Adenosine Triphosphate Levels of Mammalian Pancreatic B Cells after Stimulation with Glucose and Hypoglycemic Sulfonylureas

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SUMMARY

A microchemical technic was applied to elucidate the possible role of ATP in insulin secretion by measuring the levels of this metabolite in pancreatic islets from obese hyperglycemic mice. The β cell content of ATP was markedly reduced within the first minute after interruption of the blood supply. A steady-state level of about 5 mmoles of ATP was noted in islets incubated in the absence of glucose. The corresponding ATP level was twice as high when at least 1 mg./ml. of glucose was present in the incubation medium. While the contents of ATP and glycogen remained constant when the pancreatic islets were incubated with diazoxide, the amounts of both these metabolites were significantly reduced by concentrations of sulfonylurea compounds known to stimulate the insulin release. The sulfonylurea effect on the islet ATP content implies a change in the "phosphate potential" of the β cells, which might stimulate glycogenolysis and increase the glycolytic flux. The sulfonylurea stimulation of insulin release might thus be related to a product of glucose degradation beyond the level of glucose-6-phosphate. DIABETES *18*:509-16, August, 1969.

Little is known about the participation of adenosine triphosphate (ATP) in the events leading to release of insulin from the pancreatic β cells. Attention has been paid to the possible significance of a fast glycolytic ATP formation for this very rapid process.[1] An indirect importance of ATP is suggested by its role as parent substance for adenosine-3,5-phosphate (cyclic AMP), which is considered to act as an intracellular signal for insulin release.[2-4] It has been suggested that extramitochondrial adenosine triphosphatase (ATPase) is concerned with the storage of insulin in the β granules.[5] The stimulation of β cell function produced by repeated injections of cortisone has furthermore been found to induce a significant reduction of the islet ATPase activity.[6] R-Candela and co-workers[7] have provided in vitro data indicating that there is increased secretion of insulin from β cells exposed to ATP. On the other hand, it has been reported that the glucose stimulation of insulin release remains unimpaired under conditions in which the level of ATP presumably would be reduced by the uncoupling action of dinitrophenol.[8] In a recent publication Matschinsky and Ellerman[9] also emphasized that the β cell response to hyperglycemia cannot be correlated with the intracellular content of ATP.

The present study represents part of a microchemical approach to elucidate the possible role of ATP in insulin secretion by measuring the levels of ATP in pancreatic β cells exposed either to substances known to stimulate (glucose and hypoglycemic sulfonylurea compounds) or inhibit (diazoxide) this cellular process. In addition, evidence will be presented for a significant reduction of the glycogen content of microdissected islets exposed in vitro to sulfonylurea.

MATERIALS AND METHODS

Animals and experimental design. Forty-five nine-month-old female obese hyperglycemic mice from a colony originating from R. B. Jackson Memorial Laboratories, Bar Harbor, Maine, U.S.A., were used.[10] When not otherwise stated the animals were starved overnight before being killed by decapitation under ether anesthesia. Three series of experiments were performed:

(1) The in vivo level of ATP was measured in specimens of β cells and exocrine parenchyma dissected from frozen-dried pancreas sections from six mice allowed free access to food. In addition, the changes in the ATP levels at various intervals after interruption of the blood supply were recorded. Parallel analyses of ATP were performed in liver samples from five of these animals.

(2) The relationship between the glucose level and the ATP content of the β cells was studied in sixteen mice. A total of fifteen to twenty islets from each

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animal were incubated individually in 1 ml. Krebs-Ringer bicarbonate buffer containing 0.5 per cent albumin (pH 7.4 at 37° C.) either devoid of glucose or containing various concentrations of this substance. After preincubation for ten minutes at a glucose concentration of 1 mg./ml. the microdissected islets were exposed for 0, 5, 45 or 120 minutes to 0 or 1 mg./ml. glucose (two animals) or incubated for 120 minutes with 0, 0.2, 0.4, 0.6, 0.8 or 1.0 mg./ml. glucose (two animals). In the remaining cases the islets were exposed for forty-five minutes to glucose concentrations varying from 0-10 mg./ml. either after microdissection from the immediately excised pancreas (five animals) or from a pancreas made ischemic by immersion under mineral oil for ten to fifteen minutes (seven animals). All incubations were performed at 37° C. in a metabolic shaker (120 strokes per minute with an amplitude of 1.25 cm.) under a gas phase of 95 per cent O_2 and 5 per cent CO_2.

