BIOSYNTHESIS OF COLLAGEN

Biochemical and Physicochemical Characterization of Collagen-Synthesizing Polyribosomes

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ABSTRACT

Synthesis of collagen on polyribosomes has been demonstrated in vitro in chick embryo corium by radioisotope incorporation, zone centrifugation through sucrose gradients, and analytical ultracentrifugation. Collagen synthesis was associated with polyribosomes ranging in size, as reflected by their sedimentation constants, from about 180S to approximately 1600S. Most of the newly formed collagen, hydroxyproline, was present on the largest polyribosome aggregates (~ 350–1600S), but small polyribosomes (~180–200S) also contained collagen. On the basis of the proline-^{14}C/hydroxyproline-^{14}C ratios and the disrupting effect of collagenase, the proposal is made that the 350–1600S polyribosomes from this tissue are involved predominantly in collagen synthesis. The large polyribosomes are disrupted extensively by collagenase but only partially by ribonuclease and trypsin. Therefore, it appears that they are stabilized by the interaction of newly forming collagen chains. Evidence is presented consistent with the hypothesis that these large polyribosomes are formed by the aggregation of small polyribosomes (180–200S) through the interaction of collagen polypeptides. It is suggested that these small polyribosomes might be involved in the synthesis of subunits of the collagen alpha chain.

The biosynthesis of collagen has been assumed to occur by mechanisms similar to those described for the synthesis of other proteins (1–3). An extensive body of evidence has accumulated in the past 4 yr to support the hypothesis that protein synthesis occurs on polyribosomes (4–13). Polyribosomes may be separated according to size by sucrose density gradient sedimentation, and the suggestion has been made that, if each protein synthesized were associated with a characteristic size of polyribosome, this technique might be useful for separating different nascent proteins (8). To identify a protein associated with a particular polyribosome fraction, it is essential for the protein to have a unique composition or a specific property or activity. On the basis of these requirements, collagen should be ideal for the study of protein synthesis at the polysome level. Since it is a large molecule, its synthesis would be expected to occur on large polyribosomes. In addition, its unique amino acid composition, especially the presence of hydroxyproline (2, 14), should permit the detection of nascent collagen in the sucrose density gradient.

In the present studies, collagen biosynthesis was investigated in vitro in the chick embryo corium by following the uptake of proline-^{14}C and its conversion to hydroxyproline-^{14}C in polysomes of different sizes. Experiments were designed to determine the size of the polyribosomes synthesizing collagen, allowing for the possibility that these polysomes might produce complete alpha chains

(15) or smaller collagen subunits (16–18). The role of collagen in the stabilization of the polyribosome structure was explored by treating the polyribosomes with ribonuclease, bacterial collagenase, trypsin, and ethylenediamine tetraacetic acid (EDTA).

Since this work was begun, Kretsinger et al. (19) and Malt and Speakman (20) reported collagen synthesis on polyribosomes of 9-day chick embryos and of polyvinyl sponge-connective tissue from the guinea pig, respectively. Both groups found that the polysomes on which collagen is synthesized are large. The present work both confirms and extends these results.

MATERIALS AND METHODS

Preparation of the Ribosomal Suspension

Corium fragments from 10- and 11-day chick embryos were obtained by the method of Noble and Boucek. This tissue is composed principally of fibroblasts (21). The corium fragments were collected in cold Krebs-Ringer phosphate solution (22). The corium obtained from 30 to 100 embryos was homogenized gently in two volumes of cold standard buffer (0.01 M Tris, pH 7.4, 0.01 M KCl, 0.005 M $MgCl_2$) in a glass tissue homogenizer, with six to eight strokes of the pestle. Since preliminary experiments had shown that deoxycholate (DOC) was not needed to obtain the polyribosomes, it was omitted from the buffer. Unbroken cells, nuclei, fibers, debris, and mitochondria were removed by centrifugation at 10,000 *g* for 10 min at 4°C. The supernatant was withdrawn with a syringe and recentrifuged at 10,000 *g* for 10 min. The upper three-fourths of the supernatant, referred to as "postmitochondrial supernatant," was withdrawn with a syringe and 1 ml was layered directly on a sucrose gradient. Alternatively, the postmitochondrial supernatant was centrifuged for 60 min at 105,000 *g*. The resulting pellet then was resuspended in 2–3 ml standard buffer by homogenizing gently with six to eight strokes of a glass tissue grinder. This final ribosomal suspension was recentrifuged at 10,000 *g* for 5 min to sediment any undispersed material. The supernatant, designated "resuspended ribosomes," was aspirated carefully with a syringe, and 1 ml was layered on top of the sucrose gradient or placed in the sector cell of the analytical ultracentrifuge.

In the studies with ribonuclease, collagenase, trypsin, and EDTA, the treated and control samples were from the same ribosomal suspension and had identical volumes and concentrations of ribosomes. Crystalline pancreatic ribonuclease, highly purified bacterial collagenase, and trypsin were obtained from Worthington Biochemical Corp., Freehold, N. J., and EDTA from Matheson Co., Inc., East Rutherford, N. J.

Radioisotope Incorporation Studies

The corium strips from 30 to 100 chick embryos were suspended in 3–5 ml Krebs-Ringer phosphate solution containing 1 mg D-glucose/ml and incubated at 37°C in the Dubnoff metabolic shaker for 10 min prior to the addition of 10 μc ^{14}C-l-proline (UL) (specific activity 25 mc/mmole; Tracerlab, Waltham, Mass.). The incubation was continued for an additional 10–30 min, and the reaction was stopped by chilling in ice and adding 10 ml cold standard buffer.

Preparation of Sucrose Gradients

Linear sucrose gradients (23) were made in 34 ml cellulose nitrate centrifuge tubes, using the density gradient generating device described by Martin and Ames (24). Sucrose was dissolved in standard buffer unless otherwise specified. When a 7.5–30% sucrose gradient was used, a cushion of 3 ml of 60% sucrose (w/v) was placed in the bottom of the tube. In addition, 5–20, 15–30, and 15–60% sucrose gradients were utilized. The gradients were shown to be linear by the dye method suggested by Martin and Ames (24). The 15–60% sucrose gradient was calibrated with T2 bacteriophage, known to have a sedimentation coefficient of 710 ± 25S at neutral pH (25); on the basis of this calibration the approximate S values for the other regions of the gradient were derived (24). The other gradients were calibrated using tobacco mosaic virus (TMV), which has a sedimentation coefficient of about 200S (26).

Ultracentrifugation

Analytical ultracentrifugation was carried out in the Model E Spinco analytical ultracentrifuge with the schlieren optical system. Zone centrifugation through density gradients was carried out in a precooled SW 25.1 swinging bucket rotor of the Model L Spinco ultracentrifuge at 20,000 rpm. The gradients used and the conditions of sedimentation are noted in the legends of the figures. The temperature did not rise above 6°C during centrifugation. After centrifugation, the tubes were emptied by punching a hole in the bottom of the density gradient device with the hypodermic needle, and 30, 1 ml fractions were collected at constant flow rate.

