Effect of Diabetes on the Biosynthesis of the Renal Glomerular Basement Membrane

Studies on the Glucosyltransferase

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SUMMARY

Kidney glucosyltransferase (UDP-glucose:galactosylhydroxylysine-basement membrane glucosyltransferase), which is involved in the synthesis of the hydroxylsine-linked disaccharide units of glomerular basement membrane, has been measured in the renal cortices of normal and alloxan diabetic rats. The level of this enzyme in diabetic kidneys was found to be significantly elevated over that of age-matched controls, expressed either as specific or total activity, at all times studied (one to five months). However the difference between the normal and diabetic animals increased with the duration of the disease. When short-term diabetics were treated with insulin, the level of the glucosyltransferase could be restored to normal. Insulin treatment of long-term diabetic animals, which were more difficult to control, brought the enzyme level close to normal but did not completely restore it.

The glucosyltransferase activity in several other tissues of the alloxan diabetic rat, including lung, liver, testes, spleen, and uterus, did not show any significant elevation over that of the normal.

Measurement in kidney of a glycosyltransferase (UDP-galactose:N-acetylglucosamine-glycoprotein galactosyltransferase) involved in the synthesis of a different type of carbohydrate unit did not show the marked elevation noted for the glucosyltransferase.

It is believed that the insulin-reversible glucosyltransferase elevation of the diabetic renal cortex reflects the increased basement membrane synthesis occurring in this tissue and indicates that this process is directly or indirectly under the control of this hormone. DIABETES *20*:641-48, October, 1971.

The pathology of the renal glomerulus in diabetes mellitus is characterized by a thickening of the basement membrane and the deposition of basement membrane-like material in the mesangial region.[1-7] These changes are related to the duration of the diabetes and lead to an impairment in the capacity of the glomerulus to act as a selective filtration barrier, resulting in the serious clinical consequences of diabetic nephropathy.

It is apparent that an understanding of the pathogenesis of the diabetic glomerular alterations and a basis for their rational therapy and prevention depends on knowledge of the metabolic sequence of events leading from insulin deficiency to the basement membrane abnormality. This in turn requires an understanding of the synthesis of the basement membrane, a complex process which, after the completion of the peptide chain, includes the attachment of the carbohydrate units.

Basement membranes, along with other members of the collagen family, have been shown to contain characteristic carbohydrate units made up of glucose-galactose disaccharides (2-O-α-D-glucosyl-D-galactose) linked to hydroxylysine residues in the peptide chain.[9] The finding that the basement membranes from diabetic human glomeruli differ from normal membranes in the occurrence of an increased number of these disaccharides[9] has placed added importance on the elucidation of the biosynthesis of these units and the manner in which they might affect membrane porosity.

Recent studies have indicated that the assembly of these disaccharide units is mediated by two highly specific glycosyltransferases and have suggested that the activity of these enzymes reflects the amount of basement membrane synthesis.[10-12] This report deals with one

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of these enzymes, the glucosyltransferase, which attaches glucose to hydroxylysine-linked galactose to complete the disaccharide unit of the basement membrane. The effect of diabetes on the level of this enzyme in the rat is assessed and its response to insulin treatment is described.

EXPERIMENTAL PROCEDURES

Animals. Male albino rats of the Wistar strain (Charles River Laboratories) weighing from 200 to 300 gm. were fasted for twenty-four hours and then injected with alloxan monohydrate, 38 mg. per kilogram body weight, dissolved in physiological saline (38 mg./ml.) through the femoral vein under light ether anesthesia. For each experiment, the animals to receive alloxan were selected at random from a group of uniform age, and the remainder were kept as normal controls. One group of female albino rats weighing about 180 gm. was alloxanized under the same conditions. The diabetic animals were kept from four to twenty weeks before sacrifice and were fed ad libitum during that time. The presence of diabetes was assessed on the basis of urine and blood glucose determinations, both of which were performed by the glucose oxidase procedure. In all cases the blood glucose was over 350 mg. per 100 ml. prior to the experiment, and the average of each group was close to 500 mg. per 100 ml. (table 1).

