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IN VIVO AND *IN VITRO* EFFECTS OF A NEW HYPOGLYCEMIC AGENT, 2-(3-METHYLCINNAMYLHYDRAZONO)-PROPIONATE (BM 42.304) ON GLUCOSE METABOLISM IN GUINEA PIGS

H. F. KÜHNLE, F. H. SCHMIDT and I. V. DEACIUC
Boehringer Mannheim GmbH, Medizinische Forschung, Stoffwechsel, D-6800 Mannheim 31, F.R.G.

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Abstract—A new compound, 2-(3-methylcinnamylhydrazono)-propionate (BM 42.304), showed a dose dependent hypoglycemic effect in starved guinea pigs after both oral and intraperitoneal administration. In contrast to biguanides (phenformin and metformin) the new compound produced only a moderate increase in blood lactate concentration and did not alter the content of adenine nucleotides in the freeze-clamped liver *in vivo*. Gluconeogenesis from a variety of precursors in the perfused guinea-pig liver was also inhibited by BM 42.304. These properties suggest that the compound deserves further investigation in connection with its potential usefulness for the treatment of diabetes.

Different groups of investigators have reported in the last years on new classes of compounds having a hypoglycemic effect [1–16]. In addition to their potential usefulness for the treatment of diabetes, such compounds provide also important tools for studying the regulatory aspects of various metabolic pathways, especially of gluconeogenesis. Examples in this respect are offered by 3-mercaptopicolinate, a hypoglycemic agent [3], which is frequently used as an inhibitor of phosphoenolpyruvate carboxykinase [13, 14, 17–19] and by 2-tetradecylglycidic acid (McNeil 3802), an inhibitor of carnitine acyltransferase I [20], which has already been used to study the role of fatty acid oxidation in the regulation of gluconeogenesis [21].

A new hypoglycemic agent and an inhibitor of gluconeogenesis, 2-(3-methylcinnamylhydrazono)-propionate (BM 42.304, abbreviated here as MCHP, Fig. 1), has been synthetized and investigated in our laboratories. The results concerning its hypoglycemic action as well as its inhibitory effects on hepatic gluconeogenesis are presented in this paper. MCHP belongs to an already known class of hypoglycemic compounds described by Haeckel and Oellerich [7–9]. Two biguanides, phenformin (2-phenylethylbiguanide) and metformin (*N*,*N*-dimethylbiguanide), widely known hypoglycemic agents, were taken as a term of comparison with the new compound.

MATERIALS AND METHODS

Animals. Guinea pigs of both sexes (mixed strain), weighing 300–400 g (Meckel, Giessen) were used. The animals were appropriately housed and fed a corresponding laboratory diet. Whenever starved, the animals had free access to water.

Chemicals. MCHP and metformin were synthetized by Drs. M. Hübner and R. Heerdt of the Division of Chemistry of Boehringer Mannheim GmbH. Phenformin was purchased from Hoechst AG. All other chemicals, including substrates, enzymes and coenzymes were of the highest purity commercially available. Most of them were from Boehringer Mannhein GmbH.

In vivo *experiments*. In order to investigate the effect of the drugs on the blood glucose and lactate concentration, 24-hr-starved guinea pigs were given i.p. the drugs; MCHP as Na salt and the biguanides as HCl salts in neutral solution of NaCl (0.9%) or, for the control groups, NaCl (0.9%). At the intervals indicated on the appropriate graphs, blood samples (10–20 μl) were collected from an ear vein and assayed for glucose (see below). In experiments where blood lactate concentration was determined the animals were sacrificed by decapitation and 0.5 ml of blood was immediately treated with 0.5 ml perchloric acid (10%, w/v).

In vivo freeze-clamping experiments were performed in order to test the effect of MCHP and metformin on the content of adenine nucleotides in the liver. The compounds were administered i.p. to the guinea pigs in doses indicated in the legend to the corresponding graphs. Blood samples (see above) for glucose assay were collected as described. At the end of the third hour after the drug administration the animals were narcotized with Na pentabarbital (50 mg/kg, i.p.) and the abdomen was opened and the liver exposed for clamping with the aid of aluminium tongs precooled in liquid nitrogen. The clamps were applied always on the big left lobe of the liver.

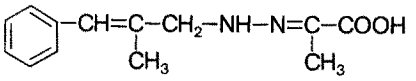

Fig. 1. Structural formula of the investigated compound, 2-(3-methylcinnamylhydrazono)-propionate (MCHP).

