Studies on Dehydrogenases of the Glucuronate-Xylulose Cycle in the Livers of Diabetic Mice and Rats

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SUMMARY

In view of reports that accessory pathways of glucose oxidation are enhanced in the diabetic state, we have determined the levels of key enzymes of the glucuronate-xylulose cycle in the livers of diabetic mice and rats. Genetically diabetic mice (db/db) were found to have increased levels of two NADP-linked enzymes of this cycle [NADP-xylitol dehydrogenase and NADP-L-hexonate dehydrogenase (aldehyde reductase II)], whereas other NAD- and NADP-linked dehydrogenase activities of the pathway were not changed. On the other hand, the livers of streptozotocin-diabetic mice and rats showed normal levels of all these enzymes. In the course of this study, evidence was obtained for the presence in db/db mouse liver of low molecular weight material inhibitory for glucose 6-phosphate dehydrogenase. The use of these animal models in diabetes research is briefly discussed.

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Two accessory pathways of glucose metabolism have been implicated in diabetes—the polyol pathway[1] and the glucuronate-xylulose cycle.[2] In both pathways, polyols are used as intermediates. Gabbay[3] has emphasized the polyol accumulation in nerve may be related to the occurrence of diabetic neuropathy, and Winegrad and his associates[4,5] have reported evidence for increased metabolism of glucose via glucuronate and L-xylulose in diabetic rats and man. The glucuronate-xylulose cycle appears to account for about 5% of the total glucose catabolized per day in man.[6] The question as to whether there is in fact enhanced use of glucose by these pathways in diabetes has been controversial. Study of the activities of various enzymes of the pathways has led to conflicting results, partly because of overlapping specificities of the enzymes. For example, aldose reductase (EC 1.1.1.21), the enzyme catalyzing the reduction of glucose to sorbitol in the polyol pathway, and NADP-linked L-hexonate dehydrogenase, the enzyme catalyzing the reduction of D-glucuronate to L-gulonate in the glucuronate-xylulose pathway, have some substrates in common. Relevant enzymatic transformations and interrelationships of the two pathways are shown in Figure 1.

Our first report[7] presented evidence for (a) the occurrence of two aldehyde reductases in mouse and rat liver and (b) the absence of aldose reductase in liver. The latter observation supports the findings of Hers,[8,9] but not of others,[10,11] on the question of the occurrence of this enzyme in mammalian liver. It is therefore evident that the polyol pathway does not function in this organ. The present report focuses on key enzymes of the glucuronate-xylulose cycle in genetically diabetic (db/db) mice, a rather widely employed experimental model for human diabetes, and in streptozotocin-treated mice and rats. Six enzyme activities were studied: (a) NAD-xylitol dehydrogenase (EC 1.1.1.14), (b) NAD-sorbitol dehydrogenase (EC 1.1.1.14), (c) NAD-L-hexonate dehydrogenase (EC 1.1.1.45), (d) NADP-xylitol dehydrogenase (EC 1.1.1.10), (e) NADP-DL-glyceraldehyde reductase (aldehyde reductases I and II), and (f) NADP-L-hexonate dehydrogenase (aldehyde reductase II) (E.C. 1.1.1.19).

Activities (a) and (b) presumably reflect the activity of the same enzyme, namely, L-iditol dehydrogenase. This enzyme catalyzes the oxidation of xylitol to D-xylulose in the glucuronate-xylose cycle (as well as the oxidation of sorbitol to D-fructose in the polyol pathway). Activity (c) is reported to be the rate-limiting enzyme of the glucuronate-xylulose pathway in rat liver,[12] while activity (d) measures the exceptionally specific enzyme catalyzing the conversion of L-xylulose to xylitol in this pathway. Activity (e) is a measure of both aldehyde reductases but not of aldose reductase, because this enzyme is absent from mouse and rat liver.[7] Activity (f), assayed with NADPH and D-glucuronate, reflects the level of NADP-L-hexonate dehydrogenase, which has recently been shown to be identical to aldehyde reductase II.[7]

