Twelve-day-old embryonic chick mandibles were cultured in vitro for 6 days. Measurements of the weights of the explants, their mineral and protein components, and the EDTA-extractable proteins established that bone tissue synthesizes O-phosphoserine- and O-phosphothreonine-containing phosphoproteins which are similar to those present in embryonic and postnatal chicken bone matrix.

The synthesis of the phosphoproteins was further confirmed by the demonstration that radioactively labeled O-phosphoserine and O-phosphothreonine were identified in bone and in the EDTA-extractable phosphoproteins after pulse-labeling chick mandibles in vitro with radioactively labeled serine and threonine, respectively.

Phosphoproteins, present in all normally and pathologically calcified vertebrate tissues (1–4), have been postulated to play a significant role in the deposition of the solid phase of calcium-inorganic phosphorus during mineralization (1–7). While tissue culture experiments (8) and indirect autoradiographic evidence (9) strongly suggest that the phosphoproteins of dentin are synthesized in the tissue, no similar biochemical evidence has been presented for the bone phosphoproteins, which are chemically quite distinct from those of dentin and enamel (10). Indeed, two major noncollagenous proteins in bone matrix, albumin and α2HS-glycoprotein, are synthesized in the liver and then transported to bone where they are adsorbed and concentrated (11–14).

From experiments utilizing bone cultured in vitro, we present data which establish for the first time that phosphoproteins containing O-phosphoserine and O-phosphothreonine, which behave chromatographically similar to well characterized bone matrix phosphoproteins from both embryonic and postnatal chickens, are synthesized by bone tissue. A preliminary report of these experiments has already been presented (14).

EXPERIMENTAL PROCEDURES

Tissue Culture Techniques

No Radioactively Labeled Acids Utilized—Portions of 12-day-old embryonic chick mandibles, 8.1 mm in length, were used as tissue culture explants in a completely defined synthetic medium according to methods previously described (15–18). In this group of experiments carried out without the use of radioactively labeled amino acids, the tissue culture medium was changed every 2 days for a total incubation period of 6 days. Twelve-day-old chick mandibles which were not incubated and those incubated for 6 days and chick mandibles from 8-day-old chick embryos grown in ovo were weighed and dried, and their dry weights, ash weights, and calcium, magnesium, inorganic orthophosphate, total protein, collagen, Ser(P), Thr(P), and Gla contents were determined as described elsewhere (19–22). From these analytic data it was possible to compute the composition of the mandibles before and after tissue culture and after growth in ovo, as well as to detect any change in the net amounts of the major inorganic and organic constituents of the tissue which occurred during growth in tissue culture and in ovo.

Samples of 12-day-old embryonic chick mandibles before and after culture and mandibles from 18-day-old chick embryos grown in ovo were freeze-dried, powdered at liquid nitrogen temperature, and extracted for 10–14 days in 0.4 M EDTA, pH 7.5, at 4 °C in the presence of protease inhibitors (19). The EDTA extracts were dialyzed free of EDTA and salts at 4 °C by ultrafiltration in an Amicon Dialyzer Concentrator with a DC2 HIP5 cartridge which retains >5000 daltons (Amicon Corp., Lexington, MA). Ammonium bicarbonate, 0.05 M, pH 7.9, was used with the same inhibitors in the final reservoir. The samples were then lyophilized.

Tissue Culture in Presence of Radioactively Labeled Amino Acids—Experiments utilizing [3H]proline to determine the conversion of [3H]proline to [3H]hydroxyproline and the proportion of the [3H]hydroxyproline which is incorporated into the tissue explant were carried out as described previously (15). In the first group of experiments utilizing [3H]serine and [3H]threonine, the radioactively labeled amino acids were added to the tissue culture in separate independent experiments and the tissue explants were exposed for 4–8 h to the radioactively labeled amino acid. The tissue culture media used during this pulse time period were made up free of serine and threonine, respectively. Media for all of the subsequent changes contained serine and threonine. [3H]Serine and [3H]threonine at 1.5 μCi/ml were used. A total of 1.5 ml was used for each tissue culture medium change in a tissue culture dish containing four mandibles. After the initial 4–8-h pulse labeling, the explants were washed with cold tissue culture medium and the washings were added to the tissue culture medium which had been removed. The explants were recultured and the procedure was repeated for the time periods shown under "Results." The extracts removed at the end of the total tissue culture period were washed and hydrolyzed appropriately (7), as were the individual tissue culture medium samples at each of the time periods, and then counted in a Beckman Model 250 scintillation counter (18).

