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Conversion of D-Mannuronolactone into D-Mannaric Acid in Mammalian Systems[1]

MICHIO MATSUI, MASASHI OKADA,[2] and MORIZO ISHIDATE[2a]

Tokyo Biochemical Research Institute

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D-Mannaric acid (II) was isolated and identified from human urine after oral administration of D-mannuronolactone (I). It was also isolated and identified from the incubation mixture containing I, nicotinamide adenine dinucleotide (NAD) and D-glucuronolactone dehydrogenase preparation purified from guinea pig liver, thus demonstrating the conversion of I into II in mammalian systems likewise the conversion of D-glucuronolactone (III) into D-glucaric acid (IV).

In connection with our study[3] on the conversion of a number of monosaccharides and their derivatives dosed to man, rat and guinea pig into D-glucaric acid (GA) (IV) in urine, it has been found that administration of D-mannuronolactone (ML) (I) to these mammals resulted in a marked increase in urinary hexaric acid excretion when assayed by the chemical method,[4] as shown in Table I. This finding strongly suggested that ML could be converted into D-mannaric acid (MA) (II) in mammalian systems likewise the conversion of D-glucuronolactone (GL) (III) into GA, which was demonstrated recently.[5] This paper deals with

TABLE I. Urinary Excretion of Hexaric Acid after Administration of D-Mannuronolactone (ML) in Different Species

Species	No.	Dose (mg)	Method of administration	Hexaric acid in urine (mg/24 hr)[a] Before ML	After ML 0—24 hr	After ML 24—48 hr
Man	1	1000	*p.o.*[b]	15	208	29
Man	2	1000	*p.o.*[b]	16	256	—
Man	3	2000	*p.o.*[b]	15	340	—
Rat	4	200	*i.p.*[c]	1.33	12.0	1.23
Rat	5	200	*i.p.*[c]	1.17	12.8	1.09
Rat	6	200	*i.p.*[c]	1.32	11.9	1.20
Guinea pig	7	200	*i.p.*[c]	1.16	7.9	0.76
Guinea pig	8	200	*i.p.*[c]	0.89	13.7	0.62
Giunea pig	9	200	*p.o.*[b]	0.95	6.8	1.10
Guinea pig	10	200	*p.o.*[b]	1.10	7.5	2.08
Guinea pig	11	200	*p.o.*[b]	1.32	9.3	1.90

a) Hexaric acid was determined by the chemical method reported earlier.[4] As indicated therein, the method is not specific for D-glucaric acid but is applicable to hexaric acid in general.
b) *per os*
c) intraperitonial injection

1) This work was presented at the 85th Annual Meeting of the Pharmaceutical Society of Japan, Tokushima, Oct. 1965. A preliminary account of some of the present data appeared in *J. Biochem.* (Tokyo), **57**, 715 (1965).
2) Location: *Takada 3-chome, Toshima-ku, Tokyo*; *a*) Present address: *National Institute of Hygienic Sciences, Tamagawayoga-machi, Setagaya-ku, Tokyo.*
3) M. Matsui, M. Okada, and M. Ishidate, *Chem. Pharm. Bull.* (Tokyo), **17**, 1064 (1969).
4) M. Ishidate, M. Matsui, and M. Okada, *Anal. Biochem.*, **11**, 176 (1965).
5) C.A. Marsh, *Biochem. J.*, **86**, 77 (1963).

