

Antinutritive effects of wheat-germ agglutinin and other N-acetylglucosamine-specific lectins

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Incorporation of N-acetylglucosamine-specific agglutinins from wheat germ (*Triticum aestivum*; WGA), thorn apple (*Datura stramonium*) or nettle (*Urtica dioica*) rhizomes in the diet at the level of 7 g/kg reduced the apparent digestibility and utilization of dietary proteins and the growth of rats, with WGA being the most damaging. As a result of their binding and endocytosis by the epithelial cells of the small intestine, all three lectins were growth factors for the gut and interfered with its metabolism and function to varying degrees. WGA was particularly effective; it induced extensive polyamine-dependent hyperplastic and hypertrophic growth of the small bowel by increasing its content of proteins, RNA and DNA. Furthermore, an appreciable portion of the endocytosed WGA was transported across the gut wall into the systemic circulation, where it was deposited in the walls of the blood and lymphatic vessels. WGA also induced the hypertrophic growth of the pancreas and caused thymus atrophy. Although the transfer of the gene of WGA into crop plants has been advocated to increase their insect resistance, as the presence of this lectin in the diet may harm higher animals at the concentrations required to be effective against most pests, its use in plants as natural insecticide is not without health risks for man.

Lectins: Antinutrients: Small intestine: Rat: Insect resistance

Lectins are generally recognized as the major antinutrient of food (Pusztai, 1991). Thus, lectins from the seeds of kidney bean (*Phaseolus vulgaris*; phytohaemagglutinin; PHA), the soya-bean agglutinin (SBA) and several others, which bind to and are endocytosed extensively by the epithelial cells of the small intestine, are nutritionally toxic for most animals (Pusztai, 1991). However, recently several lectins have been described which were only slightly deleterious for rats. Thus, diets containing the mannose-specific agglutinin from snowdrop (*Galanthus nivalis*) bulbs (GNA; Pusztai *et al.* 1990) or the mannose/glucose-specific lectin from the seed of *Vicia faba* (VFL; Rubio *et al.* 1991), were essentially non-toxic. Apparently, as membrane glycans of the brush-border epithelium contained only few saccharide chains with non-reducing terminal mannose or glucose residues, lectins with specificities for these sugars passed through the gastrointestinal tract of higher animals without binding to their brush border or inducing any damage to epithelial cells. In contrast, artificial diets into which some of these non-toxic lectins were incorporated, were highly toxic for insects *in vitro*. Thus, expectations have been raised that by utilizing differences in reactivity of the gut epithelia between species from higher and lower animals, the insect-resistance of major crop plants could be safely improved by the introduction of suitable lectin genes into the plant genome (Boulter *et al.* 1989).

Recently, it has been shown that the agglutinin from the wheat germ (WGA) has high anti-insect activity *in vitro* (Czapla & Lang, 1990; Murdock *et al.* 1990; Huesing *et al.* 1991a) and, therefore, the transfer of its gene into crop plants has been suggested to

increase their insect-resistance. However, previous limited studies have already shown that WGA causes some damage to the small intestine of rats (Pusztai *et al.* 1992), indicating that its nutritional and gastroenterological properties will have to be thoroughly tested in single-stomached animals before its use as a natural insecticide could be considered safe. Thus, as a first step towards this, the main aim of the present work was to establish the effects on the small intestine of feeding rats on diets containing WGA for 10 d and its nutritional and metabolic consequences. As other N-acetylglucosamine (GlcNAc)-specific lectins may also be considered for similar use in future, the effects of the agglutinins from thorn apple (*Datura stramonium*; DSA) and nettle rhizomes (*Urtica dioica*; UDA) on the rat gut were also investigated and the results were compared with those of WGA.

MATERIALS AND METHODS

Lectins

Lectins with specificity for GlcNAc were isolated by affinity chromatography on appropriate specific supports. Briefly, WGA, solubilized at pH 5, was heated at 60° for 10 min and the supernatant fraction absorbed on to a column of immobilized GlcNAc (Selectin 1; Pierce Chemical Co., Rockford, IL., USA). WGA was eluted from the column with 0.1 M-GlcNAc (Peumans *et al.* 1982). DSA was prepared by chromatography on chitin or fetuin-agarose and elution with acetic acid (Broekaert *et al.* 1988). UDA was also prepared by affinity chromatography on chitin (Peumans *et al.* 1984).

