GALACTOSE 1-PHOSPHATE IN THE INTESTINAL TISSUE OF THE RAT DURING GALACTOSE ABSORPTION

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(Received April 20th, 1960)

SUMMARY

The water-soluble, ethanol-insoluble barium salt mixture from a trichloracetic acid extract of the small intestines of rats assimilating galactose was resolved into several fractions on a column of Dowex-1 borate. Each of the fractions consists of several components. Galactose was identified in three of the fractions, including one in which acid-labile phosphates predominated. An enzymic method was used to confirm the presence of galactose 1-phosphate in similar fractions from intestinal extracts which had not been treated with barium. A high level of galactose 1-phosphate appears in the intestinal tissue during the absorption of galactose, but none is present when sorbose is being absorbed. Raising the rats on diets containing low levels of galactose or lactose does not influence the level of intestinal galactose 1-phosphate, measured 1 h after the administration of galactose by stomach tube. The galactose 1-phosphate is formed by the phosphorylation of free galactose in the intestinal tissue.

INTRODUCTION

In spite of the interest that attaches to a knowledge of the metabolism of galactose in intestinal tissue, it has not previously been established whether this tissue has the ability to phosphorylate galactose. HELE[1], SOLS[2], and others[3] have tested homogenates of intestinal tissue for galactokinase activity, but have obtained conflicting results. Other investigators have sought galactose phosphates in the tissue. KJERULF-JENSEN[4] found that the "hexose monophosphate fraction" from the intestinal mucosa of rats which were assimilating galactose gave a strong phloroglucinol test, and since the fraction had a high reducing power and little acid-labile organic phosphate, he suggested that galactose 6-phosphate might be present. More recently, NAGASAWA[5] administered $NaH_2{}^{32}PO_4$ to rats along with galactose, and from the intestines obtained radioactive compounds with the paper chromatographic behavior expected of galactose 1- and 6-phosphates. However, neither author completed the identification of his supposed galactose esters. A careful examination of the sugar phosphates

Abbreviations: TPN and TPNH, triphosphopyridine nucleotide and its reduced form; UDP-glucose, uridine diphosphate glucose; PGM, phosphoglucomutase.

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** Published with the approval of the Director of the Wisconsin Agricultural Experiment Station.

formed in rat intestinal tissue during galctose absorption, with the aim of positively identifying any galactose phosphates present, was therefore needed. Such an investigation is the principal subject of this paper.

A second subject is the hexose phosphate content of rat intestinal tissue. It has been known for two decades that, during the absorption of the common, well metabolized hexoses, the amounts of acid-soluble phosphates in the intestinal mucosa increase[6–8]. The evidence indicated that the extra phosphates consist at least in part of hexose phosphates. But the difficult (at the time) job of identifying the individual phosphates was not undertaken by the previous investigators. Hence, no estimates were available of the quantities of individual esters present. In the present work, the aldose 1-phosphates were determined in intestinal tissues from rats which were assimilating the "metabolically inert" sugar L-sorbose, and from those which were assimilating D-galactose. In connection with these determinations, an effort was made to detect any possible metabolic adaptation, involving galactose 1-phosphate levels in the intestine, which might result from the addition of low and non-toxic levels of galactose and lactose to the diet.

MATERIALS AND METHODS

Sugar phosphates

Barium galactose-1-phosphate was synthesized according to the directions of HANSEN *et al.*[9]. Dr. H. A. LARDY generously provided samples of dipotassium glucose 1-phosphate and barium galactose and mannose 6-phosphates. Barium glucose and fructose 6-phosphates were commercial preparations.

Coenzymes

TPN-sodium (96–98 %) and UDP-glucose sodium (90 %) were obtained from the Sigma Chemical Co., St. Louis.

Enzymes, etc.

Acid phosphatase (wheat germ) was purchased from the Mann Research Laboratories. The glucose 6-phosphate dehydrogenase routinely used was Zwischenferment, Practical, Type II, from the Sigma Chemical Co. Phosphoglucomutase was prepared from rabbit muscle by the procedure of NAJJAR[10]. The crystalline protein which was obtained reverted to amorphous form, but it retained high activity. Galactose 1-phosphate uridyl transferase was prepared according to KURAHASHI AND ANDERSON[11].

