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METABOLIC EFFECTS OF HYPOGLYCEMIC SULFONYLUREAS—III.

EFFECT OF CHLORPROPAMIDE ON ENERGY METABOLISM AND KETOGENESIS IN THE ISOLATED PERFUSED RAT LIVER*

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Abstract—The effects of 1 and 5 mM chlorpropamide were studied in the isolated perfused liver from fasted rats in the absence of exogenous substrate. 1 mM chlorpropamide caused a slight, but not significant, decrease in ATP content and in ATP/ADP ratio in the liver; 5 mM chlorpropamide caused a significant decrease in ATP, total adenine nucleotides and ATP/ADP ratio and an increase in ADP, AMP and inorganic phosphate content.

The redox states of both cytosol and mitochondria, as measured by the lactate/pyruvate and β-hydroxybutyrate/acetoacetate ratios, respectively, were unaffected. However, there was a significant and dose dependent decrease in acetoacetate and β-hydroxybutyrate content.

Livers perfused with 1 mM chlorpropamide showed a slight, but not significant, increase in oxygen uptake; 5 mM chlorpropamide had a biphasic effect: an initial stimulation, followed by a progressive inhibition of oxygen uptake. Ketone body production measured in the perfusion medium was inhibited by 1 and 5 mM chlorpropamide. No influence could be seen on ureogenesis. The measured rates of oxygen uptake by the liver compared with the changes in oxygen uptake calculated from the reduced ketogenesis indicate a mixed effect of chlorpropamide on the perfused liver; (1) a decreased oxidation of endogenous triglycerides and (2) an increased oxidation of acetyl coenzyme A through the tricarboxylic acid cycle.

The present findings suggest that chlorpropamide acts on the perfused liver as an uncoupling agent and as an inhibitor of the hepatic triglyceride lipase. (Glycerol ester hydrolase, EC 3.1.1.3.) Both effects could be related.

DURING the last two decades, sulfonylureas have been widely used in the treatment of mild maturity-onset diabetes although their mechanism of action has not been completely elucidated. Numerous *in vivo* and *in vitro* studies have clearly established that these compounds stimulate insulin secretion. Nevertheless, several clinical studies suggest that sulfonylureas also exert extrapancreatic effects since enhanced insulin secretion does not seem to account for the effects observed during long-term treatment with these drugs.[1,2] Berchtold *et al.*[3] have recently suggested that tolbutamide might have extrapancreatic effects even after acute administration. Several effects of sulfonylureas on rat liver have been described such as reduced ketogenesis in liver slices,[4]

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inhibition of triglyceride lipase in homogenates[5] and normalization of urea production in the perfused liver of alloxan diabetic animals.[6] We have demonstrated the interference of sulfonylureas with the energy metabolism of several rat tissues[7,8] and with oxidative phosphorylation[8] in isolated rat liver mitochondria. Similar effects have been observed in brown fat cells[9] and pancreatic β-cells.[10,11]

In the present work, we have examined the effect of chlorpropamide on energy metabolism, ketogenesis and urea production in the isolated perfused rat liver.

MATERIALS AND METHODS

Animals. Male Wistar rats, maintained on stock laboratory diet were used. Livers were taken from rats weighing 150–200 g, starved 24–28 hr before use.

Operative technique. The operative technique described by Hems *et al.*[12] was used except that the heparin was injected into the penis just before cannulation of the portal vein. The time lapse between portal cannulation and connection of the liver to the perfusion apparatus was less than 4 min. General appearance of the liver and bile production (0·075–0·1 ml/g liver/hr) were taken as indications of a successful perfusion.

Perfusion medium. The perfusion medium was essentially as described by Hems *et al.*[12] and contained Krebs–Henseleit bicarbonate buffer pH 7·4, 2·6% (w/v) bovine serum albumin (Armour fraction V), washed fresh human red cells to a final hemoglobin concentration of 2·5% (w/v) and 1 mg% (w/v) Ampicillin. The red cells were omitted in the oxygen uptake studies. Albumin was dialysed for 48 hr at 4° against 100 vol. of Krebs–Henseleit buffer prior to use. Hemoglobin was determined as cyanmethemoglobin.[13]

