PART IV

Biotechnological, molecular and ecophysiological aspects of nutrition

17 GMO in animal nutrition: potential benefits and risks

A. Pusztai and S. Bardocz

Consultant Biologists, Aberdeen, Scotland, UK and Norwegian Institute of Gene Ecology (GenOK), Tromso, Norway; formerly of The Rowett Research Institute, Bucksburn, Aberdeen AB21 9SB, Scotland, UK

Even a cursory look at the few papers published in peer-reviewed international journals on the potential health and metabolic effects of GM feeds/foods reveals a scarcity of published data. It is often claimed that as there are only small compositional differences between the "substantially equivalent" GM and non-GM crops, these have little biological significance. However, from the present review it has become clear that most GM and parental line crops fall short of the definition of "substantial equivalence", a concept which, in any case, has outlived its previously claimed usefulness. Thus, novel biological concepts and methods to probe into the safety of gene splicing are needed. This is made all the more urgent because the biological testing of GM feeds, as presently carried out, is rather limited in scope and mainly aimed at finding the best conditions for commercial animal production. In this review previously published animal studies have been critically examined in the light of a newly suggested testing protocol, in which the safety of GM crops is established from the effects of the GM ingredients on the physiology, pathohistology, immunology and bacterial flora of the gastrointestinal tract of young animals and the metabolic consequences of these effects. (GMO: genetically modified organisms.)

1. INTRODUCTION

1.1. Regulatory and general considerations

Assessing the production potential and value of feed components is of considerable commercial and practical importance. Indeed, in addition to safety and other nutritional considerations the effort spent by feed technologists in evaluating the feeding value of crops is considerably more extensive than that by human nutritionists to establish the nutritional value of foods for the public. Not surprisingly, this distinction is even more acute between genetically modified (GM) food or feed ingredients. Thus, except the very recently published human trial with a single dose of GM soybean-containing meal (Netherwood et al., 2004), the almost total

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absence of published data in peer-reviewed scientific literature indicates that the safety of GM foods rests more on trusting the assurances given by the biotechnology industry than on rigorous and independently verified risk assessment. A comment in Science described this in its title: "Health Risks of Genetically Modified Foods: Many Opinions but Few Data" (Domingo, 2000). Indeed, most of the attempts to establish the safety of GM food have been indirect, using animal trials with GM feed ingredients and drawing inferences from these for human health. However, even these animal studies had in most instances limited, mainly commercial, objectives, as is obvious from recent reviews (Aumaitre et al., 2002; Faust, 2002). Despite this, the main stated objective of the GM regulation is to assure the human population that GM foods are safe while animal safety is seldom discussed. The regulators, particularly in the USA, use a decision tree approach in which the authorities review the data, usually provided by the biotechnology companies, but do not carry out safety assessments of their own (Faust, 2002). Even in Europe the preferred approach is to use compositional comparisons between the GM crop and its traditional counterpart and if these results show no significant differences they are considered to be "substantially equivalent", meaning that the GM is as safe as the non-GM crop. However, even though existing legislation does not require the testing of GM crop-based feedstuffs with target animals, many new GM crops have been tested on farm animals but most of the time only to establish their effects on nutritional performance, digestibility, wholesomeness and feeding value for obvious commercial considerations (Aulrich et al., 2002; Aumaitre et al., 2002). In most of these relatively short-term and rather empirical studies the emphasis was on productivity rather than on investigating the biochemical and cell biological interactions between the GM ingredient and the digestive tract, the effect of the GM DNA and protein on the gut epithelial cellular and tissue structure, its immune and endocrine systems and bacterial ecology. This is particularly regrettable because nutritional parameters, though of great commercial interest, are rather crude measures in physiological terms of the effects of GM ingredients and may give science little guidance on what will be the likely biological consequences of long-term and heavy exposure to GM crops. Thus, as GM regulation is at present based on rather minimalistic legislative and scientific foundations, with the likely progress in the future of our understanding of the biological principles underlining the whole GM business, major efforts of clarification and updating will in time be needed.

1.2. Transgene survival in the alimentary tract and its possible consequences

In genetic modification the intended gene is incorporated into the genome of a crop, using a vector containing several other genes, including as a minimum: viral promoters, transcription terminators, antibiotic resistance or other marker genes and reporter genes. Unfortunately, the possible physiological effects on the digestive tract of these genes and their expressed proteins are seldom taken into account, even though there is some evidence that some of the other genes of the vector may have an effect on safety. This is particularly so as it is now well established that DNA does not always break down in the alimentary tract (Schubbert et al., 1994, 1997, 1998; Hohlweg and Doerfler, 2001). This opens up the possibility that the antibiotic resistance marker gene, in addition to others, may be taken up by bacteria in the digestive tract and contribute to the spreading of antibiotic resistance via human gut bacteria. In this context one potentially important observation was that a substantial proportion (6–25%) of a genetically engineered plasmid survived a 1-h exposure to human saliva (Mercer et al., 1999). Partially degraded plasmid DNA also successfully transformed *Streptococcus gordonii,* that normally lives in the human mouth. Saliva also contains factors which increase the ability of bacteria to become transformed by naked DNA. Similar results have been obtained with bacteria using artificial gut preparations (MacKenzie, 1999). Plasmid antibiotic resistance

marker gene DNA exposed to ovine saliva could transform competent *Escherichia coli* to ampicillin resistance *in vitro* (Duggan et al., 2002). Furthermore, when fed to chicks incorporated into GM maize, the plant-derived marker was shown to be present in their crop and stomach (Chambers et al., 2000). The transfer of DNA derived from GM or non-GM plant tissues to duodenal juice, lymphocytes, internal organs, etc. of animals fed on feed rations containing these is now well established (Chowdury et al., 2003; for other refs see Aumaitre et al., 2002) even though their physiological significance for humans and/or animals is unclear. However, no transgenic DNA has so far been shown to be present in milk or eggs (Phipps et al., 2003) even though it is likely that with improvements in detection techniques traces of such DNA will be found (Aumaitre et al., 2002). The only human study performed with GM soybean (Netherwood et al., 2004), to establish whether the antibiotic resistance marker gene survives in the gut and whether it is taken up by gut bacteria, confirmed the results of similar animal studies. It was shown that in the digesta of seven ileostomy patients (people whose large intestine has been surgically removed and replaced with an external pouch joined to the lower end of their small intestine) given a single meal containing GM soybean, variable but measurable amounts of the full-length transgene construct survived and that in three patients this was highly significant. Thus, transgenic DNA survives not only in mice or in artificial guts but also in humans and can be taken up by gut bacteria. Therefore, the prospect of the uptake of functional vector genes, including the antibiotic resistance gene, will have to be seriously considered. The importance of this in animal husbandry cannot be overemphasized in view of the practice of including antibiotics in animal feed in many non-European countries, although antibiotics have been banned as feed additives in the European Union from the end of 2005.

1.3. Indirect, unintended and positioning effects of genetic modification

To the generally recognized importance of the direct effects of the expression of the main transgene after its insertion into the plant genome via a gene construct, an additional concern is that this may also cause significant, indirect and unintended effects on the expression and functionality of the plant's own genes. The number of copies of the construct inserted and their location in the plant genome (positioning effect) are of particular importance. In the past, unfortunately, only scant attention has been given to their possible consequences (Ewen and Pusztai, 1999a) even though it is possible that they may cause many unexpected changes. The importance of the analysis of such unintended effects in GM foods however, has recently been recognized by including them in the *Codex Alimentarius* guidelines (Haslberger, 2003). The inadequacy of the currently used methods to detect these are frequently acknowledged (Kuiper et al., 2001, 2002). Positioning effects often occur with both conventional cross-breeding and genetic engineering and their unwanted consequences are usually eliminated by empirically selecting for the desired trait and discarding the potentially harmful ones. However, some of these changes are unpredictable. As it is only possible to compare the known properties and constituents of GM and conventional plans but not to look for, and even less analyze, unknown components, the limitations on our selection criteria are severe. Reliance based solely on chemical analysis of macro/micronutrients and known toxins is at best inadequate and, at worst, dangerous. More sophisticated analytical methods need to be devised, such as mRNA fingerprinting, proteomics, secondary metabolite profiling and other profiling techniques (Kuiper et al., 1999, 2001, 2003). However, and most importantly, there is an urgent need to develop comprehensive toxicological/physiological/nutritional methods which will equally be applicable to scientifically examine the veracity of the claimed benefits of genetic manipulation and screen for its unintended and potentially deleterious consequences

for human/animal health. The center of this effort should be the physiology of the alimentary canal, since this is the first contact point of exposure to any food/feed including those which have been genetically modified, to establish in scientific terms the short- and long-term consequences of the exposure (Ewen and Pusztai, 1999a).

