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Development of parathyroid hormone- and calcitonin-activated adenylate cyclases in embryonic chicken limb and in cultured cells from embryonic chicken limb

(osteoblast/osteoclast/development/calcium metabolism)

JAMES E. ZULL, STEVEN KRUG, DIANE ABEL, AND ARNOLD I. CAPLAN

Department of Biology, Case Western Reserve University, Cleveland, Ohio 44106

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ABSTRACT **Activation of adenylate cyclase [ATP pyrophosphate-lyase (cyclizing), EC 4.6.1.1] by parathyroid hormone (PTH) and calcitonin was measured as a function of stage of development in embryonic chicken limb buds. Responsiveness to both hormones develops in the tissue at the time when nascent bone is forming. In addition, a temporal sequence of development of hormone response was observed, with a PTH-activated adenylate cyclase appearing earlier than the calcitonin-activated enzyme. The responsiveness to the two hormones was additive, indicating the presence of two receptor populations. Undifferentiated cells obtained from limb buds prior to appearance of hormonal responsiveness were cultured and were found to develop a PTH-activated adenylate cyclase *in vitro*. However, a calcitonin-stimulated enzyme did not appear in such cultures. The PTH-activated enzyme was found to be similar to that present in bone in regard to its sensitivity to PTH. The enzyme did not respond to other hormones, and myoblast cultures did not develop a PTH-activated adenylate cyclase, indicating that a true bone adenylate cyclase was being measured.**

It has recently become clear that in bone, the adenylate cyclases [ATP pyrophosphate-lyase (cyclizing), EC 4.6.1.1] activated by parathyroid hormone (PTH) and calcitonin (CT) exist on separate cells. The most direct evidence for this conclusion comes from the work of Wong and Cohn (1, 2) who, by sequential collagenase digestions, were able to separate two populations of cells from calvaria. One population of cells (CT cells) possess both a CT- and a PTH-activated adenylate cyclase; the other population (PT cells) have an adenylate cyclase that is activated only by PTH. Later studies showed that the CT cells resorb dead bone in response to PTH and most probably are osteoclasts (3). Additional analysis of the enzymatic content and responsiveness to PTH and CT suggested that the PT cells are osteoblasts (4). Independent studies in Peck's laboratory using a mechanical separation technique also showed that adenylate cyclase of osteoblasts is activated by PTH and not by CT, whereas CT-stimulated production of cyclic AMP is observed in a separate cell population obtained from the periosteum (5). These important findings suggested to us the possibility that, by assay of the hormonally activated adenylate cyclases in developing bone tissue, it should be possible to delineate the temporal relationships for appearance of osteoblasts and osteoclasts and to define enzymatically the emergence of bone cells.

For such a study, the developing chicken limb has several advantages. First, there is a substantial literature on the development of bone in this tissue dating from the early work of Fell (6) and Fitton-Jackson (7). Second, a great deal is known about the development of muscle and cartilage in this tissue, and the development of these phenotypes can be regulated (8, 9). Third, cells from embryonic chicken limb buds can be cultured and certain developmental events reproduced *in vitro* (10, 11). Fourth, we recently observed that some bone-like ossified tissue develops in cultures of undifferentiated limb bud mesenchymal cells (12, 13), and it seemed possible that examination of osteogenesis *in vitro* could be conducted in parallel with studies of the *in vivo* development of hormonally responsive adenylate cyclases.

In this study we have asked: (*i*) Does the appearance of nascent bone in the chicken limb correspond temporally with the appearance of PTH- and CT-activated adenylate cyclases in the limb bud? and (*ii*) If so, can the appearance of these hormonally activated cyclases be observed and used as markers to study bone cell differentiation *in vitro*?

MATERIALS AND METHODS

Tissue and Cell Culture. Limb buds from chick embryos were dissected and either directly homogenized for membrane preparation and adenylate cyclase assay or treated with trypsin for preparation of cells for culture as described (10, 11).

