ENZYME ACTIVITIES AND PROTEIN CONTENTS OF ANIMAL CELLS CULTURED UNDER PERFUSION CONDITIONS*

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The metabolism of animal cells cultured in vitro can be markedly influenced by environmental changes in pH (e.g., Zwartouw and Westwood, 1958; Paul, 1962), nutrient levels (e.g., Eagle, 1955a, b; Graff et al., 1965), and accumulation of metabolic end products (e.g., Biggers et al., 1957; DeMars, 1958). In the present study of comparisons of enzyme activities among several animal cell types in vitro, these variables were largely controlled through use of a perfusion system for replicate cell cultures (Kruse, Myhr, Johnson, White, 1963). Accordingly, the enzymatic differences described herein could be correlated with changes in cell population density or with inherent characteristics of the cell types employed. In addition, cell protein contents can be markedly altered by changes in proliferation rate and population density (e.g., Salzman, 1959; Merchant, Kuchler, and Munyon, 1960; Swaffield and Foley, 1960; Kruse and White, 1961). Pronounced changes were seen here only in protein contents of fibroblast-like cells but not in epithelial-like cells.

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MATERIALS AND METHODS

WISH human amnion and HEp-2 human carcinoma epithelial-like cell lines were obtained from the American Type Culture Collection; Jensen sarcoma cell cultures were initiated from cell suspensions derived from freshly excised tumors carried in female Holtzman rats, according to procedures of McCoy and Neuman (1956). The fibroblastic WI-38 cell strain (human fetal lung) was obtained from Dr. L. Hayflick, Wistar Institute, Philadelphia, Pa.

At the time perfusion was started, each series of replicate T-60 flask cultures contained fewer than 6×10^6 cells/T-60, so that a period of ca. 3 days duration ensued in which there was rapid proliferation prior to confluency, i.e., complete saturation of the glass surface with cells. Perfusion was continued throughout 7 days so that enzyme activities in the slowly proliferating, densely populated post-confluent cultures could be compared with those in the 0-3-day pre-confluent populations. Cell counts, protein contents, and enzyme activities were determined at daily intervals.

For enzyme assays, the cells were collected by chilling or brief pronase treatment (0.05%) (Gwatkin and Thomson, 1964). Protein was determined by the Lowry method as adapted for tissue culture by Oyama and Eagle (1956). Cells were enumerated by hemocytometer counts or nuclei determinations (Sanford et al., 1951) when necessary. Lactate, malate, and G-6-P dehydrogenases were determined in intact cells with the method described previously by Slotnick (1962) but modified such that the final glycerol concentration was 5%. A unit of enzyme activity was a change in O.D. at 340 mμ of 0.001/min at 28° C as measured by the oxidation of NADH or reduction of NADP on a Beckman DU spectrophotometer. Specific activities are expressed as units/10^6 cells or per mg of protein. Enzyme activity was linear with time and cell numbers. Further, 6-phosphogluconate dehydroge-

nase activity was nil under these assay conditions and did not interfere with the measurement of G-6-PDH.

RESULTS AND DISCUSSION

The proliferation characteristics of all the cell types used are given in Table 1.

TABLE 1

PROLIFERATION CHARACTERISTICS OF PERFUSED MAMMALIAN CELLS*

	WISH	HEp-2	WI-38	Jensen
Initial Cells (x 10^6)/T-60	4.51	4.06	1.58	5.33
Final Cells (x 10^6)/T-60	51.56	39.96	25.80	129.00
Generation Time: (days)				
Pre-confluency (0-3 days)	1.3	1.6	1.3	0.8
Post-confluency (4-7 days)	3.4	4.3	2.4	1.8
Fold Increase in Cells	11	10	16	24

*All cells were cultured in Medium 7a + 10% whole calf serum (White, Smith, and Kruse, 1963).

Figure 1 illustrates that the protein content of the fibroblast-like cell types decreased markedly as the population density approached confluency; however, the protein content of the epithelial-like cells remained constant throughout the 7-day period.

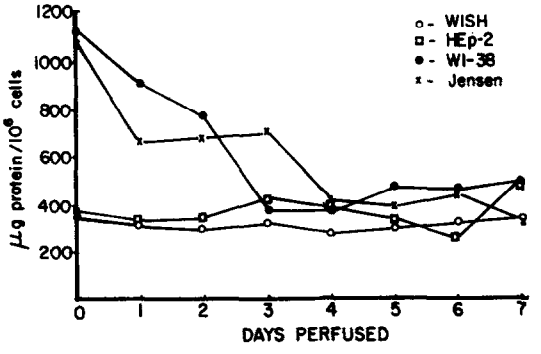

Fig. 1 - Protein Content of Proliferating Cells. Protein content as bovine serum albumin equivalents was determined daily in duplicate on cells taken from the perfusion system.

From Figure 1, it is apparent that the use of protein assays as an index of proliferation can be considerably in error for fibroblast cell types but may be valid for determining increases in numbers of epithelial-like cells.

Figure 2 summarizes LDH levels of 3 cell lines and the WI-38 strain during pre- and post-confluency phases of proliferation. As seen by the post-pre ratios, calculated from enzyme units/cell, the LDH activity was only slightly increased in the 3 cell lines, but increased 2-fold in the WI-38 cell strain. Although the WISH is derived from normal human amnion epithelium and the HEp-2 is derived from an epithelial carcinoma, their LDH specific activities (per cell) were about the same. It was also apparent that these heteroploid human epithelial cell lines exhibited several orders of magnitude greater total units of LDH (per cell) than the diploid human fibroblast-type strain, WI-38 (e.g., WISH/WI-38 in post-confluent cultures = 4).