This review will not deal with environmental issues or political considerations concerning GM food/feed regulation. The emphasis will be on discussing the significance of published scientific, physiological, histopathological and nutritional data of GM food/feed and the principles which ought to underpin the efforts aimed at widening our understanding. No opinions, unless supported by experimental results, will be discussed. The emphasis will be on papers published in peer-reviewed journals and only exceptionally will data from non-peer-reviewed sources be mentioned and only if they influenced the development of science-based ideas for GM food/feed safety. This is all the more important because even a cursory look at the list of references in recent major reviews on GM safety issues (Gasson and Burke, 2001; Kuiper et al., 2001; Pusztai et al., 2003) shows that most of the publications referred to were non-peer-reviewed institutional opinions or envisaged future scientific and methodological developments for safety assessments, but were short on actual published scientific papers on which a reliable database of safety could be founded.

As the compositional complexity of foods/feeds makes it difficult to use classical toxicological methods for the assessment of GM safety, a physiological/nutritional approach may be of more relevance (Pusztai, 2002). Reviewing previous work may therefore be rewarding in the framework of such an approach, particularly as most of the relevant information concerning the potential safety of GM food/feed has come from studies concentrating on gut histopathology and nutrition. This review will enlarge upon previously published reviews (Kuiper et al., 2001; Pusztai, 2001; Pryme and Lembcke, 2003; Pusztai et al., 2003).

2. SAFETY ASSESSMENT OF GM CROPS

2.1. Compositional studies

For diet formulation, a compositional analysis of the transformed and isogenic lines is essential and must always precede the feeding study. For this the parent and GM lines must be grown under identical conditions, treated and harvested the same way. One such example is given for GM potatoes in table 1 (Pusztai, 2002). Unfortunately, fulfilling this condition is the

Table 1

Compositional values for "Desiree" potato tubers and two GM lines expressing the snowdrop (*Galanthus nivalis*) bulb lectin, GNA, derived from them (from Pusztai, 2002)

		GM lines	
Constituent	Parent line	Line 71	Line 74
Protein (% w/w)	7.2[a]	7.2[a]	5.6[b]
Lectin (μg/g)	6.7 (0.4)[b]	7.9 (<0.1)[a]	5.8 (0.8)[c]
Trypsin inhibitor (mg/g)	3.4 (<0.1)[a]	3.1 (0.1)[b]	2.7 (0.1)[c]
Chymotrypsin inhibitor (mg/g)	2.7 (0.1)[a]	2.6 (0.1)[a]	2.2 (0.1)[b]

The plants were grown side-by-side in field tunnels. The values are means (SD) of analyses of at least four determinations of each constituent independently carried out by two workers. Values with different superscripts are significantly different ($P < 0.05$).

exception rather than the rule, as shown in some of the examples discussed below. In addition to proteins, starch, lipids, etc., of the parent and GM lines, their contents of bioactive components should also be compared by novel methods (proteomics, fingerprinting, metabolite profiling, etc.).

2.1.1. Herbicide-resistant soybeans

Based on similarities between the macronutrient composition of parent, non-GM line and glyphosate-tolerant soybean (GTS) seeds, resulting from the transformation of conventional soybean with a gene encoding for 5-enolpyruvylshikimate-3-phosphate synthase from *Agrobacterium* to make the soybean herbicide-resistant, it has been claimed that the GM and the non-GM lines are "substantially equivalent". This equally applies to GTS unsprayed with glyphosate (Padgette et al., 1996) or sprayed with this herbicide (Taylor et al., 1999). The results of proximate chemical analyses of the contents of crude protein, oil, ash, fiber, carbohydrates and amino acids of solvent-extracted and toasted or untoasted soybean meals of sprayed or unsprayed GTS and control soybean were also apparently substantially equivalent (Padgette et al., 1996; Taylor et al., 1999). Although several significant differences between GM and control lines, such as in ash, fat and carbohydrate contents were found (Table 2 in Padgette et al., 1996), these were not regarded to have biological significance by the authors. However, the statistical method for comparing the GM and non-GM lines was flawed. Instead of comparing sufficiently large numbers of samples of each individual GTS with its appropriate individual parent line grown side-by-side at the same location and harvested at the same time to establish whether they were compositionally "substantially equivalent", what the authors compared was a large number of different samples from different locations and harvest times. As growth conditions have a major influence on seed composition, the range of the amounts of constituents in the different samples was so great (± 10% or more) that the chances of finding statistically significant differences were unreal. This is all the more curious, because in the authors' experiment 1 in Puerto Rico the conventional and the GTS lines were grown at the same site but the results of their analyses on these soybean samples were not

Table 2

Results of lymphocyte proliferation assays in rats fed for 10 days diets containing raw GM-, control/non-GM potatoes, or control/non-GM potatoes supplemented with the gene product, GNA (from Pusztai, 2002)

	μg Con A/well				
Diet	0.3	1.0	3.0	6.0	9.0
Parent	10.3 (13.4)	16.0 (18.5)	4.4 (4.9)	1.9 (1.0)	1.6 (1.6)
Parent + GNA	2.5 (4.3)	2.6 (3.5)	2.0 (3.6)	1.1 (0.5)	0.9 (0.6)
GM	1.5 (0.9)	1.7 (1.1)	1.0 (0.4)	1.6 (1.1)	1.6 (1.5)
Significance ($P <$)					
Parent vs					
Parent + GNA	NS	$P < 0.05$	NS	$P < 0.05$	NS
Parent vs GM	$P < 0.05$	$P < 0.05$	$P < 0.05$	NS	NS

Rats were fed on different diets for 10 days. At the end of the experiment blood samples were taken and subjected to standard lymphocyte stimulation assay with Concanavalin A (Con A) as the mitogenic signal. The results are expressed as stimulation indexes vs control. Values are means (SD) and significance was assessed by Student t test; NS = not significant.

included in the publication based on experiments 2 and 3 from different sites (Padgette et al., 1996). The Puerto Rico results had been deposited with the American Society for Information Science, National Auxiliary Publication Service (NAPS) as supplementary information, as referred to in Padgette et al. (1996). It could also be retrieved from the archives of the *Journal of Nutrition* and the data showed that the GM soybean contained significantly less protein and the amino acid phenylalanine, amongst many other things and therefore it could not have supported the growth of animals as well as the parent line. It is possible that from a practical point of view the variation in protein concentration of samples of the three lines between 36.8–45% would fall into the normal range of agronomic variability of soybeans and therefore may not be of major concern for agronomists. However, this comparison is not strict enough to establish whether the genetic modification introduced any unintended compositional changes. What is remarkable is that even with this approach many significant changes in macronutrient levels were found. Thus, the claim of "substantial equivalence" of GTS lines with non-GM soybean is not supported by rigorous scientific evidence. Excluding the results of the soybean samples grown in Puerto Rico, no significant differences were found in the levels of antinutrients, such as trypsin inhibitors, lectin and oligosaccharide flatulance factors between solvent-extracted, toasted or untoasted GM and non-GM soybean seeds in the study by Padgette et al. (1996). However, the comparisons were made by the same method as for the macronutrients and therefore the large range of natural variability excluded the possibility of finding significant differences. Furthermore, in single soybean meal samples of two GTS and parent lines the trypsin inhibitor (also a major allergen in soybean) content was substantially higher, by almost 30%, in one of the two GTS lines, with a smaller increase in the other. No trypsin inhibitor analyses were performed on the protein isolate or protein concentrate samples originating from the meal samples. In practically all heat-treated GM soybean samples from the Puerto Rico trial the amounts of lectin and the trypsin inhibitors were significantly higher in the GM samples than in the isogenic line. Even more curiously, heat treatment appeared to have a far lesser denaturing effect on the trypsin inhibitor content of the GM lines than on the parent line samples. Although for some unexplained reason the values were from single assays on single samples (table 3), one of the GM lines (61-67-1) appeared to have almost seven times as much trypsin inhibitor per mg sample dry weight (DW) as the parent. Indeed, the values in this GM soybean approached that found in untoasted soybean

Table 3

Relative dry organ weights of rats significantly affected by feeding for 10 days with diets containing raw or boiled GM potatoes and/or parent potatoes spiked with the gene product (GNA, *Galanthus nivalis* agglutinin; from Pusztai, 2002)

	Raw potatoes		Boiled potatoes	
Diet	Pancreas	Jejunum	Prostate	Liver
Parent	0.68 (0.08)	0.62 (0.06)	0.24 (0.08)	3.78 (0.14)
GM	0.81 (0.05)	0.72 (0.07)	0.16 (0.02)	3.28 (0.21)
Parent + GNA	0.70 (0.08)	0.67 (0.04)	0.18 (0.02)	3.40 (0.28)
Significance ($P <$)				
Parent vs GM	0.01	0.03	0.05	0.001
Parent + GNA vs GM	0.03	NS	NS	NS

Rats were fed with the diets for 10 days. The values of relative dry organ weights (g organ weight/100 g dry body weight) are means (SD), $n = 6$, by multivariate statistical analysis; NS = not significant.

seed samples. Even the other GM line (40-3-2) contained three times as much trypsin inhibitor as the non-GM line. There were also other compositional differences in these processed soybean products. Although it is difficult to decide from single determinations what significance one can attach to them it is curious that these studies were not followed up to establish whether the differences were real or not.

GM soybean samples were consistently found to have significantly less isoflavones than the parent cultivars (Lappe et al., 1999) and the GM samples were considerably more variable in this respect than conventional soybeans. This high variability is particularly worrying in view of the known and powerful goitrogenic and estrogenic activities of soy isoflavones (Doerge, 2002).

