MITOTIC AND NONMITOTIC MULTIPLE-LAYERED PERFUSION CULTURES

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ABSTRACT

Cell types in addition to those previously described (Kruse et al. 1963. *J. Nat. Cancer Inst.* **31**:109; Kruse and Miedema. 1965. *J. Cell Biol.* **27**:273) were found to form multiple-layered cultures by perfusion-culture technique. Dense populations containing 43 × 10^6 embryonic rat muscle (NF-ER) cells, 23 × 10^6 diploid human tonsillar (NF-JAM) cells, 77 × 10^6 human pleural effusion isolate (RPMI 2650) cells, 35 × 10^6 embryonic diploid human lung (Flow 2000) cells, 21 × 10^6 bovine lung (FB4BM) cells, 108 × 10^6 bat lung (Tb1Lu) cells, and 81 × 10^6 SV-40 virus-transformed embryonic diploid human lung (WI-38VA13A) cells were obtained in 6–14 days from dilute inocula in T-60 or T-75 flasks; these were equivalent to about 4, 3, 3, 4, 2, 4, and eight monolayers, respectively. Perfusion of an NF-ER culture for 6 wk with medium plus 10% whole calf serum yielded a cell density equivalent to 12 monolayers (140 × 10^6 cells per T-75 flask). This culture exhibited random labeling of nuclei from bottom to top after pulsing for 90 min with thymidine-^{3}H. Medium plus 0.1% serum maintained NF-JAM cultures at constant viable cell numbers with virtual absence of thymidine-^{3}H labeling. Similar results were obtained with WI-38 cultures, but WI-38VA13A cells continued active DNA synthesis and mitosis in medium with 0.1% serum to form 16–20 layers of cells (191–239 × 10^6 cells per T-75 flask) in 27 days. WI-38VA13A cells ceased proliferation and became nonviable rapidly in serumless medium.

INTRODUCTION

Previous reports (e.g. Kruse et al., 1963; Kruse and Miedema, 1965) described a system for perfusion of replicate cell cultures, in which multiple-layered populations were produced from six different cell types. Included in these types were diploid human cells exhibiting the finite lifetime phenomenon in vitro described by Hayflick and Moorhead (1961). Multiple-layering of DON Chinese hamster cells reached the equivalent of as high as 17 monolayers, i.e. the monolayer equivalents (M.E.) = 17. This report describes similar work with other cells of diverse origins; further, it (*a*) furnishes evidence that DNA synthesis occurred throughout a culture of embryonic rat cells of M.E. = 12 produced with influent medium plus 10% serum, and (*b*) shows that, by using perfusion medium containing but 0.1% serum, multiple-layered cultures of human diploid cells could be maintained for weeks in a stationary phase, i.e. without mitosis, with retention of cell viability, and with environmental stability.

Regarding (*b*) above, certain fundamental differences between cultures of viral-infected and noninfected cells were observed in their response to medium containing 0.1% serum; the possible significance of these differences is discussed with

reference to the extensive studies reported recently on control of cell division by serum factor(s) (e.g. Amos, 1967; Temin, 1967; Todaro et al., 1967; Bürk, 1966).

MATERIALS AND METHODS

The perfusion system for replicate T-flask cultures employed in this study has been described in detail elsewhere (Kruse et al., 1963; Kruse and Miedema 1965); briefly, it consists of T-60 or T-75 flasks (60 cm^2 and 75 cm^2 floor surfaces, respectively) fitted with influent and effluent lines, the former being attached to reservoirs containing fresh medium and the latter being attached to receptacles for the effluent medium. The rate of feeding, i.e. perfusion, is controlled by compression of the influent lines under a spring-loaded clamping bar, opened to permit influent flow and closed at predetermined timed intervals by connection with a motorized shaft and cam assembly. As cell populations increase, the timing intervals are shortened and rates of perfusion are increased.

