Extracellular matrix synthesis in the chick embryo lateral plate prior to and during limb outgrowth

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SUMMARY

Little is known at the present time about the molecular basis and mechanisms of morphogenesis. The present study is an attempt to determine what influence the extracellular matrix has on the initial outgrowth of the limb bud. Stage -12 to -18 chick embryo lateral plates were examined in relation to proline and sulfate incorporation into collagen and proteoglycan. The flank and limbs incorporated the same amount of labeled proline and sulfate before stage 16. At stage 16 the flank began to incorporate more of both isotopes until at stage 18 there was twice as much incorporation into the flank as into the limbs. The flank and limbs contained the same type of collagen during the period examined. The limbs contained both large and small proteoglycans but the flank contained only small proteoglycans. These data suggest that the extracellular matrix in the flank and limb regions may play a role in limb outgrowth and that the limb buds at these stages may be more inclined toward cartilage development.

INTRODUCTION

Morphogenesis, the development of shape, is poorly understood from a biochemical, mechanistic point of view. This is partly due to the small amounts of tissue available for study and perhaps more significantly to the lack of any conceptual framework which would include possible biochemical mechanisms for morphogenesis. The developing chick limb bud provides a source of tissue which may be helpful in understanding the biochemistry of morphogenesis. The present study was confined to an investigation of the biochemical nature of the extracellular matrix in the limb primordia and intervening (flank) tissues, and how this matrix may be related to limb outgrowth.

The extracellular matrix is composed primarily of collagens and proteoglycans. Collagens are a class of triple helical proteins which are somewhat tissue specific. Types I and III occur in most tissues, Type II in hyaline cartilage.

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and Type IV in basement membrane (cf. Piez, 1972; Bornstein, 1974; Miller, 1977 for reviews). Proteoglycans consist of glycosaminoglycans attached to protein cores forming proteoglycan monomers (PGM). PGM is associated into higher order complexes, proteoglycan aggregates (PGA), by interaction with hyaluronic acid and ‘link’ proteins (see review by Muir, 1977). Proteoglycans may, like collagen, exhibit tissue-specific types and characteristics.

Several studies have suggested that the flank is capable of participation in limb development at early stages (Crosby, 1967; Dhouailly & Kieny, 1970; Saunders & Reuss, 1974). The flank begins to lose limb competence at stage 16, and by stage 19 limbs can no longer be induced in the flank. Disruption of the flank structure is necessary for its participation in limb development (Crosby 1967; Saunders & Reuss, 1974), suggesting that loss of limb competence in the flank may involve some structural component (e.g. extracellular matrix).

It has been observed that during amphibian limb development, the basement membrane in the area of the limb field is less prominent and less structured than in the flank (Balinsky, 1972; Kelly & Bluemink, 1974). Balinsky (1956) proposed that the induction of heterotopic limbs following implantation of non-limb tissues into amphibian flanks resulted from degradation of the basement membrane (i.e. basal lamina) allowing the flank mesoderm to contact the ectoderm and induce limb outgrowth.

Electron microscope studies of the embryonic chick flank and limb sub-ectodermal regions show an accumulation of collagen fibrils in the flank region after stage 14, whereas collagen fibers are scarce in the future limb region (Smith, Searls & Hilfer, 1975). These results support Balinsky’s (1956) contention that an absence of basement membrane material (i.e. basal and reticular lamina) is correlated with subsequent limb outgrowth.

The present study has revealed that there is no apparent biochemical difference in the extracellular matrix between the flank and limbs before stage 16. After stage 16 however, the flank exhibits a steady increase in matrix production over that in the limbs. The type of collagen is homogeneous throughout the lateral plate during at least stages 12–18. There was no more Type IV collagen (the type found in the basement membrane) per unit area in the flank than in the limbs (in agreement with the findings of Smith et al. 1975). At stage 18 the flank contained no proteoglycan aggregates while the limbs contained detectable levels of aggregate.

