Biochemical Pharmacology, Vol. 46, No. 5, pp. 805–811, 1993.
Printed in Great Britain.

0006-2952/93 $6.00 + 0.00

MODIFICATION OF THE GLYOXALASE SYSTEM IN STREPTOZOTOCIN-INDUCED DIABETIC RATS

EFFECT OF THE ALDOSE REDUCTASE INHIBITOR STATIL

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(*Received* 24 *March* 1993; *accepted* 7 *June* 1993)

Abstract—The glyoxalase system was characterized in tissue (liver, skeletal muscle, kidney cortex and medulla, lens and sciatic nerve) and blood from streptozotocin-induced diabetic rats and normal controls. The effect of the aldose reductase inhibitor, Statil [3-(4-bromo-2-fluorobenzyl)-4-oxo-3*H*-phthalazine-1-yl-acetic acid; ICI 128 436], was also investigated. Glyoxalase I and glyoxalase II activities were decreased in the liver and increased in skeletal muscle of diabetic rats and of Statil-treated diabetic rats, relative to normal controls. The concentration of non-protein sulphydryl (NPSH) was decreased in the liver and lens of diabetic rats, relative to normal controls; Statil prevented these effects. The concentrations of methylglyoxal in the kidney cortex and medulla, lens and blood were increased in diabetic rats, relative to normal controls. Statil prevented these increases except in the kidney cortex. The concentration of D-lactate was increased in the lens and blood of diabetic rats, relative to normal controls, which was partially prevented in blood but not in the lens by Statil. These data suggest that the glyoxalase system is modified in tissues and blood of streptozotocin-induced diabetic rats and some of the modifications may be prevented by Statil. The increased concentrations of methylglyoxal in the kidney, lens and blood, and the decreased concentration of NPSH in the lens may be related to the development of diabetic complications.

The glyoxalase system catalyses the conversion of methylglyoxal to D-lactate via the intermediate *S*-D-lactoylgluthathione. It comprises two enzymes, glyoxalase I and glyoxalase II, and a catalytic amount of reduced glutathione. Glyoxalase I (EC 4.4.1.5) catalyses the formation of *S*-D-lactoylglutathione from the hemithioacetal formed non-enzymatically from methylglyoxal and reduced glutathione (GSH‡),

$$\mathrm{MeCOCHO + GSH \leftrightharpoons MeCOCH(OH)\text{-}SG} \xrightarrow{\text{GLYOXALASE I}} \mathrm{MeCH(OH)CO\text{-}SG.}$$

Glyoxalase II (EC 3.1.2.6) catalyses the hydrolysis of *S*-D-lactoylglutathione to D-lactate, regenerating the reduced glutathione consumed in the glyoxalase I-catalysed reaction.

$$\mathrm{MeCH(OH)CO\text{-}SG + H_2O} \xrightarrow{\text{GLYOXALASE II}} \mathrm{MeCH(OH)CO_2^- + H^+ + GSH.}$$

The glyoxalase system is present in the cytosol of all cells studied [1]. The function of the glyoxalase system is unknown but may be related to the detoxification of methylglyoxal and/or the regulation of cell growth. Recent research interest has focused on the modification of the glyoxalase system during hyperglycaemia and in diabetes mellitus and a suggested link with the development of diabetic complications [2].

The concentrations of glyoxalase metabolites, methylglyoxal, *S*-D-lactoylglutathione and D-lactate, were increased in human red blood cells in culture during hyperglycaemia. This effect was attributed to increased metabolic flux through the glyoxalase pathway during hyperglycaemia [3]. The concentrations of methylglyoxal in blood from genetically obese (ob/ob) mice and streptozotocin-induced diabetic mice were increased relative to normal controls [4]. The concentrations of methylglyoxal, *S*-D-lactoylglutathione and D-lactate were also increased in blood samples from insulin-dependent and non-insulin-dependent diabetic patients [5–8], relative to normal, healthy controls. These data are consistent with an increased flux through the glyoxalase pathway during hyperglycaemia associated with diabetes mellitus.

A link between the development of diabetic complications and the glyoxalase system has been suggested at the metabolic and genetic level. Insulin-dependent diabetic patients with retinopathy had a significantly higher activity of glyoxalase I and a significantly lower activity of glyoxalase II in red blood cells than patients without retinopathy [5]. Insulin-dependent diabetic patients without complications (retinopathy, neuropathy) had a significantly higher frequency of the glyoxalase

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‡ Abbreviations: F-1-P, fructose-1-phosphate; F-6-P, fructose-6-phosphate; NPSH, non-protein sulphydryl; Statil, 3-(4-bromo-2-fluorobenzyl)-4-oxo-3*H*-phthalazine-1-yl-acetic acid (ICI 128 436); GSH, reduced glutathione.

phenotype GLO 1–1 than patients with complications [9].

