SPECIFIC PROTEIN SYNTHESIS IN CELLULAR DIFFERENTIATION

Production of Eggshell Proteins by Silkmoth Follicular Cells

M. PAUL, M. R. GOLDSMITH, J. R. HUNSLEY, and F. C. KAFATOS

From The Biological Laboratories, Harvard University, Cambridge, Massachusetts 02138

ABSTRACT

Silkmoth follicles, arranged in a precise developmental sequence within the ovariole, yield pure and uniform populations of follicular epithelial cells highly differentiated for synthesis of the proteinaceous eggshell (chorion). These cells can be maintained and labeled efficiently in organ culture; their in vitro (and cell free) protein synthetic activity reflects their activity in vivo. During differentiation the cells undergo dramatic changes in protein synthesis. For 2 days the cells are devoted almost exclusively to production of distinctive chorion proteins of low molecular weight and of unusual amino acid composition. Each protein has its own characteristic developmental kinetics of synthesis. Each is synthesized as a separate polypeptide, apparently on monocistronic messenger RNA (mRNA), and thus reflects the expression of a distinct gene. The rapid changes in this tissue do not result from corresponding changes in translational efficiency. Thus, the peptide chain elongation rate is comparable for chorion and for proteins synthesized at earlier developmental stages (1.3–1.9 amino acids/sec); moreover, the spacing of ribosomes on chorion mRNA (30–37 codons per ribosome) is similar to that encountered in other eukaryotic systems.

INTRODUCTION

The synthesis of characteristic proteins is a key aspect of cellular differentiation. Some well-known examples of differentiation-specific protein synthesis are the production of hemoglobin by erythroblasts, of myosin, actin, and myoglobin by muscle cells, of crystallins by the eye lens, and of digestive enzymes or zymogens by the pancreatic acinar epithelium. Particularly interesting are factors controlling the synthetic rates of individual proteins during differentiation and programs for sequential elaboration of several distinct products by a single cell type. The study of such processes in vertebrates is often complicated by unavailability of synchronized cells at defined developmental stages, presence of extraneous cell types, and concurrent mitotic activity or cell fusion. In this respect the exocrine glands of insects, which often consist of a single type of polyploid cell, developmentally synchronized and nondividing, have certain advantages.

The follicular epithelium of silkmoths is a particularly favorable exocrine gland. Each follicle (Fig. 1 b) consists of a single layer (Fig. 1 c) of polyploid epithelial cells, enveloping the oocyte and its seven nurse cells. In each of eight ovarioles, the follicles are arranged in a precise developmental sequence (Fig. 1 a); they are generated at the anterior end and move backward as new

follicles are added in front (King and Aggarwal, 1965). In *Hyalophora cecropia*, the developmental age difference between adjacent follicles has been estimated as 4–5 hr (Telfer and Rutberg, 1960). Thus, temporal differentiation is represented in a spatial dimension. At an early stage of vitellogenesis the oocyte grows mainly through accumulation of yolk from the blood (see Telfer, 1965). Near the end of vitellogenesis, the nurse cells degenerate (King and Aggarwal, 1965); the follicle then consists of only two cell types, the oocyte (which can be destroyed easily) and the enveloping follicular cells. In sum, pure preparations of follicular epithelial cells (Fig. 1 *c*) can be obtained conveniently in a developmental sequence.

During yolk accumulation the follicular epithelial cells play an important role, detaching laterally so as to form voluminous channels for yolk transport (King and Aggarwal, 1965). In addition, the cells synthesize and secrete into the channels a macromolecule, probably a glycoprotein, which finds its way into the yolk spheres of the oocyte (Anderson and Telfer, 1969). From experiments with trypan blue, a competitive inhibitor of yolk uptake, it has been inferred that the glycoprotein facilitates selective uptake through selective binding to yolk (Anderson and Telfer, 1969; 1970 *a*, *b*).

At the end of vitellogenesis, the follicular channels begin to collapse and blood proteins can no longer gain access to the oocyte (King and Aggarwal, 1965; Telfer and Anderson, 1968). A "terminal growth phase" ensues, during which the oocyte increases in volume by approximately 50%, primarily through the accumulation of water (Telfer and Anderson, 1968). The follicular cells apparently deposit a thickened vitelline membrane between the oocyte and themselves (Telfer and Smith, 1970). Finally, as the last step in their differentiation, they secrete a proteinaceous eggshell, the chorion, around the vitelline membrane. At maturity, the chorion accounts for approximately 30% of the dry weight of the egg (Pollack and Telfer, 1969).

Thus, follicular cell differentiation involves sequential elaboration of at least three products, the yolk-associated glycoprotein, the vitelline membrane, and the chorion. In fact, the program of differentiation is even more detailed: as will be shown later, the chorion consists of several proteins, each produced during a characteristic developmental period. The timing is apparently controlled endogenously, since a sequence of follicular stages can be found in a single ovariole, bathed in a common blood pool.

The present report focuses on chorion formation. We describe solubilization and partial characterization of the chorion proteins, which are unusually small and distinct from the proteins of the vitelline membrane. We present evidence that each chorion protein is synthesized as a distinct component; there is no interconversion and no cleavage from a larger precursor. We document sequential and rapid transitions in protein biosynthesis: synthesis of nonsecretory proteins is markedly depressed as the follicular cells embark on production of successive classes of chorion proteins. We correlate these changes in protein biosynthesis with characteristic changes in polyribosome profiles and in average translation time. Finally, we demonstrate that an active system for cell-free protein synthesis can be prepared from the follicular cells, reflecting their in vivo biosynthetic activity.

MATERIALS AND METHODS

Reagents and Proteins

Materials were purchased and treated as follows: fast green FCF R-250, trypan blue (Allied Chemical Corp., New York); glutaraldehyde (high purity, Ladd Research Industries, Inc., Burlington, Vt.); acrylamide, N,N'-methylenebisacrylamide (MBA,[1] recrystallized from acetone), N,N,N',N'-tetramethylethylenediamine (TEMED), ammonium persulfate (Biorad electrophoresis grade); phenylmethylsulfonylfluoride (PMSF) (lot 901498, Calbiochem, Los Angeles, Calif.); Siliclad (Clay-Adams, Inc., Parsippany, N. J.); guanidine hydrochloride (recrystallized, Nozaki and Tanford, 1967), 2-iodoacetamide (recrystallized from H_2O), phenylthiourea (PTU) (recrystallized from ethanol), 2-mercaptoethanol (Eastman Kodak Co., Rochester, N. Y.); gelatin, sodium heparin, sodium dodecyl sulfate (SDS) (recrystallized from

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[1] *The following abbreviations are used in this paper:* ATP, adenosine triphosphate; DOC, sodium deoxycholate; EDTA, ethylenediaminetetraacetic acid; GM, Grace's tissue culture medium without hemolymph; GTP, guanosine triphosphate; MBA, N,N'-methylenebisacrylamide; mRNA, messenger RNA; MWS, modified Weevers' saline; PMSF, phenylmethylsulfonylfluoride; PTU, phenylthiourea; S-5, 5000 *g* supernate; S-105, 105,000 *g* supernate; SB I, solubilization buffer I; SB II, solubilization buffer II; SDS, sodium dodecyl sulfate; TCA, trichloroacetic acid; TEMED, N,N,N',N'-tetramethylethylenediamine; tRNA, transfer RNA.

ethanol), sodium deoxycholate (DOC) (Fisher Scientific Company, Pittsburgh, Pa.); horse heart cytochrome *c*, sperm whale myoglobin, beef pancreas chymotrypsinogen, chicken ovalbumin, human γ-globulin (protein molecular weight kit No. 20900-8109), coomassie brilliant blue (Mann Research Laboratories Inc., New York); urea (recrystallized from ethanol), 30% H_2O_2 (Merck and Co., Inc., Rahway, N. J.); Protosol, Omnifluor, Liquifluor, Aquasol (New England Nuclear Corp., Boston, Mass.); yeast RNA, bovine serum albumin fraction V, hog stomach pepsin crystallized three times (Nutritional Biochemicals Corporation, Cleveland, Ohio); sucrose (special density gradient grade, Schwarz-Mann, Orangeburg, N. Y.); Na adenosine triphosphate (ATP), Na phosphoenolpyruvate, Na guanosine triphosphate (GTP), type II rabbit muscle pyruvate kinase, dithiothreitol, yeast soluble RNA, type III spinach ferredoxin, crystalline bovine pancreas insulin, mercaptoacetic acid (Sigma Chemical Co., St. Louis, Mo.); GF/C and 3 MM filter discs (Whatman); and bovine pancreatic ribonuclease (Rase, Worthington Biochemical Corp., Freehold, N. J.).

All other chemicals were reagent grade. Water used in these experiments was deionized and charcoal treated (Millipore Corporation, Bedford, Mass., Model Super Q). Sources of radioisotopes appear in the appropriate figure legends.

Animals

Antheraea polyphemus and *Antheraea pernyi* silkmoth pupae were stored at 2°-5°C until needed, and were then allowed to initiate adult development at 25°C. Developmental stages were determined as described by Berger and Kafatos (1971). *A. polyphemus* females of stages 14–16$_I$ were used routinely.

Dissection of Follicles and Chorion Preparation

Ovarioles were removed from developing adults into Grace's tissue culture medium without hemolymph (Grace, 1962; hereafter called GM), or into a medium based on Weevers' saline (Weevers, 1966; called Modified Weevers' Saline, MWS) containing 4.0 mm NaCl, 26.3 mm KCl, 18.0 mm $MgCl_2$, 3.0 mm $CaCl_2$, 253 mm sucrose, 10 mm potassium phosphate, pH 6.6. A crystal of PTU was routinely added to both media during dissection and culture. Follicles were cut in half and the yolky ooplasm washed away with medium. Follicular epithelium was dissected from the chorion (most easily in 7% 1-propanol). PMSF (1 mm in 7% 1-propanol or 1 mm and 1% 1-propanol in the indicated solutions) was often used in the preparation of samples for electrophoresis, although at no time did we see evidence for proteolytic activity in its absence.

Organ Culture

Follicles were cultured in GM in watch glasses with a solid square base (General Biological Supply House, Inc., Chicago, Ill.) on a rotatory shaker at 25°C. Pulse labeling was carried out routinely in GM with the total concentration of any radioactive amino acid ranging from $\frac{1}{20}$ to normal. Before labeling, the follicles were maintained for 2–5 min in GM with subnormal levels of the appropriate amino acids. Chase incubations were carried out in complete GM. Unless otherwise indicated, incorporation was terminated by transferring follicles to chilled MWS. All incubation media and glassware were sterile.

Preparation of Samples for Electrophoresis

Samples were routinely prepared for electrophoresis by heating at 100°C for 10 min in one of two buffers. In electrophoresis Method I, the sample buffer (solubilization buffer I [SB I]) contained 1% 2-mercaptoethanol, 1% SDS, 3 m urea, and 10 mm sodium phosphate, pH 7.2. For Method II, the buffer (SB II) contained 1% 2-mercaptoethanol, 1% SDS, 6 m urea, and 10 mm Tris-HCl, pH 8.4. The maximum concentration of protein was 5 mg/ml. Carboxamidomethylation of solubilized protein was carried out at room temperature with one-half volume of 1.0 m iodoacetamide in 1.2 m Tris-HCl, pH 8.4. After 30 min the reaction was terminated with excess 2-mercaptoethanol (3 µl/100 µl solution). Samples thus prepared could be electrophoresed directly without dialysis.

SDS-Acrylamide Electrophoresis

METHOD I: Electrophoresis Method I was similar to the procedure of Weber and Osborn (1969), modified to contain 15% acrylamide and 0.4% MBA. Gel tubes were treated with Siliclad for easier removal of gels. Gels were stained overnight with coomassie brilliant blue (0.008% in 5 parts methanol, 1 part acetic acid, 5 parts water), destained (in 5% methanol, 7.5% acetic acid), scanned (in 7.5% acetic acid) with a Gilford 2400 spectrophotometer, and photographed as previously described (Berger and Kafatos, 1971).

METHOD II: During the course of this study, we found that highly cross-linked gels containing urea (Swank and Munkres, 1971) produced superior resolution of chorion proteins. Gels contained 13% acrylamide, 1% MBA, 0.5% SDS, and 6 m urea. The gel solution (25 ml) was freshly made by adding 3.25 g acrylamide, 0.25 g MBA, 9 g urea, 0.125 g SDS, 9.9 ml distilled water, and 5 ml of a stock solution of 0.5 m acetic acid buffered to pH 8.4 with Tris base, and was degassed briefly. Gels were made in siliconized, 5 mm inner diameter, 8–10 cm length glass tubes. Polymerization, induced by adding 10 mg ammonium persulfate and 2 µl TEMED, generally occurred in 30

min in a water bath at room temperature. The lower reservior buffer contained 0.1% SDS and 0.1 M acetic acid adjusted to pH 8.4 with Tris base, while the upper buffer contained in addition 19 mM 2-mercaptoacetate, pH 8.4. After preelectrophoresis of the gels for 30 min, samples were layered under reservior buffer. Electrophoresis was carried out at room temperature for 18–24 hr at a constant current 2.5 ma per gel. Gels were stained, scanned, and photographed as in Method I.

