

CHANGES IN GLUCOSE OXIDATION DURING GROWTH OF EMBRYONIC HEART CELLS IN CULTURE

JOSEPH B. WARSHAW and MIRIAM D. ROSENTHAL

From the Children's Service, Massachusetts General Hospital, Shriners Burns Institute, and the Department of Pediatrics, Harvard Medical School, Boston, Massachusetts 02114

ABSTRACT

A rapid and convenient method has been utilized to investigate glucose oxidation during growth of chick embryo heart cells in tissue culture. Primary isolates of chick embryo heart cells showed exponential growth when plated at low densities and exhibited density-inhibited growth as cultures became confluent. The density-dependent growth inhibition of chick embryo heart cells is associated with a marked decrease in the specific activity of glucose oxidation to CO_2 . This decrease in glucose oxidation was observed as density increased as either a function of time in culture or as related to initial plating density. The decrease in $^{14}\text{CO}_2$ production associated with density-dependent inhibition of growth is due to a marked decrease in activity of the pentose phosphate pathway.

INTRODUCTION

The developing chick embryo is highly dependent on glucose oxidation to supply its energy needs (1). It has also been shown that the fetal heart contains the enzymes necessary for carrying out citric acid cycle oxidations (2-4) and oxidative phosphorylation (5). In the early embryonic heart there is an increased utilization of glucose by the pentose phosphate pathway (6, 7). This activity decreases as the embryonic heart reaches its adult form and cell proliferation slows. The high activity of the pentose phosphate pathway in the early embryo has been related to the rapid synthesis of nucleotides and requirement for ribose precursors during exponential growth (6).

We have measured the oxidation of glucose to CO_2 by monolayers of chick embryonic heart cells. The results indicate that the specific activity of glucose oxidation is highest when the cells are proliferating rapidly and decreases as growth plateaus. We have found that these changes in glucose oxidation are due to a marked decrease in the pentose phosphate pathway associated with

density-dependent growth inhibition. The similarity of tissue culture observations to what is observed *in vivo* makes the former an attractive system with which to study these phenomena.

METHODS

Hearts were obtained under sterile conditions from 7- and 13-day chick embryos. The cells were isolated by a minor modification of the method of DeHaan (8). Hearts from 3 to 20 dozen embryos were dissected, minced, and suspended in 10 ml of a dissociating medium consisting of 0.05% trypsin (General Biochemical, Div., Chagrin Falls, Ohio, 1:300) in calcium- and magnesium-free Tyrode's solution. After 10 min of gentle mixing at 37°C , the supernatant was discarded and the tissue resuspended in 3.3 ml of the dissociating media. The suspension was then mixed gently for 8 min and the supernatant medium containing the dissociated cells was transferred to 20 ml of F-12 medium containing 15% fetal calf serum. Two more 8 min incubations were sufficient for complete dissociation of the younger hearts; older hearts required a total of 4-5 8 min incubations. The cells were

spun gently in a clinical centrifuge, resuspended in medium, and filtered through Nitex (80 mesh) (Silk Screen Supplies, Inc., Brooklyn, N.Y.) to remove clumps. They were then counted in a hemocytometer, and plated at various densities in small Falcon flasks (Falcon Plastics, Div. of Bioquest, Oxnard, Calif.). Cultures were grown in F-12 media supplemented with 10% fetal calf serum and 5% chick embryo extract under 95% air and 5% CO₂. The initial medium contained 500 units penicillin G (E. R. Squibb and Sons, New York) per ml. Plating efficiency as determined by counting trypsinized cells in replicate flasks was 15–20%. Under these culture conditions, approximately 30–50% of the cells showed active spontaneous contractions. With increased time in culture the percentage of beating cells decreased but islands of cells were observed to beat synchronously for 6 days. Tissue culture media were changed daily to avoid changes in culture conditions.

