Regulation of Ascorbic Acid and of Xylulose Synthesis in Rat-Liver Extracts

THE EFFECT OF ALLOXAN-DIABETES ON THE GLUCURONIC ACID PATHWAY

BY F. STIRPE* AND M. COMPORTI

Istituto di Patologia generale dell'Università di Siena, Siena, Italy

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1. The synthesis of L-ascorbic acid and of L-xylulose from D-glucuronolactone, D-glucuronate, L-gulonate and L-gulonolactone has been studied with liver extracts from normal and alloxan-diabetic rats. 2. In diabetic animals the synthesis of ascorbic acid is impaired, and more from glucuronolactone and glucuronate than from gulonate and gulonolactone, whereas the formation of xylulose from gulonate and gulonolactone is enhanced. These changes are reversed by insulin therapy. 3. The activity of the NAD-linked gulonate dehydrogenase is enhanced during diabetes.

The synthesis of ascorbic acid from various precursors is impaired, and the formation of xylulose from gulonate is enhanced in liver extracts from rats subject to starvation or to acute deprivation of dietary carbohydrates (Stirpe & Comporti, 1963a, 1965). This increased formation of xylulose led to the hypothesis of an enhanced utilization of the glucuronic acid pathway of glucose metabolism during the experimental conditions examined.

The experiments described in the present paper were undertaken to study the effect of alloxan-induced diabetes, a condition of impaired utilization of glucose that has several metabolic effects in common with starvation. The results of these experiments indicated that alloxan-diabetes induced changes in the glucuronic acid pathway that are similar to those caused by starvation, and that are reversed by insulin therapy.

A preliminary communication of some of these results has been given (Stirpe & Comporti, 1963b).

EXPERIMENTAL

Chemicals. Alloxan monohydrate was obtained from Fluka A.-G., Buchs, Switzerland. Zinc-protamine-insulin was obtained from Eli Lilly and Co., Indianapolis, Ind., U.S.A. All other chemicals were obtained from the sources indicated by Stirpe & Comporti (1965).

Animals. Male rats of the Wistar-Glaxo strain weighing 200–250 g. maintained on the diet of Randoin & Causeret (1947) slightly modified were used. For the induction of diabetes the rats were deprived of food for a 24-hr. period, during which they received 20 ml. of a 20% (w/v) solution of

glucose, given in three portions by stomach tube 24, 12 and 2 hr. before the injection of alloxan. Alloxan was given by intravenous injection into a tail vein at the dosage of 35 mg./kg. body wt. (Steiner & Williams, 1959) as a 1.4% (w/v) solution in 0.9% NaCl. Immediately after the alloxan, the rats received an intraperitoneal injection of 5 ml. of 0.9% (w/v) NaCl (Bornstein & Park, 1953). With this procedure almost all rats became diabetic, and the mortality was very low. Rats were used for the experiments not less than 15 days after the injection of alloxan; only animals with marked polyuria, glycosuria and a non-starvation glycaemia of not less than 250 mg./100 ml. were used.

Insulin-treated rats received a subcutaneous injection of zinc-protamine-insulin every 12 hr. A first group of rats received 2.5 units of insulin every 12 hr.; in a second group the dosage of insulin was regulated individually every time, and the minimum quantity sufficient to prevent glycosuria was given. The insulin treatment was continued for at least 1 week before the animals were used, to avoid the interference of the temporary liver steatosis that occurs in alloxan-diabetic rats in the early days of insulin treatment (Osborn, Felts & Chaikoff, 1953). All rats were killed 12 hr. after the last injection of insulin.

Biochemical determinations. The preparations of KCl and sucrose liver extracts and subfractions, as well as all other procedures for measuring the synthesis of ascorbic acid and of xylulose and the enzyme assays, were performed as described by Stirpe & Comporti (1965). Blood glucose was determined by the glucose-oxidase method with the kits supplied by C. F. Boehringer und Soehne G.m.b.H. (Mannheim, W. Germany) or by the Istituto Sieroterapico e Vaccinogeno Toscano 'Selavo' (Siena).

RESULTS

The experiments were planned according to the general scheme followed in the study of the effects of starvation (Stirpe & Comporti, 1965).

* Present address: Istituto di Patologia generale dell'Università di Bologna, Bologna, Italy.

