Biochemical Pharmacology, Vol. 16, pp. 2355–2367. Pergamon Press Ltd. 1967. Printed in Great Britain

METABOLIC EFFECTS OF HYPOGLYCEMIC SULFONYLUREAS—II.

IN VITRO EFFECT OF SULFONYLUREAS ON CELL-FREE PROTEIN SYNTHESIS AND ENERGY METABOLISM IN RAT TISSUES*

L. DE BEER and P. J. DE SCHEPPER

Department of Pharmacology, University of Louvain, School of Medicine, Louvain, Belgium

(*Received* 19 *July* 1967; *accepted* 28 *July* 1967)

Abstract—The hypoglycemic sulfonylureas chlorpropamide, tolbutamide and carbutamide were found to decrease simultaneously and practically to the same extent, cellular levels of ATP and the incorporation of leucine into the protein of rat diaphragm. Chlorpropamide and tolbutamide also reduced ATP levels in liver slices. When diaphragm was pre-incubated with chlorpropamide or tolbutamide these effects persisted during subsequent incubation in the absence of the drugs. Cell-free mitochondrial and to a smaller extent also microsomal protein synthesis were inhibited by chlorpropamide and mitochondrial ATP was significantly decreased at 10^{-3} M drug concentration. The oxidation of sugars or Krebs-cycle substrates is not inhibited by sulfonylureas and does not explain the reduced energy stores. In diaphragm and liver the utilization of sugars is even enhanced by chlorpropamide and tolbutamide. Glucose, fructose and mannose relieve to some extent the inhibition of protein synthesis and the decrease of ATP levels by sulfonylureas. Insulin has the same effect and is therefore thought to stimulate energy conservation. Chlorpropamide and tolbutamide but not carbutamide increase oxygen uptake by rat liver mitochondria and abolish respiratory control. The fall in ATP and the inhibition of protein synthesis and other effects of sulfonylureas are supposedly the result of this uncoupling effect. The hypoglycemic activity of these drugs is discussed in the light of their effect upon mitochondrial metabolism.

THE HYPOGLYCEMIC sulfonylureas chlorpropamide, tolbutamide, carbutamide and a chemically related hypoglycemic pyrimidine (glycodiazine; 2-benzolsulfonamido-5-(β-methoxy-ethoxy)-pyrimidine) have recently been shown to be potent inhibitors of *in vitro* protein synthesis in rat tissues.[1, 2] The mechanism by which they achieve this inhibition is unknown. It is also uncertain if this newly described effect is related to the hypoglycemic activity of these compounds although the possibility can be envisaged that they are two different aspects of a more fundamental action of these drugs upon cell metabolism. Elucidation of this mechanism may also contribute to the understanding of other unexplained biochemical effects of the sulfonylureas as e.g. their effect upon lipolysis[3, 4] and the incorporation of acetate and mevalonate into cholesterol.[5] In a previous publication[1] we have shown that the inhibition by sulfonylureas of leucine incorporation into rat tissues is not the result of an interference of these drugs with leucine transport or metabolism although a significant inhibition of leucine decarboxylation was observed leading to increased tissue levels of this amino acid. It was further shown that the sulfonylureas did not, or only

* This work was supported in part by grants from the Belgian Medical Research Fund and from Chas. Pfizer & Co.

slightly, affect the endogenous respiration of rat tissues under conditions where the incorporation of leucine was severely inhibited. In an attempt to further clarify the mechanism whereby sulfonylureas inhibit protein synthesis several parameters of energy metabolism in rat tissues incubated with these drugs have been examined. In this paper we report their effect upon mitochondrial and microsomal protein synthesis, the ATP content of tissues, Krebs-cycle oxidations and oxidative phosphorylation.

MATERIALS AND METHODS

Compounds. L-leucine-1-^{14}C (sp. act. 8·5 mC/mM), D-fructose-U-^{14}C (sp. act. 86·2 mC/mM), D-glucose-U-^{14}C (sp. act. 8·5 mC/mM) and Na-acetate-U-^{14}C (sp. act. 15·7 mC/mM) have been purchased from the Radiochemical Centre, Amersham, U.K. D-glucose-U-^{14}C (sp. act. 25·7 mC/mM) was purchased from C.E.A., France. Succinic acid-2,3-^{14}C (sp. act. 8·7 mC/mM) was obtained from Calbiochem., Los Angeles, U.S.A. Glyceraldehydephosphate dehydrogenase (D-Glyceraldehyde-3-phosphate: NAD oxidoreductase, E.C. 1.2.1.12), phosphoglycerate kinase (ATP: 3-phospho-D-glycerate 1-phosphotransferase, E.C. 2.7.2.3.), creatine kinase (ATP: creatine phosphotransferase E.C. 2.7.3.2.) and the test kit (TCV 15992) to assay the latter enzyme were purchased from Boehringer GmbH., Mannheim, Germany. ATP, ADP and nicotinamide nucleotide coenzymes were obtained from Sigma chemical Co., St. Louis, Mo., U.S.A. Chlorpropamide was obtained through the courtesy of Chas. Pfizer & Co. Pfizer Ltd., Sandwich, U.K., tolbutamide and carbutamide through the courtesy of Boehringer GmbH., Mannheim, Germany, and glucagon-free insulin (P.J. 5589) through the courtesy of E. Lilly and Co. These sulfonylureas were dissolved in the minimum amount of N NaOH and made up to the appropriate concentration with bicarbonate or tris-sucrose buffer according to the experiments. PPO (2,5-diphenyloxazole) scintillation grade was purchased from E. Merck A.G., Darmstadt, Germany. Hydroxide of Hyamine 10-X (IM in methanol) was obtained from the Packard Comp., Ill., U.S.A. All other chemicals were reagent grade.

