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Formation of 4-Keto-D-aldopentoses and 4-Pentulosonates (4-Keto-D-pentonates) with Unidentified Membrane-Bound Enzymes from Acetic Acid Bacteria*

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In our previous study, a new microbial reaction yielding 4-keto-D-arabonate from 2,5-diketo-D-gluconate was identified with *Gluconacetobacter liquefaciens* RCTMR 10. It appeared that decarboxylation and dehydrogenation took place together in the reaction. To analyze the nature of the reaction, investigations were done with the membrane fraction of the organism, and 4-keto-D-arabinose was confirmed as the direct precursor of 4-keto-D-arabonate. Two novel membrane-bound enzymes, 2,5-diketo-D-gluconate decarboxylase and 4-keto-D-aldopentose 1-dehydrogenase, were involved in the reaction. Alternatively, D-arabonate was oxidized to 4-keto-D-arabonate by another membrane-bound enzyme, D-arabonate 4-dehydrogenase. More directly, D-arabinose oxidation was examined with growing cells and with the membrane fraction of *G. suboxydans* IFO 12528. 4-Keto-D-arabinose, the same intermediate as that from 2,5-diketo-D-gluconate, was detected, and it was oxidized to 4-keto-D-arabonate. Likewise, D-ribose was oxidized to 4-keto-D-ribose and then it was oxidized to 4-keto-D-ribonate. In addition to 4-keto-D-aldopentose 1-dehydrogenase, the presence of a novel membrane-bound enzyme, D-aldopentose 4-dehydrogenase, was confirmed in the membrane fraction. The formation of 4-keto-D-aldopentoses and 4-keto-D-pentonates (4-pentulosonates) was finally confirmed as reaction products of four different novel membrane-bound enzymes.

Key words: D-aldopentose 4-dehydrogenase; 2,5-diketo-D-gluconate decarboxylase; 4-keto-D-pentonate; 4-keto-D-aldopentose; 4-keto-D-aldopentose 1-dehydrogenase

4-Keto-D-arabonate (4KAB) was identified for the first time as a 4-pentulosonate derived from 2,5-diketo-D-gluconate (25DKA) in the D-glucose oxidase system of *Gluconacetobacter liquefaciens* RCTMR 10 (Fig. 1).[1)] It appeared that a single enzyme was functioning to catalyze reactions of decarboxylation and dehydrogenation. The direct metabolic intermediates derived from 25DKA were not identified, except for D-lyxuronic acid, which is remote from 25DKA by several metabolic steps.[2,3)] The finding of 4KAB potentially indicates the presence of various novel membrane-bound enzymes in acetic acid bacteria, including 25DKA decarboxylase, D-aldopentose dehydrogenases, 4-keto-D-aldopentose 1-dehydrogenase, and D-pentonate 4-dehydrogenase. Quinoprotein D-glucose dehydrogenase (GDH) is known as the major enzyme oxidizing D-aldohexoses to D-aldohexonates, but D-aldopentoses are not oxidized by GDH.[4)] No enzymes responsible for D-aldopentose oxidation have been reported. Furthermore, no report on 4-keto-D-aldopentose formation is available. When D-arabonate was incubated with the membrane fraction of *Ga. liquefaciens* RCTMR 10, 4KAB was detected as the sole reaction product. This indicates the presence of a novel D-pentonate 4-dehydrogenase that might be different from D-aldohexonate dehydrogenases, such as D-gluconate dehydrogenase (GADH), found in most *Gluconobacter* strains yielding 2-keto-D-gluconate.[5)] D-Gluconate is the sole substrate for GADH, and D-arabonate is not oxidized with GADH. There must be several membrane-bound enzymes involved in the D-aldopentose metabolism of acetic acid bacteria. This led us to the discovery of 4-keto-D-aldopentoses and 4-pentulosonates (4-keto-D-pentonates), as reported here. The ketopentoses so far identified are limited to ones having a ketone group at the C2 position, such as D-, L-ribulose and D-, L-xylulose. The reaction products after oxidation at the C1 position of these ketopentoses are not known. The formation of 4-keto-D-aldopentoses, in which a ketone group is located at the C4 position, and the formation of their aldonates after oxidation at the C1 position, including 4KAB, may be the first indication of novel pentoses and pentonates in carbohydrate chemistry. Here we report on four different unidentified membrane-bound enzymes involved in the formation of 4-keto-D-aldopentoses and 4-pentulosonates (4-keto-D-pentonates).

