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MUTAGENIC AND ANTIMUTAGENIC ACTIVITIES OF BIOFLAVONOIDS AND STRUCTURAL ANALOGUES IN THE AMES/SALMONELLA TEST.

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Structure-activity relationships between quercetin, morin, galangin, luteolin, apigenin, and chrysin in Salmonella typhimurium strains TA98 and TA100.

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SUMMARY

The studies reported here were aimed at screening for antimutagenic chemicals among a series of natural food components, bioflavonoids, which on the basis of epidemiological and experimental data are suspected to possess antagonistic properties against certain forms of human cancer. The analysis of antimutagenic properties as an indicator of anticarcinogenesis was based on the association of somatic mutagenesis with certain phases of the tumorigenesis process in animals and man. Goal of the studies was to identify those compounds with high antimutagenic (and low or no mutagenic) properties in vitro, and to make suggestions for a further assessment of their anticarcinogenic activity in animals.

Based on earlier results which, depending on the class of chemical under investigation, indicated a substantial (qualitative) degree of correlation between mutagenic activity in bacteria in vitro and tumorigenic activity in animals, the present studies were performed with the bacterial indicator strains Salmonella typhimurium TA98 and TA100. The strains were chosen because they show a sensitive, dose-dependent response to the mutagenic activity of genotoxic carcinogens occurring in human food products, such as, benzo(a)pyrene (BaP) and 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP). The former chemical was employed as reference mutagenic agent to determine antimutagenic properties among the various bioflavonoids.

The bioflavonoid compounds analysed for their mutagenic properties as well as their antimutagenic properties against BaP-induced mutagenesis in Salmonella TA98/TA100 belong to the subgroup of polyphenolic compounds present usually in minor quantities in common food products: Quercetin, morin hydrate, galangin, luteolin, apigenin, and chrysin. All are derivatives of the "parent" compound, flavone (2-phenyl-4H-1-benzopyran-4-one), and show various degrees of hydroxylation (at position 2' > 6') in the phenyl side ring and/or the 3-position of the benzopyrane moiety.

The results obtained can be summarized as follows: Some of the bioflavonoids had pronounced mutagenic properties in the presence of mammalian metabolic activation (e.g. quercetin) while others were devoid of any detectable mutagenicity (e.g. chrysin). Although limited by the number of structural analogues investigated, the present results indicate that the mutagenic activity (in Salmonella TA98 and/or TA100) is correlated with the number of (adjacent) hydroxyl residues in the phenyl ring and the benzofurane moiety (at position 3). The ranking of mutagenic potency in the presence of mammalian metabolic activation, and in the range up to 100 µg compound per plate, was in decreasing order: Quercetin > Myricetin (previous report) = Kaempferol (previous report) >>> Morin hydrate >>> Galangin / Luteolin / Apigenin / Chrysin (the last 4 compounds had no detectable mutagenic activity).

With regard to antimutagenic properties, all compounds inhibited the mutagenic activity of BaP (2 µg per plate) at concentrations of 50 µg per plate or below. Because of the relatively high mutagenic activity of Quercetin, the antimutagenic properties of this chemical against BaP-induced mutagenesis could not be adequately determined. Nevertheless, and in contrast to the situation with mutagenic properties, there was no obvious correlation between the number of hydroxyl groups in the phenyl ring (or the benzofurane moiety), and the

antimutagenic properties of the compounds. In fact the chemical with the lowest number of hydroxyl groups, Chrysin, efficiently inhibited BaP-induced mutagenesis in a dose-dependent manner in both Salmonella strains TA98 and TA100, at concentrations up to 50 μg per plate, as did Galangin or Luteolin.

Chrysin also inhibited to some extent the direct mutagenic activity of BPDE, the ultimate electrophilic and mutagenic metabolite of BaP, indicating that its inhibitory action, and possibly that of the other bioflavonoids in the present series, is at least in part due to the scavenging of reactive metabolites. Furthermore, Chrysin was shown to slightly inhibit the mutagenic activity of another bioflavonoid, Quercetin, both in the presence and absence of mammalian metabolic activation. This possibly points to the fact that the netto antimutagenic effect of natural mixtures of bioflavonoids will indeed be dependent on the relative amounts of the individual components, and their interactions.

The conclusions drawn from the present studies confirm, and in particular extend, previously published results on the antimutagenic potential of bioflavonoid compounds. Although restricted to antimutagenic properties against BaP-induced mutagenesis, the results indicate that bioflanoid compounds with low numbers of hydroxyl groups, such as Chrysin, may be as potent antimutagens as the previously described compounds with high numbers of hydroxyl groups, e.g. Quercetin and/or Kaempferol. It remains thus to be determined whether the antimutagenic properties of Chrysin oberved in vitro are also expressed in mutagenicity and/or carcinogenicity assays performed in vivo.

SAMENVATTING

Voor de van nature in voeding voorkomende "bioflavonoiden", een subgroep der polyfenolen, lijkt op grond van epidemiologische en (dier)experimentele gegevens een beschermende werking tegen bepaalde vormen van kanker aannemelijk. Vooral het voorkomen van deze niet-nutritieve stoffen in groenten en fruit wordt hierbij van belang geacht. Het in dit rapport beschreven onderzoek naar de antimutagene eigenschappen van een serie gerelateerde flavonoiden kan, vanwege de belangrijke rol van somatische mutaties in het kankerproces, als indicator dienen voor hun mogelijke anticarcinogene eigenschappen. Op deze wijze kunnen specifieke flavonoiden die sterk antimutageen zijn (in vitro) worden geidentificeerd, en vervolgens geselecteerd voor onderzoek naar hun anticarcinogene werking in dierproeven. De laatste resultaten kunnen dan benut worden voor meer gerichte epidemiologische studies, en tenslotte vertaald worden in aanbevelingen voor het gebruik van bepaalde voedingsmiddelen (c.q. produkten) die beschermen tegen kanker.

Voor de uitgevoerde mutageniteitsstudies is gebruik gemaakt van de Salmonella/microsome assay zoals beschreven door Ames en medewerkers. Als bacteriestammen zijn de TA98 en TA100 gekozen en hun gevoeligheid is voor de mutagene activiteit van in humane voeding voorkomende genotoxische carcinogene stoffen, namelijk benzo(a)pyreen (BaP) en 2-amino-1-methyl-6-fenylimidazo[4,5-b]pyridine (PhIP) getest. BaP werd tevens gebruikt als mutagene referentiestof waartegen de antimutagene eigenschappen van de verschillende flavonoiden is bepaald.

De onderzochte flavonoiden betreffen een aantal flavonen en flavonolen, die in verschillende hoeveelheden in gangbare voedingsmiddelen kunnen worden aangetroffen. Voor de flavonen zijn dit chrysin, apigenine en luteoline, en voor de flavonolen galangin, morin hydraat en quercetine. Dit zijn alle stoffen afgeleid van de basisstof flavone (2-fenyl-4H-1-benzopyraan-4-one), met verschillende mate van hydroxylering in de fenyl-zijgroep (positie 2' > 6') of de 3-positie van het benzopyraan-gedeelte.

