THE HEXOKINASE ACTIVITY OF THE INTESTINAL MUCOSA*

by

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INTRODUCTION

Two natural sugars, glucose and galactose, and a synthetic derivative, 3-methylglucose are absorbed from the small intestine of the rat and other animals at rates faster than any other sugars tested[1,2] and against a concentration gradient. Fructose is absorbed at an intermediate rate[1].

Some twenty years ago the hypothesis was formulated that this "active" absorption involved phosphorylation of the sugars[3]. This phosphorylation hypothesis has been generally accepted though direct evidence for it has been lacking. Most of the experimental work designed to test its validity has yielded data interpreted as consistent with it, but until recently little was directly known about the phosphorylating enzymes of the intestinal mucosa presumably involved in the process. Lately it has been reported by HELE[4], BISEGGER AND LASZT[5], and CSAKY[6] that the ability of intestinal mucosa homogenates or extracts to phosphorylate various sugars roughly parallels the absorption rates of these sugars.

Meanwhile the pattern of substrate specificity of an isolated animal hexokinase, that of the brain, was clearly established[7]. The information thus obtained was of assistance in undertaking a critical study of the phosphorylation of sugars by intestinal mucosa homogenates. The results presented in this paper indicate that the intestinal mucosa of the rat has a hexokinase with a pattern of specificity of the brain type, at least in a broad sense. Neither galactose nor 3-methylglucose is phosphorylated to any appreciable extent.

MATERIALS AND METHODS

The sugars and sugar derivatives, all of the D-series unless otherwise indicated, were obtained as follows: Glucose-free galactose was prepared by recrystallization from 80% ethanol of a commercial galactose (Eastman Kodak Co.); samples of 3-*o*-methyl-glucose were kindly furnished by Drs. J. SOWDEN AND T. Z. CSAKY; allose and 1,5-sorbitan by Dr. N. K. RICHTMYER; 2-deoxyglucose by Dr. F. B. CRAMER. 1,4-sorbitan was a gift from the Atlas Powder Co. Other sugars were purchased as follows: glucose, mannose, fructose, glucosamine, L-sorbose, xylose, and ribose, from the Pfanstiehl Chemical Co.; mannoheptulose from General Biochemicals, Inc.; L-arabinose from the British Drug Houses; N-acetylglucosamine from the Nutritional Biochemicals Corp. Glucose-6-phosphate was purchased from the Sigma Chemical Co. as the crystalline heptahydrate of the barium salt. Galactose-6-phosphate was synthesized by Dr. R. K. CRANE by the method of LEVENE AND RAYMOND[8].

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Adenosinetriphosphate (ATP) was purchased from the Pabst Laboratories as the disodium salt. Assay with yeast hexokinase indicated a purity of about 95 %.

Mucosa homogenate

Young albino rats of 150 to 200 g were used. They were fasted 24 hours prior to use except as specifically indicated otherwise. They were killed by decapitation; the upper two thirds of the small intestine was flushed with ice-cold saline, opened lengthwise, and the mucosa was scraped off with a spatula. After weighing, the mucosa was homogenized in a Potter-Elvehjem glass homogenizer, by hand, with 1 volume of 0.25 *M* mannitol-0.005 *M* neutralized Versene (ethylenediaminetetraacetic acid), and the resulting suspension was diluted with another volume of the same medium. Assays of phosphorylating activity were carried out within about half an hour of the preparation of the homogenates. To minimize individual variations homogenates prepared from the intestinal mucosa of 2 rats were generally mixed before use.

Measure of hexokinase activity

Most of the experiments were carried out as follows: To small tubes containing sugar (3 μM), ATP-$MgSO_4$ (15 and 7.5 μM respectively, neutralized), NaF (25 μM) and potassium phosphate and tris(hydroxymethyl)aminomethane (10 μM each, pH 8.0) in a volume of 0.3 ml was added 0.2 ml of the homogenate, and the mixture was incubated 7.5 and 15 minutes (occasionally from 2.5 to 30 minutes). The reaction was stopped by the addition of the $Ba(OH)_2$ solution of SOMOGYI's Ba-Zn deproteinization procedure[9]. Substrate disappearance in comparison with an unincubated control was estimated in the filtrates by SOMOGYI's method for sugars, increasing the boiling time, the alkalinity of the copper reagent, or both when appropriate for a particular sugar (see[10]) or by ROE's method for ketoses[11].