Before the assays, the medium was aspirated and the cells were washed with cold calcium-free Krebs-Ringer phosphate buffer. To each flask was added 2 ml of the buffer containing 1 mM glucose. Experiments were carried out using glucose-¹⁴C (uniformly labeled, UL), glucose-1-¹⁴C, or glucose-6-¹⁴C, all with a specific activity of 1 mCi/mmol. For the assay, the flasks were fitted with serum caps to which polypropylene center wells were fixed. The reaction was started by warming the cells from 3°C to 37°C. After 1 hr of gentle shaking at 37°C, the reaction was terminated by the injection of 0.5 ml of 20% perchloric acid through the serum cap. The flasks were then upturned and 0.3 ml of hyamine hydroxide was injected through the serum cap into the center well. After equilibration for 45 min to trap the evolved ¹⁴CO₂, the hyamine was transferred to scintillation vials for counting in a Beckman scintillation spectrometer (Beckman Instruments, Inc., Fullerton, Calif.) using a toluene 2,5-diphenyloxazole fluor at 70% counting efficiency. Fig. 1 illustrates how the flasks were adapted for the assays. After ¹⁴CO₂ was collected, the cells were scraped from the flasks and protein was determined by the method of Lowry et al. (9). The number of cells per flask was determined by dissociating replicate cultures with 0.05% trypsin in calcium- and magnesium-free Tyrode's solution. After complete dissociation, the cells were counted in a hemocytometer. For some experiments, the incubation medium was retained and lactate was determined spectrophotometrically (10) after neutralization with KOH and precipitation of the KClO₄. The glycogen content of the cultures was estimated by the periodic acid-Schiff (PAS)¹ stain (11) after fixation

¹ Abbreviations: G6PDH, glucose-6-phosphate dehydrogenase; LDH, lactate dehydrogenase; NADPH, reduced nicotinamide adenine dinucleotide phos-

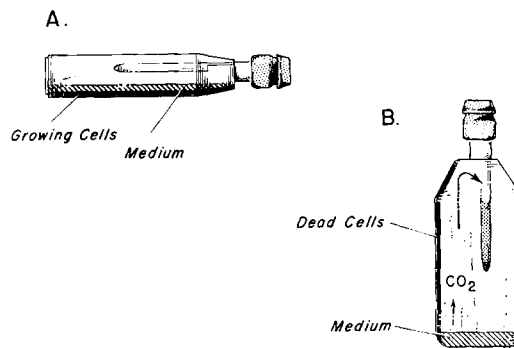


FIGURE 1 Diagram of flask adaptation for ¹⁴CO₂ collection.

for 1 hr in 10% formalin. Tissue culture media and sera were obtained from the Grand Island Biological Company, Grand Island, N. Y. Isotopes and other reagents were obtained from commercial sources.

RESULTS

Fig. 2 shows the time course of ¹⁴CO₂ production from glucose-¹⁴C (UL). The cells were plated at 1.25×10^6 cells per flask and assayed after 3 days in culture. The rate of ¹⁴CO₂ production showed a slight increase during the course of the assay. This may relate to warming of the cells to 37°C or to equilibration of substrate pools.

Cultures plated at high initial densities showed inhibited growth by day 3 in culture. As is shown in Fig. 3, both the cell number and the amount of protein per flask plateaued when the cells were plated at an initial density of above 3×10^6 cells per flask. The ratio of protein to cell number was constant over the entire range of plating densities.

¹⁴CO₂ production from glucose by 7-day embryonic heart cells was determined as a function of time in culture. Cells were plated at 1.25×10^6 and 3.75×10^6 cells per flask (Fig. 4). At both plating densities, there was a marked decrease in the specific activity of glucose oxidation measured with glucose-¹⁴C (UL) as the cultures became density inhibited. This occurred on day 3 for the higher density cultures and on day 4 in the case of those plated at a lower density. Photomicrographs of the lower density cultures (plated at 1.25×10^6 cells per flask) on days 2 and 4 in culture are shown in Fig. 5. Assays carried out after 24 hr in culture gave variable rates of ¹⁴CO₂ production, presumably because the cells

phate; PAS, periodic acid-Schiff; UL, uniformly labeled.

