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Alkaline Phosphatase and Osteogenesis *in vitro.**† (29632)

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The discovery by Robison(1) of a "monophosphoric esterase" in ossifying cartilage of young rats and rabbits led the way to further studies of this enzyme and its role in osteogenesis. This "bone phosphatase" has been found to belong to a group of phosphatases having activity at a pH optimum around 10.0 and is referred to as alkaline phosphatase. The present investigation is concerned with the alkaline phosphatase of bone and its participation in ossification and also in calcification of cartilage and bone.

The original phosphatase theory of calcification as proposed by Robison(1) has been seriously questioned, and several other roles have been assigned to this enzyme. Various studies have shown alkaline phosphatase to be involved: (a) in differentiation of both osteoblasts and chondroblasts from their mesenchymal origin(3), (b) in formation of fibrous protein and the pre-osseous matrix of bone(4), (c) in formation of mucopolysaccharides of the ground substance(5), (d) in transphosphorylation of some phosphate acceptor in the matrix(6), and (e) in removal of organic phosphates that may poison bone crystal growth(7).

Many investigators have used either normal embryonic and post fetal bone or rachitic bone slices for the study of alkaline phosphatase in osteogenesis. However, since the classical experiments of Fell and Robison (2) in 1929, impetus was given to the use of tissue culture as a tool for demonstrating and study osteogenesis of embryonic bone tissue *in vitro.*

Material and methods: Tissue culture technique. Seven-day embryonic chick femora (White Leghorn), were grown in culture on plasma clots using the roller-tube technique. The culture medium contained 3 parts of chicken serum and one part of 50% embryo extract. Each day the embryo extract was prepared fresh according to the procedure described by White(8) and using Tyrode's solution(9) as the diluent. The chicken serum was prepared from whole blood obtained by sterile heart puncture using 2- to 3-month-old White Leghorn roosters. It was stored at 4°C for use within 2 to 3 weeks. Chicken plasma was obtained in the same manner and prepared using heparin as the anticoagulant. Each pair of femora was placed in a roller-tube (16 mm × 150 mm), end to end and lengthwise to the tube. One drop of plasma was allowed to run down over the pair of femora; in this manner a very thin plasma clot was formed, due to the small amount of embryo extract still on the femora. After approximately 10 minutes, 0.80 ml of culture medium was added to each roller-tube. The tubes were stoppered with white rubber stoppers and placed in a roller drum, set at 5° angle in a 38°C incubator, and revolving at 12 rph. The culture medium was changed every two days. Sterility was maintained without the use of antibiotics.

Lyophilization and dry weight measurement. At the end of the culture period for each series of cultures, the paired femora were removed from their respective roller-

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tubes and the adhering material teased away. The femora were placed in pairs around the bottom of a lyophilization drying cup, quickly frozen in a dry ice-acetone bath and allowed to lyophilize for 3 hours. At the end of this period, they were weighed directly on the pan of a microgram balance. One femur of each pair was then placed in a small shell vial which was stoppered and stored at −15°C, until the 5 phosphorus fractions could be determined. The other femora of the pairs were used for determination of their alkaline phosphatase activity and protein nitrogen content.

Analysis of phosphorus containing fractions. Small ground-glass homogenizers were constructed, of such dimensions (5 mm × 50 mm) that a single femur could be conveniently homogenized. Extraction of the femur homogenate was carried out in the homogenizer, using the homogenizer pestle to suspend the mixture, after which the pestle was removed and the homogenizer tube centrifuged.

The method of Lowry *et al*(10) was modified by increasing quantities 5 times. The fractions determined were inorganic P, acid-soluble P, lipid P, total nucleic acid P and residual P. All fractions were determined on each femur. In this method, cold 5% trichloroacetic acid (TCA) is used to extract inorganic P and acid-soluble P. The inorganic P is determined directly on the TCA extract. The acid-soluble P is determined after wet-ashing an aliquot of the TCA extract with 0.8 N $HClO_4$ in 10 N H_2SO_4. Lipid P is extracted from the acid-insoluble residue with absolute ethanol containing 0.1 N potassium acetate. Nucleic acid P is extracted from the ethanol insoluble residue with 0.3 N $HClO_4$ at 75°C for one hour. The insoluble material after this extraction is called the residual fraction.

