rutinose on radial chromatography with propanol–ethyl acetate–water, on two dimensional chromatography with butanol–acetic acid–water and water-saturated phenol, and upon electrophoresis in 0.1 M $Na_2B_4O_7$, *p*H 9.2.

The rate of rhamnosyl transfer from TDP-L-rhamnose to 3-quercetin-β-D-glucoside is given in Figure 2.

Table I shows the effect of various compounds on the rhamnosylation reaction. There is an almost complete requirement for the rhamnosyl acceptor, 3-quercetin-β-D-glucoside, and none of the following compounds could substitute for it: D-glucose, maltose, cellobiose, salicin, phlorizin, amygdalin, and *o*-coumaric acid-β-D-glucoside.

Since 3-quercetin-β-D-glucoside is only slightly soluble in aqueous solvents, it was reasoned that the affinity of the enzyme for the compound is quite high. The effect of its concentration on the rate of L-rhamnose transfer was studied by incubating a series of reaction mixtures as described in Table I (30 minutes, 37°) except that the concentration of the acceptor was varied. Half-maximal incorporation of L-rhamnose into rutin occurred at a 3-quercetin-β-D-glucoside concentration of 6.5×10^{-6} M.

In the absence of ATP most of the radioactivity remaining after incubation of these mixtures was in free rhamnose. Thus the pronounced stimulation of the reaction by ATP may be due to the protection against hydrolysis it affords TDP-L-

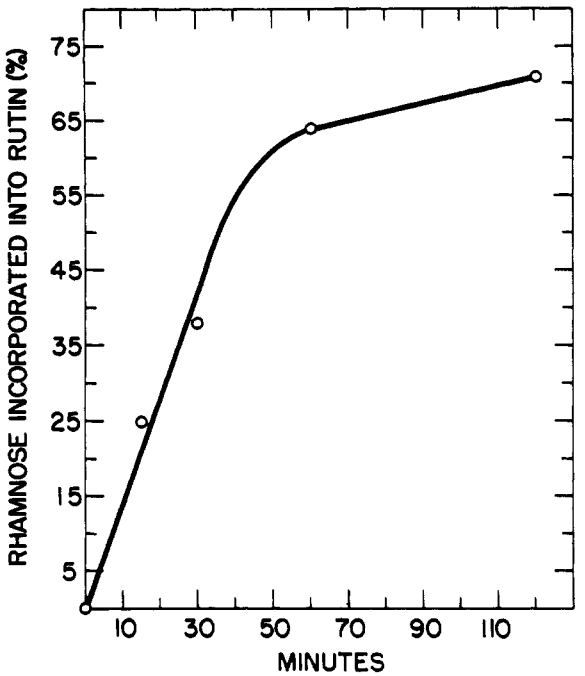

FIG. 2.—Rate of enzymic incorporation of L-rhamnose-C^{14} into rutin from TDP-L-rhamnose-C^{14}. The reaction mixtures and conditions were as described in Table I except that $MgCl_2$ was omitted. Methods used to estimate radioactivity incorporated into rutin are given in the text.

rhamnose as a competitive substrate for nucleotide pyrophosphatase and phosphatases present in the enzyme preparation. In other experiments TTP and NAD seemed as effective as ATP. Mg^{++} does not stimulate the incorporation of rhamnose into rutin. L-Rhamnose-C^{14} 1-phosphate was completely ineffective as a rhamnosyl donor.

TABLE I

REQUIREMENTS FOR THE FORMATION OF RADIOACTIVE RUTIN

The complete reaction mixture contained 6.5×10^{-4} μmole (2.4×10^{-2} μc) TDP-L-rhamnose, 0.3 μmole ATP, 0.2 μmole $MgCl_2$, 1 μl of a 1% aqueous suspension of 3-quercetin-β-D-glucoside, and 20 μl mung bean leaf enzyme (about 1.7 mg protein) in 0.025 M Tris–0.01 M β-mercaptoethanol buffer, *p*H 7.5, in a total volume of 28 μl. Mixtures were incubated in sealed capillary tubes for 2 hours at 37°. Methods used to estimate radioactivity incorporated into rutin are given in the text.

System	% Total Recovered Radioactivity Incorporated into Rutin
Complete	63
-$MgCl_2$	71
-3-Quercetin-β-glucoside	5
-ATP	6

Glucosyl Transfer.—Formation of the 3-quercetin-β-D-glucoside acceptor was also catalyzed by an enzyme in the 25–50% $(NH_4)_2SO_4$ fraction prepared from mung bean leaves. Glucose was transferred to quercetin from either TDP-D-glucose or UDP-D-glucose. C^{14}-labeled 3-quercetin-β-D-glucoside formed in the reaction was identified by the coincidence of its movement with the authentic compound upon co-chromatography in two dimensions with butanol–acetic acid–water and water-saturated phenol. It was hydrolyzed by β-glucosidase (Sigma Chemical Co.) (0.5% in 0.025 M Na acetate buffer, *p*H 5.0, 3 hours at 37°) to give C^{14}-labeled glucose which was identified chromatographically. While the glucosyl residue has not been unequivocally demonstrated to be at the 3-OH of quercetin, the ability of the compound to substitute for authentic 3-quercetin-β-D-glucoside in the subsequent transrhamnosylation reaction (see below) supports that hypothesis.

