CYTOLOGICAL EVENTS INVOLVED IN PROTEIN SYNTHESIS IN CELLULAR AND SYNCYTIAL TROPHOBLAST OF HUMAN PLACENTA

An Electron Microscope Autoradiographic Study of [³H]Leucine Incorporation

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ABSTRACT

Electron microscope autoradiography has been used to study protein synthesis in syncytial and cellular trophoblast of term human placental villi incubated in vitro with tritiated leucine ([³H]leu). Autoradiographs were analyzed using the hypothetical grain analysis of Blackett and Parry (1973. J. Cell Biol. 57:9-15). The results of this study demonstrated that both cellular and syncytial trophoblast have marked capacities for protein synthesis. Cellular trophoblast synthesized protein in both its rough endoplasmic reticulum (RER) and its ground plasm which contained abundant free ribosomes. The vast majority of ³H-proteins remained within the cell, with some of the proteins synthesized ultimately appearing in the nucleus. A small percentage of grains was ultimately associated with the trophoblast basement membrane. In syncytial trophoblast, the RER was the dominant site for protein synthesis. The autoradiographic data suggested that, as in the cellular trophoblast, the vast majority of ³H-proteins synthesized by the syncytial trophoblast remained within the syncytial trophoblast throughout the incubation period. The major portion of [³H]leu-labeling present in the syncytial trophoblast of villi incubated the longest times (4 h+) remained in association with the RER. Labeled proteins did not become concentrated in syncytial trophoblast Golgi apparatus, vesicles, or granules. In contrast to cellular trophoblast, the nuclei in the syncytium did not contain ³H-proteins at any time-point studied.

KEY WORDS placenta · electron microscopy · protein synthesis · autoradiography · trophoblast

Human placental syncytial trophoblast is unique in being the only syncytial epithelium in the body and in releasing its secretory products directly into a blood vascular space. The placenta synthesizes a number of proteins which are secreted into the maternal circulation in high quantities but are found in the fetal circulation in only small amounts (see reference 19 for review). The best characterized of these proteins are human chorionic gonad-
otropin (HCG)\(^1\) and human placental lactogen (HPL), both of which have been shown by immunocyto logic techniques to be localized to the syncytial trophoblast (37, 57, 60). Cellular trophoblast, or cytotrophoblast, is mitotically active (27, 50), and cells derived from such activity differentiate into, and fuse with, syncytium (10, 22).

Both cellular and syncytial forms of human trophoblast exhibit an ultrastructure indicating a potential for protein synthesis (see reference 67 for review). Cytotrophoblast is characterized by an electron-lucent cytoplasm containing abundant free ribosomes, profiles of undilated rough endoplasmic reticulum (RER) (often in close association with mitochondria), and a relatively large Golgi complex. Syncytial trophoblast cytoplasm is electron dense and is characterized by a predominance of dilated sacs of RER, often containing electron-dense material in their lumina. The cytoplasm also contains numerous profiles of Golgi complex, a variety of membrane-bounded granules, and abundant clear vesicles.

Electron microscope autoradiography has been applied in numerous kinetic analyses of the cyto logic organization contributing to the synthesis, intracellular translocation, and secretion of protein molecules. Although this method has been most extensively used to study protein synthesis in the acinar cells of the pancreas (8, 9, 31-34), electron microscope autoradiography has also been used to study protein synthesis in a variety of other cell types including fibroblasts (61), hepatocytes (1), plasma cells (6, 14, 69), pancreatic islet cells (30, 42), parotid acinar cells (11, 65), cartilage cells (53), odontoblasts (61), prostate cells (24), seminal vesicle cells (25), parathyroid cells (40), thyroid cells (23, 39), cells dissociated from anterior pituitary (29), and a variety of cell types in the lung (12, 36, 47). In combination with data derived from cell fractionation studies, the results of studies such as these have lead to a generally accepted theory for the intracellular pathways involved in synthesis and secretion of proteins (43, see also reference 52).

This paper reports the results of an electron microscope autoradiographic study of the cyto logical aspects of protein synthesis in both syncytial and cellular forms of human trophoblast.

