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AN IN VITRO STUDY OF THE EFFECT OF DILANTIN ON THE SURVIVAL OF S. MUTANS, A. VISCOSUS, L. CASEI AND N. CATARRHALIS

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A thesis submitted in partial requirement for the degree of Master of Dental Science.

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INTRODUCTION

Since 1938, when Merritt and Putnam\(^{(51)}\) first tested the effectiveness of 5,5-diphenylhydantoin (DPH)\(^{3}\) as an anticonvulsant, DPH has been one of the most safe and effective drugs for the control of \textit{grand mal} and psychomotor epilepsy\(^{(1)}\).

Designated chemically as 5,5-diphenylhydantoin, DPH is related to the barbiturate group of drugs and has the following structure\(^{(31)}\):

\[
\begin{array}{c}
\text{C}_6\text{H}_5 \\
\text{N} \\
\text{C}_6\text{H}_5 \\
\end{array}
\begin{array}{c}
\text{H} \\
\text{O} \\
\text{O} \\
\end{array}
\begin{array}{c}
\text{C}_6\text{H}_5 \\
\text{NH} \\
\end{array}
\]

DPH is a weak organic acid (M.W. 252.26) and although it is only sparingly soluble in water, it is readily soluble in strongly alkaline solutions\(^{(18)}\). A study by Dill \textit{et al.}\(^{(18)}\) has shown that the solubility of DPH is pH dependent. These workers found that at pH 7.0, the maximum solubility of DPH is 14.0 \(\mu\)g/ml, whilst at pH 10.0 it is 1.52 mg/ml.

\textit{a} – DPH is marketed under the proprietary name \textit{Dilantin} by Parke, Davis and Company, Sydney, Australia.
The available evidence indicates that the solubility of DPH in blood plasma is approximately 75 µg/ml. DPH is hygroscopic and exposure to CO₂ causes its conversion to the free acid form.

DPH is usually administered orally in capsules or as a suspension. Maximum absorption of the drug occurs in the duodenum. On entering the circulatory system, approximately 90 per cent of the drug is rapidly and reversibly bound to proteins. Following absorption, the unbound drug diffuses freely throughout the body. Woodbury and Swinyard have indicated that the total concentration of DPH in transcellular fluids such as saliva, bile, plasma, cerebrospinal fluid and gastro-intestinal fluids is the same as the free levels in the blood. The major portion of the drug is excreted in urine as metabolites and a minor portion is excreted in faeces.

The usefulness of DPH is limited by a number of side effects on different body systems. However, it is unusual for these reactions to be serious and fatalities are rare. One such side effect is gingival enlargement, a condition which occurs in approximately half the individuals treated and can lead to a severe, disfiguring overgrowth of the soft tissues surrounding the teeth.
Since Kimball\(^{(39)}\) first reported the effect of DPH on gingival tissues, many investigators have put forward suggestions as to the possible etiology of this condition. Early workers\(^{(25)}\)\(^{(39)}\) thought there was an association between DPH induced gingival enlargement and vitamin C deficiency. More recently, other proposals have been made to explain the action of DPH on gingival tissues. It has been suggested that the gingival response is caused by:

1. an exaggerated connective tissue reaction to local irritants in subjects with deranged adrenocortical function\(^{(1)}\)\(^{(68)}\).

2. direct contact between DPH and epithelial tissue\(^{(7)}\)\(^{(9)}\)\(^{(55)}\) or between DPH in blood and haemorrhagic gingiva with ruptured blood vessels\(^{(71)}\).

3. an allergic reaction to DPH\(^{(76)}\).

4. the combined influence of DPH and local irritating factors on gingival mast cells\(^{(3)}\).

5. the action of DPH on genetically different fibroblast cell populations\(^{(34)}\).

6. the presence of plaque\(^{(19)}\)\(^{(32)}\)\(^{(56)}\)\(^{(69)}\).

Four studies\(^{(19)}\)\(^{(32)}\)\(^{(56)}\)\(^{(69)}\) in recent years have indicated that plaque may be essential to the initiation
of the gingival tissue overgrowth. Hall\(^{(32)}\) in 1969, reported an unexpected finding in a study involving 22 patients over a two year period, in which 20 of these patients did not develop DPH induced gingival enlargement. Each of these 20 patients was seen in the first 10 days of DPH ingestion and had gingival inflammation eliminated by a strict oral hygiene routine. However, it is noteworthy that the remaining two patients were first seen six weeks after the commencement of DPH therapy. Gingival enlargement was already evident, and although the size of the overgrowth was reduced by good oral hygiene, the condition still remained.

In 1972, Nuki and Cooper\(^{(56)}\) treated cats with DPH and found that there was no overgrowth of gingival tissue if a daily brushing programme was carried out. Since then, it has been reported\(^{(69)}\) that the gingival response in monkeys can also be prevented, by inhibition of plaque formation.

It has been shown by Donnenfield \textit{et al.}\(^{(19)}\) in 1974 that, following the surgical removal of DPH induced gingival enlargement, the clinical changes will not recur when strict plaque control procedures are followed.

