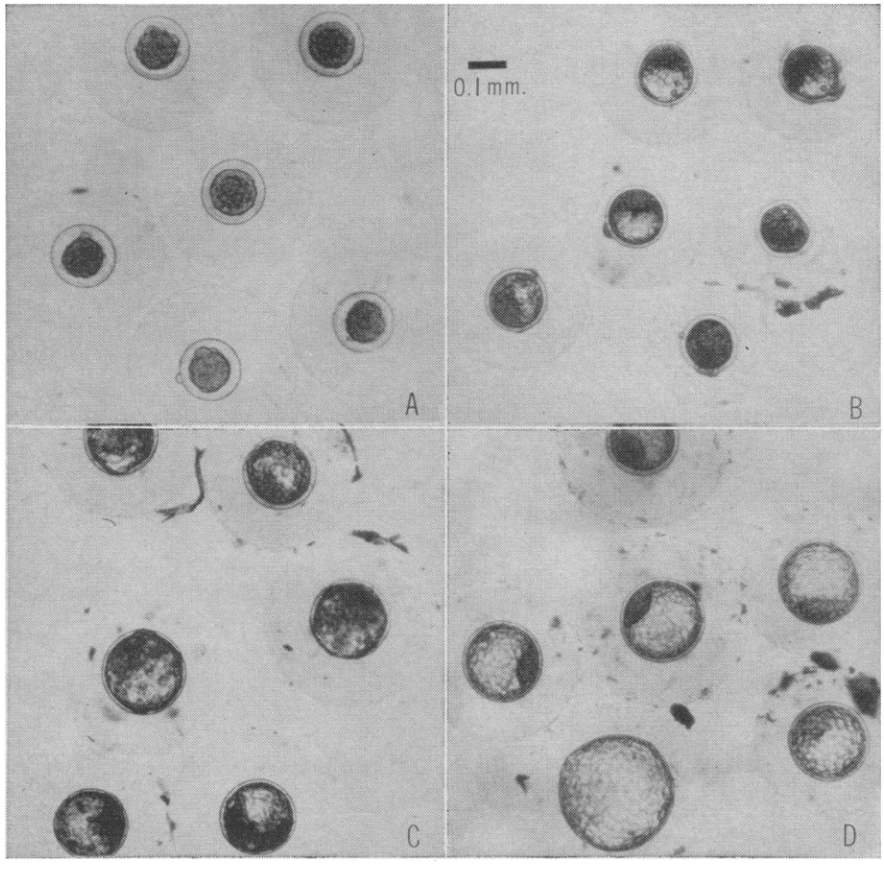

Fig. 2. Photomicrograph of 3-day rabbit morulae cultured in vitro for 24 hours in Ham's F10. (A) Normal 3-day morulae; (B) cultured in the presence of 0.3 mg of rabbit serum proteins per milliliter; (C) cultured in the presence of 0.2 mg of "blastokinin" per milliliter; (D) normal 4-day blastocysts. All pictures were taken at the same magnification.

centration of 0.5 mg/ml caused 78 percent of the embryos to expand. On the contrary, maternal serum proteins did not promote comparable expansion of embryos of these stages. This finding rules out the possibility that expansion and continued growth of blastocysts in vitro is a mere protein effect, thereby suggesting that early rabbit embryos cultured in vitro require a specific protein to continue their controlled development and growth. When one considers the absence of the specific protein from maternal serum, the ineffectiveness of supplementation with serum protein is not surprising.

Embryos cultured in vitro in the presence of the specific protein (see Fig. 2) are not exactly comparable in appearance to normal 4-day blastocysts. The trophoblast appears to be composed of extra layers of cells; nevertheless the size of the blastocysts is comparable to those grown in vivo. Although the cells have been replicating, total expansion appears limited, possibly by a tight zona pellucida. This assumption seems reasonable since cultured rabbit embryos can more easily expand when the zona pellucida is removed or weakened by the use of pronase (*2*).

The demonstration that a specific protein fraction isolated from the maternal uteri can induce and regulate blastocyst development in embryos of the same species strongly suggests the existence of endogenous substances that control blastulation. When one considers the effectiveness of appropriate concentrations of the complete uterine-fluid protein component in promoting cavitation and expansion of blastocysts, it becomes apparent that the substance (or substances) is a normal rather than a fortuitous agent. We propose that the term "blastokinin" be used to describe those substances which appear to function as endogenous regulators of blastocyst development.

