

Summaries of Toxicological Data

SAFETY EVALUATION OF THAUMATIN* (TALIN PROTEIN)

J. D. HIGGINBOTHAM and D. J. SNODIN†

Philip Lyle Memorial Research Laboratory, Tate and Lyle Group Research and Development,
The University, Reading RG6 2BX

K. K. EATON

The Cedar House, Popeswood Road, Binfield, Berkshire RG12 5AD

and

J. W. DANIEL

Life Science Research, Stock, Essex CM3 9PE, England

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Abstract—Thaumatococcus daniellii (Benth.) has been studied for its subacute toxicity in rats and dogs and its ability to produce anaphylactic antibodies following oral administration to rats and normal human subjects. Thaumatococcus was readily digested prior to absorption in rats and no adverse effects resulted from its continuous administration to rats and dogs at dietary concentrations of 0, 0.3, 1.0 and 3.0% for 13 wk. It was not teratogenic when administered orally to rats at 0, 200, 600 and 2000 mg/kg body weight/day from day 6 to 15 of gestation and was without effect on the incidence of dominant lethal mutations when administered on five consecutive days to male mice at 200 and 2000 mg/kg/day. The lack of mutagenic potential was confirmed in bacterial mutagenic assays with *Salmonella typhimurium* (strains TA1535, TA1537, TA1538, TA98 and TA100) and *Escherichia coli* WP2, at levels of addition of 0.05–50 mg/plate. In rats, thaumatococcus was found to be a weak sensitizer, comparable with egg albumen, when administered systemically but to be inactive when administered orally. Prick testing of laboratory personnel who had been intermittently exposed by inhalation to thaumatococcus for periods up to 7 yr showed that 9.3% (13/140) responded positively to commercial thaumatococcus, while 30.7% were positive to *Dermatophagoides pteronyssinus* (house dust mite). None of the subjects who gave a positive skin reaction to commercial thaumatococcus responded to the plant components remaining after removal of the specific sweet Thaumatococcus proteins. Challenge tests in man did not demonstrate any oral sensitization. The results indicate that thaumatococcus when used as a flavour modifier and extender, and partial sweetener, is unlikely to be hazardous at the anticipated level of consumption.

Introduction

The intense sweetness of fruit of the West African perennial plant *Thaumatococcus daniellii* (Benth.) was first described by a British surgeon in the *Pharmaceutical Journal* (Daniell, 1855). The sweet principle, which occurs in the aril (the soft fleshy part attached to the seeds), was identified subsequently as a mixture

of proteins, Thaumatin I, II and O, with molecular weights of 22,209, 22,293 and 22,000 $\pm$ 500 daltons, respectively, and with iso-electric points in the range 11.5–12.5. The Thaumatin proteins have a normal complement of amino acids with the exception of histidine, which is absent, and differences in the ionic charge are due to the degree of amide substitution in the glutamyl and aspartyl residues. The amino-acid sequences have been reported (Edens, Heslinga, Klok *et al.* 1982; Iyengar, Smits, van der Ouderaa *et al.* 1979) and Thaumatin I has been crystallized (van der Wel, van Soest & Royers, 1975).

Thaumatococcus has been developed both as a high-intensity sweetener and as a flavour potentiator capable of improving and extending the flavour characteristics of a variety of food products (Higginbotham, Lindley & Stephens, 1981). It has been approved for such uses without limitation in Japan and is approved for use as a sweetener in the UK (Ministry of Agricul-

*Thaumatococcus written with a lower case initial letter refers to the commercial material, which is an extract of the arils of *T. daniellii*, whereas Thaumatin, written with a capital initial letter, refers to one of the characterized proteins, for example Thaumatin I or Thaumatin II, which are the intensely sweet-tasting components of thaumatococcus.

†From whom more detailed reports of these studies may be obtained.

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ture, Fisheries and Food, 1983). This paper reports studies conducted to establish the safety of thaumatin (Talin[®] protein) when used as a food ingredient. A preliminary account of these experiments was presented at the 21st Annual Meeting of the Society of Toxicology (Higginbotham, Snodin & Daniel, 1982).

Test material

Toxicological studies were conducted over a 7-yr period, during which time various improvements were made in the production of thaumatin (US Patent No. 4,011,206; UK Patent No. 2,015,533). The most significant improvement was achieved by extraction of arils only and by the introduction of highly selective ultrafiltration techniques. Prior to the introduction of these improvements, thaumatin was manufactured to the following specification:

Protein 78–85%; moisture—max 10%; extracting salts—max 7% (principally Na citrate/citric acid); carbohydrate—max 2% (including some polysaccharide); heavy metals, below detectable levels (total as Pb < 0.5 ppm).

The present specification is as follows:

Specific absorption, $E_{1\text{cm}}^{1\%}$ —not less than 12 nor more than 12.5, determined at λ max c. 279 nm using a 0.1% (w/v) solution at pH 2.7; nitrogen—min. 16.0% (protein = N $\times$ 5.8); sulphated ash—max. 2.0%; carbohydrate—max 3.0% and aluminium—max 0.01% (all on a dry weight basis); volatile matter—max 9.0%.

