12. E. J. Farris and J. Q. Griffith, *The Rat in Laboratory Investigation* (Hafner, New York, 1967).
13. F. Rappaport, J. Fischl, N. Pinto, *Clin. Chem.* **6**, 16 (1960).
14. A. J. Eisenman, H. F. Fraser, J. Sloan, H. Isbell, *J. Pharmacol. Exp. Ther.* **124**, 305 (1958).
15. K. A. Munday and G. F. Blane, *J. Endocrinol.* **20**, 266 (1960).
16. G. Smith, *ibid.* **23**, 385 (1962).
17. J. Steinberg and R. Straus, *J. Urol.* **57**, 498 (1947); D. Sundaresivarao, *J. Pathol. Bacteriol.* **69**, 324 (1955).
18. N. B. Friedman and G. L. Garske, *J. Urol.* **62**, 363 (1949); F. J. Glassy and F. M. Mostofi, *Amer. J. Clin. Pathol.* **26**, 1303 (1956).
19. G. R. Nagamatsu, personal communication.

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Collagen Synthesis in vitro by Embryonic Spinal Cord Epithelium

Abstract. *Isolated spinal cords from chick embryos (stages 12 to 15) were incubated in vitro with radioactive proline. The proteins synthesized were fractionated by coprecipitation with added carrier collagen, followed by molecular-sieve and ion-exchange chromatography. A portion of the isotopically labeled proteins were found to be collagen molecules consisting only of α1 chains.*

There is evidence that extracellular materials, such as collagen and the acid mucopolysaccharides, which are present between interacting tissues, might be causally related to interactions between developing tissues (*1*). Independent support for this function of the extracellular matrix comes from the fact that myoblast differentiation in vitro is influenced by a macromolecule (or macromolecules) which is excreted by fibroblasts, and for which pure collagen can substitute (*2*). Similar studies (*3*) have indicated that collagen and other stromal macromolecules may be, in some as yet undefined manner, involved in inductive interactions.

Mesenchyme is the source of the connective tissue macromolecules that have been causally implicated in tissue interactions (*4*). However, epithelial cells, especially in the embryo, can also synthesize collagen (*5*). The spinal cord of the chick embryo, which is an epithelial tissue and an inducer of cartilage differentiation in adjacent somites (*6*), produces cross-striated collagen fibrils in vitro at the stage of this induction (*7*). There are at least four distinct molecular species of collagen in vertebrates (*8–11*), which differ in the primary structure of their component α chains; we studied and characterized the collagen produced by the embryonic spinal cord in vitro. Because of the small amounts of collagen produced by this tissue, we analyzed its structure by molecular-sieve and ion-exchange chromatography of radioactively labeled proteins mixed with carrier collagen.

Spinal cords were dissected from the posterior regions of chick embryos at stages 12 to 15, during which stages the posterior portion is composed of pseudostratified epithelium. Adjacent somites were removed by a brief treatment with 1 percent trypsin in Hanks balanced salt solution, at 5°C, and the isolated neuroepithelium was explanted onto a lens killed by freezing (*7*). The tissue and substratum were grown on rayon rafts and on F-10 nutrient medium supplemented with both chick embryo extract and fetal calf serum as previously described (*7*). β-Aminopropionitrile (β-APN) was added to the culture medium (50 μg/ml) in order to inhibit cross-linking in the newly synthesized collagen, thereby facilitating its solubilization for subsequent biochemical analyses. The proteins synthesized by the culture were labeled during a 16-hour incubation of the culture, from the time of explantation, with L-[2,3-^{3}H]proline (New England Nuclear, NET-323) at a concentration of 200 μc/ml.

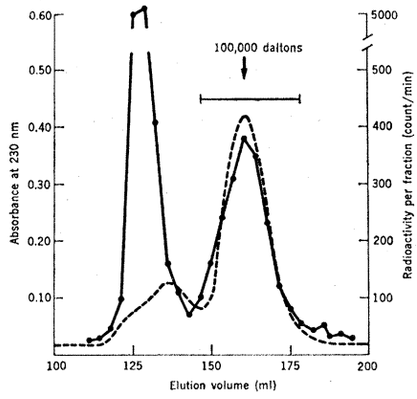

Fig. 1. Chromatogram, from a calibrated molecular sieve column (Agarose), of unlabeled α chains from collagen and of proteins, labeled with [^{3}H]proline, produced in vitro by spinal cords from stage 12 to 15 chick embryos. Absorbance, broken line; radioactivity, solid line. A portion of the labeled proteins elute with the α chains at 100,000 daltons. The bar indicates the material used for subsequent ion-exchange chromatography (Fig. 2).

