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An Inhibitory Effect of Tolbutamide and Glibenclamide (Glyburide) on the Pancreatic Islets of Normal Animals

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Summary. Tolbutamide and glibenclamide (glyburide) were administered to normal hamsters, mice or rats in daily doses proportional to their body weight and equivalent to those used in human therapy. The animals were sacrificed after 6 to 8 weeks of treatment. Pieces of pancreas or isolated pancreatic islets were incubated or perifused in a medium containing glucose or tolbutamide, with or without leucine-1^{14}C or glucose-U-^{14}C. The results indicate that the B cells of sulfonylurea treated animals synthesized and released less insulin and oxidized less glucose than those of insulin or saline treated controls. Accordingly, at least in the glibenclamide treated animals, the tolerance for glucose and the insulinogenic response to a glucose load *in vivo* were suppressed. Although insular function tended to return to normal after treatment was discontinued, the results reported in this paper do not support the generally accepted view that the lasting therapeutic effectiveness of the sulfonylureas is due to a beta-cytotrophic action.

Key words: Insulin secretion, perifusion, isolated islets, sulfonylureas, intravenous glucose tolerance, intravenous tolbutamide response, glucose utilization, tolbutamide, glibenclamide.

Pancreatic-femoral cross circulation experiments between two dogs demonstrated that, when a sulfonylurea derivative was injected into one of the animals, the second animal, recipient of the pancreatic blood of the first, becomes hypoglycemic [1, 2]. This and other indirect evidence suggests that the sulfonylureas release insulin from the pancreas [3], a notion confirmed by direct measurements of insulin *in vivo* and *in vitro* [4—7] and now generally accepted. Indeed, the rise in serum insulin levels observed following the intravenous injection of tolbutamide sodium is often used as an element in the clinical evaluation of insular function. The immediate result of this drug-induced insulin release is a depletion of the pancreatic islets, as indicated by functional [8, 9] and by morphologic [10, 11] evidence. Obviously, if this release were to continue indefinitely without adequate insulin resynthesis, the drugs would soon become ineffective and functional bankruptcy of the B cells would ensue. Clinical experience teaches that this is not the case, for no convincing or even suggestive evidence of lasting changes in insular function have been reported in man, even though countless diabetics have received oral medication for many years. In animals, however, the results of prolonged administration of sulfonylureas have been contradictory: islet hypertrophy, beta cell multiplication and increased tolerance for glucose [12, 13], but also a decrease in islet activity or no change at all [4, 8, 14, 15] have been observed. Indeed, it has been suggested that the drugs, while stimulating the release of insulin, may fail to stimulate its replacement [7]. Recently we have reported that pancreatic islets, isolated from normal hamsters after prolonged treatment with tolbutamide, secreted significantly smaller amounts of insulin than islets of control animals [16].

The purpose of the experiments reported here was to investigate further the effects of the sulfonylureas on the insulinogenic reserve of normal animals *in vivo* and on the secretory and metabolic activities of their pancreatic islets *in vitro*.

Material and Methods

Female Syrian hamsters (100 to 120 g) and female Swiss mice (20 to 25 g) were kept in individual cages and weighed once a week. Unless otherwise indicated, the animals were given a diet of Purina Rat Chow and water *ad libitum*.

In one series of experiments, hamsters received daily doses of tolbutamide (63 mg/kg, intraperitoneally) or of ultralente insulin (1.0 U/100 g, subcutaneously). Control animals were given i.p. or s.c. injections of 0.9% sodium chloride. After 6 to 8 weeks of treatment and 24 h after the last injection, the hamsters were sacrificed by cervical dislocation and their pancreata were removed, trimmed of visible fat and connective tissue and cut into small pieces. From these pieces, islets were isolated using the method of Lacy and Kostianovsky [17] and transferred to a millipore filter (diameter 13 mm; pore size 5.0 μ). The filter was placed in a millipore holder (Millipore Corporation, Bedford, Mass.) that served as a perifusion chamber and Krebs-Ringer bicarbonate, containing bovine serum albumin (0.5 g/100 ml), was pushed through it at the rate of 0.8—1.0 ml/min with a peristaltic pump. The buffer was gassed continuously with a mixture of oxygen (95%) and carbon dioxide (5%) and the pH was maintained between 7.35 and 7.45 throughout the experiment. Two chambers, containing a control and

an experimental sample, were perifused simultaneously, in parallel, while immersed in a water bath at 37° C. The buffer reservoir was kept in the same bath. The effluents were collected in graduated test tubes, their volumes were noted and aliquots were used for insulin assay, according to the method of Malaisse *et al.* [18], using a porcine insulin standard.