By virtue of their highly charged nature, the Thaumatin proteins may be readily separated from commercial thaumatin using cation resins such as Whatman CM 32, Na form. This has been done, and the non-Thaumatin components (approximately 3% of thaumatin) have been separated by ultrafiltration (using an Amicon Diaflo system) into fractions of greater and less than 5×10^4 daltons. The lower-molecular-weight fraction contained 2.5% carbohydrate and 18% protein (N $\times$ 6.25), and the higher-molecular-weight fraction contained an apparent 20% carbohydrate and 91% protein. Clearly, the carbohydrate content is overestimated using the standard analytical method (a colorimetric cysteine-sulphuric acid procedure) and actual levels of carbohydrate in commercial thaumatin are one third or less of the maximum level stated in the specification. Acid hydrolysis of the higher-molecular-weight fraction followed by paper chromatographic analysis indicated the presence of three sugars—arabinose, xylose and traces of a neutral non-amino hexose, probably a glucuronic acid derivative. It was concluded that the original polysaccharides were arabinogalactans and arabinoglucuronoxylans, which occur in all land plants and are particularly associated with pectins in the flesh of edible fruits (Aspinall, 1970), as well as being common seed mucilage components (Whistler, 1965). The mucilage, or gel, surrounding the *T. daniellii* seed has been fully characterized as an arabinoglucuronoxylan (Adesina & Higginbotham, 1977). Thus, residual gel adhering to *T. daniellii* arils is probably solubilized during the manufacturing process and contributes to the carbohydrate found in thaumatin.

Animals and diets

Male and female rats and mice of the CD strain were obtained from Charles River (UK) Ltd, Margate, Kent, and were given water and powdered rodent diet (R and M No. 1; B.P. Nutrition (UK) Ltd, London) *ad lib*. The only exception was in the digestibility study, in which a semi-synthetic diet was used. Rodents were maintained at $20 \pm 2^\circ\text{C}$ and 50–70% relative humidity. Beagle dogs were obtained from Perry Croft Farm Kennels, Colwall, and were acclimatized for a period of 4 wk. They were housed in single galvanized pens with a floor area of $1.5\text{ m} \times 1.75\text{ m}$ and were fed 400 g/day of a complete dog diet (Lab. A; B.P. Nutrition (UK) Ltd). All animals were subjected to a 12-hr light dark cycle.

Experimental diets were prepared weekly and stored at ambient temperature. Previous studies had demonstrated that thaumatin under these conditions was stable for at least 2 wk. All animal feeding studies performed after 1978 were in accordance with the requirements of the FDA regulations on Good Laboratory Practice.

Digestibility of thaumatin

Experimental procedure

Twenty male rats (body weight 140–160 g) were randomly assigned to two equal-sized groups and were housed in individual metabolism cages.

Group 1 was fed a semi-synthetic diet containing 5% maize oil, 60.45% starch, 15% cellulose, 2% vitamin and mineral pre-mix, 2.2% CaHPO_4 , 0.88% CaCO_3 , 1.27% NaCl , 0.95% KCl , 0.7% MgCO_3 and 11.42% egg albumen (diet A), while Group 2 received a similar diet but containing 5.7% egg albumen and 5.73% thaumatin (diet B). This procedure was adopted to compensate for thaumatin's lack of histidine, an essential amino acid for the rat. Each diet was fed for 10 days, the last 5 of which constituted the balance period during which food consumption was measured and excreta were analysed for nitrogen using the Kjeldahl method. Subsequently each group received the other diet for a similar 10-day period. Each animal received 30 g/day of the appropriate diet mixed with water (25 ml). Any food not consumed by an animal was combined separately for each of the four balance periods, dried at 105°C for 24 hr, weighed and analysed for nitrogen. Conversion factors of 6.7 and 5.7 were used to calculate the amount of egg albumen and thaumatin in diet.

The total nitrogen intake (IN) and the nitrogen eliminated in the faeces (FN) was calculated for each animal, and protein digestibility (D) was calculated from the expression: $D = (\text{IN} - \text{FN})/\text{FN}$.

Assuming that the digestibility of individual proteins is independent of concentration and the presence of other proteins, then the digestibility of thaumatin is given by: $D(\text{thaumatin}) = (aD(E + \text{thaumatin}) - bDE)/c$, where $D(E + \text{thaumatin})$ and DE are the digestibilities of egg albumen plus thaumatin and egg albumen diets, respectively, and a , b and c are the nitrogen content of the albumen thaumatin diet, and the nitrogen derived from albumen and from thaumatin, respectively. Since a cross-

Table 1. Nitrogen digestibility of diets containing egg albumen and egg albumen plus thaumatin fed to rats

Group no.	Dietary protein*	Nitrogen digestibility (%) in:	
		Balance period 1	Balance period 2
1	Albumen	89.1 $\pm$ 0.29	—
	Albumen + Th	—	90.3 $\pm$ 0.29
2	Albumen + Th	89.9 $\pm$ 0.53	—
	Albumen	—	89.7 $\pm$ 0.19

Th = Thaumatin

*The semi-synthetic diet contained either 11.42% egg albumen or 5.7% egg albumen plus 5.73% thaumatin.