**MATERIALS AND METHODS**

White Leghorn chick embryos obtained from George F. Shaw, Inc. (West Chester, Pa.) were incubated in a humidified egg incubator at 38 °C until the desired age had been reached and then staged according to the descriptions of Hamburger and Hamilton (1951). The extraembryonic membranes, the splanchnopleure and all tissue rostral to the 14th intersomitic space and caudal to the 32nd intersomitic space were discarded. The lateral plates were separated from
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The axial tissue (including the mesonephros) and divided into three pieces (wing, flank, and leg) of approximately equal length according to the regions delineated by Chaube (1959).

The cleaned wing, flank and leg regions were incubated for 3–24 h in the nutrient medium F12X (see Minor, 1973). L-proline [2,3-3H(N)] (20–40 Ci/mmol) and/or glycine (2-3H; 5–15 Ci/mmol), or Na₂³⁵SO₄ (all purchased from New England Nuclear, Boston, Mass.) were added to the medium in amounts of 2–50 µCi/ml. Sodium ascorbate (Sigma Chemical Co., St Louis, Mo.; 50 µg/ml) and BAPN (β-aminopropionitrile fumarate, Sigma; 100 µg/ml) were also added to some of the cultures.

Acid-extracted calf skin collagen (prepared in this laboratory) was added to some cultures as carrier and the tissue, medium and carrier collagen (1 ml total volume) were sonicated at 75 W for 30 sec. Duplicate (5–50 µl) samples were removed for DNA determinations and the remainder of the sample was assayed by various methods described below.

DNA determination was conducted according to the procedure of Kissane & Robins (1958) as modified by Santoianni & Ayala (1965) and by Hinegardner (1971). DNA concentration was determined with a Turner model 430 spectrofluorometer (Turner Associates, Riverton, N.J.; excitation 420 nm and emission 520 nm). The actual amount of DNA in the sample was determined by multiplying the value read from the fluorometer by 2.3 (determined) with known DNA standards (cf. Hinegardner, 1971).

Sonicated samples were dialyzed overnight with constant stirring against 0.5 M acetic acid. Pepsin (Sigma) was added to sonicated samples to equal 1/10 the amount of total protein in the sample determined by the method of Lowry, Rosebrough, Farr & Randall (1951). The total volume was increased by the addition of 0.5 M acetic acid to give a final pepsin concentration of 1 mg/ml and the samples were allowed to digest for 6 h at 16 °C.

The collagen was precipitated by the addition of crystalline potassium chloride (Baker Chemical Co., Phillipsburg, N.J.) to a final concentration of 15%. The mixture was kept at 4 °C for 30 min, centrifuged at 1700 g for 30 min, and the supernatant was discarded. The pellet was dissolved in various buffers depending upon the assay to be conducted with a given sample (see below).

Radioactivity was determined with scintillation cocktail consisting of toluene (Fisher) containing 6 gm/L PPO, 0.3 gm/L POPOP (Packard Instrument Co., Downers Grove, Illinois) and 10% Bio-Solv solubilizer formula BBS-3 (Beckman Instrument, Inc., Fullerton, Calif.). The resultant photon emissions were counted in an Intertechnique model SL 4200 liquid scintillation counter (Intertechnique, Fairfield, N.J.) with external standardization.

Collagenase digestion was slightly altered from the assay of Peterkofsky and Diegelmann (1971). Samples which had been labeled only with [³H]proline, pepsin digested, KCl precipitated, and resuspended in 0.5 ml 0.2 N NaOH were divided into two equal portions.
Collagenase (100 µl) was added to one sample of each pair; the other sample (control) received the same volume of buffer without collagenase. Collagenase form III (lyophilized powder) was obtained from Advance Biofactures Corp., Lynbrook, N.Y. and was reconstituted in 1 ml of the buffer prepared according to the formula supplied with the collagenase. The collagenase (0.1-0.2 mg/ml; 2500 units/ml) was determined in our laboratory to exhibit no non-specific protease activity by its failure to release more counts from tissue labeled with [³H]tryptophane (an amino acid not found in collagen) than did the buffer alone with no collagenase added.

Carboxymethyl-cellulose column chromatography was conducted according to the following procedures. Samples which had been incubated in the presence of BAPN, pepsin digested under KCl precipitated were dissolved in 1 M urea (Ultrapure; Schwartz-Mann) and 1 M Tris HCl (pH 7.2); reduced with dithioerythritol (Sigma, 50 mM final concentration); and alkylated with iodoacetamide (K and K Laboratories, Inc., Plainview, N.Y.; 0.12 M final concentration). In addition, 2-mercaptoethanol type I (Sigma) was added to the sample to further reduce and stabilize the separated Alpha chains.

