Effect of Alloxan on the Enzyme Activity of Microdissected Mammalian Pancreatic Islets

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SUMMARY

Quantitative histochemical methods have been used for the assay of 6-phosphogluconic dehydrogenase (6-PGDH), isocitric dehydrogenase (ICDH), peptidase (PEPT), and adenosine triphosphatase (ATPase) in the microdissected islets and acinar tissue obtained from normal and alloxan diabetic rats.

6-PGDH activity of the islets following alloxanization showed an increase at twelve hours; maximal change was observed at seventy-two hours. There was no consistent and significant increase in the enzyme activity of the acinar tissue following alloxanization.

ICDH activity of rat islet was increased significantly at twelve hours but decreased at twenty-four hours after alloxanization. At forty-eight and seventy-two hours the enzyme activity of the islet returned to normal. Enzyme activity of the acinar tissue showed no particular trend, although at twenty-four hours following alloxanization it was significantly lower than in the controls.

Changes in islet peptidase activity were more marked. Following alloxanization the islet enzyme activity decreased significantly up to twenty-four hours; at forty-eight hours it showed marked increase and was between two- and three-fold that of the value at twenty-four hours. It should be noted that this increase in the islet peptidase activity coincides with the period of disappearance of the beta cells.

No immediate effect of alloxan injection was noted on the ATPase activity of the rat islet tissues during the first two hours. This is contrary to the reports of some earlier workers. DIABETES *18*:589-97, September, 1969.

Since Dunn et al.[1] first reported that alloxan injection causes a necrosis of the pancreatic beta cells with consequent induction of diabetes, this compound has been widely used as a diabetogenic agent. In addition to its effect on the pancreatic beta cells, alloxan may also damage other cells such as liver, kidney and adrenal.[2] Several theories have been suggested to explain the action of alloxan,[3-5] but the mechanism by which alloxan selectively kills the beta cells remains unclear. According to one hypothesis,[6] the sensitivity of the beta cell to alloxan may be due to the inactivation of an enzyme which is present in critical amounts in the beta cell, thus interfering with an essential metabolic process.

There is as yet no clear evidence to indicate whether alloxan also acts intracellularly following its entrance into the cell or whether its action is limited to the surface of the cell membrane. Cooperstein and associates,[7,8] comparing the distribution of tracer amounts of injected C-14-labeled alloxan with that of H-3 mannitol (a compound known to remain in the extracellular space), concluded that injected alloxan did not enter the toadfish islet tissue. Other studies by these investigators[9] demonstrated that diabetogenic doses of alloxan alter the permeability of the islet tissue to mannitol, suggesting that this agent acts on the beta cell membrane. These observations do not exclude the possibility that diabetogenic doses of alloxan may also act at intracellular sites.

Histochemical studies on the mammalian pancreatic islets have been carried out by Lazarus and coworkers[10,11] and Gepts and Toussaint.[12] The latter workers have compared activities of various enzymes in the component cell types of the rat islets. Quantitative enzyme analyses were carried out on the microdissected islets of rabbits,[13,14] rats,[15] and ducks,[16] using the methods of Lowry and coworkers.[17,18]

As part of our systematic study undertaken to elucidate the mechanism of alloxan action, we have reported on the insulin content[19] and selected enzyme activities[20,21] of microdissected rat pancreatic islet tissue at various times following alloxan administration. The present study extends these investigations to include the following enzymes: 6-phosphogluconic dehydrogenase (6-PGDH), isocitric dehydrogenase (ICDH), adenosine triphosphatase (ATPase), and peptidase (PEPT).

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METHODS AND MATERIALS

Young male rats of Holtzman strain weighing between 180 and 250 gm. were fasted for sixteen to twenty-four hours. Blood samples were collected from the tail vein and glucose was determined by a modification of the Hoffman[22] procedure using the Auto-Analyzer. Alloxan was administered intravenously in doses of 40 mg. per kg. body weight.[23] In most instances the animals were decapitated seriatim at 12, 24, 48, 72 and 168 hours. At the time of sacrifice the diabetic rats had a blood sugar level greater than 300 mg. per 100 ml. Where the effects of alloxan on the islet ATPase activity were studied, the animals were sacrificed at 5, 15, 60, 120 and 720 minutes (twelve hours) after alloxan treatment. For the first sixty minutes the blood sugar levels were not affected, but at 120 minutes a slight hyperglycemia was noticed in a few animals, although this was not significant. At twelve hours the blood sugars were significantly elevated in all the animals. The control rats were injected with distilled water and sacrificed at corresponding intervals. The pancreas was rapidly removed, frozen in liquid nitrogen (−196° C.), mounted on metal blocks at −20° C. as described earlier,[24] and sectioned at twenty microns; the sections were lyophilized at −35° C.[17] and stored in vacuum jars at −20° C.