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Abbreviations: 4KAB, 4-keto-D-arabonate; 4KAR, 4-keto-D-arabinose; 4KRB, 4-keto-D-ribose; 4KRN, 4-keto-D-ribonate; 2KGA, 2-keto-D-gluconate; 25DKA, 2,5-diketo-D-gluconate

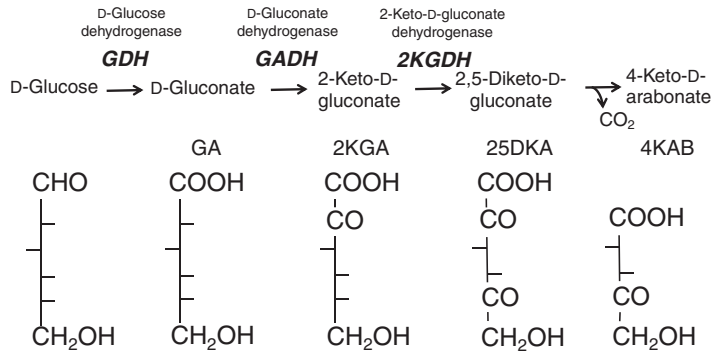

Fig. 1. D-Glucose Oxidizing Systems Leading to the Formation of 4-Keto-D-arabonate Proposed in a Previous Study.[1)]
GDH, quinoprotein D-glucose dehydrogenase; GADH, FAD-dependent D-gluconate dehydrogenase; 2KGDH, FAD-dependent 2-keto-D-gluconate dehydrogenase.

Materials and Methods

Chemicals. 25DKA and 4KAB were prepared from a cultured broth of *Ga. liquefaciens* RCTMR 10 after chromatographic separation by a Dowex 1×4 column as described previously.[1)] Yeast extract for culture media was a kind donation from the Oriental Yeast, Co. (Tokyo). Potassium D-arabonate was prepared by bromine oxidation of D-arabinose.[6)] 5-Keto-D-fructose was kindly donated from Kyowa Hakko, Co. (Tokyo). A reduced form of ubiquinone-2 (UQ_2) was a kind gift from Ezai, Co. (Tokyo).

Microorganisms and microbial growth. Ga. liquefaciens RCTMR 10 was grown on a medium containing 0.5% D-glucose, 2% Na-D-gluconate, 0.3% glycerol, 0.3% yeast extract, and 0.2% polypeptone (G/GA-medium). *G. suboxydans* IFO 12528 was grown on a D-arabinose medium containing 1% D-arabinose, 0.3% glycerol, 0.3% yeast extract, and 0.2% polypeptone (A-medium). *G. suboxydans* IFO 12528 was grown on a D-ribose medium containing 1% D-ribose, 0.3% glycerol, 0.3% yeast extract, and 0.2% polypeptone (R-medium). Cultivation was done at 30 °C on a rotary shaker at 200 rpm. Microbial growth was measured with a Klett-Summerson photoelectric colorimeter (Klett MFG, New York, NY). Under these conditions, most cultures came to the stationary phase with 350–450 Klett units after 36–48 h of incubation.

Preparation of membrane fraction. Cells were collected by centrifugation ($10{,}000 \times g$ for 10 min) at 4 °C. The wet cell paste precipitated was suspended in 10 mM acetate buffer pH 6.0, and centrifuged again. The cell suspension, 1 g of wet cell paste/10 mL buffer, was passed 2 times through a French pressure cell (Aminco, Silver Spring, MD) at 1,000 kg/cm^2. The cell homogenate was centrifuged at $10{,}000 \times g$ at 4 °C for 20 min, and unbroken cells were removed by precipitation. The supernatant was further centrifuged at $100{,}000 \times g$ at 4 °C for 60 min to collect the membrane fraction. This was homogenized with a glass homogenizer (Wheaton Ind., Millville, NJ) in 10 mM acetate buffer pH 6.0. The protein concentration was measured by a modification of the Lowry method with bovine serum albumin as standard.[7)]

TLC chromatography. To an aliquot of the culture medium and the enzyme reaction mixture, trichloroacetic acid solution (100% w/v) was added to a final concentration of 10%, and was spun down on a table-top centrifuge, and then spotted on a TLC plate. The TLC cellulose plate (aluminum sheet 20×20 cm, Merck, Darmstadt, Germany) was used. It was done at 25 °C with a solvent system of *t*-butanol:formic acid:water=4:1:1.5. Detection of oxidation products possessing ketones was done at room temperature by spraying an alkaline-ethanol solution of 2,3,5-triphenyltetrazolium chloride (TTC) over the TLC plate.[8)]

