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Effect of 1-(2-carboxy-3-chlorophenyl)pyrrole on carbohydrate metabolism in rat and mouse

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IN THE COURSE of screening for novel hypoglycemic agents, a series of 1-(2-carboxyphenyl)pyrroles and their cyclized derivatives, pyrrolo-(1,2-a)indoles, were found to have a hypoglycemic action in the rat (unpublished data). Among these compounds, 1-(2-carboxy-3-chlorophenyl)-pyrrole (T-9078) was the most active. The present report describes our biochemical studies on the mode of action of the hypoglycemics and presents evidence that the hypoglycemia is caused by a stimulation of glucose utilization in peripheral tissues.

Materials. Male SD rats and ICR mice were purchased from CLEA (Tokyo, Japan), fed a stock diet, and were used at 5–6 weeks of age. Test kits for enzyme analysis of D-glucose, L-lactate and pyruvate were obtained from Boehringer Mannheim Corp.; D-(U-^{14}C)glucose (309 mCi/m-mole), L-(U-^{14}C)alanine (173 mCi/m-mole) and insulin immunoassay kit were obtained from the Radiochemical Centre, England. Ion-exchange resins, AG 1 × 8 (acetate) and AG 50 × 4 (H), were purchased from BioRad Lab., and bovine albumin fraction V and 2-deoxyglucose from Wako Chem. Ind. Ltd. 1-(2-Carboxy-3-chlorophenyl)pyrrole was synthesized and donated for the present studies by Dr. Y. Kawamatsu of the Chemical Research Laboratories of our Division.

Methods. Blood glucose was measured by the glucose oxidase method (Test kit) as modified to fit the Autolab autoanalyser (Linson Inst. AB, Stockholm). Glycogen was determined by the anthrone method.[1]

Eviscerated, nephrectomized rats. Evisceration was performed as described by Russell.[2] The kidneys were functionally removed by ligature of the renal arteries and veins. Operated rats received 400 mg/kg of glucose subcutaneously at 1-hr intervals. The test compound was administered subcutaneously immediately after the operation. Blood samples were taken from the tail vein 3 hr after administration of the compound.

(U-^{14}C)glucose oxidation in intact mice. Fasted mice received intraperitoneal injections of the test compound and tracer doses of (U-^{14}C)glucose (2 μCi) and were placed in metabolic cages. The expired carbon-14 was continuously measured by an ionization chamber (Cary Instruments), the CO_2 excretion by infrared absorption (Horiba Ltd., Kyoto), and the specific radioactivity by a radioanalyzer (The Rikadenki Ltd.) as described by Tolbert *et al.*[3]

Uptake of 2-deoxyglucose by intact diaphragm. Intact diaphragm was taken from rats pretreated with the drug (T-9078) and incubated for 1 hr at 37° in 50 ml of Krebs–Ringer bicarbonate buffer, pH 7·4, containing 20 mM 2-deoxyglucose. The hemidiaphragms were then excised and the content of total 2-deoxyglucose and 2-deoxyglucose-6-phosphate was determined.[4]

Gluconeogenesis in vivo. Rats were injected intraperitoneally with a tracer dose of (U-^{14}C)alanine (10 μCi/rat). Blood samples were taken from the tail vein 30 min after the injection. Radioglucose was separated from radioactive ionic materials in deproteinized blood by passage through a column consisting of mixed-bed (AG 1 × 8 and AG 50 × 4) ion-exchange resins. Radioactivity was determined in a Packard liquid scintillation spectrometer.

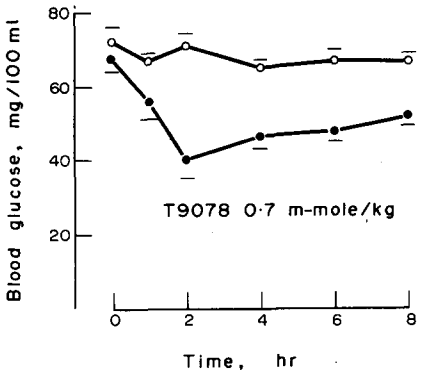

FIG. 1. Hypoglycemic activity in fasted normal rats. Rats received an intraperitoneal injection of saline (○) or T-9078 (●). Blood samples were taken from the tail vein. Each point represents the mean ± S.E. of five control or ten treated rats. Two drug-treated rats died between 2 and 4 hr.

