Homology of L-gulonolactone oxidase of species belonging to Mammalia, Aves, and Amphibia

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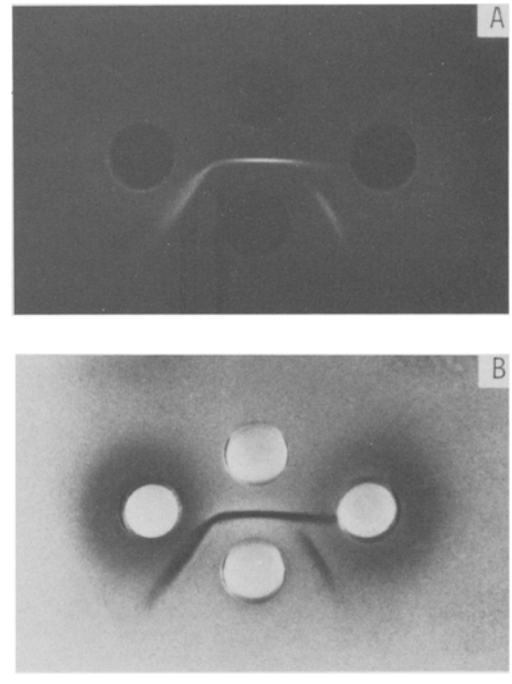
Summary. Immunological cross-reactivity of L-gulonolactone oxidase of different species (rat, chicken, and bullfrog) was tested by the Ouchterlony technique. Antiserum directed against the enzyme from chicken kidney reacted with rat liver enzyme as well as with bullfrog kidney enzyme. This finding suggests that there is, at least partly, sequence homology among the enzymes from species belonging to the three classes, Mammalia, Aves, and Amphibia.

L-Gulonolactone oxidase (L-gulono-γ-lactone: oxygen 2-oxidoreductase, EC 1.1.3.8] is the enzyme which catalyzes the last step of L-ascorbic acid biosynthesis in animal tissues (for reviews, see Burns[2] and Nishikimi[3]). Nishikimi and Udenfriend[4] demonstrated by the Ouchterlony technique that rabbit antiserum directed against rat L-gulonolactone oxidase cross-reacted with the chicken enzyme. Thus, the enzymes of the species belonging to the 2 higher classes of vertebrates, Mammalia and Aves, are immunologically homologous. On the other hand, the antiserum did not show cross-reactivity with the bullfrog enzyme[4]. It seems that during the evolution of L-gulonolactone oxidase, the amino acid sequences of both enzymes changed so diversely that their homology could not be revealed by the immunological technique. Alternatively, the frog enzyme may have evolved from an ancestral protein which is unrelated to the enzymes of mammals and birds. One of the approaches to differentiate these possibilities would be to examine the cross-reactivity by using an antiserum which recognizes a greater number of antigenic determinants than the one used previously. Such an antiserum could be obtained by immunizing a rabbit with the enzyme of an animal which is phylogenetically more distant than the enzyme of the rat. We have recently purified L-gulonolactone oxidase from chicken kidney to homogeneity, and it has become possible to perform an immunological study on these lines.

Rat liver L-gulonolactone oxidase was purified as described previously[5], and chicken L-gulonolactone oxidase was purified from the kidney by the method used for the purification of the goat enzyme[5] with some modifications. The details of the purification will be published elsewhere[6]. The purified enzyme was homogeneous as judged by polyacrylamide gel electrophoresis in the presence and absence of sodium dodecyl sulfate. The antiserum directed against this enzyme preparation was raised in albino rabbits as described previously[4] and its γ-globulin fraction was prepared by ammonium sulfate fractionation followed by DEAE cellulose column chromatography[7]. The microsomal extracts were prepared by solubilizing the microsomes of the livers and kidneys of rat and chicken by the previously published procedure[4], except that the deoxycholate concentration used was 0.35%. The Ouchterlony test was carried out in agarose plates containing 0.12 M NaCl and 0.07 M sodium phosphate (pH 7.2). The plates were developed for 20 h at 4 °C. Activity staining of L-gulonolactone oxidase in precipitin lines was done as previously described[5].

Figure A shows the result of the Ouchterlony test with rabbit antibody directed against chicken L-gulonolactone oxidase. The antibody formed a precipitin line with the extract of chicken microsomes obtained by solubilization with deoxycholate. This line fused with the precipitin line of the purified chicken enzyme, indicating that both the enzyme in the crude extract and the purified enzyme are antigenically identical with each other. The bullfrog kidney microsomal extract formed a spur with the purified chicken enzyme. This indicates that the bullfrog enzyme is antigenically related to but not identical with the chicken enzyme. The precipitin lines were stained by L-gulonolactone oxidase activity (fig. B). It is obvious, therefore, that the enzyme protein is really involved in the immunoprecipitation. These results point to the immunological homology between the chicken and bullfrog enzymes. On the other hand, the microsomal extract of bullfrog liver, which does not contain L-gulonolactone oxidase, did not form any precipitin line with the antibody directed against the chicken enzyme. The purified preparation of the enzyme from rat liver also showed a precipitin line with the antibody (result not shown), indicating the homology between the chicken and rat enzymes. This homology was previously observed by the Ouchterlony technique using the antiserum directed against the rat enzyme[4]. These findings, taken together with the above-mentioned result, indicate that the chicken enzyme is homologous with both the rat and the bullfrog enzymes. Since more than 60% of amino acid sequence should be the same for proteins to cross-react with each other[8], it is likely that the rat and bullfrog enzymes share the same sequence in substantial portion of



