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Environmental Effects on Glutathione-Insulin Transhydrogenase in Rat Liver[1] (39451)

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Glutathione-insulin transhydrogenase (GIT, thiol-protein disulfide oxidoreductase, EC 1.8.4.2) catalyzes an interchange of sulfhydryl and disulfide groups on proteins and small molecules. In practice, the enzyme catalyzes the reduction of the disulfide bridges of insulin in the presence of excess glutathione (1, 2). *In vitro*, this reduction of insulin is accompanied by the oxidation of the sulfhydryl groups of glutathione. Since it has been shown by Chandler and Varandani (3) that other sulfhydryl-containing proteins may replace glutathione in the reaction, the actual substrate pair utilized *in vivo* is not clear at this time.

The GIT-catalyzed reduction of insulin has been shown to be the rate-controlling step in insulin degradation. This raises the possibility that GIT may function to mediate insulin action (4). Glutathione-insulin transhydrogenase is widely distributed in a number of organs in several species (5). Measurements of the specific activity of GIT in various tissues have shown that those most sensitive to insulin action, i.e., heart, skeletal muscle, and fat, have the lowest levels of GIT, while insulin-insensitive tissues such as the liver and kidney exhibit the highest levels of this enzyme (6). More recently glutathione-insulin transhydrogenase has been shown to be inducible by insulin injection in diabetic rats (7).

The studies described in this paper were designed to investigate several aspects of the regulation of GIT under various dietary, developmental, and hormonal conditions. In order to relate GIT activity to the amount of enzyme present in the material analyzed, the assay for the enzyme described by Chandler and Varandani (8) utilizing ^{125}I-labeled insulin was modified accordingly.

Methods. Albino male rats were obtained from the Holtzman Rat Company, Madison, Wisconsin. For the developmental experiments pregnant rats of known gestation were used. The mothers were allowed access to a 25% protein diet *ad libitum*. The newborn rats were separated from their mothers just prior to sacrifice. Livers from the same littermates were collected and pooled. For the metabolic-regulation experiments rats were housed in a regulated light–dark room, lighted from 8:00 AM to 8:00 PM. They were allowed access to semipurified diets containing 0, 12.5, 60, or 90% protein from 8:00 AM to 4:45 PM for 10 days and sacrificed between 7:00 and 8:00 AM. For the glucagon experiments rats were allowed access to a 25% protein diet *ad libitum* for 10 days. Glucagon (Eli Lilly) was injected subcutaneously at doses of 1.0 mg/100 g body weight. Controls were injected with the diluting solution (1.6% glycine and 0.2% phenol). For the cortisone experiments rats were adrenalectomized 3 days prior to injection. They were allowed access to a 25% protein diet and water containing 0.9% NaCl *ad libitum*. Cortisone (Pfizer Corp.) was injected intraperitoneally at doses of 10 mg/100 g body weight. The animals were sacrificed at the times indicated in the results.

The rats were sacrificed by decapitation. The liver was quickly removed and homogenized in 4 vol of 0.44 *M* sucrose with 10 strokes of a Potter-Elvehjem homogenizer. The homogenate was centrifuged at 12,000*g* (SS-34 rotor, Sorvall centrifuge, 8500 rpm) for 10 min. The supernatant was centrifuged at 105,000*g* (50 Ti rotor, L2-65B Beckman ultracentrifuge, 41,000 rpm)

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for 90 min. The pellet (microsomal fraction) was resuspended and centrifuged again under the same conditions for 60 min. The microsomes were then resuspended in an adequate volume of 0.44 *M* sucrose and frozen at −70° for subsequent assay. All steps were at 0–4°.

The purification of GIT was carried out according to the method of Ansorge *et al.* (2), with the modification of the preparation of the microsomal fraction described above. The assay of GIT activity was modified from that of Ansorge *et al.* (2) and Chandler and Varandani (8). When the microsomal fraction was used as the source of enzyme, it was rehomogenized with a Polytron (Brinkman Corp.) for 45 sec at 4.5 on the rheostat before assaying the enzyme activity. The reaction mixture unless stated otherwise in the results section consisted of: 15 μM insulin; 15 μl of ^{125}I-labeled insulin (5 μCi/5 ml) (New England Nuclear); 4 μmoles of glutathione (reduced form); 5 μmoles of EDTA; and 100 μmoles of potassium phosphate buffer, pH 7.0, with a sufficient concentration of enzyme for a total volume of 1 ml. The mixture (without enzyme) was incubated at 37° for 8 min followed by incubation (with enzyme) at the same temperature for 10 min with shaking. The reaction was stopped by cooling on ice and the addition of 1 ml of bovine serum albumin (4 mg/ml) and 2 ml of 10% TCA. The mixture was centrifuged at 2500 rpm for 15 min. One milliliter of the supernatant was taken from each tube, including the controls of total radioactivity and the blank, for liquid scintillation counting. One unit of enzyme activity was taken as that amount catalyzing the breakdown of 1 μg of insulin per minute at 37°. Protein was determined by the method of Lowry *et al.* (9), using bovine serum albumin as standard.

