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In vitro and *in vivo* Evaluation of Anti-Calculus Agents

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Gem diphosphonates, vicinal polyphosphonates and polyphosphates were found to be effective inhibitors of calculus formation in rats. Sodium citrate and trisodium phosphate were found ineffective. Accumulation of calculus was inhibited by inclusion of the phosphonates in the calculus diet or by topical application of aqueous solutions. The anti-calculus effect of phosphonates was topical rather than systemic and was related to the relative effectiveness of these compounds to inhibit crystal growth of hydroxyapatite. A calculus grading method was used and was reproducible among several graders in assessing calculus production and inhibition. Calculus production in weanling animals fed the control diet occurred at a rapid rate under the conditions described, with maximum scores being reached after about 18 days.

Key words: Phosphonates — Phosphates — Calculus — Crystal — Growth — Calcium.

Des diphosphonates de gemme, des polyphosphonates voisins, et des polyphosphates semblent constituer des inhibiteurs actifs de la formation du tartre des rats. Le citrate de sodium et le phosphate trisodique sont inactifs. Les dépôts tartriques sont inhibés par adjonction des phosphonates à l'alimentation ou par application locale de solutions aqueuses. L'effet antitartre des phosphonates est local plutôt que général et semble lié à l'efficacité relative de ces composés vis à vis de l'inhibition de la croissance cristalline de l'hydroxyleapatite. Un indice de tartre est utilisé et s'avère reproductible pour divers examinateurs pour estimer le dépôt et l'inhibition du tartre. La formation de ce dernier chez des animaux sevrés, soumis à une alimentation témoin, est rapide dans les conditions décrites avec des valeurs maximales atteintes au bout de 18 jours.

Geminale Diphosphonate, vicinale Polyphosphonate und Polyphosphate erwiesen sich als wirksame Hemmer der Zahnsteinbildung in Ratten. Natriumcitrat und Trinatriumphosphat waren unwirksam. Eine Anhäufung von Zahnstein wurde durch Beigabe der Phosphonate in der Diät oder durch lokale Anwendung von wäßrigen Lösungen verhindert. Die steinhemmende Wirkung von Phosphonaten war eher lokal als systemisch und hing zusammen mit der relativen Wirksamkeit dieser Substanzen bei der Hemmung des Kristallwachstums von Hydroxyapatit. Die Steinmeßmethode wurde verwendet und war bei der Festlegung von Steinproduktion und -hemmung mit mehreren anderen Methoden reproduzierbar. Die Steinbildung bei entwöhnten Tieren, welche die Kontrollnahrung erhielten, erfolgte bei den beschriebenen Bedingungen rasch, wobei die maximalen Werte etwa nach 18 Tagen erreicht wurden.

Introduction

The formation of hydroxyapatite (HA) *in vitro* occurs in two distinct steps when calcium ion is added to orthophosphate ion at a pH of 7.4 (held constant by means of a pH-stat). The reaction can be followed titrimetrically by recording base consumption as a function of time (Francis *et al.*, 1969; Francis and Webb, 1971). The base consumed follows a standard pattern at a given concentration

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such that the first rapid consumption occurs in 1–4 min then diminishes until about 15–20 min when again rapid uptake occurs. A delay in the time of the second rapid consumption of base or a total absence of the second rapid consumption indicates an interference in the normal crystal growth of HA. Agents which effectively interfere with crystal growth of HA should be effective as anti-calculus agents and will be investigated in this report.

In a previous publication Francis and Briner (1969) described methods for the reproducible production and quantitative estimation of calculus deposition in rats. Shannon *et al.* (1970) also have investigated some of these diets. Subsequently, Francis and Briner (1973) used these methods to demonstrate an anti-calculus effect for two phosphonate salts, trisodium ethane-1-hydroxy-1,1-diphosphonate (Na_3EHDP) and trisodium methanediphosphonate (Na_3MDP). They postulated that these materials, when included in the diet, inhibited calculus formation by interference with crystal growth of HA as predicted from the phosphonate physical chemical properties (Francis, 1969). The anti-calculus potential of a large group of phosphonate compounds has been suggested by McCune (1967) and the inhibition of lower incisor supragingival calculus using Na_2EHDP in a mouthwash has been reported by Mühlemann *et al.* (1970). The inhibition of human calculus formation by Na_2EHDP in a dentifrice has been reported by Sturzenberger *et al.* (1971) and Conroy *et al.* (1972).

