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GENERAL METHODS FOR ENRICHING ALDOSES WITH OXYGEN ISOTOPES*

EDWARD L. CLARK, JR., AND ROBERT BARKER**
Division of Biological Sciences, Section of Biochemistry, Molecular and Cell Biology, Cornell University, Ithaca, NY 14853 (U.S.A.)

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ABSTRACT

Synthetic methods are described for enriching 4-, 5-, and 6-carbon aldoses with oxygen isotopes. The general approach includes exchange between $H_2{}^{18}O$ and the aldehyde group of an aldose, exchange of O-1 onto C-2 of both of the 2-epimeric aldoses formed by molybdate-resin epimerization, and chain extension using cyanide addition. These methods make possible the production of all 16 aldohexoses enriched at 5 of the 6 oxygen atoms, all 8 aldopentoses enriched at 4 of the 5 oxygen atoms, and the four aldotetroses enriched at 2 of the 4 oxygen atoms. The general applicability of these methods is illustrated by the synthesis of a group of 22 different, ^{18}O-enriched, biologically important D-aldoses having 4, 5, and 6 carbon atoms. The group includes D-[1-, 2-, 3-, 4-, and 6-^{18}O]glucose, D-[1-, 2-, 3-, 4-, and 6-^{18}O]mannose, D-[1-, 2-, 3-, and 5-^{18}O]arabinose, D-[1- and 2-^{18}O]erythrose, and D-[1- and 2-^{18}O]threose. The g.l.c.–m.s. characterization of these sugars with respect to the position and degree of ^{18}O-enrichment is reported. The potential of the methods for producing aldoses having oxygen labels at multiple positions, or aldoses labeled simultaneously with oxygen, hydrogen, and carbon isotopes is discussed.

INTRODUCTION

Monosaccharides enriched with oxygen isotopes are useful as substrates in metabolic-tracer experiments[1], as internal standards in the quantification of sugars by isotope-dilution methods, and for studying mass-spectral fragmentation-mechanisms[2], n.m.r. parameters[3,4], and kinetics and mechanisms of structural rearrangements[4]. Despite the potential utility of oxygen isotope-enriched mono-

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**To whom correspondence should be addressed.

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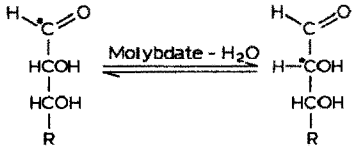
saccharides, little attention has been paid to the development of general synthetic methods. Two of the most extensive studies relied on enzymic conversions[2], or on the oxidation of isolated hydroxyl groups, isotopic exchange, and reduction[3], methods that are not generally applicable to the 4-, 5-, and 6-carbon aldoses. Serial application of the cyanohydrin synthesis[5] can be used to produce aldoses labeled at any or all positions with oxygen isotopes. However, this general applicability is compromised by the production of an epimeric pair of aldoses at each step, which leads to low yields of a specific aldose, particularly after serial applications of the synthesis.

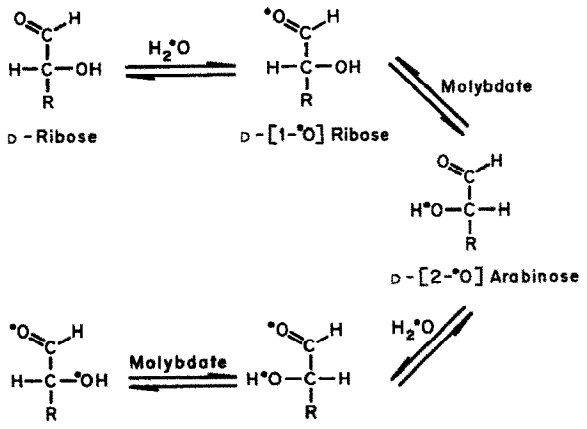
Recently, an unusual rearrangement of aldoses was discovered[6] that involves the molybdate-catalyzed inversion of the fragment comprising the first two carbon atoms of monosaccharides having four or more carbon atoms. Fragment inversion results in an epimerization of the starting aldose, as illustrated in Scheme 1.



Scheme 1.