Preparation of the perchloric acid extracts for assay of metabolites was referred to in detail in a previous paper [6].

In vitro *experiments*. These were performed on the perfused liver and isolated hepatocytes. Flow-through (i.e. non-recirculating), hemoglobin-free perfusion of the liver of 48-hr-starved guinea pigs was conducted as described in a previous paper [22]. Effluent samples were continually collected with the aid of a fraction collector set at 3 or 5 min intervals (see the graphs) and used for metabolite assay.

Isolated hepatocytes were prepared as described by Krebs *et al.* [23] from the livers of 48-hr-starved guinea pigs. Cells (30–40 mg, dry weight) were incubated in a final volume of 5 ml of Krebs–Ringer bicarbonate buffer, pH 7.4, containing 2% (w/v) bovine serum albumin with a low fatty acid content (i.e. 15 μg/g) dialysed against Krebs–Ringer bicarbonate buffer, and the additions as indicated in the appropriate figure. The incubation was carried out in a shaking water bath (80 strokes/min), at 37° in 50 ml Erlenmeyer flasks. The gaseous phase was O_2 : CO_2 (19 : 1). At 20, 40 and 60 min intervals, 1 ml of incubation mixture was collected and treated with 1 ml of 6% (w/v) of perchloric acid. After each sampling, the gaseous phase of the vessels was refreshed with the O_2 : CO_2 mixture. The perchloric acid extract was neutralized with KOH (20%, w/v) in the presence of triethanolamine (50 mM final concentration) and used for glucose assay (see below).

Metabolite assay. Glucose [24], lactate [25], AMP [26], ADP [26] and ATP [27] were assayed enzymically according to the cited procedures.

RESULTS

In vivo *experiments*. Effects on blood glucose and lactate concentration. As shown in Fig. 2, MCHP had a hypoglycemic effect dependent upon dose with respect to both intensity and duration, which compares well with that of phenformin (Fig. 3). The hypoglycemic effect of MCHP is associated with an increase of blood lactate concentration (Fig. 4). Administration of 2-chloropropionate, an activator of pyruvate dehydrogenase [28], counteracted almost totally the increase in the blood lactate concentration produced by MCHP but it was less effective in the case of phenformin treatment (Figs. 4 and 5).

Effects on the content of adenine nucleotides in liver. The hypoglycemic effect of MCHP, given i.p. in a dose of 15 mg/kg (see Fig. 6) was not associated with significant changes of the content of adenine nucleotides in liver. Metformin, given in a dose to produce a hypoglycemic effect almost identical to that of MCHP, caused a considerable decrease of ATP content coincident with an increased content of ADP and AMP in the liver (Table 1).

In vitro *experiments*. Given at increasing concentrations to the perfused liver, MCHP showed a concentration-dependent effect on glucose formation from lactate + pyruvate (Fig. 7). The lowest concentration for which we were able to obtain an inhibitory effect on hepatic gluconeogenesis in perfusion experiments was 1 μM (not shown here; provided that the inhibitor was infused for a longer period of time). However, for most of the perfusion experiments we used a concentration of 10 μM of MCHP.

Gluconeogenesis from other precursors such as alanine (Fig. 8A), propionate (Fig. 8B), glutamine (Fig. 9A), dihydroxyacetone (Fig. 9B), glycerol (Fig. 10A) and fructose (Fig. 10B) was inhibited by MCHP at 10 μM in the perfused guinea-pig liver.

As shown in Fig. 11, MCHP was almost as effective as phenformin in inhibiting gluconeogenesis from lactate by isolated hepatocytes.