In conclusion, even the results of the analytical work done to date, using mainly conventional methods have left many uncertainties about the chemical equivalence of the GM and non-GM soybeans, particularly as the design of some of the comparative studies was seriously flawed. Even more seriously, judging from the published literature, no attempt was apparently made to establish the equivalence of the GM to the conventional lines by more modern and high-resolving power technologies, such as proteomics, DNA microarray analysis using GM soybean RNA isolated from different tissues and plants grown under different but relevant conditions. No data could be found using NMR combined with chemometrics for the characterization of metabolite differences in the plants (Le Gall et al., 2003). Moreover, analysis of the possible different glycoforms of the 5-enolpyruvylshikimate-3-phosphate synthase and other proteins has not been attempted, although variability in glycosylation patterns can lead to different biochemical and antigenic properties. Furthermore, no comparison was made between the GM and non-GM forms in their contents of small RNA molecules that are emerging as very important and inheritable gene regulators. In view of these omissions no claims by the authors that the GM and non-GM soybeans are substantially equivalent can be accepted without carrying out further and more critical studies.

2.1.2. GM potatoes

The gene of soybean glycinin was transferred into potatoes with the aim to increase their protein content (Hashimoto et al., 1999a). However, as the expression level of glycinin in potatoes was only between 12–31 mg/g total soluble protein, the improvements in protein content or amino acid profile were minimal. In fact, the total protein content of the GM potatoes after the gene transfer became significantly less than that of the control line. Even more unfortunately, the contents of some vitamins were reduced while the amounts of both solanine and chaconine increased in the GM lines. In this light the claimed substantial equivalence of the GM and parent lines was not supported by the published results.

Furthermore, the finding of significant differences in a number of tuber components, the results of compositional analyses of some macro- and micronutrients of insect- and virus-resistant potatoes and those of untransformed lines (Rogan et al., 2000), also does not appear to support their substantial equivalence. However, in the absence of animal studies it is difficult to ascertain whether these differences could have any biological consequences for humans/animals, particularly as known antinutrients, such as lectins or enzyme inhibitors were not analyzed.

Modulating the adenylate pool by genetic manipulation of the plastidial adenylate kinase in transgenic potato plants has shown that it is possible to increase the level of starch in the tuber by 60%, the concentrations of several amino acids and, at the same time, increase tuber yield as well (Regierer et al., 2002). Unfortunately, no feeding studies have been reported on this GM potato. No modern analytical methods for establishing substantial equivalence between GM and non-GM potatoes have been used in either of these two studies.

2.1.3. GM rice

Transformed lines expressing the soybean glycinin gene have been developed (Momma et al., 1999) by a method similar to that used for GM potatoes. The glycinin expression level was between 40–50 mg glycinin/g total rice protein. The GM rice was claimed to contain 20% more protein, but its moisture content was less than that of the parent line and it was not clear whether the increased protein content was due to the decreased moisture content of the seeds, because it was not specified whether the values were expressed for air-dried or fully dried seeds.

2.1.4. GM cotton

Several lines of GM cotton plants have been developed, using the gene encoding an insecticidal protein from *Bacillus thuringiensis* subsp. *Kurstaki,* to increase the protection of the cotton plant against the lepidopteran insect pests. Cottonseed is an important source of oil for human consumption and of processed cottonseed meal for animal feed. The GM lines were claimed to be "substantially equivalent" to conventional lines (Berberich et al., 1996), because the levels of protein, fat, carbohydrate, moisture, ash, amino acids and fatty acids in the insect-protected lines appeared to be comparable to those found in commercial varieties. Moreover, the levels of antinutrients such as gossypol, cyclopropenoid fatty acids and aflatoxin were similar or less than those in conventional seeds. Unfortunately, the statistics used by the authors were identical to that used with glyphosate-resistant soybeans and therefore could be similarly criticized. In addition, no account was taken of environmental stress that could have had major and unpredictable effects on antinutrient, toxin and allergen levels (Novak and Haslberger, 2000). No attempts were made with modern methods to find whether the GM and non-GM cotton lines were compositionally equivalent or different due to possible unintended consequences of the genetic engineering.

2.1.5. GM maize

Except for a few minor differences, which the authors thought were unlikely to be of biological significance, the recently developed glyphosate-tolerant (Roundup Ready) corn line, GA21 collected from 16 field sites over two growing seasons, was shown by proximate analyses of fiber, amino acids, fatty acids and mineral contents of the grain and forage, to be comparable to the control line (Sidhu et al., 2000). The comparison however, was carried out by a statistical method similar to that for GTS soybean (Padgette et al., 1996) which is scientifically flawed.

The criticism raised above with GM soybean, that for characterization and establishing its substantial equivalence to non-GM soybean no modern analytical techniques were used, is also valid for GM maize.

2.1.6. GM wheat

A glyphosate-tolerant wheat variety called MON 71800, expressing 5-enolpyruvylshikimate-3-phosphate synthase from *Agrobacterium* sp. Strain CP4 (CP4 EPSPS), has been genetically engineered with reduced affinity for glyphosate in comparison with the endogenous plant EPSPS enzyme (Obert et al., 2004). Using conventional analytical techniques the authors claimed the transgenic MON 71800 wheat was substantially equivalent to the parent and other commercial wheat varieties and therefore as safe and as nutritious. While it may be understandable that a study on GM soybean carried out in 1995/6 only used rather conventional

analytical methodology for the establishment of substantial equivalence (see above), it is nevertheless clear that such a claim for GM wheat without the use of more modern and high-resolving power analytical methods is inadmissible in the present days.

2.2. Stability to degradation

It is of the utmost importance to establish the *in vivo* stability of GM products and foreign DNA, including the gene construct, promoter, antibiotic resistance marker gene, etc., to degradation in the stomach and intestines of model animals. Clearly, the safety of GM foods/feeds would be optimal if, as is often assumed, these biologically active molecules did not survive passage through the alimentary tract of animals/humans and therefore they could not interact with the gut, with possibly harmful consequences. Thus, one of the major assumptions in GM food regulation is that if the gene product protein breaks down on digestion it cannot be allergenic.

Unfortunately, at present if such studies are done at all, the stability of the gene products, DNA, etc., is only established *in vitro* by simulating gut digestion using acid and/or pepsin or other proteases in test tubes. The results of such *in vitro* digestion assays, however, can be misleading because the interactions between the digesta and the gut wall and its enzymes, which can greatly influence the stability or degradation of the components of the diet, are absent in the test tube. It is therefore questionable, for example, whether the gene product, 5-enolpyruvylshikimate-3-phosphate synthase (C4 EPSPS), that renders the soybeans glyphosate resistant (Harrison et al., 1996), truly breaks down in the gut of higher animals when in fact it has only been tested for this in a simulated digestion assay. A further complication in this study is that this *in vitro* simulated gastric/intestinal digestion assay was done with an *Escherichia coli* recombinant C4 EPSPS gene product whose stability could be different from that expressed in the soybean plant cells.

2.3. Biological, immunological and hormonal properties

To have physiologically valid results for establishing the true properties of GM plants it is essential that the gene product used for these tests must be isolated from the GM plant and that they are tested by *in vivo* assays in the rat (or other suitable animals; see Rubio et al., 1994) or in a full feeding trial. The validity of this approach can be appreciated from observations, which showed that the biological activity and stability of a gene product can be different when expressed in different life forms. Thus, it has been shown before that while the kidney bean (*Phaseolus vulgaris*) α-amylase inhibitor is fairly stable to proteolytic degradation in the rat gut (Pusztai et al., 1995, 1999), when its gene was expressed in peas (*Pisum sativum*), it was rapidly digested and inactivated in the rat stomach/small intestine *in vivo* (Pusztai et al., 1999). This may have made GM peas more safe for rats and, possibly even for other monogastric mammals.

With GM lectins, including Bt (*Bacillus thuringiensis*) toxin the presence/absence of their epithelial binding should also be demonstrated by immunohistology with the GM product isolated from the GM crop and not with a recombinant protein from *Escherichia coli,* as these two may have substantially different properties (Noteborn et al., 1995).

2.4. Nutritional testing

The genes used in present-day genetic engineering ensure that GM food is unlikely to be highly poisonous. "Toxicity" therefore is an unhelpful concept that does not easily lend itself

to quantitative assays. In contrast nutritional studies, in which GM crop-based diets are fed to young growing animals, should reveal their possible harmful effects on metabolism, organ development, immune/endocrine systems and gut flora, which together determine the safety of the GM crop and the development of the young into healthy adults. Although the validity of this approach is not always admitted, nutritional testing has often been used in the past for the safety assessment of GM foods/feeds. Indeed, historically the first such study (still unpublished) was carried out on FLAVR-SAVR™ tomato at the instigation of the FDA (Food and Drug Administration of USA). As tomatoes are of little relevance to animal nutrition and because a full review of these experiments has already been described (Pusztai et al., 2003), they will not be dealt with here.