Polysome Isolation

To ensure that the cells were actively synthesizing protein, MWS-washed epithelium was incubated in standard GM at room temperature for a minimum of 15 min before experimental manipulations. Protein synthesis was stopped rapidly by transferring the tissue to 2–4 ml of an ice slurry of hypotonic buffer containing 10 mM Tris-HCl, pH 7.4, 50 mM KCl, and 10 mM $MgCl_2$ at 0°C in a glass 7 ml Dounce homogenizer. The KCl concentration was changed where noted in the Results. All the following operations were carried out at 0°C using autoclaved glassware or sterile plastic containers to reduce nuclease contamination. The tissue was resuspended at a concentration of 12–18 follicles/0.5 ml, allowed to swell for at least 5 min, and homogenized with three to seven strokes of the loosefitting pestle and one to two strokes of the tight-fitting pestle. The homogenate was brought to a final concentration of 0.5% sodium deoxycholate and centrifuged at 5000 g for 5 min at 4°C. For analysis of polysomes, 0.5–1.0 ml of supernate was layered on 12 ml linear sucrose gradients and centrifuged in the IEC SB 283 rotor under conditions described in Results.

Gradients were prepared from RNase-free sucrose in hypotonic buffer as noted in Results. The sucrose was pretreated with bentonite (Petermann and Pavlovec, 1963) at a final concentration of 0.5 mg/ml where indicated. Gradients contained 100 μg/ml gelatin (Kaempfer, 1970) and were made in IEC polyallomer tubes treated by a modification of the method of Kaempfer et al. (1968). The tubes were heated to boiling twice in 5 mM Na ethylenediaminetetraacetic acid (EDTA), pH 8.0, and once in 100 mM magnesium acetate, 5 mM Na EDTA, pH 8.0, followed by several rinses with distilled water (Kaempfer, personal communication).

Continuous absorbance recordings were obtained with the ISCO Model UA-2 UV Analyzer (1 cm flow cell) and Honeywell Electronik strip chart recorder. Gradient fractions (0.25 ml) were collected using an ISCO Model 270 fraction collector and Model D Density Gradient Fractionator.

Polysome yields were estimated by weighing "cutouts" of the absorbance tracings. The margins of adjacent peaks were extended linearly and half the overlapping area added to each component.

Cell-Free Protein Synthesis

Halved, washed follicles (Nos. 1–5, 20/0.1 ml) were homogenized with five vertical strokes in a Bolab glass microhomogenizer using the following medium at 0°C: 50 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 30 mM KCl, 1.0 mM PTU, 300 mM sucrose, 0.5 mM dithiothreitol, and 100 μg/ml sodium heparin. Phase-contrast microscopy revealed neither whole cells nor broken nuclei. The homogenate was centrifuged for 3 min at 5000 g at 4°C and the resultant opalescent supernate was removed. Aliquots were taken for analysis of total RNA content as described in Fig. 2. A volume of supernate containing about 20 mg of protein (Warburg and Christian, 1941) was added to the following mixture at 25°C (1.00 ml): 50 μmoles Tris-HCl, pH 7.5, 5.0 μmoles $MgCl_2$, 100 μmoles KCl, 0.5 μmole dithiothreitol, 1.0 μmole Na ATP, 10 μmoles sodium phosphoenolpyruvate, 0.5 μmole Na GTP, 50 μg rabbit muscle pyruvate kinase, and a mixture of cold amino acids at $\frac{1}{10}$ the concentration in GM plus radioactive amino acids as described in Results. Samples removed from the reaction mixture up to 20 min later were pipetted onto 2.4 cm Whatman 3 MM filter discs for determination of radioactivity. The remainder was frozen in dry ice-acetone and stored at −90°C.

Radioactivity Measurements

Radioactivity was measured by liquid scintillation in a refrigerated Beckman LS-250 Liquid Scintillation Counter.

Method I polyacrylamide gels were sliced transversely, processed, and counted as previously described (Berger and Kafatos, 1971). Slices from Method II gels were dissolved in 50 μl 30% H_2O_2 overnight at 40°C and counted in 3.6 ml Aquasol. The data were analyzed by computer (Yund et al., 1971).

Sucrose graident fractions plus carrier were precipitated with 2 ml ice-cold 7% (w/v) trichloroacetic acid (TCA) containing 5 mM of each of the amino acids used in labeling. The samples were heated to 90°C for 30 min, chilled to 0°C, and filtered through 2.4 cm Whatman GF/C glass fiber filters on a glass Millipore filtration apparatus. Filters were rinsed four times with 2–3 ml each 5% TCA containing 5 mM nonradioactive precursor at 0°C and once with 2 ml 95% ethanol, air dried and transferred to glass counting vials. To each vial was added 0.30 ml of Protosol followed after at least 2 hr by 15 ml of counting mixture containing 0.4% Omnifluor and 0.33% glacial acetic acid in toluene.

Samples from cell-free protein synthesis experiments were processed by a modification of the proce-

dure of Mans and Novelli (1961). The discs were treated for 10 min at 0°C in 5% TCA containing 10 mM nonradioactive precursor, heated for 30 min at 90°C, and then washed for 2 hr in each of six changes of 5% TCA containing 10 mM nonradioactive precursor at 4°C with continuous stirring. The filters were washed once with 95% ethanol, air dried, eluted with Protosol as described above, and counted in 15 ml toluene containing 4.2% Liquifluor and 0.2% glacial acetic acid.

RESULTS

Staging of Follicles

In the ovarioles of *A. polyphemus* and *A. pernyi*, hydration at the beginning of the terminal growth phase results in a rather abrupt increase in follicle diameter (Fig. 1 *a*). At this time the color of the deep yellow (*A. polyphemus*) or green (*A. pernyi*) vitellogenic follicles changes into a more pale hue.
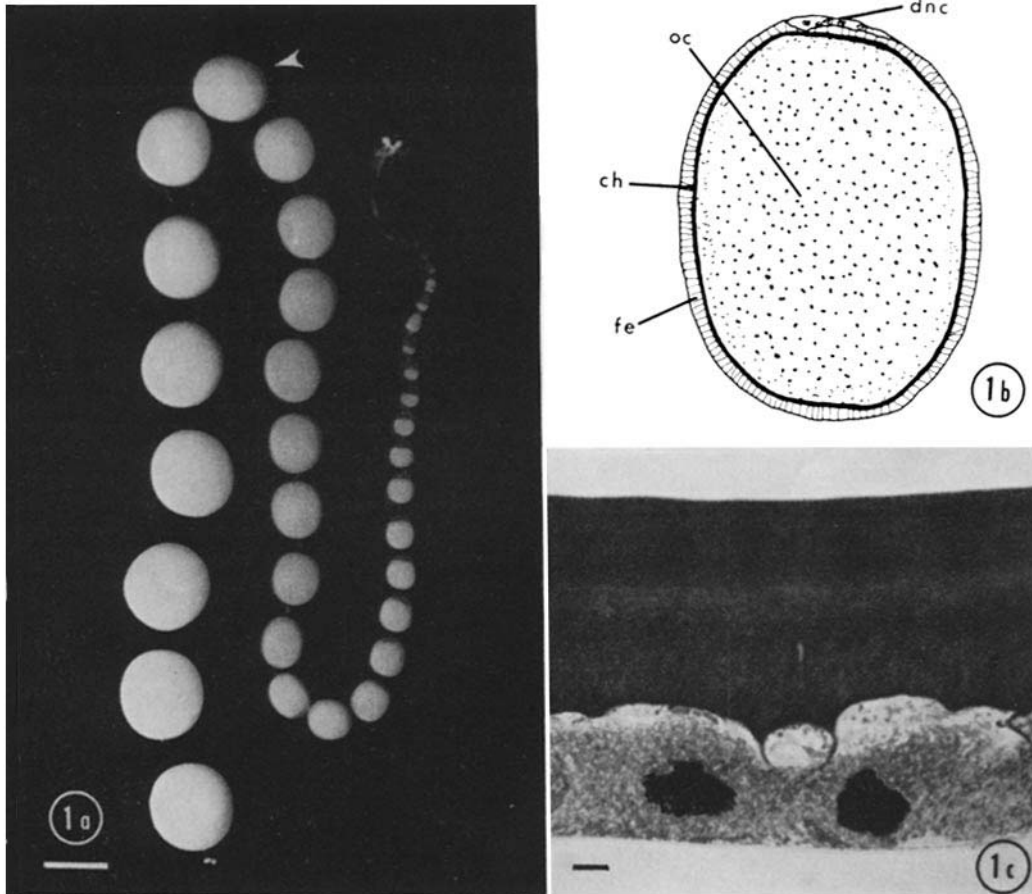
Figure 1 *a*   Ovariole dissected from a stage 15 developing adult female *A. polyphemus*. Arrowhead indicates follicle 1. Scale, 2 mm.

Figure 1 *b*   Diagram of an early chorionating follicle. *fe*, follicular epithelium; *ch*, chorion; *oc*, oocyte; *dnc*, degenerated nurse cells (after King and Aggarwal, 1965).

Figure 1 *c*   Photomicrograph of two follicle cells and their secreted chorion. A late chorionating follicle (*A. pernyi*) was fixed in ice-cold fixative (3% glutaraldehyde, 0.1 M potassium phosphate, pH 6.6), embedded in Araldite by standard procedures and sectioned at 1μ (Sjöstrand, 1967). Staining was with Mallory's azure II-methylene blue (Richardson et al., 1960). Note the substructure of the chorion, including the three horizontal zones. Scale, 10 μ.

We identify as follicle No. 1 the youngest pale follicle preceded by a deeply colored follicle substantially smaller in size. Progressively older follicles are then designated as Nos. 2, 3, 4, etc. and progressively younger ones as Nos. 0, −1, −2, etc. Vital staining with the yolk analog trypan blue (Anderson and Telfer, 1970 a) reveals that the

FIGURE 2 Dry weight, protein, and RNA content of the follicular epithelium (E) and chorion (C). Halved, washed follicles were blotted dry before being placed in individual tubes. Chorion (and the attached vitelline membrane) was separated from the follicular epithelium in 7% 1-propanol. Dry weight was determined after drying to constant weight in vacuum over P_2O_5. Total protein was determined by the biuret reaction after dissolving the sample in 1.0 M NaOH (Rutter, 1967), using dried chorion (which is about 96% protein; Kawasaki et al., 1971 a) as the standard. Values were approximately 35% higher when bovine serum albumin was used as the standard. Total RNA was estimated by a microadaptation of the method of Fleck and Munro (1962) using yeast tRNA as standard (Dawson et al., 1969). Values expressed are the mean for three follicles ±1 standard deviation.

oldest follicle staining deeply is −1; follicle 0 binds considerably less dye, indicating the end of the vitellogenic phase.

Generally, the youngest follicle bearing a visible chorion (dissectable from the epithelium after removal of the oocyte) is No. 3. As will be shown later, rapid chorion synthesis begins only slightly earlier in follicle 2. Thus, the transition from vitellogenesis to chorion production occurs within 8–10 hr.

Macromolecular Composition of Chorionating Follicles

Chorionating follicles cut in half and washed free of oocyte consist entirely of a homogeneous cell population, the follicular epithelium, and its secretory products, the chorion and the much thinner vitelline membrane (Fig. 1 c). Fig. 2 shows the dry weight, protein, and RNA content of this preparation. The mature chorion has a dry weight greater than the maximum of the epithelium itself (approximately 0.46 mg, range 0.42–0.50 mg); more than 95% of this is already deposited by follicle 10–12, i.e., in 2 days. During this time, no growth occurs in the epithelium itself. The total dry weight, protein, and RNA of the epithelium remain essentially constant for the first two-thirds of this period (approximately 370, 260, and 45 μg, respectively); thereafter, the cells regress until they are cast off at ovulation. No cell division occurs, since the cells are polyploid.
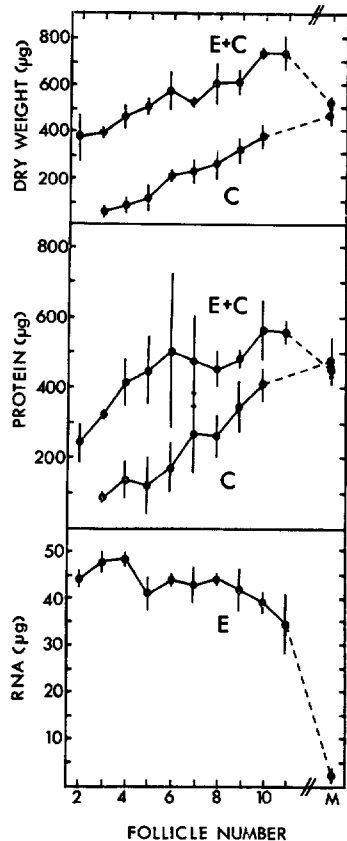
Solubilization of Chorion Proteins

A sulfhydryl reagent plus a strong denaturing agent are necessary for solubilization of chorion (Table I). High temperature and high pH favor dissolution. After this work was completed, similar methods for chorion solubilization were published by Kawasaki et al. (1971 a) and by Smith et al. (1971).