Perfusion apparatus. The perfusion apparatus is composed of an upper reservoir, with overflow, a liver chamber and an oxygenator, all thermostated at 36° by a water-jacket. From the upper reservoir the medium passes through a plastic mesh filter taken from a disposable transfusion set, flows through the liver and is collected in the oxygenator from which it is pumped back into the upper reservoir by a peristaltic pump (Watson-Marlow MHRE 2000). The height of the reservoir is adjustable to allow perfusion pressures between 10 and 25 cm H_2O. The overflow is connected to the oxygenator. In the perfusions with red cells the oxygenator used was essentially a cone, on top of which the medium leaving the liver dropped and spread as a thin film over its surface. In the hemoglobin-free perfusions a rotating disk oxygenator was used, which provided good oxygenation at the flow rates used, the oxygen concentration of the medium entering the liver being 0·7 mM. The gas mixture in both systems was 95% O_2–5% CO_2, saturated with water at 36°. pH was continuously monitored using a semi-micro combined glass electrode inserted in the medium circuit. Flow through the liver was measured by clamping the outlet of a disposable calibrated 10 ml syringe, inserted between liver outflow and oxygenator. In the hemoglobin-free perfusions oxygen consumption was measured by placing a Clark type Pt–Ag electrode before and after the liver. (Yellow Springs Instruments, model 53 Biological Oxygen Monitor.) The electrodes were calibrated against air-saturated saline at 36°. A Bunsen coefficient of 0·023 for oxygen at 36° was used in further calculations.[14] The perfusion pressure varied between 12 and 17 cm water to give a flow rate of 4–5 ml/min/g during perfusion with red cells. When oxygen uptake was measured, the pressure was adjusted to give a maximal oxygen consumption by the

liver and here the flow was always higher than 6 ml/min/g. All perfusions were carried out at 36°.

Addition of drug. Chlorpropamide, recrystallized twice from ethanol, was prepared as a stock solution in 1 N sodium hydroxyde and diluted appropriately with Krebs–Henseleit buffer before addition to the perfusion medium. In the oxygen uptake experiments the dilution was made with fresh medium and the pH was adjusted to 7·4. The drug was added through the overflow and was replaced by an equal volume of medium in control exepriments. Uniform mixing in the apparatus was achieved within 1 min.

Treatment of medium samples. Two ml medium samples were added to 6 ml ice-cold $HClO_4$ (3·3% w/v). The precipitate was removed by centrifugation in the cold at 38,000 *g* for 10 min. The supernatant was adjusted to pH 7 with 5M K_2CO_3 and after standing in the cold, the $KClO_4$ precipitate was centrifuged off.

Treatment of liver. At the end of the perfusion the liver was rapidly disconnected and pressed between metal clamps, previously cooled in liquid nitrogen.[15] The frozen liver was weighed and pulverized in a precooled mortar with frequent additions of liquid N_2. The powder was transferred to precooled centrifuge tubes containing frozen, pulverized $HClO_4$ (6% w/v). After mixing with a glass rod, ice-cold $HClO_4$ (6%, w/v) was added to a final volume of 5 ml per gram tissue, and the mixture was immediately homogenized with an Ultra–Turrax homogenizer. The pellet, obtained by centrifugation for 10 min. at 38,000 *g*, was washed with 2 ml of ice-cold $HClO_4$ (3% w/v) per g tissue. After centrifuging, the combined supernatants were adjusted to a final volume of 8 ml/g tissue and neutralized as described above. Part of the supernatant was shaken for 30 sec with acid-washed Florisil (0·1 g/ml) as described by Williamson *et al.*[16] In some experiments a portion of the powdered liver was digested with KOH and the glycogen isolated and hydrolysed as described by Good *et al.*[17]

Chemicals. Lactate, pyruvate, adenine nucleotides and all enzymes used were obtained from Boehringer Gmbh, Mannheim, Germany. NAD^+, NADH, acetoacetate,D-Lβ-hydroxybutyrate were obtained from Sigma Chemical Company, St. Louis, Missouri, U.S.A. Florisil (60–100 mesh) was obtained from Koch–Light Laboratories Ltd., Colnbrook-Bucks, U.K. Anthrone was obtained from E. Merck, A. G. Darmstadt, Germany. Chorpropamide was a gift from Pfizer Ltd., Sandwich, U.K. Commerical Ampicillin was used. All other reagents were analytical grade.

Determination of metabolites. Neutralized liver extract was used for the determination of ATP,[18] ADP,[19] AMP[19] and inorganic phosphate.[20] Lactate,[21] pyruvate,[22] β-hydroxybutyrate[23] and acetoacetate[24] were determined on Florisil-treated extract. Glycogen was determined as glucose by the anthrone method.[25] Neutralized medium samples were analysed for β-hydroxybutyrate[23] and acetoacetate;[24] urea was assayed by the urease method using a commercial test kit (Boehringer Gmbh, Mannheim, Germany).

Determination of unbound drug concentration. Unbound drug concentration was determined as described by Pedersen,[26] by centrifuging at 36° dried cellophane dialysis bags (8/32 in., Arthur H. Thomas Company, Philadelphia, Pa., U.S.A.) filled with cell-free perfusion medium containing 1 or 5 mM chlorpropamide. Appropriate dilutions of the ultrafiltrate were made in Krebs–Henseleit bicarbonate buffer and the extinction read at 232·5 nm in a Zeiss PMQ II spectrophotometer. Under these conditions the molar extinction coefficient of chlorpropamide was found to be $11{\cdot}1 \times 10^3$.

