Clin Oral Invest (1997) 1: 5–11

REVIEW

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Biological aspects of root canal filling materials – histocompatibility, cytotoxicity, and mutagenicity

Abstract In order to minimize the incidence of local and/or systemic side effects, the biocompatibility of all endodontic materials should be investigated by various *in vitro* and *in vivo* tests prior to clinical application. The battery of *in vitro* tests includes determinations of mutagenicity, cytotoxicity, and antibacterial effects. Several reports have shown that paraformaldehyde-containing ZnO-eugenol cements in particular, such as Endomethasone and N2, are antibacterial. On the other hand, it has been found that endodontic materials with strong antimicrobial activity are frequently mutagenic, i.e., primarily those which release formaldehyde. Cell culture tests clearly show significantly different cytocompatibility of the various types of endodontic sealers: in general, formaldehyde-containing ZnO-eugenol cements are classified as highly/extremely cytotoxic, whereas most $Ca(OH)_2$-based sealers are rated as possessing good or excellent cytocompatibility. These results were confirmed by numerous histological studies *in vivo*. Sealers with inferior biocompatibility, such as formaldehyde-releasing materials, should no longer be applied in practice because safer alternatives are available.

Key words Endodontic sealers · Cytocompatibility · Mutagenicity · Formaldehyde

Introduction

Various studies have revealed that elutable substances or degradation or corrosion products from root canal fillings may gain access to surrounding tissues (periodontal ligament, alveolar bone) through numerous connections, e.g., dentinal tubules, accessory and lateral canals, and apical foramina [18, 19, 43]. Araki et al. [6] investigated the diffusion of [^{14}C]-formaldehyde through radicular dentin 72 h after the application of formocresol into the root canal of cat canines. It was found that formaldehyde was distributed from the pulp space into the body.

Furthermore, the diffusion of leachable components from the pulp space to the radicular root surface is influenced significantly by the presence or absence of a dentine smear layer, which is created during any mechanical root canal preparation. Foster et al. [21] reported that removing the smear layer facilitates the diffusion of $Ca(OH)_2$ through dentinal tubules to the external root surface. It has also been described that the smear layer reduces the diffusion rate of triamcinolone and demethylchlortetracycline (from Ledermix) through radicular dentine from the pulp space to the outer root surface [1]. Breault et al. [13] investigated the effects of various medications and filling materials used to fill the canal on the attachment of human gingival fibroblasts to an exposed dentin surface free of a smear layer: Roth's sealer (ZnO-eugenol)+gutta-percha, warm gutta-percha±sealer, $Ca(OH)_2$, and formocresol. The fibroblast attachment was significantly reduced when formocresol or pure warm gutta-percha were used. The authors conclude that formocresol or warm gutta-percha applied into a root canal without a dentine smear layer may impair periodontal wound healing or regeneration. According to Tidmarsh [71] it is only necessary to remove the loose superficial layer consisting mainly of dentin chips to promote the adhesion and adaptation of the sealer to the cavity wall. With regard to the biocompatibility of a root canal filling, it is of no benefit to take away the deep and tight smear layer, which obturates the orifices of the tubules and creates a biological barrier between the endodontic material and the periodontal ligament, thus minimizing the danger of any adverse effects (Fig. 1). However, the clinical significance of this important aspect is currently under discussion and needs further clarification.

Many parameters characterize the biocompatibility of an endodontic material, such as genotoxicity/mutagenicity/carcinogenicity, cytotoxicity, histocompatibility or microbial effects. In order to evaluate these complex biological features, a variety of *in vitro* and *in vivo* tests must be performed [73, 75].

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Fig. 1 **a** Loose dentin chips (*arrows*) covering the pulpal wall would interfere with a good adaptation of the subsequent root canal filling. Therefore, these particles should be removed by sodium hypochlorite. Scanning electron micrograph (SEM), original magnification 600×. **b** This thin and compact smear layer (*arrows*) seals the orifices of the dentinal tubules and thereby creates a biological barrier, which may prevent the effects of the cytotoxic material in the adjacent periodontal ligament. SEM, original magnification 600×. **c** The smear layer has been partially removed. The orifices of the dentine tubules are open, possibly resulting in microscopical connections between the periodontal ligament, the pulp space, and the root canal filling. SEM, original magnification 600×. *Bars* 50 µm