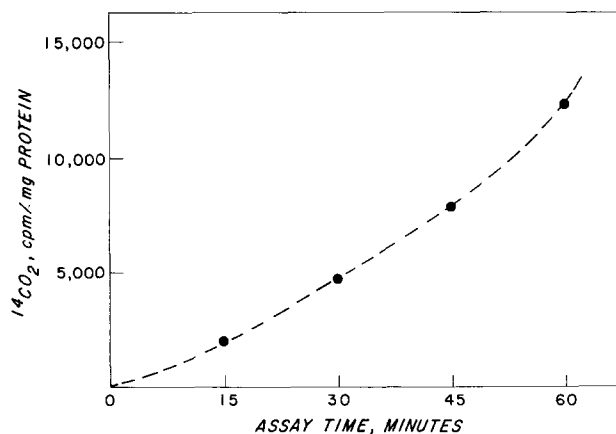


FIGURE 2 Time course of $^{14}\text{CO}_2$ production from glucose- ^{14}C (UL). 7-day embryonic heart cells were plated at an initial density of 1.25×10^6 cells per flask. The assay was carried out after 3 days in culture. The flasks contained 2 ml of calcium-free Krebs-Ringer phosphate buffer with 1 mM glucose- ^{14}C (SA 1 mCi/mmole). Other experimental conditions are as described in the text.

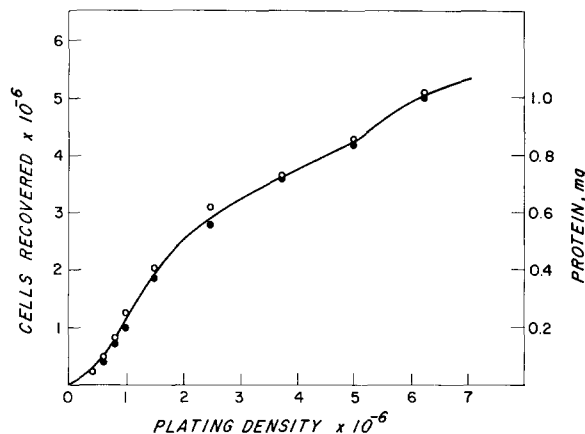


FIGURE 3 Growth of heart cell cultures of 7-day embryonic hearts plated at varying densities. \circ — \circ , cell number; \bullet — \bullet , protein. Protein and cell number per flask were determined after 3 days in culture.

had not yet fully recovered from the isolation procedure.

Glucose oxidation as a function of cell density is shown in Fig. 6. For this experiment, 7- and 13-day heart cells were plated at varying densities and assayed on day 3 in culture. Cells from 7-day embryos plated at densities above 2.5×10^6 cells showed a marked decrease in the specific activity of glucose oxidation as compared with lower density cultures still proliferating. The data for total protein indicated that cell growth was plateauing at plating densities above 4×10^6 cells per flask. The specific activity of glucose oxidation by cells isolated from 13-day hearts was about 50% lower as compared with cells from 7-

day hearts, but a similar decrease in the specific activity of glucose oxidation was observed as growth plateaued. If glucose oxidation to $^{14}\text{CO}_2$ was expressed as a function of protein, the decrease in the specific activity of $^{14}\text{CO}_2$ production from glucose at higher densities was almost identical under experimental conditions of variable initial density or variable time in culture.

In order to assess the contributions of the pentose phosphate pathway and the glycolytic-aerobic pathway to these shifts in glucose oxidation, experiments were carried out with glucose-1- ^{14}C and glucose-6- ^{14}C . A high ratio of $^{14}\text{CO}_2$ production from glucose-1- ^{14}C as compared with that from glucose-6- ^{14}C has been used as an index of

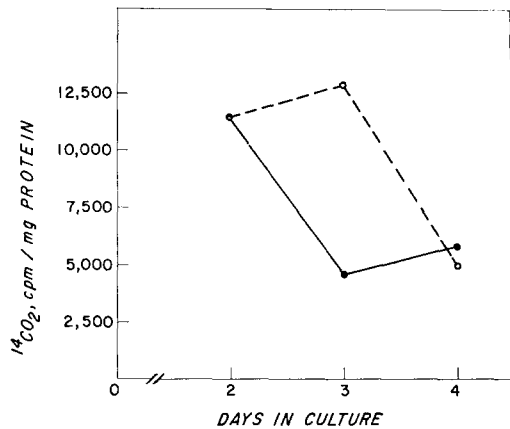


FIGURE 4 $^{14}\text{CO}_2$ production by 7-day heart cells as a function of time in culture. \circ — \circ , plating density of 1.25×10^6 cells per flask. \bullet — \bullet , plating density of 3.75×10^6 cells per flask. Assays were carried out after 2, 3, and 4 days in culture under the conditions described in the text.