Liver was perfused by the method of Exton and Park.[5] The perfusion medium was Krebs–Ringer bicarbonate buffer, pH 7·4, containing 20 mM L-lactate, 3% bovine albumin fraction V, and washed bovine erythrocytes at a packed cell volume of 22 per cent. The medium was infused at a constant rate of 7 ml/min. The circulating perfusate was sampled periodically for glucose determinations.

Hypoglycemic action. In fasted normal rats, the ED_{50} of T-9078 was about 0·74 m-mole/kg, and the action lasted for 8 hr or more (Fig. 1). In fasted mice, a higher dose of 2 m-moles/kg was necessary to obtain the same degree of hypoglycemia (Fig. 2). In alloxan diabetic mice, 3 m-moles/kg of the drug did not produce hypoglycemia (Fig. 3).

Effect on glucose utilization. In eviscerated, nephrectomized rats, the drug produced hypoglycemia at a smaller dose of 0·05 m-mole/kg (Fig. 4). The drug stimulated oxidation of (U-^{14}C)glucose in fasted mice, as expressed either in cumulative radioactivity or specific activity in expired $^{14}CO_2$ (Table 1). In this experiment specific activity of ^{14}C-glucose in the blood was markedly lowered in the treated mice at 60 min, which suggests an increase in glucose turnover rate.

Intact diaphragm was prepared 1 hr after injection of T-9078 and incubated for 60 min in Krebs–Ringer bicarbonate buffer containing 20 mM 2-deoxyglucose. Pretreatment with 0·025 to 0·2 m-mole/kg of T-9078 gave a dose-dependent stimulation of 2-deoxyglucose uptake into the diaphragm (Fig. 5). A dose of 0·2 m-mole/kg produced a response as high as that produced by 0·2 mU, insulin per ml of incubation medium. However, addition of the drug *in vitro*, without pretreatment, failed to increase the uptake of the sugar analog (Table 2).

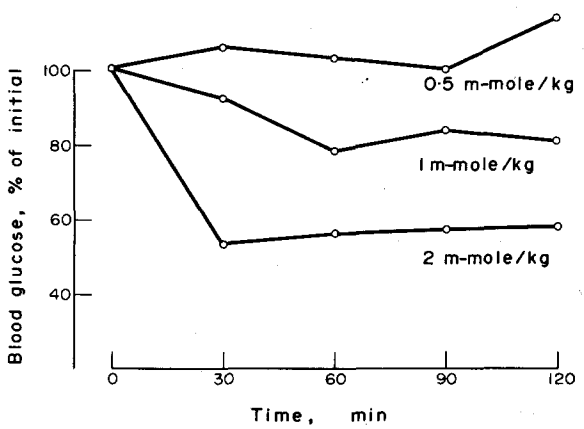

FIG. 2. Hypoglycemic activity in fasted normal mice. Fasted mice received an intraperitoneal injection of T-9078. Blood samples were taken from the orbital sinus at intervals. Each point represents the mean ± S.E. of four mice. The average blood glucose level was 107 ± 7 mg/100 ml prior to treatment.

