The hypoglycemic sulfonylureas glyburide and tolbutamide were found to be excellent inhibitors of the rat liver, heart, and skeletal muscle carnitine palmitoyltransferases, but glyburide was by far the most potent inhibitor. Carnitine, but inhibition by malonyl-CoA, a physiological inhibitor of the enzyme, but inhibition by tolbutamide was noncompetitive. Inhibition by malonyl-CoA was noncompetitive with respect to carnitine, but inhibition by glyburide was uncompetitive. These studies indicate that the hypoglycemic sulfonylureas inhibit carnitine palmitoyltransferase by a mechanism that is much different from inhibition by malonyl-CoA, but are, nevertheless, potent inhibitors of the enzyme. These results have important implications for energy metabolism in the liver and heart in relation to the use of sulfonylureas and for understanding the mechanism by which the sulfonylureas act to lower blood glucose, but there are also important implications of these results on the study of the metabolic regulation of fatty acid oxidation.

The hypoglycemic sulfonylureas are a group of drugs that are currently used in the treatment of non-insulin-dependent (type II) diabetes. Because several studies (1–4) have shown that the sulfonylureas stimulate pancreatic insulin secretion, it is thought that the primary mechanism by which these drugs act to lower blood glucose is by increasing circulating insulin levels. However, there is some question whether there is a sustained effect of the sulfonylurea on insulin secretion over a period of several weeks or months of treatment even though they are effective in lowering blood glucose over long periods of time (5, 6). Also, more recent studies have shown that there are several extra-pancreatic effects of the sulfonylureas that may be involved in the production of their hypoglycemic effects. These glucose-lowering effects of the sulfonylureas are primarily of two types: (a) they increase tissue uptake of glucose by stimulating glycolysis in the skeletal muscle (7), liver (8, 9), and heart (10, 11); and (b) they decrease hepatic output of glucose by inhibiting gluconeogenesis (8). In primary cultures of hepatocytes, glyburide increased insulin-stimulated glycogen synthesis without affecting either insulin binding or degradation (9). Even in severely diabetic rats, direct, extra-pancreatic effects of sulfonylureas have been found, e.g. in perfused rat hearts, the stimulation of glycolysis by tolbutamide was even greater in diabetic rats compared with non-diabetic controls (10).

Recently it was discovered that tolbutamide, in addition to having direct inhibitory effects on gluconeogenesis in the isolated perfused liver, also inhibited ketogenesis (12), suggesting that sulfonylureas block the oxidation of fatty acids, either directly or by inhibiting hepatic lipolysis. In light of the fact that Tan et al. (10) have concluded that tolbutamide can switch the energy metabolism of hearts of both normal and diabetic rats from a preferential dependence on fatty acid oxidation to an increased utilization of glucose as an energy source for contraction, it appears that the heart also might be susceptible to inhibition of fatty acid oxidation by sulfonylureas.

The studies reported here were initiated to ascertain whether the sulfonylureas glyburide and tolbutamide could inhibit directly the mitochondrial carnitine palmitoyltransferases of rat liver or muscle. Carnitine palmitoyltransferase is the key regulatory enzyme in the fatty acid oxidation pathway and is regulated in part by the tissue content of malonyl-CoA, a physiological inhibitor of the enzyme (13), and in part by a modulation of the enzyme that alters the inhibitory effects of malonyl-CoA (14). An interesting aspect of the control of carnitine palmitoyltransferase in relation to regulation of blood glucose is the recently discovered change that is brought about in the enzyme following treatment of rats with insulin which results in a decreased capacity for hepatic fatty acid oxidation (15). These studies indicated that insulin lowered fatty acid oxidation rates by rapidly increasing the affinity of carnitine palmitoyltransferase for malonyl-CoA. The results presented here indicate that glyburide and tolbutamide are potent inhibitors of carnitine palmitoyltransferase and that glyburide, the more potent hypoglycemic drug, is also the more potent inhibitor of carnitine palmitoyltransferase. The sulfonylureas, however, inhibit carnitine palmitoyltransferase by a mechanism quite different from inhibition by malonyl-CoA.