Animal experiments

Male Hooded Lister (Rowett) rats were weaned at 19 d of age, fed on a stock diet for 10 d and selected into groups of four of average weight 80 (SD 1) g for a full N balance experiment. For this, the rats were individually housed in metabolism cages and fed for 10 d (6 g/rat per d) on fully-balanced semi-synthetic diets containing 93 g lactalbumin protein/kg and 7 g individual pure lectin/kg. Control rats were fed on the same amount of diet containing 100 g lactalbumin protein/kg. Composition of the diets was as given previously (Rubio *et al.* 1991). Water was available *ad lib*. The animals were weighed and urine and faeces collected daily and stored at -20° until required. Faeces samples were freeze-dried and ground in a mortar for analysis.

On the morning of the 10th day rats were given 2 g diet containing 14 mg of the respective lectin and killed by diethyl ether anaesthesia 2 h later. The abdomen was cut open and the entire gastrointestinal tract together with the pancreas and spleen was removed. Stomach and small intestine were separated from the rest, rinsed with saline (9 g NaCl/l) and the washings collected for the determination of the remaining immunochemically-intact lectin by rocket immunoelectrophoresis. To stop further proteolytic breakdown the stomach contents were washed out with about 10 ml saline made to 0.05 M with phosphate buffer, pH 7.6, while for the small intestine the buffer also contained 0.1 mg aprotinin (Sigma)/ml. A weighed section of 20 mm (50 mm from the pylorus) was taken from the small intestine for histology and the following 180 mm for chemical analyses. Tissues, including pancreas, spleen, caecum, colon, liver, kidneys, thymus, heart, lungs, adrenals, testes, prostate and hind-leg muscles of soleus, plantaris and gastrocnemius, were excised, rinsed, blotted dry and weighed. The tissues and the remainder of the carcasses were freeze-dried to constant weight and weighed.

Chemical analysis

Protein. Diets, faeces, urine, carcasses and some of the tissues were analysed for total N