De verkregen resultaten kunnen als volgt worden samengevat: enkele van de flavonoiden vertonen aanzienlijke mutagene eigenschappen in aanwezigheid van metabole activering (b.v. quercetine), terwijl andere geen aantoonbare mutageniteit bezitten (b.v. chrysin). Hoewel het aantal geanalyseerde structurele analoga beperkt is, tonen de studies aan dat de mutagene activiteit in Salmonella TA98 en TA100 gecorreleerd is aan het aantal hydroxyl residuen in de fenyl-ring alsmede positie 3- van het benzofuraan gedeelte. De volgorde van afnemende mutagene actitiviteit in aanwezigheid van metabole activering, in het dosisbereik t/m 100 µg stof per plaat, was: Quercetine > myricetine (eerder gerapporteerd) ≅ kaempferol (eerder gerapporteerd) >>> morin hydraat >>> galangin / luteoline / apigenine / chrysin (deze laatste 4 stoffen vertoonden geen mutagene activiteit).

Wat betreft de antimutagene activiteit, verlaagden alle onderzochte flavonoiden de mutagene werking van BaP (2 µg per plaat) bij concentraties van 50 µg per plaat of minder. Omdat quercetine aanzienlijke mutagene activiteit vertoonde, was het niet mogelijk de antimutagene eigenschappen van deze stof tegen BaP mutageniteit nauwkeuring te bepalen. Desondanks, en in tegenstelling tot de situatie met de mutagene eigenschappen van de onderzochte

flavonoiden, werd geen duidelijk verband gevonden tussen het aantal hydroxylgroepen in de fenyl ring (en het benzofuraan gedeelte) en de antimutagene potentie van de stoffen. In feite vertoonde de stof met de kleinste aantal hydroxylgroepen, met name Chrysin, een aanzienlijke activiteit tegen BaP mutagenese in Salmonella stammen TA98 en TA100 bij concentraties t/m 50 µg per plaat; deze antimutagene activiteit was vergelijkbaar met die van b.v. Galangin of Luteoline.

Chrysin was tevens antimutageen tegen BPDE, de ultieme electrofiele en mutagene metaboliet van BaP; deze bevinding laat zien dat een deel van de mutageen-remmende werking van Chrysin, en mogelijk die van de andere onderzochte flavonoiden, berust op een interactie ("scavenging") met de reactive metabolieten van BaP. Daarnaast vertoonde Chrysin een lichte antimutagene werking tegen de mutagene activiteit van een ander flavonoid, Quercetine, in aanwezigheid en afwezigheid van metabole activering. Dit resultaat laat verwachten dat het netto antimutagene effect van natuurlijke mengsels van bioflavonoiden afhankelijk zal zijn van de relatieve hoeveelheden van de individuele stoffen, en hun interacties onderling, in deze complexe mengsels.

De conclusies getrokken op basis van de huidige resultaten bevestigen eerdere bevindingen en laten zien dat een breed spectrum van flavonoiden (flavonen en flavonolen) antimutagene eigenschappen bezit. Ondanks het feit dat alleen naar de antimutagene werking tegen BaP is gekeken, tonen de resultaten duidelijk aan dat bioflavonoiden met lage aantallen hydroxyl-groepen (zoals Chrysin) hetzelfde antimutagene potentieel kunnen hebben als stoffen met meerdere of vele hydroxyl-groepen (zoals Quercetine en/of Kaempferol). Het moet echter nog onderzocht worden of deze in vitro gedane waarnemingen ook in in vivo testen voor carcinogeniteit cq. mutageniteit kunnen worden aangetoond.

1. INTRODUCTION

The involvement of exogenous factors in the etiology of certain human cancers has been repeatedly postulated on the basis of epidemiological and experimental studies (Ames, 1989; Doll and Peto, 1981; IARC, 1990). Carcinogenic agents have been found among chemicals associated with our life-style and/or daily food consumption, and have received particular attention in the last years (Ames, 1986; IARC, 1986b, 1990). Examples are food mycotoxins (Dashwood et al., 1989), pyrolysis products arising during food processing (IARC, 1986a), and contaminants from ambient combustion processes such as polycyclic aromatic hydrocarbons, of which benzo(a)pyrene is a representative (cf. RIVM, 1989).

A substantial portion of the human exogenous carcinogens detected so far do possess mutagenic activity in vitro and/or in vivo, and are classified as "genotoxic carcinogens" because of these properties (Bartsch and Malaveille, 1989). In retrospect, the prevalence of mutagenic chemicals among human and animal carcinogens can be explained by the association of somatic mutations as rate-limiting events during the multi-step process of tumorigenesis (Bos and van Kreijl, 1992; Bos et al., 1986; Fearon and Vogelstein, 1990; Hollstein et al., 1991; Vogelstein and Kinzler, 1992; Zarbl et al., 1985). Key events identified so far in chemical-induced tumorigenesis are summarized in Fig. 1. In terms of cancer prevention, strategies have been developed at the regulatory level to minimize the exposure to chemicals identified as mutagens and/or carcinogens (Carere et al., 1995; Health Council, 1994; Kramers and Roelfzema, 1992; Kramers et al., 1991).

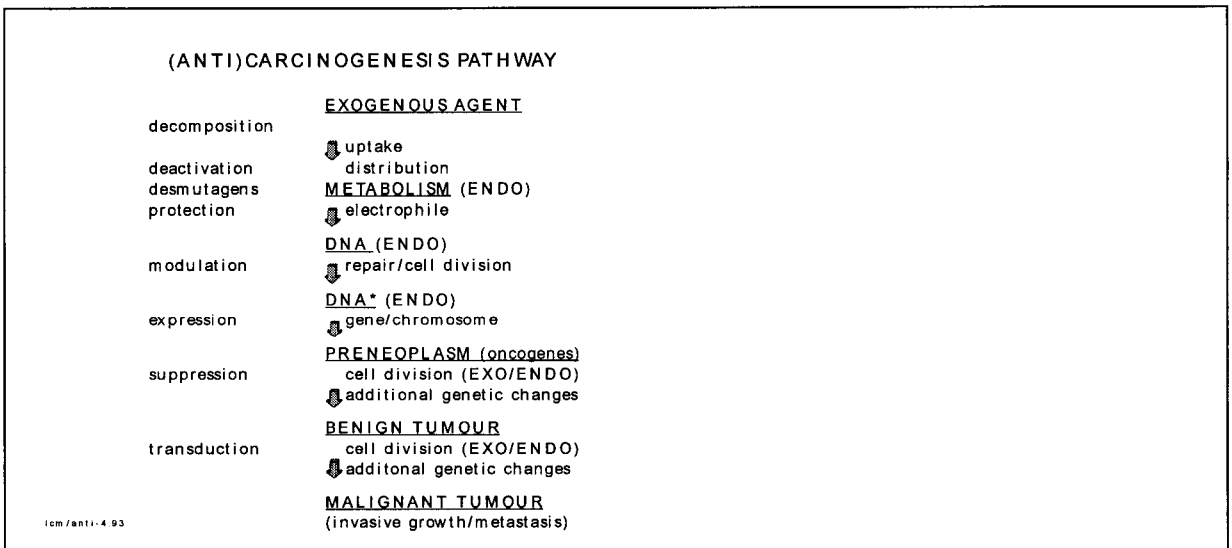


Fig. 1.
Some key events identified so far in the multi-step process of chemical tumorigenesis in animals and man, and their potential inhibition by exogenous agents. Most of the events subsequent to the formation of DNA adducts and/or damage have been associated directly or indirectly with the occurrence of genetic changes (mutations) in the affected somatic cells and/or tissues.