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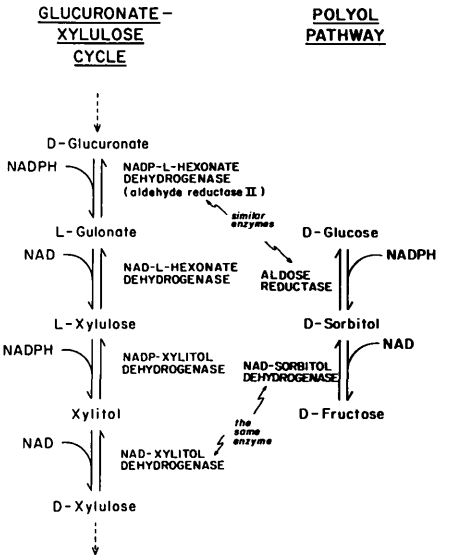


FIGURE 1. Enzyme relationships between the glucuronate-xylulose cycle and the polyol pathway.

MATERIALS AND METHODS

Chemicals were purchased from the following sources: DL-glyceraldehyde, D-glucuronic acid (sodium salt, grade II), NAD, NADH, NADP, NADPH, glucose-6-phosphate, and 6-phosphogluconic acid (trisodium salt, grade III) from Sigma; sugars and sugar alcohols (polyols) from Eastman. All other chemicals were of the highest purity available.

Animals. Male diabetic mice (db/db) and nondiabetic controls (db/+) of C57 BL/KsJ strains were bought from Jackson Laboratories, while male Wistar rats (160 to 180 g) were from Harlan Industries. Animals were allowed free access to water and to a standard laboratory rat diet.

Drug treatment. Rats were given streptozotocin (Upjohn, 65 mg/kg body weight) in about 0.2 ml of sodium citrate buffer (10 mM, pH 4.5) by tail vein injection while the animal was under light ether anesthesia. Control animals received the same volume of buffer alone. Tail vein blood glucose levels were checked 24 h after streptozotocin treatment. Only animals with glucose concentrations above 400 mg/dl at 24 h after drug treatment were used in these studies. Blood glucose levels at the time animals were killed for enzyme determinations are given in the appropriate tables.

Streptozotocin (200 mg/kg body weight) was administered intraperitoneally (i.p.) into mice (db/db and db/+) in a total volume of 0.2 ml of the above citrate buffer. The control animals received the same volume of buffer alone.

Preparation of tissue samples. Tissues were quickly excised and chilled in ice-cold homogenizing solution after the animals were stunned and killed by decapitation. Blood for measurement of glucose was obtained in heparinized tubes from the neck of the animal at decapitation. All subsequent operations were conducted at 0 to 4°C. The tissues were homogenized in 4 vol of Tris-phosphate buffer (5 mM) containing 5 mM 2-mercaptoethanol (buffer A, final pH 7.4 at room temperature) in a glass homogenizer equipped with a mechanically driven Teflon pestle (Arthur H. Thomas Co.). Moderate pressure was applied to push the pestle rotating at 1200 rpm for three up and three down passes. The homogenate was centrifuged at 27,000 rpm for 30 min in a Spinco No. 40 rotor. The supernatant was collected by aspiration and used for enzyme assays.

Enzyme assays. Coenzyme solutions were prepared fresh and used the same day. NAD-xylitol dehydrogenase and NADP-xylitol dehydrogenase were assayed spectrophotometrically as described by Touster and Montesi.[13] NAD-sorbitol dehydrogenase was assayed by the method of Gabbay and O'Sullivan.[14] NAD-L-hexonate dehydrogenase was assayed with L-gulonate as described by Ashwell et al.[15] Aldehyde reductases were assayed spectrophotometrically as follows. The standard assay mixture (1 ml) contained phosphate buffer (67 mM, pH 6.2), 2-mercaptoethanol (5 mM), NADPH (75 μM), enzyme solution, and either DL-glyceraldehyde (1 mM) for DL-glyceraldehyde reductase (aldehyde reductases I and II) or D-glucuronate (10 mM) for aldehyde reductase II. A water blank was used. Glucose 6-phosphate dehydrogenase (EC 1.1.1.47) and 6-phosphogluconate dehydrogenase (EC 1.1.1.43) were assayed as described by Kornberg and Horecker.[16] A reference cuvette, containing all components except substrate, was run simultaneously with the assays mentioned above to correct for the nonenzymatic oxidation or reduction of coenzymes. For all enzymes assayed, one unit of enzyme was defined as the change in absorbance of 0.01 per minute. Specific activity is expressed as units per milligram of protein.

