Effect of Additives on the Rate of Dissolution of Hydroxyapatite in Unstirred Acid Buffers

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A preliminary study was done on the role of some agents in inhibiting the demineralization of tooth enamel. The rate of dissolution of pressed pellets of hydroxyapatite was measured in unstirred acid buffers that contained various additives. Among several sucrose phosphates that reduced dissolution rates, the calcium salt appeared most valuable as a cariostatic agent in human beings.

Tooth enamel consists essentially of oriented crystals of hydroxyapatite embedded in an organic matrix, covered at the surface by cuticle and plaque.[1] One of the early stages in the formation of a carious lesion involves the demineralization of hydroxyapatite in the enamel. Therefore, an agent that can reduce the rate of dissolution of hydroxyapatite when the teeth are subject to demineralization may have some inhibiting effect on caries formation.

In these initial studies, for partial simulation of tooth enamel, the organic matrix, cuticle, and plaque were disregarded in favor of understanding the kinetics of relatively simple systems. With this background of information established, the degree of complexity of the systems could be increased.

The aim of the present experiments, which preceded similar investigations with stirred acid buffers,[2,3] was to study the influence of additives, in particular the sucrose phosphates, on the rate of dissolution of hydroxyapatite in unstirred acid buffers.

Materials and Methods

Enamel structure was partially simulated by the use of synthetic hydroxyapatite pellets. These pellets were prepared by subjecting hydroxyapatite powder (0.23 gm) in a die to a pressure of 5,000 atmospheres. Although pellets were probably more porous than is dental enamel, they were of sufficient mechanical strength to be readily handled.

A sample of synthetic hydroxyapatite,* 1.53M±0.05M Ca/P, has a well-defined apatitic X-ray diffraction pattern.[4,5] It appears to be a calcium-deficient hydroxyapatite, somewhat similar to that which occurs in tooth enamel.[6,7] Radiography of the pellets, and of pellets ground to a powder, showed that the hydroxyapatite remained essentially unchanged during production, although the particles of hydroxyapatite were strongly oriented within the pellet.

The peripheral edges of the pellets tended to be friable. A thin coating of a cold-curing epoxy resin was therefore placed around the periphery of each pellet, to overlap slightly onto each pellet face. The resin was cured for 24 hours at 105C prior to use. The two pellet faces, designated top and bottom, differed slightly when observed by reflected light. The top face of each pellet was always exposed to the solution. The macroscopic surface area exposed was between 1.02 to 1.08 sq cm, determined by a planimeter.

DISSOLUTION VESSELS.—The four dissolution vessels were cylindrical glass containers, 100-ml capacity, maintained at 37.0C±0.1C. An L-shaped pellet holder was supported by the lid of the vessel. A pellet was fitted neatly into a small recess in the top of the projecting arm so that it was centrally situated about the vessel's axis. A rubber gasket, attached to the lower face of the lid, prevented evaporation losses and ensured that the position of the pellet holder was identical in every experiment. The vessels were mounted on foam rubber to minimize extraneous vibration effects. All experiments were performed with unstirred systems. Although this probably simulated conditions that exist near a carious lesion, it introduced certain difficulties.

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The most serious difficulty associated with unstirred systems was the ambiguity in the interpretation of the results, compared with results obtained with stirred systems.[3]

A second difficulty was that the time required to measure the kinetics was increased by the reduced dissolution rate and the necessity for determining each point separately on the rate curve. This was partially overcome by lowering the pH and by using several dissolution vessels.

A third difficulty was that extraneous vibrations, when transmitted to the bulk of the solution, resulted in appreciable, undesirable movement of the liquid. Moreover, both thermal convective and, to a lesser degree in this system, density gradient effects could assume significant proportions. The net result of the convective and kinetic movement of the solution was to render the initial dissolution rate inaccessible to direct experimental observation.

Consequently, it was decided to measure the rate of dissolution of hydroxyapatite after various time intervals, and to extrapolate the results to zero time. This procedure is admittedly open to criticism, but seems to represent the best compromise that can be reached for this system.

BUFFER SOLUTION.—The equilibrium solubility of hydroxyapatite is greatly increased by lowering the pH. However, values less than pH 4 probably do not occur in the mouth.[3] This set a lower limit on the preliminary pH range to be studied; the upper limit was the pH of saliva (6.5 to 7.5). These initial experiments were conducted at pH 4. The buffer consisted of a solution of 0.164M acetic acid and 0.036M potassium acetate. The pH of the solution at 37C was 3.97 ± 0.05.