It appears from the studies of Hall\(^{(32)}\), Nuki and Cooper\(^{(56)}\), Staple and Reed\(^{(69)}\), and Donnenfield\(^{(19)}\) that
plaque is essential to the initiation of DPH induced gingival enlargement. Since plaque is basically a soft concentrated mass consisting mainly of a large variety of bacteria held together in a protein-carbohydrate matrix\(^{(73)}\), it is possible that DPH may induce untoward changes in gingival tissue through its effect on plaque bacteria. To date, no work appears to have been carried out to determine the effect of DPH on plaque bacteria \textit{in situ} or \textit{in vitro}.

In view of this situation, this study was undertaken to determine the effect of DPH on the survival of four species of bacteria commonly found in plaque\(^{(33)}\); \textit{S. mutans}, \textit{A. viscosus}, \textit{L. casei} and \textit{N. catarrhalis}.

\textit{S. mutans} is a Gram-positive organism which has been implicated in initiating experimental caries in a variety of animals, including rats, hamsters and monkeys\(^{(8)}\)(\(^{(22)}\)) and the presence of \textit{S. mutans} in plaque has been associated with the prevalence of dental caries\(^{(33)}\). Strains of \textit{S. mutans} have also been shown to initiate a type of periodontal disease in germ-free rats\(^{(29)}\) in which there was marked alveolar bone loss but only slight inflammatory change in the gingival tissues. \textit{A. viscosus}, a Gram-positive rod is able to form adherent deposits on solid surfaces\(^{(75)}\). It can also induce caries\(^{(75)}\) and periodontal
disease\(^{(36)}\) in monoinfected gnotobiotic laboratory animals. *L. casei*, a Gram-positive rod has long been associated with dental caries\(^{(65)}\). *N. catarrhalis* is a Gram-negative coccus. *Neisseria* species are present in early plaque, and it is suggested that they may affect the rate of plaque formation\(^{(60)}\). They are capable of producing an extracellular polymer\(^{(58)}\) which may be important in adherence to tissues.

When assessing the effect of a drug on the survival of test organisms, it is usual to add the drug to solid or liquid media inoculated with the organisms. There are basically two conventional tests that can be carried out using solid media\(^{(67)}\). In one test, the medium is pre-inoculated with the test organism and the drug is diffused into the agar and, if effective, it will produce a zone of inhibition. In the other test, the drug is incorporated into the medium and its effect on the growth of organisms plated out on the medium surface is then assessed. Tests of this nature depend on the compatibility of the test drug with the agar and other constituents of the medium. Other factors, such as the stability of the drug in relation to pH, have also to be considered\(^{(67)}\).

Liquid media tests are a more direct way of assessing inhibition, avoiding the problems of diffusion through
solid media and reaction of the test drug with the agar. These tests can be done by adding the drug to liquid media inoculated with test organisms and measuring the effect on the growth rate of the organisms. Alternatively, the serial dilution test can be used, in which the minimum concentration of drug preventing detectable growth is accepted as a measure of bacteriostatic activity.\(^{(67)}\)

All these tests require contact of the test drug with the medium, all of which contain hydrolysed protein as an essential constituent.\(^{(14)}\). Since DPH binds strongly to protein,\(^{(77)}\) the conventional methods of adding test drugs to solid or liquid media inoculated with test organisms could not be validly used in this experiment. Therefore, in order to examine the effect of DPH on the test organisms used in this study, it was necessary to develop an experimental technique which allowed the organisms to be treated in the absence of extraneous protein. The membrane filtration technique,\(^{(49)}(53)(59)\) used in this study allows washed organisms to be treated with DPH in the absence of solid or liquid media.

An additional problem for consideration was the removal of any test drug which may have remained adsorbed to the organisms. In some of the conventional methods, when the organisms are placed in a recovery medium, sufficient
of the drug may persist as an inhibitory agent and therefore hinder survival\(^{(62)}\). Many drugs can be inactivated on dilution, while others cannot\(^{(62)}\). In the latter instance, chemical or biological inactivators may be used, but to date no inactivator for DPH has been developed. The membrane filtration technique\(^{(49)}(59)\) also overcame this problem. It allows organisms to be thoroughly washed after they have been treated with the drug. The washed cells are collected on a membrane filter and can then be transferred to an agar medium for incubation and colony counting.

The concentrations of DPH in this experiment were chosen to approximate therapeutic levels of the drug in tissue fluids. Although the acceptable therapeutic concentration of DPH in plasma is 10 to 20 \(\mu g/ml\)\(^{(2)}\), in a study of 25 epileptic patients by Kutt \textit{et al.}\(^{(44)}\), 35 \(\mu g/ml\) was found to be the maximum weekly plasma level of DPH after multiple doses. On this basis an upper concentration of 35 \(\mu g/ml\) was used. A lower concentration of 9 \(\mu g/ml\) was chosen.