Requirements for the glucosylation of quercetin by UDP-D-glucose or TDP-D-glucose are shown in Table II. As when TDP-L-rhamnose is the donor, the effect of ATP is presumably due to the protection against enzymic hydrolysis it affords the sugar nucleotides. If ATP was not included in the reaction mixture, TDP-D-glucose was much more readily hydrolyzed to D-glucose than was UDP-D-glucose. This may explain the greater stimulation of the reaction by ATP when TDP-D-glucose is the glucosyl donor.

D-Glucose-C^{14} 1-phosphate was not incorporated into 3-quercetin-β-D-glucoside when incubated with the enzyme, ATP, and Mg^{++}. This rules out the possibility that TDP-D-glucose and UDP-D-glucose function merely as sources of D-glucose 1-phosphate for the reaction. The possibility

TABLE II

REQUIREMENTS FOR THE FORMATION OF RADIOACTIVE 3-QUERCETIN-β-D-GLUCOSIDE

The complete reaction mixture contained either 1.6×10^{-3} μmole (6×10^{-2} μc) TDP-D-glucose or 1.8×10^{-3} μmole (6.7×10^{-2} μc) UDP-D-glucose, 0.2 μmole $MgCl_2$, 0.3 μmole ATP, 1 μl of a 1% aqueous suspension of quercetin, 20 μl mung bean leaf enzyme (*ca* 1.0 mg protein) in 0.025 M Tris–0.01 M β-mercaptoethanol buffer, *p*H 7.5, in a total volume of 29 μl. Mixtures were incubated in sealed capillary tubes for 2 hours at 37°. Methods used to estimate radioactivity incorporated into 3-quercetin-β-D-glucoside are given in the text.

System	% Total Recovered Radioactivity Incorporated from TDP-D-Glucose C^{14} or UDP-D-Glucose-C^{14}	
	TDP-D-Glucose	UDP-D-Glucose
Complete	27	27
-$MgCl_2$	23	20
-Quercetin	4	9
-ATP	5	19

remains, however, that there is an intermediate in the glycosylation to which both TDP-D-glucose and UDP-D-glucose contribute the glucosyl moiety.

The relative activities of the L-rhamnosyl and D-glucosyl transferring systems varied from preparation to preparation, but both were generally present. In one case only, that of a preparation from leaves of the youngest seedlings used (2 weeks), transglucosylase activity could not be demonstrated while the transfer of L-rhamnose was vigorously catalyzed.

Rutin Formation.—The complete glycosylation of quercetin to rutin by the soluble enzyme system was demonstrated in the following two ways: C^{14}-labeled 3-quercetin-β-D-glucoside formed enzymically was isolated by chromatography and supplied as the acceptor to the system transferring L-rhamnose from TDP-L-rhamnose; the two glycosylations were carried out simultaneously by using quercetin as the glycosyl acceptor and supplying both TDP-L-rhamnose and TDP-D-glucose or UDP-D-glucose as glycosyl donors.

The reaction mixtures and conditions employed were as follows: 1. C^{14}-labeled 3-quercetin-β-D-glucoside was synthesized enzymically from TDP-D-glucose and from UDP-D-glucose as described in Table II, isolated chromatographically, and eluted onto planchets. The eluates were evaporated to dryness, and to each planchet was added 10 μl mung bean leaf enzyme (*ca* 0.5 mg protein) in 0.025 M Tris–0.01 M β-mercaptoethanol buffer, *p*H 7.5, 0.2 μmole ATP, and 9×10^{-4} μmoles (3.2×10^{-2} μc) C^{14}-labeled TDP-L-rhamnose in a total volume of 25 μl. The mixtures were incubated on the planchets at 37° for 2 hours.

2(*a*). Twenty μl mung bean leaf enzyme (*ca*. 1.0 mg protein) in 0.025 M Tris–0.01 M β-mercaptoethanol buffer, *p*H 7.5, 0.2 μmole $MgCl_2$, 0.3 μmole ATP, 9×10^{-4} μmole (3.2×10^{-2} μc) C^{14}-labeled TDP-L-rhamnose, and 1.6×10^{-3} μmole (6×10^{-2} μc) C^{14}-labeled UDP-D-glucose in a total volume of 30 μl were incubated in a capillary tube for 2 hours at 37°.

2(*b*). A similar mixture was incubated with 1.6×10^{-3} μmole (6.7×10^{-2} μc) of C^{14}-labeled TDP-D-glucose instead of UDP-D-glucose.

After incubation each of the four samples was mixed with 0.2 μmole of carrier rutin and applied directly to a paper chromatogram, which was developed with butanol–acetic acid–water. Rutin was eluted from the papers with methanol and treated with rhamnodiastase as described earlier. After radial chromatography of the enzymic hydrolysates with propanol–ethyl acetate–water, one radioactive band appeared in each, corresponding in position to authentic rutinose. Radioactive rutinose was eluted from the circular papers with water, hydrolyzed with HCl, and the hydrolysates chromatographed radially in the same solvent. Radioactive bands appeared for each hydrolysate in the positions of rhamnose and glucose.