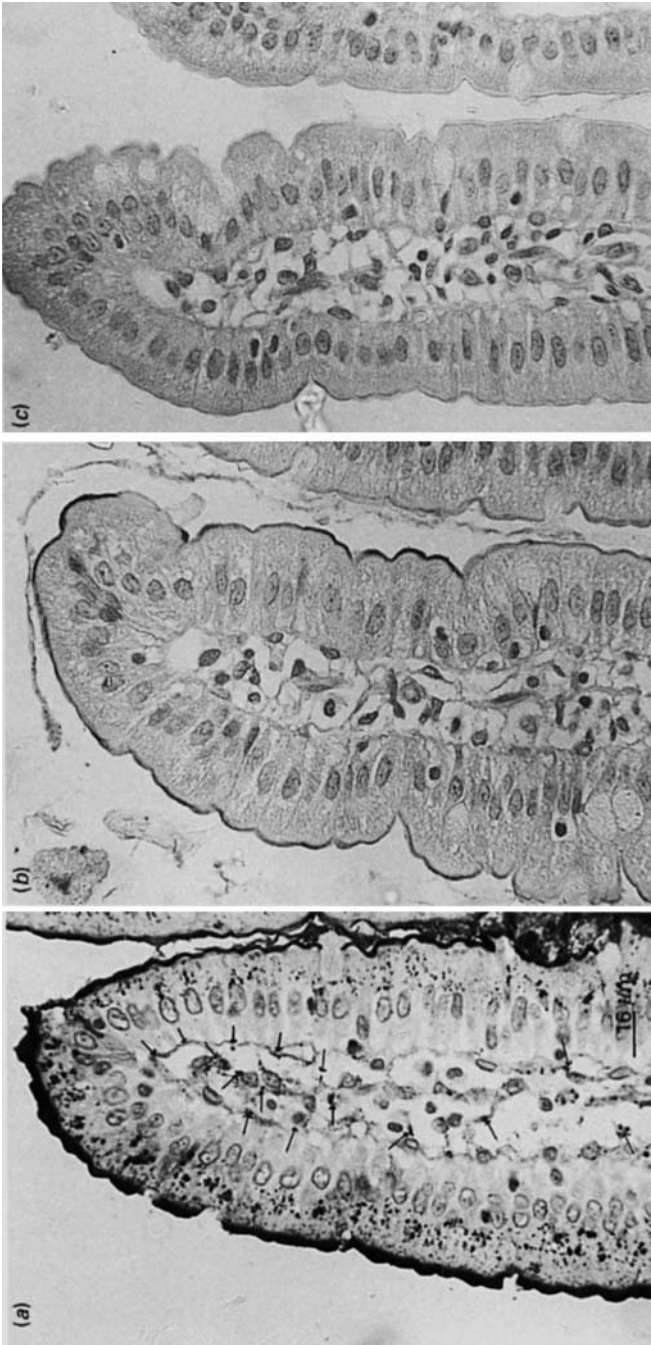


Plate 1. Specific immunoreactive PAP- (peroxidase (EC 1.11.1.7)-antiperoxidase) staining of sections obtained from the jejunum of rats fed on diets containing various N-acetylglucosamine-specific lectins for 10 d. Formalin-fixed sections after treatment with trypsin (EC 3.4.21.4) were reacted with the appropriate monospecific rabbit anti-lectin antibodies. Second antibody treatment (PAP) was followed by staining the sections with 3,3'-diaminobenzidine and counterstaining by haematoxylin. (a). Strong binding to the brush border, extensive endocytosis and staining of the basolateral membrane of enterocytes and blood and lymphatic vessels by WGA in the lamina propria (->); (b), moderately strong binding of thorn apple (*Datura stramonium*) agglutinin to brush-border membranes but no endocytosis; (c), only slight and patchy staining of brush-border membranes by nettle (*Urtica dioica*) rhizome agglutinin.

(Davidson *et al.* 1970). Urine samples were analysed for urea, creatinine, ammonia and free α -amino-N as described previously (Rubio *et al.* 1991).

Polyamine determination. Samples of pancreas and small intestine were extracted in the presence of an internal standard of 1,7-diamino heptane with perchloric acid (100 g/l; 15 mg tissue/ml) for 30 min at 0°, centrifuged (10000 g; 10 min) and individual polyamines separated and determined by a HPLC method from the supernatant fraction (Seiler & Knödgen, 1980).

The protein content of the residue insoluble in perchloric acid was determined by a modified Lowry method (Schachterle & Pollack, 1973) after solubilization in 0.3 M-NaOH. RNA (Sneider, 1957) and DNA (Lovtrup, 1962) in the tissues were estimated as before (Pusztai *et al.* 1992).

Lipids. Dried carcass samples were extracted in chloroform-methanol (2:1, v/v) for 24 h. The solvent was removed by filtration followed by drying under reduced pressure in a desiccator. Lipid content was calculated from the difference in weight before and after extraction.

The concentration of 3-hydroxybutyrate in serum was determined by Technicon AutoAnalyzer (Zivin & Snaar, 1973).

Rocket immunoelectrophoresis

Washings of the stomach and the small intestine were centrifuged and samples from the clear supernatant fractions were subjected to electrophoresis on minimum endosmosis agarose gel (Agarose C; Pharmacia GB Ltd; 10 g/l) containing the appropriate monospecific anti-lectin antibody and hapten sugar (1 g/l) in 0.07 M-Tris-glycine buffer, pH 8.6, at 0.4 V/mm for 16 h. The agarose gels were dried with filter paper, washed with saline and the rockets stained with Coomassie-blue.

Morphology

Sections of the small intestine were immediately fixed with buffered (pH 7) paraformaldehyde (40 ml/l), embedded in paraffin wax, sectioned at 3 μ m and for histological measurements were stained with haematoxylin and eosin. Ten properly oriented villi and crypts were selected at random from each animal and their length measured and the number of cells counted.