Physiologic ionic strength is 0.165,[8] which is unsuitable for interpretative experiments.[9] Addition of ionic additives may appreciably change the ionic strength of the solution, and thus alter the activity coefficients of the dissolving species. The solution used in these studies was 0.5M with respect to potassium chloride. (Addition of up to 1% of most ionized salts will change the total ionic strength by less than 10%.)

Potassium salts were used in this and in later experiments.[2,3] Potassium appears to have little tendency for preferential exchange with the calcium ions in the hydroxyapatite lattice,[3] in contrast with sodium ions.[7]

All experiments were conducted at body temperature, 37C.

DESCRIPTION OF ADDITIVES.—*CaSP, CSRC-6.*—A normal production batch of a calcium sucrose phosphates-calcium orthophosphate complex association was used. It corresponded to composition A in a published patent specification.[10]

CaSP, Inorganic Free.—This fraction was prepared from the preceding product as described in composition C in the patent specification.[10] The electrophoretic pattern and analysis showed it to be similar to the product obtained by Neuberg,[10] and it consists predominantly of component 2.

CaSP, Fraction B.—This fraction was prepared from CaSP, CSRC-6, by chromatography on an ion exchange resin.* It consisted predominantly of component 2 and was similar to the product obtained by Neuberg.[10] It contained little inorganic phosphate.

CaSP, Fraction A.—This was the other main fraction prepared from CaSP, CSRC-6, by chromatography on resin. It consisted predominantly of components 3 and 4 and contained some inorganic calcium phosphate.

K_xSP, Fraction A.—The potassium salt made from CaSP, fraction A, by ion exchange was electrophoretically similar to CaSP fraction A.

K_xSP, Fraction B.—The potassium salt made from CaSP, fraction B, by ion exchange was electrophoretically similar to CaSP fraction B.

K_xSP.—The potassium salt was made from CaSP (CSRC-6) by ion exchange. In the process it loses some inorganic phosphate but does not differ significantly from the electrophoretic pattern of CSRC-6 CaSP.

K_xSP, Inorganic Free.—The potassium salt made from inorganic-free CaSP by ion exchange was electrophoretically similar to inorganic-free CaSP.

Neuberg Product.—A calcium sucrose phosphate product was made by the original Neuberg process described under composition B (patent specification).[10] It was not specifically tested as an additive here, but as can be seen from the data, it is similar to inorganic-free CaSP.

* Dowex 50W, X-4, Bio-Rad Laboratories, Richmond, Calif.

Toy's Product.—A calcium sucrose phosphate was made by the addition of disodium hydrogen phosphate to the reaction mixture for the preparation of CaSP. It corresponded to composition D.[10] It was not used as an additive in this work, but is included because it has been used in other studies that will be reported.

Pyridine-P_2O_5 Product.—This product was obtained by phosphorylating sucrose with phosphorus pentoxide in a pyridine solution. It is included for comparison with the product made by phosphorylation in aqueous solutions.

The analysis of some of these products is shown (Table 1). The electrophoretic patterns also are shown (Fig. 1, 2).

Electrophoresis was carried out in a buffer that consisted of 5% pyridine and 0.5% glacial acetic acid in water (pH 6), using Whatman No. 54 paper*, under a voltage gradient of 16 volts/cm. Time for separation was about two hours. The position of the components was indicated by development with molybdate.

The electrophoretic pattern obtained under these conditions with CaSP consisted of five bands. Starting from the bottom of the paper, there was a faint band (component 1) believed to be a disucrose monophosphate. The next band was a major component (component 2) identified as sucrose-2-monophosphate. Third was a minor component (component 3), a sucrose monophosphate that has not been positively identified. The fourth band was a major component (component 4) and was believed to be a sucrose diphosphate. The last band, the strongest with CaSP, was inorganic orthophosphate.

PROCEDURE.—The dissolution medium was heated in sealed polythene containers at ca 50C for ten minutes. This removed enough dissolved air to prevent bubble formation in the dissolution vessel on equilibration to 37C. The solution (90.0±0.5 ml) was rapidly transferred to the dissolution vessel and equilibrated to 37C (ca 20 minutes).

The pellet, of known dry weight, was then immersed with the pellet holder into the solution. The porosity of the pellet resulted in bubble formation on the upper surface. The bubbles were readily removed by passing the pellet through the air-liquid interface.

The pellet dissolved for 6 to 65 hours. On removal from solution, the surface was carefully washed by immersion in distilled water, and the pellet dried to constant weight at 105C for one hour. The weight loss was then calculated.

