Improved Enzymatic Procedure for a Preparative-Scale Synthesis of Sialic Acid and KDN

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Sialic acid (*N*-acetylneuraminic acid) and KDN (3-deoxy-β-D-*glycero*-D-*galacto*-2-nonulosonic acid) play important roles in a variety of intercellular events. Extensive studies have been reported on the use of sialic acid aldolase as a catalyst to form the C–C bond between *N*-acetyl-D-mannosamine derivatives and pyruvic acid to give sialic acid and its analogs. However, the reported protocols have a significant drawback in terms of the tedious separation of the desired product from a large excess of pyruvic acid. We report here on a clean solution to this problem that relies on the utilization of pyruvate decarboxylase for catalyzing the decomposition of pyruvic acid into acetaldehyde and carbon dioxide to facilitate product isolation. Concerning our investigation to enhance the activity of this enzyme, we emphasized two points. One was a sufficient supply of cofactors for pyruvate decarboxylase; the other was an improvement of the enzyme reactor. As a result, the activity became nearly four-times as high as that previously reported. The combination of two enzymatic reactions worked well, affording sialic acid and KDN in 60% yield from *N*-acetyl-D-mannosamine and D-mannose, respectively. In addition, an efficient method for preparing *N*-acetyl-D-mannosamine by the epimerization of *N*-acetyl-D-glucosamine was established. Calcium hydroxide was found to be most effective for base-catalyzed epimerization.

Sialic acid (*N*-Acetylneuraminic acid, **1**) is widely distributed on the terminal of a variety of glycoproteins and glycolipids, which play important roles in the interaction and adhesion between cells.[1] For example, sialyl Lewis X (Le^X), a tetrasaccharide with a sialic acid moiety, binds to ELAM-1 and mediates inflammatory responses between leukocytes and injured tissues.[2] For a detailed biological study, the supply of natural and nonnatural sialyl oligosaccharides with a definite structure is indispensable. Accordingly, numerous chemical and chemo-enzymatic methods have recently been developed for the synthesis of sialyl oligosaccharide.[3]

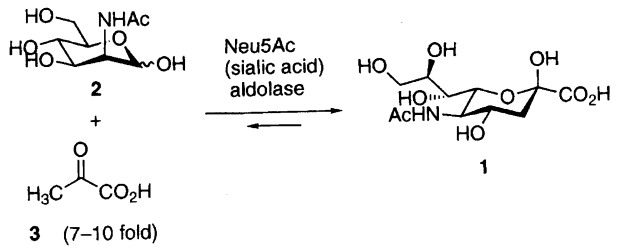
Furthermore, clinical applications are expected for sialic acid-derived molecules, such as potent inhibitors against sialidase of an influenza virus.[4] The growing importance of sialic acid and its analogs requires the establishment of methods for their preparation on a large scale.

Sialic acid has been industrially produced from natural sources, such as the nest of a swiftlet,[5] egg,[6] and milk.[7] It is also available from a sort of microorganism[8] by degradation of its homopolymer (colominic acid). In contrast to isolation from natural sources, an enzymatic preparative method has recently become attractive in terms of its applicability for preparing analogs with medicinal potential.

In 1958, sialic acid aldolase (Neu5Ac aldolase) (E.C.4.1.1.3) was reported to be the enzyme responsible for the degradation of sialic acid.[9] It catalyzes the retro-aldol decomposition of sialic acid into *N*-acetyl-D-mannosamine (ManNAc, **2**) and pyruvic acid (**3**).

In the 1980's, Augé,[10] Wong,[11] and Whitesides[12] independently demonstrated the use of aldolase for the synthesis of sialic acid from ManNAc and pyruvic acid (Scheme 1). Subsequent studies have revealed that this enzyme accepts a very broad range of substrates,[13] and many nonnatural sialic acid analogs have also been synthesized by this catalysis. The availability of a sialic acid aldolase of microbial origin has greatly increased recently via cloning and overexpression.[14] Studies concerning the stereochemical requirement of the substrates[13c,13f,13j,13l] and to establish reaction systems for large-scale preparation[13h] have also been reported.

The enzymatic aldol reaction proceeds in a reversible



Scheme 1. Synthesis of sialic acid catalyzed by sialic acid aldolase.

process; the equilibrium lies so far to the starting materials (educts). Thus, the use of pyruvic acid in large excess (normally 7—10 mol. amt.) is indispensable to shift the equilibrium to the desired aldol product. Since the pK_a's of pyruvic acid and sialic acid are nearly equal, it is extremely difficult to separate sialic acid from the pyruvic acid used in excess, and tedious ion-exchange chromatography is thus necessary. We reasoned that a prior removal of pyruvic acid before chromatography would greatly simplify the purification procedure.

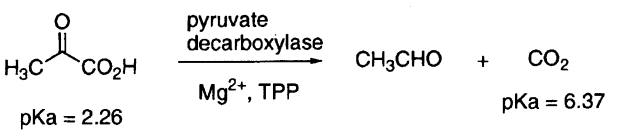
A clue to the solution was provided by the use of pyruvate decarboxylase (E.C.4.1.1.1, Scheme 2),[13l] which catalyzes the decomposition of pyruvic acid into acetaldehyde and carbon dioxide. Our expectation was that, since both products are volatile and can be readily removed, the desired product would be easily obtainable in pure form, which, indeed, proved to be the case.