After being labeled, the medium and culture were extracted overnight with 0.16*M* phosphate buffer, *p*H 7.6, at 4°C, and the resultant extract was clarified by centrifugation at 100,000*g* for 1 hour. The solubilized radioactive proteins were then mixed with a neutral solution of carrier collagen purified from whole lathyritic 13-day embryos by standard methods (*10*). The collagen and labeled proteins were precipitated twice from neutral solution by dialysis against 0.01*M* Na_2HPO_4, and were then denatured into component α chains by warming them to 45°C for 20 minutes. The denatured α chains were dialyzed to remove salt, then lyophilized, and dissolved in 1*M* $CaCl_2$ and 0.05*M* tris(hydroxymethyl)aminomethane-HCl, *p*H 7.5, and further purified by chromatography on a 2 cm by 110 cm column of Agarose (BioGel A 1.5). An aliquot of each fraction from the Agarose column was assayed for radioactivity by liquid scintillation spectrometry with Aquasol (New England Nuclear).

A typical elution pattern (Fig. 1) indicates the presence of radioactively labeled material migrating with the α chains of the carrier collagen. Approximately 15 percent of the labeled material applied to the column eluted with the α chains. The radioactive proteins from the α-chain fraction were pooled, excess salts were removed by dialysis, and the solution was then further fractionated on columns of carboxymethyl cellulose (*12*).

About 85 percent of the radioactive material applied to this column was eluted with the carrier α1 chain, whereas no significant amounts of radioactivity were present in the region of elution of the α2 chain. The remaining 15 percent of the radioactive material was equally distributed in the void volume, and in material eluted from the column with a solution of 1.0*M* NaCl and 0.05*M* NaOH (Fig. 2). The elution profile of the radioactive proteins in the α1-chain region indicates some heterogeneity of the chain, with one peak slightly preceeding that of the carrier α1 chain, another migrating precisely with the carrier, and a small shoulder slightly trailing the carrier. The presence of β-APN in the culture medium, and the prior chromatography

