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METABOLIC EFFECTS OF HYPOGLYCEMIC SULFONYLUREAS—IV

INTERFERENCE OF SULFONYLUREAS WITH MITOCHONDRIAL OXIDATIVE PHOSPHORYLATION*

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Abstract—The effects of various hypoglycemic and non-hypoglycemic sulfonylureas on succinate oxidation by rat liver mitochondria have been studied. At low succinate concentration, respiration is initially stimulated and then inhibited by all the sulfonylureas tested, except carbutamide and the compound P571. Both phenomena are dose-dependent. The respiration inhibition can be reversed by increasing the succinate concentration, but not by adding ATP plus oligomycin. Respiratory control disappears progressively in the presence of increasing concentrations of sulfonylureas, including carbutamide and P571, mainly through inhibition of state 3 respiration. This inhibition can also be lowered by increasing the succinate concentration. The effect of sulfonylureas on respiration is oligomycin-insensitive. 2,4-Dinitrophenol has the same effect as the sulfonylureas under the same experimental conditions.

It is concluded that the sulfonylureas studied, except carbutamide and P571, exert a dinitrophenol-like effect on mitochondrial respiration, and that, like dinitrophenol, all sulfonylureas, including carbutamide and P571, interfere with mitochondrial substrate uptake. A relationship between the observed effects and the clinical activity of these drugs is discussed.

IT IS well known that sulfonylureas stimulate pancreatic insulin secretion *in vivo* as well as *in vitro*. However, several clinical studies suggest that increased insulin secretion alone does not account for all the metabolic effects observed during chronic as well as acute administration of sulfonylureas.[1–3] Several extrapancreatic effects have been described including interferences with mitochondrial energy metabolism. Falcone *et al.*[4] have reported a fall of P/O ratios in rat liver mitochondria oxidizing various substrates in the presence of chlorpropamide as well as an inhibition of mitochondrial exchange reactions and a stimulation of mitochondrial ATPase activity (ATP phosphohydrolase, EC 3.6.1.4). Penttilä[5] reported similar observations. In both reports, an inhibition of mitochondrial respiration was described in the presence of 2 mM chlorpropamide. No explanation for this phenomenon was given. Earlier work in this laboratory showed that chlorpropamide and tolbutamide interfere with the respiration of various rat tissues.[6] In a subsequent report[7] it was demonstrated that these drugs

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Abbreviations: DNP—2,4-dinitrophenol; R.C.—respiratory control.

lowered the ATP content of rat tissues and stimulated the oxidation of sugars by rat liver slices. In addition, in the presence of chlorpropamide or tolbutamide, mitochondrial ATP content, P/O ratios and respiratory control index were lowered while succinate and α-ketoglutarate oxidation increased.

Hellman and Idahl[8] have proposed that uncoupling of oxidative phosphorylation by sulfonylureas might be involved in the sulfonylurea-induced insulin secretion by pancreatic β-cells of obese hyperglycemic mice. Chan and Fain[9] have reported an uncoupling effect of tolbutamide on rat brown fat cells. A possible relationship between the uncoupling and the antilipolytic activity of the drug in these cells has been advanced.[10] Recent studies from this laboratory on the effect of chlorpropamide on the isolated perfused rat liver support the hypothesis that sulfonylureas may act as uncouplers of oxidative phosphorylation in various animal tissues.[11]

The purpose of the present study was first to extend our earlier observations to a number of clinically used as well as experimental sulfonylureas, and secondly to gain insight into the mechanism of mitochondrial respiratory inhibition at various concentrations of sulfonylureas.

