very few studies. These studies have shown that a variable degree of mucosal damage with a decrease of brush border disaccharidase activities frequently accompanies childhood gastroenteritis (1). It has been shown in newborn lambs infected with rotavirus that there persists, for 4 or more days after complete clinical recovery, a state of accelerated cell turnover with functionally immature cells clothing the villi (4).

The immature intestine of neonatal rat has been shown to have a low DAO content reflected in a low serum DAO activity (6). These data aid in understanding our own results. The low DAO activity values in the healing phase of gastroenteritis probably reflect the loss of functional enterocytes replaced in the healing phase by "immature" cells. The normal values found in the acute phase of gastroenteritis probably indicate that time is needed for mucosal damage to occur. Our results further show that patients in the healing phase after severe gastroenteritis tend to have lower serum DAO activity values than patients recovering from moderate gastroenteritis. This finding supports the hypothesis that serum DAO activity might be dependent on the total mass of functional enterocytes (6), since severe gastroenteritis is most probably accompanied by a heavier loss of functional enterocytes than gastroenteritis of moderate degree. In the rat, serum DAO activity has been shown to accurately quantitate the length of acute intestinal mucosal injury (7).

The low serum DAO activity values found in healing gastroenteritis are sometimes lower than those found in acute celiac disease (unpublished data). This would logically mean that loss of functional enterocytes can be more important in gastroenteritis than in acute celiac disease. Literature data tend to support this by showing that a complete destruction of the small gut mucosa along all its length can occur in acute gastroenteritis,

whereas in celiac disease the mucosal damage is mainly localized to the proximal intestine (9). Although isolated serum DAO activity measurements are difficult to interpret because of the wide variation in normal values, we believe serial serum DAO activity measurements may be useful in evaluating the severity of small intestinal functional loss in childhood gastroenteritis. Further serial serum DAO activity measurements may turn out to be a most useful tool in the follow-up of childhood enteropathies.

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Incorporation of [^{14}C]Glucose into α -1,4 Bonds of Glycogen by Leukocytes and Fibroblasts of Patients with Type III Glycogen Storage Disease

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ABSTRACT. In two patients assay of α -1,6-amyloglucosidase activity by incorporation of [^{14}C]glucose into glycogen revealed normal activity in leukocytes, erythrocytes, and fibroblasts, whereas no activity was detected in liver and muscle. No activity in any tissue was found when enzyme activity was assayed by following the release of glucose from a phosphorylase limit dextrin. Labeling of glycogen by incubation with crude tissue homogenates according to the protocol used for the [^{14}C]glucose method

and subsequent degradation of the outer portion of the polysaccharide molecule with β -amylase showed that with tissues from normal controls more than 90% of the label of the glycogen was retained in the limit dextrin. When fibroblasts or leukocytes of the patients served as enzyme source up to 80% of the label was released after incubation with β -amylase or phosphorylase α . Addition of Tris to the assay inhibited enzyme activity in fibroblast homogenates of the patients and of controls to the same extent and had no effect on the distribution of the label between supernatant and limit dextrin after β -amylolysis of the labeled glycogen. A pH curve performed with fibroblast preparations from the patients and a normal control did not reveal

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differences in the effect of changes in pH on [^{14}C]glucose incorporation. We propose that incorporation of [^{14}C]glucose into glycogen by the enzyme present in the patients' cells was into α -1,4 linkages in glycogen. (*Pediatr Res* 19: 28-32, 1985)

Abbreviations

PBS, phosphate-buffered saline
MES, 2-(*N*-morpholino)ethanesulfonic acid
PLD, phosphorylase limit dextrin

The glycogen debranching enzyme oligo- α -1,4-glucan: α -1,4-glucan-4-glycosyltransferase (EC 2.4.1.25)/amylase (EC 3.2.1.33) is present in all tissues and a deficiency of this enzyme can be diagnosed, in most cases, by assay of enzyme activity in any available tissue. It has been known for some years that the enzyme involved in debranching of a phosphorylase limit dextrin has two different activities—a transferase activity, transferring maltotriose or maltose units, thereby exposing the α -1,6 linkage, and a hydrolytic activity, acting on the glycosidic bond at the branch point (4). Both activities seem to be located on the same macromolecule (2, 18) and further breakdown of a phosphorylase limit dextrin depends on the sequential activity of the transferase and the hydrolase.