Aliquot samples of the explants were extracted with EDTA as described above, and the salt-free extracts and calcified bone explants were analyzed independently for radioactivity. In a second group of experiments, [3H]serine (0.5 C/ml, 1.5 ml/tissue culture dish containing four explants) and [3H]threonine (1.5 μCi/ml) were added together to the explants and simultaneously used to pulse label the explants for 4–8 h. The tissue culture medium was changed at the

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Identification of O-Phosphoserine and O-Phosphothreonine

The identification of Ser(P) and Thr(P) was carried out by a slight modification of the method previously described (19, 20). It was found that equilibration and elution of the analytical amino acid analyzer column with 0.2 N citric acid, pH 7.4, greatly improved the resolution and separation of Ser(P) and Thr(P). In brief, the identification of Ser(P) and Thr(P) is carried out as follows. Partial acid hydrolysates (19, 20) and chromatographed on a preparative amino acid analyzer column (19, 20), and in the case of unlabeled samples, the aliquots eluted in the positions of standard Ser(P) and Thr(P) rechromatographed on a Beckman 121-M automatic amino acid analyzer (19, 20) to confirm their chromatographic behavior and in the case of radioactive samples, the radioactivity eluted in the positions of standard Ser(P) and Thr(P). In brief, the identification of Ser(P) and Thr(P) is carried out as follows. Partial acid hydrolysates (19, 20) and chromatographed on a preparative amino acid analyzer column (19, 20), and in the case of unlabeled samples, the aliquots eluted in the positions of standard Ser(P) and Thr(P) rechromatographed on a Beckman 121-M automatic amino acid analyzer (19, 20) to confirm their chromatographic behavior and in the case of radioactive samples, the radioactivity eluted in the positions of standard Ser(P) and Thr(P). The majority of the sample eluted from the analytical amino acid analyzer in the positions corresponding to authentic Ser(P) and Thr(P) and Thr(P) is then hydrolyzed on 6 N HCl at 106 °C for 22 h and rechromatographed on a preparative amino acid analyzer, and the quantity released of serine and threonine is determined. In the case of the radioactively labeled samples, the fractions eluting from the preparative amino acid analyzer column in the positions of authentic Ser(P) and Thr(P) are not further chromatographed on the 121-M amino acid analyzer, but hydrolyzed in 8 N HCl at 106 °C for 22 h and rechromatographed on a preparative amino acid analyzer, and the recovery of [3H]serine, [3H]threonine, and [3H]threonine is determined quantitatively. In view of the very close elution positions of many of the phosphorylated amino acids and other phosphorylated components such as phosphoethanolamine, we feel that these additional procedures are necessary for the absolute identification of Ser(P) and Thr(P). This is especially important in those instances where one is attempting to identify for the first time the nature of the phosphorylated components in whole connective tissues or in macromolecules derived from connective tissues, which we have found to always contain small amounts of phosphorylated components other than amino acids, viz. DNA, RNA, phospholipids, etc., which are tightly bound to the protein components (7, 19).

Fractionation and Purification of EDTA-soluble Nondiffusible Phosphoproteins of the Bone Matrix of Chick Mandibles

EDTA-soluble nondiffusible proteins (7, 19, 24) were extracted from 12-day-old chick mandibles, 12-day-old chick mandibles after 6 days of growth in vitro, 12-day-old chick embryos in ovo, and 12-day-old chick embryos labeled with radioactively labeled serine and/or threonine and cultured over a 6-day period. The EDTA-soluble nondiffusible proteins were fractionated as described by Lee and Glimcher (7).

