Consumption of a galactose-rich diet by nondiabetic animals affords a means to examine the role of "hyperglycemia" in the etiology of diabetic complications, because it causes elevation of blood aldohexose concentration (galactose) without causing many other metabolic abnormalities that are characteristic of diabetes (such as alterations in concentrations of insulin, glucose, fatty acids, and amino acids). Nondiabetic dogs fed a 30% galactose diet for 3 to 5 years develop a retinopathy that is morphologically indistinguishable from that seen in diabetic patients and diabetic dogs, including saccular microaneurysms, pericyte ghosts, acellular (nonperfused) capillaries, and intra-retinal microvascular abnormalities (IRMA). Thus, studies of experimentally galactosemic dogs indicate that hyperglycemia itself is capable of initiating the development of diabetic retinopathy.

Consistent with the findings in dogs, rats fed galactose have been reported to develop retinal microvascular disease. This galactose-induced retinopathy seems to develop faster in rats than in dogs, and may offer an additional animal model to investigate the role of hyperglycemia in the pathogenesis of diabetic retinopathy.

Excessive activity of aldose reductase is one sequela of hyperglycemia that is common to diabetes and galactosemia, and has been postulated to have a role in the development of diabetic complications. The role of the polyol pathway in the development of diabetic retinopathy, however, is controversial.

This controversy remains because several critical elements of the experiments (such as ensuring that hyperglycemia was comparably severe in control and inhibitor-treated groups, and documenting the extent to which polyol production and accumulation were inhibited) were neglected in some of the studies.

In the current study, we fed rats galactose-rich diets for up to 26 months to evaluate their suitability
as a model of diabetic retinopathy, and to quantitate the effects of aldose reductase inhibition on the development of such microvascular pathology, paying careful attention to documenting the comparability of glycermia between experimental groups and the biologic efficacy of the aldose reductase inhibitor therapy.

METHODS

Animals

Male Sprague-Dawley rats (200 g each) were randomly assigned to be made galactosemic or to remain as normal control subjects. Experimental galactosemia was produced by offering a diet of rat chow (Purina [Richmond, IN] 5001) enriched with 30% galactose or 50% galactose. The final concentration of protein in the galactose-enriched diets was 17% or 12%, respectively, and the normal control rats received a diet containing 18% protein (Purina 5055). All animals were caged individually, had free access to food and water, and were maintained on a 14 hours on-10 hours off light cycle with cage level light intensity at <15 foot candles. Treatment of animals conformed to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Long-term Hyperglycemia

Nonenzymatically glycated hemoglobin (GHb; an estimate of the average level of hyperglycemia during the preceding 2 to 3 months) was measured by affinity chromatography (Glyc-Affin; Pierce, Rockford, IL). The expected elevation of nonenzymatically glycated hemoglobin concentration was not detected in the galactose-fed rats using an alternate method (ion exchange chromatographic method; HbA1a), so its use was discontinued. Glycated hemoglobin, hematocrit, and blood glucose concentration were measured 2 to 3 times per year in each animal. Rats were placed in metabolism cages 5 to 7 times throughout the course of the experiment, and on each occasion, 24-hour urine volume and specific gravity were measured on each of two consecutive days. Contamination of urine by galactose-containing food prevented meaningful measurement of galactose excretion. Body weight and food consumption were measured twice per week, and average daily food intake was calculated.

