Collagen Synthesis in the Muscle of Developing Chick Embryos

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Radioactive protein was prepared from the leg muscle of chick embryos, 11, 14, 16 and 17 days old, each injected with radioactive proline and incubated for 30, 60 or 90 min afterwards. The radioactive protein was incubated with collagenase purified by chromatography on a Sephadex G-100 column. Under this condition, only collagen is digested into products soluble in trichloroacetic acid. The relative rate of collagen synthesis was determined by comparing the amount of radioactivity released into the supernatant fraction and that in the residue, by the method of Diegelmann & Peterkofsky [(1972) *Dev. Biol.* **28**, 443–453]. The results show that the rate of collagen synthesis remains at approx. 10% of the rate of synthesis of other non-collagenous proteins during the development of chick embryonic muscle from 11 to 17 days. This suggests that the synthesis of collagen and that of other proteins are co-ordinately regulated at these stages of development.

Developing chick muscle contains two major types of cells, each producing specific kinds of proteins: the myogenic cells synthesize proteins for contraction, myosin, actin, tropomyosin and troponin, and fibroblast cells secrete collagen extracellularly. It has also been shown that myoblasts and myotubes secrete into culture media proteins that have the characteristics of collagen (Schubert *et al.*, 1973). The interrelationships of these two cell types, myoblasts and fibroblasts, and of their protein products may of great importance in determining muscle differentiation *in vivo*.

Studies by Herrmann & Barry (1955) showed that the rates of accumulation of collagen and actomyosin from days 10 to 19 of development of chick embryonic muscle were very similar. It was therefore suggested that the two different cell types were responding to the same type of stimulus. In the present study we have investigated the relationship between the rate of synthesis of collagen and that of non-collagenous protein, in the leg skeletal muscle of developing chick embryos. Our results show that the rate of collagen synthesis relative to the rate of non-collagenous protein synthesis remains constant during muscle development of the chick embryo from day 11 to day 17. We infer that the synthesis of collagen and non-collagenous proteins is co-ordinately regulated.

Experimental

Materials

Leg muscle of 11-, 14-, 16- and 17-day Dekalb 171 White Leghorn embryos was used in this study.

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All chemicals were of analytical grade. Chromatographically purified collagenase prepared by Worthington Biochemical Corp. was purchased from ICN Canada, Montreal, Que., Canada. Proteinase-free ribonuclease A, tannic acid, Hepes† buffer, bovine serum albumin and Triton X-100 were obtained from Sigma Chemical Co., St. Louis, MO, U.S.A. Sodium deoxycholate was a product of BDH, Poole, Dorset, U.K., PCS solution (phase-combining solubilizer), L-[5-³H]proline (specific radioactivity 18–22 Ci/mmol) and L-[G-³H]tryptophan (specific radioactivity, 6 Ci/mmol) were purchased from Amersham-Searle Corp., Toronto, Canada.

Methods

(i) *Sephadex G-100 filtration of commercially prepared collagenase.* Collagenase was further purified by gel filtration and the absence of non-specific proteolytic activity confirmed by the method of Peterkofsky & Diegelmann (1971). The fractions eluted in the major A_{280} peak were stored in 1 ml batches at –20°C. The protein concentration of the fractions was determined by the method of Lowry *et al.* (1951), with bovine serum albumin (fraction V) as standard.

(ii) *Preparation of radioactive protein substrates (after the method described by Peterkofsky & Diegelmann, 1971).* L-[5-³H]Proline or L-[G-³H]tryptophan (0.05–0.08 ml) was injected into one of the chorio-allantoic veins and the embryos were incubated for 30, 60 or 90 min at 37°C. At the appropriate time, the legs were removed, washed in cold (0–4°C) 15 mM-NaCl, blotted dry, and the muscle was dissected free of bone. The muscle (1.0–1.5 g) of each

† Abbreviation: Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid.

embryo was separately homogenized in 2 vol. of 50 mM-Tris/HCl buffer, pH 7.6, at 0–4°C with a loose-fitting Dounce homogenizer. Proteinase-free ribonuclease A was added at a final concentration of 20 µg/ml to degrade aminoacyl-tRNA and release proline or tryptophan as acid-soluble products. All other conditions were as described by Peterkofsky & Diegelmann (1971).