In this paper, we report on a detailed study carried out to find the optimal experimental conditions of the decarboxylation of pyruvic acid and its application to the preparative-scale synthesis of sialic acid and KDN (3-deoxy-β-D-*glycero*-D-*galacto*-2-nonulosonic acid).

Results and Discussion

Decarboxylation of Pyruvic Acid. Pyruvate decarboxylase (EC 4.1.1.1)[15] is widely found in nature.[16] This enzyme has long been used as a yeast-cell bound biocatalyst in brewery and bakery industries. In a previous study,[13l] we used commercially available bakers' yeast (Sigma, type II, YSC-2) as an unpurified source of enzyme, whose activity toward pyruvic acid was estimated to be 8.3 units/g (dry cell weight). Because of the rather low activity, a quantity of dry bakers' yeast (as much as 15 g) was necessary for a smooth removal of the pyruvic acid from the reaction mixture in the preparation of 1 g of sialic acid. The yield of sialic acid could not exceed 42%. The large excess of yeast was supposed to have a deleterious effect, and to lower the yield by means of the adsorption of the product on the cells. This situation urged us to seek a higher activity of pyruvate decarboxylase. Our attempt to find a microorganism with high activity of pyruvate decarboxylase, however, gave no fruitful result.[17]

Accordingly, we decided to optimize the reaction conditions so as to secure a higher activity of pyruvate decarboxylase contained in the conventional whole cells of bakers' yeast. We focused our attention on the concentration of cofactors that are essential to the action of the enzyme. Pyruvate decarboxylase is known to require thiamine pyrophosphate (TPP) and magnesium ion (Mg^{2+}) as its cofactors. Under the physiological environment, it is present as holoenzyme,[15] i.e., a complex between the corresponding apoenzyme and these cofactors. In the present experiment, however, decarboxylation should have worked at a very high initial concentration (400—450 mM; 1 M=1 mol dm^{-3}) of pyruvic acid.[13l] We were thus anxious about the enzyme deactivation due to a loss of its cofactors.



Scheme 2. Pyruvate decarboxylase-catalyzed reaction.

We therefore examined whether the external addition of the cofactors would affect the activity of pyruvate decarboxylase or not, as shown in Table 1. Upon the addition of TPP and Mg^{2+} up to the final concentration of 0.4 mM, the activity increased nearly proportionally to the concentration of the cofactors. Further addition of the cofactors led to only a small enhancement in the activity. Thus, the optimal concentration was determined to be 0.4 mM for both TPP and Mg^{2+}.

We next focused on the improvement of an enzyme reactor. Concerning the necessity of an elaborate reactor, we should mention an important aspect of the enzymatic decarboxylation. As the reaction proceeds, the pH of the reaction system rises because pyruvic acid (pK_a 2.26) is converted to a weaker acid, carbon dioxide (pK_a 6.37, Scheme 2). To maintain the enzyme activity, the pH should always be kept at 6.0, the optimum pH for the enzyme, by the addition of a suitable acid. Apparently, this neutralization should be done by the addition of a H^+ type ion-exchange resin, since the use of a conventional acid, such as hydrochloric acid, would cause an undesirable accumulation of inorganic salts, which would cause trouble in the purification.

The enzyme reactor which we used previously is shown in Fig. 1 (A). The ion-exchange resin was directly introduced to the reaction mixture. As the reaction proceeded, the accumulated granules of the ion-exchange resin lowered the efficiency of mixing, thus hampering the efficient release of acetaldehyde from the reaction mixture. The acetaldehyde, which rapidly accumulated in the reaction mixture, inhibited the action of the pyruvate decarboxylase. We thus devised the separation of the resin from the reaction mixture to form an improved reactor, in which the reaction mixture was neutralized by passing through the cage filled with the resin [Fig. 1 (B), arrow X].

Table 1. Effect of Cofactor Concentration on Pyruvate Decarboxylase Activity

Mg^{2+}/mM[a)]	TPP/mM	Relative activity
0	0	1
0.2	0.04	1.02
0.4	0	1.19
0.4	0.04	1.33
0.4	0.4	1.62
2.0	2.0	1.78

a) Added as magnesium chloride.