activity of the pentose phosphate pathway (12). $^{14}\text{CO}_2$ production from glucose-1- ^{14}C and glucose-6- ^{14}C was determined using cells from 7- and 13-day embryonic hearts. Fig. 7 A shows an experiment with 13-day heart cells in which cultures were plated at varying densities, and assays were performed after day 3 in culture. Glucose-1- ^{14}C oxidation was very high in the low-density cultures showing exponential growth as compared with those plated at a higher density and already confluent. In the case of the lower density cultures, the ratio of glucose-1- ^{14}C to glucose-6- ^{14}C oxidation approximated 10:1; however, in the higher density plates showing inhibited growth, this ratio decreased to 1:1. In experiments with 7-day embryonic hearts, this ratio was as high as 40:1 in low-density cultures. It should be emphasized that, under the experimental conditions reported here, estimations of glucose pathways by measuring $^{14}\text{CO}_2$ production from glucose-1- ^{14}C and glucose-6- ^{14}C are approximations. However, the magnitude of the changes we report makes it extremely unlikely that pitfalls such as recycling of hexose phosphates and failure of triose phosphate pools to equilibrate (12, 13) can account for these observations. As is shown in Fig. 7 B, lactate production also decreased in the more confluent density-inhibited plates. This was also observed as density increased as a function of time in culture. Although the fall in the specific activity of lactate production mirrored changes in $^{14}\text{CO}_2$ production from

glucose-1- ^{14}C , more lactate appeared in the medium than could be accounted for by $^{14}\text{CO}_2$ production from glucose. This probably relates to dilution of exogenous glucose by endogenous glucose-6-phosphate appearing as a result of glycogenolysis. The heart cells used in our experiments had abundant PAS-positive droplets which decreased in number during the incubation. As $^{14}\text{CO}_2$ production from both glucose-1- ^{14}C and glucose-6- ^{14}C increased with time up to 120 min, the absolute availability of substrate was not rate limiting.

$^{14}\text{CO}_2$ production from glucose-1- ^{14}C relative to that from glucose-6- ^{14}C was also determined as a function of time in culture. As is shown in Fig. 8, $^{14}\text{CO}_2$ production from glucose-1- ^{14}C decreased by day 4 when the cultures became confluent. Under the same conditions, $^{14}\text{CO}_2$ production from glucose-6- ^{14}C remained constant. These results, then, indicate that the high rate of glucose oxidation during proliferative growth is primarily due to enhanced activity of the pentose phosphate pathway.

DISCUSSION

The method used in this study provides a convenient means for measuring substrate oxidations by cells growing in monolayer culture. A major advantage of performing the assays in the tissue culture flasks under the described conditions is that the stage of growth can be monitored optically and the assays can be carried out without manipulating or damaging the cells. Heart cells scraped from the flasks and then assayed showed decreased glucose oxidation. Our experiments were carried out in two ways. In one series of experiments, cells were plated at a fixed density and assays were performed on days 2, 3, and 4 in culture. Under these conditions, there were density-related restraints on growth on either day 3 or 4, depending on the initial plating density. In another series of experiments, cells were plated at varying densities and assayed after 3 days in culture. Under the latter conditions, the higher density cultures showed inhibited growth whereas those plated at a lower density still showed active proliferation. The similarity of results obtained with the use of these different growth conditions indicates that the enhanced production of CO_2 from glucose is related to proliferative growth of the heart cells.

Our results with cell culture are similar to what is observed *in situ* in the early developing chick

heart. Coffey et al. (6) measured $^{14}\text{CO}_2$ production by homogenates of embryonic hearts as a function of development age. They found a high ratio of glucose-1- ^{14}C :glucose-6- ^{14}C oxidation during the

proliferative phase of embryonic heart growth. By the time the embryonic heart reached its adult form, on days 5-6 of development, the ratio of glucose-1- ^{14}C to glucose-6- ^{14}C oxidation had de-

