The effect of phytohaemagglutinin at different dietary concentrations on the growth, body composition and plasma insulin of the rat

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Young growing rats weighing approximately 83 g were fed on diets containing kidney bean (*Phaseolus vulgaris*) lectin (phytohaemagglutinin, PHA) in the range of 0–0·45 g/kg body weight for 10 d to ascertain whether there was a minimum dose below which the lectin had no significant effect on body and skeletal muscle weights in comparison with pair-fed lectin-free controls. Averaged over all experiments, PHA doses of less than 10 mg/d (0·12 g/kg body weight) reduced body dry weight by 1·14 (SE 0·25) g when compared with controls. Between 10 and 27 mg/d (0·12–0·32 g/kg body weight) a further reduction of 0·64 (SE 0·21) g occurred, suggesting a slight but steady decline of body dry weight with increasing dose. However, above 27 mg/d the depression of growth and changes in body composition accelerated. The difference between the proportional losses of skeletal muscle and body weight was not significant at doses of PHA below 10 mg/d (0·12 g/kg body weight) but the ratio of these losses rose to 1·5–2·0 at doses above this. The proportional decrease in lipid weight exceeded that of both body and skeletal muscle weights at all lectin doses, suggesting that lipid catabolism was the first target of the PHA effect. Plasma insulin level was depressed at the PHA dose of 0·02 g/kg body weight at which growth depression and muscle atrophy were minimal but, contrary to expectations, plasma glucose levels remained stable over the whole PHA dose range. It appears that despite a PHA-induced lowering of blood insulin, glucose catabolism is elevated by an unknown, possibly hormonal, compensatory mechanism. Thus, because low insulin levels facilitate the mobilization and catabolism of lipids, it may be possible to use low doses of PHA to reduce hyperglycaemia and body fat.

Phytohaemagglutinin: Plasma: Insulin: Muscle: Body composition

Orally administered kidney-bean (*Phaseolus vulgaris*) lectin, phytohaemagglutinin (PHA), is a powerful extraneous growth factor for the rat gut, inducing fully reversible, polyamine-dependent, hyperplastic growth of the small intestine (Bardocz *et al.* 1992). The lectin binds avidly to the brush border and is partially transcytosed into the circulation (Pusztai, 1991). At high intakes (0·2–0·8 g/kg body weight per d), PHA damages the gut wall, causes coliform overgrowth in the lumen (Pusztai *et al.* 1993), increases the rates of lipid mobilization and glucose oxidation (Grant *et al.* 1987) and significantly reduces the fractional rate of protein synthesis in skeletal muscle (Palmer *et al.* 1987; Bardocz *et al.*

* For reprints.

1992). Thus, PHA is generally regarded as a nutritional toxin because at high oral doses it induces severe losses of body lipids, glycogen and muscle protein (Pusztai, 1991).

The swiftness of the metabolic shift towards catabolism on oral exposure to relatively high levels of PHA indicates that this may be due to rapid changes in the level of hormones such as insulin, and the demonstration that circulating insulin levels in rats fed on diets based on kidney bean were permanently depressed supported this view (Grant *et al.* 1987; Pusztai, 1991). However, possibly because of concomitant changes in the levels of other hormones, despite reduced insulin values, glucose concentration in plasma was not increased above normal levels and therefore the rats were not diabetic. However, as the transepithelial transport of many lectins is appreciable (Pusztai, 1989, 1991; Pusztai *et al.* 1993), it is also possible that the well-established *in vitro* insulin-mimicking effects of lectins (Cuetracasas & Teller, 1973; Pusztai & Watt, 1974; Livingstone & Purvis, 1980; Bardocz *et al.* 1993) made some contribution to the metabolic effects of bean diets.

Although PHA may exert some striking physiological effects at low daily doses (below 0·2 g/kg), these are less well-known. To the best of our knowledge, no systematic studies have been carried out to estabish the threshold levels of PHA below which the lectin can be regarded as non-toxic. Accordingly, the present study was undertaken using rats pair-fed for 10 d with diets containing different amounts of PHA and control diets to determine the concentration dependence of the effects of PHA on final body weight and composition (skeletal muscle and lipids), and to establish possibly safe, non-toxic threshold levels for the oral administration of PHA. A second objective was to determine whether circulating insulin levels were lowered at these low lectin concentrations. In these experiments the risk that results would be influenced by inadequate nutrition was minimized by using well-fed rats which were given, usually by oral administration, one single low dose of PHA or the same dose once daily for several days. This paper presents evidence that, in contrast to the damaging effects of PHA at high levels of inclusion in the diet, at lower daily doses (0·01–0·2 g/kg), loss of body weight and skeletal muscle atrophy are slight. However, as PHA can still exert its modulating effect on plasma insulin levels and lipid catabolism, the possible use of lectins for reducing obesity, hyperglycaemia and other similar metabolic disorders may now be explored.