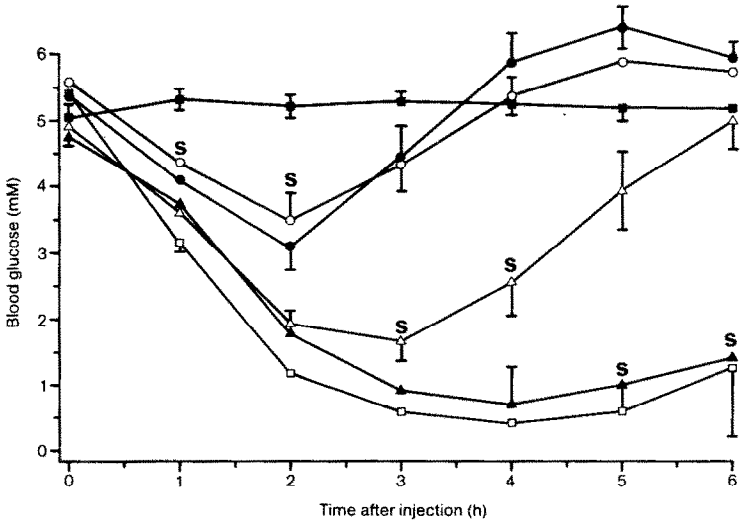

Fig. 2. Dose dependent hypoglycemic effect of MCHP in starved guinea pigs. Doses (mg/kg, i.p.): ■, Control; ○, 7.5; ●, 10; △, 15; ▲, 25; □, 35. Plotted points are mean values ± S.E.M. (vertical bars) for 10 animals, except the control (18 animals). Where not drawn, the S.E.M. were too small to be represented at the scale. All the points situated below those marked with "s", no matter on what curve, differ statistically significant ($P < 0.05$) from the points marking the control curve.

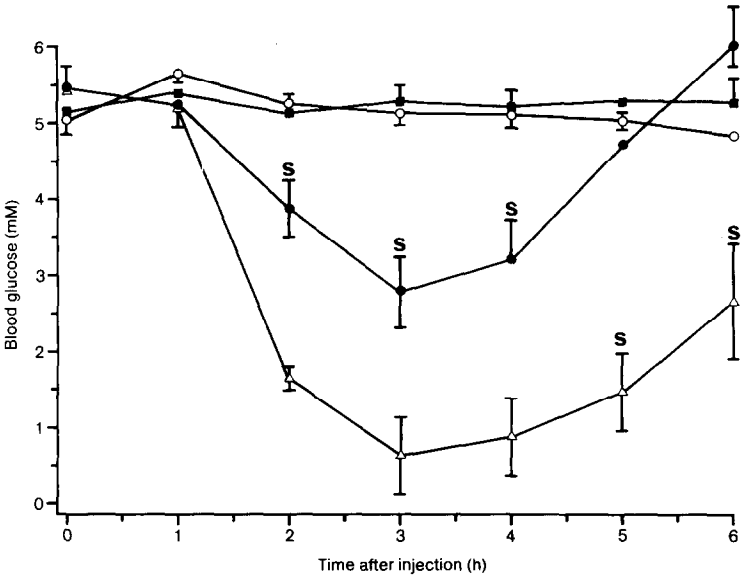

Fig. 3. Dose dependent hypoglycemic effect of phenformin in guinea pigs. Doses (mg/kg, i.p.): ■, Control; ○, 7; ●, 10; △, 15. All other details as in Fig. 2.

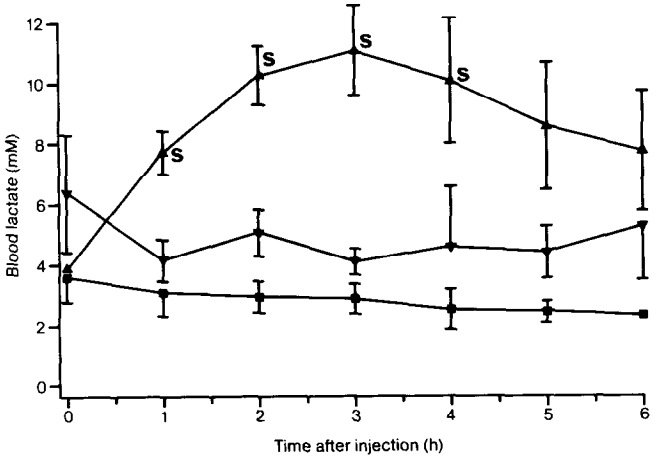

Fig. 4. Effect of MCHP alone or in association with 2-chloropropionate on the blood lactate concentration in guinea pigs. Doses (mg/kg, i.p.): ■, Control; ▲, MCHP, 10; ▼, MCHP, 10 + 2-chloropropionate, 100. Plotted points are mean values ± S.E.M. for 6 animals in each group. The points marked with "s" differ statistically significant from the corresponding points on the curve of control.