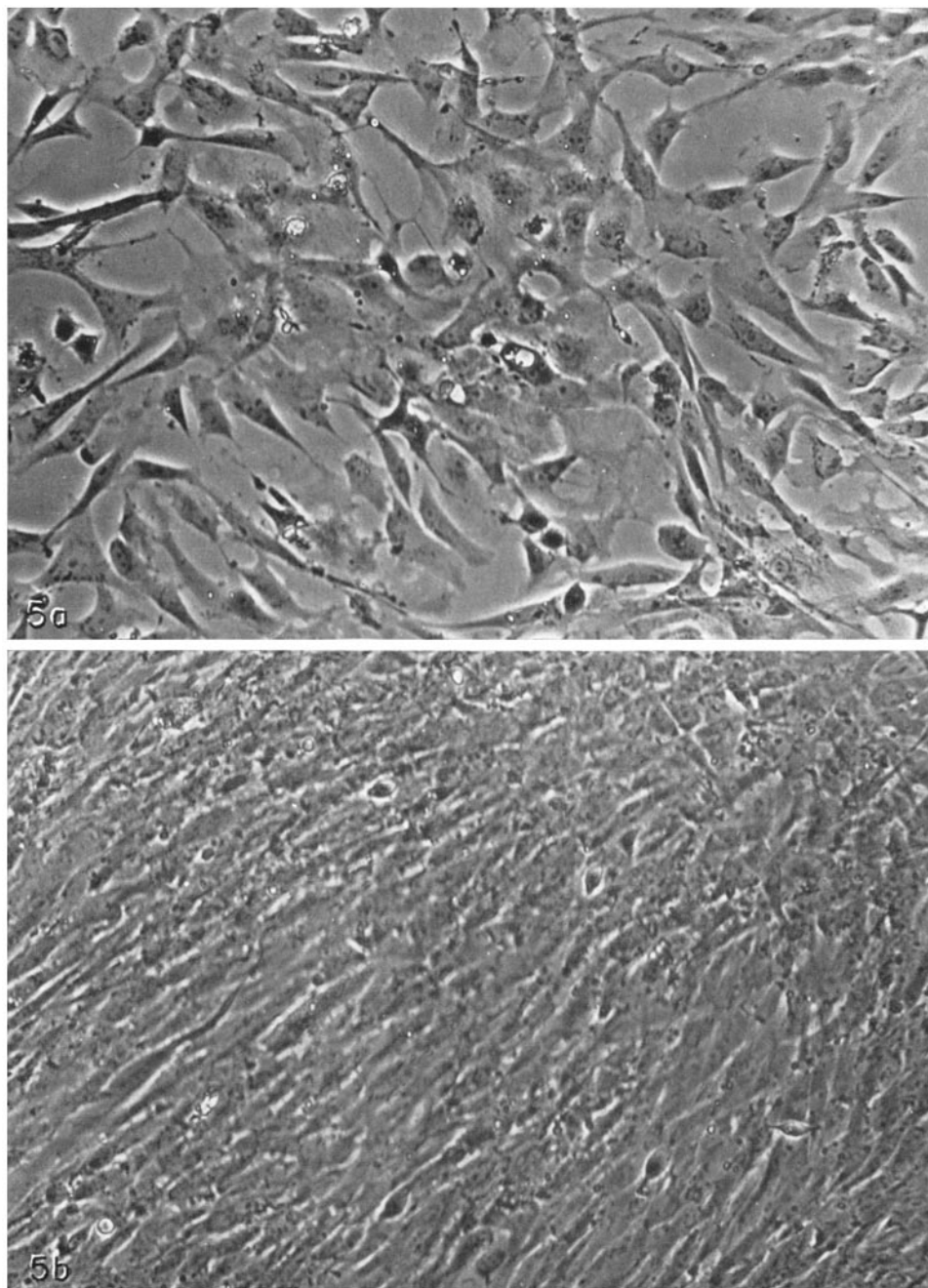


FIGURE 5 Photomicrographs of cells plated at 1.25×10^6 cells per flask after 2 and 4 days in culture. (a) 2 days in culture; (b) 4 days in culture. $\times 750$.

