available. It should be pointed out that M-H does not remove the natural agglutinins of 1-day-old chicken erythrocytes from human serum under the conditions employed here. It was necessary to absorb the M-H treated sera with erythrocytes to remove these agglutinins. For this reason it was essential to include serum-erytrocyte controls with each serum tested.

Summary. The nonspecific inhibitor(s) of rubella-virus hemagglutination were removed from human serum by the rapid, simple, and specific procedure of adding manganous chloride and heparin to the serum. This procedure was shown to precipitate preferentially β-lipoprotein without altering the immunoglobulin level in serum. The rubella-virus HI antibody titers after M-H treatment were equal to, or greater than, that after kaolin absorption and correlated in a high degree to neutralizing antibody titers.

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The Hyperglycemic and Antidiuretic Activity of a Phthalimidine Analogue: 1-Oxo-3-(4′-chlorophenyl)-3-hydroxyisoindoline (C3/76)* (33072)

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(Introduced by C. Davison)
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Diazoxide, a nondiuretic benzothiadiazine, has already been shown by several investigators to cause hyperglycemia by inhibition of insulin secretion in some species (1, 2). Continuous search for drugs with fewer side effects and a prolonged action is stimulating interest in the structure–activity relationships of diazoxide as well as in the chemical similarities of diazoxide to the diuretic benzothiadiazines. Chlorothiazide, a member of the latter group of drugs, is widely used in clinical practice and has been reported to cause hyperglycemia. Chlorothalidone, which has a different chemical structure than the benzothiadiazines, has also been reported to cause hyperglycemia and importantly causes a similar but prolonged diuresis (3).

We prepared an analogue of chlorthalidone in which the sulfamyl group had been removed. The compound is 1-Oxo-3-(4′-chlorophenyl)-3-hydroxyisoindoline (Fig.

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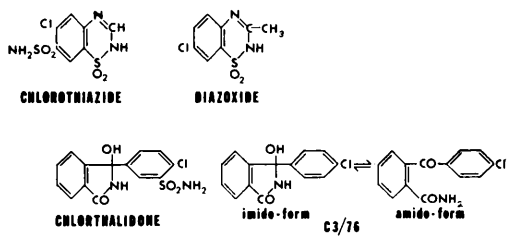

FIG. 1. Structural formula.

1) and is one of a series of analogs synthesized in our laboratories, referred to in this communication under a code C3/76. This drug has also been studied by Topliss *et al.* (4), as part of a general screening study of antihypertensive agents under the name 3-(*p*-chlorophenyl)-3-hydroxyphthalimide.

This communication deals with the effects of administration of this compound *in vivo* and *in vitro*. The implication of the results is discussed.

Methods and Materials. The C3/76 was prepared by the method of Graf *et al.* (5), by the formation of the acyl chloride of 2-(4′-chlorobenzyl)-benzoic acid and the amination and ring closure of the pseudochloride. Recrystallization from 80% ethanol yielded a white crystalline solid, mp 215°C, elemental analysis showed: C, 65.01% (64.75%); H, 3.89% (3.88%); N, 5.10% (5.39%); Cl, 13.46% (13.65%). Figures in parentheses indicate required analysis percent. The UV spectroscopy showed an absoption max. at $\lambda = 224$ mμ and IR spectroscopy confirmed the structure. The compound is only slightly soluble in water at physiological pH, but becomes more soluble at alkaline pH. It is freely soluble in dimethylformamide. However, in solution this compound probably exists in two forms (Fig. 1).

Animal studies. Rats. Male CFE[3] strain rats, weight range 200–300 gm, fed a standard laboratory cube diet, and water *ad libitum* were used. If fasted, food, but not water, was withheld from the rats for 24 hours. Capillary blood samples for sugar levels were taken from the tip of tail and estimated using the standard "Autoanalyzer" method (6).

For experiments in which urine output was measured, rats were kept in metabolic cages, having been loaded with 5 ml water by stomach tube 30 min before injection of the drug. Urine was collected for 6 hours.

