Protein synthesis *in vitro*, in the presence of Ca(OH)$_2$-containing pulp-capping medicaments

C. T. Hanks[1], G. Bergenholtz[2] and J.-S. Kim[1]

[1]Department of Oral Pathology, School of Dentistry, The University of Michigan, Ann Arbor, U.S.A., and [2]Department of Oral Diagnosis, University of Gothenburg, Gothenburg, Sweden

Four Ca(OH)$_2$-containing pulp-capping medicaments were compared for their effects on protein synthesis in early subcultures of monkey- and human-pulpal fibroblasts. While protein synthesis, as well as DNA synthesis, was depressed by three of the medicaments, the protein-synthetic rate in human-cell cultures in the presence of Life® rose to control levels when the medium was changed daily, but was depressed when the medium was not changed. This suggests that serum proteins play a protective role for pulpal tissues under inflammatory conditions, at least in the case of Life®, allowing odontoblasts to differentiate and make dentinal matrix without interference of either toxic components or excess alkalinity.

The results of a large number of studies have indicated that Ca(OH)$_2$ either in aqueous mix or in setting compounds promotes healing of dental pulp-tissue exposures (Zander 1939, Glass & Zander 1949, Nyborg 1955, Nyborg 1958, Stanley & Lundy 1972, Tronstad 1974, Schröder 1978, Fitzgerald 1979, for review see Baume & Holz 1981). This healing of the pulp is often characterized by the formation of hard tissue at the exposure site in addition to the re-establishment of normal pulp-tissue morphology.

The exact mechanism behind the anabolic effect of Ca(OH)$_2$ on the pulp is not clear. It has long been interpreted that the healing of the pulp, including the formation of new hard tissue, is the result of the caustic action of the agent. Solubilized Ca(OH)$_2$ is highly alkaline and produces superficial necrosis when placed directly on exposed pulp tissue. This necrosis is later often accompanied by the formation of dentin-like hard tissue which separates the pulp tissue from the necrotic zone and the capping agent (Glass & Zander 1949, Nyborg 1955, Schröder 1973). Thus, it has been assumed that the positive response of the pulp to capping with Ca(OH)$_2$ is the effect of the induced necrosis (Granath 1981, Baume & Holz 1981).

Recent studies have, however, demonstrated that new hard tissue may form directly at the medicament interface without being preceded by a zone of necrosis (Tronstad 1974, Fitzgerald 1979). These studies, respectively, used the Ca(OH)$_2$-containing medicaments, *Dycal®* and *Life®*.

Fitzgerald (1979) observed, in addition, that pulpal fibroblasts and endothelial cells, within a few days of experimental wounding, migrated into the wound area and lined up directly adjacent to the medicament before new odontoblasts and mineralized tissue were differentiated. This observation suggests that the Ca(OH)$_2$ in the agent used by this author (Life®) only to a minor extent, if any, produced a stimulatory effect on the pulp. The premise may exist that the reorganization of the pulpal tissue observed, including marshalled new odontoblasts, matrix formation, and mineralization of this matrix, simply was due to stimuli related to the inflammatory process initiated by the experimental wounding (Hanks & Smith 1978). In fact, Cox et al. (1982) recently reported that healing of dental pulp exposures, following capping with Life® or Dycal®, occurred as frequently in teeth of monkeys with superficial inflammatory lesions at the exposure site, initiated by a 24 h oral exposure before capping, as in teeth with normal pulps immediately capped. It seems unlikely that the capping agent, at least during the initial phases of the healing process, produced much stimulation of pulpal cells across the zone of acute inflammation. It seems reasonable to assume that the alkalinity or any other stimuli of the Ca(OH)$_2$ would have been neutralized by the inflammatory exudate released at the wound area. Hence, healing of dental pulp-tissue exposures may be the natural response of the pulp to any form of irritation, whether mechanical, chemical, or inflammatory, provided that the insult remains within tolerable limits.

Not every capping agent containing Ca(OH)$_2$ is associated with proper healing of pulpal exposures. For example, the effectiveness of Hydrex® and MPC® to support dentin bridging has been reported to be poor (Phaneuf et al. 1968, Hirschfeld et al. 1972, Pitt Ford 1979, Heys et al. 1981). The causes of the reduced healing of the pulp to these agents may be the effect of a long standing toxicity.

The objective of the present study was to use an artificial *in vitro* system to study the effects of various Ca(OH)$_2$-containing pulp-capping medicaments upon protein metabolism of isolated pulpal cells in the absence of inflammation. The question was whether any of these agents had a dramatic effect upon protein synthesis, which might, thus, support the view that they stimulate matrix formation and dentin bridging. A secondary objective was to determine whether serum components might play a role in the stimulation of protein synthesis by these Ca(OH)$_2$-containing medicaments.

Material and methods

Four different medicaments containing Ca(OH)$_2$ (New Improved Dycal® from L. D. Caulk Co., Milford, DE; Hydrex® and Life® from Kerr Manufacturing Co., Romulus, MI; and Calasept®, a non-setting Ca(OH)$_2$-salt preparation, Scania Lab, Knivsta, Sweden) were mixed according to manufacturers' directions and placed into plastic molds producing discs approximately 1.5 × 7 mm and weighing 94 mg apiece. These discs, except for Calasept®, were hardened in a moist incubator at 37°C for 10 min and, subsequently, used within 1 h (immediate set) or after gas sterilizing with ethylene oxide and degassing for 5 days in sterile dishes (long term set). These discs were then either floated in the medium above the monolayer, or were submerged, lightly touching the monolayer.