Presented in this thesis is a description of the materials and methods used in assessing the effect of DPH on the survival of \textit{S. mutans}, \textit{A. viscosus}, \textit{L. casei} and \textit{N. catarrhalis}, together with an analysis of the results
obtained. The significance of the findings in relation to the problem of DPH induced gingival enlargement is then discussed.
MATERIALS AND METHODS

MATERIALS

Organisms

Pure cultures of the four test organisms *S. mutans*, *A. viscosus*, *L. casei* and *N. catarrhalis* were obtained from the Institute of Dental Research, Sydney, N.S.W., Australia.

Table 1 lists the strains of the species and indicates the original source of the organisms that were selected.

<table>
<thead>
<tr>
<th>Species</th>
<th>Strain</th>
<th>Original Source</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>S. mutans</em></td>
<td>GS5</td>
<td>A. Bleiweis, Gainsville, Fla., U.S.A.</td>
</tr>
<tr>
<td><em>A. viscosus</em></td>
<td>NY1</td>
<td>J. Van der Hoeven</td>
</tr>
<tr>
<td><em>L. casei</em> var. rhamnosus</td>
<td>NCIB 6375</td>
<td>National Collection of Industrial Bacteria</td>
</tr>
<tr>
<td><em>N. catarrhalis</em></td>
<td></td>
<td>Institute of Dental Research, Sydney, Australia.</td>
</tr>
</tbody>
</table>
Media

Culture broths:

Trypticase soy broth (Baltimore Biological Laboratories, Cockeysville, Md., U.S.A.); brain heart infusion broth (Oxoid Ltd., London, England); M.R.S. was prepared using the formula of de Man, Rogosa and Sharpe\(^{17}\).

Solid Media:

Mitis salivarius agar (Difco Laboratories, Detroit, Mich., U.S.A.); brain heart infusion agar (Difco Laboratories); sheep blood agar (7 per cent defibrinated sheep blood in nutrient agar prepared to the formula of Cowan and Steel\(^{15}\)); Rogosa agar was prepared using the formula of Rogosa, Mitchell and Wiseman\(^{61}\).

Chemicals

Pure samples of DPH (5,5-diphenylhydantoin) were obtained from Parke, Davis and Company, Sydney, Australia. The samples were stored in a dark, moistureless container, under vacuum.

A 0.067 M phosphate buffer solution\(^{45}\) at pH 7.0 was prepared from di-sodium hydrogen orthophosphate (analytical reagent grade, Ajax Chemicals Ltd., Sydney, Australia) and potassium dihydrogen orthophosphate (analytical reagent grade, British Drug Houses Chemicals Ltd., Poole, England).
Identical solutions containing DPH at concentrations of 9 μg/ml and 35 μg/ml were also prepared. (It was found essential to first dissolve DPH in the potassium dihydrogen orthophosphate solution before the di-sodium hydrogen orthophosphate solution was added.)

All solutions were prepared with distilled, deionised water.

Apparatus

The membrane filtration apparatus (Figure 1) consisted of ten autoclavable filter holders (Filter Holder - In-Line 25 mm, Gelman Instrument Co., Ann Arbor, Mich., U.S.A.) each mounted on a manifold of copper pipe (25 mm internal diameter) by 3 cm lengths of thick walled rubber tubing. One end of the copper pipe was sealed with a rubber stopper and the other end was connected to a vacuum pump. A two-way stopcock was inserted between the filtration apparatus and vacuum pump. A cellulose membrane filter of 0.45 μm pore size with grid (HAWG 025 00, Millipore Corp., Bedford, Mass., U.S.A.) was inserted into each filter holder and the port to each filter holder was covered by a cap of aluminium foil. The ten filter holders were supported above and below by two rigid brass plates, which in turn were attached to four supports that could be adjusted to maintain the membrane filters in a horizontal plane.
FIGURE 1. (A) Membrane filtration apparatus
(B) Exploded diagram of membrane filter holder.
METHODS

Growth of Organisms

*S. mutans* strain GS5 and *A. viscosus* strain NY1 were grown anaerobically in trypticase soy broth and brain heart infusion broth respectively, for 48 hours at 37°C. *L. casei* NCIB 6375 was grown anaerobically in M.R.S. broth for 24 hours at 37°C whilst *N. catarrhalis* was grown aerobically in brain heart infusion broth for 48 hours at 37°C.

All organisms were harvested by centrifugation at 7,000 g and 4°C in a Beckman refrigerated centrifuge (Model J-21B). They were washed three times in sterile phosphate buffer pH 7.0 and resuspended each time by vortexing for 15 seconds.

In addition, *S. mutans* strain GS5 was vortexed for four minutes with sterile glass beads (0.10 mm to 0.11 mm diameter) between the first and second wash, to ensure the chains were completely broken up.

Treatment of Bacterial Pellets

Two concentrations of test solution, 9 μg/ml and 35 μg/ml DPH in pH 7.0 phosphate buffer, were used for
each organism. All buffer solutions were sterilized by filtration using a Sterifil Aseptic Filtration System (XXII 047 00, Millipore) with membrane filters of 0.22 μm pore size (GSWP 047 00, Millipore).