The present study deals with the titrimetric evaluation of the potential anti-calculus activity of 24 compounds and with evaluation of several parameters of the *in vivo* rat calculus system including:

1. Anti-calculus effect of various phosphonates and other compounds.
2. Reproducibility of the grading method (Francis and Briner, 1970) among several graders.
3. Calculus production with two different diets.
4. Rate of deposition of calculus.
5. Systemic effect of a phosphonate salt administered subcutaneously on calculus formation.
6. Residual anti-calculus effect of a phosphonate salt in offspring of rats fed the phosphonate as a dietary supplement for two generations.

Materials and Methods

In vitro *Determination of Crystal Growth Inhibitors*

Standard solutions of 0.1 M calcium chloride and 0.1 M sodium dihydrogen phosphate (J.T. Baker Chemical Co., Phillipsburg, New Jersey) were prepared. Phosphate solution (1.00 ml) was placed in a reaction flask with 22 or 23 ml of distilled water, and sweeping out with nitrogen and stirring were commenced. For control runs, 23 ml of water was added; for investigation of crystal growth inhibitors, 22 ml of water and 1 ml of compound to be tested for crystal growth inhibition were added and the pH adjusted to 7.4 ± 0.05. To these systems, 1 ml of calcium solution at pH 7.4 was added such that the final mixed concentrations before reaction were 4×10^{-3} M in calcium and phosphate ion and of varying concentrations for the additive substance. The consumption of 0.1 M standard NaOH was recorded automatically by a pH-stat (Radiometer, Copenhagen). The reaction was allowed to continue until the second rapid consumption of base was essentially complete (less than 0.005 ml consumed/30 min) or until it was evident that there was total blockage of crystal growth of HA and no significant second uptake would occur (maximum) time was 22 h). For control

systems, the second uptake of base was essentially complete after 30–40 min. The relative effectiveness of one crystal growth inhibitor versus another was assessed by determining experimentally the lowest concentration of inhibitor which would produce a delay of at least 10 min in the second rapid stage of base consumption.

In vitro *Determination of Anti-Calculus Activity*

Animals. 21–22 day old albino rats derived from the Sprague-Dawley strain supplied by the Dan Rolfsmeyer Co., Madison, Wisconsin or by Charles River Laboratories, Boston, Massachusetts were used. Rats were housed individually in open wire mesh bottom stainless steel cages. Food and distilled water were supplied *ad libitum*. Temperature and relative humidity were controlled at $24° \pm 0.5°$ and 45–50%, respectively. Sex was randomized in these experiments.

Diets. Diets RC-15 or RC-16 were used (Francis and Briner, 1969; Shannon, 1971). Their compositions are given in Table 1. Supplements were incorporated into diets by a substitution of test material for a corresponding weight of inactive Celluflour. Body weight gains were obtained in all experiments and food intake was measured in selected experiments.

Table 1. Diet compositions (% by weight)

Ingredient	RC-15	RC-16
Corn starch	50.0	50.0
Non-fat dry milk	32.0	32.0
Liver powder	3.0	3.0
Celluflour	5.0	5.0
Cottonseed oil	1.0	1.0
Sucrose (powdered)	5.0	5.0
$CaCl_2 \cdot 2H_2O$	1.0	1.0
$MgSO_4$	0.3	0.3
Na_2HPO_4	2.7	—
NaH_2PO_4	—	2.7

Treatments. Solutions were applied topically by restraining the animals in a clamp (Johansen, 1952) and swabbing the molar teeth with a cotton swab saturated with the test solution (Francis, 1966). This was done twice daily for 9 days (not on weekends) during a two-week experimental period, except in Experiment 5 and 8 where topical treatment was given twice daily for 14 days (not on weekends) during a three-week experimental period.

Grading. After sacrifice, the teeth and jaw were obtained clean and dry by autoclaving. The grading method described by Francis and Briner (1969) was used. In this method, a severity value of 0, 1, 2, or 3 is assigned to each surface graded where:

0—No calculus

1—Barely detectable (but an authentic deposit of hard calculus) to 25% of the area thinly covered.