Leucine-^{14}C incorporation. Male Wistar rats (125–200 g) maintained on stock laboratory diet were fed *ad libitum* before use unless otherwise indicated. Removal of tissues, incubation and assay of incorporated leucine were carried out as described before.[1]

$^{14}CO_2$ from labeled substrates. Tissues were incubated with radioactive glucose, fructose, acetate and succinate in flasks fitted with a removable center well. The procedure was identical with that described previously for leucine-1-^{14}C.[1]

ATP Determination. Simultaneous determination of leucine-^{14}C incorporation into tissue protein and ATP content in diaphragm and liver slices was carried out as follows. At the end of the incubation period the tissue was quickly blotted and homogenized in 1 ml of ice-cold 0·9 N perchloric acid. After centrifugation leucine-^{14}C incorporation was determined on the protein precipitate as described before. The supernatant, after neutralization with 3·75 M K_2CO_3 (methyl orange as indicator), was kept for 1 hr in an ice bath and centrifuged in the cold in order to remove precipitated perchlorate. ATP was measured on 0·2 ml of the supernatant according to the phosphoglycerate kinase method as described by Adam.[6] Changes in optical density were followed at 366 nm in an Eppendorf photometer (Netheler und Hinz,

GmbH., Hamburg, Germany) and results are expressed as μmole/100 g wet wt. tissue.

Microsomal protein synthesis. Microsomes were prepared from the liver of overnight fasted rats. After decapitation, the liver was quickly removed and placed in ice-cold Tris-sucrose medium containing 0·25 M sucrose, 0·075 M KCl, 0·01 M $MgCl_2$ and 0·035 M Tris-HCl buffer pH 7·8. The chilled whole liver was weighed, minced and homogenized in 2 vol. cold Tris-sucrose medium by means of a motor-driver Duall tissue grinder fitted with a loose teflon pestle (Kontes, Glass Co., Vineland, N.J., U.S.A.). The homogenate was centrifuged twice at 4° for 15 min at 13,500 rpm in a Spinco Ultracentrifuge (Rotor 30). The supernatant was subsequently centrifuged for 60 min at 40,000 rpm (Rotor 40). The pellet was carefully homogenized in a mixture of 0·7 ml of Tris-sucrose medium and 0·7 ml of the microsomal supernatant per g liver. Incubation flasks contained 0·45 ml of the microsomal suspension, 0·1 ml of the appropriate sulfonylurea drug in Tris-sucrose and 0·45 ml of a reaction mixture consisting of 1 μC of leucine-^{14}C, 25 μmole of phosphocreatine, 0·25 mg of creatine kinase, 1 μmole of ATP and 62 μmole of KCl. The closed flasks were incubated for 30 min at 30° in a Dubnoff metabolic shaker (100c/min). The reaction was stopped by the addition of 1 ml of cold 10% TCA containing 0·15% (w/v) of carrier leucine. After centrifugation, the incorporation of leucine-^{14}C was determined on the sediment as described before.[1]

In order to examine the effect chlorpropamide might have on the energy generating creatine kinase system utilized in microsomal protein synthesis, the activity of this enzyme was determined in the presence of increasing concentrations of chlorpropamide using the appropriate assay kit from Boehringer (TCV 15992). Creatine kinase, at a concentration of 0·285 μg/ml was not affected by 10^{-4} M chlorpropamide. It was approximately 50 per cent inhibited at 10^{-3} M and 65 per cent at 10^{-2} M chlorpropamide. In the microsomal protein synthesis experiments, creatine kinase is used in a concentration of 0·25 mg/ml which is more than 800 times the concentration used in the enzyme assay. This excludes any inhibitory effect of chlorpropamide on the energy generating system under the conditions of these experiments.

Mitochondrial protein synthesis. The effect of chlorpropamide on mitochondrial protein synthesis was studied using the preparation procedure and the optimal incubation conditions as described by Roodyn.[7] Animals were fasted overnight and succinate (10 mM) was used as substrate. At the end of the incubation period (1 hr at 30°) the reaction was stopped by the addition of 1 ml of cold 0·9 N perchloric acid. After centrifugation, leucine-^{14}C incorporation was determined on the sediment as described before,[1] and the ATP content was determined on the supernatant as detailed above for tissue experiments.

Oxidative phosphorylation. Livers of overnight fasted rats were quickly removed and placed in ice-cold sucrose (0·25 M)-EDTA (1 mM) adjusted to pH 7·4. Once cold the liver was weighed, minced and homogenized at 0–4° in 3 vol. (v/w) cold sucrose-EDTA medium using a Duall tissue grinder fitted with a loose teflon pestle. The homogenate was centrifuged at 4° for 10 min at 3,900 rpm in a Spinco Ultracentrifuge (Rotor 30). The sediment was resuspended in 3 ml of sucrose-EDTA per g liver and centrifuged at 3000 rpm for 10 min. The combined supernatants were centrifuged again at 3000 rpm for 10 min in order to remove any contaminating nuclei. Mitochondria were sedimented and washed twice by centrifuging at 12,500 rpm for 3 min.

(Rotor 40). The final pellet was suspended in 0·2 ml of sucrose-EDTA per g liver. Oxygen uptake and ADP/O ratio's were calculated from the tracings produced by a vibrating oxygen electrode (Oxygraph, Gilson Medical Electronics, Middleton, U.S.A.). Mitochondria (0·1 ml) were incubated at 30° in a final volume of 2 ml containing 0·25 M sucrose, 1 mM EDTA, 5 mM $MgSO_4$, 10 mM succinate or ketoglutarate, all adjusted to pH 7·4 with KOH, and 13 mM phosphate buffer pH 7·4. The concentration of oxygen in the air-saturated medium at the start was taken as 240 μM.[8] Mitochondrial respiration was stimulated by the addition of 0·3 μmole of ADP in the presence or absence of various concentrations of sulfonylureas.