Cells of bovine origin, designated FB4BM, and of bat origin, designated Tb1Lu, were supplied by Dr. A. Kniazeff, Naval Biological Laboratory, Oakland, Calif.; RPMI 2650 human cells were supplied by Dr. G. Moore, Roswell Park Memorial Institute, Buffalo, N. Y.; diploid embryonic human lung cells, designated Flow 2000, were purchased from Flow Laboratories, Inc., Rockville, Md; similar cells, WI-38, were supplied by Dr. L. Hayflick, Stanford University School of Medicine, Stanford, Calif.; WI-38VA13A (SV-40-virus-transformed WI-38 cells) cells were supplied by Dr. V. Cristofalo, The Wistar Institute, Philadelphia, Pa.; embryonic rat muscle cells, designated NF-ER, were obtained from Dr. M. K. Patterson, Jr., of this laboratory; human diploid cells, designated NF-JAM, were initiated in this laboratory from tonsil tissue obtained from an 8-yr-old male.

Stock cultures were carried in stoppered T-60 and T-75 flasks before subculturing to replicate flasks for attachment to the perfusion system. The medium used, 7a, was that given by Neuman and McCoy (1958) except that all the amino acids were the L-isomer and the concentrations of *i*-inositol, L-arginine·HCl, L-histidine·HCl·H_2O, L-lysine·HCl, hydroxy-L-proline, vitamin B_{12}, and glucose were changed to 36.0 μg/ml, 0.40 mM, 0.20 mM, 0.60 mM, 0.015 mM, 0.00075 μg/ml, and either 3.0 g/liter (experiments in Figs. 1, 2, 3A, 4A) or 1.0 g/liter (experiments in Figs. 3B, 4B, 5), respectively.[1] Whole calf sera supplements were 0–10% as indicated below. All media were prepared in this laboratory.

Perfusion experiments were begun with preconfluent cultures of M.E. = 0.1–0.7, i.e. 10–70% of the floor area of the T-flasks were covered with cells, and medium 7a plus 10% serum. When we desired to lower the serum content, we completely removed medium from the influent reservoirs which were recharged with fresh medium containing 1.0 or 0.1% serum or with serumless medium. Fresh medium was added to the reservoirs at 1- to 3-day intervals.

NF-ER cells in the third passage and of 1 month duration in vitro were perfusion-cultured for 6 wk; the multiple-layered culture was then pulsed for 90 min with 10 ml of medium containing 1 μc/ml of tritiated thymidine, specific activity 16.6 c/mmole. After washing with medium containing 10 mM nonradioactive thymidine, a section of the tissue was removed, overlaid on agar, fixed in 10% formalin, inbedded in paraffin, sectioned, and radioautographed.[2] In several other perfusion experiments, cultures were similarly pulsed for 30 min; after washing, the cultures were incubated in fresh medium for 30 min and aliquots of the cells were fixed on slides and radioautographed.

Cell suspensions were prepared by 15- to 30-sec treatment of the cell sheets with 5 ml of 0.05% pronase solution and aspiration in complete medium. Whole cell counts were made with a hemocytometer, and estimation of cell viabilities was made by dye-exclusion tests with trypan blue (Phillips and Terryberry, 1957) and lissamine green (Goldacre and Whisson, 1967).

RESULTS

Perfusion Data and Multiple Layering

Proliferation and formation of multiple-layered cultures of several cell types used in this study, including data on cell counts, pH control, and rates of perfusion, are illustrated in Fig. 1; the format is similar to that given for perfusion experiments with other cell types in previous reports (Kruse and Miedema, 1965; Kruse et al. 1967). Experiments were terminated at 6, 7, 8, and 14 days. Perfusion was not begun until the 3rd day in two experiments (see Fig. 1) because of relatively low initial inocula (less than 25% of the glass surface covered with cells). Confluency of the cultures was reached at

[1] A variety of media formulations designated McCoy's 5a and modified McCoy's 5a have appeared in the literature, including commercial supply catalogs; a summary of these, with appropriate corrections, is available from Dr. M. K. Patterson, Jr., The Samuel Roberts Noble Foundation, Inc., Box 878, Ardmore, Oklahoma 73401.

[2] We thank Mrs. Bettye Cox, Mr. Merle Maxwell, Dr. D. E. Kizer, and Dr. M. K. Patterson, Jr., for radioautographic portions of this work.

