prepare solutions of the free sugars. Our thanks are due to Mr L. C. Thomas, Chemical Defence Experimental Establishment, Porton, for producing the infrared-absorption spectra and to Mr D. C. Hawkins for technical assistance.

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Uridine Diphosphoglucose Dehydrogenase of Pea Seedlings

By J. L. STROMINGER
A.R.C. Virus Research Unit, Molteno Institute, University of Cambridge and Department of Pharmacology, Washington University School of Medicine, St Louis, Mo, U.S.A.

AND L. W. MAPSON
Low Temperature Station for Research in Biochemistry and Biophysics, University of Cambridge and Department of Scientific and Industrial Research

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The discovery of uridine 5′-diphosphoglucuronic acid (Dutton & Storey, 1954; Storey & Dutton, 1955) as the intermediate in the formation of 'ether-type' glucuronides by cell-free liver preparations has led to renewed interest in and a new approach to many problems of uronic acid metabolism in both plants and animals, e.g. the formation of other types of glucuronides (Isselbacher & Axelrod, 1955; Dutton, 1955, 1956), the synthesis of polysaccharides containing uronic acid (Glaser & Brown, 1955), and the biosynthesis of ascorbic acid (Isherwood, Chen & Mapson, 1954). In the liver of several animals, the synthesis of uridine diphosphoglucuronic acid is carried out by a diphospho-

pyridine nucleotide-linked dehydrogenase acting upon uridine diphosphoglucose (Strominger, Kalckar, Axelrod & Maxwell, 1954). In the present paper the purification and properties of a similar enzyme isolated from pea seedlings will be described, and its relation to uronic acid metabolism will be discussed.

EXPERIMENTAL

Materials. Uridine diphosphoglucose (UDPG) and diphosphopyridine nucleotide (DPN) were the same as those described by Strominger, Maxwell, Axelrod & Kalckar (1957). Uridine diphosphoglucuronic acid (UDPglucuronic acid) was isolated from rabbit liver according to Storey & Dutton (1955), or as the product of oxidation of UDPG by liver UDPG dehydrogenase (Strominger *et al.* 1957).

Enzyme assay. The assay for UDPG dehydrogenase, and units of measurement, were identical with those described by Strominger *et al.* (1957). In the present instance most measurements of the rate of DPN reduction (as the increase in optical density at 340 mμ) were carried out in a Hilger spectrophotometer, adapted with a pinhole diaphragm to reduce the size of the light beam. The cuvettes were fitted with an acrylic plastic insert to reduce their capacity. A volume of 0·5 ml. could be conveniently handled with the arrangement employed.

Paper electrophoresis. This was carried out as described by Markham & Smith (1951). Nucleotides were visualized by ultraviolet-absorption printing. Carbohydrates were detected by the aniline hydrogen phthalate spray (Partridge, 1949). In order to distinguish glucuronic acid and galacturonic acid the following procedure was employed. A small amount of conc. HCl was added to the dried sample, and taken to dryness over NaOH in a desiccator. The process was repeated a second time. This treatment converts glucuronic acid quantitatively into glucuronolactone. As galacturonic acid is unable to form a lactone, it remains in the acid form. Glucuronolactone and galacturonic acid were readily distinguished by electrophoresis in 0·05M-ammonium formate buffer, pH 3·4, on a strip of Whatman no. 3 filter paper, 57 cm. long. At a potential gradient of 20v/cm., galacturonic acid moves almost half the length of the paper in 1 hr. whereas glucuronolactone has no electrophoretic mobility.

RESULTS

Preparation of uridine diphosphoglucose dehydrogenase from pea seedlings

Extracts of dried peas, of acetone-dried powders of dried peas or of soaked pea seedlings all catalysed the reduction of DPN on the addition of UDPG. However, since the rate of reduction of DPN in the presence of UDPG was only 30% greater than the rate of reduction in its absence, confirmation that UDP glucuronic acid was formed was obtained by coupling the oxidation with *o*-aminophenol and the conjugating enzyme of washed guinea-pig liver microsomes (Dutton & Storey, 1954). The formation of *o*-aminophenylglucuronide from *o*-aminophenol, UDPG and DPN was dependent on both pea extract and guinea-pig liver microsomes; the pea extracts by themselves were incapable of catalysing the conjugation reaction with *o*-aminophenol.