MATERIALS AND METHODS

Preparation of mitochondria

Mitochondria were prepared from livers of overnight-fasted, male Wistar rats (150–200 g) essentially as described by Myers and Slater.[12] After decapitation, the liver was perfused *in situ* through the portal vein with approximately 6 ml of cold 0·25 M sucrose, quickly removed and placed in cold 0·25 M sucrose containing 0·01 M Tris–HCl pH 7·4. After weighing, the liver was minced and gently homogenized in 9 ml of sucrose–Tris/g liver using a Duall tissue grinder fitted with a loose Teflon pestle. The homogenate was centrifuged in the cold at 800 *g* for 5 min. Approximately 0·75 of the supernatant was centrifuged at 6500 *g* for 10 min. After suspension in 2 ml sucrose–Tris/g liver the sedimented mitochondria were centrifuged at 18,000 *g* for 10 min. The final pellet was suspended in 0·5 ml sucrose–Tris/g liver and kept on ice. Mitochondrial protein determinations were carried out according to Lowry *et al.*,[13] using bovine serum albumin as standard. Mitochondria were used within 2 hr after preparation.

Oxidative phosphorylation

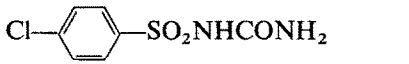
Mitochondrial oxygen uptake rates and respiratory control values (state 3/state 4)[14] were calculated from the tracings recorded by a Clark type electrode (Biological Oxygen Monitor, Yellow Springs Instrument Co., Yellow Springs, Ohio, U.S.A.). The oxygen concentration in the air-saturated medium at 30° was assumed to be 240 μM.[14] The incubation medium, adjusted to pH 7·4, contained 0·05 M KCl, 0·05 M sucrose, 0·05 M Tris–HCl, 0·01 M KH_2PO_4, 0·005 M $MgSO_4$, 0·001 M EDTA and 0·004 M Na-succinate. To 2·95 ml of this medium 0·05 ml of the mitochondrial suspension was added and the oxygen uptake measured under continuous magnetic stirring. The incubation mixture contained on the average 0·47 mg of mitochondrial protein per ml. All sulfonylureas were dissolved in dilute NaOH and made to the required concentration by dilution with water. The pH of the medium remained at 7·4 at the highest

concentrations of sulfonylureas used. 2,4-Dinitrophenol, oligomycin and rotenone were dissolved in ethanol. Appropriate controls with ethanol alone had no effect.

Chemicals

Sodium succinate, ADP and ATP, obtained from Boehringer GmbH, Mannheim, Germany, were dissolved in water and adjusted to pH 7·4. The concentration of the solutions of the nucleotides was determined spectrophotometrically at 259 nm and pH 7·4.

Tris buffer pH 7·4 (Trizma Pre-set), oligomycin, rotenone and bovine serum albumin (fraction V) were purchased from Sigma Co., St. Louis, Mo, U.S.A. 2,4-Dinitrophenol was purchased from Merck A.G., Darmstadt, Germany. Tolbutamide and carbutamide were obtained through the courtesy of Hoechst, A.G., Frankfurt a/Main, Germany. Chlorpropamide and the experimental sulfonylureas hereafter designated as P 571, P 584, P 594, P 1458 and P 2381 were obtained through the courtesy of Chas. Pfizer & Co., Groton, Conn., U.S.A. and Pfizer Ltd., Sandwich, Kent, U.K. The structural formulas of these experimental compounds are given below. All other reagents were analytical grade.



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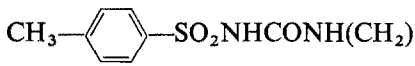

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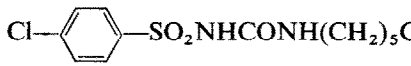

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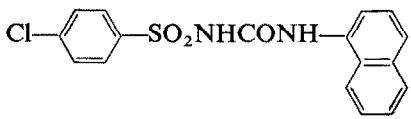
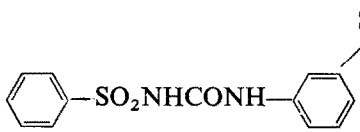


P 1458



P 2381

Statistical treatment of data

In most experiments the respiratory rates of the same mitochondria before drug addition served as control for the drug effect. Statistical analysis was applied only to the data of Table 3 where mitochondria, although obtained from the same liver, were used in separate control experiments. This statistical analysis was performed by means of analysis of variance and treatment by the method of orthogonal contrasts. More details are given in the appendix.