The aim of this study was to characterize the modification of the glyoxalase in tissue (liver, skeletal muscle, kidney cortex and medulla, lens and sciatic nerve) and blood from streptozotocin-induced diabetic rats and normal controls. The effects of the aldose reductase inhibitor, Statil [3-(4-bromo-2-fluorobenzyl)-4-oxo-3*H*-phthalazine-1-yl-acetic acid (ICI 128 436)], on changes of the glyoxalase system of diabetic rats were also investigated.

MATERIALS AND METHODS

Animals. Male albino Wistar rats (Alderley Park strain, ZENECA Pharmaceutical Ltd, Macclesfield, U.K.), weighing 125 g, were divided into three groups of six animals: control, diabetic and Statil-treated diabetic rats. Diabetes was induced in diabetic and Statil-treated diabetic rats by a single injection of streptozotocin (60 mg/kg, Sigma Chemical Co., Poole, U.K.). Statil was a gift from ZENECA Pharmaceuticals Ltd. Statil-treated diabetic rats were given Statil, 100 mg/kg, every day from 3 to 46 days post-streptozotocin injection. At 16, 32 and 46 days post-injection, animals were anaesthetized with isofluorane and *ca.* 5 mL of blood drawn from the dorsal aorta with EDTA as anti-coagulant. Animals were then killed and tissues extracted.

Characterization of the glyoxalase system. Tissues were stored at −196° until analysis within 3 months (sample storage validation studies for subsequent analyses were performed and this procedure found to preserve glyoxalase activities and metabolite concentrations [6–8, 10]). For assay of glyoxalase activities, tissue and blood samples, 50 mg, were homogenized in 1 mL of 10 mM sodium phosphate buffer with 0.02% Triton X-100, pH 7.0 and 4°. The homogenate was centrifuged at 50,000 *g* for 30 min at 4° and the supernatant used in glyoxalase activity assays as described [10]. For the determination of non-protein sulphydryl (NPSH), *ca.* 50 mg of tissue were homogenized in 2 mL of precipitating solution (1.67% glacial metaphosphoric acid, 0.2% EDTA, 30% sodium chloride), the homogenate was filtered and the filtrate used for NPSH determination as described [11]. For the determination of the concentrations of the glyoxalase metabolites, methylglyoxal, *S*-D-lactoylglutathione and D-lactate, 0.5–1.0 g of tissue was homogenized in 2 mL of 0.6 M perchloric acid. The precipitate was sedimented by centrifugation (5000 *g*, 10 min, 4°) and the supernatant used for assay of the glyoxalase metabolites as described [6–8].

Blood glucose concentration. Blood glucose measurements were made using a Technicon Autoanalyser II with glucose oxidase/peroxidase kits supplied by Boehringer Mannheim GMBH.

Statistical analysis. Statistical analysis of the results was by analysis of variance of all time point data for control, diabetic and Statil-treated diabetic groups, except blood glucose concentrations at individual time points were tested for significance by Student's *t*-test.

RESULTS

The glyoxalase system in streptozotocin-induced diabetic rats

The glyoxalase system was characterized in the liver, skeletal muscle, kidney (medulla and cortex), sciatic nerve, lens and blood samples of experimental rats. There was an active glyoxalase system (activities of glyoxalase I and glyoxalase II, glyoxalase metabolites) in all of these tissues and blood cells, methylglyoxal and D-lactate crossed cell membranes readily and were at equivalent concentrations in red blood cells and plasma. Diabetic rats and Statil-treated diabetic rats had markedly increased concentrations of blood glucose throughout the study period, relative to normal controls (Table 1).

The activity of glyoxalase I in the liver was significantly decreased ($P < 0.001$) in diabetic and Statil-treated diabetic rats and significantly increased in skeletal muscle of diabetic ($P < 0.001$) and Statil-treated diabetic rats ($P < 0.01$). There were also significant decreases in glyoxalase I activity in the kidney cortex ($P < 0.01$) and kidney medulla ($P < 0.05$) in Statil-treated diabetic rats (Table 2).

