with a safety-razor 24 h before the treatment. 0.1 ml of an ethanol solution of 8-MOP or of angelicin, at the concentration of 5×10^{-3} mol $\times$ l^{-1}, was applied to an area of skin within a polyethylene ring of 1.7 cm diameter. The solute was evaporated with a stream of air for 1-2 min[6]. Ethanol was applied to the control skin areas. The surface concentration of the substances applied was 2.2×10^{-7} mol $\times$ cm^{-2}. The skin was UV-irradiated 40 min after application of the photosensitizers. A SVD-120A high pressure mercury lamp was used, with a glass filter, and a water solution of $NiSO_4$ and $CoSO_4$ as another filter. The filters restricted the incident irradiation mainly to 334 nm. Quantum fluence rates of the incident radiation were distributed (in percentages of the total fluence rate) as follows: 10%, 63% and 27%, for the wavelengths 313, 334, and 366 nm, respectively, as estimated with a rhodamine quantum counter[7]. The total fluence rate of UV radiation was 1.82×10^{18} quanta $\times$ sec^{-1} m^{-2} (corresponding to 1.03 J $\times$ sec^{-1} m^{-2} as determined by ferrioxalate actinometry[7]. The criterion of the erythematous response was the minimal erythema dose (MED), reddening observed in 72 h after the irradiation. Five areas treated with 8-MOP or angelicin were irradiated in order to establish MED. The fluence was enhanced by 30% for every other area of skin.

Results obtained with 9 rabbits are summarized in the table. The absolute skin photosensitivity of individual animals is known to vary significantly. That is why we introduced one more parameter - the ratio of the MED of skin treated with 8-MOP ($MED_{8\text{-}MOP}$) to the MED of skin treated with angelicin (MED_{ang}), for each animal. As is seen from the table, the erythema-inducing property of angelicin under irradiation at 334 nm is comparable to that of 8-MOP. A 60-min irradiation (3700 J $\times$ m^{-2}) of the skin treated with ethanol alone caused no reddening. The known values of photosensitizing activities, namely 37 and 12 (relative units) for 8-MOP and angelicin, respectively, under irradiation at 366 nm (2) may be accounted for by the considerable difference in the ε_{366} values of these 2 substances.

In our experiments 8-MOP and angelicin were shown to exhibit approximately equal photosensitizing activities under irradiation at 334 nm. Consequently, cross-linking with DNA is not important in furocoumarin + UVA-induced erythema.

A comparison between skin photosensitizing activities of 8-MOP and angelicin. Incident irradiation is restricted mainly to 334 nm. $MED_{8\text{-}MOP}$, and MED_{ang} are minimal erythema doses for 8-MOP + UVA and angelicin + UVA, respectively

Animal No.	$MED_{8\text{-}MOP}$ (J m^{-2})	MED_{ang} (J m^{-2})	$\frac{MED_{8\text{-}MOP}}{MED_{ang}}$
1	1483	803	1.85
2	1483	1483	1.00
3	536	803	0.67
4	803	536	1.50
5	803	357	2.25
6	1112	1483	0.75
7	674	781	0.88
8*	1112	> 1483	
9*	556	< 742	
Mean ± SD	987 ± 144	892 ± 165	1.27 ± 0.23

*These values were not taken into account in the ratio $\frac{MED_{8\text{-}MOP}}{MED_{ang}}$.

1 Acknowledgment. We are grateful to Professor G. Rodighiero for his kindness in supplying furocoumarins.

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Effectiveness of phosphocitrate and N-sulpho-2-amino tricarballylate, a new analogue of phosphocitrate, in blocking hydroxyapatite induced crystal growth and calcium accumulation by matrix vesicles

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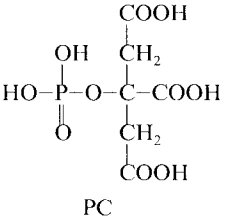
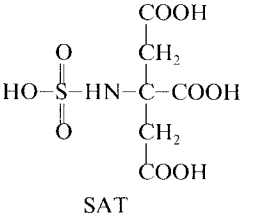
Summary. Phosphocitrate and its analogue N-sulpho-2-amino tricarballylate were compared with ethane-1-hydroxy-1,1-diphosphonate for inhibition of calcium phosphate crystallization in hydroxyapatite induced crystal growth and ^{45}Ca uptake by matrix vesicles. Phosphocitrate (1 μM) was the most potent inhibitor followed by ethane-1-hydroxy-1,1-diphosphonate and N-sulpho-2-amino tricarballylate, the latter requiring a high concentration (100 μM) to be equally effective as an inhibitor.

Biological mineralization is a complex phenomenon whereby calcium phosphate salts are transformed to hydroxyapatite (HA), a process that may be influenced by natural inhibitors in the microenvironment. Phosphocitrate (PC) is one of the most potent natural inhibitors but as yet its potential in vivo has not been thoroughly investigated. However, because PC may be prone to enzyme hydrolysis[2], a search for new stable analogues to PC was initiated. One such analogue N-sulpho-2-amino tricarballylate (SAT) has now been synthesized which is a little less active than PC as an anticalcifying agent[3]. Nevertheless, its stability in vivo could enhance its usefulness.