Two groups of diabetic rats were treated with insulin for a period of three weeks. Insulin treatment was begun in one group one week after alloxan administration and in the other group nine weeks following alloxanization. Insulin was administered once a day subcutaneously according to the following schedule: first day, 4 U. protamine zinc insulin (PZI) and 2 to 3 U. of crystalline insulin per 100 gm. of body weight; second, third, and fourth days, 1.5 to 2.5 U. of PZI per 100 gm. of body weight; and thereafter, 1 to 2 U. of PZI per 100 gm. of body weight. Each animal's dose was determined on the basis of daily urine glucose analyses and weight gain, with an attempt being made to keep the animal aglycosuric and with a normal growth pattern.

Tissues. The animals were sacrificed by decapitation and the tissues to be studied were rapidly removed and placed on ice. In all cases the kidneys were removed, weighed, and dissected to obtain the cortical material. Other tissues which were studied in some experiments included liver, lungs (dissected to remove large bronchi and blood vessels), spleen, testes, and uterus. The tissues were minced and then disrupted in an all-glass Potter-Elvehjem homogenizer in two volumes of 0.15 M Tris acetate, pH 6.8, containing 0.002 M 2-mercaptoethanol, except for the uterus, for which three volumes of this buffer were used. The homogenates were then centrifuged at 10,000 X g for ten minutes in a Spinco Model L ultracentrifuge and the supernatant was used for the enzyme assay. All procedures prior to the incubation were carried out at 2°. Protein was determined by the method of Lowry et al.[13] using bovine serum albumin as a standard.

Glycosyltransferase assay. For the assay of the glucosyltransferase (UDP-glucose:Gal-Hyl*-basement membrane glucosyltransferase), incubations were performed at 37° for two hours in a total volume of 0.1 ml. containing 15 μmoles of Tris acetate buffer, pH 6.8, 2.5 μmoles of manganese acetate, 0.2 μmoles of 2-mercapto-

*The abbreviations used are: Glc-Gal-Hyl, glucosylgalactosylhydroxylysine; Gal-Hyl, galactosylhydroxylysine; Glc-Gal-Hyl-peptides and Gal-Hyl-peptides refer to peptides containing hydroxylysine-linked glucosylgalactose or galactose respectively.

TABLE 1

Body weight, kidney weight, and blood glucose of alloxan diabetic rats and age-matched controls
(All values represent mean ± S.D.M.)

	Duration of diabetes					
	4 weeks		12 weeks		20 weeks	
	Normal (31)*	Diabetic (35)	Normal (14)	Diabetic (21)	Normal (14)	Diabetic (8)
Body weight, gm.	378 ± 60	279 ± 57	565 ± 67	336 ± 93	658 ± 71	321 ± 54
p =	<.001		<.001		<.001	
Kidney weight, gm.†	2.66 ± 0.41	3.67 ± 0.62	3.22 ± 0.34	4.61 ± 0.74	3.38 ± 0.35	4.58 ± 0.70
p =	<.001		<.001		<.005	
Blood glucose, mg./100 ml.	122 ± 8	511 ± 89	111 ± 8	474 ± 81	94 ± 7	500 ± 92
p =	<.001		<.001		<.001	

*Figures in parentheses indicate number of animals in each group.
† Represent total weight of both kidneys; the cortex weight represented the following percentages of the kidney weights: normal 82.7 per cent and diabetic 79.1 per cent.

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ethanol, 0.2 μmoles of UDP-glucose-C-14 (4.5 μCi per μmole) and as acceptor 0.25 μmoles of Gal-Hyl-peptides. The glycopeptides used as acceptor were prepared from bovine glomerular basement membrane by collagenase and Pronase digestion, followed by selective removal of the glucose by mild acid hydrolysis as previously described.[14,15] At the end of the incubation 0.4 ml. of 2.5 N NaOH was added to each tube, and the samples were transferred to polypropylene tubes (tightly capped) for hydrolysis at 105° for twenty hours to release the Glc-Gal-Hyl.