The activity of glyoxalase II was significantly decreased in the liver of diabetic ($P < 0.05$) and Statil-treated diabetic rats ($P < 0.001$). There were also significant decreases in glyoxalase II activity in the kidney cortex of diabetic and Statil-treated diabetic rats ($P < 0.001$), and in the kidney medulla of diabetic ($P < 0.001$) and Statil-treated diabetic ($P < 0.01$) rats, relative to normal controls. However, there were significant increases in glyoxalase II activity in skeletal muscle of diabetic and Statil-treated diabetic rats ($P < 0.05$) (Table 2).

The concentration of NPSH in the liver was significantly decreased in diabetic rats ($P < 0.01$). This decrease was prevented in Statil-treated diabetic rats. In the kidney cortex, there was a significant increase in the concentration of NPSH in diabetic rats ($P < 0.001$), relative to normal controls; this effect was further increased by Statil ($P < 0.01$). The concentration of NPSH was significantly decreased in the lens of diabetic rats, relative to normal controls ($P < 0.05$); this effect was also prevented by Statil (Table 2).

There were significant increases in the concentrations of methylglyoxal in the kidney cortex and kidney medulla of diabetic rats, relative to normal controls ($P < 0.001$). Statil prevented these effects completely in the kidney medulla but only partially in the kidney cortex. In red blood cells, the concentration of methylglyoxal was significantly increased in diabetic rats ($P < 0.01$); this increase was prevented in Statil-treated diabetic rats. A similar effect was found in the lens where the concentration of methylgyoxal was significantly increased in diabetic rats ($P < 0.01$) but not significantly changed in Statil-treated diabetic rats ($P > 0.05$), relative to normal controls (Table 2).

There was a significant increase in the concentration of *S*-D-lactoylglutathione in kidney cortex ($P < 0.01$) and lens ($P < 0.05$) of Statil-treated diabetic rats, relative to control and diabetic groups. The concentration of *S*-D-lactoylglutathione in whole blood was significantly decreased in diabetic and

Table 1. Blood glucose concentration in normal, diabetic and Statil-treated diabetic rats

Group	Post-injection period		
	16 days (mM)	32 days (mM)	46 days (mM)
Control	5.2 ± 1.1	7.0 ± 1.2	8.0 ± 2.0
Diabetic	22.5 ± 3.1*	26.8 ± 4.1*	24.2 ± 5.6*
Statil-treated diabetic	22.4 ± 2.7*	18.2 ± 2.7*	22.3 ± 6.4*

Data are means ± SD of six determinations. Significance test results are indicated: diabetic group relative to control group *$P < 0.001$.

Statil-treated diabetic rats, relative to normal controls (Table 2).

The concentration of D-lactate in red blood cells was markedly increased in diabetic rats ($P < 0.001$); an effect which was partially reversed by Statil ($P < 0.001$). In the lens, the concentration of D-lactate was also markedly increased in diabetic and Statil-treated diabetic rats ($P < 0.001$) (Table 2).

DISCUSSION

Until recently, the modification of the glyoxalase system in diabetes mellitus and a possible role in the development of diabetic complications had not been considered. Methylglyoxal is formed mainly from triose phosphates, dihydroxyacetone phosphate and glyceraldehyde-3-phosphate [12]; other minor sources are hydroxyacetone and aminoacetone [2] (Fig. 1). The terminal product, D-lactate, is surprisingly well-metabolized in animals. It is converted to pyruvate by 2-hydroxyacid dehydrogenase, thereby rejoining mainstream glycolysis [7]. The flux through the glyoxalase pathway normally represents only a small component of glucotriose metabolism, 0.09% in human red blood cells [3]. However, the well-established toxicity of methylglyoxal (reviewed in Refs 1 and 2), and the more recently recognised toxicity of *S*-D-lactoylglutathione [13], suggested that there may be increased metabolic flux through the glyoxalase system during periodic hyperglycaemia associated with diabetes mellitus and this may have chronic pathogenetic effects.

The activities of glyoxalase I were decreased and the activities of glyoxalase II increased in red blood cells during the onset of diabetes in (ob/ob) obese mice and streptozotocin-induced diabetic mice [4]. Similar changes in glyoxalase activities in red blood cells of streptozotocin-induced diabetic rats were not found. The activity of glyoxalase I was decreased in the liver of diabetic rats and in the liver, kidney cortex and kidney medulla of Statil-treated diabetic rats, and increased in skeletal muscle of diabetic rats, in the absence and presence of Statil. The activity of glyoxalase II was decreased in the liver, kidney cortex and kidney medulla, and increased in skeletal muscle of diabetic and Statil-treated diabetic rats. The significance and mechanism of these changes in glyoxalase activities are unknown.

