Zeitschrift für
Ernährungswissenschaft Z Ernährungswiss 28:300–309 (1989)

# Genotoxic investigations of tobacco protein using microbial and mammalian test systems

**R. Münzner and H. W. Renner**

Institute of Hygiene and Toxicology, Federal Research Centre for Nutrition, Karlsruhe, FRG

*Summary:* Tobacco protein was assayed for mutagenicity using the Ames test and three in vivo tests.

In the Salmonella strains TA 98 and TA 100 methanolic extracts of the tobacco protein and urine of rats fed tobacco protein exhibited increased revertant numbers, but extracts of feces did not. Using the micronucleus test throughout, weak mutagenic effects after feeding the tobacco protein were detected in Chinese hamsters and two inbred strains of mice, and again in Chinese hamsters when the chromosome aberration test and the SCE test were applied. The analytical specifications of the tobacco protein listed nicotine, chlorogenic acid and rutin as components. These were examined separately in a chromosome aberration test, and nicotine was discovered to be the factor or a factor responsible for the weak positive test results.

*Zusammenfassung:* Tabakprotein wurde im Ames-Test und in drei In-vivo-Tests auf Mutagenität geprüft.

In den Salmonellenstämmen TA 98 und TA 100 bewirkten Methanolextrakte des Tabakproteins und der Urin von Ratten, die mit Tabakprotein gefüttert waren, erhöhte Revertantenzahlen, nicht aber Fäzesextrakte. Im Mikrokerntest wurden durchgehend schwach positive Effekte des Tabakproteins in Chinesischen Hamstern und zwei Mäuse-Inzuchtstämmen erzielt, desgleichen im Chromosomenaberrationstest und im SCE-Test an Chinesischen Hamstern. Die in den analytischen Angaben des Tabakproteins ausgewiesenen Substanzen Nikotin, Chlorogensäure und Rutin wurden einzeln im Chromosomenaberrationstest geprüft. Dabei wurde das Nikotin als der verantwortliche Faktor oder einer der verantwortlichen Faktoren für die schwach positiven Testergebnisse erkannt.

*Key words:* tobacco protein, Ames test, micronucleus test, chromosome aberration test, SCE test

*Schlüsselwörter:* Tabakprotein, Ames-Test, Mikrokerntest, Chromosomenaberrationstest, Schwesterstrangaustauschtest

## Introduction

Anti-smoking campaigns have given rise to fears that the future economic existence of tobacco planters may be threatened by a decline in the sale of tobacco products. The high protein content of the tobacco plant (2) suggests that it may be worthwhile extracting this leaf protein and

Table 1. Composition of the diets.

| Components | Control diet C 1000 % | Test diets with tobacco protein | |
|---|---|---|---|
| Casein | 22.0 | – | – |
| Tobacco protein | – | 19.2 | 40.0 |
| Cellulose | 4.0 | 4.0 | 4.0 |
| Sucrose | 10.0 | 10.0 | 10.0 |
| Corn oil | 3.0 | 3.0 | 3.0 |
| Trace elements and minerals | 6.0 | 6.0 | 6.0 |
| Vitamins | 2.0 | 2.0 | 2.0 |
| Starch | 53.0 | 55.8 | 36.0 |

using it as a supplement in feed or food. The economic reasons are quite simple. Acre-for-acre the tobacco plant is capable of yielding approximately five-times more protein than the soybean (2).

Nutritional/physiological experiments conducted as part of a research project subsidized by the EEC Commission revealed that tobacco protein had a nutritional value close to that of milk protein and much higher than that of most other plant proteins (3). Genotoxic studies carried out on tobacco protein are described below.

## Materials and Methods

*Diets and animals*

The animals used in these experiments, Sprague Dawley rats, Chinese hamsters and the inbred strains of mice $C_3H$ and $C_{57}Bl$, were fed a semi-synthetic diet with casein as protein (Altromin C 1000) on each of the eight days prior to the experiments. During the tests the animals received for three or four days a modified version of the C 1000 diet in which casein was replaced by tobacco protein (Table 1). Taking the data given by the manufacturers of the tobacco protein*) as a basis (i.e., total N = 14.59 % in the dry mass corresponding to a protein content of 91.19) and considering the known protein content of the C 1000 diet, it was calculated that tobacco protein formed 19.2 % of the test diet. In the cytogenetic tests additional experiments using a diet containing 40 % tobacco protein were conducted.

