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Effect of reduced dietary zinc intake on carbohydrate and Zn metabolism in the genetically diabetic mouse (C57BL/KsJ *db*+/*db*+)

BY SUSAN SOUTHON, Z. KECHRID, A. J. A. WRIGHT AND SUSAN J. FAIRWEATHER-TAIT

AFRC Institute of Food Research Norwich, Colney Lane, Norwich NR4 7UA

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1. Male, 4–5-week-old, genetically diabetic mice (C57BL/KsJ *db*/*db*) and non-diabetic heterozygote littermates (C57BL/KsJ *db*/+) were fed on a diet containing 1 mg zinc/kg (low-Zn groups) or 54 mg Zn/kg (control groups) for 27 d. Food intakes and body-weight gain were recorded regularly. On day 28, after an overnight fast, animals were killed and blood glucose and insulin concentrations, liver glycogen, and femur and pancreatic Zn concentrations were determined.

2. The consumption of the low-Zn diet had only a minimal effect on the Zn status of the mice as indicated by growth rate, food intake and femur and pancreatic Zn concentrations. In fact, diabetic mice fed on the low-Zn diet had a higher total food intake than those fed on the control diet. The low-Zn diabetic mice had higher fasting blood glucose and liver glycogen levels than their control counterparts. Fasting blood insulin concentration was unaffected by dietary regimen.

3. A second experiment was performed in which the rate of loss of ^{65}Zn, injected subcutaneously, was measured by whole-body counting in the two mouse genotypes over a 28 d period, from 4 to 5 weeks of age. The influence of feeding low-Zn or control diets was also examined. At the end of the study femur and pancreatic Zn and non-fasting blood glucose levels were determined.

4. All mice fed on the low-Zn diet showed a marked reduction in whole-body ^{65}Zn loss compared with those animals fed on the control diet. In the low-Zn groups, the loss of ^{65}Zn from the diabetic mice was significantly greater than that from heterozygote mice. This difference was not observed in the control groups. Blood glucose levels were elevated in the low-Zn groups. Possible reasons for these observations are discussed.

5. The present study demonstrates an adverse effect of reduced dietary Zn intake on glucose utilization in the genetically diabetic mouse, which occurred before any significant tissue Zn depletion became apparent.

The effect of zinc deficiency on insulin and carbohydrate metabolism has been studied in some detail in laboratory animals (Boquist & Lernmark, 1969; Huber & Gershoff, 1973; Brown *et al.* 1975; Reeves & O'Dell, 1983) but in many instances the findings are difficult to interpret because of the effect of dietary Zn depletion on food intake (Quaterman *et al.* 1966). Consequently, there is no clear evidence demonstrating that inadequate dietary Zn is associated with changes in insulin secretion and action, although microscopic studies indicate that the β-cells of Zn-deficient animals have decreased granulations and histochemically detectable insulin (Boquist & Lernmark, 1969). An effect of low Zn status on hormonal action may, however, become apparent in conditions where insulin secretion and function are already abnormal. For example, it has been suggested that the altered Zn metabolism and reduced Zn status reported to occur in type II (maturity onset) diabetic subjects (Mooradian & Morley, 1987) may aggravate the insulin resistance which is characteristic of this condition (Kinlaw *et al.* 1983). It has been speculated that where there is evidence of poor Zn status a programme of Zn repletion may improve insulin sensitivity and reduce the severity of some of the complications of this disease (Levine *et al.* 1983). There appears to be little information, however, on the effect of varying dietary Zn intake on the progression and severity of this form of diabetes.

In the present study a semi-synthetic diet containing either 1 or 54 mg Zn/kg was fed to 4–5-week-old male, genetically diabetic mice (C57BL/KsJ *db*+/*db*+). Diabetes (*db*) is inherited as a unit autosomal recessive and all mice homozygous for the diabetic gene (*db*/

db) display symptoms similar to non-insulin-dependent diabetes in man: hyperglycaemia, hyperinsulinaemia, polyuria and glycosuria (Coleman & Hummel, 1967). Heterozygote mice (*db*/+) cannot be distinguished morphologically or physiologically from normal and were used for comparison in the present study. Growth rate, food intake, Zn status, blood glucose and insulin concentrations, and rate of loss of ^{65}Zn administered subcutaneously, were measured in the two dietary groups of each mouse genotype.

