879. *Cytidine Diphosphate Ribitol from* Lactobacillus arabinosus.

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Short acid hydrolysis of a nucleotide from *Lactobacillus arabinosus* gives cytidine-5′ phosphate, ribitol 1-phosphate, and smaller amounts of other substances. Ribitol 1-phosphate is also formed by enzymic hydrolysis of the nucleotide. It was identified by comparison with an authentic sample, enzymic dephosphorylation to ribitol, acid hydrolysis, and oxidation with periodate.

When heated with dilute aqueous ammonia the nucleotide gives cytidine-5′ phosphate and ribitol 1 : 2-(hydrogen phosphate), the latter being identified by comparison with authentic material and by its behaviour on acid hydrolysis. This and other evidence indicates the structure (I) for the nucleotide.

The behaviour of ribitol and its 1-phosphate towards acids is discussed. A specific method for distinguishing ribitol from other pentitols on a microgram scale has been developed.*

We described earlier the presence in *Lactobacillus arabinosus* of two new cytosine nucleotides.[1] After purification,[2] one was shown to be cytidine diphosphate glycerol (CDP-glycerol).[3] In this paper it is shown that the other cytidine nucleotide from the micro-organism is P^1-cytidine-5′ P^2-ribitol-1 pyrophosphate (I), for which we suggest the name cytidine diphosphate ribitol (CDP-ribitol).†

The nucleotide (previously called CPX) is eluted from a Dowex-2 (formate) column just before CDP-glycerol and is usually present in somewhat larger amounts than the latter in aqueous-alcoholic extracts of *L. arabinosus*. It contains two phosphate groups per cytidine residue and, like CDP-glycerol, it has a higher R_F on paper chromatography in *n*-propyl alcohol–ammonia than cytidine-5′ phosphate or cytidine-5′ pyrophosphate.[2] This suggests the presence of a substituent on one of the phosphate groups. As only a few milligrams of the nucleotide have been isolated it was not possible to perform elementary analyses and all work was carried out on material eluted from paper chromatograms.

No acidic or basic group other than those associated with a cytidine diphosphate structure was detected in this nucleotide. Its R_F in the above solvent mixture was slightly lower than that of CDP-glycerol, which suggests that the residue attached to one of the phosphate groups is more hydrophilic than the glycerol residue and so may contain more hydroxyl groups.

Like CDP-glycerol, this nucleotide gives a rapid colour reaction on paper when sprayed with periodate followed by Schiff's reagent.[4] The initial reddish-blue colour intensifies and deepens in shade during several hours. Such behaviour has been attributed to the presence of both nucleoside and acyclic 1 : 2-glycol structures in such a molecule.[3]

When the nucleotide was hydrolysed with N-hydrochloric acid at 100° for 15 minutes and then examined by paper chromatography the only product which absorbed ultraviolet light was cytidine-5′ phosphate whose identification has been described previously.[1] When the paper was sprayed with reagents for the detection of phosphates [5] the main additional non-absorbing product was an organic phosphate which, like the original nucleotide, gave a rapid reaction in the periodate–Schiff test and consequently was probably an acyclic polyhydroxy-phosphate : some inorganic phosphate and small amounts of two slightly faster-moving phosphates were also detected. One of the last two gave a rapid reaction in the periodate–Schiff test, and the other gave a yellow colour which slowly changed to

* A preliminary account of this work has been published (Baddiley, Buchanan, Carss, and Mathias, *Biochim. Biophys. Acta*, 1956, **21**, 191.

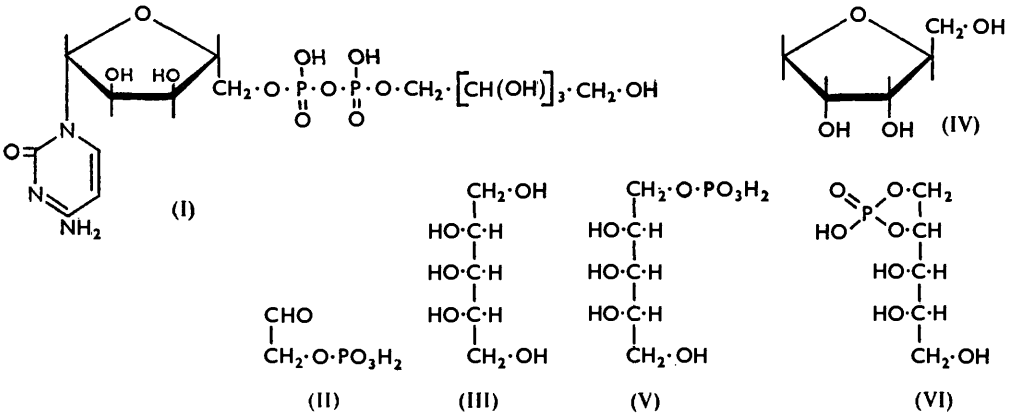
† The nomenclature of compounds of this type is discussed briefly in ref. 3.