Sections were also examined for the presence of lectins bound to the brush border by antibody-peroxidase (EC 1.11.1.7)-antiperoxidase (PAP) staining as previously (Pusztai *et al.* 1990). After inhibition of endogenous peroxidase, antigenic sites were unmasked with trypsinization and the sections were reacted with appropriate antibody solutions, followed successively by the link anti-serum and PAP serum. The label was visualized with 3,3'-diaminobenzidine and the sections were counterstained with haematoxylin.

Statistical analysis

The results were subjected to analysis of variance. Significant differences between means were determined by using Student's *t* test (Steel & Torrie, 1960).

RESULTS

Nutritional data

The weight gain of rats was reduced in the presence of lectins in the diet (Fig. 1). With WGA this amounted to a reduction by more than one-third in comparison with the paired controls. The dry body weight of the WGA-fed group was also significantly less, only about 89% of that for the control group (Table 1). Rats on the WGA-containing diet

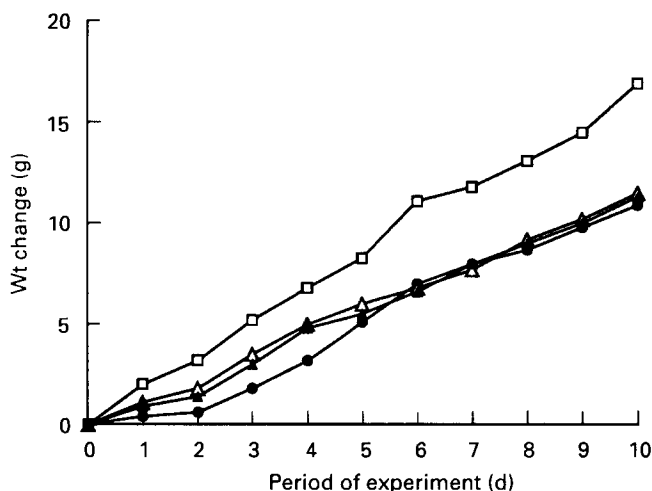


Fig. 1. Growth curve of rats fed on diets containing different N-acetylglucosamine-specific lectins (7 g/kg diet) for 10 d. For details of diets and procedures, see pp. 314–315. (□), lactalbumin; (●), wheat-germ agglutinin; (△), thorn apple (*Datura stramonium*) agglutinin; (▲), nettle (*Urtica dioica*) rhizome agglutinin.

excreted more faeces and lost more N in it than the controls or the groups with DSA or UDA in their diets. Although rats on WGA lost slightly less N through the urine than the others, overall, the N retention (not corrected for metabolic N loss) varied between 68% for WGA-, 73% for DSA- and 72% for UDA-containing diets, which was less than the corresponding value of 76% for the controls. Despite this, body N concentration of the rats of the WGA-fed group (104.8 (SD 1.2) g/kg) was increased significantly in comparison with the lactalbumin-fed controls (101.3 (SD 0.7) g/kg) which was in compensation for the reduction in the body's relative lipid content (152 (SD 9) and 177 (SD 7) g/kg for the WGA-fed group and controls respectively). The other two lectins also slightly increased the body concentration of N and reduced its lipid content but this did not reach significance. Values for apparent N digestibility were also reduced in the presence of lectins; again this was most serious with WGA (Table 1).

Tissue weight and composition

The weight of the small intestine of rats fed on diets containing the lectins was appreciably increased after feeding for 10 d. With WGA the small intestine was 45% heavier than that of the control rats (Table 2). The increase was more modest with the other two lectins; 17 and 12% with UDA and DSA respectively. Substantial growth was also indicated from morphometric measurements. Although the length of jejunal villi was similar in all groups of rats, except for the slightly smaller value of the UDA-fed group, the crypts were significantly enlarged on treatment with the lectins (Table 2). In accord with this, the number of crypt cells was about 60% higher on the WGA-containing diet (34.9 (SD 3.6)) than that on the control diet (21.2 (SD 1.2)). The increase in crypt cell numbers with the other two lectin-containing diets was less but still significant (Table 2).

