optimum level of sodium sulfate supplementation was 0.7% of the diet. Incorporation into taurine of labeled carbons occurred when cysteine-3-C^{14}, alanine-C^{14}-UL, ethanolamine-1-2-C^{14}, serine-3-C^{14}, serine-C^{14}-UL, and glycine-1-C^{14} were administered.

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Received June 20, 1966. P.S.E.B.M., 1966, v123.

Reversal of Contact-Inhibition in Primary Amnion Cultures by Hydrocortisone.* (31589)

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Surface contact by diploid cells in primary cultures results in certain alterations which in aggregate are given the term "contact-inhibition." Physical changes noted early after cells come into contact include cessation of motion of the undulating cytoplasmic membrane, decreased cellular motility and later, cementation of the intercellular membranes(1). Biological changes associated with these physical phenomena are decreased mitotic activity(2) and decreases in protein, RNA and DNA metabolism with disappearance of free ribosomes from the cytoplasm(3).

Confluent monolayers of primary human amnion cells are relatively stable in cellular density over long periods of maintenance *in vitro* under conditions where incubation is provided at 37°C in stationary racks and culture medium is replaced every 48-72 hours. That replication is present in such monolayers is difficult to determine, the mitotic index approaching 0.1% or less(4).

Reversal of contact-inhibition and growth induction were observed in amnion monolayers when hydrocortisone was added to the culture medium.

Material and methods. Amnion cultures. Cells were obtained throughout from fresh, clean amniotic membranes using versene 0.1% and trypsin 0.25%, explanted into stationary roller tubes or 3 oz prescription bottles in Eagle's minimal essential medium (EMEM) with 20% mammalian serum (10% human, 10% bovine) and incubated at 37°C. These primary cultures became confluent after periods of 5-7 days, at which time maintenance medium was added, containing 10% equine serum. In early experiments, media were completely replaced every 72-96 hours; in later experiments, every 48 hours.

Cell and nuclei counts. Initially, cells were enumerated in suspensions prepared by treating the monolayers first with 0.1% versene and then with 0.2% trypsin, which rendered

* These investigations were made possible by a contract between the Office of Naval Research NONr 3310(00) and The Variety Children's Research Foundation. During a part of the work, Dr. A. Marti was supported as a postdoctoral fellow under USPHS Training Grant CA-5075.

the suspensions relatively monodisperse. However, dispersal of cells in harvests made after long periods of cultivation was difficult; some cell destruction occurred with the periods of time necessary to break up the monolayers. Therefore the following procedure was established: monolayers were washed with phosphate buffered saline (PBS) pH 7.4, 3 times, treated with citric acid 0.1 M, 1 hour at 22°C, and scraped with a rubber spatula. Total cell removal was ascertained by microscopic inspection. Aliquots of the cell sap-nuclei suspension were centrifuged at 2000 rpm for 20 minutes and the sediments resuspended in a mixture of 0.1 M citric acid and 0.1% crystal violet for 1 hour at 22°C. Cells were enumerated in a standard hemocytometer by counting the stained nuclei 3 times in each of 2 replicate samples and taking average values. Nuclei suspensions were found to be stable for at least 4 hours at 22°C; rarely were cells with cytoplasmic membranes found in these suspensions. Nuclei counts were found to be 40-100% higher than counts of whole cells in suspensions resulting from versene and trypsin treatment.

Cell protein and DNA determinations. Protein was determined by the method of Lowry (5), as used by others(6). In one experiment, cells were solubilized *in situ* with alkali; in the remainder, replicate aliquots of the cell sap-nuclei suspensions were neutralized with 1 M NaOH and stored at —20°C until protein determinations were made. Nuclei sedimented from the cell sap-nuclei were washed with 0.2 N perchloric acid at 22°C for 20 minutes and the sediment extracted with 0.5 N perchloric acid at 90°C for 20 minutes. The supernatant of the hot acid treatment was then used for determination of cellular DNA, following the diphenylamine method of Dische(7). Replicate samples were compared to a standard high grade polymerized DNA; values for extracted nuclei varied over a narrower range than did values based upon whole cell extracts.

Mitotic index. Replicate 3 oz prescription bottle cultures of control and treated cultures were treated with colchicine for periods of 2-18 hours at 37°C. The medium was then poured off, the cells were removed by versenization and scraping, and collected by centrifugation at 1000 RPM for 20 minutes. The button was resuspended in a hypotonic solution and metaphase preparations made by a modification of the technique of Saksela and Moorhead(8). 1,000 cells at random were counted in each of 3 culture preparations in 2 experiments and the number of cells in metaphase per 1,000 cells counted was taken as the mitotic index.