Analyses of the Fractions

Ribonucleic acid (RNA) was estimated from optical density measurements at 260 mμ in cuvettes of 1 cm light-path length. The relationship of 20 optical density units at 260 mμ equivalent to 1 mg

RNA was used (27). After this measurement, 0.1 ml of 1% (w/v) bovine serum albumin (crystallized, Mann Research Labs., New York) was added to each fraction, and the nucleic acids and proteins were precipitated with an equal volume of cold 20% (w/v) trichloroacetic acid. The precipitate was collected on glass fiber filters (GF-C, W. H. Curtin & Co., Jacksonville, Fla.) and washed twice with cold 5% trichloroacetic acid and four times with 95% ethyl alcohol. The filters then were glued to aluminum planchets with one drop of 1% bovine serum albumin, and ^{14}C-radioactivity was counted (1280 counts) in a Nuclear-Chicago gas flow counter (Nuclear-Chicago Corporation, Des Plaines, Ill.) with a thin window Geiger-Müller tube. The results were not corrected for self-absorption or for the counting efficiency of the instrument since the thickness of the glass filter with the precipitated albumin was uniform and the absolute number of counts was not required. A correction for background, which varied between 10.5 and 11.1 cpm, was made.

After the total ^{14}C-radioactivity was counted, the glass filters were floated individually from the planchets with water, transferred with washings to a test tube, and dried under an air jet on the steam bath. Five ml of 6 N HCl were added to each tube, which then was sealed and heated for 16 hr at 110°C. The hydrolysates were treated with activated charcoal, filtered, and evaporated to dryness on the steam bath.

For paper chromatographic separation of proline and hydroxyproline, the amino acids of the dried hydrolysate were deaminated first (28), and the remaining imino acids were dissolved in 0.1–0.2 ml of 70% (v/v) ethanol for application to No. 1 Whatman paper (57.3 × 22.5 cm). Reference spots of proline and hydroxyproline were placed at the end of the hydrolysate streak. A descending chromatogram was developed in *n*-butanol-water-acetic acid (275:100:75) for 18 hr in the cold room, dried in air at room temperature, and redeveloped for a second 18 hr period (29, 30). The imino acids were located by spraying the reference strip with ninhydrin-isatin and developing in an oven at 80–100°C for 10 min. The corresponding areas containing proline and hydroxyproline were cut out and eluted with water by descending chromatography. The eluates were dried on planchets and counted (1280 counts) in the Nuclear-Chicago gas flow counter.

Initially, the chromatograms were mapped carefully for radioactivity and the labeled regions were shown to be confined to the areas corresponding to the proline and hydroxyproline standards developed on the same paper. A distance of 2–3 cm devoid of significant radioactivity separated proline from hydroxyproline. To prove that the radioactive material which migrated as hydroxyproline in butanol–water–acetic acid was indeed hydroxyproline, the labeled eluate was rechromatographed in water-saturated phenol in the presence of NH_3; the radioactivity was recovered only in the hydroxyproline region. Aliquots from the same hydrolysates of sucrose gradient fractions were chromatographed either in butanol–acetic acid or in water-saturated phenol; NH_3 and similar proline-^{14}C/hydroxyproline-^{14}C ratios were obtained by the two procedures.

For the colorimetric determination of hydroxyproline, the fractions from the gradient were pooled according to particle size and dialyzed overnight against distilled water to remove sucrose. The nondiffusible material was heated in 6 N HCl as described, and hydroxyproline was determined by Method II of Woessner's modification of the Stegemann procedure (31).

RESULTS

Particle Size Distribution

The ratio of the absorbancy at 260 mμ to that at 280 mμ in the resuspended ribosomes was 1.60. This ratio varied throughout the sucrose gradient, being 1.73 in the single ribosome peak and 1.40–1.50 in the 350–1600S polyribosome region.

A typical optical density profile (260 mμ) of an 11-day chick embryo corium ribosomal preparation sedimented through a 15–60% linear sucrose gradient is shown in Fig. 1, curve *a*. It is seen that material sedimenting more rapidly than the single ribosome peak ranged in sedimentation constant from 180 to 1600S. The material sedimenting in the 200S area always showed some heterogeneity, at least two components being present (fractions 17–22). Although the proportion of the total material which sedimented most rapidly (fractions 1–15) remained essentially constant, some variation was encountered in the location of the peak between fractions 7 and 11. This was probably due to differences in handling, inherent in the preparative procedure used, which affected the preservation of the aggregates of higher order.

To eliminate the possibility that the heavier aggregates were artifacts created during the resuspension of the microsomal pellet to obtain the resuspended ribosomes, the postmitochondrial supernatant was subjected directly to zone centrifugation under the same conditions (Fig. 1, curve *b*). Comparison of the two curves in Fig. 1 indicated that a smaller proportion of large aggregates (fractions 1–10) and a correspondingly larger proportion of small aggregates (fractions 17–22) were present in the resuspended ribosomes (curve

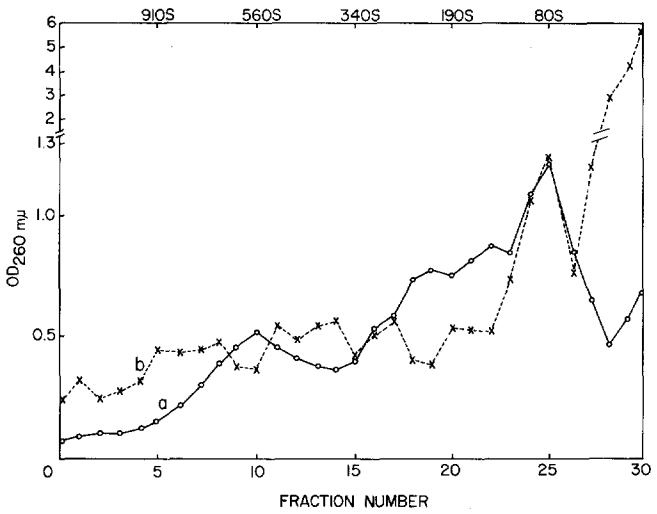

FIGURE 1 Effect of the preparative procedure on polyribosome distribution. Zone centrifugation analysis, through 15–60% sucrose density gradient, of 11-day chick embryo corium polyribosomes. 1 ml of resuspended ribosomes (curve *a*) and 1 ml of postmitochondrial supernatant from the same corium preparation (curve *b*) were layered on two sucrose gradients and centrifuged for 3 hr in the swinging bucket head of the ultracentrifuge at 20,000 rpm. The sedimentation constants throughout the gradient are relative to T2 bacteriophage marker. They are approximate and are included for descriptive purposes. The material reaching the bottom of the gradient had a sedimentation constant of approximately 1600S. The single ribosome peaks were normalized to the same optical density at 260 mμ in both experiments.

a). It would appear that the large aggregates were preserved best in the postmitochondrial supernatant and that the greater proportion of small aggregates in the resuspended ribosomes was probably the result of the mechanical disruption of large aggregates during the resuspension procedure.