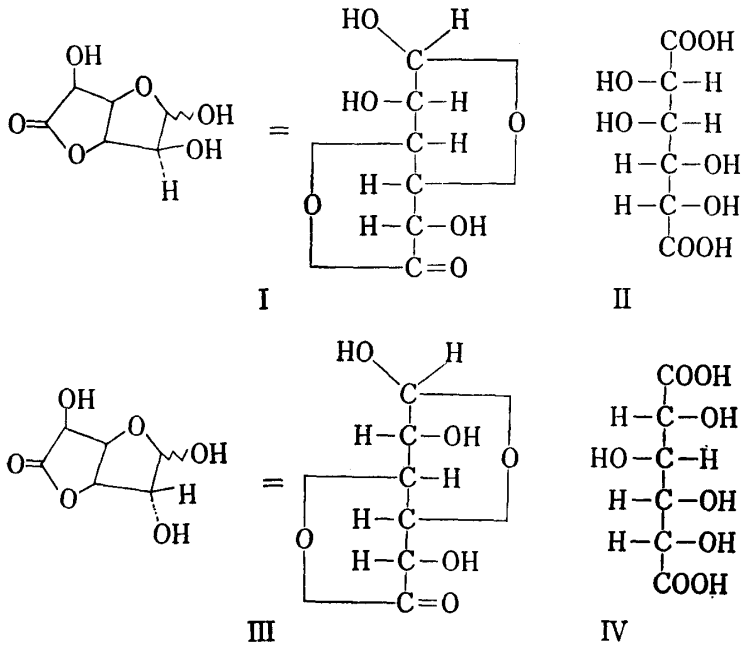

Chart 1

isolation and identification of MA from human urine after oral administration of ML, as well as from incubation mixture of ML with an enzyme preparation of D-glucuronolactone dehydrogenase[6] which has recently been purified from guinea pig liver,[7] thus demonstrating actual conversion of ML into MA in mammalian systems.

Besides the above finding indicated in Table I, paper electrophoretic examination of urines of man, rat and guinea pig collected after dosage of ML showed a spot corresponding to hexaric acid (MA or GA) together with a large spot corresponding to uronic acid (D-mannuronic or D-glucuronic acid) as shown in Figure 1, while normal urines of these mammals did not reveal the presence of any hexaric acid on paper electrophoresis. Furthermore, careful chromatographic separation of hexaric acid (MA, GA and D-galactaric acid) by one step elution on Dowex 1 X-8 borate column[4] using 0.05M sodium borate-0.02M sodium sulfate as eluant revealed that urines obtained after loading of ML exhibited quite similar peak corresponding to MA in their elution curves as shown in Figure 2, although clear-cut resolution among the hexaric acids could not be accomplished. In addition, it was found that these urines did not indicate any increase in the component (GA) compared with normal urines, which could be estimated by the enzymic assay[5] based on β-glucuronidase[EC 3.2.1.31]inhibition. From these facts, it was highly probable that the marked increase in urinary hexaric acid excretion observed after administration of ML to man, rat and ginea pig could be ascribable to MA.

Thus, isolation and identification of MA were carried out using an urine sample (No. 3 in Table I) collected from a normal human adult who had received 2 g of ML, as starting material. This 24-hour urine was estimated to contain about 340 mg of hexaric acid by the chemical assay.[4] Approximately one-tenth of the urine was subjected to column chromatography for the separation of the hexaric acid using Dowex 1 X-8 borate as reported earlier.[4] The hexaric acid fraction eluted with 0.05M sodium borate-0.1M sodium sulfate was further subjected to chromatography on Dowex 1 X-8 formate column using formic acid as eluant, in order to remove sodium and borate ions. Paper electrophoretic examination of the hexaric acid fraction thus obtained showed a spot with the same mobility as that of MA or GA (Fig. 1). Since this hexaric acid could not be crystallized it was treated with phenylhydrazine hydroch-

6) C.A. Marsh, *Biochem. J.*, **87**, 82 (1963).
7) R. Sadahiro, Y. Hinohara, A. Yamamoto, and M. Kawada, *J. Biochem.* (Tokyo), **59**, 216 (1966).

