STUDIES ON THE MECHANISM OF ACTION OF UDP-D-GLUCOSE DEHYDROGENASE FROM BEEF LIVER. II*

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ABSTRACT

Uridine 5′-(α-D-glucopyranosyl pyrophosphate) (UDP-D-glucose) was converted into UDP-D-glucuronic acid by pure bovine UDP-D-glucose:NAD oxidoreductase (E.C. 1.1.1.22) in $H_2{}^{18}O$. The D-glucosyluronate moiety of the UDP-D-glucuronate formed in the reaction was shown by mass spectrometry to contain ^{18}O. UDP-D-glucuronate carboxy-lyase (E.C. 4.1.1.35) was then used to convert the UDP-D-glucuronic acid (^{18}O) into UDP-D-xylose; the D-xylosyl moiety of the latter contained no ^{18}O when examined by mass spectrometry. These data indicate that, in the NAD-linked conversion of UDP-D-glucose into UDP-D-glucuronic acid, the new oxygen atom is derived from water and is located at C-6 of the D-glucosyluronate moiety of UDP-D-glucuronic acid. Furthermore, a mass-spectral fragmentation scheme is presented for per-*O*-trimethylsilyl-β-D-glucuronic acid, based on high-resolution, mass-spectral measurements.

INTRODUCTION

In an earlier publication we showed that, in the NAD-linked conversion of uridine 5′-(α-D-glucopyranosyl pyrophosphate) (UDP-D-Glc) into uridine 5′-(α-D-glucopyranosyluronic acid pyrophosphate) (UDP-D-GlcA), the C-6 hydrogen atoms are directly transferred to NAD and do not exchange with the medium[1]. To date it has been assumed that, in this reaction, water is the source of the oxygen atom in the carboxyl group of UDP-D-GlcA. This view is supported by the results presented in this paper.

MATERIALS AND METHODS

Materials. — D-Glucuronic acid, UDP-D-Glc, UDP-D-GlcA, and NAD were purchased from the Sigma Chemical Co., St. Louis, Missouri. $H_2{}^{18}O$ was obtained

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from Miles Laboratories, Kankakee, Illinois. UDP-*U*-^{14}C-D-Glc and UDP-*U*-^{14}C-D-GlcA were from New England Nuclear Corp., Boston, Massachusetts. Chlorotrimethylsilane and hexamethyldisilazane were from the Aldrich Chemical Co., Cedar Knolls, New Jersey, and phosphate diesterase and alkaline phosphatase were from the Worthington Biochemical Corp., Freehold, New Jersey All other chemicals were of reagent grade.

Methods

I. Reaction of UDP-D-Glc with UDP-D-Glc dehydrogenase in $H_2{}^{18}O$. — The reaction vessel contained the following concentrations of materials in 0.5 ml of 20 atom percent excess $H_2{}^{18}O$: 10 mM UDP-*U*-^{14}C-D-Glc (0.026 μCi/μmole), 9.4 mM NAD, and 0.1M glycylglycine buffer (pH 8.7). The control vessel contained the following in 0.255 ml of 20 atom percent excess $H_2{}^{18}O$: 11.6 mM UDP-*U*-^{14}C-D-GlcA (0.03 μCi/μmole) and 0.1M glycylglycine buffer (pH 8.7). UDP-D-Glc dehydrogenase[2] (28 IU/ml in $H_2{}^{16}O$) was added to the reaction (0.02 ml) and control (0.01 ml) vessels at time zero, and subsequent incubations were carried out at 30°. After 30 min, 0.05 ml of a solution of phosphate diesterase in $H_2{}^{16}O$ (E.C. 3.1.4.1) (2 IU/ml) was added to the reaction vessel and 0.025 ml to the control vessel. After 120 min, 0.03 ml of a suspension of alkaline phosphatase (E.C. 3.1.3.1) in 65% ammonium sulfate ($H_2{}^{16}O$, 310 IU/ml) was added to the reaction vessel while 0.015 ml was added to the control vessel. At 210 min, the contents of both vessels were lyophilized to recover the $H_2{}^{18}O$. The reaction and control mixtures were then taken up in a minimum volume of water and chromatographed for 17 h in solvent *A*; D-glucose and D-glucuronic acid were located by scanning center strips of each chromatogram for radioactivity and by staining each strip with silver nitrate. Appropriate areas were eluted with water, evaporated to dryness in a stream of nitrogen gas, and the residues were derivatized for gas–liquid chromatographic analysis.