Myogenic cells from 12-day embryo chicken leg muscle were isolated as described and were plated at 1×10^6 cells per 60-mm plate. Antibacterial and antimycotic agents were present in all of the salt solutions and media used in this study (1% Gibco "antimycotic antibiotic agent").

Treatment of Tissue for Assay. For adenylate cyclase assays, a crude homogenate of the cultured cells or intact tissue was prepared. With cultured cells, the procedure was as follows. One hour after feeding, the medium was decanted and the cells were rinsed with 1 ml of Tyrode's solution. Then, 3 ml of 0.1 M Tris buffer (pH 7.5) was added to one plate and the cells were removed by scraping with a rubber policeman. Cells from several plates were combined by sequential transfer of this 3-ml suspension. The cells were then centrifuged at $3000 \times g$ for 1 min, the buffer was discarded, and an additional 1.5 ml of 0.1 M Tris (pH 7.5) was added. This cell suspension was homogenized with 10 passes of a Teflon/glass homogenizer and the homogenate was centrifuged at $5000 \times g$ for 30 min. The pellet was washed once with 10 ml of Tris buffer, and the pellet from this wash was suspended in 3 ml of the same buffer. The number of plates used for each experiment varied with the time in culture and the density of plating. The range was from 4 plates (35-mm diameter) per membrane preparation to up to 24 plates in the early-time and low-density preparations.

For the intact limb bud tissue, the procedure was identical

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Abbreviations: PTH, parathyroid hormone; CT, calcitonin.

to the above, beginning with the homogenization step after dissection in Tyrode's solution. The number of limb buds used for an assay on a given day was a function of the age of the limb buds and varied from 150 to 250.

Adenylate Cyclase Assays. The medium described by Marcus and Aurbach (14) was used for all assays. The reactions with [α-^{32}P]ATP as the substrate were initiated by addition of membranes. At the end of the incubation time (up to 30 min) the reaction was stopped as described by Solomon *et al.* (15). Cyclic [^{3}H]AMP was then added. After they had been at room temperature for 1 hr, the incubation solutions were transferred to dry alumina columns in pasteur pipets as described by Ramachandran and Lee (16) for separation of cyclic AMP. The eluate from these columns was suspended in 8 ml of Aquasol (New England Nuclear) and the resultant gel was assayed for ^{3}H and ^{32}P in a liquid scintillation counter. Zero-time values were subtracted in all experiments.

Chemical Assays. Protein was measured by the method of Lowry *et al.* (17), phospholipid by the measurement of total phosphate in chloroform/methanol extracts of tissue by the method of Fiske and Subbarow (18), and DNA by the method of Burton (19).

Materials. Purified bovine PTH was prepared in our laboratory by described methods (20, 21). Salmon CT, glucagon, and insulin were obtained from Sigma. Epinephrine was a gift from Michael Maguire (Department of Pharmacology, Case Western Reserve University). All chemicals for the enzyme and other assays were of the highest purity available.

RESULTS

The developmental stages of the chicken limb studied ranged from stage 22 ($3\frac{1}{2}$ days after fertilization) to stage 34 (8 days after fertilization). Between stages 22 and 24, cells from the limb bud are undifferentiated with respect to the major phenotypes: muscle, cartilage, and bone. After stage 24 the cells are "committed" for expression of a particular phenotype (22). By stage 26 the cellular phenotypes of muscle and cartilage are present but identification of bone cells or pre-bone cells has not been reported. Osteoid is first observable at stages 28–29 and nascent calcified bone appears at or near stage 31. By stage 34, a morphologically fully developed limb is present.

Preliminary studies showed that the crude membrane fraction from both whole limb bud homogenates and cultured cells contained a fluoride-activated adenylate cyclase. Production of cyclic AMP was linear over a period of up to 30 min through a concentration range of 0 to 700 μg of protein per 100 μl for assays run in both the presence and absence of fluoride or hormones. Although the amount of protein in the various samples assayed is a complex function of the cell phenotypes present (e.g., a large amount of extracellular protein will be present after bone and cartilage development begins) and therefore activities based on amount of protein present have little meaning, all further assays were conducted at a protein concentration of approximately 500 μg per incubation mixture.