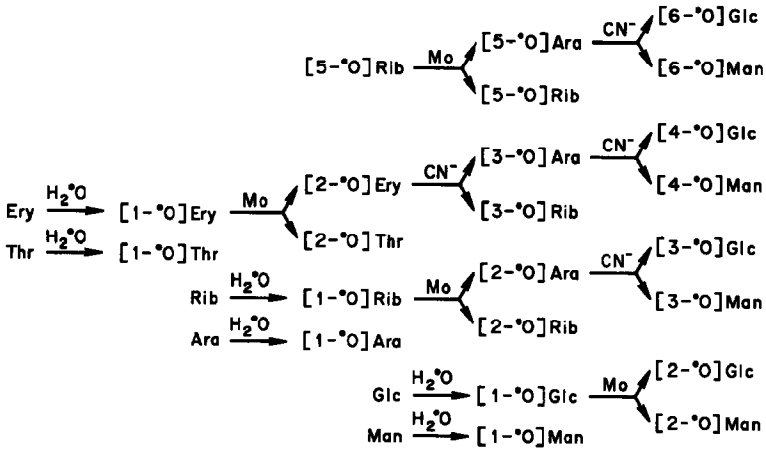
Because it had been established[6] that both carbon and hydrogen isotopes at position 1 of a starting aldose are transferred to position 2 of its 2-epimer in this epimerization, it seemed likely that this would also be the case for oxygen isotope at position 1. Should this be true, both aldoses would become labeled at position 2, as shown in Scheme 2, when the reaction is carried out in isotopically enriched water.

These considerations suggested a synthetic scheme in which molybdate



Scheme 2.

epimerization could be used in conjunction with the cyanohydrin synthesis and readily available starting-materials to produce aldo-tetroses, -pentoses, and -hexoses labeled at various sites with ^{18}O, as shown in Scheme 3.



Scheme 3.

The synthesis of the aldoses shown in Scheme 3 was undertaken in order to evaluate the potential of this approach, as it appeared to have advantages over earlier methods[2,3,5] both in general applicability and yield. Moreover, it could be adapted to the production of multi-oxygen-enriched aldoses or ketoses, or both, when combined with the cyanohydrin synthesis[5] or enzymic methods[7].

RESULTS AND DISCUSSION

Total ^{18}O-incorporation into aldoses was determined by analysis of their derived permethylated alditols using ammonia chemical-ionization (c.i.) mass spectrometry (m.s.), which gives higher yields of the protonated molecule together with the ammonia adduct. Both provided a good measure of the degree of enrichment, while electron-impact (e.i.) ionization confirmed the position of enrichment[8]. Greater than 95 atom% incorporation was demonstrated for [1-^{18}O]aldoses prepared by $H_2{}^{18}O$ exchange into the aldehydo group of monosaccharides, and for aldoses derived from D-[5-^{18}O]ribose.

Molybdate epimerizations in $H_2{}^{18}O$ produced aldoses having the total ^{18}O enrichments given in Table I. Starting with either of the nonenriched 2-epimeric aldoses, high enrichments into both epimers were achieved, except in the case of D-[2-^{18}O]ribose, where ^{18}O incorporation was 70% of that of the other compounds. This result can be understood by consideration of Scheme 2, which shows how D-[2-^{18}O]ribose was prepared. Incubation of the starting D-ribose in $H_2{}^{18}O$ before epimerization insured that the D-arabinose produced by the epimerization would be highly enriched at position 2. To insure the highest enrichment, a

TABLE I

^{18}O CONTENT OF D-ALDOSES OBTAINED BY MOLYBDATE EPIMERIZATIONS

Starting aldose	*Product aldoses*	*Incubation*		*^{18}O content (atom%)*
		Time (h)	*Temperature (°)*	
D-Erythrose	D-[2-^{18}O]threose	12	80	96
	D-[2-^{18}O]erythrose			96
D-Ribose	D-[2-^{18}O]arabinose	8	90	96
	D-[2-^{18}O]ribose			67
D-Glucose	D-[2-^{18}O]mannose	16	90	97
	D-[2-^{18}O]glucose			93

similar preincubation of the intermediate D-[2-^{18}O]arabinose from which the D-[1,2-^{18}O]ribose was derived should have been performed, but it was not. In addition, and probably more importantly, the epimerization was terminated when the arabinose:ribose ratio was 1.6:1, instead of the equilibrium value of 2.0:1. This could have left a significant fraction of the ribose that had not undergone any epimerization.

