Energy Metabolism in Pancreatic Islets of Rats

Studies with Tolbutamide and Hypoxia

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SUMMARY

Levels of ATP, phosphocreatine, glycogen and glucose of individual pancreatic islets were measured by quantitative histochemical technics. The islets were obtained from frozen-dried sections and were analyzed with enzymatic fluorometric methods coupled to enzymatic cycling and using an oil well technic. The rate of energy consumption in the islets was estimated by the rate of change in energy stores under complete ischemia. The energy use rate of the pancreatic islet was 38 mmoles of ∽ P per kg. dry tissue per min. which is approximately 50 per cent of the rate in brain. Tolbutamide (5 mg./kg. or more in vivo) resulted in a peak of insulin release one minute after administration. Regardless of the dose employed, ATP and phosphocreatine were unchanged one minute after tolbutamide, when insulin output was maximal or thirty minutes after the drug, when serum insulin levels were falling. In addition, ATP and P-creatine levels were unchanged in islets from the perfused pancreas thirty seconds after eliciting at least a sixfold increase in insulin secretion by perfusing with media containing 50 μg./ml. tolbutamide, whether glucose was present or absent in the medium. Since the readily available energy reserves were not affected by exposure to tolbutamide, the metabolic rate of the islets was either not influenced by the drug or the effect was small or well compensated. DIABETES *20*:598-606, September, 1971.

Since the molecular basis of action of sulfonylurea derivatives on pancreatic β-cells is poorly understood and since the safety of treatment with these widely used oral hypoglycemic drugs has recently been questioned,[1] a thorough investigation of the biochemical effects of these substances seemed highly desirable. Therefore we have, as a start, undertaken to assess the possible role of energy metabolism in the action of tolbutamide, a typical member of this class of compounds.

The question of whether tolbutamide-induced insulin secretion is a consequence of alterations of the phosphate potential as manifested by the tissue levels of ATP and P-creatine was examined. It had been suggested that sulfonylureas primarily decrease the phosphate potential of the β-cells by an uncoupling of oxidative phosphorylation; that this leads to stimulation of glycolysis; and that this in turn causes insulin release.[2] Evidence in support of such a mechanism of drug action was obtained from the observation that the glycogen content and ATP stores of isolated islets fell after exposure to sulfonylureas.[2] In addition, uncoupling of oxidative phosphorylation by sulfonylureas has been reported in other systems.[3,4] Like Hellman[2] we employed the biochemical approach of measuring level changes of intermediates and cofactors as indicators of molecular mechanisms. The concentrations of the energy rich compounds, ATP and P-creatine, and in some instances the glycolytic substrates, glucose and glycogen, were measured. Of particular importance seemed the analysis of P-creatine levels since it is well known from comparable studies in other excitable tissues that this compound is the most sensitive biochemical indicator of changed energy supply or demand. Initially, the effect of ischemic hypoxia on the metabolite and cofactor levels was studied to determine what changes to expect when energy demand exceeds supply and also to find out what the approximate metabolic rate of islets might be in vivo.[5]

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METHODS

Animal experiments. A total of eighty-nine male Holtzman rats (200-250 gm.) were studied. Three types of experiments were performed. In the first type, fifty-two rats which had been allowed access to food and water were anesthetized with 35 mg./kg. of pentobarbital sodium (Diabutal) by the intraperitoneal route. Fed animals were used in this group since it was found that overnight fasting resulted in variable blood sugar lowering effect of tolbutamide whereas fed animals responded more uniformly. The trachea was cannulated with PE-200 (Clay Adams) to aid breathing and the left carotid artery was cannulated with PE-60 for removal of blood samples. A cannula (PE-50) also was placed into the right jugular vein for injections of saline or tolbutamide (0.15 ml. within five seconds). The abdomen was opened and the pancreas exposed. Samples of blood (0.2-0.3 ml.) for analysis of serum glucose and serum insulin were taken at five minutes before, immediately prior to the tolbutamide injection and depending upon the duration of the experiment 1, 5, and 30 min. following the injection. Samples were allowed to clot for thirty minutes at room temperature and then spun in the cold to separate the serum, which was stored at —85° C. until assayed. It was found that storage of blood for thirty minutes at 25° did not result in altered serum glucose values. At the end of the experiment (1, 5 or 30 min. after injection), the pancreas was removed and rapidly frozen in Freon-12 (CCl_2F_2) chilled to its freezing point (—150° C.) with liquid N_2.[5]

In the second group, used for hypoxia experiments, twenty-one rats were fasted overnight to obtain uniform blood glucose levels and anesthetized as above. The pancreas was removed and frozen immediately ($<$ 5 sec.) or at 0.5, 2.5 or 10 min. after removal. During the interval between removal and freezing, the pancreases were placed under a bell jar with a constant flow of N_2 at room temperature (23° C.).