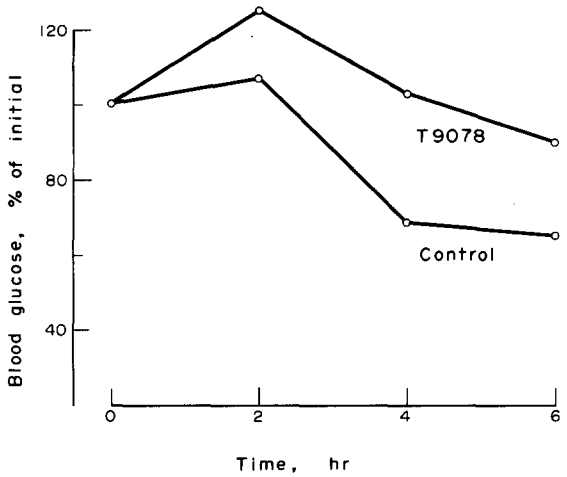

FIG. 3. Effect on alloxan diabetic mice. Fasted mice were injected with 80 mg/kg, i.v., of alloxan. On the fifth day, diabetic mice were fasted overnight and received saline or 3 m-moles/kg, i.p., of T-9078. Initial blood glucose levels were 371 ± 80 and 320 ± 23 mg/100 ml respectively.

Effect on glucose production. A tracer dose of ^{14}C-alanine was injected intraperitoneally into fasted rats and the incorporation of radioactivity into blood glucose was measured 30 min later. Table 3 shows that 0·75 m-mole/kg of T-9078 did not reduce the specific activity of radioglucose in blood.

The effect on hepatic glucose production was further studied in perfused rat liver perparations (Fig. 6). Control livers perfused with the medium containing 20 mM L-lactate gave a constant rate of glucose production during the first hr. The drug added to the medium 30 min after the initiation of perfusion did not alter the rate. In another experiment, intact fasted rats were pretreated with 0·75 m-mole/kg i.p. of T-9078. The liver, taken 30 min after the injection, was then perfused with a medium containing 20 mM L-lactate and 1 mM of the drug. The rate of glucose production in the treated liver was somewhat higher than that in the control liver. Similar results were also observed with L-alanine as a substrate (unpublished). These results indicate that T-9078 does not suppress gluconeogenesis in liver.

T-9078 is a member of a new series of hypoglycemic agents, 1-(2-carbosyphenyl)pyrroles. In this report we have presented evidence of an enhancement of glucose utilization by the drug, which includes a reduction in blood glucose in eviscerated, nephrectomized rats, a stimulation of glucose oxidation *in vivo*, and an increase of uptake of 2-deoxyglucose by diaphragm *in vitro*. On the other hand, we could not obtain any evidence for an inhibitory action of T-9078 on glucose production; that is, T-9078 neither depressed the rate of gluconeogenesis nor increased glycogen content in the liver (Table 4). Thus, the mechanism of hypoglycemic action of T-9078 may be in a stimulation of glucose utilization in peripheral tissues, which resembles one of the main actions of insulin. However, T-9078 is unlike insulin in at least two ways: (1) it shows no hypoglycemic action in the alloxan diabetic animal, and (2) it has no direct action on intact

TABLE 1. GLUCOSE OXIDATION IN FASTED MICE*

Treatment	Dose (m-moles/kg)	(% dose/hr)	Expired $^{14}CO_2$ (μCi/mg CO_2 × 10^3)	Blood ^{14}C-glucose (μCi/mg glucose × 10^3)
Control	Saline	17 ± 2†	3·1 ± 0·3	23·6 ± 5·5
T-9078	2	29 ± 5‡	5·0 ± 0·9‡	8·6 ± 3·6‡

* Fasted mice received an intraperitoneal injection of 2 μCi each of (U-^{14}C)glucose and the drug. Expired $^{14}CO_2$ and CO_2 were measured continuously during the first hr and their cumulative amounts were calculated. After 1 hr, blood samples were obtained by heart puncture, and the radioglucose was separated with a mixed-bed resin as described in Methods.

† Indicates mean ± S.D. of four mice.

‡ $P < 0·02$ by analysis of variance.