After hydrolysis, the samples were diluted with 8 ml. of water, titrated to pH 3 with hydrochloric acid, and then placed on small columns containing 2.5 gm. of Dowex 50-X4, 200-400 mesh (H+). The columns were washed with 45 ml. of water and the Glc-Gal-Hyl obtained by elution with 10 ml. of 1.5 N ammonium hydroxide. The ammonia was removed under reduced pressure on an Evapomix (Buchler) at 45°, and the samples were then streaked on 3.7 cm. strips of Whatman 1 paper and chromatographed in 1-butanol-acetic acid-water (4:1:5) for six days. Guide strips containing standard Glc-Gal-Hyl, hydroxylysine, and lysine were run alongside the samples and were stained with ninhydrin. The radioactivity on each strip was localized with a Nuclear-Chicago radioscanner and the activity of the Glc-Gal-Hyl quantitated with the use of an attached integrator. The efficiency of the scanner under the conditions employed was 13 per cent.

The assay was linear with respect to protein concentration for the three groups of animals studied, namely, normal, diabetic, and insulin-treated diabetic, up to approximately 0.8 mg. (figure 1) and the incubations were carried out so that the amount of protein present was in the range of 0.5 to 0.75 mg.

It was shown that no breakdown of the C-14-labeled Glc-Gal-Hyl-peptides due to glucosidase action occurred during the standard incubation when either normal or diabetic tissues were used as the source of the enzyme. When C-14-glucose-labeled Glc-Gal-Hyl-peptides of high specific activity were incubated at the pH, manganese, and mercaptoethanol concentrations employed in the standard assay of the glucosyltransferase, no release of

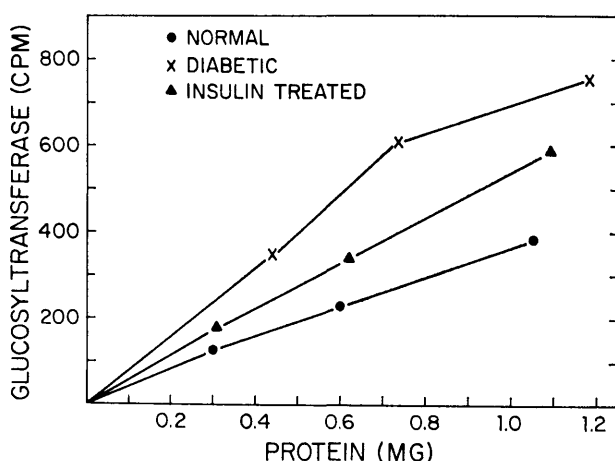

FIG. 1. Effect of protein concentration on glucosyltransferase activity of rat kidney cortex under the conditions of the standard assay procedure. Each concentration curve represents an individual animal: normal, diabetic, or insulin-treated diabetic.