(3) The third series of experiments was designed to evaluate whether the β cell level of ATP was influenced by drugs supposed to have a direct effect on insulin release. Incubation conditions were similar to those described above, twelve to eighteen islets being microdissected from each of twenty-three mice. The effects of carbutamide (BZ 55; 200 μg./ml.) and the new and more potent hypoglycemic sulfonylurea compound glybenclamid (HB 419; 50 μg./ml.) were recorded. After preincubation for forty-five minutes in the presence of 0.6 mg./ml. glucose, the islets were exposed for forty-five minutes to fresh medium supplemented with sulfonylurea (twelve animals). In other experiments the islets were exposed to HB 419 (100 μg./ml.) in the presence of 0.6 mg./ml. glucose or to the benzothiadizine compound, diazoxide (125 μg./ml.), in the presence of 3 mg./ml. of glucose for fifteen minutes after ten minutes preincubation in a medium containing 0.6 mg./ml. of glucose (eleven animals).

Preparation of tissues. The pancreatic islet material was obtained in two ways. According to the first alternative the tissue was isolated by free-hand dissection from freeze-dried sections essentially as described by Lowry.[11] Small pieces of pancreas were rapidly removed during brief ether anesthesia and dropped into isopentane chilled to its freezing point (-165° C.) with liquid nitrogen. This could be accomplished within a "zero" time of ten seconds after interruption of the blood supply. When the effects of ischemia were recorded, additional tissue specimens from the same animal were frozen after one and five minutes. Once frozen, the tissue was stored in liquid nitrogen until sectioned 20 μ. thick in a cryostat. The frozen sections were dried at -40° C. and 0.001 mm. Hg for twelve hours and stored under vacuum at -90° C. They were then brought to room temperature and microscopic samples, representing the central parts of the islets or the exocrine parenchyma or the liver, isolated under a stereomicroscope. Samples in the range of 1.0—1.5 μg. were weighed on a quartz fiber balance and transferred to micro test tubes of polyethene.

According to the second alternative pieces of pancreas were removed and placed in gassed Krebs-Ringer bicarbonate buffer (containing 0.5 per cent albumin) kept at + 2° C. The buffer was either devoid of glucose (second experiment series) or contained 0.6 mg./ml. of this substance (third experiment series). Intact and metabolically active islets were carefully dissected as described by Hellerström.[12] After the islets had been incubated under the desired conditions they were freeze-dried as described above. The frozen-dried islets were divided into pieces, equivalent to 3-10 μg., and weighed before being transferred to the micro test tubes for measurements of ATP and in some instances also glycogen.

Chemical methods. The tissue contents of ATP and glycogen were recorded by the use of enzymatic fluorometric technic in combination with enzymatic cycling.[13] In the procedure for ATP measurements interfering enzymes and glucose-6-phosphate were destroyed by heating the tissue samples for ten minutes at 60° C. in 2 to 5 μl. of 0.1 N NaOH. This treatment was initiated by twenty seconds ultrasonic disintegration (Ultrapoint 40 WST Oscillator, 32 kHz, Ultrapoint Ltd., Uppsala, Sweden) to ensure an adequate extraction of ATP. After addition of the same volume of 0.1 N HCl, the samples were incubated for thirty minutes at 28° C. with four times as much ATP reaction medium. The latter medium contained the components described by Gatfield et al.[14] in the following concentrations: 2 mM. glucose, 5 mM. $MgCl_2$, 0.125 mM. NADP, 2.5 μg./ml. hexokinase, 0.5 μg./ml. glucose-6-phosphate dehydrogenase (G-6-PDH) and 0.01 per cent albumin in 0.1 M. Tris-HCl buffer. The enzymes and other biochemicals were obtained from Boehringer, Mannheim. The procedure for the glycogen assay was identical to that previously used in our laboratory.[15] In both the ATP and glycogen assays exposure to the reaction media was followed by destruction of excess NADP by heating with NaOH and measurements of the NADPH formed by means of enzymatic cycling.[13] The cycling medium

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contained 25 μg. G-6-PDH (centrifuged and resuspended in 2 M. ammonium acetate to reduce the sulphate concentration) and 200 μg. glutamate dehydrogenase per ml. The cycling reaction was carried out in a total volume of 110-115 μl. for sixty minutes at 38° C. giving an approximate cycling rate of 8,000 ×. The final fluorometric readings were performed in an Aminco-Bowman spectrophotofluorometer.