MATERIALS AND METHODS

PHA was purified by affinity chromatography on Sepharose 4B-fetuin using the method of Pusztai & Palmer (1977) with some improvements (Carvalho, 1992). Briefly, kidney bean proteins were extracted with 0·05 M-sodium borate buffer of pH 8·0 and separated into globulins and albumins by dialysis against 0·033 M-sodium acetate buffer, pH 5·0. E-type PHA (erythroagglutinating) fractions were adsorbed on to Sepharose 4B-fetuin at pH 7·6 (0·05 M-Tris-HCl) and released by desorption with 0·05 M-glycine-HCl buffer, pH 3·0, also containing 0·5 M-NaCl, followed by dialysis and freeze-drying. For the purification of L-type (lymphoagglutinating) PHA, after the removal of small amounts of E-type PHA from the albumins by Sepharose 4B-fetuin, the non-absorbed fraction was fractionated on a sulphopropyl cation exchange HPLC column (TSK SP-5PW, 21·5 mm × 150 mm; Anachem GB Ltd) in 0·005 M-sodium acetate–acetic acid buffer, pH 3·8 containing 0·1 M-NaCl and eluted by a programmed increasing ionic strength gradient (0·1–0·5 M-NaCl). Finally, lower molecular weight impurities were removed by chromatography on Sephadex G-100, and pure L-type PHA was recovered after dialysis and freeze-drying. Recovery was 0·32 g and 0·61 g E-type and L-type PHA respectively per 100 g kidney-bean meal.

Iodination of PHA was carried out with ^{125}INa with iodobeads (Kilpatrick *et al.* 1985). Specific activities were 3·6 and 4·8 × 10^6 counts/min for E- and L-type PHA respectively.

Animal management

All management and experimental procedures in the present study were carried out in strict accordance with the requirements of UK Animals (Scientific Procedures) Act 1986 by staff licensed under this Act to carry out such procedures.

Expt 1

Three separate experiments (1a, b, c) were carried out to the same design. Rats weaned at 19 d were maintained on stock diet for 11 d and fed on a control, lactalbumin-based diet (LA-diet; Table 1) for 3 d to reach 82–84 g starting weight. The rats were then selected into groups of five rats according to body weight and within each group they were allocated at random to treatment. The rats were individually housed in the same animal room for the duration of the experiment. The experiment started on the same day for all treatment groups and rats in each group were given daily 6 g diet/rat in the morning (within less than 1 h) of the LA-diet or diets based on the LA-diet with different levels of PHA inclusion so that their daily intake was between 0·65 and 42 mg/rat (0·007 and 0·45 g/kg body weight). After 10 d the rats were fed with 2 g of the respective diets in the morning and killed precisely 2 h later (6 min interval between rats). Gastrocnemius muscles were excised and rinsed, and both bodies and muscles were freeze-dried and weighed. In Expt 1c bodies were ground to a powder and extracted with chloroform–methanol (2:1, v/v) for lipid determination.

Expts 2–5

In Expts 2–5 the insulin response of rats to PHA was tested. In Expt 2, rats were fed on diets providing 42 mg PHA/d and blood insulin levels were measured after 9 and 10 d respectively. In the other three experiments the short-term effects on insulin levels of a single dose of albumin proteins (Expt 3) or purified PHA (Expt 4) were measured in control-fed or in PHA-fed rats (Expt 5). Individually housed male Hooded Lister specific-pathogen-free rats weaned at 19 d were maintained *ad libitum* on stock diet (Special Diet Services, Manea, Cambs.) for about 14 d, followed by restricted feeding (8 g/rat per d) for 5 d on LA-diet before their use in the experiments. During a 5 d adaptation period of training of the rats to get accustomed to handling, which was necessary for blood collection without stressing the animals, the rats were handled daily and wrapped in a towel for short periods of time. During this period the rats were fed three times daily: 2·5 g at 09.00 hours, 1·0 g at 13.00 hours and 4·5 g at 18.00 hours. On the 5th day the rats were given 1·5 g LA-diet between 09.00 and 09.30 hours and pre-experimental blood samples were taken from the tail vein 2 h later. The same protocol was also used for subsequent samples during the experiments. The tail of the rats was left uncovered and immersed in warm water to increase the circulation. The tip of the tail (about 2 mm) was locally anaesthetized with a cold spray (Freez-it; Chemtronics, Inc., Hauppauge, USA) and cut off using a sterile scalpel blade. Blood was collected in heparinized tubes (25 μl heparin solution containing 26 USP units/tube) and centrifuged in a bench-top centrifuge at +1° for 15 min. Plastic granules were used to aid the separation of plasma from erythrocytes; the plasma was then divided into 100 μl portions and stored at −20° until assayed.

