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Separation of the Intermediates of D-Glucuronic Acid Metabolism by Paper Chromatography

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A selective descending paper chromatographic method was developed for the separation of intermediates (D-glucopyranuronic acid, D-glucofuranurono-6,3-lactone, L-gulonic acid, L-gulono-1,4-lactone, D-glucaric acid, D-glucaro-1,4-lactone, D-glucaro-6,3-lactone, D-glucaro-1,4-6,3-dilactone, L-ascorbic acid, and *myo*-inositol) in D-glucuronic acid metabolism. It was necessary to use two different development systems because lactone intermediates are labile in alkaline solvents whereas separation of acids requires basic solvent systems. By multiple chromatography perpendicular to paper fibres better resolution was obtained than by conventional development.

In mammals free D-glucopyranuronic acid (D-glucuronic acid) is derived from α-D-glucopyranuronic acid 1-phosphate (α-D-glucuronic acid 1-phosphate), β-D-glucopyranosiduronic acids (glucuronides), *myo*-inositol and polysaccharides. D-Glucuronic acid is converted *in vivo* into either D-glucofuranurono-6,3-lactones (D-glucurono-6,3-lactone) or L-gulonic acid. These can be metabolized to L-gulono-1,4-lactone. D-Glucurono-6,3-lactone is converted into D-glucaric acid *via* D-glucaro-1,4-6,3-dilactone, D-glucaro-1,4-lactone, and D-glucaro-6,3-lactone. L-Gulono-1,4-lactone is the substrate of 3-oxo-L-gulofuranolactone (L-ascorbic acid) biosynthesis in which 2-oxo-L-gulono-1,4-lactone is an intermediate. L-*threo*-2-Pentulose (L-xylulose) is synthesized from L-gulonic acid, 3-oxo-L-gulonic acid being a labile intermediate (for reviews, see Refs. 1 – 3).

The lability of lactones [4] and L-ascorbic acid [5] and the close structural similarity of the metabolites has hampered their separation and quantification. Methods described in the literature are useful only in studies of one or at most a few pathway intermediate. Ion exchange methods have been developed for the separation of L-ascorbic acid,[6] D-glucuronic and L-ascorbic acids,[7] L-gulonic [8] and D-glucaric acids [9] from urine. L-Ascorbic acid and *myo*-inositol have been separated by paper chromatography from the other D-glucuronic acid metabolites in reaction mixtures incubated with tissue slices and homog-

enates.[10] Fujita and Asakura [11] have described an ion exchange method for the separation of pentoses and acidic metabolites of the D-glucuronic acid pathway in liver homogenates. However, no method has been devised by which all the intermediates in glucuronic acid metabolism could be separated.

We have studied the behavior of D-glucuronic acid and its metabolites in 27 solvents and on four types of chromatographic paper in order to develop a method for their separation as the first step in the flux studies of D-glucuronic acid metabolism in the liver.

MATERIALS AND METHODS

Chromatographic papers. In the separation of L-ascorbic acid, L-xylulose, *myo*-inositol, and lactones Whatman No. 1 paper (58 × 68 cm) was used. To hinder the tailing of D-glucuronic, L-gulonic, and D-glucaric acids in alkaline solvents Whatman No. 1 paper impregnated with alginate,[12] Whatman CM 82 carboxymethylcellulose and Whatman P 20 phosphocellulose papers were used. When a solvent was developed over the paper its lower end was cut in saw-tooth fashion (each tooth *ca.* 2 cm wide).