RESULTS

The ATP level of β cell specimens obtained by dissection from frozen-dried sections of the immediately excised pancreas amounted to 6.9 ± 0.7 mmoles per kg. dry weight. This is significantly higher ($t = 3.21$; $p < 0.01$) than for the exocrine pancreas (4.0 ± 0.6 mmoles) and equivalent to the levels noted for the liver (6.2 ± 0.7 mmoles). The changes in the ATP content induced by interruption of the blood supply are illustrated in figure 1. During the first minute of ischemia the rate of ATP reduction was particularly pronounced in the pancreatic β cells. After five minutes the total loss of ATP in these cells was, however, of the same order of magnitude as in the liver parenchyma or equivalent to about 4 mmoles per kg. dry weight.

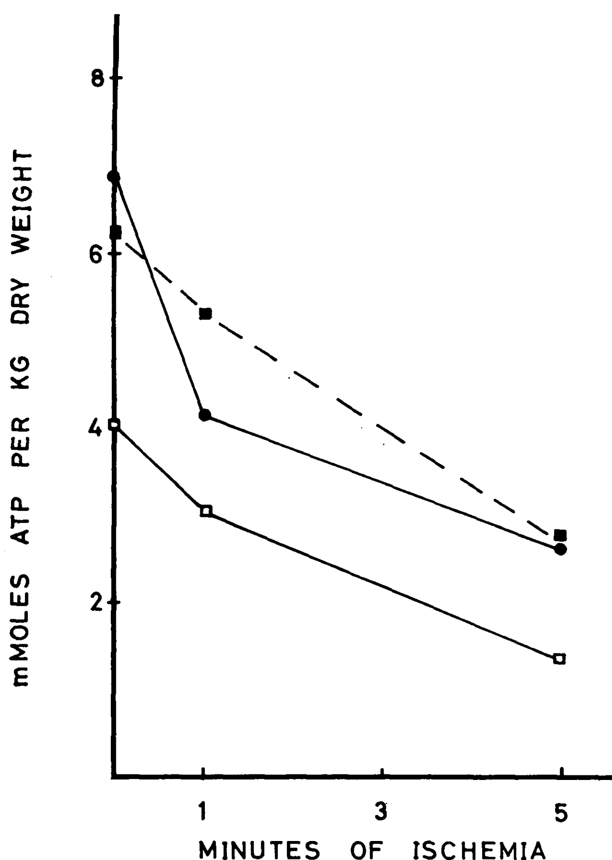

FIG. 1. The effects of ischemia on the ATP level in β cells (—●—), exocrine pancreas (—□—) and liver (—■—) from obese hyperglycemic mice. While the liver values refer to five animals the remaining analyses were performed in six individuals. Mean values ± S.E.M. During the first minute the reduction of the islet ATP content amounted to 2.80 ± 0.77 mmoles per kg. dry weight whereas the corresponding figure for the exocrine pancreas was only 0.97 ± 1.03 mmoles.

The ATP level in isolated islets incubated for various periods of time either in the absence of glucose or with fairly low concentrations of this substance is shown in figure 2. The pancreatic islets were not only able to maintain a base-line level of ATP in vitro but also to increase the amount of ATP if enough glucose was present in the medium. After forty-five minutes incubation in the presence of 1 mg./ml. glucose the islet level of ATP amounted to about 10 mmoles per kg. dry weight, i.e. 40 per cent more than recorded for β cell specimens dissected from the frozen-dried sections of a rapidly excised pancreas. In the absence of glucose the ATP content of the isolated islets persisted at a level of 5 mmoles per kg. dry weight when the incubation time was extended from forty-five to 120 minutes.

After an ischemic period of ten to fifteen minutes the isolated islets still responded with higher ATP levels when the glucose concentration was increased up to 1 mg./ml. (figure 3). The ATP concentration in these islets was, however, considerably lower than in appropriately oxygenated islets after forty-five minutes incubation. At glucose concentrations above 3 mg./ml. there was no further increase but rather a tendency to decreased levels of ATP. When the initially ischemic islets were incubated with 10 mg./ml. of glucose the ATP reduction amounted to 1.10 ± 0.28 mmoles per kg. dry weight if compared with the levels noted in the presence of 1 mg./ml. of glucose. There was, however, no statistical evidence for a depression of ATP when the appropriately oxygenated islets were incubated with the highest glucose concentration.

