BIOSYNTHESIS OF COLLAGEN AND NON-COLLAGEN PROTEIN DURING DEVELOPMENT OF THE CHICK CORNEA

JOHN R. COLEMAN, Ph.D., HEINZ HERRMANN, M.D., and BARBARA BESS

From the Institute of Cellular Biology, University of Connecticut, Storrs, Connecticut. Dr. Coleman's present address is Biology Department, Brown University, Providence, Rhode Island

ABSTRACT

The relationship between the rates of increase of corneal protein fractions and incorporation of labeled precursors has been examined during embryonic and early posthatching development of the chick corneal stroma. Non-collagen protein increased gradually from 9 through 20 days of incubation. Collagen accumulated approximately logarithmically through the 19th day, the most rapid rate occurring between 13 and 20 days of incubation. The rates at which labeled amino acids are incorporated into collagen *in vivo* and *in vitro* undergo marked changes during the last week of embryonic development, corresponding closely to the rate of collagen accumulation *in vivo*; whereas incorporation into non-collagen protein changes much less markedly. Changes in the rate of incorporation of precursors into collagen are not due to changes in the rate of conversion of collagen from the soluble to insoluble form, or to changes in the endogenous amino acid pool size. Chick embryo corneal stroma collagen turns over very slowly, if at all. Non-collagen protein turns over more rapidly. An increase in cell number, as indicated by DNA content, does not account for the increased rate of collagen synthesis between the 9th and 16th day of incubation. It is concluded that the observed changes in collagen synthesis reflect changing activities in the individual cornea fibroblasts. These activities are comparable in the intact tissue *in vivo* and in isolated corneas *in vitro*.

INTRODUCTION

The initial appearance and the changes in concentrations of cell-specific proteins in embryonic tissues have been repeatedly investigated, and the results of these studies have been the subject of several reviews (Ebert, 1955; Shen, 1958; Holtzer, 1961; Moog, 1962; Herrmann and Tootle, 1964). It was the purpose of the present work to attempt a more systematic and complete analysis of the correlation between protein formation and incorporation of labeled precursors into proteins in the intact tissues *in vivo* and tissue preparations *in vitro* at different stages of development. In approaching this problem, advantage was taken of embryonic tissues in which the cell-specific proteins become relatively stable components of the tissue structure and make up a large fraction of the total tissue protein moiety. In such tissues, the protein-forming system of the developing cells may be utilized primarily for the synthesis of one or a few characteristic protein species. Earlier work along such lines has been directed toward analysis of the rapid increase in specific muscle

proteins and in collagen of the cornea and sclera (Herrmann, 1963). In the work with the cornea, it became apparent in preliminary experiments (Herrmann, 1958) that the rate of synthesis of collagen per cell changes markedly during development of the chick embryo. The present experiments were carried out in order to check the earlier observations under *in vivo* and *in vitro* conditions and to explore several alternatives which may give rise to the observed changes.

MATERIALS AND METHODS

Fertilized eggs of the White Leghorn breed were obtained from a commercial source and incubated at 37.5°C to the desired stage, or until hatching. Embryos or chicks were killed by decapitation, the cornea was removed from the eye together with a surrounding ring of sclera by means of a fine scissors, and the ring of scleral tissue was trimmed away by scalpel under a dissecting microscope.

For studies of incorporation of labeled amino acids *in vitro*, individual corneas were placed in 10-ml beakers containing 2 ml of a defined nutrient medium (Marcus *et al.*, 1956), pH 7.3, from which bicarbonate had been omitted. Except where specified otherwise, 1 μc of isotopically labeled amino acid was added to each beaker immediately prior to incubation at 37.5°C in a Dubnoff metabolic shaker. In the final incubation mixture, the specific activity of the added glycine-1-C^{14} was 3.5 mc/mmole and proline-U-C^{14} was 2.3 mc/mmole. At the end of the incubation period, corneas were rinsed twice with Hanks' physiological saline, the epithelium was removed by scraping with a scalpel, and the remaining connective tissue layer of the cornea, the corneal stroma, was used for the preparation of either collagen or non-collagen protein. No effort was made to remove the endothelium, which may contribute a small amount of non-collagen protein. The period between isolation of an individual cornea and start of the incubation did not exceed 1 hour.

For studies of the rate of incorporation *in vivo*, glycine-1-C^{14} was injected into a chorioallantoic vein of 11- and 16-day embryos, as described earlier (Konigsberg, 1958). The dosage level was 0.3 μc per gram body weight (Yospha-Purer *et al.*, 1953), and the specific activity was 3.5 mc/mmole. For studies of the retention of label in the collagen and non-collagen protein fractions, 10-day embryos were similarly injected with 1 μc/gm body weight of glycine-1-C^{14}.

