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PURIFICATION OF A SPECIFIC D-APIITOL DEHYDROGENASE FROM A *MICROCOCCUS* ISOLATED FROM THE SURFACE OF GERMINATING PARSLEY SEEDS

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SUMMARY

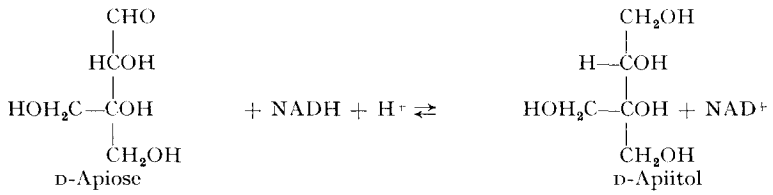
The branched-chain sugar D-apiose was oxidized to CO_2 by both *Lemna minor* and a bacterium which was isolated from the surface of germinating parsley seeds. An inducible dehydrogenase which catalyzed the interconversion of D-apiose and D-apiitol was detected in extracts of this microorganism. The enzyme which was purified about 200-fold was specific for D-apiose and D-apiitol. It oxidized *myo*-inositol and *meso*-erythritol slowly, but it was completely inactive with all of the other sugars and polyols tested. The enzyme was specific for NAD^+ and NADH as electron acceptor and donor, respectively. $NADP^+$, NADPH, ascorbate, FAD, $FADH_2$, cytochrome *c* and ferricyanide were inactive. The K_m for D-apiitol was $1.16 \cdot 10^{-2}$ M, D-apiose was $7.14 \cdot 10^{-2}$ M, NAD^+ was $3.5 \cdot 10^{-4}$ M and NADH was $1.5 \cdot 10^{-5}$ M. At high concentrations NADH inhibited the reaction. The molecular weight of the dehydrogenase determined by chromatography on Sephadex 200 and sucrose density centrifugation was 110 000. The products of the reaction were characterized by paper chromatography, periodate oxidation and gas chromatography of acetylated derivatives. A colorimetric method for the quantitative determination of small amounts of D-apiose was also developed during the course of this study.

INTRODUCTION

Many inducible enzymes which catalyze the oxidation of a wide variety of commonly occurring sugars and sugar alcohols have been obtained from microorganisms[1–3]. Bacteria also have the unique ability to metabolize more unusual sugars which are found as components of structural material in various plant tissues. D-Apiose (3-*C*-hydroxymethyl-D-tetrose) one of the best known branched-chain sugars is widely distributed in the cell wall polysaccharides of seaweeds, *Lemna minor* and certain other plants. In parsley, it is present as a component of the flavone, apiin.

Very little is known about the catabolism and utilization of this uncommon

carbohydrate in plants and microorganisms. Accordingly, it appeared of interest to examine the metabolism of this sugar, in order to determine whether it could be utilized by some microorganism or plant. A Gram-negative coccus isolated from the surface of germinating parsley seeds by enrichment culture oxidized D-apiose to CO_2 and utilized this sugar as a sole source of carbon for growth. An investigation to establish the nature of the intermediate reactions involved in the oxidation of this sugar to CO_2 was initiated. A specific inducible D-apiitol dehydrogenase dependent on NAD^+ for activity was isolated from extracts of this bacteria in order to establish a relationship between the properties of the enzyme and its function in the utilization of the sugar. The kinetic properties of the enzyme were examined. The readily reversible reaction catalyzed by this dehydrogenase is shown in the following equation. Some of the results of this study have been briefly reported in a preliminary communication[4].



EXPERIMENTAL PROCEDURES

Isolation of microorganism

Parsley seeds were germinated by placing them between two sheets of wet filter paper for 12 days. Bacteria which were growing on the top of the germinated seeds were suspended in minimal salts medium and an organism capable of utilizing D-apiose was isolated by streaking this solution on 0.5% agar plates containing minimal salts medium and 0.5% D-apiose. A culture of the Gram-negative *micrococcus* was maintained on nutrient agar slants containing 0.5% D-apiose.

Cultivation of bacteria and induction of D-apiitol dehydrogenase

The bacteria were grown on glucose, since we were able to show that they could later be incubated with D-apiose to induce the formation of the same amount of D-apiitol dehydrogenase as was present in cells grown on D-apiose. The bacteria were grown in a medium containing the following ingredients in g/l; K_2HPO_4, 7.0; KH_2PO_4, 3.0; $(NH_4)_2SO_4$, 1.0; $MgSO_4$, 1.0; glucose, 5.0. The flasks were shaken at 25 °C for 24 h. The initial culture which contained 250 ml of medium in a 500-ml erlenmeyer flask was used to inoculate six 2000-ml erlenmeyer flasks each of which contained 1200 ml of sterile medium. After 24 h the cells were harvested by centrifugation at 30 000 × *g* for 20 min and they were washed twice with 10 vol. of the sterile medium lacking glucose. The final yield was 11.6 g dry wt of cells. Growth was measured by determining the absorbance at 660 nm on samples diluted to give readings of less than 0.5 and cell yield was estimated by drying a washed sample to constant weight at 104 °C.