II. Formation of UDP-D-GlcA in $H_2{}^{18}O$ *and its subsequent decarboxylation to UDP-D-Xyl.* — The reaction vessel contained the following concentrations of materials in 0.5 ml of 18.0 atom percent excess $H_2{}^{18}O$: 1.62 mM UDP-*U*-^{14}C-D-Glc (0.99 μCi/μmole), 9.7 mM NAD, and 0.1M sodium glycylglycine buffer (pH 8.7). UDP-D-Glc dehydrogenase[2], 0.02 ml (0.56 IU) was added and, after 30 min at 30°, the contents of the reaction vessel were transferred to a tube that contained 3.5 mg of NAD. Then, 0.1 ml of UDP-D-GlcA carboxy-lyase from *Cryptococcus laurentii* (0.025 IU) purified to step 3 according to Ankel and Feingold[3] was added and the resulting solution was incubated for 65 min at 37°. The following were then added to liberate the glycosyl moieties: 0.05 ml of phosphate diesterase (E.C. 3.1.4.1, 0.1 IU) in 50 mM sodium glycylglycine buffer (pH 8.7), 0.03 ml of alkaline phosphatase (E.C. 3.1.3.1, 9.4 IU) as a 65% ammonium sulfate suspension, and 0.05 ml of 0.5M sodium glycylglycine buffer (pH 8.7). After these additions, the mixture was incubated for 120 min at 30°. The entire mixture was then streaked onto Whatman 3MM paper and the components were separated by chromatography for 17 h in solvent *A* and the free sugars localized and isolated as described in the preceding section.

III. Derivatization and gas–liquid chromatography of D-*glucuronic acid,* D-*xylose, and* D-*glucose.* — For g.l.c., D-glucuronic acid, D-xylose, and D-glucose were converted into their *O*-trimethylsilyl derivatives[4]. In a typical preparation, 0.2 ml of silylating reagent (10:2:1 pyridine–hexamethyldichlorosilane–chlorotrimethylsilane) was added to the dry sample (less than 200 μg) in a 1-dram vial. To aid in derivatization of D-glucuronic acid, the mixture was alternately heated at 70° and vigorously shaken over a 4-min period. The other derivatives were prepared as described previously[4]. All derivatives were stable for at least 3 days. G.l.c. was performed at 195° in a Perkin–Elmer 900 gas chromatograph (Perkin–Elmer Corp., Norwalk, Conn.) equipped with dual hydrogen-flame detectors. The glass chromatographic column, 6 ft × 5/32 inch (i.d.), was packed with 3% OV-1 coated onto Gas-Chrom Q (60–80 mesh). The injection and manifold temperatures were 250° and the carrier gas was nitrogen. Under these conditions the retention times, relative to the solvent front, for α-D-xylose, β-D-xylose, α-D-glucose, β-D-glucose[4], α-D-glucuronic acid, and β-D-glucuronic acid were 1.57, 1.93, 3.31, 4.80, 4.37, and 5.64 min, respectively. The peaks were sharp, symmetrical, and completely separated from one another. The trimethylsilyl derivative of sodium D-glucuronate (the β isomer[5]) yielded a single gas-chromatographic peak having a retention time of 5.64 min when chromatographed as described above.

IV. Paper chromatography. — All descending paper chromatograms were run on Whatman 3MM paper. Paper strips were scanned for radioactivity in a Tracerlab 4 pi scanner (Tracerlab, Waltham, Mass.). The chromatographic solvents used were: *A* 7:1:2 propyl alcohol–ethyl acetate–water; *B* 7:3 ethanol (95%)–ammonium formate (0.5 M).