SDS-Polyacrylamide Gel Analysis of Chorion Proteins

When solubilized chorion proteins were analyzed by SDS-acrylamide gel electrophoresis, a highly reproducible pattern of bands was observed (Fig. 3). Nearly all the material migrated in the mol wt range 7000–18,000, forming four major and 11 minor bands. For easy reference, the proteins were divided into three classes (A, B, and C, in

TABLE I

Methods for Solubilization of A. polyphemus Chorion

Method	Reducing agent (2-mercapto-ethanol)	Denaturant	pH	Time (not necessarily the minimum time required)	Temperature
	%				°C
1	1	5–8 M guanidine HCl	8.5 (with NaOH or 0.01 M Tris-HCl)	1 hr 10 min	23 100
2	1	6–8 M urea	8.0–8.5 (with NaOH) 10 (with NaOH)	10 min 2 hr	100* 23
3	1	1% SDS	8.5 (with NaOH)	10 min	100*
4‡	1	1% SDS, 6 M urea	8.4 (0.01 M Tris-HCl)	overnight several hours 10 min	2 23 100
5§	2	2% SDS, 6 M urea	7.2 (0.02 M sodium phosphate)	45 min 10 min	60 100

* Dissolution is not complete after 2.5 hr at 60°C.
‡ SB II.
§ 2× SB I.
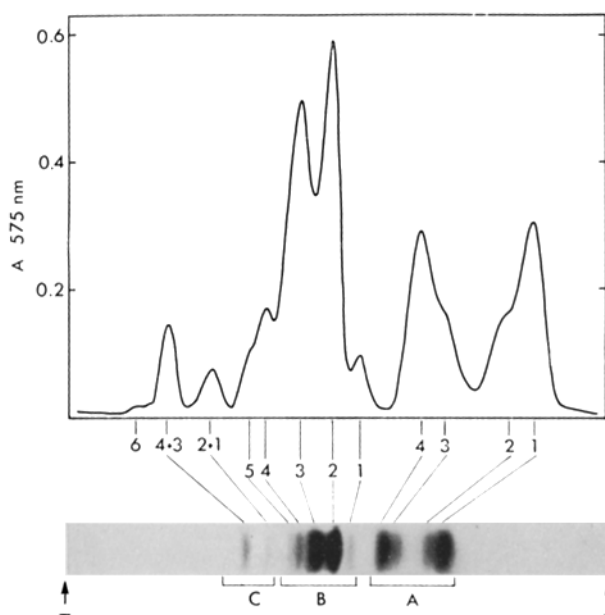
FIGURE 3 Electropherogram and densitometric scan of chorion proteins. The chorion from a nearly mature (No. 11) follicle was dissolved by heating at 100°C for 10 min in SB II and carboxamidomethylated. Electrophoresis was performed on a 9 cm gel by Method II. The photograph below shows the entire stained gel. The scan of the gel region containing all but the trace chorion components is displayed above on an expanded scale. The nomenclature of the bands is explained in the text. In this and all subsequent figures, migration is from left to right.

Apparent Molecular Weights of Chorion Proteins

Deter-mination	Chorion protein					
	A_1	A_4	B_2	B_3	C_{1+2}	C_{3+4}
I a	7,000–7,500	8,500–9,000	12,000	12,000	16,000–16,500	18,000–18,500
I b	6,500–7,500	7,500–9,000	10,000–10,500	10,000–10,500	15,500–16,000	17,000–18,000
II	7,500	9,500	13,000	14,000	18,000	20,000

Molecular weights of chorion components were interpolated from standard curves of log molecular weights *versus* relative mobility. Ranges indicate values obtained in two separate experiments. I a: Chorion proteins electrophoresed by Method I. Mature chorions were dissolved at 100°C (Table 1, Method 1), carboxamidomethylated, and dialyzed into 10 mM sodium phosphate containing 0.1% SDS and 0.5% 2-mercaptoethanol, pH 7.2. Aliquots containing $\frac{1}{20}$ of a chorion were mixed with an equal volume of 2X SB I before electrophoresis. Protein molecular weight standards were dissolved in 1X SB I at 23°C (insulin) or at 100°C for 10 min (chymotrypsinogen, myoglobin, cytochrome c, ferredoxin). Mobilities of the chorion proteins and standards were determined relative to the tracking dye on separate 10 cm gels. I b: Samples of chorion dissolved directly in SB I and analyzed by Method I as described above but without carboxamidomethylation. II: Carboxamidomethylated chorion proteins analyzed by electrophoresis Method II with the protein molecular weight standards and chorion samples mixed (six gels). Mobilities for the components of each gel were determined relative to the A_1 chorion component.

It should be noted that in this size range molecular weight estimates are imprecise, probably because of the effects of intrinsic charge and shape (Swank and Munkres, 1971).

order of increasing size). Additional trace components of higher molecular weights are present: a group of several components provisionally designated as class D (approximately 25,000–27,000 mol wt), plus a material not studied further, migrating at mol wt approximately 90,000. In mature chorion, class B (average mol wt 10,000–14,000; see Table II) predominates, with approximately 50% of the total protein; classes A and B together account for approximately 90% of the total. The four major proteins constitute approximately 75% of the chorion at maturity (B_2 and B_3 45%, A_1 15%, and A_4 15%). Quantitation with either coomassie brilliant blue or fast green (Gorovsky et al., 1970) gave essentially identical estimates.

The unusually low molecular weight of the proteins made it especially important to demonstrate that the banding profile does not result from partial proteolysis (Pringle, 1970). The banding pattern was identical when the protease inhibitor PMSF was present at all steps of sample preparation, from dissection onward. The same profile was obtained whether chorion was dissolved at 2°C (overnight), 23°C (several hours), or 100°C (10 min) in SB II.

In a further attempt to minimize proteolysis, immediately after dissolution chorion proteins were routinely reacted with iodoacetamide. Similar banding profiles were obtained with or without carboxamidomethylation, except for slight variations in relative mobility of some components (Table II). Carboxamidomethylation resulted in a sharpening of the bands, as did electrophoresis of unsubstituted proteins under reducing conditions, in gels freed of ammonium persulfate by brief pre-electrophoresis with mercaptoacetic acid.

Pulse-Label and Pulse-Chase Experiments with a Mixture of Methionine-^{3}H and Glycine-[14]C: Chorion Protein Heterogeneity, Absence of High Molecular Weight Precursors, and Kinetics of Chorion Deposition

Silkmoth chorion proteins are rich in glycine and poor in methionine (Kawasaki et al., 1969; 1971 a). The methionine/glycine ratio of electrophoretically separated proteins was used as a diagnostic tool in studies aimed at further characterization of chorion proteins, verification of their heterogeneity, evaluation of possible

precursor-product relationships, and exploration of the lag time between chorion synthesis and deposition. In general, the incorporation of two amino acids labeled with two different isotopes permits a judgment to be made about the *relative* amino acid composition of proteins even before their complete purification. In the ensuing discussion relative amino acid compositions are deduced on the assumption that leucine is equally abundant in all proteins; in fact it is comparable in chorion and in other ovarian proteins (Kawasaki et al., 1969).

Follicles were incubated in a medium containing a mixture of glycine-^{14}C and methionine-^{3}H. For one follicle, incorporation was stopped after 1 hr and the epithelium and chorion were immediately dissected apart and frozen. The other follicle was labeled for 3 hr and was then chased for 2 hr in unlabeled medium; at the end of the chase, the epithelium and the chorion were separated as before. Each of the resulting four samples was dissolved and analyzed by electrophoresis. At this stage, chorion production accounts for nearly all protein synthesis (Table III, column F: 99% of the glycine and 81–92% of the methionine incorporation). As a result, incorporation in chorion proteins could be evaluated reasonably accurately.

While glycine was extensively incorporated into both A and B proteins, high methionine incorporation was only detected in the B region (Figs. 4 *a* and

4 *b*). Thus, A proteins are either unusually rich in glycine or unusually poor in methionine or both. Differences in amino acid composition can be evaluated more directly from the ratio of incorporated amino acids (Figs. 4 *c* and 4 *d*). Clearly, compositional heterogeneity exists within classes B and C, as well as between classes. The chorion proteins are not multimers of a fundamental unit.

The ratio profiles in extracellular chorion are virtually identical for the pulse-label and pulse-chase experiments (data not shown). The ratio profiles are also similar for intracellular and extracellular chorion (Figs. 4 *c* and 4 *d*), despite the presence of small amounts of labeled nonsecretory protein in the intracellular (epithelial) samples.

It is worth noting that no major labeled peak with mol wt greater than 18,000 is present in the pulse-labeled epithelium (Fig. 4 *a*). In particular, no high molecular weight glycine-rich peak is seen, although it would be easily detectable by its characteristic isotope ratio. The lack of such a peak is a first indication that chorion proteins are not derived from a high molecular weight precursor. If such a precursor exists, its half-life must be very much shorter than 1 hr (also see below).

In the same experiment, separation of the labeled follicle into epithelium and chorion permitted a preliminary estimate of the time needed for chorion transport to the site of extracellular deposition. The average protein molecule requires

Table III

Synthesis of Chorion and Nonchorion Proteins, and Transport of Chorion

Experiment	Isotope	(Amino acid)	(A) Total incorporation	(B) Label in extracellular chorion	(C) Label in intracellular chorion	(D) Total incorporation in chorion	(E) Total incorporation in nonchorion	(F) Incorporation in chorion as % of total incorporation	(G) % of labeled chorion transported
			cpm	*cpm*	*cpm*	*cpm*	*cpm*		
1 hr pulse	$\lbrace\begin{array}{l}{}^{14}C\\ {}^{3}H\end{array}$	(Glycine) (Methionine)	120,000 92,300	57,800 39,700	60,600 45,400	118,000 85,100	1,600 7,200	99 92	$\left.\begin{array}{l}49\\47\end{array}\right\}$ 48
3 hr pulse 2 hr chase	$\lbrace\begin{array}{l}{}^{14}C\\ {}^{3}H\end{array}$	(Glycine) (Methionine)	318,000 247,000	294,000 178,000	21,700 22,600	316,000 200,000	2,300 46,400	99 81	$\left.\begin{array}{l}93\\89\end{array}\right\}$ 91

See text and Fig. 4 for experimental details. Follicular tissue was either pulse labeled or pulse chased with a mixture of glycine-^{14}C and methionine-^{3}H. Each tissue was separated into epithelium and transported extracellular chorion, and each of the four samples was analyzed by electrophoresis (see Fig. 4). A: sum of the total counts in the two gels (epithelium and chorion) for each tissue. B: total counts in each chorion gel. C: counts in the chorion region of the epithelium gel (slices 18–46 and 20–44 for the pulsed and pulsed-chased samples, respectively). Correction for overlapping nonchorion proteins was made by extrapolation of the incorporation on either side of the chorion region. D: B + C. E: A − D. F: D/A × 100. G: B/D × 100.
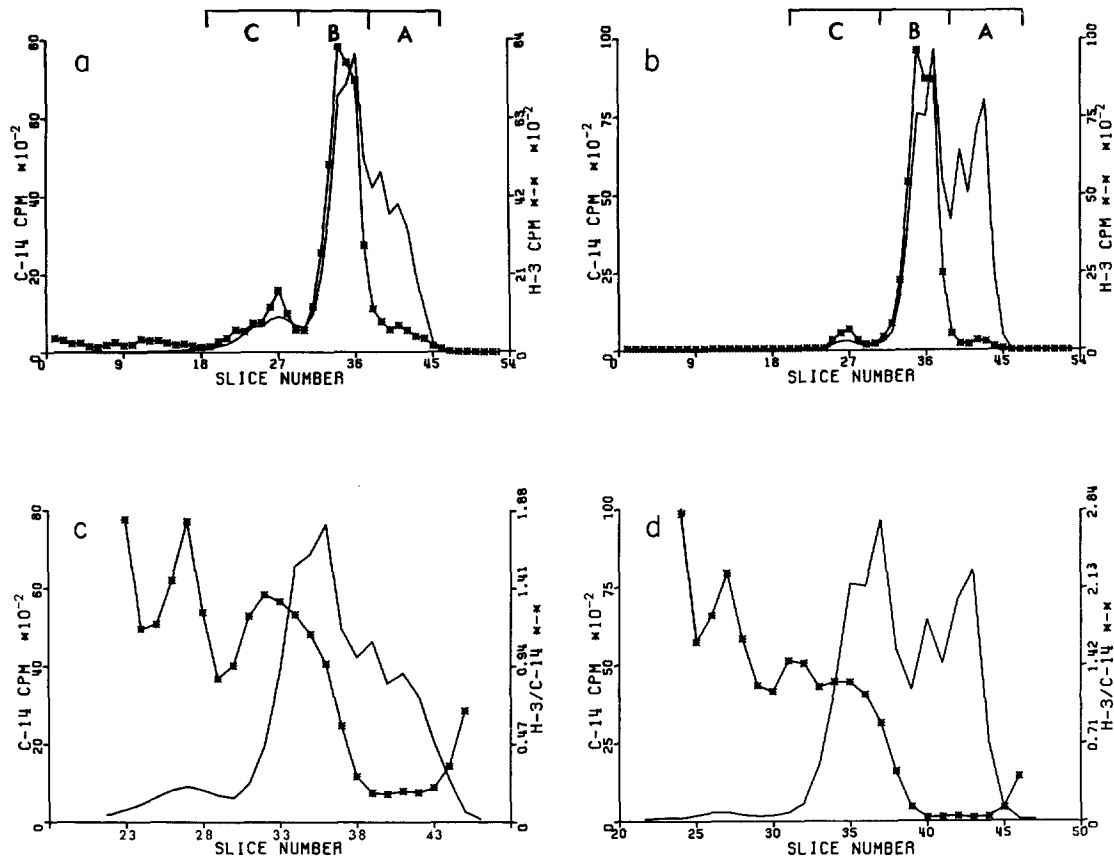
FIGURE 4 Incorporation of methionine and glycine in chorion proteins. Chorionating follicles (No. 5 or 6) were incubated in 0.6 ml GM containing glycine-^{14}C (uniformly labeled, 20 Ci/mole, 20 μCi/ml, Schwarz-Mann lot 6901) and methyl-methionine-^{3}H (2.5 Ci/mmole, 420 μCi/ml, Schwarz-Mann lot 2165). After 1 hr of labeling, one follicle was transferred to chilled MWS, cut in half, and washed. The follicular epithelium was separated from the chorion in 50 μl distilled water and the samples frozen separately. The other follicle was labeled for 3 hr and chased for 2 hr in GM, transferred to chilled MWS, and dissected as above. The samples were dissolved in SB I (containing 1 mM PMSF) by heating at 100°C for 10 min, and analyzed by Method I. *Upper:* ^{14}C (——) and ^{3}H (∗——∗) profiles of the pulsed epithelium (a) and pulse-chased extracellular chorion (b). Higher molecular weight proteins are on the left. The locations of the three protein classes A, B, and C are indicated. *Lower:* ^{14}C profiles (——) of the pulsed epithelium (c) and pulse-chased extracellular chorion (d) together with the ^{3}H/^{14}C ratios (∗——∗). Only the chorion region of each gel is shown (cf. a and c or b and d).

less than 1 hr for transport: of the chorion synthesized throughout the 1 hr labeling period, 48% is already extracellular by the end of the hour (Table III). Of the chorion synthesized during 3 hr, 91% is extracellular after two additional hr of chase. Rather similar transport kinetics are observed in other insect as well as vertebrate systems (Kafatos and Kiortsis, 1971). The nonsecretory, large, methionine-rich proteins are retained within the epithelium while chorion is being deposited (Table III; cf. columns C and E).