The following procedure has led to a 1000-fold purification of the UDPG dehydrogenase from an extract of pea seedlings.

Step 1: *preparation of extract.* English Wonder peas were soaked for 12 hr. in tap water and then germinated for 1–3 days at 20°. The pea seedlings (600 g.) were ground in an end-runner mill, previously cooled at −20°, with 600 ml. of phosphate buffer (0·025M), pH 6·8, and a trace of silicone antifoam. The extract was pressed through muslin, and the pH adjusted to 5·5 with N-acetic acid. After centrifuging at 20000 *g* for 15 min., the clear supernatant was brought back to pH 6·8 with N-NaOH.

Step 2: *ammonium sulphate* 1. To the cooled supernatant (3°) was added solid $(NH_4)_2SO_4$ to 55% saturation (35 g./100 ml. of soln.). After 10 min. the supernatant was centrifuged, and the active precipitate dissolved in about 60 ml. of phosphate buffer (0·01M), pH 6·8, containing mM-ethylenediaminetetra-acetic acid (EDTA); in the subsequent steps in the purification EDTA was maintained at this level.

Step 3: *heat.* The dissolved precipitate was adjusted to pH 5·5 with N-acetic acid, heated rapidly to 50°, and maintained at this temperature for 1·5 min. After rapid cooling to 3° the precipitate was centrifuged and discarded.

Step 4: *ammonium sulphate* 2. The supernatant from the previous step was adjusted to pH 6·8 with NaOH, and the $(NH_4)_2SO_4$ concentration (about 7% saturation) determined on a conductivity meter. Saturated $(NH_4)_2SO_4$ solution was added until a saturation level of 21% was reached. The precipitate was centrifuged and discarded. The supernatant was made 32% saturated, centrifuged and the precipitate dissolved in about 40 ml. of phosphate buffer (0·01M), pH 6·6. This preparation could be stored at −20° for several weeks without significant loss of activity.

Step 5: *acetone.* The solution was dialysed for 4 hr. against 3 l. of phosphate buffer (0·01M), pH 6·6, at 1°. Longer dialysis caused a loss of activity, possibly due to the removal of $(NH_4)_2SO_4$, which was found to stabilize the enzyme. After dialysis the solution was centrifuged and the clear supernatant cooled in a brine bath kept at −3°. Chilled acetone was added, drop by drop down the side of the flask, until a concentration of 35% (v/v) was reached. After equilibration for 10 min. the precipitate was collected by centrifuging at 20000 *g* for 5 min. and discarded. Further acetone was added to bring the concentration to 50% (v/v). After 10 min. the precipitate was centrifuged, dried *in vacuo* and dissolved in 40 ml. of phosphate buffer (0·01M), pH 6·6, containing 1% of $(NH_4)_2SO_4$. This solution was dialysed at once, against 3 l. of phosphate buffer (0·01M), pH 6·6, for 3 hr., to remove traces of acetone. After centrifuging to clarify the enzyme, EDTA (mM) was added.

Step 6: *calcium phosphate gel.* The solution was treated with an equal volume of calcium phosphate gel (17·2 mg./ml.) prepared according to Keilin & Hartree (1938), left for 15 min. in the cold and centrifuged. The gel was washed with phosphate buffer (0·01M), pH 6·6, and the enzyme eluted with 15 ml. of phosphate buffer (0·1M), pH 6·6, for 15 min.; this was repeated, the two eluates being combined.

Step 7: *ammonium sulphate* 3. Saturated $(NH_4)_2SO_4$ solution was added until a saturation level of 42% was reached. The precipitate was centrifuged and dissolved in 10 ml. of phosphate buffer (0·01M), pH 6·6, and stored at −20°. After storage for several weeks a precipitate formed and was removed by centrifuging.

The recovery and specific activity of the enzyme at the different stages of the procedures are given in Table 1.

Table 1. *Purification of uridine diphosphoglucose dehydrogenase*

Stage of purification	Total units (units/100 g. fresh wt. of pea seedlings)	Total protein (mg./100 g. fresh wt. of pea seedlings)	Specific activity (units/mg. of protein)
Pea extract	16 300	3000	5
Ammonium sulphate 2	11 000	54	204
Acetone	9 100	19	480
Calcium phosphate	3 600	3	1250
Ammonium sulphate 3	3 400	0·7	4850

Table 2. *Stoicheiometry of reduction of diphosphopyridine nucleotide*

Reduction of DPN was followed by measuring the increase in absorption at 340 mμ in a Hilger spectrophotometer. The reaction mixture contained enzyme, DPN (0·5 μmole) and varying amounts of UDPG in a total volume of 0·5 ml. of 0·1 M-glycine buffer, pH 8·9.

