NADP DEPENDENT OXIDATION OF TDP-GLUCOSE BY AN ENZYME SYSTEM FROM SUGAR BEETS

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Analysis of the free nucleotide pool in the mature sugar beet root demonstrated the presence of TDP-D-glucose (TDPG) and TDP-galacturonic acid (TDPGalA) among other sugar nucleotides (Katan and Avigad, 1966). TDP-glucose was also found to be formed in the 'sucrose synthetase' reaction by transglucosylation from sucrose to TDP (Milner and Avigad, 1965). Further experiments have now shown that extracts prepared from sugar beet roots catalyzed the reduction of NADP when TDPG was added as a substrate. A preliminary analysis of this enzymic system is described in this communication.

Materials and Methods. Mature, fresh sugar beet roots were kept at 4° until utilized. TDPG and other nucleotides were purchased from Calbiochem. In some experiments TDPG and TDPG-^{14}C were synthesized in the 'sucrose synthetase' reaction starting with ^{12}C or ^{14}C-sucrose, and TDP as the glucosyl acceptor (Milner and Avigad, 1965). NADPH formation was measured at 340 mμ in the Gilford Model 2000 multiple sample Absorbance Recorder in cells of 2 or 10 mm light path. Glucose was estimated with glucose oxidase (Glucostat reagent, Worthington Biochemical Corp.), and galactose with galactose oxidase (Avigad et al., 1962; Roth et al., 1965). Uronic acid was determined colorimetrically in the carbazol-H_2SO_4

reaction (Dische, 1962).

Chromatography of nucleotides and of sugar phosphates was carried out on Whatman No. 1 filter paper using ethanol: 1 M ammonium acetate pH 7.5 (7:3 v/v) as the solvent (Paladini and Leloir, 1952). Thin layer chromatography of sugars was carried on Cellulose-D (Camag AG, Muttenz, Switzerland) with pyridine, ethyl acetate, acetic acid and water (5:5:1:3 v/v) as the solvent. Phosphates were detected by the molybdate spray reagent (Bandurski and Axelrod, 1951) and sugars with the 2-amino-biphenyl reagent (Timell et al., 1956). A selection of other chromatographic, electrophoretic and analytical procedures occasionally employed for the identification of the nucleotides and sugars in the reaction system was as described by Neufeld (1962), Kalina and Avigad (1963), Feingold et al. (1964), Preiss (1964) and Lin and Hassid (1964).

A unit of enzyme activity was defined as that amount which catalyzes the formation of 1 mμ mole NADPH/min at 30°. The assay system contained 0.1 M Tris-HCl buffer, pH 8.5; 0.1 mM sodium diethyldithiocarbamate (DEDTC); 5 mM $MgSO_4$; 2 mM NADP and 1 mM TDPG in a final volume of 1.0 ml.

Experimental. Fresh extracts prepared from sugar beet roots were found to epimerize TDPG and UDPG to the corresponding nucleoside diphosphate galactose (Katan and Avigad, unpublished experiments) similar to the reactions observed in other plant preparations (Neufeld, 1962; Feingold et al., 1964). When NADP was added to such a system with TDPG as a substrate, reduction of the pyridine nucleotide occurred. NAD could not substitute NADP. The catalytic activity in the crude extract declined rapidly with a half-life of about one hour. In addition, browning, precipitate formation and high phosphatase activity in the extracts obliged us to try to obtain a partially

purified, more stable preparation in an endeavour to characterize this enzyme system.

The following procedure was adopted for a rapid partial purification (all steps carried out at 4°). 120 g of washed and peeled sugar beet roots were sliced and homogenized in a Waring Blendor with 120 ml of 50 mM phosphate buffer, pH 7.5, containing 1 mM EDTA. The filtrate obtained through gauze and glass woolwas reused for the homogenization of an additional 100 g of root tissue. The filtrate (125 ml, 600 mg protein, 900 units enzyme) was centrifuged at 12,000 g for 20 min. Solid $(NH_4)_2SO_4$ was added to obtain the fraction precipitating between 0.6-0.9 saturation. 20 min of stirring at each concentration was allowed before collecting the protein fraction by centrifugation at 15,000 g for 20 min. The precipitate was dissolved in 3.5 ml of 0.1 M Tris buffer, pH 8.5, containing 5 mM $MgSO_4$ and 0.1 mM DEDTC, and added to a Sephadex G-25 column (28x1.6 cm) preequilibrated with the same solution. The column was eluted with the sameTris solution and the protein-containing fraction (8 ml) was loaded on a DEAE-cellulose column (5x0.8 cm). A linear gradient of Tris buffer, pH 8.5, 0.1-1.0 N containing 0.1 mM DEDTC was applied and twenty 0.5 ml fractions were collected. Most of the TDPG oxidizing activity was recovered in fractions (Nos. 11-16) containing 0.4-0.8 M Tris. The enzyme, obtained in about 30% yield, was purified 5-10 fold (8-16 units/mg protein) whereas only about 2% of the total protein was recovered in these fractions. This result is indicative of a significant enzyme inactivation during the purification procedure. The half-life of the dehydrogenase activity in these preparations was about 10 hr at 4°. Freezing and thawing inactivated the enzyme, whereas incubation with 1 mM TDPG increased its stability 2 to 3 fold. Addition of 1 mM 2-mercaptoethanol, dithiothreitol, reduced glutathione or 0.1% serum

albumin was only slightly beneficial in stabilizing the enzyme.

