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Evaluation of the mutagenic potential of root canal sealers using the salmonella/microsome assay

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Three root canal filling materials and their components were tested for mutagenic activity with the *Salmonella*/microsome test using tester strains TA100 and TA98 both in the spot test and in the plate incorporation test. Eluates of mixed Diaket and mixed N2, as well as their components, showed no mutagenicity. Dimethylsulphoxide (DMSO) eluates of freshly mixed AH26 were mutagenic to strain TA100, whereas those of the completely set material were not. The liquid component of AH26 is considered to be the mutagenic substance since it exhibited the same mutagenic effect as the mixed material. In both cases, mutagenicity was reduced by rat liver microsomes.

1. Introduction

Dental materials, especially root canal filling cements, usually remain in close contact with living oral tissues over a long period of time. Therefore, quality control of dental materials should address not only the usefulness but also the biological safety of these materials in order to minimize potential risks to health [1, 2].

Even though extensive *in vitro* and *in vivo* studies have been made during the last decade concerning the toxicological and tissue-irritating properties of dental materials, relatively little information is presently available regarding mutagenicity or carcinogenicity testing. Athas *et al.* [3] discovered alterations of DNA caused by catalyst and resin solutions of an orthodontic bonding resin thus indicating a genotoxic potential of these materials. As a consequence of these findings, the material was replaced by a slightly different formulation that was not carcinogenic in experimental animals [4]. By means of bacterial mutagenicity tests (Ames test), positive results were obtained with other orthodontic bonding materials [5, 6], a chemical component of a dental material [7], and an endodontic sealer [8].

The *Salmonella*/microsome assay developed by Ames and co-workers [9] is a widely used *in vitro* bacterial assay for screening genotoxic properties of chemicals. It was initially developed as a substitute for carcinogenicity testing [10]. Because this short-term screening test is inexpensive, rapid and easy to perform, it is capable of screening a large number of chemicals and it may in some cases limit the need for expensive and prolonged animal bioassays. Therefore it has also become part of recommended national and international standard testing protocols for dental materials [11–14].

In this study, three endodontic sealers and their components were tested for mutagenic activity by means of the *Salmonella*/microsome assay in both spot test and plate incorporation modes.

2. Materials and methods

2.1. Test materials

The three root-canal filling cements and their components that were screened for mutagenicity are listed in Table I. The composition of the test materials, as declared by the manufacturers, is presented in Table II.

2.2. Mutagenicity assays

The plate incorporation test and the spot test were carried out according to the procedure described by Maron and Ames [15]. *Salmonella typhimurium* strains TA98 and TA100 were used for the detection of frame-shift and base-pair mutations. The tester strains were kindly provided by Dr B. N. Ames, Department of Biochemistry, University of California at Berkeley. The genotype characteristics of the bacterial strains were confirmed within each experiment.

2.3. Plate incorporation test

After mixing according to the manufacturer's instructions, 1.5 g of each material were set for 1 h, 1 day or 1 week at 37 °C in a humidified chamber. The set materials were crushed in a mortar and 1 g of the resulting powder was eluted for 4 h or 4 days in 20 ml of physiological saline or in organic solvents dimethylsulphoxide (DMSO), and for Diaket dimethylformamide (DMF). DMF was used for Diaket, because it is recommended as an appropriate solvent by the manufacturer. 1 g of the sealer components (liquid and powder) was eluted as described above. Different dilutions

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TABLE I Test materials used

Material	Manufacturer	Batch No.
1. AH26	DeTrey, Konstanz, Germany	870309 871210 880525 188350 203560
2. N2	Indrag Agsa, Lausanne, Switzerland	BN 805/BN 808 7704/488
3. Diaket	ESPE, Seefeld, Germany	MO24 LO44 M100

TABLE II Composition of test materials according to manufacturers' descriptions

Material		Ingredients
1. AH26	Powder	8 g contain: 0.8 g powdered silver 4.8 g bismuth oxide 2.0 g methenamine 0.4 g titanium(IV)-oxide
	Liquid	7.5 g contain: 7.5 g epoxy-bisphenol-resin
2. N2	Powder	1 g contains: 0.630 g zinc oxide 0.036 g titanium(IV)-oxide 0.150 g basic bismuth nitrate 0.100 g basic bismuth carbonate 0.070 g paraformaldehyde 0.014 g dye
	Liquid	1 g contains: 0.770 g eugenol 0.018 g rose oil 0.012 g lavender oil 0.200 g peanut oil (PhHVI)
3. Diaket	Powder	1 g contains: 0.300 g bismuth phosphate 0.700 g zinc oxide
	Liquid	1 g contains: 0.005 g dichlorophene 0.002 g triethanolamine 0.760 g propionyl-acetophenone 0.233 g copolymer of vinyl acetate, vinyl chloride, vinyl iso butyl ether

of the original eluates of the test materials were prepared and tested for mutagenicity.

