Hepatic metabolism of genetically diabetic (db/db) mice. 1. Carbohydrate metabolism

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In the genetically diabetic strain of mice C57BL/Ks-db/db first recognized in 1965 in The Jackson Laboratory, Bar Harbor, Maine (10, 11), the disease is inherited as a single autosomal recessive gene with complete penetrance. The earliest abnormalities are a tendency towards obesity, an abnormally high level of circulating insulin, and hyperglycemia (10). As the disease progresses, the body weight increases rapidly due mostly to excessive fat deposition, the blood glucose rises to very high values, whereas the plasma insulin declines toward normal.

There have been very few studies of the metabolism of the db/db mice and the biochemical basis of the syndrome is largely unknown. It has been reported that the activities of several lipogenic enzymes in the liver and adipose tissue of db/db mice younger than 10 wk of age are elevated above normal (11). Hepatic glycogenic and gluconeogenic enzyme activities are also increased in db/db mice aged 2 mo or older (8, 11).

In vivo experiments have provided evidence that mildly hyperglycemic db/db mice oxidize $^{14}C$-glucose to $^{14}CO_2$ more rapidly than normal, whereas a subnormal rate is observed in severely hyperglycemic mice (10). The rate of $[1-^{14}C]$glucose metabolism in adipose tissue from 4 wk-old diabetic mice is similarly higher than normal and is further elevated by insulin (43). However, it declines to below normal at approximately 9 wk of age with a concomitant loss of insulin sensitivity.

The idea that excessive gluconeogenesis contributes to the hyperglycemia of db/db mice has been proposed by several workers, including Chang and Schneider (8) who found increased incorporation in vivo of radioactivity from $[3-^{14}C]$pyruvate into serum glucose in db/db mice. However, insufficient data were presented to permit clear interpretation of their findings. In view of the possibility that glucose utilization was reduced (43) and because of the subnormal blood volume/body weight ratio (3) in these animals, the conclusion that gluconeogenesis was enhanced is equivocal.

The present study was undertaken to determine more precisely the role of the liver in the development of hyperglycemia and fat deposition in db/db mice. The results are presented in this report which describes the quantitative changes in several hepatic metabolic processes prior to and following the onset of hyperglycemia and/or obesity.

Data from in vivo and liver perfusion experiments are given.

Materials and Methods

Animals. C57BL/Ks-J and C57BL/Ks-db/db mice were obtained at 3-7 wk of age from The Jackson Laboratory, Bar Harbor, Maine. They were fed Purina rat chow ad libitum for at least 1 wk prior to use.

All purified enzymes used in analyses were purchased from Boehringer Mannheim Corp. Radioactive tracers were purchased from Amersham/Searle Corp. and biochemicals from Sigma Chemical Co.

General analyses. Liver and serum lipids were extracted according to Folch et al. (17). Serum free fatty acids and fatty acids extracted from saponified liver lipids according to Exton et al. (13) were determined colorimetrically (22). Serum glucose was determined either by glucose oxidase (28) or by the ferricyanide method using the Technicon AutoAnalyzer (13). Serum lactate and pyruvate were determined enzymatically after deproteinization with ZnSO4 and Ba(OH)2 (13). Serum acetooacetate and β-OHbutyrate were also determined in the Ba/Zn filtrates by

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fluorometric adaptations of the enzymatic methods of Williamson et al. (41).

Liver glycogen was isolated from frozen or fresh liver samples by alkali digestion followed by ethanol precipitation (20). The glycogen suspension was subsequently hydrolyzed enzymatically (26), and the glucose was determined by either ferricyanide or glucose oxidase methods. Liver protein was determined by the biuret method using a bovine serum albumin standard. The method described by Schneider (33) was followed for extraction and quantitation of DNA. For tissue water content determinations, liver samples were thoroughly minced, placed on preweighed aluminum foil, weighed, and heated at 100°C for 4 h. The same procedure was followed in determining total body water except that glass petri dishes were used instead of aluminum foil. The 4H2O method described by Crowdon et al. (21) was also used to determine body water in mice of 5 and 8 wk of age. Methodology for extraction and determination of liver cyclic AMP has been described (7).

For total body potassium determinations, animal carcasses were placed in crucibles and ashed in muffle furnace at 800°C. The residues were dissolved in concentrated HCl and appropriate dilutions were made for potassium analysis by flame photometry.

Enzyme assays. Fresh or rapidly frozen liver samples obtained in vivo were homogenized in 20 vol of cold buffer (pH 7.5) containing 20 mM Tris, 120 mM KCl, 5 mM MgSO4, and 0.1 mM EDTA. The homogenate was centrifuged at 104,000 X g for 60 min at 4°C and the resultant supernatant fraction was used for measurements of lactate dehydrogenase (2), pyruvate kinase (4), fructose-1,6-P2-aldolase (38), fructose-1,6-bisphosphatase (30), and glucokinase plus hexokinase (40) activities. Glucose-6-phosphatase activity was assayed according to the method described by Nordlie and Arion (28). 14C02 fixation assays were performed using liver homogenate subfractions prepared according to Exton et al. (14).

