The Effects of Thyroid State on Rat Liver Hexokinase Isozymes[1] (36509)

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The liver is unique in its metabolism of carbohydrates. Liver parenchyma cells contain a stereospecific facilitative carrier for glucose which is not thought to limit the rate of glucose entry into the cell, even in the absence of insulin (1). Therefore, the hepatocyte must rely upon the rates of glucose phosphorylation, dephosphorylation, glycogenesis, glycogenolysis, gluconeogenesis, and glucose metabolism to direct the net movement of glucose within the cell and to regulate blood sugar levels.

Thyroxine (T_4) has a marked effect upon liver carbohydrate metabolism. Thyrotoxic animals frequently have livers that are glycogen depleted and susceptible to chemical injury. In addition, many hepatic enzymes involved in glucose metabolism and the homeostasis of blood sugar are greatly affected by thyroxine. Thyrotoxic animals exhibit an altered tolerance for glucose that is often diabetic in nature.

Until recently, little attention has been given to the effects of thyroxine upon liver glucose phosphorylation, especially with respect to each of the hexokinase isozymes involved (2). The present study investigates the effect of different thyroid states upon each of the four isozymes of liver hexokinase, including glucokinase.

Materials and Methods. Male Sprague-Dawley derived rats purchased from Charles River Breeding Laboratory, Inc. weighing 150 ± 20 g were housed 2 to a cage in a temperature (25 ± 2°) and light controlled room (14 hr of light from 4 a.m. to 6 p.m. and 10 hr of darkness) and maintained on Purina Lab Chow[3] and tap water (*ad libitum*) for 7 days, after which they were ear-marked and divided into 3 groups:

1. Hypothyroid group. Animals designated as hypothyroid (Hypo) were placed on an iodine deficient diet[4] and deionized water for 7 days. On day 8 radiothyroidectomies were performed by sc injection of 1.0 mCi of ^{131}I. The iodine-deficient regimen was continued for an additional 2 days, the diet was then changed to Purina Lab Chow and tap water, the animals were weighed and daily sc injections of 0.1 ml of 2% propylene glycol in 0.9% saline (PG) were initiated.[5]

2. Euthyroid group. This group was maintained on a normal diet and tap water for the duration of the experiment. After assignment to a treatment group, they were weighed and given daily sc injections of PG. The injections were continued for 13 days.

3. Hyperthyroid group. These animals were maintained on a normal diet and tap water for the duration of the experiment. Upon assignment to a treatment group, they were weighed and daily sc injections of 100 μg L-thyroxine (Calbiochem) in 0.1 ml of the PG solution were administered.

General procedure. To confirm the completeness of the thyroidectomies all animals were injected sc with a tracer dose of ^{125}I on the afternoon prior to the day of sacrifice (day 13

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[3] Purina Lab Chow with a stable iodine content of 1.3–1.5 μg/g food.

[4] Remington Iodine Deficient Diet with a stable iodine content of 0.03 μg/g food.

[5] The solvent for T_4 used in the hyperthyroid animals.

of injections). The following morning the animals were weighed and decapitated. The entire trachea, including the larynx and thyroid gland area of each animal, was removed at the time of sacrificing and placed in a test tube (containing a 10% formalin solution) for isotope counting and fixation for subsequent histological processing.

Hexokinase isozyme chromatography. Immediately after exanguination of the animal, its liver was removed, quickly cleaned of extraneous fat and connective tissue, blotted, weighed and homogenized in ice-cold 0.01 *M* Tris–HCl buffer (pH 7.0), containing 0.001 *M* EDTA and 0.005 *M* mercaptoethanol. A 1:1 ratio (w/v) of liver and homogenizing buffer was used in a Potter-Elvehjem homogenizer. In most assays 2 livers were pooled for chromatography.

Liver homogenates were treated and chromatographed on a DEAE-cellulose column according to the method of Gonzalez, *et al.* (3) with the following modifications: ultracentrifugation was done in a Beckman Model 12-65B ultracentrifuge at 65,000 rpm (264,243*g*) for 20 min, a larger chromatographic column was used (2.0 cm i.d. × 20 cm) with a flow rate of approximately 40 ml/hr, 0.005 *M* mercaptoethanol and 0.001 *M* EDTA were included in the elution buffer to stabilize some of the isozymes (4–7), and 10.0 ml fractions were collected. The elutions were carried out in a cold room at 4°. Chloride ion concentration was monitored using an Industrial Instrument, Inc. Model RC-16-B2 conductivity bridge and a Yellow Spring Instrument Co. Model 3403 conductivity cell.