The final pellet was resuspended in 20 ml of phosphate buffer by vortexing for 20 seconds. Serial dilutions of the bacterial suspension in both control (phosphate buffer, pH 7.0) and test (phosphate buffered DPH, pH 7.0) solutions were made aseptically to give a final concentration of approximately $10^{-6}$ organisms per ml. Duplicate 0.1 ml aliquots of each suspension were transferred and spread out on the appropriate agar plates.

Replicate test samples were prepared by aseptically pipetting 10 ml of the test bacterial suspension into five separate sterile test tubes. The test tubes were then placed in a mechanical shaker in a water bath at 37°C and agitated well for 30 minutes.

**Filtration of Bacterial Suspensions**

On completion of shaking, each bacterial suspension was vortexed for 20 seconds and 1 ml aseptically pipetted using the Oxford Sampler Micropipetting System (Oxford Laboratories, Foster City, Calif., U.S.A.) into a separate membrane filter holder. The apparatus was lifted and
tilted gently in several directions to ensure an even spread of the fluid over each membrane filter surface.

A vacuum was applied for 15 seconds to filter the bacterial suspensions. When the vacuum was stopped, 2 ml of washing solution (sterile quarter-strength Ringer's solution at 37°C) was pipetted into the port of each filter holder, left for 3 minutes and the vacuum re-applied for 25 seconds. After this washing procedure, 1 ml of the appropriate sterile culture broth at 37°C was passed through the filter membranes by applying the vacuum for a further 15 seconds.

Recovery of Organisms

When the vacuum was stopped, the membrane filters were removed from the holders and, using sterile tweezers, were transferred to solid media and anaerobically incubated (5 per cent carbon dioxide, 95 per cent nitrogen) at 37°C.

*S. mutans* strain GS5 was grown on mitis salivarius agar, *A. viscosus* strain NY1 on brain heart infusion agar and *N. catarrhalis* on sheep blood agar, all for 48 hours, whilst *E. casei* NCIB 6375 was grown on Rogosa agar for 24 hours.
After incubation, a preserving solution of 15 per cent glycerine, 50 per cent formaldehyde and 35 per cent water\(^{(53)}\) was used to saturate the membrane filters on the agar, in order to preserve colony morphology. The plates were stored at 4°C.

The number of colonies of organisms on the membrane filters were counted using a plate culture microscope.
RESULTS

Overall, the number of colonies of organisms on each membrane filter ranged from 94 to 227 for the organisms treated with the test solutions, and from 72 to 249 for the organisms treated with the control solution.

A representative example of colonies of organisms on the membrane filters is shown in Figure 2. In this instance, the organisms are *S. mutans* strain GS5, and the number of colonies of DPH treated organisms (Figure 2-A) ranged from 122 to 165. The number of colonies of *S. mutans* strain GS5 treated with the control solution (Figure 2-B) ranged from 141 to 193.

Table 2 shows the mean number of colonies of organisms that survived the 30 minute period of exposure to (i) DPH at concentration of 9 µg/ml and 35 µg/ml in pH 7.0 phosphate buffer, or (ii) the control solution, pH 7.0 phosphate buffer. DPH treatments did not appreciably affect the survival of the organisms. The greatest differences between the organisms treated with the test solutions and the corresponding organisms treated with the control solution, occurred with *S. mutans* strain GS5 and *A. viscosus* strain NY1 (14 per cent and 12 per cent respectively) when DPH was used at a concentration of 9 µg/ml. However these
FIGURE 2. An example of the number of colonies of organisms (S. mutans strain GS5) treated with 35 μg/ml DPH (A) and control (B) solutions on the membrane filters.
NUMBER OF COLONIES OF ORGANISMS SURVIVING TREATMENTS WITH DPH

TABLE 2

<table>
<thead>
<tr>
<th>Organism</th>
<th>DPH Concentration (μg/ml)</th>
<th>Mean Number of Colonies&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Percentage Difference from Control</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Organisms treated with control solution</td>
<td>Organisms treated with test solutions</td>
</tr>
<tr>
<td><em>S. mutans</em> strain GS5</td>
<td>9</td>
<td>174 (24)</td>
<td>150 (18)</td>
</tr>
<tr>
<td></td>
<td>35</td>
<td>150 (37)</td>
<td>136 (27)</td>
</tr>
<tr>
<td><em>A. viscosus</em> strain NY1</td>
<td>9</td>
<td>143 (8)</td>
<td>126 (30)</td>
</tr>
<tr>
<td></td>
<td>35</td>
<td>157 (75)</td>
<td>168 (36)</td>
</tr>
<tr>
<td><em>L. casei</em> var. rhamnosus</td>
<td>9</td>
<td>194 (23)</td>
<td>198 (23)</td>
</tr>
<tr>
<td>NCIB 6375</td>
<td>35</td>
<td>133 (4)</td>
<td>135 (12)</td>
</tr>
<tr>
<td><em>N. catarrhalis</em></td>
<td>9</td>
<td>144 (31)</td>
<td>131 (11)</td>
</tr>
<tr>
<td></td>
<td>35</td>
<td>144 (31)</td>
<td>156 (2)</td>
</tr>
</tbody>
</table>

<sup>a</sup> Each value represents the mean of five samples.