Digestibility figures are means $\pm$ 1 SD for groups of ten rats.

over study design was used each animal served as its own control.

Results

The nitrogen digestibility of each of the experimental diets is shown in Table 1. As no attempt was made to measure the loss of endogenous nitrogen, these estimates of the amount of protein digested are to be considered minimal. The digestibility of thaumatin calculated for the two balance periods was 89.9 ± 0.52 and 91.2 ± 0.48 , respectively.

Subacute toxicity in rats and dogs

Experimental

Thaumatin was fed to groups of 20 male and 20 female rats, housed five/cage, and to groups of four male and four female dogs at dietary levels of 0, 0.3, 1.0 and 3.0% for 13 wk. The condition of all animals was assessed daily and weekly records of body weights and food consumption were maintained. Ophthalmoscopy was performed on all animals before treatment and on those in the control and top-dose groups during wk 12. Water intake was measured in the rats on three consecutive days before treatment and again during wk 6 and 12.

Blood samples were obtained during wk 4 and 12 from the retro-orbital sinus of ten male and ten female rats/group and from the jugular vein of all dogs and were analysed for haemoglobin, packed cell volume (PCV), erythrocytes, platelets and leucocytes (total and differential). Dog blood was also analysed for reticulocytes and erythrocyte sedimentation rate. Clotting characteristics were assessed in both species by measuring prothrombin and partial thromboplastin times.

Serum was separated and analysed for glucose, urea nitrogen, total protein, albumin, globulin, cholesterol, triglycerides, total bilirubin, sodium, potassium, alkaline phosphatase, glutamic-oxalacetic transaminase and glutamic-pyruvic transaminase. Urine was collected during the final week of treatment and examined for the presence of blood, glucose, protein, ketones and bilirubin.

At the end of treatment, animals were exsanguinated under barbiturate anaesthesia (dogs) or CO₂ asphyxiation (rats), a post-mortem examination was performed and any macroscopic abnormalities were recorded. The brain, pituitary, caecum, lungs, liver, spleen, kidneys, thyroids, adrenals, prostate, uterus and gonads were weighed. Samples of these organs,

together with the aorta, cervical and mesenteric lymph nodes, colon, duodenum, epididymides, eyes and optic nerve, ileum, jejunum, mammary gland, oesophagus, pancreas, salivary gland, seminal vesicles (rat), skeletal muscle, spinal cord, sternum, stomach, thymus, tongue and urinary bladder were preserved in 10% buffered formal saline or Davidson's fluid. Sections were stained with haematoxylin and eosin and examined by light microscopy. Liver, kidneys, heart, lung, spleen and brain from all rats were examined, while examination of the remaining tissues was restricted to rats in the control and top-dose groups. In the dog study, all tissues from all animals were examined.

Statistical analyses were performed using Student's *t* test.

Results

The effect of thaumatin ingestion on the growth of rats and of dogs is shown in Tables 2 and 3, respectively. Male rats fed diets containing 3% thaumatin had a mean body weight higher than that of the corresponding controls at wk 4, whereas the body weight of females fed 1 or 3% thaumatin was reduced at wk 8 and 12. There was no evidence that treatment had any effect on the growth of dogs despite the significant increase observed during wk 12 in males fed 1% thaumatin.

Food consumption was similar in all groups and the average daily intakes of thaumatin for rats were estimated to be 243, 861 and 2417 mg/kg for males and 293, 1000 and 2822 mg/kg for females. The corresponding values for the dog were 140, 450 and 1400 mg/kg for both sexes.

The principal change detected in the haemato-

Table 2. Mean body weights of rats fed 0-3% thaumatin in the diet for 13 wk

Dietary level (%)	Mean body weight (g) at wk:			
	0	4	8	12
Males				
0	114	287	395	447
0.3	114	282	387	436
1.0	114	293	398	452
3.0	113	304*	415	466
Females				
0	103	187	240	263
0.3	103	185	230	250
1.0	103	182	225*	244*
3.0	102	188	233	253

Values are means for groups of 20 rats and those marked with an asterisk differ significantly from the control: **P* < 0.05 by Student's *t* test.