The samples were dialyzed against 0.04 M sodium acetate and 4.0 M ultra pure urea, pH 4.8 (start buffer); calf skin collagen (5 mg) was added as carrier (heat denatured) and the sample with carrier was chromatographed on a 15 mm x 100 mm jacketed carboxymethyl-cellulose column (Whatman CM52; Reeve Angel and Co., Inc., Clifton, N.J.) with a gradient of 0.0 M-NaCl to 0.08 M-NaCl in 0.4 M sodium acetate and 4.0 M urea, pH 4.8 (150 ml total volume).

The location of standard Type I chick Alpha chains was determined by chromatographing radioactively labeled, 13-day-old chick embryo tendon together with calf skin carrier. All experimental samples were then compared to the standardized chick tendon Type I collagen.

The amount of 3-hydroxyproline, 4-hydroxyproline, and proline was determined according to the procedure of Clark et al. (1975) on the long column (12 mm x 450 mm) of a Joel JCL-6AH amino acid analyzer (Japan Electron Optics Lab., Co., Ltd., Tokyo, Japan).

Collagenase activity in the tissues was determined as follows. Wing, flank and leg tissues were labeled with [³H]proline for 18 h as described above. The radioactive medium was replaced with medium devoid of radioactive label. Samples were removed after 6 h and 24 h and assayed for the amount of collagenase sensitive radioactivity remaining in the tissue per ng DNA.

Determination of sulfate incorporation in tissues which had been cultured in medium containing Na₂³⁵SO₄ (2-100 µCi/ml) and sonicated was according to the methods of Daniel, Kosher, Lash & Hertz (1973).

An aliquot from sulfate labeled, purified glycosaminoglycans was incubated at 37 °C for 3 h with 0.1 unit chondroitinase ABC (Miles Laboratories, Inc.) in 10 µl enriched Tris buffer and chromatographed according to the procedures described by Saito, Yamagata & Suzuki (1968).
Table 1. Collagenase-sensitive, pepsin-resistant, and 15%-potassium-chloride-precipitable [3H]proline in the wing, flank, and leg regions of chick embryo lateral plates expressed as DPM per ng DNA

<table>
<thead>
<tr>
<th>Stage</th>
<th>Tissues</th>
<th>Experiments</th>
<th>Wing*</th>
<th>Flank*</th>
<th>Leg*</th>
<th>F-W†</th>
<th>F-L†</th>
<th>W-L†</th>
</tr>
</thead>
<tbody>
<tr>
<td>12</td>
<td>237</td>
<td>4</td>
<td>2·9</td>
<td>1·8</td>
<td>1·9</td>
<td>Sig</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>15</td>
<td>311</td>
<td>8</td>
<td>2·5</td>
<td>3·1</td>
<td>2·8</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>18</td>
<td>462</td>
<td>6</td>
<td>7·0</td>
<td>15·0</td>
<td>5·3</td>
<td>Sig</td>
<td>Sig</td>
<td>NS</td>
</tr>
</tbody>
</table>

* Expressed as DPM/ng DNA.
† Determined by paired t test (Sig: P > 0·05).

Differential guanidine hydrochloride extraction was accomplished according to the techniques described by Vasan & Lash (1977) and Lash & Vasan (1978). Controlled-pore glass beads (CPG; Electro-nucleonics Inc., Fairfield, N.J.) were used to separate 35SO4 labeled proteoglycans according to the method of Lever & Goetinck (1976), as modified by Lash & Vasan (1978). The sample was applied to an 0·8 cm x 100 cm column containing CPG-10-2500B (mean pore diameter of 257·3 nm) and elution was accomplished at 4 °C with 0·5 M-NaCl and 0·02% sodium azide (Sigma).