<sup>b</sup> None of the differences were shown to be statistically significant at the 0.05 level.

Standard Deviations are shown in parenthesis.
differences were not statistically significant at the 0.05 level.

Examples of colonies of each of the four organisms used are shown in Figures 3, 4, 5 and 6.

The typically high colonies of *S. mutans* strain GS5, which were soft and dark blue in colour, are shown in Figure 3.

The colonies of *A. viscosus* strain NY1 shown in Figure 4, were grey to white in colour, soft, slightly sticky and shiny.

The typical dome shape colony morphology of *L. casei* NCIB 6375 is illustrated in Figure 5. The colonies were white to translucent in colour.

Colonies of *N. catarrhalis*, which were greyish yellow and porcelain-like, are shown in Figure 6.
FIGURE 3. Photomicrographs of colonies of *S. mutans* strain GS5 on membrane filters. The typical high rough surfaced colonies observed with organisms treated with DPH test solutions (A) and those treated with control solution (B) are shown. x 20.
FIGURE 4. Photomicrographs of colonies *A. viscosus* strain NY1 on membrane filters. The flattened convex colonies with a smooth entire are observed with organisms treated with DPH test solutions (A) and those treated with control solution (B) are shown. x 20.
FIGURE 5. Photomicrographs of colonies of *L. casei* NCIB 6375 on membrane filters. The high, regular dome shape colonies are observed with organisms treated with DPH test solutions (A) and those treated with control solution (B) are shown. x 20.
FIGURE 6. Photomicrographs of colonies of *N. catarrhalis* on membrane filters. The flattened convex colonies are observed with organisms treated with DPH test solutions (A) and those treated with control solution (B) are shown. x 20.
DISCUSSION

The ability of a drug to affect the survival of organisms, appears to depend on its chemical reactivity with various groups on or in the organisms. The following generalizations can be made about the effect of antimicrobial compounds on bacteria (26):

1. Antimicrobial compounds bind readily to bacteria, the amount absorbed increasing with an increasing concentration in solution. The site of absorption is almost exclusively the cytoplasmic membrane.

2. The extent of killing of bacteria is governed by three principal factors: (a) concentration of the antimicrobial compound (b) bacterial cell density (c) time of contact.

3. The lowest concentration of an antimicrobial compound that causes death of the organism also brings about leakage of cytoplasmic constituents of low molecular weight. The most immediate effect is loss of potassium ions. Gram-positive cells show leakage of amino acids. Increased permeability is a sign of changes in the membrane which are initially reversible but become irreversible on prolonged treatment.
4. There is a low and rather narrow concentration range in which antimicrobial compounds have a bacteriostatic effect. At these low concentrations certain biochemical functions associated with the cell membrane may be inhibited.

5. In the presence of higher concentrations of antimicrobial compounds and after prolonged treatment, the compound usually penetrates the cell and brings about damage to the biochemical mechanism.

The primary effect of antimicrobial compounds on the cytoplasmic membrane is established beyond doubt, but secondary actions on cytoplasmic processes are less defined and may vary from one compound to another \(^{(26)}\).

Various factors govern the behaviour of an antimicrobial compound when it is administered systemically. In order that the compound's activity be maintained, it must reach the site of action in adequate concentration and remain there for a sufficient period of time. It is also affected by the body's enzymes which produce metabolic changes. Metabolism usually inactivates the drug, however several examples are known where metabolism is necessary to convert an inactive compound into an active circulating drug \(^{(26)}\). The degree of drug binding to various tissues may also be important. Some drugs, such as DPH \(^{(77)}\), are firmly bound
to plasma proteins. This increases their persistence in the body, but may also lower their effectiveness if activity depends on the concentrations of free (unbound) compound present in the blood\(^{26}\). Approximately 90 per cent\(^{48}\) of DPH is strongly bound to plasma protein and thus only a small amount is free in solution.

Under the conditions of this \textit{in vitro} study, DPH did not have any discernable effect on the survival of \textit{S. mutans} strain GS5, \textit{A. viscosus} strain NY1, \textit{L. casei} NCIB 6375 or \textit{N. catarrhalis}.

Despite the results obtained, it is possible that (i) a longer contact time between DPH and the test organisms could have produced different results, and (ii) organisms \textit{in situ} in dental plaque may be affected to a greater degree than the test organisms used in this study. Since DPH is excreted in saliva soon after ingestion of the drug and is present in saliva for several hours at reasonably high concentrations\(^{55}\), plaque organisms are undoubtedly exposed to DPH for longer periods of time than that used in this experiment, and longer exposure times may affect the survival of the organisms.

