PRODUCTION AND CHARACTERIZATION OF MULTIPLE-LAYERED POPULATIONS OF ANIMAL CELLS

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ABSTRACT

Dense populations containing 129 × 10^6 Jensen sarcoma, 134 × 10^6 DON Chinese hamster, 28.9 × 10^6 WI-38 human diploid, 61.8 × 10^6 HEp-2 human carcinoma, and 67.4 × 10^6 WISH human amnion cells were produced from dilute inocula, 0.85 to 5.33 × 10^6, in 7 to 8 days in a perfusion system using replicate T-60 flasks. Perfusion rates as high as 560 ml medium/day/T-60 were required to maintain pH (to *ca* ±0.1 unit) and adequate nutrient supplies. The cell densities encountered are described by the term "monolayer equivalents" (M.E.), defined as number of cells per culture divided by number of cells in a monolayer. The M.E.'s for T-60 cultures containing unusually dense populations of 40 × 10^6 WI-38 and 250 × 10^6 DON cells (9-day perfusion) were 5 and 17, respectively, and numbers of cells in illustrations of stained cross-sections of membranes from these cultures were in excellent agreement. Threshold M.E.'s exist below which proliferation is the chief cellular activity and above which one or more cell functions may predominate even though proliferation persists. Cellular nutrition and metabolism may change with changes in M.E., as illustrated in different patterns of glutamic acid, proline, and glycine utilization or production in dense *vs.* dilute WI-38 cell populations. The results indicated that the role of contact inhibition phenomena in arresting cellular proliferation was diminished in perfusion system environments.

Considerable knowledge of proliferation, nutrition, and metabolism of animal cells *in vitro* has accumulated to date. Much of this has derived from cell cultures of low population densities in dispersed, "monolayer," and suspension-type systems. Since it is known that the density of cell populations *in vitro* may profoundly affect these aspects (*e.g.*, Neuman and McCoy, 1958; Eagle and Piez, 1962; Green and Goldberg, 1963; Whittaker, 1963), there appears a need for further study of them in densely populated cultures. Recently a technique for culturing dense cell aggregates (Moskowitz, 1964) has been described and presumably this would be adaptable to certain nutritional and metabolic studies. The usual cell culture techniques, however, do not lend themselves to experimentation with dense tissue-like cell populations. Reasons for this include the very frequent medium changes which would be required in stationary cultures and the limitations in cell-to-cell proximities inherent in any suspended cell system. To circumvent these limitations, a perfusion system for replicate stationary cultures was developed (Kruse, Myhr, Johnson, and White, 1963). The present paper describes application of an improved version of the system to a number of animal cell types derived from normal and malignant tissue and ones possessing fibroblast- *vs.* epithelial-like morphology. Because the population densities encountered could not be described ade-

quately with existing cell culture nomenclature, the term "monolayer equivalents" is introduced. This expression places the relationship between the sticking cell populations observed and the physical arrangement of the culture vessels on a quantitative basis. Its use is substantiated with illustrations of tissue membranes having thicknesses equivalent to as many as 17 cell layers.

In addition, an example of differences in amino acid utilization and production between dilute and dense cell populations is illustrated. Also, the report indicates that the role of contact inhibition in arresting cellular proliferation may be greatly diminished in perfusion system environments.

MATERIALS AND METHODS

Jensen sarcoma cultures were initiated from cell suspensions derived from freshly excised 7- to 8-day tumors carried in Holtzman rats. WI-38 human diploid cells were obtained through the courtesy of Dr. L. Hayflick, Wistar Institute, Philadelphia; DON Chinese hamster cells (CCL 16), HEp-2 human carcinoma cells (CCL 23), and WISH human amnion cells (CCL 25) were purchased from the American Type Culture Collection, Rockville, Maryland. Periodically, several tests for the presence of bacteria and mycoplasma (our appreciation is extended to Dr. Hayflick for some of these) were made with each of the cell types and found to be negative.