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References and Notes

1. N. Purshottam and G. Pincus, *Anat. Rec.* **140**, 51 (1961); R. L. Brinster, *Exp. Cell Res.* **32**, 205 (1963); J. D. Biggers, B. D. Moore, D. G. Whittingham, *Nature* **206**, 734 (1965).
2. R. J. Cole, R. G. Edwards, J. Paul, *Dev. Biol.* **13**, 385 (1966).
3. R. L. Huff, *Amer. Zool.* **2**, 415 (1962).
4. J. C. Daniel, *J. Embryol. Exp. Morphol.* **13**, 83 (1965).
5. R. G. Ham, *Exp. Cell Res.* **29**, 515 (1963).
6. O. H. Lowry, N. J. Rosebrough, A. L. Farr, R. J. Randall, *J. Biol. Chem.* **193**, 265 (1951).
7. Supported by AEC contract AT(11-1)-1597.

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Intracellular Pool of Unhydroxylated Polypeptide Precursors of Collagen

Abstract. *The hydroxyproline and hydroxylysine in collagen are synthesized by an apparently unique pathway in which proline and lysine are hydroxylated after they are incorporated into a large polypeptide precursor of collagen called protocollagen. When the hydroxylation of protocollagen in isolated tissues is intermittently interrupted, hydroxylation can occur after complete polypeptides are released from ribosomal complexes. Cartilage from chick embryos was incubated with the iron chelator α,α'-dipyridyl for 2 hours to inhibit protocollagen hydroxylase, and then the inhibition was reversed by transferring the tissues to medium containing ferrous iron and no α,α'-dipyridyl. "Pulse labeling" of the tissues during these two periods indicated that both the accumulated protocollagen and the polypeptides synthesized after reversal of the inhibition were hydroxylated at the same rate. Even when no measures are taken to inhibit the hydroxylation of protocollagen, most of the hydroxyproline in collagen is probably synthesized after complete protocollagen polypeptides are released from ribosomes.*

Hydroxyproline (*1*) and hydroxylysine (*2*) in collagen are synthesized by an apparently unique pathway in which proline and lysine are hydroxylated after they have been incorporated into a large polypeptide precursor of collagen called protocollagen. The enzyme protocollagen hydroxylase hydroxylates the proline and probably the lysine in protocollagen (*3*), and both hydroxylations require ascorbate, α-ketoglutarate, atmospheric oxygen, and ferrous iron (*1*, *3*, *4*). When tissues synthesizing collagen are incubated under anaerobic conditions or

with the iron chelator α,α'-dipyridyl, protocollagen polypeptides comparable in size to the complete α-chains of collagen (*5*) accumulate within the cells (*6*). Under these conditions, protein synthesis continues at 60 to 90 percent of the control rate for over 4 hours. When the hydroxylase activity of the tissues is restored, the appropriate proline and lysine residues in the accumulated protocollagen are hydroxylated, and the molecules are extruded into the extracellular matrix (*6*). Because it is unlikely that significant amounts of complete protocollagen polypeptides can accumulate on ribosomes without a marked decrease in the rate of protein synthesis, these observations suggest that, when the hydroxylation of protocollagen is intermittently interrupted, hydroxylation can occur after complete polypeptides are released from ribosomal complexes. The evidence is conflicting (*7–9*), however, as to whether hydroxylation occurs while nascent polypeptide chains are still attached to ribosomal complexes when protocollagen hydroxylase is not inhibited.