Table 3. Mean body weights of dogs fed 0-3% thaumatin in the diet for 13 wk

Dietary level (%)	Mean body weight (g) at wk:			
	0	4	8	12
Males				
0	7.2	8.5	9.3	9.8
0.3	7.1	8.4	9.5	10.3
1.0	7.1	8.6	9.7	10.8*
3.0	7.2	8.6	9.8	10.8
Females				
0	6.5	7.7	8.6	9.2
0.3	6.4	7.5	8.5	9.2
1.0	6.5	7.6	8.4	8.8
3.0	6.5	7.7	8.5	9.1

Values are means for groups of four dogs and that marked with an asterisk differs significantly from the control: * $P < 0.05$ by Student's t test.

logical parameters was an increase in the PCV determined at wk 12 in male rats fed 1 or 3% thaumatin (Table 4). A reduction in the PCV in female rats achieved statistical significance only in the low and intermediate groups. There was a decrease at wk 12 in the haemoglobin concentration, erythrocyte count and PCV in male dogs fed 3% thaumatin. Apart from a dose-related reduction in the triglyceride levels in the serum of female rats there were no changes in biochemical parameters that could be attributed to the ingestion of thaumatin (Table 5). Water intake and the composition of urine was similar for all groups and ophthalmoscopic examination did not reveal any treatment-related abnormalities.

The weight of the thyroid relative to body weight was increased in male but reduced in female rats (Table 6). The only other organ-weight increase compared with controls was in the relative kidney weight in treated females. There were no treatment related effects on organ weights in dogs.

Microscopic examination in rats revealed, in both treatment and control groups, peribronchial and perivascular lymphoid reactions in the lungs and interstitial chronic infiltration of inflammatory cells in the kidney (Table 7). Similar changes were observed in dogs (Table 8), but as with other, less common, reactions their distribution was similar in treated and control animals.

Teratogenicity

Experimental

Thaumatococcus was administered by oral gavage to groups of 20 pregnant female rats from day 6 to day 15 of gestation at dosages of 0, 200, 600 and 2000 mg/kg/day. Body weights were recorded throughout the study and animals were killed by asphyxiation with CO₂ on the day prior to term. The number of corpora lutea in each ovary was recorded, as were the number, position and condition of implantations. The time of death of non-viable implantations was estimated. One third of each litter was placed in Bouin's fixative for subsequent free-hand sectioning (Wilson, 1965). The thoracic and abdominal cavities of the remainder were dissected and examined under low-power magnification. After evisceration they were placed in 95% industrial methylated spirit prior to skeletal examination.

Results

Treatment with thaumatin was without effect on body weight, and the numbers of litters and foetal weights in the treated groups were similar to those in the controls. One female receiving thaumatin at 2000 mg/kg/day displayed total litter loss but the behaviour of other animals in this group was comparable to that in controls. Implantation losses were essentially the same for all groups (Table 9).

A few abnormalities were present in control and treated animals when foetuses were examined *post mortem* by free-hand serial sectioning or after skeletal processing. These were of types that occur spontaneously in the CD rat and their incidence showed no treatment-related association or trend.

Mutagenic evaluation

Experimental

The ability of thaumatin to induce gene mutations was investigated using the histidine-dependent strains of *Salmonella typhimurium* TA1535, TA1537, TA1538, TA98 and TA100 and the tryptophan-auxotroph *Escherichia coli* WP2. Tests were conducted both in the absence and presence of an activating

Table 4. Haematological values (at wk 12) in rats fed 0-3% thaumatin in the diet for 13 wk

Dietary level (%)	Hb (g/100 ml)	PCV (%)	RBC (10 ⁶ /mm ³)	Total (10 ⁹ /mm ³)	Leucocytes				Platelets (10 ³ /mm ³)	PT (sec)	PTT (sec)
					Differential count (%)						
					N	E	L	M			
Males											
0	15.8	46.9	7.88	14.1	17	1	79	3	604	13.8	14.7
0.3	15.9	47.5	7.91	14.7	17	1	79	3	565	13.9	14.9
1.0	15.8	50.8*	8.14	12.3	17	1	80	3	634	13.9	14.7
3.0	15.3*	53.7**	8.09	13.0	17	1	78	4*	557	13.8	14.7
Females											
0	14.9	43.8	6.96	10.3	16	1	80	3	570	13.7	14.7
0.3	15.1	39.5***	6.83	11.6	16	1	80	3	551	13.8	14.8
1.0	15.2	38.9***	6.64*	9.3	16	1	80	3	533	13.8	14.6
3.0	15.0	41.7	6.56	7.8*	16	1	80	3	554	13.8	14.8

Hb = Haemoglobin PCV = Packed cell volume RBC = Red blood cells N = Neutrophils E = Eosinophils
L = Lymphocytes M = Monocytes PT = Prothrombin time PTT = Partial thromboplastin time

Values are means for groups of ten rats and those marked with asterisks differ significantly (Student's t test) from the control value: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

Table 5. Biochemical values for wk-12 serum samples of rats fed 0-3% thaumatin in the diet for 13 wk