Expt 2. In the morning of the 5th day of the adaptation period the rats were given 1·5 g LA-diet between 09.00 and 09.30 hours and pre-experimental blood samples were taken from the tail vein exactly 2 h later. The rats were then randomly divided into two groups of thirteen animals and individually housed for the experiment. Group 1 was fed exclusively on a diet containing kidney bean (KB-diet; Table 1) for 10 d (8 g diet/rat per d, divided

Table 1. *Composition of diets (g/kg diet)*

	Lactalbumin (LA)	Kidney bean (KB)	KB albumins (KBA)
Maize starch	373	177	372
Potato starch	100	100	100
Glucose	150	150	150
Maize oil	150	150	150
Vitamin mix*	50	50	50
Mineral mix*	50	50	50
Lactalbumin	127	63	102
Kidney-bean meal	—	260	—
Kidney-bean albumin	—	—	26
L-Methionine	—	2·1	0·9
L-Tryptophan	—	0·25	0·13
Silicic acid	0·4	0·4	0·4

* For composition of vitamin and mineral mixes, see Carvalho (1992).

into three meals; 2·5 g at 09.00 hours, 1 g at 13.00 hours and 4·5 g at 18.00 hours) while the control group was pair-fed on LA-diet under the same conditions. On the 9th day, blood samples were taken in the morning exactly 2 h after the morning feed of 1·5 g KB-diet and this protocol was repeated on the 10th (last) day of the experiment, after which the rats were killed under diethyl ether anaesthesia, their abdomens were cut open and the rest of their blood was collected from the heart. The gatrointestinal tract and the pancreas were removed and after a quick rinse with ice-cold water they were frozen in liquid N_2. Control rats were treated the same way except that they were given 1·5 g LA-diet before the blood samples were taken. Plasma samples were kept frozen until assayed for insulin.

Insulin was extracted from the pancreas (six randomly selected rats of each group) after homogenization of a sample of this tissue (about 25–50 mg dry weight) with 10 ml acidified ethanol (ethanol–water–18 M-H_2SO_4; 96:18:2·5, by vol.) overnight in a cold room and then centrifuged at 1500 *g* for 10 min in the cold. The clear supernatant fractions were diluted to about 1:200 (v/v) with insulin assay buffer before including them in the insulin radioimmunoassay.

Expts 3 and 4. Rats were weaned at 19 d, kept in groups of eight to ten rats and fed on stock diet for 12 d. They were then randomly selected into two groups (thirteen rats in each group), individually housed for the rest of the experiment and fed on stock diet for another 8 d. Rats, after fasting overnight, were given 2 g stock diet in the morning and 2 h later blood-sampled (pre-experimental sample). Immediately after this the rats in Expt 3 were intragastrically intubated with a 1 ml extract of kidney bean (50 mg; 5–7 mg PHA) while the controls were dosed with 1 ml 0·01 M-phosphate buffered saline (9 g NaCl/l; PBS). Blood samples were obtained from each animal at 15, 60 and 120 min after the intubation. Expt 4 was done the same way as Expt 3 but the test animals were administered a 1 ml solution of either 5 mg E-type or L-type lectins. Some of these lectin samples were labelled with ^{125}I (total counts of 2·5–3 million counts/min). The controls received PBS. Blood samples were taken at 0, 15, 60 and 120 min as before. To measure the actual amounts of PHA delivered into the duodenum, radioactivity was measured in both stomach and small intestinal washes in some rats killed 1 or 2 h after intubation.

Expt 5. Sixteen rats weaned at 19 d and housed individually during the experiment were fed on stock diet for 15 d, followed by 5 d on LA-diet (8 g diet/rat per d). Twelve randomly selected rats were then switched to a diet containing kidney-bean albumin (KBA-

diet; Table 1) for the next 3 d, with a daily intake of about 30 mg PHA/rat, while four control rats continued with the LA-diet. In the evening of the 3rd day, instead of the evening meal, the rats on KBA-diet were intragastrically intubated with a 1 ml solution of 100 mg kidney-bean albumin sample containing 25 mg PHA, while the controls were given the evening portion of their LA-diet. After this the animals were not fed again until the following morning when they were all given 2 g LA-diet to boost their plasma insulin level. Exactly 2 h later the rats were blood-sampled (pre-experimental sample), those which had been pre-fed on kidney-bean albumin were randomly selected into groups of four and were administered 1 ml solutions of either 20 mg kidney-bean albumins, or 5 mg E-type or 5 mg L-type PHA lectins, some of which were ^{125}I-labelled. The four control rats were administered kidney-bean albumin (40 mg; 4–6 mg PHA) for comparison. Rats were blood sampled at 120 min, killed and the pancreas removed and frozen for insulin assays.

Insulin assay

Immunoreactive plasma insulin concentrations were measured using a double-antibody precipitation technique (MacRae *et al.* 1991) and a rat insulin standard (Incstar Corporation, Stillwater, MN, USA). ^{125}I-labelled bovine insulin, 0·185 MBq/0·1 μg (ref. IM38), was supplied by Amersham International plc (Amersham, Bucks.) and antiserum to porcine insulin raised in guinea-pigs by Miles Scientific (Stoke Poges, Slough, Berks.). Anti-guinea-pig immunoglobulin G (IgG) serum and normal guinea-pig serum were from the Scottish Antibody Production Unit (Law Hospital, Carluke, Lanarkshire).

