Short Communications

Decreased Activities of Enzymes Associated with Sialic Acid Synthesis in Livers of Diabetic Rats

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(Received 12 July 1971)

Diabetes is a disease associated with profound changes in fat and carbohydrate metabolism, changes influenced mainly by specific enzyme alterations in these pathways (Renold & Cahill, 1966; Lardy, 1966; Randle, Garland, Hales & Newsholme, 1969). A satisfactory explanation, however, for other afflictions promoted by this disease, such as retinopathy and glomerulosclerosis that result from defective capillary basement-membrane synthesis or degradation, is less apparent. Undoubtedly, altered enzyme activities participate also in the pathogenesis of these defects, since diabetic subjects are found often to have elevated glycoprotein concentrations in their sera and capillary basement membranes (Jacobs, 1949; Berkman, Rifkin & Ross, 1953; Törnblom & Nordström, 1954; Andreani & Gray, 1956). Evidence in partial support of this thesis was provided by the observation by Spiro & Spiro (1968) that the activities of hydroxylysine glycosyl-transferases are elevated in the renal cortex of diabetic animals, a finding that agrees well with the significantly elevated hydroxylysine disaccharide content reported for human diabetic glomerular basement membranes (Beisswenger & Spiro, 1970).

In the present paper we show that a group of enzymes involved in the biosynthesis of sialic acid, an important constituent of many glycoproteins and gangliosides, is diminished and may in some as yet unexplained manner contribute to some of the pathology associated with diabetes.

Materials. *N*-Acetyl-D-[1-¹⁴C]neuraminic acid 9-phosphate was prepared enzymically from *N*-acetyl-D-[1-¹⁴C]mannosamine 6-phosphate by modifying the purification procedure of Watson, Jourdan & Roseman (1966) to include a cellulose phosphate elution step after their described DEAE-cellulose column. The final specific activity of the enzyme was 8.9 units/mg of protein. *N*-Acetyl-D-[1-¹⁴C]mannosamine and *N*-acetyl-D-[1-¹⁴C]glucosamine were prepared by the *N*-acetylation of the free hexosamines with [1-¹⁴C]acetic anhydride

(Amersham/Searle, Des Plaines, Ill., U.S.A.). *N*-Acetyl-D-[1-¹⁴C]glucosamine 1-phosphate was synthesized similarly from D-glucosamine 1-phosphate (Maley, Maley & Lardy, 1956), and UDP-*N*-acetyl-D-[1-¹⁴C]glucosamine was synthesized by the procedure of Roseman, Distler, Moffatt & Khorana (1961).

Methods. (a) Assay of sialic acid 9-phosphatase activity. The assay mixture contained the following components in 125 μ l: 0.48 mM-*N*-acetyl-D-[1-¹⁴C]neuraminic acid 9-phosphate (Watson *et al.* 1966) (2.20×10^6 c.p.m./ μ mol); 100 mM-potassium phosphate buffer, pH 7.1; 50 μ l of a supernatant fraction from a 30% (w/v) homogenate in iso-osmotic potassium chloride solution centrifuged at 144000 *g* for 1 h. The extent to which subcellular organelle membrane was disrupted by this procedure is not known. When imidazole was substituted for the phosphate, the supernatant fraction was diluted eight- to ten-fold. Separate reaction tubes were incubated for 10 min and 20 min, and the reactions were stopped by heating at 100°C for 2 min. The contents of each tube were washed on to Dowex 1 (formate form) columns (1 cm $\times$ 4 cm), which were eluted successively with 10 ml of water and 20 ml of 1 M-formic acid. The latter eluate contained the sialic acid. Portions (0.5 ml) were each mixed with 10 ml of a modified Bray's (1960) solution and measured for radioactivity in a Nuclear-Chicago 701 series scintillation counter. The average of the two time-intervals was taken in each case, although the reactions were essentially linear. Chromatography of the freeze-dried formic acid eluates in ethanol-ammonium acetate (5:2, v/v) medium, pH 7.5, and *n*-butyl acetate-acetic acid-water (3:2:1, by vol.) verified the presence of *N*-acetylneuraminic acid.