Collagen was prepared by extraction of individual corneal stromas with 0.1 N NaOH followed by dissolution in 0.5 ml of glass-distilled water in a pressure cooker as described previously (Herrmann and Barry, 1955). The samples were centrifuged at 750 RCF for 5 minutes and aliquots were removed for N determinations (Britt and Herrmann, 1959), counting, or hydroxyproline determinations (Martin and Axelrod, 1953).

Comparison of the nitrogen and hydroxyproline content of the collagen preparation permits an estimate of its purity, assuming that the composition of collagen does not change with development. Calculations based on the amino acid composition of chicken tendon collagen (Leach, 1957) show a hydroxyproline-to-N ratio of 0.79. Collagen preparations from 16- and 21-day corneas have ratios of 0.79 and 0.76, respectively (Table I), suggesting a high degree of purity. A relatively low ratio (0.55) was found for 11-day cornea collagen, probably indicating contamination by some non-collagen N-containing material.

Non-collagen protein was prepared by removal of soluble components from individual stromas in several washes of cold 5 per cent TCA, extraction of collagen and nucleic acids in 5 per cent TCA at 90°C for 15 minutes, and extraction of lipid in chloroform-ethanol (1:3) at 70°C for 15 minutes. The sediment remaining was hydrolyzed overnight at 140°C in sealed tubes containing 0.5 ml of 6 N HCl. The hydrolyzed samples were dried under vacuum and redissolved in 0.5 ml of glass-distilled water. Aliquots were removed for counting and N

TABLE I

Hydroxyproline and Nitrogen Content of Corneal Stroma Collagen Preparations

Time of incubation	μg hydroxyproline/ Stroma	μg N/Stroma	μg hydroxyproline/ μg N
Days			
11	3.2 ± 0.2*(13)‡	5.9 ± 0.4*(6)‡	0.54 ± 0.05*
16	20.7 ± 0.7 (16)	26.1 ± 0.4 (18)	0.79 ± 0.01
21	36.6 ± 0.7 (20)	48.1 ± 0.6 (20)	0.76 ± 0.01

* Standard error of the mean.

‡ Number of determinations.

determinations. DNA was determined in the supernatant from the hot TCA extraction by the diphenylamine reaction (Burton, 1956).

The level of radioactivity in the collagen and non-collagen protein samples was determined by plating 0.1 ml aliquots at infinite thinness on aluminum planchettes and counting in a gas-flow counter (Nuclear Chicago, Model D-47).

RESULTS

1. *Increase in the Quantities of Collagen and Non-Collagen Protein in the Developing Cornea (Protein Accumulation)*

Collagen and non-collagen protein were found to exhibit quite different patterns of accumulation in the developing corneal stroma (Table II). Non-collagen protein increased gradually from 9 through 20 days, whereas plots of collagen accumulation produced a sigmoid curve with the most rapid rate of accumulation occurring between 13 and 20 days (Fig. 1). Concomitantly, collagen increased from a level only one-third that of the non-collagen protein at 9 days to nearly three times the level of non-collagen protein at 20 days. Both fractions were somewhat variable after hatching.

2. *Incorporation of Glycine-1-C^{14} and Proline-U-C^{14} in vitro and in vivo into Collagen and Non-Collagen Protein of the Cornea*

The data for incorporation *in vitro* of glycine-1-C^{14} into collagen and non-collagen protein are given in Table III and Fig. 1. The period from 13 to 20 days, when collagen was accumulating most rapidly, was also the period of most active incorporation of glycine-1-C^{14} into collagen. Incorporation into non-collagen protein exhibited a peak of activity at about the same time, but this was of a lower order of magnitude than that found with collagen (Table III). The rate of incorporation into collagen increased 14.5-fold per stroma between days 9 and 17, whereas the rate into non-collagen protein increased only 2.5-fold during the same period.

An increase in the rate of protein synthesis in a developing system such as the cornea can be a

TABLE II

Accumulation of DNA, Collagen N, and Non-Collagen Protein N in the Corneal Stroma During Development of the Chick