Glycogen synthetase was assayed by a modification of the method of Thomas, Schlender, and Larner (37). A 5 or 20 % homogenate (wt/vol) was prepared from frozen liver samples in a buffer containing 0.4 M sucrose, 5 mM EDTA, 50 mM NaF, 20 mM cysteine, and 20 mM Tris-HCl, pH 8.0. An aliquot (50 μl) of the homogenate was then incubated at 30°C for 20 min with 50 μl of an assay mixture containing 100 mg/ml repurified oyster glycogen, 10 mM UDP-[14C]glucose (approximately 0.03 μCi/μmol), 2.5 mM Na3PO4, 50 mM KF, 27 mM EDTA, 50 mM Tris-HCl, pH 7.6 in the presence or absence of 10 mM glucose-6-P. At the end of the incubation period, 50 μl of the mixture was removed and spotted on filter paper and dropped into a 66 % ethanol solution. The units of activity are expressed as micromoles of glucose-1-P incorporated into glycogen per gram liver per minute.

Liver perfusion. The apparatus used for mouse liver perfusion was essentially similar to that described by Exton and Park (15). The mouse liver perfusion technique of Assimacopoulos-Jeannet et al. (1) was adopted with minor modifications. A 19-gauge needle from a butterfly infusion set was used for the portal cannula and firm Teflon tubing (0.5 mm ID) was used for the inferior vena cava cannula. The final hematocrit of the perfusion medium was brought to 40 % with thoroughly washed bovine red blood cells. The perfusate flow rate was maintained at about 2 ml/min per g of liver.1 Unless otherwise stated, a nonrecirculating perfusion system was used in all perfusion experiments. Substrates and hormones were either added directly to the perfusion medium or infused into the portal line approximately 8 cm from the portal cannula. Perfusate samples were usually collected every 10 min from the vena cava outflow, deproteinized with WC104 (final concentration 0.5 M), neutralized with KOH, and analyzed for glucose, lactate, pyruvate, and [14C]glucose (15).

For 14CO2 analysis, 0.5-ml perfusate samples were collected with a hypodermic syringe from a point midway along the vena cava outflow tubing and immediately injected into plastic counting vials. Each of these contained 1.0 ml of 1 M HC104 solution, was tightly sealed with a serum cap, and had a suspended plastic center well containing a strip of filter paper and 0.3 ml of either 1 N HAOH or hyamine hydroxide. The plastic vials containing added samples were incubated at 37°C with shaking for 30 min. At the end of the incubation period, they were cooled to room temperature and the center wells dropped into glass counting vials containing 10 ml of a dioxane-toluene counting fluid (13), and counted in a Packard Tri-Carb scintillation spectrometer.

At the end of each perfusion, the liver was frozen immediately with a pair of aluminum tongs cooled in liquid nitrogen (42). Methods for treatment of frozen livers and analyses for glycogen, [14C]glycogen and gluconeogenic intermediates have been described elsewhere (16).

In vivo [3-3H]glucose turnover. Food was removed from the animals at 5 A.M. They were lightly anesthetized with an intraperitoneal injection of Nembutal (20 mg/100 g animal) 5 h later, followed by an injection of 25 μCi (50 μl) of [3-3H]-glucose via the femoral vein. Blood samples (10–13 μl) were taken alternately from the left and right periorbital sinuses using heparinized capillary tubes. Samples were immediately deproteinized with 20 μl each of ZnSO4 and Ba(OH)2 and diluted with distilled H2O.

Aliquots (100 μl) of the diluted deproteinized samples

1 This flow rate was chosen on the basis of measurements in vivo of portal blood flow in normal and db/db mice (unpublished findings).
were evaporated completely in glass counting vials under a heat lamp. One milliliter of H₂O and 10 ml of a Triton-
toluene scintillation fluid were added to each vial and the radioactivity remaining after evaporation of ³H₂O was determined. Glucose contents in the deproteinized samples were determined by the glucose oxidase method. Blood glucose levels in these animals remained relatively constant throughout the experiment. Estimates of glucose turnover and clearance were carried out according to Growdon et al. (21).

RESULTS

General characteristics of C57BL/−Ks−J and C57BL/Ks−
db/db mice. Several physical parameters were examined in normal and db/db mice aged 4−9 wk. No significant abnormality could be detected in the body weight (Fig. 1) and serum glucose (Fig. 2) of db/db mice at ages 4 and 5 wk. The body weight of the db/db mice began to deviate from normal after the 5th wk, and they became twice the size of normal by the 9th wk (Fig. 1). Concomitant with the abnormal weight gain, the serum glucose rose dramatically (Fig. 2).