TABLE 1. EFFECT OF SULFONYLUREAS ON SUCCINATE OXIDATION IN ISOLATED RAT LIVER MITOCHONDRIA

Drug	(M)	State 4	SU_1	SU_2
Chlorpropamide	10^{-5} (13)	0·066	0·063	0·057
	10^{-4} (7)	0·069	0·070	0·063
	10^{-3} (8)	0·064	0·072	0·069
	5×10^{-3} (7)	0·057	0·100	0·052
	10^{-2} (5)	0·059	0·116	0·024
Carbutamide	10^{-5} (8)	0·054	0·055	0·053
	10^{-4} (8)	0·069	0·069	0·063
	10^{-3} (8)	0·065	0·064	0·062
	10^{-2} (8)	0·068	0·070	0·064
Glibenclamide	10^{-6} (8)	0·077	0·077	0·071
	10^{-5} (8)	0·081	0·083	0·081
	10^{-4} (8)	0·073	0·100	0·086
P 1458	5×10^{-7} (6)	0·057	0·054	0·049
	5×10^{-6} (12)	0·061	0·077	0·070
	5×10^{-5} (5)	0·056	0·179	0·067
	5×10^{-4} (5)	0·059	0·227	0·030
P 571	5×10^{-5} (5)	0·072	0·076	0·073
	5×10^{-4} (8)	0·072	0·076	0·068
	5×10^{-3} (7)	0·064	0·064	0·056

Rat liver mitochondria were preincubated for 1 min before sulfonylurea addition with: 50 mM sucrose; 50 mM Tris–HCl; 10 mM KH_2PO_4; 5 mM $MgSO_4$; 1 mM EDTA; and 4 mM sodium succinate. Mitochondrial protein was 0·47 mg/ml (range 0·45–0·51). Final volume: 3 ml; pH: 7·4; temp.: 30°. Oxygen uptake was measured polarographically. Respiration rates are expressed as μ atoms oxygen/min. State 4: respiratory rate before drug addition. SU_1: respiratory rate during 1st min after drug addition. SU_2: respiratory rate during 6th min after drug addition. Results are expressed as means of the number of experiments given in parentheses.

RESULTS

Effect of sulfonylureas on succinate oxidation

The effect of several sulfonylureas on respiration of mitochondria oxidizing succinate (4 mM) was examined. After an initial preincubation period of 1 min (state 4 according to Chance and Williams[14]), sulfonylureas were added in various concentrations. Respiratory rates during the first minute (SU_1) and the sixth minute (SU_2) after drug addition are given in Table 1. The same type of effect is seen with all compounds except carbutamide and P 571. The immediate effect of sulfonylureas (SU_1) is a dose-dependent stimulation of succinate oxidation. The subsequent effect (SU_2) is variable. The slight inhibition of respiration, observed in the presence of the lowest concentrations of the drugs is also seen in the absence of drug (data not shown). At intermediate drug concentrations the initial stimulation gradually levels off but the respiratory rate remains higher than during state 4. At high concentrations the drugs cause a strong inhibition of respiration. This corresponds to the inhibitory effect reported by Falcone *et al.*[4] and Penttilä,[5] using chlorpropamide and various oxidizable substrates. Tolbutamide was found to be of the same potency as chlorpropamide while

TABLE 2. EFFECT OF DIFFERENT PREINCUBATION CONDITIONS ON SUCCINATE OXIDATION IN THE PRESENCE OF 2,4-DINITROPHENOL AND CHLORPROPAMIDE IN RAT LIVER MITOCHONDRIA