*Salmonella/mammalian-microsome mutagenicity test*

24 male rats (140–160 g body weight) were housed individually (in order to enable a separate collection of urine and feces) in metabolism cages with free access to drinking water and food for three days. One group (eight control animals) was given the semisynthetic diet with casein, and the other group (16 experimental animals) was fed the diet in which tobacco protein took the place of casein.

The 24 h-urine and feces were collected every morning on three consecutive days and were stored at −30 °C until assayed.

*) Supplier of the tobacco protein concentrate, fraction 1: Instituto Industrie Agrarie, Dipartimento Science e Technologie Alimentari e della Nutrizione, Universita di Perugia/Italy.

Table 2. Animal species/strains and mutagenicity test systems used.

| | Sprague-Dawley rats | Chinese hamsters | Mice $C_3H$ | Mice $C_{57}Bl$ |
|---|---|---|---|---|
| Salmonella test | × | – | – | – |
| Micronucleus test | – | × | × | × |
| Chromosome aberration test | – | × | – | – |
| Sister chromatid exchange test | – | × | – | – |

*Preparation of urine and extraction of feces and tobacco protein*

The thawed urine of four animals and the feces of eight animals each were pooled. After centrifugation the urine was filter-sterilized.
Aqueous fecal extracts were prepared by grinding 1 g of feces with 3 ml of warm distilled water. The slurry was centrifuged and the supernatant was sterilized by successive passage through filters with 1.2, 0.45, and 0.2 µm pore size. For the alcoholic extraction 1 g of feces and samples of tobacco protein were extracted with 9 ml of methanol for 20 h. After centrifugation the solvent was evaporated and the residue was weighed and dissolved in dimethyl sulfoxide (DMSO).

*Mutagenicity testing*

The urine and extracts of feces and tobacco protein were tested for mutagenic activity in the Salmonella/mammalian-microsome test with Salmonella typhimurium strains TA 98 and TA 100. The test was conducted according to the original method (1). The plates were run in triplicate. Assays were made with or without the addition of S9-mix prepared from three pooled livers of male Sprague-Dawley rats induced with Aroclor 1254. The amount of S9 was 50 µl per plate. To detect compounds which are excreted as conjugates 1000 Sigma units of β-glucuronidase/aryl sulfatase (Typ H-2S, Sigma) were added to the top agar. A reproducible dose-related, at least two-fold, increase in the number of revertants over the spontaneous number (control) was considered to be a positive result.

In the cytogenetic tests Chinese hamsters (aged 16–20 weeks, body weight $32 \pm 2$ g) and the inbred strains of mice $C_3H$ and $C_{57}Bl$ (aged 20–28 weeks, body weight 25–30 g) were used. Test groups, positive and negative controls were made up of equal numbers of males and females so that any possible sex differences would be distinguishable. After the tobacco protein diet had been administered, bone marrow cells of the test animals were examined for chromosomal damage and the genetic endpoints micronuclei, chromosome aberrations (structural and numerical) and sister chromatid exchanges were evaluated. The bone marrow cells were obtained by flushing out both femora.

*Micronucleus test*

The bone marrow cells were flushed out with fetal calf serum. After centrifuging the cell sediment was spread onto slides and dyed with the May-Grünwald- and Giemsa solution (6). 1000 polychromatic erythrocytes/slide and animal were scored for micronuclei, and about 1000 normochromatic erythrocytes/slide and animal were scored for control purposes.