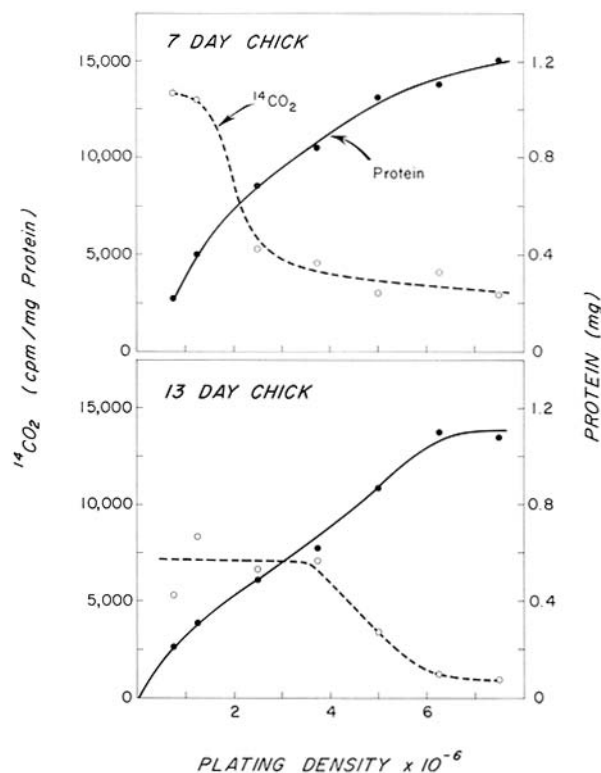


FIGURE 6 $^{14}\text{CO}_2$ production by 7- and 13-day heart cells plated at varying initial densities. $\circ-\circ$, $^{14}\text{CO}_2$ production from glucose- ^{14}C (UL); $\bullet-\bullet$, protein per flask. Assays were carried out after 3 days of culture as described in the text.

creased to 1 and did not change thereafter. In our experiments primary cultures were initiated from 7- and 13-day embryonic hearts, and both showed increased ratios of glucose-1- ^{14}C relative to glucose-6- ^{14}C oxidation. It is apparent that pentose phosphate pathway activity can be reactivated under conditions of low-density plating and cell proliferation and is consistent with the notion that a rapidly proliferating system has increased requirements for ribose phosphate precursors as well as for nicotinamide adenine dinucleotide phosphate (NADPH), a necessary cofactor for fatty acid synthesis. The constancy of $^{14}\text{CO}_2$ production from glucose-6- ^{14}C as compared with decreased $^{14}\text{CO}_2$ production from glucose-1- ^{14}C rules out an over-all impairment of cellular function. Furthermore, the decrease in the specific activity of lactate production by high-density cultures indicates that the decreased oxidation of glucose-1- ^{14}C to $^{14}\text{CO}_2$ is not due to an increase in anaerobic metabolism. Rather, it is likely that a decrease in pentose phosphate pathway activity results in less glyceralde-

hyde-3-phosphate production and subsequently less lactate.

A number of enzyme activities relating to carbohydrate metabolism have been shown to change during tissue culture growth (14). DeLuca (15) has reported that lactate dehydrogenase (LDH) and glucose-6-phosphate dehydrogenase (G6PDH) activities of fibroblasts can be influenced by the mode of culture, growth media, or stage of growth. The activities of LDH and G6PDH were found to be higher in suspension cultures as compared with monolayer cultures, and the activities of both enzymes increased with cell growth. Using a human diploid line, Cristofalo and Kritchevsky (16) showed a direct correlation between glucose utilization and cell division. As G6PDH has been reported to rise during tissue culture growth, it is unlikely that changes in the specific activity of this enzyme control the marked decrease in pentose phosphate pathway activity observed under our high-density conditions.