To induce the formation of D-apiitol dehydrogenase the washed cells were suspended in an equal volume of minimal salts medium and they were shaken for

24 h at 30 °C. Then 0.4 vol. of the starved cell suspension was mixed with 0.5 vol. of minimal salts medium containing 3% casein hydrolysate (Casamino Acids, Difco) and 40 μmoles/ml of D-apiose. This mixture was shaken for 24 h at 30 °C. More D-apiose (40 μmoles/ml) was added after 12 h. The cells were harvested by centrifugation and they were washed 3 times with 10 vol. of distilled water and the final pellet was suspended in 2 vol. of 0.05 M Tris–HCl, pH 7.5.

Enzyme assays

D-Apiitol dehydrogenase was assayed spectrophotometrically. The absorbance change at 340 nm resulting from the reduction of NAD^+ in the presence of D-apiitol or the oxidation of NADH in the presence of D-apiose was measured. The standard reaction mixture for measuring the rate of oxidation of D-apiitol contained in 0.5 ml; 80 mM glycylglycine–NaOH (pH 9.5), 20 mM D-apiitol, 1 mM NAD^+ and the enzyme. The reduction of D-apiose was measured in a 0.5-ml reaction mixture which contained 80 mM glycylglycine–NaOH (pH 7.5), 20 mM D-apiose, 0.1 mM NADH and enzyme. The change in absorbance at 340 nm with time was linear with respect to the amount of enzyme added when less than 10% of the substrate was utilized. One unit of activity in these assays is defined as the amount of enzyme required to oxidize 1 μmole of D-apiitol per min or the amount necessary to reduce 1 μmole of D-apiose per min. Specific activity is expressed as units per mg of protein.

Materials and methods

Enzyme grade Tris (General Biochemicals) and $(NH_4)_2SO_4$ (Mann) were used. Acetylated sugar derivatives were provided by Dr Derek Horton of the Ohio State University. D-Apiose was prepared from acid hydrolysates of *Lemna minor* and *Zostera marina* L., by the procedure of Picken and Mendicino[5]. The sugar was also isolated from parsley seeds by a modification of the procedure of Gupta and Seshadri[6,7]. D-[U-^{14}C]Apiose was prepared by incubating 10 g wet wt of *Lemna minor* with 25 μCi of $^{14}CO_2$ under conditions of continuous light for 2 days. Labeled D-apiose with a specific activity of $6.1 \cdot 10^5$ cpm/μmole was isolated by a procedure described in a previous report[6]. D-Apiitol and D-[U-^{14}C]apiitol were synthesized by reducing the sugars with sodium borohydride[5]. 40 mg of sodium borohydride and 90 mg of D-apiose were dissolved in 30 ml of water and the mixture was left at room temperature for 2 h. The pH was then adjusted to 6 with 1 M acetic acid and the solution was passed through a column of Dowex 50(H^+). The D-apiitol was isolated by paper chromatography with 2 solvent systems[9,10]. Radioactivity was measured in a scintillation counter with the solvent system of Patterson and Greene[8]. Analysis of sugars by paper chromatography was carried out with Whatman No. 3 MM paper using the following solvent systems with the volumes of solvents indicated in parenthesis: ethyl acetate–pyridine–water (12:5:4, by vol.)[10]; 2-propanol–water (9:1, by vol.)[9]; 1-butanol–ethanol–water (52:32:16, by vol.)[10]; 1-butanol–pyridine–water (6:4:3, by vol.)[11]; 1-propanol–ethylacetate–water (7:1:2, by vol.)[12]. The $AgNO_3$ and alcoholic NaOH reagent[13], anisidine[14] and benzidine–trichloroacetic acid spray reagents[15] were used to detect sugars on paper chromatograms. Apiose reacts with the latter reagent to yield a yellow area with an intense white fluorescence under ultraviolet light.

Two colorimetric assays were used to determine the concentration of D-apiose[6,16,17]. D-Apiitol was assayed spectrophotometrically by quantitative periodate

oxidation[18,19]. The amount of periodate ion was measured at 222.5 nm[18,20]. Formaldehyde was determined by the chromotropic acid method[21]. Glycolic acid was measured by the procedure of Calkins[22] and formic acid was assayed by the method of Gabriel[23]. Reduction with borohydride was carried out according to Abdel-Akher *et al.*[24]. Protein was determined by biuret[25] and spectrophotometric procedures[26]. Molecular weight determinations on Sephadex G-200 columns were carried out according to Andrews[27]. Sucrose density centrifugation was performed according to Martin and Ames[28].