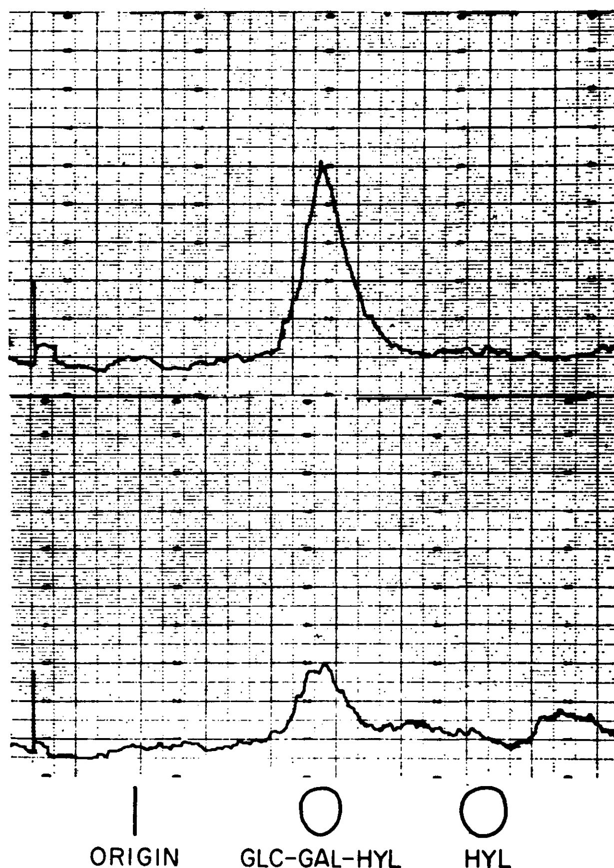

FIG. 2. Radioscans showing the formation of C-14-labeled Glc-Gal-Hyl by glucosyltransferase from diabetic kidney cortex (upper) and normal age-matched kidney cortex (lower) under the conditions of the standard assay (see text). The incubations contained 0.53 mg. of protein from the diabetic kidney and 0.66 mg. of protein from the normal kidney. Chromatography was performed in 1-butanol-acetic acid-water (4:1:5) for six days, and the position of the Glc-Gal-Hyl and hydroxylysine (Hyl) on a guide strip is shown. When incubation of either the normal or diabetic kidney enzyme preparations was carried out without the addition of Gal-Hyl-peptides as acceptor, no radioactive component was observed in the Glc-Gal-Hyl region.

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TABLE 2
Effect of diabetes on glucosyltransferase activity of rat kidney cortex

Duration of diabetes	Normal	Diabetic	Diabetic/ Normal
weeks	cpm × 10^{-4}		
4	10.0 ± 0.3 (31)*	13.2 ± 0.7 (35) p <.001	1.32
10	8.8 ± 0.5 (14)	15.0 ± 1.2 (21) p <.001	1.71
20	8.7 ± 0.5 (14)	21.4 ± 3.1 (8) p <.001	2.47

* All values given as mean ± S.D.M.; figures in parentheses indicate number of animals in each group.

C-14-labeled glucose was detected by radioactive scanning of paper chromatograms after desalting and deproteinization of the incubation mixtures.

In some experiments the activity of another glycosyltransferase (UDP-galactose:N-acetylglucosamine-glycoprotein transferase) was measured. The assay conditions for this enzyme were similar to those previously employed and involved the use of N-acetylglucosamine as an acceptor.[16] In this assay, the N-acetyllactosamine (4-o-β-D-galactosyl-N-acetyl-D-glucosamine) formed was separated by paper chromatography in 1-butanol-ethanol-water (10:1:2) and determined by radioscanning.

The results of the enzyme assays were expressed as activity per mg. of protein and also as activity per total cortex. The latter manner of expression takes into account the significant increase in the weight of the diabetic kidneys (table 1), as well as the decrease in the protein content (85 per cent of the normal) in the diabetic renal cortex.

RESULTS

Glucosyltransferase activity of diabetic kidneys. The activity of this enzyme was increased in the renal cortex of diabetic animals (figure 2). This increase over the normal age-matched controls was highly significant at all times studied (table 2), but became greater with increasing length of the disease, with the ratio of normal to diabetic changing from 1.32 at 4 wks. to 2.47 at 20 wks. This significant alteration from the normal was observed whether the activity was expressed per total cortex (figure 3) or per mg. of protein (figure 4). It is apparent that the specific activity (cpm per mg. of protein) of the normal animals decreased with age, while that of the diabetics stayed constant (figure 4). In contrast, the total activity of the diabetic increased with age, while that of the normal stayed fairly constant (figure 3).