Assay method

The tracer:antibody ratio was adjusted to a total binding of 30–40%. Samples (100 μl) were incubated overnight at room temperature (RT) with 500 μl anti-porcine insulin serum (diluted 1:37 500), followed by the addition of 100 μl ^{125}I-labelled insulin (diluted 1:20) and incubated for a further 4 h at RT. After the addition of 500 μl of second antibody solution (670 μl anti-guinea-pig IgG serum and 150 μl normal guinea-pig serum diluted with 50 ml assay buffer) the mixture was kept at +4° overnight, centrifuged in a Damon/IEC DPR-6000 centrifuge at 1600 ***g*** for 20 min and the bound fraction was counted by a Cobra auto-gamma counter, model no. 5010, equipped with a PC compatible computer (Packard Instrument Co., IL, USA).

Standard curves were prepared for every radioimmunoassay. Total binding capacity (counts/min in the insulin-free tube) and non-specific binding (in the antiserum-free tube) were determined. The total specific binding (100%) was defined as total binding minus non-specific binding. Specific binding of the sample was measured as counts/min in the sample tube minus the non-specific binding value. Logit b, which equals $\log_e (b/100 - b)$, where b is the proportion of tracer bound expressed as a percentage of that in the zero standard, was calculated and a standard curve of logit b *v.* log insulin concentration was plotted. The goodness of fit of straight line through the points was judged from the correlation coefficient (*r* value) of a linear regression. After calculation of logit b of the sample its insulin concentration was determined from the standard curve.

The means sensitivity of the assay measured as 2 SD of the zero standard was $8{\cdot}97 \pm 2{\cdot}18$ pmol/l. The inter-assay coefficient of variation for a bulk plasma sample assayed ten times over a 6-month period was 9·4%. The intra-assay coefficient of variation was 4·6% for eight replicates of the same sample.

Plasma glucose

Concentrations of glucose in plasma samples were measured by the autoanalyser method of Trinder (1967).

Statistical analysis

For each of Expts 1a, 1b and 1c the data were subjected to ANOVA to yield the means and pooled standard errors. Thereafter the means for each treatment were expressed as a proportion of the control for the same experiment and these 'relative' dry body weights (excluding control groups) were related to the logarithm of the PHA dose. This method is similar to omitting the control (zero dose) means and relating dry body weights to the logarithm of the PHA dose on a within-experiment basis, but it ignores the error in the estimates of the control means. Because of the known damage to the gut wall and consequent coliform overgrowth at high PHA doses, it was anticipated that the response would be biphasic linear with a join point in the region of 25 mg PHA/d (curve fitting in fact suggested a join at 27 mg PHA).

In the present paper, unless otherwise stated, body and muscle weights are dry weights with the exception of the starting weights of rats and the PHA dose expressed as g PHA/kg body weight in which they are live weights.

For Expts 2–5 the post-treatment insulin levels were expressed as a percentage of the pre-treatment levels and the means were compared with 100%. Unless otherwise stated, all significance tests were performed at the $P = 0{\cdot}05$ probability level.

RESULTS

Expt 1

The mean body weights for the control groups in Expts 1a, b and c were very similar lying between 23·2 and 24·0 g. Feeding rats with diets containing PHA in the range of 0–42 mg/rat per d (0–0·45 g/kg body weight) reduced their body weight in a biphasic manner (Fig. 1; Tables 2 and 3). There was a small reduction in body weight even at low levels of PHA (e.g. 4% at 3·5 mg/rat per d; 0·04 g/kg body weight), after which relatively large increases in the lectin dose (to about 0·32 g/kg) produced only modest further reductions in the body weight. Thus, averaged over all the experiments at doses below 10 mg PHA/d (0·12 g/kg body weight) the mean body-weight reduction was 1·14 (SE 0·25) g when compared with control (4·9% of control body weight). At daily doses of PHA between 10 and 27 mg (0·12 and 0·32 g/kg body weight) there was a further reduction of 0·64 (SE 0·12) g (2·7% of control body weight). However, at higher doses (0·20–0·45 g/kg body weight) the reduction became more appreciable. The relationship between relative body dry weight (as proportion of zero dose control; RBDW) and the PHA dose expressed as mg/d from three separate feeding trials (Expts 1a, b and c) below PHA dose of 27 mg/d was:

$$\text{RBDW} = 0{\cdot}918\ (\text{SE}\ 0{\cdot}008) - 0{\cdot}0334\ (\text{SE}\ 0{\cdot}0062) \times \log_{10}(\text{dose PHA}/27).$$

Above this PHA dose the equation was:

$$\text{RBDW} = 0{\cdot}918\ (\text{SE}\ 0{\cdot}008) - 0{\cdot}5138\ (\text{SE}\ 0{\cdot}0876) \times \log_{10}(\text{dose PHA}/27).$$

Changes in the dry weight of the gastrocnemius (skeletal) muscles followed a similar trend with increasing lectin input (Tables 2 and 3). The proportional loss of muscle weight compared with control rats tended to be about 1·5–2·0 times that of the equivalent loss of body weight (Tables 2 and 3), but at daily doses of less than 10 mg PHA (0·12 g/kg body weight) the difference between the proportional loss of body and muscle weights was not significant ($P > 0{\cdot}05$).