Blood glucose was assayed by the glucose oxidase/peroxidase method[17] using the glucostat reagent (Worthington). Protein was determined by the method of Lowry et al.[18] as described by Miller.[19] Gel filtration with Sephadex G-25 (Pharmacia) and Bio-gel P10 (Bio-Rad Laboratories) was performed according to the manufacturers' instructions.

RESULTS

INITIAL EVIDENCE FOR CHANGES IN NADP-LINKED DEHYDROGENASES IN GENETICALLY DIABETIC MICE

In preliminary experiments, several enzymes of the glucuronate-xylulose pathway were assayed in the supernatant fraction (see section on MATERIALS AND METHODS) obtained from different organs (liver, kidney, brain, spinal cord, pancreas, sciatic nerve, and lens) of 10-wk-old genetically diabetic mice (db/db) and their nondiabetic controls (db/+). Only liver and kidney showed substantial levels of these enzymes, and only NADP- and NADPH-linked enzymes were elevated in the diabetic animals. A developmental study was then undertaken to determine whether there is any relationship between changes of enzyme activities, on one hand, and the development of hyperglycemia on the other. Since the hepatic enzymes showed greater increases than those in kidney, the developmental study was continued only with liver enzymes. To determine whether NADP-linked enzymes that are not involved in steps of glucuronate-xylulose pathway were also elevated in liver, two enzymes of the phosphogluconate pathway—glucose 6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase—were also studied.

AGE-RELATED CHANGES IN BLOOD GLUCOSE, BODY WEIGHT, AND LIVER WEIGHT OF GENETICALLY DIABETIC MICE

Mice received from Jackson laboratories were kept in our animal facilities for at least 3 days before any experiment

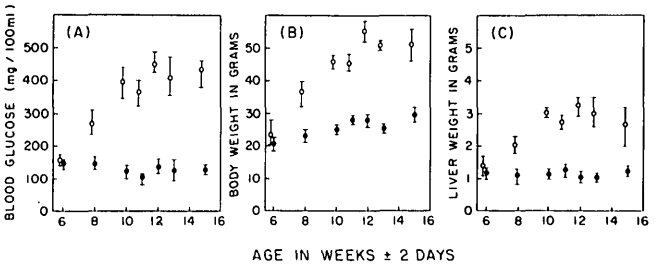


FIGURE 2. Blood glucose (A), body weight (B), and liver weight (C) of db/+ (●) and db/db (○) mice. The points represent the mean value; the vertical bars indicate the range of values obtained with four experimental animals.

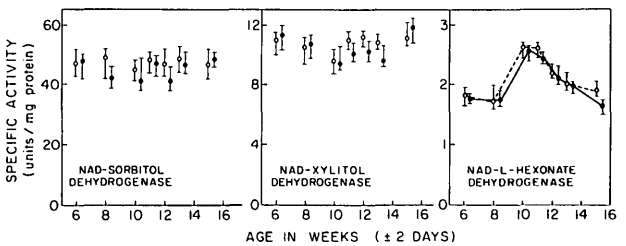
was conducted. Blood glucose of control animals (db/+) remained between 100 and 150 mg/dl of blood between 6 and 15 wk of development (Figure 2A). The diabetic animals (db/db) showed blood glucose levels similar to those of control animals at 6 wk of age, but they had considerably higher levels (250 mg/dl) by the time they were 8 wk old. The increase in blood glucose level plateaued at 12 wk of age (average of 450 mg/dl).

The diabetic mice were slightly heavier than the nondiabetic controls at 6 wk of age (Figure 2B). The increase in body weight of diabetic mice was gradual after 6 wk of age, until it plateaued at 12 wk, at about 55 g. Control animals, however, gained weight slowly, reaching a maximum of 30 g at 12 wk. Diabetic mice consumed more food than the corresponding nondiabetic animals of the same age. Changes in liver weight of animals during development showed similar patterns as those in body weight (Figure 2C). The diabetic and control animals had the same liver weight (1.25 g) at 6 wk of age. The weight remained constant in control animals, but gradually increased in diabetic animals until it reached a maximum of 3.0 g at 12 wk.