METHODS

Animals

Two experiments were performed in which 4–5-week-old, male diabetic mice and non-diabetic heterozygote littermates (Harlan Olac, Bicester, Oxon.) were each randomly allocated into two groups. Approximately half of each genotype received a diet containing 1 mg Zn/kg (low-Zn groups) and the remaining animals received a diet containing 54 mg Zn/kg (control groups). The recommended dietary Zn concentration for both mice and rats is 12–30 mg/kg diet, depending on the protein source (American Institute of Nutrition, 1977). The composition of the diet was similar to that described previously (Southon *et al.* 1984) but with egg-albumin (G. Fiske, Richmond, Surrey) as the protein source. The low-Zn diet was prepared by omitting zinc carbonate from the mineral mix. The number of mice in each group is shown in the tables of results.

In the first experiment mice were caged singly in polypropylene cages with stainless-steel gridded tops and bottoms and stainless-steel food hoppers, in a room at 21° having a 12 h light–12 h dark cycle. Trays were placed under each food hopper to collect spilt food. Food and distilled water were provided *ad lib.* Food intake was measured daily and body-weights recorded twice weekly for 28 d.

In the second experiment it was necessary to house the mice three to four per cage. Food was provided *ad lib.* but, because of excessive spillage and contamination of food with urine, it was not possible to estimate food intake. Body-weights were recorded twice weekly over an experimental period of 28 d.

Expt 1

The effect of reduced dietary Zn intake on the growth, food intake, Zn status, fasting blood glucose and insulin concentration, and fasting liver glycogen content of diabetic mice and heterozygote littermates was investigated. Mice were maintained on the appropriate experimental diet *ad lib.* for 26 d, fasted overnight and on day 27 given access to food for two periods of 1 h between 11·00 and 12·00 hours and 17·00 and 18·00 hours, so that time of feeding on the day before death was similar for all groups. Mice were then killed beween 11·00 and 12·30 hours on day 28, one animal from each group being killed at approximately the same time by exsanguination from the heart whilst under diethyl ether anaesthesia. Blood was transferred into an ice-cold heparinized vial and a portion taken for whole-blood glucose analysis which was performed promptly after exsanguination. The remaining blood was centrifuged and the plasma stored at −20° before insulin assay. Livers were rapidly excised, weighed, freeze-clamped at −196°, ground under liquid nitrogen and stored at −20° before glycogen analysis. The pancreas and right femur were removed, weighed, dried at 80° for 16 h and Zn concentration determined.

Expt 2

The effect of dietary Zn intake on ^{65}Zn loss from diabetic mice was investigated and compared with that of heterozygote mice. Mice were ear-clipped, housed three or four per cage and all animals fed on the control semi-synthetic diet *ad lib.* for 5 d, after which time

mice were injected subcutaneously into the scruff with 37 kBq ^{65}Zn (3·7–92·5 GBq/g Zn; Amersham International, Amersham, Bucks.) in 0·1 ml saline (9 g sodium chloride/l). Immediately after injection, the mice were restrained within a well-ventilated perspex box (100 × 100 × 120 mm) and counted in a small-animal whole-body gamma counter (NE8112; Nuclear Enterprises, Edinburgh) to determine the exact dose of isotope given to each animal. Counting geometry was such that the count from a radioactive 'point' source at any position in the box was within ±0·5% of the overall mean.

After the injection, half these animals continued to receive the control diet and half were given the low-Zn diet, and the amount of whole-body ^{65}Zn lost over a 27 or 28 d period was measured by whole-body counting as described previously (Southon *et al.* 1988*b*). On days 27 and 28 of the experiment the mice were killed between 10·00 and 13·00 hours by intraperitoneal injection of an overdose of sodium pentabarbitone (0·3 ml, 200 mg/ml; Euthatal; May & Baker, Dagenham, Essex), one mouse from each group being killed at approximately the same time.

Before the administration of pentabarbitone a tail-blood sample was taken from each mouse and, after death, the pancreas and right femur were removed from a random selection of mice from each group, for Zn analysis. The number of observations made for each measurement in both experiments is given in the tables of results.

Analytical methods

Blood glucose was measured in 10 μl samples of fresh whole-blood by the glucose oxidase (*EC* 1.1.3.4) method, using a YSI Model 27 glucose analyser (Yellow Springs Inst. Inc., Ohio, USA).

Plasma insulin was determined by a radioimmunoassay as described previously (Southon *et al.* 1988*a*).

Glycogen was determined as glucose following enzymic hydrolysis with amyloglucosidase (*EC* 3.2.1.3) (Keppler & Dekker, 1974).