Further Evidence for Chorion Protein Heterogeneity

In a similar experiment, follicle 4 was labeled for 2 hr in a mixture of leucine-^{3}H and cysteine-^{14}C. The labeled follicle was exposed to a 30 min chase in unlabeled medium and then the chorion was freed of epithelium. Fig. 5 a shows the incorporation profiles of chorion proteins, and Fig. 5 b shows the profile of leucine plus the leucine/cysteine ratio for those gel slices with sufficient radioactivity.
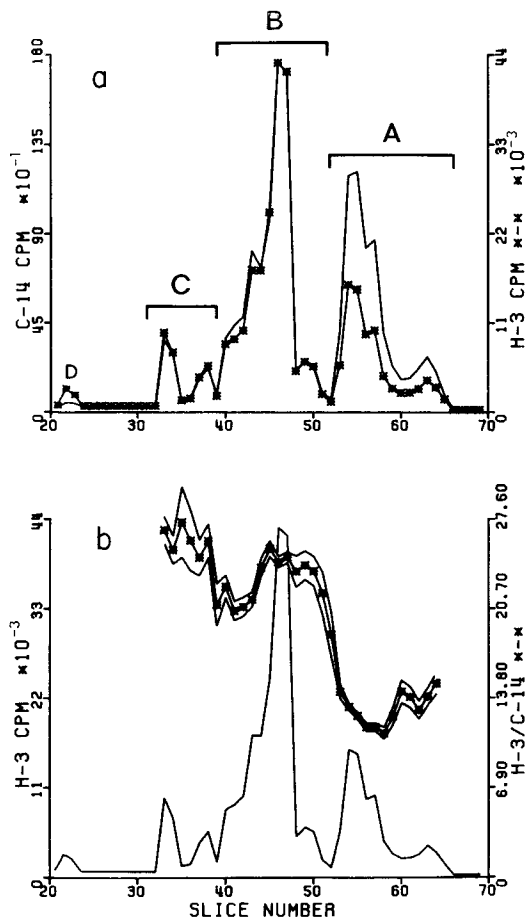
FIGURE 5 a Incorporation of cysteine and leucine in chorion proteins. A follicle 4 was labeled for 2 hr in 0.3 ml GM containing L-leucine-4,5-^{3}H (5 Ci/mmole, 133 μCi/ml, Schwarz-Mann lot WR 2568) and cysteine-^{14}C (uniformly labeled, 330 Ci/mole, 33 μCi/ml, New England Nuclear Corp. lot 605-185). Labeling was followed by a chase in unlabeled GM for 30 min to permit deposition of the labeled chorion. The follicle was halved and washed, and the chorion separated from the epithelium, dissolved in SB II, carboxamidomethylated, and analyzed by Method II. (——), cysteine-^{14}C; (*——*), leucine-^{3}H.

FIGURE 5 b The same experiment as Fig. 5 a. (——), leucine-^{3}H; (*——*), ^{3}H/^{14}C ratio, plotted with the 95% confidence interval for counting error.

In this run electrophoretic separation was adequate for resolution of 10 labeled components. All A proteins, and especially A_3 and A_4, showed a very low leucine/cysteine ratio. Proteins B_1, B_2, and C_1-C_4 showed a relatively high ratio, whereas B_3-B_5 had an intermediate value. Finally, D proteins were much poorer in cysteine than any other chorion component (Fig. 5 a).

Similar experiments were performed with glycine-^{14}C and either lysine-^{3}H or leucine-^{3}H. In an experiment with lysine and glycine, a lysine-rich component was detected within class C, apparently corresponding to $C_{1 + 2}$; incorporation of lysine was too low to permit meaningful comparisons among the other chorion proteins. However, the lysine/glycine ratio was uniformly high in nonchorion proteins, being two to three times higher than even in the lysine-rich chorion component.

In several experiments, the ratio of glycine to leucine was two to three times lower in the nonchorion as compared to the chorion proteins. The ratios for class A, B, and C proteins were similar, though slightly higher for A and particularly for A^1; the ratio of the class D components was twofold lower.

These experiments revealed that chorion proteins are distinct from each other in terms of amino acid composition. As compared with other follicular cell proteins, they are unusually rich in glycine and cysteine and poor in methionine and lysine. Class A proteins are the most distinctive, and D proteins are the most similar to nonchorion proteins.

Double-Label Experiments with Radioactive Glycine or Leucine: Identity of Newly Synthesized and Older Chorion Proteins

In the preceding experiments, the amino acid ratio profiles indicated both the heterogeneity of chorion and a close similarity of corresponding proteins before and after extracellular deposition (Fig. 4). However, because chorion and epithelium were analyzed on separate gels, it was impossible to assert unequivocally that secreted and newly synthesized proteins were identical in molecular weight.

A follicle 6 was cut in half. One-half was pulse labeled for 45 min in glycine-^{14}C, and its epithelium was immediately dissected free of deposited chorion. The other half was labeled in glycine-^{3}H for 1 hr, chased in cold medium for 3 hr, and its chorion was dissected free of epithelium. Pulse-labeled epithelium and pulse-chased chorion were mixed and analyzed on a single acrylamide gel. In the chorion region the profiles of the two isotopes were essentially identical (Fig. 6 a). Similar results
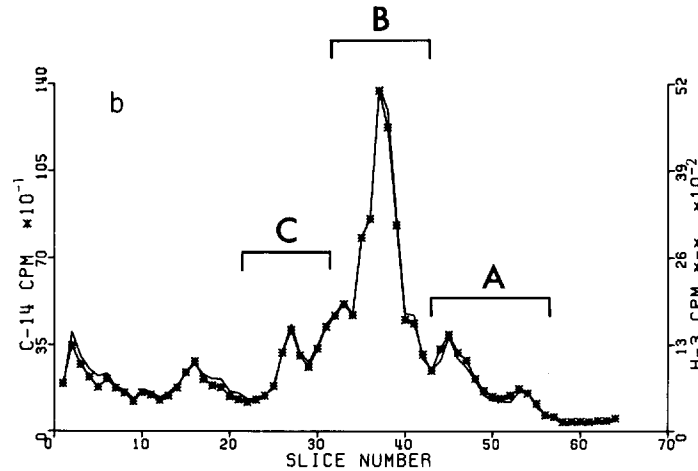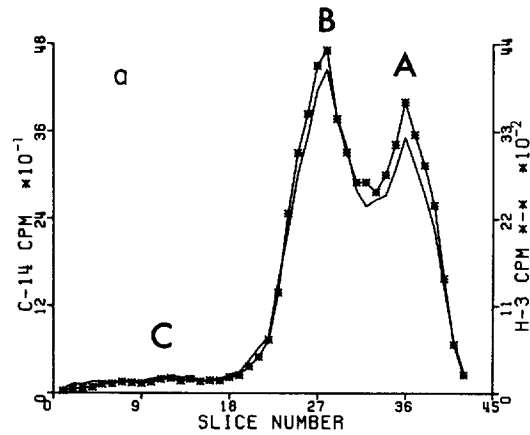
FIGURE 6 *a* ^{14}C pulse-labeled epithelium and ^{3}H pulse-chased extracellular chorion mixed and analyzed on a single gel. A follicle 6 was dissected into MWS, cut in half, and the oocyte washed away. One-half was incubated in 0.1 ml GM containing glycine-^{14}C (uniformly labeled, 36 Ci/mole, 20 μCi/ml, Schwarz-Mann lot 6901) for 45 min. At the end of the pulse the sample was chilled, and the epithelium separated from the chorion in 50 μl 7% 1-propanol containing 1 mM PMSF and frozen. The other half was incubated in 0.1 ml GM containing glycine-2-H^{3} (1.8 Ci/mmole, 1.0 mCi/ml, New England Nuclear Corp. lot 281-137) for 1 hr, and was then chased for 3 hr in normal GM. The chorion was separated and cleaned of cytoplasm in 7% 1-propanol containing 1 mM PMSF; it was then added to the frozen ^{14}C-labeled epithelium. The mixture was dissolved in SB I by heating at 100°C for 15 min, and was analyzed on a 10 cm gel by Method I. The region of the gel containing the chorion proteins was sliced and counted. *A*, *B*, and *C* indicate the respective classes; the individual proteins are not resolved in this experiment. (——), ^{14}C; (*——*), ^{3}H.

FIGURE 6 *b* ^{3}H pulse-labeled and ^{14}C pulse-chased follicular proteins. A follicle 4 was dissected into MWS, halved, and the oocyte washed away. Both halves were incubated in 0.2 ml GM containing leucine-^{14}C (uniformly labeled, 260 Ci/mole, 130 μCi/ml, New England Nuclear Corp. lot 605-025) for 12 min. They were then transferred into unlabeled GM with three washes, chased for 10 min, and incubated in 0.2 ml GM containing L-leucine-4,5-^{3}H (4.7 Ci/mmole, 3 mCi/ml, Schwarz-Mann lot WR 2568) for 10 min. Finally, the tissue was washed in ice-cold GM and frozen (within 1 min of pulse termination) in a dry ice-acetone bath. The sample was dissolved, carboxamidomethylated, and analyzed on an 8 cm gel by Method II. Brackets indicate the *A*, *B*, and *C* classes of chorion protein.

were obtained in a second experiment involving two follicles 6, from two ovarioles of the same animal. The results indicate that chorion proteins do not change molecular weight at the time of deposition, and that there is no interconversion between different chorion proteins during the 3 hr chase. Finally, this experiment reveals no major disparities in the kinetics of transport, such as prolonged retention of a particular protein class within the cytoplasm.

A second experiment was designed to detect any interconversions or other processing which might occur during the first 20 min after completion of a particular polypeptide chain. Follicle 4 tissue was incubated for 12 min in leucine-^{14}C. The cells were then chased for 10 min, labeled with leucine-^{3}H for a further 10 min, and frozen in a dry ice-acetone bath within 1 min from the end of ^{3}H-labeling. In this short pulse-label/pulse-chase experiment, ^{3}H represents newly synthesized proteins (produced, on the average, 5 min before freezing) and ^{14}C represents older proteins (produced, on the average, 26 min before freezing). When the proteins were displayed on an acrylamide gel, the two isotope profiles were virtually identical (Fig. 6 b). In particular, it is clear that no chorion protein accumulates (high ^{14}C/^{3}H ratio) at the expense of another (low ^{14}C/^{3}H ratio); in other words, no interconversion of chorion proteins occurs. Moreover, there is no indication of a high molecular weight precursor: such a precursor would have been depleted during the chase and would be identified as a ^{3}H peak with low ^{14}C/^{3}H ratio. Finally, the superimposable isotope profiles in the chorion region indicate that, after their synthesis, chorion proteins do not "mature" either by removal of a peptide fragment or by covalent attachment of an oligosaccharide moiety large enough to affect electrophoretic mobility. From previous experience (Berger and Kafatos, 1971) we estimate that this experiment would have easily detected a change in molecular weight of about 200 daltons (a shift of peaks by half a slice, with one slice in the chorion region corresponding to 300–500 daltons).

In sum, the experiments in this section and those described earlier indicate that, after synthesis, chorion proteins are packaged in the cytoplasm and deposited extracellularly without interconversion, cleavage from a high molecular weight precursor, or other major maturation step. Each

chorion protein can be studied independently with reasonable confidence that its biosynthesis reflects the expression of a discrete gene. Any maturation step would have to be completed in considerably less than 5 min.

Changes in Chorion Composition during Development

A developmental series of follicles (Nos. 3–11) was obtained from a single ovariole. The chorions were dissected, washed and dissolved, and aliquots containing comparable amounts of protein were analyzed on SDS-acrylamide gels. The stained gels (Fig. 7) revealed that the proportions of chorion proteins change dramatically with developmental stage. Early chorions are rich in C proteins, whereas A and B proteins become progressively more dominant. Detailed examination leads to the conclusion that each protein resolved by our methods has its own characteristic kinetics of accumulation. For example, component A_4 is dominant in early chorions while A_1 accumulates rapidly after follicle 7, and accumulation of B_3 lags behind that of B_2.