After drying to constant weight, the pellet was dissolved in hydrochloric acid. The total weight of potassium in the pellet was determined by atomic absorption spectrophotometry. Thus, the weight of buffer solids trapped in the pellet on drying could be calculated. The true decrease in weight was then obtained by adding the weight of the included buffer solids to the observed weight loss. The pH of the dissolving solution was remeasured, and the solution was analyzed for calcium and inorganic phosphate ions.

The results were all corrected at 3% or less to a standard surface area of 1.05 sq cm, by assuming a direct proportionality between the measured surface area and the weight loss. The total weight loss was determined for a given set of dissolution conditions for four or five different time intervals. Each point on the dissolution curve required a completely separate experiment.

In the presence of additives, the procedure was identical with that in the preceding description. However, the additives often altered the pH of the standard solution by as much as 0.3 pH units. The pH of the dissolution medium was always adjusted to 3.97±0.05 by the addition of ca 0.1 ml of 9N hydrochloric acid or 9N potassium hydroxide. The presence of a high concentration of potassium chloride in the solution blanketed any influence of added acid or base.

Standard curves (ie, without the addition

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TABLE 1

ANALYSIS OF ADDITIVES

Additive	% Ca	% P Total	P, % Inorganic
CaSP, CSRC-6	12.5	9.3	2.8
CaSP, inorganic free	6.9	7.0	0.2
CaSP, fraction A	6.2	7.1	0.5
CaSP, fraction B	7.8	6.3	0.005
K_2SP, fraction A	0.0	7.5	0.6
K_2SP, fraction B	0.0	6.8	0.01
K_2SP	0.0	7.0	0.7
K_2SP, inorganic free	0.0	7.3	0.2
CaSP (Neuberg product)*	8.1	6.8	0.05

* Included for comparison.