Statistical methods. All values are given as arithmetical means ± standard error of the mean, unless otherwise stated. The significance of differences between means was established by the Student *t*-test. The oxygen uptake experiments were treated as paired data and tested for significance by Student's *t*-test.

RESULTS

Concentration of unbound drug in the perfusion medium. The concentration of unbound chlorpropamide in the perfusion medium was found to be 0·3 and 3·3 mM at total concentrations of 1 and 5 mM respectively.

Effect of chlorpropamide on adenine nucleotides and inorganic phosphate. The control values in Table 1 show that during the perfusion, without substrate, of livers from fasted rats the concentration of adenine nucleotides remains constant after 35, 45 and 90 min of perfusion. When the perfusion medium contained 1 mM chlorpropamide after 30 min of perfusion, ATP content and ATP/ADP ratio decreased, although not significantly, after 15 and 60 min of perfusion with chlorpropamide. When livers were perfused with 5 mM chlorpropamide for 90 min, ATP, total nucleotides and ATP/ADP ratio were significantly decreased while ADP, AMP and inorganic phosphate increased.

Effect of chlorpropamide on lactate/pyruvate and β-hydroxybutyrate/acetoacetate ratios. The lactate/pyruvate and β-hydroxybutyrate/acetoacetate ratios are a measure of the redox state of the liver cytosol and mitochondria respectively.[16] Table 2 shows that chlorpropamide has little or no effect on these ratios in the perfused rat liver. When the *t*-test was applied to the lactate/pyruvate ratios of the 35 min experiments, the difference between the means appeared significant. However, the *t*-test cannot be applied to ratios. The data in Table 2 clearly shows that the individual components of the ratios in the control and the sulfonylurea groups belong to the same population. Therefore, their ratio cannot be significantly different. This was again demonstrated when the ratios were compared by means of variance analysis and orthogonal contrasts. However, after perfusion with chlorpropamide the concentration of acetoacetate and β-hydroxybutyrate in the liver decreased in a dose dependent way. Inhibition of ketogenesis in rat liver slices by tolbutamide, another hypoglycemic sulfonylurea, has been reported by Boshell *et al.*[4] No explanation has been found for the increase of pyruvate content of the liver after 90 min perfusion with 5 mM chlorpropamide.

Effect of chlorpropamide on oxygen uptake. Livers of fasted rats were perfused with a cell-free medium and without substrate as described under Materials and Methods. Preliminary experiments (data not shown) have indicated that, after an initial equilibration period of 12–15 min, the oxygen uptake of the perfused livers remained constant for up to 90 min. Therefore, in the following experiments each liver was taken as its own control (paired data), and the stabilized oxygen consumption at minute 30 was used as control value. The effect of chlorpropamide on oxygen uptake is presented in Table 3.

When chlorpropamide (1 mM final concentration) was added to the medium at minute 30 a slight but not significant increase in oxygen uptake occurred. Raising the concentration at 2 mM at minute 50 resulted in an additional but still not significant

TABLE 1. EFFECT OF CHLORPROPAMIDE ON ADENINE NUCLEOTIDES AND INORGANIC PHOSPHATE CONTENT OF THE ISOLATED RAT LIVER PERFUSED WITHOUT SUBSTRATE

Total duration of perfusion time (min)	Experimental conditions	ATP	ADP	AMP	Sum nucleotides	P_i	ATP / ADP
		[μmoles/g liver (wet weight)]					
35	Control (4)	2·83 ± 0·09	1·04 ± 0·06	0·14 ± 0·01	4·02 ± 0·16	4·49 ± 0·07	2·72 [2·64–2·87]
	1 mM drug at min 30 (4)	2·85 ± 0·07	0·92 ± 0·06*	0·12 ± 0·01*	3·89 ± 0·11	4·18 ± 0·26	3·09 [2·75–3·44]
45	Control (4)	2·85 ± 0·16	1·01 ± 0·04	0·11 ± 0·06	3·97 ± 0·19	4·49 ± 0·19	2·82 [2·49–3·17]
	1 mM drug at min 30 (4)	2·60 ± 0·11	1·15 ± 0·09	0·16 ± 0·02†	3·91 ± 0·10	4·46 ± 0·12	2·27 [1·75–2·85]
90	Control (4)	2·78 ± 0·07	1·04 ± 0·04	0·13 ± 0·01	3·95 ± 0·12	3·77 ± 0·19	2·67 [2·56–2·74]
	1 mM drug at min 30 (4)	2·58 ± 0·11*	0·98 ⊥ 0·11	0·14 ± 0·01	3·70 ± 0·23	3·52 ± 0·29	2·62 [2·22–3·37]
	5 mM drug at min 0 (4)	2·37 ± 0·07†	1·15 ± 0·03†	0·16 ± 0·01*	3·68 ± 0·08†	4·75 ± 0·23†	2·07 [1·92–2·36]

Rats were fasted 24–28 hr before perfusion. Chlorpropamide (1 mM final concentration) was added to the perfusion medium after 30 min and the livers perfused for different times. When the medium contained 5 mM in chlorpropamide, the drug was added at the beginning of the perfusion and the livers perfused for 90 min. Results are given as means ± S.E.M. Ratios are given as means with ranges between brackets. Figures in parentheses indicate number of experiments. Significance is indicated as: * $P < 0·10$; † $P < 0·05$.