RESULTS

Determination of D-*apiose by a sensitive colorimetric assay*

In order to measure the concentration of D-apiose in growth medium and crude enzyme preparations a quantitative procedure for the estimation of this sugar was developed. We noticed that this pentose, which has a 3-C-hydroxymethyltetrose structure, reacted in the specific fructose–H_2SO_4 test for tetroses[19,29]. The absorption spectra of the products formed from D-apiose, D-apiose-1-*P*, apiosides and D-erythrose in this assay are shown in Fig. 1. The sample in 0.5 ml and 0.5 ml of 1% fructose

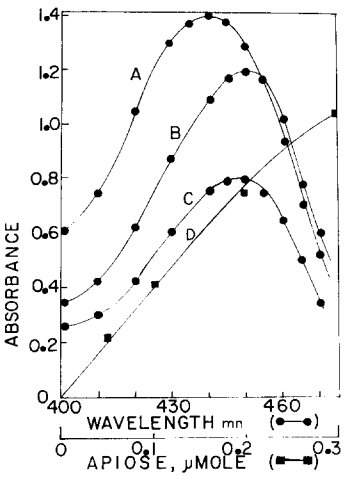


Fig. 1. The absorption spectra of D-apiose, D-apiose-1-*P* and D-erythrose in the fructose–H_2SO_4 assay. Curve A was obtained with 0.3 μmole of D-erythrose; Curve B, with 0.3 μmole of α-D-apio-L-furanosyl-1-*P*. Curve C, with 0.2 μmole of D-apiose or the apiosides, furcation [D-apiosyl)-1 → 6(β-D-glucosyl)]-*P*-vinylphenol or apiin 7-*O*-[β-D-apiosyl)-1 → 2(β-D-glucosyl)]apigenin. The proportional relation of the concentration of D-apiose to absorbance at 450 nm is shown in Curve D.

were cooled in ice bath and 4.5 ml of 7.5 M H_2SO_4 were added and the solution was mixed. It was then heated at 100 °C for 3 min and quickly cooled to 26 °C. After 2 h its absorption was measured. Under these conditions D-apiose and its derivatives all showed an absorption maximum at 450 nm, whereas D-erythrose, a typical tetrose, had a maximum at 440 nm. The color yield at 450 nm was directly proportional to the concentration of D-apiose up to about 0.3 μmole. The molar extinction coefficient for D-apiose was calculated to be $19.5 \cdot 10^3$. The esters and glycoside derivatives of

D-apiose are labile to acid[6,19] and they would be completely hydrolyzed in this assay mixture. When the volume of the reaction mixture is halved, as little as 0.01 μmole of D-apiose can be detected. The color yield in this assay could also be increased by adding cysteine[29].

The conversion of D-*apiose to* CO_2 *by bacteria and plant tissues*

Although large amounts of D-apiose are present in some higher plants, there is no information available on mechanism of utilization of this sugar. Accordingly, studies were undertaken to examine the metabolism of this sugar in bacteria and plants such as *Lemna minor* where it occurs as a constituent of cell wall pectins.

A microorganism isolated from the surface of germinating parsley seeds used D-apiose as the sole source of carbon for growth and it converted the sugar to CO_2. This *micrococcus* also grew on hydrocarbons, such as dodecane. The rate of formation of $^{14}CO_2$ from D-[U-^{14}C]apiose was examined. About 36.5 mg dry wt of the bacteria which had been induced in the presence of D-apiose was suspended in 14 ml of medium containing 43 μmoles of D-[U-^{14}C]apiose ($3 \cdot 10^5$ cpm/μmole) in a sealed flask. The $^{14}CO_2$ evolved was continuously monitored by passing air through the flask and into an alkali trap. The initial rate of formation of $^{14}CO_2$ was 10 μmoles/h and the rate of utilization of D-apiose, estimated by assaying aliquots removed from the medium, was 2.1 μmoles/h. Since 1 mole of D-apoise would yield 5 moles of CO_2, these results indicated that most of the sugar was being completely oxidized to CO_2. About 95% ($1.23 \cdot 10^7$ cpm) of the sugar was converted to CO_2 in 24 h. These results indicate that this microorganism contains genetic information for the induced formation of an enzyme system capable of converting D-apiose to CO_2.