Dietary level (%)	BUN (mg/100 ml)	Glucose (mg/100 ml)	Protein (g/100 ml)	Albumin (g/100 ml)	Globulin (g/100 ml)	AP (U/litre)	GOT (U/litre)	GPT (U/litre)	Cations (mequiv./litre)			Ch (mg/100 ml)	TG (mg/100 ml)	BR (mg/100 ml)	
									Na	K					
Males															
0	11.8	175	6.9	3.3	3.7	115	109	45	147	3.9		53	177	0.4	
0.3	13.3	171	6.6	3.0	3.6	113	114	44	148	4.1		57	189	0.5	
1.0	13.3	172	6.9	3.2	3.7	114	110	39	148	4.2		65	202	0.6	
3.0	14.8	182	6.9	3.1	3.8	84	105	39	149	4.6		61	202	0.6	
Females															
0	14.1	167	7.9	3.6	4.3	82	157	48	147	3.5		69	263	0.3	
0.3	13.4	162	7.6	3.4	4.2	97	139	41	147	3.6		59	146**	0.3	
1.0	13.2	165	7.3	3.4	3.9	76	150	44	138	3.6		62	136**	0.2	
3.0	16.2	182	7.1	3.2	3.9	75	132	29	142	3.6		63	102***	0.2	
BUN = Blood urea nitrogen AP = Alkaline phosphatase GOT = Glutamic-oxalacetic transaminase															
GPT = Glutamic-pyruvic transaminase Ch = Cholesterol TG = Triglycerides BR = Bilirubin															

Values are means for groups of ten rats and those marked with asterisks differ significantly from the control value: ***P* < 0.01; ****P* < 0.001 by Student's *t* test.

Values are means for groups of ten rats and those marked with asterisks differ significantly from the control value: ** $P < 0.01$; *** $P < 0.001$ by Student's t test.

AP = Alkaline phosphatase
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system (S-9) derived from the livers of rats pretreated with Aroclor 1254 as described by Ames, McCann & Yamasaki (1977) but modified by the inclusion of $4 \mu\text{mol NADH/ml}$ in the S-9 mix.

Thaumatococcus was also examined for its ability to induce dominant lethal mutations in the germ cells of male mice. Groups of 15 male mice of proven fertility were intubated daily on five consecutive days with either thaumatococcus (200 or 2000 mg/kg/day), trimethyl phosphate (100 mg/kg/day) or distilled water (10 ml/kg). Each male was then paired with three sexually mature virgin females until mating was confirmed by the presence of a vaginal plug. This was designated day 1 of pregnancy. Any female that had not mated was removed after 7 days and new females were introduced on the following day. This procedure was repeated for a total of 7 wk. The females from each mating period were randomly assigned to one of three sub-groups which were killed on days 4, 11 and 18 of pregnancy. The contents of each uterine horn of animals killed on day 4 were collected by flushing with 0.4 ml sterile saline and the washings were examined for the number of eggs recovered, the number cleaved and the developmental stage. For animals killed on days 11 and 18 the numbers of corpora lutea, implantation sites and resorptions were recorded and in addition, on day 18, the sex of individual foetuses was noted.

Results

Thaumatococcus did not induce gene mutations in either *S. typhimurium* or *E. coli* WP2 at concentrations ranging from 0.05 to 50 mg/plate. The sensitivity of the individual strains was confirmed by the use of ethylnitrosoguanidine, 2-nitrofluorene, 9-amino-acridine, benzo[a]pyrene and 2-aminoanthracene as positive controls. The results of the test for the induction of dominant lethal mutations in male mice are summarized in Table 10. Treatment with thaumatococcus was without effect on litter size, or pre- and post-implantation survival, while the development of zygotes examined on day 4 was comparable to that in the negative controls. Trimethyl phosphate had an adverse effect on all parameters, particularly during wk 1 and 2. This was in accord with an increased number of abnormal zygotes observed during this period.

Allergenic potency

Experimental—Animal studies

Thaumatococcus was examined for its ability to induce sensitization in guinea-pigs, an anaphylactic antibody response in rats and non-immunological histamine release *in vivo* (in the baboon) and *in vitro* (from rat mast cells).

Groups of five guinea-pigs received an intramuscular injection of either thaumatococcus or egg albumen (50 mg in 0.1 ml) together with 0.1 ml of either Freund's Complete Adjuvant or 1.3% alhydrogel. Animals were killed between day 12 and day 30 and three sections of ileum from each animal were transferred to a Schultz-Dale bath and challenged with a range of doses of the appropriate protein allergen.

