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Note

Paper chromatography–enzyme spray technique for the detection of sugar nucleotides with galactose and N-acetyl-galactosamine residues

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Paper chromatography (PC) in suitable solvents coupled with spraying the chromatogram with appropriate enzyme solutions[1] is a sensitive method for identifying sugars and sugar nucleotides which are oxidizable by galactose oxidase[2–4]. In the technique developed in our laboratory, the sugars and sugar nucleotides are first separated from other reaction products by PC and the dried chromatogram is then sprayed lightly with a buffered solution of galactose oxidase and peroxidase followed by an alcoholic solution of *o*-tolidine. D-Galactose and N-acetyl-D-galactosamine, as well as compounds which contain these moieties as terminal units, appear as blue spots on the chromatogram. From the rate of appearance and the intensities of the colored spots, relative rates for the oxidation of the various compounds can be calculated. Such calculations yield information on the types of functional groups involved in the formation of the enzyme–substrate complex and the types of sugar moieties in polymers which can be detected by use of galactose oxidase.

In the present study it has been found that uridine diphosphate galactose, thymidine diphosphate galactose and uridine diphosphate N-acetyl-D-galactosamine are oxidized by galactose oxidase at rates nearly equal to the rate for galactose. Since in the first tests with uridine diphosphate galactose[2] an oxidation of this compound was not detectable, it is not generally recognized that sugar nucleotides are so readily oxidized by galactose oxidase. The chromatography–spray technique is especially suitable for detecting sugar nucleotide interconversions that are effected by 4-epimerases. Thus while uridine diphosphate glucose and uridine diphosphate N-acetyl-glucosamine are not oxidized by galactose oxidase, the products of epimerase action on these substrates, uridine diphosphate galactose and uridine diphosphate N-acetyl-galactosamine, are both rapidly oxidized and are easily detected by the chromatography–spray method. In view of the foregoing observations and methodology, assays and kinetic experiments for studying the epimerases should be facilitated.

EXPERIMENTAL

The sources of compounds were as follows. Uridine diphosphate glucose, uridine diphosphate galactose and uridine diphosphate N-acetyl-glucosamine were

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purchased from Sigma (St. Louis, Mo., U.S.A.). The thymidine diphosphate glucose was purchased from Calbiochem (La Jolla, Calif., U.S.A.). The uridine diphosphate N-acetyl-galactosamine was kindly provided by Dr. S. Roth, Biology Department, The Johns Hopkins University, Baltimore, Md., U.S.A. The thymidine diphosphate galactose was prepared in this laboratory from thymidine triphosphate and α-D-galactose-1-phosphate utilizing the inducible TDPgalactose pyrophosphorylase from *Streptococcus faecalis*[5]. The D-talose was provided by Dr. H. Ebertova of the Czechoslovak Academy of Science, Prague, Czechoslovakia, and L-galactose was provided by Dr. H. Isbell, American University, Washington, D.C., U.S.A. The other sugars were reagent grade laboratory samples.

PC of the carbohydrates and sugar nucleotides was carried out on Whatman No. 1 paper (27 × 25 cm) by the ascending method[5] at room temperature in two different solvent systems. Solvent system I consisted of *n*-butyl alcohol–pyridine–water (6:4:3, v/v) and solvent system II consisted of ethyl alcohol–1.0 *M* ammonium acetate (7:3, v/v) of pH 7.5. The sugar nucleotides were located on the paper by irradiation with short-wave UV light (Mineralight UVS.12, Ultra-violet Products, San Gabriel, Calif., U.S.A.) and the sugars were detected by the silver nitrate staining procedure[6].