Experiments designed to study the incorporation of D-[U-^{14}C]apiose into cell wall polysaccharides of *Lemna minor* yielded unexpected results which showed that this tissue could also convert D-apiose to CO_2. *Lemna minor* (5 g wet wt) were incubated with D-[U-^{14}C]apiose (2.1 μmoles, $1 \cdot 10^6$ cpm/μmole) under sterile conditions in 5 ml of the medium of Norris *et al.*[30] in a closed flask under continuous light. The formation of $^{14}CO_2$ was measured and plants were harvested after 24 h and 48 h. Cell walls, cell wall polysaccharides and D-apiose were isolated from these samples[6]. D-[U-^{14}C]Apiose was not efficiently utilized for the synthesis of cell wall polysaccharide. Instead about 51% of the D-apiose was oxidized to CO_2 in 24 h. Less than 0.01% of the labeled sugar was found in cell wall polysaccharide even after 48 h. [U-^{14}C]-Glucose is converted to the D-apiose moiety of cell wall polysaccharides under these conditions[5]. After 48 h almost all of the D-apiose was assimilated and converted to CO_2 or other metabolic intermediates. The oxidation of this sugar to CO_2 in this tissue may be important in the degradation of free D-apiose after hydrolytic cleavage of the sugar during turnover of cell wall constituents. Other plants including lettuce, carrots, spinach were unable to utilize D-apiose.

Influence of D-*apiose on the rate of induction of* D-*apiitol dehydrogenase*

The characterization of enzymes involved in the conversion of D-apiose to CO_2 was further examined in the bacterium isolated from the surface of germinating parsley seeds. The concentration of D-apiose effected the rate of induction of D-apiitol dehydrogenase. The results summarized in Table I show that no enzyme was formed in the absence of D-apiose and that the amount of enzyme formed increased with

TABLE I

INFLUENCE ON THE CONCENTRATION OF D-APIOSE ON THE RATE OF INDUCTION OF D-APIITOL DEHYDROGENASE

The incubation mixtures contained in a final volume of 10 ml; 36.6 mg dry wt .of bacteria, grown in a minimal salts medium on D-glucose and starved for 24 h, 8 ml of minimal salts medium and the amount of D-apiose indicated. The reaction mixture was shaken for 0.5, 1, and 2 h at 25 °C and the cells were collected by centrifugation at 35 000 × *g* for 10 min. The pellet was washed three times with 10 vol. of 0.05 M Tris–HCl, pH 7.5. The final precipitate was suspended in 5 ml of buffer and it was sonicated for 5 min. The preparation was then centrifuged at 100 000 × *g* for 1 h to remove a small inactive precipitate and the D-apiitol dehydrogenase activity was measured using the standard assay for the reduction of D-apiose. The total volume of the extract was 4.6 ml in each case. The D-apiitol dehydrogenase activity is expressed in μmoles/min.

Time (h)	D-*Apiose added (μmoles)*						
	0	*6.4*	*19*	*38*	*129*	*258*	*516*
0.5	0	0.003	0.005	0.007	0.010	0.015	0.028
1.0	0	0.005	0.017	0.034	0.072	0.085	0.088
2.0	0	0.012	0.055	0.080	0.087	0.087	0.089

increasing concentrations of D-apiose. There was a lag period of about 30 min. The amount of enzyme formed after 2 h increased with respect to the concentration of D-apiose up to 12.9 μmoles/ml. The maximum amount of activity consistantly observed was between 0.13 and 0.089 μmole/min per 36.6 mg dry wt of bacteria.

Effect of the concentration of D-*apiose on the rate of utilization of substrate by fully-induced cells*

Bacteria were grown on D-glucose, harvested and washed with sterile medium. The cells were starved and then induced with D-apiose as described in Experimental Procedures. Fully-induced cells 18.3 mg dry wt in each case, were washed with sterile medium and they were suspended in 2 ml of sterile medium containing varying amounts of D-apiose. The flasks were shaken at 25 °C and aliquots of 0.1 ml were removed at various times and they were diluted to 1.0 ml with distilled water, centrifuged at 3 °C and assayed for D-apiose. At concentrations of 0.006, 0.013, 0.026, 0.064, 0.128 and 0.192 M the rate of utilization of D-apiose was 0.01, 0.017, 0.030, 0.090, 0.110 and 0.130 μmole/min per 18.3 mg of cells, respectively. The very strong dependence of the uptake and utilization of the sugar on its concentration in the medium indicates that there is probably no system for the active transport of this sugar into the bacteria. The K_m of D-apiitol dehydrogenase for D-apiose is about 0.071 M, and it is likely that this factor is the rate limiting component in the utilization of the sugar.

Purification and properties of D-*apiitol dehydrogenase*

Crude extracts were prepared from 11.6 g dry wt of cells grown and fully induced with D-apiose as described in Experimental Procedures. All of the procedures were carried out at 3 °C. The cells were suspended in 250 ml of 0.05 M Tris-HCl, pH 7.5, and they were broken in a French press. The resulting snspension was centrifuged at 34 000 × *g* for 20 min and the supernatant was centrifuged again at

TABLE II

ISOLATION OF D-APIITOL DEHYDROGENASE FROM INDUCED CELLS

The enzyme was assayed by the standard procedure described in the text.