In addition to the presence of mutagenic cq. carcinogenic chemicals in products associated with our life-style and/or food patterns, compounds with antagonistic properties have been identified in these mixtures as well. Both at the epidemiological and experimental level, classes of chemicals were postulated or demonstrated as having antimutagenic and/or anticarcinogenic properties, and their mode of action can be ascribed to the inhibition of specific steps within the tumorigenic process, as summarized in Fig. 1 (see also De Flora and Ramel, 1988; Kada and Shimoi, 1987; Ramel et al., 1986; Wattenberg, 1980). The presence of anticarcinogenic factors in food products was inferred from epidemiological studies (see for example Steinmetz and Potter, 1991a, 1991b) and specific classes of chemicals could be subsequently identified as possessing antimutagenic cq. anticarcinogenic activity in experimental systems. Such chemicals often belong to the classes of "micronutrients" or "minor anutrients", such as, polyphenols, retinoids and associated vitamins (Ames, 1986; Das et al., 1994; Dashwood et al., 1989; De Luca et al., 1989; Moon et al., 1989; Mohr and Lewkowski, 1989; Wattenberg, 1990; Wattenberg and Leon, 1970).

On the basis of the above findings, a new dimension can then be added to cancer preventive measures, consisting of recommendations for the increased consumption of food products enriched in natural ingredients with demonstrated antimutagenic cq. anticarcinogenic properties. Suggestions in this direction have already been made (Block et al., 1992; Freudenheim, 1989; Henderson et al., 1991; Palmer, 1983; Whittemore, 1989), and strategies for the identification and quantification of the antagonistic properties of natural food chemicals have been proposed (De Flora, 1988). In analogy with the strategies employed for mutagens and/or carcinogens, strategies aimed at elucidating the anticarcinogenic properties of chemicals are based first on a screening for antimutagenesis in in vitro tests (Kada et al., 1986; Kroese et al., 1990), followed by confirmatory tests at the genotoxicity and/or tumorigenicity level in vivo (Heo et al., 1992, 1994; Kerklaan et al., 1986; Raj and Katz, 1983; Wattenberg and Leong, 1970. These approaches have the additional advantage that information is obtained about possible antagonistic effects for germ cell mutagenesis as well (Raj and Katz, 1984; for recent reviews see Ferguson, 1994; Van Kreijl et al., 1995).

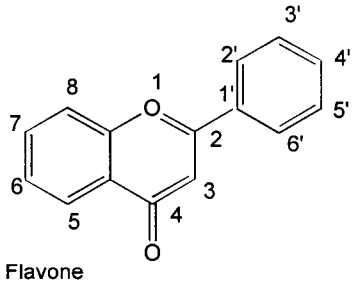
Following the above-mentioned lines of reasoning, the present experiments were directed towards an analysis of mutagenic and antimutagenic properties in vitro within a series of structurally related bioflavonoids, namely, flavones and flavonols. The choice for this class of compounds was made on the basis of (i) recommendations expressed at the workshop "Non-Nutritive Anticarcinogens in Food" (see Hertog and Hollman, 1990), (ii) epidemiological studies which indicated a protective effect of vegetable and fruit in common forms of human cancer (Bueno de Mesquita, 1992; Hertog, 1994; Steinmetz and Potter, 1991a, 1991b), and (iii) earlier studies in vitro and in vivo which demonstrated the antimutagenic and/or anticarcinogenic properties of some bioflavonoid compounds (Buening et al., 1981; Huang et al., 1983; MacGregor et al., 1983; Wattenberg, 1970). The chemical structures of some flavonoids included in our in vitro studies are depicted in Table 1.

TABLE 1.

Chemical structure of bioflavonoids and analogues.

S[1]	Trivial name	HYDROXYL RESIDUE AT POSITION: 5,7	3	2'	3'	4'	5'	6'
flavones								
	Flavone[2]							
X	Chrysin[3]	OH						
X	Apigenin	OH				OH		
	Norartocarpetin	OH		OH		OH		
X	Luteolin	OH			OH	OH		
flavonols								
X	Galangin	OH	OH					
	Datiscetin	OH	OH	OH				
	Kaempferol	OH	OH			OH		
X	Morin	OH	OH	OH		OH		
X	Quercetin	OH	OH		OH	OH		
	Myricetine	OH	OH		OH	OH	OH	

[1] S: Chemicals included in this report are indicated by "X".
[2] Flavone = 2-phenyl-4H-1-benzopyran-4-one; chemical structure see below.
[3] Chrysin = 5,7-dihydroxyflavone.



Flavone
2-phenyl-4H-1-benzopyran-4-one
2-phenyl-1,4-benzopyrone

The chemicals were chosen in order to obtain a systematic overview of those structures associated with mutagenic as opposed to antimutagenic properties. In the antimutagenesis part of the studies, the mutagenic compound used was a persistent chemical, known to be present as a contaminant in food products and to be metabolized in mammalian organisms to reactive, electrophilic, mutagenic and carcinogenic species: benzo(a)pyrene (BaP), as representative of the class of polycyclic aromatic hydrocarbons (see RIVM, 1989). Some antimutagenesis experiments were performed using benzo(a)pyrene-7,8-diol-9,10-epoxide, BPDE, the directly acting mutagenic metabolite of BaP.

In view of data obtained earlier (Voogd and van der Stel, 1993; Voogd et al., 1993) the bacterial in vitro mutagenesis system used in this study was the Salmonella/microsome assay developed by Ames and coworkers (see Maron and Ames, 1984). In the present studies, the Salmonella strains TA98 and TA100 served as sensitive indicators of the mutagenic activity of BaP in the presence of a mammalian metabolic activation system (Aroclor 1254-induced rat liver S9 fraction). The sensitivity of the strains was further assayed with a representative of food pyrolysis products, PhIP (2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine).

Results on the mutagenic and antimutagenic properties of Myricetin and Kaempferol were reported earlier (Voogd and Van der Stel, 1993; Voogd et al., 1993). The present report includes the results of experiments aimed at obtaining information on the mutagenic properties of Quercetin, Morin , Galangin, Luteolin, Apigenin, and Chrysin in Salmonella strains TA98 and TA100. Results on the antimutagenic properties of the latter chemicals against BaP-induced mutagenesis are reported as well. Finally, in combination with the results published earlier, some tentative conclusions are drawn about the chemical structures associated with in vitro mutagenic and/or antimutagenic properties within the present series of bioflavonoid compounds.