The analytical sedimentation pattern of the resuspended ribosomes from the 11-day embryo (Fig. 2) had features similar to those observed in the optical density profile of the sucrose gradient (Fig. 1). Ahead of the single ribosome peak (*A*) there were two main peaks (*B*) and (*C*). The slower of these two peaks (*B*) was sharp, while the faster material (*C*) formed a rapidly spreading boundary. Table I shows the sedimentation constants of the single ribosome peak (*A*) at different concentrations of ribosomes. The s_{20,w,c_0} value of 64.5 did not vary significantly with concentration. This peak had a sedimentation constant close to 80S if it first were isolated from the other components by sucrose gradient centrifugation and then studied in the analytical ultracentrifuge. In calibrated sucrose gradients, the single ribosomes also sedimented at approximately 80S. Measurements of the sedimentation rate of peak B in the multicomponent system (Fig. 2 and Table I) gave S_{20,w,c_0} values of about 160S, while sucrose gradient analysis indicated values between 180 and 200S (Fig. 1, curve *a*) for the polyribosome material sedimenting just ahead of the single ribosomes. The Johnston-Ogston effect (32), whereby a fast moving component causes retardation and enhances the apparent concentration of a slower sedimenting component, may explain the lower apparent sedimentation constants calculated for the single ribosome and for the slower polyribosome peak in the ultracentrifugal analysis. The complex nature of this multicomponent ribosomal system does not permit a mathematical calculation of these corrections. It was decided, therefore, to investigate the behavior of a material of known sedimentation constant in the multicomponent system. For this purpose, TMV dissolved in standard buffer was mixed at several concentrations with the resuspended ribosomes from 11-day chick embryo corium, and the mixture was studied in the wedge window cell of the analytical ultracentrifuge. The same ribosomal preparation without added virus was studied in the flat window cell. The S value of the virus was found to be reduced in the multi-

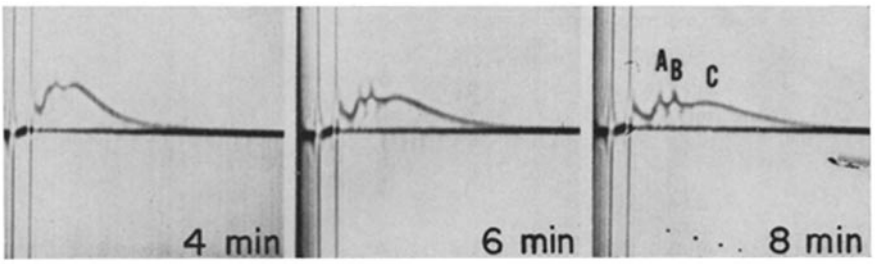

FIGURE 2 Analytical sedimentation pattern of chick embryo corium resuspended ribosomes. 11-day chick embryo corium resuspended ribosomes in standard buffer. Rotor speed, 20,410 rpm; bar angle, 70°; temperature, 9°C; exposure interval, 2 min; ribosome concentration, 3 mg/ml. Sedimentation from left to right: (*A*) single ribosome peak; (*B*) small polyribosome peak; (*C*) rapidly spreading boundary.

TABLE I

Analytical Ultracentrifugation of the Resuspended Ribosomes

Ribosomes	Single ribosome peak			Small polysome component		
(*mg/ml*)	$S_{20,w}$	$1/S(\times 10^{11})$	S_{20,w,c_0}	$S_{20,w}$	$1/S(\times 10^{11})$	S_{20,w,c_0}
2.25	64.2	15.5763		159.9	6.2507	
3.5	64.5	15.5038		139.3	7.1782	
5.0	65.0	15.3846		136.9	7.3019	
10.5	63.8	15.6739		126.5	7.9057	
			64.5			159.5

Sedimentation constant of TMV added to the resuspended ribosomes

mg TMV/ml	$S_{20,w}$	$1/S(\times 10^{11})$	S_{20,w,c_0}
0.5	155.0	6.4503	
1.0	147.0	6.8012	
1.5	140.3	7.1280	
			163.3

The conditions of sedimentation velocity experiments are described in the legend of Fig. 2. All the values were obtained from photographs taken during the first 10 min of the run. 3.5 mg ribosomes/ml were used with each concentration of TMV. S_{20,w,c_0} for TMV alone was found to be 203.7. Sedimentation constants of the components of the ribosomal suspension were calculated from the schlieren pattern obtained at several concentrations of ribosomes by ploting 1/S against concentration. The value at 0 concentration was obtained by extrapolation of the linear regression line derived from the data by the method of least squares.

component ribosomal system when compared to the value obtained for the virus alone (Table I). Because of the likelihood of the occurrence of the Johnston-Ogston effect, the sedimentation constant of 180–200S obtained from calibrated sucrose gradients, rather than the value obtained by analytical ultracentrifugation, has been used to define the slower polysome peak.

Schlieren patterns of resuspended ribosomes from 11-day chick embryo corium showed only one sharp peak (*B*, Fig. 2) sedimenting ahead of the single ribosomes. However, when TMV was added to this ribosomal system, two peaks were seen sedimenting behind the virus. The sedimentation rate of TMV was measured independently, and the virus was found to migrate as a single peak at all concentrations studied. It is known that, as a result of the Johnston-Ogston effect, the detection of small amounts of material with sedimentation rates lower than the principal components is possible (32–34). The counterparts of these two peaks sedimenting behind the virus are probably the two small peaks previously described on the sucrose gradients in the 180–200S area (Fig. 1, curve *a*).

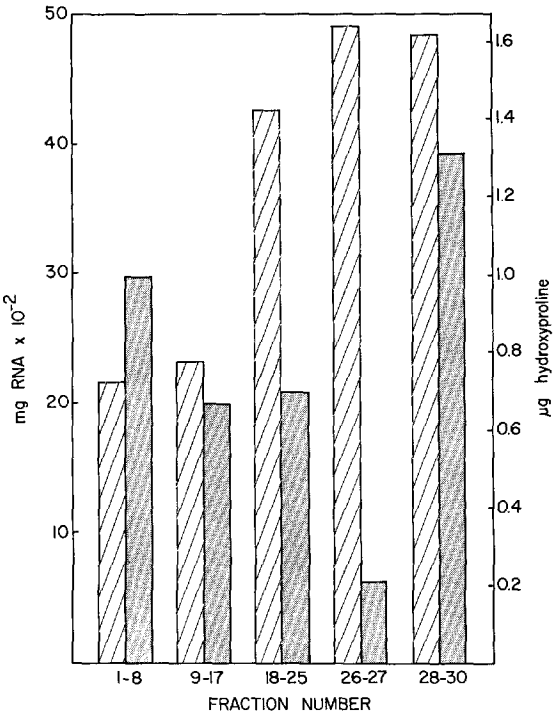

FIGURE 3 Hydroxyproline in chick embryo corium resuspended ribosomes. 10-day chick embryo corium fragments were prepared and the ribosomes layered on a 7.5–30% linear sucrose gradient with a cushion of 3 ml of 60% sucrose in the bottom of the tube. Centrifugation was carried out for 1.5 hr in the swinging bucket head of the ultracentrifuge. Hydroxyproline was determined colorimetrically on the fractions pooled as indicated. Fractions 1–8, large polysomes; 9–17, intermediate size polysomes; 18–25, small polysomes; 26–27, single ribosomes; 28–30, top of the gradient. Diagonal hatching, mg RNA $\times$ 10^{-2}; dotted bars, μg hydroxyproline.

The counterpart of the rapidly spreading boundary (*C*) in the schlieren pattern (Fig. 2) is apparently the material sedimenting in the 350–1600S region previously described in the 15–60% sucrose gradients calibrated with T2 bacteriophage (Fig. 1).