PC



SAT

While growth and aggregation of HA or calcium oxalate crystals are negatively affected by PC and SAT, their ability to prevent heterogeneously nucleated crystal growth has not been assessed. Nucleation is an early stage proposed in calcification and epiphyseal cartilage matrix vesicles (MV) are thought to participate in this early event by accumulating and transferring calcium and phosphate as HA to selected extracellular matrix sites[4-6]. In order to compare the physicochemical and biological effects of the new anticalcifying agents in terms of nucleation and crystal growth, we have compared the responses of the inhibitors in a seeded crystal growth system and in the accumulation of calcium by isolated epiphyseal MV.

Materials and Methods. Inhibitor assays systems. a) Seeded crystal growth: The system was essentially that developed in the laboratories of Fleisch[7]. Metastable calcium phosphate solutions (0.5-ml aliquots) ranging in (Ca)×(Pi) product from 1.73 mM^2 to 6.73 mM^2 were seeded with 16 µg of HA crystals[8] in the presence and absence of various concentrations of inhibitors. The mixtures were shaken in 5-ml screw-capped plastic vials for 24 h at 37 °C and then centrifuged at 12,000× g for 10 min. Total calcium in the supernatant was determined by atomic absorption spectrophotometry and inorganic phosphate by the method of Le Bel et al[9]. In this system, the initial (Ca)×(Pi) product necessary to induce 50% precipitation was the criterion by which the different inhibitors were judged and was previously found to be sensitive to inhibitors provided the incubation times were not too short[10].

b) Matrix vesicles: MV were isolated non-enzymatically from chicken epiphyseal cartilage by the method of Wuthier et al.[11] which resulted in homogeneous vesicles in lighter sucrose density fractions A and B. Confirmation of purity was obtained through electron microscopy and alkaline phosphatase activity. ^{45}Ca uptake by MV was determined by the method of Warner and Wuthier[12] using the Millipore filtration technique on pooled fractions A and B incubated in the presence and absence of inhibitors.

Assays and reagents. Protein associated with MV was estimated by the Coomassie blue dye binding method of Bradford[13]. PC was synthesized by the method of Williams and Sallis[14] by coupling triethyl citrate to cyanoethyl phosphate and subsequent alkaline hydrolysis whilst SAT was prepared by the sulphonation of 2-amino tricarballylate with pyridine-sulphur trioxide[3]. EHDP was a generous gift from the Proctor and Gamble Co. (Cincinnatti, Ohio).

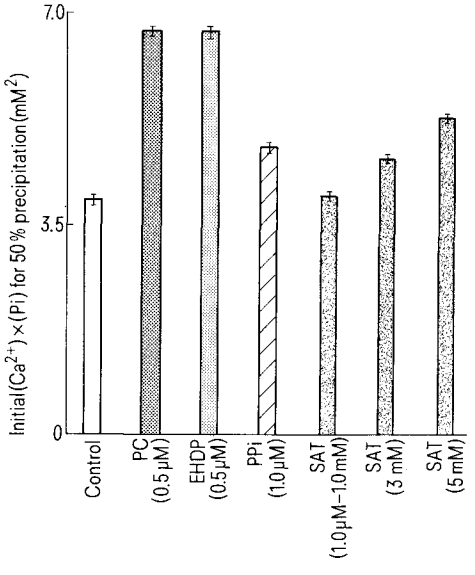


Figure 1. The effect of inhibitors of heterogeneously induced crystal growth. Phosphocitrate, N-sulpho-2-amino tricarballylate, ethane-1 hydroxy-1,1-diphosphonate and pyrophosphate were the inhibitors tested and a control value is included for comparison. The results shown are the mean ± 1SD of triplicate assays from 3 experiments.

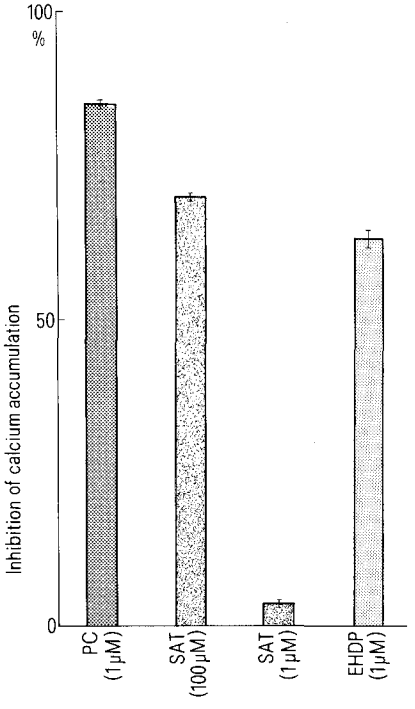


Figure 2. Comparative effectiveness of phosphocitrate, N-sulpho-2-amino tricarballylate, ethane-1-hydroxy-1, 1-diphosphonate on the inhibition of calcium accumulation by matrix vesicles. The data shown are the mean ± 1 SD from 4 experiments.

