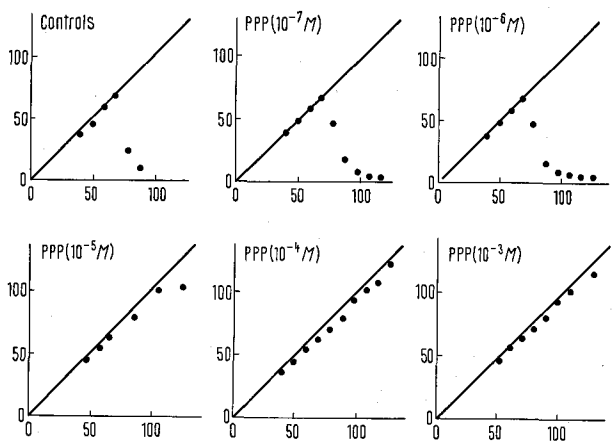
The Effect of Polyphloretin Phosphate, Polyoestradiol Phosphate, a Diphosphonate and a Polyphosphate on Calcification Induced by Dihydrotachysterol in Skin, Aorta and Kidney of Rats

Condensed phosphates such as inorganic pyrophosphate and long-chain polyphosphates at concentrations as low as 10^{-6} *M* are able to block the precipitation of calcium phosphate in vitro[1]. They also prevent mineralization of chick embryo femurs grown in tissue culture[2], and block vitamin D_3-induced aortic calcification[3] and the skin calcification following oral administration of dihydrotachysterol and epilation[4] in rats. Since pyrophosphate (PP_i) is present normally in body fluids[5], it has been suggested that one of its roles is to prevent soft tissue calcification. The initiation of calcification in tissues would require the removal of PP_i by pyrophosphatases[6]. Indeed, the injection of alkaline phosphatase, which is known to possess pyrophosphatase activity[7–9] causes local skin calcification in rats, whereas heat-inactivated enzyme does not[10]. It was therefore of interest to examine whether experimentally induced soft tissue calcification could be prevented by the administration of phosphatase inhibitors. In these studies we have used 2 such compounds, polyphloretin phosphate (PPP) and polyoestradiol phosphate (PEP)[11,12] against calcification induced by dihydrotachysterol. Since it was possible that any effect of PPP and PEP might involve direct inhibition of crystal deposition, we also tested these compounds on the precipitation of hydroxyapatite in vitro. The results were compared with those obtained by administering a long-chain polyphosphate (hexamataphosphate) and a diphosphonate (EHDP) to the rats. The diphosphonates are related in structure to the condensed phosphates but contain P-C-P bonds instead of P-O-P bonds and are therefore resistant to enzymic and chemical hydrolysis. They are also known to inhibit calcium phosphate precipitation in vitro[13–16] and to prevent aortic and kidney calcification in rats given large amounts of vitamin D[13,15,16]. In contrast to condensed phosphates, they are also active when given orally.

Materials and methods. Calcification in vivo. 75 female Wistar rats weighing between 130 and 150 g were bred in this laboratory and allocated into 6 groups. Allocation of rats to cages was by randomized selection with 3 rats per cage. Throughout the experiment the animals were fed on standard laboratory chow diet ad libitum (Nafag, Gossau, No. 194, containing 10.1 g Ca/kg, 8.5 g P/kg) and tap water ad libitum. The experiment lasted 10 days. The groups I (control with DHT alone) and II (control without DHT) received single s.c. injections of NaCl (0.9% w/v) 0.2 ml/100 g body weight daily for 9 days. Groups III–VI received the following compounds daily, over a period of 9 days, at doses of 10 mg P/kg body weight: Group III – polyphloretin phosphate (PPP) (AB Leo, Hälsingborg, Sweden); Group IV – polyoestradiol phosphate (PEP) (AB Leo); Group V – hexametaphosphate (Benckiser, Ludwigshafen/Rh, Germany); Group VI – ethane-1-hydroxy-1,1-diphosphonate (EHDP) (Procter and Gamble Company, Cincinnati, Ohio, USA). The doses were administered s.c., a solution containing 5 mg P/ml being used (= 0.2 ml/100 g body wt.). On day 3, all rats in groups I, III, IV, V and VI received by stomach tube a single dose of dihydrotachysterol (Wander AG, Berne, Switzerland) in arachis oil (0.5% w/v; 0.2 ml/ 100 g body wt., i.e. 10 mg DHT/kg body wt.). On day 4, all rats were anaesthetized with diethyl ether and hair was completely removed from an interscapular area of skin approximately 9 cm^2 in size. Rats were weighed on days 1 and 10 of the experiment and mortality in each treatment group was recorded. To assess skin calcification in epilated areas they were removed following sacrifice of the rat on day 10 and were dried to constant weight at 100 °C. They were then ashed dry at 600 °C for 8 h and the ash was dissolved in 2 ml of 2 *N*-HCl. The calcium content of these solutions was measured by atomic absorption spectrophotometry (Perkin Elmer 290 B). Renal and aortic calcification was assessed chemically. Kidneys and descending thoracic aortas were removed on day 10 and subjected to drying, ashing and calcium estimation as for the skins. Serum calcium was measured on days 4 and 10 of the experiment by atomic absorption spectrophotometry.

