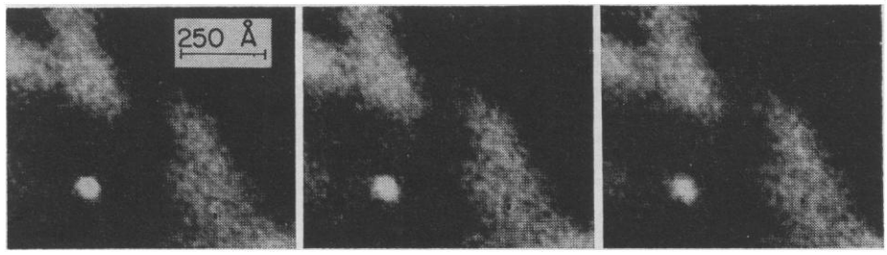

Fig. 3. Polytropic montage made by photographic superimposition of the component images in three different ways so as to accentuate the details lying at different levels in the specimen. With change in level, the gold-sol particle (bottom left) goes out of focus; virus particles, while maintaining a constant general arrangement, are changed with respect to their fine detail.

(Fig. 2D) by application of a simple correlative test to the 12 components of each montage point: let N be the number of component points with gray value higher than 5, and let the gray value of the montage point equal $(N-5)$ if $(N-5)$ is positive or let the gray value of the montage point be 0 if $(N-5)$ is negative or 0.

As a means of enhancing contrast, the usefulness of the polytropic montage is well demonstrated by Figs. 2 and 3. Good, high-contrast images have been obtained of unstained, unshadowed particles of tobacco mosaic virus. I anticipate that even smaller biological particles may be similarly imaged by use of a larger number of component micrographs taken of a specimen in various directions.

Although I have not yet objectively tested resolution for a polytropic montage, there is every reason to expect that it will be limited only by the resolution quality of the original micrographs. By comparison other factors, such as the finite size and number of scan points, nonlinearity in the cathode-ray tube of the Eyeball, and errors of image displacement, can be made negligible simply by increasing the magnification of the micrographs and scanning a correspondingly smaller area of the specimen.

Closely related to the question of resolution is that of depth discrimination. From geometrical considerations one can see that the montage has a certain "depth of focus" analogous to that of a camera. Two factors determine the degree of depth discrimination: the fineness of the details under consideration, and the angle of tilt, which is geometrically analogous to the F number. For example, if the tilt angle were increased from its present 20 deg to 45 deg, the depth discrimination, for the finest details observable, would be comparable to the lateral resolution and would be limited by only the quality and number of the original micrographs.

Thus the polytropic montage seems to offer a means of determining the three-dimensional structures of low-contrast biological specimens at a resolution of 3 Å, or the best resolution attainable with existing electron microscopes. I have not yet reached this point, but preliminary efforts have produced images of tobacco mosaic virus comparable in fineness of detail to those obtained by shadowing a similar specimen with tungsten or rhenium (*8*). Still to be determined is the extent to which the fine details appearing in the montage represent real structures of the virus rather than residual noise that may have survived this attempt at its elimination.

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1. Other sources of pictorial noise are photographic grain, and the ultimate inhomogeneity of the electron beam composed as it is of discrete particles. If the specimen is stable in the electron beam, both these kinds of noise can be reduced to any desired level by superimposition of a sufficient number of micrographs of the same area of the specimen.
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9. Performed under the auspices of the AEC. I thank James Yoshiyama for help with all phases of the experiments, especially electron miscroscopy; Landon Bruce for writing the computer programs for digital superimposition and parallax computation; and John Beard who was largely responsible for the high performance of the Eyeball. Other contributors of advice and assistance were K. Bertran, R. Bjorklund, R. De Saussure, D. Dixon, G. Michael, and S. Stone.

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Human Hormone Production in vitro

Abstract. *A new in vitro hormone-synthesizing cell system, line BeWO, derived from human gestational choriocarcinoma, has been permanently established; a high degree of synthesis of functional hormone has been maintained for 18 months in tissue culture. Predominantly glycolytic metabolism, dependent on the level of glucose in the medium, has been observed. Many facets of placental and tumor-cell metabolism, growth, and differentiation are found in this unique multipotential cell.*

The trophoblast of the human hemochorial placenta performs many functions essential to support of the developing embryo. Most fundamental among the absorptive, nutritive, and biosynthetic functions is the production of glycoprotein hormone. With the tissue-culture system it was demonstrated (*1*) that the pregnancy gonadotropin was synthesized in the placenta rather than in the maternal pituitary. This hormone closely resembles the luteinizing hormone of the anterior pituitary in both biological and molecular characteristics.