HEPATIC ENZYME LEVELS IN db/db AND db/+ MICE DURING DEVELOPMENT

Figure 3 shows that the three NAD-linked dehydrogenase activities showed similar developmental patterns in the genetically diabetic mice and their controls. Both groups of animals possessed rather constant levels of NAD-sorbitol dehydrogenase (Figure 3A) and NAD-xylitol dehydrogenase (Figure 3B) activities and similar age-related patterns for NAD-L-gulonate dehydrogenase (Figure 3C), a maximum occurring at 10 to 12 wk. On the other hand, the three NADP-linked dehydrogenase activities of the glucuronate-xylulose cycle all showed more marked changes in the diabetic mice than in the control animals (Figure 4).

FIGURE 3. NAD-linked dehydrogenase activities in livers of db/+ (●) and db/db (○) mice. The points represent the mean value for the specific activities; the vertical bars indicate the range of values obtained with four enzyme preparations. Details of enzyme assays are described under MATERIALS AND METHODS.



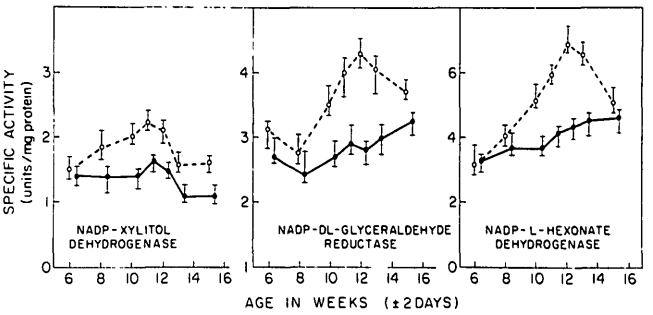


FIGURE 4. NADP-linked dehydrogenase activities in livers of db/+ (●) and db/db (○) mice. The points represent the mean value for the specific activities; the vertical bars indicate the range of values obtained with four enzyme preparations. Details of enzyme assays are described under MATERIALS AND METHODS.

Of the two NADP-linked enzymes of the 6-phosphogluconate pathway, glucose 6-phosphate dehydrogenase showed depressed levels in diabetic mouse liver (Figure 5A), whereas 6-phosphogluconate dehydrogenase was markedly elevated (Figure 5B). As indicated below, the apparent decrease in glucose 6-phosphate dehydrogenase is a result of the presence of a low molecular weight inhibitor. When this enzyme was assayed after removing the inhibitor, it showed similar activities in db/db and db/+ mouse livers.

When changes in enzyme levels are observed in crude tissue extracts, the possible presence of activators or inhibitors must be investigated. Consequently, mixing experiments were conducted using control and diabetic extracts. The results for the NADP-dependent enzymes were always similar to the activities calculated to be the sum of the activities composing the mixture, except for glucose 6-phosphate dehydrogenase, which gave lower values than the calculated sum. To determine whether db/db mouse liver preparations showing depressed levels of this dehydrogenase (Figure 5A) contained inhibitors, the crude enzyme solution was passed through a Bio-gel P10 column equilibrated with buffer A. With these extracts, the enzyme recovered in the void volume of the column was always in excess of 100%; the recoveries from the livers of db/+ mice of similar age were considerably lower (Table 1). These results suggest

FIGURE 5. NADP-linked dehydrogenases of 6-phosphogluconate pathway in livers of db/+ (●) and db/db (○) mice. Each point represents mean value for the specific activity; the vertical bars indicate the range of values obtained with four enzyme preparations. Details of enzyme assays are described under MATERIALS AND METHODS.