Exp. No.	Preincubation	Respiratory rate	Addition I	Respiratory rate	Addition II	Respiratory rate
1	Succinate 4 mM	0·130	ADP	0·317–0·115	—	
2	Succinate 4 mM	0·115	DNP 10^{-5} M	0·187–0·158	—	
		0·101	DNP 10^{-4} M	0·302–0·043	ATP + oligomycin	0·101
		0·115	DNP 10^{-3} M	0·173–0·050	ATP + oligomycin	0·058
3	Succinate 4 mM + rotenone	0·108	DNP 10^{-4} M	0·173–0·122	—	
4	Succinate 40 mM + rotenone	0·108	DNP 10^{-5} M	0·202	—	
		0·101	DNP 10^{-4} M	0·518	—	
		0·072	DNP 10^{-3} M	0·374	—	
5	Succinate 4 mM	0·101	ADP	0·245–0·079	—	
6	Succinate 4 mM	0·108	Chlorprop. 10^{-4} M	0·072	ATP + oligomycin	0·058
		0·086	Chlorprop. 10^{-3} M	0·115–0·079	ATP + oligomycin	0·072
		0·072	Chlorprop. 5×10^{-3} M	0·101–0·036	ATP + oligomycin	0·050
		0·086	Chlorprop. 10^{-2} M	0·130–0·022	ATP + oligomycin	0·036
7	Succinate 4 mM + rotenone	0·101	Chlorprop. 5×10^{-3} M	0·202–0·050	—	
8	Succinate 40 mM	0·122	Chlorprop. 5×10^{-3} M	0·209	ATP + oligomycin	0·209
9	Succinate 40 mM + rotenone	0·101	Chlorprop. 10^{-3} M	0·115	—	
		0·086	Chlorprop. 5×10^{-3}M	0·187	—	
		0·101	Chlorprop. 10^{-2} M	0·230	—	

Experimental conditions as in Table 1, except that the preincubation time was 3 min and 4 or 40 mM sodium succinate was used as indicated. First addition after 3 min of preincubation; second addition 6 min after first addition. Where indicated the following compounds were present: ATP 2 mM, ADP 0·125 mM, oligomycin 1·33 μg/ml, rotenone 0·33 μg/ml. When the rate of respiration declined with time, both the initial and final rates are given. Respiratory rates are given as μatoms oxygen/min.

TABLE 3. EFFECT OF SULFONYLUREAS ON RESPIRATORY CONTROL AND UNCOUPLING OF OXIDATIVE PHOSPHORYLATION BY DINITROPHENOL IN ISOLATED RAT LIVER MITOCHONDRIA

Drug	(M)	Respiratory control		Dinitrophenol/State 4	
		Control	S.U.	Control	S.U.
Chlorpropamide	10^{-5} (5)	3·58	3·60	2·25	2·26
	10^{-4} (7)	4·66	3·44*	2·18	2·07*
	10^{-3} (8)	4·54	2·70†	2·61	1·98†
	5×10^{-3} (6)	3·99	1·03†	3·77	0·78‡
	10^{-2} (11)	3·90	1·05‡	3·17	0·87‡
Carbutamide	10^{-4} (8)	3·32	3·00	2·09	2·25
	10^{-3} (8)	4·55	3·62*	2·22	1·95*
	10^{-2} (8)	3·19	1·54‡	2·27	1·28‡
Glibenclamide	10^{-6} (8)	3·59	3·35	2·01	1·94
	10^{-5} (8)	3·77	2·80†	2·05	1·87*
	10^{-4} (8)	3·54	1·23‡	1·81	1·05‡
P 1458	5×10^{-7} (6)	3·29	3·37	2·55	2·51
	5×10^{-6} (12)	3·60	2·74*	2·25	1·85†
	5×10^{-5} (5)	3·76	1·00‡	2·61	1·15‡
	5×10^{-4} (5)	3·05	1·00‡	2·29	1·00‡
P 571	5×10^{-5} (5)	3·97	3·97	2·09	1·88
	5×10^{-4} (8)	4·47	3·46	1·91	1·62
	5×10^{-3} (7)	4·86	2·90*	2·22	1·62†

Experimental conditions as described in Table 1. ADP 0·125 mM was added after 6 min of incubation without (control) or with sulfonylureas (S.U.). 2,4-Dinitrophenol (DNP) 10^{-5} M was added 5 min after ADP addition. Results are expressed as ratios of oxygen uptake rates: respiratory control (State 3/State 4) and DNP/State 4. Results are means of the number of experiments given in parentheses. Statistical analysis of data: see materials and methods.