The dried pancreas and femur were heated in silica crucibles at 480° for 48 h and the ash taken up in hot hydrochloric acid (11.7 M) for Zn analysis by atomic absorption (PU 9000; Pye Unicam, Cambridge).

Statistical analysis

Comparisons between the effect of diet and genotype were made using Student's unpaired *t* test.

RESULTS

Expt 1

Body-weight gain of the diabetic and heterozygote mice fed on a low-Zn diet for 28 d was not significantly different from their control counterparts (Table 1). Although the growth rate of diabetic mice was similar in the two dietary groups, the total food intake of the low-Zn diabetic animals was 7–8% higher ($P < 0·01$) than that of the control diabetic mice. The amount of food consumed by the low-Zn and control heterzygote mice was not significantly different. Values for body-weight gain and food intake were significantly higher for diabetic animals compared with the heterozygotes (Table 1).

Femur and pancreatic Zn concentrations, taken as an index of Zn status, indicated that both the low-Zn diabetic and heterozygote mice were able to maintain a similar status to that of their control group (Table 1). Total pancreatic Zn, however, was significantly lower in low-Zn diabetic mice compared with the controls. Pancreatic Zn concentration was significantly lower in all the diabetic animals compared with the heterozygotes. However,

Table 1. *Expt 1. Body-weight gain, total food intake, femur zinc concentration, weight of pancreas and pancreatic Zn content of diabetic and heterozygote mice given a low-Zn (1 mg Zn/kg) or control (54 mg Zn/kg) semi-synthetic diet for 28 d**

(Values are means with their standard errors for *n* observations)

Genotype...	Diabetic				Heterozygote			
Diet...	Control (*n* 20)		Low-Zn (*n* 19)		Control (*n* 8)		Low-Zn (*n* 8)	
	Mean	SE	Mean	SE	Mean	SE	Mean	SE
Initial body-wt (g)	19·6[a]	0·3	19·5[a]	0·1	17·4[b]	0·5	17·2[b]	0·8
Body-wt gain (g)	11·9[a]	0·8	12·7[a]	0·7	6·9[b]	1·2	4·9[b]	0·8
Total food intake (g)	121[a]	2	130[b]	2	88[c]	3	88[c]	4
Femur Zn (μg/g dry wt)	120[a]	3	121[a,b]	6	130[b]	4	132[a,b]	6
Pancreas dry wt (mg)	59[a]	3	57[a]	3	40[b]	4	38[b]	3
Pancreatic Zn:								
Total (μg)	3·2[a]	0·1	2·7[b]	0·1	3·6[a,c]	0·2	3·9[c]	0·3
Concentration (μg/g dry wt)	56·5[a]	4·1	49·0[a]	3·8	92·5[b]	7·8	104·7[b]	7·7

[a,b,c] Values within a horizontal line with different superscript letters were significantly different ($P < 0·05$).
* For details of animals and diets, see p. 500.

Table 2. *Expt 1. Fasting blood glucose and plasma insulin concentration, liver weight and liver glycogen content of diabetic and heterozygote mice given a low-zinc (1 mg Zn/kg) or control (54 mg Zn/kg) semi-synthetic diet for 28 d**

(Values are means with their standard errors for *n* observations)

Genotype...	Diabetic				Heterozygote			
Diet...	Control (*n* 20)		Low-Zn (*n* 19)		Control (*n* 8)		Low-Zn (*n* 8)	
	Mean	SE	Mean	SE	Mean	SE	Mean	SE
Blood glucose (mmol/l whole blood)	12·4[a]	0·4	15·3[b]	0·9	5·6[c]	0·9	4·7[c]	0·6
Plasma insulin (μunits/ml)	52·1[a]	5·6	46·0[a]	5·4	9·8[b]	2·2	8·0[b]	3·2
Liver (g fresh wt)	1·86[a]	0·08	2·10[b]	0·05	0·78[c]	0·03	0·74[c]	0·02
Liver glucogen:								
Total (g)	22·1[a]	3·5	32·7[b]	3·9	1·6[c]	1·1	1·6[c]	1·2
Concentration (mg/g fresh wt)	11·4[a]	1·5	15·7[a]	1·9	1·9[b]	1·2	1·8[b]	1·3

[a,b,c] Values within a horizontal line with different superscript letters were significantly different ($P < 0·05$).
* For details of animals and diets, see p. 500.

because of the increased dry weight of pancreases from diabetic animals, differences in total Zn content were not so marked. In both experiments mean femur Zn concentrations for diabetic mice were lower than those for heterozygotes, but the difference was not always significant (Tables 1 and 3).