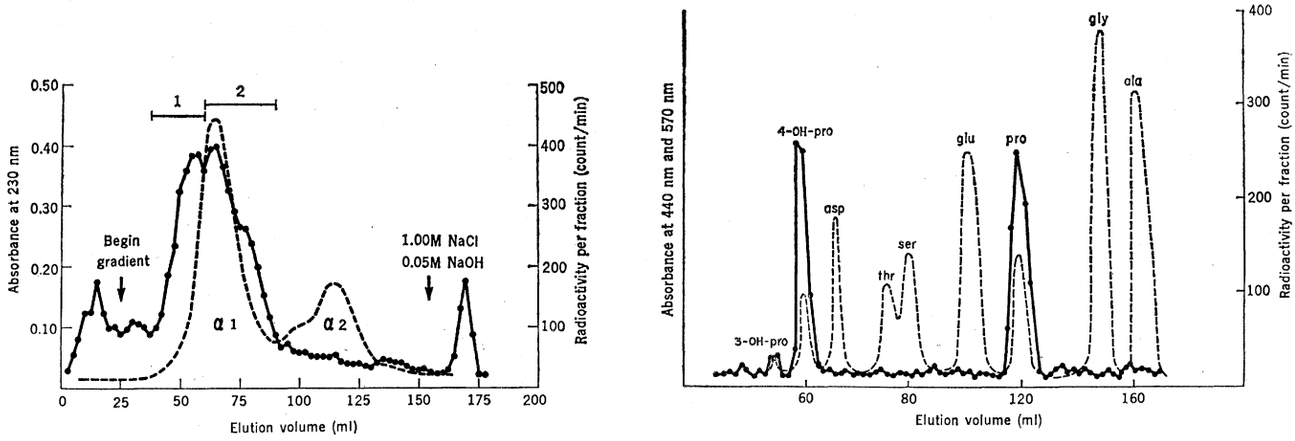

Fig. 2 (left). Chromatogram, from carboxymethyl cellulose column, of the α-chain peak seen in Fig. 1. Absorbance, broken line; radioactivity, solid line. Fractions 1 and 2 were later separately assayed for radioactive hydroxyproline (Fig. 3). Fig. 3 (right). Chromatogram, from the amino acid analyzer, of the α1 chains seen in Fig. 2 after acid hydrolysis. The results with fractions 1 and 2 (Fig. 2) were nearly identical. Abbreviations used: asp, aspartic acid; thr, threonine; ser, serine; glu, glutamic acid; gly, glycine; ala, alanine; pro, proline; 3-OH-pro, 3-hydroxyproline; 4-OH-pro, 4-hydroxyproline. Absorbance, broken line; radioactivity, solid line.

on Agarose, preclude any of these peaks being a cross-linked β component. The possibility that one of these peaks represents a recently described precursor of the collagen α1 chain or pro-α1 (*13*) cannot be excluded, but it is unlikely as the extraction and chromatographic conditions used in our study were unfavorable for the isolation and detection of pro-α1.

In order to further establish the identity of the radioactive material eluting in the α1-chain region, we separately collected fractions from the two major peaks, removed the salt by dialysis, and hydrolyzed them for 24 hours in constantly boiling 6*N* HCl (108°C) in tubes sealed under nitrogen. The amino acids were separated on an amino acid analyzer (Beckman 120C) equipped with a stream splitting device. A portion of the effluent was continuously monitored for ninhydrin reactivity, and the remainder was collected in fractions of 1 ml, and was assayed for radioactivity as described above. The amount of label in hydroxyproline was nearly equal to that in proline (Fig. 3). Most of the radioactivity in hydroxyproline was present in the 4-hydroxy residue. The labeling patterns obtained from the two major peaks of the chromatogram from carboxymethyl cellulose gave identical results.

These data indicate that embryonic spinal cord epithelium can synthesize collagen containing only the polypeptide chains of the α1 type. Although most collagens characterized so far in vertebrates, including dermis, bone, and tendon, consist of two α1 type I chains and one α2 chain, that is, $[\alpha 1\ (I)]_2\alpha 2$ (*8, 9*), the major collagen molecules in cartilage and in basement membranes consist only of α1 chains (*10, 11*). The collagen molecule from cartilage is composed of three α1 type II chains, or $[\alpha 1\ (II)]_3$ (*10*), which differ in primary structure both from α1 type I chains, and from α1 chains, not fully characterized, from basement membranes (*11*). The origin of the chromatographic heterogeneity in the α1 chains made by the spinal cord is not known, but it is possible that either the triple helical collagen molecule contains two different α1 chains, or that two different triple helical molecules are being made, each with a unique α1 chain. However, microheterogeneity of this degree has been previously observed because of variable oxidation of certain lysyl residues or absence of a few NH_2-terminal residues (*9, 14*). At present, therefore, it is impossible to determine whether the observed heterogeneity represents α chains of different primary structure or microheterogeneity. We can conclude, however, that the spinal cord epithelium synthesizes polypeptide chains of the α1 type only.

Tissue interactions occur in the embryo within precisely defined anatomical regions often described as fields or gradients. The molecular basis for such gradients is not known but could relate, in part, to the spatial distribution of the extracellular matrix. Embryonic connective tissues might thus be involved in tissue interactions, and, simultaneously, in the establishment of regional anatomical differences, both resulting in the coordinated cytodifferentiation and morphogenesis of a tissue. Our results indicate that the embryonic spinal cord produces collagen at the same time it induces the adjacent mesenchyme to form the segmentally arranged axial cartilages, but the results provide no direct evidence that these events are causally related.