Time of development	μg DNA/Stroma	Collagen N/Stroma	Non-collagen protein N/Stroma
Days			
8	1.8 ± 0.2*(9)‡	—	—
9	2.8 ± 0.1 (12)	2.3 ± 0.8*(2)‡	6.0 ± 0.6*(3)‡
10	4.0 ± 0.1 (16)	4.2 ± 0.8 (2)	6.5 ± 0.4 (2)
11	4.6 ± 0.2 (10)	6.8 ± 0.5 (2)	7.7 ± 0.5 (4)
12	5.2 ± 0.1 (15)	8.8 ± 0.0 (2)	8.0 ± 1.0 (4)
13	5.7 ± 0.2 (16)	10.0 ± 1.7 (3)	9.1 ± 0.6 (3)
14	5.0 ± 0.1 (12)	16.5 ± 1.0 (4)	10.4 ± 0.5 (3)
15	5.9 ± 0.2 (11)	20.5 ± 0.6 (4)	13.3 ± 1.2 (4)
16	5.8 ± 0.2 (11)	33.4 ± 1.6 (12)	12.7 ± 0.6 (6)
17	6.8 ± 0.2 (11)	37.1 ± 1.4 (9)	15.2 ± 1.4 (6)
18	6.1 ± 0.1 (12)	47.4 ± 2.9 (8)	16.8 ± 1.2 (8)
19	6.3 ± 0.1 (12)	54.3 ± 1.7 (12)	17.8 ± 1.3 (6)
20	5.4 ± 0.2 (12)	57.4 ± 2.1 (12)	19.5 ± 1.1 (4)
21§	4.9 ± 0.1 (15)	56.7 ± 2.2 (12)	18.5 ± 1.5 (6)
22	6.2 ± 0.2 (11)	51.9 ± 2.8 (12)	19.4 ± 1.2 (6)
23	5.7 ± 0.1 (11)	62.4 ± 2.7 (12)	14.6 ± 2.9 (6)
24	5.3 ± 0.2 (15)	70.4 ± 3.1 (12)	18.6 ± 1.6 (3)
25	5.4 ± 0.1 (16)	65.7 ± 3.5 (12)	25.1 ± 3.2 (4)

* Standard error of the mean.

‡ Number of determinations.

§ Age at hatching.

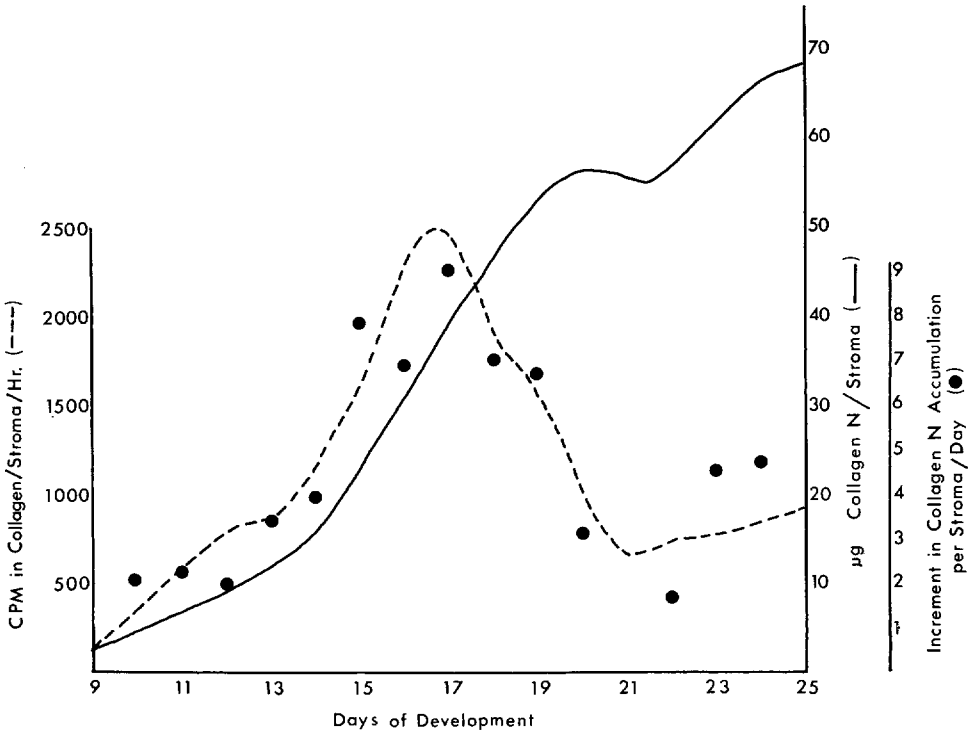

FIGURE 1 Comparison of the accumulation of collagen N with the rate of incorporation of glycine-1-C^{14} into collagen in the developing corneal stroma. The accumulation and incorporation curves are representations of the data in Tables I and II. Increments (in micrograms) in collagen accumulation were calculated from the graphed curve, rather than from Table I.

TABLE III

Incorporation in vitro of Glycine-1-C^{14} into Collagen and Non-Collagen Protein Fractions of the Developing Corneal Stroma