**MATERIALS AND METHODS**

**Tissue Preparation and Incubation**

A total of four human term placentas obtained within 5 min of normal vaginal delivery or elective Cesarean section after uncomplicated pregnancies were used. Three pieces of tissue were dissected from three different cotyledons. The tissue selected was free of visible calcification, infarct, hematoma, and/or injury and excluded the margins of the placental disc, chorionic and basal plates. Each piece was rinsed in a separate vial of ice-cold medium 199 (M199) (Gibco). Each of the three specimens was separately diced under fresh, cold M199 into 3-5-mm pieces. One piece of tissue was taken from each of the three specimens and distributed to each of six 2-ml polystyrene tubes (=3 tissue pieces, 15-30 mg wet wt per tube) containing about 1 ml of fresh M199. All tubes were kept on ice until incubation in radioactive medium. Incubation in radioactive medium was begun by sequentially removing the three pieces of tissue and gently transferring them into a new tube containing 0.5 ml of [\(^{1}\)H]leucine-free M199 containing 250 \(\mu\)Ci/ml [\(^{1}\)H]leucine ([\(^{1}\)H]leu) (New England Nuclear, Boston, Mass.; lot no. 798-194; sp act, 50.0 Ci/mmol). Each tube was gassed with 95% air-5% CO\(_2\), stoppered, and incubated in a Dubnoff metabolic shaker maintained at 25°C and agitated at approx. 60 cycles/min. "Pulse" tissues were incubated in radioactive medium for 5, 20, or 40 min while "chase" tissues were incubated in nonradioactive medium for 20-40 min after a 20-min pulse incubation in radioactive medium. After an appropriate pulse time, tissue was either rinsed with three changes (5-ml each) of ice-cold M199 (8 min total) before aldehyde fixation or rinsed in two changes (2-ml each) of 25°C M199 before incubation in chase medium for 20-40 min. Chase medium was M199 containing 10% heat-inactivated fetal calf serum (FCS) (Grand Island Biological Co., Grand Island, N. Y.), antibiotic, and an approx. 1,000-fold higher concentration of [\(^{1}\)H]leu than in pulse medium. Vials incubated for the longer time intervals were rinsed every 30-40 min. After incubating in chase medium and rinsing in two changes (5-ml each) of ice-cold M199, tissue was transferred to 5-ml glass vials containing fixative.

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\(^1\) Abbreviations used in this paper: BL, basal lamina; BPM, basal plasma membrane; BM, basement membrane; CT, connective tissue; FCS, heat-inactivated (56°C, 30 min) fetal calf serum; GP, ground plasm; HCG, human chorionic gonadotropin; HPL, human placental lactogen (also known as human chorionic somatomammotropin); [\(^{3}\)H]leu, \(\tau\)-[\(^{3}\)H]leucine; [\(^{1}\)H]leu, \(\tau\)-[\(^{1}\)H]leucine; M199, medium 199 with Earle's salts gassed with 95% air-5% CO\(_2\) and containing 100 \(\mu\)g/ml penicillin, 100 \(\mu\)g/ml streptomycin, and 0.25 \(\mu\)g/ml Fungizone; MS, microvillose surface of syncytium; Mit, mitochondria; N, nucleus; NEn, nuclear envelope; PM, all cytotrophoblast surface membrane except that adjacent to basal lamina; RER, rough endoplasmic reticulum; and V, Golgi-associated vesicles.
Light Microscope Autoradiography

Selection of Optimal Fixative, Fixation, Dehydration, and Embedding

To determine whether or not aldehyde fixation caused significant nonspecific binding of [3H]leu to tissue, two experiments were performed. Tissues were either incubated for 5 or 40 min at 25°C or incubated for 5 min at 4°C in M199 containing 250 μCi/ml of [3H]leu. After incubation, the were rinsed 3 times in ice-cold M199, and samples of tissue from each incubation were fixed in either three changes of 3% glutaraldehyde or 4% formaldehyde (freshly prepared from paraformaldehyde) each buffered with 0.1 M phosphate buffer (pH 7.3) containing 0.05% CaCl2. After fixation for 1 h at room temperature, tissues were rinsed for 4-12 h in three changes of cold 0.1 M phosphate buffer (pH 7.3) containing 5% (wt/vol) sucrose, postfixed for 1 h in cold 2% OsO4 in 0.1 M phosphate buffer (pH 7.2) containing 0.05% CaCl2, dehydrated in a graded series of cold ethanol, cleared in propylene oxide, and embedded in Araldite. All tissues used in preparing electron microscope autoradiographs used in the grain analysis were fixed in the glutaraldehyde-containing fixative but otherwise handled exactly as described above.

Light Microscope Autoradiography

For each time-point in each experiment, sections (approx. 0.5 μm in thickness) from two or more blocks of tissue were cut onto water, using glass knives, and transferred to each of two clean slides. Slides were dipped by hand in 40°C NTB-2 emulsion (Kodak), diluted five parts emulsion to three parts water and dessicant. Transferred to each of two clean slides. Slides were stored at 4°C for periods of 2 wk-5 mo. Control grids simultaneously exposed in each LKB box included coated grids containing nonradioactive tissue sections stored with or without prior exposure to light.

Grid Development: After removing the Formvar coating by immersion in 1,2 dichloroethane (2 min), grids were processed, 3-4 at a time, by immersion sequentially in the following solutions, all maintained at 20°C: D-19 (2 min), distilled water (30 s), 25% Ektadil distilled with water (30 s), and finally three distilled water rinses (1 min each).