Synthesis and destruction of ascorbic acid and synthesis of xylulose. According to Kanda (1960) the destruction of ascorbic acid was decreased and the synthesis from D-glucuronolactone and from L-gulonolactone was unchanged in liver preparations from alloxan-diabetic rats. We reported (Stirpe & Comporti, 1965) that in the system used for the synthesis of ascorbic acid the destruction of added ascorbic acid by liver preparations from normal and starved rats was almost nil. The destruction of ascorbic acid by liver extracts from alloxan-diabetic rats was studied with the same experimental system, and the added ascorbic acid was quantitatively recovered after an incubation period of 2 hr.

The synthesis of ascorbic acid was depressed in diabetic rats, and more from the uronic than from the aldonic precursors (Table 1). The formation of xylulose from gulonate and from gulonolactone was markedly enhanced during diabetes, whereas no changes were observed with glucuronolactone or glucuronate as substrate. The administration of insulin to a first group of rats (I in Table 1) decreased the formation of xylulose to almost normal values, whereas the amount of ascorbic acid formed, although enhanced as compared with untreated diabetics, was still below normal values. Since this

could be attributed to the very low blood glucose concentration of these animals, the dosage of insulin was regulated individually in a second group of rats (II in Table 1) to maintain glycaemia at about the normal level. In these rats the formation of ascorbic acid from all substrates and of xylulose from gulonate and gulonolactone was almost normal. A high formation of xylulose from glucuronolactone and glucuronate was observed in these animals.

Gulonolactone oxidase. The activity of this enzyme was not significantly decreased in diabetic rats (Table 2). However, in these animals the synthesis of ascorbic acid from gulonolactone in the presence of the whole extract was decreased much less than in the other experiments.

NADP-linked hexonate dehydrogenase and NAD-linked gulonate dehydrogenase. The activity of these enzymes was measured in the supernatant preparations from perfused liver (Stirpe & Comporti, 1965). The activity of hexonate dehydrogenase was unchanged, whereas that of gulonate dehydrogenase was about 20% higher in the preparations from diabetic rats (Table 3).

Fractionation experiments. These experiments were performed to ascertain whether the cause of the alterations observed in the synthesis of ascorbic

Table 1. *Synthesis of ascorbic acid and of xylulose from various substrates in normal, alloxan-diabetic and diabetic insulin-treated rats*

Incubation mixtures contained in a final volume of 2.5 ml.: 50 μ moles of potassium phosphate buffer, pH 7.4, 4 μ moles of NAD ⁺ , 4 μ moles of ATP, 75 μ moles of nicotinamide, 5 μ moles of MgCl ₂ , 20 μ moles of substrate and 0.7 ml. of KCl extract. Incubation was at 37°, with air as gas phase, with shaking. The incubation period optimum for ascorbic acid formation from each substrate was chosen. The results given are mean values $\pm$ s.e.m. The numbers of experiments are indicated in parentheses.								
Substrate ... Treatment				Mean concn. of blood glucose (mg./100 ml.)	Ascorbic acid formed			
					$(\mu\text{mole/mg. of N/2hr.})$		$(\mu\text{mole/mg. of N/hr.})$	
					Glucuronolactone	Glucuronate	Gulonate	Gulonolactone
Control (9)				90*	0.172 $\pm$ 0.022	0.170 $\pm$ 0.024	0.172 $\pm$ 0.009	0.202 $\pm$ 0.014
Diabetic (10)				387	0.044 $\pm$ 0.008†	0.048 $\pm$ 0.012†	0.121 $\pm$ 0.008†	0.167 $\pm$ 0.007‡
Diabetic + insulin	{	I (4)		21	0.082 $\pm$ 0.020	0.106 $\pm$ 0.033	0.127 $\pm$ 0.018	0.136 $\pm$ 0.028
		II (4)		70	0.129 $\pm$ 0.025	0.165 $\pm$ 0.019	0.148 $\pm$ 0.016	0.181 $\pm$ 0.022
Xylulose formed								
Substrate ...					$(\mu\text{moles/mg. of N/2hr.})$		$(\mu\text{moles/mg. of N/hr.})$	
					Glucuronolactone	Glucuronate	Gulonate	Gulonolactone
Control (9)					1.89 $\pm$ 0.14	1.86 $\pm$ 0.15	0.92 $\pm$ 0.06	0.90 $\pm$ 0.06
Diabetic (10)					1.93 $\pm$ 0.10	1.59 $\pm$ 0.19	1.84 $\pm$ 0.21§	1.60 $\pm$ 0.12†
Diabetic + insulin	{	I (4)			1.86 $\pm$ 0.14	1.77 $\pm$ 0.18	0.87 $\pm$ 0.05	1.17 $\pm$ 0.15
		II (4)			3.07 $\pm$ 0.30	2.18 $\pm$ 0.12	1.29 $\pm$ 0.18	1.23 $\pm$ 0.11

* Determined in four cases only.