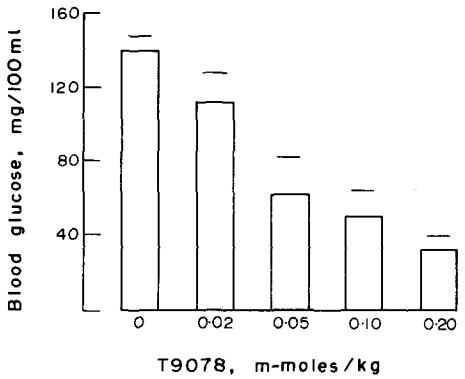

FIG. 4. Hypoglycemic activity in eviscerated, nephrectomized rats. Operated rats received 400 mg/kg, s.c., of glucose every hr. T-9078 was subcutaneously injected immediately after the operation. Blood samples were taken 3 hr after the injection. Each column represents the mean ± S.E. of six to eight rats.

diaphragm. The biological activities of the drug are seen only when it is administered to donor animals *in vivo*. The underlying mechanism is not clear. T-9078 differs from sulfonylureas and biguanides in its mode of action, because the latter agents have no action on glucose oxidation in intact animals or on glucose uptake by diaphragm.

The mechanism of the stimulating action of the drug on glucose utilization is not clear. Two lines of evidence suggest that enhanced anaerobic glycolysis can be excluded. First, T-9078 showed no uncoupling action on oxidative phosphorylation in rat liver mitochondria even at the higher concentration of 4 mM (unpublished data). Second, T-9078 did not increase the ratio of plasma lactate and pyruvate concentration in spite of a remarkable increase of lactate level (Table 4). These properties are different from the action of phenethylbiguanide, which increases both the lactate level and the lactate/pyruvate ratio in experimental animals.[6]

TABLE 2. UPTAKE OF 2-DEOXYGLUCOSE IN INTACT DIAPHRAGM INCUBATED *in vitro**

Treatment	Total 2-DG (μmoles/g tissue)	2-DG-6-P (μmoles/g tissue)
Control	6·8 ± 1·2†	3·7 ± 1·5
T-9078	6·6 ± 1·2	3·6 ± 1·7
Insulin	34·6 ± 4·2‡	22·4 ± 3·9‡

* Intact diaphragms were prepared from untreated fasted rats and incubated in KRB buffer containing 20 mM 2-deoxyglucose (2-DG) with or without 1 mM T-9078, or with 0·4 U/ml of insulin for 60 min.

† Indicates mean ± S.D. of seven or more diaphragms.

‡ $P < 0.01$ by analysis of variance.

TABLE 3. GLUCONEOGENESIS *in vivo* IN FASTED RATS*

Treatment	Blood ^{14}C-glucose (cpm × 10^{-6}/100 ml)	(cpm × 10^{-4}/mg glucose)
Control	2·7 ± 0·5†	5·6 ± 1·0
T-9078	1·0 ± 0·2‡	5·1 ± 1·0

* Fasted rats received an intraperitoneal injection of saline or 0·75 m-mole/kg of T-9078 and, 40 min later, 10 μCi ^{14}C-alanine. Blood samples were obtained 30 min after the isotope injection. Radioglucose was separated with a mixed-bed resin as described in Methods.

† Indicates mean ± S.D. of four control rats and six treated rats.

‡ $P < 0.01$ by analysis of variance.

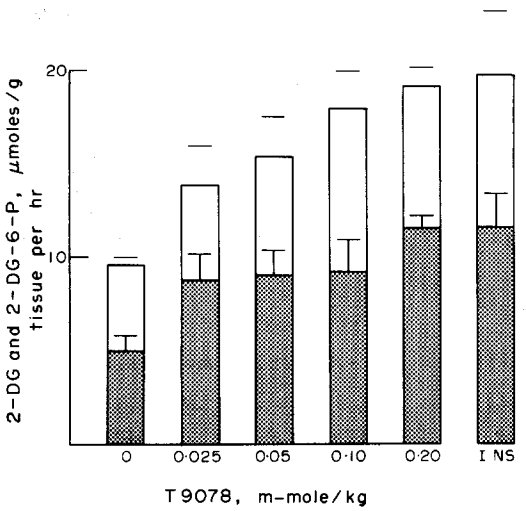

FIG. 5. Uptake of 2-deoxyglucose by intact diaphragm. Intact diaphragm was taken from rats pretreated with T-9078 and incubated for 1 hr at 37° under 95% O_2–5% CO_2 in 50 ml Krebs–Ringer bicarbonate buffer, pH 7·4, containing 20 mM 2-deoxyglucose. Insulin was not given to rats but was added to the incubation medium (0·2 mU/ml). The hemidiaphragms were then excised and the content of total 2-deoxyglucose (open column) and 2-deoxyglucose-6-phosphate (shadowed column) was determined. Each column represents the mean ± S.E. of four diaphragms.

