PRECURSOR SPECIFIC INHIBITION OF HEPATIC GLUCONEOGENESIS BY GLISOXEPIDE, AN INHIBITOR OF THE L-ASPARTATE/ L-GLUTAMATE ANTIPORT SYSTEM

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1. Introduction

Blood glucose lowering sulfonamides do not only stimulate the release of insulin from the β-cell of the pancreas but have also extra-pancreatic effects. One of these effects is the inhibition of hepatic ketogenesis [1–3]. During recent studies of a new hypoglycemic sulfonylurea we observed not only an inhibition of ketogenesis but also of hepatic glucogeneogenesis. We present now data which show that glisoxepide, a new blood glucose lowering sulfonylurea, inhibits in rat liver specifically gluconeogenesis from L-lactate and L-alanine but not from pyruvate. Urea production from endogenous precursors as well as from L-alanine is likewise diminished. Gluconeogenesis from L-lactate is only slightly inhibited in pigeon liver. In isolated rat liver mitochondria glisoxepide inhibits specifically the L-aspartate/L-glutamate exchange.

2. Materials and methods

Male Wistar rats (Winkelmann, Kirchborchen/ Germany) fed a standard diet were used throughout the experiments. Pigeons between 15 and 20 weeks of age were purchased from Henning, Selters/Germany. Liver cells from 48 hr starved rats and 72 hr starved pigeons were prepared by a modification of the method of Berry and Friend [4]. No hyaluronidase was used. The isolated liver cells were incubated in Krebs-Ringer bicarbonate buffer, pH 7.4 equilibrated with carbogen (95% O_2, 5% CO_2) under the conditions mentioned in the legends to tables 1–3. Mitochondria from rat liver were isolated according to Chappel and Hansford [5].

For measuring the malonate/α-oxoglutarate and the L-aspartate/L-glutamate exchange the change of the reduction of mitochondrial NAD(P) was registered by dual wavelength spectrophotometry with an Aminco-Chance DW 2 spectrophotometer as described by Azzi et al. [6]. In a similar way the transport of L-glutamate [6] and D-isocitrate [7] was studied. Inhibition of soluble and mitochondrial glutamate-oxaloacetate aminotransferase (EC 2.6.1.1) from rat liver by glisoxepide was determined in the direction of oxaloacetate formation. The test mixture contained (final concentration): K-phosphate 85 mM; NADH 0.2 mM; α-oxoglutarate 15 mM; malate dehydrogenase (NH_4^+-free) 17 μg; appropiate amounts of S_3- or mitochondrial protein; L-aspartate (0.125–20 mM). Glucose, L-lactate, pyruvate, L-alanine, L-aspartate, and urea were determined enzymic-optically by standard methods [8]. Enzymes and coenzymes came from Boehringer Mannheim Corp., Mannheim, Germany, carbonyl cyanide, *m*-chlorophenyl hydrazone (CCCP) from Calbiochem, Luzern, Switzerland, antimycin A and rotenone from Sigma Chem. Co., St. Louis, Md., USA, and DL-isocitrate from Roth, Karlsruhe, Germany. All other reagents were ordered from E. Merck A.G., Darmstadt, Germany. Glisoxepide (1-hexahydro-1-H-azepin-1-yl)-3-((*p*-[2-(5-methylisooxalol-3-carboxamido)-ethyl]-phenyl-sulfonyl))-urea) was a gift of Dr N. N. Kiesselbach, Farbenfabriken Bayer A. G., Pharmazeutisches Forschungszentrum, Wuppertal-E., Germany. Avenaciolide was kindly donated by Dr Turner Biochemistry Dept. Pharmaceutic. Div. ICI Ltd., Macclesfield Cheshire, England.