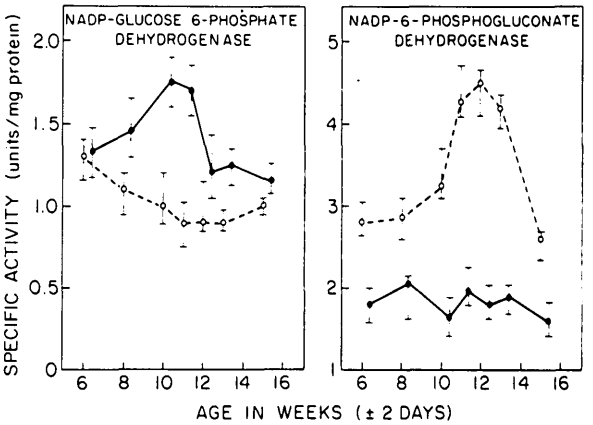


TABLE 1
Recovery of mouse liver glucose 6-phosphate dehydrogenase from Bio-gel P10 columns*

Age (wk)	(% recovery) db/+	(% recovery) db/db	db/db ÷ db/+
8	79.6	112.2	1.41
9	82.6	117.3	1.42
10	88.9	122.2	1.37

* Mouse liver was homogenized and centrifuged as described under MATERIALS AND METHODS. The crude extract (1 ml) was applied to the P10 column (0.7 × 12 cm), equilibrated with buffer A. Fractions (0.5 ml) were collected and analyzed for glucose 6-phosphate dehydrogenase. Data reported are the average of two separate experiments for each age group.

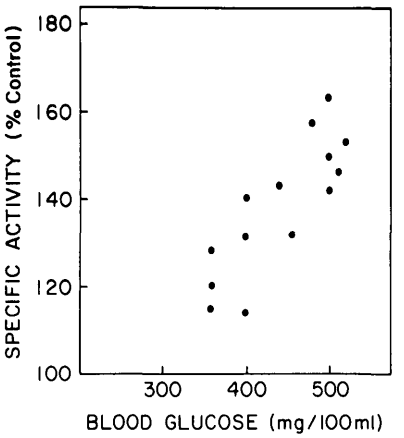


FIGURE 6. Relationship between blood glucose and liver NADP-L-hexonate dehydrogenase (aldehyde reductase II) in db/db mice. Each point represents specific activity (% control) of the enzyme and blood glucose level in the same animal.

the presence of a low molecular weight inhibitory material in the db/db liver extract that influences the measurable activity of glucose 6-phosphate dehydrogenase.

NADP-DL-glyceraldehyde reductase and NADP-L-hexonate dehydrogenase showed similar patterns of developmental changes in the two groups of mice. When it was discovered that the former activity is a reflection of both aldehyde reductases I and II,[7] it was necessary to determine whether one, or both, of these reductases is elevated in genetically diabetic (db/db) mouse liver. Consequently, the two enzymes were separated by chromatography on a DEAE-cellulose (DE-52) column.[7] Quantitation of the two enzymes after separation showed that aldehyde reductase II, but not aldehyde reductase I, is elevated in genetically diabetic mouse liver. In fact, as shown in Figure 6 for animals between 8 and 10 wk of age, direct assay of aldehyde reductase II activity with D-glucuronate as substrate shows that the enzyme level in these animals is directly related to the blood glucose levels.

Enzyme activities in db/+ and db/db mice treated with streptozotocin. To determine whether the changes in NADP-linked dehydrogenases in db/db mice were specific responses to the diabetic condition, the influence of streptozotocin was studied. Hepatic enzyme levels in db/+ and db/db mice treated with i.p. injections of streptozotocin (200 mg/kg body weight) are shown in Table 2. Only the two 6-phosphogluconate pathway enzymes were increased by this treatment in the db/+ mice, and only glucose 6-phosphate dehydrogenase was increased in the db/db mice, the NADP-linked dehydrogenases of the glucuronate-xylulose cycle remaining unchanged.