The third group consisted of sixteen rats which were fasted overnight and anesthetized with 35 mg./kg. pentobarbital. Preparation for perfusion of the pancreas was as described by Landgraf, Kotler-Brajtburg and Matschinsky.[6] A flow rate of approximately 7 ml./min. was employed. Samples of perfusate were removed at 10 min., 12.5 min. and 14.5 min. of perfusion. At fifteen minutes the perfusate was switched to one containing 50 μg./ml. of tolbutamide. In the control experiments the switch-over from one circulation to the other was performed without changing the composition of the medium. Samples of the perfusate were taken from 15 to 18 sec. and 27 to 30 sec. after the switch. At thirty seconds the pancreas was removed and frozen in liquid Freon-12 chilled to its freezing point with liquid nitrogen.

All frozen tissue was stored at —85° C. until sectioned at 20 μm in a cryostat at —20° C. Sections were subjected to freeze drying at —40° C. for forty-eight hours with a vacuum of less than 0.001 mm. Hg. Frozen dried sections were stored under vacuum at —20° C. Dissection of islets was performed at 18-20° C. with a relative humidity of approximately 50 per cent.[7,8] A fish pole balance was employed to weigh samples of 0.1 to 0.3 μg.[7]

Analytical procedures. Serum glucose was measured in duplicate by an enzymatic fluorometric method.[5] Serum insulin was measured in duplicate by the Hales and Randle[9] double antibody technic with use of a kit purchased from Amersham/Searle and with use of porcine insulin as a standard. The assay was linear at several dilutions of rat serum and of perfusate. The insulin levels found with porcine insulin as standard amounted to approximately 30 per cent of the levels obtainable, when crystalline rat insulin* was used for comparison.

Tissue levels of ATP, P-creatine, glycogen and glucose were measured with the aid of an oil well method and enzymatic cycling by enzymatic fluorometric methods.[8] Two of these methods were modified: in the glycogen reagent, 0.15 M glycylglycine, pH 8.65, was substituted for imidazole as the buffer since the kinetics of the assay were more favorable in this buffer; in the P-creatine assay the method for removal of NADPH after completion of the ATP step was altered as indicated below. The latter change was introduced to improve recovery and reproducibility of the P-creatine step which was unsatisfactory because of the relatively low tissue levels of P-creatine (20 per cent of the ATP concentration) and to alleviate the difficulty in cooling the oil well rack during the acid treatment. An aliquot of 0.2 μl. from the ATP step was transferred to another oil well rack, at room temperature, and 0.05 μl. of Tris-HCl (0.1 M, pH 8) containing 12.5 μg./ml. glutathione reductase and 100 μM oxidized glutathione (GSSG) was added. Under these conditions NADPH was rapidly oxidized by the GSSG (half-time of 6 sec.) whereas the GSSG was slowly but completely reduced (half-time of 6.5 min.) by dithiothreitol, which was present at a concentration of 1.5 mM in the ATP reagent. (Dithiothreitol does not affect the ATP assay.)

*Rat insulin was kindly provided by Dr. Schlichtkrull (Novo, Copenhagen).

After twenty minutes at room temperature the oil well rack was heated for twenty minutes at 75° to destroy the glutathione reductase. The P-creatine was measured by adding 0.14 μl. of glycine-HCl (0.1 M, pH 9) containing 6 mg./ml. of creatine kinase,* 9 μg./ml. of hexokinase and 9 μg./ml. of glucose-6-P dehydrogenase. Following the destruction of excess $NADP^+$ (20 min. at 75°, pH 13), the NADPH formed in both racks (ATP and P-creatine) was measured by enzymatic cycling in a volume of 6.5 μl. (60 min. at 38° gave 1,500 cycles) and the product, 6-P-gluconate, was measured enzymatically.[10] An additional advantage of this procedure is that the entire process can be more conveniently followed fluorometrically in a 1 ml. volume. This was done routinely.