Hexokinase assay. Glucose phosphorylating activity of each DEAE-cellulose elution fraction was measured by the enzyme coupled method of Joshi and Jagannathan [cited in Ref.(8)] with the exception that 2 substrate concentrations were used: 7.0×10^{-5} *M* glucose and 0.12 *M* glucose. These substrate levels were 5 times the substrate concentrations at which maximum velocity is achieved for hexokinase (Hex) III and IV, respectively (7). Such substrate concentrations allowed the measurement of enzymatic activity, within the limits of the assay, without substrate depletion effects and also permitted the effective resolution of the activities of isozymes III and IV, whose peaks overlap after DEAE-cellulose chromatography (Fig. 1).

A Gilford 240 spectrophotometer equipped with a 221 recorder was used to monitor changes in absorbance produced by the reduction of $NADP^+$ (Sigma Chemical Co.). One unit of hexokinase activity was defined as that amount of enzyme that would catalyze the conversion of 1.0 μM of glucose to glucose-6-phosphate/min at 30°.

Characterization of hexokinase isozymes. Kinetic data was gathered on peaks I and and II of the DEAE-cellulose chromatography and subjected to graphic analysis using the method of Lineweaver and Burk (9). Because peaks III and IV overlapped after chromatography (Fig. 1), further processing was necessary before kinetic analysis of these peaks could be performed. The molecular weights of Hex III and Hex IV differ appreciably: mol wt Hex III is 96,000; mol

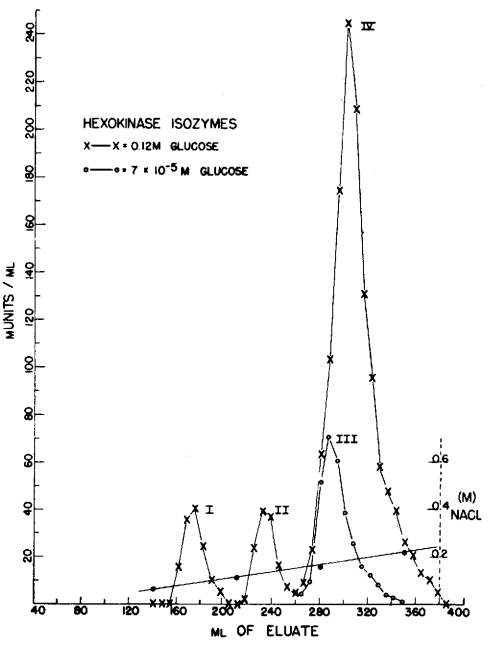

FIG. 1. Typical elution pattern of rat liver hexokinase isozymes I, II, III, and IV eluted with NaCl from a DEAE-cellulose column and assayed at two substrate concentrations: 0.12 *M* glucose and 7×10^{-5} *M* glucose. (●) *M* NaCl.

wt Hex IV is 50,000 (7, 10). Gel filtration was used to resolve these two isozymes. Several DEAE-cellulose chromatographic fractions corresponding to peaks III and IV were combined, and their volume was reduced by swelling Sephadex G-25 in the mixture. The Sephadex G-25 was removed by centrifugation, and the supernate was chromatographed on a Sephadex G-100 column (1.1 cm i.d. $\times$ 18 cm) using the same buffer that was used in the DEAE-cellulose chromatography. Two peaks of glucose phosphorylating activity were resolved, and these were subjected to graphic analysis.

Confirmation of thyroid state. Radioactivity in each thyroid gland area (removed at sacrifice) was counted in a Packard Auto-Gamma well-type scintillation counter equipped with a 2 channel spectrometer, and the percentage uptake of ^{125}I was determined. Appropriate corrections for residual ^{131}I that registered in the ^{125}I channel were made.

Serial sections were prepared from the thyroid areas of each animal and stained with hematoxylin–eosin. The functional state of the thyroid was evaluated on the basis of its histological appearance.

Results. Hexokinase isozyme chromatography. The elution of each Hex from the DEAE-cellulose column occurred at the following NaCl concentrations (Fig. 1): Hex I, 0.04–0.11 *M*; Hex II, 0.11–0.16 *M*; Hex III and Hex IV, 0.16–0.28 *M*.