RESULTS

Source and extent of incorporation of ^{18}O *into UDP-*D-*GlcA.* — UDP-D-Glc was converted into UDP-D-GlcA in 20 atom percent excess $H_2{}^{18}O$ and the products were analyzed by combined g.l.c.–m.s. as described in *Methods*. As is shown by the data presented in the following paragraphs, the results obtained are consistent with the introduction of one atom of ^{18}O into the glycose portion of UDP-D-Glc during its conversion into UDP-D-GlcA. Table I shows the mass spectra of the pertrimethylsilyl derivatives of standard D-glucuronic acid and of the D-glucuronic acid formed during the enzymic dehydrogenation. The elemental composition of selected fragments is also shown in the table. The extent of enrichment due to ^{18}O was calculated as follows. For the unenriched species, let (i) equal the relative intensity of any mass-spectral fragment at an integral m/e value, and let $(i+2)$ equal the relative intensity of the fragment at two mass units greater than (i). For the enriched population, the terms $(i)'$ and $(i+2)'$ are used. To calculate the extent of enrichment due to the introduction of one atom of ^{18}O, a condition that must hold in the simplest case is that $(i)+(i+2)=(i)'+(i+2)'$. This relationship assumes that the base peak at m/e 217 is not enriched. Examination of the spectra in Table I indicate that this assumption is probably true. It is also necessary that there be no loss of intensity from $(i+2)'$ to

$(i+4)'$ as well as no added intensity at $(i)'$ due to a contribution from $(i-2)'$. Since the atom percent excess ^{18}O in an enriched sample is the atom percent in the enriched sample minus the atom percent in the unenriched sample (here taken as negligible), the expression $\frac{(i+2)'-(i+2)}{(i)} \times 100$ is equivalent to atom percent excess ^{18}O in the fragments at *m/e* values of 233, 292, and 375 for which the foregoing conditions are satisfied (Table I): for *m/e* 292, $(i) = 20.8$, $(i+2) = 2.7$, $(i)' = 17.2$, and $(i+2)' = 6.4$. In this case, the calculated atom percent excess is 17.8%, which agrees reasonably with the atom percent excess of the $H_2{}^{18}O$ (20 atom percent excess) in the reaction medium. A similar calculation for the enrichment at *m/e* 233 and 375 yields 18.4 and 17.9 atom percent excess. Furthermore, the mass-spectral fragments at *m/e* 233 and 292 have proposed structures (chart 1, see the following sections) that contain the carboxyl function of D-glucuronic acid. Although all the fragments at *m/e* values greater than 375 might contain ^{18}O, they cannot be used as a basis for significant calculations, since the already-mentioned summation condition does not hold for them. Repeated and slow scanning would be necessary to obtain useful data from them; however, at this level, impurities present in the samples would make interpretation difficult. Similar difficulties would be expected with the fragments at *m/e* values of 245 and 333.

The following shows that the enrichment in D-glucuronic acid occurred as a result of the enzymic reaction only. Firstly, a control reaction vessel that contained enzyme, buffer, $H_2{}^{18}O$, and UDP-D-GlcA was otherwise treated exactly as the reaction vessel. The D-glucuronic acid obtained from this solution showed no enrichment with respect to ^{18}O. This rules out a non-enzymic exchange reaction between the oxygen atoms of D-glucuronic acid and the medium. Secondly, no exchange occurred between the $H_2{}^{18}O$ in the medium and the D-glucosyl moiety of UDP-D-Glc. This was shown by isolating D-glucose from the reaction medium (the source being unreacted UDP-D-Glc) and comparing the mass spectrum of its trimethylsilyl ether with that of standard D-glucose.

Position of ^{18}O incorporated into UDP-D-GlcA. — As already demonstrated, one atom of ^{18}O is incorporated into the D-glucosyluronate moiety when UDP-D-Glc is converted into UDP-D-GlcA in $H_2{}^{18}O$. The results presented in the following section show that D-xylose prepared from the UDP-D-Xyl formed by decarboxylation of UDP-D-GlcA with UDP-D-GlcA carboxy-lyase contains no excess ^{18}O. This establishes the position of the ^{18}O at C-6 of the D-glucosyluronate moiety.

As described in *Methods*, the D-glucosyl moiety of UDP-D-Glc was converted into D-xylose via a series of enzymic reactions, all conducted in $H_2{}^{18}O$. This D-xylose was shown by mass spectrometry to be unenriched with respect to ^{18}O. Furthermore, all of these reactions, with the exception of the conversion of UDP-D-Glc into UDP-D-GlcA, went to completion, as judged by paper chromatography of aliquots of the reaction mixture at the appropriate time-intervals. To demonstrate that there was no exchange of ^{18}O between chromatographic solvent *A* and D-xylose, this solvent was prepared with 22.5 atom percent excess $H_2{}^{18}O$. There was no observable incorpor-