* $P < 0.05$.
† $P < 0.01$.
‡ $P < 0.001$.

the experimental sulfonylureas were more active by several orders of magnitude (P 1458 > P 594 ⩾ P 584 > P 2381). Carbutamide and P 571 have little or no effect on state 4 oxidation. No qualitative differences could be detected between the four clinically used sulfonylureas and the experimental compounds which are only slightly or not hypoglycemic when tested in acute animal experiments.[15]

Effect of different preincubation conditions on succinate oxidation in the presence of dinitrophenol or chlorpropamide

Inhibition of mitochondrial respiration in the presence of uncouplers of oxidative phosphorylation has been reported.[16–20] In the case of succinate oxidation, several explanations have been offered: (1) succinate dehydrogenase [succinate: (acceptor) oxidoreductase, EC 1.3.99.1] can be gradually inhibited by the rising concentrations of oxaloacetate and this inhibition can be reversed by adding oligomycin and ATP;[21–22] (2) oxidized coenzyme Q_{10} could also inhibit succinate dehydrogenase,[23] and ATP

abolishes this inhibition;[24] (3) succinate oxidation could be inhibited through competition of uncouplers with the uptake of substrate.[25–28] This phenomenon is also seen with other anionic substrates. Table 2 shows the results of experiments designed to gain insight into the above mentioned respiratory inhibition. Comparison between the data in Table 1 and those in experiment 2 of Table 2 shows that, using 4 mM succinate, DNP and sulfonylureas exert the same effect, DNP being more active. Experiments 2 and 6 indicate that the addition of oligomycin and ATP either during the inhibitory phase or during the preincubation period (data not shown) induces a stimulation of respiration but does not completely reverse the inhibition by DNP or chlorpropamide. Addition of rotenone, which prevents the formation of oxaloacetate, is not sufficient either to reverse the inhibition produced by 5×10^{-3} M chlorpropamide (experiment 7) or to allow a sustained stimulation of respiration in the presence of 10^{-4} M DNP (experiment 3). Inhibition of respiration by DNP or chlorpropamide is no longer seen in the presence of 40 mM succinate with (experiments 4 and 9) or without (experiment 8) the addition of rotenone. These results suggest that the inhibition of respiration is the consequence of a competitive inhibition of substrate uptake.

Effect of sulfonylureas on respiratory control and on DNP effect

Table 3 shows that mitochondrial respiratory control progressively disappears in the presence of increasing concentrations of sulfonylureas. Tolbutamide produced the same results as chlorpropamide and the experimental sulfonylureas displayed the same order of potency as in Table 1: P 1458 > P 594 ⩾ P 584 > P 2381. In addition, carbutamide and P 571, which did not affect succinate oxidation (Table 1) do lower the respiratory control. The tracings of oxygen uptake in state 3 and state 4 (data not shown) indicate that, in the presence of low concentrations of sulfonylureas, only state 3 respiration is inhibited. At increasing drug concentrations, the inhibition of state 3 respiration increases while state 4 respiration is affected as described under SU_2 in Table 1: stimulation at intermediate concentrations and inhibition at high concentrations of sulfonylureas. Inhibition of state 3 by concentrations of uncouplers which stimulate state 4 has also been observed with classical uncouplers of oxidative phosphorylation.[26,29] The effect of carbutamide and P 571 on respiratory control is also the result of an inhibition of state 3 without any effect on state 4. This could indicate that these compounds inhibit mitochondrial substrate uptake which becomes rate limiting under the high respiratory rates of state 3.