Time of development	CPM in collagen per:		CPM in non-collagen protein per:	
	Stroma/hr.	µg DNA/Stroma/hr.	Stroma/hr.	µg DNA/Stroma/hr.
days				
9	165 ± 21* (12)‡	59 ± 7* (12)‡	313 ± 22* (12)‡	112 ± 8* (12)‡
10	376 ± 18 (12)	94 ± 5 (12)	320 ± 30 (7)	80 ± 7 (7)
11	579 ± 31 (12)	126 ± 7 (12)	400 ± 36 (12)	87 ± 8 (12)
12	838 ± 71 (12)	162 ± 14 (12)	354 ± 36 (11)	68 ± 7 (11)
13	840 ± 82 (12)	147 ± 14 (12)	433 ± 37 (12)	76 ± 6 (12)
14	1144 ± 50 (12)	228 ± 10 (12)	530 ± 44 (12)	106 ± 9 (12)
15	1648 ± 120 (12)	279 ± 20 (12)	632 ± 55 (12)	107 ± 9 (12)
16	2346 ± 115 (24)	405 ± 20 (24)	672 ± 26 (12)	116 ± 5 (12)
17	2407 ± 91 (17)	354 ± 13 (17)	787 ± 47 (12)	116 ± 7 (12)
18	1852 ± 85 (17)	304 ± 14 (17)	790 ± 39 (16)	129 ± 6 (16)
19	1589 ± 113 (12)	252 ± 19 (12)	622 ± 50 (12)	98 ± 8 (12)
20	912 ± 51 (12)	178 ± 10 (12)	457 ± 48 (8)	85 ± 9 (8)
21§	664 ± 24 (12)	136 ± 5 (12)	382 ± 25 (12)	78 ± 5 (12)
22	764 ± 26 (12)	123 ± 4 (12)	404 ± 25 (12)	65 ± 4 (12)
23	791 ± 46 (12)	139 ± 8 (12)	417 ± 31 (12)	73 ± 5 (12)
24	854 ± 28 (12)	161 ± 5 (12)	444 ± 55 (8)	84 ± 10 (8)
25	937 ± 46 (12)	173 ± 9 (12)	469 ± 35 (8)	87 ± 6 (8)

* Standard error of the mean.
‡ Number of determinations.
§ Age of hatching.

function of an increase in the number of actively synthesizing cells or of an increase in synthetic activity of the individual cells. Since DNA content of the stroma should be proportional to the number of cells present, DNA determinations were carried out to eliminate cell number as a variable parameter (Table II). DNA per stroma increased regularly through day 13 and was relatively constant thereafter. Expression of the incorporation of glycine-1-C^{14} into collagen and non-collagen protein on a per μg DNA basis still shows marked changes in the rate of incorporation into collagen *in vitro* (Table III), increasing sevenfold between day 9 and day 16, whereas the rate of incorporation into non-collagen protein undergoes relatively minor changes in the same period (Table III).

The incorporation of proline-U-C^{14} into collagen during the standard 2-hour period of incubation follows a straight line which can be extrapolated back to the origin (Fig. 2). The rate of incorporation at 16 days was 4.6 times that at 11 days, thus confirming the results obtained with glycine-1-C^{14} as a precursor *in vitro*.

Incorporation of glycine-1-C^{14} into corneal proteins *in vivo* was determined at various times following injection of glycine-1-C^{14} into the chorioallantoic veins of 11- and 16-day old embryos (Table IV). Incorporation by 11-day stroma was linear for 60 minutes following injection, while that by 16-day stroma was linear for only 30 minutes. Comparison of the slopes of the linear portions of these two curves shows that the incorporation rate is 4.9-fold greater per μg DNA at 16 days than at 11 days. Thus, comparable differences in rate of incorporation with developmental age are demonstrable *in vivo* and *in vitro*.

TABLE IV

Time Course of Glycine-1-C^{14} Incorporation into Corneal Stroma Collagen in vivo

Time of development	Time after glycine-1-C^{14} injection	CPM in collagen/μg DNA/Stroma
Days	*min*	
11	30	7.7 ± 0.4* (4)‡
	60	16.9 ± 0.9 (4)
	120	25.9 ± 1.0 (4)
16	15	19.9 ± 2.4 (4)
	30	40.0 ± 2.4 (8)
	60	55.1 ± 4.7 (8)
	120	82.5 ± 2.3 (4)

* Standard error of the mean.

‡ Number of determinations.

3. *Endogenous Precursor Pool Size*

Changes in the size of the endogenous glycine pool with development could influence the rate of labeled glycine incorporation into protein if the amount of labeled glycine added is not at a saturation level. To test this possibility, increasing amounts of glycine-1-C^{14} were added and the

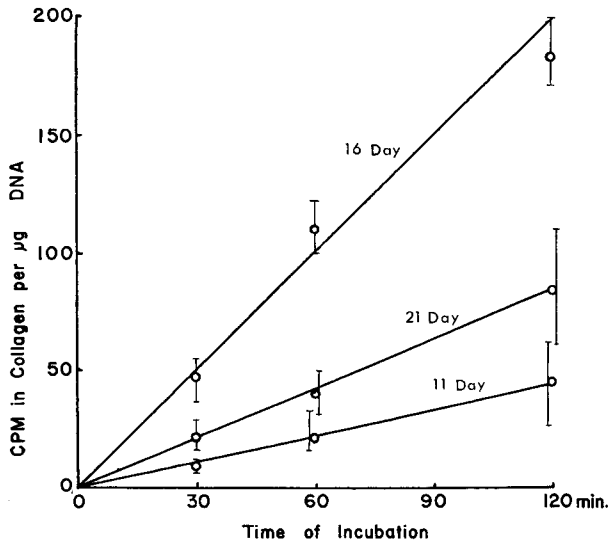

FIGURE 2 The time course of proline-U-C^{14} incorporation into corneal stroma collagen *in vitro*. Each point represents an average of 8 determinations except the 60- and 120-minute values for the 16-day embryos, which are averages of 7 determinations. The vertical lines at each point represent the respective ranges of values. The ordinate indicates CPM in collagen per μg stroma DNA.

amount of radioactivity in collagen determined after a 2-hour incubation period (Table V). It was found that a threefold increase in the concentration of labeled glycine in the incubation medium led to an increase in the amount of label incorporated into collagen of only about 20 per cent at all ages tested. Therefore, the lowest concentration used must be very near the saturation point, and the endogenous pool size must be quite similar at all five ages tested.