TABLE 2
Hepatic enzymes in control and streptozotocin (STZ)-treated db/+ and db/db mice*

Enzyme†	Specific activity¶ db/+‡	db/+ + STZ§	db/db‡	db/db + STZ§
NAD-L-gulonate dehydrogenase	2.3 ± 0.2	2.4 ± 0.3	2.2 ± 0.3	2.5 ± 0.4
NAD-xylitol dehydrogenase	8.7 ± 0.8	9.0 ± 0.3	9.1 ± 0.4	8.7 ± 0.5
NAD-sorbitol dehydrogenase	39.9 ± 2.8	38.2 ± 3.0	34.6 ± 6.0	38.5 ± 4.7
NADP-xylitol dehydrogenase	1.0 ± 0.1	1.1 ± 0.1	1.6 ± 0.2	1.7 ± 0.2
NADP-DL-glyceraldehyde reductase‖	2.6 ± 0.4	2.8 ± 0.2	3.7 ± 0.3	3.5 ± 0.3
NADP-L-hexonate dehydrogenase (aldehyde reductase II)	5.1 ± 0.8	4.9 ± 0.6	7.3 ± 0.6	6.5 ± 0.5
Glucose 6-phosphate dehydrogenase (NADP)	1.5 ± 0.3	3.0 ± 0.2#	1.1 ± 0.3	2.9 ± 0.2#
6-Phosphogluconate dehydrogenase (NADP)	1.2 ± 0.2	3.9 ± 0.8#	4.2 ± 0.4	4.5 ± 0.4

* Data reported are the average of four separate experiments (± indicates the range of values obtained).

† All enzymes were assayed as described under MATERIALS AND METHODS.

‡ Eight week old db/+ and db/db mice were injected i.p. with saline in 10 mM citrate buffer (pH 4.5) as described under MATERIALS AND METHODS. Animals were killed 2 wk after start of experiment. Average blood glucose: db/+ mice, 117 mg/dl; db/db mice, 376 mg/dl.

§ Eight week old db/+ and db/db mice were given streptozotocin (200 mg/kg body wt) intravenously as described under MATERIALS AND METHODS. Animals were killed 2 wk after start of the experiment. Average blood glucose: STZ-treated db/+ mice, 342 mg/dl; STZ-treated db/db mice, 262 mg/dl. db/db Mice treated with the drug lost 7 g body weight in 2 wk and their livers weighed only 61% of those of db/db mice.

‖ As indicated in the text, (1) DL-glyceraldehyde is utilized by both aldehyde reductase I and aldehyde reductase II, and (2) the elevated db/db activities observed here are caused by the presence of aldehyde reductase II.

¶ Specific activity represents units of enzyme per milligram protein. The average amount of soluble protein per gram liver in these animals ranged from 82.6 to 90 mg.

These values differed significantly ($P < 0.001$) from those of the corresponding animals not treated with STZ. Levels of other enzymes do not differ significantly from their controls.

Enzyme activities in rats with streptozotocin-induced diabetes. The results of the analysis of enzyme activities in liver extracts of streptozotocin-induced diabetic rats are reported in Table 3. All activities, with the exception of 6-phosphogluconate dehydrogenase, remained unchanged in the induced diabetic rat liver. Depressed levels of 6-phosphogluconate dehydrogenase were observed in diabetic rats, an observation opposite to that found for db/db mice and for streptozotocin-treated db/db and db/+ mice.

TABLE 3
Hepatic enzymes in control and streptozotocin-induced diabetic rats*

Enzyme†	Specific activity‖ Control rats‡	Specific activity‖ Streptozotocin rats§	P-value
NAD-L-gulonate dehydrogenase	1.1 ± 0.2	1.1 ± 0.4	NS¶
NAD-xylitol dehydrogenase	4.9 ± 0.8	4.4 ± 1.2	NS
NAD-sorbitol dehydrogenase	6.3 ± 2.8	6.1 ± 2.6	NS
NADP-xylitol dehydrogenase	1.3 ± 0.2	1.3 ± 0.4	NS
NADP-DL-glyceraldehyde reductase	3.2 ± 0.7	2.5 ± 0.7	NS
NADP-L-hexonate dehydrogenase	3.3 ± 0.6	2.4 ± 0.6	NS
Glucose 6-phosphate dehydrogenase (NADP)	2.1 ± 0.5	1.9 ± 0.4	NS
6-Phosphogluconate dehydrogenase (NADP)	11.1 ± 1.2	5.4 ± 0.8	<0.001

* Data reported are the average of four separate experiments (± indicates the range of values obtained).

† All enzymes were assayed as described under MATERIALS AND METHODS.