RESULTS

As the inhibitory effect of the sulfonylureas on protein synthesis could be the result of an interference of these drugs with the energy metabolism of tissues, we measured simultaneously leucine incorporation into protein and the ATP content of rat diaphragm at the end of incubation periods of various length. The results are shown in Table 1. Except at the lowest concentration of chlorpropamide and tolbutamide

TABLE 1. EFFECT OF SULFONYLUREAS ON ATP CONTENT AND PROTEIN SYNTHESIS IN RAT DIAPHRAGM

Drug concentration	Incub. time (min)	ATP (μmole/100 g wet wt. tissue)			Leucine-^{14}C incorporation (cpm/mg)		
		Control	Drug	% effect	Control	Drug	% effect
Chlorpropamide							
$2·5 \times 10^{-3}$ M	15 (9)	370	341 ± 18 $0·1 < P < 0·2$	− 8	42	46 ± 3 $0·2 < P < 0·3$	+ 9
	30 (6)	285	229 ± 12 $P < 0·01$	−20	78	59 ± 7 $P < 0·05$	−24
	120 (8)	206	102 ± 11 $P < 0·001$	−50	140	95 ± 8 $P < 0·01$	−32
5×10^{-3} M	15 (6)	324	227 ± 19 $P < 0·01$	−30	52	37 ± 5 $P < 0·05$	−29
	120 (11)	186	74 ± 10 $P < 0·001$	−60	178	104 ± 13 $P < 0·001$	−42
Tolbutamide							
$2·5 \times 10^{-3}$ M	30 (6)	347	348 ± 21 $P > 0·9$	0	120	130 ± 6 $P > 0·1$	+ 8
	120 (11)	216	184 ± 6 $P < 0·001$	−15	177	131 ± 7 $P < 0·001$	−31
5×10^{-3} M	15 (8)	319	245 ± 15 $P < 0·01$	−23	55	43 ± 3 $P < 0·01$	−22
	120 (6)	267	150 ± 17 $P < 0·01$	−44	306	154 ± 8 $P < 0·001$	−50
Carbutamide							
5×10^{-3} M	120 (12)	178	170 ± 15 $0·5 < P < 0·7$	− 5	233	202 ± 18 $0·1 < P < 0·2$	−14

Leucine incorporation and ATP content were measured in the same diaphragm after incubation for the stated periods. Figures in parentheses indicate the number of observations.

which together with the shorter incubation periods did not permit a significant effect on leucine incorporation or ATP concentration to appear, the results at higher drug concentrations and/or longer incubation show a rather striking correspondence between the inhibition of protein synthesis and the reduction of cellular ATP. After only 15 min of incubation these effects may be observed. It is important that none

of the results show a significant effect on protein synthesis without a simultaneous fall in cellular ATP and vice-versa. This would seem to indicate a causal relationship between these two effects, resulting from a primary interference of the sulfonylureas with the energy metabolism of diaphragm. The data with carbutamide, although less complete, support this conclusion. This compound which was previously shown to be a less potent inhibitor of protein synthesis, when compared to chlorpropamide or tolbutamide, did not significantly affect either the ATP concentration or leucine incorporation in the present experiments although the changes observed are in the expected direction of a fall in cellular ATP. This will be discussed again later (Table 8). A similar fall in ATP was observed when liver slices were incubated with chlorpropamide or tolbutamide. The effect of carbutamide was again less significant (Table 2).

TABLE 2. EFFECT OF SULFONYLUREAS ON ATP CONTENT IN RAT LIVER SLICES

Drug concentration and number of observations	ATP (μmole/100 g wet wt. tissue)		
	Control	Drug	% effect
Chlorpropamide			
$2{\cdot}5 \times 10^{-3}$ M (12)	67	54 ± 5 $P < 0{\cdot}05$	−20
5×10^{-3} M (8)	75	46 ± 3 $P < 0{\cdot}001$	−39
Tolbutamide			
$2{\cdot}5 \times 10^{-3}$ M (12)	86	71 ± 6 $P < 0{\cdot}05$	−18
5×10^{-3} M (8)	56	42 ± 4 $P < 0{\cdot}05$	−25
Carbutamide			
5×10^{-3} M (8)	63	65 ± 5 $P > 0{\cdot}7$	+ 3
10^{-2} M (8)	81	71 ± 7 $0{\cdot}1 < P < 0{\cdot}2$	−13

Incubation time 120 min. Figures in parentheses represent number of observations.

It is interesting to note (Table 1) that with increasing incubation times, the ATP content of the control tissues decreases. Although this is not unexpected under the conditions of these experiments where the oxidation of endogenous substrate is the only source of energy, it contrasts with earlier observations that diaphragm incorporates leucine practically linearly during more than 2 hr. This could raise some doubt about the significance of the observed fall in ATP as the cause for the inhibition of protein synthesis by sulfonylureas although it should be recognized that tissue levels of ATP may not closely reflect total ATP synthesis over a given period of time.

Diaphragm was pre-incubated with chlorpropamide or tolbutamide in the absence of labeled leucine and subsequently further incubated with leucine but in the absence of the drugs. Under these conditions a weak and reversible binding of the sulfonylurea at their site of action is expected to show as a reduced inhibitory effect upon protein synthesis and ATP content. This does not occur (Table 3). The magnitude of the sulfonylurea effect on leucine incorporation and ATP concentration is comparable to the effect shown in Table 1 indicating that the drugs are rather firmly bound or that their effect is largely irreversible.