In the range of protein loads used in this experiment, there was a linear relationship between the amount of chorion protein loaded on the gel and the density of the coomassie brilliant blue-stained bands. Thus, it was possible to derive cumulative deposition curves by integrating the absorbance of appropriate bands in gels of progressively older chorions. Fig. 8 a shows the accumulation of class B and C proteins, as well as the accumulation of two A subclasses. The derivatives of these curves measure the $rates$ of accumulation (Fig. 8 b), which reflect the respective protein-synthetic rates (on the reasonable assumption that there is no differential turnover and no asynchrony in the transport of individual components; see Fig. 6 a). The results suggest that during follicular cell differentiation major changes in specific protein synthesis take place between the initiation and termination of chorion deposition. Synthesis of C proteins is maximal in follicle 3 and progressively declines thereafter to a vanishingly low level by follicle 7 or 8. By contrast, the synthesis of B proteins increases rapidly and then declines; the A proteins show similar kinetics, except that the curves are less steep and the synthetic peaks occur at different times.
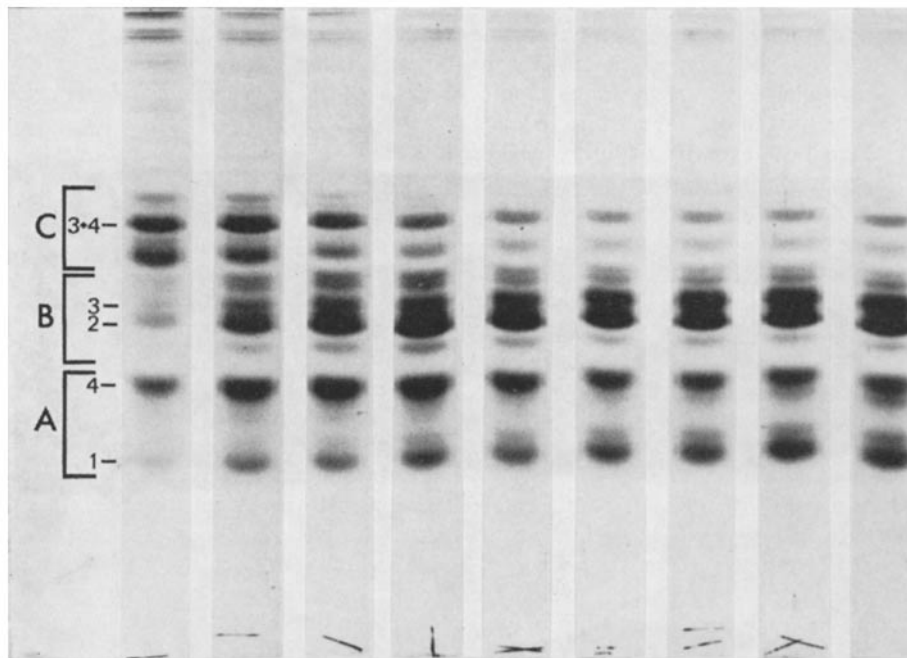
Figure 7   Electropherograms of chorions dissected from a developmental series of follicles (Nos. 3–11, from left to right). Follicle 3 has the first dissectable chorion. The origin is at the top; markings at the bottom are codes for identification of gels. Chorions were dissected from sequential follicles of a single ovariole in 7% 1-propanol containing 1 mM PMSF, dissolved in SB II by heating at 100°C for 5 min, carboxamidomethylated, and analyzed electrophoretically on 8 cm gels by Method II. The fraction of the total chorion loaded was $\frac{1}{2}$, $\frac{1}{3}$, $\frac{1}{6}$, $\frac{1}{10}$, $\frac{1}{15}$, $\frac{1}{20}$, $\frac{1}{20}$, $\frac{1}{20}$, and $\frac{1}{20}$ for Nos. 3–11, respectively.

Changes in Follicular Protein Synthesis during Development

As the kinetics of accumulation predicted (Fig. 8), major developmental changes in protein synthesis were detected when staged follicles were pulse labeled with radioactive amino acids in short-term organ culture. Fig. 9 shows typical results, from an experiment in which staged follicles were exposed to leucine-^{3}H for 2 hr. Since our electrophoretic conditions are designed to maximize resolution in the mol wt range 7000–25,000, the normally large proteins synthesized by pre-chorionating follicles penetrate only a short distance into the gel (Fig. 9, follicle 0); in such follicles, there is very little incorporation in the chorion region, and none of it can be assigned specifically to chorion components. By contrast, in follicle 2 the labeling of nonspecific proteins has declined considerably, and labeling of chorion proteins, especially C, has become prominent. The trend toward reduction of nonspecific protein synthesis and accentuation of chorion synthesis continues in subsequent stages.

In this experiment, the electrophoretic resolution was sufficient to display unambiguously 10 chorion peaks (Fig. 9, follicle 3). Careful attention to the incorporation profiles reveals that every single chorion peak shows distinct developmental kinetics. Moreover, these kinetics are in very close agreement with those expected from the analysis of accumulated proteins in a developmental series of follicles (Fig. 7). C proteins, and especially $C_{1 + 2}$ and $C_{5 + 6}$, are the earliest synthesized. Production of D proteins occurs somewhat later. Of the two major B proteins, B_2 is dominant in early incorporation profiles; B_3 becomes evident as a prominent shoulder by follicle 6, and is the dominant component synthesized in follicle 10. Finally, component $A_{1 + 2}$ is synthesized later than component $A_{3 + 4}$.

These incorporation kinetics are highly reproducible. They have been observed in three separate experiments involving labeling and analysis of a
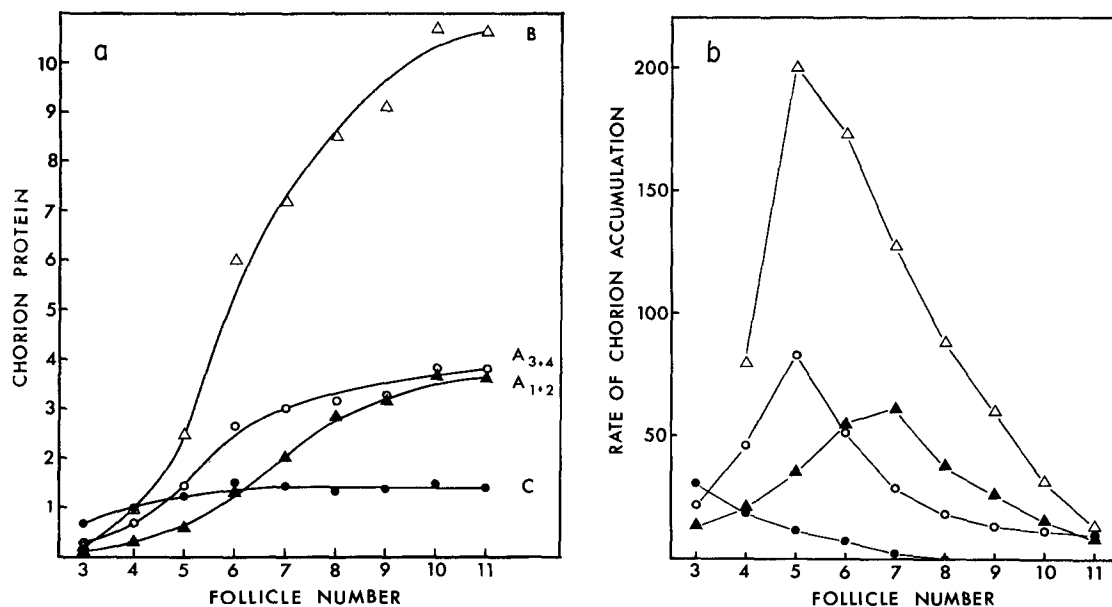
Figure 8 Accumulation of chorion proteins (a), and rates of accumulation (b) during follicular development. To determine the cumulative amount of protein (a), the stained gels of a developmental series of chorions (Fig. 7) were scanned, and groups of peaks were cut out, weighed, and corrected for the fraction of chorion used. Rates of accumulation (b) were determined by measuring the slope of the accumulation curves. The scale of each ordinate is in arbitrary units, but the same for all protein classes.

complete developmental series of follicles. They have been observed after labeling with either leucine or glycine; in fact, because of the amino acid composition of chorion, glycine profiles are even more dramatic.

Figure 10 a emphasizes the over-all switch to chorion synthesis. In a brief span of 4–10 hr, the follicular cells shift 60–70% of their protein synthesis to chorion production. In 16–20 hr (from follicles 0 to 4), the shift is complete, from undetectable to approximately 90% chorion synthesis.

Figure 10 b summarizes the relative kinetics of synthesis for specific chorion proteins, as determined from incorporation experiments. Since the specific activity of the intracellular labeled amino acid was not measured, it is not possible to express the data in terms of absolute rates. Very similar relative kinetics can be derived from Fig. 8 b. Thus, the same precise developmental program of specific chorion protein synthesis can be documented both by in vivo accumulation and by in vitro labeling.

Proteins of the Vitelline Membrane

The vitelline membrane is an extracellular layer bordering the oocyte and secreted before chorion

deposition (Telfer and Anderson, 1968; Telfer and Smith, 1970). Electrophoretic analysis of vitelline membrane dissected from a follicle 3 revealed three major components approximately 55,000, 70,000, and 100,000 mol wt, all substantially larger than the chorion proteins.

Changes in Follicular
Polysomes during Development

Paralleling the change in synthetic activity from large cellular proteins to small chorion components, there is a shift in the size distribution of polysomes. Prechorionating cells have a polydisperse polysome profile; most abundant are moderately large polysomes (7–12 ribosomes, the largest size resolved by our conditions). By contrast, chorionating follicles have almost exclusively small polysomes, especially tri- to heptamers, and show a sharp cutoff above the heptamer region.

The unusually small polysomes of older follicles do not result from selective breakdown during sample manipulations. Prechorionating (−5 to 0) and late-chorionating (4 to 8) follicles were labeled separately with leucine-^{3}H and leucine-^{14}C, respectively, for 5 min. The tissues were then mixed,
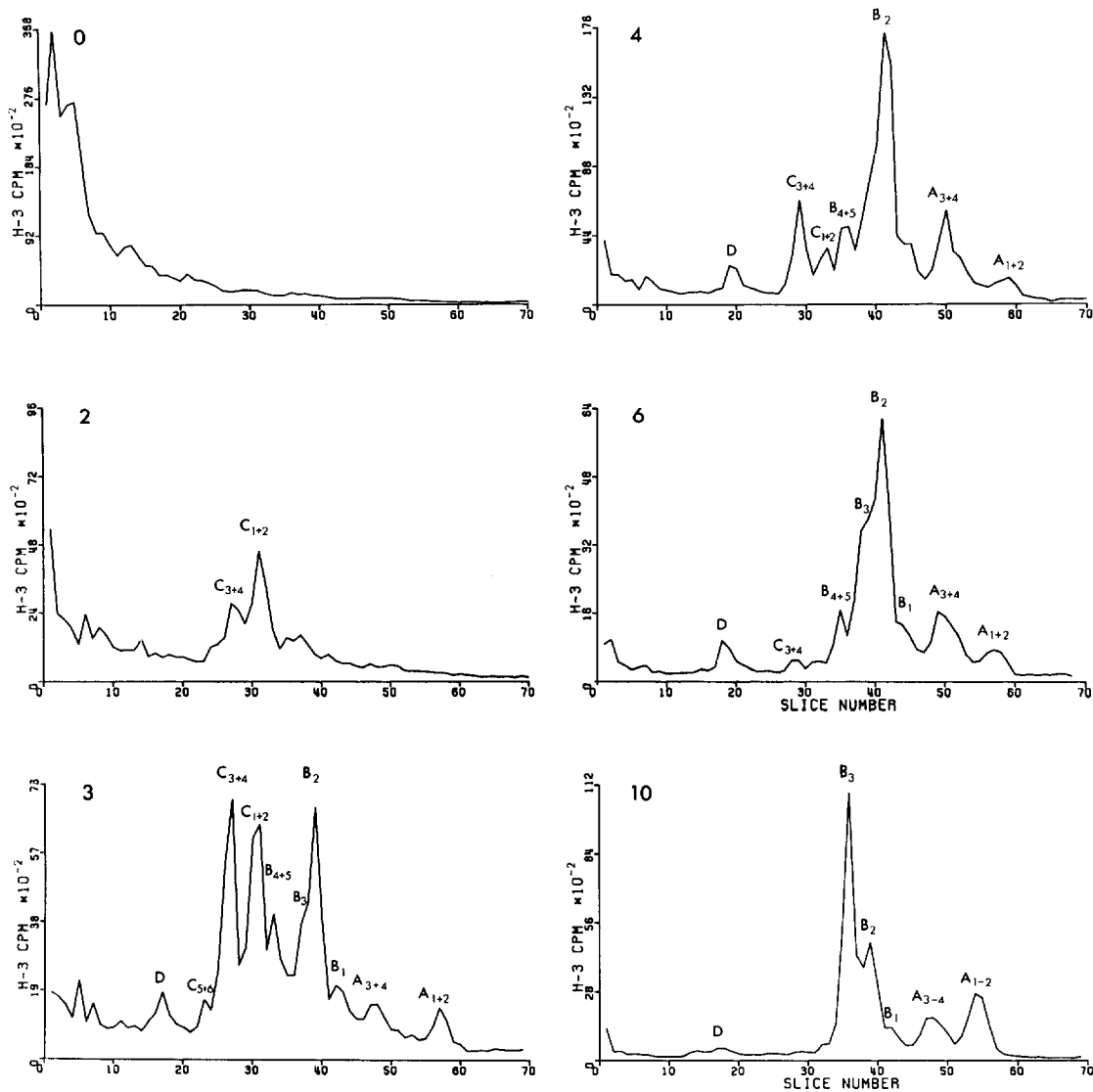
FIGURE 9 The proteins synthesized by follicular cells in short-term organ culture. A complete developmental series of follicles (Nos. −2 through 10) was incubated in 0.4 ml GM containing L-leucine-4,5-^{3}H (5.4 Ci/mmole, 188 μCi/ml, Schwarz-Mann lot WR 2568) and glycine-^{14}C (uniformly labeled, 100 Ci/mole, 50 μCi/ml, Schwarz-Mann lot VR 2216) for 2 hr. The follicles were chilled, cut in half, and the oocyte washed away. Each sample (epithelium plus chorion, if present) was dissolved in SB II, carboxamidomethylated, and analyzed on an 8 cm gel by Method II. Only representative leucine-^{3}H profiles are shown, but the glycine-^{14}C profiles were similar. The number at upper left indicates the follicle number. The major labeled chorion protein peaks are identified. The bulk of the nonchorion protein in each sample, as seen in the stained gels (not shown here) is between slices 1 and 30, although this region accounts for little of the incorporation in chorionating follicles.