Table 1 Composition of Apexit (Vivadent, Liechtenstein)

Component	Percentage of weight
Paste (batch 460299)	
$Ca(OH)_2$	32
Colophonium hydrated	31.5
SiO_2	8
CaO	5.6
ZnO	5.5
Tricalcium phosphate	4
Polydimethylsiloxane	2.5
Zinc stearate	2.3
Activator (batch 640432)	
Trimethylhexandiol disalicylate	25
Bismuth carbonate	18
Bismuth oxide	18
SiO_2	15
1,3-Butanediol disalicylate	11
Colophonium hydrated	5.4
Tricalcium phosphate	5
Zinc stearate	1.4

Genotoxicity/mutagenicity of endodontic materials

Genotoxicity/mutagenicity and carcinogenicity are very important factors affecting the systemic compatibility of an endodontic material. In general, genotoxicity means the presence of a DNA-reactive component which may result in mutagenicity and carcinogenicity [39]. Due to the extremely serious and life-threatening consequences, mutagenicity and carcinogenicity are gaining increasing public interest. The recent European Standard "Biological evaluation of medical devices – Part 3: Tests for genotoxicity, carcinogenicity and reproductive toxicity" therefore recommends various methods for their evaluation which are listed in the "OECD guidelines for the testing of chemicals". Additionally, for certain aspects, alternative methods can be accepted [24].

In vitro test systems for genotoxicity can be differentiated into bacterial tests, e.g. the classic Ames test and the newly developed *umu* test, and eukaryotic tests such as the hypoxanthine-guanine phosphoribosyltransferase test (HPGRT), the chromosomal aberration test, and the newer DNA synthesis inhibition test, the DIT [24, 39, 45, 48, 51, 58, 59, 68]. Since various dental or endodontic materials are highly cytotoxic, it is a basic requirement that genotoxicity tests easily quantify cytotoxicity simultaneously, in order to avoid misinterpretation of the data. The prokaryotic *umu* test and the eukaryotic DIT both fulfill this important criterion, whereas the results of the bacterial Ames test, for example, are usually published without adequate toxicity data [24].

Moreover, it must be considered that various endodontic filling materials reveal a strong antibacterial activity as has been described by Orstavik [47] and by Stea et al. [68]. This fact, among others, indicates that a bacterial test system cannot be the only basis for the assessment of the DNA-damaging activity of a dental material. Therefore a combination of a bacterial and a eukaryotic test, e.g., the bacterial *umu* test [45] with the eukaryotic DIT [51], is necessary in order to gain more reliable results with respect to the genotoxicity of a root canal filling material.

Today, numerous root canal sealers are available, based on various formulae and containing a variety of different and partly mutagenic components, such as epoxy resin sealers, e.g., AH26 and AH Plus, $Ca(OH)_2$ based materials such as Sealapex and Apexit, and ZnO-eugenol cements, e.g., N2 and Endomethasone (Tables 1–4).

The epoxy resin sealer AH26 is based on bisphenol-A-diglycidylether. Additionally, the powder contains hexamethylene-tetramine, which is synthesized from formaldehyde and ammonia. Spangberg et al. [65] found that AH26 releases formaldehyde after mixing, with a maximum release after 2 days. Apart from eugenol, N2 contains a variety of aromatic oils which are cytotoxic. The high concentration of formaldehyde in this material represents a particular risk. According to Spangberg et al. [65] the concentration of formaldehyde released from N2 is more

Table 2 Composition of Sealapex (Kerr, USA)

Component	Percentage of weight
Paste (batch 31270)	
$Ca(OH)_2$	25
ZnO	6.5
Activator (batch 31272)	
Polymethylene salicylate resin	33
$BaSO_4$	19
TiO_2	5
Additionally	
Ethyltoluene sulfonamide	No specification
Various salicylates	No specification
Pigments	No specification

Table 3 Composition of AH26 (De Trey/Dentsply, Germany)

Component	Percentage of weight
Powder (batch 900907)	
Bismuth (III) oxide	60
Hexamethylene tetramine	25
Ag	10
TiO_2	5
Liquid (batch 900727)	
Bisphenol-A-diglycidylether	100

Table 4 Composition of N2 (Indrag Agsa, Switzerland)

Component	Percentage of weight
Powder (batch 7704)	
ZnO	63
Bismuth nitrate	15
Bismuth carbonate	10
Paraformaldehyde	7
TiO_2	3.6
Liquid (batch 489)	
Eugenol	77
Peanut oil	20
Rose oil	1.8
Lavender oil	1.2

than 300-fold that of the concentration of AH26 2 days after mixing. No formaldehyde or other marked cytotoxic and mutagenic ingredients are known to be released from the $Ca(OH)_2$-based sealers, e.g., Sealapex and Apexit. The setting reaction of these materials is based on various salicylates.