Determination of enzyme activities. (i) 2,5-Diketo-D-gluconate decarboxylase (25DKA decarboxylase) giving 4-keto-D-arabinose (4KAR) from 25DKA was assayed in a reaction mixture containing 25DKA and a membrane fraction having 4-keto-D-aldopentose 1-dehydrogenase and 25DKA decarboxylase in 1 mL of total reaction mixture. The reaction was conducted in 0.1 M acetate buffer pH 6.0. After incubation of the reaction mixture at 25 °C, the enzyme activity of 4-keto-D-aldopentose 1-dehydrogenase was assayed with an aliquot of the reaction mixture for 25DKA decarboxylase in the presence of potassium ferricyanide as electron acceptor.[5)] The reaction was started by the addition of 10 µmol of potassium ferricyanide, and was terminated by the addition of ferric-Dupanol reagent.[9)] When 25DKA decarboxylase is absent in enzyme solution to be checked, no appreciable Prussian blue color of the Dupanol solution appears. (ii) D-Arabonate 4-dehydrogenase was assayed in a reaction mixture containing 100 µmol of acetate buffer pH 4.0, 100 µmol of potassium D-arabonate, and the enzyme in 1.0 mL of the total reaction mixture. The reaction was started by the addition of 10 µmol of potassium ferricyanide and terminated by the addition of ferric-Dupanol reagent.[9)] (iii) D-Aldopentose 4-dehydrogenase together with 4-keto-D-aldopentose 1-dehydrogenase was measured on the basis of the following facts: D-aldopentoses such as D-arabinose and D-ribose are not oxidized by GDH from *G. suboxydans* IFO 12528.[10)] *Pseudomonas fluorescens* GDH[11)] and *Escherichia coli* GDH[12)] show broad substrate specificity to various aldoses but neither D-arabinose nor D-ribose is oxidized with purified GDHs. 4KAB and 4-keto-D-ribonate (4KRN) are finally formed after successive oxidation of D-arabinose and D-ribose by D-aldopentose 4-dehydrogenase and 4-keto-D-aldopentose 1-dehydrogenase. D-Aldopentose 4-dehydrogenase yielding 4-keto-D-aldopentose, was assayed with D-ribose or D-arabinose as primary substrate in 0.1 M acetate buffer pH 6.0, in the presence of 10 µmol potassium ferricyanide. Since GDHs from various sources reported to date do not oxidize D-ribose and D-arabinose,[10–12)] there must be an enzyme catalyzing the oxidation of D-arabinose and D-ribose, yielding 4KAR and 4-keto-D-ribose (4KRB). 4KAR and 4KRB are not commercially available. Therefore, the enzyme activities of D-arabinose and D-ribose oxidation are difficult to differentiate from those of 4KAR oxidation and 4KRB oxidation, catalyzed by 4-keto-D-aldopentose 1-dehydrogenase. Hence, two sequential enzyme activities combining D-aldopentose 4-dehydrogenase with 4-keto-D-aldopentose 1-dehydrogenase were measured.

Inactivation of quinoproteins in the membrane fraction by EDTA treatment. The membrane fraction of *G. suboxydans* IFO 12528 was adjusted to 20 mg of protein/mL and dialyzed in a cold room overnight against 10 mM acetate buffer pH 6.0, containing 20 mM EDTA. The dialyzed membrane fraction was centrifuged at $100{,}000 \times g$ at 4 °C for 60 min to remove excess EDTA. The precipitate was re-suspended and homogenized in 10 mM acetate buffer pH 6.0, and centrifuged to collect the membrane fraction. The same procedure was repeated 2 times to remove EDTA. The precipitated membrane fraction was finally homogenized in 10 mM acetate buffer pH 6.0, and designated apo-enzyme. Many different quinoproteins are known to lose their enzyme activities after EDTA treatment.[13,14)] Coenzyme PQQ interacts with the apo-protein *via* divalent ions, and EDTA removes the divalent ions inactivating the quinoproteins. Most apo-enzymes of quinoproteins can be restored to the original enzyme activity by subsequent addition of divalent metal ions and PQQ.