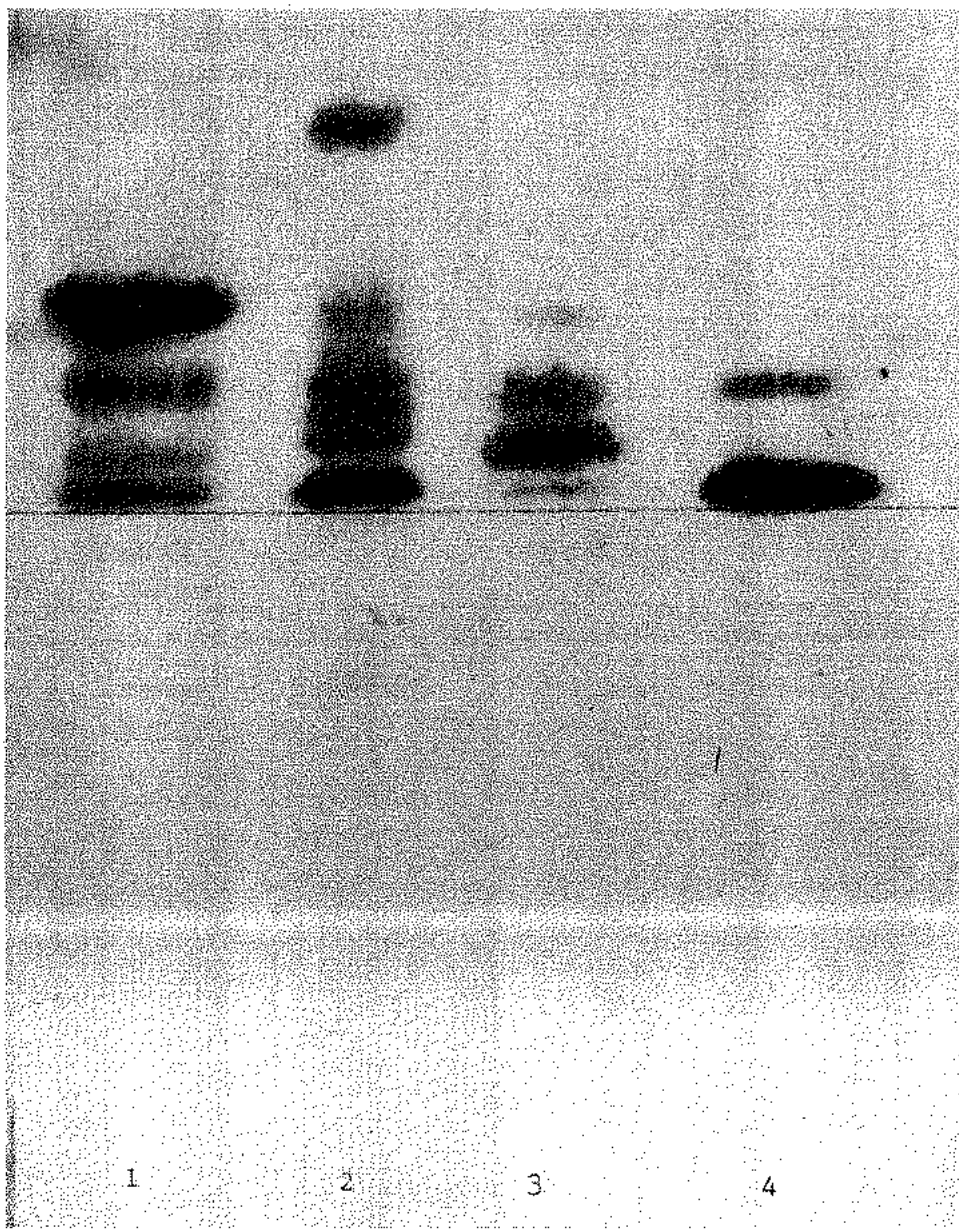

FIG 1.—Electrophoretic pattern of various sucrose phosphate products. *1*, CaSP (CSRC-6); *2*, pyridine- P_2O_5 product; *3*, CaSP fraction A; *4*, CaSP fraction B.

of additives) were obtained by duplicating each point. However, subsequent curves were not duplicated point by point because each point, except for the longest, checks the preceding point.

Initially, it was necessary to demonstrate that the sucrose phosphate anion did not undergo hydrolysis during the dissolution experiments. A 0.5% solution of CaSP fraction B was allowed to stand under standard conditions in a vessel for 65 hours, and the solution was subsequently analyzed for inorganic phosphate and fructose.

