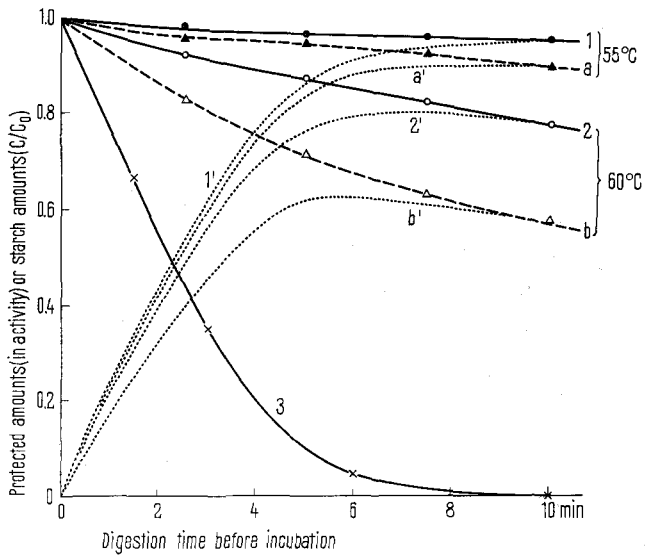

Fig. 2. Changes of protected amounts (difference between activities of TAA heat-treated in the absence and presence of starch) with digestion time before heat-treatment. Curves 1, 2, and a, b, heat-treated at pH 5.6 and 9.0, respectively; curve 3, starch concentrations at various digestion times before incubation, and also means the amount protected by starch; curves 1′, 2′, and a′, b′, amounts protected by decomposition products from starch, corresponding to the cases of curves 1, 2, and a, b, respectively. The digestion was made at pH 7.0 at 20 °C in all cases. The heat-treatment was made for 10 min at the temperature shown and at pH 5.6 or 9.0, immediately after digestion. The protected amount at zero digestion time is normalized to unity. The activity was measured at pH 5.6. Concentrations of TAA and starch, 0.036 and 6 mg/ml, respectively.

concentrations employed in the present investigation, it is possible roughly to separate the total protected amount into two parts: the amount protected by starch and that protected by decomposition products. These are shown in Figure 2, curves 3 and 1′ or 2′, or a′ or b′ respectively. In the short period of digestion, the protection is mainly due to the starch effect. The proportion of protection by digestion products to that by starch at pH 5.6 is larger than that at pH 9.0, as seen in Figure 2. This means that digestion products are less effective for the protection from heat inactivation of TAA in pH-inactivated or -denatured states.

Further details will be published in a future paper.

Zusammenfassung. Nachweis eines Schutzes der Eiweissstruktur, der Taka-Amylase A, gegen Wärme-Inaktivierung und -Denaturierung durch das Substrat. Auf Grund der pH-Abhängigkeit dieser Schutzwirkung kann angenommen werden, dass sie die Folge der Stabilisierung der Sekundärstruktur des Proteins durch Enzym-Substrat- und -Produkt-Komplexe ist.

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July 12, 1965.

Inhibition of Skin Calcification (Calciphylaxis) by Polyphosphates

Condensed phosphates, such as inorganic pyrophosphate and long chain polyphosphates (e.g. Graham salt), are able to block collagen-induced calcium phosphate precipitation in vitro in concentrations as low as $10^{-6} M$[1]. Condensed phosphates can also prevent the mineralization of chick embryo femurs grown in tissue culture[2]. These facts, together with the presence of pyrophosphate in both urine[3] and plasma[4] suggest that pyrophosphate may play an important role in the biological regulation of calcification in vivo. Recently it has been shown that pyrophosphate and long chain polyphosphates can indeed inhibit calcification in vivo, as they are able to block vitamin D_3-induced aortic calcification in rats[5].

In the present study we have investigated the inhibitory effect of Graham salt on another calcification system in vivo. As our model of calcification, the calciphylactic skin reaction described by Selye[6] was chosen. Graham salt was used as the condensed phosphate because pyrophosphate was found to cause skin necrosis at the site of injection.