Primary monkey-pulp fibroblasts (pulpoblasts) were obtained from extracted molars from young *Macaca mulatta* (Rhesus monkey), after cleaning the outside of the teeth with 70% ethanol and cracking the teeth in half. Pulpal tissue was removed with sterile forceps from each tooth and minced. Small

explants of this tissue were placed into chicken-embryo extract (Grand Island Biological Co., Grand Island, NY) and then into drops of reconstituted CO_2-treated chicken plasma. Primary human-pulpoblasts cultures were begun in the same way from non-carious premolars taken from young patients for orthodontic treatment. Primary cultures of both cell types were grown to confluency in the alpha formulation of minimum essential medium (alpha-MEM; Grand Island Biological Co., Grand Island, NY) with 10% heat-inactivated fetal calf serum (FCS), glutamine (300 µg/ml), and penicillin/streptomycin (100 IU and 100 µg/ml, respectively). Both types of pulpoblast cultures often took 2–3 months to develop dense primary cultures. These cells were fibroblastic in morphology when growing on plastic. The medium was changed every 3 days. At confluency, these cells were subcultured at 50,000 cells/cm^2 to 24-well plastic dishes (Falcon 3008; Becton, Dickinson & Co., Cockeysville, MD) in 1 ml of alpha-MEM with appropriate levels of FCS.

Four experiments were performed in this study. In the *first experiment,* the monkey cells were used to compare the effects of the four kinds of medicaments (either floating or submerged) upon protein synthesis with continuous labelling with tritiated leucine (^{3}H-leucine; 3 µCi/ml; New England Nuclear Corp., Boston, MA). Dishes containing experimental samples were maintained for 2, 6, and 24 h in the presence of alpha-MEM and 10% FCS. When necessary, surface tension of the medium was able to keep the discs floating and centered in the dish.

In the *second experiment,* discs of each medicament were placed in 24-well dishes of freshly subcultured monkey-pulpal cells. The pulpoblasts had been plated at the same density and with the same medium except that 20% FCS was used and the complete medium was exchanged every 24 h. This was done to determine the effects of higher levels of serum

proteins upon DNA synthesis. Three 24-well dishes containing experimental and control wells were labelled with tritiated thymidine (^{3}H-TdR; 5 µCi/ml; New England Nuclear Corp., Boston, MA) 24 h before death. One dish was harvested at 24 h, another at 48 h and the last at 72 h after introduction of the discs. Observation of the effects of the medicaments upon early growth (logarithmic) permitted measurement without the interference of contact inhibition.

In the *third experiment,* monkey-pulpoblast subcultures, plated at 50,000 cells/cm^2 in alpha-MEM and 10% FCS, were allowed to grow to confluency so that contact inhibition would reduce their proliferative rate, and their metabolic activity would be pushed toward protein synthetic activity, especially of secreted proteins. Therefore, experimental discs and alpha-MEM medium with 20% FCS were introduced after 3 days incubation. The complete medium including 20% FCS was changed each day after introduction of the sample discs. As in the second experiment, this medium exchange was introduced to mimic the presence of increased amounts of serum protein in inflammatory exudate. ^{3}H-leucine was added in the medium 24 h before death. The dishes were harvested at 1, 2, and 3 days after introduction of the discs.

The *fourth experiment* was similar to the third experiment except that human pulpoblasts were used, and experimental dishes were harvested at 1 and 7 days, the medium with 20% FCS being changed each 24 h. As a second kind of control, the alpha-MEM/10% FCS was allowed to remain in another set of wells without change for the entire 7 days. Twenty-four hours before death, the medium was drawn off all wells. Media for a particular medicament or control was pooled and ^{3}H-leucine was added. The respective media for each medicament was then replaced back into the wells. This protocol was used to determine if daily changes of medium with 20% FCS

were a major contributing factor for increased synthesis of new proteins.

The 24-well dishes were stored frozen after after rinsing each well twice with phosphate-buffered saline (PBS). At the time of analysis, cells in each well were broken up by freeze-thawing four to six times in the presence of 250 μl of a 1:9 solution of 0.05% Triton X-100 and 0.1 N NaOH. The contents of the wells and one wash (PBS) were removed to a polyethylene microcentrifuge tube (1.5 ml; Fisher Scientific Co., Pittsburgh, PA) and trichloroacetic acid was added to a final concentration of 12.5%. The samples were centrifuged at $15,000 \times 6$ for 15 min at 4°C. In those samples incubated with ^{3}H-leucine, the pellet was solubilized in 1 N NaOH and protein determination was performed according to Lowry et al. (1951). In those samples incubated with ^{3}H-TdR, the pellets were dried and prepared by the method of Puzas and Goodman (1978). To each dried pellet, 100 μl of diaminobenzoic acid (DABA, 2 M; Aldrich Chemical Co., Milwaukee, WI), followed by hydrolysis at 100°C for 30 min. After addition of 4 ml of 0.6 N perchloric acid, each sample was analyzed by a Turner Model 111 fluorometer for DNA. A 2 cm^2 well from a 24-well dish contained between 0.35 and 0.58 μg DNA.