Establishment of continuous strains from the normal trophoblast that would provide a reliable source of hormone for clinical and research purposes has been unsuccessful. The placental cell type associated with production of human chorionic gonadatropine (HCG) has been identified (*2*), but it has not yet been possible to propagate continuously this cytotrophoblastic stem cell of the normal placenta.

Of the two villus epithelial cells, the cytotrophoblast and syncytiotrophoblast, the former represents a stem cell and gives rise to the latter by a process of differentiation (*3*). The earliest and most fundamental property of the trophoblast is synthesis of HCG. This glycoprotein is capable of maintaining the corpus luteum function of the ovary until the time of complete placental autonomy.

A rare and aggressive malignant tumor of the placenta, chorioepithelioma, is characterized by production of large quantities of the pregnancy hormone detected in patients bearing this tumor immediately postpartum. Histologically this tumor comprises many mitotically active cytotrophoblasts with moderate degrees of syncytial differentiation. No biological differences have

been shown between the HCG from this tumor and that of normal pregnancy.

It has long been recognized that the multipotential nature, high proliferative capacity, absence of connective-tissue stroma, stem-cell predominance, and synthesis of a unique hormone as a marker render this cell most desirable for in vitro isolation and propagation. Using the roller-tube method of culture (*4*), employing a reconstituted tropocollagen matrix, we have established the first human hormone-synthesizing cell line; it has been in continuous culture for 18 months (Fig. 1). Similar functional lines of rat and mouse endocrine tumors in vitro have been reported (*5*). The BeWo line thus provides an important in vitro human-cell model for the study of differentiation and hormone synthesis, and one model for testing cancer-chemotherapeutic agents.

Table 1. Hormone assays; I.U., international units.

Triplicate cell counts (×100)	Mean value, three animals (I.U.)	Gonadotropin per cell (10^{-5} I.U./24 hr)
	Flask A	
15,572	16.70	1.05
15,742		
16,130		
	Flask B	
12,244	11.70	0.95
12,204		
12,359		

The line was derived from a human choriocarcinoma previously transplanted and maintained by serial passage in the hamster cheek pouch (*6*); it has been permanently established and propagated continuously in vitro. Production of the pregnancy hormone HCG has remained uninterrupted through more than 50 passages, with an available fourfold increase in mass every 7 days.

Animal bioassays and immunoassays of the medium in which the cells are grown show positive responses, with as little as 0.1 ml of culture fluid, in all currently used pregnancy test systems. The Delfs immature-rat bioassay (*7*) has been used to evaluate the level of hormone response. The medium in contact with the cultures for 24 hours is washed with equal volumes of ether to remove estrogenic steroids that would erroneously increase the uterine weight. The gonadotropin is precipitated with five volumes of 95-percent ethyl alcohol, dried in a partial vacuum, and stored at 5°C. Rats of the hooded strain maintained by Delfs, aged 21 to 22 days and weighing 36 to 40 g, are injected with dilutions of the precipitate dissolved in normal saline over a 36-hour period, and killed at 72 hours. The uteri are weighed, and their increase in weight, representing stimulation by way of the ovary from injected gonadotropin, is converted to international units of gonadotropin from a standard dose-response curve.

The assays and cell counts were conducted in the following manner. The cellular contents of duplicate flasks, upon reaching maximum cell density 10 to 11 days after subculture, were dispersed with 0.25 percent trypsin and counted with a Coulter counter. The fluid in contact with these cells for 24 hours was extracted and assayed for chorionic gonadotropin. Hormone yield per cell was then calculated (Table 1). With this bioassay about 10^{-5} international unit of gonadotropin is produced per cultured cell over a 24-hour test period. This value varies, however, depending on the period of growth during which the fluid is sampled; young, freshly subcultured cells give lower, and exponentially growing cultures give higher values.

The tissue was initially grown on reconstituted rat-tail collagen matrix by a modification of a reported method (*6*). Gradual adaptation to glass and Falcon plastic flasks has since been accomplished. The medium initially consisted of Waymouth's MB 752/1 diluted with Gey's balanced salt solution and supplemented with placental-cord serum stored until all biologically active HCG normally present was no longer detectable by bioassay.