When dinitrophenol (10^{-5} M) was added 5 min after the addition of ADP, a progressively decreasing uncoupling effect by DNP was observed, in the presence of increasing concentrations of sulfonylureas. In some cases DNP even induced an additional inhibition of state 4 respiration. The decrease in the uncoupling effect of DNP could be the result either of a stronger inhibition of substrate uptake in the presence of sulfonylureas or of a competition between sulfonylureas and DNP, as will be further discussed in the following paper.

Effect of sulfonylureas on ADP-stimulated succinate oxidation (state 3)

Table 4 presents more data on state 3 respiration induced by 0·625 mM ADP. These data clearly show inhibition of state 3 by carbutamide as well as by 10^{-4} M chlorpropamide and by 10^{-5} M and 10^{-4} M tolbutamide. At these concentrations, no effect on state 4 respiration could be detected (Table 1).

TABLE 4. EFFECT OF SULFONYLUREAS ON MITOCHONDRIAL RESPIRATION STIMULATED BY ADP (STATE 3)

Drug	(M)	State 4	State 3	State 3 + SU	Inhibition of state 3 (%)
Control (8)		0·060	0·314	0·219	31
Chlorpropamide	10^{-5} (5)	0·060	0·263	0·203	23
	10^{-4} (5)	0·057	0·259	0·158	39
	10^{-3} (6)	0·056	0·253	0·099	61
Tolbutamide	10^{-5} (4)	0·071	0·349	0·199	43
	10^{-4} (5)	0·060	0·348	0·157	55
	10^{-3} (5)	0·066	0·313	0·100	69
Carbutamide	10^{-5} (4)	0·059	0·318	0·193	40
	10^{-4} (4)	0·061	0·307	0·185	40
	10^{-3} (4)	0·058	0·356	0·164	54

Experimental conditions as in Table 1. Sulfonylureas (SU) were added 1 min after inducing state 3 respiration by 0·625 mM ADP. Effect of SU was measured 4 min after their addition. Respiratory rates are expressed as μatoms oxygen/min and are means of the number of experiments given in parentheses.

Effect of various preincubation conditions on DNP- or chlorpropamide-inhibited state 3

Table 5, experiment 2 shows that increasing concentrations of DNP exert the same effect on state 3 respiration as sulfonylureas. The effect of chlorpropamide was studied in the presence of rotenone in order to exclude any inhibitory effect of oxaloacetate. A comparison of Table 4 with experiment 4 of Table 5 indicates that little or no inhibition of state 3 by chlorpropamide can be assigned to oxaloacetate accumulation. However, raising the succinate concentration from 4 to 40 mM produces both a higher state 3 respiratory rate in control experiments as well as a release of the spontaneous slowing-down of state 3 respiration observed in control experiments (Table 5, experiments 3 and 5). The inhibition of state 3 respiration by 10^{-3} M chlorpropamide disappears while a concentration of 5×10^{-3} M chlorpropamide remains inhibitory (Table 5, experiment 6). These experiments strongly suggest that in the presence of chlorpropamide as well as in control experiments substrate uptake is the limiting factor since the ADP concentration was the same in all experiments.

Effect of sulfonylureas on succinate oxidation by oligomycin-treated mitochondria

Uncoupling of oxidative phosphorylation is not affected by oligomycin, an inhibitor of oxidative phosphorylation.[30] Oligomycin (1·33 μg/ml) had practically no effect on state 4 respiration and in its presence, sulfonylureas exerted the same effect as that observed in the absence of oligomycin (Table 3). Identical results were obtained when oligomycin was added to the reaction mixture after addition of sulfonylureas.