For determination of radioactivity in the samples utilized for protein determination, 300 μl of the NaOH-solubilized pellet was placed into 7 ml liquid scintillation vials with 5 ml of aqueous counting scintillant (ACS; Amersham Corp., Arlington Heights, IL). For DNA samples, 300 μl of the DABA hydrolysate was added to the liquid scintillation vials with ACS. Readings were expressed as CPM/μg protein or CPM/μg DNA. Statistical comparison of the samples utilized the Student 't' test.

Results

For the most part, our model utilized small discs of pulp-capping medicaments lying directly on top of the cell layer. While the effect of the weight of the disc was minimal, as was apparent when the no disc and Teflon disc controls were compared (Figs. 1 and 5), it was felt that this model might approximate to some extent the direct-contact relationship often observed between the Ca(OH)$_2$-containing medicament and the pulpal tissue from which pulpoblasts develop into new odontoblasts and produce dentin matrix.

In the first experiment, discs of the pulp-capping medicaments were gently placed over monkey-pulpal pulpoblasts and were compared at 2, 6, and 24 h of continuous labelling with ^{3}H-leucine for new protein. Fig. 1 shows the relationship of immediate set, submerged discs of the four pulp-capping medicaments to control wells (no disc and Teflon discs). In 24 h, Hydrex® allowed synthesis of about 66% as much new protein as produced in the control wells; Life® allowed only 38%. Protein synthesis by the cells in the presence of Dycal® as well as Calasept® (either in disc form or suspended) was negligible.

This experiment was repeated three times and the same rank-order relationship occurred, whether the discs were floating or submerged, and whether they were used immediately after preparation or 5 days after preparation (long term set; Fig. 2). Calasept® was not used in all studies because the discs did not set up like other medicaments, but rather dried by dehydration. Consequently, the discs frequently fell apart. Also, from Fig. 2 it can be seen that, in general, new protein synthesis occurred at greater levels for a given medicament if the sample discs were floating rather than submerged on top of the cell layer. At least for the floating sample discs, the samples which had set for 5 days (long-term set)

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allowed more new protein synthesis than the samples which were used immediately.

It was also observed that the medium, which contained a color indicator, remained more alkaline in certain groups of samples than in others. Therefore, pH readings of samples with media were taken at the same surface area:volume ratio (94 mg:1 ml) over 48 h, with changes of medium plus 10% FCS after 24 h (Fig. 3). The incubation conditions were also the same, i.e., in a humid, 37°C oven