Two millilitre of molten top agar containing minimal histidine and biotin were routinely mixed with 0.1 ml of graded doses of eluates from set materials or their components. Following the addition of 0.1 ml bacterial suspension and, if appropriate, 0.5 ml of S9-mix, the mixture was plated on bottom agar containing minimal glucose. The plates were incubated upside down for 72 h at 37 °C and the prototroph revertant colonies were counted. Positive controls (Na-azide, trinitrofluorenone, 2-aminoanthracene), negative controls (solvent), and test samples were run concurrently in triplicate.

2.4. Spot test

Two millilitre of molten top agar which contained minimal histidine and biotin were mixed with 0.1 ml of bacterial suspension and 0.5 ml of S9-mix, if appropriate, and the mixture was plated on bottom agar containing minimal glucose. Specimens of the set materials were prepared as pieces of about 1 cm^2 and placed directly on the solidified top agar. In a modified procedure, the set materials were crushed in a mortar and eluates were prepared as described above. Ten microlitre of each eluate were pipetted onto a sterile filter disc (15 mm diameter), which then was placed on the solidified top agar.

2.5. Liver homogenate fraction (S9)

A liver microsomal fraction was prepared according to the method of Matsushima *et al.* [16] using phenobarbital/naphthoflavone induced Sprague–Dawley male rats. The prepared microsomal fraction was examined both for sterility and activity, and the protein content was determined [17]. Cofactors were added to obtain the S9-mix [15] and the mix was adjusted to 2.5 mg protein ml^{-1}.

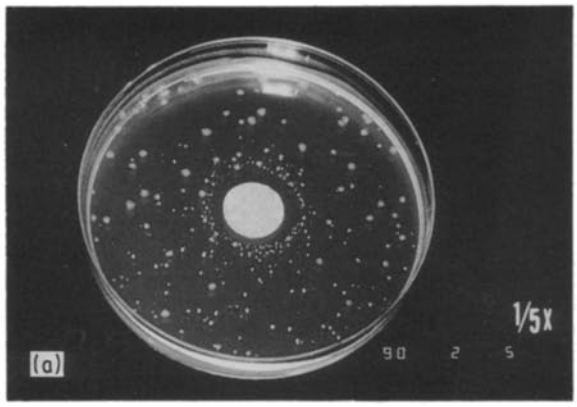
3. Results

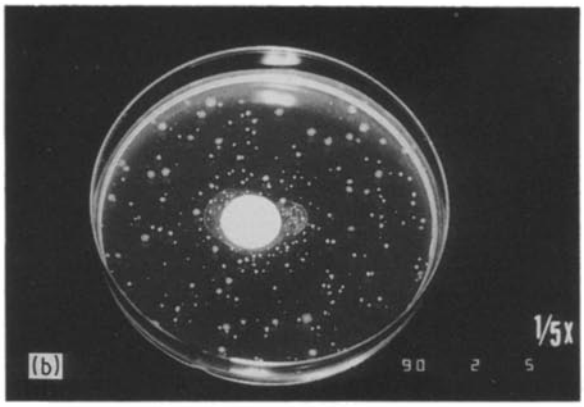
3.1. Spot tests

Spot tests were carried out on all mixed materials and their components, and with AH26 also on eluates with strains TA98 and TA100. When inspected by eye, the mixed N2 and Diaket as well as their components were toxic in both tester strains. This was indicated by a decreased or even completely absent background lawn on the experimental plates. The freshly mixed AH26 and its liquid component produced no or only a slight increase of the number of revertant colonies. The reaction of TA100 to DMSO-eluates, however, gave evidence for a positive effect compared to the solvent control (Fig. 1). No difference in mutagenicity was observed between the different batches of each test material.