For the first 50 min the islets were perifused with buffer containing glucose at a concentration of 50 mg/100 ml and samples of effluent were collected every 5 min. After this period of equilibration one of the chambers was switched to a buffer containing glucose at a concentration 300 mg/100 ml, while the other continued to be perifused with the initial buffer. Thereafter, samples of effluent were collected every min for 20 min; then every 5 min for an additional 100 min.

In a second series of experiments, hamsters that had been treated with tolbutamide (63 mg/kg/day for 6 to 8 weeks) or with ultralente insulin (1.0 U/100 g/day for 8 weeks) and mice that had been treated with glibenclamide (2.0 mg/kg/day for 8 weeks) were killed 24 or 48 h after receiving the last dose of the respective drug. Pieces of pancreas were prepared as described above and 50 to 70 mg of pancreatic tissue (not of isolated islets) were placed in 25 ml Erlenmeyer flasks with 2 ml of Krebs-Ringer bicarbonate buffer, containing bovine serum albumin (1 g/100 ml), glucose (30 or 250 mg/100 ml), leucine-1-^{14}C (28 mc/mM, 0.5 μc/ml), with or without tolbutamide sodium (20 mg/100 ml). The samples were equilibrated for 5 min with O_2 (95%) and CO_2 (5%) and incubated for 2 h, in a Dubnoff metabolic shaker, under this gas mixture. After incubation, the pieces of tissue were removed and placed in 10% trichloracetic acid. Insulin was extracted according to the method of Grodsky *et al.* [19], and purified, using a modification of the double antibody techniques of Taylor *et al.* [20] and Howell *et al.* [21], as follows: 1.0 ml of the crude insulin solution was incubated with 0.5 ml of guinea pig anti-insulin serum and 2.0 ml of rabbit anti-guinea pig gamma globulin and the flocculent precipitate was re-extracted with 1.0 ml of acid alcohol (ethanol: water: HCl = 3:1:0.3; v/v/v). One aliquot of the extract was used for the determination of its insulin content, as stated above, the rest was mixed with Bray's scintillation fluid for the measurement of radioactivity. Pieces of liver were incubated and extracted under the same conditions and their radioactivity was subtracted from that of the pancreatic extracts. The specific activity (cpm/mU) of the insulin rich extract, corrected for the contribution of non-insulin impurities, was used as a measure of insulin synthesis.

In a third series of experiments, after 6 to 8 weeks of treatment with tolbutamide (63 mg/kg), with insulin (1.0 U/100 g) or with suitable volumes of saline, hamsters were sacrificed and their islets isolated as described above. Samples of 15 to 25 islets were placed in 15 ml Erlenmeyer flasks containing 1 ml of Krebs-Ringer bicarbonate buffer with bovine serum albumin (0.5 g/100 ml), glucose (100 or 300 mg/100 ml) and glucose-U-^{14}C (1.0 μc/ml). After equilibration for 5 min with the O_2-CO_2 mixture, the flasks were closed with a rubber stopper, holding a small plastic well, and incubated for 3 h at 37° C in a Dubnoff metabolic shaker. These experiments were terminated by injecting 0.2 ml of 10N H_2SO_4 into the medium and 0.5 ml of hyamine hydroxide 10× into the plastic well. After 1 h, the hyamine was transferred to a scintillation vial for measurement of radioactivity. Two flasks, containing medium and labelled glucose, but no islets, were carried through the entire procedure to provide background counts.