The combined action of the two activities as well as each one separately can be assayed by various methods (11, 12). Many of these methods are either tedious and cumbersome or require special substrates which are not commercially available (12). As a result of these limitations the assay based on incorporation of labeled glucose into glycogen has been widely used for diagnosis of α -1,6-amyloglucosidase deficiency (type III glycogen storage disease). This assay, which had originally been introduced by Hers (9) on the basis of earlier observations by Larner and Schlisfeld (16), is based on the slight reversibility of the hydrolytic activity of α -1,6-amyloglucosidase (10) but its exact mechanism is poorly understood. The assay had initially been applied by Hers to muscle and liver biopsies only (9) but was subsequently used for assays on red (22) and white blood cells (21, 24) and on fibroblasts (14). With this method the enzyme activity in leukocytes of proven cases of type III glycogen storage disease has been reported as normal by Brandt and DeLuca (3) and in two siblings described by us several years ago (7). In fibroblasts obtained from two patients DiMauro *et al.* (8) described only slightly reduced activity and low but significant activity was found by Justice *et al.* (14) in fibroblasts of another patient. Furthermore, the general "unsuitability" of this assay for assay of amyloglucosidase deficiency in fibroblasts has been reported by Brown *et al.* (6) and by Maire and Mathieu (17).

We have recently examined two patients with α -1,6-amyloglucosidase deficiency with apparently normal incorporation of [^{14}C]glucose into glycogen in leukocytes and in cultured fibroblasts and have been able to show that the observed incorporation of [^{14}C]glucose into glycogen was due to the formation of linkages which could be degraded by the action of β -amylase or of phosphorylase and could therefore be presumed to be α -1,4 bonds.

PATIENTS AND METHODS

Patients. A. H. The patient was a 2-yr-old Arab boy whose parents were first cousins. Physical examination revealed a thin boy with moonface and a protuberant abdomen due to a grossly enlarged liver. Laboratory examinations were unremarkable except for a blood glucose concentration of 1–2 mM/liter on different occasions and serum aspartate aminotransferase activity of 1000 IU/liter (normal up to 50 IU/liter). Blood lactate was 1.1 mM/liter. A fasting glucagon test was flat whereas a post-

prandial test showed a rise in blood glucose from 2 to 6.2 mM/liter. An intravenous galactose tolerance test showed a normal rise in blood glucose.

E. A. A 13-month-old Arab boy was admitted for evaluation of hepatomegaly. E. A. was born after a normal pregnancy and delivery. At the age of 7 months he had a generalized seizure which lasted 10 min and 2 months later he had a similar, although milder, seizure. Both seizures occurred at 0400 h.

On examination he was fat, with round cheeks. His weight was 10.300 kg and his height was 75 cm. The abdomen was distended and the liver was firm, smooth, nontender with a span of 18 cm. The tip of the spleen was palpable. Neurologic examination revealed hypotonia with reduced tendon reflexes.

Laboratory examinations revealed a blood glucose of 2.0–2.5 mM/liter on different occasions, cholesterol 5.8 mM/liter, bilirubin 15–30 $\mu\text{mol/liter}$, aspartate aminotransferase 240–750 IU/liter, creatine phosphokinase 183 IU/liter, lactate dehydrogenase 470 IU/liter. All other laboratory examinations, including blood lactate and pyruvate, were unremarkable. Fasting and postprandial glucagon tests were flat. ECG showed right ventricular hypertrophy.

Liver biopsy revealed marked swelling of hepatocytes and accumulation of glycogen. Muscle biopsy (left quadriceps) showed differences in fiber diameter with many central nuclei and perinuclear vacuolization. Type I fibers were large and scarce in comparison to type II fibers. Stains for glycogen were positive.