[1] Baddiley and Mathias, *J.*, 1954, 2723.
[2] Baddiley, Buchanan, Carss, Mathias, and Sanderson, *Biochem. J.*, in the press.
[3] Baddiley, Buchanan, Mathias, and Sanderson, *J.*, 1956, 4186.
[4] Buchanan, Dekker, and Long, *J.*, 1950, 3162; Baddiley, Buchanan, Handschumacher, and Prescott, *J.*, 1956, 2818.
[5] Hanes and Isherwood, *Nature*, 1949, **164**, 1107.

grey-green. These are possibly isomers arising through acid-catalysed phosphate migration (cf. the formation of a trace of β-glycerophosphate during the hydrolysis of CDP-glycerol).

The presence of a hydroxyl group in proximity to a phosphate group was suggested by the behaviour of the nucleotide towards ammonia at 100°. After 30 minutes hydrolysis was complete, the products being cytidine-5′ phosphate and a phosphate with a relatively high R_F value in basic solvents. The latter product was recognised as a cyclic phosphate containing a 5-membered ring from its behaviour on chromatography before and after acid hydrolysis. It was readily converted by dilute acids into the same mixture of products as was obtained by the action of acids on the original nucleotide. A 6-membered cyclic phosphate would be stable under such conditions.[6]

The behaviour of the nucleotide towards acids, and especially towards ammonia, is typical of a pyrophosphoric ester which bears a hydroxyl group on a carbon atom adjacent to that bearing the pyrophosphate residue. Several nucleotide coenzymes possess this structural feature and all yield cyclic phosphates on treatment with ammonia.[3, 7, 8]



(I) (II) (III) (IV) (V) (VI)

In the reactions described so far the nucleotide shows some similarity to CDP-glycerol. The main differences are (1) the formation under acid conditions of inorganic orthophosphate and the two unidentified substances, and (2) the greater ease with which a cyclic phosphate is formed in ammonia. The relative ease of cyclic phosphate formation from pyrophosphates will be governed largely by steric factors. In uridine diphosphate glucose,[7] which is a derivative of α-glucose 1-pyrophosphate, and in flavin adenine dinucleotide,[8] where the pyrophosphate group is attached to the terminal position of an acyclic ribityl residue, the adjacent hydroxyl groups are in a sterically favourable position for cyclic phosphate formation. Consequently, these coenzymes are very unstable in ammonia, giving 5-membered cyclic phosphates. In coenzyme A only a 6-membered cyclic phosphate can be formed and so the pyrophosphate linkage in this nucleotide is fairly stable towards alkali.[9] In CDP-glycerol the relative difficulty experienced in forming a cyclic phosphate with ammonia was attributed to the adoption in the glycerol residue of a preferred *trans*-conformation of hydroxyl groups. It is probable that as the length of a polyol 1-phosphate chain increases then the preferred conformation of the hydroxyl group at position 2 will approach the *cis*-relation with respect to the phosphate at position 1. This would facilitate cyclic phosphate formation and hence explain the observed difference in stability of flavin adenine dinucleotide and CDP-glycerol. The conductivity of boric acid solution in the presence of polyols increases very considerably as the length of the polyol chain is increased.

[6] Baddiley and Thain, *J.*, 1951, 3421; Baddiley, Buchanan, and Szabó, *J.*, 1954, 3826.
[7] Paladini and Leloir, *Biochem. J.*, 1952, **51**, 426.
[8] Forrest and Todd, *J.*, 1950, 3295.
[9] Baddiley and Thain, *J.*, 1952, 3782.

This is due to the formation of cyclic boric esters, and it has been observed [10] that ethylene glycol and glycerol show only very small effects compared with pentitols and hexitols.