Each of the bound phosphorus fractions was evaporated to dryness and wet-ashed. The phosphorus content of each fraction was determined in the same manner, using standards which were carried through the same drying and wet-ashing procedure. Optical densities were read in Bessey-Lowry cuvettes (11) with a Beckmann DU Spectrophotometer. For details of the procedure the original paper should be consulted(10).

Alkaline phosphatase determination. The method used was essentially that of Bessey, Lowry and Brock(12), however the bicarbonate buffer of Harris and Mehl(13) was substituted for glycine buffer. Each femur was homogenized with 25 μl of cold buffer and the pestle rinsed with another 25 μl of buffer. Then 50 μl of cold 1% p-nitrophenylphosphate was added and the mixture incubated for 15 minutes at 37°C. The reaction was stopped by addition of 100 μl of 5% TCA. After centrifugation, the supernatant was removed and diluted with 5% TCA according to the phosphatase activity. A 100 μl aliquot of the diluted supernatant was added to 3.0 ml of 0.02 N NaOH and the absorbance read at 410 mμ. A standard curve was prepared with p-nitrophenol, with a range of 0.02 μm to 0.14 μm P-NP in 3.1 ml 0.02 N NaOH. Activity was expressed as μm P-NP released after 15 minutes incubation.

Protein nitrogen determination. The precipitates left after the alkaline phosphatase determinations were stored in 5% TCA at 4°C and used for this analysis. The same extractions that were used for the phosphorus fractions were performed on these precipitates. The residue remaining after these extractions was considered to contain little nitrogen except for that in protein. The nitrogen content of the residue was determined by the method of Lang(14).

Statistical method. The data obtained from the various analyses described for each culture period were statistically analyzed using the Student's t-test. The 99% confidence limits shown in Fig. 1 through 6 were calculated from the standard error of the mean according to Alder and Roessler(15).

Histochemical studies on alkaline phosphatase. Fixation and clearing of the bones was performed in a cold room at 4°C. After cooling to 4°C for 30 minutes, they were fixed in 3 changes of absolute acetone, for one hour each, *in vacuo*. They then were transferred through one change of absolute ethyl alcohol and 2 changes of benzene, again