RESULTS

Proline incorporation. Stage-12 wing primordia incorporated considerably more proline than did the flank and leg (2·9 DPM/ng DNA in the wing vs. 1·8 and 1·9 DPM/ng DNA in the flank and leg respectively; Table 1). If stage-15 tissues were incubated for 18 h, incorporation into the flank was greater than that into wing and leg (3·1 CPM/ng DNA in the flank, 2·5 DPM/ng DNA in the wing, and 2·8 DPM/ng DNA in the leg; Table 1). If stage-15 tissues were incubated for 3 h in culture the flank incorporated the same amount of label as did the wing. Stage-12 tissues incubated for 3 h gave the same results as 18 h cultures. Stage-18 flank incorporated more than twice as much label (15·0 DPM/ng DNA) as either the wing or the leg (7·0 and 5·3 DPM/ng DNA respectively) whether incubated for 18 h or 3 h. Pulses of various lengths (Fig. 1) demonstrated that the flank did not begin to increase in relation to the wing and leg until approximately stage 16. The flank continued to increase linearly (in relation to the limbs) from stage 16 to 18. Flank tissue which was incubated at stage 15 for 18 h thus achieved some threshold age (that age at which the flank showed a higher level of matrix production than the limbs). Pulse experiments demonstrated that this threshold stage is 16+. Whatever changes occur in the lateral plate to cause a difference in matrix formation between the flank and limbs can occur in vitro and can occur when the flank and limbs are separated from each other and adjacent tissues.
Fig. 1. Summary of data from experiments with radioactive proline plotted as a function of embryonic age. The points plotted were based upon collagenase-released radioactive label from pepsin-resistant material per ng DNA. Tissues were incubated for short 3-6 h pulses beginning at various developmental stages and then plotted on a continuum of incubation time in hours.

Table 2. Total $^{35}$S incorporated into TCA-precipitable glycosaminoglycans of the wing, flank, and leg regions of chick embryo lateral plates expressed as DPM per ng DNA

<table>
<thead>
<tr>
<th>Stage</th>
<th>Tissues</th>
<th>Experiments</th>
<th>Wing*</th>
<th>Flank*</th>
<th>Leg*</th>
<th>F-W†</th>
<th>F-L†</th>
<th>W-L†</th>
</tr>
</thead>
<tbody>
<tr>
<td>12</td>
<td>434</td>
<td>3</td>
<td>0.2</td>
<td>0.1</td>
<td>0.2</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>18</td>
<td>660</td>
<td>4</td>
<td>0.4</td>
<td>0.5</td>
<td>0.3</td>
<td>Sig</td>
<td>Sig</td>
<td>NS</td>
</tr>
</tbody>
</table>

* Expressed as DPM/ng DNA.
† Determined by paired t test (Sig: $P > 0.05$).

Sulfate incorporation. The incorporation of $^{35}$S into the limbs and flank was similar to that of $[^3]$H]proline but the differences between the regions were less marked (Table 2). At stage 12 the three regions were quite similar in the amount of label incorporated (wing 0.2 DPM/ng DNA, flank 0.1 DMP/ng DNA, and leg 0.2 DPM/ng DNA). The flank incorporated significantly more radioactivity at stage 18 (0.5 DPM/ng DNA) than did the wing (0.4 DMP/ng DNA) or leg (0.3 DPM/ng DNA). The differences in $^{35}$S incorporation between the flank and limbs was less than one and one-half fold while the differences in $[^3]$H]proline incorporation was over two fold.

Carboxymethyl-cellulose column chromatography. The elution patterns of the collagen Alpha chains from stage-12, -16, -18 and -23 wing, flank and leg regions exhibited almost identical profiles (Fig. 2). The last peak of the profiles (peak No. 6, Fig. 2, Table 3), 5.8% of the total collagen, contained 14.4% of its total
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Fig. 2. CMC chromatograms of labeled wing, flank, and leg regions from stage-18 chick embryo lateral plates (dotted line) and chick tendon Type 1 collagen (solid line). Chromatography was performed at 43 °C on a 15 mm x 100 mm jacketed column with a flow rate of 30 ml/hour. The fractions were eluted in 0.04 M sodium acetate, pH 4.8, containing 4.0 M urea with a linear gradient of 0.0 to 0.08 M NaCl over a total volume of 150 ml and 1 ml fractions were collected. The sample was applied between fraction 1 and 5 the gradient was started at approximately fraction 18, and the base wash was started at approximately fraction 86. Stages 12, 16, and 23 gave almost identical profiles. Peaks from embryonic tissue were labeled consecutively from 1 to 6 beginning after application of the sample. Standard Type 1 collagen profile peaks were labeled as \( \alpha_1, \beta_1, \beta_2, \) and \( \alpha_2 \).