*Chromosome aberration test*

2 h before sacrifice animals were injected with colchicine (1 mg/kg b.w. subcutaneously). After flushing out the bone marrow cells into hypotonic solution,

Table 3. Mutagenicity testing of the methanolic extract of tobacco protein in Salmonella typhimurium strains TA 98 and TA 100 with and without the addition of S9-mix and β-glucuronidase/aryl sulfatase.

| Methanolic extract (ml/plate) | TA 98 | | | | TA 100 | | | |
|---|---|---|---|---|---|---|---|---|
| | −S9 | | +S9 | | −S9 | | +S9 | |
| | −βG/AS[b] | +βG/AS | −βG/AS | +βG/AS | −βG/AS | +βG/AS | −βG/AS | +βG/AS |
| 6.25 | 108[a] | 159 | 391 | 432 | 130 | 165 | 362 | 417 |
| 1.25 | 32 | 42 | 109 | 127 | 116 | 129 | 170 | 201 |
| 0.625 | 24 | 27 | 57 | 81 | 114 | 110 | 131 | 148 |
| Control | 22 | 19 | 41 | 39 | 107 | 109 | 115 | 121 |

[a] Values are means of three plates
[b] β-glucuronidase/aryl sulfatase

centrifugation steps and fixation, the cells were dropped on chilled slides followed by flame fixation and staining with 2% aceto-orcein solution (7). 100 well-spread metaphase cells/animal were evaluated for chromosome aberrations.

*Sister chromatid exchange test*

In addition to the procedure described for the chromosome aberration test the SCE-test was performed using the method of implantation of a bromodeoxyuridine tablet (5). In this way about 60% well-spread and differential stained metaphase chromosomes in the second replication cycle were scorable. 100 cells/animal were evaluated for exchanges.

## Results

Without metabolic activation the methanolic extract of tobacco protein showed in strain TA 98 at the lower concentrations and in strain TA 100 at all investigated concentrations only a slight elevation in the number of revertants; in the presence of S9-mix (with and without β-glucuronidase/aryl sulfatase) an increased response was observed in both the strains (Table 3).

Urine from rats fed the diet containing tobacco protein and urine from the control animals were tested for mutagenicity in strains TA 98 and TA 100 with and without S9-mix and with and without β-glucuronidase/aryl sulfatase (Table 4). The results showed a volume-dependent increase in the number of revertants in both the strains for control urines, presumably as a consequence of histidine or histidine-related growth factors in the urine. This increase in revertants did not differ from day to day during the experiments.

From the second day of the experiments urine from rats fed the tobacco protein diet produced a slight increase in revertants in strain TA 98 and TA 100 under all four test conditions with the greatest response evident in the presence of S9-mix with or without β-glucuronidase/aryl sulfatase when compared to the control urines. This excretion of mutagenic activity in the urine of the experimental rats increased slightly on the third day of the feeding period. As sufficient quantities of tobacco protein were not avail-

Table 4. Mutagenicity testing of urine from rats fed a tobacco protein diet for 3 days and of urine from rats fed a control diet (data shown are from the last day of feeding).

| Volume of urine (mg/plate) | Test urine / Control urine | TA 98 | | | | | | | | TA 100 | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | –S9 | | | | +S9 | | | | –S9 | | | | +S9 | | | |
| | | –βG/AS[b] | | +βG/AS | | –βG/AS | | +βG/AS | | –βG/AS | | +βG/AS | | –βG/AS | | +βG/AS | |
| 0 | | 24 | 17[a] | 20 | 22 | 27 | 35 | 37 | 35 | 110 | 108 | 118 | 109 | 115 | 103 | 109 | 112 |
| | | 20 | 23 | 22 | 22 | 33 | 31 | 35 | 34 | 106 | 108 | 115 | 122 | 109 | 113 | 115 | 121 |
| 0.2 | | 56 | 38 | 39 | 44 | 92 | 69 | 119 | 81 | 192 | 140 | 169 | 186 | 210 | 186 | 183 | 192 |
| | | | | 25 | 30 | 44 | 37 | 46 | 50 | 116 | 134 | 126 | 136 | 120 | 133 | 120 | 196 |
| 0.4 | | 71 | 63 | 50 | 60 | 146 | 117 | 170 | 116 | 256 | 178 | 248 | 200 | 287 | 215 | 233 | 231 |
| | | 33 | 36 | 27 | 35 | 51 | 47 | 52 | 55 | 142 | 164 | 134 | 159 | 148 | 161 | 148 | 202 |
| 0.6 | | 82 | 73 | 79 | 69 | 196 | 141 | 224 | 170 | 272 | 198 | 274 | 242 | 312 | 254 | 383 | 273 |
| | | 40 | 38 | 40 | 38 | 63 | 54 | 59 | 65 | 155 | 176 | 140 | 170 | 155 | 173 | 162 | 205 |
| 0.8 | | 114 | 104 | 89 | 77 | 253 | 220 | 310 | 199 | 283 | 270 | 324 | 289 | 448 | 360 | 395 | 361 |
| | | 42 | 40 | 44 | 41 | 66 | 68 | 63 | 73 | 165 | 186 | 160 | 192 | 167 | 192 | 171 | 206 |