Light and Electron Microscopy

Animals were killed after an overnight fast at durations of study of up to 26 months. The left eye was placed into formalin for preparation of trypsin-digested retinas,10 the superior portion of the right eye was fixed in glutaraldehyde for electron microscopy using previously described methods,11 and the inferior portion was frozen for chemical analysis. The trypsinized retinal vessels were first stained with Oil Red O for sudanophilia, and later restained with periodic acid-Schiff and hematoxylin for histologic evaluation. Retinal cells and lesions and basement membrane thickness were quantitated in a masked fashion. Sudanophilia was graded semiquantitatively (0 = none; 3 = much). Endothelial cell and pericyte counts were determined in the mid-retina on approximately 1300 capillary cells per retina, and the number of acellular capillaries was counted in multiple mid-retinal fields (one field adjacent to each of the 5 to 7 retinal arterioles radiating out from the optic disc) and expressed per square millimeter of retinal area examined. No more than 10 acellular capillaries were counted in any one field, so that focal areas of acellularity would not unduly bias the results. The maximum number of acellular capillaries by this method thus was 55 per mm². Pericyte "ghosts," indicating where pericytes had been lost, were counted only on capillaries that possessed one or more endothelial cells. Capillary basement membrane width was determined in the superior portion of the retina (mid-retina of the right eye) for representative animals using the method of Sipos-erstein.12 Fifteen capillaries (3 from each of 5 blocks) of the outer plexiform or inner nuclear layer were measured per animal. Only patent capillaries cut in cross section with a diameter of approximately 3.3 μm or less were photographed and printed at a final magnification of about 23500×. Coded photographs from animals were intermixed before measurement to ensure unbiased evaluation of the basement membrane width. Identical conclusions were reached using the minimum thickness method13 (not shown) on the same micrographs.

Aldose Reductase Inhibition

Rats assigned to the 50% galactose group were further randomly subdivided into untreated and Sorbinil-treated (0.04% w/w in the diet) groups. This dose of Sorbinil (Pfizer, Groton, CT) was selected because of its reported beneficial effects on galactose-induced retinal disease in rats.14-17 To estimate the extent of aldose reductase inhibition during the experiment, galactitol was determined two to four times in blood (100 to 500 μL whole blood) by gas chromatography/mass spectroscopy.18,19 Galactitol was determined also in retina and sciatic nerve, and myoinositol quantitated in retina, of representative animals at autopsy. Uniformly deuterated galactitol and myoinositol (donated by Drs. J. Williamson and W. Sherman, Washington University) were added to each sample as internal standards. Blood and tissue was always collected from rats after an overnight fast, so that no Sorbinil had been ingested for the preceding 12 to 14 hours. Polyol concentrations reported for Sorbinil-treated animals thus are believed to represent the minimum degree
TABLE 1. Comparison of Galactosemia Severity During the Duration of Study*  

<table>
<thead>
<tr>
<th></th>
<th>GHB (%)</th>
<th>Urine Excretion (ml/day)</th>
<th>Plasma Glucose (mg/dl)</th>
<th>Body Weight (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal (16)</td>
<td>3.2 ± 0.3</td>
<td>7 ± 2</td>
<td>87 ± 11</td>
<td>515 ± 77</td>
</tr>
<tr>
<td>30% Galactose (11)</td>
<td>5.4 ± 0.9</td>
<td>35 ± 7</td>
<td>102 ± 11</td>
<td>437 ± 38</td>
</tr>
<tr>
<td>50% Galactose (13)</td>
<td>8.5 ± 1.0</td>
<td>80 ± 14</td>
<td>85 ± 14</td>
<td>340 ± 32</td>
</tr>
<tr>
<td>50% Galactose + 0.04% Sorbinil (16)</td>
<td>8.9 ± 0.8</td>
<td>84 ± 10</td>
<td>90 ± 7</td>
<td>324 ± 38</td>
</tr>
</tbody>
</table>

* Data from animals studied 20 months or longer.

of inhibition that occurred throughout the day. Daily consumption of food and Sorbinil are reported herein for three representative periods (months 3, 13 and 19). Lens clarity was assessed after mydriasis at 20 months of galactosemia, and graded semiquantitatively (0 = no lens changes; 4 = dense cataract). Retinopathy was evaluated as described earlier using coded specimens.

Experimental groups were compared statistically using analysis of variance followed by Tukey-Kramer tests. The nonparametric Kruskal-Wallis test followed by Mann-Whitney U tests yielded similar results. Results are expressed as mean ± SD.

RESULTS

Glycated hemoglobin and daily urine excretion were consistently elevated throughout the experiment in all galactosemic groups, and galactosemia was most severe in animals consuming the 50% galactose diet (Table 1). The elevated glycated hemoglobin in galactose-fed rats was not due to an increase in blood glucose concentration, because the plasma glucose concentration remained normal in all galactosemic rats throughout the study. Galactose-fed rats gained weight at less than the normal rate, the defect being most severe in rats receiving the higher concentration of galactose. Daily food consumption (per kg body weight) tended to diminish in all groups with increasing age, but was about 25% and 85% greater than normal in animals fed the 30% and 50% galactose diets, respectively.