Table 1. Effect of MCHP and of metformin on the content of adenine nucleotides in the guinea-pig liver *in vivo*

Conditions	ATP	ADP	AMP
		(μmoles/g tissue, wet wt)	
Control (N = 10)	2.12 ± 0.08	1.33 ± 0.05	0.35 ± 0.02
MCHP (N = 5) 15 mg/kg, i.p.	2.34 ± 0.10	1.28 ± 0.08	0.37 ± 0.02
Metformin (N = 5) 50 mg/kg, i.p.	1.31 ± 0.08*	1.90 ± 0.06*	0.73 ± 0.04*

Values are mean ± S.E.M with the number of animals shown in parantheses.
* Shows a statistically significant difference from the control at $P < 0.01$.

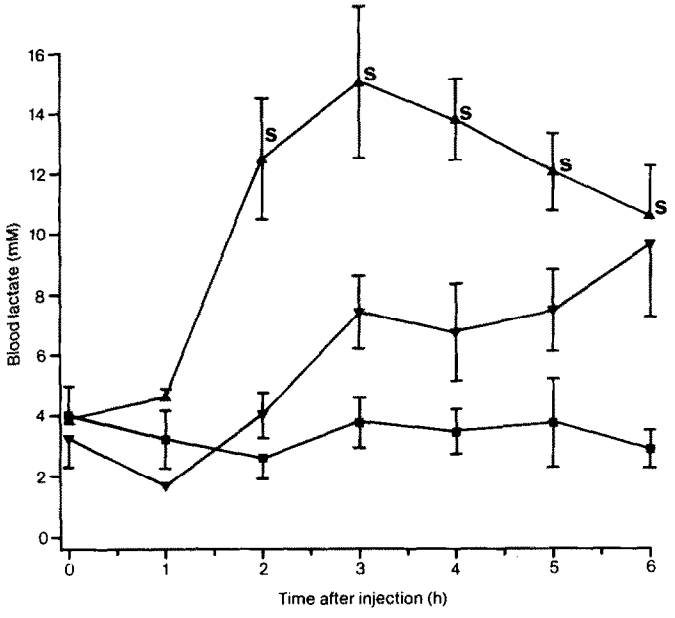

Fig. 5. Effect of phenformin alone or in association with 2-chloropropionate on the blood lactate concentration in guinea pigs. Doses (mg/kg, i.p.): ■, Control; ▼, phenformin, 12; ▼, phenformin, 12 + 2-chloropropionate, 100. Plotted points are mean values ± S.E.M. for 4–6 animals in each group. For other details, see the legend to Fig. 4.

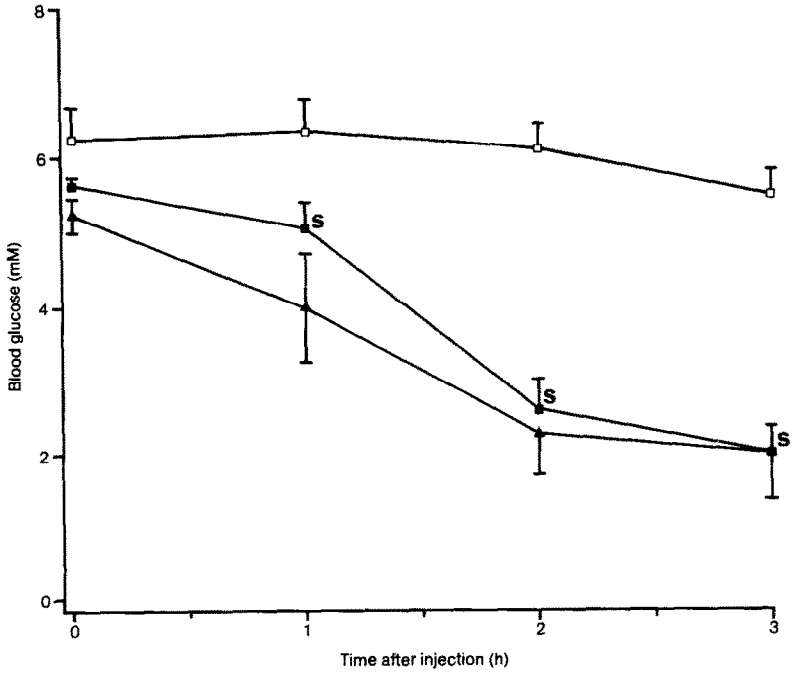

Fig. 6. Evolution of the blood glucose concentration in guinea pigs used for *in vivo* freeze-clamping experiments. Doses (mg/kg): □, Control; △, MCHP, 15; ■, Metformin, 50. The plotted points are mean values ± S.E.M. for 4–6 animals in each group. Other details, as in Fig. 2.