Table 6. Relative organ weights of rats fed 0–3% thaumatin in the diet for 13 wk

Table 1. Relative organ weights of rats fed a 5% thiamin diet for 12 wk											
Dietary level (%)	Relative organ weight (g/100 g body weight)										Prostate or Uterus
	Brain	Pituitary†	Caecum	Lungs	Liver	Spleen	Kidneys	Thyroids†	Adrenals†	Gonads‡	
						Males					
0	0.45	2.69	0.32	0.40	4.06	0.17	0.76	2.86	13.13	0.70	0.17
0.3	0.47	2.57	0.31	0.39	4.17	0.17	0.70**	4.78***	12.44	0.75	0.17
1.0	0.45	2.23**	0.32	0.40	3.96	0.17	0.72	4.74***	12.20	0.69	0.16
3.0	0.44	2.73	0.31	0.42	4.32	0.17	0.76	4.49***	13.00	0.69	0.15
						Females					
0	0.73	4.52	0.41	0.50	3.80	0.18	0.74	6.79	27.54	39.50	0.23
0.3	0.74	4.38	0.40	0.52	3.75	0.19	0.80***	4.23***	27.58	38.31	0.22
1.0	0.76	4.91	0.43	0.53	3.73	0.18	0.84***	5.32**	26.03	39.88	0.23
3.0	0.73	4.78	0.40	0.57	3.69	0.18	0.82***	3.36***	26.39	40.67	0.23

†Values for this organ are expressed in mg/100 g body weight.

‡Values for female gonads are expressed in mg/100 g body weight.

Values are means of approximately 20 determinations in each group. Those marked with asterisks differ significantly (Student's *t* test) from the control value: ***P* < 0.01; ****P* < 0.001.

Table 7. Microscopic pathology in rats fed 0–3% thaumatin in the diet for 13 wk

Organ and finding	Dietary concn (%), ...	No. of rats affected/group of 20							
		Males				Females			
		0	0.3	1.0	3.0	0	0.3	1.0	3.0
Liver									
Minimal chronic inflammation		6	9	10	16	13	13	13	16
Vacuolation of hepatocytes		1	2	5	2	0	0	0	0
Kidney									
Minimal chronic inflammation		7	4	6	1	0	5	1	0
Basophilic tubules		4	3	4	2	1	0	0	0
Hydronephrosis		0	0	1	2	4	4	4	3
Lungs									
Minimal chronic inflammation		12	15	14	15	9	12	13	9
Patchy congestion		2	5	9	2	2	2	4	2
Mammary glands									
Minimal duct proliferation		1	NE	NE	4	13	NE	NE	17
Minimal acinar development		4	NE	NE	4	0	NE	NE	0
Thyroids									
Epithelial whorls		7	NE	NE	8	7	NE	NE	5
Lymph nodes									
Mesenteric hyperplasia		2	NE	NE	0	0	NE	NE	0
congestion		0	1	1	0	0	NE	NE	0
Cervical hyperplasia		0	2	NE	0	0	NE	NE	0

NE = Not examined

Table 8. Microscopic pathology in dogs fed 0–3% thaumatin for 13 wk

Organ and finding	Dietary concn (%), ...	No. of dogs affected/group of four							
		Males				Females			
		0	0.3	1.0	3.0	0	0.3	1.0	3.0
Liver									
Minimal chronic inflammation		4	4	3	4	4	3	3	3
Kidney									
Minimal chronic inflammation		3	1	3	3	1	2	2	2
Focal mineralization		0	1	0	0	2	0	1	0
Pyelonephritis		0	0	0	0	0	1	1	1
Lungs									
Minimal chronic inflammation		2	4	4	3	4	2	2	3
Focal bronchitis		0	0	0	0	0	1	1	1
Mammary glands									
Minimal duct proliferation		0	0	0	0	1	0	1	1
Lymph nodes									
Peribronchiolar hyperplasia		0	0	1	0	0	1	1	1
congestion		0	1	1	1	1	0	0	0

Table 9. Effect of thaumatin on pregnancy in the rat

Dose* (mg/ kg/day)	No. of pregnant animals	No. of implantations	Total foetal deaths		Mean litter weight (g)	Mean foetal weight (g)
			Early	Late		
0	20	276	15	1	49.2	3.8
200	20	278	19	1	48.3	3.7
600	19	280	18	0	50.4	3.7
2000	19	259	14	1	48.3	3.7

*Given orally on days 6–15 of gestation.

The contractions recorded for thaumatin were compared with those for egg albumen.

Groups of five male rats were sensitized by a subcutaneous injection of thaumatin or egg albumen with Freund's Complete Adjuvant at four separate sites (10 µg protein at each site). Animals were killed after 11 or 12 days and the levels of anaphylactic antibody in the serum were determined by passive cutaneous anaphylactic (PCA) dilution titration. The serum (0.1 ml) was administered intradermally to rats and the animals were challenged after an interval of 48 hr with an intravenous injection of allergen protein (2 mg) and Evans blue dye (2%).

Suspensions of approximately 10⁵ peritoneal mast cells from control rats were incubated at 37°C for 5 min with a range of concentrations of thaumatin or the ACTH β^{1-24} synthetic polypeptide, synacthen. The amount of histamine released into the incubation medium was measured fluorimetrically (Evans, Lewis & Thompson, 1973). Additionally, a range of concentrations of thaumatin (0.1 ml) were injected into

duplicate sites of the shaved abdomen of a baboon immediately after an intravenous injection of indicator dye. The area of 'blueing' was measured and compared with the responses obtained with isotonic saline and with synacthen.