TABLE I. Effects of Cortisol upon Mitosis of Contact-Inhibited Amnion Cells.

Day*	Control	Cortisol
0	0/1000†	
13	0/1000	13/1000

* Incubation period in days after cortisol added to the medium.
† Ratio of metaphase arrests/cells in interphase.

Hydrocortisone. Hydrocortisone phosphate (SoluCortef, Upjohn) for intravenous use was diluted in distilled water at a standard concentration and used in preparation of medium by dilution for maintenance of the cultures. Hydrocortisone alcohol (cortisol) was put into solution with ethyl alcohol 10% and incorporated into the medium by dilution at the time of preparation. The final concentration of alcohol in the medium was 0.01%; this concentration alone failed to influence cell growth.

Results. The first morphological evidence of replication in the treated monolayers was appreciated at approximately 8-10 days after steroid was added to the medium; distinct lines of separation between individual cells in the monolayers became evident. At this time and after longer periods of treatment, it was also apparent that cells in the treated monolayers were of smaller diameter than those of the controls (Fig. 1).

The maximal proportion of dividing cells in the treated cultures was determined to be 2%. In contrast to this, it was difficult to find a single cell in metaphase in a monolayer which was maintained in medium containing no steroid (Table I).

Further proof of cell growth was obtained in a series of 4 experiments in which hydrocortisone phosphate was incorporated into the medium of amnion monolayers in roller tube cultures. As little as 10 μg% in the medium seemed to have the same effect to induce

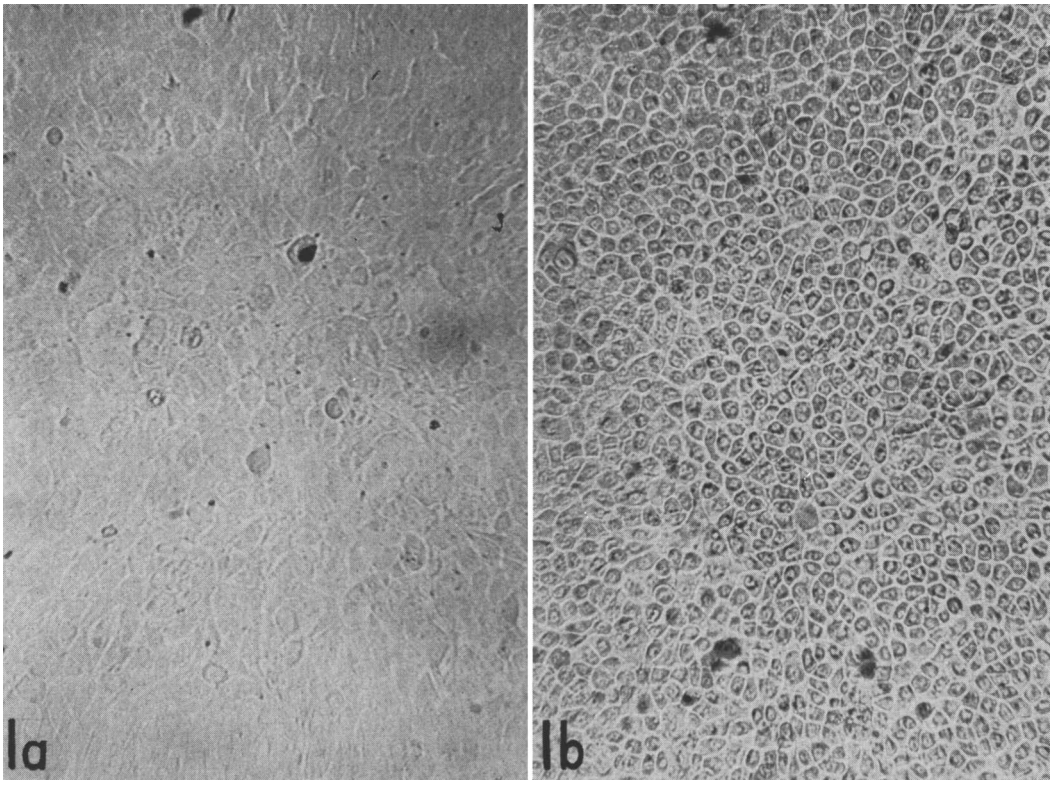

FIG. 1a, b. Primary human amnion cells in monolayers *in situ*. a, untreated control monolayers. b, monolayers treated with hydrocortisone phosphate over a 28-day period, with complete replacement of media every 3-4 days (× 600).

