Hepatic Glucose Phosphotransferases

Variations Among Species

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SUMMARY

Glucokinase activity (ATP:D-glucose-6-phosphotransferase, E.C. 2.7.1.2) was assayed in various species, including man (*Homo sapiens*). Low or absent activities were found in the toadfish (*Opsanus tau*), cat (*Felis domesticus*), guinea pig (*Cavia porcellus*), calf (*Bos taurus*), and man, and higher activities in the bullfrog (*Rana catesbeiana*), dog (*Canis familiaris*), rabbit (*Oryctolagus cuniculus*), mouse (*Mus musculus*), rat (*Rattus norwegicus*), and sand rat (*Psammomys obesus*). Fasted mice and dogs failed to show a decrease in activity. Sand rats with spontaneous diabetes or rendered diabetic by diet also failed to show a reduction in activity. The role of the enzyme in the regulation of blood glucose concentration remains speculative. DIABETES *15*:475-79, July, 1966.

Previous studies demonstrated both increased glucose utilization by liver slices with increasing glucose concentrations as high as 0.08 M[1,2] and a cell membrane very permeable to glucose.[3] A glucose phosphorylating mechanism differing from other hexokinases by nature of its low affinity for glucose was demonstrated by DiPietro and Weinhouse,[4] resolved into two enzymes by Vinuela, Salas and Sols,[5] by Walker,[6] and by Weinhouse and his group[7] and has been characterized further by a number of investigators. One enzyme has a low apparent Km, is a nonspecific hexokinase (ATP:D-hexose 6-phosphotransferase, E. C. 2.7.1.1) and is stable under dietary and hormonal changes, and the other has a high apparent Km, is a more specific glucokinase (ATP:D-glucose 6-phosphotransferase, E.C. 2.7.1.2), and is dependent upon the nutritional state of the animal and the presence of insulin. High activities of this latter enzyme have been found in carbohydrate feeding and low activities in fasting and diabetes.[4-22]

The capacity of the liver to regulate the concentration of glucose in blood was demonstrated by Soskin et al.[23] and has been correlated with the relative activities of glucokinase and glucose-6-phosphatase (D-glucose-6-phosphate phosphohydrolase, E.C. 3.1.3.9).[24] These facts, particularly those involving enzyme activities, having been obtained mainly from rodent experiments, prompted a comparative search for phosphotransferase activities in different species, particularly in man, with both clinical and general biological interests in mind.

METHODS

Assays for glucokinase and hexokinase were performed in every detail according to the methods of Sharma et al.[8] These involved the determination of NADP* reduction in the presence of glucose-6-phosphate dehydrogenase (D-glucose-6-phosphate; NADP oxidoreductase, E.C. 1.1.1.49) in supernatant fluids obtained from 33 per cent liver homogenates in EDTA-mercaptoethanol buffer and centrifuged for forty-five minutes at 105,000 G in a Model L Spinco. Glucose 5×10^{-4} M and 10^{-1} M was used as substrate to determine hexokinase and hexokinase plus glucokinase activities using a third cuvette containing 5×10^{-2} M N-acetylglucosamine and homogenate as a tissue blank. For these determinations, ATP, NADP* and glucose-6-phosphate dehydrogenase were all obtained from Boehringer Mannheim Corporation, New York, EDTA and glycylglycine from Sigma Chemical, St. Louis, Mo., and β-mercaptoethanol from Eastman Kodak Co., Rochester, N.Y. Absorption was determined at 340 mμ in a Zeiss PMQ spectrophotometer at 25° C. for ten minutes, of which the last five minutes were always linear and used for rate calculations. In each case, other activity determinations using different glucose concentrations were performed to guarantee that changing levels of enzyme activity were not due to alterations in apparent Km. No such instance was found and these data will not be presented.

Other determinations included glucose-6-phosphatase, performed on a diluted aliquot of the total homogenate

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*Enzymes and identification code are according to the Report of the Commission on Enzymes of the International Union of Biochemistry, 1961, Pergamon Press, Oxford, England.

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before centrifugation, by the method of Swanson[25] using the determination of inorganic phosphate[26] as index of activity with glucose-6-phosphate (Sigma Chemical, St. Louis, Mo.) as substrate. Appropriate time and reagent blanks were subtracted from final rates. Liver glycogen was measured by the method of Good, Kramer and Somogyi[27] and glucose determined by glucose oxidase (β-D-glucose:O_2 oxidoreductase, E.C. 1.1.3.4)[28] or standard reduction.[29]

Diabetes was induced in rats (*Rattus norwegicus*) by a single intravenous injection of 40 mg. per kg. alloxan after a twenty-four-hour fast. In mice (*Mus musculus*) the alloxan was administered intraperitoneally, 200 mg. per kg. Only rats with a blood glucose value greater than 300 mg. per 100 ml. were selected. All the diabetic mice had glucose levels over 600 mg. per 100 ml.