2—25–75% of area covered, but not thick.

3—50–100% of area covered with a thick, heavy deposit.

The scoring is done destructively with a dental explorer since it is impossible to otherwise determine the thickness of the deposit. A binocular microscope at 30X magnification was used. Data were recorded on cards as described by Francis and Briner (1969) or punched directly on keypunch cards. The total severity score per animal was determined and defined as the CSSI—Calculus Surface Severity Index. The incidence score (presence or absence of calculus on a surface) was obtained and defined as the CSI—Calculus Surface Index, in a manner analogous to that used in calculus scoring in man (Conroy and Sturzenberger, 1968). Both data are presented. Third molars were not assayed in tests of two-week duration because all third molars were not in place at termination of the experiments. Animals were coded from random number tables so that during grading group bias would be avoided.

Statistical Analysis. All computations and analyses of variance were carried out by computer, and the treatments were ranked according to the method of Newman and Kuels outlined by Snedecor and Cochran (1967) which allows for all possible comparisons among treatments while maintaining a constant α risk of 0.05.

Test Designs. A balanced complete block test design was used in all experiments. In experiments consisting of 10 treatments, 20 litters of 10 rats/litter were used, one rat from each litter per treatment; in 9 treatment experiments, litters of 9 rats were used. In experiments consisting of 4 treatments, 10 litters of 8 rats/litter were used, 2 rats from each litter per treatment. Designs of the test with only 2 treatment groups varied and are discussed under the separate experiment which follows.

Anti-Calculus Effects of Various Compounds

The 23 compounds listed in Table 2 were examined for anti-calculus activity in Experiments 1 through 5. Experiments 1 and 2 were exclusively dietary studies and contained 10 treatment groups in each experiment. Experiment 3 was a combination of both diet supplementation and topical treatment and contained nine treatment groups. Experiments 4 and 5 were topical studies and contained 4 treatment groups each.

Reproducibility of Calculus Grading and Calculus Incidence

Experiment 6 contained 10 treatment groups. Four treatment groups were evaluated by one grader, 4 similar treatment groups by a second grader, a third grader examined 2 treatment groups which were included in the other 2 groups of 4 treatments. Since the calculus deposit is chipped away during grading, multiple grading of a single specimen is not feasible, therefore, grader variability is inclusive of both group to group and grader to grader within experiment and within treatment error.

Experiment 7 contained 9 treatment groups. Three graders evaluated 3 similar treatments which included a comparison of Diets RC-15 and RC-16.

Rate of Deposition of Calculus

In Experiment 8, 10 treatment groups were established. Seven of these groups were fed Diet RC-15 and swabbed thrice daily with water. From these 7 identically treated groups, one group was sacrificed every 3 days to evaluate the rate of accretion of calculus. Three additional groups were fed Diet RC-15 supplemented with 1% sodium penicillin G (Nutritional Biochemical Co., Cleveland, Ohio). One of these latter 3 groups was sacrificed every 7 days. At the termination of the experiment, the jaws were randomized and graded as usual. A regression analysis of CSSI or CSI scores versus time by the method of least squares was performed by computer.

Systemic Anti-Calculus Effect of Na_2EHDP

In Experiment 9, two treatment groups were established using 20 rats per treatment. Four rats from each of 5 litters (8 rats per litter) were allocated to each group. One group received 30 mg/kg/day of Na_2EHDP administered subcutaneously; the other group received an equimolar amount of sodium administered as NaCl. Solutions were prepared aseptically and contained 5 mg/ml Na_2EHDP or the sodium chloride equivalent in sodium at pH 7.4. Body weights were taken daily and used to calculate volume of injection. Diet RC-16 was fed throughout this 2-week study.

Residual Anti-Calculus Effect in Offspring of Rats Fed Na_2EHDP

In Experiment 10, twenty rats (litter and sex random) were selected from 2 different pools of rats. One pool had received a diet of Purina Lab Chow (Ralston Purina Company, St. Louis, Missouri) for 2 generations; the other a diet of Purina Lab Chow supplemented with 0.5% Na_2EHDP for 2 generations. After weaning both treatment groups (the F_2 generation) were fed Diet RC-16 for two weeks, sacrificed and graded in the usual manner.