The specific procedure utilized for the detection of compounds which reacted with galactose oxidase was as follows. Samples of 5 μl containing 5 to 25 μg of the sugar or the sugar nucleotides were chromatographed in one of the above solvent systems. In the case of the sugar nucleotides, the dried chromatogram was irradiated with UV light to locate the sugar nucleotides. A print of the UV irradiated chromatogram was obtained on Kodagraph KC5 contact paper by placing the chromatogram on the emulsion side of the paper, irradiating with short wave UV light for 2 to 3 sec and developing the contact paper in Kodak Dektol as directed by the manufacturer. The chromatogram was then sprayed with 10 ml of a solution containing 125 units of galactose oxidase (1.5 mg of a preparation from Sigma) and 500 units of horse radish peroxidase (6 mg of a preparation from Sigma) in 10 ml of 0.2 *M* sodium phosphate buffer of pH 7.5. The moist chromatogram was immediately sprayed with 20 ml of a 0.5% solution of *o*-tolidine in 95% ethyl alcohol. In a few seconds those compounds which react with galactose oxidase appear as blue spots. For a permanent record, the chromatogram and the UV prints should be photographed on Polaroid black and while film. In the case of sugars, duplicate chromatograms were prepared and one chromatogram was sprayed by the above method and the other was stained by the silver nitrate method[6]. Figs. 1 and 2 show photographs of typical chromatograms and UV prints obtained with different sugars and sugar nucleotides.

Use of the spray technique was made for investigating the types and levels of 4-epimerase activities in cell free extracts of *Streptococcus faecalis*. The extracts were prepared from cells collected from 500 ml of freshly grown cultures of *S. faecalis*. These cells were broken in 1 ml of phosphate buffer of pH 7.2 containing 0.05 *M* $MgCl_2$ in a Micle disintegrator. Centrifugation at 10,000 *g* yielded a clear supernatant solution which constituted the cell free extract. Samples of 3.5 mg of uridine diphosphate glucose or uridine diphosphate N-acetyl-glucosamine were dissolved in 0.1 ml of the extracts. Samples (5 μl) of each digest were placed on Whatman No. 1 paper at 0 h and after incubation for 8 or 18 h. Reference sugar nucleotides were also placed on the paper and the chromatograms were then developed in solvent system II.

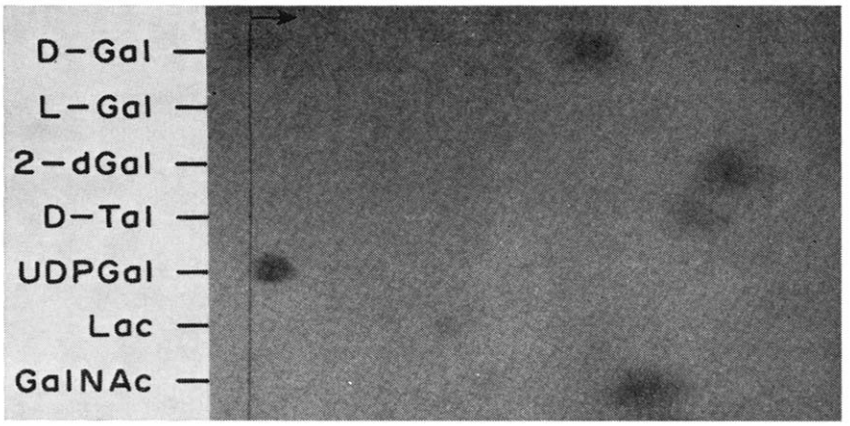

Fig. 1. A photograph of a paper chromatogram of sugars and uridine diphosphate galactose developed in solvent system I and sprayed with galactose oxidase, peroxidase and *o*-tolidine: 2-dGal = 2 deoxy-D-galactose, Lac = lactose, the other abbreviations are in common usage.

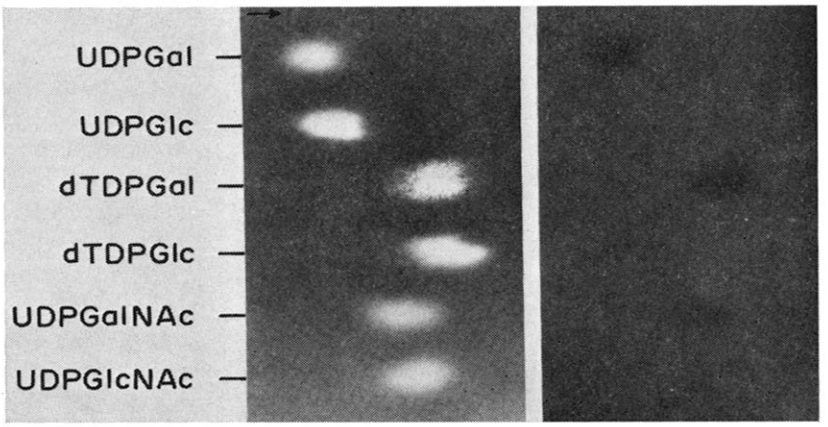

Fig. 2. Photographs of a UV contact print (left) and the enzyme-sprayed chromatogram (right) of several sugar nucleotides developed in solvent system II.