Validation of methods: Preliminary studies demonstrated a marked lability of glucokinase activity; so in all experiments livers were removed and prepared as expeditiously as possible. The humans had been fed over 200 gm. carbohydrate daily up to the operation, were anesthetized with cyclopropane and nitrous oxide and were given intravenous glucose during the procedure. None had liver disease as demonstrated by clinical tests and by microscopic examination of a sample of the excised liver specimen. The operation itself was for nonrelated gastrointestinal surgery. A 1- to 3-gm. wedge of liver was excised after hemostatic sutures had been placed but not tightened; therefore, only one to five seconds elapsed between in vivo perfusion and immersion in the iced EDTA-mercaptoethanol buffer.[8] Within fifteen minutes these samples were homogenized and placed in the ultracentrifuge. For the analyses on calf (*Bos taurus*), cat (*Felis domesticus*), rabbit (*Oryctolagus cuniculus*) and dog (*Canis familiaris*), immediate death was obtained by a captive bolt pistol. Rats and mice were narcotized with 50 per cent O_2 : 50 per cent CO_2, this mixture having been shown previously[30] to have no known acute metabolic effects on carbohydrate metabolism. Cold-blooded species were anesthetized with trimercaine methane sulfate (Sandoz), 1 gm. in one inch of fresh water for frogs (*Rana catesbeiana*) and in sea water for fish (*Opsanus tau*).

The above precautions were taken since it was found that rat glucokinase was extremely labile; for example, incubating rat liver at 37° C. for thirty or sixty minutes resulted in much loss of activity (figure 1). Likewise, freezing and thawing the liver caused loss of almost all activity. The enzyme, however, was very stable in the supernatant fluid after centrifugation.

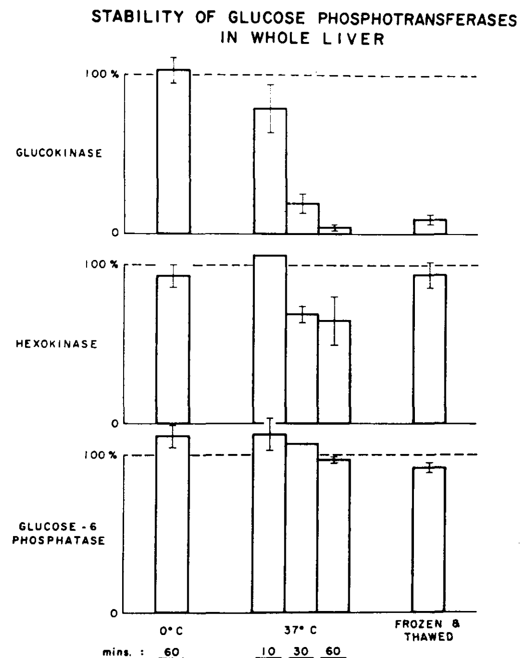

FIG. 1. Stability of glucose phosphotransferases in rat liver expressed as a per cent of the activity of fresh liver immediately chilled and homogenized. The column on the left is whole liver kept in iced buffer for sixty minutes and then homogenized, showing no loss of activity (n = 2). Columns in the center represent whole liver incubated at 37° C. for ten, thirty or sixty minutes (n = 2, 2, 5), showing a rapid and significant decay of glucokinase and, to a lesser extent, hexokinase activities. The column on the right represents fresh liver rapidly frozen and thawed at the time of homogenization, demonstrating a marked loss of glucokinase activity (n = 10).

Storage of frozen supernatant fluid at —20° C. or repeated freezing or thawing of the supernatant fluid did not result in loss of glucokinase activity. Also, no change in activity was noted in whole liver if kept cold in iced buffer for one hour. Hexokinase and glucose-6-phosphatase activities were unchanged by these various maneuvers.