4. *Turnover of Collagen and Non-Collagen Protein*

Accumulation of a protein and the rate of incorporation of a precursor into it can be quite independent phenomena if a significant degree of breakdown occurs while synthesis is going on. To determine the importance of turnover as a factor in these studies, experiments were carried out in which a 60-minute labeling period of corneas *in vitro* was followed by incubation in a tenfold excess of unlabeled glycine for an additional 30-, 60-, or 90-minute period (Table VI). At all ages studied, the amount of label in collagen was near its maximum level within 30 minutes after the wash and did not change significantly thereafter. The same type of experiment followed by isolation of non-collagen protein yielded essentially similar results, but with greater variability (Table VI).

In experiments *in vivo*, glycine-1-C^{14} was injected into the chorioallantoic circulation of 10-day embryos. At various periods after the initial injection, corneas were isolated and the amount of tracer in collagen or non-collagen stroma protein determined (Table VII). If a breakdown of any substantial extent occurred after the 10th day, protein with a high specific activity should be replaced by protein with a much lower specific activity. In the case of the collagen fraction, no such decrease was observed. On the other hand, non-collagen protein showed a very marked decrease within 4 days after the injection. It is tentatively concluded that no significant degree of collagen turnover occurred during the period studied, but a considerable turnover of non-collagen protein may take place. These conclusions must remain tentative until information is available on the efficiency of reutilization of breakdown products in this system.

TABLE V

Effect of Glycine-1-C^{14} Concentration on Appearance of Label in Collagen after a 2-Hour Incubation Period

Time of development	Glycine-1-C^{14} concentration	CPM in collagen/μg Stroma DNA/hr.
Days	μM	
11	144	100 ± 7* (12)‡
	284	110 ± 8 (12)
	422	114 ± 9 (12)
15	144	242 ± 13 (12)
	284	245 ± 18 (12)
	422	303 ± 17 (12)
17	144	287 ± 25 (12)
	284	298 ± 27 (12)
	422	359 ± 36 (12)
21	144	151 ± 14 (12)
	284	177 ± 12 (12)
	422	198 ± 18 (12)
23	144	158 ± 6 (12)
	284	206 ± 13 (12)
	422	174 ± 16 (12)

* Standard error of the mean.
‡ Number of determinations.

DISCUSSION

The data obtained in these experiments show a close relationship between the changes in the rates of accumulation of collagen and of incorporation of labeled precursors into collagen during development of the cornea. The incorporation pattern is similar to what would be expected of the first derivative of the accumulation curve (Fig. 1).

The rate of accumulation of a protein such as collagen can be influenced by its rate of synthesis and breakdown. In turn, the rate of synthesis is determined by the number of actively synthesizing cells, the synthetic activity of the individual cells, the rate of conversion of soluble collagen to an insoluble form, or any combination of these. The rate of incorporation of labeled precursors into insoluble collagen would be dependent upon the same factors, as well as changes in the endogenous precursor pool size, the rate of equilibration with exogenous precursor, and differences between *in vivo* and *in vitro* conditions. The experiments reported above permit an evaluation of the relative contribution of each of these factors to the observed results.

TABLE VI

Effect of Incubation in 1.33 mM Glycine-C^{12} Following 60 Minutes in 0.144 mM Glycine-1-C^{14} on Appearance of Label in Collagen and Non-Collagen Protein Fractions

Total time	Time in glycine-C^{12}	Time of development	CPM in collagen/μg Stroma DNA	CPM in non-collagen protein/μg Stroma DNA
min.	*min.*	*Days*		
60	0	11	66 ± 7*(8)‡	102 ± 10*(12)‡
90	30		103 ± 13 (8)	101 ± 11 (12)
120	60		93 ± 8 (8)	110 ± 13 (12)
150	90		105 ± 14 (8)	121 ± 13 (12)
60	0	15	191 ± 16 (12)	164 ± 22 (7)
90	30		279 ± 23 (12)	135 ± 10 (8)
120	60		299 ± 14 (12)	120 ± 9 (7)
150	90		224 ± 20 (12)	124 ± 9 (8)
60	0	17	253 ± 21 (12)	124 ± 10 (4)
90	30		309 ± 25 (12)	151 ± 28 (4)
120	60		333 ± 18 (12)	144 ± 13 (4)
150	90		273 ± 15 (12)	162 ± 7 (4)
60	0	21	125 ± 11 (8)	119 ± 11 (12)
90	30		165 ± 14 (8)	130 ± 10 (12)
120	60		184 ± 11 (8)	128 ± 10 (12)
150	90		184 ± 8 (8)	128 ± 11 (11)
60	0	23	127 ± 8 (8)	128 ± 17 (8)
90	30		136 ± 8 (8)	119 ± 10 (8)
120	60		138 ± 14 (8)	128 ± 8 (8)
150	90		147 ± 8 (8)	144 ± 14 (8)