Similar to the reduction in body and muscle weights, the lipid content of the body of rats was reduced by increasing the dose of PHA in the diet (Table 3; Expt 1c). However,

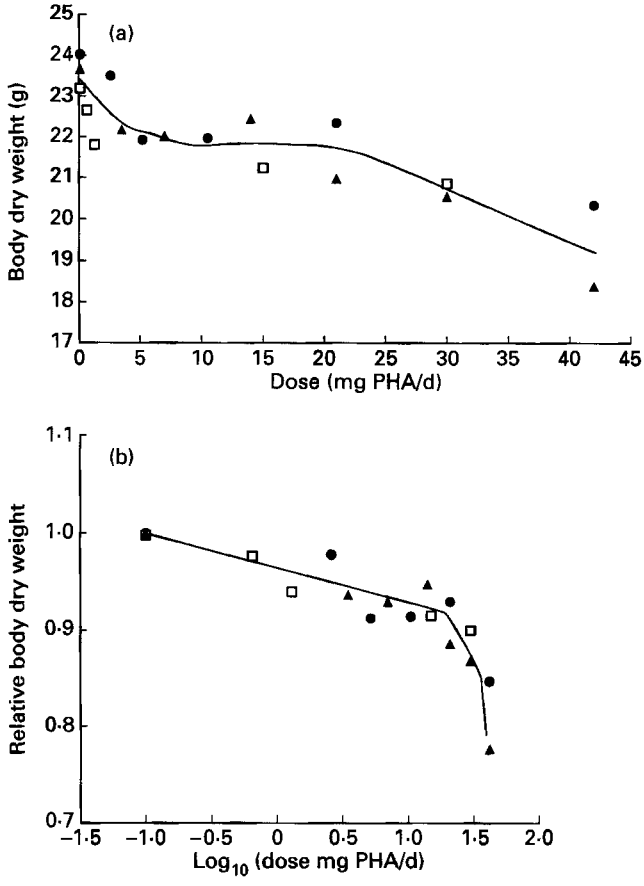

Fig. 1. Expt 1a, b, c. Dry body weights of rats fed on diets containing different amounts of phytohaemagglutinin (PHA) for 10 d. Results are expressed as (a) body dry weight *v.* mg PHA/d and (b) relative body weight *v.* $\log_{10}$ (mg PHA/d). (●), Expt 1a; (□), Expt 1b; (▲), Expt 1c. For details of diets and procedures, see Table 1 and pp. 614–615.

proportionally the lipid loss exceeded that of both the body and skeletal muscle although the ratio of the losses remained roughly constant for all doses.

Expt 2

Feeding rats with KB-diet providing 42 mg PHA/rat per d (0·45 g/kg body weight) for 10 d significantly reduced the plasma insulin concentration from the pre-experimental level of 2·97 (SD 0·84) to 0·36 (SD 0·05) ng/ml on the 9th day of the experiment. The depression was apparently permanent during PHA exposure because blood samples taken on the 10th day were similarly low, 0·23 (SD 0·06) ng insulin/ml. In contrast, the plasma insulin levels in controls remained high, 3·31 (SD 0·30) and 1·55 (SD 0·21) ng/ml on the 9th and 10th days of the feeding respectively.

The absolute and relative dry weights of the pancreas of rats fed on KB-diet at the highest dose of PHA for 10 d were significantly increased in comparison with pair-fed controls (Expt 2; Table 4). In contrast, the insulin content of the pancreas expressed either as μg/pancreas or μg/g protein was significantly decreased (Table 4).

Despite the highly significant reduction in insulin levels, plasma glucose concentrations

Table 2. *Expts 1a and 1b. Body weights and gastrocnemius muscle weights of rats fed on diets containing different levels of phytohaemagglutinin (PHA) for 10 d**

(Mean values for five rats per group)

Dose of PHA (mg/rat per d)	Body dry weight		Gastrocnemius dry weight	
	g	Loss *v.* control (%)	g	Loss *v.* control (%)
Expt 1a				
0	24·0	—	0·184	—
2·6	23·5	2·2	0·183	0·7
5·2	21·9	8·7	0·166	9·7
10·5	22·0	8·5	0·155	15·5
21·0	22·4	7·0	0·153	16·5
42·0	20·4	15·2	0·129	29·8
SED	0·61		0·0074	
Expt 1b				
0	23·2	—	0·178	—
0·6	22·7	2·3	0·174	2·1
1·3	21·8	6·0	0·161	9·4
15·0	21·2	8·4	0·151	15·3
30·0	20·9	10·0	0·143	19·9
SED	0·35		0·0039	

* For details of diets, see Table 1.