To observe what proportion of the reduc-

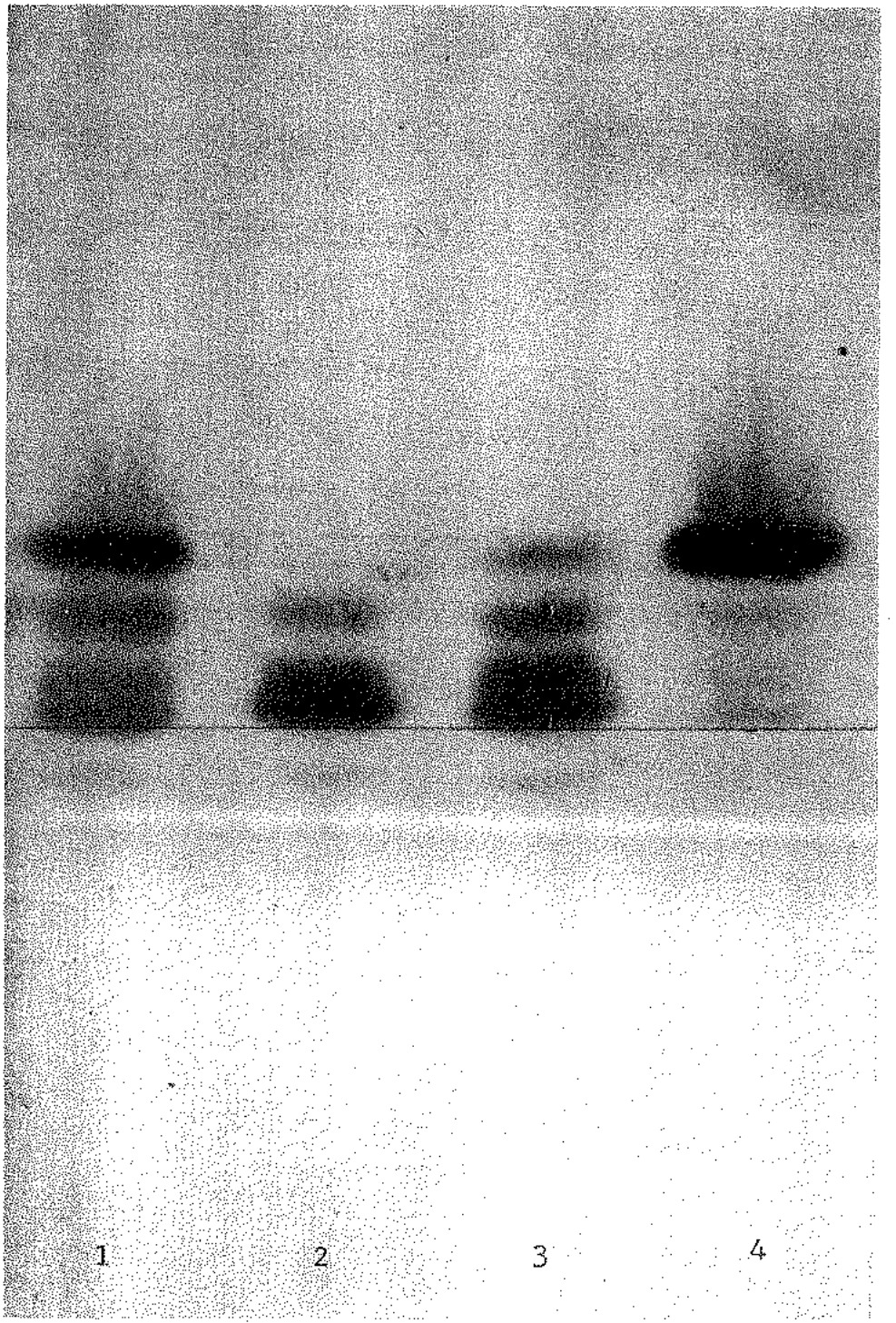

FIG 2.—Electrophoretic pattern of various sucrose phosphate products. *1*, CaSP, CSRC-6; *2*, CaSP, Neuberg product; *3*, CaSP, inorganic free; *4*, CaSP, Toy's product.

tion in the hydroxyapatite dissolution rate obtained with CaSP fraction B was attributable to the common ion effect of the calcium which it contains, the effect of $9.79M\times10^{-3}$ $CaCl_2$ (the concentration of calcium in 0.5% solution of CaSP fraction B) on the rate of dissolution was determined. Similarly, to observe the effect of inorganic phosphate contained in some of these products, the effect of $9.79M\times10^{-3}$ KH_2PO_4 was determined. A lower concentration, $8.45M\times10^{-4}$ KH_2PO_4, which corresponded to the inorganic phosphate level in a 0.5% solution of CaSP fraction A, also was studied.

MASS BALANCE AND REPRODUCIBILITY.—The analysis of the hydroxyapatite showed that the weight loss, ΔW_A, may be calculated from the analyses of the dissolution medium by means of the formula

$$\Delta W_A(gm) = 3.79\ (\Delta[Ca] + 9.0\ (\Delta[P])$$

where $\Delta[Ca]$ and $\Delta[P]$ represent the change in the molar concentration of calcium and inorganic phosphorus in the dissolution medium. For stoichiometric dissolution, $\Delta[Ca]/\Delta[P]$ is 1.53, and thus $\Delta W_A\ (gm) = 9.73\ (\Delta[Ca])$.

Results

Results of mass balance calculations for five experiments show that the weight loss, ΔW_B, determined directly, and ΔW_A agree to at least 5%, which is the probable accuracy of the methods used.

The duplication of points for the standard curves has shown that the reproducibility of the weight loss for any given vessel is about 5%. The reproducibility of experimental results was reasonable in any one vessel. However, under identical dissolution medium conditions, differences as large as 20% were observed from vessel to vessel. In this study, the relative, and not the absolute, rates were of major interest.

Standard curves were obtained for each vessel in the absence of additives. Under the standard conditions, the $\Delta[Ca]/\Delta[P]$ observed for pellet dissolution was always identical with the $[Ca]/[P]$ for the hydroxyapatite (ie, 1.53 ± 0.05). Thus, stoichiometric dissolution of hydroxyapatite always occurred. The pH change observed in the dissolution medium was less than 0.2 pH units, and thus may be neglected.

RATES OF DISSOLUTION.—The rates of dissolution of hydroxyapatite pellets obtained in the four different vessels, both in the absence and presence of various additives, are shown (Fig 3-6). These curves of weight loss versus times are nonlinear; the rate of dissolution decreased with time. These rates of dissolution were similar to those of human dental enamel under the same experimental conditions.