Electron Microscope Autoradiography

Section Coating: Serial thin sections (silver-pale gold interference color) cut on a Sorvall MT-2 ultramicrotome (Sorvall Operations, DuPont Instruments, Newtown, Conn.) were picked up on uncoated copper grids (75 x 300 mesh). Comparison of samples was achieved by taking a large number of grids (6-10/ block; 4-8 sections/grid) from each block. Grids were coated with Ilford L4 emulsion (Polysciences, Inc., Warrington, Pa.) with a modification of the stripping film method of Williamson and van den Bosch (66). Emulsion was melted at 50°C, diluted to 33% with water, gently mixed by stirring with a glass rod, and allowed to cool to 26°C in a water bath. Sheets of emulsion-coated Formvar were floated onto water, and grids were then gently placed, section side down, onto the sheet. Formvar with attached grids was picked up by rolling the back side of a rectangular sheet of undeveloped photographic paper over the water surface, thereby adsorbing the Formvar sheet. The sheets were then hung vertically to dry for at least 15 min. After drying, coated grids were detached from the sheet by scratching around their edges using sharp forceps, being careful not to tear the emulsion covering the grid. All manipulations were done at least 3 feet away from a Duplex Super Safelight (Thomas Instrument Company, Inc., Charlottesville, Va.).

Test grids thus coated were then (a) viewed in the electron microscope without development to verify the presence of a tightly packed monolayer of silver bromide crystals, (b) developed (see below) before viewing in the electron microscope to check background, or (c) exposed to light before development and viewing to verify that grains would develop after exposure of the emulsion to an energy source. When these controls proved satisfactory, the experimental grids were coated, put into LKB grid storage boxes (LKB Instruments, Inc., Rockville, Md.) that were then placed in black boxes containing dessicant, sealed with tape, and stored at 4°C for periods of 2 wk-5 mo. Control grids simultaneously exposed in each LKB box included coated grids containing nonradioactive tissue sections stored with or without prior exposure to light.

Grid Development: After removing the Formvar coating by immersion in 1,2 dichloroethane (2 min), grids were processed, 3-4 at a time, by immersion sequentially in the following solutions, all maintained at 20°C: D-19 (2 min), distilled water (30 s), 25% Ektadil distilled with water (30 s), and finally three distilled water rinses (1 min each).

Heavy Metal Staining and Electron Microscope Examination: Developed grids were stained for 10 min with lead citrate by floating a grid, section side up, on a drop of stain. The following sampling procedure was followed in obtaining a set of micrographs for analysis: for each tissue sample, at least three grids were studied, and areas with reasonable tissue visibility were photographed randomly. No more than six micrographs of any one cell type was taken from each grid. Magnification calibration (using a carbon grating replica with 2,160 lines/mm (Ernest F. Fullam, Inc., Schenectady, N.Y.) of the RCA-EMU 3G electron microscope used for grid examination was completed twice during the course of this study. Control grids were...
examined for the presence of positive or negative chemography. Background in regions of experimental grids not containing tissue was determined to be 1 grain/50 μm² of section.

**GRAIN ANALYSIS:** Grain analysis was carried out by the method of Blackett and Parry (5). This method combines the circle analysis of Williams (64) with the mathematical considerations of resolution reported by Salpeter et al. (54) to make estimates, based on grain data, for the activity within structures in autoradiographs. 8-10 micrographs from each of the five to six time-points (for a given cell type and tracer) were pooled, and a hypothetical grain analysis was done on this set of micrographs. A grid of equally spaced points was used to overlay each of the micrographs in the set, and each grid point was assigned a distance and random direction. The grid point is assumed to be a source of a radioactive disintegration while the distance and direction are used to generate the location that a grain might develop as the result of the disintegration of a tritiated source. Further details of the analysis are given in the original papers (5, 44). The half distance assumed under the autoradiographic conditions outlined was 1,450 Å (56) which gives rise to a 50% probability circle of 14.8 mm for prints of 30,000 magnification.

For grain analysis, organelles in cellular and syncytial trophoblast were grouped as shown in Tables I and II, respectively. Ground plasm¹ is assumed to be a component of all circled compartments. Note that RER included nuclear envelope (NEn). Golgi structures included obvious stacks of Golgi cisterns and Golgi-associated vesicles that may have been, but were not necessarily, adjacent to a stack of Golgi cisterns. For the analysis of syncytial trophoblast, circles overlying microvilli or surface membrane between their bases were assigned to the microvillous surface (MS) compartment, irrespective of what other organelles were present within the limits of the 50% probability circle. Similarly, basement membrane (BM) included circles overlying basal plasma membrane (BPM) or basal lamina (BL) without regard to what other organelles were present.¹

¹ Syncytial inclusions (I) included all membrane-bounded granules and lipidlike bodies found in syncytial cytoplasm.

**RESULTS**

**Selection of Optimal Fixative**

Light microscope autoradiographs of tissue fixed in glutaraldehyde had <8% higher numbers of grains than tissue fixed in formaldehyde. These grains were randomly distributed throughout the villi. Since morphologic preservation was markedly inferior when formaldehyde was used, glutaraldehyde-containing fixative was used for primary fixation of all tissues processed for electron microscope autoradiography.