Materials. Most biochemicals, including enzymes, were purchased from Boehringer and Sons. Phosphorylase *a*, glutathione reductase and dithiothreitol were obtained from Sigma Chemical Co. Glycogen was purchased from Mann. Tolbutamide sodium (Orinase Diagnostic) was obtained from Upjohn.

RESULTS

Evaluation of micro methods for ATP and P-creatine. The assays were linear between 1 and 4 x 10^{-12} moles of ATP or P-creatine as indicated by two randomly selected standard curves (figure 1). Recoveries of authentic ATP and P-creatine as compared to NADPH were 94 and 79 per cent, respectively. Recoveries of standards added to tissue samples were satisfactory, ranging from 81 to 105 per cent.

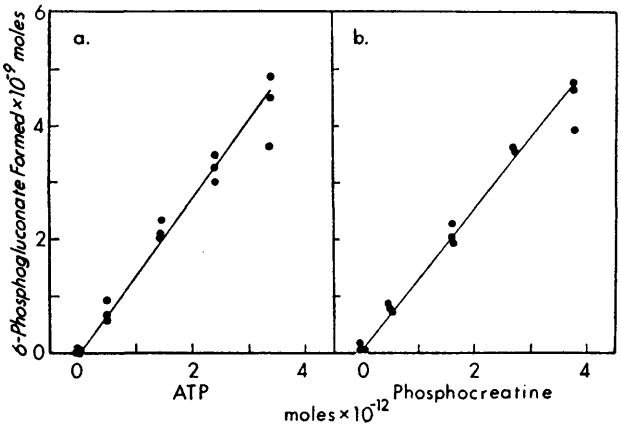

FIG. 1. Yield of product in the assays for ATP and P-creatine. Each point indicates the content of one oil well. The over-all blanks were equivalent to 1.2 X 10^{-12} moles of ATP and 1.6 X 10^{-12} moles of P-creatine.

Energy stores for anaerobic use and metabolic rate of pancreatic islets. In order to test whether ATP and P-creatine could serve as useful indicators of alterations of energy use in islet tissue, hypoxia studies according to the procedure described[5] for brain were performed. For that purpose we measured the changes in ATP, P-creatine, glycogen and glucose at intervals of 0.5, 2.5 and 10 min. after interruption of blood flow (figure 2). Hypoxia had very different effects on these four compounds. During the first thirty seconds the changes were: P-creatine, —65 per cent; ATP, —23 per cent; glucose, —17 per cent; and glycogen did not fall at all. As time went on, only ATP continued to decrease markedly whereas P-creatine, glucose and glycogen showed relatively small declines.

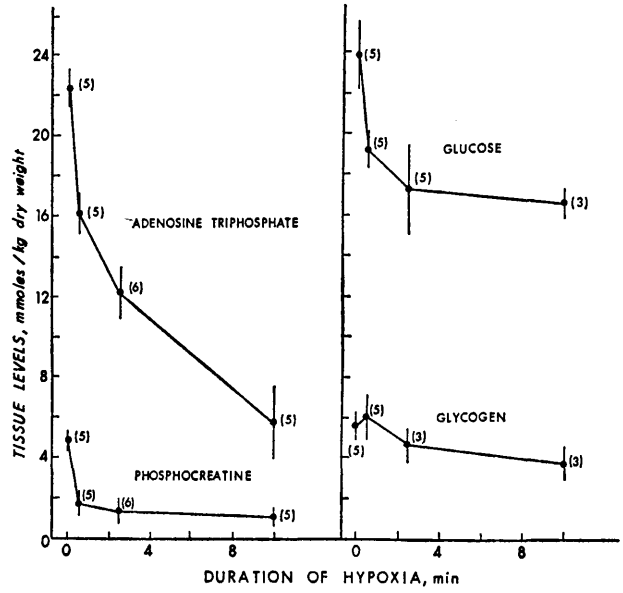

FIG. 2. Effect of hypoxia on levels of ATP, P-creatine, glucose and glycogen in pancreatic islets of rat. Hypoxia was induced by removal of the pancreas and placing it in an N_2 atmosphere for the indicated period of time. The number of pancreases analyzed at each point is presented in parentheses. The standard errors are shown. Six analyses were carried out with each pancreas. Glycogen levels are in terms of glucosyl units.