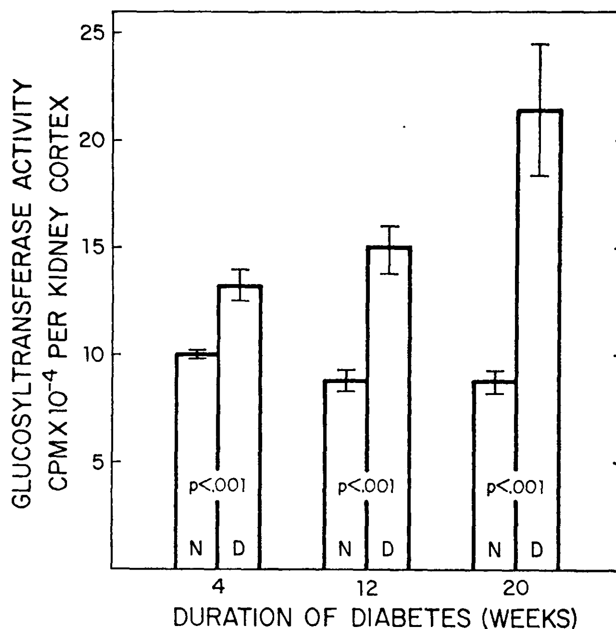

FIG. 3. Effect of diabetes on the total glucosyltransferase activity of rat kidney cortex at various times after the administration of alloxan. The diabetic animals (D) are compared to age-matched controls (N). The details in regard to the animals used in these experiments are given in table 1. The standard assay procedure was employed for the determination of the glucosyltransferase activity (see text). Mean values are plotted, and the standard error of the mean and the p values are indicated.

In order to determine how rapidly the elevation of glucosyltransferase activity becomes apparent in the diabetic rats, animals were studied at varying times shortly after alloxan administration. Although the hyperglycemia became established by twenty-four hours, the enzyme level responded more slowly. The ratio of diabetic glucosyltransferase activity per total cortex to that of the normal was 1.0 at 24 hrs., 1.06 at 48 hrs., 1.11 at 72 hrs., 1.17 at 6 days, and 1.37 at 8 days. These values represent an average at each time of two diabetic animals compared to a group of nine normal animals sacrificed over the same period of time.

Effect of insulin treatment on glucosyltransferase activity. When animals with diabetes for one week were treated with insulin for a period of three weeks, their blood glucose, kidney weight, and total body weight could be restored to normal (table 3). This treatment also achieved a return of the glucosyltransferase activity to a level indistinguishable from the normal (figure 5).

To confirm that the treated animals were still diabetic, a group of nine rats which had received insulin for a three-week period were studied after withdrawal

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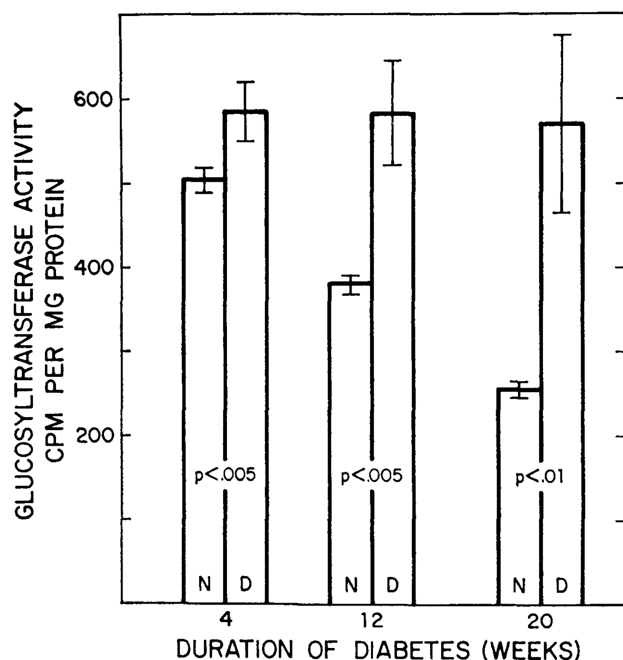

FIG. 4. Effect of diabetes on the specific activity of the glucosyltransferase of rat kidney cortex at varying times after the administration of alloxan. Diabetic animals (D) are compared to age-matched controls (N). Details in regard to the animals used in these experiments are reported in table 1. The assay procedure for the glucosyltransferase was the standard one described in the text. Mean values are plotted, and the standard error of the mean and the p values are shown.

of this hormone. After one day, all animals became glycosuric, and the average blood glucose level after two days was 479 mg. per 100 ml., and ranged from 425 to 620 mg. per 100 ml. The assay of the glucosyltransferase of the kidney cortex of these insulin-treated and withdrawn animals, however, indicated that this enzyme had not risen from the treated (normal) level after two days.