Typical adenylate cyclase data from the limb bud tissue obtained before (stage 26), during (stage 29), and after (stage 33) bone development are shown in Table 1. At the earliest stage, no hormonally activated adenylate cyclase was observed, although an approximately 7-fold stimulation by fluoride was seen. In fact, CT induced a significant decrease in cyclic AMP after 30 min of incubation. In contrast to stage 26, by stage 29 a clearly significant PTH activation of adenylate cyclase was observed, in agreement with previous indications that bone begins to form at or near this stage in limb development. However, at this stage there was still no CT-activated adenylate cyclase, although the apparent inhibition by this hormone was not detected. Fluoride stimulated the basal activity approximately 8-fold in tissue from stage 29. At stage 33, a significant increase in adenylate cyclase activity was produced by addition of either CT or PTH, and the effects of the individual hormones were additive. The lower basal activity at this stage probably represents the presence of the much larger amounts of noncellular protein in the much more fully developed limb. Nonetheless, there was a distinct increase above basal levels of enzyme activity upon addition of the hormones and it is clear that both PTH- and CT- activated adenylate cyclases were now present in the tissue.

Fig. 1 presents a more complete developmental study of the activity of adenylate cyclase in chicken limb buds. Because it in no way alters the conclusions drawn, these data are shown as ratios of the hormone- or fluoride-stimulated activity to the basal activity in order to more clearly indicate the time at which hormonal responses appear in the tissue. Again, prior to stage 26, we found a large fluoride-stimulated adenylate cyclase but little responsiveness to either PTH or CT. However, between stages 26 and 31 significant activation by PTH became evident (accompanied by an apparent inhibition by CT) and after stage 31 both PTH- and CT-activated adenylate cyclase were present. Of interest is the time sequence for development of the responsiveness to the two hormones, the PTH-activated enzyme apparently *preceding* the CT-activated enzyme cyclase. For example, at stage 28 the tissue clearly had a PTH-activated enzyme but, in contrast, CT significantly decreased cyclic AMP accumulation to below basal levels. However, by stage 31, significant activation by both hormones was observed. The maximal responsiveness to PTH was seen at stage 32 and the PTH stimulation was generally greater than the CT stimulation. Again, after development of activation by both hormones, their effects were additive. Both the magnitude and pattern of a large increase followed by a decrease in the PTH-responsive enzyme were reproducible, suggesting that major changes in the balance of hormonally sensitive adenylate cyclases take place near stage 32 in limb development. The data also suggest the presence of separate receptors and, most likely, separate cell types, each responsive to one hormone and each developing at a different time during differentiation in the limb.

We next investigated whether the same hormonal responses would develop in cultured cells obtained from early stages of limb development and, if so, whether the PTH activation would also precede the CT activation *in vitro*.

In Fig. 2, the data obtained from cultured stage 24 limb bud cells and assayed for 5 subsequent days for adenylate cyclase are presented both on a specific activity basis and as S/B ratios for comparison with Fig. 1. Specific activities are expressed on a total phospholipid basis in order to correct for possible changes in membrane amount per cell during the experimental period. The basal enzyme activity (specific activity) increased up to days 3–4 in culture and then decreased. Other experiments showed that this pattern was consistent and that, in general, although the *total* basal activity increased slowly for up to 20 days in culture, specific activity tended to increase slowly or not at all after day 4. Of interest was the appearance of a significant PTH stimulation of adenylate cyclase over the basal activity on days 2, 3, and 4 in culture, a time that corresponds generally with the *in vivo* development of hormonal responses. Because the parent cells (stage 24 limb buds) have no PTH-activated adenylate cyclase, this result must arise from the differentiation of a new cell type *in vitro*. Of equal interest was the observation that no CT-activation of adenylate cyclase was