Galactose-induced Retinal Lesions

Retinal vascular abnormalities were found at 15 months in the rats fed the 50% galactose diet (Table 2), including a significantly greater than normal incidence of pericyte ghosts (indicating sites of pericyte loss; P < 0.001) and a trend toward increased frequency of acellular capillaries. Individual capillaries and small groups of capillaries became devoid of endothelial and pericyte nuclei, and therefore seemed acellular. Many acellular capillaries, as well as occasional capillaries containing seemingly viable pericytes, exhibited sudanophilic deposits in their vessel wall and/or lumen. The incidence of these and other lesions tended to increase with longer durations of galactosemia. The ratio of capillary endothelial cells to pericytes tended to become slightly higher than normal after 15 months of galactosemia, but this ratio is unreliable because of difficulties in differentiating all capillary cells in rats. Similar lesions were observed in animals receiving the 30% galactose diet.

Acellular (presumably nonperfused) capillaries and sudanophilic capillaries became more numerous with increasing duration of galactosemia. In 4 of 10 rats fed 50% galactose (and no drug) for longer than 22 months, the acellular capillaries were so numerous that much of the microvasculature of the posterior and mid-retina was obliterated. Animals with extensive capillary loss generally possessed some dilated, hypercellular vessels or IRMAs consistent with possible neovascularization (Fig. 1). These abnormal vessels were not distributed uniformly across the posterior pole, but were found in patches with seemingly normal vessels nearby. Saccular capillary microaneurysms such as are characteristic of diabetic humans were not observed, although some vaguely similar focal accumulations of vascular cells were seen, usually at intersections of capillaries (Fig. 2). The significance of these focal accumulations is not clear. Retinal capillary basement membrane thickened as a result of the experimental galactosemia, and was significantly greater than normal at both durations of 50% galactosemia examined (P > 0.01). No retinal folds were seen.

Mortality

No animals died until the second year of study, and then 10 of 32 normal rats, 12 of 31 rats fed 50% galactose, and 3 of 14 fed 30% galactose died spontaneously. The cause of death was not always apparent, but intestinal obstruction apparently secondary to the diet was found at autopsy in four rats fed the 50% galactose diet but in none of the animals receiving 30% galactose diet or the normal diet.

Effect of Aldose Reductase Inhibition

Rats in the Sorbinil-treated or untreated groups receiving 50% galactose were compared at a mean duration of 23 months (range, 20 to 26 months; Table 2). The
Retinal Microvascular Abnormalities in Galactose-fed Rats (mean ± SD)

<table>
<thead>
<tr>
<th>Duration (months)</th>
<th>Acellular Capillaries (per mm²)</th>
<th>Pericyte Ghosts (/1000 cells)</th>
<th>Sudanophilia (grades 1–3)</th>
<th>Basement Membrane Thickness (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>16 ± 4</td>
<td>6</td>
<td>4 ± 3</td>
<td>0.3 ± 0.4</td>
</tr>
<tr>
<td>30% Galactose</td>
<td>22 ± 1</td>
<td>16</td>
<td>2 ± 2</td>
<td>0 ± 0.2</td>
</tr>
<tr>
<td>50% Galactose</td>
<td>23 ± 1</td>
<td>11</td>
<td>16 ± 12*</td>
<td>12.5 ± 7.9*</td>
</tr>
<tr>
<td>50% Galactose + Sorbinil</td>
<td>23 ± 2</td>
<td>15</td>
<td>24 ± 12*</td>
<td>12.6 ± 6.9*</td>
</tr>
</tbody>
</table>

*P < 0.01 compared to normal.
†P < 0.01 compared to age-matched 50% galactose group.
Values in parentheses represent n.