growth as the maximal amount employed, 25 mg% (Fig. 2). Only the highest concentration used, 25 mg%, appeared to cause some granularity and vacuolization in the cells, but the monolayers remained intact. In the experiments shown and others, at time of initial hydrocortisone treatment, the cell density varied from 2×10^4 to 8×10^4 per ml; at these low densities, the magnitude of the growth response to hydrocortisone was a 4-8-fold increase in cells over a 28-day period of cultivation. In contrast, the cell density of the untreated cultures slowly but progressively decreased despite frequent replacement of maintenance medium containing 10% uninactivated equine serum. Cells in all cultures were in good condition throughout the periods of cultivation. The cell borders in treated cultures could be readily distinguished microscopically, without fixation or staining, even at the highest cell densities. In one experiment (Fig. 2b), monolayers were washed 3 times with buffered saline, treated *in situ* with Lowry's alkali reagent and aliquots used for determination of cellular protein. The content of protein per 1 ml tube culture generally reflected the increase in cell density of the culture, with approximately 3 times as much protein present in treated cultures as in the untreated controls.

Incorporation of a standard amount (100 μg%) of cortisol (hydrocortisone alcohol) into the medium of monolayers with cell densities of $3\text{-}7 \times 10^5$ resulted also in reversal of contact-inhibition and growth in the monolayers.

At a density of 3×10^5 cells per ml the response was a 3-fold increase in cell density and in total protein over a 30-day period, while in the untreated cultures a 50% cell loss was evident (Fig. 3a). At the higher density of approximately 7×10^5 per ml, it was evident again that the gradual but progressive cell loss in the heavy monolayers could be reversed by hydrocortisone. After

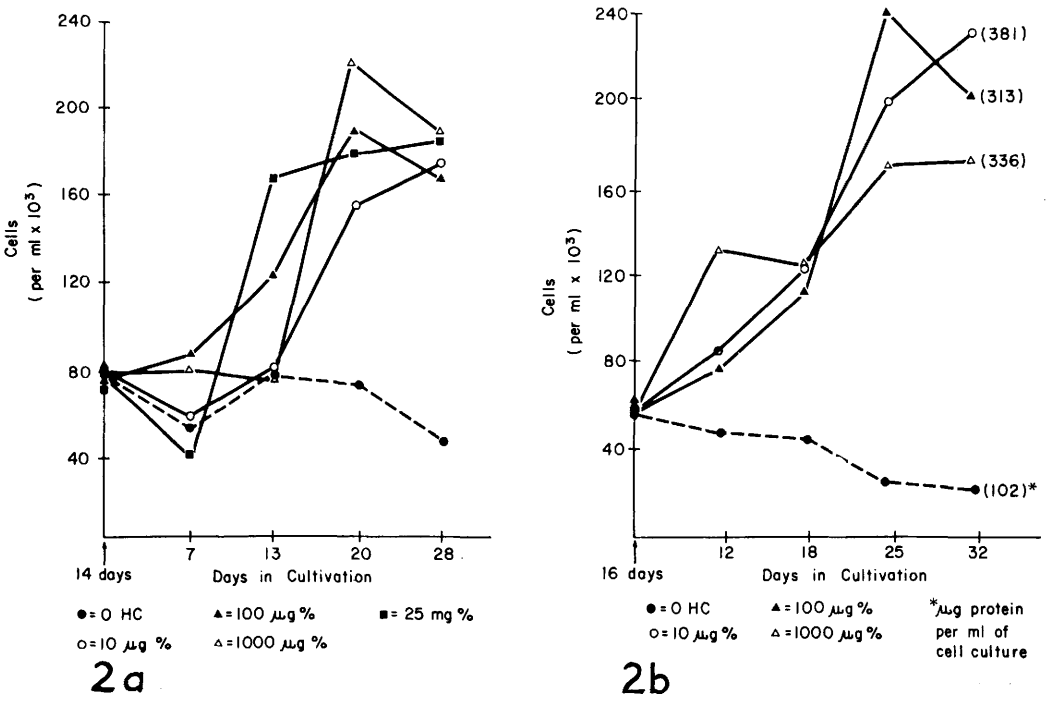

FIG. 2a, b. Effects of hydrocortisone phosphate upon monolayers of primary human amnion cells. Cell counts referred to original volume of fluid in culture are indicated on ordinate. Periods of cultivation after steroid was added to the medium are indicated on abscissa. Arrow (↑) indicates age of confluent monolayers at time experiment was initiated. Broken lines indicate control cultures, solid lines indicate treated cultures.

a lag period of at least 48 hours there was general evidence of increases in cell density or in total cellular protein. The maximal cell density reached in these and other cultures in a series of 5 experiments in response to hydrocortisone was approximately 1×10^6 per ml or 2.7×10^5 per cm^2 of substrate surface.