The concentration of NPSH, of which the major component is normally GSH, was decreased in the liver of diabetic rats. This may be due to increased export of GSH from the liver, under hormonal control [14] but has also been attributed to nutritional deprivation [15]. Statil inhibited this effect. The demand for NADPH in the formation of sorbitol from glucose, catalysed by aldose reductase, in the lens (and other tissues) may lead to a depletion in NADPH and decreased *in situ* glutathione reductase activity. This, and increased oxidative stress associated with the oxidative degradation of fructosamines of glycated protein [16], may lead to a decrease in tissue GSH concentration, prompting an increased export of GSH from the liver [17, 18]. Inhibition of aldose reductase by Statil may prevent this. The increased NPSH in the kidney may be due to scavenging of increased circulatory GSH (converted to cysteinylglycine by the high γ-glutamyltranspeptidase activity of the kidney) [19].

The concentration of methylglyoxal was increased in red blood cells, kidney (medulla and cortex) and lens. Statil reversed this effect except in the kidney cortex. This suggests that there was increased methylglyoxal formation in diabetes (although decreased glyoxalase I activity in the kidney cortex in Statil-treated diabetic rats may also contribute to the increase in methylglyoxal in this tissue). The prevention of this increase by Statil in the lens may be related to the inhibition of aldose reductase. Aldose reductase activity may influence the concentration of methylglyoxal in tissues by at least three mechanisms: (i) by conversion of methylglyoxal to hydroxyacetone [20] (Fig. 2), (ii) by increased triosephosphate formation from metabolic flux through the polyol pathway [21], and (iii) by consumption of NADPH, leading to decreased GSH levels and decreased *in situ* glyoxalase pathway activity [1, 17]. Inhibition of aldose reductase activity by Statil from (i) would be expected to increase tissue methylglyoxal concentration and from (ii) and (iii) would be expected to decrease tissue methylglyoxal concentration. Restoration of tissue methylglyoxal concentrations to control levels is expected to be beneficial, see below.

The concentration of *S*-D-lactoylglutathione was increased in the kidney cortex and lens of Statil-treated diabetic rats only. The increased concentrations of methylglyoxal and/or D-lactate without concomitant increase in *S*-D-lactoylglutathione in the lens and kidney in diabetic rats is attributed to efficient formation and metabolism of the intermediate by the glyoxalase system. The low

Table 2. Characteristics of the glyoxalase system in tissues and blood of normal, streptozotocin-induced diabetic and Statil-treated streptozotocin-induced diabetic rats

Tissue	Glyoxalase I activity (U/g)	Glyoxalase II activity (U/g)	Non-protein sulphydryl (μmol/g)	Methylglyoxal (nmol/g)	*S*-D-Lactoyl-glutathione (nmol/g)	D-Lactate (nmol/g)
Liver						
Mean (N)						
Control	162.8 (18)	89.9 (18)	5.81 (18)	1.10 (18)	56.5 (4)	232.4 (18)
Diabetic	134.5 (17)	79.1 (16)	5.10 (17)	1.07 (17)	42.3 (4)	218.1 (16)
Statil	133.6 (18)	75.5 (18)	5.44 (18)	1.04 (17)	32.7 (4)	202.4 (18)
ST:						
D vs C	0.0001	0.013	0.009	—	—	—
D vs S	—	—	—	—	—	—
S vs C	0.0001	0.0008	—	—	—	—
Skeletal muscle						
Mean (N)						
Control	165.2 (18)	2.05 (18)	0.89 (18)	ND	ND	92.0 (12)
Diabetic	207.7 (18)	2.67 (15)	0.90 (18)	ND	ND	85.0 (11)
Statil	194.0 (18)	2.63 (18)	0.85 (18)	ND	ND	100.5 (12)
ST:						
D vs C	0.0001	0.012	—	ND	ND	—
D vs S	—	—	—	ND	ND	—
S vs C	0.004	0.014	—	ND	ND	—
Kidney cortex						
Mean (N)						
Control	125.7 (18)	55.8 (18)	2.89 (18)	1.02 (18)	6.92 (8)	257.5 (18)
Diabetic	115.3 (17)	37.5 (18)	3.46 (17)	1.40 (17)	6.93 (8)	277.3 (17)
Statil	106.3 (18)	34.9 (18)	3.89 (18)	1.33 (17)	9.78 (8)	240.2 (17)
ST:						
D vs C	—	0.0001	0.0002	0.0001	—	—
D vs S	—	—	0.003	—	0.004	0.036
S vs C	0.003	0.0001	0.0001	0.0004	0.004	—
Kidney medulla						
Mean (N)						
Control	85.1 (18)	32.2 (18)	2.39 (18)	0.94 (18)	7.00 (4)	170.0 (16)
Diabetic	78.2 (18)	21.7 (18)	2.31 (18)	1.27 (17)	9.17 (4)	160.4 (17)
Statil	75.5 (18)	25.3 (18)	2.53 (18)	1.10 (17)	4.76 (4)	132.9 (18)
ST:						
D vs C	—	0.0001	—	0.0009	—	—
D vs S	—	—	—	—	—	—
S vs C	0.028	0.003	—	—	—	0.011