Sorbinil-treated group was not significantly different from the untreated galactosemic group with respect to the severity of galactosemia as estimated by glycated hemoglobin and 24-hour urine excretion. Body weight in Sorbinil-treated galactosemic rats was slightly but significantly less than that in untreated galactosemic animals (P < 0.05). Consumption of food (and thus galactose) relative to body weight decreased with increasing age in the two 50% galactose groups, but remained greater than normal throughout the experiment, and did not differ between the two 50% galactose groups at any time. Average daily food intake in the Sorbinil-treated and untreated 50% galactosemic groups, respectively was: during month 3, 100 g/kg body weight/day ± 7 and 97 ± 9; during month 12, 79 ± 7 and 74 ± 7; and during month 19, 71 ± 4 and 69 ± 6. Sorbinil intake in the Sorbinil-treated animals during months 3, 12, and 19, respectively was 39 ± 4 mg/kg body weight/day, 29 ± 2, and 28 ± 3.

Dense cataract developed within 2 months in the untreated 50% galactose group. Cataractogenesis was markedly inhibited in Sorbinil-treated animals, and consisted of no more than slight vacuolization or haziness of the lens at 20 months of galactosemia (semi-quantitative scores of 0 ± 0, 4 ± 0, and 1.4 ± 0.5, respectively for normal [n = 15], untreated galactosemic [n = 13], and Sorbinil-treated galactosemic [n = 16] groups). Galactitol concentration in blood and sciatic nerve in the untreated galactosemia group was, respectively, 123-fold and 43-fold greater than normal, and both of these very large increases in tissue galactitol were inhibited more than 90% by Sorbinil treatment (Table 3). Galactitol accumulation in retinas of galactosemic rats rose to 13-fold that of normal rats. Sorbinil treatment significantly inhibited 62% of this increase (P < 0.001), although this level of inhibition did not prevent the accumulation of retinal galactitol (P < 0.001 compared to normal). Free myoinositol in the retina was not affected by long-term galactosemia (54 ± 25 nmol/g protein versus 50 ± 18 in normal and galactosemic groups, respectively), and values in

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FIGURE 1. Posterior/mid-region of trypsin-digested retinas from (left) experimentally galactosemic rat (24 months), and (right) age-matched normal rat. Dilated, hypercellular vessels are visible and numerous degenerate, acellular capillaries lacking nuclei are widespread in the galactosemic. The optic nerve head is out of the micrograph to the left. (periodic acid-Schiff and hematoxylin; original magnification ×100).
Sorbinil-treated animals (37 ± 22), although not significantly different, tended to be lower than those in normal or galactosemic animals.

Retinal lesions in Sorbinil-treated galactosemic animals were equally or more severe than those in the untreated galactosemic rats (Table 2), and retinal capillary basement membrane thickening was not inhibited in our Sorbinil-treated rats. IRMAs were observed in animals receiving the aldose reductase inhibitor (Fig. 3), and all capillaries in large areas of the posterior and mid-retina were acellular in 9 of the 11 Sorbinil-treated rats receiving 50% galactose for more than 21 months.

DISCUSSION

Experimental galactosemia has proved to be a valuable tool in the study of the pathogenesis of diabetic complications because it reproduces some of the anatomic complications of diabetes in the absence of many of the metabolic abnormalities characteristic of diabetes. The development of cataract and nerve abnormalities have been extensively probed using the galactosemic rat model. Evidence that experimental galactosemia was capable of producing a diabetes-like retinopathy first was reported by us in 1982 in dogs, and galactose-induced thickening of retinal capillary basement membranes in rats was reported the next year.

Robison et al. reported that three rats fed a diet enriched with 50% galactose for 28 months developed a retinopathy that they regarded as diabetic-like in appearance. The current study confirms that prolonged consumption of a 50% galactose diet by nondiabetic rats leads to the development of a retinal microangiopathy that resembles the early stages of diabetic retinopathy in important respects. The retinal lesions in our animals include pericyte loss, acellular capillaries, thickened capillary basement membrane and IRMAs. The IRMAs consist of hypercellular foci of vascular cells, usually adjacent to a large area of acellular (presumably nonperfused) capillaries. Retinal folds, observed by others in rats at 20 months of galactose-feeding, and retinal hemorrhages were not seen by us either grossly or in retinal cross sections. Saccular capillary microaneurysms were absent in our