Total ketose was estimated in some cases by applying ROE's method to trichloroacetic acid-Norit-A charcoal filtrates[7].

Other methods occasionally used will be described with the corresponding experiments.

EXPERIMENTAL

Preliminary work

The conditions for a reliable assay of hexokinase activity were explored with glucose as substrate.

The rats were fasted to minimize endogenous sugar in the preparations. The hexokinase activity of the homogenates appeared to be very labile, more than half of the activity being lost within 24 hours at 4°. Among a number of agents tested as possible stabilizers, Versene has a definite though small stabilizing effect, and mannitol at isotonic concentration provided some stabilization. The conditions of assay were chosen so as to minimize phosphomonoesterase activity and to allow for the rapid ATP loss (Table I). Substrate concentration was kept low enough to permit a high relative utilization within the limits of time imposed by the instability of the enzyme and the destruction of the ATP. For comparable results equality of both sugar con-

TABLE I

PHOSPHOMONOESTERASE ACTIVITY AND ATP DISAPPEARANCE IN THE STANDARD ASSAY CONDITIONS FOR HEXOKINASE

	µM/15 minutes
Hexokinase (on glucose)	2.4
Phosphomonoesterase*	*ca.* 0.1
ATP disappearance**	5.2

* Galactose-6-phosphate (2.5 μM) as substrate. Hydrolysis was estimated from the appearance of free sugar.

** No sugar added. Residual ATP was estimated (after heating the mixture in boiling water for 3 minutes) with myokinase-free yeast hexokinase and excess glucose.

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centration and total volume was maintained. With glucose at the initial concentration of $6 \cdot 10^{-3}$ M the enzyme is saturated with substrate until well over 50% of the glucose has been utilized (see below).

Under these conditions the utilization of glucose is of the order of 1.7 to 2.5 μM in 15 minutes. This corresponds to a specific activity of 0.35 to 0.50 μM/mg/15 minutes (see [12]).

Sugars which are phosphorylated

The sugars under investigation were tested under the standard assay conditions in parallel experiments; an assay with glucose was always included for reference. Fig. 1 presents an experiment with glucose, galactose, and mannose. Results with all the sugars tested are summarized in Table II.

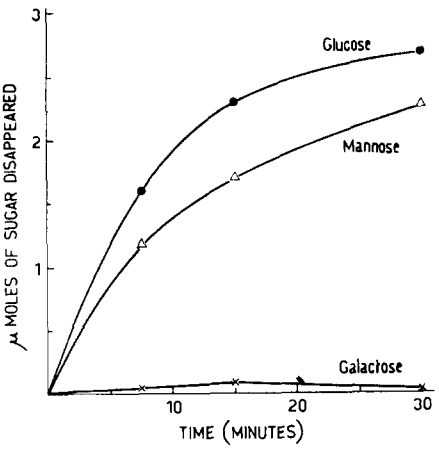

Fig. 1. Utilization of glucose and mannose and non-utilization of galactose in a parallel experiment.

In our studies with glucose, the difficulties which attend the use of substrate disappearance as an assay of activity at widely different initial glucose concentrations were avoided by the use of an indirect method in which the formation of ketose ester is detected. Under our standard conditions of incubation, phosphofructokinase is of the same order of activity as hexokinase and phosphoglucose isomerase is considerably higher[13]. Thus, glucose phosphorylation is accompanied by a proportional formation of ketose ester, and no ketose ester is formed when either glucose or ATP is omitted. When, using this method, an influence of glucose concentration on the rate of the reaction was looked for none was found in the range of glucose concentrations from $2 \cdot 10^{-3}$ M to $4 \cdot 10^{-2}$ M, as shown in Fig. 2. The results of this experiment indicate that the Michaelis constant (K_m) for glucose is of the order of $2 \cdot 10^{-4}$ M or less and do not confirm the anomalous rates of glucose phosphorylation at the higher concentrations which have been reported by HELE[4].

TABLE II

PHOSPHORYLATION OF DIFFERENT SUGARS RELATIVE TO GLUCOSE AT $6 \cdot 10^{-3} M$ SUGAR CONCENTRATION

	Relative rate		*Relative rate*
Fructose	120	3-Methylglucose	$< 5^*$
Glucose	100	L-Sorbose	$< 5^*$
2-Deoxyglucose	90	Mannoheptulose	$< 5^*$
Glucosamine	75	N-Acetylglucosamine	$< 5^*$
Mannose	75	Xylose	$< 5^*$
Allose	10	Ribose	$< 5^*$
Galactose	$< 5^*$	L-Arabinose	$< 5^*$

Assay under standard conditions. Rates were calculated from the sugar which disappeared during 10 minutes of incubation, except for allose which was incubated for 30 minutes.