Table 2 continued.

Tissue	Glyoxalase I activity (U/g)	Glyoxalase II activity (U/g)	Non-protein sulphydryl (μmol/g)	Methylglyoxal (nmol/g)	*S*-D-Lactoyl-glutathione (nmol/g)	D-Lactate (nmol/g)
Lens						
Mean (N)						
Control	46.0 (17)	1.14 (12)	2.59 (10)	2.10 (15)	15.48 (8)	25.9 (12)
Diabetic	50.3 (16)	1.09 (12)	1.79 (11)	4.54 (14)	15.03 (8)	73.7 (12)
Statil	43.8 (17)	1.14 (12)	3.08 (9)	2.80 (15)	19.35 (8)	68.7 (12)
ST:						
D vs C	—	—	0.023	0.0026	—	0.0001
D vs S	—	—	0.0004	0.028	0.008	—
S vs C	—	—	—	—	0.016	0.0002
Red blood cells (or whole blood)*						
Mean (N)						
Control	104.8 (16)	10.6 (15)	2.14 (15)	0.16 (15)	4.28 (12)	28.7 (14)
Diabetic	108.8 (17)	11.0 (17)	2.29 (18)	0.22 (15)	2.95 (12)	66.6 (15)
Statil	101.7 (15)	11.4 (15)	2.15 (13)	0.17 (15)	3.48 (12)	47.4 (14)
ST:						
D vs C	—	—	—	0.003	0.0004	0.0001
D vs S	—	—	—	0.011	—	0.0004
S vs C	—	—	—	—	0.024	0.0006
Sciatic nerve						
Mean (N)						
Control	41.6 (18)	6.13 (15)	0.97 (18)	2.40 (16)	3.60 (4)	60.4 (12)
Diabetic	37.3 (17)	5.93 (16)	0.94 (18)	2.75 (15)	5.08 (4)	59.3 (12)
Statil	42.2 (17)	5.93 (16)	0.96 (18)	2.85 (14)	10.0 (4)	72.8 (12)
ST:						
D vs C	—	—	—	—	—	—
D vs S	—	—	—	—	—	—
S vs C	—	—	—	—	—	—

Key: ND, not determined. ST, analysis of variance significance tests with presentation of P values; C, control group; D, streptozotocin-induced diabetic group; S, Statil-treated streptozotocin-induced diabetic group. Blank significance test result indicates $P > 0.05$.

* Concentration units for methylglyoxal, *S*-D-lactoylglutathione and D-lactate determinations are nmol/mL of whole blood.

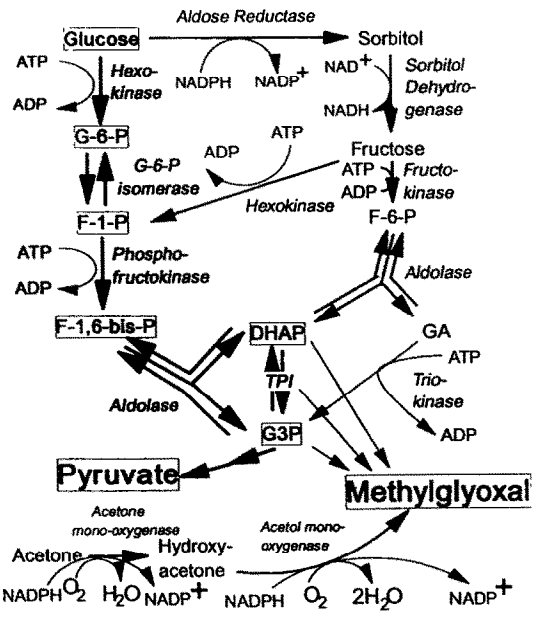

Fig. 1. Metabolic pathways for the formation of methylglyoxal in diabetes.

concentration of *S*-D-lactoylglutathione in whole blood may be due partly to decreased haematocrit since *S*-D-lactoylglutathione is found only in blood cells.