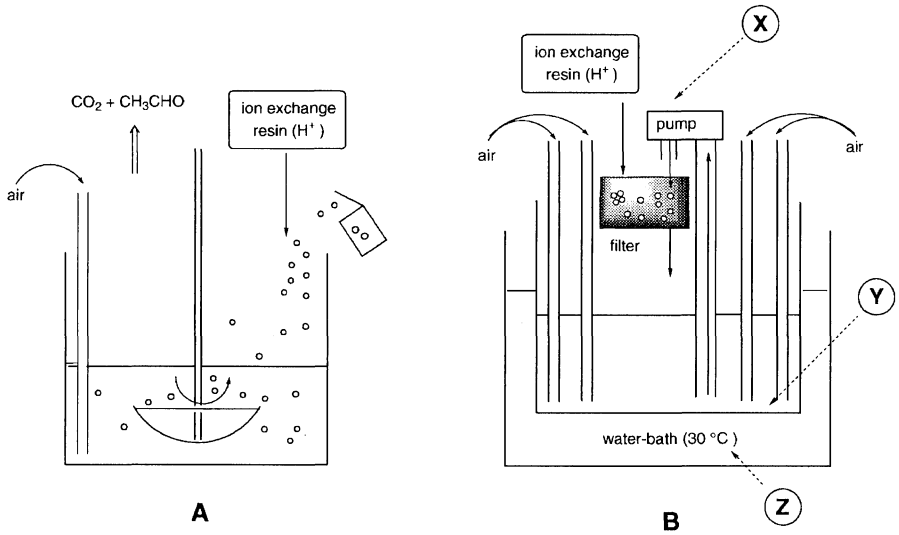


Fig. 1. Enzyme-reactor for the decarboxylative removal of pyruvate.

The method of stirring the reaction mixture proved to be important. In previous experiments, we often encountered problems with the centrifugal precipitation of the yeast and the ion-exchange resin, that stick to the side wall of the reactor due to excessively vigorous stirring with a propeller. Thus, mechanical stirring was replaced by mixing by aeration through bubbling tubes [Fig. 1 (B), arrow Y]. Efficient mixing was accomplished by enhancing the rate of aeration from 10000 ml min^{-1} to 18000 ml min^{-1}. This aeration also serves to purge the resulting acetaldehyde from the reaction system. The solution could remain essentially homogeneous, which is one of the advantage of this mixing method.

Finally, the optimum temperature (30 °C) was maintained by external heating with a water bath [Fig. 1 (B), arrow Z]. At a temperature higher than 35 °C, further acceleration of the enzymatic reaction was observed during the initial stage. However, the activity dropped off quickly.

As a result of the above improvements, the activity of pyruvate decarboxylase was successfully raised to 32.8 units/g (dry cell weight), which was nearly four-times as high as the one that had originally been reported [8.3 units/g (dry cell weight)]. The results are summarized in Table 2. In this way, the amount of bakers' yeast needed for the decarboxylation was reduced, which led, indeed, to improvements in the yields of sialic acid and KDN.

Table 2. Improvement of the Conditions of Pyruvate Decarboxylase Reaction

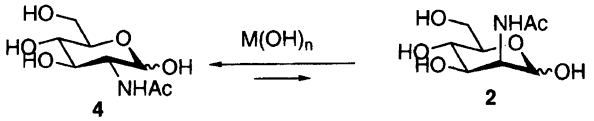
	Original (A)	Improved (B)
Resin (H^+)	In solvent	Out of solvent
Aeration/ml min^{-1}	1000	18000
Cofactor	None	Mg^{2+}, TPP
Temperature/°C	22—25	30
Enzyme activity /unit·(g of yeast)$^{-1}$	8.3	32.8

Improved Procedure for *N*-Acetyl-D-mannosamine by the Epimerization of *N*-Acetyl-D-glucosamine. The substrate for the enzymatic synthesis of sialic acid is *N*-acetyl-D-mannosamine. Because *N*-acetyl-D-mannosamine (**2**) is expensive (Sigma $ 82.50/5 g), the preparation by the C(2)-epimerization of *N*-acetyl-D-glucosamine (GlcNAc) (**4**) (Sigma $ 8.20/5 g) has so far been extensively studied by way of chemical (Scheme 3)[12,13w,18] or enzymatic[13h] means.

Among the procedures used for the epimerization of carbohydrates catalyzed by metal salt,[19] Yanagihara, Osanai, and co-workers have reported that the addition of calcium salt is effective for the epimerization of aldoses and ketoses.[20] The epimerization of the 2-deoxy-2-amino sugar, whose amino group was acyl-protected, however, has not been attempted.

We therefore investigated the effect of calcium hydroxide on the epimerization of *N*-acetyl-D-glucosamine (**4**, Scheme 3). Firstly, it was revealed that the reaction smoothly proceeded in the case of water being used as a solvent, while no reaction was observed either in a 95% ethanol solution or without any solvent (the solid state reaction). Accordingly, the reaction was carried out at pH 11.2 in saturated aqueous calcium hydroxide.

After 1 d at room temperature, the reaction had already reached equilibrium between GlcNAc (**4**) and ManNAc (**2**) in a ratio of ca. 4:1. This ratio was in good accordance with that which had been observed under the conventional alkaline epimerization with sodium



Scheme 3. Epimerization of *N*-acetyl-D-glucosamine to *N*-acetyl-D-mannosamine.

hydroxide.[12,18] At higher temperature, although the period required to reach equilibrium could be shortened, the yield of ManNAc (**2**) diminished, probably because of the self-condensation of the starting material and the product.