**EXPERIMENTAL PROCEDURES**

Male Wistar rats weighing 200–250 g were fed *ad libitum* (Purina Lab Chow for Rats, Ralston Purina Co., Richmond, IN) or fasted for 72 h. Diabetic rats were male BB Wistar spontaneously diabetic rats that were maintained on insulin after development of diabetes until 48 h before rats were used for experiments. All animals were allowed free access to water. Diabetic rats were checked for the presence of ketone bodies in the urine (determined with Bili-Labstix, Miles

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were checked at the time of removal of organs for experiments to be certain that diabetic and ad libitum fed rats were well fed and that fasting rats had not inadvertently received food.

Mitochondria from the liver, heart, or skeletal (quadriceps) muscle were isolated by the method of Johnson and Lardy (16) with the modifications previously published. Skeletal muscle mitochondria were isolated exactly as previously reported for heart (14–15). The final mitochondrial pellet was resuspended in distilled water to rupture the mitochondria and centrifuged at pH 7.2. Protein was determined by a biuret procedure (17). Respiratory control ratios (18), determined with 10 mm glutamate and 0.5 mM malate as substrates, were 5 or greater for all preparations used. The outer carnitine palmitoyltransferase was measured as described previously (15). Each assay contained, in a total volume of 1 ml, 82 mM sucrose, 70 mM KCl, 35 mM Hepes, 35 mM imidazole, 5 mM reduced glutathione, 2 mg of bovine serum albumin, L-carnitine (0.4 μCi of L-[methyl-3H]carnitine/μmol) at the concentrations indicated, 1 μg of antymicine A, palmitoyl-CoA and inhibitors at concentrations indicated, 2 mM ATP; and 2 mM MgCl2. ATP and MgCl2 were added because of a previous report that acyl-CoA concentrations were maintained better in their presence (19). Reactions were carried out at pH 7 and 30°C for 5 min following a 5-min preincubation period in the presence of all components except carnitine, which was added to initiate the reaction.

Solubilized carnitine palmitoyltransferase was prepared from mitochondria isolated as described above. The mitochondrial pellet of Johnson and Lardy (16) was resuspended in distilled water to rupture the mitochondria and then centrifuged at 45,000 g for 30 min to release soluble proteins and substrates and sediment mitochondrial membranes. This pellet was resuspended in 0.5% Triton X-100 to solubilize membrane proteins before centrifuging at 105,000 g for 1 h. The supernatant solution was used for carnitine palmitoyltransferase inhibition experiments.

Potassium glyburide, sodium tolbutamide, and carboxytolbutamide were gifts from The Upjohn Co. Malonyl-CoA, palmitoyl-CoA, reduced glutathione, imidazole, Hepes, L-carnitine hydrochloride, EDTA, ATP, and essentially fatty acid-free bovine serum albumin were purchased from Sigma. L-[methyl-3H]Carnitine hydrochloride was obtained from Amersham Corp. Normal Wistar rats were obtained from Harlan Industries (Indianapolis, IN). BB Wistar spontaneously diabetic rats were provided by Dr. Solomon S. Solomon of the University of Tennessee, Memphis, and the Veterans Administration Research Service.