DISCUSSION

Falcone *et al.*[4] and Penttilä[5] reported an uncoupling effect of chlorpropamide on oxidative phosphorylation in rat liver mitochondria. Similar observations have been made in this laboratory with chlorpropamide and tolbutamide.[7]

TABLE 5. EFFECT OF DIFFERENT PREINCUBATIONS ON STATE 3 INHIBITION BY 2,4-DINITROPHENOL OR CHLORPROPAMIDE

Exp. No.	Preincubation	State 4	State 3	Addition	Resp. rate	Inhibition of State 3 (%)
1	Succinate 4 mM	0·108	0·281	—	0·202	28
2	Succinate 4 mM	0·115	0·288	DNP 10^{-5} M	0·187	35
		0·122	0·331	DNP 10^{-4} M	0·130	61
		0·086	0·302	DNP 10^{-3} M	0·094	69
3	Succinate 4 mM + Rotenone	0·086	0·202	—	0·151	25
4	Succinate 4 mM	0·094	0·202	chlorprop. 10^{-4} M	0·137	32
	+ Rotenone	0·115	0·230	chlorprop. 10^{-3} M	0·101	56
5	Succinate 40 mM + Rotenone	0·094	0·432	—	0·432	0
6	Succinate 40 mM	0·144	0·446	chlorprop. 10^{-3} M	0·446	0
	+ Rotenone	0·072	0·374	chlorprop. 5×10^{-3} M	0·274	27

Experimental conditions as in Table 1. Preincubation time was 1 min and 4 mM or 40 mM sodium succinate was used as indicated. Rotenone, where present, was 0·33 μg/ml. 2,4-Dinitrophenol (DNP) or chlorpropamide were added 1 min after inducing state 3 respiration by 0·625 mM ADP. Drug effect was measured 4 min after its addition. Respiratory rates are expressed as μatoms oxygen/min.

The present report demonstrates a dinitrophenol-like effect of both clinically used and experimental sulfonylureas. Except for carbutamide and P 571, sulfonylureas, like DNP, stimulate and subsequently inhibit state 4 respiration in the presence of low substrate concentrations. This dual effect is dose-related and oligomycin-insensitive. In addition, dinitrophenol and all sulfonylureas examined including carbutamide and P 571 inhibit state 3 respiration at low substrate concentrations.

In the interpretation of the observed effect it should be noted that competitive inhibition of substrate uptake by uncouplers of oxidative phosphorylation has been described.[25–28] According to Veldsema-Currie and Slater[31] uncouplers such as dinitrophenol enter the mitochondria as the anion via a number of specific substrate carriers and thereby inhibit substrate uptake. Sulfonylureas seem to have this property in common with classical uncouplers. Indeed, data in Tables 2 and 5 show that the inhibition of both state 4 and state 3 respiration can be abolished by raising substrate concentration. Kraayenhof and Van Dam[28] proposed that the uncoupling anion, once inside, would react with a proton to form the undissociated acid, which diffuses outwards. This proton would be obtained by splitting a water molecule at the expense of a high-energy bond. This bond could represent the high energy intermediate thought to be hydrolyzed by uncoupling agents according to the classical chemical coupling hypothesis.[32,33] This energy dissipating process would result in a stimulatory effect on state 4 respiration, until this effect becomes eventually limited by the above mentioned inhibition of substrate uptake. This mechanism of action could also be applied to the sulfonylureas which, like the classical uncouplers, have a lipophilic and weak acid character.[34] Nevertheless, in comparison with dinitrophenol the sulfonylureas display

a more pronounced inhibitory effect on respiration relative to their stimulatory activity. This could reflect a limited mobility of the drug–substrate carrier complex across the mitochondrial membrane.

Our data indicate that the concentrations of drugs required to produce a marked effect on state 4 respiration exceed the therapeutic blood levels of the clinically used sulfonylureas. However, respiratory control index and state 3 respiration are influenced by concentrations of 10^{-4} M chlorpropamide or tolbutamide which are well within the clinically effective range. Although no data are available on the intracellular distribution of sulfonylureas, especially during their chronic administration, our data indicate that not only *in vitro* vs *in vivo* drug concentrations but also substrate concentrations have to be taken into account.