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Table 1
Effect of glisoxepide on gluconeogenesis from L-lactate, L-alanine, and L-lactate by isolated rat liver cells

Experimental condition	Concentration of glisoxepide (M)	Glucose production	Formation of L-lactate + pyruvate	L/P
		(μmol·g dry weight^{-1} · min^{-1})		(ratio)
Control no substrate	ϕ	0.37 ± 0.01	0.31 ± 0.03	3.2 ± 0.5
L-Alanine control	ϕ	1.54 ± 0.05	1.91 ± 0.21	3.3 ± 0.2
L-Alanine	$5 \cdot 10^{-4}$	0.85 ± 0.01	2.28 ± 0.22	5.4 ± 0.4
L-Alanine	$1 \cdot 10^{-4}$	0.88 ± 0.03	2.07 ± 0.08	5.1 ± 0.4
L-Alanine	$5 \cdot 10^{-5}$	1.20 ± 0.01	1.86 ± 0.01	4.5 ± 0.1
L-Alanine	$2.5 \cdot 10^{-5}$	1.35 ± 0.04	1.88 ± 0.09	3.8 ± 0.3
L-Lactate control	ϕ	2.53 ± 0.02	–	10.6 ± 0.9
L-Lactate	$5 \cdot 10^{-4}$	1.23 ± 0.02	–	20.8 ± 1.1
L-Lactate	$1 \cdot 10^{-4}$	1.77 ± 0.10	–	15.6 ± 0.3
Pyruvate control	ϕ	2.79 ± 0.02	–	1.0 ± 0.2
Pyruvate	$5 \cdot 10^{-4}$	2.56 ± 0.01	–	1.0 ± 0.2

The initial precursor concentration was always 10 mM. Cells were isolated as described in Materials and methods. Cells corresponding to 10 to 15 mg dry weight were incubated in a total vol of 2 ml and shaken in a waterbath at 37°C and 120 strokes/min for 60 min. The incubation was stopped by addition of 1 ml of 1 N $HClO_4$ (mean values ± SEM) (n = 4).

Table 2
Comparative effects of glisoxepide (1 mM) and avenaciolide (0.5 mM) on gluconeogenesis and ureogenesis from endogenous precursors and from L-alanine (10 mM) in experiments with isolated rat liver cells from 48 hr starved rats

Experimental condition	Net formation of glucose	Net formation of urea
	(μmol·g dry weight^{-1} · min^{-1})	
No external precursor	0.70 ± 0.03	0.51 ± 0.02
L-Alanine	1.67 ± 0.02	2.40 ± 0.03
L-Alanine + glisoxepide	0.85 ± 0.01	1.67 ± 0.03
L-Alanine + avenaciolide	0.72 ± 0.02	1.30 ± 0.04

The conditions for isolation and incubation of cells were the same as mentioned in Materials and methods and in the legend to table 1. (n = 5). (Mean values ± SEM).

3. Results

3.1. *Effects on gluconeogenesis*

In isolated rat liver cells glisoxepide inhibited significantly gluconeogenesis from L-lactate and L-alanine, but not from pyruvate (table 1). In the experiments with L-lactate the lactate/pyruvate ratio was significantly higher in the presence of the inhibitor. Production of pyruvate + L-lactate from L-alanine was not diminished indicating that transamination was not significantly affected. Urea production in the presence of 10 mM L-lactate or pyruvate was inhibited by 26% and 36% resp. in the presence of $1 \cdot 10^{-4}$ M glisoxepide. Urea production from L-alanine was likewise inhibited (table 2) although less than by avenaciolide at a concentration which was about equieffective with respect to inhibition of

Table 3
Effects of glisoxepide on gluconeogenesis from L-lactate (initial concentration 10 mM) by isolated pigeon liver cells

Experimental condition	Concentration of glisoxepide (M)	Glucose formation (μmol·g dry weight^{-1} · min^{-1})	L/P (ratio)
Control no substrate	ϕ	0.48 ± 0.02	–
L-lactate	ϕ	9.60 ± 0.06	25 ± 0.8
L-lactate	$1 \cdot 10^{-3}$	7.59 ± 0.05	36 ± 3.2
L-lactate	$5 \cdot 10^{-4}$	8.33 ± 0.27	36 ± 1.2
L-lactate	$1 \cdot 10^{-4}$	9.43 ± 0.04	33 ± 0.1

Isolation and incubation was performed as described in the legend to table 1. Each incubation vessel contained cells corresponding to 3–5 mg dry weight (Mean values ± SEM) (n = 4).

gluconeogenesis. In isolated pigeon liver cells glisoxepide had only a weak inhibitory effect on gluconeogenesis from L-lactate (table 3) although the lactate/pyruvate ratio became also significantly reduced.

