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## Quantitative assessment of the genotoxicity of fecapentaenes

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### Summary

Fecapentaenes are a group of fecal mutagens of microbial origin isolated from human stools. Fecapentaene-12 (F-12) and fecapentaene-14 (F-14), differing only in two carbon atoms in the side chain, are glyceryl ethers with a highly reactive chromophoric aliphatic side chain incorporating a conjugated pentaene moiety. Although these compounds are known for their genotoxicity, no test systems have been developed to precisely assess their relative genotoxicity. In this study F-12 and F-14 were assayed for their genotoxicity using the SOS Chromotest in which the induction of  $\beta$ -galactosidase in *E. coli* PQ37 was used as a quantitative measure of biological activity. The activity obtained with F-12 and F-14 was compared with that of 4-nitroquinoline oxide (4-NQO) as the reference standard of a direct acting mutagen. While F-14 was almost as active as 4-NQO, F-12 was only about 25% as active as F-14, the higher analog.

The fecapentaenes are a group of fecal mutagens of microbial origin isolated from human stools (Bruce et al., 1981; Lederman et al., 1980; Van Tassell et al., 1982). They are, structurally, glyceryl ethers with a highly reactive, chromophoric, aliphatic side chain (12 or 14 carbons) incorporating a conjugated pentaene moiety (Gupta et al., 1983; Hirai et al., 1982).

These compounds (fecapentaene-12 and fecapentaene-14) are characterized by their mutagenicity in a number of test systems (Ricchio et al., 1987; Peters et al., 1988); they cause DNA damage in human and animal cells (Curren et al., 1987; Plummer et al., 1986; Hinzman et al., 1987) and have been implicated as a potential risk factor associated with human colon cancer (Schiffman, 1986). A number of studies reporting the biological activity of the naturally occurring fecapentaenes and their synthetic analogs have demonstrated their mutagenicity in *in vitro* systems (Goggelman et al., 1986; Govindan et al., 1987).

In this report we describe the quantitative assessment of the genotoxicity of F-12 and F-14 using the SOS Chromotest with 4-nitroquinoline oxide (4-NQO) as the reference standard of the

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**Abbreviations:** F-12, fecapentaene-12; F-14, fecapentaene-14; 4-NQO, 4-nitroquinoline oxide; X-Gal, 5-bromo-4-chloro-3-indolyl- $\beta$ -D-galactopyranoside; SOSIP, SOS inducing potency.

direct acting mutagen. The approach utilizes the induction of  $\beta$ -galactosidase in *E. coli* PQ37 (Quillardet et al., 1982; Quillardet and Hofnung, 1985; Quillardet et al., 1985) as a measure of genotoxicity, a procedure that is quantitative and highly reproducible.

### Materials and methods

**Reagents.** *E. coli* PQ37 was obtained as part of the Chromotest kit (Thomas and Thomas Technologies, Inc.). 4-NQO and X-gal were purchased from Aldrich and Boehringer Mannheim respectively. Bactopeptone and Bacto Yeast extract for LB medium were obtained from Difco. F-12 and F-14, synthesized by Stanford Research Institute by the general procedure of Nicolaou et al. (1984) consisted of a mixture of 5 *cis-trans* isomers of which the all-*trans* form represented 80% of the total.

**Bacterial cultures.** The tester strain (*E. coli* PQ37) is rehydrated with LB medium (Miller, 1972) and grown overnight at 37°C. The bacterial suspension is diluted with LB medium to a working concentration of 0.04 O.D.<sub>600 nm</sub>.

**SOS Chromotest.** The  $\beta$ -galactosidase gene, *lacZ*, in *E. coli* PQ37, is placed under the control of *SulA*, one of the SOS genes (Huisman and D'Ari, 1981). In addition to the *SulA::lacZ* operon fusion, there is a deletion in the *lac* region so that the activity of  $\beta$ -galactosidase is entirely dependent upon *SulA* expression (Quillardet et al., 1982; Quillardet and Hofnung, 1985).

The assay of genotoxic activity of 4-NQO, the reference standard, and that of F-12 and F-14 are carried out in flat-bottomed 96 well ELISA plates (Falcon #3072). A Pro/pette liquid handling system (Perkin Elmer/Cetus) is used for all the dilutions and additions so that the time required for each step in the procedure is uniform from run to run. A standardized stock solution of 4-NQO at a concentration of 2 mg/ml in pure DMSO is freshly prepared, and 50  $\mu$ l of a 1:60 dilution is used to make 7 two-fold serial dilutions in duplicate wells. A 3- $\mu$ l aliquot of each dilution is transferred to a second test plate and incubated at 37°C for 3 h with 100- $\mu$ l aliquots of a working suspension of *E.*