3.2. Plate incorporation test

Because the data with eluates of the set AH26 obtained from the spot test did not exclude the possibility of a mutagenic effect to TA100, all materials were investigated in the plate incorporation assay to demonstrate a dose–response relationship. When the mixed AH26 was eluted for 4 h and 4 days in DMSO after a setting time of 1 h, it directly elicited mutagenicity in the *Salmonella* strain TA100. The number of histidine prototroph bacterial colonies was about four times higher than the number of colonies representing the solvent control (Fig. 2). The mutagenic effect caused by the mixed material decreased as setting time increased. Mutagenicity was clearly reduced after a setting time of up to 1 day and was completely eliminated when the material had set for 1 week. At higher concentrations the material was toxic to the bacterial cells (Fig. 2). Toxicity was not elicited by higher concentrations of organic solvents,





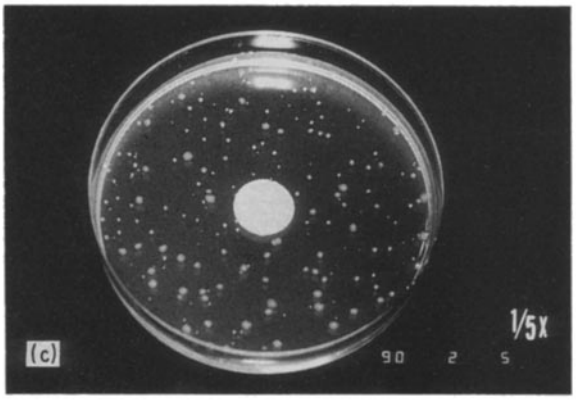


Figure 1 Results from eluates (4 days) of (a) mixed AH26 (1 h after mixing). (b) Liquid component; and (c) solvent control in the spot test (TA100).

as could be demonstrated in control experiments. No mutagenic effect was found with the mixed AH26 after elution in physiological saline (Fig. 2)

The liquid component of AH26, an epoxy-*bis*-phenol resin, but not the powder, proved to be mutagenic. The mutagenic effect evoked in TA100 by the epoxy-*bis*-phenol resin was depressed in the presence of S9-mix (Fig. 3a). Similar effect of the S9-mix were obtained with eluates of the mixed material after a setting time of 1 h (Fig. 3b).

No mutagenic activity to TA98 and TA100 could be observed with eluates of mixed Diaket and mixed N2 or their components, either in the presence or absence of S9-mix. With the exception of Diaket powder extracts, the materials were toxic at higher concentrations. The reversion rate of TA100 by saline eluates of the paraformaldehyde containing N2 powder was only slightly enhanced both with and without S9; DMSO eluates of the eugenol containing liquid were toxic (Fig. 4).

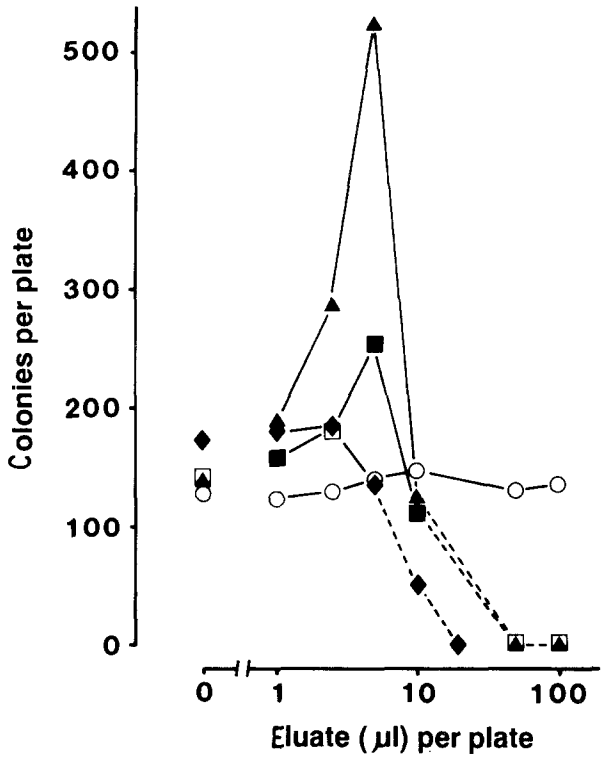


Figure 2 Mutagenicity of mixed AH26 in TA100. DMSO eluates (4 days) of the mixed material set for (▲) 1 h, for (■) 24 h, and (◆) 7 days; and saline eluates (4 days) of the 1 h set material are shown (○).