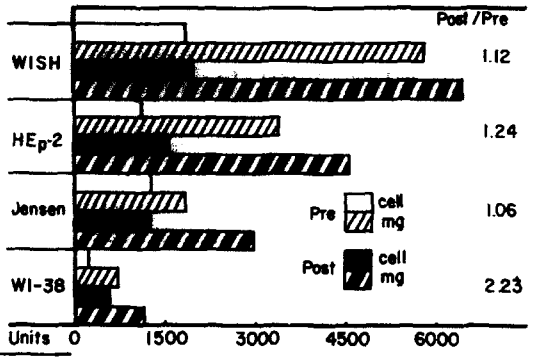

Fig. 2 - LDH Activity in Perfused Cell Cultures. Reaction mixture contained 0.5-1.5 x 10^6 cells (in 1 ml of phosphate buffered saline/glycerol, 85/15 vv), 0.2 ml NADH (0.32 mg), 0.1 ml sodium pyruvate (0.1 M), and phosphate buffered saline to 3 ml (pH 7.3). Pre = pre-confluent cultures; post = post-confluent cultures. Post/pre ratios calculated from units/cell.

DeLuca and Nitowsky (1964) reported that LDH underwent a characteristic decline in specific activity in non-perfused, aged human cell line cultures, and this decline was accelerated by the addition of 10 mM lactic acid to the culturing medium. No such decline in LDH activity was noted in any of the experiments conducted in the present study, since the perfusion system automatically removed lactic acid and other spent media products as they were formed. It should be noted here also that Lieberman et al. (1964) demonstrated that LDH may be substrate inducible in primary kidney cell

cultures, with glucose as the hexose source. Thus, if the level of glucose were allowed to fall appreciably, as it does in non-perfused cell culture systems, one might see a corresponding decline in the activity of LDH, as well as other enzymes associated with glycolysis. However, in the present perfusion system, glucose concentrations were held quite constant, e.g., influent at 280 mg/100 ml and effluent at 258 ± 10 mg/100 ml throughout the 7-day experiment with WISH cells.

Figure 3 depicts the specific activity of MDH during pre- and post-confluent proliferation. Its specific activity (per cell) in all four cases exhibited very little change and was considered to remain constant relative to population density changes.

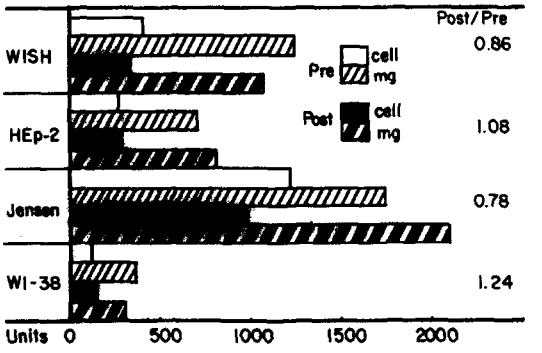

Fig. 3 - MDH Activity in Perfused Mammalian Cells. Conditions as for Fig. 2, except 0.2 ml oxalacetate (0.01 M) as substrate.

Figure 4 illustrates the levels of G-6-PDH in the same pre- and post-confluent phases. Only the WI-38 cell strain exhibited an increased specific activity (per cell) in the post-confluent cultures. Reasons for the increased G-6-PDH activity - and, therefore, available levels of reduced triphosphopyridine nucleotide - in the dense WI-38 cultures were not investigated further in the present study. However, DeMars (1964) has reported similar findings employing primary skin cell cultures from normal and electrophoretically variant G-6-PDH human subjects, wherein the normal cultures showed a 2-fold increase in the level of G-6-PDH when the cultures had achieved 1/3 to 1/2 maximal density. The increased G-6-PDH activities may

have been associated with collagen production, since Shimizu, McCann, and

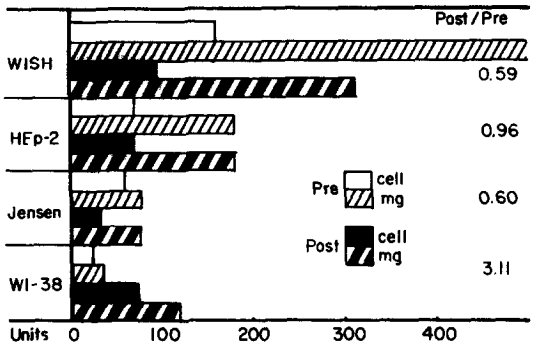

Fig. 4 - G-6-PDH Activity in Perfused Mammalian Cells. Conditions as for Fig. 2, except 0.15 ml of TPN (0.54 mg) and 0.1 ml of 0.1 M G-6-P (Na-salt).

Keech (1965) related the need for aerobiosis and energy production with collagen synthesis in human fibroblast cell cultures. However, whether other diploid fibroblast strains would behave similarly under the perfusion environment is not known; thus, before these changes in enzyme activities with population density can be related to the ploidy or function of cells, additional studies with other cell lines and strains are needed.

The present results do indicate rather pronounced metabolic differences between pre- and post-confluent cultures and between cell lines and strains. Also they suggest that metabolic studies of animal cells in vitro - in particular enzymatic ones - are facilitated when variables such as culture pH, nutrient levels, and accumulation of end products have been minimized.

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