Solubilization of the various unidentified enzymes. To the membrane fractions of *Ga. liquefaciens* RCTMR 10 and *G. suboxydans* IFO 12528 in 10 mM acetate buffer pH 6.0, *n*-dodecyl-*β*-D-maltoside (DM) was added to 1% v/v. After it was left overnight under gentle stirring in a cold room, the mixture was centrifuged at 100,000 × *g* for 60 min, and a clear supernatant containing the enzyme activities was separated from the residual membrane.

Results and Discussion

Detection of 4-keto-D-arabinose as the precursor of 4KAB

In the early growth phase of *Ga. liquefaciens* RCTMR 10 grown on G/GA-medium, 25DKA accumulated as the major oxidation product in the culture medium. Most of the 25DKA that accumulated was converted to 4KAB at the later growth stage, as shown in Fig. 2.[1)] It appeared to be catalyzed by a single reaction from 25DKA to 4KAB, but faint TTC-positive spots with slightly higher R*f* values than 25DKA appeared after 4–6 d of culture. It is possible that 4-keto-D-arabinose (4KAR) appeared in the culture medium and then disappeared along with the conversion of 25DKA to 4KAB. When the membrane fraction of the organism was reacted with 25DKA at various pHs, an unidentified spot, presumably corresponding to 4KAR, was observed (Fig. 2). The spots near the front line up to 2 d of cultivation corresponded to dihydroxyacetone (DHA) formed from glycerol oxidation during cultivation. These observations indicate the occurrence of 4KAR as the direct precursor of 4KAB derived from 25DKA. This is the first time that a spot corresponding to 4KAR was detected. Based on the observations described above, two novel membrane-bound enzymes in the organism were postulated. As in Fig. 3, 25DKA decarboxylase was assigned to *Enz 1* and 4-keto-D-arabinose 1-dehydrogenase to *Enz 2* in the D-glucose oxidizing system leading to 4KAB.

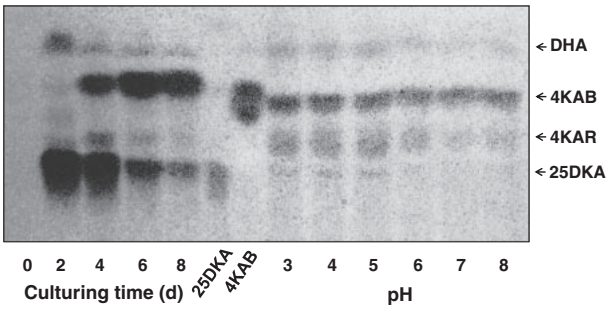

Fig. 2. TLC Chromatogram of 4-Keto-D-arabonate Formation with *Ga. liquefaciens*.

Ga. liquefaciens RCTMR 10 was grown on G/GA-medium containing 0.5% D-glucose and 2% Na-D-gluconate. An aliquot of the culture medium was taken at 2-d intervals as indicated by the numbers along the base line on the left side of the TLC plate. 25DKA and 4KAB prepared as in the previous study[1)] were used as standards. On the right side of the TLC plate, reaction mixtures done with the membrane fraction at various pHs of McIlvaine buffer are shown. The reaction mixture contained 20 µL of membrane fraction of 30 mg of protein/mL in 10 mM acetate buffer pH 6.0, 30 µL of 0.5 M 25DKA, and 50 µL of McIlvaine buffer of different pH, as indicated. The enzyme reaction was carried out for 18 h at 30 °C with gentle stirring.

Detection of 4-keto-D-aldopentoses as direct precursors of 4-pentulosonates (4-keto-D-pentonates) in culture media

Gluconobacter suboxydans IFO 12528 was grown on A-medium and also on R-medium, separately. Cultivation continued for 6 d, and TLC chromatography was done with an aliquot of the corresponding supernatants. In both cases, the formation of DHA from glycerol predominated. As shown in Fig. 4, in the A-medium, most of the D-arabinose was converted to 4KAB that was identical with that found in the culture broth of *Ga. liquefaciens* grown on G/GA-medium and in the reaction product from the membrane fraction (Fig. 2), and in a previous study.[1)] In most of the cultured broths examined up to 6 d, faint TTC-sensitive spots corresponding to 4KAR, the same as those shown in Fig. 2, were detected, although the intensity was not so strong as 4KAB. Likewise, from the supernatant broth from R-medium, similar TTC-positive spots corresponding to 4KRB were found, with slightly lower R*f* values than 4KAR. The R*f* value of 4KRN was always slightly higher than 4KAB (Fig. 4). Other aldopentoses, including L-arabinose, L-ribose, D- and L-lyxose, and D- and L-xylose, on the other hand, gave no TTC-positive spots, and the sugar oxidation examined here appeared specific only to the substrate structure having the D-*erythro* configuration. At present, standard compounds of 4KAR, 4KRB, and 4KRN are not commercially available, except for 4KAB, and the formation of such novel compounds is proposed for the first time here. Hence, the formation and location of 4KAR, 4KRB, and 4KRN, except for 4KAB, were deduced to be the most probable.