3.2. *Anion transport in isolated mitochondria*

The oxidation of NAD(P)H following the addition of L-aspartate was significantly decreased by glisoxepide (fig.1a and 1b). In addition the final redox state at which the NAD(P)/NAD(P)H system arrived after the addition of L-aspartate was shifted towards a less oxidized state. When the concentration of L-aspartate was 0.1 mM, the inhibition could still be seen at a glisoxepide concentration as low as $5 \cdot 10^{-7}$ M (figure 2a and 2b). The inhibition ($K_i = 1.5 \cdot 10^{-4}$ M) was of the competitive type (insert in fig.2). The malonate/α-oxoglutarate exchange was not affected by varying the outer pH between 7.21 and 7.76 (fig.1c) whereas the aspartate/glutamate exchange was inhibited at lower pH values (fig.1c). The inhibition of the aspartate/glutamate exchange by glisoxepide decreased with increasing pH (fig.1d). The uptake and/or adsorption of [^{3}H]glisoxepide (not shown here) was not affected by pH changes between 7.20 and 7.80.

The rate of reduction of NAD(P) following the addition of malonate was not affected by $1 \cdot 10^{-4}$ glisoxepide and only slightly diminished by $5 \cdot 10^{-4}$ M glisoxepide (insert in fig.3). The oxidation of L-gluta-

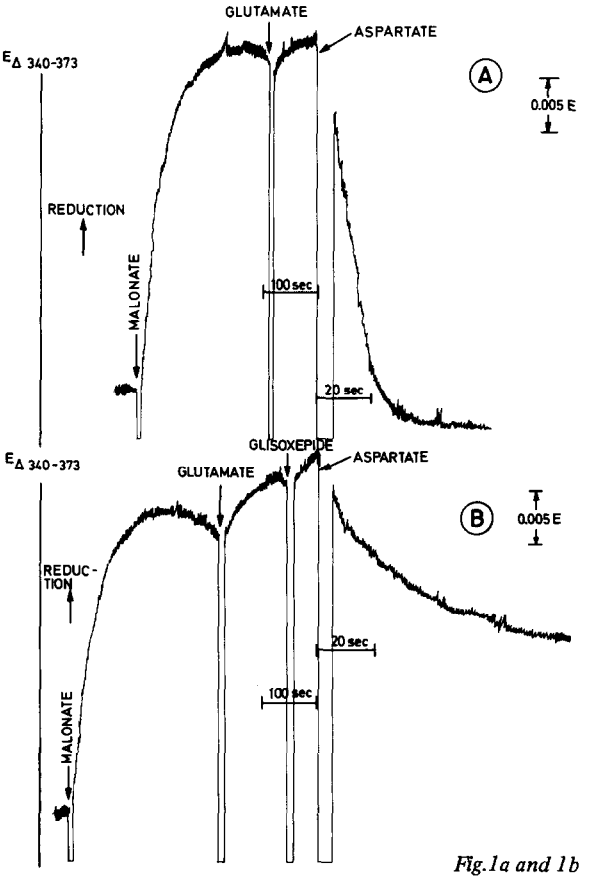

Fig.1a and 1b

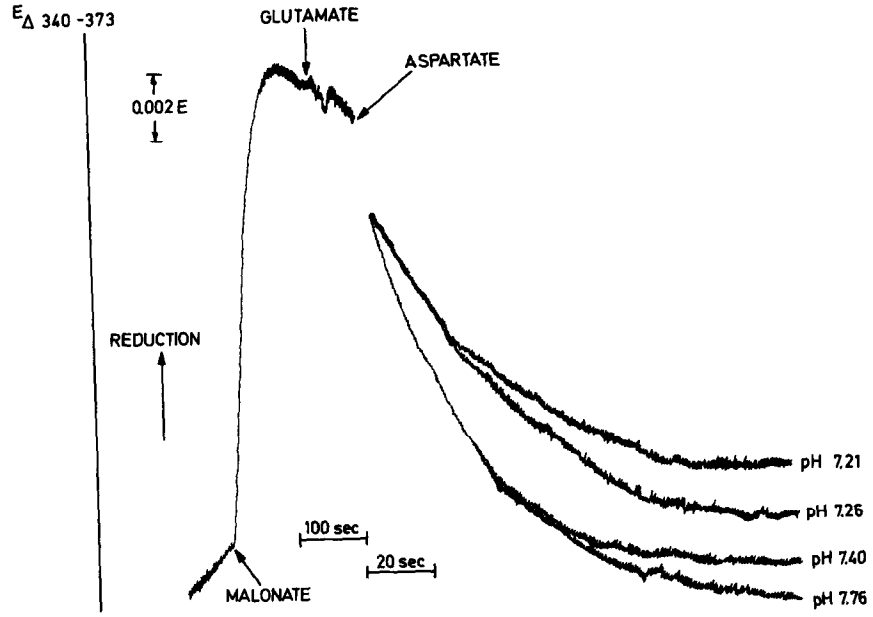
$E_{\Delta\ 340\ -373}$
GLUTAMATE
ASPARTATE
0.002 E
REDUCTION
100 sec
20 sec
MALONATE
pH 7.21
pH 7.26
pH 7.40
pH 7.76