When animals with diabetes for nine weeks were treated with insulin for the three-week period, the body weight and kidney weight were restored toward normal (table 3), but manifested a wide spread of values, indicating that this period of insulin treatment had been less effective than in the case of the diabetic animals with a shorter duration of the untreated disease. This partial restoration toward normal was also reflected in the assay of the glucosyltransferase activity, the average level of which was clearly restored toward normal (figure 5), but which manifested a large spread of values leading to a p value of < 0.1.

Effect of diabetes on glucosyltransferase activity of other tissues of the rat. When the glucosyltransferase was measured in several other tissues of the diabetic rats, no significant elevation over the normal was observed (table 4).

Effect of diabetes on UDP-galactose:N-acetylglucosamine-glycoprotein galactosyltransferase. The activity of another glycosyltransferase not specifically involved in the synthesis of basement membranes but operative in the synthesis of the asparagine-linked carbohydrate units common to many glycoproteins, was studied in the kidney cortex of rats with diabetes of twelve weeks duration and their age-matched controls.

When the kidneys of nineteen diabetic and sixteen normal rats were assayed for this enzyme, it was found that in contrast to the glucosyltransferase, no increase in the specific activity (cpm per mg. of protein) was found in the kidneys of the diabetic rats, and only a small increase in the total activity per kidney cortex with a ratio of diabetic to normal of 1.35 ($p < .05$) was found.

TABLE 3

Effect of insulin administration on body weight, kidney weight and blood glucose of alloxan diabetic rats (All values represent mean ± S.D.M.)

	Short-term diabetes*			Long-term diabetes†		
	Normal (7)‡	Diabetic (10)	Treated (9)	Normal (11)	Diabetic (17)	Treated (17)
Body weight, gm.	435 ± 14	336 ± 12	430 ± 7	565 ± 22	326 ± 23	464 ± 12
p =		<.001§	<.001		<.001	<.001
Kidney weight, gm.	3.10 ± 0.12	3.95 ± 0.16	3.13 ± 0.08	3.24 ± 0.12	4.73 ± 0.61	3.39 ± 0.79
p =		<.001	<.001		<.001	<.001
Blood glucose, mg./100 ml.	126 ± 3	489 ± 31	100 ± 22	114 ± 2	492 ± 13	152 ± 78
p =		<.001	<.001		<.001	<.001

* Animals with diabetes of one-week duration prior to receiving a three-week course of insulin treatment (see text).
† Animals with diabetes of nine weeks duration prior to receiving insulin treatment for three weeks.
‡ Figures in parentheses indicate number of animals in group.
§ P values give a comparison of normal and diabetic animals and of diabetic and insulin-treated animals. All the animals in these experiments were age-matched.

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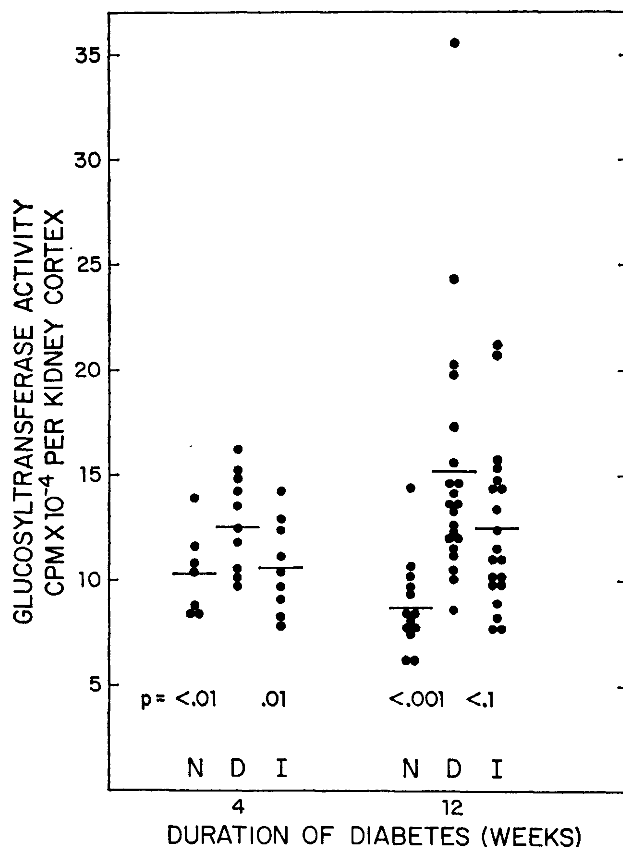