2.4.1. Diets

For animal feeding tests iso-proteinic and iso-energetic diets need to be formulated in which most of the dietary protein is derived from the GM crop. The composition of the control diets should be the same as the GM diet, but containing the parent line with or without supplementation with the isolated gene product at the same level as expressed in the GM line. Unfortunately, although the use of the gene product-spiked control diet ought to be mandatory in these nutritional tests, they have rarely been used.

2.4.2. Feeding protocol

Groups of animals (5–6 or more animals per group) of closely similar weights, should be pair-fed in short- and long-term experiments. It is of particular importance to perform long-term nutritional experiments with GM feed components because small changes in the nutritional value of GM crops are more likely to show up with extended feeding. For example, the effect on the growth rate of rats fed GM potato-based diets was too small to be seen in the 10-day feeding experiments but a GM potato-induced reduction in rat growth was readily demonstrated in 110-day feeding trials, even when the potatoes were fully cooked (these results were published by the Rowett Research Institute (Bucksburn, Aberdeen, UK) against the authors' wishes on the institute's website (http://www.rri.sari.ac.uk)). In this experiment the GM protein in the diet was only diluted 2-fold by other dietary proteins, and the GM diet had to be supplemented with an extra 12 g lactalbumin/kg diet in order to equalize the growth rate of the rats on cooked GM potatoes to that of the control non-GM potato diets. This extra protein gives a quantitative measure of the difference in the nutritional value between GM and non-GM potatoes. Even at these similar growth rates the weights of some of the rats' vital organs, such as the gut and particularly the small intestine, the liver and kidneys were still significantly different.

In feeding experiments urine and feces should be collected for determination of net protein utilization (NPU), nitrogen balance, and feed utilization ratios. Blood samples should be taken before, during and at the end of the experiments for immune studies, such as lymphocyte proliferation assays. An example of this with GM potatoes is given in Table 2. Other assays, such as Elispot, hormone assays (insulin, CCK, etc.) and the determination of other blood constituents should also be performed. The animals should be weighed daily and any abnormalities observed. After killing the animals, the bodies are dissected, their gut rinsed and its contents saved for further studies (enzymes, GM products, DNA), gut sections taken for histology (see, for example, Pusztai et al., 2003), wet and dry weights of organs recorded and analyzed as in the example given in table 3, taken from a previous paper (Pusztai, 2002).

This recommended optimal protocol has a long history of usage for evaluating the nutritional value of conventional feedstuffs. Some of the pertinent important nutritional studies with GM crops will be fully reviewed here, to see how these previous studies were conducted and how they compare to this recommended optimal protocol and whether these methods could be incorporated into the general risk assessment procedures of GM foods/feeds.

2.5. Published nutritional/toxicological studies

2.5.1. Herbicide-resistant soybean

For the safety assessment of glyphosate-resistant soybean (GTS), the feeding value, wholesomeness (Hammond et al., 1996) and possible toxicity (Harrison et al., 1996) of two major GM lines of GTS were compared to that of the parent line. Processed GTS meal-based diets were fed to rats, broiler chickens, catfish and dairy cows for between 4–10 weeks at the same concentrations as in commercial non-GM soybean rations. According to the authors, the growth and feed conversion efficiency in rats, catfish and broilers, the fillet composition in catfish, the breast muscle and fat pad weights in broilers and milk production and composition, rumen fermentation and digestibilities in dairy cows, were similar for the GTS and parental lines. These results were therefore taken by the authors to suggest that the GTS and parental lines had similar feeding values.

2.5.1.1. Rat studies Although this study (Hammond et al., 1996) had a wider and more academic scope than many of the production-type studies (e.g. Cromwell et al., 2002; see Aumaitre et al., 2002; Faust, 2002) its design and execution could be criticized. Thus, in the published paper no primary individual data were given and no full description of the rat diet. It only reported on experiments carried out with soybean samples from experiments 2 and 3 but did not include those obtained with the equally relevant samples grown in Puerto Rico. It appears that the total protein content of the diets was adjusted to 24.7 g protein/100 g diet to be iso-nitrogenous with Purina Laboratory Rat Chow by the addition of 24.8 g of GTS and parent soybean meals, respectively (about 10% protein), to a base diet. All comparisons were made to rats fed commercial Purina Chow. The protein concentration in these diets was, however, appreciably higher than the usual 10–16% crude protein, regarded as optimal for the rat. This extra protein could have potentially masked any possible transgene product effects, particularly with the raw unprocessed soybean diets in which the GM meals were incorporated only at the level of 5% or 10% of the diet. Thus, these meals only replaced 8.5% and 17%, respectively, of the total protein of 24.7 g/100 g diet. In other words, the GM protein was diluted by other dietary proteins by 12-fold and 6-fold, respectively, producing another possible masking effect. The composition of the control Purina Chow diet in the ground raw soybean feeding study was not given and the identity of the raw control soybeans not specified.

In a 28-day feeding study four groups of singly housed rats (10 males and 10 females in each group) were fed diets containing the parental line or the GTS lines (40-3-2 or 61-67-1) for 28 days. No individual values (or their ranges) for feed intake or body weight were given. The bar diagrams of the combined bodyweights of rats were crude and uninformative. However, the Purina Chow-fed control male rats grew significantly better than most of the three experimental groups fed toasted soybeans (including the parental line). The bar diagrams also indicated that the growth of the 61-67-1 GTS line-fed rats was probably equal to that of the Purina Chow-fed control and therefore, by inference, these rats also grew significantly better than the other two experimental lines, the GM 40-3-2 GTS line and the parental line, indicating

significant differences in their nutritional value. There were no individual data for organ weights in the paper, but the kidney weights of the raw GTS line-fed (and parental control?) male rats were significantly higher than those of the controls, while the testes of the parental line-fed rats was significantly enlarged. According to the authors as these differences were not dose-related and were also shown by the parental line, they were therefore not caused by genetic modification. Unfortunately, in a major omission, no stomach or intestinal weights were recorded in the paper. No histology appears to have been done on these tissues either, apart from some qualitative microscopic observations on the pancreas that has been described as showing some minimal to mild lesions, which were claimed to be common to all groups. The absence of pancreatic hypertrophy, however, was not surprising because the unusually high dietary protein concentration, as pointed out by the authors, masked and/or diluted the biological effect of the trypsin inhibitors. This is of particular concern because the trypsin inhibitor content of GTS lines in unprocessed soybean was significantly higher than in the control line (Padgette et al., 1996). Thus, because of the major omissions in the feeding study and the lack of gut histology, more critical work is needed to decide whether the feeding value of GM and non-GM soybeans is equal or not.

2.5.1.2. Chicken study The broiler chicken feeding study's experimental design closely followed commercial practice and the results therefore at best could only be indicative of commercial feeding and production values of the various soybean lines which, according to the authors, were practically equal for both GTS and parental soybean lines.

2.5.1.3. Catfish experiment Similar to the findings with rats, one of the GTS lines, 61-67-1, was superior to the other lines (GTS 40-3-2 and the parental line) in most respects. Thus, fish ate more on GTS line 61-67-1, had better weight gain and gain/feed ratio and weighed more at the end of the 10 weeks study than the others, even though the composition of the fillets from these fish was not significantly different. Accordingly, genetic modification may not be as reproducible as claimed and the feeding value and metabolic effects of GM and parent lines are not always "substantially equivalent".

2.5.1.4. Lactating cows Milk production and composition and performance data in the lactating cow study showed some significant differences between cows fed diets containing the different lines of soybean, indicating a lack of their "substantial equivalence".

2.5.1.5. Testing the stability of the gene product In the acute gavage studies the EPSPS enzyme used was not isolated from the GTS lines but was an *Escherichia coli* recombinant product. This is a major flaw in the experimental design because, as even the authors themselves pointed out, post-translational modifications, such as amidation, acetylation and proteolytic processing of the completed polypeptide chains emerging from the ribosomes is so different in two such evolutionary distinct life forms as higher plants and prokaryotic bacteria that major differences in the conformation of the protein can be expected. As a result, these two products of the same gene may behave differently in *vivo* in the digestive system, putting a question mark to the authors' conclusion that the gene product from soybean could not have had any toxic effects because it broke down in an artificially simulated digestion test. Moreover, in gavage studies, unlike in the work described, young, rapidly growing animals must be used to establish whether the gene product has any toxic effect, affecting the growth of the animal. With older animals any effect on growth could only be shown if they were gavaged with potent toxins that by definition did not apply in this case.

The results of a separate study (Teshima et al., 2000) with toasted glyphosate-resistant GM soybean, in which rats and mice were fed with this GM soybean at 30% inclusion level in the diet for 15 weeks, could not be seriously considered because rat growth was minimal (less than 30 g over 105 days) and mice did not grow at all on either the test or control diets. This invalidates the authors' observations of finding no significant differences in nutritional performance, organ development, histopathology of the thymus, liver, spleen, mesenteric lymph nodes, Peyer's patches and small intestine and the production of IgE and IgG humoral antibodies between GM and non-GM line diets.