There is only scant information about the mutagenicity of endodontic filling materials. Schweikl et al. [59] investigated the mutagenicity of AH26 in the V79/HGPRT mammalian cell assay. They found that this material elicits mutagenic effects 24 h after mixing which significantly decrease within 1 week. These observations are in accordance with the declining release of formaldehyde from the setting material with a maximum 2 days after mixing [65]. In contrast to these data, Stea et al. [68] (Ames test) as well as Heil et al. [24] (*umu*, DIT) found mutagenic substances even in the set material (presumably an epoxy derivative of bisphenol-A-diglicidylether).

Diaket and N2 were not found to be mutagenic in the *Salmonella*/microsome test [58], whereas Heil et al. [24] reported that N2 produces alterations in the *umu* test and in the DIT, which also is indicative of genotoxic activity. Further studies are needed to clarify whether eugenol, the mutagenic formaldehyde [37] or both substances cause these effects, since eugenol has been found to be genotoxic in various *in vitro* systems [56]. No genotoxicity was determined for the $Ca(OH)_2$-based sealer Apexit [24].

Cytotoxicity of root canal filling materials

Cell culture studies have been performed for more than 30 years for the investigation of cytotoxic reactions induced by endodontic sealers [31, 55]. Permanent cell lines, e.g., HeLa, 3T3 or L929 cells, and primary cells, mainly oral fibroblasts, are used for these experiments [3, 7, 36, 41]. Furthermore, various biological endpoints are used for the investigation of cytotoxic effects. These are growth inhibition or the determination of the ED_{50}, membrane integrity, DNA, RNA or protein synthesis, or the determination of alterations of cellular morphology by light or electron microscopy [3, 8, 11, 36, 41, 42].

There is still controversy over whether permanent cell lines or primary cell cultures derived from the tissue of the target (the periodontal ligament primarily) should be preferred [35, 38]. Al-Nazhan and Spangberg [3] used human periodontal ligament fibroblasts and L929 cells in order to compare the effects of extracts of the resin base of polymeric endodontic sealers on the morphology of both cell types by scanning and transmission electron microscopy as well as on chromium release. It was found that chromium release and the degree of cell damage were related. However, the periodontal ligament fibroblasts were more resistant to the cytotoxic effects than were L929 cells. Therefore, these authors conclude that periodontally derived primary cells should be preferred for the determination of the cytotoxicity of endodontic filling materials. These cells will have contact primarily with extruded endodontic materials. Similar results were reported by Leinenbach et al. [36].

Due to the fact that an endodontic sealer or material may impair the tissues adjacent to the root by releasing components [1, 6, 21] as well as by direct contact, for example, in the case of overfilling, it is necessary to test extracts as well as solid specimens for biocompatibility [3, 4, 11, 36, 41]. Several authors report a comparably good or even excellent cytocompatibility of various $Ca(OH)_2$-based sealers [36, 41, 44, 77]. Matsumoto et al. [41] tested three experimental [two $Ca(OH)_2$ based, one ZnO-glycol based]

and five commercially available sealers (AH26, Diaket, Canals, Tubli-Seal, Sealapex) in primary rat dental pulp cells (DNA synthesis by [^{3}H]-thymidine incorporation). Strong, almost complete inhibition of DNA synthesis was found for fresh Diaket and AH26, whereas no such effects were noted with the experimental materials and Sealapex. Having set for 6 days, the $Ca(OH)_2$ materials and Diaket revealed only little cytotoxicity but AH26 still induced marked irritation. In another study [11], the cytotoxicity of three $Ca(OH)_2$-based sealers (Sealapex, CRCS, Apexit) after 1 day of setting was investigated in L929 and BHK21/C13 cells by dye exclusion tests with trypan blue. Sealapex was highly cytotoxic compared to CRCS and Apexit, which revealed the least cytotoxicity.