Discussion

A previous example of the enzymic glycosylation of phenolic compounds in two steps was given by Yamaha and Cardini (1960). They showed that in the presence of an enzyme preparation from wheat germ D-glucose is transferred from UDP-D-glucose to hydroquinone and various other phenols. The compound is first glucosylated, forming the β-monoglucoside. It can then accept a second molecule of D-glucose to produce the gentiobioside.

Watkin and Neish (1961) studied the biosynthesis of rutin *in vivo* by administering differentially labeled D-glucose or L-rhamnose-1-C^{14} to buckwheat seedlings. They found that L-rhamnose was not incorporated into rutin to any significant extent, but D-glucose-C^{14} labeled the D-glucose and L-rhamnose moieties of rutin about equally. Furthermore, randomization of C^{14} in D-glucose, which occurred to a considerable extent, was essentially reproduced in the carbon chain of L-rhamnose recovered from rutin. Thus it appears that D-glucose is a direct precursor of L-rhamnose *in vivo*. This would be expected if the interconversion of the two sugars were carried out by the TDP-D-glucose → TDP-L-rhamnose pathway which has been shown to occur in some microorganisms (Glaser and Kornfeld, 1961; Pazur and Shuey, 1961). A TDP-D-glucose pyrophosphorylase has been found in higher plants (Pazur and Shuey, 1961), but the transformation of TDP-D-glucose to TDP-L-rhamnose has yet to be demonstrated.

The recent discovery of Recondo and Leloir (1961) that ADP-D-glucose as well as UDP-D-glucose is a donor of D-glucose to starch suggests that enzymes involved in such transglycosylations may be relatively nonspecific. Further support of this hypothesis is afforded by the data given here showing that either TDP-D-glucose or UDP-D-glucose will act as a glucose donor for the enzymic synthesis of 3-quercetin-β-D-glucoside. It is theoretically possible that there are at least eight different sugar nucleotides of each monosaccharide, and a number of these have been isolated from natural sources or produced enzymically, *e.g.*, UDP-D-glucose, TDP-D-glucose, and GDP-D-glucose, and TDP-D-mannose and GDP-D-mannose (Hassid, 1961). Therefore if a transglycosylase is of low nucleotide specificity, the question of which sugar nucleotide serves as its substrate *in vivo* cannot be readily answered.

Acknowledgment

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Carbohydrates and Nucleotides in the Red Alga *Porphyra perforata*.* I. Isolation and Identification of Carbohydrates

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An investigation of the marine red alga *Porphyra perforata* showed that taurine, laminitol, *scyllo*-inositol and 1-*O*-α-D-galactopyranosyl-D-glycerol were present in the ethanolic extract of the plant; these were isolated in crystalline form. Although no isolation could be achieved, indirect evidence indicated that 2-*O*-α-D-galactopyranosylglycerol was also present. The algae also contained an insoluble polysaccharide. Hydrolysis of the latter with acid produced D- and L-galactose and 6-*O*-methyl-D-galactose, all of which were isolated in crystalline form. Evidence was also presented for the presence of 3,6-anhydro-L-galactose. The polysaccharide was shown to contain sulfate in the ester form. Analysis of the sodium salt of the polysaccharide indicated that the polysaccharide consists of D-galactose, 6-*O*-methyl-D-galactose, 3,6-anhydro-L-galactose, and sulfate in the molar ratio of approximately 1:1:2:1. The galactose is present in the polysaccharide as a mixture of the D- and L-isomers in the ratio of 1.3:1. A microquantitative method for estimating D-galactose with the specific D-galactose dehydrogenase from *Pseudomonas saccharophila* was developed in connection with this study.

Seaweeds belonging to the order *Bangiales* are known to contain an intercellular polysaccharide consisting chiefly of D- and L-galactosyl residues. *Porphyra crispata*, a member of this order, was shown to contain a DL-galactan in its hot-water extract, while a D-galactoside was present in the ethanolic extract (Su, 1956).

This paper represents the results of an investigation of the various carbohydrates present in the ethanolic extract and of the ethanol-insoluble galactan isolated from this marine alga.

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Experimental Procedure

Materials and Methods.—The algae were collected from the east side of the Golden Gate Bridge, San Francisco, California, in October, 1959. After collection, the algae were placed in sea water and brought to the laboratory immediately. A 5.2-kg quantity of the algae (700 g dry weight) was washed with tap water, drained, and dropped into 5.2 liters of boiling ethanol. The mixture was brought to boiling again and kept at that temperature for 2 minutes. The algae were kept in ethanol at −10° until used.

A culture of *Pseudomonas saccharophila* from which the specific D-galactose dehydrogenase was prepared (Doudoroff *et al.*, 1958) was provided by Dr. M. Doudoroff of the Bacteriology Department. Floridoside and its hexa-acetate were provided by Dr. E. W. Putman. *scyllo*-Inositol and its hexa-acetate were furnished by Dr. C. E.