* Standard error of the mean.
‡ Number of determinations.

TABLE VII

Retention of Label in Collagen and Non-Collagen Protein at Various Times Following Injection of Glycine-1-C^{14} into the Chorioallantoic Circulation of 10-Day Chick Embryos

Time of development	CPM in collagen/Stroma	CPM in non-collagen protein/Stroma
Days		
11	989 ± 32* (12)‡	305 ± 34* (4)‡
14	1028 ± 17 (8)	189 ± 53 (6)
16	1014 ± 46 (8)	191 ± 19 (6)
18	1040 ± 34 (8)	154 ± 12 (6)
19	1045 ± 62 (6)	—

* Standard error of the mean.
‡ Number of determinations.

The increase in the amount of DNA per stroma from the 9th through the 14th day of incubation probably reflects an increase in cell number; hence, an increase in the number of actively synthesizing cells may be an important factor during that period. It is not the only factor operating, however, since the rate of incorporation of precursors into collagen increases faster than the DNA even then (Table III). The level of DNA is fairly constant after the 14th day. This is the time during which collagen accumulation is most rapid and the changes in rate of incorporation of precursors are most dramatic. Thus, it appears that these marked changes occur in the absence of any significant change in cell number. Since the most active period of collagen synthesis occurs after DNA accumulation has ceased, the synthesis of collagen and DNA may be mutually exclusive processes, as suggested by Stockdale and Holtzer (1961) for myosin synthesis in developing muscle.

It should be noted that in the present series of experiments the DNA content per cornea ceases to increase on the 13th day of development, whereas in an earlier series of experiments the rate of increase did not fall off until about the 16th day of development (Herrmann, 1958). No methodological discrepancies could be discovered between the two series of experiments. Therefore, the possibility is being considered that the differences in the corneal DNA accumulation represent properties of the different chick strains used in the two sets of experiments.

The rate of conversion of soluble collagen precursors to insoluble collagen has been ruled out as a major contributing factor by the kinetics of label incorporation and the cold glycine-chase experiments. If a significant period of time were spent in the soluble form, there should have been a lag period before the labeled precursors appeared in a 0.1 N NaOH insoluble form. Since the rate of incorporation of proline-U-C^{14} was linear for a 2-hour period at all ages studied and could be extrapolated back to the origin (Fig. 2), the rate of equilibration of the corneal tissues with the exogenous proline must be fairly rapid and the half-life of the soluble collagen precursors of insoluble collagen must be quite short relative to the time period of the experiments. It should be pointed out, however, that neither the points in Fig. 2 nor earlier data (Herrmann, 1957; Buckingham and Herrmann, unpublished) obtained with glycine exclude rigorously the possibility of a short lag in incorporation for the prehatching embryo. Such a lag, if it exists, could not account for the marked differences in the rates of incorporation at different stages of development. Also, in the presence of a sizeable collagen precursor pool, incorporation of label after substitution of an unlabeled precursor would be expected to continue for a longer period than has actually been observed in our experiments. Smith and Schuster (1962) and Levene and Gross (1959) were unable to prepare soluble collagen from 15- and 16-day chick embryos, respectively. It is likely that this failure was due to a very rapid conversion of soluble collagen to the insoluble form. In contrast to our results, Herrmann *et al.* (1958) found a lag period in the incorporation of glycine-1-C^{14} into the collagen fraction of 14-day chick embryo leg muscle, which they interpreted as being due to a soluble pro-collagen intermediate.

The size of the endogenous precursor pool appears to be relatively constant in the chick embryo cornea stroma from the 11th day of incubation through 2 days post-hatching (Table V). Furthermore, the concentration of glycine used in all of the above incorporation experiments was very near the saturation level. These facts permit the conclusion that the rate of incorporation of tracer into the collagen or non-collagen protein fraction is a direct measure of the rate of synthesis of collagen and non-collagen protein, respectively, at a given developmental age.