The large polyribosomes from the chick embryo corium proved to be very stable. The heavy fraction (30–60% sucrose interface) was isolated, dialyzed against standard buffer, and rerun on sucrose gradients. Some degradation occurred, producing polysomes of smaller size and single ribosomes, but the bulk of the material still reached the 30–60% sucrose interface as it had originally. The large polyribosomes isolated from sucrose gradients were treated with 0.5% DOC and recentrifuged through sucrose gradients. DOC did not modify appreciably the proportion or the sedimentation behavior of the large polyribosomes from the chick embryo corium.

Hydroxyproline Content and Radioisotope Incorporation

Detectable amounts of hydroxyproline were found in pooled sucrose gradient fractions throughout the gradient. The amount of hydroxyproline per mg RNA was maximal in the large polyribosome region of the gradient, decreased progressively in the intermediate and small size polyribosomes, was minimal in the single ribosome peak, and increased in the top of the gradient (Fig. 3).

Resuspended ribosomes prepared from 10- and 11-day chick embryo corium fragments incubated for 15 min with ^{14}C-l-proline showed incorporation of the isotope in all fractions of the sucrose gradient which had sedimentation constants greater than 80S. The single ribosomes were labeled minimally and radioactivity increased again toward the top of the gradient. When ^{14}C-radioactivity was expressed on the basis of counts per minute per milligram RNA, the largest aggregates (350–1600S) were found to be the most active (Fig. 4). The activity was lower in the 180–200S polyribosomes and minimal in the single ribosomes. The degree of labeling was high in the top of the gradient. Similar results were obtained in 10 additional experiments. Fig. 5 shows the proline-^{14}C and hydroxyproline-^{14}C profiles of sucrose gradient fractions of resuspended ribosomes prepared from 10-day chick embryo corium incubated with proline-^{14}C. It should be noted that hydroxyproline-^{14}C was found in all fractions.

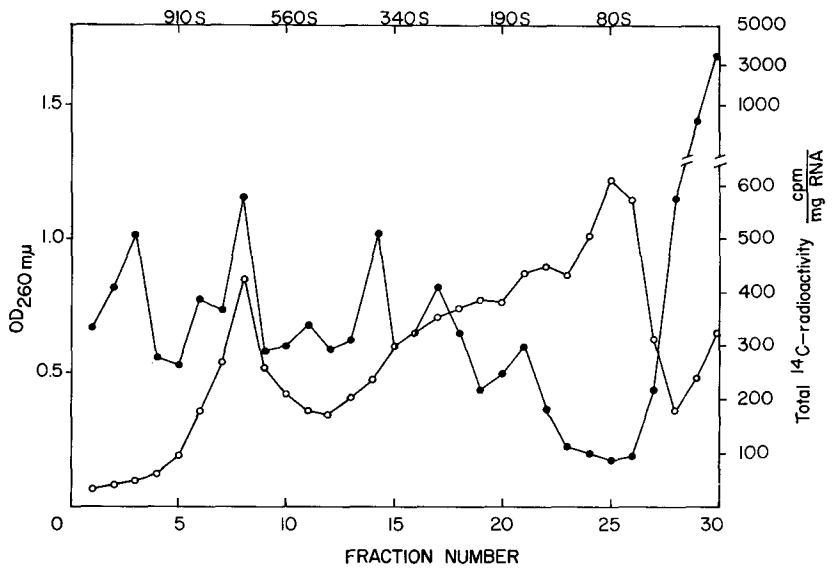

FIGURE 4 Proline-^{14}C incorporation per mg RNA into chick embryo corium resuspended ribosomes. Zone centrifugation analysis through 15–60% sucrose density gradient. The corium fragments from 10-day chick embryos were incubated with 10 μc proline-^{14}C for 15 min prior to homogenization. Sucrose gradients and centrifugation as in Fig. 1. Open circles, $OD_{260m\mu}$; closed circles, total ^{14}C-radioactivity (cpm/mg RNA).

The ratios of labeled proline to labeled hydroxyproline in the sucrose gradient fractions shown in Table II were obtained from measurements made in eight experiments. The nascent protein on the largest polyribosomes (fractions 1–10) had a proline-^{14}C/hydroxyproline-^{14}C value within the range expected for collagen on the basis of total proline and hydroxyproline (2, 14), while this ratio in the 180–200S region (fractions 21–24) was higher. The difference between the ratio in the large polyribosomes (fractions 1–20) and that in the smaller aggregates (fractions 21–24) was significant ($p < 0.001$).

Effect of Ribonuclease

The ribonuclease preparations used in these experiments were shown to be active by the assay of Kunitz (35). One aliquot of resuspended ribosomes prepared from 11-day chick embryo corium fragments was treated with 2 μg of ribonuclease in the cold (4°C) for 0.5 hr, and another aliquot kept in the cold served as control. The samples were layered on sucrose gradients and centrifuged under conditions specified in the caption of Fig. 6. The ribonuclease-treated suspension showed a 30–40% decrease in the amount of polyribosome aggregates and a corresponding increase in the single ribosome peak (curves *a*, *b*). The radioactivity removed from the polyribosomes accumulated mainly at the single ribosome peak (curves *c*, *d*). The extent of the RNase effect was similar whether the experiments were performed at room temperature or at 37°C. By increasing the amount of enzyme to 5 μg, it was possible to degrade 50% of the 350–1600S polyribosomes.

Effect of EDTA

Corium fragments from 10-day chick embryos were incubated for 10 min at 37°C in medium containing 10 μc proline-^{14}C, and the resuspended ribosomes were prepared as described in Materials and Methods. 1 ml of the ribosomal suspension was dialyzed against standard buffer, and another aliquot was dialyzed against standard buffer containing 0.01 M EDTA but no Mg^{++}. After dialysis for 3 hr at 6°C (three buffer changes), both samples were layered on top of 15–60% linear sucrose gradients (in standard buffer with and without Mg^{++}, respectively) and centrifuged for 3 hr. The treatment with EDTA caused a breakdown of the polyribosome region; the bulk of the material sedimented at rates slower than 80S. By using a

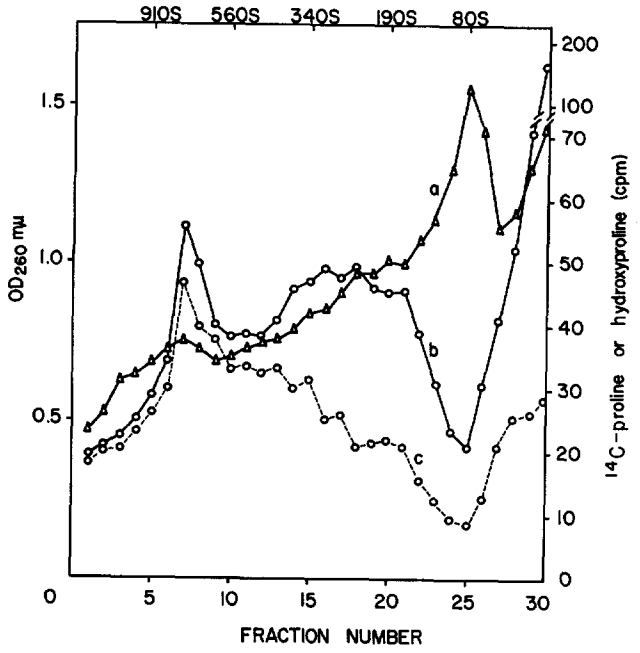

FIGURE 5 Distribution of proline-^{14}C and hydroxyproline-^{14}C in 10-day chick embryo corium polyribosomes following incubation with proline-^{14}C. Sucrose gradients and centrifugation as in Fig. 1. Proline and hydroxyproline of the hydrolyzed individual fractions were separated by paper chromatography as described in Materials and Methods. (*a*) $OD_{260m\mu}$; (*b*) proline-^{14}C; (*c*) hydroxyproline-^{14}C.