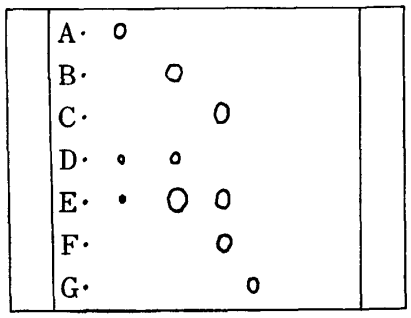

Fig. 1. Paper Electrophoresis of Urines after Administration of D-Mannuronolactone (ML) and of Hexaric Acid Fractions obtained from the Urines

A: sorbitol
B: uronic acid (D-mannuronic or D-glucuronic acid)
C: hexaric acid (D-mannaric or D-glucaric acid)
D: normal urines of man, rat, and guinea pig[a)]
E: urines after administration of ML to man, rat, and guinea pig[a)]
F: hexaric acid fractions obtained by column chromatography from urines after administration of ML to man, rat, and guinea pig
G: tartaric acid
a) Since urines of rat and guinea pig gave entirely similar figure to that of human urine, only the latter was indicated here.

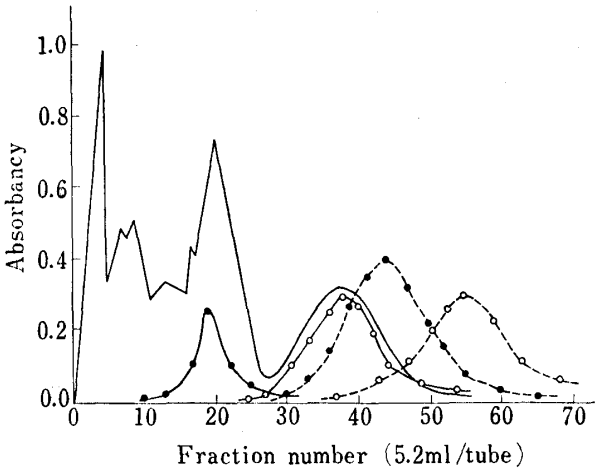

Fig. 2. Chromatography of D-Hexaric Acids and Urines after Administration of D-Mannuronolactone (ML) on Dowex 1 X-8 Borate using 0.05M $Na_2B_4O_7$–0.02M Na_2SO_4 as Eluant

——: human urine collected after administration of ML[a)]
—○—: D-mannaric acid
---●---: D-glucaric acid
---○---: D-galactaric acid
—●—: D-mannuronic acid
a) Since the patterns of urines after administration of ML to rat and guinea pig were essentially similar to that of the human urine, only the latter was shown here.

loride and sodium acetate to give a crystalline product, mp 214—216° (decomp.), which was shown to be identical with MA bisphenylhydrazide by direct comparison with authentic sample.[8)]

In the course of his studies[5,6,9,10)] on the conversion of GL into GA, Marsh examined substrate specificity of the enzyme, D-glucuronolactone dehydrogenase [EC 1.1.1. 70], which is involved in the conversion of GL into GA, and found that among several monosaccharides tested *in vitro* only GL was substrate for the enzyme while ML was not.[6,9)] Because conversion of ML into MA in man and other mammals was demonstrated as described above, this finding of Marsh concerning substrate specificity of D-glucuronolactone dehydrogenase was open to question. In the mean time, Sadahiro, *et al.*[7)] purified the enzyme from guinea pig liver and indicated that ML could be dehydrogenated by the same enzyme in the presence of nicotinamide adenine dinucleotide (NAD) which catalyzes the conversion of GL into GA.

Then, an attempt was made to isolate and identify MA from incubation mixture containing ML, NAD and the D-glucuronolactone dehydrogenase preparation. The incubation was carried out using procedure described in "Experimental" section which was essentially similar to that employed in the conversion of GL by rat liver preparation into GA.[6)] Paper electrophoretic examination of the incubation solution after dialysis revealed a spot corresponding to MA or GA together with a large spot corresponding to D-mannuronic or D-glucuronic acid as shown in Figure 3. Furthermore, the elution curve of the diffusate exhibited a sharp and entirely similar peak corresponding to MA when it was subjected to one step elution chromatography on Dowex 1 X-8 borate using 0.05M sodium borate–0.02M sodium sulfate as eluant, as indicated in Figure 4.

8) E. Fischer, *Ber.*, **24**, 539 (1891).
9) C.A. Marsh, *Biochem. J.*, **89**, 108 (1963).
10) C.A. Marsh, *Biochem. J.*, **99**, 22 (1966).

