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Energy balance in rats given chronic hormone treatment

1. Effects of long-acting insulin

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1. Sprague–Dawley rats were injected for 16 d with long-acting insulin, and energy balance was calculated using the comparative carcass technique. Two experiments were carried out with females (starting weights 150 and 90 g respectively), and one with males (starting weight 150 g). In a fourth experiment, cytochrome *c* oxidase (*EC* 1.9.3.1) activity was measured as an indicator of the capacity for substrate oxidation.

2. Insulin increased weight gain by up to 57% ($P < 0.01$ for all studies). Metabolizable energy intake (kJ/d) was also consistently higher in the treated groups, by up to 34% ($P < 0.01$ for all studies). The excess weight gained by the insulin-treated rats was predominantly due to fat deposition.

3. Energy expenditure, calculated as the difference between metabolizable intake and carcass energy gain, was expressed on a whole-body basis, or relative to either metabolic body size (kg body-weight$^{0.75}$) or fat-free mass. Insulin consistently raised energy expenditure, regardless of the method of expression, but this change reached statistical significance in only two of the nine comparisons.

4. Cytochrome *c* oxidase activity was not affected by insulin treatment in either interscapular brown adipose tissue or gastrocnemius muscle. In liver, total enzyme activity (U/tissue) was increased from 2928 (SE 162) in the controls to 3940 (SE 294) in the treated group ($P < 0.02$), but specific activity (U/mg protein) was unchanged.

5. It is concluded that, despite causing substantial hyperphagia, insulin treatment only slightly increases energy expenditure in rats. The costs of increased tissue deposition may account for this change.

Long-acting insulin causes hyperphagia and weight gain in rats, eventually producing a substantial degree of obesity (Mackay *et al.* 1940; Hausberger & Hausberger, 1958). Hyperphagia has also been demonstrated when insulin is used in conjunction with a protein-deficient diet (Beaton *et al.* 1965), during cold exposure (Morrison, 1984), and in rats with cancer cachexia (Moley *et al.* 1985) or hypothalamic hypophagia (Bernardis *et al.* 1981).

The effects of insulin on energy expenditure are less clear. Oxygen consumption was not altered in rats given either acute (Moley *et al.* 1985) or chronic (Beaton *et al.* 1965) insulin treatment. However, low doses of insulin were found to increase body-weight (Bartness *et al.* 1986) and fat content (Torbay *et al.* 1985) without causing hyperphagia, suggesting that energy expenditure may have been reduced.

Accordingly, the present study was carried out to measure energy balance during chronic insulin treatment, using the comparative carcass technique. In addition cytochrome *c* oxidase (*EC* 1.9.3.1) activity was analysed in various tissues, as an indicator of the capacity for substrate oxidation. A preliminary report of the present work has already been published (Woodward & Emery, 1985).

MATERIALS AND METHODS

Chemicals

Isophane insulin (Insulatard; 80 U/ml) was obtained from the Wellcome Foundation Ltd, Crewe, Cheshire. Cytochrome *c* Type III and digitonin were bought from Sigma Chemical Co., Poole, Dorset.

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Table 1. *Composition of semi-purified diet (g/kg)*

Ingredient	
Casein	210
Maize starch	290
Sucrose	290
Maize oil	100
Mineral mixture*	40
Vitamin mixture†	20
Powdered cellulose	50

* Providing (/kg diet): calcium 6·7 g, phosphorus 4·2 g, potassium 4·3 g, sodium 2·7 g, magnesium 560 mg, manganese 66 mg, iron 40 mg, zinc 16 mg, copper 6·0 mg, iodine 0·18 mg.

† Made up in powdered cellulose and providing (mg/kg diet): thiamin hydrochloride 10, pyridoxine 10, riboflavin 10, pteroylmonoglutamic acid 5, calcium pantothenate 40, nicotinic acid 60, ascorbic acid 75, cyanocobalamin 0·05, biotin 1·0, choline bitartrate 1800, menadione 1·0, α-tocopheryl acetate 75, retinyl acetate 4·0, cholecalciferol 0·037.