A UV print of the chromatogram was obtained and subsequently the chromatogram was sprayed as described above. Photographs of the chromatogram and UV print for the two digests and reference compounds are reproduced in Fig. 3.

RESULTS AND DISCUSSION

A photograph of a chromatogram which had been developed in solvent system I and sprayed with galactose oxidase, peroxidase and *o*-tolidine is shown in Fig. 1. The dark spots on the chromatogram show those compounds that were oxidized by galactose oxidase and the intensities of the spots indicate the relative rates of oxidation. Thus D-galactose, N-acetyl-D-galactosamine, 2-deoxy-D-galactose and uridine diphosphate galactose were oxidized at very fast rates while D-talose and lactose were oxidized slowly and L-galactose was not oxidized at all by the enzyme.

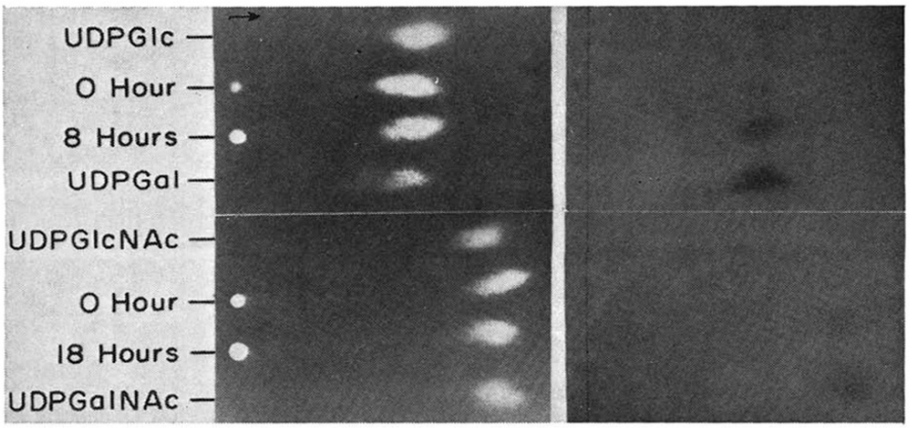

Fig. 3. Photographs of a UV contact print (left) and the enzyme-sprayed chromatogram (right) showing the action of 4-epimerases on uridine diphosphate glucose and uridine diphosphate N-acetyl-glucosamine.

The location of L-galactose and other reducing sugars on the chromatogram was determined by staining a duplicate chromatogram by the silver nitrate method. The conclusion in an early study[2] that uridine diphosphate galactose is not oxidizable by galactose oxidase was based on results of a colorimetric assay on solutions of the substrate and enzyme. It is possible that an inhibition of the galactose oxidase had occurred in these tests or perhaps sufficient levels of galactose oxidase or substrate were not employed to obtain a measurable amount of oxidation. It is clear from the results in Fig. 1 that uridine diphosphate galactose is indeed oxidized by galactose oxidase. In fact, the enzyme has been used in a recent study[7] to prepare tritium labeled uridine diphosphate galactose and carbon-14 labeled uridine diphosphate galacturonic acid.

The chromatograms in Fig. 2 show that galactose oxidase oxidizes not only uridine diphosphate galactose but also thymidine diphosphate galactose and uridine diphosphate N-acetyl-galactosamine. On the other hand, uridine diphosphate glucose, thymidine diphosphate glucose and uridine diphosphate N-acetyl-glucosamine were not oxidized. It should be noted in this figure that on the basis of the intensities of the spots on the chromatogram on the right in Fig. 2, uridine diphosphate N-acetyl-galactosamine appears to be oxidized at a somewhat slower rate than the other two sugar nucleotides.

