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Metabolic Effects of Phenethyldiguanide, A New Hypoglycemic Compound.* (23386)

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With the introduction of the sulfonylureas for treatment of diabetes mellitus, renewed attention has been paid to the use of orally effective compounds. The sulfonylureas have proven to be useful in selected diabetic patients, but in a significant number of subjects they have failed to control the metabolic imbalance. Therefore, a considerable effort has been made to find other compounds which might be more effective than the sulfonylureas. In a search for such compounds, Ungar (1) recently discovered that phenethyldiguanide (PEDG), administered orally or parenterally, caused profound hypoglycemia in certain animals. This interesting observation prompted investigation of the mechanism by which hypoglycemia is produced. An understanding of the mode of action of PEDG would be of great help in its clinical applications. In an attempt to elucidate this problem, we have made studies of the effect of PEDG on glucose, glycogen, lactic acid, urea and non-protein-nitrogen *in vivo* and *in vitro*.

Materials and methods. Guinea pigs weighing between 450 and 750 g were used in these studies because they have been shown to be more sensitive to PEDG† than other animal species(1,2). In each type of experiment at least 24 animals were sacrificed, 12 serving as experimentals and 12 as controls. The drug was injected subcutaneously because responses after administration by this route were more uniform than after oral or intraperitoneal intake. The dose used was 20 mg/kg.

The following determinations were made 4 hours after PEDG administration as well as at longer and shorter intervals: blood glucose by the method of Somogyi(3), liver and muscle glycogen, using KOH for tissue digestion by the anthrone reagent of Carroll *et al.*(4), blood urea nitrogen by the method of Barker (5) and blood lactic acid by the method of Barker and Summerson(6). Non-protein-nitrogen in the total amount of urine produced during the 4 hours following PEDG injection was determined by the method of Fee *et al.*(7). In order to collect all of the urine, a small abdominal incision was made, the bladder emptied and the urethra ligated just before PEDG was given. Four hours later the urine in the bladder was collected with a needle and syringe.

The first part of the study *in vitro* was carried out on guinea pig liver slices. Slices weighing approximately 100 mg were incubated for 45 minutes at 37° in 2 ml of a solution containing 4 parts of 0.9% NaCl and one part of 0.1 M potassium phosphate buffer, pH 7.5. The beakers were placed in a Dubnoff apparatus and shaken at a rate of 110 oscillations per minute. Three different concentrations of PEDG were tested, at 50, 250 and 500 μg per ml of medium. At the end of the incubation, glucose and lactic acid concentration in the medium and glycogen concentration in the tissue were measured, using the same chemical methods mentioned in the *in vivo* studies.

The second part of the *in vitro* study was conducted with isolated rat diaphragms.

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† PEDG has been supplied through the courtesy of Dr. Louis Freedman, U. S. Vitamin Corp., Yonkers, N. Y.

TABLE I. Effect of PEDG on Blood Glucose, on Liver and Muscle Glycogen.

	Blood glucose, mg %	Liver glycogen, mg/g		Muscle glycogen, mg/g	
		Non-fasted	Fasted	Non-fasted	Fasted
Experimentals	31 ± 4*(12)†	.6 ± .06(19)	.3 ± .01(19)	7.3 ± .4 (11)	5.5 ± .1 (20)
Controls	103 ± 7 (14)	20.5 ±5.4 (18)	2.0 ± .4 (18)	10.7 ± .2 (11)	5.4 ± .1 (21)
p	<.001	<.001	<.001	<.001	

* Stand. error of mean. † No. of animals.

Hemidiaphragms, weighing approximately 125 mg were incubated in 2 ml of Gey and Gey bicarbonate buffer containing 300 mg glucose/100 ml. One-half of the hemidiaphragm served as a control, while to the other half, 500 μg PEDG/ml was added. The Warburg flasks were flushed for 5 minutes with 95% O_2 and 5% CO_2. After incubation of the hemidiaphragms at 37° for 90 minutes, glucose and lactic acid concentration in the medium and glycogen concentration in the tissue were determined. In the experiments where oxygen consumption was measured, the hemidiaphragms were incubated in a potassium phosphate sodium chloride buffer solution and the flasks were flushed with air or with oxygen.