The cells were cultured in the perfusion system as described previously (Kruse *et al.*, 1963). Jensen cells were counted by chilling them off the glass into replacement media, and the WI-38, DON hamster, HEp-2, and WISH cells were removed with 0.10 per cent trypsin or 0.02 to 0.05 per cent pronase. Initially, pH of the cultures was controlled at 7.1–7.3 by gassing the T-60 flasks with 5 per cent CO_2, 75 per cent N_2, 20 per cent O_2, or 8 per cent CO_2, 72 per cent N_2, 20 per cent O_2. Thereafter, pH was maintained by gassing the influent medium reservoirs upon addition of fresh medium and also by periodic increases in the rates of perfusion.

Cell numbers were determined by whole cell counts in a hemocytometer. Portions of the dense cell sheets from two experiments were overlaid with agar, fixed in 10 per cent formalin, processed in an Autotechnicon, imbedded in paraffin, sectioned, and stained with hemotoxylin-eosin for microscopic evaluation of thickness and number of cells per cross-section.

The motor-clamp assembly described previously (Kruse *et al.*, 1963, Fig. 3), for control of the perfusion rate by alternately opening and closing the influent media lines, was replaced in the course of this work with the modification shown in Fig. 1, which describes its specifications and operation. At the most rapid perfusion rates employed in this study, the clamp was opened once each 6 seconds, or 600 times per hour; approximately 0.04 ml of medium flowed at each opening.

Concentrations of glutamic acid, proline, and glycine in influent and effluent media were determined with a Technicon Corp. automatic amino acid analyzer. The rates of amino acid utilization or production were calculated as μmoles $\times 10^{-9}$/hour/cell (McCarty, 1962).

RESULTS

Chief advantages of the modifications in the perfusion system have been (*a*) greater mechanical reliability in continuous operation of the motor-clamp assembly over several thousand hours, and (*b*) prevention of loss of carbon dioxide from the influent lines by substitution of teflon for silicone-base tubing.

All of the data pertinent to this report, except that for amino acids, have been summarized in Fig. 2, including proliferation curves, numbers of doublings of the cell population ($\log_2$), generation times of dispersed and dense populations, cell counts, pH control, and rates of perfusion of influent media. Also, an expression called monolayer equivalents (M.E.) is introduced in Fig. 2 to relate the population densities at each daily interval with the physical arrangement of the culture system. This term is defined here as:

$$\frac{\text{Total number of cells per flask}}{\text{Number of cells required to cover the supporting glass surface}}$$

The denominator in this expression was found to be about 19×10^6, 15×10^6, 8×10^6, 20×10^6, and 20×10^6 for T-60 flask cultures (60 cm^2 surface) of Jensen sarcoma, DON, WI-38, HEp-2, and WISH, respectively. These values were obtained from cell counts on cultures estimated by microscopic examination to be 50, 90, 100, and 110 to 150 per cent confluent. Also, cultures containing regions of two layers of cells were split to two flasks and estimates of confluency were made on each. Monolayer equivalents ranged from 0.06 to 9 in the experiments shown in Fig. 2.

The close correlation of calculated M.E.'s with actual cell population density is shown in Fig. 3. This illustrates stained cross-sections of cell membranes from two additional experiments. The T-60 cultures contained 40×10^6 WI-38 and 250×10^6 DON (9-day perfusion) cells, or M.E.'s of 5 and 17, respectively. Cell (nuclei) counts across the two