Enumeration of cells, and determinations of cellular protein and DNA from the same individual samples of treated and untreated replicate monolayers in culture flasks harvested at intervals in a series of 5 experiments further documented the effects of hydrocortisone upon non-replicating, high density amnion cultures (Fig. 3b). DNA per culture increased in a linear relationship to cell density, thus implying that the predominant cell of the sample was diploid. On the other hand, protein per culture increased more rapidly than cell density and this was found to be the case in the majority of experiments, regardless of initial cell density. Likewise, the protein curve of untreated cultures was observed not to follow the slope of the curve of loss of cells from the monolayer. Calculations of all protein values revealed that generally, protein per cell (μg protein/10^6 cells) in all treated and untreated cultures gradually increased as the age *in vitro* of the cells increased.

Beginning with a standard concentration, $\log_{10}$ dilutions of hydrocortisone were prepared, added to the medium of replicate flask cultures and completely replaced 6 times over a total period of 14 days, in order to determine the actual cumulative amount of the corticosteroid required to allow growth which resulted in 2-fold increases in cell density, in that period.

Cumulative amounts of steroid were referred to average cell counts of controls, and calculations were made to determine arbitrarily the number of molecules of steroid that were available to the cells throughout the experiment (Fig. 4). Based upon these calculations, approximately 10^8 molecules of hy-

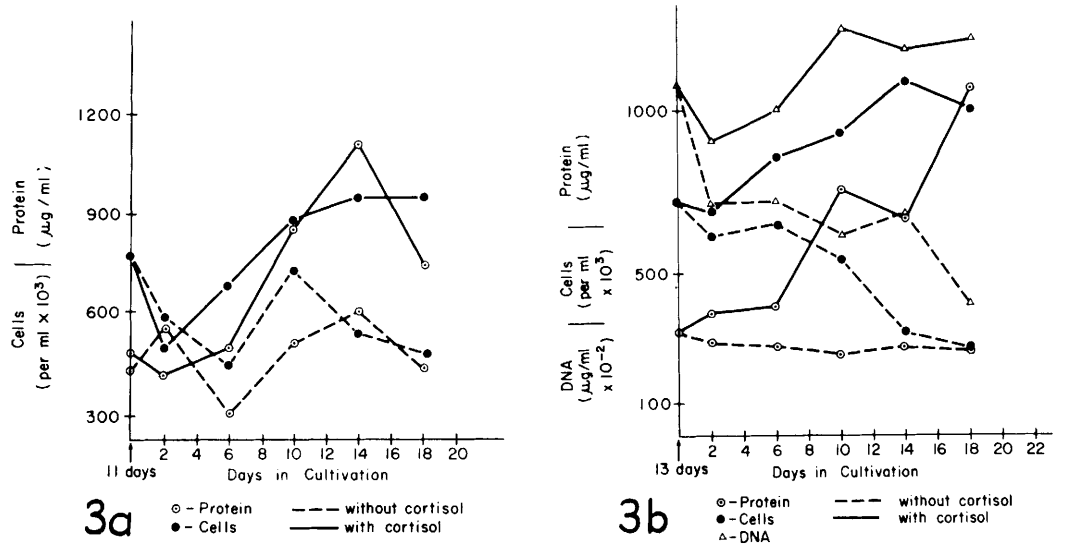

FIG. 3a, b. Effects of hydrocortisone upon amnion monolayers of high cell density. Cell count, protein and DNA values were referred to milliliter aliquots of cell sap-nuclei suspensions, recorded on ordinate. Arrow at junction of ordinate and abscissa indicates age *in vitro* of the monolayers at beginning of experiments.

drocortisone per cell were required to allow growth resulting in 100% increase in the cells. This corresponds roughly to 2×10^{-7} molar solution of hydrocortisone, a value similar to that found by Ambrose for initiating secondary antibody responses in cultures of lymph node fragments, maintained in a protein-free medium(9).