Fraction	*Volume (ml)*	*Protein (mg/ml)*	*Total activity (units)*	*Specific activity (units/mg)*
1. Crude extract	183	458	36.6	0.00043
2. Treatment with streptomycin sulfate	175	64	27.9	0.00248
3. Treatment with calcium phosphate gel	160	48	24.0	0.0032
4. Precipitation with ethanol	40	58	24.0	0.0103
5. Chromatography on DEAE-cellulose	35	23	19.5	0.024
6. Chromatography on Sephadex G-200	32	4	13.0	0.102

100 000 × *g* for 1 h. The D-apiitol dehydrogenase activity was present in the supernatant solution (Fraction 1, Table II).

About 11.3 ml of 20% streptomycin sulfate were added to 183 ml of the crude extract. The mixture was stirred for 15 min and an inactive precipitate was removed by centrifugation and discarded. Excess streptomycin sulfate was removed by passing the supernatant solution through a small Rexyon 102(H^+) column (2.2 cm × 3 cm) and the eluant was assayed (Fraction 2).

Calcium phosphate gel (0.5 mg per mg protein) prepared by the method of Keilin and Hartree[31] and aged for 2 weeks was slowly added to the solution from the previous step and the suspension was stirred for 30 min and centrifuged.

The supernatant solution (Fraction 3) was diluted with 2 vol. of 0.05 M Tris–HCl, pH 7.5. This solution, 480 ml, was cooled to 0 °C and 480 ml of 95% ethanol at −20 °C were slowly added with stirring. Afterwards the precipitate was collected by centrifugation and it was dissolved in 35 ml of 0.05 M Tris–HCl, pH 7.5. Some insoluble material was removed by centrifugation and the clear supernatant solution was assayed (Fraction 4).

The enzyme was then adsorbed to a DEAE-cellulose column (2.2 cm × 7 cm). It was eluted from the column with a solution formed as a linear gradient from 250 ml of 0.03 Tris–HCl, pH 7.5, in the mixing chamber and 250 ml of the buffer with 0.5 M NaCl in the reservoir. The D-apiitol dehydrogenase was found in a protein peak which emerged from the column after about 100 ml of the eluting solution had been collected. The fractions containing activity were pooled and concentrated by the use of a Diaflo membrane. The solution was dialyzed against 1 l of 0.05 M Tris–HCl, pH 7.5 (Fraction 5, Table II).

The enzyme in 5-ml fractions was applied to 7 Sephadex G-200 columns (2.2 cm × 35 cm) previously equilibrated against 0.05 M Tris–HCl, pH 7.5. The enzyme was eluted with this buffer and the activity was found in the first protein peak after about 80 ml of buffer had been collected. The active fractions from each column were combined and the solution was concentrated and dialyzed as described previously (Fraction 6). The results obtained in a typical preparation are summarized in Table II. The enzyme was purified 200-fold from the crude extract with a yield of about 30%. The preparation remained active when it was frozen at −20 °C for at least several weeks. The enzyme had a broad pH profile with an optimum at 7.5 in

0.08 M Tris–HCl or 0.08 M glycylglycine buffer when assayed in the direction of D-apiose reduction. A pH optimum of 10 was observed when the enzyme activity was measured in the direction of D-apiitol oxidation using 0.08 M glycine–NaOH buffer.

The molecular weight of the purified enzyme, determined by sucrose density centrifugation, was 115 000. Samples were spun in a SW-39 rotor for 8 h at 37 000 rev./min. The rate of sedimentation of the dehydrogenase was compared with those of alcohol dehydrogenase (150 000) and catalase (250 000) as described by Martin and Ames[28].

The elution volume of D-apiitol dehydrogenase on Sephadex G-200 columns (2.2 cm × 27 cm) was 69 ml. These columns had bed volumes of 103 ml and void volumes of 41 ml. The molecular weight of the enzyme was measured by using lactate dehydrogenase, hexokinase and aldolase as standards. The molecular weight of D-apiitol dehydrogenase based on plots of the log of the molecular weight against elution volume obtained from several determinations according to the method of Andrews[27] was calculated to be 110 000 ± 5000. The value obtained by this procedure was in good agreement with the molecular weight determined by sucrose density gradient centrifugation.

Specificity of D-*apiitol dehydrogenase*

The enzyme was highly specific for D-apiose. No activity was detected in the standard assay system when the same concentration of D-glucose, D-fructose, D-mannose, D-glucosamine, D-galactose, D-ribose, D-xylose, L-rhamnose, L-fucose, D-fucose, D-arabinose, L-arabinose, D-erythrose and D-glyceraldehyde was used. The enzyme was also specific for D-apiitol with few possible exceptions. D-Ribitol, D-sorbitol, D-arabitol, L-arabitol, D-mannitol, D-xylitol and D-galactitol were inactive when tested in the standard assay. The activity obtained with *myo*-inositol and *meso*-erythritol was 38 and 13%, respectively, of that obtained with D-apiitol.