Fig. 1c

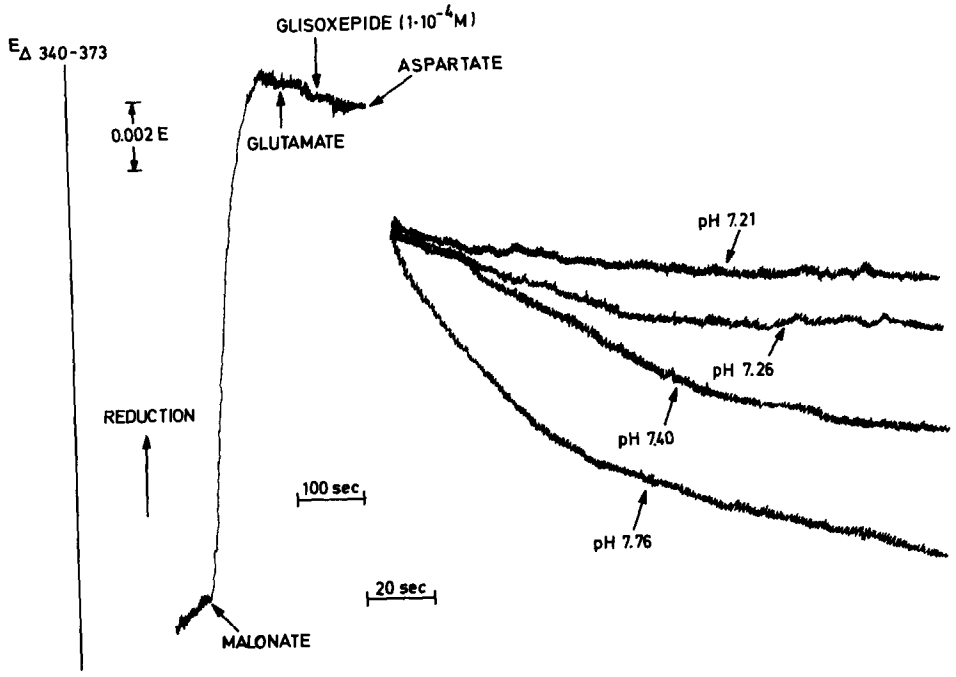
GLISOXEPIDE ($1 \cdot 10^{-4}$ M)
$E_{\Delta\ 340-373}$
ASPARTATE
0.002 E
GLUTAMATE
pH 7.21
pH 7.26
pH 7.40
pH 7.76
REDUCTION
100 sec
20 sec
MALONATE