Table 1. Effects of PTH, CT, and fluoride on adenylate cyclase activity in chicken limb buds at various stages of development

Stage	Adenylate cyclase activity* Basal	+ PTH (1 μM)	+ CT (0.1 μM)	+ PTH + CT	+ F^- (10 mM)
26	49.8 ± 2.2	52.2 ± 2.5	38.7 ± 1.8	—	352 ± 2.5
29	40.5 ± 1.3	49.2 ± 0.7†	42.3 ± 0.9	51.1 ± 0.6‡	363 ± 4.9
33	26.4 ± 0.7	53.5 ± 1.3†	37.4 ± 0.7†	62.8 ± 0.6‡	171 ± 8.4

* Data are expressed as pmol of cyclic AMP/30 min (mean ± SEM); each value is the mean of 10 replicates. Mean zero-time values are subtracted from all data. For example, stage 26, zero-time = 18.2 ± 1.9 pmol cyclic AMP, basal = 68.0 ± 2.2, etc.

† $P < 0.001$ for difference between hormone-treated and basal activities.

‡ $P < 0.001$ for difference between both hormones and one hormone.

seen in the cultured cells even at the later stages corresponding to the time when a maximal activation was seen in the limb buds. However, a significant decrease in cyclic AMP accumulation below basal levels was again observed (days 4 and 5) with CT.

The data shown in Fig. 2 were obtained with cells plated at a density of 1500 cells per mm^2. Because the phenotype expressed in cultured cells from chicken limb buds depends on the density of plating (23), similar experiments were conducted with cells plated at a higher density (6000 cells per mm^2). This density leads to increased expression of the cartilage phenotype and decreased amounts of muscle cells in culture (10, 11). The higher-density cultures (Fig. 3) gave results similar to those seen with the lower density cultures in a qualitative sense but the hormonal response developed earlier, was larger, and disappeared more rapidly. Additionally, no significant CT effects were observed in the high-density plates, some of which were cultured for up to 20 days before assay.

The specificity and sensitivity of the PTH-responsive adenylate cyclase in high-density plates after 2 days of culture were characterized further. The enzyme was activated by Mg^{2+} in the expected manner with maximal activity being reached at 5 mM $MgCl_2$ (data not shown). As indicated in Fig. 4, the cells were highly sensitive to PTH, significant activation being observed at hormone concentrations as low as 10 nM and a maximal response at 0.5 μM. This is comparable to the sensitivity seen in other isolated bone cell systems (24) and to the PTH-responsive adenylate cyclase in isolated kidney membranes (25). In addition, at no time in the development of the tissue was a response to another hormone observed: CT, insulin, glucagon, and epinephrine had no effect on the adenylate cyclase in day 2 cultures from high-density plates over a concentration range of 1–1000 nM.

The tissue specificity of the PTH response in cultured cells was investigated by comparing the response in cultured myogenic cells under identical conditions to those used for the