The degree to which these rates are related to the accumulation of collagen or non-collagen protein is indicated by studies of the rate of turnover of these two fractions *in vivo*. No indication was found of any significant degradation of corneal stroma collagen during the last week of embryonic life. Therefore, the rate of incorporation of precursors in short-term experiments should be directly related to the rate of collagen accumulation. Comparison of these rates indicates that this is indeed the case for collagen (Fig. 1). This relationship does not hold for non-collagen protein, however, since similar experiments indicated a relatively high rate of turnover in this fraction. Conclusions drawn from these experiments must be tentative, since labeled precursors of small molecular size may linger for an undetermined period and the efficiency of re-utilization of breakdown products in this system is unknown. A slow turnover of collagen has been demonstrated in young rats (Kao *et al.*, 1961; Gerber *et al.*, 1960) and is not excluded by our results with chick tissues.

The results presented above strongly suggest that the cells of the corneal stroma exhibit marked changes during development in the rate at which they synthesize a specific protein, collagen. The most active period of collagen synthesis, 15 to 19 days of incubation, coincides with the period during which progressive dehydration and development of transparency of the cornea occurs (Coulombre and Coulombre, 1961), but the relationship between these two processes is not understood. It should be noted that the accumulation of actomyosin (Csapo and Herrmann, 1951) and collagen (Herrmann and Barry, 1955) in the chick embryo leg musculature has been demonstrated to be most rapid from the 12th through the 18th day of incubation, following a pattern not unlike that described in the present paper. Thus, the last week of embryonic development appears

to be an active period for the synthesis of cell-specific structural proteins in several tissues.

Protein formation during the last week of chick embryo development has been found to be hormonally controlled in certain instances (Love and Konigsberg, 1958; Konigsberg, 1958). Collagen formation in the rat uterus is increased in response to estrogen injection (Hurley and Herrmann, 1955; Harkness *et al.*, 1957). The incorporation *in vitro* of proline into collagen is decreased by parathyroid hormone (Johnston *et al.*, 1962). Therefore, changes in the rate of corneal collagen synthesis may also be influenced by hormone levels.

A relatively conservative approach to the analysis of protein synthesis in embryonic tissues, using whole cells, may be justified in view of recent reports which indicate that amino acid incorporation as measured in cell-free preparations represents only about 1 to 5 per cent of the activity observed in the intact cell. Measurements with such systems may merely reflect the completion of peptide chains, the synthesis of which was initiated in the intact cell (Dintzis *et al.*, 1963; Turba and Hilpert, 1961; Hu *et al.*, 1962). Nevertheless, it will be of interest to see whether the rates of amino acid incorporation into collagen in cell-free systems obtained from the cornea parallel the marked changes observed in the intact cell. Measurements of amino acid incorporation into collagen by cell-free preparations from embryonic connective tissues have been carried out by several investigators (Urivetzky *et al.*, 1963; Peterkofsky and Udenfriend, 1963).

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REFERENCES

BRITT, G. L., and HERRMANN, H., Protein accumulation in early chick embryos grown under different conditions of explantation, *J. Embryol. and Exp. Morphol.*, 1959, **7**, 66.

BURTON, K., A study of the conditions and mechanism of the diphenylamine reaction for the colorimetric estimation of deoxyribonucleic acid, *Biochem. J.*, 1956, **62**, 315.

COULOMBRE, A. J., and COULOMBRE, J. L., The development of the structural and optical properties of the cornea, *in* The Structure of the Eye, (G. K. Smelser, editor), New York, Academic Press, Inc., 1961, 405.

CSAPO, A., and HERRMANN, H., Quantitative changes in contractile proteins of chick skeletal muscle during and after embryonic development, *Am. J. Physiol.*, 1951, **165**, 701.

DINTZIS, M., and KNOPF, P. M., Cell-free synthesis of hemoglobin, *in* Informational Macromolecules, (H. J. Vogel, V. Bryson, and J. O. Lampen, editors), New York, Academic Press, Inc., 1963, 375.

EBERT, J. D., Some aspects of protein biosynthesis in development, *in* Aspects of Synthesis and Order in Growth, (D. Rudnick, editor), Princeton University Press, 1955, 69.

GERBER, G., GERBER, G., and ALTMAN, K. I., Studies on the metabolism of tissue proteins. I. Turnover of collagen labeled with proline-U-C^{14} in young rats, *J. Biol. Chem.*, 1960, **235**, 2653.

HARKNESS, M. L. R., HARKNESS, R. D., and MORALEE, B. E. The effect of the oestrous cycle and of hormones on the collagen content of the uterus of the rat, *J. Physiol.*, 1957, **135**, 270.

HERRMANN, H., Protein synthesis and tissue integrity in the cornea of the developing chick embryo, *Proc. Nat. Acad. Sc.*, 1957, **43**, 1007.

HERRMANN, H., Some problems of protein formation in the sclera and cornea of the chick embryo, *in* The Chemical Basis of Development, (W. D. McElroy and B. Glass, editors), Baltimore, The Johns Hopkins Press, 1958, 329.

HERRMANN, H., Quantitative studies of protein synthesis in some embryonic tissues, *in* Cytodifferentiation and Macromolecular Synthesis, (M. Locke, editor), New York, Academic Press, Inc., 1963, 85.