In an interesting paper, it was shown that the liver of mice fed on diets containing GM soybean underwent significant modifications in some nuclear features in comparison with livers from mice fed conventional soybean-based diets (Malatesta et al., 2002). Hepatocytes in GM soybean-fed mice showed irregularly shaped nuclei, indicating high metabolic rates, increased numbers of nuclear pores, suggestive of intense molecular trafficking and more irregular nucleoli with numerous small fibrillar centers, typical of increased metabolic rates. Nucleoplasmic and nucleolar splicing factors were also more abundant in GM-fed mice than in controls. Unfortunately, the design and execution of the feeding part of this study was poor which may put a question mark to the final conclusions. There is a general lack of appreciation by non-nutritionally oriented scientists that if the nutritional part of their study is not correctly designed and executed this will have an overriding influence on the results of the follow-up investigations, no matter how sophisticated these may be. Test and control diets must be iso-proteinic and iso-energetic and the starting weight of the animals must be closely similar and they must be strictly pair-fed before valid conclusions could be drawn from the studies carried out on internal organs and their ultrastructure.

An extensive production study with growing-finishing swine indicated the essential compositional and nutritional value equivalence of Roundup Ready and conventional soybeans (Cromwell et al., 2002). The results of this commercial production study are obviously useful for the industry even though their contribution to our understanding of the possible interactions between GM feedstuffs and the animal gut is limited.

Finally, as there is an inseparable link between glyphosate-resistant GM crops and the obligatory use of glyphosate with the GM plants a general warning must be given here. Although glyphosate is generally regarded as one of the most benign, wide-spectrum herbicides, there are some unconfirmed claims by Canadian scientists that spraying food crops with glyphosate may lead to elevated amounts of the toxic fungal mold, fusarium headblight, in the food crops. More serious was the published finding of French scientists (Marc et al., 2002) showing that there was a synergistic and concentration-dependent interaction between glyphosate and some of the chemicals used in herbicide formulations to delay the entry of cells into the M-phase of the cell cycle. Roundup inhibited the activation of CDK1/cyclin B *in vivo,* affecting cell cycle regulation by delaying the activation of the CDK1/cyclin B complex. These results may question the safety for human health of the widespread use of glyphosate and Roundup formulations with glyphosate-resistant GM crops.

2.5.2. GM corn

Rations containing transgenic Event 176 derived Bt corn were tested in a study involving 1280 birds (Brake and Vlachos, 1998). However, the results of this study are more relevant to commercial than to academic scientific studies. Similar conclusions could be drawn from other poultry feeding studies, such as that carried out with GA21 Roundup Ready corn-based

diets (Sidhu et al., 2000) or that with the maize line expressing the PAT protein (Flachowsky and Aulrich, 2001).

The conclusion by Kramer et al. (2000), that the GM corn developed by transferring the gene of egg white avidin to make the seed resistant to storage insect pests was safe for mice because they suffered no ill effects, can at best be regarded as premature. As the authors fed mice solely on GM or non-GM corn instead of on a balanced diet, it is not surprisingly that the mice did not grow at all with either.

The results of a study (Teshima et al., 2002) with GM corn (CBH351) expressing *Bt. thuringiensis* toxin Cry9C, in which rats and mice were fed with this GM corn at 50% inclusion level in the diet for 13 weeks in similar manner to their GM soya feeding study (Teshima et al., 2000), are open to the same criticisms as the latter study.

In a very interesting study, in which the testicular development in young mice fed on Bt-corn diets was estimated by dual parameter flow cytometry, it was shown that the GM corn had no measurable or observable effect on fetal, postnatal, pubertal or adult testicular development (Brake et al., 2004). According to the authors, if the results of this study were extrapolated to humans the consumption of Bt-corn cannot be regarded to have harmful effects on human reproductive development.

Finally, there have been some worrying reports of infertility in pigs given Bt-corn in Iowa, USA (www.organicconsumers.org/ge/pigfertility012703.cfm). However, there is no conclusive evidence whether this is linked to the genetic engineering of the corn or to the high levels of fusarium mold growth on the Bt-corn.

2.5.3. GM peas

In a 10-day feeding trial, the nutritional performance of rats fed diets containing transgenic peas expressing the transgene for insecticidal bean α-amylase inhibitor (about 3 g/kg peas!) at an inclusion level in the diet of 30% (Pusztai et al., 1999) was comparable to that of rats pair-fed iso-proteinic and iso-energetic diets containing parent-line peas and also lactalbumin diets spiked with isolated bean and pea α-amylase inhibitors, respectively. At this inclusion level, the nutritional value of diets containing transgenic or parent peas were not significantly different. Even at a 65% inclusion level the differences were small, mainly because the transgenically expressed recombinant α-amylase inhibitor in the pea was quickly (in less than 10 min) degraded in the rat digestive tract and therefore its antinutritive effect was abolished. In contrast, spiking the parental line pea diet with the stable, bean α-amylase inhibitor, as expected, reduced its nutritional value (Pusztai et al., 1995, 1999). Unfortunately, neither gut histology nor lymphocyte responsiveness assays were done and nutritional data alone are rather insensitive for finding possible differences in metabolic responses between GM and conventional food components: therefore, the results can only be regarded as preliminary. Although there were significant differences in the development of some organs of rats fed GM pea diets, mainly the enlargement of the cecum and pancreas (and a substantial but not significant increase in the size of the small intestine), most other organ weights were similar. At the end of the study cautious optimism was expressed that GM peas could be used in the diets of farm animals, particularly at the low/moderate levels recommended in commercial practice and if careful monitoring was made of the progress of the animals for the entire feeding period. However, the study cannot be taken to show that GM peas are safe for human consumption. This requires that further and more specific risk assessment will have to be designed and carried out. Moreover, only one particular line of GM peas was tested in which the endogenous antinutrient levels were selected to be similar to those of the parent peas.

In some other GM lines, however, lectin levels varied, up or down, by a factor of four and the concentration of trypsin and chymotrypsin inhibitors was usually significantly increased compared with their parent line (Pusztai, unpublished data). Therefore, to assess safety it is important that many GM lines are investigated and from the results of a single GM line no blanket approval should be given to other GM lines.

2.5.4. GM potatoes

2.5.4.1. Glycinin-expressing potatoes In a 4-week rat-feeding study both the experimental and control groups were fed the same commercial diet but they were also given by gavage 2 g/day of the respective potato lines/kg body weight. These were the parental control line and two transformed GM lines, one with the glycinin gene and another one with a designed glycinin gene (coding for four additional methionines in the gene product), respectively. Commendably, the authors (Hashimoto et al., 1999b) measured the growth, feed intake, blood cell count and blood composition and internal organ weights of the rats. However, it is unclear whether the animals were fed with raw or boiled/baked potatoes and this makes the interpretation of the results difficult.

2.5.4.2. Bt toxin potatoes A mainly histologic study was made of the ileum of mice fed with potatoes transformed with a *Bacillus thuringiensis* var. *kurstaki* CryI toxin gene compared to control mice fed potatoes treated with the toxin itself (Fares and El-Sayed, 1998). It was shown that both the δ-endotoxin and, to a lesser extent the Bt-potato, caused villus epithelial cell hypertrophy and multinucleation, disrupted microvilli, mitochondrial degeneration and increased numbers of lysosomes and autophagic vacuoles and the activation of crypt Paneth cells. Unfortunately, there were some flaws in the experimental design, such as the lack of proper description of the Bt potatoes and their gene expression level, or the uncertainty of whether the potatoes in the diet were cooked/baked or raw, or the failure to describe the amount of Bt toxin used to supplement the control potato diet. This makes it difficult to quantitatively compare the effects on the ileum of the Bt potato with the spiked control potato diets. All the same, this was an important study because once and for all it established that, in contrast to general belief, exposure of the mouse gut (ileum) to the CryI gene product caused profound hypertrophic and hyperplastic changes in the cells of the gut absorptive epithelium and these could lead to mucosal sensitization, as was later demonstrated (Vazquez Padron et al., 1999, 2000). These changes could only have occurred because, in contrast to the artificial stability shown in the *in vitro* simulated gut proteolysis tests, the Bt toxin did in fact survive, in a biologically active form, the passage through the digestive tract. Clearly, concerns about the possible biological consequences of exposure to GM food, such as those expressing the Bt toxin, should be addressed under *in vivo* conditions. As a result it was recommended that "thorough tests of these new types of genetically engineered crops must be made to avoid risks before marketing".

2.5.4.3. GNA GM potatoes Work concerning effects on the histology of the different gut compartments of feeding rats with diets based on GM potatoes expressing the snowdrop (*Galanthus nivalis*) bulb lectin (GNA) gene (Ewen and Pusztai, 1999b) revealed some major changes in gut structure and function. The significance of these results was further expanded in the authors' reply (Ewen and Pusztai, 1999a) to the invited comments by the *Lancet* (Kuiper et al., 1999) and also in a recent review (Pusztai et al., 2003). Some other selected results of the nutritional/metabolic studies published on the website of the Rowett Research Institute

(http://www.rri.sari.ac.uk), where most of the work was done (Bucksburn, Aberdeen, Scotland, UK), will only be briefly mentioned.