In all experiments on rats, the compounds C3/76 and chlorthalidone were administered intraperitoneally, suspended in 1% gelatin due to their low solubility. Regular insulin (Squibb) was injected intraperitoneally at a dose of 5 U/kg to ascertain the effect of exogenous insulin on the hyperglycemic activity of C3/76.

Dogs. Nine mongrel dogs were used. After overnight fasting, anesthesia was induced with sodium pentobarbital 30 mg/kg intravenously. Mean arterial blood pressure was measured by mercury manometer connected to the femoral artery and venous blood samples taken from the femoral vein.

Following a 1 hour equilibration period, 25 mg/kg of C3/76 in 9–14 ml of 70% dimethylformamide (DMF) was infused slowly intravenously during 10 min, in 3 dogs. For 1 hour before and 2 hours after the infusion venous blood was sampled for blood glucose, plasma insulin (8), serum FFA (9), and catecholamines (10) estimation. In three other dogs a similar volume of control solution of 70% DMF was injected, followed by a slow infusion of 20% glucose solution, to produce a similar change in blood sugar to that produced by C3/76.

In vitro *experiments.* Pieces of rabbit pancreas were prepared and incubated according to the method of Coore and Randle (7). Each experiment was performed in three phases. In each phase, the tissue was incubated in Krebs bicarbonate buffer at 37°C in a Dubnoff incubation bath for 30 min. In phase I, the "resting" phase, the medium contained a low glucose concentration (50 mg/100 ml). In phase 2, the tissue was transferred into medium with the same glucose concentration but containing C3/76 or chlorthalidone at a concentration of 50 μg/ml. In phase 3, the tissue was transferred into medium with a high glucose concentration (500 mg/100 ml) and the same concentration of the drugs. As both C3/76 and chlorthalidone required 1–2 drops of 1 N NaOH to dissolve in the medium, back

[3] Supplied by Carworth Farms, Inc.

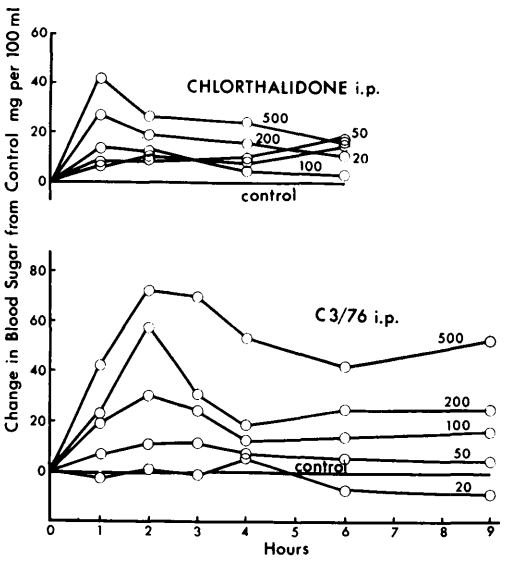

FIG. 2. Comparison of hyperglycemic response to chlorthalidone and C3/76 injected i.p. into rats. Doses shown as mg/kg. Response expressed as change in blood sugar from rats injected with control solution. Each group comprises eight rats.

titration with 0.1 *N* HCl was required to achieve a pH 7.4. Control media were similarly prepared. In the second experiment, C3/76 and chlorthalidone were used at a concentration of 100 μg/ml. Insulin released by the pancreatic tissue into the incubation medium was measured at the end of phases 2 and 3 by the double antibody radio immunoassay of Hales and Randle (8).

The insulin production is expressed in $\mu\mu$g/mg of tissue per 30 min. By comparing the amounts of insulin production in experimental and control incubation media, one can estimate the degree of inhibition of insulin production caused by the drugs.