The concentration of D-lactate was increased in lens and blood; an effect that was partially reversed by Statil in blood only. Decreased D-lactate formation concomitant with inhibition of aldose reductase may reflect control of metabolic flux through the glyoxalase pathway by tissue GSH levels and/or metabolic flux for D-lactate formation originating from the polyol pathway (Fig. 1).

The glyoxalase system was modified in the liver, skeletal muscle, lens, kidney and blood of diabetic rats. The increase in methylglyoxal and D-lactate levels in the lens, kidney and blood is consistent with an increased metabolic flux through the glyoxalase pathway in hyperglycaemia in lens and kidney tissue and red blood cells. Methylglyoxal and D-lactate readily cross cell plasma membranes, hence, it is not possible to define the precise origin of the pools of these metabolites; they are also both excreted in the urine [22].

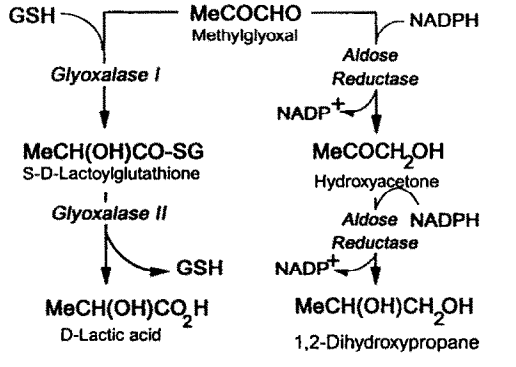

Fig. 2. The glyoxalase pathway and metabolism of methylglyoxal by aldose reductase (see Refs 1 and 20).

The formation of methylglyoxal in diabetes may originate from increased formation of triose phosphates directly in the Embden–Meyerhof pathway [12] and indirectly via the polyol pathway [21]. Fructose formed in the polyol pathway from glucose may be converted to triose phosphates, via fructose-6-phosphate (F-6-P) or fructose-1-phosphate (F-1-P) and glyceraldehyde [23]. Methylglyoxal may also be formed from the metabolism of acetone in ketoacidosis [24]. Oxidative stress may also increase the formation of methylglyoxal from the accumulation of triose phosphates following the oxidative inactivation of glyceraldehyde-3-phosphate dehydrogenase [25].

The increase in tissue concentration of methylglyoxal may be of pathological significance. Methylglyoxal binds and modifies proteins, thought to be predominantly on arginine residues [26], producing fluorescent adducts [20] and crosslinks [27]. Recent research has shown that physiological concentrations of methylglyoxal found in blood samples in diabetes mellitus bind and modify plasma proteins [28]. Non-sulphydryl protein crosslinks and loss of protein structure–function characteristics are thought to be important biochemical characteristics of the development of diabetic complications [29, 30]. Aminoguanidine, an agent which prevents diabetes-induced arterial wall protein cross-linking [30], is an efficient scavenger of methylglyoxal [28, 31]. The formation of methylglyoxal–protein adducts in diabetes now deserves investigation.

This study demonstrates a modification of the glyoxalase system in the liver, skeletal muscle, kidney, lens and blood of streptozotocin-induced diabetic rats. Increases in the concentrations of methylglyoxal, *S*-D-lactoylglutathione and D-lactate, in insulin-dependent and non-insulin-dependent diabetic patients have recently been reported [6–8]. The protein binding activity of methylglyoxal, the suppression of increased tissue concentrations of methylglyoxal by aldose reductase inhibitor, and the scavenging of methylglyoxal by aminoguanidine, suggests that the involvement of methylglyoxal metabolism by the glyoxalase system and aldose reductase in the development of diabetic complications may be a promising area for future research.

Acknowledgements—S.A.P. thanks the Science and Engineering Research Council for a CASE PhD studentship in collaboration with ZENECA Pharmaceuticals Ltd, Macclesfield, Cheshire, U.K. This is a contribution from the Glyoxalase Research Group at the University of Essex, U.K.

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