Results

In vitro *Determination of Crystal Growth Inhibitors*

The effect of a large group of compounds on the crystal growth inhibition of HA is shown in Table 2 with the compounds arranged in approximate order of the least to greatest concentration which produced a visible delaying effect on the HA reaction as determined on the pH-stat. The most effective compound methanecyclohexylhydroxydiphosphonate (MCHDP) required only one-two hundredths (2×10^{-5} M) the initial concentration of the calcium and phosphate ions (4×10^{-3} M) to inhibit crystal growth of apatite while sodium citrate, a known calcium complexing agent, had no effect on the HA reaction at one fourth (1×10^{-3} M) the concentration of calcium and phosphate (4×10^{-3} M). Other compounds between these two extremes of action are given in Table 2.

In vitro *Anti-Calculus Effects of Various Compounds*

The results from Experiments 1 to 5 are presented in Tables 3–4. Table 3 shows the decreasing anti-calculus effect resulting from decreasing the dietary con-

Table 2. Compounds investigated *in vitro* and *in vivo*

Chemical name	Abbreviation	Concentration (M)[a]	Delay min	Calculus experiment(s)
1. Trisodium methanecyclohexylhydroxydiphosphonate	MCHDP	2.0×10^{-5}	16	3
2. Disodium phenylaminomethanediphosphonate	PAMDP	3.1×10^{-5}	75	3
3. Trisodium ethane-1-hydroxy-1,1-diphosphonate	Na_3EHDP	3.5×10^{-5}	58	1,2
4. Disodium ethane-1-hydroxy-1,1-diphosphonate	Na_2EHDP	2.0×10^{-4}	Total	4,6,7,9,10
5. Pentasodium nitrilotri(methylenephosphonate)	Dequest	6.8×10^{-5}	Total	2
6. Trisodium dichloromethanediphosphonate	Cl_2MDP	5×10^{-5}	57	1
7. Tetrasodium condensate of EHDP	Cond II	5×10^{-5}	50	2
8. Disodium methanehydroxydiphosphonate	MHDP	6.0×10^{-5}	30	1
9. Hexasodium hexane-1,2,3,4,5,6-hexaphosphonate	HHP	5.1×10^{-5}	16	2
10. Tetrasodium pyrophosphate	PP	2×10^{-4} [b]	Total	5
11. Pentasodium tripolyphosphatehexahydrate	STP	2×10^{-4} [b]	Total	1
12. Pentasodium ethane-1-hydroxy-1,1,2-triphosphonate	E-1-HTP	1.5×10^{-4}	16	1
13. Disodium 1,2-dicarboxy-1-phosphonoethane	1,2-DPE	2.0×10^{-4}	110	3
14. Hexasodium propane-1,1,3,3-tetraphosphonate	PTeP	1.2×10^{-3}	Total	1
15. Disodium ethane-1,2-diphosphonate	E-1,2-DP	1.0×10^{-3}	130	2
16. Disodium phosphonoacetate	PAA	1.1×10^{-3}	80	2
17. Sodium citrate	Na citrate	1.0×10^{-3}	None	4
18. Sodium ethylenedinitrilotetraacetate	EDTA	2.4×10^{-3}	None	—
19. Distannous ethane-1-hydroxy-1,1-diphosphonate	Sn_2EHDP	not tested		2
20. Dicalcium ethane-1-hydroxy-1,1-diphosphonate	Ca_2EHDP	not tested		2
21. Disodium zinc ethane-1-hydroxy-1,1-diphosphonate	Na_2ZnEHDP	not tested		3
22. Tetrasodium ethane-2-carboxy-1,1-diphosphonate	E-2-CDP	not tested		2
23. Trisodium phosphate	Na_3PO_4	not tested		4
24. Linear polyphosphate ($Na_{13}P_{11}O_{34}$)	LPP	not tested		5

[a] Lowest concentration determined experimentally which produces total inhibition or a significant delay of at least 10 min in the crystal growth of forming hydroxyapatite as measured by the rate of bases uptake. Differences in time of delay are not considered significant only that a delay is effected.

[b] Hydrolyzed slowly.