[a] Values are means of three plates; two separate assays were run
[b] β-glucuronidase/aryl sulfatase

able it was not investigated whether or not after a longer time of feeding the urinary mutagenicity was further increased.

Feces were also collected during the three days when the rats were fed with tobacco protein and with control diet, and were extracted with distilled water. The aqueous fecal extracts of every day were tested with and without S9-mix and with and without β-glucuronidase/aryl sulfatase.

As no mutagenicity was detectable under these test conditions, further investigations were carried out in which the feces of the experimental animals fed with tobacco protein for three days were pooled and extracted with methanol. But also in the methanolic fecal extracts no indications of an increase in the number of revertant colonies were found. (Data are not shown.)

By checking the weight of the feed in the experiments with Chinese hamsters and mice, acceptance and intake of the test diet (19.2 % tobacco protein) was tested and compared with the intake of the control diet (C 1000). No notable differences between the intake of the control and the test diet were observed. It was important to have experimental proof of this presupposition before going on to consider the results of the genotoxicity tests described below.

Sampling of bone marrow cells for the cytogenetic tests was carried out on the animals given the diet containing 19.2 % tobacco protein after four days of feeding, and on the animals fed the diet with 40 % tobacco protein after three days.

Table 5. Micronucleus test (figures represent mean values ± SD).

| | Chinese hamsters | | | $C_3H$-Mice | | | $C_{57}$ Bl-Mice | | |
|---|---|---|---|---|---|---|---|---|---|
| | ‰ micronucleated erythrocytes | | | ‰ micronucleated erythrocytes | | | ‰ micronucleated erythrocytes | | |
| | normo-chromatic | poly-chromatic | (n) | normo-chromatic | poly-chromatic | (n) | normo-chromatic | poly-chromatic | (n) |
| Negative control<br>Control diet C 1000 | 2.0 ± 0.7 | 2.5 ± 0.5 | (10) | 2.3 ± 0.7 | 2.5 ± 0.9 | (10) | 2.1 ± 0.6 | 2.6 ± 0.8 | (10) |
| Positive control:<br>Cyclophosphamide<br>20 mg/kg b.w. p.o. | 2.0 ± 1.1 | 10.9 ± 1.8 | (10) | 3.0 ± 0.7 | 18.4 ± 1.2 | (10) | 2.8 ± 1.1 | 18.1 ± 1.1 | (10) |
| Test diet<br>with 19.2 % tobacco protein | 1.9 ± 0.8 | 2.2 ± 0.9 | (20) | 2.1 ± 0.8 | 2.3 ± 0.8 | (20) | 2.2 ± 1.2 | 2.6 ± 0.8 | (20) |
| Test diet<br>with 40 % tobacco protein | 2.0 ± 0.9 | 3.8 ± 1.2** | (6) | 2.2 ± 0.8 | 4.5 ± 1.2** | (6) | 2.2 ± 0.8 | 4.0 ± 1.1* | (6) |

* $p < 0.05$ (t-test, control diet: test diet)
** $p < 0.01$

Table 6. Chromosome aberration test (figures represent mean values ± SD).

| | Chinese hamsters | | |
|---|---|---|---|
| | % gaps | % aberrant cells | (n) |
| Negative control<br>Control diet C 1000 | 0.5 ± 0.5 | 0.3 ± 0.5 | (10) |
| Positive control:<br>Cyclophosphamide 18 mg/kg b.w. p.o. | 30.2 ± 3.5 | 10.7 ± 1.2 | (10) |
| Test diet<br>with 19.2 % tobacco protein | 4.1 ± 1.0 | 1.0 ± 0.7** | (20) |
| Test diet<br>with 40 % tobacco protein | 4.7 ± 0.8 | 1.7 ± 0.5** | (6) |

In the micronucleus test the results obtained after the test diet, in which the sole source of protein came from tobacco (19.2 %), with Chinese hamsters, and with the two inbred strains of mice, were not different from the results obtained after the control diet (C 1000). When, however, the tobacco protein content of the test diet was raised to 40 % then the number of micronucleated polychromatic erythrocytes in both animal species was slightly higher (Table 5).

