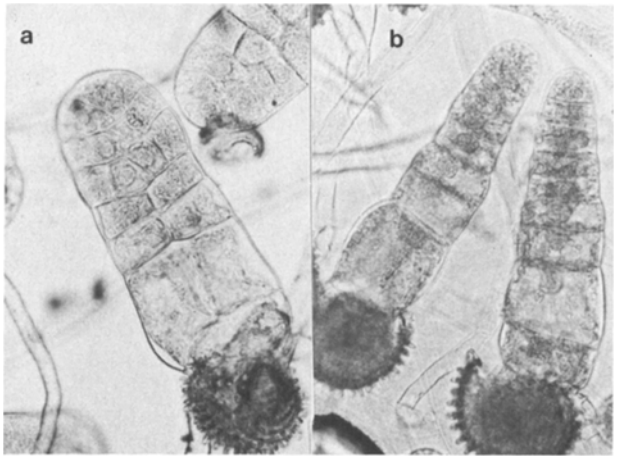

Growth inhibition and morphogenetic influence of TA on gametophytes of *A. phyllitidis*. a) control, b) retardation of cell division and induction of two-dimensional prothallium form by 10^{-4} M TA, 12-day cultures in continuous white light; 22 ± 0.1 C°.

Table 3

	dpm/g fresh wt
control	11060
	11020
10^{-4} M TA	10350
	10100

Effects of 10^{-4} M TA on the uptake of GA_3 (1.5×10^{-5} M + 0.1 μC C^{14} GA_3; spec. act. 1,7,12,18^{-14}C 5–15mCi/mmol). Application of hormone and hormone + TA respectively to groups of 15-day-old prothallia. Culture in continuous white light at 22 ± 0.1 °C. Extraction after 7 days of feeding.

and gibberellins in the rice assay should be based on an interaction of the reactions leading to shoot growth rather than on a synergistic influence at the hormone binding site.

Indeed, Takeno et al.[3] explained the synergistic effects of TA in the dwarf rice test by a combined action of this substance on the uptake and the metabolism of GA_4. The determination of these two processes is very difficult in the *Anemia* system because, compared to tissues of higher plants, the rate of gibberllin uptake into cells of the fern gametophyte is extremely low. After 7 days of culture on a medium containing a hormone concentration of 1.5×10^{-5} M TA as compared with untreated controls on the basis of total uptake per prothallium or g fresh weight (table 3) although this TA concentration already inhibits cell division of the prothallia.

Despite a far reaching correspondence of the reaction-system between fern antheridiogen and many gibberellin-mediated reactions of spermatophytes, there are distinct differences with respect to TA-treatment in the reactivity of the mechanisms of the pheromone and hormone transport.

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1 Ogawa, M., Matusi, T., Oyamada, K., and Tobisuga, J., Pl. Cell Physiol. *18* (1977) 841.
2 Satoh, Y., Yamane, H., Takahashi, N., and Ogawa, M., Pl. Cell Physiol. *22* (1981) 1003.
3 Takeno, K., Koshioka, M., and Pharis, R.P., Plant Physiol. *72*, suppl. (1983) 72.
4 Schraudolf, H., Naturwissenschaften *69* (1982) 286.
5 Schraudolf, H., Z. Pflanzenphysiol. *110* (1983) 469.
6 Schraudolf, H., Pl. Cell Physiol. *7* (1966) 277.

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Partial purification and some characteristics of hamster molar alkaline phosphatase

A.A. Dogterom, D.M. Lyaruu, A. Doderer and J.H.M. Wöltgens[1]

Laboratory of Preventive Dentistry, and Department of Biology, Vrije Universiteit, P.O.Box 7161, NL-1007 MC Amsterdam (The Netherlands), 5 September 1983

Summary. A rapid 3-step method is given to purify partially hamster molar alkaline phosphatase. Molecular weight was 50,200 and isoelectric point 3.7. The alkaline phosphatases in the mesenchymal and ectodermal parts of the tooth are probably identical.
Key words. Hamster, Syrian; *Mesocricetus auratus;* alkaline phosphatase, molar.

Alkaline phosphatase (AP) has been related to the process of mineralization in bone[2] and teeth[3]. Isolation and characterization of AP from various tissues revealed the heterogeneity of the enzyme[4] with respect to molecular weight, isoelectric point, substrate specificity and stability. Little is known, however, about the characteristics of AP from dental tissue[5–7]. Moreover, the purification methods described are laborious. For future immunohistochemical localization of AP in hamster molar tooth germs, it will be necessary to obtain relatively pure enzyme. Therefore, we developed a simple and rapid procedure to purify hamster molar AP up to 121 times.