Animals

Sprague–Dawley rats from the College animal colony were housed singly in plastic cages with wire-mesh bases. Groups of five to nine animals were used. The animal house was maintained at 20–25° with a 12 h light–12 h dark cycle. From at least 4 d before the start of each experiment, the animals were given, *ad lib.*, water and a powdered semi-synthetic diet (Table 1). Food intake and body-weights were recorded every 2–4 d. Insulin was injected subcutaneously once daily at 15.00–17.00 hours for 16 d. The initial dose was 20 U/kg per d increasing to a plateau over the course of the 1st week. For the first two experiments, in which energy balance was determined in female rats, the plateau dose was 50 U/kg per d. However, since this caused some mortality, a dose of 40 U/kg per d was used in the remaining two studies. Control animals were injected with saline solution (9 g sodium chloride/l water). The following experiments were carried out.

Expt 1: female rats weighing approximately 150 g were injected with insulin (maximum dose 50 U/kg per d) before calculating energy balance.

Expt 2: female rats weighing approximately 90 g were injected with insulin (maximum dose 50 U/kg per d) before calculating energy balance.

Expt 3: male rats weighing approximately 150 g were injected with insulin (maximum dose 40 U/kg per d) before calculating energy balance.

Expt 4: female rats weighing approximately 150 g were injected with insulin (maximum dose 40 U/kg per d) before determining cytochrome *c* oxidase activity.

Carcass analysis

After killing the animals by diethyl ether anaesthesia, their gastrointestinal tracts were washed out with water. The rats were oven-dried at 105° to constant weight, the weight loss being taken as water. Drying time was 4 d in almost all cases. The carcasses were then minced and stored in a desiccator. Duplicate samples of 10–15 g were extracted in a Soxhlet apparatus with light petroleum (b.p. 60–80°). Carcass fat content was calculated from the weight loss during extraction, a typical coefficient of variation being 1·6% (*n* 12 duplicate samples). Carcass crude protein content was found during pilot analyses, using the Kjeldahl method, to be a constant fraction of defatted dry matter (DDM) which did not change significantly with insulin treatment, sex or age of rat. Carcass crude protein content was therefore routinely calculated as weight of DDM × 0·81.

Table 2. *Carcass composition of basal rats*

(Values are means with their standard errors for the number of rats indicated)

					Carcass content (g/kg)							
			Body-wt (g)		Water		Fat		DDM		Crude protein†	
Expt no.*	Sex	*n*	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE
1	Female	6	147	4·0	680	8	105	8	216	2	175	2
2	Female	7	89	5·8	702	8	89	9	210	2	170	2
3	Male	5	145	4·1	718	5	67	5	226	4	183	3

DDM, defatted dried matter

* For details of individual experiments, see p. 438.

† Calculated as weight of DDM × 0·81.

Calculation of carcass gains and energy balance

Initial composition was estimated using the percentage composition of basal groups of appropriate weight (Table 2). Gains in carcass components were calculated by difference. The metabolizable energy content of the diet, calculated using Atwater factors (Passmore & Eastwood, 1986) and without drying, was 16·1 kJ/g, a value which agreed with that measured directly in pilot experiments using metabolism cages. Carcass energy content was calculated factorially, assuming energy densities of 39 and 19 kJ/g for carcass fat and DDM respectively. These factors were obtained from ballistic-bomb calorimetry of pooled samples. Energy expenditure, calculated by subtracting carcass energy gain from metabolizable energy intake, was expressed in three different ways: per whole rat, relative to metabolic body size (kg body-weight$^{0.75}$) or relative to fat-free mass.