(iii) *Hydrolysis of protein labelled with L-[G-³H]tryptophan.* L-[G-³H]Tryptophan was injected into 17-day embryos, which were then incubated at 37°C for 1 h. Radioactive protein was prepared as described above and dissolved in 0.1 M-NaOH, at a final concentration of 36 mg/ml. Radioactively labelled protein (3.6 mg), 60 µmol of Hepes buffer, pH 7.2, 1.25 µmol of *N*-ethylmaleimide, 125 µmol of CaCl₂, 0.1 ml of 0.08 M-HCl and 270 mg of collagenase were incubated with shaking at 37°C for 1 h. Bovine serum albumin (1%, w/v) (2 or 3 drops) was added to each tube followed immediately by the addition of an equal volume of 10% (w/v) trichloroacetic acid/0.5% tannic acid (at 0–4°C). All other conditions were similar to those described by Peterkofsky & Diegelmann (1971).

(iv) *Time-course of hydrolysis of radioactive protein substrates by collagenase.* L-[5-³H]Proline was injected into 11- and 17-day chick embryos and radioactive protein was prepared. It was dissolved in 0.1 M-NaOH by warming in a water bath at 37°C at a final protein concentration of 9.3 mg/ml (for radioactive protein from 11-day old embryos) and 29.6 mg/ml (for radioactive protein from 17-day embryos). The standard incubation mixture (0.53 ml) contained 60 µmol of Hepes buffer, pH 7.2, 1.25 µmol of *N*-ethylmaleimide, 125 µmol of CaCl₂, radioactively labelled protein substrate (0.9–5.6 mg) and 0.08 M-HCl to neutralize the excess of NaOH. The reaction was started by the addition of collagenase (0.17–0.19 mg/ml) and the tubes were incubated at 37°C for 0, 10, 30, 60 and 120 min. Enzyme was omitted from the blanks; instead, an equal volume of 50 mM-Tris/HCl, pH 7.2, was added. After incubation, 2 or 3 drops of bovine serum albumin (1%) were added and the tubes incubated on ice for 5 min. An equal volume of 10% trichloroacetic acid/0.5% tannic acid was added and the tubes were incubated on ice for 15 min. The tubes were centrifuged at 10000g for 10 min at 0°C; the supernatant was transferred to a scintillation vial and the pellet washed in 1 ml of 5% trichloroacetic acid/0.25% tannic acid by centrifugation. The resulting supernatant was mixed with the initial one, and the pellet resuspended in 1 ml of 5% (w/v) trichloroacetic acid/0.25% tannic acid. The radioactivity of the supernatant fractions and the pellet was determined after the addition of 10 ml of PCS/xylene (3:1, v/v) to each.

(v) *Determination of relative collagen synthesis.* The amount of collagen synthesized relative to that of

other proteins was calculated from the formula, presented by Diegelmann & Peterkofsky (1972), based on the fact that collagen contains 5.4 times as much proline as an average non-collagen protein (Diegelmann & Peterkofsky, 1972).

(vi) *Radioactivity determination.* The radioactivity of the samples was measured in the presence of PCS/xylene mixture (3:1, v/v) with a Tri-Carb liquid-scintillation spectrometer (³H counting efficiency 17%).

Results

Determination of purity and specificity of collagenase

The success and reliability of the method described by Peterkofsky & Diegelmann (1971) for the specific assay of radioactive collagen in the presence of other proteins depend to a great extent on the purity of the collagenase preparation used in the enzymic hydrolysis. As previously reported by Goldberg & Green (1967), commercial preparations of chromatographically purified collagenase are contaminated with other proteolytic enzymes. The collagenase used in this study was further purified by gel filtration on a column of Sephadex G-100. Only the first major peak had any collagenase activity. To inhibit any non-collagenase proteolytic activity, *N*-ethylmaleimide was always added to all incubation mixtures for the hydrolysis of radioactively labelled protein (Peterkofsky & Diegelmann, 1971).