Table 3. *Expt 1c. Body composition of rats fed on diets containing different levels of phytohaemagglutinin (PHA) for 10 d**

(Mean values for five rats per group)

PHA dose (mg/rat per d)	Body dry weight (BDW)		Gastrocnemius dry weight		Lipid weight (LW)		Total muscle dry weight (TMDW)†	Other dry weight (OW)‡	Ratio loss of gastrocnemius % *v.* loss of lipid (%)
	g	Loss *v.* control (%)	g	Loss *v.* control (%)	g	Loss *v.* control (%)	g	g	
0	23·2	—	0·196	—	4·18	—	9·27	9·75	
3·5	22·2	4·3	0·188	4·1	3·90	6·7	8·89	10·39	0·61
7·0	22·0	5·2	0·184	6·1	3·86	7·7	8·70	9·46	0·79
14·0	22·4	3·4	0·185	5·6	3·56	14·8	8·75	10·14	0·38
21·0	21·0	9·5	0·167	14·8	3·39	18·9	7·90	9·69	0·79
30·0	20·6	11·2	0·154	21·4	2·82	32·5	7·28	10·46	0·66
42·0	18·4	20·7	0·134	31·6	2·25	46·1	6·39	9·75	0·69
SED	0·47		0·0061		0·17				

* For details of diets and procedures, see Table 1 and pp. 614–615.

† Based on the assumptions: (1) gastrocnemius dry weight/TMDW is the same for all treatments, and (2) TMDW/BDW = 0·4 for the control group.

‡ Calculated by subtracting the sum of LW and TMDW from the total weight.

Table 4. *Expt 2. Weight and insulin content of pancreas of rats fed with diets containing kidney beans or lactalbumin (control) for 10 d*†

(Mean values and standard deviations for six animals per group)

	Pancreas dry wt				Insulin			
	mg		mg/kg body wt		μg/pancreas		μg/g protein	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Kidney-bean diet	162**	20	9820**	1220	22·38**	7·63	182**	80
Lactalbumin diet	138	16	6050	630	40·60	12·71	354	152

** Mean values were significantly different from those for the lactalbumin diet, $P < 0.01$.
† For details of diets and procedures, see Table 1 and pp. 614–617.

were not significantly altered in kidney-bean-fed animals with an overall mean value of 1·7 (SD 0·1) mg glucose/ml for both treated and control rats.

Expt 3

In acute experiments a single dose of a soluble kidney-bean protein sample caused a gradual decrease in plasma insulin concentration. The pre-experimental value of 1·78 (SD 0·22) ng insulin/ml plasma decreased to 1·05 (SD 0·22) ng/ml after 120 min, i.e. about 59% of the initial value (Table 5). In control rats the insulin level remained roughly constant within experimental errors at all time points (1·76 (SD 0·42) ng/ml).

Expt 4

In rats intubated with pure E-type PHA the pre-experimental plasma insulin levels were also decreased in the first 60 min to 1·03 (SD 0·32) ng/ml in a similar way to that found in animals administered kidney-bean proteins (Table 5). However, there appeared to be a slight recovery in the next 60 min which rendered the change in insulin level at 120 min not significantly different ($P > 0.05$) from the pre-experimental value. A single dose of L-type PHA also appeared to cause a gradual reduction in plasma insulin but the changes were not significant at any of the time points during the 120 min of the experiment (1·39 (SD 0·35) ng/ml at 120 min). The rates of stomach emptying in rats intubated with the lectins were slow and not significantly different for the two types of PHA ($P > 0.05$). With E-type about 52% of the initial dose reached the small intestine after 120 min, while with L-type PHA this was slightly more, about 63%. Plasma glucose levels were slightly reduced on acute exposure to PHA and/or kidney-bean albumins from 1·6 (SD 0·2) to 1·4 (SD 0·2) mg/ml but the reduction was not significant ($P > 0.05$).

Expt 5

Intragastric intubation with a single dose of purified PHA isolectins of rats pre-fed with diets containing kidney-bean albumin proteins or control diet and intubated with doses of PHA for 3 d substantially reduced the concentration of plasma insulin in the circulation. With both E-type and L-type lectins the difference in insulin levels between pre-experimental (0·59 (SD 0·05) ng/ml) and 120 min values (0·15 (SD 0·09) ng/ml) was significant ($P < 0.05$). Pre-feeding also appeared to speed up the rate of stomach emptying of intragastrically administered E-type lectins as over 80% of the dose reached the duodenum in the first 60 min. In contrast, L-type lectins were still slow to reach the duodenum and after the first

Table 5. *Expts 3 and 4. Relative insulin levels (expressed as a percentage of control) in rats after acute intragastric exposure to kidney-bean proteins or purified E-type or L-type lectins*†

(Mean values and standard deviations for thirteen rats per group)

Time (min)	Expt 3				Expt 4			
	Control		Kidney-bean protein		E-type		L-type	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
15	109	36	82	34	76*	27	98	32
60	97	36	65*	21	58*	23	86	34
120	89	28	59*	15	80	39	81	32

* Mean values were significantly different from 100 ($P < 0.05$). Differences between means for different treatments may be tested for significance using approximate t tests (see e.g. Cochran & Cox, 1958).

† For details of procedures, see pp. 614–617.

