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Effect of an Enzyme-Resistant Phosphopeptide on Calcification of Embryo Chicken Bone in Vitro

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ABSTRACT

Cultivation of embryonic chicken bone in vitro enables substances to be added to the culture medium in order to ascertain how they affect the histological development of the bone. This method has been adopted for studying an enzyme-resistant phosphopeptide extracted by Mellander from casein. By cultivating paired bones that from one side can be used as a control for the contralateral bone. The test group was given calcium complexly bound to the phosphopeptide and the control group calcium as $CaCl_2$. Studies of bones from embryos of different incubation ages after cultivation for various periods in media containing different concentration of calcium revealed that similar degress of ossification and rates of osteoid tissue formation were achieved when the phosphopeptide was the source of calcium as when it was $CaCl_2$. These experiments have demonstrated that calcium bound to a phosphopeptide can be utilized in the ossification process just as well as readily soluble inorganic calcium.

It has long been known that phosphoproteins are present in such products as milk—there in the form of casein—and that considerable amounts of phosphorylated peptides are formed when these proteins are hydrolyzed in the human intestinal tract (1). Using experimental animals with rickets fed special diets, Mellander discovered an antirachitic effect of such phosphorylated peptides (2). This effect has been interpreted as the result of increased intestinal absorption of calcium complexly bound to phosphorylated peptides (3). We have in the present investigation studied their direct action on the ossification of embryonic chicken bone in an *in vitro* system.

Most previous trials with bone cultivation have been done with chicken bone. They have revealed that bone from 6-day chick embryos incubated at 36°C can be explanted, that histologically they continue to differentiate normally, and that they exhibit considerable longitudinal growth (4). Bone tissue from 6-day chick embryos have also been used for studying the effects *in vitro* of various hormones on longitudinal growth and ossification (5, 6, 7, 8, 9, 10, 11, 12, 13). Such experiments, all using a mixture of plasma and chicken embryo extract as culture medium, have disclosed that both longitudinal growth and ossification are significantly affected by insulin, thyroxin, cortisone, vitamin A and parathyroid hormone.

Embryo chicken bone, incubated at 36°, develop *in vivo* from commencing differentiation at 6 days to a bone with well developed marrow cavity at 12 to 16 days of age (14). Pilot studies in this laboratory on embryonic chick tibiae aged 6 to 17 days and cultivated for 1 to 15 days demonstraeted that 10 to 14 days was a suitable age for studying ossification and the formation of osteoid tissue.

TECHNIQUES

Dissection was carried out with a cataract knife and tweezers under a dissection microscope. All bones were measured before and after cultivation by means of Trowell's grid technique (15). According to Franks, the ambient gas phase may well be air for cultivation of embryonic organs, so we chose air (16). The medium was »Eagle's minimum essential medium for Spinner cultures» with 10% calf serum (17). To avoid cells attaching themselves to the grid, we placed the bones on millipore paper which accompanied them to fixation. The specimens were fixed in 10% formalin neutralized with calcium, dehydrated, embedded in paraffine, sectioned longitudinally in 5 to 10 slices, and stained with haematoxylin-eosine according to van Gieson and von Kossa.

First the tibia was dissected out from 6 embryos in each age group of 10, 12 and 14 days. Thus each embryo yielded 2 bones, one of which was cultivated in medium plus calcium chloride and the other in medium plus phosphopeptide.

The amounts of $CaCl_2$ and phosphopeptide were such as to yield concentrations of calcium of 100 mg/litre. Two of the 6 embryos in each age group were cultivated for 5, 10 and 15 days respectively.

RESULTS

The specimens were examined after staining and judged on the basis of the degree of ossification and the amount of osteoid tissue. Both variables were classified according to a scale from 0 to 3, where 0 implies no ossification or osteoid tissue and 3 denotes marked ossification and plenty of osteoid tissue respectively.

With respect to both controls and experimental bones it appeared that:

(i) both ossification and osteoid tissue were equal to 3 in the group incubated for 14 days regardless of how long they were cultivated;

(ii) both ossification and osteoid tissue were rated 0.5 in the group incubated for 10 days, regardless of how long they were cultivated; and

(iii) osteoid tissue rated 2.5 in the experimental group and 2.7 in the controls while ossification was 3 in the experimental group and 2 in the controls among embryos incubated for 12 days.

The differences observed between experimental and control groups were confined to those embryos incubated for 10 days and cultivated for 10 days and to those incubated for 12 and cultivated for 5 days.

Embryos 10 to 12 days old are evidently in a critical state with respect to ossification and formation of osteoid tissue. Hence we examined 20 additional embryos incubated for 10 days and cultivated for 6 and 8 days, as described above. The results were as follows:

It will be seen that no differences in amount of osteoid tissue and degree of ossification could be demonstrated between embryonic chicken bones cultivated in a medium containing 100 mg calcium per litre in the form of $CaCl_2$ and similar cultures in a medium containing calcium complexly bound to a phosphopeptide.

To exclude the possibility that a concentration of 100 mg of calcium per litre was too high to allow any differences to appear, we carried out another experiment in which the calcium concentration was reduced to 25 mg per litre. For this latter experiment we chose embryos incubated for 9 days and cultivated them for 8 days in the same way as before.

It turned out that longitudinal growth in the experimental group was 1.1 ± 0.5 mm and 1.3 ± 0.6 mm in the control group. The amount of osteoid tissue in the two groups was 2.2 and 2.3 respectively. No bone exhibited any calcification.

Consequently not even a calcium concentration which is extremely low for tissue cultivation purposes could expose any differences between the phosphopeptide and calcium chloride in either ossification or the rate of osteoid tissue formation in embryonic chicken bone.

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Ca source	No. of bones	Incub. time, days	Cultiv. time days	Growth, mm	Osteoid tissue	Ossification
$CaCl_2$	10	10	6	1.0 ± 0.4	2.9	2.9
Phospho-peptide	10	10	6	1.4 ± 0.4	2.9	2.9
$CaCl_2$	10	10	8	0.9 ± 0.4	2.9	2.9
Phospho-peptide	10	10	8	1.0 ± 0.5	2.9	2.9

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