Rats were obtained from the Holtzman Animal Laboratories, Madison.

Ion exchange chromatography was carried out on columns of Dowex-1 borate as described by DIEDRICH[12]. The basic procedure is a modification of that of GOODMAN, BENSON AND CALVIN[13].

Hexose analyses

The anthrone method of ROE[14] was employed for the quantitative determination of the hexose content of pooled fractions, etc. This method was also used initially for the detection of chromatographic peaks. Later, a more sensitive and selective colorimetric method for hexoses, developed in this laboratory[12], was used for monitoring the effluents from the ion exchange columns.

Phosphate analyses

Phosphate analyses were carried out by the FISKE AND SUBBAROW method[15]. When hexose-1-phosphates were present, samples for the inorganic phosphate determination were diluted to 9 ml with water and cooled to 20° before the acid molybdate and reducing agent were added. By letting the color development proceed at 20 ± 1° (for exactly 15 min) the hydrolysis of hexose 1-phosphate was held to a minimum (0.4 % for glucose 1-phosphate)[16]. Samples for the total phosphate determination were first digested with nitric acid, then perchloric acid. After dilution with water and boiling to hydrolyze pyrophosphate, the samples were neutralized with NaOH and analyzed for inorganic phosphate.

The enzymic assay of hexose 1-phosphates is discussed in the EXPERIMENTAL section.

Preparation of intestinal extracts

The entire procedure was carried out in a cold room, and cold solutions were used. The animal was killed by a blow on the head, decapitated, and exsanguinated. The small intestine was cut at the pyloric sphincter and pulled free of the adhering mesentery, and the upper two thirds was taken for extraction. Isotonic NaCl was pumped into the duodenal end of the dissected organ from a wash bottle, with momentary clamping of the ileal end to cause distension, and the exterior was rinsed in a beaker of saline. Excess saline was blotted off, the intestine was weighed to the nearest 0.1 g, cut into strips 2–3 cm long, and homogenized in 20 ml of 10 % trichloracetic acid in a small Waring blendor. The homogenate was transferred to a plastic tube with distilled water and centrifuged, and the precipitate washed once with 20 ml of 5 % trichloracetic acid. The combined supernatants were kept frozen at —10°. When further workup was to be started, the frozen solutions were thawed overnight at *ca* 10°, filtered through glass wool, and extracted three times with their own volume of ether to remove trichloracetic acid and lipids. The solutions were then neutralized and the dissolved ether was flashed off *in vacuo*.

EXPERIMENTAL

Identification of galactose 1-phosphate in intestinal tissue

By ion exchange chromatography: A number of preliminary experiments were conducted in which a single rat was allowed to assimilate a dose of galactose for 1 h, and the intestinal extract was subjected to ion exchange chromatography. Sugar phosphate appeared in the eluates in the position corresponding to galactose 1-phosphate, but the amounts were too small for further characterization. It was therefore necessary to pool the extracts from a number of intestines, even though the pooled extracts could not be chromatographed directly. The large amount of inorganic salts present would have made a very large column necessary.

Forty adult female rats were fasted for 10–15 h, then each rat was given 4 ml of 0.75 *M* D-galactose by gavage. Extracts were prepared from the intestines and pooled, and the resulting solution, free of trichloracetic acid, was neutralized and concentrated *in vacuo* to a volume of 100 ml. The water-soluble, ethanol-insoluble barium salt fraction was prepared from the concentrate in the usual way[17]. Barium

was then removed from an aqueous solution of the salts by precipitation with a slight excess of K_2SO_4, and the supernatant was chromatographed on a column of Dowex-1 borate.

