da COLBURN e MASS[1] tra sostanze fenoliche e ATP, chelazione che noi pensiamo possa ritenersi estendibile anche a sostanze 5-idrossindoliche.

D'altra parte esiste un enorme numero di dati sul significato che lo Zn può assumere negli esseri viventi, animali e vegetali, come attivatore di sistemi enzimatici e come costituente legato a proteine[7]. In particolare ricorderemo che per quanto non esista una comparabilità diretta tra i veleni degli anfibi e quelli dei rettili, DELEZENNE[8] ha dimostrato la presenza di Zn nelle ghiandole velenose dei serpenti.

Zusammenfassung. Mit Hilfe der Farbreaktion mit Dithizon und polarographischer Analysen wurde die Anwesenheit von Zink im Gift von *Triton cristatus*, *Salamandra maculosa* und *Bombinator pachypus* nachgewiesen. Die Möglichkeit einer Chelatbildung durch Zink wird diskutiert.

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[8] C. DELEZENNE, Annls Inst. Pasteur Lille *33*, 68 (1919).

The Role of Pyrophosphate and Alkaline Phosphatase in Skin Calcification (Calciphylaxis)

$KMnO_4$ and several other metal salts produce heterotopic calcifications when injected s.c. SELYE[1] called this mechanism direct calcification and the metal salts direct calcifiers. The deposition of calcium salts in skin calcification occurs in the collagenous tissue (SELYE[1]) in the form of hydroxyapatite (GLIMCHER[2], POSNER[3]). This process of experimental calcification is, therefore, similar to the formation of normal bone. Using this type of calcification, we were able to test in vivo under experimental conditions the theory of FLEISCH and NEUMAN[4] concerning the significance of pyrophosphate and alkaline phosphatase in the mineralization process of collagenous fibres.

Material and methods. Male and female Wistar rats weighing about 150 g were given 'Lats' diet (Lats, Germany) and tap water ad libitum throughout the experiment. 3 groups of rats, each containing 5 animals, were studied. The basic treatment for all animals consisted of a single s.c. injection of 0.2 mg $KMnO_4$ in 0.2 ml distilled water at 4 different sites on the back. As a result of this treatment, there appeared within 6 days visible and radiologically demonstrable calcifications at the sites of injection. 10 of the 15 animals received i.p. injections of 2.5 mg pyrophosphate (per 100 g rat) twice daily during the 6 day duration of the experiment (the pyrophosphate solution was adjusted with hydrochloric acid to pH 7.4). This treatment with pyrophosphate was found to be effective in preventing calcifications in group 2. The remaining 5 rats were injected s.c. with 16 U daily of alkaline phosphatase (dissolved in 0.5 ml water, pH 7.0) at the sites of previous $KMnO_4$ injections. Experimental procedure and results are shown in the Table.

Discussion. The s.c. injection of potassium permanganate produces an alteration of the connective tissue, causing the calcification of collagenous fibres. It seems probable that $KMnO_4$ has a depolymerizing effect on the ground substance of connective tissue (unpublished histochemical observations of SCHWARZ and DIEZEL[5]). As a result of this change in connective tissue structure, the permeability to extracellular phosphates and calcium increases and their diffusion to the collagenous fibres is facilitated.

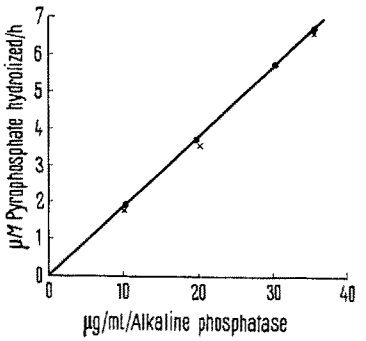
The experiments described above show that the heterotopic skin calcification produced by potassium permanganate may be prevented by pyrophosphate injections (attempts were not made to assess the lowest pyrophosphate dose necessary to produce this effect). These observations correspond well with the in vitro experiments of FLEISCH and NEUMAN[4], who found that pyrophosphate in small amounts inhibits calcium phosphate nucleation and calcium phosphate precipitation. Similar observations were made by SCHIBLER and FLEISCH[6], who supposed that

Group	Treatment (5 animals)	Result
I	Subcutaneous injection of 0.2 mg $KMnO_4$ in 0.2 ml distilled water at 4 different locations on the back[a]	Visible and radiologically demonstrable calcifications at the sites of injection
II	Initial preparation of the skin sites as described above. In addition, these animals received i.p. injections of pyrophosphate (2.5 mg/100 g rat) twice daily[a]	No calcifications
III	Initial preparation of the skin sites and pyrophosphate treatment as described above. In addition, these animals received daily s.c. injections of alkaline phosphatase (Böhringer Mannheim) in a single dose of 16 U dissolved in water (pH 7.0) at the sites where the $KMnO_4$ solution had previously been injected. As a control 0.5 ml of a 0.9% saline solution was injected at 1 of the 4 prepared skin sites[a]	Visible and radiologically demonstrable calcifications at the sites of injection with $KMnO_4$ and alkaline phosphatase, but not in those control sites injected with $KMnO_4$ and 0.9% saline solution

[a] The animals were sacrificed 6 days later.