Encouraged by the viability of calcium hydroxide-catalyzed epimerization, we further examined the effect of the reaction period. The results of the conversion of the epimerization [ManNAc (**2**)/[ManNAc (**2**)+GlcNAc (**4**)]] during the epimerization by calcium and sodium hydroxides are shown in Fig. 2. The epimerization catalyzed by calcium ion was 5.6-times as fast as that by sodium ion at the same pH during the initial stage, and the reaction reached equilibrium after 8 h at room temperature. The equilibration ratio of GlcNAc and ManNAc is not different between the use of calcium and sodium hydroxide. Indeed, the yield of ManNAc (**4**) from the starting material in the case of a calcium hydroxide catalyzed reaction was almost the same as that obtained by a sodium hydroxide catalyzed reaction. A prolonged reaction of more than 24 h had no effect to change the yield. The best result was obtained when the reaction was carried out at room temperature for 8—10 h. These results suggest the significance of the interaction[20] between calcium ion and carbohydrates, as has been documented[21] for the separation of an epimeric mixture of a carbohydrate by means of ion-exchange chromatography (Ca^{2+} form resin).

The great practical advantage of using the calcium ion-catalyzed epimerization reaction is noteworthy. ManNAc serves as the starting material for preparing sialic acid. For purifying sialic acid at a later stage, the contamination of inorganic salt in ManNAc should be controlled to be as low as possible. In the present study, calcium hydroxide, which had been used for the epimerization of GlcNAc, could be easily removed, by only passing carbon dioxide gas and the subsequent centrifugation or filtration of the precipitated calcium carbonate (Scheme 4).[22]

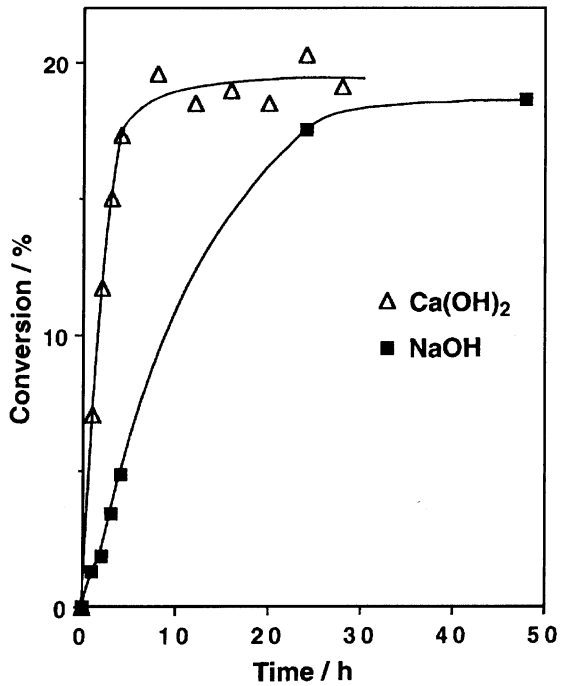
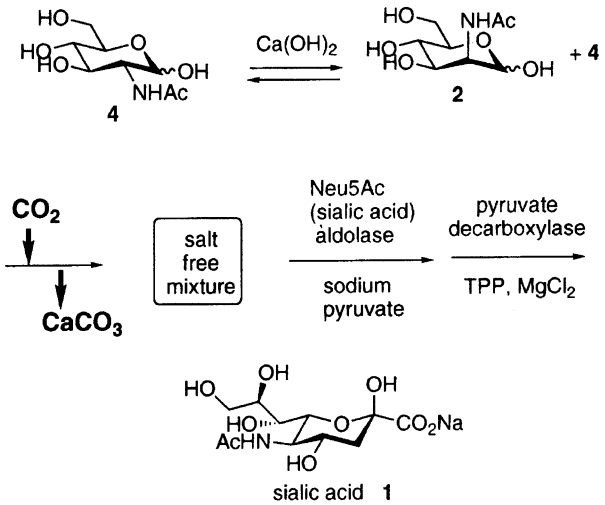


Fig. 2. The epimerization of *N*-acetyl-D-glucosamine to *N*-acetyl-D-mannosamine by calcium and sodium hydroxides.

Synthesis of Sialic Acid. The above-mentioned three reactions were subsequently combined, i.e., the calcium hydroxide-catalyzed epimerization of GlcNAc (**4**) to ManNAc (**2**), the aldolase-catalyzed reaction with pyruvic acid, and the degradation of excess pyruvic acid catalyzed by pyruvate decarboxylase (Scheme 4). The procedure worked well, resulting in sialic acid in 60% yield from ManNAc (**2**) and in 7.6% from GlcNAc (**4**).[23] Because GlcNAc (**4**) is intact either in the step of a sialic acid aldolase-catalyzed reaction and in the step to remove pyruvic acid, the unreacted GlcNAc (**4**) can be recycled as the starting material for epimerization.

Synthesis of KDN. Encouraged by this success, we turned our attention to the next target, D-KDN (3-deoxy-D-*glycero*-*β*-D-*galacto*-2-nonulosonic acid, **5**), which is a sialic acid-related compound with a structure in which the C(5)-*N*-acetamido group in a sialic acid is replaced by a hydroxyl group, and has been isolated from the egg of trouts.[24] Because of the distribution in gangliosides and biological interests as an alternative of sialic acid,[25] studies toward the preparation of its derivatives,[26] as well as its incorporation into oligosaccharides,[27] have been investigated. So far, KDN has been synthesized by chemical[28] and enzymatic methods.[11,13d,13e,13f,13i,13j,13l] Among the reported procedures, the most practical way seems to be the Cornforth synthesis reported by Ogura and coworkers,[28b] which is based on the addition of oxalacetic acid to D-mannose. The problem is that this ad-