Results. In the rat. Figure 2 shows the dose response relationship between hyperglycemia and the dose levels of C3/76 and chlorthalidone injected intraperitoneally. The hyperglycemic response to C3/76 is greater and more prolonged than that due to chlorthalidone. At the dose levels used, chlorthalidone is diuretic, while C3/76 reduces urine output (Fig. 3). Twenty-four hours after the injection of C3/76 blood sugar levels are significantly higher than in rats which received injection of control solution only

TABLE I. The 24-Hour Blood Sugar Levels in Rats Given One Injection of C3/76, or Diazoxide, or Control (i.p.).[a]

Drug	Dose (mg/kg i.p.)	24-hour blood sugar (mg/100 ml ± SE)	Significance
C3/76	100	78 ± 2.9	$p < 0.01$
	200	72 ± 1.8	$p < 0.01$
	500	77 ± 2.2	$p < 0.01$
Control		61 ± 1.2	
Diazoxide	100	67 ± 0.8	NS
	200	69 ± 1.8	NS
	500	68 ± 3.1	NS
Control		68 ± 3.5	

[a] p values derived from Student's *t* test with 6 animals in each group, at each dose level.

(1% gelatin), which is not the case with diazoxide treated rats (Table I).

The hyperglycemia due to C3/76 is reversed by exofienous insulin (Table II and Fig. 4).

In an experiment comparing the effect of C3/76 injected intraperitoneally into fed and fasted rats, the rise in blood sugar levels was more pronounced in the fasted group (Table III).

In the dog. The C3/76 in 70% DMF causes a prompt rise in blood sugar levels ($p < 0.01$ for 2 hours after infusion) and a fall in mean arterial blood pressure ($p < 0.05$ for 105 min after infusion). The hyperglycemic response is maintained for 2 hours (Fig. 5), while the blood pressure steadily returns towards normal (Fig. 6).

Even though there is a marked rise in

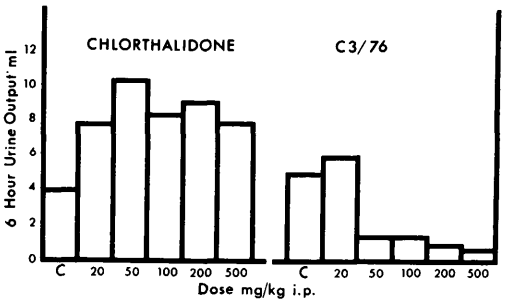

FIG. 3. Comparison of effects of C3/76 and chlorthalidone on 6 hour urine output in rats; C = control injection; 6 rats/group.

TABLE II. Effect of Insulin on Hyperglycemia Due to C3/76.[a]

Group	Mean blood sugar levels (mg/100 ml)						
Min:	0	60	75	90	120	180	240
1	120	163	91	83	55	38	30
2	116	159	179	188	172	180	177
3	117	118	58	63	56	55	44

[a] Each group consisted of six rats. Group 1 received 200 mg/kg of C3/76 in 1% gelatin i.p. at 0 min and insulin 5 U/kg in saline i.p. at 60 min. Group 2 received the same dose of C3/76 i.p. at 0 min and insulin control solution (saline) at 60 min. Group 3 received 1% gelatin at 0 min and 5 U/kg of insulin at 60 min.

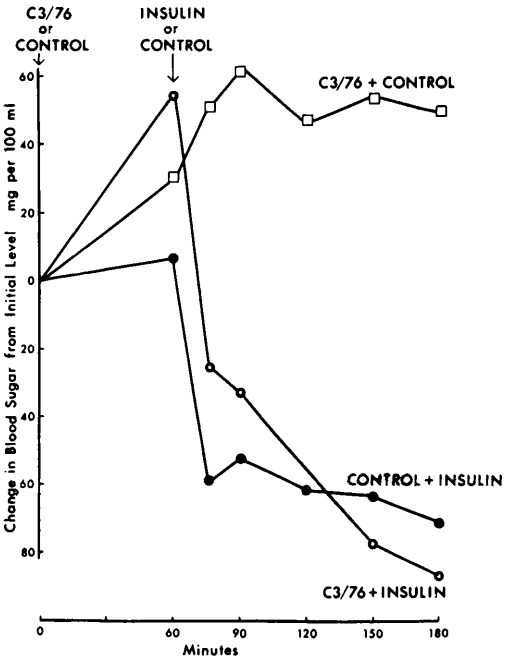

FIG. 4. Effect of insulin on blood sugar changes (mg%) due to C3/76 and control. Groups of six rats. Based on values in Table II.

blood sugar levels there was no significant change in plasma insulin levels (Fig. 5). These changes are accompanied by a rise in serum FFA levels ($p < 0.05$ at 1 hour after infusion) Fig. 6. Serum catecholamine levels showed no significant difference in the dogs receiving C3/76 from animals receiving control infusion of 70% DMF. A 70% DMF infusion alone caused no significant change in any parameter.