Materials and methods. First maxillary and mandibulary molars were dissected from 3–4-day-old Syrian hamsters *(Mesocricetus auratus)* and collected on solid CO_2. Between 100 and 150 molars were used for each experiment. They were homogenized in 10 ml bidistilled water at 0–4 °C, using a Potter tube with glass pestle. After homogenization 4 ml n-butanol was added[4] and the mixture stirred for 2 h at 0–4 °C. The phases were separated by centrifugation (15 min, 3000 × g, 0–4 °C) and the water phase collected. This water phase was centrifuged at high speed (30 min, 190,000 × g, 0–4 °C) and the resulting supernatant reduced in volume by lyophilization (this procedure also removes the remaining butanol). This supernatant was used for assessing some properties of AP and for further purification. To determine the molecular weight of the AP, the high-speed supernatant was further purified by means of high pressure gel permeation chromatography. The G 2000 SW column was calibrated with molecular weight markers in the range 94 kD to 255 D. To determine the isoelectric point of the AP a high-speed supernatant was chromatofocussed at 0–4 °C. Since hamster molar AP activity decreased with time at low pH, the activities found in the eluate were corrected for the time they had been at low pH. In the various fractions AP activity was determined using p-nitrophe-

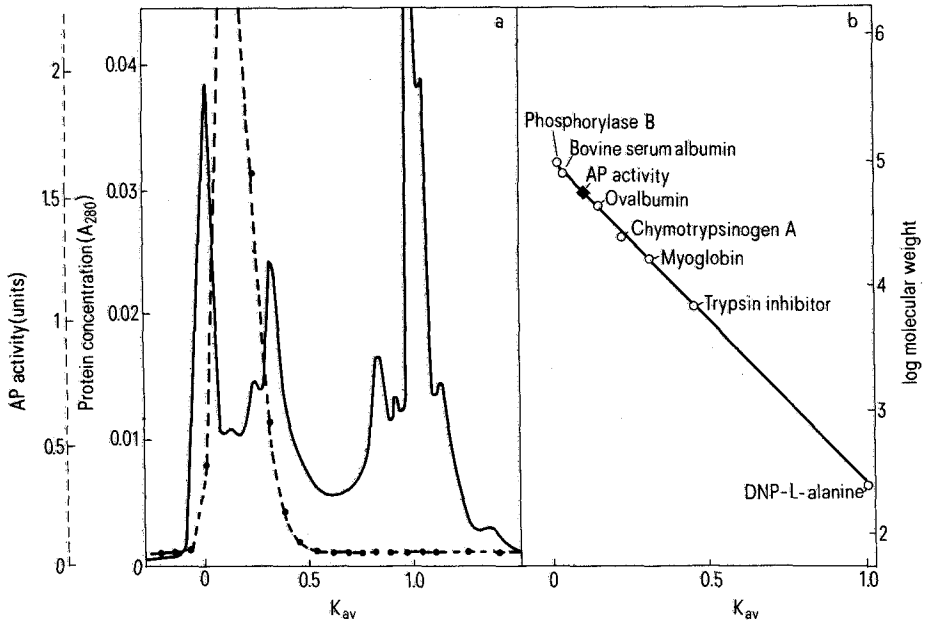

Figure 1. *A* High pressure gel permeation chromatography (HPGPC) of a high-speed supernatant of 100 butanol-extracted hamster molars on G 2000 SW (LKB; 2135 Ultropac TSK column; 7.5 × 300 mm). The sample was applied to the column and eluted with 0.1 M triethylamine acetic acid buffer pH 7.0 at 1 ml·min^{-1}. Extinction at 280 nm was continuously recorded and the AP activity of the individual fractions determined with p-nitrophenylphosphate as a substrate[8]. K_{av}: relative elution volume.
B Determination of the molecular weight of the AP possessing fractions of the G 2000 SW column in a separate experiment. Molecular weight of the markers; phosphorylase B (from Pharmacia): 94 kD; bovine serum albumin (from Serva): 67 kD; ovalbumin (from Pharmacia): 43 kD; chymotrypsinogen A (from Pharmacia): 25 kD; myoglobin (horse skeletal muscle, from Serva): 17.8 kD; trypsin inhibitor (bovine lung, from Serva): 6.5 kD; dinitrophenyl-L-alanine (from Serva): 255 D.

nylphosphate as a substrate[8]; protein content was determined with the Lowry method[9] using bovine serum albumin as a standard.