The above considerations suggest that the cytidine nucleotide from *L. arabinosus* is a derivative of a polyol pyrophosphate and that the polyol chain should be larger than that of glycerol. These conclusions are confirmed by the observation that the cyclic phosphate obtained from the nucleotide gives a positive reaction for an acyclic glycol in the periodate–Schiff test. It follows that the polyol must contain at least one 1 : 2-glycol system or its equivalent, in addition to that involved in cyclic phosphate formation. The simplest structure consistent with these observations would be a pyrophosphate formed from cytidine-5′ phosphate and a tetritol phosphate.

The unfractionated phosphatases of *Crotalus atrox* venom hydrolysed the nucleotide readily. The products were cytidine, inorganic phosphate, and an organic phosphate which was indistinguishable from that obtained as a major product of mild acid hydrolysis. This is consistent with a P^1-cytidine-5′ P^2-polyol pyrophosphate structure, as the venom is known to exhibit pyrophosphatase and nucleoside-5′ phosphatase activity.

The organic phosphate produced by enzymic hydrolysis of the nucleotide was eluted from a paper chromatogram and oxidised with periodate. When the products were examined by paper chromatography glycollaldehyde phosphate (II) was detected and identified by comparison with an authentic sample. This indicates that the grouping $H_2O_3P{\cdot}O{\cdot}CH_2{\cdot}CH(OH){\cdot}CH(OH)-$ is present in the hydrolysis product, and that the nucleotide must be a P^1-cytidine-5′ P^2-polyol-1 pyrophosphate.

Further enzymic hydrolysis of the organic phosphate by a mixed phosphatase preparation (Polidase-S) gave inorganic phosphate and a polyol. The polyol was indistinguishable on paper chromatography from authentic ribitol (adonitol) (III), but the differences in R_F values of ribitol and the other two pentitols (xylitol and arabitol) were small (cf. Table).

A more satisfactory identification of ribitol as a product of enzymic hydrolysis of the nucleotide was highly desirable, particularly in view of the complex nature of the acid hydrolysis products. The liberation of inorganic phosphate under relatively mild conditions was somewhat unexpected for a compound of the type shown in formula (I). Cytidine-5′ phosphate and α-glycerophosphate were not hydrolysed under these conditions. This had led us at first to suspect the presence of a centre of unsaturation (double bond or carbonyl group) in the polyol residue. However, no such grouping could be detected, and a more detailed study of the acid hydrolysis of the polyol 1-phosphate was undertaken.

With N-hydrochloric acid at 100° for 1 hour the phosphate was partially hydrolysed to give inorganic phosphate, small amounts of the compound which shows a yellow colour reaction in the periodate–Schiff test, and the other presumably isomeric phosphate, and a new phosphorus-free compound with an R_F higher than that of ribitol. The new compound reacted slowly in the periodate–Schiff test, giving a blue-violet colour. This is consistent with a cyclic structure (cf. nucleosides and other glycosides) and it is probable that an anhydroribitol had been formed. The most likely structure for this product is 1 : 4-anhydroribitol (IV). 1 : 4-Anhydroxylitol has been prepared by acid-catalysed dehydration of xylitol under anhydrous conditions,[11] and it appears that in the hexitols too furan rings are more readily formed than are pyrans.

The ready liberation of inorganic phosphate from the polyol 1-phosphate is almost certainly related in part to the simultaneous formation of an anhydro-compound. The mechanism of this hydrolysis and the structure of the anhydroribitol are under investigation but in the meantime use has been made of these reactions for the full identification of the polyol.

It was found that conversion of ribitol itself into this anhydro-compound in 2N-hydrochloric acid at 100° was substantial in 3 hours (as determined by paper chromatography) and complete in 24 hours. The polyol obtained by enzymic dephosphorylation of the nucleotide behaved in exactly the same way as did authentic ribitol under these conditions and it is concluded that the two compounds are identical. Xylitol was unaffected by 2N-hydrochloric acid at 100° after 24 hours, and arabitol was only very slightly affected.

[10] Böeseken and Furnée, *Rec. Trav. chim.*, 1940, **59**, 97.
[11] Carson and Maclay, *J. Amer. Chem. Soc.*, 1945, **67**, 1808.

The reason for the relative ease with which ribitol is converted into an anhydro-compound, as compared with the other pentitols, is not clear, but here again the adoption of preferred conformations is probably a driving factor. This test is the only one known whereby ribitol can be easily distinguished from other pentitols on a microgram scale.