Furthermore, on the basis of the findings of Ellwood \textit{et al.}\(^{20}\), it is possible that DPH may have a greater effect on the metabolic activity of organisms with a lower
mean generation time than those grown in batch culture. Ellwood et al. (20) showed that slow growing organisms were more susceptible to fluoride than fast growing organisms. It is now believed that dental plaque organisms grow far more slowly than the same organisms grown in batch culture (74).

Although the four organisms used in this study, *S. mutans* strain GS5, *A. viscosus* strain NY1, *L. casei* NCIB 6375 and *N. catarrhalis* were not affected by DPH, they represent only a small portion of the organisms isolated from dental plaque. In view of their suggested role in periodontal disease (38), spirochetes, *B. melaninogenicus* and *V. parvula* would also be worthwhile testing, using the same technique, with modifications to ensure maintenance of anaerobic conditions.

The membrane filtration technique used in this study has advantages over other methods when testing the antimicrobial properties of drugs which bind readily to protein (as is the case with DPH):

1. it allows organisms to be treated with a test drug in the absence of extraneous protein. This overcomes the problems associated with conventional methods in which the organisms are exposed to the test drug in the presence of liquid or solid media.
2. carry over of the test drug which may hinder survival of the organisms, into the recovery media is minimised by washing the organisms after exposure to the drug.

The simple nature of this technique, together with its adaptability to suit the conditions required for the particular organism being tested, makes membrane filtration an ideal method for testing potential antimicrobial agents.

DPH induced gingival enlargement was recognized shortly after the introduction of the drug in 1938. Although it is not a problem of major clinical significance, the fact that DPH remains an important anticonvulsant drug means that this side effect should be investigated in order to prevent its occurrence.

Kimball (39), in 1939 and later Frankel (25) in 1940, reported a decreased ascorbic acid level in serum of patients with gingival enlargement, which was thought to be induced by the drug. However, there was insufficient evidence for this hypothesis, especially since subsequent administration of large dosages of vitamin C did not alter the condition (40). Merritt and Foster (50) established that the initial low level of vitamin C in the plasma of these patients was caused by the patients consuming a diet
low in vitamin C and was not related to administration of the drug.

Later, in 1945\(^{21}\) it was suggested that DPH induced gingival enlargement was associated with an endocrine disturbance. Salama and Hilmy\(^{63}\), in 1950, presented a case report but they had no proof of any connection between the endocrine abnormality and the gingival enlargement.

Staple\(^{68}\) in 1953, felt that enlarged gingiva was due to local irritating factors occurring in patients with deranged adrenocortical function caused by DPH medication. He suggested that, as hormones of the pituitary-adrenal complex affect collagen synthesis, a change in the hormone levels, due to adrenal derangement, may cause the altered response in the gingiva.

Van der Kwast\(^{76}\) noted histologically the diffuse infiltrations of plasma cells, in 1956. He observed an associated hypergammaglobulinemia in the majority of his cases and suggested an allergic basis for the condition. Gaillard\(^{27}\) in 1957, reported success in decreasing DPH induced gingival enlargement by treatment with antihistamine therapy in two patients. He suspected that this corrected or counteracted the deranged adrenocortical function.
Other workers\textsuperscript{(10(11)} however, have shown this treatment to be ineffective.

Histamine and serotonin levels of DPH induced gingival enlargement in dogs and ferrets was studied by Francis and Melville\textsuperscript{(23)(24)} in 1958. They concluded that a hypersensitive response was occurring.

In 1958, Noach \textit{et al.}\textsuperscript{(55)} first showed that DPH was secreted in saliva and suggested that this could cause the gingival response. It had been hinted at 10 years earlier when Brandon\textsuperscript{(9)} suggested that DPH "by-products" on contact with the epithelium could cause a "proliferation" of this tissue. Babcock and Nelson\textsuperscript{(7)} in 1964, showed a correlation between severe gingival enlargement and high concentrations of DPH in saliva.

Strean and Leoni\textsuperscript{(71)} in 1959, advocated the use of a topical anti-inflammatory agent, as they believed that the gingival enlargement started as an inflammatory reaction. They thought that DPH particles lodge in the gingiva and act as an irritant to the tissues. The retention of this irritant increased the inflammatory response and promoted a fibroblastic proliferation.

The role of mast cells in DPH gingival enlargement has been examined by Angelopoulos\textsuperscript{(3)}. He suggested that
DPH has a direct effect on these cells, resulting in degranulation and liberation of their cytoplasmic constituents (histamine, heparin and hyaluronic acid) into the surrounding tissues. These substances are taken up and metabolized by the surrounding fibroblasts, which in turn are stimulated to reproduce themselves and specific mucopolysaccharides of the ground substance as well as collagen fibrils, thus building up connective tissue. Local irritating factors in the mouth have a similar effect on mast cells, being capable of inducing inflammation in the gingival tissues. Angelopoulos concluded that it was the combined influence of DPH and plaque on the gingival mast cells which caused the gingival overgrowth.

Recently, Hassel et al. \(^{(34)}\) suggested that there could be a genetic basis for the selection of a susceptible group of fibroblasts, characterised by enhanced levels of protein synthesis and collagen production. However, it seems that the current knowledge of the mechanism by which DPH induces its effects on connective tissue fibroblasts remains obscure.