In a fourth series of experiments, female Sprague-Dawley rats, weighing 250 to 300 g were treated with single or with daily injections of tolbutamide (63 mg/kg) or glibenclamide (1.2 mg/kg) for 8 weeks. Twenty-four and 48 h after the last injection, respectively, the rats were given an intravenous dose of glucose (1.0 g/kg) or of tolbutamide (20 mg/kg) and blood samples were taken for glucose and insulin determinations at various intervals of time. Glucose was measured using an oxidase method adapted to the Auto Analyzer [22]; insulin was measured as stated above.

The intervals of time between the last dose and the sacrifice of the animal were deemed sufficient to reduce the concentration of the drugs in the serum to pharmacologically insignificant levels, given a biologic half-life of 3.5 h for tolbutamide and of 6 h for glibenclamide [23]. The doses of tolbutamide and glibenclamide used in these experiments were comparable to those used in human therapy and were much smaller than those necessary to alter thyroid function and metabolic rate [24] and thus complicate the interpretation of the results.

Results

During the first 30 min of perifusion, the concentration of insulin in a buffer containing glucose at a concentration of 50 mg/100 ml, fell to a plateau that lasted as long as the medium remained the same. No significant differences were noted between control and tolbutamide or insulin treated animals (Fig. 1). When the medium was exchanged for one containing glucose at a concentration of 300 mg/100 ml, the rate of insulin secretion increased rapidly and 30 to 45 min later reached a new plateau, where it remained for the duration of the experiment. Under these experimental conditions, insulin secretion was unaffected by previous insulin treatment, but was markedly depressed by treatment with tolbutamide (Fig. 2). The biphasic response to glucose stimulation was obliterated by the process of averaging, but could be demonstrated in individual experiments. Table 1 shows that insulin treatment did not alter the pattern of insulin secretion of unstimulated islets (glucose concentration 50 mg/100 ml of medium) and of islets stimulated with glucose

(300 mg/100 ml of medium). Table 1 shows also that treatment with tolbutamide did not decrease significantly the total amount of insulin released by unstimulated islets, but reduced the total amount of insulin secreted in response to glucose to almost one half and the maximum rate of insulin release attained at any time during the experiment by about 40%.

The effect of treatment with tolbutamide or with

Table 1. *Insulin released by perifused islets of control, tolbutamide-treated and insulin-treated hamsters. Number of experiments in parentheses*

	Total insulin released μu/islet Ave. ± S.E.			Maximum rate* μu/islet/min	
	Glucose concentration in the medium				
	50 mg/100 ml	300 mg/100 ml			
Control	153.5± 9.1 (9)	931.4± 69.6 (9)	$p<0.001$	9.58 (9)	$p<0.02$
Tolbutamide-treated	127.1± 8.1 (9)	491.5± 59.4 (9)		5.93 (9)	
Insulin-treated	172.5±18.9 (6)	972.8±148.1 (6)		9.54 (6)	

* At any time during the experiment

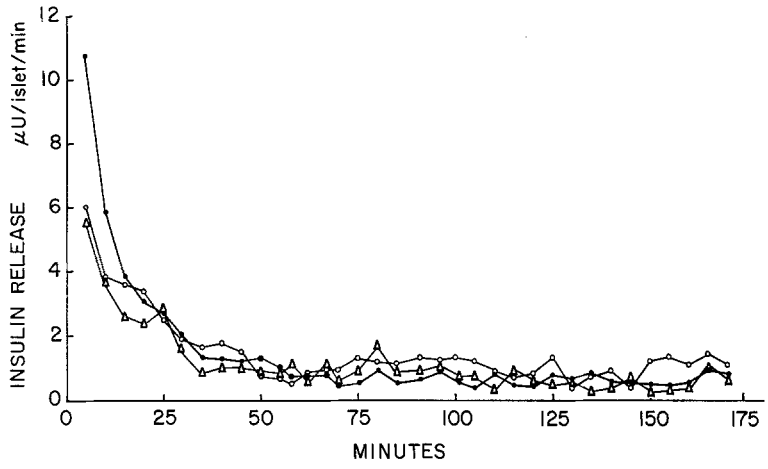

Fig. 1. Insulin released by perifused pancreatic islets of the Syrian hamster. 50 islets per sample. Glucose content of the perifusion medium: 50 mg/100 ml. ● Control ○ Tolbutamide treated △ Insulin treated