TABLE 2.—EFFECT OF CHLORPROPAMIDE ON LACTATE, PYRUVATE, β-HYDROXYBUTYRATE AND ACETOACETATE CONTENT, AND "REDOX-STATE" OF THE PERFUSED RAT LIVER

Time (min)	Experimental conditions	Lactate	Pyruvate	β-Hydroxybutyrate	Acetoacetate	Lactate / Pyruvate	β-Hydroxybutyrate / acetoacetate
		[μmoles/g liver (wet weight)]					
35	Control	0·315 ± 0·039	0·046 ± 0·002	0·160 ± 0·023	0·478 ± 0·057	6·80 [3·85– 8·62]	0·33 [0·25–0·43]
	1 mM drug at min 30 (4)	0·556 ± 0·108	0·041 ± 0·003	0·077 ± 0·008*	0·244 ± 0·047	13·55 [9·08–17·65]	0·22 [0·14–0·36]
45	Control	0·373 ± 0·042	0·039 ± 0·004	0·135 ± 0·014	0·506 ± 0·122	9·57 [7·08–13·87]	0·27 [0·19–0·76]
	1 mM drug at min 30 (4)	0·427 ± 0·026	0·043 ± 0·003	0·166 ± 0·028	0·430 ± 0·019	9·82 [8·42–12·32]	0·39 [0·26–0·61]
90	Control	0·282 ± 0·019	0·049 ± 0·003	0·123 ± 0·004	0·529 ± 0·052	5·73 [4·72– 7·95]	0·23 [0·21–0·29]
	1 mM drug at min 30 (4)	0·409 ± 0·089	0 047 ± 0 005	0 064 ± 0·008†	0·280 ± 0·045*	8·46 [4·48–17·14]	0·23 [0·15–0·39]
	5 mM drug at min 0 (4)	0·282 ± 0·090	0·063 ± 0·001†	0·024 ± 0·003†	0·122 ± 0·009†	4·09 [2·45– 6·68]	0·20 [0·16–0·24]

Same livers and perfusion conditions as in Table 1. Results are given as means ± S.E.M. Ratios are given as means with the range between brackets. Figures in parentheses indicate number of experiments. Significance is indicated as: * $P < 0·02$. † $P < 0·001$.

TABLE 3. EFFECT OF CHLORPROPAMIDE ON OXYGEN UPTAKE BY THE PERFUSED RAT LIVER

Experimental conditions	O_2-uptake [μmoles/min/g liver (wet weight)]
Control (5)	2·751 ± 0·256
1 mM drug	+0·051 ± 0·068
2 mM drug	+0·079 ± 0·109
Control (4)	2·786 ± 0·204
5 mM drug (stimul.)*	+0·164 ± 0·035†
5 mM drug (inhib.)*	−0·265 ± 0·057†
10 mM drug	−0·469 ± 0·022†

Rats were fasted 24–28 hr before perfusion. The livers were perfused without substrate with a cell free medium as described in "Materials and Methods". The first drug dose was added at min 30. The stabilized oxygen uptake at min 30 has been used as the control value (paired data). Results are given as means ± S.E.M. Figures in parentheses indicate number of experiments. * See text for explanation. † $P < 0·05$.

stimulation of oxygen consumption which lasted during the next 20 min of observation time. In another series of experiments the perfusion medium was made 5 mM in chlorpropamide at minute 30 which resulted in a biphasic effect on oxygen uptake. After an initial rise in oxygen consumption lasting for approximately 20 min (maximal stimulation reported in Table 3) a progressive inhibition of oxygen uptake developed which stabilized after about 10 min (inhibition reported in Table 3). When, at minute 75, the chlorpropamide concentration was raised to 10 mM, oxygen uptake by the liver was further inhibited.