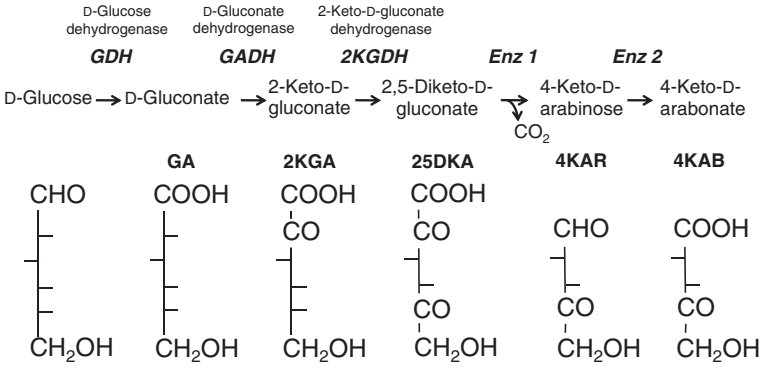

Fig. 3. Proposed Scheme of D-Glucose Oxidizing Systems Leading to the Formation of 4-Keto-D-arabonate *via* 4-Keto-D-arabinose.

The enzymatic steps focused on are shown as *Enz 1* and *Enz 2*. *Enz 1*, 2,5-diketo-D-gluconate decarboxylase; *Enz 2*, 4-keto-D-aldopentose 1-dehydrogenase.

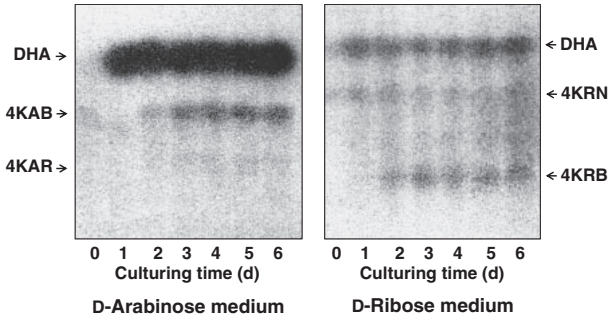

Fig. 4. TLC Chromatogram of Culture Media Grown on D-Arabinose and D-Ribose.

G. suboxydans IFO 12528 was grown on A-medium containing 1% D-arabinose and on R-medium containing 1% D-ribose. Aliquots of the culture medium were taken at 1-d intervals, as indicated by the numbers along the base line of the chromatogram.

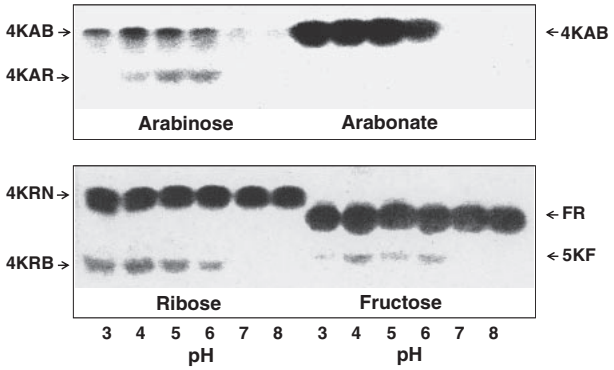

Fig. 5. TLC Chromatogram of Reaction Mixtures Done with the Membrane Fraction with Four Different Substrates.

The membrane fraction of *G. suboxydans* IFO 12528 was incubated with four different substrates at various pHs, as indicated. The reaction conditions were almost the same as in Fig. 2. FR, D-fructose; 5KF, 5-keto-D-fructose.