To further explore the relationship between the decreased ATP concentration and the inhibition of leucine incorporation, cell-free protein synthesis in liver mitochondria and microsomes was studied in the presence of chlorpropamide. The results, in Table 4, show that mitochondrial ATP is reduced by 25 per cent after 30 min

TABLE 3. EFFECT OF PRE-INCUBATION OF RAT DIAPHRAGM WITH CHLORPROPAMIDE OR TOLBUTAMIDE ON THE ATP CONTENT AND LEUCINE-^{14}C INCORPORATION INTO PROTEIN

Drug concentration	ATP (μmole/100 g wet wt. tissue)		Leucine incorporation (cpm/mg)	
	Control	Drug	Control	Drug
Chlorpropamide 5×10^{-3} M (7)	84	39 ± 8 $P < 0.01$	58	31 ± 4 $P < 0.001$
Tolbutamide 5×10^{-3} M (8)	118	81 ± 11 $P < 0.02$	63	43 ± 7 $P < 0.05$

Diaphragms were pre-incubated during 60 min with chlorpropamide or tolbutamide in the absence of leucine-^{14}C. Tissues were removed from the flasks, quickly washed in buffer, blotted and re-incubated during 120 min in fresh medium containing leucine-^{14}C but no drug. Figures in parentheses indicate the number of observations.

TABLE 4. *IN VITRO* EFFECT OF CHLORPROPAMIDE ON LEUCINE-^{14}C INCORPORATION INTO RAT LIVER MICROSOMAL AND MITOCHONDRIAL PROTEIN AND ON MITOCHONDRIAL ATP CONTENT

Chlorpropamide concentration	Mitochondria				Microsomes	
	ATP (μmole ATP/100 g wet wt. tissue)	Mitochondrial	Leucine incorporation (cpm/mg)		Leucine incorporation (cpm/mg)	
	Control	Drug	Control	Drug	Control	Drug
10^{-4} M	—	—	—	—	491 (20)	511 ± 19 $P > 0.3$
10^{-3} M	99 (8)	74 ± 6 $P < 0.01$	52	48 ± 4 $P < 0.3$	564 (18)	523 ± 18 $P < 0.05$
2.5×10^{-3} M	128 (6)	59 ± 6 $P < 0.001$	61	49 ± 4 $P < 0.05$	—	—
5×10^{-3} M	98 (8)	8 ± 2.3 $P < 0.001$	55	20 ± 4 $P < 0.01$	713 (12)	501 ± 49 $P < 0.01$
10^{-2} M	—	—	—	—	467 (10)	166 ± 30 $P < 0.001$

Flasks contained microsomes from 0·3 g of liver in 1 ml final volume or mitochondria from 2·5 g of liver in 1·5 ml final volume. Incubation time 30 min for microsomes and 60 min for mitochondria at 30°. Mitochondrial protein synthesis and ATP content were measured in the same mitochondria of each flask. Figures in parentheses indicate number of experiments. For details see materials and methods.

incubation at a chlorpropamide concentration of 10^{-3} M. At higher drug concentrations mitochondrial ATP is reduced to extremely low levels. The magnitude of this effect, as compared to tissue experiments, is undoubtedly related to a higher effective concentration of chlorpropamide at its mitochondrial site of action due to the absence of tissular permeability barriers. Mitochondrial protein synthesis, although severely

depressed, is not reduced to the same extent as mitochondrial ATP while microsomal protein synthesis is severely affected only by higher drug concentrations. The observation that microsomal protein synthesis is affected at all will be discussed later.

The apparent interference of the sulfonylureas with oxidative energy metabolism together with the previously reported observation[1] that the endogenous respiration of rat tissues was either stimulated (diaphragm, liver) or inhibited (kidney) by these drugs led us to examine their effect on the oxidation of glucose, fructose, acetate and succinate (Tables 5 and 6). In diaphragm the oxidation of glucose and fructose was

TABLE 5. EFFECT OF CHLORPROPAMIDE AND TOLBUTAMIDE ON $^{14}CO_2$ PRODUCTION FROM GLUCOSE-U-^{14}C AND FRUCTOSE-U-^{14}C IN RAT TISSUES

Tissue	Substrate	$^{14}CO_2$ Production (cpm/mg wet wt. tissue)			
		Control	Chlorpropamide 5×10^{-3} M	Control	Tolbutamide 5×10^{-3} M
Diaphragm	Glucose	76 (11)	201 ± 9 $P < 0.001$	70 (15)	138 ± 10 $P < 0.001$
	Fructose	49 (8)	101 ± 10·8 $P < 0.01$	62 (8)	81 ± 4·7 $P < 0.01$
Liver	Glucose	45 (12)	53 ± 1·7 $P < 0.001$	46 (12)	49 ± 2·8 $P > 0.3$
	Fructose	170 (11)	192 ± 11·5 $0.05 < P < 0.1$	168 (12)	184 ± 5·6 $P < 0.02$
Kidney	Glucose	429 (7)	484 ± 26 $0.05 < P < 0.1$	498 (8)	544 ± 29 $0.1 < P < 0.2$
Adipose tissue	Glucose	27 (8)	18 ± 1·7 $P < 0.01$	23 (7)	18 ± 1·1 $P < 0.01$

Final concentration of glucose or fructose: 100 mg %. Each flask contained 0·4 μC of glucose-U-^{14}C or fructose U-^{14}C in a total incubation volume of 4 ml. Figures in parentheses indicate the number of observations.