Temin (17) and Steck et al. (18) have reported a

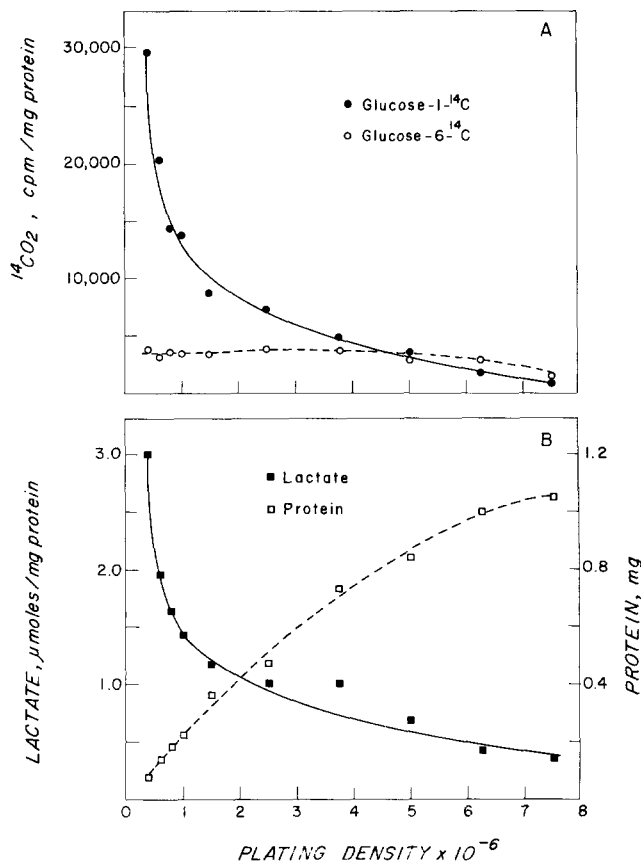


FIGURE 7 (A) $^{14}\text{CO}_2$ production from glucose-1- ^{14}C and glucose-6- ^{14}C by 7-day heart cells plated at varying densities; ●—●, glucose-1- ^{14}C ; ○—○, glucose-6- ^{14}C . Assays were carried out after 3 days in culture as described in the text. (B) Lactate production by 7-day heart cells plated at varying densities. ■—■, lactate; □—□, protein per flask.

decrease in glycolytic activity of normal and Rous sarcoma virus-transformed chick embryo cell cultures commensurate with high-density growth inhibition. However, their results did not discriminate the pathways of glucose catabolism involved. Condon et al. (19) have investigated glycolytic activity of fetal chick fibroblasts. In their study $^{14}\text{CO}_2$ production from glucose-1- ^{14}C was highest when assays were carried out within 48 hr after subculture, presumably when the cells were showing maximum proliferation.

Stoker (20) has used the term "density dependent inhibition" to describe the slowing of growth as cells are crowded in tissue culture. He has suggested that the term "contact inhibition" (21) be reserved to describe inhibition of movement of cells. A number of studies have recently been described which show that cells can be re-

leased from density dependent inhibition under appropriate conditions. Gurney (22) showed that confluent, slowly growing cells could be stimulated to make DNA by a wounding procedure which allowed cells to proliferate in the area of the wound. Enhancement of growth has also been described following media replenishment (23) and also after exposing dense cell populations to trypsin (24). The pattern of growth after trypsinization and culture of heart cells from intact 7- and 13-day embryonic hearts may be analogous to that in the above situations.

At the present time, the mechanism by which high cell density inhibits the oxidation of glucose-1- ^{14}C to $^{14}\text{CO}_2$ can only be speculated. It is likely that, under our high density culture conditions, there are inhibition of growth, a decrease in the requirement for ribose phosphate precursors for

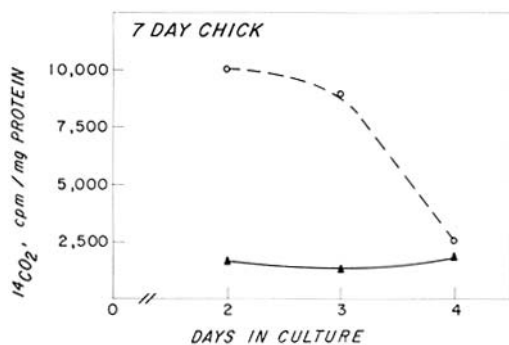


FIGURE 8 ¹⁴CO₂ production from glucose-1-¹⁴C and glucose-6-¹⁴C by 7-day heart cells as a function of time in culture. ○—○, glucose-1-¹⁴C; ▲—▲, glucose-6-¹⁴C. Cultures were plated at 1.5×10^6 cells per flask and assayed on days 2, 3, and 4.

nucleotide synthesis, decreased NADPH production, and a related fall in ¹⁴CO₂ production via the pentose phosphate pathway.

This research was supported by United States Public Health Service grant HD03610 from the National Institute of Child Health and Human Development and the Shriners Burns Institute.

Received for publication 23 July 1971, and in revised form 30 September 1971.