In most cases, longer epimerization will yield both 2-epimeric [1,2-^{18}O]aldoses with the same degree of ^{18}O-enrichment at both sites as that of the solvent. With the aldopentoses, however, longer reaction-times lead to a significant accumulation of other diastereomeric forms. These accounted for 1.2% of the total in the experiment reported, but can accumulate with prolonged incubation to constitute 50% of the reaction mixture. After completion of this study, an improved method for conducting aqueous molybdate epimerizations was developed that allows longer epimerizations with substantially less formation of side products. This method is described in the accompanying paper[9], and should be used with aldopentoses and whenever side-product formation needs to be minimized.

The methods described herein for the incorporation of oxygen isotope have some advantage, in terms of their more general applicability, over the approaches used by Caprioli and Seifert[2] and Gorin and Mazurek[3]. In addition, they are advantageous, in terms of product yields, over the cyanohydrin synthesis[5], in which the carbonyl oxygen atom of an aldose is exchanged with isotopically enriched H_2O, and cyanide is then added to the exchanged aldose in order to trap the oxygen isotope and produce two epimeric cyanohydrins that are reduced to epimeric aldoses that are one carbon atom longer than the starting aldose.

Although serial application of the cyanohydrin synthesis could be used to produce all manner of aldoses, having an oxygen isotope at any or all desired positions, it can be very inefficient for producing a particular ^{18}O-enriched aldose, due to the formation of aldonate and amine side-products and of the unwanted,

epimeric aldose at each step. These losses are ameliorated in the molybdate-epimerization approach used in this study. For example, D-[2-^{18}O]glucose was prepared in 68% overall yield from D-mannose, whereas an overall yield of 29% would be expected from the addition of cyanide to [1-^{18}O]arabinose. The molybdate epimerization generally produces an equilibrium between the pair of 2-epimeric aldoses that favors the biologically more important (and thus, more desirable) of the two. This is often not the case with the cyanohydrin synthesis. For example, the production of D-[2-^{18}O]glucose *via* the cyanohydrin synthesis yields D-glucose and D-mannose in the ratio of 1:2, whereas the molybdate epimerization gives them in the ratio of 5:2.

The methods described herein could be used in a similar fashion to produce many other aldo-hexoses and -pentoses. Three examples are given.

1. To produce pentoses or hexoses enriched in the primary hydroxyl group: 1,2-*O*-isopropylidene-α-D-*xylo*-pentodialdo-1,4-furanose[10] could be exchanged with $H_2{}^{18}O$ and then reduced with $NaBH_4$, to afford 1,2-*O*-isopropylidene-α-D-[5-^{18}O]xylofuranose. After hydrolysis, the resulting D-[5-^{18}O]xylose could be epimerized with molybdate, to give a mixture of D-[5-^{18}O]lyxose and D-[5-^{18}O]xylose which, in turn, could be converted into [6-^{18}O]hexoses by the cyanohydrin synthesis[11].

2. To produce aldoses having multiple, ^{18}O enrichments: [2-^{18}O]aldoses produced by molybdate epimerization could be exchanged with $H_2{}^{18}O$ at the carbonyl position, and then converted into epimeric [2,3-di-^{18}O]aldoses *via* the cyanohydrin synthesis[11].

3. To prepare aldoses enriched simultaneously with oxygen, carbon, and hydrogen isotopes: D-[1-^{13}C,1-^{2}H]glucose could be epimerized to D-[1-^{13}C,1-^{2}H,2-^{18}O]glucose and D-[2-^{13}C,2-^{2}H,2-^{18}O]mannose after epimerization by molybdate in $H_2{}^{18}O$. In the first phase of reaction, D-[1-^{13}C,1-^{2}H,1-^{18}O]glucose formed by exchange with H_2O is converted into D-[2-^{13}C,2-^{2}H,2-^{18}O]mannose, which then equilibrates with the $H_2{}^{18}O$ to afford D-[2-^{13}C,2-^{2}H,1,2-di-^{18}O]mannose. The latter is converted by molybdate into D-[1-^{13}C,1-^{2}H,1,2-di-^{18}O]glucose, so that, at equilibrium, both the glucose and the mannose epimers have ^{18}O at C-1 and C-2. The 1-^{18}O is then removed by exchange with $H_2{}^{16}O$ in the absence of molybdate.

