FORMATION AND EPIMERIZATION OF dTDP-D-GALACTOSE CATALYZED BY PLANT ENZYMES*

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Following the original discovery by Leloir and co-workers in 1951, it has been shown in many laboratories that animals, microorganisms, and higher plants contain enzyme systems which catalyze the formation of uridine diphosphate (UDP) D-galactose and the 4-epimerization of this sugar nucleotide to UDP-D-glucose (Leloir and Cardini, 1960). Evidence is now presented for similar reactions involving thymidine diphosphate (dTDP) derivatives, which are catalyzed by extracts of germinating seeds of Phaseolus aureus:

1) dTTP + α-D-galactose 1-phosphate $\rightleftharpoons$ dTDP-D-galactose + PP

2) dTDP-D-galactose $\rightleftharpoons$ dTDP-D-glucose

While this manuscript was in preparation, we were informed of the occurrence of dTDP-D-galactose 4-epimerase (reaction 2) in several bacterial genera (Tinelli et al., 1962). The existence of systems for synthesizing dTDP-D-galactose in both plants and microorganisms suggests a role for this compound in the metabolism of D-galactose, perhaps as a specific donor in transgalactosylations.

Preparation of Substrates. UTP, dTTP and dUTP were purchased from Pabst or Sigma chemical companies. Radioactive α-D-galactose 1-phosphate (7 μc/μmole), prepared by the action of the galactokinase of Saccharomyces fragilis on 1-C^{14} D-galactose, was incorporated into UDP-D-galactose in the

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presence of UTP and UDP-D-galactose pyrophosphorylase from Phaseolus aureus (Neufeld et al., 1957). Enzyme preparation B, described below, was used for this purpose. The preparation of UDP-D-glucose and of dTDP-D-glucose labeled in the D-glucose moiety (37 μc/μmole) have been previously described (Feingold et al., 1958; Barber and Neufeld, 1961).

Analytical methods have been previously described (Neufeld et al., 1961).

Preparation of dTDP-D-Galactose Pyrophosphorylase and Epimerase. Seeds of Phaseolus aureus were germinated overnight in water, with aeration. The germinated seeds (50 g) were ground in a chilled mortar with 50 ml of a solution consisting of 0.5 M sucrose - 0.01 M phosphate, pH 7.0 - 0.01 M mercaptoethanol. After centrifugation at 20,000 X g, the supernatant fluid was fractionated with a saturated solution of $(NH_4)_2SO_4$ at pH 7.0.

The precipitate obtained at 0-50% saturation was suspended in 5 ml of 0.05 M Tris, pH 7.5 - 0.01 M mercaptoethanol, dialyzed against 2 liters of that buffer for at least 3 hours, with stirring, and the dialyzed solution was treated with 0.1 volume of 1 M $MnCl_2$. After 30 minutes, a voluminous precipitate was removed by centrifugation. The supernatant fluid was dialyzed for 18 hours against 2 liters of Tris-mercaptoethanol, clarified by centrifugation, and made 30% saturated with respect to $(NH_4)_2SO_4$. The precipitate was taken up in 1 ml of Tris-mercaptoethanol, dialyzed overnight against 1 liter of buffer, and used for epimerase experiments. This preparation will be referred to as Fraction A.

The 50-70% ammonium sulfate precipitate was suspended in 2 ml of Tris-mercaptoethanol buffer and subjected to dialysis, $MnCl_2$ treatment, and subsequent dialysis as described above. After the second dialysis the solution (about 4 ml) was lyophilized. The dry powder (Fraction B) was reconstituted in either water or 0.05 M mercaptoethanol and used as the source of pyrophosphorylase.

Evidence for dTDP-D-Galactose Pyrophosphorylase Activity. A typical mixture, containing 0.2 μmole dTTP, 0.5 μmole α-D-galactose 1-phosphate, 0.13 μmole $MgCl_2$, 3 μg crystalline inorganic pyrophosphatase (Kunitz, 1962),

and 10 µl of Fraction B reconstituted in 0.05 M mercaptoethanol, was incubated in a total volume of 25 µl. After 30-60 minutes at 37°, the mixture was subjected to paper electrophoresis in 0.2 M ammonium formate buffer at pH 3.7 and examined with the aid of ultraviolet light. A new compound ($R_{picrate}$ = 1.1), presumed to be dTDP-D-galactose, had been formed. A sufficient quantity was prepared for identification by performing the incubation on a larger scale. The compound was purified by paper electrophoresis at pH 3.7 followed by chromatography in ethanol - 1 M ammonium acetate (70:30).

The spectrum of this compound corresponded to that of thymidine nucleotides (Pabst Circular OR-17) at pH 7 and 11.5. Hydrolysis of 1 N HCl at 100° for 15 min. liberated 0.96 µmole inorganic phosphate and 1.17 µmole reducing sugar, calculated as galactose, per µmole of thymidine nucleotide. The nucleotide liberated by the acid hydrolysis had an electrophoretic mobility at pH 3.7 and chromatographic mobility in ethanol - 1 M ammonium acetate identical with that of dTMP.