Fig. 1d

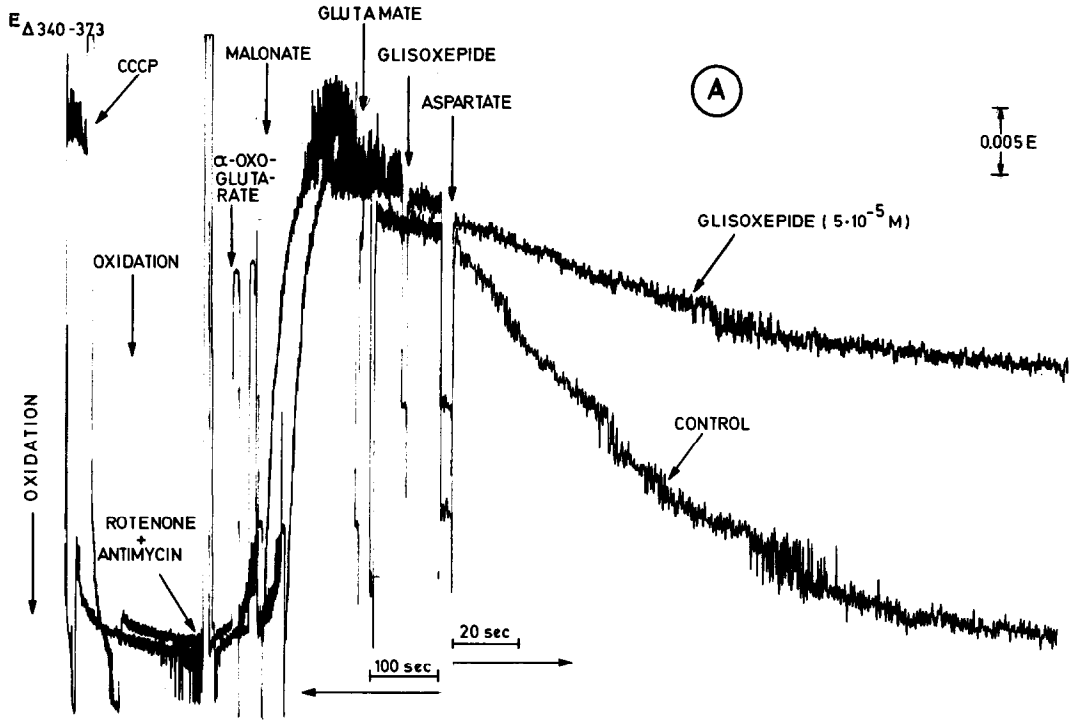
E Δ 340-373
CCCP
MALONATE
GLUTAMATE
GLISOXEPIDE
ASPARTATE
α-OXO-GLUTA-RATE
A
0.005 E
GLISOXEPIDE (5·10⁻⁵ M)
OXIDATION
CONTROL
OXIDATION
ROTENONE + ANTIMYCIN
20 sec
100 sec

Fig. 2a

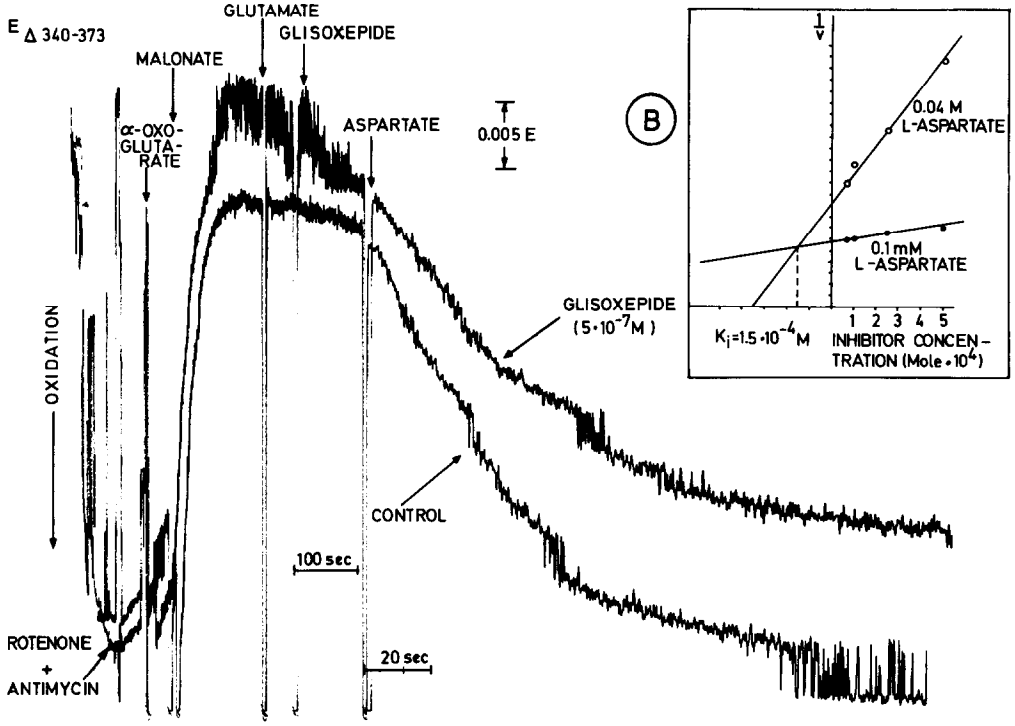
E Δ 340-373
GLUTAMATE
GLISOXEPIDE
MALONATE
α-OXO-GLUTA-RATE
ASPARTATE
0.005 E
B
0.04 M L-ASPARTATE
0.1 mM L-ASPARTATE
GLISOXEPIDE (5·10⁻⁷ M)
Kᵢ=1.5·10⁻⁴ M
1 2 3 4 5
INHIBITOR CONCENTRATION (Mole·10⁴)
OXIDATION
CONTROL
100 sec
ROTENONE + ANTIMYCIN
20 sec