TABLE 6. EFFECT OF TOLBUTAMIDE ON $^{14}CO_2$ PRODUCTION FROM ACETATE-U-^{14}C AND SUCCINATE-2,3-^{14}C IN RAT TISSUES

Drug concentration	$^{14}CO_2$ from Acetate-U-^{14}C (cpm/mg wet wt. tissue)		$^{14}CO_2$ from Succinate-2,3-^{14}C (cpm/mg wet wt. tissue)	
	Control	Tolbutamide	Control	Tolbutamide
Diaphragm				
$2·5 \times 10^{-3}$ M	1·118 (8)	1·331 ± 38 $P < 0.001$	249 (9)	245 ± 19 $P > 0.7$
5×10^{-3} M	1·362 (14)	1·373 ± 68 $P > 0.9$	279 (16)	224 ± 11 $P < 0.001$
Kidney				
5×10^{-3} M	3·931 (6)	3·964 ± 110 $P > 0.9$	5·499 (5)	5·802 ± 255 $0.1 < P < 0.2$
Adipose tissue				
$2·5 \times 10^{-3}$ M	131 (7)	154 ± 27 $P > 0.9$	—	—
5×10^{-3} M	121	108 ± 9 $0.1 < P < 0.2$	—	—
Liver				
5×10^{-3} M	—	—	351 (5)	222 ± 10 $P < 0.001$

Flasks contained 0·1 μC/ml of Acetate-U-^{14}C or 0·2 μC/ml of Succinate-2,3-^{14}C.
Final concentration of both substrates was 10^{-3} M. Total incubation volume 4 ml.
Figures in parentheses indicate the number of observations. Incubation time 120 min.

substantially stimulated by chlorpropamide and tolbutamide. A similar but smaller stimulation was observed in liver and kidney while glucose oxidation was inhibited in adipose tissue.

With Krebs-cycle substrates (Table 6) no such stimulation was observed except with acetate in diaphragm, at low concentrations of tolbutamide. In all other cases the stimulation was either not significant or an inhibition of substrate oxidation was found. These results, as those observed before with endogenous respiration, indicate that the effects of sulfonylureas on substrate oxidation is variable according to the concentration of the drug utilized and the tissue studied.

Glucose and insulin have been shown to reduce the inhibition of leucine incorporation by sulfonylureas.[1] In Table 7 it is shown that this relative stimulation of protein synthesis is accompanied by an increase in the ATP concentration of diaphragm. This again demonstrates that the inhibition of protein synthesis by sulfonylureas is the result of a decreased energy metabolism. Direct evidence for this is apparent from the observations on mitochondrial oxidative phosphorylation reported in Table 8. Chlorpropamide and tolbutamide at the concentrations used throughout

TABLE 7. EFFECT OF SUGAR SUBSTRATES AND OF INSULIN ON THE ATP CONTENT AND LEUCINE-^{14}C INCORPORATION IN RAT DIAPHRAGM INCUBATED WITH CHLORPROPAMIDE

Additions and incubation time		Leucine-^{14}C incorporation (cpm/mg)		ATP (μmole/100 g wet wt. tissue)	
		Chlorpropamide	Chlorpropamide + additions	Chlorpropamide	Chlorpropamide + additions
D-Glucose	30 min (20)	46	50 ± 9 $0{\cdot}05 < P < 0{\cdot}1$	153	180 ± 21 $P < 0{\cdot}001$
	120 min (8)	45	59 ± 5 $P < 0{\cdot}02$	47	61 ± 16 $P < 0{\cdot}05$
D-Fructose	30 min (13)	51	57 ± 3 $0{\cdot}05 < P < 0{\cdot}1$	177	200 ± 9 $P < 0{\cdot}05$
D-Mannose	30 min (11)	40	49 ± 2 $P < 0{\cdot}01$	171	194 ± 14 $0{\cdot}1 < P < 0{\cdot}2$
Insulin	60 min (13)	113	143 ± 9 $P < 0{\cdot}01$	147	168 ± 7 $P < 0{\cdot}01$

Mean control values for the incorporation of leucine after 30 and 120 min of incubation in the absence of drugs or substrate were respectively 72 and 144 cpm/mg. The corresponding mean ATP values were 291 and 203 μmole/100 g. Figures in parentheses indicate the number of observations. Chlorpropamide concentration 5×10^{-3} M in experiments with sugar substrates and $2{\cdot}5 \times 10^{-3}$ M in experiments with insulin. Concentration of sugars 3×10^{-2} M. Insulin 0·4 U per ml.

these experiments, stimulate mitochondrial oxygen uptake both with succinate and ketoglutarate as substrates. Respiratory control gradually disappears with increasing concentrations of these drugs and the ADP/O ratio's decrease, indirectly indicating uncoupling of oxidation and phosphorylation. At identical drug concentrations the effect of carbutamide on mitochondria appears to be different. With ketoglutrate as substrate, inhibition of oxygen consumption was observed instead of a stimulation and both the respiratory control and ADP/O ratio's are only moderately affected.

DISCUSSION

The *in vitro* inhibition of protein synthesis by sulfonylureas has been observed before but no explanation has thus far been offered.[2, 10] To our knowledge, the

concommitant fall in cellular ATP levels which we described has not been reported before. The observation that both protein synthesis and ATP levels in tissues are affected together and roughly to the same extent, can be interpreted as an indication that the primary effect of the sulfonylureas is to bring about a fall of the ATP concentration of tissues. It can be expected that a substantial deficiency in cellular ATP by whatever mechanism it is achieved, will affect energy requiring processes to the