While assaying the DEAE-cellulose elution fractions for Hex activity, a substance that interfered with the assay was found to elute from the column at the same NaCl concentration as Hex I. This substance caused a rapid $NADP^+$ reduction in the blank tubes of the Hex assay which did not occur in the absence of the highly specific coupling enzyme, glucose-6-phosphate dehydrogenase (zwischenferment-Sigma Chemical Co.), and which was not dependent upon the presence of glucose. It was, therefore, presumed to be glucose-6-phosphate.

Recovery of Hex from DEAE-cellulose chromatography was found to be variable, but it usually ranged between 40 to 50% of the total Hex activity. A value representing recovery from the DEAE column was determined by summing the Hex activity of all DEAE fractions measured with the high glucose concentration (0.12 *M* glucose) assay and dividing this value by the total Hex activity present in the supernate of the ultracentrifuged liver homogenate (also measured at the high glucose concentration).

Characterization of Hex isozymes. 1. Michaelis constants. Lineweaver–Burk plots were done on each resolved Hex. The values for the Michaelis constants (9) obtained were consistent with the data reported by other investigators for Hex I, II, III and IV, respectively (3, 4, 7, 10).

2. Substrate inhibition. An occasion arose whereby substrate inhibition of Hex III became apparent. In one Hex assay, the high substrate concentration assay showed less activity than the low substrate concentration assay (Fig. 2), suggesting that in this particular assay Hex III was in excess of Hex IV and substrate inhibited. Substrate inhibition of Hex III has been reported by other investigators (7).

Confirmation of thyroid state. Radioiodine uptake and histological studies confirmed each respective thyroid state. Histological examination of the thyroid glands of rats thyroidectomized with ^{131}I (Hypo group) showed only connective tissue scars with no intact follicles. These glands were nonfunctional as indicated by their inability to concentrate ^{125}I injected 18 hr previously.

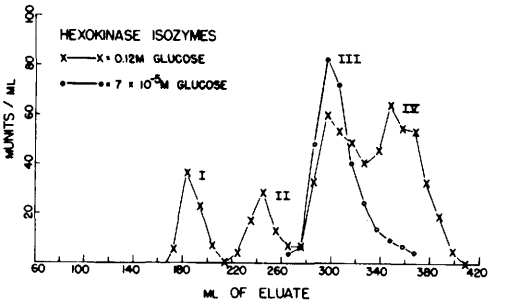

FIG. 2. Substrate inhibition of hexokinase isozyme III. Isozyme III apparently had greater activity than isozyme IV, and when isozyme III was measured at the high substrate concentration, substrate inhibition became evident. Activity is expressed as milliunits per milliliter of DEAE-cellulose eluate.

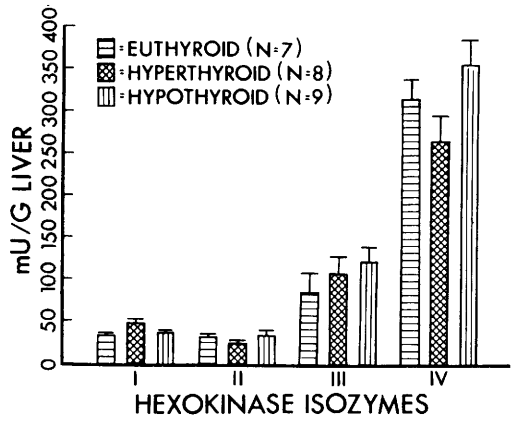

FIG. 3. Liver hexokinase isozyme activities in rats of various thyroid states (mU/g liver). Bars represent group means ± SE.

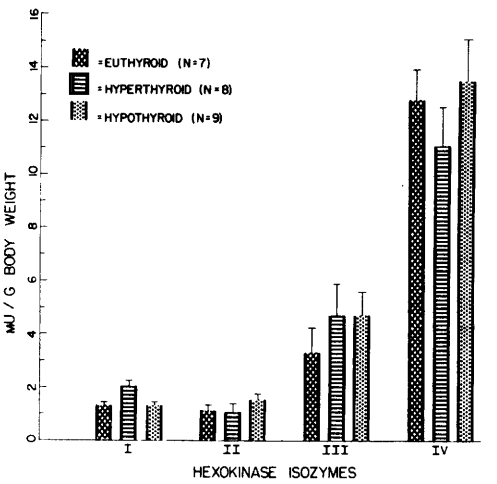

FIG. 4. Liver hexokinase isozyme activities in rats of various thyroid states (mU/g body wt). Bars represent group means ± SE.