Table 3. Experiment 1: Anti-calculus effect of compounds added to the diet. Length of study: 2 weeks. Diet: RC-16

Additive[a]	Mean CSSI score/rat	Reduction (%)	Mean CSI score/rat	Reduction (%)
0.25% Na_3EHDP	14.8	79.3	13.6	61.0
0.50% Na_3EHDP	18.9	73.6	18.1	48.1
0.25% Cl_2MDP	27.6	61.5	24.5	29.8
0.10% Na_3EHDP	32.4	54.7	23.9	31.5
0.25% MHDP	32.6	54.5	26.6	23.8
0.25% STP	34.9	51.3	26.8	23.5
0.25% E-1-HTP[b]	46.4	35.2	31.6	9.5
0.25% PTeP[b,c]	46.4	35.2	31.0	11.1
0.05% Na_3EHDP	52.0	27.4	32.2	−7.7
Control	71.6	—	34.9	—

Treatments within brackets are significantly different from those outside brackets at $\alpha = 0.05$.
[a] See Table 2 for abbreviations.
[b] Body weight gain significantly lower than control.
[c] Food intake significantly lower than control.

Table 4. Experiment 2: Anti-calculus effect of phosphonates added to the diet. Length of study 2 weeks. Diet: RC-16

Additive[a]	Mean CSSI score/rat	Reduction (%)	Mean CSI score/rat	Reduction (%)
0.19% Na_3EHDP	16.7	75.5	14.6	56.5
0.19% Sn_2EHDP	21.0	69.3	16.7	50.3
0.25% dequest	36.6	46.4	26.5	21.1
0.25% cond. II	36.8	46.1	27.0	19.6
0.25% E-2-CDP	38.8	43.1	27.1	19.3
0.25% HHP	42.1	38.4	28.5	15.2
0.25% PAA[b,c]	47.1	31.0	30.4	9.5
0.19% Ca_2EHDP	52.9	22.5	30.4	9.5
0.25% E-1,2-DP	66.0	3.4	33.6	0
Control	68.3	—	33.6	—

Treatments within brackets are significantly different from those outside brackets at $\alpha = 0.05$
[a] See Table 2 for abbreviations.
[b] Body weight gain significantly lower than control.
[c] Food intake significantly lower than control.

tent of Na_3EHDP from 0.25 to 0.1 to 0.05%. All three concentrations gave significantly different calculus reductions from each other and all three produced a significant reduction compared to the control diet. There was no significant difference between the two highest levels of Na_3EHDP, 0.5 and 0.25%.

Table 5. Experiment 3: Anti-calculus effect of phosphonates added to the diet or applied topically. Length of study, 2 weeks. Diet: RC-16

Compound tested[a]	Application method	Mean CSSI score/rat	Reduction (%)	Mean CSI score/rat	Reduction (%)
0.12% PAMDP[b]	Diet	4.6	88.1	3.8	85.6
0.14% MCHDP[b]	Diet	9.2	76.2	8.2	68.9
0.10% Na_2EHDP	Diet	9.3	75.9	7.5	71.6
0.07% MCHDP	Diet	19.1	50.5	14.5	45.2
0.06% PAMDP	Diet	19.2	50.3	14.1	46.9
0.25% 1,2-DPE	Diet	21.5	44.4	15.5	41.4
0.05% Na_2EHDP	Diet	23.0	40.4	17.0	35.9
1.5% Na_2Zn EHDP	Topical	25.6	33.7	20.8	21.4
Water	Topical	38.6	—	26.4	—

Treatments within brackets are significantly different from those outside brackets at $\alpha=0.05$.
[a] See Table 2 for abbreviations.
[b] Body weight gain significantly lower than control.

E-1-HTP and PTeP produced significant reductions in calculus but were less effective on an equal weight basis than Na_3EHDP, Cl_2MDP, MHDP or STP as could be anticipated from the *in vitro* data in Table 2.

The data in Table 4 show that the most effective compounds were Na_2EHDP and Sn_2EHDP, PAA and Ca_2EHDP were only minimally effective, while E-1,2-DP was the only compound not significantly different from the control. This agrees well with the ranking in Table 2. In Table 5, PAMDP, MCHDP and Na_2EHDP at two equimolar levels in the diet were all highly effective anti-calculus agents and were not different from one another at equal molar concentration levels. As in Table 3, however, the higher concentration level produced the greater reduction in calculus. The Na_2ZnEHDP, although it produced a significant reduction topically, was the least effective. 1,2-DPE was the least active dietary additive, as determined by the higher level required in the diet to produce an effect comparable to the other compounds.