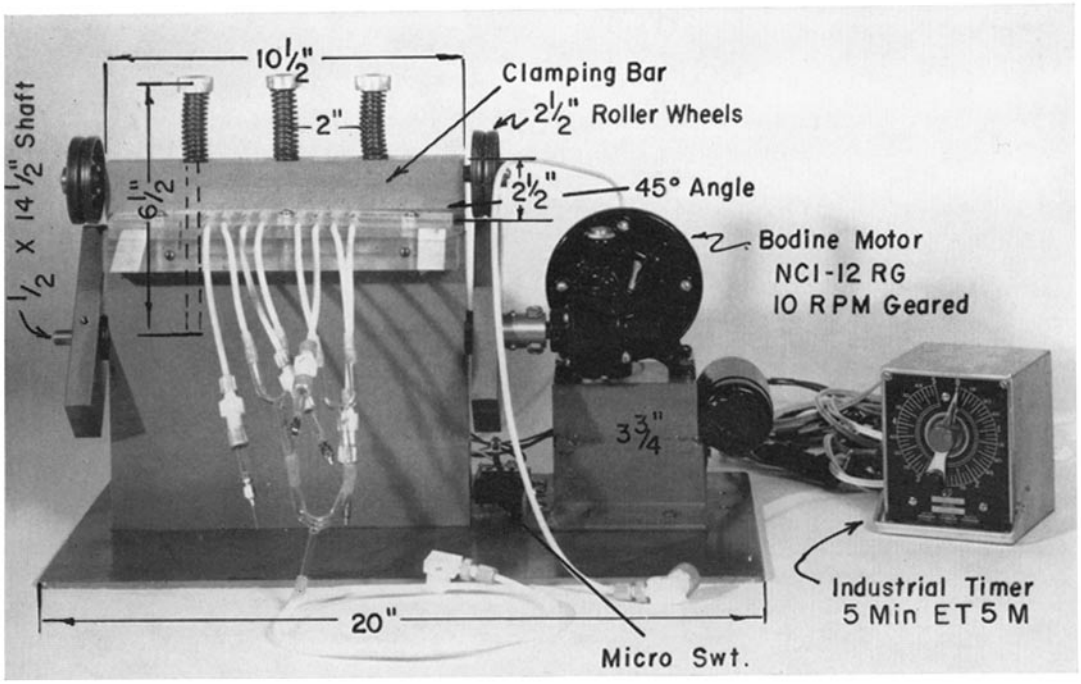


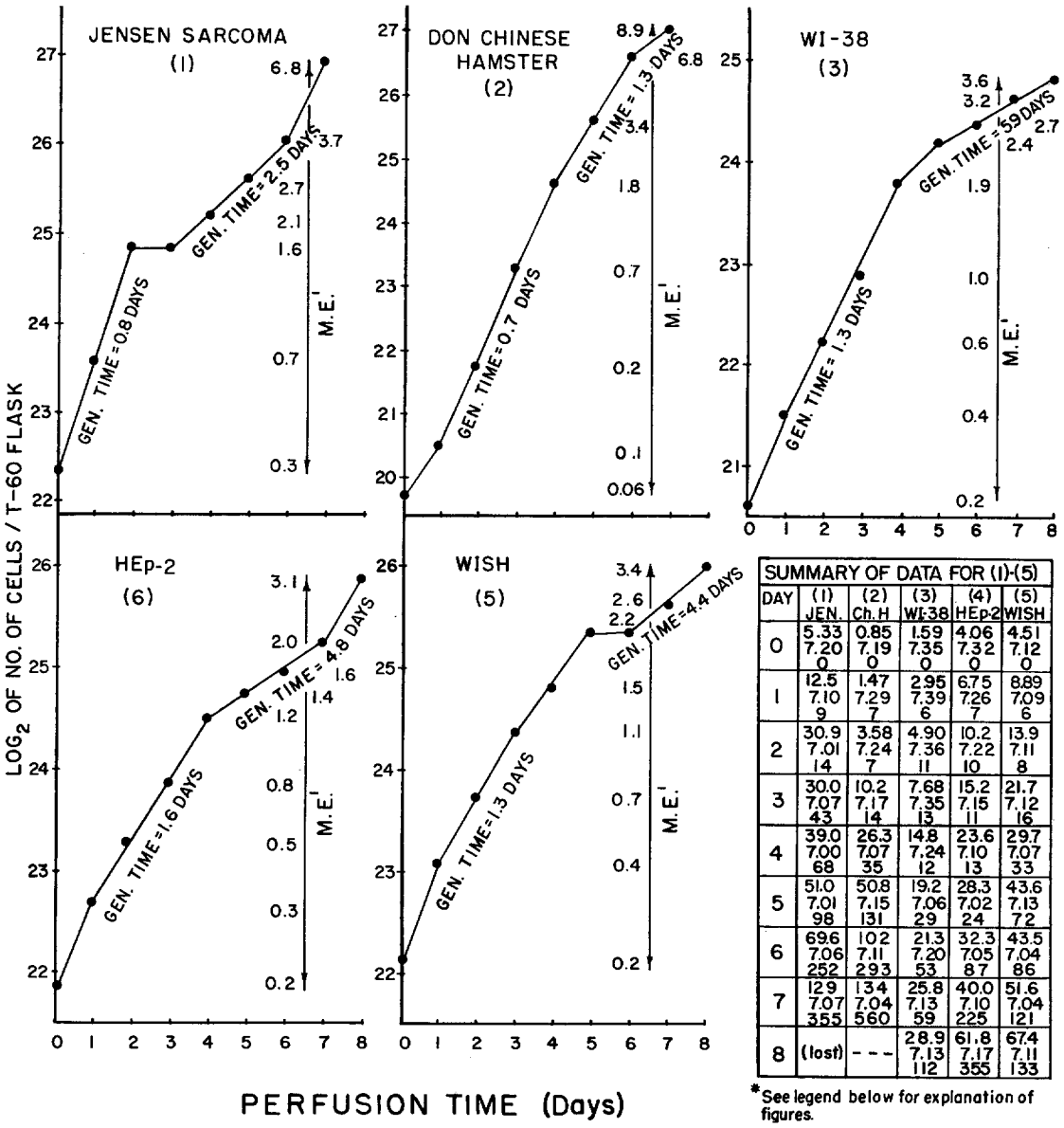
FIGURE 1 Motor-clamp assembly for controlling perfusion rate in replicate culture perfusion system (*cf.* Kruse, Myhr, Johnson, and White, 1963). The influent tubing leading to 8 T-60 flasks (not shown) is 1/8 inch I.D. × 0.185 inch O.D. × 0.030 inch wall thickness flexible teflon except for 1.0 inch section of 1/8 × 7/32 × 3/64 inch Silatube positioned under the clamping bar (tubing from Labtician Products Co., Hollis, New York). Four influent lines lead into and 4 out from plane of photograph. Each train of 4 lines is connected to culture medium reservoirs (not shown). The bottom of the clamping bar is machined at 45° angle to a dull knife edge to compress the 1-inch Silatube sections in the closed position of the clamp as shown. Actuation of the timer-controlled motor opens the clamp and influent lines by rotating the end-mounted shafts through 360°. Rates of openings and closings of the clamps are programmed with the timer.

sections yield values agreeing closely with the calculated M.E.'s.[1] Some evidence of cell injury was apparent in the bottom three to four layers of the M.E. = 17 section from the DON hamster culture (Fig. 3 *a*), manifested as cytoplasmic granularity with increased eosinophilic staining and occasional vacuolization. Nuclear staining did not appear to be altered except for occasional evidence of pycnosis. Whether the injury was due to inadequate nutrition at the bottom of the tissue, differences among cell types, etc., or to damage in removal from the flask and preparation for staining was not determined. No evidence of cell injury was apparent in the M.E. = 5 section from the WI-38 culture (Fig. 3 *b*); the tissue appeared healthy throughout, with good basophilic staining of both cytoplasm and nuclei.[2, 3]

[1] Thus, the term M.E. is not meant to imply numbers of distinct layers. Some cells—especially the fibroblast types—exhibit marked changes in cell size and protein content as they become crowded. It seems that the term is considerably more descriptive, however, than "dilute," "dense," "confluent," etc. Although its use is confined here to T-60 flask cultures, it should be possible to apply it to most culture devices in which cells proliferate on a plane supporting surface. It may not be applicable for cultures on curved surfaces, since they probably would not be covered uniformly (*cf.* Curtis and Varde, 1964).

[2] Our appreciation is extended to Dr. R. C. Reynolds, Department of Pathology, University of Texas Southwestern Medical School, Dallas, Texas, for these histological examinations.