Before opening, the jars were brought to room temperature under vacuum. Islet and acinar tissues were microdissected under a binocular microscope at a magnification of 60 ×. The tissue samples were weighed on a quartz fiber balance[25] and placed in microtest tubes using a mechanical loading device.[26] Measurements of 6-phosphogluconic dehydrogenase (6-PGDH)[27] and isocitric dehydrogenase (ICDH)[28] were carried out by the fluorometric procedures. Peptidase (PEPT) was determined by the fluorometric method of Robins and Lowe,[29] using glycyl-l-phenylalanine as a substrate. Adenosine triphosphatase (ATPase) activity was determined by measuring the inorganic phosphorus liberated[18] during incubation of the tissue in the reaction medium at pH 8.8. A pooled sample of over 100 islets weighing approximately 5 μg. was used for the ATPase determination. A number of animals were used at each time period, and multiple samples of islets and acinar tissue were analyzed from each rat. Since the enzyme values of all animals in any particular group were similar, the standard error of the mean was calculated for each time period using all of the determinations. The difference between the corresponding means of the experimental and control groups was determined using the Student *t* test, and the p value was found by referring to the probability chart.

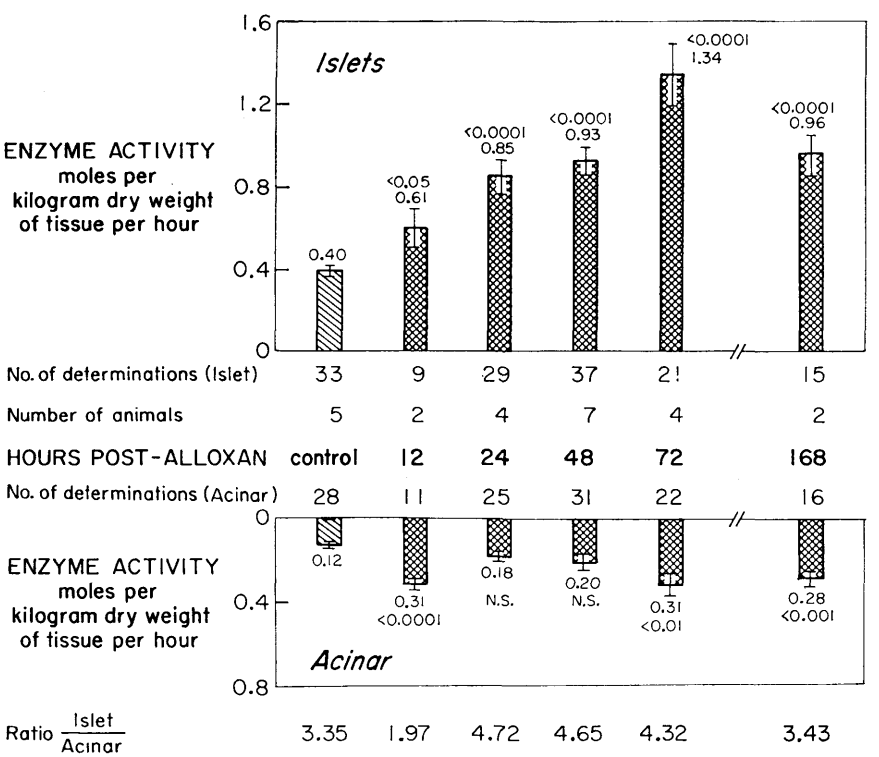

FIG. 1. Effect of alloxan on 6-phosphogluconic dehydrogenase (6-PGDH) activity of microdissected rat islet and acinar tissue. Vertical lines denote standard error of mean, while the number above the bars indicates the mean value. The p value between the mean of the control and experimental groups is also given at the top of the bar.