Results and discussion. As indicated in Methods, glutathione-insulin transhydrogenase was purified by a slight modification of the method described by Ansorge and his associates (2). Fourteen milliliters of the concentrated active fraction (80.5 mg of protein) obtained from the Sephadex G-75 column was placed on a 3.0 × 30-cm column of DEAE-Sephadex A-50 which had equilibrated with 0.1 *N* Tris buffer, pH 7.82, containing 2 m*M* 2-mercaptoethanol, and 0.1 *N* KCl. Elution was initiated with 100 ml of the same buffer and continued with a linear gradient of KCl (0.1–0.4 *M*) in 0.1 *M* Tris buffer, pH 7.82, containing 2 m*M* 2-mercaptoethanol using an LKB gradient former. The flow rate was 19 ml/hr,

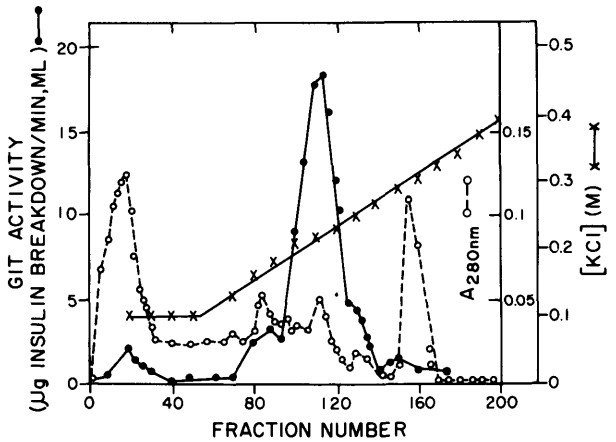

FIG. 1. Chromatography of glutathione-insulin transhydrogenase (GIT) fractions from Sephadex G-75 of DEAE-Sephadex A-50. Fourteen milliliters of the concentrated active fraction (80.5 mg of protein) obtained from chromatography on Sephadex G-75 was applied to a column (3.0 × 30 cm) of DEAE-Sephadex A-50 equilibrated with 0.1 *M* Tris buffer, pH 7.82, containing 2 m*M* 2-mercaptoethanol and 0.1 *M* KCl. The elution was begun with 100 ml of the same buffer and continued with a linear gradient of KCl (0.1–0.4 *M*) in 0.1 *M* Tris buffer, pH 7.82, with mercaptoethanol using an LKB gradient former. The flow rate was 16 ml/hr, and 5-ml fractions were collected at 4°.

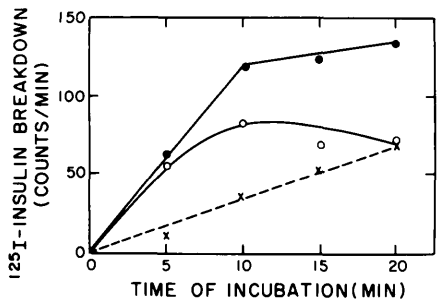

FIG. 2. Degradation of ^{125}I-labeled insulin as a function of time. One-hundred microliters of solution (44 μg of insulin and 100 μl of ^{125}I insulin (5 μCi/5ml) were mixed with 40 μl of 25 mM glutathione and 500 μl of 0.2 M potassium phosphate buffer, pH 7.0, containing 10 mM EDTA. The mixture was preincubated for 8 min at 37°. After this time (designated as 0 time in the figure) 0.632 μg of protein as GIT was added and the solutions incubated at 37°. Phosphate buffer was added in place of GIT in the blanks. See Methods for further details. (●), Total degraded insulin; (×), nonenzymatic degraded insulin; (○), GIT-catalyzed insulin breakdown (total amount of enzymatically degraded insulin minus that nonenzymatically degraded).