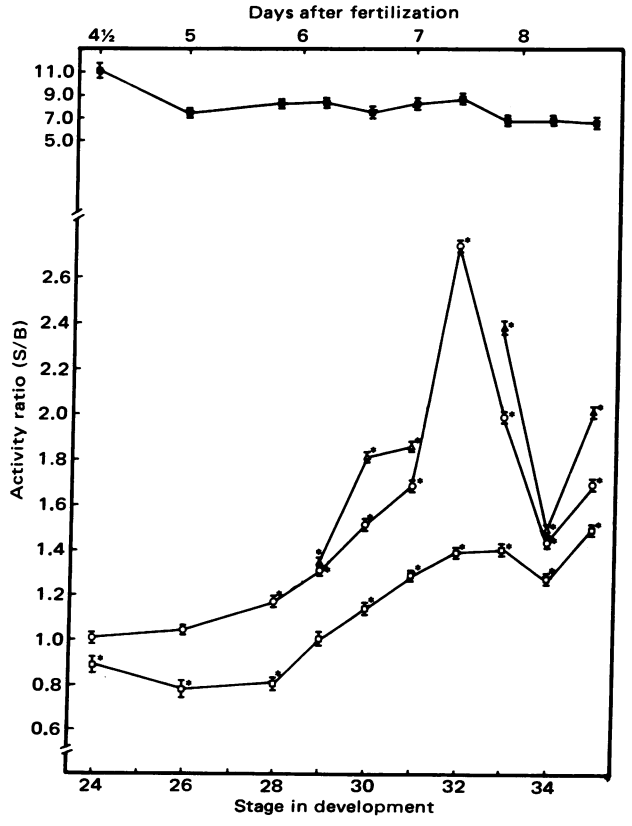

FIG. 1. Development of hormone-stimulated adenylate cyclases in the chicken limb bud. The ratio of the stimulated to basal (S/B) activity is plotted as a function of stage in development (bottom axis) and days after fertilization (top axis). The tissue from each stage was assayed for basal, hormonally activated, and fluoride-activated activity in the same experiment. Each point is the mean of a minimum of 30 replicates ± SEM. ■, Fluoride (10 mM); ○, PTH (1 μM); □, CT (1 μM); △, CT + PTH. *, $P < 0.001$ for difference between hormone-responsive and basal activities ($n > 30$ in all cases).

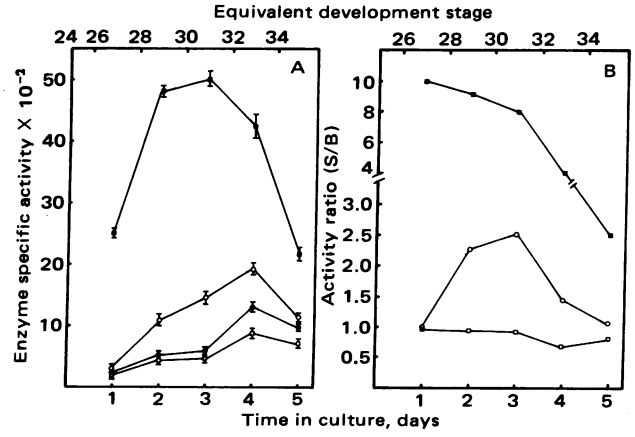

FIG. 2. Appearance of PTH-activated adenylate cyclase in cultured mesodermal cells from stage 24 limb buds plated at 1500 cells per mm^2. The data are presented as specific activities (pmol of cyclic AMP/30 min per μg of phospholipid) (*A*) and as ratios (see Fig. 1) (*B*). For comparison, the equivalent stage for *in vivo* development is plotted on the top of each figure. Each point represents the mean (± SEM) of at least five replicates. ■, Fluoride (10 mM); ○, PTH (1 μM); □, CT (1 μM); ●, basal activity.

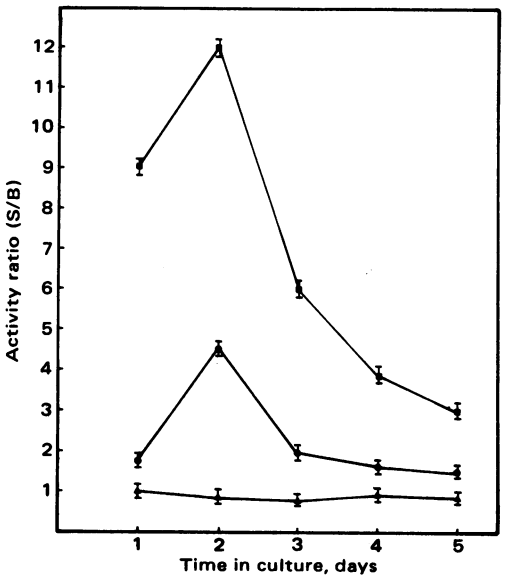

FIG. 3. Appearance of PTH-stimulated adenylate cyclase in cultured mesodermal cells from stage 24 chicken limb buds plated at high density (6000 cells per mm^2). Experimental conditions and data presentation are as in Fig. 2. Each point is the mean (± SEM) of at least 10 replicates. ■, Fluoride; ●, PTH; ▲, CT.

isolated limb bud cells. Such cells developed no PTH-sensitive adenylate cyclase for periods of up to 6 days in culture.