RESULTS

A typical experiment demonstrating the mineral and organic constituents of 12-day-old embryonic chick mandible and chick mandible after 6 days of growth in tissue culture and in ovo is shown in Table 1. Changes in the major mineral components (Ca, P, Mg) and in the total protein and collagen contents of the bone were also very similar to those of mandibles grown over a similar period of time in ovo and to the changes accompanying mineralization in vivo in postnatal animals (25). The data demonstrate that active synthesis of bone matrix protein as well as calcification of bone matrix have occurred in the bone cultured in vivo although not quite to the extent that they have in the mandibles grown in ovo. Freeze-thawed specimens of mandible incubated in tissue culture medium for similar periods of time showed a maximum of 5% increase in weight of the mineral component and organic constituents.

From 96-99% of the Ser(P) and Thr(P) in mandibular bone before and after growth in tissue culture and in ovo was extracted in EDTA and was nondialyzable, properties similar to those of the phosphoproteins of adult chicken bone matrix (19). This establishes that almost all of the Ser(P) and Thr(P) synthesized in organ culture and in vivo is present in EDTA-extractable nondiffusible proteins (7-19).

Molecular Sieve and Chromatographic Behavior of EDTA-extracted Proteins Containing Ser(P) and Thr(P)—The molecular sieve and ion exchange chromatographic behavior of the EDTA extracts from 12-day-old chick mandibles and from 12-day-old chick mandibles after 6 days of growth in vitro and in vivo was similar and had the same characteristic behavior previously described for the EDTA-extractable phosphoproteins of 10-14-week-old chicken bone (7, 24). For example, the concentration of Ser(P) in the second peak from the Sephadex S-200 column (Fig. 3) corresponding to the approximately M, = 12,000 purified phosphoprotein of postnatal chicken bone matrix (7) was 12.0 nmol/mg and that of Thr(P) was 2.7 nmol/mg, an approximate 6-fold increase from the Ser(P) concentration in the crude EDTA extract. (No corrections were made for destruction and maximum yield for the phosphoamino acids.) Peak b (Fig. 3) was not purified to homogeneity. The position of this peak corresponded to the M, = 28,000-30,000 fraction isolated from postnatal chick bone (7). The Ser(P) concentration in peak b varied somewhat in different preparations, but in general was from 1.5 to 2.0 times that of peak a (7, 26).

The last peak eluted from the Sephadex G-100 column contains the majority of the Gla applied to the column (27). This confirms earlier work that the Gla-containing protein, osteocalcin, is also synthesized in bone (27, 28). Further purification of this fraction on DEAE-cellulose ion exchange chromatography resulted in the elution of a peak which behaved as a single band by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Ser(P) was detected in this fraction and in a single component eluted by high precision liquid chromatography from a DEAE-cellulose purified fraction whose amino acid composition and concentration of Gla are identical with those of authentic osteocalcin (27). This subject is being further investigated at this time.

Incorporation of Radioactively Labeled Serine and Threonine into Bone Matrix Proteins During Tissue Culture of 12-day-old Chick Mandibles—In the first group of experiments, [3H]Ser(P) and [3H]Thr(P) were identified in the partial acid hydrolysates of whole chick mandible bone and in the EDTA-extractable nondiffusible proteins after the mandibles had been cultured in vitro with [3H]serine and [3H]threonine for 4-8 h. 95-99% of the [3H]Ser(P) and [3H]Thr(P) was extracted in the EDTA solutions as nondialyzable components.
In a typical experiment, approximately 2% of the total counts/min incorporated into the mandibles was recovered as [3H]Ser(P) (11,500 cpm/mandible) and [3H]Thr(P) (3,208 cpm/mandible) as determined by preparative amino acid chromatography. When these putative [3H]Ser(P) and [3H]Thr(P) fractions were further hydrolyzed in 6 N HCl at 106 °C for 22 h, 87.2% of the counts/min were recovered as [3H]serine and [3H]threonine, respectively. In comparison, approximately 90% of authentic Ser(P) and Thr(P) was recovered as serine and threonine, respectively, under similar conditions.

Table II presents the results of a typical group of the second type of experiments carried out which demonstrate that very little of [3H]Ser(P) or [3H]Thr(P) synthesized in the bone is released as such into the tissue culture medium. In contrast and in keeping with previous findings (15), approximately 50% of the hydroxyproline synthesized during the same time period was recovered into the tissue culture medium.