* No significant sugar disappearance within 30 minutes incubation.

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The experiment in Fig. 3 indicates that the K_m for fructose is approximately $4 \cdot 10^{-3}$ M. The ratio V_{max} fructose/V_{max} glucose would be about 1.8.

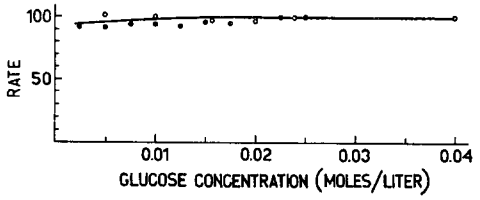
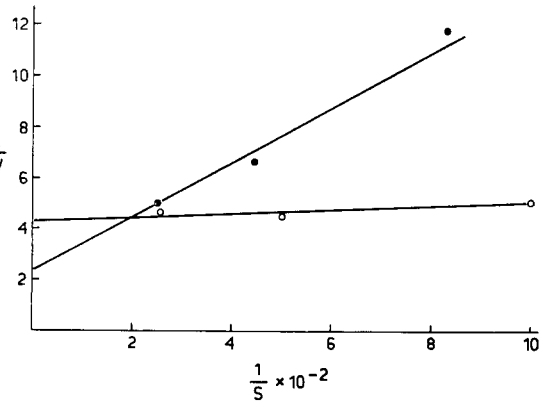


Fig. 2. The effect of the concentration of glucose on the phosphorylation rate. Assay under standard conditions except for substrate concentration as indicated and total volume which in one series (●) was raised to 1.0 ml by dilution with water. Incubation time 10 minutes. Unincubated controls were prepared for each glucose concentration. Estimation of total ketose. Free sugar disappearance at the lowest concentration was 1.0 μM; ketose formation throughout was *ca.* 0.8 μM.

Fig. 3. The effect of the concentration of fructose on the phosphorylation rate. The standard assay mixture (0.5 ml) was diluted with 0, 0.5, and 1.5 ml of water so as to obtain the initial concentrations 6, 3, and $1.5 \cdot 10^{-3}$ M. As a precaution against unspecific dilution effects glucose was tested similarly. Time of incubation, 15 minutes. Fructose phosphorylation was estimated by ketose disappearance; glucose phosphorylation by sugar disappearance. Rates plotted[14] against average substrate concentrations. Fructose phosphorylation in the undiluted experiment was 2.0 μM.



Some of the sugars were also tested at high concentration through isolation of the phosphorylated products, as shown in Table III. The results confirm the high rate of fructose phosphorylation. The rate of allose phosphorylation appears to be increased with the concentration. At this high substrate concentration there is a perceptible phosphorylation of L-sorbose.

TABLE III

PHOSPHORYLATION AT $5 \cdot 10^{-2}$ M SUGAR CONCENTRATION

	Incubation minutes	*Total ester isolated* μM
Fructose	10	2.20
Glucose	10	1.15
Glucose	30	2.35
Allose	30	0.90
L-Sorbose	30	0.60
Galactose	30	$< 0.10^*$
3-Methylglucose	30	$< 0.10^*$
Xylose	30	$< 0.10^*$

Incubation under standard conditions except for the sugar concentration. TCA-Norit filtrates were treated with excess barium acetate and neutralized to pH *ca.* 8.2 (phenolphthalein); after addition of 4 volumes of 96 % ethanol the tubes were chilled, centrifuged and thoroughly decanted; the precipitates were washed with 80 % ethanol containing barium acetate; the washed precipitates were treated with 0.1 N H_2SO_4 and centrifuged; the supernatant fluids were analysed for carbohydrate with anthrone[15]. Unincubated controls for every sugar gave essentially uniform low values. Results with fructose and glucose were calculated with fructose-1,6-diphosphate as standard; the others with standards of the corresponding sugars. The figures for 10 and 30 minutes incubation were obtained with different homogenates.

* After correction for a 0.7 % glucose impurity.