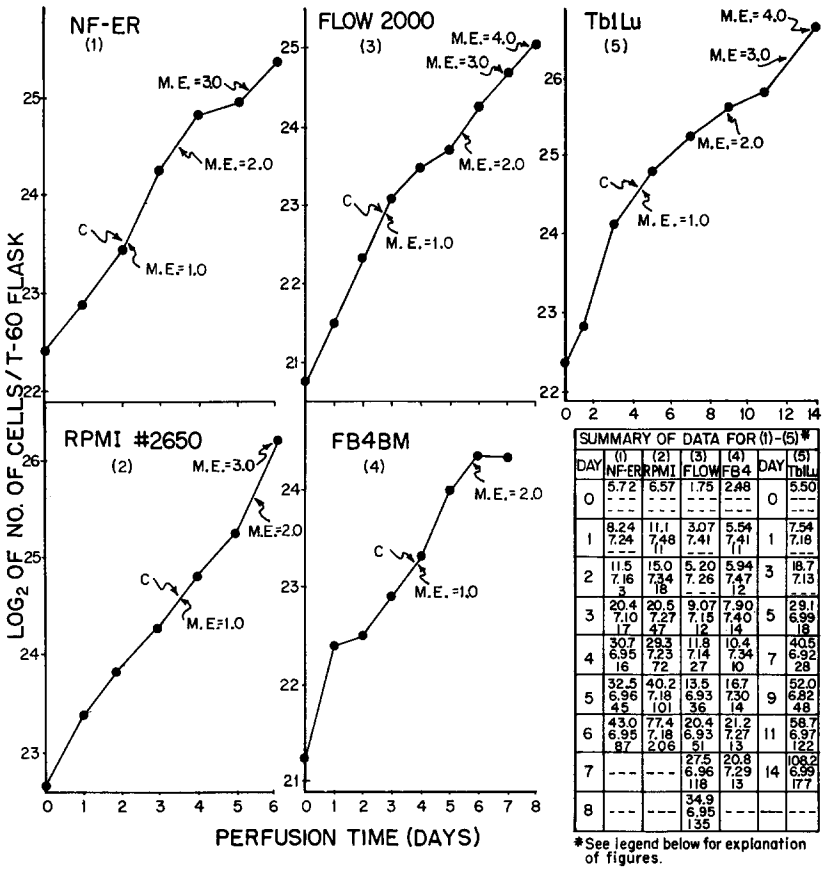

FIGURE 1 Illustrating proliferation curves, number of doublings (log_2), cell counts, pH control, and perfusion rates during production of multiple-layered populations of rat, human, bovine, and bat cells in a perfusion system for replicate T-60 flask cultures. The data in each square in the inset signify (from the top) cell numbers (millions T-60), culture pH, and perfusion rate in ml/day of medium 7a plus 10% whole calf sera. In each experiment, proliferating cultures in stoppered flasks were transferred to perfusion (day 0). *M.E.* = monolayer equivalents; this expression indicates how much the cell population developed beyond a monolayer (confluency) and is defined as: the total number of cells per flask divided by the number of cells required to cover the supporting glass surface. *C* = point of confluency (monolayer).

points designated *C* on the curves, followed by extensive population increases.

The permanent cell line RPMI 2650, derived from fresh pleural effusion from a human with a malignancy and found to be quasi-diploid (Moorhead, 1965), proliferated at a nearly constant rate. It reached a population density equivalent to three monolayers in the 6 day experiment, and rapid proliferation was still taking place when the experiment was stopped. Nearly identical results were obtained in a 6 day perfusion of embryonic rat muscle cells, NF-ER (Fig. 1). The diploid Flow 2000 line, of human embryonic lung origin and of finite lifetime in vitro, formed the equivalent of more than four monolayers in 8 days and was still proliferating when the experiment was stopped. Data and results from this experiment are nearly identical to those reported previously (Kruse and Miedema, 1965) with WI-38 cells. Since the FB4BM bovine cells had a very low glycolytic rate, a low perfusion rate of 10–14 ml per day per culture was employed which was sufficient to maintain pH of the cultures between 7.27 and 7.47 for the 7 day period, while the cell population developed to the equivalent of two monolayers.

The bat lung cells exhibited a pronounced tendency to form networks of interconnected cells and

to pile up in clusters before covering the floor area of the T-flasks. Estimation of the number of cells required to form one monolayer was therefore difficult, but appeared to be about 25 × 10^6 cells per T-60. Perfusion was continued for 14 days (last column in inset, Fig. 1) and the yield of cells was 108 × 10^6 cells per T-60, or M.E. = 4.3. A similar experiment with Tb1Lu cells, not illustrated in Fig. 1, yielded 112 × 10^6 cells per T-60 in 12 days.