TABLE I

MASS SPECTRUM OF PER-*O*-TRIMETHYLSILYL-β-D-GLUCURONIC ACID

m/e	*Elemental composition*	*Relative intensities*[a]		m/e	*Elemental composition*	*Relative intensities*[a]	
		I	*II*			*I*	*II*
72		3.2	3.3	291		2.8	2.8
73		80.5	75.0	292	$C_{11}H_{28}Si_3O_3$	20.8	17.2
74		15.5	16.4	293		5.8	5.5
75		16.6	19.1	294		2.7	6.4
101		1.9	1.9	295		0.57	1.8
103	$C_4H_{11}SiO$	5.1	5.4	303		21.7	19.1
116		2.8	3.3	304		6.3	6.0
129	$C_5H_9SiO_2$	5.1	5.0	305	$C_{12}H_{29}Si_3O_3$	3.1	5.6
131		3.9	3.9	306		0.65	1.5
133		8.1	8.3	307		0.21	0.58
143		8.1	7.5	331		1.7	1.6
147	$C_5H_{15}Si_2O$	41.5	42.0	333	$C_{13}H_{29}Si_3O_4$	4.1	4.3
148		7.0	7.3	334		1.2	1.3
149		4.9	6.7	335		1.9	2.5
169		3.2	3.3	359	$C_{14}H_{27}Si_3O_5$	2.2	2.0
189	$C_7H_{17}Si_2O_2$	6.3	6.6	361		0.46	0.99
190		1.9	1.9	375	$C_{15}H_{31}Si_3O_5$	1.4	1.2
191	$C_7H_{19}Si_2O_2$	29.3	30.7	376		0.46	0.58
192		5.5	7.1	377		0.46	0.71
193		3.0	3.3	378		0.11	0.21
204	$C_8H_{20}Si_2O_2$	81.5	84.5	407		0.44	0.53
205		17.3	17.5	409		0.12	0.20
206		7.9	7.9	421	$C_{16}H_{37}Si_4O_5$	0.71	0.78
207		2.5	2.7	422		0.32	0.35
217	$C_9H_{21}Si_2O_2$	100.0	100.0	423		0.18	0.34
218		21.3	21.6	424		0.05	0.14
219		9.7	12.0	449	$C_{17}H_{37}Si_4O_6$	0.48	0.47
221		3.0	2.9	450		0.19	0.20
232		2.5	2.1	451		0.11	0.24
233	$C_9H_{21}Si_2O_3$	10.5	8.6	452		0.03	0.08
234		2.3	2.1	464	$C_{18}H_{40}Si_4O_6$	0.14	0.14
235		0.97	2.9	466		0.04	0.07
243		1.1	1.1	539	$C_{20}H_{47}Si_5O_7$	0.38	0.35
245	$C_{10}H_{21}Si_2O_3$	2.0	1.9	540		0.18	0.19
247		0.33	0.52	541		0.11	0.18
257		1.7	1.9	542		0.06	0 10

[a]I: Standard β-D-glucuronic acid. II: β-D-glucuronic acid obtained from enzymic conversion of UDP-D-Glc into UDP-D-GlcA in $H_2{}^{18}O$ (20 atom percent excess). An aliquot of the pyridine solution containing the appropriate per-*O*-trimethylsilyl-D-glucuronic acid was injected into a glass column, 6 feet × 1/8 inch, packed with 3% OV-101 that was coated onto Gas Chrom Q (60–80 mesh) and maintained at 210°. The column was operated in an LKB 9000 combined gas–liquid chromatograph and mass spectrometer. Spectra were recorded at 70 eV. The electron current and accelerating voltage were 60 μA and 3.5 kV, respectively. The molecular separator and ion-source temperatures were 250° and 270°, respectively. After the spectra had been corrected for background, the table was prepared by assigning an intensity of 100% to the ions having *m/e* values of 217. The values obtained from two spectra were averaged in each instance. The appropriate *m/e* values were assigned to the various fragments by calibration of the spectrometer with perfluorokerosene. Fragments having relative intensities of less than 1% were omitted from the table unless they appeared to be enriched