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Competition between sugars

The principal substrates were tested in pairs to obtain evidence that a single enzyme is involved and to obtain some indication of relative apparent affinities. Table IV shows that glucose strongly inhibits the phosphorylation of fructose, while fructose inhibition of glucose is hardly detectable. The effect of different sugars on fructose utilization is shown in Table V.

TABLE IV

COMPETITION BETWEEN GLUCOSE AND FRUCTOSE

	μM phosphorylated	
	Glucose	*Fructose*
Glucose alone	1.4	—
Fructose alone	—	1.75
Glucose + fructose	1.3	0.1

3 *μM* of each sugar under otherwise standard conditions. Incubation time, 10 minutes. Fructose phosphorylation estimated by ketose disappearance; glucose phosphorylation calculated from sugar disappearance minus ketose disappearance.

TABLE V

INHIBITION OF THE PHOSPHORYLATION OF FRUCTOSE BY SUBSTRATES OF HEXOKINASE AND RELATED COMPOUNDS

Compound	*μM/ml*	*Inhibition per cent*
Glucose	6	90
Mannose	6	80
N-Acetylglucosamine	2	75
Mannoheptulose	2	65
2-Deoxyglucose	6	60
Glucosamine	6	60
Xylose	6	50
Allose	6	none
Galactose	6	none
3-Methylglucose	6	none
1,5-Sorbitan	100	40
1,4-Sorbitan	100	none

Assay under standard conditions with $6 \cdot 10^{-3} M$ fructose plus other compounds at the initial concentration indicated. Ketose disappearance was measured.

Sugar disappearance from a mixture of 3 *μM* of glucose, mannose, or 2-deoxyglucose and 10 *μM* of N-acetylglucosamine was smaller than from the corresponding substrates alone; that from glucose plus galactose or 3-methylglucose at equimolar concentrations was equal to that from glucose alone. Both N-acetylglucosamine and xylose inhibit the phosphorylation of allose.

The inhibition of glucose phosphorylation by N-acetylglucosamine decreases with increasing substrate concentration. On the assumption that the inhibition is strictly competitive, the experiment of Fig. 4 would indicate the ratio K_m N-acetylglucosamine/K_m glucose to be approximately 4.

Phosphorylation of L-sorbose at $5 \cdot 10^{-2}$ *M* concentration as in the experiment reported in Table III was unaffected by $1 \cdot 10^{-2}$ *M* N-acetylglucosamine.

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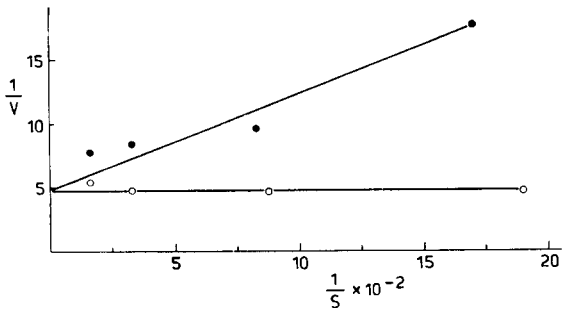

Fig. 4. Inhibition of glucose phosphorylation by N- acetylglucosamine. Incubation (15′) as in the standard assay method except for the amount of sugar and the total volume which was 0.65 ml. Analysis by the total ketose method. Ketose formation without N-acetylglucosamine was 1.1 μM. Rates plotted[14] against average glucose concentration. ○ = glucose alone. ● = glucose plus $6.1 \cdot 10^{-2}$ M N-acetylglucosamine.

These observations suggest that two phosphorylating enzymes are present in mucosa homogenates. Substrates other than L-sorbose are phosphorylated by a hexokinase for which they and certain structurally related compounds compete. The order of relative affinities appears to be as follows: glucose, mannose > 2-deoxyglucose, mannoheptulose, glucosamine, N-acetylglucosamine > xylose, fructose > allose, 1,5-sorbitan. The enzyme that phosphorylates L-sorbose at high concentration is probably similar to that found by SLEIN, CORI AND CORI in muscle[16] though its activity cannot be very great in amount relative to hexokinase since glucose inhibits fructose phosphorylation by at least 90% (Table V).