5–20% sucrose gradient and extending centrifugation time to 12 hr, the large peak of degraded ribosomal material was resolved into a 50 and a 30S peak (Fig. 7). Some ultraviolet-absorbing material sedimented at a faster rate than the 50S component (Fig. 7, fractions 1–12), and this ribosomal material resistant to EDTA contained hydroxyproline-^{14}C. The extent of disruption of the large polyribosomes depends on the conditions employed. A 1–3 hr treatment with 0.01 M EDTA disrupts 90% of the heavy fraction. Prolonging the treatment with EDTA to 8–10 hr produces a complete disruption of the preparation to 50 and 30S particles.

Effect of Collagenase

Chick embryo corium fragments were incubated for 30 min with 10 μc of proline-^{14}C, and the resuspended ribosomes were prepared as described. To 1 ml of the ribosomal suspension was added either 0.3 ml of a solution of bacterial collagenase containing 1 mg enzyme/ml in standard buffer or the same volume of boiled enzyme as control. The highly purified preparation of bacterial collagenase (CLSP 631013, Worthington Biochemical Corp., Freehold, N. J.), prepared by chromatography on DEAE-cellulose, had no significant effect on elastin or casein, or ribonuclease activity.

TABLE II

Proline-^{14}C/Hydroxyproline-^{14}C Ratios in Chick Embryo Corium Polyribosomes

Fraction	Proline-^{14}C/hydroxyproline-^{14}C
1–10	1.47 ± 0.75 (54)
11–20	1.50 ± 0.92 (16)
21–24	2.81 ± 1.15 (9)
25–26	3.60 ± 2.14 (5)
27–30	3.30 ± 1.47 (13)

10-day chick embryo corium fragments were incubated with proline-^{14}C for 15 min. The resuspended ribosomes were prepared, layered on a 7.5–30% sucrose gradient with a 60% sucrose cushion, and centrifuged as described in Fig. 3. Proline-^{14}C and hydroxyproline-^{14}C were determined on individual sucrose gradient fractions, and several fractions were pooled for statistical analysis. Mean value ± SD is given, with number of determinations in parentheses.

During exposure to the enzyme, both samples were dialyzed at room temperature against standard buffer made 0.001 M with $CaCl_2$. The samples then were layered on top of a sucrose density gradient and centrifuged as previously described, or analyzed in the analytical ultracentrifuge. 1 ml of the diffusate was dried on a planchet, and ^{14}C-

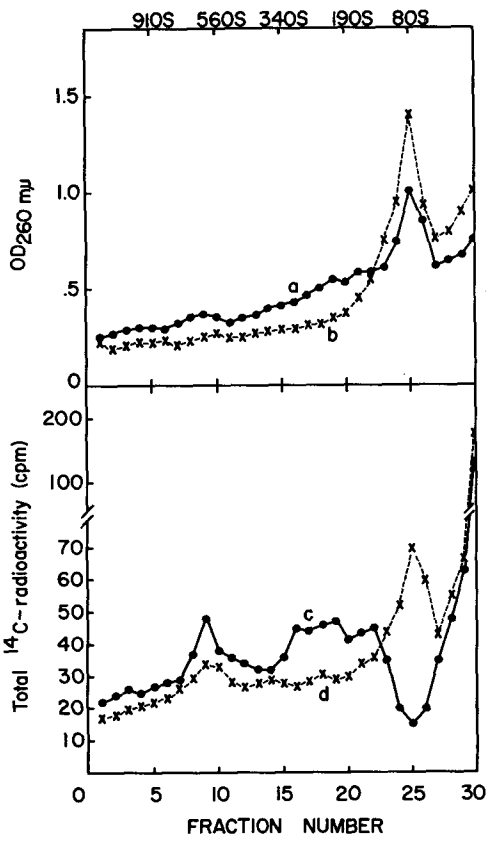

FIGURE 6 Effect of ribonuclease on 10-day chick embryo corium polyribosomes. Incubation with proline-^{14}C, sucrose gradient and centrifugation were as described in Fig. 4. (*a*) $OD_{260m\mu}$, control; (*b*) $OD_{260m\mu}$, sample treated with ribonuclease as described in the text; (*c*) total ^{14}C-radioactivity (cpm), control; (*d*) total ^{14}C-radioactivity (cpm), sample treated with ribonuclease.

radioactivity, was counted. The diffusate was lyophilized, hydrolyzed, and chromatographed as before.

Fig. 8, curve *b*, shows the optical density profile of 10-day chick embryo corium resuspended ribosomes treated for 1 hr with collagenase at room temperature. Total incorporation in the unfractionated resuspended ribosomes was 8,700 cpm for the control and 9,600 cpm for the collagenase-treated. A 50–60% decrease occurred both in the optical density (*b* compared with *a*) and in radioactivity (*d* compared with *c*) in the 350–1600S polyribosome region. A corresponding increase in optical density units occurred in the 80S and the top of the gradient. The greater proportion of the released radioactivity was diffusible, i.e. control 1,200 cpm and treated 4,100 cpm. The ratio of proline-^{14}C to hydroxyproline-^{14}C in the diffusate was 10:1 in the control and 4:1 in the collagenase-treated.

The use of even milder conditions of digestion than described above was necessary to demonstrate an increase of the small polysome peak (~180S) as a consequence of collagenase treatment. In Fig. 9, the analytical sedimentation pattern of the crude ribosomal suspension after treatment with collagenase for only 15 min at room temperature (*b*) is compared with that of the control (*a*). The rapidly spreading boundary (*C* in Fig. 9), corresponding to the polyribosomes with highest Svedberg constants, was decreased in the sample treated with collagenase. In addition, the treated sample, which contained a considerable amount of material sedimenting more slowly than the 80S peak, showed more prominent single ribosome (*A*) and small polysome (*B*) peaks.

This enhancement of the concentration of the small polysomes displayed in the analytical sedimentation pattern was confirmed by sucrose gradient analyses of collagenase-treated ribosomal suspension. Fig. 10 shows the variation in the extent of polysome disruption by collagenase in experiments in which temperature and time of incubation differed. A 10 min exposure to the enzyme caused only moderate degradation of the large polysomes (pellet), with a corresponding increase in the 200S area, single ribosomes, and lighter fractions (Fig. 10, curve *c*). However, a 45 min incubation at 37°C (Fig. 10, curve *b*) resulted in almost total degradation of the large polysomes (about 95%) as well as degradation of the 200S area. The control at 37°C for 45 min is very similar to the one at room temperature for 10 min; no significant degradation of the preparation is produced by raising the temperature.