Hydroxyproline as 3-hydroxyproline (Table 3). Type IV collagen is the only type described which exhibits a 3-hydroxyproline level greater than 5% (Grant & Harwood, 1974; Clark et al. 1975; Chung, Rhodes & Miller, 1976). The other five peaks contained less than 3% 3-hydroxyproline (Table 3). Type IV collagen contains 69.2% of its total proline as hydroxyproline (Kefalides, 1973). The presence of only 21.2% hydroxyproline in the last peak (peak 6) may suggest
Table 3. Summary of the analysis of each peak obtained after CMC acetate column chromatography. The peaks were numbered from the earliest eluted to the latest eluted. Each peak was expressed as a percentage of the total collagen eluted in the profile. The total proline which existed as hydroxyproline and the percentage of hydroxyproline that existed as 3-hydroxyproline was listed for each except peak No. 5 for which enough material was not available for analysis

<table>
<thead>
<tr>
<th>Peak No.</th>
<th>Percentage of Total</th>
<th>Percentage Prol as Hypro</th>
<th>Percentage Hyp as 3-Hypro</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>12.8</td>
<td>32.7</td>
<td>2.4</td>
</tr>
<tr>
<td>2</td>
<td>4.0</td>
<td>48.3</td>
<td>1.7</td>
</tr>
<tr>
<td>3</td>
<td>43.5</td>
<td>51.3</td>
<td>1.9</td>
</tr>
<tr>
<td>4</td>
<td>10.0</td>
<td>57.0</td>
<td>2.5</td>
</tr>
<tr>
<td>5</td>
<td>23.9</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>6</td>
<td>5.8</td>
<td>21.2</td>
<td>14.4</td>
</tr>
</tbody>
</table>

Fig. 3. Tissue collagenase activity in the wing (dashed line), flank (solid line), and leg (broken line) of stage-18 chick embryo lateral plates. The tissues were incubated for 18 h in medium containing [3H]proline, the medium was removed, and the tissues were washed. Medium with no radioactive label was added and samples were taken at 0, 6 and 24 h. The points plotted were based upon collagenase-released label per ng DNA.

that only 31.4% of the material is collagen. Therefore, although the last peak contained 16.5% of the total radioactivity it accounted for only 5.8% of the total collagen.

Tissue collagenase activity. Pulse, chase experiments demonstrated that the levels of labeled collagen decreased slightly after a 24 h chase (Fig. 3) but that this decrease was the same in the wing, flank and leg.

Glycosaminoglycan content. The relative amount of chondroitin 4-sulfate in the lateral plate increased from stage 12 to 18 as the relative amount of chondroitin 6-sulfate decreased (Table 4). This data was consistent with previous
Table 4. Chondroitinase ABC sensitivity of $^{35}S$ incorporated into the wing, flank, and leg regions of chick embryo lateral plates.

Material which remained at the origin was termed resistant; material which co-chromatographed with $\Delta$Di-6S was identified as chondroitin 6-sulfate; and material which co-chromatographed with $\Delta$Di-4S was identified as chondroitin 4-sulfate.

<table>
<thead>
<tr>
<th>Stage</th>
<th>Tissues</th>
<th>Sensitivity</th>
<th>Exp.</th>
<th>Flank*</th>
<th>Leg*</th>
<th>Wing*</th>
<th>F-L-†</th>
<th>F-W-†</th>
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<tbody>
<tr>
<td>12</td>
<td>596</td>
<td>Resistant</td>
<td>3</td>
<td>32-9</td>
<td>26-7</td>
<td>24-0</td>
<td>22-1</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Chon 6-SO$_4$</td>
<td>65-9</td>
<td>59-7</td>
<td>77-8</td>
<td>17-7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>18</td>
<td>420</td>
<td>Resistant</td>
<td>2</td>
<td>27-6</td>
<td>20-3</td>
<td>72-650</td>
<td>22-1</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td>Chon 6-SO$_4$</td>
<td>27-3</td>
<td>50-3</td>
<td>57-2</td>
<td>15-5</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Expressed as % of total
† Determined by paired $t$ test (Sig. $P > 0.05$).
Table 5. Guanidine hydrochloride extraction of incorporated $^{35}$S from the wing, flank, and leg regions of chick embryo lateral plates. Radioactive label removed from the tissues by 0-4 M and 4-0 M GuHCL were each expressed as a percentage of the total extractable label. Each experiment was repeated twice and the data averaged.