The specificity of the collagenase, purified by chromatography on a Sephadex G-100 column, was verified by incubating the enzyme with protein labelled with [³H]tryptophan and measuring the degree of digestion. Since collagen contains no detectable tryptophan (Piez, 1965), any radioactive tryptophan released would result from the degradation of other proteins by a non-collagenase proteolytic enzyme. The results show that the collagenase preparation digested only 0.5% of the [³H]tryptophan-labelled chick embryo protein: we conclude that the purified collagenase is free of contaminating non-collagenase proteinases.

Optimum conditions for assay of collagenase activity

We determined the optimum conditions for enzymic hydrolysis of radioactive collagen in the presence of other proteins, particularly the length of time for complete substrate digestion and the optimum substrate concentration. The enzyme concentration used in the assays was in excess of that used by Peterkofsky & Diegelmann (1971). The amounts of radioactive protein substrate (prepared from 11-day embryonic muscle) in separate incubation mixtures were 0.93 and 1.87 mg. The time-course of the reaction was extended to 120 min.

Fig. 1. Time-course of release of radioactivity from radioactive protein substrate of 11-day embryonic chick muscle

Radioactive protein was prepared from the leg muscle of 11-day embryos as described under 'Methods', section (ii): 0.93mg of protein (2.5×10^5 d.p.m.) or 1.87mg of protein (5×10^5 d.p.m.) was incubated with 169 μ g of collagenase and other components of the standard incubation mixture. At the appropriate time, the radioactivity released in the reaction and that in the residue were determined and the values were used to calculate relative collagen synthesis (Fig. 1, inset) as described by Diegelmann & Peterkofsky (1972). ●, 0.93 mg of protein; ○, 1.87 mg of protein.

Table 1. Relative collagen synthesis in 11-, 14-, 16- and 17-day chick embryonic muscle

Chick embryos were injected with [3 H]proline and incubated for 30, 60 and 90min. Leg muscle from each embryo was analysed separately. After digestion of collagen with purified collagenase, the radioactivity released and the radioactivity in the residue were determined and the relative amount of collagen synthesized was calculated by using the formula given by Diegelmann & Peterkofsky (1972). For collagen synthesis each value represents the mean $\pm$ 1 s.d. of five separate experiments, each of which was carried out in duplicate. For mean collagen synthesis each value is the mean $\pm$ 1 s.d. for all values for the time points 30, 60 and 90min for any given age.

Day	Time (min)	Collagen synthesis (%)	Mean collagen synthesis (%)
11	30	10.0 $\pm$ 0.25	10.2 $\pm$ 0.18
	60	10.0 $\pm$ 0.12	
	90	10.5 $\pm$ 0.22	
14	30	10.2 $\pm$ 0.25	9.9 $\pm$ 0.22
	60	9.5 $\pm$ 0.25	
	90	10.0 $\pm$ 0.2	
16	30	9.8 $\pm$ 0.25	9.7 $\pm$ 0.22
	60	9.6 $\pm$ 0.2	
	90	9.6 $\pm$ 0.2	
17	30	10.0 $\pm$ 0.22	9.9 $\pm$ 0.18
	60	10.0 $\pm$ 0.18	
	90	9.7 $\pm$ 0.15	

Also, relative collagen synthesis remained at approx. 10% at the plateau of the reaction.

As 11- and 17-day embryos represent the youngest and the oldest stages studied respectively, we have assumed that the time-course of the reaction reaches a plateau in 60min, and that for all stages of development between the 11- and the 17-day embryo the amount of released radioactivity is directly proportional to the amount of radioactive protein substrate up to a maximum of 5.6mg. However, for practical reasons, the amount of radioactive protein substrate used in subsequent experiments ranged from 1.8 to 2.5mg.