Fig. 2b

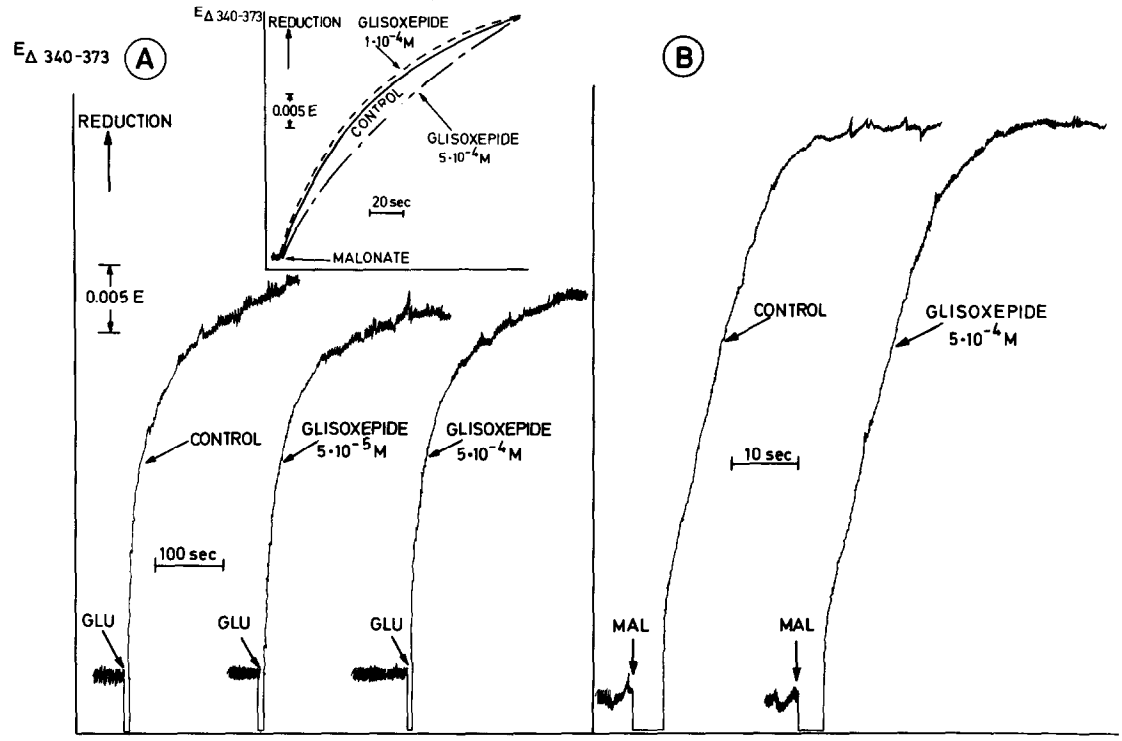

Fig.3. A) Lack of inhibition of L-glutamate transport by $5\cdot10^{-4}$ glisoxepide. Rat liver mitochondria (2.3 mg) were added to potassium hydroxyethylpiperazine-ethanesulphonate (80 mM; pH 6.8). Oxidation of intramitochondrial NAD(P)H was achieved by addition of CCCP (1 μM). Antimycin (0.5 μg/ml) and rotenone 2 μg/ml were added. Reduction of NAD(P) was initiated by addition (GLU) of L-glutamate (2.5 mM). Glisoexepide had been added 2 minutes prior to the addition of L-glutamate. The total volume was 3 ml. Reaction temperature 30°C. *Insert in figure 3A*: Effect of glisoxepide on the malonate induced transport of α-oxoglutarate. Conditions were as given in the legend to fig.1A except that glisoxepide was added before the addition of α-oxoglutarate. Concentration of mitochondria was 0.8 mg/ml. B) Lack of inhibition of transport of isocitrate by glisoxepide. Rat liver mitochondria (2.3 mg) were suspended in the same medium as given in the legend to figure 1A. Oxidation of NAD(P)H was achieved by CCCP, and rotenone and antimycin were added as described in the same legend. A slow reduction of NAD(P) was initiated by the addition of D, L-isocitrate (2.5 mM). A rapid reduction followed immediately after addition (MAL) of L-malate (0.42 mM). Glisoxepide ($5\cdot10^{-4}$ M) was added 2 minutes prior to the addition of D, L-isocitrate. The total volume was 3.0 ml, the reaction temperature 26°C. Figures 3A and 3B were redrawn from original curves.