TABLE 8. EFFECT OF SULFONYLUREAS ON RESPIRATION AND PHOSPHORYLATION IN RAT LIVER MITOCHONDRIA

Drug concentration	Substrate					
	Succinate (10^{-2} M)			Ketoglutarate (10^{-2} M)		
	O_2 uptake % stimulation	ADP/O	R.C.	O_2 uptake % stimulation	ADP/O	R.C.
Carbutamide						
0	—	1·77	4·00	—	3·20	3·17
$0·5 \times 10^{-3}$ M	—	—	—	0	3·31	2·94
1×10^{-3} M	0	1·67	2·63	− 7	3·14	2·90
$2·5 \times 10^{-3}$ M	+ 10	1·50	2·60	−10	3·09	2·41
5×10^{-3} M	0	1·26	2·50	−17	2·72	2·31
Tolbutamide						
0	—	1·70	3·60	—	3·30	3·10
1×10^{-3} M	+ 30	1·51	1·95	+12	2·65	2·10
$2·5 \times 10^{-3}$ M	+ 47	1·23	1·80	+36	2·00	1·51
5×10^{-3} M	+127	0	1·00	+75	0	1·00
Chlorpropamide						
0	—	1·70	3·60	—	3·27	2·82
$0·5 \times 10^{-3}$ M	—	—	—	+20	2·75	2·08
1×10^{-3} M	+ 25	1·44	1·93	+43	2·50	1·88
$2·5 \times 10^{-3}$ M	+ 74	0·90	1·20	+62	0·81	1·16
5×10^{-3} M	+127	0	1·00	—	—	—

Mitochondria corresponding to 500 mg of liver were incubated at 30° in a medium containing 0·25 M sucrose, 1 mM EDTA, 5 mM $MgSO_4$, 10 mM substrate and 13 mM phosphate buffer pH 7·4. Total volume 2 ml. Sulfonylureas were added during 'state 4' respiration and the stimulation of oxygen uptake was recorded. The ADP/O ratio and respiratory control index (R.C.) were measured upon subsequent addition of 0·3 μmole of ADP. Figures are the means of two experiments.

extent that these are more or less tightly dependent upon available ATP or other energy rich intermediates. It is indeed recognized that in the absence of ADP phosphorylation, energy derived from the respiratory chain oxidations may be conserved and utilized in various energy requiring processes. Reversal of the respiratory chain and protein and phospholipid synthesis have been observed in the presence of oligomycin, an inhibitor of ADP phosphorylation and of mitochondrial ATP-ase.[10–12] In view of this a decrease in ATP does not necessarily indicate an impaired biosynthetic capacity, especially in short term experiments. On the other hand, unpublished observations from our laboratory and from other sources[13] have shown that 2-deoxyglucose, a strong inhibitor of *in vitro* protein synthesis, depletes the tissues of ATP as the result of its phosphorylation which is not followed by further metabolism and energy return. A similar energy trapping and inhibition of protein synthesis by the phosphorylation of glucosamine has been observed by us (to be published). Other conditions which are known to deplete cellular ATP such as

anaerobiosis or dinitrophenol also inhibit *in vitro* protein synthesis. The hypoglycemic phenylethylbiguanide (DBI) which inhibits mitochondrial electron transport and therefore should affect available ATP, has also been shown to inhibit *in vitro* protein synthesis.[2]

It is likely that other *in vitro* and perhaps *in vivo* observations with sulfonylureas are related to the fall of cellular ATP. The lethal effect on HeLa cells in tissue culture and the growth inhibition of transplanted tumors,[14] the decreased ketogenesis from acetate in liver slices[15] and the decreased incorporation of glucose into fat in adipose tissue,[16] the reduced glucose output by liver slices[17] and the inhibition of lipolysis[4] may all, directly or indirectly, reflect the reduced energy stores.

Our data of Table 4 show that mitochondrial protein synthesis and ATP concentration are strongly affected by chlorpropamide. This could result from a simultaneous inhibition of ATP generation and an increase in mitochondrial ATP-ase activity which could be expected if the sulfonylureas, at the concentrations utilized, act as true uncouplers of oxidative phosphorylation. The inhibition of microsomal protein synthesis by chlorpropamide, although less marked than the inhibition of mitochondrial protein synthesis, is somewhat surprising in view of the fact that the creatine phosphate energy generating system itself is, according to our results, unlikely to be affected at the ratio of inhibitor to enzyme utilized. McDonald and DeChatelet[18] reported a similar inhibition of leucine incorporation into microsomal protein by tolbutamide and chlorpropamide. The possibility can be envisaged that at high concentration of sulfonylureas, other effects on the reaction sequences of protein synthesis such as on amino acid activation or transfer become operative. The unmasking of a microsomal ATP-ase by sulfonylureas would also lead to the observed inhibition and the significance of ATP hydrolysis in microsomal protein synthesis has recently been emphasized.[19] As ribosomes have a low ATP-ase activity, comparative studies of microsomal versus ribosomal protein synthesis and of microsomal ATP-ase activity should provide more information. On the other hand it should be recalled that sulfonylureas have been shown to interfere with the activity of several enzyme systems in homogenates or microsomal preparations. Berthet *et al.*[17] and Cahill *et al.*[20] reported the inhibition of microsomal glucose-6-phosphatase. Inhibition of the incorporation of acetate into cholesterol in liver homogenate and of mevalonate in a microsomal-soluble enzyme system was reported by McDonald *et al.*[5] The phosphorylase-reactivation system and its stimulation by glucagon or epinephrine are inhibited in liver homogenate.[17] These effects have all been shown at drug concentrations between 10^{-3} and 10^{-2} M which is the range we have used in microsomal protein synthesis but it would seem that there is at this moment no unifying concept on the basis of which these effects could be explained.

With regard to the mechanism whereby sulfonylureas interfere with energy metabolism we examined the oxidation of carbohydrate and Krebs-cycle substrates by rat tissues. The observed stimulation of glucose and fructose oxidation to CO_2 in diaphragm and liver is in agreement with the earlier observed increased respiration in these tissues[1] and with other reports in the literature.[21–23] In kidney slices the slight increase in CO_2 release from glucose does not correspond however to the previously observed inhibition of endogenous respiration at the same drug concentration. On the other hand, acetate and succinate oxidation in this tissue remain practically unchanged. The most important aspect of these substrate oxidations is that only in a

few experiments an inhibition of CO_2 release was observed after two hours of incubation. Although an inhibited succinate oxidation in rat liver homogenate by sulfonylureas was reported before by Clarke[24] our data on endogenous respiration have shown that an initial stimulation of oxygen uptake may be followed by an inhibition of respiration later during incubation. It would therefore appear that the fall in ATP observed after 30 min of incubation is not the result of an inhibition of substrate oxidation by the sulfonylureas.