The effect of incubating the pancreatic islets for forty-five minutes in media containing hypoglycemic sulfonylurea compounds is shown in table 1. The ATP content decreased by 22 per cent in the presence of carbutamide and by no less than 40 per cent in the presence of HB 419. The carbutamide depression is also a statistically significant effect. The mean reduction noted for the individual animal amounted to 1.76 ± 0.49 mmoles per kg. islet dry weight in the presence of carbutamide ($t = 3.59$; $p < 0.01$). In accordance with the above-

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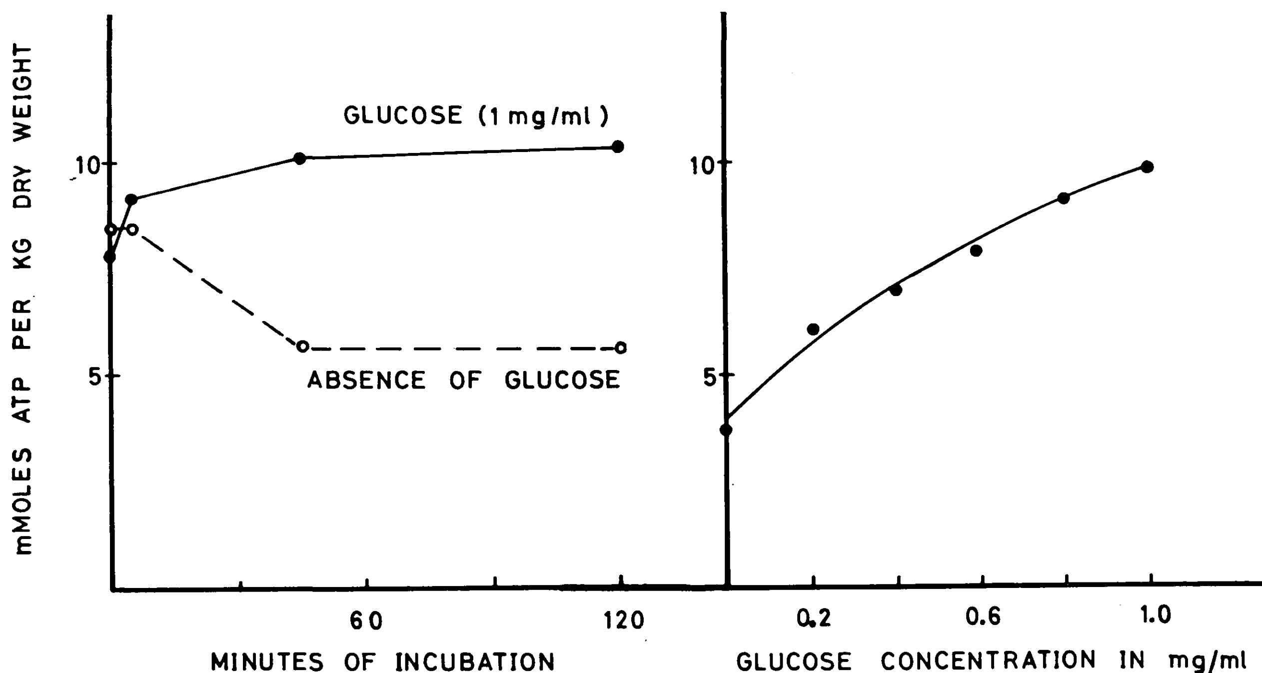

FIG. 2. The in vitro effect of glucose on the ATP content of microdissected pancreatic islets. After ten minutes preincubation the islets in the left part of the figure were exposed for various periods of time to 0 (—O—) or 1 mg./ml. glucose (—●—). The right part of the figure shows the effect of incubating the islets for 120 minutes either in the absence of glucose or with concentrations of this compound up to 1 mg./ml.