The nature of the sugar moiety was demonstrated most effectively when C^{14}-D-galactose 1-phosphate was substituted for the unlabeled ester. The radioactive sugar liberated on acid hydrolysis (1 N HCl, 100°, 15 min.) was identified as galactose by a) chromatographic mobility equal to that of galactose in butanol-pyridine-H_2O, 6:4:3; b) co-chromatography with galactose in two-dimensional chromatography, using water-saturated phenol and butanol-acetic acid-water (52:13:35) as solvents; c) co-electrophoresis of the product of $NaBH_4$ reduction with galactitol in 0.05 M sodium tetraborate.

The reversibility of the reaction was shown by the partial conversion of dTDP-D-galactose prepared as described above to a compound with the electrophoretic mobility of dTTP in the presence of an excess of sodium pyrophosphate (but not of phosphate), $MgCl_2$ and Fraction B.

dTDP-D-galactose was not a substrate for the dTDP-D-glucose pyrophosphorylase of Pseudomonas aeruginosa (Kornfeld and Glaser, 1961), nor was it converted to dTDP-L-rhamnose by extracts of the same microorganism (Glaser and Kornfeld, 1961).

Formation of Other Sugar Nucleotides Catalyzed by Fraction B. Fraction B was tested for its ability to catalyze the formation of other dTDP sugars. The formation of dTDP-D-glucose was observed in the presence of dTTP and α-D-glucose 1-phosphate [dTDP-D-glucose pyrophosphorylase activity had previously been found in higher plants by Pazur and Shuey (1961)]. A small amount of a sugar nucleotide, presumably dTDP-L-arabinose, was formed from dTTP and β-L-arabinose 1-phosphate. No detectable dTDP-sugar formation occurred in the presence of the 1-phosphate derivatives of β-D-galactose, α-D-mannose, α-D-glucuronic acid, α-D-galacturonic acid, α-D-xylose, or N-acetyl-α-D-glucosamine.

The formation of a number of UDP-sugars, including UDP-D-galactose, was catalyzed by Fraction B. This is to be expected, since Fraction B is similar to preparations in which the UDP-sugar pyrophosphorylases had been previously detected (Hassid, Neufeld and Feingold, 1959). The rate of formation of dTDP-D-galactose as measured by phosphate released in the presence of inorganic pyrophosphatase, was 1-5% that of UDP-D-galactose. Recently, however, a preparation has been obtained in which the ratio was close to unity (Frydman, unpublished experiments).

No activity was observed when Fraction B was incubated in the presence of α-D-galactose 1-phosphate and ATP, GTP, ITP, or CTP. The substitution of dUTP for UTP or dTTP, however, led to the rapid formation of a sugar nucleotide tentatively identified as dUDP-D-galactose.[1]

Evidence for dTDP-D-Galactose Epimerase Activity. Fraction A (10 µl, containing 0.2 mg protein) was incubated with radioactive dTDP-D-galactose (0.025 µc), 0.13 µmole $MgCl_2$, and 0.5 µmole of dTTP (added to protect the sugar nucleotide from the action of nucleotide pyrophosphatase) in a total volume of 23 µl. After 90 min. at 37°, the dTDP-sugar was isolated by

[1]Fraction B also catalyzed the formation of dUDP-D-glucose. Preliminary experiments show that dUDP derivatives can function in some systems as analogs of UDP derivatives, as for yeast UDP-D-glucose pyrophosphorylase, while in others they function as analogs of dTDP derivatives, as for the dTDP-L-rhamnose-forming system of Ps. aeruginosa (Barber and Neufeld, unpublished experiments).

electrophoresis and chromatography and hydrolyzed in 1 N HCl (15 min., 100°). When the hydrolysis products were chromatographed in butanol-pyridine-water (6:4:3), two radioactive spots in a ratio of approximately 4:1, with the mobility of glucose and galactose, respectively, were observed. These were eluted and identified by a) co-chromatography with glucose or galactose in water-saturated phenol and in butanol-acetic acid-water, 52:13:35; b) co-electrophoresis of the products of $NaBH_4$ reduction with glucitol or galactitol, respectively.

The reaction was also carried out in the reverse direction, using C^{14}-labeled dTDP-D-glucose as substrate, and the products were identified in the same manner. The amount of galactose formed was about one-fourth that of the residual glucose.

Similar reactions were carried out using labeled UDP-D-galactose and dUDP-D-galactose, except that UTP and dUTP, respectively, were used as protective nucleotides instead of dTTP. On the basis of evidence similar to that described above, Fraction A was shown to catalyze the epimerization of these two nucleotides as well as that of dTDP-D-galactose.

We are presently attempting to determine whether the formation and epimerization of TDP-D-galactose are catalyzed by enzymes specific for the thymidine configuration.

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