Results. It was found that the hypoglycemia caused by PEDG was accompanied, in fed as well as in fasted animals, by a rapid fall in glycogen content of the liver. This fall was readily demonstrable 2 hours after PEDG injection but was most marked after 4 hours (Table I). Muscle glycogen determinations made at several time intervals failed to show any difference in concentration between control and experimental groups if the animals were fasted for 24 hours prior to the injection. In fed animals there was a slight but significant drop in glycogen concentration 4 hours after injection (Table I).

Table II indicates that the non-protein-nitrogen excretion in the urine which was collected during the 4 hours following PEDG administration, was markedly lower than in the controls. Blood urea nitrogen was slightly elevated by PEDG treatment. In order to eliminate the effect of PEDG on the kidney, blood urea nitrogen was determined in nephrectomized animals. Table II shows that in such animals PEDG was associated with less rise in blood urea nitrogen concentration.

PEDG caused a rise in blood lactic acid. (Table III). This rise was observed one hour after PEDG injection and even before the blood glucose started to drop, and was also evident 4 hours after PEDG administration and was even more marked in nephrectomized animals.

Addition of PEDG to the incubation medium of guinea pig liver slices caused a small but significant rise in lactic acid production. There was a decrease in glycogen concentration in the tissue but no significant increase in glucose output (Table IV). (These effects are obtained with a concentration of 250 μg PEDG per ml incubation medium, but they are more pronounced if 500 μg per ml is added.)

In isolated rat diaphragms, PEDG in a concentration of 500 μg per ml caused an increase in lactic acid formation, an increased glucose uptake, a decreased glycogen formation, and a decreased oxygen consumption (Table V).

TABLE II. Effect of PEDG on Nitrogen Metabolism.

	4 hr urinary NPN excretion, mg	Blood urea nitrogen, mg %	Blood urea nitrogen, mg % Nephrectomized animals
Experimentals	12.5 ± 2.4*(9)†	31.8 ± 5.4 (16)	42 ± 2.8 (11)
Controls	39.2 ± 14.6 (9)	20.1 ± 7.0 (15)	64.6 ± 2.6 (11)
p	<.01	<.01	<.001

* Stand. error of mean. † No. of animals.

TABLE III. Effect of PEDG on Blood Lactic Acid (mg %).

	1 hr	4 hr	4 hr Nephrectomized animals
Experimentals	20.7 ± 1.7*(10)†	27.3 ± 2.3 (9)	75.6 ± 3.5 (6)
Controls	13.5 ± .3 (10)	15.8 ± 1.5 (10)	14.1 ± 3.1 (6)
p	<.01	<.001	<.001

* Stand. error of mean. † No. of animals.

Discussion. The observation that PEDG causes an early and marked decrease in liver glycogen concentration rules out the possibility that hypoglycemia is produced by a decreased glycogenolysis in the liver. The fact that no rise in muscle glycogen concentration is observed after PEDG administration indicates that hypoglycemia cannot be explained on the basis of an increased glycogen deposition in the muscle. Williams *et al.* reported that $C^{14}O_2$ production is not significantly different in either PEDG treated or control animals following administration of glucose-U-C^{14}(8), suggesting that hypoglycemia is not produced by an increased glucose oxidation.

Since there was evidence that PEDG did not cause an increased glucose utilization, the possibility of a decreased gluconeogenesis was then investigated. It was apparent from the studies on nitrogen metabolism that PEDG decreases urea formation. A decreased gluconeogenesis from protein is consistent with such a finding and may contribute significantly to the hypoglycemic action of PEDG.

It is likely, however, that this is not the only mechanism by which hypoglycemia is produced. It was found, indeed, that PEDG had a marked effect on lactic acid production. An increased lactic acid formation was observed in the total guinea pig, in surviving guinea pig liver slices, and in isolated rat diaphragm. These changes suggest that an increased anaerobic glycolysis might also account for the hypoglycemic action of PEDG. The increased glucose uptake, decreased glycogen deposition and oxygen consumption in the diaphragms and the decrease in liver glycogen concentration observed *in vivo* and *in vitro* are consistent with such a hypothesis.