Material and methods. Female Wistar rats weighing about 130 g were given Altromin-R rat diet (Altromin, Lage, Germany) and tap water ad libitum throughout the experiment. 42 rats were allotted to 5 groups as follows: Group I: a single dose (10 mg/kg) of dihydrotachysterol (DHT), obtained from Wander AG, Berne, Switzerland, was dissolved in arachis oil and given by stomach tube. 24 h later epilation was performed under ether anaesthesia on an area of skin of 9 cm² in the intrascapular region. On the 7th day after DHT the animals were killed with ether. Group II: similar to group I, but the animals were in addition given daily subcutaneous injections of Graham salt (J. A. Benckiser, Ludwigshafen a. Rh., Germany) at a dose of 1 mg P/kg/day, beginning 2 days before the administration of DHT and continuing throughout the course of the experiment. The Graham salt was

[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., in press.
[3] H. Fleisch and S. Bisaz, Am. J. Physiol. *203*, 671 (1962).
[4] H. Fleisch and S. Bisaz, Nature *195*, 911 (1962).
[5] H. Fleisch, D. Schibler, J. Maerki, and I. Frossard, Nature *207*, 1300 (1965).
[6] H. Selye, *Calciphylaxis* (University of Chicago Press, Chicago 1962).

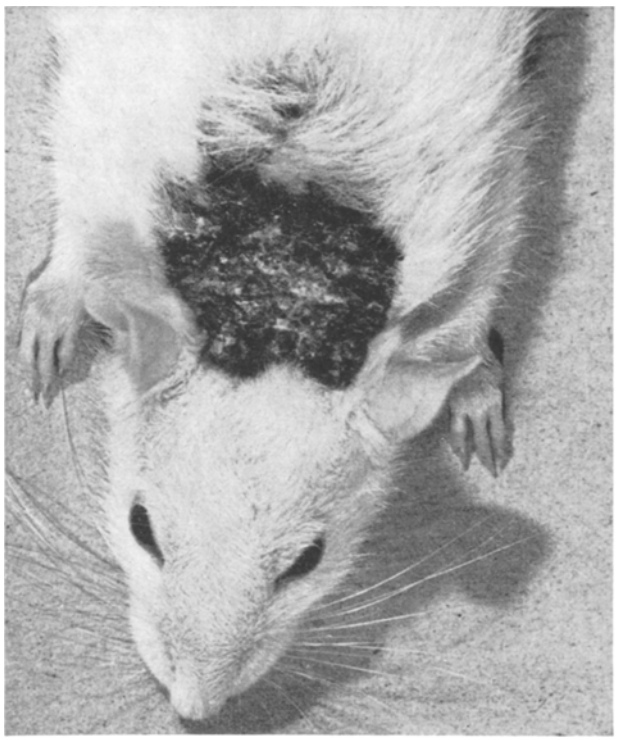

Fig. 1a

Fig. 1b

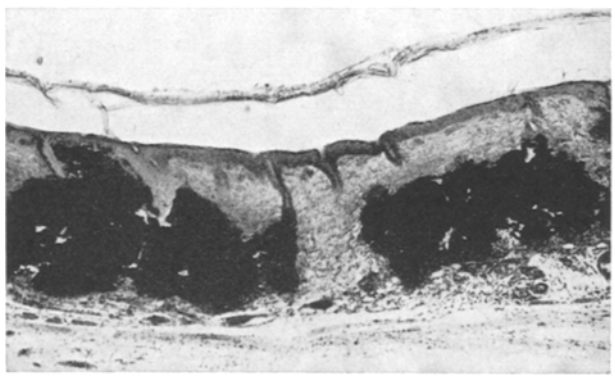

Fig. 2a

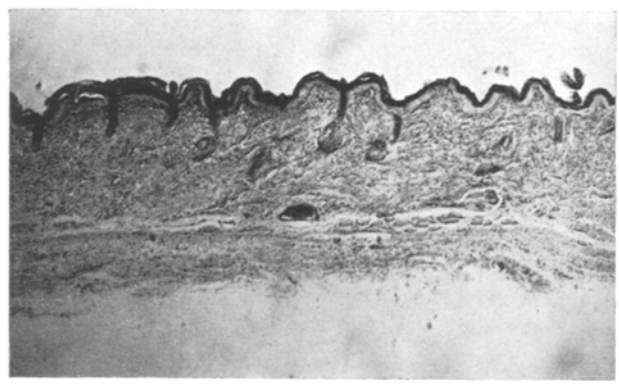

Fig. 2b

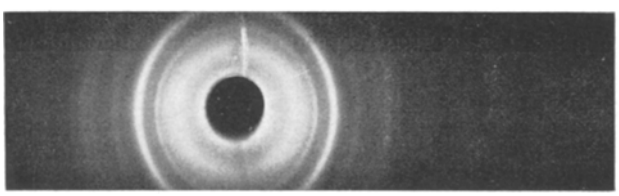

Fig. 3a

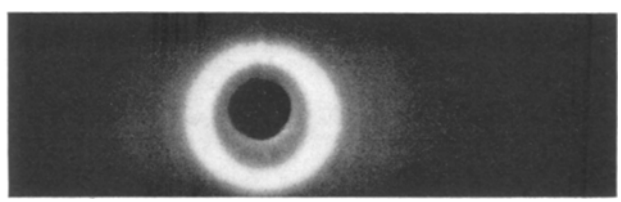

Fig. 3b

Fig. 1. Example of the effect of Graham salt on the macroscopic appearance of a calciphylactic skin reaction induced by dihydrotachysterol and epilation. (a) Without Graham salt; (b) with Graham salt (10 mg P/kg/day).

Fig. 2. Example of the effect of Graham salt on histology of a calciphylactic skin reaction induced by dihydrotachysterol and epilation. (a) Without Graham salt; (b) with Graham salt (10 mg P/kg/day). ($AgNO_3$, × 120).

Fig. 3. Example of the effect of Graham salt on the X-ray diffraction patterns of a calciphylactic skin reaction induced by dihydrotachysterol and epilation. (a) Without Graham salt; (b) with Graham salt (10 mg P/kg/day).