With the chromosome aberration test, carried out on Chinese hamsters, the tendency revealed in the micronucleus test became even clearer: the percentage of cells with gaps and of cells with aberrations increased markedly even in the group which received 19.2 % tobacco protein and was more pronounced in the group which was fed the 40 % tobacco protein diet (Table 6).

The results of the sister chromatid exchange test (SCE test) produced a similar picture: a slight rise in the chromatid exchanges in the 19.2 % group and a greater increase in the 40 % group (Table 7).

Table 7. Sister chromatid exchange (SCE) test (figures represent mean values ± SD).

| | Chinese hamsters | |
|---|---|---|
| | SCEs/metaphase | (n) |
| Negative control<br>Control diet C 1000 | 5.1 ± 0.1 | (4) |
| Positive control:<br>Cyclophosphamide 5 mg/kg b.w. p.o. | 10.4 ± 0.5 | (6) |
| Test diet<br>with 19.2 % tabacco protein | 6.0 ± 0.3*** | (20) |
| Test diet<br>with 40 % tobacco protein | 7.0 ± 0.2*** | (6) |

** $p < 0.01$ (t-test, control diet: test diet)
*** $p < 0.001$

Table 8. Examination of three components of tobacco protein – Chromosome aberration test, Chinese hamsters, bone marrow cells, six animals/test, 100 cells/animal.

| Substance, dose mg/kg b.w. p.o. | Treatment time 24 hr. | % (± SD) cells with gaps | aberrations |
|---|---|---|---|
| Nicotine | 0.5 mg | 1.2 ± 0.8 | 0.7 ± 0.5 |
| | 1 mg | 1.2 ± 1.2 | 1.2 ± 1.0 |
| | 2.5 mg | 2.0 ± 0.6 | 1.0 ± 0.6 |
| | 5 mg | 2.8 ± 0.8 | 1.2 ± 0.8 |
| Chlorogenic acid | 100 mg | 4.0 ± 1.3 | 0.2 ± 0.4 |
| Rutin | 100 mg | 1.5 ± 1.4 | 0.3 ± 0.5 |
| Untreated control | | 0.5 ± 0.5 | 0.3 ± 0.5 |

As the quantity of tobacco protein available was limited, it was not possible to conduct all the experiments in the various mutagenicity test systems with the same number of animals.

The data supplied by the manufacturers of the tobacco protein contains the information: "total phenolics 6.02 mg/g of sample". This content is mainly due to chlorogenic acid and rutin. Furthermore a nicotine content of 94.12 mg/kg of tobacco protein is given. Taking this and the previously ascertained feed consumption as a basis, it was calculated that each animal received on average 2.3 mg nicotine/kg b.w./day with the 19.2 % tobacco protein diet and 4.7 mg nicotine/kg b.w./day with the 40 % tobacco protein diet. In order to discover whether any chromosomal damage could be induced under in vivo conditions by these particular tobacco protein ingredients, a chromosome aberration test with Chinese hamsters was performed using nicotine base (research grade), chlorogenic acid (pure) and rutin cryst. (research grade, all three compounds purchased from Serva, Heidelberg). The test substances were administered orally as aqueous solutions via stomach tube and different treatment times were checked. Results show that chlorogenic acid, and rutin did not cause any clastogenic effects whereas nicotine induced (at a treatment time of 24 h) a corresponding increase in gaps and chromosome aberrations (Table 8). This effect was not clearly connected with a dose relationship because a dose exceeding 2.5 mg nicotine/kg may provoke initial toxic effects in bone marrow.