From these data, approximate initial

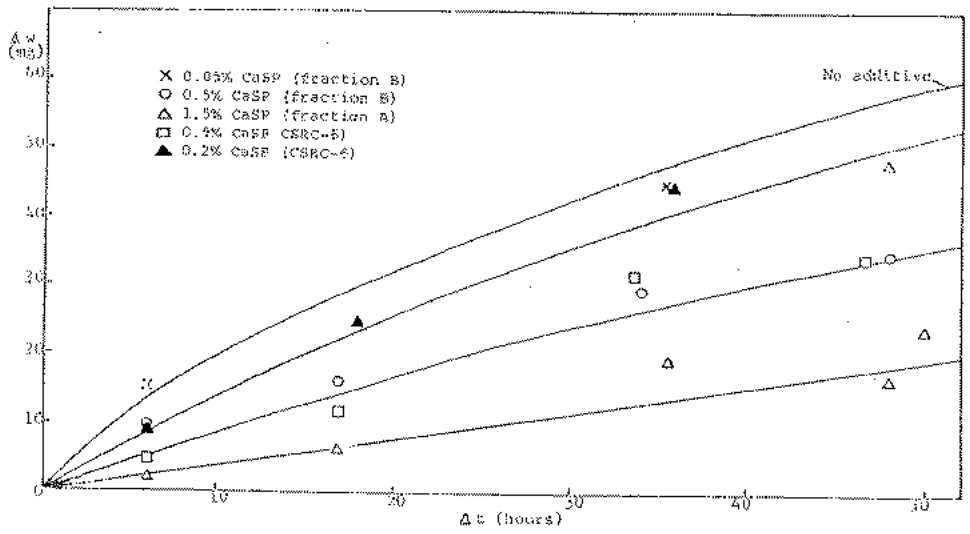

FIG 3.—Rates of dissolution in vessel 1. *Top curve*, no additive.

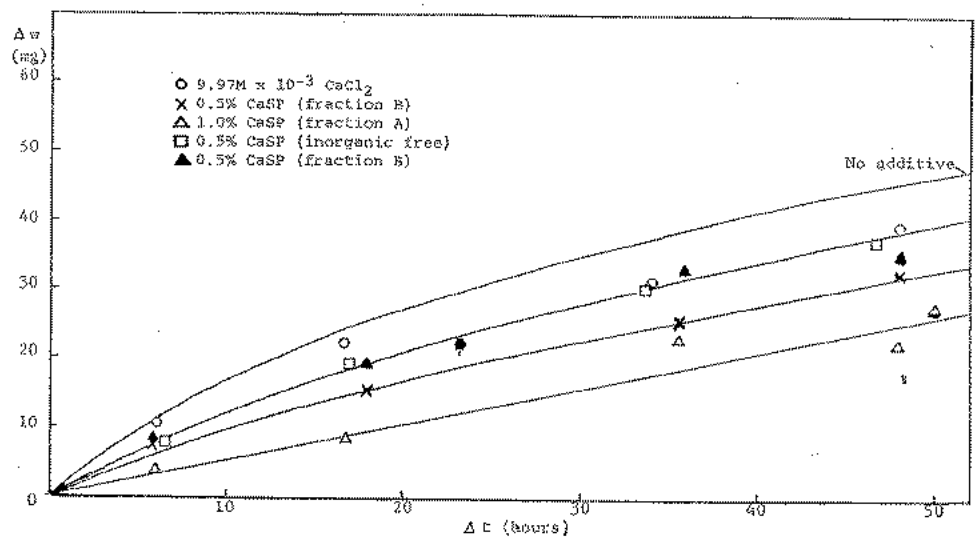

FIG 4.—Rates of dissolution in vessel 2. *Top curve*, no additive.

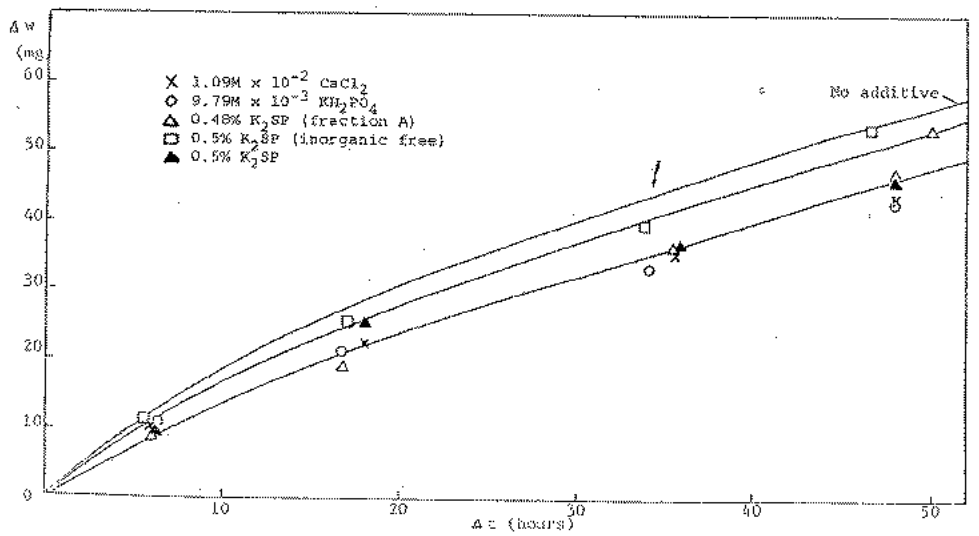

FIG 5.—Rates of dissolution in vessel 3. *Top curve*, no additive.