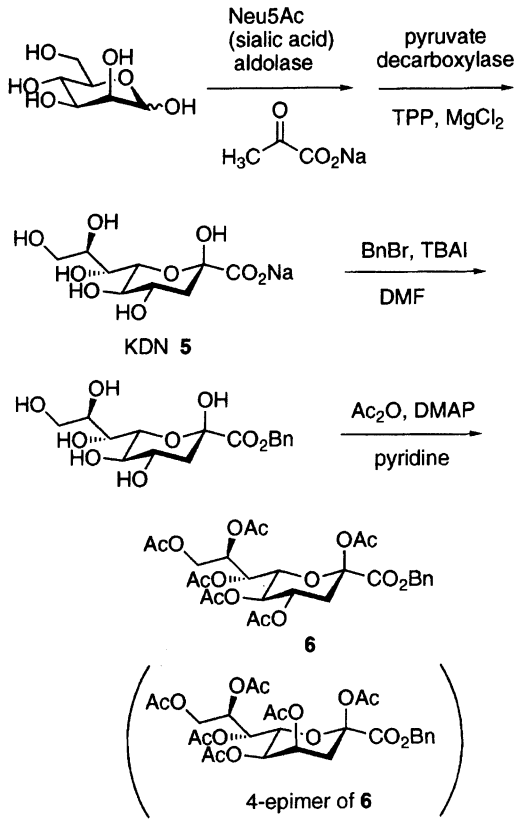


Scheme 4. Preparation of salt free mixture of *N*-acetyl-D-glucosamine and *N*-acetyl-D-mannosamine and subsequent enzymatic procedure to sialic acid.

dition gives an inseparable 4:1 mixture of KDN and an undesirable epimer on the C-4 position. The only way to remove any undesirable epimer has been tedious chromatography at the stage of the protected form (**6**). These situations prompted us to establish a large-scale preparation of KDN and its derivatives based on an enzymatic procedure.

We were pleased to find that the combination of aldolase and decarboxylase worked well to give KDN in 60% yield from D-mannose. The total procedure could be carried out on a preparative (ca. 10 g of KDN) scale. As a large amount of KDN was in hand, we embarked upon the synthesis of an important derivative (**6**) for an oligosaccharide synthesis.

A notable advantage is that the crude sodium salt of KDN, obtained just after removing pyruvic acid by way of a pyruvate decarboxylase catalyzed reaction, could be used directly for preparing its benzyl ester. Subsequent acetylation gave a fully protected derivative (**6**)[26a] in 53% yield from D-mannose (Scheme 5). This derivative was obtained in a crystalline form (mp 97—98 °C) for the first time. Thanks to the stereoselectivity of the enzyme-mediated aldol reaction, there is no need for a tedious chromatographic separation of an undesired epimer at the C-4 position of (**6**), thereby leading to a higher yield compared with that of the previous method



Scheme 5. Combined aldolase- and pyruvate decarboxylase-catalyzed synthesis of KDN and chemical conversion to its derivatives.

(35% from D-mannose).[28b]

Experimental

The melting point (mp) was measured with a Yanaco MP-S3 and was uncorrected. ^{1}H NMR spectra were measured in chloroform-*d* with tetramethylsilane as the internal standard or in D_2O with methyl β-D-glucopyranoside as the internal standard at 270 MHz with a JEOL JNM EX-270 spectrometer, or at 400 MHz with a JEOL JNM GX-400 spectrometer and JEOL α-400 spectrometer, unless otherwise stated. The optical-rotation values were recorded by using a JASCO DIP 360 polarimeter with chloroform as the solvent. Wako gel B-5F and silica gel 60 K070-WH (70-230 mesh) from Katayama Chemical Co. were used for preparative TLC and column chromatography, respectively. Malt extract, peptone, and yeast extract were purchased from Kyokuto Pharmaceutical Co., and were used for incubating microorganisms. Deionized water was used throughout experiments.

Assay Procedure for the Activity of Pyruvate Decarboxylase in Yeast Cells. For the screening of yeast with a high activity of pyruvate decarboxylase, the yeast was incubated in a medium (pH 6.5, 10 ml, in 50 ml volume conical tube) containing malt extract (1.0%), peptone (0.5%), glucose (0.2%), yeast extract (0.3%), potassium dihydrogen phosphate (0.3%), dipotassium hydrogen phosphate (0.2%), and antifoam AF emulsion [Nacalai Tesque, 10% in water, 20 μl (1 μl=10^{-6} dm^3)], for 1.5 d at 30 °C with shaking. The yeast cells from two tubes were collected by centrifugation (2000 rpm for 20 min), re-suspended in water (4 °C, 10 ml), and centrifuged again. At this stage, the dry weight of the cells was estimated by comparing its absorbance at 660 nm with an authentic sample made by bakers' yeast (Sigma, type II, YSC-2).