2. MATERIAL AND METHODS

2.1. Bacterial strains:

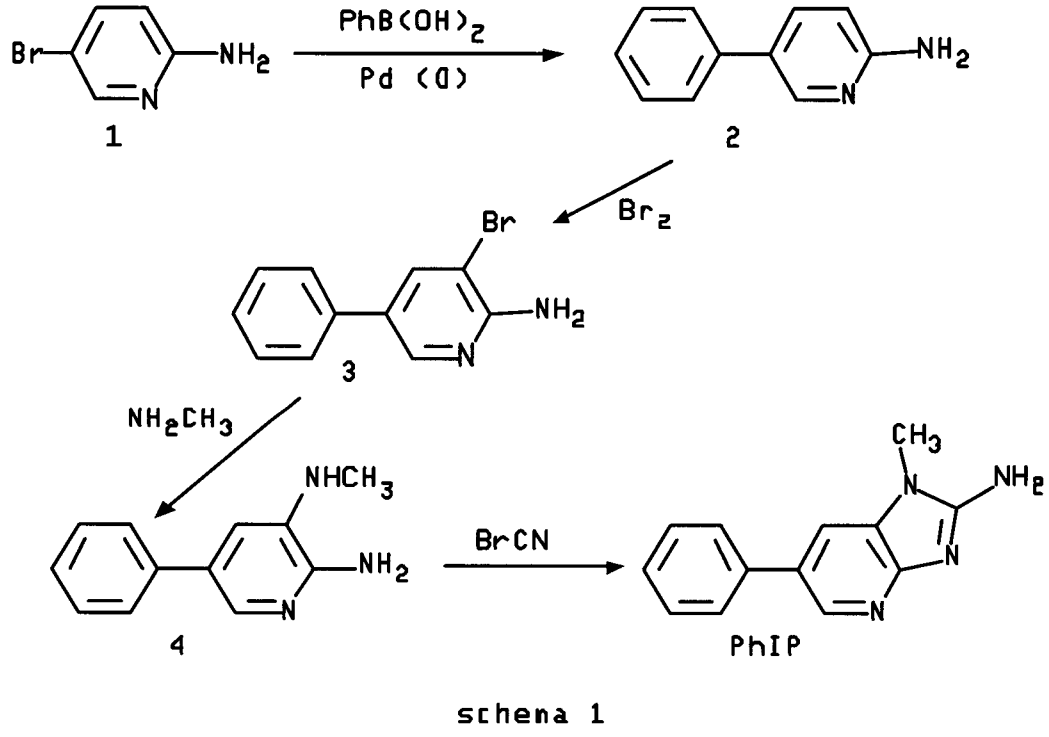
The bacterial strains used were Salmonella typhimurium TA98 and TA100, as described by Maron and Ames (1984); derivatives of both strains were kindly provided by Prof. B.N. Ames, University of California at Berkeley, USA, and were kept as frozen aliquots since their receipt. Methods for handling and culturing the strains, as well as compositions of the bacteriological media and reagents used for performing the (anti-)mutagenicity tests were described earlier (Voogd and Van der Stel, 1993; Voogd et al., 1993).

2.2. Test chemicals:

Benzo(a)pyrene (BaP; lot nr. 24022; purity 98,64%) was purchased from Serva, Heidelberg; its directly active metabolite, benzo(a)pyrene-7,8-diol-9,10 epoxide (BPDE; batch nr. 02) was obtained from Midwest Research Institute. PhiP was synthesized in our laboratory, as described in Scheme 1.

SCHEME 1.

Outline of the synthesis of 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP) according to Knize and Felton (1986), with modifications.



schema 1

The synthesis consisted of four steps, following basically the procedures of Knize and Felton (1986) with substantial modifications. Compound 2 was prepared from 1 (10,38 g, 60 mmol) by the procedure of Stavenuiter et al. (1987). The product was not isolated, but extracted from

the organic layer of the reaction mixture into a 2N H_2SO_4 solution. To this acid solution 3.1 ml (60 mmol) of Br_2 was added at room temperature to convert compound 2 to 3.
After the reaction had been completed, as judged from TLC, the solution was basified with a 25% NH_4OH solution and extracted with ethyl acetate. The ethyl acetate extracts were washed with water and dried over anhydrous magnesium sulphate. After evaporation of the solvent, crude 3 was obtained.
For the preparation of 4 the crude 3 was treated together with 1 g $CuSO_4$ in 180 ml of 40% aqueous methylamine at 220°C in a PTFE-lined pressure bomb for 4 hours.
The product was extracted with chloroform. After evaporation of 90% of the solvent, the residue was purified by flash chromatography on silica gel (Still et al., 1978). Yield: 5.5 g (27.6 mmol, 46%). For the final step, the cyclisation of 4 to PhIP, 2.75 g (13.8 mmol) of 4 was dissolved in 180 ml of 25% phosphoric acid, 5 g of CNBr was added, and the mixture was heated in a PTFE-lined pressure bomb for 5 hours at 180°C. After cooling, the reaction mixture was poured out over ice and extracted with chloroform. The aqueous phase was basified by the careful addition of 1 N NaOH and extracted with 1-butanol. Silica gel was added to the butanol extract. The butanol extract was evaporated and the residue was purified by flash chromatography on silica gel, to give PhIP. Yield: 1.2 g (5.4 mmol, 39 %).

The chemicals used in the antimutagenesis studies were obtained as follows: Quercetin dihydrate, purity 99%, was purchased from Janssen Chimica, lot nr. 53173/1; Morin dihydrate, lot nr. 60985/1, was purchased from Janssen Chimica; Galangin, Batch nr. 1344592 was purchased from Aldrich Chemicals; Luteolin, charge nr. 12210842, was purchased from Roth Chemicals, as was Apigenin, Charge nr. 1929410; Chrysin, batch nr. 7259225 was purchased from Aldrich Chemicals.

2.3. Mutagenesis and antimutagenesis test procedures:
The test procedures were basically as described by Voogd and Van der Stel (1993) and Voogd et al. (1993). In both test procedures, use was made of the plate incorporation test. In brief, the bacterial indicator cells were mixed together with the mutagenic test substance, an active fraction of mammalian liver homogenate (Aroclor-induced rat liver S9 *plus* cofactors), and eventually the anti-mutagenic test substance, and then poured in soft agar over the surface of selective agar plates. After solidification of the soft agar, and upon incubation of the plates in the dark at 37°C for 3 days, the numbers of mutant (HIS+ revertant) colonies were counted and plotted against the concentration of test chemical in the plate.

3. RESULTS

3.1. Mutagenesis studies with BaP, BPDE, and PhIP.

A first series of experiments was aimed at establishing the optimum mutagenic concentrations of BaP or BPDE to be further used in the antimutagenesis studies. Some experiments were also performed with PhIP to confirm its mutagenic activity towards Salmonella strains TA98 and TA100. The dose-dependent induction of HIS+ revertants in TA98 and TA100 exposed to BaP at concentrations up to 100 μg per plate in the presence of mammalian metabolic activation is shown in Fig. 2a (see APPENDIX).