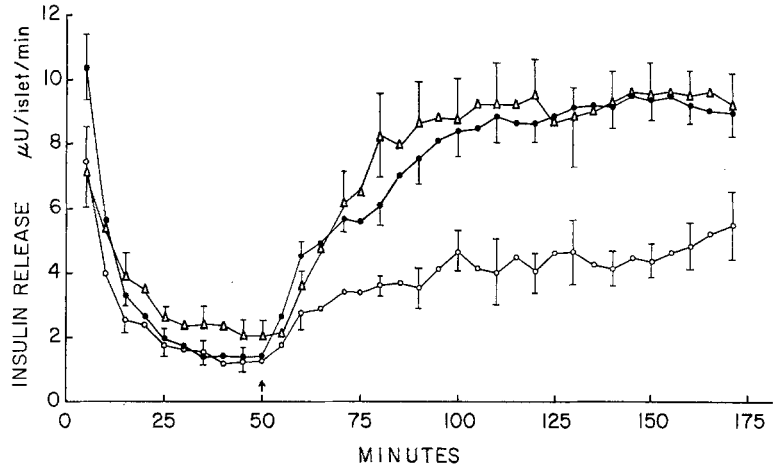

Fig. 2. Insulin released by perifused pancreatic islets of the Syrian hamster. 50 islets per sample. Arrow indicates change in the perifusion medium from one containing glucose at the concentration of 50 mg/100 ml to one containing glucose at the concentration of 300 mg/100 ml. ● Control (N = 9) ○ Tolbutamide treated (N = 9) △ Insulin treated (N = 6)

Table 2. *Incorporation of leucine-1-^{14}C into an insulin-rich extract of pancreas of control hamsters and of hamsters treated with tolbutamide or with insulin. Leucine concentration in the medium 1.78 × 10^{-2} μM/ml, corresponding to 0.5 μc/ml. Number of animals in parentheses*

Additions	Specific activity of insulin CPM × 10^3/U Ave. ± S.E. Control	Tolbutamide-treated	Insulin-treated
Glucose 30 ml/100 ml	2.21±0.25 (12)	1.99±0.22 (14)	1.93±0.20 (13)
	$p<0.005$	$p<0.01$	$p<0.01$
Glucose 250 mg/100 ml	5.75±0.89 (10)	NS* ($p<0.06$) 3.98±0.60 (15)	NS 4.04±0.37 (15)
		$p<0.05$	
Glucose 30 mg/100 ml, Tolbutamide 200 μg/ml	2.46±0.26 (9)	NS 2.08±0.30 (10)	

* NS = Not Significant

Table 3. *Incorporation of leucine-1-^{14}C into an insulin-rich extract of the pancreas of control mice and of mice treated with glibenclamide. Leucine concentration in the medium 1.78 × 10^{-2} μM/ml, corresponding to 0.5 μc/ml. Number of animals in parentheses*

Additions	Specific activity of insulin CPM × 10^3/U Ave. ± S.E. Control	Experimental	Recovery*
Glucose 30 mg%	3.94±0.91 (8)	3.69±0.79 (9)	3.97±2.67 (5)
	$p<0.005$	NS**	NS
Glucose 250 mg%	6.73±0.74 (15)	$p<0.01$ 4.26±0.43 (18)	5.68±2.31 (5)
		NS	
Glucose 30 mg%, Tolbutamide 200 μg/ml	4.13±0.90 (8)	4.77±0.93 (9)	

* Six days after cessation of treatment
** NS = Not Significant

Table 4. *Production of $^{14}CO_2$ from glucose-U-^{14}C in vitro by islets of control, tolbutamide-treated and insulin-treated hamsters. Number of experiments in parentheses*

	CPM/25 Islets Ave. ± S.E. Glucose 100 mg%	Glucose 300 mg%
Control	777.28±93 (15)	1762.14±185 (15)
	NS	$p<0.05$
Tolbutamide-treated	NS* 558.60±93 (12)	$p<0.001$ 1251.06±155 (13) NS
	$p<0.01$	$p<0.005$
Insulin-treated	925.06±81 (10)	$p<0.001$ 1970.67±126 (10)