This fractionation of pooled extracts from the intestines of a large number of rats was repeated a number of times, with similar results each time. A typical elutogram is shown in Fig. 1, and the information which was obtained about the composition of the peaks is summarized in the following paragraph. In addition to the assays for carbohydrate (anthrone), analyses for acid-labile and acid-stable organic phosphate were performed on the pooled fractions representing each peak. Portions of the material from each peak were also converted to the free sugars for paper chromatographic analysis. Hydrolysis of the acid-labile phosphates was accomplished by making the solutions 1 *N* in HCl and heating at 100° for 10 min. Fractions containing acid-stable phosphate were first freed of cations by passage over Dowex-50 (H^+), then freed

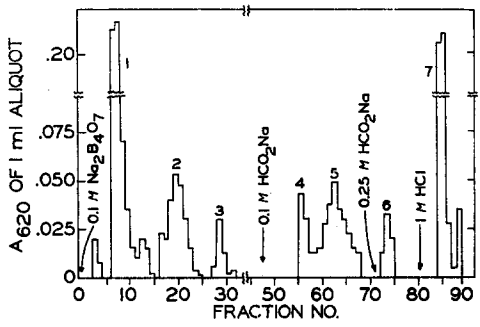

Fig. 1. Chromatography of the water-soluble, ethanol-insoluble barium salt fraction of pooled intestinal extracts. Resin, Dowex-1 X-8, 50–100 mesh, borate form. Column size, 1.2 cm (diameter) × 26 cm. Fraction volume, 40 ml. "A_{620} of 1 ml aliquot" designates the absorbancy, at 620 mμ, found when a 1 ml aliquot was analyzed by the anthrone method. An A_{620} value of 0.1 is given by 22 μg of sugar, calculated as glucose.

of boric acid by extraction with isoamyl alcohol[12]. Wheat germ phosphatase (which is strongly inhibited by boric acid) was used to hydrolyze the phosphates. All hydrolyzates were deionized by successive passages over Dowex-50 (H^+) and Dowex-1 (OH^-), concentrated, and spotted on Whatman No. 1 paper. The solvent mixture of DEWHALLEY *et al.*[18], *n*-butanol–pyridine–water–benzene (5:3:3:1), was used for the chromatography (descending). The aniline hydrogen phthalate reagent of PARTRIDGE[19] and WOLFROM AND MILLER'S[20] reagents for vicinal hydroxyl groups were used (on separate papers) to reveal the spots.

In peak 1, the WOLFROM-MILLER reagents disclosed at least 6 compounds, including glucose and galactose. Peak 2, in which the phosphate was largely acid-labile, contained galactose and an unidentified sugar. Peak 3 contained both acid-labile and acid-stable phosphates and a number of unidentified compounds which reacted with the WOLFROM-MILLER reagents. It was not further studied. Peak 4, in which the phosphate was largely acid-stable, gave a prominent spot with the R_F of galactose. Peak 5 (acid-stable phosphates) yielded sugars with the R_F values of glucose and ribose, and an unidentified sugar. Peak 6 (acid-stable phosphates) gave three spots, one with the R_F of ribose. The latter two peaks had nucleotide-like u.v. absorp-

tion spectra. Peak 7 represents the material which was stripped from the column by 1 *N* HCl. It was not further investigated.

Enzymic identification

In view of the difficulties encountered in the foregoing attempts to resolve the ethanol-insoluble barium salt fraction of intestinal extract by ion exchange chromatography, an enzymic method for the positive identification of galactose 1-phosphate was indicated. Preliminary work was done on a system containing the hexose 1-phosphate epimerizing system of calf liver[21], phosphoglucomutase, and glucose 6-phosphate dehydrogenase (Zwischenferment). This system should convert galactose 1-phosphate to 6-phosphogluconate with the concomitant generation of TPNH. At this time, Dr. ELIZABETH ANDERSON informed us that she and Dr. K. KURAHASHI had already developed a Zwischenferment assay for galactose 1-phosphate, using galactose 1-phosphate uridyl transferase, and UDP-glucose at the substrate level, instead of the complete epimerizing system. Drs. ANDERSON AND KURAHASHI kindly furnished us a copy of their then unpublished manuscript, and we used essentially their procedure[11], slightly modified to permit the determination of the glucose 1-phosphate present in the sample.