HERRMANN, H., and BARRY, S. R., Accumulation of collagen in skeletal muscle, heart and liver of the chick embryo, *Arch. Biochem. and Biophys.*, 1955, **55**, 526.

HERRMANN, H., LERMAN, L., and WHITE, B. N., Uptake of glycine-1-C^{14} into the actomyosin and collagen fractions of developing chick muscle, *Biochim. et Biophysica Acta*, 1958, **27**, 161.

HERRMANN, H., and TOOTLE, M. L., Specific and general aspects of the development of enzymes and metabolic pathways, *Physiol. Rev.*, 1964, **44**, 289.

HERRMANN, H., WHITE, B. N., and COOPER, M., The accumulation of tissue components in the leg muscle of the developing chick, *J. Cell. and Comp. Physiol.*, 1957, **49**, 227.

HOLTZER, H., Aspects of chondrogenesis and myogenesis, *in* Synthesis of Molecular and Cellular

Structure, (D. Rudnick, editor), New York, The Ronald Press, 1961, 35.

HU, A. S. L., BOCK, R. M., and HALVORSON, H. O., Separation of labeled from unlabeled proteins by equilibrium density gradient sedimentation, *Anal. Biochem.*, 1962, **4,** 489.

HURLEY, L. S., and HERRMANN, H., Effect of estrogen and progesterone on collagen content of rat uterus, heart and skeletal muscle, *Am. J. Physiol.*, 1955, **183,** 67.

JOHNSTON, C. C., JR., DEISS, W. P., JR., and MINER, E. B., Bone matrix biosynthesis *in vitro*. II. Effects of parathyroid hormone, *J. Biol. Chem.*, 1962, **237,** 3560.

KAO, KUNG-YING TANG, HILKER, D. M., and MCGAVACK, T. H., Connective tissue. V. Comparison of synthesis and turnover of collagen and elastin in tissues of rats at several ages, *Proc. Soc. Exp. Biol. and Med.*, 1961, **106,** 335.

KONIGSBERG, I. R., Thyroid regulation of protein and nucleic acid accumulation in developing skeletal muscle of the chick embryo, *J. Cell. and Comp. Physiol.*, 1958, **52,** 13.

LEACH, A. A., The amino acid composition of amphibian, reptile and avian gelatins, *Biochem. J.*, 1957, **67,** 83.

LEVENE, C. I., and GROSS, J., Alteration in state of molecular aggregation of collagen induced in chick embryos by β-aminopropionitrile (Lathyrus factor), *J. Exp. Med.*, 1959, **110,** 771.

LOVE, D. S., and KONIGSBERG, I. R., Enhanced desoxyribonucleic acid accumulation and retarded protein accumulation in skeletal muscle of "hypophysectomized" chick embryos, *Endocrinology*, 1958, **62,** 378.

MARCUS, P. I., CIECIURA, S. J., and PUCK, T. T., Clonal growth *in vitro* of epithelial cells from normal human tissues, *J. Exp. Med.*, 1956, **104,** 615.

MARTIN, C. J., and AXELROD, A. E., A modified method for determination of hydroxyproline, *Proc. Soc. Exp. Biol. and Med.*, 1953, **83,** 461.

MOOG, F., Enzyme adaptation in the differentiation of animal cells, *in* Biological Interactions in Normal and Neoplastic Growth, (M. J. Brennan and W. L. Simpson, editors), Boston, Little, Brown & Co., 1962, 439.

PETERKOFSKY, B., and UDENFRIEND, S., Conversion of proline to collagen hydroxyproline in a cell-free system from chick embryos, *J. Biol. Chem.*, 1963, **238,** 3966.

SHEN, S. C., Changes in enzymatic patterns during development, *in* The Chemical Basis of Development, (W. D. McElroy and B. Glass, editors), Baltimore, The Johns Hopkins Press, 1958, 416.

SMITH, D. J., and SHUSTER, R. C., Biochemistry of lathyrism. I. Collagen biosynthesis in normal and lathyritic chick embryos, *Arch. Biochem. and Biophys.*, 1962, **98,** 498.

STOCKDALE, F. E., and HOLTZER, H., DNA synthesis and myogenesis, *Exp. Cell Research*, 1961, **24,** 508.

TURBA, F., and HILPERT, H., Proteinsynthese in der Milchdrüse: Gewinnung und Vergleich der Funktion von Drüsenschnitten, Zellen und Zellfraktionen, *Biochem. Z.*, 1961, **334,** 487.

URIVETZKY, M., KRANZ, V., and MEILMAN, E., Studies with a cell-free system of proline incorporation into protein cleaved by collagenase, *Arch. Biochem. and Biophys.*, 1963, **100,** 478.

YOSPHA-PURER, Y., FENDRICH, J., and DAVIES, A. M., Estimation of the blood volumes of embryonated hen eggs at different ages, *Am. J. Physiol.*, 1953, **175,** 178.