Not all metabolic effects of hypoglycemic sulfonylureas can be accounted for by increased insulin secretion alone, even during acute administration.[1-3] Several authors have described an uncoupling effect of sulfonylureas in various animal tissues. This uncoupling effect has been discussed in relationship with their antilipolytic activity in rat fat cells,[9,10] their antiketogenic effect in rat liver,[11] and even their stimulation of insulin secretion by pancreatic islets of obese hyperglycemic mice.[8] However, our results do not show qualitative differences in the response of rat liver mitochondria to hypoglycemic vs experimental sulfonylureas. These experimental compounds are slightly or not hypoglycemic when tested in acute animal experiments.[15] The hypoglycemic carbutamide and the non-hypoglycemic P 571 do not stimulate state 4 respiration, while the other little or not hypoglycemic compounds are more potent stimulators of state 4 respiration than the clinically active sulfonylureas. This would indicate that uncoupling of oxidative phosphorylation cannot be the main determinant in their hypoglycemic activity after acute administration but does not preclude a role of their uncoupling activity in their long-term extrapancreatic effects. In an attempt to assess how the uncoupling activity of sulfonylureas could explain some of these long-term effects, it might be of interest to administer chronically to animals these experimental compounds which are not hypoglycemic in acute experiments.

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APPENDIX

Table 3 in the foregoing paper deals with the comparison of ratios. Student's *t*-test or the common methods of analysis of variance cannot be applied to ratios. Hereafter we will briefly discuss the statistical basis used in the comparison of the respiratory control indices (state 3/state 4).* The same discussion applies to the dinitrophenol/state 4 ratios.

Testing the significance of differences between the above mentioned ratios, the fact that no difference should occur between these ratios was taken as null hypothesis. Translated in mathematical terms this null hypothesis becomes:

$$\left(\frac{\text{state 3}}{\text{state 4}}\right)_C = \left(\frac{\text{state 3}}{\text{state 4}}\right)_{SU} \tag{1}$$

$$\text{or: } \left(\frac{\text{state 3}}{\text{state 4}}\right)_C \Big/ \left(\frac{\text{state 3}}{\text{state 4}}\right)_{SU} = 1. \tag{2}$$

The use of respiratory control indices implies that treatment effects are not additive, but multiplicative, i.e. addition of ADP, SU or combinations of both to state 4, or addition of SU to state 3, increases or decreases the original respiration rates by a certain percentage. In such cases a logarithmic transformation is indicated.[35]

After logarithmic transformation equation (2) becomes:

$$(\ln \text{state}_3 - \ln \text{state}_4)_C - (\ln \text{state}_3 - \ln \text{state}_4)_{SU} = 0 \tag{3}$$

Now, a usual analysis of variance can be applied to these individually logarithmic transformed respiration rates. A two way classification[36] was chosen with four columns (state_{3C}, state_{4C}, state_{3SU}, state_{4SU}) and with a number of rows identical to the number of experiments. After isolating the row effect (effect between livers since each control experiment was carried out with mitochondria of the same liver as the corresponding SU experiment), the column effect was compared with the residual error by a *F*-test. When the column effect is significantly different from the residual error, it can be further subdivided and compared using the method of orthogonal contrasts and the following coefficients:[37]

* Abbreviations. State 4—state 4 respiration rate; State 3—state 3 respiration rate; C—control; SU—sulfonylureas.

	$State_{3C}$	$State_{4C}$	$State_{3SU}$	$State_{4SU}$
SU vs C	−1	−1	+1	+1
$State_4$ vs $State_3$	−1	+1	−1	+1
Interaction	+1	−1	−1	+1

The coefficients of the third row, called "Interaction", are identical to those of equation (3). Hence, to test significance of differences between the above mentioned ratios the interaction effect was compared by the *F*-test with the residual error obtained through analysis of variance.

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