rates of dissolution in the presence of the additive can be obtained by extrapolation. In Table 2, these are expressed as a percentage of the initial rate of dissolution in the absence of any additive for each vessel.

These results show that all calcium sucrose phosphates at a concentration of 0.5% significantly reduce the rate of dissolution of hydroxyapatite. The commercial product, CaSP CSRC-6, thus appears to be more effective than the various fractions obtained from it.

Analyses of the buffer solution that contained 0.5% CaSP fraction B showed that hydrolysis of the calcium sucrose phosphate was negligible after 65 hours.

Results obtained in the presence of calcium chloride and potassium dihydrogen phosphate showed that the percentage reductions in dissolution rate obtained in the presence of the various calcium sucrose phosphate additives were only in part attributable to the calcium and inorganic phosphate that they contained.

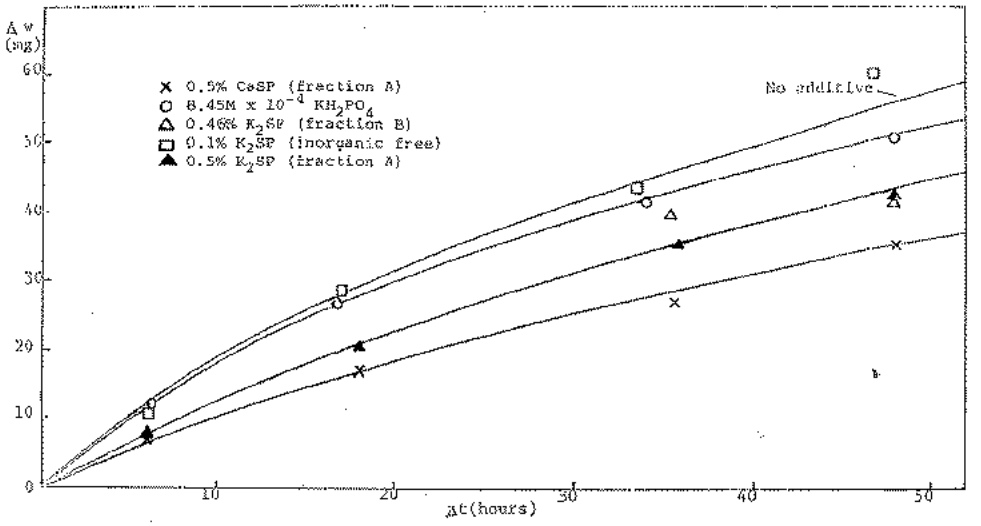

FIG 6.—Rates of dissolution in vessel 4. *Top curve,* no additive.

TABLE 2

EFFECT OF ADDITIVES ON THE INITIAL RATE OF DISSOLUTION OF HYDROXYAPATITE PELLETS UNDER UNSTIRRED CONDITIONS

Concentration	Additive	% of the Initial Rate with No Additive
....	None	100
0.2%	CaSP, CSRC-6	65
0.5%	CaSP, CSRC-6	35
0.05%	CaSP, fraction B	100
0.5%	CaSP, fraction B	55
0.5%	CaSP, fraction B	65
0.5%	CaSP, fraction B	55
0.5%	CaSP, inorganic free	65
0.5%	CaSP, fraction A	55
1.0%	CaSP, fraction A	30
1.5%	CaSP, fraction A	20
0.1%	K_2SP, inorganic free	100
0.5%	K_2SP, inorganic free	90
0.5%	K_2SP	70
0.46%	K_2SP, fraction B	75
0.5%	K_2SP, fraction A	65
0.48%	K_2SP, fraction A	70
$8.45M \times 10^{-4}$	KH_2PO_4	90
$9.79M \times 10^{-3}$	KH_2PO_4	80
$9.79M \times 10^{-3}$	$CaCl_2$	90
$1.09M \times 10^{-2}$	$CaCl_2$	80

The various potassium sucrose phosphates at a concentration of 0.5% also reduced the initial rate of dissolution of hydroxyapatite under these conditions. However, the effect was not as pronounced as with the calcium salts. As with the calcium salts, the decrease in rate was greater in those materials containing the higher levels of inorganic phosphate.