Tissue analysis

At the end of Expt 4, the interscapular brown adipose tissue, liver and left-leg gastrocnemius muscle were rapidly dissected out and frozen in liquid N_2. The tissues were kept at $-15°$ in a deep-freeze and analysed within 1 month. Tissue samples were homogenized in 0·1 M-potassium dihydrogen phosphate–dipotassium hydrogen phosphate buffer, pH 7·0, and diluted to approximately 1 g tissue/l. Duplicate portions were taken for protein determination (Markwell *et al.* 1978) using bovine serum albumin as standard. A second pair of portions was incubated with digitonin (5 g/l) for 2 h at 0° to disrupt the mitochondrial membranes. This pre-treatment increased apparent enzyme activity approximately fivefold. Cytochrome *c* oxidase activity was then determined using the method of Wharton & Tzagoloff (1967). Enzyme activity was calculated from the decrease in absorbance of reduced cytochrome *c* at 550 nm, using a SP8-100 recording spectrophotometer (Pye-Unicam Ltd, Cambridge). A typical coefficient of variation for this assay was 5·3% (*n* 10 duplicate samples). Enzyme concentrations are expressed as units of activity, where one unit (U) is equivalent to 1 μmol substrate converted/min at 30°.

Statistical analyses

Results are expressed as means with their standard errors. Means were compared using Student's unpaired *t* test. Where the Fisher test indicated that variances were unequal, Cochran's modified *t* test was used (Snedecor & Cochran, 1978).

Table 3. *Body-weight and carcass composition of rats injected with insulin for 16 d and their controls*

(Values are means with their standard errors for the number of rats indicated)

Expt no†	Sex	Starting wt (g)	Treatment	*n*	Body-wt: Final (g) Mean	SE	Body-wt: Gain (g/d) Mean	SE	Gains of carcass components (g/d): Water Mean	SE	Fat Mean	SE	DDM Mean	SE	Crude protein‡ Mean	SE
1	Female	150	Insulin	5	262***	3·6	6·6***	0·3	2·94**	0·11	2·56***	0·30	1·12*	0·06	0·91*	0·05
			Control	6	217	5·6	4·2	0·2	2·24	0·12	0·97	0·12	0·94	0·04	0·76	0·03
2	Female	90	Insulin	8	194*	3·9	6·3**	0·2	3·11	0·10	1·91***	0·14	1·32*	0·04	1·07*	0·03
			Control	6	175	7·0	5·0	0·3	2·73	0·21	1·09	0·09	1·16	0·06	0·94	0·05
3	Male	150	Insulin	5	256*	9·9	7·4**	0·3	3·24	0·27	2·67***	0·21	1·51	0·06	1·22	0·05
			Control	5	229	4·9	5·8	0·3	3·40	0·26	1·02	0·10	1·41	0·07	1·14	0·06

DDM, defatted dried matter.
Mean values were significantly different from those of the corresponding control group: * $P < 0·05$, ** $P < 0·01$, *** $P < 0·001$.
† For details of individual experiments, see p. 438.
‡ Calculated as weight of DDM × 0·81.

Table 4. *Energy balance of rats injected with insulin for 16 d and their controls*

(Values are means with their standard errors for five to eight rats per group, as indicated in Table 3

Expt no.†	Sex	Starting wt (g)	Treatment	Metabolizable energy intake: kJ/d Mean	SE	kJ/kg $W^{0·75}$ per d Mean	SE	kJ/kg FFM per d Mean	SE	Carcass gain (kJ/d) Mean	SE	Energy expenditure: kJ/d Mean	SE	kJ/kg $W^{0·75}$ per d Mean	SE	kJ/kg FFM per d Mean	SE
1	Female	150	Insulin	368***	8·7	1194***	31	2151***	83	121***	11·1	248*	9·0	806	26	1444	41
			Control	275	7·9	978	21	1716	37	55	4·6	219	7·2	781	23	1369	38
2	Female	90	Insulin	311**	8·4	1348***	30	2601***	74	99***	5·9	212	5·4	919	22	1769	44
			Control	264	8·6	1193	16	2233	40	64	3·6	200	6·7	900	16	1687	32
3	Male	150	Insulin	397**	17·4	1345**	38	2391**	89	133***	8·1	265	11·7	894	19	1587*	44
			Control	308	8·6	1102	12	1861	19	66	3·9	242	5·4	869	8	1462	16

W, body-weight; FFM, fat-free mass.
Mean values were significantly different from those of the corresponding control group: * $P < 0·05$, ** $P < 0·01$, *** $P < 0.001$.
† For details of individual experiments, see p. 438.

RESULTS

Expts 1–3. Body composition

Since the designs of these experiments were similar, their results are presented together. In Expts 1 and 3, one rat died during insulin treatment, presumably because of hypoglycaemia. In Expt 2 one insulin-treated rat was killed after developing proteinuria.