The total anaerobically usable energy reserves (table 1) were equivalent to 109 mmoles of ~ P/kg. dry tissue. This reserve dropped to about one half within ten minutes of ischemia.

*The level of creatine kinase can be lowered to a final concentration of 400 μg./ml. provided 0.1 per cent bovine serum albumin and 25 μM ADP (final concentrations) are added with the enzyme. This was the procedure employed for the analysis of the pancreatic islets from perfused tissue.

TABLE 1

Energy use in pancreatic islets from rats

Time of hypoxia	PCr*	ATP	Glucose	Glycogen†	~ P Total available‡	~ P use cumulative	~ P use separate periods
min.		mmoles/kg. dry weight§				mmoles/kg. dry tissue/min.	
< 0.05	4.92	20.92	23.00	5.54	109.4	—	—
0.5	1.75	16.02	19.20	6.04	90.3	38.1	38.1 (0 to 0.5 min.)
2.5	1.28	12.14	17.20	4.72	74.1	14.1	8.10 (0.5 to 2.5 min.)
10	1.12	5.76	16.50	3.78	56.98	5.24	2.28 (2.5 to 10 min.)

* P-creatine

† Glycogen levels in terms of glucosyl units.

‡ High energy phosphate (~ P) was calculated by the following formula: PCr + 2(ATP) + 2(glucose) + 3(glycogen) = ~ P.

§ ATP, PCr, glycogen and glucose data were taken from figure 1.

From the initial rate of decrease of this energy pool (0-30 sec. of ischemia) the metabolic rate of the tissue at the time of sampling can be estimated.[5] It was equivalent to the consumption of 38.1 mmoles ~ P/kg. dry tissue/min., which is approximately half the rate observed with brain[11] and five times faster than reported for nerve.[12] Our value of the metabolic rate is only an approximation and probably an underestimation since lowering of the tissue temperature (4.5° C. during the first 30 sec. after extirpation of the pancreas) undoubtedly slackened ATP consumption. Nevertheless, the results of this indirect determination of the metabolic rate are in fair agreement with the values attained with the cartesian diver technic.[13,14] The $-Q_{O_2}$ measured[13,14] was 6.9 while the $-Q_{O_2}$ calculated from our data is 8.7. These data, in particular the rapid response of P-creatine to hypoxia, serve to establish the validity of P-creatine and ATP levels as indices of the energy state possibly altered during tolbutamide-induced insulin secretion.

Lack of effect of tolbutamide on energy reserves of islets.

Studies in vivo: It is well known that insulin release following parenteral injection of tolbutamide is rapid and transient. This was confirmed under our experimental conditions with administration of 10 or 100 mg./kg. of tolbutamide (figure 3). The dose-response data obtained at one minute after tolbutamide administration (figure 4) show that the threshold for insulin release lies between 1 and 5 mg./kg. of tolbutamide. The

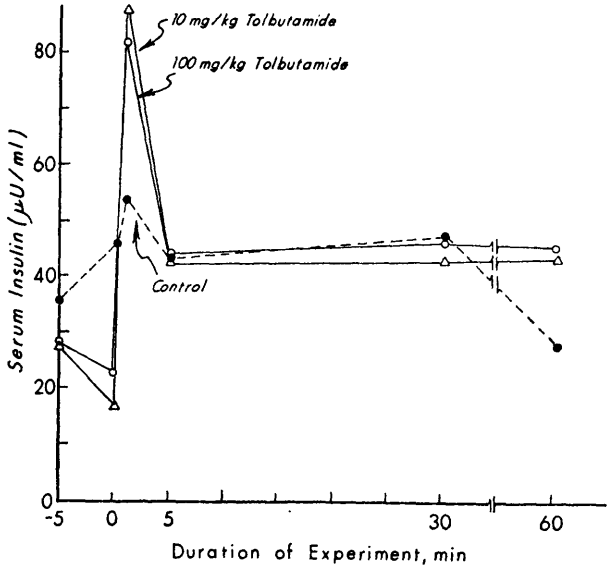

FIG. 3. Tolbutamide-induced insulin release as a function of time. Each line represents data from one animal.