* NS = Not Significant

glibenclamide on leucine-1-^{14}C incorporation into insulin by pancreatic tissues *in vitro* is shown in Tables 2 and 3. Pancreatic tissue from control animals produced insulin of higher specific activity when stimulated with glucose, while tolbutamide, added to a medium containing small amounts of glucose, had no effect. Treatment with insulin did not alter significantly the specific activity of insulin extracted from the pancreas incubated under basal or under stimulated conditions, the response to glucose was prevented by treatment with glibenclamide and depressed, almost to the level of statistical significance ($p < 0.06$) by treatment with tolbutamide. Table 3 shows also that when the mice were allowed to survive for 6 days without treatment, the specific activity of insulin was no longer significantly different from that of the pancreatic insulin of untreated animals, under basal conditions or following glucose stimulation. This could be interpreted as a sign of recovery. Table 4 shows that the production of $^{14}CO_2$ from glucose-U-^{14}C by isolated islets was greater when the concentration of glucose in the incubation medium was 300 mg/100 ml, than when it was 100 mg/100 ml. This effect of glucose was significantly smaller when the islets were obtained from animals treated with tolbutamide. The metabolic activity of the islets of tolbutamide and of insulin treated animals, incubated in the presence of low glucose concentrations was not significantly different from that of the control islets, although they were different from each other. Table 5 shows that treatment with tolbutamide or with insulin resulted in a comparable, mild degree of fasting hypoglycemia, but did not modify significantly the blood glucose concentration of fed animals. Nevertheless, the serum insulin level was lower in the tolbutamide treated than in the control, fed animals.

After a single injection or after 8 weeks of treatment with glibenclamide, but not with tolbutamide, the tolerance of rats for intravenous glucose was decreased (Figs. 3 and 4) and the hypoglycemic effect of intravenous tolbutamide was abolished (Fig. 5). Both drugs suppressed the insulinogenic response to intravenous glucose and to intravenous tolbutamide (Figs. 6—8). Table 6 shows that the various forms of treatment did not affect the weight of the animals significantly.

Discussion

The release of insulin by isolated perifused pancreatic islets was characterized by a rapid decline to a relatively stable plateau, followed by a secondary rise, when the glucose concentration in the medium was increased. Although, in individual experiments, this secondary rise was preceded by a small peak, no clear cut biphasic response was obtained. Similar secretory kinetics have been noted also by other investigators

Table 5. *Serum glucose and immunoreactive insulin (IRI) levels in control, tolbutamide-treated and insulin-treated hamsters. Number of animals in parentheses*

	Glucose mg/100 ml Ave. ± S.E.		IRI μu/ml Ave. ± S.E.	
Fasted				
Control	84.4±10.2 (16)	$p<0.02$	52.80± 6.65 (15)	NS
Tolbutamide-treated	60.7± 3.5 (24)	NS*	41.73± 5.40 (15)	
Insulin-treated	60.6± 4.96 (21)			
Non-fasted				
Control	112.0± 7.83 (20)		150.49±16.41 (20)	$p<0.05$
Tolbutamide-treated	106.6± 5.96 (20)		110.90± 7.05 (20)	
Insulin-treated	110.3± 4.76 (14)			

* NS = Not Significant

Table 6. *Changes in the average body weight of control and sulfonylurea-treated animals-grams*

	Initial	Final	% Change
		Young Hamsters	
Control	113	149	31
Tolbutamide-treated	115	148	28
		Mature Hamsters	
Control	149	167	12
Tolbutamide-treated	143	153	7
Insulin-treated	146	161	10
		Mice	
Control	25	28	12
Glibenclamide-treated	26	27	4

using perifused islets [25]. The initial decline and the lack of a distinct first phase in response to glucose stimulation may represent evidence that exposure to collagenase [26] and other manipulations required to prepare the islets for perifusion, may predispose them to the release of preformed insulin. Thus, their secretory activity probably was more truly reflected in the prolonged secondary response. This response was significantly altered by pretreatment with either tolbutamide or glibenclamide. Both drugs reduced not only the total amount of insulin secreted by the perifused islets, but also the maximum rate of secretion attained at any time during the experiment, confirming the results obtained in experiments with perfused rat pancreas [4] and with isolated islets [16]. The marked degranulation [27] and the decrease in islet volume [16] following prolonged treatment with sulfonylureas may represent the morphologic counterparts of this functional impairment.