Immunological cross-reactivity of chicken and bullfrog L-gulonolactone oxidase. The Ouchterlony test was carried out in agarose plates at 4 °C for 20 h. 5 µl of the following enzyme samples and antibody solution were placed in wells. Left: the chicken kidney microsomal extract (25 mg protein/ml); upper: the purified chicken enzyme (0.4 mg/ml); right: the bullfrog kidney microsomal extract (25 mg protein/ml); and bottom: the rabbit antibody directed against the chicken enzyme (100 µg/ml). *A* The precipitin lines; *B* the lines stained for enzymic activity.

the protein structure. Moreover, we demonstrated previously that the enzymes of rat, chicken, and bullfrog all have practically the same molecular weight, and that they contain a flavin covalently bound to the apoprotein[9]. Accordingly, it is reasonable to consider that the L-gulonolactone oxidase of species belonging to the 3 classes Mammalia, Aves, and Amphibia, evolved from a common ancestral protein.

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The effect of calcitonin on calcium uptake in mouse molars in vitro

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Summary. Mouse maxillary second molars were removed at either 24 or 96 h of age and maintained in vitro. Half of the teeth, of each age group, were treated with 50 m-units of synthetic salmon calcitonin. By comparing the initial and final calcium concentrations in the medium, the net uptake or release of calcium was inferred. The treated molars took up significantly more calcium than the untreated groups.

Calcitonin (CT), a hormone secreted by the parafollicular cells of the mammalian thyroid, acts as a hypocalcemic factor[1-3]. This effect is due to the ability of CT to suppress osteoclastic-induced bone resorption[4-6]. CT has also been shown to decrease calcium (Ca) efflux from the bone cells, increase the formation of a type of short-term storage Ca-phosphate material in bone fluid, and increase the uptake of Ca into bone cell mitochondria[7-10]. In this study the in vitro effects of synthetic salmon CT on Ca and phosphate uptake by unerupted maxillary 2nd molars was assessed.

Methods. Maxillary 2nd molars were taken from 24- or 96-h-old white mouse pups. A total of 100 molars were used in each age group. The molars were maintained on a culture medium composed of 90% Weymouth medium 722/1 and 10% fetal calf serum supplemented with 15 mg ascorbic acid/100 ml of medium[11]. Ca was added to the medium to a concentration of 10.5 mg%, and phosphate to levels of 5.8 mg%, both within the normal range. Antibiotic coverage consisting of penicillin (50 U/ml) and streptomycin (50 µg/ml) was supplied. Half the teeth within each age group were cultured on this medium; the other half on the same medium to which 50 m-units of synthetic salmon CT (supplied by Armour Pharmaceutical Co.) dissolved in 0.1% highly purified bovine serum albumin was added. An equal volume of the CT vehicle was added to the medium of the untreated teeth.

2 molars were cultured in each dish containing 2.5 ml medium. The molars were incubated in an atmosphere of 50% O_2, 45% N_2, and 5% CO_2 at 37 °C for 48 h[12]. The media were assayed for Ca using a fluorometric method[13] and phosphate using a colorimetric method[14]. The initial and final media were assayed for both Ca and phosphate as was the medium in 1 dish incubated without teeth. The net release or uptake of Ca or phosphate by the molars was determined by comparing the initial and final concentrations. The mean changes in the Ca and phosphate concentrations of the untreated and treated molars, of the same age, were compared using the Student t-test.

After incubation teeth from both age groups and treatments were decalcified and prepared for histological examination to verify their viability and to detect possible morphological differences.

Results. The results are summarized in the table. Each group of molars started with an equal number of samples, i.e. 100. A total of 22 specimens were discarded due to bacterial contamination.

The CT-treated media, both for 24-h and 96-h age groups, were found to contain significantly ($p < 0.05$) less Ca than the untreated groups. The CT-treated media, from both age groups, contained less phosphate, but not at significant levels.

20 molars, randomly selected from all groups after incubation, were examined histologically. All molars exhibited a normal healthy appearance and no morphological differences were observed between experimental and control molars of the same age.

Discussion. At birth the maxillary 2nd molars lie in their bony cripts and have begun to lay down dentin[15]. As the molars are actively laying down predentin and dentin at this period of development, if CT affects the rate of calcification its addition to the culture media should produce a detectable effect.

The CT-treated molars had a significantly greater uptake of Ca from the media but not phosphate. This uptake presumably involved increased Ca deposition in the dentin, and

Average calcium levels (in mg% with SD) in the culture media

	Initial concentration	Final concentration
Group I (24-h mice)		
Untreated	(n = 100) 10.5 ± 0.10	(n = 96) 11.1 ± 0.12
Treated	(n = 100) 10.6 ± 0.11	(n = 94) 10.0 ± 0.13
		($p < 0.05$)
Group II (96-h mice)		
Untreated	(n = 100) 10.3 ± 0.08	(n = 92) 11.5 ± 0.11
Treated	(n = 100) 10.4 ± 0.10	(n = 96) 9.8 ± 0.13
		($p < 0.05$)