Young, rapidly growing rats (starting weight of 84 ± 1 g) were strictly pair-fed on iso-proteinic (60 g total protein/kg diet; most of which was from potatoes) and iso-caloric diets supplemented with vitamins and minerals for 10 days. The test diets contained GM potatoes, either raw or boiled. The control diets contained the same amount of parental line potatoes (raw or boiled) alone or supplemented with GNA at the same concentration as expressed in the GM potatoes. As a system control a lactalbumin group of rats was also included. Samples of stomach, jejunum, ileum, cecum and colon were, after fixation and staining with hematoxylin and eosin, subjected to full quantitative histological evaluation. This revealed that the thickness of the stomach mucosa was increased, partly due to GNA, the gene product (Ewen and Pusztai, 1999b). However, the proliferative hyperplastic growth of the rat small intestine leading to crypt enlargement and a part of the stomach enlargement was not a GNA lectin effect. Instead this was probably due either to some other component of the gene vector used for the genetic modification and/or the disruption caused by the incorporation of the vector in the plant genome. Indeed, unlike the strongly mitotic lectins such as the kidney bean phytohemagglutinin, GNA from snowdrops is a nonmitotic lectin whose binding to, and growth-promoting activity for, the small intestinal epithelium is slight and not significant (Pusztai et al., 1990) and as measured by staining with antiGNA antibody + PAP (peroxidase–antiperoxidase), it remains unchanged after the expression of its gene in GM potatoes. Hyperplasia was also confirmed by measuring the increase in crypt cell numbers and crypt mitotic figures in the jejunum of GM potato-fed rats (Pusztai et al., 2003). However, as the solanine glycoalkaloid content of the GM potatoes was significantly less than that of the parent lines (Birch et al., 2002) the suggestion that the jejunal growth was caused by potato glycoalkaloids could be ruled out. Overall the results suggested that crypt hyperplasia and the observed epithelial T lymphocyte infiltration caused by GM potatoes might also occur with other GM plants which had been developed using the same or similar genetic vectors and method of insertion. It is therefore imperative that the effects on the gut structure and metabolism of all GM crops should be thoroughly examined as part of the regulatory process before their release into the human food chain.

2.5.4.4. Potatoes expressing cationic peptide chimeras Desiree and Russet Burbank potatoes expressing N-terminus modified cecropin-melittin cationic peptide chimeras and control line potatoes fed to mice caused severe weight loss (Osusky et al., 2000). The animals did not grow even after supplementing these potatoes with Rodent Laboratory Chow. Apparently, mice fed with tubers from transgenic potatoes were as healthy and vital (sic) as those from the control group and their fecal pellets were comparable. The severe weight loss seriously questioned the value of the results of this poorly designed feeding experiment.

2.5.5. GM tomatoes

Tomatoes are of little importance in animal nutrition, but some of the ideas in this non-peer-reviewed book chapter describing the properties of a GM tomato line developed using the *Bacillus thuringiensis* crystal protein CRYIA(b) gene may have some general relevance in GM studies. Instead of the cauliflower mosaic virus 35 s promoter (CaMV 35 s), which is used in practically all first-generation GM crops, a potentially safer plant promoter was used (Noteborn et al., 1995). However, with this promoter the expression level of the Bt toxin was only about 1/20th of that found with CaMV 35 s and therefore the validity of the results of the

feeding studies may be questionable. However, this study is still of interest because, in contrast to most other studies with GM crops, there was a commendable attempt to use immunohistology to measure the binding of the gene product to the rat gut surface *in vivo,* rather than using spurious arguments about why the gene product should not bind. Unfortunately, instead of the Bt toxin isolated from GM tomatoes, an *Escherichia coli* recombinant and potentially less stable form of the gene product was tested, which puts a serious question mark over the results. However, even with this recombinant form the *in vitro* binding of the Bt toxin to gut sections, including the cecum and colon of humans and rhesus monkeys, was demonstrated by immunocytochemistry.

More recently, a Chinese study on the safety assessment of GM tomato and GM sweet pepper expressing the coat protein (CP) gene of cucumber mosaic virus (CMV) has been published. It appears that the expression of the CMV CP gene makes these plants resistant to CMV (Chen et al., 2003). It was claimed that these two GM products showed no genotoxicity either *in vitro* or *in vivo* by the micronucleus test, sperm aberration and Ames tests. According to the authors, the 30-day animal feeding studies showed no significant differences in growth, body weight gain, food consumption, hematology, blood biochemical indices, organ weights and histopathology in rats or mice of either sex, fed with either GM sweet pepper or tomato diets compared with those on non-GM diets, and therefore it was claimed that the GM crops appeared to be as safe as their comparable non-GM counterparts. However, some of these sweeping claims are difficult to accept on the basis of the actual data in the published paper. Additionally, there is a lack of precision in defining some of the parameters measured in the work. Thus, one of the major omissions is that the coat protein expression level in the plants is not given and in the toxicity tests it is impossible to see what is measured without making comparisons with equivalent amounts of CP, particularly as no attempt has been made to isolate CP from the two GM plants. The nutrition study has not been described adequately, no starting or during-the-experiment weights of the individual animals are given. Means are no substitute, particularly when as in Figure 3 the standard deviations in the bar diagram are so big (e.g. in 3 A at 3 weeks the mean weight of the rats is about 150 ± 50 g) that makes the in-between group comparisons meaningless. No diet composition and no animal management data are described, even though without pair-feeding no valid conclusions about weight gain, organ weights, biochemical blood indices, etc., can be arrived at. The graphs and data are uninformative. The size of the most important tissues, such as the small and large intestines, pancreas, etc., has not been recorded. The methods used for histological evaluation are not detailed and therefore it is impossible to see whether the authors used appropriate methods or not. In view of these deficiencies it is difficult to accept the authors' conclusions that these GM plants are as safe as their conventional counterparts.

3. EVALUATION AND CONCLUSIONS

For the evaluation of the safety of GM food/feed the significance of differences, if any, in the parameters outlined above should be established by suitable statistical analyses (ANOVA, multiple comparisons and/or multivariate analysis). If the experiments show up differences between animals fed GM and parent-line diets this indicates that the genetic modification must have had a significant effect on the utilization and nutritional value of the GM crop and therefore this cannot be accepted as it is without further more detailed studies. However, if both the GM diet and the parent-line diet spiked with the gene product show differences, the use of this gene in GM food/feed is not acceptable. Finally, if negative effects are observed with the GM crop but not with the parent line diet containing the isolated gene product, it is

likely that the harm is caused by the use of the particular construct or by an unwanted or unforeseen effect of the gene insertion on the genome.

In this overview, peer-reviewed and published biological studies carried out with GM materials have been described and discussed in the framework of a suggested safety testing protocol (Pusztai, 2002). However, this can only be regarded as a start. There is a compelling need to further develop the concepts of biological testing, particularly for the investigation of potential long-term GM effects. Moreover, since the GM potato work with male rats showed abnormalities in the development of their sexual organs, it is imperative that similar experiments should also be done with female rats, to be followed by studies of the effects on reproductive performance of rats (or other animals) reared and maintained on GM vs non-GM diets for several generations.

4. FUTURE PERSPECTIVES

Apart from the practical considerations discussed in this review, the conceptual framework of the safety of genetically modified products also needs to move forward. In this respect it would be helpful in the interests of science to generally recognize and accept that views such as that "transgenic varieties expressing a single agronomic trait (are) not expected to (have an) altered nutrient composition" (Aumaitre et al., 2002) fly in the face of the facts and published data. The adherence of some plant molecular biologists to the almost mythical concept of "substantial equivalence" with single-trait first-generation GM crops is now a serious impediment for further progress in GM studies, as has also been recognized by the *Codex Alimentarius* guidelines. In any case, with multiple traits of the coming GM generations it will be impossible to use this as a conceptual basis for risk assessment. However, even with the present single-trait GM crops, there is a need to put some of the most important and well-established principles in the center of the science of genetic modification because, in addition to their general validity, they also apply to the recombinant gene technology work (Schubert, 2002). Thus, it needs to be recognized that the introduction of the same gene into two different cell types can produce two proteins, which can have distinct properties. Moreover, with this the overall gene expression and the phenotype of the cell may also change and it is possible that the genetically introduced enzymatic pathways could interact with endogenous pathways of the cell. Genetic engineering is therefore able to produce novel, possibly harmful, toxic, allergenic or carcinogenic molecules. As this is unpredictable, their presence can only be established from biological testing experiments, particularly from those carried out for extended time periods. Although not necessarily for the same reasons, one has to agree with the views of some biotechnologists that relatively short-term animal feeding/production experiments, particularly as they are presently carried out, do not contribute much to GM safety.