McNamara et al. [42] studied freshly polymerized compared to prepolymerized Hydron (poly-2-hydroxyethyl methacrylate hydrogel) and freshly mixed AH26 compared to Tubli-Seal in L929 fibroblast cultures ([^{35}S]-sulfate and [^{3}H]-thymidine incorporation). During polymerization, Hydron proved to be as highly cytotoxic as Tubli-Seal. Set Hydron inhibited cell functions to the same extent as AH26. All materials induced marked alterations of the cellular metabolism. AH26 was also found to be highly cytotoxic in several cell culture systems by various other investigators [36, 52, 64] (Fig. 2b). These observations were corroborated by *in vivo* investigations in monkeys, in which the periapical reactions to $Ca(OH)_2$-based sealers and AH26 were compared [69]. The high cytotoxicity of this epoxy resin sealer is mainly caused by the formaldehyde it contains. This is released during the initial period of the setting reaction [65]. Accordingly, it was found that AH26 induced severe tissue inflammation and necrosis when freshly implanted into animals [55]. However, after 6 weeks of incubation, only moderate alterations were observed [73]. These data indicate that AH26 is characterized by a marked acute toxicity which is reduced in time as the concentration of leachable formaldehyde decreases.

Furthermore, various ZnO-eugenol sealers, such as N2, were classified as highly/extremely cytotoxic [5, 14, 36, 55]. Additionally, it was found in experiments that these materials, especially N2, reveal cytotoxic effects even after several elutions of the hardened specimens [36]. This is indicative of the high and long-term cytotoxic potential of root canal fillings for which ZnO-eugenol sealers have been used, primarily those containing formaldehyde (e.g., N2, Endomethasone). These conclusions are supported by clinical observations [28] as well as by animal investigations of the effects of formocresol on the marginal and periapical periodontal ligament. The histological results of these studies reveal that formaldehyde, which is distributed through root canals locally and systemically, significantly irritates the surrounding tissue and clearly delays the healing of the periapical periodontal ligament [6, 22, 37, 40, 76].

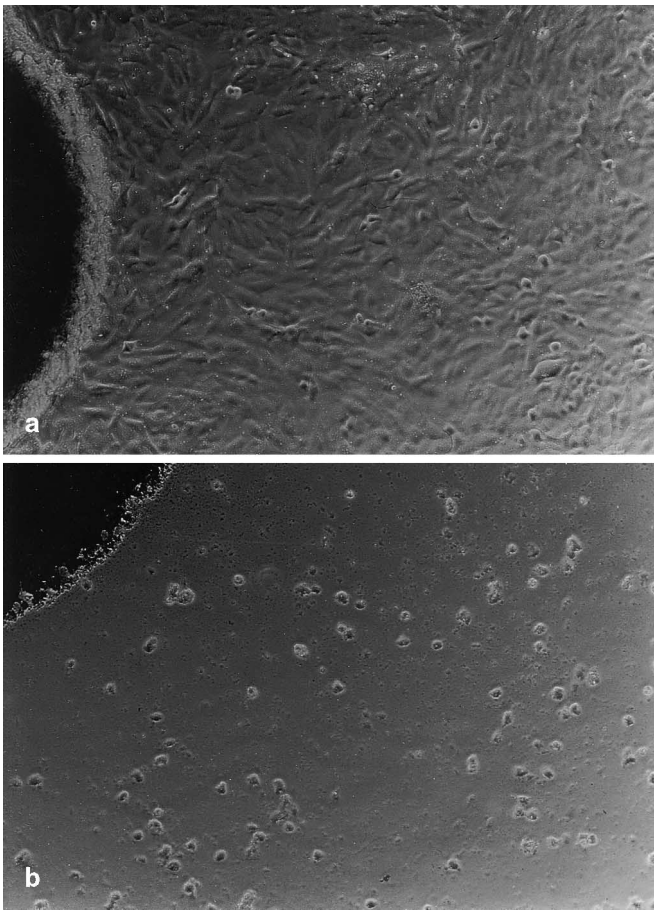

Fig. 2 **a** 3T3 fibroblasts have been grown into tight contact with this Apexit specimen after an incubation period of 48 h. This indicates an excellent cytocompatibility of the $Ca(OH)^2$-based endodontic sealer. **b** In contrast to **a**, this 3T3 culture is extremely altered by the AH26 specimen after 48 h. Only a few viable cells are present and the morphology of the fibroblasts is severely altered, indicating a very high cytotoxic potential of this endodontic sealer