It is probable that lesser amounts of hydrocortisone are required to initiate the metabolic events that result in cell growth, in this system, for by measuring total cellular protein in a treated culture, increases could be detected as early as 48 hours after addition of the corticosteroid.

Discussion. In previous studies, when corticosteroids have been incorporated into the medium of mammalian cells growing in tissue cultures, some of the changes assumed to be the result of the action of the steroid have been lessened viability, inhibition of growth, lysis, enhanced viability and increased growth (10). Such effects have been noted to vary with the type of cells used and the concentration of steroid applied(11,12,13). Recently, Polet(14) demonstrated that hydrocortisone acted upon primary amnion cells in a manner such that trypsin and versene disruption of intact monolayers was prolonged and solubilization of cells by lauryl sulfate was prevented.

The data reported here clearly demonstrate that hydrocortisone in physiological concen-

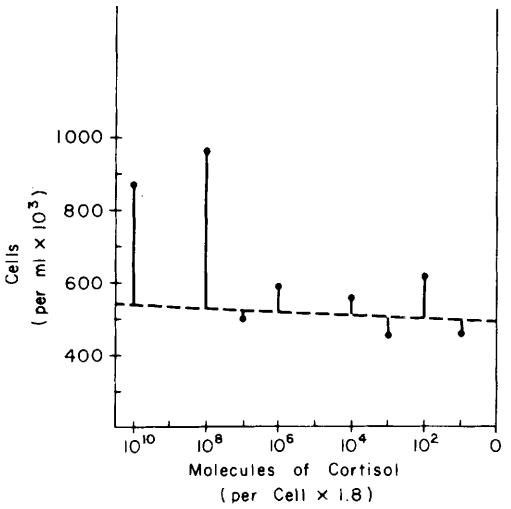

FIG. 4. Calculated cumulative amount of hydrocortisone per cell required to induce 100% increase in cells in a 14-day period. - - - - represents cells in untreated cultures. Vertical lines indicate net increase or loss of cells in replicate-treated cultures at the end of 14 days. Abscissa shows calculated values on a molecular basis (molecules of hydrocortisone per cell), referred to average cell density of control cultures, 0.5×10^6/ml.

trations in the medium of primary amnion cells induces a profound change in the state of contact inhibition of the culture. The early morphological evidence of the change is the development in the monolayer of distinct lines of cleavage between individual cells; as the density of the monolayers increases, it is apparent that the average diameter of the cells is less in the tightly packed culture.These morphological alterations are accompanied by the reappearance in the monolayers of dividing cells and the magnitude of the replicative response (mitotic index) is approximately equal to the rate of division of primary amniotic cells freshly explanted into culture medium and not yet in a state of contact inhibition. This rate of mitotic activity accounts for the slow increase in cell density over the periods of study employed. Removal of steroid from the treated cultures generally resulted in a rapid deterioration with cell loss.

The morphological changes are accompanied by increases in total protein and DNA in the cultures, and these metabolic alterations may be noted as early as 48 hours after hydrocortisone is incorporated into the medium.

It is concluded that the cell growth apparent in the treated cultures was composed of new diploid cells for the following reasons: 1) at no time did there appear areas of cell transformation in the cultures, 2) growth continued to be in an organized monolayer, 3) the rate of mitosis of treated cultures (2%) did not approach that rate of mitotic activity found in cultures of transformed cells, and 4) it was not possible to passage cells of the treated replicating monolayers.

The magnitude of the response to hydrocortisone was related to the density of the monolayers at the time of initial treatment: a 3-8-fold increase in cells occurring in those of lowest density and a 2-fold increase occuring in the ones of highest density. Obviously, the confines of the substrate area available for the growth of the cells imposed a limit upon maximal cell proliferation in the monolayer. These maximum densities were accommodated by a decrease in cell size, and, perhaps, by alterations in surface configurations of the initially flat cells.

These studies do not define the temporal relationships of the various events influenced by the steroid, but they do indicate that increased protein synthesis was the first manifestation of the metabolic changes, which were observed under the experimental conditions.

Summary. Contact-inhibited, non-replicating primary amnion cell monolayers responded to physiological concentrations of hydrocortisone with replication and increase in cell density, total cellular protein and DNA in the cultures. There was no evidence of cell transformation over long periods of steroid treatment. It is concluded from these data that hydrocortisone provided a stimulus to metabolic activity sufficient to overcome contact-inhibition, or induced alterations in the cytoplasmic membranes *in situ* which allowed expression of the normal growth potential of the primary cells.

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Received June 24, 1966. P.S.E.B.M., 1966, v123.