DISCUSSION

All of the experiments described here were done with highly heterogeneous cell populations. Therefore, interpretations of the data in terms of bone cell development depends on the assumption that, of all the cells present in either the whole limb buds or the cultured cells, only bone cells possess PTH- and CT-activated adenylate cyclase. Of the many cell types present in the limb bud tissue, the major phenotypes are muscle, cartilage, and bone. In addition, significant numbers of undifferentiated mesodermal cells and fibroblasts are present, but these cells obviously do not possess hormonally activated adenylated cyclase because they exist at all stages of development including those which show no hormonal sensitivity (Figs. 1–3). We tested muscle directly and the results clearly showed that myogenic tissue does not develop such cyclases.

It has been reported that cartilage cells have a PTH-sensitive adenylate cyclase (23), raising the possibility that we are observing the differentiation of cartilage. However, the reported PTH-responsive adenylate cyclases in cartilage shows significant differences from bone cell systems and from the response that develops in the cultured chicken limb bud cells. The

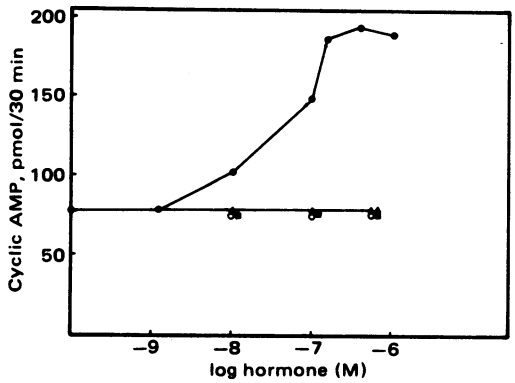

FIG. 4. Hormone concentration dependency and specificity for activation of the adenylate cyclase in cultured chicken limb bud cells. The experiment was conducted with cells plated at high density and cultured for 2 days. Each point is the mean of at least three replicates. ●, PTH; ○, CT; ■, insulin; ▲, glucogen; △, epinephrine.

chicken limb enzyme in our developing bone system shows a PTH concentration-dependence of a similar order of magnitude to that reported for cultured bone cells (Fig. 4) (24), whereas the apparent K_A for PTH activation of the enzyme detected in cartilage cells was 1 μM and no significant effect was seen with hormone concentrations below 0.1 μM. Furthermore, the hormone responses to PTH and CT are additive in bone cell systems and in the limb bud cell system, but such additivity was not observed with the cartilage cells. In addition, if the responses observed in cultured cells are due to development of a cartilage phenotype, these responses should be greatest during the period when the cartilage phenotype becomes dominant—i.e., from days 5–8 in culture (10). Because the responses we see are earlier than this and have virtually disappeared by day 6 (Figs. 2 and 3), we believe it most likely that we are observing bone cell differentiation *in vitro*. However, it is possible that a common precursor cell to chondroblasts and osteoblasts (26) develops in culture. If this were so, and if the development of a PTH-sensitive cyclase were a very early event in osteogenesis and chondrogenesis, then the question might well be moot—i.e., we see the common precursor cell.