When crude extracts of intestine were tested in this assay system, TPNH was formed, but it was quickly reoxidized. This suggested that the extracts contained an interfering substance, presumably the oxidized substrate of some dehydrogenase present as a contaminant in one of the enzyme preparations used. It could be shown that the contaminating dehydrogenase was in the Zwischenferment*, and that it was present in all the available samples of this enzyme**. The solution to the problem thus lay in removing the interfering substrate from the intestinal extracts. Since a simple purification by adsorption on Dowex-1 and elution with formate did not accomplish the desired results, the samples were chromatographed in the Dowex-1 borate system.

The samples were prepared as described above, except that each sample consisted of the crude extract of a single rat intestine, freed of trichloracetic acid, but not fractionated with barium. Samples were adjusted to pH 4 before being put on the column, since the resin does not completely adsorb sugar phosphates from neutral solutions containing large amounts of inorganic salts. The fractions of the eluate (from an individual sample) corresponding to peaks 1 and 2 were pooled and an ion exchange method[12] was used to remove sodium ions and boric acid, which strongly inhibits Zwischenferment. The borate-free eluate, which was now in 0.1 *N* ammonium formate at pH 6, was concentrated from the frozen state to a small volume, made up to 10 ml, and an aliquot was taken for enzymic assay. The addition of phosphoglucomutase gave an increment of TPNH, as expected for samples containing glucose 1-phosphate. In samples from rats which had received a dose of galactose 1 h before the intestines were removed, the addition of galactose 1-phosphate uridyl transferase gave a second increment of TPNH, as expected for samples containing galactose 1-phosphate. Fig. 2 records the results of a typical experiment.

* It is quite likely that the interfering substrate was oxidized glutathione. Glutathione reductase was found[22] in samples of Sigma Zwischenferment which were on the market when the present work was being carried out.

** We thank Dr. S. A. KUBY of the Institute for Enzyme Research, Madison, for a sample of Zwischenferment prepared by an unpublished procedure.

Quantitative determination of hexose 1-phosphates in rat intestine

Forty weanling, female rats were divided into four groups of ten. The animals were maintained for 30 days on diets consisting of a standard stock ration containing no supplement, 8 % D-glucose, 8 % D-galactose, and 16 % lactose, respectively. Diet and water were given *ad libitum*, and each animal received a dose of Haliver oil weekly. Within experimental error, the average gains of the animals in the different groups were identical and none showed any toxic effects. The average weight achieved was 125 g.

At the end of the 30 day period, the animals were fasted 10–15 h. Five animals from each group received 4 ml of 0.75 *M* L-sorbose by stomach tube, and the remaining five animals received 4 ml of 0.75 *M* D-galactose by the same route. One hour after the sugar was administered, the animals were killed and the intestines were extracted as described under MATERIALS AND METHODS. The individual extracts were then

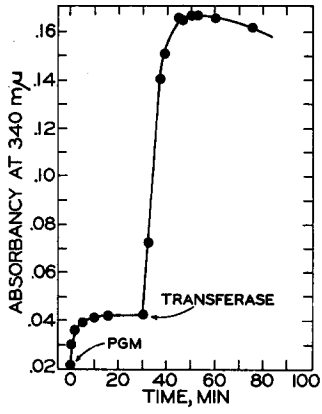

Fig. 2. TPNH production by glucose 1-phosphate and galactose 1-phosphate in intestinal extract. The incubation mixture contained: 0.17 *M* glycylglycine buffer, pH 8.7, 0.6 ml; 0.03 *M* disodium ethylenediaminetetraacetate, 0.1 ml; 0.3 *M* $MgCl_2$, 0.1 ml; 0.15 *M* cysteine at pH 8.5, 0.2 ml; 0.00125 *M* TPN, 0.2 ml; 0.0015 *M* UDP-glucose, 0.1 ml; Zwischenferment, 1 Kornberg unit/ml, 0.1 ml; and water and/or sample, 1.4 ml. Total volume, 2.8 ml. Enzyme additions (0.1 ml each) were: phosphoglucomutase, 0.5 mg protein/ml; and galactose 1-phosphate uridyl transferase, 12.5 mg of lyophilized powder/ml of 0.001 *M* phosphate buffer, pH 6.0. Absorbancy was read in an unthermostated Beckman model DU spectrophotometer.