[1] H. SELYE, *Calciphylaxis* (The University of Chicago Press, Chicago 1962).
[2] M. J. GLIMCHER, Recent Prog. Horm. Res. *20*, 56 (1964).
[3] P. POSNER, Recent Prog. Horm. Res. *20*, 56 (1964).
[4] H. FLEISCH and W. F. NEUMAN, Am. J. Physiol. *200*, 1296 (1961).
[5] G. SCHWARZ and B. P. DIEZEL, to be published.
[6] D. SCHIBLER and H. FLEISCH, Experientia *22*, 367 (1966).

pyrophosphate, or condensed phosphates in general (the authors used Graham salt), 'prevent the initiation and/or growth of calcium phosphate crystals by acting directly at what would otherwise be a site of calcification'. In their experiments the possibility can be excluded that polyphosphates act by lowering the extracellular concentration of biologically active calcium by binding this mineral in a complex form. The presumptive local inhibitory action of pyrophosphate on calcium phosphate precipitation could be demonstrated in the third group of our experimental animals. It was demonstrated that the inhibitory action of pyrophosphate on calcium phosphate precipitation could be destroyed by alkaline phosphatase. This enzyme proved to be capable of hydrolyzing pyrophosphate to orthophosphate (the Figure shows this hydrolytic activity). 16 U of alkaline phosphatase (Böhringer, Mannheim[7]) proved to be capable of hydrolyzing 7.2 mg of pyrophosphate. In our experiments the local concentration of alkaline phosphatase was, therefore, high enough to hydrolyze all of the pyrophosphate available for the inhibition of calcification.



Hydrolytic activity of alkaline phosphatase on pyrophosphate. TRA-buffer 0.1 *M*, pH 7.0; pyrophosphate 6,6 μM, 37 °C.

Injection of pyrophosphate in an amount sufficient to prevent heterotopic skin calcification has no influence on the formation and growth of normal bone. Treatment of normal growing rats with pyrophosphate does not affect the final size and weight of these animals when compared with control rats receiving sham injections of 0.9% saline instead of pyrophosphate (author's unpublished experiments). We are convinced that in normal bone and epiphyseal plate there is enough alkaline phosphatase present to hydrolyze all pyrophosphate and, thereby, destroy this calcification inhibitor.

The most probable local action of pyrophosphate inhibition consists of an interference with the initial step of calcification – the binding of collagen and phosphate. It may be that a collagen-pyrophosphate binding occurs instead of the normal collagen-phosphate binding.

Zusammenfassung. Durch $KMnO_4$-Injektionen hervorgerufene lokale Hautverkalkung kann durch Pyrophosphatinjektionen verhindert werden. Durch alkalische Phosphatase wird die Pyrophosphatwirkung aufgehoben.

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[7] Alkaline phosphatase acetone dry powder from calf intestine, 8 U/mg, *p*-nitrophenylphosphate-substrate.

Effect of Irradiation on the Properties of Pollen in Austrian and Scotch Pines

It is known that several pine species belonging either to the same or different branches of the same subgenus cannot be mutually crossed. Such incompatibility exists, for instance, between the Austrian pine (*Pinus nigra* Aru.) and the red pine (*Pinus resinosa* Ait.) and between the Austrian pine and Scotch pine (*Pinus silvestris* L.). The problem of incompatibility between red pine and Austrian pine was studied by MCWILLIAM[1], who analysed sugars and amino acids in the ovules of these pines.

VIDAKOVIĆ[2], in a bid to overcome the incompatibility in the crossing of Scotch and Austrian pines, irradiated the pollens just prior to pollination, on the assumption that certain changes that were apt to occur in the irradiated pollens would help to circumvent the 'incompatibility barrier', resulting in the fecundation of the female flower of other species.

However, the effects of such a mutagen causing cytogenetic damage or modification are apt to be reflected also in the pollen biochemistry, i.e. sugars, amylase, amino acids, etc., present in mature pollens, are likely to undergo changes with irradiation. The present communication is concerned with the determination of sugars, amylase, and free and bound amino acids in irradiated as well as non-irradiated pollens of Austrian and Scotch pines with a view to demonstrating the differential effects of irradiation on the pollen of the 2 pine species.

Collection and irradiation of pollen. The male flower-branches were collected 1 day prior to pollen dusting. The pollen was collected after 24 h and dispersed pollen irradiated by means of a Co^{60} source of about 60 Curies. The irradiation doses were 200, 800, 1000 and 1200 r.

Total reducing sugar was determined by the SCHOORL-LUFF[3] method after the extraction of sugars from pollen with water.

Individual sugars. Qualitative determination of the individual sugars has been made by paper chromatography using the descending technique in the solvent system *n*-butanol-pyridine-benzene-water (5:3:1:5). The time of development of the chromatogram was 48 h. Detection was carried out by 2,3,5-triphenyl-tetrazolium chloride.

[1] J. R. MCWILLIAM, Am. J. Bot. *43*, 6, 425 (1959).
[2] M. VIDAKOVIĆ, World Consultation on Forest Genetics and Tree Improvement, Stockholm. F.A.O.For.Gen.-63, Vol. I, 2b/5:1–5 (1963).
[3] N. SCHOORL, Zeitschrift für Chemie *57*, 566 (1929).