UDPG added (μmole)	DPN reduced (μmole)	DPN reduced (moles/mole of UDPG added)
0·01	0·022	2·2
0·02	0·036	1·80
0·02	0·039	1·95
0·02	0·035	1·75
0·02	0·037	1·85
0·04	0·074	1·85
0·04	0·075	1·88

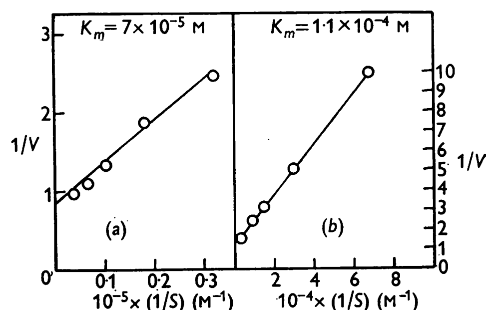

Fig. 2. Plots to determine the Michaelis constants for (*a*) UDPG, (*b*) DPN. Velocity V is expressed as ΔE_{340}/min. Measurements were made in 0·1 M-glycine buffer, pH 8·8, at 17°.

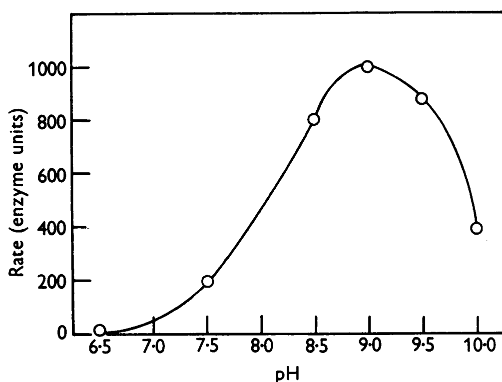

Fig. 1. Activity of uridine disphosphoglucose dehydrogenase as influenced by pH. The reaction was studied in a mixed buffer consisting of phosphate and glycine, each 0·1 M at the pH indicated; temp. 17°.

Properties of the purified enzyme

Stoicheiometry of diphosphopyridine nucleotide reduction. With the most purified enzyme 1·8–1·9 moles of DPN were reduced/mole of UDPG added (Table 2).

Reversibility. When UDPglucuronic acid and reduced DPN were incubated with enzyme at pH 7·2 or 8·8, no enzymic oxidation of reduced DPN could be observed.

pH *Optimum.* A mixed buffer consisting of phosphate and glycine, each 0·1 M final concentration, adjusted to the desired pH with HCl or NaOH, was used throughout the H^+-ion concentration range studied. The pH optimum for the purified enzyme was found to be 9·0 (Fig. 1).

Substrate affinity constants. The Michaelis constants for UDPG and DPN, determined by the method of Lineweaver & Burk (1934), measured in 0·1 M-glycine at pH 8·8, were 7×10^{-5} M and $1{\cdot}15 \times 10^{-4}$ M respectively (Fig. 2).

Specificity. The following substances were not oxidized by the purified UDPG dehydrogenase with DPN as electron acceptor: uridine diphosphogalactose, uridine diphosphoacetylglucosamine, uridine diphosphoacetylgalactosamine, guanosine diphosphomannose, glucose, α-glucose 1-phosphate and ethanol. With crude preparations of the enzyme uridine diphosphogalactase was oxidized at the same rate as UDPG.

The two following UDPG analogues, prepared as intermediates in the synthesis of UDPG by Michelson & Todd (1956), were kindly supplied by Dr A. M. Michelson. The 2′:3′-*OO*-dibenzyl ether of UDPG was not detectably oxidized. However, a compound presumed to be the 2′:3′-*OO*-diacetyl derivative of UDPG was oxidized at about one-third of the rate of UDPG. This compound is as yet incompletely characterized. In neutral ethanol–ammonium acetate (Paladini & Leloir, 1952) the

compound has a mobility much greater than that of UDPG, and no UDPG could be detected in the preparation.

With UDPG as substrate, no reduction of triphosphopyridine nucleotide could be detected.