RESULTS

Figure 1 indicates that islet 6-PGDH activity increases progressively during the first seventy-two hours following alloxan administration; at twelve hours the increase was 50 per cent and at seventy-two hours it was over 200 per cent. The 6-PGDH activity of the normal acinar tissue was approximately one third that of the islet. Following alloxan administration there was a variable increase in the enzyme activity of the acinar tissue; this was significant at 12, 72 and 168 hours, but not at twenty-four and forty-eight hours.

The ICDH activity of rat islet showed small fluctuations following alloxan administration (figure 2). The observed 30 per cent increase at twelve hours and 26 per cent decrease at twenty-four hours are of borderline significance. The ICDH activity in the islet tissue is approximately 25 per cent lower than in the acinar pancreas.

No changes were found in the acinar tissue at twelve, forty-eight, and seventy-two hours after alloxan treatment.

There was a progressive decrease in islet PEPT activity to 60 per cent of the control value at twenty-four hours ($p = < 0.0001$) (figure 3). At forty-eight hours the activity was increased to 158 per cent of the control value ($p = < 0.0001$), and 265 per cent of the twenty-four-hour value. Subsequently the PEPT activity returned to the control value.

In contrast to the islet tissue, the peptidase activity of the acinar pancreas showed little change following alloxanization. In the normal rat the ratio of PEPT activity in the islet to acinar tissue was 2.40.

As shown in figure 4, there was no significant change in the ATPase activity of either the islet or acinar tissue in the initial periods, that is, from five minutes to two hours following alloxan administration.

DISCUSSION

Table 1 demonstrates the close correspondence in the islet and acinar tissue enzyme activities as reported by different investigators for various species. In most instances the enzyme patterns in the rabbit and mouse parallel those found in the rat. In the rat the enzymes of the hexose monophosphate shunt (glucose-6-phos-

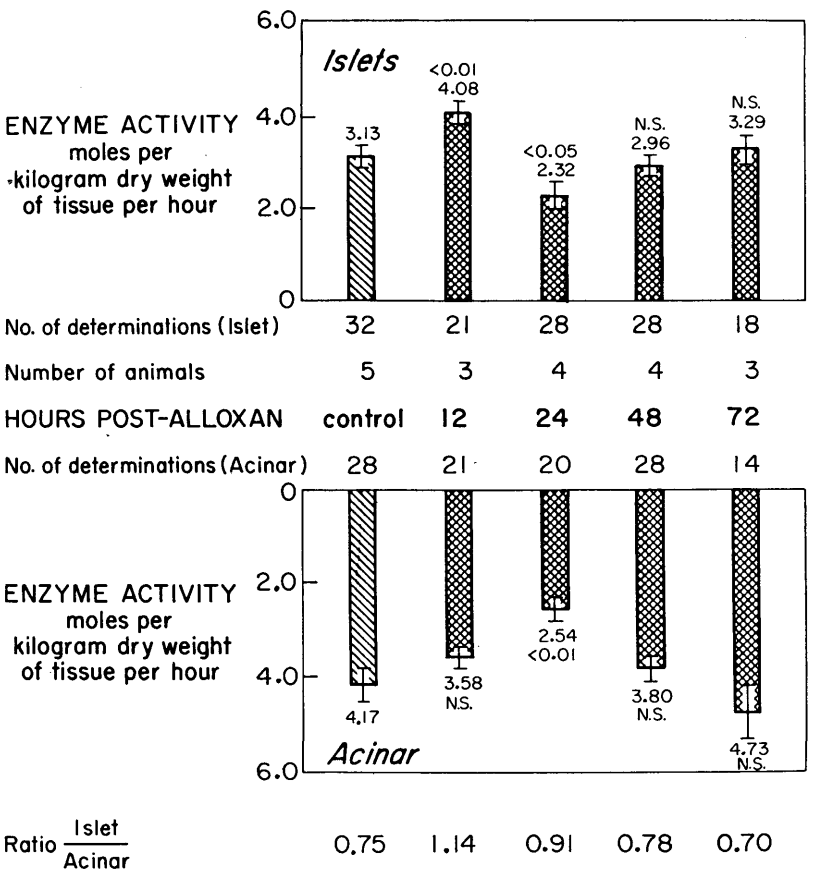

FIG. 2. Effect of alloxan on isocitric dehydrogenase (ICDH) activity of microdissected rat islet and acinar tissue. Other details as in figure 1.