We have confirmed the observation that the incorporation of labelled amino acids into insulin is increased by glucose [28], but not by tolbutamide added *in vitro*

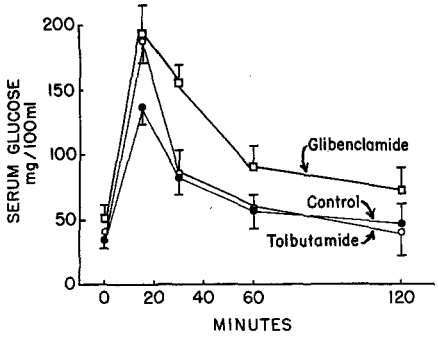

Fig. 3. Serum glucose response to a glucose load (1 g/kg i.v.) in 6 rats treated with a single dose of tolbutamide or glibenclamide. Ave. ± SEM

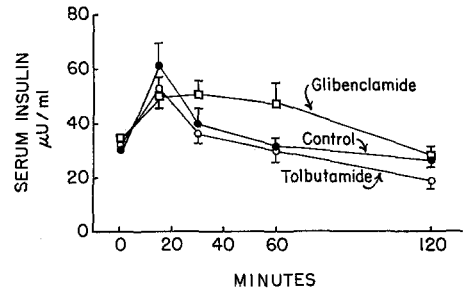

Fig. 6. Serum insulin response to a glucose load (1 g/kg i.v.) in 6 rats treated with a single dose of tolbutamide or glibenclamide. Ave. ± SEM

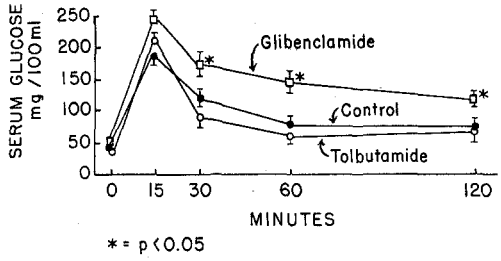

Fig. 4. Serum glucose response to a glucose load (1 g/kg i.v.) following treatment with tolbutamide or glibenclamide for 8 weeks. Ave. ± SEM. 8 rats

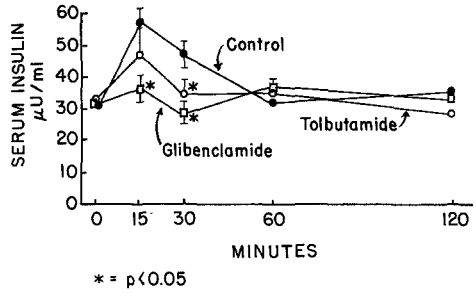

Fig. 7. Serum insulin response to a glucose load (1 g/kg i.v.) following treatment with tolbutamide or glibenclamide for 8 weeks. Ave. ± SEM. 8 rats

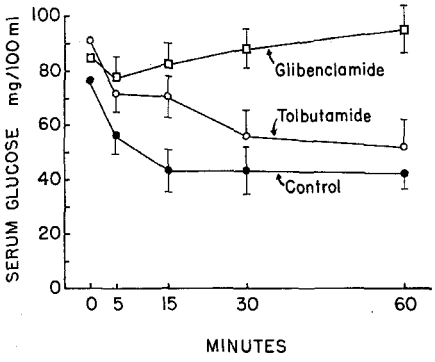

Fig. 5. Serum glucose response to tolbutamide (20 mg/kg i.v.) following treatment with tolbutamide or glibenclamide for 8 weeks. Ave. ± SEM. 8 rats