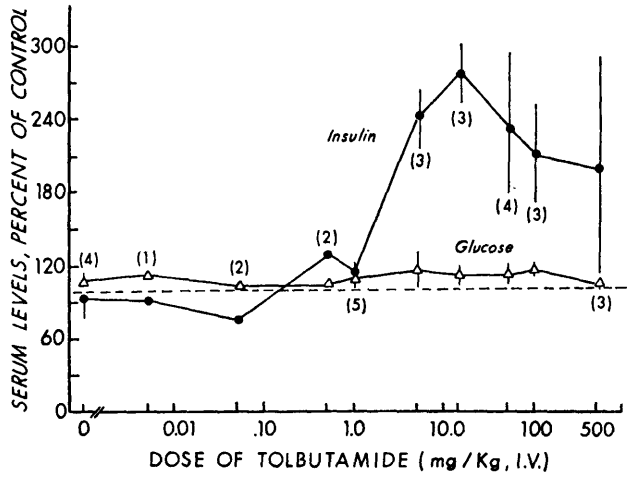

FIG. 4. Dose dependency of insulin release following tolbutamide. Samples were taken 1 min. after injection. The 100 per cent (control) values for serum insulin and serum glucose are 29 μU./ml. and 7.76 mM, respectively. The number of animals at each point and the S.E. are shown.

maximal response was reached following 15 mg./kg. of tolbutamide. Blood glucose was unaltered at this time. The decreasing efficacy of excess amount of drug can also be inferred from the blood glucose patterns seen in other studies, where large amounts of sulfonylurea were administered.[15]

ATP and P-creatine levels were little changed by tolbutamide at one minute when the insulin release was maximal (figure 5). Compared to controls the ATP levels were not altered significantly by 1, 5, 50, 100 or 500 mg./kg. of tolbutamide. Only with 15 mg./kg. was a significant change (an increase) observed ($0.025<P<0.05$). P-creatine levels seemed to be somewhat lower at the most effective drug dose (5 to 15 mg./kg.). This apparent change in P-creatine had no statistical significance when compared with control ($0.05<P<0.1$). Doses larger than 500 mg./kg. were injected but the rapid injections, necessary to make comparisons at one minute valid, were always fatal. Also, in about one third of the animals a dose of 500 mg./kg. was fatal and therefore these animals were excluded from the series.

At present we have no good explanation for the comparatively low control levels of ATP found in this group of animals (figure 5). The difference does not seem to be due to analytical difficulties, since the P-creatine levels, which are an extremely sensitive monitor of possible faults in the procedures were in the normal range. It is also unlikely that feeding or fasting overnight is the cause of the difference, since in a much larger group of fed animals ATP levels ranged from 16 to 20 mmoles/kg. dry tissue.[16] Because of the high variability of the ATP content of islets from group to group encountered in our studies a separate control group was included for each type of experiment.

The time course of tolbutamide action was investigated (figure 6) using one low (10 mg./kg.) and one high (200 mg./kg.) dose of the drug. The serum insulin profile observed following the smaller dosage was typical with an 84 per cent rise at one minute. In contrast the release was not increased as much (38 per cent) after injection of the larger amount. In agreement with the insulin data only the smaller dosage caused a relative decrease in blood sugar (at 30 min., —21 per cent compared to control group levels). But it should not be overlooked, that the absolute glucose levels of the serum in the control animals were actually rising by 54 per cent during this half hour long period probably due to the particular experimental conditions employed here. The ATP and P-creatine levels showed only minor alterations. They certainly were not lower and, at times, were elevated. The only significant