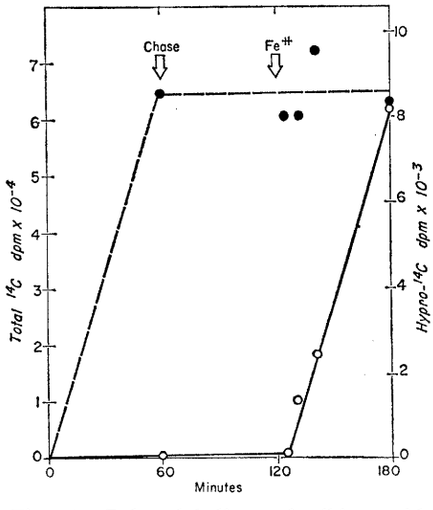

Fig. 1. Pulse labeling of tibiae with proline-^{14}C during inhibition of protocollagen hydroxylase with α,α'-dipyridyl. Four tibiae from 10-day-old chick embryos were incubated in 2.5 ml of medium for 30 minutes, and then α,α'-dipyridyl was added to a final concentration of 1 m*M*. After incubation for 30 minutes, 2 μc of proline-^{14}C was added for a labeling period of 60 minutes; then the label was chased for 60 minutes by transferring the tibiae to fresh medium containing 100 μg of proline-^{12}C per milliliter and 1 m*M* α,α'-dipyridyl. After the chase period, the inhibition of protocollagen hydroxylase was reversed by transferring the tibiae to medium containing no α,α'-dipyridyl and 1 m*M* $FeSO_4$. Closed circles, total dpm of nondialyzable ^{14}C in the tissues; open circles, dpm of hydroxyproline-^{14}C content.

We now present observations which suggest that, even in uninhibited tissues, most of the synthesis of collagen hydroxyproline occurs after complete protocollagen molecules are released from ribosomal complexes.

Tibiae were removed from 10-day-old embryos under sterile conditions and incubated in 2.5 ml of medium at 37°C, as described previously (*1*, *8*). After the incubations, the tissues were immediately homogenized in distilled water containing 10 m*M* α,α'-dipyridyl, and the homogenates were dialyzed against running tap water. The dialyzed samples were then hydrolyzed in HCl and evaporated to dryness in a vacuum (*8*). Total ^{14}C was assayed with a liquid-scintillation counter, and hydroxyproline-^{14}C was assayed with a specific chemical procedure (*10*). Observed counts per minute were converted to disintegrations per minute (dpm) by correcting for counting efficiencies of 58 to 60 percent and a background of 10 count/min as described previously (*10*).

Chick embryo tibiae synthesize collagen at a rapid rate in vitro (*8*), and, when the tissue is incubated with proline-^{14}C, essentially all the incorporated radioactivity is recovered as proline-^{14}C and hydroxyproline-^{14}C. Tibiae were first incubated with α,α'-dipyridyl, proline-^{14}C was added for a labeling period of up to 60 minutes, and the proline-^{14}C was then "chased" for 60 minutes by replacing the medium with fresh medium containing carrier L-proline and α,α'-dipyridyl (Fig. 1). The average incorporation of ^{14}C during the 60-minute labeling period was 64 percent of the value observed in control samples incubated without α,α'-dipyridyl. Essentially no hydroxyproline-^{14}C was synthesized in the presence of α,α'-dipyridyl. After inhibition of the hydroxylase was reversed by removing the α,α'-dipyridyl and adding 1 m*M* $FeSO_4$, proline-^{14}C in the pulse-labeled protocollagen was converted to hydroxyproline-^{14}C. There was no apparent lag in the hydroxylation of the accumulated protocollagen-^{14}C, an indication that the activity of protocollagen hydroxylase was promptly restored. The rate of hydroxylation was linear for about 60 minutes after reversal of the inhibition.

To determine whether newly synthesized protocollagen polypeptides were hydroxylated more rapidly than protocollagen accumulated during inhibition of the hydroxylase, we incubated

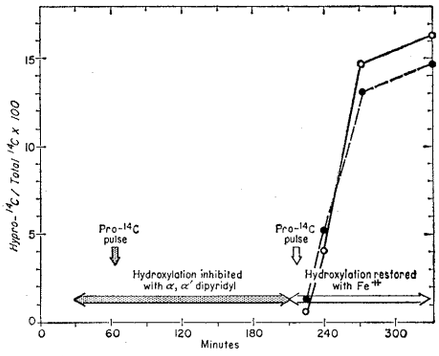

Fig. 2. Pulse labeling of tibiae with proline-^{14}C during inhibition of protocollagen hydroxylase and after reversal of the inhibition (for outline of the experiment, see Table 1). Closed circles, values for the ratio of hydroxyproline-^{14}C to total ^{14}C in tibiae pulse-labeled during the inhibition period; open circles, values for the ratio in tibiae labeled after reversal of the inhibition.

matched pairs of tibiae from the same embryos under identical conditions, except that one set was "pulse-labeled" with proline-^{14}C at the beginning of the inhibition period, and the other set was pulse-labeled after reversal of the inhibition (Table 1). The experimental conditions were similar to those for the experiment in Fig. 1, except that a shorter pulse-labeling period with 5 μc of proline-^{14}C was