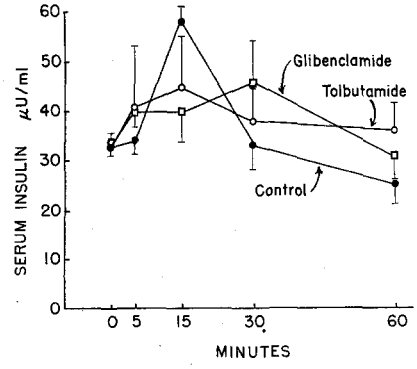

Fig. 8. Serum insulin response to tolbutamide (20 mg/kg i.v.) following treatment with tolbutamide or glibenclamide for 8 weeks. Ave. ± SEM. 8 rats

[29—31]. In addition, we have found that following prolonged treatment with sulfonylureas the pancreas, when incubated in a glucose containing medium, produced an insulin of similar or significantly lower specific activity than the pancreas of control animals. Abundant direct [32—35] and indirect [36, 37] evidence demonstrates that pancreatic islets or pancreatic slices metabolize glucose *in vitro*. Under the conditions of our experiments, the production of $^{14}CO_2$ from labelled glucose by the islets of animals treated with tolbutamide was significantly depressed, although it was not affected by treatment with insulin.

Thus, our experiments indicated that the islets of animals treated with sulfonylureas were functionally depressed, that is, they synthesized and secreted less insulin and oxidized less glucose than the islets of control animals. Six days after cessation of treatment, partial recovery from the sulfonylurea-induced suppression appeared to have occurred. These results extend those previously reported from our laboratory, indicating that prolonged treatment with tolbutamide decreased the insulin content of the pancreas [16]. Thus, it would appear that at least two sulfonylurea drugs, when administered in amounts proportional to those used in human therapy, tended to suppress three parameters of B cell function (insulin release, insulin synthesis, glucose metabolism) in one or the other of three animal species. This conclusion stands in sharp contrast with that of other investigators [1, 5, 6, 13, 37, 39] who found evidence of islet hyperfunction after prolonged treatment with sulfonylureas. Differences in animal species, in the intensity and duration of therapy and the use of normal instead of alloxan diabetic animals are possible, albeit unsatisfactory, explanations for this contrast. Perhaps the answer lies in the fact that most of the evidence for a lasting beta-cytotrophic action of the sulfonylureas was derived from morphologic observations and most of the evidence against it from biochemical and physiologic evidence.

The mechanism by which, in our experiments, the sulfonylureas suppressed islet function remains to be clarified. Four factors may be considered: hypoglycemia, anorexia and malnutrition, elevated serum insulin levels and a direct effect of the drugs on the beta cells. Hypoglycemia and anorexia are not probable explanations because, when fasting hypoglycemia was induced by insulin or by phlorizin [40], the function of the beta cells was not modified significantly and, above all, because hypoglycemia was not observed when our animals had free access to food, which, judging by the changes in body weight, they consumed in normal amounts [16]. High serum insulin levels could have inhibited islet function [41—45]; however, no significant hyperinsulinism was noted in our sulfonylurea treated animals. Thus, a direct action of the drugs on the beta cells emerges as the most likely cause of these observations. In support of this conclusion, the following evidence may be cited: carbutamide and glibenclamide lower the ATP [46] and the glucose-6-phosphate content of isolated islets [46, 47], even as glibenclamide and tolbutamide increase their oxygen consumption and lactate production [33, 48]. Thus, although there is some evidence to the contrary [37, 49], the sulfonylureas may uncouple oxidative phosphorylation in the islets, as they appear to do in liver, diaphragm and adipose tissue [50, 51]. In so doing, these drugs inhibit the energy producing reactions required for the synthesis of insulin. The decrease in glucose tolerance and in the insulinogenic effect of glucose, observed in animals treated with a single injection of glibenclamide, does not help in the interpretation of the results, for it was due, most likely, to acute insulin depletion. What our results indicate is that this depletion continues as long as the drug is being administered to the animal.

The mechanism whereby the protracted use of sulfonylureas has a lasting effect on the concentration of blood glucose remains unresolved: normal rodents are probably different from diabetic patients in whom hyperglycemia may stimulate the islets directly or alter their response to the drug; nevertheless, we believe that the evidence presented in this paper reinforces the argument that the mechanism may be, in part, extrapancreatic [3, 52, 53].

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