RESULTS

Tolbutamide and glyburide were excellent inhibitors of the activity of carnitine palmitoyltransferase that is expressed in intact mitochondria (on the outside of the mitochondrial inner membrane), but tolbutamide was much more potent as an inhibitor than tolbutamide (Fig. 1). The concentration of tolbutamide that produced 50% inhibition of the enzyme was 35 times greater than the concentration of glyburide producing equivalent inhibition (1.4 mM and 40 μM, respectively). Glyburide is a much more potent hypoglycemic agent than tolbutamide, and these observations indicate that the potency of the two compounds for inhibition of carnitine palmitoyltransferase occurs in a very similar ratio to the concentrations producing their hypoglycemic actions; however, comparing the exact potencies of the compounds for these actions is difficult because glyburide binds more tightly than tolbutamide to albumin (20). The percentage inhibition of enzyme activity was not modified by increasing the time of preincubation (0–20 min) of mitochondria with tolbutamide or glyburide or by addition of the inhibitors after preincubation of mitochondria with palmitoyl-CoA at the time of starting the reaction by the addition of carnitine (no preincubation with inhibitor). Washing mitochondria by centrifuging and resuspending in mitochondrial isolation medium after preincubating the mitochondrial isolation medium after preincubating the mitochondria with tolbutamide or glyburide was not modified by increasing the time of preincubation (0–20 min) of mitochondria with tolbutamide or glyburide or by addition of the inhibitors after preincubation of mitochondria with palmitoyl-CoA at the time of starting the reaction by the addition of carnitine (no preincubation with inhibitor). Washing mitochondria by centrifuging and resuspending in mitochondrial isolation medium after preincubating the mitochondria, but tolbutamide was much more potent as an inhibitor than tolbutamide.

Interestingly, the phenomenon occurring during fasting and diabetes that causes loss of sensitivity to inhibition is not restricted to inhibition by malonyl-CoA.

Because the ability of malonyl-CoA to inhibit hepatic carnitine palmitoyltransferase is greatly decreased after freezing and thawing mitochondria (24), the inhibitory effects of the sulfonylureas were tested on carnitine palmitoyltransferase of frozen-thawed mitochondria. Fig. 3 indicates that freezing and thawing had little effect on the inhibition of carnitine palmitoyltransferase by glyburide or tolbutamide when assayed under conditions identical to those for Fig. 1. It is

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1 The abbreviation used is: Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.
Sulfonylureas Inhibit Carnitine Palmitoyltransferase

FIG. 2. Comparison of the inhibitory effects of glyburide and tolbutamide in genetically diabetic BB Wistar rats and their nondiabetic littersmates. Inhibition of carnitine palmitoyltransferase by glyburide, panel A, and tolbutamide, panel B, was compared in liver mitochondria from diabetic (●) and nondiabetic (○) BB Wistar rats under the same conditions as in Fig. 1. Results are means ± S.E. for four animals in each group. Specific activity of the outer carnitine palmitoyltransferase of diabetic and nondiabetic rats, measured under the optimum conditions indicated in Fig. 1, was 13.3 ± 0.6 and 7.6 ± 0.5 nmol/min/mg protein, respectively.

FIG. 3. Inhibition of carnitine palmitoyltransferase (CPT) in frozen and thawed rat liver mitochondria. Inhibition by glyburide (●), tolbutamide (○), and carboxytolbutamide (■) was compared in rat liver mitochondria that had been frozen at −80 °C, thawed, then refrozen and thawed. Assays were conducted under conditions identical to those in Fig. 1. Results are presented as means ± S.E. for three preparations of mitochondria. The specific activity of these preparations measured at 2.5 mM L-carnitine and 0.15 mM palmitoyl-CoA (determined to be optimal conditions for these preparations) was 31 ± 3 nmol/min/mg protein.

FIG. 4. Comparison of the inhibitory effects of malonyl-CoA and glyburide in rat liver mitochondria. Inhibition by glyburide (●) and malonyl-CoA (■) was compared with activity in the absence of inhibitors (○). Carnitine palmitoyltransferase (CPT) was assayed at 0.5 mM carnitine in panel A and at 0.1 mM palmitoyl-CoA in panel B. Other conditions are described under "Experimental Procedures." Results are presented as means ± S.E. for three preparations of mitochondria.
Fig. 5. Inhibition of rat liver carnitine palmitoyltransferase (CPT) by glyburide. Carnitine palmitoyltransferase was assayed without inhibitor (○), with 50 μM glyburide (●), or with 100 μM glyburide (■) under the conditions described under "Experimental Procedures," except that in panel A carnitine was 0.2 mM and in panel B palmitoyl-CoA was 40 μM. The experiments illustrated here are representative of three different preparations of rat liver mitochondria.