Table 1. Pulse labeling of tibiae with proline-^{14}C during inhibition (samples A, C, E, and G) hydroxylase and after reversal (samples B, D, F, and H) of the inhibition. Each flask contained four tibiae from 10-day-old chick embryos. The tibiae were incubated in 2.5 ml of medium for 30 minutes. Then α,α'-dipyridyl was added to a final concentration of 1 m*M*, and the incubation was continued for 2.5 hours. The tibiae were transferred to fresh medium containing no α,α'-dipyridyl and 1 m*M* $FeSO_4$ for a further incubation period of 15 to 120 minutes. Sets of tibiae were pulse-labeled for 10 minutes by the addition of 5 μc of proline-^{14}C, and then the label was chased by multiple changes of medium containing 30 μg per milliliter of proline-^{12}C. One set of tibiae was pulse-labeled and chased beginning 30 minutes after α,α'-dipyridyl. The other set was pulse-labeled and chased beginning 5 minutes after the tibiae were removed from medium containing α,α'-dipyridyl and transferred to medium containing $FeSO_4$.

Sample	Reversal time with Fe^{+2} (min)	Total ^{14}C (10^{-4} dpm)	Hypro-^{14}C (10^{-3} dpm)
A	15	4.6	0.58
B	15	3.1	.17
C	30	4.0	2.2
D	30	4.2	2.0
E	60	5.0	7.0
F	60	4.3	6.3
G	120	4.7	7.5
H	120	3.8	6.6

used. The total incorporation of ^{14}C was similar in samples labeled early during inhibition of protocollagen hydroxylase and in samples labeled after reversal of the inhibition, an indication that the rate of polypeptide synthesis was essentially the same during the two labeling periods. After reversal of the inhibition, the synthesis of hydroxyproline-^{14}C proceeded at about the same rate in both sets of samples, and there was no significant difference in the rate at which the ratios of hydroxyproline-^{14}C to total ^{14}C increased during the reversal period (Fig. 2).

In tibiae that were pulse-labeled after reversal of the inhibition (Fig. 2), essentially all the proline-^{14}C was in polypeptides synthesized in the presence of active protocollagen hydroxylase. In tibiae that were pulse-labeled early in the inhibition period, all the proline-^{14}C should have been in complete polypeptides released from ribosomal complexes. Under the conditions of the experiment, it is unlikely that the separate pools of growing polypeptides on ribosomes and the complete polypeptides released from ribosomes would be hydroxylated at the same rate. If the hydroxylation of proline occurred preferentially in nascent polypeptides attached to ribosomal complexes, the proline-^{14}C in tibiae pulse-labeled after reversal of the inhibition of protocollagen hydroxylase should have been hydroxylated more rapidly than the proline-^{14}C in tibiae pulse-labeled early in the inhibition period. In that both sets of pulse-labeled polypeptides were hydroxylated at essentially the same rate, the results suggest that the hydroxylation of both the newly synthesized and the accumulated polypeptides occurred in the same intracellular pool. From these observations as well as from our previous studies on the effects of puromycin (*8*) and with pulse-labeling techniques in cartilage (*9*), it appears that, even when no measures are taken to inhibit protocollagen hydroxylase, most of the hydroxyproline in collagen is synthesized after complete protocollagen polypeptides are released from ribosomes.