Of the three compounds applied topically at concentrations of 1.5% (Table 6) only Na_2EHDP was effective, producing a 52.4 and 29.9% reduction by CSSI and CSI scorings, respectively. There was no significant effect from sodium phosphate or sodium citrate. A comparison of the condensed phosphates is shown in Table 7. All three compounds produced a significant reduction in calculus of approximately 38 and 11% by CSSI and CSI scores, respectively.

Reproducibility of Calculus Grading and Calculus Incidence

The two experiments on grading reproducibility are presented in Tables 8 and 9. Four graders in the two experiments ranked treatments similarly and there was no significant difference among graders within experiments and across experiments (Graders 1 and 2). In Experiment 6 (Table 8), 0.5% Na_2EHDP given in the RC-15

Table 6. Experiment 4: Anti-calculus effect of agents applied topically. Length of study, 2 weeks. Diet: RC-16

Solution applied[a]	Mean CSSI score/rat	Reduction (%)	Mean CSI score/rat	Reduction (%)
1.5% Na_2EHDP	34.9	52.4	24.5	29.9
1.5% Na_3PO_4	65.1	11.2	32.1	38.3
1.5% Na citrate	71.3	2.7	34.5	1.5
Water	73.3	—	35.0	—

Treatments within brackets are significantly different from those outside brackets at $\alpha=0.05$.
[a] See Table 2 for abbreviations.

Table 7. Experiment 5: Anti-calculus effect of phosphates applied topically. Length of study, 3 weeks. Diet: RC-15

Solution applied[a]	Mean CSSI score/rat	Reduction (%)	Mean CSI score/rat	Reduction (%)
1% PP	45.4	38.2	33.1	11.7
1% LPP	45.4	38.2	34.2	10.9
1% STP	44.1	39.9	33.9	11.7
Water	73.4	—	38.4	—

Treatments within brackets are significantly different from those outside brackets at $\alpha=0.05$.
[a] See Table 2 for abbreviations.

diet produced a calculus reduction of 89% (CSSI) and 84% (CSI) using two graders while 1% Na_2EHDP applied topically produced a reduction of 62% (CSSI) and 39% (CSI) using three graders. In Experiment 7 (Table 9), 0.25% Na_2EHDP given in RC-16 diet produced a calculus reduction of 88 and 83% (CSSI and CSI, respectively). As reported previously (Francis and Briner, 1969), there was a significant difference in calculus production between the two diets RC-15 and RC-16 with the acid phosphate salt (NaH_2PO_4) in diet RC-16 inducing more calculus than the basic salt (Na_2HPO_4) in diet RC-15.

Rate of Deposition of Calculus

The calculus severity (CSSI) and incidence (CSI) scores plotted by the method of least squares (see Fig. 1 for CSSI plot) revealed that there was a steady rise in CSSI score from day 3 on diet RC-15 through day 18. There was no significant rise in deposition or accumulation of calculus after day 18 indicating a maximum accumulation of calculus (CSSI score 62) had been reached. The penicillin supplemented diet produced significantly lower calculus scores but calculus did increase significantly as follows: 7 days CSSI-3.2, CSI-2.7; 14 days CSSI-7.0, CSI-6.0; 21 days CSSI-12.6, CSI-12.0.

Table 8. Experiment 6: Reproducibility of grading. Length of study, 3 weeks. Diet: RC-15

Compound tested	Application method	Grader No.	Mean CSSI score/rat	Reduction (%)	Mean CSI score/rat	Reduction (%)
Control	—	1	63.9	—	38.3	—
0.5% Na_2EHDP	Diet	1	7.7	87.9	7.1	81.6
Water	—	1	78.3	—	40.1	—
1.0% Na_2EHDP	Solution	1	29.0	62.9	24.7	38.2
Control	—	2	63.9	—	39.0	—
0.5% Na_2EHDP	Diet	2	6.2	90.3	5.3	86.5
Water	—	2	73.9	—	37.4	—
1.0% Na_2EHDP	Solution	2	26.6	64.1	20.2	46.0
Water	—	3	73.9	—	39.6	—
1.0% Na_2EHDP	Solution	3	29.5	60.1	26.4	33.2

Treatments within brackets are significantly different from those outside brackets at $\alpha=0.05$.