Sulphydryl requirement. The enzyme was 55% inhibited by μM-*p*-chloromercuribenzoate and this inhibition was not reversed by 0·01 M-cysteine. Oxidized glutathione (0·01 M) inhibited the enzyme 42% when incubated at 20° for 1·5 hr., and this inhibition was increased to 65% on leaving for 18 hr. No reactivation could be obtained with reduced glutathione (0·05 M). Iodosobenzoic acid (mM) inhibited 34% when incubated with the enzyme for 10 min. at 20°, but no inhibition was produced by iodoacetate under similar conditions.

An activating effect of cysteine or thioglycollate was observed only with low concentrations of enzyme. With an enzyme preparation (activity 120 units) no effect of cysteine or thioglycollate was observed. With one-fourth this amount of enzyme the rate of reaction was increased, after the addition to the assay mixture of cysteine (0·01 M) and thioglycollate (0·01 M) by 2 and 2½ times respectively.

Other inhibitors. Sodium pyrophosphate, when added to the enzyme before DPN at levels of 0·02 M and 0·004 M, inhibited to the extent of 40 and 17% respectively, but no inhibition occurred when DPN and pyrophosphate were added together and the reaction was started by the addition of enzyme.

Sodium fluoride (mM), when incubated with the enzyme at 20° for 10 min., produced 50% inhibition, but potassium cyanide in a similar concentration and under similar conditions had no effect.

Stability. In the early stage of purification the enzyme was unstable but became more stable as it was purified. The preparations of greater purity have been freeze-dried without any loss of activity, and no loss occurred during 3 months' storage of the dried preparation at −20°. A freeze-dried preparation was sent by air mail from Cambridge, England, to St Louis, Mo, U.S.A., without loss of activity.

Isolation and identification of UDPglucuronic acid as the product of the enzymic oxidation of uridine diphosphoglucose

When the purified enzyme from pea seedlings was incubated with UDPG, DPN, *o*-aminophenol and washed guinea-pig liver microsomes, formation of *o*-aminophenylglucuronide occurred (Table 3). Presumably therefore the product of oxidation of UDPG by the pea enzyme was UDPglucuronic acid.

To isolate the product, 0·8 μmole of UDPG, 3 μmoles of DPN and 1500 units of purified enzyme were incubated in 0·5 ml. of glycine buffer (0·1 M), pH 8·8. After 30 min. the pH was adjusted to 5 with 2 N-acetic acid, and the solution was streaked side to side about 9 cm. from one end on a strip of Whatman no. 3 filter paper, 2·5 cm. × 57 cm., previously wetted with ammonium acetate buffer (0·1 M), pH 5·2, containing EDTA (0·001 M). Electrophoresis was carried out at a potential gradient of about 20 v/cm. for 2 hr. in the

Table 3. *Formation of* o-*aminophenylglucuronide by pea or liver preparations in the presence of guinea-pig liver microsomes*

Each incubation mixture contained phosphate buffer (0·5 M), pH 7·6, 50 μl. of $MgCl_2$ (0·05 M), *o*-aminophenol (50 μl., 6 mg./25 ml.) and microsomes from 100 mg. of guinea-pig liver (100 μl.). To some incubations, UDPG (0·095 μmole), DPN (0·5 μmole) and UDPG dehydrogenase (from liver or from peas) were added. To other incubations UDPglucuronic acid (prepared from UDPG by means of UDPG dehydrogenase from liver or from peas) was added. The total volume was adjusted to 300 μl. and the samples were incubated for 60 min. at 38°. Blanks were obtained by omitting the nucleotides, and calculations were with reference to internal standards of *o*-aminophenylglucuronide. The reactions were stopped by the addition of 300 μl. of 2 M-phosphate–1·25 M-trichloroacetic acid, pH 2·25. The preparations were centrifuged and to 300 μl. of supernatant, 100 μl. of 0·05% $NaNO_2$, 100 μl. of 0·5% ammonium sulphamate and 100 μl. of 0·1% naphthylethylenediamine dihydrochloride were added. After 2 hr. in the dark, the colour was read at 535 mμ.