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References and Notes

1. C. Grobstein, *Nat. Can. Inst. Monograph* **26**, 279 (1967).
2. I. R. Konigsberg and S. K. Hauschka, in *Reproduction: Molecular, Subcellular, and Cellular*, M. Locke, Ed. (Academic Press, New York, 1965), p. 243.
3. N. K. Wessels, *Proc. Nat. Acad. Sci. U.S.A.* **52**, 252 (1964); J. W. Dodson, *J. Embryol. Exp. Morph.* **17**, 83 (1967); D. W. O'Hare, *ibid.* **27**, 235 (1972).
4. F. Kallman and C. Grobstein, *Develop. Biol.* **11**, 169 (1965); M. R. Bernfield, *ibid.* **22**, 213 (1970).
5. J. W. Dodson and E. D. Hay, *Exp. Cell. Res.* **65**, 215 (1971).
6. H. Holtzer and S. Detwiler, *J. Exp. Zool.* **123**, 335 (1953); R. L. Waterson, I. Fowler, B. J. Fowler, *Amer. J. Anat.* **95**, 337 (1954); C. Grobstein and G. Parker, *Proc. Soc. Exp. Biol. Med.* **85**, 477 (1954); G. Strudel, *Develop. Biol.* **4**, 67 (1962); J. W. Lash, in *Epithelial-Mesenchymal Interactions*, R. Fleischmajer and R. E. Billingham, Eds. (Williams & Wilkins, Baltimore, 1968), p. 165.
7. A. M. Cohen and E. D. Hay, *Develop. Biol.* **26**, 578 (1972).
8. E. J. Miller, G. R. Martin, K. A. Peiz, M. R. Powers, *J. Biol. Chem.* **242**, 5481 (1967).
9. A. H. Kang, K. A. Piez, J. Gross, *Biochemistry* **8**, 3648 (1969).
10. E. J. Miller and V. J. Matukas, *Proc. Nat. Acad. Sci. U.S.A.* **64**, 1264 (1969); R. L. Trelstad, A. H. Kang, S. Igarashi, J. Gross, *Biochemistry* **9**, 4993 (1970); E. J. Miller, E. H. Epstein, K. A. Piez, *Biochem. Biophys. Res. Commun.* **42**, 1024 (1971); E. J. Miller, *Biochemistry* **10**, 1652 (1971).

11. N. Kefalides, *Biochem. Biophys. Res. Commun.* **45**, 226 (1971).
12. K. A. Piez, E. A. Eigner, M. S. Lewis, *Biochemistry* **2**, 58 (1963).
→ D. Layman, E. B. McGoodwin, G. R. Martin, *Proc. Nat. Acad. Sci. U.S.A.* **68**, 454 (1971);
→ G. Bellamy and P. Bornstein, *ibid.*, p. 1138.
14. K. A. Piez, G. R. Martin, P. Bornstein, *Biochemistry* **5**, 3813 (1966).

15. Publication No. 582 of the Robert W. Lovett Memorial Group for the study of Diseases Causing Deformities. Supported by NIH grants AM 3564 and AM 12785 and by the National Foundation–March of Dimes grant CRBS-229. R.L.T. is a fellow of the Helen Hay Whitney Foundation; A.H.K. is Cecil L. Russell Fellow of the Arthritis Foundation.

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Induction of Choline Phosphotransferase and Lecithin Synthesis in the Fetal Lung by Corticosteroids

Abstract. *Rabbit fetuses 23 to 24 days of gestation were injected with either 9-fluoroprednisolone acetate or saline. Three days later the lungs of steroid-treated animals showed a significant increase in lecithin concentration and cholinephosphotransferase activity. In addition, lung slices from these animals incorporated more [^{14}C]choline into lecithin. The rise in enzyme activity and [^{14}C]choline incorporation was blocked by prior treatment of fetuses with cycloheximide but not by treatment with actinomycin D. It is proposed that the corticosteroids induce de novo synthesis of the lung enzyme, which in turn leads to increased synthesis of lecithin through the choline incorporation pathway. Furthermore, it appears that the site of regulation involves translation of messenger RNA.*

Respiratory distress syndrome is a developmental disease of prematurely delivered animals characterized by progressive atelectasis of the lungs in association with a marked reduction in the concentration of surface-active lecithin, that is, surfactant (*1*). At the present time only supportive measures such as administration of oxygen and alkali are available therapeutically. Current evidence suggests that there are two principal pathways for pulmonary lecithin biosynthesis—incorporation of cytidine diphosphocholine (CDP-choline) into 1,2-diglyceride and trimethylation of phosphatidyl ethanolamine. The former pathway appears to develop late in gestation, and its deficiency in the premature animal may well be etiologically related to the respiratory distress syndrome (*2*).

Kotas and Avery (*3*) and Motoyama *et al.* (*4*) reported that rabbit fetuses treated with corticosteroids showed evidence of increased pulmonary maturation as determined by pressure-volume curves obtained during deflation, bubble stability ratios, and surface tension properties of lung extracts. These results are consistent with the proposal that glucocorticoid administration leads to earlier development of alveolar surface-active phospholipid. However, the above factors provide a relatively crude assessment of the capability of fetal lung for lecithin biosynthesis.