The dose-dependent induction kinetics of HIS+ mutations are very similar to those observed under the same experimental conditions in earlier studies (RIVM, 1993a, 1993b) or in other laboratories (Maron and Ames, 1984), and indicate a saturation of mutagenic activity at BaP concentrations at or above 10 μg per plate which is probably due to the limited metabolic capacity of the rat liver fraction. As also expected from earlier results, no mutagenic activity of BaP was apparent in the absence of mammalian metabolic activation (data not shown). In order to remain within the BaP dose range in which the kinetics of mutation induction are compatible with linearity (see Fig. 2b), a BaP concentration of 2 μg per plate was chosen for the antimutagenesis studies.

The mutagenic activity of BPDE for Salmonella TA100 in the absence of mammalian activation is shown in Fig. 3. As expected from previous results, the mutagenic potency of BPDE in this system is much higher than that of the parent compound BaP. This again indicates that the in vitro metabolic activation of BaP by mammalian liver S9 is not complete under the present conditions. BPDE was mutagenic in Salmonella TA98 as well (data not shown).

The mutagenic activity of PhIP in the presence of mammalian metabolic activation in Salmonella strains TA98 and TA100 is shown in Fig. 4. As in the case of BaP, the results obtained with PhIP are similar to those observed earlier (Voogd et al., 1993b). No mutagenic activity was observed in PhIP-exposed bacteria in the absence of mammalian activation (data not shown). A notable difference between BaP and PhIP mutagenesis in the present system is the response of strains TA98 and TA100 for the two compounds, BaP inducing at equivalent exposures more mutations in TA100 than in TA98, while the contrary is observed in PhIP-exposed bacteria. As expected from earlier studies, this phenomenon is very probably due to the quite different metabolic products formed by BaP and PhIP, and the different probabilities of the individual metabolites to result in HIS+ mutations (Holme et al., 1989; RIVM, 1989).

3.2. Mutagenesis studies with bioflavonoids.

Some of the bioflavonoids chosen in the present studies were already described as possessing mutagenic properties in various test systems (Carver et al., 1983; Das et al., 1994; Mac Gregor et al., 1983; Voogd et al., 1993b). In order to obtain a systematic overview of their activity under identical experimental conditions, all compounds were tested for mutagenic activity in strains TA98 and TA100, at concentrations up to 100 μg per plate, and in the presence and/or absence of mammalian metabolic activation.

Results of mutagenesis experiments with Quercetin, Morin hydrate, Galangin, Luteolin, Apigenin, and Chrysin in Salmonella strain TA98 and/or TA100 in the presence and/or absence of mammalian activation are depicted in Fig. 5, 6, 7, 8, 9, and 10, respectively (see APPENDIX). Essentially comparable results, and conclusions about mutagenic activity, were obtained in the two Salmonella strains, but the data for only one of either strain are presented.

In confirmation of earlier experiments (Voogd et al., 1993b), Quercetin was clearly mutagenic for Salmonella strain TA98 (and TA100, data not shown) and induced a dose-dependent increase of HIS+ mutations in the range up to 100 μg per plate. Its mutagenic activity was not dependent upon, but greatly enhanced by, the addition of mammalian liver S9 homogenate, as shown in Fig. 5. Results of mutagenesis experiments with Morin hydrate are shown in Fig. 6. Although of substantially lower mutagenic activity than Quercetin, this former compound induced a significant number of HIS+ mutations at the highest dose tested (100 μg per plate) in the presence of mammalian metabolic activation, but not in its absence. Analogous results were obtained in Salmonella strain TA100 (data not shown). However, it cannot be decided on the basis of the present results whether Morin hydrate can indeed be classified as "mutagenic" for both strains, until confirmatory experiments (including higher dose levels) have been performed. The remaining compounds Galangin, Luteolin, Apigenin, and Chrysin, all were devoid of mutagenic activity under the present experimental conditions, i.e. at concentrations up to 100 μg per plate, as shown in Fig. 7 to 10 (see APPENDIX).

3.3. Antimutagenesis studies with bioflavonoids.

The antimutagenic properties of the various bioflavonoids were tested against BaP-induced mutagenesis in the presence of mammalian metabolic activation. The standard BaP concentration in Salmonella strains TA98 and TA100 was 2.0 μg per plate, and the bioflavonoid concentrations assayed were 100 μg per plate. The results are summarized in Table 2 (Salmonella strain TA98) and Table 3 (Salmonella strain TA100). They demonstrate that all bioflavonoids of the present series do possess antimutagenic activity against BaP-induced mutagenesis in both strains. This is shown by the lower number of observed HIS+ mutants as expected on the basis of BaP mutagenesis alone.

Because of the relatively high mutagenic activity of Quercetin in the system, the antimutagenic properties of this compound are somewhat obscured compared to the remaining bioflavonoids. Nevertheless, a slight reduction of the number of BaP-induced mutants was consistently seen in TA98 and TA100 (cf. Table 2 and 3). The potential mutagenic effects of Morin hydrate also may have been responsible for a smaller reduction of BaP-induced mutations, as compared to the remaining compounds of the series, which reach spontaneous levels of HIS+ mutations in the case of Galangin and Chrysin.

TABLE 2

Antimutagenic effects of bioflavonoids against the mutagenic activity of benzo(a)pyrene (BaP) in Salmonella TA98 in the presence of mammalian metabolic activation.

SUBSTANCE	HIS+ REVERTANTS PER PLATE		
	-BaP	+BaP	expected
spontaneous	47	-	
2-AA[(1)]	996	-	
BaP[(2)]	-	203	
Chrysin[(3)]	41	62	± 200
Apigenin	49	111	± 200
Luteolin	53	102	± 200
Galangin	39	46	± 200
Morin hydrate	91	124	± 200
Quercetin	1143	1128	± 1300

[(1)]2-AA (2-aminoanthracene; control mutagen) conc.: 2.0 µg per plate
[(2)]BaP (benzo(a)pyrene; inducing mutagen) conc.: 2.0 µg per plate
[(3)]Chrysin and other bioflavonoids conc.: 100 µg per plate

Since Chrysin was the compound with the lowest number of hydroxyl residues in the present series, and still exhibited a strong antimutagenic activity in the first experiments (at 100 µg per plate), further tests were performed to assess more quantitatively its antimutagenic potential against BaP. Results of the dose-dependent inhibition of BaP-mutagenesis (2.0 µg per plate) by Chrysin in Salmonella strains TA98 and TA100 are shown in Fig. 11 (see APPENDIX). They demonstrate that a significant reduction of BaP-induced HIS+ mutants occurs already at 5.0 µg per plate (and probably lower), and that a reduction to spontaneous mutation levels is reached at 50 µg per plate.