Fig. 3 contains results which show the utility of the chromatography–spray technique for detecting the presence of sugar nucleotide 4-epimerases in extracts of *Streptococcus faecalis*. It will be noted in this figure in the photograph of the enzyme-sprayed chromatogram that cell-free extracts of *S. faecalis* convert uridine diphosphate glucose rapidly to uridine diphosphate galactose. Thus even in the 0-h sample some conversion had occurred in the time interval required for inactivation of the epimerase. In the 8-h reaction period, a high percentage of uridine diphosphate glucose was converted to uridine diphosphate galactose indicating a high level of the uridine diphosphate galactose-4-epimerase. However, the extracts contained only a low level of the epimerase for converting uridine diphosphate N-acetyl-glucosamine to uridine diphosphate N-acetyl-galactosamine as no conversion was observable in the 0-h sample and only a small amount of conversion was observable in the 18-h sample.

Further it should be noted that uridine diphosphate glucose and uridine diphosphate N-acetyl-glucosamine are not oxidized by galactose oxidase. Hence, even though these sugar nucleotides are not separated from the corresponding galactose and N-acetyl-galactosamine compounds (Fig. 3, left), it is still possible to detect the reaction products by the chromatography–spray technique because of the specificity of the enzyme (Fig. 3, right).

Sugar nucleotides are utilized by *S. faecalis* for the synthesis of important cell wall components such as peptido-glycans and immunogenic glycans. The peptido-glycan of *S. faecalis* occurs as a single polymer composed of N-acetyl-glucosamine, muramic acid and oligopeptides[8]. However, the immunogenic glycans occur as two polymers, a diheteroglycan of glucose and galactose and a tetraheteroglycan of rhamnose, glucose, galactose and N-acetyl-galactosamine[9]. The biosynthesis of the peptido-glycan occurs via the uridine diphosphate hexose pathway in which uridine diphosphate N-acetyl-glucosamine probably functions as donor of the amino sugar moiety[10]. However, the synthesis of the immunogenic glycans involves the participation of thymidine diphosphate hexoses which function as donors of rhamnose, galactose and glucose moieties for the glycans[11]. In the synthesis of the tetraheteroglycan, the N-acetyl-galactosamine is probably donated by uridine diphosphate N-acetyl-galactosamine since the corresponding thymidine compound[12] is not produced by extracts from this organism[5]. Thus, *S. faecalis* has evolved the system of enzymes for synthesizing thymidine diphosphate hexoses of the common sugars, glucose, galactose and rhamnose of the glycans but not for the less common sugar, N-acetyl-galactosamine. With the thymidine diphosphate hexose pathway, a regulatory mechanism for the control of synthesis of the cell wall glycans has likely evolved since the pyrophosphorylases of this pathway are under feedback control[13,14]. The paper chromatography–enzyme spray technique described in this note should be of value for investigating aspects of the regulatory processes involving feedback control by sugar nucleotides.

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REFERENCES

1 D. French, A. P. Pulley, M. Abdullah and J. C. Linden, *J. Chromatogr.*, 24 (1966) 271.
2 G. Avigad, D. Amaral, C. Asensio and B. L. Horecker, *J. Biol. Chem.*, 237 (1962) 2736.
3 G. L. Nelsestuen and S. Kirkwood, *Anal. Biochem.*, 40 (1971) 359.
4 J. H. Pazur, H. R. Knull and G. E. Chevalier, *J. Carbohydr. Nucleos. Nucleot.*, 4 (1977) 129.
5 J. H. Pazur and J. S. Anderson, *J. Biol. Chem.*, 238 (1963) 3155.
6 F. C. Mayer and J. Larner, *J. Amer. Chem. Soc.*, 81 (1959) 188.
7 A. K. Rao and J. Mendicino, *Anal. Biochem.*, 72 (1976) 400.
8 M. Ikawa, *J. Biol. Chem.*, 236 (1961) 1087.
9 J. H. Pazur, J. S. Anderson and W. W. Karakawa, *J. Biol. Chem.*, 246 (1971) 1793.
10 S. G. Nathenson and J. L. Strominger, *J. Biol. Chem.*, 237 (1962) 3839.
11 J. H. Pazur and J. S. Anderson, *Biochim. Biophys. Acta*, 74 (1963) 788
12 S. Kornfeld and L. Glaser, *J. Biol. Chem.*, 237 (1962) 3052.
13 R. L. Bernstein and P. W. Robbins, *J. Biol. Chem.*, 240 (1965) 391.
14 A. Malo and L. Glaser, *J. Biol. Chem.*, 240 (1965) 398.