The formation of anhydroribitol during acid-treatment of ribitol or its 1-phosphate probably occurs by different mechanisms in the two cases. The phosphate reacts much more rapidly under standard conditions than does the polyol itself. Consequently, it is unlikely that hydrolysis to ribitol and inorganic phosphate represents the first stage in this reaction. This is supported by our failure to detect even traces of free ribitol during the hydrolysis of its phosphate.

Confirmation of the structure of the ribitol 1-phosphate from the nucleotide was obtained by comparison with synthetic L-ribitol 1-phosphate (V) (obtained by reduction of D-ribose 5-phosphate with sodium borohydride). The substances were indistinguishable on paper and gave indistinguishable hydrolysis products in identical relative amounts when treated with acid for varying times. Synthetic ribitol phosphate was converted into its 1 : 2-(hydrogen phosphate) (VI) by trifluoroacetic anhydride. This product was indistinguishable on paper chromatography and electrophoresis from that obtained by the action of ammonia on CDP-ribitol.

It has not been possible, with the quantities of CDP-ribitol available, to determine the configuration of the ribitol phosphate residue. It is interesting that if this is D-ribitol 5-phosphate (L-ribitol 1-phosphate) it could be related biochemically to either D-ribose 5-phosphate or D-ribulose 5-phosphate, whereas if it is D-ribitol 1-phosphate (L-ribitol 5-phosphate) it may be related to D-ribose 1-phosphate or D-ribulose 1-phosphate. As most of these pentose phosphates occur naturally it is not possible to predict the configuration of CDP-ribitol.

Although ribitol has been detected in various plants, little is known about its metabolism. It might be predicted on general grounds that CDP-ribitol would be involved in enzymic transformations concerning ribitol 1-phosphate. However, neither this nor any other pentitol phosphate has been detected hitherto in living cells. On the other hand, D-mannitol 1-phosphate has been shown recently to be formed by reduction of fructose 6-phosphate in the presence of enzymes from various bacteria.[12] Moreover, we have isolated D-mannitol 1-phosphate in substantial amounts from *L. arabinosus*.[2] The existence of related metabolic processes involving ribitol phosphates must now be considered.

Experimental

The almost pure nucleotide was isolated from a 100 l. batch of bacterial culture by chromatography on Dowex-2 resin, adsorption on charcoal, and elution with ammonia. Details are described elsewhere.[2] It was purified further by paper chromatography in solvent system *A*, eluted from the paper, and used as a 2% aqueous solution for the following experiments.

Acid Hydrolysis.—A sample of nucleotide was hydrolysed for 15 min. at 100° with N-hydrochloric acid in a sealed tube. Solvent was removed in a desiccator *in vacuo*, and the hydrolysate was examined by paper chromatography in solvent system *A*. Hydrolysis products were identified whenever possible by comparison with authentic substances on adjacent tracks. Cytidine-5′ phosphate and ribitol 1-phosphate were the main products. Some inorganic phosphate, a little " anhydroribitol," and traces of the unidentified " organic phosphate " were detected in addition to the phosphate which gave a yellow spot with the periodate–Schiff spray reagents.[4] For R_F values see Table.

Hydrolysis with Ammonia.—A sample of nucleotide solution was heated with an equal volume of ammonia (*d* 0·88) in a sealed tube at 100° for 30 min. Hydrolysis was complete, the products being cytidine-5′ phosphate and ribitol 1 : 2-(hydrogen phosphate). The cyclic phosphate was eluted from the paper and a sample was shown to be unchanged after 1 hr. at 100° in ammonia (*d* 0·88). A further sample of cyclic phosphate was dissolved in a little N-hydrochloric acid and heated for 5 min. at 100°. The solution was evaporated to dryness in a desiccator, and the products were examined by paper chromatography in solvent system *A*. No starting material was detected. The components of the mixture were indistinguishable

[12] Wolff and Kaplan, *J. Biol. Chem.*, 1956, **218**, 849.

from the non-nucleotide products of short acid hydrolysis of CDP-ribitol or of synthetic ribitol 1-phosphate.