There has been considerable discussion in the literature as to what part plaque control plays in the initiation and progression of DPH induced gingival enlargement. With only
a few exceptions \(^{(21)}(52)\) investigators agree that local factors do affect the degree of gingival overgrowth.

However, there has been less agreement as to whether plaque plays a primary or secondary etiological role. Prevention of the gingival enlargement has been achieved in cats \(^{(56)}\) by daily plaque control and in monkeys \(^{(69)}\) with the aid of chlorhexidine. In two clinical studies \(^{(19)}(41)\) post-surgical recurrence has been eliminated or minimised by careful daily supervision of oral hygiene procedures. A positive pressure appliance constructed post-surgically and worn only during the night has also prevented recurrence of the gingival enlargement \(^{(5)}(16)(64)\). The reason for success with this appliance is not apparent, however, it has been suggested \(^{(5)}\) that the gingiva was protected at night from saliva containing DPH, and that better oral hygiene habits developed by the test group may also have contributed.

Although it has been established that an intimate relationship exists between the presence of plaque, an inflammatory reaction in the gingiva and the initiation of the DPH induced gingival enlargement, the precise nature of the mechanism is still unclear. It is known that the gingival enlargement does not occur clinically in edentulous areas \(^{(52)}\). It has also been shown by
Donnenfield et al. (19) that although recurrence of tissue overgrowth post-surgically can be overcome by meticulous plaque control, the characteristic histological changes are still present.

The DPH induced reaction in the gingiva is mainly observed in the connective tissue which extends to the epithelium, with loss of distinction between the papillary and reticular layers and proliferation, elongation and reticulation of the rete pegs (19). It is interesting to note that these changes occur only rarely in other connective tissues of the body (34). Most animals are not susceptible to gingival tissue changes (28a), but it has been induced in cats (35), ferrets (42) and recently in monkeys (69).

Within a year of a tooth or teeth being extracted, gingival enlargement will regress in that area, despite continuation of the medication (78). The gingival sulcus, the important distinguishing anatomical feature between an edentulous and dentulous region and an ideal niche for plaque accumulation, appears essential for the clinical response to occur. Following its absorption, DPH becomes a component of transcellular fluids (77). Consequently, it would seem that micro-organisms in the gingival sulcus region are constantly exposed to some level
of DPH. This drug is found in saliva\textsuperscript{(55)(72)} and is most probably also present in gingival fluid. However, it must be pointed out that as yet no reports have appeared on the levels of DPH in gingival fluid. When this is combined with the evidence that meticulous daily oral hygiene following gingivectomy will clinically prevent recurrence of the gingival overgrowth\textsuperscript{(19)}, the action of DPH on plaque organisms of the gingival sulcus region appears vital to the initiation of the lesion.

DPH induced gingival enlargement does not occur in all who ingest the drug. The number of individuals actually affected is difficult to assess, due to large discrepancies in epidemiological data relating to this condition. The more recent studies have stated that the incidence is between 40\textsuperscript{(47)} and 60\textsuperscript{(37)} per cent, with probably about 30 per cent of individuals affected sufficiently to warrant surgical removal of the tissue\textsuperscript{(1)}.

The lesion usually develops in the first six months of DPH administration\textsuperscript{(46)}. Several investigators\textsuperscript{(4)(21)(57)(68)} have suggested that the period of medication is of no significance, whilst some\textsuperscript{(1)(70)(78)} say that it is of importance, with longer periods of medication resulting in a larger incidence and greater degree of gingival enlargement. Opinion is also divided as to whether a
relationship exists between the incidence and severity of the gingival response and the size of DPH dosage. Many workers\(^{(1)}(6)(21)(25)(37)(43)(46)(57)\) have stated that a connection exists whereas others\(^{(4)}(28)(47)\) have not observed such a relationship.

Epidemiological studies\(^{(6)}(21)(43)(52)\) indicate that the lesion does exhibit age and site specificity. The gingiva on the facial aspect of upper and lower anterior teeth is more severely and more frequently affected than that around posterior teeth\(^{(57)}\).

DPH induced gingival enlargement occurs primarily in young individuals, and is seen only rarely in persons over 35 years of age\(^{(6)}\). In a clinical study by Klar\(^{(43)}\) of 312 individuals, most of whom were under the age of 20, it was observed that with increasing age of the patient, a corresponding increase in the number of patients with any amount of gingival enlargement was detected. A peak was reached in the 11-15 year old group.

Socransky and Manganiello\(^{(66)}\) have indicated that changes occur in the oral microbiota with increasing age. They noted that the predominant cultivable organisms of the gingival crevice in a school child generally resembled that of an adult with the exception of spirochaetes and \textit{B. melaninogenicus}, which were not present in all children.
It is also known that with loss of teeth, there is a marked reduction of spirochaetes, as well as a reduction in lactobacilli, certain yeasts, S. mutans and S. sanguis\(^{66}\).