injected at various sites on the back and the abdomen, as far as possible from the site of epilation. Group III: similar to group II, but given Graham salt at a dose of 10 mg P/kg/day. Group IV: these animals were given no DHT or Graham salt, but were subjected to epilation and killed 6 days later. Group V: control animals not given any treatment.

On the day of death the epilated skin was cut out and divided into two portions, one of which was taken for histological examination and the other was lyophilized and weighed. Ashing of the lyophilized skin was then performed at 400 °C using concentrated hydrochloric acid, followed by concentrated sulphuric acid and hydrogen peroxide. Calcium was measured by titration with EDTA in an alkaline medium uscing calcein as indicator. For histological examination the skin was fixed in 10% neutral formol, embedded in paraffin and stained by the von Kossa technique modified according to KRUTSAY[7]. X-ray diffraction patterns were obtained from fresh skin after dehydration in alcohol using a Debye-Scherrer camera.

Results. General appearance: In group I (DHT and epilation) 10 of the 12 rats showed extensive calciphylactic lesions, the epilated skin regions being transformed into hard, whitish patches (Figure 1a). The animals receiving the low dose of condensed phosphates presented calciphylactic lesions less frequently, while those which received 10 mg P/kg/day were entirely normal (Figure 1b).

Chemistry: The calcium values from the epilated skins are summarized in the Table. The skins from the animals of group I (DHT and epilation) had markedly increased calcium levels compared with those from animals of group IV (epilation only) and V (controls). In group II (DHT, epilation and low dose of Graham salt) the skin calcification was reduced in incidence, the skin of 5 of the 8 rats now containing normal amounts of calcium. The calcium content of the skin of those animals in this group that did calcify was as high as that of animals receiving no condensed phosphates. With the higher dose (10 mg P/kg/day) of Graham salt (group III) the skin calcification was completely prevented in all the animals, the calcium values being similar to those of group IV (epilation only) and V (controls).