Effect of chlorpropamide on ketone body production. Inhibition of ketogenesis in rat liver slices[4] and of triglyceride lipase in rat liver homogenates[5] by tolbutamide have been reported. No effect on ketogenesis in the isolated perfused rat liver could be demonstrated by Willms *et al.*[6] using glibenclamide and Söling *et al.*[27] using carbutamide. However, the data presented in Table 4 clearly show that ketone body output by the cell-free perfused rat liver is inhibtted by chlorpropamide in a dose dependent

TABLE 4. EFFECT OF CHLORPROPAMIDE ON KETONE BODY PRODUCTION BY THE PERFUSED RAT LIVER

	Sum of ketone bodies [μmoles/g liver (wet weight)]			
	Time (min)			
	0	30	60	90
Control (4)	0·00	10·24 ± 1·54	22·53 ± 6·25	31·92 ± 4·06
1 mM drug at min 30 (4)	0·00	7·66 ± 1·52	13·71 ± 1·22	18·98 ± 1·90*
5 mM drug at min 0 (4)	0·00	1·82 ± 0·61‡	3·48 ± 0·43†	5·11 ± 0·61‡

Ketone body production was measured in the perfusate of livers perfused for 90 min with a cell free medium. Ketones were taken as the sum of β-hydroxybutyrate and acetoacetate production. Results are given as means ± S.E.M. Figures in parentheses indicate number of experiments. Significance is indicated as * $P < 0·02$. † $P < 0·01$. ‡ $P < 0·001$.

way. The discrepancy between our results and those mentioned above could be explained by a specific effect of chlorpropamide, not seen with other hypoglycemic sulfonylureas. A more likely explanation is that the perfusion medium used by Willms *et al.*[6] and Söling *et al.*[27] contained 100 mg % glucose which is markedly antiketogenic. When livers of fasted rats were perfused in our laboratory with 10 mM lactate, no effect of chlorpropamide (1 mM) on ketogenesis could be demonstrated (unpublished results).

Effect of chlorpropamide on urea production. As shown in Table 5, chlorpropamide has no effect on urea production by the perfused rat liver.

TABLE 5. EFFECT OF CHLORPROPAMIDE ON UREA PRODUCTION BY THE PERFUSED RAT LIVER

	Urea [μmoles/g liver (wet weight)]			
	Time (min)			
	0	30	60	90
Control (4)	0·00	8·89 ± 0·51	15·80 ± 1·39	20·13 ± 2·42
1 mM drug at min 30 (4)	0·00	8·53 ± 0·68	13·25 ± 1·26	19·98 ± 1·50
5 mM drug at min 0 (4)	0·00	11·18 ± 1·20	16·01 ± 1·93	18·09 ± 1·43

Urea production was measured in the perfusate of the same livers as described in Table 4. Results are given as means ± S.E.M. Figures in parentheses indicate number of experiments.

Comparison of the effect of chlorpropamide on ketogenesis and oxygen consumption. The rate of ketone body production by the perfused rat liver (Table 4) remained constant in the control group (minute 0–90) as well as in the presence of chlorpropamide 1 mM (minute 30–90) and 5 mM (minute 0–90). These rates are shown in columns 1 and 2 of Table 6 for acetoacetate and β-hydroxybutyrate production. The observed decrease in ketogenesis could be the result of two main mechanisms. Chlorpropamide could affect directly or indirectly ketogenesis through inhibition of triglyceride lipase and/or fatty acid oxidation. In this case there should be a corresponding decrease in oxygen consumption by the liver. This decrease can be calculated from the measured decrease in ketone body production, assuming that oleate resembles the average composition of fatty acids in the liver.[28] The overall reactions used for the calculation are:

$$C_{18}H_{34}O_2 + 7{\cdot}5\ O_2 \rightarrow 4{\cdot}5 \text{ acetoacetate}$$

$$C_{18}H_{34}O_2 + 5{\cdot}25\ O_2 \rightarrow 4{\cdot}5\ \beta\text{-hydroxybutyrate}$$

The results of these calculations are shown in Table 6, column 3. On the other hand, chlorpropamide could decrease ketogenesis by stimulating the oxidation of acetyl coA in the tricarboxylic acid cycle. In this case, there should be a corresponding increase in oxygen uptake. The reactions are:

$$\text{acetoacetate} + 4O_2 \rightarrow 4CO_2 + 3H_2O$$

$$\beta\text{-hydroxybutyrate} + 4{\cdot}5\ O_2 \rightarrow 4CO_2 + 4H_2O$$

TABLE 6. KETONE BODY FORMATION AND OXYGEN UPTAKE BY THE PERFUSED RAT LIVER

	Acetoacetate formed (μmoles/g/min)	β-Hydroxybutyrate formed (μmoles/g/min)	Difference in O_2-uptake against control, calculated from difference in ketone body production, assuming a decreased oxidation of fatty acids (μmoles O_2/g/min)	Difference in O_2-uptake against control, calculated from difference in ketone body production, assuming an increased oxidation of acetyl coA (μmoles O_2/g/min)	Measured difference in O_2-uptake against control (data from Table 3) (μmoles O_2/g/min)
Control (4)	0·316 ± 0·037	0·045 ± 0·005			
1 mM drug (4)	0·186 ± 0·022	0·002 ± 0·008	− 0·286 ± 0·077	+ 0·731 ± 0·364	+ 0·051 ± 0·068
5mM drug (4)	0·052 ± 0·006	0·005 ± 0·001	− 0·487 ± 0·065	+ 1·238 ± 0·150	− 0·265 ± 0·057