As an additional control, polyribosomes isolated from sea urchin eggs 30 min after fertilization were treated with the same highly purified enzyme under the same conditions found to degrade the polyribosomes of the chick embryo corium. Collagenase modified neither the proportion nor the sedimentation behavior of the sea urchin egg polyribosomes. These polyribosomes are sensitive to ribonuclease (36). The effect of collagenase on the chick embryo polysomes in the absence of bentonite was identical to that in its presence (1 mg/ml).

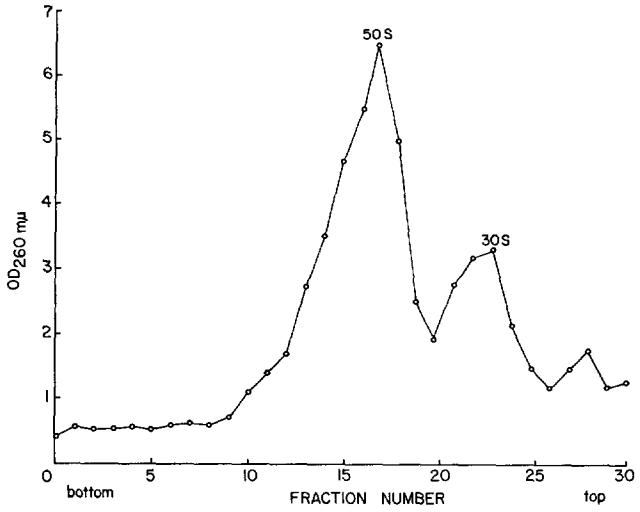

FIGURE 7 Release of 50 and 30S ribosomal subunits from resuspended ribosomes by EDTA. Zone centrifugation analysis, through sucrose density gradient, of 10-day chick embryo corium resuspended ribosomes dialyzed overnight against 0.01 M EDTA, layered on a 5–20% linear sucrose gradient, and centrifuged for 12 hr at 20,000 rpm. Optical density profile at 260 mμ.

Effect of Trypsin

10-day chick embryo corium fragments were incubated for 30 min with 10 μc of proline-^{14}C. The resuspended ribosomes were prepared as before and divided into two 1 ml aliquots. To 1 ml of the ribosomal suspension was added either 0.3 ml of a solution of trypsin (twice crystallized) containing 0.5 mg enzyme/ml in standard buffer or the same volume of boiled enzyme as control. The samples were dialyzed against standard buffer at 25°C for 60 min, layered on top of sucrose gradients, and centrifuged as previously described. The sucrose gradient fractions were analyzed for absorbancy at 260 mμ and for total ^{14}C-radioactivity.

Fig. 11 shows that trypsin does not diminish appreciably the proportion of polyribosomes larger than 500S although it partially degrades smaller size aggregates (curves *a* and *b*). The degraded material sediments much more slowly than the single ribosomes. Thus, the effect of trypsin on these aggregates is different from that observed with collagenase. Apparently, trypsin only partially digests the nascent protein on the large polyribosomes (Fig. 11, curves *c* and *d*), but about 90% of these polysome aggregates are preserved (Fig. 11, curves *a* and *b*).

DISCUSSION

Several lines of evidence indicate that collagen is being synthesized by large polyribosomes of the chick embryo corium (350–1600S). The rapid and extensive incorporation of proline-^{14}C into the large polyribosomes and a proline-^{14}C/hydroxyproline-^{14}C ratio similar to the value reported for collagen would appear to indicate little if any synthesis of other proteins by the large polyribosomes. This demonstration of large polyribosome aggregates synthesizing only collagen is possible because of (*a*) the uniqueness of the system employed, the chick embryo corium which synthesizes collagen very actively at this stage of development; and (*b*) the selective separation of large polyribosomes synthesizing collagen by zone centrifugation through sucrose gradients. Noncollagenous proteins presumably are synthesized on polysomes of smaller size.

Preliminary kinetic studies during a 60 min incubation period indicate that the proline-^{14}C incorporation was greater in the 350–1600S polyribosomes than in the 180–200S area. This may be indicative of a greater rate of protein synthesis in the large aggregates. Alternatively, the polyribosomes in the 200S area may be engaged in the synthesis of both collagen and noncollagen protein containing lower levels of proline and no hydroxyproline; the relatively high ratio of proline to hydroxyproline in the small polyribosome region compared with that in the large polyribosomes would support this alternative. On the other hand, if in this tissue the extent of synthesis of noncollagen protein was small compared with that of collagen, the higher ratio on the small polyribosomes might be explained by the incorporation of proline into a nascent polypeptide precursor of collagen having low hydroxyproline content. The data on proline-

^{14}C incorporation and its conversion to hydroxyproline are compatible with hydroxylation of proline either in a step prior to (37, 38) or after peptide bond formation (39). The determination of the proportion of noncollagen protein in the different fractions and the isolation and characterization of the nascent protein from these polyribosomes may help to clarify this point.

Additional evidence supporting the view that these large polyribosomes are engaged principally in collagen synthesis is obtained by studying the effect of trypsin. This enzyme has been reported to attack randomly coiled polypeptide chains of denatured collagen, but it appears to be unable to degrade the intact three-chain helical, tropocollagen molecule (40). Nascent collagen polypeptides at the stage prior to the formation of the triple helix should be susceptible to the enzyme. The finding that radioactivity is removed extensively by trypsin, though the large aggregates are not disrupted, is consistent with the proposal that the presence of finished tropocollagen molecules contributes to the stability of these large polysomes.

The specific action of collagenase in causing the degradation of the large polyribosomes indicates that collagen is present on them and contributes to their stability. Quantitative analyses also have shown that hydroxyproline is associated with the polyribosomes; the greater proportion relative to RNA was found with the aggregates sedimenting at rates between 350 and 1600S. A smaller proportion of hydroxyproline was present in polyribosomes with sedimentation constants of 180–200S. The comparison of the optical density profile of the postmitochondrial supernatant with that of the resuspended ribosomes (Fig. 1, curves *a* and *b*) suggests that a large proportion of the small aggregates ($\sim$ 200S) in the resuspended ribosomes are derived from disruption of the large polyribosomes. It is thus possible that these small polysomes observed by zone centrifugation are really an integral part of the large polysomes in natural conditions.