Our previous report[1] that glucose relieves to some extent the inhibition of protein synthesis by sulfonylureas has been further substantiated in the present experiments and extended to other metabolically active sugars. It is probable that this effect is mediated through the increased ATP concentration which is observed in the presence of these carbohydrates. Less expected was the observation that in the absence of added carbohydrate insulin also enhances tissue levels of ATP which would indicate that insulin improves the conservation of energy. While the nature of this insulin effect is not clear it opens perspectives on the mechanism of action of this hormone which deserve further investigation (see also discussion in ref. 1).

The uncoupling of mitochondrial oxidation and phosphorylation by chlorpropamide has recently been described by Penttilä[25] and is confirmed by our findings. While this uncoupling effect is undoubtedly relevant to many *in vitro* observations with sulfonylureas its significance with respect to the hypoglycemic action of these drugs is not clear. Some comments may however be appropriate. A direct effect of the sulfonylureas on glucose uptake or metabolism is generally considered to be lacking since these drugs exert no hypoglycemic effect in eviscerated or depancreatized animals unless recently given insulin. The only direct extra-pancreatic effect of possible significance is a reduced glucose output by the liver, although the liver is not required for sulfonylurea hypoglycemia to develop (for reviews see refs. 26, 27). A number of authors have nevertheless reported an increased glucose uptake by the isolated diaphragm while others could not detect such an effect. Our results on glucose oxidation, while consistent with an increased glucose uptake, may primarily reflect an impaired mitochondrial energy conservation. It is well documented that glucose uptake is restrained in respiring muscle and that anaerobiosis and a number of substances which interfere with mitochondrial respiration or energy transfer such as azide, cyanide and dinitrophenol also stimulate glucose uptake and utilization. According to Randle and Smith[28] these substances may act by removing energy rich phosphate from membrane sites where it controls the uptake of glucose. Some of these findings, however, have recently been challenged by Short *et al.*[29] particularly in the case of dinitrophenol and salicylate. The latter substance exerts multiple biochemical actions among which the uncoupling of oxidative phosphorylation,[30] inhibition of *in vitro* and *in vivo* protein synthesis,[31–33] interference with soluble and mitochondrial enzymes.[34, 35] Early reports of a hypoglycemic effect of salicylates and related compounds have been re-investigated by Hecht and Goldner[36] who found a marked blood sugar lowering effect both in diabetics and non-diabetics. In some cases diabetic control could be established by salicylate replacing insulin. The mechanism of this hypoglycemic effect of salicylate is unknown but it is tempting to speculate, as in the case of the sulfonylureas, that it may be secondary to an altered mitochondrial homeostasis.

The observation of a gross correlation between the degree of hyperglycemia and

the hypoglycemic effect of salicylate recalls somewhat similar observations with the biguanides which lower the blood sugar only in diabetics. In contrast to tolbutamide and chlorpropamide, phenylethyl biguanide inhibits the oxygen uptake by diaphragm and liver slices and by isolated mitochondria. According to Steiner and Williams[37] this would lead to a stimulation of anaerobic glycolysis and would explain the increased uptake of glucose observed with this drug. Later observations that the inhibition of mitochondrial respiration by DBI does not occur at drug concentrations corresponding to therapeutic blood levels and that the clinically effective dimethylbiguanide did not produce tissue anoxia has raised much controversy about the therapeutic significance of the inhibition of mitochondrial respiration.[38] Recently, Jangaard[39] has reexamined this problem and reported a good correlation between the inhibition of mitochondrial electron transport and increased glucose uptake by rat tissues. The fact that the sulfonylureas stimulate while the biguanides mainly inhibit mitochondrial respiration is perhaps relatively unimportant as the critical factor involved would probably be the decrease in cellular high energy compounds, the change in ATP/ADP/AMP ratio's and in oxidized versus reduced pyridine nucleotides. These would in turn lead to the inhibition of biosynthetic processes such as protein synthesis, to the redistribution of substrates between mitochondrial and extra-mitochondrial compartments, to alterations in the activity of enzymes which utilize these substrates and the adenine or pyridine nucleotides or which are subject to allosteric control by these substances. Two processes which are particularly interesting in this respect are gluconeogenesis and the metabolism of the branched chain amino acids. Gluconeogenesis is to a large extent responsible for the diabetic hyperglycemia and both Jangaard[39] and Meyer[40] *et al.* have recently reported its inhibition by several biguanides at concentrations occurring *in vivo*. Although a decrease in available high energy or a shift in mitochondrial oxido-reduction systems would be expected to inhibit gluconeogenesis the possibility remains that the biguanides have a direct effect on the carboxylation and transamination steps involved in gluconeogenesis. There is a little information on sulfonylureas and gluconeogenesis although several authors have reported indirect evidence of an inhibitory effect.[41, 42]

The inhibition of $^{14}CO_2$ formation from leucine-1-^{14}C by sulfonylureas has been reported by us and other authors[1, 2, 9, 25] and a similar effect was described for DBI.[2] This decarboxylation occurs in mitochondria. Penttilä[25] showed that the inhibition by sulfonylureas is not due to a direct action of these compounds on the decarboxylase but rather to an impaired mitochondrial function in the presence of sulfonylureas. The fall in mitochondrial ATP by uncoupling of oxidative phosphorylation in the case of sulfonylureas or by decreased electron transport in the case of DBI could explain the inhibition of leucine catabolism. As leucine itself can produce hypoglycemia the significance of high tissue levels of this amino with respect to mitochondrial function and the mechanism of action of oral hypoglycemic compounds deserves further investigation. Contrary to the pronounced effect of chlorpropamide and tolbutamide on ATP levels, protein synthesis and mitochondrial respiration, carbutamide has a much smaller effect and inhibits rather than stimulates mitochondrial oxygen uptake. Our earlier observation[1] that carbutamide does not increase the tissue levels of labeled leucine in diaphragm is probably a direct result of the relatively small effect of this compound on cellular ATP. Since carbutamide is however an equally effective hypoglycemic agent as chlorpropamide or tolbutamide it

seems indicated to further explore how these substances interfere with mitochondrial metabolism. Such comparative studies are now in progress.