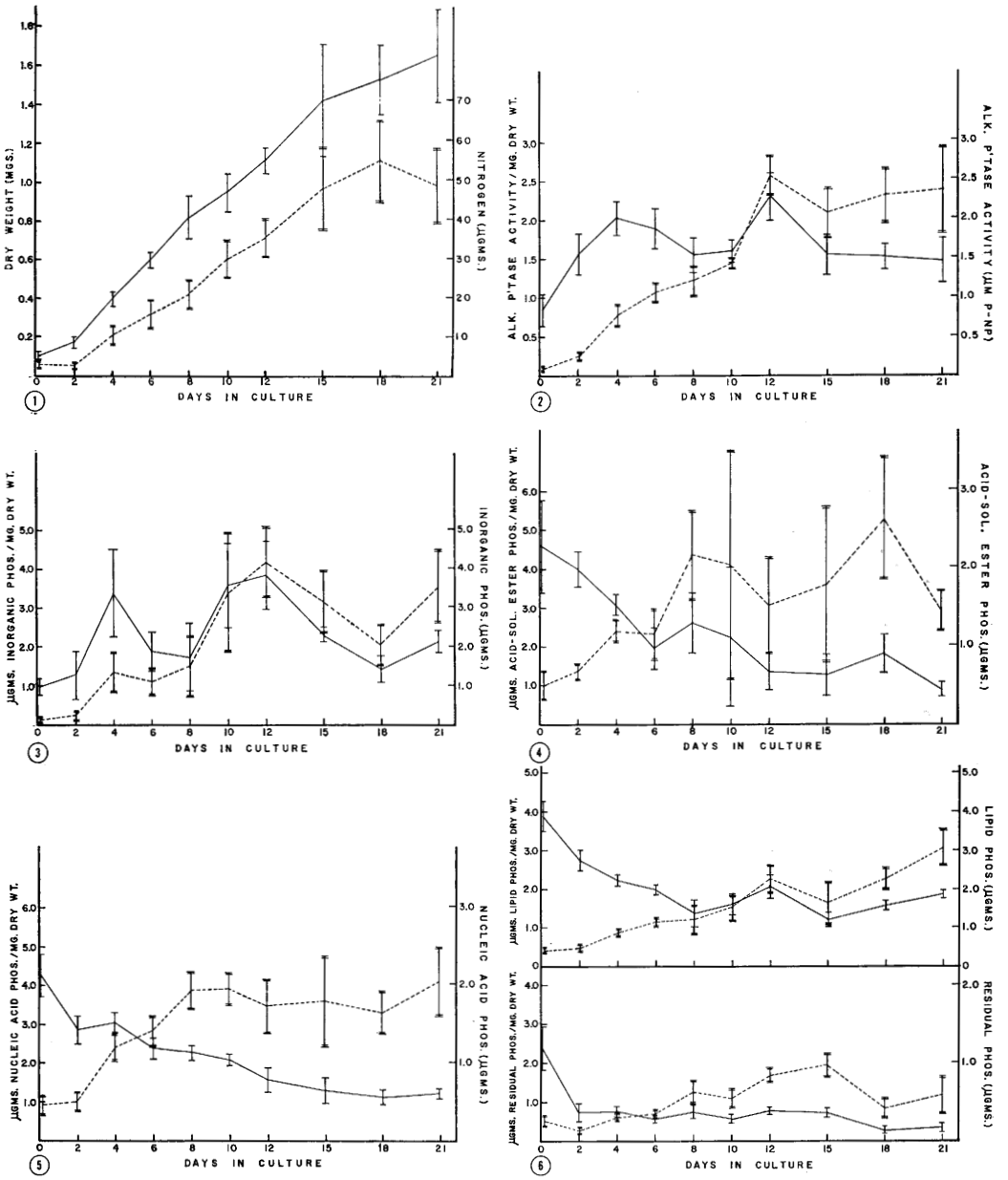

FIG. 1-6. Data obtained from cultivation of 7-day embryonic chick femora in rooster serum and embryo extract (3:1). Curves in all the Figures are drawn through the mean values obtained by analysis of 10 to 12 femora for each culture period. Vertical lines represent 99% confidence limits. In Fig. 2-6, broken line curves represent total or absolute values per femur for the parameter measured; solid line curves represent the amount in relation to dry weight of femur. The Figures represent the following:

FIG. 1. Growth curve of 7-day embryonic chick femora based on either dry weight (solid line) or protein nitrogen (broken line).

FIG. 2. Changes in alkaline phosphatase activity.

FIG. 3. Changes in inorganic phosphorus content.

FIG. 4. Changes in acid-soluble ester phosphorus.

FIG. 5. Changes in nucleic acid phosphorus.

FIG. 6. Changes in lipid phosphorus (top graph) and changes in residual phosphorus (bottom graph).