The body water content of the db/db mice was lower than normal even at 4 wk of age (Fig. 1). Body water expressed as percent of body weight declined from 83% of normal at 4 wk to 50% of normal at 9 wk of age. Similar observations have been reported for ob/ob mice (3). Since the water content of adipose tissue is very low relative to other tissues, these observations are consistent with the excessive weight gain being due to the accumulation of fat. The reduced body water content of the db/db mice reflects decreases in both extracellular (3) and intracellular water and indicates that the mice have a decreased metabolic mass.

Total body potassium contents differ dramatically between normal and diabetic mice. At 8 wk of age, total body potassium was 24.3 and 9.19 meq/kg for the normal and db/db mice, respectively. It therefore appears that in accord with the lower metabolic mass, total intracellular fluid was also reduced in the db/db mice.

The liver weight/body weight ratio of both normal and db/db mice was constant with age (Fig. 3) with that of the db/db mice being consistently higher. The ratio of liver weight to body water content remained relatively constant with age in the normal mice, while that of the diabetic mice rose to a value which was twice normal at 8 wk (Fig. 3). These data indicate that the liver of the db/db mice is inappropriately large in relation to the metabolic mass.

Several other metabolic parameters were also altered in db/db mice at 8 wk of age, i.e., when hyperglycemia and obesity were clearly expressed. Total serum ketone bodies were 0.27 ± 0.02 μmol/ml in the db/db mice and 0.40 ± 0.02 μmol/ml in normal mice, despite the fact that the serum free fatty acid levels were almost 3 times normal in db/db mice (2.69 ± 0.80 μeq/ml compared to 0.91 ± 0.26 μeq/ml in normal mice). There was also significant elevation of serum lactate in the db/db mice (3.77 ± 0.30 μmol/ml compared to 2.25 ± 0.16 μmol/ml in normal mice).
Table 1 shows that the liver weight was twice normal in the db/db mice and that the total water content of the liver was unchanged. DNA content was only 70% of normal, suggesting a lower cellularity in the diabetic liver. Both liver lipids and glycogen were markedly elevated in the db/db mice, whereas no significant alteration was detected in liver protein. Total cyclic AMP levels were not significantly changed in the diabetic livers.

Hepatic glycogen metabolism. In db/db mice aged 7 wk or younger, there was little or no significant increase in the level of glycogen in the liver, but at 8 wk, the diabetic mice showed an almost threefold increase above normal (64 ± 3 mg/g liver compared to 22 ± 3 mg/g liver in normal mice). When expressed per 100 g of body weight, liver glycogen was greater in the db/db mice at all ages.

To investigate the cause of the increased glycogen content of the db/db mice, livers of 8-wk-old normal and diabetic mice were perfused with media containing different levels of glucose, and glycogen breakdown and synthesis were measured. Diabetic livers perfused with medium containing no added glucose released more glucose and broke down more glycogen than normal livers, the rates being three- to fourfold higher on a body water basis (Table 2). Glucose release was also greater in the livers of 5-wk-old db/db mice perfused under the same conditions even though the initial glycogen content was similar to normal.

Since it has been well demonstrated that the blood glucose level per se can control liver glycogen breakdown and synthesis in vivo (11) and in vitro (6, 19, 35), it was of interest to examine glucose release from normal and diabetic livers perfused with recirculating medium containing various concentrations of glucose. The results of these experiments are shown in Fig. 4. In agreement with the foregoing results obtained using nonrecirculating medium, the rate of glucose release from diabetic livers perfused with media containing no added glucose was greatly increased above normal. When the livers were perfused with 8.5 mM glucose, a physiological concentration for normal mice, normal livers exhibited a small net glycogenolysis during the first 20 min, but thereafter showed no net change in glucose balance, whereas diabetic livers released glucose at a steady rate for 90 min. When the livers were perfused with 17 mM glucose, a gradual net uptake of glucose by the normal livers was observed, whereas there was a slow release of glucose from the livers of the db/db mice. When the diabetic livers were perfused with 25 mM glucose, a concentration close to physiological for 8-wk-old db/db mice, a rise in medium glucose level was observed during the first 20 min, but no net glucose output occurred thereafter. Thus, Fig. 4 shows that when livers from normal and diabetic mice are perfused with their respective physiological glucose concentrations, a steady state is rapidly achieved in which there is no net glucose release or uptake.

In order to see whether liver glycogen synthesis per se was altered in db/db mice, livers of 8-wk-old mice were perfused with recirculating media containing initially 14 or 25 mM [U-14C]glucose. It was found that with the lower concentration of glucose, labeling of glycogen per gram liver was similar in normal and db/db mice (4.8 ± 1.0 μmol glucose per hour per gram liver compared to 3.5 ± 0.5 for normal mice). With the higher glucose level, however, the incorporation of isotope into liver glycogen in the db/db mice was twice normal (21.5 ± 3.0 μmol glucose per hour per gram liver compared to 22.5 ± 3.0 μmol glucose per hour per gram liver in normal mice).