chromatographed and assayed enzymically for glucose 1-phosphate and galactose 1-phosphate as described in the preceding section. When known amounts of the hexose 1-phosphates were added to crude intestinal extracts, the procedure gave recoveries of 90 % for glucose 1-phosphate and 60 % for galactose 1-phosphate.

Prior to the finding that the sugar phosphates of intestinal extracts must be purified chromatographically before they can be assayed in the Zwischenferment system, it was hoped that glucose 6-phosphate could be determined, along with the 1-phosphates. This idea was given up because the continuation of the chromatography until glucose 6-phosphate is eluted is laborious, and there is no assurance that the interfering substrate mentioned above would be separated from the glucose 6-phosphate.

The results of the assays are shown in Table I. It may be seen that the intestines of the animals which received sorbose had a low but readily measurable level of

TABLE I

HEXOSE 1-PHOSPHATE LEVELS IN RAT INTESTINAL TISSUE DURING SUGAR ABSORPTION

Details of the analytical method are given under Fig. 2.

Diet supplement	*Sugar administered*	*No. of animals*	*Hexose 1-phosphate (μmoles/100 g fresh tissue)**		
			Glucose 1-phosphate found	*Galactose 1-phosphate found*	*Galactose 1-phosphate corrected***
None	Galactose	5	0.9 (0.8–1.0)***	14 (11–19)	23 (18–32)
8% glucose	Galactose	3	1.1 (0.9–1.2)	11 (8–15)	18 (13–25)
8% galactose	Galactose	3	1.4 (1.3–1.5)	14 (10–17)	23 (17–28)
16% lactose	Galactose	4	0.6 (0.4–1.0)	6 (2–13)	10 (3–22)
All galactose groups		15	1.0 (0.4–1.5)§	11 (2–19)	18 (3–32)
None	Sorbose	2	0.8 (0.8–0.9)	0.2 (0.1–0.2)§§	—
8% glucose	Sorbose	3	0.9 (0.3–1.4)	0.0 (0.0–0.1)	—
8% galactose	Sorbose	2	1.4 (1.3–1.5)	0.0 (0.0)	—
16% lactose	Sorbose	2	0.6 (0.6–0.7)	0.0 (0.0–0.1)	—
All sorbose groups		9	1.0 (0.3–1.5)	0.0 (0.0–0.2)	—

* Figures given are means for the numbers of animals indicated, and (in parentheses) ranges.
** For 60% recovery in the analytical procedure.
*** 4 values.
§ 14 values.
§§ Value not considered significantly different from zero.

glucose 1-phosphate; presumably this represents the fasting level. The average value is 1.0 μmole/100 g of wet tissue, and this value is not increased during galactose absorption. In the intestines of rats which had not received galactose, the concentration of galactose 1-phosphate was zero, within experimental error. But during the absorption of galactose, the ester was present in intestinal tissue in considerable concentration (eleven times the value for glucose 1-phosphate or eighteen times, if the galactose 1-phosphate values are corrected).

In order to rule out the possibility that the galactose 1-phosphate found in rat intestinal tissue is formed in some other organ and brought to the intestine in the blood stream, blood plasma was assayed. Neither galactose 1-phosphate nor glucose 1-phosphate could be detected in a sample of plasma from animals assimilating galactose.

DISCUSSION

From the evidence presented in the foregoing paragraphs, it seems conclusively established that galactose 1-phosphate is formed during the intestinal absorption of galactose in the rat. Galactose 1-phosphate gives a characteristic peak on chromatography in the Dowex-1 borate system. When extracts were made from the intestines of rats assimilating galactose, and the water-soluble, alcohol-insoluble barium salt fraction of the pooled extracts chromatographed in this system, the corresponding peak contained acid-labile organic phosphate and, after hydrolysis, a sugar with the R_F of galactose.