It is of interest to compare the timing of the appearance of these enzymes *in vivo* with other known events related to osteogenesis in the chicken limb. This is done in Table 2. From this table and from the data in Fig. 1, it is possible to divide bone development into three stages: (*i*) early stages when no bone cells are present but a slight apparent inhibition of adenylate cyclase by CT is observed; (*ii*) a middle stage when only osteoblasts are present, nascent bone formation is taking place, and PTH-activated adenylate cyclase is present (possibly associated with CT inhibition); and (*iii*) a late stage when vascular invasion occurs, resorption begins, and a CT-activated cyclase

Table 2. Bone development in the chicken limb

Day	Stage	Bone cell types observed*	Observed osteogenic events	Action on adenylate cyclase	
				PTH	CT
4½	24	None	None	None	Slight inhibition
5	26	None	None	Slight activation	Slight inhibition
6	28–29	Ob	First osteoid	Significant activation	Slight inhibition
7	31	Ob + Oc	First periosteal bone	Maximal activation	Slight activation
7½	32	Ob + Oc + Ocl	Cartilage resorption, vascular invasion of bone	Activation	Maximal activation

* Histologically observed. Ob, osteoblasts; Oc, osteocytes, Ocl, osteoclasts.

is added to the tissue. These correlations lend support to the conclusions of Cohn's group and Peck *et al.* (1–5) that the PTH-activated adenylate cyclase is characteristic of osteoblasts and the CT-activated cyclase is a marker enzyme for osteoclasts.

From Fig. 1 and Table 2, it is clear that PTH-sensitive cells can be detected by the adenylate cyclase assay as soon as, or slightly sooner than, any other measureable osteogenic event. Therefore, we suggest that osteoblasts possess PTH receptors as a stable, original feature of the phenotype and that the PTH-activated cyclase is a valid, sensitive, and precise marker for osteoblasts. Although it has been known for some time that fetal bone is sensitive to PTH, this is by far the earliest reported response to the hormone and we believe that it marks the primary osteogenic event in the developing tissue. Parallel conclusions also seem possible for CT-stimulated adenylate cyclase and osteoclasts, the appearance of the CT-activation of adenylate cyclase marking the primary resorptive event in bone development. However, this conclusion is somewhat clouded by the apparent inhibition of cyclic AMP accumulation in the assays done on tissue from early stages of development, because this phenomenon makes it difficult to identify the exact stage of development at which CT-activated adenylate cyclase actually appears.

The development of a PTH-activated adenylate cyclase from undifferentiated mesodermal cells when cultured *in vitro* suggests that the pre-osteoblast is mesodermal in orgin. On the other hand, the lack of development of CT-activated adenylate cyclase under similar conditions *in vitro* is in agreement with the recent conclusions of Walker (27) that pre-osteoclasts are not a part of and do not originate in mesodermal tissue but rather are vascular in origin. However, it is possible that pre-osteoclasts in the limb bud do not survive the preparative steps of early culture events or that some biochemical event or signal that stimulates their differentiation is missing. Nonetheless, the following working hypothesis seems reasonable for the sequence of steps leading to bone development in the chicken: (*i*) undifferentiated mesodermal cells differentiate into pre-osteoblasts and then into osteoblasts which begin to make nascent bone; (*ii*) nascent bone is invaded by osteoclasts which probably originate in the vascular system (27) and which add the final cellular phenotype to produce "complete" bone in terms of cellular content of the tissue. This interpretation of the *in vivo* development of hormone-activated adenylate cyclases (Fig. 1) is supported by the *in vitro* data in Figs. 3 and 4 but will require further testing for its substantiation.

Two observations remain unexplained at the moment and will require further work. First, an explanation for the decreasing amount of PTH-activated adenylate cyclase after 2–3 days in culture (Figs. 2 and 3) is not available. It may be due to an overgrowth of the osteoblasts by other cell types in this system or to assay problems resulting from the increasing complexity of the system with time in culture (e.g., the PTH-sensitive cells are buried in cartilage matrix). Alternatively, it may directly reflect the normal life-span of osteoblasts in culture. Second, the effects of CT on the production of cyclic AMP in the very early stages (stages 26–28) of development need to be explored further. The clearly significant reduction in the amount of cyclic AMP below basal levels by tissue obtained from those stages of development (Table 1, Fig. 1) could be due to an inhibition of adenylate cyclase, an activation of phosphodiesterase, or to the action of CT on some unknown regulatory component in the system (e.g., Ca^{2+}-dependent regulator, see ref. 28). To our knowledge no reports of any such action of CT have appeared in the literature, and it is clear that further study of this phenomenon and its biological significance will be required.