DNA Synthesis and Nutrition of Multiple Layers

A random pattern of thymidine-^{3}H-labeled nuclei was found throughout the multiple layered tissue culture of embryonic rat cells, as illustrated in Fig. 2. Even though these cells had been perfused for 6 wk and had formed the equivalent of about 12 monolayers (140 × 10^6 cells per T-60 flask), DNA synthesis was detected throughout from bottom to top (see legend, Fig. 2). This result furnished evidence that the nutrition of multilayered cultures undergoing perfusion was apparently adequate throughout for DNA synthesis.

Response of NF-JAM, WI-38, and WI-38V13A Cells to Minimal Serum Media

When NF-JAM cells were perfusion-cultured in medium containing 10% serum for 6 days, they readily proliferated beyond confluency as shown in Fig. 3A. These cells, which were derived from the tonsil tissue from an 8-yr-old-male, have the normal number of 46 chromosomes and exhibit the phase III phenomenon of finite lifetime in vitro described by Hayflick and Moorhead (1961) at about 20 passages. After proliferation had ensued well above confluence, the serum content of the influent medium was lowered from 10 to 1.0, 0.1, or 0% with the results shown in Fig. 3B. The cultures ceased proliferation and could be maintained thereafter as stationary phase, tissue-like cultures, except for the ones without serum which underwent a 30% decline in cell numbers and a rise in pH. The cultures perfused with 1.0 or 0.1% serum were terminated at 18 and 25 days, respectively.

When WI-38 and WI-38VA13A cells were perfusion-cultured with medium 7a plus 10% serum for 7–8 days, they, too, readily proliferated beyond confluency as shown in Fig. 4A. The rates of proliferation of the human diploid WI-38 cells after confluency were slower than the rates of their SV-40 viral-transformed progeny, WI-38VA13A cells, and the final 7- to 8-day cultures contained the equivalent of about 4 vs 8 monolayers, respectively. Duplicate experiments were made with both cell types. A photomicrograph of a stained cross-section preparation of multiple-layered WI-38 cells produced in a similar perfusion-type experiment has been illustrated previously (Kruse and Miedema, 1965).

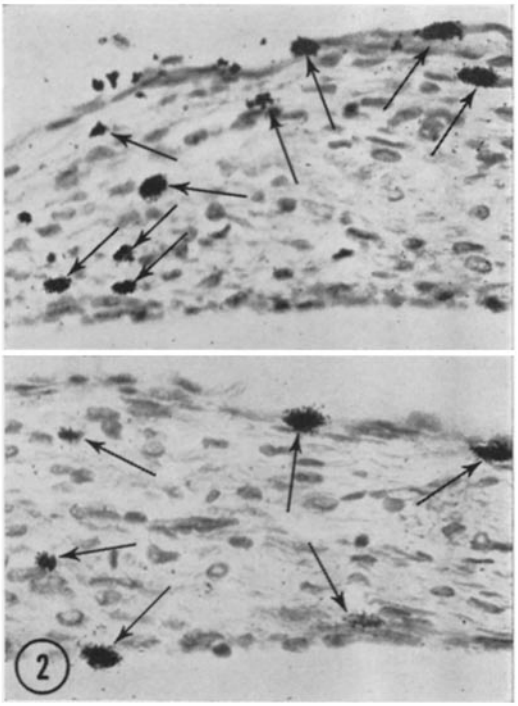

FIGURE 2 Two views of cross-section of tissue from a culture of embryonic rat cells after 6 wk of perfusion; the cell count was about 140 × 10^6 cells per T-60 flask, equivalent to about 12 monolayers (confluency in T-60 reached at 12 × 10^6 cells). Cells were in the third passage at start of perfusion. The tissue was pulsed with thymidine-^{3}H before sectioning and autoradiography. Of 138 labeled nuclei (such as indicated by arrows) which were counted, 52 were in the top few layers, 42 were in bottom few layers, and 44 were in between; this result showed that DNA synthesis was occurring randomly throughout the tissue and furnished evidence of adequate nutrition throughout, i.e. from top to bottom of the multiple-layered culture. Perfusion rate was 100 ml of medium 7a plus 10% whole calf serum per day.