In line with the growth of the small intestine, its protein, RNA, DNA and polyamine contents were also increased (Table 2). In contrast, values for dry weight on a per kg dry body weight basis of other parts of the gut, including stomach (5.0 (SD 0.2)), caecum (3.3 (SD 0.3)) or colon (3.8 (SD 0.4)) were similar irrespective of whether the rats were fed on control or lectin-containing diets.

Table 1. Nutritional performance of rats on diets containing various *N*-acetylglucosamine-specific lectins and control diets*

(Mean values and standard deviations for four rats per treatment)

Dietary treatment ...	Lactalbumin		WGA		DSA		UDA	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Wt change (g/10 d)	16.9 ^a	1.0	10.9 ^b	0.7	11.5 ^b	1.0	11.3 ^b	0.8
N intake (g/10 d)	1.05 ^a	0.01	1.02 ^a	0.02	0.94 ^b	0.02	0.97 ^b	0.02
Faeces (g/10 d)	3.32 ^a	0.29	4.09 ^b	0.13	3.43 ^a	0.46	3.51 ^a	0.56
Faecal N (g/10 d)	0.14 ^a	0.01	0.23 ^b	0.01	0.14 ^a	0.01	0.17 ^b	0.01
Urine (ml/10 d)	17.2 ^a	0.5	12.0 ^b	1.2	7.3 ^c	0.8	10.9 ^b	3.4
Urine N (g/10 d)	0.12 ^a	0.01	0.10 ^a	0.01	0.11 ^a	0.02	0.11 ^a	0.01
Urea-N (g/10 d)	0.03 ^a	0.01	0.02 ^a	0.01	0.03 ^a	0.01	0.03 ^a	0.01
Ammonia-N (g/10 d)	0.01	0	0.01	0	0.01	0	0.01	0
Creatinine-N (g/10 d)	< 0.01	—	< 0.01	—	< 0.01	—	< 0.01	—
Free amino-N (g/10 d)	< 0.01	—	< 0.01	—	< 0.01	—	< 0.01	—
3-hydroxybutyrate (μmol/10 d)	55.4 ^a	4.9	44.5 ^a	6.5	22.5 ^b	5.9	25.7 ^b	5.9
N balance (g)	0.79 ^a	0.01	0.70 ^b	0.01	0.69 ^b	0.01	0.70 ^b	0.01
N retention (g retained/g intake)	0.756	—	0.676	—	0.733	—	0.712	—
Dry body wt (g)	27.8 ^a	0.3	24.8 ^b	0.7	25.4 ^b	0.3	25.5 ^b	0.8
N digestibility (apparent, g/g)	0.87 ^a	0.02	0.77 ^b	0.02	0.85 ^a	0.02	0.82 ^b	0.02

^{a, b, c} Mean values in the same row with different superscript letters were significantly different ($P < 0.01$).
WGA, wheat-germ agglutinin; DSA, thorn apple (*Datura stramonium*) agglutinin; UDA, nettle (*Urtica dioica*) rhizome agglutinin.
* For details of diets, see p. 314.

Table 2. *The effect of dietary lectins on the weight, composition and morphology of the small intestine (jejunum) of rats*
(Mean values and standard deviations for four rats per treatment)