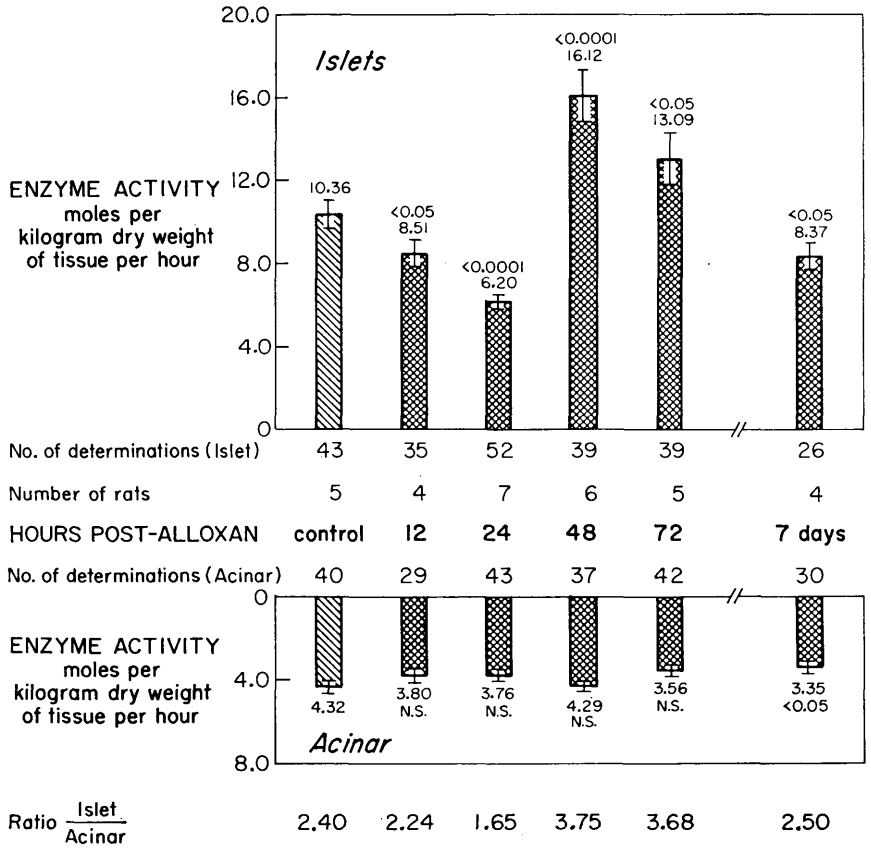

FIG. 3. Effect of alloxan on the dipeptidase (PEPT) activity of microdissected rat islet and acinar tissue. Other details as in figure 1.

phate dehydrogenase [G-6-PDH] and 6-PGDH), tricarboxylic acid cycle (MDH), alkaline phosphatase, glutamine-oxaloacetate transaminase (GOT), and peptidase were greater in the islet than in acinar tissue. The activity of LDH—an enzyme which functions under anaerobic conditions—was much lower in islet tissue than in acinar tissue.

We have compared the insulin content and the enzyme activities of the islet tissue in rat and toadfish (figure 5). Both the TCA cycle enzymes studied (MDH and ICDH) showed considerably greater enzyme activity in the rat islets than in the corresponding fish tissue. Of the two HMP shunt enzymes studied, the G-6-PDH activity of rat islets was approximately ten-fold that of the fish, whereas the 6-PGDH activity in rat islets was only slightly greater than that in the fish. By contrast the fish islets contained larger amounts of LDH.

It is known that alloxan combines with sulfhydryl groups,[35] and it is also well established that the activity of a number of enzymes is dependent upon the presence of free sulfhydryl groups.[36] It seems reasonable to suggest that the observed changes in the enzyme activity of the islet beta cells following alloxan administration may provide a clue to understanding the mechanism of alloxan action.