4. Discussion

In this study the mutagenic potential of three endodontic sealers and their components was tested using the Ames spot test and the plate incorporation assay. With the spot test, none of the materials could be considered unequivocally mutagenic. In the cases of mixed N2 and mixed Diaket, the spot test qualitatively indicates extreme toxicity to the bacteria. This might prevent the detection of weak mutagenic responses. The results with DMSO eluates of the mixed AH26 and its liquid component were ambiguous concerning mutagenicity to tester strain TA100. No clear mutagenic effect of AH26 and its liquid component could be seen in the spot test, although we demonstrated a distinct dose–response relationship in the incorporation assay. This might be explained by the fact that water solubility of the tested chemical is an indispensable prerequisite for the reliable use of the spot test [15]. Therefore, genotoxic effects of hydrophobic substances are hardly detectable.

As was expected from the results with the spot test, the mixed Diaket and the mixed N2 were toxic but not mutagenic in the plate incorporation assay. The results with the paraformaldehyde-containing powder of N2 (Table II) are less definitive. Saline extracts of N2

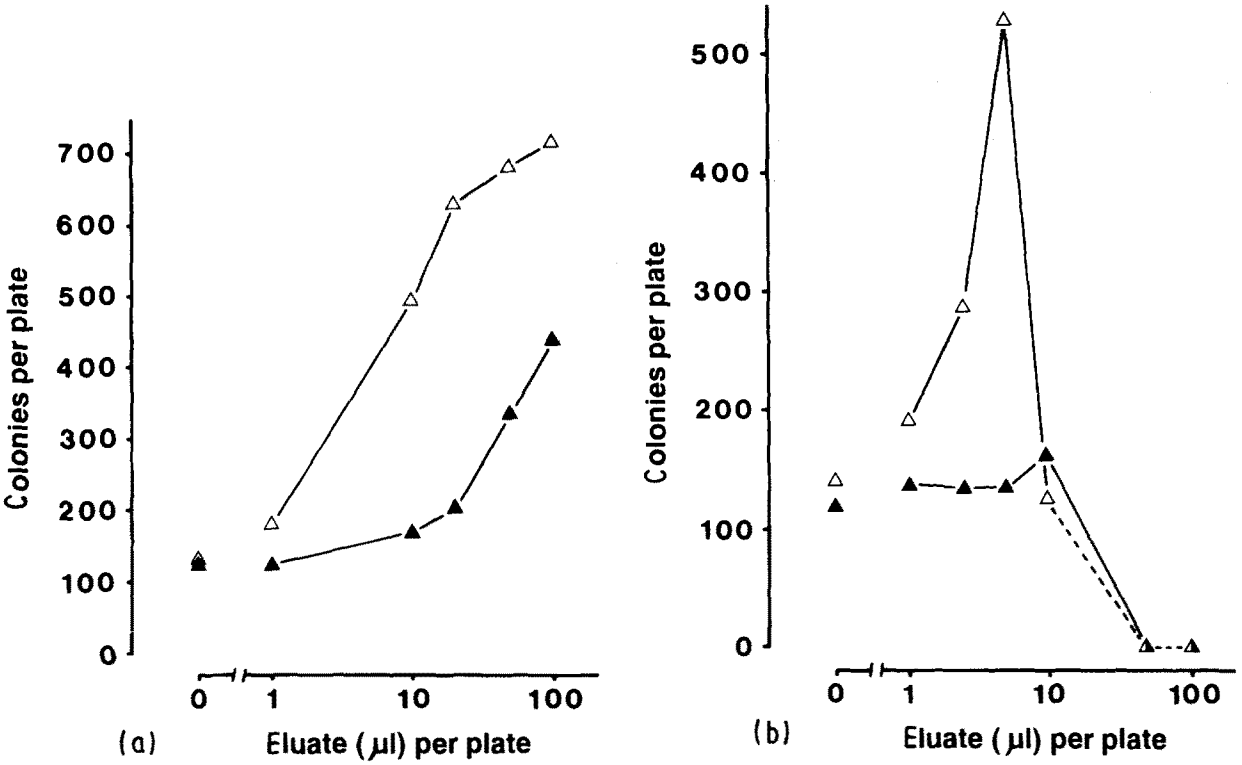


Figure 3 Mutagenicity of DMSO eluates (4 h) of (a) AH26 liquid and (b) DMSO eluates (4 days) of mixed AH26 set for 1 h. Mutagenic effects towards TA100 are recorded in the presence (▲) and absence (△) of S9-mix.