Dietary treatment* ...	Lactalbumin		WGA		DSA		UDA	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Dry wt of small intestine (g/kg dry body wt)	22.8 ^a	0.6	33.5 ^c	1.5	25.8 ^b	1.2	26.3 ^b	1.1
Dry wt of jejunum (200 mm) (g/kg dry body wt)	5.8 ^a	0.4	7.2 ^b	0.2	6.9 ^b	0.4	6.6 ^b	0.2
Protein (mg/200 mm)	71 ^a	5	85 ^b	3	84 ^b	7	77 ^{ab}	7
RNA (mg/200 mm)	7.3 ^a	0.3	12.6 ^d	0.5	11.2 ^c	0.3	9.3 ^b	0.5
DNA (mg/200 mm)	2.16 ^a	0.05	2.30 ^b	0.05	2.34 ^b	0.10	2.40 ^b	0.08
Putrescine (nmol/200 mm)	160 ^a	9	201 ^b	19	133 ^c	7	133 ^c	13
Spermidine (nmol/200 mm)	878 ^a	9	1097 ^b	25	1127 ^b	63	1044 ^b	67
Spermine (nmol/200 mm)	412 ^a	16	492 ^b	5	531 ^b	68	507 ^b	67
Acetylspermine (nmol/200 mm)	33 ^a	10	72 ^b	8	nd	nd	nd	nd
Villus length (μ m)	577 ^a	12	543 ^a	11	549 ^a	11	448 ^b	20
Crypt length (μ m)	119 ^a	2	190 ^d	4	145 ^b	3	167 ^c	5
Crypt cell number	21.2 ^a	1.2	34.9 ^c	3.6	28.7 ^b	3.2	27.0 ^b	3.6

^{a, b, c, d} Mean values with different superscript letters in the same row were significantly different ($P < 0.01$).

WGA, wheat-germ agglutinin; DSA, thorn apple (*Datura stramonium*) agglutinin; UDA, nettle (*Urtica dioica*) rhizome agglutinin; nd, not determined.

* For details of diets, see p. 314.

WGA in the diet induced pancreatic enlargement. After feeding for 10 d the dry weight of the pancreas of rats, relative to their body weight (5.3 (SD 0.1) g/kg), was about 18% heavier than that of the controls. There was also a corresponding increase (15–20%) in its protein, RNA and polyamine contents (results not given). The enlargement with the other two lectins was not significant (g dry weight/kg dry body weight values were: 4.8 (SD 0.4) for UDA and DSA and 4.4 (SD 0.4) for the control group).

The weight of the thymus was significantly reduced (by over 20%) with WGA in the diet, but no other tissues of the body were affected. The other two lectins had no effect on the thymus or other tissues (results not given).

Survival of immunoreactive lectins in the small intestine

Sections of the jejunum, reacted with the appropriate monospecific antibodies followed by PAP staining, showed that all three lectins survived in functionally- and immunoreactively-intact form and were bound to the brush-border epithelium (Plate 1). The binding was extensive with WGA, moderate with DSA and slight with UDA. Furthermore, with WGA significant binding was observed, not only on the luminal membrane of jejunal epithelial cells but staining by the PAP method showed that this lectin was also bound by the basolateral membrane and the walls of the venules and lacteals. However, no endocytosis or binding to subepithelial tissues was observed with the other two lectins.

Appreciable amounts of all three lectins were shown by rocket immunoelectrophoresis to have survived the passage through the small intestine. Thus, 2 h after the last feed intake over 60% of WGA was found in the small-intestinal wash. The corresponding values for DSA and UDA were similar, 50 and 60% respectively. The lectin content of the caecum was not tested.

DISCUSSION

The inclusion of WGA or other GlcNAc-specific lectins in the diet significantly depressed the growth of rats (Fig. 1; Table 1). On similar intakes of food and N, both the live weight of the rats and the dry weight of their carcass were significantly reduced in comparison with the lactalbumin-fed controls. The lectin-fed animals appeared to digest the dietary proteins less efficiently and, consequently, retained less of the ingested N. In fact, the less favourable N balance of the rats on the lectin-containing diets appeared to be due entirely to the faecal loss of N, a part of which may have been of endogenous origin, as the N content of urine was similar in all groups of rats, including the controls. This suggested that, in contrast to the effects of other toxic lectins such as the kidney bean lectin (Pusztai *et al.* 1981), soya-bean agglutinin and others (Pusztai, 1991) the antinutritional effects of GlcNAc-specific lectins, particularly WGA, were mainly the result of their interaction with the digestive tract.

WGA was a potent growth factor for the gut by significantly increasing both crypt size and crypt cell proliferation in the small intestine. The other two GlcNAc-specific lectins also induced significant, but slightly less extensive, growth of this tissue by hyperplasia (Table 2). Although it is difficult to measure it precisely, the nutritional cost of this compulsive gut growth contributes to the overall antinutritive effects of the lectins (Pusztai *et al.* 1991).