Intravenous glucose in 70% DMF sufficient to obtain a rise in blood sugar level similar to that caused by C3/76 was accompanied by a rise in plasma insulin levels ($p < 0.05$ for 2 hours after infusion) and a fall in serum FFA levels (not significant) and no change in serum catecholamine levels. The level of serum insulin is significantly higher in the dogs receiving glucose than in those receiving C3/76 even though blood sugar levels are comparable.

In Vitro *experiments*. In the incubation of pieces of rabbit pancreas neither chlorthalidone nor C3/76 at either concentration (5 or 10 mg/100 ml) caused inhibition of insulin release in low glucose medium (Table IV), compared to control incubation. At high glucose concentration chlorthalidone also showed no significant difference from control. How-

TABLE III. Effects of Fasting for 24 Hours on the Response to C3/76 (injected i.p.) in Comparison to Fed Rats.[a]

Drug	Food	Dose (mg/kg)	Initial blood sugar (mg/100 ml ± SE)	Rise in blood sugar over initial level (mg/100 ml) (hours after injection)				
				1	2	3	4	6
C3/76	Fasted	50	86 ± 3.4	9	10	9	13	8
C3/76	Fasted	200	85 ± 3.2	20	38	48	52	54
Control	Fasted	0	76 ± 4.4	7	4	5	10	1
C3/76	Fed	50	120 ± 5.6	5	2	4	1	—8
C3/76	Fed	200	122 ± 2.1	19	52	24	19	20
Control	Fed	0	124 ± 2.9	—2	—6	—5	—7	—14

[a] Six rats used in each group.

TABLE IV. Effect of C3/76 and Chlorthalidone (50 and 100 μg/ml) on Mean Insulin Production (μμg/mg of tissue/30 min) from Rabbit Pancreas Pieces Incubated in Media Containing Low or High Glucose Concentration.[a]

Drug in medium	Mean insulin production (μμg/mg of tissue/30 min) in glucose medium		Percentage of insulin production (control = 100%)
	Low glucose (50 mg/100 ml)	High glucose (500 mg/100 ml)	
Control (8)	671 ± 183[b]	1632 ± 198	100
C3/76 (50 μg/ml) (8)	747 ± 125	1553 ± 704	86
Chlorthalidone (50 μg/ml) (8)	824 ± 201	2334 ± 711	130
Control (8)	573 ± 88	2636 ± 386	100
C3/76 (100 μg/ml) (8)	657 ± 163	1678 ± 263	46[c]
Chlorthalidone (100 μg/ml) (8)	520 ± 94	2232 ± 351	91

[a] No. of observations in parentheses.
[b] Mean ± SE.
[c] Significance $p < 0.05$.

ever, 10 mg/100 ml of C3/76 produced over 50% reduction ($p < 0.05$) of insulin response to the increase in glucose concentration in the medium. At the lower concentration of C3/76 the effect was not marked. This result demonstrates a direct effect of C3/76 on insulin production in response to increased glucose concentration similar to that shown by Howell and Taylor (11) with diazoxide at a concentration of 12.5 mg/100 ml.

Discussion. The experiments presented have shown that C3/76, with a structure quite different from diazoxide, causes hyperglycemia in both starved or fed rats. It reduced urine output in the rat, an effect shared with diazoxide.

The recurring problem in evaluating drug-induced hyperglycemia in both fed and starved animals is well seen in these experiments. Both the rise in absolute blood sugar levels and the percentage change from the initial level due to C3/76 are greater in fasted animals. These animals probably have an increased rate of gluconeogenesis, while in fed rats increased breakdown of glycogen seems to be the prevalent mechanism.