homogenized together, and displayed on a sucrose gradient (Fig. 11). Thus, all polysomes were exposed to identical conditions from the time of cell rupture onward. Large aggregates predominated in prechorionating epithelium; the ^{3}H/^{14}C ratio revealed that chorionating follicles have an excess of dimers through hexamers (median size, four ribosomes). Samples that include both types of
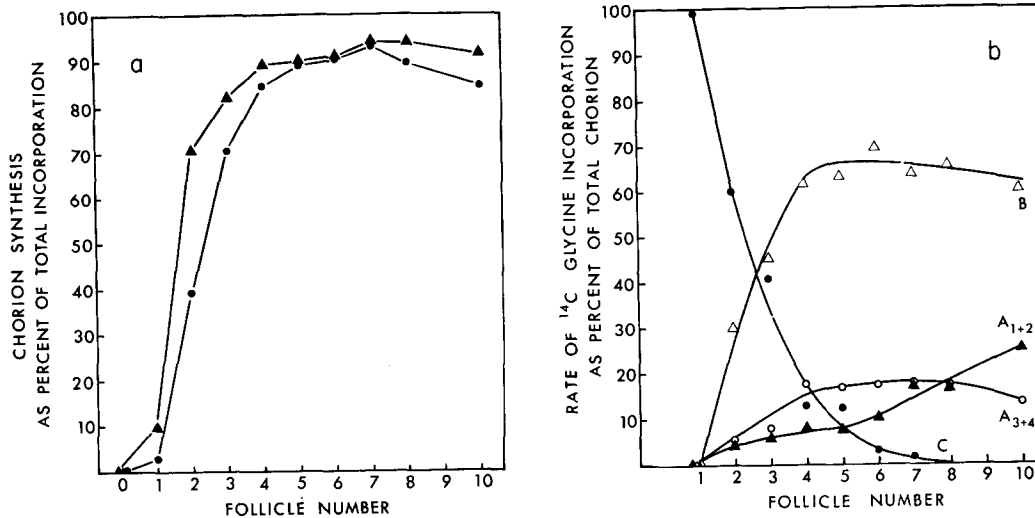
FIGURE 10 a Relative rate of total chorion protein synthesis, as revealed by amino acid incorporation in chorion relative to incorporation in the total protein during a standard labeling period (for details of the experiment see Fig. 9). The amount of incorporation in each stage was determined by weighing cutouts of the total incorporation profile and of all the chorion peaks (after correcting by extrapolation for the small amount of overlapping nonchorionr poteins). (▲——▲), glycine-^{14}C; (●——●), leucine-^{3}H. The difference between the curves is a result of the higher glycine content of chorion as compared to nonspecific proteins.

FIGURE 10 b Relative rate of synthesis for particular chorion proteins, as revealed by incorporation of glycine-^{14}C in specific classes relative to total incorporation in chorion during a standard labeling period. Incorporation was determined as above, by weighing cutouts of the appropriate labeled peaks (see Fig. 9).

tissue (follicles 1–6, Fig. 13) contain both types of polysomes.

An even better correlation between nascent protein size and polysome size was revealed from study of more closely staged follicles (Fig. 12). As expected from earlier results (Figs. 9, 10 b), polysomes from follicle 2 (which synthesizes predominantly C proteins) included major peaks in a size range (seven to nine ribosomes in this experiment) just higher than the cutoff of polysomes from follicles 5 and 6 (which synthesize almost exclusively A and B proteins). Again as expected (see Fig. 9), follicle 2 also had larger polysomes. The contrast between the two types of tissue is emphasized by the ratio profile (bottom ratio panel, Fig. 12). Apparently, A and B proteins are synthesized predominantly in three- to five-member polysomes. As expected (see Fig. 9), polysomes of follicles 3 or 4 were not substantially different from those of follicles 5 and 6 (Fig. 12, middle and top ratio panels).

The high level of monosomes (40–60% of the

total ribosomes) apparently reflects in vivo conditions (see Bosches, 1970): several criteria indicate that monosomes do not result from polysome breakdown during isolation. First, after brief labeling with amino acids, both the level of incorporation and the specific activity in the monosome peak are very low (Fig. 13). Further, in common with native monosomes and in contrast to particles engaged in protein synthesis, 80% of follicular monosomes are dissociated by 0.5 M KCl (Zylber and Penman, 1970); by contrast, addition of 2 μg/ml of RNase converts follicular polysomes quantitatively to salt-resistant monosomes. Finally, endogenous nuclease activity in crude follicular homogenates is exceptionally low. Incubation of high specific activity transfer RNA (tRNA)-^{32}P from Staphylococcus epidemidis (10^5 cpm, less than 1 μg) with the cytoplasmic fraction of follicle homogenate (60 follicles/ml) for 20 min at 37°C converts less than 1% of the tRNA to oligonucleotides smaller than eight residues long. Under identical conditions, more than 95% of the
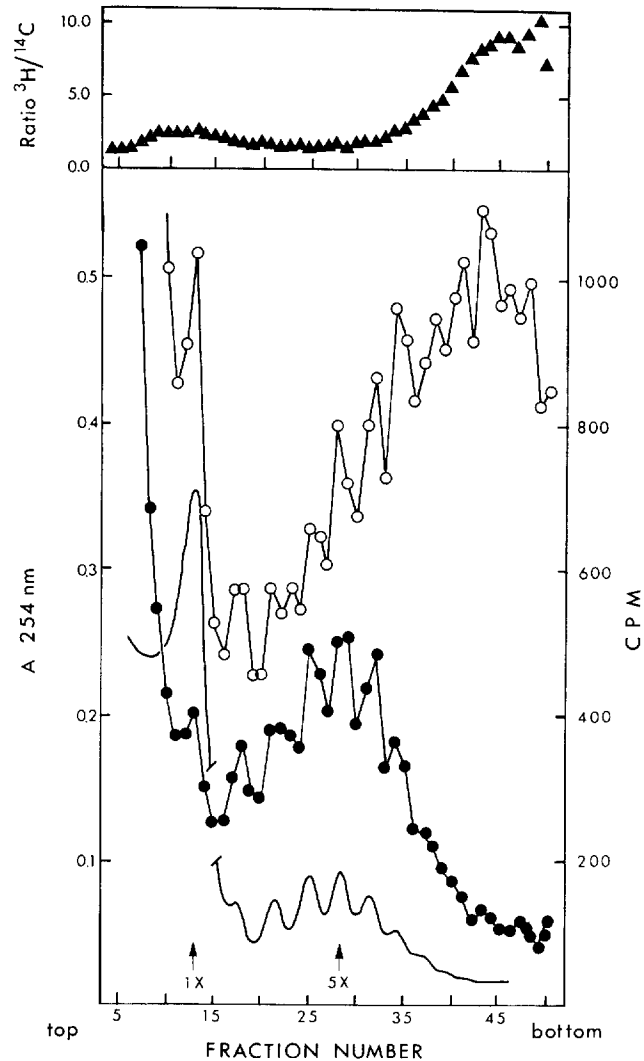
Figure 11 Polysomes from prechorionating and chorionating follicles. Cells from follicles −5 to 0 (from a single ovariole) were incubated for 5 min in GM containing L-leucine-4,5-^{3}H (50 Ci/mmole, 200 μCi/ml, Schwarz-Mann lot WR 2568); cells from follicles 4–8 were incubated for 5 min in GM containing leucine-^{14}C (uniformly labeled, 312 Ci/mole, 50 μCi/ml, Schwarz-Mann lot WR 2168). Homogenization and sedimentation were carried out in standard buffer with the KCl raised to 0.25 M. Linear sucrose gradients (20–40% w/w) were centrifuged at 39,000 rpm for 90 min. All solutions were treated with bentonite. (o——o), ^{3}H-counts per minute (polysomes of prechorionating follicles); (●——●), ^{14}C-counts per minute (polysomes from chorionating follicles); (———), absorbance. The profile is dominated by the more abundant polysomes of chorionating cells. Maximum absorbance of the monosome peak is 1.2. Arrows indicate the position of the monosomes (1 X) and pentasome (5 X). (▲▲▲), ^{3}H/^{14}C ratio, representing for each fraction the relative abundance of polypeptide chains from prechorionating and chorionating cells.

labeled tRNA is degraded by *S. epidemidis* lysates.[2] Low RNase activity is also indicated by the pro-

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[2] We are indebted to R. Roberts and R. Gelinas for this determination.

longed retention of protein synthetic capacity by follicular cell extracts stored at 0°C (see below). Indeed, we routinely use follicular cell homogenates to preserve labile polysomes from other insect tissues, in a manner analogous to the use of
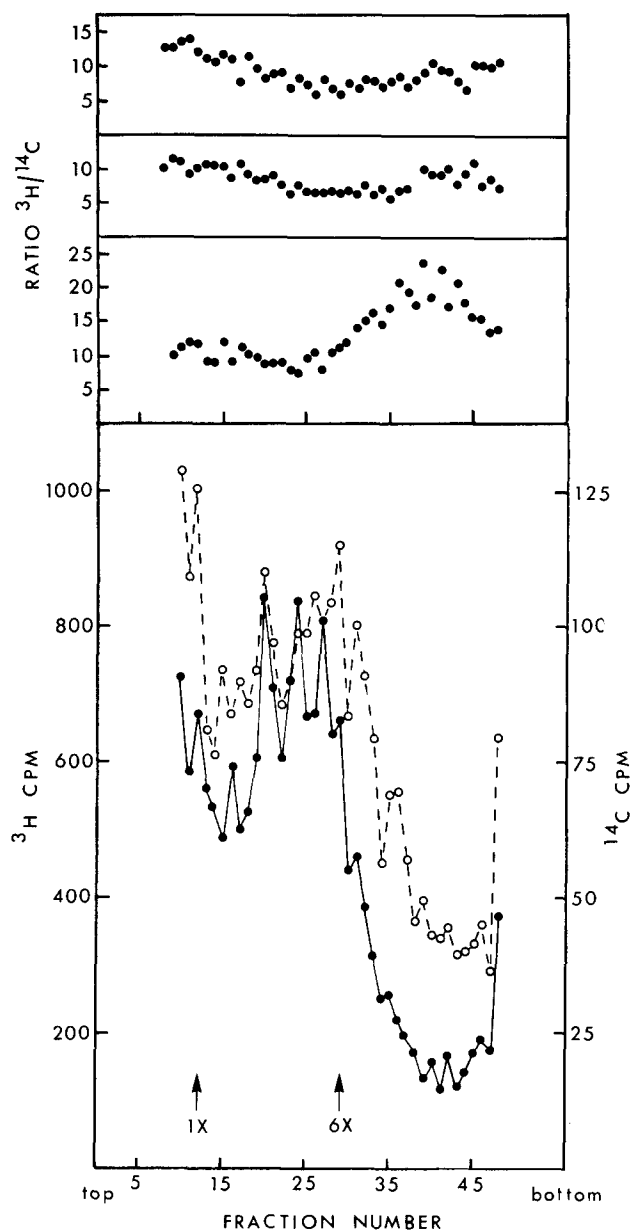
FIGURE 12   Stage-specific polysomes. Groups of follicles (5 each for Nos. 2, 3, or 4, from a single animal) were labeled for 9.5, 10, and 10.5 min, respectively, in GM containing L-leucine-4,5-^{3}H (50 Ci/mmole, 100 μCi/ml, Schwarz-Mann lot WR 2568). Follicles 5 and 6 (five each) were incubated together for 11 min in leucine-^{14}C (uniformly labeled 259 Ci/mole, 20 μCi/ml, New England Nuclear Corp. lot 605-025). After homogenization, 0.2 ml aliquots of the follicle 5 plus 6 homogenate were added to each of the other three samples before layering on gradients. Sedimentation was carried out on 20–40% (w/w) linear sucrose gradients in standard buffer at 39,000 rpm for 90 min. All solutions were treated with bentonite. (O——O), polysomes from follicle 2; (●——●), polysomes from follicles 5 plus 6. Arrows indicate the position of monosomes (1 X) and hexasomes (6 X). (● ● ●), ^{3}H/^{14}C ratios; bottom ratio panel, polysomes from follicle 2 *versus* 5 plus 6; middle, follicle 3 *versus* 5 plus 6; top, follicle 4 *versus* 5 plus 6.
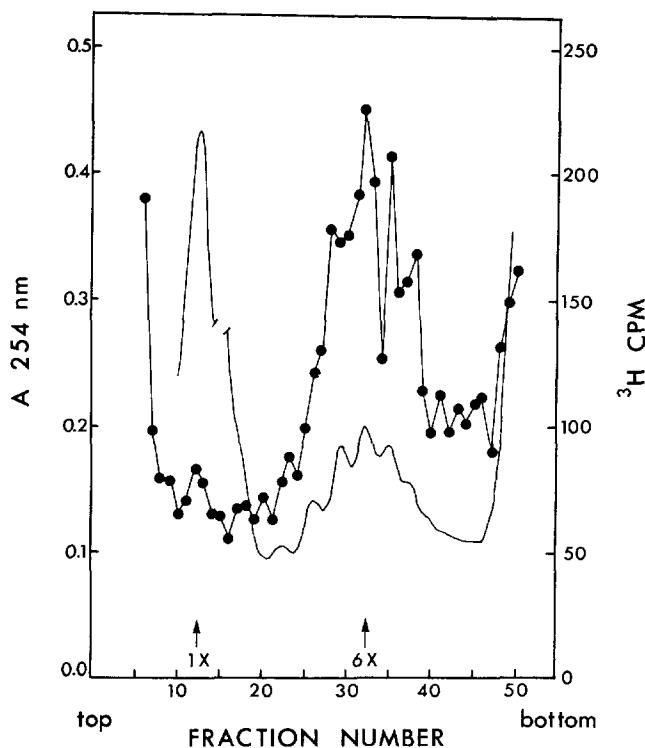
Figure 13   Polysomes isolated from follicular epithelium. Tissue from follicles 1–6, taken from a single animal, was labeled for 2 min in GM containing glycine-2-^{3}H (4.3 Ci/mmole, 200 μCi/ml, Schwarz-Mann lot 281-137). Centrifugation was carried out on linear 20–35% (w/v) sucrose gradients in standard buffer at 39,000 rpm for 2 hr. Arrows indicate the position of monosomes (1 X) and hexasomes (6 X). Absorbance maximum of the monosome peak was 1.6. (——), absorbance; (●——●), radioactivity.

rat liver supernates (Roth, 1956). In summary, mechanical or enzymatic degradation of polysomes seems minimal in this tissue.