FIG. 5. Effect of insulin treatment on the glucosyltransferase activity of the kidney cortex of diabetic rats. Individual values of insulin-treated diabetic rats (I), untreated diabetic rats (D), and normal age-matched controls (N) are plotted, and the mean is indicated for each group. The p values between two adjoining groups are given. The duration of diabetes refers to the time elapsed since alloxan administration. The schedule of insulin treatment is described in the text. Glucosyltransferase activity was measured by the standard assay.

DISCUSSION

These studies demonstrate that the glucosyltransferase of kidney cortex, an important enzyme in the machinery of basement membrane assembly, is elevated in diabetes, suggesting that enhanced synthesis of this membrane occurs in this disease. This finding is consistent with the morphological and chemical evidence for the presence of increased amounts of glomerular basement membrane material in diabetic kidneys, both in the peripheral capillary loops and in the mesangial region.[2-7] Such an increase in this enzyme would also be expected from the finding that the basement membrane isolated from human diabetics manifests a distinct chemical alteration characterized by an increased number of glucosylgalactose units linked to hydroxylysine residues in the peptide chain.[9]

It is important to note that the activity of the enzymes involved in the synthesis of the hydroxylysine-linked carbohydrate units of basement membranes declines with age, and comparisons of enzyme levels in diabetic animals have to be made to age-matched normal controls. The present studies have indicated that the diabetic animals do not appear to undergo this decline in glucosyltransferase and continue to maintain a high level of this activity.

In this study the activity of the glucosyltransferase was used to assess the rate of basement membrane synthesis rather than the other enzyme involved in the assembly of the disaccharide unit, namely the galactosyltransferase responsible for attaching galactose to hydroxylysine.[10,11] Although our studies performed on the latter enzyme have suggested that it is also elevated in diabetic animals, its activity in normals falls to such

TABLE 4

Glucosyltransferase activity in several tissues of diabetic* and normal rats
(All values given as mean ± S.D.M.)

	Glucosyltransferase activity				Tissue weight	
	Per tissue		Per mg. protein			
	Normal	Diabetic	Normal	Diabetic	Normal	Diabetic
Tissue	cpm × 10^{-4}		cpm		gm.	
Liver (14N, 23D)†	104 ± 17‡	116 ± 8	417 ± 60	419 ± 23	17.2	15.7
Testes (5N, 9D)	13.4 ± 4.2	16.7 ± 1.9	873 ± 210	1014 ± 87	3.29	3.25
Spleen (7N, 9D)	1.03 ± 0.07	0.84 ± 0.05	1212 ± 91	1453 ± 142	0.95	0.78
Lung (10N, 15D)	2.77 ± 0.19	2.91 ± 0.14	1515 ± 147	1958 ± 190	2.28	2.07
Uterus (5N, 7D)§	6.06 ± 1.82	4.74 ± 1.22	2392 ± 564	2686 ± 378	0.43	0.33

* All values reported are for rats with diabetes of four weeks duration and their age-matched controls except for liver and lung which also include values from animals with twelve weeks of diabetes. No differences were observed between these two times in these tissues. All tissues except the uterus were obtained from males.

† Figures in parentheses indicate number of animals in each group. N = normal; D = diabetic.

‡ P value of all groups indicated no significant differences between normal and diabetics.

§ The kidney cortex of these diabetic female rats had a glucosyltransferase activity 1.78 times that of their normal age-matched controls.

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low levels with increasing age that the accuracy of the measurements is impaired.