Nucleotide added	Enzyme added	*o*-Aminophenylglucuronide formed (μmole)	Conversion of uridine nucleotide into glucuronide (%)
1. UDPG, DPN	Pea UDPG dehydrogenase (50 units)	0·042	44
2. UDPG, DPN	Liver UDPG dehydrogenase (205 units)	0·049	52
3. UDPglucuronic acid (0·029 μmole) prepared with pea UDPG dehydrogenase	—	0·016	55
4. UDPglucuronic acid (0·018 μmole) prepared with liver UDPG dehydrogenase	—	0·013	72

Quantitative conversion of UDPG or of UDPglucuronic acid into the glucuronide was not observed (cf. Strominger *et al.* 1957); no explanation for this has been obtained.

apparatus described by Markham & Smith (1951). The resulting ultraviolet-absorbing bands (Fig. 3*a*) were eluted and transferred directly to another sheet of filter paper, and chromatographed (Fig. 3*b*) in neutral ethanol–ammonium acetate (Paladini & Leloir, 1952). Band 1 had the R_F expected for UDPglucuronic acid in this solvent (Smith & Mills, 1954) and was further identified as UDPglucuronic acid by (i) the formation of *o*-aminophenylglucuronide (Table 3), with guinea-pig liver microsomes (Levvy & Storey, 1949); (ii) the characteristic ultraviolet-absorption spectrum of a uridine nucleotide in acid and alkali; (iii) the content of glucuronic acid (0·86 μmole/μmole of uridine) estimated by the carbazole reaction (Dische, 1950).

Further identification of the glucuronic acid moiety was obtained by boiling a nucleotide sample in 0·1 N-HCl for 5 min. to hydrolyse the glycoside band, and then carrying out the differentiation of glucuronic and galacturonic acids described in Methods. No trace of galacturonic acid was observed.

Band 2 (Fig. 3*a*) was found to be due to the presence of another uridine nucleotide, which was chromatographically identical with UDPG; it may represent a small amount of unoxidized nucleotide. Bands 3, 4 and 5 were due to adenine-containing compounds representing oxidized and reduced DPN and their degradation products. Band 6 was contributed by the enzyme preparation.

DISCUSSION

These experiments have established the presence in pea seedlings of an enzyme uridine diphosphoglucose dehydrogenase, which catalyses the formation of uridine diphosphoglucuronic acid. It seems likely that UDPglucuronic acid may be an intermediate in the biosynthesis of those simple glucuronides (Marsh & Levvy, 1956) and of glucuronic acid-containing polysaccharides (Whistler & Smart, 1953), which occur in peas and other plants.

The possible relation of this biosynthetic mechanism to ascorbic acid formation was our primary reason for undertaking this investigation. It has been established that ascorbic acid is formed in animals from a hexose precursor without degradation and recombination of the carbon chain (Jackel, Mosbach, Burns & King, 1950). Carbon atom 6 of D-glucose becomes C-1 of L-ascorbic acid (Horowitz, Doerschuk & King, 1952; Horowitz & King, 1953). The pathway from hexose to ascorbic acid appears to lead through a uronic acid intermediate. However, in animals the intermediate appears to be a derivative of glucuronic acid (Isherwood *et al.* 1954), whereas in plants a derivative of galacturonic acid has been implicated (Mapson, Isherwood & Chen, 1954). A major point of interest in the present study was therefore the specificity of the uridine nucleotide dehydrogenase of pea seedlings. Like its counterpart in liver, UDPG dehydrogenase of pea seedlings has a strict specificity for the configuration C-4 of the hexose and is incapable of oxidizing uridine diphosphogalactose. So far, attempts to demonstrate the synthesis of L-ascorbic acid from UDPG or from UDPglucuronic acid by pea preparations have been unsuccessful.