TABLE VI

INHIBITION BY GLUCOSE-6-PHOSPHATE

Additions	*Average concentration*, µM/ml*		*Hexokinase activity, µM free sugar disappeared*
	glucose-6-P	*galactose-6-P*	
None	0.2	—	0.98
Glucose-6-phosphate	5.5	—	0.48
Galactose-6-phosphate	0.2	8.7	0.92

Assay with glucose as substrate under standard conditions, with additions (4.4 μM) as indicated.

* Calculated from the activities of phosphoglucose isomerase, phosphofructokinase, and phosphomannose isomerase. Phosphomonoesterase activity under these conditions is so low (Table I) that it can be neglected.

The product of reaction

Phosphoglucomutase, although present[13], is not active under the standard conditions of incubation. This observation and the fact that phosphorylated glucose is rapidly transformed into ketose esters (described above) point to glucose-6-phosphate as the primary product of the phosphorylation of glucose. It also may be noted that mannose phosphorylation is likewise accompanied by ketose formation and that the intestinal mucosa contains a phosphomannose isomerase[17]. Glucosamine, on the contrary, does not give rise to ketose formation, which indicates that glucosamine can be phosphorylated without prior deamination. The product of allose phosphorylation accumulates as an aldose reducing ester. These facts indicate that the phosphoric group is introduced into position 6 of the substrate. Inhibition of the phosphorylation of fructose by 1,5-sorbitan and not by 1,4-sorbitan further indicates that the substrates are the pyranose forms of the aldoses and the furanose form of fructose.

Added glucose-6-phosphate can inhibit the hexokinase activity of the homoge-

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nates. The experiment of Table VI suggests that the inhibition is of the same type as that of brain hexokinase[18], although the concentration required for 50% inhibition, *ca.* $6 \cdot 10^{-3}$ *M*, is ten times higher than that required for 50% inhibition of the particulate brain hexokinase. The low apparent affinity of glucose-6-phosphate for the enzyme together with the high activities of phosphoglucose isomerase and phosphofructokinase permit negligible accumulation of and autoinhibition by glucose-6-phosphate under our assay conditions.

Phosphorylation in non-fasted animals

In contrast to results reported by others with mucosa homogenates or extracts from non-fasted rats[4,5,6], we could not demonstrate a phosphorylation of galactose, xylose or 3-methylglucose in mucosa homogenates prepared from fasted rats. This difference in results is striking and would seem to have two possible explanations. Either the phosphorylation of these sugars is accomplished by specific enzymes (other than hexokinase) which are labile and are not active in our preparations or the reported phosphorylations are not real and result from an unsuspected artefact. The latter possibility was strongly suggested by our early observation that Ba-Zn filtrates prepared from mucosa homogenates of *non-fasted* rats contain significant amounts of reducing substances. Moreover, these reducing substances increase on incubation of the homogenates in the absence of added ATP and decrease when ATP is added. Thus, incubation even in the absence of added substrate of an otherwise completely fortified homogenate prepared from *non-fasted* rats will always provide data indicative of phosphorylation. When this endogenous phosphorylation is appropriately corrected for, it is found that only those compounds which are substrates for hexokinase have any influence on sugar disappearance over the endogenous values.

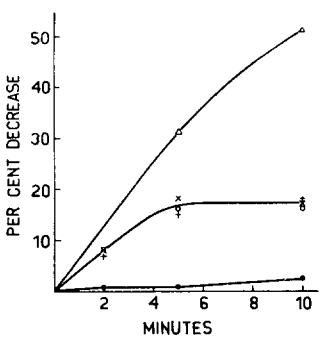

Fig. 5. Sugar and ketose disappearance with homogenate of intestinal mucosa of a non-fasted rat. Assay under standard conditions except for the sugars (2 μM) as indicated below. Filtrates analysed for sugar disappearance with the aldoses and for both sugar and ketose disappearance with the ketose. Results plotted as per cent of the values at zero time.
+ = galactose
× = incubation without added sugar; galactose added *after* the $Ba(OH)_2$
L-sorbose:
○ = sugar disappearance
● = ketose disappearance
△ = glucose.