The 350–1600S aggregates possess several unusual characteristics for polyribosomes, namely, their partial resistance to ribonuclease attack, their unexpectedly large sedimentation constants, and their remarkable thermal stability. In spite of these peculiarities, several lines of evidence support the conclusion that these aggregates are indeed polyribosomes. The aggregates, like conventional polyribosomes, incorporate labeled amino acids

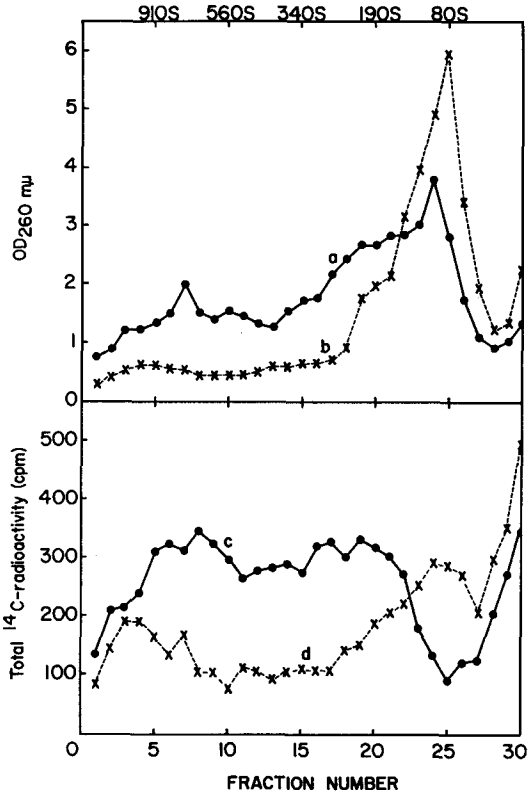

FIGURE 8 Effect of collagenase on chick embryo corium polyribosomes. Zone centrifugation analysis, through 15–60% sucrose density gradient, of 10-day chick embryo corium resuspended ribosomes. Incubation with proline-^{14}C, sucrose gradient and centrifugation were as described in Fig. 4. (*a*) $OD_{260m\mu}$, control; (*b*) $OD_{260m\mu}$, sample treated with collagenase as described in the text; (*c*) cpm, control; (*d*) cpm, sample treated with collagenase.

at a rapid rate. EDTA effects the degradation of the large aggregates, releasing 50 and 30S ribosomal subunits. Extensive treatment of ribosomal preparations with collagenase prior to zone centrifugation completely disrupts the large polyribosomes, and material accumulates mainly in the single ribosome peak. Thus, the polyribosomes appear to be stabilized by nascent collagen. Experiments with both EDTA and collagenase clearly demonstrate that, although the large aggregates are partially resistant to ribonuclease, they are formed of ribosomes. In addition, the absence of any demonstrable effect of DOC on the large polysomes speaks against association of these large aggregates with lipoprotein membranes.

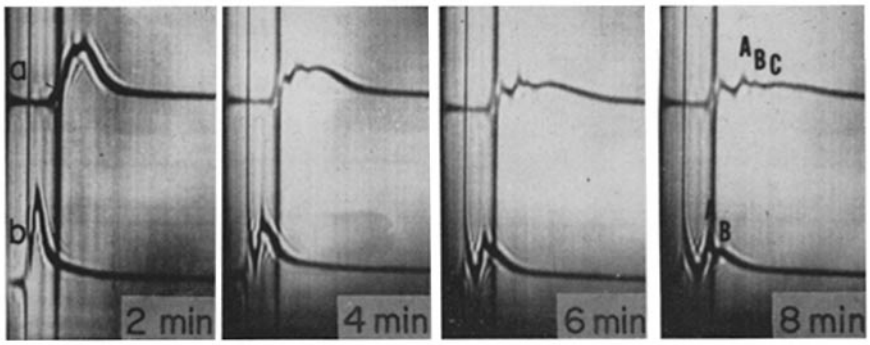

FIGURE 9 Effect of collagenase on chick embryo corium polyribosomes. Analytical sedimentation pattern of 10-day chick embryo corium resuspended ribosomes. Experimental conditions as in Fig. 2. (*a*) control; (*b*) treated with 0.3 mg of purified bacterial collagenase for 15 min at room temperature; (*A*) single ribosome peak; (*B*) small polyribosome peak; (*C*) rapidly spreading boundary.

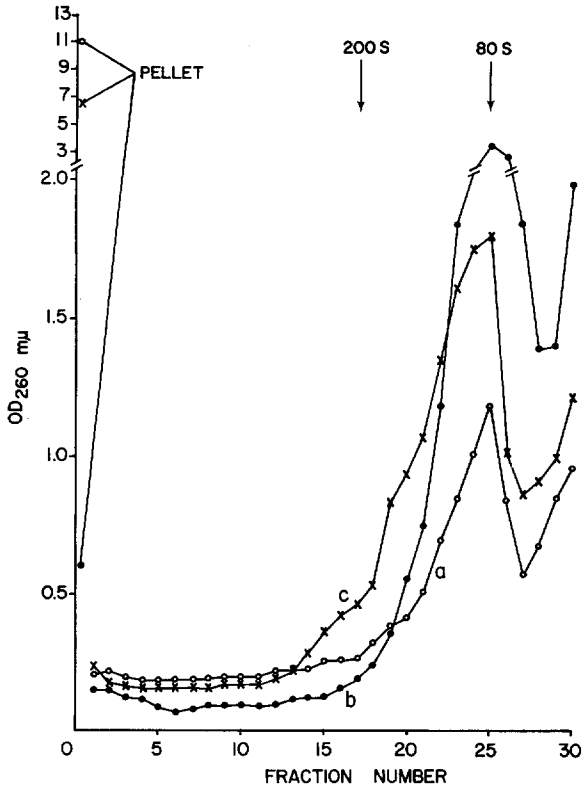

FIGURE 10 Effect of collagenase on chick embryo corium polyribosomes. To each of three aliquots of resuspended ribosomes was added 0.15 ml of 0.001 M $CaCl_2$. One tube received 0.3 ml of a solution of purified bacterial collagenase (0.3 mg) and was incubated for 45 min at 37°C; the second, incubated at room temperature (22–25°C) for 10 min, received the same amount of enzyme, and the third tube, which served as control, received 0.3 mg of boiled enzyme and was incubated at 22–25°C for 10 min. A 45-min control at 37°C was run independently. Incubation was terminated by layering on 15–30% sucrose gradients and centrifuging for 3 hr in the swinging bucket head. (*a*) $OD_{260m\mu}$ control, 10 min at 22–25°C; (*b*) $OD_{260m\mu}$ collagenase, 45 min at 37°C; (c) $OD_{260m\mu}$ collagenase, 10 min at 22–25°C.

The question as to why collagenase action primarily increases the absorbancy at the single ribosome peak could be raised. This effect would appear more like that of RNase. However, RNase itself does not produce such a large effect at room temperature or even at 37°C. It is possible that, when the large polysomes are intact, the collagen chains joining the aggregates prevent an extensive ribonuclease breakdown. However, when this protection provided by the collagen chains is removed by the action of collagenase, even trace amounts of ribonuclease, or perhaps endogenous