Fig. 6. Comparison of the effects of malonyl-CoA and tolbutamide on detergent-solubilized carnitine palmitoyltransferase from rat liver mitochondria. Carnitine palmitoyltransferase was assayed at 40 μM palmitoyl-CoA and 0.5 mM carnitine to determine the effect of malonyl-CoA (●), and at 0.1 mM palmitoyl-CoA and 0.1 mM carnitine to determine the effects of tolbutamide (○). Data are reported as means ± S.E. for three different preparations of enzyme. The specific activity of these preparations, measured at optimal concentrations of 0.15 mM palmitoyl-CoA and 2.5 mM carnitine, was 89 ± 8 nmol/min/mg protein. This enzyme preparation was not inhibited by tetradeetylglcylic acid in the absence of CoA, Mg²⁺, and ATP.

Fig. 7. Comparison of the effects of malonyl-CoA and tolbutamide on detergent-solubilized carnitine palmitoyltransferase from rat liver mitochondria. Carnitine palmitoyltransferase was assayed at 40 μM palmitoyl-CoA and 0.5 mM carnitine to determine the effect of malonyl-CoA (●), and at 0.1 mM palmitoyl-CoA and 0.1 mM carnitine to determine the effects of tolbutamide (○). Data are reported as means ± S.E. for three different preparations of enzyme. The specific activity of these preparations, measured at optimal concentrations of 0.15 mM palmitoyl-CoA and 2.5 mM carnitine, was 89 ± 8 nmol/min/mg protein. This enzyme preparation was not inhibited by tetradeetylglcylic acid in the absence of CoA, Mg²⁺, and ATP.

DISCUSSION

The data presented here indicate that the sulfonylureas are potent, reversible inhibitors of liver, heart, and skeletal muscle carnitine palmitoyltransferases. Glyburide, which is a much more potent hypoglycemic drug than tolbutamide, is also a much better inhibitor of carnitine palmitoyltransferase, but carboxytolbutamide, which is an inactive metabolite of tolbutamide, has no effect on enzyme activity. Two other inhibitors of carnitine palmitoyltransferase, (+)-decanoylcarnitine (25) and pentaenoic acid (26), are known to inhibit hepatic gluconeogenesis by inhibiting fatty acid oxidation. The studies of Menahan and Wieland (27) have established that maximal rates of hepatic gluconeogenesis require the oxidation of fatty acids to meet the energy demand. A recent investigation has demonstrated that the carnitine palmitoyltransferase inhibitor 2-(5-(4-chlorophenyl)-pentyl)-oxirane-2-carboxylate not only inhibits fatty acid oxidation but also stimulates glycolysis (28). The mechanism for stimulation of glycolysis may be by the activation of pyruvate dehydrogenase, since recent investigations have shown that oxidation of fatty acids inactivates pyruvate dehydrogenase in liver (29) and heart (30) by modulating pyruvate dehydrogenase kinase activity. The proposed mechanism is that inhibition of carnitine palmitoyltransferase inhibits oxidation of fatty acids causing the mitochondrial [NAD⁺]/[NADH] ratio to become more oxidized and the mitochondrial [acetyl-CoA]/[CoA] ratio to be decreased. Such changes in these mitochondrial ratios inactivate pyruvate dehydrogenase kinase and lead to increased conversion of pyruvate dehydrogenase to the active form (29). These considerations suggest that at least a part of the hypoglycemic action of the sulfonylureas could be explained by the inhibition of carnitine palmitoyltransferase which would result in (a) inhibition of hepatic gluconeogenesis by decreasing energy supply and (b) stimulation of glycolysis by increasing the relative amount of the active form of pyruvate dehydrogenase. This idea is supported by the recent work of Tutwiler et al. (31) which indicated that tetradeetylglcylic acid, when administered to animals in vivo, not only inhibited ketogenesis but also lowered blood glucose.