Enzymatic synthesis of 4KAR, 4KRB, and 4KRN should be done in order to confirm their existence by mass spectroscopic analysis. For this purpose, a purified D-aldopentose 4-dehydrogenase devoid of D-pentonate 4-dehydrogenase, the terminal oxidase, and ubiquinone-2 (UQ_2), should be reconstituted into proteoliposomes,[4] as explained below. The solvent system used for TLC chromatography gave the clearest separation of the various compounds, although it took 8–10 h until the front line came up to the top of a TLC plate. Column chromatographic separation of 4KAR from 4KAB and of 4KRB from 4KRN was unsuccessful after many times of trial and error. Differently from a clear separation of 4-ketoaldopentoses and 4-pentulosonates (4-keto-D-pentonates) by TLC, 4KAR moved close to 4KAB and 4KRB moved close to 4KRN in most column chromatographic trials, making it difficult to separate the two compounds in significant amounts. Extraction of 4KAR and 4KRB from a developed TLC plate appeared to be the only successful way, but the samples eluted from the TLC plate were colored dark after subsequent handling. This made it difficult to bring the intermediate compounds to a highly purified state.

Detection of 4-keto-D-aldopentoses and 4-pentulosonates (4-keto-D-pentonates) in reaction mixtures conducted with the membrane fraction

Two unidentified enzymes, tentatively named D-aldopentose 4-dehydrogenase and 4-keto-D-aldopentose 1-dehydrogenase, predominated in the membrane fraction of *G. suboxydans* IFO 12528. The membrane fraction was reacted with D-arabinose and D-ribose separately at various pHs, as shown in Fig. 5. TTC-positive spots of 4KAR from D-arabinose and of 4KRB from D-ribose were clearly detected. The R*f* values of 4KAR were always higher than those of 4KRB, and the R*f* values of 4KRN were always higher than those of 4KAB (Fig. 5), as seen for TLC examined with the supernatant. 4KAR formation was clearly observed from pH 3 to 6, but no appreciable TTC spots corresponding to 4KAB and 4KAR were detected for the reaction mixtures done at pH 7 and 8. D-Ribose oxidation to 4KRB and 4KRN was clearly shown at various pHs. 4KRB was detected with the reaction mixtures done at pH 3–6, and 4KRN formation appeared clearer than those of 4KAB formation from D-arabinose. D-Arabinose and D-ribose did not give any TTC-positive spots under the reaction conditions. Judging from the color intensity that appeared on a TLC-plate, it was concluded that D-ribose was a better substrate than D-arabinose. Formation of 5-keto-D-fructose (5KF) was detected when D-fructose was reacted with the membrane fraction, as shown in Fig. 5. Major TTC-positive spots that corresponded to D-fructose remained in the reaction mixture and did not react with the membrane. In addition, a series of slight TTC-positive spots from pH 3 to 6 with lower R*f* values that corresponded to 5KF were detected. When 2KGA was reacted with the membrane fraction, 25DKA was formed, but it gave fainter spots than those of 5KF from D-fructose (data not shown). The formation of 5KF or 25DKA was detected only *in vitro* when the membrane fraction was incubated with high concentrations of D-fructose or 2KGA. Under conventional culturing conditions, neither 5KF nor 25DKA was produced by *G. suboxydans* IFO 12528. This means that D-aldopentose 4-dehydrogenase recognized the chemical structure common to D-arabinose, D-ribose, and the C2–C6 moiety of D-fructose and 2KGA. It recognized the secondary alcoholic group in the D-*erythro* configuration adjacent to the primary alcoholic group. The secondary alcoholic group at the C4 position of D-arabinose and D-ribose was oxidized to the ketone group. It is known that the oxidation of D-fructose is catalyzed only by the membrane-bound D-fructose dehydrogenase (FDH) of *G. industrius* IFO 3260.[15] Bacteria possessing FDH are limited to only a few strains, and FDH activity is not expressed in *G. suboxydans* IFO 12528. Likewise, 2KGA oxidation was catalyzed only with 2-keto-D-gluconate dehydrogenase (2KGDH), and formed only by a few strains of *G. melanogenes*.[16] Therefore, a new enzyme corresponding to D-aldopentose 4-dehydrogenase was postulated from the data above and it was distinct from FDH and 2KGDH.