Of some interest is the finding that an enzyme involved in the synthesis of the asparagine-linked carbohydrate units of glycoproteins (UDP-galactose:N-acetylglucosamine-glycoprotein galactosyltransferase) does not show the marked increase observed with the glucosyltransferase operative in the synthesis of the hydroxylysine-linked units. This would be consistent with the chemical observations recently made on the diabetic glomerular basement membrane which shows a marked increase in hydroxylysine-linked carbohydrate units but no increase in the asparagine-linked heteropolysaccharide units.[9]

The finding that the glucosyltransferase activity of a number of other tissues of the diabetic rat was not elevated, in contrast to the kidney, is consistent with the pronounced involvement, both morphologically and clinically, of kidney basement membranes in diabetes.

This study addresses itself to a central clinical problem of diabetes, namely, whether the basement membrane alterations of diabetic microangiopathy are the ultimate result of a sequence of events originating in insulin deficiency and can consequently be prevented or minimized by careful administration of this hormone. It was shown that the glucosyltransferase, which can be considered an indicator of basement membrane synthesis, can be returned to normal by insulin administration to diabetic animals. It appears that institution of therapy early after the onset of diabetes results in a greater success in establishing normal enzyme levels than treatment instituted after a more prolonged exposure of the animal to the diabetic state. It was found that the effectiveness of insulin in restoring glucosyltransferase activity to normal reflected the over-all efficacy of the hormone therapy, as indicated by less subtle parameters, such as blood glucose levels, as well as body and kidney weights. This is consistent with the clinical observation that the incidence of microangiopathy can be reduced in very carefully controlled human diabetics.[17] It would appear that insulin administration must be conducted in a meticulous manner in order to restore to normal the machinery of basement membrane synthesis and thereby to prevent the small blood vessel alterations.

The response to insulin was not immediate and the regimen adopted in this study was a three-week period of treatment. Conversely, after the onset of insulin deficiency, either through cessation of hormone treatment of the diabetic animals, or the destruction of the β-cells by alloxan administration, the early blood glucose elevation was not accompanied by a measurable elevation of the enzyme.

The fact that the glucosyltransferase level responded to insulin treatment indicates that the elevation observed in this enzyme was due to a deficiency of the hormone rather than to a direct effect of alloxan on the kidney.

The effect of insulin deficiency on basement membrane metabolism in the kidney of alloxan diabetic rats is consistent with the finding that basement membrane lesions similar to those found in idiopathic diabetes occur in the glomeruli of patients with secondary diabetes due to hemochromatosis or chronic pancreatitis.[18-20] The glomerular basement membrane lesions have also now been observed in a variety of experimental diabetic animals, including alloxan-treated rats,[6,21-23] dogs,[24,25] and monkeys.[26] Although these lesions differ somewhat in their appearance from those observed in human diabetics, this is not unexpected, as there may be species variation in the peripheral and mesangial contributions.

These findings, however, are not consistent with the concept that the basement membrane pathology of diabetes is unrelated to insulin deficiency and may actually be the causative factor of the hormonal disturbance.[27] Moreover such a theory does not take into account the properties of basement membranes, which are coarse filters and should be freely permeable to molecules the size of glucose and its metabolites or to insulin itself.[28,29] The defect in diabetes, certainly in the kidney, appears to increase the porosity of this membrane.

While a growing body of clinical, pathological, and biochemical evidence indicates that the basement membrane alterations of diabetes are the sequelae of insulin deficiency, the mechanism by which this is mediated is not clear. While it is possible that insulin could play a direct role on basement membrane synthesis, it seems more likely that secondary phenomena are responsible. It is possible that the elevated levels of glucose or one of its metabolites could stimulate basement membrane synthesis by influencing one or more of the postribosomal enzymatic steps, such as hydroxylation or glycosylation.[8,30] In this regard it is of interest that in the kidneys of uncontrolled diabetics, either human or experimental animal, glycogen, another glucose-containing polymer, is deposited in increased amounts in the tubular cells.[5,31]

The possibility should also be considered that the kidney may respond to some cellular injury imposed by the abnormal environment resulting from the hyperglycemia, glycosuria and polyuria of diabetes through the increased production of its major form of collagen, basement membrane.

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