**Light Microscope Autoradiography**

Light microscope autoradiographs of tissue incubated with [3H]leu had silver grains located over cytoplasm of both syncytiotrophoblast and cellular trophoblast. Over cytotrophoblast, grains were distributed throughout the cytoplasm with no apparent clustering in any portion of the cell. Grains overlying syncytial cytoplasm of a single villus cut in cross section were also distributed in a relatively uniform pattern. However, syncytiotrophoblast nuclei had numerous overlying grains autoradiographs of tissue incubated for 4 h.²

**Electron Microscope Autoradiography:**

**Cytotrophoblast**

The compartments analyzed as sources and sites in the hypothetical grain analysis are presented in Table III along with the numerical distribution of hypothetical grains actually recorded. The total number of grains in each row provides an estimate of the relative area occupied by the organelle in the cells represented by micrographs while the summation of each column provides the circle analysis for a distribution of radioactivity assumed to be uniform (5, 64). By use of sequential computer analysis of the data,² it was possible to

² Our sincere thanks go to Dr. Nick Blackett, of the Institute of Cancer Research, Sutton, Surrey, Great Britain, for performing the analysis of the data by computer.

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¹ The list of distances and directions were kindly supplied by Dr. Nick Blackett.
² "Ground plasm," a compartment used in the Blackett and Parry analysis (5, 44), can be considered synonymous with the phrase "cytoplasmic matrix" or "ground substance."
³ In the circle analysis, the 50% probability circle centered on grains overlying basal lamina generally included the basal plasma membrane and/or the collagen fibers of the reticular lamina. The phrase "basement membrane" is used in this study to be inclusive of the above-mentioned structures in designating the interface of the epithelium and stroma.
Grouping of organelles for circle analysis of cytotrophoblast (Table I) and syncytial trophoblast (Table II). A grid of equally spaced points was used to overlay a set of micrographs representative of either cytotrophoblast or syncytial trophoblast. A distance and direction was assigned each grid point, providing the site at which a (hypothetical) grain might develop from the emission of a tritiated source. All organelles in the micrograph underlying the limits of a 50% probability circle centered on the hypothetical grain were recorded as a "compartment" (left column). Compartments containing <5 grains (e.g., RER/NEn/GP) were pooled with an appropriate similar compartment (e.g., RER/GP) yielding a "pooled compartment" (e.g., RER) which was used for the analysis of the real grains. Because the 50% probability circle is generally larger than individual organelles, ground plasm is assumed to be a component of each of the pooled compartments except the nucleus.

make estimates of the relative volumes occupied by each organelle and to use this in combination with the hypothetical grain data to determine an estimate of the relative activity in each organelle at each time-point. The estimates of relative activity for each organelle as a function of time, i.e., reading across each row of the table, is presented for convenient comparison with the relative activity at each time-point and so that all time-points analyzed can be conveniently presented. (All "chase" incubations were preceded by a 20-min "pulse" in radioactive medium.)
in Table IV. Labeling of both ground plasm and RER was highest in tissue incubated for 5 min, and decreased to its lowest level in tissue incubated for 4 h in chase medium (Figs. 1, 4, and 5). The number of grains present overlying Golgi structures exhibited a pattern opposite to this.

**TABLE III**

| Position of Grid Point (Source of Hypothetical Grain) | GP | RER | RER/ Mit | RER/ Golgi | N | N/ Golgi | Golgi/ RER | BM | PM | Total |
|------------------------------------------------------|----|-----|----------|------------|---|---------|------------|-----|-----|-------|
| Ground Plasm (GP)                                   | 11 | 16  | 12        | 15         | 2 | 1       | 0          | 10 | 22 | 101   |
| Basement Membrane (BM)                              | 0  | 4   | 1         | 0          | 0 | 0       | 0          | 0  | 24 | 33    |
| Rough Endoplasmic Reticulum (RER)                   | 1  | 43  | 41        | 21         | 9 | 2       | 3          | 0  | 8  | 143   |
| Mitochondria (Mit)                                  | 0  | 1   | 26        | 2           | 1 | 2       | 0          | 3  | 3  | 46    |
| Golgi                                                | 1  | 5   | 4         | 17          | 0 | 2       | 3          | 6  | 34 | 87    |
| Nucleus (N)                                         | 0  | 1   | 0         | 1           | 18| 59      | 3          | 0  | 0  | 82    |
| Total                                                | 13 | 70  | 87        | 57          | 30| 66      | 9          | 10 | 55 | 492   |

**DISTRIBUTION OF REAL GRAINS**

|              | 5 min Pulse | 20 min Pulse | 40 min Pulse | 20 min Chase | 40 min Chase | 4 h Chase |
|--------------|-------------|--------------|--------------|--------------|--------------|----------|
| GP           | 20±2        | 42±12        | 40±12        | 17±10        | 6±9          | 19±15    | 0±8     |
| BM           | 7±1         | 1±3          | 5±4          | 13±5         | 23±6         | 17±3     | 17±4    |
| RER          | 30±3        | 47±11        | 40±10        | 28±12        | 26±8         | 20±12    | 3±6     |
| Mit          | 9±1         | 0±1          | 11±5         | 9±7          | 4±6          | 8±5      | 5±6     |
| Golgi        | 18±2        | 6±4          | 1±3          | 16±7         | 31±9         | 26±8     | 45±8    |
| N            | 16±2        | 4±2          | 3±2          | 17±3         | 10±4         | 10±3     | 30±3    |