Table 2. Glycogenolysis and endogenous gluconeogenesis during 1 h perfusion of livers from fed 8-wk-old mice

<table>
<thead>
<tr>
<th>Glucose released</th>
<th>db/db Mice</th>
<th>Normal Mice</th>
</tr>
</thead>
<tbody>
<tr>
<td>μmol/g liver</td>
<td>μmol/100 g</td>
<td>μmol/100 g</td>
</tr>
<tr>
<td>body H2O</td>
<td>body H2O</td>
<td></td>
</tr>
<tr>
<td>Glucose released</td>
<td>93.0±7.6</td>
<td>690±56</td>
</tr>
<tr>
<td>Decrease in glycogen</td>
<td>63.2±5.4</td>
<td>465±40</td>
</tr>
<tr>
<td>Endogenous gluconeogenesis</td>
<td>30.6±8.3</td>
<td>225±61</td>
</tr>
</tbody>
</table>

One lobe of the liver was ligated, and immediately frozen prior to cannulation. The remaining lobes were perfused without recirculation for 1 h, and then frozen in situ. Glycogen content was determined in both the nonperfused and perfused portion of the same liver. Each value is mean ± SE of five livers. Glucose release, total glucose found in perfusate after 1 h perfusion, decrease in glycogen, glycogen-glucose in nonperfused lobe – glycogen-glucose in perfused lobe, Endogenous gluconeogenesis, glycogen release – decrease in glycogen.
per hour per gram liver compared to 10.1 ± 1.0 for normal mice).

These data and those from Table 2 and Fig. 4 suggest that hepatic glycogen turns over more rapidly in the db/db mice. Possible alterations in hepatic glycogen metabolizing enzymes were tested for. Hepatic glycogen synthetase and phosphorylase activities are shown in Table 3. Total glycogen synthetase activity increased about 70%, when normal mice advanced from 5 to 8 wk of age, whereas glycogen synthetase I activity nearly doubled. In both the 5- and 8-wk-old diabetic mice glycogen synthetase 1 and total synthetase activities were greatly increased above normal. Paradoxically, glycogen phosphorylase activities were also elevated in both young and old diabetic livers, to 130 and 177% of normal, respectively. Thus, the perfusion and enzyme data suggest a substantially greater rate of hepatic glycogen turnover in the db/db mice.

**Hepatic gluconeogenesis.** As illustrated in Table 2, glucose production by perfused livers of fed rats was not fully accounted for by the changes in liver glycogen and the difference is due to endogenous gluconeogenesis. It is seen further in Table 2 that the contribution to glucose production from endogenous gluconeogenesis was greater than normal in the db/db mouse livers when expressed on the basis of total body water. As considered further in the discussion, comparison of hepatic metabolic data on the basis of 100 g of body water is more valid and meaningful than on the basis of body weight or liver weight. Description and discussion of results will hereafter be made mainly on this basis.

Gluconeogenesis from added physiological substrates was also studied. Figure 5 shows that [14C]glucose production from L-[U-14C]lactate at 10 mM concentration was greater in the perfused livers of db/db mice at all ages studied. The rate of gluconeogenesis in the 15-wk-old db/db mouse livers was greater than twice that of normal mouse livers. Lactate gluconeogenesis was also examined under more physiological conditions; i.e., at lower concentrations and the presence of pyruvate at 0.1 the concentration of lactate (Fig. 6). Higher rates of gluconeogenesis in the db/db mouse livers were observed at lactate concentrations greater than 2 mM. It is interesting to note that the lactate concentrations for half-maximal gluconeogenesis were 2.3 mM for normal livers and 3–4 mM for diabetic livers. These values correspond to the serum lactate concentrations observed in these animals in vivo.

Further experiments were carried out with other gluconeogenic substrates. Table 4 compares the rates of [14C]glucose and 14CO2 production in livers perfused with 14C-labeled 3 mM L-lactate, pyruvate, or L-alanine. Unlike results obtained in the perfused rat liver (34), pyruvate and alanine were better gluconeogenic precursors than lactate at this concentration. Production of [14C]glucose and 14CO2 in these experiments was substantially greater in the livers of db/db mice. The livers of fasted diabetic mice also converted fructose to glucose at a rate which was almost threefold higher than normal: addition of 10 mM fructose to the medium increased glucose production in normal and diabetic mice by 674 ± 11 and 1,742 ± 79 μmol/h per 100 g of body water, respectively.