Fig.1. A) Control experiment. About 2.5 mg of rat liver mitochondria were incubated in the following medium; Tris–Cl 20 mM, KCl 80 mM, K-phosphate 1 mM, pH 7.4. Oxidation of NAD(P) was initiated by carbonyl cyanide, *m*-chlorophenyl hydrazone (CCCP) (1 μM). After completion of oxidation antimycin (0.5 μg/ml) and rotenone (2 μg/ml) were added, followed by the addition of α-oxoglutarate (1 mM). Reduction of NAD(P) was initiated by addition of malonate (1 mM). After completion of reduction glutamate (1 mM) was added, Re-oxidation was initiated by addition of L-aspartate (1 mM). The total vol was 3 ml. Reaction temperature 30°C. B) Same experiment as in (A) with the exception that glisoxepide ($5\cdot10^{-4}$ M) was added before the addition of L-aspartate. (C) Same type of experiment as in (A). The concentration of L-aspartate was 0.1 mM. The pH values were varied between 7.21 and 7.76. Note that NAD(P) reduction is not affected by the pH changes. D) These experiments were carried out as those shown in (C) but $1\cdot10^{-4}$ glisoxepide was added before the addition of L-aspartate.

Fig.2. A) Inhibitory effect of $5\cdot10^{-5}$ M glisoxepide on the transport of L-aspartate at a low concentration of aspartate. Rat liver mitochondria (2.1 mg) were incubated under the same condition as described in the legend to fig.1(A) and (B) with the exception that the concentration of aspartate was only 0.1 M. Reaction temperature 30°C. B) Same experiment as in (A), but the concentration of glisoxepide was reduced to $5\cdot10^{-7}$ M. The insert represents the graphical evaluation of K_i by a Dixon plot.

