Studies on Gluconeogenesis in Galactosamine Induced Hepatitis[1]
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DANIELLE MONIER AND S. R. WAGLE
Department of Pharmacology, Indiana University School of Medicine, Indianapolis, Indiana 46202

Keppler *et al.* (1) reported that administration of D-galactosamine hydrochloride to rats induced histological modifications similar to viral hepatitis. Prominent features of this syndrome are the complete depletion of liver glycogen, absence of fatty infiltration, elevation of serum transaminase and decrease of serum proteins. However, this drug-induced hepatitis is reversible: normal histology being found 4 weeks later.

In the liver galactosamine (GalN) is first phosphorylated to GalN-1-PO_4 (2, 3) which then is metabolized to UDP-GalN by UDPG: galactose-1-PO_4 uridyltransferase (EC 2.7.7.12). UDP-GalN can be further converted to UDP-N-acetyl GalN or epimerized (4) to UDP-GlucN. In contrast to galactose metabolism, UDPG is not regenerated in this pathway as UDP is kept bound to the metabolites; this situation leads to depletion of UTP pool as demonstrated in liver perfusions with GalN by Keppler *et al.* (5). Inhibition of UDPG pyrophosphorylase by accumulation of GalN-1-PO_4 (3) constitutes a third factor involved in lowering UDPG level.

Even though glycogen stores were depleted no significant hypoglycemia was observed in these animals. We wish to report studies on gluconeogenesis, using this experimental model in which liver damage similar to human viral hepatitis is present.

Experimental Procedures. Induction of hepatitis. Normal male albino rats, weighing 120–190 g Cox-Holtzman strain, were used. Hepatitis was induced by intraperitoneal (ip) injection of D-galactosamine·HCl (obtained through Sigma), 1.5 g/kg over a period of 24 hr; injections of ¼ the total dose, diluted in 0.5 ml of NaCl (0.154 *M*) and adjusted to pH 7.0–7.2 with NaOH (1 *N*), were administered at time 0, 4, 8, and 22 hr. Eighty to 90% of the treated animals exhibited hepatitis upon macroscopic examination with SGOT values $>$ 1500U/ml. Controls received ip injections of 0.5 ml NaCl (0.154 *M*) at the same time interval and were fasted for 18 hr to lower the liver glycogen content. All rats were sacrificed 24 hr after the first injection by decapitation. Blood was collected in heparinized beakers, centrifuged, for determination of serum glucose (6), serum (SGOT) transaminase (7), and serum proteins (8).

Determination of liver glycogen. Two-hundred mg of liver were digested in 30% KOH and glycogen was precipitated with alcohol. It was hydrolyzed with H_2SO_4 (5 *N*) as described by Good *et al.* (9). The glycogen content is expressed as micromoles of glycogen glucose per gram of tissue.

Studies with liver slices. Liver slices from controls and GalN-treated animals were prepared as described previously (10) with a Stadie-Riggs hand microtome. Five-hundred mg of liver slices were incubated in 6 ml of Umbreit–Ringer HCO_3 medium (121 m*M* NaCl, 5 m*M* KCl, 0.5 m*M* $MgCl_2$, 1.0 m*M* $CaCl_2$, 25 m*M* $NaHCO_3$) previously equilibrated with 95% O_2:5% CO_2. ^{14}C-labeled substrates (60 μmoles; 2 μCi) were added to each of the incubation flasks. The incubation was carried out at 37° for 90 min in a Dubnoff metabolic shaker. Incorporation of alanine-UL-^{14}C (New England Nuclear), Na pyruvate-2-^{14}C (Nuclear-Chicago) and glutamic-3,4-^{14}C acid (New England Nuclear) into

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glucose and CO_2 was studied. Incorporation of $H^{14}CO_3$ (5 μCi/flask) into glucose with nonradioactive Na pyruvate (10 m*M*) was also measured. Medium glucose was assayed with the glucose oxidase reagent of Huggett and Nixon (6) and values are expressed as micromoles of glucose/g of wet liver. Glucose-^{14}C was isolated from the medium as the phenylosazone (11); radioactivity was measured in a gas-flow counter and values are reported as cpm/g of liver. When pyruvate was used as a substrate, it was removed from the deproteinized medium by absorption and elution on a Dowex-1 Column (Sigma Chemical Co., Lot 24B, 1270). $^{14}CO_2$ was captured in NaOH and precipitated as the $Ba^{14}CO_3$, plated on a planchette and assayed for radioactivity (cpm/g of liver) in a gas-flow scintillation counter (Nuclear Chicago) (12).