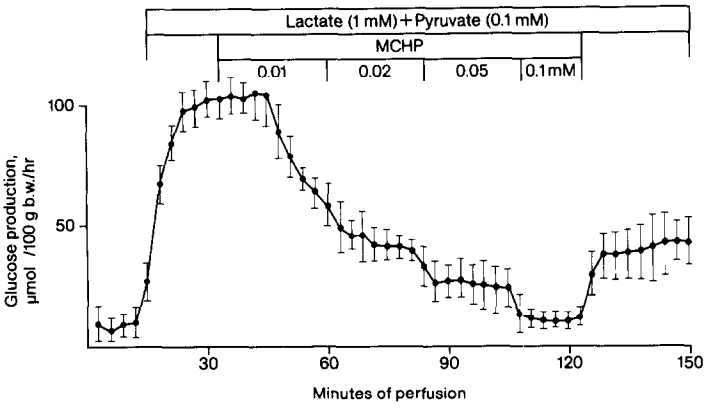

Fig. 7. The effect of different concentrations of MCHP on the rate of glucose formation from lactate + pyruvate by the perfused guinea-pig liver. The horizontal columns in this and subsequent similar graphs mark the period of time for which a compound, mentioned inside the columns, was being infused to the perfusate entering the liver so as to give the final concentration specified inside the columns. Each point on the graphs representing perfusion experiments is the mean value for at least 4 perfusions. The vertical bars, as in Fig. 2.

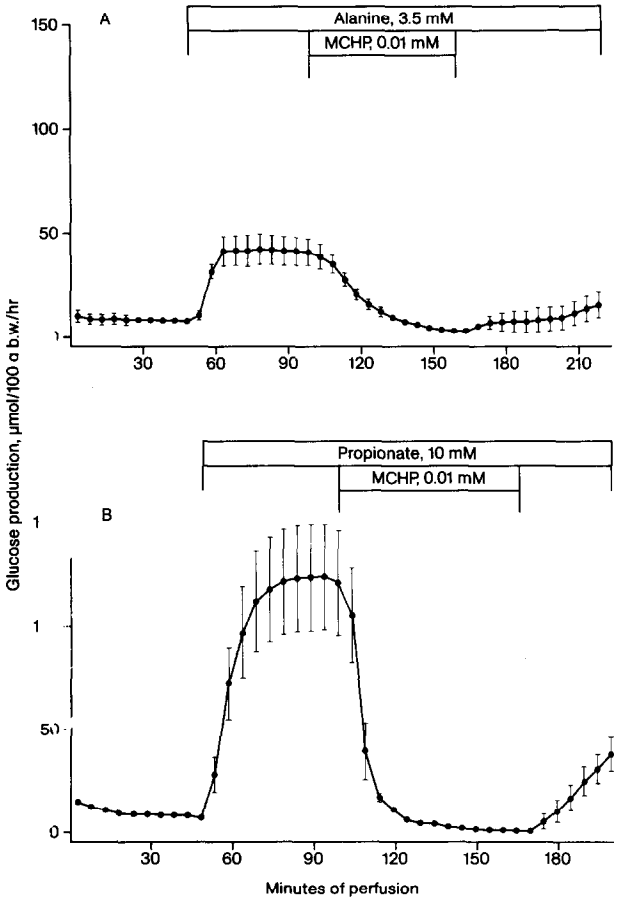

Fig. 8. Effect of MCHP on the rate of gluconeogenesis from alanine (A) and propionate (B) in the perfused guinea-pig liver. For other details see the legend to Fig. 7.