On day 27 of the study, time of feeding was controlled to allow a more accurate comparison of blood glucose and insulin concentrations between groups on day 28 when the mice were killed following an overnight fast. Food-intake measurements showed that

Table 3. *Expt 2. Body-weight gain, femur and pancreatic zinc concentrations and blood glucose concentration of diabetic and heterozygote mice given a low-Zn (1 mg Zn/kg) or control (54 mg Zn/kg) semi-synthetic diet for 28 d**

(Values are means with their standard errors for *n* observations)

Genotype...	Diabetic				Heterozygote			
Diet...	Control (*n* 20)		Low-Zn (*n* 19)		Control (*n* 8)		Low-Zn (*n* 8)	
	Mean	SE	Mean	SE	Mean	SE	Mean	SE
Initial body-wt (g)	24·6[a]	0·7	23·0[a]	0·5	19·1[b]	0·4	20·4[b]	0·2
Body-wt gain (g)	8·8[a]	0·4	7·4[a]	0·6	4·0[b]	0·5	4·2[b]	0·4
Femur Zn (μg/g dry wt)	152[a,b]	9	140[a]	6	174[b,c]	12	193[c]	17
Pancreatic Zn (μg/g fresh wt)	20·9[a]	2·4	19·5[a]	2·1	24·3[a,b]	1·5	28·5[b]	1·7
Blood glucose (mmol/l whole-blood)	22·2[a]	2·6	29·9[b]	2·7	5·8[c]	0·3	7·6[d]	0·4

[a,b,c,d] Values within a horizontal line with different superscript letters were significantly different ($P < 0·05$).
* For details of animals and diets, see pp. 500–501.

Table 4. *Expt 2. Whole-body ^{65}Zn loss, 27 d after a subcutaneous dose (37 kBq), from diabetic and heterozygote mice given a low-Zn (1 mg Zn/kg) or control (54 mg Zn/kg) semi-synthetic diet**

(Values are means with their standard errors for *n* observations)

		Whole-body ^{65}Zn loss (kBq)		
Genotype	Diet	Mean	SE	*n*
Diabetic	Control	27·0[a]	1·1	13
	Low-Zn	18·6[b]	1·0	14
Heterozygote	Control	28·0[a]	0·3	9
	Low-Zn	14·5[c]	0·4	10

[a,b,c] Values within a vertical column with different superscript letters were significantly different ($P < 0·05$).
* For details of animals and diets, see pp. 500–501.

there was no difference between genotypes or dietary groups in the amount of food consumed at either of the two meals on the day before sampling. Weights of food consumed at the last meal (mean with SEM) were (g) control groups: diabetic 0·9 (SEM 0·05), heterozygote 1·0 (SEM 0·1); low-Zn groups: diabetic 0·8 (SEM 0·07), heterozygote 0·8 (SEM 0·02). Analysis of fasting blood glucose and plasma insulin concentration and liver glycogen content, indicated that low-Zn diabetic mice had a similar plasma insulin concentration, a higher fasting blood glucose level ($P < 0·05$) and an increased liver glycogen content ($P < 0·05$) compared with the control diabetic animals (Table 2). The higher liver glycogen values in the low-Zn diabetic group were associated with a significant increase in liver weight ($P < 0·05$) together with a trend towards a higher glycogen concentration ($P < 0·1$) (Table 2). Blood glucose and plasma insulin concentrations, and liver glycogen content were similar in both low-Zn and control groups of heterozygote mice (Table 2).