<table>
<thead>
<tr>
<th>Stage</th>
<th>GuHCL (molar)</th>
<th>Wing*</th>
<th>Flank*</th>
<th>Leg*</th>
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<td>12</td>
<td>0.4</td>
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<td>87.0</td>
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<td></td>
<td>4.0</td>
<td>22.0</td>
<td>19.3</td>
<td>16.5</td>
</tr>
<tr>
<td>18</td>
<td>0.4</td>
<td>84.4</td>
<td>87.8</td>
<td>74.4</td>
</tr>
<tr>
<td></td>
<td>4.0</td>
<td>15.6</td>
<td>12.2</td>
<td>25.6</td>
</tr>
</tbody>
</table>

* Expressed as percent of total.

reports (cf. Meyer, 1969; Abrahamson, Lash, Kosher & Minor, 1975; Kosher & Lash, 1975). The amount of chondroitin 4-sulfate, chondroitin 6-sulfate, and chondroitinate-resistant material did not differ between the wing, flank and leg at either stage examined.

Guanidine hydrochloride extraction. The amount of proteoglycan aggregate in the wing, flank and leg regions of the lateral plate at both stage 12 and 18 was 15–20% (Table 5). This data is consistent with previous reports for limbs (Goetinck, Pennypacker & Royal, 1974; Vasan & Lash, 1979). The amount in the wing, flank and leg did not differ significantly.

Controlled-pore glass column chromatography. Goetinck et al. (1974) designated three fractions obtained from chick limb-bud cultures after agarose molecular sieve chromatography as aggregate (Ia), large monomer (Ib), and small monomer (II). Lever & Goetinck (1976) demonstrated that a similar profile can be obtained with CPG molecular sieve chromatography. They described peak Ia as an aggregate and Ib and II as monomers of different size (Goetinck et al. 1974). Although the designation of these fractions has become more complicated (McKeown & Goetinck, 1979; Ovadia, Parker & Lash, 1980), the molecular size of the extracted proteoglycans is a useful index of chondrogenesis.

In the present study, three sizes were observed (Fig. 4). Peak No. 1 may have contained large aggregates and corresponded to Ia, peak No. 2 (perhaps Ib) contained intermediates and peak No. 3 (perhaps II) low molecular weight proteoglycans. These suppositions have been confirmed by work in this laboratory (cf. Vasan & Lash, 1977 and Lash & Vasan, 1978). Peak No. 3 (Fig. 4) contained approximately 80% of the total proteoglycans from the wing and leg (in agreement with previous reports; Goetinck et al., 1974; Vasan & Lash, 1977) which agreed with the data obtained from guanidine hydrochloride extraction (above). Peak No. 3 (Fig. 4) from the flank, however, contained 93% of the total proteoglycans.

The limbs exhibited approximately 10–15% of the total proteoglycans in
Fig. 4. CPG-2500 molecular sieve column chromatography of labeled wing, flank, and leg regions of stage-18 chick embryo lateral plates. Chromatography was performed at 4 °C on a 0.8 cm x 100 cm column with a flow rate of 20 ml/h. The fractions were eluted in 0.5 M NaCl and 0.02 % sodium azide and 0.7 ml fractions were collected. Peak No. 1 eluted in the region expected for higher molecular weight PGA and peak No. 3 eluted in the region expected for PGM.

peak No. 1 (aggregates), but the flank contained no detectable molecules of such large size. These data suggest that the limbs produce proteoglycan aggregates by stage 18, but that the flank does not.