Chromatographic separation of MA fraction from the diffusate was performed on Dowex 1 X-8 columns in the same manner as outlined above with the urine. The hexaric acid fraction thus obtained revealed a spot with the same mobility as MA or GA (Fig. 3). Treatment of the hexaric acid with phenylhydrazine afforded a crystalline product identical with MA bisphenylhydrazide.

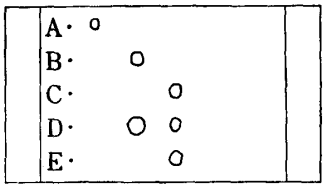

Fig. 3. Paper Electrophoresis of the Incubation Solution and of the Hexaric Acid Fraction obtained from the Incubation Solution

A: sorbitol
B: uronic acid(D-mannuronic or D-glucuronic acid)
C: hexaric acid (D-mannaric or D-glucaric acid)
D: incubation solution after dialysis
E: hexaric acid fraction obtained by column chromatography from the incubation solution

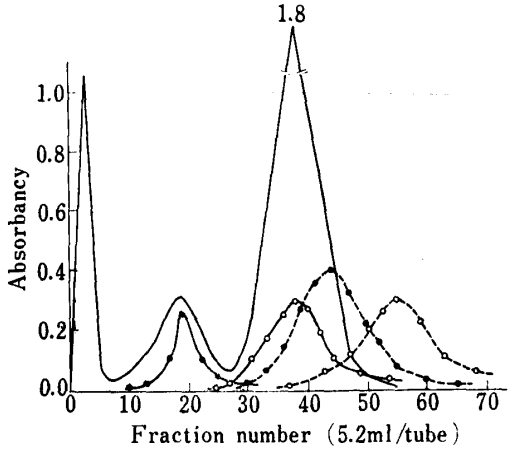

Fig. 4. Chromatography of the Incubation Solution on Dowex 1 X-8 Borate using 0.05M $Na_2B_4O_7$–0.02M Na_2SO_4 as Eluant

——— : incubation solution after dialysis and concentration
—○— : D-mannaric acid
---●--- : -D-glucaric acid
---○--- : D-galactaric acid
—●— : D-mannuronic acid

From the results presented in this paper, it has been definitely established that ML is metabolized to MA in man and other mammals likewise the metabolism of GL to GA. Moreover, it has been demonstrated that contrary to the finding of Marsh D-glucuronolactone dehydrogenase purified from guinea pig liver is not specific for GL and can oxidize also ML to MA in the presence of NAD, thus adducing evidence that the enzyme is actually involved in the conversion of ML into MA in mammals. The Marsh's finding on substrate specificity of D-glucuronolactone dehydrogenase is based principally on his experimental result obtained by using an enzymic assay depending on β-glucuronidase inhibition, which could really hold good for GA or D-galactaric acid, not for MA.

Experimental

Determination of D-Mannaric Acid (MA)(II)——Determination of MA in urine and incubation mixture was performed according to "Procedure I" of the method reported earlier[4] except that oxidation time with HIO_4 was extended to 30 min in place of 15 min, since ML resisted to the oxidation as compared with GA.

Paper Electrophoresis——Toyo Roshi No. 51, 12.5×26 cm; solvent, 0.05M Na_2CO_3; 90 min runs at 9 V/cm (200 V and 8—10 mA). Spots were revealed by benzidine–HIO_4 reagent after spraying paper with 10% AcOH followed by drying.

Removal of Sodium and Borate Ions from Hexaric Acid Fraction separated on Dowex 1 X-8 Borate Column——A column of dimensions 1.0 cm (diameter)×23 cm was packed with 6.0 ml of Dowex 1 X-8 (200—400 mesh) formate. Twenty ml of hexaric acid fraction separated on Dowex 1 X-8 borate column according to the procedure described elsewhere[4] was put on the column cited above. First 40 ml of 0.5N formic acid was passed through the column to remove sodium and borate ions, and then hexaric acid was eluted with 50 ml of 2N formic acid. The effluent was concentrated under reduced pressure at about 50° to a small volume and then assayed. Recoveries of GA as well as MA added to urines were found to be approximately 90%.