Paper electrophoresis at 400 v in 0·05M-ammonium acetate buffer (pH 8) of a sample of the cyclic phosphate produced from the nucleotide by ammoniacal hydrolysis showed its identity with the synthetic material. It moved towards the anode at a velocity just over half that of ribitol-1 phosphate.

Action of Crotalus atrox *Venom.*—A solution of the nucleotide was treated with a 1% solution of the venom (kindly supplied by Dr. D. M. Brown) in a glycine–ammonia buffer (pH 9) for 16 hr. at 37°. The products, identified by chromatography in solvent system *A*, were cytidine, ribitol 1-phosphate, and inorganic phosphate.

Enzymic Dephosphorylation of Ribitol 1-Phosphate.—Ribitol 1-phosphate was obtained by the action of snake venom on CDP-ribitol, paper chromatography of the products, and elution of the appropriate area of the paper with water. Traces of inorganic phosphate were removed as barium phosphate with a slight excess of 0·1N-barium hydroxide, followed by a little Dowex-50 (H^+ form) resin to remove barium ions. The resulting ribitol 1-phosphate solution was mixed with a 1% solution of " Polidase-S " (Schwarz Laboratories) in an ammonium acetate buffer (pH 7) and incubated at 37° for 16 hr. The products were inorganic phosphate and ribitol, the latter being identified from its R_F in solvent systems *A*, *B*, *C*, *D*, *E*, and *H*, and by the action of hot dilute hydrochloric acid (see below).

Action of Acid on Ribitol 1-Phosphate.—Ribitol 1-phosphate obtained from the nucleotide by short acid hydrolysis, followed by chromatography (solvent system *A*) and elution, was treated with N-hydrochloric acid at 100° for 15 min. Solvent was removed *in vacuo* and the products were run on paper in solvent system *A*. The products were the same in kind and relative amount as those resulting from a similar treatment of the nucleotide itself (cytidine-5′ phosphate was, of course, absent), the cyclic phosphate, or authentic ribitol 1-phosphate.

Ribitol (100 μg.) in N-hydrochloric acid (20 μl.) was heated in a sealed tube for 1 hr. at 100°. An equivalent amount of synthetic ribitol 1-phosphate was treated similarly. The solutions were evaporated in a desiccator, and products were examined by paper chromatography in solvent system *A*. Under these conditions a weak spot corresponding to anhydroribitol was seen in the ribitol hydrolysis products, whereas the phosphate gave much more anhydro-compound. It was estimated that the phosphate decomposed about 10 times faster than did the free polyol.

Oxidation with Periodate.—To a solution of ribitol 1-phosphate derived from the nucleotide by treatment with snake venom was added a slight excess of 4% sodium metaperiodate solution. After 3 hr. at room temperature sodium ions were removed with a small amount of Dowex-50 (H^+ form) resin, and the products were run on paper in solvent systems *F* and *G*. The spot corresponding to ribitol 1-phosphate was no longer visible but one which was indistinguishable from glycollaldehyde phosphate was present.

Synthesis of Pentitols.—Ribitol, arabitol, and xylitol were obtained from the corresponding pentoses by reduction with sodium borohydride.[13] The pentitols were characterised by the m. p.s of their penta-*O*-acetyl derivatives. Samples were regenerated therefrom by sodium methoxide in methanol. Sodium ions were removed with an excess of Dowex-50 (H^+ form) resin, and evaporation of solvent yielded crystalline products except in the case of xylitol. These were pure when examined by paper chromatography in solvent systems *A*, *B*, *C*, *D*, *E*, and *H*. None of these solvent systems distinguished satisfactorily between the three pentitols.

Action of Acid on Ribitol, Arabitol, and Xylitol.—1% Solutions in water of each pentitol were treated with 2N-hydrochloric acid in sealed tubes at 100° and samples were withdrawn after 3 hr. and 24 hr. After evaporation of solvent the products were run on paper in solvent system *A* and examined with the periodate–Schiff spray reagents. Arabitol and xylitol were unchanged after 3 hr., but about half of the ribitol had been converted into " anhydroribitol." After 24 hr. ribitol had been completely converted into " anhydroribitol," whereas arabitol and xylitol gave only traces of products. Ribitol obtained from CDP-ribitol by the successive action of snake venom and Polidase-S was indistinguishable in behaviour from authentic ribitol.