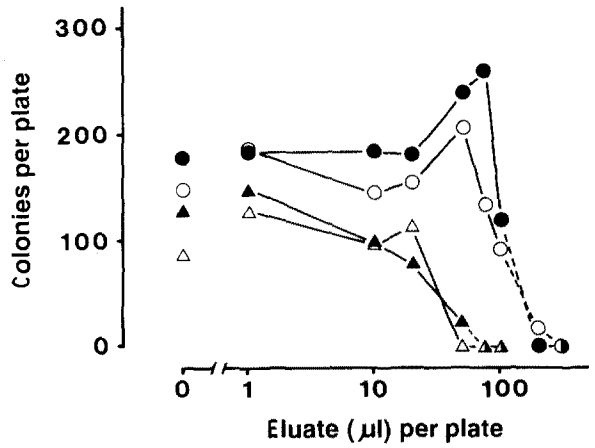


Figure 4 Number of revertants of TA100 by DMSO eluates (4 h) of N2 liquid (●, ○); and saline eluates (4 h) of N2 powder (▲, △). Filled symbols: S9-mix.

powder failed to increase the reversion rate of TA100 at least twofold compared to the spontaneous reversion rate, a criterion often used to demonstrate unequivocal mutagenicity. However, mutagenicity of formaldehyde, with paraformaldehyde being one of its sources, has been repeatedly demonstrated [8, 18–20]. Eugenol, which may have selective toxicity to eukaryotic tissues [21–23], is the main component of the N2 liquid. The N2 liquid was toxic to the bacteria; however we could not demonstrate any mutagenic activity in contrast to the previous findings with pure eugenol [24, 25].

DMSO eluates of the mixed AH26 were mutagenic whereas saline eluates were not. This is in agreement with the results of Orstavik and Hangslo [8]. However the mutagenic potency of DMSO eluates of the mixed AH26 was directly related to the setting time of the material. Eluates of the freshly mixed cement were strongly mutagenic; those derived from the material which had set for 1 week were not. The mutagenic effect of AH26 even 7 days after mixing, as reported by Orstavik and Hangslo [8], may arise from residual liquid component as a consequence of differences in sample preparation and extraction method.

The mutagenicity of the freshly mixed AH26 may be due to the liquid component as this epoxy-*bis*-phenol resin exhibited the same characteristics as the mixed material. There was a steep rise of revertant bacterial cells within a very narrow concentration range of DMSO eluates with both substances, which dropped in the presence of metabolically active rat liver microsomes. Regarding the mutagenicity of the liquid of AH26, our results agree with the findings of Andersen *et al.* [26] who detected mutagenic activity of aromatic epoxy resins with TA100 which was reduced by S9-mix.

Equal amounts of eluates of mixed AH26 stored for 7 days, for 1 day or for 1 h exhibited the same toxicity to the bacteria, whereas only eluates of the freshly mixed material were mutagenic. Furthermore, the toxic dose of the freshly mixed AH26 was far below the highest concentration of AH26 liquid that proved to be mutagenic but not toxic. Therefore our results indicate that the mutagenicity and toxicity are due to different components of the mixed AH26. Toxicity of the root canal sealers which we tested has been reported [27–31]. The fact that the liquid component and the freshly mixed AH26, but not the set material are mutagenic may be explained by the polymerization reaction of the liquid epoxy-*bis*-phenol resin.

This reaction is catalysed (Table II) by methenamine (hexamethylene tetramine) and after 24–36 h at body temperature, and 7 days at room temperature, the liquid resin polymerizes to a solid material [31].

Since the bacterial mutagenicity of AH26 was elicited only by DMSO eluates of the material after a short setting time and the mutagenicity was also reduced by microsomal enzyme activity, it is clear that AH26 should be further evaluated by appropriate *in vivo* mutation assays and long-term animal bioassays to establish its mutagenic–carcinogenic status. This is necessary to minimize health risks to patients, dental professionals handling the material, and those preparing and manufacturing the components.

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