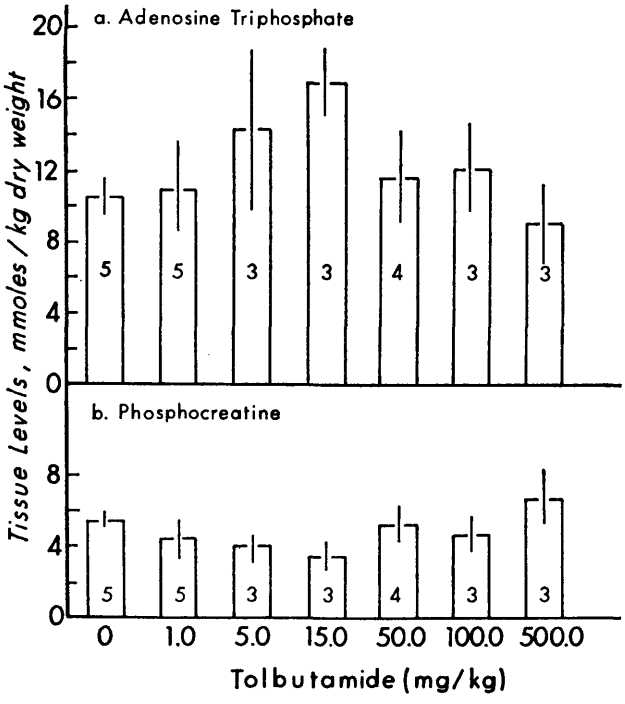

FIG. 5. Levels of ATP and P-creatine in rat islets one minute after tolbutamide (dose response curve). The numbers of animals analyzed are recorded. Standard errors are indicated. Determinations were made with six islet samples from each animal.

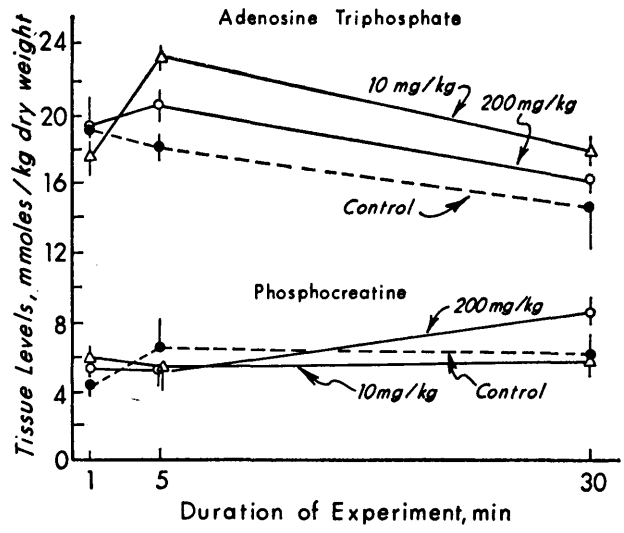

FIG. 6. ATP and P-creatine levels in pancreatic islets of rats as influenced by the time course of tolbutamide action. Each point is the mean from three animals. For each animal six islet samples were analyzed. The S.E. are shown.

change was an elevated ATP level at five minutes with a dose of 10 mg./kg. of tolbutamide. This change was very similar to the increase noted at one minute in animals treated with 15 mg./kg. of tolbutamide (compare figure 6 with figure 5).

Glycogen levels were not altered substantially by tolbutamide at one minute after administration of a dose of 500 mg./kg. nor at thirty minutes after administration of 200 mg./kg. (table 2). This statement seems justified even in view of the large variation in the glycogen levels obtained in the last two groups.

increase of activity. The ATP levels in the islets from the perfused pancreas are as a rule lower than found in the majority of islets sampled in vivo. This difference might be due to a loss of adenine nucleotides most likely occurring in the period of complete ischemia (3 to 5 min.) encountered when the pancreas is prepared for perfusion.

DISCUSSION

Significance of the P-creatine system of islet tissue. Fiske and Subbarow reported that P-creatine was present

TABLE 2

Lack of effect of tolbutamide on pancreatic islet glycogen levels*

Experimental conditions	Individual animals	Mean ± S.E.
	mmoles/kg. dry tissue*	
1 min. after injection of saline (controls)	7.45 ± 0.81†	
	10.12 ± 1.03	
	7.56 ± 1.24	9.12 ± 0.69
	9.49 ± 2.56	
	10.89 ± 0.60	
1 min. after injection of 500 mg./kg. of tolbutamide	9.55 ± 0.51	
	8.45 ± 0.58	8.69 ± 0.49
	8.07 ± 1.70	
30 min. after injection of saline (controls)	10.17 ± 1.50	
	11.31 ± 1.06	8.79 ± 2.13
	4.90 ± 2.12	
30 min. after injection of 200 mg./kg. of tolbutamide	19.75 ± 0.95	
	9.96 ± 0.58	12.51 ± 3.97
	7.83 ± 1.47	

* Glycogen levels in terms of glucosyl units.
† Each value is the mean, ± S.E., of six samples from each pancreas.