Many other labeling arrangements can be obtained by combining the methods described in this study with cyanohydrin methods[5,11] for introducing carbon and hydrogen isotopes into aldoses and with enzymic methods for inclusion of isotopes[2] or for structural rearrangements[7].

EXPERIMENTAL

Materials. — D-Erythrose and D-threose were prepared by the methods of Perlin[12] and Ball[13], respectively.

Methyl 2,3-*O*-isopropylidene-β-D-ribofuranoside, D-ribose, and D-mannose were from Pfanstiehl Laboratories, Inc., D-glucose was from MCB Corporation,

and D-arabinose and $NaBH_4$ were from Sigma Chemical Co. Potassium *tert*-butoxide, methyl iodide, and chromium trioxide were Aldrich Chemical Co. reagents. 2-Propanol (Mallinckrodt, Inc.) was distilled after refluxing with CaH_2, and stored over 4Å molecular sieves. Pyridine (Mallinckrodt) was distilled after refluxing with BaO, and stored over 4Å sieves. AG MP-1 chloride resin (200–400 mesh) was purchased from Bio Rad Laboratories, and ammonium molybdate $[(NH_4)_6Mo_7O_{24}]$ was a Mallinckrodt reagent. Me_2SO (Fisher Scientific Co.) was refluxed with BaO, distilled at 650 Pa, and then stored under dry argon over 4Å sieves. Oxygen-18-enriched water (>95 atom% of ^{18}O) was provided by the Los Alamos Scientific Laboratory, University of California, Los Alamos, NM.

Molybdate AG MP-1 resin was prepared by eluting a column of the resin in the chloride form with 0.1M $(NH_4)_6Mo_7O_{24}$ until the column effluent gave a negative test for Cl^- with 5% $AgNO_3$ in 5M HNO_3. The $AgNO_3$ reagent produces a white precipitate with $(NH_4)_6Mo_7O_{24}$ that dissolves on vigorous shaking. After conversion of the resin was complete, the column was rinsed well with distilled H_2O. The unpacked resin was dried for 3 h at 60°, and then stored in a sealed vial over Drierite.

General methods. — The progress of molybdate epimerizations was monitored by g.l.c. analysis of the alditol acetates from the reaction products. The reaction mixture, containing suspended resin, was centrifuged at low speed in a clinical centrifuge, and a sample (0.5 μL) containing ~0.5–1 μmol of total sugar was injected into a 100-μL Reacti-Vial (Pierce) containing 60 μL of a 2 mg/mL solution of $NaBH_4$ in dry 2-propanol. After sealing the vial and mixing the contents thoroughly, the mixture was kept for 30 min at room temperature. The excess of $NaBH_4$ was decomposed by adding 25 μL of 1.7M acetic acid. Volatile reactants were removed at 65° under a N_2 stream. Boric acid was removed by mixing the dry residue with methanol (100 μL) and evaporating to dryness as before. After repeating the last step three times, the dry residue was derivatized with dry pyridine (7 μL) and acetic anhydride (10 μL) and heated for 30 min at 80°; 1-μL aliquots were injected for g.l.c. analysis. A column (2 mm × 2 m) of 3% of OV-255 on Gas Chrom Q (Applied Science) was used to separate the alditol acetates by means of a temperature program of 170 to 225° at 2°/min.

The amount of ^{18}O enrichment in the aldose products was determined by g.l.c.–m.s. analysis of their permethylated alditols using ammonia c.i.-m.s. Each aldose (~0.5 μmol) was reduced to its alditol as already described. After removal of boric acid, the dry residue was dissolved in distilled H_2O, and the solution passed through ~100 μL of Dowex-50 X-8 (H^+) resin (200–400 mesh) in a Pasteur pipet. The column effluent plus water was evaporated 2–3 times, to remove acetic acid, and the alditol syrup was mixed with absolute ethanol in a 1-dram, screw-capped vial and dried for several hours in an oven at 90°. The vial was then capped tightly and stored over Drierite. Potassium dimsyl reagent was prepared by dissolving potassium *tert*-butoxide (1 g) in dry Me_2SO (3 mL) under argon in a tightly sealed, screw-capped vial at room temperature. The clear, yellow reagent containing a

small amount of white solid was stored at 4° over Drierite, and thawed and centrifuged before use. Samples of dried alditol were permethylated by the method of Finne *et al.*[14].