The perfusion conditions are as described previously. Ketone body production was linear during the studied period (Table 4). The O_2-uptake required for the formation of ketone bodies was calculated on the assumption that oleate resembles the average composition of the endogenous fatty acids. Results are given as means ± S.E.M. Figures in parentheses indicate number of experiments. Data of column 5 are significantly different from the corresponding values in both columns 3 and 4, with $P < 0{\cdot}02$.

The results of these calculations are shown in Table 6, column 4. Our experimental data on the effect of chlorpropamide on oxygen uptake by the perfused rat liver, presented in Table 3, are summarized in Table 6, column 5. These values lie between the calculated values of columns 3 and 4. They suggest that in these experiments chlorpropamide exerts a mixed effect on the perfused rat liver: an inhibition of oxidation of fatty acids which could be a result of the described inhibition of triglyceride lipase,[5] and a stimulation of oxidation through the tricarboxylic acid cycle which could be the result of the uncoupling effect of sulfonylureas demonstrated by us on isolated rat liver mitochondria.[8] That the changes in oxygen uptake brought about by chlorpropamide result mainly from an interference with fatty acid metabolism can be derived from the fact that ureogenesis was unaffected (Table 5) and that the fasted perfused livers contained very little glycogen: 0·94 ± 0·06 μmoles glucose/g wet weight ($n = 7$) after 30 min of perfusion.

DISCUSSION

Earlier work from this laboratory described an uncoupling effect of chlorpropamide and tolbutamide on isolated rat liver mitochondria.[8]

In this work, we have attempted to study the effect of chlorpropamide on energy metabolism under more physiological conditions and have therefore used the isolated perfused rat liver. Although a dose of 5 mM chlorpropamide (135 mg %) exceeds the therapeutic dose, a concentration of 1 mM (27 mg %) lies well within the range of the therapeutic blood levels. The mean level reported by Häussler and Wicha[29] is 10–20 mg % and the range is 2·5–32 mg %. The concentration of unbound drug in our perfusion system is 0·3 mM at a total concentration of 1 mM (27 mg %) and compares well with the plasma concentration of 0·13 mM computed from the binding data of Judis[30] for human serum albumin using 20 mg % chlorpropamide as total concentration. The data presented here seem to extend the uncoupling effect of chlorpropamide observed on isolated liver mitochondria[8] to the isolated perfused rat liver. The rates of oxygen uptake measured under chlorpropamide lie between the values calculated for the two above mentioned mechanisms of reduced ketogenesis, namely a decreased oxidation of fatty acids or an increased oxidation of acetyl CoA through the Krebs cycle (Table 6). This would point towards a mixed effect of chlorpropamide on the perfused rat liver. Similar observations have been made in rat liver slices with glibornuride, another blood sugar lowering sulfonamide.[31] The inhibition of oxidation of fatty acids could be explained by a diminished hepatic trigylceride lipase activity. Bewsher *et al.*[5] have described in rat liver homogenates the inhibition by tolbutamide of a triglyceride lipase with a pH optimum of 7. The existence of several triglyceride lipases in liver with different pH optimum has been established by Guder *et al.*[32] An inhibition of the β-oxidation system as such seems unlikely in view of the findings of Hasselblatt[33] that in liver slices glycodiazin (0·8 mM) does not inhibit ketogenesis from exogenous long-chain fatty acids. In addition it is reasonable to assume that in our perfusions with not defatted albumin, the ketones originate from endogenous triglycerides as shown by Krebs and Hems.[34] An inhibition of fatty acid activation seems to be excluded despite the significant drop in ATP content of livers perfused with 5 mM chlorpropamide. Indeed, livers perfused with oleate by Williamson[35] and with octanoate or oleate in this laboratory (unpublished results) revealed a marked drop in ATP content concomitant with high rates of ketogenesis.

An increase of oxidation of acetyl CoA could be explained by a stimulation of the Krebs cycle activity. Indeed, inhibition of triglyceride lipase should result in a lower concentration of acyl CoA esters in the liver. This in turn might release a relative inhibition of the mitochondrial adenine nucleotide translocase,[36] lower the phosphorylation state of the intramitochondrial adenine nucleotides and hence stimulate the Krebs cycle. However, earlier work from this laboratory on isolated mitochondria[8] and our measurements of adenine nucleotides suggest an uncoupling effect of chlorpropamide on the perfused liver. Under 5 mM chlorpropamide, ATP and total nucleotide concentrations and ATP/ADP ratio are significantly lowered while ADP, AMP and inorganic phosphate concentrations increase. Similar effects, although not statistically significant, are already seen with 1 mM chlorpropamide (Table 1). That no effect was observed on the redox state of the cytosol or the mitochondria is rather unexpected.