mentioned observations more islet ATP was found when the glucose concentration was increased from 0.6 to 3.0 mg./ml. This is a statistically significant effect as shown by the fact that the mean individual increase was 1.63 ± 0.48 mmoles per kg. islet dry weight. It is evident from table 2 that fifteen minutes incubation with HB 419 was enough to reduce the islet ATP concentration by 25 per cent. This is equivalent to a mean reduction of 1.57 ± 0.77 mmoles per kg. islet dry weight for the individual animal. Furthermore, exposure of the pancreatic islets to HB 419 resulted in a depletion of their glycogen stores. After fifteen minutes incubation the glycogen content was reduced by 40 per cent, which is

TABLE 1

ATP content in mmoles per kg. dry weight in microdissected islets exposed for forty-five minutes to HB 419 (50 μg./ml.) and carbutamide (200 μg./ml.) in the presence of 0.6 mg./ml. glucose. In addition, the effect of increasing the glucose concentration to 3 mg./ml. is shown. The figures in the first column denote the ATP content immediately after dissection of the islets. Mean values ± S.E.M. The number of animals studied is given within brackets.

Initial value	Glucose (0.6 mg./ml.) Controls	HB 419	Carbutamide	Glucose (3.0 mg./ml.)
8.84±0.64 (11)	8.69±0.64 (12)	5.23±0.40 (10)	6.76±0.56 (11)	10.11±0.73 (9)

equivalent to a loss of 2.99 ± 1.00 mmoles glucosyl residues per kg. islet dry weight. However, neither the ATP nor the glycogen content was significantly affected when the isolated islets were incubated with diazoxide for fifteen minutes.

DISCUSSION

The results essentially reflect the metabolism of the β cells, since this cell type comprises more than 90 per cent of the islets of the obese hyperglycemic mice.[10] The occurrence of islet hyperplasia in these animals may be interpreted as a reaction of the β cells to counteract a hyperglycemia of extrapancreatic origin.[10,16] The fact that the isolated pancreatic islets provide a satisfactory model for metabolic studies of mammalian β cells is particularly evident from the recent demonstration that these islets respond adequately with insulin secretion when stimulated with glucose in vitro.[17]

While a steady-state level of ATP of about 5 mmoles per kg. dry weight was recorded in islets incubated in the absence of glucose, this amount was considerably increased when the glucose concentration was raised to 1 mg./ml. At the latter glucose concentration the ATP content attained a plateau of about 10 mmoles per kg. dry weight. These observations give rise to the question

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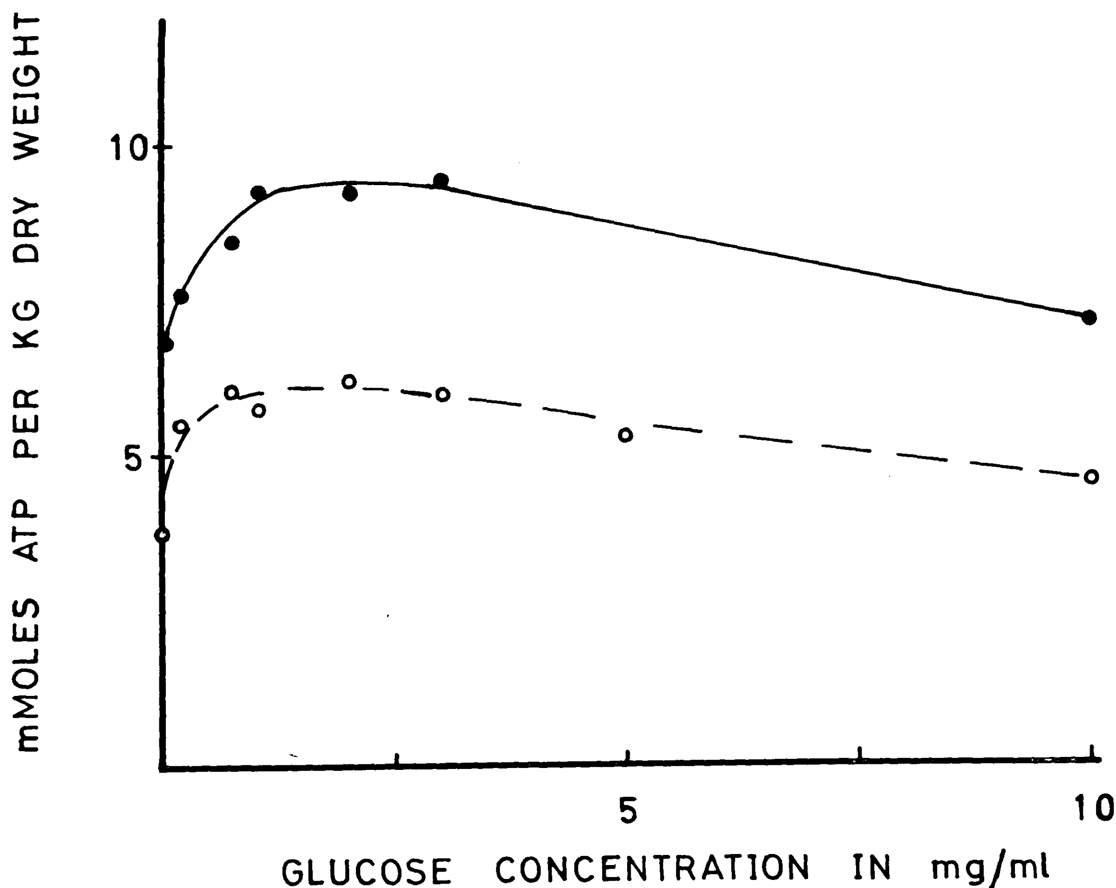