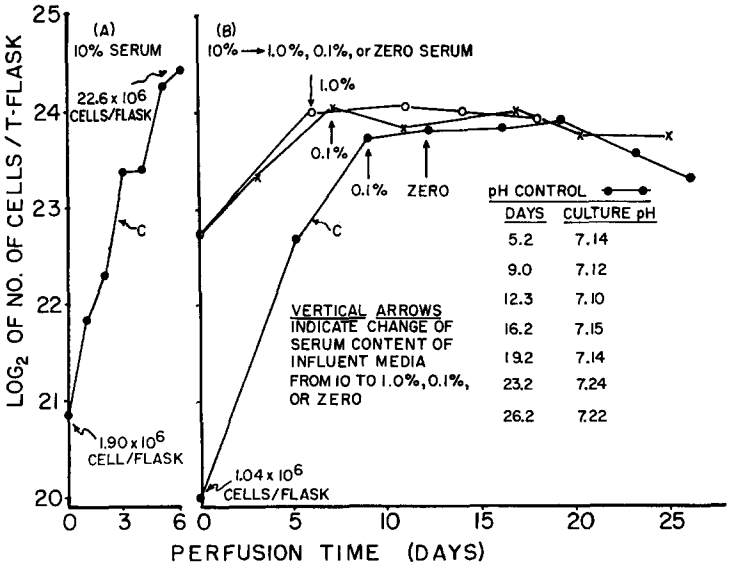

FIGURE 3 (A) Proliferation of NF-JAM diploid human tonsillar fibroblasts perfused with medium 7a plus 10% whole calf serum. (B) Illustrating maintenance of NF-JAM tissue cultures when the serum content of the influent medium was changed to 1.0 or 0.1%, and their decline in cell numbers after 7–8 days without serum; *C* = confluency (monolayer). Cell viability in the maintenance phases was above 85% as determined with dye-exclusion tests, and less than 0.5% of cell nuclei were labeled after pulsing with thymidine-^{3}H. In the experiment with 0.1% serum, stationary phase cell counts at 7, 11, 13, 17, 20, and 25 days were 17.5-, 14.9-, 15.9-, 16.7-, 14.3-, and 14.0 × 10^6 cells per culture, respectively.

The profound difference in response of the parent and viral-infected cells to lowering the serum content 100-fold to 0.1% is illustrated in Fig. 4B; duplicate experiments were made, all of about 1 month duration. Whereas this medium, containing but 1 ml of serum per liter, caused WI-38 cells to cease proliferation, it had no such effect on WI-38VA13A cultures. The latter continued to increase in population during the entire extended time period. Initial and final WI-38VA13A cell counts were 3.03–191 × 10^6 ($\log_2$ 21.5–27.5) cells and 3.96–239 × 10^6 ($\log_2$ 21.9–27.8) cells per T-75 flask in the 27-day experiments. Thus, the equivalent of 16–20 layers of cells was reached since about 12 × 10^6 of these cells form a single confluent sheet in T-75 flasks.

Since mitosis ceased in WI-38 cells and continued in WI-38VA13A cells in medium with 0.1% serum, it was obviously necessary to perfuse cultures of the latter faster to maintain pH, eliminate build-up of lactic acid and other metabolic products, and furnish adequate nutrition. For example, the perfusion rates in a WI-38 experiment (Fig. 4B) were 9 ml per culture per day for the first 4 days and 32 ml per culture per day for the remaining 23 days with 0.1% serum medium; the average pH of the cultures was 7.13 (range from 0 to 27 days was 7.09–7.17). Similar data for a WI-38VA13A experiment (Fig. 4B) were 9 ml for the first 3 days and 38, 52, 68, 85, 138, 133, and 142 ml per culture per day at days 5, 10, 13, 17, 21, 24, and 27, respectively; the average pH of the cultures was 7.01 (range from 0 to 27 days was 6.86–7.34). At the highest perfusion rate of 142 ml per day, the 16–20 layer WI-38VA13A cultures were receiving only 0.142 ml of serum per day. This amount of serum was but a fraction of the level necessary to effect proliferation in parent WI-38 cultures. All cultures contained 35 ml of medium in the flasks; as shown in Fig. 3B, for example, 1.0% serum, or 0.35 ml in the flask, did not support proliferation of diploid NF-JAM fibroblast cultures.