For the assay of pyruvate decarboxylase, the following procedure was applied. A solution of magnesium chloride [0.2 M, 20 μl] and a solution of thiamine pyrophosphate hydrochloride (abbreviated as TPP-HCl in the following text, 0.2 M, 20 μl) were added to a sodium pyruvate solution [10 ml, prepared from sodium pyruvate (11 g, 100 mmol), sodium dihydrogen phosphate dihydrate (3.9 g, 25 mmol) in water and adjusted to 250 ml of total volume]. The yeast cells, which were obtained as described above, were re-suspended with this solution, and antifoam (20 μl) was added. After its pH was adjusted to 6.0, the mixture was bubbled with air at 1000 ml min^{-1} at 30 °C. The analytical sample (200 μl) was taken periodically after 0, 1, 2, and 4 h, and the pH of the solution was readjusted to 6.0. The activity of pyruvate decarboxylase was determined as follows by combining the lactate dehydrogenase (LDH)-NADH method and carrying out a rough observation by monitoring the increase in the pH of the reaction.

The sample (200 μl) was centrifuged at 12000 rpm for 5 min, and 20 μl of the supernatant was taken. It was then diluted with phosphate buffer (0.1 M, pH 7.5, 980 μl). A portion (25 μl) of the diluted solution was taken and added to the assay solution (1.4 ml) in a micro poly(methacrylate) (l=1.0 cm) UV measurement cell. In a separated manner, the assay solution had been prepared as follows in advance. A lactate dehydrogenase solution (each 250 units) had been prepared by diluting lactate dehydrogenase (Sigma, L-1254) in a phosphate buffer (0.1 M, pH 7.5) to a concentration of 1000 units ml^{-1}; it was then divided into 250 μl portions,

and preserved in refrigerator at −80 °C. One 250 μl portion of the LDH solution and NADH (Oriental, 305-50451, ca. 3 mg) was dissolved in a phosphate buffer (0.1 M, pH 7.5, 20 ml). A portion (1.4 ml) of this solution was taken into the cell, and the absorbance (A_A) at 340 nm was confirmed to be between 1.4 and 1.6. The sample of sodium pyruvate solution (25 μl, as above) was then put into the cell and the absorbance (A_B) at 340 nm was measured after mixing; it was then left to stand at 23 °C for 3 min. The concentration of sodium pyruvate in the yeast-mediated reaction mixture was calculated based on the difference ($\Delta A = A_A \times 1.400/1.425 - A_B$) in the following equation:

$$c(\mathrm{mM}) = \Delta A/(6.22\times10^3)\times(1.425\times10^{-3})/(2.5\times10^{-5})\times10^3\times50.$$

From the change in the concentration of sodium pyruvate which remained in the yeast-mediated reaction, and the weight of the cell estimated as above, the activity of pyruvate decarboxylase per dry weight of the cell was calculated.

Effect of Cofactors. Dry bakers' yeast (Sigma type II, YSC-2, 100 mg) was suspended in water (4 °C, 20 ml) and centrifuged at 2000 rpm for 20 min at 4 °C. The supernatant was decanted off, and the residue was further washed with water (4 °C, 10 ml) in the same manner. The experiment was performed in essentially the same way as described above. In the standard case, the concentration of TPP, as added TPP–HCl, and magnesium ion, as added magnesium chloride, were 0.4 mM and 0.4 mM, respectively. The other experiments were carried out by changing the concentrations of the cofactors. The results are given in Table 1.

Large-Scale Decarboxylation of Pyruvic Acid by Bakers' Yeast. Bakers' yeast cells were pre-treated as follows. The cells (Sigma type II, YSC-2, 75 g) were suspended in water (4 °C, 360 ml) and then harvested by centrifugation at 3000 rpm for 20 min at 5 °C. This procedure was repeated three times. To a solution of sodium pyruvate (54.0 g, 491 mmol) in water (1200 ml), magnesium chloride (2.4 ml of 0.2 M solution, 0.48 mmol), TPP–HCl (2.4 ml of 0.2 M solution, 0.48 mmol), antifoam (2.4 ml), and the yeast cells (60 g, suspended in 100 ml of water) were suspended. The suspension was agitated with the bubbling of air (18000 ml min^{-1}), and the pH was kept between 5.8—6.2 by using Amberlite IR-120B (H^+ form, 20—50 mesh), as illustrated in Fig. 1 (B). The decomposition of pyruvic acid was monitored as mentioned above. After 4 h, an additional amount of yeast cells (15 g) were added again. After 6 h from the start of the reaction, the complete consumption of pyruvic acid was confirmed by combining LDH-NADH method.