ation of ^{18}O into the sugar after incubation of D-xylose (0.62%, *w/v*) in the ^{18}O-enriched solvent for 24 h at room temperature. As the final isolation of D-xylose from the paper chromatogram was accomplished by elution with water and subsequent evaporation of the eluent, another control experiment was carried out. In this instance a 0.012M solution of D-xylose was prepared in 22.5 atom percent excess $H_2{}^{18}O$ and kept for 3.5 h at 25°, the time necessary to elute D-xylose and evaporate the solvent. Under these conditions a small enrichment (1.5–4.7 atom percent excess) was seen in several mass-spectral fragments obtained from the pertrimethylsilylated D-xylose. This enrichment probably results from an exchange of the C-1 oxygen atom of D-xylose with solvent ^{18}O. This is consistent with enrichment of the fragment at *m/e* 193 (3.5 atom percent excess) by a contribution from *m/e* 191. This is significant because *m/e* 191 also occurs in the mass spectra of both pertrimethylsilylated α-D-glucose and β-D-glucuronic acid. As shown by DeJongh *et al.*[6] for α-D-glucose, this fragment has the structure $(CH_3)_3SiO\text{–}\overset{1}{C}H{=}\overset{+}{O}Si(CH_3)_3$ where the C-1 ring atom is retained together with its $(CH_3)_3SiO$ grouping. In all cases, a metastable peak is present at *m/e* 27.9 for the decomposition of this fragment to *m/e* 73 $(\overset{+}{S}i(CH_3)_3 + (CH_3)_3SiO\overset{1}{C}H{=}O$. In any event, the extent of enrichment found in D-xylose is much too small to account for the complete absence of ^{18}O in the material isolated from the reaction medium.

The mass spectrum of pertrimethylsilyl β-D-xylose is similar to that of other trimethylsilylated carbohydrates[6] in that a molecular ion of very low intensity may be seen at *m/e* 438, together with the characteristic $M^+ - 15$ peak at *m/e* 423. Other common fragments having high intensities appear at *m/e* values of 73, 147, 191, 204, and 217.

Mass-spectral fragmentation pattern of pertrimethylsilylated β-D-glucuronic acid. — The elemental compositions of eighteen of the mass spectral fragments obtained from pertrimethylsilylated β-D-glucuronic acid were determined by high-resolution mass spectrometry and are shown in Table I. These measurements support the following fragmentation-scheme.

Characterization of the trimethylsilylated derivative of β-D-glucuronic acid as the pertrimethylsilylated species (mol.wt. 554) is based on the existence of the fragment at *m/e* 539 which corresponds to the loss of $\cdot CH_3$ from the molecular ion (M^+), as noted by DeJongh *et al.*[6] in the spectrum of pertrimethylsilylated D-glucose and related carbohydrates. Several other fragments characteristic of the mass spectra of pertrimethylsilylated carbohydrates[6] also appear. One of these is *m/e* 464, which

with respect to ^{18}O, in which case both the enriched species and its precursor are shown. High-resolution, mass-spectral data were obtained from an AEI MS-9 mass spectrometer by peak matching versus perfluorokerosene. The sample was maintained in the direct-insertion probe and spectra were recorded at an ionizing potential of 70 eV and an ionizing current of 100 mA. The resolution was 10,000 (10% valley). Fourteen peaks were within ±1.0 m mass units of the calculated value and four peaks were within ±2.7 m mass units.

corresponds to $M^+ - TMSiOH$ mol.wt. = 90). This loss is probably followed by further elimination of ·TMSiO to yield *m/e* 375. The loss of $\cdot CH_3$ from M^+ to yield *m/e* 539 might be followed by elimination of (O=CH–OTMSi) from C-1 to yield *m/e* 421. The stepwise elimination of two molecules of TMSiOH from *m/e* 539 would yield fragments at *m/e* 449 and 359.

The fragments at *m/e* 217 and 305 are also present in the spectrum of pertrimethylsilylated α-D-glucose; in the latter case each fragment has been proposed[6] to contain C-2, C-3, and C-4 of the glucose ring. Also present in the spectrum obtained from D-glucuronic acid is a fragment at *m/e* 204; with D-glucose this fragment originates mainly from C-2–C-3 and C-3–C-4. The presence of a metastable peak at *m/e* 175.1 suggests the decomposition of *m/e* 204 to *m/e* 189 by loss of a methyl group. Another metastable peak at *m/e* 27.9 would account in part for the formation of *m/e* 73 $[(CH_3)_3\overset{+}{S}i]$ from *m/e* 191, just as it does in the case of D-glucose. The ion at *m/e* 147 is commonly obtained in the mass spectra of carbohydrates[6] and its proposed structure is $[(CH_3)_3SiO\overset{+}{S}i(CH_3)_2]$. The fragment at *m/e* 103 $[CH_2{=}\overset{+}{O}Si(CH_3)_3]$ must contain one carbon atom of the ring as well as one rearranged hydrogen atom.