Mass spectra were recorded with a Hewlett–Packard 5985 GCMS instrument equipped with a DB1 55-m, wide-bore, capillary column operated at 200°, and with a source temperature of 150°. The carrier gas was He at 2.5 mL min^{-1}. Chemical ionization was carried out with NH_3 at 45 Pa. Spectra of the permethylated alditols are simple, consisting almost entirely of the proton $(M + H)^+$ and ammonia $(M + NH_4)^+$ adduct ions. For enriched hexitols, these ions occur at *m/z* 267 and 284, respectively, and, with ^{18}O-enrichment, at *m/z* 269 and 286. For pentitols, the enriched ions occur at *m/z* 237 and 254; for tetritols, at *m/z* 205 and 222.

Pentoses were quantified by the anthrone method of Bailey[15]. Hexoses were assayed by the anthrone method of Barlett[16], and tetroses were estimated by weighing desiccated syrups.

General syntheses. — Cyanohydrin syntheses and separations of epimeric aldoses were performed according to Serianni *et al.*[11]. Total yields of aldose were typical of those reported.

Epimerizations were carried out by dissolving a dry sample of the starting aldose in a screw-cap vial in enough $H_2{}^{18}O$ to give a M solution. The vial contained a magnetic stirring bar and was tightly sealed with a screw cap and Teflon-faced, silicone-rubber septum. The starting aldose was incubated with $H_2{}^{18}O$ for various times, as detailed, and then molybdate resin (17 mg of dry resin per mmol of starting aldose) that had been rehydrated in a small volume of $H_2{}^{18}O$ was added. Epimerization was initiated by heating the stirred solution in an oil bath at 90° for hexoses and pentoses, or 80° for tetroses, for various times, as given in Table I. The reaction was monitored by removing the vial from the oil bath, cooling, centrifuging, and then withdrawing a sample (0.5–1.0 μL) of the clarified solution through the silicone septum, using a micro-syringe. Alditol acetate analysis was performed as already described, and the reaction was stopped when the equilibrium concentrations of the 2-epimeric alditols were observed.

When the reaction was complete, $H_2{}^{18}O$ was recovered by lyophilization. The dry residue was mixed with distilled H_2O, and the suspension filtered through a pad of glass wool or a GFD glass-fiber filter (Whatman) in the tip of a Pasteur pipet. After carefully rinsing the filtered resin with H_2O, the combined filtrate and rinsing were stirred with Dowex-1 X-8 (CO_3^-) resin to remove ions. The epimeric aldoses were separated on columns[11,17] of Dowex 50 (Ca^{2+}) resin.

To be certain that ^{18}O in the carbonyl oxygen atoms of the separated aldoses was removed, the pH of 0.5M aqueous ($H_2{}^{16}O$) solutions of aldose was adjusted to 1.95 with HCl, and they were heated in sealed vials for 8 h at 60°. Most of the H_2O was removed in a rotary evaporator at 40°. The volume was restored with fresh $H_2{}^{16}O$, and the solution was heated for 6 h at 60°. After cooling, the HCl was removed by neutralization with Dowex-1 (CO_3^-) resin. Aldoses prepared in this way can be used without further purification in the cyanohydrin synthesis.

Total aldose recoveries after molybdate epimerizations were typically >92%.

[1-^{18}O]Aldoses were prepared for g.l.c.–m.s. analysis by dissolving dry aldose samples in enough $H_2{}^{18}O$ to give a 0.5M solution, using $H_2{}^{18}O$ whose pH had been adjusted to 1.95 with concentrated HCl. The aldose solutions, in tightly capped vials, were then heated in an oil bath for 24 h at 60°. Samples (1 μL) were taken directly from the reaction mixture for g.l.c.–m.s. analysis of the permethylated alditols as already described.