Experimental—Human studies

Altogether, 140 laboratory and pilot-plant staff, intermittently exposed to thaumatin over periods of up to 7 yr, were screened to common inhalant allergens. Materials used were standard skin-testing extracts (E. Merck Ltd, Alton, Hants.) and a standard modified prick-test technique was used throughout. Test solutions of thaumatin were prepared by dissolving the commercial material in glycerol-saline (50% glycerine, 0.4% phenol) at a strength of 10,000 pnu/ml, and subjecting the solutions so obtained to sterile filtration. The optimum strength of solution was determined by the weal diameters produced with strengths of 1000–10,000 pnu in three skin-test-positive volunteers. Standard European

Table 10. Effects of thaumatin on the frequency of dominant lethal mutations in male mice*

Treatment†	Wk no.	No. of females		Total implantations	Foetal deaths		Mutagenic index§
		Mated‡	With implantations		Early	Late	
Distilled water (10 ml/kg/day)	1	15	14	161	10	6	10.0
	2	15	13	156	12	4	10.3
	3	15	13	172	12	4	9.3
	4	15	12	148	7	5	8.1
	5	15	13	152	7	1	5.3
	6	15	11	134	9	1	7.5
	7	15	11	112	6	1	6.3
Thaumatin (200 mg/kg/day)	1	11	9	118	3	4	5.9
	2	15	14	169	11	12	13.6
	3	13	12	143	13	1	10.0
	4	14	13	147	5	11	10.9
	5	14	11	120	3	2	4.2
	6	11	10	125	10	2	9.6
	7	11	10	127	4	1	3.9
Thaumatin (2000 mg/kg/day)	1	13	9	103	6	0	5.8
	2	15	14	172	11	3	8.1
	3	10	10	110	6	0	5.5
	4	14	12	145	4	1	3.4
	5	13	13	153	9	0	5.9
	6	12	12	148	10	2	8.1
	7	13	13	157	8	0	5.1
Trimethyl phosphate (100 mg/kg/day)	1	15	1	13	6	5	84.6
	2	13	3	36	9	0	25.0
	3	14	6	67	9	0	25.0
	4	12	12	149	4	4	5.4
	5	11	9	99	7	0	7.0
	6	13	11	134	6	5	8.2
	7	12	11	136	13	2	11.0

*Data from animals killed on day 18 of gestation.

†By intubation on five consecutive days.

‡Corrected for those that showed no evidence of mating.

§Total deaths as a percentage of total implantations.

Bacterial Contamination Tests were performed on all extracts. Materials were stored at 4–8°C. Skin-testing extracts of thaumatin fractions and formulations were prepared by the same technique.

The following solutions were used for skin testing: (1) commercial thaumatin; (2) purified Thaumatin I and Thaumatin II as separate solutions; (3) freeze-dried thaumatin–gum arabic mixture, 1:9, w/w; (4) non-Thaumatin plant constituents; (5) spray-dried thaumatin–gum arabic mixture, 1:9, w/w. A standard gum arabic skin-test extract was obtained from Dome–Hollister–Stier, Farnham Common, Slough.

Material for solutions (2) and (4) above was prepared by treating a solution of commercial thaumatin with several lots of Sephadex SP C50 cation-exchange resin (acid form) to remove the basic Thaumatin proteins and leave behind the residual plant constituents. The residual material amounted to approximately 2.5% (w/w) of the original thaumatin and still contained nitrogenous substances equivalent to 25% protein. Thaumatin I and II were eluted from the resin using a solution of sodium chloride, and were isolated from the eluate after dialysis and freeze drying. Skin-testing solutions were prepared at a concentration of 0.59 mg/ml, equivalent to 10,000 pnu/ml for Thaumatin I and II.

Thaumatin (100 mg) or lactose (100 mg) was administered in gelatin capsules daily for 14 days to ten volunteers (six male and four female) followed by a cross-over period of a further 14 days. The volunteers were randomly assigned to two groups of five and were allocated test substance or placebo in a double-blind manner.

All volunteers were prick tested for common allergens and with thaumatin solution before the study. Seven were tested with thaumatin a second time before the study commenced to determine whether sensitization to thaumatin could result from prick testing alone. No sensitization was observed. At the completion of the study, a further prick test was performed to determine whether sensitization had occurred following thaumatin ingestion.

Blood (20 ml) was taken from the volunteers at commencement and again after the 28-day study period. Sera were tested both before and after incubation for 2 hr at 56°C to distinguish between heat-labile IgE and heat-stable IgG antibodies. Duplicate aliquots of unheated serum from each donor were injected intradermally into sites on the shaved abdomen of either a baboon or rhesus monkey. Similar amounts of serum from a normal individual and one sensitive to grass pollen were also injected to serve as negative and positive controls, respectively. Portions (0.1 ml) of heated sera were injected after 24 hr. A solution (3 ml) of Evans blue dye (2%) was injected intravenously after an interval of 3 hr and was followed 10 min later by a solution (1 ml) of thaumatin (10 µg) given by the same route. Thaumatin (100 mg) was administered subsequently by stomach tube to each animal.