Reference intermediates. D-Glucuronic acid and D-glucurono-6,3-lactone were purchased from Sigma Chemical Co. (St. Louis, USA), L-gulono-1,4-lactone from ICN Nutritional Biochemicals (Ohio, USA), potassium hydrogen-D-glucarate (D-glucaric acid) from British Drug Houses (Poole, England), D-glucaro-1,4-lactone from Pfizer, (Folkestone, England) and D-glucaro-6,3-lactone from J. T. Baker Chemicals N. V. (Deventer, Holland); L-ascorbic acid and *myo*-inositol were obtained from E. Merck AG (Darmstadt, Germany) and L-xylulose from Mann Research Laboratories (New York, USA). Potassium L-gulonate (L-gulonic acid) was prepared by hydrolyzing L-gulono-1,4-lactone with an equivalent amount of aqueous potassium hydroxide for 24 h at 38°C.[3] D-Glucaro-1,4-6,3-dilactone was synthesized from D-glucaro-1,4-lactone monohydrate purchased form Calbiochem AG (Lucerne, Switzerland) as described by Harigaya.[13]

References were applied to the paper, 50 μg/spot, as aqueous solutions (5 mg/ml), 3 cm apart and at a distance of 8 cm from the end of the paper.

Solvents and development. In the separation of the intermediates the following solvent systems were used: 1-butanol : acetic acid : water (12 : 3 : 5) [10] and (6 : 1 : 4), 1-butanol : propionic acid : water (94 : 45 : 61), 1-butanol : ethanol : water (60 : 28 : 75) and (40 : 11 : 19),[14] ethyl acetate : acetic acid : water (2 : 1 :2) and (7 : 2 : 1), phenol : water (4 : 1 v/v),[15] pyridine : 1-butanol : water (30 : 45 : 22), pyridine : amyl alcohol : water (40 : 35 : 30), pyridine : ethyl methyl ketone : formic acid : water (20 : 20 : 12 : 12), pyridine : 2-propanol : acetic acid : water (40 : 40 : 5 : 20), pyridine : formic acid : water (5 : 1 : 1), pyridine : ethyl acetate : water (34 : 80 : 12) and (11 : 40 : 6),[4] pyridine : ethyl acetate : acetic acid : water (5 : 5 : 1 : 3),[4] (5 : 10 : 1 : 1), (5 : 2 : 1 : 2), (30 : 7 : 12 : 14), and (3 : 1 : 2 : 2), 1-propanol : methyl benzoate : formic acid : water (7 : 3 : 2 : 5),[16] 1-propanol : methyl benzoate : acetic acid : water (7 : 3 : 2 : 5) and (50 : 20 : 20 : 35). In the separation of D-glucuronic, D-glucaric and L-gulonic acids the following alkaline solvents were used: acetone : ethanol : 1-propanol : 2 % ammonia (3 : 1 : 1 : 1), 1-propanol : ethyl methyl ketone : 25 % ammonia : water (20 : 15 : 6 : 10), 1-propanol : ethyl acetate : 25 % ammonia : water (5 : 1 : 1 : 3),[17] and 2-propanol : ethyl acetate : 25 % ammonia : water (23 : 15 : 8 : 5). The papers were developed from 8 to 70 h at room temperature in a glass chamber (60 × 70 × 20 cm) saturated with the vapor of the solvent used. Descending ordinary, flowing and multiple chromatography were used.

Detection of spots. All the intermediates studied could be visualized by spraying the chromatographic paper after drying with alkaline silver nitrate.[18]

RESULTS

When the chromatographic papers were developed perpendicular to the fibres, resolution was in general better than when they were developed parallel to the fibres. With multiple chromatography the intermediates were separated

much better than with flowing chromatography (Fig. 2 (A)). Adequate differences in R_F values of D-glucuronic, D-glucaric, and L-gulonic acids were only obtained in alkaline solvents, but in these there was tailing, if normal chromatographic paper was used. Of the acidic papers used, phosphocellulose paper was found to be the best to prevent tailing, and with this the best resolution was achieved.