Studies with the perfused pancreas: The perfused pancreas was employed in order to assess whether the glucose level might modify the behavior of the ATP and P-creatine stores of islets, when exposed to tolbutamide. It was found that tolbutamide released insulin equally well in the presence and absence* of glucose in the perfusion medium (table 3). The levels of ATP and P-creatine in pancreas islets were not influenced by the glucose content of the perfusate and were not changed thirty seconds after addition of the drug, although the β-cells showed a marked (sixfold)

exclusively in striated muscle and nervous tissue,[18] and the Eggletons[19] had suggested that the availability of this "phosphagen" in tissue might be correlated "with its ability to respond to sudden demands of violent activity." Because this concept is so plausible the possible involvement of the P-creatine system in the energy metabolism of other tissues, which apparently have a more constant energy demand, seemed to have received little or no attention. Since we had previously found substantial amounts of P-creatine in the epithelial layer of the retina[8] and in the stria vascularis,[20] both tissues involved in secretory processes, the levels of P-creatine were determined in a wide variety of organs. Many of the secretory organs as for instance the parotid, sublingual and lacrimal glands and the pancreas had sizable P-creatine stores (4-8 mmoles/kg. dry tissue).[21] In another secretory tissue, the meningeal structures covering the lamprey brain, Rovainen et al. recently de-

*It seems worth pointing out that with sensitive fluorometric methods, traces of glucose (≦ 0.1 mM) were always detectable in the perfusate and that in accordance with this finding the apparent glucose content of islets sampled during perfusion with "glucose free" medium varied from 0 to 1.1 mmole/kg. dry tissue.[17]

TABLE 3

ATP and P-creatine levels in islets of the perfused rat pancreas before and after brief stimulation with tolbutamide

Group	Glucose in perfusate (mM)	Insulin Basal*	Insulin 15 sec.	Insulin 30 sec.	ATP	P-creatine
			μU./ml. perfusate		mmoles/kg. dry tissue	
Control	0	7.3 ± 2.1	9.3 ± 1.5	6.3 ± 0.9	12.96 ± 1.38	3.76 ± 0.34
Tolbutamide (50 μg/ml. perfusate)	0	7.6 ± 0.8	52† ± 15	97† ± 23	13.71 ± 0.52	4.16 ± 1.08
Control	3	10.0 ± 1.6	7.5 ± 0.8	10.4 ± 1.5	11.83 ± 0.60	4.43 ± 0.31
Tolbutamide (50 μg./ml. perfusate)	3	11.4 ± 1.3	52† ± 10	73† ± 7	12.01 ± 0.31	4.47 ± 0.30

The pancreas was removed from the perfusion chamber and frozen quickly at 30 sec. after switching the perfusion medium (see method section). For tissue analysis six islet samples were assayed and averaged for each pancreas. Variability for each individual pancreas was the same as found in tissue sampled in vivo (usual standard error less than 10 per cent). The means and standard errors of the means obtained from four animals per group are recorded.

* Insulin secretion observed in each experiment at 5, 2.5 and 0.5 min. before the change of the perfusion medium was averaged and used to calculate the basal values. Insulin was also measured 15 and 30 sec. after changing the perfusion fluid. The values recorded represent the means and standard errors ($n = 4$).

† Only the increase in insulin secretion after tolbutamide in the presence as well as in the absence of glucose is statistically significant compared to the respective control ($p < 0.05$).

tected as much as 40 mmoles of P-creatine/kg. dry tissue.[22] In view of these observations it is not surprising that the pancreatic islets are similarly provided with P-creatine stores, too large (20 to 30 per cent of the ATP levels) to be neglected when investigating the energy metabolism of islet tissue (figures 2, 5 and 6; tables 1 and 3, see also reference 16). At present it is not clear how important the additional energy reserve present in the form of P-creatine might be for islet tissue function. Studies with 1-fluoro-2,4-dinitrobenzene, which can completely inhibit creatine kinase,[23] promise to be useful in answering this question.