Tissues. Primary cultures of skin fibroblasts were obtained by biopsy. Cell sheets were washed twice with PBS and were detached by trypsinization. The cells were centrifuged and washed 3 times with PBS, suspended in 0.05 M MES buffer, and disrupted by sonication with a Kontes Micro Ultrasonic Cell Disruptor (Kontes, Vineland NJ) for three periods of 1 min at maximal output.

Leukocytes and erythrocytes were isolated as previously described (7), suspended in 0.05 M MES buffer, and disrupted by sonication as described for fibroblasts.

Enzyme Assays. Activity of α -1,6-amyloglucosidase was assayed: 1) by measuring the incorporation of [^{14}C]glucose into glycogen as described by Nelson and Larner (19) using 0.05 M MES buffer pH 6.5; and 2) by measuring the release of glucose from a PLD as described by Hers (11) using 0.05 M MES buffer instead of histidine buffer in the original method. Phosphorylase limit dextrin was prepared according to Hers *et al.* (11) and kept lyophilized at -70°C until used.

^{14}C -labeled glycogen was prepared by using the assay system for assay of α -1,6-amyloglucosidase by incorporation of [^{14}C]glucose on a larger scale (using 10–20 times the standard amounts of enzyme and substrate) and purifying the labeled glycogen by repeated precipitation with ethanol, as originally described by Hers (9).

Glycogen labeled in the α -1,4 linkage (used for control experiments) was prepared by incubation of glycogen with UDP-[^{14}C]glucose under the conditions used for assay of glycogen synthase (1).

Acid and neutral α -1,4-glucosidase was assayed at pH 4.5 and 6.5 (8), with maltose, glycogen, or PLD as substrates. Incubations were carried out for 2 h and the released glucose was determined by a hexokinase method using a commercial kit obtained from Boehringer, Mannheim, F. R. G. and a Centrifichem (Union Carbide, Rye, NY) centrifugal analyzer.

Degradation of labeled glycogen. Glycogen labeled by incubation with [^{14}C]glucose or UDP-[^{14}C]glucose (0.5–2 mg) was suspended in 0.05 M acetate buffer, pH 5.0, and incubated with 2 units of β -amylase in a final volume of 0.4 ml for 60 min at 37°C in a shaking water bath. In preliminary experiments the course of the reaction was followed by taking portions of the incubation mixture at intervals of 15 min for assay of maltose [measured as reducing substances by the neocuproine method on a Technicon Autoanalyzer (Technicon Corp., Tarrytown, NY)].

The release of labeled glucose was determined by precipitation of the limit dextrin with 2 volumes of 96% ethanol and meas-

urement of ^{14}C radioactivity of the supernatant and of the (twice reprecipitated) precipitate.

RESULTS

Assay of α -1,6-amyloglucosidase activity of various tissues by incorporation of $[\text{U-}^{14}\text{C}]$ glucose into glycogen (^{14}C method) revealed normal activity in leukocytes, erythrocytes, and fibroblasts, whereas no activity was detectable in liver or muscle biopsies (Table 1). No activity in any of the tissues examined was found when enzyme activity was assayed by following the release of glucose from PLD (PLD method).

The observed discrepancies between blood cells and fibroblasts on one hand and liver and muscle on the other hand, as well as the discrepancies between results obtained in leukocytes and fibroblasts when using the two methods, prompted a reexamination of the specificity of the ^{14}C method. Labeling of glycogen by incubation of crude tissue homogenates according to the protocol used for the ^{14}C method and subsequent degradation of the outer portion of the polysaccharide molecule with β -amylase showed that 96–100% of the label initially present in glycogen was retained in the β -amylase limit dextrin. Completeness of β -amylolysis was confirmed by chemical assay of the products as well as by a control experiment which showed that almost all the label could be removed by β -amylase when the initial labeling of glycogen had been carried out by incubation with UDP- $[\text{U-}^{14}\text{C}]$ glucose under the conditions used for assay of glycogen synthase (Table 2).

β -Amylolysis of glycogen labeled by incubation with the patients fibroblasts (Table 3) or leukocytes (Table 4) resulted in release of up to 80% of the label, in contrast to the almost complete recovery of the label in the β -amylase limit dextrin when cells from normal controls served as enzyme source.