<table>
<thead>
<tr>
<th>Table 3. Tissue Galactitol Concentration in Rats Fed 50% Galactose (mean ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Erythrocytes</strong> (nmol/mg Hb)</td>
</tr>
<tr>
<td>----------------</td>
</tr>
<tr>
<td>Normal</td>
</tr>
<tr>
<td>50% Galactose</td>
</tr>
<tr>
<td>50% Galactose + Sorbinil</td>
</tr>
</tbody>
</table>

*P < 0.001 for each comparison between groups in a column.*

Values in parentheses represent n.
galactose-fed rats, although focal accumulations of vascular cells that might be mistaken for aneurysms were seen. Thus, 2 years of galactosemia in rats seems to reproduce only a portion of the spectrum of lesions characteristic of diabetic retinopathy in patients or dogs, but experimentally galactosemic rats are likely to be a useful model of at least the early stages of diabetic retinopathy. Whether or not unequivocal vascular microaneurysms and more advanced lesions would eventually develop reproducibly in rats as they do in patients and dogs is not clear.

The acellular capillaries and pericyte ghosts that develop reproducibly in galactose-fed rats are among the earliest structural abnormalities demonstrable in diabetic retinopathy or galactose-induced retinopathy. Pericyte loss has long been recognized to occur in the early stages of retinopathy, but the significance of early development of individual or small groups of acellular, nonperfused capillaries has been appreciated less. A progressive increase in the number of nonperfused capillaries can lead to retinal ischemia, and is likely to be an important factor leading to neovascularization and proliferative retinopathy in diabetes. The cause of retinal capillary occlusion and acellularity is unknown.

Similar to the retinal lesions caused by 50% galactose, retinal microvascular disease also developed in rats fed 30% galactose. Daily, these animals ingested less than half the quantity of galactose consumed by rats fed the 50% galactose diet. A diet of 30% galactose is less costly, but most importantly, seems to result in healthier rats than those receiving the 50% galactose diet. The 30% galactose diet was associated with less evidence of galactose toxicity such as impaired growth and intestinal obstruction than seen with the 50% galactose diet.

In our laboratory, retinopathy induced by 50% galactose in rats was not inhibited by administration of 0.04% Sorbinil. Sorbinil-treated galactosemic rats were found to have no fewer pericyte ghosts, acellular capillaries, shunt vessels, or hypercellular foci of vascular cells than their untreated galactosemic counterparts, and thickening of capillary basement membrane was not inhibited in the Sorbinil-treated animals. Our findings are contrary to those of previous studies of galactosemic rats in which Sorbinil added to the diet at the same or lower concentration was reported to inhibit thickening of retinal capillary basement membranes.\(^{14-16}\) Moreover, retinopathy has reportedly been prevented in 3 galactosemic rats fed an aldose reductase inhibitor, tolrestat, for 28 months.\(^{1,24}\)

The reason for the disparate results about the ability of aldose reductase inhibitors to prevent retinal pathology is not clear. The dose of Sorbinil given herein is equal to or higher than that reported by others to inhibit galactose-induced thickening of retinal capillary basement membrane or alterations in retinal blood flow and permeability in rats.\(^{14-17}\) Administration of Sorbinil to our animals inhibited galactitol accumulation in erythrocytes and extracellular tissues of our galactosemic rats by more than 90%, and cataract was markedly inhibited. However, polyol pathway inhibition in the retina seemed less than in blood, sciatic nerve, and lens, and the concentration of galactitol in retinas of Sorbinil-treated rats remained greater than normal. The polyol concentration in retina and other tissues has not been measured by other workers studying the relationship of the polyol pathway to retinopathy, and the lack of such data from their reports prevents meaningful comparison with our observations. Inhibition of the polyol pathway to the extent achieved in the rats in our study clearly did not inhibit the development of galactose-induced retinopathy. Studies in diabetic dogs and galactosemic dogs (where polyol accumulation in the retina was inhibited by 100% and 96%, respectively) and in diabetic humans have revealed no effect\(^{7-9}\) or a possibly transient effect\(^6\) of available aldose reductase inhibitors on the development of retinopathy.

**Key Words**
retinopathy, galactosemia, rat, polyol pathway, aldose reductase inhibitor

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