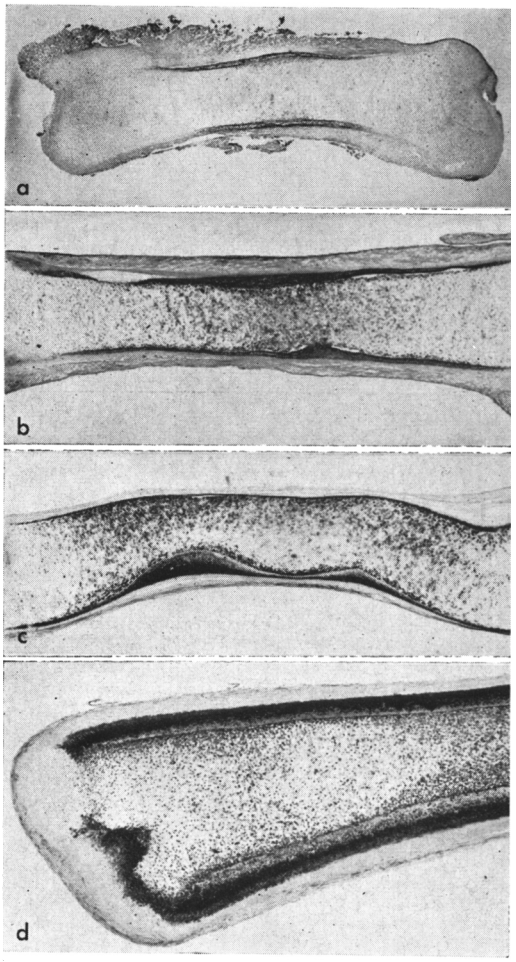

FIG. 7. Alkaline phosphatase distribution in cultured femora. (a) 7-Day embryo femur. 31 ×. (b) 7-Day embryo femur cultured 4 days. 31 ×. (c) 7-Day embryo femur cultured 8 days. 31 ×. (d) 7-Day embryo femur cultured 18 days. 31 ×.

for one hour in each bath. Embedding in paraffin was at 55°C *in vacuo*. Three changes of paraffin were used, for 15 minutes each. Sectioning was performed at 8 to 9 μ. The histochemical staining procedure was essentially the simultaneous coupling azo dye method described by Pearse(16). The substrate was α-naphthyl phosphate and the stable diazotate was Naphthanil Diazo Red RC (4-chloro-o-anisidine, Dajac). The sections were incubated in the reaction mixture for 30 minutes at 26°C, and after washing were mounted in 50% (aqueous) polyvinyl pyrrolidone.

Results. All of the data obtained are shown in Fig. 1 through 6, and the results of the histochemical studies of alkaline phosphatase are shown in Fig. 7. In the first 6 Figures, the lines are drawn through the average values in all cases. Two typical biological growth curves are seen in Fig. 1, where either total dry weight of the femora or protein nitrogen is a measure of growth during the cultivation period.

Fig. 2 shows changes in alkaline phosphatase activity during the cultivation period, both in absolute values and in terms of activity per dry weight of the femora. Two distinct peaks of activity relative to dry weight are seen, one at 4 days and the other at 12 days. When total phosphatase activity is plotted, only the peak at 12 days is demonstrated.

During the cultivation of embryonic chick femora, 5 different phosphorus fractions were determined. These fractions are plotted in Fig. 3 through 6 as absolute values and also as μg/mg dry weight of the femora. The values for acid-soluble ester P are the differences between total acid-soluble P and inorganic P. When Fig. 2 and 3 are compared, changes in the ratios of inorganic P to dry weight seem to correlate well with the peaks of alkaline phosphatase activity after 4 and 12 days in culture. The amount of acid-soluble ester P per mg dry weight as shown in Fig. 4, appears to be related to alkaline phosphatase activity in a reciprocal manner at about 4 and 12 days in culture. The curves in Fig. 5 and 6 show a gradual decline and then a leveling off, when the ratios of nucleic acid and lipid P respectively to dry weight are plotted against length of time in culture. The ratio of residual P to dry weight is rather constant during the cultivation period. The rather sharp decrease from zero to two days in culture in Fig. 4, 5, and 6 may be due to acclimatization of the femora to *in vitro* conditions.

Discussion. Since it was not the purpose of this study to confirm previously established optimum conditions for cultivation of embryonic chick femora, a simple natural medium with a pH range of 7.3-7.7 proved satisfactory, as evidenced by a lag of only 2 days

in the growth curve. An overall increase in size of the femora was noted macroscopically as early as 3 days after cultivation, and the explanted femora increased to approximately 3 or 4 times their original length during the course of 21 days in culture. On the basis of macroscopic observation, the femora seemed to show very little increase in length after 12 to 15 days in culture, but the increase in weight continued until the end of the culture period.