It should be noted that peak No. 1 is not observed if the proteoglycan extract is immediately placed upon the CPG column after dialysis (cf. Lash & Vasan, 1978; Ovadia et al. 1980). In the experiments reported in this paper, the extracts were stored a week between dialysis and column chromatography. During this period of time, a small amount of aggregates formed. The appearance of aggregates in this manner has also been observed in older cartilages.
DISCUSSION

The present study has revealed three characteristics of the chick lateral plate extracellular matrix prior to and during the initiation of limb outgrowth (i.e. when the limb regions begin to expand laterally in relation to the adjacent tissues). First, the entire lateral plate (wing flank and leg) contains similar collagen(s), as determined by Alpha chain analysis (from stage 12 through stage 18). Second, the limbs contain both small proteoglycans and proteoglycan aggregates but the flank contains only small proteoglycans (at stage 18). Third, from stage 16 through at least stage 18, the flank produces more extracellular matrix (both collagens and proteoglycans) than the limbs. The question of what imparts the quality of ‘limbness’ to specific regions of the lateral plate has been approached in this study by examining the extracellular matrix between the limb regions and the flank when the limbs first begin to develop.

Is flank outgrowth inhibited?

It is well known that a mesodermal-ectodermal interaction is involved in limb outgrowth, but we know very little about the mechanisms involved (cf. Zwilling, 1972). Does limb outgrowth occur because of the lack of some ‘inhibition’, or does it occur as the result of a positive inductive influence which results in ‘limbness’, or perhaps both? The presence of an inhibitor effect in the flank would imply that the entire lateral plate has an outgrowth potential, and indeed, it has been reported that the flank can be stimulated to develop supernumerary limbs (Balinsky, 1956; Crosby, 1967).

It has been fairly well established that the tetrapod limb has evolved from a continuous lateral fin fold which existed in pregnathostomes (Jarvik, 1965). Thus, it would seem that the forerunners of the modern tetrapods had lateral plates with a uniform potential for outgrowth. Such an equality of potential could be reflected in the initial development of the lateral plate. Searls & Janners (1971) found that limb outgrowth is not due to an increased proliferation in the limb mesoderm but rather a decreased proliferation in the flank. It would appear that the flank is initially proliferating as rapidly as the limb region but is subsequently inhibited.

There are reports in the literature which suggest that an inhibitory influence can be detected in the flank (Balinsky, 1956; Crosby, 1967; Dhouailly & Kieny, 1970; Singer, 1972; Saunders & Reuss, 1974). The cells themselves do not appear to inhibit limb development since flank cells can participate in a developing limb (Dhouailly & Kieny, 1972). Most of these reports have presented evidence that the inhibition may have a structural basis. Crosby (1967) observed that intact flank mesoderm, placed inside a wing ectodermal jacket and grafted, produced no limb structures whereas fragmented or dissociated flank mesoderm similarly grafted did develop limb structures (as many as 45% of the grafts at stage 14). These data can be interpreted as the flank material having an influence that is disrupted by tissue fragmentation or dissociation.
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The 'structural' basis of inhibition

One aspect of the hypothesized 'structural' basis may be the extracellular matrix. The extracellular matrix surrounding the mesenchymal cells can provide microheterogeneities that may be correlated with regional differences (cf. Lash, Ovadia & Vasan, 1978). The extracellular matrix components which could be implicated are the basement membrane (presumably containing Type IV collagen) and the matrix synthesized by the mesenchymal cells, which contains collagen (of uncharacterized type) and proteoglycans. In the experiments of Crosby (1967), the intact flank mesoderm, with its extracellular matrix undisturbed, did not produce limb structures when grafted in a wing jacket. The mesenchyme which had its extracellular matrix perturbed by fragmentation of cell dissociation was able to contribute to limb structures, and these perturbations would have stimulated the cells to synthesize and secrete matrix products. Might the matrix components normally found in flank tissue contribute to the 'structural' basis of inhibition, whereas matrix products synthesized in association with the ectodermal limb jacket synthesize products more compatible with limb outgrowth?