Tolbutamide and the energy metabolism of islets

There seems to be some discrepancy between the in vitro data reported here and comparable studies reported by Hellman's group.[2,24-29] These investigators observed a decrease of ATP and glycogen contents of isolated islets on exposure to sulfonylurea, whereas the data obtained here with the perfused rat pancreas do not provide evidence for a decrease of either ATP or, more important, of P-creatine. Admittedly, there are many differences between the two in vitro studies. The drugs used were different (tolbutamide vs carbutamide and glybenclamide) the species were not the same (rats vs obese mice), and the preparation of islets was different (the perfused pancreas system vs islets dissected freehand from fresh tissue). But the outcome of the perfusion experiments is well supported by our in vivo studies which also showed no indication for decreases of ATP, P-creatine or glycogen with doses of tolbutamide ranging from an apparently ineffective dose of 1 mg./kg. to a nearly lethal dose (500 mg./kg.). Neither were decreases seen in vivo at a time when insulin release was maximal (1 min. after administration of tolbutamide) nor at times when serum insulin was declining or had fallen back to control levels. It is noteworthy in this context that Hellman[25] also found only an insignificant fall of ATP (—25 per cent) when experiments were conducted in vivo.

While the studies were under way we considered the possibility that the glucose supply to the islets might have a marked influence on the results as suggested by the findings of other investigators. Malaisse et al.[30] have stated that tolbutamide has its most prominent action at 1 to 1.5 mg./ml. of glucose. This concentration is approximately the level found in the serum of the rats we studied here (1.4 mg./ml.). Stork et al.[14] have demonstrated that tolbutamide (0.37 mM) and glybenclamide (0.18 mM) increase O_2 consumption of isolated islets from obese hyperglycemic mice when no glucose was present in the medium but inhibit O_2 consumption when glucose is 3 mg./ml. Hellman and Idahl[27] also show that sulfonylureas (glybenclamide) affect ATP levels more at low (0.6 mg./ml.) than at high (3 mg./ml.) glucose concentrations. The results of our perfusion studies, however, seem to suggest that glucose metabolism is of minor or no importance for tolbutamide action, at least not for the initial response of the β-cells, since the rapidity of onset and the rate of in-

sulin release was high, regardless of the presence or absence of glucose in the perfusion medium. This interpretation was further strengthened by the results of the ATP and P-creatine measurements in islets. The results showed no difference between the groups perfused either with or without 3 mM glucose. At least three explanations can be offered that might resolve the apparently contradictory data: (1) the early major response elicited in islets by tolbutamide may be independent of glucose metabolism in contrast to the delayed weaker response to the drug presumably studied by the other investigators. (2) A possible interaction between tolbutamide and glucose may depend upon a critical glucose level ($>$ 3 mM), not reached in the perfusion system. (3) Any drug induced change of the energy metabolism might manifest itself more readily if the energy supply should become limiting due to depletion of endogenous fuel (e.g., glycogen) or due to damage to the β-cells resulting during the in vitro experiment. The preservation of the islet tissue in the perfusion experiments is presumably better than in *most* other in vitro conditions,* and therefore the islets of the perfused pancreas may be able to cope more easily with the excessive work load imposed by the drug.

Whatever the explanations for the discrepancies, results reported here demonstrate that a primary ATP deficit does not seem to be the common cause of action of sulfonylurea derivatives. The relative constancy of the islet energy stores observed in the various experiments with tolbutamide does not imply an unaltered turnover of $\sim$ P, but rather that if a change of flux occurred it was well compensated. It seems more likely that a decrease of ATP level when observed is an expression of a side action rather than a primary effect. This seems plausible in view of several other findings. De Beer and De Schepper[4] and De Schepper[31] have demonstrated that sulfonylurea compounds at high concentrations are uncouplers of oxidative phosphorylation in vitro (2.5 mM tolbutamide lead to a 15 per cent decrease in ATP content of rat diaphragm after 120 min. of incubation). The same concentration inhibited C-14-leucine incorporation into protein of liver, diaphragm or adipose tissue by approximately 30 per cent. Pentillä[3] concluded that the capacity of tolbutamide and chloropropamide to decrease protein synthesis or to uncouple oxidative phosphorylation cannot be primarily involved in the therapeutic actions of the drugs because of the high concentration needed to observe these effects (1 mM). A dose of 10 mg./kg. (tolbutamide) which is sufficient to cause insulin release would give a concentration of 0.16 mM in a 250 gm. rat if it is assumed that tolbutamide is confined to the extracellular fluid.[32] This concentration is approximately one-tenth the level reported[4] to have a marginal (—15 per cent) effect on ATP levels. In humans tolbutamide is effective at blood levels of 0.13 mM.[33] It is of course possible that the pancreatic islets concentrate tolbutamide but there is no evidence for this.

*Electron photomicrographs of perfused pancreas tissue show the ultrastructure of the islets to be optimal. (L. Nagel, unpublished).

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