[3] In addition, while this paper was submitted for publication another perfusion experiment with Jensen sarcoma cells yielded 350 × 10^6 cells/T-60 flask,



SUMMARY OF DATA FOR (1)-(5)					
DAY	(1) JEN.	(2) Ch. H	(3) WI-38	(4) HEp-2	(5) WISH
0	5.33 7.20 0	0.85 7.19 0	1.59 7.35 0	4.06 7.32 0	4.51 7.12 0
1	12.5 7.10 9	1.47 7.29 7	2.95 7.39 6	6.75 7.26 7	8.89 7.09 6
2	30.9 7.01 14	3.58 7.24 7	4.90 7.36 11	10.2 7.22 10	13.9 7.11 8
3	30.0 7.07 43	10.2 7.17 14	7.68 7.35 13	15.2 7.15 11	21.7 7.12 16
4	39.0 7.00 68	26.3 7.07 35	14.8 7.24 12	23.6 7.10 13	29.7 7.07 33
5	51.0 7.01 98	50.8 7.15 131	19.2 7.06 29	28.3 7.02 24	43.6 7.13 72
6	69.6 7.06 252	102 7.11 293	21.3 7.20 53	32.3 7.05 87	43.5 7.04 86
7	129 7.07 355	134 7.04 560	25.8 7.13 59	40.0 7.10 225	51.6 7.04 121
8	(lost)	- - -	28.9 7.13 112	61.8 7.17 355	67.4 7.11 133

*See legend below for explanation of figures.

FIGURE 2 Illustrating proliferation curves, number of doublings (log_2), generation times, monolayer equivalents (see text and definition below), cell counts, pH control, and perfusion rates during production of dense populations of 5 animal cell types in a perfusion system for replicte T-60 flasks cultures. The data in each square in the inset signify cell numbers (millions/T-60), culture pH, and perfusion rate in ml/day of Medium 7a plus 10 per cent whole calf sera. In each experiment, proliferating cultures in stoppered flasks were transferred to perfusion (day zero).

M.E. = Monolayer Equivalent (see text), defined as

$$\frac{\text{Total number of cells per flask}}{\text{Number of cells required to cover the supporting glass surface}}$$

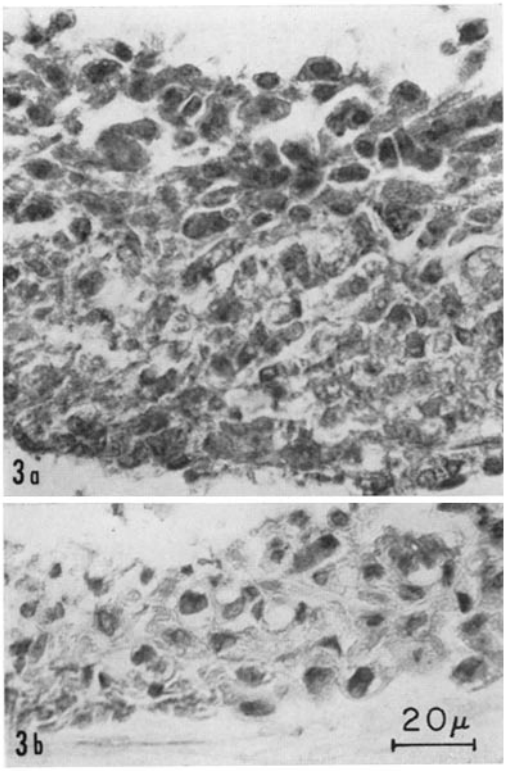


FIGURE 3 Cross-sections of cell membranes from DON hamster (*a*) and WI-38 (*b*) T-60 flask cultures containing 250 × 10^6 and 40 × 10^6 cells, respectively. Approximate numbers of cells across each membrane are 14 to 17 (*a*) and 4 to 6 (*b*), agreeing closely with calculated monolayer equivalents of 17 and 5 (see text). Hematoxylin and eosin stain. For description of histological examination of the tissue, see text under Results. × 480.