Chan and Fain[9] have described an uncoupling effect of tolbutamide on rat brown fat cells comparable to the effect of carbonyl cyanide *m*-chlorophenyl hydrazone (*m*-Cl CCP), a typical uncoupler of oxidative phosphorylation. Tolbutamide (1·85 mM) as well as *m*-Cl CCP (0·5 mg %) inhibited lipolysis without significantly affecting the total ATP content of the cells.[37] This is what occurs when the rat liver is perfused with 1 mM chlorpropamide (Tables 1 and 4). Fain and Loken[38] have described a similar inhibition of lipolysis in brown fat cells by valinomycin, another uncoupler of oxidative phosphorylation. These authors suggest that the uncouplers could interfere with the energy-dependent steps in the activation of triglyceride lipase. This could also be the case for the hepatic triglyceride lipase, although Bewsher[5] reported a direct inhibition of the enzyme by tolbutamide in rat liver homogenates. However, the concentration of tolbutamide used by this author is not stated. Our results support the hypothesis of an interference of uncouplers with the energy-dependent activation of triglyceride lipase. Indeed, the initial stimulation of oxygen uptake by the liver in the presence of 5 mM chlorpropamide (Table 3) suggests that uncoupling of oxidative phosphorylation precedes the maximal effect on triglyceride lipase, which would then be responsible for the later inhibition of oxygen consumption.

Another possibility is that both effects occur simultaneously and independently and that the initial stimulation of oxygen uptake results from an accelerated oxidation of the preformed acyl coA esters in the liver. However, this seems unlikely since after 30 min perfusion under similar conditions Williamson *et al.*[39] found that the liver contains only 86 nmoles fatty acyl coA esters/g dry weight which gives 21 nmoles/g wet weight, assuming that the liver contains 75% water. Using our rate of ketogenesis, 71 nmoles of acyl coA esters would be used per min per g liver (wet weight) for ketone body production alone.

Although a concentration of 5 mM chlorpropamide exceeds the therapeutic blood levels, a concentration of 1 mM chlorpropamide, which lies within the range of therapeutic plasma levels,[29] has similar although less pronounced effects. Whether or not the hepatic antiketogenic effect of the sulfonylureas is involved in their therapeutic action is still a matter of debate (for review see Ref. 40). Indeed, an inhibition of the hepatic triglyceride lipase activity could hardly reduce ketogenesis in patients, since ketogenesis *in vivo* seems to be dependent on the free fatty acid supply to the liver. However, one must keep in mind that several workers described an antilipolytic action of sulfonylureas on white adipose tissue.[41–43] The stimulation

of acetyl CoA oxidation through the Krebs cycle, as suggested by us, seems too small to account for a significant reduction of ketogenesis under a continuous supply of free fatty acids *in vivo*. It has still to be established to what extent an increased Krebs cycle activity could stimulate carbohydrate oxidation in the liver. Furthermore an alteration of hepatic lipid metabolism, especially of hepatic triglyceride metabolism, could have profound implications in view of the recent findings of the University Group Diabetes Program.[44]

We conclude from this work that, in the perfused rat liver, chlorpropamide acts as an uncoupler of oxidative phosphorylation and inhibits ketogenesis probably through inhibition of the triglyceride lipase. Both effects could well be related.