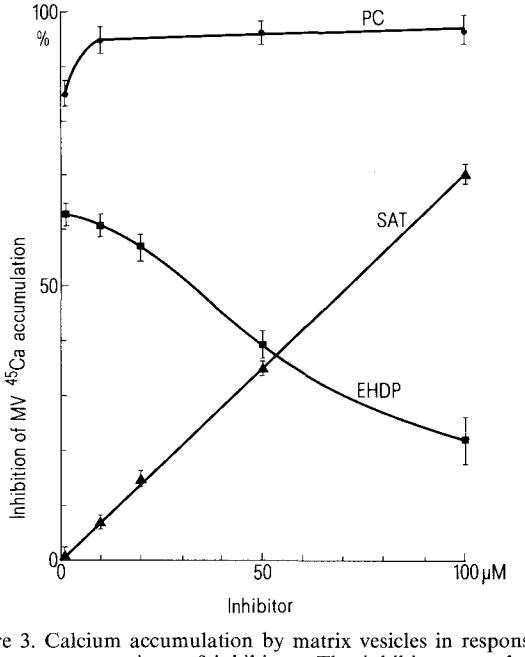


Figure 3. Calcium accumulation by matrix vesicles in response to different concentrations of inhibitors. The inhibitors tested were phosphocitrate, N-sulpho-2-amino tricarballylate and ethane-1-hydroxy-1,1-diphosphonate. The data shown are the mean ± 1 SD from 3 experiments.

Results and discussion. The comparative effectiveness of PC, SAT and EHDP and pyrophosphate to block heterogeneously induced crystal growth is shown in figure 1. Both PC and EHDP at 0.5 μM concentration were equally potent as inhibitors; SAT and pyrophosphate required much higher concentrations to exert similar effects. Despite the reduced response for SAT in this physicochemical system, the compound could still be a useful controller of events in the initiation of calcification.

In studies with calcium uptake by MV, purified fractions were used in preference to heterogeneous MV enriched microsomes because the characteristics of calcium uptake differ. It is known that microsomes accumulate calcium actively whereas MV are believed to use a non-energy dependent process[12,15]. The data (fig. 2) reveal that PC was the most effective of the inhibitors, reducing accumulation by 85%. This effect could not be attributed to possible cleavage products as in separate experiments, Pi and citrate at concentrations up to 10 μM had no effect. This is interesting, that despite a high alkaline phosphatase activity associated with the MV, PC was obviously still active. Inhibition (63%) was also seen with 1 μM EHDP but 100 μM SAT was required to inhibit to the same degree.

The effect of different concentrations of inhibitors on calcium accumulation by MV is shown in figure 3. PC exhibited a maximal response at 1 μM which remained unaffected with increased concentrations up to 100 μM. By contrast, EHDP which also exhibited maximal response at 1 μM showed declining effectiveness at concentrations in the range 10-100 μM. A different pattern was seen with SAT, where a linear increase in inhibition up to 100 μM was observed. Thereafter (not shown here) no change in effectiveness was apparent up to 200 μM.

The differences noted may be in response to their various mechanisms of action because many factors interplay to determine the inhibitor potency of a compound[16]. The fact that SAT is less effective than PC in both test systems is not surprising considering their structural differences. Another area yet to be investigated is the ability of PC und SAT to bind to target cell membranes and cross the plasma membrane of cells. The present studies in highlighting the potential of SAT as a calcification inhibitor do suggest that if the compound proves to be non-toxic during long term administration, it could be useful as a therapeutic agent in some disorders of calcium metabolism.

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Identification of calcium antagonist receptor binding sites using (^{3}H)nitrendipine in bovine tracheal smooth muscle membranes

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Summary. (^{3}H)Nitrendipine binding to the bovine tracheal muscle membrane at 25 °C was rapid, saturable (B_{max} = 14.8 ± 3.9 fmol/mg protein) and of high affinity (K_d = 0.15 ± 0.04 nM). The rank order of Ca^{2+} antagonists competing for airway (^{3}H)nitrendipine binding was nitrendipine ≒ nisoldipine ≒ nifedipine ≫ verapamil. Cromolyn, however, neither inhibited nor increased the binding.

Calcium antagonists such as nifedipine, verapamil and cromolyn have recently been shown to be effective in preventing exercise- or deep inspiration-induced bronchospasm in asthmatics[2-5]. In addition to their stabilizing effect on the mast cell membrane against degranulation of the cell[6-8], the antagonists, except for cromolyn, an antiasthmatic agent used prophylactically, have a direct influence on the tone of the airway muscle. Thus nifedipine produces a potent relaxation of isolated tracheal muscle either with intrinsic tone present[9,10] or precontracted by a bronchocon-