GROWTH AND METABOLISM OF L CELLS IN A CHEMICALLY DEFINED MEDIUM IN A CONTROLLED ENVIRONMENT CULTURE SYSTEM

I. EFFECTS OF O2 TENSION ON L-CELL CULTURES

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Six water-jacketed 500-ml Bellco spinner flasks were equipped to monitor and control environmental variables to study their effects on the growth and metabolism of mammalian cells. Studies with automated control of pH levels of L-cell cultures, grown at pH 6.9 ± 0.1, showed that dissolved O2 tensions of ca. 9% were optimal for cell growth. At pH values of 5 and 20%, maximum cell yields as well as growth rates were reduced by approximately 20%. Peak yields of L-cell cultures exceeded $5 \times 10^4$ cells/ml when grown for 4 days without medium renewal from inocula of ca. $10^6$ cells/ml in a defined medium sparged with 5% CO2 and maintained at 9% dissolved O2 tension. The redox potentials of L-cell cultures reflected the $pO_2$ levels in the medium and ranged from $-45$ to $+160$ mv (versus calomel reference) for $O_2$ values ranging from 2 to 20% dissolved oxygen tension. Increased utilization of glucose per cell occurred in the presence of increased $pO_2$, whereas minimal accumulation of ammonia occurred with a $pO_2$ value maintained at 9%

Workers in cell culture research have long recognized that factors such as temperature, pH, partial O2 and CO2 pressures and redox potential have significant effects on cell physiology. These investigators (4-6, 10, 12, 13, 15, 16) have employed various procedures to study the effects of one or more of these factors on the growth and metabolism of mammalian cells. However, much more remains to be done to understand fully the effects of these variables on cellular physiology. We present a description of a system developed for studies of the effects of varied environmental conditions on mammalian cells grown in a chemically defined medium. Results obtained in this system concerning the effects of oxygen tension on cell growth and related data on culture pH, redox potential, and carbohydrate and NH3 metabolism will be described.

MATERIALS AND METHODS

Culture medium and cell line. The chemically defined medium of Nagle et al. (14) was modified (Table 1) to permit improved yields of L cells (8). The strain of L cell was obtained from W. F. Daniels at Fort Detrick and designated L-DR. Stock cultures were examined periodically (7) for mycoplasma and other contaminants.

Preparation of inoculum. Inocula for flasks (Bellco Glass, Inc., Vineland, N.J.) were prepared in two stages. First, cultures were grown in 25 ml of medium in 100-ml serum bottles (Fig. 1) on a gyroratory incubator shaker (model G25; New Brunswick Scientific Co., New Brunswick, N.J.) as described previously (8). When a population of approximately $10 \times 10^6$ cells/ml was attained, this culture was transferred to 100 ml of medium in 250-ml Woulff bottles (Fig. 1) and incubated in a water bath shaker (model G76; New Brunswick Scientific Co.) at 35 ± 0.5 C. Inocula of less than $10^6$ cells/ml in this system yielded 4 × $10^6$ to $5 \times 10^6$ cells/ml in 4 days without a medium change. The Woulff bottle cultures were used to inoculate the Bellco flask system described below.

Spinner flasks. Six water-jacketed, 500-ml spinner flasks (Bellco Glass Inc.) were arranged on a table with magnetic stirrer drives (Multi-Magnistir, Lab-Line Instruments, Inc., Melrose Park, Ill.). Each flask containing 500 ml of medium was inoculated with $1.0 \pm 0.03 \times 10^6$ cells/ml. The incubation temperature was maintained at 35 ± 0.5 C with a Haake constant temperature water circulator (Haake, Berlin, West Germany—Brinkmann Instruments, Inc., Westbury, N.Y.). Each spinner flask contained four 16-mm ports (Fig. 2) for an oxygen probe, a platinum probe,
TABLE 1. Modified, chemically defined medium for the growth of L cells in suspension