**Effects of glucagon on glycogenolysis and gluconeogenesis in livers of normal and db/db mice.** Glucagon is a potent stimulator of hepatic glycogenolysis and gluconeogenesis in many species, but has been reported to be without effect on gluconeogenesis in one strain of mice (1). The hormone also has been found to have negligible effect on the high rate of gluconeogenesis in livers of alloxa- n-streptozotocin diabetic rats (29). It was therefore of interest to measure its effect in db/db mice. Figure 7 shows the results of a series of experiments in which livers of 8-wk-old mice were perfused with glucagon at concentrations within the range 11–10–7 M. It is seen that the hormone produced a larger than normal stimulation of glucose release in the diabetic livers, due presumably to their higher glycogen and glycogen phosphorylase contents (Table 3). There appeared to be no difference in the sensitivity of the normal

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**Table 3. Hepatic glycogen synthetase and glycogen phosphorylase activities of fed mice**

<table>
<thead>
<tr>
<th>Animals</th>
<th>Glycogen Synthetase Units</th>
<th>Glycogen Phosphorylase Units</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>G-6-P</td>
<td>+G-6-P</td>
</tr>
<tr>
<td>Normal (5 wk old)</td>
<td>5.13 ± 1.46</td>
<td>31.8 ± 10.3</td>
</tr>
<tr>
<td>db/db (5 wk old)</td>
<td>20.1 ± 4.26</td>
<td>105 ± 16.4</td>
</tr>
<tr>
<td>Normal (8 wk old)</td>
<td>10.6 ± 3.05</td>
<td>54.3 ± 16.9</td>
</tr>
<tr>
<td>db/db (8 wk old)</td>
<td>19.2 ± 3.14</td>
<td>138 ± 22.2</td>
</tr>
</tbody>
</table>

Each value is mean ± SE of five to eight livers. * 1 unit activity, μmol UDP-glucose incorporated per g per liver per h.
† 1 unit activity, μmol G-1-P incorporated per g per liver per min.
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FIG. 6. [14C]glucose production in livers perfused with various concentrations of 1-[U-14C]lactate plus pyruvate. Each point is mean ± SE of at least 4 observations. All livers were perfused without recirculation. Substrates were infused directly into portal inflow at different rates to give final substrate concentrations indicated. All substrate concentrations were verified by enzymatic analysis of perfusion medium.

TABLE 4. [14C]glucose and [14CO2] productions from physiological substrates in perfused livers from 8-wk-old fed mice

<table>
<thead>
<tr>
<th>Substrate</th>
<th>14C/Glucose</th>
<th>14CO2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Normal mice</td>
<td>db/db mice</td>
</tr>
<tr>
<td>L-[U-14C]alanine</td>
<td>250±15</td>
<td>487±39</td>
</tr>
<tr>
<td>L-[U-14C]pyruvate</td>
<td>296±24</td>
<td>752±33</td>
</tr>
<tr>
<td>L-[U-14C]lactate</td>
<td>142±13</td>
<td>215±15</td>
</tr>
</tbody>
</table>

All values are means of four observations ± SE. Livers were perfused without recirculation with substrates infused directly into the portal inflow to achieve a concentration of 3 mM. * One hundred grams body water is equivalent to 7.5 g liver in normal mice and 17 g liver in db/db mice.

and db/db mice to glucagon as measured by hepatic glycogenolysis; i.e., in both types of animal, half-maximum glycogenolysis was observed with 5 × 10^{-10} M glucagon, a concentration with the range reported for portal venous blood in humans (5).

Figure 8 shows the response of lactate gluconeogenesis to glucagon in perfused livers of 8-wk-old normal and db/db mice. Glucagon stimulation of gluconeogenesis was clearly present in both types of animals and was about 250% of basal with maximal levels of the hormone. Half-maximum gluconeogenesis was achieved with about 10^{-9} M glucagon in both normal and db/db mice. Thus, 8-wk-old C57BL/6J mice respond strongly to the glycogenolytic and gluconeogenic actions of glucagon, and the db/db mutants show no impairment in their response to the hormone.

Changes in levels of gluconeogenic intermediates in livers of mice perfused with lactate. Since changes in tissue metabolite levels can be useful in locating control points in a pathway, gluconeogenic intermediates were measured in livers of 5- and 8-wk-old normal and db/db mice perfused with 10 mM l-lactate. Figure 9 presents the metabolite levels found in diabetic livers expressed as percentages of those found in normal livers. A striking similarity is evident in metabolite changes in the 5- and 8-wk old db/db mice. A marked reduction in P-enolpyruvate was seen in the diabetic livers and there were decreases in pyruvate, aspartate, P-glycerates, and fructose-1,6-P2. In contrast, glucose and hexose-P were elevated and a crossover point was observed between fructose-1,6-P2 and fructose-6-P. Judging from the lactate/pyruvate and P glyceraldehyde-3-phosphate ratios, the NAD couple in the liver cytoplasm of the db/db mice appeared to be more reduced than normal.
Changes above the triose phosphates are consistent with facilitation of the fructose-1,6-bisphosphatase reaction (or restraint of the P-fructokinase reaction) in the livers of db/db mice.

However, it should be noted that the metabolite levels are expressed per gram of liver and, on this basis, the gluconeogenic rates from lactate are similar in normal and db/db mice. Thus, the apparent facilitation of the net conversion of fructose-1,6-P2 to fructose-6-P does not lead to a higher rate of gluconeogenesis per gram liver from this substrate. This is presumably due to the fact that gluconeogenesis from lactate, pyruvate, and alanine is limited by a reaction(s) between pyruvate and P-enolpyruvate in the pathway. In the case of fructose, which enters the gluconeogenic pathway at various sites between 2-P-glycerate and triose-P, the rate of glucose synthesis is significantly higher per gram liver in the db/db mice.