The dehydrogenase was specific for NAD^+ as the electron acceptor and NADH as the electron donor. $NADP^+$, NADPH, ascorbate, FAD, $FADH_2$, cytochrome *c* and ferricyanide were all inactive.

Characterization of the products of the reaction

For the large scale preparation of the reduced product 100 μmoles of D-[U-^{14}C]-apiose ($6.1 \cdot 10^5$ cpm/μmole), 5 units of D-apiitol dehydrogenase, 100 μmoles of glycylglycine–NaOH, pH 7.5, 0.5 μmole of NADH, a NADH-regenerating system containing 10 μmoles of $MgCl_2$, 200 μmoles of glyceraldehyde-3-*P*, 5 units of glyceraldehyde-3-*P* dehydrogenase and 200 μmoles of P_i in a volume of 10 ml were incubated for 2 h at 30 °C. The reaction mixture was deionized by passing it through a Dowex 1 (acetate; 2.5 cm × 3 cm) and Dowex 50(H^+) column (2.5 cm × 3 cm). The solution was concentrated under reduced pressure and the product was isolated[19]. Upon paper chromatography in four different solvent systems, the polyol formed in the reaction was recovered almost quantitatively in a single well defined region, with an R_F in each case identical to that of D-apiitol. As shown in Fig. 2, all of the radioactivity in the isolated sample was found in the same area as D-apiitol. When the product was oxidized with sodium metaperiodate, there was a rapid uptake of 3 moles of periodate and 1 mole of glycolic acid, 1 mole of formic acid and 2 moles of formaldehyde were formed based on the equivalent weight of a mole of apiitol. The specific activities of

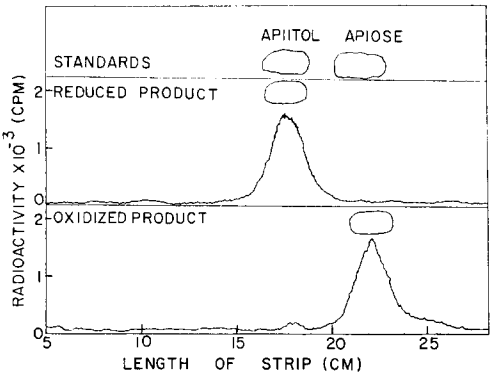


Fig. 2. Paper chromatography and radioactivity scans of the products of the dehydrogenase reaction. Standards are shown in the first lane. The reduced product formed from D-[U-^{14}C]-apiose and the scan for radioactivity are shown in the center lane. The oxidized product formed from D-[U-^{14}C]-apiitol and the corresponding scan are shown in the bottom lane. Chromatograms were developed with a solvent containing 2-propanol–water (9:1, by vol.) and they were treated with the alkaline $AgNO_3$ reagent after scanning for radioactivity in a Packard Model 7201 strip counter. Similar results were obtained when the paper was developed with ethylacetate–pyridine–water (12:5:4, by vol.).

the glycolic acid, formic acid and two equivalents of formaldehyde were about 40, 20 and 40%, respectively of that of the substrate, D-apiose. The data clearly indicate that the reduced product is D-apiitol, since very few other naturally occurring polyols yield these products[5].

For the large scale preparation of the oxidized product, 400 μmoles of D-apiitol ($6 \cdot 10^5$ cpm/μmole), 0.5 μmole of NADH, 100 μmoles of glycylglycine–NaOH, pH 9.0, 5 units of purified D-apiitol dehydrogenase, 600 μmoles of pyruvate and 10 units of lactate dehydrogenase in a volume of 10 ml were incubated at 30 °C for 2 h. The product was isolated[19] and examined by paper chromatography in four solvent systems. It had exactly the same mobility as D-apiose and all of the radioactivity was found in this area, as shown in Fig. 2. The product had the same absorption as D-apiose in the fructose–H_2SO_4 colorimetric test and it reacted with the benzidine–trichloroacetic acid reagent on paper chromatograms to give a yellow spot with a white fluorescence under ultraviolet light. In agreement with expected reactions[5], the oxidation of the isolated product resulted in the utilization of 3 equiv of periodate and the formation of 1 equiv of glycolic acid and formaldehyde and 2 equiv of formic acid.