Insulin treatment significantly increased final body-weight and weight gain in all three studies (Table 3). Weight gain was raised by 57, 26 and 28% in Expts 1–3 respectively. Insulin also substantially increased the rate of deposition of carcass fat. This increase was 2·6-fold in both Expts 1 and 3 and 1·7-fold in Expt 2. In Expt 1 the insulin-treated rats also showed significantly higher rates of accretion of carcass water and DDM compared with the controls. In Expt 2 DDM gain was higher in the treated group than in the controls, but the difference in water gain did not reach statistical significance. The male rats in Expt 3 showed no increase in either water or DDM accretion after insulin treatment. In Expts 1–3, fat deposition accounted for 66, 63 and 103% respectively of the increased weight gain induced by insulin.

Expts 1–3. Energy balance

Metabolizable energy intake, calculated factorially from food consumption and expressed as kJ/d, was significantly increased by insulin treatment in each experiment (Table 4). The increases were 34, 18 and 29% in Expts 1–3 respectively. The differences were also highly significant when metabolizable intake was expressed relative to metabolic body size or fat-free mass. The rate of carcass energy gain was substantially higher in the treated groups than in the controls by 2·2-, 1·5- and 2·0-fold respectively in Expts 1–3.

Energy expenditure was expressed in three different ways for the three experiments. In all nine comparisons, the insulin-treated rats showed higher expenditures. However, the differences were significant in only two cases, i.e. in Expt 1 expressed as kJ/d (+13%; $P < 0{\cdot}05$) and in Expt 3 expressed as kJ/kg fat-free mass per d (+9%; $P < 0{\cdot}05$).

Expt 4. Tissue composition

The final weights and gains of the treated rats in Expt 4 were significantly increased by insulin (Table 5). Interscapular brown adipose tissue had a more fatty appearance in the treated rats and weighed significantly more than that in the controls. However, the protein content of this tissue was not altered by insulin, and cytochrome *c* oxidase activity did not differ significantly between the groups when expressed either per whole tissue or relative to protein content. Liver weight, protein content and total enzyme activity (U/tissue) were all significantly increased by 33–35%. Specific enzyme activity (U/mg protein) did not differ between the groups. None of the variables of muscle composition was affected by insulin treatment.

DISCUSSION

Rats injected with high doses of insulin die unless they eat sufficient carbohydrate to counteract hypoglycaemia (Lotter & Woods, 1977; Vasselli & Sclafani, 1979). Treated animals must, therefore, have continuous access to food. Acclimation to the diet is also required, since insulin treatment causes hyper-reactivity to novel or aversive feeds (Vasselli & Sclafani, 1979). In the present study an incremental-dose regimen was used to reduce stress and mortality. It is not certain that insulin-induced hyperhagia reflects any physiological mechanism of appetite control. Indeed, there is evidence that it is a pathological response to hypoglycaemia, accompanied by abnormal behaviour patterns including ataxia and reduced locomotion (Brandes, 1977). Mild behavioural changes of this type were also observed in the present studies.

Table 5. *Expt 4. Tissue composition in rats injected with insulin for 16 d and their controls*

(Values are means with their standard errors for six females per group)

	Treated		Control	
	Mean	SE	Mean	SE
Final body-wt (g)	263**	5·8	223	6·9
Wt gain (g/d)	8·7**	0·36	5·3	0·45
Interscapular brown adipose tissue				
Tissue wt (mg)	1779**	161	1036	49
Protein (mg)	128	12	102	5
COX: U/tissue	511	43	435	25
U/mg protein	4·08	0·33	4·32	0·45
Liver				
Tissue wt (g)	14·8*	1·2	11·1	0·4
Protein (g)	1·96†	0·15	1·45	0·07
COX: U//tissue	3940†	294	2928	162
U/mg protein	2·04	0·15	2·01	0·04
Gastrocnemius muscle				
Tissue wt (mg)	1330	69	1308	49
Protein (mg)	291	17	278	13
COX: U/tissue	138	15	136	8
U/mg protein	0·46	0·03	0·49	0·02

COX, cytochrome *c* oxidase activity (*EC* 1.9.3.1).
Mean values were significantly different from those of the control group: * $P < 0·05$, † $P < 0·02$, ** $P < 0·01$.