It also appears that the polysome profiles do not result from disassembly through continuing chain elongation and termination without reinitiation. Nearly identical absorbance profiles are obtained, whether rapidly chilled cells are disrupted immediately or after 30 min in hypotonic homogenization buffer at 0°C. Moreover, the polysome absorbance profile is stable when protein synthesis is abruptly stopped at 0°C, even if homogenates are subsequently incubated at 18°C for up to 30 min. "Runoff" is only observed if the initial chilling in homogenization buffer is insufficiently rapid. It appears that the profiles obtained by our standard conditions are a reasonable reflection of the polysome size distribution in vivo.

Finally, it seems clear that the polysome-associated radioactivity after brief labeling with radioactive amino acids represents nascent poly-

peptides, rather than completed and secondarily adsorbed chains. The distribution of label is virtually identical, whether polysomes are prepared with the standard low ionic strength buffer or in the presence of 0.25–0.5 M KCl, which should reduce nonspecific protein binding (Warner and Pene, 1966).

Changes in Translation Time during Follicular Cell Development

The average translation time for proteins synthesized by prechorionating and chorionating follicles was determined by a modification of the method of Fan and Penman (1970). Since nascent peptide chains can be assumed to be half finished, on the average (Hunt et al., 1968), once the precursor pool specific activity has stabilized, the time required for one set of labeled nascent chains to be released is equal to one-half the average translation time, $t_{1/2}$. Operationally, this time is

determined from the progressive increase of TCA-precipitable amino acid label in the total cell extract and, separately, in released chains. The half-translation time is the time required for all the radioactivity initially found in the total sample (polysome bound plus released) to become associated with released chains alone.

Follicular epithelium was prepared from prechorionating (−5 to 0) and chorionating (4 to 8) follicles. Each type of tissue was labeled separately at 23.5°C for various times (5–25 min), and was then homogenized. Follicles from a single ovariole were used for each time point. Nascent polypeptides were visualized directly by displaying the tissue homogenates on sucrose gradients in 0.25 M KCl (to minimize nonspecific binding of completed chains to ribosomes). The radioactivity sedimenting slower than monosomes was attributed to released chains. The prechorionating epithelium was labeled in leucine-^{3}H and the chorionating epithelium in leucine-^{14}C, and tissues labeled for corresponding periods of time were mixed before homogenization. This reduced the total number of samples processed and made the two tissues more comparable. To reduce the scatter from variable amounts of tissue in each aliquot, total or released radioactivity was normalized by dividing by the ratio of total radioactivity to time of labeling. The normalized data (now expressed in time units) were plotted against the time of labeling (Fig. 14). Because of the normalization, the data for total counts gave a straight line through the origin, at an angle of 45°. Best fit lines were then calculated by regression analysis for the released chain data. For both types of tissue the best fit line had a slope in good agreement with the expected value of 1 (0.998 and 0.991 for chorionating and prechorionating samples, respectively). The horizontal distance between the best fit line and the 45° line gave the value for the half-translation time, 1.0 and 2.3 min for chorionating and prechorionating tissues, respectively.[3]

The calculated translation times were consistent with the hypothesis that the average time needed to add a single amino acid to a growing polypeptide chain is similar for chorion and for cellular proteins. The rate of peptide chain elongation is the number of amino acids in a protein divided by

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[3] This difference in translation times causes a low ^{3}H/^{14}C ratio near the top of the gradient in Fig. 11.
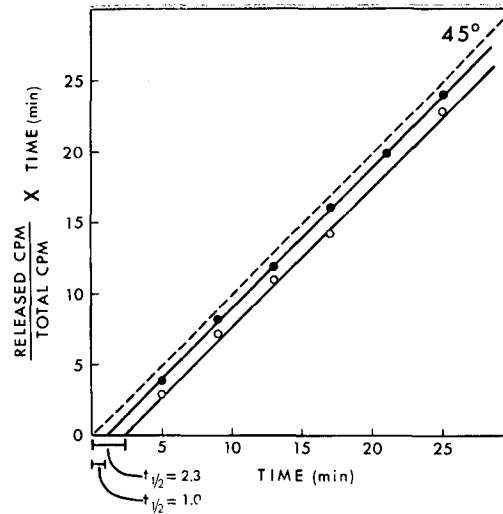

FIGURE 14 Average translation time for proteins in prechorionating (Nos. −5 to 0) and chorionating (Nos. 4–8) follicles. Follicles were selected, incubated in radioactive GM for the indicated time, and homogenates displayed on sucrose gradients as described in Fig. 11; prechorionating (^{3}H, o——o) and chorionating (^{14}C, ●——●) samples for each time point were displayed on the same gradient. All but the 5 min gradient (shown in Fig. 11) were collected from the bottom. The first 10 fractions were designated as released chains, and correction for the additional chains overlapping with monosomes was made by adding to the released chain total the radioactivity below the line extrapolated from the top of the gradient through the monosome region (fractions 12–14 in Fig. 11). Data for each time point were normalized by dividing by the corresponding ratio (total counts per minute)/(duration of labeling). Best fit lines were determined by regression analysis.

the translation time. Aliquots of total homogenate from the 25 min sample were dissolved and electrophoresed on SDS-acrylamide gels together with protein molecular weight standards. The median molecular weight of the labeled proteins was determined, as the weight above and below which there were equal amounts of radioactivity in the gel.[4] The values for prechorionating and chorionating tissue were 58,000 ± 4000 and 13,800 ± 1000, respectively (Fig. 15). The molecular weight of the average amino acid residue in the nonspecific

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[4] This is the correct formula for the average molecular weight to be used in conjunction with the average translation time as calculated by the method of Fan and Penman (1970).
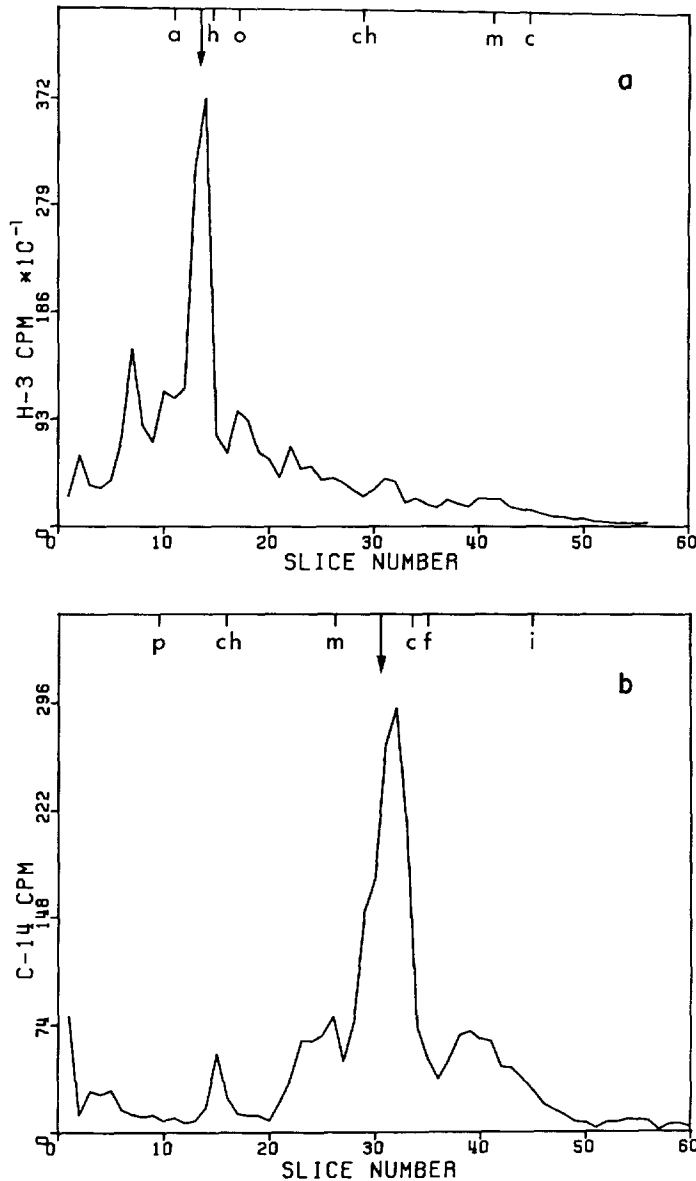
Figure 15   Electrophoretic analysis of released plus nascent chains from pulse-labeled chorionating (lower) and prechorionating (upper) follicular cells. Aliquots of the 25 min homogenate (Fig. 14) were dissolved in SB II by heating at 100°C for 10 min. Samples were analyzed either by electrophoresis Method II (b) or on standard 10% acrylamide gels (a; Weber and Osborn, 1969) for optimal resolution of chorionating and prechorionating proteins, respectively. Gels were processed and radioactivity counted by Method II. Proteins of known molecular weight were run both in parallel and as internal standards in the same gels as the samples. Arrows point to the average molecular weight of the labeled proteins (58,000 in a and 13,800 in b); a, bovine serum albumin; h, heavy γ-immunoglobulin chain; o, ovalbumin; p, pepsin; ch, chymotrypsinogen; m, myoglobin; c, cytochrome c; f, ferredoxin; i, insulin.

proteins is 110 (Kawasaki et al., 1969; see also Lehninger, 1970) and in chorion proteins is 93 (Kawasaki et al., 1969, 1971 a). Thus, the average size is 530 amino acids per protein for the non-chorionating and 150 for the chorionating cells. These values, divided by the respective translation times, yielded values for the rate of peptide chain elongation, 1.9 amino acids/sec and 1.3

amino acids/sec for prechorionating and chorionating tissue, respectively.

Cell-Free Follicular Protein Synthesis

The cytoplasmic fraction (5000 g supernate [S-5]) of homogenized follicular cells is highly active in cell-free protein synthesis when supplemented with an ATP-generating source, appropriate cofactors, and amino acids. Incorporation of labeled amino acids into TCA-precipitable material is nearly linear for 20 min (Fig. 16). The crude cell-free system incorporates at 16–23% of the rate of whole cells incubated in the same concentration of isotope (Table IV [A]).

The crude cytoplasmic fraction (S-5) was further fractionated into a microsomal pellet and a postmicrosomal 105,000 g supernate (S-105). By itself the S-105 fraction was nearly devoid of incorporating ability, but the microsomal fraction was significantly active, presumably because it contained low levels of the supernate factors; when the two subfractions were recombined, the reconstituted system had an incorporating ability
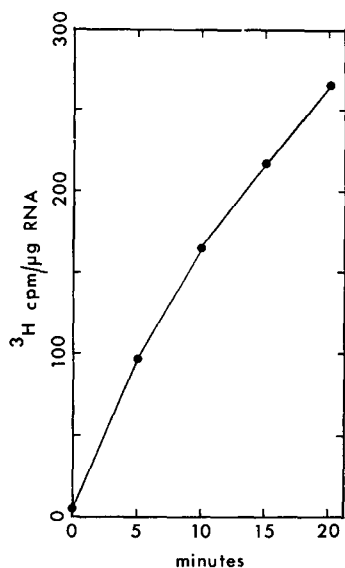

Figure 16   Incorporation of leucine-^{3}H by a reconstituted cell-free system. Microsomal and postmicrosomal supernate (S-105) fractions stored at −90°C (Table IV, experiment B) were combined and incubated at 25°C in the cell-free reaction mixture containing L-leucine-4,5-^{3}H (55.2 Ci/mmole, 400 μCi/ml, New England Nuclear Corp. lot 577-027). Aliquots were withdrawn at the indicated times and radioactivity determined as described in Methods. Similar kinetics were shown by the S-5 cell-free system.

fourfold higher than that of the microsomal pellet alone (Table IV [B]). On the average, the reconstituted system showed one-fourth the incorporating ability of the S-5 crude cytoplasmic fraction (Table IV [C]). Both the S-5 preparation and its subfractions were active after storage at −90°C for as long as 1 yr. In addition, the S-5 fraction retained at least 50% of its activity after storage at 0°C for 24 hr, indicating that ribonuclease activity is unusually low in these cells. Both the crude and the reconstituted systems displayed a sharp pH optimum at 7.5 to 8.0, and optimal K^+ and Mg^{2+} concentrations of 30 mM and 10 mM, respectively.