*coli* PQ37. The plate is vigorously agitated on an orbital shaker placed inside the incubator. After 3 h, 100  $\mu$ l of X-gal solution (2.0 mg/ml in 20% DMSO in 0.3 M phosphate buffer, pH 7.5, containing 0.003 M magnesium chloride and 5% methanol) is dispensed into each of the wells, the contents are mixed thoroughly and read immediately in a microplate reader (Titertek Multiscan, Flow Laboratories) at 615 nm to establish baseline readings. The plate is incubated for an additional three hours before reading at 615 nm. The net increase in OD is entered into a computer program to generate a dose-response curve from which a linear regression plot of the linear portion of the graph is constructed. The SOS Inducing Potency (SOSIP) of 4-NQO or of F-12 and F-14 were calculated and expressed as the SOSIP of 1  $\mu$ g of 4-NQO under standard conditions (Quillardet et al., 1982).

These experiments were conducted over a period of a year, and F-12, F-14 and 4-NQO were routinely assayed simultaneously. The activity of alkaline phosphatase, the constitutive enzyme, was frequently monitored for viability of the test organism as described by Quillardet et al. (1982). Furthermore, in each assay, duplicate wells were run at each dose level, along with duplicate controls consisting only of the reagents. The assays of relative potency carried out over a period of a year established the constancy of the response characteristics of the test organism.

**Reference materials.** Stock solutions of F-12 and F-14 were stored at -70°C in amber vials sealed under an atmosphere of argon. Aliquots of the stock solution in methanol or DMSO were diluted with methylene chloride (1:1) and spectral scans were carried out in a Cary Model 2200 Double Beam Recording Spectrophotometer to obtain a profile of the characteristic UV/visible absorption spectrum of the undegraded compound. In order to obtain the precise concentrations of 4-NQO in stock solutions, UV/visible absorption spectra were obtained and its molar absorptivity ( $\epsilon$ ) determined at its absorption maximum (Montgomery and Swenson, 1969).

**Definition of unit of activity.** The SOS Inducing Potency (SOSIP) is the slope of the linear

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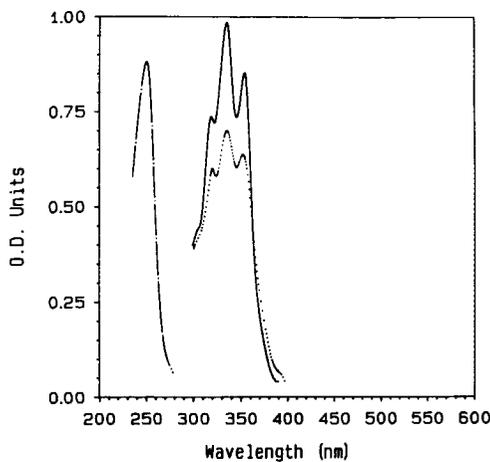


Fig. 1. Ultraviolet absorption spectra of 4-nitroquinoline oxide, fecapentaene-12 and fecapentaene-14. —○—, 4-NQO; —, F-14; ·····, F-12.

TABLE 1

MOLAR ABSORPTIVITY ( $\epsilon$ ) OF THE FECAPENTAENES AND 4-NQO

The test compounds were carefully weighed and dissolved in HPLC grade methanol:methylene chloride (1:1). Spectra were obtained in a Cary 2200 Double Beam Recording Spectrophotometer and the absorbances at peak maxima were used to compute molar absorptivity ( $\epsilon$ ).

Compound	Wavelength ( $\lambda$ )	$\epsilon \times 10^{-3}$ ( $M^{-1}cm^{-1}$ )
Fecapentaene-12	333 nm	93.9
Fecapentaene-14	335 nm	110
4-Nitroquinoline oxide	255 nm	17.2

Molar absorptivity ( $\epsilon$ ) is defined as the absorbance of a one molar solution of the test substance at the stated wavelength maximum using a cell of 1-cm pathlength (Montgomery and Swenson, 1969).

**Mutagenicity of 4-NQO.** The genotoxicity of 4-NQO in the SOS test system is represented by the dose response curve shown in Fig. 2. The linear segment of this curve is used to construct the linear regression plot shown in the same figure. The toxicity of 4-NQO at higher concentrations is reflected by a drop in the dose response curve. The values of SOSIP for 4-NQO monitored over a one year period ranged between 45 and 55 ( $47.3 \pm 2.5$ ; mean + SE).