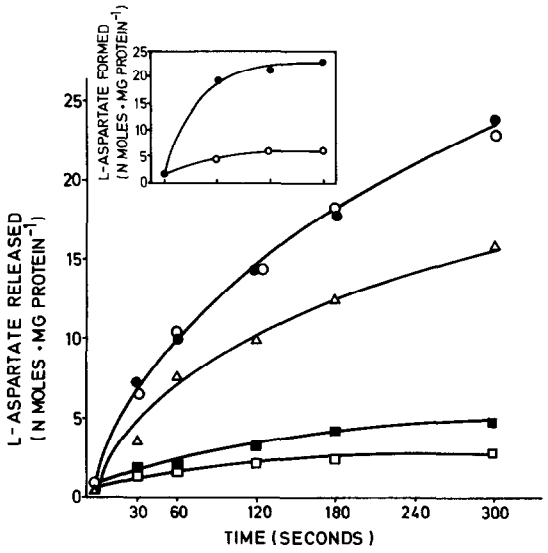

Fig.4. Effect of glisoxepide on the L-glutamate induced efflux of L-aspartate from isolated rat liver mitochondria (4.5 mg/ml). Mitochondria were isolated in mannitol (0.225 M) sucrose (75 mM), EDTA (0.1 mM) at pH 7.4. The mitochondria were loaded with L-glutamate and the intramitochondrial formation of L-aspartate was induced by addition of oxaloacetate (2 mM) as described by LaNoue et al. [9]. At zero time L-glutamate (5 mM) was added to the incubation medium. At the time points indicated in the figure aliquots of the incubation mixture were layered on top of a silicon oil phase and mitochondria were separated by rapid centrifugation in an Eppendorf-centrifuge with swing-out rotor. •———• Controls + L-glutamate; ○———○ glisoxepide, 0.25 mM, + L-glutamate; △———△ glisoxepide, 5 mM, + L-glutamate; ■———■ Controls without L-glutamate (0.25 mM glisoxepide without L-glutamate gave identical values); □———□ glisoxepide, 5 mM, without L-glutamate. *Insert*: Net formation of L-aspartate (extra- plus intramitochondrial) at 1, 2, and 3 minutes after addition of 2 mM oxaloacetate to L-glutamate loaded mitochondria in the absence (•———•) and presence (○———○) of 5 mM glisoxepide.

mate (fig.3a) or D-isocitrate (fig.3b) was not affected by glisoxepide up to concentrations of $5 \cdot 10^{-4}$ M. No inhibition of soluble or mitochondrial glutamate-oxaloacetate aminotransferase could be observed at L-aspartate concentrations from 0.125 to 20 mM when the concentration of glisoxepide was varied between $5 \cdot 10^{-6}$ and $5 \cdot 10^{-3}$ M. Only concentrations exceeding 5 mM caused some inhibition of soluble and mitochondrial glutamate-oxaloacetate amino transferase.

When isolated rat liver mitochondria were charged with L-aspartate as described by La-Noue et al. [9] and the outward transport of L-aspartate after addition of L-glutamate was measured 0.25 mM glisoxepide had no significant effect (fig.4). However, the concentration of L-aspartate in the matrix water space was between 10 and 20 mM under these conditions. This explains the inefficiency of the inhibitor since, according to the experiments described above, the inhibition by glisoxepide is competitive with aspartate. When the outward transport of L-aspartate was measured in the presence of 5 mM glisoxepide (fig.4) the inhibition of formation of L-aspartate interfered with the inhibition of transport (insert in fig.4). Further experiments are underway in order to discriminate between these two phenomena.

4. Discussion

Glisoxepide is a rather specific inhibitor of the L-aspartate/L-glutamate antiport system. According to LaNoue et al. [9] the L-aspartate/L-glutamate exchange is electrogenic and proceeds under physiological conditions unidirectionally from the matrix space to the outside. However, under our conditions the transmembrane pH-gradient had been abolished by the use of an uncoupler in combination with inhibitors of respiration. Under this condition, external L-aspartate can exchange with internal L-glutamate. In accordance with this is the finding that lowering of the external pH inhibited the L-aspartate/L-glutamate exchange but increased the inhibitory action of glisoxepide. The drug did not only diminish the rate of oxidation of intramitochondrial NAD(P)H but shifted also the final redox state of the $NAD(P)^+/NAD(P)H$ system towards a higher degree of reduction. Since the redox state results from the oxidation of α-oxoglutarate and the reduction of oxaloacetate, an inhibition of intramitochondrial transamination due to a lack of incoming L-aspartate will diminish formation of NAD(P)H in the malate dehydrogenase reaction and leave more α-oxoglutarate and L-glutamate for oxidation. The rather selective inhibition of gluconeogenesis from L-lactate and L-alanine together with an inhibition of ureogenesis would be in line with the concept that gluconeogenesis from these two precursors but not that from pyruvate involves transamination and transport of L-aspartate out of the mitochondria [10–12]. The low sensitivity of

gluconeogenesis from L-lactate towards the drug in isolated pigeon liver cells fits also this concept, since in this species no transamination cycle is involved in gluconeogenesis from L-lactate [13]. Glisoxepide inhibited ureogenesis from endogenous precursors and from L-alanine. The higher degree of inhibition of ureogenesis by avenaciolide reflects most probably additional unspecific effects as is indicated by an inhibition (−22%) of gluconeogenesis from pyruvate under the same conditions.

Sulfonylureas could contribute to an inhibition of gluconeogenesis also by affecting oxidative phosphorylation [14–16]. However, if this were the main reason for the results obtained in this study one would expect a similar inhibition of gluconeogenesis from pyruvate.

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