**Molecular Sieving and Ion Exchange Chromatography of Radioactively Labeled Phosphoproteins of Chicken Bone Matrix Synthesized in Tissue Culture**—Typical molecular sieve and ion exchange elution patterns of the EDTA extracts of the 6-day-old cultured mandibles labeled simultaneously with [14C]serine and [3H]threonine are shown in Figs. 1-3. The molecular sieve and ion exchange chromatographic behavior of the labeled proteins extracted from the cultured mandibles is similar to that of the proteins extracted from embryonic bone.

**TABLE II**

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Ser(P)</th>
<th>Thr(P)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Media</td>
<td>Explant</td>
</tr>
<tr>
<td>Experiment 1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>8 h</td>
<td>185</td>
<td>106</td>
</tr>
<tr>
<td>24 h</td>
<td>209</td>
<td>106</td>
</tr>
<tr>
<td>Total in media</td>
<td>384</td>
<td>106</td>
</tr>
<tr>
<td>Experiment 2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>8 h</td>
<td>217</td>
<td>159</td>
</tr>
<tr>
<td>24 h</td>
<td>254</td>
<td>112</td>
</tr>
<tr>
<td>48 h</td>
<td>193</td>
<td>2,915</td>
</tr>
<tr>
<td>Total in media</td>
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<td>2,915</td>
</tr>
<tr>
<td>Experiment 3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>8 h</td>
<td>297</td>
<td>116</td>
</tr>
<tr>
<td>24 h</td>
<td>298</td>
<td>105</td>
</tr>
<tr>
<td>48 h</td>
<td>167</td>
<td>2,637</td>
</tr>
<tr>
<td>96 h</td>
<td>184</td>
<td></td>
</tr>
<tr>
<td>Total in media</td>
<td>946</td>
<td>2,637</td>
</tr>
<tr>
<td>Experiment 4</td>
<td></td>
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</tr>
<tr>
<td>8 h</td>
<td>263</td>
<td>125</td>
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<tr>
<td>24 h</td>
<td>332</td>
<td>116</td>
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<tr>
<td>48 h</td>
<td>255</td>
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<tr>
<td>96 h</td>
<td>130</td>
<td></td>
</tr>
<tr>
<td>6 days</td>
<td>450</td>
<td></td>
</tr>
<tr>
<td>Total in media</td>
<td>980</td>
<td>3,320</td>
</tr>
</tbody>
</table>
One of the postulated roles of the phosphorylated bone matrix proteins is their participation in the initiation of the formation of a solid phase of calcium-inorganic phosphorus from solution (heterogeneous nucleation) (28, 30). For such components to play the critical role postulated, it is certainly almost mandatory that these proteins be synthesized in bone and be available at the sites in the extracellular organic matrix where calcification occurs. The present experiments, which have established that the characteristic phosphorylated proteins of bone matrix are indeed synthesized in bone, are therefore consistent with the concept that the phosphoproteins of bone matrix play a significant role in the initiation and formation of a solid mineral phase of calcium-inorganic phosphorus (2, 30, 31). The similarities in the basic physical chemical characteristics of the phosphoproteins of all the other vertebrate mineralized tissues thus far studied (5, 6, 32–35) suggest that the postulated role of phosphoproteins in the mineralization of bone may apply in general sense to all of the calcified vertebrate tissues (2–4).

From the data presented in this report, it is impossible to state which cell is responsible for the synthesis of the bone phosphoproteins. What is not well appreciated is the fact that the vast majority of cells present in bone as a tissue or organ are marrow and connective tissue cells and not the classical bone cells (osteoblast, osteocytes, and osteoclasts). This is especially true in the case of young growing animals. In a series of recent experiments, however, we have been able to obtain autoradiographic and biochemical evidence that osteoblasts are the principal cell type synthesizing the phosphorylated extracellular matrix proteins of bone (36, 37).

REFERENCES

FIG. 3. Elution profile of molecular sieving of DEAE-cellulose-purified fractions through Sephacyr S-200 in 5 mM guanidine HCl. Top, peak II (Fig. 2) from DEAE-cellulose chromatography; bottom, refiltering of peak b (see top).
Phosphoproteins of chicken bone matrix. Proof of synthesis in bone tissue.
M J Glimcher, D Kossiva and D Brickley-Parsons


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