As described in this short overview, it is clear that most of our understanding of the possible consequences of GM food/feed ingestion has come from academic, basic research studies in which the digestive tract was investigated as the main target organ for the GM ingredients and then followed up by looking for the general metabolic consequences of this initial interaction between food/feed and the gut. Although, regretfully, there are only a few examples of such published studies, even these few have clearly indicated that methods based on the striking biological effects of the transgenic DNA and proteins on the gastrointestinal tract could be developed into potentially powerful tools in GM food/feed risk analysis. There is therefore a need to extend the present commercial, ad-hoc, production-oriented research of relatively crude comparisons of GM or non-GM feed rations performed with farm animals. More basic

studies are needed, in order to establish the scientific principles of the possible GM effects on the gut, including not only that of the transferred gene but also of other parts of the genetic construct, particularly the promiscuous viral promoters such as the cauliflower mosaic virus promoter, and the effect of their incorporation into, and their position in, the plant genome. It is no longer acceptable to have bland assurances, such as that CaMV 35 s promoter is specific to plants and will not work in animal cells, when indeed this is against the observed biological wisdom. It is also unacceptable to expect that the behavior and the potential hazard of the CaMV 35 s promoter incorporated into the chromosome of transgenic plants is the same as the replication and behavior of the virus whose DNA possibly never integrates into the chromosomes of the infected plant cell. These issues are open to experimentation and as such ought to be decided by *in vivo* observations.

With the development of a thorough understanding of the possible changes in the physiology, nutrition, immunology and metabolism of the animals receiving GM products, a more rational basis for further advances in animal health and production can be and indeed needs to be established. Unfortunately, considering the strikingly poor record of peer-reviewed publications of GM studies in the scientific literature, the often-publicized great potential of the genetic manipulation of our food/feed crops, is apparently based less on scientific achievements than on future promises. This is all the more curious, because some of the science and ethical considerations which appear to impede the efforts to establish risk assessment methods for human health safety with GM foods most of the time do not apply to animal work. Even a cursory look at the huge numbers of published papers on animal nutrition research with conventional feedstuffs reveals that most of the methods for investigating the interactions between novel protein-containing feeds and the gut and their metabolic consequences for the animals are well established. It is hoped, therefore, that if sufficient funding is made available, scientists working with GM will be keen to make good use of these methods, for our benefit.

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ADDENDUM

Although the release of new GM products has continued unabated in the 10 months since the main body of our review was completed, the number of papers describing the underpinning science and principles that supported their safety does not appear to have increased substantially. Nevertheless, our review could not be regarded complete without evaluating some of these.

1. SAFETY ASSESSMENT OF GM CROPS

1.1. Compositional studies

Despite great advances in analytical methodology with the introduction of proteomics, metabolite and DNA/RNA profiling, fingerprinting, microarrays, etc. into basic laboratory science, regrettably, judging from the papers published or the submissions of biotechnology companies, scientists engaged in the evaluation of the safety of GM crops have apparently been rather reluctant to rely on these novel techniques. Accordingly, rather than to aim for completeness in this review only selected examples will be given to demonstrate the rather sterile and static conventional analytical approach to safety that still appears to be the norm

and juxtapose these with the occasional examples of more novel research work attempting to unravel the actual molecular mechanisms involved in genetic modification.

1.1.1. Herbicide-resistant soybeans

With the publication of a major paper comparing the chemical composition and protein quality of soybeans from five leading soybean-producing countries (Karr-Lilienthal et al., 2004) the often-predicted quality/yield drag of genetic modification appears to have received some, albeit indirect, support by showing that the protein, some amino acid and mineral contents of the almost exclusively GM soybeans from Argentina were the lowest of the five countries. It was particularly interesting that the phenylalanine content of the Argentinian soybeans, similar to that of the Puerto Rican-produced GM soybeans (Padgette et al., 1996), was particularly low, signaling potential problems in the synthesis of some key aromatic metabolites.

1.1.2. GM potatoes

The composition of two GM lines G2 and G3 expressing *Cry V* gene from *Bacillus thuringiensis* was compared by conventional analytical methods with that of the Egyptian parent line called Spunta in a recently published paper (El Sanhoty et al., 2004). A general comment: it would have been helpful if the authors had made clear whether the potatoes used for the analyses were from the Egyptian markets as stated in the introduction or from Michigan field plots in the USA as indicated under materials and methods. According to the authors, none of the 14 main chemical components in the two GM lines and the parent potatoes was significantly different. However, a closer examination of the results in tables 2–5 of El-Sanhoty et al. (2004) showed that the contents of some components of the control and either or both of the GM lines may have differed significantly. Thus, the ascorbic acid, phosphorus and calcium contents in table 2 (El-Sanhoty et al.) appeared to be different. The contents of some amino acids in the three lines (table 3, El-Sanhoty et al.) also showed major differences, chief amongst them were methionine, cystine, tryptophan and histidine. Curiously, phenylalanine was not listed in table 3 (El-Sanhoty et al.) and each of the methionine and tryptophan contents exceeded that of aspartic acid. Both genetically modified potato lines contained significantly higher protease inhibitor activity than the parent line (table 5, El-Sanhoty et al.). The general state of the presentation of the results was rather poor and left the reader confused whether to believe the text or the tables. In fact, the results appeared to contradict the conclusions of the paper that the parent and GM potatoes were substantially equivalent.

In contrast to the traditional approach of using standard analytical techniques to establish the "substantial equivalence" of GM and non-GM lines as above, the great value of the molecular approach was demonstrated in some new research papers. These included publications in which the mechanism of the potential nutritional enhancement by genetic engineering by increasing the contents of β-carotene and other carotenoids of various crops has been investigated. A good example is the GM potato plants expressing an *Erwinia uredovora crtB* gene encoding phytoene synthase in the tuber of *Solanum tuberosum* cv. *Desiree* (Ducreux et al., 2004). Clearly, the main aim of the researchers was to understand the molecular basis of the gene transfer and the working mechanism of the genes involved in carotene biosynthesis and their regulation but not to show whether the potato tubers of higher carotenoid content were as safe as the conventional tubers. However, it would have been advantageous to use the microarray analysis not only to establish changes, up- and down-regulation in the transcription of cDNAs involved in carotenogenesis, such as that of fibrillin but also other more

nutritionally important proteins. Hopefully, together with other compositional parameters, these will come from future studies, the results of which would allow us to judge the safety of these GM potatoes, particularly if and when they will be subjected to short- and long-term animal feeding safety studies.

1.2. Published nutritional/toxicological studies

In the absence of generally agreed safety testing procedures for GM crops intended for animal/human nutrition the papers published in the last year have followed previous patterns. Whilst in some of them, particularly those associated with testing the safety of new biotechnology GM products, the methods used were similar to those established earlier for Roundup Ready soybeans (Hammond et al., 1996; Harrison et al., 1996), some others tried to find new and more functional and/or cellular methods to establish the effects on animal health of the GM crops of nutritional importance.

1.2.1. Herbicide-resistant soybean

The Malatesta group continued with their histology work aimed at establishing what effect, if any, the long-term feeding of GM-soybean-based diets had on the structure and function of some important body organs of mice. Their previous finding of reduced digestive enzyme synthesis and secretion in the pancreas (Malatesta et al., 2002b) in GM-soybean-fed mice has now been confirmed and extended by showing that in these animals the accumulation of nucleoplasmic and nucleolar splicing factors and perichromatin granules was also significantly reduced, suggesting reduced post-transcriptional hnRNA processing and/or nuclear transport (Malatesta et al., 2003) that may be the underlying mechanism of the deficiency in digestive enzyme synthesis and secretion by pancreatic acinar cells.

Due to the ever-increasing area of cultivation of glyphosate-resistant RoundupReady GM crops, the use of glyphosate has also increased. For this reason some newly published work on the possible health-damaging effects of glyphosate need to be dealt with in this review. Further findings by French scientists (Marc et al., 2005) have confirmed and extended their previous results by showing that the main ingredient of commercial Roundup formulations, glyphosate, in a millimolar concentration range, particularly when used together with the obligatory polyoxyethylene amine surfactant, significantly delayed the hatching of sea urchin embryos by inhibiting the transcription of one of the enzymes involved in hatching. As inhalation of herbicide sprays in which the active ingredient concentration exceeds by about 25 times that used in the transcription inhibition studies, health concerns associated with the use of glyphosate may have to be taken truly seriously. In another study it was shown that the oral treatment of Wistar rats to increasing concentrations of the herbicide Glyphosate-Biocarb, a formulation used in many countries such as Brazil, the number of Kupffer cells in hepatic sinusoids increased, followed by large deposition of reticulin fibers and the leakage of hepatic aspartate-aminotransferase and alanine-aminotransferase into the circulation, indicating hepatic damage in these animals (Benedetti et al., 2004). In further studies by another group of French researchers it was shown that glyphosate, particularly as used together with polyoxyethylene amine surfactant in Roundup formulations, was toxic to human placental JEG3 cells at concentrations lower than that used in agriculture. Even at subtoxic concentrations Roundup was an endocrine disruptor on aromatase activity and its mRNA level and glyphosate interacted with the active site of the purified enzyme (Richard et al., 2005). It is possible that the pregnancy problems in agricultural workers using Roundup may be traced

back to the exposure to this herbicide (Savitz et al., 2000). All these findings indicate that there is an urgent need to carry out systematic and direct studies, independent of the biotech industry, on the short- and long-term effects on animal (and human) health of exposure to glyphosate and its more effective commercial formulations. With the presently cultivated huge areas of Roundup Ready crops and the anticipated even larger future extensions of this glyphosate-dependent GM crop technology the potential danger for animal/human health needs to be dealt with in advance and not if or when it occurs.