The point is illustrated by the experiment depicted in Fig. 5. In this experiment, galactose, glucose, and L-sorbose were separately incubated with a mucosal homogenate from a non-fasted rat. When assayed by sugar disappearance with the sugar present from the beginning of the incubation, a decrease in reducing power was observed in all instances. However, the decrease in the presence of glucose continued at a good rate for at least 10 minutes whereas in the presence of galactose and L-sorbose it ceased abruptly at 5 minutes. Moreover, incubation of the homogenate in the absence of added sugar, galactose being added after the termination of the incubation with $Ba(OH)_2$, gave precisely the same result as when galactose was present throughout. As a further control, ketose was assayed in the tubes to which L-sorbose had been added and it was found not to have decreased to a significant extent. It is, thus, clear that the endogenous phosphorylation can cause, if not corrected for, a considerable

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error, especially with short incubation periods such as those used by HELE[4] and CSAKY[19].

DISCUSSION

Our findings that the hexokinase of the intestinal mucosa has a substrate specificity similar to that of the hexokinases of other animal tissues[7, 20] and dissimilar to that of the absorption of sugar by the intestinal mucosa[1, 2] do not support the hypothesis that hexokinase is involved in intestinal absorption and, indeed, suggest that phosphorylation is not a part of the absorptive process. Our experiments on mucosal homogenates from non-fasted rats not only failed to provide us with evidence of "labile" hexose phosphorylating enzymes but also indicated that the reports of HELE[4], BISSEGGER AND LASZT[5] and CSAKY[6] which claim a specificity for phosphorylation similar, if not identical, to the specificity of intestinal absorption are not free of the experimental complications resulting from endogenous phosphorylation in these preparations.

The quantitative aspects of our data indicate that the normal role of mucosal hexokinase is solely to initiate glycolysis for the tissue's own needs. The hexokinase activity of the intestinal mucosa appears to be about the same as in most animal tissues[21] although less than that of brain[12]. In the mucosa it is of the same order of magnitude as the phosphofructokinase and phosphoglucomutase and much less than the phosphoglucose isomerase. With this in mind, the increase in hexose phosphates in the mucosa during absorption of certain sugars[22, 23*], which has been cited as evidence to link absorption with phosphorylation, becomes only an expected consequence of the presence of hexokinase.

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SUMMARY

Homogenates of rat intestinal mucosa phosphorylate glucose, fructose, mannose, glucosamine, 2-deoxyglucose, allose and L-sorbose. No phosphorylation of galactose or 3-methylglucose has been observed. With the exception of L-sorbose, the phosphorylations are carried out by a single enzyme with a pattern of specificity similar to that of brain hexokinase. The hexoses appear to be phosphorylated in the 6 position. Glucose-6-phosphate inhibits the enzyme.

The data indicate that hexokinase is not a part of the mechanism of intestinal absorption of sugars.

* KJERULF-JENSEN[23] has reported an ester suggesting galactose-6-phosphate during galactose absorption. The concentration of galactose in the cells of the intestinal mucosa might become so high as to allow for an appreciable "marginal" phosphorylation (see[7]). A non-metabolizable ester even if formed very slowly could easily attain a concentration of an order of magnitude similar to that of those in a highly dynamic state. But since other possibilities are open, identification of the accumulated compound with more specific methods would be interesting.

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RÉSUMÉ

Les homogénéisats de muqueuse intestinale de rat phosphorylent les sucres suivants: glucose, fructose, mannose, glucosamine, 2-desoxyglucose, allose et L-sorbose. On n'a pas observé de phosphorylation de galactose ni de 3-méthylglucose. Avec l'exception du L-sorbose, les phosphorylations sont réalisées par un seul enzyme avec une spécificité semblable à celle de l'hexokinase du cerveau. Les hexoses semblent être phosphorylés dans la position 6. Le glucose-6-phosphate inhibe l'enzyme.

Les résultats indiquent que l'hexokinase ne joue aucun rôle dans le mécanisme de l'absorption intestinale des sucres.

ZUSAMMENFASSUNG

Homogenate der Darmschleimhaut der Ratte phosphorylieren Glucose, Fructose, Mannose, Glucosamin, 2-Desoxyglucose, Allose und L-Sorbose. Es wurde weder Galactose- noch 3-Methylglucose-Phosphorylierung beobachtet. Diese Phosphorylierungen wenden, mit Ausnahme der L-Sorbose, von einem einzigen Enzym mit einem Spezifitätstyp der dem der Gehirn-Hexokinase ähnelt zu stande gebracht. Die Hexosen scheinen in der Stellung 6 phosphoryliert zu werden. Glucose-6-phosphat hemmt das Enzym.

Die Ergebnisse zeigen, dass die Hexokinase nicht an dem Mechanismus der Darmresorption der Zucker beteiligt ist.

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