Isolation and Identification of MA from Human Urine after Oral Administration of D-Mannuronolactone (ML)——After oral administration of 2 g of ML to normal male human adult 24-hour urine (1970 ml) (No. 3

in Table I) was collected without any preservative, which was estimated by the chemical assay to contain about 340 mg of MA. It revealed spots corresponding MA as well as D-mannuronic acid on paper eletrophoresis (Fig. 1). When 0.6 ml of this urine was subjected to one step elution chromatography on Dowex 1 X-8 borate column using 0.05M sodium borate–0.02M Na_2SO_4 as eluant, the elution curve indicated in Fig. 2 was obtained.

On the other hand, 3.5 ml of this urine containing about 600 μg of MA was first subjected to column chromatography using Dowex 1 X-8 borate according to the procedure reported earlier[4] to afford 20 ml of MA fraction eluted with 0.05M sodium borate–0.1M Na_2SO_4, which was further chromatographed on Dowex 1 X-8 formate as outlined above. Paper electrophoretic analysis of MA fraction thus obtained showed one spot corresponding to MA (Fig. 1). The above separation procedure using 3.5 ml of urine was repeated fifty times, thus amounting to 175 ml. The pooled MA fraction was concentrated under reduced pressure at about 50° to give a light yellow sirup, which was expected to contain about 30 mg of MA. As the sirup could not be crystallized it was dissolved in water (2 ml). The solution was heated with phenylhydrazine (200 mg) and anhydrous AcONa (50 mg) for 2.5 hr at 100°. After cooling the reaction mixture was kept overnight in a refrigerator to afford light yellow plates (21.8 mg) which were washed with 99% EtOH and dried, mp 214—216° (decomp.). The melting point did not change by recrystallization from dimethylformamide–EtOH. *Anal.* Calcd. for $C_{18}H_{22}O_6N_4$: C, 55.38; H, 5.68; N, 14.35. Found: C, 55.22; H, 5.47; N, 14.55. The melting point of the mixture with authentic MA bisphenylhydrazide,[8] mp 214—216° (decomp.), showed no depression and IR spectra of the two samples were identical in all respects. For comparison, GA bisphenylhydrazide, mp 208—210° (decomp.), and D-galactaric acid bisphenylhydrazide, mp 236—238° (decomp.), were prepared by similar methods. They found to be different from the MA bisphenylhydrazide obtained from the urine in mixed melting point and comparison of IR spectrum.

Conversion of ML by Guinea Pig Liver Preparation into MA——A partially purified guinea pig liver preparation[7] (42 ml, about 2 mg/ml protein) was incubated for 10 hr at 37° with ML (50 mM, 25 ml), NAD (20 mM, 10 ml) and 0.2M phosphate buffer (pH 7.2, 10 ml). This incubation mixture was dialyzed twice against each 500 ml of water for 15 hr at 0°. The diffusate revealed two spots corresponding to MA and D-mannuronic acid on paper electrophoresis (Fig. 3). It was concentrated under reduced pressure to 35 ml containing about 20 mg of MA in the chemical assay. When 1.0 ml of this concentrated diffusate was subjected to one step elution chromatography on Dowex 1 X-8 borate column using 0.05M sodium borate–0.02M Na_2SO_4 as eluant, the elution curve indicated in Fig. 4 was obtained.

On the other hand, 1.0 ml of the concentrated diffusate containing about 600 μg of MA was subjected to column chromatography using Dowex 1 X-8 borate and formate successively as outlined above with urine. This separation procedure was repeated thirty times, thus totaling to 30 ml. The pooled MA fraction was concentrated under reduced pressure to afford a yellow sirup. It was then treated with phenylhydrazine hydrochloride (160 mg) and anhydrous AcONa (40 mg) in the same way as outlined above to give light yellow plates (8 mg), mp 215—217° (decomp.), identical with MA bisphenylhydrazide in mixed melting point and comparison of IR spectrum.

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