| Component                   | Conc (mg/liter) |
|------------------------------|-----------------|
| L-Amino acids                |                 |
| Arginine-HCl                 | 100             |
| Asparaginase-H$_2$O          | 150             |
| Cysteine-HCl-H$_2$O          | 75              |
| Histidine-HCl-H$_2$O         | 60              |
| Isoleucine                   | 150             |
| Leucine                      | 300             |
| Lysine-HCl                   | 300             |
| Methionine                   | 60              |
| Proline                      | 115             |
| Phenylalanine                | 120             |
| Proline                      | 105             |
| Serine                       | 135             |
| Threonine                    | 60              |
| Tyrosine                     | 120             |
| Valine                       | 150             |
| Glutamine                    | 450             |
| Inorganic salts              |                 |
| NaCl                         | 7,400           |
| KCl                          | 400             |
| Na$_2$HPO$_4$-H$_2$O         | 276             |
| NaHCO$_3$                    | 500*            |
| CaCl$_2$-2H$_2$O             | 265             |
| MgCl$_2$-6H$_2$O             | 275             |
| Ferric ammonium citrate-3H$_2$O | 3.14          |
| ZnSO$_4$-7H$_2$O             | 0.287           |
| Vitamins                     |                 |
| d-Biotin                     | 0.004           |
| Choline chloride             | 2.0             |
| Folic acid                   | 2.0             |
| Niacinamide                  | 2.0             |
| Calcium pantothenate         | 4.0             |
| Pyridoxal-HCl                | 2.0             |
| Thiamine-HCl                 | 2.0             |
| Inositol                     | 2.0             |
| Riboflavin                   | 0.2             |
| Vitamin B$_6$                |                 |
| Carbohydrate and derivatives |                 |
| Glucose                      | 3,000           |
| Sodium                       | 110             |
| Sodium gluconate             | 17.8            |
| Miscellaneous                |                 |
| Methylcellulose$^b$          | 1,000           |
| Kanamycin                    | 50              |
| Sodium penicillin G          | 60              |
| Phenol red                   | 10              |

$^a$ This level of NaHCO$_3$ in the medium is added aseptically as a 5% NaHCO$_3$ solution autoclaved in nearly filled, tightly stoppered serum bottles. $^b$ Fifteen centipoise.

and a combination pH probe. The remaining port was used for sampling, gas sparging lines, and to vent exhaust gases. The spinners were rotated at 250 to 300 rev per min as measured by the method of Borkowski and Johnson (2).

Cell enumeration. Viable cell numbers were determined each day in the hemocytometer by the trypan blue exclusion procedure of McLimans et al. (11).

Oxygen electrode. The Clark polarographic-type sensor (model 5331) supplied by Yellow Springs Instrument Co., Yellow Springs, Ohio, was employed. The assembled probe was not toxic to L-DR cells in suspension culture and was stable in culture for at least 4 days. The probe was connected through an amplifier (model 5520-3; Yellow Springs Instrument Co.) to a recorder (model EUW-20A; Heath Co., Benton Harbor, Mich.).

pH electrode. The Sargent/Jena (E. H. Sargent and Co., Springfield, N.J.) nonpolarizable combination electrode, with low electrolyte leakage rate (15 to 40 μliters/hr), was used in conjunction with the pH recorder-controller (Analytical Measurements, Inc., Chatham, N.J.).

Oxidation-reduction electrode. The potential developed on a miniature platinum electrode was read against the reference cell of the pH electrode and recorded on a modified pH recorder-controller (Analytical Measurements, Inc.). The pH recorder was modified to read redox potential by plugging a 100 ohm resistor into the internal meter shunt and calibrating with a standard d-c voltage source. Each

![Fig. 1. Culture vessels employed for preparation of inoculum. The 100-ml serum bottle (right) was used for initial cultivation of inoculum. The larger 250-ml Woolff bottle (left) was used for second-stage inoculum build-up for the 500-ml Belco spinner culture vessels.](image-url)
electrode system was compared with a standard platinum-calomel reference system to insure uniformity. Each electrode was polished with scouring powder prior to sterilization.

Sterilization. All probes were sealed in rubber stoppers with Silastic (RTV731; Dow Corning Corp., Midland, Mich.) and sterilized in test tubes containing an acid-alcohol-formaldehyde solution (3% HCl-70% isopropanol-0.05% formaldehyde) for 10 min. The probes were then rinsed in similar test tubes containing sterile distilled water or sterile pH 7.0 buffer before aseptic transfer to culture vessels.