Rate of collagen synthesis

The rate of collagen synthesis relative to that of other proteins was determined. Embryos were given a pulse of [3 H]proline for 30, 60 and 90min. The results presented in Table 1 show that: (a) there is no significant difference in the relative rates of collagen synthesis up to the 90min-labelling time point for each particular stage studied; (b) there are no statistically significant differences in relative collagen synthesis in developing chick muscle of 11-, 14-, 16- and 17-day embryos; collagen synthesis relative to that of other proteins remains at approx. 10%.

Fig. 2. Polyribosome profile of S-10 supernatant fraction of 14-day embryonic chick leg muscle

Chick embryos (14 days old) were injected with [^3H]proline and incubated for 1 h at 37°C. Leg muscle (2.5 g) was homogenized in buffer [250 mM-KCl/7.5 mM-MgCl₂/20 mM-Tris/HCl (pH 7.6)/0.5% Triton X-100/0.2% sodium deoxycholate] (1 g fresh wt. of muscle/ml of buffer) with three strokes of a loose-fitting Dounce homogenizer at 0–4°C. The homogenate was centrifuged at 10000g at 0°C, and the resulting supernatant layered on a 15–40% (w/v) linear sucrose density gradient (12 ml) and centrifuged at 41000 rev./min for 60 min at 0°C in the IEC model SB-48 rotor. The A_{260} profile of the gradients was analysed with a Gilford recording spectrophotometer model 2400 and fractions (1.5 ml) were collected simultaneously. Fractions corresponding to A, B, C, D and E were pooled separately for identification of collagen-synthesizing polyribosomes and determination of relative collagen synthesis by the polyribosomes and ribosomes as described in Table 2.

Collagen synthesis on polyribosomes

In an attempt to follow the synthesis of collagen *de novo* in developing chick muscle, we have identified collagen-synthesizing polyribosomes and have determined relative collagen synthesis on these polyribosomes. The procedure of digesting collagen with collagenase was applied to the polyribosome and ribosome fractions, A, B, C and D, and to the non-ribosomal fraction, E (Fig. 2). Table 2 shows

Table 2. Relative collagen synthesis by fractions A–D, and relative collagen content of non-ribosomal fraction, E, of Fig. 2

Fractions A, B, C, D and E (Fig. 2) were each separately incubated with ribonuclease (20 μg) at 37°C for 5 min. A 0.5 ml portion (500 μg of protein) of S-10 supernatant fraction from 14-day embryonic chick muscle (Nwagwu, 1975) was added to each fraction as carrier protein, followed by an equal volume of cold (0–4°C) 10% (w/v) trichloroacetic acid. The suspension was mixed thoroughly, incubated on ice for at least 10 min and centrifuged at 10000g for 10 min at 0°C. The pellet was resuspended in 5% trichloroacetic acid, re-centrifuged as before and the pellet washed in ethanol/ether (3:1, v/v). The washing procedure was repeated twice and the protein pellet slowly dried in a desiccator for 48 h. The protein substrate was then incubated with purified collagenase (150–190 μg) and other components of the standard incubation mixture at 37°C for 60 min. Relative collagen content of each fraction was analysed as described under 'Methods', sections (iv) and (v). Values are averages of two experiments.

Fraction	Relative collagen content (%)
A	17
B	2.1
C	2.7
D	0.3
E	6

that the collagen-synthesizing polyribosomes sedimented in the heavy regions of the gradient, in fraction A. The results also show that approx. 17% of the polypeptides synthesized by the polyribosomes in fraction A is collagen.

Discussion

A major advantage of the method adopted in the present work for studying collagen synthesis is its relative ease. It is assumed that the same precursor pool supplied amino acids for the synthesis of both collagen and non-collagenous proteins. We therefore need not consider changes in the amino acid pool at different stages of embryonic development. Also, by exploiting the specific enzymic action of purified collagenase on collagen, we can avoid the more laborious techniques of hydrolysing the collagen and determining the hydroxyproline content.