Histology: As already described by SELYE[8] the calcification appeared to start in the hair follicles and surrounding areas of fibrous tissue. As calcification proceeded, these areas became confluent and large calcareous masses were seen extending throughout the dermis, destroying its structure (Figure 2a). There was a good correlation between the histological and chemical findings. Thus in all the animals of group II and group III (low and high dose of Graham salt respectively) in which skin calcification was prevented as assessed by the chemical results, the histological sections showed a complete lack of calcium deposition in the hair follicles and in the fibrous tissue (Figure 2b) and were indistinguishable from sections from the animals which were subjected to epilation only (group IV).

X-ray diffraction: The calcified skin presented an X-ray diffraction pattern characteristic of hydroxyapatite (Figure 3a), in agreement with Moss and URIST[9]. After heating to 900 °C the X-ray diffraction pattern showed the appearance of whitlockite, suggesting that a partially calcium-deficient apatite was present. The skin of animals treated with Graham salt showed only a collagen pattern, without any sign of apatite (Figure 3b).

Discussion. These results show that Graham salt, given subcutaneously, can prevent DHT-induced skin calcification in rats. The inhibition with larger doses of Graham salt appears to be complete, and this effect can be seen equally well in the general appearance, and by chemical analysis, histology and X-ray diffraction. The inhibitory effect of Graham salt on pathological calcifications is unlikely to be due to either an interference with the hypercalcaemic action of DHT or to a complexing of calcium in the extracellular fluid. Indeed, in some of the animals of groups I, II and III, where total calcium levels in the plasma were measured, Graham salt had practically no effect in reducing the hypercalcaemia induced by DHT. Furthermore, it was found in a separate study that the administration of 10 mg P/kg of Graham salt also has no effect on the level of ionized calcium in the plasma of rats receiving high doses of vitamin D_3[10]. It is therefore probable that the condensed phosphates prevent the initiation and/or growth of calcium phosphate crystals by acting directly at what would otherwise be a site of calcification.

It is an attractive possibility that this property might be used to prevent pathological calcifications in man, especially since the doses of polyphosphates required to prevent ectopic calcification are apparently unable to affect the normal calcification process in bone[11,12].

Zusammenfassung. Grahamsalz, ein langkettiges Polyphosphat, ist bei subcutaner Verabreichung imstande, eine pathologische Verkalkung der Haut (Calciphylaxe) bei der Ratte zu hemmen. Der Wirkungsmechanismus dieser Hemmung wird kurz diskutiert.

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Effect of Graham salt on skin calcification induced by dihydrotachysterol and epilation

Group	Treatment	mg (Ca/g lyophilized skin
I	DHT 10 mg/kg + epilation	49.5 ± 7.46 (12)
II	DHT 10 mg/kg + epilation + 1 mg P/kg Graham salt	17.6 ± 9.13 (8)
III	DHT 10 mg/kg + epilation + Graham salt 10 mg P/kg	4.8 ± 0.56 (10)
IV	Epilation only	3.5 ± 0.83 (6)
V	Controls, no treatment	2.7 ± 0.36 (6)

All tabulated values shown are means ± standard error. Number of experiments in brackets.

[7] M. KRUTSAY, Acta histochem. *15*, 189 (1963).
[8] H. SELYE and K. NIELSEN, Acta morph. hung. *10*, 327 (1961).
[9] M. J. Moss and M. R. URIST, Arch. Path. *78*, 127 (1964).
[10] D. SCHIBLER and H. FLEISCH, in preparation.
[11] J. T. IRVING, D. SCHIBLER, and H. FLEISCH, submitted for publication.
[12] This work was supported by Grant No. AM-07266 from the National Institute of Arthritis and Metabolic Diseases, by Grant No. 2618 and No. 3567 from the Schweizerischer Nationalfonds zur Förderung der wissenschaftlichen Forschung, and by the Sandoz-Stiftung zur Förderung der medizinisch-biologischen Wissenschaften. We are grateful to Ing. F. STRAUMANN for the X-ray diffraction studies, to MARIANNA LATSCHA and ISABELLE FROSSARD for their skilled assistance, and to R. G. G. RUSSELL for helpful advice.