Activities of gluconeogenic and glycolytic enzymes in livers of normal and db/db mice. To obtain more direct evidence of facilitation of the hepatic fructose-1,6-bisphosphatase reaction in db/db mice, several gluconeogenic and glycolytic enzymes were assayed in 4-, 50, 7-, and 8-wk-old mice. The activity of lactate dehydrogenase per gram liver remained constant at all ages and there was no difference between normal and db/db mice. Aldolase showed little change with age in the normal mice. In the db/db mice, aldolase was not markedly elevated until 8 wks of age (data not shown). No difference in P-enolpyruvate carboxykinase was detected at all ages examined. Pyruvate carboxylase also showed little change with age in the normal mice. Fructose-bisphosphatase and glucose-6-phosphatase were higher in the db/db mice at all ages (Fig. 10, upper panel), there being twofold increases above normal in the 8-wk-old mice.

In contrast to lactate dehydrogenase, aldolase, and the gluconeogenic enzymes which remained relatively constant with age in the normal mice, pyruvate kinase and glucokinase plus hexokinase increased steadily from 4 to 8 wk in the livers of normal and db/db mice (Fig. 10, lower panel). At 7 wk the levels of the enzymes were clearly elevated in the db/db mice and at 8 wk they were increased twofold.

These data agree with those of Coleman and Hummel (11) and Chang and Schneider (8) who measured some of these enzymes in mice grouped less precisely according to age. The finding that the activities of pyruvate carboxylase and P-enolpyruvate carboxykinase are similar per gram liver in normal and diabetic mice is consistent with the observation that gluconeogenesis from lactate, alanine, or pyruvate is not increased per gram liver in the diabetic mice since these enzymes are believed to be rate limiting for glucose synthesis from these substrates. The increased hepatic fructose bisphosphatase activity of the db/db mice accounts for the crossover seen in the metabolite profiles of Fig. 9 and is presumably responsible for the high rate of
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To obtain further information about the rates of pyruvate dehydrogenation in db/db mice, their livers were perfused with [1-14C]lactate.

Table 5 shows that isotope incorporation into CO2 was much more extensive than into glucose in livers perfused with [1-14C]lactate. 14CO2 formation by livers of db/db mice was increased about 4.5-fold and [14C]glucose production was enhanced about 3.5-fold. These data suggest that pyruvate dehydrogenase activity is increased along with gluconeogenesis in the diabetic livers and this conclusion is supported by measurements of this enzyme reported in a separate report.

**DISCUSSION**

The data of the present study confirm the pattern of development of hyperglycemia and adiposity reported for C57BL/Ks-db/db mice by Coleman and Hummel (10, 11). As indicated by reduced body water content (Fig. 1), elevated serum free fatty acid (unpublished observations) and insulin (19) levels, and increased activities of certain hepatic gluconeogenic (Fig. 10) and lipogenic (11) enzymes (unpublished observations), the metabolism of the db/db mice is disordered as early as 4 wk of age. At this time the blood sugar is normal (Fig. 2 of ref 43), indicating that hyperglycemia is not a primary factor in the development of the syndrome.

Any study of the role of the liver in the etiology of the hyperglycemia and excessive fat deposition of the db/db mice necessitates a consideration of the abnormal relationship between the liver mass and the metabolic mass of these animals. Analysis of data in Fig. 1 indicates that, despite a doubling of body mass, the amount of body water in 8 wk old db/db mice is normal and that the extra body weight is largely, if not entirely, due to adipose tissue, which has an extremely low water content. Since fat contains much less cytoplasm and is much less metabolically active than tissues with a relatively high water content such as muscle, heart, intestine, liver, and kidney, it would appear that the body water content is a more valid and meaningful index of the metabolic mass of db/db mice than is the body weight. As illustrated in Fig. 3, the liver mass of db/db mice aged from 4 to 8 wk is proportional to the body mass, but becomes abnormally large in relation to the body water. It was concluded that the body water was the most valid basis on which to compare data from normal and db/db mice, particularly those dealing with carbohydrate metabolism. It is not likely that metabolic differences are exaggerated by this approach since body potassium contents

**Table 5. Metabolism of [1-14C]lactate in perfused livers of 8-wk-old mice**

<table>
<thead>
<tr>
<th></th>
<th>Normal Mice</th>
<th>db/db Mice</th>
</tr>
</thead>
<tbody>
<tr>
<td>14Cglucose</td>
<td>2.48 ± 0.08</td>
<td>8.70 ± 1.73</td>
</tr>
<tr>
<td>14CO2</td>
<td>20.9 ± 2.59</td>
<td>92.8 ± 6.30</td>
</tr>
<tr>
<td>14CO2:14Cglucose</td>
<td>8.45 ± 0.40</td>
<td>10.7 ± 0.51</td>
</tr>
</tbody>
</table>

Livers were perfused without recirculation with 5 mM l-[1-14C]lactate of identical specific radioactivity for 45 min. Each value is mean ± SF of four livers. * Refer to Table 4 for body water-liver conversion factors.