Since each feeding of the perfusion system delivered about 0.04 ml of medium via 22-gauge needles into each culture, the two extremes of perfusion rates, i.e. 9 and 142 ml per culture per day, represented 225 and 3,550 feedings per day. The stationary WI-38 cultures were receiving the equivalent of about one medium change per day

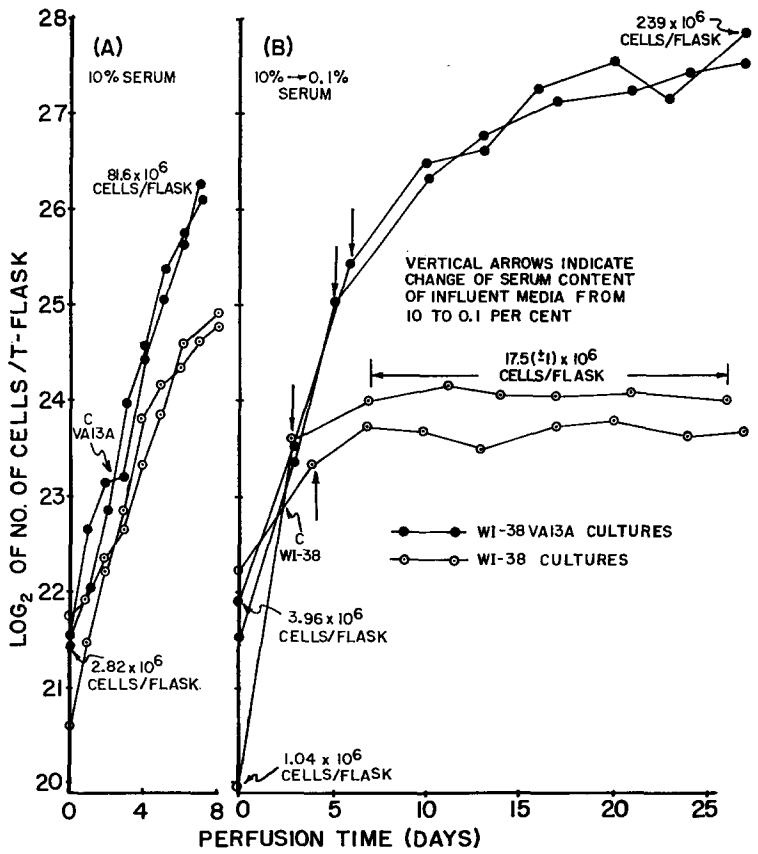

FIG. 4 (A) Proliferation of WI-38VA13A and WI-38 cells when perfused with medium 7a plus 10% whole calf serum. (B) Proliferation of WI-38VA13A cells and nonproliferation (extended maintenance) of WI-38 cells in medium 7a plus 0.1% whole calf serum. Population densities reached the equivalent of about 8 and 4 monolayers of WI-38VA13A and WI-38 cells, respectively, in (A) and 16–20 and 2 monolayers in (B). The several cell numbers given illustrate initial, final, and constant populations; *C* = points of confluency (single sheet of cells) of WI-38VA13A and WI-38 cultures. A discussion of perfusion rates, pH control, cell viability, and DNA synthesis is given in the text.

(32 ml), and the equivalent of about four changes (142 ml) were made towards the end of the WI-38VA13A experiments.

After changing the serum content from 10 to 0.1% in the influent medium reservoirs, it was important to determine how soon the change was reflected completely in the T-flask culture medium. By drying and weighing serum proteins precipitated from aliquots of culture media, it was found that the shift in serum concentration was completed in 1–2 days, at the perfusion rates used (as given above). Thus, mixing of influent media with culture flask media was very efficient.

Viability of the cells was estimated from dye-exclusion tests with trypan blue and lissamine green and found to average 90% (range 81–99) and 88% (range 75–98) throughout the WI-38 and WI-38VA13A experiments, respectively. Some of the dye uptake probably resulted from damage to the cells incurred in the brief pronase treatment and aspiration necessary to prepare cell suspensions for counting.