All three lectins were bound by the small intestinal epithelium (Plate 1). The binding of WGA to the luminal membrane of enterocytes was particularly avid, leading to extensive endocytosis. This was in line with the recently observed firm correlation between the strength of the binding of lectins to the brush border and their effectiveness as antinutrients (Pusztai *et al.* 1990). Furthermore, there was also a relatively strong and specific staining

by anti-WGA antibodies of the basolateral membrane and the walls of the small blood vessels and lacteals in the subepithelium. This suggested that WGA was not only transported through the entire cellular domain of the enterocytes by an unknown mechanism but that it also was transcytosed and may have reached the systemic circulation (Plate 1).

The physiological consequences of the transepithelial transport of detectable amounts of intact WGA from the diet are unknown. However, as the lectin appeared to be fully active and fairly stable against proteolytic breakdown, its potentially harmful effects on both metabolism and health need to be explored, particularly when diets containing WGA are fed for extended periods of time. In this context it is particularly significant that WGA (but not the other GlcNAc-specific lectins) induced appreciable growth of the pancreas during the 10 d feeding period. The consequences of the continuous stimulation of the pancreas, particularly in the long term, may be harmful for both of its vital exocrine and endocrine functions. Moreover, as one of the previously shown toxic effects of dietary PHA is due to its interference with the functioning of the immune system (Pusztai *et al.* 1981), it is of special concern what effects the systemically-absorbed WGA may have on the gut and the body's immune systems. The thymus atrophy observed in rats fed with diets containing WGA may be particularly damaging for the proper functioning of the immune system.

It is possible to express selected lectin genes at high levels and reach a concentration of up to 1% of lectin in the tissues of transgenic plants by the use of appropriate promoters. Moreover, it has been shown by incorporating lectins into artificial insect diets that at this concentration a number of lectins, notably WGA, are highly toxic for some insect species (Czapla & Lang, 1990; Murdock *et al.* 1990; Huesing *et al.* 1991 *a*). This has raised the hope that if the transferred WGA gene can be expressed at high level in the tissues of economically important crop plants, the insecticidal activity of the lectin will confer a high degree of protection for the transgenic plant. Moreover, as WGA occurs naturally in wheat germs and is consumed by man and animals without any apparent harmful nutritional consequences, its use as a natural insecticide may be safe.

Clearly, from the results of the present work, the use of the WGA gene in transgenic crop plants will have to be viewed with considerable caution. Although WGA is present in staple foods derived from cereals its concentration is only about 300 mg/kg wheat germ, which is far below the level of the 10 g/kg shown to be effective against insects. It is not unexpected that at this low natural level, particularly when also diluted with other food ingredients, no toxic effects of WGA have been observed. However, the nutritional evaluation of the effects of pure WGA at the dietary inclusion of 7 g/kg clearly showed that the lectin reduces the utilization of dietary proteins, induces wasteful growth of both the small intestine and the pancreas, causes thymus atrophy and depresses the growth of rats. Moreover, it is particularly worrying that detectable amounts of functionally- and immunochemically-intact WGA are transported across the intestinal wall and may reach the systemic circulation. The long-term effects of this systemic absorption of WGA on immune function, metabolism and health are unknown. Thus, the general use of WGA in edible parts of crop plants as a natural insecticide is not without dangers for potential consumers and its safety may still have to be established. Due to the heat stability of WGA and its resistance to proteolytic breakdown, this same consideration applies also to the expression of the WGA gene in the leaves of crop plants (e.g. maize) which are used as winter feed for cattle in many countries. From the limited comparison of the properties of the three GlcNAc-specific lectins in the present study and, particularly, from the absence (or the low level) of the transepithelial transport of DSA and UDA, the antinutritional effects of these two lectins appear to be less damaging. However, it may be of particular significance that when tested *in vitro*, and in line with their diminished toxicity for higher

animals, DSA and UDA also appeared to be less active against insects (Czapla & Lang, 1990; Murdock *et al.* 1990; Huesing *et al.* 1991*a, b*).

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