Except for the DON hamster cultures, the different cell types exhibited marked changes in rates of proliferation (Fig. 2) as the cell populations progressed from dispersed cells to dense tissue. For example, the generation time of WI-38 cultures with M.E.'s less than unity was 4.5-fold faster than in the population densities of 2.4 to 3.6 M.E. During the last 3 days, the WI-38 population increased from 19.2 to 28.9 × 10^6 cells per T-60 flask, or at the rate of 16 per cent increase per day. This was in comparison with an average daily rate of increase of 70 per cent during the first 3 days, 1.59 to 7.68 × 10^6 cells per T-60 flask.

Generally, the pH was held within ±0.1 unit. In a few instances, the variation was greater than this, whereas it was considerably less in others; *e.g.*, mean pH and variation in the WISH experiment was 7.09 ± 0.05, and in days 1 to 7 in the Jensen cultures it was 7.05 ± 0.05. Each daily pH measurement and cell count served as the basis for estimating the frequency and magnitude of increases in perfusion rates for the ensuing 24-hour period. This procedure resulted in maintenance of relatively constant glucose concentrations throughout the experimental period even though population increases were as much as 157-fold (DON hamster, Fig. 2). The concentration of glucose in the influent medium was 280 mg per cent in all of the experiments; the glucose concentration in the effluents collected daily in the WISH experiment, for example, ranged from 249 to 268 mg per cent. Under these conditions, rates of glucose utilization were related to cell numbers and proliferation (Kruse and Miedema, 1965).

The rates of perfusion (Fig. 2) were not necessarily increased in proportion to increases in numbers of cells, nor were they of the same magnitude among the different cell types. Reasons for this included (*a*) marked differences among the cell types in amount of acid production, and (*b*) changes in glucose utilization occurring in transition from dispersed to dense epithelial-like cultures. The most extreme example was the DON hamster which required 560 ml perfusate during the last, 6th to 7th, day to maintain good pH control. This volume was delivered through the system by approximately 14,000 additions (openings of the clamp assembly, Fig. 1) of 0.04 ml each, throughout the daily interval. The HEp-2 carcinoma populations required a greater volume of perfusate than either of the two cell types derived from normal human tissue (WI-38 and WISH).

Although Fig. 2 illustrates performance data from 5 perfusion experiments, 40 such experiments have been performed to date with similar results.

In the WI-38 experiment from 0- to 3-day and 5- to 8-day periods, notable differences in patterns of utilization or production of 3 amino acids were observed (Table I, Experiment 1). In the 0- to 3-day period, dispersed cells were multiplying to form an initial layer; and glutamic acid, proline,

from 6.5 × 10^6 cells/T-60 initial inoculum, requiring 950 ml of perfusate during the 7th to 8th day. This tissue sheet, M.E. = 18, contained dense aggregates of cells. After harvesting, the cells appeared uniformly in good condition, with sharp outlines upon examination for cell counting in a hemocytometer.

TABLE I

*Changes in Rate of Amino Acid Utilization or Production (+) in Post- vs. Pre-Confluent WI-38 Human Diploid Cell Cultures**

Amino acid	Utilization or production (+)‡			
	Pre-confluent		Post-confluent	
	Exp. 1	Exp. 2	Exp. 1	Exp. 2
Glutamic acid	+39	+44	+11	+9
Proline	+2	+2	6	2
Glycine	+1	0	0	4

* Cells were cultured in a perfusion system for replicate T-60 flasks (see text).

‡ Calculated as μmoles $\times$ 10^{-9}/hour/cell (*cf.* McCarty, 1962). About 8 $\times$ 10^6 cells covered the floor of a T-60 flask; in Exp. 1, duration of the pre-confluent phase was 3 days and cell numbers increased from 1.59 to 7.68 $\times$ 10^6 cells/T-60; similar data for Exp. 2 were 2 days and 2.88 to 8.39 $\times$ 10^6 cells/T-60. In post-confluent populations the time intervals were 3 days (Exp. 1) and 1 day (Exp. 2), and cells increased from 19.2 to 28.9 $\times$ 10^6 and 8.90 to 19.8 $\times$ 10^6 cells/T-60, respectively.

and glycine were produced at the rates of 39, 2, and 1 μmoles $\times$ 10^{-9}/hour/cell, respectively. The pattern changed markedly during the 5- to 8-day period when multiple layers of cells were present. Here glutamic acid production was diminished to 11 μmoles $\times$ 10^{-9}/hour/cell, while proline was extensively utilized and production of glycine ceased. The pattern of these changes was confirmed in a second experiment, as shown in Table I. Proline utilization was higher in the most dense population, Experiment 1.