Table 9. Experiment 7: Reproducibility of grading and comparison of diets RC-15 and RC-16. Length of study 2 weeks. Diets: RC-15, RC-16

Diet	Additive	Grader No.	Mean CSSI score/rat	Reduction (%)	Mean CSI score/rat	Reduction (%)
RC-16	0.25% Na_2EHDP	1	2.5	95.9	2.4	92.7
RC-15	—	1	38.4	—	25.0	—
RC-16	—	1	60.3	—	33.0	—
RC-16	0.25% Na_2EHDP	2	6.7	88.9	5.6	83.6
RC-15	—	2	38.3	—	27.1	—
RC-16	—	2	60.3	—	34.2	—
RC-16	0.25% Na_2EHDP	4	9.7	78.3	8.5	72.7
RC-15	—	4	31.1	—	23.7	—
RC-16	—	4	44.7	—	31.1	—

Treatments within brackets are significantly different from those outside brackets for each grader at $\alpha=0.05$.

Systemic Effect of Na_2EHDP

The mean CSSI score for the Na_2EHDP-treated rats (subcutaneously) was 62.6; the score for the control rats was 65.3. The difference was not significant at $\alpha = 0.05$. The CSI results were similar in showing no difference between treated and control rats.

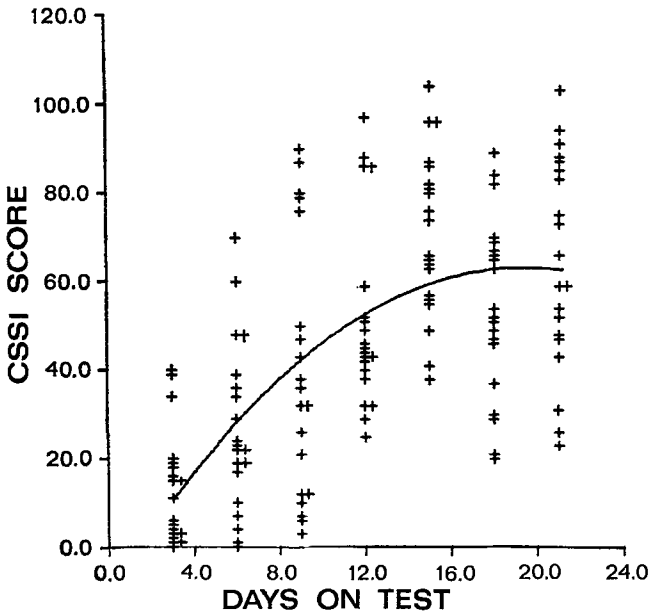

Fig. 1. Graphical representation of the increase in calculus surface severity index (CSSI) score as a function of time on calculus diet RC-15 (Table 1)

Residual Anti-Calculus Effect in Offspring of Rats Fed Na_2EHDP

The mean CSSI score of the group on RC-16 diet whose parents and grandparents had received the 0.5% Na_2EHDP supplemented diet was 82.9; the chow diet control group placed on RC-16 was 73.4. The difference was not significant at $\alpha = 0.05$. The CSI results were similar in showing no difference between the supplemented and control (chow) groups.

Discussion

Crystal Growth Inhibition and Anti-Calculus Effects

All the gem diphosphonates and the vicinal polyphosphonates where more than two adjacent carbon atoms had phosphonate groups (e.g., propane-1,2,3-triphosphonic acid[1] and butane-1,2,3,4-tetraphosphonic acid[1], not shown in Table 2, and hexane-1,2,3,4,5,6-hexaphosphonic acid, HHP — Table 2), were effective crystal growth inhibitors. The vicinal diphosphonate, E-1,2-DP, was a very poor crystal growth inhibitor and produced a delay of 130 min only after the concentration was raised to 1×10^{-3} M. This is 30 to 50 times the concentration required to produce a delay by MCHDP (MCHDP produced a delay of 300 min at 3×10^{-5} M and 16 min at 2×10^{-5} M). From construction of molecular models of the vicinal phosphonates, the most probable reason for the loss of crystal growth effect of the E-1,2-DP molecule is the free rotation and lack of proximity

1 Note: These two compounds were effective crystal growth inhibitors at 2×10^{-4} M but were not investigated at lower concentration to determine minimal effective level and therefore were not put in Table 2.

of the phosphonate groups in this molecule. In the polyvicinal molecules, there is strong hindered rotation and forced closer proximity of adjacent paired phosphonate groups and hence favorable configuration for adsorption on HA exists. Current work involves comparison of cis- and trans-vicinal phosphonates to shed more light on this postulation.