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REFERENCES

1. M. FELDMAN and H. E. LEBOVITZ, *Diabetes* **20,** 745 (1971).
2. P. BERCHTOLD, P. BJÖRNTORP, A. GUSTAFSON, A. JONSSON and S. F. FAGERBERG, *Eur. J. Clin. Pharmac.* **4,** 22 (1971).
3. P. BERCHTOLD, V. BÜBER, V. MEIER, J.-P. FELBER and G. KEISER, *Diabetologia* **7,** 77 (1971).
4. B. R. BOSHELL, G. R. ZAHND and A. E. RENOLD, *Metabolism* **9,** 21(1960).
5. P. D. BEWSHER and J. ASHMORE, *Biochem. biophys. Res. Commun.* **24,** 431 (1966).
6. B. WILLMS, H. JELLINGHOUS, J. KLEINEKE and H. D. SÖLING, *Arzneimittelforsch* **19,** 1479 (1969).
7. P. J. DE SCHEPPER, *Biochem. Pharmac.* **16,** 2337 (1967).
8. L. DEBEER and P. J. DE SCHEPPER, *Biochem. Pharmac.* **16,** 2355 (1967).
9. S. S. CHAN and J. N. FAIN, *Molec. Pharmac.* **6,** 513 (1970).
10. B. HELLMAN, *Acta diabetol. Lat.* **6,** Suppl. 1, 597 (1970).
11. C. HELLERSTRÖM and R. GUNNARSSON, *Acta diabetol. Lat.* **7,** Suppl. 1, 127 (1970).
12. R. HEMS, B. D. ROSS, M. N. BERRY and H. A. KREBS, *Biochem. J.* **101,** 284, (1966).
13. D. DRABKIN and J. AUSTIN, *J. biol. Chem.* **98,** 719 (1932).
14. R. SCHOLZ, in *Stoffwechsel der Isoliert perfundierten Leber* (Ed. W. STAIB und R. SCHOLZ), p. 25. Springer, Berlin (1968).
15. A. WOLLENBERGER, O. RISTAU and G. SCHOFFA, *Pflügers Arch. ges. Physiol.* **270,** 399 (1960).
16. D. H. WILLIAMSON, P. LUND and H. A. KREBS, *Biochem. J.* **103,** 514 (1967).
17. C. A. GOOD, H. KRAMER and M. SOMOGYI, *J. biol. Chem.* **100,** 485 (1933).
18. H. ADAM in *Methods of Enzymatic Analysis* (Ed. H. U. BERGMEYER), 2nd edn, p. 539. Academic Press, New York (1965).
19. H. ADAM, in *Methods of Enzymatic Analysis* (Ed. H. U. BERGMEYER), 2nd edn, p. 573. Academic Press, New York (1965).
20. C. H. FISKE and Y. SUBBAROW, *J. biol. Chem.* **66,** 375 (1925).
21. H. J. HOHORST, in *Methods of Enzymatic Analysis* (Ed. H. U. BERGMEYER), 2nd edn, p. 266. Academic Press, New York (1965).
22. Th. BÜCHER, R. CZOK, W. LAMPRECHT and E. LATZKO, in *Methods of Enzymatic Analysis* (Ed. H. U. BERGMEYER), 2nd edn, p. 253. Academic Press, New York (1965).
23. D. H. WILLIAMSON and J. MELLANBY, in *Methods of Enzymatic Analysis* (Ed. H. U. BERGMEYER), 2nd edn, p. 459. Academic Press, New York (1965).
24. H. U. BERGMEYER and E. BERNT, *Enzym. biol. Clin.* **5,** 65 (1965).
25. W. Z. HASSID and S. ABRAHAM, in *Methods in Enzymology* (Eds. S. COLOWICK and N. O. KAPLAN), Vol. III, p. 34. Academic Press, New York (1957).
26. K. O. PEDERSON, *Scand. J. Clin. Lab. Invest.* **28,** 57 (1971).
27. H. D. SÖLING, *Acta diabetol. Lat.* **6,** Suppl. I, 396 (1969).
28. J. P. FLATT, *Diabetes* **21,** 50 (1972).
29. A. HÄUSSLER and H. WICHA, in *Handbuch der Experimentellen Pharmakologie* (Ed. H. MASKE), Vol. XXIX, p. 305. Springer, Berlin (1971).
30. J. JUDIS, *J. Pharm. Sci.* **61,** 89 (1972).
31. H. D. SÖLING, *Communication to the Vth Int. Congress on Pharmacology*, San Francisco (1972.)
32. W. GUDER, L. WEISS and O. WIELAND, *Biochim. biophys. Acta* **187,** 173 (1969).

33. A. HASSELBLATT, *Naunyn-Schmiedeberg's Arch. Pharm. exp. Path.* **262,** 152 (1969).
34. H. A. KREBS and R. HEMS, *Biochem. J.* **119,** 525 (1970).
35. J. R. WILLIAMSON, R. A. KREISBERG and P. W. FELTS, *Proc. natn. Acad. Sci., U.S.A.* **56,** 247 (1966).
36. S. V. PANDE and M. C. BLANCHAER, *J. biol. Chem.* **246,** 420 (1971).
37. J. N. FAIN, J. W. ROSENTHAL and W. F. WARD, *Endocrinology* **90,** 52 (1972).
38. J. N. FAIN and S. LOKEN, *Mol. Pharmac.* **7,** 455 (1971).
39. J. R. WILLIAMSON, R. SCHOLTZ and E. T. BROWNING, *J. biol. Chem.* **244,** 4617 (1969).
40. J. M. FELDMAN and H. E. LEBOVITZ, *Arch. intern. Med.* **123,** 314 (1969).
41. D. B. STONE and J. D. BROWN, *Diabetes* **15,** 314 (1966).
42. D. B. STONE, J. D. BROWN and C. P. COX, *Am. J. Physiol.* **210,** 26 (1966).
43. J. D. BROWN and D. B. STONE, *Endocrynology* **81,** 71 (1967).
44. THE UNIVERSITY GROUP DIABETES PROGRAM, *Diabetes*, Suppl. 2, **19,** 747 (1970).