Addition of Tris, which is known to be a specific inhibitor of α -1,6-amyloglucosidase activity (20), inhibited incorporation of $[\text{U-}^{14}\text{C}]$ glucose into glycogen with fibroblasts from the two patients and from a normal control to a similar extent (42 and 72%,

Table 1. Incorporation of $[\text{U-}^{14}\text{C}]$ glucose into glycogen by tissues of patients and of normal controls*

Patient	White blood cells	Fibroblasts	Red blood cells	Muscle	Liver
1	16–22	37–46	3.9–4.3	0	0
2	21–27	34–41	3.5–4.2	0	0
Controls (n = 15)	6–20	30–50	1.5–3.5	68–110	30–400

* Values given are nmol/min per g Hb for red blood cells and nmol/min per g protein for all other tissues. Each assay on patients' blood cells and fibroblasts was carried out at least five times.

Table 2. Degradation of $[\text{U-}^{14}\text{C}]$ glycogen by β -amylase*

Enzyme source	Limit dextrin (%)	Supernatant (%)
Muscle	96	4
Liver	98	2
Leukocytes	100	0
Erythrocytes	97	3
Fibroblasts	96	4
Muscle†	4	96

* Glycogen was labeled by incubation with $[\text{U-}^{14}\text{C}]$ glucose and a crude tissue homogenate, purified by repeated precipitation with 70% ethanol, and incubated with β -amylase as described in "Patients and Methods". All glycogen samples were also incubated without β -amylase (blanks). The radioactivity found in the supernatant was corrected for these blank values, which ranged from 1–5% of total radioactivity.

† Labeled with UDP- $[\text{U-}^{14}\text{C}]$ glucose, as described in "Patients and Methods".

Table 3. Degradation of $[\text{U-}^{14}\text{C}]$ glycogen labeled by incubation with patients' fibroblasts*

Patient	Tris	Initial (nmol/min/g protein)	Limit dextrin (%)	Supernatant (%)
1	–	39.8	15	82
	+	22.9	25	77
2	–	44.2	45	57
	+	12.4		
Control	–	33.7	99	3
	+	13.1	92	9

* Glycogen was labeled by incubation with a fibroblast homogenate with or without the addition of 0.01 M Tris. Other experimental details as in Table 1 and "Patients and Methods".

Table 4. Degradation of $[\text{U-}^{14}\text{C}]$ glycogen labeled by incubation with leukocyte homogenates; effects of pH and Tris on incorporation*

pH	Tris	Patient 1		Patient 2		Control	
		cpm	PLD (%)	cpm	PLD (%)	cpm	PLD (%)
5.6	–	680	52	250		2500	99
	+					685	86
6.5	–	6480	32	1180		5750	98
	+	1800	21			1428	70
7.5	–	6300		1500		6720	99
	+					1692	70

* Phosphorylase *a* was used for degradation of labeled glycogen. Other experimental details as described in Table 3.

† Leukocytes obtained from pooled blood of five normal volunteers.

respectively, versus 61%) (Table 3). The distribution of the $[\text{U-}^{14}\text{C}]$ glucose label between the limit dextrin and the supernatant in preparations obtained by incubation of glycogen and fibroblast extracts in the presence of Tris was similar to that obtained without the inhibitor (not shown).

The effects of Tris and of changes in pH of the incubation medium on the labeling pattern obtained by incubation of glycogen and $[\text{U-}^{14}\text{C}]$ glucose with a leukocyte homogenate as enzyme source is shown in Table 4. In all preparations incorporation was below pH 6 and a plateau was obtained at pH 6.5–7.5. Addition of 0.01 M Tris inhibited incorporation by about two-thirds in patients and in normal controls. Up to 30% of the $[\text{U-}^{14}\text{C}]$ glucose label incorporated by incubation of leukocyte homogenates from normal controls in the presence of Tris could be removed by subsequent incubation with β -amylase (not shown) or phosphorylase *a*. When leukocytes of the patients served as enzyme source the proportion removed by β -amylase or phosphorylase was even higher than in preparations labeled without added Tris (Table 4).