It is evident from the data presented that PEDG acts like Synthalin A in many respects, but is different from insulin and the sulfonylureas.

Summary. When phenethyldiguanide (PEDG) is administered to guinea pigs, it produces hypoglycemia accompanied by depletion of liver glycogen with no increase in muscle glycogen. PEDG causes decreased

TABLE IV. Effect of PEDG on Glucose and Lactic Acid Output and on Glycogen in Guinea Pig Liver Slices.

	Glucose output, mg/g	Lactic acid output, mg/g	Glycogen at end of incubation, mg/g
Experimentals	11.7 ± 7.9*(13)†	1.14 ± .09 (13)	39 ± 2.9 (7)
Controls	10.5 ± 6.0 (13)	.65 ± .04 (13)	47 ± 2.0 (7)
p	<.5	<.001	<.05

* Stand. error of mean.
† No. of liver slices. Not more than 6 slices (3 controls - 3 experimentals) were made out of one liver.

TABLE V. Effects of PEDG on Glucose Uptake, Lactic Acid Output, Oxygen Uptake, and Glycogen in Isolated Rat Diaphragms.

	Glucose uptake, mg/g	Lactic acid output, mg/g	Glycogen at end of incubation, mg/g	Oxygen uptake, μl/g
Experimentals	8.1 ± .6*(12)†	5.3 ± .5 (12)	1.4 ± .3 (12)	1330 ± 93 (8)
Controls	5.6 ± .5 (12)	2.0 ± .3 (12)	2.8 ± .2 (12)	1910 ± 93 (8)
p	<.01	<.001	<.001	<.001

* Stand. error of mean. † No. of hemidiaphragms.

blood urea formation and decreased non-protein nitrogen excretion in the urine. It also produces an early and significant rise in blood lactic acid. From studies made on surviving slices of guinea pig liver, it is evident that PEDG increases lactic acid formation and increases glycogen depletion, but it does not affect glucose output. PEDG also exerts an effect on isolated rat diaphragm, increasing glucose uptake, increasing lactic acid production, decreasing glycogen formation, and decreasing oxygen uptake.

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Calcification XVIII. Lack of Correlation between Calcification *in vitro* and Glycolytic Enzymes.* (23387)

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Harris(1) reported that the glycogen present in preosseous cartilage played a role in calcification. Robison and Rosenheim(2) found that certain enzyme inhibitors such as iodoacetate, cyanide and fluoride caused inhibition of calcification *in vitro*. From these observations it was suggested that a phosphatase enzyme system in preosseous cartilage was responsible for calcification. Later, Gutman(3,4) stated that one of the major difficulties of the Robison postulate was its failure adequately to demonstrate the presence of significant amounts of phosphoric ester substrate at site of phosphatase activity in the hypertrophic cartilage. Gutman then presented the hypothesis that a phosphorylative enzyme initiated the degradation of glycogen to glucose-l-phosphate which then passed through the glycolytic cycle of hexose and triose phosphates to phosphopyruvate and that one of these phosphoric esters acted as substrate for the phosphatase present in the preosseous cartilage. In a study of the effect of glycolytic enzyme inhibitors on calcification *in vitro* it was concluded that calcification was directly dependent, in some way, on glycogenolysis. More recently, Albaum, Hirshfeld and Sobel(5) reported that most of the enzymes of glycolysis present in muscle and yeast appeared to be present in epiphyseal cartilage.

The aim of this investigation was to explore the relationship that may exist between calcification *in vitro* and the glycolytic enzymes. Our previous studies, utilizing an *in vitro* calcification technic, revealed that freezing(6), heating(7) and demineralizing(8) rachitic rat tibial slices evoked inactivation of the calcifying mechanism in preosseous cartilage which could be reactivated following appropriate treatment with $CaCl_2$.

In the case of the demineralized bone it was necessary to treat first with chondroitin

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