Following alloxan administration, the activity of 6-PGDH in rat islet tissue was markedly elevated (figure 1); this was similar to changes in the LDH activity (see table 2) previously reported.[21] However, the islet LDH activity was maximum at forty-eight hours after alloxan administration, whereas 6-PGDH activity was higher at seventy-two hours. The observed increase in 6-PGDH activity in the islet tissue following alloxan could be explained by the selective disappearance of the beta cell if this enzyme were likewise present in the normal alpha cell in amounts greater than that found in normal beta cells. However, the observed increase in the acinar 6-PGDH activity at seventy-two and 168 hours indicates that other factors may also be influencing the enzyme activity.

Hellerström and Hellman[37] have demonstrated a significantly higher level of alanylglycine peptidase activity in the pancreatic islets of obese-hyperglycemic mice as compared to that of lean controls. We have found a

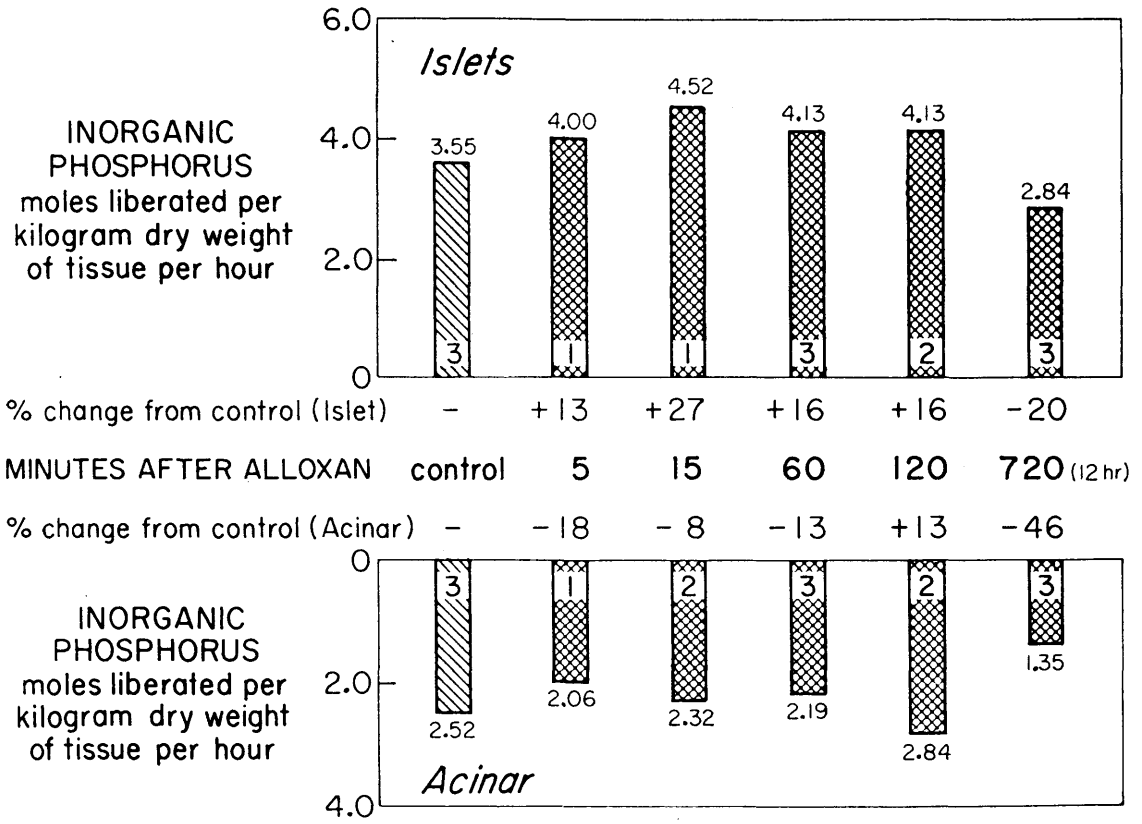

FIG. 4. Effect of alloxan on adenosine triphosphatase (ATPase) activity of microdissected rat islet and acinar tissue.

significant decrease in the PEPT activity of the islet tissue at twelve and twenty-four hours after alloxan administration (figure 3, table 2); at this time period the beta cells are shrunken and pyknotic.[2] Between twenty-four and forty-eight hours following the drug administration there was a two- to three-fold rise in the islet peptidase activity. This coincides with the time the beta cells are undergoing autolysis and disappearance. We have likewise shown[19] that the insulin content of the rat islet tissue disappears between twenty-four and forty-eight hours after alloxan administration (see table 2). In contrast to the islet tissue, alloxan did not affect the peptidase activity of acinar tissue at the corresponding time periods.