These cultures receive new fluid me-

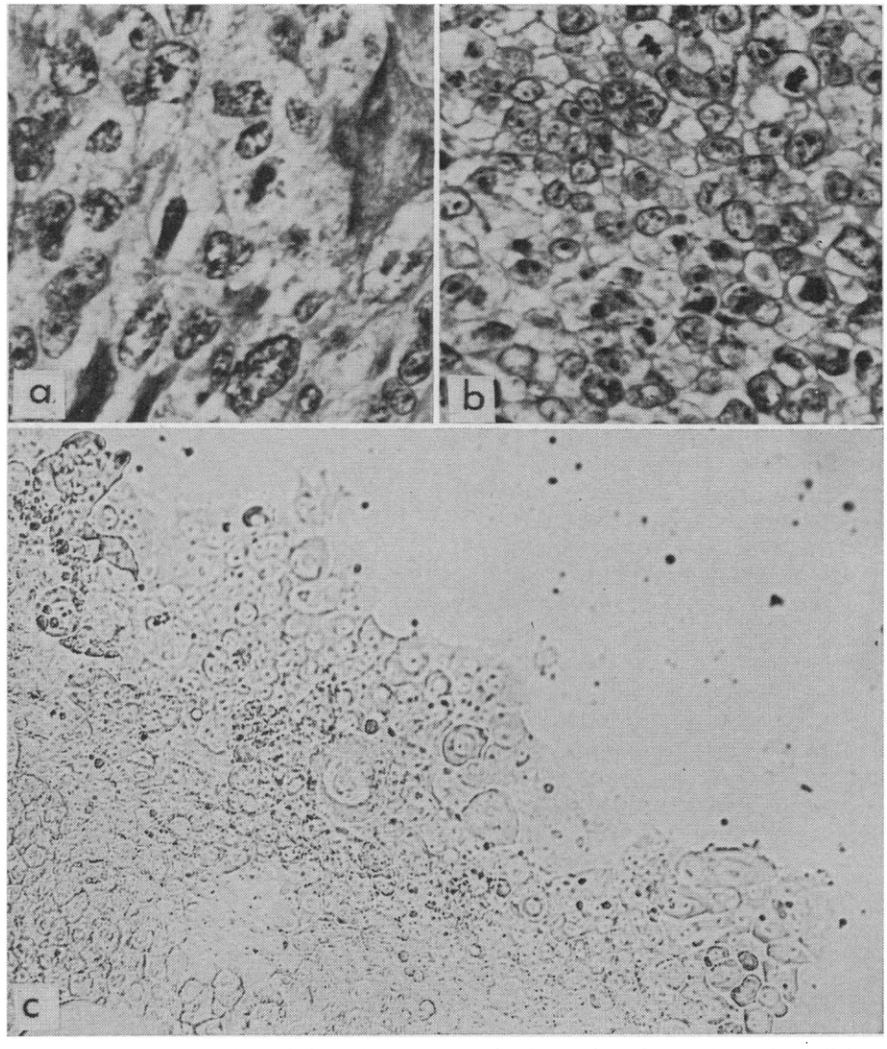

Fig. 1. (a) Tissue section of the primary tumor in the patient from whom the cell line was derived (× 500). (b) Multilayered colony removed from a roller-tube subculture, fixed, and sectioned; the section contains only cytotrophoblast without evidence of maturation to syncytial layers under the present culture conditions of rapid replication (× 500). (c) The living culture in the roller tube after 18 months of continuous culture and weekly subculturing (× 150). The pregnancy hormone is abundant in the medium in which these cells are grown; the cells grow as multilayered organoid colonies, with only a limited monolayer proceeding from the central mass.

dium every 24 hours because of the high rates of metabolism and production of CO_2. Subculture is performed weekly by microsurgical dissection as the cells grow in tissue organoid form not unlike normal cytotrophoblast; in culture they reach a multilayer thickness of six to seven cells within 1 week. Dispersion with trypsin and other proteolytic agents has been possible, but subsequent viability is poor.

The BeWo line has maintained the cellular characteristics of the cytotrophoblast of the patient's original tumor, with no differentiation to syncytium in the rapidly growing log phase of growth. The line is highly aneuploid, varying widely in chromosome numbers from hypertriploid to octoploid.

Variations in the level of glucose in the medium exert profound changes on cellular growth and on glycogen content as shown by periodic acid–Schiff staining; the intensity of glycogen staining shown by this technique is very high in cultures maintained with glucose at 340 mg percent in the medium, whereas much less glycogen and less-vigorous growth are seen in cultures maintained on glucose at 140 mg percent. This decrease in growth rate can be seen in the increased time required by cultures on lower concentrations of glucose to reach the approximately 8-mm-diameter colony size when subculture is performed: with glucose at 340 mg percent in the medium, eight subcultures are possible within 60 days; with glucose at 140 mg percent, only four subcultures are possible. The utilization of glucose, the synthesis and breakdown of glycogen, and the participation of the key enzymes of the glycolytic cycle remain to be evaluated.