‡ Male rats (160–180 g) were given saline in citrate buffer (10 mM, pH 4.5) i.v. as described under MATERIALS AND METHODS. Animals were killed 3 wk after start of the experiment. The average weight gained in 3 wk was 125 g. At that time the average liver weight was 10.6 g and the average blood glucose was 101 mg/dl.

§ Male rats (160–180 g) were given streptozotocin (65 mg/100 g body weight) in citrate buffer (10 mM, pH 4.5) i.v. as described under MATERIALS AND METHODS. Animals were killed 3 wk after start of experiment. The average weight gained in 3 wk was 73 g, the average liver weighed 10.4 g, and the average blood glucose was 450 mg/dl.

‖ Specific activity is units enzyme per milligram protein. The average protein per gram liver in control and streptozotocin-treated rats ranged from 79.6 to 84.9 mg.

¶ NS = not significant ($P > 0.1$).

DISCUSSION

The study of dehydrogenase levels in the livers of genetically diabetic mice yielded the interesting result that, whereas two NADP-linked dehydrogenases were elevated, NAD-linked enzyme activities were not. The exceptionally high substrate specificity of NADP-xylitol dehydrogenase makes the increase in this enzyme worthy of note. Similarly, the increase in NADP-L-hexonate dehydrogenase (aldehyde reductase II) is of interest because it is reported to be the rate-limiting enzyme of the glucuronate-xylulose cycle in rats.[12] The elevation observed in this enzyme in genetically diabetic mice is consistent with reports suggesting that C_6-oxidation pathway is enhanced in diabetes.[4,5] However, neither of these two NADP-linked enzymes, nor other enzymes of the cycle, were increased in mice or rats made diabetic by treatment with streptozotocin. Our results therefore provide no information on a specific relationship of this glucose oxidation pathway to the diabetic state. The greatly different results obtained with genetically diabetic mice, as opposed to chemically induced diabetic animals, suggest that fundamentally different metabolic processes are altered in the two types of diabetes. It is of considerable interest that, in a study of plasma lysosomal hydrolase levels, we have found that streptozotocin-treated rats, like diabetic patients,[20–24] show elevated plasma hydrolase activities, whereas streptozotocin-treated mice and genetically diabetic mice show depressed levels.[25] In the present study, the two types of diabetic mice gave different results. In the study of lysosomal enzymes, the divergent results were species dependent. Obviously, we are lacking in a fundamental understanding of the metabolic abnormalities.

Two points concerning analytic procedures should be noted. First, the use of glyceraldehyde in assays of NADP-linked dehydrogenase[3,9,11] of accessory pathways of glucose oxidation requires caution because it serves as substrate for three enzymes, namely, aldehyde reductase I, aldehyde reductase II, and aldose reductase.[7] Second, glucose 6-phosphate dehydrogenase determinations in crude tissue extracts may be unreliable because of the presence of a low molecular weight inhibitory material.

The increase observed in hepatic levels of 6-phosphogluconate dehydrogenase of db/db mice is generally similar to the results of Chang and Schneider,[26] who stated that glycolytic and pentose phosphate shunt enzymes are elevated in young db/db mice, when the rate of glucose oxidation is elevated. It should be emphasized that the elevated levels of NADP-linked enzymes observed in the present study are even more impressive when the increased liver size of db/db mice is borne in mind. It is also of interest that, since the livers of these animals are especially high in lipid content and show greatly enhanced rates of fatty acid biosynthesis,[27] it is possible that the changes in NADP-linked dehydrogenase activities are related to an increased requirement for NADPH.

The elevated hepatic levels of NADP-linked dehydrogenases reported in the present paper are a further indication of the abnormal metabolism of genetically diabetic mice. The absence of such changes in streptozotocin-induced diabetic animals again emphasizes the fundamental difference in the pathology of diabetes of genetic origin as compared with the condition resulting from B-cell dysfunction. Also relevant are the findings that genetically diabetic mice show opposite changes in plasma acid hydrolase levels to those observed in diabetic patients and diabetic rats.[25] Since in many ways genetically diabetic mice are a rather useful model of human diabetes,[28–30] the present report provides additional information on which to base the selection of a test animal for investigation of particular questions relevant to our understanding of diabetes.

ACKNOWLEDGMENTS

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