In agreement with previous work (Hausberger & Hausberger, 1958; Beaton *et al.* 1965), insulin substantially increased body fat content in the present experiments. However, in contrast with previous reports, increased protein and water accretion were observed with females. It is possible that the sex and age of the rats may have influenced this response: previous workers have in general used males at a higher weight than the animals described here. A further experiment in our laboratory, in which male rats weighing 90 g were given insulin, also showed no change in protein and water accretion (Amirthanayagam, 1985). Female rats also gain lean body mass after ovariectomy (Hervey & Hervey, 1981; Schemmel *et al.* 1982) or force-feeding (Harris *et al.* 1986; McCracken, 1986), but there is little evidence that lean body mass can be increased in males. However, McCracken (1986) has argued that overfeeding increases lean body mass in adult rats of both sexes, but not in younger animals. Further work is needed to resolve this question.

Insulin substantially raised food consumption and energy gain in Expts 1–3, but caused only small increases of energy expenditure. An increment in energy expenditure would normally be expected when growth rate is increased, because of the energy costs of tissue deposition. The efficiencies of deposition for carcass protein and fat in normal rats eating a carbohydrate-based diet are thought to be approximately 0·44 and 0·75 respectively (Pullar & Webster, 1977). Assuming that these factors are applicable during insulin treatment, it is possible to calculate the energy expenditures associated with the tissue deposition rates given in Table 3. On this basis insulin treatment would be expected to raise energy expenditure by 27, 15 and 26 kJ/d in Expts 1, 2 and 3 respectively. These predicted increments are in fact very close to the increases observed (see Table 4).

Voluntary locomotion appeared to be reduced in the rats treated with insulin, and this may have influenced overall energy expenditure. However, physical activity is thought to make only a minor contribution to total energy expenditure in small species such as the rat

(Miller & Mumford, 1966). Beaton *et al.* (1965) found a small and non-significant decrease in spontaneous running activity in insulin-treated rats fed on a diet with adequate protein. Rats consuming a protein-deficient diet showed high spontaneous running activity, and this was substantially reduced by insulin treatment. Torbay *et al.* (1985) achieved equal activities in insulin-treated and control rats by using sedation.

Cytochrome *c* oxidase activity was determined as an indicator of the capacity for substrate oxidation. No change in enzyme activity was found in either interscapular brown adipose tissue or gastrocnemius muscle. In liver, proportional increases of weight, protein content and oxidative capacity were observed. These may be adaptive changes enabling the metabolism of a higher food intake and its storage as fat. It has been estimated that the liver accounts for 20% of resting heat production in the rat (Webster, 1981). The increases of 33–35% observed in liver size and enzyme activity might, therefore, suggest an elevation of whole-body energy expenditure of about 7%. This value is of approximately the same magnitude as the observed increases in expenditure.

To our knowledge, there are no previous long-term measurements of energy expenditure in insulin-treated rats. However Beaton *et al.* (1965) measured resting oxygen consumption in rats after 7 and 14 d of insulin treatment. In each case small but non-significant increases were observed of similar magnitude to those found here. Moley *et al.* (1985) found no change in O_2 consumption in rats given insulin acutely. Low doses of insulin may reduce energy expenditure when food intake is limited to that of control animals (Torbay *et al.* 1985; Bartness *et al.* 1986). However, this change could be caused by the reduced lean body mass found under such conditions (Torbay *et al.* 1985).

Insulin has been implicated in the thermic effect of carbohydrate feeding in rats (Rothwell *et al.* 1985), but not in man (Christin *et al.* 1986). It may also increase O_2 consumption in skeletal muscle after exercise (Balon *et al.* 1984). The present findings do not preclude the possibility that normal or sub-normal insulin concentrations influence energy expenditure. It is clear, however, that sustained hyperinsulinaemia in rats does not alter energy expenditure more than would be predicted from consideration of growth and body composition.

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