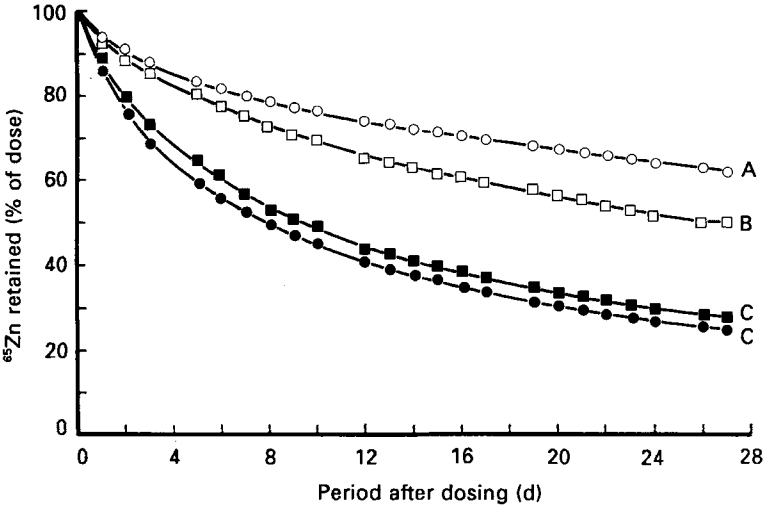

Fig. 1. Whole-body ^{65}Zn loss from a subcutaneous dose (37 kBq) given to 4–5-week-old male diabetic (■, □) and non-diabetic (●, ○) mice fed on a control (■, ●) or low-Zn (□, ○) diet immediately after dosing. For details of animals and diets, see pp. 500–501. A, B, C Values for day 27 having a different letter were significantly different ($P < 0{\cdot}05$).

Expt 2

The body-weight gain was similar in the low-Zn and control groups, with the diabetic mice having the greatest weight gain (Table 3). Pancreatic and femur Zn concentrations in both diabetic and heterozygote mice were unaffected by the level of dietary Zn (Table 3).

In this second study, blood glucose concentration was measured following normal *ad lib.* feeding and, under these conditions, whole-blood glucose concentration was found to be significantly higher in each of the low-Zn groups killed after a 27 d feeding period ($P < 0{\cdot}05$), compared with mice fed on the control diet (Table 3).

A whole-body ^{65}Zn study, performed between approximately 4 and 8 weeks of age, showed that ^{65}Zn loss was markedly lower at all time-points for animals fed on the low-Zn diet (Table 4, Fig. 1). Rate of ^{65}Zn loss was similar in both the control groups, but low-Zn diabetic mice had a significantly greater whole-body ^{65}Zn loss ($P < 0{\cdot}05$) over the 28 d period of study compared with heterozygotes fed on the same diet (Fig. 1). Regression analysis of the $\log_{10}$ per cent rate of ^{65}Zn loss, over the last 9 d indicated that the mean daily ^{65}Zn loss was 2 and 1·3% for low-Zn diabetic and heterozygote mice respectively.

DISCUSSION

Findings on growth rate, food intake and femur and pancreatic Zn concentrations indicated that the long-term consumption of a low-Zn semi-synthetic diet (1 mg Zn/kg) resulted in only a very mild deficiency state in the mice used in the present study, compared with the very severe deficiency which would have been observed in the laboratory rat fed on this level of Zn (Southon *et al.* 1984). It appears, therefore, that although the recommended dietary Zn concentration for mice is similar to that for rats (American Institute of Nutrition, 1977), the minimum requirement of the mouse is very much lower.

In both experiments the body-weight gain of diabetic and heterozygote mice was not significantly affected by dietary Zn concentration (1 or 54 mg Zn/kg). The amount of food

consumed by the heterozygote non-diabetic animals was also unaffected by dietary Zn concentration, but the diabetic mice fed on the low-Zn diet had a higher food intake than those fed on the control diet. This is surprising in view of the many studies demonstrating reduced appetite in animals fed on low-Zn diets (Mills *et al.* 1969) and raises the possibility that the degree and direction of response to dietary Zn depletion may be influenced by the metabolic state of the animal.

Pancreatic Zn concentrations were similar in dietary groups of the same genotype, despite the fact that this tissue is generally regarded to be one of the most sensitive to variation in dietary Zn intake (Williams & Mills, 1970). There was, however, an indication of reduced total pancreatic Zn in diabetic mice fed on the low-Zn diet. The lower pancreatic Zn concentration observed in the diabetic mice fed at both levels of Zn compared with heterozygotes, is probably related to their hyperinsulinaemia, the early onset of β-cell degranulation and other pathological changes in this tissue associated with the progression of the condition (Coleman & Hummel, 1967), and is consistent with human studies showing that the pancreatic Zn concentration of diabetics is depressed compared with normals (Scott & Fisher, 1938). The marked reduction in femur Zn concentrations in genetically diabetic mice, compared with heterozygote littermates, reported by Levine *et al.* (1983), was not confirmed by the present study. There were indications that bone Zn concentration was slightly lower in the diabetic animals, but the difference was not always significant.