The fragments at *m/e* values of 233, 245, 292, and 333 are of considerable interest in the spectrum of pertrimethylsilylated D-glucuronic acid, as all of the proposed structures (Chart I) contain the carboxyl group. To aid in visualizing the formation of these four fragments during the fragmentation process, the structure of pertrimethylsilyl D-glucuronic acid also is presented in Chart I.

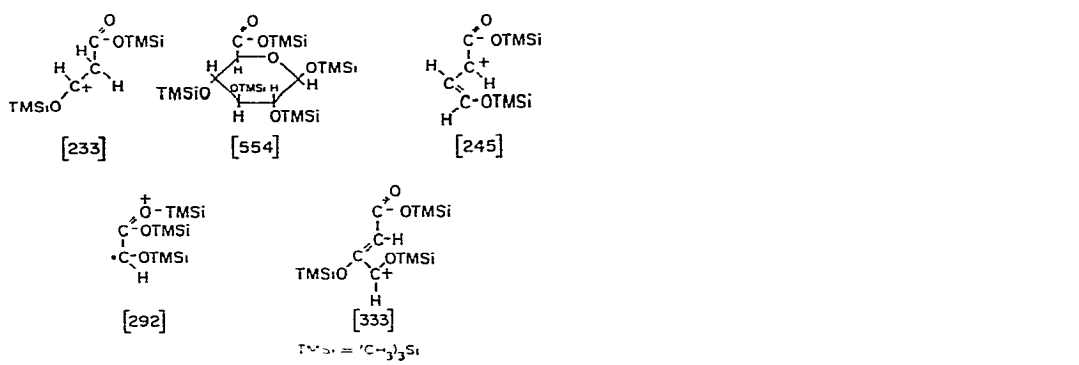

Chart I. The chart illustrates four mass-spectral fragments obtained from per-*O*-trimethylsilyl-β-D-glucuronic acid (mol. wt. 554) that are proposed to contain the carboxyl group. The elemental compositions of these fragments are supported by high-resolution, mass-spectral measurements.

DISCUSSION

D-Glucuronic acid, obtained from UDP-D-GlcA (prepared by dehydrogenation of UDP-D-Glc in 20 atom percent excess $H_2{}^{18}O$) contains approximately 18 atom percent excess ^{18}O. These data indicate that each molecule of D-glucuronic acid

contains one atom of oxygen from the solvent. As no label is present in UDP-D-Xyl obtained from the UDP-^{18}O-D-GlcA, the ^{18}O must have been located in the carboxyl group of UDP-D-GlcA. In other words, during the conversion of UDP-D-Glc into UDP-D-GlcA, one oxygen atom of the carboxyl group is derived from water.

During the course of our studies, Nelsestuen and Kirkwood[7] demonstrated that UDP-α-D-*gluco*-hexodialdose (UDP-D-Glc-6-CHO) is an intermediate in the conversion of UDP-D-Glc into UDP-D-GlcA by bovine liver dehydrogenase. This indicates that the enzymic reaction begins by direct transfer of a C-6 hydrogen atom of UDP-D-Glc to NAD. Subsequent loss of a C-6 hydroxyl hydrogen ion to the medium would result in the formation of UDP-D-Glc-6-CHO. From this point there are at least three mechanisms by which the aldehyde could be converted into UDP-D-GlcA. The first requires that the aldehyde become hydrated. The resulting diol would then be dehydrogenated to yield the carboxylic acid. The second mechanism requires that the aldehyde react with a thiol group of the enzyme to form a hemiacetal, as in the case of glyceraldehyde 3-phosphate dehydrogenase[8]. The thioester resulting from oxidation of the latter would then be hydrolyzed to yield the free acid. The third mechanism calls for the direct removal of a hydride ion from the aldehyde and its subsequent replacement by a hydroxyl ion from the medium. This mechanism is most probably operative in the action of human liver aldehyde dehydrogenase[9] as well as of xanthine oxidase obtained from milk[10], in which the free carboxyl forms of the aldehydes employed are the true substrates. From the data presently available, it is not possible to determine which mechanism is involved in the reaction catalyzed by UDP-D-Glc dehydrogenase.

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