In order to elucidate whether the antimutagenic action of Chrysin was due to inhibition of mammalian metabolic anzymes or to the scavenging of reactive BaP metabolites, dose-dependent mutagenesis experiments were carried out with BPDE (up to 0.5 µg per plate) in strains TA98 and TA100, in which Chrysin was simultaneously added at concentrations of 50 or 100 µg per plate. The results are depicted in Fig. 12 (see APPENDIX) and show that the mutagenic action of BPDE can indeed be inhibited by Chrysin. However, its antimutagenic activity becomes apparent only above relatively high BPDE-concentrations (0.1 - 0.2 µg per plate) and seems similar for the two chrysin concentrations used. Analogous results were obtained with Salmonella strain TA98 (data not shown).

TABLE 3

Antimutagenic effects of bioflavonoids against the mutagenic activity of benzo(a)pyrene (BaP) in Salmonella TA100 in the presence of mammalian metabolic activation

SUBSTANCE	HIS+ REVERTANTS PER PLATE		
	-BaP	+BaP	expected
spontaneous	136	-	
2-AA[(1)]	1222	-	
BaP[(2)]	-	490	
Chrysin[(3)]	121	122	± 500
Apigenin	155	220	± 500
Luteolin	163	230	± 500
Galangin	86	106	± 500
Morin hydrate	166	308	± 500
Quercetin	1061	1020	± 1500

[(1)]2-AA conc. : 2.0 µg per plate (control mutagen)
[(2)]BaP conc. : 2.0 µg per plate (inducing mutagen)
[(3)]Chrysin and other bioflavonoids: 100 µg per plate

Finally, experiments were performed in order to assess the antimutagenic potential of Chrysin against the mutagenic effects induced by another bioflavonoid of the present series, Quercetin. The results of a representative experiment in Salmonella TA98, in which Chrysin was added at 50 µg or 100 µg per plate in the presence of mammalian activation, are shown in Fig. 13. As in the case of BPDE they demonstrate a slight inhibition of Quercetin-induced mutagenesis. The inhibition of Quercetin-induced mutagenesis in Salmonella strain TA98 (and strain TA100, data not shown) was also observed in the absence of mammalian activation, as depicted in Fig. 14 (see APPENDIX).

4. DISCUSSION AND CONCLUSIONS

4.1. Mutagenic activity of bioflavonoids.

In combination with data reported earlier (Voogd and Van der 1993b), the present series of experiments show a diversity of mutagenic activity among the bioflavonoids for Salmonella strains TA98 and TA100 in vitro. On the basis of equivalent exposure expressed in μg per plate, and in comparison with the reference mutagens BaP and PhIP, the mutagenic potency ranged from relatively high (Quercetin) to non-detectable (e.g. Chrysin), the ranking in decreasing order being Quercetin > Myricetine = Kaempferol >>> Morin hydrate >>> Galangin, Luteolin, Apigenin, Chrysin; the last 4 chemicals being devoid of mutagenic activity in the present in vitro system. The present results confirm and systematically expand the available database on the mutagenic potential of bioflavonoids (Camoirano et al., 1994; Carver et al., 1983; Das et al., 1994; De Flora, 1988).

With regard to the mechanism(s) behind the in vitro mutagenicity of bioflavonoids, such as Quercetin, Myricetine, and Kaempferol, some conclusions can be drawn on the basis of the structure-mutagenicity relationships observed. As a general rule, increasing hydroxylation of the parent molecule leads to increasing mutagenic properties. Hydroxylation of the benzopyrane ring at C-3 position appears to be a prerequisite for mutagenicity, as demonstrated by the fact that Quercetin is mutagenic whereas Luteolin is not. In addition, hydroxylation of the phenyl side ring at C-4' (and C-3') appears to be necessary (Quercetin, Myricetine, Kaempferol), but not sufficient in the absence of hydroxylation at C-3 (Luteolin, Apigenin). As already observed by others (Laughton et al., 1989), the dependence of mutagenicity on (several) hydroxyl residues may point to the pro-oxidant and antioxidant state of the molecule as being associated with its mutagenic potential. In particular the accelerated formation of hydroxyl radicals and the induction of DNA damage under aerobic conditions in the presence of iron (III) or copper (II) ions appear to be responsible for the in vitro mutagenic properties of bioflavonoids, including those of the present studies (Laughton et al., 1989; Lee et al., 1994; Rahman et al., 1989, 1992; Sahu and Gray, 1993; Said Ahmad et al. 1992). This hypothesis is also compatible with the earlier findings that bioflavonoids are of doubtful mutagenic activity in vivo, possibly due to the scavenging of active oxygen species by catalase and/or superoxide dismutase in mammalian cells (MacGregor et al., 1983; Sahu and Gray, 1993; Rahman et al., 1992).

4.2. Antimutagenic properties of bioflavonoids.

With regard to antimutagenic properties, there was a definite tendency towards antimutagenic effects against BaP-induced mutagenesis among all the bioflavonoids investigated so far. In preliminary experiments, the antimutagenic effects of Chrysin against PhIP-induced mutagenesis could be ascertained as well. Because of the relatively high mutagenic properties of Quercetin in the present system, the antimutagenic activity of this compound could not be efficiently determined; however, other studies have demonstrated its antimutagenicity in vitro (see for example Das et al., 1994).

In general, the results obtained so far allow to draw the following conclusions: In clear contrast to the mutagenic properties among bioflavonoids, the expression of antimutagenic properties does not appear to be correlated with the number and/or the location of hydroxyl residues in the flavone molecule; this becomes evident in view of the antimutagenic activites of both the flavone and flavonol derivatives. In the present series, the chemical with the lowest number of hydroxyl groups, Chrysin, was as efficient against BaP-induced mutagenesis as, for example, Galangin or Luteolin. On the basis of the present results with BaP and BPDE, and of results obtained by others (see also Voogd et al., 1993b), it seems possible that the antimutagenic activity of bioflavonoids is associated with a dual mode of action, namely,
(i) inhibition of mammalian enzymes (very probably cytochrome P-450 isozymes) involved in the bio-activation of BaP (Lee et al., 1994; Buening et al., 1981; Das et al., 1994) , and
(ii) direct interaction with and scavenging of reactive PAH-metabolites, such as BPDE, as already postulated by Huang et al. (1993), De Flora and Ramel (1988), Kada and Simoi (1987), and Ramel et al. (1986). Finally, it should be noted that the combined exposure of Quercetin and Chrysin resulted in decreased mutagenesis of the former compound for Salmonella strains TA98 and TA100. This result confirms experimentally that the biological effects of natural mixtures of bioflavonoids will very likely represent the netto results of mutagenic and antimutagenic properties of the individual compounds, as well as their interreactivity.

Taken together, the present studies have confirmed and extended the range of bioflavonoid compounds which exhibit antimutagenic activities against BaP-induced mutagenesis in vitro. The results demonstrate that bioflavonoids with low numbers of hydroxyl groups, e.g. Chrysin, are as potent antimutagens as those with high hydroxyl group numbers, e.g. Quercetin and/or Kaempferol, in the Salmonella TA98 and/or TA100 system. Whether this antimutagenic activity in vitro is expressed in whole animal mutagenesis and/or carcinogenesis studies as well remains to be determined.