Fig. 1. Protein synthesis in wells of monkey pulpoblast after 2, 6, and 24 h continuous labelling with ^{3}H-leucine. Quadruplicate wells contained immediate set, submerged discs of Hydrex®, Life®, Dycal®, Calasept® (Ca(OH)$_2$ disc and Ca(OH)$_2$ susp., Teflon (control) or no discs (control).
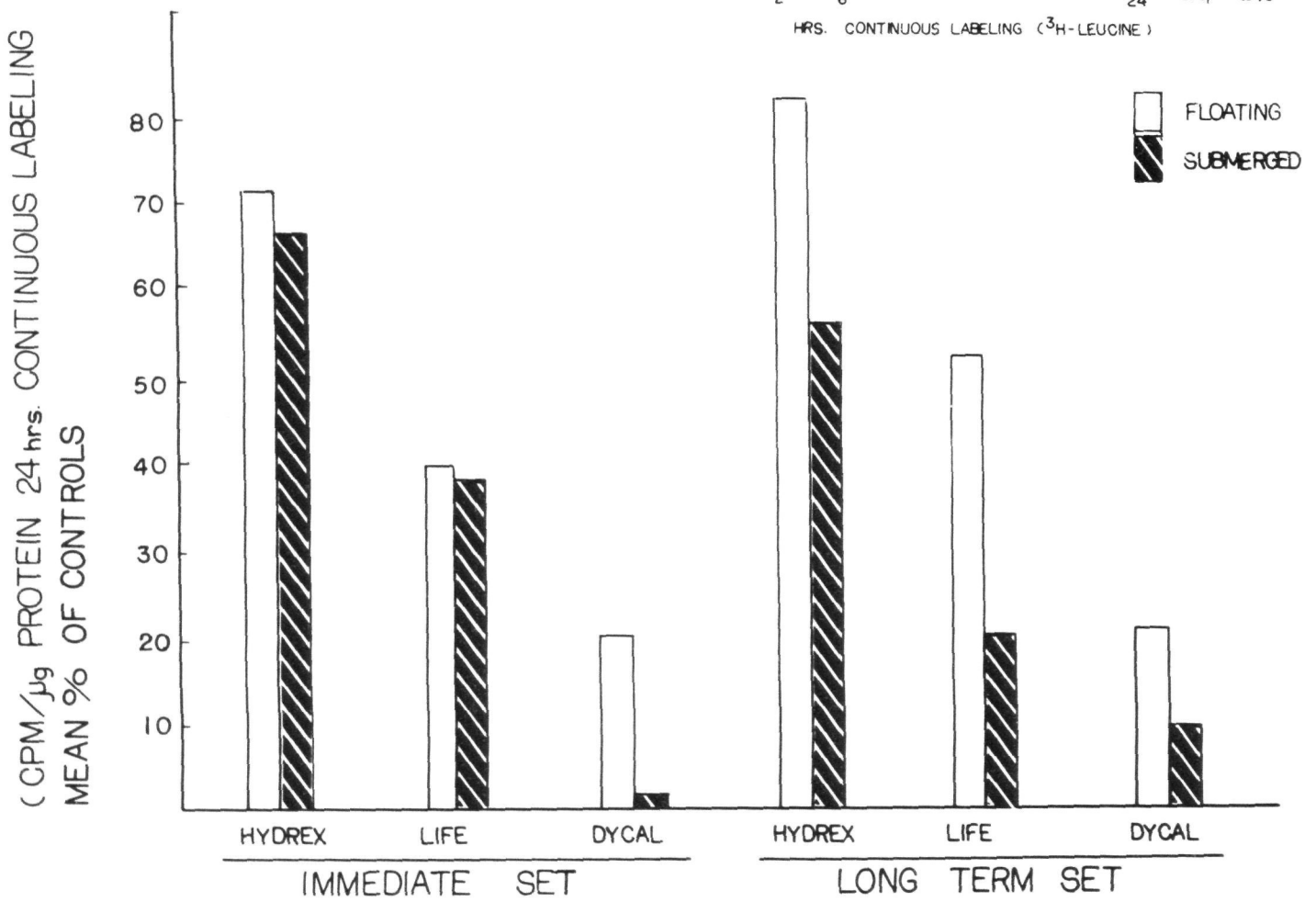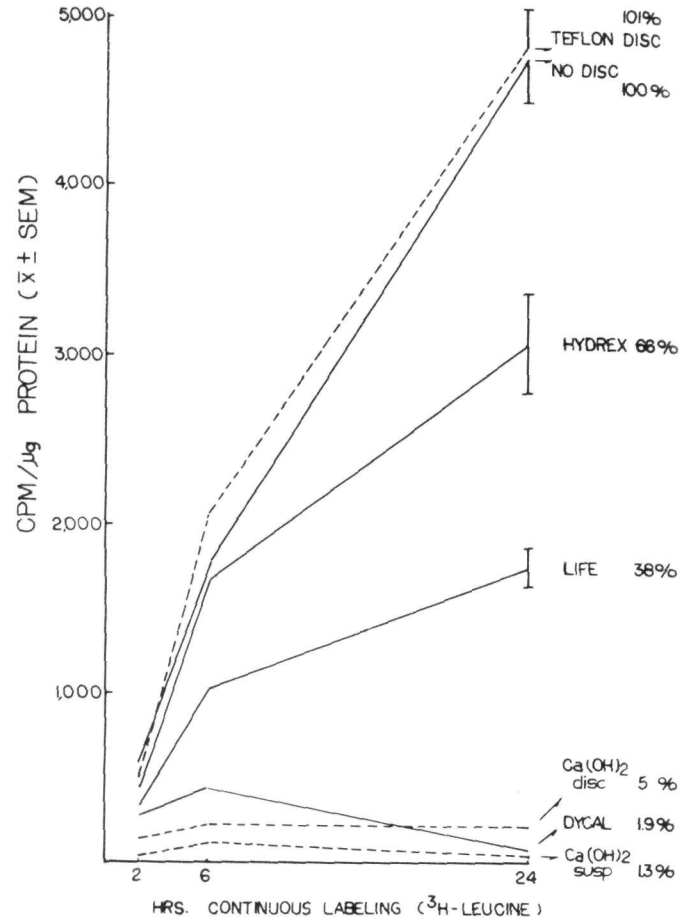

Fig. 2. Protein synthesis after 24 h labelling of monkey pulpoblasts with ^{3}H-leucine for three pulp-capping medicaments.
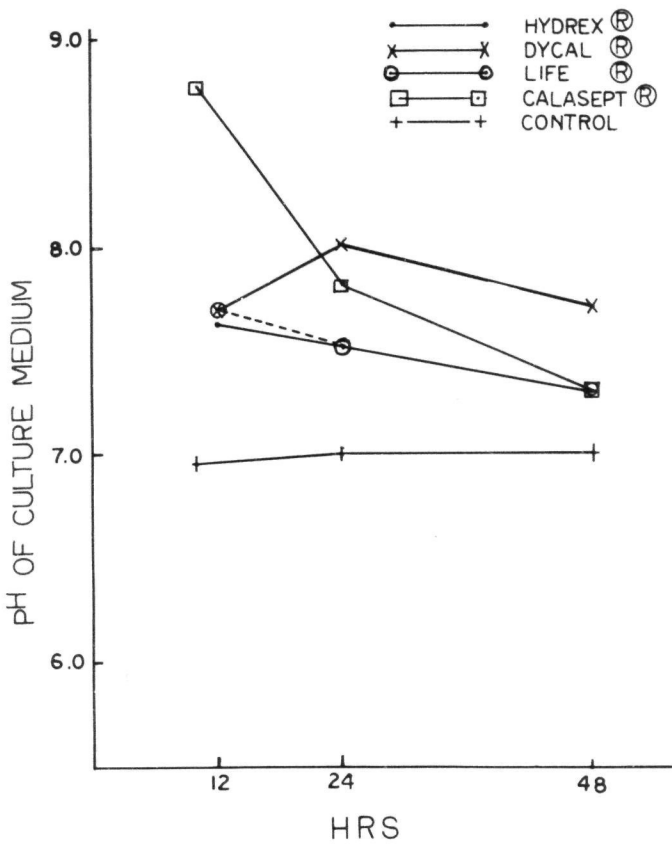
Fig. 3. pH values for wells with four pulp-capping medicaments and no disc controls for a 48 h period, with one change of medium plus 10% FCS at 24 h. No cells present in any of the wells.