Histological studies with endodontic materials

Various histological investigations indicate that components leaching from an endodontic material may induce local side effects. Yesiloy et al. [77] injected Grossman sealer (ZnO-eugenol), eucapercha (with eucalyptol), Endo-Fill, CRCS [$Ca(OH)_2$-eugenol-eucalyptol], Sealapex [$Ca(OH)_2$-polymeric resin], Hypocal [aqueous $Ca(OH)_2$] into the subdermal tissue of 12 guinea pigs. Histological examination was performed after 6, 15, and 80 days. Overall, Sealapex and Endo-Fill (silicone resin-based) induced less severe inflammatory alterations than the other test materials.

Wayman et al. [74] reported that 17 out of 58 periapical lesions investigated contained extruded sealer. These histological observations were confirmed by experimental studies in animals. Holland [25] compared the periapical reaction following the application of a ZnO-eugenol sealer of Grossman's formulation using of the $Ca(OH)_2$-based sealer Sealapex in ferrets. He found that all teeth with ZnO-eugenol fillings had inflammatory lesions at their api-

ces, whereas only 3 of the 12 teeth treated with the $Ca(OH)_2$ sealer showed similar alterations. Comparable results were published by Tepel et al. [70], who reported that the ZnO-eugenol root canal sealers Endomethasone and N2 significantly impaired periapical repair. These data were corroberated by Hong et al. [27], who deliberately overfilled root canals of monkey incisors with calcium phosphate cement, Grossman's sealer, and N2. N2 induced severe periapical inflammations even after 6 months. Grossman's sealer induced milder but persisting alterations over 6 months, whereas the calcium phosphate cement showed only mild irritation after 1 month and minimal alteration thereafter. These observations indicate the bioincompatibility of the ZnO-eugenol-based root canal sealers, especially that of N2. An inferior biocompatibility of these materials was also reported by Lambjerg-Hansen [33], who applied Endomethasone and N2 after vital pulpectomies on 16 pairs of human teeth. The residual pulp tissue was investigated after 4–8 months. Necrosis was found most frequently with Endomethasone. The author recommends that the two materials not be used routinely due to their formaldehyde content and complex composition.

Furthermore it was found that gutta-percha may also induce periapical lesions. Oswald and Friedman [50] packed dentin chips into the apical openings of one canine of cats, whereas in the contralateral tooth a dentin plug was not formed. Then, all canals were obturated with gutta-percha. Healing was quicker at the apices where dentin plugs were deposited, whereas all control teeth demonstrated periapical inflammations at the apex. Sonat et al. [62] studied the histological response of the periapical tissue of dogs to root canal fillings with various materials: pure $Ca(OH)_2$, pure gutta-percha, Sealapex and gutta-percha. The most pronounced hard tissue formation was found after application of Sealapex followed by $Ca(OH)_2$, whereas pure gutta-percha induced the least periapical healing.

In vitro data indicate that the inflammatory periapical reaction after application of gutta-percha might be due to serum complement activation. Serene et al. [60] tested the serum complement activation (C3) induced by four brands of gutta-percha and the nine ingredients of one product as an indicator of possible inflammatory potential. It was found that all materials led to complement activation and thus may impair periapical healing in cases of overfilling. However, histological investigations revealed that these adverse effects are not characteristic of gutta-percha in general but are typical for certain types. Holland et al. [26] implanted silver points and two brands of gutta-percha into rat connective tissue. After 1 year it was found that one brand of gutta-percha as well as the silver points were well tolerated, whereas the other gutta-percha type elicited a moderate to severe chronic inflammatory reaction. The authors suggest that different types of gutta-percha be carefully evaluated in order to determine which are most favorable to healing of the periapical tissue. Sjögren et al. [61] implanted three forms of gutta-percha subcutaneously in guinea pigs. It was found that fine gutta-percha particles evoked an intense localized tissue response, characterized by the presence of macrophages and multinucleated giant cells. They conclude that these alterations may be a cofactor in the impairment of the healing of periapical lesions when canals are overfilled.