Results. After butanol extraction of homogenized molars even more AP was present than in the original crude homogenate (a typical example of one of the procedures is given in the table). A simple high-speed centrifugation even resulted in a 17 times purer preparation, again without much loss of activity. Addition of 10.5% butanol (the maximum concentration soluble in water) to the lyophilized and butanol-free supernatant did not change the AP activity (data not shown). Figure 1A shows that chromatography of the supernatant on G 2000 SW resulted in many peaks of which only a small one immediately after the excluded volume showed AP activity. With molecular weight markers the apparent molecular weight was estimated to be 50,200 (fig. 1B).

After a single column run, the active fractions ($0.06 < K_{av} < 0.21$) consisted of a 121 times more pure AP preparation containing 87% of the original activity (table). During a first chromatofocussing run on a pH gradient from 7 to 4 no AP activity was found in the eluate. Even in a gradient from 6 to 3 little activity was found. AP activity was found to be destroyed by low pH. However, when the time at low pH was shorter than 5 h, AP activity could recover partly from this treatment (data not shown). When we again ran a high speed supernatant on a chromatofocussing column (pH 6–3) and neutralized the fractions immediately after pH determination, we found, after correction for pH inactivation, a single peak eluting at pH 3.8–3.6 (fig. 2), indicating a pI for hamster molar AP of about 3.7.

Discussion. The reported sequence of relatively simple techniques resulted in a 121-fold purification of hamster molar AP within 1 day. In contrast to other more laborious methods[5–7] almost no loss of AP activity occurs. Since the use of the G 2000 SW column is highly reproducible, several runs can be performed without changing the tubes of the fraction collector.

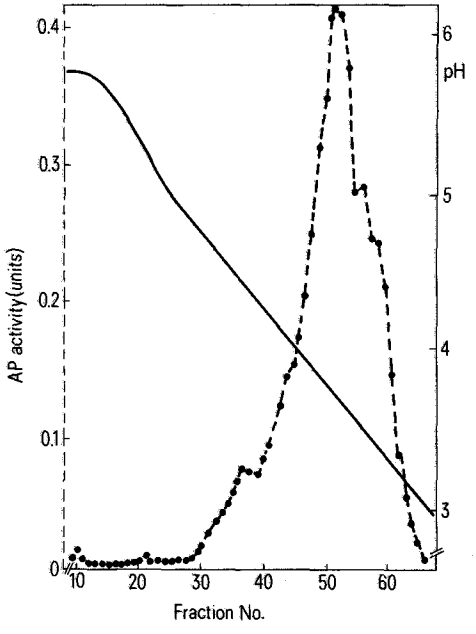

Figure 2. Chromatofocussing of a high speed supernatant of 100 butanol-extracted hamster molars on a pH gradient from 6 to 3[10]. Before chromatofocussing the high speed supernatant was gel filtrated on Bio-Gel P6 (BioRad) in a 9 × 600 mm column and eluted with bidistilled water at 10 ml·h^{-1}. The excluded volume (mol.wt > 6 kD) was applied to the chromatofocussing column (9 × 300 mm) and eluted with polybuffer 74 (set at pH 3.0) at 40 ml·h^{-1}. Starting buffer was 0.025 M histidine. HCl pH 6.2. The pH of the fractions was measured immediately after termination of that particular fraction and after alkalization of the fractions to pH 9–10 with 0.5 M 2-amino-2-methyl-propanol buffer pH 10.3, AP activity was measured with p-nitrophenylphosphate as a substrate[8].

Purification and recovery of AP during the various steps of isolation

Fraction	Protein (μg)	AP activity (U)	AP specific activity* (U · mg protein^{-1})	Yield (%)
Crude homogenate	25000	25.2	1	100
Butanol extract	3250	30.2	9.3	120
190,000 × g supernatant	1545	25.7	16.6	102
Active fractions of G 2000 SW ($0.06 < K_{av} < 0.21$)	180	21.8	121.3	87

Protein was determined according to Lowry et al.[9] and AP activity according to Wöltgens et al.[8]. *This column also represents the purification factor.

This results in AP quantities sufficient to raise antibodies for future ultrastructural localization of AP. The purity of AP obtained in the present study is the highest reported from crude tooth homogenates. Molecular weights reported for AP from dental tissue from other species than the Syrian hamster, range from 55,000 to 240,000[4-6, 11]. Especially since different techniques are employed, some of them not being the most appropriate for this purpose, it is difficult to compare our value of 50,200 with the previous reported ones. Whether di- or polymer forms are involved and whether this is due to the butanol treatment, is not clear. The observed pI of 3.7 is very low compared with values for most mammalian AP's, although values around and below 4 have been reported[4, 12]. Comparison with other dental tissue AP is not possible, since no other pI values are available. The fact that in gel permeation as well as in chromatofocussing only 1 AP peak is found, strongly suggests that in hamster molars one, or several very similar forms occur. This means that the mesenchymal dentine-producing tissue and the epithelial enamel-producing tissue make use of the same AP isoenzyme.