Using a biochemically directed approach, we examined the effect of corticosteroids on surface-active lecithin by assaying the compound directly and by measuring the incorporation of isotopic precursors into lecithin by lung slices. The capacity of hydrocortisone to induce synthesis of several enzymes involved in gluconeogenesis has been documented (*5*). We postulated that a similar mechanism might be operative for the lecithin biosynthetic pathway, and in order to assess this possibility the activities of four enzymes in two pathways were also examined. These included choline kinase (E.C. 2.7.1.32) and choline phosphotransferase (E.C. 2.7.8.2) of the choline incorporation pathway and methionine adenosyltransferase (E.C. 2.5.1.6) and phosphatidyl ethanolamine methyltransferase of the methylation pathway. Only choline phosphotransferase was elevated.

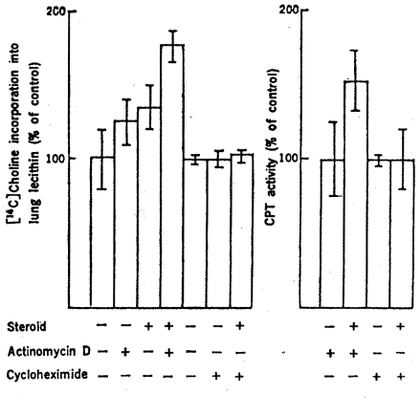

Fig. 1. Effects of actinomycin D and cycloheximide on the corticosteroid-associated changes in choline phosphotransferase (*CPT*) activity and in lecithin synthesis in fetal rabbit lung. The latter was assayed as incorporation of [^{14}C]choline into lecithin by lung slices. Each group contains two to four rabbit fetuses. Standard errors of the mean are indicated.

Pregnant rabbits 23 to 24 days of gestation, for which conception time was known within 2 hours, were anesthetized with barbiturates and subjected to laparotomy. The fetuses were injected intramuscularly through the transparent uterine wall with 50 μg of 9-fluoroprednisolone acetate as Predef (*6*) or with saline. Three days later, unless otherwise noted, the animals were delivered by cesarean section, and breathing was prevented by the application of a ligature around the neck. The lungs were rapidly removed, blotted free of blood, and extracted by the technique of Folch *et al.* (*7*) for lecithin analysis. The latter was performed after thin-layer chromatography by measuring the char density of spots sprayed with sulfuric acid. Fresh lungs were also sliced in a Stadie-Riggs microtome and incubated for 1 to 2 hours at 37°C in a Krebs-Ringer bicarbonate solution containing 1 μc of either [^{14}C]choline or [^{14}C]methionine. Incorporation of radioactivity was subsequently measured in either lipid extract or isolated lecithin. In the case of [^{14}C]choline incorporation, thin-layer chromatography demonstrated that more than 99 percent of the radioactivity was found in lecithin. Choline phosphotransferase activity was measured in lung homogenates at *p*H 8.5 by incubation of dipalmitin (1 m*M*) with CDP-[^{14}C]choline. The assay system also contained 0.1*M* tris(hydroxymethyl)aminomethane hydrochloride, 5 m*M* $MgCl_2$, and 0.006 percent Triton X-100. Radioactivity of isolated lecithin served as a measure of enzyme

Table 1. Synthesis of lung lecithin in the rabbit fetus after injection of corticosteroid or saline. The lecithin fraction precipitated by cold acetone is represented as surface-active lecithin (*9*). Incorporation of isotopic precursors by lung slices is given as counts per minute in lecithin per milligram of wet lung per hour of incubation. Standard errors of the mean are given. The numbers of animals are in parentheses. Abbreviations: Met., methionine; Chol., choline.

Lecithin (milligrams per gram of dry lung)		Incorporation of	
Total	Surface-active	[^{14}C]Met.	[^{14}C]Chol.
		NaCl	
70 ± 3 (17)	59 ± 2 (17)	72 ± 8 (5)	1043 ± 114 (6)
		Steroid	
96 ± 7* (15)	86 ± 2* (15)	69 ± 11 (5)	1589 ± 167† (6)

* $P < .005$. † $P < .05$.