Table IV presenting the values for the relative area of each organelle assumed to be a source of radioactivity in cytotrophoblast and the relative activity in each organelle for each time period. The relative activity of each organelle as a function of time can be studied by reading across each row. All “chase” incubations followed a 20-min “pulse” incubation in radioactive medium.
FIGURE 1  Electron micrograph of a portion of a cytotrophoblast cell in an autoradiograph of tissue incubated for 5 min in [3H]leu-containing medium. Some silver grains are associated with the RER and Mit, while others appear to overlie free ribosomes. Golgi stacks are devoid of grains. Exposure, 1 mo. Bar, 0.5 μm. × 30,000.

FIGURE 2  Electron micrograph of a portion of a cytotrophoblast cell in an autoradiograph of tissue incubated for 20 min in [3H]leu-containing medium and then an additional 4 h in nonradioactive medium. Most silver grains overlie stacks and Golgi-associated vesicles. Exposure, 1 mo. Bar, 0.5 μm. × 30,000.

FIGURE 3  Electron micrograph of a cytotrophoblast process in an autoradiograph of tissue incubated for 20 min in [3H]leu-containing medium and then an additional 20 min in nonradioactive medium. Numerous grains straddle the basal lamina and immediately adjacent structures. Exposure, 20 mo. Bar, 0.5 μm. × 30,000.
having a low relative activity in tissue incubated the shortest times and the highest value for tissue incubated the longest time (Figs. 1, 2, and 5). The relative activity of both the nucleus and basement membrane was low in tissues incubated for 5 and 20 min, but significant labeling of both compartments was present in tissue incubated for longer than 20 min (Figs. 3 and 5). The relative activity of mitochondria was present at a low but persistent level at all time-points (Figs. 1 and 4).

Table V presents the results of the chi-squared test. Values <19 or 20 with 10 or 11 degrees of freedom are reasonable data fits at values of $P = 0.05$ or higher.


### Table V

| Incubation Time | $X^2±S.E.$ | Degrees of Freedom |
|-----------------|------------|--------------------|
| 5 min Pulse     | 11±15      | 10                 |
| 20 min Pulse    | 21±15      | 11                 |
| 40 min Pulse    | 22±14      | 11                 |
| 20 min Chase    | 17±14      | 11                 |
| 40 min Chase    | 9±8        | 11                 |
| 4 h Chase       | 35±29      | 10                 |

Table V presenting the results of the chi-squared analysis of the grain data for cytotrophoblast. The high value at 4 h is probably not significant in light of the high standard error. The latter indicates that lower values were often obtained in the analysis of individual compartments at a given time point.

**Electron Microscope Autoradiography:**

**Syncytiotrophoblast**

Table VI presents the compartments analyzed as sources and sites in the hypothetical grain analysis of syncytiotrophoblast and gives the numerical distribution of hypothetical and real grains actually recorded in the analysis. These data were used to calculate a relative area (5) which is presented in Table VII. RER is the dominant organelle in syncytiotrophoblast, occupying almost half of the syncytiotrophoblast mass. The relative activity of each organelle is also presented in Table VII and can be studied as a function of time by reading across each row. A majority of the grains recorded at all time-points were associated with the RER (Figs. 6 and 7). Grains often straddled the ribosome-studded RER membrane (Figs. 6 and 7) while grains overlying RER cisternae were especially prevalent in syncytiotrophoblast incubated 4 h (Fig. 8). The Golgi complex exhibited its highest relative activity in syncytiotrophoblast incubated the longest time period (Fig. 9). At no time were syncytial inclusions, such as the lipidlike inclusion in Fig. 9, or the Golgi-associated granule illustrated in Fig. 10, significantly labeled. Similarly, neither basement membrane directly adjacent to syncytiotrophoblast nor nuclei in the syncytiotrophoblast had selective concentration of labeled materials (Fig. 8). The microvillous surface had its highest relative activity in tissue incubated either for 40 min in pulse medium or for 4 h in chase medium (Figs. 8 and 11). The low relative activity of the microvillous surface at 40-min chase reflects the fact that different villi, even in the same tissue block, exhibited varying amounts of microvillous surface labeling. The total amount of labeling of syncytiotrophoblast also varied from villus to villus in tissue incubated for the longer time periods. Surface labeling was usually associated with microvilli or with membrane located between the bases of the microvilli. However, there were also some surface protrusions that had grains associated with the surface membrane (Fig. 8). Both ground plasm and mitochondria were labeled at a low but rather consistent level at all time-points studied.