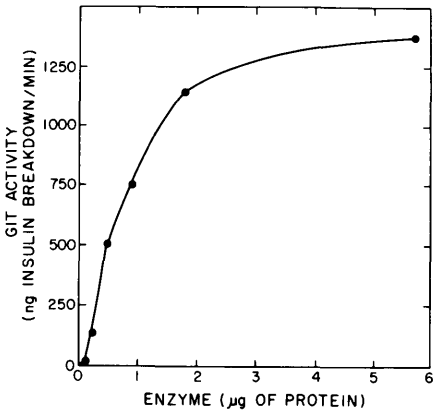

FIG. 3. Relationship of purified GIT amount (protein) and the rate of insulin degradation. See Methods for details of the assay.

with 5-ml fractions being collected at 4°. As can be seen from Fig. 1 a peak of enzyme activity was obtained between the 90th and 120th fraction. This enzyme was used to determine the relationships seen in Figs. 2 and 3.

Figure 2 shows the relationship of enzymatic to nonenzymatic degradation of ^{125}I-labeled insulin as a function of time. The greatest difference between the levels of enzymatic and nonenzymatic insulin degradation occurred at 10 min; thus, this time was selected for the incubation time for the standard assay. Use of shorter times (5 min) for assay did not alter the K_m values seen in the tables. A plot of the rate of enzymatic insulin breakdown versus the amount of GIT protein is linear only at low concentrations of enzyme (Fig. 3). In order to approximate zero-order reaction conditions and thereby have a quantitative assay for the amount of GIT present, different concentrations of insulin and glutathione were tested (Fig. 4). Concentrations of 4 mM glutathione and 15 μM insulin gave linear results up to 56 μg of microsomal protein. Polytron treatment of the microsomal fraction after homogenization with the Potter-Elvehjem homogenizer resulted in an increase in GIT activity from 2.7 to 8.0 units/mg of protein. Thus, the specific activity for the enzyme was measured after Polytron treatment in all subsequent assays in a reaction mixture containing 4 mM glutathione and 15 μM insulin and at concentrations of less than 56 μg of microsomal protein.

The specific activity of GIT was determined as a function of rat age (Fig. 5). The newborn rats were separated from their mothers immediately before sacrifice, with livers from the same littermates being pooled for each time point until the 12th day after birth subsequent to which the liv-

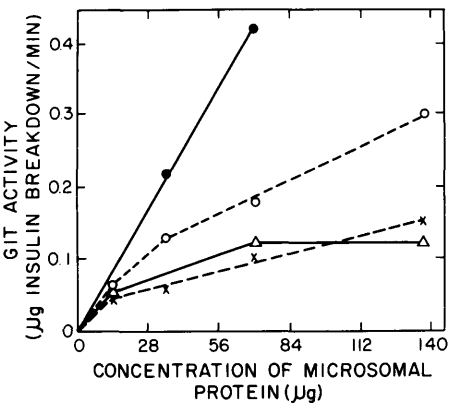

FIG. 4. Relationship of the concentration of microsomal proteins with GIT activity at different substrate concentrations. (×), 4 mM glutathione, 44 μg of insulin; (●), 4 mM glutathione, 88 μg of insulin; (○), 2 mM glutathione, 44 μg of insulin; (△), 2 mM glutathione, 88 μg of insulin. See Methods for further experimental details.

ers were assayed individually. Fetal rats had levels of GIT close to the adult (62-day) levels. However, upon birth the GIT specific activity was double that of the adult, with a gradual decay to adult levels by the second week after birth. It is of interest that the GIT activity in the Morris 7800 hepatoma was 3.1 ± 0.9 on the same basis as seen in Fig. 5. Thus, unlike many other enzymes which are low in activity in fetal liver and also in hepatomas (10), GIT activity in the fetal liver is the same as that in the adult during the latter stages of fetal life in contrast to the activity of the enzyme in the hepatoma which is less than half that seen in the adult. GIT activity in the host liver of the tumor-bearing animal was 5.5 ± 1.1 (average ± SEM of animals).

The effect of dietary and hormonal alterations on the specific activity of microsomal glutathione-insulin transhydrogenase can be seen in Table I. Increasing the protein content of the diet from 0 to 90% and allowing animals to eat such diets for a period of 10 days did not result in any change in the specific activity of GIT in microsomal protein. However, there was undoubtedly a total increase in the enzyme since other investigations (11) have demonstrated significant increases in the amount of microsomal protein per liver cell as the protein content of the diet increases. In animals fed a laboratory chow diet the administration of glucagon at the dose seen in Table I resulted in no significant change or a slight increase in the specific activity of the enzyme during the time interval investigated. Cortisone administration to adrenalectomized rats also had no effect on the specific activity of GIT in the microsomes 4 hr after its administration. However, as can be seen from the data in

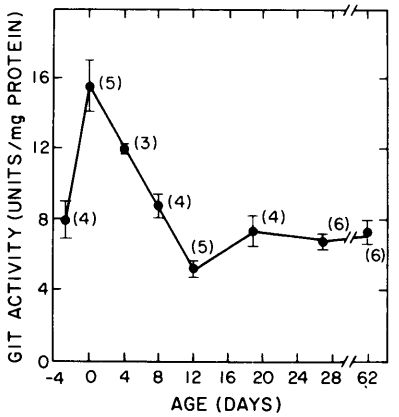

FIG. 5. The specific activity of GIT as a function of age in rats. The treatment of animals and the assay of GIT are described in the methods section. The numbers in parentheses indicate the number of animals utilized for each assay point beyond the 12th day. The numbers at the 12th and earlier days refer to the number of batches of pooled livers from littermates.