**Oxygen control system.** Control of dissolved oxygen tensions was achieved by automated blending of a gas mixture of a higher oxygen content with a basic sparging gas (Fig. 3). The flow into the flask of the resultant gas mixture was maintained between 60 and 80 cc/min with a flowmeter (model 620PB; Matheson Gas Products, East Rutherford, N.J.). The amount of blending gas added was controlled by a Matheson flowmeter and a bidirectional motorized valve. The bidirectional valve was a microflow valve (model 150, Matheson Gas Products) coupled through a 3:1 ratio gear set to a 2 rev/min, synchronous, reversible motor (Hurst model DA, Allied Electronics, Chicago, Ill.). This valve opened or closed as a result of changes of the pO2 values reflected by the oxygen probes in the culture vessels. The motorized valve was coupled to the oxygen probe-amplifier-recorder system through a delay timer (model T-RE-1; Clare-

![Fig. 2. A 500-ml Bellco spinner flask showing positioning of various electrodes. Also visible is the filtered coupling for the gas sparging lines. The flask is jacketed to allow circulation of thermostatically controlled water.](image)

Fig. 3. Block diagram of automated oxygen control system for cell cultures, showing the coupling of the oxygen probe to the motorized valve through the adjustable limit-switch and delay timer and the relative positions of the pressure gauges, flowmeters, and humidifier to each other.

Electroseal Corp., Des Plaines, Ill.) and an adjustable limit switch. The limit switch was made by connecting in series a resistive-sensitive relay (model B-1212, Industrial Solid State Control Inc., York, Pa.), a linear motion 50 K ohm potentiometer, and a linear motion 100 K ohm potentiometer mounted on the drive shaft of the Heath recorder. The purpose of the adjustable limit switch was to provide a means for controlling at any desired oxygen tension.

**Chemical analyses.** Glucose was determined in the Technicon AutoAnalyzer (Technicon Instruments Corp., Tarrytown, N.Y.) by the potassium ferri-ferrocyanide oxidation-reduction reaction.

Ammonia was determined by the method of Johnson (9), substituting KOH for NaOH and preparing the standards in 0.001% phenol red to allow for the dye present in cultures. The assay was performed with the AutoAnalyzer. The color was developed for 5 min at room temperature and measured at 490 nm. This method gave results that compared reasonably well with the Conway Diffusion Test (3) when determining ammonia in the presence of glutamine.

Lactic acid was determined manually by the method of Barker and Summerseon (1).

**Data acquisition.** Stepping switches (type 44, Automatic Electric Sales Corp., North Lake, Ill.) advanced by a 5-min recycling timer (series CM; Industrial Timer Corp., Los Angeles, Calif.) sequentially recorded redox potential and monitored and controlled dissolved oxygen tension in each flask (Fig. 4). The pH of each flask was recorded continuously on individual pH recorder-controllers. A 20-position programmer (Selectroswitch, Selectro Corp., Mamaroneck, N.Y.) was employed to control valve operation in the sampling of culture media for glucose and ammonia analyses. Data from the glucose and
ammonia analyses were collected with a master scanner (model 453M, Cohu Electronics Inc., San Diego, Calif.) and printed on a digital recorder (model 562A, Hewlett-Packard, Palo Alto, Calif.).

RESULTS

The growth curve of L cells obtained in this system with the pO₂ maintained at 9%, together with continuously recorded pH and redox values, is shown in Fig. 5. Relatively uniform values of medium pH and redox potential were maintained during the growth cycle under controlled pO₂ condition. In our culture system, pH values generally remained within 6.9 ± 0.1 pH units, even at varied pO₂ levels without further adjustment as long as the CO₂ content of the sparging gas mixture was maintained at 5%. When growth of L-cell cultures was compared over a pO₂ range of 2 to 20%, optimum growth was obtained at a pO₂ value of approximately 9% (Fig. 6). This value agrees with results reported by other workers cited earlier.