† Significantly different from control ($P < 0.001$).

‡ Significantly different from control ($P < 0.02$).

§ Significantly different from control ($P < 0.01$).

Table 2. *Formation of ascorbic acid by microsomal fraction (gulonolactone oxidase) and by sucrose extracts in normal and alloxan-diabetic rats*

Experimental conditions were as given in Table 1, with 20 μ moles of gulonolactone as substrate and 1 ml. of microsomal fraction or of sucrose extract. The average glycaemia of diabetic rats was 385 mg./100 ml. The results given are mean values $\pm$ S.E.M. of five experiments in each group.

Treatment	Ascorbic acid formed (μ mole/mg. of N/hr.)	
	Microsomal fraction	Sucrose extract
Control	0.872 $\pm$ 0.042	0.186 $\pm$ 0.008
Diabetic	0.789 $\pm$ 0.156	0.152 $\pm$ 0.016

Table 3. *NADP-linked hexonate-dehydrogenase and NAD-linked gulonate-dehydrogenase activities in liver supernatant preparations from normal and alloxan-diabetic rats*

Reaction mixtures contained: for the hexonate-dehydrogenase assay: 150 μ moles of tris-HCl buffer, pH 7.6, 30 μ moles of KCl, 30 μ moles of nicotinamide, 9 μ moles of potassium glucuronate, 0.3 μ mole of NADPH and 0.2 ml. of sucrose supernatant from perfused rat liver, in a final volume of 3.0 ml.; for the gulonate-dehydrogenase assay: 50 μ moles of tris-HCl buffer, pH 8.5, 5 μ moles of cysteine, 50 μ moles of potassium gulonate, 1.25 μ moles of NAD⁺ and 0.2 ml. of sucrose supernatant as above, in a total volume of 2.5 ml. Reactions were performed at 37°. The results given are mean values $\pm$ S.E.M. of five experiments for each group.

Treatment	Hexonate-dehydro- genase activity (μ mole of NADPH oxidized/mg. of N/10 min.)	Gulonate-dehydro- genase activity (μ moles of NAD ⁺ reduced/mg. of N/10 min.)
Normal	0.626 $\pm$ 0.023	0.923 $\pm$ 0.047
Alloxan-diabetic	0.610 $\pm$ 0.027	1.110 $\pm$ 0.018*

* Significantly different from normal ($P < 0.05$).

acid and of xylulose was in the sedimentable or in the supernatant fraction of the liver extracts. It appeared (Table 4) that mixtures of 'diabetic' sediment and of normal supernatant gave results very similar to those observed with 'diabetic' extracts; however, the synthesis of ascorbic acid and of xylulose were considerably altered also in mixtures of normal sediment and of 'diabetic' supernatant. A complication in these experiments was that the fractionation and subsequent recombination of liver extracts themselves caused an enhancement of xylulose formation; this enhancement was more evident in 'diabetic' than in normal extracts. The synthesis of ascorbic acid was only slightly decreased

after fractionation and recombination. This alteration in the synthesis of xylulose should be attributed to the fractionation procedure and not to the 'aging' of the preparations, because the unfractionated portion was kept in ice while the other one was fractionated, and incubations were started at the same time.

Experiments with mixed extracts. The quantity of ascorbic acid formed by 50% mixtures of extracts from normal and diabetic rats was almost exactly the average between the values obtained with the same extracts incubated separately (Table 5). The quantity of xylulose formed by the same mixtures was very close to that formed by normal extracts.

DISCUSSION

The alterations caused by alloxan-induced diabetes in the reactions examined are very similar to those observed previously in starved rats. The experiments with recombined fractions indicated that the cause(s) of the changes in diabetic animals are predominantly, although not entirely, in the sedimentable fraction. A complete similarity with starvation exists in the experiments with mixed extracts from normal and diabetic animals, in which the same amount of xylulose is formed as in normal extracts. This reinforces the hypothesis (Stirpe & Comporti, 1965) of the occurrence in normal liver of a 'factor' inhibiting xylulose formation. A difference between starved and diabetic animals is that in the latter the quantity of xylulose formed from glucuronolactone or glucuronate is not decreased. This makes it more reasonable to suppose that an increased utilization of the glucuronic acid pathway occurs in diabetic liver. This hypothesis is in agreement with the observation of a higher concentration of glucuronic acid in the blood of a high percentage of diabetic patients (Saltzman, Caraway & Beck, 1954) and with the evidence given by Winegrad & Shaw (1964) for an increased utilization of glucose through the glucuronic acid pathway in the adipose tissue of starved and alloxan-diabetic rats. The occurrence of these changes in different tissues such as fat and liver suggests that the glucuronic acid pathway may be used as an emergency route in conditions of altered glucose metabolism, although this shift is difficult to interpret since the physiological role of the glucuronic acid pathway is still not completely known. The glucuronic acid pathway leads to the formation of xylose (Touster, 1963), which has antiketogenic action (Exton & Edson, 1964), and, consequently, an increased utilization of glucose through this metabolic channel would be useful under conditions of enhanced production of ketone bodies, e.g. during starvation and diabetes. The ultimate result of an increased metabolism of glucose via the glucuronic