Hexokinase assay. Hex I showed significant differences (mU/g of liver) in the various thyroid states (Fig. 3). It was increased 55% ($p < .025$) in the Hyper group (48.6 ± 6.0 mU/g) when this group was compared to the Eu animals (31.3 ± 2.2 mU/g), and it was increased 47% ($p < .025$) when the Hyper group was compared to the Hypo animals (33.1 ± 2.2 mU/g). No significant difference in the levels of Hex I was found between the Hypo group and Eu group. The different thyroid states produced no significant differences in the Hex activity per gram of liver for Hex II or Hex III. Hex IV activity (mU/g liver) was lowest in the Hyper animals (264.8 ± 31.2 mU/g) being reduced 16 and 26% from the values seen in the Eu (316.0 ± 21.2 mU/g) and Hypo (357.0 ± 29.7 mU/g) group, respectively. These differences in Hex IV activity (mU/g liver) were not statistically significant.

When the activities of Hex I (mU/g body wt) were compared, it was found to be increased 58% ($p < .02$) in the Hyper group (1.97 ± 0.23 mU/g) when compared to the Eu animals (1.25 ± 0.09 mU/g) and increased 60% ($p < .01$) when the Hyper group was compared to the Hypo group (1.23 ± 0.07 mU/g). No significant difference was found when the Hypo and Eu groups were compared. No significant differences were noted (mU/g body wt) for Hex isozymes II, III, and IV with respect to thyroid state (Fig. 4).

When the total activity of each isozyme (mU/animal) was compared with respect to thyroid state, different results were apparent. Instead of the significant differences being found in Hex I activity they were found in Hex IV, the glucokinase (Fig. 5). Glucokinase was found to have less activity in the Hyper group than in the Eu or Hypo animals. It was reduced 43% ($p < .01$) when the Hyper group (5010.4 ± 836.2 mU) was compared to the Eu group (8847.0 ± 828.7 mU), and it was reduced 39% ($p < .05$) when the Hyper animals were compared to the Hypo animals (8194.9 ± 1157.7). No significant difference was found when the Eu and Hypo values for Hex IV were compared.

Liver weights and body weights of animals. The liver and body weight of each animal was recorded at the time of sacrifice. The results are presented in Table I. No significant differences were found between the 3 respective treatment groups in absolute liver weight or when liver weight was expressed as percentage of body weight. Treatments had some effect on body weight. The mean body weight of the Hyper animals was 15.3% less ($p < .02$) than that of the Eu group. The body weight of Hypo animals and Eu controls were not significantly different (Table I).

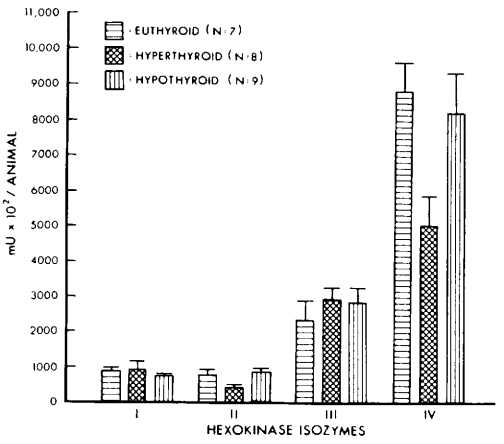

FIG. 5. Liver hexokinase isozyme activities in rats of various thyroid states (mU/animal). Bars represent group means ± SE.

Discussion. The effect of thyroid state upon each of the four isozymes of liver hexokinase was investigated in this study. Hex I activity was found to be increased, and Hex IV activity was decreased in thyrotoxic animals. The reduction in Hex IV activity is of particular interest. At the postprandial glucose level of portal blood (200 mg/100 ml), Hex IV operates at 1/2 its maximum velocity (11). A reduction in Hex IV activity of the magnitude observed could have considerable effect upon the liver's ability to "trap" and store glucose after a meal. This curtailment of glucose phosphorylation coupled with increased glycolysis, and gluconeogenesis (12) could be partly responsible for the altered tolerance for glucose seen in thyrotoxicosis. Intestinal glucose absorption may also contribute, but this is controversial (13).