Acetate derivatives of the isolated product were prepared. A 50-μmole sample was dissolved in pyridine and acetic anhydride[19], and it was allowed to react for 13 days at 3 °C. The rate and extent of acetylation was measured and compared with a sample of D-apiose treated in the same way. The products were analyzed by chromatography on paper impregnated with dimethylsulfoxide and developed with isopropyl ether[32]. Different anomeric forms of a number of fully acetylated sugars are readily separated by this procedure[19]. As shown in Table III, four acetates were formed from both D-apiose and the isolated product. The relative rates of migration of the four acetates formed from the oxidized product were essentially the same as those formed from D-apiose. The slow conversion of two partially acetylated deriva-

TABLE III

IDENTIFICATION OF THE OXIDIZED PRODUCT AS D-APIOSE BY ITS RATE OF ACETYLATION AND CHROMATOGRAPHY OF THE ACETATE DERIVATIVES

Samples containing 1 μmole of D-apiose or oxidized product were removed from the acetylation reaction mixture at the times shown in the table, and they were spotted on Whatman 3MM paper impregnated with 25% (v/v) of dimethylsulfoxide in toluene and the paper was developed with isopropyl ether. The separated acetates were located on small stripes cut from the paper with the alkaline $AgNO_3$ reagent, and the untreated areas were then eluted with methylene-chloride. The samples were concentrated, and the amount of D-apiose in each sample was determined as described in the text. The results are calculated as the % of the total sample present in each area on the chromatogram. $R_{\alpha\text{-}D\text{-}glucose\ pentaacetate}$ represents the distance of migration of the compound relative to 1,2,3,4,6-pentaacetyl-α-D-glucose.

Time (days)	D-*Apiose standard I plus II (%)*	*Oxidized product I plus II (%)*	D-*Apiose standard III plus IV (%)*	*Oxidized product III plus IV (%)*
5	51	49	48	48
9	46	47	52	48
11	37	33	60	58
13	24	20	72	74

Acetylated samples	$R_{\alpha\text{-}D\text{-}glucose\ pentaacetate}$
α-D-Glucopyranoside	1.00
β-D-Glucopyranoside	0.69
D-Apiose, Fraction I	0.14
D-Apiose, Fraction II	0.28
β-D-Apio-L-furanosyltetraacetate, Fraction III	0.98
β-D-Apio-D-furanosyltetraacetate, Fraction IV	1.22
Oxidized product, Fraction I	0.13
Oxidized product, Fraction II	0.28
Oxidized product, Fraction III	0.93
Oxidized product, Fraction IV	1.25

tives (I and II) to fully acetylated sugars (III and IV) is a characteristic feature of this branched-chain sugar. The rate of disappearance of Fractions I and II with time resulted in an almost stoichiometric increase in the amount of Fractions III and IV for both the oxidized product and D-apiose. The acetates were isolated from the reaction mixture after 13 days[19] and they were examined by gas chromatography. The retention times for the compounds present in Fractions I, II, III and IV are shown in Fig. 3. The same peaks were observed with samples derived from the isolated product and D-apiose. These results further confirm the observation that the oxidized product of the reaction is D-apiose.

Kinetic and equilibrium studies

The influence of the concentration of D-apiose, D-apiitol, NADH and NAD^+ upon the rate of the reaction is shown in Figs 4 and 5. The apparent K_m for D-apiose is $7.14 \cdot 10^{-2}$ M; D-apiitol, $1.16 \cdot 10^{-2}$ M; NAD^+, $3.5 \cdot 10^{-4}$ M; NADH, $1.5 \cdot 10^{-5}$ M. High concentrations ($1 \cdot 10^{-4}$ M) of NADH inhibited the reaction. The apparent equilibrium constant of the dehydrogenase reaction was determined at pH 9.5 and pH 7.5. When a reaction mixture was incubated at pH 9.5 with 0.2 μmole of NAD^+, 2.0 μmoles of D-apiitol and an excess of enzyme only 0.019 μmole of NADH and D-apiose were

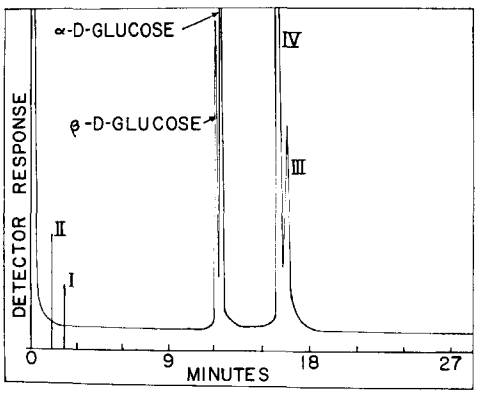
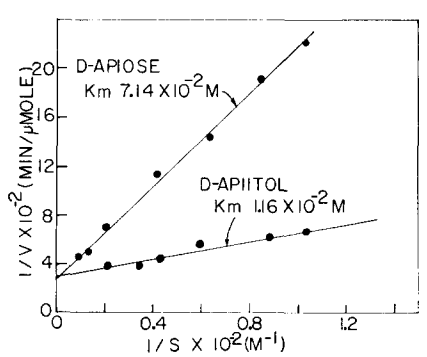