1.2.2. GM corn

A 13-week rat-feeding safety assurance study with Roundup Ready GM corn (NK 603) was carried out to a design similar to that used previously with Roundup Ready soybean (Hammond et al., 2004). Indeed, the work could be similarly criticized. Thus, no precise composition of the test or control diets was described in the published paper. No starting weight range of the rats was given, only the mean weight of the groups with the assurance that their standard deviations were within 95% confidence limit. However, the wider the starting weight range the more difficult it is to find significant differences in growth during the feeding experiment. This would have been difficult to establish in any case because the growth and feed intake graphs were crude and uninformative. Without error bars one has to take the assurances given in the paper that there were no significant differences between the test and control groups. The low values of about 12–13% for feed conversion efficiency indicate problems with the diet. Indeed, the fact that feed intake remained static or even declined in the second part of the experiment pointed to a similar conclusion. Some of the critical hematology mean values, such as WBC (white blood cell count), NEU (leukocyte differential count) and LYC (lymphocyte count) that could indicate problems in immune responses, had huge SD values (standard deviations; almost ± 30% in some cases), so the authors' claim was not surprising that there were no significant differences between the groups. Some of the serum chemistry SD values were only little better. The two most important tissues, the small intestine and the pancreas, were not weighed. The acute mouse gavage study was conducted with the CP4 EPSPS surrogate recombinant protein and not that isolated from the GM corn although it is known that this could lead to erroneous conclusions. In view of all these flaws in the experimental design and the execution of the study it is difficult to accept the conclusion of the authors that the Roundup Ready GM corn is equivalent to its traditional counterpart. In view of the lack of experimental evidence presented in the paper, such as the use of a second control group in which the parent line is supplemented with the gene product isolated from GM corn or a third zero control group in which the parent corn was transformed with the empty vector, it is even more difficult to understand how one can accept the conclusion of the authors that their study did not detect any pleitropic effect.

The survival of transgenes and/or their products in the animal gastrointestinal tract and their uptake into body organs (e.g. Klotz et al., 2002; Chowdury et al., 2003) is a hotly debated issue with clear implications for human/animal safety. However, this issue cannot be separated from taking an account of the sensitivity of the detection methods used as shown by finding that amplifiable fragments of rabbit retrotransposon and mitochonrial DNAs were taken up from the gastrointestinal tract in human volunteers (Forsman et al., 2003). Although it is not possible to say whether one can expect any physiological effects as a result of this DNA uptake across the species barrier, it cannot be assumed either that the DNA transferred from the gut is nonfunctional. Indeed, DNA vaccines are known to have been successfully developed and oral vaccination has been achieved with an artificial promoter-driven DNA coding for an immunogenic protein (Jones et al., 1997).

Some of the possible problems in the detection of surviving gene products have been highlighted by a recent finding that the amounts of intact Cry1Ab protein in the bovine gastrointestinal tract of cows fed GM maize measured by ELISA appeared to be appreciable but only fragments of the Bt toxin could be detected by immunoblotting (Lutz et al., 2005). However, without establishing what, if any, physiological effect and/or possible toxicity for the animals of these immunoreactive Cry1Ab protein fragments can have, the assurance that GM corn is safe implied in finding no intact Bt-toxin in the bovine intestines cannot be accepted without further studies.

1.2.3. GM potatoes

GM potatoes expressing a cysteine-proteinase inhibitor, rice cystatin, are partially (or in some cases fully) resistant to nematodes without apparently harming nontarget anthropods or perturbing soil microbial communities (Urwin et al., 2003). As it occurs naturally in rice, maize and even in potatoes, cystatin is not new for the mammalian digestive system. Moreover, the expression of cystatin can be limited to the roots, further reducing the already potentially low concern of toxicity or allergenicity with these GM potatoes. According to recent claims by the Atkinson group, their nutritional studies presented *prima facie* evidence that cystatin-expressing GM potatoes do not present toxic risks to mammals (Atkinson et al., 2004). However, in reality these authors did not test the safety of the GM potatoes. Neither did they carry out a proper nutritional evaluation of them. What they actually tested was the *in vitro* stability to digestion with simulated gastric fluid at pH 1.2 and at abnormally high concentrations of pepsin, of a surrogate recombinant *E. coli* protein and not that isolated from GM potatoes or, even more importantly, not of the GM potatoes themselves. Thus, this *in vitro* simulation test was flawed both in principle and execution. Instead of a proper nutritional evaluation of the GM potatoes the authors used this recombinant surrogate protein in a LD_{50}-type oral toxicity study, the design of which was seriously flawed. Rats of unknown starting weight were kept in groups of five, and not individually, making it impossible to follow their individual feed intakes and growth and to relate therefore their body tissue weights and other measured parameters to final bodyweights. Although some body organs were weighed and some, such as the liver showed differences, the only part of the gastrointestinal tract that was measured was the cecum, but not the small intestine or the colon, the very tissues that are the most sensitive indicators of dietary effects. This omission is the more serious because the weight of the cecum, which is a relatively small part of the large intestine, was found to be significantly increased (unrelated to the final body weights!). For all these flaws the authors' conclusion that these cystatin-expressing GM potatoes do not present toxicity risks to mammals may need to be reassessed by further studies.

Raw Punta G2 and G3 GM and non-GM potatoes whose analytical composition was given above were also subjected to a 30-day feeding study with rats (El Sanhoty et al., 2004). Some of the annoying discrepancies between data in the text and the tables should have been removed during the peer-review and editing. As the potato inclusion in the diet was only 30%, less than 15% of the protein in the diet was provided by the GM potatoes. More seriously, the composition of control group 1 was substantially different from the potato-containing diets as its protein content was about 15% less. It was not made clear why all the diets were supplemented with 0.3% methionine when the potatoes were not deficient in this amino acid. Differences in rat starting weights (± 10%) were too high although they improved after prefeeding. However, despite assurances by the authors that feed efficiency values were not significantly different between the test and the control groups this cannot be accepted on the

basis of the data in table 6 of El-Sanhoty et al. (2004). Similar considerations apply to testes weights in table 7 (El-Sanhoty et al.). Apart from the specific criticisms above, the design of the feeding study was rather simplistic and static without looking at more dynamic novel features in the GM-fed rats, such as possible changes in immune, hormonal and metabolic functions, and no attempt was made to use histopathology methods to detect and/or exclude potential alterations in the cells or tissues of the rats on different diets. A particularly serious omission in the work is the absence of a critical look at the structure and function of the small intestinal epithelium.

1.2.4. GM crops expressing bioactive proteins to replace antibiotics in animal nutrition

The cultivation of a new class of GM crops expressing bioactive or pharmaceutical proteins has been on the increase in the last few years. One of the first examples of these was GM rice lines expressing lactoferrin or lysozyme designed to protect the gastrointestinal tract against bacterial infection in place of antibiotics in the feed (Humphrey et al., 2002). In a chicken-feeding study of rather limited scope and design in which some of the nutritional and physiological parameters of chickens fed on diets containing antibiotics such as bacitracin and roxarsone included in a conventional rice diet, were compared with diets containing GM rice expressing lactoferrin or lysozyme or a mixture of both. The most serious major flaw in the two experiments carried out to the same design was that in the feeding studies not all necessary control groups were included. Thus, the effects of the two GM rice lines were only compared to a conventional rice diet with or without added antibiotics but not to that of conventional rice control diets in which lactoferrin or lysozyme or both at the same concentration as they were expressed in the appropriate GM rice lines were also included. In these controls lactoferrin and/or lysozyme supplementation should have been done with the proteins isolated from the appropriate transgenic rice line. However, even the inclusion of these two proteins from conventional sources could have demonstrated whether the authors were justified in suggesting a truly "antibiotics effect" on the bacterial flora in the GM-rice-fed chickens as an explanation or whether what they measured was a direct effect of these two proteins on the gut epithelium. Although the gut histology indices were suggestive that these two proteins through their effect on the bacterial flora may have indeed been indirectly responsible for the modest nutritional improvement described in the paper but direct evidence by monitoring the gut bacterial population and counts could have given a more convincing proof for the authors' thesis. In the absence of such evidence further work is needed to substantiate the authors' suggested action mechanism. On a separate issue, the long-term human/animal safety of GM rice expressing bioactive proteins also needs to be established, particularly in view of the possibility of their cross-pollination with non-GM rice.

2. CONCLUSIONS AND PERSPECTIVES

Although there has been no great improvement in the science underpinning GM crop safety in the actual papers published in the intervening year since the completion of the main part of this review, some hopeful signs have appeared. Scientists funded by the European Union as part of the Thematic Network ENTRANSFOOD have recently reviewed the work focusing on the evaluation of current approaches to risk assessment of GM-crop-derived foods and potential needs for further improvements in safety testing methods and have come up with their recommendation of adopting a step-wise approach to safety assessment. Their recommendations have been summarized in a "Concluding Remarks" paper by Kuiper et al. (2004), together with references to the individual papers dealing with safety issues in detail.

It is hoped that this will take us a step closer to having an agreed procedure for risk assessment and safety testing of GM crops that will be based on solid scientific principles. One can but hope that in this testing procedure the recommendations of two recent influential reviews (Wilson et al., 2004; Freese and Schubert, 2003) will also be incorporated.

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