A more detailed pH curve performed with fibroblast preparations from the two patients and from a normal control (Fig. 1) did not reveal differences in the effect of pH on $[\text{U-}^{14}\text{C}]$ glucose incorporation.

Although incorporation of glucose into glycogen decreased at acid pH, the possibility was considered that the hydrolytic activity of acid (lysosomal) or neutral α -1,4-glucosidase known to be active in fibroblasts (6) and leukocytes (15) may also be slightly reversible and that an unusually high activity of these enzymes could have resulted in the observed findings. Table 5 shows that α -glucosidase activity assayed with maltase, glycogen, or PLD as substrates was similar in patients and controls. Table 5 also shows that in cells of the controls, but not of the patient, hydrolysis of PLD at pH 6.5 exceeded that of glycogen.

DISCUSSION

We have shown that an enzyme activity present in leukocytes and fibroblasts of two patients with type III glycogen storage disease could catalyze incorporation of [^{14} C]glucose into linkages on glycogen which could be removed by subsequent incubation with β -amylase or phosphorylase. We initially preferred β -amylase since even highly purified phosphorylase might contain traces of α -1,6-amyloglucosidase, but results with both enzymes were similar. Since chemical analysis of the extent of degradation of glycogen by either enzyme ruled out the possibility that degradation had proceeded beyond the outermost branch points and no significant radioactivity was released from glycogen labeled by incubation with enzyme from normal controls, we assume that incorporation of [^{14} C]glucose by the enzyme present in the patients' cells was into α -1,4 linkages in glycogen.

The question whether only the glucosidase or both glucosidase and transferase was involved in the incorporation of [^{14} C]glucose has been the subject of controversy. The data presented by Van Hoof and Hers (23) on patients with incomplete α -1,6-amyloglucosidase deficiency (type IIIB) included one patient with low-normal [^{14} C]glucose incorporation, normal hydrolytic activity on a glucosyl-Schardinger dextrin, and rudimentary or absent transferase activity in muscle and liver but above normal incor-

poration in erythrocytes. In several other patients muscle and liver glucosidase activity was also in the low-normal range but [^{14} C]glucose incorporation was negligible. These findings led Van Hoof and Hers (23) to conclude that incorporation of [^{14} C]glucose depended on the hydrolytic activity but that this in itself was not sufficient to allow incorporation. Cohen and his colleagues (2) found that transferase activity was not required for the net incorporation of [^{14} C]glucose residues into glycogen. On the other hand, Brown *et al.* (5), who studied the structure of the product obtained by incubation of glycogen with [^{14} C]glucose and purified amyloglucosidase concluded that both the hydrolytic and the transferase activity were involved in the reaction.

Although our data point to the formation of α -1,4 rather than 1,6 bonds by an enzyme found in leukocyte and fibroblast homogenates of some patients with type III glycogen storage disease, no evidence for the existence of an enzyme with properties differing from those of α -1,6-amyloglucosidase (pH, inhibition by Tris) was obtained. The only suggestion that an additional enzyme may be involved came from the experiments on the leukocyte enzyme (from normal controls) in the presence of Tris which showed some incorporation of glucose into bonds which could be hydrolyzed by β -amylase. Since this phenomenon was found only in leukocytes (80% granulocytes) one may speculate that it is related to the very high neutral α -1,4-glucosidase activity found in granulocytes (15).

If the observed incorporation of [^{14} C]glucose is not due to the activity of an additional enzyme, further work will be necessary to determine whether this "altered" enzyme is limited to blood cells and fibroblasts or whether it is present in all cells, but can express itself only in certain tissues. The data of van Hoof and Hers (23) on glucosidase activity and ^{14} C incorporation in liver and muscle of some patients may be taken as support for the latter possibility.

Our results support the conclusion of Brown *et al.* (6) and Maire and Mathieu (17) that normal [^{14} C]glucose incorporation in fibroblasts does not rule out type III glycogen storage disease and suggest that the same conclusion should apply to leukocytes.