Using histochemical methods, Lazarus and coworkers[11] have reported a decreased ATPase reaction in the rabbit beta cell within five to fifteen minutes after intravenous injection of alloxan (100 mg. per kg. body weight). By eight hours the enzyme activity was markedly decreased and at eighteen hours there was no apparent enzyme activity in the beta cells. They postulated that the beta cells contain both mitochondrial and extramitochondrial -SH dependent ATPase enzyme, and that following alloxan treatment there is a more rapid disappearance of the extramitochondrial ATPase. Hellman,[38] using microchemical methods for ATPase determination, reported a 50 per cent reduction in the enzyme activity at five to sixty minutes when alloxan was injected intravenously in mice in doses of 200 mg. per kg. body weight. It should be noted, however, that this dose is several-fold greater than the usual diabetogenic dose of alloxan in the mouse.[39]

We did not find significant changes in the rat islet ATPase activity at 5, 15, 60 and 120 minutes after the administration of diabetogenic doses of alloxan (40 mg./kg.). It is difficult to explain the discrepancy between our observations and those of Lazarus and coworkers[11] and Hellman.[38] In our experiments the ATPase activity was determined at a pH of 8.8 and, although this is slightly lower than the pH maximum (namely 9.1),[38] this probably does not account for the observed differences. Since the cellular integrity is altered in lyophilized tissue, the microchemical assay used by us would not detect selective changes in the extramitochondrial ATPase activity. The effects of the larger doses of alloxan reported by Hellman[38]

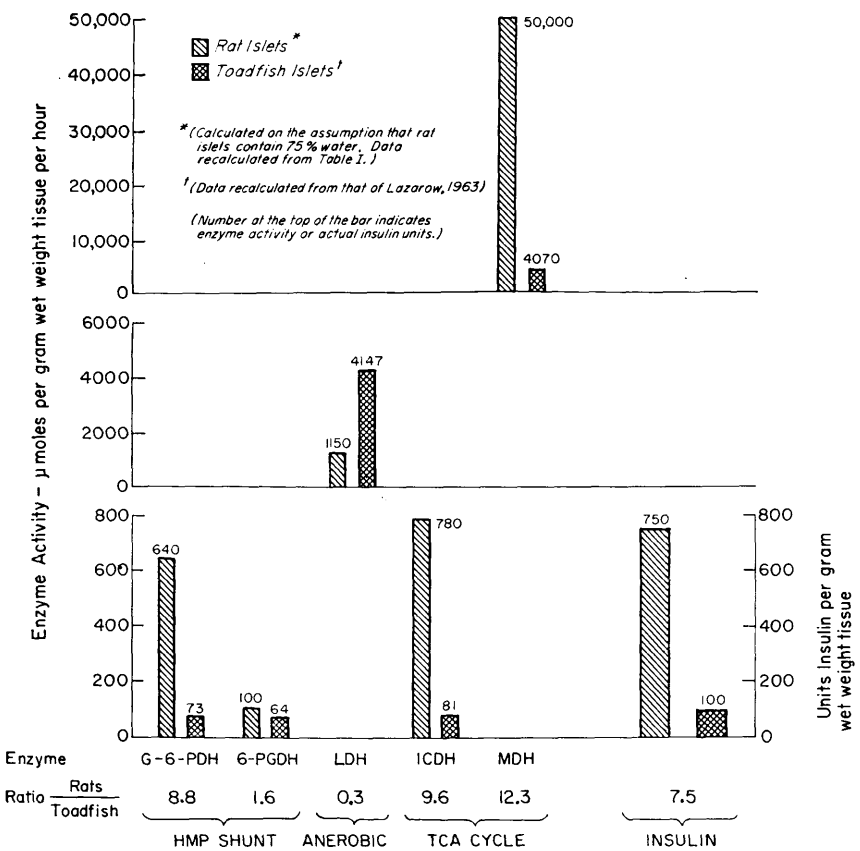

FIG. 5. Comparative activity of enzymes in rat and toadfish islets. N.B.: Enzyme activity and insulin content of the rat islet were calculated on the assumption that rat islet tissue contains 75 per cent water. In the case of toadfish islets, the data were recalculated from those of Lazarow.[34]

may reflect the toxic effect of alloxan rather than its diabetogenic action.*

Since the larger doses of alloxan may affect other tissues like liver, kidney and adrenal,[3,5] its diabetogenic mechanism must explain the selectivity of alloxan for the beta cell enzymes.