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17 January 1968

Ethanol Increases Hepatic Smooth Endoplasmic Reticulum and Drug-Metabolizing Enzymes

Abstract. *Rats were fed ethanol for 2 weeks along with diets either adequate or deficient in protein and choline, the latter intake being similar to that of many alcoholics. Hepatic lipids, smooth endoplasmic reticulum, and the activities of drug-metabolizing enzymes (aniline hydroxylase and nitroreductase) were increased with the adequate diet but more so with the deficient one. These results may explain the increased tolerance by alcoholics of drugs such as sedatives.*

The association between drug-induced hypertrophy of smooth-surfaced endoplasmic reticulum (SER) in hepatocytes and increased activities of hepatic drug-metabolizing enzymes is well documented (*1*). Both in the rat (*2*) and in man (*3*) chronic administration of ethanol also leads to increase in and vesiculation of SER in hepatocytes. Because of these morphologic alterations and the possible clinical implications, it was of interest to determine whether chronic administration of ethanol affects hepatic microsomal drug-metabolizing enzymes.

Thirty-two male Sprague-Dawley rats, initially weighing about 250 g, were studied. One ("nondeficient") group of 12 animals was fed a described (*4*) complete diet in liquid form, with 18 percent of total calories as protein; the remaining 20 (the "deficient" group) received a deficient diet.

The two diets were identical except that the deficient diet lacked choline and 75 percent of casein, methionine, and cystine; these shortages were isocalorically replaced by carbohydrate. Ethanol was isocalorically substituted for carbohydrate and comprised 36 percent of total calories (*2*). Each rat fed ethanol was matched with a control rat that received the appropriate diet without ethanol.

After 15 days the animals were killed by exsanguination, and portions of the livers were homogenized in 0.25*M* sucrose at 0°C. In four pairs of animals fed the deficient diet, the microsomal fraction was isolated by centrifugation, total microsomal protein was measured, and the enzyme activities in washed microsomes were determined. The concentration of cytochrome P450 in isolated microsomes was measured in one pair from each group (*5*).

Contents of DNA, total lipids, and triglycerides (*4*) also were determined. Small blocks of liver were fixed in ice-cold 1-percent buffered osmic acid and embedded in Epon; ultrathin sections were then prepared for examination with the electron microscope.

The mean concentration of triglycerides in control animals was 2.5 times greater in the deficient group than in the nondeficient group ($P < .05$) (Table 1). After administration of ethanol, mean hepatic triglycerides increased fourfold in both groups ($P < .001$). The mean increase in hepatic triglycerides induced by ethanol was 2.4 times greater in the deficient group than in the other. Because of the greater content of hepatic lipids in the deficient animals, enzyme activities were expressed in terms of fat-free wet weight of the liver.

After administration of ethanol the mean aniline hydroxylase activity was strikingly increased in both groups; that of nitroreductase was moderately increased (Table 1). When the difference in aniline hydroxylase activity between control and alcohol-treated animals was calculated for individual pairs, the increase after administration of alcohol averaged 658 ± 140 units ($P < .001$) in the nondeficient and 1231 ± 181 units ($P < .001$) in the deficient group (means $\pm$ S.E.). The mean increase in aniline hydroxylase activity after administration of alcohol in the deficient group was therefore double that in the other group. Aniline hydroxylase activi-

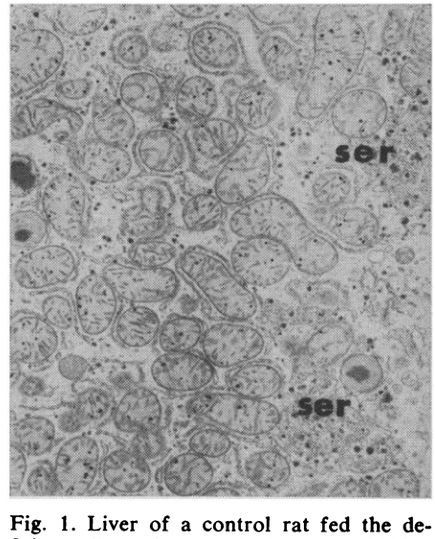

Fig. 1. Liver of a control rat fed the deficient diet without alcohol (× 9000). The smooth endoplasmic reticulum (*ser*) is not increased; mitochondria appear normal.