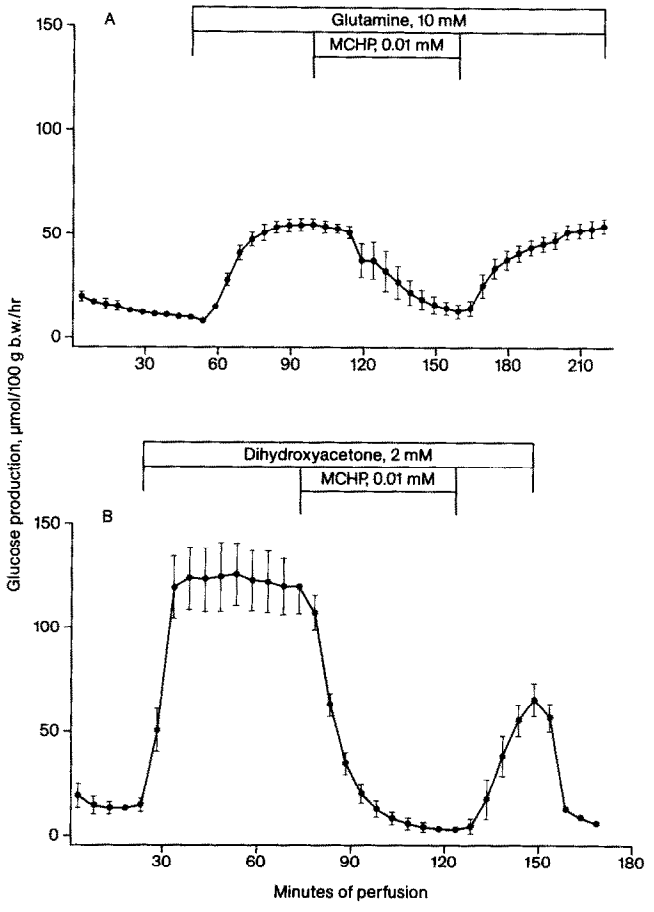

Fig. 9. Effect of MCHP on the rate of gluconeogenesis from glutamine (A) and dihydroxyacetone (B). For other details, see the legend to Fig. 7.

DISCUSSION

Amongst the plausible suppositions to be made in connection with the mechanism of action of a hypoglycemic agent is that it might either stimulate insulin secretion and/or release, or somehow inhibit hepatic gluconeogenesis. The second was the main idea that underlay the investigation reported in this paper. Indeed, the *in vivo* experiments, showing that hypoglycemic effect of MCHP was associated with an increased lactatemia favours such a supposition. It seems, however, that the way MCHP affects the rate of hepatic gluconeogenesis is quite different from that of phenformin and, probably, of other biguanides. This is supported by the fact that activation of pyruvate dehydrogenase complex with the aid of 2-chloropropionate led to an efficient removal of lactate from the blood in the presence of MCHP but not of phenformin. This should be connected to the fact that phenformin was shown to be an inhibitor of pyruvate oxidation by different tissues [29]. Our data show that such an inhibition is not counteracted by 2-chloropropionate, an activator of pyruvate dehydrogenase [28]. Therefore, the increase in blood lactate concentration produced by MCHP seems to be a consequence of a decreased lactate conversion into glucose rather than a consequence of an impaired utilization of pyruvate by different tissues. With this respect one should notice that the main mechanism of action of phenformin and, in general, of biguanides, consists in an unspecifical inhibition of the energy generating and transferring processes at the mitochondrial level [30, 31]. This is also supported by our data on the metformin effect on ATP concentration in liver *in vivo*. MCHP, in spite of producing a pronounced hypoglycemia, did not affect the content of adenine nucleotides in the liver. This suggests that the site of its attack on gluconeogenesis is not an impairment of ATP generation in the liver.

Since the new compound reported here exerts an inhibitory effect on glucose formation from a broad variety of precursors that enter the gluconeogenic pathway at different enzymatic steps, a direct inhibitory effect of MCHP on any of the gluconeogenic enzymes seems to be ruled out.

The data reported here are in agreement with those obtained by Haeckel and Oellerich [7–9] with other two compounds, i.e. 2-(phenylethylhydrazono)- and 2-(2-cyclohexylethylhydrazono)-propionate, belonging to the same class of compounds. MCHP seems, however, to be a more