Table VIII presents the chi-squared values obtained from the grain analysis of syncytiotrophoblast. All chi-squared values are less than 16 (with 8-9 degrees of freedom) and, thus, good data fits were obtained for the real grain data.

**DISCUSSION**

This study has demonstrated that both cellular and syncytiotrophoblast have marked capacities for protein synthesis. Cytotrophoblast was capable of synthesizing protein in both its RER and ground plasm which contained abundant free ribosomes. The vast majority of H-proteins remained within the cell, with some ultimately appearing in the nucleus. A small percentage of grains was also associated with the trophoblast basement membrane. The RER was the dominant
TABLE VI
Distribution of Hypothetical Grains for Uniform Activity throughout the Tissue

| Position of Grid Point (Source of Hypothetical Grain) | SITE OF HYPOTHETICAL GRAIN (POSITION OF CIRCLE) | BM | Golgi | Mit | RER | I | N | RER | Mit | MS | Total |
|-------------------------------------------------------|-------------------------------------------------|----|-------|-----|-----|---|---|-----|-----|----|-------|
| Basement Membrane (BM)                                | 8                                              | 2  | 0     | 1   | 0   | 0 | 0 | 0   | 0   | 0  | 12    |
| Golgi                                                 | 0                                              | 15 | 7     | 24  | 8   | 2 | 0 | 0   | 1   | 4  | 63    |
| Ground Plasm (GP)                                     | 0                                              | 2  | 0     | 1   | 1   | 0 | 1 | 2   | 13  | 0  | 28    |
| Syncytial Inclusions (I)                              | 0                                              | 1  | 0     | 0   | 0   | 0 | 0 | 0   | 0   | 4  | 12    |
| Mitochondria (Mit)                                    | 0                                              | 1  | 1     | 1   | 0   | 0 | 0 | 0   | 2   | 17 | 42    |
| Nucleus (N)                                           | 0                                              | 1  | 0     | 0   | 0   | 0 | 2 | 51  | 17  | 3  | 83    |
| Rough Endoplasmic Reticulum (RER)                     | 0                                              | 2  | 2     | 26  | 1   | 1 | 2 | 20  | 81  | 59 | 203   |
| Microvillous Surface (MS)                             | 0                                              | 0  | 0     | 3   | 0   | 0 | 0 | 0   | 0   | 41 | 44    |
| Total                                                 | 8                                              | 24 | 10    | 56  | 7   | 15| 56| 40  | 104 | 101| 66   | 481  |

DISTRIBUTION OF REAL GRAINS

| Time Period | Number of Real Grains |
|-------------|-----------------------|
| 5 min Pulse | 13 17 6 47 7 16 20 42 121 95 26 410 |
| 20 min Pulse| 4 3 3 37 2 7 6 31 139 92 21 345 |
| 40 min Pulse| 7 21 7 59 0 14 20 29 86 62 95 400 |
| 40 min Chase| 9 16 1 29 0 8 5 28 78 75 31 280 |
| 4 h Chase   | 2 33 9 37 1 10 5 18 115 36 70 336 |

Table VI presenting the sites and sources used in the hypothetical and real grain analyses of syncytiotrophoblast and the numerical grain totals for each compartment. The column on the right shows the total number of hypothetical grains analyzed and the total number of real grains recorded from autoradiographs of tissue incubated for the indicated periods of time. In the hypothetical grain analysis, the total number of grains in each row provides an estimate for the volume occupied by the organelle assumed to be a source of radioactivity in the syncytiotrophoblast while the total in each column provides the circle analysis for a grain distribution obtainable from tissue assumed to contain radioactivity uniformly distributed. All "chase" incubations followed a 20-min "pulse" incubation. Site for protein synthesis in the syncytiotrophoblast. The vast majority of [3H]-proteins synthesized by the syncytiotrophoblast remained within the syncytiotrophoblast, especially in association with the RER. Tritium-labeled proteins did not become concentrated in syncytiotrophoblast Golgi apparatus, or in vesicles or granules in the syncytiotrophoblast. Unlike cytotrophoblast nuclei, the nuclei in the syncytiotrophoblast did not contain significant amounts of [3H]-proteins at any time-point studied. A small percentage of the labeled proteins of syncytiotrophoblast incubated for 4 h was associated with the microvillous surface. Whether this protein became associated with the cytoplasm subjacent to the plasmalemma, contributed to the plasmalemma itself, or represented a secretory product that subsequently adsorbed to the surface (18, 20) could not be determined.