Table 4. *Formation of ascorbic acid and of xylulose by potassium chloride extracts and recombined subfractions from normal and alloxan-diabetic rats*

Experimental conditions were as given in Table 1, with 20 μ moles of potassium gulonate as substrate and 0.7 ml. of KCl extract or subfraction. The results given are the mean values $\pm$ S.E.M. of four experiments for each group. The average glycaemia of diabetic rats was 438 mg./100 ml.

Sediment	Supernatant	Ascorbic acid formed		Xylulose formed	
		(μ mole/200 mg. wet wt. of liver/hr.)	(μ mole/mg. of N/hr.)	(μ moles/200 mg. wet wt. of liver/hr.)	(μ moles/mg. of N/hr.)
Normal (Unfractionated)	Normal	0.622 $\pm$ 0.007	0.156 $\pm$ 0.002	6.07 $\pm$ 0.78	1.52 $\pm$ 0.20
Normal (Recombined)	Normal	0.564 $\pm$ 0.010	0.137 $\pm$ 0.006	8.01 $\pm$ 0.91	1.93 $\pm$ 0.18
Diabetic (Unfractionated)	Diabetic	0.368 $\pm$ 0.080	0.080 $\pm$ 0.014	12.48 $\pm$ 2.54	2.80 $\pm$ 0.64
Diabetic (Recombined)	Diabetic	0.316 $\pm$ 0.053	0.070 $\pm$ 0.012	18.31 $\pm$ 1.21	4.02 $\pm$ 0.20
Normal	Diabetic	0.446 $\pm$ 0.042		11.13 $\pm$ 1.50	
Diabetic	Normal	0.390 $\pm$ 0.052		13.53 $\pm$ 1.19	

Table 5. *Formation of ascorbic acid and of xylulose by mixtures of potassium chloride extracts from normal and alloxan-diabetic rats*

Experimental conditions were as given in Table 1, with 20 μ moles of potassium gulonate as substrate. The results given are mean values $\pm$ S.E.M. of six experiments.

Treatment	Concn. of blood glucose (mg./100 ml.)	Ascorbic acid formed		Xylulose formed	
		(μ mole/200 mg. wet wt. of liver/hr.)	(μ mole/mg. of N/hr.)	(μ moles/200 mg. wet wt. of liver/hr.)	(μ moles/mg. of N/hr.)
Control	75*	0.775 $\pm$ 0.050	0.195 $\pm$ 0.014	3.75 $\pm$ 0.59	0.94 $\pm$ 0.16
Diabetic	410	0.386 $\pm$ 0.060	0.093 $\pm$ 0.014	7.46 $\pm$ 1.05	1.82 $\pm$ 0.38
50% Control + 50% diabetic		0.599 $\pm$ 0.052	0.148 $\pm$ 0.013	4.30 $\pm$ 0.54	1.06 $\pm$ 0.13

* Determined in two cases only.

acid pathway would be enhanced utilization of the pentose phosphate pathway. According to Ashmore, Cahill & Hastings (1960) the pentose phosphate pathway accounts for the utilization of 2% of the phosphorylated glucose in normal rat liver, and of 6% in diabetics. These calculations were based on the assumption that the activity of the pentose phosphate pathway could be measured by the amount of $^{14}\text{CO}_2$ produced from [$1\text{-}^{14}\text{C}$]glucose. However, carbon dioxide is produced from C-6 when glucose enters the pentose phosphate pathway via the glucuronic acid pathway, and therefore it is possible that a larger amount of glucose is metabolized through the pentose phosphate pathway in starved and diabetic rats, if the alterations described by Winegrad & Shaw (1964) and in this and the previous paper (Stirpe & Comporti, 1965) occur in the living animal.

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