When phosphatase activity was expressed per mg dry weight we obtained 2 peaks of activity, at 4 and 12 days, as contrasted with a single peak at 12 days when the activity per bone was plotted (Fig. 2). Since the bones are growing most rapidly between 2 and 8 days (Fig. 1), the drop in relative activity after 4 days probably means that growth is exceeding osteoblastic activity. At 12 days, there is a peak both in total and relative phosphatase activity. Histochemistry bears out the fact that concomitant with increasing osteoblastic activity there is degeneration of hypertrophic cartilage cells which contributes to the phosphatase activity of the homogenates. We believe that the peak of phosphatase activity at 12 days reflects increased cartilage cell degeneration at this time in addition to the continuing increase in osteoblastic activity.

The inorganic P content of the femora parallels in time the phosphatase activity, and there is a roughly reciprocal relationship with the acid-soluble ester P content. This may indicate that the substrate for the enzyme is an acid-soluble ester of phosphoric acid and is in agreement with the work of Whitehead and Weidmann(19,20), in which they demonstrated the significance of the acid-soluble phosphates as compared with the acid-insoluble phosphates of ossifying cartilage. Changes in lipid P and in nucleic acid P seem to have little significance or correlation with alkaline phosphatase activity. The gradual decrease in amount of the nucleic acid P fraction in relation to dry weight until the fifteenth day of cultivation may be explained by the fact that the cartilage cells hypertrophy during this period with little increase in cell numbers, but dry weight of the femora increases due to continued elaboration of the organic matrix. Residual P also seems to have no apparent correlation with alkaline phosphatase activity.

Histochemical studies of the distribution of alkaline phosphatase were done to supplement the quantitative biochemical data and aid in its interpretation. Fig. 7 shows sections made from 7-day femora, before and after cultivation for 4, 8, and 18 days. These cultures were not run simultaneously with those used for the chemical analysis, and therefore the changes noted here may not correspond exactly in time with those noted in the previous cultures. In the 7-day femur, alkaline phosphatase activity is seen to be localized in the diaphysis, in those cells that will shortly differentiate into osteoblasts. There is little alkaline phosphatase activity in the cartilage. After 4 days in culture, osteoblasts have differentiated, and are demonstrating intense alkaline phosphatase activity. By this time, the hypertrophic cartilage cells in the diaphysis have degenerated, and are now clearly showing the alkaline phosphatase activity observed by Fell and Robison(2,21), and Henrichsen(22). As seen in the sections made from bones cultured for 8 and 18 days, we have a progressive extension of the same sequence of events towards the ends of the bones. However, at all times the most intense alkaline phosphatase activity is seen to occur in the perichondrium and subadjacent osteoid. This agrees with the results of Henrichsen, who observed the same distribution *in vivo*. Henrichsen pointed out that the increase in cartilaginous distribution of alkaline phosphatase probably reflects the progressive degeneration of the hypertrophic cartilage. We think it is significant, however, that the intensity of the reaction never approaches that associated with osteoblasts, either in our own study, or in that of Henrichsen. The principal conclusion that we wish to make from the histochemical study, is that the total alkaline phosphatase activity of a homogenate of these cultures is primarily a measure of osteoblastic activity,

with some smaller activity due to cartilage cell degeneration.

Summary. A study has been made of the role of alkaline phosphatase in the osteogenesis of 7-day embryonic chick femora during 21 days in organ culture. The femora were analyzed for their content of inorganic P, acid-soluble ester P, lipid P, total nucleic acid P and residual P, after increasing culture periods. The results indicate that there is a close parallel between inorganic P content and alkaline phosphatase activity. Acid soluble ester P content shows a roughly reciprocal relationship to phosphatase activity, while the other P fractions do not appear to be related.

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Viruses and Mammalian Chromosomes. III. Effect of Herpes Zoster Virus on Human Embryonal Lung Cultures.* (29633)

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Evidence accumulated during the past years supports the concept that some viruses can induce chromosome aberrations(1-4) in host cells; this often leads to formation of new cell types with abnormal chromosome complements(5-8). However, our knowledge of this problem is still fragmentary. It is not known whether the ability of a virus to induce chromosome damage is correlated with its composition, structure, site of multiplication, virulence, toxic products, or with other aspects of virus-cell interaction. It is, therefore, important to survey the virus-chromosome relationships of a variety of virus-host cell systems in order to elucidate these possibilities. The present paper describes several cytological and chromosomal aberrations observed in diploid human cells infected in

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