60 min about 50% of the initial amount of the PHA remained in the stomach. Despite the differences in the absolute plasma insulin concentrations between rats pre-fed on KBA-diet (low) and those kept on a control diet (moderately high), after administration of kidney-bean albumin proteins the proportional decrease in plasma insulin was similar in both groups of rats. The insulin content of the pancreas was slightly but not significantly reduced after the 3 d pre-feeding with KBA-diet from 58·3 (SD 10·8) in controls to 42·5 (SD 8·3) μg insulin/pancreas. However, there were no significant changes in the pre-experimental mean plasma glucose levels of about 1·6 (SD 0·2) mg/ml, although the values decreased slightly during the experiment. There were no significant changes in body and muscle weights after exposure to PHA for 3 d.

DISCUSSION

PHA is widely regarded as a nutritional toxin because rats fed exclusively on kidney-bean proteins containing high levels of PHA (0·8–1·0 g/kg body weight) die within a few days (Pusztai, 1991). However, as PHA is not harmful for germ-free rats its toxic effects in rats with a conventional microflora are likely to be the consequence of the dramatic *Escherichia coli* overgrowth in the small-intestinal lumen which is negligible below 0·2 g/kg but rises sharply in proportion with the increase in the level of PHA in the diet (Pusztai *et al.* 1993). In the present paper, evidence is presented that in the low concentration range where no bacterial overgrowth occurs (below 0·2 g/kg) the antinutritive effects of PHA are also slight in rats harbouring a conventional microflora, at least by the simple criterion that reduction in body weight is minimal after a 10 d exposure to the lectin. Moreover, in contrast to muscle atrophy observed at high doses (0·45 g/kg and above), reduction in skeletal muscle weights below PHA doses of 0·10 g/kg body weight was also slight and proportional to loss in final body weight (Tables 2 and 3).

With the assumptions that (1) the ratio of dry weight of the gastrocnemius muscle to total skeletal muscle dry weight (TMDW) was the same for all treatments and (2) that TMDW constituted 40% of the total body weight for the control treatment, it was possible to obtain an estimate that TMDW = 47 × dry weight of gastrocnemius muscle. Using this estimate the total body weight could be partitioned into three components: lipids, muscle and 'other' and the relationship between them and TMDW could then be assessed at different PHA doses (Table 3). For Expt 1c where lipid weights (LW) were assessed, plots

of LW, TMDW and 'other' weights (OW) against the body dry weight (BDW) showed near linear relationships described by:

$$LW = -5{\cdot}26\ (\text{SE}\ 1{\cdot}19) + 0{\cdot}406\ (\text{SE}\ 0{\cdot}056) \times BDW,$$
$$TMDW = -5{\cdot}42\ (\text{SE}\ 1{\cdot}17) + 0{\cdot}635\ (\text{SE}\ 0{\cdot}055) \times BDW,$$

then by difference we get:

$$OW = 10{\cdot}68 - 0{\cdot}041 \times BDW.$$

Although the relationships between LW, TMDW and DBW were linear, the plots had negative intercepts. Moreover, relative to the control, the proportional loss of lipid was higher than the proportional loss of muscle although the ratio between them remained roughly constant (Table 3), confirming previous results that the first effect of the lectin is a stimulation of body lipid catabolism (Grant *et al.* 1987).

The previously suggested link between the strong catabolic effects of high doses of PHA on body metabolism of lipids, carbohydrates and proteins and its lowering of plasma insulin levels (Pusztai, 1991) has now been confirmed. In fact, insulin levels were depressed not only in the blood circulation during the 10 d oral exposure of rats to PHA but also the insulin content of the pancreas was significantly reduced in these animals.

Insulin levels in the circulation are known to be strongly influenced by the state of nutrition of the animals, the protein content and quality of the diet and the time between the last feed intake and blood sampling. Although most of these factors were strictly standardized it is still conceivable that the plasma insulin was low because bean proteins are nutritionally of poor quality and/or that PHA may have interfered with the absorption of dietary proteins in the small intestine. However, E-type PHA significantly lowered insulin levels at very low luminal lectin concentrations (below 0·02 g/kg body weight; calculated from ^{125}I-labelled PHA left in the stomach) in acute experiments with well-fed rats. In fact it is possible that the metabolic consequences of this interference with insulin levels, and possibly other endocrine hormones, are responsible for the relatively large (about 4%) apparent loss of body weight at relatively low PHA inclusion levels in the diet such as 0·04 g/kg body weight, followed by a disproportionately low response (less than doubling of the weight loss of that at 0·04 g PHA/kg) to an 8-fold increase in the PHA dose. At these low doses toxic effects of PHA are expected to be minimal and nutritional influences on plasma insulin must be marginal, suggesting that PHA probably acts as a metabolic signal. However, as epithelial cell receptors in the proximal small intestine, including those of enteroendocrine cells (King *et al.* 1980, 1986), are saturated at the lowest PHA inclusion levels used in the present study, only slight further metabolic effects could be expected after the initial changes even at substantially increased PHA doses. Although the reduction was only significant with E-type PHA in acute experiments, the insulin-lowering effect of L-type PHA also became significant in rats which had been prefed with PHA diets for 3 d before intubation with this lectin.