with a 5% CO_2-95% air atmosphere. Calasept® discs, which continued to break down over this period initially gave the highest pH value. The 12-h pH value was 8.8 because this alpha-MEM medium contains both a bicarbonate and phosphate buffer system. Over the 48-h period, after one medium/FCS change, the pH of the medium containing Calasept® was reduced toward neutrality (7.8 at 24 h, 7.3 at 48 h). On the other hand, the pH of Dycal®, which began at 7.7 at 12 h, rose to 8.0 by 24 h. After changing the medium/FCS, the pH was buffered back only to 7.8 by 48 h. The pH of Life® and Hydrex® began at 7.7 and 7.6, respectively, and dropped to 7.5 by 24 h. After the medium/FCS change, the pH continued to drop to 7.3 by 48h. This pattern of buffering is somewhat reflective of the relative aqueous solubility of these four medicaments. The control wells maintained a pH

of 7.0 throughout the time period. At the end of 48 h, a hard crystalline material had formed in the center of the well containing Calasept®. Further observations with a scanning electron microscope and elemental analysis showed orderly crystal formation associated with the Dycal® and Calasept® discs (unpublished data). These crystals were found to contain calcium and phosphorus by elemental analysis. Wells containing Life® discs contained much smaller, amorphous crystals.

In the second experiment, the effects of these medicaments upon the synthesis of new DNA were assessed. The level of DNA in each of the wells in the presence of all sample
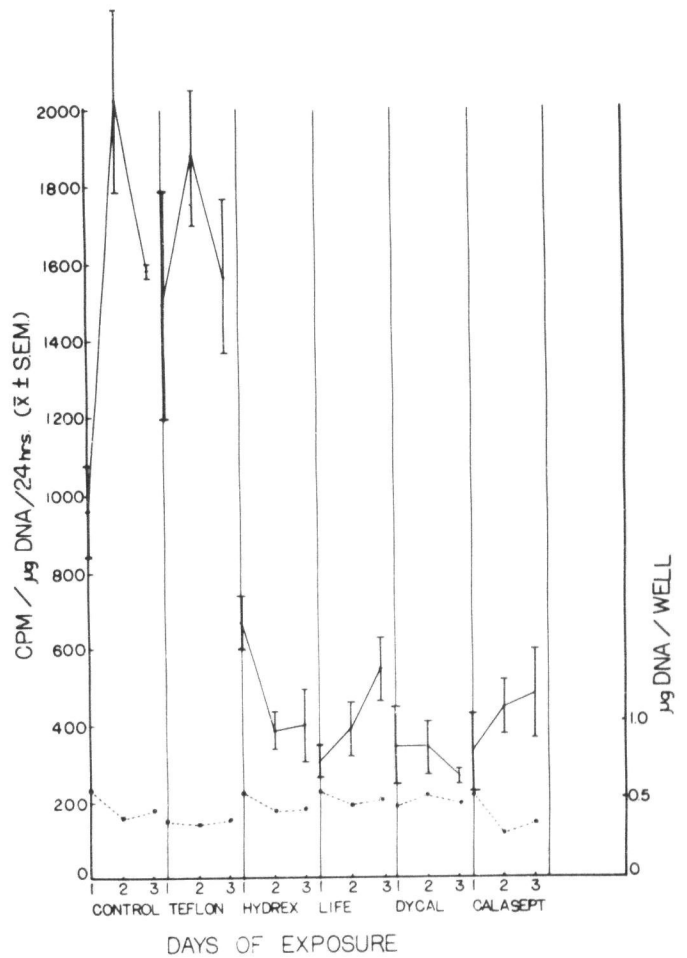