Fig. 17 demonstrates that both chorion and nonchorion proteins are synthesized by the cell-free system. Just as in whole-cell experiments, determination of chorion synthesis in a cell-free preparation is made possible by the unusually low molecular weight and characteristic amino acid composition (low lysine-^{3}H/glycine-^{14}C ratio) of chorion.

DISCUSSION

The Proteins of Chorion

Morphologically, the chorion of insects is a distinctive structure (Smith et al., 1971); we now know that it is equally distinctive biochemically. In *A. polyphemus*, as in other related moths (Paul et al., 1972), it consists of a small number of proteins of unusually low molecular weight.

The moth chorion is almost exclusively proteinaceous (96% according to Kawasaki et al., 1971 *a*; see also Tichomiroff, 1885; Webster, 1910; McFarlane, 1962). On the basis of solubility characteristics, Kawasaki and his coworkers (1971 *a*) distinguished one major and one or two minor chorion proteins, depending on the moth species. In the present study, we detected four predominant proteins (three in other saturniids and in *Bombyx mori*; Paul et al., 1972), 11 minor components, and a number of trace constituents. Preliminary isoelectric focusing experiments indicated only limited additional heterogeneity. Electrophoretic patterns are similar in all saturniids examined and in *B. mori* (members of the superfamily Bombycoidea); the pattern in the tobacco hornworm, *Manduca sexta* (a member of the related superfamily Sphingoidea), is somewhat different (Paul et al., 1972). Morphological (for references see Smith et al., 1971) as well as amino acid composition data (Kawasaki et al., 1971 *b*) indicate

Whole-Cell and Cell-Free Incorporation of Leucine-^3H

Incorporating system	Rate of incorporation (cpm/min/μg RNA)	Rate of incorporation (cpm/min/ total assay mixture)
A Whole cells	560	
Cytoplasmic fraction (S-5)	112	
B Microsomal pellet		261
Postmicrosomal supernate (S-105)		5
Microsomal pellet + S-105		961
C Cytoplasmic fraction (S-5)	95 ± 61 (six experiments)	
Microsomal pellet + S-105	26 ± 8 (three experiments)	

A: Follicles (Nos. 1–5) from one animal were divided into two groups. One was incubated at 25°C in 0.5 ml of GM containing L-leucine-4,5-^3H (55.2 Ci/mmole, 400 μCi/ml, New England Nuclear Corp. lot 577-027). After 20 min, the epithelial tissue was chilled, rinsed three times with MWS, homogenized in distilled water at 0°C, and the TCA-precipitable radioactivity and RNA content determined. An S-5 cytoplasmic fraction was prepared from the remaining follicles, and an aliquot (containing 112 μg RNA) was used in 0.5 ml of incorporation mixture with leucine-^3H at the same concentration and specific activity as above. Incubation was carried out at 25°C for 20 min; the value given is the average rate of incorporation between 0 and 10 min, which is at least 70% of the initial rate of synthesis. B: An S-5 fraction (follicles 1–5) was centrifuged at 105,000 g for 90 min at 4°C in an IEC SB 405 rotor. The microsomal pellet (resuspended in homogenization medium; see Methods), and the postmicrosomal supernate (S-105) were both stored at −90°C. Aliquots of the frozen fractions, alone and in combination, were incubated at 25°C in 0.10 ml of the incorporation mixture containing leucine-^3H (as above). In terms of the number of follicle equivalents, the microsomal fraction used was double the S-105 fraction. Incorporation values are the average rates between 0 and 10 min. The RNA content was 59.2 μg/0.1 ml assay mixture for the microsomal pellet, 1.44 μg for S-105, and 60.6 μg for the two combined. C: Either S-5 cytoplasmic fractions or a reconstituted system (microsomal pellet plus S-105 supernate) were assayed in the incorporation mixture as in A and B. Incorporation rates (0–10 min) are the mean ± one standard deviation. The RNA content was 262 ± 67 for S-5 experiments and 637 ± 44 for reconstitution experiments (0.5 ml assay volume).

FIGURE 17 Radioactivity profiles of protein labeled with lysine-^3H and glycine-^{14}C in a cell-free system and analyzed electrophoretically. Cell-free protein synthesis was carried out for 15 min at 25°C in 1 ml of reaction mixture (containing S-5 cytoplasmic fraction with 223 μg RNA/ml). The label was L-lysine-^3H (generally labeled, 6.5 Ci/mmole, 0.75 mCi/ml, Schwarz-Mann lot WR 2394) and glycine-^{14}C (uniformly labeled, 104 Ci/mole, 75 μCi/ml, Schwarz-Mann lot WR 2198). The reaction was stopped at 0°C and part of the sample precipitated with an equal volume of ice cold 10% (w/v) TCA. The precipitate was washed twice with ethanol, air dried, analyzed electrophoretically on an 8 cm gel by Method II, and the radioactivity profiles determined. Chorion proteins can be identified by the low $^3H/^{14}C$ ratio, as well as by their characteristically low molecular weight. The ratio plot includes the 95% confidence limits for counting error. Radioactivity profiles of labeled whole cells, homogenized and then precipitated as above, were similar to the profiles obtained routinely by directly dissolving samples in sample buffer (see Fig. 9).
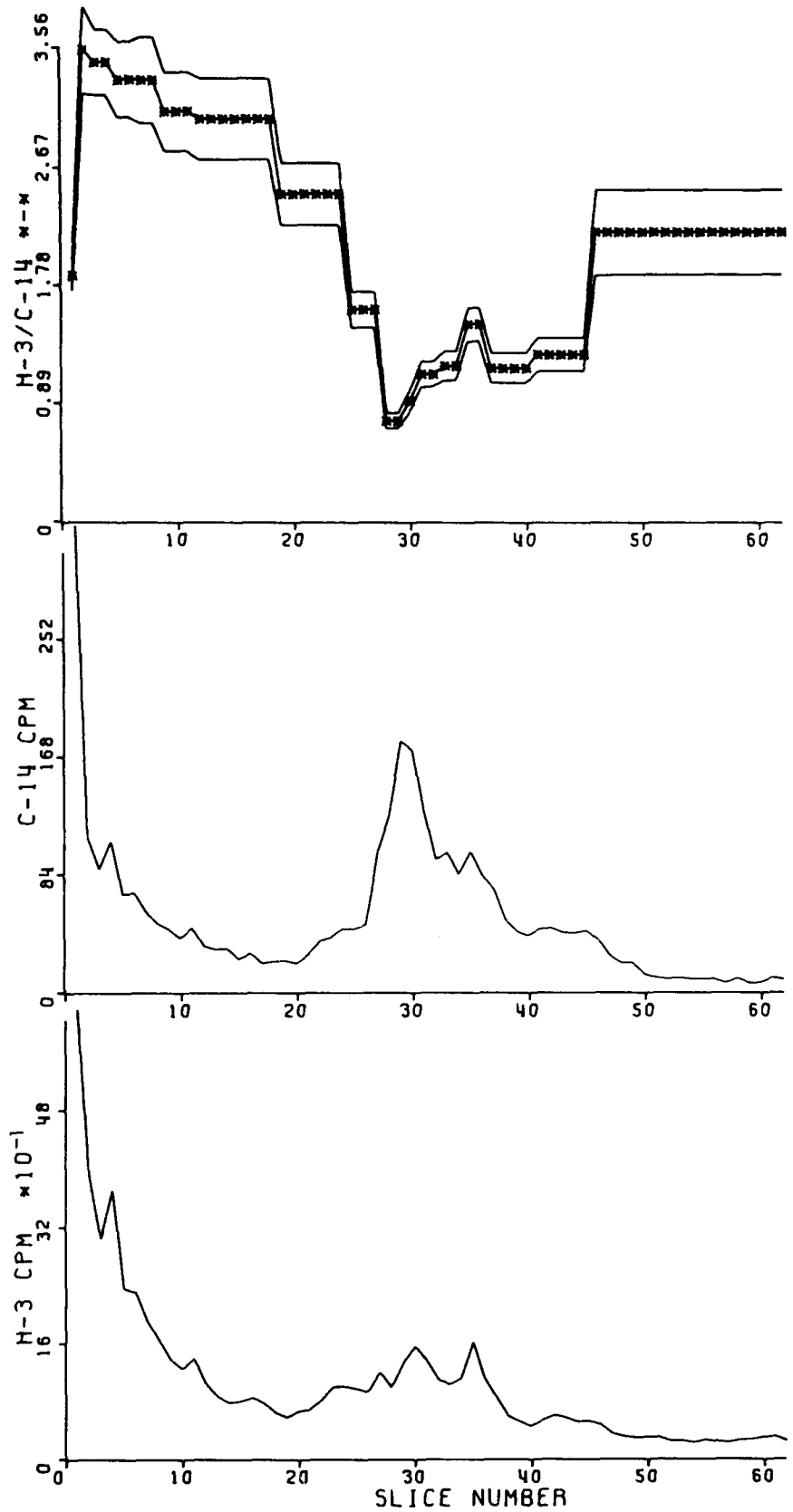
that the chorion may be substantially different in other insect orders.

The experiments reported here document that the *A. polyphemus* chorion proteins resolved by our procedures are synthesized as distinct proteins by the secretory follicular cells. They are not artifacts of partial proteolysis. They are diverse in amino acid composition, and hence not multimers of a fundamental unit. They are not cleaved from a large precursor nor do they undergo any interconversion or other detectable maturation step, either during their sojourn in the cytoplasm or during extracellular deposition. Their translation time and the size of the polysomes on which they are synthesized are consistent with the hypothesis that they are synthesized on monocistronic mRNAs. Finally, their separate kinetics of synthesis during follicle development testify to their distinctness. Most likely, each resolvable protein can thus be studied as the expression of a distinct gene.

Differentiation-Specific Protein Synthesis

Cellular differentiation is commonly characterized by major changes in the rate of synthesis of cell-specific proteins (Kafatos, 1972 *a*). Typically, the rate of synthesis of a specific protein begins to accelerate rapidly at a characteristic period of differentiation, reaches a peak value, and is then further "modulated," often downwards (Rutter et al., 1968; Kafatos, 1972 *a*). Follicular cell differentiation conforms to these generalizations and appears unusually promising for study. The "turning on" of chorion synthesis is very rapid and occurs in parallel with a precipitous drop in incorporation into nonchorion proteins (Fig. 9). Most important, in this system the synthesis of each of a number of cell-specific proteins is regulated separately, according to a precise developmental program. We have recently found that this program continues to unfurl in cells maintained in organ culture (Paul and Kafatos, in preparation).

The precision and specificity of the synthetic program raises the question of its control. We hope that the follicular system will be useful in answering this type of question, so central to an understanding of cellular differentiation. Obviously, it is still too early to present hypotheses with any degree of confidence. Nevertheless, we may speculate that specific protein synthesis in the follicular cells will ultimately prove to be controlled either at the level of specific mRNA production or at the level of specific mRNA recruitment, from an inactive into an active pool. By analogy with other highly differentiated cell types (Kafatos, 1972 *a*), we do not anticipate gene amplification in the follicular cells. As detailed below, control does not seem to reside in the efficiency of translation per se. In view of the massive rate of chorion production, and the rapidity with which newly synthesized chorion is exported, control at the level of protein degradation appears unlikely. Finally, in view of the rapidity of the changes in protein synthesis it will be interesting to determine whether significant control is exerted through differential mRNA stability, which apparently plays a crucial role in a number of other, more slowly developing highly differentiated cell types (Kafatos, 1972 *a*, *b*).

The massive shift in follicular protein synthesis does not occur as a result of a corresponding change in the efficiency of translation. Translational efficiency, i.e. the number of polypeptide chains produced per polysome-bound mRNA molecule per minute, can be calculated as the ratio of the rate of peptide chain elongation divided by the average spacing of ribosomes on the mRNA. The rate of peptide chain elongation is comparable in chorionating and prechorionating follicles, within experimental error; it is also very similar to that of other eukaryotic systems at 25°C (Kafatos, 1972 *a*). The average spacing of ribosomes on the chorion mRNAs can be estimated as 30–37 codons per ribosome, based on the average size of the protein (150 amino acids; see above) and the average size of the polysomes (four to five ribosomes; see above). This agrees with the very uniform spacing determined for other eukaryotic polysomes, both differentiation-specific and nonspecific (e.g., Staehelin et al., 1964; see Kafatos, 1972 *a*); under our conditions, determination of the average size of the large polysomes in prechorionating follicles was not feasible. The calculated translational efficiency for chorion, 2.1–2.6 polypeptide chains per minute per mRNA, is typical of eukaryotic systems at 25°C (Kafatos, 1972 *a*).

Whether control resides in the production or the activation of mRNA, it will be important to detect and quantify the specific mRNAs directly. The characterization of the mRNAs corresponding to the three or four major chorion proteins

should be greatly facilitated by their preponderance in the cells, their expected low molecular weight (which should cause them to separate easily from ribosomal RNA upon acrylamide gel electrophoresis), their occurrence in polysomes of a defined size class, and the relative scarcity of ribonuclease in the follicular tissue. RNA species of the expected properties have recently been observed (R. Gelinas, unpublished observations). Hopefully, our system for cell-free protein synthesis can be made initiation dependent and refined to the point of permitting assay of specific chorion mRNA, in conjunction with double labeling and SDS-acrylamide electrophoresis of the cell-free product (Fig. 17). Our long-term aim is to characterize a number of the chorion mRNA species and to relate their appearance and metabolism to the changing rate of synthesis of the corresponding protein.

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