REFERENCES

- NEEDHAM, J., and H. LEHMANN. 1937. Intermediary carbohydrate metabolism in embryonic life. *Biochem. J.* **31**:1210.
- MAHLER, H. R., N. H. WITTENBURGER, and L. BRAND. 1958. Biochemical studies of the developing avian embryo. II. Enzymes of the citric acid cycle. *J. Biol. Chem.* **233**:770.
- BRAND, L., and H. R. MAHLER. 1960. Biochemical studies of the developing avian embryo. III. The oxidation of reduced pyridine nucleotide. *J. Biol. Chem.* **234**:1615.
- BRAND, L., and H. R. MAHLER. 1960. Biochemical studies of the developing avian embryo. IV. Some respiratory pigments. *J. Biol. Chem.* **235**:2456.
- WARSHAW, J. B. 1969. Cellular energy metabolism during fetal development. I. Oxidative phosphorylation in the fetal heart. *J. Cell Biol.* **41**:651.
- COFFEY, R. G., V. H. CHELDELIN, and R. W. NEWBURGH. 1964. Glucose utilization by chick embryo heart homogenates. *J. Gen. Physiol.* **48**:105.
- JOLLEY, R. L., JR., V. H. CHELDELIN, and R. W. NEWBURGH. 1958. Glucose catabolism in fetal and adult heart. *J. Biol. Chem.* **233**:1289.
- DEHAAN, R. L. 1967. Regulation of spontaneous activity and growth of embryonic chick heart cells in tissue culture. *Develop. Biol.* **16**:216.
- LOWRY, O. H., N. J. ROSEBROUGH, A. L. FARR, and R. J. RANDALL. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**:265.
- HÖRST, H. H. 1965. L(+) Lactate: determination with lactic dehydrogenase and DPN. In *Methods of Enzymatic Analysis*. H. H. Bergmeyer, editor. Academic Press Inc., New York. 266.
- MOWRY, R. W. 1958. Improved procedure for the staining of acidic polysaccharides by Muller's colloidal (hydrous) ferric oxide and its combination with the Feulgen and the periodic acid-Schiff reactions. *Lab. Invest.* **7**:566.
- KATZ, J., and H. G. WOOD. 1963. The use of ¹⁴CO₂ yields from glucose-1- and -6-¹⁴C for the evaluation of pathways of glucose metabolism. *J. Biol. Chem.* **238**:517.
- WOOD, H. G., J. KATZ, and B. R. LANDAU. 1963. Estimation of pathways of carbohydrate metabolism. *Biochem. Z.* **338**:809.
- PAN, Y. L., and R. S. KROOTH. 1968. The influence of progressive growth on the specific catalase activity of human diploid cell strains. *J. Cell. Physiol.* **71**:151.
- DELUCA, C. 1966. Effects of mode of culture and nutrient medium on cyclic variations in enzyme activities of mammalian cells cultures *in vitro*. *Exp. Cell Res.* **43**:39.
- CRISTOFALO, V. J., and D. KRITCHEVSKY. 1965. Growth and glycolysis in the human diploid WI-38. *Proc. Soc. Exp. Biol. Med.* **118**:1109.
- TEMIN, H. 1968. Studies on carcinogenesis by avian sarcoma viruses. VIII. Glycolysis and cell multiplication. *Int. J. Cancer.* **3**:273.
- STECK, T. L., S. KAUFMAN, and J. P. BADER. 1968. Glycolysis in chick embryo cell cultures transformed by Rous sarcoma virus. *Cancer Res.* **28**:1611.
- CONDON, M. A. A., F. A. OSKI, S. DIMAURO, and W. J. MELLMAN. 1971. Glycolytic differences between foetal and non-foetal human fibroblast lines. *Nature (London)*. **229**:214.
- STOKER, M. G. P., and H. RUBIN. 1967. Density dependent inhibition of cell growth in culture. *Nature (London)*. **215**:171.
- ABERGROMBIE, J. 1970. Contact inhibition in tissue culture. *In Vitro*. **6**:128.

22. GURNEY, T., JR. 1969. Local stimulation of growth in primary cultures of chick embryo fibroblasts. *Proc. Nat. Acad. Sci. U.S.A.* **62**:906.
23. TODARO, G. J., G. K. LAZAR, and H. GREEN. 1965. The initiation of cell division in a contact-inhibited mammalian cell line. *J. Cell. Comp. Physiol.* **66**:225.
24. SEFTON, B. M., and H. RUBIN. 1970. Release from density dependent growth inhibition by proteolytic enzymes. *Nature (London)*. **227**:843.