The decrease in liver weight and body weight observed in the Hyper group was most likely due to the general catabolic effects of high doses of T_4. Since the decrease in body weight was only 15.3%, and the decrease in liver weight was not statistically significant (although the liver weight did tend to decrease in proportion to the decrease in body weight), the 43% decrease in Hex IV activity/animal in the Hyper group was not merely a reflection of a decrease in animal size.

The increase in Hex I activity observed in the Hyper group is difficult to interpret at this time. A relatively small amount of Hex I activity was found in each group, and its contribution to total hepatocyte glucose phosphorylation is probably small. Also, this enzyme functions at its maximum velocity at a glucose concentration well below that normally found in the fasting animal, therefore it probably does not contribute significantly to postprandial blood sugar homeostasis. However, since glucokinase activity at fasting blood sugar levels is very low, it is possible that Hex I and the other two Hex isozymes function to maintain the hepatocyte's need for glucose substrate during postabsorptive periods. Since glucose is reported to be "preferentially" oxidized via the pentose shunt in thyrotoxicosis (14), the change in Hex I activity observed could represent a shifting of glucose substrate into a specific pathway of intermediary metabolism. Further kinetic studies are necessary before the function of each of these Hex isozymes can be elucidated.

Summary. A chromatographic procedure and different substrate concentrations were used to resolve the activities of the four hexokinase isozymes of rat liver. Animals rendered hypothyroid by radiothyroidectomy showed no significant changes in hexokinase isozyme activities after 14 days. Treatment of animals with high doses of thyroxine resulted in in-

TABLE I. Effect of Thyroid Status on Liver and Body Weight.[a]

	Euthyroid	No.	Hyperthyroid	No.	Hypothyroid	No.
Wet wt of liver (g)	13.9 ± .5	(14)	11.8 ± .4	(13)	11.8 ± .5	(18)
Body wt (g)	347.1 ± 14.3	(14)	293.9 ± 14.4	(13)	323.0 ± 11.6	(20)
Liver (% body wt)	4.0 ± .4	(14)	4.1 ± .1	(13)	3.7 ± .1	(18)

[a] Each value represents group mean ± SE.

creased Hex I activity and decreased Hex IV (glucokinase) activity. Possible physiological consequences of these changes were discussed.

1. Williams, T. F., Exton, J. H., and Park, C. R., Amer. J. Physiol. **215**, 1200 (1968).

2. Winnick, S., Endocrinology **87**, 124 (1970).

3. Gonzalez, C., Ureta, T., Sanchez, R., and Niemeyer, H., Biochem. Biophys. Res. Commun. **16**, 347 (1964).

4. Katzen, H. M., and Schimke, R. T., Proc. Nat. Acad. Sci. U.S.A. **54**, 1218 (1965).

5. Katzen, H. M., Soderman, D. D., and Nitowsky, H. M., Biochem. Biophys. Res. Commun. **19**, 377 (1965).

6. Katzen, H. M., Soderman, D. D., and Cirillo, V. T., Ann. N.Y. Acad. Sci. **151**, 351 (1968).

7. Schimke, R. T., and Grossbard, L., Ann. N.Y. Acad. Sci. **151**, 332 (1968).

8. Wood, W. A. (ed.), *in* "Methods in Enzymology" (S. P. Colowick and N. O. Kaplan, eds.), Vol. 9, p. 371. Academic Press, New York.

9. Lineweaver, H., and Burk, D., J. Amer. Chem. Soc. **56**, 658 (1934).

10. Grossbard, L., and Schimke, R. T., J. Biol. Chem. **241**, 3546 (1966).

11. Sharma, C., Marjeshwar, R., and Weinhouse, S., Advan. Enzyme Regul. **2**, 189 (1964).

12. Böttger, I., Kriegel, H., Wieland, O., Eur. J. Biochem. **13**, 253 (1970).

13. Middleton, W. R. J., Gut **12**, 172 (1971).

14. Ashmore, J., Cahill, G. F., Jr., and Hastings, A. B., *in* "Recent Progress in Hormone Research" (G. Pincus, ed.), Vol. 16, p. 547. Academic Press, New York (1960).

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