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gluconeogenesis from fructose. The elevated levels of pyruvate kinase, P-enolpyruvate carboxykinase, glucose-6-phosphatase, and glucokinase plus hexokinase suggest that there may be increased recycling between P-pyruvate and pyruvate and between glucose-6-P and glucose in the db/db mice. The increase in glucokinase plus hexokinase could also explain why the elevation in glucose-6-phosphatase did not lead to a crossover between glucose-6-P and glucose.

Levels of adenine nucleotides in livers of normal and db/db mice. It has been proposed that AMP and ATP may be important regulators of gluconeogenesis and glycolysis (24, 27, 34). AMP is an inhibitor of fructose bisphosphatase and a stimulator of P-fructokinase, whereas ATP is an inhibitor of P-fructokinase (see ref. 12). To determine whether changes in hepatic adenine nucleotides contributed to the accelerated gluconeogenesis of db/db mice, livers from 8-wk-old mice were freeze clamped in vivo or after perfusion for 1 h with 10 mM L-lactate. No significant differences between normal and diabetic livers as regards ATP, ADP, AM, and total adenine nucleotide levels were detected. The data thus give no support to the possibility that fructose bisphosphatase is stimulated or P-fructokinase is inhibited in the diabetic livers due to a decrease in AMP or rise in ATP.

In vivo glucose turnover. The enzyme assays and data from perfusion experiments presented thus far indicate that the livers of db/db mice have a higher gluconeogenic capacity and can produce more glucose than is appropriate for the metabolic mass of these animals. However, these findings do not necessarily mean that gluconeogenesis and hepatic glucose production are increased in db/db mice in vivo. To obtain evidence on this point, glucose turnover, which at steady state should reflect glucose production, was determined in normal and db/db mice in vivo using [3-3H]glucose. The turnover of this form of isotopic glucose followed since the tritium at carbon 3 is irreversibly lost via the Emden-Meyerhof pathway at the triose phosphate isomerase step, without further catabolism of the six-carbon skeleton.

The t1/2 values for [3-3H]glucose obtained for normal and diabetic mice were 31 ± 1 and 36 ± 1 min, respectively. The fractional turnover rates were 2.25 ± 0.09% min⁻¹ for normal mice and 1.91 ± 0.06 min⁻¹ for diabetic mice. Glucose clearance was estimated to be 201 and 341 μmol glucose per min per kg body weight for normal and db/db mice, respectively. It therefore follows that hepatic glucose production in the diabetic mice is greater than normal in agreement with the liver perfusion results.

Metabolism of specifically labeled lactate. Earlier findings (Table 4) indicated a markedly increased conversion of 14C-labeled lactate, pyruvate, and alanine to 14CO2 in the perfused livers of db/db mice, but the reasons for this were not clear. Isotope from this type of labeled substrate can appear in CO2 as a result of the oxidative decarboxylation of pyruvate, the operation of the Krebs cycle, and the conversion of oxalacetate to P-enolpyruvate in gluconeogenesis.

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* Liver perfusion was not attempted in mice younger than 3 wk.
suggest that metabolic mass differences between normal and db/db mice may even be greater than those indicated by body water content.

**Hepatic glycogen metabolism.** The present study indicates that livers from db/db mice contain more glycogen and produce more glucose by gluconeogenesis per 100 g body water than do normal mice. The increase in liver glycogen with age appears to parallel the rise in blood sugar. As shown in Fig. 4, massive glycogenolysis occurs when livers of db/db mice are perfused with low concentrations of glucose, but glycogen is retained when the perfusate glucose concentration is set at the physiological level for these animals. Thus it seems that the increase in liver glycogen may be largely the consequence of hyperglycemia. However, the elevated liver glycogen in younger db/db mice which are normoglycemic suggests that a factor(s) other than high blood glucose plays a role in increased glycogen synthesis. Since serum insulin levels have been shown to be elevated even before 4 wk of age, it is conceivable that hyperinsulinemia may be a primary cause for the higher hepatic glycogen. High insulin levels could be responsible for the elevated hepatic glycogen synthetase activity (I form and total) in the db/db mice at both 5 and 8 wk of age (Table 3). Although glycogen levels are higher than normal in the livers of db/db mice, the perfusion data and the assays of glycogen synthetase and phosphorylase suggest a high rate of turnover of glycogen in these animals. It should be noted that enhanced hepatic glycogenolysis could not contribute on a long-term basis to the increased hepatic glucose production of db/db mice.