Another possible factor is the basement membrane. Balinsky (1956) has proposed that the basement membrane inhibits supernumerary limb development in the amphibian flank. He has also reported (1972) that the amphibian flank contained a more prominent basement membrane than did the limb regions. It was therefore of considerable interest in the present study to analyze the collagen in the chick embryo flank and limb regions. Analysis of the peaks obtained after CMC chromatography showed that the peak containing 14.4% of the hydroxy proline as 3-hydroxy proline (characteristic of Type IV collagen) did not differ in relative amount between the flank and limbs. These data are not conclusive since the peak also contained some non-collagenous material which could mask slight differences between the regions. It does however suggest that the amount of basement membrane alone may have no bearing on limb development in the chick, but that limb inhibition in the flank may result from an increase in total collagen. These data agree with previous observations of Smith et al. (1975) who reported that the flank and limbs have similar relative amounts of basement membrane but the total collagen in the flank is increased.

It has been suggested that proteoglycan aggregates are characteristic of tissues which will produce cartilage (Goetinck et al. 1974; Lash & Vasan, 1978; Ovadia et al. 1980). The presence of proteoglycan aggregates in the wing and leg regions is indicative of the eventual development of cartilage in those tissues. The flank, on the other hand, does not form cartilage (Pinot, 1969; Sweeney & Watterson, 1969) and the absence of proteoglycan aggregates is correlated with the absence of cartilage production in that tissue. It is possible that inhibition in the flank is not merely a result of quantitative differences between the flank and limb extracellular material, but may also result from qualitative differences. This is
suggested by the observations that limb inhibition (Crosby, 1967) as well as decreased cartilage production (Pinot, 1969) can be observed in the flank at stage 14, before this tissue begins to produce more extracellular matrix than the limbs (stage 16).

In the present study, the proteoglycans were observed to differ in type as well as in amount. Pinot (1969) observed that stage-14 to -16 wing tissues in culture produced cartilage in 84% of the cases while the flank produced cartilage in only 35% of the cases. The decreased production of cartilage in the flank may in some way relate to the inhibition of limb development in the same tissue. Since the type of collagen in the flank and limbs is similar from stage 12 through 18, differences observed at stage 14 could not be attributed to collagen (at that stage both the amount and type of collagen is the same). Stage-18 wing and leg tissues exhibited approximately 15% of their total proteoglycans as large aggregates. The flank on the other hand, contained no detectable aggregates. Thus, there is a correlation between the lack of proteoglycan aggregates in the flank, the meager amount of cartilage production in culture (Pinot, 1969), and the inhibition of limb development by flank mesenchyme (cf. Singer, 1972). The proteoglycans then may play a major role in the inhibition of limb development in the flank.

It should be noted that while our data from proline incorporation studies agree with observations of other morphogenetic systems (Bernfield & Wessells, 1970), our sulfate incorporation data does not. In contrast to the present observation that the morphogenetically quiescent flank incorporated the greatest amounts of sulfate, Bernfield & Wessells (1970) reported that the morphogenetically active regions of the developing salivary gland incorporated the greatest amount of glucosamine. Furthermore, Searls (1965) observed that at later stages the morphogenetically active sub-ridge region of the developing limb bud exhibited somewhat elevated levels of sulfate incorporation. Lunt & Seegmiller (1977) have shown that autoradiographic studies of sulfate incorporation reveal higher levels of activity in the limbs than in the flank at stages comparable to those examined in the present study. That there are regional and specific interactions between glycosaminoglycans and collagen (David & Bernfield, 1979) and proteoglycans and cell aggregation (Morris, 1979) is further support for the concept that extracellular matrix components play a role in the regulation of morphogenesis and differentiation. The meaning of these seemingly disparate results will have to await further experimentation and knowledge of the role of extracellular matrices during development.

We have attempted to determine some of the biochemical differences between the limb primordia and the intervening (flank) tissues, and to relate these differences to what little is known about the acquisition of 'limbness' (the morphogenetic properties resulting in a limb, cf. Zwilling, 1972). With regard to the extracellular matrix, 'limbness' may be defined as the relatively early presence of large proteoglycan aggregates accompanied by lower levels of matrix
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production than intervening tissues. This definition is neither "exclusive nor all encompassing, but it is a beginning. As more data become available concerning the nature of cellular and other differences between the limbs and flank, a more accurate biochemical definition of 'limbness' may be possible.

Supported by NIH grants TO1 GM 00281 and RO1 HD 00380.

REFERENCES


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(Received 15 October, 1979, revised 1 March 1980)