Results

The tests of ileum preparations from guinea-pigs sensitized with protein and incomplete adjuvant (which is preferred for evoking an IgE antibody response) revealed that the minimum dose of thau-

matin capable of eliciting a response was 250 ng. This was comparable to the minimum dose of egg albumen needed to evoke a similar response. Tests on ileum preparations from guinea-pigs sensitized with protein and complete (Freund's) adjuvant gave an essentially similar result, although under these conditions thaumatin was possibly slightly more sensitizing than egg albumen.

The PCA titre, as indicated by the maximum dilution of the serum required to induce a 'blueing' response varied from 1 in 8 to 1 in 32 for animals treated with thaumatin and from 1 in 32 to 1 in 128 for those treated with egg albumen.

The concentration of thaumatin required to induce the liberation of 50% of the available histamine from purified rat mast cells was in the region of 1 mM whereas synacthen was equipotent at a concentration of 2 µM. The intradermal injection of thaumatin at a concentration of 1 mM provoked a weak response, the area of 'blueing' being 0.19 mm², whereas synacthen gave a measurable response at a concentration of 1 µM.

Prick testing of personnel who had been exposed to thaumatin dust revealed that about one half (67/140) responded to common inhalant allergens (Eaton, Higginbotham, Snodin *et al.* 1982). The incidence of atopy (21.4%) was high when compared with an estimate of 12.1% for the general population (Eaton, 1982). A positive response to thaumatin was observed in 13 subjects, all except one of whom were atopic or allergic. This 9.3% (13/140) incidence compared with a 30.7% incidence of positive response to the house-dust mite (*Dermatophagoides pteronyssinus*). The one thaumatin-positive individual who was neither atopic nor allergic gave only a borderline response to *D. pteronyssinus*. None of the nine subjects who gave positive prick tests to thaumatin and who were tested with separated fractions of commercial thaumatin responded to the non-Thaumatin component. The results with purified Thaumatin I and II were comparable to those with the commercial material, indicating that these proteins account for the inhalant immunological activity of thaumatin and that the non-Thaumatin plant constituents are non-allergenic. All of the nine subjects who were prick-test-positive to thaumatin also gave positive tests to mixtures of thaumatin with gum arabic. Therefore, although bulking of thaumatin with gum arabic reduces airborne dust levels, if any of the mixture is inhaled, its allergenic potency relative to thaumatin is unlikely to be diminished.

Sera separated from the blood of volunteers participating in the oral-challenge capsule study showed no anaphylactic antibodies to thaumatin when subjected to the PCA test in monkeys. Additionally, no sensitization was detected in prick tests at the end of the study.

Discussion

Despite the fact that thaumatin is some 2000 times sweeter than sucrose, it is probable that it will be used principally as a flavour modifier and extender, resulting in an average daily intake from all sources of 2 mg per head (equivalent to 0.03 mg/kg for a 60-kg adult).

The absence of any adverse reactions following the administration of thaumatin to rats and dogs can be attributed to the purity of the product, which results from the use of an ultrafiltration technique designed to exclude components with molecular weights of less than 2×10^4 daltons, and to the ease with which it is digested prior to absorption. The failure to detect the presence of anaphylactic antibodies in the sera of rats and human volunteers following oral ingestion of thaumatin is confirmatory evidence that the intact constituent proteins are not absorbed. Several preparations of thaumatin of differing geographical origin and purity were used in these investigations and the essential uniformity of the biological response indicates the absence of any toxic component. Unpublished studies with material containing only 78–85% protein (compared with 95–98% protein (dry weight) in the current material) provided no evidence of any treatment-related change in either tissue function or structure when the material was administered to rats for 90 days at dietary concentrations up to 8%. The results of these various studies indicate that the dietary 'no-effect level' of thaumatin in rats and dogs is 3%, corresponding to an average daily intake of between 1.5 and 3 g/kg. Applying a safety factor of 100, which may be considered excessive for a readily assimilated protein, would give a value for the acceptable daily intake of 15–30 mg/kg. It is clear that this permits a considerable margin of safety when compared with the anticipated levels of exposure (see above). Since the sole dietary effect of thaumatin is to make an insignificant contribution to the existing metabolic load from food proteins (approximately 0.0025% of the average protein intake per head), it would appear reasonable to base the safety evaluation of thaumatin on the results of subacute studies. This strategy is wholly consistent with criteria established, *inter alia*, by the US Bureau of Foods (Food and Drug Administration, 1982) and the World Health Organization (Joint FAO/WHO Expert Committee on Food Additives, 1957 & 1974).

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