When WI-38VA13A cultures were pulsed with thymidine-^{3}H, as described above in Methods, subsequent radioautographs showed that the percentage of labeled nuclei during rapid proliferation, at 3 days (Fig. 4B), was 29%; after introduction of the medium with 0.1% serum at 6 days, labeling indices were 3, 35, 10, 9, 18, and 13% on days 10, 13, 16, 20, 23, and 27, respectively. Thus, DNA synthesis proceeded throughout the entire period of multiple layering and exposure to 0.1%

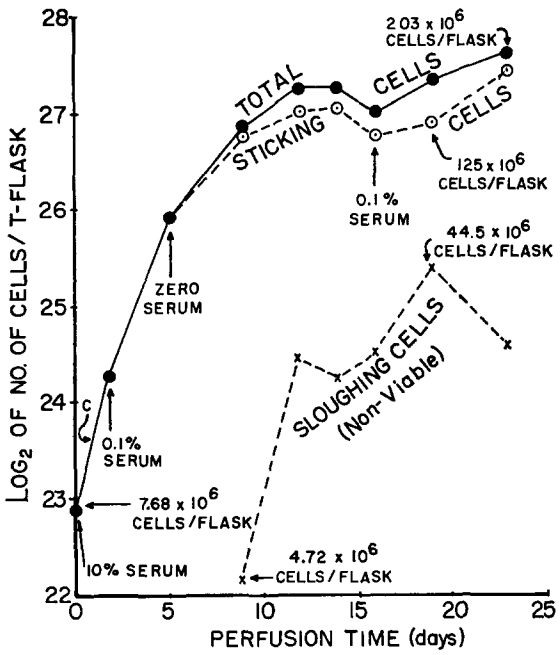

FIGURE 5 Illustrating the decline and recovery of proliferation and viability of WI-38VA13A (SV-40 transformed) T-75 flask cultures when perfused with medium containing 0 and 0.1% whole calf serum; C = point of confluency (single sheet of cells).

serum. In contrast, the labeling index of WI-38 stationary phase cells was found to be less than 0.5%, and here the nuclei which were counted as "labeled" were responsible for development of only a few grains on the radiographs.

An experiment with NF-JAM cells had indicated that cell numbers began to decline after exposure for 7 days to serumless medium (see Fig. 3B). Since WI-38VA13A cells proliferated luxuriantly in the presence of as little as 0.1% serum (Fig. 4B), it was of interest to determine whether they would do so in the absence of serum. As Fig. 5 shows, soon after the 0.1% serum medium was changed to serumless medium, the WI-38VA13A cells began to slough and the total cell population began to decline. The sloughed cells, amounting to as much as 26% of the total cell population, were nearly all nonviable as judged with dye-exclusion tests. After 11 days of perfusion with serumless medium (from days 5 to 16, Fig. 5), the influent medium was changed back to contain 0.1% serum; the cell populations began again to proliferate, reaching a final density of 203 $\times$ 10^6 cells per culture. Thus, the trace of serum represented by a concentration of 0.1% was essential to proliferation and viability of the virus-transformed cells.

DISCUSSION

Holley and Kiernan (1968) reported recently that multiple layering in cultures of mouse 3T3 cells occurred upon frequent medium changes and serum contents above 10%. This observation is directly related to our previously published results (Kruse et al., 1963; Kruse and Miedema, 1965) showing multiple layering of a variety of cell types by perfusion cultures from influent fresh media reservoirs, to previous results of others (e.g. Rubin, 1966), and to the results described here in Figs. 1–5. With the possible exception of a pig kidney cell line currently under investigation, no cell type has been found as yet which does not form multiple-layered cultures in the perfusion system with medium containing 10% serum. Thus, cessation of mitosis upon confluency of cells in vitro, frequently observed in stoppered flasks and Petri dish cultures, is more a reflection of the in vitro culture methods employed than it is a property derived from monolayer cell-to-cell contact (see Stoker and Rubin, 1967). Cell-to-cell contact can, however, exert other types of metabolic control, e.g. control of alkaline phosphatase activity (Miedema and Kruse, 1967).

Although there is probably an upper limit of population density, i.e. multiple layering, beyond which nutrition from perfusion over the surface becomes inadequate, this upper limit was apparently not reached in the embryonic rat tissue culture having the equivalent of 12 monolayers. Thymidine-^{3}H labeling of nuclei was randomly distributed throughout the tissue-like mass. How-

ever, different patterns of nuclear labeling have been observed in recent work (Kruse et al., unpublished data) with multiple-layered cultures of malignant Jensen sarcoma and SV-40 virus-transformed human WI-38 cultures. Here the nuclei appeared to be labeled by thymidine-^{3}H mostly in the upper layers of the sarcoma tissue culture and mostly on the bottom and top layers of the virus-transformed tissue culture. These apparent differences among cell types are being investigated further.