Enzymatic studies. Livers from fed and fasted animals were homogenized in 10 vol of cold KCl, 0.154 *M*. Glucose-6-phosphatase was measured at 37° in the whole homogenate (13) and the activity reported as P_i liberated (μmoles/g of liver/hr). Fructose-1-6-diphosphatase activity was measured at 37° (14) in the supernatant obtained from the homogenate that was centrifuged at 100,000*g* for 60 min and activity reported as P_i liberated (μmoles/g of liver/hr). Inorganic phosphorus determination was done as described by Fiske and Subbarow (15) using a Klett-Summerson colorimeter.

For CO_2 fixating enzymes livers were homogenized in 5 vol of isotonic NaCl. Nuclei and cell debris were removed by centrifugation at 600*g* for 5 min. The mitochondria were isolated by centrifugation at 10,000*g* for 10 min. Isolated mitochondria were resuspended in 3 ml of sucrose and were sonicated for 1 min in a Raytheon sonicator and then recentrifuged for 15 min at 15,000*g* and the supernatant obtained was used for assay of pyruvate carboxylase (16, 17). The enzymatic activity being expressed as CO_2 fixed (cpm/g/min). Phosphoenol pyruvate carboxykinase activity was measured in the supernatant obtained from the homogenate that was centrifuged at 100,000*g* for 60 min, as described previously (18) and activity reported as CO_2 fixed (cpm/g/min).

Results and Discussion. Galactosamine-treated animals showed a fivefold increase in SGOT values (>1500 IU) as compared to normal controls. The treated animals showed a lowering in serum glucose which was further reduced on fasting and these values were comparable to normal fasted controls. As reported by Keppler *et al.* extremely low liver glycogen values and decreased serum proteins were observed (Table I).

Liver slices from GalN-treated animals, incubated with alanine-UL-^{14}C, pyruvate-2-^{14}C, or glutamic-3,4-^{14}C acid were found to release 2–3 times less glucose in the medium

TABLE I. Biochemical Changes in Liver and Serum after GalN Treatment.[a]

	Glycogen (μmoles of glycogen glucose/g of liver)	Serum glucose (mg/100 ml)	Serum proteins (g/100 ml)	Serum transaminase (SGOT) (IU)
Controls				
Fed	303.8 ± 20.2 (6)	133.8 ± 9.8 (5)	6.3 ± 0.2 (5)	234.5 ± 25.9 (4)
Fasted	34 ± 7 (6)	68.8 ± 6.4 (6)	6.5 ± 0.24 (11)	267.6 ± 26.7 (6)
GalN treated				
Fed	6.05 ± 3.82[b] (8)	102.6 ± 12.7 (8)	4.6 ± 0.14[b] (11)	>1500[b] (8)
Fasted	Not detectable[b]	63.6 ± 2.9 (5)	4.6 ± 0.22[b] (5)	>1500[b] (4)

[a] Values are expressed as mean ± SE; no. of animals is given in parentheses.

[b] $p < 0.01$.

TABLE II. Incorporation of ^{14}C-Labeled Substrates into Glucose and CO_2 in Liver Slices from Normal and Galactosamine-Treated Animals.^{a}

	Medium glucose (μmoles/g of liver)	Glucose-^{14}C (cpm/g of liver)	$^{14}CO_2$ (cpm/g of liver)
		Pyruvate-2-^{14}C	
Controls	45.9 ± 4.2 (9)	81,416 ± 3617 (9)	67,811 ± 4504 (9)
GalN treated	17.3 ± 2.6^{b} (7)	20,695 ± 3157^{b} (7)	30,613 ± 4112^{b} (7)
		Alanine-UL-^{14}C	
Controls	31.9 ± 3.5 (7)	57,348 ± 2767 (6)	48,926 ± 5273 (6)
GalN treated	9.7 ± 1.1^{b} (5)	11,000 ± 5094^{b} (5)	15,395 ± 8003^{b} (4)
		Glutamic acid-3,4-^{14}C	
Controls	31.9 ± 4.2 (13)	53,402 ± 3037 (13)	23,580 ± 1660 (12)
GalN treated	13.7 ± 2.9^{b} (7)	12,095 ± 2678^{b} (7)	10,396 ± 1272^{b} (7)
		Bicarbonate-^{14}C	
Controls	28.4 ± 1.4 (7)	57,750 ± 2484 (7)	—
GalN treated	8.3 ± 0.92^{b}(5)	4600 ± 1123^{b} (5)	—