FIG. 3. The ATP content after incubating appropriately oxygenated islets (—●—) or initially ischemic islets (—O—) for forty-five minutes in media either devoid of glucose or containing up to 10 mg./ml. of this substance.

of whether a high level of ATP must be reached in the β cells before glucose can stimulate insulin release. A significant glucose stimulation of insulin release has been reported only for glucose concentrations above 0.5 — 1.0 mg./ml.[18-19]

When exposed to physiological concentrations of glucose the ATP content of the microdissected islets was 3 mmoles higher than that of the β cell specimens dissected from frozen dried sections of the pancreas. The demonstration of a marked reduction of the β cell content of ATP within the first minute after interruption of the blood supply suggests that this difference is due to ischemia. The supposition that the ATP value for the incubated islets is more relevant to in vivo conditions is also consistent with the data recently provided by Matschinsky and Ellerman.[9] These authors

TABLE 2

ATP and glycogen contents in mmoles per kg. dry weight for microdissected islets incubated for fifteen minutes with HB 419 (100 μg./ml.) or diazoxide (125 μg./ml.). The figures in the left hand column denote the substrate levels recorded after the ten-minute period of preincubation. Mean values ± S.E.M. The number of animals studied is given within brackets.

Metabolite	Initial value	Glucose (0.6 mg./ml.) Controls	Glucose (0.6 mg./ml.) HB 419	Glucose (3.0 mg./ml.) Controls	Glucose (3.0 mg./ml.) Diazoxide
ATP	5.17±0.53 (8)	6.29±0.59 (9)	4.72±0.47 (9)	6.51±0.28 (9)	5.83±0.76 (8)
Glycogen	7.57±1.40 (10)	7.39±1.46 (11)	4.40±0.53 (11)	8.36±0.93 (10)	7.61±1.03 (10)

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found no less than 11.3 mmoles ATP per kg. dry weight in β cell samples from anesthetized obese hyperglycemic mice. Stimulation of insulin release is known to be an extremely rapid process; a detectable rise in insulin occurs within thirty seconds after introducing either glucose or glucagon in a nonrecycling perfusion system of rat pancreas.[20] The presence of a considerably higher ATP content in the islets than in the remaining part of the mouse pancreas might consequently be a useful arrangement to ensure readily mobilized energy, if this is a necessary prerequisite for insulin secretion. The enzyme profile of the β cells furthermore suggests a great capacity for rapid regeneration of ATP. While the substrate for glycolytic ATP formation might be preferably derived from the pentose phosphate pathway,[1,21-22] the subsequent degradation of glucose in the β cells is characterized by high enzyme activities in the ATP regenerating steps.[23]

There are some indications that very high concentrations of glucose may damage the β cells by overstimulation. For example, Dohan and Lukens[24-25] reported hydropic changes in the β cells and production of permanent diabetes in cats given repeated intraperitoneal injections of 20 per cent glucose. It has been repeatedly observed with a wide variety of tissue preparations that the rate of glycolysis is lower under aerobic than under anaerobic conditions.[4] Although there is no definite proof for the existence of such a Pasteur effect in the β cells, it is worthy of note that the initially ischemic islets reacted to the highest glucose concentration with a significantly lower ATP value. It remains to be settled whether this reduction is due to a "toxic" effect on the β cells, and if so, whether this can be related more specifically to the glucose molecule or only to the increase of the osmotic pressure in the incubation medium.