## Summing up and evaluation of the results

The mutagenicity testing of the tobacco protein was carried out using one microbial test system (Ames test) and three in vivo tests on small rodents. Mutagenicity could thus be measured in its various manifestations and at various levels. By using different animal species it was possible to compare and evaluate possible species-specific differences. The use of different percentages of tobacco protein in the diets also made it easier to evaluate the results. While the Salmonella/microsome test reveals point mutations, the other test systems make chromosome aberra-

tions visible under the microscope: the micronucleus test indirectly permits the detection of major chromosomal damage; in the chromosome aberration test all forms of chromosomal damage can be detected; in the SCE test the special, independent form of the sister chromatid exchanges can be observed. In these studies the values of the said genetic endpoints (e.g., SCEs) after administration of the tobacco protein diet were compared with the values after administration of the control diet.

First, slight mutagenic activity was observed in the alcoholic extract of the tobacco protein in the Salmonella test. Using the same test system mutagenicity was also detectable in rat urine (increasing during three days of diet). In the micronucleus test effects were only observed in the 40 % tobacco protein group; this was the case for both of the animal species, hamster and mouse. The chromosome aberration test was more sensitive to mutagenic effects caused by the tobacco protein and revealed an increase in chromosomal damage even in the animals fed the 19.2 % diet. This increase was more marked in the animals fed the 40 % diet. The clastogenic damage consisted solely of chromatid and chromosome breaks. Evidence of mutagenic substances in the tobacco protein was also provided by the increased appearance of sister chromatid exchanges in Chinese hamsters; their quantity varying with percentage of tobacco protein in the diet which the animals received. The development of SCEs depends on other molecular-genetic processes, i.e., chemomutagens which induce chromosomal breaks need not necessarily induce SCEs.

Further studies were conducted using the chromosome aberration test to examine three individual components of tobacco protein: nicotine, chlorogenic acid, and rutin. While the latter two showed no signs of chromosome damaging effects, nicotine led to a four-fold increase in the spontaneous aberration rate in bone marrow cells (0.3 to 1.2 %). This value is approximately equal to that obtained in the same test system with the same animal species after feeding with the tobacco protein diet (Table 6).

It was, therefore, concluded that the nicotine content of the tobacco protein sample used was a cause and probably the sole cause of the observed mutagenic effects. This could be settled beyond doubt by conducting a mutagenicity test with tobacco protein from which all nicotine has been removed, if such a purified protein were available.

*Acknowledgement*

The authors are indebted to Ms. G. Dubberke for her most valuable assistance in the microbial tests, and to Ms. R. Lambertz and Mr. M. Knoll for their excellent technical assistance in the laboratory tasks and in handling the animals.

*References*

1. Ames BN, McCann J, Yamasaki E (1975) Methods for detecting carcinogens and mutagens with the Salmonella/mammalian-microsome mutagenicity test. Mutation Res 31:347–364
2. Fantozzi P, Sensidoni A (1983) Protein extraction from tobacco leaves: technological, nutritional and agronomical aspects. Qual Plant, Plant Foods Hum Nutr 32:351–368

3. Harmuth-Hoene AE, Diehl JF (1986) Nutritional value of tobacco protein fractions. In: Fantozzi P (ed) Tobacco protein utilization perspectives. Bioscience Ediprint Inc Geneva, Switzerland, pp 117–123
4. Miles RD, Campbell DR, Janky DM, White CE, Rich JR (1989) Evaluation of tobacco leaf protein concentrate as a supplement in broiler diets. Nutrition Rep Int 39:649–657
5. Renner HW (1979) Zur Bewertung des in vivo-sister chromatid exchange Tests, dargestellt an verschiedenen Zytostatika. Arzneim Forsch/Drug Res 29: 1871–1875
6. Schmid W (1975) The micronucleus test. Mutation Res 31:9–15
7. Schwarzacher HG, Wolf U (1974) Methods in Human Cytogenetics. Springer, Berlin Heidelberg New York, pp 78–80

Authors' address:

Dr. R. Münzner, Bundesforschungsanstalt für Ernährung, Engesserstraße 20, 7500 Karlsruhe, F.R.G.