^{a} Each value is mean ± SE.
^{b} $p <.01$.

than the 18-hr fasted controls (Table II). Impairment of incorporation of these labeled substrates into glucose, as evidenced by the lower radioactivity of glucose was also noticeable, suggesting that synthesis of glucose is inhibited from these substrates. As the incorporation of alanine and pyruvate is reduced to the same extent, it rules out the possibility that alterations in transamination be responsible for decreased gluconeogenesis.

Further studies involving the so-called "key-gluconeogenic enzymes" showed a significant decrease in activity of the various enzymes measured. However, decrease in PEP carboxykinase and pyruvate carboxylase was far more striking as compared to the controls (Table III). It may be speculated that accumulation of GalN metabolites could have a direct effect on these two enzymes or that the mere presence of these metabolites would interfere with cofactors levels or alter the nucleotide pools. Another possibility, of

TABLE III. Effect of Galactosamine on Gluconeogenic Enzymes.^{a}

	G-6-Pase (μmoles of P_i/g/hr)	F-1,6-di-Pase (μmoles of P_i/g/hr)	PEP carboxykinase (cpm/g/min)	Pyr. carboxylase (cpm/g/min)
Controls				
Fed	953 ± 50.7 (5)	544 ± 25.4 (5)	4138 ± 280 (4)	9860 ± 900 (4)
Fasted 18 hr	1874 ± 99.6 (7)	602 ± 25.8 (7)	4659 ± 303 (5)	10,050 ± 890 (4)
GalN treated				
Fed	759 ± 46.8^{c} (8)	413 ± 40.9^{c} (8)	2058 ± 181^{b} (5)	3960 ± 315^{b} (4)
Fasted 18 hr	743 ± 34.0^{b} (5)	267 ± 42.2^{b} (5)	—	—

^{a} Values are mean ± SE.
^{b} $p <.01$.
^{c} $p <0.05$.

course, would be inhibition of protein synthesis with a consequent decrease in enzyme synthesis. However, further *in vitro* studies are in progress to ascertain whether the presence of GalN (or of GalN metabolites) would inhibit these two enzymes as GalN-1-PO_4 is known to inhibit UDPG pyrophosphorylase.

Both phosphatases, fructose-1, 6-diphosphatase and glucose-6-phosphatase demonstrate only a small but significant reduction in activity. Upon fasting, an increase in activity of these enzymes was noted in controls but did not appear in the GalN-treated ones. A similar behavior has been observed in some hepatomas where gluconeogenesis is also reduced but the glycolytic pathway is stimulated.

Labeled CO_2 produced by liver slices with various substrates was measured. In animals treated with GalN, the CO_2 production was found to be reduced by 50–70% (Table II) suggesting impaired citric acid cycle activity with possible mitochondrial damage. Electron micrograph studies revealed that the outer membrane of the mitochondria is intact while the cristae are hardly visible (19).

The present study shows that the GalN-induced hepatic damage is accompanied by an overall inhibition of gluconeogenesis, the CO_2 fixation step being the more affected. However, no direct or single effect can be ascribed to the amino sugar for inhibition of gluconeogenesis.

Summary. Hepatitis was induced in animals treated with D-GalN·HCl (1.5 g/kg) *in vitro*. Incorporation of alanine-UL-^{14}C, pyruvate-2-^{14}C, and glutamic-3,4-^{14}C acid into glucose by liver slices from GalN-treated animals was found decreased to 20–25% of the control values. The CO_2 production from these substrates was impaired by 50%. Activity of gluconeogenic enzymes was measured. PEP carboxykinase and pyruvate carboxylase exhibited the most important changes and FDPase and G-6-Pase were also significantly decreased but failed to respond to fasting. It is suggested that this GalN-induced hepatitis might be a good model to use to reproduce a study of hypoglycemia found sometimes in human viral hepatitis and other liver damage.

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