Fig. 3. Gas chromatogram of the acetylated oxidized product of the D-apiitol dehydrogenase reaction. Samples were analyzed on a Varian Aerograph 204 using an 8 ft × $^1/_8$ inch column packed with 3% PO17 Analabs, Inc., Hamden, Conn. on 60–70 mesh Anakrom ABS, Analabs, Inc., Hamden, Conn. The column temperature varied from 75 to 240 °C (6 °C/min). The injection port temperature was 225 °C and the carrier gas was H_2 at 40 ml/min. The time scale corresponds to 3 min/inch. α-D-Glucopyranosyl pentaacetate and β-D-glucopyranosyl pentaacetate were used as standards. The structures of the compounds in Peaks III and IV were assigned on the basis of their NMR spectra[19]. The positions of the peaks of the partially acetylated derivatives of D-apiose are labeled I and II.

formed after equilibrium was attained. Increasing the concentration of NAD^+ to 0.4 μmole and D-apiitol to 4.0 μmoles resulted in the formation of 0.039 μmole of NADH and D-apiose. An apparent equilibrium constant of 950 at pH 9.5 was calculated from values taken from several experiments with the following equation: K_{app} = [D-apiitol] [NAD]/[D-apiose] [NADH]. The equilibrium constant calculated from reactions run in both directions using D-[U-^{14}C]apiose and D-[U-^{14}C]apiitol was greater than $1 \cdot 10^5$ at pH 7.5. Since a H^+ is involved in the reaction, it is expected



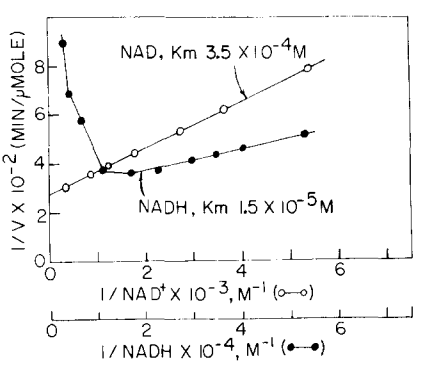


Fig. 4. Influence of the concentration of D-apiose and D-apiitol on the activity of the enzyme. Varying amounts of D-apiose were added to a reaction mixture which contained in 1 ml, 30 mM glycylglycine buffer, pH 7.5, 0.4 mM NAD^+ and 0.01 unit of enzyme. D-Apiitol was assayed in a reaction mixture which contained in 1 ml, 30 mM glycylglycine buffer, pH 9.5, 0.05 mM NADH and 0.01 unit of enzyme.

Fig. 5. Influence of the concentration of NAD^+ and NADH on the activity of the enzyme. The conditions of the assay are the same as those described in Fig. 4. The concentration of NAD^+ was varied with 60 mM D-apiitol. The amount of NADH was varied in the presence of 60 mM D-apiose.

that a shift of pH from 9.5 to 7.5 would alter the equilibrium in favor of D-apiitol formation. These results indicate that the equilibrium of the D-apiitol dehydrogenase reaction under physiological conditions greatly favors the formation and accumulation of D-apiitol.

DISCUSSION

Many polyol dehydrogenases found in bacteria are inducible enzymes and they are formed only when the microorganism is grown in the presence of a specific inducer which is usually the substrate. The *micrococcus* isolated from the surface of germinating parsley seeds was able to utilize D-apiose as the only source of carbon for growth and an unusual inducible D-apiitol dehydrogenase was isolated from extracts of this bacteria. The purified enzyme was relatively specific for D-apiose, D-apiitol, NAD^+ and NADH. The enzyme has a very low apparent K_m for NAD^+ and NADH and a relatively high K_m for D-apiose and D-apiitol. The bacteria does not actively transport D-apiose, however, the equilibrium of the dehydrogenase reaction would tend to favor the accumulation of D-aptiiol inside the cell. In all of the experiments which were carried out to study the utilization of D-apiose by whole cells, no D-apiitol was ever detected in the medium. These observations strongly indicate that D-apiitol does not pass through the cell wall or that the polyol is immediately metabolized to CO_2.

The metabolism of branched chain sugars in plants has received little attention compared to the many studies related to their distribution. The data obtained in this study show that when D-[U-^{14}C]apiose was incubated with *Lemna minor* less than 0.01% of the sugar was incorporated into the cell wall polysaccharide of this plant in 48 h, even through cell wall synthesis from D-glucose did occur under these conditions. Instead after 24 h about 50% of the D-apiose was converted to CO_2. It appears that this plant can utilize D-apiose as a source of energy after it is released from polysaccharides during turnover of cell wall constituents. There is probably no mechanism for the reutilization of the free sugar, as such, for the synthesis of cell wall polysaccharide in this tissue.

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