ACKNOWLEDGMENT

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*In a more recent study, Hellman (personal communication) found that smaller (diabetogenic) doses of alloxan did not selectively affect the islet ATPase activity.

TABLE 1

Enzyme activities in the islet and acinar tissue from mammalian pancreas

Enzyme	Animal species	Enzyme activity (moles/kg. dry wt./hr.) Islet	Acinar	Ratio Islets: Acinar	Author reference
Tricarboxylic acid cycle enzymes					
MDH	Rabbit	277	89	3.11	Lacy[13]
	Rabbit	273	117	2.33	Smith and Lacy[14]
	Rat	230	72	3.19	Kissane and Brolin[15]
	Rat	174	61	2.85	Dixit et al.[20]
	Rat	200	60	3.34	Dixit (unpublished)
	Mice	111	65	1.71	Dixit (unpublished)
ICDH	Rat	3.13	4.17	0.75	Dixit and Lazarow (this paper)
Hexose monophosphate shunt enzymes					
G-6-PDH	Rabbit	0.49	0.57	0.86	Lacy[13]
	Rabbit	0.51	0.54	0.94	Smith and Lacy[14]
	Rat	2.57	0.67	3.84	Kissane and Brolin[15]
6-PGDH	Rabbit	1.17	0.74	1.58	Lacy[13]
	Rabbit	0.92	0.56	1.64	Smith and Lacy[14]
	Rat	0.40	0.12	3.33	Dixit and Lazarow (this paper)
Anaerobic enzyme					
LDH	Rabbit	7.41	21.01	0.35	Lacy[13]
	Rat	2.97	13.00	0.23	Kissane and Brolin[15]
	Rat	4.60	10.80	0.43	Dixit et al.[21]
	Rat	3.92	10.92	0.36	Dixit (unpublished)
	Mice	7.58	18.72	0.41	Dixit (unpublished)
Transaminase					
Glutamic oxalacetic transaminase (GOT)	Rat	16.60	4.98	3.34	Kissane and Brolin[15]
Phosphatases					
Alkaline phosphatase (β-glycerophosphate as substrate)	Rat	0.56	0.24	2.33	Taljedal et al.[30]
Acid phosphatase (pH 5.3; p-nitrophenylphosphate as substrate)	Rat	0.47	1.65	0.28	Taljedal et al.[31]
ATPase	Mice	2.94	7.42	0.40	Taljedal et al.[32]
	Rat	3.06	5.78	0.53	Hellerström et al.[33]
	Rat	3.55	2.52	1.41	Dixit and Lazarow (this paper)
ATPase	Rat	0.91	1.78	0.51	Taljedal et al.[31]
ITPase	Rat	0.78	0.76	1.03	Taljedal et al.[31]
Protease					
PEPT	Rat	10.36	4.32	2.40	Dixit and Lazarow (this paper)

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TABLE 2

Percentage change from normal in the enzyme activity and insulin content of microdissected rat islet and acinar tissue following alloxan administration