DISCUSSION

Applicability of a perfusion system for replicate cultures has been demonstrated for a number of animal cell types. It provides for maintaining cultures in controlled environments with apparently plentiful supplies of nutrients at all times, pH control, and elimination of "waste" products. Dense cell populations were produced from relatively small, dispersed-cell inocula. From the proliferation curves in Fig. 2, it is apparent that the system can be adapted conveniently to comparative nutritional and biochemical studies among various cell types. In some experiments, periods of (*a*) rapid proliferation, (*b*) slow proliferation, (*c*) non-proliferation, (*d*) multiplication of single cells into a monolayer sheet, and (*e*) formation of multi-cell thick membranes, could be selected for these purposes. Previously, the study of densely packed cell populations has been impractical in most culture systems. For example, the medium would have to be changed about 16 times daily in a stoppered flask system containing cell populations equivalent to that requiring 560 ml per day in the present study.

Although marked changes in rates of proliferation usually occurred as the cultures became crowded, they continued increasing in population, even to forming the equivalent of 17 layers of cells in one instance (Fig. 3). These results implied that the perfusion system environment diminished contact inhibition effects, which have been cited in other instances (*e.g.*, Abercrombie and Ambrose, 1962; Golde, 1962; Levine, Becker, Boone, and Eagle, 1965) as chief factors in arresting cell proliferation and overlap.

The term "confluent sheet" has been applied to monolayers of human diploid cells (Hayflick and Moorhead, 1961). If this term is expanded to signify any culture of animal cells that has covered the supporting surface area, cell culture investigations could conveniently be classified as those conducted in (*a*) pre-confluency and (*b*) post-confluency. Further definition within each of these classifications can be made easily by the monolayer equivalent (M.E.) term introduced here. As an example, an experiment described as having been conducted in post-confluency of M.E. 7 tells the reader immediately a great deal about the culture system employed and serves as a definitive basis upon which to compare results among experiments of a similar nature. It is probable that cell cultures have a threshold M.E. below which proliferation is their chief activity and above which one or more functions, such as collagen production, may predominate. In the striking demonstrations of collagen production (Green and Goldberg, 1963; Goldberg and Green, 1964), one can calculate from the data given for Petri dish cultures that the probable M.E. threshold for this function was approximately 2 to 3. Another potential advantage of the use of M.E.'s is to avoid terms of "cells per milliliter of culture fluid" for population densities in cultures requiring supporting surfaces, as is sometimes done.

Cultures such as those illustrated in Fig. 3 obviously have population densities greater than those possible in systems containing free floating cells. In the 60-cm^2 flask containing 250 $\times$ 10^6

cells the thickness of the sheet of tissue was about 100 microns (Fig. 3 *a*); thus the tissue volume was 0.6 cm^3, equivalent to 4.2×10^8 cells per cm^3. Similar calculations for the 40-micron-thick WI-38 culture containing 40×10^6 cells (Fig. 3 *b*) gave 0.24 cm^3, or 1.7×10^8 cells per cm^3. The cell population density in liver was found to be 1.3×10^8 cells per gram (Allard, Mathieu, Lamirande, and Cantero, 1952).

The changes in glutamic acid, proline, and glycine utilization or production in dense WI-38 cultures probably reflected cellular activity in collagen production, which has been demonstrated (Hayflick and Moorhead, 1961) in WI-38 cultures. These 3 amino acids are major constituents (or precursors) of collagenous proteins, and the changes observed exemplify an influence of population density upon cellular nutrition and metabolism.

Post-confluent populations with high M.E.'s *in vitro* may more closely resemble *in vivo* animal cell tissues than do the usually encountered less dense cell culture populations. A means of perfusion, such as employed herein, would appear necessary to control culture environments during the production and study of high M.E. populations over extended time intervals.

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