In the dogs, the results suggest that C3/76 produces hyperglycemia by inhibiting insulin release but without any measurable change in serum catecholamine levels. This is of interest as catecholamines have been shown to inhibit insulin (12), and some workers believe that diazoxide-induced inhibition of insulin secretion is mediated by

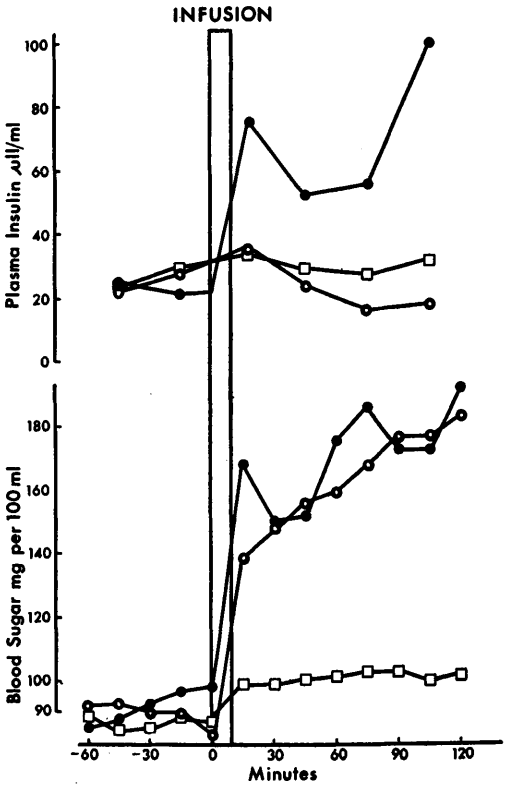

FIG. 5. Response in plasma insulin (μU/ml) and blood sugar (mg/100 ml) in the dog to 10-min infusion of C3/76 in 70% DMF: Each group of 3 dogs received: 70% DMF infusion (□); plus either 25 mg/kg of C3/76 (○); or 20% glucose (●).

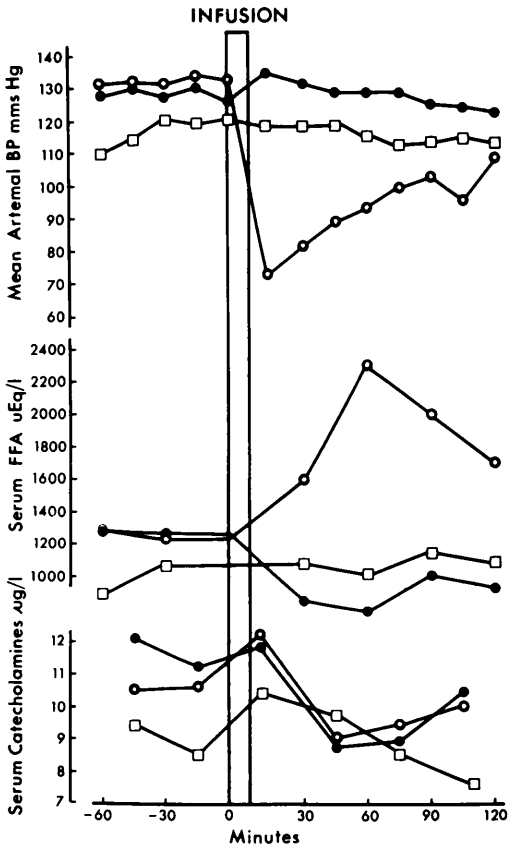

FIG. 6. Response in mean arterial blood pressure (mm Hg), serum FFA (μeq/liter), and serum catecholamines in the dog to 10-min infusion of C3/76 in 70% DMF; same dosage as Fig. 5.

catecholamines (2), as the effect can be attenuated by adrenergic blocking agents. The direct action of C3/76 on the B-cell is further substantiated by incubation studies of pancreatic tissue, where the drug decreases the insulin-secretion response to glucose.