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APPENDIX

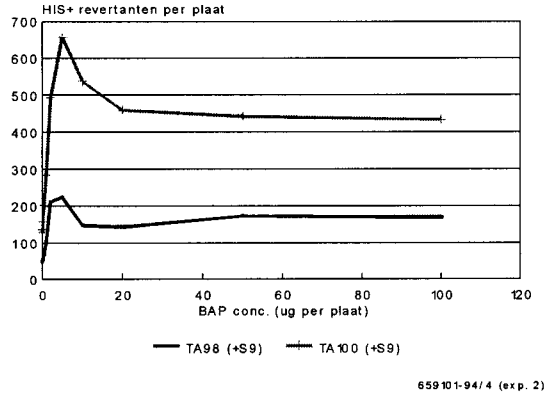

Fig. 2a.
Mutagenic activity of benzo(a)pyrene (BaP) at concentrations up to 100 µg per plate for Salmonella typhimurium strains TA98 and TA100 in the presence of mammalian metabolic activation (liver S9 fraction).

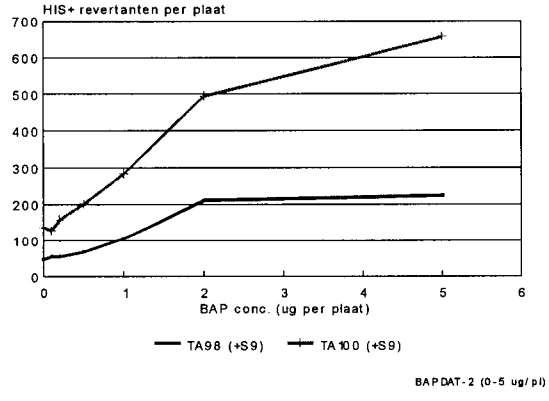

Fig. 2b.
Mutagenic activity of BaP at concentrations up to 5 µg per plate for Salmonella typhimurium strains TA98 and TA100 in the presence of mammalian metabolic activation.

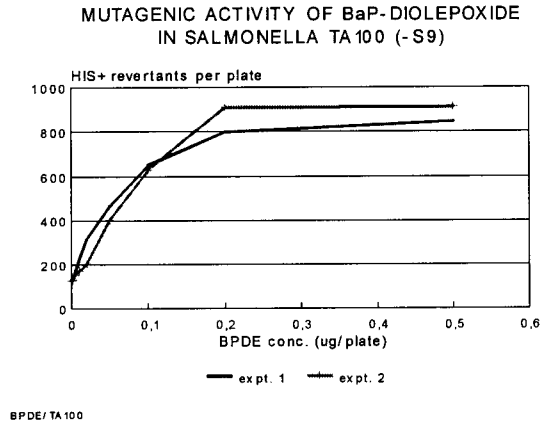

Fig. 3.
Mutagenic activity of benzo(a)pyrene-7,8-diol-9,10-epoxide (BPDE) at concentrations up to 0.5 µg per plate for Salmonella strain TA100 in the absence of mammalian metabolic activation.

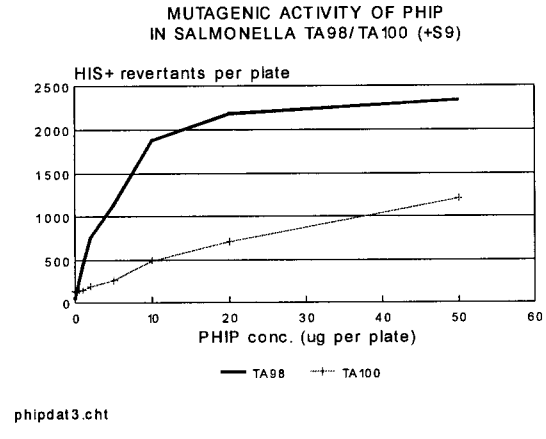

Fig. 4.
Mutagenic activity of PHIP at concentrations up to 50 µg per plate in Salmonella strains TA98 and TA100 in the presence of mammalian metabolic activation.

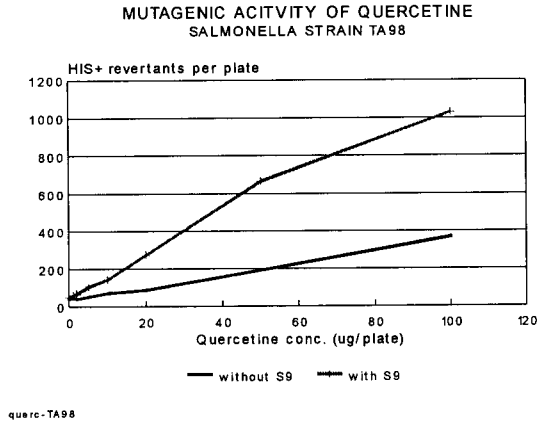

Fig. 5.
Mutagenic activity of Quercetin at concentrations up to 100 µg per plate for Salmonella strain TA98 in the presence and/or absence of mammalian metabolic activation.

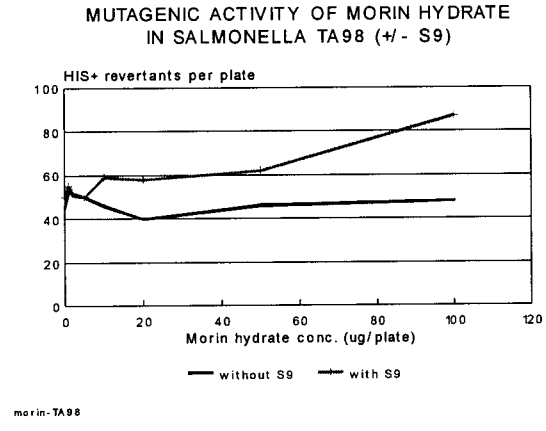

Fig. 6.
Mutagenic activity of Morin hydrate at concentrations up to 100 µg per plate for Salmonella strain TA98 in the presence and/or absence of mammalian metablic activation.

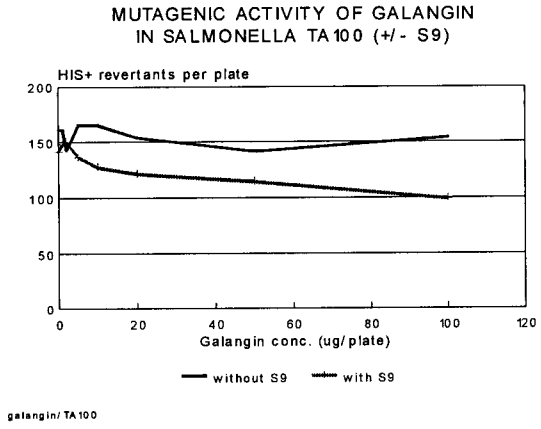

Fig. 7.
Mutagenic activity of Galangin at concentrations up to 100 µg per plate for Salmonella strain TA100 in the presence and/or absence of mammalian metabolic activation.