Altogether, the various histological data indicate that endodontic sealers, especially those containing paraformaldehyde and ZnO-eugenol, as well as gutta-percha may induce periapical irritations. This was mainly observed when there was a marked direct contact between the soft tissues adjacent to the root and the endodontic filling material due to overfilling. Furthermore, neurotoxic effects due to direct contact between endodontic sealers and nerves have been determined *in vivo* and *in vitro* [2, 9, 12, 20, 23, 29, 30, 57, 66, 67].

Microbial effects of root canal sealers

Another aspect which must be considered when discussing the biocompatibility of a root canal filling is possible interactions between an endodontic material and/or its components with microorganisms. These may persist within the pulp cavity after root canal obturation or they may proliferate in adjacent tissues. Even after careful cleaning and shaping of the pulp space, bacteria will often remain in the tubules or lateral canals and may repopulate the former root canal [46]. Furthermore, Torabinejad et al. [72] have found that bacteria may penetrate an obturated root canal within a few days if the access cavity is not sealed sufficiently. These persisting or reinfecting bacteria may enhance possible adverse effects. Therefore, it would be of great benefit if an endodontic material were biocompatible as well as antibacterial. Numerous authors have studied the antibacterial properties of various endodontic materials against different microorganisms [15–17, 47, 53, 54].

Pumarola et al. [53] investigated the antimicrobial activity of seven endodontic sealers by means of *Streptococcus aureus* (agar diffusion and agar dilution tests): Traitement Spad, Endomethasone, N2 Universal, Diaket-A, AH26 with silver, Tubli-Seal, and Sealapex. A good antibacterial activity was determined for Diaket-A, Traitement-Spad, N2, and Endomethasone. These results confirmed the data published by Pupo et al. [54] who studied the antimicrobial effects of five root canal cements on microorganisms derived from infected root canals. The most active material immediately after mixing was cement which contained paraformaldehyde (Endomethasone). Similar results were reported by Canalda and Pumarola [16], who determined the effects of five sealers [two with $Ca(OH)_2$, one with paraformaldehyde (Endomethasone)] on the growth of six microorganisms. Again, the best inhibition was determined for the paraformaldehyde-containing material. Additionally, Broisman et al. [15] had previously described that N2 has a strong antibacterial activity (bactericidal to *Streptococcus mutans*) and releases antimicrobial gases which inhibit the growth of oral microorganisms. These data were confirmed by Cox et al. [17], who found that formocresol and paraformaldehyde enhance the antimicrobial activity of endodontic materials.

All these reports show that, in particular, formaldehyde-containing ZnO-eugenol cements such as Endomethasone and N2, are antibacterial. In contrast, it has been determined that ZnO-containing sealers may induce aspergillosis of the maxillary sinus if the upper posterior teeth are overfilled [10, 32, 34]. This indicates that a material which inhibits certain species of bacteria may, on the other hand, promote the growth of other microorganisms. Furthermore, it must be emphasized that endodontic filling materials with strong antibacterial activity have frequently been found to induce adverse effects during and after treatment and were also cytotoxic or even mutagenic [24, 48, 49, 63].

Summary and conclusions

The results of histological studies, cytotoxicity tests, and the investigations for mutagenicity/genotoxicitiy support considerations that, in addition to their benefits, several root canal filling materials also possess undesirable properties which may pose a threat to human health. Certainly, it is very difficult to determine whether the cytotoxic activity of an endodontic material or insufficient root canal preparation, resulting in persisting microorganisms in spaces inaccessible to mechanical and/or chemical cleaning, is the cause of endodontic failure. Adverse material effects may play an important role in failures in many cases in which no major fault in treatment can be identified.

The data described in this paper support the demand for a much better preclinical evaluation of the biocompatibility of endodontic materials by histological studies and *in vitro* tests for the determination of cytotoxicity, mutagenicity, and microbial effects. The dental profession must accept that biocompatibility is at least as important as physical and chemical features when deciding on a root canal filling material. Finally, only those root canal filling materials should be used which are characterized by an at least acceptable biocompatibility. Formaldehyde-releasing root canal sealers are no longer to be recommended.

Acknowledgements Our thanks are due to Dr. E. H. Ehrmann, Melbourne, Australia, for constructive criticism of the manuscript.

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