Epimerization of *N*-Acetyl-D-glucosamine (4) by Calcium Hydroxide. *N*-Acetyl-D-glucosamine (**4**, 3.00 g, 13.6 mmol) was dissolved in water (12 ml). To this solution, calcium hydroxide (0.61 g, 8.2 mmol) was added with stirring until saturation. At this time, the pH of the mixture showed 11.2. The mixture was stirred at 25 °C and the sample used for a ^{1}H NMR measurement was occasionally taken from the reaction mixture. The amounts of *N*-acetyl-D-glucosamine (**4**) and *N*-acetyl-D-mannosamine (**2**) in the sample were estimated by ^{1}H NMR as follows. The sample (100 μl) was mixed with a solution of methyl β-D-glucopyranoside (270 mg in water, whose total volume was adjusted to 10 ml, 13.3 mM solution, 400 μl). Carbon dioxide gas was passed through the mixture until no further precipitation of calcium carbonate was observed. The precipitate was removed by centrifugation (12000 rpm, 5 min). From this supernatant, a portion (100 μl) was taken and concentrated in vacuo. The residue was dissolved in D_2O (0.5 ml) and concentrated in vacuo. The substitution of exchangeable protons with deuterium was carried out once again. The determination of *N*-acetyl-D-glucosamine (**4**) and *N*-acetyl-D-mannosamine (**2**) was performed by comparing the integration of signals in ^{1}H NMR with that of methyl β-D-glucopyranoside. The chemical shifts (δ) and coupling constants (J, Hz) of those signals are given below: *N*-acetyl-D-glucosamine (**4**) δ=4.68 (d, J=8.6 Hz, H-1, β-isomer), 5.17 (d, J=3.7 Hz, H-1, α-isomer); *N*-acetyl-D-mannosamine (**2**) δ=5.00 (d, J=1.7 Hz, H-1), 5.10 (d, J=1.5 Hz, H-1); methyl β-D-glucopyranoside δ=4.35 (d, J=8.1 Hz, H-1).

The determination was also possible by an HPLC analysis of the mixture after neutralization. HPLC condition: column, Tosoh AMID 80 at 75 °C; eluent, CH_3CN–H_2O (95 : 5, v/v); flow rate, 1.2 ml min^{-1}; detected at 210 nm.

In the case of sodium ion-mediated epimerization, sodium hydroxide was added to the mixture and neutralization was carried out by adding Amberite IR-120B (H^+ form, 20—50 mesh). Barium ion-mediated epimerization was performed by adding of barium hydroxide in a similar manner to that of calcium ion-mediated epimerization.

Sialic Acid (*N*-Acetylneuraminic Acid) (1). According to the reported procedure, the proportion of *N*-acetyl-D-mannosamine in the epimeric mixture was further enriched by fractional crystallization.[12,18] A mixture containing *N*-acetyl-D-mannosamine (13.3 g, 55.5 mmol) and *N*-acetyl-D-glucosamine (1.3 g, 5.5 mmol) was treated with sodium pyruvate (45.8 g, 416 mmol), sodium azide (19.2 mg, 0.3 mmol), phosphate buffer (0.1 M, 19.2 ml, pH 7.5), and Neu5Ac aldolase (Toyobo NAL-301, 150 units) in water (280 ml) at pH 7.5 and stirred at 30 °C for 4 d. The pH was occasionally adjusted to 7.5 by 10 M sodium hydroxide.

The solution of sialic acid and pyruvic acid, which had already been obtained, was treated with pyruvate decarboxylase in a similar manner as already described.

After confirming the consumption of pyruvic acid, the yeast cells were removed by centrifugation at 3000 rpm for 20 min at 5 °C; the cells were washed three times with cold water (300 ml). The pH of the combined extract was adjusted to 2.6 by using Amberlite IR-120B (H^+ form, 20—50 mesh, 300 ml); this solution was concentrated in vacuo. The residual solid was suspended in 50% aqueous THF (500 ml) and left standing overnight at 4 °C. The precipitate was removed by centrifugation at 10000 rpm for 30 min at 4 °C. The supernatant was concentrated in vacuo. The residue was dissolved in a mixture of water (25 ml) and acetic acid (75 ml), and the resultant solution was allowed to stand overnight at 4 °C. The precipitated Neu5Ac was collected by centrifugation at 10000 rpm for 30 min at 4 °C and washed successively with 75% aqueous acetic acid (100 ml), methanol (100 ml), and diethyl ether (100 ml). This was lyophilized twice from water to give sialic acid (11.0 g, 60%, 94% purity). The purity of sialic acid was determined by TBA (2-thiobarbituric acid) method.[29]

^{1}H NMR (D_2O) δ=1.88 (dd, J=12.7 and 11.7 Hz, 1H), 2.05 (s, 3H), 2.31 (dd, J=12.7 and 5.4 Hz, 1H), 3.55 (d, J=8.3 Hz, 1H), 3.61 (dd, J=11.7 and 6.4 Hz, 1H), 3.75 (ddd, J=8.3, 6.4 and 2.4 Hz, 1H), 3.84 (dd, J=11.7 and 2.4

Hz, 1H), 3.93 (dd, J=10.3 and 10.3 Hz, 1H), 4.03—4.10 (m, 2H). Its NMR spectrum was identical with that reported previously.[12)]

D-KDN Ammonium Salt (Ammonium 3-Deoxy-D-*glycero*-β-D-*galacto*-2-nonulosonate) (5). According to the procedure for sialic acid, D-mannose (10.0 g, 55.5 mmol) and sodium pyruvate were treated with Neu5Ac aldolase for 24 h at 30 °C. The yield of KDN from D-mannose reached to 83%, judging from ^{1}H NMR spectrum of the reaction mixture. The integration of the signals for KDN (δ=1.73) and methyl β-D-glucopyranoside (δ=4.36, internal standard) were compared, as stated above.