(b) Assay of UDP-*N*-acetyl-D-glucosamine 2'-epimerase activity. The following components were incubated at 37°C for 10 min and 20 min in a final volume of 0.25 ml: 40 mM-tris-HCl buffer, pH 8.0; 1.28 mM-UDP-*N*-acetyl-D-[1-¹⁴C]glucosamine (1.13×10^6 c.p.m./ μ mol); 50 μ l of the rat liver supernatant fraction described above. The reactions were stopped by heating, and the contents

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of each tube were passed through Dowex 1 (formate form) columns (1cm×4cm) and washed with water until 15ml was collected. Portions (0.5ml) were taken for measurement of radioactivity. The only radioactive component that passed through the column migrated with carrier *N*-acetyl-D-mannosamine on electrophoresis in 1% (w/v) sodium tetraborate decahydrate (Maley & Maley, 1959).

(c) Assay of *N*-acetyl-D-glucosamine kinase and *N*-acetyl-D-mannosamine kinase activities. The incubation mixtures contained the following components in a volume of 0.25 ml at 37°C: 1.68 mM-*N*-acetyl-D-[1-¹⁴C]mannosamine (8.13×10^5 c.p.m./μmol) or 2.64 mM-*N*-acetyl-D-[1-¹⁴C]glucosamine (5.23×10^5 c.p.m./μmol); 10.2 mM-ATP; 1.2 mM-magnesium chloride; 24 mM-sodium fluoride; 120 mM-tris-HCl buffer, pH 8.0; 50 μl of a rat liver supernatant fraction prepared as above. Portions (10 μl) were removed at 0 min, 10 min and 20 min and applied to a 20.3 cm×25.4 cm sheet of DE-81 anion-exchange paper (H. Reeve Angel and Co. Inc., Clifton, N.J., U.S.A.). The sheet was placed in a chromatographic chamber and subjected to descending elution with 5 mM-formic acid until the solvent had migrated three-quarters of the length of the paper. The DE-81 anion-exchange paper sheets were dried and strips (1 cm above, 2 cm below and 1 cm on each side of the applied sample) were cut from the origin and placed in scintillation vials with 10 ml of Liquifluor (New England Nuclear Corp., Boston, Mass., U.S.A.). The reactions were essentially linear after correction for the zero-time value. The quench of the paper amounted to 25.8% of the applied radioactivity. *N*-Acyl-D-glucosamine 2'-epimerase was not a problem, as its activity is low in rat liver (Kundig, Ghosh & Roseman, 1966).

(d) Determination of protein. This was carried out by the method of Lowry, Rosebrough, Farr & Randall (1951), with bovine serum albumin as standard.

(e) Treatment of animals. Female rats (150–200 g, a Wistar germ-free albino strain) were made

diabetic by the injection of streptozotocin (a generous gift from the Upjohn Co., Kalamazoo, Mich., U.S.A.) into the tail vein (5 mg/100 g body wt.). This compound was preferred to alloxan because of its relatively low toxicity (Rakieten, Rakieten & Nadkarni, 1963). The rats were considered diabetic when their blood glucose concentration was greater than 400 mg/100 ml. Appearance, urine glucose and increased water consumption were found to be valid symptoms also. The rats were permitted to adapt to the diabetic state for at least 2 weeks before being used.