Calcification in vitro. The model system as previously described[1] was used. It consists in the determination of the minimum concentration of calcium and phosphate necessary to induce precipitation of calcium phosphate within 3 days at pH 7.4 and 37 °C. The concentration [Ca] × [P] in the solution before the experiment (initial



Effect of polyphloretin phosphate (PPP) on the minimum product [Ca] × [P] expressed as (mg/100 ml)^2 required to induce crystal formation in vitro. Abscissa: initial [Ca] × [P] concentration. Ordinate: final [Ca] × [P] concentration.

[1] H. Fleisch and W. F. Neuman, Am. J. Physiol. *200*, 1296 (1961).
[2] H. Fleisch, F. Straumann, R. Schenk, S. Bisaz and M. Allgöwer, Am. J. Physiol. *211*, 821 (1966).
[3] D. Schibler, R. G. G. Russell and H. Fleisch, Clin. Sci. *35*, 363 (1968).
[4] D. Schibler and H. Fleisch, Experientia *22*, 367 (1966).
[5] R. G. G. Russell, S. Bisaz, A. Donath, D. B. Morgan and H. Fleisch, J. clin. Invest. *50*, 961 (1971).
[6] H. Fleisch and R. G. G. Russell, *Encyclopaedia of Pharmacology and Therapeutics* (Pergamon Press, London 1970), vol. 51, p. 61.
[7] H. N. Fernley and S. Bisaz, Biochem. J. *107*, 279 (1968).
[8] R. G. G. Russell, S. Bisaz, P. Casey and H. Fleisch, Proc. V. Symp. Calc. Tiss. Sedes Paris (1968), p. 65.
[9] D. W. Moss, R. H. Eaton, J. R. Smith and L. G. Whitby, Biochem. J. *102*, 53 (1967).
[10] R. D. Solomon, B. B. Nadkarni and L. Richardson, Arch. Path. *82*, 60 (1964).
[11] E. Diczfalusy, O. Fernö, H. Fex, B. Hogberg, T. Linderot and Th. Rosenberg, Acta chem. scand. *7*, 913 (1953).
[12] O. Fernö, H. Fex, B. Hogberg, T. Linderot, S. Veige and E. Diczfalusy, Acta chem. scand. *12*, 1675 (1958).

Effects of polyphloretin phosphate (PPP), polyoestradiol phosphate (PEP), a polyphosphate and a diphosphonate (EHDP) on calcification induced by DHT and epilation

	Treatment group	Weight change (g) during 9 days of experiment	Survival	Serum Ca (mg/100 ml)		Kidney dry wt. (mg)	Calcium content (mg/g dry wt)		
				Day 4	Day 10		Skin	Aorta	Kidney
I	Control treated with DHT alone	−19.4± 3.3	$^{17}/_{25}$	14.5±0.4	13.6±0.2	320± 9	74.1 (2.4–154.8)	45.9 (0.3–331)	35.9 (6.6–75.6)
II	NaCl alone without DHT	+28.0± 2.6[a]	$^{10}/_{10}$[c]	11.2±0.2[a]	11.9±0.4[a]	290±12	0.45 (0.3– 0.7)	4.3 (0.5–16.5)	2.1 (0.3–15.3)
III	DHT + polyphloretin phosphate (PPP)	−3.1 ± 3.7[b]	$^{9}/_{11}$	14.0±0.5	12.0±0.5[b]	336± 6	21.7 (1.2–62.4)	46.0 (6.1–98.7)	23.3 (9.6–46.4)
IV	DHT + polyoestradiol phosphate (PEP)	−21.0± 2.9	$^{10}/_{10}$[c]	14.5±0.4	12.8±0.2	268±10[c]	15.6 (0.8– 46.7)	28.6 (1.1–77.6)	23.6 (3.1–78.0)
V	DHT + a polyphosphate	−6.7 ± 10.3	$^{6}/_{9}$	13.8±0.5	11.3±0.2[a]	278±21[c]	3.0 (0.3– 11.9)	2.9 (1.4– 7.8)	19.8 (0.6–46.0)
VI	DHT + a diphosphonate (EHDP)	−5.6 ± 2.4[b]	$^{9}/_{10}$	16.5±1.2	14.3±1.0	289±10	0.8 (0.3– 1.6)	3.5 (0.4–10.5)	1.5 (1.0– 4.2)