Fig. 4. DNA levels and 24-h DNA synthetic rates of monkey pulpoblasts in quadruplicate wells with 1, 2, and 3 days exposure to discs of the four medicaments, Teflon, or no discs (controls). The medium with 20% FCS was changed every 24 h and ³H-Tdr was added for the last 24 h before harvest of the cells.
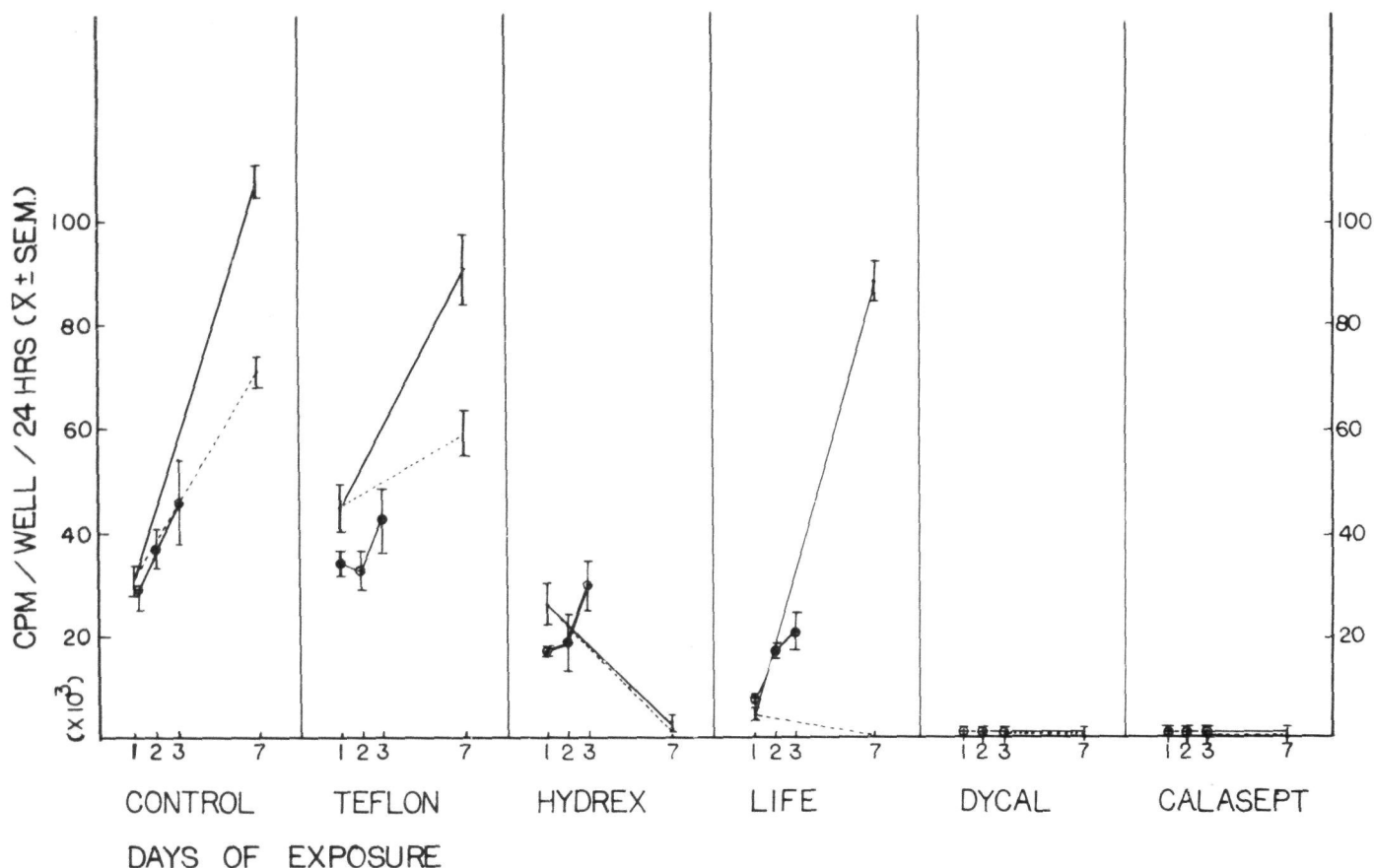
Fig. 5. Protein synthetic rates (24 h) in wells of monkey pulpoblasts (●——●) and human pulpoblasts with (·——·) and without (·----·) daily changes of medium with 20% FCS, when exposed to the four medicaments, Teflon or no disc (controls).

discs averaged about 0.5 μg. In Fig. 4, the 24-h ^{3}H-TdR incorporation rate into new DNA in control wells (no discs or Teflon discs) peaked at 2 days, which probably reflects when the wells became confluent, and then dropped off by 3 days. The levels of newly synthesized DNA were lower for all the pulp-capping medicaments than for the controls. Although the standard errors were small, the slight variation from one type of medicament to another is probably not as significant as the generally reduced DNA synthesis. Hydrex® showed a 2.5 fold decrease in thymidine incorporation between the first and second day (P < 0.1). For the other medicaments, the fairly wide standard error values and 't' values suggest that there are no real differences in the DNA synthetic rates between 1 and 3 days.

The results of the third and fourth experi-

ments are combined in Fig. 5 in order to compare the protein synthesis of monkey and human cells in the presence of these medicaments. It can be seen that for control wells (no discs or Teflon discs), monkey pulpoblasts (Days 1–3) and human pulpoblasts (Days 1–7) increased their rate of incorporation of ^{3}H-leucine dramatically. For human pulpoblasts, if the medium with 20% FCS was not changed daily, the protein synthetic rate was depressed for all pulp-capping medicaments. For the wells containing Life® discs, again both monkey pulpoblasts and human pulpoblasts showed dramatic increases in 24-h rates of new protein synthesis. Although the rate for human cells with Life® discs was not quite as high as the rate for control wells of human cells with no discs, they were about the same as control wells of human cells with Teflon discs. Between 1 and 7 days, the increase in

protein synthetic rate was the most dramatic (21-fold) found with any of the pulp-capping medicaments (P < 0.001). Again, daily changes of alpha-MEM with 20% FCS were essential for this protein synthesis. The results for Hydrex® discs were different in the two experiments. Although monkey cells showed an increasing new protein-synthetic rate over the 3 day period, protein synthesis in the human cells dropped off dramatically over a 7 day period, as if the daily changes of FCS made no difference or were not sufficient to withstand the effects of Hydrex®. Finally, the protein-synthetic rates in monkey- and human-pulpal pulpoblasts in the presence of Dycal® and Calasept® were similar, i.e., negligible. There was only minimal protein synthesis in the presence of either medicament, which resembles the results of the first study, at 3 days and 7 days, even with daily changes of serum-rich medium.