Table 1 shows that, at each stage of development studied, (a) the relative rate of collagen synthesis up to 90 min labelling time is constant; (b) relative collagen synthesis remains constant in the muscle of 11-, 14-, 16- and 17-day chick embryos: approx. 10% of newly synthesized polypeptides is represented by collagen.

The present data do not represent accumulation of collagen. The labelling period of 30–90 min is a measure of synthesis only, since it represents only a small fraction of the expected half-life of the proteins (Schimke, 1970). Thus the labelled proteins have not had a chance to be degraded. At any of the time points studied, the ratio collagen/non-collagenous protein remains the same. Diegelmann & Peterkofsky (1972) made a similar observation: they determined that, over a period of 6–48 h, once the embryonic tissue is labelled with [^3H]proline, the label is incorporated into both collagen and non-collagenous protein with no apparent redistribution into collagen.

This is the first report of the relative rate of collagen synthesis in developing chick muscle. Diegelmann & Peterkofsky (1972) have determined the relative rate of collagen synthesis in the intact chick embryo and some isolated tissues. Their results showed that between 8 and 14 days of development the relative rate of collagen synthesis increased by more than fivefold in the lower limb. Since the lower limb at this time contains mainly skin, bone, tendon and little muscle, the tissue examined by Diegelmann & Peterkofsky (1972) is probably not comparable with that studied here.

Schubert *et al.* (1973) have shown that myoblasts and myotubes secrete at least 12 distinct proteins extracellularly: of these, three proteins were identified as collagen. Schubert *et al.* (1973) stated that the rate of appearance of extracellular soluble protein is about 2% of the rate of protein synthesis by the cells. We find that the rate of synthesis of collagen relative to that of non-collagenous protein is approx. 10%. This higher value is probably due to the added activity of fibroblasts, which are known to secrete large quantities of collagen, in whole muscle tissue. It has also been shown in other systems that the relative amount of collagen synthesis is much lower in cultured cells than in similar types of cells *in vivo*. For example, collagen constitutes 20–25% of the embryonic parietal yolk-sac basement membrane from day 14.5 to day 17.5 (Clark *et al.*, 1975a). But in freshly isolated parietal yolk-sac endoderm collagen synthesis decreased from approx. 7% of the total protein synthesized at day 14 to approx. 3% of that synthesized by endoderm at day 16.5 (Clark *et al.*, 1975b). In other tissues, such as lens and glomeruli (Clark *et al.*, 1975a) and 'parietal yolk-sac carcinoma' (Priest, 1970), only 0.5% of the newly synthesized protein is basement-membrane collagen. It has been suggested that the lower value for the relative content of collagen in tissue cultures is probably due to increased breakdown of basement-membrane collagen *in vitro* (Minor *et al.*, 1976). These differences may also apply to muscle tissue *in vivo* and muscle cell cultures.

The collagen-synthesizing polyribosomes sedimented in fraction A of the sucrose gradient (Fig. 2),

in agreement with the findings of Lazarides & Lukens (1971). After analysis of the released polypeptides synthesized *in vitro*, it was shown that 11–14% of the radioactive proline residues incorporated by the collagen-synthesizing polyribosomes could be hydroxylated by the collagen proline hydroxylase (Lazarides & Lukens, 1971). This suggests that 11–14% of the polypeptides synthesized and released by these polyribosomes is represented by collagen. By contrast we find that approx. 17% of the polypeptides synthesized by polyribosomes of fraction A (Fig. 2) is collagen. This higher value may be attributable to the fact that 14-day embryos were used in this particular aspect of our study. There is an increase in the quantity of myosin-synthesizing polyribosomes in the leg muscle of chick embryos during development from 11 to 17 days and an increase in released myosin chains (Herrmann *et al.* 1970). The same may hold true for collagen-synthesizing polyribosomes and the released collagen.