With the slowly running solvents 1-butanol : acetic acid : water (12 : 3 : 5) and (6 : 1 : 4) and 1-butanol : propionic acid : water (94 : 45 : 61), which are often used in carbohydrate chromatography, only the fastest compound, L-ascorbic acid, and the slowest, *myo*-inositol, could be separated. D-Glucurono-6,3-lactone and L-xylulose had almost the same R_F values, and L-gulono-1,4- and D-glucaro-1,4-lactones ran also at the same speed. L-Gulonic, D-glucuronic, and D-glucaric acids could not be separated from each other (Fig. 1 (A)). When the solvents 1-butanol : ethanol : water (60 : 28 : 75) and (40 : 11 :19) were used, the spots were large and tailing took place. With the fast-running solvents ethyl acetate : acetic acid : water (2 : 1 : 2) and (7 : 2 : 1) the intermediates were poorly separated. When a phenol : water (4 : 1) mixture was used, the faster-moving L-xylulose, D-glucurono-6,3-lactone, and L-gulono-1,4-lactone ran at almost the same speed and the slower L-ascorbic acid, L-gulonic acid, D-glucaro-1,4-lactone, and D-glucuronic and D-glucaric acids could not be separated from each other (Fig. 1 (B)).

In the pyridine-containing solvents, which are also common in carbohydrate chromatography, partial decomposition of L-ascorbic acid and lactones took place. Of the solvents used, the pyridine : ethyl acetate : water and pyridine : ethyl acetate : acetic acid : water mixtures gave the best resolution of metabolites. With the fast-running solvent pyridine : ethyl acetate : water (34 : 80 : 12) most compounds were separated satisfactorily, but D-glucuronic and L-gulonic acids had almost the same R_F value (Fig. 1 (C)). If acetic acid was added to the solvent, *e.g.* pyridine : ethyl acetate : acetic acid : water (5 : 5 : 1 : 3), similar separation was achieved but the hydrolysis of D-glucurono- and L-gulonolactones could be prevented (Fig. 1 (D)). If the solvent composition was adjusted to 3 : 1 : 2 : 2, also the decomposition of L-ascorbic acid and D-glucarolactones was much diminished.

With the solvent 1-propanol : methyl benzoate : formic acid : water (7 : 3 : 2 : 5) and 3×20 h multiple chromatography, the intermediates with the exception of L-gulonic, D-glucuronic, and D-glucaric acids were separated (Fig. 1 (E)). During development partial lactonization of L-gulonic acid was, however, observed. If the developing time exceeded 20 h an oily emulsion ran down the paper. When formic acid was replaced with acetic acid, there was no lactonization or emulsion formation. The resolution was also somewhat better. D-Glucaro-1,4-lactone ran much slower than in the presence of formic acid (Fig. 2 (A)).

In the final method the lactones, L-xylulose, *myo*-inositol, and L-ascorbic acid were separated on Whatman No. 1 paper with solvent 1-propanol : methyl benzoate : acetic acid : water (50 : 20 : 20 : 35), developing 3×20 h. The drying time in a hood between developments was cut to 1/2 h to prevent oxidation of L-ascorbic acid. The mobilities of the metabolites decreased in the following order: L-ascorbic acid, D-glucurono-6,3-lactone, L-xylulose, L-gulono-1,4-lac-