An interesting finding during these experiments concerned the difference in the appearances of the dried pellets at the end of an experiment, after partial dissolution. In the absence of additives, the dried surfaces were heavily scored. The presence of the sucrose phosphates in the dissolution medium gave rise to pellets with surfaces that, after drying, were scarcely cracked and had a distinct sheen.

Discussion

Although these results on the influence of additives on the rate of dissolution of hydroxyapatite under unstirred conditions are of a pragmatic nature, certain general features are apparent.

The dissolution of hydroxyapatite involves the release of the hydroxyl ion. Thus, it is necessary to buffer the dissolving solution against pH changes, and so obviate pH effects. Lactate ions probably play an important role in buffering human saliva.[3] However, acetate ions were preferred in these initial studies because of their weaker calcium-chelating properties.

The dissolution of hydroxyapatite pellets both in the absence and presence of certain additives was stoichiometric. This would be expected in a system that is greatly undersaturated with respect to the dissolving species. Also as expected, the addition of calcium or orthophosphate ions reduced the degree of undersaturation in the buffer solu-

tion and concomitantly reduces the rate of dissolution. Their effect appeared to be less pronounced at the beginning of the experiment.

During the progress of dissolution under these static conditions, a concentration gradient would be expected to be progressively built up from the dissolving face of the pellet into the bulk of the solution. Apparently, under these conditions, the rate of mass transfer of the ionic species away from the pellet face becomes the effective rate-controlling step. However, the failure of the data to obey the first-order Nernst equation,[11] describing diffusion-controlled dissolution, was probably because the unstirred solution did not attain uniform solute concentration. This solute nonhomogeneity was readily demonstrable by sampling of the buffer solution. In stirred systems,[2,3] the disengagement of ions from the surface of the pellet become important in controlling the rate of dissolution.

It appears likely that the present experimental conditions provide a changing situation with respect to the relative contributions of ion disengagement and mass transfer. In the initial stages, the rate of ion disengagement could influence the rate of dissolution to a greater extent than at larger time intervals, when a greater diffusion barrier has been built up. This could be a reason why the sucrose phosphates, which are adsorbed on the hydroxyapatite surface and retard the ion disengagement step,[3] have a greater effect on the initial dissolution rate than do calcium and orthophosphate.

Obviously, the kinetics of dissolution of hydroxyapatite under these unstirred conditions are more complex and less amenable to quantitative treatment than under stirred conditions. Under the former conditions, the kinetics would be expected to be markedly dependent on the geometry of the system, which probably accounts for the differences in the experimental results in different vessels.

Nevertheless, these experiments demonstrate that calcium ions and phosphate ions, both inorganic and organic, when present in sufficient concentrations are capable of inhibiting the rate of dissolution of hydroxyapatite in unstirred acid buffers.

The particular advantage of the calcium sucrose phosphates in this role lies in the fact that these salts, unlike the calcium salts of many other phosphates, are extremely soluble in water at all pH values. Moreover, they are also capable of forming soluble complex associations with other phosphates such as the calcium phosphates which are normally sparingly soluble at physiological pH values. In solution, such compositions provide calcium, inorganic phosphate and sucrose phosphates, all of which can inhibit the rate of dissolution of hydroxyapatite.

Laboratory tests which have been carried out on a number of sugar phosphate salts as cariostatic additives to the diet of rats have indicated that these substances can inhibit dental caries in animals.[12] The results of laboratory tests such as those described in this report have been used to select a sugar phosphate product which would be expected to be most effective in reducing dental caries in man. The first year of a clinical trial to test calcium sucrose phosphates (corresponding to CaSP [CSRC-6]) as a cariostatic additive to carbohydrate foods in man has shown that a reduction of about 25% was obtained in the number of new decayed, missing or filled tooth surfaces in the group of children receiving the additive.[13,14]

Conclusions

These experiments demonstrate that sucrose phosphates, particularly when present as the calcium salt in a soluble, complex association with inorganic calcium phosphate, are capable of inhibiting the rate of dissolution of hydroxyapatite in unstirred acid buffers. This suggests that these substances, when added to carbohydrate foods, could well inhibit the demineralization of tooth enamel.

In combination with other in vitro experiments, and a clinical trial, these results provide encouraging evidence for the value of calcium sucrose phosphates as cariostatic agents in man.

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