All figures are means ± S.E. [a] Difference from control group treated with DHT alone significant at 0.1% level. [b] Difference from control group treated with DHT alone significant at 1.0% level. [c] Difference from control group treated with DHT alone significant at 5.0% level. When the results do not follow a normal distribution, ranges are given instead of standard errors and significance values.

product) is plotted against the concentration after the incubation and after filtration through Millipore filters (final product). The break in the curve indicates the minimum concentration [Ca]×[P] necessary for the precipitation to occur. An increase in this product indicates the presence of an inhibitory substance.

Results. The results of DHT-induced changes in rats are given in the Table. The results of the experiments on calcium phosphate precipitation in vitro are given in the Figure. PPP produced a strong inhibition at 10^{-3}, 10^{-4} and $10^{-5}M$, but not at 10^{-6} and $10^{-7}M$. PEP did not inhibit at 10^{-5} and $10^{-6}M$. Higher concentrations of PEP could not be tested because it precipitated in the test system. It should be noted that these concentrations refer to phosphorus content and not to the true molar concentration of the polymers, which would be some hundred-fold less.

Discussion. Like condensed phosphates[1], polyphloretin phosphate and polyoestradiol phosphate inhibit soft tissue calcification in vivo, albeit less effectively. This effect might be due to the inhibitory action on phosphatases, thus increasing the local concentration of pyrophosphate. In the case of PPP it could also be due to its inhibition of crystal formation, although the greater effectiveness of PEP contrasts with its failure to inhibit crystal formation in vitro. Other mechanisms of action are possible. Thus PPP has been shown to have effects on prostaglandin $F_{2\alpha}$[16] and PEP could conceivably work by releasing oestradiol.

From the therapeutic point of view it is relevant that none of the phosphatase inhibitors were as active as the polyphosphates themselves and especially as the diphosphonate EHDP. This result supports earlier experiments showing that diphosphonates inhibit other types of calcification[13,15,16] and strengthens the rationale of using these compounds in man. First results in patients with myositis ossificans show that they are also active in human conditions[17,18].

Zusammenfassung. Zwei Phosphatasehemmer (Polyphloretinphosphat und Polyoestradiolphosphat), ein Polyphosphat (Hexametaphosphat) und ein Diphosphonat (Ethan-1-hydroxy-1,1-diphosphonat) wurden auf ihre Fähigkeit untersucht, Verkalkungen zu verhindern, welche in der Haut, der Aorta und den Nieren von Ratten durch Dihydrotachysterol (DHT) hervorgerufen wurden. Das Diphosphonat erwies sich als der stärkste Hemmer; dann folgten das Polyphosphat und die zwei Phosphatasehemmer. Die Phosphatasehemmer wurden zusätzlich auf ihre Fähigkeit geprüft, die Calciumphosphat-Ausfällung in vitro zu verlangsamen. Polyphloretinphosphat besitzt diese Fähigkeit bei Konzentrationen von 10^{-5} M und mehr; Polyoestradiolphosphat ist unwirksam.

P.A. CASEY, G. CASEY, H. FLEISCH and R.G.G. RUSSEL[19]

Department of Pathophysiology, University of Berne, Hügelweg 2, CH-3012 Berne (Switzerland); and Laboratory for Experimental Surgery, Davos (Switzerland), 26 November 1971.

[13] H. FLEISCH, R. G. G. RUSSELL, S. BISAZ, P. A. CASEY and R. C. MÜHLBAUER, Calc. Tiss. Res. *2*, Suppl. 10 (1968).
[14] M. D. FRANCIS, Calc. Tiss. Res. *3*, 151 (1969).
[15] M. D. FRANCIS, R. G. G. RUSSELL and H. FLEISCH, Science *165*, 1264 (1969).
[16] H. FLEISCH. R. G. G. RUSSELL, S. BISAZ, R. C. MÜHLBAUER and D. A. WILLIAMS, Europ. J. clin. Ivest. *1*, 12 (1970).
[17] A. A. MATHE, K. STRANDBERG and A. ASTRON, Nature, Lond. *230*, 215 (1971).
[18] C. A. L. BASSETT, A. DONATH, F. MACAGNO, R. PREISIG, H. FLEISCH and M. D. FRANCIS, Lancet *2*, 845 (1969).
[19] Acknowledgments. This work has been supported by the Swiss National Fund, Grant No. 4564, the U.S. Public Health Service, Grant No. 07266, AB Leo, Hälsingborg, Sweden, and Procter and Gamble Company, Cincinnati, USA.