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1. Wong, G. & Cohn, D. (1974) *Nature* **252,** 713–714.
2. Wong, G. & Cohn, D. V. (1975) *Proc. Natl. Acad. Sci. USA* **67,** 305–307.
3. Luben, R. A., Wong, G. L. & Cohn, D. A. (1977) *Nature* **265,** 629–630.
4. Luben, R. A., Wong, A. & Cohn, O. V. (1976) *Endocrinology* **99,** 526–534.
5. Peck, W. A., Burks, J. K., Wilkins, J., Rodan, S. B. & Rodan, G. A. (1977) *Endocrinology* **100,** 1357–1364.
6. Fell, H. B., (1925) *J. Morph. Physiol.* **40,** 418–459.
7. Fitton-Jackson, S. (1956) *Proc. Roy. Soc. (London)* **146,** 270–285.
8. Zwilling, E. (1968) *Dev. Biol. Suppl.* **2,** 184–207.
9. Caplan, A. I. & Rosenberg, M. J. (1975) in *Extracellular Matrix Influences on Gene Experession,* eds. Slavkin, H. & Gruelich, R. (Academic, New York), pp. 47–55.
10. Caplan, A. I., Zwilling, E. & Kapan, N. O. (1968) *Science* **160,** 1009–1010.
11. Caplan, A. I. (1970) *Exp. Cell Res.* **62,** 341–355.
12. Osdoby, P. & Caplan, A. I. (1976) *Dev. Biol.* **52,** 283–299.
13. Caplan, A. I. (1978) in *Somite and Limb Morphogenesis,* eds. Ede, D. A., Hinchcliffe, J. R. & Balls, M. (Cambridge Univ. Press, NY), in press.
14. Marcus, R. & Aurbach, A. D. (1971) *Biochim. Biophys. Acta* **242,** 410–421.
15. Solomon, R., Londes, C. & Rodbell, M. (1974) *Anal. Biochem.* 541–548.
16. Ramachandron, J. & Lee, V. (1970) *Biochem. Biophys. Res. Commun.* **41,** 358–366.
17. Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193,** 265–275.
18. Fiske, C. H. & SubbaRow, Y. (1925) *J. Biol. Chem.* **66,** 375.
19. Burton, K. (1968) *Methods Enzymol.* **12,** 163–166.
20. Zull, J. E., & Chuang, J. (1975) *J. Biol. Chem.* **250,** 1668–1675.
21. Zull, J. E. & Malbon, C. C. (1976) in *Methods in Molecular Biology-Methods in Receptor Research,* ed. Bletcher, M. (Dekker, New York), Vol. 9, Part 2, pp. 533–564.
22. Searls, R. L. & Janners, N. Y. (1969) *J. Exp. Zool.* **170,** 365–375.
23. Smith, D. M., Roth, L. M. & Johnston, C. C., Jr. (1976) *Endocrinology* **98,** 242–246.
24. Peck, W. A., Carpenter, J., Messinger, R. & DeBro, D. (1973) *Endocrinology* **92,** 692–697.
25. Zull, J. E., Malbon, C. C. & Chuang, J. (1977) *J. Biol. Chem.* **252,** 1071–1078.
26. Hall, B. K. (1972) *Calcif. Tissue Res.* **8,** 131.
27. Walker, D. Q. (1975) *Science* **190,** 784–787.
28. Dedman, J. R., Jackson, R. C., Schreiber, W. E. & Means, A. R. (1978) *J. Biol. Chem.* **253,** 343–346.