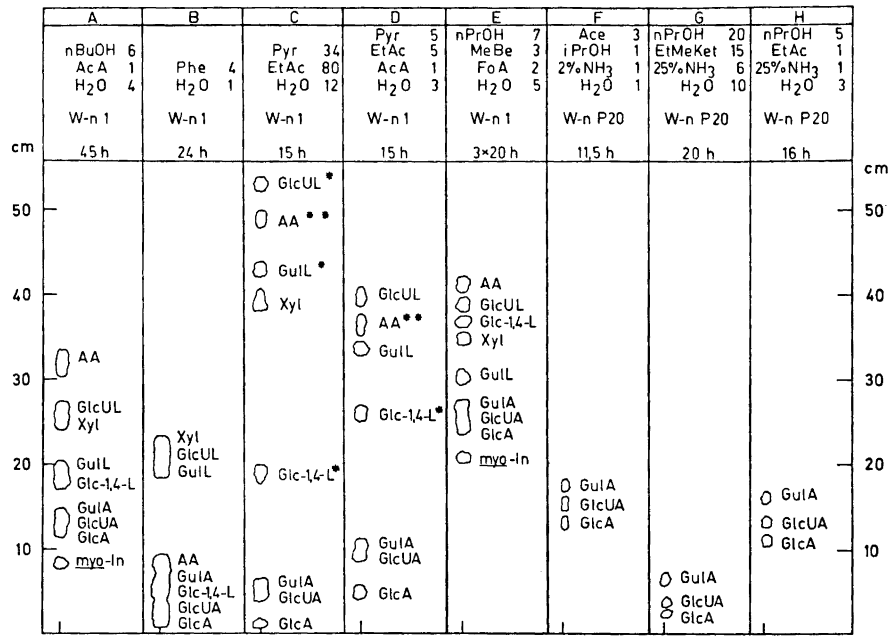

Fig. 1. Separation of the intermediates of D-glucuronic acid metabolism by paper chromatography in various solvent systems. Since flowing development was used in many cases, the distances which the metabolites had traveled are indicated in cm. The solvent systems (nBuOH = 1-butanol, AcA = acetic acid, Phe = phenol, Pyr = pyridine, EtAc = ethyl acetate, nPrOH = 1-propanol, MeBe = methyl benzoate, FoA = formic acid, Ace = acetone, iPrOH = 2-propanol, EtMeKet = ethyl methyl ketone), the development time and the type of chromatographic paper (W-n 1 and W-n P20 are Whatman No. 1 and P20, respectively) are given. The following abbreviations for the metabolites have been used: GlcUA = D-glucuronic acid, GlcUL = D-glucurono-6,3-lactone, GulA = L-gulonic acid, GulL = L-gulono-1,4-lactone, GlcA = D-glucaric acid, Glc-1, 4-L = D-glucaro-1,4-lactone, Glc-6,3-L = D-glucaro-6,3-lactone, Glc-1,4-6,3-L = D-glucaro-1,4-6,3-dilactone, AA = L-ascorbic acid, Xyl = L-xylulose and *myo*-In = *myo*-inositol. * The lactone is partly decomposed to the respective acid. ** The compound is tailing.

tone, D-glucaro-1,4-lactone, D-glucaro-1,4-6,3-dilactone, D-glucaro-6,3-lactone, and *myo*-inositol. However, some overlapping of the various D-glucarolactones could not be avoided. The separation of D-glucaro-6,3-lactone and L-gulonic acid also presented some difficulties. L-Gulonic, D-glucuronic, and D-glucaric acids moved slower than the other intermediates (except *myo*-inositol) (Fig. 2 (B), I). No decomposition of the compounds took place during chromatography.

L-Gulonic, D-glucuronic, and D-glucaric acids could be separated (Fig. 1 (F, G and H)) with the alkaline solvents acetone : ethanol : 2-propanol : 2 % ammonia (3 : 1 : 1 : 1), 1-propanol : ethyl methyl ketone : 25 % ammonia : water (20 : 15 : 6 : 10), and 1-propanol : ethyl acetate : 25 % ammonia : water (5 : 1 : 1 : 3).