Results and discussion. The apparent defect in sialic acid synthesis was discovered during studies on the utilization of D-[1-¹⁴C]glucosamine by perfused livers from normal and diabetic rats. Gradient-elution chromatography of the acid-soluble extracts from these livers on Dowex 1 (formate form) (McGarrahan & Maley, 1962; DelGiacco & Maley, 1964) revealed definitive differences in the quantities of the radioactive compounds eluted. The most obvious of these was a 50% decrease in the sialic acid pool and a 30–50% increase in the hexosamine phosphate and UDP-*N*-acetyl-D-glucosamine pools of the livers from the diabetic animals. These results suggested that one or more of the following reactions in the sialic acid pathway (Roseman, 1962; Warren & Felsenfeld, 1962; Watson *et al.* 1966) were altered in the diabetic rat: UDP-*N*-acetyl-D-glucosamine $\xrightarrow{1}$ *N*-acetyl-D-mannosamine $\xrightarrow{2}$ *N*-acetyl-D-mannosamine 6-phosphate $\xrightarrow{3}$ sialic acid 9-phosphate $\xrightarrow{4}$ sialic acid $\xrightarrow{5}$ CMP-sialic acid. The activities of enzymes 1, 2 and 4 were compared with that of *N*-acetyl-D-glucosamine kinase, since this last enzyme, although related, is not directly involved in this pathway. Sialic acid 9-phosphatase was measured separately in the presence of imidazole buffer and phosphate buffer to provide an estimate of enzyme activity under optimum and sub-optimum conditions.

The first indication of a difference in enzyme activity between the livers of normal and diabetic rats was obtained with sialic acid 9-phosphatase.

Table 1. *Activity of enzymes in livers of normal and diabetic rats*

Experimental conditions are described in the text under 'Methods'. Results are given as means±S.E.M., with numbers of rats in parentheses. N.S., Not significant.

Enzyme	Sp. activity (nmol/10 min per mg of protein)		P
	Normal	Diabetic	
Sialic acid 9-phosphatase (in phosphate buffer)	2.98±0.20 (7)	1.46±0.10 (7)	<0.05
Sialic acid 9-phosphatase (in imidazole buffer)	45.3±3.3 (5)	37.0±2.5 (5)	N.S.
UDP- <i>N</i> -acetyl-D-glucosamine 2'-epimerase	18.5±0.6 (20)	11.4±0.5 (20)	<0.001
<i>N</i> -Acetyl-D-mannosamine kinase	23.0±0.8 (13)	14.2±1.6 (12)	<0.01
<i>N</i> -Acetyl-D-glucosamine kinase	41.7±2.3 (13)	39.8±2.7 (12)	N.S.

As shown in Table 1, a much greater percentage decrease in sialic acid 9-phosphatase activity was observed in the presence of phosphate than in imidazole and, although a small difference was obtained in the latter case, it was not statistically significant ($P < 0.5$). Why the sialic acid 9-phosphatase in the diabetic rat is more susceptible to inhibition by phosphate than by imidazole is not known, but the possibility that the enzyme is structurally different in the two cases cannot be ruled out. Multiple forms of the enzyme activity, as with glucokinase and hexokinase, with different susceptibilities to inhibitors, dietary conditions and hormones, cannot be excluded at present either (Viñuela, Salas & Sols, 1963; Sharma, Manjeshwar & Weinhouse, 1963). Pertinent to the studies with sialic acid 9-phosphatase was the finding that the non-specific phosphatase activity of the normal liver was no different from that of the liver of diabetic rats, as measured by hydrolysis of *p*-nitrophenyl phosphate at pH 7.1.

Comparison of UDP-*N*-acetyl-D-glucosamine 2'-epimerase and the *N*-acetyl-D-mannosamine kinase activities in livers of normal and diabetic rats revealed significant differences in the respective enzyme activities. The selective nature of the decrease in the sialic acid enzymes is emphasized by the finding that no significant difference between the livers of normal and diabetic rats was observed for *N*-acetyl-D-glucosamine kinase activity. The diminished activity of the sialic acid enzymes, particularly UDP-*N*-acetyl-D-glucosamine 2'-epimerase, could in part explain the elevated UDP-*N*-acetyl-D-glucosamine content and the decreased sialic acid content found in livers of diabetic animals. Low-molecular-weight inhibitors do not appear to be involved, as neither dialysis nor passage through Sephadex G-25 improved the enzyme activities.

Although the differences in enzyme activities are not large, most of the pathology in diabetes results from the affliction with this disease over long periods of time. Cumulative effects of small differences thus cannot be ignored.

This investigation was supported in part by Research Grant CA-06406 from the National Cancer Institute, U.S. Public Health Service.

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