D-Arabonate oxidation was examined with the membrane fraction, as shown in Fig. 5, and 4KAB formation was observed over the pH ranges of 3–6. However, no TTC-positive spots appeared when the reaction was done at pH 7 and 8. This is very similar to the pH profile of D-aldohexonate oxidation by GADH, which is strong at pH 3–5.[5] D-Arabonate itself showed no positive

colorization with TTC. When assayed by D-arabonate oxidation with potassium ferricyanide, the highest color intensity of the Dupanol-reagent was found when the reaction was done at pH 4.0, indicating good coincidence with TLC chromatography. This means that D-arabonate oxidation is catalyzed by D-arabonate 4-dehydrogenase as distinct from D-aldopentose 4-dehydrogenase. The oxidation product of D-arabonate was identified as 4KAB, the same as that from 25DKA.[1] GADH did not oxidize D-arabonate at all, and D-gluconate was the sole substrate for GADH, as extensively found in the genera *Gluconobacter* and *Gluconacetobacter*.[5] Therefore, there must be an alternative enzyme that oxidizes D-arabonate to 4KAB as reaction product. Similarly, if D-ribonate is available, 4KRN may be the reaction product. It is necessary to determine whether D-pentonate 4-dehydrogenase is found extensively in the genera *Gluconobacter* and *Gluconacetobacter*. Both D-arabonate and D-ribonate might be oxidized by the same membrane-bound enzyme, D-pentonate 4-dehydrogenase, although this should be confirmed with a purified enzyme. As has been proposed, 4KAR and 4KRB might be oxidized to 4KAB and 4KRN by a single membrane-bound enzyme, 4-keto-D-aldopentose 1-dehydrogenase, which is distinct from D-pentonate 4-dehydrogenase. Thus, the two different enzymes presumably give the same ultimate oxidation products. All the above should be clarified in experiments using D-ribonate, which can be obtained from bromine oxidation of D-ribose.

Effect of EDTA on enzyme activities

Glycerol and D-sorbitol are well known favorite substrates to the membrane-bound polyol dehydrogenase of acetic acid bacteria.[4] This enzyme is the major one in the bacterial membrane, and it is a typical quinoprotein oxidizing various sugars and sugar alcohols according to the Bertrand-Hudson rule.[16] PQQ, involved in most quinoproteins, can be removed with EDTA treatment, thus converting the active holo-enzyme to an inactive apo-enzyme. The enzyme activity of apo-enzyme can be restored to the active quinoprotein by adding divalent ions such as Ca^{2+} or Mg^{2+} and PQQ.[4,13,14,17] To characterize the enzyme responsible for D-arabinose and D-ribose oxidation, the membrane fraction of *G. suboxydans* IFO 12528 was treated with EDTA. Various substrates were reacted with the EDTA-treated membrane (apo-enzyme) and the native enzyme (holo-enzyme) in the original membrane. As shown in Fig. 6, polyol dehydrogenase was inactivated, and decreased intensity of TTC-positive spots was observed as expected with the enzymatic oxidation of glycerol, *meso*-erythritol, ribitol, and D-allose. Clear enzymatic formation of dihydroxyacetone (DHA) from glycerol, L-erythrulose from *meso*-erythritol, 5-keto-D-allose from D-allose, and L-ribulose from ribitol was observed with the holo-enzyme, but the intensity of the TTC spots decreased in the reactions done with the apo-enzyme. The enzyme activities involved in these substrate oxidations are catalyzed by the quinoprotein polyol dehydrogenase.[17] On the other hand, the enzyme activities for D-arabinose and D-ribose oxidation were not affected by EDTA treatment of the membrane. This means that the coenzyme accommodated in the membrane-bound dehydrogenase for the oxidation of D-arabinose and D-ribose is not PQQ, and presumably a covalently bound FAD as similarly seen with GADH and 2KGDH.[18] These data strongly support the assumption that one of the unidentified enzymes is D-aldopentose 4-dehydrogenase, and not the major quinoprotein polyol dehydrogenase in acetic acid bacteria. D-Pentonate 4-dehydrogenase was classified as another unidentified enzyme because of the clear formation of 4KAB and 4KRN.

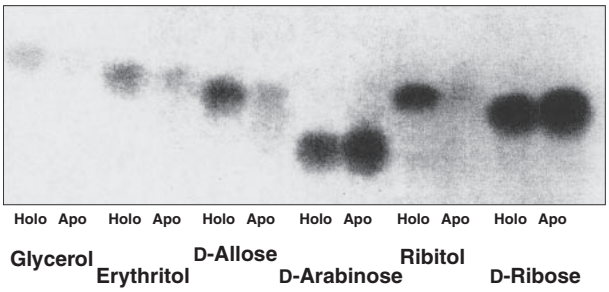

Fig. 6. Effects of EDTA on the Oxidation of Various Substrates.
The apo-enzyme was prepared after EDTA treatment. The enzyme activities of the apo-enzyme and holo-enzyme were examined with various substrates, as indicated. The reaction conditions were almost the same as in Fig. 2.