**Hepatic gluconeogenesis.** The present data indicates that enhanced gluconeogenesis is a major factor in the hyperglycemia of db/db mice and probably contributes to the high liver glycogen. Glucose production in the perfused livers on a per gram liver basis was greater than normal in the 5 wk-old but not in the 8-wk-old db/db mice. Nevertheless, due to the abnormally high ratio of liver mass to body water in the db/db mice, glucose production was inappropriately high at all ages. The in vitro evidence of enhanced glucose production was supported by in vivo glucose turnover studies, which revealed that although the fractional glucose turnover was lower in the db/db mice, the actual mass of glucose turning over was substantially greater in these animals.

The increased gluconogenic capacity of the livers of db/db mice was clearly present at 5 wk (Fig. 5), the earliest age examined, i.e., at a time when hyperglycemia was not evident (Fig. 2). At this age and at 4 wk when the ratio of liver mass to body water is 40–50% higher than normal, the activities of pyruvate carboxylase and P-enolpyruvate carboxykinase in db/db mice are increased in parallel with the increased liver mass, whereas the activities of glucose-6-phosphatase and fructose bisphosphatase are increased to a greater extent. These changes suggest that gluconeogenesis in vivo is increased in 4- and 5-wk-old db/db mice. Since the blood glucose is not elevated, this would also mean that glucose utilization is concurrently increased.

It is evident from Fig. 10 that certain glycolytic enzymes are also increased in the db/db mice; in particular, pyruvate kinase and glucokinase plus hexokinase. Unless these increases are occurring in nonparenchymal cells or are accompanied by changes in allosteric regulators, it would seem that recycling between P-enolpyruvate and pyruvate and between glucose-6-P and glucose would be increased in the db/db mice. It is possible that the decreased levels of fructose-1,6-P_2, which is an activator of liver pyruvate kinase (36), result in diminished recycling at this level in the db/db mice. As alluded to already, there was no evidence of altered adenine nucleotide levels in the diabetic mice. Thus, changes in the control of fructose bisphosphatase, P-fructokinase, and other enzymes by these effects did not appear to be involved in the altered gluconeogenesis of these animals.

**Possible factors involved in etiology of obese-hyperglycemic syndrome in db/db mice.** This report is concerned primarily with describing the alterations in hepatic carbohydrate metabolism in db/db mice. It is clear that an important change is the rate of gluconeogenesis which is inappropriately high in relation to the metabolic mass of these animals. Altered gluconeogenesis is an early disorder in these animals which clearly precedes the hyperglycemia. Increased gluconeogenesis cannot be sustained unless there is a concurrent increase in the supply of gluconeogenic precursors. Two glucose precursors which are present in plasma in increased amounts and are probably produced at an increased rate in db/db mice are lactate and pyruvate. However, these substrates would not contribute to an increase in the total carbohydrate content of the animal since they are derived from glucose and glycogen. Amino acids derived from the diet are presumably the major substrates for gluconeogenesis in the db/db mice. Consistent with this view is the fact that the concentrations of gluconeogenic amino acids (lysine, serine, threonine, glycine, and arginine) are significantly reduced in the plasma of db/db mice (D. M. Regen and P. W. Felts, personal communication).

It is not possible to explain the disordered metabolism of the db/db mice merely on the basis of the inappropriately large size of the liver. For example, the contents per gram liver of glycogen, lipid, certain gluconeogenic, glycolytic, and lipogenic enzymes are markedly increased in the db/db mice. Furthermore, in young db/db mice, the rate of hepatic gluconeogenesis and the activities of glycogen synthetase and phosphorylase are elevated.

The increased gluconeogenesis of db/db mice is paradoxical in view of the hyperinsulinemia. In man and experimental animals, enhanced gluconeogenesis is usually associated with a fall in plasma insulin or insensitivity to the hormone. Although it might be proposed that the livers of db/db mice are resistant to insulin, this does not fit with the enhanced lipogenesis (11) and increased activities of glucokinase and pyruvate kinase that are simultaneously present and are consistent with hyperinsulinism. Presumably, insulin resistance does develop in the livers of older diabetic mice, but we have no data on this.

One possible explanation for the anomalous situation is that the secretion of glucagon is also enhanced in db/db mice. This possibility is supported by one report (25) in which it was found that glucagon release from perfused pancreas of db/db mice was much greater than normal.

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*Hyperinsulinemia could not account for the higher phosphorylase activity in the livers of the db/db mice.*
and was not suppressed by glucose. If it is proposed that enhanced secretion of both insulin and glucagon play a role in the hepatic changes in db/db mice, it is necessary to postulate that these hormones have differential effects on gluconeogenesis, lipogenesis, and on the induction of key lipogenic, gluconeogenic, and glycolytic enzymes in the liver.

Further studies are in progress to determine whether or not a pancreatic defect(s) is primarily responsible for the development of the obese-hyperglycemic syndrome in db/db mice. The possible role of hepatic insulin resistance in the progress of the syndrome is also under study. Since db/db mice cannot be distinguished from normal mice at early ages, another mutant strain in which the diabetes gene (db) is coupled with a misty coat color gene (m) will be employed in most of these studies.

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REFERENCES


40. Vinuela, E., M. Salas, and A. Sols. Preliminary communica-