L-*Ribitol 1-Phosphate.*—To a solution of D-ribose 5-phosphate (180 mg.) in water (4 ml.) was added sodium borohydride (23 mg.) in water (2 ml.). After 12 hr. at room temperature acetic acid (0·3 ml.) was added, followed by dilute ammonia solution (to pH 8—9). The solution was diluted to 20 ml. and passed through a small Amberlite IRA-400 (acetate form) resin column,

[13] Abdel-Akher, Hamilton, and Smith, *J. Amer. Chem. Soc.*, 1951. **73**. 4691.

which was then washed with water. Ribitol phosphate was eluted from the resin with N-ammonium acetate solution (30 ml.). Cations were removed by adding an excess of Dowex-50 (H^+ form) resin, and the eluate was evaporated below 25° with repeated additions of water to remove acetic acid, and finally to dryness. Traces of borate which remained in the product interfered with its chromatography. These were removed by evaporation under reduced pressure with anhydrous methanol (4 × 25 ml.). The ribitol 1-phosphate was isolated as its barium salt from aqueous solution (3 ml.) by the addition of a slight excess of 0·1N-barium hydroxide followed by a small piece of solid carbon dioxide. Some water was removed by evaporation under reduced pressure, traces of barium carbonate by centrifugation, and the *barium salt* was precipitated by addition to the centrifugate of an equal volume of ethanol. It was purified by reprecipitation from water with ethanol, washed with acetone, and dried at 80° *in vacuo* (Found : P, 8·4; Ba, 37·2. $C_5H_{11}O_8BaP$ requires P, 8·4; Ba, 37·4%).

L-*Ribitol* 1 : 2-(*Hydrogen Phosphate*).—The barium salt of ribitol 1-phosphate (35 mg.) in water (1·5 ml.) was passed through a small Dowex-50 (H^+ form) resin column and the eluate was freeze-dried. The residue, after drying (P_2O_5) *in vacuo*, was shaken during 1 hr. with trifluoroacetic anhydride (1 ml.) at room temperature. The mixture was evaporated to dryness *in vacuo* and to the residue was added water (1 ml.) and ammonia (1 ml.; *d* 0·88) with shaking. Paper chromatography of the solution in solvent system *A* and electrophoresis showed that the principal product was the cyclic phosphate.

Paper Chromatography.—Unless otherwise stated, ascending front chromatography was carried out on Whatman No. 4 paper which had been washed with 2N-acetic acid, then water. The following solvent systems were used :

A, *n*-Propyl alcohol–ammonia (*d* 0·88)–water (6 : 3 : 1).
B, Pyridine–ethyl acetate–water (2 : 7 : 1).
C, Pyridine–ethyl acetate–water (organic layer) (1 : 2 : 2).
D, *n*-Butyl alcohol–benzene–pyridine–water (organic layer) (5 : 1 : 3 : 3).
E, *n*-Butyl alcohol–acetic acid–water (organic layer) (4 : 1 : 5).
F, *iso*Butyric acid–ammonia (10 : 6 ml.; 0·5N-ammonia).
G, *tert.*-Butyl alcohol–picric acid–water (80 ml. : 2 g. : 20 ml.).
H, *n*-Butyl alcohol–ethanol–water (organic layer) (4 : 1 : 5).

	R_F in solvents								IO_4^-
	A	B	C	D	E	F *	G *	H	Schiff reaction †
Cytidine	0·61	—	—	—	—	—	—	—	S
Cytidine-5′ phosphate	0·20	—	—	—	—	0·53	—	—	S
CDP-ribitol	0·33	—	—	—	—	0·39	—	—	R
Ribitol 1-phosphate	0·34	—	—	—	—	0·39	0·50	—	R
" Organic phosphate "	0·39	—	—	—	—	—	—	—	R
" Yellow spot "	0·39	—	—	—	—	—	—	—	S
" Anhydroribitol "	0·87	—	—	—	—	—	—	—	S
Ribitol 1 : 2-(hydrogen phosphate)	0·53	—	—	—	—	—	—	—	R
Glycollaldehyde phosphate	—	—	—	—	—	0·33	0·50	—	S
Ribitol	0·76	0·22	0·62	0·36	0·34	—	—	0·34	R
Arabitol	0·76	0·20	0·61	0·36	0·35	—	—	0·33	R
Xylitol	0·74	0·21	0·58	0·33	0·34	—	—	0·33	R

* Descending front chromatography. † S = slow reaction, R = rapid reaction.

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