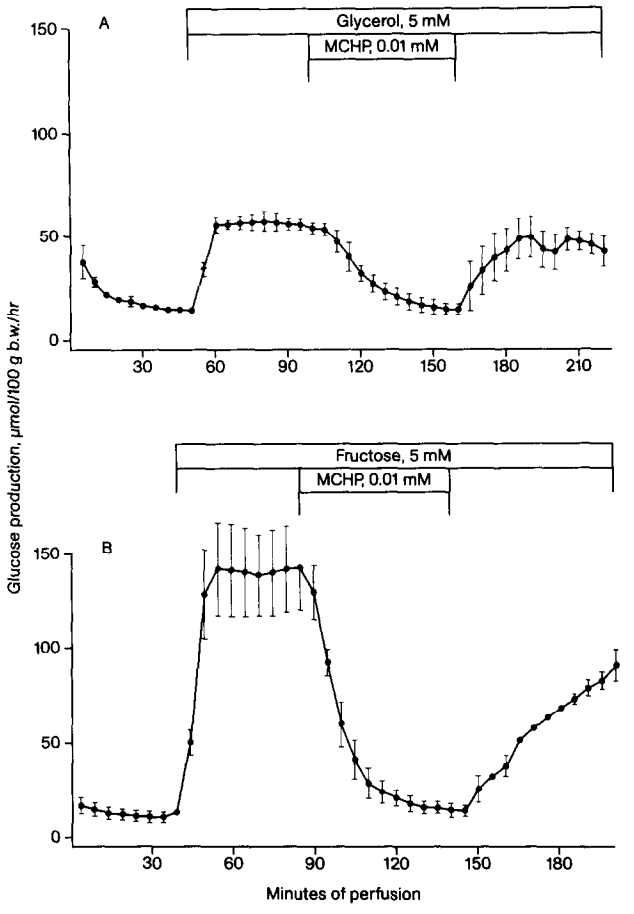

Fig. 10. Effect of MCHP on the rate of gluconeogenesis from glycerol (A) and fructose (B). For other details see the legend to Fig. 7.

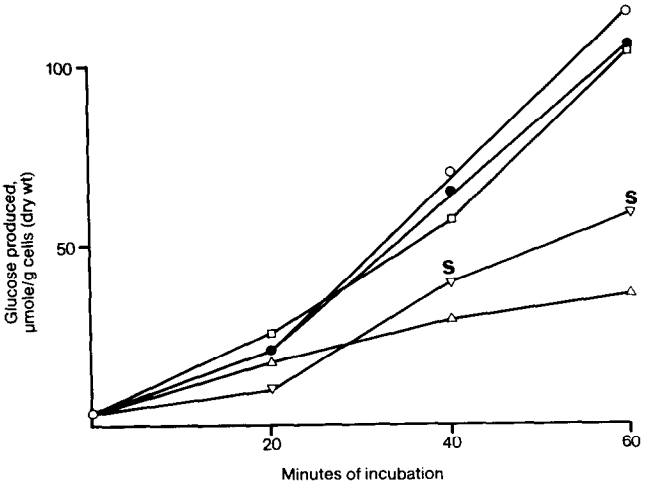

Fig. 11. Effect of MCHP and of phenformin on the glucose formation from lactate by isolated guinea-pig hepatocytes. □, Lactate (8 mM); ●, lactate + MCHP (0.01 mM); ▽, lactate + MCHP (0.05 mM); ○, lactate + phenformin (0.01 mM); △, lactate + phenformin (0.05 mM). Plotted points are mean value for 4 animals. S.E.M. were not drawn. For "s" see the legend to Fig. 2.

powerful hypoglycemic agent than the two analogs mentioned above. In addition and unlike the other two compounds MCHP does not exert an inhibitory effect on the activity of monoaminooxydase *in vivo* or *in vitro* (H. F. Kühnle, unpublished observations).

Experiments performed in our laboratory on isolated guinea-pig liver mitochondria showed that neither oxidative phosphorylation nor the rate of phosphoenolpyruvate synthesis were affected by the inhibitor at concentrations exceedingly higher (0.5 mM) than those necessary to produce an impairment of glucose formation in the perfused liver (manuscript submitted for publication).

In addition we obtained experimental data showing that the compound is an inhibitor of the transfer of long-chain fatty acids across the mitochondrial inner membrane of isolated guinea-pig liver mitochondria. In full agreement with such a mechanism of action are experimental data showing that MCHP inhibits QO_2 of isolated guinea-pig liver mitochondria in the presence of palmitoyl-carnitine and palmitoyl-CoA plus L-(−)-carnitine but not in the presence of octanoate, glutamate and malate; it also inhibited the ketogenesis by the perfused guinea-pig liver with oleate but not with octanoate. Finally, an increased concentration of free fatty acids in the blood of guinea-pigs associated with a hypoketonemia were also observed after administration of the compound. All these experimental data (soon to be published) offer a plausible explanation for the hypoglycemic effect of MCHP described in this paper.

The data presented in this paper suggest that MCHP has properties that make it an efficient hypoglycemic agent deserving further investigation in the view of its usefulness for the treatment of diabetes.

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