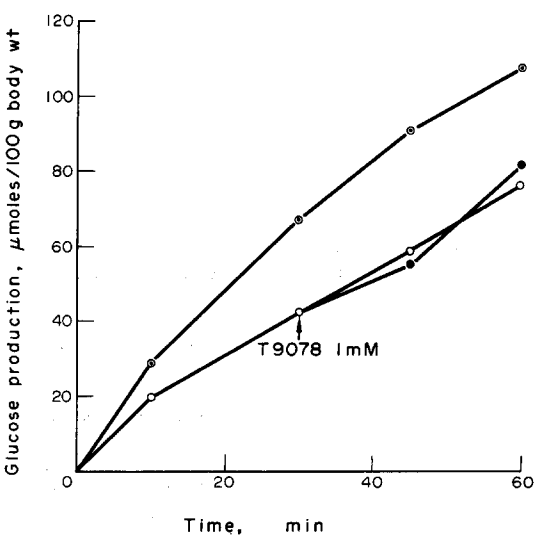

FIG. 6. Glucose production in perfused rat liver. The perfusion medium was Krebs–Ringer bicarbonate buffer, pH 7·4 containing 20 mM L-lactate, 3% bovine albumin fraction V and washed bovine erythrocytes. The medium was infused at a constant rate of 7 ml/min. Glucose production in untreated liver, ○; liver perfused with 1 mM T-9078 added in the medium at the time indicated by the arrow, ●; liver from rats injected with T-9078 (0·75 m-mole/kg) and perfused with 1 mM T-9078 added in the medium at zero time, ⊙. Mean values of four rats in each group.

TABLE 4. LIVER GLYCOGEN AND PLASMA LACTATE AND PYRUVATE IN FASTED RATS*

Treatment	Liver glycogen (mg/g wet tissue)	Lactate (mg/100 ml)	Plasma Pyruvate (mg/100 ml)	Lactate/pyruvate ratio
Control	5·0 ± 1·0†	19 ± 1	0·47 ± 0·08	41 ± 7
T-9078	3·6 ± 0·6	43 ± 7‡	0·92 ± 0·20‡	45 ± 10

* Fasted rats received an intraperitoneal injection of saline or 0·75 m-mole/kg of T-9078 and were killed by decapitation 2 hr after the injection.

† Indicates mean ± S.D. of five rats.

‡ $P < 0.01$ by analysis of variance.

The development of tachyphylaxis in experimental animals precludes clinical trials for antidiabetics.

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Amphetamine-induced changes in striatal dopamine and acetylcholine levels and relationship to rotation (circling behavior) in rats

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UNILATERAL lesions of the nigro-striatal system cause rats to turn in circles toward the side of the lesion, soon after recovering from the surgery. The rotational behavior is potentiated by amphetamine and can also be induced by amphetamine long after the animals have recovered from their tendency to rotate spontaneously.[1,2] This behavior has been attributed[2] to a neurochemical imbalance between the sides ipsilateral and contralateral to the lesion in the nigro-striatal system. Amphetamine presumably stimulates the intact system (by releasing dopamine), further enhancing the bilateral imbalance. Recently, high doses (15–25 mg/kg of the *d*-isomer) of amphetamine have been found to induce rotation in normal rats resembling that induced by lower doses (1–5 mg/kg of the *d*-isomer) in lesioned rats. As in lesioned rats, the direction of rotation is consistent for normal rats: when tested on 3 different days, some rats consistently rotated to the left, while others consistently rotated to the right.[3] The former results suggest the presence of an intrinsic and normal bilateral imbalance in the dopamine content of left and right nigro-striatal systems which is accentuated by amphetamine. The work described here was designed to test this hypothesis and to study the possible relationships to rotation of dopamine, acetylcholine and norepinephrine.