TABLE I. THE EFFECTS OF CORTISONE, GLUCAGON, AND DIFFERENT PROTEIN DIETS ON THE SPECIFIC ACTIVITY OF MICROSOMAL GLUTATHIONE-INSULIN TRANSHYDROGENASE.

Conditions	Units/mg of GIT microsomal protein[a]
0% Protein diet, 10 days	9.2 ± 2.9 (6)
12.5% Protein diet, 10 days	7.2 ± 1.9 (6)
60.0% Protein diet, 10 days	7.1 ± 1.0 (6)
90.0% Protein diet, 10 days	9.3 ± 2.3 (6)
Glucagon (1 mg/100 g B.W.), 4 hr	13.7 ± 0.8 (3)
Control, 4 hr	10.7 ± 1.6 (4)
Cortisone (10 mg/100 g body weight), adrenalectomized rats, 4 hr	8.4 ± 2.6 (4)
Control, adrenalectomized rats, 4 hr	8.0 ± 0.9 (4)

[a] Values represent the means ± standard deviation, with the number of rats used in parentheses. See text for further details.

TABLE II. THE EFFECTS OF CORTISONE AND GLUCAGON ON THE K_m AND V_{max} OF GLUTATHIONE-INSULIN TRANSHYDROGENASE.

Conditions	V_{max} (nmoles/min)[a]	K_m (μM)[a]	Microsomal protein (mg)
Glucagon (1 mg/100 g body weight), 4 hr	0.172 ± 0.038	11.2 ± 0.3	0.23
Control, 4 hr	0.174 ± 0.016	7.5 ± 2.0	0.18
Cortisone (10 mg/100 g body weight), adrenalectomized rats, 4 hr	0.288 ± 0.078	12.5 ± 4.7	0.23
Control, adrenalectomized rats, 4 hr	0.686 ± 0.038	40.2 ± 14.5	0.21

[a] Values represent the means ± standard deviation of three rats. These kinetic assays were run with the same rats used in Table I. See text for further details. V_{max} values were the rates under conditions of zero-order kinetics (Fig. 4).

Table II, while glucagon did not significantly change the K_m of GIT after administration, that of the enzyme in the adrenalectomized animal exhibited significant differences from the control. Hepatic microsomal GIT of adrenalectomized animals showed a fourfold increase in its K_m and a two- to threefold increase in V_{max} as compared to that of the enzyme in control or glucagon-treated intact animals. Administration of cortisone to adrenalectomized animals resulted in a drop of both the K_m and the V_{max} of hepatic microsomal GIT to essentially normal levels within 4 hr.

While it is relatively easy to understand the insensitivity of the levels of GIT to dietary and hormonal changes, the alteration in V_{max} and K_m in the adrenalectomized animal presents some interesting possibilities. Previous theoretical (12) and kinetic (13, 14) studies of enzymes associated with membranes have demonstrated alterations in enzymatic behavior resulting from the membrane environment of the enzyme. It is interesting to speculate that in the adrenalectomized animal, the change in hormonal environment may result in changes in membrane synthesis and structure leading to the altered kinetic constants of this microsomal enzyme. The effect of hormones on membrane synthesis and membrane enzymes has been previously reported both for the liver and the kidney (15). The effect of other environmental changes on the kinetic constants of GIT is presently under investigation in this laboratory.

Summary. The dietary and hormonal regulation of the level of glutathione-insulin transhydrogenase in rat liver was investigated in these studies. In order to make valid comparisons, the assay of glutathione-insulin transhydrogenase was performed at near zero-order kinetics wherein enzyme rate was proportional to enzyme amount. Changing the protein content of the diet or administration of glucagon or cortisone did not significantly affect the specific activity of glutathione-insulin transhydrogenase in microsomes from rat liver. However, the V_{max} and K_m of this enzyme in the livers of adrenalectomized rats were increased three- and fourfold over these values in microsomes from normal liver. Administration of cortisone resulted in a return to the normal kinetic constants of microsomal GIT within 4 hr.

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