Despite the apparently negligible effects of consuming the low-Zn diet on growth and Zn status of mice used in the present study, significant differences in glucose metabolism were observed. In the first experiment, where the time of feeding was strictly controlled and the amount of food eaten by each animal before an overnight fast was known to be similar, the mean fasting blood glucose concentration in the low-Zn diabetic mice was found to be 23% higher than that for the control diabetic group. This suggests that the lower Zn intake had exacerbated the reduced ability of the diabetic mice to utilize glucose. In the second experiment, where blood glucose was measured after normal *ad lib.* feeding, low-Zn diabetic mice were again found to have elevated blood glucose concentrations and there was some indication of abnormal glucose utilization in the low-Zn heterozygote animals. Although there was no evidence from the first experiment of a difference in feeding pattern between dietary groups, investigations to verify the increased blood glucose concentrations observed in the second experiment should be repeated under conditions of strict dietary control. Results from the present study, and those of a previous study showing increased blood glucose concentations after oral dosing in rats fed on a marginal-Zn diet in late pregnancy (Southon *et al.* 1988*a*), suggest a relation between carbohydrate utilization and dietary Zn supply, particularly in conditions associated with hyperinsulinaemia and tissue insulin resistance. Since circulating insulin levels in both studies were unaffected by the reduced dietary Zn intake, the possibility of increased insulin resistance or reduced physiological potency of the hormone should be considered.

Aside from the well-known large accumulation of subcutaneous fat in the genetically diabetic mice, the most striking anatomical deviation is the size of the liver, which is in part due to metabolic defects resulting in increased fat and glycogen deposition (Coleman & Hummel, 1967). In the present study, the livers of 8–9-week-old diabetic mice were two to three times heavier than their heterozygote littermates and it was noted that livers from low-Zn diabetic mice were significantly heavier than those from their control counterparts. In addition, the fasting glycogen content of the low-Zn diabetic mice was approximately 48% higher than in the control diabetic animals, although food intake on the day before death was similar for these two groups. This again indicates that the carbohydrate metabolism of these animals is sensitive to reduced Zn intake. It is interesting to note that Reeves & O'Dell (1983) also found evidence of increased glycogen synthesis in Zn-deficient

rats. In the present study it was difficult to ascertain whether there was any effect of diet on glycogen deposition in the heterozygote mice since only small amounts of glycogen were detectable in the fasted state, and no analyses were performed in the 'fed' animals because of the difficulty of determining food intake in the second study.

Results from the ^{65}Zn study clearly demonstrated the ability of both mouse genotypes to reduce ^{65}Zn loss when dietary Zn intake was restricted. This may have been achieved by decreased endogenous Zn secretion into the gastrointestinal tract (Jackson *et al.* 1984) or increased efficiency of absorption of Zn from the diet (Wada *et al.* 1985), or both. The diabetic condition also had a significant effect on ^{65}Zn loss, in that the loss was significantly greater in low-Zn diabetic mice than in the non-diabetic heterozygote. It appears, therefore, that the diabetic mice were less able to adapt to conditions of reduced dietary Zn intake. In contrast, ^{65}Zn loss from both genotypes fed on the control diet was similar. It was also noted that, whereas the low-Zn diabetic mice had a significantly lower femur Zn concentration than low-Zn heterozygotes in the present experiment, there was no significant difference between control groups (Table 3). Although the rate of whole-body ^{65}Zn turnover is known to be directly related to dietary Zn intake (Huber & Gershoff, 1970), it is unlikely that the increased loss of ^{65}Zn from low-Zn diabetic mice was related to their higher food intake compared with heterozygotes; first, because the Zn content of the low-Zn diet was such that the observed increase in food consumption would not have influenced the rate of body Zn turnover and second, because no difference in ^{65}Zn loss was found between genotypes fed on the control diet. The route of loss of ^{65}Zn was not determined in the present experiment, but there are reports in the literature describing a higher Zn concentration in the urine of genetically diabetic mice (Levine *et al.* 1983). However, little is known about the efficiency of Zn absorption in these animals, and any difference in absorption between genotypes could have influenced rates of ^{65}Zn loss. Clearly more detailed studies are required to gain a better understanding of the complex metabolic changes observed.

In conclusion, findings presented in the present paper demonstrate that reduced Zn intake had an adverse effect on glucose utilization in the genetically diabetic mouse, although changes in Zn status appeared to be minimal. It appears, therefore, that abnormalities in carbohydrate metabolism may occur before tissue Zn depletion becomes apparent.

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