Specific syntheses

Aldoses derived from [5-^{18}O]ribose. — Methyl 2,3-*O*-isopropylidene-β-D-*ribo*-pentodialdo-1,4-furanoside was synthesized from methyl 2,3-*O*-isopropylidene-β-D-ribofuranoside by the CrO_3–dipyridine method of Arrick *et al.*[18]. The crystalline aldehyde was stored over a desiccant if it was not to be immediately exchanged in $H_2{}^{18}O$. In a sealed vial, 400 mg of the dialdofuranoside was dissolved in $H_2{}^{18}O$ (2 mL) and dry tetrahydrofuran (0.6 mL). After 24 h at room temperature, the exchanged aldehyde was reduced by careful addition of $NaBH_4$ (75 mg) with stirring. After 2 h, the excess of $NaBH_4$ was decomposed by addition of glacial acetic acid (50 μL) and the methyl 2,3-*O*-isopropylidene-β-D-[5-^{18}O]ribofuranoside resulting was extracted 3 times with equal volumes of $CHCl_3$. The remaining $H_2{}^{18}O$ was recovered by lyophilization. The $CHCl_3$ extract was evaporated at 40°, to yield the [5-^{18}O]ribose derivative as a colorless syrup from which the isopropylidene group was removed by dissolving in 0.04M HCl (5 mL) and heating the solution for 2 h at 100°. The resulting D-[5-^{18}O]ribose solution was de-ionized with Dowex-50 (H^+) and Dowex-1 (CO_3^-) resins.

D-[5-^{18}O]Ribose (M) in $H_2{}^{16}O$ was treated with molybdate resin as described under *General syntheses*, to produce a mixture of D-[5-^{18}O]ribose and D-[5-^{18}O]arabinose. After 12 h at 90°, g.l.c. analysis of the alditol acetates showed that the reaction had reached equilibrium, with arabinose:ribose = 2:1, and with ~0.5% of xylose as a side product. The [5-^{18}O]pentoses were separated as already described, and a portion of the D-[5-^{18}O]arabinose was used to produce D-[6-^{18}O]glucose and D-[6-^{18}O]mannose *via* the cyanohydrin synthesis[11].

Aldoses derived from [2-^{18}O]aldotetroses. — Desiccated, syrupy D-erythrose was dissolved in $H_2{}^{18}O$, to give a M solution that was kept for 3 h at 80° in a sealed vial. Molybdate resin hydrated in a small volume of $H_2{}^{18}O$ was added, and the stirred solution was kept for 12 h at 80°. After recovery of $H_2{}^{18}O$ by lyophilization, the mixture was de-ionized, and the tetrose products were separated and then incubated in $H_2{}^{16}O$ to remove exchangeable ^{18}O from the anomeric center. A portion of the D-[2-^{18}O]erythrose was used to produce D-[3-^{18}O]ribose and D-[3-^{18}O]arabinose *via* the cyanohydrin synthesis[11]. After purification and separation of the pentose mixture, the cyanohydrin synthesis was applied to a portion of the D-[3-^{18}O]arabinose, to produce D-[4-^{18}O]glucose and D-[4-^{18}O]mannose.

Aldoses derived from [2-^{18}O]aldopentoses. — D-Ribose (M) in $H_2{}^{18}O$ was incubated for 12 h at 60°. After addition of molybdate resin, the stirred solution was

heated for 8 h at 90°. At this time, g.l.c. analysis of the alditol acetates showed an arabinose:ribose ratio of 1.6:1, with ~0.8% of xylose as a side product. After recovery of $H_2{}^{18}O$, the [2-^{18}O]pentoses were isolated in the usual way. After exchanging the purified pentoses by means of $H_2{}^{16}O$, a portion of the D-[2-^{18}O]arabinose was used to produce D-[3-^{18}O]glucose and D-[3-^{18}O]mannose by the cyanohydrin synthesis.

[2-^{18}O]Aldohexoses. — D-Glucose (M) in $H_2{}^{18}O$ was incubated for 24 h at 90° and then treated with molybdate resin for 16 h at 90°. The final ratio of glucose to mannose was 2.5:1.0, as determined by alditol acetate analysis. The mixture of D-[2-^{18}O]glucose and D-[2-^{18}O]mannose was purified, separated, and exchanged with $H_2{}^{16}O$ as already described.

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