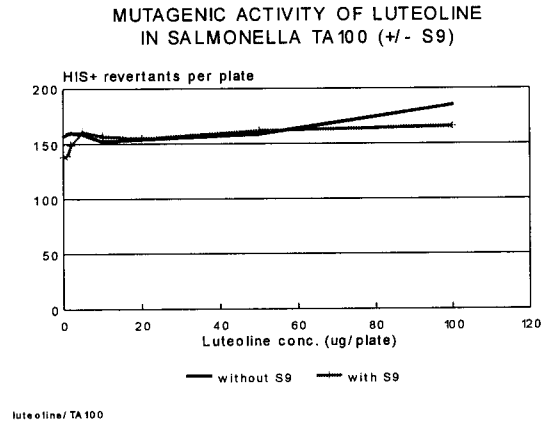

Fig. 8.
Mutagenic activity of Luteolin at concentrations up to 100 µg per plate for Salmonella strain TA100 in the presence and/or absence of mammalian metabolic activation.

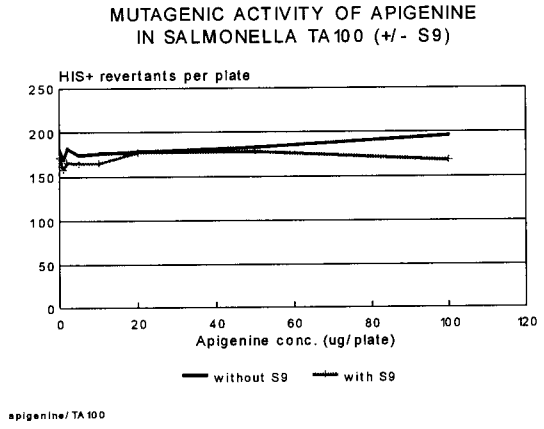

Fig. 9.
Mutagenic activity of Apigenin at concentrations up to 100 µg per plate for Salmonella strain TA100 in the presence and/or absence of mammalian metabolic activation.

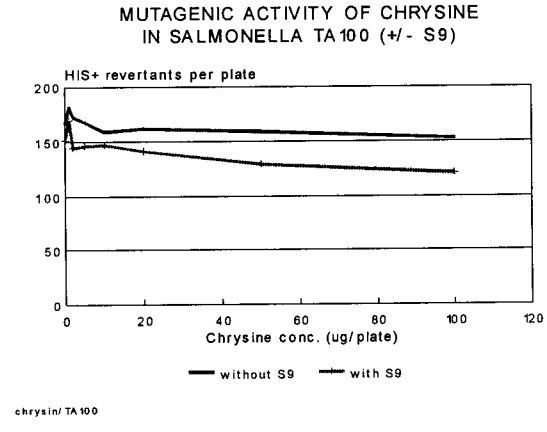

Fig. 10.
Mutagenic activity of Chrysin at concentrations up to 100 µg per plate for Salmonella strain TA100 in the presence and/or absence of mammalian metabolic activation.

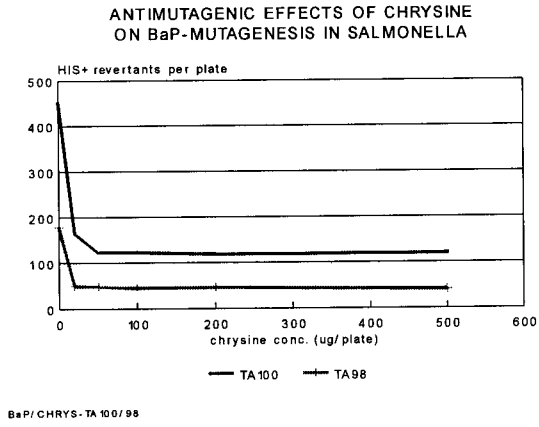

Fig. 11.
Antimutagenic effect of Chrysin at concentrations up to 500 µg per plate on BAP-induced mutagenesis in Salmonella strains TA98 and TA100 in the presence of mammalian metabolic activation.

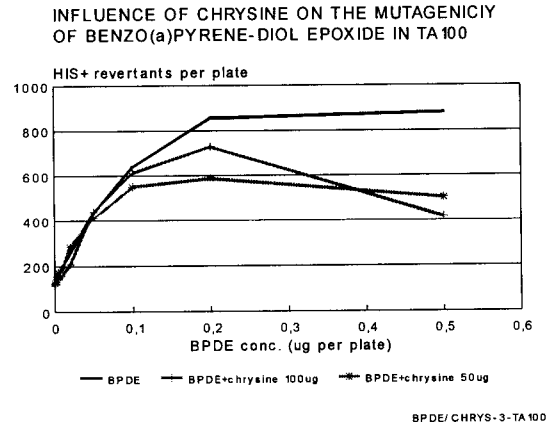

Fig. 12.
Antimutagenic effect of Chrysin at concentrations of 50 and/or 100 µg per plate on BPDE-induced mutagenesis in Salmonella strain TA100 in the absence of mammalian metabolic activation.

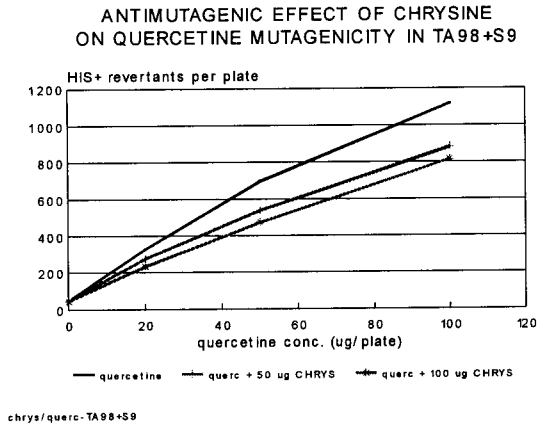

Fig. 13.
Antimutagenic effect of Chrysin at concentrations of 50 and/or 100 μg per plate on Quercetin-induced mutagenesis in Salmonella TA98 in the presence of mammalian metabolic activation.

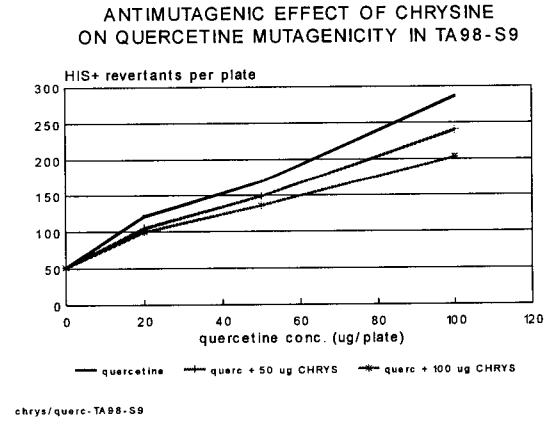

Fig. 14.
Antimutagenic effect of Chrysin at concentrations of 50 and/or 100 μg per plate on Quercetin-induced mutagenesis in Salmonella TA98 in the absence of mammalian metabolic activation.