A subsequent decomposition of excess sodium pyruvate was performed in the same way as in the case of sialic acid to give KDN sodium salt (23.9 g, 32.4 mmol, 39% purity, 59% yield from D-mannose). The resulting KDN sodium salt was pure enough to prepare its derivatives. The purity of KDN sodium salt was determined by combining the LDH-NADH method toward the product that was decomposed by Neu5Ac aldolase.

The purification of KDN was achieved by column chromatography on Dowex-1 (HCO_3^- form, 20—50 mesh; 210 ml, 3 cm×30 cm) with ammonium hydrogen carbonate (0—0.2 M); the eluate was concentrated in vacuo and lyophilized to give KDN ammonium salt (5.5 g, 12.2 mmol, 22%, 85% purity) as a colorless amorphous solid. The purity of KDN ammonium salt was determined by ^{1}H NMR with an internal standard (methyl β-D-glucopyranoside, as stated above). No significant signals from organic compounds were observed by ^{1}H NMR measurements.

D-KDN ammonium salt: ^{1}H NMR (D_2O) δ=1.81 (dd, J=12.9 and 12.2 Hz, 1H), 2.10 (dd, J=12.9 and 5.1 Hz, 1H), 3.61 (dd, J=9.8 and 9.3 Hz, 1H), 3.68 (dd, J=11.7 and 6.4 Hz, 1H), 3.81 (ddd, J=9.0, 6.4 and 2.4 Hz, 1H), 3.87 (d, J=9.0 Hz, 1H), 3.90 (dd, J=11.7 and 2.4 Hz, 1H), 3.96 (d, J=9.8 Hz, 1H), 4.01 (ddd, J=12.2, 9.3 and 5.1 Hz, 1H). Its NMR spectrum was identical with that reported previously.[26a)]

Benzyl 2,4,5,7,8,9-Hexa-*O*-acetyl-D-*glycero*-β-D-*galacto*-2-nonulopyranosonate (6). D-KDN sodium salt (11.56 g, 15.5 mmol, 39% purity) was thoroughly dried by repeated concentration and lyophilization. The amorphous residue was suspended in DMF (60 ml), and benzyl bromide (9 ml, 73.7 mmol) and tetrabutylammonium iodide (480 mg, 1.3 mmol) were added. The reaction mixture was stirred for 48 h at room temperature. After the disappearance of the starting material judging from TLC [isopropyl alcohol–acetone–0.5 M lactic acid in water (5 : 5 : 4)], acetic anhydride (52 ml), DMAP (480 mg), and pyridine (60 ml) were added at 0 °C. The mixture was stirred for 12 h at room temperature, poured into ice-water (150 ml) and extracted with toluene (120 ml) five times. The organic layer was washed with water, saturated aqueous sodium hydrogen carbonate and brine, twice respectively, then dried over anhydrous sodium sulfate, and concentrated in vacuo. The residual syrup was purified on a column of silica gel (580 g, 5 cm×60 cm) with diethyl ether–hexane (3 : 2) and recrystallized from diisopropyl ether to yield **6** (8.79 g, 14.4 mmol, 53% yield from D-mannose) as colorless needles, mp 97.0—98.0 °C; see note.[30)] R_f 0.43 (silica gel, hexane–diethyl ether 1 : 5); $[\alpha]_D^{20}$ −27.5° (c 1.0, $CHCl_3$) [lit,[26a)] $[\alpha]_D^{25}$ −17.4° (c 1.0, $CHCl_3$); see note[30)]]; ^{1}H NMR ($CDCl_3$) δ=2.00 (s, 3H), 2.01 (s, 3H), 2.02 (s, 2×3H), 2.08 (dd, J=13.7 and 11.7 Hz, 1H), 2.09 (s, 3H), 2.11 (s, 3H), 2.61 (dd, J=13.7 and 5.4 Hz, 1H), 4.14 (dd, J=12.5 and 5.9 Hz, 1H), 4.19 (dd, J=10.1 and 2.4 Hz, 1H), 4.40 (dd, J=12.5 and 2.6 Hz, 1H), 4.96 (dd, J=10.1 and 9.8 Hz, 1H), 5.15 (ddd, J=6.1, 5.9 and 2.6 Hz, 1H), 5.16 (d, J=12.2 Hz, 1H), 5.21 (d, J=12.2 Hz, 1H), 5.39 (dd, J=6.1 and 2.4 Hz, 1H), 7.33—7.37 (m, 5H). Its NMR spectrum was identical with that reported previously.[26a)] Found: C, 54.85; H, 5.87%. Calcd for $C_{28}H_{34}O_{15}$: C, 55.08; H, 5.61%.

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22) The same effect was expected in the case that barium ion was used as the catalyst. Barium ion, however, turned out to be inferior to calcium ion, because the rate of epimerization of GlcNAc was similar to that catalyzed by sodium ion.

23) It has been reported that the enzyme-catalyzed aldol reaction works under the presence of excess GlcNAc.[13w] In this case, however, prior to the enzyme-catalyzed aldol reaction, the proportion of *N*-acetyl-D-mannosamine in the epimeric mixture was further enriched by fractional crystallization.[12,18]

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30) Formerly, the derivative (**6**) was reported as amorphous solid[26a] and the crystalline nature of our sample suggests the reliability of the value of specific rotation of the present sample.