		Time after alloxanization								
		Minutes				Hours				
		5	15	1	2	12	24	48	72	168
TCA cycle enzymes										
MDH	Islet	—	—	—	—	+14 (N.S.)	−46 (0.001)	−58 (0.001)	−65 (0.001)	−63 (0.001)
	Acinar	—	—	—	—	−24 (N.S.)	−10 (N.S.)	−25 (N.S.)	−13 (N.S.)	−34 (0.01)
ICDH	Islet	—	—	—	—	+30 (0.01)	−26 (0.05)	−5 (N.S.)	+5 (N.S.)	—
	Acinar	—	—	—	—	+14 (N.S.)	−39 (0.01)	−9 (N.S.)	+13 (N.S.)	—
HMP shunt enzyme										
6-PGDH	Islet	—	—	—	—	+52 (0.05)	+112 (0.001)	+132 (0.0001)	+234 (0.0001)	+140 (0.0001)
	Acinar	—	—	—	—	+158 (0.0001)	+50 (N.S.)	+67 (N.S.)	+158 (0.01)	+134 (0.001)
Anaerobic enzymes										
LDH	Islet	—	—	—	—	−9 (N.S.)	+98 (0.0001)	+312 (0.0001)	+156 (0.0001)	+77 (0.005)
	Acinar	—	—	—	—	+13 (N.S.)	+17 (N.S.)	+16 (N.S.)	+0 (N.S.)	+23 (N.S.)
Phosphatase										
ATPase	Islet	+13	+27	+16	+16	−20	—	—	—	—
	Acinar	−18	− 8	−13	+13	−46	—	—	—	—
Proteinase										
PEPT	Islet	—	—	—	—	−18 (0.05)	−40 (0.0001)	+56 (0.0001)	+26 (0.05)	−19 (0.05)
	Acinar	—	—	—	—	−12 (N.S.)	−13 (N.S.)	−1 (N.S.)	−17 (N.S.)	−22 (0.05)
Insulin										
	Islet	—	—	—	+13 (N.S.)	—	+1 (N.S.)	−96 (0.0001)	−97 (0.0001)	

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Overfeeding Lean and Obese Individuals

Leanness and the ability to eat apparently unlimited amounts of food appear to be the antithesis of the obese state. For this reason, a number of investigators have studied individuals who were chronically underweight, hoping to determine the reason for their subjects' good fortune.

One such study was made about a decade ago by R. Passmore and co-workers (*see Nutrition Reviews 14:* 267, 1956). They persuaded three lean young men to consume an extra 2000 kcal. above their daily requirement. Despite the abrupt change in caloric intake, there was no alteration in the percentage absorption of carbohydrates, fats, or proteins. During the period of overeating, there was an increased energy production of 6 to 16 per cent of the excess calories. This was attributed to the higher protein intake and the consequent increased specific dynamic effect. During the ten to fourteen days of overeating the subjects gained some weight, but it did not account for all the extra calories.

The same group of investigators (J. A. Strong, D. Shirling, and Passmore *Brit. J. Nutrition 21*:909, 1967) have performed similar experiments, but of shorter duration. The subjects for these studies, men and women, were from nineteen to thirty-eight years old. Six were from 74 to 86 per cent of standard weight, while nine were from 23 to 126 per cent above standard weight.

Each subject received a diet that maintained his body weight for an unspecified number of days. Caloric intake during this control period was calculated. The maintenance diet was then supplemented with foods the subject thought he could tolerate in large amounts. For four days each subject received approximately 1500 extra calories per day.

The thin subjects consumed an average of 6590 extra calories, while the obese subjects consumed an extra 4550. The obese subjects had difficulty eating more food, despite the fact that the lean subjects ate an average of 3882 kcal. per day while the obese consumed 3711 kcal. during the overfeeding period.

The extra caloric intake produced an increase in body weight of 1.6 kg. for the lean subjects and 1.7 kg. for the obese. This increase was attributed to 80 per cent of the extra calories. On the basis of respiratory quotient and nitrogen balance studies, it appeared that 9 per cent, or 88 gm., was deposited as protein, 35 per cent, or 315 gm., as fat, and 36 per cent, or 470 gm., as glycogen.

Overfeeding caused no change in the digestibility of calories as evidenced by the percentage of the caloric intake appearing in the stools. Furthermore, there was no difference during the overfeeding period between the thin and the obese on that score. Thus, the thin individuals had no advantage over the obese in disposing of surplus calories through the feces.

The metabolic rate during the night was 8.5 per cent higher during the overfeeding period than in the control period. The greatest difference between the two periods occurred early in the evening and was attributed to the specific dynamic action of the preceding meal. For twenty-four hours there was, during the overfeeding period, an increase of 300 kcal. in the subjects' heat production. This was about 20 per cent of the excess caolries. Since the remaining 80 per cent was deposited as body tissue, all the excess calories were accounted for. . . .

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