Discussion

The present study showed that with early subcultures of monkey pulpoblasts, the relative ability of several pulp-capping medicaments to support cellular protein synthesis is as follows: Controls > Hydrex® > Dycal® and Calasept®. This was true for monkey-pulpoblast cultures through 3 days exposure to these medicaments. This was also true for human-pulpoblast cultures at 24 h after exposure. However, for one medicament, Hydrex®, the protein-synthetic rate in human pulpoblasts was depressed, even with daily changes of medium plus 20% FCS, almost as much as when the medium was not changed. Therefore, for human pulpoblasts, by 7 days, the rank order of Life® and Hydrex® was reversed.

The findings in the present study parallel some reports of direct pulp-capping tests with Life® in monkeys (Figzgerald 1979, Heys et al. 1981, Cox et al. 1982), and with Hydrex® in humans (Phaneuf 1968), but not those with Dycal® in monkeys (Heys et al. 1981) or in humans (Stanley 1971). *In vitro* tests are usually much more sensitive to the detrimental effects of materials on cellular metabolism than are cells and tissues *in situ*. Since Hydrex® is relatively insoluble, the reduced protein synthesis in our cultured human cells in the presence of Hydrex® is probably related more to toxic effects of the medicament than to alkalinity. Likely candidates for the "factors" toxic to human pulpoblasts in our system may be mineral oil or natural resin components released from Hydrex® into the medium. This may explain the poor *in vivo* results obtained with this medicament (Phaneuf 1968, Hirschfeld et al. 1972). Likewise, Dycal® was extremely toxic to protein synthesis in our system for both human and monkey pulpoblasts, confirming the findings of Tronstad et al. (1978). Dycal® contains a plasticizer, ethyl toluene sulfonamide, which is used as a "catalyst" (Pitt Ford 1979). It has been suggested that the release of this plasticizer is responsible for increased cell-membrane permeability and subsequent decreased cellular respiration in cultured rat pulp-tissue (Jones 1979). Life® also contains ethyl toluene sulfonamide (Emil Jandourek, Kerr Manfacturing Company, personal communication). However, it may be released at different rates from Life® and Dycal® because of the greater density of Life®. Further, Dycal® is more soluble than either Life® or Hydrex®, and the alkalinity is not well buffered (Fig. 3). Therefore, toxin concentration as well as alkalinity may be responsible for Dycal®'s effect upon both species of pulpoblasts. Finally, the toxicity of Calasept® in our system may be attributed to the "localized alkalinity" caused by the solubility of this material. This alkalinity has been associated with the superficial necrosis produced in pulpal tissue (Nyborg 1958,

Schröder 1973, Granath 1981). Consequently, although it eventually permits hard tissue formation *in vivo*, it would not seem to serve as well as a direct pulp-capping medicament in which the Ca(OH)$_2$ is less soluble.

Without daily changes of medium with 20% FCS (which stimulated protein synthesis in controls), the protein-synthetic rate in the human cells was depressed for all of the Ca(OH)$_2$-containing medicaments over the 7 day period (Fig. 5). Clearly, factors in the serum had a beneficial value for the cells, and these factors were depleted with time in culture. Likewise, serum factors such as proteins released from dilated, leaky capillaries may play an important role in maintaining the health of the pulpal tissue at the site of pulp capping. Although we did not test mechanisms by which daily changes of FCS aided protein synthesis in our cell cultures, we may speculate that at least three mechanisms may be operative: (1) buffering of +H and −OH ions by several buffer systems in serum (Granath 1981), (2) serum peptide and hormone stimulation (Mather & Sato 1979, Price & Gregory 1982), (3) detoxification through complexing to such proteins as albumin globulins and fibrinogen (Fugisawa & Masuhara 1981).

In the present study, the buffering capacity of the medium/FCS was sufficient to reduce the alkalinity of these incubating solutions containing Life®, Hydrex® and Calasept® samples toward neutrality between 12 and 24 h (Fig. 3). It was not adequate to keep the pH from rising from 12 to 24 h in the incubating solutions containing Dycal®. This resultant alkalinity within the first 24 h was probably responsible for the depressed DNA and protein synthesis for Dycal® and Calasept®. For Life®, after one change of medium/FCS at 24 h, the pH of the medium was near neutrality by 48 h. Further, the protein-synthetic rate in human cells at the end of 7 days of daily medium/FCS changes was the same as that in

Teflon-disc control wells. Also, if the medium/FCS were not changed daily, the protein-synthetic rate for Life® discs decreased over the 7 day period. Consequently, the new medium/FCS was protecting the human cells from some toxic substance in Life®. Finally, our experimental model could not discriminate between mechanisms responsible for the protection given by the medium/FCS against these protein synthesis-depressing effects, whether they occurred in Hydrex®, Dycal® or Life®.