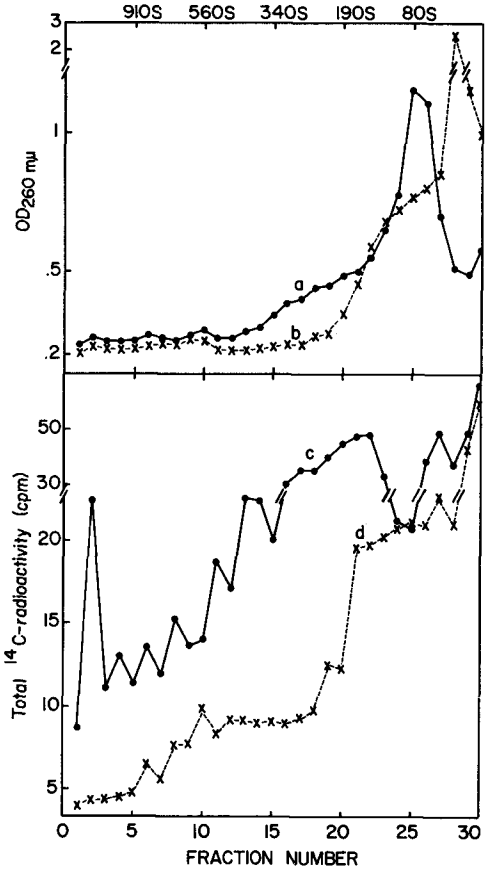

FIGURE 11 Effect of trypsin on 10-day chick embryo corium polyribosomes. Incubation with proline-^{14}C and treatment with trypsin are described in the text. Sucrose gradients and centrifugation were as described in Fig. 4. (*a*) $OD_{260m\mu}$, control; (b) $OD_{260m\mu}$, sample treated with trypsin; (*c*) total ^{14}C-radioactivity (cpm), control; (*d*) total ^{14}C-radioactivity (cpm), sample treated with trypsin.

ribonuclease linked to ribosomes (41, 42), may complete the disruption of the aggregates. Also, it could be speculated that nascent collagen polypeptides might contribute, perhaps by hydrogen bonding with the ribosomal protein, to the stability of the 180–200S polyribosomes. Gilbert has proposed that the nascent protein stabilizes the polyribosome structure through hydrogen bonding (9). Thus, digestion of collagen chains by collagenase might destabilize the small polysomes, thereby releasing ribosomal monomers.

Collagen synthesis has been reported on 9-day chick embryo polyribosomes by Kretsinger et al., (19) who described a diffuse peak of material containing hydroxyproline-^{14}C and sedimenting at about 400S. The hydroxyproline-containing pellet reported by these investigators is probably like the material described here as the pellet in 15–30% sucrose gradients, as the interface between 30 and 60% sucrose layers, and as the aggregates heavier than 700S in 15–60% sucrose gradients. Kretsinger et al. reported ratios of proline-^{14}C to hydroxyproline-^{14}C on the largest polysomes of between 6:1 and 10:1; the ratio decreased to about 4:1 after ribonuclease treatment. It was properly concluded, therefore, that the ribonuclease-resistant fraction contained a greater proportion of collagen-like material. The high proline to hydroxyproline ratios observed by these authors are probably related to the fact that they studied ribosomes of the whole chick embryo, which synthesize a vast array of proteins in addition to collagen.

The findings reported here are in accord with those reported by Kretsinger et al. on the sucrose gradient centrifugation and on the effect of ribonuclease on chick embryo polyribosomes. Also in agreement is the proposal that nascent collagen contributes to the stability of the polyribosomes. However the present data may not agree with Kretsinger et al. as to the nature of the fundamental polyribosomal unit. These authors suggested that each of the three alpha chains of collagen is synthesized on a polyribosome containing approximately 30 ribosomes and that these polyribosomes are held together by the folding of the three growing polypeptide chains into a partially completed, triple stranded molecule. This model of collagen biosynthesis on the polysomes is consistent with the alpha chain's being the elementary collagen subunit. Sedimentation equilibrium studies indicate that the molecular weight of the alpha chains is probably about 100,000 (43).

However, it has been reported that the alpha chains themselves may be the product of interaction of small molecular weight collagen polypeptides. Gallop observed that exposure of denatured tropocollagen (alpha chains) to hydrazine or hydroxylamine under mild conditions resulted in further fragmentation of alpha chains into fragments of small molecular weight (16). More recently, another subunit model for the tropocollagen macromolecule has been proposed in which the three alpha chains are built up from a repeating sequence of five, five and six subunits, respectively (17, 18). The evidence for this model rests

primarily on an analysis of the band pattern in segment-long spacing crystallites of the calf skin tropocollagen macromolecule observed in the electron microscope after phosphotungstic acid staining.

If the large aggregates are joined together by both mRNA and interacting nascent collagen polypeptides, the highly specific enzyme bacterial collagenase might be expected to release smaller polyribosomes stabilized only by mRNA, revealing indirectly the size of the elementary collagen subunits. Under the present experimental conditions, following collagenase degradation of the large polysomes, material does not accumulate in the 300–400S region of the sucrose gradient, the size that would be expected for a polyribosome responsible for the synthesis of one alpha chain. Rather, our data show that very mild collagenase treatment results in the breakdown of the large polyribosomes, with the release of smaller polyribosomes (180–200S; Figs. 9 and 10). Finally, this 180–200S polysome region contained newly formed hydroxyproline which was susceptible to collagenase. The length of the peptide chains synthesized, calculated on the basis of the size of the small polysome(s) ($\sim$ 180–200S), corresponds to that proposed for subunits of the collagen alpha chain (16–18). These results are consistent with the hypothesis that these polyribosomes are engaged in the synthesis of small molecular weight collagen subunits. The interaction of these nascent subunits by protein-protein interaction may lead to polyribosome aggregation. Thus, the small polysomes might be considered "monomers" of the large polyribosomes (350–1600S), i.e., "polymers" which are stabilized by the interacting collagen chains. However, a word of caution should be introduced. There remains the possibility of a preferential production of small polyribosomes from the large ones by collagenase treatment as the result of some effect other than the splitting of collagen chains.

Interaction of nascent collagen chains while still attached to the ribosomes may lead to a consequence in addition to the formation of large polyribosomes (350–1600S), i.e., the aggregation of finished tropocollagen molecules still linked to the polysomes. This is suggested by the fact that the aggregates sedimenting in the leading edge of the particle size distribution (> 500S) have the highest hydroxyproline content and are larger than those which would be expected to be necessary for the synthesis of individual tropocollagen molecules (300,000 molecular weight), approximately 100 ribosomes. The findings with trypsin also support the existence of triple helical tropocollagen on these aggregates.

Current ideas on the structure of proteins indicate that the full information necessary to attain the complete tertiary and quaternary configurations of proteins resides in the primary amino acid sequence (44). The assembly of protein subunits to reconstitute biologically active proteins occurs very rapidly in solution (45). Therefore, it is not surprising that aggregation of protein subunits may occur in some cases at the site of synthesis, on the polysomes.

It can be concluded that the synthesis of collagen occurs on polyribosomes, following the same general mechanism initially postulated for polyribosome protein synthesis. The two apparently unusual features of these polyribosomes, i.e. the extremely large size and the partial resistance to ribonuclease, are probably due to the polypeptide-polypeptide interactions on the polysomes.

I am greatly indebted to Dr. R. J. Boucek for continuing support, encouragement, and discussion, and to Doctors J. F. Woessner, Jr., and N. L. Noble for generous help in experimental procedures and preparation of the manuscript. Dr. Ray Iverson kindly performed the control experiment with sea urchin egg polysomes. TMV was a gift from Dr. C. A. Knight, and T2 bacteriophage was kindly provided by Dr. W. Pond.

This investigation was supported in part by Public Health Service Training Grants T1 HE 5463 from the National Heart Institute and T1 GM 649 from the Division of General Medical Science.

The material in this report has been taken from the dissertation submitted to the Graduate School of the University of Miami by F. Fernández-Madrid, in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

Received for publication 17 June 1966.

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