This alteration in oral microflora with age, plus differences that occur both between individuals and within the same individual in different locations of the mouth\(^{66}\) may be a contributing factor to the apparent age and site specificity\(^{6}(43)\) of DPH induced gingival enlargement.

As there is an increased incidence of this lesion during the period of puberty\(^{43}\), more understanding of the mechanism of DPH induced gingival enlargement may be gained by closer examination of plaque samples and gingival fluid taken from different regions of affected mouths and comparisons made with similar samples from unaffected mouths, both from individuals taking DPH and others who are not.

In this study, only one aspect of the possible effect that DPH may have on plaque organisms, that is, on their survival, was assessed. DPH may have affected cell metabolism in ways not measured in this study. The drug may be adsorbed by the cells or cross the cell membrane, thus interfering with normal metabolic processes.

Since the major metabolite of DPH in man, 5-(p-hydroxyphenyl)-5-phenylhydantoin, (HPPH), is secreted in saliva\(^{13}\),
an assessment should be made of its effect on selected oral organisms. In addition, if the concentrations of DPH and HPPH were measured in gingival fluid and the levels absorbed by dental plaque established, then greater understanding of DPH induced gingival enlargement may be gained. As all the evidence suggests that the presence of dental plaque is required to produce the gingival response, further work on the effect of DPH on dental plaque is definitely indicated.
SUMMARY AND CONCLUSIONS

DPH induced gingival enlargement is a disfiguring, sometimes severe, overgrowth of the gingiva, which occurs in approximately 50 per cent of individuals who ingest DPH. Although this side effect is not a major clinical problem, DPH continues to be an important anticonvulsant drug and prevention of this adverse gingival response is desirable.

The gingival enlargement occurs more frequently in the young, with the greatest incidence in the circumpubertal age group. It occurs more often in the anterior region of the mouth, and to a greater degree on the facial aspect. There is no definite correlation with size of dosage or duration of treatment.

There is evidence to suggest that the presence of plaque plays a primary etiological role. It has been shown both in animal and human studies, that good plaque control, and thus elimination of gingivitis, prior to or at the time of DPH administration, will prevent development of DPH induced gingival enlargement. It has also been shown that post-surgical recurrence can be prevented, at least on a clinical level, by meticulous daily plaque control. To date, there have been no reports of studies that have
examined the effect of DPH on plaque bacteria *in situ* or *in vitro*.

This study was therefore undertaken to assess the effect of DPH on the survival of four organisms commonly found in plaque; *S. mutans* strain GS5, *A. viscosus* strain NY1, *L. casei* NCIB 6375 and *N. catarrhalis*.

The membrane filtration technique used in this study has advantages over other methods when testing the antimicrobial properties of drugs which bind readily to protein (as is the case with DPH). Firstly, it allows the organisms to be treated with DPH in the absence of extraneous protein. Secondly, it permits the test organisms to be washed after exposure to the drug, thereby minimising the carry over of DPH which may hinder survival of the organisms, into the recovery media.

The selected organisms were grown in batch culture and after centrifuging and washing, the organisms were added to pH 7.0 phosphate buffer at 37°C containing DPH at a concentration of 9 µg or 35 µg/ml. After 30 minutes, aliquots from each buffer solution were pipetted onto membrane filters. After filtration, the organisms, which were spread over the filter surface, were washed and the filters transferred onto solid media. Following an incubation
period of 24 or 48 hours, the colonies of organisms on the membrane filters were counted using a plate culture microscope.

Under the conditions of this experiment, DPH did not have any discernable effect on the survival of *S. mutans* strain GS5, *A. viscosus* strain NY1, *L. casei* NCIB 6375 and *N. catarrhalis*. However, as only one parameter was investigated in this study, further work should be done. Organisms in situ in dental plaque are probably in contact with DPH for longer periods, and may be more susceptible to chemical agents than organisms grown in vitro. As the membrane filtration technique proved an effective way of testing the antimicrobial potential of DPH, the method should be used to further test the effect of DPH on the survival of other oral organisms implicated in periodontal disease, such as *V. parvula*, *B. melaninogenicus* and spirochaetes.

An examination of plaque samples, both from areas involved and those that are not, in an affected individual, may indicate some differences in the nature of plaque. Comparisons should be made with plaque samples from individuals not affected by DPH, and also from individuals who are not taking the drug.
It is possible that DPH affects plaque organisms in ways not measured in this study. It may interfere with normal cell metabolism or alter excretory products. Since it is known that DPH is in saliva, the level of the drug in plaque may be of importance. As DPH is contained in all transcellular fluids in the free form, the levels of DPH in gingival fluid should also be established.

The major metabolite of DPH in man, 5-(p-hydroxyphenyl)-5-phenylhydantoin may be capable of producing the necessary changes in plaque organisms, since it is also present in saliva. A study to test its effect on the survival of selected plaque organisms may provide important information.

The etiology of DPH induced gingival enlargement remains unknown. Further investigation of the effect of DPH on dental plaque may provide the answer to this adverse gingival response.
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