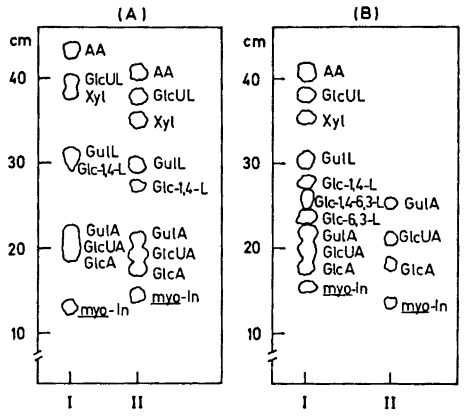

Fig. 2. (*A*) Chromatography of the D-glucuronic acid metabolites by (I) flowing (47 h) and (II) multiple (3 × 20 h) development on Whatman No. 1 paper with a solvent system 1-propanol : methyl benzoate : acetic acid : water (7 : 3 : 2 : 5). (*B*) Separation of (I) the lactones, L-ascorbic acid, L-xylulose, and *myo*-inositol by multiple chromatography (3 × 20 h) on Whatman No. 1 paper with 1-propanol : methyl benzoate : acetic acid : water (50 : 20 : 20 : 35) as solvent, and (II) of D-glucuronic, L-gulonic, D-glucaric acids and *myo*-inositol after prechromatography with pyridine : ethyl acetate : acetic acid : water (3 : 1 : 2 : 2) (12 h), followed by development with 1-propanol : ethyl acetate : 25 % ammonia : water (23 : 15 : 8 : 5) (2 × 20h) on Whatman P20 phosphocellulose paper.
For other explanations, see Fig. 1.

In the method developed the separation of D-glucuronic, D-glucaric, and L-gulonic acids was still better, and it was achieved by using Whatman P 20 phosphocellulose paper and 2 × 20 h developments with 2-propanol : ethyl acetate : 25 % ammonia : water (23 : 15 : 8 : 5) as solvent. But it was necessary first to wash apart L-xylulose and the alkali-labile L-ascorbic acid and lactones of the intermediate mixture with a 12 h prechromatography with pyridine : ethyl acetate : acetic acid : water (3 : 1 : 2 : 2) as solvent. With this method very slow-moving *myo*-inositol could be separated, too (Fig. 2 (B), II). The lactones, L-ascorbic acid, and L-xylulose were usually eluted completely from the paper during the procedure.

DISCUSSION

A number of different solvents used in the paper chromatography of sugars and their derivatives were studied in order to develop a separation method for D-glucuronic acid and its various metabolites. No solvent system suitable for this purpose has previously been described in the literature. In the ion exchange methods described for the separation of certain single metabolites,[6–9] the other intermediates are eluted in large volumes and usually with overlapping. For instance, in the method described by Ishidate *et al.*[9] for the separation of D-glucaric acid, L-gulonic, D-glucuronic, and L-ascorbic acids are eluted together. Fujita and co-workers[11] were able to separate the acids of the D-glucuronic acid pathway and pentoses, but the D-glucuronic and L-gulonic acid fraction also contained the respective lactones. But their separation is necessary in studies on the flux of the D-glucuronic acid pathway. The new method described in the present report provides a means for such studies.

In the method described, the separation of the lactones, L-ascorbic acid, and L-xylulose is carried out with an acidic solvent system, since alkaline

solvents promote the decomposition of L-ascorbic acid [5] and the hydrolysis of lactones.[4] The separation of D-glucuronic, L-gulonic, and D-glucaric acids was carried out with an alkaline solvent system and acidic phosphocellulose paper. The labile metabolites were, however, first separated by prechromatography with pyridine : ethyl acetate : acetic acid : water. This solvent rapidly carries the labile metabolites to a considerable distance, with minimal decomposition and this allows subsequent separation of the slowly moving D-glucuronic, L-gulonic, and D-glucaric acids with an alkaline solvent system. The error caused by the decomposition of the alkali-labile metabolites can thus be avoided.

During the separation of D-glucuronic, L-gulonic, and D-glucaric acids by the method described, slight hydrolysis (10 – 15 %) of D-glucarolactones takes place. The D-glucarolactones are even *in vivo* labile intermediates in the biosynthesis of D-glucaric acid, which makes the small error in D-glucaric acid separation less significant in the flux studies of D-glucuronic acid metabolism.

The separation of D-glucuronic acid and its metabolites by the method developed is reproducible, and has been applied successfully to studies of the metabolism of D-glucuronic acid-6-^{14}C and D-glucurono-6,3-lactone-6-^{14}C in rat liver homogenates and slices.

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