It was obvious that this *in vitro* model was not suitable for Calasept®, since the discs broke down and formed a suspension. This medicament was highly soluble, producing a pH of almost 9 in the buffered system at 12 h. By 24 and 48 h, a crystallization reaction occurred which was associated with a reduction of pH. While Ca(OH)$_2$ may be responsible for the superficial zone of necrosis caused *in situ* at the tissue interface, this crystallization process may account for the limited exposure of newly marshalled odontoblasts to hydroxyl ions, so that they are able to synthesize a dentin matrix. While it has been shown that Ca++ from the Ca(OH)$_2$ medicament does not contribute to calcification of the dentin bridge in dogs (Sciaky & Pizanti 1960), it has also been suggested that an alkaline environment in the vicinity of the highly soluble Ca(OH)$_2$ may contribute to mineralization of a tissue with a suitable matrix, the calcium and phosphate ions coming from the blood supply. Perhaps this crystallization on the surface of the discs and early crystallization of necrotic, non-matrix tissue represents the same phenomenon, and this area of "dystrophic calcification" forms a barrier, protecting the odontoblasts from the highly alkaline and lethal environment of Dycal® and Calasept®.

Acknowledgements

References

Baume, L. J. & Holz, J. (1981) Long term clinical assessment of direct pulp capping. *Int Dent J* **31** (4), 251–260.

Cox, C. F., Bergenholtz, G., Fitzgerald, M., Heys, D. R., Heys, R. J., Avery, J. K. & Baker, J. A. (1982) Capping of the dental pulp mechanically exposed to the oral microflora – a 5 week observation of wound healing in the monkey. *J Oral Pathol* **11**, 327–339.

Fitzgerald, M. (1979) Cellular mechanisms of dentinal bridge repair using ^{3}H-thymidine. *J Dent Res* **58** (Sp. Issue D), 2198–2206.

Fugisawa, S. & Masuhara, E. (1981) Binding of eugenol and O-ethoxybenzoic acid to bovine serum albumin. *J Dent Res* **60** (4), 860–864.

Glass, R. L. & Zander, H. A. (1949) Pulp healing. *J Dent Res* **28**, 97–107.

Granath, L. (1981) Pulp-capping materials. In: *Biocompatibility of Dental Materials*, eds. Smith, D. C. & Williams, D. F., Vol. 2, pp. 253–267. Boca Ratan, Florida: CRC Press, Inc.

Hanks, C. T. & Smith, E. O. (1978) Inhibition of connective-tissue proliferation by dermal extract. tion by dermal extract. *J Invest Dermatol* **71**, 172–176.

Heys, D. R., Cox, C. F., Heys, R. J. & Avery, J. K. (1981) Histological considerations of direct pulp-capping agents. *J Dent Res* **60** (7), 1371–1379.

Hirschfeld, Z., Sela, J. & Ulmansky, M. (1972) Hydrex and its effect on the pulp. *Oral Surg* **34** (2), 364–371.

Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) Protein measurement with the Folin phenol reagent. *J Biol Chem* **197**, 265–275.

Mather, J. P. & Sato, G. H. (1979) The use of hormone-supplement serum-free media in primary cultures. *Exp Cell Res* **124**, 215–221.

Nyborg, H. (1955) Healing processes in the pulp on capping. *Acta Odontol* **13** (Suppl. 16), 9–130.

Nyborg, H. (1958) Capping of the pulp. The processes involved and their outcome. A report of the follow-ups of a clinical series. *Odontol Tids* **66**, 296–386.

Phaneuf, R. A., Frankl, S. N. & Ruben, M. P. (1968) A comparative histological evaluation of three calcium hydroxide preparations on the human primary dental pulp. *J Dent Child* **35** (1), 61–76.

Pitt Ford, T. R. (1979) Pulpal response to Procal for capping exposures in dog's teeth. *J Br Endodont Soc* **12** (2), 67–72.

Price, P. J. & Gregory, E. A. (1982) Relationship between *in vitro* promotion and biophysical and biochemical properties of the serum supplement. *In vitro* **18** (6), 576–584.

Puzas, J. E. & Goodman, D. B. P. (1978) A rapid assay for cellular deoxyribonucleic acid. *Anal Biochem* **86**, 50–55.

Schröder, U. (1973) Reaction of human dental pulp to experimental pulpotomy and capping with calcium hydroxide. *Odontol Rev* **24** (Suppl. 25), 1–33.

Schröder, U. (1978) A two year follow-up of primary molars pulpotomized with a gentle technique and capped with calcium hydroxide. *Scand J Dent* **86**, 173–178.

Sciaky, I. & Pizanti, S. (1960) Localization of calcium placed over amputated pulps in dog's teeth. *J Dent Res* **39**, 1128–1132.

Stanley, H. R. (1971) Dycal therapy for pulp exposures. *Programs and Abstracts of the 49th General Session, Int Asso Dent Res*, p. 189.

Stanley, H. R. & Lundy, T. (1972) Dycal therapy for pulp exposures. *Oral Surg* **34**, 818–827.

Tronstad, L. (1974) Reaction of the exposed pulp to Dycal treatment. *Oral Surg* **38**, 945–953.

Tronstad, L., Hasselgren, G. & Wennberg, A. (1978) Cytotoxicity of calcium hydroxide-containing materials and their solubility as pulp-capping agents. *J Dent Res* **57** (Sp. Issue A), p. 385.

Zander, H. A. (1939) Reaction of the pulp to calcium hydroxide. *J Dent Res* **18**, 373–379.

Address:
Dr. Carl T. Hanks,
5224 School of Dentistry,
The University of Michigan,
Ann Arbor,
Michigan 48109,
U.S.A.