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References and Notes

1. B. Peterkofsky and S. Udenfriend, *J. Biol. Chem.* **238**, 3966 (1963); D. J. Prockop and K. Juva, *Biochem. Biophys. Res. Commun.* **18**, 54 (1965); ———, *Proc. Nat. Acad. Sci. U.S.* **53**, 661 (1965); L. N. Lukens, *J. Biol. Chem.* **240**, 1661 (1965).
2. D. J. Prockop, E. Weinstein, T. Mulveny, *Biochem. Biophys. Res. Commun.* **22**, 124 (1966).
3. K. I. Kivirikko and D. J. Prockop, *Arch. Biochem. Biophys.* **118**, 611 (1967); ———, *Proc. Nat. Acad. Sci. U.S.* **57**, 782 (1967).
4. K. Juva and D. J. Prockop, in *Biochemie et Physiologie du Tissu Conjonctif*, P. Compte, Ed. (Société Ormeco et Imprimerie du Sud-Est à Lyon, 1966), p. 417; J. Hurych and M. Chvapil, *Biochim. Biophys. Acta* **97**, 361 (1965); E. Hausmann, *Fed. Proc.* **26**, 669 (1967).
5. L. N. Lukens, *Proc. Nat. Acad. Sci. U.S.* **55**, 1235 (1966); K. I. Kivirikko and D. J. Prockop, *Biochem. J.* **102**, 432 (1967).
6. K. Juva, D. J. Prockop, G. W. Cooper, J. W. Lash, *Science* **152**, 92 (1966); G. W. Cooper, *Federation Proc.* **26**, 828 (1967).
7. R. H. Kretsinger, G. Manner, B. S. Gould, A. Rich, *Nature* **202**, 438 (1964); G. Manner, R. H. Kretsinger, B. S. Gould, A. Rich, *Biochim. Biophys. Acta* **134**, 411 (1967); R. Fernandez-Madrid, *J. Cell Biol.* **33**, 21 (1967); B. Goldberg and H. Green, *Federation Proc.* **25**, 663 (1966); I. Bekhor, Z. Mohseni, L. A. Bavetta, *ibid.* **25**, 715 (1966); J. M. Manning and A. Meister, *Biochemistry* **5**, 1154 (1966); S. Udenfriend, *Science* **152**, 1335 (1966).
8. K. Juva and D. J. Prockop, *J. Biol. Chem.* **241**, 4419 (1966).
9. R. S. Bhatnagar, J. Rosenbloom, D. J. Prockop, *Federation Proc.* **26**, 669 (1967).
10. D. J. Prockop and P. S. Ebert, *Anal. Biochem.* **6**, 263 (1963); K. Juva and D. J. Prockop, *ibid.* **15**, 77 (1966).
11. We thank Mrs. Rosalie Stein and Geraldine Zuka for technical asistance. Supported in part by research grants FR-107, HD-183, AM-5459, and GM-14583 from NIH.

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Polycystic Renal Disease: A New Experimental Model

Abstract. *A single injection of any one of several long-acting adrenal corticosteroids at birth induces progressive cystic changes in nephrons which develop in the subcapsular zone of the rabbit kidney until 10 days after birth. These cystic lesions enlarge progressively and become visible within a few days after birth. When the animal is 2 weeks old, renal size has become three times as large as that of uninjected littermates. Adrenal corticosteroids prolong the duration of nephrogenic activity in the renal cortex. The cysts are blind and represent dilatation of the developing end of the collecting ducts. When the steroid-induced hypokalemia is prevented with repeated potassium chloride injections, renal cystic disease is almost completely prevented. Certain long-acting steroids induce cystic renal changes without systemic signs of toxicity.*

In the search for an experimental model of polycystic kidney disease (*1*), several investigators have produced cystic lesions in kidneys of experimental animals (*2*) and induced tubular dilatation in rats and rabbits through repeated injections of large doses of steroids in young adult animals (*3*). We studied the effect of a single injection of long-acting adrenal corticosteroids in newborn rabbits and observed persistence and progression of the cysts in the renal cortex and prolongation of the period during which new nephrons developed. Furthermore, we found that 9-fluoroprednisolone acetate (*4*) induces cystic renal changes without signs of systemic toxicity when injected in the proper dosage during the neonatal period; this permits study of the progression of the cystic disease in otherwise intact animals. We obtained evidence which strongly suggests potassium deficiency as the cause for the cystic lesions.

Litters of New Zealand white rabbits (40 to 50 g) were obtained on the day of birth. Some of each litter were given an intramuscular injection of one of nine long-acting steroids (Table 1), while others were not injected and served as controls. After injection, the animals were returned to the care of their mother. One group, injected with 0.5 mg of 9-fluoroprednisolone acetate at birth, was given an intraperitoneal injection of 0.25 meq of potassium chloride (KCl) daily for 14 days. They were provided with as much Purina rabbit chow and water as they wanted. Qualitative urinalyses were done on fresh urine obtained by manually compressing the lower abdomen of the young rabbits. Concentrations of sodium, potassium, creatinine, and urea nitrogen in the serum were determined by routine methods. Urinary osmolalities were measured (on a Fiske Osmometer) in 6-week-old rabbits after they had been deprived of water and food for 36 hours. Microdissection was performed with the method of Oliver *et al.* (*5*). Cystic changes observed on kidney sections stained with hematoxylin and eosin were subjectively graded as zero to four pluses. The majority of long-acting steroids induced cystic changes in renal cortical tubules (Table