Synthesis of Proteins in Cytotrophoblast

Through a process of division, differentiation and fusion, cytotrophoblast contributes to the formation of syncytiotrophoblast (10, 22, 50). Free ribosomes are often associated with the formation of constitutive, or nonsecretory, proteins of the cell, and protein synthesis by RER is generally associated with the formation of secretory products (28, 48). The nuclear proteins synthesized by cytotrophoblast may be an end product of synthesis by either free ribosomes or RER. The latter possibility exists since not all proteins synthesized...
Table VII

| Relative Area | 5 min Pulse | 20 min Pulse | 40 min Pulse | 40 min Chase | 4 h Chase |
|---------------|-------------|--------------|--------------|--------------|----------|
| BM            | 2±1         | 4±2          | 0±1          | 1±1          | 5±2      | 1±1      |
| Golgi         | 13±2        | 9±5          | 0±3          | 16±3         | 5±4      | 18±7     |
| GP            | 6±1         | 11±8         | 7±4          | 3±6          | 18±11    | 5±4      |
| I             | 2±1         | 0±2          | 0±1          | 0±1          | 0±1      | 0±2      |
| Mit           | 9±2         | 12±9         | 10±7         | 5±5          | 17±7     | 8±5      |
| N             | 17±2        | 7±3          | 1±1          | 6±3          | 1±2      | 0±1      |
| RER           | 42±3        | 57±12        | 82±7         | 47±8         | 50±15    | 48±12    |
| MS            | 9±1         | 0±1          | 0±2          | 21±2         | 3±3      | 20±16    |

Table VII presenting the values of the relative area of each organelle assumed to be a source of radioactivity in syncytial trophoblast and the relative activity of each organelle for each incubation period. The relative activity of each organelle as a function of time can be studied by reading across each row. All “chase” incubations followed a 20-min “pulse” incubation in radioactive medium.

by RER are destined for secretion, as illustrated in the following study of cytotrophoblast (41) and by other investigators studying liver cells (2, 3, 21). The constitutive proteins most likely contribute to the process of division and/or differentiation of cytotrophoblast. At term, the cytotrophoblast showed minimal evidence of secretion. It is probable that formation of trophoblast BM is more active early in gestation when cytotrophoblast cells are more numerous and placental growth is more active. The small amount of BM synthesized by cytotrophoblast at term may represent a residual capacity of these cells to synthesize BM proteins, and may also have been derived from either free ribosome or RER synthetic activity. The relatively constant amount of labeled protein associated with the BM of tissue incubated for 20-min chase or longer may result from a steady state in the addition and breakdown of 3H-labeled BM components.

Protein Synthesis by Syncytial Trophoblast

Our observations indicated that the vast majority of newly synthesized proteins in syncytial trophoblast remained within the syncytial trophoblast. The frequent appearance of grains overlying membrane of RER in syncytium incubated for 4 h suggests that some newly synthesized proteins were not released from the membrane-bound polysome (49) or were constitutive proteins that became structural components of the RER. Formation of RER membrane protein has been demonstrated in liver cells (16, 17). The observations of numerous autoradiographic studies have suggested the formation of constitutive proteins (4, 39, 40, 45, 46) some of which were proteins that remained associated with the RER (45, 46). Unlike cytotrophoblast, nuclear proteins were not among the constitutive proteins formed by syncytium which is not surprising since nuclei of syncytium do not divide (50).

In electron microscope autoradiographic studies of amino acid incorporation into a variety of hormone-secreting cells, 3H-labeled proteins were transported from the site of synthesis in the RER to the Golgi complex and, finally, to secretory granules or vesicles that discharged the secretory product to the extracellular space (23, 29, 30, 39, 42, 45, 46, 63). The autoradiographic data presented in this study are inconclusive with regard to evidence demonstrating the pathway of transport and the presence of active secretion of protein by human syncytial trophoblast. Proteins secreted by syncytial trophoblast would be released into a location (the intervillous space)
Electron micrographs of syncytiotrophoblast in autoradiographs of tissue incubated either for 5 min in [3H]leu-containing medium (Fig. 6) or for 20 min in this medium and an additional 4 h in nonradioactive medium (Fig. 7). In both micrographs, grains are associated predominantly with the RER, often overlying its ribosome-studded membrane. Exposure, 2.5 mo. (Fig. 6); 1.5 mo. (Fig. 7). Bar, 0.5 μm. × 30,000.
where they would not be retained for autoradiographic analysis. This absence of a "secretory wave" of radioactivity did not become apparent until after the grain analysis was completed. In the absence of definite autoradiographic evidence of secretion, a pathway for intracellular protein transport leading to secretion cannot be formulated. The only point that can be made is that the absence of $^3$H-protein concentration in granules and the lack of a peak concentration of $^3$H-labeled products in the Golgi complex suggests that, if these organelles are involved in processing synthesized proteins, the rate of movement through them precludes accumulation of proteins. Since the tissue used in these in vitro experiments was from term placentas, the trophoblast studied was nearing the end of its "normal" lifespan. It may well be that term placenta is not so efficient in intracellular transport and discharge of secretory product as placenta from an earlier period in gestation. However, the syncytial trophoblast does clearly continue to secrete some proteins at term as evidenced by the presence of the highest levels of HPL in maternal blood at term (68). Immuno-cytochemical studies have localized HPL to the syncytial trophoblast (57). In addition, Suwa and Freisen (26, 58, 59) and Choy and Watkins (13) have shown that synthesis and secretion of $^3$H-HPL occurs in term placental villi incubated with $[3H]$leu in vitro. Thus, although some protein secretion occurs in term placental villi, the level does not appear to be sufficient to allow the study of pathways of intracellular processing of secretory protein by electron microscope autoradiography. Autoradiographic study of protein synthesis by placentas obtained earlier in gestation, or by placentas of other primates, might provide more