The effects of oxygen tension on glucose utilization rates are shown in Fig. 6. The glucose utilization rates were calculated by the method of Zwartouw and Westwood (17) and represent the amount of glucose (in micrograms) disappearing from the medium during 24 hr divided by the average number of cells (in millions) present during that period. The results indicate that the glucose utilization rate increased with increasing oxygen tension; e.g., even though the growth rates for cultures grown at 5 and 20% oxygen tension were approximately the same, the glucose
utilization rate at 20% oxygen was about 2.5 times that at 5% O₂.

Another correlation between dissolved oxygen tensions and cell activity was observed in a study of ammonia accumulation in the experimental cultures (Fig. 6). The curves represent data on daily levels of ammonia in each culture and indicated that NH₃ production was at a minimum in cultures grown at the optimal oxygen tension of 9%.

Approximately 45 to 50 µg of lactic acid per ml were accumulated in all cultures during the first 24 hr, regardless of oxygen tension; however, on subsequent days cultures with the higher rates of glucose utilization tended to accumulate more lactic acid. Lactic acid levels never exceeded 100 µg/ml during the 4-day growth period of any culture.

Fig. 7 is a plot of the redox potentials obtained in cultures grown at various dissolved oxygen tensions. The redox potential value that corresponds to the optimal pO₂ value of 9% was approximately +90 mv. This redox value for optimal cell growth is in essential agreement with the results obtained by Daniels et al. (5) and Wiles and Smith (16).

DISCUSSION

Cooper et al. (4) extrapolated the data they obtained on the growth of embryo rabbit kidney suspension cultures, at pH 6.9 to 7.1 for various oxygen tensions, and suggested that the optimal oxygen concentration for cell growth in the liquid phase was equivalent to equilibrium with about 9% oxygen in the gas phase. Kilburn and Webb (10) reported on the growth of mouse LS-cell suspension cultures, at pH 7.4 ± 0.1 for various controlled oxygen tensions, and showed that maximum viable cell populations were obtained when the dissolved oxygen tension was controlled at values between 40 and 100 mm of Hg (approximately 5 to 13% pO₂). Our data, therefore, are in good agreement with the results obtained by other workers.

Daniels et al. (5) and Wiles and Smith (16) demonstrated the use of redox potential as a means of controlling the culture environment when growing mammalian cells in submerged cultures. Their data indicated that the optimum redox potential for growth of L-DR cells, at pH

FIG. 6. Yields (solid line), rate of glucose utilization (broken line), and ammonia accumulation (dotted line) of L-DR cell cultures grown at various oxygen tensions. Each point represents an average of values obtained from four to six cultures.

FIG. 7. Effects of dissolved oxygen tension on redox potentials of L-cell cultures at pH 6.9 (±0.1). Average recorded redox potentials during 4 days of growth at various dissolved oxygen tensions are presented. Fluctuations in redox values during the 4 days of incubation were less than ±20 mv for each pO₂ value. Values presented are potentials obtained against a standard calomel reference cell.
6.9 to 7.1, was between 75 and 100 mv based on a silver-silver chloride reference cell, which is equal to an $E_h$ range of approximately +292 to +322 mv. Our work differed from that of Daniels and Wiles because we employed a totally synthetic culture medium; however, our results are in essential agreement with their work. The optimal oxygen partial pressure of approximately 9% corresponded to a redox potential of approximately 90 mv (based on a saturated calomel reference) which equals an $E_h$ value of approximately +334 mv.

The data presented on glucose utilization indicated that regardless of $pO_2$ value, L cells showed increased rates of carbohydrate metabolism as the culture reached maximum population density (Fig. 6). These results differed from those reported by Zwartouw and Westwood (17), who found that rates of glucose uptake by MK-2 cells decreased with culture age. The almost zero level of glucose uptake by all our cultures during the first day of incubation cannot be explained; however, repeated analyses of many samples have consistently shown that only negligible amounts of glucose are utilized by L cells during the early part of the growth cycle.

The finding that oxygen tension in culture media markedly affected NH$_3$ accumulation by L cells (Fig. 6) may be of considerable significance in view of the possibility suggested by Higuchi (8) that accumulation of NH$_3$ is a factor that impairs the growth of L cells.

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