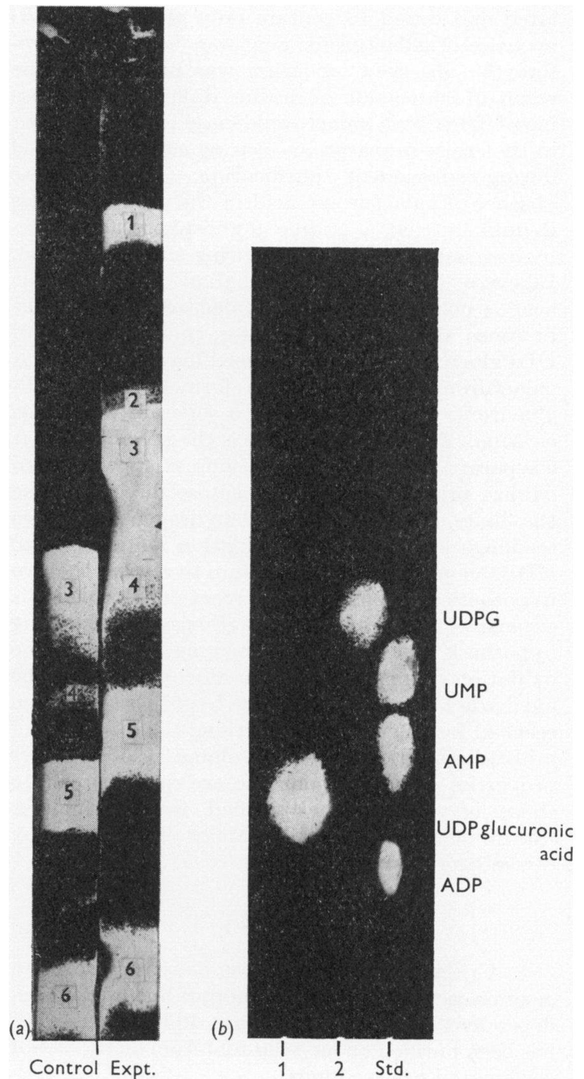

Fig. 3 (*a*). Separation of UDPglucuronic acid by paper electrophoresis from a digest containing pea UDPG dehydrogenase, UDPG and DPN; UDPG was omitted from the control digest. Electrolyte, ammonium acetate buffer (0·1 M), pH 5·2; potential, 20 v/cm.; duration, 2 hr. Band 1, UDPglucuronic acid; band 2, UDPG; bands 3, 4, 5, oxidized and reduced DPN and degradation products; band 6, contributed by enzyme preparation. (*b*) Identification of UDPglucuronic acid by paper chromatography. Bands 1 and 2 (Fig. 3*a*) were eluted and chromatographed with 7·5 vol. of 95% ethanol +3 vol. of ammonium acetate (M), pH 7·5. Standards: UMP, uridine 5′-phosphate; AMP, adenosine 5′-phosphate; ADP, adenosine diphosphate.

With the more impure preparations of the enzyme it was observed that uridine diphosphogalactose was oxidized at the same rate as UDPG. The nucleotide product of the oxidation was isolated and found to contain only glucuronic acid; no trace of galacturonic acid was detected. Therefore the observed oxidation was presumably the result of conversion of uridine diphosphogalactose into UDPG by a galactowaldenase enzyme present in the crude preparations, but which was removed during subsequent purification. The complete absence of galacturonic acid in the product makes it unlikely that a uridine diphosphogalactose dehydrogenase was removed during the purification. Likewise, it may be inferred that UDPglucuronic acid is not a substrate for galactowaldenase. The presence of a mechanism for the formation of UDPglucuronic acid in pea seedlings suggests that galacturonic acid might be formed from UDP-glucuronic acid by means of a separate waldenase enzyme. Alternatively, there is the possibility that a separate dehydrogenase enzyme exists which can oxidize uridine diphosphogalactose directly. Like the liver enzyme, UDPG dehydrogenase of pea seedlings catalyses the oxidation of UDPG to UDPglucuronic acid. The failure to resolve the two hypothetical steps of the reaction during this extensive purification strengthens the working hypothesis that a single enzyme catalyses the oxidation of an alcohol to an aldehyde and of the aldehyde to an acid. The same hypothesis has been reached for the enzyme catalysing the oxidation of L-histidinol to L-histidine (Adams, 1955). Other properties of the pea enzyme are quite similar to those of the enzyme purified from calf liver (Strominger *et al.* 1957; Maxwell, Kalckar & Strominger, 1956).

SUMMARY

1. Uridine diphosphoglucose dehydrogenase, an enzyme catalysing a diphosphopyridine nucleotide-dependent oxidation of uridine diphosphoglucose, has been purified about 1000-fold from an extract of germinated pea seedlings.

2. Some of the properties of the purified enzyme have been examined. Like its counterpart in liver, the purified enzyme catalyses a two-step oxidation of uridine diphosphoglucose but does not oxidize uridine diphosphogalactose.

3. The product of the oxidation was isolated and identified as uridine diphosphoglucuronic acid.

4. The relationship of this enzymic mechanism to uronic acid metabolism in plants has been discussed.

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