A principal objective of this study was to establish perfusion conditions whereby multiple-layered cultures could be maintained in a nonmitotic and viable condition (and in an environment characterized by constant nutrient input, metabolic product concentrations, and pH). This objective was accomplished with the perfusion system by lowering the influent medium concentration of serum to 0.1%. Presumably, such cultures could be held for long periods of time, with perfusion rates equivalent to one or more medium changes per day; the cultures demonstrated here were terminated arbitrarily after several weeks in the stationary phase. Hayflick and Moorhead (1961) have shown that diploid human cells in confluent sheets survived successfully for 4 wk with no added serum. Our results indicate that a trace of serum, i.e. 0.1%, was necessary, under perfusion conditions, for maintenance of constant cell numbers and viability. The methodology of tissue culture has been directed almost exclusively to cell proliferation in vitro, and relatively little attention has been given to means for maintaining cells in nonmitotic and viable populations of tissue-like density. Such cultures should enhance retention of phenotype of differentiated cells in vitro, and will likely require impingement of a constant supply of nutritional factors as suggested recently (Amos, 1967). Confluent sheets of highly differentiated chick cells have been maintained for months in a precisely controlled and automated circumfusion culture system described recently (Rose, 1967), one characteristic of which is a high ratio of medium volume to cell numbers. In all probability, stationary phase cultures of high density and adequate nutritional status can be expected to elicit maximal specific cellular functions also. For example, steroid synthesis was maximal in stationary phase cultures of mouse testicular interstitial cells undergoing frequent medium changes (Shin, 1967).

The influence of serum factor(s) on cell protein synthesis and growth has been the subject of a number of reports by Amos and coworkers (e.g. Amos and Moore, 1963). Primary cultures of embryonic chick fibroblasts were found to require serum not only for initiation and maintenance of protein synthesis but also for retention of ribosomal capacities (Soeiro and Amos, 1966). At least one of the recurrent results in our perfusion cultures in which medium plus 10% serum was used—namely, multiple layering of both diploid and heteroploid cell types—can be interpreted on the mechanistic basis of cell "governors" of protein synthesis and their neutralization by serum "signals" as outlined by Amos (1967). Accordingly, the perfusion system presents a nearly constant supply of serum signals such that protein synthesis, and proliferation, occurs despite intimate cell contact and crowding. In this regard, we have observed that cultures of Jensen sarcoma cells synthesized protein at a constant rate while progressing from rapidly proliferating cell cultures to slowly proliferating multiple-layered tissue cultures (Kruse et al., 1967); when proliferation slowed, protein was excreted into the surrounding medium. For certain cell types the promotion of protein synthesis and proliferation by serum factor(s) is definitely a function of serum concentration (e.g. Amos, 1967; Temin, 1967; Todaro et al., 1967; Bürk, 1967).

Interpretation of the fact that virus-transformed and uninfected parent cells differ in their requirement for serum factor(s) is difficult both from present results and previous ones (e.g. Todaro et al., 1967; Temin, 1967; Bürk, 1967; Holley and Kiernan, 1968). A trace of serum, 0.1%, was sufficient to permit WI-38VA13A cells to proliferate and form cultures equivalent to 20 mono layers in 27 days of perfusion; without serum, many of the viral-transformed cells soon died. Even a level of 0.1% serum, however, contains much physiological potential. For instance, 142 ml of medium containing 0.142 ml of serum were perfused in 1 day's time over a culture containing about 191×10^6 cells. At a serum protein concentration of 70 mg %, 0.142 ml would provide in this case about 10^6 molecules of protein, assuming 10^5 average molecular weight, to each cell; further, at protein and peptide hormone concentrations in serum in the range of 10^{-10} M, as found by Berson et al. (1964), 0.142 ml of serum could supply to each of 191×10^6 cells about 45 molecules of hormone(s). Clearly, elucidation of the factor(s) in serum which exert regulatory effects on cells in

vitro is a formidable, but not insurmountable, task. Insulin, or insulin-like activity in serum, has been found in part to replace the stimulatory activity of serum for cell proliferation (e.g. Gey and Thalhimer, 1924; Temin, 1967; Schwartz and Amos, 1968). Highly fractionated and partially characterized serum components have also been found to exert considerable such activity in very small concentrations (Holmes, 1967; Puck et al., 1968).

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