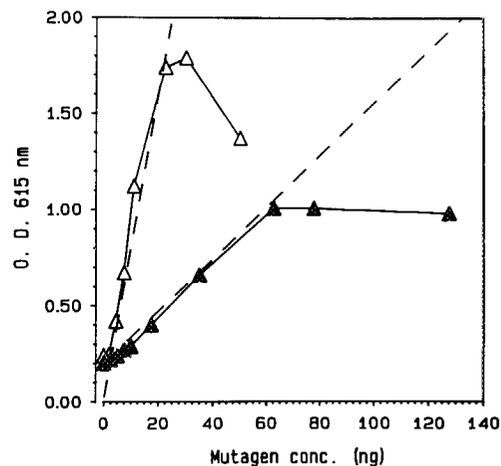


Fig. 2. Dose response curves and linear regression plots for mutagenicity of 4-nitroquinoline oxide and fecapentaene-12.  $\Delta$ , 4-nitroquinoline oxide;  $\blacktriangle$ , fecapentaene-12; -----, linear regression plots.

agitated on an incubator. After 3 ng/ml in 20% , pH 7.5, con- and 5% of the wells, the and read im- Titertek Multi- m to establish ubated for an ng at 615 nm. to a computer ise curve from linear portion SOS Inducing F-12 and F-14 e SOSIP of 1 ditions (Quil-

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portion of the dose-response curve. A unit of activity is defined as the SOSIP of 1  $\mu$ g of 4-NQO under standard conditions (Quillardet et al., 1982).

**Safety precautions.** Since these compounds are suspected carcinogens and are mutagenic, they were handled in safety cabinets and all personnel wore protective outer garments and gloves. All waste material was collected separately for special disposal.

## Results

**Spectral characteristics and molar absorptivity of 4-NQO and of the fecapentaenes.** The spectral characteristics of 4-NQO, F-12 and F-14 are shown in Fig. 1. The experimentally determined values for the molar absorptivity ( $\epsilon$ ) at their respective wavelength of maximum absorption are presented in Table 1. Since the triplet exhibited by the fecapentaenes in the 320–360 nm range is a characteristic of the undegraded compound, all test solutions are initially checked by this criterion before being assayed for mutagenicity. The absolute concentrations of the test substances are established by reference to the values of molar absorptivity ( $\epsilon$ ), the extinction coefficient of a 1 M solution.

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TABLE 2  
MUTAGENICITY OF FECAPENTAENES RELATIVE TO  
THAT OF 4-NITROQUINOLINE OXIDE

The SOSIP of 4-NQO = 55.

Compound	Relative activity
4-Nitroquinoline oxide	1.0
Fecapentaene-12	0.23
Fecapentaene-14	0.91

*Mutagenicity of F-12 and F-14.* The mutagenicity of F-12, the predominant form in human stools (unpublished observations), is shown in Fig. 2. The dose response curve and linear regression plot were similar in appearance to those obtained with 4-NQO. F-14 also gave reproducible dose response curves and linear regression plots as in the case of F-12. The values of SOSIP for F-14 and F-12 were  $50.1 \pm 1.8$  and  $12.7 \pm 0.6$  (means  $\pm$  SE) respectively for a set of four replicates.

*Relative Mutagenic Potency of F-12 and F-14.* In order to determine, quantitatively, the relative mutagenic potency of F-12 and F-14, their respective SOSIP values were transformed to units relative to that of 4-NQO. The results presented in Table 2 show that F-14 is as potent a mutagen as 4-NQO and that in comparison F-12 is a significantly weaker mutagen.

## Discussion

The fecapentaenes are an important class of naturally occurring mutagens in stools (Van Tassell et al., 1982). Several studies reporting their genotoxicity using the Ames/Salmonella assay (Gupta et al., 1983; Riccio et al., 1987; Peters et al., 1988; Govindan et al., 1987) have shown them to be direct acting mutagens. A recent report comparing F-12, F-14 and cis-FP-12 showed that all of them yielded similar genotoxic activities in the Ames assay, using the strain TA 100 as the test organism (Peters et al., 1988), although our results showed F-14 to be significantly more active than F-12. The apparent difference between the two studies is probably a reflection of the relative sensitivity of the test organisms to compounds with different lipid solubilities. The methodology

developed in the present study extends these observations for the assessment of genotoxicity in more precise quantitative terms. It is now feasible to express genotoxicity in terms of units relative to that of a known direct acting mutagen, viz. 4-NQO. The molar absorptivity ( $\epsilon$ ) of 4-NQO at 255 nm in the solvent system used in this study was consistent with published data for aqueous systems at 250 nm (Winkle and Tinoco, 1978). Since F-14 appears to be several fold more potent than F-12 in the present test system the total mutagenic load in fecal specimens will be dictated by the relative concentrations of each of these mutagens in the specimen.

Although F-12 and F-14 concentrations vary greatly from sample to sample (Baptista et al., 1984), in some of our human studies (unpublished observations), F-12 appeared to be the predominant form of fecapentaene with a few exceptions. F-12 which differs from F-14 only in the length of the side chain by a two-carbon unit, is less lipophilic. With appropriate modifications this approach could be applied to the quantitative assessment of genotoxicity in biological samples and permit statistical analyses of the data as a continuous variable.

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