The level of insulin in the plasma is dependent on its rates of synthesis and secretion by the pancreas and the speed of clearance from the circulation. The insulin content of the pancreas was unchanged by a single dose of PHA and therefore the depression of plasma insulin was unlikely to be due to a PHA-induced acceleration of the rate of degradation in the circulation because increased secretion from the pancreas should have quickly restored it to normal levels. It is more likely that in the short term PHA interfered with the secretion of insulin from the pancreas by some unknown mechanism. In contrast, PHA also interfered with the synthesis of insulin in the islets under chronic conditions, reducing both its content and concentration in the tissue (Table 4). This is more significant in the light of the growth of the pancreas under these conditions.

The mechanism of the interference by PHA with the synthesis and/or secretion of insulin is not clear at present. However, it is possible that although the rapid PHA-induced growth of the pancreas affects mainly acinar tissues (Pusztai, 1991), this may also have secondary, indirect effects on the islets and their insulin content. As other pancreatic hormones have, in the past, been suggested to be affected by PHA (Grant *et al.* 1987), their paracrine effects may interfere with insulin release (Samols *et al.* 1986). PHA is also known to bind to small-intestinal endocrine cells (King *et al.* 1986) and therefore its effects may be mediated indirectly by hormones (CCK) released from these cells into the systemic circulation. Although it has been suggested that most gastrointestinal peptide hormones such as CCK, vasoactive intestinal peptide, gastric inhibitory peptide and others actually stimulate insulin release, a few neurotransmitters and hormones, such as the calcitonin-gene-related peptide, opioids, Substance P and pancreastatin, can inhibit insulin secretion (Ashcroft & Ashcroft, 1992). Accordingly, if any of these are released by PHA, they can selectively, or in combination, affect the functioning of the endocrine pancreas.

PHA is able to cross the gut wall and reach the systemic circulation (Pusztai *et al.* 1989; Pusztai, 1989). By binding to insulin receptors of cell membranes (Bardocz *et al.* 1993), PHA can mimick insulin effects *in vitro* (Pusztai & Watt, 1974). However, dependent on the glycosylation of the insulin receptor, PHA may not stimulate the cell but rather act as an antagonist. Thus, it is not possible to predict at present whether the systemically absorbed PHA can directly affect body metabolism or not.

Rats exposed to PHA were not diabetic but, by increasing the metabolic utilization of glucose, maintained euglycaemia. With the availability of excess glucose due to the low circulating insulin levels, the body's glycogen stores were expected to remain at normal levels or increase. In fact, as shown before, the opposite happened and the glycogen content of the liver was halved on prolonged feeding with KB-diet (Grant *et al.* 1987). However, the overall mechanism of regulation of glycaemia is complex and involves many hormones in addition to insulin: amongst others glucagon, cortisol, adrenaline, growth hormone, thyroid hormone, vasoactive intestinal peptide and somatostatin. In fact, as most of these hormones are also involved in the regulation of lipid metabolism, stimulation of lipid catabolism may be one of the means to provide substrates for gluconeogenesis and maintain functional glycogen levels in the liver. As very little is known about the changes in the levels of these hormones during PHA exposure, speculation concerning their involvement in the maintenance of stable blood glucose levels is clearly unprofitable for the time being. However, the possible contribution to glucose homeostasis of the experimentally well-established (Knott *et al.* 1992) upregulation of the levels of both insulin receptor and insulin-responsive glucose transporter, GLUT-4, manifests clearly how effectively the organs and tissues of the body can respond to changing physiological conditions (Gould & Bell, 1990).

Although the precise mechanism of the PHA effect on insulin and glucose levels is unknown at present, the evidence presented in the current paper shows that homeostasis can be maintained in rats fed on diets containing essentially non-toxic (or slightly antinutritive) levels of PHA. The nutritional importance of this is further underlined by recent findings which showed that most changes, including the PHA-induced dramatic growth of the gut and pancreas, are reversed after the removal of the lectin from the diet (Bardocz *et al.* 1995). Accordingly, as it is dangerous to extrapolate from one species to another, it may now be interesting to explore whether it is possible to use low doses of PHA safely for the reduction of hyperglycaemia and perhaps even obesity in other animal species, including possibly humans. This is clearly based on the findings presented here which suggest that appreciable mobilization of body fat is possible under conditions when changes in the body and skeletal muscle weights are slight. Furthermore, the need to replace

glucose and glycogen which are increasingly utilized because of a probable shift in the endocrine hormone balance of the body may also make a contribution to the total lipid loss. However, the end result of using up excess glucose (carbohydrates) at the expense of body fat by the oral administration of low doses of PHA presents a potentially more effective and less dangerous pharmacological means of reducing hyperglycaemia and obesity than the suggested use of inhibitors of α-amylase and/or α-glucosidase (Bischoff, 1994) which, because of the accumulation of undigested starch, will lead to the blockage of passage of the digesta in the gastrointestinal tract (Pusztai *et al.* 1995).

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