The sulfonylureas are apparently different from other known carnitine palmitoyltransferase inhibitors like malonyl-CoA and tetradeetylglcylic acid, which inhibit in the form of the coenzyme A ester (32–33), and they are also different from oxefinicine, which must be converted to the free carboxylic acid form before inhibition will occur (34). The mechanisms by which these compounds produce their inhibitory effects are all quite different. While malonyl-CoA is cooperatively competitive with respect to palmitoyl-CoA and non-

an explanation of this method). The solubilized enzyme was not inhibited by carboxytolbutamide or tetradeetylglcylic acid in the absence of added coenzyme A, Mg²⁺, or ATP; the addition of these substrates resulted in inhibition by tetradeetylglcylic acid, but had no effect on the results obtained with tolbutamide or carboxytolbutamide (results not shown).

The rat heart and skeletal muscle carnitine palmitoyltransferases are very much more sensitive than the liver enzyme to inhibition by malonyl-CoA (14, 19), but when the enzymes from the heart and skeletal muscle were assayed under conditions identical to those in Fig. 1, 100 μM glyburide inhibited the heart enzyme by 57 ± 6% (n = 4) and the skeletal muscle enzyme by 60 ± 7% (n = 3), indicating that the heart, skeletal muscle, and liver enzymes were very similar in their response to inhibition by glyburide. Carboxytolbutamide had very little inhibitory effect on carnitine palmitoyltransferases from heart and skeletal muscle (less than 10% inhibition).
competitive with respect to carnitine, the sulfonylureas are noncompetitive with respect to palmitoyl-CoA and uncompetitive with respect to carnitine. The metabolite of oxenine, hydroxyphenylglyoxylate, is competitive with carnitine and noncompetitive with palmitoyl-CoA (34). These observations suggest that carnitine palmitoyltransferase is a complex enzyme that is capable of binding a number of different inhibitors at apparently different sites. Recent data (33, 35–36) have suggested that palmitoyl-CoA binds at a site different from the active site and possibly on a regulatory subunit (33). Also, the cooperativity of inhibition by palmitoyl-CoA seen by us (14–15) points to some interaction between subunits of the enzyme. It is thought that freezing and thawing or detergent treatment separates the regulatory and catalytic subunits and prevents inhibition by palmitoyl-CoA (33). The sulfonylureas apparently behave in an entirely different manner from that of malonyl-CoA since freezing and thawing had little effect on inhibition by tolbutamide or glyburide. Furthermore, a preparation of carnitine palmitoyltransferase that was solubilized by detergent treatment could be completely inhibited by tolbutamide, but malonyl-CoA did not inhibit this enzyme preparation even though assay conditions were used for each particular inhibitor.

Results presented here indicate that there are some differences and some similarities between inhibition of carnitine palmitoyltransferase by the hypoglycemic sulfonylureas and the other known inhibitors of this enzyme. Inhibition by the sulfonylureas occurs by a much different mechanism than inhibition by malonyl-CoA, hydroxyphenylglyoxylate, or tridecylglycidic acid. Also, the hypoglycemic sulfonylureas inhibit both the inner and outer carnitine palmitoyltransferase activities while malonyl-CoA apparently inhibits only the outer activity. There are some similarities in inhibition by the sulfonylureas and malonyl-CoA, however, because fasting and diabetes decrease the sensitivity of carnitine palmitoyltransferase to both inhibitors. The implications of the changes occurring in the fasting and diabetes are not yet clear but may reflect changes in the enzyme itself, possibly by covalent modification of the enzyme (37).

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Note Added in Proof—The colony of spontaneously diabetic rats at the University of Tennessee, Memphis, which was the source of animals used in this paper, has recently been officially designated BB/WorUtM by the Institute of Laboratory Animals of the National Science Foundation. The designation indicates the source of the substrain (UtM for the University of Tennessee, Memphis, and Wor for the University of Massachusetts at Worcester) of the original colony of spontaneously diabetic rats (BB for the BioBreeding Laboratories of Ottawa, Canada). This colony was developed and maintained by funds provided in part by the Tennessee Affiliate and the Memphis Chapter of the American Diabetes Association.

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