**FIGURE 8** Electron micrograph of syncytiotrophoblast in an autoradiograph of tissue incubated for 20 min in $[3H]$leu-containing medium and then 4 h in nonradioactive medium. Most grains are associated with the RER while some overlie the membrane of an RER-containing protrusion emanating from the microvillous surface. Relatively few grains overlie inclusions (arrows), nuclei, or basal lamina. Exposure, 1 mo. Bar, 0.5 μm. × 10,700.

**FIGURE 9** Electron micrograph of a Golgi complex in syncytiotrophoblast in an autoradiograph of tissue incubated for 20 min in $[3H]$leu-containing medium and an additional 4 h in nonradioactive medium. The Golgi complex has overlying grains but the inclusion (I) is devoid of them. Exposure, 1 mo. Bar, 0.5 μm. × 30,000.
Figure 10. Electron micrograph of syncytiotrophoblast in an autoradiograph of tissue incubated for 20 min in [H]leu-containing medium and then an additional 40 min in nonradioactive medium. The Golgi complex contains an example of a granular inclusion often found in the syncytiotrophoblast. Exposure, 2 mo. Bar, 0.5 μm. × 30,000.

Figure 11. Electron micrograph of syncytiotrophoblast in an autoradiograph of tissue incubated for 40 min in [H]leu-containing medium. Grains are distributed throughout the syncytiotrophoblast but not in the nuclei. However, the nuclear envelope, a part of the RER, does have overlying grains. Numerous grains overlie the microvilli which extend into the intervillous space. Exposure, 2 wk. Bar, 0.5 μm. × 10,700.
Table VIII presenting the results of the chi-squared
analysis of the grain data for syncytial trophoblast. All
values are <16, indicating that good data fits were
obtained at all time-points.

| Incubation Time | X²±SE | Degrees of Freedom |
|-----------------|--------|--------------------|
| 5 min Pulse     | 6±12   | 9                  |
| 20 min Pulse    | 4±4    | 8                  |
| 40 min Pulse    | 2±7    | 8                  |
| 40 min Chase    | 8±6    | 9                  |
| 4 h Chase       | 8±12   | 9                  |

Table VIII of the intracellular pathways followed by
proteins secreted by the placenta.

Use of Hypothetical Grain Analysis

The hypothetical grain analysis, described by
Blackett and Parry (5) and used in autoradi-
ographic studies by Parry and Blackett (44) and
Meyrick and Reid (36), was chosen to analyze
the data in this study and the following one (41).
This method of analysis was selected primarily
because the organelles in both cellular and syncy-
tial trophoblast are distributed throughout the
cytoplasm in a pattern that is more nearly random
than that in many secretory cells. This method
combines the circle analysis of Williams (64) with
the mathematical considerations of resolution
reported by Salpeter et al. (54). As pointed out
in the original description of the method (5), the
hypothetical grain analysis has two particular ad-
vantages over previous methods used to analyze
autoradiographs (see reference 55 for a review of
the methods of analysis of autoradiographs). First,
the method takes into account the actual shapes
and sizes of subcellular components observed in
the set of autoradiographs being analyzed and
thus avoids the need for constructing theoretical
models of subcellular structures in order to take
into account “cross-fire” effects of tritium emis-
sions that cause grains to develop over organelles
that are nonradioactive. An example of the use-
fulness of this aspect of the analysis is the fact
that the grains overlying the compartment RER/
Mit could not be assigned to RER only in this
study but could be assigned to RER in the follow-
ing study of glycoprotein synthesis in trophoblast
(41). The results of other autoradiographic (1,
15) and biochemical (35, 62) studies of a variety
of tissues, including human placenta (38), are consistent with mitochondrial protein synthesis.

The second advantage of the hypothetical grain
analysis is to make estimates for the activity within
different structures that are consistent with the
distribution of autoradiographic grains observed.
In addition, the method also allows one to calcu-
late estimates for the error of activity per unit
area and total activity in each structure assumed
to be a source (5). The standard errors for many
of the values in this study were rather high. This
is attributable to the large number of sites and
sources chosen for analysis. Because most grain
analysis methods are essentially empirical, a pre-
diction must be made as to where the activity in
the autoradiographs is likely to be located. When
more sources are assumed to contain activity, it
becomes easier to obtain low chi-squared values,
but the uncertainty in the activity estimates in-
creases. Since this was the first electron micro-
scope autoradiographic study of trophoblast, ar-itrary pooling of compartments was kept to a
minimum, thus yielding the relatively large num-
ber of sites and sources for analysis with the
attendant high error in the activity measurements.

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