The peaks from the Dowex column are clearly mixtures, but the presence of both glucose 1-phosphate and galactose 1-phosphate in eluates from single intestinal extracts which had not been fractionated with barium and alcohol was confirmed by the response which these eluates gave in the enzymic assay. Reduction of TPN was

observed when phosphoglucomutase was added to the system, and again when galactose 1-phosphate uridyl transferase was added. The first rise in absorbancy is attributed to glucose 1-phosphate and the second to galactose 1-phosphate. Phosphoglucomutase, galactose 1-phosphate uridyl transferase, and Zwischenferment are quite specific, and even though the enzyme preparations employed were not pure, it is highly unlikely that the observed responses could be given by substances other than the two hexose 1-phosphates, particularly in a sample which had been subjected to ion exchange chromatography. The fact that a response for galactose 1-phosphate is obtained only in extracts from rats which are assimilating galactose is an additional argument in support of this conclusion. And since the blood plasma is free of galactose 1-phosphate (and glucose 1-phosphate), it may be further concluded that the galactose ester is formed by the phosphorylation of free galactose in the intestinal tissue.

It now becomes of interest to examine the physiological significance of the intestinal phosphorylation of galactose. However, the fact that galactose 1-phosphate appears in the tissue during galactose absorption does not of itself answer questions of this kind. In particular, it reveals nothing about the possible participation of phosphorylation in the process of intestinal absorption. The ester which is found in the tissue could, *a priori*, represent galactose which was in transit when the tissue was excised, or a *cul de sac* into which some galactose is diverted when it is available, or a portion of the transient galactose which has been diverted to furnish metabolic energy to the tissue.

With regard to the latter possibility, the ability to metabolize galactose has been well documented for intestinal tissue from two species of animals. Everted sacs of hamster intestine placed in galactose solutions convert some of the galactose to lactic acid[23], some to glucose, and some to CO_2 (see ref. 24). The conversion of galactose to CO_2 has also been demonstrated in slices of rat intestine[25]. In discussing these conversions, LANDAU AND WILSON[24] suggest that phosphorylation must be involved, with the implication that galactose is metabolized in intestine by the same pathways as in liver and erythrocytes[26]. The results reported here provide an experimental foundation for these suggestions.

In considering the results of the quantitative determinations, one may inquire whether the hexose 1-phosphates present in intestinal tissue during galactose absorption represent a significant proportion of the organic phosphates of the tissue. Since the present data applies to whole (small) intestine, and earlier workers have analyzed only the mucosa, comparison is difficult. However, if a tissue dry matter content of 20% is assumed, one may calculate that 19 μmoles of glucose 1-phosphate plus galactose 1-phosphate/100 g fresh tissue is equivalent to 29 mg hexose 1-phosphate P/100 g dry tissue. LASZT AND SÜLLMANN[6] quoted a value of 96 mg and LUNDSGAARD[7] a value of 346 mg for total acid-soluble organic P/100 g dry wt. in rat intestinal mucosa during glucose absorption.

The levels of intestinal galactose 1-phosphate achieved during galactose absorption were not significantly different in animals raised on stock, glucose-supplemented, and galactose-supplemented diets. Three animals of the lactose-fed group had below average levels of both hexose 1-phosphates during galactose absorption. However, the data are not extensive enough to warrant the conclusion that this is a typical effect of lactose feeding.

The finding that one of the fractions (peak 4, Fig. 1) from the pooled intestinal

extracts consists largely of acid-stable phosphate, with galactose apparently the major sugar, suggests that galactose 6-phosphate may be present in intestinal tissue during the absorption of galactose. In view of the fact that two previous investigators[4,5] have obtained similar indications, this possibility should be further explored.

ACKNOWLEDGEMENT

This investigation was supported in part by the Research Committee of the Graduate School from funds supplied by the Wisconsin Alumni Research Foundation.

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