Diazoxide, the most potent inhibitor of insulin release in the benzothiadiazine group, has been used both as a therapeutic agent in hypoglycemia[26] and as a tool in the study of experimental diabetes.[27] Divergent opinions have been expressed as to whether the diazoxide inhibition of insulin release is mediated by a stimulation of the α-receptor sites of the β cells[28] or whether it represents a more complex mechanism.[29] In further exploration of this matter attention should be paid to the fact that the amounts of both ATP and glycogen remained constant under the present incubation conditions. Diazoxide inhibition of the phosphodiesterase activity in the β cells, similar to that previously reported for muscle cells,[30] might give rise to increased intracellular levels of cyclic AMP with subsequent possibilities for stimulation of glycogenolysis.

A marked reduction of the ATP content occurred when the microdissected islets were exposed to hypoglycemic sulfonylurea compounds. This is consistent with our previous observation of a tendency to lower ATP levels in the mouse pancreatic β cells fifteen minutes after an intravenous injection of 0.3 ml. 0.2 M. carbutamide solution.[31] The more reproducible sulfonylurea effects in the present study may be an illustration of the experimental advantage of using an in vitro approach for recording changes of this metabolically labile metabolite in the pancreatic islets. Both carbutamide and HB 419 significantly depressed the ATP levels of the isolated islets. The concentration of carbutamide employed (200 μg./ml.) is equivalent to the plasma level at which blood glucose decrease commences in most species.[32] The more potent HB 419 was, however, tested in concentrations (50-100 μg./ml.) far above this level.[33] While 100 μg./ml. of HB 419 is known to induce significant stimulation of insulin release from the microdissected islets from obese hyperglycemic mice,[34] the minimum effective concentration still remains to be determined in this species. It is of interest to note that maximal stimulation was not yet reached with 100 μg./ml. of HB 419 in a recent study of insulin release from slices of rat pancreas.[35]

An accelerated breakdown of ATP might represent a primary sulfonylurea effect or be secondarily related to a consumption of energy in the events leading to insulin secretion. The possibility that the low ATP level could be due to impaired regeneration should, however, also be considered. An uncoupling of the oxidative phosphorylation has been reported after exposing liver and diaphragm preparations to therapeutic concentrations of various hypoglycemic sulfonylurea compounds.[36,37] Recent observations have shown that hypoglycemic sulfonylureas stimulate rather than inhibit respiration when microdissected islets are exposed to low or moderate concentrations of glucose.[38]

It is reasonable to assume that the reduced ATP content implies a change of the "phosphate potential" in the sense that increased amounts of ADP, AMP and inorganic phosphate will be available for the β cell metabolism. Since this alteration of the "phosphate potential" is known to stimulate the process of glycogenolysis in other tissues it might also well explain the striking sulfonylurea reduction of the amounts of glycogen stored in the β cells. Furthermore, if the glycolytic pathway of the β cells is similar to that in most other tissues

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in working, under normal conditions, far below its maximal capacity,[4] the modified "phosphate potential" would also be expected to result in an increased glycolytic flux. Experimental results indicate that insulin release is triggered by a product of glucose metabolism rather than by the glucose molecule itself.[39] The proposed stimulation of the glycolytic flux in the β cells might therefore be of direct importance for the rate of insulin secretion. Mannoheptulose is known to interfere with the phosphorylation of glucose in the pancreatic islets.[40] The previous observation that mannoheptulose inhibits the stimulation of insulin release obtained with glucose but not that obtained with tolbutamide has been considered to reflect a fundamental difference in the mechanisms by which these compounds affect β cell function.[39,41] However, the demonstration of a marked sulfonylurea reduction of the glycogen stores of the β cells makes it necessary to reconsider this view in favor of a common trigger for insulin release related to glucose degradation beyond the level of glucose-6-phosphate.

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Erratum

The following references were omitted from the article entitled "Insulin Response to Glucagon: The Opposing Effects of Diabetes and Obesity," by Peter M. Crockford, M.D., William R. Hazzard, M.D., and Robert H. Williams, M.D., which appeared in the April 1969 issue of this Journal.

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