Solubilization of the respective membrane-bound enzymes

The solubilization of the respective membrane-bound enzymes was examined with the membrane fractions of *Ga. liquefaciens* RCTMR 10 and *G. suboxydans* IFO 12528. Under the solubilization conditions employed using DM as detergent, most of the membrane-bound enzymes having peripheral interaction with the cytoplasmic membrane were solubilized, giving a pale red characteristic of the cytochrome components. On the other hand, highly hydrophobic transmembraneous proteins such as the terminal oxidase remained in the membrane.[4] Reaction mixtures of D-arabinose, D-ribose, and 25DKA with the solubilized enzyme showed TTC-positive spots. 4KAB was given from 25DKA and D-arabinose and 4KRN from D-ribose, although the color intensity was stronger for the reaction mixture done with the original membrane fraction (data not shown). Reconstitution experiments with the solubilized enzymes and the terminal oxidase in the presence of reduced ubiquinone-2 (UQ_2) were also examined. Low oxidase activities for D-arabinose and D-ribose were also detected with the reconstituted oxidase systems. This indicates the possibility of limited enzymatic synthesis of 4KAR and 4KRB. For a successful enzymatic synthesis of 4KAR and 4KRB, a purified D-aldopentose 4-dehydrogenase devoid of 4-keto-D-aldopentose 1-dehydrogenase and the terminal oxidase should be reconstituted into proteoliposomes in the presence of UQ_2. Then enzymatic synthesis of 4KAR and 4KRB from D-arabinose and D-ribose becomes possible.[4] Optimization of solubilization, purification, and characterization of the individual enzymes is in progress for detailed descriptions of the metabolic pathway proposed here.

In summary, the four different unidentified membrane-bound enzymes involved in the formation of

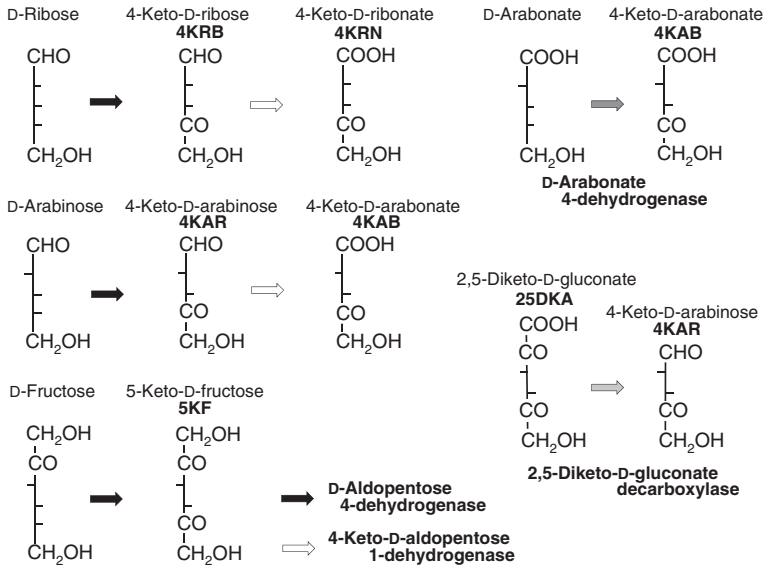

Fig. 7. Proposed Scheme of the Membrane-Bound Enzymes Involved in the Formation of 4-Keto-D-aldopentoses and 4-Pentulosonates (4-Keto-D-pentonates).

In the left, D-aldopentose 4-dehydrogenase yielding 4-keto-D-aldopentoses and 4-keto-D-aldopentose 1-dehydrogenae oxidizing 4-keto-D-aldopentose to 4-pentulosonates (4-keto-D-pentonastes) are illustrated. The oxidation of D-fructose to 5-keto-D-fructose with D-aldopentose 4-dehydrogenase is added to indicate that the responsible enzyme can recognize the chemical structure in common among these three substrates.

4-keto-D-aldopentose and 4-pentulosonates (4-keto-D-pentonates) are given with tentative names to the respective reactions in Fig. 7.

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