RESULTS

As shown in table 1, there was a significant reduction in liver glucokinase activity in fasted and alloxan diabetic rats, in agreement with previous observations by many investigators. Also, as previously reported, there was no change in hexokinase activity, particularly when expressed per 100 gm. body weight. Glucose-6-phosphatase activity in diabetes was markedly elevated, as previously demonstrated.[31]

In contrast, in laboratory mice (table 2) fasting resulted in little change in glucokinase activity and only with diabetes did it decrease significantly ($p <$

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TABLE 1

Laboratory rats, dietary effects on liver glucose phosphotransferases

	Hexokinase*		Glucokinase*		Glucose-6-phosphatase†	
	U./gm. liver	U./100 gm. body weight	U./gm. liver	U./100 gm. body weight	U./gm. liver	U./100 gm. body weight
Normal, fed (26)	0.6± 0.03	2.4± 0.20	1.9± 0.16	8.2± 0.76	8.4± 0.24	36± 1.1
Fasted, forty-eight hours (4)	0.8± 0.15	2.4± 0.44	0.7± 0.17	2.2± 0.53	13.5± 0.39	42± 1.6
Fasted, seventy-two hours (4)	1.3± 0.31	3.4± 1.58	0.1± 0.1	0.4± 0.28	12.3± 0.09	39± 2.7
Alloxan diabetes (11)	0.6± 0.06	2.9± 0.33	0	0	19.4± 1.01	100± 8.2

*Units of hexokinase and glucokinase = μmoles of NADPH/min. at 25° C.
†Units of glucose-6-phosphatase = μmoles phosphorus/min. at 30° C.
Figures in parentheses = number of animals.

TABLE 2

Laboratory mice, dietary effects on liver glucose phosphotransferases

	Hexokinase*		Glucokinase*		Glucose-6-phosphatase†	
	U./gm. liver	U./100 gm. body weight	U./gm. liver	U./100 gm. body weight	U./gm. liver	U./100 gm. body weight
Normal, fed (10)	0.3± 0	1.5± 0.16	1.6± 0.12	8.8± 0.81	12± 0.6	65± 3.0
Fasted, twenty-four hours (2)	0.3± 0	1.5± 0.07	2.2± 0.25	10.2± 1.15	13± 0.6	63± 1.1
Fasted, forty-eight hours (2)	0.4± 0.1	1.5± 0.09	1.6± 0.12	6.1± 0.07	13± 0.9	50± 0.5
Fasted, seventy-two hours (11)	0.2± 0.02	0.9± 0.07	1.9± 0.17	7.6± 0.73	11± 0.4	44± 2.5
Alloxan diabetes (21)	0.3± 0.03	1.6± 0.19	0.5± 0.08	3.0± 0.45	16± 0.5	87± 3.3

*Units of hexokinase and glucokinase = μmoles of NADPH/min. at 25° C.
†Units of glucose-6-phosphatase = μmoles phosphorus/min. at 30° C.
Figures in parentheses = number of animals.

0.001). Glucose-6-phosphatase, as in rats, increased significantly with diabetes ($p < 0.001$). Since these results on glucokinase activity differed somewhat from those observed in the rat, blood glucose and liver glycogen were determined to document that these animals were indeed fasting. These results are shown in table 3.

The disparity in dietary response of glucokinase between rats and mice prompted assays in other species. Of particular interest seemed the Egyptian sand rat (*Psammomys obesus*) which may develop diabetes mellitus when fed a synthetic laboratory ration[32] instead of its usual fare of leafy vegetables. Animals were obtained from Egypt,* placed on Purina Laboratory Chow for four days during which time one developed mild diabetes, and interestingly exhibited no decrease in glucokinase activity (table 4, animal No. 4).

*Obtained through the courtesy of Dr. Harry Hoogstrahl of NAMRU-3, in Egypt and Drs. Hackel, Prange and Schmidt-Nielsen at Duke University, Durham, N.C.

TABLE 3

Laboratory mice, dietary effects on blood glucose and liver glycogen

	Number of animals	Blood glucose mg./100 ml.	Liver glycogen mg./gm. liver
Normal fed	12	148±6	54±4
Fasted			
Twenty-four hours	7	69±6	2±0.4
Forty-eight hours	7	58±8	4±1.0
Seventy-two hours	6	95±10	10±1.4
Ninety-six hours	7	69±7	16±0.9
Alloxan diabetes	9	731±32	17±1.8

Another sand rat, diabetic on arrival from Egypt (table 4, animal No. 7), with an initial blood glucose of 322 mg. per 100 ml. and with hyperlipemia and ketonuria, also demonstrated normal activity of glucokinase.