There is a definite correlation between crystal growth inhibitor properties and anti-calculus effectiveness as seen from the order of compounds in Table 2 and anti-calculus effectiveness (Tables 3–4). The HA crystal inhibition effect on depositing calculus *per se* when Na_3EHDP and Na_3MDP were included in the diet has previously been shown by electron microscopy (Francis and Briner, 1973). It is clear, also, that chelation or complexation of calcium is not the mechanism of action since neither sodium citrate nor sodium ethylenedinitrilotetraacetate (Table 2) produced a significant crystal growth-inhibition effect even at the highest concentrations tested. In addition, the latter materials are relatively damaging to polished enamel whereas the di- and polyphosphonates are not (Francis and Briner, 1973). The crystal growth inhibiting and anti-calculus properties of the linear polyphosphates (Tables 2 and 7) are in good agreement with previously reported work on transformation of amorphous to crystalline calcium phosphate (Fleisch *et al.*, 1968; Francis, 1969) and on inhibition of calculus formation *in vitro* (Draus, 1970). Vogel and Amdur (1967) found that higher levels of pyrophosphate in saliva corresponded to lower levels of calculus formation in adults. Pyrophosphate and linear polyphosphates, however, are easily hydrolyzed, chemically and enzymatically, whereas the phosphonates are very stable.

Interpretation of calculus data was complicated for two compounds PTeP and PAA (Tables 3 and 4) by the lower food consumption in animals fed these compounds. The lower food intake (less frequent contact with the strong calculus inducing diet) could be the reason these compounds appeared to have an anti-calculus effect (lower calculus) in Experiments 1 and 2 although the data in Table 2 suggest they should not be as effective as the other materials tested.

Reproducibility of Calculus Grading

The results were remarkably consistent among graders. Graders in both experiments ranked treatments in the same order and the difference among graders was not significant at $\alpha = 0.05$ even though each grader evaluated a similarly treated, but not identical group.

Rate of Deposition of Calculus

During the first twelve days, initiation of calculus (CSI) and growth of "already initiated" calculus occurred at a relatively constant rate. After this time, the rates of initiation and growth slowed with CSI and CSSI scores reaching a maximum at about eighteen days with no significant change thereafter. On the basis of these data, a two-week experimental period was selected for some experiments realizing that calculus production was probably not quite maximum at this time, but this disadvantage was outweighed by the convenience of the shorter time period.

Inclusion of 1% penicillin in the diet reduced calculus significantly. Since penicillin is effective mainly against gram-positive microorganisms and neisseria, it seems likely that these organisms were involved in calculus formation in our rats. Earlier inoculation studies using neisseria in rats failed to enhance calculus production (Francis and Briner, 1969), but the work of Baer *et al.* (1968) suggests that bacteria have a large role in the etiology and transmissability of calculus.

Systemic and Residual Anti-Calculus Effects of Na_2EHDP

Na_2EHDP administered subcutaneously had no significant effect on calculus production indicating that the anti-calculus effects observed in the preceding experiments (1–7) were due primarily to a topical effect. The lack of an effect of Na_2EHDP in F_2 generation feeding (Experiment 10) was not surprising in view of the topical effect described. If, indeed, EHDP was accumulated in the fetus *in utero* the compound was not mobilized after birth and secreted in sufficient amount in the saliva to affect accumulation of calculus. Since the anti-calculus effect appears to be exclusively topical, the phosphonate compounds have potential as anti-calculus agents for human use. One of these compounds, Na_2EHDP, has been shown to be efficacious for human calculus prevention in clinical trials by Sturzenberger *et al.* (1971) and Conroy *et al.* (1972).

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