1 Reprint requests to: J.H.M. Wöltgens, Laboratory of Preventive Dentistry, Vrije Universiteit, P.O. Box 7161, 1007 MC Amsterdam, The Netherlands.
2 Robison, R., Biochem. J. *24* (1931) 1927.
3 Wöltgens, J.H.M., and Bonting, S.L., in: Proc. 9th Eur. Symp. Calcif. Tiss., p. 311. Eds H. Czitober and J. Eschberger. Facta Publication, 1973.
4 McComb, R.M., Bowers, G.N., and Posen, S., Alkaline phosphatase. Plenum Press, New York 1979.
5 Tojyo, Y., Saida, K., and Suzuki, A., Archs oral Biol. *26* (1981) 1099.
6 Harada, M., Hiraoka, B.Y., Fukasawa, K., and Fukasawa, K.M., Archs oral Biol. *27* (1982) 69.
7 Finley, S.L., and Hollinger, J.O., J. dent. Res. *61* (1982) 194.
8 Wöltgens, J.H.M., Bonting, S.L., and Bijvoet, O.L.M., Calcif. Tissue Res. *5* (1970) 333.
9 Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randal, R.J., J. biol. Chem. *193* (1951) 265.
10 Performed to the manufacturer's (Pharmacia) instructions, except that in some experiments the pH of the polybuffer was set at 3.0.
11 Granström, G., Jontell, M., and Linde, A., Calcif. Tissue Int. *27* (1979) 211.
12 Stinson, R.A., and Seargeant, L.E., Clinica chim. Acta *110* (1981) 261.

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Freeze tolerance in the frog, *Rana sylvatica*

K.B. Storey[1]

Institute of Biochemistry and Department of Biology, Carleton University, Ottawa (Ontario, Canada K1S 5B6), 3 October 1983

Summary. Wood frogs survive extracellular freezing at moderate subzero temperatures (−4 °C) for at least 11 days. Freezing survival is aided by the accumulation of high concentrations of glucose as a cryoprotectant in blood and tissues. Glucose production was accompanied by a rapid decline in liver, but not muscle, glycogen levels suggesting that liver is the organ controlling cryoprotectant synthesis.
Key words. Rana sylvatica; frog, freeze tolerance; cryoprotectant synthesis; glycogen levels, liver; glucose levels, cryoprotectant.

A natural tolerance of extracellular freezing during overwintering has recently been reported for several species of terrestrial frogs[2, 3]. While freeze tolerance occurs quite widely amongst terrestrial insect groups[4, 5] these frogs are the only known vertebrate animals which survive freezing. As such they present us with what may be the optimal model system for studying problems related to tissue and organ cryopreservation in mammals. The present report discusses our initial investigations of the biochemical adaptations for freeze tolerance in the wood frog, *Rana sylvatica.*

Materials and methods. Specimens of *R. sylvatica* were collected during September 1982 from woodlands around Ottawa. Frogs were held in the laboratory at 23 °C for 3 weeks and were fed crickets. Feeding was then discontinued and animals were transferred to a cold room at 3 °C (range 1–4 °C) and held for up to 12 weeks. Frogs were held in plastic boxes with damp sphagnum moss to maintain humidity. Animals were sampled at intervals over the course of this cold acclimation to monitor cryoprotectant production. Cold acclimated frogs were then transferred to an incubator at 3 °C and temperature was lowered 1 °C per day until −4 °C was reached.

Results and discussion. Freezing occurred between −2° and −3 °C (Schmid[2] reported a supercooling point of −1.9 °C for *R. sylvatica*). Cold acclimated animals survived freezing at −4 °C for at least 11 days. External evidence of freezing included stiff and brittle limbs, solid abdomens and opaque eyes. Internally ice crystals were found surrounding the leg muscles and a solid mass of ice filled the abdominal cavity. Organs were surrounded by ice but were not frozen themselves. Heart beat and breathing were not observed. Animals could survive repeated freeze/thaw cycles; when thawed limb movement returned within 12–24 h at 3 °C.

The percentage of b.wt as ice was estimated by the method of Schmid[2]. Briefly, frozen frogs are transferred to an insulated container with 20 ml of water at 20 °C and allowed to thaw. The cooling of the surrounding water is measured and is compared to the cooling values of comparable weights of ice or water at −4 °C. The results showed an average of 47.8 ± 2.5%