A survey of other species, easily obtained in the laboratory, was therefore instituted. Glucokinase activity

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TABLE 4

Sand rats, dietary effects on liver glucose phosphotransferases

Diet	Hexokinase* U./gm. liver	Hexokinase* U./100 gm. body wt.	Glucokinase* U./gm. liver	Glucokinase* U./100 gm. body wt.	Glucose-6-phosphatase† U./gm. liver	Glucose-6-phosphatase† U./100 gm. body wt.	Liver glycogen mg./gm. tissue	Blood glucose mg./100 ml.
1. Vegetables	0.27	1.3	1.6	7.8	5.6	28	40	51
2. Vegetables	0.27	1.6	2.5	14.2	5.0	29	55	78
3. Vegetables	0.20	1.0	2.0	10.5	6.5	33	38	51
4. Laboratory chow four days	0.33	2.0	2.3	13.9	9.3	55	45	239
5. Laboratory chow four days	0.23	1.4	2.4	14.1	10.1	60	41	84
6. Laboratory chow four days	0.20	1.2	3.4	20.7	8.3	50	57	86
7. Spontaneous diabetes	0.20	1.0	2.0	9.6	6.7	33	—	322

*Units of hexokinase and glucokinase = μmoles of NADPH/min. at 25°C.
†Units of glucose-6-phosphatase = μmoles of phosphorus/min. at 30° C.

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was from extremely low to absent in the toadfish, guinea pig, cat and man. The toadfish were well-fed with either carbohydrate (glucose) or protein (scallops) and in other experiments received insulin and glucose intraperitoneally for a period of four to six days without any increase in glucokinase activity. The cats, guinea pigs and three humans also possessed adequate quantities of liver glycogen (> 20 mg. per gm.) and were well-fed with diets high in carbohydrate without evoking an increase in activity. Altering glucose concentration in the assay did not reveal an alteration in affinity as a cause of the low activity, and enzymes from all species exerted a similar temperature response; thus the species differences could not be explained on these bases. Five dogs, fed or fasted for four days, also failed to show an alteration in glucokinase activity similar to the experiments on mice. Liver specimens from six calves obtained at a local slaughterhouse, contained 0.22 ± 0.01 U. per gm. hexokinase, 0.16 ± 0.02 U. per gm. glucokinase, and 14.9 ± 0.14 U. per gm. glucose-6-phosphatase activities. Glycogen values were less than 10 mg. per gm., thus these animals were presumably in a fasting state, as reflected by the glycogen values, and it cannot be stated from this series whether or not they would have had higher glucokinase levels when fed.

DISCUSSION

These studies have revealed a divergence among hepatic phosphoglucotransferases in several different species. If liver has a membrane very permeable to glucose, and the concentration of glucose inside the cell is equal or close to the extracellular concentration, the net flow of glucose into and out of the pool (or pools) of glucose-6-phosphate is dependent upon the respective activities of the two kinases and glucose-6-phosphatase, the enzymes regulating glycogen synthesis, glycolysis and the direct oxidative pathway, and also glucose dehydrogenase (β-D-glucose: NAD (P) oxidoreductase, E.C. 1.1.1.47) if such an enzyme be present.[21] The lack of any decrease in glucokinase activity in fasting mice and dogs and diabetic sand rats and its relative absence in certain other species, well-fed at the time, all question whether the assay itself is a representation of the activity of the enzyme in situ, or whether the assay reflects a difference in the lability of the enzyme, or whether the enzyme is even important in the regulation of carbohydrate metabolism. No definite answers to these queries can be offered except where further studies on enzyme affinities, stability and temperature effects were examined; these suggested an intrinsic and not an artifactual change in activity. Intracellular compartmentalization of enzyme and substrate appear to be a logical explanation, but at present this is only speculative.

Ballard and Oliver[13] reported an absence of glucokinase in fetal and adult sheep (*Ovis aries*), explaining this observation in adult sheep as due to the conversion of carbohydrate in the rumen to volatile fatty acid; thus the animals are on a high fat diet. A similar explanation cannot be offered for cat and man, however. Likewise, Sols et al.[20] failed to find glucokinase activity in the chicken (*Gallus domesticus*), also a nonruminant. In a report published after the completion of this paper, Ballard[21] listed other species which he has assayed, finding low or absent glucokinase activities in cat, sheep, cow, quokka (*Setonix brachyurus*) and tammar (*Wallabie eugenii*) and significant amounts of activity in rat, mouse, possum (*Trichosurus vulpecula*), pig (*Sus scrofa*) and dog. Finally, the presence of ac-

tivity in frog was previously reported by Sharma, Manjeshwar and Weinhouse,[19] even when fasted for a period of several months. These conflicting and contrasting interspecific findings indicate the necessity for further studies, biochemical, comparative and nutritional, before the physiological role of the enzyme can be interpreted.

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