Incubation of the purified protein fraction with TDPG and NADP (but not with NAD) resulted in NADPH formation at a molar ratio of 2:1 in relation to the amount of substrate oxidized or to the amount of uronic acid which appeared (Table 1). This product yielded the same spectrum of

TABLE 1

STOICHIOMETRY OF TDPG OXIDATION

Experiment No.	TDPG added	NADPH formed	Uronic acid formed	NADPH / uronic acid
1	0.21	0.39	0.21	1.85
2	0.19	0.33	0.17	1.95
3	0.38	0.66	0.34	1.94
4	0.36	0.46	0.21	2.18

The reaction mixture (1 ml) at 30° contained (in μmoles): Tris pH 8.5, 90; DEDTC, 0.1; $MgSO_4$, 5; NADP, 2; TDPG as indicated in the table and about 5-10 units of partially purified enzyme from different preparations obtained after fractionation on DEAE-cellulose. Increase of absorption at 340 mμ was followed until it slowed down significantly (40-60 min). Samples were assayed colorimetrically for uronic acid against the proper blank systems. Values indicate μmoles/ml reaction solution.

absorption as galacturonic acid in the carbazol reagent. NADP reduction started usually with a short lag of 1 to 5 min with different enzyme fractions. This lag could be cancelled if the system was preincubated with TDPG about 5 min prior to the addition of NADP. As all the enzyme preparations contained TDPG 4-epimerase, it is conceivable that the length of the lag period was the expression of the relative amount of this enzyme in the system. Table 2 presents a quantitative analysis of the reaction mixture which shows the formation of TDPGal as well as hexuronic acid.

TABLE 2

ASSAY OF PRODUCTS IN THE TDPG OXIDIZING SYSTEM

System	NADPH	Galactose	Glucose	Hexuronic acid
Complete	1.09	1.00	1.20	0.42
Without NADP	0	0.60	2.15	0

Reaction mixture (4 ml) at 30° contained (in mM): Tris, pH 8.5, 82; $MgSO_4$, 4; DEDTC, 0.1; NADP, 3.3; TDPG, 2.82 and enzyme, 54 units/ml (DEAE-cellulose purified fraction). After 150 min, when the increase in absorption at 340 mμ slowed down significantly, the system was acidified to pH 2.0 and placed for 15 min at 96°. After neutralization with dilute NaOH, the solution was analyzed colorimetrically for uronic acid and enzymatically for glucose and galactose. Values indicate μmoles/ml reaction solution.

In order to identify products of the reaction, 5 ml of a standard reaction mixture (with 26 units dehydrogenase/ml) were incubated for 40 min at 30°. Ethanol was then added to 90%. The precipitate was removed by filtration on a Millipore filter and the solution concentrated under reduced pressure at 30°. The contents of this reaction mixture was compared with control systems without NADP or TDPG. Chromatographic and electrophoretic analysis (Feingold et al., 1964) failed to reveal the presence of any TDP-uronic acid. However, a spot with the properties of galacturonic acid 1-phosphate in mobility and color reactions was detected. After hydrolysis in 0.1 N HCl for 15 min it yielded a hexuronic acid with chromatographic mobility similar to galacturonic acid. The product after acid hydrolysis failed to form a stable lactone in contrast to glucuronic acid under the same conditions. In addition, some free galacturonic acid, TMP and traces of TDP were also observed in the reaction system. In control systems without NADP, only very small amounts of TDP, TMP or free

aldohexoses could be detected in addition to TDPG and TDPGal. It is thus evident that TDP-galacturonic acid formed in this enzyme system was cleaved by some contaminating enzymes such as a pyrophosphatase or transglycosylation activities which have not yet been characterized.

Discussion. Many efforts invested in order to obtain a highly purified enzyme which catalyzes an NADP dependent oxidation of TDPG or TDPGal to the corresponding TDP-hexuronic acid, were unsuccessful. This activity, as present in the sugar beet root extracts, was found to be too labile to allow for a purification procedure which extended over several hours. The partially purified preparation obtained was relatively free of interfering nucleotides, sucrose, pectin and other contaminants but contained, in addition to the dehydrogenase, TDPG 4-epimerase as well as activities which decompose the TDP hexuronic acid produced. The fact that glucuronic acid or its lactone were not detected in the reaction mixture as well as the observation that an initial lag in the reaction could be abolished by the epimerization of TDPG to TDPGal, tentatively suggests the reaction sequence TDPG $\longrightarrow$ TDPGal $\longrightarrow$ $\longrightarrow$ TDPGalA to occur in the described system. This pathway could thus be considered complementary to the already well known reactions leading to the formation of UDP-hexuronic acids in plants (Strominger and Mapson, 1957; Feingold et al., 1960). As was recently shown for UDPGalA (Villemez et al., 1965), TDPGalA may also be an intermediate in the biosynthesis of pectin or other acidic plant polysaccharides.

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