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Fig. 1. Incorporation of [^{14} C]glucose into glycogen as a function of pH. Fibroblasts of the patients and of a control without metabolic disease served as enzyme source. Buffers used were: MES (pH 5.5–7.0) and [4-(2-hydroxyethyl)-1-piperazinepropanesulfonic acid] (pH 7.0–9.0). At pH 7.0 results with both buffers were identical. Protein concentration of the homogenates was 1.52, 2.17, and 1.60 mg/ml for patients 1 and 2 and the control, respectively.

Table 5. Neutral and acid glucosidase activity in fibroblasts

	pH	Substrate			
		Glycogen (nmol/min × mg protein)	PLD	Maltose	PLD/glycogen
Patient	4.0	6.0	3.3	6.3	0.55
Control*	4.0	6.8	3.6	6.0	0.73
Control	4.0	5.9	2.9	5.7	0.59
Patient	6.5	1.6	1.6	2.0	1.00
Control*	6.5	1.9	3.6	2.1	1.90
Control	6.5	1.2	2.7	1.7	2.20

* Fibroblasts from cultures grown from skin biopsies of children without metabolic diseases were used as controls.

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Altered Growth, Hypoglycemia, Hypoalaninemia, and Ketonemia in the Young Rat: Postnatal Consequences of Intrauterine Growth Retardation

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ABSTRACT. We characterized some of the consequences of intrauterine growth retardation in rat pups growth retarded [small for gestational age (SGA)] due to bilateral maternal uterine artery ligation. Pups of sham and nonoperated (normal) mothers served as controls. SGA pups had significantly reduced body and carcass mass throughout the study while body mass did not differ between sham and normal pups after 4 days. Brain mass was similar in the three groups at any age, while at 21 days and later, SGA liver weight as % body mass exceeded that of sham or normals. At 21 days, a 48-h fast reduced plasma glucose significantly in SGA compared to sham and normal pups; SGA plasma insulin was decreased and glucagon increased. Hepatic phosphoenolpyruvate carboxykinase activity and glycogen content were similar among groups. SGA pups did have significantly reduced plasma alanine and elevated betahydroxybutyrate levels. No differences in the responses to fasting occurred at 28 or 35 days. These data indicate that intrauterine growth retardation has profound effects on postnatal growth and metabolism. (*Pediatr Res* 19: 32-37, 1985).

Abbreviations

SGA, small for gestational age
RIA, radioimmunoassay
BOHB, β -hydroxybutyrate
PEPCK, phosphoenolpyruvate carboxykinase

The consequences of intrauterine growth retardation on postnatal growth and metabolic development are not well understood. Human SGA neonates born to women with placental insufficiency are likely to remain small during later life (28). The alterations in organ growth associated with this diminished postnatal growth pattern are not known. In addition, while it is well recognized that the SGA newborn is at risk for hypoglycemia (5), it is unclear whether they have later difficulty with glucoregulation.

To address these questions, we used the technique of maternal uterine artery ligation to induce intrauterine growth retardation in the rat (29). Limited data indicate that SGA rat pups remain smaller than controls during early life in a manner similar to human infants (23), but no information is available concerning their glucoregulatory capabilities during later life. For this reason, we characterized the growth of SGA pups raised under well-defined conditions during the first 35 days of postnatal life. Also, the pups were fasted to determine their glucoregulatory abilities. Our findings suggest that the postnatal consequences of intrauterine growth retardation include both altered growth and diminished glucoregulatory capacity.

MATERIALS AND METHODS

Animal techniques. Time-dated pregnant Sprague Dawley rats were purchased on day 10 of gestation from Harlan Laboratories, Madison, WI. The rats had been mated at 9-10 wk of age and weighed 160-190 g at time of surgery. We modified Wigglesworth's (29) unilateral uterine artery ligation technique to bilateral ligation. On day 18 of their 21-day gestation, we anesthetized the rats with an intraperitoneal injection of chloral hydrate (60 mg/100 g body weight). The abdomen was incised under sterile conditions, and the uterus was isolated. We ligated both the right and left uterine arteries with 3-0 silk suture, returned the uterus to the abdomen, and closed the incision with suture and wound

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