It is surprising that the fall in blood pressure accompanying the administration of C3/76 in the dog did not evoke a catecholamine response. The increase of serum FFA levels does not clarify the problem as this change could have accompanied either the fall in insulin level or a small rise in catecholamine levels which we were not able to measure.

Whether C3/76 induces hyperglycemia by insulin inhibition alone has yet to be proven. As insulin is the only hormone which causes the blood sugar to fall, it is generally believed that if the homeostatic secretion of this hormone is reduced, hyperglycemia will occur. However, C3/76 may have two simultaneous effects; the first to cause hyperglycemia by action elsewhere, i.e., in liver and the second to block the ability of the B-cell to respond to the hyperglycemia.

Our findings with regard to blood pressure and antidiuresis agree with those reported by Topliss *et al.* (4). C3/76 at a dose of 10 mg/kg intravenously in the anesthetized dog produced a fall in blood pressure of an extent and duration similar to that produced by the same dose of diazoxide. In their experiments, the drug was reported as antidiuretic on oral administration to saline-loaded rats. However, they did not report blood sugar values in either of these experiments.

Despite the differences in chemical structure, there is a remarkable degree of similarity in activity between C3/76 and diazoxide. The same can also be said for chlorothiazide and chlorthalidone of which diazoxide and C3/76, respectively, are analogs. However, both C3/76 and chlorthalidone act for a longer period, the former as a hyperglycemic agent, the latter as a diuretic agent. Chlorthalidone is believed to act longer than chlorothiazide by virtue of its slow metabolism and renal handling (13) and C3/76 may be similarly effected.

The use of drugs causing inhibition of insulin secretion is as yet limited to the treatment of patients with hypoglycemia secondary to insulinoma (14) and hypoglycemic states in children (15). The evaluation of such drugs in functional hypoglycemia is being investigated in our clinic and the experimental use of these drugs in hyperinsulinemic obese diabetic patients may also be of interest in studying the kinetics of insulin secretion.

While C3/76 has a more prolonged action than diazoxide, the modifications of its structure have not removed the undesirable antidiuretic effect which follows diazoxide therapy. It is of theoretical as well as practical interest that small changes in the chlorthalidone structure change activity from diuresis to an-

tidiuresis and greatly enhance hyperglycemic activity.

Summary. An analogue of chlorthalidone (C3/76) has been studied in an attempt to obtain a long acting hyperglycemic agent. In rats C3/76 (administered i.p.) caused a dose-related hyperglycemia which lasted longer than that of diazoxide and was reversed by insulin. The drug was effective in both fed and fasted animals and the hyperglycemia was accompanied by a reduced urine output. In the dog, i.v. administration of C3/76 caused hyperglycemia, a fall in blood pressure, an inhibition of insulin secretion, a rise in serum free fatty acid levels, and no significant change in serum catecholamine levels. In *in vitro* studies with isolated rabbit pancreas, C3/76 reduced insulin secretion, while chlorthalidone had no effect. It can therefore be concluded, that C3/76 produces hyperglycemia by inhibition of insulin secretion similar to one of the reported modes of action of diazoxide. It has similar side effects, hypotension and antidiuresis, even though its chemical structure is quite dissimilar to that of diazoxide.

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Immunofluorescent Study on Early Virus-Cell Interaction in Shope Papilloma *in Vitro* System* (33073)

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Appearance of a new antigen, now widely referred to as T antigen, in cells infected with SV40, polyoma and adenovirus has been well established (1–3). The role of this new virus-related antigenic substance in lytic infection or in the neoplastic transformation has not been well understood as yet. Mainly due to the fact that a suitable *in vitro* experimental system was not available, few studies have been carried out on the "early events" of the virus-cell interaction in the Shope papilloma system. In a previous communication (4), we have presented some preliminary observations on the appearance of T antigen-like immunofluorescence in the nuclei of cells of embryonic skin culture approximately 20 hours after their exposure to the Shope papilloma virus (SPV). The present paper describes the results of more detailed findings suggesting that such a new antigen, which could be clearly distinguished from the viral structural antigen, does appear in the early

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