Collagen constitutes only 6% of the released polypeptides obtained in the non-ribosomal cell supernatant fraction, E (Fig. 2). If the polyribosome fractions are combined with the cell supernatant fraction, one would expect that 12.5% of the newly synthesized polypeptides would be represented by collagen. This value of 12.5% is similar to that of 10% obtained when total muscle protein is analysed for collagen synthesis (Fig. 2).

The specific interactions between collagen, myoblasts and myotubes are not yet clear (see Hauschka, 1972). Earlier studies of muscle-cell cultures showed that myoblasts required a substratum of collagen for differentiation (Ehrmann & Gey, 1956; Konigsberg & Hauschka, 1965; Hauschka & Konigsberg, 1966; Hauschka, 1966). However, it is now known that muscle-cell culture can be maintained, and that myoblasts can differentiate, in the absence of an added collagen substrate (Yaffe, 1968, 1969; Schubert *et al.*, 1973). Nevertheless these findings do not necessarily mean that collagen is not required for muscle differentiation, since myoblasts themselves also secrete collagen (Schubert *et al.*, 1973). That the relative rate of collagen synthesis remains constant during muscle development suggests that the rate of synthesis of muscle non-collagenous proteins is adjusted to keep pace with the rate of collagen synthesis, and vice versa. The regulatory mechanisms that determine these rates need to be elucidated.

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References

- Clark, C. C., Minor, R. R., Koszalka, T. R., Brent, R. L. & Kefalides, N. A. (1975a) *Dev. Biol.* **46**, 243–261

- Clark, C. C., Tomichek, E. A., Koszalka, T. R., Minor, R. R. & Kefalides, N. A. (1975b) *J. Biol. Chem.* **250**, 5259–5267
- Diegelmann, R. F. & Peterkofsky, B. (1972) *Dev. Biol.* **28**, 443–453
- Ehrmann, R. L. & Gey, G. O. (1956) *J. Natl. Cancer Inst.* **16**, 1375–1390
- Goldberg, B. & Green, H. (1967) *J. Mol. Biol.* **26**, 1–18
- Hauschka, S. D. (1966) Ph.D. Thesis, Johns Hopkins University, Baltimore
- Hauschka, S. D. (1972) in *Growth, Nutrition and Metabolism of Cells in Culture* (Rothblat, G. H. & Cristofalos V. J., eds.), vol. 2, pp. 67–130, Academic Press, New York
- Hauschka, S. D. & Konigsberg, I. R. (1966) *Proc. Natl. Acad. Sci. U.S.A.* **55**, 119–126
- Herrmann, H. & Barry, S. R. (1955) *Arch. Biochem. Biophys.* **55**, 526–533
- Herrmann, H., Heywood, S. M. & Marchok, A. C. (1970) *Curr. Top. Dev. Biol.* **5**, 181–234
- Konigsberg, I. R. & Hauschka, S. D. (1965) in *Reproduction, Molecular, Subcellular and Cellular* (Locke, M., ed.), pp. 243–290, Academic Press, New York
- Lazarides, E. & Lukens, L. N. (1971) *Nature (London)* **232**, 37–40
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
- Minor, R. R., Strause, E. L., Koszalka, T. R., Brent, R. L. & Kefalides, N. A. (1976) *Dev. Biol.* **48**, 365–376
- Nwagwu, M. (1975) *Biochem. J.* **146**, 395–400
- Peterkofsky, B. & Diegelmann, R. (1971) *Biochemistry* **10**, 988–994
- Piez, K. (1965) *Biochemistry* **4**, 2590–2596
- Priest, R. E. (1970) *Nature (London)* **225**, 744–745
- Schimke, R. T. (1970) in *Mammalian Protein Metabolism* (Munro, H. N., ed.), vol. 4, pp. 177–228, Academic Press, New York
- Schubert, D., Terikas, H., Humphreys, S., Heinemann, S. & Patrick, J. (1973) *Dev. Biol.* **33**, 18–37
- Yaffe, D. (1968) *Proc. Natl. Acad. Sci. U.S.A.* **61**, 477–483
- Yaffe, D. (1969) *Curr. Top. Dev. Biol.* **4**, 37–77