Acknowledgments—The author wishes to thank Miss H. Verhelst and Miss H. Souren for their excellent technical assistance; Chas. Pfizer & Co., Boehringer GmbH for the generous supplies of chlorpropamide, tolbutamide and carbutamide; E. Lilly and Co. for their gift of insulin.

REFERENCES

1. P. J. De Schepper, *Biochem. Pharmac.* **16**, MS. 1789 (1967).
2. L. R. DeChatelet and H. J. McDonald, *Proc. Soc. exp. Biol. Med.* **122**, 765 (1966).
3. D. B. Stone and J. D. Brown, *Diabetes* **15**, 314 (1966).
4. D. B. Stone, J. D. Brown and C. P. Cox, *Am. J. Physiol.* **210**, 26 (1966).
5. H. J. McDonald and J. E. Dalidowicz, *Biochemistry, N.Y.* **1**, 1187 (1962).
6. H. Adam, in *Methods of Enzymatic Analysis* (Ed. H. U. Bergmeyer), p. 539. Academic Press, New York (1963).
7. D. B. Roodyn, *Biochem. J.* **97**, 782 (1965).
8. B. Chance and G. R. Williams, *J. biol. Chem.* **217**, 383 (1955).
9. R. J. Jarett and W. J. H. Butterfield, *Br. Med. J.* **1**, 865 (1964).
10. A. M. Kroon, *Biochim. biophys. Acta* **72**, 391 (1963).
11. J. R. Bronk, *Proc. Natn Acad. Sci. U.S.A.* **50**, 524 (1963).
12. J. Garbus, H. F. DeLuca, M. E. Loomans and F. M. Strong, *J. biol. Chem.* **238**, 59 (1963).
13. O. Søvik, I. Øye and M. Rossell-Perez, *Biochim. biophys. Acta* **124**, 26 (1966).
14. J. L. Davenport, unpublished observations; John L. Smith Memorial for Cancer Research Maywood, N.J., U.S.A.
15. B. R. Boshell, G. R. Zahnd and A. E. Renold, *Metabolism* **9**, 21 (1960).
16. A. E. Renold, G. R. Zahnd, B. Jeanrenaud and B. R. Boshell, *Ann. N.Y. Acad. Sci.* **74**, 490 (1959).
17. J. Berthet, E. W. Sutherland and M. H. Makman, *Metabolism* **5**, 768 (1956).
18. H. J. McDonald and L. R. DeChatelet, *Life Sci.* **6**, 183 (1967).
19. J. Hamburger-Heyd, A. Halbreich and J. Mager, *Biochem. biophys. Res. Commun.* **26**, 471 (1967).
20. G. F. Cahill, A. B. Hastings, J. Ashmore, *Diabetes* **6**, 26 (1957).
21. A. Pletscher and K. F. Gey, *Experientia* **13**, 447 (1957).
22. S. Garantini, R. Paoletti and L. Tessari, *Arzneimittel Forsch.* **8**, 447 (1958).
23. Y. Goto and F. D. W. Lukens, *Diabetes* **10**, 52 (1961).
24. D. W. Clarke, *Ann. N.Y. Acad. Sci.* **74**, 478 (1959).
25. I. M. Penttilä, *Annls Med. exp. biol. Fenn.* **44**, *suppl.* 11 (1966).
26. J. D. H. Slater, *Progr. med. Chem.* **1**, 187 (1961).
27. L. J. P. Duncan and B. F. Clarke, *Ann. Rev. Pharmac.* **5**, 151 (1965).
28. P. J. Randle and G. H. Smith, *Biochem. J.* **70**, 490 (1958).
29. A. L. Short, F. E. Wright and J. E. Whitney, *Diabetes* **14**, 128 (1965).
30. J. E. Leader and M. W. Whitehouse, *Biochem. Pharmac.* **15**, 1379 (1966).
31. P. D. Dawkins, B. J. Gould and M. J. H. Smith. *Biochem. J.* **99**, 703 (1966).
32. P. D. Dawkins, P. Luzio, M. Burleigh, B. J. Gould and M. J. H. Smith, *Biochem. J.* **102**, 18 P (1967).
33. M. Reunanen, O. Hänninen and K. Hartiala, *Nature, Lond.* **213**, 918 (1967).
34. B. J. Gould, A. K. Huggins and M. J. H. Smith, *Biochem. J.* **88**, 346 (1963).
35. J. A. Sturman and M. J. H. Smith, *Biochem. Pharmac.* **15**, 1857 (1966).
36. A. Hecht and M. G. Goldner, *Metabolism* **8**, 418 (1959).
37. D. F. Steiner and R. H. Williams, *Diabetes* **8**, 154 (1959).
38. J. Sterne, *Metabolism* **13**, 791 (1964).
39. N. O. Jangaard, *Fedn Proc.* **26**, 507 (1967).
40. F. Meyer, M. Ipaktchi and H. Clauser, *Nature, Lond.* **213**, 203 (1967).
41. R. C. De Meutter and W. W. Shreeve, *J. clin. Invest.* **42**, 525 (1963).
42. Y. Goto and F. D. W. Lukens, *Diabetes* **10**, 52 (1961).